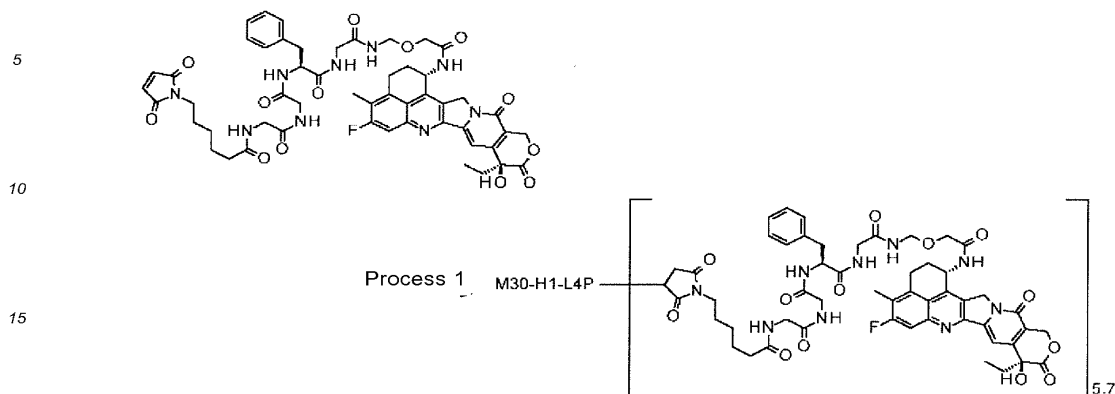


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[Formula 133]



20 Process 1: Antibody-drug conjugate (61)

[0630] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (4 mL, 40 mg of the antibody) was placed in a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.14 mL; 5.2 equivalents per antibody molecule). After confirming that the solution had pH near 7.0 by using a pH meter, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour. Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.232 mL; 8.6 equivalents per antibody molecule) to the above solution, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 60 minutes. Next, an aqueous solution (0.035 mL; 12.9 equivalents per antibody molecule) of 100 mM NAC was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0631] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 13 mL of a solution containing the titled antibody-drug conjugate.

[0632] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

[0633] Antibody concentration: 2.03 mg/mL, antibody yield: 26 mg (66%), and average number of conjugated drug molecules (n) per antibody molecule: 5.7.

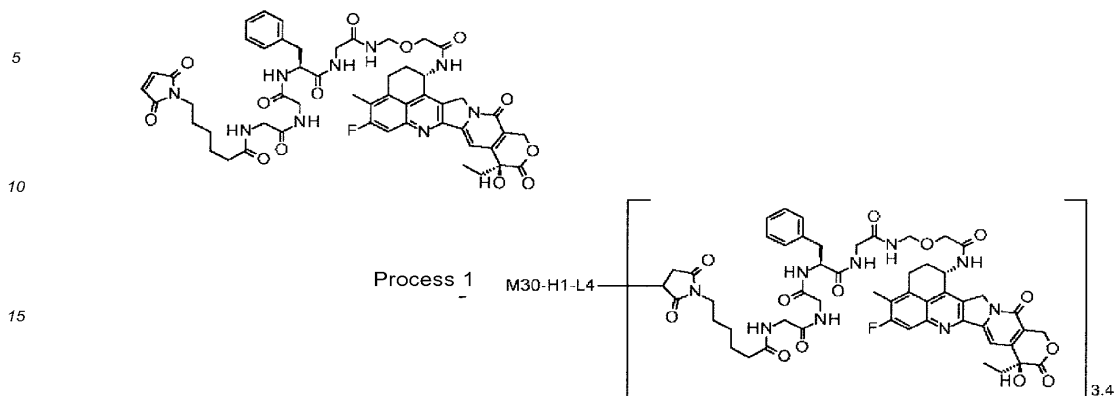
Example 65 Antibody-drug conjugate (62)

45 [0634]

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[Formula 134]



[0635] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (1.25 mL, 12.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0287 mL; 3.4 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

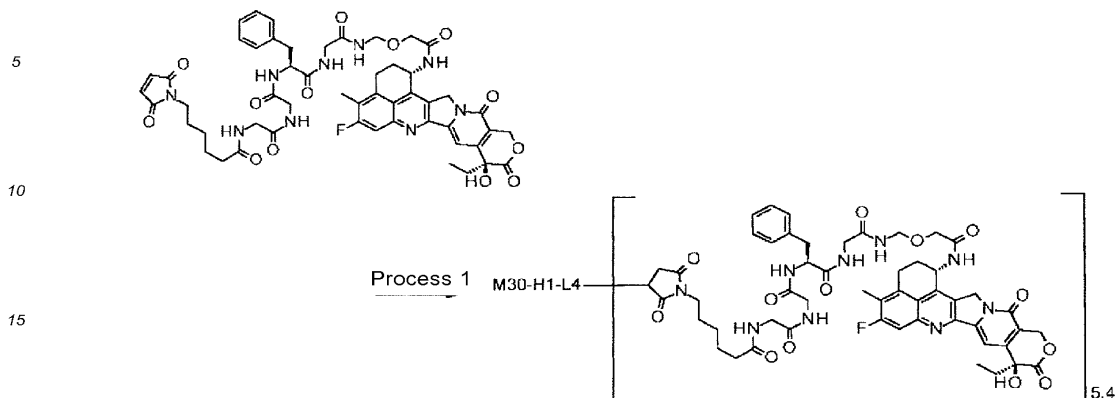
[0637] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

[0639] Antibody concentration: 10.0 mg/mL, antibody yield: 7.8 mg (62%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

[0640]

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[Formula 135]



Process 1: Antibody-drug conjugate (63)

[0641] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.25 mL, 12.5 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0439 mL; 5.2 equivalents per antibody molecule) (0.0287 mL; 3.4 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0642] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.0726 mL; 8.6 equivalents per antibody molecule) to the above solution at room temperature, it was incubated for conjugating the drug linker to the antibody in a water bath at 15°C for 1 hour. Next, an aqueous solution (0.011 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated to terminate the drug linker reaction at room temperature for another 20 minutes.

[0643] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

[0644] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

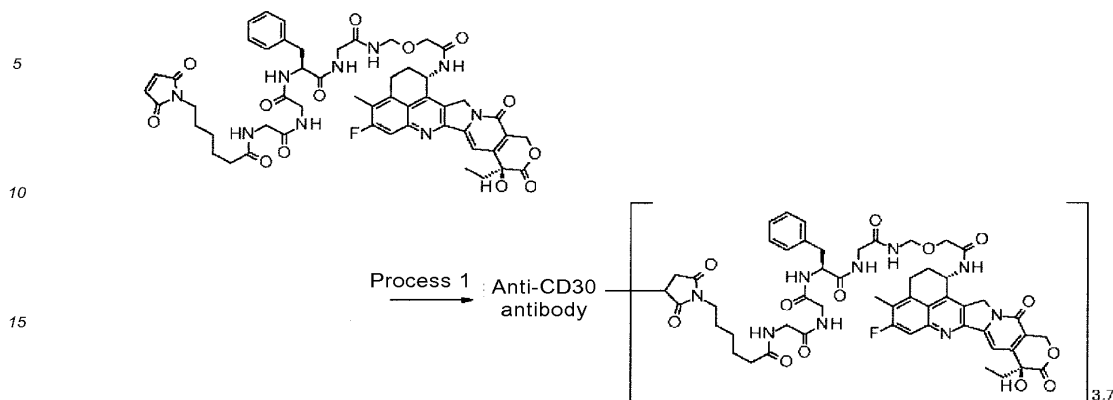
[0645] Antibody concentration: 10.0 mg/mL, antibody yield: 7.3 mg (58%), and average number of conjugated drug molecules (n) per antibody molecule: 5.4.

Example 67 Antibody-drug conjugate (64)

[0646]

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[Formula 136]



Process 1: Antibody-drug conjugate (64)

[0647] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.75 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0065 mL; 2.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0058 mL). After confirming that the solution had pH of 7.0 ± 0.1 , the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0648] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.0116 mL; 4.5 equivalents per antibody molecule) and dimethyl sulfoxide (0.0101 mL) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0017 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

[0649] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 270400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

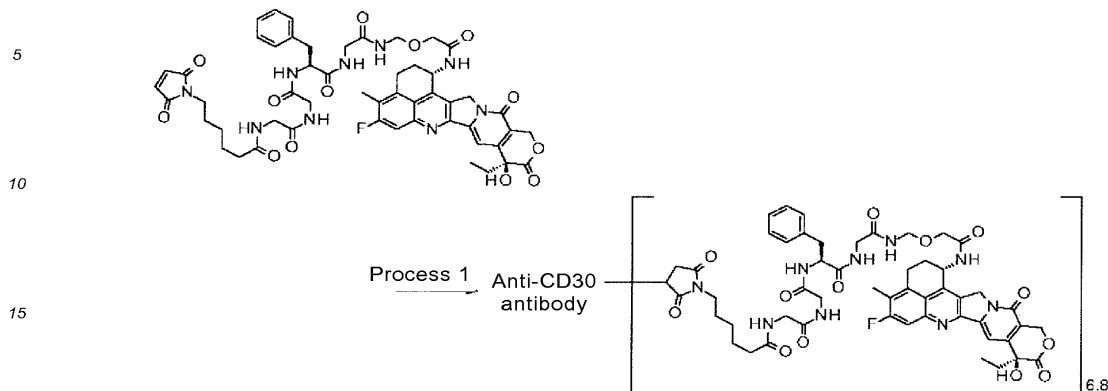
[0650] Antibody concentration: 0.96 mg/mL, antibody yield: 2.4 mg (60%), and average number of conjugated drug molecules (n) per antibody molecule: 3.7.

Example 68 Antibody-drug conjugate (65)

[0651]

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[Formula 137]



20 Process 1: Antibody-drug conjugate (65)

[0652] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.75 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0129 mL; 5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.006 mL). After confirming that the solution had pH of 7.0 ± 0.1 , the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0653] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.0233 mL; 9 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0035 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0654] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

[0655] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 270400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

[0656] Antibody concentration: 0.39 mg/mL, antibody yield: 1.0 mg (24%), and average number of conjugated drug molecules (n) per antibody molecule: 6.8.

45 Example 69 Antibody-drug conjugate (66)

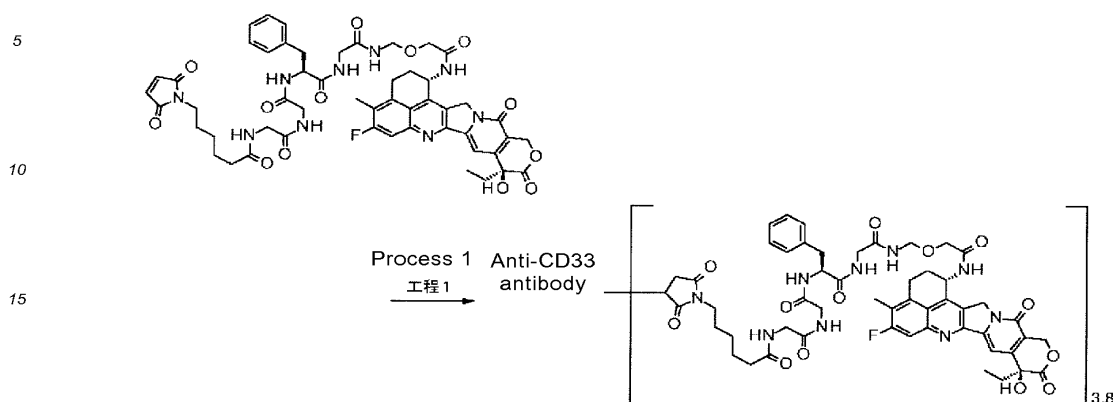
[0657]

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[Formula 138]



Process 1: Antibody-drug conjugate (66)

[0658] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.66 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0065 mL; 2.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0058 mL). After confirming that the solution had pH of 7.0 ± 0.1 , the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0659] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.0116 mL; 4.5 equivalents per antibody molecule) and dimethyl sulfoxide (0.0101 mL) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0017 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

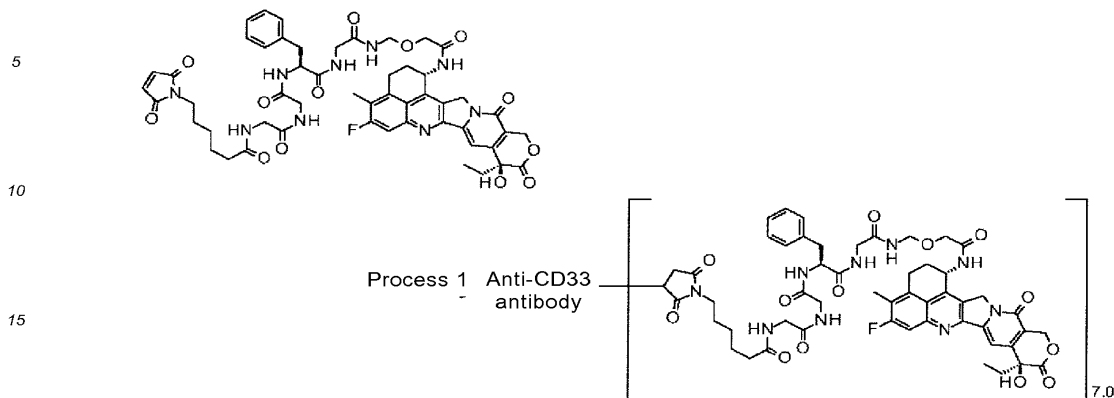
[0660] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 256400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

[0661] Antibody concentration: 1.19 mg/mL, antibody yield: 3.0 mg (74%), and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

Example 70 Antibody-drug conjugate (67)

[0662]

[Formula 139]



[0663] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.66 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0129 mL; 5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.006 mL). After confirming that the solution had pH of 7.0 ± 0.1, the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

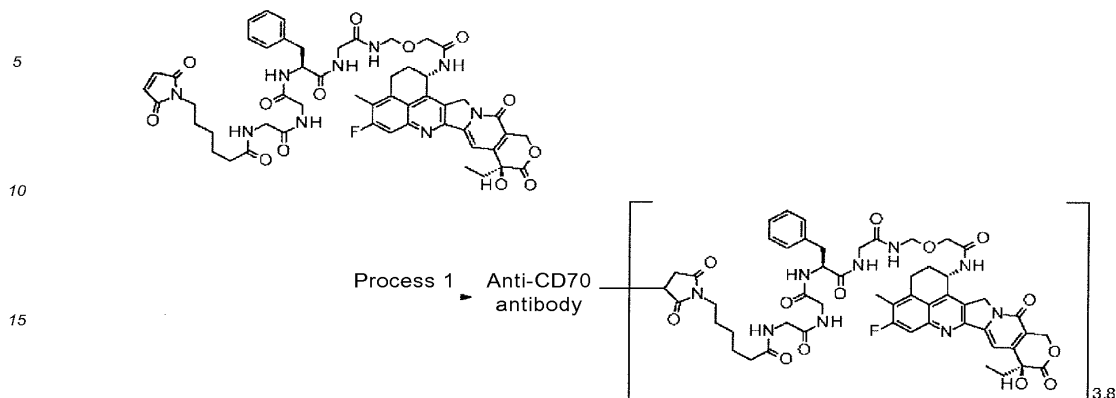
[0665] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

[0667] Antibody concentration: 1.24 mg/mL, antibody yield: 3.1 mg (78%), and average number of conjugated drug molecules (n) per antibody molecule: 7.0.

[0668]

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[Formula 140]



Process 1: Antibody-drug conjugate (68)

[0669] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.69 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0065 mL; 2.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0058 mL). After confirming that the solution had pH of 7.0 ± 0.1 , the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0670] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.0116 mL; 4.5 equivalents per antibody molecule) and dimethyl sulfoxide (0.0101 mL) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0017 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes. Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

[0671] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 262400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

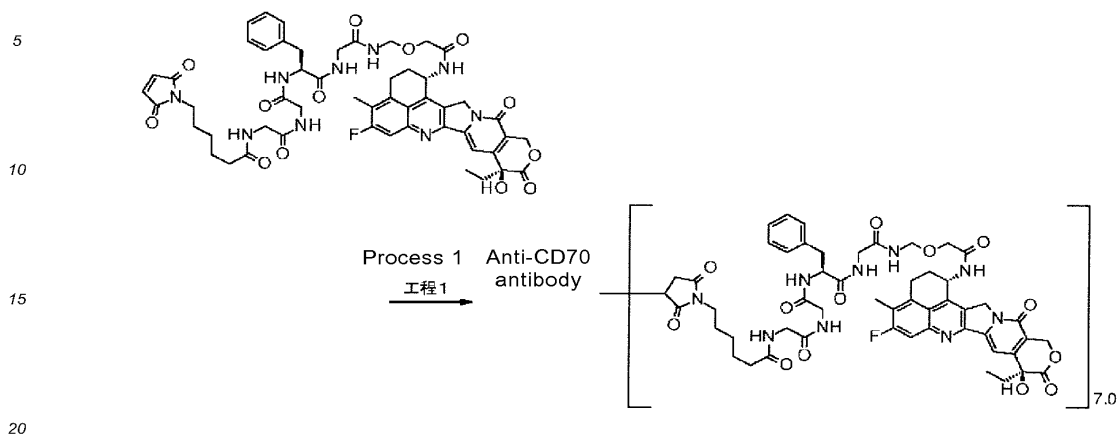
[0672] Antibody concentration: 1.10 mg/mL, antibody yield: 2.8 mg (69%), and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

Example 72 Antibody-drug conjugate (69)

[0673]

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[Formula 141]



Process 1: Antibody-drug conjugate (69)

[0674] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.5/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.69 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (0.4 mL, 4 mg of the antibody) was placed in a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0129 mL; 5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.006 mL). After confirming that the solution had pH of 7.0 ± 0.1 , the disulfide bond in hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0675] Conjugation between antibody and drug linker: After adding a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 8 of Example 58 (0.0233 mL; 9 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) for conjugating the drug linker to the antibody at room temperature for 1 hour. Next, an aqueous solution (0.0035 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and further incubated to terminate the drug linker reaction at room temperature for 20 minutes.

[0676] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 2.5 mL of a solution containing the titled antibody-drug conjugate.

[0677] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 262400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5178$ (measured value), and $\epsilon_{D,370} = 20217$ (measured value) were used), the following characteristic values were obtained.

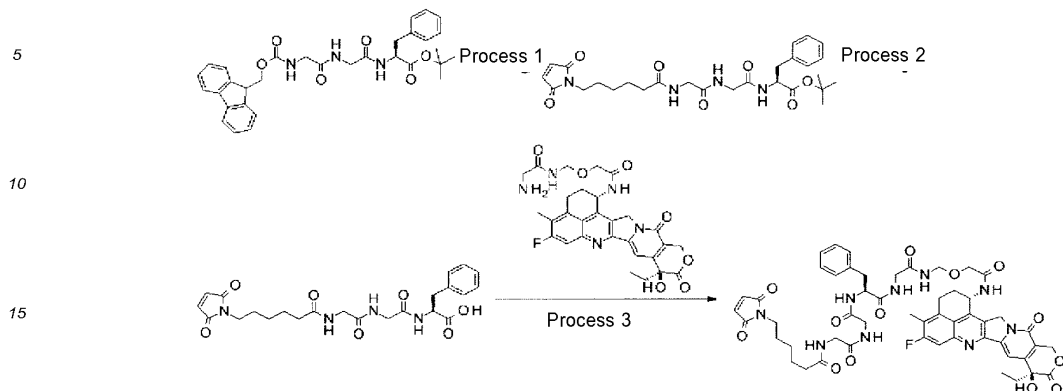
[0678] Antibody concentration: 1.16 mg/mL, antibody yield: 2.9 mg (73%), and average number of conjugated drug molecules (n) per antibody molecule: 7.0.

Example 73 (Another method for synthesizing compound of Process 8 of Example 58)

[0679]

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[Formula 142]



Process 1: Tert-butyl N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenyl alaninate

[0680] Under ice cooling, to THF (12.0 ml) solution of tert-butyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycylglycyl-L-phenyl alaninate (J. Pept. Res., 1999, vol. 53, pp. 393) (0.400 g, 0.717 mmol), 1,8-diazabicyclo[5.4.0]-7-undecene (0.400 ml) was added and stirred at room temperature for 4 days, and then N-succinimidyl 6-maleimide hexanoate (0.221 g, 0.717 mmol) was further added and stirred for 3 hours. The reaction solution was diluted with ethyl acetate and washed with an aqueous solution of 10% citric acid, a saturated aqueous solution of sodium hydrogen carbonate, and saturated brine, and then the organic layer was dried over anhydrous magnesium sulfate. After the solvent was removed under reduced pressure, the residue obtained were purified by silica gel column chromatography [chloroform - chloroform : methanol = 9 : 1 (v/v)] to yield the titled compound as a pale yellow solid (0.295 g, 78%).

¹H-NMR (400 MHz, CDCl₃) δ: 1.28-1.36 (2H, m), 1.41 (9H, s), 1.57-1.71 (4H, m), 2.23 (2H, t, J=7.6 Hz), 3.09 (2H, d, J=6.0 Hz), 3.51 (2H, t, J=7.6 Hz), 3.85-4.02 (4H, m), 4.69-4.78 (1H, m), 6.15 (1H, t, J=4.6 Hz), 6.33 (1H, d, J=7.3 Hz), 6.60 (1H, t, J=5.0 Hz), 6.68 (2H, s), 7.10-7.16 (2H, m), 7.22-7.31 (3H, m).

MS (ESI) m/z: 529 (M+H)⁺

Process 2: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanine

[0681] To dichloromethane (8.00 ml) solution of the compound (0.295 g, 0.558 mmol) obtained in Process 1, trifluoroacetic acid (4.00 mL) was added and stirred at room temperature for 18 hours. The solvent was removed under reduced pressure to yield the titled compound as a pale yellow solid (0.240 g, 91%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 1.15-1.23 (2H, m), 1.40-1.53 (4H, m), 2.10 (2H, t, J=7.6 Hz), 2.88 (1H, dd, J=13.7, 8.9 Hz), 3.04 (1H, dd, J=13.7, 5.0 Hz), 3.35-3.43 (2H, m), 3.58-3.77 (4H, m), 4.41 (1H, td, J=7.8, 5.0 Hz), 7.00 (2H, s), 7.16-7.31 (5H, m), 8.00 (1H, t, J=5.7 Hz), 8.06 (1H, t, J=5.7 Hz), 8.13 (1H, d, J=7.8 Hz).

Process 3: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanine-N-[(2-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino)-2-oxoethoxy)methyl]glycinamide

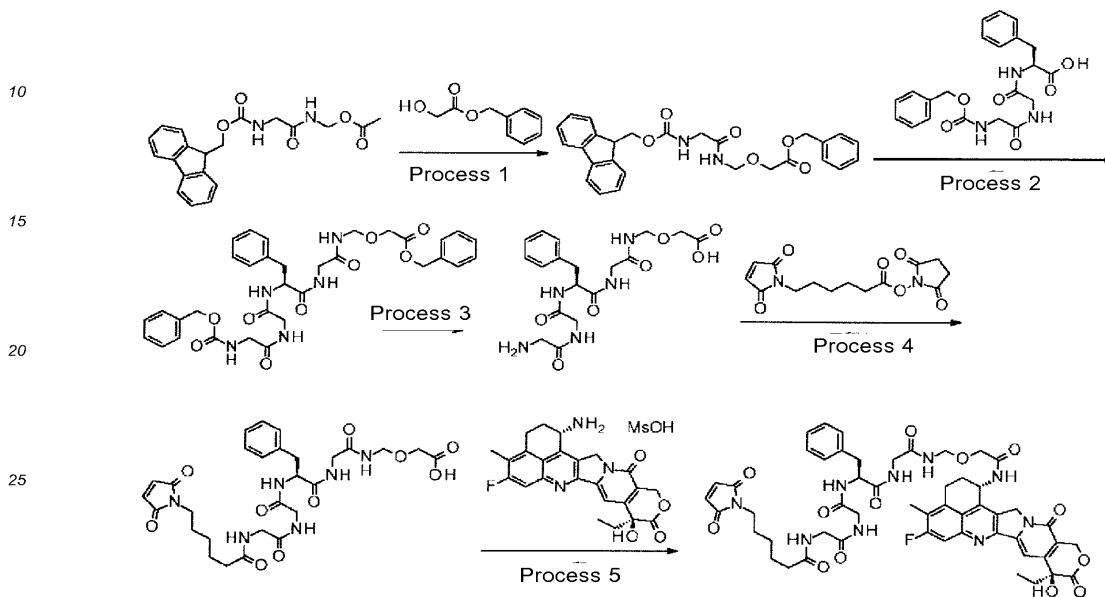
[0682] The compound (0.572 g, 1.21 mmol) obtained in Process 2 was dissolved in dichloromethane (12.0 mL), charged with N-hydroxysuccinimide (0.152 g, 1.32 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.253 g, 1.32 mmol), and stirred for 1 hour. The reaction solution was added to an N,N-dimethyl formamide (22.0 mL) solution of the mixture (1.10 mmol) obtained in Process 5 of Example 58, and stirred at room temperature for 3 hours. The reaction solution was charged with an aqueous solution of 10% citric acid and extracted with chloroform. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound as a pale yellow solid (0.351 g, 31%). The instrumental data of the compound was the same as that of the compound of Process 8 of Example 58.

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Example 74 (Another method for synthesizing compound of Process 8 of Example 58)

[0683]

[Formula 143]



Process 1: Benzyl [(N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycyl)amino]methoxy]acetate

[0684] To tetrahydrofuran (200 ml) solution of the compound (7.37 g, 20.0 mmol) obtained in Process 1 of Example 58, benzyl glycolate (6.65 g, 40.0 mmol) and p-toluene sulfonic acid monohydrate (0.381 g, 2.00 mmol) were added at 0°C and stirred at room temperature for 2 hours and 30 minutes. The reaction solution was charged with a saturated aqueous solution of sodium hydrogen carbonate and extracted with ethyl acetate. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [hexane : ethyl acetate = 100 : 0 (v/v) - 0 : 100] to yield the titled compound as a colorless solid (6.75 g, 71%). The instrumental data of the compound was the same as that of the compound of Process 2 of Example 58.

Process 2: N-[(Benzyloxy)carbonyl]glycylglycyl-L-phenylalanine-N-[(2-(benzyloxy)-2-oxoethoxy)methyl]glycinamide

[0685] To an N,N-dimethyl formamide (140 mL) solution of the compound (6.60 g, 13.9 mmol) obtained in Process 1, 1,8-diazabicyclo[5.4.0]undec-7-ene (2.22 g, 14.6 mmol) was added at 0°C and stirred at room temperature for 15 minutes. The reaction solution was charged with an N,N-dimethyl formamide (140 mL) solution of N-[(benzyloxy)carbonyl]glycylglycyl-L-phenylalanine (6.33 g, 15.3 mmol), N-hydroxysuccinimide (1.92 g, 16.7 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.20 g, 16.7 mmol) stirred in advance at room temperature for 1 hour, and stirred at room temperature for 4 hours. The reaction solution was charged with 0.1 N hydrochloric acid and extracted with chloroform. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound as a colorless solid (7.10 g, 79%).

¹H-NMR (DMSO-D₆) δ: 2.78 (1H, dd, J=13.9, 9.6 Hz), 3.05 (1H, dd, J=13.9, 4.5 Hz), 3.56-3.80 (6H, m), 4.15 (2H, s), 4.47-4.55 (1H, m), 4.63 (2H, d, J=6.6 Hz), 5.03 (2H, s), 5.15 (2H, s), 7.16-7.38 (15H, m), 7.52 (1H, t, J=5.9 Hz), 8.03 (1H, t, J=5.5 Hz), 8.17 (1H, d, J=8.2 Hz), 8.36 (1H, t, J=5.7 Hz), 8.61 (1H, t, J=6.6 Hz).

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Process 3: Glycylglycyl-L-phenylalanyl-N-[(carboxymethoxy)methyl]glycinamide

[0686] To an N,N-dimethyl formamide (216 mL) solution of the compound (7.00 g, 10.8 mmol) obtained in Process 2, palladium carbon catalyst (7.00 g) was added and stirred under hydrogen atmosphere at room temperature for 24 hours. The insolubles were removed by filtration through Celite, and the solvent was removed under reduced pressure. The residue obtained were dissolved in water, the insolubles were removed by filtration through Celite, and the solvent was removed under reduced pressure. This procedure was repeated twice to yield the titled compound as a colorless solid (3.77 g, 82%).

¹H-NMR (DMSO-D₆) δ: 2.84 (1H, dd, J=13.7, 9.8 Hz), 3.08 (1H, dd, J=13.7, 4.7 Hz), 3.50-3.72 (4H, m), 3.77-3.86 (2H, m), 3.87 (2H, s), 4.52-4.43 (1H, m), 4.61 (2H, d, J=6.6 Hz), 7.12-7.30 (5H, m), 8.43 (1H, t, J=5.9 Hz), 8.54 (1H, d, J=7.8 Hz), 8.70 (1H, t, J=6.3 Hz), 8.79 (1H, t, J=5.5 Hz).

Process 4: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-[(carboxymethoxy)methyl]glycinamide

[0687] To an N,N-dimethyl formamide (85.0 mL) solution of the compound (3.59 g, 8.48 mmol) obtained in Process 3, N-succinimidyl 6-maleimide hexanoic acid (2.88 g, 9.33 mmol) and triethylamine (0.858 g, 8.48 mmol) were added and stirred at room temperature for 1 hour. The reaction solution was charged with 0.1 N hydrochloric acid and extracted with chloroform and a mixed solvent of chloroform and methanol [chloroform : methanol = 4 : 1 (v/v)]. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - partitioned organic layer of chloroform : methanol : water = 7 : 3 : 1 (v/v/v)] to yield the titled compound as a colorless solid (3.70 g, 71%).

¹H-NMR (DMSO-D₆) δ: 1.13-1.24 (2H, m), 1.42-1.53 (4H, m), 2.11 (2H, t, J=7.4 Hz), 2.80 (1H, dd, J=13.7, 9.8 Hz), 3.06 (1H, dd, J=13.9, 4.5 Hz), 3.37 (2H, t, J=7.2 Hz), 3.56-3.78 (6H, m), 3.97 (2H, s), 4.46-4.53 (1H, m), 4.61 (2H, d, J=6.3 Hz), 7.00 (2H, s), 7.15-7.29 (5H, m), 8.03-8.20 (3H, m), 8.32 (1H, t, J=5.9 Hz), 8.60 (1H, t, J=6.7 Hz).

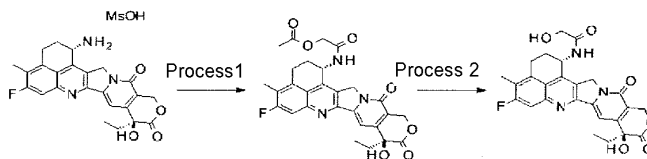
Process 5: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanyl-N-[(2-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino)-2-oxoethoxy)methyl]glycinamide

[0688] To an N,N-dimethyl formamide (40.0 mL) solution of mesylate of the compound (4) (1.14 g, 2.00 mmol), triethylamine (0.202 g, 2.00 mmol), the compound (1.48 g, 2.40 mmol) obtained in Process 4, and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (0.993 g, 3.00 mmol) containing 16.4% water were added at 0°C and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform - chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound as a pale yellow solid (1.69 g, 82%). The instrumental data of the compound was the same as that of the compound of Process 8 of Example 58.

Example 75 N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-2-hydroxyacetamide

[0689]

[Formula 144]



Process 1: 2-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino)-2-oxoethylacetate

[0690] Under ice cooling, to an N,N-dimethyl formamide (20.0 mL) suspension of mesylate of the compound (4) (0.500 g, 0.941 mmol), N,N-diisopropylethylamine (0.492 mL, 2.82 mmol) and acetoxyacetyl chloride (0.121 mL, 1.13 mmol)

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were added and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform-partitioned organic layer of chloroform : methanol : water = 7 : 3 : 1 (v/v/v)] to yield the titled compound as a pale yellow solid (0.505 g, quantitative).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.81-1.92 (2H, m), 2.08 (3H, s), 2.08-2.22 (2H, m), 2.41 (3H, s), 3.14-3.21 (2H, m), 4.51 (2H, dd, J=19.4, 14.7 Hz), 5.22 (2H, dd, J=40.1, 19.0 Hz), 5.43 (2H, s), 5.56-5.61 (1H, m), 6.53 (1H, s), 7.31 (1H, s), 7.81 (1H, d, J=11.0 Hz), 8.67 (1H, d, J=8.6 Hz).

MS (ESI) m/z: 536 (M+H)⁺

Process 2: N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-2-hydroxyacetamide

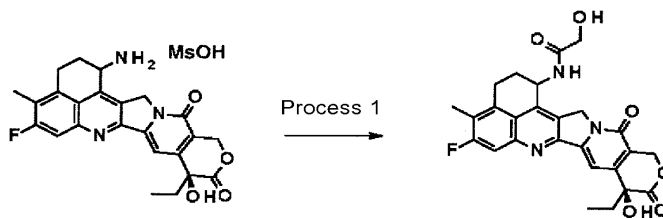
[0691] To methanol (50.0 mL) suspension of the compound (0.504 g, 0.941 mmol) obtained in Process 1, tetrahydrofuran (20.0 mL) and an aqueous solution of 1 N sodium hydroxide (4.00 mL, 4.00 mmol) were added and stirred at room temperature for 1 hour. The reaction was terminated by the addition of 1 N hydrochloric acid (5.00 mL, 5.00 mmol), and the solvent was removed under reduced pressure. The residue obtained were purified by silica gel column chromatography [chloroform-partitioned organic layer of chloroform : methanol : water = 7 : 3 : 1 (v/v/v)] to yield the titled compound as a pale yellow solid (0.412 g, 89%). This compound was confirmed in the tumor of a cancer-bearing mouse that received the antibody-drug conjugate (55) or (56).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.3 Hz), 1.78-1.95 (2H, m), 2.09-2.28 (2H, m), 2.39 (3H, s), 3.07-3.27 (2H, m), 3.96 (2H, d, J=6.0 Hz), 5.11-5.26 (2H, m), 5.42 (2H, s), 5.46-5.54 (1H, m), 5.55-5.63 (1H, m), 6.52 (1H, s), 7.30 (1H, s), 7.78 (1H, d, J=10.9 Hz), 8.41 (1H, d, J=9.1 Hz). MS (ESI) m/z: 494 (M+H)⁺

Example 76 (Another method for synthesizing compound of Example 75)

[0692]

[Formula 145]



Process 1: N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-2-hydroxyacetamide

[0693] Glycolic acid (0.0201 g, 0.27 mmol) was dissolved in N,N-dimethyl formamide (1.0 mL), charged with N-hydroxysuccinimide (0.0302 g, 0.27 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.0508 g, 0.27 mmol), and stirred for 1 hour. The reaction solution was added to an N,N-dimethyl formamide suspension (1.0 mL) charged with mesylate of the compound (4) (0.1 g, 0.176 mmol) and triethylamine (0.025 mL, 0.18 mmol) and stirred at room temperature for 24 hours. The solvent was removed under reduced pressure and the residue obtained were purified by silica gel column chromatography [chloroform-chloroform : methanol = 10 : 1 (v/v)] to yield the titled compound as a pale yellow solid (0.080 g, 92%). The instrumental data of the compound was the same as that of the compound obtained in Process 2 of Example 75.

(Test Example 1) Production of full-length human B7-H3 variant 1 expression vector

[0694] cDNA encoding human B7-H3 variant 1 was amplified by PCR reaction using cDNA synthesized from LNCaP cell (American Type Culture Collection: ATCC) total RNA as a template and the following primer set:

primer 1:

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5'-ctatagggagacccaagctggctagcatgctgcgctcggcggggcag-

3' (SEQ ID NO: 22) and

primer 2:

5' -

aacggggccctctagactcgagcggcgcgtcaggctatcttctgtccatcatcttc

tttgctgtcag-3' (SEQ ID NO: 23).

[0695] Next, the obtained PCR product was purified by using MagExtractor PCR & Gel cleanup (Toyobo Co., Ltd.). The purified product was further digested with restriction enzymes (NheI/NotI) and thereafter purified by using MagExtractor PCR & Gel cleanup (Toyobo Co., Ltd.). pcDNA3.1 (+) plasmid DNA (Life Technologies) was digested with the same restriction enzymes as above (NheI/NotI) and thereafter purified by using MagExtractor PCR & Gel cleanup (Toyobo Co., Ltd.).

[0696] These purified DNA solutions were mixed, further charged with Ligation high (Toyobo Co., Ltd.), and incubated for ligation at 16°C for 8 hours.

[0697] *Escherichia coli* DH5 α competent cells (Life Technologies) were transformed by the addition of the obtained reaction product.

[0698] The colonies thus obtained were subjected to colony direct PCR using PCR primers and BGH reverse primer to select candidate clones.

[0699] The obtained candidate clones were cultured in a liquid medium (LB/Amp), and plasmid DNA was extracted with MagExtractor-Plasmid- (Toyobo Co., Ltd.).

[0700] Each obtained clone was compared with the provided CDS sequence by the sequencing analysis between primer 3 (CMV promoter primer):

5'-cgcaaatggcggtaggcgtg-3' (SEQ ID NO: 24) and primer 4 (BGH reverse primer):

5'-tagaaggcacagtcgagg-3' (SEQ ID NO: 25)

with the obtained plasmid DNA as a template.

[0701] After confirming the sequence, the obtained clone was cultured in 200 mL of LB/Amp medium, and plasmid DNA was extracted by using VioGene Plasmid Midi V-100 kit.

[0702] The vector was designated as pcDNA3.1-B7-H3. The sequence of an ORF site of the B7-H3 variant 1 gene cloned in the vector is shown in nucleotide positions 1 to 1602 of SEQ ID NO: 26 (Figure 16) in the Sequence Listing. Also, the amino acid sequence of the B7-H3 variant 1 is shown in SEQ ID NO: 1 in the Sequence Listing.

(Test Example 2) Preparation of CCRF-CEM cell stably expressing B7-H3 variant 1 gene

[0703] pcDNA3.1-B7-H3 produced in Test Example 1 was transfected into CCRF-CEM cells (ATCC) by electroporation using Nucleofector II (manufactured by Lonza Group Ltd.). Then, the cells were further cultured for two nights in RPMI1640 medium (Life Technologies) containing 10% fetal bovine serum (FBS) (hereinafter, referred to as 10%FBS-RPMI1640) under conditions of 37°C and 5% CO₂.

[0704] After the 2-day culture, culture was started in 10%FBS-RPMI1640 containing 750 μ g/mL G418 (Life Technologies) in order to select CCRF-CEM cells in which pcDNA3.1-B7-H3 was stably integrated.

[0705] After the 1-month culture, cloning was carried out by the limiting dilution method in order to yield a single cell clone. Specifically, cells having resistance to G418 were diluted into 10 cells/mL, inoculated to a 96-well plate at a concentration of 100 μ L/well, and cultured, and cells allowed to proliferate were recovered from individual wells.

[0706] Flow cytometry was used for confirming B7-H3 expression in each recovered clone. Specifically, each recovered clone was washed twice with PBS containing 5% FBS, thereafter suspended by the addition of PBS containing 5% FBS and 10 μ g/mL M30, and left standing at 4°C for 30 minutes. The clone was washed twice with PBS containing 5% FBS, thereafter suspended by the addition of Fluorescein-conjugated goat IgG fraction to mouse IgG (Whole Molecule) (#55493, manufactured by ICN Pharmaceuticals, Inc.) diluted 1000-fold with PBS containing 5% FBS, and left standing at 4°C for 30 minutes. The clone was washed twice with PBS containing 5% FBS, thereafter resuspended in PBS containing 5% FBS, and detected by using a flow cytometer (FC500: Beckman Coulter, Inc.).

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[0707] The CCRF-CEM cells stably expressing the B7-H3 variant 1 gene thus obtained by these procedures were designated as CEM_V1_3.1_2 cells. The parent line CCRF-CEM cells were used as a cell line lacking B7-H3 expression.

(Test Example 3) Cytotoxicity test (1) of antibody-drug conjugate

[0708] The CEM_V1_3.1_2 cells produced in Test Example 2 or CCRF-CEM cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The CEM_V1_3.1_2 cells or CCRF-CEM cells were prepared to have a concentration of 8×10^4 cells/mL by using a medium, added at a concentration of 25 μ L/well to a 96-well microplate for cell culture charged with 65 μ L/well of a medium, and cultured overnight. On the next day, the M30-H1-L4 antibody, M30-H1-L4P antibody, and antibody-drug conjugate each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO₂ at 37°C for 3 days. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC₅₀ value was calculated according to the following equation:

$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

a: Concentration a of the test substance

b: Concentration b of the test substance

c: Ratio of live cells supplemented with the test substance having the concentration a

d: Ratio of live cells supplemented with the test substance having the concentration b

[0709] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0710] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

a: Average amount of light emission from the test substance-supplemented wells (n = 2)

b: Average amount of light emission from the test substance non-supplemented wells (n = 10)

[0711] The antibody-drug conjugates (5), (16), (21), (32), (44), (45), (46), (52), and (54) exhibited a cytotoxic activity of IC₅₀ < 0.1 (nM) against the CEM_V1_3.1_2 cells. The antibody-drug conjugates (1), (12), (13), (20), (28), (29), (35), (36), (37), (41), (49), and (53) exhibited a cytotoxic activity of $0.1 < IC_{50} < 1$ (nM) against the cells. The antibody-drug conjugates (33), (34), (47), (48), (50), and (51) exhibited a cytotoxic activity of $1 < IC_{50} < 100$ (nM) against the cells. On the other hand, none of these antibody-drug conjugates exhibited a cytotoxic activity against the CCRF-CEM cells (> 100 (nM)). Neither of the M30-H1-L4 antibody nor the M30-H1-L4P antibody exhibited a cytotoxic activity against both of the cells (> 100 (nM)).

(Test Example 4) Cytotoxicity test (2) of antibody-drug conjugate

[0712] Antigen-positive cells SR cells (ATCC) or antigen-negative cells Daudi cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The SR cells or Daudi cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium and added at a concentration of 90 μ L/well to a 96-well microplate for cell culture. Two hours later, the anti-CD30 antibody and antibody-drug conjugates (6) and (7) each diluted into 40 nM, 8 nM, 1.6 nM, 320 pM, 64 pM, 12.8 pM, and 2.6 pM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO₂ at 37°C for 3 days. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader

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(PerkinElmer). The IC_{50} value was calculated according to the following equation:

$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

a: Concentration a of the test substance

b: Concentration b of the test substance

c: Ratio of live cells supplemented with the test substance having the concentration a

d: Ratio of live cells supplemented with the test substance having the concentration b

[0713] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0714] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

a: Average amount of light emission from the test substance-supplemented wells (n = 2)

b: Average amount of light emission from the test substance non-supplemented wells (n = 12)

[0715] The antibody-drug conjugates (6) and (7) exhibited a cytotoxic activity of $IC_{50} < 0.01$ (nM) against the SR cells. On the other hand, the antibody-drug conjugates (6) and (7) exhibited no cytotoxic activity against the Daudi cells (> 4.0 (nM)). The anti-CD30 antibody exhibited no cytotoxic activity against both of the cells (> 4.0 (nM)).

(Test Example 5) Cytotoxicity test (3) of antibody-drug conjugate

[0716] Antigen-positive cells SR cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The SR cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium and added at a concentration of 90 μL /well to a 96-well microplate for cell culture. Two hours later, the anti-CD30 antibody and antibody-drug conjugates (22), (23), (38), (64), and (65) each diluted into 1000 nM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and 1 pM by using a medium were added at a concentration of 10 μL /well to the microplate. A medium was added at a concentration of 10 μL /well to test substance non-supplemented wells. The cells were cultured under 5% CO_2 at 37°C for 6 days. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

a: Concentration a of the test substance

b: Concentration b of the test substance

c: Ratio of live cells supplemented with the test substance having the concentration a

d: Ratio of live cells supplemented with the test substance having the concentration b

[0717] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0718] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

a: Average amount of light emission from the test substance-supplemented wells (n = 2)

b: Average amount of light emission from the test substance non-supplemented wells (n = 12)

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[0719] The antibody-drug conjugates (23), (38), (64), and (65) exhibited a cytotoxic activity of $IC_{50} < 0.01$ (nM) against the SR cells. The antibody-drug conjugate (22) exhibited a cytotoxic activity of $IC_{50} < 0.1$ (nM) against the SR cells. The anti-CD30 antibody exhibited no cytotoxic activity against the SR cells (> 4.0 (nM)).

5 (Test Example 6) Cytotoxicity test (4) of antibody-drug conjugate

[0720] Antigen-positive cells HL-60 cells (ATCC) or antigen-negative cells Raji cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The HL-60 cells or Raji cells were prepared to have a concentration of 8×10^4 cells/mL by using a medium and added at a concentration of 25 μ L/well to a 96-well microplate for cell culture charged with 65 μ L/well of a medium. The anti-CD33 antibody and antibody-drug conjugates (8) and (9) each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured for 3 days under 5% CO_2 at 37°C. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

$$20 \quad IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

25 a: Concentration a of the test substance
b: Concentration b of the test substance
c: Ratio of live cells supplemented with the test substance having the concentration a
d: Ratio of live cells supplemented with the test substance having the concentration b

[0721] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

30 [0722] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

35 a: Average amount of light emission from the test substance-supplemented wells (n = 2)
b: Average amount of light emission from the test substance non-supplemented wells (n = 5)

[0723] The antibody-drug conjugates (8) and (9) exhibited a cytotoxic activity of $IC_{50} < 0.1$ (nM) against the HL-60 cells. On the other hand, the antibody-drug conjugates (8) and (9) exhibited no cytotoxic activity against the Raji cells (> 100 (nM)). The anti-CD33 antibody exhibited no cytotoxic activity against both of the cells (> 100 (nM)).

(Test Example 7) Cytotoxicity test (5) of antibody-drug conjugate

45 [0724] Antigen-positive cells NOMO-1 cells (HSRRB) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The NOMO-1 cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium and added at a concentration of 90 μ L/well to a 96-well microplate for cell culture. Two hours later, the anti-CD33 antibody and antibody-drug conjugates (24), (25), and (67) each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO_2 at 37°C for 6 days. After the culture, the microplate was taken out from the incubator and left standing for 30 minutes at room temperature. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

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$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

- a: Concentration a of the test substance
 b: Concentration b of the test substance
 c: Ratio of live cells supplemented with the test substance having the concentration a
 d: Ratio of live cells supplemented with the test substance having the concentration b

[0725] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0726] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

- a: Average amount of light emission from the test substance-supplemented wells (n = 2)
 b: Average amount of light emission from the test substance non-supplemented wells (n = 5)

[0727] The antibody-drug conjugate (25) exhibited a cytotoxic activity of $IC_{50} < 0.1$ (nM) against the NOMO-1 cells. The antibody-drug conjugates (24) and (67) exhibited a cytotoxic activity of $1 < IC_{50} < 100$ (nM) against the cells. The anti-CD33 antibody exhibited no cytotoxic activity against the NOMO-1 cells (> 100 (nM)).

(Test Example 8) Cytotoxicity test (6) of antibody-drug conjugate

[0728] Antigen-positive cells U251 cells (ATCC) or antigen-negative cells MCF-7 cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The U251 cells or MCF-7 cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium, added at a concentration of 90 μ L/well to a 96-well microplate for cell culture, and cultured overnight. On the next day, the anti-CD70 antibody and antibody-drug conjugates (10) and (11) each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO₂ at 37°C for 6 days. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

- a: Concentration a of the test substance
 b: Concentration b of the test substance
 c: Ratio of live cells supplemented with the test substance having the concentration a
 d: Ratio of live cells supplemented with the test substance having the concentration b

[0729] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0730] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

- a: Average amount of light emission from the test substance-supplemented wells (n = 2)
 b: Average amount of light emission from the test substance non-supplemented wells (n = 12)

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[0731] The antibody-drug conjugates (10) and (11) exhibited a cytotoxic activity of $IC_{50} < 1$ (nM) against the U251 cells. On the other hand, the antibody-drug conjugates (10) and (11) exhibited no cytotoxic activity against the MCF-7 cells (≥ 90 (nM)). The anti-CD70 antibody exhibited no cytotoxic activity against both of the cells (> 100 (nM)).

5 (Test Example 9) Cytotoxicity test (7) of antibody-drug conjugate

[0732] Antigen-positive cells U251 cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The U251 cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium and added at a concentration of 90 μ L/well to a 96-well microplate for cell culture. Two hours later, the anti-CD70 antibody and antibody-drug conjugates (26), (27), (40), (68), and (69) each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO_2 at 37°C for 6 days. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

$$20 \quad IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

25 a: Concentration a of the test substance
b: Concentration b of the test substance
c: Ratio of live cells supplemented with the test substance having the concentration a
d: Ratio of live cells supplemented with the test substance having the concentration b

[0733] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

30 **[0734]** The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

35 a: Average amount of light emission from the test substance-supplemented wells (n = 2)
b: Average amount of light emission from the test substance non-supplemented wells (n = 12)

[0735] The antibody-drug conjugates (26), (27), (40), and (69) exhibited a cytotoxic activity of $1 < IC_{50} < 10$ (nM) against the U251 cells. The antibody-drug conjugate (68) exhibited a cytotoxic activity of $10 < IC_{50} < 100$ (nM) against the cells. The anti-CD70 antibody exhibited no cytotoxic activity against the U251 cells (> 100 (nM)).

(Test Example 10) Cytotoxicity test (8) of released drug

[0736] A375 cells (ATCC) were cultured in DMEM (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The A375 cells were prepared to have a concentration of 4×10^4 cells/mL by using a medium, added at a concentration of 25 μ L/well to a 96-well microplate for cell culture (CORNING) charged with 65 μ L/well of a medium, and cultured overnight. On the next day, each test substance diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using DMSO was added at a concentration of 0.5 μ L/well to the microplate. DMSO was added at a concentration of 0.5 μ L/well to test substance non-supplemented wells. The volume of the medium in each well was adjusted to 100 μ L by the addition of 10 μ L/well of a medium, and the cells were cultured for 6 days under 5% CO_2 at 37°C. After the culture, the microplate was taken out from the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader. The IC_{50} value was calculated according to the following equation:

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$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

- a: Concentration a of the test substance
 b: Concentration b of the test substance
 c: Ratio of live cells supplemented with the test substance having the concentration a
 d: Ratio of live cells supplemented with the test substance having the concentration b

[0737] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0738] The survival rate of cells was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

- a: Average amount of light emission from the test substance-supplemented wells ($n = 2$)
 b: Average amount of light emission from the test substance non-supplemented wells ($n = 10$)

[0739] The compound of Example (75) and exatecan exhibited a cytotoxic activity of $0.1 < IC_{50} < 1$ (nM) against the A375 cells. The compound of Example (42) exhibited a cytotoxic activity against $1 < IC_{50} < 10$ (nM) against the cells. The compound of Example (1) exhibited a cytotoxic activity against $10 < IC_{50} < 100$ (nM) against the cells.

(Test Example 11) Antitumor test (1)

[0740] Mouse: 5- to 6-week-old female BALB/c nude mice (Charles River Laboratories Japan, Inc.) were acclimatized for 4 to 7 days under SPF conditions before use in the experiment. The mice were fed with sterilized solid feed (FR-2, Funabashi Farms Co., Ltd) and given sterilized tap water (prepared by the addition of 5 to 15 ppm sodium hypochlorite solution).

[0741] Assay and calculation expression: In all studies, the major axis and minor axis of tumor were measured twice a week by using an electronic digital caliper (CD-15C, Mitutoyo Corp.), and the tumor volume (mm^3) was calculated. The calculation expression is as shown below.

$$\text{Tumor volume (mm}^3\text{)} = 1/2 \times \text{Major axis (mm)} \times [\text{Minor axis (mm)}]^2$$

[0742] All of the antibody-drug conjugates were diluted with physiological saline (Otsuka Pharmaceutical Factory, Inc.) and used at a volume of 10 mL/kg for intravenous administration to the tail of each mouse. Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 8×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 11. The M30-H1-L4P antibody and antibody-drug conjugate (2) were each intravenously administered at a dose of 10 mg/kg to the tail of each mouse at Days 11, 18, and 25.

[0743] The results are shown in Figure 17. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with open triangles depicts the effect of the M30-H1-L4P antibody, and the line with open circles depicts the effect of the antibody-drug conjugate (2).

[0744] As seen from these results, the administration of the antibody-drug conjugate (2) remarkably decreased the tumor volume, and no further tumor growth was observed after the final administration. By contrast, the administration of the M30-H1-L4P antibody resulted in the progression of tumor growth.

[0745] In addition, the mice that received the antibody-drug conjugate (2) were free from notable signs such as weight loss, suggesting that the antibody-drug conjugate (2) is low toxic and highly safe.

(Test Example 12) Antitumor test (2)

[0746] Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 6×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude

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mouse (Day 0), and the mice were randomly grouped at Day 18. The antibody-drug conjugate (2) was intravenously administered at each dose (0.1, 0.3, and 1.3 mg/kg) to the tail of each mouse at Days 18, 25, and 32 in a schedule of $qw \times 3$. [0747] The results are shown in Figure 18. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with filled squares depicts the effect of the antibody-drug conjugate (2) administered at 0.1 mg/kg, the line with X marks depicts the effect of the antibody-drug conjugate (2) administered at 0.3 mg/kg, the line with filled triangles depicts the effect of the antibody-drug conjugate (2) administered at 1 mg/kg, and the line with open circles depicts the effect of the antibody-drug conjugate (2) administered at 3 mg/kg. The antibody-drug conjugate (2) was effective for shrinking tumor in a dose-dependent manner.

(Test Example 13) Antitumor test (3)

[0748] Human non-small cell lung cancer line Calu-6 cells were purchased from ATCC (American Type Culture Collection). 5×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 11. The M30-H1-L4P antibody and antibody-drug conjugate (2) were each intravenously administered at a dose of 10 mg/kg to the tail of each mouse at Days 11, 18, and 25 in a schedule of $qw \times 3$.

[0749] The results are shown in Figure 19. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with open triangles depicts the effect of the M30-H1-L4P antibody, and the line with open circles depicts the effect of the antibody-drug conjugate (2). The administration of the antibody-drug conjugate (2) remarkably decreased the tumor volume, and no further tumor growth was observed after the final administration. By contrast, the administration of the M30-H1-L4P antibody resulted in the progression of tumor growth.

[0750] In addition, the mice that received the antibody-drug conjugate (2) were free from notable signs such as weight loss, suggesting that the antibody-drug conjugate (2) is low toxic and highly safe.

(Test Example 14) Antitumor test (4)

[0751] Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 8×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 21. The antibody-drug conjugates (1), (13), (41), and (55) were each intravenously administered at a dose of 10 mg/kg to the tail of each mouse at Day 21.

[0752] The results are shown in Figure 20. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with open circles depicts the effect of the administered antibody-drug conjugate (1), the line with open triangles depicts the effect of the administered antibody-drug conjugate (13), the line with X marks depicts the effect of the administered antibody-drug conjugate (41), and the line with open squares depicts the effect of the administered antibody-drug conjugate (55). The administration of the antibody-drug conjugate (1), (13), (41), or (55) remarkably decreased the tumor volume, and all of these antibody-drug conjugates exerted a tumor growth inhibitory effect.

[0753] In addition, the mice that received the antibody-drug conjugate (1), (13), (41), or (55) were free from notable signs such as weight loss, suggesting that the antibody-drug conjugates (1), (13), (41), and (55) are low toxic and highly safe.

(Test Example 15) Antitumor test (5)

[0754] Human non-small cell lung cancer line Calu-6 cells were purchased from ATCC (American Type Culture Collection). 5×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 12. The antibody-drug conjugates (13), (41), and (55) were each intravenously administered at a dose of 10 mg/kg to the tail of each mouse at Day 12. As a comparative control, DE-310 was intravenously administered at a dose of 0.1 mg/kg to the tail of each mouse at Day 12. Here, the aforementioned dose of the antibody-drug conjugate was based on the amount of the antibody in the conjugate and the aforementioned dose of DE-310 was based on the amount of the drug contained therein. In this respect, the amounts of the drugs respectively contained in the antibody-drug conjugate and DE-310 were about 1 : 100. This means that the doses of the antibody-drug conjugate and DE-310 were equal in terms of the amounts of the drugs contained therein.

[0755] The results are shown in Figure 21. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with open circles depicts the effect of DE-310, the line with open triangles depicts the effect of the antibody-drug conjugate (13), the line with X marks depicts the effect of the antibody-drug conjugate (41), and the line with open squares depicts the effect of the antibody-drug conjugate (55). The administration of the antibody-drug conjugate (13), (41), or (55) remarkably decreased the tumor volume, whereas the administration of DE-310 exhibited no reduction in tumor volume.

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[0756] In addition, the mice that received the antibody-drug conjugate (13), (41), or (55) were free from notable signs such as weight loss, suggesting that these antibody-drug conjugates are low toxic and highly safe.

(Test Example 16) Antitumor test (6)

5

[0757] Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 1×10^7 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 11. The antibody-drug conjugates (17), (18), (19), (59), (60), and (61) were each intravenously administered at a dose of 3 mg/kg to the tail of each mouse at Days 11 and 18 in a schedule of $qw \times 2$.

10

[0758] The results are shown in Figure 22. In the drawing, the line with filled rhombuses depicts the results about untreated tumor, the line with filled squares depicts the effect of the administered antibody-drug conjugate (17), the line with open triangles depicts the effect of the administered antibody-drug conjugate (18), the line with open circles depicts the effect of the administered antibody-drug conjugate (19), the line with filled triangles depicts the effect of the administered antibody-drug conjugate (59), the line with open squares depicts the effect of the administered antibody-drug conjugate (60), and the line with X marks depicts the effect of the administered antibody-drug conjugate (61).

15

[0759] The administration of the antibody-drug conjugate (17), (18), (19), (59), (60), or (61) remarkably decreased the tumor volume, and all of these antibody-drug conjugates exerted a tumor growth inhibitory effect.

20

[0760] In addition, the mice that received the antibody-drug conjugate (17), (18), (19), (59), (60), or (61) were free from notable signs such as weight loss, suggesting that these antibody-drug conjugates are low toxic and highly safe.

Free Text of Sequence Listing

[0761]

25

SEQ ID NO: 1 - Amino acid sequence of the B7-H3 variant 1
 SEQ ID NO: 2 - Amino acid sequence of the B7-H3 variant 2
 SEQ ID NO: 3 - Amino acid sequence of CDRH1 of the M30 antibody
 SEQ ID NO: 4 - Amino acid sequence of CDRH2 of the M30 antibody
 SEQ ID NO: 5 - Amino acid sequence of CDRH3 of the M30 antibody
 SEQ ID NO: 6 - Amino acid sequence of CDRL1 of the M30 antibody
 SEQ ID NO: 7 - Amino acid sequence of CDRL2 of the M30 antibody
 SEQ ID NO: 8 - Amino acid sequence of CDRL3 of the M30 antibody
 SEQ ID NO: 9 - Amino acid sequence of the M30-H1-type heavy chain
 SEQ ID NO: 10 - Amino acid sequence of the M30-H2-type heavy chain
 SEQ ID NO: 11 - Amino acid sequence of the M30-H3-type heavy chain
 SEQ ID NO: 12 - Amino acid sequence of the M30-H4-type heavy chain
 SEQ ID NO: 13 - Amino acid sequence of the M30-L1-type light chain
 SEQ ID NO: 14 - Amino acid sequence of the M30-L2-type light chain
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 SEQ ID NO: 17 - Amino acid sequence of the M30-L5-type light chain
 SEQ ID NO: 18 - Amino acid sequence of the M30-L6-type light chain
 SEQ ID NO: 19 - Amino acid sequence of the M30-L7-type light chain
 SEQ ID NO: 20 - Amino acid sequence of a heavy chain of the M30 antibody
 SEQ ID NO: 21 - Amino acid sequence of a light chain of the M30 antibody
 SEQ ID NO: 22 - PCR primer 1
 SEQ ID NO: 23 - PCR primer 2
 SEQ ID NO: 24 - CMV promoter primer: primer 3
 SEQ ID NO: 25 - BGH reverse primer: primer 4
 SEQ ID NO: 26 - Nucleotide sequence of the B7-H3 variant 1
 SEQ ID NO: 27 - Amino acid sequence of a heavy chain of the anti-CD30 antibody
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5	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	
	210						215					220					
	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Arg	Val	Glu	
	225					230					235					240	
10																	
	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	
					245					250					255		
15	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	
				260					265					270			
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	
20			275					280					285				
	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	
	290						295					300					
25																	
	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
	305					310					315					320	
30	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
					325					330					335		
	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
35				340					345					350			
	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
			355					360					365				
40																	
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	
	370						375					380					
45	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	
	385					390					395					400	
	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	
50					405					410					415		
	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	
					420				425					430			
55	Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	
			435					440					445				

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Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
 450 455 460

5 Leu Ser Leu Ser Pro Gly Lys
 465 470

<210> 11
 <211> 471
 10 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Humanized heavy chain of M30

15 <400> 11

Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
 1 5 10 15

20 Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
 20 25 30

25 Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
 35 40 45

Thr Asn Tyr Val Met His Trp Val Lys Gln Ala Pro Gly Gln Gly Leu
 50 55 60

30 Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Asp Val Lys Tyr Asn
 65 70 75 80

35 Glu Lys Phe Lys Gly Lys Ala Thr Ile Thr Ala Asp Glu Ser Thr Ser
 85 90 95

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
 100 105 110

40 Tyr Tyr Cys Ala Arg Trp Gly Tyr Tyr Gly Ser Pro Leu Tyr Tyr Phe
 115 120 125

45 Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
 130 135 140

50 Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
 145 150 155 160

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu
 165 170 175

55 Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His

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	180		185		190
5	Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser	195	200	205	
10	Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys	210	215	220	
15	Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu	225	230	235	240
20	Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro	245	250	255	
25	Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys	260	265	270	
30	Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val	275	280	285	
35	Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp	290	295	300	
40	Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr	305	310	315	320
45	Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp	325	330	335	
50	Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu	340	345	350	
55	Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg	355	360	365	
60	Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys	370	375	380	
65	Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp	385	390	395	400
70	Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys	405	410	415	
75	Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser	420	425	430	

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Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
 435 440 445

5 Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
 450 455 460

Leu Ser Leu Ser Pro Gly Lys
 465 470

10 <210> 12
 <211> 471
 <212> PRT
 <213> Artificial Sequence

15 <220>
 <223> Humanized heavy chain of M30

20 <400> 12

Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
 1 5 10 15

25 Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
 20 25 30

Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
 35 40 45

30 Thr Asn Tyr Val Met His Trp Val Lys Gln Ala Pro Gly Gln Gly Leu
 50 55 60

35 Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Asp Val Lys Tyr Asn
 65 70 75 80

40 Glu Lys Phe Lys Gly Lys Ala Thr Gln Thr Ser Asp Lys Ser Thr Ser
 85 90 95

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
 100 105 110

45 Tyr Tyr Cys Ala Arg Trp Gly Tyr Tyr Gly Ser Pro Leu Tyr Tyr Phe
 115 120 125

50 Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
 130 135 140

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
 145 150 155 160

55 Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu

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	165	170	175
5	Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His 180 185 190		
10	Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser 195 200 205		
15	Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys 210 215 220		
20	Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu 225 230 235 240		
25	Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro 245 250 255		
30	Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys 260 265 270		
35	Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val 275 280 285		
40	Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp 290 295 300		
45	Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr 305 310 315 320		
50	Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp 325 330 335		
55	Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu 340 345 350		
	Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg 355 360 365		
	Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys 370 375 380		
	Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp 385 390 395 400		
	Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys 405 410 415		

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Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
 420 425 430
 5 Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
 435 440 445
 10 Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
 450 455 460
 Leu Ser Leu Ser Pro Gly Lys
 465 470
 15 <210> 13
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 <212> PRT
 <213> Artificial Sequence
 20 <220>
 <223> Humanized light chain of M30
 <400> 13
 25 Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15
 Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 20 25 30
 30 Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 35 40 45
 35 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 55 60
 40 Leu Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80
 Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg
 85 90 95
 45 Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110
 50 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125
 Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140
 55 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro

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145 150 155 160
 Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 5 165 170 175
 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 10 180 185 190
 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 15 195 200 205
 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220
 Thr Lys Ser Phe Asn Arg Gly Glu Cys
 20 225 230
 <210> 14
 <211> 233
 <212> PRT
 25 <213> Artificial Sequence
 <220>
 <223> Humanized light chain of M30
 <400> 14
 30 Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15
 Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 35 20 25 30
 Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 40 35 40 45
 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 50 55 60
 Leu Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 45 65 70 75 80
 Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Arg
 50 85 90 95
 Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110
 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 55 115 120 125

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Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
130 135 140

5 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
145 150 155 160

10 Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
165 170 175

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
180 185 190

15 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
195 200 205

20 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
210 215 220

25 Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230

<210> 15
<211> 233
30 <212> PRT
<213> Artificial Sequence

<220>
<223> Humanized light chain of M30

35 <400> 15

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
1 5 10 15

40 Gly Ala Tyr Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Thr Leu Ser
20 25 30

45 Leu Ser Pro Gly Glu Arg Ala Thr Leu Thr Cys Arg Ala Ser Ser Arg
35 40 45

Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ala Pro Lys
50 55 60

Leu Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
65 70 75 80

55 Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Thr Leu Thr Ile Ser Arg
85 90 95

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Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

5 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125

10 Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140

15 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160

Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175

20 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190

25 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205

30 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220

35 Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230

<210> 16
 <211> 233
 <212> PRT
 <213> Artificial Sequence

40 <220>
 <223> Humanized light chain of M30
 <400> 16

45 Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15

50 Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 35 40 45

55 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 55 60

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Pro Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80

5 Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser
 85 90 95

10 Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125

15 Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140

20 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160

Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175

25 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190

30 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205

35 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220

Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230

40 <210> 17
 <211> 233
 <212> PRT
 <213> Artificial Sequence

45 <220>
 <223> Humanized light chain of M30
 <400> 17

50 Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15

Gly Ala Tyr Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Thr Leu Ser
 20 25 30

55 Leu Ser Pro Gly Glu Arg Ala Thr Leu Thr Cys Arg Ala Ser Ser Arg

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	35	40	45
5	Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ala Pro Lys 50 55 60		
10	Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg 65 70 75 80		
15	Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Thr Leu Thr Ile Ser Arg 85 90 95		
20	Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser 100 105 110		
25	Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr 115 120 125		
30	Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu 130 135 140		
35	Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro 145 150 155 160		
40	Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly 165 170 175		
45	Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr 180 185 190		
50	Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His 195 200 205		
55	Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val 210 215 220		
	Thr Lys Ser Phe Asn Arg Gly Glu Cys 225 230		
	<210> 18 <211> 233 <212> PRT <213> Artificial Sequence		
	<220> <223> Humanized light chain of M30		
	<400> 18		
	Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser 1 5 10 15		

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Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 20 25 30

5 Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 35 40 45

10 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 55 60

Pro Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80

15 Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg
 85 90 95

20 Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

25 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125

Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140

30 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160

35 Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175

40 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190

Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205

45 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220

50 Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230

55 <210> 19
 <211> 233
 <212> PRT
 <213> Artificial Sequence

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<220>

<223> Humanized light chain of M30

<400> 19

5

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
1 5 10 15

10

Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
35 40 45

15

Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
50 55 60

20

Pro Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
65 70 75 80

25

Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Arg
85 90 95

Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
100 105 110

30

Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
115 120 125

35

Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
130 135 140

Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
145 150 155 160

40

Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
165 170 175

45

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
180 185 190

50

Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
195 200 205

Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
210 215 220

55

Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230

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<210> 20
 <211> 471
 <212> PRT
 <213> Mus musculus

5

<400> 20

10

Met Glu Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
 1 5 10 15

Val His Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
 20 25 30

15

Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe
 35 40 45

20

Thr Asn Tyr Val Met His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu
 50 55 60

Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Asp Val Lys Tyr Asn
 65 70 75 80

25

Glu Lys Phe Lys Gly Lys Ala Thr Gln Thr Ser Asp Lys Ser Ser Ser
 85 90 95

30

Thr Ala Tyr Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
 100 105 110

35

Tyr Tyr Cys Ala Arg Trp Gly Tyr Tyr Gly Ser Pro Leu Tyr Tyr Phe
 115 120 125

Asp Tyr Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser Ala Lys Thr
 130 135 140

40

Thr Ala Pro Ser Val Tyr Pro Leu Ala Pro Val Cys Gly Asp Thr Thr
 145 150 155 160

45

Gly Ser Ser Val Thr Leu Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu
 165 170 175

50

Pro Val Thr Leu Thr Trp Asn Ser Gly Ser Leu Ser Ser Gly Val His
 180 185 190

Thr Phe Pro Ala Val Leu Gln Ser Asp Leu Tyr Thr Leu Ser Ser Ser
 195 200 205

55

Val Thr Val Thr Ser Ser Thr Trp Pro Ser Gln Ser Ile Thr Cys Asn
 210 215 220

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	Val	Ala	His	Pro	Ala	Ser	Ser	Thr	Lys	Val	Asp	Lys	Lys	Ile	Glu	Pro
	225					230					235					240
5	Arg	Gly	Pro	Thr	Ile	Lys	Pro	Cys	Pro	Pro	Cys	Lys	Cys	Pro	Ala	Pro
					245					250					255	
10	Asn	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Lys	Ile	Lys
				260					265					270		
15	Asp	Val	Leu	Met	Ile	Ser	Leu	Ser	Pro	Ile	Val	Thr	Cys	Val	Val	Val
			275					280					285			
20	Asp	Val	Ser	Glu	Asp	Asp	Pro	Asp	Val	Gln	Ile	Ser	Trp	Phe	Val	Asn
			290				295					300				
25	Asn	Val	Glu	Val	His	Thr	Ala	Gln	Thr	Gln	Thr	His	Arg	Glu	Asp	Tyr
						310					315					320
30	Asn	Ser	Thr	Leu	Arg	Val	Val	Ser	Ala	Leu	Pro	Ile	Gln	His	Gln	Asp
					325					330					335	
35	Trp	Met	Ser	Gly	Lys	Glu	Phe	Lys	Cys	Lys	Val	Asn	Asn	Lys	Asp	Leu
				340					345					350		
40	Pro	Ala	Pro	Ile	Glu	Arg	Thr	Ile	Ser	Lys	Pro	Lys	Gly	Ser	Val	Arg
			355					360					365			
45	Ala	Pro	Gln	Val	Tyr	Val	Leu	Pro	Pro	Pro	Glu	Glu	Glu	Met	Thr	Lys
		370					375					380				
50	Lys	Gln	Val	Thr	Leu	Thr	Cys	Met	Val	Thr	Asp	Phe	Met	Pro	Glu	Asp
	385					390					395					400
55	Ile	Tyr	Val	Glu	Trp	Thr	Asn	Asn	Gly	Lys	Thr	Glu	Leu	Asn	Tyr	Lys
					405					410					415	
60	Asn	Thr	Glu	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Tyr	Phe	Met	Tyr	Ser
				420					425					430		
65	Lys	Leu	Arg	Val	Glu	Lys	Lys	Asn	Trp	Val	Glu	Arg	Asn	Ser	Tyr	Ser
			435					440					445			
70	Cys	Ser	Val	Val	His	Glu	Gly	Leu	His	Asn	His	His	Thr	Thr	Lys	Ser
							455					460				
75	Phe	Ser	Arg	Thr	Pro	Gly	Lys									

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465

470

5 <210> 21
 <211> 235
 <212> PRT
 <213> Mus musculus

 <400> 21

 10 Met Asp Phe Leu Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
 1 5 10 15

 15 Val Ile Met Ser Arg Gly Gln Ile Val Leu Ser Gln Ser Pro Thr Ile
 20 25 30

 Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala Ser
 35 40 45

 20 Ser Arg Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ser
 50 55 60

 25 Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro
 65 70 75 80

 Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
 85 90 95

 30 Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp
 100 105 110

 35 Asn Ser Asn Pro Pro Thr Phe Gly Thr Gly Thr Lys Leu Glu Leu Lys
 115 120 125

 Arg Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu
 130 135 140

 40 Gln Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe
 145 150 155 160

 45 Tyr Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg
 165 170 175

 50 Gln Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser
 180 185 190

 Thr Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu
 195 200 205

 55 Arg His Asn Ser Tyr Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser

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	210	215	220	
5	Pro Ile Val Lys Ser Phe Asn Arg Asn Glu Cys			
	225	230	235	
	<210> 22			
	<211> 46			
	<212> DNA			
10	<213> Artificial Sequence			
	<220>			
	<223> PCR primer			
15	<400> 22			
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	<210> 23			
	<211> 66			
20	<212> DNA			
	<213> Artificial Sequence			
	<220>			
	<223> PCR primer			
25	<400> 23			
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	tgtcag			66
30	<210> 24			
	<211> 21			
	<212> DNA			
	<213> Artificial Sequence			
35	<220>			
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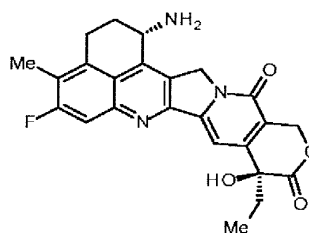
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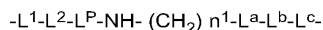
Claims

1. An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 1]



is conjugated to an antibody via a linker having a structure represented by the following formula:



wherein the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c with the nitrogen atom of the amino group at position 1 as the connecting position,

wherein

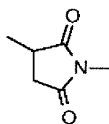
n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$, $-CH_2-C(=O)-NH-(CH_2)_{n^3}-C(=O)-$, $-C(=O)-\text{cyc.Hex}(1,4)-CH_2-(N\text{-ly-3-dimincuccuS})-$, or $-C(=O)-(CH_2)_{n^4}-C(=O)-$,

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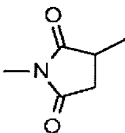
wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, L^2 represents $-NH-(CH_2-CH_2-O)n^5-CH_2-CH_2-C(=O)-$, $-S-(CH_2)n^6-C(=O)-$, or a single bond, wherein n^5 represents an integer of 1 to 6, n^6 represents an integer of 1 to 6, L^P represents a peptide residue consisting of 2 to 7 amino acids, L^a represents $-C(=O)-NH-$, $-NR^1-(CH_2)n^7-$, $-O-$, or a single bond, wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)n^8-COOH$, or $-(CH_2)n^9-OH$, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6, L^b represents $-CR^2(-R^3)-$, $-O-$, $-NR^4-$, or a single bond, wherein R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)n^a-NH_2$, $-(CH_2)n^b-COOH$, or $-(CH_2)n^c-OH$, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other, L^c represents $-CH_2-$ or $-C(=O)-$, $-(Succinimid-3-yl-N)-$ has a structure represented by the following formula:

[Formula 2]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, $-(N-ly-3-diminiccuS)-$ has a structure represented by the following formula:

[Formula 3]



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and when L^2 is $-S-(CH_2)n^6-C(=O)-$, L^1 is $-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-$.

2. The antibody-drug conjugate according to claim 1, wherein the peptide residue of L^P is an amino acid residue comprising an amino acid selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid.

3. The antibody-drug conjugate according to claim 1 or 2, wherein

L^P represents a tetrapeptide residue,

L^a represents $-O-$ or a single bond,

L^b represents $-CR^2(-R^3)-$ or a single bond,

wherein R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)n^a-NH_2$, $-(CH_2)n^b-COOH$, or $-(CH_2)n^c-OH$, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other, and

L^c represents $-C(=O)-$.

4. The antibody-drug conjugate according to any one of claims 1 to 3, wherein

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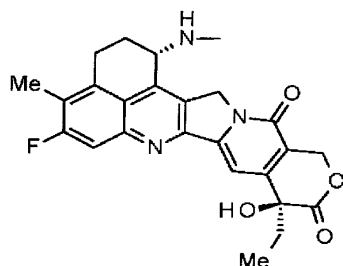
L^P represents a tetrapeptide residue of GGFG,
 L^a represents -O- or a single bond,
 L^b represents -CR²(-R³)- or a single bond,
 wherein R² and R³ each represents a hydrogen atom,
 and
 L^c represents -C(=O)-.

- 5 5. The antibody-drug conjugate according to any one of claims 1 to 4, wherein L¹ is -(Succinimid-3-yl-N)-(CH₂)^{n²}-C(=O)- or -CH₂-C(=O)-NH-(CH₂)^{n³}-C(=O)-.
- 10 6. The antibody-drug conjugate according to any one of claims 1 to 4, wherein L¹ is -(Succinimid-3-yl-N)-(CH₂)^{n²}-C(=O)-.
- 15 7. The antibody-drug conjugate according to any one of claims 1 to 4, wherein L¹ is -C((=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-dimincucS)- or -C(=O)-(CH₂)^{n⁴}-C(=O)-.
- 20 8. The antibody-drug conjugate according to any one of claims 1 to 6, wherein n² is an integer of 2 to 5, and L² is a single bond.
- 25 9. The antibody-drug conjugate according to any one of claims 1 to 6, wherein n² is an integer of 2 to 5, L² is -NH-(CH₂CH₂O)^{n⁵}-CH₂-CH₂-C(=O)-, and n⁵ is 2 or 4.
- 30 10. The antibody-drug conjugate according to any one of claims 1 to 9, wherein the following formula:
 -NH-(CH₂)^{n¹}-L^a-L^b-L^c- is a partial structure having a chain length of 4 to 7 atoms.
- 35 11. The antibody-drug conjugate according to any one of claims 1 to 9, wherein the following formula:
 -NH-(CH₂)^{n¹}-L^a-L^b-L^c- is a partial structure having a chain length of 5 or 6 atoms.
- 40 12. The antibody-drug conjugate according to any one of claims 1 to 11, wherein the following formula:
 -NH-(CH₂)^{n¹}-L^a-L^b-L^c- is
 -NH-(CH₂)₃-C(=O)-,
 -NH-CH₂-O-CH₂-C(=O)-, or
 -NH-(CH₂)₂-O-CH₂-C(=O)-.
- 45 13. The antibody-drug conjugate according to any one of claims 1 to 12, wherein the structure of the linker is one structure selected from the following group:
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-dimincucS)-S-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)

wherein -(NH-DX) represents a group represented by the following formula:

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[Formula 4]



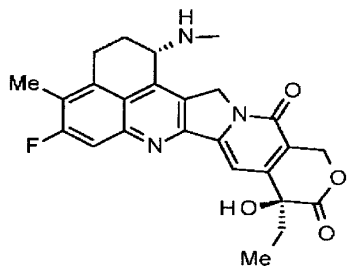
wherein the nitrogen atom of the amino group at position 1 is the connectig position, and
-GGFG- represents a peptide residue of -Gly-Gly-Phe-Gly-.

14. The antibody-drug conjugate according to any one of claims 1 to 6 and 8 to 11, wherein the structure of the linker is one structure selected from the following group:

-(Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 -(Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-
 CH₂CH₂CH₂-C(=O)-(NH-DX)

wherein -(NH-DX) represents a group represented by the following formula:

[Formula 5]

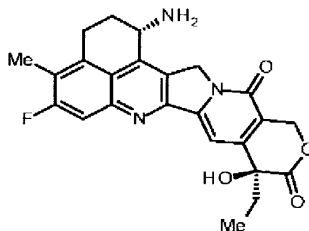


wherein the nitrogen atom of the amino group at position 1 is the connecting position.

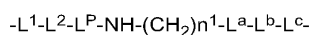
15. An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

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[Formula 6]



is conjugated to an antibody via a linker having a structure represented by the following formula:



wherein the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c , wherein

n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$ and is connected to the antibody via a thioether bond which is formed at a disulfide bond moiety present in a hinge part of the antibody,

wherein n^2 represents an integer of 2 to 8,

L^2 represents $-NH-(CH_2-CH_2-O)_{n^5}-CH_2-CH_2-C(=O)-$ or a single bond,

wherein n^5 represents an integer of 1 to 6,

L^P represents a tetrapeptide residue of GGFG,

L^a represents $-O-$ or a single bond,

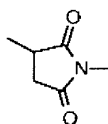
L^b represents $-CR^2(-R^3)-$ or a single bond,

wherein R^2 and R^3 each represents a hydrogen atom,

L^c represents $-C(=O)-$, and

$-(\text{Succinimid-3-yl-N})-$ has a structure represented by the following formula:

[Formula 7]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1.

16. The antibody-drug conjugate according to claim 15, wherein

n^2 is 2, L^2 is $-NH-(CH_2-CH_2-O)_{n^5}-CH_2-CH_2-C(=O)-$, n^5 is 2, n^1 is 3, and both of L^a and L^b are single bonds,

n^2 is 5, L^2 is a single bond, n^1 is 1, L^a is $-O-$, and L^b is $-CR^2(-R^3)-$, or

n^2 is 5, L^2 is a single bond, n^1 is 2, L^a is $-O-$, and L^b is $-CR^2(-R^3)-$.

17. The antibody-drug conjugate according to claim 15 or 16, wherein n^2 is an integer of 2 to 5, and L^2 is a single bond.

18. The antibody-drug conjugate according to claim 15 or 16, wherein n^2 is an integer of 2 to 5, L^2 is $-NH-(CH_2CH_2O)_{n^5}-CH_2-CH_2-C(=O)-$, and n^5 is 2 or 4.

19. The antibody-drug conjugate according to any one of claims 15 to 18, wherein the following formula: $-NH-(CH_2)_{n^1}-L^a-L^b-L^c-$ is

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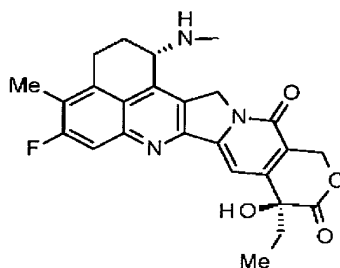
-NH-(CH₂)₃-C(=O)-,
 -NH-CH₂-O-CH₂-C(=O)-, or
 -NH-(CH₂)₂-O-CH₂-C(=O)-.

- 5 **20.** The antibody-drug conjugate according to any one of claims 15 to 19, wherein the structure of the linker is one structure selected from the following group:

10 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 15 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 20 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)

wherein -(NH-DX) represents a group represented by the following formula:

[Formula 8]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

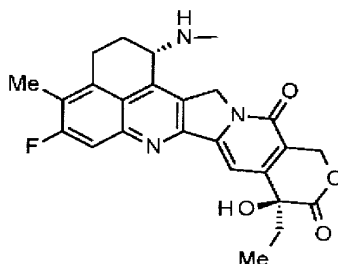
21. The antibody-drug conjugate according to any one of claims 15 to 19, wherein the structure of the linker is one structure selected from the following group:

45 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 50

wherein -(NH-DX) represents a group represented by the following formula:

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[Formula 9]



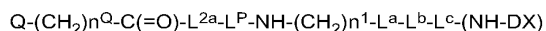
wherein the nitrogen atom of the amino group at position 1 is a connecting position.

22. The antibody-drug conjugate according to any one of claims 1 to 21, wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 1 to 10.
23. The antibody-drug conjugate according to any one of claims 1 to 21, wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 2 to 8.
24. The antibody-drug conjugate according to any one of claims 1 to 21, wherein an average number of units of the selected one drug-linker structure conjugated per antibody is in a range of from 3 to 8.
25. The antibody-drug conjugate according to any one of claims 1 to 24, wherein the antibody is an antibody having one or more of a property of recognizing a target cell, a property of binding to a target cell, a property of internalizing in a target cell, and a property of damaging a target cell.
26. The antibody-drug conjugate according to any one of claims 1 to 24, wherein a cell which is targeted by the antibody-drug conjugate is a tumor cell.
27. The antibody-drug conjugate according to any one of claims 1 to 24, wherein the antibody is an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, or an anti-mesothelin antibody.
28. The antibody-drug conjugate according to any one of claims 1 to 24, wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.
29. The antibody-drug conjugate according to any one of claims 1 to 24, wherein the antibody is an anti-B7-H3 antibody.
30. A drug containing the antibody-drug conjugate according to any one of claims 1 to 29 or a salt thereof.
31. An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to any one of claims 1 to 29 or a salt thereof.
32. The antitumor drug and/or anticancer drug according to claim 31, which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.
33. A pharmaceutical composition containing the antibody-drug conjugate according to any one of claims 1 to 29 or a salt thereof as an active component, and a pharmaceutically acceptable formulation component.

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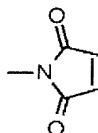
34. A method for treating tumor and/or cancer comprising administering the antibody-drug conjugate according to any one of claims 1 to 29 or a salt thereof.

35. A drug-linker intermediate compound represented by the following formula:



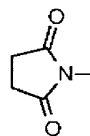
wherein Q represents (maleimid-N-yl)-, HS-, X-CH₂-C(=O)-NH-, or (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, X represents a bromine atom or an iodine atom,
 n^Q represents an integer of 2 to 8,
 L^{2a} represents -NH-(CH₂-CH₂-O) n^5 -CH₂-CH₂-C(=O)- or a single bond,
 wherein n^5 represents an integer of 1 to 6,
 L^P represents a peptide residue consisting of 2 to 7 amino acids selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid,
 n^1 represents an integer of 0 to 6,
 L^a represents -C(=O)-NH-, -NR¹-(CH₂) n^7 -, -O-, or a single bond,
 wherein n^7 represents an integer of 1 to 6, R¹ represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂) n^8 -COOH, or -(CH₂) n^9 -OH, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,
 L^b represents -CR²(-R³)-, -O-, -NR⁴-, or a single bond,
 wherein R² and R³ each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂) n^a -NH₂, -(CH₂) n^b -COOH, or -(CH₂) n^c -OH, R⁴ represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R² and R³ are not the same as each other,
 L^c represents -CH₂- or -C(=O)-,
 (maleimid-N-yl)- is a group represented by the following formula:

[Formula 10]



wherein the nitrogen atom is a connecting position, (Pyrrolidine-2,5-dione-N-yl) is a group represented by the following formula:

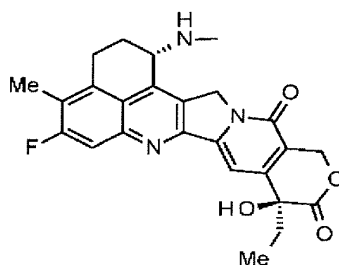
[Formula 11]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

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[Formula 12]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

36. The drug-linker intermediate compound according to claim 35, wherein L^c is $-C(=O)-$.

37. The drug-linker intermediate compound according to claim 35 or 36, wherein L^P is a peptide residue consisting of 4 amino acids.

38. The drug-linker intermediate compound according to any one of claims 35 to 37, wherein L^P is $-GGFG-$.

39. The drug-linker intermediate compound according to any one of claims 35 to 38, wherein the following formula:
 $-NH-(CH_2)_n-L^a-L^b-$ is

$-NH-CH_2CH_2-$,
 $-NH-CH_2CH_2CH_2-$,
 $-NH-CH_2CH_2CH_2CH_2-$,
 $-NH-CH_2CH_2CH_2CH_2CH_2-$,
 $-NH-CH_2-O-CH_2-$, or
 $-NH-CH_2CH_2-O-CH_2-$.

40. The drug-linker intermediate compound according to any one of claims 35 to 38, wherein the following formula:
 $-NH-(CH_2)_n-L^a-L^b-$ is

$-NH-CH_2CH_2CH_2-$,
 $-NH-CH_2-O-CH_2-$, or
 $-NH-(CH_2)_2-O-CH_2-$.

41. The drug-linker intermediate compound according to any one of claims 35 to 40, wherein n^Q is an integer of 2 to 6.

42. The drug-linker intermediate compound according to claim 39, wherein

Q is (maleimid-N-yl)-,
 n^Q is an integer of 2 to 5, and
 L^{2a} is a single bond.

43. The drug-linker intermediate compound according to claim 40, wherein

Q is (maleimid-N-yl)-,
 n^Q is an integer of 2 to 5, and
 L^{2a} is a single bond.

44. The drug-linker intermediate compound according to any one of claims 35 to 38, wherein

Q is (maleimid-N-yl)-,

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45. The drug-linker intermediate compound according to claim 44, wherein

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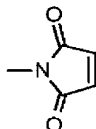
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[illegible]

wherein (maleimid-N-yl)- is a group represented by the following formula:

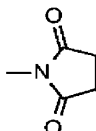
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[Formula 13]



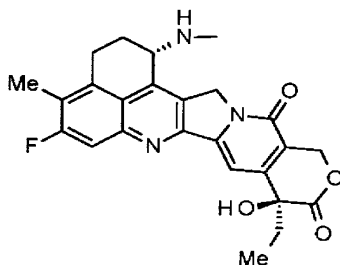
wherein the nitrogen atom is a connecting position, X represents a halogen atom, (Pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

[Formula 14]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 15]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

47. A compound of the following:

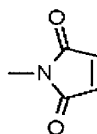
(maleimid-N-yl)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)

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(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-
 CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-
 CH₂CH₂CH₂-C(=O)-(NH-DX), or
 (maleimid-N-yl)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-
 CH₂CH₂CH₂-C(=O)-(NH-DX)

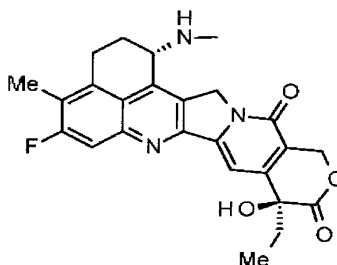
wherein (maleimid-N-yl)- is a group represented by the following formula:

[Formula 16]



wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 17]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

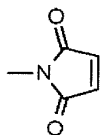
48. A compound of the following:

(maleimid-N-yl)-CH₂CH₂-C(=O)-NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-GGFG-NH-
 CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX) or
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),

wherein (maleimid-N-yl)- is a group represented by the following formula:

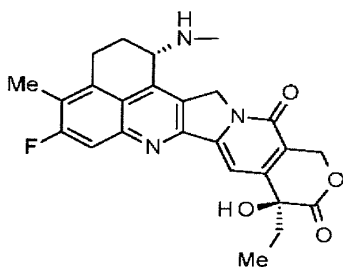
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[Formula 18]



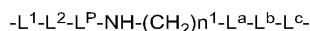
wherein the nitrogen atom is a connecting position, and -(NH-DX) is a group represented by the following formula:

[Formula 19]



wherein the nitrogen atom of the amino group at position 1 is a connecting position.

49. A linker represented by the following formula:



for obtaining an antibody-drug conjugate in which a drug is conjugated to an antibody via the linker, wherein L^1 is a connecting position for the antibody, L^c is a connecting position for an antitumor compound, wherein n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$, $-CH_2-C(=O)-NH-(CH_2)_{n^3}-C(=O)-$, $-C(=O)-\text{cyc.Hex}(1,4)-CH_2-(N\text{-ly-3-dimimiccuS})-$, or $-C(=O)-(CH_2)_{n^4}-C(=O)-$,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, L^2 represents $-NH-(CH_2-CH_2-O)_{n^5}-CH_2-CH_2-C(=O)-$, $-S-(CH_2)_{n^6}-C(=O)-$, or a single bond,

wherein n^5 represents an integer of 1 to 6, n^6 represents an integer of 1 to 6,

L^P represents a peptide residue consisting of 2 to 7 amino acids,

L^a represents $-C(=O)-NH-$, $-NR^1-(CH_2)_{n^7}-$, $-O-$, or a single bond,

wherein n^7 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)_{n^8}-COOH$, or $-(CH_2)_{n^9}-OH$, n^8 represents an integer of 1 to 4, n^9 represents an integer of 1 to 6,

L^b represents $-CR^2(-R^3)-$, $-O-$, $-NR^4-$, or a single bond,

wherein R^2 and R^3 each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)_{n^a}-NH_2$, $-(CH_2)_{n^b}-COOH$, or $-(CH_2)_{n^c}-OH$, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^a represents an integer of 0 to 6, n^b represents an integer of 1 to 4, n^c represents an integer of 1 to 4, provided that when n^a is 0, R^2 and R^3 are not the same as each other,

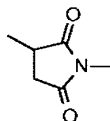
L^c represents $-CH_2-$ or $-C(=O)-$,

$-(\text{Succinimid-3-yl-N})-$ has a structure represented by the following formula:

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[Formula 20]

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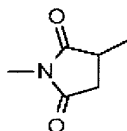
10

which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, -(N-ly-3-diminiccuS)- has a structure represented by the following formula:

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[Formula 21]

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which is connected to L² at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and when L² is -S-(CH₂)_n-C(=O)-, L¹ is -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-.

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Fig. 1

Amino acid sequence of B7-H3 variant 1 (SEQ ID NO: 1)

```
MLRRRGSPGMGVHVGAAALGALWFCLTGALEVQVPEDPVVALVGTD
ATLCCSFSPPEPGFSLAQLNLIWQLTDTKQLVHSFAEGQDQGSAYA
NRTALFPDLLAQGNASLRLQRVRVADEGSFTCFVSI RDFGSAAVS
LQVAAPYSKPSMTLEPNKDLRPGDVTITCSSYQGYPEAEVFWQD
GQGVPLTGNVTTSQMANEQGLFDVHSILRVVLGANGTYSCLVRNP
VLQQDAHSSVTITPQRSPTGAVEVQVPEDPVVALVGTDATLRCSF
SPEPGFSLAQLNLIWQLTDTKQLVHSFTEGRDQGSAYANRTALFP
DLLAQGNASLRLQRVRVADEGSFTCFVSI RDFGSAAVSLQVAAPY
SKPSMTLEPNKDLRPGDVTITCSSYRGYPEAEVFWQDGQGVPLT
GNVTTSQMANEQGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAH
GSVTITGQPMTFPPEALWVTVGLSVCLIALLLVALAFVCWRRIKQS
CEEENAGAEDQDGE GEGSKTALQPLKHSDSKEDDGQEIA
```

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Fig. 2

Amino acid sequence of B7-H3 variant 2 (SEQ ID NO: 2)

```
MLRRRGSPGMGVHVGAAALGALWFCLTGALEVQVPEDPVVALVGTD
ATLCCSFSPPEPGFSLAQLNLIWQLTDTKQLVHSFAEGQDQGSAYA
NRTALFPDLLAQGNASLRLQRVRVADEGSFTCFVSI RDFGSAAVS
LQVAAPYSKPSMTLEPNKDLRPGDTVTITCSSYRGYPEAEVFWQD
GQGVPLTGNTTSQMANEQGLFDVHSVLRVVLGANGTYSCLVRNP
VLQQDAHGSVTITGQPMTFPPEALWVTVGLSVCLIALLLVALAFVC
WRKIKQSCEENAGAEDQDGE GEGSKTALQPLKHSDSKEDDGQE I
A
```

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Fig. 3

Amino acid sequence of M30-H1-type heavy chain (SEQ ID NO: 9)

MKHLWFFLLLVAAPRWVLSQVQLVQSGAEVKKPGSSVKVSCKASG
YTFTNYVMHWVRQAPGQGLEWMGYINPYNDDVKYNEKFKGRVTIT
ADESTSTAYMELSSLRSEDTAVYYCARWGYGSPLYYFDYWGGGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT I
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
CS VMHEALHNHYTQKSLSLSPGK

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Fig. 4

Amino acid sequence of M30-H2-type heavy chain (SEQ ID NO: 10)

MKHLWFFLLLVAA PRWVLSQVQLVQSGAEVKKPGSSVKV SCKASG
YTFTNYVMHWVRQAPGQGLEWIGYINPYNDDVKYNEKFKGRVTIT
ADESTSTAYMELSSLRSEDTAVYYCARWGYGSPLYYFDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT I
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCS
VMHEALHNHYTQKSLSLSPGK

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Fig. 5

Amino acid sequence of M30-H3-type heavy chain (SEQ ID NO: 11)

MKHLWFFLLLVAAPRWVLSEVQLVQSGAEVKKPGSSVKVSKASG
YTFTNYVMHWVKQAPGQGLEWIGYINPYNDDVKYNEKFKGKATIT
ADESTSTAYMELSSLRSEDVAVYYCARWGYYGSPLYFDFYWGQGT
LTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
CSVMHEALHNHYTQKSLSLSPGK

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Fig. 6

Amino acid sequence of M30-H4-type heavy chain (SEQ ID NO: 12)

MKHLWFFLLLVAA PRWVLSEVQLVQSGAEVKKPGSSVKV SCKASG
YTFTNYVMHWVKQAPGQGLEWIGYINPYNDDVKYNEKFKGKATQT
SDKSTSTAYMELSSLRSEDTAVYYCARWGYYGSPLYYFDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT I
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFFLYSKLTVDKSRWQQGNVFS CS
VMHEALHNHYTQKSLSLSPGK

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Fig. 7

Amino acid sequence of M30-L1-type light chain (SEQ ID NO: 13)

MVLQTQVFISLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA
SSRLIYMHWYQQKPGQAPRLLIYATSNLASGIPARFSGSGSGTDF
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVT
KSFNRGEC

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Fig. 8

Amino acid sequence of M30-L2-type light chain (SEQ ID NO: 14)

```
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA  
SSRLIYMHWYQQKPGQAPRLWIYATSNLASGIPARFSGSGSGTDY  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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Fig. 9

Amino acid sequence of M30-L3-type light chain (SEQ ID NO: 15)

```
MVLQTQVFISLLLWISGAYGQIVLSQSPATLSLSPGERATLTCRA  
SSRLIYMHWYQQKPGSAPKLWIYATSNLASGIPARFSGSGSGTSY  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTEKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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Fig. 10

Amino acid sequence of M30-L4-type light chain (SEQ ID NO: 16)

```
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA  
SSRLIYMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDF  
TLTISSLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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Fig. 11

Amino acid sequence of M30-L5-type light chain (SEQ ID NO: 17)

```
MVLQTQVFISLLLWISGAYGQIVLSQSPATLSLSPGERATLTCRA  
SSRLIYMHWYQQKPGSAPKPWIIYATSNLASGIPARFSGSGSGTSY  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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Fig. 12

Amino acid sequence of M30-L6-type light chain (SEQ ID NO: 18)

```
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA  
SSRLIYMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDF  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```


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Fig. 13

Amino acid sequence of M30-L7-type light chain (SEQ ID NO: 19)

MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA
SSRLIYMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDY
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT
KSFNRGEC

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Fig. 14

Amino acid sequence of M30 antibody heavy chain (SEQ ID NO: 20)

```
MEWSWIFLFLLSGTAGVHSEVQLQQSGPELVKPGASVKMSCKASG  
YTFTNYVMHWVKQKPGQGLEWIGYINPYNDDVKYNEKFKGKATQT  
SDKSSSTAYMELSSLTSEDSAVYYCARWGYGSPLYYFDYWGGGT  
TLTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTL  
TWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNV  
AHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFI FPK  
IKDVLMI SLSPIVTCVVVDVSEDDPDVQISW FVNNVEVHTAQTQT  
HREDYNSTLRVVSALPIQH QDWMSGKEFKCKVNNKDLPAPIERTI  
SKPKGSVRAPQVYVLP PPEEEMTKKQVTLT CMVTDFMPEDIYVEW  
TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCS  
VVHEGLHNHHTTKSFSRTPGK
```

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Fig. 15

Amino acid sequence of M30 antibody light chain (SEQ ID NO: 21)

```
MDFLVQIFSFLISASVIMSRGQIVLSQSPTILSASPGEKVTMTC  
RASSRLIYMHYQQKPGSSPKPWYATSNLASGVPARFSGSGSGT  
SYSLTISRVEAEDAATYYCQQWNSNPPTFGTGTKLELKRADAAPT  
VSIFFPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGV  
LNSWTDQDSKDYSTYSMSSTLTLTKEYERHNSYTCEATHKTSTSP  
IVKSFNRNEC
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Fig. 16

Nucleotide sequence of B7-H3 variant 1 (SEQ ID NO: 26)

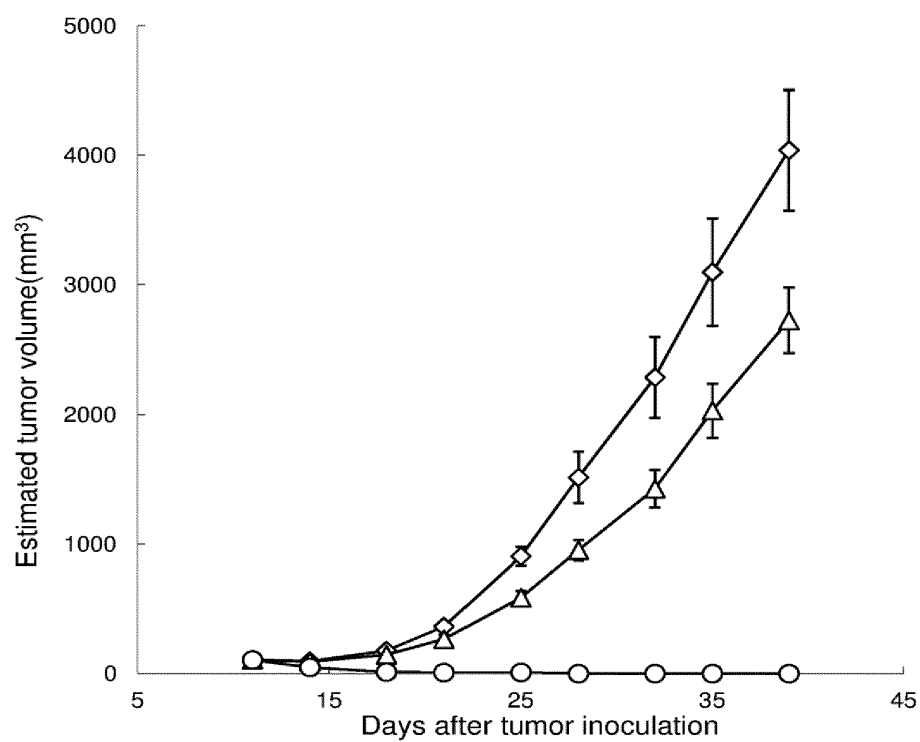
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a a a g a a g a t g a t g g a c a a g a a a t a g c c t g a g c g g c c g c c a c t g t g
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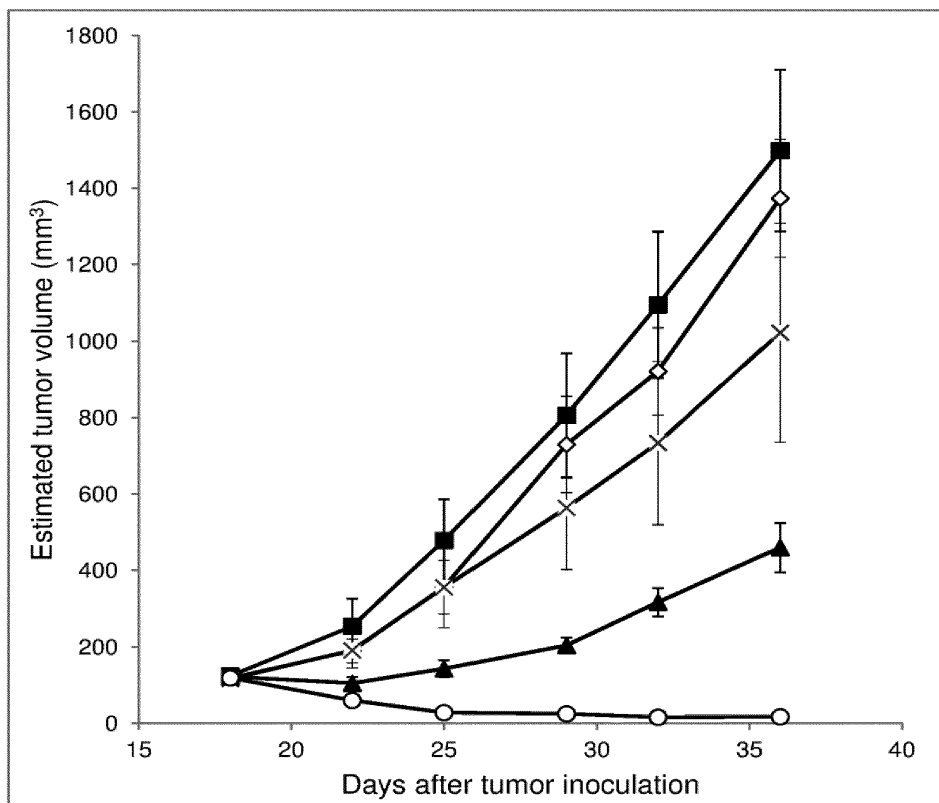
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Fig. 17



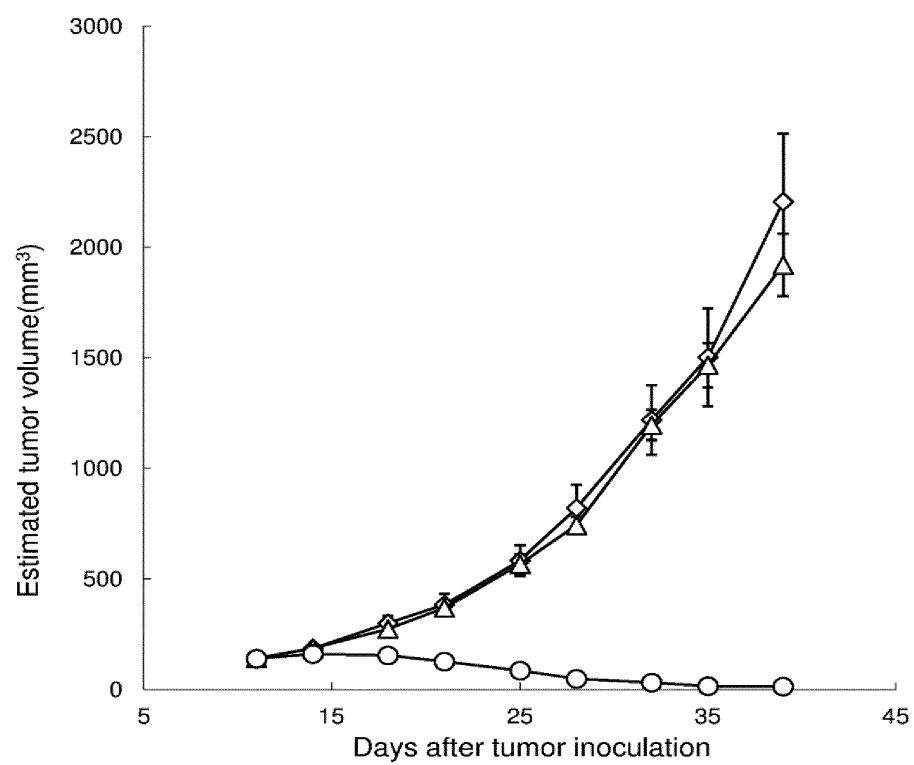
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Fig. 18



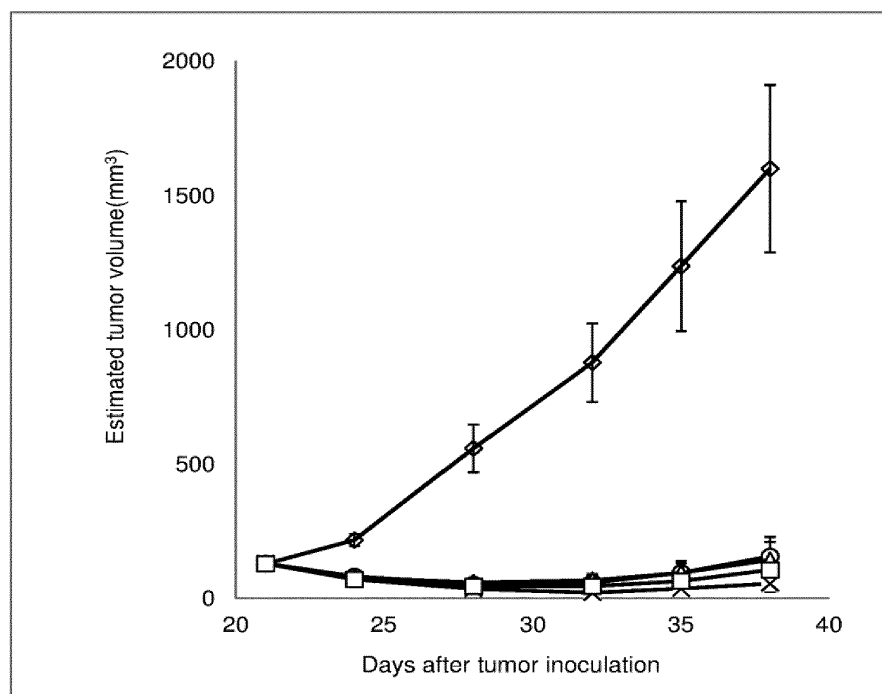
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Fig. 19



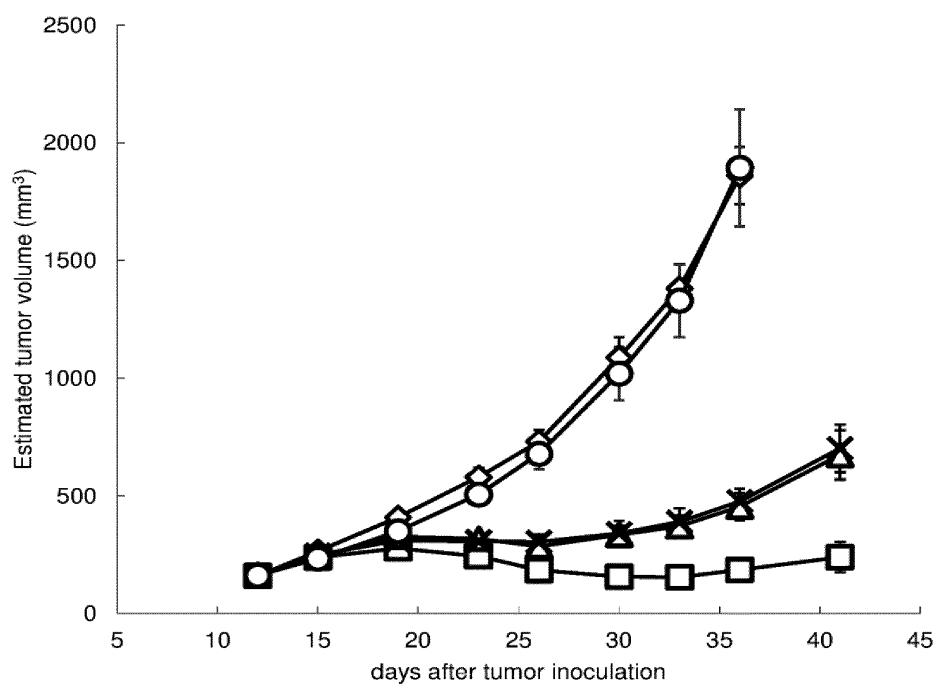
EP 2 907 824 A1

Fig. 20



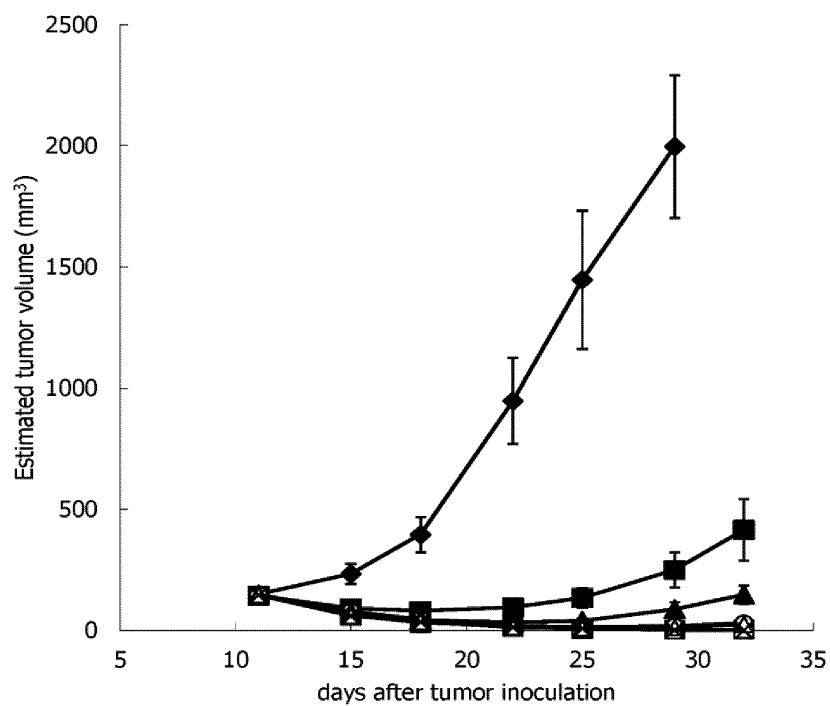
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Fig. 21



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Fig. 22



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INTERNATIONAL SEARCH REPORT		International application No. PCT/JP2013/006069												
5	A. CLASSIFICATION OF SUBJECT MATTER C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P35/00(2006.01)i, A61K31/4745(2006.01)n, A61K31/48(2006.01)n According to International Patent Classification (IPC) or to both national classification and IPC													
10	B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2013 Kokai Jitsuyo Shinan Koho 1971-2013 Toroku Jitsuyo Shinan Koho 1994-2013 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CAPLUS/REGISTRY (STN), PubMed													
20	C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td> <div>X</div> <div>Y</div> </td> <td> JP 2009-538629 A (Genentech, Inc.), 12 November 2009 (12.11.2009), entire text & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A </td> <td> <div>49</div> 1-33, 35-48 </td> </tr> <tr> <td> <div>X</div> <div>Y</div> </td> <td> JP 2008-521828 A (Seattle Genetics, Inc.), 26 June 2008 (26.06.2008), entire text & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2 </td> <td> <div>49</div> 1-33, 35-48 </td> </tr> <tr> <td> <div>X</div> <div>Y</div> </td> <td> JP 2005-511627 A (Seattle Genetics, Inc.), 28 April 2005 (28.04.2005), entire text & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2 </td> <td> <div>49</div> 1-33, 35-48 </td> </tr> </tbody> </table>		Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	<div>X</div> <div>Y</div>	JP 2009-538629 A (Genentech, Inc.), 12 November 2009 (12.11.2009), entire text & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A	<div>49</div> 1-33, 35-48	<div>X</div> <div>Y</div>	JP 2008-521828 A (Seattle Genetics, Inc.), 26 June 2008 (26.06.2008), entire text & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2	<div>49</div> 1-33, 35-48	<div>X</div> <div>Y</div>	JP 2005-511627 A (Seattle Genetics, Inc.), 28 April 2005 (28.04.2005), entire text & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2	<div>49</div> 1-33, 35-48
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40	☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.													
45	<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>		* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
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50	Date of the actual completion of the international search 04 December, 2013 (04.12.13)	Date of mailing of the international search report 17 December, 2013 (17.12.13)												
55	Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer												
	Facsimile No.	Telephone No.												

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/006069

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2002/000734 A1 (Daiichi Pharmaceutical Co., Ltd.), 03 January 2002 (03.01.2002), entire text & US 2003/0166513 A1 & EP 1298145 A1 & CN 1449412 A	1-33,35-48
Y	JP 11-092405 A (Daiichi Pharmaceutical Co., Ltd.), 06 April 1999 (06.04.1999), entire text (Family: none)	1-33,35-48
Y	JP 10-095802 A (Tanabe Seiyaku Co., Ltd.), 14 April 1998 (14.04.1998), entire text & US 5892043 A & EP 781781 A2 & CN 1158334 A	1-33,35-48
A	DOSIO, F. et al., "Antibody-targeted leucinostatin A.", JOURNAL OF CONTROLLED RELEASE, 1994.11, Vol.32, No.1, P.37-44	49

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/006069

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 34
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 34 pertains to a therapeutic method for a human being and thus relates to a subject matter on which this International Searching Authority is not required to carry out an international search under the provisions of PCT Article 17(2)(a)(i) and [PCT Rule 39.1(iv)].
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

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REFERENCES CITED IN THE DESCRIPTION

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(11) **EP 2 910 573 A1**

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(54) **ANTIBODY-DRUG CONJUGATE PRODUCED BY BINDING THROUGH LINKER HAVING HYDROPHILIC STRUCTURE**

(57) As an antitumor drug which is excellent in terms of antitumor effect and safety, there is provided an antibody-drug conjugate in which an antitumor compound represented by the following formula (I) is conjugated to an antibody via a linker having a structure represented by the following formula: $-L^1-L^2-L^P-NH-(CH_2)$

$n^1-L^a-L^b-L^c-$ or $-L^1-L^2-L^P-$ wherein the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c or L^P , and any one or two or more of linkers of L^1 , L^2 , and L^P has a hydrophilic structure.

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Description

Technical Field

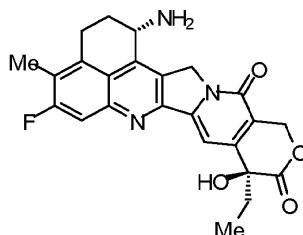
5 [0001] The present invention relates to an antibody-drug conjugate having an antitumor compound conjugated to an antibody capable of targeting tumor cells via a linker structure moiety having a hydrophilic structure, the conjugate being useful as an antitumor drug.

Background Art

10 [0002] An antibody-drug conjugate (ADC) having a drug with cytotoxicity conjugated to an antibody, whose antigen is expressed on a surface of cancer cells and which also binds to an antigen capable of cellular internalization, and therefore can deliver the drug selectively to cancer cells and is thus expected to cause accumulation of the drug within cancer cells and to kill the cancer cells (see, Non Patent Literatures 1 to 3). As an ADC, Mylotarg (Gemtuzumab ozogamicin) in which calicheamicin is conjugated to an anti-CD33 antibody is approved as a therapeutic agent for acute myeloid leukemia. Further, Adcetris (Brentuximab vedotin), in which auristatin E is conjugated to an anti-CD30 antibody, has recently been approved as a therapeutic agent for Hodgkin's lymphoma and anaplastic large cell lymphoma (see, Non Patent Literature 4). The drugs contained in ADCs which have been approved until now target DNA or tubulin.

20 [0003] With regard to an antitumor, low-molecular-weight compounds, camptothecin derivatives, compounds that inhibit topoisomerase I to exhibit an antitumor effect, are known. Among them, an antitumor compound represented by the formula below

[Formula 1]



(exatecan, chemical name: (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-10,13(9H,15H)-dione) is a water soluble derivative of camptothecin (Patent Literature 1 and 2). Unlike irinotecan currently used in clinical settings, an activation by an enzyme is unnecessary. Further, the inhibitory activity on topoisomerase I is higher than SN-38 which is a main pharmaceutically active substance of irinotecan and topotecan also used in clinical settings, and higher in vitro cytotoxic activity is yielded for against various cancer cells. In particular, it exhibits the effect against cancer cells which have resistance to SN-38 or the like due to expression of P-glycoprotein. Further, in a human tumor subcutaneously transplanted mouse model, it exhibited a potent antitumor effect, and thus has undergone the clinical studies, but has not been put on the market yet (see, Non Patent Literatures 5 to 10). However, it remains unclear whether or not exatecan functions effectively as an ADC.

45 [0004] DE-310 is a complex in which exatecan is conjugated to a biodegradable carboxymethyl dextran polyalcohol polymer via a GGFG peptide spacer (Patent Literature 3). By converting exatecan into a form of a polymer prodrug, so that a high blood retention property can be maintained and also a high targetable property to a tumor area is passively increased by utilizing the increased permeability of newly formed blood vessels within tumor and retention property in tumor tissues. With DE-310, through a cleavage of the peptide spacer by enzyme, exatecan and exatecan with glycine connected to an amino group are continuously released as a main active substance. As a result, the pharmacokinetics are improved and DE-310 was found to have higher effectiveness than exatecan administered alone even though the dosage of exatecan is lower than the case of administration of exatecan alone according to various tumor evaluation models in non-clinical studies. A clinical study was conducted for DE-310, and effective cases were confirmed in humans, in which a report suggesting that the main active substance accumulates in a tumor than in normal tissues was present, however, there is also a report indicating that the accumulation of DE-310 and the main active substance in a tumor is not much different from the accumulation in normal tissues in humans, and thus no passive targeting is observed in humans (see, Non Patent Literatures 11 to 14). As a result, DE-310 was not also commercialized, and it remains unclear whether or not exatecan effectively functions as a drug oriented for such targeting.

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[0005] As a compound relating to DE-310, a complex in which -NH(CH₂)₄C(=O)- is inserted between -GGFG-spacer and exatecan to form -GGFG-NH(CH₂)₄C(=O)- spacer structure is also known (Patent Literature 4). However, the antitumor effect of the complex is not known at all.

5 [Citation List]

[Patent Literature]

[0006]

10

[Patent Literature 1] Japanese Patent Laid-Open No. 5-59061
 [Patent Literature 2] Japanese Patent Laid-Open No. 8-337584
 [Patent Literature 3] International Publication No. WO 1997/46260
 [Patent Literature 4] International Publication No. WO 2000/25825

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[Non Patent Literature]

[0007]

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[Non Patent Literature 8] Joto, N. et al. Int J Cancer (1997) 72, 680-686.; DX-8951f, a water-soluble camptothecin analog, exhibits potent antitumor activity against a human lung cancer cell line and its SN-38-resistant variant.

[Non Patent Literature 9] Kumazawa, E. et al. Cancer Chemother. Pharmacol. (1998) 42, 210-220.; Potent and broad antitumor effects of DX-8951f, a water-soluble camptothecin derivative, against various human tumors xenografted in nude mice.

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[Non Patent Literature 12] Kumazawa, E. et al. Cancer Sci (2004) 95, 168-175.; DE-310, a novel macromolecular carrier system for the camptothecin analog DX-8951f: Potent antitumor activities in various murine tumor models.

[Non Patent Literature 13] Soepenberg, O. et al. Clinical Cancer Research, (2005) 11, 703-711.; Phase I and pharmacokinetic study of DE-310 in Patients with Advanced Solid Tumors.

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[Non Patent Literature 14] Wente M. N. et al. Investigational New Drugs (2005) 23, 339-347.; DE-310, a macromolecular prodrug of the topoisomerase-I-inhibitor exatecan (DX-8951), in patients with operable solid tumors.

[Summary of Invention]

55

[Technical Problem]

[0008] With regard to the treatment of tumor by an antibody, an insufficient antitumor effect may be observed even when the antibody recognizes an antigen and binds to tumor cells, and there is a case in which a more effective antitumor

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antibody is needed. Further, many antitumor low-molecular-weight compounds have a problem in safety like side effect and toxicity even the compounds have an excellent antitumor effect, it remains as a subject to achieve a superior therapeutic effect by further enhancing the safety. Thus, an object of the present invention is to yield to provide an antitumor drug having an excellent therapeutic effect, which is excellent in terms of antitumor effect and safety.

[Means to solve the Problem]

[0009] The inventors thought that, when an antitumor compound exatecan is converted into an antibody-drug conjugate, via a linker, by conjugation to the antibody, which is capable of targeting tumor cells, that is having a property of recognizing tumor cells, a property of binding to tumor cells, a property of internalizing within tumor cells, a cytotoxic activity against tumor cells, or the like, the antitumor compound can be more surely delivered to tumor cells to specifically exhibit the antitumor effect of the compound in tumor cells, and thus the antitumor effect can be surely exhibited and also an enhanced cytotoxic effect of the antibody is expected, and a dose of the antitumor compound can be reduced compared to a case of administering the compound alone, and thus an influence of the antitumor compound on normal cells can be alleviated so that higher safety can be achieved.

[0010] In this connection, the inventors created a linker with a specific structure.

[0011] The present inventors have particularly constructed a linker having:

a linker structure in which a hydrophilic amino acid other than glycine is connected at the N terminal of a peptide moiety of the linker;

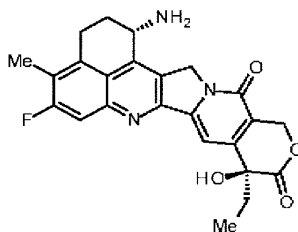
a linker structure in which glycine or glycyglycine is connected at the C terminal of a peptide moiety of the linker; or a linker structure in which a linker element having a hydrophilic structure is inserted between a peptide moiety in the linker and an antibody;

and successfully obtained an antibody-drug conjugate having exatecan conjugated to an antibody via such a linker. The present inventors have further found that this antibody-drug conjugate is excellent in the release of the drug component having an antitumor effect, and as a result, the antibody-drug conjugate of the present invention exerts an excellent antitumor effect, leading to the completion of the present invention.

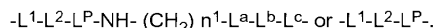
[0012] Specifically, the present invention relates to the followings.

[1] An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 2]



is conjugated to an antibody via a linker having a structure represented by the following formula:



[0013] Here, the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c or L^P , wherein

n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$, $-(\text{Succinimid-3-yl-N})-CH[(CH_2)_{n^3}-COOH]-C(=O)-CH_2-C(=O)-NH-(CH_2)_{n^4}-C(=O)-$, $-C(=O)-\text{cyc.Hex}(1,4)-CH_2-(N\text{-ly-3-dimino})-$, or $-C(=O)-(CH_2)_{n^5}-C(=O)-$,

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wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, n^5 represents an integer of 1 to 8,

L^2 represents $-NH-(CH_2-CH_2-O)n^6-CH_2-CH_2-C(=O)-$, $-N[-(CH_2CH_2-O)n^7-CH_2CH_2-OH]-CH_2-C(=O)-$, $-S-(CH_2)n^8-C(=O)-$, or a single bond,

5 wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4, n^8 represents an integer of 1 to 6,

L^P represents a peptide residue consisting of 3 to 8 amino acids,

L^a represents $-C(=O)-NH-$, $-NR^1-(CH_2)n^9-$, $-O-$, or a single bond,

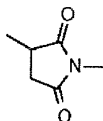
wherein n^9 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)n^a-COOH$, or $-(CH_2)n^b-OH$, n^a represents an integer of 1 to 4, n^b represents an integer of 1 to 6, L^b represents

10 $-CR^2(-R^3)-$, $-O-$, $-NR^4-$, or a single bond,

wherein R^2 and R^3 each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, $-(CH_2)n^c-NH_2$, $-(CH_2)n^d-COOH$, or $-(CH_2)n^e-OH$, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^c represents an integer of 0 to 6, n^d represents an integer of 1 to 4, n^e represents an integer of 1 to 4, provided that when n^c is 0, R^2 and R^3 are not the same as each other,

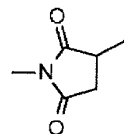
15 L^c represents $-CH_2-$ or $-C(=O)-$, $-(Succinimid-3-yl-N)-$ has a structure represented by the following formula:

[Formula 3]



20 which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, $-(N-ly-3-diminiccuS)-$ has a structure represented by the following formula:

[Formula 4]



40 which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,

$cyc.Hex(1,4)$ represents a 1,4-cyclohexylene group, and when L^2 is $-S-(CH_2)n^8-C(=O)-$, L^1 is $-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-$.

provided that any one or two or more of linkers of L^1 , L^2 , and L^P have a structure containing a hydrophilic structure, and said hydrophilic structure means,

45 as for linker L^P , the case in which,

L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or

L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, or

50 as for linker L^1 , the case in which L^1 is $-(Succinimid-3-yl-N)-CH[-(CH_2)n^3-COOH]-C(=O)-$, or

as for linker L^2 , the case in which L^2 is $-N[-(CH_2CH_2-O)n^7-CH_2CH_2-OH]-CH_2-C(=O)-$.

[0014] The present invention also relates to each of the followings.

[2] The antibody-drug conjugate according to [1],

55 wherein

L^1 is $-(Succinimid-3-yl-N)-(CH_2)n^2-C(=O)-$, $-(Succinimid-3-yl-N)-CH[-(CH_2)n^3-COOH]-C(=O)-$, or $-CH_2-C(=O)-NH-(CH_2)n^4-C(=O)-$,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8,

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L^2 is $-\text{NH}-(\text{CH}_2-\text{CH}_2-\text{O})^{n^6}-\text{CH}_2-\text{CH}_2-\text{C}(=\text{O})-$, $-\text{N}[(\text{CH}_2\text{CH}_2-\text{O})^{n^7}-\text{CH}_2\text{CH}_2-\text{OH}]-\text{CH}_2-\text{C}(=\text{O})-$, or a single bond, wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4,

L^P is a peptide residue consisting of 3 to 8 amino acids, each of L^a and L^b is a single bond, and

L^c is $-\text{C}(=\text{O})-$.

5 [3] The antibody-drug conjugate according to [1] or [2], wherein any one of linkers of L^1 , L^2 , and L^P is the linker containing the hydrophilic structure.

[4] The antibody-drug conjugate according to [3], wherein the linker containing the hydrophilic structure is L^P .

[5] The antibody-drug conjugate according to [4], wherein L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal.

10 [6] The antibody-drug conjugate according to [5], wherein the hydrophilic amino acid other than glycine is aspartic acid, glutamic acid, lysine, serine, threonine, glutamine, asparagine, histidine, tyrosine, or arginine.

[7] The antibody-drug conjugate according to [5], wherein the N-terminal hydrophilic amino acid other than glycine in L^P is glutamic acid, aspartic acid, or lysine.

15 [8] The antibody-drug conjugate according to [6] or [7], wherein a peptide residue following the N-terminal hydrophilic amino acid in L^P is amino acid residue consisting of amino acids selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid.

[9] The antibody-drug conjugate according to [8], wherein a peptide residue following the N-terminal hydrophilic amino acid in L^P is a peptide residue consisting of 3 or 4 amino acids.

20 [10] The antibody-drug conjugate according to [9], wherein the peptide residue following the N-terminal hydrophilic amino acid in L^P is GGF or GGFG.

[11] The antibody-drug conjugate according to any one of [5] to [10], wherein L^P is DGGF, KGGF, EGGF, DGGFG, KGGFG, or EGGFG.

[12] The antibody-drug conjugate according to any one of [5] to [10], wherein L^P is DGGFG, KGGFG, or EGGFG.

25 [13] The antibody-drug conjugate according to any one of [1] to [12], wherein the linker is a linker having a structure represented by $-\text{L}^1-\text{L}^2-\text{L}^P-\text{NH}-(\text{CH}_2)^{n^1}-\text{L}^a-\text{L}^b-\text{L}^c-$.

[14] The antibody-drug conjugate according to [13], wherein L^c is $-\text{C}(=\text{O})-$.

[15] The antibody-drug conjugate according to [14], wherein L^1 is $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)^{n^2}-\text{C}(=\text{O})-$, n^2 is an integer of 2 to 5, and L^2 is a single bond.

[16] The antibody-drug conjugate according to [15], wherein n^2 is 5.

30 [17] The antibody-drug conjugate according to [15] or [16], wherein n^1 is 1 to 3.

[18] The antibody-drug conjugate according to any one of [5] to [17], wherein the structure of the $-\text{NH}-(\text{CH}_2)^{n^1}-\text{L}^a-\text{L}^b-\text{L}^c-$ moiety in the linker is

35 $-\text{NH}-\text{CH}_2-\text{C}(=\text{O})-$,

$-\text{NH}-(\text{CH}_2)_2-\text{C}(=\text{O})-$,

$-\text{NH}-(\text{CH}_2)_3-\text{C}(=\text{O})-$,

40 $-\text{NH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$,

or

45 $-\text{NH}-(\text{CH}_2)_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$.

[19] The antibody-drug conjugate according to any one of [5] to [17], wherein the structure of the $-\text{NH}-(\text{CH}_2)^{n^1}-\text{L}^a-\text{L}^b-\text{L}^c-$ moiety in the linker is

50 $-\text{NH}-\text{CH}_2-\text{C}(=\text{O})-$,

$-\text{NH}-(\text{CH}_2)_2-\text{C}(=\text{O})-$,

or

55 $-\text{NH}-(\text{CH}_2)_3-\text{C}(=\text{O})-$.

[20] The antibody-drug conjugate according to any one of [1] to [12], wherein the linker is a linker having a structure represented by $-\text{L}^1-\text{L}^2-\text{L}^P-$.

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- [21] The antibody-drug conjugate according to [20], wherein L^1 is $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)_{n^2}-\text{C}(=\text{O})-$, n^2 is an integer of 2 to 5, and L^2 is a single bond.
- [22] The antibody-drug conjugate according to [21], wherein n^2 is 5.
- [23] The antibody-drug conjugate according to [4], wherein L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the antitumor compound, and the N terminal is not a hydrophilic amino acid other than glycine, even in case that a hydrophilic amino acid is present at N terminal.
- [24] The antibody-drug conjugate according to [23], wherein the peptide residue is a linker consists of 4 to 8 amino acids.
- [25] The antibody-drug conjugate according to [23], wherein the C-terminal glycine oligopeptide is an oligopeptide consisting of 2 or 3 glycines.
- [26] The antibody-drug conjugate according to any one of [23] to [25], wherein the peptide residue of the linker is GGFGG or GGFGGG.
- [27] The antibody-drug conjugate according to any one of [23] to [26], wherein L^1 is $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)_{n^2}-\text{C}(=\text{O})-$, and n^2 is an integer of 2 to 5.
- [28] The antibody-drug conjugate according to [27], wherein n^2 is 5.
- [29] The antibody-drug conjugate according to [3], wherein L^1 is $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)_{n^3}-\text{COOH}]-\text{C}(=\text{O})-$.
- [30] The antibody-drug conjugate according to [29], wherein n^3 is 2 or 3.
- [31] The antibody-drug conjugate according to [3], wherein L^2 is $-\text{N}[(\text{CH}_2\text{CH}_2-\text{O})_{n^7}-\text{CH}_2\text{CH}_2-\text{OH}]-\text{CH}_2-\text{C}(=\text{O})-$.
- [32] The antibody-drug conjugate according to [31], wherein n^7 is 2 to 4.
- [33] The antibody-drug conjugate according to any one of [29] to [32], wherein the linker is a linker having a structure represented by $-L^1-L^2-L^P-\text{NH}-(\text{CH}_2)_{n^1}-L^a-L^b-L^c-$.
- [34] The antibody-drug conjugate according to [33], wherein L^c is $-\text{C}(=\text{O})-$.
- [35] The antibody-drug conjugate according to any one of [29] to [34], wherein n^1 is 1 to 3.
- [36] The antibody-drug conjugate according to any one of [29] to [35], wherein L^P is GGFG.
- [37] The antibody-drug conjugate according to any one of [29] to [36], wherein the structure of the $-\text{NH}-(\text{CH}_2)_{n^1}-L^a-L^b-L^c-$ moiety in the linker is
- $-\text{NH}-\text{CH}_2-\text{C}(=\text{O})-$,
- $-\text{NH}-(\text{CH}_2)_2-\text{C}(=\text{O})-$,
- $-\text{NH}-(\text{CH}_2)_3-\text{C}(=\text{O})-$,
- $-\text{NH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$,
- or
- $-\text{NH}-(\text{CH}_2)_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$.
- [38] The antibody-drug conjugate according to any one of [29] to [36], wherein the structure of the $-\text{NH}-(\text{CH}_2)_{n^1}-L^a-L^b-L^c-$ moiety in the linker is
- $-\text{NH}-\text{CH}_2-\text{C}(=\text{O})-$,
- $-\text{NH}-(\text{CH}_2)_2-\text{C}(=\text{O})-$,
- or
- $-\text{NH}-(\text{CH}_2)_3-\text{C}(=\text{O})-$.
- [39] The antibody-drug conjugate according to any one of [1] to [38], wherein the bond between the antibody and L^1 is a thioether bond which is formed at a disulfide bond moiety present in a hinge part of the antibody, a disulfide bond which is formed at a disulfide bond moiety present in a hinge part of the antibody, or an amide bond which is formed at an amino group present on a side chain of an amino acid constituting the antibody or at the terminal amino group.
- [40] The antibody-drug conjugate according to any one of [1] to [38], wherein the bond between the antibody and L^1 is a thioether bond which is formed at a disulfide bond moiety present in a hinge part of the antibody, or an amide bond which is formed at an amino group present on a side chain of an amino acid constituting the antibody or at

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the terminal amino group.

[41] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- 5
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - 10
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - 20
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - 30
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - 35
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - 40
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[42] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- 45
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - 50
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

[43] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- 55
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

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- [44] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).
- [45] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX).
- [46] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX).
- [47] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX).
- [48] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-(NH-DX).
- [49] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-(NH-DX).
- [50] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-(NH-DX).
- [51] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-(NH-DX).
- [52] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-(NH-DX),

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- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-(NH-DX).

[53] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-(NH-DX).

[54] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX).

[55] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[56] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:

- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

[57] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:

- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-(NH-DX).

[58] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[59] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-(NH-DX).

[60] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

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- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$,
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$,
- 5 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$,
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$.

10 [61] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-(NH-DX)}$,

15 [62] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is one structure selected from the following group:

- 20 - $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$,
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$,
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$,
- 25 - $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N(-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$.

[63] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is the following structure:

- 30 - $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-(NH-DX)}$.

35 [64] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:

- $\text{(Succinimid-3-yl-N)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-GGFGG-(NH-DX)}$,
- $\text{(Succinimid-3-yl-N)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-GGFGGG-(NH-DX)}$.

40 [65] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-GGFGG-(NH-DX)}$,
- 45 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-GGFGGG-(NH-DX)}$.

[66] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:

- 50 - $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-GGFGG-(NH-DX)}$,
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-GGFGGG-(NH-DX)}$.

[67] The antibody-drug conjugate according to [1], [2], [39], or [40], wherein the drug-linker structure moiety in the antibody-drug conjugate is any of the following structures:

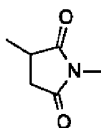
- 55 - $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-GGFGG-(NH-DX)}$,
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-GGFGGG-(NH-DX)}$.

[0015] Here, according to any one of [41] to [67], - (Succinimid-3-yl-N)- has a structure represented by the following

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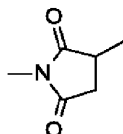
formula:

[Formula 5]



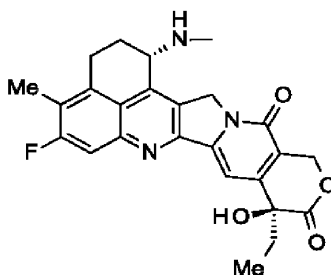
which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, cyc.Hex(1,4) represents a 1,4-cyclohexylene group, -(N-ly-3-diminiccuS)- has a structure represented by the following formula:

[Formula 6]



which is connected to L² at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and -(NH-DX) is a group represented by the following formula:

[Formula 7]



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

[68] The antibody-drug conjugate according to any one of [1] to [67], wherein an average number of conjugated antitumor compounds per antibody is in a range of from 1 to 10.

[69] The antibody-drug conjugate according to any one of [1] to [67], wherein an average number of conjugated antitumor compounds per antibody is in a range of from 1 to 8.

[70] The antibody-drug conjugate according to any one of [1] to [67], wherein an average number of conjugated antitumor compounds per antibody is in a range of from 3 to 8.

[71] The antibody-drug conjugate according to any one of [1] to [70], wherein the antibody is an antibody having one or more of a property of recognizing a target cell, a property of binding to a target cell, a property of internalizing in a target cell, and a property of damaging a target cell.

[72] The antibody-drug conjugate according to [71], wherein the target cell is a tumor cell.

[73] The antibody-drug conjugate according to any one of [1] to [72], wherein the antibody is an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-

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Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, or an anti-mesothelin antibody.

[74] The antibody-drug conjugate according to any one of [1] to [72], wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

[75] The antibody-drug conjugate according to any one of [1] to [72], wherein the antibody is an anti-B7-H3 antibody.

[76] A drug containing the antibody-drug conjugate according to any one of [1] to [75], a salt thereof or a hydrate thereof.

[77] An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to any one of [1] to [75], a salt thereof or a hydrate thereof.

[78] The antitumor drug and/or anticancer drug according to [77], which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.

[79] A pharmaceutical composition containing the antibody-drug conjugate according to any one of [1] to [75], a salt thereof or a hydrate thereof as an active component, and a pharmaceutically acceptable formulation component.

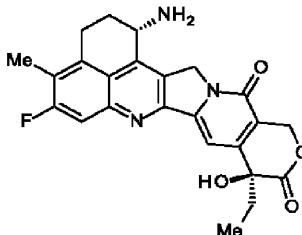
[80] The pharmaceutical composition according to [79], which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.

[81] A method for treating tumor and/or cancer comprising administering the antibody-drug conjugate according to any one of [1] to [75], a salt thereof or a hydrate thereof.

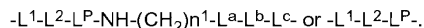
[82] The pharmaceutical composition according to [81], which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.

[83] An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 8]



is conjugated to an antibody via a linker having a structure represented by the following formula:



[0016] Here, the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c or L^P ,

wherein

n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$, $-(\text{Succinimid-3-yl-N})-CH[-(CH_2)_{n^3}-COOH]-C(=O)-$, or $-CH_2-C(=O)-NH-(CH_2)_{n^4}-C(=O)-$,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8,

L^2 represents $-NH-(CH_2-CH_2-O)_{n^6}-CH_2-CH_2-C(=O)-$, $-N[-(CH_2CH_2-O)_{n^7}-CH_2CH_2-OH]-CH_2-C(=O)-$, or a single bond,

wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4,

L^P represents a peptide residue consisting of 3 to 8 amino acids,

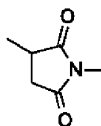
L^a represents $-O-$ or a single bond,

L^b represents $CR^2(-R^3)-$ or a single bond,

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wherein R^2 and R^3 each independently represent a hydrogen atom,
 L^c represents $-C(=O)-$,
 $-(\text{Succinimid-3-yl-N})-$ has a structure represented by the following formula:

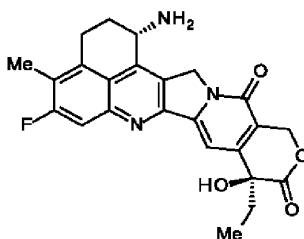
[Formula 9]



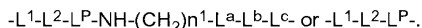
which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, provided that any one or two or more of linkers of L^1 , L^2 , and L^P have a structure containing a hydrophilic structure, said hydrophilic structure means, as for linker L^P , the case in which, L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, or as for linker L^1 , in case that L^1 is $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)_{n^3}-\text{COOH}]-\text{C}(=O)-$, or as for linker L^2 , in case that L^2 is $-\text{N}[(\text{CH}_2\text{CH}_2-\text{O})_{n^7}-\text{CH}_2\text{CH}_2-\text{OH}]-\text{CH}_2-\text{C}(=O)-$.

[84] An antibody-drug conjugate wherein an antitumor compound represented by the following formula:

[Formula 10]



is conjugated to an antibody via a linker having a structure represented by the following formula:



[0017] Here, the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c or L^P with the nitrogen atom of the amino group at position 1 as the connecting position, wherein

n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)_{n^2}-\text{C}(=O)-$ or -

$(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)_{n^3}-\text{COOH}]-\text{C}(=O)-$ and is connected to the antibody via a thioether bond at a disulfide bond moiety in a hinge part of the antibody,

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8,

L^2 represents $-\text{N}[(\text{CH}_2\text{CH}_2-\text{O})_{n^7}-\text{CH}_2\text{CH}_2-\text{OH}]-\text{CH}_2-\text{C}(=O)-$ or a single bond,

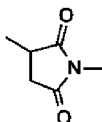
wherein n^7 represents an integer of 1 to 4,

L^P represents a peptide residue consisting of 3 to 8 amino acids,

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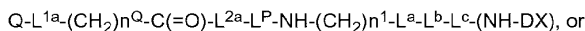
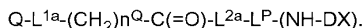
L^a represents a single bond,
 L^b represents a single bond,
 L^c represents -C(=O)-,
 -(Succinimid-3-yl-N)- has a structure represented by the following formula:

[Formula 11]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, provided that any one or two or more of linkers of L¹, L², and L^P have a structure containing a hydrophilic structure, and said hydrophilic structure means, as for linker L^P, the case in which, L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, or as for linker L¹, the case in which L¹ is -(Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-, or as for linker L², the case in which L² is -N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)-.

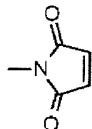
[85] A drug-linker intermediate compound represented by the following formula:



[0018] In the formula, Q represents (maleimid-N-yl)-, HS-, X-CH₂-C(=O)-NH-, or (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, X represents a bromine atom or an iodine atom, L^{1a} represents -CH[-(CH₂)ⁿ³-COOH]- or a single bond, n^Q represents an integer of 0 to 8, L^{2a} represents -NH-(CH₂-CH₂-O)ⁿ⁶-CH₂-CH₂-C(=O)-, -N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)-, or a single bond, wherein n⁶ represents an integer of 0 to 6, n⁷ represents an integer of 1 to 4, L^P represents a peptide residue consisting of 3 to 8 amino acids, n¹ represents an integer of 0 to 6, L^a represents -C(=O)-NH-, -NR¹-(CH₂)ⁿ⁹-, -O-, or a single bond, wherein n⁹ represents an integer of 1 to 6, R¹ represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂)^{n^a}-COOH, or -(CH₂)^{n^b}-OH, n^a represents an integer of 1 to 4, n^b represents an integer of 1 to 6, L^b represents -CR²(-R³)-, -O-, -NR⁴-, or a single bond, wherein R² and R³ each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂)^{n^c}-NH₂, -(CH₂)^{n^d}-COOH, or -(CH₂)^{n^e}-OH, R⁴ represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^c represents an integer of 0 to 6, n^d represents an integer of 1 to 4, n^e represents an integer of 1 to 4, provided that when n^c is 0, R² and R³ are not the same as each other, L^c represents -CH₂- or -C(=O)-, In the above, (maleimid-N-yl)- is a group represented by the following formula:

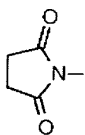
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[Formula 12]



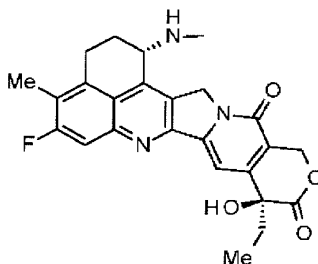
wherein the nitrogen atom is the connecting position, (Pyrrolidine-2,5-dione-N-yl) is a group represented by the following formula:

[Formula 13]



wherein the nitrogen atom is the connecting position, and- (NH-DX) is a group represented by the following formula:

[Formula 14]



wherein the nitrogen atom of the amino group at position 1 is the connecting position, provided that any one or two or more of linkers of $L^{1a}-(CH_2)_n^Q-C(=O)-$, L^2 and L^P have a structure containing hydrophilic structure,

and said hydrophilic structure means,

as for linker L^P , the case in which,

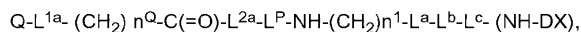
L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or

L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, or

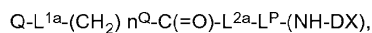
as for linker $L^{1a}-(CH_2)_n^Q-C(=O)-$, the case in which $L^{1a}-(CH_2)_n^Q-C(=O)-$ is $-CH[-(CH_2)_n^3-COOH]-C(=O)-$, or

as for linker L^{2a} , the case in which L^{2a} is $-N[-(CH_2CH_2-O)_n^7-CH_2CH_2-OH]-CH_2-C(=O)-$.

[86] A drug-linker intermediate compound represented by the following formula:



or



wherein Q represents (maleimid-N-yl)-, HS-, $X-CH_2-C(=O)-NH-$, or (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-,

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X represents a bromine atom or an iodine atom,

L^{1a} represents -CH[-(CH₂)^{n³}-COOH]- or a single bond,

n^Q represents an integer of 0 to 8,

L^{2a} represents -NH-(CH₂-CH₂-O)^{n⁶}-CH₂-CH₂-C(=O)-, -N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-, or a single bond,

wherein n⁶ represents an integer of 0 to 6, n⁷ represents an integer of 1 to 4,

L^P represents a peptide residue consisting of 3 to 8 amino acids,

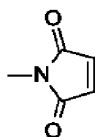
n¹ represents an integer of 0 to 6,

L^a and L^b each independently represent a single bond,

L^c represents -C(=O)-,

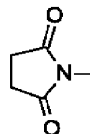
(maleimid-N-yl)- is a group represented by the following formula:

[Formula 15]



wherein the nitrogen atom is the connecting position, (Pyrrolidine-2,5-dione-N-yl) is a group represented by the following formula:

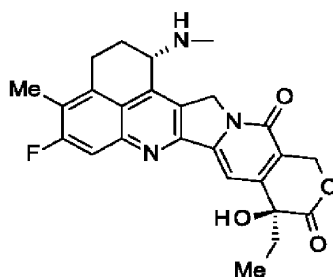
[Formula 16]



wherein the nitrogen atom is the connecting position, and

-(NH-DX) is a group represented by the following formula:

[Formula 17]



wherein the nitrogen atom of the amino group at position 1 is the connecting position.

[87] The compound according to [86], which is selected from the following group:

(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),

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(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 5 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 10 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[88] The compound according to [86], which is selected from the following group:

15 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 20 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

[89] The compound according to [86], which is selected from the following group:

25 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 30 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 35 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 40 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[90] The compound according to [86], which is selected from the following group:

45 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 50 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

[91] The compound according to [86], which is selected from the following group:

55 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),

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- (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 5 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 10 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 15 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 20 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 25 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[92] The compound according to [86], which is selected from the following group:

- 30 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 35 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 40 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

[93] The compound according to [86], which is selected from the following group:

- 45 HS-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 50 HS-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 55 HS-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),

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HS-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

5 [94] The compound according to [86], which is selected from the following group:

HS-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 10 HS-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX).

15

[95] The compound according to [86], which is selected from the following group:

(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
 20 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX).

[96] The compound according to [86], which is any of the followings:

25 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-(NH-DX).

[97] The compound according to [86], which is selected from the following group:

30 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-(NH-DX).

35 [98] The compound according to [86], which is any of the followings:

X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFGGG-(NH-DX).

40 [99] The compound according to [86], which is selected from the following group:

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 45 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX).

[100] The compound according to [86], which is any of the followings:

50 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-(NH-DX).

[101] The compound according to [86], which is selected from the following group:

55 HS-CH₂CH₂-C(=O)-DGGF-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGF-(NH-DX),
 HS-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 HS-CH₂CH₂-C(=O)-KGGFG-(NH-DX).

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[102] The compound according to [86], which is selected from the following group:

(maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX) .

[103] The compound according to [86], which has a structure of the following formula:

(maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-(NH-DX).

[104] The compound according to [86], which is selected from the following group:

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX) .

[105] The compound according to [86], which is selected from the following group:

(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[106] The compound according to [86], which is selected from the following group:

X-CH₂-C(=O)-NH-CH₂CH₂-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 X-CH₂-C(=O)-NH-CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[107] The compound according to [86], which is selected from the following group:

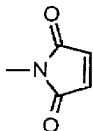
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[0019] In the linker according to any one of [87] to [107], (maleimid-N-yl)- is a group represented by the following formula:

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[Formula 18]

5

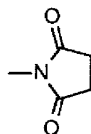


10 wherein the nitrogen atom is the connecting position,
X represents a halogen atom,
(Pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

15

[Formula 19]

20

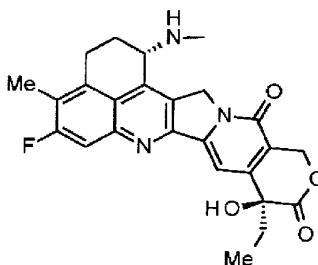


25 wherein the nitrogen atom is the connecting position, and -(NH-DX) is a group represented by the following formula:

25

[Formula 20]

30

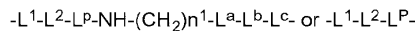


35

40 wherein the nitrogen atom of the amino group at position 1 is the connecting position.

[108] A linker having a structure represented by the following formula:

45



for obtaining an antibody-drug conjugate in which a antitumor compound is conjugated to an antibody via the linker.

[0020] In the above, the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c or L^P ,

50

wherein

n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$, $-(\text{Succinimid-3-yl-N})-CH[-(CH_2)_{n^3}-COOH]-C(=O)-$, $-CH_2-C(=O)-NH-(CH_2)_{n^4}-C(=O)-$, $-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-$, or $-C(=O)-(CH_2)_{n^5}-C(=O)-$,

55

wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, n^5

represents an integer of 1 to 8,

L^2 represents $-NH-(CH_2-CH_2-O)_{n^6}-CH_2-CH_2-C(=O)-$, $-N[-(CH_2CH_2-O)_{n^7}-CH_2CH_2-OH]-CH_2-C(=O)-$, $-S-(CH_2)_{n^8}-C(=O)-$, or a single bond,

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wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4, n^8 represents an integer of 1 to 6,

L^P represents a peptide residue consisting of 3 to 8 amino acids,

L^a represents $-C(=O)-NH-$, $-NR^1-(CH_2)n^9-$, $-O-$, or a single bond,

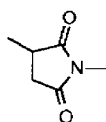
wherein n^9 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms,
 5 $-(CH_2)n^a-COOH$, or $-(CH_2)n^b-OH$, n^a represents an integer of 1 to 4, n^b represents an integer of 1 to 6, L^b represents $-CR^2(-R^3)-$, $-O-$, $-NR^4-$, or a single bond,

wherein R^2 and R^3 each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms,
 $-(CH_2)n^c-NH_2$, $-(CH_2)n^d-COOH$, or $-(CH_2)n^e-OH$, R^4 represents a hydrogen atom or an alkyl group having 1 to 6 carbon

atoms, n^c represents an integer of 0 to 6, n^d represents an integer of 1 to 4, n^e represents an integer of 1 to 4, provided
 10 that when n^c is 0, R^2 and R^3 are not the same as each other,

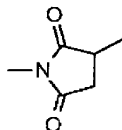
L^c represents $-CH_2-$ or $-C(=O)-$, $-(Succinimid-3-yl-N)-$ has a structure represented by the following formula:

[Formula 21]



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure
 containing this structure on the nitrogen atom at position 1, $-(N-ly-3-diminiccuS)-$ has a structure represented by the
 following formula:

[Formula 22]



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing
 35 this structure on the nitrogen atom at position 1,

$cyc.Hex(1,4)$ represents a 1,4-cyclohexylene group, and when L^2 is $-S-(CH_2)n^8-C(=O)-$, L^1 is
 $-C(=O)-cyc.Hex(1,4)-CH_2-(N-ly-3-diminiccuS)-$.

provided that any one or two or more of linkers of L^1 , L^2 , and L^P have a structure containing a hydrophilic structure,
 said hydrophilic structure means,

40 as for linker L^P , the case in which,

L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or

L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected
 to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal,
 no other hydrophilic amino acid than glycine is present thereat,

45 as for linker L^1 , the case in which L^1 is $-(Succinimid-3-yl-N)-CH[-(CH_2)n^3-COOH]-C(=O)-$, or

as for linker L^2 , the case in which L^2 is $-N[-(CH_2CH_2-O)n^7-CH_2CH_2-OH]-CH_2-C(=O)-$.

[109] The linker according to [108], which is selected from the following group, provided that the left terminal is a
 connecting position to the antibody and the right terminal is a connecting position to the antitumor compound:

- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-DGGF-NH-CH_2CH_2CH_2-C(=O)-$,
- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-DGGF-NH-CH_2-O-CH_2-C(=O)-$,
- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-DGGF-NH-CH_2CH_2-O-CH_2-C(=O)-$,
- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-DGGFG-NH-CH_2CH_2CH_2-C(=O)-$,
- 55 - (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-DGGFG-NH-CH_2-O-CH_2-C(=O)-$,
- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-DGGFG-NH-CH_2CH_2-O-CH_2-C(=O)-$,
- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-KGGF-NH-CH_2CH_2CH_2-C(=O)-$,
- (Succinimid-3-yl-N)- $CH_2CH_2CH_2CH_2CH_2-C(=O)-KGGF-NH-CH_2-O-CH_2-C(=O)-$,

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- 5
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-,
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-,
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-,
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-,
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-.
- 30 [110] The linker according to [108], which is selected from the following group, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:
- 35
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-,
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-,
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-,
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-.
- 40 [111] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:
- 45
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-, and
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-.
- 50 [112] The linker according to [108], which is selected from the following group, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:
- 55
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-, and
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-.
- [113] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:
- [114] The linker according to [108], which is selected from the following group, provided that the left terminal is the

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connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-.

[115] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-, and
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-.

[116] The linker according to [108], which is selected from the following group, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-,
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-,
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-,
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-,
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-,
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-,
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-,
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-,
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-,
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-.

[117] The linker according to [108], which is selected from the following group, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-.

[118] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-, and
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-.

[119] The linker according to [108], which has the following structure, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-.

[120] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-, and
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-.

[121] The linker according to [108], which has the following structure, provided that the left terminal is the connecting

position to the antibody and the right terminal is the connecting position to the antitumor compound:

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-}$.

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-, and
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-.

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-

- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-,
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-,
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-,
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-.

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-CH₂-C(=O)-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-,
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-.

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-}$,
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-}$,
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-}$,
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-}$.

- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-}$,
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-}$,
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-}$,
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-N-(CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-O-CH}_2\text{CH}_2\text{-OH)-CH}_2\text{-C(=O)-GGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-}$.

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[128] The linker according to [108], which has the following structure, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N) -CH (CH₂CH₂-COOH) -C(=O)-NH-CH₂CH₂-C(=O)-GGFG-

[129] The linker according to [108], which has the following structure, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-

[130] The linker according to [108], which has the following structure, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-

[131] The linker according to [108], which has the following structure, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- C (CH₂CH₂-OH)-CH₂-C(=O)-GGFG- (CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-C(=O)-N(CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-N(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-

[132] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-, and
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-

[133] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFGG-, and
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFGGG-

[134] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

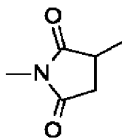
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-GGFGG-, and
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-GGFGGG-

[135] The linker according to [108], which is any of the followings, provided that the left terminal is the connecting position to the antibody and the right terminal is the connecting position to the antitumor compound:

- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-, and
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-

[0021] Here, in the linker according to any one of [109] to [135], -(N-ly-3-diminiccuS)- has a structure represented by the following formula:

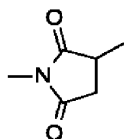
[Formula 23]



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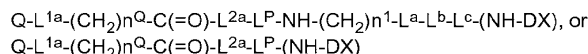
which is connected to L² at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1,
 -(N-ly-3-diminiccuS)- has a structure represented by the following formula:

[Formula 24]



which is connected to L² at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and
 cyc.Hex(1,4) represents a 1,4-cyclohexylene group.

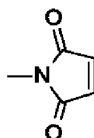
[136] A method for producing an antibody-drug conjugate comprising reacting a compound represented by the following formula:



with an antibody or a reactive derivative thereof and conjugating a drug-linker moiety to the antibody by a method for forming a thioether bond at a disulfide bond moiety present in a hinge part of the antibody, or by a method for forming an amide bond at an amino group present on a side chain of an amino acid constituting the antibody or at the terminal amino group.

[0022] In the formula, Q represents (maleimid-N-yl)-, HS-, X-CH₂-C(=O)-NH-, or (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, X represents a bromine atom or an iodine atom,
 L^{1a} represents -CH[-(CH₂)_{n³}-COOH]- or a single bond,
 n^Q represents an integer of 0 to 8,
 L^{2a} represents -NH-(CH₂-CH₂-O)_{n⁶}-CH₂-CH₂-C(=O)-, -N[-(CH₂CH₂-O)_{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-, or a single bond, wherein n⁶ represents an integer of 0 to 6, n⁷ represents an integer of 1 to 4,
 L^P represents a peptide residue consisting of 3 to 8 amino acids,
 n¹ represents an integer of 0 to 6,
 L^a represents -C(=O)-NH-, -NR¹-(CH₂)_{n⁹}-, -O-, or a single bond, wherein n⁹ represents an integer of 1 to 6, R¹ represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂)_{n^a}-COOH, or -(CH₂)_{n^b}-OH, n^a represents an integer of 1 to 4, n^b represents an integer of 1 to 6, L^b represents -CR²(-R³)-, -O-, -NR⁴-, or a single bond, wherein R² and R³ each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂)_{n^c}-NH₂, -(CH₂)_{n^d}-COOH, or -(CH₂)_{n^e}-OH, R⁴ represents a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^c represents an integer of 0 to 6, n^d represents an integer of 1 to 4, n^e represents an integer of 1 to 4, provided that when n^c is 0, R² and R³ are not the same as each other,
 L^c represents -CH₂- or -C(=O)-,
 (maleimid-N-yl)- is a group represented by the following formula:

[Formula 25]



wherein the nitrogen atom is the connecting position, (Pyrrolidine-2,5-dione-N-yl) is a group represented by the following formula:

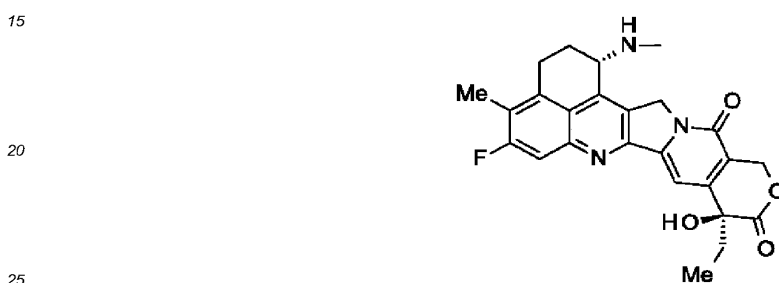
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[Formula 26]



10 wherein the nitrogen atom is the connecting position, $-(NH-DX)$ is a group represented by the following formula:

[Formula 27]



wherein the nitrogen atom of the amino group at position 1 is the connecting position, provided that any one or two or more of linkers of $L^{1a}-(CH_2)n^Q-C(=O)-$, L^2 , and L^P have a structure containing a hydrophilic structure,

30 said hydrophilic structure means, when L^P has this structure,

L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or

L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the drug, and the even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, as for linker $L^{1a}-(CH_2)n^Q-C(=O)-$, the case in which $L^{1a}-(CH_2)n^Q-C(=O)-$ is $-CH[-(CH_2)n^3-COOH]-C(=O)-$, or as for linker L^{2a} , the case in which L^{2a} is $-N[-(CH_2CH_2-O)n^7-CH_2CH_2-OH]-CH_2-C(=O)-$.

[137] The method for producing an antibody-drug conjugate according to [136], wherein the method for conjugating a drug-linker moiety to an antibody is a method of reducing the antibody and thereafter forming a thioether bond by the reaction of the antibody with the compound in which Q is a maleimidyl group or $X-CH_2-C(=O)-NH-$, a method of forming an amide bond by the reaction of the antibody with the compound in which Q is (Pyrrolidine-2, 5-dione-N-yl)-O-C(=O)-, or

40 a method of reacting the antibody with a compound represented by the formula $Q^1-L^{1a}-(CH_2)n^Q-C(=O)-Q^2$ wherein Q^1 represents (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, (3-Sulfo-pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, $R^Q-O-C(=N)-$, or $O=C=N-$,

L^{1a} represents -cyc.Hex(1,4)-CH₂-, an alkylene group having 1 to 10 carbon atoms, a phenylene group, $-(CH_2)n^{4a}-C(=O)-$, $-(CH_2)n^{4a}-NH-C(=O)-$, $-(CH_2)n^{4b}-$, or $-(CH_2)n^{4a}-NH-C(=O)-cyc.Hex(1,4)-CH_2-$, Q^2 represents (maleimid-N-yl), a halogen atom, or -S-S-(2-Pyridyl),

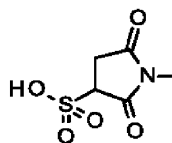
45 R^Q represents an alkyl group having 1 to 6 carbon atoms, n^4 represents an integer of 1 to 8, n^{4a} represents an integer of 0 to 6, n^{4b} represents an integer of 1 to 6, (3-Sulfo-pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

50 the following formula:

55

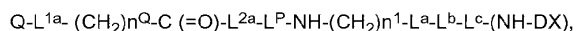
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[Formula 28]

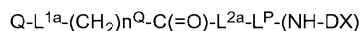


wherein the nitrogen atom is the connecting position, this sulfonic acid is capable of forming a lithium salt, sodium salt, or potassium salt, cyc.Hex(1,4) represents a 1,4-cyclohexylene group, and (2-Pyridyl) represents a 2-pyridyl group and thereafter reacting the antibody with the compound in which Q is SH to form a drug-linker structure by an amide bond.

[138] The method for producing an antibody-drug conjugate according to [136] or [137], wherein the compound represented by the following formula:



or



is a compound according to any one of [87] to [107].

[139] A method for producing an antibody-drug conjugate produced by a production method according to any one of [136] to [138].

Advantageous Effects of Invention

[0023] With an antibody-drug conjugate having an antitumor compound exatecan conjugated via a linker with a specific structure, an excellent antitumor effect can be achieved.

Brief Description of Drawings

[0024]

[Figure 1] Figure 1 shows an amino acid sequence of B7-H3 variant 1 (SEQ ID NO: 1).

[Figure 2] Figure 2 shows an amino acid sequence of B7-H3 variant 2 (SEQ ID NO: 2).

[Figure 3] Figure 3 shows an amino acid sequence of an M30-H1-type heavy chain (SEQ ID NO: 9).

[Figure 4] Figure 4 shows an amino acid sequence of an M30-H2-type heavy chain (SEQ ID NO: 10).

[Figure 5] Figure 5 shows an amino acid sequence of an M30-H3-type heavy chain (SEQ ID NO: 11).

[Figure 6] Figure 6 shows an amino acid sequence of an M30-H4-type heavy chain (SEQ ID NO: 12).

[Figure 7] Figure 7 shows an amino acid sequence of an M30-L1-type light chain (SEQ ID NO: 13).

[Figure 8] Figure 8 shows an amino acid sequence of an M30-L2-type light chain (SEQ ID NO: 14).

[Figure 9] Figure 9 shows an amino acid sequence of an M30-L3-type light chain (SEQ ID NO: 15).

[Figure 10] Figure 10 shows an amino acid sequence of an M30-L4-type light chain (SEQ ID NO: 16).

[Figure 11] Figure 11 shows an amino acid sequence of an M30-L5-type light chain (SEQ ID NO: 17).

[Figure 12] Figure 12 shows an amino acid sequence of an M30-L6-type light chain (SEQ ID NO: 18).

[Figure 13] Figure 13 shows an amino acid sequence of an M30-L7-type light chain (SEQ ID NO: 19).

[Figure 14] Figure 14 shows an amino acid sequence of an M30 antibody heavy chain (SEQ ID NO: 20).

[Figure 15] Figure 15 shows an amino acid sequence of an M30 antibody light chain (SEQ ID NO: 21).

[Figure 16] Figure 16 shows a nucleotide sequence of B7-H3 variant 1 (SEQ ID NO: 26).

[Figure 17] Figure 17 shows the effects of an M30-H1-L4P antibody and antibody-drug conjugates (1), (2), (18), and (19) administered at 10 mg/kg on human melanoma line A375 cells transplanted in mice. The line with open rhombuses depicts results about untreated tumor, the line with filled rhombuses depicts the effect of the M30-H1-L4P antibody, the line with filled squares depicts the effect of the antibody-drug conjugate (1), the line with open squares depicts the effect of the antibody-drug conjugate (2), the line with filled triangles depicts the effect of the antibody-

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drug conjugate (18), and the line with open triangles depicts the effect of the antibody-drug conjugate (19).
 [Figure 18] Figure 18 shows the effects of the antibody-drug conjugates (2) and (19) administered at 1 mg/kg and 3 mg/kg on human melanoma line A375 cells transplanted in mice. The line with open rhombuses depicts results about untreated tumor, the line with filled squares depicts the effect of the antibody-drug conjugate (2) administered at 1 mg/kg, the line with open squares depicts the effect of the antibody-drug conjugate (2) administered at 3 mg/kg, the line with filled circles depicts the effect of the antibody-drug conjugate (19) administered at 1 mg/kg, and the line with open circles depicts the effect of the antibody-drug conjugate (19) administered at 3 mg/kg.
 [Figure 19] Figure 19 shows the effects of an M30-H1-L4P antibody and the antibody-drug conjugates (1), (2), (18), and (19) administered at 10 mg/kg on human non-small cell lung cancer line Calu-6 cells transplanted in mice. The line with open rhombuses depicts results about untreated tumor, the line with filled rhombuses depicts the effect of the M30-H1-L4P antibody, the line with filled squares depicts the effect of the antibody-drug conjugate (1), the line with open squares depicts the effect of the antibody-drug conjugate (2), the line with filled triangles depicts the effect of the antibody-drug conjugate (18), and the line with open triangles depicts the effect of the antibody-drug conjugate (19).
 [Figure 20] Figure 20 shows the effects of antibody-drug conjugates (3), (20), and (30) administered at 3 mg/kg and 10 mg/kg on human melanoma line A375 cells transplanted in mice. The line with open rhombuses depicts results about untreated tumor, the dotted line with filled squares depicts the effect of the antibody-drug conjugate (3) administered at 3 mg/kg, the solid line with filled squares depicts the effect of the antibody-drug conjugate (3) administered at 10 mg/kg, the dotted line with filled triangles depicts the effect of the antibody-drug conjugate (20) administered at 3 mg/kg, the solid line with filled triangles depicts the effect of the antibody-drug conjugate (20) administered at 10 mg/kg, the dotted line with filled circles depicts the effect of the antibody-drug conjugate (30) administered at 3 mg/kg, and the solid line with filled circles depicts the effect of the antibody-drug conjugate (30) administered at 10 mg/kg.

25 Description of Embodiments

[0025] The antibody-drug conjugate of the present invention is an antitumor drug in which an antitumor antibody is conjugated to an antitumor compound via a linker structure moiety and explained in detail hereinbelow.

30 [Antibody]

[0026] The antibody used in the antibody-drug conjugate of the present invention means an immunoglobulin and is a molecule containing an antigen-binding site immunospecifically binding to an antigen. The class of the antibody of the present invention may be any of IgG, IgE, IgM, IgD, IgA, and IgY and is preferably IgG. The subclass of the antibody of the present invention may be any of IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2 and is preferably IgG1 or IgG2. The antibody may be derived from any species, and preferred examples of the species can include humans, rats, mice, and rabbits. In case when derived from other than human species, it is preferably chimerized or humanized using a well known technique. The antibody of the present invention may be a polyclonal antibody or a monoclonal antibody and is preferably a monoclonal antibody.

[0027] The antibody of the present invention may be those which is capable of targeting tumor cells. Since the antibody of the present invention is conjugated with a drug having antitumor activity via a linker, the antibody preferably possesses one or more of a property of recognizing a tumor cell, a property of binding to a tumor cell, a property of internalizing in a tumor cell, and a property of damaging a tumor cell.

[0028] The binding activity of the antibody against tumor cells can be confirmed using flow cytometry. The internalization of the antibody into tumor cells can be confirmed using (1) an assay of visualizing an antibody incorporated in cells under a fluorescence microscope using a secondary antibody (fluorescently labeled) binding to the therapeutic antibody (Cell Death and Differentiation (2008) 15, 751-761), (2) an assay of measuring the amount of fluorescence incorporated in cells using a secondary antibody (fluorescently labeled) binding to the therapeutic antibody (Molecular Biology of the Cell, Vol. 15, 5268-5282, December 2004), or (3) a Mab-ZAP assay using an immunotoxin binding to the therapeutic antibody wherein the toxin is released upon incorporation into cells to inhibit cell growth (Bio Techniques 28: 162-165, January 2000).

[0029] The antitumor activity of the antibody refers to a cytotoxic activity or cytotoxic effect against tumor cells and can be confirmed *in vitro* by determining inhibitory activity against cell growth. For example, a cancer cell line overexpressing a target protein for the antibody is cultured, and the antibody is added at varying concentrations into the culture system to determine an inhibitory activity against focus formation, colony formation, and spheroid growth. The antitumor activity can be confirmed *in vivo*, for example, by administering the antibody to a nude mouse with a transplanted tumor cell line highly expressing the target protein, and determining change in the cancer cell. Since the drug conjugated in the antibody-drug conjugate exerts an antitumor effect, it is more preferred but not essential that the antibody itself

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should have an antitumor effect. For exerting the antitumor effect and also for specifically and selectively damaging tumor cells by the drug, it is important and also preferred that the antibody should have the property of internalizing to migrate into tumor cells.

[0030] Examples of such an antibody can include, but not limited to, an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, and an anti-mesothelin antibody.

[0031] The antibody of the present invention is preferably an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD70 antibody, or an anti-B7-H3 antibody, and more preferably an anti-B7-H3 antibody.

[0032] The antibody of the present invention can be yielded using a method usually carried out in the art, which involves immunizing animals with an antigenic polypeptide and collecting and purifying antibodies produced *in vivo*. The origin of the antigen is not limited to humans, and the animals may be immunized with an antigen derived from a non-human animal such as a mouse, a rat and the like. In this case, the cross-reactivity of antibodies binding to the yielded heterologous antigen with human antigens can be tested to screen for an antibody applicable to a human disease.

[0033] Alternatively, antibody-producing cells which produce antibodies against the antigen are fused with myeloma cells according to a method known in the art (e.g., Kohler and Milstein, *Nature* (1975) 256, p. 495-497; and Kennet, R. ed., *Monoclonal Antibodies*, p. 365-367, Plenum Press, N.Y. (1980)) to establish hybridomas, from which monoclonal antibodies can in turn be yielded.

[0034] The antigen can be yielded by genetically engineering host cells to produce a gene encoding the antigenic protein. Specifically, vectors that permit expression of the antigen gene are prepared and transferred to host cells so that the gene is expressed. The antigen thus expressed can be purified.

[0035] The anti-CD30 antibody, the anti-CD33 antibody, and the anti-CD70 antibody can be yielded by an approach known in the art with reference to WO2002/043661, U.S. Patent No. 5,773,001, and WO2006/113909, respectively.

[0036] The B7-H3 antibody used in the present invention is preferably those having properties as described below.

(1) An antibody having the following properties:

- (a) specifically binding to B7-H3,
- (b) having antibody-dependent cell-mediated phagocytosis (ADCP) activity, and
- (c) having antitumor activity *in vivo*.

(2) The antibody according to (1), wherein B7-H3 is a molecule comprising the amino acid sequence represented by SEQ ID NO: 1 or 2.

(3) The antibody according to (1) or (2), wherein the antibody has CDRH1 comprising the amino acid sequence represented by SEQ ID NO: 3, CDRH2 comprising the amino acid sequence represented by SEQ ID NO: 4, and CDRH3 comprising the amino acid sequence represented by SEQ ID NO: 5 as heavy chain complementarity determining regions, and CDRL1 comprising the amino acid sequence represented by SEQ ID NO: 6, CDRL2 comprising the amino acid sequence represented by SEQ ID NO: 7, and CDRL3 comprising the amino acid sequence represented by SEQ ID NO: 8 as light chain complementarity determining regions.

(4) The antibody according to any of (1) to (3), wherein the constant region thereof is a human-derived constant region.

(5) The antibody according to any of (1) to (4), wherein the antibody is a humanized antibody.

(6) The antibody according to (5), wherein the antibody has a heavy chain variable region comprising an amino acid sequence selected from the group consisting of (a) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 9, (b) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 10, (c) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 11, (d) an amino acid sequence described in amino acid positions 20 to 141 in SEQ ID NO: 12, (e) an amino acid sequence having at least 95% or higher homology to any of the sequences (a) to (d), and (f) an amino acid sequence derived from any of the sequences (a) to (d) by the deletions, replacements, or additions of at least one amino acid, and a light chain variable region comprising an amino acid sequence selected from the group consisting of (g) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 13, (h) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 14, (i) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 15, (j) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 16, (k) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 17, (l) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 18, (m) an amino acid sequence described in amino acid positions 21 to 128 in SEQ ID NO: 19, (n) an amino acid sequence having at least 95% or higher homology to any of the sequences (g) to (m), and (o) an amino acid sequence derived from any of the sequences (g) to (m) by the deletions, replacements, or additions of at least one amino acid.

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- represented by SEQ ID NO: 16, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 17, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 18, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 9 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 19, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 13, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 14, a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 15, and a heavy chain comprising the amino acid sequence represented by SEQ ID NO: 12 and a light chain comprising the amino acid sequence represented by SEQ ID NO: 16.
- (10) The antibody according to (8) or (9), wherein the antibody lacks an amino acid at the carboxy terminus of the amino acid sequence represented by SEQ ID NO: 9 or 12 in the heavy chain.
- (11) An antibody yielded by a method for producing the antibody according to any of (1) to (10), the method comprising the steps of: culturing a host cell transformed with an expression vector containing a polynucleotide encoding the antibody; and collecting the antibody of interest from the cultures yielded in the preceding step.
- (12) The antibody according to any of (1) to (11), wherein the modification of a glycan is regulated in order to enhance antibody-dependent cytotoxic activity.
- [0037] Hereinafter, the B7-H3 antibody used in the invention is described.
- [0038] The terms "cancer" and "tumor" as used herein are used with the same meaning.
- [0039] The term "gene" as used herein includes not only DNA, but also mRNA thereof, cDNA thereof and cRNA thereof.
- [0040] The term "polynucleotide" as used herein is used with the same meaning as a nucleic acid and also includes DNA, RNA, probes, oligonucleotides, and primers.
- [0041] The terms "polypeptide" and "protein" as used herein are used without distinction.
- [0042] The term "cell" as used herein also includes cells in an animal individual and cultured cells.
- [0043] The term "B7-H3" as used herein is used in the same meaning as B7-H3 protein, and also refers to B7-H3 variant 1 and/or B7-H3 variant 2.
- [0044] The term "CDR" as used herein refers to a complementarity determining region (CDR), and it is known that each heavy and light chain of an antibody molecule has three complementarity determining regions (CDRs). The CDR is also called the hypervariable region, and is present in a variable region of each heavy and light chain of an antibody. It is a site which has unusually high variability in its primary structure, and there are three separate CDRs in the primary structure of each heavy and light polypeptide chain. In this specification, as for the CDRs of an antibody, the CDRs of the heavy chain are represented by CDRH1, CDRH2, and CDRH3 from the amino-terminal side of the amino acid sequence of the heavy chain, and the CDRs of the light chain are represented by CDRL1, CDRL2, and CDRL3 from the amino-terminal side of the amino acid sequence of the light chain. These sites are proximate to one another in the tertiary structure and determine the specificity for an antigen to which the antibody binds.
- [0045] The phrase "hybridization is performed under stringent conditions" as used herein refers to a process in which hybridization is performed under conditions under which identification can be achieved by performing hybridization at 68°C in a commercially available hybridization solution ExpressHyb Hybridization Solution (manufactured by Clontech, Inc.) or by performing hybridization at 68°C in the presence of 0.7 to 1.0 M NaCl using a filter having DNA immobilized thereon, followed by performing washing at 68°C using 0.1 to 2 x SSC solution (1 x SSC solution is composed of 150 mM NaCl and 15 mM sodium citrate) or under conditions equivalent thereto.
1. B7-H3
- [0046] B7-H3 is a member of the B7 family expressed on antigen-presenting cells as a co-stimulatory molecule, and is considered to act on a receptor on T cells to enhance or suppress immune activity.
- [0047] B7-H3 is a protein having a single-pass transmembrane structure, and the N-terminal extracellular domain of B7-H3 contains two variants. The B7-H3 variant 1 (4Ig-B7-H3) contains a V-like or C-like Ig domain at two sites, respectively, and the B7-H3 variant 2 (2Ig-B7-H3) contains a V-like or C-like Ig domain at one site, respectively.
- [0048] As for B7-H3 to be used in the invention, B7-H3 can be directly purified from B7-H3-expressing cells of a human or a non-human mammal (such as a rat or a mouse) and used, or a cell membrane fraction of the above-described cells can be prepared and used. Further, B7-H3 can be yielded by in vitro synthesis thereof or production thereof in a host cell through genetic engineering. In the genetic engineering, specifically, after B7-H3 cDNA is integrated into a vector capable of expressing B7-H3 cDNA, B7-H3 can be yielded by synthesizing it in a solution containing an enzyme, a substrate and an energy substance required for transcription and translation, or by expressing B7-H3 in another prokaryotic or eucaryotic transformed host cell.

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[0049] The amino acid sequence of an open reading frame (ORF) of a human B7-H3 variant 1 gene is represented by SEQ ID NO: 1 in the Sequence Listing. Further, the sequence of SEQ ID NO: 1 is shown in Fig. 1.

[0050] The amino acid sequence of an ORF of a human B7-H3 variant 2 gene is represented by SEQ ID NO: 2 in the Sequence Listing. Further, the sequence of SEQ ID NO: 2 is shown in Fig. 2.

5 [0051] Further, a protein which consists of an amino acid sequence wherein one or several amino acids are substituted, deleted and/or added in any of the above-described amino acid sequences of B7-H3 and also has a biological activity equivalent to that of the protein is also included in B7-H3.

[0052] Mature human B7-H3 variant 1 from which the signal sequence has been removed corresponds to an amino acid sequence consisting of amino acid residues 27 to 534 of the amino acid sequence represented by SEQ ID NO: 1. Further, mature human B7-H3 variant 2 from which the signal sequence has been removed corresponds to an amino acid sequence consisting of amino acid residues 27 to 316 of the amino acid sequence represented by SEQ ID NO: 2.

2. Production of anti-B7-H3 antibody

15 [0053] The antibody against B7-H3 of the invention can be yielded by immunizing an animal with B7-H3 or an arbitrary polypeptide selected from the amino acid sequence of B7-H3, and collecting and purifying the antibody produced in vivo according to a common procedure. The biological species of B7-H3 to be used as an antigen is not limited to being human, and an animal can be immunized with B7-H3 derived from an animal other than humans such as a mouse or a rat. In this case, by examining the cross-reactivity between an antibody binding to the yielded heterologous B7-H3 and human B7-H3, an antibody applicable to a human disease can be selected.

20 [0054] Further, a monoclonal antibody can be yielded from a hybridoma established by fusing antibody-producing cells which produce an antibody against B7-H3 with myeloma cells according to a known method (for example, Kohler and Milstein, *Nature*, (1975) 256, pp. 495-497; Kennet, R. ed., *Monoclonal Antibodies*, pp. 365-367, Plenum Press, N.Y. (1980)).

25 [0055] B7-H3 to be used as an antigen can be yielded by expressing B7-H3 gene in a host cell using genetic engineering. [0056] Specifically, a vector capable of expressing B7-H3 gene is produced, and the resulting vector is transfected into a host cell to express the gene, and then, the expressed B7-H3 is purified. Hereinafter, a method of yielding an antibody against B7-H3 is specifically described.

30 (1) Preparation of antigen

[0057] Examples of the antigen to be used for producing the anti-B7-H3 antibody include B7-H3, a polypeptide consisting of a partial amino acid sequence comprising at least 6 consecutive amino acids of B7-H3, and a derivative yielded by adding a given amino acid sequence or carrier thereto.

35 [0058] B7-H3 can be purified directly from human tumor tissues or tumor cells and used. Further, B7-H3 can be yielded by synthesizing it in vitro or by producing it in a host cell by genetic engineering.

[0059] With respect to the genetic engineering, specifically, after B7-H3 cDNA is integrated into a vector capable of expressing B7-H3 cDNA, B7-H3 can be yielded by synthesizing it in a solution containing an enzyme, a substrate and an energy substance required for transcription and translation, or by expressing B7-H3 in another prokaryotic or eucaryotic transformed host cell.

40 [0060] Further, the antigen can also be yielded as a secretory protein by expressing a fusion protein yielded by ligating the extracellular domain of B7-H3, which is a membrane protein, to the constant region of an antibody in an appropriate host-vector system.

45 [0061] B7-H3 cDNA can be yielded by, for example, a so-called PCR method in which a polymerase chain reaction (hereinafter referred to as "PCR") is performed using a cDNA library expressing B7-H3 cDNA as a template and primers which specifically amplify B7-H3 cDNA (see Saiki, R. K., et al., *Science*, (1988) 239, pp. 487-489).

[0062] As the in vitro synthesis of the polypeptide, for example, Rapid Translation System (RTS) manufactured by Roche Diagnostics, Inc. can be exemplified, but it is not limited thereto.

50 [0063] Examples of the prokaryotic host cells include *Escherichia coli* and *Bacillus subtilis*. In order to transform the host cells with a target gene, the host cells are transformed by a plasmid vector comprising a replicon, i.e., a replication origin derived from a species compatible with the host, and a regulatory sequence. Further, the vector preferably has a sequence capable of imposing phenotypic selectivity on the transformed cell.

[0064] Examples of the eucaryotic host cells include vertebrate cells, insect cells, and yeast cells. As the vertebrate cells, for example, simian COS cells (Gluzman, Y., *Cell*, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., *Proc. Natl. Acad. Sci. USA* (1980) 77, pp. 4126-4220) of Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61); and the like are often used, however, the cells are not limited thereto.

[0065] The thus yielded transformant can be cultured according to a common procedure, and by the culturing of the

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transformant, a target polypeptide is produced intracellularly or extracellularly.

[0066] A suitable medium to be used for the culturing can be selected from various commonly used culture media depending on the employed host cells. If *Escherichia coli* is employed, for example, an LB medium supplemented with an antibiotic such as ampicillin or IPMG as needed can be used.

5 [0067] A recombinant protein produced intracellularly or extracellularly by the transformant through such culturing can be separated and purified by any of various known separation methods utilizing the physical or chemical property of the protein.

[0068] Specific examples of the methods include treatment with a common protein precipitant, ultrafiltration, various types of liquid chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, and affinity chromatography, dialysis, and a combination thereof.

10 [0069] Further, by attaching a tag of six histidine residues to a recombinant protein to be expressed, the protein can be efficiently purified with a nickel affinity column. Alternatively, by attaching the IgG Fc region to a recombinant protein to be expressed, the protein can be efficiently purified with a protein A column.

15 [0070] By combining the above-described methods, a large amount of a target polypeptide can be easily produced in high yield and high purity.

(2) Production of anti-B7-H3 monoclonal antibody

20 [0071] Examples of the antibody specific binding to B7-H3 include a monoclonal antibody specific binding to B7-H3, and a method of yielding the antibody is as described below.

[0072] The production of a monoclonal antibody generally requires the following operational steps of:

- (a) purifying a biopolymer to be used as an antigen;
- 25 (b) preparing antibody-producing cells by immunizing an animal by injection of the antigen, collecting the blood, assaying its antibody titer to determine when the spleen is excised;
- (c) preparing myeloma cells (hereinafter referred to as "myeloma");
- (d) fusing the antibody-producing cells with the myeloma;
- (e) screening a group of hybridomas producing a desired antibody;
- 30 (f) dividing the hybridomas into single cell clones (cloning);
- (g) optionally, culturing the hybridoma or rearing an animal implanted with the hybridoma for producing a large amount of a monoclonal antibody;
- (h) examining the thus produced monoclonal antibody for biological activity and binding specificity, or assaying the same for properties as a labeled reagent; and the like.

35 [0073] Hereinafter, the method of producing a monoclonal antibody will be described in detail following the above steps, however, the method is not limited thereto, and, for example, antibody-producing cells other than spleen cells and myeloma can be used.

(a) Purification of antigen

40 [0074] As the antigen, B7-H3 prepared by the method as described above or a partial peptide thereof can be used.

[0075] Further, a membrane fraction prepared from recombinant cells expressing B7-H3 or the recombinant cells expressing B7-H3 themselves, and also a partial peptide of the protein of the invention chemically synthesized by a method known to those skilled in the art can also be used as the antigen.

45 (b) Preparation of antibody-producing cells

[0076] The antigen yielded in the step (a) is mixed with an adjuvant such as Freund's complete or incomplete adjuvant or aluminum potassium sulfate and the resulting mixture is used as an immunogen to immunize an experimental animal. As the experimental animal, any animal used in a known hybridoma production method can be used without any trouble. Specifically, for example, a mouse, a rat, a goat, sheep, cattle, a horse, or the like can be used. However, from the viewpoint of ease of availability of myeloma cells to be fused with the extracted antibody-producing cells, a mouse or a rat is preferably used as the animal to be immunized.

55 [0077] Further, the strain of a mouse or a rat to be used is not particularly limited, and in the case of a mouse, for example, various strains such as A, AKR, BALB/c, BDP, BA, CE, C3H, 57BL, C57BL, C57L, DBA, FL, HTH, HT1, LP, NZB, NZW, RF, R III, SJL, SWR, WB, and 129 and the like can be used, and in the case of a rat, for example, Wistar, Low, Lewis, Sprague, Dawley, ACI, BN, Fischer and the like can be used.

[0078] These mice and rats are commercially available from breeders/distributors of experimental animals, for example,

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CLEA Japan, Inc. and Charles River Laboratories Japan, Inc.

[0079] Among these, in consideration of compatibility of fusing with myeloma cells described below, in the case of a mouse, BALB/c strain, and in the case of a rat, Wistar and Low strains are particularly preferred as the animal to be immunized.

5 **[0080]** Further, in consideration of antigenic homology between humans and mice, it is also preferred to use a mouse having decreased biological function to remove auto-antibodies, that is, a mouse with an autoimmune disease.

[0081] The age of such mouse or rat at the time of immunization is preferably 5 to 12 weeks of age, more preferably 6 to 8 weeks of age.

10 **[0082]** In order to immunize an animal with B7-H3 or a recombinant thereof, for example, a known method described in detail in, for example, Weir, D. M., Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher Springfield, Illinois (1964) or the like can be used.

[0083] Among these immunization methods, a preferred specific method in the invention is, for example, as follows.

15 **[0084]** That is, first, a membrane protein fraction serving as the antigen or cells caused to express the antigen is/are intradermally or intraperitoneally administrated to an animal.

[0085] However, the combination of both routes of administration is preferred for increasing the immunization efficiency, and when intradermal administration is performed in the first half and intraperitoneal administration is performed in the latter half or only at the last dosing, the immunization efficiency can be particularly increased.

20 **[0086]** The administration schedule of the antigen varies depending on the type of animal to be immunized, individual difference or the like. However, in general, an administration schedule in which the frequency of administration of the antigen is 3 to 6 times and the dosing interval is 2 to 6 weeks is preferred, and an administration schedule in which the frequency of administration of the antigen is 3 to 4 times and the dosing interval is 2 to 4 weeks is more preferred.

[0087] Further, the dose of the antigen varies depending on the type of animal, individual differences or the like, however, the dose is generally set to 0.05 to 5 mg, preferably about 0.1 to 0.5 mg.

25 **[0088]** A booster immunization is performed 1 to 6 weeks, preferably 2 to 4 weeks, more preferably 2 to 3 weeks after the administration of the antigen as described above.

[0089] The dose of the antigen at the time of performing the booster immunization varies depending on the type or size of animal or the like, however, in the case of, for example, a mouse, the dose is generally set to 0.05 to 5 mg, preferably 0.1 to 0.5 mg, more preferably about 0.1 to 0.2 mg.

30 **[0090]** Spleen cells or lymphocytes including antibody-producing cells are aseptically removed from the immunized animal 1 to 10 days, preferably 2 to 5 days, more preferably 2 to 3 days after the booster immunization. At this time, the antibody titer is measured, and if an animal having a sufficiently increased antibody titer is used as a supply source of the antibody-producing cells, the subsequent procedure can be carried out more efficiently.

35 **[0091]** Examples of the method of measuring the antibody titer to be used here include an RIA method and an ELISA method, but the method is not limited thereto.

[0092] For example, if an ELISA method is employed, the measurement of the antibody titer in the invention can be carried out according to the procedures as described below.

40 **[0093]** First, a purified or partially purified antigen is adsorbed to the surface of a solid phase such as a 96-well plate for ELISA, and the surface of the solid phase having no antigen adsorbed thereto is covered with a protein unrelated to the antigen such as bovine serum albumin (hereinafter referred to as "BSA"). After washing the surface, the surface is brought into contact with a serially-diluted sample (for example, mouse serum) as a primary antibody to allow the antibody in the sample to bind to the antigen.

45 **[0094]** Further, as a secondary antibody, an antibody labeled with an enzyme against a mouse antibody is added and is allowed to bind to the mouse antibody. After washing, a substrate for the enzyme is added and a change in absorbance which occurs due to color development induced by degradation of the substrate or the like is measured and the antibody titer is calculated based on the measurement.

50 **[0095]** The separation of the antibody-producing cells from the spleen cells or lymphocytes of the immunized animal can be carried out according to a known method (for example, Kohler et al., Nature (1975), 256, p. 495; Kohler et al., Eur. J. Immunol. (1977), 6, p. 511; Milstein et al., Nature (1977), 266, p. 550; Walsh, Nature (1977), 266, p. 495). For example, in the case of spleen cells, a general method in which the antibody-producing cells are separated by homogenizing the spleen to yield the cells through filtration with a stainless steel mesh and suspending the cells in Eagle's Minimum Essential Medium (MEM) can be employed.

(c) Preparation of myeloma cells (hereinafter referred to as "myeloma")

55 **[0096]** The myeloma cells to be used for cell fusion are not particularly limited and suitable cells can be selected from known cell lines. However, in consideration of convenience when a hybridoma is selected from fused cells, it is preferred to use an HGPRT (hypoxanthine-guanine phosphoribosyl transferase) deficient strain whose selection procedure has

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been established.

[0097] More specifically, examples of the HGPRT-deficient strain include X63-Ag8(X63), NS1-ANS/1(NS1), P3X63-Ag8.U1(P3U1), X63-Ag8.653(X63.653), SP2/0-Ag14(SP2/0), MPC11-45.6TG1.7(45.6TG), FO, S149/5XXO, and BU.1 derived from mice; 210.RSY3.Ag.1.2.3(Y3) derived from rats; and U266AR(SKO-007), GM1500-GTG-A12(GM1500), UC729-6, LICR-LOW-HMy2(HMy2) and 8226AR/NIP4-1(NP41) derived from humans. These HGPRT-deficient strains

are available from, for example, the American Type Culture Collection (ATCC) or the like.
[0098] These cell strains are subcultured in an appropriate medium such as an 8-azaguanine medium [a medium yielded by adding 8-azaguanine to an RPMI 1640 medium supplemented with glutamine, 2-mercaptoethanol, gentamicin, and fetal calf serum (hereinafter referred to as "FCS")], Iscove's Modified Dulbecco's Medium (hereinafter referred to as "IMDM"), or Dulbecco's Modified Eagle Medium (hereinafter referred to as "DMEM"). In this case, 3 to 4 days before performing cell fusion, the cells are subcultured in a normal medium [for example, an ASF104 medium (manufactured by Ajinomoto Co., Ltd.) containing 10% FCS] to ensure not less than 2×10^7 cells on the day of cell fusion.

(d) Cell fusion

[0099] Fusion between the antibody-producing cells and the myeloma cells can be appropriately performed according to a known method (Weir, D. M. Handbook of Experimental Immunology Vol. I. II. III., Blackwell Scientific Publications, Oxford (1987), Kabat, E. A. and Mayer, M. M., Experimental Immunochemistry, Charles C Thomas Publisher, Springfield, Illinois (1964), etc.), under conditions such that the survival rate of cells is not excessively reduced.

[0100] As such a method, for example, a chemical method in which the antibody-producing cells and the myeloma cells are mixed in a solution containing a polymer such as polyethylene glycol at a high concentration, a physical method using electric stimulation, or the like can be used. Among these methods, a specific example of the chemical method is as described below.

[0101] That is, in the case where polyethylene glycol is used in the solution containing a polymer at a high concentration, the antibody-producing cells and the myeloma cells are mixed in a solution of polyethylene glycol having a molecular weight of 1500 to 6000, more preferably 2000 to 4000 at a temperature of from 30 to 40°C, preferably from 35 to 38°C for 1 to 10 minutes, preferably 5 to 8 minutes.

(e) Selection of a group of hybridomas

[0102] The method of selecting hybridomas yielded by the above-described cell fusion is not particularly limited. Usually, an HAT (hypoxanthine, aminopterin, thymidine) selection method (Kohler et al., Nature (1975), 256, p. 495; Milstein et al., Nature (1977), 266, p. 550) is used.

[0103] This method is effective when hybridomas are yielded using the myeloma cells of an HGPRT-deficient strain which cannot survive in the presence of aminopterin.

[0104] That is, by culturing unfused cells and hybridomas in an HAT medium, only hybridomas resistant to aminopterin are selectively allowed to survive and proliferate.

(f) Division into single cell clone (cloning)

[0105] As a cloning method for hybridomas, a known method such as a methylcellulose method, a soft agarose method, or a limiting dilution method can be used (see, for example, Barbara, B. M. and Stanley, M. S.: Selected Methods in Cellular Immunology, W. H. Freeman and Company, San Francisco (1980)). Among these methods, particularly, a three-dimensional culture method such as a methylcellulose method is preferred. For example, the group of hybridomas produced by cell fusion are suspended in a methylcellulose medium such as ClonaCell-HY Selection Medium D (manufactured by StemCell Technologies, inc., #03804) and cultured. Then, the formed hybridoma colonies are collected, whereby monoclonal hybridomas can be yielded. The collected respective hybridoma colonies are cultured, and a hybridoma which has been confirmed to have a stable antibody titer in an yielded hybridoma culture supernatant is selected as a B7-H3 monoclonal antibody-producing hybridoma strain.

[0106] Examples of the thus established hybridoma strain include B7-H3 hybridoma M30. In this specification, an antibody produced by the B7-H3 hybridoma M30 is referred to as "M30 antibody" or simply "M30".

[0107] The heavy chain of the M30 antibody has an amino acid sequence represented by SEQ ID NO: 20 in the Sequence Listing. Further, the light chain of the M30 antibody has an amino acid sequence represented by SEQ ID NO: 21 in the Sequence Listing. In the heavy chain amino acid sequence represented by SEQ ID NO: 20 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 19 is a signal sequence, an amino acid sequence consisting of amino acid residues 20 to 141 is a variable region, and an amino acid sequence consisting of amino acid residues 142 to 471 is a constant region. Further, in the light chain amino acid sequence represented by SEQ ID NO: 21 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 22 is a signal sequence, an

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amino acid sequence consisting of amino acid residues 23 to 130 is a variable region, and an amino acid sequence consisting of amino acid residues 131 to 235 is a constant region.

(g) Preparation of monoclonal antibody by culturing hybridoma

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[0108] By culturing the thus selected hybridoma, a monoclonal antibody can be efficiently yielded. However, prior to culturing, it is preferred to perform screening of a hybridoma which produces a target monoclonal antibody.

[0109] In such screening, a known method can be employed.

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[0110] The measurement of the antibody titer in the invention can be carried out by, for example, an ELISA method explained in item (b) described above.

[0111] The hybridoma yielded by the method described above can be stored in a frozen state in liquid nitrogen or in a freezer at -80°C or below.

[0112] After completion of cloning, the medium is changed from an HT medium to a normal medium, and the hybridoma is cultured.

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[0113] Large-scale culture is performed by rotation culture using a large culture bottle or by spinner culture. From the supernatant yielded by the large-scale culture, a monoclonal antibody which specifically binds to the protein of the invention can be yielded by purification using a method known to those skilled in the art such as gel filtration.

[0114] Further, the hybridoma is injected into the abdominal cavity of a mouse of the same strain as the hybridoma (for example, the above-described BALB/c) or a Nu/Nu mouse to proliferate the hybridoma, whereby the ascites containing a large amount of the monoclonal antibody of the invention can be yielded.

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[0115] In the case where the hybridoma is administrated in the abdominal cavity, if a mineral oil such as 2,6,10,14-tetramethylpentadecane (pristane) is administrated 3 to 7 days prior thereto, a larger amount of the ascites can be yielded.

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[0116] For example, an immunosuppressant is previously injected into the abdominal cavity of a mouse of the same strain as the hybridoma to inactivate T cells. 20 days thereafter, 10^6 to 10^7 hybridoma clone cells are suspended in a serum-free medium (0.5 ml), and the suspension is administrated in the abdominal cavity of the mouse. In general, when the abdomen is expanded and filled with the ascites, the ascites is collected from the mouse. By this method, the monoclonal antibody can be yielded at a concentration which is about 100 times or much higher than that in the culture solution.

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[0117] The monoclonal antibody yielded by the above-described method can be purified by a method described in, for example, Weir, D. M.: Handbook of Experimental Immunology Vol. I, II, III, Blackwell Scientific Publications, Oxford (1978).

[0118] The thus yielded monoclonal antibody has high antigen specificity for B7-H3.

(h) Assay of monoclonal antibody

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[0119] The isotype and subclass of the thus yielded monoclonal antibody can be determined as follows.

[0120] First, examples of the identification method include an Ouchterlony method, an ELISA method, and an RIA method.

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[0121] An Ouchterlony method is simple, but when the concentration of the monoclonal antibody is low, a condensation operation is required.

[0122] On the other hand, when an ELISA method or an RIA method is used, by directly reacting the culture supernatant with an antigen-adsorbed solid phase and using antibodies corresponding to various types of immunoglobulin isotypes and subclasses as secondary antibodies, the isotype and subclass of the monoclonal antibody can be identified.

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[0123] In addition, as a simpler method, a commercially available identification kit (for example, Mouse Typer Kit manufactured by Bio-Rad Laboratories, Inc.) or the like can also be used.

[0124] Further, the quantitative determination of a protein can be performed by the Folin Lowry method and a method of calculation based on the absorbance at 280 nm [$1.4 \text{ (OD 280)} = \text{Immunoglobulin } 1 \text{ mg/ml}$].

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[0125] Further, even when the monoclonal antibody is separately and independently yielded by performing again the steps of (a) to (h) in (2), it is possible to yield an antibody having a cytotoxic activity equivalent to that of the M30 antibody.

As one example of such an antibody, an antibody which binds to the same epitope as the M30 antibody can be exemplified. The M30 recognizes an epitope in the IgC1 or IgC2 domain, which is a domain in the B7-H3 extracellular domain, and binds to the IgC1 domain or the IgC2 domain or both. Therefore, as the epitope for the antibody of the invention, particularly, an epitope present in the IgC1 or IgC2 domain of B7-H3 can be exemplified. If a newly produced monoclonal antibody binds to a partial peptide or a partial tertiary structure to which the M30 antibody binds, it can be determined

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that the monoclonal antibody binds to the same epitope as the M30 antibody. Further, by confirming that the monoclonal antibody competes with the M30 antibody for the binding to B7-H3 (that is, the monoclonal antibody inhibits the binding between the M30 antibody and B7-H3), it can be determined that the monoclonal antibody binds to the same epitope as the M30 antibody even if the specific epitope sequence or structure has not been determined. When it is confirmed

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that the monoclonal antibody binds to the same epitope as the M30 antibody, the monoclonal antibody is strongly expected to have a cytotoxic activity equivalent to that of the M30 antibody.

(3) Other antibodies

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[0126] The antibody of the invention includes not only the above-described monoclonal antibody against B7-H3 but also a recombinant antibody yielded by artificial modification for the purpose of decreasing heterologous antigenicity to humans such as a chimeric antibody, a humanized antibody and a human antibody. These antibodies can be produced using a known method.

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[0127] As the chimeric antibody, an antibody in which antibody variable and constant regions are derived from different species, for example, a chimeric antibody in which a mouse- or rat-derived antibody variable region is connected to a human-derived antibody constant region can be exemplified (see Proc. Natl. Acad. Sci. USA, 81, 6851-6855, (1984)).

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[0128] As the humanized antibody, an antibody yielded by integrating only a complementarity determining region (CDR) into a human-derived antibody (see Nature (1986) 321, pp. 522-525), and an antibody yielded by grafting a part of the amino acid residues of the framework as well as the CDR sequence to a human antibody by a CDR-grafting method (WO 90/07861) can be exemplified.

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[0129] However, the humanized antibody derived from the M30 antibody is not limited to a specific humanized antibody as long as the humanized antibody has all 6 types of CDR sequences of the M30 antibody and has an antitumor activity. The heavy chain variable region of the M30 antibody has CDRH1 (NYVMH) consisting of an amino acid sequence represented by SEQ ID NO: 3 in the Sequence Listing, CDRH2 (YINPYNDDVKYNEKFKG) consisting of an amino acid sequence represented by SEQ ID NO: 4 in the Sequence Listing, and CDRH3 (WGYYGSPLYFFDY) consisting of an amino acid sequence represented by SEQ ID NO: 5 in the Sequence Listing. Further, the light chain variable region of the M30 antibody has CDRL1 (RASSRLIYMH) consisting of an amino acid sequence represented by SEQ ID NO: 6 in the Sequence Listing, CDRL2 (ATSNLAS) consisting of an amino acid sequence represented by SEQ ID NO: 7 in the Sequence Listing, and CDRL3 (QQWNSNPPT) consisting of an amino acid sequence represented by SEQ ID NO: 8 in the Sequence Listing.

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[0130] As an example of the humanized antibody of a mouse antibody M30, an arbitrary combination of a heavy chain comprising a heavy chain variable region consisting of any one of (1) an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9, 10, 11, or 12 in the Sequence Listing, (2) an amino acid sequence having a homology of at least 95% or more with the amino acid sequence (1) described above, and (3) an amino acid sequence wherein one or several amino acids in the amino acid sequence (1) described above are deleted, substituted or added and a light chain comprising a light chain variable region consisting of any one of (4) an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 13, 14, 15, 16, 17, 18, or 19 in the Sequence Listing, (5) an amino acid sequence having a homology of at least 95% or more with the amino acid sequence (4) described above, and (6) an amino acid sequence wherein one or several amino acids in the amino acid sequence (4) described above are deleted, substituted or added can be exemplified.

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[0131] The term "several" as used herein refers to 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 or 2.

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[0132] As the amino acid substitution in this specification, a conservative amino acid substitution is preferred. The conservative amino acid substitution refers to a substitution occurring within a group of amino acids related to amino acid side chains. Preferred amino acid groups are as follows: an acidic group (aspartic acid and glutamic acid); a basic group (lysine, arginine, and histidine); a non-polar group (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan); and an uncharged polar family (glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine). More preferred amino acid groups are as follows: an aliphatic hydroxy group (serine and threonine); an amide-containing group (asparagine and glutamine); an aliphatic group (alanine, valine, leucine, and isoleucine); and an aromatic group (phenylalanine, tryptophan, and tyrosine). Such an amino acid substitution is preferably performed within a range which does not impair the properties of a substance having the original amino acid sequence.

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[0133] As an antibody which has a preferred combination of a heavy chain and a light chain described above, an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 13; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 14; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence consisting of amino acid residues 21 to 128 of SEQ ID NO: 15; an antibody consisting of a heavy chain comprising a heavy chain variable region consisting of an amino acid sequence consisting of amino acid residues 20 to 141 of SEQ ID NO: 9 and a light chain comprising a light chain variable region consisting of an amino acid sequence

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sequence of SEQ ID NO: 19; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 13; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 14; an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 15; and an antibody consisting of a heavy chain consisting of an amino acid sequence of SEQ ID NO: 12 and a light chain consisting of an amino acid sequence of SEQ ID NO: 16 can be exemplified.

[0136] By combining a sequence having a high homology with the above-described heavy chain amino acid sequence with a sequence having a high homology with the above-described light chain amino acid sequence, it is possible to select an antibody having a cytotoxic activity equivalent to that of each of the above-described antibodies. Such a homology is generally a homology of 80% or more, preferably a homology of 90% or more, more preferably a homology of 95% or more, most preferably a homology of 99% or more. Further, by combining an amino acid sequence wherein one to several amino acid residues are substituted, deleted or added in the heavy chain or light chain amino acid sequence, it is also possible to select an antibody having a cytotoxic activity equivalent to that of each of the above-described antibodies.

[0137] The homology between two amino acid sequences can be determined using default parameters of Blast algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25: 3389-3402). The Blast algorithm can be used also through the Internet by accessing the site www.ncbi.nlm.nih.gov/blast.

[0138] In the heavy chain amino acid sequence represented by SEQ ID NO: 9, 10, 11 or 12 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 19 is a signal sequence, an amino acid sequence consisting of amino acid residues 20 to 141 is a variable region, and an amino acid sequence consisting of amino acid residues 142 to 471 is a constant region. The sequence of SEQ ID NO: 9, 10, 11 and 12 are shown in Fig. 3, 4, 5 and 6 respectively. Further, in the light chain amino acid sequence represented by SEQ ID NO: 13, 14, 15, 16, 17, 18 or 19 in the Sequence Listing, an amino acid sequence consisting of amino acid residues 1 to 20 is a signal sequence, an amino acid sequence consisting of amino acid residues 21 to 128 is a variable region, and an amino acid sequence consisting of amino acid residues 129 to 233 is a constant region. The sequence of SEQ ID NO: 13, 14, 15, 16, 17, 18 and 19 are shown in Fig. 7, 8, 9, 10, 11, 12 and 13 respectively.

[0139] Further, the antibody of the invention includes a human antibody which binds to the same epitope as the M30 antibody. An anti-B7-H3 human antibody refers to a human antibody having only a sequence of an antibody derived from a human chromosome. The anti-B7-H3 human antibody can be yielded by a method using a human antibody-producing mouse having a human chromosome fragment comprising heavy and light chain genes of a human antibody (see Tomizuka, K. et al., *Nature Genetics* (1997) 16, pp. 133-143; Kuroiwa, Y. et al., *Nucl. Acids Res.* (1998) 26, pp. 3447-3448; Yoshida, H. et al., *Animal Cell Technology: Basic and Applied Aspects* vol. 10, pp. 69-73 (Kitagawa, Y., Matuda, T. and Iijima, S. eds.), Kluwer Academic Publishers, 1999; Tomizuka, K. et al., *Proc. Natl. Acad. Sci. USA* (2000) 97, pp. 722-727, etc.).

[0140] Such a human antibody-producing mouse can be created specifically as follows. A genetically modified animal in which endogenous immunoglobulin heavy and light chain gene loci have been disrupted, and instead, human immunoglobulin heavy and light chain gene loci have been introduced via a yeast artificial chromosome (YAC) vector or the like is created by producing a knockout animal and a transgenic animal and mating these animals.

[0141] Further, according to a recombinant DNA technique, by using cDNAs encoding each of such a heavy chain and a light chain of a human antibody, and preferably a vector comprising such cDNAs, eukaryotic cells are transformed, and a transformant cell which produces a recombinant human monoclonal antibody is cultured, whereby the antibody can also be yielded from the culture supernatant.

[0142] Here, as the host, for example, eukaryotic cells, preferably mammalian cells such as CHO cells, lymphocytes, or myeloma cells can be used.

[0143] Further, a method of yielding a phage display-derived human antibody selected from a human antibody library (see Wormstone, I. M. et al., *Investigative Ophthalmology & Visual Science*. (2002) 43 (7), pp. 2301-2308; Carmen, S. et al., *Briefings in Functional Genomics and Proteomics* (2002), 1 (2), pp. 189-203; Siriwardena, D. et al., *Ophthalmology* (2002) 109 (3), pp. 427-431, etc.) is also known.

[0144] For example, a phage display method in which a variable region of a human antibody is expressed on the surface of a phage as a single-chain antibody (scFv), and a phage which binds to an antigen is selected (*Nature Biotechnology* (2005), 23, (9), pp. 1105-1116) can be used.

[0145] By analyzing the gene of the phage selected based on the binding to an antigen, a DNA sequence encoding the variable region of a human antibody which binds to an antigen can be determined.

[0146] If the DNA sequence of scFv which binds to an antigen is determined, a human antibody can be yielded by preparing an expression vector comprising the sequence and introducing the vector into an appropriate host to express

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it (WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, WO 95/15388, Annu. Rev. Immunol. (1994) 12, pp. 433-455, Nature Biotechnology (2005) 23 (9), pp. 1105-1116).

[0147] If a newly produced human antibody binds to a partial peptide or a partial tertiary structure to which the M30 antibody binds, it can be determined that the human antibody binds to the same epitope as the M30 antibody. Further, by confirming that the human antibody competes with the M30 antibody for the binding to B7-H3 (that is, the human antibody inhibits the binding between the M30 antibody and B7-H3), it can be determined that the human antibody binds to the same epitope as the M30 antibody even if the specific epitope sequence or structure has not been determined. When it is confirmed that the human antibody binds to the same epitope as the M30 antibody, the human antibody is strongly expected to have a cytotoxic activity equivalent to that of the M30 antibody.

[0148] The chimeric antibodies, humanized antibodies, or human antibodies yielded by the above-described method are evaluated for the binding property to an antigen by a known method or the like, and a preferred antibody can be selected.

[0149] As one example of another index for use in the comparison of the properties of antibodies, the stability of antibodies can be exemplified. The differential scanning calorimetry (DSC) is a device capable of quickly and accurately measuring a thermal denaturation midpoint temperature (T_m) to be used as a favorable index of the relative conformational stability of proteins. By measuring the T_m values using DSC and comparing the values, a difference in thermal stability can be compared. It is known that the storage stability of antibodies shows some correlation with the thermal stability of antibodies (Lori Burton, et. al., Pharmaceutical Development and Technology (2007) 12, pp. 265-273), and a preferred antibody can be selected by using thermal stability as an index. Examples of other indices for selecting antibodies include the following features: the yield in an appropriate host cell is high; and the aggregability in an aqueous solution is low. For example, an antibody which shows the highest yield does not always show the highest thermal stability, and therefore, it is necessary to select an antibody most suitable for the administration to humans by making comprehensive evaluation based on the above-described indices.

[0150] In the invention, a modified variant of the antibody is also included. The modified variant refers to a variant yielded by subjecting the antibody of the invention to chemical or biological modification. Examples of the chemically modified variant include variants chemically modified by linking a chemical moiety to an amino acid skeleton, variants chemically modified with an N-linked or O-linked carbohydrate chain, etc. Examples of the biologically modified variant include variants yielded by post-translational modification (such as N-linked or O-linked glycosylation, N- or C-terminal processing, deamidation, isomerization of aspartic acid, or oxidation of methionine), and variants in which a methionine residue has been added to the N terminus by being expressed in a prokaryotic host cell.

[0151] Further, an antibody labeled so as to enable the detection or isolation of the antibody or an antigen of the invention, for example, an enzyme-labeled antibody, a fluorescence-labeled antibody, and an affinity-labeled antibody are also included in the meaning of the modified variant. Such a modified variant of the antibody of the invention is useful for improving the stability and blood retention of the original antibody of the invention, reducing the antigenicity thereof, detecting or isolating such an antibody or an antigen, and so on.

[0152] Further, by regulating the modification of a glycan which is linked to the antibody of the invention (glycosylation, defucosylation, etc.), it is possible to enhance an antibody-dependent cellular cytotoxic activity. As the technique for regulating the modification of a glycan of antibodies, WO 99/54342, WO 00/61739, WO 02/31140, etc. are known. However, the technique is not limited thereto. In the antibody of the invention, an antibody in which the modification of a glycan is regulated is also included.

[0153] In the case where an antibody is produced by first isolating an antibody gene and then introducing the gene into an appropriate host, a combination of an appropriate host and an appropriate expression vector can be used. Specific examples of the antibody gene include a combination of a gene encoding a heavy chain sequence of an antibody described in this specification and a gene encoding a light chain sequence thereof. When a host cell is transformed, it is possible to insert the heavy chain sequence gene and the light chain sequence gene into the same expression vector, and also into different expression vectors separately.

[0154] In the case where eukaryotic cells are used as the host, animal cells, plant cells, and eukaryotic microorganisms can be used. As the animal cells, mammalian cells, for example, simian COS cells (Gluzman, Y., Cell, (1981) 23, pp. 175-182, ATCC CRL-1650), murine fibroblasts NIH3T3 (ATCC No. CRL-1658), and dihydrofolate reductase-deficient strains (Urlaub, G. and Chasin, L. A., Proc. Natl. Acad. Sci. USA (1980) 77, pp. 4126-4220) of Chinese hamster ovarian cells (CHO cells; ATCC: CCL-61) can be exemplified.

[0155] In the case where prokaryotic cells are used, for example, Escherichia coli and Bacillus subtilis can be exemplified.

[0156] By introducing a desired antibody gene into these cells through transformation, and culturing the thus transformed cells in vitro, the antibody can be yielded. In the above-described culture method, the yield may sometimes vary depending on the sequence of the antibody, and therefore, it is possible to select an antibody which is easily produced as a pharmaceutical by using the yield as an index among the antibodies having an equivalent binding activity. Therefore, in the antibody of the invention, an antibody yielded by a method of producing an antibody, characterized by including

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a step of culturing the transformed host cell and a step of collecting a desired antibody from a cultured product yielded in the culturing step is also included.

[0157] It is known that a lysine residue at the carboxyl terminus of the heavy chain of an antibody produced in a cultured mammalian cell is deleted (Journal of Chromatography A, 705: 129-134 (1995)), and it is also known that two amino acid residues (glycine and lysine) at the carboxyl terminus of the heavy chain of an antibody produced in a cultured mammalian cell are deleted and a proline residue newly located at the carboxyl terminus is amidated (Analytical Biochemistry, 360: 75-83 (2007)). However, such deletion and modification of the heavy chain sequence do not affect the antigen-binding affinity and the effector function (the activation of a complement, the antibody-dependent cellular cytotoxicity, etc.) of the antibody. Therefore, in the invention, an antibody subjected to such modification is also included, and a deletion variant in which one or two amino acids have been deleted at the carboxyl terminus of the heavy chain, a variant yielded by amidation of the deletion variant (for example, a heavy chain in which the carboxyl terminal proline residue has been amidated), and the like can be exemplified. The type of deletion variant having a deletion at the carboxyl terminus of the heavy chain of the antibody according to the invention is not limited to the above variants as long as the antigen-binding affinity and the effector function are conserved. The two heavy chains constituting the antibody according to the invention may be of one type selected from the group consisting of a full-length heavy chain and the above-described deletion variant, or may be of two types in combination selected therefrom. The ratio of the amount of each deletion variant can be affected by the type of cultured mammalian cells which produce the antibody according to the invention and the culture conditions, however, a case where one amino acid residue at the carboxyl terminus has been deleted in both of the two heavy chains contained as main components in the antibody according to the invention can be exemplified.

[0158] As isotype of the antibody of the invention, for example, IgG (IgG1, IgG2, IgG3, IgG4) can be exemplified, and IgG1 or IgG2 can be exemplified preferably.

[0159] As the function of the antibody, generally an antigen-binding activity, an activity of neutralizing the activity of an antigen, an activity of enhancing the activity of an antigen, an antibody-dependent cellular cytotoxicity (ADCC) activity and a complement-dependent cytotoxicity (CDC) activity can be exemplified. The function of the antibody of the invention is a binding activity to B7-H3, preferably an antibody-dependent cell-mediated phagocytosis (ADCP) activity, more preferably a cytotoxicity activity (antitumor activity) to tumor cell mediated by an ADCP activity. Further, the antibody of the invention may have an ADCC activity and/or a CDC activity in addition to an ADCP activity.

[0160] The yielded antibody can be purified to homogeneity. The separation and purification of the antibody may be performed employing a conventional protein separation and purification method. For example, the antibody can be separated and purified by appropriately selecting and combining column chromatography, filter filtration, ultrafiltration, salt precipitation, dialysis, preparative polyacrylamide gel electrophoresis, isoelectric focusing electrophoresis, and the like (Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Daniel R. Marshak et al. eds., Cold Spring Harbor Laboratory Press (1996); Antibodies: A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988)), but the method is not limited thereto.

[0161] Examples of such chromatography include affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, reverse phase chromatography, and adsorption chromatography.

[0162] Such chromatography can be performed employing liquid chromatography such as HPLC or FPLC.

[0163] As a column to be used in affinity chromatography, a Protein A column and a Protein G column can be exemplified. For example, as a column using a Protein A column, Hyper D, POROS, Sepharose FF (Pharmacia) and the like can be exemplified.

[0164] Further, by using a carrier having an antigen immobilized thereon, the antibody can also be purified utilizing the binding property of the antibody to the antigen.

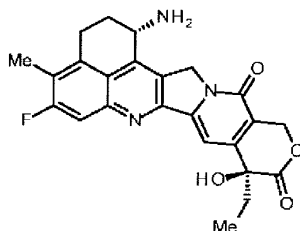
[Antitumor compound]

[0165] The antitumor compound to be conjugated to the antibody-drug conjugate of the present invention is explained. The antitumor compound is not particularly limited if it is a compound having an antitumor effect and a substituent group or a partial structure allowing connecting to a linker structure. When a part or whole linker is cleaved in tumor cells, the antitumor compound moiety is released to exhibit the antitumor effect of the antitumor compound. As the linker is cleaved at a connecting position to drug, the antitumor compound is released in its intrinsic structure to exhibit its intrinsic antitumor effect.

[0166] Examples of the antitumor compound can include doxorubicin, daunorubicin, mitomycin C, bleomycin, cyclocytidine, vincristine, vinblastine, methotrexate, platinum-based antitumor agent (cisplatin or derivatives thereof), taxol or derivatives thereof, and camptothecin or derivatives thereof (antitumor agent described in Japanese Patent Laid-Open No. 6-87746). In the antibody-drug conjugate of the present invention, exatecan as a camptothecin derivative (((1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione; shown in the following formula) can be preferably used.

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[Formula 29]



Although having an excellent antitumor effect, exatecan has not been commercialized as an antitumor drug. The compound can be easily obtained by a known method and the amino group at position 1 can be preferably used as a connecting position to the linker structure. Further, although exatecan can be also released in tumor cells while part of the linker is still attached thereto, it is an excellent compound exhibiting an excellent antitumor effect even in such case.

[0167] With regard to the antibody-drug conjugate, the number of conjugated drug molecules per antibody molecule is a key factor having an influence on the efficacy and safety. Production of the antibody-drug conjugate is performed by defining the reaction condition including the amounts of use of raw materials and reagents for reaction so as to have a constant number of conjugated drug molecules, a mixture containing different numbers of conjugated drug molecules is generally obtained unlike the chemical reaction of a low-molecular-weight compound. The number of drugs conjugated in an antibody molecule is expressed or specified by the average value, that is, the average number of conjugated drug molecules. Unless specifically described otherwise as a principle, the number of conjugated drug molecules means an average value except in a case in which it represents an antibody-drug conjugate having a specific number of conjugated drug molecules that is included in an antibody-drug conjugate mixture having different number of conjugated drug molecules. The number of exatecan molecules conjugated to an antibody molecule is controllable, and as an average number of conjugated drug molecules per antibody, about 1 to 10 exatecans can be bound. Preferably, it is 2 to 8, and more preferably 3 to 8. Meanwhile, a person skilled in the art can design a reaction for conjugating a required number of drug molecules to an antibody molecule based on the description of the Examples of the present application and can obtain an antibody conjugated with a controlled number of exatecan molecules.

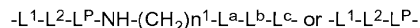
[0168] Because exatecan has a camptothecin structure, it is known that the equilibrium shifts to a structure with a closed lactone ring (closed ring) in an aqueous acidic medium (for example, pH 3 or so) but it shifts to a structure with an open lactone ring (open ring) in an aqueous basic medium (for example, pH 10 or so). A drug conjugate being introduced with an exatecan residue corresponding to the closed ring structure and the open ring structure is also expected to have the same antitumor effect and it is needless to say that any of them is within the scope of the present invention.

[Linker structure]

1. Linker having hydrophilic structure

[0169] The antibody-drug conjugate of the present invention is characterized by a linker structure in which a hydrophilic structure moiety is formed. This hydrophilic structure moiety is present in the L^P moiety, L^1 moiety, or L^2 moiety of the linker and plural of them may have the hydrophilic structure. This hydrophilic structure corresponds to the following cases. In case of linker L^P , either of the two following forms corresponds to, i.e., L^P is a peptide residue having a hydrophilic amino acid other than glycine at its N terminal, or L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the drug, and further, even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, in case of L^1 , L^1 corresponds to the form of - (Succinimid-3-yl-N)-CH[-(CH₂)_n-COOH]-C(=O)-, in case of L^2 , L^2 corresponds to the form of -N[-(CH₂CH₂-O)_n-CH₂CH₂-OH]-CH₂-C(=O)-.

[0170] Hereinbelow, the linker of the present invention is described. The linker of the present invention has a structure represented by the following formula:



The antibody is connected to the terminal of L^1 , terminal opposite to the connecting position to L^2 . The antitumor drug is connected to the terminal of L^c , terminal opposite to the connecting position to L^b , or the terminal of L^P , terminal

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opposite to the connecting position to L².

[0171] n¹ represents an integer of 0 to 6 and is preferably an integer of 1 to 5, and more preferably 1 to 3.

2. Hydrophilic structure in L^P

[0172] The hydrophilic structure of each moiety in the linker is described. As for linker L^P, two forms are present as the hydrophilic structure. One of them is a structure in which the peptide residue L^P is a peptide residue having a hydrophilic amino acid at its N terminal and, also, this N-terminal hydrophilic amino acid is a hydrophilic amino acid other than glycine.

[0173] The total number of amino acids constituting such a peptide linker can be in a range of from 3 to 8. The hydrophilic amino acid other than glycine can be aspartic acid, glutamic acid, lysine, serine, threonine, glutamine, asparagine, histidine, tyrosine, or arginine. Among them, glutamic acid, aspartic acid, or lysine is preferred, and aspartic acid is more preferred. The number of this hydrophilic amino acid can be 1 or more and is preferably 1 or 2, and more preferably 1.

[0174] A peptide following this hydrophilic amino acid can be in a range of from 2 to 7 in total and more preferably consists of 3 or 4 amino acids. This peptide can be a peptide consisting of amino acids selected from phenylalanine (Phe; F), tyrosine (Tyr; Y), leucine (Leu; L), glycine (Gly; G), alanine (Ala; A), valine (Val; V), lysine (Lys; K), citrulline (Cit), serine (Ser; S), glutamic acid (Glu; E), aspartic acid (Asp; D), and the like. Further, the amino acids constituting the peptide can be any of L- and D-amino acids and are preferably L-amino acids. Further, the amino acids may be amino acids having a structure such as β-alanine, ε-aminocaproic acid, or γ-aminobutyric acid in addition to α-amino acids. Further, they can be non-natural type amino acids such as N-methylated amino acids. Among them, preferred examples can include phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. The peptide after the second amino acid counted from the N terminal in the peptide residue of the linker is preferably GGF or GGFG.

[0175] The peptide residue L^P of the linker having a hydrophilic amino acid at the N terminal is preferably DGGF, KGGF, EGGF, DGGFG, KGGFG, or EGGFG, more preferably DGGF, KGGF, DGGFG, or KGGFG, and further preferably DGGF or DGGFG.

[0176] Another form in which L^P becomes a hydrophilic linker can include a case in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the drug, and also, even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat. Although glycine is also classified as a hydrophilic amino acid, it is preferable that a plurality of glycines are connected to the C terminal when glycine is selected as the hydrophilic amino acid in the peptide linker. Such a glycine oligopeptide can consist of 2 or 3 or more glycines and is preferably glycine di- or tri-peptide.

[0177] When linker L^P has the hydrophilic structure containing the glycine oligopeptide, preferred examples thereof can include GGFGG and GGFGGG.

[0178] Also, when L^P has the hydrophilic structure containing the glycine oligopeptide at the C terminal, a further feature of this peptide residue of the linker is that L^P having this structure is directly connected to the drug.

[0179] When oligopeptide consisting of 2 or 3 or more glycines is present at the C terminal and is connected to the drug, a peptide sequence other than this moiety in the peptide linker L^P can be a peptide consisting of amino acids selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. However, in this case, the N terminal of the peptide residue of the linker is not aspartic acid, glutamic acid, lysine, serine, threonine, glutamine, asparagine, histidine, tyrosine, or arginine listed above as the hydrophilic amino acid.

[0180] The amino acids constituting the peptide can be any of L- and D-amino acids and are preferably L-amino acids. Further, the amino acids may be amino acids having a structure such as β-alanine, ε-aminocaproic acid, or γ-aminobutyric acid in addition to α-amino acids. Further, they can be non-natural type amino acids such as N-methylated amino acids. As for the number of the amino acids, the peptide can consist of 4 to 8 amino acids and more preferably consists of 4 to 6 amino acids.

3. L^P

[0181] The linker L^P may be in a form that does not have the hydrophilic structure, as mentioned below. Specifically, even when linker L^P does not have the hydrophilic structure, linker L^P can consist of an oligopeptide residue in which 3 to 8 amino acids are linked by a peptide bonding. L^P is connected to L² at its N terminal and is connected to the -NH-(CH₂)^{n¹}-L^a-L^b-L^c moiety of the linker at its C terminal or directly to the antitumor compound at its C terminal, in case of exatecan, it is connected to the amino group at position 1 thereof.

[0182] The amino acid constituting L^P that does not have the hydrophilic linker is not particularly limited as long as it is not the amino acid constituting L^P having the hydrophilic structure. The amino acid can be any of L- and D-amino acids and is preferably an L-amino acid. Further, it can be an amino acid having a structure such as β-alanine, ε-ami-

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nocaproic acid, or γ -aminobutyric acid in addition to an α -amino acid. Further, it can be a non-natural type amino acid such as N-methylated amino acid.

[0183] The amino acid sequence of such L^P is not particularly limited, but examples of the constituting amino acid can include phenylalanine, tyrosine, leucine, glycine, alanine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Among them, preferred examples can include phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Depending on the type of the amino acid, drug release pattern can be controlled. The number of the amino acid can be between 3 to 8.

[0184] Specific examples of L^P that does not form the hydrophilic structure in the linker can include -GGF-,
-GGFG-,
-GFLG-.

Among them, -GGF- or -GGFG- can be preferably used.

[0185] In the structure moiety represented by $-\text{NH}-(\text{CH}_2)_{n^1}-$ in the linker, n^1 is an integer of 0 to 6 and is preferably an integer of 1 to 5, and more preferably 1 to 3. The amino group of this moiety is connected to the C terminal of L^P .

4. Hydrophilic structure in L^1 or L^2

[0186] When linker L^1 takes a form of $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)_{n^3}-\text{COOH}]-\text{C}(=\text{O})-$, this moiety corresponds to the hydrophilic linker according to the present invention. n^3 is an integer of 1 to 8 and is preferably 2 to 4, and more preferably 2. In $-(\text{CH}_2)_{n^3}-\text{COOH}$ of this hydrophilic structure moiety, the carboxy group may be a hydroxy group or an amino group. When L^1 in the linker is $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)_{n^3}-\text{COOH}]-\text{C}(=\text{O})-$, L^2 in the linker is $-\text{NH}-(\text{CH}_2-\text{CH}_2-\text{O})_{n^6}-\text{CH}_2-\text{CH}_2-\text{C}(=\text{O})-$ wherein n^6 is 0 to 4, preferably.

[0187] When linker L^2 takes a form of $-\text{N}[(\text{CH}_2\text{CH}_2-\text{O})_{n^7}-\text{CH}_2\text{CH}_2-\text{OH}]-\text{CH}_2-\text{C}(=\text{O})-$, this moiety corresponds to the hydrophilic structure according to the present invention. n^7 is an integer of 1 to 4 and is preferably 3 or 4.

This linker is connected to L^1 at its terminal amino group and is connected to the N terminal of L^P at its carbonyl group at the other terminal.

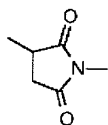
[0188] The presence of the linker having the hydrophilic structure described above can achieve the excellent release of the drug component having an antitumor effect.

5. L^1

[0189] The linker L^1 is the linker represented by the following structure of $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)_{n^2}-\text{C}(=\text{O})-$, $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)_{n^3}-\text{COOH}]-\text{C}(=\text{O})-$, $-\text{CH}_2-\text{C}(=\text{O})-\text{NH}-(\text{CH}_2)_{n^4}-\text{C}(=\text{O})-$, $-\text{C}(=\text{O})-\text{cyc.Hex}(1,4)-\text{CH}_2-(\text{N-ly-3-dimethylaminocyclohexyl})-$, or $-\text{C}(=\text{O})-(\text{CH}_2)_{n^5}-\text{C}(=\text{O})-$. Wherein, n^2 is an integer of 2 to 8, n^3 is an integer of 1 to 8, and n^4 is an integer of 1 to 8, and n^5 is an integer of 1 to 8.

[0190] In the linker having a structure represented by $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)_{n^2}-\text{C}(=\text{O})-$ among L^1 , $-(\text{Succinimid-3-yl-N})-$ has a structure represented by the following formula:

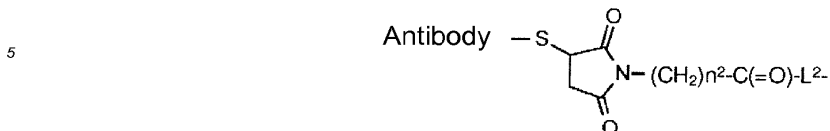
[Formula 30]



[0191] Position 3 of the above partial structure is a connecting position to the antibody. And further, the bond to the antibody at this position 3 is characterized by connecting with the formation of thioether at a disulfide bond moiety in a hinge part of the antibody. On the other hand, the nitrogen atom at position 1 of this structure moiety is connected to the carbon atom of methylene which is present within the linker including this structure. Specifically, $-(\text{Succinimid-3-yl-N})-(\text{CH}_2)_{n^2}-\text{C}(=\text{O})-\text{L}^2-$ is a structure represented by the following formula (herein, "antibody-S-" originates from an antibody).

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[Formula 31]



10 [0192] In the formula, n^2 is an integer of 2 to 8, and preferably 2 to 5.

[0193] Among L^1 , $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)n^3-\text{COOH}]-\text{C}(=\text{O})-$ is the hydrophilic structure mentioned above.

15 [0194] In the linker having a structure represented by $-\text{CH}_2-\text{C}(=\text{O})-\text{NH}-(\text{CH}_2)n^4-\text{C}(=\text{O})-$ among L^1 , n^4 is an integer of 1 to 8, preferably 2 to 6. This linker is connected to the antibody at its carbon atom of terminal methylene and, as with the preceding, has the following structure for connection by forming thioether, (herein, "antibody-S-" originates from an antibody).

Antibody-S- $\text{CH}_2-\text{C}(=\text{O})-\text{NH}-(\text{CH}_2)n^4-\text{C}(=\text{O})-\text{L}^2$.

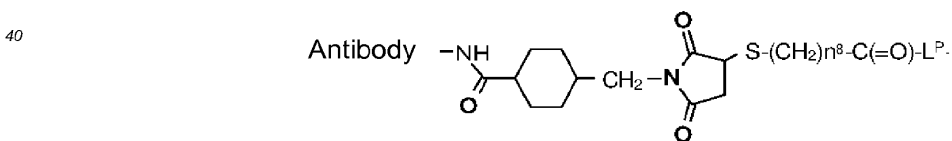
[0195] In the linker having a structure represented by $-\text{C}(=\text{O})-\text{cyc.Hex}(1,4)-\text{CH}_2-(\text{N-ly-3-diminiccuS})-$ among L^1 , $-(\text{N-ly-3-diminiccuS})-$ has a structure represented by the following formula:

20 [Formula 32]



30 [0196] In this structure moiety, the nitrogen atom at position 1 is connected to the carbon atom of methylene present in the linker structure containing this structure. The carbon atom at position 3 is connected to $-\text{S}-(\text{CH}_2)n^8-\text{C}(=\text{O})-$ among linker L^2 at its terminal sulfur atom. This $-\text{S}-(\text{CH}_2)n^8-\text{C}(=\text{O})-$ among linker L^2 forms a linker structure only combined with $-\text{C}(=\text{O})\text{cyc.Hex}(1,4)-\text{CH}_2-(\text{N-ly-3-diminiccuS})-$ among linker L^1 . In the above, $-\text{cyc.Hex}(1,4)-$ contained in the linker represents a 1,4-cyclohexylene group. The linker $-\text{C}(=\text{O})-\text{cyc.Hex}(1,4)-\text{CH}_2-(\text{N-ly-3-diminiccuS})-$ is connected to the antibody by forming amide bond at its terminal carbonyl carbon (herein, "antibody-NH-" originates from an antibody).

35 [Formula 33]



45 [0197] The amino group of the antibody forming this amide bond may be an amino group in a lysine residue in the antibody or that at the N terminal of the antibody. The linker can be conneted by amide bond as well as with ester bond formation with the hydroxy group carried by an amino acid in the antibody.

50 [0198] The structure moiety $-\text{cyc.Hex}(1,4)-$ in the linker may be a divalent saturated cyclic alkylene group other than the 1,4-cyclohexylene group, i.e., a divalent cyclic saturated hydrocarbon group such as a cyclobutylene group, a cyclopentylene group, a cycloheptylene group, or a cyclooctylene group. The moiety may also be a divalent aromatic hydrocarbon group such as a phenylene group or a naphthylene group, or a 5- or 6-membered saturated, partially saturated, or aromatic divalent heterocyclic group containing 1 or 2 heteroatoms. Alternatively, this moiety may be a divalent alkylene group having 1 to 4 carbon atoms. The divalent group may be at adjacent positions or at distant positions.

55 [0199] In the linker having a structure represented by $-\text{C}(=\text{O})-(\text{CH}_2)n^5-\text{C}(=\text{O})-$ among L^1 , n^5 is an integer of 1 to 8, and preferably 2 to 6. This linker is also connected by forming amide bond at its terminal carbonyl group with an amino group of an amino acid in the antibody, as with the linker mentioned above (see the following formula; in the structure thereof, "antibody-NH-" originates from an antibody).

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Antibody-NH-C(=O)-(CH₂)_n⁵-C(=O)-L².

[0200] Specific examples of L¹ in the linker can include

- (Succinimid-3-yl-N)-CH₂CH₂-C(=O)-
- (Succinimid-3-yl-N)-CH₂CH₂CH₂-C(=O)-
- 5 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂-C(=O)-
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-
- (Succinimid-3-yl-N)-CH(-CH₂-COOH)-C(=O)-
- (Succinimid-3-yl-N)-CH(-CH₂CH₂-COOH)-C(=O)-
- (Succinimid-3-yl-N)-CH(-CH₂CH₂CH₂-COOH)-C(=O)-
- 10 - (Succinimid-3-yl-N)-CH(-CH₂CH₂CH₂CH₂-COOH)-C(=O)-
- (Succinimid-3-yl-N)-CH(-CH₂CH₂CH₂CH₂CH₂-COOH)-C(=O)-
- (Succinimid-3-yl-N)-CH(-CH₂CH₂CH₂CH₂CH₂CH₂-COOH)-C(=O)-
- (Succinimid-3-yl-N)-CH(-CH₂CH₂CH₂CH₂CH₂CH₂CH₂-COOH)-C(=O)-
- 15 - CH₂-C(=O)-NH-CH₂-C(=O)-
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-
- CH₂-C(=O)-NH-CH₂CH₂CH₂-C(=O)-
- CH₂-C(=O)-NH-CH₂CH₂CH₂CH₂-C(=O)-
- 20 - CH₂-C(=O)-NH-CH₂CH₂CH₂CH₂CH₂-C(=O)-
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-
- C(=O)-Aryl-CH₂-(N-ly-3-diminiccuS)-
- C(=O)-cyc.Het-CH₂-(N-ly-3-diminiccuS)-
- C(=O)-CH₂-C(=O)-
- C(=O)-CH₂CH₂-C(=O)-
- 25 - C(=O)-CH₂CH₂CH₂-C(=O)-
- C(=O)-CH₂CH₂CH₂CH₂-C(=O)-
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-
- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-

(Aryl represents a divalent aromatic hydrocarbon group, and cyc.Het represents a divalent cyclic heterocyclic group).

6. L²

- [0201] Linker L² is a linker represented by the following structure: -NH-(CH₂-CH₂-O)_n⁶-CH₂-CH₂-C(=O)-, -N[-(CH₂CH₂-O)_n⁷-CH₂CH₂-OH]-CH₂-C(=O)-, or -S-(CH₂)_n⁸-C(=O)-, L² may not be present, and in such a case, L² is a single bond. And, n⁶ is an integer of 0 to 6, n⁷ is an integer of 1 to 4, and n⁸ is an integer of 1 to 6.

- [0202] In the linker having a structure represented by -NH-(CH₂CH₂O)_n⁶-CH₂-CH₂-C(=O)- among L², n⁶ is an integer of 0 to 6, and preferably 2 to 4. Said linker is connected to L¹ at the nitrogen atom of its terminal amino group and is connected to the N terminal of L^P at its carbonyl group at the other terminal. When L¹ is -(Succinimid-3-yl-N)-CH[-(CH₂)_n³-COOH]-C(=O)-, only -NH-(CH₂CH₂O)_n⁶-CH₂-CH₂-C(=O)- wintin L² is connected to this and also n⁶ is 0.

- [0203] Among L², the linker represented by -N[-(CH₂CH₂-O)_n⁷-CH₂CH₂-OH]-CH₂-C(=O)- is the hydrophilic structure previously mentioned.

[0204] In the linker having a structure represented by -S-(CH₂)_n⁸-C(=O)- among L², n⁸ is an integer of 1 to 6, and preferably 2 to 4.

[0205] Specific examples of L² in the linker can include

- 45 -NH-CH₂CH₂-C(=O)-
- NH-CH₂CH₂O-CH₂CH₂-C(=O)-
- NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-
- NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-
- NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-
- 50 -NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-
- NH-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂O-CH₂CH₂-C(=O)-
- N-(CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-
- N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-
- N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-
- 55 -N-(CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-.

[0206] When L² is -S-(CH₂)_n⁸-C(=O)-, L¹ to be combined therewith is -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-.

So the specific examples of -L¹-L²- in the linker can include

-C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂-C(=O)-

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-C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-
 -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂CH₂-C(=O)-
 -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂CH₂CH₂-C(=O)-
 -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂CH₂CH₂CH₂-C(=O)-
 5 -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-.

7. L^a

[0207] The linker L^a is any of structures -C(=O)-NH-, -NR¹-(CH₂)^{n⁹}-, and -O- or is a single bond, wherein, n⁹ is an integer of 1 to 6, R¹ is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂)^{n^a}-COOH, or -(CH₂)^{n^b}-OH, n^a is an integer of 1 to 4, and n^b is an integer of 1 to 6.

[0208] The amide structure -C(=O)-NH- among linker L^a is connected to L^b at its nitrogen atom side. In the structure moiety -NR¹-(CH₂)^{n⁹}- among linker L^a, n⁹ is an integer of 1 to 6, and preferably 1 to 3. This moiety is connected to L^b at its methylene side. R¹ is a hydrogen atom or an alkyl group having 1 to 6 carbon atoms. The alkyl group having 1 to 6 carbon atoms may be linear or branched. Examples thereof can include a methyl group, an ethyl group, a propyl group, an isopropyl group, a butyl group, an isobutyl group, a sec-butyl group, a tert-butyl group, a pentyl group, an isopentyl group, a 2-methylbutyl group, a neopentyl group, a 1-ethylpropyl group, a hexyl group, an isohexyl group, a 4-methylpentyl group, a 3-methylpentyl group, a 2-methylpentyl group, a 1-methylpentyl group, a 3,3-dimethylbutyl group, a 2,2-dimethylbutyl group, a 1,1-dimethylbutyl group, a 1,2-dimethylbutyl group, a 1,3-dimethylbutyl group, a 2,3-dimethylbutyl group, and a 2-ethylbutyl group. Of them, a methyl group or an ethyl group is preferred. When R¹ has a structure represented by -(CH₂)^{n^a}-COOH, n^a is an integer of 1 to 4, and preferably 1 or 2. When R¹ has a structure represented by -(CH₂)^{n^b}-OH, n^b is an integer of 1 to 6, and preferably 1 or 2. R¹ is preferably a hydrogen atom, a methyl group, an ethyl group, -CH₂COOH, -CH₂CH₂-COOH, or -CH₂CH₂-OH, and more preferably a hydrogen atom, a methyl group, or -CH₂COOH. It is further preferably a hydrogen atom. The L^a moiety of the linker may be -O- or a single bond.

8. L^b

[0209] The linker L^b is any of structures -CR²-(R³)-, -O-, and -NR⁴- or is a single bond. In the above, R² and R³ each independently represents a hydrogen atom, an alkyl group having 1 to 6 carbon atoms, -(CH₂)^{n^c}-NH₂, -(CH₂)^{n^d}-COOH, or -(CH₂)^{n^e}-OH, R⁴ is a hydrogen atom or an alkyl group having 1 to 6 carbon atoms, n^c is an integer of 0 to 6, n^d is an integer of 1 to 4, and n^e is an integer of 0 to 4. When n^c or n^e is 0, R² and R³ are not the same as each other.

[0210] When each of R² and R³ is an alkyl group, this alkyl group is interpreted as defined in the alkyl group of R¹. When each of R² and R³ has a structure of -(CH₂)^{n^c}-NH₂, n^c is an integer of 0 to 6, and preferably 0, or is 3 to 5. When n^c is 0, R² and R³ are not the same as each other. When each of R² and R³ has a structure of -(CH₂)^{n^d}-COOH, n^d is an integer of 1 to 4, and preferably 1 or 2. When each of R² and R³ has a structure of -(CH₂)^{n^e}-OH, n^e is an integer of 0 to 4, and preferably 1 or 2.

[0211] Each of R² and R³ is preferably a hydrogen atom, a methyl group, an ethyl group, -NH₂, -CH₂CH₂CH₂NH₂, -CH₂CH₂CH₂CH₂NH₂, -CH₂CH₂CH₂CH₂CH₂NH₂, -CH₂COOH, -CH₂CH₂-COOH, -CH₂OH, or -CH₂CH₂-OH, and more preferably a hydrogen atom, a methyl group, -NH₂, -CH₂CH₂CH₂CH₂NH₂, -CH₂COOH, -CH₂CH₂-COOH, -CH₂OH, or -CH₂CH₂-OH. They are further preferably hydrogen atoms.

[0212] When R⁴ is an alkyl group having 1 to 6 carbon atoms, this alkyl group is interpreted as defined in the alkyl group of R¹. R⁴ is preferably a hydrogen atom or a methyl group, and more preferably a hydrogen atom.

[0213] Specific examples of the structure represented by linker -NH-(CH₂)^{n¹}-L^a-L^b- include

-NH-CH₂-
 45 -NH-CH(-Me)-
 -NH-C(-Me)₂-
 -NH-CH₂-CHMe-
 -NH-CH(-CH₂OH)-
 -NH-CH(-CH₂COOH)-
 50 -NH-CH(-CH₂CH₂COOH)-
 -NH-CH(-CH₂CH₂CH₂CH₂NH₂)-
 -NH-CH₂CH₂-
 -NH-CH₂-O-CH₂-
 -NH-CH₂CH₂-O-
 55 -NH-CH₂CH₂-O-CH₂-
 -NH-CH₂CH₂C(-Me)₂-
 -NH-CH₂CH₂NH-
 -NH-CH₂CH₂NH-CH₂-

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- NH-CH₂CH₂NMe-CH₂-
 -NH-CH₂CH₂NH-CH₂CH₂-
 -NH-CH₂CH₂NMe-CH₂CH₂-
 -NH-CH₂CH₂N(-CH₂COOH)-CH₂-
 5 -NH-CH₂CH₂N(-CH₂CH₂OH)-CH₂-
 -NH-CH₂CH₂N(-CH₂CH₂OH)-CH₂CH₂-
 -NH-CH₂CH₂CH₂C(=O)-NHCH(-CH₂OH)-
 -NH-CH₂CH₂CH₂C(=O)-NHCH(-CH₂COOH)-
 -NH-CH₂CH₂CH₂C(=O)-NHCH(-CH₂CH₂CH₂NH₂)-
 10 -NH-CH₂CH₂CH₂-
 -NH-CH₂CH₂CH₂CH₂-
 -NH-CH₂CH₂CH₂CH₂CH₂-
 -NH-CH₂CH₂CH₂CH₂CH(NH₂)-.
- [0214]** Of them, preferred examples thereof can include
- 15 -NH-CH₂-
 -NH-CH₂-CH(Me)-
 -NH-CH(-CH₂OH)-
 -NH-CH(-CH₂CH₂COOH)-
 -NH-CH₂CH₂-
 20 -NH-CH₂-O-CH₂-
 -NH-CH₂CH₂-O-
 -NH-CH₂CH₂-O-CH₂-
 -NH-CH₂CH₂C(-Me)₂-
 -NH-CH₂CH₂NH-
 25 -NH-CH₂CH₂NH-CH₂-
 -NH-CH₂CH₂NMe-CH₂-
 -NH-CH₂CH₂NMe-CH₂CH₂-
 -NH-CH₂CH₂N(-CH₂COOH)-CH₂-
 -NH-CH₂CH₂N(-CH₂CH₂OH)-CH₂-
 30 -NH-CH₂CH₂N(-CH₂CH₂OH)-CH₂CH₂-
 -NH-CH₂CH₂CH₂C(=O)-NHCH(-CH₂OH)-
 -NH-CH₂CH₂CH₂C(=O)-NHCH(-CH₂COOH)-
 -NH-CH₂CH₂CH₂-
 -NH-CH₂CH₂CH₂CH₂-
 35 -NH-CH₂CH₂CH₂CH₂CH₂-.
- [0215]** More preferred examples thereof can include
- NH-CH₂-
 -NH-CH₂CH₂-
 -NH-CH₂-O-CH₂-
 40 -NH-CH₂CH₂-O-
 -NH-CH₂CH₂-O-CH₂-
 -NH-CH₂CH₂NH-
 -NH-CH₂CH₂NH-CH₂-
 -NH-CH₂CH₂N(-CH₂COOH)-CH₂-
 45 -NH-CH₂CH₂N(-CH₂CH₂OH)-CH₂CH₂-
 -NH-CH₂CH₂CH₂C(=O)-NHCH(-CH₂COOH)-
 -NH-CH₂CH₂CH₂-
 -NH-CH₂CH₂CH₂CH₂-
 -NH-CH₂CH₂CH₂CH₂CH₂-.
- [0216]** Further preferred examples thereof can include
- 50 -NH-CH₂-
 -NH-(CH₂)₂-
 -NH-(CH₂)₃-
 -NH-CH₂-O-CH₂- and
 55 -NH-(CH₂)₂-O-CH₂-.

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9. L^c

[0217] The linker L^c is -CH₂- or -C(=O)-. Said linker is connected to the antitumor compound. The linker L^c is more preferably -C(=O)-.

5 [0218] The chain length of the linker -NH-(CH₂)ⁿ¹-L^a-L^b-L^c moiety is preferably a chain length of 4 to 7 atoms, and more preferably a chain length of 5 or 6 atoms.

[0219] With regard to the antibody-drug conjugate of the present invention, when it is transferred to the inside of tumor cells, the linker moiety is cleaved and the drug derivative having a structure represented by NH₂-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX) is released to express an antitumor activity. Examples of the antitumor derivative exhibiting an antitumor effect by releasing from the antibody-drug conjugate of the present invention include an antitumor derivative having a structure moiety in which the structure represented by -NH-(CH₂)ⁿ¹-L^a-L^b- of the linker is bound with L^c and has a terminal amino group, and the particularly preferred include the followings.

NH₂-CH₂-C(=O)-(NH-DX)

NH₂-CH₂CH₂-C(=O)-(NH-DX)

15 NH₂-CH₂CH₂CH₂-C(=O)-(NH-DX)

NH₂-CH₂-O-CH₂-C(=O)-(NH-DX)

NH₂-CHCH₂-O-CH₂-C(=O)-(NH-DX)

[0220] Meanwhile, in case of NH₂-CH₂-O-CH₂-C(=O)-(NH-DX), as the amina structure in the molecule is unstable, it again undergoes a self-decomposition to release the following

20 HO-CH₂-C(=O)-(NH-DX). Those compounds can be also preferably used as a production intermediate of the antibody-drug conjugate of the present invention.

[0221] For the antibody-drug conjugate of the present invention in which exatecan is used as a drug, it is preferable that the drug-linker structure moiety having the structure described below is connected to an antibody. The average connected number of the drug-linker structure moiety per antibody can be 1 to 10. Preferably, it is 2 to 8, and more preferably 3 to 8. Preferred examples of the drug-linker structure moiety can include the followings.

10. Specific example when L^P has hydrophilic structure

[0222] A drug-linker structure moiety in which L^P is a peptide residue having a hydrophilic amino acid at the N terminal is present in the form of -L¹-L²-L^P-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX) or -L¹-L²-L^P-(NH-DX). The combinations of each part of the linkers constituting this drug-linker structure moiety are the followings.

[0223] When the drug-linker structure moiety is connected to the antibody via a thioether bond, L¹ is -(Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)- or -CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-. When the drug-linker structure moiety is connected to the antibody via an amide bond, it is -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-dimincus)- or -C(=O)-(CH₂)ⁿ⁵-C(=O)-.

35 [0224] As for the linker L², when L¹ is -(Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)- or -CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-, this can be a single bond, when linker L¹ is -C(=O)-(CH₂)ⁿ⁵-C(=O)-, this is selected from -NH-(CH₂-CH₂-O)ⁿ⁶-CH₂-CH₂-C(=O)- and a single bond. And the linker -S-(CH₂)ⁿ⁸-C(=O)- is used in combination with -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-dimincus)- among L¹.

40 [0225] The linker -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety preferably has a chain length of 3 to 7 atoms. The moiety more preferably has a chain length of 4 to 7 atoms in the linker and further preferably has a chain length of 5 or 6 atoms. Specific examples of the linker -NH-(CH₂)ⁿ¹-L^a-L^b- are as described above, -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)- is particularly preferred one.

[0226] The linker -L^c- moiety is preferably -C(=O)-.

45 [0227] Specific examples of the drug-linker structure moiety containing the peptide having a hydrophilic amino acid at the N terminal can include the followings.

[0228] Examples of the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via a thioether bond can include the following formulas:

- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGF-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- 50 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGF-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-EGGF-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- 55 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-EGGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)

In the above formula, preferably, n² is 2 to 5, and the -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety is -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly connected to the drug are preferable.

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[0229] More specifically, it is preferably represented by the following formula:

- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- 5 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- 10 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- 15 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

20 or the following formulas:

- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGF-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGF-(NH-DX)
- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-DGGFG-(NH-DX)
- 25 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-KGGFG-(NH-DX)

More preferably, n² is 2 or 5.

[0230] Further preferably, n² is 5, and it is represented by the following formulas:

- 30 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- 35 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- 40 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- 45 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formulas:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX)
- 50 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX)

[0231] Among them, it is further preferably represented by the following formulas:

- 55 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)

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- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- 5 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formulas:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX)
- 10 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX)

[0232] Examples of another form of the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via a thioether bond can include the following formulas:

- 15 - CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGF-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGF-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-EGGF-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)
- 20 - CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-EGGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)

In the above formula, preferably, n⁴ is 2 to 5, and the-NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety is-NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly connected to the drug are preferable.

- 25 **[0233]** More specifically, it is preferably

- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- 30 - CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- 35 - CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- 40 - CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formulas:

- 45 - CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGF-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGF-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-DGGFG-(NH-DX)
- CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-KGGFG-(NH-DX)
- 50

[0234] More preferably, n⁴ is 2, and it is represented by

- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- 55 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)

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- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGF-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGF-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- 5 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- 10 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$

or the following formulas:

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGF-(NH-DX)}$
- 15 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGF-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-KGGFG-(NH-DX)}$

[0235] Further preferably, it is

- 20 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- 25 - $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$

30 or the following formulas:

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGF-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-(NH-DX)}$

35 [0236] Alternatively, examples of the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via an amide bond can include the following formulas:

- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}^n\text{-C(=O)-DGGF-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}^n\text{-C(=O)-KGGF-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-(NH-DX)}$
- 40 - $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}^n\text{-C(=O)-EGGF-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}^n\text{-C(=O)-DGGFG-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}^n\text{-C(=O)-KGGFG-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}^n\text{-C(=O)-EGGF-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-(NH-DX)}$

45 In the above formula, n^8 is preferably 1 to 6, and more preferably 2. The $\text{-NH-(CH}_2\text{)}^n\text{-L}^a\text{-L}^b\text{-L}^c\text{-}$ moiety is preferably $\text{-NH-CH}_2\text{CH}_2\text{-C(=O)-}$, $\text{-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-}$, $\text{-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-}$, or $\text{-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-}$. Also, those which L^P is directly connected to the drug are preferable.

[0237] More specifically, it is more preferably

- 50 - $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-DGGF-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-KGGF-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- 55 - $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-KGGF-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-KGGF-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-KGGF-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-CH}_2\text{CH}_2\text{-C(=O)-DGGFG-NH-CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$

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- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- 5 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formulas:

- 10 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-(NH-DX)
- 15 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-(NH-DX)

[0238] Among them, it is further preferably represented by the following formula:

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- 20 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

25 or the following formulas:

- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-(NH-DX)

30 [0239] Examples of another form of the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via an amide bond can include the following formulas:

- C(=O)-(CH₂)^{n⁵}-C(=O)-L²-DGGF-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
- C(=O)-(CH₂)^{n⁵}-C(=O)-L²-KGGF-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
- 35 - C(=O)-(CH₂)^{n⁵}-C(=O)-L²-EGGF-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
- C(=O)-(CH₂)^{n⁵}-C(=O)-L²-DGGFG-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
- C(=O)-(CH₂)^{n⁵}-C(=O)-L²-KGGFG-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
- C(=O)-(CH₂)^{n⁵}-C(=O)-L²-EGGFG-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)

40 In the above formula, n⁵ is preferably 6, and L² is -NH-(CH₂-CH₂-O)n⁶-CH₂-CH₂-C(=O)- or a single bond and is preferably a single bond. n⁶ is preferably 0, 2, or 4, and more preferably 0. The -NH-(CH₂)^{n¹}-L^a-L^b-L^c moiety is preferably -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly connected to the drug are preferable.

[0240] More specifically, it is preferably

- 45 - C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- 50 - C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- 55 - C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
- C(=O)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)

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- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-KGGFG-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-(NH-DX)}$

5 or the following formulas:

- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-DGGF-(NH-DX)}$
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-KGGF-(NH-DX)}$
- $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-DGGFG-(NH-DX)}$
- 10 - $\text{C(=O)-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-KGGFG-(NH-DX)}$

[0241] When the linker L^P is a peptide residue having a glycine oligopeptide at the C terminal, the drug-linker structure moiety is present in a form in which L^P is directly connected to the drug, as in $-L^1-L^2-L^P\text{-(NH-DX)}$. When L^P in the linker is a peptide residue having a glycine oligopeptide at the C terminal, the combinations of each linker moiety constituting the drug-linker structure moiety are as follows.

[0242] When the drug-linker structure moiety is connected to the antibody via a thioether bond, the linker L^1 is $-(\text{Succinimid-3-yl-N})\text{-(CH}_2\text{)}_n\text{-C(=O)-}$ or $-\text{CH}_2\text{-C(=O)-NH-(CH}_2\text{)}_n\text{-C(=O)-}$ and when the drug-linker structure moiety is connected to the antibody via an amide bond, is selected from $-\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-}$ or $-\text{C(=O)-(CH}_2\text{)}_n\text{-C(=O)-}$.

[0243] As for the linker L^2 , when linker L^1 is $-(\text{Succinimid-3-yl-N})\text{-(CH}_2\text{)}_n\text{-C(=O)-}$ or $-\text{CH}_2\text{-C(=O)-NH-(CH}_2\text{)}_n\text{-C(=O)-}$, it can be $-\text{NH-(CH}_2\text{-CH}_2\text{-O)}_n\text{-CH}_2\text{-CH}_2\text{-C(=O)-}$ or a single bond, and when linker L^1 in $-\text{C(=O)-(CH}_2\text{)}_n\text{-C(=O)-}$, it is selected from single bonds. And the linker $-\text{S-(CH}_2\text{)}_n\text{-C(=O)-}$ is used in combination with $-\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-}$ among L^1 .

[0244] Specific examples of the drug-linker structure moiety containing the peptide having a glycine oligopeptide at the C terminal can include the followings.

[0245] Examples of the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via a thioether bond can include following formulas:

- $(\text{Succinimid-3-yl-N})\text{-(CH}_2\text{)}_n\text{-C(=O)-GGFGG-(NH-DX)}$
- 30 - $(\text{Succinimid-3-yl-N})\text{-(CH}_2\text{)}_n\text{-C(=O)-GGFGGG-(NH-DX)}$

In the above formula, preferably, n^2 is 2 or 5.

[0246] More preferably, n^2 is 5, and it is represented by the following formula:

- 35 - $(\text{Succinimid-3-yl-N})\text{-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-GGFGG-(NH-DX)}$
- $(\text{Succinimid-3-yl-N})\text{-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-GGFGGG-(NH-DX)}$

[0247] Examples of the drug-linker structure moiety in another form for conjugating the drug-linker structure moiety to the antibody via a thioether bond can include the following formulas:

- 40 - $\text{CH}_2\text{-C(=O)-NH-(CH}_2\text{)}_n\text{-C(=O)-GGFGG-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-(CH}_2\text{)}_n\text{-C(=O)-GGFGGG-(NH-DX)}$

In the above formula, more preferably, n^4 is 2 or 5.

[0248] Among them, more preferably, n^4 is 2, and it is preferably represented by the following formula:

- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-GGFGG-(NH-DX)}$
- $\text{CH}_2\text{-C(=O)-NH-CH}_2\text{CH}_2\text{-C(=O)-GGFGGG-(NH-DX)}$

[0249] Alternatively, examples of the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via an amide bond or an ester bond can include the following formulas:

- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}_n\text{-C(=O)-GGFGG-(NH-DX)}$
- $\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-(N-ly-3-diminiccuS)-S-(CH}_2\text{)}_n\text{-C(=O)-GGFGGG-(NH-DX)}$

55 In the above formula, n^8 is preferably 2.
[0250] Specifically, it is

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- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-GGFGG-(NH-DX)
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-GGFGGG-(NH-DX)

5 [0251] Examples of the drug-linker structure moiety in another form for conjugating the drug-linker structure moiety to the antibody via an amide bond can include the following formulas:

- C(=O)-(CH₂)ⁿ⁵-C(=O)-L²-GGFGG-(NH-DX)
- C(=O)-(CH₂)ⁿ⁵-C(=O)-L²-GGFGGG-(NH-DX)

10 In the above formula, n⁵ is preferably 6, and L² is -NH-(CH₂-CH₂-O)ⁿ⁶-CH₂-CH₂-C(=O)- or a single bond and is preferably a single bond. n⁶ is preferably 0, 2, or 4, and more preferably 0.

[0252] More specifically, it is

- C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-(NH-DX)
- 15 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-(NH-DX)

11. Specific example when L¹ has hydrophilic structure

20 [0253] When linker L¹ is -(Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-, the drug-linker structure moiety is connected to the antibody via a thioether bond, and is present in the form of -L¹-L²-L^P-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX) or -L¹-L²-L^P-(NH-DX). The combinations of each linker moiety constituting this drug-linker structure are as follows.

[0254] The linker L² is in NH-(CH₂-CH₂-O)ⁿ⁶-CH₂-CH₂-C(=O)- or a single bond and is preferably a single bond. n⁶ is preferably 0, 2, or 4, and more preferably 0.

25 [0255] The amino acid sequence of linker L^P is not particularly limited, but examples of the constituting amino acid include phenylalanine, tyrosine, leucine, glycine, alanine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Among them, preferred examples include phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Depending on the type of the amino acid, drug release pattern can be controlled. The number of the amino acid can be between 3 to 8. Specific examples thereof are as listed above, and GGFG is particularly preferred.

30 [0256] The linker -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety preferably has a chain length of 4 to 7 atoms. The moiety more preferably has a chain length of 5 or 6 atoms in the linker. Specific examples of the -NH-(CH₂)ⁿ¹-L^a-L^b-moiety in the linker are as described above.

[0257] The -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety may be a single bond.

[0258] The linker -L^c- moiety is preferably -C(=O)-.

35 [0259] The drug-linker structure moiety in which linker L¹ is -(Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)- is connected to the antibody via a thioether bond and has a structure represented by the following formula:

- (Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-(CH₂-CH₂-O)ⁿ⁶-CH₂-CH₂-C(=O)-GGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX) In the above formula, n⁶ is 0. Preferably, n³ is 2 to 4, and the -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety is -NH-CH₂CH₂-C(=O)-,
- 40 - NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly connected to the drug are preferable.

[0260] More specifically, it is represented by the following formula:

- 45 - (Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

50 or the following formula:

- (Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-(NH-DX)

[0261] In the above formula, n³ is preferably 2, and it is preferably represented by the following formula:

- 55 - (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
- (Succinimid-3-yl-N)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)

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- (Succinimid-3-yl-N)-(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formula:

5 - (Succinimid-3-yl-N)-(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-(NH-DX)

12. Specific example when L² has hydrophilic structure

10 **[0262]** When linker L² is -N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)-, the drug-linker structure moiety is present in the form of -L¹-L²-LP-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX) or -L¹-L²-LP-(NH-DX). The combinations of each linker moiety constituting this drug-linker structure moiety are as follows.

[0263] When the drug-linker structure moiety is connected to the antibody via a thioether bond, linker L¹ is - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)- or -CH₂-C(=O)-NH-(CH₂)ⁿ⁴-C(=O)-, and when the drug-linker structure moiety is connected to the antibody via an amide bond, it is selected from -C(=O)-(CH₂)ⁿ⁵-C(=O)-.

15 **[0264]** The amino acid sequence of linker L^P is not particularly limited, but examples of the constituting amino acid can include phenylalanine, tyrosine, leucine, glycine, alanine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Among them, preferred examples can include phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid. Depending on the type of the amino acid, drug release pattern can be controlled. The number of the amino acid can be between 3 to 8. Specific examples thereof are as listed above. GGFG is particularly preferred.

20 **[0265]** The linker -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety preferably has a chain length of 4 to 7 atoms. The moiety more preferably has a chain length of 5 or 6 atoms in the linker. Specific examples of the -NH-(CH₂)ⁿ¹-L^a-L^b-moiety in the linker are as described above.

[0266] The -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety may be a single bond.

[0267] The linker -L^c- moiety is preferably -C(=O)-.

25 **[0268]** The drug-linker structure moiety in which linker L² is -N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)- is preferably represented by the following formula:

- L¹-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)-LP-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)

30 **[0269]** The drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via a thioether bond is represented by the following formula:

- (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH] -CH₂-C(=O)-GGFG-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)

35 In the above formula, preferably, n² is 2 or 5, n⁷ is 3 or 4, and the -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety is -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly connected to the drug are preferable.

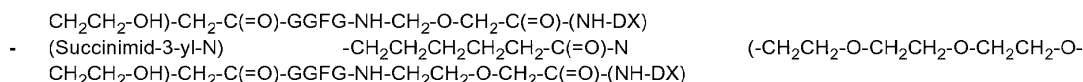
[0270] More specifically, it is represented by the following formula:

40 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH] -CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 45 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH] -CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH] -CH₂-C(=O) -GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX) or the following formula:
 - (Succinimid-3-yl-N)-(CH₂)ⁿ²-C(=O)-N[-(CH₂CH₂-O)ⁿ⁷-CH₂CH₂-OH]-CH₂-C(=O)-GGFG-(NH-DX)

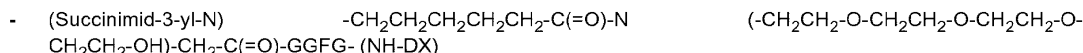
50 **[0271]** Further preferably, n² is 5, n⁷ is 3 or 4, and more preferably 3, and it is preferably represented by the following formulas:

55 - (Succinimid-3-yl-N) -CH₂CH₂CH₂CH₂CH₂-C(=O)-N (-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N) -CH₂CH₂CH₂CH₂CH₂-C(=O)-N (-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 - (Succinimid-3-yl-N) -CH₂CH₂CH₂CH₂CH₂-C(=O)-N (-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-

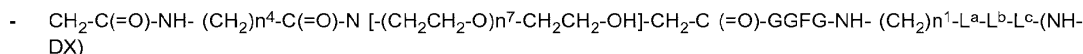
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5 or the following formula:



10 **[0272]** Examples of the drug-linker structure moiety as another form for conjugating the drug-linker structure moiety to the antibody via a thioether bond can include the following formula:

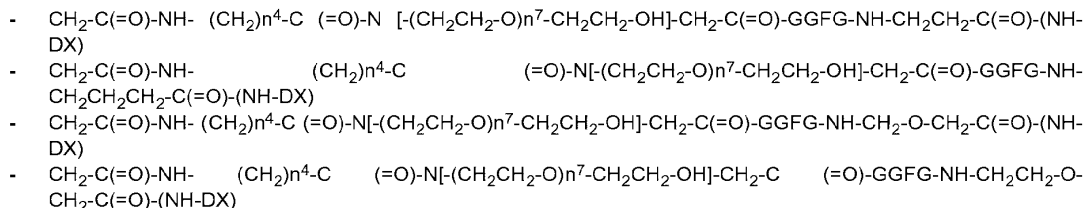


15

[0273] In the above formula, preferably, n^4 is 2 or 5, n^7 is 3 or 4, and the $\text{-NH- (CH}_2\text{)}^{n^1}\text{-L}^a\text{-L}^b\text{-L}^c\text{-}$ moiety is $\text{-NH-CH}_2\text{CH}_2\text{-C(=O)-}$, $\text{-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-}$, $\text{-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-}$, or $\text{-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-}$. Also, those which L^p is directly connected to the drug are preferable.

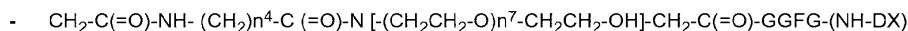
[0274] Specifically, it is represented by the following formula:

20



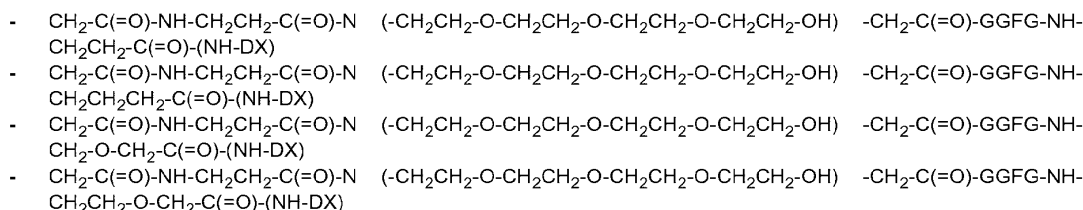
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30 or the following formula:



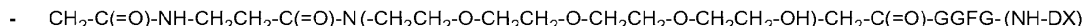
[0275] In the above formula, preferably, n^4 is 2, n^7 is 3 or 4, and more preferably 3, and it is represented by the following formulas:

35



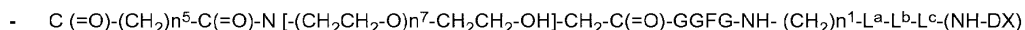
45

or the following formula:



50

[0276] Alternatively, the drug-linker structure moiety for conjugating the drug-linker structure moiety to the antibody via an amide bond is represented by the following formula:



55

In the above formula, preferably, n^5 is an integer of 1 to 8 and is more preferably 2 to 6, n^7 is 3 or 4, and the $\text{-NH-(CH}_2\text{)}^{n^1}\text{-L}^a\text{-L}^b\text{-L}^c\text{-}$ moiety is $\text{-NH-CH}_2\text{CH}_2\text{-C(=O)-}$, $\text{-NH-CH}_2\text{CH}_2\text{CH}_2\text{-C(=O)-}$, $\text{-NH-CH}_2\text{-O-CH}_2\text{-C(=O)-}$, or $\text{-NH-CH}_2\text{CH}_2\text{-O-CH}_2\text{-C(=O)-}$. Also, those which L^p is directly connected to the drug are preferable.

[0277] More specifically, it is represented by the following formula:

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- $C(=O)-(CH_2)_n^5-C(=O)-N[-(CH_2CH_2-O)_n^7-CH_2CH_2-OH]-CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)$
- $C(=O)-(CH_2)_n^5-C(=O)-N[-(CH_2CH_2-O)_n^7-CH_2CH_2-OH]-CH_2-C(=O)-GGFG-NH-CH_2CH_2CH_2-C(=O)-(NH-DX)$
- $C(=O)-(CH_2)_n^5-C(=O)-N[-(CH_2CH_2-O)_n^7-CH_2CH_2-OH]-CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)$
- $C(=O)-(CH_2)_n^5-C(=O)-N[-(CH_2CH_2-O)_n^7-CH_2CH_2-OH]-CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)$

5

or the following formula:

- $C(=O)-(CH_2)_n^5-C(=O)-N[-(CH_2CH_2-O)_n^7-CH_2CH_2-OH]-CH_2-C(=O)-GGFG-(NH-DX)$

10 **[0278]** Further preferably, n^5 is 6, n^7 is 3 or 4, and more preferably 3, and it is represented by the following formulas:

- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)$
- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-NH-CH_2CH_2CH_2-C(=O)-(NH-DX)$
- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX)$
- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-NH-CH_2CH_2-O-CH_2-C(=O)-(NH-DX)$

15

20

or the following formula:

- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-(NH-DX)$

25

[0279] Further preferably, it is represented by the following formula:

- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-NH-CH_2CH_2-C(=O)-(NH-DX)$
- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-NH-CH_2CH_2CH_2-C(=O)-(NH-DX)$

30

or the following formula:

- $C(=O)-CH_2CH_2CH_2CH_2CH_2CH_2-C(=O)-N(-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-O-CH_2CH_2-OH)-CH_2-C(=O)-GGFG-(NH-DX)$

35

[Production method]

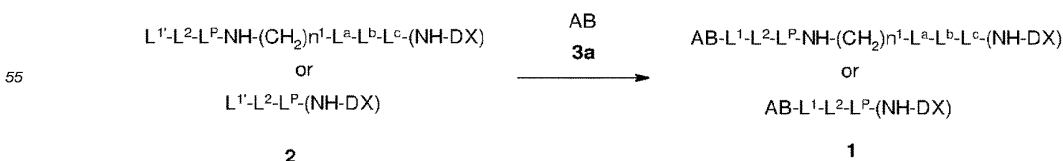
40 **[0280]** Next, explanations are given for the representative method for producing the antibody-drug conjugate of the present invention or a production intermediate thereof. Meanwhile, the compounds are hereinbelow described with the number shown in each reaction formula. Specifically, they are referred to as a "compound of the formula (1)", a "compound (1)", or the like. The compounds with numbers other than those are also described similarly.

45 1. Production method 1

[0281] The antibody-drug conjugate represented by the formula (1) in which the antibody is connected to the linker structure via thioether can be produced by the following method, for example.

50

[Formula 34]

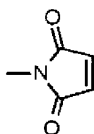


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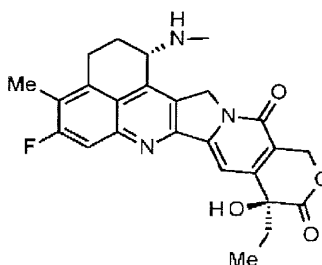
[0282] In the formula, AB represents an antibody with a sulfhydryl group, and L¹ represents an L¹ linker structure in which the linker terminal has a maleimidyl group (formula shown below) (in the formula, the nitrogen atom is the connecting position)

[Formula 35]



or the terminal has a halogen, and represents a group in which the -(Succinimid-3-yl-N)- moiety in -(Succinimid-3-yl-N)-(CH₂)_n²-C(=O)- among L¹ is a maleimidyl group or a halogen-CH₂C(=O)NH-(CH₂)_n³-C(=O)- group in which terminal methylene in -CH₂C(=O)NH-(CH₂)_n⁴-C(=O)- among L¹ is halogenated to form haloacetamide. Further, the -(NH-DX) represents a structure represented by the following formula:

[Formula 36]



and it represents a group that is produced by removing one hydrogen atom of the amino group at position 1 of the antitumor drug. Meanwhile, the compound of the formula (1) in the above reaction formula is described as a structure in which one structure moiety from drug to the linker terminal is connected to one antibody. However, it is only the description given for the sake of convenience, and there are actually many cases in which a plurality of the structure moieties are connected to one antibody molecule. The same also applies to the explanation of the production method described below.

[0283] Specifically, the antibody-drug conjugate (1) can be produced by reacting the compound (2), which is obtainable by the method described below, with the antibody (3a) having a sulfhydryl group.

[0284] The antibody (3a) having a sulfhydryl group can be obtained by a method well known in the art (Hermanson, G.T., Bioconjugate Techniques, pp. 56-136, pp. 456-493, Academic Press (1996)). Examples include: Traut's reagent is reacted with the amino group of the antibody; N-succinimidyl S-acetylthioalkanoates are reacted with the amino group of the antibody followed by reaction with hydroxylamine; after reacting with N-succinimidyl 3-(pyridyldithio)propionate, it is reacted with a reducing agent; the antibody is reacted with a reducing agent such as dithiothreitol, 2-mercaptoethanol, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to reduce the disulfide bond at a hinge part in the antibody, but it is not limited thereto.

[0285] Specifically, the antibody-drug conjugate (1) can be produced by adding dimethyl sulfoxide solution of the compound (2) into a phosphate-buffered sodium chloride aqueous solution (pH 7.2) containing the antibody (3a) having a sulfhydryl group. Then, as is ordinary used in the reaction for the production of antibody-drug bond formation, unreacted compound (2) was deactivated by the addition of N-acetyl-L-cysteine (NAC). The produced antibody-drug conjugate (1) can be subjected to the following procedures such as, concentration, buffer exchange, conducting purification, measurement of antibody concentration and average number of conjugated drug molecules per antibody molecule, and calculation of aggregate content, identification of the antibody-drug conjugate (1).

[0286] Common procedure A: Concentration of aqueous solution of antibody or antibody-drug conjugate To a Amicon Ultra (50,000 MWCO, Millipore Corporation) container, a solution of antibody or antibody-drug conjugate was added and the solution of the antibody or antibody-drug conjugate was concentrated by centrifugation (centrifuge for 5 to 20 minutes at 2000 G to 3800 G) using a centrifuge (Allegra X-15R, Beckman Coulter, Inc.).

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Common procedure B: Measurement of antibody concentration

[0287] Using a UV detector (Nanodrop 1000, Thermo Fisher Scientific Inc), measurement of the antibody concentration was performed according to the method defined by the manufacturer. At that time, 280 nm absorption coefficient different for each antibody was used (1.3 to 1.8 /mg/mL).

[0288] Common procedure C-1: NAP-25 column (Cat. No. 17-0852-02, GE Healthcare Japan Corporation) using Sephadex G-25 carrier was equilibrated with phosphate buffer (10 mM, pH 6.0) (it is referred to as PBS6.0/EDTA in the specification) containing sodium chloride (137 mM) and ethylene diamine tetraacetic acid (EDTA, 5 mM) according to the method defined by the manufacturer's instruction manual. Aqueous solution of the antibody was applied in an amount of 2.5 mL to single NAP-25 column, and then the fraction (3.5 mL) eluted with 3.5 mL of PBS6.0/EDTA was collected. The resulting fraction was concentrated by the Common procedure A. After measuring the concentration of the antibody using the Common procedure B, the antibody concentration was adjusted to 10 mg/mL using PBS6.0/EDTA.

Common procedure C-2: Buffer Exchange for antibody

[0289] NAP-25 column (Cat. No. 17-0852-02, GE Healthcare Japan Corporation) using Sephadex G-25 carrier was equilibrated with phosphate buffer (50 mM, pH 6.5) (it is referred to as PBS6.5/EDTA in the specification) containing sodium chloride (50 mM) and EDTA (2 mM) according to the method defined by the manufacturer. Aqueous solution of the antibody was applied in an amount of 2.5 mL to single NAP-25 column, and then the fraction (3.5 mL) eluted with 3.5 mL of PBS6.5/EDTA was collected. The resulting fraction was concentrated by the Common procedure A. After measuring the concentration of the antibody using the Common procedure B, the antibody concentration was adjusted to 20 mg/mL using PBS6.5/EDTA.

Common procedure D-1: Purification of antibody-drug conjugate

[0290] NAP-25 column was equilibrated with any buffer selected from commercially available phosphate buffer (PBS7.4, Cat. No. 10010-023, Invitrogen), sodium phosphate buffer (10 mM, pH 6.0; it is referred to as PBS6.0) containing sodium chloride (137 mM), and acetate buffer containing sorbitol (5%) (10 mM, pH 5.5; it is referred to as ABS in the specification). Aqueous solution of the antibody-drug conjugate reaction was applied in an amount of about 1.5 mL to the NAP-25 column, and then eluted with the buffer in an amount defined by the manufacturer to collect the antibody fraction. The collected fraction was again applied to the NAP-25 column and, by repeating 2 to 3 times in total the gel filtration purification process for eluting with buffer, the antibody-drug conjugate excluding non-conjugated drug linker and a low-molecular-weight compound (tris(2-carboxyethyl)phosphine hydrochloride (TCEP), N-acetyl-L-cysteine (NAC), and dimethyl sulfoxide) was obtained.

Common procedure D-2: Purification of succinimidyl 4-(N-maleimidylmethyl)-cyclohexane-1-carboxylate (SMCC)-derivatized antibody

[0291] NAP-25 column was equilibrated with PBS6.5/EDTA. To the NAP-25 column, reaction solution (about 0.5 mL) containing the succinimidyl 4-(N-maleimidylmethyl)-cyclohexane-1-carboxylate (herein, referred to as SMCC)-derivatized antibody was applied, and then eluted with the buffer in an amount defined by the manufacturer to collect the antibody fraction for purification.

Common procedure E: Measurement of antibody concentration in antibody-drug conjugate and average number of conjugated drug molecules per antibody molecule.

[0292] The conjugated drug concentration in the antibody-drug conjugate can be calculated by measuring UV absorbance of an aqueous solution of the antibody-drug conjugate at two wavelengths of 280 nm and 370 nm, followed by performing the calculation shown below.

[0293] Because the total absorbance at any wavelength is equal to the sum of the absorbance of every light-absorbing chemical species that are present in a system [additivity of absorbance], when the molar absorption coefficients of the antibody and the drug remain the same before and after conjugation between the antibody and the drug, the antibody concentration and the drug concentration in the antibody-drug conjugate are expressed with the following equations.

$$A_{280} = A_{D,280} + A_{A,280} = \epsilon_{D,280}C_D + \epsilon_{A,280}C_A \quad \text{Equation (1)}$$

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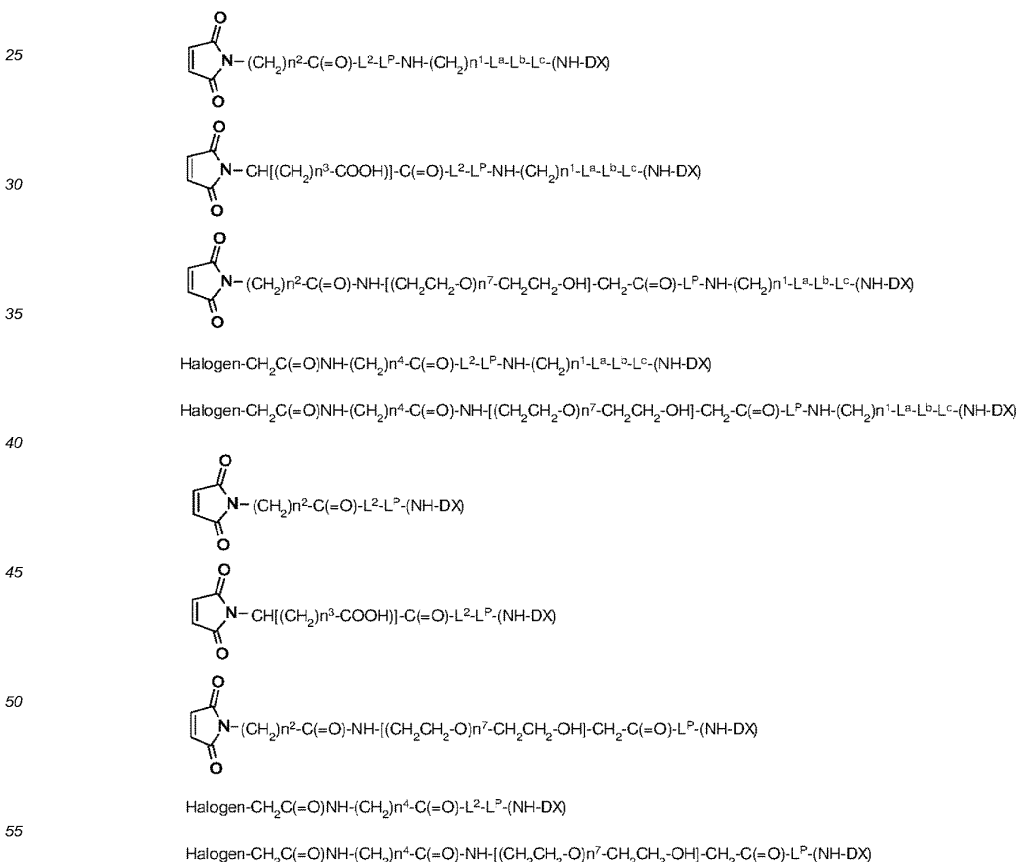
$$A_{S,370} = A_{D,370} + A_{A,370} = \epsilon_{D,370}C_D + \epsilon_{A,370}C_A \quad \text{Equation (2)}$$

5 [0294] In the above, A_{280} represents the absorbance of an aqueous solution of the antibody-drug conjugate at 280 nm; A_{370} represents the absorbance of an aqueous solution of the antibody-drug conjugate at 370 nm; $A_{A,280}$ represents the absorbance of an antibody at 280 nm; $A_{A,370}$ represents the absorbance of an antibody at 370 nm; $A_{D,280}$ represents the absorbance of a conjugate precursor at 280 nm; $A_{D,370}$ represents the absorbance of a conjugate precursor at 370 nm; $\epsilon_{A,280}$ represents the molar absorption coefficient of an antibody at 280 nm; $\epsilon_{A,370}$ represents the molar absorption coefficient of an antibody at 370 nm; $\epsilon_{D,280}$ represents the molar absorption coefficient of a conjugate precursor at 280 nm; $\epsilon_{D,370}$ represents the molar absorption coefficient of a conjugate precursor at 370 nm; C_A represents the antibody concentration in an antibody-drug conjugate; and C_D represent the drug concentration in an antibody-drug conjugate.

10 [0295] $\epsilon_{A,280}$, $\epsilon_{A,370}$, $\epsilon_{D,280}$, and $\epsilon_{D,370}$ in the above are known from the previous measurements. By measuring A_{280} and A_{370} of an aqueous solution of the antibody-drug conjugate and solving the simultaneous equations (1) and (2) using the values, C_A and C_D can be obtained. Further, by dividing C_D by C_A , the average drug conjugated number per antibody can be obtained.

15 [0296] The compound represented by the formula (2) in Production method 1 is any compound represented by the following formula:

20 [Formula 37]



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[0297] In the formula, n^1 , n^2 , n^3 , n^4 , n^7 , L^2 , L^P , L^a , L^b , and L^c are as already defined, and L^P or L^c is a connecting position to the drug.

[0298] In an intermediate useful in producing such an antibody-drug conjugate of the present invention, preferably, n^2 is an integer of 2 to 5,

5 when L^1 in the linker is not $-(\text{Succinimid-3-yl-N})-\text{CH}[(\text{CH}_2)n^3-\text{COOH}]-\text{C}(=\text{O})\text{L}^2$ is $-\text{N}[(\text{-CH}_2\text{CH}_2\text{-O})n^7-\text{CH}_2\text{CH}_2\text{-OH}]-\text{CH}_2-\text{C}(=\text{O})-$, and n^7 is 3 or 4, or a single bond, what is preferable for L^P ,

when when this is a peptide residue having a hydrophilic amino acid at the N terminal, is DGGF, KGGF, EGGF, DGGFG, KGGFG, or EGGFG, or

10 when this is a peptide linker having a glycine oligopeptide at the C terminal, is GGFGG or GGFGGG and the $-\text{NH}-(\text{CH}_2)n^1-\text{L}^a-\text{L}^b-\text{L}^c-$ moiety is a partial structure of $-\text{NH}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-$, $-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-$, $-\text{NH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$, or $-\text{NH}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$.

[0299] And, those which L^P is directly connected to the drug are preferable, in case the peptide linker has a glycine oligopeptide at its C terminal, this C terminal is directly connected to the drug.

15 [0300] Specific examples of these compounds can include the followings [herein, (maleimid-N-yl) represents a maleimidyl group (2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl group)].

[0301] When linker L^P is a peptide linker which has a hydrophilic amino acid at the N terminal, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

20 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGF-NH- $(\text{CH}_2)n^1-\text{L}^a-\text{L}^b-\text{L}^c$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGF-NH- $(\text{CH}_2)n^1-\text{L}^a-\text{L}^b-\text{L}^c$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGFG-NH- $(\text{CH}_2)n^1-\text{L}^a-\text{L}^b-\text{L}^c$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGFG-NH- $(\text{CH}_2)n^1-\text{L}^a-\text{L}^b-\text{L}^c$ -(NH-DX)

25 [0302] In the above formula, preferably, n^2 is 2 to 5, and the $-\text{NH}-(\text{CH}_2)n^1-\text{L}^a-\text{L}^b-\text{L}^c-$ moiety is $-\text{NH}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-$, $-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-$, $-\text{NH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$, or $-\text{NH}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$. Also, those which L^P is directly connected to the drug are preferable.

[0303] More specifically, it is represented by the following formula:

30 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGF-NH- $\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGF-NH- $\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGF-NH- $\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGF-NH- $\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGF-NH- $\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 35 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGF-NH- $\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGF-NH- $\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGF-NH- $\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGFG-NH- $\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGFG-NH- $\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 40 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGFG-NH- $\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGFG-NH- $\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGFG-NH- $\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGFG-NH- $\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGFG-NH- $\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 45 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGFG-NH- $\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)

or the following formulas:

50 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGF-(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGF-(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -DGGFG-(NH-DX)
 (maleimid-N-yl)- $(\text{CH}_2)n^2-\text{C}(=\text{O})$ -KGGFG-(NH-DX)

In the above formula, preferably, n^2 is 2 or 5.

55 [0304] Among them, further preferably, n^2 is 5, and it is represented by the following formulas:

(maleimid-N-yl)- $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -DGGF-NH- $\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)
 (maleimid-N-yl)- $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -DGGF-NH- $\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})$ -(NH-DX)

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(maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 5 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 10 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 15 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formulas:

(maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O) -DGGF- (NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O) -KGGF- (NH-DX)
 20 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O) -DGGFG- (NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O) -KGGFG- (NH-DX)

[0305] When linker L^P is a peptide linker which has a glycine oligopeptide at the C terminal, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

(maleimid-N-yl)-(CH₂)ⁿ²-C(=O)-GGFGG-(NH-DX)
 (maleimid-N-yl)-(CH₂)ⁿ²-C(=O)-GGFGGG-(NH-DX)

In the above formula, n² is preferably 2 to 5.

[0306] Among them, further preferably, n² is 5, and it is represented by the following formula:

(maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O) -GGFGG- (NH-DX)
 (maleimid-N-yl) -CH₂CH₂CH₂CH₂CH₂-C(=O) -GGFGGG- (NH-DX)

[0307] When linker L¹ in -(Succinimid-3-yl-N)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

(maleimid-N-yl)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-(CH₂-CH₂-O)ⁿ⁶-CH₂-CH₂-C(=O)-L^P-NH-(CH₂)ⁿ¹-L^a-L^b-L^c-(NH-DX)

In the above formula, n⁶ is 0. Preferably, n³ is 2 to 4, and the -NH-(CH₂)ⁿ¹-L^a-L^b-L^c- moiety is -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly connected to the drug are preferable. L^P is preferably GGFG.

[0308] More specifically, it is represented by the following formula:

(maleimid-N-yl)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 50 (maleimid-N-yl)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formula:

(maleimid-N-yl)-CH[-(CH₂)ⁿ³-COOH]-C(=O)-NH-CH₂CH₂-C(=O)-GGFG- (NH-DX)

[0309] In the above formula, n³ is preferably 2, and it is preferably represented by the following formula:

(maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O) - (NH-DX)
 (maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)

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(maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formula:

(maleimid-N-yl)-CH(CH₂CH₂-COOH)-C(=O)-NH-CH₂CH₂-C(=O)-GGFG- (NH-DX)

[0310] When linker L² is -N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-, a compound having a structure represented by the following formula can be preferably used as production intermediate:

(maleimid-N-yl)-(CH₂)^{n²}-C(=O)-N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-L^P-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)

[0311] In the above formula, preferably, n² is 2 to 5, n⁷ is 3 or 4, and the -NH- (CH₂)^{n¹}-L^a-L^b-L^c- moiety is -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also those which L^P is directly connected to the drug are preferable. L^P is preferably GGFG.

[0312] More specifically, it is represented by the following formulas:

(maleimid-N-yl)-(CH₂)^{n²}-C(=O)-N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-(CH₂)^{n²}-C(=O)-N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-(CH₂)^{n²}-C(=O)-N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-(CH₂)^{n²}-C(=O)-N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formula:

(maleimid-N-yl)-(CH₂)^{n²}-C(=O)-N[-(CH₂CH₂-O)^{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-GGFG- (NH-DX)

[0313] Further preferably, n² is 5, n⁷ is 3 or 4, and more preferably 3, and it is represented by the following formulas;

(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX)
 (maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX)

or the following formula:

(maleimid-N-yl)-CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG- (NH-DX)

[0314] In case when a halogenoacetyl group is present and peptide linker has a hydrophilic amino acid at the N terminal, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

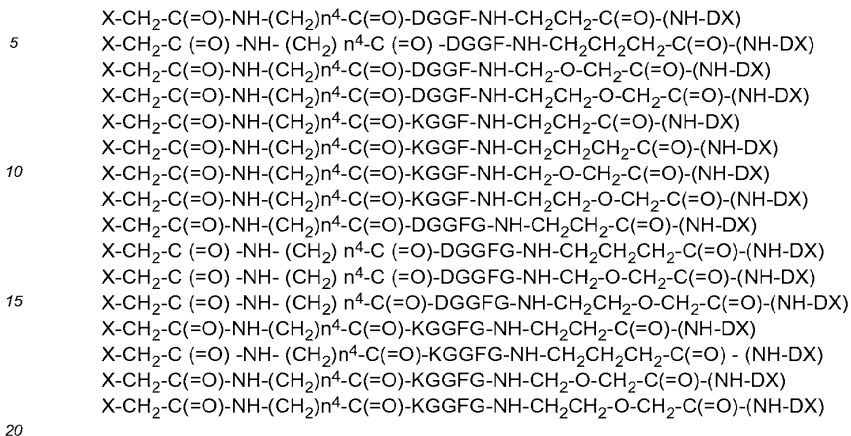
X-CH₂-C(=O)-NH-(CH₂)^{n⁴}-C(=O)-DGGF-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
 X-CH₂-C(=O)-NH-(CH₂)^{n⁴}-C(=O)-KGGF-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
 X-CH₂-C(=O)-NH-(CH₂)^{n⁴}-C(=O)-EGGF-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
 X-CH₂-C(=O)-NH-(CH₂)^{n⁴}-C(=O)-DGGFG-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
 X-CH₂-C(=O)-NH-(CH₂)^{n⁴}-C(=O)-KGGFG-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)
 X-CH₂-C(=O)-NH-(CH₂)^{n⁴}-C(=O)-EGGFG-NH-(CH₂)^{n¹}-L^a-L^b-L^c-(NH-DX)

[0315] In the above formula, preferably, n⁴ is 2 to 5, and the -NH-(CH₂)^{n¹}-L^a-L^b-L^c- moiety is -NH-CH₂CH₂-C(=O)-, -NH-CH₂CH₂CH₂-C(=O)-, -NH-CH₂-O-CH₂-C(=O)-, or -NH-CH₂CH₂-O-CH₂-C(=O)-. Also, those which L^P is directly

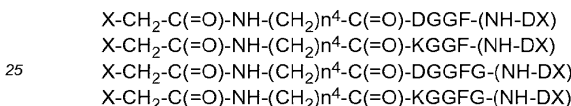
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connected to the drug are preferable. X is preferably bromine or iodine.

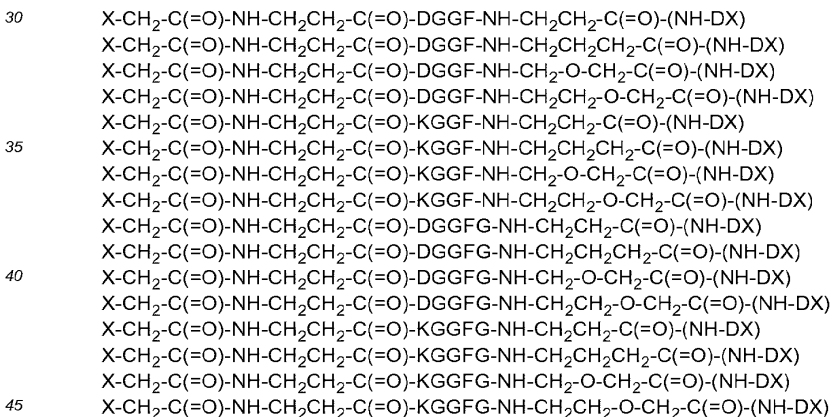
[0316] More specifically, it is more preferably represented by the following formulas:



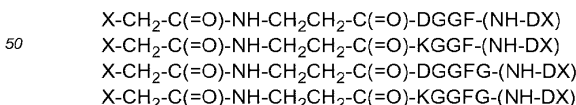
or the following formulas:



[0317] Further preferably, n⁴ is 2, and it is represented by the following formulas:



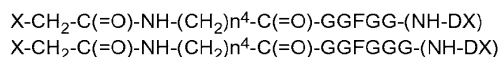
or the following formulas:



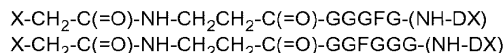
[0318] In the above formula, X represents a bromine atom or an iodine atom. All of these bromine and iodine compounds can be preferably used as production intermediates.

[0319] In case when halogenoacetyl group is present and the peptide linker has a glycine oligopeptide at the C terminal, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

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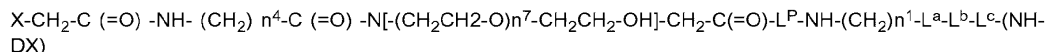


[0320] Preferably, n^4 is 2 or 5, and more preferably 2, and it is represented by the following formula:



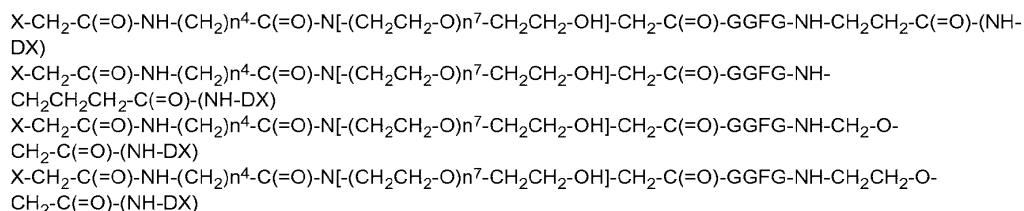
[0321] In the above formula, X represents a bromine atom or an iodine atom. All of these bromine and iodine compounds can be preferably used as production intermediates.

[0322] When linker L^2 is $-N[-(CH_2CH_2-O)n^7-CH_2CH_2-OH]-CH_2-C(=O)-$, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

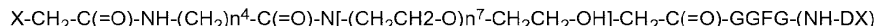


[0323] In the above formula, preferably, n^4 is 2 to 5, n^7 is 3 or 4, and the $-NH-(CH_2)n^1-L^a-L^b-L^c-$ moiety is $-NH-CH_2CH_2-C(=O)-$, $-NH-CH_2CH_2CH_2-C(=O)-$, $-NH-CH_2-O-CH_2-C(=O)-$, or $-NH-CH_2CH_2-O-CH_2-C(=O)-$. Also, those which L^P is directly connected to the drug are preferable. L^P is preferably GGFG.

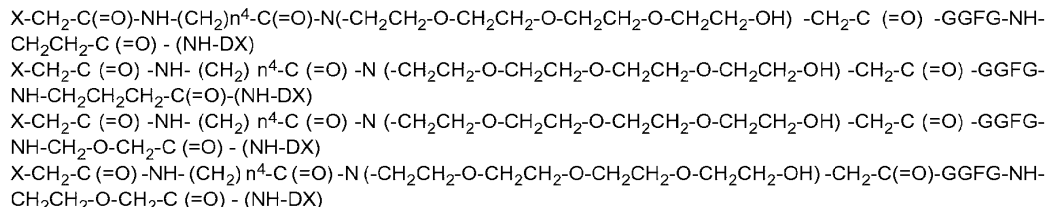
[0324] Specifically, it is represented by the following formula:



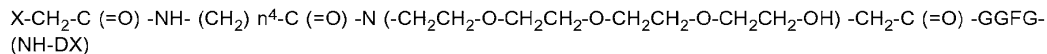
or the following formula:



[0325] Further preferably, n^4 is 2, n^7 is 3 or 4, and more preferably 3, and it is represented by the following formulas:



or the following formula:



[0326] In order to secure the amount of the conjugate, a plurality of conjugates obtained under similar production conditions to have an equivalent number of drugs (e.g., about ± 1) can be mixed to prepare new lots. In this case, the average number of drugs falls between the average numbers of drugs in the conjugates before the mixing.

2. Production method 2

[0327] The antibody-drug conjugate represented by the formula (1) or a pharmacologically acceptable salt thereof, in which the bond to the antibody is amide group and has thioether bond within the linker, specifically, a structure in which

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-L¹-L²- is -C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-dimiccuS)-S-(CH₂)ⁿ⁸-C(=O)-, can be also produced by the following method.

[Formula 38]

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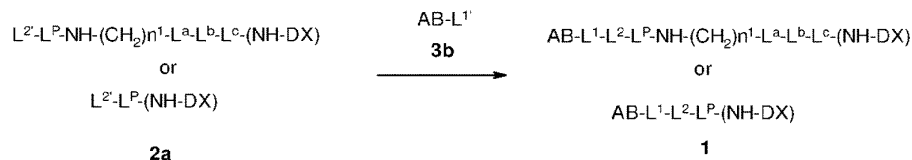
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[In the formula, AB-L¹ represents that the antibody is connected to linker L¹, and the terminal of L¹ is converted to a N-maleimidyl group. This group specifically has a structure in which -(N-ly-3-dimiccuS)-in AB-C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-dimiccuS)- is converted to a maleimidyl group. L² represents a HS-(CH₂)ⁿ⁸-C(=O) - group in which the terminal is a mercapto group, and AB represents the antibody.]

[0328] Specifically, the antibody-drug conjugate (1) can be produced by reacting the compound (2a), which is obtainable by the method described below, with the antibody (3b) to which the linker having a maleimidyl group is connected.

[0329] The antibody (3b) having a maleimidyl group can be also obtained by a method well known in the art (Hermanson, G.T, Bioconjugate Techniques, pp. 56-136, pp. 456-493, Academic Press (1996)). Example includes a method in which a bifunctional linker such as succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), which is capable of connecting to an amino group or a hydroxyl group, is allowed to act on the amino group of the antibody so as to introduce a maleimidyl group, but it is not limited thereto.

[0330] For example, a compound having an amino group-reactive moiety and a thiol group-reactive moiety are connected via a linker can be preferably used. Here, the amino group-reactive moiety can be active ester, imide ester, or the like, and the thiol-reactive moiety can be maleimidyl, halogenated acetyl, halogenated alkyl, dithiopyridyl, or the like.

[0331] As a method for constructing the linker via an amide bond with the amino group or hydroxy group, particularly, the amino group, of an amino acid constituting antibody, the compound to be first reacted with the antibody can be a compound represented by the following formula: Q¹-L^{1a}-Q².

[0332] In the formula, Q¹ represents (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, (3-Sulfo-pyrrolidine-2,5-dione-N-yl)-O-C(=O)-, R^Q-O-C(=N)-, or O=C=N-, L^{1a} represents -cyc.Hex(1,4)-CH₂-, an alkylene group having 1 to 10 carbon atoms, a phenylene group, -(CH₂)ⁿ⁴-C(=O)-, -(CH₂)^{n4a}-NH-C(=O)-(CH₂)^{n4b}-, or -(CH₂)^{n4a}-NH-C(=O)-cyc.Hex(1,4)-CH₂-, Q² represents (maleimid-N-yl), a halogen atom, or -S-S-(2-Pyridyl),

R^Q represents an alkyl group having 1 to 6 carbon atoms, n⁴ represents an integer of 1 to 8, n^{4a} represents an integer of 0 to 6, and n^{4b} represents an integer of 1 to 6.

[0333] In the above, R^Q is an alkyl group having 1 to 6 carbon atoms, and more preferably a methyl group or an ethyl group.

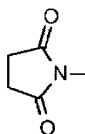
[0334] The alkylene group of L^{1a} may be those having 1 to 10 carbon atoms. The phenylene group may be any of ortho, meta, and para and is more preferably a para- or meta-phenylene group.

[0335] Preferred examples of L^{1a} can include -cyc.Hex(1,4)-CH₂-, -(CH₂)₅-NH-C(=O)-cyc.Hex(1,4)-CH₂-, -(CH₂)₂-NH-C(=O)-CH₂-, -(CH₂)₅-NH-C(=O)-(CH₂)₂-, -CH₂-, -(CH₂)₂-, -(CH₂)₃-, -(CH₂)₅-, -(CH₂)₁₀-, -(para-Ph)-, -(meta-Ph)-, -(para-Ph)-CH(-CH₃)-, -(CH₂)₃-(meta-Ph)-, and -(meta-Ph)-NH-C(=O)-CH₂-.

[0336] Q¹ is preferably (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)- and Q² is preferably (maleimid-N-yl), or -S-S-(2-Pyridyl) can be used when a disulfide bond is to be formed.

[0337] In the above, (Pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

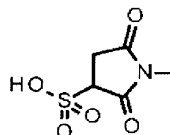
[Formula 39]



wherein the nitrogen atom is the connecting position, (3-Sulfo-pyrrolidine-2,5-dione-N-yl)- is a group represented by the following formula:

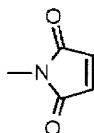
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[Formula 40]



wherein the nitrogen atom is the connecting position, and this sulfonic acid is capable of forming a lithium salt, sodium salt, or potassium salt, and preferably sodium salt, cyc.Hex(1,4) represents a 1,4-cyclohexylene group, (maleimid-N-yl) is a group represented by the following formula:

[Formula 41]



wherein the nitrogen atom is the connecting position, (2-Pyridyl) represents a 2-pyridyl group, (para-Ph) represents a para-phenylene group, and (meta-Ph) represents a meta-phenylene group.

[0338] As for such a compound other than the compounds described above, sulfosuccinimidyl-4-(N-maleimidylmethyl)cyclohexane-1-carboxylate (sulfo-SMCC), N-succinimidyl-4-(N-maleimidylmethyl)-cyclohexane-1-carboxy-(6-aminodocaproate) (LC-SMCC), κ -maleimidyl undecanoic acid N-succinimidyl ester (KMUA), γ -maleimidyl butyric acid N-succinimidyl ester (GMBS), ϵ -maleimidyl caproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidylbenzoyl-N-hydroxysuccinimide ester (MBS), N-(α -maleimidylacetoxyl)-succinimide ester [AMAS], succinimidyl-6-(β -maleimidylpropionamide)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidylphenyl)-butyrate (SMPB), N-(p-maleimidylphenyl)isocyanate (PMPi), N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), N-succinimidyl 3-(bromoacetamide)propionate (SBAP), N-succinimidyl-3-(2-pyridylthio)propionate (SP-DP), and succinimidylloxycarbonyl- α -methyl- α -(2-pyridylthio)toluene (SMPT) can be used.

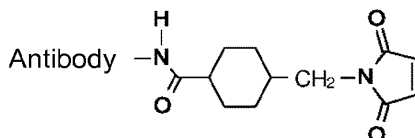
[0339] Specifically, for example, by reacting 2 to 6 equivalents of SMCC with the antibody (3) in a phosphate buffer of pH 6 to 7 at room temperature for 1 to 6 hours, the active ester of SMCC can react with the antibody to yield the antibody (3b) having a maleimidyl group. The obtained antibody (3b) can be purified by Common procedure D-2 described below, and used for the next reaction with the compound (2).

[0340] The amino group and hydroxyl group of the antibody refer to, for example, a N-terminal amino group carried by the antibody and/or an amino group carried by a lysine residue and a hydroxy group carried by a serine residue, respectively, but they are not limited thereto.

[0341] For the produced antibody-drug conjugate (1), concentration, buffer exchange, purification, and identification of the antibody-drug conjugate (1) by the measurement of antibody concentration and an average number of conjugated drug molecules per antibody molecule and calculation of aggregate content can be performed in the same manner as Production method 1.

[0342] The compound represented by the formula (3b) in Production method 2 has the following structure (see the following formula; in the structure thereof, "antibody-NH-" originates from an antibody).

[Formula 42]



[0343] A compound which is an intermediate for producing the antibody-drug conjugate of the present invention and

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has the above structure is as described below (in the formula, n is an integer of 1 to 10, preferably 2 to 8, and more preferably 3 to 7).

[Formula 43]

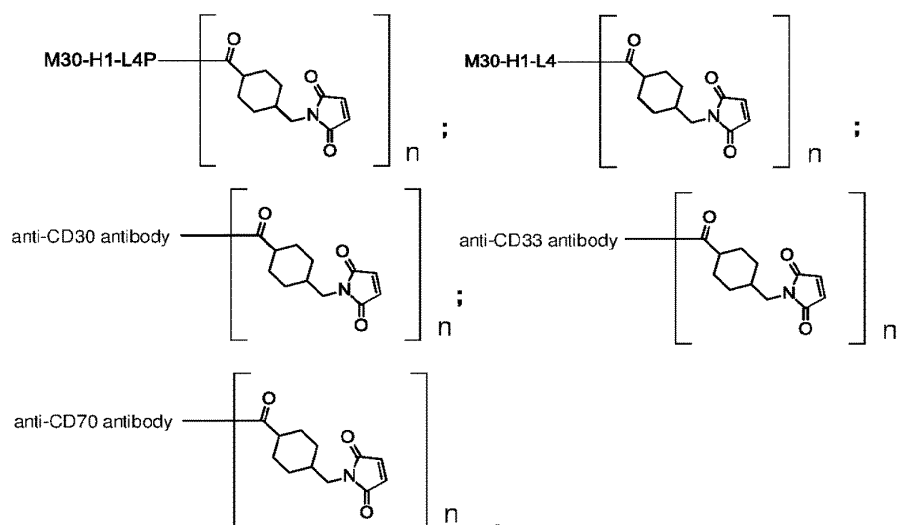
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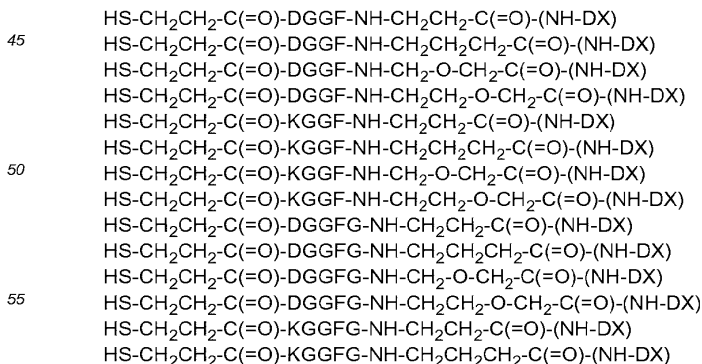
[0344] Further, examples of the compound of the present invention in which the terminal is a mercapto group can include the followings.

[0345] When the peptide linker has a hydrophilic amino acid at the N terminal, a compound having a structure represented by the following formula can be preferably used as a production intermediate:

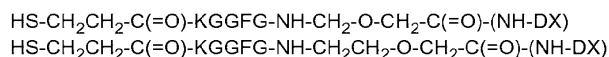


[0346] In the above formula, preferably, n^8 is 2 to 5, and the $-\text{NH}-(\text{CH}_2)^{n^1}-\text{L}^{\text{a}}-\text{L}^{\text{b}}-\text{L}^{\text{c}}-$ moiety is $-\text{NH}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-$, $-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-$, $-\text{NH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$, or $-\text{NH}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2-\text{C}(=\text{O})-$. Also, those which LP is directly connected to the drug are preferable.

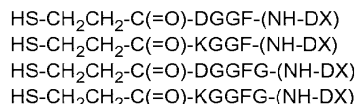
[0347] More specifically, n^8 is preferably 2, and it is represented by the following formula:



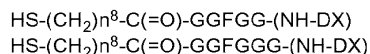
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or the following formulas:

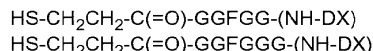


[0348] When the peptide residue of the linker has a glycine oligopeptide at the C terminal, a compound having a structure represented by the following formula can be preferably used as a production intermediate:



[0349] In the above formula, n^8 is preferably 2 or 5.

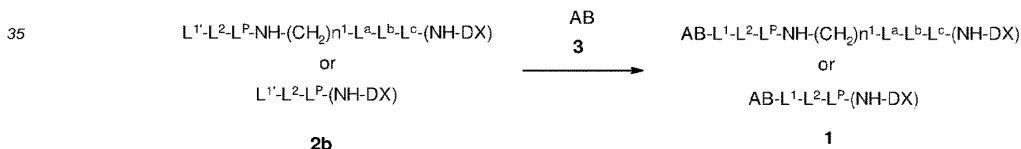
[0350] More preferably, n^8 is 2, and it is represented by the following formula:



3. Production method 3

[0351] The antibody-drug conjugate represented by the formula (1) or a pharmacologically acceptable salt thereof in which the antibody is conjugated to the drug linker moiety via an amide bond can be produced by a method described below. For example, L^1 in which L^1 is $\text{-C(=O)-(CH}_2\text{)}^n\text{-C(=O)-}$, and this is converted to active ester, for example, (Pyrrolidine-2, 5-dione-N-yl)-O-C(=O)-(CH₂)ⁿ-C(=O)-, can be preferably used. Further, when L^2 is a single bond, the antibody-drug conjugate (1) can be produced by the following method, for example.

[Formula 44]



[0352] Specifically, the antibody-drug conjugate (1) can be produced by reacting the compound (2b), which is obtainable by the method described below, with the antibody (3).

[0353] The compound (2b) has a property capable of connecting to the amino group or hydroxyl group of the antibody. The amino group and hydroxyl group of the antibody refer to, as described in Production method 2, for example, a N-terminal amino group carried by the antibody and/or an amino group carried by a lysine residue and a hydroxy group carried by a serine residue, respectively, but they are not limited thereto.

[0354] The compound (2b) is an active ester composed of a N-hydroxysuccinimidyl ester group, and alternatively, other active esters, for example, a sulfosuccinimidyl ester group, N-hydroxyphthalimidyl ester, N-hydroxysulphophthalimidyl ester, ortho-nitrophenyl ester, para-nitrophenyl ester, 2,4-dinitrophenyl ester, 3-sulfonyl-4-nitrophenyl ester, 3-carboxy-4-nitrophenyl ester, and pentafluorophenyl ester, may be used.

[0355] As the reaction between compound (2b) and antibody (3), using 2 to 20 molar equivalents of the compound (2b) per the antibody (3) in the reaction of the compound (2b) with the antibody (3), the antibody-drug conjugate (1) in which 1 to 10 drug molecules are conjugated per antibody can be produced. Specifically, the solution containing the compound (2b) dissolved therein can be added to a buffer solution containing the antibody (3) for the reaction to produce the antibody-drug conjugate (1). Herein, examples of the buffer solution which may be used include sodium acetate solution, sodium phosphate, and sodium borate. pH for the reaction can be 5 to 9, and more preferably the reaction is performed near pH 7. Examples of the solvent for dissolving the compound (2b) include an organic solvent such as

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dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide (DMA), and N-methyl-2-pyrindone (NMP). It is sufficient that the organic solvent solution containing the compound (2b) dissolved therein is added at 1 to 20% v/v to a buffer solution containing the antibody (3) for the reaction. The reaction temperature is 0 to 37°C, more preferably 10 to 25°C, and the reaction time is 0.5 to 20 hours.

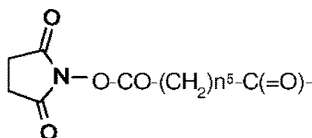
5 [0356] For the produced antibody-drug conjugate (1), concentration, buffer exchange, purification, and identification of the antibody-drug conjugate (1) by the measurement of antibody concentration and an average number of conjugated drug molecules per antibody molecule can be performed in the same manner as Production method 1.

[0357] In Production method 3, (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-(CH₂)ⁿ-C(=O)- has the following structure.

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[Formula 45]

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[0358] Examples of the compound having the above partial structure and having the peptide linker having a hydrophilic amino acid at the N terminal can include the followings.

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(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[0359] Among them, the followings are more preferred.

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(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-C(=O)-(NH-DX).

[0360] Examples of the compound having the above partial structure and having the peptide linker having a hydrophilic amino acid at the N terminal, which is directly connected to the drug can include the followings.

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(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
 (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),

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(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX).

[0361] Examples of the compound having the above partial structure and having the peptide linker having a glycine oligopeptide at the C terminal, which is directly connected to the drug can include the followings.

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-(NH-DX).

[0362] Examples of the compound having the above partial structure and having the hydrophilic structure in L¹ can include the followings.

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[0363] Examples of the compound having the above partial structure, having the hydrophilic structure in L¹, and having the peptide linker directly connected to the drug can include the followings.

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH(CH₂CH₂-COOH)-CH₂-C(=O)-GGFG-(NH-DX).

[0364] Examples of the compound having the above partial structure and having the hydrophilic structure in L² can include the followings.

(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-C(=O)-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX).

[0365] Examples of the compound having the above partial structure, having the hydrophilic structure in L², and the peptide linker directly connected to the drug can include the followings.

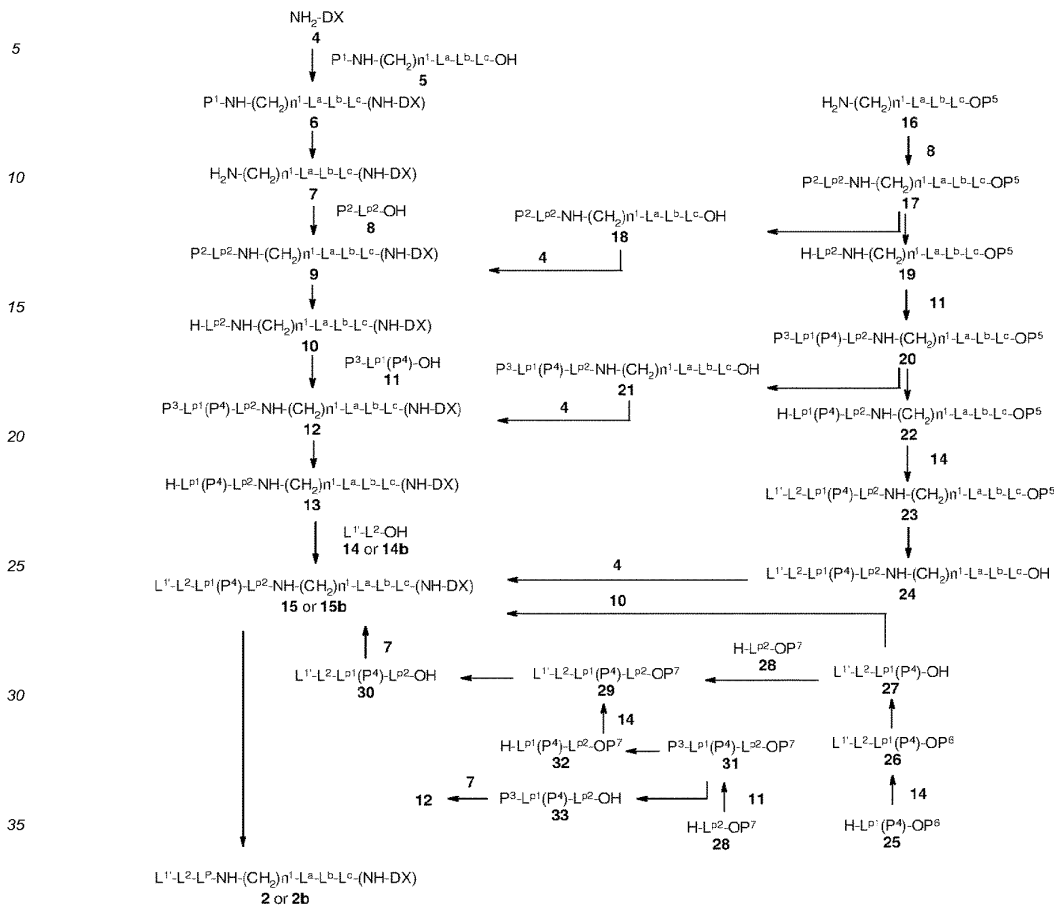
(Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-N(-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-O-CH₂CH₂-OH)-CH₂-C(=O)-GGFG-(NH-DX).

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[0366] Among the compound represented by the formula (2) or (2b) used as an intermediate in the previous production method and a pharmacologically acceptable salt thereof, those in which the linker has a structure represented by -L¹-L²-L^P-NH-(CH₂)_n-L^a-L^b-L^c-, and L^P has a hydrophilic amino acid other than glycine at the N terminal, for example, can be produced by the following method.

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[Formula 46-1]



[0367] In the formula, L^c is a -C(=O)- group, L^{1'} represents L¹ structure in which the terminal is a maleimidy group or a haloacetyl group, or L¹ is converted to (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-(CH₂)_n⁵-C(=O)-, L^p represents a structure of -L^{p1}-L^{p2}-, and P¹, P², P³, P⁴, P⁵, P⁶, and P⁷ each represent a protecting group.

[0368] Since L^p is formed by connecting L^{p1} and L^{p2}, the N-terminal hydrophilic amino acid of L^p is derived from L^{p1}, therefore, L^{p1} having a hydrophilic amino acid at the N terminal can be used. A plurality of hydrophilic amino acids may be present therein. If L^{p2} having a hydrophilic amino acid is used, according to its position, L^p can be produced so as to contain a plurality of hydrophilic amino acids at the N terminal of L^p or at the N terminal and other positions.

[0369] The compound (6) can be produced by derivatizing the carboxylic acid compound (5) having the terminal amino group protected with P¹ into active ester, mixed acid anhydride, acid halide, or the like and reacting it with NH₂-DX [which represents exatecan; chemical name: (1S, 9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolino[1,2-b]quinoline-10,13(9H,15H)-dione] (that is, the pharmaceutical compound described in claim 2 of Japanese Patent Laid-Open No. 6-87746) (4) or a pharmacologically acceptable salt thereof in the presence of a base.

[0370] For this reaction, reagents and conditions commonly used for amidation and peptide synthesis can be employed. There are various kinds of active ester, for example, it can be produced by reacting phenols such as p-nitrophenol, N-hydroxy benzotriazole, N-hydroxy succinimide, or the like, with the carboxylic acid compound (5) using a condensation agent such as N,N'-dicyclohexylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. Further, the active ester can be also produced by a reaction of the carboxylic acid compound (5) with pentafluorophenyl trifluoroacetate or the like; a reaction of the carboxylic acid compound (5) with 1-benzotriazolyl oxytripyrrolidinophosphonium

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hexafluorophosphite; a reaction of the carboxylic acid compound (5) with diethyl cyanophosphonate (Shioiri method); a reaction of the carboxylic acid compound (5) with triphenylphosphine and 2,2'-dipyridyl disulfide (Mukaiyama's method); a reaction of the carboxylic acid compound (5) with a triazine derivative such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM); or the like. Further, the reaction can be also performed by, e.g., an acid halide method by which the carboxylic acid compound (5) is treated with acid halide such as thionyl chloride or oxalyl chloride in the presence of a base. By reacting the active ester, mixed acid anhydride, or acid halide of the carboxylic acid compound (5) obtained above with the compound (4) in the presence of a suitable base in a solvent which does not inhibit a reaction at -78°C to 150°C, the compound (6) can be produced.

[0371] Specific examples of the base used for each step described above include carbonate of an alkali metal or an alkali earth metal, an alkali metal alkoxide, hydroxide or hydride of an alkali metal including sodium carbonate, potassium carbonate, sodium ethoxide, potassium butoxide, sodium hydroxide, potassium hydroxide, sodium hydride, and potassium hydride, organometallic base represented by an alkyl lithium including n-butyl lithium, dialkylamino lithium including lithium diisopropylamide; organometallic base of bissilylamine including lithium bis(trimethylsilyl)amide; and organic base including pyridine, 2,6-lutidine, collidine, 4-dimethylaminopyridine, triethylamine, N-methylmorpholine, diisopropylethylamine, and diazabicyclo[5.4.0]undec-7-ene (DBU).

[0372] Examples of the solvent, which is used for the reaction of the present invention and does not inhibit the reaction, include a halogenated hydrocarbon solvent such as dichloromethane, chloroform, and carbon tetrachloride; an ether solvent such as tetrahydrofuran, 1,2-dimethoxyethane, and dioxane; an aromatic solvent such as benzene and toluene; and an amide solvent such as N,N-dimethylformamide, N,N-dimethylacetamide, and N-methylpyrrolidin-2-one. In addition to them, a sulfoxide solvent such as dimethyl sulfoxide and sulfolane; an alcohol solvent such as methanol and ethanol; and a ketone solvent such as acetone and methyl ethyl ketone may be used depending on a case.

[0373] The hydroxy group, carboxy group, amino group, or the like of L^a and L^b in the compound (6) may be protected by a protecting group which is commonly used in organic compound synthesis, as mentioned later. Specifically, examples of the protecting group for a hydroxyl group include an alkoxymethyl group such as methoxymethyl group; an arylmethyl group such as benzyl group, 4-methoxybenzyl group, and triphenylmethyl group; an alkanoyl group such as acetyl group; an aroyl group such as benzoyl group; and a silyl group such as tert-butyl diphenylsilyl group. Carboxy group can be protected, e.g., as an ester with an alkyl group such as methyl group, ethyl group, and tert-butyl group, an allyl group, or an arylmethyl group such as benzyl group. Amino group can be protected with a protecting group for an amino group which is generally used for peptide synthesis, for example, an alkoxy carbonyl group such as tert-butyloxy carbonyl group, methoxycarbonyl group, ethoxycarbonyl group, and 2-(trimethylsilyl)ethoxycarbonyl group; an arylmethyl group such as allyloxycarbonyl, 9-fluorenylmethyloxy carbonyl group, benzyloxy carbonyl group, paramethoxybenzyloxy carbonyl group, and para (or ortho)nitrobenzyloxy carbonyl group; an alkanoyl group such as acetyl group; an arylmethyl group such as benzyl group and triphenyl methyl group; an aroyl group such as benzoyl group; and an aryl sulfonyl group such as 2,4-dinitrobenzene sulfonyl group or orthonitrobenzene sulfonyl group. Protection with and deprotection of the protecting group can be performed according to a method commonly carried out in the art.

[0374] As for the protecting group P¹ for the terminal amino group of the compound (6), a protecting group for an amino group which is generally used for peptide synthesis, for example, tert-butyloxy carbonyl group, 9-fluorenylmethyloxy carbonyl group, and benzyloxy carbonyl group, can be used. Examples of the other protecting group for an amino group include an alkanoyl group such as acetyl group; an alkoxycarbonyl group such as methoxycarbonyl group and ethoxycarbonyl group; an arylmethoxy carbonyl group such as paramethoxybenzyloxy carbonyl group, and para (or ortho)nitrobenzyloxy carbonyl group; an arylmethyl group such as benzyl group and triphenyl methyl group; an aroyl group such as benzoyl group; and an aryl sulfonyl group such as 2,4-dinitrobenzene sulfonyl group and orthonitrobenzene sulfonyl group. The protecting group P¹ can be selected depending on, e.g., properties of a compound having an amino group to be protected.

[0375] By deprotecting the protecting group P¹ for the terminal amino group of the compound (6) obtained, the compound (7) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0376] By derivatizing the peptide or amino acid (8), which is protected by P² at its N terminal, into an active ester, mixed acid anhydride, or the like and reacting it with the compound (7) obtained, the compound (9) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the peptide or amino acid (8) and the compound (7) are not limited as long as they do not inhibit a reaction, and can be suitably selected from those described for the synthesis of the compound (6). The protecting group P² for an amino group can be suitably selected from those described for the protecting group of the compound (6), and the selection can be made based on, e.g., the properties of the compound. As it is generally used for peptide synthesis, by repeating sequentially the reaction and deprotection of the amino acid or peptide constituting the peptide or amino acid (8) for elongation, the compound (9) can be also produced.

[0377] By deprotecting P² as the protecting group for the amino group of the compound (9) obtained, the compound (10) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0378] By derivatizing the amino acid or peptide (11) having the N terminal protected with P³ and a side chain carboxy

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group, hydroxy group, or amino group protected with P⁴ into an active ester, mixed acid anhydride, or the like and reacting it with the compound (10) obtained, the compound (12) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the amino acid or peptide (11) and the compound (10) can be suitably selected from those described for the synthesis of the compound (6). The protecting groups P³ and P⁴ can be suitably selected from those described for the protecting group for the amino group, carboxy group, or hydroxy group of the compound (6). However, in such case, it is necessary that the protecting group P³ for an amino group and the protecting group P⁴ for a side chain functional group can be removed by a different method or different conditions. For example, a representative example includes a combination in which P³ is a 9-fluorenylmethyloxycarbonyl group and P⁴ is a tert-butyl group or the like for a carboxy group, a methoxymethyl group or the like for a hydroxy group, or a tert-butyloxycarbonyl group or the like for an amino group. The protecting group P⁴ for a side chain functional group is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be selected from the aforementioned ones depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group. As it is generally used for peptide synthesis, by repeating sequentially the reaction and deprotection of the constituting amino acid or peptide for elongation, the compound (12) can be also produced.

[0379] By deprotecting P³ as the protecting group for the terminal amino group of the compound (12) obtained, the compound (13) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0380] By derivatizing the carboxylic acid derivative (14) or (14b) into an active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (13) obtained, the compound (15) or (15b) can be produced. Here, the carboxylic acid derivative (14) is a compound having a structure of L^{1'} in which the linker terminal is a maleimidyl group or a haloacetyl group, and the carboxylic acid derivative (14b) is a compound having a structure of L^{1'} in which the linker terminal has (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-.

[0381] The reaction conditions, reagents, base, and solvent used for a peptide bond formation between the carboxylic acid derivative (14) or (14b) and the compound (13) can be suitably selected from those described for the synthesis of the compound (6).

[0382] By deprotecting P⁴ as the protecting group for the amino acid side chain carboxy group, hydroxy group or amino group of the peptide moiety of the compound (15) or (15b) obtained, the compound (2) or (2b) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0383] The compound (9) can be also produced by the following method, for example.

[0384] By derivatizing the peptide or amino acid (8) having the N terminal protected with P² into active ester, mixed acid anhydride, or the like and reacting it with the amine compound (16) having the terminal carboxy group protected with P⁵ in the presence of a base, the compound (17) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the peptide or amino acid (8) and the compound (16) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P² for the amino group of the compound (17) can be suitably selected from those described for the protecting group of the compound (6). As for the protecting group P⁵ for a carboxy group, a protecting group commonly used as a protecting group for a carboxy group in organic synthetic chemistry, in particular, peptide synthesis can be used. Specifically, it can be suitably selected from those described for the protecting group of the compound (6), for example, esters with an alkyl group such as a methyl group, an ethyl group, or a tert-butyl, allyl esters, and benzyl esters. In such case, it is necessary that the protecting group P² for an amino group and the protecting group P⁵ for a carboxy group can be removed by a different method or different conditions. For example, a representative example includes a combination in which P² is a tert-butyloxy carbonyl group and P⁵ is a benzyl group. The protecting groups can be selected from the aforementioned ones depending on, e.g., the properties of a compound having an amino group and a carboxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group.

[0385] By deprotecting the protecting group P⁵ for the carboxy group of the compound (17) obtained, the compound (18) can be produced. Reagents and conditions are selected depending on the protecting group.

[0386] By derivatizing the compound (18) obtained into active ester, mixed acid anhydride, acid halide, or the like and reacting with the compound (4) in the presence of a base, the compound (9) can be produced. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

[0387] The compound (12) can be also produced by the following method, for example.

[0388] By deprotecting the protecting group P² for the amino group of the compound (17), the compound (19) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0389] By derivatizing the amino acid or peptide (11) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (19) obtained in the presence of a base, the compound (20) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the amino acid or peptide (11) and the compound (19) can be suitably selected from those described for the synthesis of the compound (6). Here,

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it is necessary that the protecting groups P³ and P⁴ of the amino acid or peptide (11) and the protecting group P⁵ of the compound (19) can be removed by a different method or different conditions. For example, a representative example includes a combination in which P³ is a 9-fluorenylmethoxycarbonyl group, P⁴ is a tert-butyloxycarbonyl group, tert-butyl group, or methoxymethyl group, and P⁵ is a benzyl group. As mentioned above, the protecting group P⁴ for a side chain functional group is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be selected from the aforementioned ones depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group.

[0390] By deprotecting the protecting group P⁵ for the carboxy group of the compound (20) obtained, the compound (21) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0391] By derivatizing the compound (21) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) in the presence of a base, the compound (12) can be produced. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

[0392] The compound (15) can be also produced by the following method, for example.

[0393] By deprotecting the protecting group P³ for the amino group of the compound (20), the compound (22) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0394] By derivatizing the carboxylic acid derivative (14) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (22) obtained in the presence of a base, the compound (23) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the carboxylic acid derivative (14) and the compound (22) can be suitably selected from those described for the synthesis of the compound (6).

[0395] By deprotecting the protecting group P⁵ for the carboxy group of the compound (23) obtained, the compound (24) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0396] The compound (15) can be produced by derivatizing the compound (24) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) in the presence of a base. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

[0397] The compound (15) can be also produced by the following method, for example.

[0398] By derivatizing the carboxylic acid derivative (14) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the amino acid or peptide (25) having a carboxy group protected with P⁶ and a side chain carboxy group, hydroxy group, or amino group protected with P⁴ in the presence of a base, the compound (26) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the carboxylic acid derivative (14) and the compound (25) can be suitably selected from those described for the synthesis of the compound (6). Here, the protecting groups P⁴ and P⁶ of the compound (26) can be suitably selected from those described for the protecting group for the carboxy group, hydroxy group, or amino group of the compound (6). However, in such case, it is necessary that the protecting group P⁶ for a carboxy group and the protecting group P⁴ for a side chain functional group can be removed by a different method or different conditions. For example, a representative example includes a combination in which P⁶ is a benzyl group and P⁴ is a tert-butyl group or the like for a carboxy group, a methoxymethyl group or the like for a hydroxy group, or a tert-butyloxycarbonyl group or the like for an amino group. The protecting group P⁴ for a side chain functional group is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be selected from the aforementioned ones depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group.

[0399] By deprotecting the protecting group P⁶ for the carboxy group of the compound (26) obtained, the compound (27) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0400] By derivatizing the compound (27) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (10) in the presence of a base, the compound (15) can be produced. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

[0401] By derivatizing the compound (27) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the amino acid or peptide (28) having a carboxy group protected with P⁷ in the presence of a base, the compound (29) can be produced. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6). Here, the protecting groups P⁴ and P⁷ of the compound (29) can be suitably selected from those described for the protecting group for the carboxy group, hydroxy group, or

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amino group of the compound (6). However, in such case, it is necessary that the protecting group P⁷ for a carboxy group and the protecting group P⁴ for a side chain functional group can be removed by a different method or different conditions. For example, a representative example includes a combination in which P⁷ is a benzyl group and P⁴ is a tert-butyl group or the like for a carboxy group, a methoxymethyl group or the like for a hydroxy group, or a tert-butyloxycarbonyl group or the like for an amino group. The protecting group P⁴ for a side chain functional group is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be selected from the aforementioned ones depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group. By repeating sequentially the reaction and deprotection of the constituting amino acid or peptide for elongation, the compound (29) can be also produced.

[0402] By deprotecting the protecting group P⁷ for the carboxy group of the compound (29) obtained, the compound (30) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0403] By derivatizing the compound (30) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (7) in the presence of a base, the compound (15) can be produced. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

[0404] The compound (29) can be also produced by the following method, for example.

[0405] By derivatizing the amino acid or peptide (28) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the amino acid or peptide (11) having the N terminal protected with P³ and a side chain carboxy group, hydroxy group, or amino group protected with P⁴ in the presence of a base, the peptide (31) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the amino acid or peptide (28) and the amino acid or peptide (11) can be suitably selected from those described for the synthesis of the compound (6). Here, as mentioned above, it is necessary that the protecting group P⁷ for the carboxy group of the amino acid or peptide (28) and the protecting groups P³ and P⁴ of the amino acid or peptide (11) can be removed by a different method or different conditions. For example, a representative example includes a combination in which P³ is a 9-fluorenylmethyloxycarbonyl group, P⁴ is a tert-butyl group or the like for a carboxy group, a methoxymethyl group or the like for a hydroxy group, or a tert-butyloxycarbonyl group or the like for an amino group, and P⁷ is a benzyl group. The protecting group P⁴ for a side chain functional group is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be selected from the aforementioned ones depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group.

[0406] By deprotecting P³ as the protecting group for the N-terminal of the peptide (31) obtained, the peptide (32) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0407] By derivatizing the carboxylic acid derivative (14) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the peptide (32) obtained in the presence of a base, the compound (29) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the carboxylic acid derivative (14) and the peptide (32) can be suitably selected from those described for the synthesis of the compound (6).

[0408] The compound (12) can be also produced by the following method, for example.

[0409] By deprotecting P⁷ as the protecting group for the C terminal of the peptide (31), the peptide (33) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0410] By derivatizing the peptide (33) obtained into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (7) in the presence of a base, the compound (12) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the peptide (33) and the compound (7) can be suitably selected from those described for the synthesis of the compound (6).

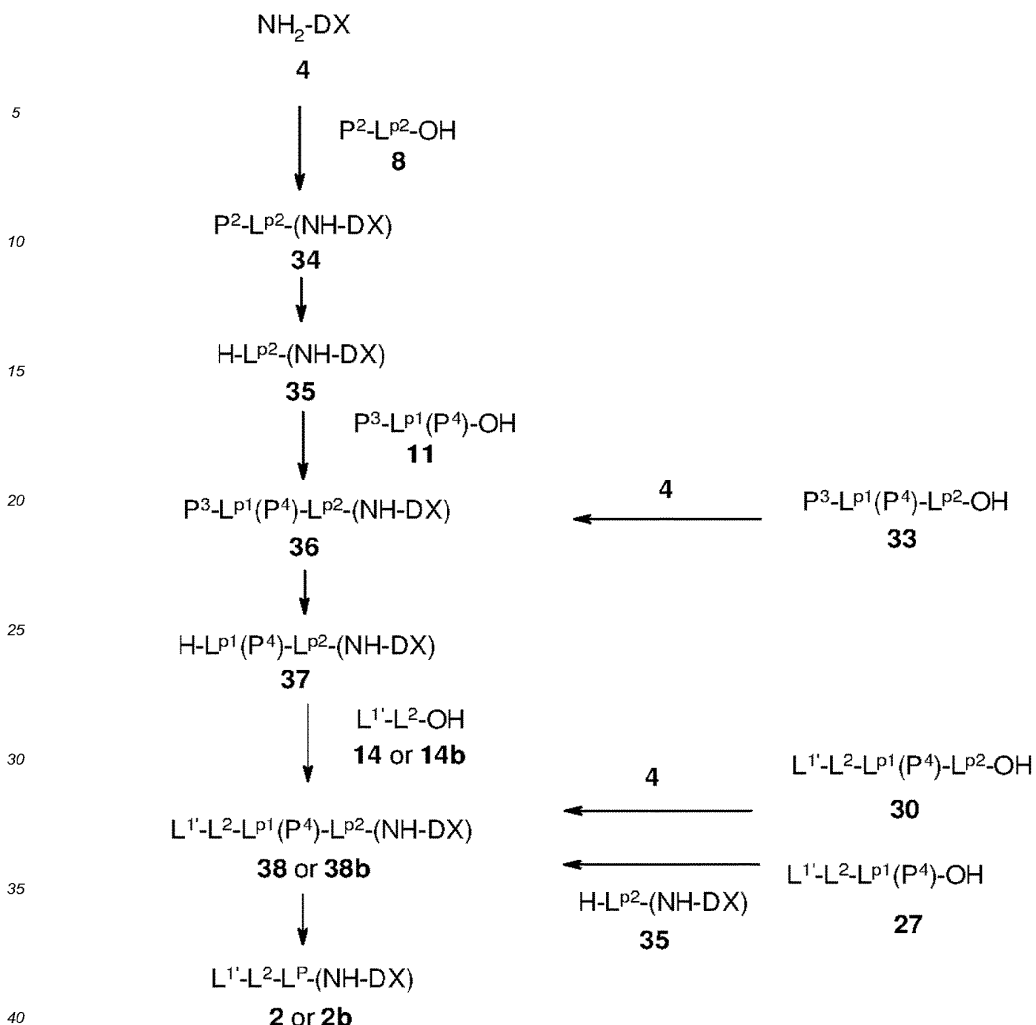
5. Production method 5

[0411] Among the production intermediate represented by the formula (2) or (2b) in which the linker has a structure represented by -L¹-L²-L^P-, and L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal can be also produced by the following method.

[Formula 46-2]

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[0412] In the formula, L^{1'} represents L¹ structure in which the terminal is a maleimidyl group or a haloacetyl group, or L¹ is converted to (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-(CH₂)_n-C(=O)-, L^P represents a structure of -L^{P1}-L^{P2}-, and P², P³, P⁴, and P⁷ each represent a protecting group.

[0413] Since L^P is formed by connecting L^{P1} and L^{P2}, the N-terminal hydrophilic amino acid of L^P is derived from L^{P1}, therefore, L^{P1} having a hydrophilic amino acid at the N terminal can be employed. L^P may have a plurality of hydrophilic amino acids. If L^{P2} having a hydrophilic amino acid is used, according to the position thereof, L^P can be produced so as to contain hydrophilic amino acids at the N terminal of L^P or at the N terminal and other positions.

[0414] By derivatizing the peptide or amino acid (8) described in Production method 4 having the N terminal protected with P² into an active ester, mixed acid anhydride, or the like and reacting it with the compound (4) or a salt thereof, the compound (34) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the peptide or amino acid (8) and the compound (4) are not limited as long as they do not inhibit a reaction, and can be suitably selected from those described for the synthesis of the compound (6). The protecting group P² can be suitably selected from those described for the protecting group of the compound (6), and the selection can be made based on, e.g., the properties of the compound having an amino group to be protected. As it is generally used for peptide synthesis, by repeating sequentially the reaction and deprotection of the amino acid or peptide constituting the peptide or amino acid (8) for elongation, the compound (34) can be also produced.

[0415] By deprotecting P² as the protecting group for the amino group of the compound (34) obtained, the compound

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(35) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0416] By derivatizing the amino acid or peptide (11) described in Production method 4 having the N terminal protected with P³ and a side chain carboxy group, hydroxy group, or amino group protected with P⁴ into an active ester, mixed acid anhydride, or the like and reacting it with the compound (35) obtained, the compound (36) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the amino acid or peptide (11) and the compound (35) can be suitably selected from those described for the synthesis of the compound (6). The protecting groups P³ and P⁴ are as described in Production method 4. As it is generally used for peptide synthesis, by repeating sequentially the reaction and deprotection of the constituting amino acid or peptide for elongation, the compound (36) can be also produced.

[0417] By deprotecting P³ as the protecting group for the amino group of the compound (36) obtained, the compound (37) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0418] By derivatizing the carboxylic acid derivative (14) or (14b) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (37) obtained, the compound (38) or (38b) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the carboxylic acid derivative (14) or (14b) and the compound (37) can be suitably selected from those described for the synthesis of the compound (6).

[0419] By deprotecting P⁴ as the protecting group for the carboxy group or hydroxy group, or amino group of the compound (38) or (38b) obtained, the compound (2) or (2b) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0420] The compound (36) can be also produced by the following method, for example.

[0421] By derivatizing the peptide (33) described in Production method 4 into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) or a salt thereof, the compound (36) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the peptide (33) and the compound (4) can be suitably selected from those described for the synthesis of the compound (6).

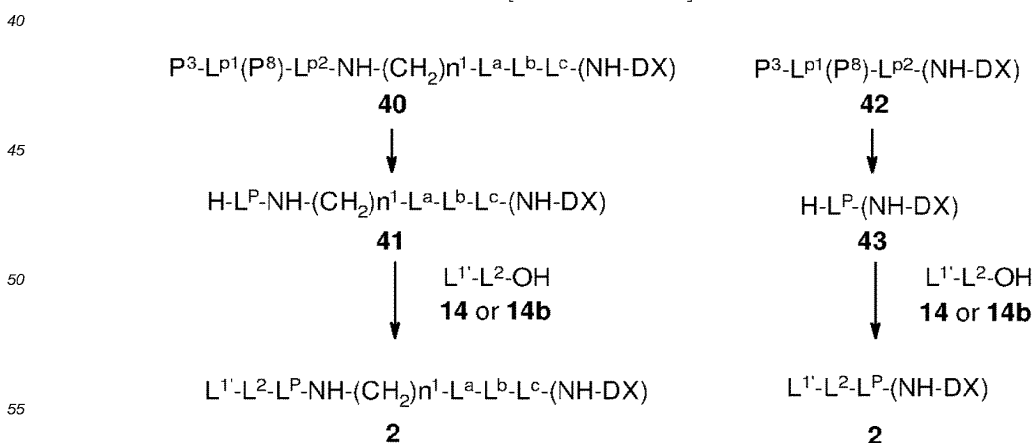
[0422] The compound (38) can be also produced by the following method, for example.

[0423] The compound (38) can be produced by derivatizing the compound (30) described in Production method 4 into an active ester, mixed acid anhydride, or the like and reacting it with the compound (4) in the presence of a base, or by derivatizing the amino acid or peptide (27) described in Production method 4 into an active ester, mixed acid anhydride, or the like and reacting it with the compound (35) in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming a peptide bond can be suitably selected from those described for the synthesis of the compound (6).

6. Production method 6

[0424] Among the production intermediate represented by the formula (2), those which have a structure of -L¹-L²-L^P-NH-(CH₂)_n-L^a-L^b-L^c- or -L¹-L²-L^P-, and L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal can be also produced by the following method, for example.

[Formula 47]



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[0425] In the formula, L¹ represents L¹ structure in which the terminal is a maleimidy group or a haloacetyl group, or L¹ is converted to (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-(CH₂)ⁿ-C(=O)-, L^P represents a structure of -L^{P1}-L^{P2}-, and P³ and P⁸ each represent a protecting group.

[0426] The production intermediate represented by the formula (2) has two forms of the linker: a structure represented by -L¹-L²-L^P-NH-(CH₂)ⁿ-L^a-L^b-L^c- and a structure represented by -L¹-L²-L^P-.

[0427] The compound (2) in which the linker has the structure represented by -L¹-L²-L^P-NH-(CH₂)ⁿ-L^a-L^b-L^c- can be produced as follows.

[0428] The compound (40) can be synthesized in the same manner as in the compound (12) described in Production method 4. Unlike the compound (12), it may be unnecessary that the protecting group P³ for an amino group and the protecting group P⁸ for a side chain functional group can be removed by a different method or different conditions. The functional group of the side chain is a carboxy group or a hydroxy group, and the protecting group P³ for an amino group and the protecting group P⁸ for a side chain carboxy group or hydroxy group can be deprotected simultaneously. For example, a representative example includes a combination in which P³ is a tert-butyloxycarbonyl group, and P⁸ is a tert-butyl group or a trityl group, or P³ is a benzyloxycarbonyl group, and P⁸ is a benzyl group. These protecting groups can be suitably selected from those described for the protecting group of the compound (6) depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group. The compound (40) can be synthesized in the same manner as Production method 4 by using the protected amino acid or peptide that satisfies the properties described above.

[0429] By sequentially or simultaneously deprotecting the protecting groups P³ and P⁸ of the compound (40), the compound (41) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0430] Although the functional group in the hydrophilic side chain of L^P in the compound (41) is not particularly protected, the compound (2) can be produced by reacting it with the compound (14) or (14b) derivatized into an active ester, mixed acid anhydride, or the like in the presence of a base. The reaction conditions, reagents, base, and solvent used for a peptide bond formation can be suitably selected from those described for the synthesis of the compound (6).

[0431] The compound (2) in which the linker has the structure represented by -L¹-L²-L^P- can be produced as follows.

[0432] The compound (42) can be also synthesized as in the same manner as the compound (36) described in Production method 5. Unlike the compound (36), it may be unnecessary that the protecting group P³ for an amino group and the protecting group P⁸ for the functional group of the side chain can be removed by a different method or different conditions. The functional group of the side chain is a carboxy group or a hydroxy group, and the protecting group P³ for an amino group and the protecting group P⁸ for a side chain carboxy group or hydroxy group can be also deprotected simultaneously. For example, a representative example includes a combination in which P³ is a tert-butyloxycarbonyl group, and P⁸ is a tert-butyl group or a trityl group, or P³ is a benzyloxycarbonyl group, and P⁸ is a benzyl group. These protecting groups can be suitably selected from those described for the protecting group of the compound (6) depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. For removal of the protecting groups, reagents and conditions can be selected depending on the protecting group. The compound (42) can be synthesized in the same manner as Production method 5 by using the protected amino acid or peptide that satisfies the properties described above.

[0433] By sequentially or simultaneously deprotecting the protecting groups P³ and P⁸ of the compound (42), the compound (43) can be produced. Reagents and conditions can be selected depending on the protecting group.

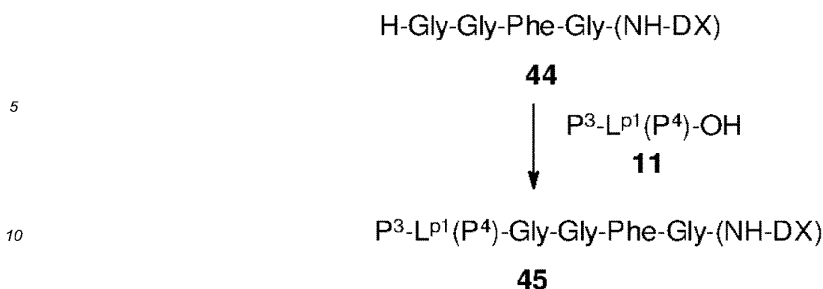
[0434] Although the functional group in the hydrophilic side chain of L^P in the compound (43) is not particularly protected, the compound (2) can be produced by reacting it with the compound (14) or (14b) derivatized into an active ester, mixed acid anhydride, or the like in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming a peptide bond can be suitably selected from those described for the synthesis of the compound (6).

7. Production method 7

[0435] The compound (36) shown in Production method 5 in which linker -L^P- has a structure of -L^{P1}-Gly-Gly-Phe-Gly can be also produced by the following method.

[Formula 48]

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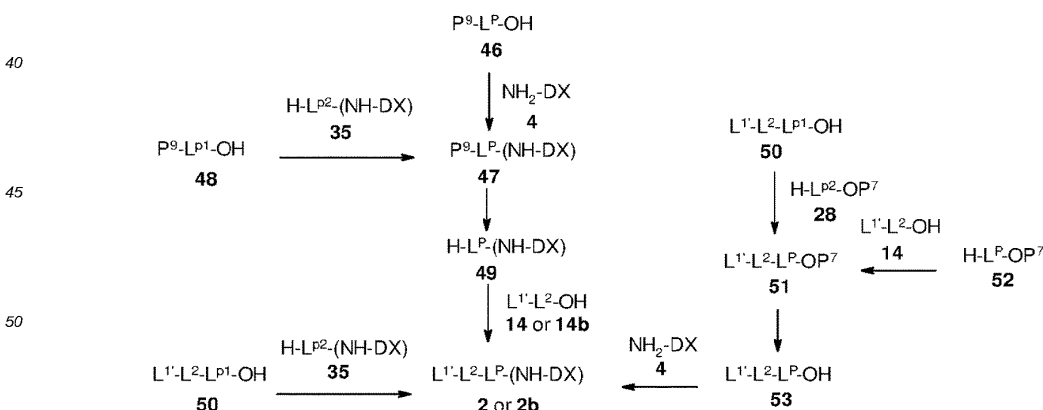
[0436] In the formula, L^1 represents L^1 structure in which the terminal is a maleimidyl group or a haloacetyl group, or L^1 is converted to (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-(CH₂)_n-C(=O)-, $-L^P$ - represents a structure of $-L^{P^1}-Gly-Gly-Phe-Gly-$, and P^3 and P^4 each represent a protecting group.

[0437] The compound (45) can be produced by derivatizing the amino acid or peptide (11) described in Production method 4 into active ester, mixed acid anhydride, acid halide, or the like and reacting it with glycyglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzozo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycineamide (that is, a free form of the pharmaceutical compound described in WO97/46260) (44) or a salt thereof in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the amino acid or peptide (11) and the compound (44) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^3 for the N-terminal and the protecting group P^4 for a side chain functional group are as mentioned above in Production method 4. The protecting group P^4 for a side chain functional group may be absent, and the compound (45) can be obtained by the reaction using the amino acid or peptide (11) protected only at the N-terminal.

8. Production method 8

[0438] Among the compound represented by the formula (2) or (2b), those which have the linker structure represented by $-L^1-L^2-L^P-$, and L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the drug, and even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, can be also produced by the following method, for example.

[Formula 49]



[0439] In the formula, L^1 represents L^1 structure in which the terminal is a maleimidyl group or a haloacetyl group, or L^1 is converted to (Pyrrolidine-2,5-dione-N-yl)-O-C(=O)-(CH₂)_n-C(=O)-, L^P represents a structure of $-L^{P^1}-L^{P^2}-$, and P^7 and P^9 each represent a protecting group.

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[0440] Since L^P is formed by connecting L^{P1} and L^{P2} , the number of glycines contained therein for constituting the C terminal of L^P can be determined by considering the number of C-terminal glycines of L^P and further, the number of repetitive uses thereof for the reaction.

5 [0441] The peptide (46) is an oligopeptide in which the C terminal is consisting of 2 or 3 or more glycines, and in case when the N terminal is optionally a hydrophilic amino acid but it is not a hydrophilic amino acid other than glycine, and this N terminal is protected with P^9 . The peptide (46) can be synthesized by repeating sequentially the condensation reaction and deprotection of the amino acid or peptide constituting it, as it is generally used for peptide synthesis.

10 [0442] By derivatizing the peptide (46) into an active ester, mixed acid anhydride, or the like and reacting it with the compound (4) or a salt thereof, the compound (47) can be produced. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the peptide (46) and the compound (4) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^9 can be suitably selected from those described for the synthesis of the compound (6).

15 [0443] The compound (47) can be also produced by derivatizing the amino acid or peptide (48) having the N terminal protected with P^9 into an active ester, mixed acid anhydride, or the like and reacting it with the compound (35) described in Production method 5. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the amino acid or peptide (48) and the compound (35) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^9 can be suitably selected from those described for the synthesis of the compound (6).

20 [0444] By deprotecting the protecting group P^9 for the amino group of the compound (47) obtained, the compound (49) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0445] By derivatizing the carboxylic acid derivative (14) or (14b) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (49) obtained, the compound (2) or (2b) can be produced. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the carboxylic acid derivative (14) or (14b) and the compound (49) can be suitably selected from those described for the synthesis of the compound (6).

25 [0446] The compound (2) can be also produced by the following method.

30 [0447] The compound (50) in which the N-terminal glycine of L^{P1} is connected to L^2 can be synthesized as in the same manner as the compound (27) described in Production method 4. By derivatizing the amino acid or peptide (28) described in Production method 4 into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (50), the compound (51) can be produced. Here, the amino acid or peptide (28) is glycine or an oligopeptide having the C-terminal consisting of 2 or 3 or more glycines, and its C terminal is protected with P^7 . The reaction conditions, reagents, base, and solvent used for forming an amide bond between the amino acid or peptide (28) and the compound (50) can be suitably selected from those described for the synthesis of the compound (6).

35 [0448] The compound (51) can be also produced by derivatizing the compound (14) into an active ester, mixed acid anhydride, or the like and reacting it with the peptide (52) having the C terminal protected with P^7 . Here, the peptide (52) is an oligopeptide in which the C terminal is consisting of 2 or 3 or more glycines, and the N terminal is optionally a hydrophilic amino acid but no other hydrophilic amino acid than glycine is present thereat. The peptide (52) can be synthesized by repeating sequentially the condensation reaction and deprotection of the amino acid or peptide constituting it, as it is generally used for peptide synthesis. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the peptide (52) and the compound (14) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P^7 is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be suitably selected from those described for the synthesis of the compound (6).

40 [0449] By deprotecting the protecting group P^7 for the carboxy group of the compound (51) obtained, the compound (53) can be produced. Reagents and conditions can be selected depending on the protecting group.

45 [0450] The compound (2) can be produced by derivatizing the compound (53) into an active ester, mixed acid anhydride, or the like and reacting it with the compound (4) or a salt thereof. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the compound (53) and the compound (4) can be suitably selected from those described for the synthesis of the compound (6).

50 [0451] Alternatively, the compound (2) can be also produced by the following method.

[0452] The compound (2) can be produced by derivatizing the compound (35) described in Production method 5 into an active ester, mixed acid anhydride, or the like and reacting it with the compound (50) in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the compound (50) and the compound (35) can be suitably selected from those described for the synthesis of the compound (6).

55 9. Production method 9

[0453] The production intermediate represented by the formula (2a) described in Production method 2 in which $L^{2'}$ corresponds to L^2 having a structure in which the terminal is converted to a mercaptoalkanoyl group can be produced

by the following method.

5



[0457] The compound (55) can be produced by derivatizing the carboxylic acid compound (54) having a terminal mercapto group protected with P¹⁰ into an active ester, mixed acid anhydride, or the like and reacting it with the compound (13) described in Production method 4. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6). As for the protecting group P¹⁰ for a mercapto group, a protecting group commonly used as a protecting group for a mercapto group in organic synthetic chemistry can be used. Specifically, it can be suitably selected from sulfide groups such as a S-methyl sulfide group, a S-ethyl sulfide group, and a S-2-pyridyl sulfide group, ester groups such as an acetyl group, aryl methyl ether groups such as a benzyl group, a 9-fluorenylmethyl group, and a trityl group, ethyl ether groups such as a S-2-cyanoethyl group, and the like. In this case, the protecting group P⁴ for the side chain amino group, carboxy group, or hydroxy group of LP¹ is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto, and can be selected from the aforementioned ones depending on, e.g., the properties of the compound having an amino group, carboxy group, or hydroxy group to be protected. However, it is necessary that the protecting group P¹⁰ for a mercapto group and the protecting group P⁴ for the side chain carboxy group, hydroxy group, or amino group of LP¹ can be removed

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by a different method or different conditions. For example, a representative example includes a combination in which the protecting group P⁴ is a tert-butyl group for a carboxy group, and the protecting group P¹⁰ is a S-methyl sulfide group. The protecting group P¹⁰ may be absent. In this case, the mercapto group of the compound (55) is unprotected.

5 **[0458]** By deprotecting the protecting group P⁴ for the side chain carboxy group, hydroxy group, or amino group of LP¹ in the compound (55) obtained, the compound (56) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0459] By deprotecting the protecting group P¹⁰ for the mercapto group of the compound (56) obtained, the compound (2a) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0460] The compound (55) can be also produced by the following method.

10 **[0461]** The compound (57) can be produced by derivatizing the carboxylic acid compound (54) having a mercapto group protected with P¹⁰ into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (22) described in Production method 4. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6). The protecting groups P⁴ and P¹⁰ are as mentioned above. The protecting group P⁵ for a carboxy group can be suitably selected from those described for the protecting group of the compound (6). However, it is necessary that the protecting group P¹⁰ for a mercapto group and the protecting group P⁴ for a side chain functional group can be removed by a different method or different conditions from those for the protecting group P⁵ for a carboxy group. For example, a representative example includes a combination in which P⁴ is a tert-butyl group for a carboxy group, P¹⁰ is a S-methyl sulfide group, and P⁵ is an allyl group. The protecting group P¹⁰ may be absent. In this case, the mercapto group of the compound (57) is unprotected.

20 **[0462]** The compound (58) can be produced by deprotecting the protecting group P⁵ for the carboxy group of the compound (57) obtained. Reagents and conditions can be selected depending on the protecting group.

25 **[0463]** The compound (55) can be produced by derivatizing the compound (58) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) in the presence of a base. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

[0464] The compound (2a) in which the linker has the structure represented by -L¹-L²-LP-, and LP is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal can be produced as follows.

30 **[0465]** The compound (59) can be produced by derivatizing the carboxylic acid compound (54) having a mercapto group protected with P¹⁰ into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (37) described in Production method 5. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6). The protecting groups P⁴ and P¹⁰ are as mentioned above.

35 **[0466]** The compound (59) can be also produced by the following method, for example.

40 **[0467]** The compound (60) can be produced by derivatizing the carboxylic acid compound (54) having a mercapto group protected with P¹⁰ into active ester, mixed acid anhydride, or the like and reacting it with the compound (32) described in Production method 4. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis. The protecting groups P⁴, P⁷, and P¹⁰ are as mentioned above and can be suitably selected from those described for the protecting group of the compound (6). However, it is necessary that the protecting group P¹⁰ for a mercapto group and the protecting group P⁴ for a side chain functional group can be removed by a different method or different conditions from those for the protecting group P⁷ for a carboxy group. For example, a representative example includes a combination in which P⁴ is a tert-butyl group for a carboxy group, P¹⁰ is a S-methyl sulfide group, and P⁷ is an allyl group. The protecting group P¹⁰ may be absent. In this case, the mercapto group of the compound (60) is unprotected.

[0468] By deprotecting the protecting group P⁷ for the carboxy group of the peptide in the compound (60) obtained, the compound (61) can be produced. Reagents and conditions can be selected depending on the protecting group.

50 **[0469]** The compound (59) can be produced by derivatizing the compound (61) obtained into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (4) or a salt thereof in the presence of a base. For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

55 **[0470]** By deprotecting the protecting group P⁴ for the carboxy group of LP¹ in the compound (59) obtained, the compound (62) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0471] By deprotecting the protecting group P¹⁰ for the mercapto group of the compound (62) obtained, the compound (2a) can be produced. Reagents and conditions can be selected depending on the protecting group.

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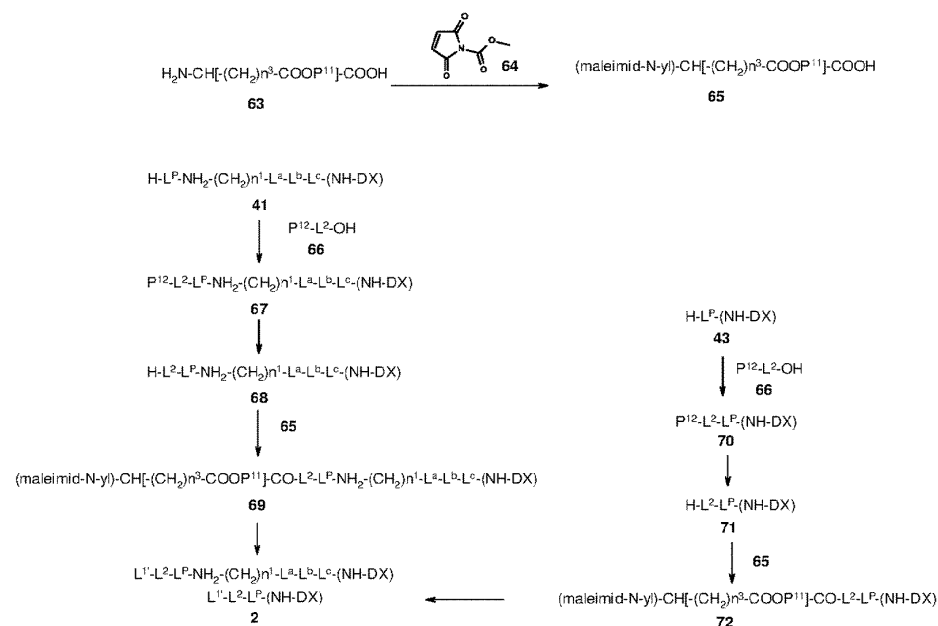
[0472] The compound (2a) in which the linker has the structure represented by $-L^1-L^2-L^P-$, and L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the drug, and even in case when a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat, can be produced as follows.

[0473] The compound (2a) can be produced by derivatizing the carboxylic acid compound (54) into active ester, mixed acid anhydride, or the like and reacting it with the compound (49) described in Production method 8. Here, the mercapto group may not be protected with P^{10} . For the reaction, reaction reagents and conditions that are generally used for peptide synthesis can be also used, and the reaction conditions, reagents, base, and solvent used for the reaction can be suitably selected from those described for the synthesis of the compound (6).

10. Production method 10

[0474] Among the production intermediate represented by the formula (2), L^1 in which L^1 is converted to have a structure of terminal (maleimid-N-yl)- $CH[-(CH_2)n^3-COOH]-C(=O)-$ can be produced by the following method.

[Formula 51]



[0475] In the formula, P^{11} and P^{12} each represents a protecting group.

[0476] The production intermediate represented by the formula (2) has two forms of the linker: a structure represented by $-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$ and a structure represented by $-L^1-L^2-L^P-$.

[0477] The compound (2) in which the linker has the structure represented by $-L^1-L^2-L^P-NH-(CH_2)n^1-L^a-L^b-L^c-$ can be produced as follows.

[0478] The (maleimid-N-yl)- compound (65) can be produced by reacting the amino acid (63) having a protected side chain carboxy group by P^{11} with the N-methoxycarbonylmaleimide (64) at -40°C to 100°C in the presence of a base such as sodium bicarbonate in water. The maleimidyl compound can be synthesized from a compound having an amino group by a method known in the art using N-methoxycarbonylmaleimide (e.g., Keller, O.; Rudinger, J. *Helv. Chem. Acta* 1975, 58 (2), 531-541.) or a method equivalent thereto. As for the protecting group P^{11} for a carboxy group, a protecting group commonly used as a protecting group for a carboxy group in organic synthetic chemistry can be used. It is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto.

[0479] The compound (67) can be produced by derivatizing the compound (66) having a terminal amino group protected by P^{12} into an active ester, mixed acid anhydride, or the like and reacting it with the compound (41) described in Production method 6 in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming an amide

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bond between the compound (67) and the compound (41) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P¹² for the amino group of the compound (66) can be suitably selected from those described for the protecting group of the compound (6).

5 [0480] By deprotecting the protecting group P¹² for the amino group of the compound (67) obtained, the compound (68) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0481] The compound (69) can be produced by derivatizing the compound (65) into an active ester, mixed acid anhydride, or the like and reacting it with the compound (68) obtained in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the compound (65) and the compound (68) can be suitably selected from those described for the synthesis of the compound (6).

10 [0482] By deprotecting the protecting group P¹¹ for the carboxy group of the compound (69) obtained, the compound (2) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0483] The compound (2) in which the linker has the structure represented by -L¹-L²-L^P- can be produced as follows.

15 [0484] Similarly, the compound (70) can be produced by derivatizing the compound (66) having a protected terminal amino group by P¹² into an active ester, mixed acid anhydride, or the like and reacting it with the compound (43) described in Production method 6 in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the compound (66) and the compound (43) can be suitably selected from those described for the synthesis of the compound (6). The protecting group P¹² is as mentioned above.

[0485] By deprotecting the protecting group P¹² for the amino group of the compound (70) obtained, the compound (71) can be produced. Reagents and conditions can be selected depending on the protecting group.

20 [0486] The compound (72) can be produced by derivatizing the compound (65) into an active ester, mixed acid anhydride, or the like and reacting it with the compound (71) obtained in the presence of a base. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the compound (65) and the compound (71) can be suitably selected from those described for the synthesis of the compound (6).

25 [0487] By deprotecting the protecting group P¹¹ for the carboxy group of the compound (72) obtained, the compound (2) can be produced. Reagents and conditions can be selected depending on the protecting group.

11. Production method 11

30 [0488] Among the production intermediate represented by the formula (2), those having L¹ in which L¹ is converted to have a structure of terminal maleimidyl group or terminal haloacetyl group, and L² is -N[-(CH₂CH₂-O)_n-CH₂CH₂-OH]-CH₂-C(=O)- can be produced by the following method.

[Formula 52]

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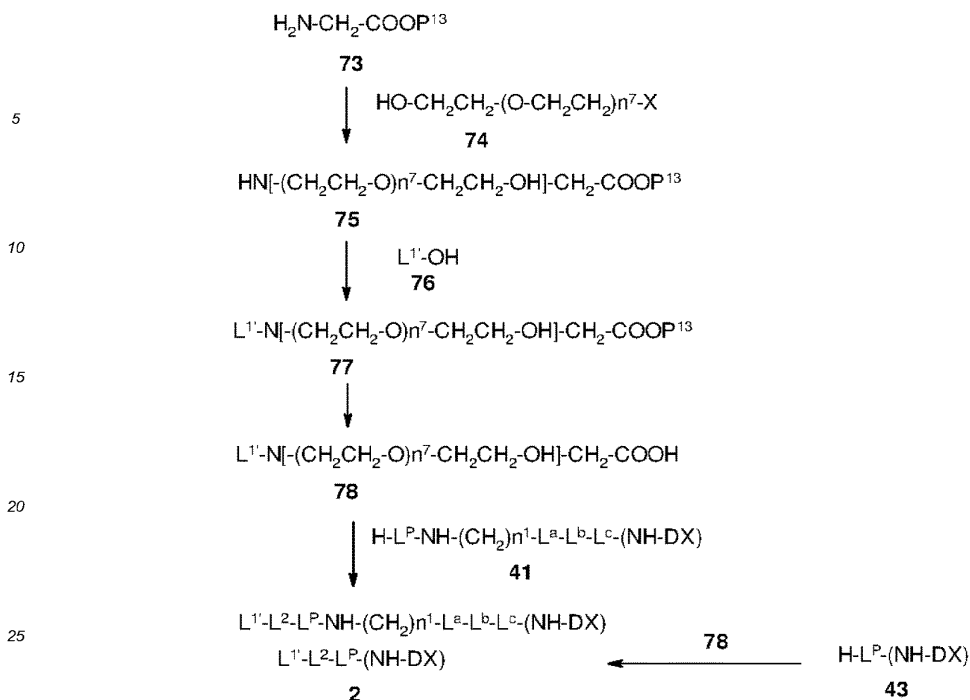
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[0489] In the formula, P^{13} represents a protecting group, and X represents a leaving group.

[0490] The production intermediate represented by the formula (2) has two forms as the linker: a structure represented by $-\text{L}^1-\text{L}^2-\text{L}^p-\text{NH}-(\text{CH}_2)_n-\text{L}^a-\text{L}^b-\text{L}^c-$ and a structure represented by $-\text{L}^1-\text{L}^2-\text{L}^p-$.

[0491] The compound (2) in which the linker has the structure represented by $-\text{L}^1-\text{L}^2-\text{L}^p-\text{NH}-(\text{CH}_2)_n-\text{L}^a-\text{L}^b-\text{L}^c-$ can be produced as follows.

[0492] The compound (75) can be produced by reacting the glycine derivative (73) having the protected C terminal by P^{13} with the compound (74) in the presence of a base. The protecting group P^{13} for a carboxy group is preferably a protecting group that can be deprotected under acidic conditions, but it is not limited thereto. Examples of the leaving group X of the compound (74) can include sulfonic acid esters such as p-toluene sulfonate, methyl sulfonate, and trifluoromethyl sulfonate as well as halides such as iodide, bromide, and chloride. For this reaction, reaction conditions that are generally used for N-alkylation can be also used, and the base and solvent used for the reaction can be selected from those described for the synthesis of the compound (6).

[0493] The compound (77) can be produced by derivatizing the carboxylic acid derivative (76) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (75) obtained. The reaction conditions, reagents, base, and solvent used for forming a peptide bond between the carboxylic acid derivative (76) and the compound (75) can be suitably selected from those described for the synthesis of the compound (6).

[0494] By deprotecting the protecting group P^{13} for the carboxy group of the compound (77) obtained, the compound (78) can be produced. Reagents and conditions can be selected depending on the protecting group.

[0495] The compound (2) can be produced by derivatizing the compound (78) obtained into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (41) described in Production method 6. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the carboxylic acid derivative (78) and the compound (41) can be suitably selected from those described for the synthesis of the compound (6).

[0496] The compound (2) in which the linker has the structure represented by $-\text{L}^1-\text{L}^2-\text{L}^p-$ can be produced as follows.

[0497] Similarly, the compound (2) can be produced by derivatizing the compound (78) into active ester, mixed acid anhydride, acid halide, or the like and reacting it with the compound (43) described in Production method 6. The reaction conditions, reagents, base, and solvent used for forming an amide bond between the carboxylic acid derivative (78) and the compound (43) can be suitably selected from those described for the synthesis of the compound (6).

[0498] All of the intermediate compounds of Production methods 1 to 11 may form salts.

[0499] Meanwhile, the antibody-drug conjugate of the present invention, when it is left in air or recrystallized, may absorb moisture to have adsorption water or turn into a hydrate, and such a compound and a salt containing water are

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also included in the present invention.

[0500] A compound labeled with various radioactive or nonradioactive isotopes is also included in the present invention. One or more atoms constituting the antibody-drug conjugate of the present invention may contain an atomic isotope at non-natural ratio. Examples of the atomic isotope include deuterium (^2H), tritium (^3H), iodine-125 (^{125}I), and carbon-13 (^{13}C). Further, the compound of the present invention may be radioactive-labeled with a radioactive isotope such as tritium (^3H), iodine-125 (^{125}I), carbon-14 (^{14}C), copper-64 (^{64}Cu), zirconium-89 (^{89}Zr), iodine-124 (^{124}I), fluorine-18 (^{18}F), indium-111 (^{111}In), carbon-11 (^{11}C) and iodine-131 (^{131}I). The compound labeled with a radioactive isotope is useful as a therapeutic or prophylactic agent, a reagent for research such as an assay reagent and an agent for diagnosis such as an in vivo diagnostic imaging agent. Without being related to radioactivity, any isotope variant type of the antibody-drug conjugate of the present invention is within the scope of the present invention.

[Drugs]

[0501] The antibody-drug conjugate of the present invention exhibits a cytotoxic activity against cancer cells, and thus, it can be used as a drug, particularly as a therapeutic agent and/or prophylactic agent for cancer.

[0502] Examples of the cancer type to which the antibody-drug conjugate of the present invention is applied include lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer, however, it is not limited to them as long as it is a cancer cell expressing, in a cancer cell as a treatment subject, a protein which the antibody within the antibody-drug conjugate can recognize.

[0503] The antibody-drug conjugate of the present invention can be preferably administered to a mammal, but it is more preferably administered to a human.

[0504] Substances used in a pharmaceutical composition containing antibody-drug conjugate of the present invention can be suitably selected and applied from formulation additives or the like that are generally used in the art, in view of the dosage or administration concentration.

[0505] The antibody-drug conjugate of the present invention can be administered as a pharmaceutical composition containing at least one pharmaceutically suitable ingredient. For example, the pharmaceutical composition above typically contains at least one pharmaceutical carrier (for example, sterilized liquid). for example, water and oil (petroleum oil and oil of animal origin, plant origin, or synthetic origin (the oil may be, for example, peanut oil, soybean oil, mineral oil, sesame oil or the like)). Water is a more typical carrier when the pharmaceutical composition above is intravenously administered. Saline solution, an aqueous dextrose solution, and an aqueous glycerol solution can be also used as a liquid carrier, in particular, for an injection solution. A suitable pharmaceutical vehicle is known in the art. If desired, the composition above may also contain a trace amount of a moisturizing agent, an emulsifying agent, or a pH buffering agent. Examples of suitable pharmaceutical carrier are disclosed in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulations correspond to an administration mode.

[0506] Various delivery systems are known and they can be used for administering the antibody-drug conjugate of the present invention. Examples of the administration route include intradermal, intramuscular, intraperitoneal, intravenous, and subcutaneous routes, but not limited thereto. The administration can be made by injection or bolus injection, for example. According to a specific preferred embodiment, the administration of the antibody-drug conjugate is performed by injection. Parenteral administration is a preferred administration route.

[0507] According to a representative embodiment, the pharmaceutical composition is prescribed, as a pharmaceutical composition suitable for intravenous administration to human, according to the conventional procedures. The composition for intravenous administration is typically a solution in a sterile and isotonic aqueous buffer solution. If necessary, the drug may contain a solubilizing agent and local anesthetics to alleviate pain at injection site (for example, lignocaine). Generally, the ingredient above is provided individually as any one of lyophilized powder or an anhydrous concentrate contained in a container which is yielded by sealing in an ampoule or a sachet having an amount of the active agent or as a mixture in a unit dosage form. When the drug is to be administered by injection, it may be administered from an injection bottle containing water or saline of sterile pharmaceutical grade. When the drug is administered by injection, an ampoule of sterile water or saline for injection may be provided such that the aforementioned ingredients are admixed with each other before administration.

[0508] The pharmaceutical composition of the present invention may be a pharmaceutical composition containing only the antibody-drug conjugate of the present invention or a pharmaceutical composition containing the antibody-drug conjugate and at least one cancer treating agent other than the conjugate. The antibody-drug conjugate of the present invention can be administered with other cancer treating agent. The anti-cancer effect may be enhanced accordingly. Another anti-cancer agent used for such purpose may be administered to an individual simultaneously with, separately from, or subsequently to the antibody-drug conjugate, and it may be administered while varying the administration interval for each. Examples of the cancer treating agent include abraxane, carboplatin, cisplatin, gemcitabine, irinotecan (CPT-11), paclitaxel, pemetrexed, sorafenib, vinorelbine, drugs described in International Publication No. WO 2003/038043,

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LH-RH analogues (leuprorelin, goserelin, or the like), estramustine phosphate, estrogen antagonist (tamoxifen, raloxifene, or the like), and an aromatase inhibitor (anastrozole, letrozole, exemestane, or the like), but it is not limited as long as it is a drug having an antitumor activity.

5 [0509] The pharmaceutical composition can be formulated into a lyophilization formulation or a liquid formulation as a formulation having desired composition and required purity. When formulated as a lyophilization formulation, it may be a formulation containing suitable formulation additives that are used in the art. Also for a liquid formulation, it can be formulated as a liquid formulation containing various formulation additives that are used in the art.

10 [0510] Composition and concentration of the pharmaceutical composition may vary depending on administration method. However, the antibody-drug conjugate contained in the pharmaceutical composition of the present invention can exhibit the pharmaceutical effect even at a small dosage when the antibody-drug conjugate has higher affinity for an antigen, that is, higher affinity (= lower K_d value) in terms of the dissociation constant (that is, K_d value) for the antigen. Thus, for determining dosage of the antibody-drug conjugate, the dosage can be determined in view of a situation relating to the affinity between the antibody-drug conjugate and antigen. When the antibody-drug conjugate of the present invention is administered to a human, for example, about 0.001 to 100 mg/kg can be administered once or administered several times with an interval of one time for 1 to 180 days.

[Examples]

20 [0511] The present invention is specifically described in view of the examples shown below, however, the present invention is not limited to them, and further, it is by no means interpreted in a limited sense. Further, unless specifically described otherwise, the reagent, solvent, and starting material described in the specification can be easily obtained from a commercial supplier.

Reference Example 1 M30-H1-L4 antibody

25 [0512] Among humanized antibodies of an anti-B7-H3 antibody, an antibody composed of a heavy chain consisting of an amino acid sequence described in amino acid positions 20 to 471 in SEQ ID NO: 9 and a light chain consisting of an amino acid sequence described in amino acid positions 21 to 233 in SEQ ID NO: 16 was produced in accordance with a method known in the art. The obtained humanized anti-B7-H3 antibody was designated as an M30-H1-L4 antibody.

Reference Example 2 M30-H1-L4P antibody

30 [0513] The modification of a glycan linked to the M30-H1-L4 antibody obtained above was regulated by defucosylation in accordance with a method known in the art. The obtained antibody with the regulated modification of a glycan was designated as an M30-H1-L4P antibody.

Reference Example 3 Anti-CD30 antibody

40 [0514] An anti-CD30 antibody was produced with reference to National Publication of International Patent Application No. 2005-506035. Its sequence is shown in SEQ ID NOs: 27 and 28.

Reference Example 4 Anti-CD33 antibody

45 [0515] An anti-CD33 antibody was produced with reference to Japanese Patent Laid-Open No. 8-48637. Its sequence is shown in SEQ ID NOs: 29 and 30.

Reference Example 5 Anti-CD70 antibody

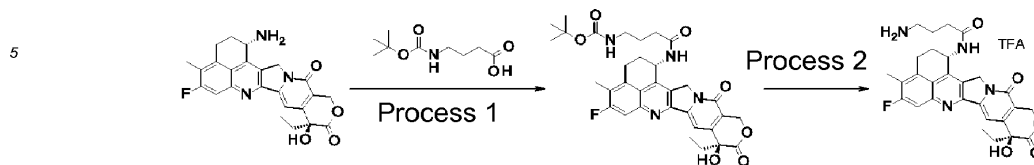
50 [0516] An anti-CD70 antibody was produced with reference to National Publication of International Patent Application No. 2008-538292. Its sequence is shown in SEQ ID NOs: 31 and 32.

Example 1: 4-Amino-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]butanamide

55 [0517]

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[Formula 53]



Process 1: tert-Butyl 4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]carbamate

[0518] 4-(tert-Butoxycarbonylamino)butanoic acid (0.237 g, 1.13 mmol) was dissolved in dichloromethane (10 mL), charged with N-hydroxysuccinimide (0.130 g, 1.13 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.216 g, 1.13 mmol), and stirred for 1 hour. The reaction solution was added dropwise to an N,N'-dimethylformamide solution (10.0 mL) charged with methanesulfonate of the compound (4) (0.500 g, 0.941 mmol) and triethylamine (0.157 mL, 1.13 mmol), and stirred at room temperature for 1 day. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform - chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound as a deep yellow solid (0.595 g, quantitative).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.31 (9H, s), 1.58 (1H, t, J=7.2 Hz), 1.66 (2H, t, J=7.2 Hz), 1.82-1.89 (2H, m), 2.12-2.21 (3H, m), 2.39 (3H, s), 2.92 (2H, t, J=6.5 Hz), 3.17 (2H, s), 5.16 (1H, d, J=18.8 Hz), 5.24 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.55-5.59 (1H, m), 6.53 (1H, s), 6.78 (1H, t, J=6.3 Hz), 7.30 (1H, s), 7.79 (1H, d, J=11.0 Hz), 8.40 (1H, d, J=8.6 Hz).

MS (APCI) m/z: 621 (M+H)⁺

Process 2: 4-Amino-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]butanamide

[0519] The compound (0.388 g, 0.626 mmol) obtained in Process 1 above was dissolved in dichloromethane (9.00 mL). Trifluoroacetic acid (9.00 mL) was added to be stirred for 4 hours. The solvent was removed under reduced pressure and the obtained residue was purified by silica gel column chromatography [chloroform - partitioned organic layer of chloroform : methanol : water = 7 : 3 : 1 (v/v/v)] to yield trifluoroacetate of the titled compound as a yellow solid (0.343 g, quantitative).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.79-1.92 (4H, m), 2.10-2.17 (2H, m), 2.27 (2H, t, J=7.0 Hz), 2.40 (3H, s), 2.80-2.86 (2H, m), 3.15-3.20 (2H, m), 5.15 (1H, d, J=18.8 Hz), 5.26 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.54-5.61 (1H, m), 6.55 (1H, s), 7.32 (1H, s), 7.72 (3H, brs), 7.82 (1H, d, J=11.0 Hz), 8.54 (1H, d, J=8.6 Hz).

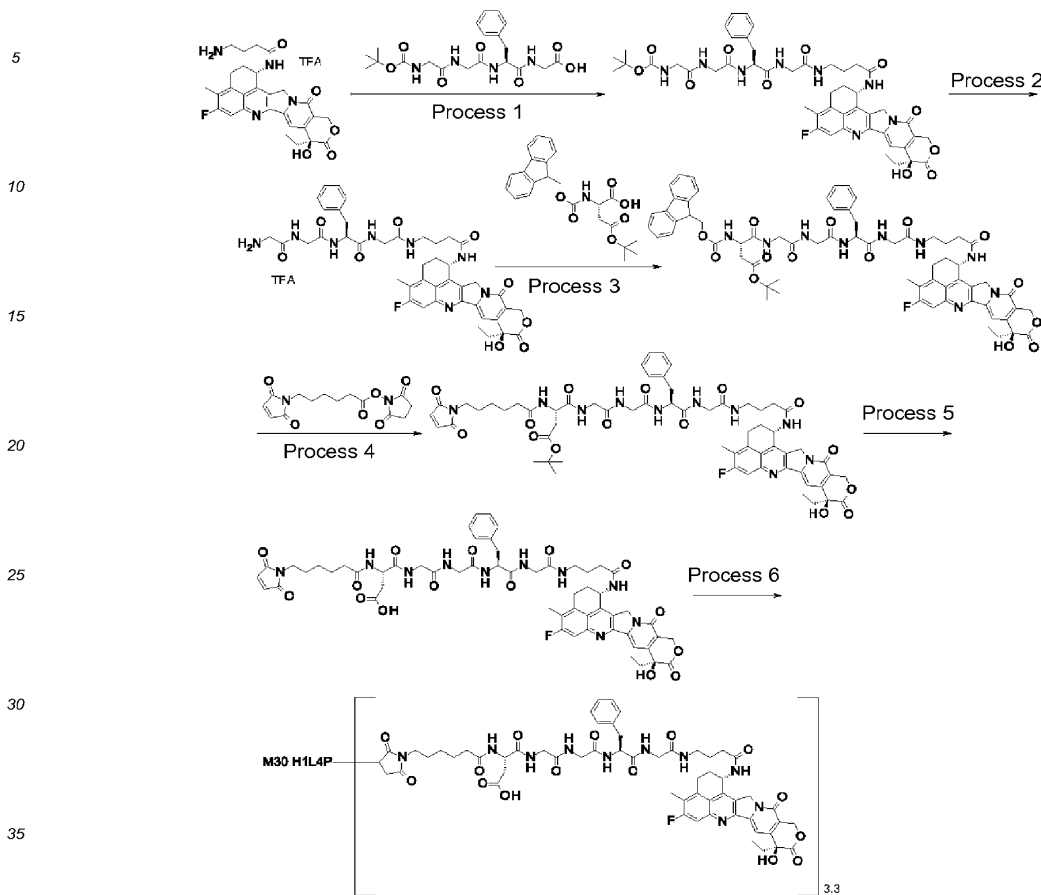
MS (APCI) m/z: 521 (M+H)⁺

Example 2: Antibody-drug conjugate (1)

[0520]

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[Formula 54]



Process 1: N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0521] N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanylglycine (80.9 g, 0.185 mmol) was dissolved in dichloromethane (3.00 mL), charged with N-hydroxysuccinimide (21.3 mg, 0.185 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (35.5 mg, 0.185 mmol), and stirred for 3.5 hours. The reaction solution was added dropwise to an N,N'-dimethylformamide solution (1.50 mL) charged with the compound (80.4 mg, 0.154 mmol) of Example 1, and stirred at room temperature for 4 hours. The solvent was removed under reduced pressure and the obtained residue was purified by silica gel column chromatography [chloroform - chloroform : methanol = 8 : 2 (v/v)] to yield the titled compound as a pale yellow solid (0.106 g, 73%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.36 (9H, s), 1.71 (2H, m), 1.86 (2H, t, J=7.8 Hz), 2.15-2.19 (4H, m), 2.40 (3H, s), 2.77 (1H, dd, J=12.7, 8.8 Hz), 3.02 (1H, dd, J=14.1, 4.7 Hz), 3.08-3.11 (2H, m), 3.16-3.19 (2H, m), 3.54 (2H, d, J=5.9 Hz), 3.57-3.77 (4H, m), 4.46-4.48 (1H, m), 5.16 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 6.53 (1H, s), 7.00 (1H, t, J=6.3 Hz), 7.17-7.26 (5H, m), 7.31 (1H, s), 7.71 (1H, t, J=5.7 Hz), 7.80 (1H, d, J=11.0 Hz), 7.92 (1H, t, J=5.7 Hz), 8.15 (1H, d, J=8.2 Hz), 8.27 (1H, t, J=5.5 Hz), 8.46 (1H, d, J=8.2 Hz).

MS (APCI) m/z: 939 (M+H)⁺

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Process 2: Glycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide trifluoroacetate

5 [0522] The compound (72.6 mg, 77.3 μ mol) obtained in Process 1 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a yellow solid (64.8 g, quantitative).

¹H-NMR (400 MHz, DMSO-d₆) δ : 0.87 (3H, t, J=7.4 Hz), 1.71-1.73 (2H, m), 1.82-1.90 (2H, m), 2.12-2.20 (4H, m), 2.40 (3H, s), 2.75 (1H, dd, J=13.7, 9.4 Hz), 3.03-3.09 (3H, m), 3.18-3.19 (2H, m), 3.58-3.60 (2H, m), 3.64 (1H, d, J=5.9 Hz), 3.69 (1H, d, J=5.9 Hz), 3.72 (1H, d, J=5.5 Hz), 3.87 (1H, dd, J=16.8, 5.9 Hz), 4.50-4.56 (1H, m), 5.16 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 7.17-7.27 (5H, m), 7.32 (1H, s), 7.78-7.81 (2H, m), 7.95-7.97 (3H, m), 8.33-8.35 (2H, m), 8.48-8.51 (2H, m).

MS (APCI) m/z: 839 (M+H)⁺

15 Process 3: tert-Butyl (3S,12S)-12-benzyl-21-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-3-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-4,7,10,13,16,21-hexaoxo-5,8,11,14,17-pentaazaheneicosan-1-olate

20 [0523] (2S)-4-tert-Butoxy-2-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-4-oxobutanoic acid (0.625 g, 1.52 mmol) was dissolved in dichloromethane (10.0 mL), charged with N-hydroxysuccinimide (0.175 g, 1.52 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.291 g, 1.52 mmol), and stirred for 1 hour. The reaction solution was added dropwise to an N,N'-dimethylformamide solution (10.0 mL) charged with the compound (1.00 g, 1.01 mmol) obtained in Process 2 above, and stirred at room temperature for 20 hours. The solvent was removed under reduced pressure and the obtained residue was purified by silica gel column chromatography [chloroform to chloroform : methanol = 8:2 (v/v)] to yield the titled compound as a pale yellow solid (0.873 g, 70%).

25 ¹H-NMR (400 MHz, DMSO-d₆) δ : 0.88 (3H, t, J=7.4 Hz), 1.37 (9H, s), 1.68-1.78 (2H, m), 1.81-1.93 (2H, m), 2.10-2.23 (4H, m), 2.41 (3H, s), 2.68-2.85 (3H, m), 2.99-3.22 (5H, m), 3.58-3.81 (6H, m), 4.19-4.36 (3H, m), 4.38-4.52 (2H, m), 5.17 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=19.2 Hz), 5.43 (2H, s), 5.54-5.62 (1H, m), 6.55 (1H, s), 7.15-7.34 (8H, m), 7.41 (2H, t, J=7.2 Hz), 7.66-7.75 (4H, m), 7.81 (1H, d, J=11.0 Hz), 7.88 (2H, d, J=7.4 Hz), 8.01-8.06 (1H, m), 8.14 (1H, d, J=8.2 Hz), 8.17-8.22 (1H, m), 8.25-8.30 (1H, m), 8.47 (1H, d, J=8.6 Hz).

30 MS (APCI) m/z: 1232 (M+H)⁺

35 Process 4: tert-Butyl (3S,12S)-12-benzyl-3-[[[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]amino]-21-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4,7,10,13,16,21-hexaoxo-5,8,11,14,17-pentaazaheneicosan-1-olate

[0524] The compound (0.800 g, 0.649 mmol) obtained in Process 3 above was dissolved in N,N'-dimethylformamide (3.00 mL), charged with piperidine (0.643 mL, 6.49 mmol), and stirred for 1 hour. The solvent was removed by drying under reduced pressure, and the obtained residue was dissolved in N,N'-dimethylformamide (10 mL). N-Succinimidy 6-maleimide hexanoate (0.300 g, 0.974 mmol) was added thereto and stirred for 20 hours. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 8:2 (v/v)] to yield the titled compound as a pale yellow solid (0.224 g, 29%).

40 ¹H-NMR (400 MHz, DMSO-d₆) δ : 0.87 (3H, t, J=7.6 Hz), 1.15-1.22 (2H, m), 1.35 (9H, s), 1.44-1.47 (4H, m), 1.71-1.73 (2H, m), 1.80-1.91 (2H, m), 2.08 (2H, t, J=7.6 Hz), 2.13-2.20 (4H, m), 2.40 (3H, s), 2.67 (1H, dt, J=11.1, 4.8 Hz), 2.78 (1H, dd, J=13.6, 9.4 Hz), 2.99-3.17 (6H, m), 3.31-3.36 (2H, m), 3.57-3.76 (6H, m), 4.45-4.47 (1H, m), 4.57-4.60 (1H, m), 5.16 (1H, d, J=18.7 Hz), 5.25 (1H, d, J=18.7 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.15-7.27 (5H, m), 7.31 (1H, s), 7.70 (1H, t, J=5.4 Hz), 7.80 (1H, d, J=10.9 Hz), 7.99 (1H, t, J=5.7 Hz), 8.09-8.12 (3H, m), 8.25 (1H, t, J=6.0 Hz), 8.45 (1H, d, J=9.1 Hz).

MS (APCI) m/z: 1203 (M+H)⁺

50 Process 5: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L- α -aspartylglycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0525] The compound (0.224 g, 0.186 mmol) obtained in Process 4 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a pale yellow solid (21.2 mg, 10%).

55 ¹H-NMR (400 MHz, DMSO-d₆) δ : 0.87 (3H, t, J=7.2 Hz), 1.13-1.21 (2H, m), 1.42-1.45 (6H, m), 1.70-1.72 (2H, m), 1.85-1.88 (2H, m), 2.06-2.20 (6H, m), 2.39 (3H, s), 2.63-2.67 (1H, m), 2.78-2.81 (1H, m), 3.04-3.12 (6H, m), 3.63-3.70 (6H, m), 4.46-4.52 (2H, m), 5.16 (1H, d, J=18.8 Hz), 5.25 (1H, d, J=18.8 Hz), 5.42 (2H, s), 5.55-5.58 (1H, m), 6.53 (1H,

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s), 6.99 (2H, s), 7.18-7.23 (6H, m), 7.30 (1H, s), 7.71 (1H, t, $J=5.5$ Hz), 7.79 (1H, d, $J=10.9$ Hz), 7.99-8.02 (1H, m), 8.10-8.11 (3H, m), 8.27-8.30 (1H, m), 8.47-8.50 (1H, m).
MS (APCI) m/z : 1147 ($M+H$)⁺

5 Process 6: Antibody-drug conjugate (1)

[0526] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution
10 (8.0 mL) was collected into a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.124 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.400 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0527] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.249 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 above was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.050 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0528] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 18.5 mL of a solution containing the titled antibody-drug conjugate.

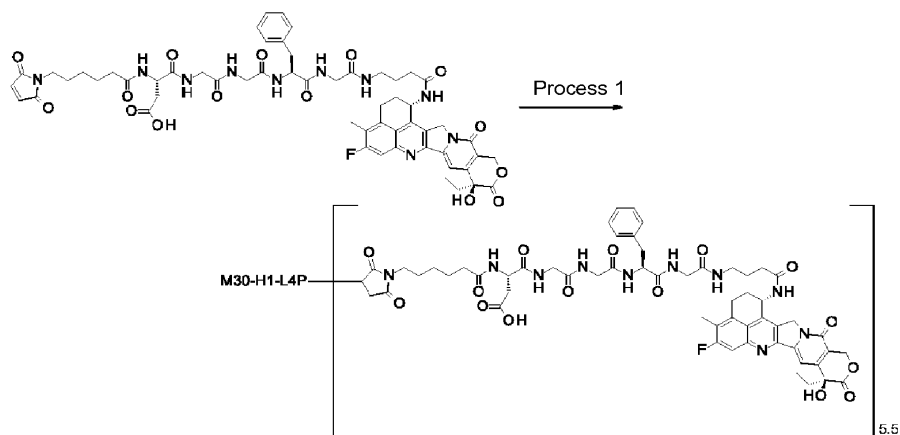
[0529] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0530] Antibody concentration: 3.56 mg/mL, antibody yield: 66 mg (83%), and average number of conjugated drug molecules (n) per antibody molecule: 3.3.

Example 3: Antibody-drug conjugate (2)

30 [0531]

[Formula 55]



55 Process 1: Antibody-drug conjugate (2)

[0532] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution

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(8.0 mL) was collected into a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.187 mL; 3.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.400 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

5 **[0533]** Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.373 mL; 6.9 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 of Example 2 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.075 mL; 13.8 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

10 **[0534]** Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 16 mL of a solution containing the titled antibody-drug conjugate.

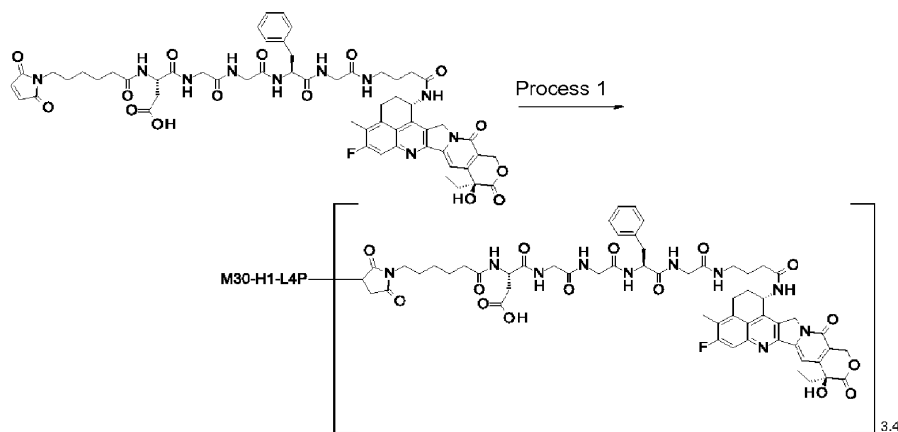
15 **[0535]** Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0536] Antibody concentration: 3.55 mg/mL, antibody yield: 57 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 5.5.

20 Example 4: Antibody-drug conjugate (3)

[0537]

25 [Formula 56]



45 Process 1: Antibody-drug conjugate (3)

[0538] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

50 **[0539]** Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.109 mL) and a dimethyl sulfoxide solution containing 10 mM (0.039 mL; 4.6 equivalents per antibody molecule) of the compound obtained in Process 5 of Example 2 to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.008 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto

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and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

[0540] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

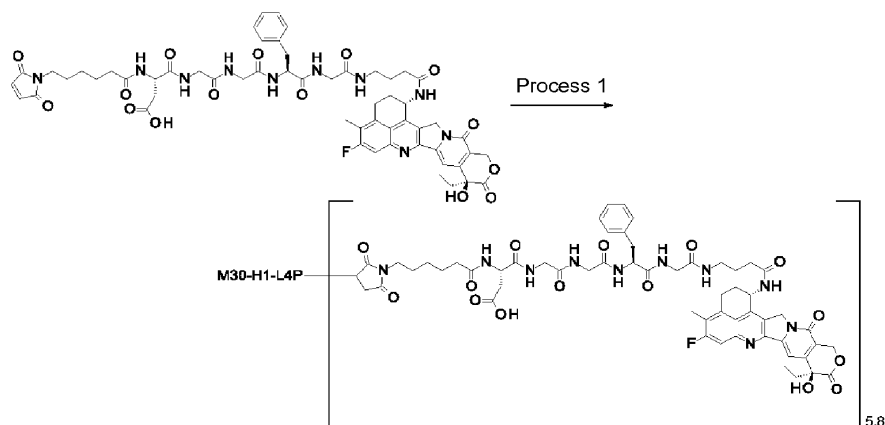
5 **[0541]** Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

10 **[0542]** Antibody concentration: 10.63 mg/mL, antibody yield: 7.4 mg (59%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 5: Antibody-drug conjugate (4)

[0543]

[Formula 57]



Process 1: Antibody-drug conjugate (4)

40 **[0544]** Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

45 **[0545]** Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.067 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 5 of Example 2 (0.085 mL; 10.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 60 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.013 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

50 **[0546]** Purification: The above solution was subjected to purification using the Common procedure D (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

55 **[0547]** Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0548] Antibody concentration: 1.48 mg/mL, antibody yield: 8.88 mg (71%), and average number of conjugated drug

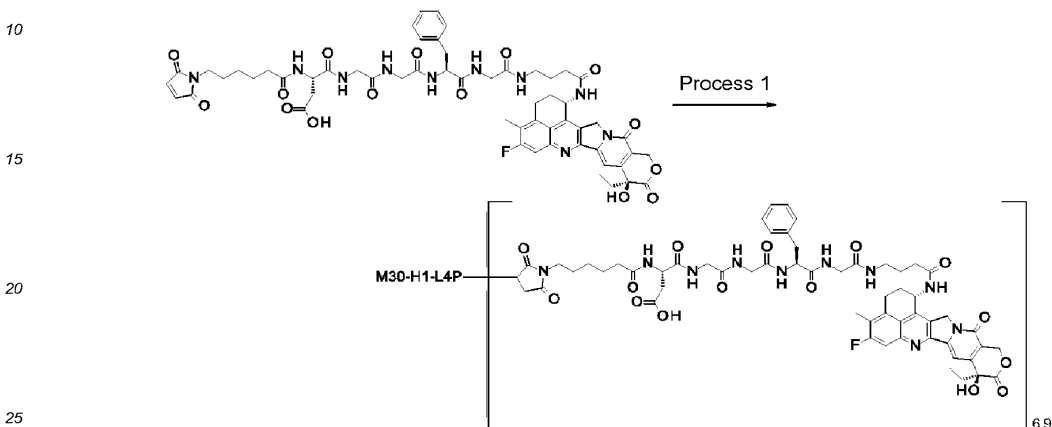
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molecules (n) per antibody molecule: 5.8.

Example 6: Antibody-drug conjugate (5)

5 [0549]

[Formula 58]



Process 1: Antibody-drug conjugate (5)

30 [0550] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 mLmg⁻¹cm⁻¹ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution

35 had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour. [0551] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.025 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 5 of Example 2 (0.127 mL; 15.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 60 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.019 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

40 [0552] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

45 [0553] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

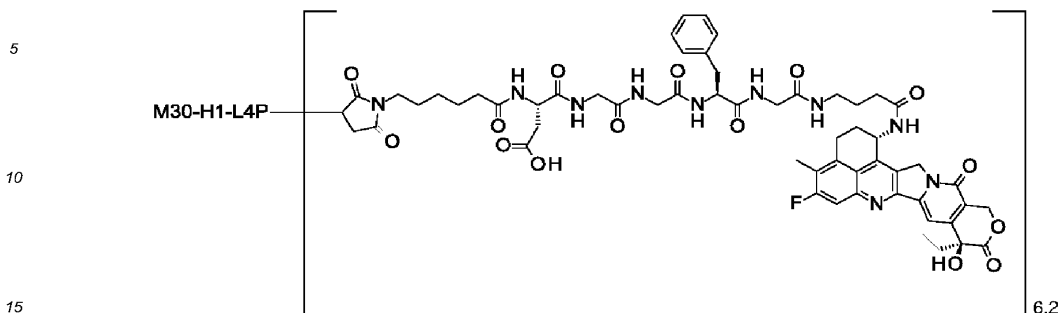
50 [0554] Antibody concentration: 0.99 mg/mL, antibody yield: 5.94 mg (48%), and average number of conjugated drug molecules (n) per antibody molecule: 6.9.

Example 7: Antibody-drug conjugate (6)

55 [0555]

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[Formula 59]



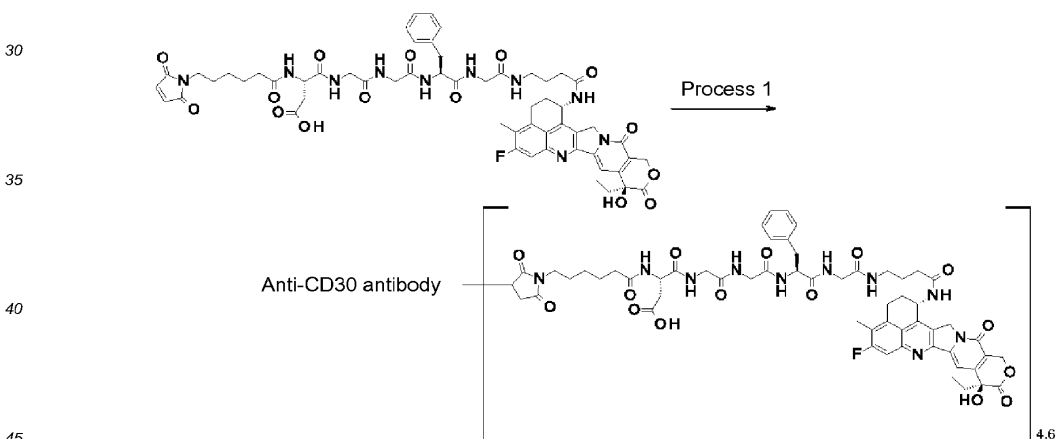
[0556] Almost the whole amounts of the antibody-drug conjugates of Examples 5 and 6 were mixed and the solution was concentrated by the Common procedure A to yield the titled antibody-drug conjugate.

[0557] Antibody concentration: 10.0 mg/mL, antibody yield: 14.36 mg, and average number of conjugated drug molecules (n) per antibody molecule: 6.2.

Example 8: Antibody-drug conjugate (7)

[0558]

[Formula 60]



Process 1: Antibody-drug conjugate (7)

[0559] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.75 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0560] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 of Example 2 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug

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linker to the antibody. Next, an aqueous solution (0.00593 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

5 **[0561]** Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

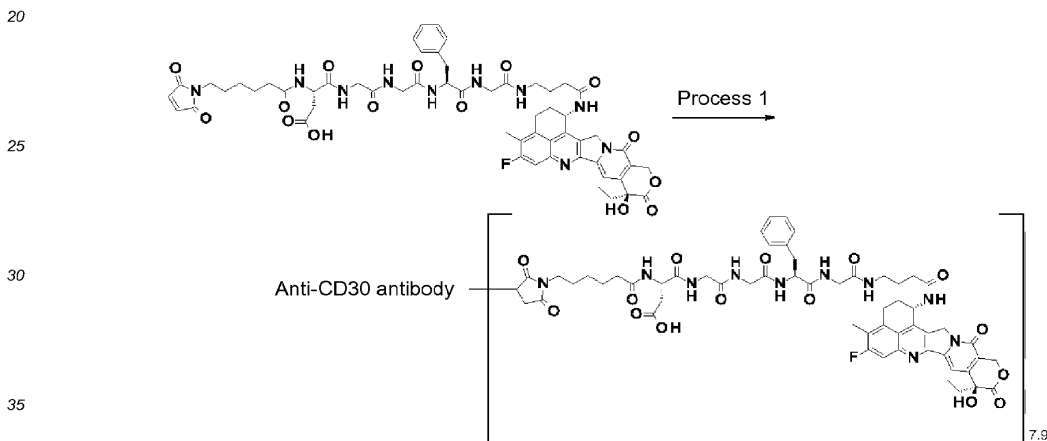
[0562] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 270400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following

10 characteristic values were obtained.
[0563] Antibody concentration: 1.18 mg/mL, antibody yield: 7.08 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 4.6.

Example 9: Antibody-drug conjugate (8)

[0564]

[Formula 61]



Process 1: Antibody-drug conjugate (8)

40 **[0565]** Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.75 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen

45 phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.
[0566] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 of Example 2 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug

50 linker to the antibody. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.
[0567] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug

55 conjugate.
[0568] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 270400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following

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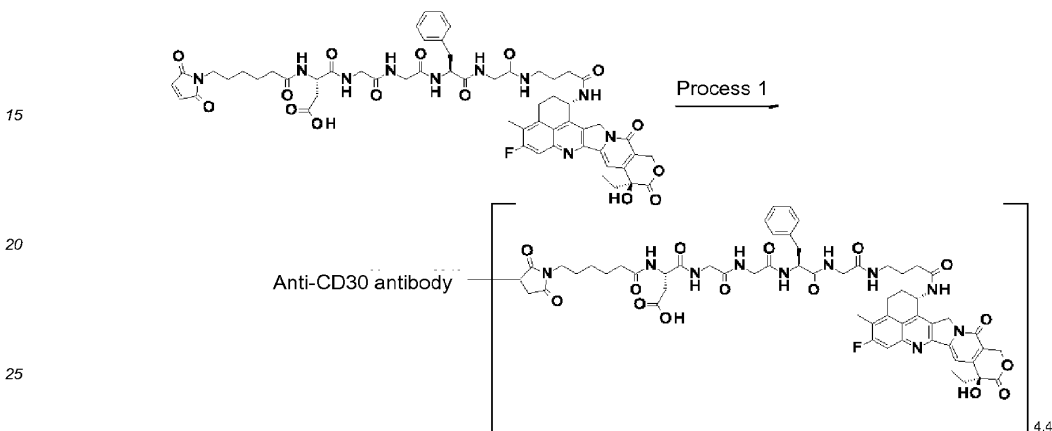
characteristic values were obtained.

[0569] Antibody concentration: 1.07 mg/mL, antibody yield: 6.42 mg (64%), and average number of conjugated drug molecules (n) per antibody molecule: 7.9.

5 Example 10: Antibody-drug conjugate (9)

[0570]

[Formula 62]



Process 1: Antibody-drug conjugate (9)

30 [0571] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.66 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

35 [0572] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 of Example 2 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00593 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

40 [0573] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

45 [0574] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 256400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

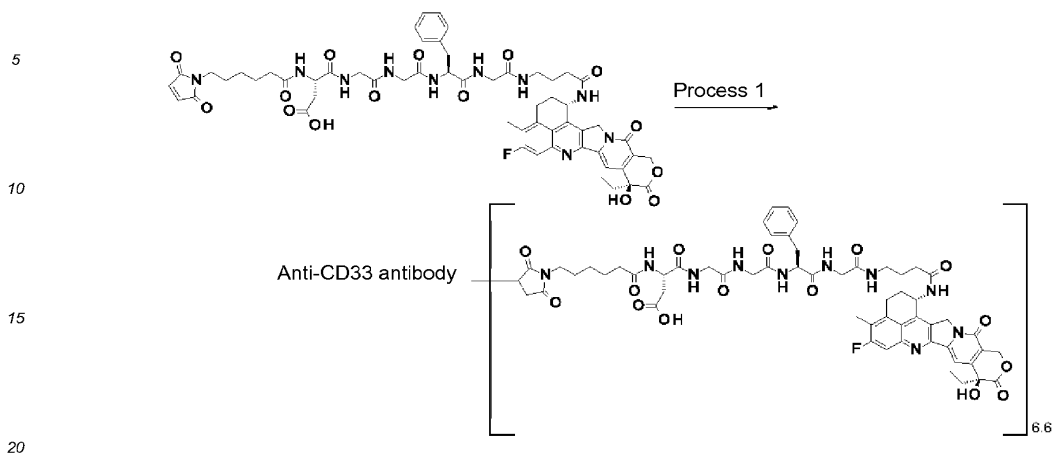
50 [0575] Antibody concentration: 1.09 mg/mL, antibody yield: 6.54 mg (65%), and average number of conjugated drug molecules (n) per antibody molecule: 4.4.

Example 11: Antibody-drug conjugate (10)

[0576]

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[Formula 63]



Process 1: Antibody-drug conjugate (10)

[0577] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.66 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0578] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 of Example 2 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0579] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0580] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 256400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

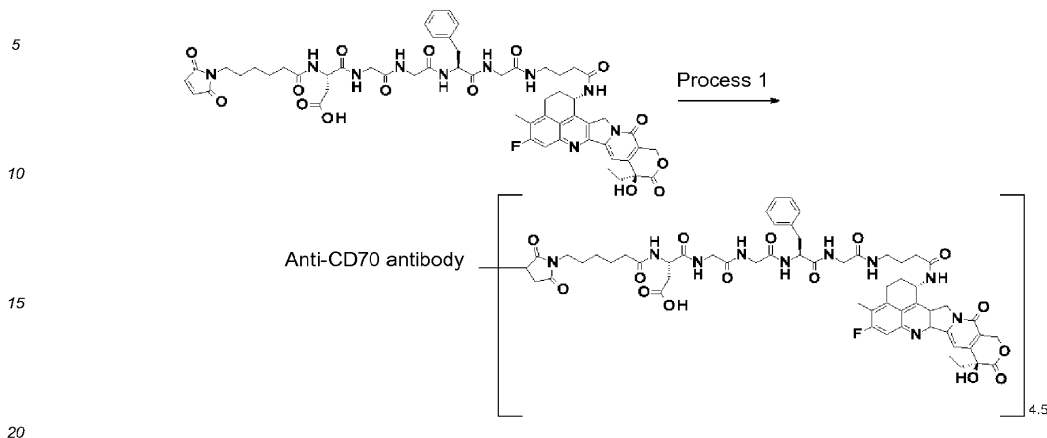
[0581] Antibody concentration: 1.04 mg/mL, antibody yield: 6.24 mg (62%), and average number of conjugated drug molecules (n) per antibody molecule: 6.6.

Example 12: Antibody-drug conjugate (11)

[0582]

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[Formula 64]



Process 1: Antibody-drug conjugate (11)

[0583] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.66 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0584] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 5 of Example 2 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00593 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0585] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0586] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 262400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

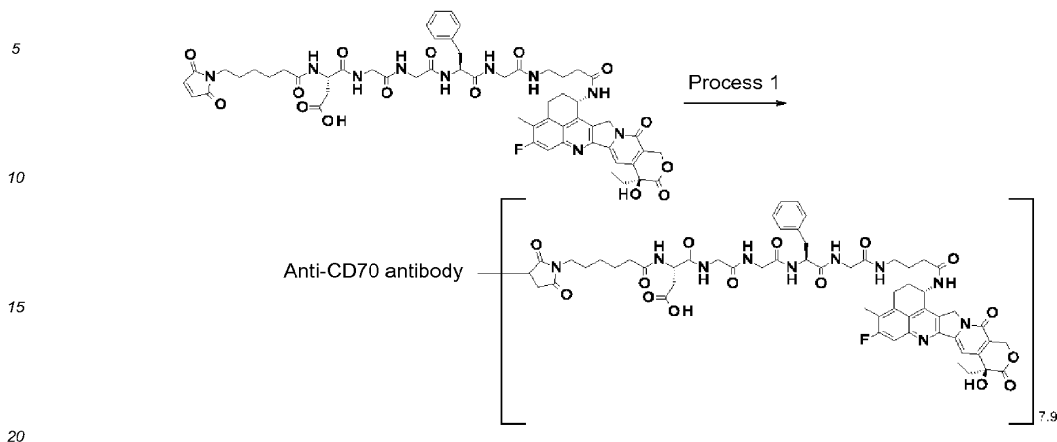
[0587] Antibody concentration: 1.12 mg/mL, antibody yield: 6.72 mg (67%), and average number of conjugated drug molecules (n) per antibody molecule: 4.5.

Example 13: Antibody-drug conjugate (12)

[0588]

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[Formula 65]



Process 1: Antibody-drug conjugate (12)

[0589] Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.69 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0590] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 7 of Example 1 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0591] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0592] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 262400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

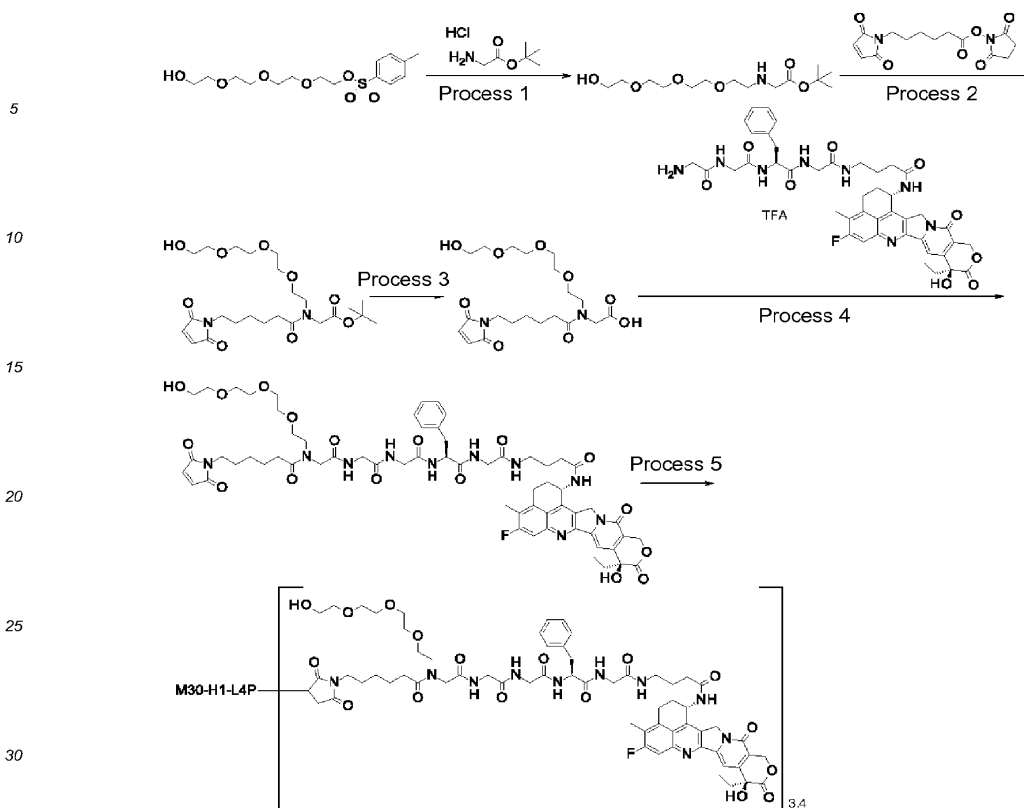
[0593] Antibody concentration: 1.03 mg/mL, antibody yield: 6.18 mg (62%), and average number of conjugated drug molecules (n) per antibody molecule: 7.9.

Example 14: Antibody-drug conjugate (13)

[0594]

[Formula 66]

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Process 1: tert-Butyl N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)glycinate

[0595] To an N,N-dimethylformamide (50.0 mL) solution of 2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl 4-methylbenzene sulfonate (Bioorg. Med. Chem. Lett., 2011, Vol. 21, p. 550; 1.75 g, 5.00 mmol) and glycine tert-butyl hydrochloride (1.26 g, 7.52 mmol), N,N-diisopropylethylamine (1.94 g, 15.0 mmol) was added and stirred at 60°C for 10 hours. Chloroform was added to the reaction solution, the organic layer was washed with 1 N hydrochloric acid, and the obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 8:1 (v/v)] to yield the titled compound in colorless oily substance (426 mg, 28%).

¹H-NMR (400 MHz, CDCl₃) δ: 1.47 (9H, s), 2.80 (2H, t, J=5.3 Hz), 3.32 (2H, s), 3.76-3.54 (17H, m).

Process 2: tert-Butyl N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)glycinate

[0596] The compound (426 mg, 1.39 mmol) obtained in Process 1 above was reacted in the same manner as Process 4 of Example 2 to yield the titled compound in colorless oily substance (489 mg, 70%).

¹H-NMR (400 MHz, CDCl₃) δ: 1.28-1.36 (2H, m), 1.45 (9H, s), 1.57-1.71 (4H, m), 2.39 (2H, t, J=7.3 Hz), 3.48-3.76 (3H, m), 4.02 (2H, s), 6.68 (2H, s).

Process 3: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)glycine

[0597] The compound (489 mg, 0.977 mmol) obtained in Process 2 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a colorless solid (211 mg, 49%).

¹H-NMR (400 MHz, CDCl₃) δ: 1.38-1.28 (2H, m), 1.73-1.55 (4H, m), 2.28 (2H, t, J=7.0 Hz), 3.50-3.79 (18H, m), 4.12

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(2H, s), 6.68 (2H, s).

Process 4: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-N-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)glycylglycylglycyl-L-phenylalanyl-N-(4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl])glycinamide

[0598] The compound (48.9 mg, 0.110 mmol) obtained in Process 3 above was reacted in the same manner as Process 1 of Example 1 by using the compound (84.0 mg, 0.100 mmol) obtained in Process 2 of Example 2 instead of methanesulfonate of the compound (4) to yield the titled compound as a pale yellow solid (54.0 mg, 43%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.14-1.26 (2H, m), 1.39-1.51 (4H, m), 1.68-1.76 (2H, m), 1.81-1.91 (2H, m), 2.08-2.23 (4H, m), 2.40 (3H, s), 2.73-2.84 (1H, m), 2.98-3.21 (5H, m), 3.25-3.79 (26H, m), 3.93 (2H, s), 4.43-4.49 (1H, m), 4.54-4.61 (1H, m), 5.21 (2H, q, J=18.6 Hz), 5.42 (2H, s), 5.54-5.60 (1H, m), 6.53 (1H, s), 7.00 (2H, s), 7.14-7.27 (5H, m), 7.31 (1H, s), 7.68-7.74 (1H, m), 7.80 (1H, d, J=11.0 Hz), 8.02-8.32 (4H, m), 8.46 (1H, d, J=8.6 Hz).

MS (ESI) m/z: 1265 (M+H)⁺

Process 5: Antibody-drug conjugate (13)

[0599] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 4 above, the titled antibody-drug conjugate was obtained in the same manner as Process 6 of Example 2.

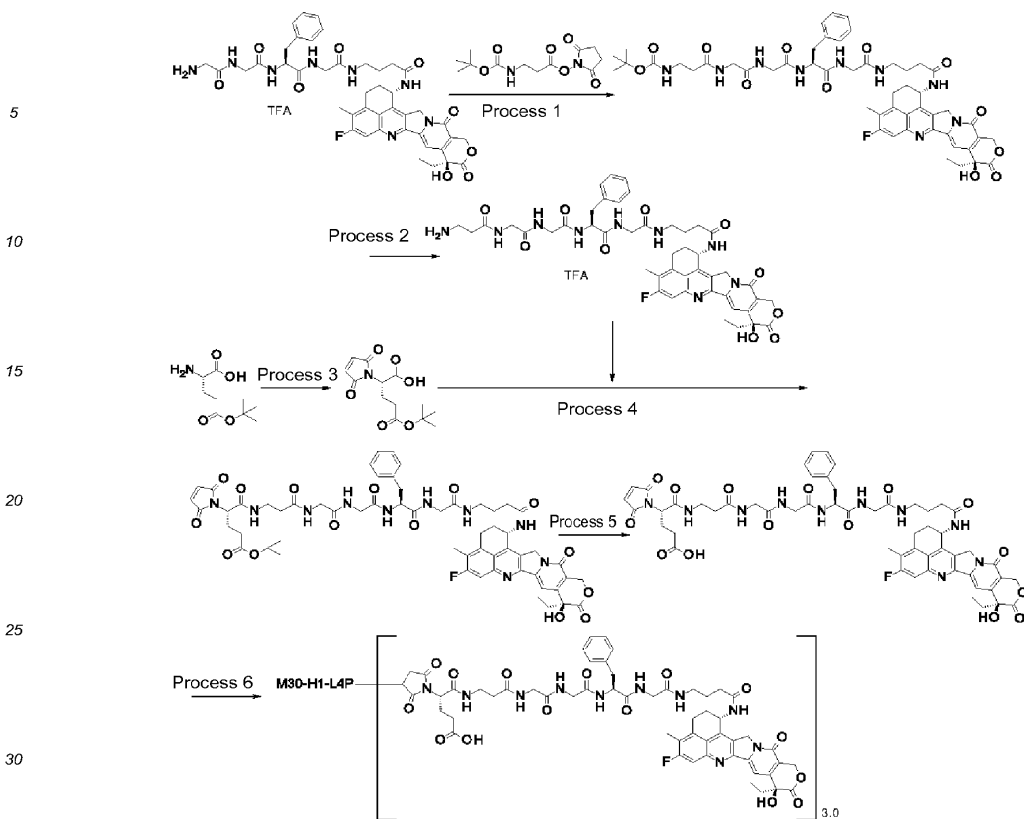
[0600] Antibody concentration: 13.13 mg/mL, antibody yield: 9.2 mg (74%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

Example 15: Antibody-drug conjugate (14)

[0601]

[Formula 67]

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Process 1: N-(tert-butoxycarbonyl)-β-alanylglycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0602] The compound (0.839 g, 1.00 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 3 of Example 2 by using N-(tert-butoxycarbonyl)-β-alanine instead of N-succinimidyl 6-maleimide hexanoate. The obtained crude product was used in the next process without purification.

Process 2: β-Alanylglycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0603] The crude product obtained in Process 1 was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a pale yellow solid (0.610 g, 67%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.67-1.77 (2H, m), 1.79-1.92 (2H, m), 2.09-2.22 (4H, m), 2.40 (3H, s), 2.46-2.55 (2H, m), 2.82-2.73 (1H, m), 2.95-3.13 (5H, m), 3.14-3.21 (2H, m), 3.55-3.80 (6H, m), 4.44-4.52 (1H, m), 5.20 (2H, dd, J=35.0, 19.0 Hz), 5.42 (2H, s), 5.53-5.60 (1H, m), 6.54 (1H, s), 7.14-7.28 (5H, m), 7.31 (1H, s), 7.67 (2H, brs), 7.72-7.78 (1H, m), 7.80 (1H, d, J=11.0 Hz), 8.10-8.17 (2H, m), 8.29 (1H, t, J=5.9 Hz), 8.42 (1H, t, J=5.7 Hz), 8.47 (1H, d, J=8.6 Hz).

Process 3: (2S)-5-tert-Butoxy-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopentanoic acid

[0604] 5-tert-Butyl L-glutamate (1.02 g, 5.00 mmol) was dissolved in a saturated aqueous solution of sodium hydrogen carbonate (20.0 mL), charged with N-methoxycarbonylmaleimide (0.775 g, 5.00 mmol) at 0°C, and stirred at 0°C for 30 minutes and then stirred at room temperature for 1 hour. The reaction solution was rendered acidic by the addition of 5

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N hydrochloric acid at 0°C and then extracted with ethyl acetate. The obtained organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure to yield a crude product. The obtained crude product was used in the next process without purification.

5 Process 4: N-[(2S)-5-tert-butoxy-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-oxopentanoyl]-β-alanyl-glycyl-glycyl-L-phenylalanyl-N-(4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benz[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl)glycinamide

10 [0605] The crude product (85.0 mg, 0.300 mmol) obtained in Process 3 above was reacted in the same manner as Process 1 of Example 1 by using the compound (182 mg, 0.200 mmol) obtained in Process 2 above instead of methanesulfonate of the compound (4) to yield the titled compound as a pale yellow solid (102 mg, 43%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.35 (9H, s), 1.67-1.76 (2H, m), 1.81-1.90 (2H, m), 2.35-2.05 (10H, m), 2.40 (3H, s), 2.75-2.83 (1H, m), 2.99-3.13 (3H, m), 3.14-3.26 (4H, m), 3.55-3.76 (6H, m), 4.36-4.50 (2H, m), 5.21 (2H, q, J=18.9 Hz), 5.42 (2H, s), 5.54-5.61 (1H, m), 6.53 (1H, s), 7.03 (2H, s), 7.17-7.26 (5H, m), 7.31 (1H, s), 7.68-7.73 (1H, m), 7.80 (1H, d, J=10.6 Hz), 8.00-8.05 (2H, m), 8.12 (1H, d, J=7.8 Hz), 8.16-8.20 (1H, m), 8.23-8.28 (1H, m), 8.46 (1H, d, J=8.6 Hz).

20 Process 5: N-[(2S)-4-carboxy-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)butanoyl]-β-alanyl-glycyl-glycyl-L-phenylalanyl-N-(4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benz[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl)glycinamide

[0606] The compound (102 mg, 86.8 μmol) obtained in Process 4 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a pale yellow solid (76.0 mg, 78%).

25 ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.68-1.75 (2H, m), 1.84-1.91 (2H, m), 2.35-2.05 (10H, m), 2.40 (3H, s), 2.74-2.83 (1H, m), 2.99-3.12 (3H, m), 3.14-3.26 (4H, m), 3.55-3.77 (6H, m), 4.41-4.49 (2H, m), 5.21 (2H, dd, J=38.7, 18.8 Hz), 5.42 (2H, s), 5.54-5.61 (1H, m), 6.54 (1H, s), 7.03 (2H, s), 7.15-7.27 (5H, m), 7.31 (1H, s), 7.69-7.74 (1H, m), 7.80 (1H, d, J=10.9 Hz), 8.01-8.07 (2H, m), 8.12 (1H, d, J=8.2 Hz), 8.19 (1H, t, J=5.5 Hz), 8.27 (1H, t, J=6.3 Hz), 8.47 (1H, d, J=8.6 Hz), 12.12 (1H, s).

MS (ESI) m/z: 1119 (M+H)⁺

30 Process 6: Antibody-drug conjugate (14)

[0607] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 5 above, the titled antibody-drug conjugate was obtained in the same manner as Process 6 of Example 2.

35 [0608] Antibody concentration: 12.77 mg/mL, antibody yield: 8.9 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 3.0.

Example 16: Antibody-drug conjugate (15)

40 [0609]

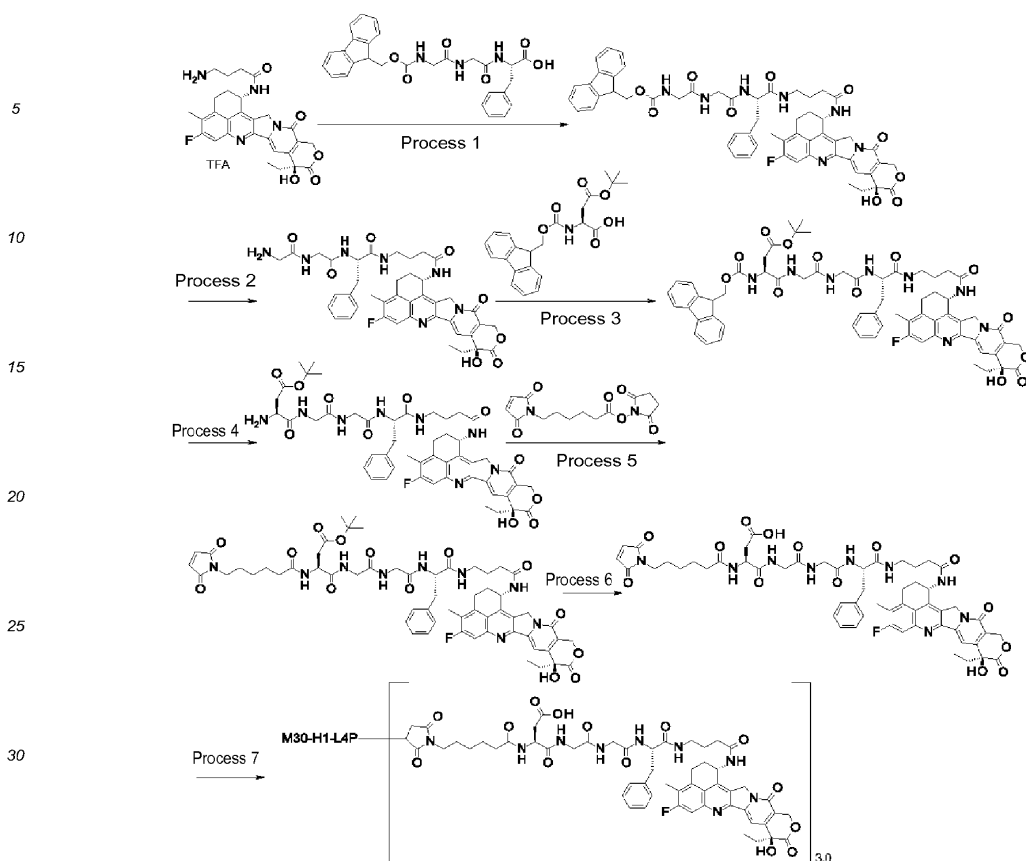
[Formula 68]

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Process 1: N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycylglycyl-L-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]-L-phenylalaninamide

[0610] The compound (300 mg, 0.473 mmol) obtained in Process 2 of Example 1 was reacted in the same manner as Process 1 of Example 1 by using N-[(9H-fluoren-9-ylmethoxy)carbonyl]glycylglycyl-L-phenylalanine (the compound described in Japanese Patent Laid-Open No. 2002-60351; 346 mg, 0.691 mmol) instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a pale yellow solid (230 mg, 40%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.85 (3H, t, J=7.2 Hz), 1.67-1.68 (2H, m), 1.81-1.84 (2H, m), 2.13 (4H, t, J=6.8 Hz), 2.39 (3H, s), 2.76 (1H, t, J=11.4 Hz), 2.96-3.08 (4H, m), 3.16-3.17 (2H, m), 3.59-3.74 (4H, m), 4.22-4.28 (2H, m), 4.39-4.42 (1H, m), 5.16-5.22 (2H, m), 5.36-5.41 (2H, m), 5.56-5.59 (1H, m), 6.52 (1H, s), 7.14-7.20 (5H, m), 7.29-7.31 (3H, m), 7.38-7.41 (2H, m), 7.61 (1H, t, J=6.0 Hz), 7.69 (2H, d, J=7.4 Hz), 7.79 (1H, d, J=11.0 Hz), 7.87 (2H, d, J=7.8 Hz), 7.95 (1H, s), 8.07 (2H, t, J=4.3 Hz), 8.42 (1H, d, J=8.6 Hz).

MS (APCI) m/z: 1004 (M+H)⁺

Process 2: Glycylglycyl-L-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]-L-phenylalaninamide

[0611] To an N,N-dimethylformamide (1.00 mL) solution of the compound (226 mg, 0.225 mmol) obtained in Process 1, piperidine (0.223 mL, 2.25 mmol) was added and stirred at room temperature for 5 hours. The solvent was removed under reduced pressure to yield a mixture containing the titled compound. The mixture was used for the next reaction without further purification.

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Process 3: tert-Butyl (3S,12S)-12-benzyl-18-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-3-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-4,7,10,13,18-pentaoxo-5,8,11,14-tetraazaoctadecan-1-oate

5 **[0612]** The compound (0.225 mmol) obtained in Process 2 above was reacted in the same manner as Process 1 of Example 1 by using 4-tert-butyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (104 mg, 0.337 mmol) instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a pale yellow solid (114 mg, 43%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.86 (3H, t, J=7.2 Hz), 1.35 (9H, s), 1.66-1.69 (2H, m), 1.84-1.85 (2H, m), 2.11-2.13 (4H, m), 2.39 (3H, s), 2.43-2.45 (1H, m), 2.68-2.79 (2H, m), 2.94-3.16 (5H, m), 3.66 (5H, tt, J=30.5, 10.0 Hz), 4.23-4.30 (3H, m), 4.39-4.41 (1H, m), 5.15 (1H, d, J=19.2 Hz), 5.21 (1H, d, J=18.8 Hz), 5.37 (1H, d, J=17.2 Hz), 5.42 (1H, d, J=16.0 Hz), 5.53-5.57 (1H, m), 6.54 (1H, s), 7.15-7.22 (5H, m), 7.26-7.34 (3H, m), 7.38-7.40 (2H, m), 7.68-7.70 (2H, m), 7.79 (1H, d, J=10.9 Hz), 7.86-7.87 (2H, m), 7.88-7.90 (1H, m), 7.96 (1H, t, J=6.3 Hz), 8.03-8.07 (2H, m), 8.20 (1H, t, J=5.5 Hz), 8.43 (1H, d, J=8.6 Hz).

MS (APCI) m/z: 1175 (M+H)⁺

15 Process 4: tert-Butyl (3S,12S)-3-amino-12-benzyl-18-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4,7,10,13,18-pentaoxo-5,8,11,14-tetraazaoctadecan-1-oate

20 **[0613]** The compound (110 mg, 0.0936 mmol) obtained in Process 3 above was reacted in the same manner as Process 2 to yield a mixture containing the titled compound. The mixture was used for the next reaction without further purification.

25 Process 5: tert-Butyl (3S,12S)-12-benzyl-3-[[[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]amino]-18-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4,7,10,13,18-pentaoxo-5,8,11,14-tetraazaoctadecan-1-oate

[0614] The compound (0.0936 mmol) obtained in Process 4 above was reacted in the same manner as Process 4 of Example 2 to yield the titled compound as a pale yellow solid (40.2 mg, 38%).

30 ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.86 (3H, t, J=7.4 Hz), 1.17-1.19 (2H, m), 1.35 (9H, s), 1.44-1.47 (4H, m), 1.66-1.67 (2H, m), 1.81-1.88 (2H, m), 2.06-2.13 (6H, m), 2.39-2.41 (1H, m), 2.40 (3H, s), 2.67 (1H, dd, J=16.0, 5.5 Hz), 2.76 (1H, dd, J=13.3, 9.0 Hz), 2.96 (1H, dd, J=13.5, 4.9 Hz), 3.04 (2H, td, J=13.4, 6.6 Hz), 3.18 (2H, s), 3.36 (2H, d, J=7.0 Hz), 3.58 (1H, dd, J=16.8, 5.5 Hz), 3.70 (3H, dt, J=21.5, 7.2 Hz), 4.38-4.41 (1H, m), 4.57-4.59 (1H, m), 5.16 (1H, d, J=18.8 Hz), 5.24 (1H, d, J=19.2 Hz), 5.38 (1H, d, J=16.4 Hz), 5.43 (1H, d, J=16.0 Hz), 5.57-5.58 (1H, m), 6.54 (1H, s), 6.99 (2H, s), 7.13-7.25 (5H, m), 7.31 (1H, s), 7.80 (1H, d, J=10.9 Hz), 7.94-8.04 (3H, m), 8.13-8.16 (2H, m), 8.43 (1H, d, J=8.6 Hz).

MS (APCI) m/z: 1146 (M+H)⁺

40 Process 6: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-α-aspartylglycylglycyl-N-(4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl)-L-phenylalaninamide

[0615] The compound (40.0 mg, 0.0349 mmol) obtained in Process 5 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a pale yellow solid (33.6 g, 88%).

45 ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, q, J=7.2 Hz), 1.14-1.20 (2H, m), 1.46 (4H, td, J=14.8, 7.3 Hz), 1.67 (2H, td, J=12.9, 6.3 Hz), 1.84 (2H, dq, J=25.5, 7.2 Hz), 2.11 (6H, dt, J=23.4, 7.3 Hz), 2.39 (3H, s), 2.45-2.47 (1H, m), 2.69 (1H, dd, J=16.5, 5.5 Hz), 2.76 (1H, dd, J=13.7, 9.3 Hz), 2.94-3.01 (1H, m), 3.05 (2H, dq, J=25.1, 6.4 Hz), 3.17-3.19 (1H, m), 3.34-3.46 (4H, m), 3.59 (1H, dd, J=16.6, 5.6 Hz), 3.69 (2H, dt, J=20.1, 6.8 Hz), 4.37-4.41 (1H, m), 4.55 (1H, dd, J=13.5, 7.7 Hz), 5.16 (1H, d, J=19.0 Hz), 5.22 (1H, d, J=18.6 Hz), 5.38 (1H, d, J=16.4 Hz), 5.43 (1H, d, J=16.4 Hz), 5.55-5.59 (1H, m), 6.54 (1H, s), 6.99 (2H, s), 7.19 (5H, dq, J=31.6, 7.9 Hz), 7.31 (1H, s), 7.79 (1H, d, J=11.0 Hz), 7.99 (3H, ddd, J=25.1, 14.2, 6.2 Hz), 8.11 (1H, t, J=5.5 Hz), 8.17 (1H, d, J=7.6 Hz), 8.44 (1H, d, J=8.5 Hz), 12.32 (1H, s).

MS (APCI) m/z: 1090 (M+H)⁺

Process 7: Antibody-drug conjugate (15)

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[0616] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 mL·mg⁻¹·cm⁻¹ was used) described in Production

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method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

5 **[0617]** Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.102 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in above Process 6 (0.047 mL; 5.5 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.009 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

10 **[0618]** Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A.

15 **[0619]** Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

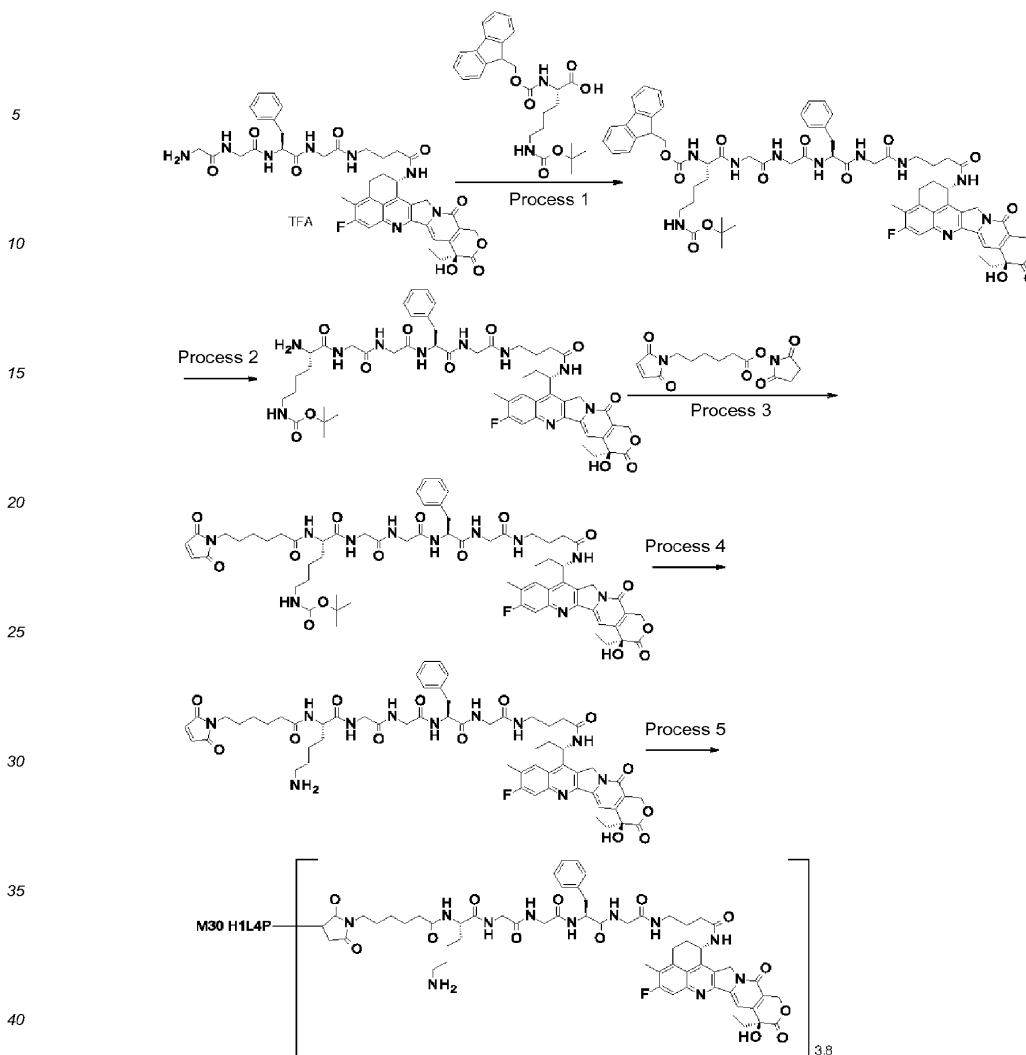
[0620] Antibody concentration: 9.16 mg/mL, antibody yield: 6.4 mg (51%), and average number of conjugated drug molecules (n) per antibody molecule: 3.0.

20 Example 17: Antibody-drug conjugate (16)

[0621]

25 [Formula 69]

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Process 1: N⁶-(tert-butoxycarbonyl)-N²-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-lysylglycylglycyl-L-phenylalanyl-N-(4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0622] The compound (167 mg, 0.176 mmol) obtained in Process 2 of Example 2 was reacted in the same manner as Process 1 of Example 1 by using N⁶-(tert-butoxycarbonyl)-N^α-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-lysine (103 mg, 0.22 mmol) instead of 4-(tert-butoxycarbonylamino)butanoic acid. The obtained crude product was used in the next process without purification.

Process 2: N⁶-(tert-butoxycarbonyl)-L-lysylglycylglycyl-L-phenylalanyl-N-(4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0623] To an N,N-dimethylformamide (4.00 mL) solution of the crude product obtained in Process 1 above, piperidine (0.400 mL) was added and stirred at room temperature for 2 hours. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to partitioned organic layer of

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chloroform : methanol : water = 7:3:1 (v/v/v),] to yield the titled compound as a pale yellow solid (113 mg, 60%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.18-1.49 (5H, m), 1.36 (9H, s), 1.51-1.60 (1H, m), 1.67-1.76 (2H, m), 1.80-1.91 (2H, m), 2.09-2.20 (4H, m), 2.39 (3H, s), 2.76-2.89 (3H, m), 2.99-3.22 (6H, m), 3.58-3.77 (6H, m), 4.43-4.49 (1H, m), 5.20 (2H, q, J=18.5 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 6.54 (1H, s), 6.76 (1H, t, J=5.5 Hz), 7.15-7.26 (5H, m), 7.31 (1H, s), 7.69-7.74 (1H, m), 7.80 (1H, d, J=10.9 Hz), 8.08 (1H, t, J=5.7 Hz), 8.14 (1H, d, J=7.8 Hz), 8.22-8.30 (2H, m), 8.47 (1H, d, J=8.6 Hz).

Process 3: N⁶-(tert-butoxycarbonyl)-N²-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-lysylglycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0624] The compound (113 mg, 0.106 mmol) obtained in Process 2 above was reacted in the same manner as Process 4 of Example 2 to yield the titled compound as a pale yellow solid (102 mg, 61%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.11-1.53 (11H, m), 1.35 (9H, s), 1.56-1.65 (1H, m), 1.68-1.76 (2H, m), 1.81-1.92 (2H, m), 2.06-2.20 (6H, m), 2.40 (3H, s), 2.74-2.90 (3H, m), 2.96-3.39 (7H, m), 3.57-3.74 (6H, m), 4.14-4.21 (1H, m), 4.42-4.49 (1H, m), 5.20 (2H, q, J=18.9 Hz), 5.42 (2H, s), 5.55-5.60 (1H, m), 6.54 (1H, s), 6.72-6.78 (1H, m), 7.00 (2H, s), 7.15-7.26 (5H, m), 7.31 (1H, s), 7.69-7.72 (1H, m), 7.80 (1H, d, J=10.9 Hz), 7.93 (1H, d, J=7.4 Hz), 7.99-8.04 (1H, m), 8.10-8.18 (2H, m), 8.26 (1H, t, J=6.1 Hz), 8.46 (1H, d, J=8.2 Hz).

Process 4: N²-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-lysylglycylglycyl-L-phenylalanyl-N-4-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-4-oxobutyl]glycinamide

[0625] To a dichloromethane (4.00 mL) solution of the compound (102 mg, 80.9 μmol) obtained in Process 3 above, trifluoroacetic acid (1.00 mL) was added and stirred at room temperature for 2 hours. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to partitioned organic layer of chloroform : methanol : water = 7:3:1 (v/v/v)] to yield the titled compound as a pale yellow solid (57.0 mg, 61%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.12-1.35 (4H, m), 1.41-1.55 (7H, m), 1.61-1.77 (3H, m), 1.80-1.91 (2H, m), 2.07-2.22 (6H, m), 2.40 (3H, s), 2.84-2.71 (3H, m), 2.97-3.40 (7H, m), 3.59-3.76 (6H, m), 4.20-4.25 (1H, m), 4.45-4.50 (1H, m), 5.20 (2H, q, J=18.5 Hz), 5.42 (2H, s), 5.54-5.60 (1H, m), 6.55 (1H, s), 7.01 (2H, s), 7.15-7.26 (5H, m), 7.31 (1H, s), 7.74 (1H, t, J=5.7 Hz), 7.81 (1H, d, J=10.9 Hz), 7.97 (1H, d, J=7.8 Hz), 8.05 (1H, t, J=6.1 Hz), 8.13-8.18 (2H, m), 8.28 (1H, t, J=5.7 Hz), 8.47 (1H, d, J=8.6 Hz).

MS (ESI) m/z: 1160 (M+H)⁺

Process 5: Antibody-drug conjugate (16)

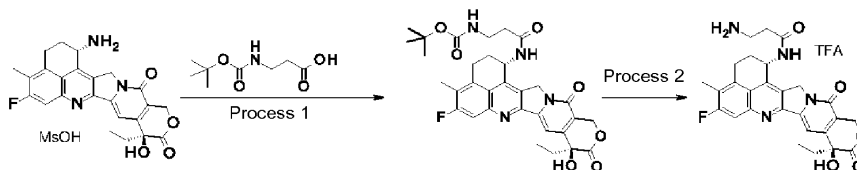
[0626] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 4 above, the titled antibody-drug conjugate was obtained in the same manner as Process 7 of Example 16.

[0627] Antibody concentration: 19.26 mg/mL, and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

Example 18 N-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0628]

[Formula 70]



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Process 1: tert-Butyl (3-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-3-oxopropyl)carbamate

5 **[0629]** Mesylate of the compound (4) (500 mg, 0.941 mmol) was reacted in the same manner as Process 1 of Example 1 by using N-(tert-butoxycarbonyl)- β -alanine instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a yellow-brown solid (616 mg, quantitative).

$^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ : 0.87 (3H, t, J=7.2 Hz), 1.29 (9H, s), 1.86 (2H, dt, J=15.1, 7.3 Hz), 2.04-2.22 (2H, m), 2.31 (2H, t, J=6.8 Hz), 2.40 (3H, s), 3.10-3.26 (4H, m), 5.15 (1H, d, J=18.8 Hz), 5.26 (1H, d, J=19.2 Hz), 5.42 (2H, dd, J=18.8, 16.4 Hz), 5.57 (1H, dt, J=8.5, 4.2 Hz), 6.53 (1H, s), 6.78 (1H, t, J=5.5 Hz), 7.30 (1H, s), 7.80 (1H, d, J=11.0 Hz), 8.46 (1H, d, J=8.6 Hz).

10 MS (ESI) m/z: 607 (M+H)⁺

Process 2: N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]- β -alaninamide

15 **[0630]** The compound obtained in Process 1 above was reacted in the same manner as Process 2 of Example 1 to yield trifluoroacetate of the titled compound as a yellow solid (499 mg, 86%).

$^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ : 0.87 (3H, t, J=7.2 Hz), 1.86 (2H, dquin, J=14.6, 7.2, 7.2, 7.2, 7.2 Hz), 2.06-2.27 (1H, m), 2.41 (3H, s), 2.46-2.57 (2H, m), 3.08 (2H, t, J=6.8 Hz), 3.14-3.24 (2H, m), 5.22 (1H, d, J=18.8 Hz), 5.29 (1H, d, J=18.8 Hz), 5.43 (2H, s), 5.58 (1H, dt, J=8.5, 4.5 Hz), 6.55 (1H, s), 7.32 (1H, s), 7.74 (3H, brs), 7.82 (1H, d, J=11.0 Hz), 8.67 (1H, d, J=8.6 Hz).

20 MS (ESI) m/z: 507 (M+H)⁺

Example 19: Antibody-drug conjugate (17)

25 **[0631]**

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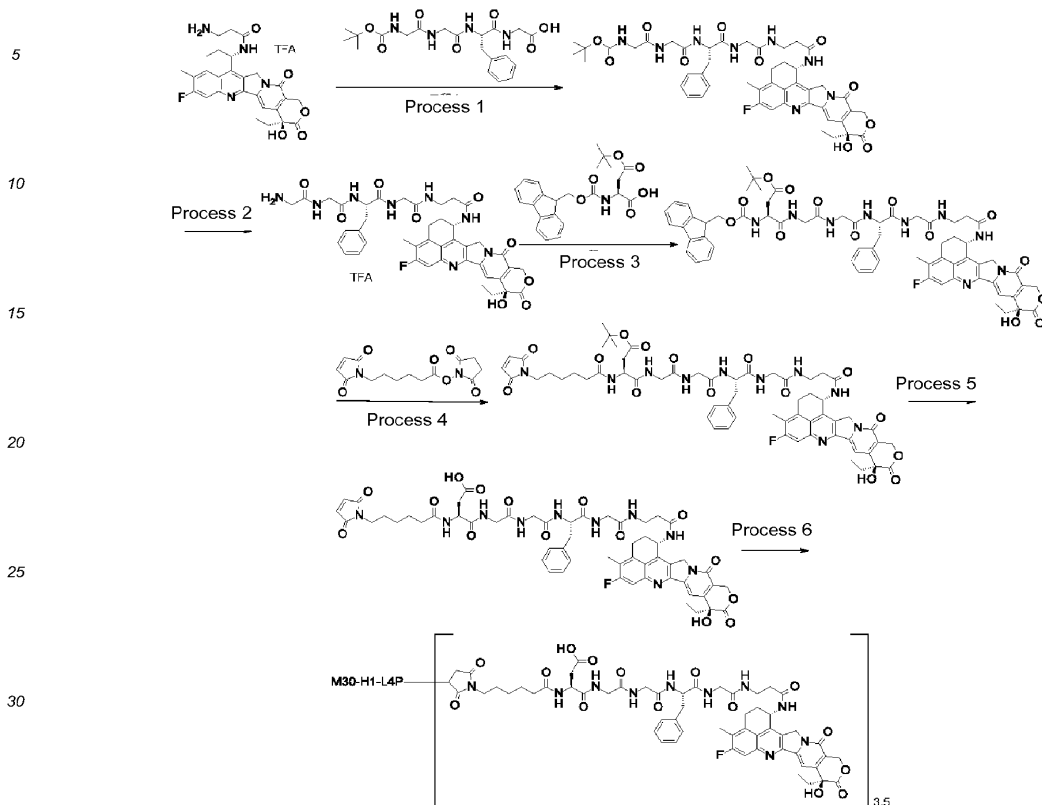
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[Formula 71]



Process 1: N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alanineamide

[0632] The compound (484 mg, 0.780 mmol) of Example 18 was reacted in the same manner as Process 1 of Example 2 to yield the titled compound as a pale yellow solid (626 mg, 87%).

¹H-NMR (400 MHz, 400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.27-1.42 (9H, m), 1.77-1.93 (2H, m), 2.06-2.22 (2H, m), 2.36 (2H, t, J=7.2 Hz), 2.40 (3H, d, J=1.6 Hz), 2.44-2.54 (2H, m), 2.76 (1H, dd, J=14.5, 10.2 Hz), 3.02 (1H, dd, J=13.9, 4.5 Hz), 3.12-3.22 (2H, m), 3.52 (6H, d, J=6.3 Hz), 4.42-4.54 (1H, m), 5.19 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.4 Hz), 5.42 (1H, dd, J=18.4, 16.4 Hz), 5.57 (1H, dt, J=8.7, 4.4 Hz), 6.53 (1H, s), 6.98 (1H, t, J=5.9 Hz), 7.14-7.28 (5H, m), 7.31 (1H, s), 7.77-7.84 (1H, m), 7.91 (1H, t, J=5.5 Hz), 8.16 (1H, d, J=7.8 Hz), 8.27 (1H, t, J=5.1 Hz), 8.52 (1H, d, J=9.0 Hz).

Process 2: Glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alanineamide trifluoroacetate

[0633] The compound (624 mg, 0.675 mmol) obtained in Process 1 above was reacted in the same manner as Process 2 of Example 2 to yield the titled compound as a yellow solid (626 mg, 92%).

¹H-NMR (400 MHz, 400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.86 (2H, tt, J=14.5, 7.2 Hz), 2.07-2.22 (2H, m), 2.36 (2H, t, J=7.2 Hz), 2.40 (3H, s), 2.44-2.54 (2H, m), 2.75 (1H, dd, J=13.7, 9.8 Hz), 3.04 (1H, dd, J=13.7, 4.3 Hz), 3.12-3.22 (2H, m), 3.58 (2H, d, J=4.7 Hz), 3.69 (3H, td, J=11.2, 5.7 Hz), 3.87 (1H, dd, J=17.0, 5.7 Hz), 4.54 (1H, m, J=17.8, 4.5 Hz), 5.19 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.8 Hz), 5.43 (2H, s), 5.51-5.60 (1H, m), 6.55 (1H, s), 7.14-7.29 (5H, m), 7.32 (1H, s), 7.81 (1H, d, J=10.9 Hz), 7.88 (1H, t, J=5.7 Hz), 7.97 (3H, br.s.), 8.29-8.38 (2H, m), 8.50 (1H, t, J=5.7 Hz),

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8.55 (1H, d, J=8.6 Hz).
MS (ESI) m/z: 825 (M+H)⁺

Process 3: tert-Butyl (9S,18S)-9-benzyl-1-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-18-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-1,5,8,11,14,17-hexaoxo-4,7,10,13,16-pentaazaicosan-20-noate

[0634] The compound (150 mg, 0.182 mmol) obtained in Process 2 above was reacted in the same manner as Process 1 of Example 2 by using (2S)-4-tert-butoxy-2-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-4-oxobutanoic acid (90.0 mg, 0.219 mmol) instead of N-(tert-butoxycarbonyl)glycylglycyl-L-phenylalanylglycine to yield the titled compound as a pale yellow solid (84.0 mg, 38%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.82-0.91 (3H, m), 1.35 (9H, s), 1.85 (2H, tt, J=14.0, 7.3 Hz), 2.06-2.21 (2H, m), 2.39 (3H, s), 2.31-2.53 (5H, m), 2.64-2.73 (1H, m), 2.78 (1H, dd, J=13.7, 9.8 Hz), 3.02 (1H, dd, J=13.9, 4.5 Hz), 3.11-3.20 (2H, m), 3.55-3.80 (6H, m), 4.17-4.35 (3H, m), 4.35-4.43 (1H, m), 4.44-4.51 (1H, m), 5.18 (1H, d, J=19.2 Hz), 5.24 (1H, d, J=19.2 Hz), 5.41 (2H, dd, J=18.8, 16.4 Hz), 5.51-5.60 (1H, m), 6.53 (1H, s), 7.13-7.20 (1H, m), 7.20-7.27 (4H, m), 7.27-7.34 (3H, m), 7.39 (2H, t, J=7.2 Hz), 7.65-7.73 (3H, m), 7.79 (2H, d, J=10.6 Hz), 7.87 (2H, d, J=7.4 Hz), 8.00 (1H, t, J=6.1 Hz), 8.08-8.20 (2H, m), 8.22-8.31 (1H, m), 8.52 (1H, d, J=8.2 Hz).

MS (ESI) m/z: 1218 (M+H)⁺

Process 4: tert-Butyl (9S,18S)-9-benzyl-18-[[[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]amino]-1-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]aminol-1,5,8,11,14,17-hexaoxo-4,7,10,13,16-pentaazaicosan-20-noate

[0635] The compound (81.0 mg, 0.0665 mmol) obtained in Process 3 above was reacted in the same manner as Process 4 of Example 2 to yield the titled compound (56.0 mg, 71%).

MS (ESI) m/z: 1189.5 (M+H)⁺

Process 5: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-α-aspartylglycylglycyl-L-phenylalanylglycyl-N-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]-β-alaninamide

[0636] The compound (52.0 mg, 0.0437 mmol) obtained in Process 4 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a pale yellow solid (35.0 mg, 71%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.12-1.22 (2H, m), 1.39-1.51 (4H, m), 1.78-1.92 (2H, m), 2.04-2.19 (2H, m), 2.08 (2H, t, J=7.2 Hz), 2.40 (3H, s), 2.31-2.46 (6H, m), 2.61-2.72 (1H, m), 2.73-2.85 (1H, m), 3.02 (1H, dd, J=14.1, 4.7 Hz), 3.17 (2H, m, J=5.5 Hz), 3.26-3.43 (2H, m), 3.55-3.77 (6H, m), 4.42-4.50 (1H, m), 4.51-4.58 (1H, m), 5.19 (1H, d, J=18.4 Hz), 5.26 (1H, d, J=18.4 Hz), 5.42 (2H, brs), 5.52-5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.12-7.27 (5H, m), 7.31 (1H, s), 7.80 (2H, d, J=10.9 Hz), 7.93-8.02 (1H, m), 8.03-8.17 (3H, m), 8.22-8.31 (1H, m), 8.53 (1H, d, J=8.6 Hz).

MS (ESI) m/z: 1133 (M+H)⁺

Process 6: Antibody-drug conjugate (17)

[0637] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 5 above, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 4.

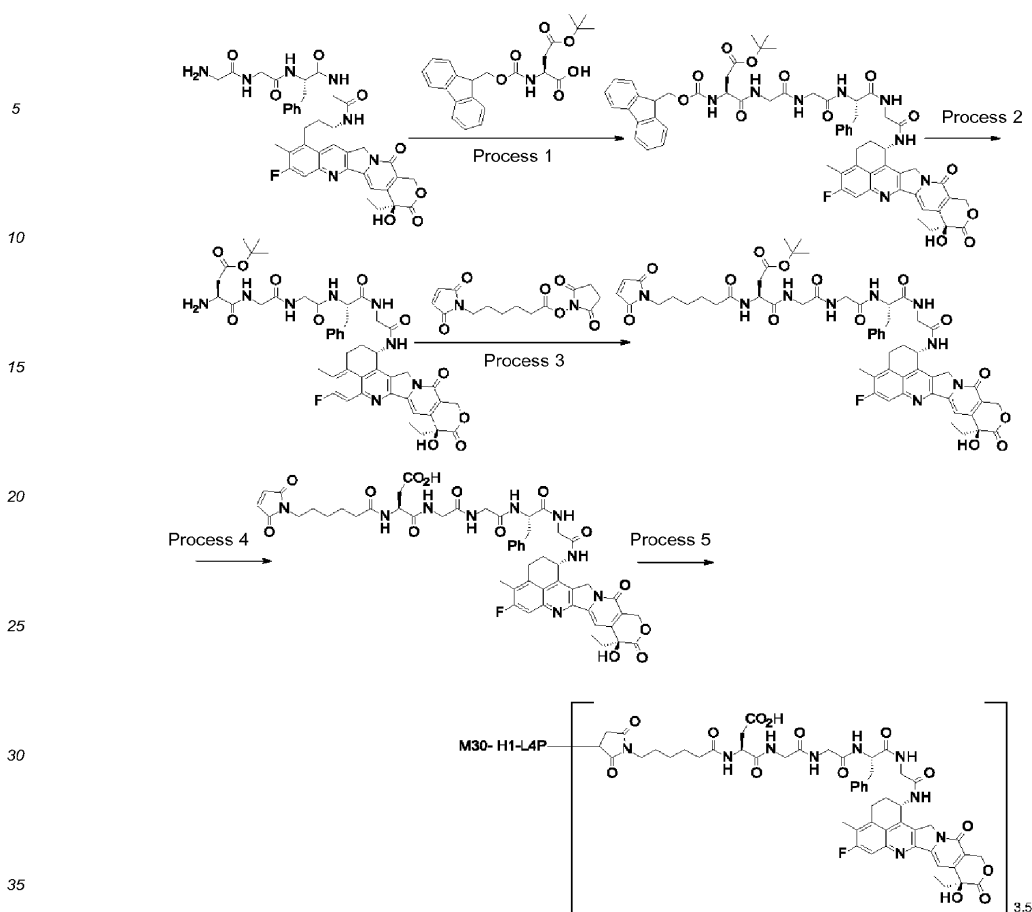
[0638] Antibody concentration: 9.56 mg/mL, antibody yield: 6.7 mg (54%), and average number of conjugated drug molecules (n) per antibody molecule: 3.5.

Example 20: Antibody-drug conjugate (18)

[0639]

[Formula 72]

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Process 1: tert-Butyl (5S,14S)-5-benzyl-1-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-14-[[[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-1,4,7,10,13-pentaoxo-3,6,9,12-tetraaza-hexadecan-16-oate

[0640] Under ice cooling, to an N,N-dimethylformamide (10.0 mL) solution of glycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide (a free form of the pharmaceutical compound described in WO97/46260; 0.250 g, 0.332 mmol), N-hydroxysuccinimide (57.2 mg, 0.497 mmol), and 4-tert-butyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-aspartate (0.205 g, 0.497 mmol), N,N'-dicyclohexylcarbodiimide (0.123 g, 0.497 mmol) was added and stirred at room temperature for 2 days. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 9:1 (v/v)] to yield the titled compound as a pale yellow solid (0.278 g, 73%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.86 (3H, t, J=7.1 Hz), 1.35 (9H, s), 1.79-1.90 (2H, m), 2.03-2.25 (2H, m), 2.40 (3H, s), 2.40-2.51 (2H, m), 2.64-2.82 (2H, m), 2.98 (1H, dd, J=13.7, 4.6 Hz), 3.16 (2H, brs), 3.55 (1H, dd, J=16.7, 5.7 Hz), 3.63-3.80 (4H, m), 4.16-4.34 (3H, m), 4.36-4.50 (2H, m), 5.23 (2H, s), 5.37 (1H, d, J=16.5 Hz), 5.43 (1H, d, J=16.5 Hz), 5.51-5.62 (1H, m), 6.52 (1H, s), 7.10-7.25 (5H, m), 7.26-7.33 (3H, m), 7.39 (2H, t, J=7.3 Hz), 7.65-7.72 (3H, m), 7.80 (1H, d, J=11.0 Hz), 7.86 (2H, d, J=7.3 Hz), 7.98 (1H, t, J=5.5 Hz), 8.07 (1H, d, J=7.8 Hz), 8.15 (1H, t, J=5.5 Hz), 8.31 (1H, t, J=5.5 Hz), 8.41 (1H, d, J=8.7 Hz).

MS (ESI) m/z: 1147 (M+H)⁺

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Process 2: tert-Butyl (5S,14S)-14-amino-5-benzyl-1-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-1,4,7,10,13-pentaoxo-3,6,9,12-tetraazahexadecan-16-oate

- 5 **[0641]** To an N,N-dimethylformamide (2.00 mL) solution of the compound (0.279 g, 0.242 mmol) obtained in Process 1 above, piperidine (0.240 mL, 2.42 mmol) was added and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 2:1 (v/v)] to yield the titled compound as a pale yellow solid (0.265 g, quantitative).
 10 ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.88 (3H, t, J=7.2 Hz), 1.39 (9H, s), 1.81-1.94 (1H, m), 2.07-2.28 (2H, m), 2.37 (1H, dd, J=15.8, 8.0 Hz), 2.43 (3H, s), 2.60 (1H, dd, J=15.8, 4.9 Hz), 2.75-2.82 (1H, m), 3.00 (1H, dd, J=13.9, 4.5 Hz), 3.16-3.25 (2H, m), 3.50-3.61 (2H, m), 3.65-3.81 (5H, m), 4.40-4.51 (1H, m), 5.27 (2H, dd, J=24.1, 19.0 Hz), 5.43 (2H, dd, J=21.3, 16.2 Hz), 5.56-5.65 (1H, m), 6.55 (1H, s), 7.15-7.28 (5H, m), 7.33 (1H, s), 7.83 (1H, d, J=11.0 Hz), 8.04 (1H, t, J=5.7 Hz), 8.09 (1H, d, J=8.2 Hz), 8.26-8.39 (2H, m), 8.44 (1H, d, J=8.2 Hz).

- 15 Process 3: tert-Butyl (5S,14S)-5-benzyl-14-[[[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]amino]-1-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino]-1,4,7,10,13-pentaoxo-3,6,9,12-tetraazahexadecan-16-oate

- 20 **[0642]** To an N,N-dimethylformamide (2.00 mL) solution of the compound (0.100 g, 0.108 mmol) obtained in Process 2 above, N-succinimidyl 6-maleimide hexanoate (40.0 mg, 0.130 mmol) was added and stirred at room temperature for 2 days. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 9:1 (v/v)] to yield the titled compound as a pale yellow solid (80.0 mg, 66%).
 25 ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.88 (3H, t, J=7.2 Hz), 1.13-1.23 (2H, m), 1.37 (9H, s), 1.42-1.54 (4H, m), 1.80-1.96 (2H, m), 2.08-2.25 (4H, m), 2.35-3.76 (15H, m), 2.43 (3H, s), 4.39-4.49 (1H, m), 4.55-4.67 (1H, m), 5.21-5.34 (2H, m), 5.43 (2H, dd, J=21.1, 16.4 Hz), 5.56-5.64 (1H, m), 6.55 (1H, s), 7.01 (2H, d, J=0.8 Hz), 7.16-7.26 (5H, m), 7.33 (1H, s), 7.83 (1H, d, J=11.3 Hz), 8.04-8.18 (3H, m), 8.30-8.37 (1H, m), 8.43 (1H, d, J=8.6 Hz).
 MS (ESI) m/z: 1118 (M+H)⁺

- 30 Process 4: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-α-aspartylglycylglycyl-L-phenylalanyl-N-[[[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

- 35 **[0643]** Under ice cooling, to the compound (70.0 mg, 62.6 μmol) obtained in Process 3 above, trifluoroacetic acid (4.00 mL) was added and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure to yield the titled compound as a pale yellow solid (55.0 mg, 83%).
 40 ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.88 (3H, t, J=7.4 Hz), 1.14-1.24 (2H, m), 1.41-1.53 (4H, m), 1.79-1.95 (2H, m), 2.08-2.28 (4H, m), 2.37-2.60 (2H, m), 2.42 (3H, s), 2.63-2.82 (2H, m), 2.99 (1H, dd, J=14.1, 5.1 Hz), 3.12-3.25 (2H, m), 3.29-3.44 (1H, m), 3.52-3.80 (6H, m), 4.38-4.48 (1H, m), 4.56 (1H, dd, J=13.7, 7.4 Hz), 5.27 (2H, dd, J=24.3, 18.8 Hz), 5.43 (2H, dd, J=21.5, 16.4 Hz), 5.57-5.62 (1H, m), 6.55 (1H, s), 7.01 (2H, s), 7.15-7.26 (5H, m), 7.33 (1H, s), 7.82 (1H, d, J=11.0 Hz), 7.98 (1H, brs), 8.08 (1H, d, J=6.7 Hz), 8.15 (1H, d, J=7.8 Hz), 8.34 (1H, brs), 8.44 (1H, d, J=8.6 Hz), 12.26 (1H, brs).
 MS (ESI) m/z: 1062 (M+H)⁺

- 45 Process 5: Antibody-drug conjugate (18)

- [0644]** Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (8.0 mL) was collected into a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.124 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.400 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

- 55 **[0645]** Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.249 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 above was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.050 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

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[0646] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 17.5 mL of a solution containing the titled antibody-drug conjugate.

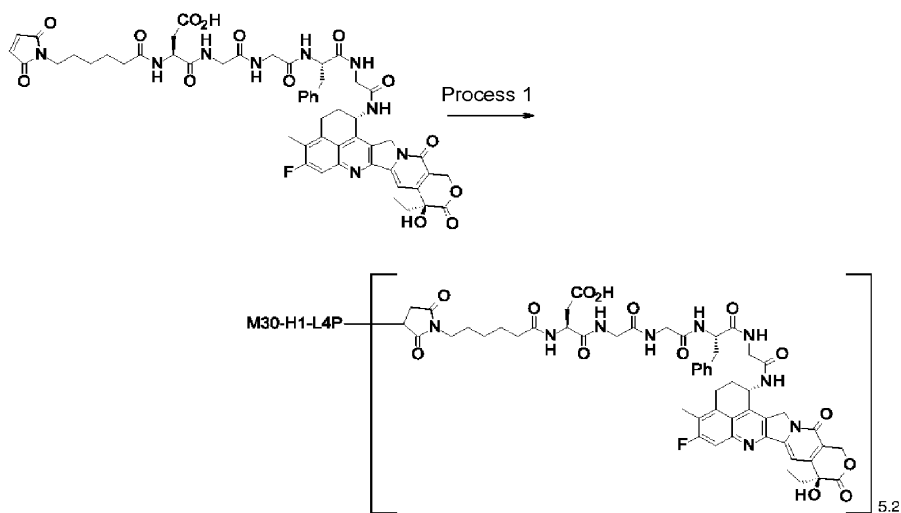
[0647] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0648] Antibody concentration: 3.56 mg/mL, antibody yield: 62 mg (77%), and average number of conjugated drug molecules (n) per antibody molecule: 3.5.

Example 21: Antibody-drug conjugate (19)

[0649]

[Formula 73]



Process 1: Antibody-drug conjugate (19)

[0650] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (8.0 mL) was collected into a 15 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.187 mL; 3.5 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.400 mL). After confirming that the solution had pH near 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0651] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.373 mL; 6.9 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.075 mL; 13.8 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0652] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 16 mL of a solution containing the titled antibody-drug conjugate.

[0653] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value),

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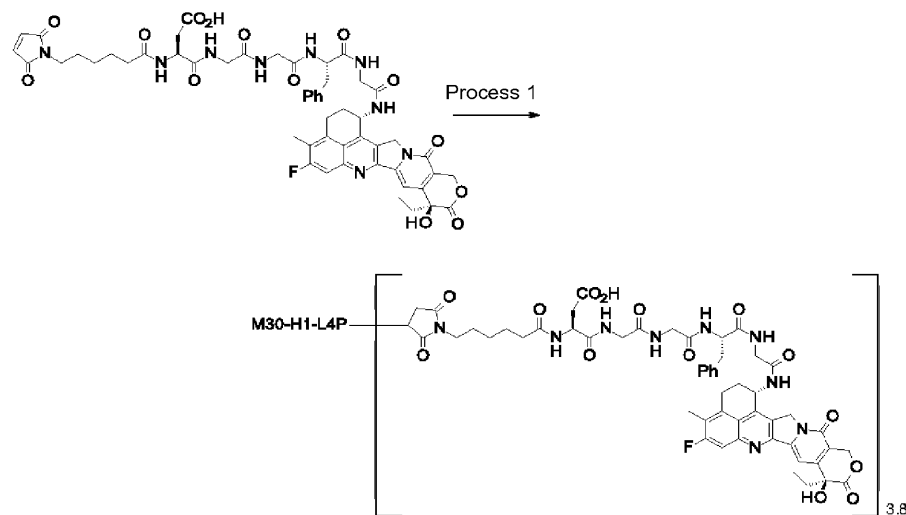
$\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0654] Antibody concentration: 3.66 mg/mL, antibody yield: 59 mg (74%), and average number of conjugated drug molecules (n) per antibody molecule: 5.2.

Example 22: Antibody-drug conjugate (20)

[0655]

[Formula 74]



Process 1: Antibody-drug conjugate (20)

[0656] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0657] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.109 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 4 of Example 20 (0.039 mL; 4.6 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution of 100 mM NAC (Sigma-Aldrich Co. LLC; 0.008 mL) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

[0658] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A described in Production method 1.

[0659] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A, 280} = 235300$ (estimated calculation value), $\epsilon_{A, 370} = 0$ (estimated calculation value), $\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

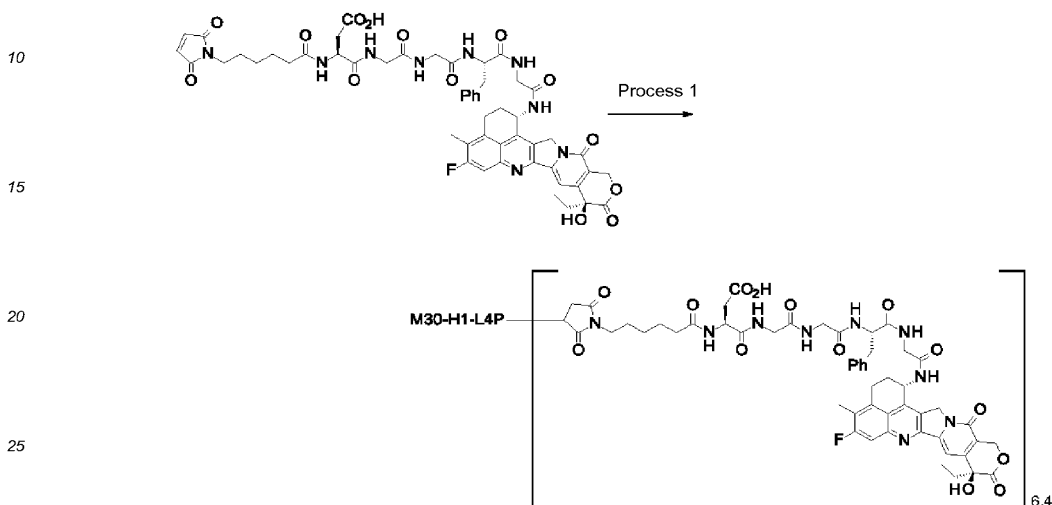
[0660] Antibody concentration: 11.14 mg/mL, antibody yield: 7.8 mg (62%), and average number of conjugated drug molecules (n) per antibody molecule: 3.8.

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Example 23: Antibody-drug conjugate (21)

[0661]

[Formula 75]



Process 1: Antibody-drug conjugate (21)

[0662] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0663] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.067 mL) and a dimethyl sulfoxide solution containing 10 mM of the compound obtained in Process 4 of Example 20 (0.085 mL; 10.0 equivalents per antibody molecule) to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 60 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.013 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

[0664] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0665] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A, 280} = 235300$ (estimated calculation value), $\epsilon_{A, 370} = 0$ (estimated calculation value), $\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

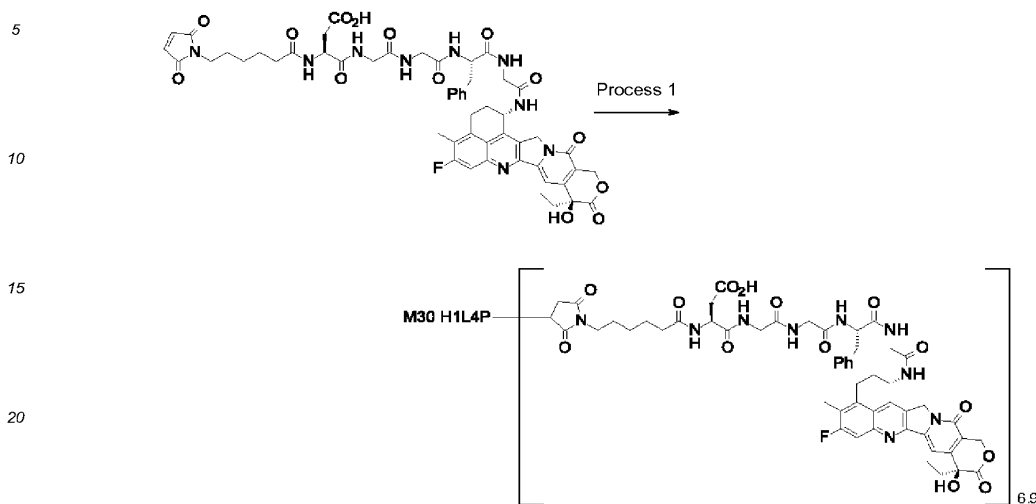
[0666] Antibody concentration: 0.88 mg/mL, antibody yield: 5.28 mg (42%), and average number of conjugated drug molecules (n) per antibody molecule: 6.4.

Example 24: Antibody-drug conjugate (22)

[0667]

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[Formula 76]



25 Process 1: Antibody-drug conjugate (22)

[0668] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.051 mL; 6.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0669] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.025 mL) and a dimethyl sulfoxide solution (0.127 mL; 15.0 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 60 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.019 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

[0670] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A described in Production method 1.

[0671] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0672] Antibody concentration: 1.19 mg/mL, antibody yield: 7.14 mg (57%), and average number of conjugated drug molecules (n) per antibody molecule: 6.9.

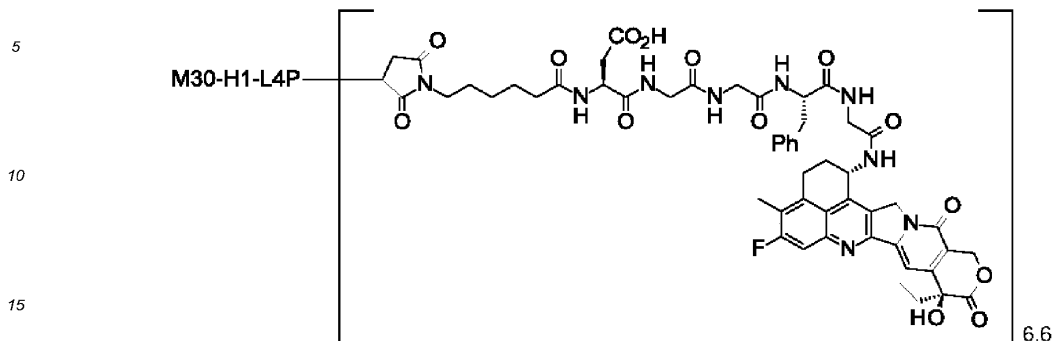
50 Example 25: Antibody-drug conjugate (23)

[0673]

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[Formula 77]



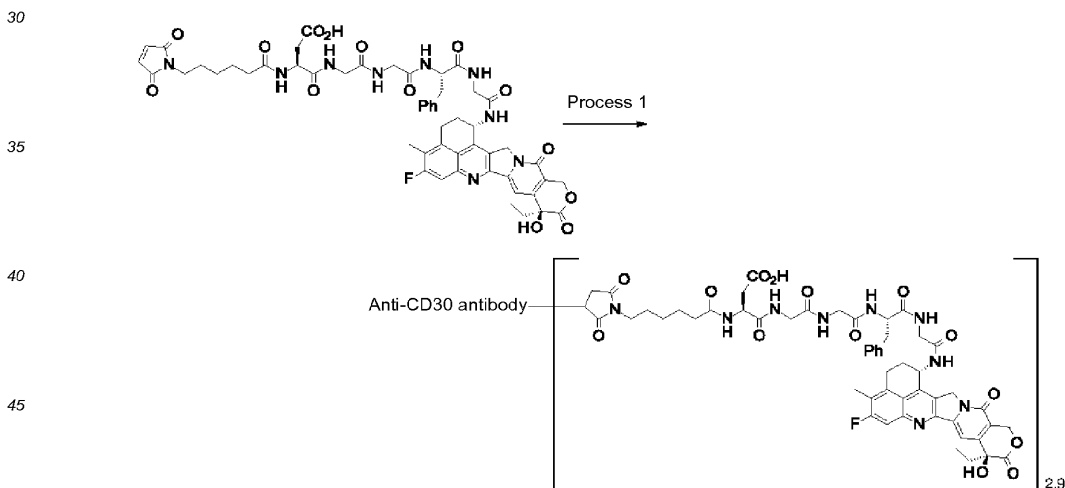
[0674] Almost the whole amounts of the antibody-drug conjugates of Examples 23 and 24 were mixed and the solution was concentrated by the Common procedure A described in Production method 1 to yield the titled antibody-drug conjugate.

[0675] Antibody concentration: 10.0 mg/mL, antibody yield: 9.07 mg, and average number of conjugated drug molecules (n) per antibody molecule: 6.6.

Example 26: Antibody-drug conjugate (24)

[0676]

[Formula 78]



Process 1: Antibody-drug conjugate (24)

[0677] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.75 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

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[06778] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00593 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0679] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

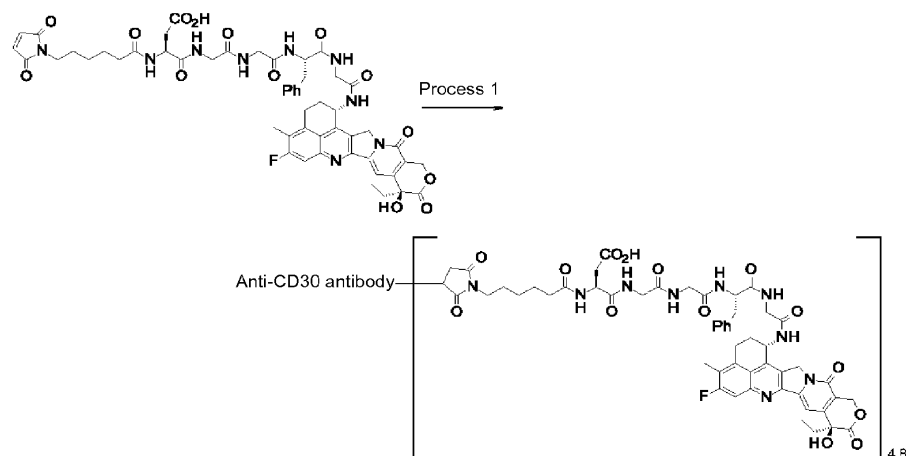
[0680] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A, 280} = 270400$ (estimated calculation value), $\epsilon_{A, 370} = 0$ (estimated calculation value), $\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0681] Antibody concentration: 0.95 mg/mL, antibody yield: 5.70 mg (57%), and average number of conjugated drug molecules (n) per antibody molecule: 2.9.

Example 27: Antibody-drug conjugate (25)

[0682]

[Formula 79]



Process 1: Antibody-drug conjugate (25)

[0683] Reduction of the antibody: The anti-CD30 antibody produced in Reference Example 3 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.75 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0684] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0685] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used

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as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

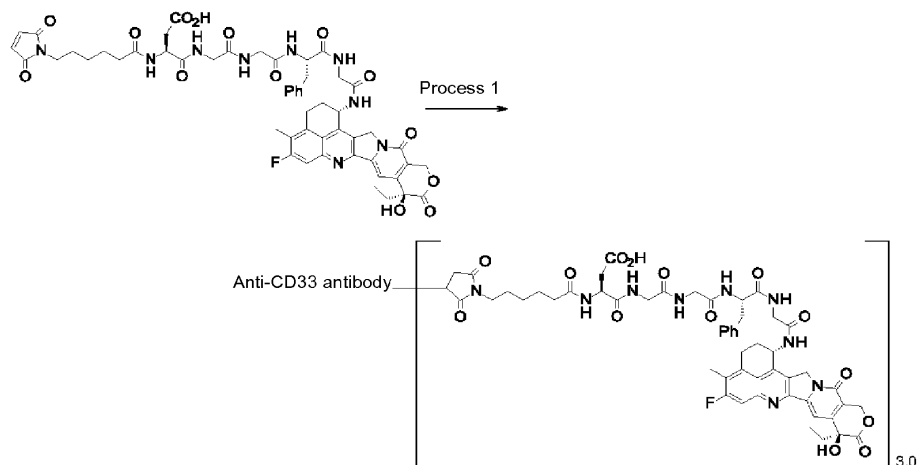
[0686] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A, 280} = 270400$ (estimated calculation value), $\epsilon_{A, 370} = 0$ (estimated calculation value), $\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0687] Antibody concentration: 1.04 mg/mL, antibody yield: 6.24 mg (62%), and average number of conjugated drug molecules (n) per antibody molecule: 4.8.

Example 28: Antibody-drug conjugate (26)

[0688]

[Formula 80]



Process 1: Antibody-drug conjugate (26)

[0689] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.66 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0690] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00593 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0691] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0692] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A, 280} = 256400$ (estimated calculation value), $\epsilon_{A, 370} = 0$ (estimated calculation value), $\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristics values were obtained.

[0693] Antibody concentration: 0.96 mg/mL, antibody yield: 5.76 mg (58%), and average number of conjugated drug

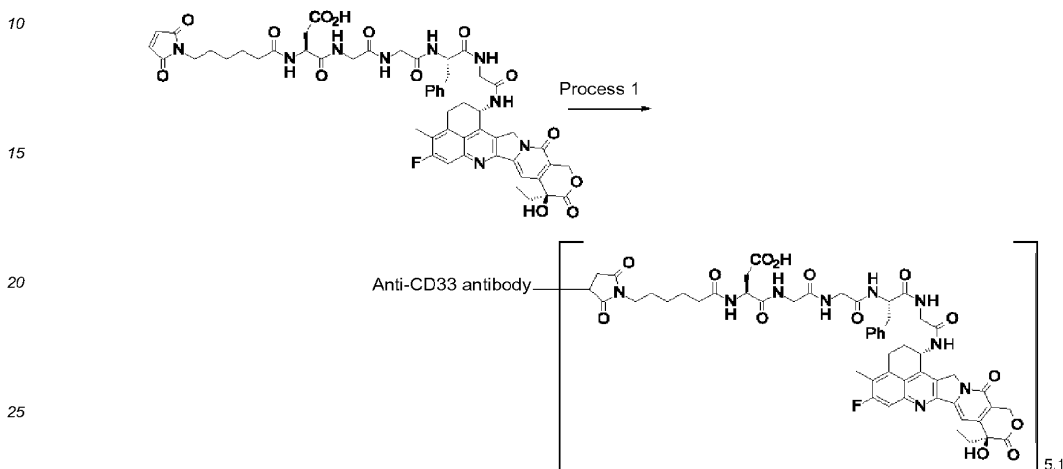
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molecules (n) per antibody molecule: 3.0.

Example 29: Antibody-drug conjugate (27)

5 [0694]

[Formula 81]



Process 1: Antibody-drug conjugate (27)

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[0695] Reduction of the antibody: The anti-CD33 antibody produced in Reference Example 4 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.66 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

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[0696] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

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[0697] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

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[0698] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 256400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

50

[0699] Antibody concentration: 0.95 mg/mL, antibody yield: 5.70 mg (57%), and average number of conjugated drug molecules (n) per antibody molecule: 5.1.

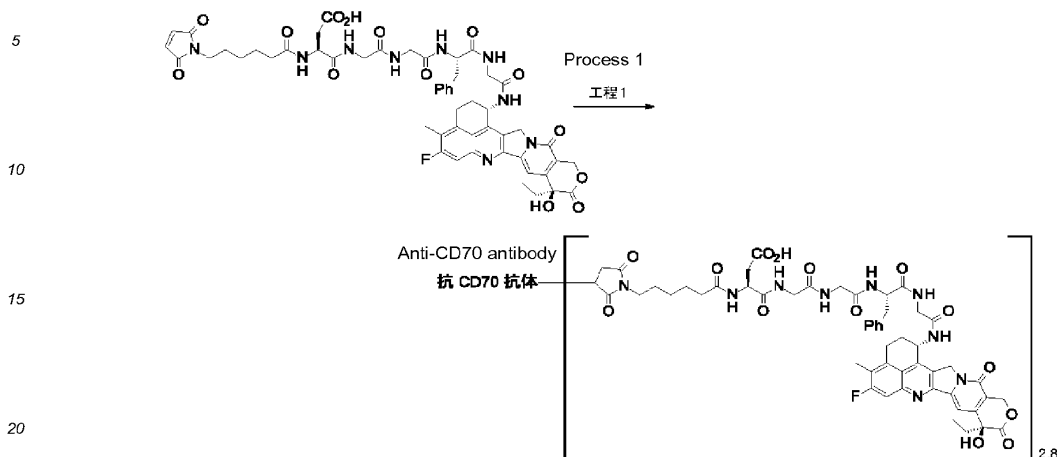
Example 30: Antibody-drug conjugate (28)

55

[0700]

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[Formula 82]



Process 1: Antibody-drug conjugate (28)

25 **[0701]** Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.69 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0148 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen

30 phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0702] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0297 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00593 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0703] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0704] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 262400$ (estimated calculation value), $\epsilon_{A,370} = 0$ (estimated calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristics values were obtained.

45 **[0705]** Antibody concentration: 1.01 mg/mL, antibody yield: 6.06 mg (61%), and average number of conjugated drug molecules (n) per antibody molecule: 2.8.

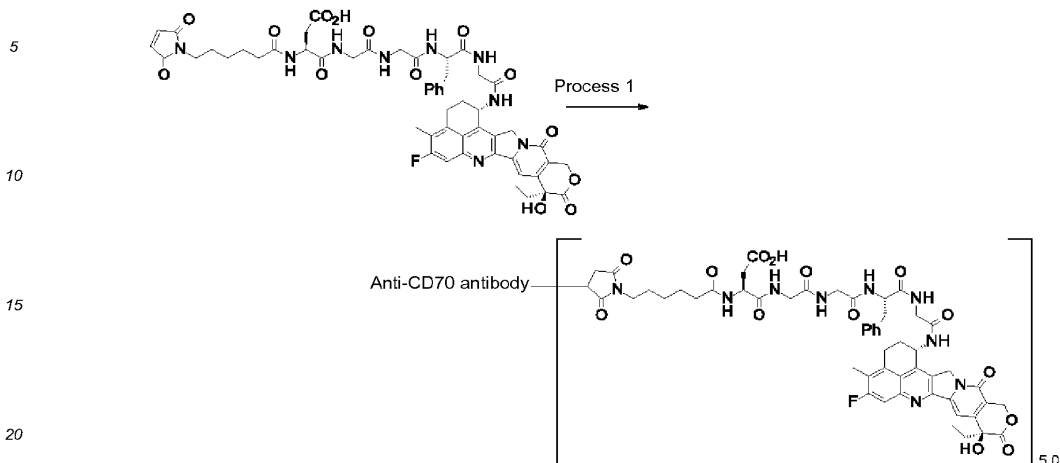
Example 31: Antibody-drug conjugate (29)

50 **[0706]**

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[Formula 83]



Process 1: Antibody-drug conjugate (29)

25 **[0707]** Reduction of the antibody: The anti-CD70 antibody produced in Reference Example 5 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.69 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 2 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0297 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

30 **[0708]** Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0593 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 4 of Example 20 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0119 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

35 **[0709]** Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

40 **[0710]** Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A, 280} = 262400$ (estimated calculation value), $\epsilon_{A, 370} = 0$ (estimated calculation value), $\epsilon_{D, 280} = 5000$ (measured average value), and $\epsilon_{D, 370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

45 **[0711]** Antibody concentration: 1.18 mg/mL, antibody yield: 7.08 mg (71%), and average number of conjugated drug molecules (n) per antibody molecule: 5.0.

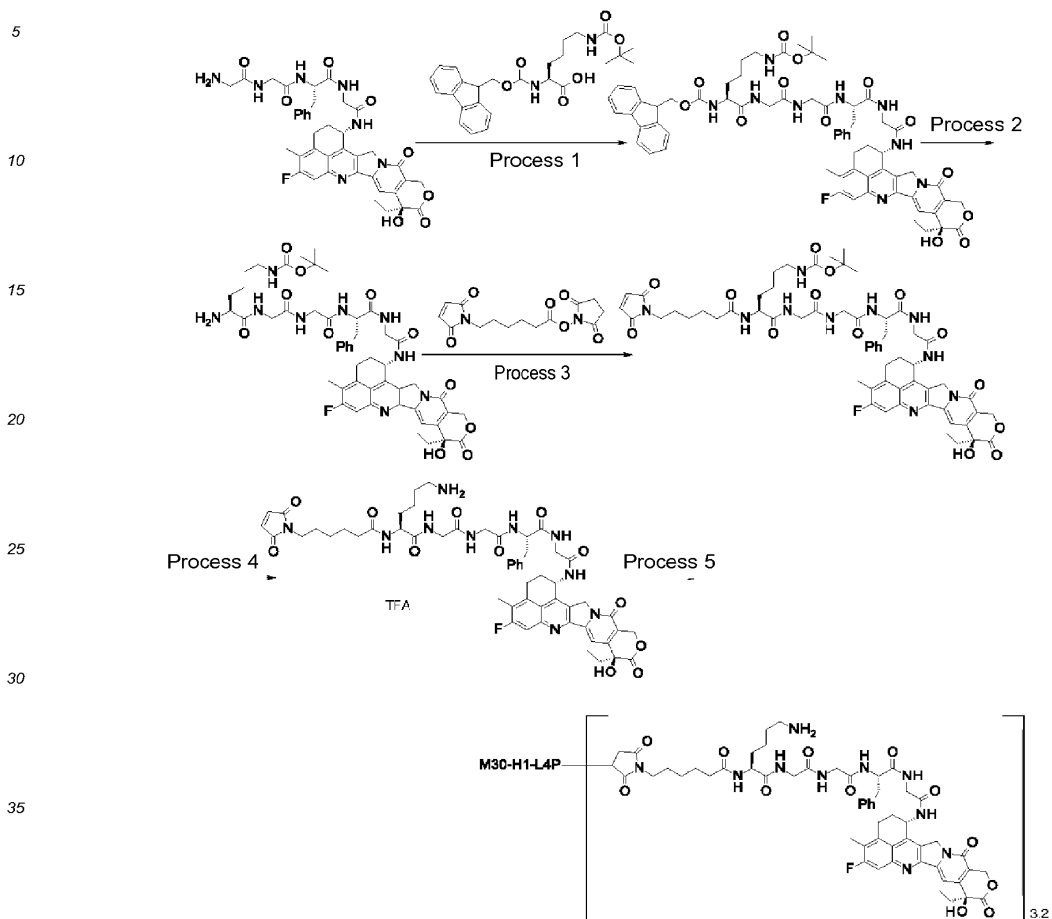
Example 32: Antibody-drug conjugate (30)

50 **[0712]**

55

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[Formula 84]



Process 1: N⁶-(tert-butoxycarbonyl)-N²-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-lysylglycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0713] Glycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide (a free form of the pharmaceutical compound described in WO97/46260; 0.300 g, 0.397 mmol) was reacted in the same manner as Process 1 of Example 20 by using N⁶-(tert-butoxycarbonyl)-N²-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-lysine instead of 4-tert-butyl N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-aspartate to yield the titled compound as a yellow solid (0.471 g, 98%).

MS (ESI) m/z : 1204 (M+H)⁺

Process 2: N⁶-(tert-butoxycarbonyl)-L-lysylglycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0714] The compound (0.417 g, 0.391 mmol) obtained in Process 1 above was reacted in the same manner as Process 2 of Example 20 to yield the titled compound as a pale yellow solid (0.272 g, 71%).

MS (ESI) m/z : 1062 (M+H)⁺

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Process 3: N⁶-(tert-butoxycarbonyl)-N²-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-lysylglycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

5 [0715] The compound (0.210 g, 0.213 mmol) obtained in Process 2 above was reacted in the same manner as Process 3 of Example 20 to yield the titled compound as a pale yellow solid (63.0 mg, 21%).
MS (ESI) m/z: 1175 (M+H)⁺

10 Process 4: N²-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]-L-lysylglycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide trifluoroacetate

15 [0716] Under ice cooling, to the compound (63.0 mg, 53.6 μmol) obtained in Process 3 above, trifluoroacetic acid (2.00 mL) was added and stirred at room temperature for 1 hour. The solvent was removed under reduced pressure to yield the titled compound as a yellow solid (50.0 mg, 78%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.89 (3H, t, J=7.2 Hz), 1.13-1.39 (4H, m), 1.43-1.58 (7H, m), 1.61-1.73 (1H, m), 1.80-1.94 (2H, m), 2.07-2.28 (4H, m), 2.43 (3H, s), 2.72-2.84 (4H, m), 3.00 (1H, dd, J=13.7, 3.9 Hz), 3.20 (2H, brs), 3.55-3.80 (6H, m), 4.20-4.30 (1H, m), 4.42-4.52 (1H, m), 5.27 (2H, dd, J=23.7, 19.8 Hz), 5.43 (2H, dd, J=21.9, 16.4 Hz), 5.55-5.65 (1H, m), 6.56 (1H, s), 7.02 (2H, s), 7.15-7.27 (5H, m), 7.34 (1H, s), 7.64 (3H, brs), 7.83 (1H, d, J=10.6 Hz), 7.98-8.04 (2H, m), 8.09-8.20 (2H, m), 8.37 (1H, t, J=5.5 Hz), 8.47 (1H, d, J=8.6 Hz).

MS (ESI) m/z: 1075 (M+H)⁺

Process 5: Antibody-drug conjugate (30)

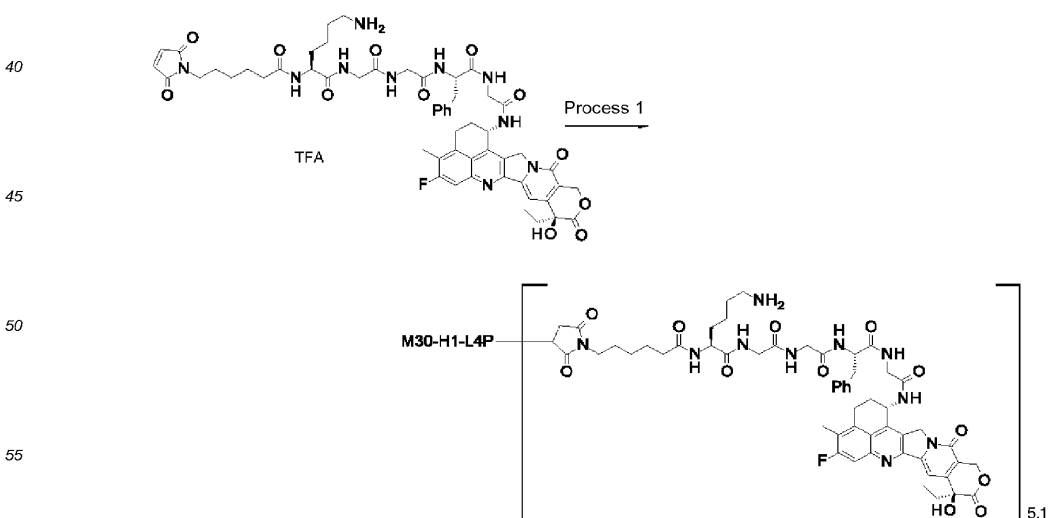
25 [0717] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 4, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 4.

[0718] Antibody concentration: 12.04 mg/mL, antibody yield: 8.4 mg (67%), and average number of conjugated drug molecules (n) per antibody molecule: 3.2.

30 Example 33: Antibody-drug conjugate (31)

[0719]

35 [Formula 85]



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Process 1: Antibody-drug conjugate (31)

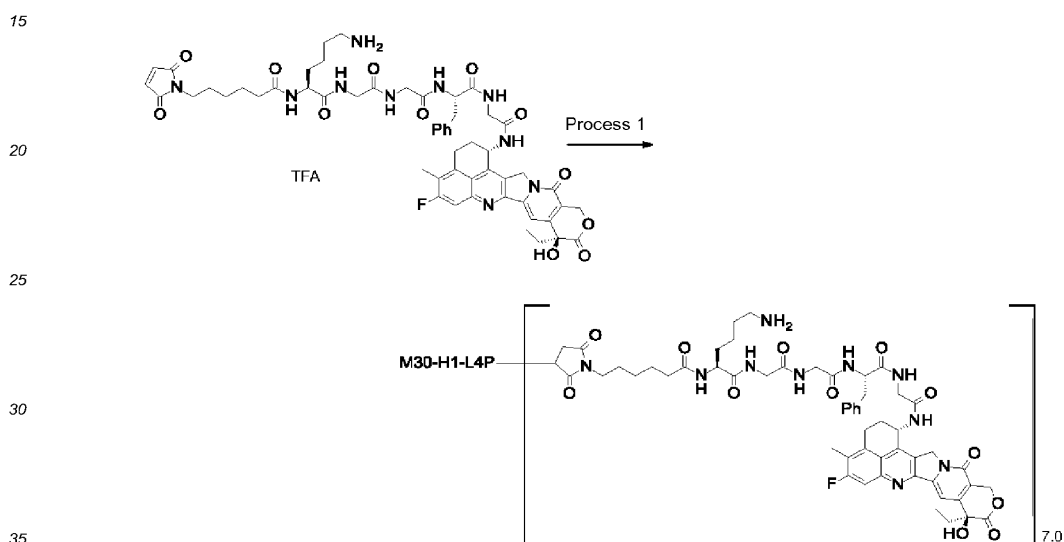
[0720] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 4 of Example 32, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 5.

5 [0721] Antibody concentration: 1.79 mg/mL, antibody yield: 10.74 mg (86%), and average number of conjugated drug molecules (n) per antibody molecule: 5.1.

Example 34: Antibody-drug conjugate (32)

10 [0722]

[Formula 86]



Process 1: Antibody-drug conjugate (32)

40 [0723] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 4 of Example 32, the titled antibody-drug conjugate was obtained in the same manner as Process 1 of Example 6.

[0724] Antibody concentration: 1.87 mg/mL, antibody yield: 11.22 mg (90%), and average number of conjugated drug molecules (n) per antibody molecule: 7.0.

Example 35: Antibody-drug conjugate (33)

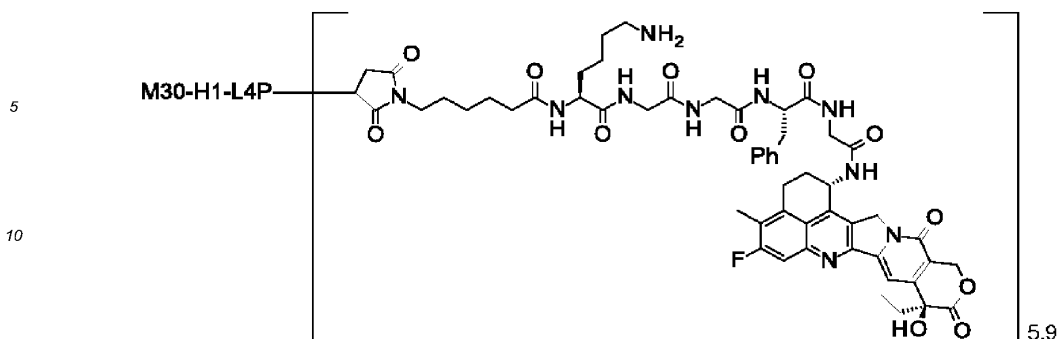
45 [0725]

[Formula 87]

50

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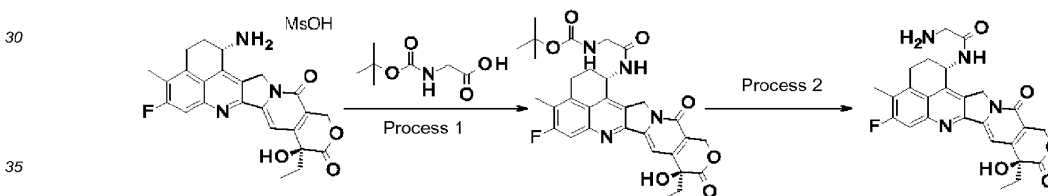
[0726] Almost the whole amounts of the antibody-drug conjugates of Examples 33 and 34 were mixed and the solution was concentrated by the Common procedure A described in Production method 1 to yield the titled antibody-drug conjugate.

[0727] Antibody concentration: 10.0 mg/mL, antibody yield: 22.21 mg, and average number of conjugated drug molecules (n) per antibody molecule: 5.9.

Example 36: N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0728]

[Formula 88]



Process 1: tert-Butyl (2-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]amino)-2-oxoethyl)carbamate

[0729] To a dichloromethane (3.00 mL) solution of N-(tert-butoxycarbonyl)-glycine (0.395 g, 2.26 mmol), N-hydroxy-succinimide (0.260 g, 2.26 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.433 mg, 2.26 mmol) were added and stirred at room temperature for 1 hour. This solution was added to a solution consisting of methanesulfonate of the compound (4) (1.00 g, 1.88 mmol), triethylamine (0.315 mL, 2.26 mmol), and N,N-dimethylformamide (3.00 mL) and stirred at room temperature for 16.5 hours. The reaction solution was diluted with chloroform and washed with 10% citric acid solution, and then the organic layer was dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 9:1 (v/v)] to yield the titled compound as a yellow solid (1.16 g, 99%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.86 (3H, t, J=7.2 Hz), 1.30 (9H, s), 1.81-1.89 (2H, m), 2.09-2.21 (2H, m), 2.38 (3H, s), 3.15-3.17 (2H, m), 3.55-3.56 (2H, m), 5.15 (1H, d, J=18.8 Hz), 5.23 (1H, d, J=19.2 Hz), 5.41 (2H, s), 5.55-5.56 (1H, m), 6.53 (1H, s), 6.95 (1H, t, J=5.5 Hz), 7.28 (1H, s), 7.77 (1H, d, J=11.0 Hz), 8.39 (1H, d, J=8.6 Hz). MS (APCI) m/z: 593 (M+H)⁺

Process 2: N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0730] The compound (0.513 g, 1.01 mmol) obtained in Process 1 above was reacted in the same manner as Process 4 of Example 17 to yield the titled compound as a yellow solid (0.463 g, 93%).

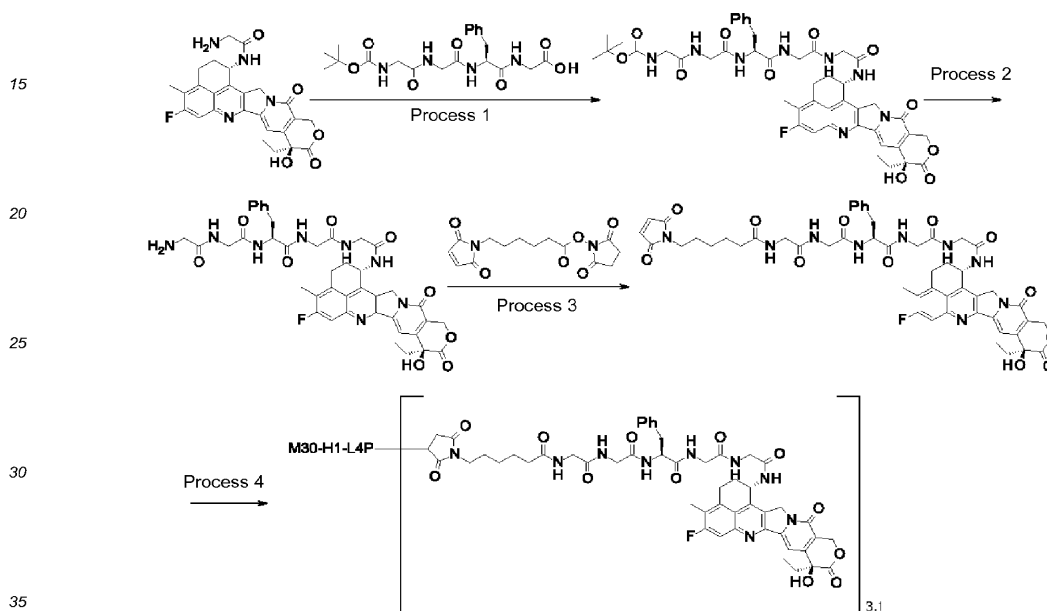
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¹H-NMR (400 MHz, CD₃OD) δ: 0.96 (3H, t, J=7.0 Hz), 1.89-1.91 (2H, m), 2.14-2.16 (1H, m), 2.30 (3H, s), 2.40-2.42 (1H, m), 3.15-3.21 (2H, m), 3.79-3.86 (2H, m), 4.63-4.67 (1H, m), 5.00-5.05 (1H, m), 5.23 (1H, d, J=16.0 Hz), 5.48 (1H, d, J=16.0 Hz), 5.62-5.64 (1H, m), 7.40-7.45 (2H, m). MS (APCI) m/z: 493 (M+H)⁺

5 Example 37: Antibody-drug conjugate (34)

[0731]

[Formula 89]



Process 1: N-(tert-butoxycarbonyl)-glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

40 [0732] N-(tert-butoxycarbonyl)-glycylglycyl-L-phenylalanylglycine (0.292 mg, 0.669 mmol) was dissolved in dichloromethane (5.00 mL), charged with N-hydroxysuccinimide (77.0 mg, 0.669 mmol) and N,N'-dicyclohexylcarbodiimide (128 mg, 0.669 mmol), and stirred for 1 hour and 20 minutes. The reaction solution was added dropwise to an N,N-dimethylformamide solution (5.00 mL) of the compound (0.275 g, 0.558 mmol) of Example 36 and stirred at room temperature for 1 day. An aqueous solution of 10% citric acid (20.0 mL) was added thereto and extracted with 20 mL of chloroform three times. The obtained organic layer was evaporated under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to chloroform : methanol = 8:2 (v/v)] to yield the titled compound as a pale yellow solid (0.430 g, 85%).

45 ¹H-NMR (400 MHz, CD₃OD) δ: 0.94 (3H, t, J=7.2 Hz), 1.43 (9H, s), 1.83-1.85 (2H, m), 2.20-2.22 (1H, m), 2.29 (3H, s), 2.36-2.39 (2H, m), 2.50-2.53 (1H, m), 2.67 (1H, s), 3.08-3.11 (1H, m), 3.18-3.21 (1H, m), 3.63-3.67 (4H, m), 3.78-3.82 (1H, m), 3.99 (2H, dd, J=23.5, 16.8 Hz), 4.16 (1H, s), 4.58 (1H, d, J=18.8 Hz), 5.15 (1H, d, J=19.2 Hz), 5.25 (1H, d, J=16.4 Hz), 5.52 (1H, d, J=16.4 Hz), 5.59-5.61 (1H, m), 6.89 (2H, d, J=6.7 Hz), 7.15-7.17 (3H, m), 7.28 (1H, d, J=10.6 Hz), 7.41 (1H, s).

MS (APCI) m/z: 911 (M+H)⁺

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Process 2: Glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0733] The compound (0.227 g, 0.249 mmol) obtained in Process 1 above was dissolved in dichloromethane (1.00 mL). Trifluoroacetic acid (3.00 mL) was added thereto and stirred for 1 hour. The solvent was removed under reduced pressure and the obtained residues were purified by silica gel column chromatography [chloroform to partitioned organic layer of chloroform : methanol : water = 7:3:1 (v/v/v)] to yield the titled compound as a pale yellow solid (0.200 g, 99%).
¹H-NMR (400 MHz, CD₃OD) δ: 0.93 (3H, t, J=7.4 Hz), 1.85 (2H, q, J=7.3 Hz), 2.24-2.45 (5H, m), 2.32 (3H, s), 2.56 (1H, dd, J=13.7, 5.5 Hz), 3.09-3.25 (2H, m), 3.66-3.76 (6H, m), 4.18-4.24 (1H, m), 4.76 (1H, d, J=19.2 Hz), 5.18 (1H, d, J=18.8 Hz), 5.30 (1H, t, J=18.4 Hz), 5.52 (1H, d, J=16.0 Hz), 5.63 (1H, t, J=5.9 Hz), 6.93 (2H, d, J=6.6 Hz), 7.17 (3H, q, J=7.3 Hz), 7.30 (1H, d, J=10.9 Hz), 7.42 (1H, s).
 MS (APCI) m/z: 811 (M+H)⁺

Process 3: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0734] The compound (0.125 g, 0.154 mmol) obtained in Process 2 above was reacted in the same manner as Process 3 of Example 20 to yield the titled compound as a pale yellow solid (0.0775 g, 50%).
¹H-NMR (400 MHz, DMSO-d₆) δ: 0.86 (3H, t, J=7.2 Hz), 1.18-1.19 (2H, m), 1.45-1.48 (4H, m), 1.83-1.85 (2H, m), 2.12-2.17 (4H, m), 2.39 (3H, s), 2.68 (1H, dd, J=24.4, 14.7 Hz), 2.83-2.87 (1H, m), 3.17-3.78 (12H, m), 4.42-4.45 (1H, m), 5.23 (2H, s), 5.41 (2H, s), 5.58-5.60 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.15-7.29 (6H, m), 7.76 (1H, d, J=10.9 Hz), 7.97-8.00 (1H, m), 8.09-8.12 (3H, m), 8.25-8.28 (1H, m), 8.44 (1H, d, J=8.2 Hz).
 MS (APCI) m/z: 1004 (M+H)⁺

Process 4: Antibody-drug conjugate (34)

[0735] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL by replacing the medium with PBS6.0/EDTA by using the Common procedure C-1 and Common procedure B (as absorption coefficient at 280 nm, 1.61 mLmg⁻¹cm⁻¹ was used) described in Production method 1. The solution (1.25 mL) was added to a 1.5 mL polypropylene tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.025 mL; 3.0 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.0625 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0736] Conjugation between antibody and drug linker: After adding dimethyl sulfoxide (Sigma-Aldrich Co. LLC; 0.102 mL) and a dimethyl sulfoxide solution (0.047 mL; 5.5 equivalents per antibody molecule) containing 10 mM of the compound obtained in above Process 3 to the above solution at room temperature, it was stirred by using a tube rotator (MTR-103, manufactured by AS ONE Corporation) at room temperature for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.009 mL) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and stirred at room temperature for another 20 minutes to terminate the reaction of drug linker.

[0737] Purification: The above solution was subjected to purification using the Common procedure D-1 (ABS was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate. After that, the solution was concentrated by the Common procedure A described in Production method 1.

[0738] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, ε_{A,280} = 235300 (calculation value), ε_{A,370} = 0 (calculation value), ε_{D,280} = 5000 (measured average value), and ε_{D,370} = 19000 (measured average value) were used), the following characteristic values were obtained.

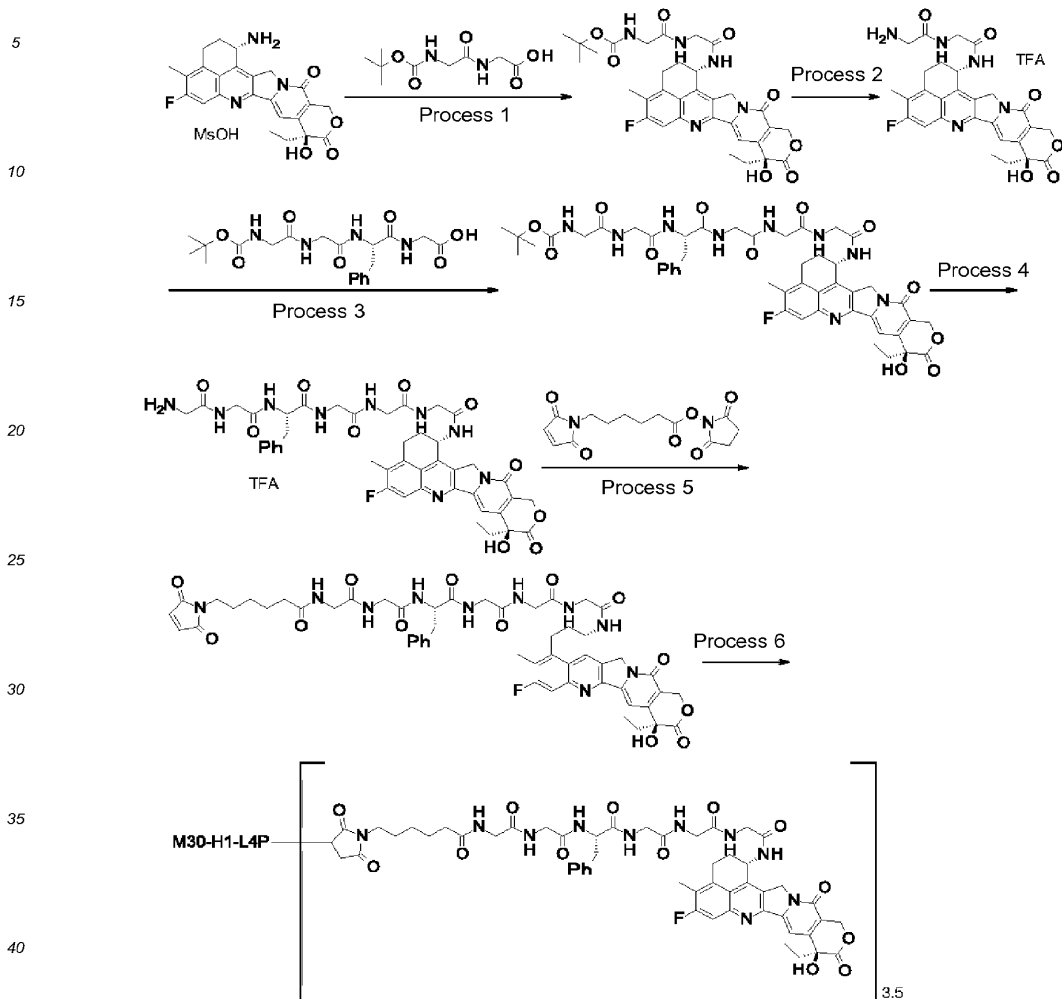
[0739] Antibody concentration: 12.4 mg/mL, antibody yield: 8.7 mg (70%), and average number of conjugated drug molecules (n) per antibody molecule: 3.1.

Example 38: Antibody-drug conjugate (35)

[0740]

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[Formula 90]



Process 1: N-(tert-butoxycarbonyl)glycyl-L-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0741] Methanesulfonate of the compound (4) (0.800 g, 1.51 mmol) was reacted in the same manner as Process 1 of Example 1 by using N-(tert-butoxycarbonyl)-glycylglycine (0.419 g, 1.81 mmol) instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a yellow solid (0.965 g, 99%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.23 (9H, s), 1.82-1.89 (2H, m), 2.11-2.19 (2H, m), 2.40 (3H, s), 3.16-3.17 (2H, m), 3.52 (2H, ddd, J=21.3, 15.5, 4.7 Hz), 3.77 (2H, ddd, J=24.3, 16.8, 5.9 Hz), 5.23 (2H, s), 5.43 (2H, s), 5.56-5.60 (1H, m), 6.53 (1H, s), 7.04 (1H, t, J=5.9 Hz), 7.31 (1H, s), 7.80 (1H, d, J=11.0 Hz), 8.12 (1H, t, J=5.5 Hz), 8.31 (1H, d, J=8.6 Hz).

MS (APCI) m/z: 650 (M+H)⁺

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Process 2: Glycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycineamide trifluoroacetate

[0742] The compound (0.884 g, 1.36 mmol) obtained in Process 1 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a yellow solid (0.787 g, quantitative).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.82-1.89 (2H, m), 2.11-2.18 (2H, m), 2.41 (3H, s), 3.17-3.18 (2H, m), 3.63 (2H, s), 3.88 (2H, d, J=5.5 Hz), 5.19 (1H, d, J=18.8 Hz), 5.25 (1H, d, J=19.2 Hz), 5.42 (2H, s), 5.56-5.61 (1H, m), 6.56 (1H, s), 7.32 (1H, s), 7.81 (1H, d, J=11.0 Hz), 8.01 (3H, brs), 8.65 (1H, d, J=8.6 Hz), 8.72 (1H, t, J=5.5 Hz). MS (APCI) m/z: 550 (M+H)⁺

Process 3: N-(tert-butoxycarbonyl)glycylglycylphenylalanylglycylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0743] The compound (0.400 g, 0.728 mmol) obtained in Process 2 above was reacted in the same manner as Process 1 of Example 1 by using N-(tert-butoxycarbonyl)-glycylglycyl-L-phenylalanylglycine (0.381 mg, 0.873 mmol) instead of 4-(tert-butoxycarbonylamino)butanoic acid to yield the titled compound as a pale yellow solid (0.545 g, 77%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.37 (9H, s), 1.80-1.90 (2H, m), 2.09-2.11 (1H, m), 2.18-2.21 (1H, m), 2.40 (3H, s), 2.72-2.77 (1H, m), 3.01 (1H, dd, J=13.7, 4.3 Hz), 3.16-3.17 (2H, m), 3.52-3.83 (10H, m), 4.48-4.51 (1H, m), 5.21 (1H, d, J=19.2 Hz), 5.26 (1H, d, J=18.8 Hz), 5.43 (2H, s), 5.55-5.59 (1H, m), 6.53 (1H, s), 6.99 (1H, t, J=5.9 Hz), 7.18-7.24 (5H, m), 7.31 (1H, s), 7.80 (1H, d, J=11.0 Hz), 7.90 (1H, t, J=5.3 Hz), 8.02 (1H, t, J=5.5 Hz), 8.15-8.19 (2H, m), 8.30 (1H, t, J=5.5 Hz), 8.43 (1H, d, J=8.6 Hz). MS (APCI) m/z: 968 (M+H)⁺

Process 4: Glycylglycylphenylalanylglycylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycineamide trifluoroacetate

[0744] The compound (0.429 g, 0.443 mmol) obtained in Process 3 above was reacted in the same manner as Process 2 of Example 1 to yield the titled compound as a yellow solid (0.385 g, quantitative).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.82-1.89 (2H, m), 2.11-2.19 (2H, m), 2.40 (3H, s), 2.74 (1H, dd, J=13.7, 9.8 Hz), 3.03 (1H, dd, J=13.7, 4.3 Hz), 3.16-3.18 (2H, m), 3.57-3.58 (2H, m), 3.67-3.76 (7H, m), 3.82-3.90 (1H, m), 4.53-4.56 (1H, m), 5.23 (2H, s), 5.43 (2H, s), 5.55-5.59 (1H, m), 6.55 (1H, s), 7.17-7.19 (1H, m), 7.22-7.29 (4H, m), 7.31 (1H, s), 7.80 (1H, d, J=10.9 Hz), 8.00 (3H, brs), 8.07 (1H, t, J=5.7 Hz), 8.22 (1H, t, J=5.7 Hz), 8.36 (2H, dd, J=10.9, 7.0 Hz), 8.47-8.52 (2H, m).

MS (APCI) m/z: 868 (M+H)⁺

Process 5: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycylphenylalanylglycylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0745] The compound (0.278 g, 0.320 mmol) obtained in Process 4 above was reacted in the same manner as Process 3 of Example 20 to yield the titled compound as a pale yellow solid (0.166 g, 49%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.2 Hz), 1.14-1.22 (2H, m), 1.44-1.49 (4H, m), 1.80-1.90 (2H, m), 2.06-2.13 (3H, m), 2.20 (1H, d, J=14.1 Hz), 2.40 (3H, s), 2.77 (1H, dd, J=13.3, 8.7 Hz), 3.01 (1H, dd, J=13.3, 4.3 Hz), 3.17 (2H, t, J=6.7 Hz), 3.35-3.38 (2H, m), 3.56-3.84 (10H, m), 4.48 (1H, dd, J=13.1, 9.2 Hz), 5.23 (2H, s), 5.43 (2H, s), 5.55-5.59 (1H, m), 6.53 (1H, s), 6.99 (2H, s), 7.20-7.24 (5H, m), 7.31 (1H, s), 7.80 (1H, d, J=11.0 Hz), 8.00 (2H, q, J=5.5 Hz), 8.06 (1H, t, J=5.9 Hz), 8.13 (1H, d, J=8.2 Hz), 8.18 (1H, t, J=5.7 Hz), 8.28 (1H, t, J=5.7 Hz), 8.43 (1H, d, J=8.6 Hz). MS (APCI) m/z: 1061 (M+H)⁺

Process 6: Antibody-drug conjugate (35)

[0746] By using the M30-H1-L4P antibody produced in Reference Example 2 and the compound obtained in Process 5 above, the titled antibody-drug conjugate was yielded in the same manner as Process 7 of Example 16.

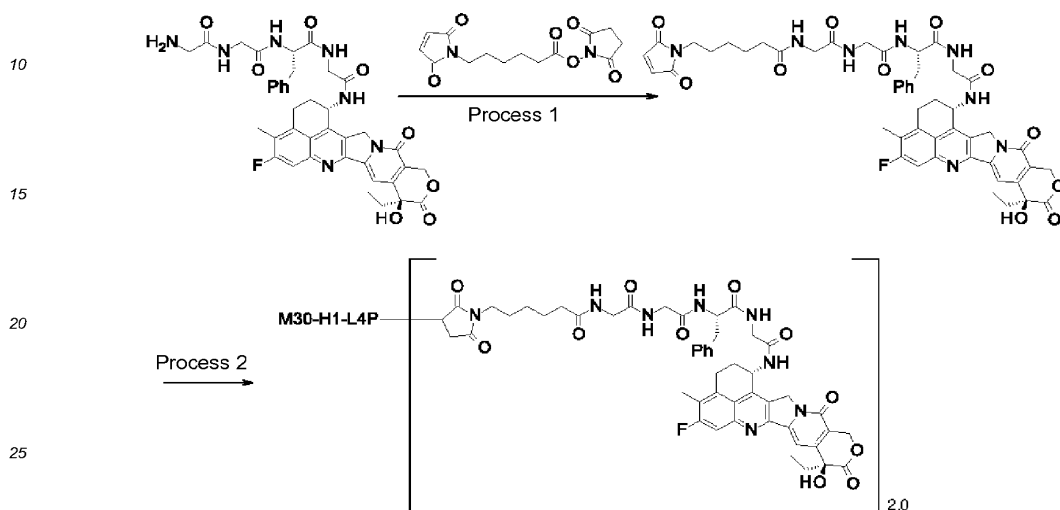
[0747] Antibody concentration: 11.7 mg/mL, antibody yield: 8.2 mg (66%), and average number of conjugated drug molecules (n) per antibody molecule: 3.5.

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Example 39: Antibody-drug conjugate (36)

[0748]

[Formula 91]



Process 1: N-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycylglycyl-L-phenylalanylglycyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide

[0749] Glycylglycyl-L-phenylalanyl-N-[(1S,9S)-9-ethyl-5-fluoro-9-hydroxy-4-methyl-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-1-yl]glycinamide (a free form of the pharmaceutical compound described in WO97/46260; 0.150 g, 0.200 mol) was reacted in the same manner as Process 3 of Example 20 to yield the titled compound as a pale yellow solid (70.0 mg, 37%).

¹H-NMR (400 MHz, DMSO-d₆) δ: 0.87 (3H, t, J=7.4 Hz), 1.15-1.21 (2H, m), 1.41-1.50 (4H, m), 1.80-1.90 (2H, m), 2.07-2.12 (4H, m), 2.17-2.23 (1H, m), 2.35-2.40 (1H, m), 2.41 (3H, s), 2.73-2.81 (1H, m), 2.98 (1H, dd, J=13.7, 4.6 Hz), 3.15-3.20 (2H, m), 3.53 (1H, dd, J=16.6, 5.7 Hz), 3.62-3.77 (5H, m), 4.39-4.45 (1H, m), 5.22 (1H, d, J=18.9 Hz), 5.27 (1H, d, J=18.9 Hz), 5.39 (1H, d, J=16.0 Hz), 5.44 (1H, d, J=16.0 Hz), 5.55-5.60 (1H, m), 6.53 (1H, s), 6.98 (2H, s), 7.13-7.24 (5H, m), 7.32 (1H, s), 7.81 (1H, d, J=10.3 Hz), 7.95-8.00 (1H, m), 8.05-8.09 (2H, m), 8.28-8.31 (1H, m), 8.41 (1H, d, J=8.6 Hz).

MS (APCI) m/z: 947 (M+H)⁺

Process 2: Antibody-drug conjugate (36)

[0750] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, 1.61 mLmg⁻¹cm⁻¹ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0147 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1, the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0751] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0295 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 1 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00590 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

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[0752] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

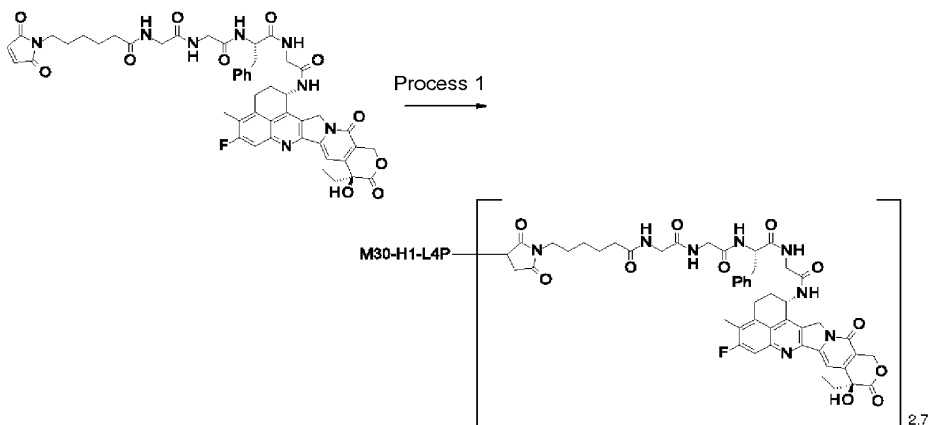
[0753] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (calculation value), $\epsilon_{A,370} = 0$ (calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

[0754] Antibody concentration: 1.23 mg/mL, antibody yield: 7.38 mg (74%), and average number of conjugated drug molecules (n) per antibody molecule: 2.0.

Example 40: Antibody-drug conjugate (37)

[0755]

[Formula 92]



Process 1: Antibody-drug conjugate (37)

[0756] Reduction of the antibody: The M30-H1-L4P antibody produced in Reference Example 2 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0295 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0757] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0590 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 1 of Example 39 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0118 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0758] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0759] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (calculation value), $\epsilon_{A,370} = 0$ (calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

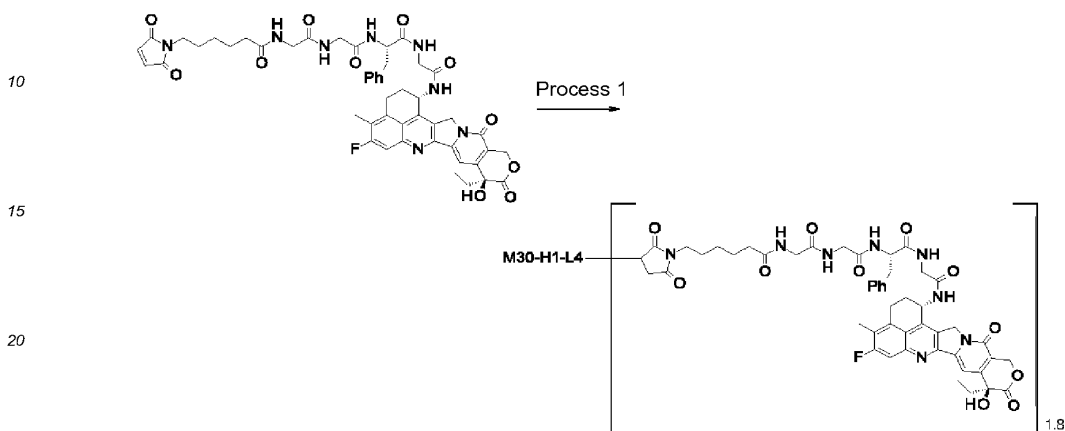
[0760] Antibody concentration: 1.22 mg/mL, antibody yield: 7.32 mg (73%), and average number of conjugated drug molecules (n) per antibody molecule: 2.7.

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Example 41: Antibody-drug conjugate (38)

[0761]

[Formula 93]



Process 1: Antibody-drug conjugate (38)

[0762] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0147 mL; 2.3 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the disulfide bond at hinge part in the antibody was reduced by incubating at 37°C for 1 hour.

[0763] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0295 mL; 4.6 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 1 of Example 39 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.00590 mL; 9.2 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

[0764] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

[0765] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (calculation value), $\epsilon_{A,370} = 0$ (calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristic values were obtained.

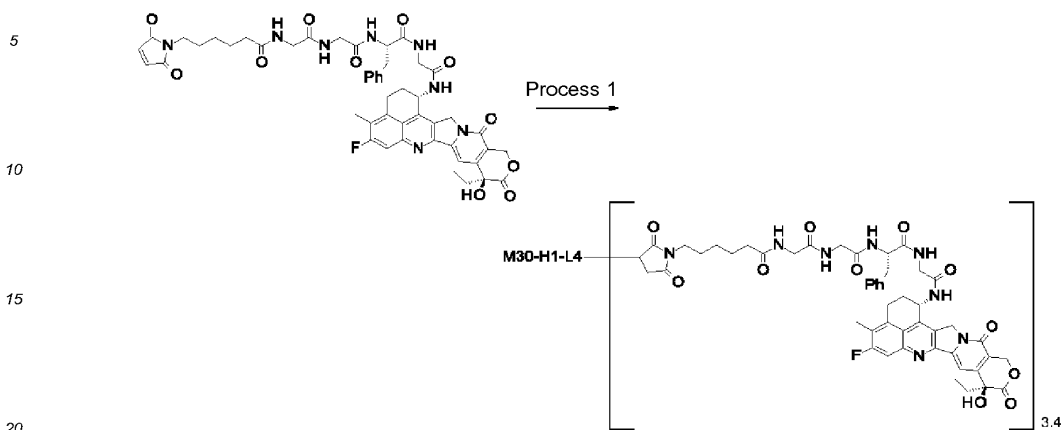
[0766] Antibody concentration: 1.11 mg/mL, antibody yield: 6.66 mg (67%), and average number of conjugated drug molecules (n) per antibody molecule: 1.8.

Example 42: Antibody-drug conjugate (39)

[0767]

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[Formula 94]



Process 1: Antibody-drug conjugate (39)

25 [0768] Reduction of the antibody: The M30-H1-L4 antibody produced in Reference Example 1 was prepared to have antibody concentration of 10 mg/mL with PBS6.0/EDTA by using the Common procedure B (as absorption coefficient at 280 nm, $1.61 \text{ mLmg}^{-1}\text{cm}^{-1}$ was used) and Common procedure C-1 described in Production method 1. The solution (1.0 mL) was collected into a 1.5 mL tube and charged with an aqueous solution of 10 mM TCEP (Tokyo Chemical Industry Co., Ltd.) (0.0295 mL; 4.6 equivalents per antibody molecule) and an aqueous solution of 1 M dipotassium hydrogen phosphate (Nacalai Tesque, Inc.; 0.050 mL). After confirming that the solution had pH of 7.4 ± 0.1 , the

30 [0769] Conjugation between antibody and drug linker: After incubating the above solution at 22°C for 10 minutes, a dimethyl sulfoxide solution (0.0590 mL; 9.2 equivalents per antibody molecule) containing 10 mM of the compound obtained in Process 1 of Example 39 was added thereto and incubated at 22°C for 40 minutes for conjugating the drug linker to the antibody. Next, an aqueous solution (0.0118 mL; 18.4 equivalents per antibody molecule) of 100 mM NAC (Sigma-Aldrich Co. LLC) was added thereto and incubated at 22°C for another 20 minutes to terminate the reaction of drug linker.

35 [0770] Purification: The above solution was subjected to purification using the Common procedure D-1 (PBS7.4 was used as buffer solution) described in Production method 1 to yield 6 mL of a solution containing the titled antibody-drug conjugate.

40 [0771] Physicochemical characterization: By using the Common procedure E described in Production method 1 (as molar absorption coefficient, $\epsilon_{A,280} = 235300$ (calculation value), $\epsilon_{A,370} = 0$ (calculation value), $\epsilon_{D,280} = 5000$ (measured average value), and $\epsilon_{D,370} = 19000$ (measured average value) were used), the following characteristics values were obtained.

45 [0772] Antibody concentration: 1.00 mg/mL, antibody yield: 6.00 mg (60%), and average number of conjugated drug molecules (n) per antibody molecule: 3.4.

(Test Example 1) Production of full-length human B7-H3 variant 1 expression vector

50 [0773] cDNA encoding human B7-H3 variant 1 was amplified by PCR reaction using cDNA synthesized from LNCaP cell (American Type Culture Collection: ATCC) total RNA as a template and the following primer set:

primer 1:

5'-ctatagggagacccaagctggctagcatgctgcgtcgccggggcag-3' (SEQ ID NO: 22) and

55 primer 2:

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5' -

aacggggccctctagactcgagcgccgctcaggctatttcttgccatcatcttctt
 tgctgtcag-3' (SEQ ID NO: 23).

- [0774] Next, the obtained PCR product was purified by using MagExtractor PCR & Gel cleanup (Toyobo Co., Ltd.). The purified product was further digested with restriction enzymes (NheI/NotI) and thereafter purified by using MagExtractor PCR & Gel cleanup (Toyobo Co., Ltd.). pcDNA3.1 (+) plasmid DNA (Life Technologies) was digested with the same restriction enzymes as above (NheI/NotI) and thereafter purified by using MagExtractor PCR & Gel cleanup (Toyobo Co., Ltd.).
- [0775] These purified DNA solutions were mixed, further charged with Ligation high (Toyobo Co., Ltd.), and incubated at 16°C for 8 hours for ligation.
- [0776] *Escherichia coli* DHS α competent cells (Life Technologies) were transformed by the addition of the obtained reaction product.
- [0777] The colonies thus obtained were subjected to colony direct PCR using PCR primers and BGH reverse primer to select candidate clones.
- [0778] The obtained candidate clones were cultured in a liquid medium (LB/Amp), and plasmid DNA was extracted with MagExtractor-Plasmid- (Toyobo Co., Ltd.).
- [0779] Each obtained clone was compared with the provided CDS sequence by the sequencing analysis between primer 3 (CMV promoter primer):

5'-cgcaaatggcggtaggcgtg-3' (SEQ ID NO: 24) and

primer 4 (BGH reverse primer):

5'-tagaaggcacagtcgagg-3' (SEQ ID NO: 25)

with the obtained plasmid DNA as a template.

- [0780] After confirming the sequence, the obtained clone was cultured in 200 mL of LB/Amp medium, and plasmid DNA was extracted by using VioGene Plasmid Midi V-100 kit.
- [0781] The vector was designated as pcDNA3.1-B7-H3. The sequence of an ORF site of the B7-H3 variant 1 gene cloned in the vector is shown in nucleotide positions 1 to 1602 of SEQ ID NO: 26 (Figure 16) in the Sequence Listing. Also, the amino acid sequence of the B7-H3 variant 1 is shown in SEQ ID NO: 1 in the Sequence Listing.

(Test Example 2) Preparation of CCRF-CEM cell stably expressing B7-H3 variant 1 gene

- [0782] pcDNA3.1-B7-H3 produced in Test Example 1 was transfected into CCRF-CEM cells (ATCC) by electroporation using Nucleofector II (manufactured by Lonza Group Ltd.). Then, the cells were further cultured for two nights in RPMI1640 medium (Life Technologies) containing 10% fetal bovine serum (FBS) (hereinafter, referred to as 10%FBS-RPMI1640) under conditions of 37°C and 5% CO₂.
- [0783] After the 2-day culture, culture was started in 10%FBS-RPMI1640 containing 750 μ g/mL G418 (Life Technologies) in order to select CCRF-CEM cells in which pcDNA3.1-B7-H3 was stably integrated.
- [0784] After the 1-month culture, cloning was carried out by the limiting dilution method in order to yield a single cell clone. Specifically, cells having resistance to G418 were diluted into 10 cells/mL, inoculated to a 96-well plate at a concentration of 100 μ L/well, and cultured, and cells allowed to proliferate were recovered from individual wells.
- [0785] Flow cytometry was used for confirming B7-H3 expression in each recovered clone. Specifically, each recovered clone was washed twice with PBS containing 5% FBS, thereafter suspended by the addition of PBS containing 5% FBS and 10 μ g/mL M30, and left standing at 4°C for 30 minutes. The clone was washed twice with PBS containing 5% FBS, thereafter suspended by the addition of Fluorescein-conjugated goat IgG fraction to mouse IgG (Whole Molecule) (#55493, manufactured by ICN Pharmaceuticals, Inc.) diluted 1000-fold with PBS containing 5% FBS, and left standing at 4°C for 30 minutes. The clone was washed twice with PBS containing 5% FBS, thereafter resuspended in PBS containing 5% FBS, and detected by using a flow cytometer (FC500: Beckman Coulter, Inc.).
- [0786] The CCRF-CEM cells stably expressing the B7-H3 variant 1 gene thus obtained by these procedures were designated as CEM_V1_3.1_2 cells. The parent line CCRF-CEM cells were used as a cell line lacking B7-H3 expression.

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(Test Example 3) Cell growth inhibition assay (1) of antibody-drug conjugate

[0787] The CEM_V1_3.1_2 cells produced in Test Example 2 or CCRF-CEM cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The CEM_V1_3.1_2 cells or CCRF-CEM cells were prepared to have a concentration of 8×10^4 cells/mL by using a medium, added at a concentration of 25 μ L/well to a 96-well microplate for cell culture charged with 65 μ L/well of a medium, and cultured overnight. On the next day, the M30-H1-L4 antibody, M30-H1-L4P antibody, and antibody-drug conjugate each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO₂ at 37°C for 3 days. After the culture, the microplate was taken out of the incubator and left standing at room temperature for 30 minutes.

[0788] The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC₅₀ value was calculated according to the following equation:

$$\text{IC}_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

- a: Concentration a of the test substance
- b: Concentration b of the test substance
- c: Ratio of live cells supplemented with the test substance having the concentration a
- d: Ratio of live cells supplemented with the test substance having the concentration b

[0789] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0790] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

- a: Average amount of light emission from the test substance-supplemented wells (n = 2)
- b: Average amount of light emission from the test substance non-supplemented wells (n = 10)

[0791] The antibody-drug conjugates (6) and (23) exhibited an anticellular effect of IC₅₀ < 0.1 (nM) against the CEM_V1_3.1_2 cells. The antibody-drug conjugates (3), (33), and (39) exhibited an anticellular effect of $0.1 < \text{IC}_{50} < 1$ (nM) against the cells. The antibody-drug conjugates (13), (14), (15), (16), (17), (20), (30), (35), and (37) exhibited an anticellular effect of $1 < \text{IC}_{50} < 100$ (nM) against the cells. On the other hand, none of the antibody-drug conjugates exhibited an anticellular effect against the CCRF-CEM cells (> 100 (nM)). Neither of the M30-H1-L4 antibody nor the M30-H1-L4P antibody exhibited a cytotoxic activity against both of the cells (> 100 (nM)).

(Test Example 4) Cell growth inhibition assay (2) of antibody-drug conjugate

[0792] Antigen-positive cells SR cells (ATCC) or antigen-negative cells Daudi cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The SR cells or Daudi cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium and added at a concentration of 90 μ L/well to a 96-well microplate for cell culture. Two hours later, the anti-CD30 antibody and antibody-drug conjugates (7), (8), (24), and (25) each diluted into 40 nM, 8 nM, 1.6 nM, 320 pM, 64 pM, 12.8 pM, and 2.6 pM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO₂ at 37°C for 3 days. After the culture, the microplate was taken out of the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC₅₀ value was calculated according to the following equation:

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$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

5

- a: Concentration a of the test substance
 b: Concentration b of the test substance
 c: Ratio of live cells supplemented with the test substance having the concentration a
 d: Ratio of live cells supplemented with the test substance having the concentration b

10

[0793] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0794] The survival rate of cells at each concentration was calculated according to the following equation:

15

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

- a: Average amount of light emission from the test substance-supplemented wells (n = 2)
 b: Average amount of light emission from the test substance non-supplemented wells (n = 12)

20

[0795] The antibody-drug conjugates (7), (8), (24), and (25) exhibited an anticellular effect of $IC_{50} < 0.01$ (nM) against the SR cells. On the other hand, none of the antibody-drug conjugates exhibited an anticellular effect against the Daudi cells (> 4.0 (nM)). The anti-CD30 antibody exhibited no anticellular effect against both of the cells (> 4.0 (nM)).

(Test Example 5) Cell growth inhibition assay (3) of antibody-drug conjugate

25

[0796] Antigen-positive cells HL-60 cells (ATCC) or antigen-negative cells Raji cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). The HL-60 cells or Raji cells were prepared to have a concentration of 8×10^4 cells/mL by using a medium and added at a concentration of 25 μ L/well to a 96-well microplate for cell culture containing 65 μ L/well of medium. The anti-CD33 antibody and antibody-drug conjugates (9), (10), (26), and (27) each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO_2 at 37°C for 3 days. After the culture, the microplate was taken out of the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

40

$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

45

- a: Concentration a of the test substance
 b: Concentration b of the test substance
 c: Ratio of live cells supplemented with the test substance having the concentration a
 d: Ratio of live cells supplemented with the test substance having the concentration b

[0797] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

50

[0798] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

55

- a: Average amount of light emission from the test substance-supplemented wells (n = 2)
 b: Average amount of light emission from the test substance non-supplemented wells (n = 5)

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[0799] The antibody-drug conjugate (10) exhibited a cytotoxic effect of $IC_{50} < 1$ (nM) against the HL-60 cells. The antibody-drug conjugates (9), (26), and (27) exhibited an anticellular effect of $1 < IC_{50} < 100$ (nM). On the other hand, all of the antibody-drug conjugates exhibited no anticellular effect against the Raji cells (> 100 (nM)). The anti-CD33 antibody exhibited no anticellular effect against both of the cells (> 100 (nM)).

(Test Example 6) Cytotoxicity test (4) of antibody-drug conjugate

[0800] Antigen-positive cells U251 cells (ATCC) or antigen-negative cells MCF-7 cells (ATCC) were cultured in RPMI1640 (GIBCO) containing 10% fetal bovine serum (MOREGATE) (hereinafter, referred to as a medium). U251 cells and MCF-7 cells were prepared to have a concentration of 2.8×10^4 cells/mL by using a medium and added at a concentration of 90 μ L/well to a 96-well microplate for cell culture, and cultured overnight. On the next day, the anti-CD70 antibody and antibody-drug conjugates (11), (12), (28), and (29) each diluted into 1000 nM, 200 nM, 40 nM, 8 nM, 1.6 nM, 0.32 nM, and 0.064 nM by using a medium were added at a concentration of 10 μ L/well to the microplate. A medium was added at a concentration of 10 μ L/well to test substance non-supplemented wells. The cells were cultured under 5% CO_2 at 37°C for 6 days. After the culture, the microplate was taken out of the incubator and left standing at room temperature for 30 minutes. The culture solution was charged with an equal amount of CellTiter-Glo Luminescent Cell Viability Assay (Promega) and stirred. After the microplate was left standing at room temperature for 10 minutes, the amount of light emission was measured by using a plate reader (PerkinElmer). The IC_{50} value was calculated according to the following equation:

$$IC_{50} \text{ (nM)} = \text{antilog}((50 - d) \times (\text{LOG}_{10}b - \text{LOG}_{10}a) / (d - c) + \text{LOG}_{10}b)$$

a: Concentration a of the test substance

b: Concentration b of the test substance

c: Ratio of live cells supplemented with the test substance having the concentration a

d: Ratio of live cells supplemented with the test substance having the concentration b

[0801] The concentrations a and b establish the relation $a > b$ crossing 50% ratio of live cells.

[0802] The survival rate of cells at each concentration was calculated according to the following equation:

$$\text{Survival rate of cells (\%)} = a / b \times 100$$

a: Average amount of light emission from the test substance-supplemented wells (n = 2)

b: Average amount of light emission from the test substance non-supplemented wells (n = 12)

[0803] The antibody-drug conjugate (12) and (29) exhibited a cytotoxic effect of $1 < IC_{50} < 10$ (nM) against the U251 cells. The antibody-drug conjugates (11) and (28) exhibited a cytotoxic effect of $10 < IC_{50} < 100$ (nM). On the other hand, all of the antibody-drug conjugates exhibited no cytotoxic effect against the MCF-7 cells (≥ 90 (nM)). The anti-CD70 antibody exhibited no cytotoxic effect against both of the cells (> 100 (nM)).

(Test Example 7) Antitumor test (1)

[0804] Mouse: 5- to 6-week-old female BALB/c nude mice (Charles River Laboratories Japan, Inc.) were acclimatized for 4 to 7 days under SPF conditions before use in the experiment. The mice were fed with sterilized solid feed (FR-2, Funabashi Farms Co., Ltd) and given sterilized tap water (prepared by the addition of 5 to 15 ppm sodium hypochlorite solution).

[0805] Assay and calculation expression: In all studies, the major axis and minor axis of tumor were measured twice a week by using an electronic digital caliper (CD-15C, Mitutoyo Corp.), and the tumor volume (mm^3) was calculated. The calculation expression is as shown below.

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$$\text{Tumor volume (mm}^3\text{)} = 1/2 \times \text{Major axis (mm)} \times [\text{Minor axis (mm)}]^2$$

5

[0806] All of the antibody-drug conjugates were diluted with physiological saline (Otsuka Pharmaceutical Factory, Inc.) and used at a volume of 10 mL/kg for intravenous administration to the tail of each mouse. Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 8×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 11. The M30-H1-L4P antibody and antibody-drug conjugate (1), (2), (18), and (19) were each intravenously administered at a dose of 10 mg/kg to the tail of each mouse at Days 11, 18, and 25 in a schedule of $qw \times 3$.

10

[0807] The results are shown in Figure 17. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with filled rhombuses depicts the effect of the M30-H1-L4P antibody, the line with filled squares depicts the effect of the administered antibody-drug conjugate (1), the line with open squares depicts the effect of the administered antibody-drug conjugate (2), the line with filled triangles depicts the effect of the administered antibody-drug conjugate (18), and the line with open triangles depicts the effect of the administered antibody-drug conjugate (19).

15

[0808] The administration of the antibody-drug conjugate (1), (2), (18), or (19) remarkably decreased the tumor volume. Particularly, as a result of the administration of the antibody-drug conjugate (2) or (19), the tumor completely regressed by Day 18 and was not confirmed to recur even after Day 39.

20

[0809] In addition, the mice that received the antibody-drug conjugate (1), (2), (18), or (19) were free from notable signs such as weight loss, suggesting that these antibody-drug conjugates are low toxic and highly safe.

(Test Example 8) Antitumor test (2)

25

[0810] Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 6×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 18. The antibody-drug conjugate (2) and (19) were each intravenously administered at a dose of 1.3 mg/kg to the tail of each mouse at Days 18, 25, and 32 in a schedule of $qw \times 3$.

30

[0811] The results are shown in Figure 18. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with filled squares depicts the effect of the antibody-drug conjugate (2) administered at 1 mg/kg, the line with open squares depicts the effect of the antibody-drug conjugate (2) administered at 3 mg/kg, the line with filled circles depicts the effect of the antibody-drug conjugate (19) administered at 1 mg/kg, and the line with open circles depicts the effect of the antibody-drug conjugate (19) administered at 3 mg/kg. The antibody-drug conjugates (2) and (19) exhibited a tumor growth inhibitory effect in a dose-dependent manner.

35

(Test Example 9) Antitumor test (3)

40

[0812] Human non-small cell lung cancer line Calu-6 cells were purchased from ATCC (American Type Culture Collection). 5×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 11. The M30-H1-L4P antibody and antibody-drug conjugate (1), (2), (18), or (19) were each intravenously administered at a dose of 10 mg/kg to the tail of each mouse at Days 11, 18, and 25 in a schedule of $qw \times 3$.

45

[0813] The results are shown in Figure 19. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the line with filled rhombuses depicts the effect of the M30-H1-L4P antibody, the line with filled squares depicts the effect of the administered antibody-drug conjugate (1), the line with open squares depicts the effect of the administered antibody-drug conjugate (2), the line with filled triangles depicts the effect of the administered antibody-drug conjugate (18), and the line with open triangles depicts the effect of the administered antibody-drug conjugate (19).

[0814] The administration of the antibody-drug conjugate (1), (2), (18), or (19) remarkably decreased the tumor volume, and no further tumor growth was observed after the final administration.

50

[0815] In addition, the mice that received the antibody-drug conjugate (1), (2), (18), or (19) were free from notable signs such as weight loss, suggesting that these antibody-drug conjugates are low toxic and highly safe.

(Test Example 10) Antitumor test (4)

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[0816] Human melanoma line A375 cells were purchased from ATCC (American Type Culture Collection). 8×10^6 cells suspended in physiological saline were subcutaneously transplanted to the right abdomen of each female nude mouse (Day 0), and the mice were randomly grouped at Day 14. The antibody-drug conjugates (3), (20), and (30) were each intravenously administered at each dose (3 and 10 mg/kg) to the tail of each mouse at Day 14 in a schedule of $qd \times 1$.

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[0817] The results are shown in Figure 20. In the drawing, the line with open rhombuses depicts the results about untreated tumor, the dotted line with filled squares depicts the effect of the antibody-drug conjugate (3) administered at 3 mg/kg, the solid line with filled squares depicts the effect of the antibody-drug conjugate (3) administered at 10 mg/kg, the dotted line with filled triangles depicts the effect of the antibody-drug conjugate (20) administered at 3 mg/kg, the solid line with filled triangles depicts the effect of the antibody-drug conjugate (20) administered at 10 mg/kg, the dotted line with filled circles depicts the effect of the antibody-drug conjugate (30) administered at 3 mg/kg, and the solid line with filled circles depicts the effect of the antibody-drug conjugate (30) administered at 10 mg/kg.

[0818] The administration of the antibody-drug conjugate (3), (20), or (30) remarkably decreased the tumor volume, and all of these antibody-drug conjugates exerted a tumor growth inhibitory effect in a dose-dependent manner.

[0819] In addition, the mice that received the antibody-drug conjugate (3), (20), or (30) were free from notable signs such as weight loss, suggesting that these antibody-drug conjugates are low toxic and highly safe.

Free Text of Sequence Listing

[0820]

SEQ ID NO: 1 - Amino acid sequence of the B7-H3 variant 1
 SEQ ID NO: 2 - Amino acid sequence of the B7-H3 variant 2
 SEQ ID NO: 3 - Amino acid sequence of CDRH1 of the M30 antibody
 SEQ ID NO: 4 - Amino acid sequence of CDRH2 of the M30 antibody
 SEQ ID NO: 5 - Amino acid sequence of CDRH3 of the M30 antibody
 SEQ ID NO: 6 - Amino acid sequence of CDRL1 of the M30 antibody
 SEQ ID NO: 7 - Amino acid sequence of CDRL2 of the M30 antibody
 SEQ ID NO: 8 - Amino acid sequence of CDRL3 of the M30 antibody
 SEQ ID NO: 9 - Amino acid sequence of the M30-H1-type heavy chain
 SEQ ID NO: 10 - Amino acid sequence of the M30-H2-type heavy chain
 SEQ ID NO: 11 - Amino acid sequence of the M30-H3-type heavy chain
 SEQ ID NO: 12 - Amino acid sequence of the M30-H4-type heavy chain
 SEQ ID NO: 13 - Amino acid sequence of the M30-L1-type light chain
 SEQ ID NO: 14 - Amino acid sequence of the M30-L2-type light chain
 SEQ ID NO: 15 - Amino acid sequence of the M30-L3-type light chain
 SEQ ID NO: 16 - Amino acid sequence of the M30-L4-type light chain
 SEQ ID NO: 17 - Amino acid sequence of the M30-L5-type light chain
 SEQ ID NO: 18 - Amino acid sequence of the M30-L6-type light chain
 SEQ ID NO: 19 - Amino acid sequence of the M30-L7-type light chain
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 SEQ ID NO: 21 - Amino acid sequence of a light chain of the M30 antibody
 SEQ ID NO: 22 - PCR primer 1
 SEQ ID NO: 23 - PCR primer2
 SEQ ID NO: 24 - CMV promoter primer: primer 3
 SEQ ID NO: 25 - BGH reverse primer: primer 4
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 SEQ ID NO: 27 - Amino acid sequence of a heavy chain of the anti-CD30 antibody
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 SEQ ID NO: 31 - Amino acid sequence of a heavy chain of the anti-CD70 antibody
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SEQUENCE LISTING

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30	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
				340					345					350			
	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
35			355					360					365				
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	
		370					375					380					
40	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	
	385					390					395					400	
	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	
45					405					410					415		
	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	
50					420				425					430			
	Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	
			435					440					445				
55	Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	
	450						455					460					

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Leu Ser Leu Ser Pro Gly Lys
 465 470

5 <210> 10
 <211> 471
 <212> PRT
 <213> Artificial Sequence

10 <220>
 <223> Humanized heavy chain of M30
 <400> 10

15 Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
 1 5 10 15

20 Val Leu Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
 20 25 30

25 Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
 35 40 45

30 Thr Asn Tyr Val Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
 50 55 60

35 Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Asp Val Lys Tyr Asn
 65 70 75 80

40 Glu Lys Phe Lys Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser
 85 90 95

45 Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
 100 105 110

50 Tyr Tyr Cys Ala Arg Trp Gly Tyr Tyr Gly Ser Pro Leu Tyr Tyr Phe
 115 120 125

55 Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
 130 135 140

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
 145 150 155 160

Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu
 165 170 175

Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His
 180 185 190

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	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	
			195					200					205				
5	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	
		210					215					220					
	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Arg	Val	Glu	
10	225					230					235					240	
	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	
					245					250					255		
15	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	
				260					265					270			
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	
20			275					280					285				
	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	
		290					295					300					
25																	
	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
	305					310					315					320	
30																	
	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
					325					330					335		
35	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
				340					345					350			
	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
			355					360					365				
40																	
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	
		370					375					380					
45	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	
	385					390					395					400	
	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	
50					405					410					415		
	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	
					420				425					430			
55																	
	Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	
				435				440					445				

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Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
 450 455 460

5 Leu Ser Leu Ser Pro Gly Lys
 465 470

10 <210> 11
 <211> 471
 <212> PRT
 <213> Artificial Sequence

15 <220>
 <223> Humanized heavy chain of M30

<400> 11

20 Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
 1 5 10 15

Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
 20 25 30

25 Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
 35 40 45

30 Thr Asn Tyr Val Met His Trp Val Lys Gln Ala Pro Gly Gln Gly Leu
 50 55 60

35 Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Asp Val Lys Tyr Asn
 65 70 75 80

Glu Lys Phe Lys Gly Lys Ala Thr Ile Thr Ala Asp Glu Ser Thr Ser
 85 90 95

40 Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
 100 105 110

45 Tyr Tyr Cys Ala Arg Trp Gly Tyr Tyr Gly Ser Pro Leu Tyr Tyr Phe
 115 120 125

50 Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
 130 135 140

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
 145 150 155 160

55 Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu
 165 170 175

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	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	
				180					185					190			
5	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	
			195					200					205				
	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	
10		210					215					220					
	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Arg	Val	Glu	
	225					230					235					240	
15	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	
					245					250					255		
	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	
20				260					265					270			
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	
			275					280					285				
25	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	
		290					295					300					
	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
30		305				310					315					320	
	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
35					325					330					335		
	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
				340					345					350			
40	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
			355					360					365				
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	
45			370				375					380					
	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	
	385					390					395					400	
50	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	
				405						410					415		
55	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	
				420					425					430			

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Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
 435 440 445

5 Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
 450 455 460

10 Leu Ser Leu Ser Pro Gly Lys
 465 470

15 <210> 12
 <211> 471
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Humanized heavy chain of M30

20 <400> 12

Met Lys His Leu Trp Phe Phe Leu Leu Leu Val Ala Ala Pro Arg Trp
 1 5 10 15

25 Val Leu Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
 20 25 30

30 Pro Gly Ser Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
 35 40 45

35 Thr Asn Tyr Val Met His Trp Val Lys Gln Ala Pro Gly Gln Gly Leu
 50 55 60

Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Asp Val Lys Tyr Asn
 65 70 75 80

40 Glu Lys Phe Lys Gly Lys Ala Thr Gln Thr Ser Asp Lys Ser Thr Ser
 85 90 95

45 Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
 100 105 110

Tyr Tyr Cys Ala Arg Trp Gly Tyr Tyr Gly Ser Pro Leu Tyr Tyr Phe
 115 120 125

50 Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
 130 135 140

55 Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
 145 150 155 160

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	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	
					165					170					175		
5	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	
				180					185					190			
	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	
10			195				200						205				
	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	
		210					215					220					
15	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Arg	Val	Glu	
	225					230					235					240	
	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	
20					245					250					255		
	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	
25				260					265					270			
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	
			275				280						285				
30	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	
		290					295					300					
	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
35		305				310					315					320	
	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
					325					330					335		
40	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
				340					345					350			
	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
45			355					360					365				
	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	
50			370				375					380					
	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	
		385				390					395					400	
55	Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	
					405					410					415		

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Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
 420 425 430

5 Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
 435 440 445

10 Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
 450 455 460

Leu Ser Leu Ser Pro Gly Lys
 465 470

15 <210> 13
 <211> 233
 <212> PRT
 <213> Artificial Sequence

20 <220>
 <223> Humanized light chain of M30

25 <400> 13

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15

30 Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 35 40 45

Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 55 60

40 Leu Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80

45 Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg
 85 90 95

Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

50 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125

55 Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140

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Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160

5 Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175

10 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190

Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205

15 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220

20 Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230

<210> 14
 <211> 233
 <212> PRT
 25 <213> Artificial Sequence

<220>
 <223> Humanized light chain of M30

30 <400> 14

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15

35 Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 20 25 30

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 35 40 45

40 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 55 60

45 Leu Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80

50 Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Arg
 85 90 95

Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

55 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr

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115
120
125
Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
130 135 140
5
Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
145 150 155 160
10
Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
165 170 175
15
Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
180 185 190
20
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
195 200 205
25
Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
210 215 220
30
Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230
<210> 15
<211> 233
<212> PRT
<213> Artificial Sequence
<220>
<223> Humanized light chain of M30
35
<400> 15
Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
1 5 10 15
40
Gly Ala Tyr Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Thr Leu Ser
20 25 30
45
Leu Ser Pro Gly Glu Arg Ala Thr Leu Thr Cys Arg Ala Ser Ser Arg
35 40 45
50
Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ala Pro Lys
50 55 60
55
Leu Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
65 70 75 80
60
Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Thr Leu Thr Ile Ser Arg
85 90 95

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Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

5 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125

10 Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140

15 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160

Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175

20 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190

25 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205

30 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220

35 Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230

40 <210> 16
 <211> 233
 <212> PRT
 <213> Artificial Sequence

45 <220>
 <223> Humanized light chain of M30
 <400> 16

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15

Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
 20 25 30

50 Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
 35 40 45

55 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
 50 55 60

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Pro Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80

5 Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser
 85 90 95

10 Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110

15 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125

Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140

20 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160

25 Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175

30 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190

35 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205

40 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220

Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230

<210> 17
 <211> 233
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Humanized light chain of M30

50 <400> 17

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
 1 5 10 15

55 Gly Ala Tyr Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Thr Leu Ser
 20 25 30

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Leu Ser Pro Gly Glu Arg Ala Thr Leu Thr Cys Arg Ala Ser Ser Arg
 35 40 45
 5 Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ala Pro Lys
 50 55 60
 Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
 65 70 75 80
 10 Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Thr Leu Thr Ile Ser Arg
 85 90 95
 15 Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
 100 105 110
 Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
 115 120 125
 20 Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
 130 135 140
 25 Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
 145 150 155 160
 30 Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
 165 170 175
 Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
 180 185 190
 35 Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
 195 200 205
 40 Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
 210 215 220
 45 Thr Lys Ser Phe Asn Arg Gly Glu Cys
 225 230
 <210> 18
 <211> 233
 <212> PRT
 50 <213> Artificial Sequence
 <220>
 <223> Humanized light chain of M30
 55 <400> 18
 Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser

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1          5          10          15
5      Gly Ala Tyr  Gly Glu Ile Val  Leu Thr Gln Ser Pro Ala Thr Leu Ser
      20          25          30
10     Leu Ser Pro  Gly Glu Arg Ala  Thr Leu Ser Cys Arg Ala Ser Ser Arg
      35          40          45
15     Leu Ile Tyr  Met His Trp Tyr  Gln Gln Lys Pro Gly Gln Ala Pro Arg
      50          55          60
20     Pro Leu Ile Tyr Ala  Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
      65          70          75          80
25     Phe Ser Gly  Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg
      85          90          95
30     Leu Glu Pro  Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
      100          105          110
35     Asn Pro Pro  Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
      115          120          125
40     Val Ala Ala  Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
      130          135          140
45     Lys Ser Gly  Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
      145          150          155          160
50     Arg Glu Ala  Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
      165          170          175
55     Asn Ser Gln  Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
      180          185          190
60     Ser Leu Ser  Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
      195          200          205
65     Lys Val Tyr  Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
      210          215          220
70     Thr Lys Ser  Phe Asn Arg Gly Glu Cys
      225          230
75     <210> 19
      <211> 233
      <212> PRT
      <213> Artificial Sequence

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<220>

<223> Humanized light chain of M30

<400> 19

5

Met Val Leu Gln Thr Gln Val Phe Ile Ser Leu Leu Leu Trp Ile Ser
1 5 10 15

10

Gly Ala Tyr Gly Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser
20 25 30

15

Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Ser Arg
35 40 45

Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
50 55 60

20

Pro Leu Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Ile Pro Ala Arg
65 70 75 80

25

Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Arg
85 90 95

Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Trp Asn Ser
100 105 110

30

Asn Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
115 120 125

35

Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
130 135 140

40

Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
145 150 155 160

Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
165 170 175

45

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
180 185 190

50

Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
195 200 205

Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
210 215 220

55

Thr Lys Ser Phe Asn Arg Gly Glu Cys

225

230

5

10

15

20

25

30

35

40

45

50

55

Val Thr Val Thr Ser Ser Thr Trp Pro Ser Gln Ser Ile Thr Cys Asn

210 215 220

170

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Phe Ser Arg Thr Pro Gly Lys
 465 470

5 <210> 21
 <211> 235
 <212> PRT
 <213> Mus musculus

10 <400> 21

Met Asp Phe Leu Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
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20 Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala Ser
 35 40 45

Ser Arg Leu Ile Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser Ser
 50 55 60

25 Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro
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30 Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile
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35 Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp
 100 105 110

Asn Ser Asn Pro Pro Thr Phe Gly Thr Gly Thr Lys Leu Glu Leu Lys
 115 120 125

40 Arg Ala Asp Ala Ala Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu
 130 135 140

45 Gln Leu Thr Ser Gly Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe
 145 150 155 160

Tyr Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg
 165 170 175

50 Gln Asn Gly Val Leu Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser
 180 185 190

55 Thr Tyr Ser Met Ser Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu
 195 200 205

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 20 Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
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 50 55 60
 25 Glu Trp Ile Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn
 65 70 75 80
 30 Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser
 85 90 95
 Thr Ala Phe Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
 100 105 110
 35 Tyr Phe Cys Ala Asn Tyr Gly Asn Tyr Trp Phe Ala Tyr Trp Gly Gln
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 40 Gly Thr Gln Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val
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 45 Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
 145 150 155 160
 Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
 165 170 175
 50 Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
 180 185 190
 55 Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
 195 200 205

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	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly
10					245					250					255	
	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile
				260					265					270		
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	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His
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	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg
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	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys
					325					330					335	
30	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu
				340					345					350		
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	Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val	Ser	Leu
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	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val
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	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp
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Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Lys Ala Ser Gln Ser
35 40 45

25 Val Asp Phe Asp Gly Asp Ser Tyr Met Asn Trp Tyr Gln Gln Lys Pro
50 55 60

30 Gly Gln Pro Pro Lys Val Leu Ile Tyr Ala Ala Ser Asn Leu Glu Ser
65 70 75 80

Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
35 85 90 95

Leu Asn Ile His Pro Val Glu Glu Glu Asp Ala Ala Thr Tyr Tyr Cys
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40 Gln Gln Ser Asn Gln Asp Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu
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45 Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
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55 Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
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 5 Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly
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 35 Thr Asp Ser Asn Ile His Trp Val Arg Gln Ala Pro Gly Gln Ser Leu
 50 55 60
 40 Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Gly Gly Thr Asp Tyr Asn
 65 70 75 80
 45 Gln Lys Phe Lys Asn Arg Ala Thr Leu Thr Val Asp Asn Pro Thr Asn
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 50 Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Phe
 100 105 110
 55 Tyr Tyr Cys Val Asn Gly Asn Pro Trp Leu Ala Tyr Trp Gly Gln Gly
 115 120 125
 60 Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
 130 135 140
 65 Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu
 145 150 155 160
 70 Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp

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	165	170	175
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10	Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser 195 200 205		
15	Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro 210 215 220		
20	Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys Ser Cys Asp Lys 225 230 235 240		
25	Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro 245 250 255		
30	Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser 260 265 270		
35	Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp 275 280 285		
40	Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn 290 295 300		
45	Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val 305 310 315 320		
50	Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu 325 330 335		
55	Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys 340 345 350		
	Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr 355 360 365		
	Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr 370 375 380		
	Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu 385 390 395 400		
	Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu 405 410 415		

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Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
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5 Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
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Lys
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30 Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser
 35 40 45

35 Leu Asp Asn Tyr Gly Ile Arg Phe Leu Thr Trp Phe Gln Gln Lys Pro
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40 Gly Lys Ala Pro Lys Leu Leu Met Tyr Ala Ala Ser Asn Gln Gly Ser
 65 70 75 80

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr
 85 90 95

45 Leu Thr Ile Ser Ser Leu Gln Pro Asp Asp Phe Ala Thr Tyr Tyr Cys
 100 105 110

50 Gln Gln Thr Lys Glu Val Pro Trp Ser Phe Gly Gln Gly Thr Lys Val
 115 120 125

Glu Val Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
 130 135 140

55 Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu

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10	Ala	Leu	Gln	Ser	Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln	Asp	Ser
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15	Lys	Asp	Ser	Thr	Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	Ala
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45	Val	Leu	Ser	Gln	Val	Gln	Leu	Val	Gln	Ser	Gly	Ala	Glu	Val	Lys	Lys
				20					25					30		
50	Pro	Gly	Ala	Ser	Val	Lys	Val	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Thr	Phe
			35				40						45			
55	Thr	Asn	Tyr	Gly	Met	Asn	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu
		50				55						60				
60	Lys	Trp	Met	Gly	Trp	Ile	Asn	Thr	Tyr	Thr	Gly	Glu	Pro	Thr	Tyr	Ala
	65					70					75					80
65	Asp	Ala	Phe	Lys	Gly	Arg	Val	Thr	Met	Thr	Arg	Asp	Thr	Ser	Ile	Ser
				85						90					95	
70	Thr	Ala	Tyr	Met	Glu	Leu	Ser	Arg	Leu	Arg	Ser	Asp	Asp	Thr	Ala	Val
				100					105					110		
75	Tyr	Tyr	Cys	Ala	Arg	Asp	Tyr	Gly	Asp	Tyr	Gly	Met	Asp	Tyr	Trp	Gly
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5	Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala	145	150	155
10	Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val	165	170	175
15	Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala	180	185	190
20	Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val	195	200	205
25	Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His	210	215	220
30	Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys Ser Cys	225	230	235
35	Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly	245	250	255
40	Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met	260	265	270
45	Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His	275	280	285
50	Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val	290	295	300
55	His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr	305	310	315
60	Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly	325	330	335
65	Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile	340	345	350
70	Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val	355	360	365
75	Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser			

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	370		375		380	
5	Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu					
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	Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro					
		405		410		415
10	Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val					
		420		425		430
15	Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met					
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20	His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser					
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	Pro Gly Lys					
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	Gly Ala Tyr Gly Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala					
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40	Val Ser Leu Gly Glu Arg Ala Thr Ile Asn Cys Arg Ala Ser Lys Ser					
		35		40		45
45	Val Ser Thr Ser Gly Tyr Ser Phe Met His Trp Tyr Gln Gln Lys Pro					
		50		55		60
50	Gly Gln Pro Pro Lys Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu Ser					
		65		70		75
	Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr					
		85		90		95
55	Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys					
		100		105		110

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Gln His Ser Arg Glu Val Pro Trp Thr Phe Gly Gln Gly Thr Lys Val
115 120 125

5 Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
130 135 140

10 Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu
145 150 155 160

15 Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn
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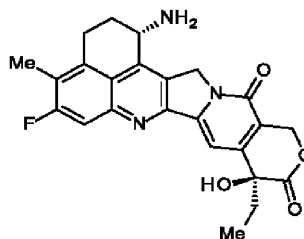
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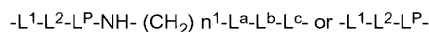
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Claims

1. An antibody-drug conjugate wherein an antitumor compound represented by the following formula:



is conjugated to an antibody via a linker having a structure represented by the following formula:



wherein the antibody is connected to the terminal of L^1 , the antitumor compound is connected to the terminal of L^c or L^P ,

wherein

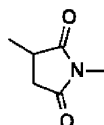
n^1 represents an integer of 0 to 6,

L^1 represents $-(\text{Succinimid-3-yl-N})-(CH_2)_{n^2}-C(=O)-$, $-(\text{Succinimid-3-yl-N})-CH[-(CH_2)_{n^3}-COOH]-C(=O)-$, $-CH_2-C(=O)-NH-(CH_2)_{n^4}-C(=O)-$, $-C(=O)-\text{cyc.Hex}(1,4)-CH_2-(N\text{-ly-3-dimethyl})$ or $-C(=O)-(CH_2)_{n^5}-C(=O)-$,

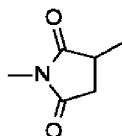
wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, n^5

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represents an integer of 1 to 8,
 L^2 represents $-\text{NH}-(\text{CH}_2\text{-CH}_2\text{-O})_{n^6}\text{-CH}_2\text{-CH}_2\text{-C(=O)-}$, $-\text{N}[(\text{CH}_2\text{CH}_2\text{-O})_{n^7}\text{-CH}_2\text{CH}_2\text{-OH}]\text{-CH}_2\text{-C(=O)-}$,
 $-\text{S}-(\text{CH}_2)_{n^8}\text{-C(=O)-}$, or a single bond,
 wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4, n^8 represents an integer of 1 to 6,
 L^P represents a peptide residue consisting of 3 to 8 amino acids, L^a represents $-\text{C(=O)-NH-}$, $-\text{NR}^1\text{-(CH}_2)_{n^9}\text{-}$, $-\text{O-}$,
 or a single bond, wherein n^9 represents an integer of 1 to 6, R^1 represents a hydrogen atom, an alkyl group having
 1 to 6 carbon atoms, $-(\text{CH}_2)_{n^a}\text{-COOH}$, or $-(\text{CH}_2)_{n^b}\text{-OH}$, n^a represents an integer of 1 to 4, n^b represents an integer
 of 1 to 6, L^b represents $-\text{CR}^2\text{(-R}^3)\text{-}$, $-\text{O-}$, $-\text{NR}^4\text{-}$, or a single bond,
 wherein R^2 and R^3 each independently represent a hydrogen atom, an alkyl group having 1 to 6 carbon atoms,
 $-(\text{CH}_2)_{n^c}\text{-NH}_2$, $-(\text{CH}_2)_{n^d}\text{-COOH}$, or $-(\text{CH}_2)_{n^e}\text{-OH}$, R^4 represents a hydrogen atom or an alkyl group having 1 to 6
 carbon atoms, n^c represents an integer of 0 to 6, n^d represents an integer of 1 to 4, n^e represents an integer of 1 to
 4, provided that when n^c is 0, R^2 and R^3 are not the same as each other, L^c represents $-\text{CH}_2\text{-}$ or $-\text{C(=O)-}$,
 $-(\text{Succinimid-3-yl-N})\text{-}$ has a structure represented by the following formula:



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure
 containing this structure on the nitrogen atom at position 1,
 $-(\text{N-ly-3-diminnicuS})\text{-}$ has a structure represented by the following formula:



which is connected to L^2 at position 3 thereof and is connected to a methylene group in the linker structure containing
 this structure on the nitrogen atom at position 1,
 cyc.Hex(1,4) represents a 1,4-cyclohexylene group,
 when L^2 is $-\text{S}-(\text{CH}_2)_{n^8}\text{-C(=O)-}$, L^1 is $-\text{C(=O)-cyc.Hex(1,4)-CH}_2\text{-}$ (N-ly-3-diminnicuS),
 provided that any one or two or more of linkers of L^1 , L^2 ,
 and L^P have a structure containing a hydrophilic structure, and
 said hydrophilic structure means,
 as for linker L^P , the case in which,
 L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal, or
 L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is
 connected to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal,
 no other hydrophilic amino acid than glycine is present thereat, or
 as for linker L^1 , the case in which L^1 is $-(\text{Succinimid-3-yl-N})\text{-CH}[(\text{CH}_2)_{n^3}\text{-COOH}]\text{-C(=O)-}$, or
 as for linker L^2 , the case in which L^2 is $-\text{N}[(\text{CH}_2\text{CH}_2\text{-O})_{n^7}\text{-CH}_2\text{CH}_2\text{-OH}]\text{-CH}_2\text{-C(=O)-}$.

2. The antibody-drug conjugate according to claim 1, wherein
 L^1 is $-(\text{Succinimid-3-yl-N})\text{-(CH}_2)_{n^2}\text{-C(=O)-}$, $-(\text{Succinimid-3-yl-N})\text{-CH}[(\text{CH}_2)_{n^3}\text{-COOH}]\text{-C(=O)-}$, or $-\text{CH}_2\text{-C(=O)-NH}-(\text{CH}_2)_{n^4}\text{-C(=O)-}$,
 wherein n^2 represents an integer of 2 to 8, n^3 represents an integer of 1 to 8, n^4 represents an integer of 1 to 8, L^2
 is $-\text{NH}-(\text{CH}_2\text{-CH}_2\text{-O})_{n^6}\text{-CH}_2\text{-CH}_2\text{-C(=O)-}$, $-\text{N}[(\text{CH}_2\text{CH}_2\text{-O})_{n^7}\text{-CH}_2\text{CH}_2\text{-OH}]\text{-CH}_2\text{-C(=O)-}$, or a single bond,
 wherein n^6 represents an integer of 0 to 6, n^7 represents an integer of 1 to 4,
 L^P is a peptide residue consisting of 3 to 8 amino acids,
 each of L^a and L^b is a single bond, and
 L^c is $-\text{C(=O)-}$.

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3. The antibody-drug conjugate according to claim 1 or 2, wherein any one of linkers of L^1 , L^2 , and L^P is the linker containing the hydrophilic structure.
- 5 4. The antibody-drug conjugate according to claim 3, wherein the linker containing the hydrophilic structure is L^P .
5. The antibody-drug conjugate according to claim 4, wherein L^P is a peptide residue having a hydrophilic amino acid other than glycine at the N terminal.
- 10 6. The antibody-drug conjugate according to claim 5, wherein the hydrophilic amino acid other than glycine is aspartic acid, glutamic acid, lysine, serine, threonine, glutamine, asparagine, histidine, tyrosine, or arginine.
7. The antibody-drug conjugate according to claim 5, wherein the N-terminal hydrophilic amino acid other than glycine in L^P is glutamic acid, aspartic acid, or lysine.
- 15 8. The antibody-drug conjugate according to claim 6 or 7, wherein the peptide residue following the N-terminal hydrophilic amino acid in L^P is an amino acid residue comprising an amino acid selected from phenylalanine, glycine, valine, lysine, citrulline, serine, glutamic acid, and aspartic acid.
- 20 9. The antibody-drug conjugate according to claim 8, wherein a peptide residue following the N-terminal hydrophilic amino acid in L^P is a peptide residue consisting of 3 or 4 amino acids.
10. The antibody-drug conjugate according to claim 9, wherein the peptide residue following the N-terminal hydrophilic amino acid in L^P is GGF or GGFG.
- 25 11. The antibody-drug conjugate according to any one of claims 5 to 10, wherein L^P is DGGF, KGGF, EGGF, DGGFG, KGGFG, or EGGFG.
12. The antibody-drug conjugate according to any one of claims 5 to 10, wherein L^P is DGGFG, KGGFG, or EGGFG.
- 30 13. The antibody-drug conjugate according to any one of claims 1 to 12, wherein the linker is a linker having a structure represented by $-L^1-L^2-L^P-NH-(CH_2)_{n^1}-L^a-L^b-L^c-$.
14. The antibody-drug conjugate according to claim 13, wherein L^c is $-C(=O)-$.
- 35 15. The antibody-drug conjugate according to claim 14, wherein L^1 is $-(Succinimid-3-yl-N)-(CH_2)_{n^2}-C(=O)-$, n^2 is an integer of 2 to 5, and L^2 is a single bond.
16. The antibody-drug conjugate according to claim 15, wherein n^2 is 5.
- 40 17. The antibody-drug conjugate according to claim 15 or 16, wherein n^1 is 1 to 3.
18. The antibody-drug conjugate according to any one of claims 5 to 17, wherein the structure of the $-NH-(CH_2)_{n^1}-L^a-L^b-L^c-$ moiety in the linker is

$-NH-CH_2-C(=O)-$,
 $-NH-(CH_2)_2-C(=O)-$,
 $-NH-(CH_2)_3-C(=O)-$,
 $-NH-CH_2-O-CH_2-C(=O)-$,

 or
 $-NH-(CH_2)_2-O-CH_2-C(=O)-$.
- 45 19. The antibody-drug conjugate according to any one of claims 5 to 17, wherein the structure of the

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-NH-(CH₂)^{n¹}-L^a-L^b-L^c- moiety in the linker is

-NH-CH₂-C(=O)-,

5 -NH-(CH₂)₂-C(=O)-,

or

10 -NH-(CH₂)₃-C(=O)-.

20. The antibody-drug conjugate according to any one of claims 1 to 12, wherein the linker is a linker having a structure represented by -L¹-L²-L^P-.
21. The antibody-drug conjugate according to claim 20, wherein L¹ is -(Succinimid-3-yl-N)-(CH₂)_{n²}-C(=O)-, n² is an integer of 2 to 5, and L² is a single bond.
22. The antibody-drug conjugate according to claim 21, wherein n² is 5.
23. The antibody-drug conjugate according to claim 4, wherein L^P is a peptide residue in which the C terminal is an oligopeptide consisting of 2 or 3 or more glycines and is connected to the antitumor compound, and even in case that a hydrophilic amino acid is present at N terminal, no other hydrophilic amino acid than glycine is present thereat.
24. The antibody-drug conjugate according to claim 23, wherein the peptide residue consists of 4 to 8 amino acids in the linker.
25. The antibody-drug conjugate according to claim 23, wherein the C-terminal glycine oligopeptide is an oligopeptide consisting of 2 or 3 glycines.
26. The antibody-drug conjugate according to any one of claims 23 to 25, wherein the peptide residue of the linker is GGFGG or GGFGGG.
27. The antibody-drug conjugate according to any one of claims 23 to 26, wherein L¹ is -(Succinimid-3-yl-N)-(CH₂)_{n²}-C(=O)- and n² is an integer of 2 to 5.
28. The antibody-drug conjugate according to claim 27, wherein n² is 5.
29. The antibody-drug conjugate according to claim 3, wherein L¹ is -(Succinimid-3-yl-N)-CH[-(CH₂)_{n³}-COOH]-C(=O)-.
30. The antibody-drug conjugate according to claim 29, wherein n³ is 2 or 3.
31. The antibody-drug conjugate according to claim 3, wherein L² is -N[-(CH₂CH₂-O)_{n⁷}-CH₂CH₂-OH]-CH₂-C(=O)-.
32. The antibody-drug conjugate according to claim 31, wherein n⁷ is 2 to 4.
33. The antibody-drug conjugate according to any one of claims 29 to 32, wherein the linker is a linker having a structure represented by -L¹-L²-L^P-NH-(CH₂)_{n¹}-L^a-L^b-L^c-.
34. The antibody-drug conjugate according to claim 33, wherein L^c is -C(=O)-.
35. The antibody-drug conjugate according to any one of claims 29 to 34, wherein n¹ is 1 to 3.
36. The antibody-drug conjugate according to any one of claims 29 to 35, wherein L^P is GGFG.
37. The antibody-drug conjugate according to any one of claims 29 to 36, wherein the structure of the

-NH-CH₂-C(=O)-,

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-NH-(CH₂)₂-C(=O)-,

-NH-(CH₂)₃-C(=O)-,

-NH-CH₂-O-CH₂-C(=O)-,

or

-NH-(CH₂)₂-O-CH₂-C(=O)-.

38. The antibody-drug conjugate according to any one of claims 29 to 36, wherein the structure of the -NH-(CH₂)_n¹-L^a-L^b-L^c- moiety in the linker is

-NH-CH₂-C(=O)-,

-NH-(CH₂)₂-C(=O)-,

or

-NH-(CH₂)₃-C(=O)-.

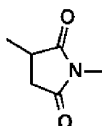
39. The antibody-drug conjugate according to claim 1 or 2, wherein the drug-linker structure moiety in the antibody-drug conjugate has one structure selected from the following drug-linker structure group:

- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),

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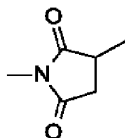
- C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂-O-CH₂-C(=O)-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-NH-CH₂CH₂-O-CH₂-C(=O)-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-KGGFG-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-KGGFG-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGF-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGF-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-DGGFG-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-KGGFG-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 - (Succinimid-3-yl-N)-CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 - CH₂-C(=O)-NH-CH₂CH₂-C(=O)-GGFGGG-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 - C(=O)-cyc.Hex(1,4)-CH₂-(N-ly-3-diminiccuS)-S-CH₂CH₂-C(=O)-GGFGGG-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGG-(NH-DX),
 - C(=O)-CH₂CH₂CH₂CH₂CH₂CH₂-C(=O)-GGFGGG-(NH-DX)

wherein -(Succinimid-3-yl-N)- has a structure represented by the following formula:



which is connected to the antibody at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, cyc.Hex(1,4) represents a 1,4-cyclohexylene group, -(N-ly-3-diminiccuS)- has a structure represented by the following formula:

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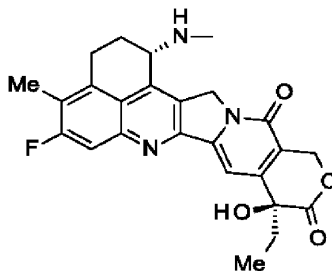
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which is connected to L² at position 3 thereof and is connected to a methylene group in the linker structure containing this structure on the nitrogen atom at position 1, and
 -(NH-DX) is a group represented by the following formula:

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wherein the nitrogen atom of the amino group at position 1 is the connecting position.

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40. The antibody-drug conjugate according to any one of claims 1 to 39, wherein an average number of conjugated antitumor compounds per antibody is in a range of from 1 to 10.

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41. The antibody-drug conjugate according to any one of claims 1 to 39, wherein an average number of conjugated antitumor compounds per antibody is in a range of from 1 to 8.

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42. The antibody-drug conjugate according to any one of claims 1 to 39, wherein an average number of conjugated antitumor compounds per antibody is in a range of from 3 to 8.

43. The antibody-drug conjugate according to any one of claims 1 to 42, wherein the antibody is an antibody having one or more of a property of recognizing a target cell, a property of binding to a target cell, a property of internalizing in a target cell, and a property of damaging a target cell.

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44. The antibody-drug conjugate according to claims 43, wherein the target cell is a tumor cell.

45. The antibody-drug conjugate according to any one of claims 1 to 44, wherein the antibody is an anti-A33 antibody, an anti-B7-H3 antibody, an anti-CanAg antibody, an anti-CD20 antibody, an anti-CD22 antibody, an anti-CD30 antibody, an anti-CD33 antibody, an anti-CD56 antibody, an anti-CD70 antibody, an anti-CEA antibody, an anti-Cripto antibody, an anti-EphA2 antibody, an anti-G250 antibody, an anti-MUC1 antibody, an anti-GPNMB antibody, an anti-integrin antibody, an anti-PSMA antibody, an anti-tenascin-C antibody, an anti-SLC44A4 antibody, or an anti-mesothelin antibody.

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46. The antibody-drug conjugate according to any one of claims 1 to 44, wherein the antibody is an anti-B7-H3 antibody, an anti-CD30 antibody, an anti-CD33 antibody, or an anti-CD70 antibody.

47. The antibody-drug conjugate according to any one of claims 1 to 44, wherein the antibody is an anti-B7-H3 antibody.

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48. A drug containing the antibody-drug conjugate according to any one of claims 1 to 44 or a salt thereof.

49. An antitumor drug and/or anticancer drug containing the antibody-drug conjugate according to any one of claims 1 to 44 or a salt thereof.

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50. The antitumor drug and/or anticancer drug according to claim 49, which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.
- 5 51. A pharmaceutical composition containing the antibody-drug conjugate according to any one of claims 1 to 44, a salt thereof or a hydrate thereof as an active component, and a pharmaceutically acceptable formulation component.
52. The pharmaceutical composition according to claim 51, which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.
- 10 53. A method for treating tumor and/or cancer comprising administering the antibody-drug conjugate according to any one of claims 1 to 44, a salt thereof or a hydrate thereof.
- 15 54. The treatment method according to claim 53, which is applied to lung cancer, kidney cancer, urothelial cancer, colorectal cancer, prostate cancer, glioblastoma multiforme, ovarian cancer, pancreatic cancer, breast cancer, melanoma, liver cancer, bladder cancer, stomach cancer, or esophageal cancer.
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FIG.1

Amino acid sequence of B7-H3 variant 1 (SEQ ID NO: 1)

MLRRRGSPGMGVHVGAAALGALWFCLTGALEVQVPEDPVVALVGTD
ATLCCSFSPPEPGFSLAQLNLIWQLTDTKQLVHSFAEGQDQGSAYA
NRTALFPDLLAQGNASLRLQRRVVADEGSFTCFVSI RDFGSAAVS
LQVAAPYSKPSMTLEPNKDLRPGDVTITCSSYQGYPEAEVFWQD
GQGVPLTGNTTTSQMANEQGLFDVHSILRVVLGANGTYSCLVRNP
VLQQDAHSSVTITPQRSPTGAVEVQVPEDPVVALVGTDATLRCSF
SPEPGFSLAQLNLIWQLTDTKQLVHSFTEGRDQGSAYANRTALFP
DLLAQGNASLRLQRRVVADEGSFTCFVSI RDFGSAAVSLQVAAPY
SKPSMTLEPNKDLRPGDVTITCSSYRGYPEAEVFWQDQGVPLT
GNTTTSQMANEQGLFDVHSVLRVVLGANGTYSCLVRNPVLQQDAH
GSVTITGQPMTFPPEALWVTVGLSVCLIALLVALAFVCWRKIKQS
CEEENAGAEDQDGE GEGSKTALQPLKHS DSKEDDGQEIA

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FIG.2

Amino acid sequence of B7-H3 variant 2 (SEQ ID NO: 2)

MLRRRGSPGMGVHVGAAALGALWFCLTGALEVQVPEDPVVALVGTD
ATLCCSFSPPEPGFSLAQLNLIWQLTDTKQLVHSFAEGQDQGSAYA
NRTALFPDLLAQGNASLRLQRVRVADEGSFTCFVSI RDFGSAAVS
LQVAAPYSKPSMTLEPNKDLRPGDTVITC SSYRGYPEAEVFWQD
GQGVPLTG NVTTSQMANEQGLFDVIISVLRVVLGANGTYSCLVRNP
VLQQDAHGSVTITGQPMTFPPEALWVTVGLSVCLIAL LVALAFVC
WRKIKQSCEEENAGAEDQDGE GEGSGKTALQPLKHSDSKEDDGQE I
A

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FIG.3

Amino acid sequence of M30-H1-type heavy chain (SEQ ID NO: 9)

MKHLWFFLLLVAAPRWVLSQVQLVQSGAEVKKPGSSVKVSCKASG
YTFTNYVMHWVRQAPGQGLEWMGYINPYNDDVKYNEKFKGRVTIT
ADESTSTAYMELSSLRSED TAVYYCARWGYYSPLYFFDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK

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FIG.4

Amino acid sequence of M30-H2-type heavy chain (SEQ ID NO: 10)

MKHLWFFLLLVAAPRWVLSQVQLVQSGAEVKKPGSSVKVSCKASG
YTFTNYVMHWVRQAPGQGLEWIGYINPYNDDVKYNEKFKGRVTIT
ADESTSTAYMELSSLRSED TAVYYCARWGYYGSPLYYFDYWGGGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
CS
VMHEALHNHYTQKSLSLSPGK

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FIG.5

Amino acid sequence of M30-H3-type heavy chain (SEQ ID NO: 11)

MKHLWFFLLLVAAPRWVLSEVQLVQSGAEVKKPGSSVKVSCKASG
YTFTNYVMHWVKQAPGQGLEWIGYINPYNDDVKYNEKFKGKATIT
ADESTSTAYMELSSLRSED TAVYYCARWGYYGSPLYFFDYWGQGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRV EPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
CS
VMHEALHNHYTQKSLSLSPGK

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FIG.6

Amino acid sequence of M30-H4-type heavy chain (SEQ ID NO: 12)

MKHLWFFLLLVAA PRWVLSEVQLVQSGAEVKKPGSSVKV SCKASG
YTFTNYVMHWVKQAPGQGLEWIGYINPYNDDVKYNEKFKGKATQT
SDKSTSTAYMELSSLRSED TAVYYCARWGYYGSPLY YFDYWGGGT
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICN
VNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCS
VMHEALHNHYTQKSLSLSPGK

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FIG.7

Amino acid sequence of M30-L1-type light chain (SEQ ID NO: 13)

MVLQTQVFISLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA
SSRLIYMHWYQQKPGQAPRLLIYATSNLASGIPARFSGSGSGTDF
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSITYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVT
KSFNRGEC

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FIG.8

Amino acid sequence of M30-L2-type light chain (SEQ ID NO: 14)

MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA
SSRLIYMHWYQQKPGQAPRLWIYATSNLASGIPARFSGSGSGTDY
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVT
KSFNRGEC

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FIG.9

Amino acid sequence of M30-L3-type light chain (SEQ ID NO: 15)

```
MVLQTQVFISLLWISGAYGQIVLSQSPATLSLSPGERATLTCRA  
SSRLIYMHWYQQKPGSAPKLWIYATSNLASGIPARFSGSGSGTSY  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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FIG.10

Amino acid sequence of M30-L4-type light chain (SEQ ID NO: 16)

```
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA  
SSRLIYMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDF  
TLTISSLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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FIG.11

Amino acid sequence of M30-L5-type light chain (SEQ ID NO: 17)

```
MVLQTQVFISLLLWISGAYGQIVLSQSPATLSLSPGERATLTCRA  
SSRLIYMHWYQQKPGSAPKPWIIYATSNLASGIPARFSGSGSGTSY  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```


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FIG. 12

Amino acid sequence of M30-L6-type light chain (SEQ ID NO: 18)

```
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA  
SSRLIYMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDF  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDYSLSSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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FIG. 13

Amino acid sequence of M30-L7-type light chain (SEQ ID NO: 19)

```
MVLQTQVFISLLLWISGAYGEIVLTQSPATLSLSPGERATLSCRA  
SSRLIYMHWYQQKPGQAPRPLIYATSNLASGIPARFSGSGSGTDY  
TLTISRLEPEDFAVYYCQQWNSNPPTFGQGTKVEIKRTVAAPSVF  
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE  
SVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT  
KSFNRGEC
```

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FIG.14

Amino acid sequence of M30 antibody heavy chain (SEQ ID NO: 20)

MEWSWIFLFLLSGTAGVHSEVQLQQSGPELVKPGASVKMSCKASG
YTFTNYVMHWVKQKPGQGLEWIGYINPYNDDVKYNEKFKGKATQT
SDKSSSTAYMELSSLTSEDSAVYYCARWGYYGSPLYFDFYWGQGT
TLTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTL
TWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNV
AHPASSTKVDKKIEPRGPTIKFCPPCKCPAPNLLGGPSVFI FPPK
IKDVLMI SLSPIVTCVVVDVSEDDPDVQISW FVNNVEVHTAQTQT
HREDYNSTLRVVSALPIQH QDWMSGKEFKCKVNNKDLPAPIERTI
SKPKGSVRAPQVYVLP PPEEEMTKKQVTLTCMVTD FMPEDIYVEW
TNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCS
VVHEGLHNHHTTKSF SRTPCK

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FIG.15

Amino acid sequence of M30 antibody light chain (SEQ ID NO: 21)

```
MDFLVQIFSFLLISASVIMSRGQIVLSQSPTILSASPGEKVTMTC  
RASSRLIYMHWYQQKPGSSPKPWIYATSNLASGVPARFSGSGSGT  
SYSLTISRVEAEDAATYYCQQWNSNPPTFGTGTKLELKRADAAPT  
VSIFFPSSEQLTSGGASVVCFLNMFYPKDINVKWKIDGSEKQNGV  
LNSWTDQDSKDYSTYSMSSTLTLTKEDEYERHNSYTCEATHKTSTP  
IVKSFNRNEC
```

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FIG.16

Nucleotide sequence of B7-H3 variant 1 (SEQ ID NO: 26)

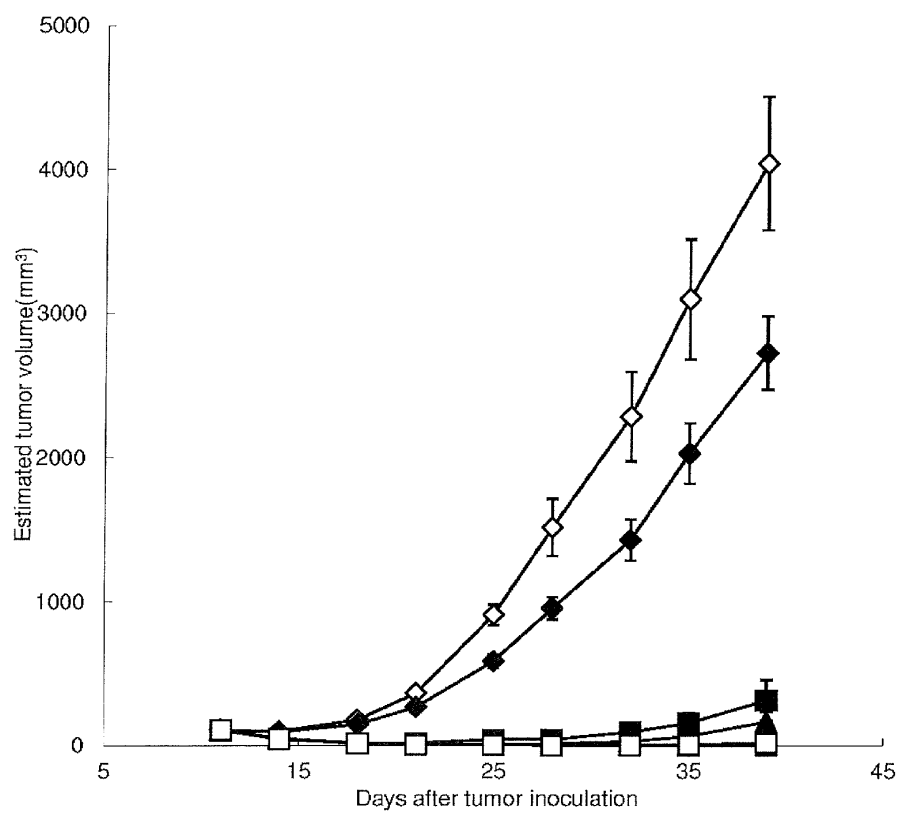
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atgctgcgtcggcggggcagccctggcatgggtgtgcatgtgggt
gcagccctgggagcactgttggttctgectcacaggagccctggag
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gccacccctgtgctgctccttctcccttgagccctggccttcagccctg
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tcagctaccaggggctacccctgaggctgagggtgttctggcaggat
gggcagggtgtgccccctgactggcaacgtgacacgtgcagatg
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agaagccccacaggagccgtgggagggtccagggtccctgagggaaccg
gtgggtggccctagtgggcaccgatgccacccctgcgctgctccttc
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ggggacacgggtgaccatcacgtgctccagctacccggggctacct
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gatgtgcacagcgtcctgcgggtgggtgctgggtgcggaatggcacc
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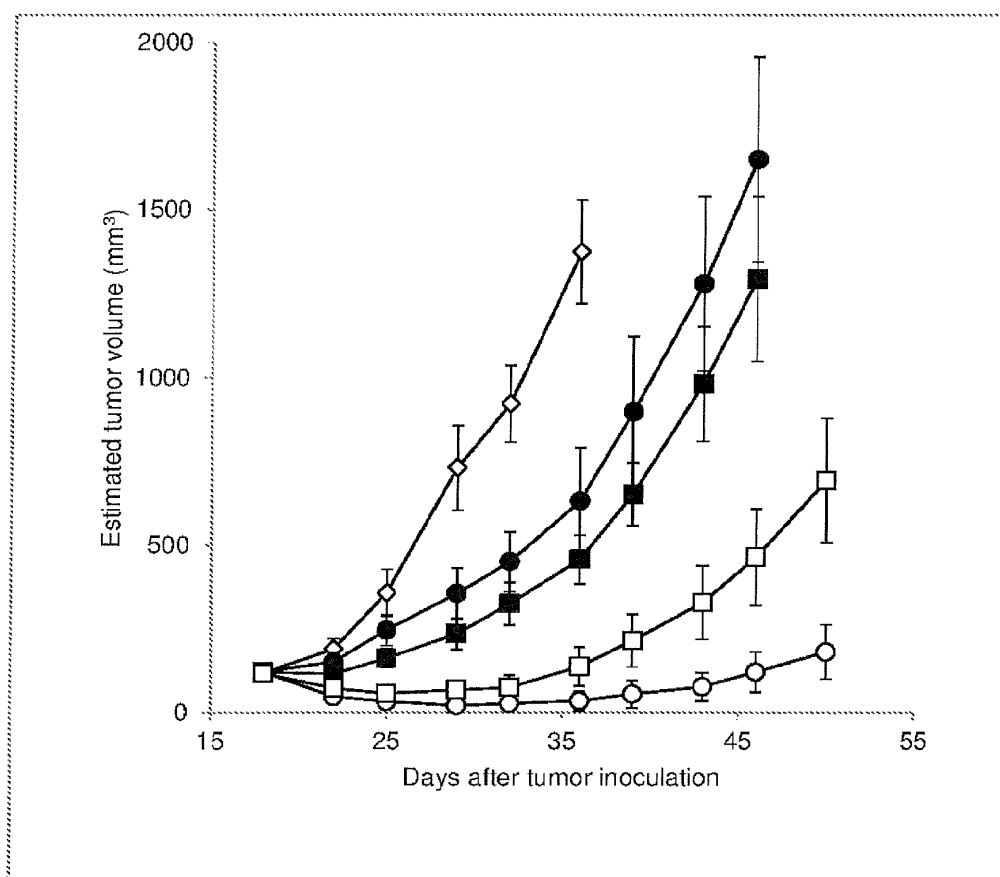
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FIG.17



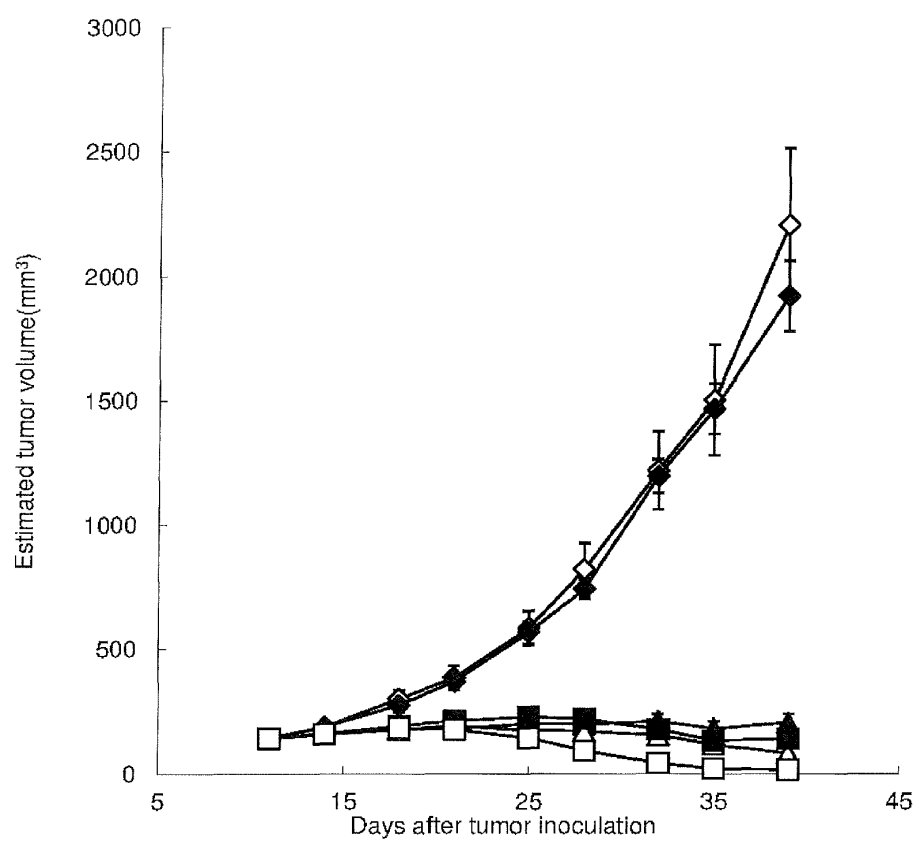
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FIG.18



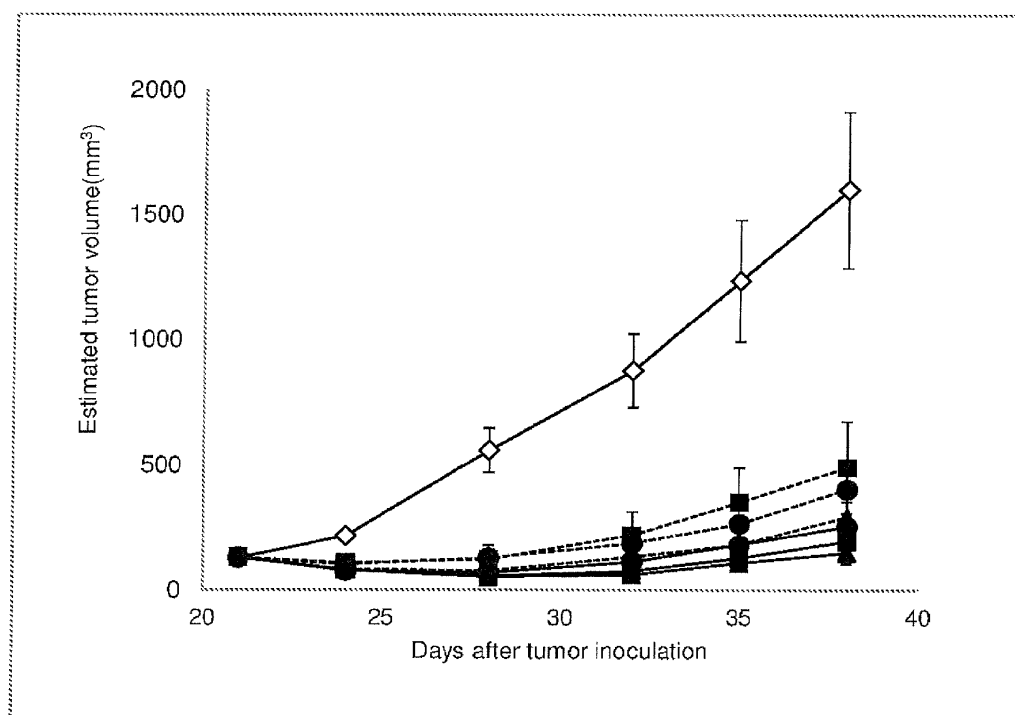
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FIG.19



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FIG.20



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/006178

A. CLASSIFICATION OF SUBJECT MATTER

C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P35/00(2006.01)i,
A61K31/4745(2006.01)n, A61K31/48(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2013
Kokai Jitsuyo Shinan Koho 1971-2013 Toroku Jitsuyo Shinan Koho 1994-2013Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS/REGISTRY (STN), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>Y</u> A	JP 2009-538629 A (Genentech, Inc.), 12 November 2009 (12.11.2009), entire text & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A	<u>1-30, 33-52</u> 31-32
<u>Y</u> A	JP 2008-521828 A (Seattle Genetics, Inc.), 26 June 2008 (26.06.2008), entire text & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2	<u>1-30, 33-52</u> 31-32
<u>Y</u> A	JP 2005-511627 A (Seattle Genetics, Inc.), 28 April 2005 (28.04.2005), entire text & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2	<u>1-30, 33-52</u> 31-32

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
04 December, 2013 (04.12.13)Date of mailing of the international search report
17 December, 2013 (17.12.13)Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/006178

5	C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
	Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
10	<u>Y</u> A	WO 2002/000734 A1 (Daiichi Pharmaceutical Co., Ltd.), 03 January 2002 (03.01.2002), entire text & US 2003/0166513 A1 & EP 1298145 A1 & CN 1449412 A	<u>1-30, 33-52</u> 31-32
15	<u>Y</u> A	JP 11-092405 A (Daiichi Pharmaceutical Co., Ltd.), 06 April 1999 (06.04.1999), entire text (Family: none)	<u>1-30, 33-52</u> 31-32
20	<u>Y</u> A	JP 10-095802 A (Tanabe Seiyaku Co., Ltd.), 14 April 1998 (14.04.1998), entire text & US 5892043 A & EP 781781 A2 & CN 1158334 A	<u>1-30, 33-52</u> 31-32
25			
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Form PCT/ISA/210 (continuation of second sheet) (July 2009)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/006178

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 53-54

because they relate to subject matter not required to be searched by this Authority, namely:

Claims 53-54 pertain to therapeutic methods for a human being and thus relate to a subject matter on which this International Searching Authority is not required to carry out an international search under the provisions of PCT Article 17(2)(a)(i) and [PCT Rule 39.1(iv)].

2. ☐ Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

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REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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- WO 199746260 A [0006]
- WO 200025825 A [0006]
- WO 2002043661 A [0035]
- US 5773001 A [0035]
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- WO 9220791 A [0146]
- WO 9306213 A [0146]
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- **KUMAZAWA, E. ; TOHGO, A.** Antitumour activity of DX-8951f: a new camptothecin derivative. *Exp. Opin. Invest. Drugs*, 1998, vol. 7, 625-632 [0007]
- **MITSUI, I. ; KUMAZAWA, E. ; HIROTA, Y. et al.** A new water-soluble camptothecin derivative, DX-8951f, exhibits potent antitumor activity against human tumors in vitro and in vivo. *Jpn J. Cancer Res.*, 1995, vol. 86, 776-786 [0007]
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Description

BACKGROUND OF THE INVENTION

5 Field of the Invention:

This invention relates to a novel compound having an antitumor activity and a process for preparing this compound.

10 Description of the Background:

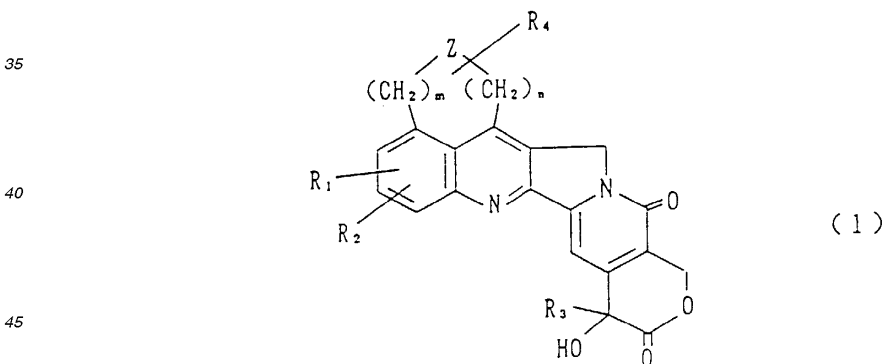
Camptothecin is a penta-cyclic alkaloid isolated from barks, roots, fruits, or leaves of camptotheca acuminata. This compound is known to exhibit an antitumor activity because of its capability of inhibiting nucleic acid synthesis. According to the results of clinical tests conducted in the United States, however, the compound was found to have a problem in view of safety, and its research and development as a medicine have been discontinued. Thereafter, research on derivatives of camptothecin possessing better activity and reduced toxicity has been undertaken worldwide. However, no report has surfaced so far on the derivative with satisfactory clinical results. JP-A-2-138186 discloses a Camptothecin derivative having an amino group in 9-position instead of a hydroxyl group, but the derivative exhibited poor solubility in water.

20 The scarce solubility of camptothecin in water is another problem of this compound in administering it as a medicine. A method of opening the lactone ring and converting it into the sodium carbonate is known as one of the means for making camptothecin water-soluble. The product obtained by this method, however, exhibits a very reduced antitumor activity. The development of a water-soluble camptothecin derivative with the lactone ring being retained as is has therefore been desired.

25 The present inventors have conducted extensive studies for the purpose of obtaining camptothecin derivatives with more excellent activity and higher safety, as well as excellent characteristics required for a drug to be administered, and found that hexa-cyclic compounds obtained by the addition of a water-soluble ring to camptothecin had characteristics superior to camptothecin. This finding has led to the completion of this invention.

SUMMARY OF THE INVENTION

30 Accordingly, an object of this invention is to provide a hexa-cyclic compound represented by the following general formula:



wherein R₁ and R₂ individually represent a hydrogen atom, a hydroxyl group, a C₁₋₆ alkyl group ("C₁₋₆ alkyl group means an alkyl group having 1 to 6 carbon atoms. Hereinafter defined in the same manner.) which may contain a halogen atom, a nitro or cyano group, a C₁₋₆ alkenyl group, a C₁₋₆ alkynyl group, a C₁₋₆ alkoxy group, a C₁₋₆ aminoalkoxy group, a halogen atom, a nitro group, a cyano group, a mercapto group, an alkylthio group, an amino group which may contain a protective group, a C₁₋₆ aminoalkyl groups which may contain a protective group or a C₁₋₆ alkyl group at the amino-position, a C₁₋₆ aminoalkylamino group which may contain a protective group or a C₁₋₆ alkyl group at the amino-position, a C₁₋₆ alkyl group with a heterocyclic ring which may contain a C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro, or cyano group, a heterocyclic carbonyl group which may contain a C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, micro, or cyano group, a C₁₋₆ alkylamino group with a heterocyclic ring which may contain C₁₋₆ alkyl, C₁₋₆ alkoxy, amino (which may contain a protective group), halogeno, nitro, cyano or a protective group, an amino-heterocyclic group which may contain a protective group or a C₁₋₆ alkyl group at the

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nitrogen atom of the heterocyclic ring moiety or at the amino position, a heterocyclic-amino group which may contain a protective group or a C₁₋₆ alkyl group at the nitrogen atom of the heterocyclic ring moiety or at the amino position, or a carbamoyl group which may contain a protective group or a C₁₋₆ alkyl group; R₃ represents a C₁₋₆ alkyl group; R₄ represents an amino group which may contain a protective group, a quaternary trialkyl ammonium such as -N⁺(CH₃)₃, a C₁₋₆ alkylamino group which may contain a protective group, a C₁₋₆ aminoalkyl group which may contain a protective group, a C₁₋₆ alkylaminoalkyl group which may contain a protective group, a sulfonic acid group, or a carboxyl group; Z represents an oxygen atom, a sulfur atom, CR₅R₆, wherein R₅ and R₆ individually represent a hydrogen atom or a C₁₋₆ alkyl, or N-R₇, wherein R₇ stands for a hydrogen atom, a C₁₋₆ alkyl group, a C₁₋₆ aminoalkyl group which may contain a protective group, a C₁₋₆ aminoalkyl group which may contain a protective group, a C₁₋₆ alkylaminoalkyl group which may contain a protective group, or a protective group for the amino group; and m and n individually represent 0, 1 or 2.

Other objects, features and advantages of the invention will hereinafter become more readily apparent from the following description.

15 DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

Preferred examples of groups represented by R₁ or R₂ in formula (I) are C₁₋₃ alkyl, C₁₋₃ alkenyl, hydroxymethyl, hydroxyl, C₁₋₃ alkoxy, halogen, nitro, amino, C₁₋₃ alkylamino, cyano-C₁₋₃ alkyl, aminomethyl, dimethylhydrazino, morpholine-1-yl, piperidine-1-yl, and the like.

Ethyl group and the like are given as preferable groups for R₃.

Amino group, C₁₋₆ alkylamino, amino-C₁₋₆ alkyl, C₁₋₆ alkylamino-C₁₋₆ alkyl groups are given as preferred groups for R₄. Among these, especially preferable are methylamino, dimethylamino, aminomethyl, ethylamino, diethylamino, aminoethyl, methylaminomethyl, dimethylaminomethyl, hydroxy ethylamino.

When substituent R₄ and substituent R₁ or R₂ of compound (I) are suitably selected, the compound possesses sufficient water-solubility and a suitable degree of lipophilicity, and thus exhibits excellent properties. The lipophilicity is a parameter of cell membrane permeability of the compound, and the improved cell membrane permeability promises cytotoxicity against cancerous cells.

Examples of preferable combination of R₄, R₁ and R₂ include such that:

R₄ is NH₂, NHCH₃ or N(CH₃)₂, and
R₁ or R₂ is CH₃ or C₂H₅

Among them, most preferred is a combination where R₄ is NH₂, R₁ is 4-methyl and R₅ is 5-fluoro.

When R₄ is NH₂, and R₁ or R₂ is OH, only an insufficient cell membrane permeability is obtained, and therefore, it is preferred that the compound be converted to a pro-drug, in other words, the OH group is converted to -O-Y or -O-CO-Y. Here, Y is amino, dialkylamino, dialkylaminoalkylamino, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, heterocyclic group which may contain one or more C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro or cyano group, or alkyl group with heterocyclic ring which may contain one or more C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro or cyano groups.

A quaternary trialkyl ammonium such as -N⁺(CH₃)₃ is also preferred as R₄.

Methylene group, oxygen atom, sulfur atom, imino(-NH-), alkylimino(-N(alkyl)-), and the like are given as preferable groups represented by Z.

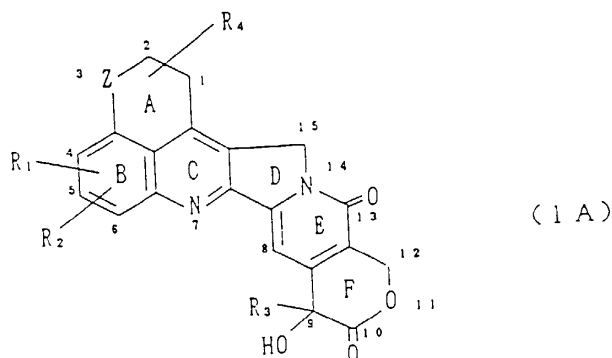
As preferred protective groups for amino group, given are formyl, acetyl, trityl, tert-butoxycarbonyl, benzyl, p-methoxybenzyloxycarbonyl.

Given as preferred examples for heterocyclic groups are 4-7 membered rings having one or more nitrogen atoms which may contain one or more oxygen atoms or sulfur atoms, such as azetidine, piperazine, morpholine, pyrrolidine, piperidine, imiazole, thiazole, oxazole, pyridine, and the like. Among them, those having 5 or 6 membered rings such as pyrrolidine, piperidine, piperazine, morpholine are especially preferred.

Among the compounds of formula (I), those having a six-membered ring for the A-ring, which are represented by the following formula (IA) is particularly preferable.

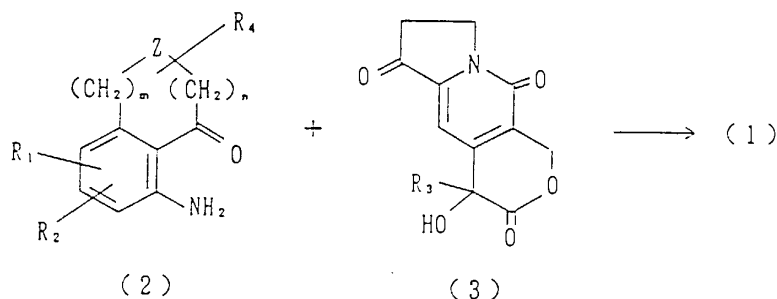
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Furthermore, among the compounds of formula (I), those in which the asymmetric carbon at 9 position of the F-ring takes the S-type configuration are preferable from the aspect of medicinal activity.

The compounds of the present invention can be prepared according to the process exemplified by the following reaction scheme.



According to the above process scheme, an aminoketone compound (2) and a pyranindolizine compound (3) are condensed by the Friedlaender reaction to produce the compound (I).

Aminoketone compounds (2) are known compounds and can be readily prepared according to the methods known in the art. The conditions of this condensation ring-closing reaction of compounds (2) and (3) can be suitably selected from the conditions, wherein the reaction is conducted at room temperature or an elevated temperature in the presence of an acid or a base.

There is no specific limitation to the kind of the solvent used, so long as the solvent is inert to the reaction. Examples of such solvents include aromatic hydrocarbons such as benzene, toluene, xylene, halogenated hydrocarbons such as dichloromethane, chloroform, 1,2-dichloroethane, and the like, ethers such as diethyl ether, diisopropyl ether, tetrahydrofuran, dimethyl cellosolve, diethyl cellosolve, diglyme, and the like, lower alcohols such as methanol, ethanol, propanol, tert-butanol, and the like, amides such as acetamide, dimethylacetamide, N,N-dimethylformamide, and acetic acid. Particularly preferable solvents are benzene, toluene, and acetic acid.

Either an organic acid or inorganic acid can be used for the reaction. Hydrochloric acid and sulfuric acid are typical examples given of inorganic acids. Organic acids which can be used include sulfonic acids such as methanesulfonic acid, trifluoromethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, pyridine-p-toluene sulfonate; carboxylic acids such as acetic acid. Among these especially preferable are p-toluenesulfonic acid, pyridine-p-toluene sulfonate, acetic acid. Here, acetic acid can function also as a solvent.

A base to be employed in the reaction may be either an inorganic or organic base. Given as examples of inorganic bases are hydroxides, carbonates, bicarbonates, and hydrides of alkali metal such as lithium hydroxide, sodium hydroxide, potassium hydroxide, lithium carbonate, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, sodium hydride, and the like. Organic bases include alkoxide of alkali metal such as sodium methoxide, sodium ethoxide, potassium tert-butoxide, and the like; tert-alkyl amines such as triethylamine, N,N-diisopropylethylamine; aromatic tertiary amines such as N,N-dimethylaniline, N,N-diethylaniline, N,N-dimethylaminopyridine; pyridine; 1,8-diazabicycloundecene. Preferred bases are potassium carbonate and triethylamine.

Some compounds of formula (3) are unstable against basic compounds. Deliberate considerations therefore must be given to the reaction conditions when a base is used. For example, measures must be taken such as carrying out

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the reaction at a relatively low temperature, for a shorter period of time, or under acidic conditions.

The reaction is carried out at a temperature usually of 20-150°C, and preferably 80-120°C. Depending on the characteristics of compound (3), however, the reaction under ice-cooling is desirable. The reaction time may be between 1 hour and 48 hours. Usually, the reaction is completed within 1-24 hours.

5 A typical example of performing the reaction is refluxing the reaction mixture in benzene, toluene, or acetic acid in the presence of pyridine p-toluenesulfonate.

When a group R₁, R₂, or R₄, or their substituent is an amino group with a protective group, such a protective group can be removed by the reduction or hydrolysis with an acid or alkali.

10 A compounds having an alkoxyl group can be converted into the corresponding hydroxyl compounds by treating them with aluminum chloride or aluminum bromide in an inert solvent such as toluene, benzene, or by heating in a solution of hydrobromic acid.

A compound having a nitro group can be converted into the corresponding amino compound by catalytic reduction using platinum, palladium.

15 A compound having an amino group can be converted into the corresponding hydroxyl compound via a diazonium compound by the treatment with sodium nitrite in an acidic solvent at a low temperature, followed by hydrolysis of the diazonium salt.

A compound having an amino group can also be converted into the corresponding halogeno compound by the Sandmeyer reaction via diazonium salt mentioned above. General Sandmeyer reaction conditions can be applicable to this reaction using cuprous chloride, cuprous bromide.

20 The compound of this invention can optionally be converted into a form of physiologically acceptable salt, e.g., a salt of an alkali metal or alkali earth metal, by using a hydroxide of these metals; or when such a compound is a basic compound such as that possessing an amino group or the like, may be converted into an inorganic or organic salt using an inorganic acid such as hydrochloric acid, sulfuric acid, phosphoric acid, or the like, or an organic acid such as formic acid, acetic acid, methanesulfonic acid.

25 Antitumor effects of the compound of this invention thus prepared are hereinafter described by way of experimental examples.

Experimental Example 1

30 P388 murine leukemia cells were spread over a 96-well microplate, 2.5x10³ cells per well. A sample to be tested was added after 24 hours. Cells were cultivated under the conditions of 5% CO₂ at 37°C for 3 days. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added 4 hours after the addition of the sample. Upon the addition of 200 µl/ml of isopropyl alcohol containing 0.04 N HCl the absorption at 540 nm was measured to determine IC₅₀.

35 The results are shown in Table 1.

TABLE 1

	IC ₅₀ (ng/ml)
40 Compound of Example 2 (Isomer A)	3.38
Compound of Example 2 (Isomer B)	12.40
Compound of Example 4 (Isomer A)	9.21
Compound of Example 4 (Isomer B)	27.80
45 Compound of Example 7 (Isomer A)	11.40
Compound of Example 7 (Isomer B)	11.90

As shown in Table 1, the compounds of this invention have an excellent antitumor activity and a high degree of safety, and are water-soluble. They are useful as an antitumor medicine.

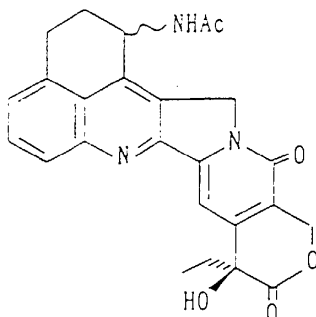
50 Other features of the invention will become apparent in the course of the following description of the exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1

55 Preparation of (9S)-1-acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino [1,2-b]-quinoline-10,13(9H,15H)-dione

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(1) 8-Acetylamino-1-tetralone

10 gm of 1-acetylamino-tetraline was dissolved into a mixed solvent of 400 ml acetone and 40 ml of 15% aqueous solution of magnesium sulfate. The solution was kept at 0°C to add 42 gm of potassium permanganate, followed by stirring for 20 minutes at the same temperature. 800 ml of water was added to the residue obtained by concentrating the solvent. Precipitates thus obtained was extracted with chloroform and the extract was washed with brine and dried over magnesium sulfate. The residue obtained by the concentration of the solvent was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 5.46 gm of the title compound.

NMR(CDCl₃)δ: 2.08-2.14(2H, m), 2.23(3H, s), 2.70(2H, t, J=6.8Hz), 2.97(2H, t, J=6.8Hz), 6.93(1H, d, J=6.8Hz), 7.44(1H, t, J=8.3Hz), 8.59(1H, d, J=8.3Hz)

(2) 8-Acetylamino-2-hydroxyimino-1-tetralone

To a reaction solution obtained by suspending 316 mg of potassium tert-butoxide in 18 ml of tetrahydrofuran (hereinafter abbreviated as THF) and cooling the suspension to 0°C under a nitrogen stream was added a solution of 500 mg of the compound prepared in (1) above in 2 ml of THF, a bit at a time. After stirring for 10 minutes at the same temperature and an addition of 0.35 ml of butyl nitrite, the mixture was heated at 50°C for 1 hour while stirring. Precipitates obtained by the addition of diethyl ether to the reaction mixture were collected by filtration. The powder thus obtained was suspended into a 10% aqueous solution of hydrochloric acid and extracted with ethyl acetate. The extract was washed with brine and dried over magnesium sulfate. The residue obtained by the concentration of the solvent was subjected to silica gel column chromatography using an ethyl acetate-hexane (1:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 320 mg of the title compound.

IR ν_{max}^{KBr} cm⁻¹: 3440, 1698, 1678, 1608, 1580, 1518 NMR(CDCl₃)δ: 2.26(3H, s), 3.08(4H, s), 6.98(1H, d, J=7.4Hz), 7.53(1H, t, J=7.7Hz), 8.64(1H, d, J=8.5Hz) MASS m/z: 232(M⁺)

(3) 2,8-Diacetylamino-1-tetralone

1 gm of zinc powder was added to a solution of 300 mg of the compound prepared in (2) above dissolved in a mixed solvent of 10 ml of acetic acid and 10 ml of acetic anhydride, and the mixture was stirred for 40 minutes at room temperature. Insoluble substances were removed by filtration and filtrate was concentrated. The residue was recrystallized in ethyl acetate and hexane to obtain 263 mg of the title compound.

IR ν_{max}^{KBr} cm⁻¹: 3280, 1660, 1596, 1516
NMR(CDCl₃)δ: 1.6-2.0(1H, m), 2.11(3H, s), 2.23(3H, s), 2.6-3.0(1H, m), 3.1-3.4(2H, m), 4.6-4.8(1H, m), 6.93(1H, d, J=6.8Hz), 7.49(1H, t, J=8.3Hz), 8.59(1H, d, J=8.3Hz)
MASS m/z: 260(M⁺)

(4) 2-Acetylamino-8-amino-1-tetralone

245 mg of the compound prepared in (3) above was dissolved into 40 ml of 3 N hydrochloric acid and heated at

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60°C for 1 hour while stirring. After cooling to 0°C, the reaction solution was neutralized with sodium carbonate and extracted with chloroform. The extract was dried over magnesium sulfate. The residue obtained after the removal of the solvent was recrystallized in ethyl acetate and hexane to obtain 150 mg of the title compound.

5 IR_v^{KBr}_{max} cm⁻¹ : 3424, 3304, 1668, 1632, 1558, 1508
 NMR(CDCI₃)δ: 1.6-2.0(1H, m), 2.09(3H, s), 2.6-3.2(3H, m), 4.41-4.67(1H, m), 6.47-6.71(2H, m), 7.12-7.26(1H, m)
 MASS m/z: 218(M⁺)

10 (5) (9S)-1-Acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino[1,2-b]quinoline-10,13-(9H,15H)-dione

361 mg of (S)-4-ethyl-4-hydroxy-7,8-dihydro-1H-pyrano-[3,4-f]indolizine-3,6,10(4H)-trione (hereinafter abbreviated as "trione") was added to the solution of 300 mg of the compound prepared in (4) above in 200 ml of toluene. The mixture was heated under reflux using a Deanstark apparatus for 10 minutes. To the mixture was added 1 mg of pyridinium-p-toluenesulfonate (hereinafter abbreviated as "PPTS") and the mixture was refluxed while stirring for a further 24 hours. After cooling, the residue obtained by removing toluene by concentration was suspended into 300 ml of chloroform-methanol (10:1) solvent. Insoluble substances were removed and the residue was powdered with methanol to obtain 336 mg of the title compound.

20 mp: above 240°C (decomposed)
 IR_v^{KBr}_{max} cm⁻¹ : 3452, 1750, 1660
 NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.2Hz), 1.83-1.92(2H, m), 1.94(3H, d, J=4Hz), 2.09-2.17(2H, m), 3.14-3.31(2H, m), 5.21(2H, d, J=5.6Hz), 5.42(2H, d, J=5.6Hz), 5.58-5.61(1H, d), 6.50(1H, br s), 7.35(1H, d, J=2.4Hz), 7.52(1H, d, J=7.2Hz), 7.79(1H, t, J=7.2Hz), 8.02(1H, d, J=8.7Hz), 8.52(1H, t, J=9.5Hz)
 25 MASS m/z: 445(M⁺)

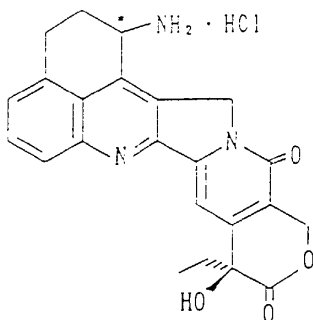
Example 2

30 Preparation of (9S)-1-amino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

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A mixture obtained by the addition of 300 mg of the compound prepared in Example 1 to 100 ml of 6N aqueous solution of hydrochloric acid was reacted under reflux for 4 hours. After having been allowed to cool, the reaction mixture was concentrated. 100 ml of water was added to the residue to remove insoluble substance by filtration using FALCON 7105 (0.22 μm). The residue obtained by concentrating the filtrate was purified with reverse HPLC (CAPCELL PAK C18: trademark, manufactured by Shiseido Co.) using a mixture of acetonitrile-water-1 N hydrochloric acid (20: 80:2) to separate two kinds of diastereoisomers. The residue obtained by concentrating the fraction first eluted was precipitated in methanol and acetonitrile to obtain 74 mg of the title compound (Isomer A). In the same manner, 90 mg of the title compound (Isomer B) was obtained from the fraction next eluted. In the following Examples, isomers first eluted from the reverse HPLC are designated as Isomer A and second eluted as Isomer B.

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Isomer A

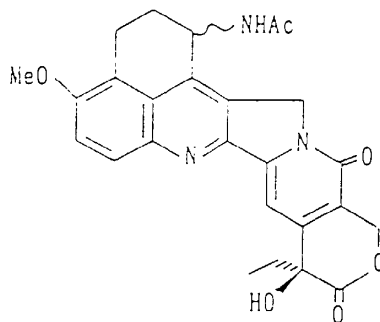
mp: above 240°C (decomposed) $[\alpha]_D^{20} = +178^\circ$ (c=0.25, in H₂O)
 IR_v^{KBr}_{max} cm⁻¹: 3440, 1738, 1658
 5 NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.2Hz), 1.85-1.94(2H, m), 2.17-2.23(1H, m), 3.20-3.23(1H, m), 3.36-3.43(1H, m),
 5.12(1H, br s), 5.42-5.46(3H, m), 5.94(1H, d, J=19Hz), 6.53(1H, s), 7.38(1H, s), 7.61(1H, d, J=7.2Hz), 7.85(1H, t, J=7.2Hz), 8.09(1H, d, J=8.8Hz), 8.77(3H, br)
 MASS m/z: 403(M⁺)

10 Isomer B

mp: above 240°C (decomposed)
 $[\alpha]_D^{20} = -38^\circ$ (c=0.25, in H₂O)
 IR_v^{KBr}_{max} cm⁻¹: 3444, 1740, 1658
 15 NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.2Hz), 1.84-1.93(2H, m), 2.16-2.23(1H, m), 3.21-3.24(1H, m), 3.38-3.45(1H, m),
 5.13(1H, br s), 5.42-5.49(3H, m), 5.98(1H, d, J=19Hz), 7.38(1H, s), 7.61(1H, d, J=7.2Hz), 7.86(1H, t, J=7.2Hz), 8.09(1H, d, J=8.8Hz), 8.77(3H, br)
 MASS m/z: 403(M⁺)

20 Example 3

Preparation of (9S)-1-acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



40 (1) 2-Hydroxyimino-5-methoxy-8-nitro-1-tetralone

The same procedure as in Example 1-(2) was carried out, except that 1.5 gm of 5-methoxy-8-nitro-1-tetralone was used instead of 8-acetylamino-1-tetralone. Upon the posttreatment in the same manner as in Example 1-(2), 740 mg of the title compound was obtained.

45 IR_v^{KBr}_{max} cm⁻¹: 3428, 3256, 1696, 1604, 1580, 1534
 NMR(DMSO-d₆)δ: 2.95(4H, s), 3.94(3H, s), 7.32(1H, d, J=8.7Hz), 7.78(1H, d, J=8.7Hz)
 MASS m/z: 251(M⁺)

50 (2) 2,8-Diacetylamino-5-methoxy-1-tetralone

The same procedure as in Example 1-(3) was carried out, except that 500 mg of the compound prepared in (1) above was used instead of 8-acetylamino-2-hydroxyimino-1-tetralone. Upon the post-treatment in the same manner as in Example 1-(3), 225 mg of the title compound was obtained.

55 IR_v^{KBr}_{max} cm⁻¹: 3432, 1696, 1642, 1532
 NMR(CDCl₃)δ: 1.6-2.0(1H, m), 2.11(3H, s), 2.21(3H, s), 2.6-3.2(3H, m), 3.85(3H, s), 4.5-4.8(1H, m), 7.09(1H, d, J=9.2Hz), 8.55(1H, d, J=9.2Hz)

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MASS m/z: 290(M⁺)

(3) 2-Acetylamino-6-amino-5-methoxy-1-tetralone

5 The same procedure as in Example 1-(4) was carried out, except that 200 mg of the compound prepared in (2) above was used instead of 2,8-diacetylamino-1-tetralone. Upon the post-treatment in the same manner as in Example 1-(4), 130 mg of the title compound was obtained.

10 IR_{max}^{KBr} cm⁻¹: 3444, 2940, 1632, 1564, 1534
 NMR(CDC₃)δ: 1.6-2.0(1H, m), 2.08(3H, s), 2.6-3.4(3H, m), 3.77(3H, s), 4.52-4.61(1H, m), 6.52(1H, d, J=9.2Hz), 6.98(1H, d, J=9.2Hz)
 MASS m/z: 248(M⁺)

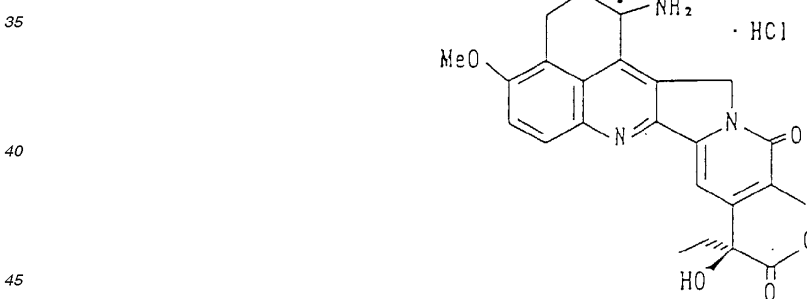
15 (4) (9S)-1-Acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino [1,2-b]-quinoline-10,13(9H,15H)-dione

125 mg of the compound prepared in (3) above was reacted with 133 mg of trione for 24 hours in the same manner as in Example 1-(5). Upon the post-treatment in the same manner as in Example 1-(5), 207 mg of the title compound was obtained.

20 mp: above 240°C (decomposed)
 IR_{max}^{KBr} cm⁻¹: 3448, 1748, 1660, 1600
 NMR(DMSO-d₆)δ: 0.88(3H, dt, J=3.2, 7.2Hz), 1.84-1.89(2H, m), 1.92(3H, d, J=4.8Hz), 2.06-2.07(2H, m), 3.07-3.08(2H, m), 4.00(3H, s), 5.20(2H, d, J=4.8Hz), 5.41(2H, d, J=4.8Hz), 5.52-5.54 (1H, m), 6.48(1H, d, J=1.6Hz), 7.28(1H, d, J=2.4Hz), 7.77(1H, d, J=9.5Hz), 8.08(1H, d, J=9.5Hz), 8.44(1H, t, J=9.5Hz)
 25 MASS m/z: 475(M⁺)

Example 4

30 Preparation of (9S)-1-amino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino [1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



102 mg of the compound prepared in Example 3-(4) was reacted and post-treated in the same manner as in Example 2 to obtain 50 mg of Isomer A and 44 mg of Isomer B of the title compound.

50 Isomer A

mp: above 240°C (decomposed)
 [α]_D²⁰ = +78° (c=0.25, in H₂O)
 55 IR_{max}^{KBr} cm⁻¹: 3448, 2936, 1740, 1658, 1598
 NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.2Hz), 1.84-1.93(2H, m), 2.07-2.12(1H, m), 2.94-3.00(1H, m), 3.25-3.33(1H, m), 4.03(3H, s), 5.07(1H, br), 5.40-5.44(3H, m), 5.91(1H, d, J=19Hz), 7.32(1H, s), 7.83(1H, d, J=9.5Hz), 8.15(1H, d, J=8.8Hz), 8.75(3H, br)

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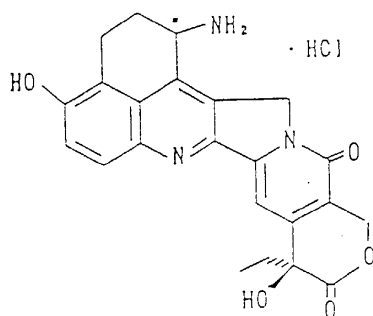
MASS m/z: 433(M⁺)

Isomer B

5 mp: above 240°C (decomposed)
 $[\alpha]_D^{20} = -34^\circ$ (c=0.25, in H₂O)
 IR_v^{max} ^{KBr} cm⁻¹: 3448, 1744, 1654
 NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.2Hz), 1.84-1.91(2H, m), 2.06-2.12(1H, m), 2.95-3.01(1H, m), 4.03(3H, s), 5.07(1H, br), 5.41-5.44(3H, br s), 5.93(1H, d, J=19Hz), 7.32(1H, s), 7.84(1H, d, J=9.5Hz), 8.16(1H, d, J=8.8Hz), 8.78(3H, br)
 10 MASS m/z: 433(M⁺)

Example 5

15 Preparation of (9S)-1-amino-9-ethyl-2,3-dihydro-4,9-dihydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



20 90 mg of the compound prepared in Example 3-(4) was added to 30 ml of 47% aqueous solution of hydrobromic acid and the mixture was heated under reflux for 3 hours. To the residue obtained by removing solvent under reduced pressure 30 ml of water was added. Insoluble substances were removed by filtration using FALCON 7105 (0.22 μm).
 25 The residue obtained by concentrating the filtrate was purified with reverse HPLC (CAPCELL PAK C18: trademark, manufactured by Shiseido Co.) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:2) to obtain 34 mg
 30 of Isomer A and 35 mg of Isomer B of the title compound.

Isomer A

40 mp: above 240°C (decomposed)
 $[\alpha]_D^{20} = +135^\circ$ (c=0.25, in H₂O)
 NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.2Hz), 1.83-1.92(2H, m), 2.04-2.09(1H, m), 2.88-2.95(1H, m), 3.20-3.24(1H, m), 5.04(1H, br), 5.43(3H, m), 5.89(1H, d, J=19Hz), 7.29(1H, s), 7.61(1H, d, J=8.7Hz), 7.99(1H, d, J=9.5Hz), 8.71(3H, br), 10.5(1H, br)
 45 MASS m/z: 419(M⁺)

Isomer B

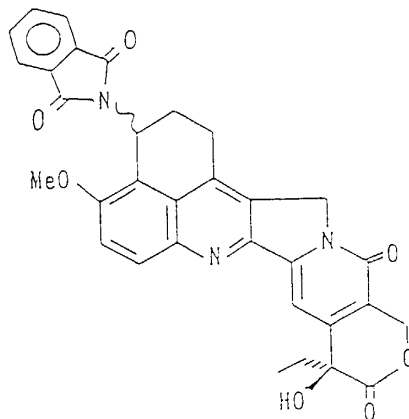
50 mp: above 240°C (decomposed) $[\alpha]_D^{20} = -6.0^\circ$ (c=0.2, in H₂O)
 NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.2Hz), 1.84-1.91(2H, m), 2.07-2.09(1H, m), 2.88-2.95(1H, m), 3.21-3.24(1H, m), 5.05(1H, br), 5.39-5.47(3H, m), 5.88(1H, d, J=19Hz), 7.29(1H, s), 7.61(1H, d, J=8.7Hz), 7.98(1H, d, J=8.7Hz), 8.68(3H, br), 10.5(1H, br)
 MASS m/z: 419(M⁺)

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Example 6

Preparation of (9S)-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-3-(1,3-dioxoisindolin-2-yl)-1H,12H-benzo[de]pyrano
[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 4-Hydroxy-5-methoxy-8-nitro-1-tetralone

2.0 gm of 5-methoxy-8-nitro-1-tetralone (described in Japanese Patent Laid-open (ko-kai) 279891/1989) and 2.05 gm of N-bromosuccinic imide were dissolved in 50 ml of carbon tetrachloride. After the addition of a catalytic amount of benzoyl peroxide, the mixture was heated under reflux for 4 hours and then cooled to room temperature, followed by the addition of 50 ml of chloroform. The mixture was washed with 10% aqueous solution of sodium hydroxide, water, and saturated brine in this order, and dried over anhydrous sodium sulfate. To the residue obtained by removing the solvent were added 5 ml of tetrahydrofuran, 5 ml of ethanol, 8 ml of water, and 250 mg of calcium carbonate, and the mixture was heated under reflux. A residue was obtained by removing the solvent from the reaction mixture, which, after the addition of 50 ml of water, was extracted with chloroform. The extract was washed with saturated brine, dried over anhydrous sodium sulfate, and subjected to silica gel column chromatography using chloroform-ethyl acetate (4:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 1.49 gm of light yellow powder of the title compound.

NMR(CDC₃) δ : 2.2-2.4(2H, m), 2.5-2.7(2H, m), 3.0-3.2(1H, m), 4.00(3H, s), 5.29(1H, s), 7.09(1H, d, J=8.8Hz), 7.52(1H, d, J=8.8Hz)

(2) 8-Amino-5-methoxy-4-(1,3-dioxoisindolin-2-yl)-1-tetralone

424 mg of the compound obtained in (1) above, 288 mg of phthalimide, and 517 mg of triphenylphosphine was added to 20 ml of dried THF. While cooling in an ice-water bath, a THF solution of 0.34 ml of diethylazodicarboxylate (hereinafter abbreviated as "DEAD") was slowly added to the above mixture. After stirring for 30 minutes at the same temperature and an addition of 30 ml of water, the mixture was extracted with chloroform. The chloroform layer was washed with saturated brine and dried over magnesium sulfate. To the residue obtained by the concentration of the solvent were added 50 ml of dioxane, 50 ml of ethanol, and 280 mg of 10% palladium-on-carbon to effect catalytic hydrogenation. After removal of the catalyst by filtration and concentration of the filtrate, the residue was subjected to silica gel column chromatography using a chloroform-ethyl acetate (9:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 306 mg of the title compound.

NMR(CDC₃) δ : 2.1-3.2(4H, m), 3.36(3H, s), 5.70(1H, m), 6.56(1H, d, J=9Hz), 6.86(1H, d, J=9Hz), 7.6-7.8(4H, m)

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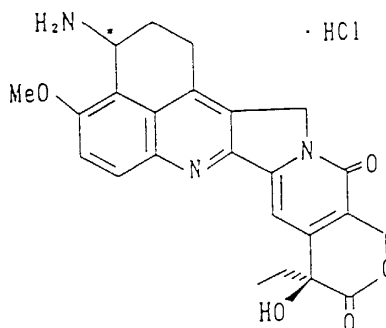
(3) (9S)-9-Ethyl-2,3-dihydro-9-hydroxy-4-methoxy- 3-(1,3-dioxoisindolin-2-yl)-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione

To 30 ml of toluene were added 306 mg of the compound prepared in (2) above and 220 mg of trione. To this was added a catalytic amount of PPTS to react the mixture in a Deanstark apparatus under heating with refluxing for 6 hours. The reaction mixture was cooled to collect the precipitate by filtration, thus obtaining 390 mg of the title compound.

NMR(CDCl₃)δ: 1.04(3H, t, J=7.6Hz), 1.8-2.0(2H, m), 2.3-2.4(1H, m), 2.5-2.56(1H, m), 3.1-3.2 (1H, m), 3.3-3.41 (1H, m), 3.87(3H, s), 5.25(2H, s), 5.31,5.75(2H, ABq, J=15.9Hz), 6.05(1H, m), 7.52(1H, d, J=9.5Hz), 7.6-7.8(5H, m), 8.28(1H, d, J=9.5Hz)

Example 7

Preparation of (9S)-3-amino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



224 mg of the compound prepared in Example 6-(3) was dissolved in 14 ml of chloroform. After the addition of 10 ml of methanol, followed by 1.2 ml of hydrazine monohydrate, the mixture was stirred for 2 hours. After removing the solvent, 7 ml of 5 N hydrochloric acid was added. The mixture was heated under reflux for 1 hour, followed by cooling. 10 ml of water was added to remove insoluble substances by filtration. The residue obtained by concentrating the filtrate was purified with reverse HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:2) to obtain 94 mg of Isomer A and 80 mg of Isomer B of the title compound.

Isomer A

mp: above-215°C (decomposed)
NMR(CD₃OD)δ: 1.00(3H, t, J=7.2Hz), 1.95(2H, m), 2.4-2.5(1H, m), 2.5-2.6(1H, m), 3.2-3.4(2H, m), 4.12(3H, s), 5.17(1H, m), 5.23,5.32(2H, ABq, J=19.1Hz), 5.37,5.55(2H, ABq, J=16.7Hz), 7.62(1H, s), 7.85(1H, d, J=9.5Hz), 8.28(1H, d, J=9.5Hz)

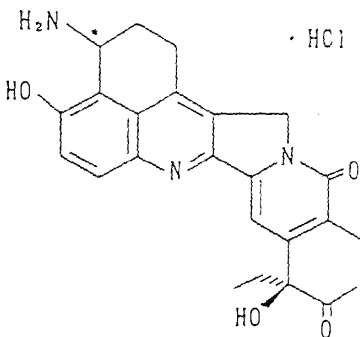
Isomer B

mp: above 180°C (decomposed)
NMR(CD₃OD)δ: 1.01(3H, t, J=7.1Hz), 1.97(2H, m), 2.3-2.45(1H, m), 2.5-2.6(1H, m), 3.2-3.4(2H, m), 4.16(3H, s), 5.18(1H, m), 5.25,5.38(2H, ABq, J=19.1Hz), 5.39,5.57(2H, ABq, J=16.7Hz), 7.67(1H, s), 7.89(1H, d, J=9.5Hz), 8.31(1H, d, J=9.5Hz)

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Example 8

Preparation of (9S)-3-amino-9-ethyl-2,3-dihydro-4,9-dihydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



94 mg of Isomer A prepared in Example 7 was dissolved in 25 ml of acetic acid and heated under reflux for 30 hours. After removing the solvent, 10 ml of water was added to the residue to remove insoluble substances by filtration. The filtrate was purified with reverse HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:2). The fractions containing the target compound was concentrated. 17 mg of Isomer A of the title compound was obtained by precipitation of the residue in a methanol-ethanol-ethyl acetate mixture. Following the same procedure as above, 11 mg of Isomer B of the title compound was prepared from 68 mg of Isomer B of the compound which was prepared in Example 7.

Isomer A

mp: above 170°C (decomposed)
NMR(DMSO- d_6) δ : 0.89(3H, t, $J=7.2$ Hz), 1.82(2H, m), 2.1-2.2(1H, m), 2.4-2.6(1H, m), 3.15-3.35(2H, m), 4.93(1H, m), 5.21, 5.33(2H, ABq, $J=19.1$ Hz), 5.41, 5.45(2H, ASq, $J=15.9$ Hz), 7.28(1H, s), 7.64(1H, d, $J=9.5$ Hz), 8.20(1H, d, $J=9.5$ Hz) 8.25-8.35(3H, m)

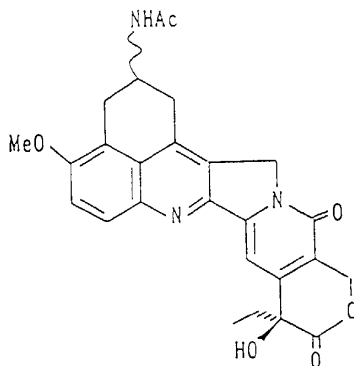
Isomer B

mp: above 195°C (decomposed)
NMR (DMSO- d_6) δ : 0.89(3H, t, $J=7.6$ Hz), 1.8-2.0(2H, m), 2.1-2.2(1H, m), 2.4-2.6(1H, m), 3.15-3.35(2H, m), 4.94(1H, m), 5.21, 5.34(2H, ABq, $J=19.1$ Hz), 5.43(2H, s), 7.28(1H, s), 7.65(1H, d, $J=9.5$ Hz), 8.10(1H, d, $J=9.5$ Hz), 8.25-8.35(3H, m)

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Example 9

Preparation of (95)-2-acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 2-Acetylamino-8-methoxytetraline

To a solution of 5.18 gm of 8-methoxy-2-tetralone [described in J. Chem. Soc. 2636 (1965)] dissolved in 100 ml of methanol, were added 560 mg of sodium cyanoborohydride and 4.86 gm of ammonium acetate, and the mixture was stirred for 89 hours at room temperature. The reaction mixture was ice-cooled and adjusted to below pH 1, followed by the addition of 500 ml of water. After washing with ether, the water layer was adjusted to greater than pH 10 with potassium hydroxide and extracted with ether. The extract was dried over anhydrous magnesium sulfate, and the residue obtained by removing the solvent was dissolved in 50 ml of methylene chloride. 2 ml of pyridine and 2.4 ml of acetic anhydride were added to the solution, followed by stirring at room temperature for 1 hour. The reaction mixture was washed with 10% aqueous solution of hydrochloric acid, saturated aqueous solution of sodium bicarbonate, and saturated brine in this order, and dried over anhydrous magnesium sulfate. After removal of the solvent, the residue was crystallized in ethyl acetate and hexane to produce 2.47 gm of the title compound.

NMR(CDCl₃) δ : 1.75-2.11(2H, m), 1.98(3H, s), 2.49-2.58(1H, m), 2.79-3.32(3H, m), 3.80(3H, s), 4.21-4.37(1H, m), 6.62-6.76(2H, m), 7.12(1H, t, J=7.9Hz)

(2) 2-Acetylamino-8-methoxy-5-nitrotetraline

To 20 ml of acetic anhydride cooled to 0°C, was slowly added 1.75 ml fuming nitric acid and further was added a drop of concentrated sulfuric acid. To this solution was added 2.2 mg of the compound obtained in (1) above, and the mixture was stirred for 20 minutes. 40 ml of 25% aqueous solution of sodium hydroxide was added to the reaction mixture, followed by stirring for a further 30 minutes. The precipitate was collected by filtration and washed with water. The organic layer was dried over magnesium sulfate. The residue obtained by the concentration of the solvent was subjected to silica gel column chromatography using a chloroform-methanol (80:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 770 mg of the title compound.

mp: 207-210°C

NMR(CDCl₃) δ : 1.6-2.2(2H, m), 2.01(3H, s), 2.3-2.6(1H, m), 3.10-3.24(3H, m), 3.89(3H, s), 4.26(1H, m), 6.76(1H, d, J=9. Hz), 7.96(1H, d, J=8.9Hz)

(3) 2,5-Diacetylamino-8-methoxytetraline

320 mg of the compound prepared in (2) above was dissolved in a mixed solvent of 5 ml of acetic anhydride and palladium-on-carbon, the mixture was catalytically hydrogenated for 5 hours. After removal of the catalyst by filtration and concentration of the filtrate, the residue was crystallized from chloroform to produce 190 mg of the title compound.

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NMR(CDCL₃)δ: 1.6-2.2(2H, m), 1.96(3H, s), 2.16(3H, s), 2.4-2.9(4H, m), 3.80(3H, s), 4.0-4.4(1H, m), 6.68(1H, d, J=8.5Hz), 7.18(1H, d, J=8.5Hz)

(4) 3,8-Diacetylamino-5-methoxy-1-tetralone

190 mg of the compound prepared in (3) above was suspended into a mixture of 16 ml of acetone and 4 ml of 15% aqueous solution of magnesium sulfate, and to the solution was added 543 mg of potassium permanganate, followed by stirring for 1.5 hours at room temperature. After the addition of 150 ml of water, the reaction mixture was extracted with chloroform. The extract was washed with saturated brine and dried over magnesium sulfate. After removal of the solvent, the residue was crystallized in ethyl acetate and hexane to produce 124 mg of the title compound.

NMR(CDCL₃)δ: 1.96(3H, s), 2.21(3H, s), 2.6-3.4(4H, m), 3.84(3H, s), 4.4-4.7(1H, m), 7.11(1H, d, J=9.2Hz), 8.61(1H, d, J=9.2Hz)

(5) 3-Acetylamino-8-amino-5-methoxy-1-tetralone

102 mg of the compound prepared in (4) above was charged into 20 ml of 3 N hydrochloric acid aqueous solution, and the mixture was heated at 60°C for 2 hours while stirring. After cooling to 0°C, the reaction mixture was neutralized with sodium carbonate and extracted with chloroform. The extract was dried over magnesium sulfate, and subjected to silica gel column chromatography using a chloroform-methanol (40:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 66 mg of the title compound.

NMR(CDCL₃)δ: 1.94(3H, s), 2.51-3.34(4H, m), 3.76(3H, s), 4.06-4.75(1H, m), 6.55(1H, d, J=9.0Hz), 7.00(1H, d, J=9.0Hz)

(6) (9S)-2-Acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino [1,2-b]-quinoline-10,13(9H, 15H)-dione

126 mg of the compound prepared in (5) and 134 mg of triene were reacted for 24 hours and post-treated in the same manner as in Example 1-(5) to obtain 103 mg of the title compound.

IR_{max}^{KBr} cm⁻¹: 3392, 1748, 1660

NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.85(3H, s), 1.87-1.93(2H, m), 2.67-2.81(1H, m), 3.02-3.09(1H, m), 3.97(3H, s), 4.22(1H, br), 5.09-5.25(2H, m), 5.37-5.46(2H, m), 6.47(1H, br, s), 7.29(1H, s), 7.71(1H, d, J=9.3Hz), 8.03(1H, d, J=9.3Hz), 8.13(1H, br)

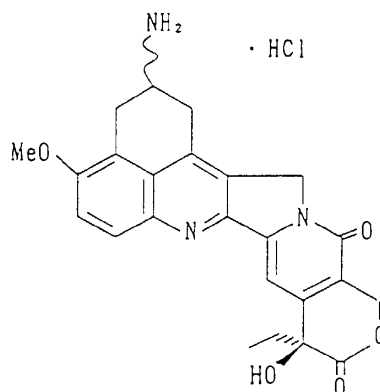
MASS m/z: 475(M⁺)

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Example 10

Preparation of (9S)-2-amino-9-ethyl-2,3-dihydro-9-hydroxy-4-methoxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino

[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

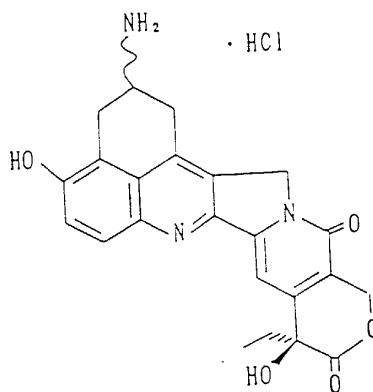


48 mg of the compound prepared in Example 9 was reacted and post-treated in the same manner as in Example 2 to obtain 42 mg of the title compound.

mp: above 240°C (decomposed)
 $\text{IR}_{\text{max}}^{\text{KBr}} \text{ cm}^{-1}$: 3440, 1744, 1658, 1592
 $\text{NMR}(\text{DMSO-d}_6)\delta$: 0.89(3H, t, $J=7.3\text{Hz}$), 1.83-1.93(2H, m), 2.95-3.02(1H, m), 3.26-3.33(1H, m), 3.76(1H, br), 4.00(3H, s), 5.15-5.26(2H, m), 5.43(2H, s), 7.03(1H, s), 7.77(1H, d, $J=9.3\text{Hz}$), 8.09(1H, d, $J=9.3\text{Hz}$), 8.55(3H, br)
 MASS m/z : 433(M^+)

Example 11

Preparation of (9S)-2-amino-9-ethyl-2,3-dihydro-4,9-dihydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 9 (46 mg) was reacted and post-treated in the same manner as in Example 5 to obtain 18 mg of the title compound.

mp: above 240°C (decomposed)
 $\text{IR}_{\text{max}}^{\text{KBr}} \text{ cm}^{-1}$: 3420, 1742, 1658, 1590

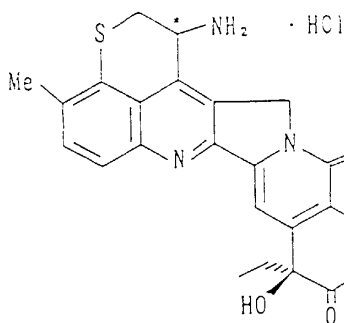
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NMR(DMSO- d_6) δ : 0.89(3H, t, $J=7.3$ Hz), 1.85-1.92(2H, m), 2.91-2.98(1H, m), 3.24-3.31(1H, m), 3.75(1H, br), 5.23(2H, d, $J=6.4$ Hz), 5.42(2H, s), 7.27(1H, s), 7.55(1H, d, $J=9.3$ Hz), 7.93(1H, d, $J=8.8$ Hz), 8.48(3H, br s), 10.5(1H, br s)

MASS m/z : 419(M^+)

Example 12

Preparation of (9S)-1-amino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride:



(1) 3,5-Diacetylamino-8-methyl-4-thiochromanone

To 3 ml of a THF solution containing 153 mg of potassium-*t*-butoxide was added 9 ml of a THF solution containing 291 mg of 5-acetylamino-8-methyl-4-thiochromanone [J. Heterocyclic Chem., 11, 515 (1974)]. After stirring for 5 minutes, 0.22 ml of *n*-butyl nitrite was added to the mixture, followed by further stirring for 1 hour at room temperature. After the addition of 20 ml of ether, the reaction mixture was stirred for another 1 hour. The deposited precipitate was collected by filtration, washed thoroughly with ether, and dissolved into a mixture of 20 ml of acetic acid and 20 ml of acetic anhydride. To the solution was added about 200 mg of zinc powder at room temperature while stirring. After the addition, the stirring was continued for a further 0.5 hour. Insoluble substances were removed by filtration, the solvent was evaporated, and 20 ml of chloroform was added to the residue. The residue, after washing it water, saturated aqueous solution of sodium hydroxide, and saturated brine in this order, was dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was subjected to silica gel column chromatography using a hexane-ethyl acetate (4:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 109 mg of the title compound.

NMR($CDCl_3$) δ : 2.12(3H, s), 2.23(3H, s), 2.26(3H, s), 3.06(1H, dd, $J=12.5$ Hz, 13.7Hz), 3.60(1H, dd, $J=4.4$ Hz, 12.5Hz), 4.94(1H, ddd, $J=4.4$ Hz, 4.9Hz, 13.7Hz), 6.82(1H, m), 7.29(1H, d, $J=8.8$ Hz), 8.39(1H, d, $J=8.8$ Hz)

(2) 3-Acetylamino-5-amino-8-methyl-4-thiochromanone

109 mg of the compound prepared in (1) above was added to 10 ml of 6 N hydrochloric acid, and the mixture was stirred at 90°C for 20 minutes. After cooling, the mixture was alkalized by the addition of sodium hydroxide and extracted with chloroform. The chloroform layer was dried over anhydrous sodium sulfate. The residue obtained after removal of the solvent was subjected to silica gel column chromatography using a chloroform-ethyl acetate (4:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 51 mg of the title compound.

NMR($CDCl_3$) δ : 2.09(3H, s), 2.16(3H, s), 2.98(1H, m), 3.60(1H, m), 4.6-5.0(1H, m), 6.33(1H, d, $J=9$ Hz), 7.02(1H, d, $J=9$ Hz)

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(3) (9S)-1-Amino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride

45 mg of the compound prepared in (2) above and 47 mg of trione was added to 10 ml of toluene. To this was added a catalytic amount of PPTS to react the mixture in a Deanstark apparatus under heating with refluxing for 15 hours. The reaction mixture was cooled and the solvent was evaporated. The residue was subjected to silica gel column chromatography using a chloroform-methanol (98:2) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated by evaporating the solvent. 10 ml of hydrochloric acid was added to the residue and the mixture was stirred for 4 hours. After concentration, 10 ml of water was added to the resulting product to remove insoluble substances by filtration. The filtrate was purified with HPLC (CAPCELL PAK C18: trademark, manufactured by Shiseido Co.) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:1) to obtain 37 mg of Isomer A and 36 mg of Isomer B of the title compound.

Isomer A

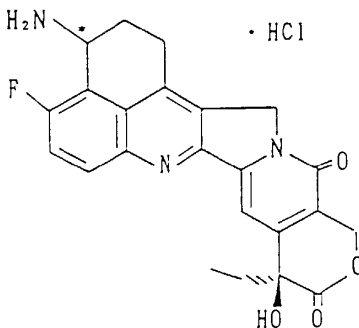
mp: above 210°C (decomposed)
NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.8-1.95(2H, m), 3.5-3.65(2H, m), 5.38(1H, m), 5.43, 5.89(2H, ABq, J=19.5Hz), 5.44(2H, s), 6.53(1H, s), 7.34(1H, s), 7.78(1H, d, J=8.3Hz), 7.96(1H, d, J=8.3Hz), 8.88(3H, m)

Isomer B

mp: above 224°C (decomposed)
NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.8-1.95(2H, m), 3.54(1H, dd, J=14.2Hz, 1.5Hz), 3.63(1H, dd, J=14.2Hz, 3.4Hz), 5.39(1H, m), 5.45(2H, m), 5.47, 5.92(2H, ABq, J=19.5Hz), 6.54(1H, s), 7.36(1H, s), 7.79(1H, d, J=8.8Hz), 7.97(1H, d, J=8.8Hz), 8.94(3H, m)

Example 13

Preparation of (9S)-3-amino-4-fluoro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



(1) Methyl 2-fluorocinnamate

15 gm of 2-fluorocinnamic acid was suspended into 100 ml of methanol. While maintaining the suspension at 0°C, 1.7 gm of thionyl chloride was added slowly. After the addition, the mixture was stirred for 30 minutes at 0°C and for 4 hour at room temperature. The residue obtained by the removal of the solvent was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 17.8 gm of the title compound.

NMR(CDCl₃)δ: 3.82(3H, s), 6.54(1H, d, J=16.2Hz), 6.97-7.63(4H, m), 7.82(1H, d, 16.2Hz)

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(2) Methyl 3-(2-fluorophenyl)propanoate

17.8 gm of the compound prepared in (1) above was dissolved in 200 ml of methanol. The mixture was catalytically hydrogenated using 6 gm of 10% palladium-on-carbon as a catalyst. After the removal of the catalyst by filtration and the solvent was evaporated to produce 14.7 gm of the title compound.

NMR(CDCl₃) δ : 2.53-2.72(2H, m), 2.8-3.1(2H, m), 3.67(3H, s), 6.97-7.28(4H, m)

(3) 3-(2-Fluorophenyl)propanol

2.2 gm of lithium aluminum hydride was suspended in 110 ml of dried THF and stirred for 1 hour at room temperature. To the suspension was slowly added over 15 minutes a solution of 5 gm of the compound prepared in (2) above in 50 ml of THF. The mixture was stirred for 12 hours at room temperature, and after the addition of 20 ml of ethyl acetate, for a further 3 hours. Then, 5 ml of saturated aqueous solution of ammonium chloride was added to the mixture, followed by further stirring for 30 minutes. After removing the precipitate by filtration, the filtrate was washed with water and then with saturated brine, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 4.27 gm of the title compound.

NMR(CDCl₃) δ : 1.6-2.1(2H, m), 2.75(2H, t, J=7.5Hz), 3.68(2H, t, J=6.34Hz), 6.8-7.6(4H, m)

(4) 3-(2-Fluorophenyl)propyl tosylate

17.75 gm of the compound prepared in (3) above was dissolved into 200 ml of dry pyridine. To this was slowly added 24.25 gm of p-toluenesulfonyl chloride at 0°C and the mixture was stirred for 3 hours at room temperature. After the addition of 300 ml of ether, the mixture was washed with water, followed by the addition of 6 N hydrochloric acid to acidify it and washing with saturated brine. The product was dried over anhydrous sodium sulfate and the solvent was evaporated to obtain 31.3 gm of the title compound.

NMR(CDCl₃) δ : 1.8-2.2(2H, m), 2.45(3H, s), 2.68(2H, t, J=7.8Hz), 4.04(2H, t, J=6.2Hz), 6.8-7.4(4H, m), 7.33(2H, d, J=8Hz), 7.79(2H, d, J=8Hz)

(5) 4-(2-Fluorophenyl)butanonitrile

To a solution of 9.89 gm of sodium cyanate in 35 ml of dimethylsulfoxide (hereinafter abbreviated as DMSO) was added dropwise a solution of 31.2 gm of the compound prepared in (4) above in 70 ml of DMSO. The mixture was stirred for 12 hours at room temperature. After the addition of 500 ml of water, the mixture was extracted twice with 200 ml of ether. The extract was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 15.4 gm of the title compound.

NMR(CDCl₃) δ : 1.8-2.2(2H, m), 2.15-2.42(2H, m), 2.81(2H, t, J=7.65Hz), 6.8-7.3(4H, m)

(6) 4-(2-Fluorophenyl)butanoic acid

15.4 gm of the compound prepared in (5) above was added to a mixture of 400 ml of 5% aqueous solution of sodium hydroxide and 400 ml of diethylene glycol monoethyl ether. The mixture was heated under reflux for 3.5 hours, cooled to room temperature, and washed with ether. After the addition of concentrated hydrochloric acid to acidify, the water layer was extracted twice with 500 ml of ethyl acetate. The extract was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 15.9 gm of the title compound.

NMR(CDCl₃) δ : 1.75-2.15(2H, m), 2.28-2.55(2H, m), 2.71(2H, t, J=7.43Hz), 6.8-7.4(4H, m)

(7) 5-Fluoro-1-tetralone

To 200 gm of polyphosphoric acid heated at 80°C was added 15.9 gm of the compound prepared in (6) above over 1 hour while stirring. The stirring was continued for another 1 hour at the same temperature. The resultant reaction mixture was poured into ice-cooled water and extracted with chloroform. The extract was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 14.1 gm

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of the title compound.

NMR(CDCI₃)δ: 2.16(2H, m), 2.67(2H, t, J=6.3Hz), 2.96(2H, t, J=6.3Hz), 7.20-7.31(2H, m), 7.84(1H, d, J=7.81Hz)

5 (8) 5-Fluoro-8-nitro-1-tetralone

14 gm of the compound prepared in (7) above was dissolved into 100 ml of concentrated sulfuric acid. After cooling the solution, a solution of 9.05 gm of potassium nitrate in 80 ml of concentrated sulfuric acid was added dropwise while maintaining the internal temperature below 5°C. The stirring was continued for a further 30 minutes. The resultant reaction mixture was poured into ice-cooled water and extracted with chloroform. The extract was washed with saturated aqueous solution of sodium bicarbonate, water, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and residue was recrystallized in methanol to obtain 9.15 gm of the title compound.

15 NMR(CDCI₃)δ: 2.18-2.24(2H, m), 2.74(2H, t, J=6.3Hz), 2.99(2H, t, J=6.3Hz), 7.29(1H, t, J=8.3Hz), 7.38(1H, dd, J=4.4Hz, 8.8Hz)

(9) 5-Fluoro-8-nitro-4-(1,3-dioxoisindoline-2-yl)-1-tetralone

20 323 mg of the compound prepared in (8) above, 323 mg of N-bromosuccinimide, and a catalytic amount of benzoyl peroxide were added to 20 ml of carbon tetrachloride. The mixture was heated under reflux for 4.5 hours, cooled to room temperature, and after the addition of 30 ml of chloroform, washed with cold 3% aqueous solution of sodium hydroxide, water, and saturated brine in this order, and dried over anhydrous sodium sulfate, followed by removal of the solvent by evaporation. The residue was dissolved into 15 ml of dimethylformamide (hereinafter abbreviated as DMF) and cooled to 0°C, followed by the addition of 100 mg of sodium azide, a bit at a time. The mixture was stirred for 30 minutes at 0°C and for a further 1 hour at room temperature, and after the addition of 30 ml of water, was extracted twice with ether, washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate, followed by removal of the solvent by evaporation. The residue was subjected to silica gel column chromatography using hexane-ethyl acetate (4:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 308 mg of 4-azide isomer. To the isomer were added 10 ml of benzene, 390 mg of triphenylphosphine, and 220 mg of phthal anhydride. The mixture was heated under reflux for 7 hours, and a further 12 hours after the addition of 37 mg of tetra-n-butylammonium cyanide. The mixture was cooled to room temperature, and after the addition of 20 ml of ethyl acetate, washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate, followed by removal of the solvent by evaporation. The residue was subjected to silica gel column chromatography using hexane-ethyl acetate (2:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 395 mg of the title compound.

40 NMR(CDCI₃)δ: 2.51-2.62(2H, m), 2.78-2.84(1H, m), 3.06-3.10(1H, m), 5.87(1H, t, J=5.0Hz), 7.28(1H, t, J=8.8Hz), 7.51(1H, dd, J=4.4Hz, 8.8Hz), 7.74-7.78(2H, m), 7.82-7.87(2H, m)

(10) 8-Amino-5-fluoro-4-(1,3-dioxoisindoline-2-yl)-1-tetralone

45 To 320 mg of the compound prepared in (9) above were added 9 ml of dioxane and 15 ml of ethanol. The mixture was catalytically hydrogenated with 290 mg of 10% palladium-on-carbon. The catalyst was removed by filtration, the filtrate was concentrated, and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (2:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 206 mg of the title compound.

50 NMR(CDCI₃)δ: 2.3-2.55(2H, m), 2.61-2.70(1H, m), 2.85-2.30(1H, m), 5.78(1H, m), 6.59(1H, dd, J=4.4Hz, 8.8Hz), 6.96(1H, t, J=8.8Hz), 7.65-7.75(2H, m), 7.75-7.85(2H, m)

(11) (9S)-9-Ethyl-4-fluoro-2,3-dihydro-9-hydroxy-3-(1,3-dioxoisindolin-2-yl)-1H,12H-benzo[de]pyrano[3',4': 6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

55 10 ml of toluene, 74 mg of the compound prepared in (10) above, and 66 mg of trione were reacted for 15 hours and treated in the same manner as in Example 1-(5) to obtain 120 mg of the title compound.

NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.3Hz), 1.89(2H, m), 2.36(1H, m), 2.45-2.5(1H, m), 3.25-3.4(2H, m), 5.34(2H, s),

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5.45(2H, s), 5.99(1H, m), 7.36(0.5H, s), 7.37(0.5H, s), 7.69(1H, t, J=9.3Hz), 7.86(4H, s), 8.18(1H, dd, J=5.4Hz, 9.3Hz)

(12) (9S)-3-Amino-4-fluoro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride

120 mg of the compound prepared in (11) above was dissolved in a mixed solvent of 7 ml of chloroform and 5 ml of methanol. After the addition of 0.7 ml of hydrazine monohydrate, the mixture was heated under reflux for 1 hour. The reaction mixture was concentrated to dryness and to the residue was added 7 ml of 4 N hydrochloric acid, and the mixture was heated under reflux for 1 hour. After concentration to dryness, 10 ml of water to the residue to remove the insoluble substances by filtration. The filtrate was purified in the same manner as in Example 7 to obtain 32 mg of Isomer A and 20 mg of Isomer B of the title compound.

Isomer A

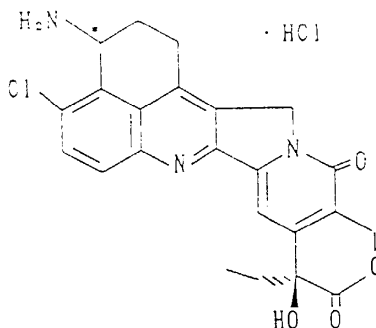
mp: above 196°C (decomposed)
NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.3Hz), 1.87(2H, m), 2.15-2.30(1H, m), 2.4-2.6(1H, m), 3.3-3.4(2H, m), 5.08(1H, m), 5.26, 5.40(2H, ABq, J=19Hz), 5.44(2H, s), 7.35(1H, s), 7.88(1H, t, J=9.3Hz), 8.30(1H, dd, J=5.5Hz, 9.3Hz), 8.70(3H, m)

Isomer B

mp: above 215°C (decomposed)
NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.88(2H, m), 2.15-2.30(1H, m), 2.4-2.6(1H, m), 3.3-3.4(2H, m), 5.08(1H, m), 5.28, 5.40(2H, ABq, J=19Hz), 5.45(2H, s), 7.36(1H, s), 7.89(1H, t, J=9.3Hz), 8.30(1H, dd, J=5.4Hz, 9.3Hz), 8.65(3H, m)

Example 14

Preparation of (9S)-3-amino-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4': 6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



(1) 5-Chloro-8-nitro-4-(1,3-dioxoisindoline-2-yl)-1-tetralone

403 mg of 5-chloro-8-nitro-1-tetralone, 412 mg of N-bromosuccinimide, and a catalytic amount of benzoyl peroxide were added to 20 ml of carbon tetrachloride. The mixture was heated under reflux for 6 hours, cooled to room temperature, and after the addition of 30 ml of chloroform, washed with cold 3% aqueous solution of sodium hydroxide, water, and saturated brine in this order, and dried over anhydrous sodium sulfate, followed by removal of the solvent by evaporation. The residue was dissolved into 10 ml of DMF and cooled to 0°C, followed by the addition of 140 mg of sodium azide, a bit at a time. The mixture was stirred for 30 minutes at 0°C and for a further 1 hour at room temperature, and after the addition of 30 ml of water, was extracted twice with ether, washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate, followed by removal of the solvent by evaporation. The residue was

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subjected to silica gel column chromatography using hexane-ethyl acetate (4:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 450 mg of 4-azide isomer. To the isomer were added 20 ml of benzene, 487 mg of triphenylphosphine, and 275 mg of phthal anhydride. The mixture was heated under reflux for 7 hours, and a further 12 hours after the addition of 40 mg of tetra-n-butylammonium cyanide. After evaporating the solvent, the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (2:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 356 mg of the title compound.

NMR(CDCl₃)δ: 2.40-2.48(1H, m), 2.56-2.67(1H, m), 2.75-2.83(1H, m), 2.96-3.75(1H, m), 5.83(1H, dd, J=2.9Hz, 4.9Hz), 7.46(1H, d, J=8.3Hz), 7.63(1H, d, J=8.3Hz), 7.74-7.78(2H, m), 7.82-7.84(2H, m)

(2) 8-Amino-5-chloro-4-(1,3-dioxoisindoline-2-yl)-1-tetralone.

To 320 mg of the compound prepared in (1) above were added 9 ml of dioxane and 15 ml of ethanol. The mixture was catalytically hydrogenated with 200 mg of 10% palladium-on-carbon. The catalyst was removed by filtration, the filtrate was concentrated, and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (2:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 176 mg of the title compound.

NMR(CDCl₃)δ: 2.4-3.3(4H, m), 5.72(1H, m), 6.60(1H, d, J=8.3Hz), 7.17(1H, d, J=8.3Hz), 7.6-7.9(4H, m)

(3) (9S)-3-Amino-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride

15 ml of toluene, 176 mg of the compound prepared in (2) above, and 135 mg of trione were reacted for 16 hours and post-treated to obtain 193 mg of (9S)-9-ethyl-4-chloro-2,3-dihydro-9-hydroxy-3-(1,3-dioxoisindoline-2-yl)-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione as a mixture of 3-position isomer. 171 mg of this mixture was processed in the same manner as in Example 13-(12) to obtain 63 mg of Isomer A and 59 mg of Isomer B of the title compound.

Isomer A

mp: above 190°C (decomposed)

NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.86(2H, m), 2.24(1H, m), 2.61(1H, m), 3.2-3.5(2H, m), 5.09(1H, m), 5.23,5.39(2H, ABq, J=19.5Hz), 5.44(2H, s), 7.35(1H, s), 7.98(1H, d, J=9.3Hz), 8.23(1H, d, J=9.3Hz), 8.69(3H, m)

Isomer B

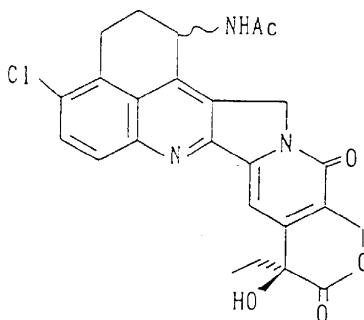
mp: above 215°C (decomposed)

NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.88(2H, m), 2.24(1H, m), 2.54(1H, m), 3.3-3.5(2H, m), 5.12(1H, m), 5.27, 5.42(2H, ABq, J=19.0Hz), 5.45(2H, s), 7.36(1H, s), 8.00(1H, d, J=9.3Hz), 8.26(1H, d, J=9.3Hz), 8.56(3H, m)

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Example 15

Preparation of (9S)-1-acetylaminoethyl-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 8-Acetylamino-5-chloro-2-hydroxymethylene-1-tetralone

960 mg of 60% aqueous solution of sodium hydride was added to 8 ml of ethyl formate under ice-cooling, and 5 minutes after the mixture was cooled to room temperature, a solution of 1.43 gm of 8-acetylamino-5-chloro-1-tetralone dissolved in 30 ml of dimethoxyethane was slowly added. After the addition of 0.06 ml of ethanol, the mixture was stirred for 30 minutes. The reaction mixture was poured into 300 ml of 14% aqueous solution of sodium chloride, extracted with ethyl acetate, washed with 10% aqueous solution of citric acid and saturated brine, and dried over anhydrous sodium sulfate. After concentration, ether was added to the residue to collect the precipitate of 1.30 gm of the title compound by filtration.

IR_v^{KBr} cm^{-1} : 1650, 1574, 1502, 1194
 NMR(CDCl₃) δ : 2.23(s, 3H), 2.49(t, 2H, J=7Hz), 3.03(t, 2H, J=7Hz), 7.50(d, 1H, J=9Hz), 8.56(d, 1H, J=9Hz),
 MASS m/z: 265(M⁺), 267(M⁺ + 2)

(2) 9-Acetylamino-6-chloro-4,5-dihydronaphtho[1,2-d]-isoxazole

1.29 gm of the compound prepared in (1) above was dissolved into 30 ml of acetic acid. After the addition of 339 mg of hydroxylamine hydrochloride, the mixture was heated for 10 minutes at 120°C while stirring. After cooling, water was added to collect the precipitate by filtration. The precipitate was washed with water and hexane to obtain 1.1 gm of the title compound.

IR_v^{KBr} cm^{-1} : 1664, 1524, 1390, 1288
 NMR(CDCl₃) δ : 2.29(s, 3H), 2.80(t, 2H, J=8Hz), 3.18(t, 2H, J=8Hz), 7.37(d, 1H, J=9Hz), 8.25(s, 1H), 8.30(d, 1H, J=9Hz), 8.80(s, 1H)
 MASS m/z: 262(M⁺), 264(M⁺ + 2)

(3) 8-Acetylamino-5-chloro-2-cyano-1-tetralone

To a solution obtained by dissolving 1.54 gm of the compound prepared in (1) above in 20 ml of anhydrous ethanol, was slowly added a solution of 460 mg of sodium methoxide in 50 ml of anhydrous ethanol. After stirring 3 hours at room temperature, 10 ml of 1 N hydrochloric acid and water were added to the reaction mixture, followed by extraction with chloroform. The extract was washed with saturated brine, dried over anhydrous sodium sulfate, and concentrated to obtain 1.36 gm of the title compound.

IR_v^{KBr} cm^{-1} : 2251, 1702, 1658, 1598, 1522
 NMR(CDCl₃) δ : 2.26(s, 3H), 2.3-2.7(m, 2H), 2.9-3.5(m, 2H), 3.83(dd, 1H, J=12Hz, 5Hz), 7.61(d, 1H, J=9Hz), 8.70(d, 1H, J=9Hz), 11.56(s, 1H)
 MASS m/z: 262(M⁺), 264(M⁺ + 2)

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(4) 8-Acetylaminomethyl-2-acetylaminomethyl-5-chloro-1-tetralone

1.36 gm of the compound prepared in (3) above was reacted in the same manner as in Example 28-(3) and post-treated to obtain 1.20 gm of the title compound.

NMR(CDCI₃)δ: 2.01(s, 3H), 2.25(s, 3H), 1.7-2.4(m, 2H), 2.6-3.0(m, 2H), 3.1-3.6(m, 2H), 3.6-3.8(m, 1H), 6.23(br. s, 1H), 7.52(d, 1H, J=9Hz), 8.60(d, 1H, J=9Hz), 11.89(s, 1H)
 MASS m/z: 308(M⁺), 310(M⁺ + 2)

(5) 2-Acetylaminomethyl-8-amino-5-chloro-1-tetralone

The procedure of Example 1-(4) was followed by using 1.20 gm of the compound prepared in (3) above, instead of 2,8-diacetylaminomethyl-1-tetralone. The reaction product was post-treated to obtain 1.20 gm of the title compound.

NMR(CDCI₃)δ: 1.98(s, 3H), 1.7-2.3(m, 2H), 2.5-3.0(m, 2H), 3.0-3.5(m, 2H), 3.7-3.9(m, 1H), 6.37(br.s, 2H), 6.48(d, 1H, J=9Hz), 7.23(d, 1H, J=9Hz)
 MASS m/z: 266(M⁺), 268(M⁺ + 2)

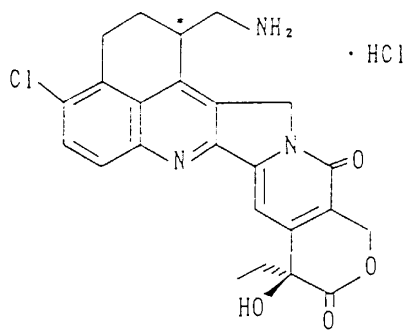
(6) (9S)-1-Acetylaminomethyl-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino [1,2-b]quinoline-10,13(9H,15H)-dione

820 mg of the compound prepared in (5) above and 808 mg of trione were reacted for 24 hours in the same manner as in Example 1-(5). The reaction mixture was concentrated and the residue was subjected to silica gel column chromatography using chloroform-methanol (40:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 420 mg of the title compound.

NMR(DMSO-d₆)δ: 0.88(t, 3H, J=7Hz), 1.87(s, 3H), 1.7-2.0(m, 2H), 2.2-3.6(m, 7H), 5.37(s, 2H), 5.45(s, 2H), 6.52(br. s, 2H), 7.34(s, 1H), 7.88(d, 1H, J=9Hz), 8.04(d, 1H, J=9Hz)
 MASS m/z: 493(M⁺), 495(M⁺ + 2)

Example 16

Preparation of (9S)-1-aminomethyl-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



100 mg of the compound prepared in Example 15-(6) was reacted in the same manner as in Example 2 and post-treated to obtain 25 mg of Isomer A and 22 mg of Isomer B of the title compound.

Isomer A

mp: 230-240°C (decomposed)

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NMR(DMSO- d_6) δ : 0.88(t, 3H, J=7Hz), 1.8-2.0(m, 2H), 1.9-3.8(m, 7H), 5.32, 5.48(ABq, 2H, J=17Hz), 5.46(s, 2H), 6.56 (br.s, 1H), 7.35(s, 1H), 7.91(d, 1H, J=9Hz), 8.07(d, 1H, J=9Hz) 8.14(s, 3H)

MASS m/z: 451(M^+), 453($M^+ + 2$)

5 Isomer B

mp: 250-255°C (decomposed)

NMR(DMSO- d_6) δ : 0.88(t, 3H, J=7Hz), 1.8-2.0(m, 2H), 1.9-3.8(m, 7H), 5.32, 5.48(ABq, 2H, J=19Hz), 5.45(s, 2H), 6.57 (br.s, 1H), 7.35(s, 1H), 7.90(d, 1H, J=9Hz), 8.06(d, 1H, J=9Hz)

10 MASS m/z: 451(M^+), 453($M^+ + 2$)

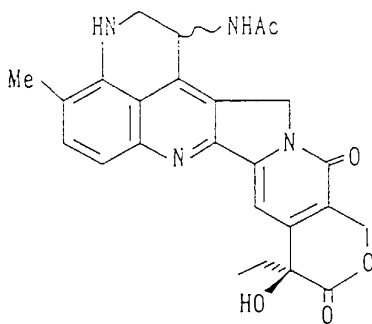
Example 17

Preparation of (9S)-1-acetylamino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c] benzo[ij][2,7]naphthyridine-10,13(9H,15H)-dione

20

25

30



(1) 4-Acetyl-5-acetylamino-8-methyl-2,3-dihydroquinoline-4-one

35 To a solution of 7.0 gm of 5-amino-8-methyl-2,3-dihydroquinoline-4-one (described in Japanese Patent Laid-open (ko-kai) 279891/1989) in a mixed solvent of 30 ml of dichloromethane and 80 ml of dioxane was added 15 ml of acetyl chloride, and the mixture was heated under reflux for 2 hours. 200 ml of ethyl acetate was added to the residue obtained by removing the solvent, and mixture was washed with saturated aqueous solution of sodium bicarbonate and saturated brine, and dried over anhydrous sodium sulfate. After concentration, the residue was recrystallized from ether to obtain 7.45 gm of the title compound.

40

NMR(CDC $_3$) δ : 1.95(s, 3H), 2.23(s, 3H), 2.27(s, 3H), 2.0-5.5(m, 5H), 7.45(d, 1H, J=8Hz), 8.59(d, 1H, J=8Hz)

(2) 1-Acetyl-5-acetylamino-3-hydroxyimino-8-methyl-2,3-dihydroquinoline-4-one

45 The procedure of Example 1-(2) was followed by using 2.6 gm of the compound prepared in (1) above instead of 8-acetylamino-1-tetralone. The reaction product was post-treated to obtain 2.89 gm of the title compound.

mp: 195-205°C (decomposed)

50 IR $_{\max}^{\text{KBR}}$ cm^{-1} : 1674, 1590, 1518, 1404

NMR(CDC $_3$) δ : 2.24(s, 9H), 1.8-5.5(m, 3H), 7.54(d, 1H, J=8Hz), 8.62(d, 1H, J=8Hz)

MASS m/z: 289(M^+)

(3) 1-Acetyl-3,5-diacetylamino-8-methyl-2,3-dihydroquinoline-4-one

55 The procedure of Example 1-(3) was followed by using 2.89 gm of the compound prepared in (2) above instead of 8-acetylamino-2-hydroxyimino-1-tetralone. The reaction product was post-treated to obtain 1.65 gm of the title compound.

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mp: 216-221°C (decomposed)
 IR_v^{KBr} cm^{-1} : 1662, 1594, 1518
 NMR(CDCl₃) δ : 2.12(s, 3H), 2.23(s, 6H), 2.54(s, 3H), 3.3-5.0(m, 1H), 6.1-6.6(br. s, 1H), 7.44(d, 1H, J=8Hz), 7.60(d, 1H, J=8Hz)
 5 MASS m/z: 317(M⁺)

(4) 3-Acetylamino-5-amino-8-methyl-2,3-dihydroquinoline-4-one

The procedure of Example 1-(4) was followed by using 1.40 gm of the compound prepared in (3) above instead of 2,8-diacetylamino-1-tetralone. The reaction product was post-treated to obtain 0.79 gm of the title compound.

NMR(CDCl₃) δ : 2.01(s, 3H), 2.08(s, 3H), 3.0-4.5(m, 3H), 5.89(d, 1H, J=8Hz), 6.95(d, 1H, J=8Hz) 5.7-6.8(br.s, 2H)
 MASS m/z: 233(M⁺)

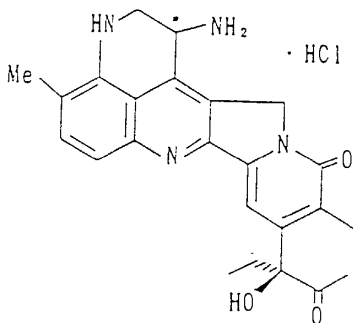
15 (5) (9S)-1-Acetylamino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo[ij][2,7]naphthyridine-10,13(9H,15H)-dione

900 mg of trione, 3 mg of PPTS, and 20 ml of acetic acid were added to 790 mg of the compound prepared in (4) above, and the mixture was heated in a nitrogen stream at 100°C for 7 hours while stirring. The reaction mixture was concentrated, and the residue was subjected to silica gel column chromatography using chloroform-methanol (20:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 210 mg of the title compound.

mp: 225-235°C
 25 IR_v^{KBr} cm^{-1} : 1746, 1658, 1596, 1156
 NMR(DMSO-d₆) δ : 0.87(t, 3H, J=7Hz), 1.8-2.0(m, 2H), 1.90, 1.91(each s, 3H), 2.30(s, 3H), 5.16, 5.25(ABq, 2H, J=18Hz), 5.42(s, 2H), 5.3-5.6(m, 1H), 6.17(br.s, 1H), 7.26(s, 1H), 7.36(d, 1H, J=8Hz), 7.52(d, 1H, J=8Hz)
 MASS m/z: 460(M⁺)
 30

Example 18

Preparation of (9S)-1-amino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo-[ij][2,7]naphthyridine-10,13(9H,15H)-dione hydrochloride



50 The compound obtained in Example 17-(5) (400 mg) was reacted for 4 hours in the same manner as in Example 2 and post-treated to produce 80 mg of Isomer A and 55 mg of Isomer B of the title compound.

Isomer A

mp: 230-250°C (decomposed)
 55 IR_v^{KBr} cm^{-1} : 1756, 1658, 1614
 NMR(DMSO-d₆) δ : 0.88(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 2.35(s, 3H), 3.50, 3.87(each d, 1H, J=12Hz), 5.0-5.2(m, 1H),

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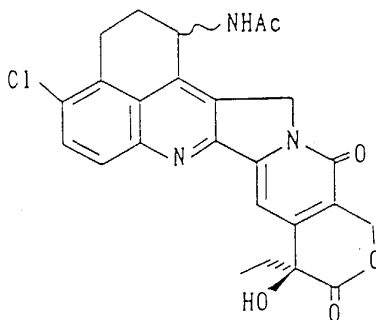
5.44(s, 2H), 5.37, 5.83(ABq, 2H, J=10Hz), 7.31(s, 1H), 7.44(d, 1H, J=8Hz), 7.59(d, 1H, J=8Hz), 8.77(br.s, 3H)
 MASS m/z: 418(M⁺)

5 Isomer B

mp: 220-240°C (decomposed)
 IR_{max}^{KBr} cm⁻¹: 1746, 1658, 1592
 NMR(DMSO-d₆)δ: 0.88(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 2.35(s, 3H), 3.50, 3.86(each d, 1H, J=12Hz), 5.0-5.2(m, 1H), 5.45(s, 2H), 5.39, 5.79(ABq, 2H, J=9Hz), 6.3-6.5(br, 1H), 6.54(s, 1H), 7.32(s, 1H), 7.45(d, 1H, J=8Hz), 7.60(d, 1H, J=8Hz), 8.70(br. s, 3H)
 MASS m/z: 418(M⁺)

Example 19

Preparation of (9S)-1-acetyl-amino-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 2,8-Diacetyl-5-chloro-1-tetralone

The reaction was carried out in the same manner as in Example 1-(2), except that 600 mg of 8-acetyl-amino-5-chloro-1-tetralone was used instead of 8-acetyl-amino-1-tetralone of Example 1-(2). Crude 8-acetyl-amino-5-chloro-2-hydroxyimino-1-tetralone which was obtained by the post-treatment was reacted in the same manner as in Example 1-(3) and post-treated to produce 304 mg of the title compound.

IR_{max}^{KBr} cm⁻¹: 3296, 1652, 1574, 1464
 NMR(CDCl₃)δ: 1.75-2.04(1H, m), 2.12(3H, s), 2.23(3H, s), 2.70-3.18(3H, m), 4.55-4.83(1H, m), 6.4(1H, br), 7.55(1H, d, J=9.0Hz), 8.61(1H, d, 9.2Hz)
 MASS m/z: 294(M⁺)

(2) 2-Acetyl-amino-8-amino-5-chloro-1-tetralone

The reaction was carried out in the same manner as in Example 1-(4), except that 270 mg of the compound prepared in (1) above was used instead of 2,8-diacetyl-amino-1-tetralone of Example 1-(4), and post-treated to produce 160 mg of the title compound.

IR_{max}^{KBr} cm⁻¹: 3448, 1614, 1458
 NMR(CDCl₃)δ: 1.73-1.87(1H, m), 2.09(3H, s), 2.66-3.15(3H, m), 4.53-4.74(1H, m), 6.50(1H, d, J=9.0Hz), 7.26(1H, d, J=9.0Hz)
 MASS m/z: 252(M⁺)

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(3) (9S)-1-Acetylamino-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

130 mg of the compound prepared in (2) above and 136 mg of trione were reacted for 24 hours in the same manner as in Example 1-(5) and post-treated to produce 174 mg of the title compound.

mp: above 240°C (decomposed)

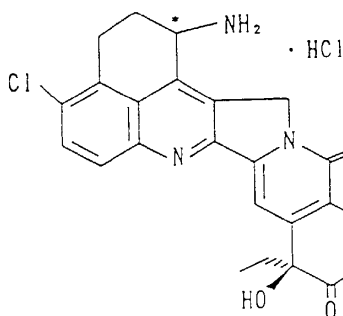
IR_{max}^{KBr} cm⁻¹: 3416, 1660, 1494

NMR(DMSO-d₆)δ: 0.86-0.90(3H, m), 1.85-1.89(2H, m), 1.91(3/2H, s), 1.92(3/2H, s), 2.16(2H, br s), 3.24(2H, br s), 5.18-5.30(2H, m), 5.43(2H, s), 5.57-5.62(1H, m), 6.52(1H, s), 7.33(1H, s), 7.89(1H, d, J=8.8Hz), 8.05(1H, d, J=8.8Hz), 8.46-8.50(1H, m)

MASS m/z: 479(M⁺)

Example 20

Preparation of (9S)-1-amino-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 19-(3) (124 mg) above was reacted for 6 hours in the same manner as in Example 2 and post-treated to produce Isomer A (8.2 mg) and Isomer B (8.2 mg) of the title compound.

Isomer A

mp: above 240°C (decomposed)

NMR(DMSO-d₆)δ: 0.89(3H, t, J=6.8Hz), 1.81-1.92(2H, m), 2.18-2.25(1H, m), 3.16-3.25(1H, m), 5.13(1H, br), 5.45-5.49(3H, m), 5.95(1H, d, J=19Hz), 6.56(1H, s), 7.37(1H, s), 7.97(1H, d, J=9.3Hz), 8.13(1H, d, J=9.3Hz), 8.79(1H, br)

Isomer B

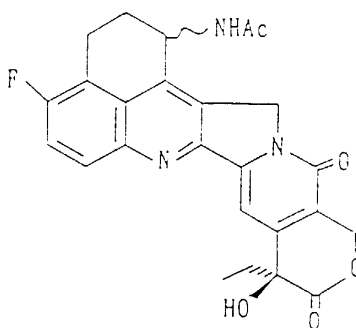
mp: above 240°C (decomposed)

NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.81-1.92(2H, m), 2.18-2.25(1H, m), 3.16-3.25(1H, m), 5.14(1H, br), 5.46-5.50(3H, m), 5.90(1H, d, J=19Hz), 6.56(1H, s), 7.38(1H, s), 7.98(1H, d, J=9.3Hz), 8.14(1H, d, J=9.3Hz), 8.66(1H, br)

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Example 21

Preparation of (9S)-1-acetylamino-9-ethyl-4-fluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 2,8-Diacetyl-5-fluoro-1-tetralone

The reaction was carried out in the same manner as in Example 1-(2), except that 600 mg of 8-acetylamino-5-fluoro-1-tetralone was used instead of 8-acetylamino-1-tetralone of Example 1-(2). Crude 8-acetylamino-5-fluoro-2-hydroxyimino-1-tetralone which was obtained by the post-treatment was reacted in the same manner as in Example 1-(3) and post-treated to produce 372 mg of the title compound.

IR_{max}^{KBr} cm⁻¹: 3264, 1698, 1614, 1538, 1400
 NMR(CDCl₃)δ: 1.83-2.04(1H, m), 2.11(3H, s), 2.22(3H, s), 2.65-3.19(3H, m), 4.61-4.82(1H, m), 6.5(1H, br s), 7.26(1H, t, J=9.2Hz), 8.62(1H, dd, J=4.6, 9.6Hz)
 MASS m/z: 278(M⁺)

(2) 2-Acetylamino-8-amino-5-fluoro-1-tetralone

The reaction was carried out in the same manner as in Example 1-(4), except that 300 mg of the compound prepared in (1) above was used instead of 2,8-diacetylamino-1-tetralone of Example 1-(4), and post-treated to produce 182 mg of the title compound.

IR_{max}^{KBr} cm⁻¹: 3428, 2960, 1622, 1472
 NMR(CDCl₃)δ: 1.73-1.88(1H, m), 2.10(3H, s), 2.69-3.08(3H, m), 4.49-4.69(1H, m), 6.43-6.54(1H, m), 7.06(1H, t, J=8.8Hz),
 MASS m/z: 236(M⁺)

(3) (9S)-1-Acetylamino-9-ethyl-4-fluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione

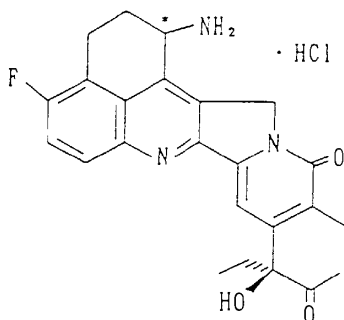
160 mg of the compound prepared in (2) above and 179 mg of trione were reacted for 24 hours in the same manner as in Example 1-(5) and post-treated to produce 224 mg of the title compound.

mp: above 240°C (decomposed)
 IR_{max}^{KBr} cm⁻¹: 3292, 2944, 1750, 1660, 1598
 NMR(DMSO-d₆)δ: 0.88(3H, t, J=8.8Hz), 1.82-1.92(2H, m), 1.93(3H, s), 2.09-2.14(2H, m), 3.16-3.19(2H, m), 5.18-5.29(2H, m), 5.43(2H, s), 5.57-5.62(1H, m), 6.51(1H, s), 7.33(1H, s), 7.78(1H, t, J=9.3Hz), 8.11(1H, dd, J=5.4, 9.3Hz), 8.51(1H, d, J=8.8Hz)
 MASS m/z: 463(M⁺)

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Example 22

Preparation of (9S)-1-amino-9-ethyl-4-fluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino
[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 21-(3) (180 mg) above was reacted for 5 hours in the same manner as in Example 2 and post-treated to produce Isomer A (10 mg) and Isomer B (14 mg) of the title compound.

Isomer A

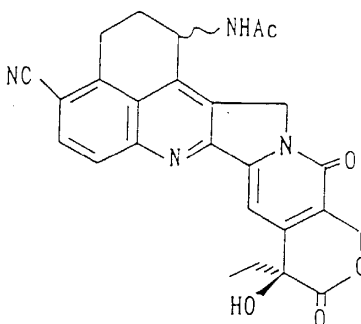
mp: above 240°C (decomposed)
NMR(DMSO- d_6) δ : 0.89(3H, t, $J=7.3$ Hz), 1.81-1.94(2H, m), 2.13-2.21(1H, m), 3.14-3.22(1H, m), 5.13(1H, br), 5.43-5.48(3H, m), 5.92(1H, d, $J=19$ Hz), 6.55(1H, s), 7.37(1H, s), 7.85(1H, t, $J=9.3$ Hz), 8.18(1H, dd, $J=5.4, 9.3$ Hz), 8.68(1H, br)

Isomer B

mp: above 240°C (decomposed)
NMR(DMSO- d_6) δ : 0.89(3H, t, $J=7.3$ Hz), 1.83-1.93(2H, m), 2.16-2.20(1H, m), 3.15-3.25(1H, m), 5.16(1H, br), 5.45-5.50(3H, m), 5.95(1H, d, $J=19$ Hz), 7.37(1H, s), 7.85(1H, t, $J=9.3$ Hz), 8.19(1H, dd, $J=5.4, 9.3$ Hz), 8.81(1H, br)

Example 23

Preparation of (9S)-1-acetylamino-4-cyano-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



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(1) 2,8-Diacetyl-5-cyano-1-tetralone

The reaction was carried out in the same manner as in Example 1-(2), except that 1 gm of 8-acetylamino-5-cyano-1-tetralone was used instead of 8-acetylamino-1-tetralone of Example 1-(2). Crude 8-acetylamino-5-cyano-2-hydroxyimino-1-tetralone which was obtained by the post-treatment was reacted in the same manner as in Example 1-(3) and post-treated to produce 270 mg of the title compound.

IR_{v_{max}}^{KBr} cm⁻¹ : 3292, 2228, 1708, 1666, 1588, 1518
 NMR(CDCl₃)δ: 1.71-2.05(1H, m), 2.12(3H, s), 2.27(3H, s), 2.66-3.43(3H, m), 4.60-4.87(1H, m), 6.3(1H, br), 7.77(1H, d, J=8.7Hz), 8.78(1H, d, J=9.0Hz)
 MASS m/z: 285(M⁺)

(2) 2-Acetylamino-8-amino-5-cyano-1-tetralone

The reaction was carried out in the same manner as in Example 1-(4), except that 250 mg of the compound prepared in (1) above was used instead of 2,8-diacetylamino-1-tetralone of Example 1-(4), and post-treated to produce 182 mg of the title compound.

IR_{v_{max}}^{KBr} cm⁻¹ : 3428, 3336, 2216, 1652, 1614, 1542
 NMR(CDCl₃)δ: 1.6-2.0(1H, m), 2.09(3H, s), 2.56-3.39(3H, m), 4.51-4.71(1H, m), 6.59(1H, d, J=9.0Hz), 7.40(1H, d, J=9.0Hz)
 MASS m/z: 243(M⁺)

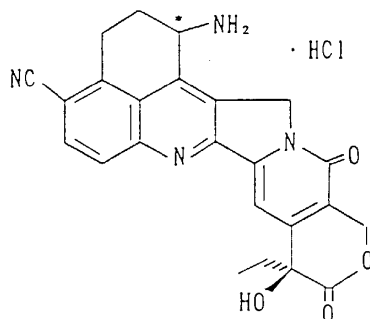
(3) (9S)-1-Acetylamino-4-cyano-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

165 mg of the compound prepared in (2) above and 179 mg of trione were reacted for 43 hours in the same manner as in Example 1-(5) and post-treated to produce 135 mg of the title compound.

mp: above 240°C (decomposed)
 IR_{v_{max}}^{KBr} cm⁻¹ : 3296, 2940, 2228, 1752, 1662, 1602
 NMR(DMSO-d₆)δ: 0.86-0.90(3H, m), 1.81-1.91(2H, m), 1.93(3/2H, s), 1.94(3/2H, s), 2.16-2.34(2H, m), 3.38-3.51(2H, m), 5.20-5.31(2H, m), 5.43-5.44(2H, m), 5.61-5.66(1H, m), 6.55(1H, s), 7.37(1/2H, s), 7.38(1/2H, s), 8.09(1H, d, J=8.8Hz), 8.14(1H, d, J=8.8Hz), 8.51-8.54(1H, m)
 MASS m/z: 470(M⁺)

Example 24

Preparation of (9S)-1-amino-4-cyano-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 21-(3) (124 mg) above was reacted for 3 hours in the same manner as in Example 2 and post-treated to produce Isomer A (29 mg) and Isomer B (30 mg) of the title compound.

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Isomer A

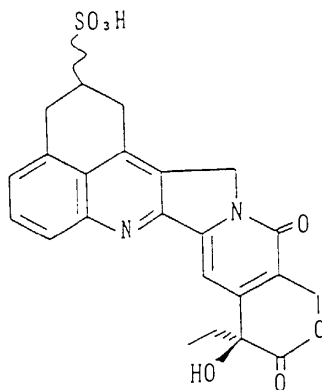
mp: above 240°C (decomposed)
 IR_{v^{max}}^{KBr} cm⁻¹: 3428, 2928, 2232, 1740, 1660, 1600
 5 NMR(DMSO-d₆)δ: 0.89(3H, t, J=6.8Hz), 1.87-1.91(2H, m), 2.2-2.4(1H, m), 2.6-2.7(1H, m), 3.4-3.6(2H, m), 5.18(1H, br), 5.46-5.51(3H, m), 5.96(1H, d, J=19Hz), 6.58(1H, s), 7.42(1H, s), 8.17(1H, d, J=8.8Hz), 8.22(1H, d, J=8.8Hz), 8.79(1H, br)
 MASS m/z: 428(M⁺)

10 Isomer B

mp: above 240°C (decomposed)
 IR_{v^{max}}^{KBr} cm⁻¹: 2884, 2228, 1754, 1658, 1590
 15 NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.83-1.94(2H, m), 2.26-2.33(1H, m), 2.54-2.68(1H, m), 3.38-3.57(2H, m), 5.20(1H, br), 5.47-5.52(3H, m), 5.97(1H, d, J=19Hz), 6.58(1H, s), 7.38(1H, s), 8.18(1H, d, J=8.8Hz), 8.23(1H, d, J=8.8Hz), 8.85(1H, br)
 MASS m/z: 428(M⁺)

Example 25

20 Preparation of (9S)-9-ethyl-9-hydroxy-2-sulfonyl-2,3-dihydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



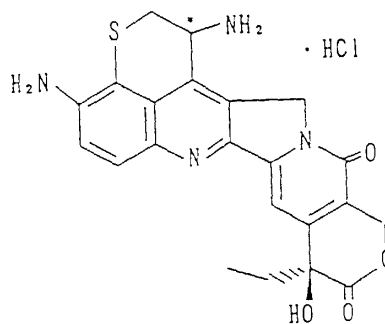
45 500 mg of 3-sulfonyl-8-amino-tetralone [Ann. 638, 43-56 (1960)] and 355 mg of trione was reacted for 16 hours in the same manner as in Example 17-(5). After cooling, the reaction mixture was concentrated, the residue was diluted with water to submit it to Diaion HP-20 column chromatography using 25% aqueous ethanol as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 220 mg of the title compound.

mp: 190-200°C (decomposed)
 IR_{v^{max}}^{KBr} cm⁻¹: 1744, 1660, 1164, 1038
 50 NMR(DMSO-d₆)δ: 0.89(t, 3H, J=7Hz), 1.8-2.0(m, 2H), 2.9-3.6(m, 5H), 5.1-5.4(ABq, 2H), 5.43(s, 2H), 6.50(br, 1H), 7.31, 7.32(each s, 1H), 7.49(d, 1H, J=7Hz), 7.73(t, 1H, J=7Hz), 7.96(d, 1H, J=7Hz)
 MASS m/z: 468(M⁺)

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Example 26

Preparation of (9S)-1,4-diamino-9-ethyl-1,2-dihydro-9-hydroxy-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



(1) 8-Acetylamino-5-benzoyloxycarbonylamino-4

To 40 ml of dichloromethane 570 mg of 8-acetylamino-5-amino-4-thiochromanone and 1 ml of pyridine were added. While stirring at 0°C, 0.69 ml of carbobenzoylchloride was added and the mixture was stirred for 1 hour. The reaction mixture was washed with dilute hydrochloric acid, water, and saturated brine in this order, and dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was recrystallized from methanol to obtain 710 mg of the title compound.

NMR(CDCl₃)δ: 2.21(3H, s), 3.03(2H, m), 3.16(2H, m), 5.21(2H, s), 6.97(1H, br.s), 7.3-7.5(5H, m), 7.79(1H, d, J=8.8Hz), 8.30(1H, d, J=8.8Hz), 11.60(1H, br.s)

(2) 3,8-Diacetylamino-5-amino-4-thiochromanone

To 5 ml of a THF solution containing 168 mg of potassium-t-butoxide was added 5 ml of a THF solution containing 370 mg of the compound prepared in (1) above. After stirring for 5 minutes, 0.24 ml of n-butyl nitrite was added to the mixture, followed by further stirring for 1 hour at room temperature. After the addition of 20 ml of ether, the reaction mixture was stirred for another 1 hour. The deposited precipitate was collected by filtration, washed thoroughly with ether, and dissolved into a mixture of 20 ml of acetic acid and 20 ml of acetic anhydride. To the solution was slowly added about 200 mg of zinc powder at room temperature while stirring. After the addition, the stirring was continued for a further 0.5 hour. Insoluble substances were removed by filtration, the solvent was evaporated, and 20 ml of chloroform was added to the residue. The residue, after washing with water, saturated aqueous solution of sodium hydroxide, and saturated brine in this order, was dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was subjected to silica gel column chromatography using a hexane-ethyl acetate (4:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated and the residue was dissolved in 20 ml of a mixed solvent of dioxane-methanol (1:1) and catalytically hydrogenated using 100 mg of palladium-on-carbon. After evaporating the solvent, the residue was subjected to silica gel column chromatography using a hexane-ethyl acetate (4:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 23 mg of the title compound.

NMR(CDCl₃)δ: 2.10(3H, s), 2.19(3H, s), 2.99(1H, t, J=13Hz), 3.56(1H, dd, J=4Hz,12Hz), 4.79(1H, dt, J=4Hz, 13Hz), 6.40(1H, d, J=8.8Hz), 6.85(1H, m), 6.92(1H, d, J=4Hz), 7.35(1H, d, J=8.8Hz)

(3) (9S)-1,4-Diamino-9-ethyl-1,2-dihydro-9-hydroxy-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indolizino-[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride

23 mg of the compound prepared in (2) above and 20 mg of triene was added to 5 ml of toluene. To this was added a catalytic amount of PPTS to react the mixture in a Deanstark apparatus under heating with refluxing for 20 hours.

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The reaction mixture was cooled and the solvent was evaporated. The residue was subjected to silica gel column chromatography using a chloroform-methanol (95:5) mixed solvent as an eluant to obtain fractions containing the target compound. 4 ml of 6 N hydrochloric acid was added to the residue obtained by concentrating the fractions and the mixture was stirred for 1.5 hours. After concentrating the resulting mixture, 4 ml of water was added to remove insoluble substances by filtration. The filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:1) to obtain 10 mg of Isomer A and 7.4 mg of Isomer B of the title compound.

Isomer A

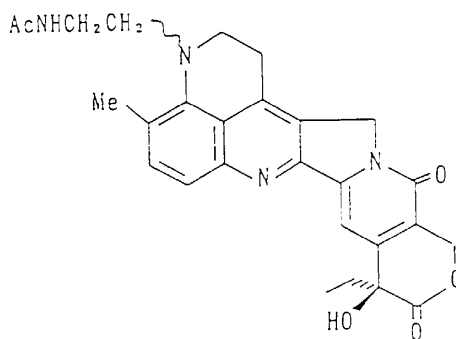
mp: above 220°C (decomposed)
NMR(DMSO- d_6) δ : 0.88(3H, t, J=7.3Hz), 1.86(2H, m), 3.34(1H, d, J=14.0Hz), 3.50(1H, dd, J=14.0Hz, 3.2Hz), 5.29(1H, m), 5.36, 5.76(2H, ABq, J=19.5Hz), 5.43(2H, s), 6.11(2H, s), 6.49(1H, s), 7.22(1H, s), 7.42(1H, d, J=9.3Hz), 7.82(1H, d, J=9.3Hz), 8.66(3H, m)

Isomer B

mp: above 220°C (decomposed)
NMR(DMSO- d_6) δ : 0.88(3H, t, J=7.3Hz), 1.87(2H, m), 3.35(1H, d, J=13.7Hz), 3.48(1H, dd, J=13.7Hz, 3.0Hz), 5.28(1H, m), 5.35, 5.74(2H, ABq, J=19.5Hz), 5.43(2H, s), 6.11(2H, s), 6.49(1H, s), 7.22(1H, s), 7.41(1H, d, J=9.3Hz), 7.82(1H, d, J=9.3Hz), 8.61(3H, m)

Example 27

Preparation of (9S)-3-(2-acetylaminoethyl)-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-3H,12H-pyrano[3',4':6,7]-indolizino[1,2-c]benzo[ij][2,7]naphthylidene-10,13(9H,15H)-dione



(1) 5-Acetylamino-8-methyl-2,3-dihydroquinoline-4-one

In a mixed solvent of 20 ml of dichloromethane and 1.3 ml of pyridine was dissolved 2.0 gm of 5-amino-8-methyl-2,3-dihydroquinoline-4-one. 1.2 ml of acetyl chloride was added to the solution under ice-cooling while stirring, followed by further stirring for 4 hours. After the addition of water, the reaction mixture was extracted with chloroform, washed with saturated brine, and dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was crystallized in ether to obtain 1.24 gm of the title compound.

NMR(CDCl₃) δ : 2.01(s, 3H), 2.20(s, 3H), 2.72(t, 2H, J=7Hz), 3.61(t, 2H, J=7Hz), 7.19(d, 1H, J=8Hz), 7.94(d, 1H, J=8Hz)

(2) 5-Acetylamino-1-cyanomethyl-8-methyl-2,3-dihydroquinoline-4-one

The compound prepared in (1) above (1.95 gm) was dissolved into 40 ml of DMF, and 6 ml of bromoacetonitrile was added to the solution. After heating under reflux for 3 hours, the reaction mixture was concentrated, and chloroform

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was added to the residue. The residue, after washing with saturated brine, was dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was subjected to silica gel column chromatography using a chloroform-methanol (100:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 605 mg of the title compound.

5

mp: 190-193°C
 IR_v^{KBr} cm^{-1} : 1696, 1644, 1506, 1392, 1248
 NMR(CDC₃) δ : 2.22(s, 3H), 2.29(s, 3H), 2.93(t, 2H, J=6.5Hz), 3.68(t, 2H, J=6.5Hz), 4.01(s, 2H), 7.38(d, 1H, J=8Hz), 8.43(d, 1H, J=8Hz)

10

MASS m/z: 257(M⁺)

(3) 5-Acetylamino-1-(2-aminoethyl)-8-methyl-2,3-dihydroquinoline-4-one

The compound prepared in (2) above (650 mg) was dissolved into a mixed solvent of 10 ml of acetic acid and 30 ml of acetic anhydride. The mixture was catalytically hydrogenated with the addition of 2 ml of Raney nickel. After removing the catalyst by filtration, the reaction mixture was concentrated and chloroform was added to the residue, which was washed with saturated aqueous solution of sodium bicarbonate and saturated brine, and dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was recrystallized from chloroform and ether to obtain 510 mg of the title compound.

20

mp: 151-153°C (decomposed)
 IR_v^{KBr} cm^{-1} : 1690, 1646, 1520
 NMR(CDC₃) δ : 2.00(s, 3H), 2.21(s, 3H), 2.26(s, 3H), 2.76(t, 2H, J=7Hz), 3.14(t, 2H, J=7Hz), 3.4-3.7(m, 4H), 5.98(br, 1H), 7.30(d, 1H, J=8Hz), 8.27(d, 1H, J=8Hz)

25

MASS m/z: 303(M⁺)

(4) 5-Amino-1-(2-aminoethyl)-8-methyl-2,3-dihydroquinoline-4-one

The reaction was carried out in the same manner as in Example 1-(4), except that 510 mg of the compound prepared in (1) above was used instead of 2,8-diacetylamino-1-tetralone of Example 1-(4), and post-treated to produce 220 mg of the title compound.

30

NMR(CDC₃) δ : 1.99(s, 3H), 2.16(s, 3H), 2.6-2.9(m, 4H), 3.3-3.7(m, 4H), 5.8-6.8(br, 1H), 6.20(d, 1H, J=8Hz), 7.04(d, 1H, J=8Hz)
 MASS m/z: 261(M⁺)

35

(5) (9S)-3-(2-Acetylaminoethyl)-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo[ij][2,7]naphthyridine-10,13(9H,15H)-dione

210 mg of the compound prepared in (2) above and 230 mg of trione were reacted for 7 hours in the same manner as in Example 17-(5) and post-treated to produce 195 mg of the title compound.

40

mp: 155-165°C (decomposed)
 NMR(DMSO-d₆) δ : 0.88(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 1.82(s, 3H), 2.47(s, 3H), 2.9-3.1(m, 2H), 3.2-3.3(m, 2H), 3.4-3.7(m, 4H), 5.26(s, 2H), 5.3-5.5(m, 2H), 7.34(s, 1H), 7.67(d, 1H, J=7Hz), 7.74(d, 1H, J=7Hz), 8.0-8.1(br.s, 1H)
 MASS m/z: 488(M⁺)

45

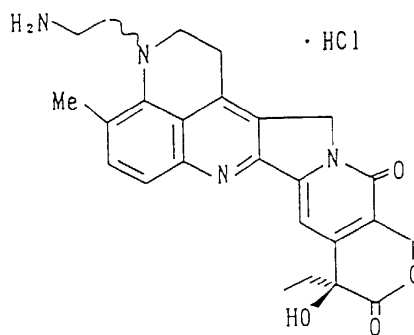
50

55

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Example 28

Preparation of (9S)-3-(2-aminoethyl)-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo[*ij*][2,7]naphthyridine-10,13(9H,15H)dione

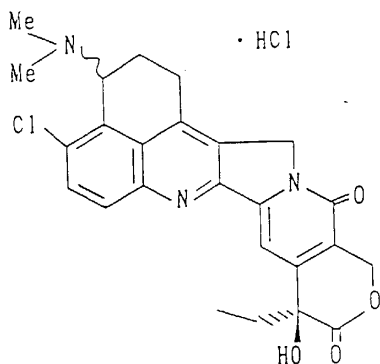


The compound prepared in Example 27-(5) (190 mg) was reacted in the same manner as in Example 2 and post-treated to produce 120 mg of the title compound.

mp: 210-230°C (decomposed)
 IR_v^{KBr} cm⁻¹: 1746, 1660, 1594
 NMR(DMSO-d₆)δ: 0.88(t, 3H, J=5.5Hz), 1.8-1.9(m, 2H), 2.49(s, 3H), 3.1-3.5(m, 8H), 5.26(s, 2H), 5.43(s, 2H), 7.32(s, 1H), 7.69(d, 1H, J=9Hz), 7.78(d, 1H, J=9Hz), 8.1-8.3(br.s, 3H)
 MASS m/z: 446(M⁺)

Example 29

Preparation of (9S)-4-chloro-9-ethyl-2,3-dihydro-9-hydroxy-3-dimethylamino-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)dione hydrochloride



To a solution of 44 mg of Isomer B prepared in Example 14 dissolved in 5 ml of 50% aqueous methanol were added 0.5 ml of 35% aqueous solution of formalin and 50 mg of 10% palladium-on-carbon to effect catalytic hydrogenation. After the reaction, the catalyst was removed by filtration, and the solvent was evaporated. 4 ml of water was added to remove insoluble substance by filtration. The filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:1) to obtain 8 mg of the title compound.

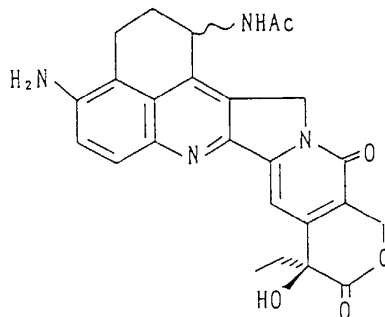
NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.88(2H, m), 2.2-2.4(1H, m), 2.4-2.6(1H, m), 2.67(3H, m), 3.06(3H, m),

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3.3-3.5(2H, m), 5.19(1H, m), 5.21, 5.43(2H, ABq, J=18.6Hz), 5.45(2H, s), 6.56(1H, s), 7.37(1H, s), 8.03(1H, d, J=9.3Hz), 8.31(1H, d, J=9.3Hz)

Example 30

Preparation of (9S)-1-acetylamino-4-amino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 2,5,8-Triacetylamino-1-tetralone

The reaction was carried out in the same manner as in Example 1-(2), except that 1.65 gm of 5,8-diacetylamino-1-tetralone was used instead of 8-acetylamino-1-tetralone of Example 1-(2). Crude 5,8-diacetylamino-2-hydroxyimino-1-tetralone which was obtained by the post-treatment was reacted in the same manner as in Example 1-(3) and post-treated to produce 950 mg of the title compound.

IR_v^{KBr} cm^{-1} : 3280, 1660, 1596, 1516
 NMR(CDCl₃) δ : 1.79-2.02(1H, m), 2.11(3H, s), 2.18(3H, s), 2.23(3H, s), 2.4-3.0(3H, m), 4.54-4.69(1H, m), 7.60(1H, d, J=9.0Hz), 8.55(1H, d, J=9.0Hz)
 MASS m/z: 317(M⁺)

(2) 2-Acetylamino-5,8-diamino-1-tetralone

The reaction was carried out in the same manner as in Example 1-(4), except that 500 mg of the compound prepared in (1) above was used instead of 2,8-diacetylamino-1-tetralone of Example 1-(4), and post-treated to produce 290 mg of the title compound.

IR_v^{KBr} cm^{-1} : 3436, 3352, 3296, 2444, 1626, 1556
 NMR(DMSO-d₆) δ : 1.74-1.87(1H, m), 1.90(3H, s), 2.14-2.16(1H, m), 2.54-2.77(2H, m), 4.41-4.47(1H, m), 6.48(1H, d, J=8.3Hz), 6.83(1H, d, J=8.3Hz), 8.04(1H, d, J=7.8Hz)
 MASS m/z: 233(M⁺)

(3) (9S)-1-Acetylamino-4-amino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

234 mg of the compound prepared in (2) above and 261 mg of triene were dissolved in 30 ml of acetic acid and heated under reflux for 14 hours in a nitrogen atmosphere. The reaction product was post-treated in the same manner as in Example 1-(5) to produce 134 mg of the title compound.

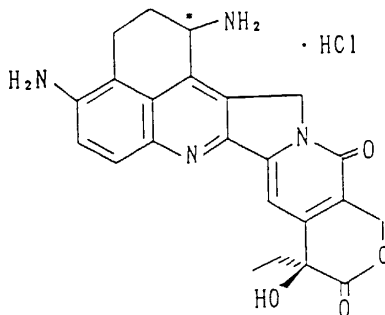
mp: above 240°C (decomposed)
 IR_v^{KBr} cm^{-1} : 3380, 2984, 2940, 1748, 1662, 1602
 NMR(DMSO-d₆) δ : 0.89(3H, t, J=7.2Hz), 1.82-1.92(2H, m), 1.93(3/2H, s), 1.94(3/2H, s), 2.08-2.09(2H, m), 3.08(2H, br), 5.16-5.26(2H, m), 5.39-5.46(2H, m), 5.53-5.56(1H, m), 6.51(1H, s), 7.32(2H, s), 7.96(1H, d,

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MASS m/z: J=9.5Hz), 7.99(1H, d, J=8.7Hz), 8.54(1H, t, J=8.7Hz)
458(M⁺)

Example 31

Preparation of (9S)-1,4-diamino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 21-(3) (113 mg) was reacted for 6 hours in the same manner as in Example 2 and post-treated to produce Isomer A (28 mg) and Isomer (B) (28 mg) of the title compound.

Isomer A

mp: above 240°C (decomposed)
IR_{max}^{KBr} cm⁻¹: 3392, 3232, 2936, 1742, 1652, 1590
NMR(DMSO-d₆)δ: 0.89(3H, t, J=6.8Hz), 1.82-1.92(2H, m), 2.03-2.09(1H, m), 2.67-2.93(2H, m), 4.97(1H, br), 5.32-5.46(3H, m), 5.74-5.81(1H, m), 6.47(1H, s), 7.21(1H, s), 7.39(1H, d, J=8.8Hz), 7.83(1H, d, J=8.8Hz), 8.60(1H, br)
MASS m/z: 418(M⁺)

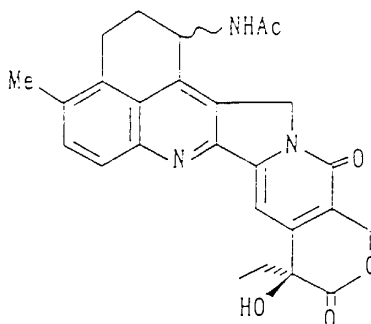
Isomer B

mp: above 240°C (decomposed)
IR_{max}^{KBr} cm⁻¹: 3436, 3232, 1746, 1658, 1592
NMR(DMSO-d₆)δ: 0.89(3H, t, J=6.8Hz), 1.81-1.92(2H, m), 2.03-2.09(1H, m), 2.68-2.95(2H, m), 4.99(1H, br), 5.37-5.47(3H, m), 5.81(1H, d, J=19Hz), 7.23(1H, s), 7.43(1H, d, J=8.8Hz), 7.85(1H, d, J=8.8Hz), 8.64(1H, br)
MASS m/z: 418(M⁺)

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Example 32

Preparation of (9S)-1-acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) Methyl (2'-tolyl)acetate

To a solution of 25 gm of o-tolylacetic acid in 350 ml of methanol 1 ml of concentrated hydrochloric acid was added. The mixture was heated under reflux overnight. 200 ml of water was added to the residue obtained by removing the solvent. The precipitate was extracted with chloroform and the extract was washed with saturated brine, and dried over anhydrous magnesium sulfate. The solvent was removed to obtain 27.2 gm of the title compound.

NMR(CDCl₃)δ: 2.30(3H, s), 3.64(2H, s), 3.68(3H, s), 7.17(4H, s)

(2) Methyl 4-(2'-tolyl)-3-butenate

The solution of 13.1 gm of the compound prepared in (1) above in 100 ml of toluene was cooled to -65°C, and to this was dropwise added over 1 hour 80 ml of 1 M diisobutylaluminum hydride in toluene and the mixture was stirred for 1 hour at the same temperature, following which the reaction was terminated by the addition of methanol. The reaction mixture was diluted with ethyl acetate, washed with 10% aqueous solution of hydrochloric acid, water, and saturated brine in this order, and dried over anhydrous magnesium sulfate. After removing the solvent, the residue was dissolved into 100 ml of benzene. To this solution was added 26.5 gm of methyl(triphenylphosphoranilidene)acetate, and the mixture was stirred overnight at room temperature. The solvent was evaporated, and a mixed solvent of ethyl acetate-n-hexane (1:9) was added to the residue to remove insoluble substance by filtration. The filtrate was concentrated and the residue was subjected to silica gel column chromatography using an ethyl acetate-n-hexane (1:9) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 12.7 mg of the title compound.

NMR(CDCl₃)δ: 2.27(3H, s), 3.46-3.54(2H, m), 3.70(3H, s), 5.70(1H, d, 15.5Hz), 6.95-7.24(5H, m)

(3) 4-(2'-Tolyl)butyric acid

A solution of 12.6 gm of the compound prepared in (2) above in 200 ml of methanol was catalytically hydrogenated for 20 minutes with 10% palladium-on-carbon. After the catalyst was removed by filtration, the reaction liquid was concentrated to a volume of 150 ml. 70 ml of 1 N sodium hydroxide solution was added to the concentrate, followed by heating at 50°C for 1 hour while stirring. After cooling to room temperature, the reaction product was adjusted below pH 1 with hydrochloric acid while ice-cooling. The precipitate was extracted with chloroform, washed with saturated brine, and dried over anhydrous magnesium sulfate. The solvent was removed to obtain 11.4 gm of the title compound.

mp: 54-56°C (decomposed)
IR_{max}^{KBr} cm⁻¹: 3416, 2944, 1748, 1660, 1602
NMR(CDCl₃)δ: 1.83-1.99(2H, m), 2.31(3H, s), 2.34-2.75(4H, m), 7.12(4H, m)

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MASS m/z: 178(M⁺)

(4) 5-Methyl-1-tetralone

To 120 gm of polyphosphoric acid heated at 70°C 10.0 gm of the powder prepared in (3) above was added over 30 minutes, and the mixture was stirred for 20 minutes at the same temperature. the reaction mixture was added to 600 ml of ice water to collect the precipitate by filtration, followed by washing with water. The powder thus obtained was dissolved into ethyl acetate, washed with brine, and dried over anhydrous magnesium sulfate. The solvent was removed to obtain 8.19 gm of the title compound.

mp: 43-47°C (decomposed)
IR_v^{KBr} cm⁻¹ : 3452, 2952, 1676, 1592, 1464
NMR(CDCI₃)δ: 2.07-2.27(2H, m), 2.31(3H, s), 2.57-2.93(4H, m), 7.11-7.33(2H, m), 7.89-7.98(1H, m)
MASS m/z: 160(M⁺)

(5) 5-Methyl-8-nitro-1-tetralone

A solution of 8.1 gm of the compound obtained in (4) above in 70 ml of concentrated sulfuric acid was cooled to -5°C. To this solution was dropwise added a solution of 5.37 gm of potassium nitrate in 50 ml of concentrated sulfuric acid while controlling the temperature below 5°C, followed by stirring for 30 minutes at the same temperature. The reaction mixture was poured to 600 ml of ice-cold water and extracted with chloroform, washed with saturated aqueous sodium bicarbonate and saturated brine, and dried over anhydrous magnesium sulfate. After evaporating the solvent, the residue was subjected to silica gel column chromatography using an ethyl acetate-n-hexane (1:15) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 5.82 gm of the title compound.

mp: 104-107°C
IR_v^{KBr} cm⁻¹ : 3452, 2964, 1690, 1596
NMR(CDCI₃)δ: 2.17-2.22(2H, m), 2.37(3H, s), 2.69-2.72(2H, m), 2.89(2H, t, J=5.9Hz), 7.25(1H, d, J=8.3Hz), 7.40(1H, d, J=7.8Hz)
MASS m/z: 205(M⁺)

(6) 8-Acetylamino-5-methyl-1-tetralone

The compound prepared in (5) above (2.5 gm) was dissolved into a mixed solvent of 50 ml of acetic acid and 50 ml of acetic anhydride. The mixture was catalytically hydrogenated for 1 hour in a hydrogen stream with the addition of 800 mg of 10% palladium-on-carbon. After removing the catalyst by filtration, the reaction mixture was concentrated. The residue was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 2.22 gm of the title compound.

mp: 92-94°C
IR_v^{KBr} cm⁻¹ : 3512, 3176, 2940, 1690, 1650, 1604
NMR(CDCI₃)δ: 1.95-2.18(2H, m), 2.22(3H, s), 2.25(3H, s), 2.61-2.93(4H, m), 7.33(1H, d, J=8.5Hz), 8.51(1H, d, J=8.5Hz)
MASS m/z: 217(M⁺)

(7) 2,8-Diacetylamino-5-methyl-1-tetralone

The reaction was carried out in the same manner as in Example 1-(2), except that 1 gm of 8-acetylamino-5-methyl-1-tetralone was used instead of 8-acetylamino-1-tetralone of Example 1-(2). Crude 8-acetylamino-2-hydroxyimino-5-methyl-1-tetralone which was obtained by the post-treatment was reacted in the same manner as in Example 1-(3) and post-treated to produce 476 mg of the title compound.

mp: 195-198°C (decomposed)
IR_v^{KBr} cm⁻¹ : 3312, 2928, 1712, 1638, 1596, 1520
NMR(CDCI₃)δ: 1.74-1.98(1H, m), 2.11(3H, s), 2.22(3H, s), 2.24(3H, s), 2.66-3.03(3H, m), 4.52-4.79(1H, m), 6.5(1H, br), 7.36(1H, d, J=8.5Hz), 8.50(1H, d, 8.5Hz)
MASS m/z: 274(M⁺)

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(8) 2-Acetylamino-8-amino-5-methyl-1-tetralone

The reaction was carried out in the same manner as in Example 1-(4), except that 400 mg of the compound prepared in (7) above was used instead of 2,8-diacetylamino-1-tetralone of Example 1-(4), and post-treated to produce 265 mg of the title compound.

mp: 192-194°C (decomposed)
 IR_{max}^{KBr} cm⁻¹: 3460, 3360, 2924, 1618, 1566, 1536
 NMR(CDCI₃)δ: 1.76-1.87(1H, m), 2.10(3H, s), 2.16(3H, s), 2.63-2.69(1H, m), 2.91-2.97(2H, m), 4.55-4.56(1H, m), 6.55(1H, d, J=8.3Hz), 7.13(1H, d, J=8.3Hz)
 MASS m/z: 232(M⁺)

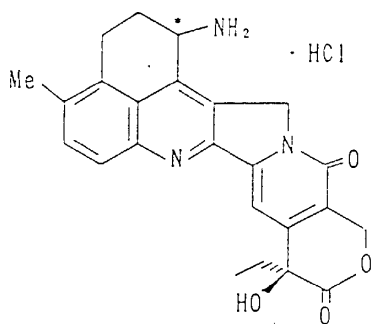
(9) (9S)-1-Acetylamino-9-ethyl-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

200 mg of the compound prepared in (8)-above and 227 mg of trione were reacted for 23 hours in the same manner as in Example 1-(5) and the reaction product was post-treated to produce 287 mg of the title compound.

mp: above 270°C (decomposed)
 IR_{max}^{KBr} cm⁻¹: 3428, 2944, 1754, 1660, 1608, 1556
 NMR(DMSO-d₆)δ: 0.86-0.90(3H, m), 1.75-1.89(2H, m), 1.91(3/2H, s), 1.92(3/2H, s), 2.11-2.14(2H, m), 2.47(3/2H, s), 2.48(3/2H, s), 3.08-3.11(2H, m), 5.19-5.21(2H, m), 5.43(2H, s), 5.52-5.57(1H, m), 6.50(1/2H, s), 6.51(1/2H, s), 7.31(1H, s), 7.71(1H, d, J=8.3Hz), 7.94(1H, d, J=8.3Hz), 8.47(1H, t, J=8.3Hz)
 MASS m/z: 459(M⁺)

Example 33

Preparation of (9S)-1-amino-9-ethyl-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 32-(9) (150 mg) was reacted for 7 hours in the same manner as in Example 2 and post-treated to produce Isomer A (58 mg) and Isomer B (62 mg) of the title compound.

Isomer A

mp: above 250°C (decomposed)
 IR_{max}^{KBr} cm⁻¹: 3432, 2936, 1746, 1658, 1594
 NMR(DMSO-d₆)δ: 0.90(3H, t, J=6.8Hz), 1.83-1.91(2H, m), 2.15-2.22(1H, m), 2.56-2.59(1H, m), 3.12-3.28(2H, m), 5.09(1H, br), 5.40-5.45(3H, m), 5.90(1H, d, J=19Hz), 6.52(1H, s), 7.34(1H, s), 7.76(1H, d, J=8.8Hz), 8.01(1H, d, J=8.8Hz), 8.73(3H, br)
 MASS m/z: 417(M⁺)

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Isomer B

mp: above 250°C (decomposed)

IR_{max}^{KBr} cm⁻¹: 3448, 2936, 1746, 1658, 1596

5 NMR(DMSO-d₆)δ: 0.89(3H, t, J=6.8Hz), 1.83-1.93(2H, m), 2.17(1H, br), 2.61-2.68(1H, m), 3.10-3.21(2H, m), 5.10(1H, br), 5.43-5.48(3H, m), 5.93(1H, d, J=19Hz), 7.35(1H, s), 7.78(1H, d, J=8.8Hz), 8.02(1H, d, J=8.8Hz), 8.80(3H, br)

MASS m/z: 417(M⁺)

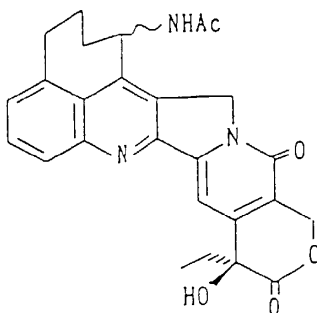
10 Example 34

Preparation of (10S)-1-acetylamino-10-ethyl-1,2,3,4-tetrahydro-10-hydroxy-13H-cyclohepto[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-11,14(10H,16H)-dione

15

20

25



30 (1) 1-Acetylamino-6,7,8,9-tetrahydro-5H-benzocycloheptene

8.7 gm of 1-nitro-6,7,8,9-tetrahydro-5H-benzocycloheptene [J. Am. Chem. Soc., 5820 (9169)] was dissolved into a mixed solvent of 150 ml of acetic anhydride and 50 ml of acetic acid. The mixture was catalytically hydrogenated with the addition of 10 ml of raney nickel. After removing the catalyst by filtration, the reaction mixture was concentrated, the residue was washed with saturated aqueous solution of sodium bicarbonate and saturated brine, and dried over anhydrous sodium sulfate. The product was concentrated to produce 6.17 gm of the title compound.

35 NMR(CDCl₃)δ: 1.5-1.9(m, 6H), 2.15, 2.20(each s, 3H), 2.6-2.9(m, 4H), 6.9-7.4(m, 3H)

40 MASS m/z: 203(M⁺)

(2) 1-Acetylamino-6,7,8,9-tetrahydro-5H-benzocycloheptene-9-one

The reaction was carried out in the same manner as in Example 1-(1), except that 200 mg of the compound prepared in (1) above was used instead of 1-acetylaminotetraline of Example 1-(1). The reaction product was post-treated to produce 55 mg of the title compound.

45 mp: 155-162°C

NMR(CDCl₃)δ: 1.5-2.0(m, 6H), 2.17(s, 3H), 2.6-2.9(m, 4H), 6.93(d, 1H, J=8Hz), 7.38(t, 1H, J=8Hz), 8.30(d, 1H, J=8Hz)

50 MASS m/z: 217(M⁺)

(3) 1,8-Diacetylamino-6,7,8,9-tetrahydro-5H-benzocycloheptene-9-one

The reaction was carried out in the same manner as in Example 1-(2), except that 270 mg of the compound prepared in (2) above was used instead of 8-acetylamino-1-tetralone of Example 1-(2). The reaction mixture obtained by the post-treatment was further reacted in the same manner as in Example 1-(3) and post-treated to produce 126 mg of the title compound.

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mp: 220-223°C (decomposed)
 IR_v^{KBr} cm^{-1} : 1722, 1682, 1616, 1548
 NMR(CDC₃) δ : 2.08(s, 3H), 2.19(s, 3H), 1.5-2.4(m, 4H) 2.7-3.1(m, 2H), 4.7-4.9(m, 1H), 6.1-6.3(br.s, 1H), 6.94(d, 1H, J=8Hz), 7.34(t, 1H, J=8Hz), 8.32(d, 1H, J=8Hz), 9.01(br.s, 1H)
 5 MASS m/z: 274(M⁺)

(4) 8-Acetylamino-1-amino-6,7,8,9-tetrahydro-5H-benzocycloheptene-9-one

10 The reaction was carried out in the same manner as in Example 1-(4), except that 115 mg of the compound prepared in (3) above was used instead of 2,8-diacetylamino-1-tetralone of Example 1-(4). The product was post-treated to produce 90 mg of the title compound.

NMR(CDC₃) δ : 2.04(s, 3H), 1.5-1.7(m, 2H), 2.0-3.2(m, 4H), 4.9-5.1(m, 1H), 5.98(br.s, 1H), 6.53(d, 1H, J=8Hz), 6.54(d, 1H, J=8Hz), 6.72(br.s, 1H), 7.23(t, 1H, J=8Hz)

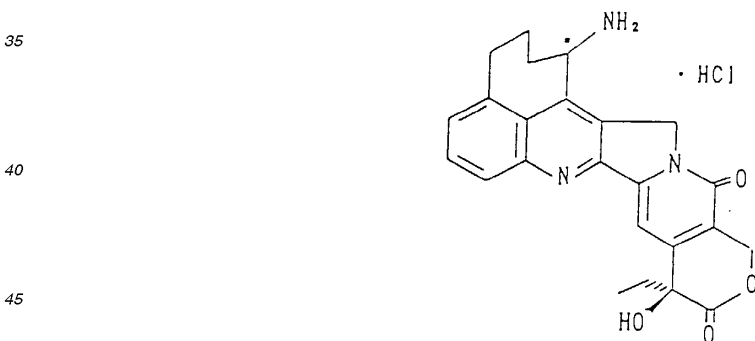
15 (5) (10S)-1-Acetylamino-10-ethyl-1,2,3,4-tetrahydro-10-hydroxy-13H-cyclohepto[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-11,14(10H,16H)-dione

20 The compound prepared in (4) above (90 mg) and 105 mg of trione were reacted for 21 hours in the same manner as in Example 1-(5) and the product was post-treated to obtain 72 mg of the title compound.

mp: 203-206°C (decomposed)
 IR_v^{KBr} cm^{-1} : 1748, 1660, 1600, 1160
 NMR(CDC₃) δ : 0.96, 1.03(each t, 3H, J=7Hz), 1.5-4.0(m, 6H), 2.15, 2.27(each s, 3H), 5.0-6.0(m, 2H), 7.3-8.2(m, 4H)
 25 MASS m/z: 459(M⁺)

Example 35

30 Preparation of (10S)-1-amino-10-ethyl-1,2,3,4-tetrahydro-10-hydroxy-13H-cyclohepto[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-11,14(10H,16H)-dione hydrochloride



The compound prepared in Example 34-(5) (65 mg) was reacted for 4 hours in the same manner as in Example 2 and the product was post-treated to obtain Isomer A (17 mg) and Isomer B (21 mg) of the title compound.

50 Isomer A

mp: 200-220°C (decomposed)
 IR_v^{KBr} cm^{-1} : 1746, 1660, 1600, 1164
 55 NMR(DMSO-d₆) δ : 0.88(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 2.0-3.5(m, 6H), 5.15(s, 1H), 5.46(s, 2H), 5.53, 5.73(ABq, 2H, J=19Hz), 6.56(s, 1H), 7.35(s, 1H), 7.63(d, 1H, J=8Hz), 7.79(t, 1H, J=8Hz), 8.09(d, 1H, J=8Hz), 8.79(br.s, 3H)
 MASS m/z: 417(M⁺)

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Isomer B

mp: 210-230°C (decomposed)

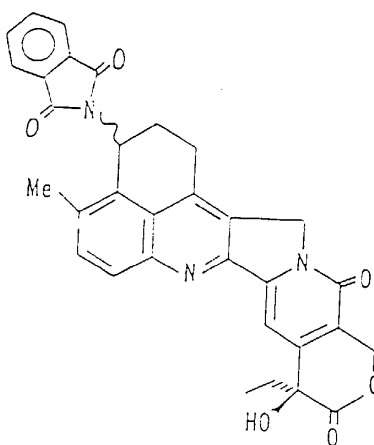
IR_v^{KBr} cm⁻¹: 1744, 1664, 1600, 1160

5 NMR(DMSO-d₆)δ: 0.88(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 2.0-3.5(m, 6H), 5.0-5.2(m, 1H), 5.46(s, 2H), 5.54, 5.72(ABq, 2H, J=19Hz), 6.56(s, 1H), 7.35(s, 1H), 7.62(d, 1H, J=8Hz), 7.77(t, 1H, J=8Hz), 8.09(d, 1H, J=8Hz), 8.61(br.s, 3H)

MASS m/z: 417(M⁺)

10 Example 36

Preparation of (9S)-9-ethyl-2,3-dihydro-3-(1,3-dioxoisindoline-2-yl)-9-hydroxy-4-methyl-1H, 12H-benzo[de]pyrano [3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione



(1) 4-Azide-5-methyl-8-nitro-1-tetralone

35 The reaction was carried out in the same manner as in Example 13-(9), except that 410 mg of 5-methyl-8-nitro-1-tetralone was used instead of 5-fluoro-8-nitro-1-tetralone of Example 13-(9). The product was post-treated to produce 490 mg of the title compound.

40 NMR(CDCl₃)δ: 2.33-2.50(2H, m), 2.55(3H, s), 2.69-2.75(1H, m), 2.98-3.07(1H, m), 5.07(1H, t, J=2.9Hz), 7.42(1H, d, J=7.8Hz), 7.54(1H, d, J=8.8Hz)

(2) 5-Methyl-8-nitro-4-(1,3-dioxoisindoline-2-yl)-1-tetralone

45 The reaction was carried out in the same manner as in Example 13-(9), except that 490 mg of the compound prepared in (1) above was used instead of 4-azide-5-fluoro-8-nitro-1-tetralone of Example 13-(9). The reaction product was post-treated to produce 366 mg of the title compound.

mp: 220-230°C

IR_v^{KBr} cm⁻¹: 3456, 3084, 2940, 1770, 1712, 1596

50 NMR(CDCl₃)δ: 2.26(3H, s), 2.38-2.44(1H, m), 2.55-2.65(1H, m), 2.72-2.79(1H, m), 2.93-3.02(1H, m), 5.70-5.72(1H, m), 7.39(1H, d, J=8.3Hz), 7.42(1H, d, J=8.3Hz), 7.74-7.84(4H, m)

MASS m/z: 350(M⁺)

55 (3) 8-Amino-5-methyl-4-(1,3-dioxoisindoline-2-yl)-1-tetralone

The reaction was carried out in the same manner as in Example 13-(10), except that 145 mg of the compound

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prepared in (2) above was used instead of 5-fluoro-8-nitro-4-(1,3-dioxoisindoline-2-yl)-1-tetralone of Example 13-(10). The reaction product was post-treated to produce 75 mg of the title compound.

5 IR_v^{KBr}_{max} cm⁻¹: 3456, 3344, 2952, 1770, 1710, 1622
 NMR(CDC₃)δ: 2.02(3H, s), 2.25-2.32(1H, m), 2.41-2.50(1H, m), 2.58-2.64(1H, m), 2.88-2.98(1H, m), 5.60-5.62(1H, m), 6.58(1H, d, J=8.3Hz), 7.05(1H, d, J=8.3Hz), 7.70-7.81(4H, m)
 MASS m/z: 320(M⁺)

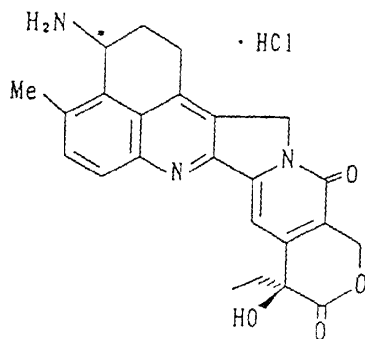
10 (4) (9S)-9-Ethyl-2,3-dihydro-9-hydroxy-4-methyl-3-(1,3-dioxindoline-2-yl)-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

The compound prepared in (3) above (62 mg) and 51 mg of trione was reacted for 18 hours in the same manner as in Example 6-(3) to obtain 86 mg of the title compound.

15 mp: 285-290°C (decomposed)
 IR_v^{KBr}_{max} cm⁻¹: 3448, 2944, 1748, 1714, 1660, 1600
 NMR(DMSO-d₆)δ: 0.89-0.93(3H, m), 1.82-1.93(2H, m), 2.32(3H, s), 2.46(1H, br), 3.18-3.22(2H, m), 5.22-5.48(4H, m), 5.85(1H, br), 6.49(1/2H, s), 6.51(1/2H, s), 7.36(1H, s), 7.65(1H, d, J=8.3Hz), 7.79-7.84(4H, m), 8.04(1H, d, J=8.3Hz)
 20 MASS m/z: 547(M⁺)

Example 37

25 Preparation of (9S)-3-amino-9-ethyl-2,3-dihydro-9-hydroxy-4-methyl-1H, 12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



40 The compound prepared in Example 36-(4) (74 mg) was reacted in the same manner as in Example 7 and post-treated to produce Isomer A (14 mg) and Isomer B (15 mg) of the title compound.

Isomer A

45 mp: above 250°C (decomposed)
 IR_v^{KBr}_{max} cm⁻¹: 3448, 2936, 1742, 1656, 1592
 NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.3Hz), 1.84-1.91(2H, m), 2.13-2.20(1H, m), 2.54-2.58(1H, m), 2.68(3H, s), 5.05(1H, br), 5.19-5.44(4H, m), 6.50(1H, s), 7.33(1H, s), 7.76(1H, d, J=8.8Hz), 8.10(1H, d, J=8.8Hz), 8.27(3H, br)
 50 MASS m/z: 417(M⁺)

Isomer B

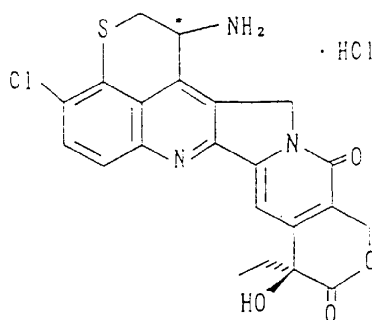
55 mp: above 250°C (decomposed)
 IR_v^{KBr}_{max} cm⁻¹: 3400, 3236, 2976, 1746, 1662, 1614
 NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.83-1.94(2H, m), 2.13-2.20(1H, m), 2.59-2.62(1H, m), 2.69(3H, s), 3.27-3.42(2H, m), 5.07(1H, br), 5.19-5.44(4H, m), 7.34(1H, s), 7.78(1H, d, J=8.8Hz), 8.12(1H, d, J=8.8Hz),

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MASS m/z: 8.53(3H, br)
417(M⁺)

Example 38

Preparation of (9S)-1-amino-4-chloro-9-ethyl-1,2-dihydro-9-hydroxy-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



(1) 5-Acetylamino-8-chloro-4-thiochromanone

Into a solution of 500 mg of 5-acetylamino-8-amino-4-thiochromanone (described in Japanese Patent Laid-open (ko-kai) 279891/1989) suspended in a mixed solvent of 12 ml of concentrated hydrochloric acid and 3 ml of water cooled to 0°C was slowly dropped a solution of 153 mg of sodium nitrite in 2 ml of water. The mixture was stirred for 5 minutes, whereupon a solution of copper (I) chloride in 3 ml of concentrated hydrochloric acid was added to it at 0°C. The mixture was allowed to stand at room temperature overnight and stirred for 20 minutes at 70°C. The reaction mixture was extracted with chloroform, the extract was dried over anhydrous magnesium sulfate. After concentration, the residue was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 478 mg of the title compound.

NMR(CDCl₃)δ: 2.22(3H, s), 2.45-3.42(4H, m), 7.43(1H, d, J=9Hz), 8.50(1H, d, J=9Hz)

(2) 3,5-Diacetylamino-8-chloro-4-thiochromanone

The reaction was carried out in the same manner as in Example 12-(1), except that 477 mg of the compound prepared in (1) above was used instead of 5-acetylamino-8-methyl-4-thiochromanone of Example 12-(1). The reaction product was post-treated to produce 171 mg of the title compound.

NMR(CDCl₃)δ: 2.08(3H, s), 2.20(3H, s), 3.20(1H, d, J=13Hz), 3.51(1H, dd, J=13.9Hz), 4.7-4.9(1H, m), 7.50(1H, d, J=9.2Hz), 8.54(1H, d, J=9.2Hz)

(3) 3-Acetylamino-5-amino-8-chloro-4-thiochromanone

The compound obtained in (2) above (171 mg) was reacted in the same manner as in Example 12-(2) and the reaction product was post-treated to produce 22 mg of the title compound.

NMR(CDCl₃)δ: 2.09(3H, s), 3.07(1H, d, J=13Hz), 3.60(1H, dd, J=12.5Hz, 4.5Hz), 4.7-4.9(1H, m), 6.36(1H, d, J=9Hz), 7.00(1H, d, J=9Hz)

(4) (9S)-1-Amino-4-chloro-9-ethyl-1,2-dihydro-9-hydroxy-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

The compound prepared in (3) above (22 mg) and 23 mg of trione was reacted in the same manner as in Example

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12-(3) and post-treated to produce Isomer A (12 mg) and Isomer B (11 mg) of the title compound.

Isomer A

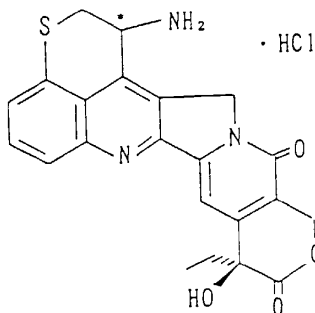
IR_v^{KBr} cm^{-1} : 3444, 1744, 1660, 1596, 1556
 NMR(DMSO- d_6) δ : 0.90(3H, t, J=7.3Hz), 1.89(2H, m), 3.62(2H, d, J=1.95Hz), 5.46(2H, s), 5.47,5.85(1H, ABq, J=19.5Hz), 6.54-6.58(1H, br.s), 7.39(1H, s), 7.97(1H, d, J=8.8Hz), 8.06(1H, d, J=8.8Hz), 8.80-8.87(3H, m)
 MASS m/z: 456(M⁺ +1)

Isomer B

IR_v^{KBr} cm^{-1} : 3452, 1746, 1662, 1596, 1556
 NMR(DMSO- d_6) δ : 0.89(3H, t, J=7.3Hz), 1.88(2H, m), 3.60-3.63(2H, m), 5.46(2H, s), 5.48,5.81(2H, ABq, J=19.5Hz), 6.54-6.57(1H, br.s), 7.40(1H, s), 7.97(1H, d, J=8.8Hz), 8.06(1H, d, J=8.8Hz), 8.75-8.82(3H, m)
 MASS m/z: 456(M⁺ +1)

Example 39

Preparation of (9S)-1-amino-9-ethyl-1,2-dihydro-9-hydroxy-12H-thiino[4,3,2-de]pyrano[3',4':6,7]indo1izino[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride



(1) 3,5-Diacetylamino-4-thiochromanone

The reaction was carried out in the same manner as in Example 12-(1), except that 360 mg of 5-acetylamino-4-thiochromanone was used instead of 5-acetylamino-8-methyl-4-thiochromanone of Example 12-(1). The reaction product was post-treated to produce 133 mg of the title compound.

NMR(CDCl₃) δ : 2.11(3H, s), 2.23(3H, s), 3.06(1H, d, J=12.7Hz), 3.52(1H, dd, J=12.5Hz, 4.8Hz), 4.83-5.09(1H, m), 6.7-6.9(1H, br), 6.93(1H, dd, J=7.9Hz, 1.3Hz), 7.2-7.4(1H, br), 7.27-7.46(1H, m), 8.46(1H, dd, J=8.4Hz, 1.2Hz)

(2) 3-Acetylamino-5-amino-4-thiochromanone

The compound obtained in (1) above (132 mg) was reacted in the same manner as in Example 12-(2) and the reaction product was post-treated to produce 62 mg of the title compound.

NMR(CDCl₃) δ : 2.09(3H, s), 3.13(1H, d, J=13Hz), 3.52(1H, dd, J=13Hz, 9Hz), 4.72-4.97(1H, m), 6.36(1H, dd, J=8.3Hz, 1Hz), 6.50(1H, dd, J=7.66Hz, 1.09Hz), 6.75-7.1(1H, br), 7.09(1H, t, J=8Hz)

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(3) (9S)-1-Amino-9-ethyl-1,2-dihydro-9-hydroxy-12H-thiino-[4,3,2-de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13 (9H,15H)-dione hydrochloride

The compound prepared in (2) above (62 mg) and 76 mg of trione was reacted in the same manner as in Example 12-(3) and post-treated to produce Isomer A (19 mg) and Isomer B (11 mg) of the title compound.

Isomer A

IR_{max}^{KBr} cm⁻¹: 3444, 1746, 1660, 1596, 1502

¹H NMR(DMSO-d₆)δ: 0.94(3H, t, J=7.3Hz), 1.89(2H, m), 3.58(2H, ABq, J=15Hz,14Hz), 5.43(2H, s), 5.49(1H, d, J=16.1Hz), 5.91(1H, d, J=19.5Hz), 6.47-6.55(1H, br.s), 7.43(1H, s), 7.69(1H, d, J=6.84Hz), 7.78-7.82(1H, m), 8.04(1H, d, J=8.3Hz), 8.86-8.95(3H, br)

MASS m/z: 422(M⁺ +1)

Isomer B

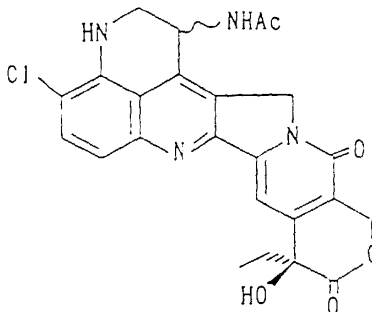
IR_{max}^{KBr} cm⁻¹: 3420, 1746, 1660, 1598, 1504

¹H NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.89(2H, m), 3.51-3.63(1H, m), 5.39-5.52(4H, m), 5.91(1H, d, J=19.5Hz), 6.49-6.60(1H, br), 7.40(1H, s), 7.71(1H, d, J=7.33Hz), 7.79-7.83(1H, m), 8.04(1H, d, J=8.3Hz), 8.87-9.0(3H, br)

MASS m/z: 422(M⁺ +1)

Example 40

Preparation of (9S)-1-acetylamino-9-ethyl-4-chloro-1,2-dihydro-9-hydroxy-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo[*ij*][2,7]naphthyridine-10,13(9H,15H)-dione



(1) 1-Acetyl-5-acetylamino-8-chloro-2,3-dihydroquinoline-4-one

The reaction was carried out in the same manner as in Example 17-(1), except that 3.8 gm of 5-amino-8-chloro-2,3-dihydroquinoline-4-one (described in Japanese Patent Laid-open (ko-kai) 279891/1989) was used instead of 5-amino-8-methyl-2,3-dihydroquinoline-4-one of Example 17-(1). The reaction product was post-treated to obtain 2.8 gm of the title compound.

mp: 175-177°C

¹H NMR(CDC₃)δ: 2.14(s, 3H), 2.26(s, 3H), 2.0-6.2(m, 5H), 7.60(d, 1H, J=9Hz), 8.66(d, 1H, J=9Hz)

(2) 1-Acetyl-3,5-diacetylamino-8-chloro-2,3-dihydroquinoline-4-one

The reaction was carried out in the same manner as in Example 1-(2), except that 1.4 gm of the compound prepared in (1) above was used instead of 8-acetylamino-1-tetralone of Example 1-(2). The reaction mixture obtained by the post-treatment was further reacted in the same manner as in Example 1-(3) and post-treated to produce 0.62 gm of

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the title compound.

mp: 206-210°C
 IR_v^{KBr} cm⁻¹: 1680, 1662, 1576, 1512, 1284
 5 NMR(CDCl₃)δ: 2.06(s, 3H), 2.12(s, 3H), 2.25(s, 3H), 3.5-6.5(m, 3H), 7.5-8.7(m, 2H), 11.44(br.s, 1H)
 MASS m/z: 337(M⁺), 339(M⁺ +2)

(3) 3,5-Diamino-8-chloro-2,3-dihydro-quinoline-4-one

10 8 ml of 6 N hydrochloric acid was added to 514 mg of the compound prepared in (2) above, and the mixture was heated at 110°C for 1.5 hours while stirring. After cooling chloroform was added to it, followed by the addition of 50 ml of 1N aqueous solution of sodium hydroxide while stirring. The chloroform layer was extracted, the extract was washed with saturated brine and dried over anhydrous sodium sulfate. The product was concentrated to produce 260 mg of the title compound.

15 NMR(CDCl₃)δ: 3.2-3.5(m, 1H), 3.6-3.8(m, 2H), 5.00(br.s, 2H), 5.89(d, 1H, J=9Hz), 6.37(br.s, 2H), 7.09(d, 1H, J=9Hz)

(4) 3-Acetylamino-5-amino-8-chloro-2,3-dihydroquinoline-4-one

20 260 mg of the compound prepared in (3) above was dissolved into a mixed solvent of 5 ml of dichloromethane and 5 ml of THF. To this solution were added 0.16 ml of pyridine and 0.13 ml of acetic anhydride while stirring under ice cooling, and the mixture was stirred for 20 minutes. The reaction product was concentrated, and after the addition of chloroform, the residue was washed with saturated sodium bicarbonate and saturated brine, and dried over anhydrous sodium sulfate. The product was concentrated and the residue was charged into ether and petroleum ether in to produce 287 mg powder of the title compound.

25 IR_v^{KBr} cm⁻¹: 3428, 1614, 1498, 1316, 1146
 30 NMR(CDCl₃)δ: 2.09(s, 3H), 3.0-4.7(m, 3H), 5.10(br.s, 1H), 5.89(d, 1H, J=9Hz), 6.51(br.s, 1H), 7.11(d, 1H, J=9Hz)
 MASS m/z: 253(M⁺), 255(M⁺ +2)

(5) (9S)-1-Acetylamino-9-ethyl-4-chloro-1,2-dihydro-9-hydroxy-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo-[ij][2,7]naphthyridine-10,13(9H,15H)-dione

35 The compound prepared in (4) above (267 mg) and 277 mg of trione werte reacted for 7 hours in the same manner as in Example 17-(5) and post-treated to obtain 202 mg of the title compound.

40 mp: 250-270°C
 IR_v^{KBr} cm⁻¹: 1748, 1662, 1608, 1158
 NMR(DMSO-d₆)δ: 0.87(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 2.50(s, 3H), 3.4-3.6(m, 2H), 5.19,5.27(ABq, 2H, J=19Hz), 5.43(s, 2H), 5.4-5.6(m, 1H), 6.52(s, 1H), 7.29,7.30(each s, 1H), 7.40(d, 1H, J=9Hz), 7.71(d, 1H, J=9Hz), 8.52(t, 1H, J=8Hz)
 MASS m/z: 480(M⁺), 482(M⁺ +2)

45

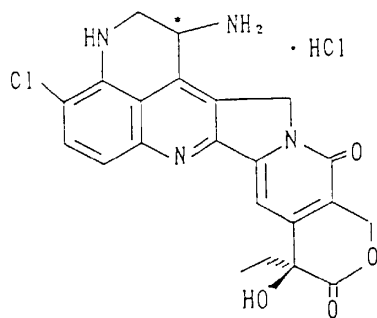
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Example 41

Preparation of (9S)-1-amino-9-ethyl-4-chloro-1,2-dihydro-9-hydroxy-3H,12H-pyrano[3',4':6,7]indolizino[1,2-c]benzo
[ij]-[2,7]naphthyridine-10,13(9H,15H)-dione hydrochloride



The compound prepared in Example 40-(5) (190 mg) was reacted for 3 hours in the same manner as in Example 2 and post-treated to produce Isomer A (67 mg) and Isomer B (40 mg) of the title compound.

Isomer A

mp: 130-150°C
 $\text{IR}_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1744, 1658, 1606, 1162
 $\text{NMR}(\text{DMSO-}d_6)\delta$: 0.88(t, 3H, J=7Hz), 1.7-2.0(m, 2H), 3.45(q, 1H, J=7Hz), 3.57,3.89(each d, 1H, J=12Hz), 4.2-4.5 (br, 1H), 5.15(s, 1H), 5.39,5.79(ABq, 2H, J=19Hz), 5.45(s, 2H), 6.55(s, 1H), 6.85(s, 1H), 7.34(s, 1H), 7.49(d, 1H, J=9Hz), 7.78(d, 1H, J=9Hz), 8.68(br.s, 3H)
 MASS m/z: 438(M⁺), 440(M⁺ +2)

Isomer B

mp: 250-270°C
 $\text{IR}_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1750, 1600, 1608, 1160
 $\text{NMR}(\text{DMSO-}d_6)\delta$: 0.88(t, 3H, J=7Hz), 1.7-1.9(m, 2H), 3.56,3.89(ABq, 1H, J=12Hz), 5.16(s, 1H), 5.41,5.78(ABq, 2H, J=19Hz), 5.45(s, 2H), 6.56(s, 1H), 6.86(s, 1H), 7.34(s, 1H), 7.49(d, 1H, J=9Hz), 7.79(d, 1H, J=9Hz), 8.67(br.s, 3H)
 MASS m/z: 438(M⁺), 440(M⁺ +2)

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Example 42

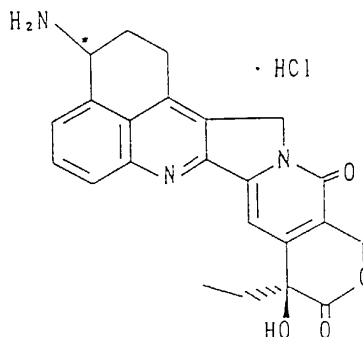
Preparation of (9S)-3-amino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

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(1) 8-Acetylamino-4-(1,3'-dioxoisindoline-2-yl)-1-tetralone

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1.07 gm of 8-acetylamino-1-tetralone, 1.28 gm of N-bromosuccinimide, and a catalytic amount of benzoyl peroxide were added to 60 ml of carbon tetrachloride. The mixture was heated under reflux for 1 hour, and cooled to room temperature. The precipitate was removed by filtration and the solvent was evaporated. The residue was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing bromine isomer of the target compound. The bromine isomer was dissolved in 20 ml of DMF and cooled to 0°C, followed by the addition 440 ml of sodium azide, a bit at a time. After stirring for 30 minutes at 0°C and for 1 hour at room temperature, and an addition of 30 ml of water, the mixture was extracted twice with ether. The extract was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (4:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 1.17 mg of 4-azide isomer. To the azide isomer were added 50 ml of benzene, 1.38 gm of triphenylphosphine, and 781 mg of phthalic anhydride. The mixture was heated under reflux for 12 hours, and a further 4 hours after the addition of 40 mg of tetra-n-butylammonium cyanide. After evaporating the solvent, the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (2:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 1.25 mg of the title compound.

40

NMR(CDCl₃)δ: 2.26(3H, s), 2.2-2.3(1H, m), 2.75-3.0(3H, m), 5.69(1H, dd, J=5.4Hz, 10.8Hz), 6.74(1H, d, J=7.8Hz), 7.44(1H, t, J=7.8Hz), 7.75-7.80(2H, m), 7.85-7.82(2H, m), 8.71(1H, d, J=7.8Hz), 12.18(1H, s),

(2) 8-Amino-4-(1,3'-dioxoisindoline-2-yl)-1-tetralone

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The compound prepared in (1) above (339 mg) was added to 10 ml of 1 N hydrochloric acid and heated under reflux for 1 hour. The mixture was cooled to room temperature, alkalized with the addition of sodium bicarbonate, and extracted with chloroform. The extract was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (4:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 190 mg of the title compound.

NMR(CDCl₃)δ: 2.15-2.21(1H, m), 2.7-3.0(3H, m), 5.60(1H, dd, J=4.6Hz, 11.5Hz), 6.22(1H, d, J=7.8Hz), 6.55(1H, d, J=7.8Hz), 7.12(1H, t, J=7.8Hz), 7.74-7.79(2H, m), 7.86-7.90(2H, m)

55

(3) (9S)-3-Amino-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

20 ml of toluene, 190 mg of the compound prepared in (2) above, and 163 mg of trione were reacted for 15 hours

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in the same manner as in Example 1-(5) and post-treated to obtain 263 mg of (9S)-9-ethyl-2,3-dihydro-9-hydroxy-3-(1,3-dioxoisindoline-2-yl)-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione as a mixture of 3-position isomer. 223 mg of this compound was processed in the same manner as in Example 13-(12) to obtain 73 mg of Isomer A and 58 mg of Isomer B of the title compound.

Isomer A

mp: above 190°C (decomposed)

NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.3Hz), 1.87(2H, m), 2.2-2.5(2H, m), 3.2-3.45(2H, m), 4.98(1H, m), 5.30(2H, s), 5.44(2H, s), 7.36(1H, s), 7.85-7.93(2H, m), 8.17(1H, d, J=8.3Hz), 8.88(3H, m)

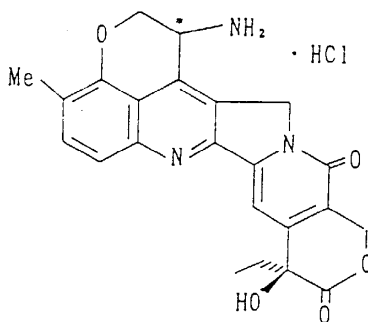
Isomer B

mp: above 218°C (decomposed)

NMR(DMSO-d₆)δ: 0.90(3H, t, J=7.3Hz), 1.88(2H, m), 2.3-2.5(2H, m), 3.3-3.4(2H, m), 4.91(1H, m), 5.32(2H, s), 5.45(2H, s), 7.37(1H, s), 7.85(1H, d, J=6.8Hz), 7.92(1H, dd, J=6.8Hz, 7.7Hz), 8.19(1H, d, J=7.7Hz), 8.80(3H, m)

Example 43

Preparation of (9S)-1-amino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-12H-pyrano[4,3,2-de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



(1) 3,5-Diacetylamino-8-methyl-4-chromanone

The reaction was carried out in the same manner as in Example 12-(1), except that 1 gm of 5-acetylamino-8-methyl-4-chromanone was used instead of 5-acetylamino-8-methyl-4-thiochromanone of Example 12-(1). The reaction product was post-treated to produce 105 mg of the title compound.

NMR(CDCl₃)δ: 2.11(3H, s), 2.17(3H, s), 2.22(3H, s), 3.98(1H, dd, J=12.1Hz, 15.1Hz), 4.70-5.05(2H, m), 6.26(1H, m), 7.34(1H, d, J=8.3Hz), 8.18(1H, d, J=8.3Hz)

(2) 3-Acetylamino-5-amino-8-methyl-4-chromanone

The compound prepared in (1) above (100 mg) was added to 3 ml of concentrated hydrochloric acid and heated at 80°C with stirring for 1 hour. The mixture was cooled, alkalized with the addition of sodium bicarbonate, and extracted with chloroform. The chloroform layer was dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using chloroform-ethyl acetate (4:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 48 mg of the title compound.

mp: 178-180°C

IR_{max}^{KBr} cm⁻¹: 3448, 3328, 1650, 1628, 1484

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NMR(CDC_l₃) δ : 2.06(3H, s), 2.08(3H, s), 3.7-4.1(1H, m), 4.65-5.05(2H, m), 6.14(1H, d, J=8.3Hz), 7.06(1H, d, J=8.3Hz)

(3) (9S)-1-Amino-9-ethyl-1,2-dihydro-9-hydroxy-4-methyl-12H-pyrano[4,3,2-de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride

The compound prepared in (2) above (139 mg) and 156 mg of trione was added to 12 ml of acetic acid and heated under reflux for 6 hours. The reaction mixture was cooled, solvent was evaporated, and the residue was subjected to silica gel column chromatography using chloroform-methanol (98:2) as an eluant to obtain fractions containing the target compound. The fractions were concentrated and the residue, after the addition of 5 ml of concentrated hydrochloric acid, was stirred for 2 hours. The reaction mixture was concentrated and 20 ml of water was added to the residue to remove insoluble substances. The filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (18:82:1) to obtain 77 mg of Isomer A and 93 mg of Isomer B of the title compound.

Isomer A

mp: above 200°C (decomposed)

IR_v^{KBr} cm^{-1} : 3432, 1756, 1658, 1602

NMR(DMSO- d_6) δ : 0.89(3H, t, J=6.8Hz), 1.83-1.92(2H, m), 2.44(3H, s), 4.44(1H, d, J=12.2Hz), 4.91(1H, d, J=12.2Hz), 5.20(1H, brs), 5.39,5.78(2H, ABq, J=19Hz), 5.44(2H, s), 7.37(1H, s), 7.78(1H, d, J=8.8Hz), 7.80(1H, d, J=8.8Hz), 8.88(3H, br)

MASS m/z: 420(M⁺⁺¹)

Isomer B

mp: above 200°C (decomposed)

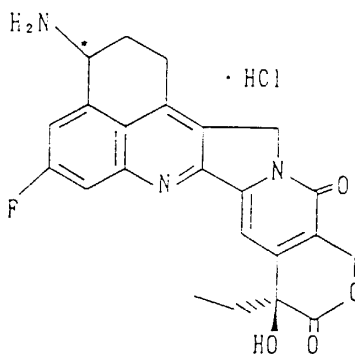
IR_v^{KBr} cm^{-1} : 3444, 1746, 1658, 1596

N(DMSO- d_6) δ : 0.90(3H, t, J=7.3Hz), 1.83-1.92(2H, m), 2.45(3H, s), 4.44(1H, d, J=11.7Hz), 4.91(1H, d, J=11.7Hz), 5.20(1H, brs), 5.41,5.78(2H, ABq, J=19.5Hz), 5.44(2H, s), 5.44,5.48(2H, ABq, J=16.6Hz), 7.40(1H, s), 7.78(1H, d, J=8.8Hz), 7.82(1H, d, J=8.8Hz), 8.97(3H, br)

MASS m/z: 420(M⁺⁺¹)

Example 44

Preparation of (9S)-3-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione



(1) 4-(4-Fluorophenyl)-4-oxobutanoic acid

50 gm of succinic anhydride and 133.3 gm of aluminum chloride was added to 79.6 gm of fluorobenzene, and the

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mixture was heated under reflux for 6 hours. After evaporating the surplus fluorobenzene, the residue was added to 2 l of 1% hydrochloric acid aqueous solution. The precipitate was collected by filtration, washed with water, and dried to obtain 73 gm of the title compound.

5 NMR(CDCl₃)δ: 2.82(2H, t, J=6.6Hz), 3.29(2H, t, J=6.6Hz), 7.1-7.2(2H, m), 7.9-8.1(2H, m)

(2) Methyl 4-(4-fluorophenyl)butanoate

10 The compound obtained in (1) above 73 gm was dissolved into 600 ml of acetic acid. To the solution were added 20 ml of 40% perchloric acid and 10% palladium-on-carbon to effect catalytic hydrogenation under 5 atm. After the evaporation of acetic acid and the addition of 100 ml of water, the residue was extracted with ethyl acetate. The extract was washed with saturated sodium bicarbonate and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and 100 ml of dichloromethane, 74 gm of sodium carbonate, and 30 ml of thionyl chloride were added to the residue. The mixture was heated under reflux for 3 hours and then cooled to 0°C, and 150 ml of methanol was slowly added, followed by stirring for 12 hours at room temperature. After the addition of 300 ml of ethyl acetate, the resulting product was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 66.7 gm of the title compound.

20 NMR(CDCl₃)δ: 1.93(2H, quintet, J=7.8Hz), 2.32(2H, t, J=7.8Hz), 2.62(2H, t, J=7.8Hz), 3.66(3H, s), 6.96(2H, m), 7.13(2H, m)

(3) Methyl 4-(4-fluoro-2-nitrophenyl)butanoate

25 The compound obtained in (2) above (66.7 gm) was added to 600 ml of cold concentrated sulfuric acid, and to the mixture was dropwise added a solution of 50 gm of potassium sulfate in 300 ml concentrated sulfuric acid while maintaining the internal temperature below 5°C. After the addition, the mixture was stirred for a further 30 minutes. The reaction product was poured into ice-cooled water and extracted with ethyl acetate. The ethyl acetate layer was washed with water, saturated sodium bicarbonate, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (40:3) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 39.3 gm of the title compound.

35 NMR(CDCl₃)δ: 1.98(2H, m), 2.40(2H, t, 7.3Hz), 2.91(2H, t, J=7.8Hz), 3.69(3H, s), 7.27(1H, ddd, J=2.9Hz, 7.8Hz, 8.3Hz), 7.37(1H, dd, J=5.3Hz, 8.3Hz), 7.66(1H, dd, J=2.9Hz, 8.8Hz)

(4) Methyl 4-(2-acetylamino-4-fluorophenyl)butanoate

40 The compound obtained in (3) above (39.3 gm) and 3 gm of 10% palladium-on-carbon were added to 200 ml of methanol, and the mixture was catalytically hydrogenated for 6 hours. The catalyst was removed by filtration and the solvent was evaporated. To the residue were added 50 ml of chloroform and, while stirring at room temperature, 50 ml of acetic anhydride. After further stirring for 3 hours, the reaction mixture was concentrated to dryness to obtain 42.0 gm of the title compound.

45 NMR(CDCl₃)δ: 1.72-1.85(2H, m), 2.31(3H, s), 2.45(2H, m), 2.56(2H, m), 3.76(3H, s), 6.71(1H, dt, J=2.5Hz, 8.3Hz), 7.04(1H, dd, J=6.3Hz, 8.3Hz), 8.08(1H, dd, J=2.5Hz, 10.8Hz)

(5) 5-Acetylamino-7-fluoro-1-tetralone

50 The compound obtained in (4) above (42 gm) was dissolved into 100 ml of methanol. After the addition of 200 ml of 1 N hydrochloric acid, the mixture was stirred for 3 hours at room temperature. The reaction mixture was concentrated to 200 ml and, after acidifying with the addition of concentrated hydrochloric acid, extracted three times with ethyl acetate. The ethyl acetate layer was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated, and to the residue were added 100 ml of dichloromethane, then 17 gm of sodium carbonate and 12 ml of thionyl chloride. The mixture was heated under reflux for 2 hours, and then cooled to room temperature to remove insoluble substances by filtration. The filtrate was concentrated and 200 ml of 1,2-dichloromethane and 40 gm of aluminum chloride were added to the residue. The mixture was stirred for 3 hours at 70°C, then for 1 hour at 90°C. The reaction product was poured into ice-cooled water and extracted with chloroform. The extract was washed with water, saturated sodium bicarbonate, and saturated brine in this order, and dried over anhy-

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drous sodium sulfate. The solvent was evaporated, and the residue was subjected to silica gel column chromatography using chloroform-ethyl acetate (7:3) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 17 gm of the title compound.

5 NMR(CDCl₃)δ: 2.16(2H, m), 2.24(3H, s), 2.66(2H, t, J=6.83Hz), 2.78(2H, t, J=6.0Hz), 7.08(1H, br.s), 7.59(1H, dd, J=2.4Hz, 8.3Hz), 7.84(1H, dd, J=2.4Hz, 7.8Hz) (6)

(6) 5-Acetylamino-7-fluoro-1,2,3,4-tetrahydronaphthalene

10 The compound obtained in (5) above (10.5 gm), 5 gm of 10% palladium-on-carbon, and 5 ml of perchloric acid were added to 300 ml of acetic acid, and the mixture was catalytically hydrogenated at 5 atm. for 6 hours. The catalyst was removed by filtration and the filtrate was concentrated to 50 ml. Upon addition of 100 ml of water, the concentrate was extracted 3 times with chloroform. The chloroform layer was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 9.5 gm of the title compound.

15 NMR(CDCl₃)δ: 1.7-1.9(4H, m), 2.20(3H, s), 2.52(2H, m), 2.75(2H, m), 6.61(1H, d, J=8.8Hz), 6.94(1H, m), 7.59(1H, d, J=9.8Hz)

(7) 8-Acetylamino-6-fluoro-1-tetralone

20 To absorption of the compound obtained in (6) above (9.46 gm) in 420 ml of acetone was added 42 ml of 15% aqueous solution of magnesium sulfate. After cooling to 0°C, 21.7 gm of potassium permanganate was added to the mixture, a bit at a time. The mixture was stirred for 50 minutes at 0°C and for 1 hour at room temperature. Upon addition of 1 l of water, the reaction product was extracted 3 times with chloroform. The chloroform layer was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (3:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 7.98 gm of the title compound.

25 NMR(CDCl₃)δ: 2.08(2H, m), 2.24(3H, s), 2.69(2H, t, J=6.4Hz), 2.96(2H, t, J=6.1Hz), 6.64(1H, dd, J=2.4Hz, 8.3Hz), 8.42(1H, dd, J=2.4Hz, 12.0Hz), 12.35(1H, br.s)

30 (8) 6-Fluoro-4-triphenylmethylamino-1-tetralone

35 10 ml of 4 N hydrochloric acid was added to 287 mg of the compound obtained in (7) above, and the mixture was heated under reflux for 1 hour. The reaction mixture was poured into ice-cooled water extracted with ethyl acetate. The ethyl acetate layer was washed with water, saturated aqueous solution of sodium bicarbonate, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (7:3) as an eluant to obtain fractions containing the target compound. 5 ml of dichloromethane, 0.8 ml of triethylamine, and 1.5 gm of triphenylmethyl chloride were added to the residue, and the mixture was heated under reflux for 4 hour, then cooled to room temperature. Upon addition of 20 ml of dichloromethane, the reaction mixture was washed with water, saturated aqueous solution of sodium bicarbonate, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using hexane-ethyl acetate (3:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 706 mg of the title compound.

45 NMR(CDCl₃)δ: 2.03(2H, m), 2.66(2H, t, J=6.4Hz), 2.84(2H, t, J=6.1Hz), 5.67(1H, dd, J=2.4Hz, 12.7Hz), 6.02(1H, dd, J=2.4Hz, 8.8Hz), 7.2-7.4(15H, m), 11.29(1H, s)

50 (9) 6-Fluoro-8-triphenylmethylamino-4-(1,3-dioxoisindolin-2-yl)-1-tetralone

55 706 mg of the compound obtained in (8) above, 231 mg of N-bromosuccinimide, and a catalytic amount of benzoyl peroxide were added to 30 ml of carbon tetrachloride. The mixture was heated under reflux for 30 minutes, and cooled to room temperature. After the addition of 30 ml of chloroform, the reaction product was washed with 1 N sodium hydroxide, water, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was dissolved into 5 ml of DMF and cooled to 0°C. 160 mg of sodium azide was added to the solution, a bit at a time, followed by stirring for 1 hour at room temperature. After the addition of 30 ml of water, the reaction mixture was extracted with ether and washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chroma-

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5 tography using hexane-ethyl acetate (9:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 344 mg of 4-azide isomer. To the azide isomer were added 20 ml of benzene, 205 mg of triphenylphosphine, and 116 mg of phthalic anhydride. The mixture was heated under reflux for 12 hours, after the addition of 40 mg of tetra-n-butylammonium cyanide for a further 8 hours. After heating under reflux for a further 8 hours, the solvent was evaporated, and the residue was subjected to silica gel column chromatography using chloroform-hexane (1:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 440 mg of the title compound.

10 NMR(CDCI₃)δ: 2.15(1H, m), 2.7-3.1(3H, m), 5.55(1H, dd, J=4.40Hz, 12.2Hz), 5.75(1H, dd, J=2.4Hz, 7.8Hz), 5.77(1H, dd, J=2.4Hz, 10.2Hz), 7.2-7.4(15H, m), 7.67(2H, m), 7.88(2H, m), 11.41(1H, s)

(10) 8-Amino-6-fluoro-(1,3-dioxoisindolin-2-yl)-1-tetralone

15 The compound obtained in (1) above (423 mg) was added to 8 ml of formic acid which were cooled to 0°C, and while stirring, one drop of concentrated hydrochloric acid was added to the mixture. After stirring for 1 hour at room temperature the solvent was evaporated. The residue was dissolved into chloroform and concentrated. A mixed solvent of ether and hexane was added to the concentrate to obtain crystals, which were collected by filtration to obtain 132 mg of the title compound.

20 NMR(CDCI₃)δ: 2.16(1H, m), 2.7-3.05(3H, m), 5.56(1H, dd, J=4.4Hz, 11.7Hz), 5.95(1H, dm, J=9.8Hz), 6.21(1H, dd, J=2.0Hz, 10.8Hz), 7.78(2H, m), 7.90(2H, m)

(11) (9S)-3-Amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione hydrochloride

25 10 ml of toluene, 125 mg of the compound obtained in (10) above, and 104 mg of trione were reacted for 22 hours in the same manner as in Example 1-(5) and post-treated to obtain 119 mg of (9S)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-3-(1,3-dioxoisindolin-2-yl)-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione as a mixture of 3-position isomers. 119 mg of this compound was treated in the same manner as in Example 13-(12) to obtain 31 mg of Isomer A and 35 mg of Isomer B of the title compound.

Isomer A

mp: gradually colored from 206°C (decomposed)

35 NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.87(2H, m), 2.21-2.33(1H, m), 2.40-2.55(1H, m), 3.25-3.40(2H, m), 4.92(1H, m), 5.31(2H, s), 5.45(2H, s), 6.54(1H, s), 7.35(1H, s), 7.81(1H, dd, J=2.4Hz, 9.3Hz), 7.97(1H, dd, J=2.4Hz, 10.3Hz), 8.70(3H, br.s)

Isomer B

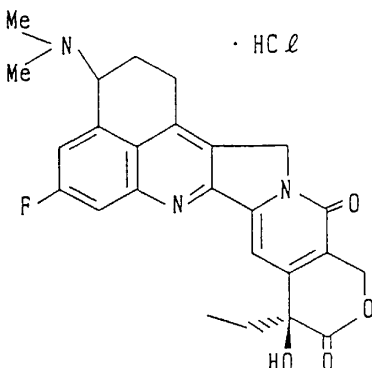
40 mp: gradually colored from 194°C (decomposed)

NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.88(2H, m), 2.2-2.3(1H, m), 2.4-2.5(1H, m), 3.3-3.4(2H, m), 4.94(1H, m), 5.31(2H, s), 5.45(2H, s), 7.36(1H, s), 7.82(1H, dd, J=2.4Hz, 9.3Hz), 7.98(1H, dd, J=2.4Hz, 10.2Hz), 8.81(3H, br.s)

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Example 45

Preparation of (9S)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-3-dimethylamino-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

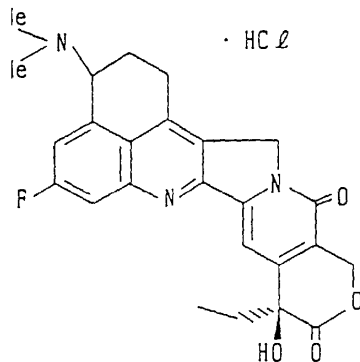


To 15 mg of Isomer A prepared in Example 44 were added 1 ml of 35% aqueous solution of formaline, 0.08 ml of formic acid and 0.03 ml of 1 N sodium hydroxide aqueous solution, and the mixture was heated under reflux for 1 hour. After evaporating the solvent, 2 ml of 1N hydrochloric acid aqueous solution was added to the residue. The solvent was evaporated and 4 ml of water was added to remove insoluble substances by filtration. The filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (17:83:1) to obtain 9 mg of the title compound.

mp: above 182°C (decomposed)
NMR(DMSO- d_6) δ : 0.88(3H, t, J=7.3Hz), 1.87(2H, m), 2.3-2.7(2H, m), 2.85(3H, d, J=4.4Hz), 2.87(3H, d, J=4.4Hz), 3.2-3.6(2H, m), 4.99(1H, m), 5.25,5.36(2H, ABq, J=19Hz), 5.45(2H, s), 6.55(1H, m), 7.36(1H, s), 8.0-8.1(2H, m), 10.42(1H, m)

Example 46

Preparation of (9S)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-3-dimethylamino-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



Isomer B prepared in Example 44 (20 mg) was treated in the same manner as in Example 45 and purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (21:79:1) to obtain 9 mg of the title compound.

mp: above 230°C (decomposed)

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NMR(DMSO- d_6) δ : 0.88(3H, t, $J=7.3$ Hz), 1.88(2H, m), 2.3-2.7(2H, m), 2.86(3H, d, $J=4.4$ Hz), 2.89(3H, d, $J=4.9$ Hz), 3.3-3.5(2H, m), 4.99(1H, m), 5.26, 5.37(2H, ABq, $J=19$ Hz), 5.45(2H, s), 6.55(1H, m), 7.36(1H, s), 8.01(1H, dm, $J=9.3$ Hz), 8.05(1H, dm, $J=9.8$ Hz), 10.25(1H, m)

5 Example 47

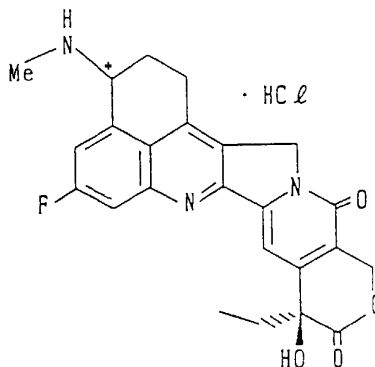
Preparation of (9S)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-3-methylamino-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

10

15

20

25



(1) 5-Acetoamino-7-fluoro-1-methyltrifluoroacetylaminotetralone

30

35

The compound obtained in Example 44-(5) (1.02 gm), 11 ml of 40% aqueous solution of methylamine, and 1.2 gm of 10% palladium-on-carbon were added to 20 ml of ethanol and catalytically hydrogenated for 9.5 hours. The catalyst was removed by filtration and the filtrate was concentrated to dryness. The residue was dissolved into 20 ml of chloroform 2 ml of triethylamine was added to the solution. Then, 4 ml of trifluoroacetic anhydride was added at 0°C while stirring. After stirring for a further 1 hour at room temperature, the resulting reaction mixture was washed with dilute hydrochloric acid, water, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using chloroform-ethyl acetate (40:3) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 1.33 gm of the title compound.

40

NMR(CDCl₃) δ : 1.7-2.0(1.6H, m), 2.0-2.3(2.4H, m), 2.22(3H, s), 2.5-2.7(2H, m), 2.71, 2.84(3H, each s), 5.15(0.4H, m), 5.79(0.6H, m), 6.54(0.6H, d, $J=8.3$ Hz), 6.65(0.4H, d, $J=8.3$ Hz), 6.97(1H, m), 7.65-7.75(1H, m)

(2) 8-Acetoamino-6-fluoro-4-methyltrifluoroacetylaminotetralone

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To a solution of the compound obtained in (1) above (1.32 gm) in 60 ml of acetone was added 6 ml of 15% aqueous solution of magnesium sulfate. 1.9 gm of potassium permanganate was slowly added to the mixture at 0°C while stirring. After the addition, the mixture was stirred for 30 minutes at the same temperature and another 30 minutes at room temperature. Upon addition of 60 ml of water, the reaction product was extracted 3 times with chloroform. The chloroform layer was washed with water and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using chloroform-ethyl acetate (9:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 748 mg of the title compound.

55

NMR(CDCl₃) δ : 2.0-2.4(2H, m), 2.26(3H, s), 2.7-3.0(2H, m), 2.84, 2.97(3H, each small m), 5.30(0.3H, dd, $J=4.9$ Hz, 11.7Hz), 5.96(0.7H, dd, $J=4.4$ Hz, 11.7Hz), 6.43(0.7H, dm, $J=7.3$ Hz), 6.56(0.3H, dm, $J=7.3$ Hz), 8.57(0.7H, dd, $J=2.4$ Hz, 11.2Hz), 8.60(0.3H, dd, $J=2.9$ Hz, 11.7Hz), 12.3(1H, br.s)

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(3) 8-Amino-6-fluoro-4-methyltrifluoroacetylamino-1-tetralone

20 ml of 4 N hydrochloric acid was added to 722 mg of the compound obtained in (2) above, and the mixture was heated under reflux for 5 hours. After cooling to room temperature, the mixture was weakly alkalized by the addition of saturated sodium carbonate and extracted three times with chloroform. The extract was washed with saturated brine, and dried over anhydrous sodium sulfate. The solvent was evaporated, and to the residue were added 50 ml of dichloromethane and 0.29 ml of triethylamine. Then, 15 ml of a dichloromethane solution containing 0.29 ml of trifluoroacetic anhydride was added dropwise to the mixture while stirring and cooling in an ice-salt bath. After stirring for 1 hour at the same temperature, the mixture was washed with water, saturated aqueous solution of citric acid, water, and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using chloroform-ethyl acetate (19:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 421 mg of the title compound.

NMR(CDCI₃)δ: 2.0-2.4(2H, m), 2.6-2.9(2H, m), 2.86,2.98(3H, each s), 5.20(0.35H, dd, J=4.4Hz,11.7Hz), 5.87(0.65H, dd, J=4.4Hz,11.7Hz), 5.97(0.65H, dm, J=9.3Hz), 6.09(0.35H, dm, J=9.3Hz), 6.26(0.65H, dd, J=2.4Hz, 8.7Hz), 6.28(0.35H, dd, J=2.0Hz, 8.8Hz)

(4) (9S)-9-Ethyl-5-fluoro-2,3-dihydro-9-hydroxy-3-methylamino-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino [1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

To 24 ml of toluene were added 378 mg of the compound obtained in (3) above and 330 mg of trione, and the mixture was reacted in the same manner as in Example 1-(5) and post-treated to obtain 340 mg of (9S)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-3-methyltrifluoroacetylamino-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione as a mixture of 3-position isomers. 30 ml of concentrated hydrochloric acid was added to 300 mg of this compound, and the mixture was heated at 80°C for 2.5 hours while stirring. The solvent was evaporated, 15 ml of water was added to the residue to remove insoluble substances by filtration. The filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:1) to obtain 83 mg of Isomer A and 103 mg of Isomer B of the title compound.

Isomer A

mp: above 230°C (decomposed)
NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.87(2H, m), 2.3-2.6(2H, m), 2.70(3H, br.s), 3.3-3.5(2H, m), 4.81(1H, m), 5.26,5.37(2H, ABq, J=19.1Hz), 5.45(2H, s), 6.55(1H, s), 7.36(1H, s), 7.90(1H, dd, J=2.4Hz,9.3Hz), 8.03(1H, dd, 2.4Hz, J=9.8Hz), 9.2-9.4(2H, m)

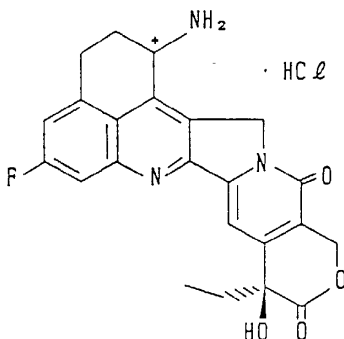
Isomer B

mp: above 230°C (decomposed)
NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.88(2H, m), 2.3-2.6(2H, m), 2.70(3H, br.s), 3.3-3.5(2H, m), 4.82(1H, m), 5.26,5.37(2H, ABq, J=19Hz), 5.45(2H, s), 6.54(1H, s), 7.36(1H, s), 7.91(1H, dd, J=2.4Hz,9.3Hz), 8.03(1H, dd, 2.4Hz, J=10.3Hz), 9.2-9.5(2H, m)

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Example 48

Preparation of (9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino
[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride



(1) 2,8-Diacetylamino-6-fluoro-1-tetralone

To 60 ml of a THF solution containing 2.28 gm of potassium-t-butoxide was slowly added 90 ml of a THF solution containing 3 gm of the compound obtained in Example 44-(7). After stirring for 10 minutes, 2.44 ml of n-butyl nitrite was added to the mixture, followed by further stirring for 1 hour at room temperature. The reaction mixture was poured into a dilute hydrochloric acid solution and extracted 3 times with chloroform. The extract was washed with water and saturated brine and dried over anhydrous sodium sulfate. The solvent was evaporated, and to the residue were added 87 ml of acetic acid and 87 ml of acetic anhydride, followed by adding thereto 11 gm of zinc powder at room temperature while stirring. After the addition, the stirring was continued for a further 10 minutes. Insoluble substances were removed by filtration, the solvent was evaporated, and the residue was dissolved into 100 ml of chloroform. This solution was washed with water, saturated aqueous solution of sodium hydroxide, and saturated brine in this order, was dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was subjected to silica gel column chromatography using a hexane-ethyl acetate (2:3) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 2.82 gm of the title compound.

NMR(CDCl₃)δ: 1.8-2.0(1H, m), 2.11(3H, s), 2.14(3H, s), 2.67(1H, m), 3.02(1H, m), 3.25(1H, m), 4.65(1H, ddd, J=4.9Hz, 5.4Hz, 13.2Hz), 6.48(1H, br.s), 6.64(1H, dd, J=8.3Hz, 2.4Hz), 8.43(1H, dd, J=8.2Hz, 2.4Hz)

(2) 8-Amino-6-fluoro-2-trifluoroacetylamino-1-tetralone

20 ml of concentrated hydrochloric acid was added to 1.23 gm of the compound prepared in (1) above, and the mixture was stirred at 100°C for 12 hours. After cooling to the room temperature, 200 ml of water was added and the mixture washed twice with chloroform. The water layer was alkalized by the addition of sodium hydroxide and extracted 4 times with chloroform. The extract was washed with saturated brine and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was dissolved into 50 ml of dried THF. To the solution was added 0.59 ml of triethylamine. Then, 10 ml of THF solution containing 0.6 ml of trifluoroacetic anhydride was added dropwise to the mixture while stirring and cooling in an ice-salt bath. After stirring for 1 hour at the same temperature, 5 ml of water was added and the solvent was evaporated. 30 ml of water was added to the residue and the mixture was extracted 3 times with chloroform. The extract was dried over anhydrous sodium sulfate. After removal of the solvent by evaporation, the residue was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 1.1 gm of the title compound.

NMR(CDCl₃)δ: 1.88(1H, ddd, J=4.4Hz, 13.2Hz, 25.4Hz), 2.79(1H, m), 2.97(1H, m), 3.15(1H, m), 4.50(1H, ddd, J=4.4Hz, 4.9Hz, 13.2Hz), 6.21(2H, m)

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(3) (9S)-1-Amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

144 mg of the compound prepared in (2) above and 130 mg of trione were added to 30 ml of toluene. To this was added a catalytic amount of PPTS to react the mixture in a Deanstark apparatus under heating with refluxing for 41 hours. The reaction mixture was cooled and the solvent was evaporated. The residue was subjected to silica gel column chromatography using a chloroform-methanol (99:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated, and 5 ml of methanol, 5 ml of water, and 4 ml of 4 N hydrochloric acid were added to the concentrate, followed by the stirring at 80°C for 5 hours. After concentration, 4 ml of water was added to the resulting product to remove insoluble substances by filtration. The filtrate was purified with HPLC (CAP-CELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (22:78:1) to obtain 19 mg of Isomer A and 16 mg of Isomer B of the title compound.

Isomer A

mp: above 200°C (decomposed)
NMR(DMSO-d₆)δ: 0.89(3H, t, J=7.3Hz), 1.89(2H, m), 2.15-2.27(1H, m), 2.40-2.55(1H, m), 3.20-3.45(2H, m), 5.07(1H, m), 5.42,5.84(2H, ABq, J=19.5Hz), 5.46(2H, s), 6.55(1H, s), 7.38(1H, s), 5.46(2H, s), 6.55(1H, s), 7.38(1H, s), 7.60(1H, dd, J=2.4Hz,9.3Hz), 7.86(1H, dd, 2.4Hz, J=10.3Hz), 8.2-8.6(3H, m)

Isomer B

mp: above 210°C (decomposed)
NMR(DMSO-d₆)δ: 0.88(3H, t, J=7.3Hz), 1.88(2H, m), 2.15-2.27(1H, m), 2.45-2.60(1H, m), 3.20-3.45(2H, m), 5.12(1H, m), 5.42,5.83(2H, ABq, J=19.1Hz), 5.46(2H, s), 6.55(1H, s), 7.38(1H, s), 7.62(1H, dd, J=2.4Hz,8.8Hz), 7.86(1H, dd,2.4Hz, J=10.2Hz), 8.4-8.6(3H, m), 7.86(1H, dd, 2.4Hz, J=10.2Hz), 8.4-8.6(3H, m)

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Example 49

Preparation of (9S)-1-amino-9-ethyl-4,5-difluoro-9-hydroxy-2,3-dihydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino-
[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

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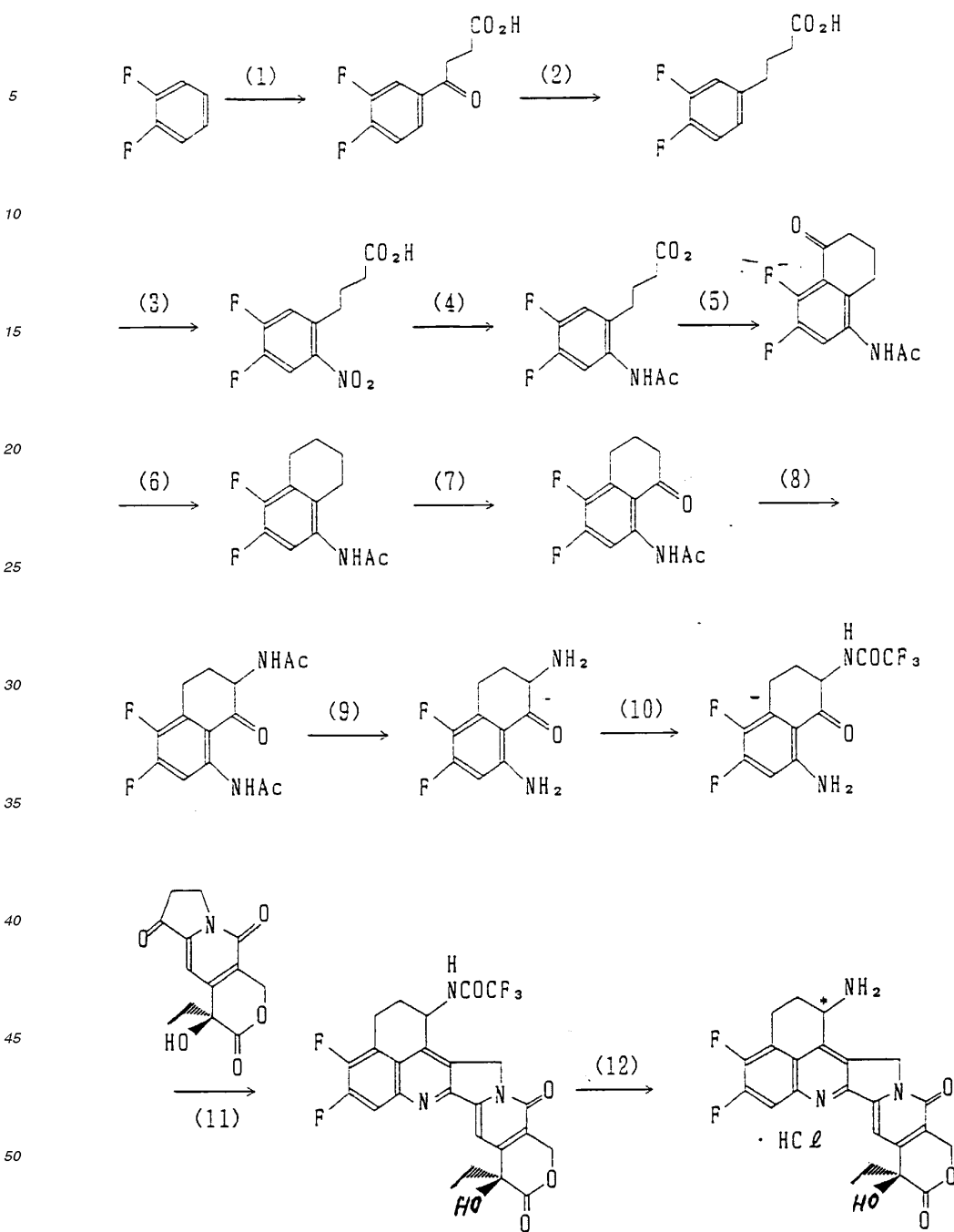
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(1) 4-(3,4-Difluorophenyl)-4-oxobutanoic acid

45 gm of succinic anhydride and 130.7 gm of aluminum chloride were added to 57 gm of difluorobenzene, and the

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mixture was heated under reflux for 4 hours. Upon the addition of 200 ml of 1% hydrochloric acid aqueous solution, the reaction mixture was extracted with chloroform, washed with water, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using a chloroform-methanol (30:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 30 gm of the title compound.

NMR(CDCl₃)δ: 2.82(2H, t, J=7Hz), 3.26(2H, t, J=7Hz), 7.0-7.9(3H, m)

(2) 4-(3,4-Difluorophenyl)-4-butanolic acid

The compound obtained in (1) above (30 gm) was dissolved into 200 ml of acetic acid. To the solution were added 8 ml of 60% perchloric acid and 10% palladium-on-carbon to effect catalytic hydrogenation under 6.5 atm. The catalyst was removed by filtration and acetic acid was evaporated. Upon the addition of 600 ml of ethyl acetate, the residue was washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 26 gm of the title compound.

NMR(CDCl₃)δ: 1.8-2.1(2H, m), 2.37(2H, t, J=7Hz), 2.64(2H, t, J=7Hz), 6.9-7.1(3H, m)

(3) 4-(4,5-Difluoro-2-nitrophenyl)butanoic acid

The compound obtained in (2) above (500 mg) was added to 4 ml of cold concentrated sulfuric acid, and to the mixture was dropwise added 2.5 ml concentrated sulfuric acid solution containing 371 mg of potassium sulfate while maintaining the internal temperature below 5°C. After the addition, the mixture was stirred for a further 30 minutes. The reaction product was poured into ice-cold water and extracted with 100 ml of chloroform. The chloroform layer was dried over anhydrous sodium sulfate and the solvent was evaporated to obtain 480 mg of the title compound.

NMR(CDCl₃)δ: 1.8-2.1(2H, m), 2.48(2H, t, J=7Hz), 2.9-3.0(2H, m), 7.21(1H, dd, J=7Hz, 10Hz), 7.90(1H, dd, J=7Hz, 10Hz)

(4) 4-(2-Acetylamino-4,5-difluorophenyl)butanoic acid

The compound obtained in (3) above (380 mg) was dissolved in a mixed solvent of 5 ml of acetic acid and 10 ml of acetic anhydride. Upon the addition of 60 mg of 10% palladium-on-carbon, the mixture was catalytically hydrogenated. The catalyst was removed by filtration, the solvent was evaporated, and the residue was subjected to silica gel column chromatography using a chloroform-methanol (10:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 90 mg of the title compound.

NMR(CDCl₃)δ: 1.7-1.9(2H, m), 2.29(3H, s), 2.4-2.6(4H, m), 6.93(1H, dd, J=8Hz, 12Hz), 8.09(1H, dd, J=8Hz, 12Hz), 8.53(1H, br s)

(5) 5-Acetylamino-7,8-difluoro-1-tetralone

The compound obtained in (4) above (9.07 gm) was dissolved into 450 ml of dichloromethane. To the mixture was added 7.71 gm of phosphorous pentachloride while stirring at room temperature. After stirring for a further 1 hour, the reaction mixture was concentrated and dichloromethane was added to the residue. Dichloromethane was evaporated again and 500 ml of 1,2-dichloroethane and 9.88 gm of anhydrous aluminum chloride were added to the residue. The mixture was stirred for 1 hour at 70°C and gently heated under reflux for 15 hours. The reaction product was poured into ice-cold water, extracted with chloroform, washed with saturated brine, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was subjected to silica gel column chromatography using chloroform-methanol (50:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 2.31 gm of the title compound.

NMR(CDCl₃)δ: 2.1-2.2(2H, m), 2.23(3H, s), 2.5-2.7(2H, m), 2.7-2.9(2H, m), 6.93(1H, br s), 7.84(1H, dd, J=7Hz, 11Hz)

(6) 5-Acetylamino-7,8-difluoro-1,2,3,4-tetrahydronaphthalene

The compound obtained in (5) above (1 gm) was dissolved into 20 ml of ethanol. To the solution was added 166

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mg of sodium borohydride while stirring at room temperature. After stirring for 20 minutes, chloroform and 10% citric acid were added to the reaction mixture. The chloroform layer was extracted and concentrated. To the concentrate were added 20 ml of toluene and a small amount of p-TsOH, followed by heating under reflux for 1 hour. Upon addition of 100 ml of ethyl acetate, the reaction product was washed with saturated brine and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was dissolved into a mixed solvent of 20 ml of ethanol, 20 ml of dioxane, and 0.2 ml of acetic acid. The mixture was catalytically hydrogenated with the addition of 200 mg of platinum oxide. The catalyst was removed, the filtrate was concentrated, and ether was added to the concentrate. 0.8 gm of the title compound was obtained by collecting the precipitate by filtration.

NMR(CDCl₃)δ: 1.79(4H, br s), 2.18(3H, s), 2.52(2H, br s) 2.73(2H, br s), 6.91(1H, br s), 7.4-7.6(1H, m)

(7) 8-Acetylamino-5,6-difluoro-1-tetralone

To a solution of the compound obtained in (6) above (810 mg) in 30 ml of acetone was added 3 ml of 15% aqueous solution of magnesium sulfate. 1.17 gm of potassium permanganate was slowly added to the mixture while stirring. After 1 hour, the reaction product was extracted with chloroform, the extract was washed with water and saturated brine, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 806 mg of the title compound.

NMR(CDCl₃)δ: 2.05-2.17(2H, m), 2.23(3H, s), 2.68(2H, t, J=5.85Hz), 3.00(2H, t, J=5.85Hz), 8.61(1H, dd, J=7.8Hz, 13.2Hz), 12.1-12.23(1H, br s)

(8) 2,8-Diacetylamino-5,6-difluoro-1-tetralone

To a mixed solution of 15 ml of a THF solution and 1.5 ml of tert-butanol containing 309 mg of potassium-butoxide was added slowly in a nitrogen stream at 0°C 300 mg of the compound prepared in (7) above dissolved in 7.5 ml of THF. After stirring for 10 minutes at the same temperature, 0.22 ml of n-butyl nitrite was dropwise added to the mixture, followed by further stirring for 1.5 hours, during which the temperature was slowly raised to 20°C. The reaction mixture was adjusted to pH 1 with the addition of 1 N hydrochloric acid, extracted with chloroform, washed with saturated brine, and dried over anhydrous sodium sulfate. To the residue obtained by evaporating the solvent was added a mixture of 15 ml of acetic acid and 15 ml of acetic anhydride, and was added about 1 gm of zinc powder, followed by stirring at 20°C for 18 hours. Insoluble substances were removed by filtration, the solvent was evaporated, and the residue was extracted with chloroform. The extract was washed with saturated brine and dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was subjected to silica gel column chromatography using a chloroform-methanol (97:3) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 311 mg of the title compound.

NMR(CDCl₃)δ: 1.83-1.94(1H, m), 2.10(3H, s), 2.21(3H, s), 2.59-2.67(1H, m), 2.92-3.01(1H, m), 3.18-3.26(1H, m), 4.62-4.68(1H, m), 6.70(1H, d, J=5.9Hz), 8.56(1H, dd, J=7.3Hz, 13.2Hz), 11.64-11.67(1H, br s)

(9) 2,8-Diamino-5,6-difluoro-1-tetralone

50 ml of 3 N hydrochloric acid was added to 300 mg of the compound obtained in (8) above, and the mixture was stirred at 60°C for 3.5 hours. After the addition of 15 ml of concentrated hydrochloric acid, the mixture was stirred at 90°C for 30 minutes, cooled, and neutralized with sodium bicarbonate. The product was extracted with 300 ml of chloroform, washed with saturated brine, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 140 mg of the title compound.

NMR(CDCl₃)δ: 1.79-1.93(1H, m), 2.68-2.81(2H, m), 3.16-3.20(1H, m), 3.53-3.56(1H, m), 6.26-6.29(2H, m), 6.34-6.45(2H, m), 7.28(1H, s)

(10) 8-Amino-5,6-difluoro-2-trifluoroacetylamino-1-tetralone

The compound obtained in (8) above (140 mg) was dissolved into 10 ml of THF and cooled to 0°C. To the solution were added 75 mg of triethylamine, and then 155 mg trifluoroacetic anhydride slowly, followed by stirring for 1.5 hour.. After the addition of saturated aqueous solution of sodium bicarbonate, the mixture was extracted with chloroform, washed with saturated brine and dried over anhydrous sodium sulfate. After removal of the solvent by evaporation, the residue was subjected to silica gel column chromatography using chloroform-methanol (97:3) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 93 mg of the title compound.

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NMR(CDCI₃)δ: 1.85(1H, ddd, J=4.4Hz, 13Hz, 26.1Hz), 2.75-2.84(2H, m), 3.20-3.26(1H, m), 4.5(1H, dd, J=4.9Hz, 13.7Hz), 6.31(1H, dd, J=6.3Hz, 11.7Hz), 6.35-6.44(2H, br s), 7.54-7.6(1H, br s)

(11) (9S)-9-Ethyl-4,5-difluoro-2,3-dihydro-9-hydroxy-1-trifluoroacetyl-amino-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

The compound obtained in (10) above (90 mg) was dissolved into 20 ml of toluene, and, after the addition of 81 mg of trione, the mixture was heated under reflux for 111 hours in a nitrogen stream. The reaction product was concentrated and the residue was subjected to silica gel column chromatography using chloroform-methanol (30:1) as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 98 mg of the title compound.

NMR(CDCI₃)δ: 1.01(3H, t, J=7Hz), 1.80-1.83(2H, m), 2.01(1H, s), 2.33(1H, s), 2.96(1H, dd, J=6.35Hz, 7.32Hz), 4.32-4.36(1H, m), 5.23(3H, dd, J=6.35Hz, 17.1Hz), 5.67(1H, d, J=17.1Hz), 7.55(1H, s)

(12) (9S)-1-Amino-9-ethyl-4,5-difluoro-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

30 ml of 1 N hydrochloric acid was added to 95 mg of the compound prepared in (11) above, and the mixture was heated under reflux for 1 hour. The solvent was evaporated and 30 ml of water was added to remove insoluble substances by filtration. The filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:1) to obtain 6.2 mg of Isomer A and 5.9 mg Isomer B of the title compound.

Isomer A

IR_{v_{max}}^{KBr} cm⁻¹: 3416, 1746, 1660, 1602, 1512
NMR(DMSO-d₆)δ: 0.87(3H, t, J=7.3Hz), 1.82-1.91(2H, m), 2.15-2.26(1H, m), 5.11-5.18(1H, br s), 5.44,5.84(2H, ABq, J=19Hz), 5.45(2H, s), 7.35(1H, s), 8.18(1H, dd, J=7.8Hz, 11.5Hz), 8.55-8.64(3H, br)
MASS m/z: 439(M⁺)

Isomer B

IR_{v_{max}}^{KBr} cm⁻¹: 2932, 1750, 1658, 1596, 1508
NMR(DMSO-d₆)δ: 0.86(3H, t, J=7.3Hz), 1.83-1.9(2H, m), 2.15-2.24(1H, m), 5.12-5.16(1H, br s), 5.44,5.82(2H, ABq, J=19Hz), 5.45(2H, s), 6.55(1H, s), 7.35(1H, s), 8.19(1H, dd, J=7.8Hz, 11.5Hz), 8.53-8.66(3H, br)
MASS m/z: 439(M⁺)

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Example 50

Preparation of (9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

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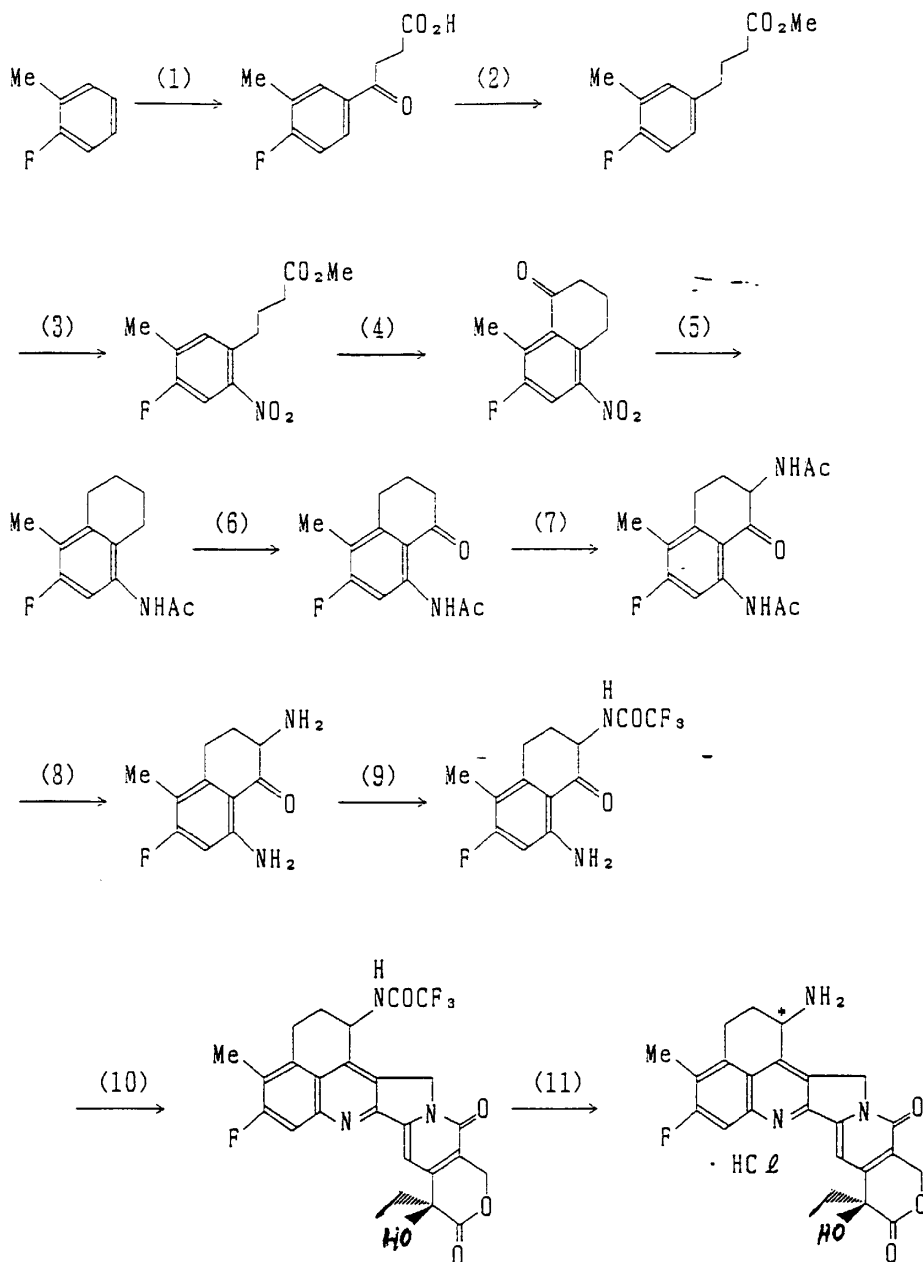
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(1) 4-(4-Fluoro-3-methylphenyl)-4-oxobutanoic acid

200 gm of succinic anhydride and 800 gm of aluminum chloride were added to 250 ml of 2-fluorotoluene, and the mixture was heated at 80°C for 1 hour. Upon the addition of 5 l of 1% cold hydrochloric acid aqueous solution, the reaction mixture was extracted with ethyl acetate, washed with 1 N hydrochloric acid and water, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 345 gm of the title compound.

NMR(CDCl₃)δ: 1.89-1.97(2H, m), 2.24(3H, s), 2.36(2H, t, J=7.3Hz), 2.60(2H, t, J=7.3Hz), 6.84-6.99(3H, m)

(2) Methyl 4-(4-fluoro-3-methylphenyl)butanoate

The compound obtained in (1) above (172 gm) was dissolved into 700 ml of acetic acid. To the solution were added 10 ml of 40% perchloric acid and 30 gm of 10% palladium-on-carbon to effect catalytic hydrogenation under 6 atm. The catalyst was removed by filtration and acetic acid was evaporated. Upon the addition of 2 l of water, the residue was extracted with chloroform, washed with saturated brine, and dried over anhydrous sodium sulfate. The solvent was evaporated and 1 l of methanol was added to the residue. After cooling to 0°C, 269 gm of thionyl chloride was slowly dropped to the solution, followed by the addition of 10 ml of dimethylformamide. The mixture was stirred for 12 hours at room temperature, the reaction product was concentrated, and extracted with chloroform. The extract was washed with water and saturated brine, and dried over anhydrous magnesium sulfate. The solvent was evaporated to obtain 153 gm of the title compound.

NMR(CDCl₃)δ: 1.91(2H, quintet, J=7.5Hz), 2.24(3H, d, J=2Hz), 2.31(2H, t, J=7.5Hz), 2.57(2H, t, J=7.5Hz), 3.66(3H, s), 6.89(1H, t, J=9Hz), 6.93(1H, ddd, J=9Hz, 5Hz, 3Hz), 6.97(1H, dd, J=7Hz, 3Hz)

(3) Methyl 4-(4-fluoro-3-methyl-6-nitrophenyl)butanoate

The compound obtained in (2) above (10.9 gm) was added to 6 ml of cold concentrated sulfuric acid, and to the mixture was dropwise added 5.8 gm of potassium nitrate in 15 ml of concentrated sulfuric acid, while maintaining the internal temperature below 5°C. After the addition, the mixture was stirred for a further 20 minutes. The reaction product was poured into ice-cooled water and extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and the solvent was evaporated. The residue was subjected to silica gel column chromatography using a hexane-ethyl acetate (90:7) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 7.1 gm of the title compound.

NMR(CDCl₃)δ: 1.97(2H, quintet, J=8Hz), 2.34(3H, d, J=2Hz), 2.41(2H, t, J=8Hz), 2.90(2H, t, J=8Hz), 3.69(3H, s), 7.18(1H, d, J=7Hz), 7.66(1H, d, J=9Hz)

(4) 7-Fluoro-8-methyl-5-nitro-1-tetralone

The compound obtained in (3) above (7.1 gm) was dissolved in 20 ml of methanol. To the solution was added 10 ml of 15% aqueous solution of sodium hydroxide and the mixture was stirred for 3 hours at room temperature. The reaction mixture was concentrated, acidified with concentrated hydrochloric acid, and extracted with chloroform. The chloroform layer was washed with water and saturated brine in this order and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was slowly added to 50 ml of polyphosphoric acid heated to 110°C and stirred for 4.5 hours. After the addition of 100 ml of ice-water, the reaction product was extracted with ethyl acetate, washed with water, saturated sodium bicarbonate and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was subjected to silica gel column chromatography using a hexane-ethyl acetate (1:1) mixed solvent as an eluant to obtain fractions containing the target compound. The fractions were concentrated to produce 1.7 gm of the title compound.

NMR(CDCl₃)δ: 2.11(2H, quintet, J=7Hz), 2.60(3H, d, J=2Hz), 2.72(2H, t, J=7Hz), 3.14(2H, t, J=6.0Hz), 7.74(1H, d, J=9Hz)

(5) 5-Acetylamino-7-fluoro-8-methyl-1,2,3,4-tetrahydro-naphthalene

The compound obtained in (4) above (1.35 gm) was dissolved into 5 ml of ethanol and 15 ml of THF. To the solution was added 114 mg of sodium borohydride while stirring at room temperature. After stirring for 20 minutes, the reaction mixture was concentrated. To the concentrate were added 25 ml of toluene and 840 mg of p-TsOH, followed by heating

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under reflux for 30 minutes. Upon the addition of 100 ml of ethyl acetate, the reaction product was washed with saturated brine and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was dissolved into 20 ml of ethyl acetate and catalytically hydrogenated with the addition of 400 mg of platinum oxide for 4 hours. The catalyst was removed, the filtrate was concentrated, and 10 ml of dichloromethane was added to the concentrate. After the further addition of 1.2 ml of triethylamine and 0.81 ml of acetic anhydride, the mixture was stirred for 40 minutes. The reaction product was diluted with 50 ml of chloroform, washed with dilute hydrochloric acid, saturated sodium bicarbonate and saturated brine in this order, and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 1.23 gm of the title compound.

NMR(CDCl₃)δ: 1.79(4H, s), 2.10(3H, s), 2.19(3H, s), 2.53(2H, s), 2.62(2H, s), 7.46(1H, d, J=9Hz)

(6) 8-Acetylamino-6-fluoro-5-methyl-1-tetralone

1.2 gm of the compound obtained in (5) above was reacted in the same manner as in Example 49-(7) and post-treated to obtain 825 mg of the title compound.

NMR(CDCl₃)δ: 2.08(2H, quintet, J=7Hz), 2.15(3H, d, J=2Hz), 2.22(3H, s), 2.66(2H, t, J=7Hz), 2.88(2H, t, J=6Hz), 8.42(1H, d, J=13Hz)

(7) 2,8-Diacetylamino-6-fluoro-5-methyl-1-tetralone

4.7 gm of the compound obtained in (6) above was reacted in the same manner as in Example 49-(8) and post-treated to obtain 3.85 gm of the title compound.

NMR(CDCl₃)δ: 1.7-1.9(1H, m), 2.11(3H, s), 2.15(3H, s), 2.23(3H, s), 2.7-2.8(1H, m), 2.9-3.1(2H, m), 4.5-4.7(1H, m), 6.53(1H, br s), 8.43(1H, dq, J=13Hz), 11.76(1H, s)

(8) 2,8-Diamino-6-fluoro-5-methyl-1-tetralone

3.85 gm of the compound obtained in (7) above was added to 100 ml of 6 N hydrochloric acid, and the mixture was stirred at 80°C for 6 hours. The reaction mixture was poured into 100 ml of water, adjusted to pH 10 with the addition of 15% aqueous solution of sodium hydroxide, and extracted with chloroform. The extract was washed with water and saturated brine in this order and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain 1.66 gm of the title compound.

NMR(CDCl₃)δ: 1.83(1H, dq, J=13Hz, 4Hz), 2.04(3H, s), 2.25-2.4(1H, m), 2.75(1H, ddd, J=14Hz, 13Hz, 4Hz), 2.98(1H, ddd, J=14Hz, 4Hz, 3Hz), 3.53(1H, dd, J=13Hz, 4Hz), 6.20(1H, d, J=12Hz), 6.42(1H, br s)

(9) 8-Amino-6-fluoro-5-methyl-2-trifluoroacetylamino-1-tetralone

The compound obtained in (8) above (1.66 gm) was dissolved into a mixed solvent of 70 ml of ethanol and 10 ml of THF. To the solution were added 2 ml of triethylamine and 1.5 ml of ethyltrifluoroacetate, followed by stirring for 20 hours at 20°C. The reaction product was added to dilute aqueous solution of hydrochloric acid and extracted with chloroform. The extract was washed with water, saturated sodium bicarbonate and saturated brine in this order, and dried over anhydrous sodium sulfate. After removal of the solvent by evaporation, the residue was subjected to silica gel column chromatography using chloroform as an eluant to obtain fractions containing the target compound. The fractions were concentrated to obtain 1.79 gm of the title compound.

NMR(CDCl₃)δ: 1.81(1H, dq, J=13Hz, 5Hz), 2.06(3H, d, J=0.5Hz), 2.8-3.1(3H, m), 4.48(1H, dt, 13Hz, 4Hz), 6.26(1H, d, J=13Hz), 7.64(1H, br s)

(10) (9S)-9-Ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1-trifluoroacetylamino-1H,12H-benzo[de]pyrano [3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione

The compound obtained in (9) above (4 gm) and trione (4.57 gm) were reacted for 47 hours in the same manner as Example 49-(11) and post-treated to obtain 4.21 gm of the title compound.

NMR(MeOH-d₄+CDCl₃)δ: 0.94, 0.96(3H, each t, J=7Hz), 1.75-1.95(2H, m), 2.36(3H, s), 2.3-2.5(2H, m), 3.05-3.35

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(2H, m), 5.05-5.75(5H, m), 7.30(0.5H, d, J=11Hz), 7.45(0.5H, s), 7.56(0.5H, d, J=11Hz), 7.57(0.5H, s)

(11) (9S)-1-Amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino [1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride

625 mg of the compound prepared in (10) above was added to a mixed solution of 7.5 ml-of concentrated hydrochloric acid, 18 ml of methanol and 12 ml of water, and stirred at 80°C for 7 hours. Insoluble substances were removed by filtration and the filtrate was purified with HPLC (CAPCELL PAK C18) using a mixture of acetonitrile-water-1 N hydrochloric acid (20:80:1) to obtain 138 mg of Isomer A and 143 mg Isomer B of the title compound.

Isomer A

mp: 220-250°C (Decomposed)
 $[\alpha]_D^{20} = +198^\circ$ (c=0.42, in H₂O)
 IR_{max}^{KBr} cm⁻¹: 3400, 1748, 1660, 1592
 NMR(D₂O)δ: 0.73(3H, t, J=7.3Hz), 1.74(2H, q, J=7.3Hz), 2.13(3H, s), 2.45-2.55(1H, m), 2.6-2.7(1H, m), 2.85-3.0(1H, m), 3.2-3.3(1H, m), 5.11(1H, m), 5.18, 5.25(2H, ABq, J=19Hz), 5.18, 5.32(2H, ABq, J=16Hz), 7.05(1H, s), 7.09(1H, d, J=11Hz)

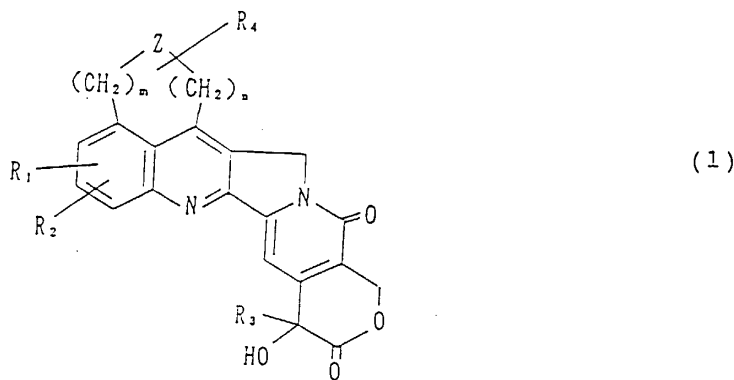
Isomer B

mp: 220-230°C (Decomposed)
 NMR(D₂O)δ: 0.82(3H, t, J=7.3Hz), 1.83(2H, q, J=7.3Hz), 2.13(3H, s), 2.1-2.3(1H, m), 2.5-2.6(1H, m), 2.9-3.1(1H, m), 3.1-3.3(1H, m), 4.97(1H, br s), 5.06, 5.32(2H, ABq, J=19Hz), 5.24, 5.37(2H, ABq, J=16Hz), 6.99(1H, d, J=11Hz), 7.16(1H, s)

Claims

Claims for the following Contracting States : AT, BE, CH, LI, DE, DK, FR, GB, IT, LU, NL, PT, SE

1. A hexa-cyclic compound represented by the following general formula:



wherein R₁ and R₂ individually represent

- a hydrogen atom,
- a hydroxyl group,
- a C₁₋₆ alkyl group which may contain one or more hydroxyl groups, halogen atoms, nitro groups or cyano groups,
- a C₁₋₆ alkenyl group,
- a C₁₋₆ alkynyl group,

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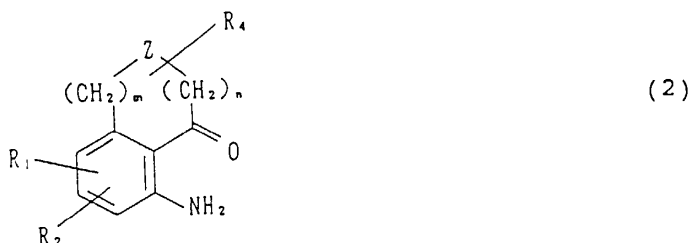
- a C₁₋₆ alkoxy group,
 a C₁₋₆ aminoalkoxy group,
 a halogen atom,
 a nitro group,
 5 a cyano group,
 a mercapto group,
 an alkylthio group,
 an amino group which may contain a protective group,
 a C₁₋₆ aminoalkyl group which may contain a protective group or a C₁₋₆ alkyl group at the amino-moiety,
 10 a C₁₋₆ aminoalkylamino group which may contain a protective group or a C₁₋₆ alkyl group at the amino-moiety,
 a C₁₋₆ alkyl group with a heterocyclic ring which may contain one or more C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro or cyano groups,
 a heterocyclic carbonyl group which may contain one or more C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro or cyano groups,
 15 a C₁₋₆ alkylamino group with a heterocyclic ring which may contain C₁₋₆ alkyl, C₁₋₆ alkoxy, amino (which may contain a protective group), halogeno, nitro, cyano group, or a protective group,
 an amino-heterocyclic group which may contain a protective group or one or more C₁₋₆ alkyl groups at the nitrogen atom of the heterocyclic ring moiety or at the amino moiety,
 a heterocyclic-amino group which may contain a protective group or a C₁₋₆ alkyl group at the nitrogen atom of the heterocyclic ring moiety or at the amino moiety, or
 20 a carbamoyl group which may contain a protective group or a C₁₋₆ alkyl group;
- R₃ represents a C₁₋₆ alkyl group;
 R₄ represents -N⁺(CH₃)₃,
 25
- an amino group which may contain a protective group,
 a C₁₋₆ alkylamino group which may contain a protective group,
 a C₁₋₆ aminoalkyl group which may contain a protective group,
 a C₁₋₆ alkylaminoalkyl group which may contain a protective group,
 30 a sulfonic acid group, or
 a carboxyl group;
- Z represents an oxygen atom,
 35
- a sulfur atom, CR₅R₆, wherein R₅ and R₆ individually represent a hydrogen atom or a C₁₋₆ alkyl, or N-R₇, wherein R₇ stands for a hydrogen atom, a C₁₋₆ alkyl group, a C₁₋₆ aminoalkyl group (which may contain a protective group), a C₁₋₆ alkylaminoalkyl group (which may contain a protective group), or a protective group for the amino group; and
 40
- m and n individually represent 0, 1 or 2;
 or a salt thereof.
- 45 2. The compound according to Claim 1, wherein R₁ and R₂ individually represent a group selected from the group consisting of C₁₋₃ alkyl, C₁₋₃ alkenyl, C₁₋₃ alkoxy, hydroxymethyl, hydroxyl, halogen atom, nitro, amino, C₁₋₃ alkylamino, cyano, cyano-C₁₋₃ alkyl, aminomethyl, dimethylhydrazino, morpholin-1-yl and piperidin-1-yl.
 3. The compound according to Claim 1, wherein R₃ represents an ethyl group.
 - 50 4. The compound according to Claim 1, wherein R₄ represents a group selected from the group consisting of C₁₋₆ alkylamino, amino-C₁₋₆ alkyl and C₁₋₆ alkylamino-C₁₋₆ alkyl.
 - 55 5. The compound according to Claim 1, wherein Z represents a group selected from the group consisting of methylene group, oxygen atom, sulfur atom, imino group and alkylimino group.
 6. The compound according to Claim 1, wherein Z represents a methylene group.
 7. The compound according to Claim 1, having S-type configuration with respect to the substituent groups at F-ring

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in formula (1) defined in Claim 1.

8. (9S)-1-Amino-9-ethyl-4-methyl-9-hydroxy-2,3-dihydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione.
9. (9S)-1-Amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6'7]indolizino [1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride.
10. A process for the preparation of the compound defined in Claim 1 comprising subjecting an aminoketone compound

of the following formula (2),

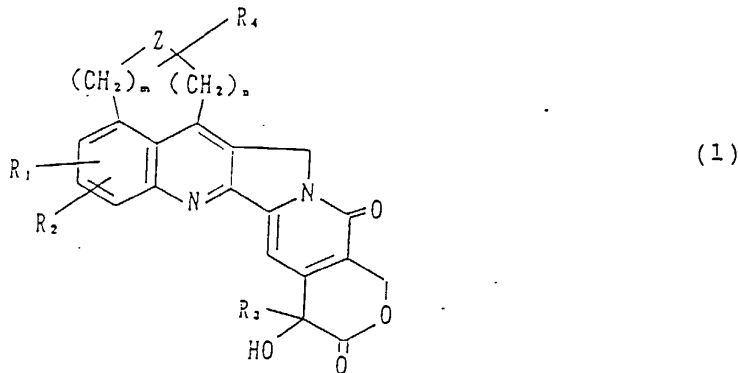


and a pyranoindolizine compound of the following formula (3) to the condensation-ring closing reaction in the presence of an acid or a base.



Claims for the following Contracting States : ES, GR

1. A process for the preparation of a hexa-cyclic compound represented by the following formula:



wherein R_1 and R_2 individually represent

a hydrogen atom,

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a hydroxyl group,
 a C₁₋₆ alkyl group which may contain one or more hydroxyl groups, halogen atoms, nitro groups or cyano groups,
 a C₁₋₆ alkenyl group,
 5 a C₁₋₆ alkynyl group,
 a C₁₋₆ alkoxy group,
 a C₁₋₆ aminoalkoxy group,
 a halogen atom,
 a nitro group,
 10 a cyano group,
 a mercapto group;
 an alkylthio group,
 an amino group which may contain a protective group,
 a C₁₋₆ aminoalkyl group which may contain a protective group or a C₁₋₆ alkyl group at the amino-moiety,
 15 a C₁₋₆ aminoalkylamino group which may contain a protective group or a C₁₋₆ alkyl group at the amino-moiety,
 a C₁₋₆ alkyl group with a heterocyclic ring which may contain one or more C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro or cyano groups,
 a heterocyclic carbonyl group which may contain one or more C₁₋₆ alkyl, C₁₋₆ alkoxy, amino, halogeno, nitro or cyano groups,
 20 a C₁₋₆ alkylamino group with a heterocyclic ring which may contain C₁₋₆ alkyl, C₁₋₆ alkoxy, amino (which may contain a protective group), halogeno, nitro, cyano group, or a protective group,
 an amino-heterocyclic group which may contain a protective group or one or more C₁₋₆ alkyl groups at the nitrogen atom of the heterocyclic ring moiety or at the amino moiety,
 a heterocyclic-amino group which may contain a protective group or a C₁₋₆ alkyl group at the nitrogen atom
 25 of the heterocyclic ring moiety or at the amino moiety, or
 a carbamoyl group which may contain a protective group or a C₁₋₆ alkyl group;

R₃ represents a C₁₋₆ alkyl group;
 30 R₄ represents -N⁺(CH₃)₃.

an amino group which may contain a protective group,
 a C₁₋₆ alkylamino group which may contain a protective group,
 a C₁₋₆ aminoalkyl group which may contain a protective group,
 35 a C₁₋₆ alkylaminoalkyl group which may contain a protective group,
 a sulfonic acid group, or
 a carboxyl group;

Z represents an oxygen atom,
 40 a sulfur atom,
 CR₅R₆, wherein R₅ and R₆ individually represent a hydrogen atom or a C₁₋₆ alkyl, or
 N-R₇, wherein R₇ stands for a hydrogen atom, a C₁₋₆ alkyl group, a C₁₋₆ aminoalkyl group (which may contain a protective group), a C₁₋₆ alkylaminoalkyl group (which may contain a protective group), or a protective group
 45 for the amino group; and

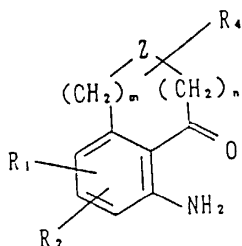
m and n individually represent 0, 1 or 2;

or a salt thereof, comprising subjecting an aminoketone compound of the following formula (2):

50

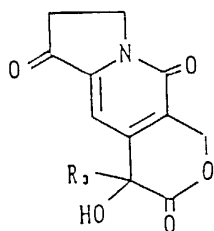
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(2)

wherein R_1 , R_2 , R_4 , m and n are as defined above, and a pyranoindolizine compound of the following formula (3) to the condensation-ring closing reaction in the presence of an acid or a base,



(3)

wherein R_3 is as defined above.

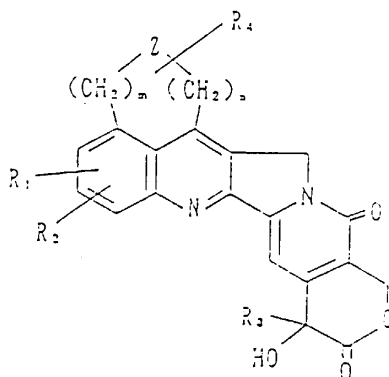
2. The process according to Claim 1, wherein R_1 and R_2 individually represent a group selected from the group consisting of C_{1-3} alkyl, C_{1-3} alkenyl, C_{1-3} alkoxy, hydroxymethyl, hydroxyl, halogen atom, nitro, amino, C_{1-3} alkylamino, cyano, cyano- C_{1-3} alkyl, aminomethyl, dimethylhydrazino, morpholin-1-yl and piperidin-1-yl.
3. The process according to Claim 1, wherein R_3 represents an ethyl group.
4. The process according to Claim 1, wherein R_4 represents a group selected from the group consisting of C_{1-6} alkylamino, amino- C_{1-6} alkyl and C_{1-6} alkylamino C_{1-6} alkyl.
5. The process according to Claim 1, wherein Z represents a group selected from the group consisting of methylene group, oxygen atom, sulfur atom, imino group and alkylimino group.
6. The process according to claim 1, wherein Z represents a methylene group.
7. The process according to Claim 1, having S-type configuration with respect to the substituent groups at F-ring in formula (I) defined in Claim 1.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, LI, DE, DK, FR, GB, IT, LU, NL, PT, SE

1. Hexacyclische Verbindung, die durch die folgende allgemeine Formel dargestellt wird:

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(1)

in der R_1 und R_2 einzeln darstellen:

ein Wasserstoffatom,
 eine Hydroxylgruppe,
 eine C_{1-6} -Alkylgruppe, die eine oder mehrere Hydroxylgruppen, Halogenatome, Nitrogruppen oder Cyano-
 gruppen enthalten kann,
 eine C_{1-6} -Alkenylgruppe,
 eine C_{1-6} -Alkynylgruppe,
 eine C_{1-6} -Alkoxygruppe,
 eine C_{1-6} -Aminoalkoxygruppe,
 ein Halogenatom,
 eine Nitrogruppe,
 eine Cyanogruppe,
 eine Mercaptogruppe,
 eine Alkylthiogruppe,
 eine Aminogruppe, die eine Schutzgruppe enthalten kann,
 eine C_{1-6} -Aminoalkylgruppe, die eine Schutzgruppe oder eine C_{1-6} -Alkylgruppe an der Aminoereinheit enthalten
 kann,
 eine C_{1-6} -Aminoalkylaminogruppe, die eine Schutzgruppe oder eine C_{1-6} -Alkylgruppe an der Aminoereinheit
 enthalten kann,
 eine C_{1-6} -Alkylgruppe mit einem heterocyclischen Ring, die eine oder mehrere C_{1-6} -Alkyl-, C_{1-6} -Alkoxy-,
 Amino-, Halogen-, Nitro- oder Cyanogruppen enthalten kann,
 eine heterocyclische Carbonylgruppe, die eine oder mehrere C_{1-6} -Alkyl-, C_{1-6} -Alkoxy-, Amino-, Halogen-,
 Nitro- oder Cyanogruppen enthalten kann,
 eine C_{1-6} -Alkylaminogruppe mit einem heterocyclischen Ring, die C_{1-6} -Alkyl-, C_{1-6} -Alkoxy-, Aminogruppen
 (die
 eine Schutzgruppe enthalten können), Halogen-, Nitro-, Cyanogruppen oder eine Schutzgruppe enthalten
 kann,
 eine aminoheterocyclische Gruppe, die eine Schutzgruppe oder eine oder mehrere C_{1-6} -Alkylgruppen am
 Stickstoffatom der heterocyclischen Ringeinheit oder an der Aminoereinheit enthalten kann,
 eine heterocyclische Aminogruppe, die eine Schutzgruppe oder eine C_{1-6} -Alkylgruppe am Stickstoffatom der
 heterocyclischen Ringeinheit oder an der Aminoereinheit enthalten kann, oder
 eine Carbamoylgruppe, die eine Schutzgruppe oder
 eine C_{1-6} -Alkylgruppe enthalten kann;

R_3 eine C_{1-6} -Alkylgruppe darstellt;

R_4 $-N^+(CH_3)_3$,

eine Aminogruppe, die eine Schutzgruppe enthalten kann,
 eine C_{1-6} -Alkylaminogruppe, die eine Schutzgruppe enthalten kann,
 eine C_{1-6} -Aminoalkylgruppe, die eine Schutzgruppe enthalten kann,
 eine C_{1-6} -Alkylaminoalkylgruppe, die eine Schutzgruppe enthalten kann,
 eine Sulfonsäuregruppe oder

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eine Carboxylgruppe darstellt;

Z ein Sauerstoffatom,

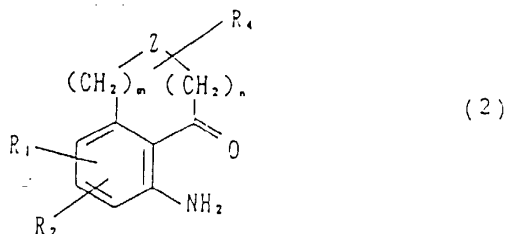
ein Schwefelatom,

CR_5R_6 , worin R_5 und R_6 einzeln ein Wasserstoffatom oder ein C_{1-6} -Alkyl darstellen, oder NR_7 darstellt, worin R_7 für ein Wasserstoffatom, eine C_{1-6} -Alkylgruppe, eine C_{1-6} -Aminoamkyogruppe (die eine Schutzgruppe enthalten kann), eine C_{1-6} -Alkylaminoalkylgruppe (die eine Schutzgruppe enthalten kann) oder eine Schutzgruppe für die Aminogruppe steht; und

m und n einzeln 0, 1 oder 2 darstellen;

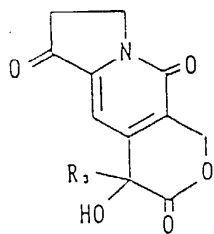
oder ein Salz derselben.

2. Verbindung nach Anspruch 1, in der R_1 und R_2 einzeln eine Gruppe darstellen, die aus der Gruppe ausgewählt ist, die aus C_{1-3} -Alkyl, C_{1-3} -Alkenyl, C_{1-3} -Alkoxy, Hydroxymethyl, Hydroxyl, einem Halogenatom, Nitro, Amino, C_{1-3} -Alkylamino, Cyano, Cyano- C_{1-3} -alkyl, Aminomethyl, Dimethylhydrazino, Morpholin-1-yl und Piperidin-1-yl besteht.
3. Verbindung nach Anspruch 1, in der R_3 eine Ethylgruppe darstellt.
4. Verbindung nach Anspruch 1, in der R_4 eine Gruppe darstellt, die aus der Gruppe ausgewählt ist, die aus C_{1-6} -Alkylamino, Amino- C_{1-6} -alkyl und C_{1-6} -Alkylamino- C_{1-6} -alkyl besteht.
5. Verbindung nach Anspruch 1, in der Z eine Gruppe darstellt, die aus der Gruppe ausgewählt ist, die aus einer Methylengruppe, einem Sauerstoffatom, einem Schwefelatom, einer Iminogruppe und einer Alkyliminogruppe besteht.
6. Verbindung nach Anspruch 1, in der Z eine Methylengruppe darstellt.
7. Verbindung nach Anspruch 1, die eine Konfiguration vom S-Typ bezüglich der Substituentengruppen am Ring F in der in Anspruch 1 definierten Formel (I) aufweist.
8. (9S)-1-Amino-9-ethyl-4-methyl-9-hydroxy-2,3-dihydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]chinolin-10,13(9H,15H)-dion.
9. (9S)-1-Amino-9-ethyl-5-fluor-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]chinolin-10,13(9H,15H)-dionhydrochlorid.
10. Verfahren zur Herstellung der in Anspruch 1 definierten Verbindung, umfassend, daß man eine Aminoketon-Verbindung der folgenden Formel (2)



und eine Pyranoindolizin-Verbindung der folgenden Formel (3) der Kondensations-Ringschluß-Reaktion in Anwesenheit einer Säure oder einer Base unterzieht.

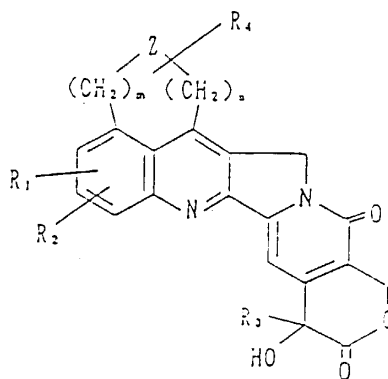
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(3)

Patentansprüche für folgende Vertragsstaaten : ES, GR

1. Verfahren zur Herstellung einer hexacyclischen Verbindung, die durch die folgende Formel dargestellt wird:



(1)

in der R₁ und R₂ einzeln darstellen:

ein Wasserstoffatom,
 eine Hydroxylgruppe,
 eine C₁₋₆-Alkylgruppe, die eine oder mehrere Hydroxylgruppen, Halogenatome, Nitrogruppen oder Cyano-
 gruppen enthalten kann,
 eine C₁₋₆-Alkenylgruppe,
 eine C₁₋₆-Alkynylgruppe,
 eine C₁₋₆-Alkoxygruppe,
 eine C₁₋₆-Aminoalkoxygruppe,
 ein Halogenatom,
 eine Nitrogruppe,
 eine Cyanogruppe,
 eine Mercaptogruppe,
 eine Alkylthiogruppe,
 eine Aminogruppe, die eine Schutzgruppe enthalten kann,
 eine C₁₋₆-Aminoalkylgruppe, die eine Schutzgruppe
 oder eine C₁₋₆-Alkylgruppe an der Aminoeinheit enthalten kann,
 eine C₁₋₆-Aminoalkylaminogruppe, die eine Schutzgruppe oder eine C₁₋₆-Alkylgruppe an der Aminoeinheit
 enthalten kann,
 eine C₁₋₆-Alkylgruppe mit einem heterocyclischen Ring, die eine oder mehrere C₁₋₆-Alkyl-, C₁₋₆-Alkoxy-,
 Amino-, Halogen-, Nitro- oder Cyanogruppen enthalten kann,
 eine heterocyclische Carbonylgruppe, die eine oder mehrere C₁₋₆-Alkyl-, C₁₋₆-Alkoxy-, Amino-, Halogen-,
 Nitro- oder Cyanogruppen enthalten kann,
 eine C₁₋₆-Alkylaminogruppe mit einem heterocyclischen Ring, die C₁₋₆-Alkyl-, C₁₋₆-Alkoxy-, Aminogruppen
 (die
 eine Schutzgruppe enthalten können), Halogen-, Nitro-, Cyanogruppen oder eine Schutzgruppe enthalten

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kann,
eine aminoheterocyclische Gruppe, die eine Schutzgruppe oder eine oder mehrere C₁₋₆-Alkylgruppen am Stickstoffatom der heterocyclischen Ringeinheit oder an der Aminoereinheit enthalten kann,
eine heterocyclische Aminogruppe, die eine Schutzgruppe oder eine C₁₋₆-Alkylgruppe am Stickstoffatom der heterocyclischen Ringeinheit oder an der Aminoereinheit enthalten kann, oder
eine Carbamoylgruppe, die eine Schutzgruppe oder eine C₁₋₆-Alkylgruppe enthalten kann;

R₃ eine C₁₋₆-Alkylgruppe darstellt;

R₄ -N⁺(CH₃)₃,

eine Aminogruppe, die eine Schutzgruppe enthalten kann,
eine C₁₋₆-Alkylaminogruppe, die eine Schutzgruppe enthalten kann,
eine C₁₋₆-Aminoalkylgruppe, die eine Schutzgruppe enthalten kann,
eine C₁₋₆-Alkylaminoalkylgruppe, die eine Schutzgruppe enthalten kann,
eine Sulfonsäuregruppe oder
eine Carboxylgruppe darstellt;

Z ein Sauerstoffatom,

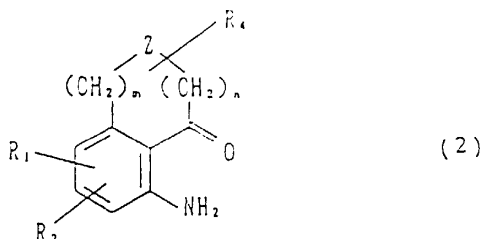
ein Schwefelatom,

CR₅R₆, worin R₅ und R₆ einzeln ein Wasserstoffatom oder ein C₁₋₆-Alkyl darstellen, oder

NR₇ darstellt, worin R₇ für ein Wasserstoffatom, eine C₁₋₆-Alkylgruppe, eine C₁₋₆-Aminoamkyoaruppe (die eine Schutzgruppe enthalten kann), eine C₁₋₆-Alkylaminoalkylgruppe (die eine Schutzgruppe enthalten kann) oder eine Schutzgruppe für die Aminogruppe steht; und

m und n einzeln 0, 1 oder 2 darstellen;

oder eines Salzes derselben,
umfassend, daß man eine Aminoketon-Verbindung der folgenden Formel (2)



in der R₁, R₂, R₄, m und n wie oben definiert sind, und eine Pyranoindolizin-Verbindung der folgenden Formel (3) der Kondensations-Ringschluß-Reaktion in Anwesenheit einer Säure oder einer Base unterzieht,



worin R₃ wie oben definiert ist.

2. Verfahren nach Anspruch 1, in dem R₁ und R₂ einzeln eine Gruppe darstellen, die aus der Gruppe ausgewählt ist, die aus C₁₋₃-Alkyl, C₁₋₃-Alkenyl, C₁₋₃-Alkoxy, Hydroxymethyl, Hydroxyl, einem Halogenatom, Nitro, Amino,

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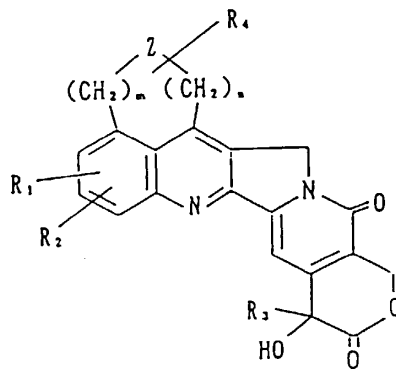
C₁₋₃-Alkylamino, Cyano, cyano-C₁₋₃-alkyl, Aminomethyl, Dimethylhydrazino, Morpholin-1-yl und Piperidin-1-yl besteht.

3. Verfahren nach Anspruch 1, in dem R₃ eine Ethylgruppe darstellt.
4. Verfahren nach Anspruch 1, in dem R₄ eine Gruppe darstellt, die aus der Gruppe ausgewählt ist, die aus C₁₋₆-Alkylamino, Amino-C₁₋₆-alkyl und C₁₋₆-Alkylamino-C₁₋₆-alkyl besteht.
5. Verfahren nach Anspruch 1, in dem Z eine Gruppe darstellt, die aus der Gruppe ausgewählt ist, die aus einer Methylengruppe, einem Sauerstoffatom, einem Schwefelatom, einer Iminogruppe und einer Alkyliminogruppe besteht.
6. Verfahren nach Anspruch 1, in dem Z eine Methylengruppe darstellt.
7. Verfahren nach Anspruch 1, wobei eine Konfiguration vom S-Typ bezüglich der Substituentengruppen am Ring F in der in Anspruch 1 definierten Formel (I) vorliegt.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, LI, DE, DK, FR, GB, IT, LU, NL, PT, SE

1. Composé hexacyclique représenté par la formule générale suivante :



(1)

dans laquelle R₁ et R₂ représentent individuellement

un atome d'hydrogène,
 un groupe hydroxyle,
 un groupe alkyle en C₁₋₆ pouvant contenir un ou plusieurs groupes hydroxyle, atomes d'halogène, groupes
 nitro ou groupes cyano,
 un groupe alcényle en C₁₋₆,
 un groupe alcynyle en C₁₋₆,
 un groupe alcoxyle en C₁₋₆,
 un groupe aminoalcoxyle en C₁₋₆,
 un atome d'halogène,
 un groupe nitro,
 un groupe cyano,
 un groupe mercapto,
 un groupe alkylthio,
 un groupe amino pouvant contenir un groupe protecteur,
 un groupe aminoalkyle en C₁₋₆ pouvant contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ au niveau
 du fragment amino,

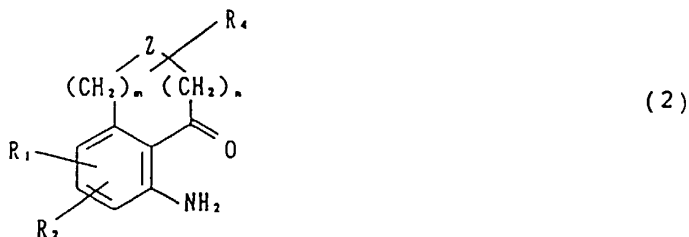
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- un groupe aminoalkylamino en C₁₋₆ pouvant contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ au niveau du fragment amino,
 un groupe alkyle en C₁₋₆, avec un groupe hétérocyclique pouvant contenir un ou plusieurs groupes alkyle en C₁₋₆, alcoyle en C₁₋₆, amino, halogéno, nitro ou cyano,
 5 un groupe hétérocyclique carbonyle pouvant contenir un ou plusieurs groupes alkyle en C₁₋₆, alcoyle en C₁₋₆, amino, halogéno, nitro ou cyano,
 un groupe alkylamino en C₁₋₆ avec un groupe hétérocyclique pouvant contenir un groupe alkyle en C₁₋₆, alcoyle en C₁₋₆, amino (qui peut contenir un groupe protecteur), halogéno, nitro, cyano, ou un groupe protecteur,
 10 un groupe amino-hétérocyclique, qui peut contenir un groupe protecteur ou un ou plusieurs groupes alkyle en C₁₋₆ au niveau de l'atome d'azote du fragment groupe hétérocyclique ou au niveau du fragment amino,
 un groupe hétérocyclique-amino, qui peut contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ au niveau de l'atome d'azote du fragment groupe hétérocyclique ou au niveau du fragment amino, ou
 un groupe carbamoyle, qui peut contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ ;
 15 R₃ représente un groupe alkyle en C₁₋₆ ;
 R₄ représente -N⁺(CH₃)₃,
 un groupe amino qui peut contenir un groupe protecteur,
 20 un groupe alkylamino en C₁₋₆ qui peut contenir un groupe protecteur,
 un groupe aminoalkyle en C₁₋₆ qui peut contenir un groupe protecteur,
 un groupe (alkylamino en C₁₋₆)-alkyle, qui peut contenir un groupe protecteur,
 un groupe acide sulfonique, ou
 un groupe carboxyle ;
 25 Z représente un atome d'oxygène,
 un atome de soufre,
 CR₅R₆, où R₅ et R₆, représentent individuellement un atome d'hydrogène ou un groupe alkyle en C₁₋₆, ou
 30 N-R₇, où R₇ est un atome d'hydrogène, un groupe alkyle en C₁₋₆, un groupe aminoalkyle en C₁₋₆ (qui peut contenir un groupe protecteur), un groupe (alkylamino en C₁₋₆)-alkyle (qui peut contenir un groupe protecteur),
 ou un groupe protecteur pour le groupe amino ; et
 m et n représentent individuellement 0, 1 ou 2 ;
 35 ou un sel de celui-ci.
2. Composé selon la revendication 1, dans lequel R₁ et R₂ représentent individuellement un groupe choisi parmi l'ensemble constitué par alkyle en C₁₋₃, alcényle en C₁₋₃, alcoyle en C₁₋₃, hydroxyméthyle, hydroxyle, halogène, nitro, amino, alkylamino en C₁₋₃, cyano, cyano-(alkyle en C₁₋₃), aminométhyle, diméthylhydrazino, morpholine-1-yle et pipéridine-1-yle.
 40
 3. Composé selon la revendication 1, dans lequel R₃ est un groupe éthyle.
 4. Composé selon la revendication 1, dans lequel R₄ représente un groupe choisi parmi l'ensemble constitué par alkylamino en C₁₋₆, amino-alkyle en C₁₋₆ et (alkylamino en C₁₋₆)-alkyle en C₁₋₆.
 45
 5. Composé selon la revendication 1, dans lequel Z représente un groupe choisi parmi l'ensemble constitué par le groupe méthylène, l'atome d'oxygène, l'atome de soufre, le groupe imino et un groupe alkylimino.
 50
 6. Composé selon la revendication 1, dans lequel Z représente un groupe méthylène.
 7. Composé selon la revendication 1, ayant une configuration de type S pour ce qui est des groupes substituants au niveau du noyau F dans la formule (I) selon la revendication 1.
 55
 8. (9S)-1-amino-9-éthyl-4-méthyl-9-hydroxy-2,3-dihydro-1H,12H-benzo[de]pyranno[3',4':6,7]indolizino-[1,2-b]quinoléine-10,13(9H,15H)-dione.

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9. Chlorhydrate de la (9S)-1-amino-9-éthyl-5-fluoro-2,3-dihydro-9-hydroxy-4-méthyl-1H,12H-benzo[de]pyranno[3',4':6,7]indolizino-[1,2-b]quinoléine-10,13(9H,15H)-dione.

10. Procédé pour préparer le composé selon la revendication 1, qui comprend l'opération consistant à soumettre une aminocétone ayant la formule (2) ci-après

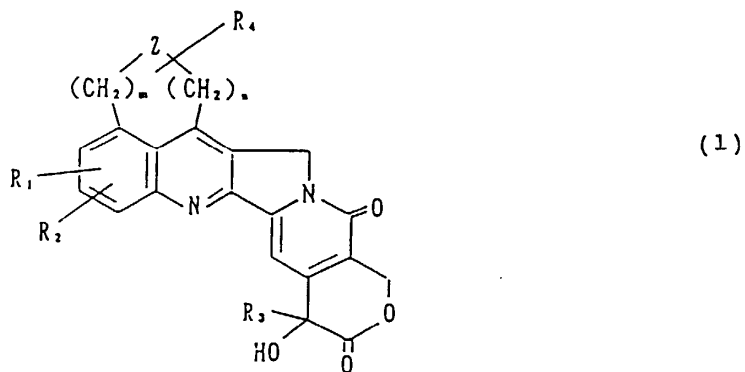


et une pyrannindolizine ayant la formule (3) ci-après, à la réaction de cyclisation par condensation, en présence d'un acide ou d'une base.



Revendications pour les Etats contractants suivants : ES, GR

1. Procédé pour préparer un composé hexacyclique représenté par la formule générale suivante :



dans laquelle R_1 et R_2 représentent individuellement

- un atome d'hydrogène,
- un groupe hydroxyle,
- un groupe alkyle en C_{1-6} pouvant contenir un ou plusieurs groupes hydroxyle, atomes d'halogène, groupes nitro ou groupes cyano,
- un groupe alcényle en C_{1-6} ,
- un groupe alcynyle en C_{1-6} ,
- un groupe alcoyle en C_{1-6} ,
- un groupe aminoalcoyle en C_{1-6} .

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un atome d'halogène,
 un groupe nitro,
 un groupe cyano,
 un groupe mercapto,
 5 un groupe alkylthio,
 un groupe amino pouvant contenir un groupe protecteur,
 un groupe aminoalkyle en C₁₋₆ pouvant contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ au niveau
 du fragment amino,
 10 un groupe aminoalkylamino en C₁₋₆ pouvant contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ au
 niveau du fragment amino,
 un groupe alkyle en C₁₋₆ avec un groupe hétérocyclique pouvant contenir un ou plusieurs groupes alkyle en
 C₁₋₆, alcoyle en C₁₋₆, amino, halogéno, nitro ou cyano,
 un groupe hétérocyclique carbonyle pouvant contenir un ou plusieurs groupes alkyle en C₁₋₆, alcoyle en
 C₁₋₆, amino, halogéno, nitro ou cyano,
 15 un groupe alkylamino en C₁₋₆ avec un groupe hétérocyclique pouvant contenir un groupe alkyle en C₁₋₆,
 alcoyle en C₁₋₆, amino (qui peut contenir un groupe protecteur), halogéno, nitro, cyano, ou un groupe pro-
 tecteur,
 un groupe amino-hétérocyclique, qui peut contenir un groupe protecteur ou un ou plusieurs groupes alkyle
 en C₁₋₆ au niveau de l'atome d'azote du fragment groupe hétérocyclique ou au niveau du fragment amino,
 20 un groupe hétérocyclique-amino, qui peut contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ au niveau
 de l'atome d'azote du fragment groupe hétérocyclique ou au niveau du fragment amino, ou
 un groupe carbamoyle, qui peut contenir un groupe protecteur ou un groupe alkyle en C₁₋₆ ;

R₃ représente un groupe alkyle en C₁₋₆ ;
 25 R₄ représente -N⁺(CH₃)₃,

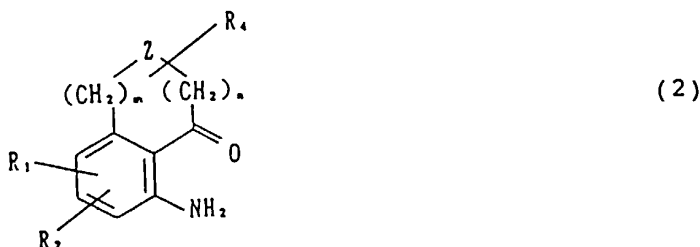
un groupe amino qui peut contenir un groupe protecteur,
 un groupe alkylamino en C₁₋₆ qui peut contenir un groupe protecteur,
 un groupe aminoalkyle en C₁₋₆ qui peut contenir un groupe protecteur,
 30 un groupe (alkylamino en C₁₋₆)-alkyle qui peut contenir un groupe protecteur,
 un groupe acide sulfonique, ou
 un groupe carboxyle ;

35 Z représente un atome d'oxygène,

un atome de soufre,
 CR₅R₆, où R₅ et R₆ représentent individuellement un atome d'hydrogène ou un groupe alkyle en C₁₋₆, ou
 N-R₇, où R₇ est un atome d'hydrogène, un groupe alkyle en C₁₋₆, un groupe aminoalkyle en C₁₋₆ (qui peut
 contenir un groupe protecteur), un groupe (alkylamino en C₁₋₆)-alkyle (qui peut contenir un groupe protecteur),
 40 ou un groupe protecteur pour le groupe amino ; et

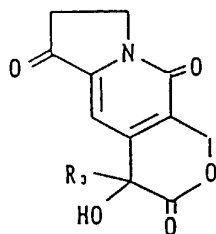
m et n représentent individuellement 0, 1 ou 2 ;

45 ou un sel de celui-ci, qui comprend l'opération consistant à soumettre une aminocétone ayant la formule (2)
 ci-après :



55 dans laquelle R₁, R₂, R₄, m et n sont tels que définis ci-dessus, et une pyranoidolizine ayant la formule
 (3) ci-après, à la réaction de cyclisation par condensation en présence d'un acide ou d'une base,

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(3)

où R_3 est tel que défini ci-dessus.

2. Procédé selon la revendication 1, dans lequel R_1 et R_2 représentent individuellement un groupe choisi parmi l'ensemble constitué par alkyle en C_{1-3} , alcényle en C_{1-3} , alcoxyle en C_{1-3} , hydroxyméthyle, hydroxyle, halogène, nitro, amino, alkylamino en C_{1-3} , cyano, cyano-(alkyle en C_{1-3}), aminométhyle, diméthylhydrazino, morpholine-1-yle et pipéridine-1-yle.
3. Procédé selon la revendication 1, dans lequel R_3 est un groupe éthyle.
4. Procédé selon la revendication 1, dans lequel R_4 représente un groupe choisi parmi l'ensemble constitué par alkylamino en C_{1-6} , amino-alkyle en C_{1-6} et (alkylamino en C_{1-6})-alkyle en C_{1-6} .
5. Procédé selon la revendication 1, dans lequel Z représente un groupe choisi parmi l'ensemble constitué par le groupe méthylène, l'atome d'oxygène, l'atome de soufre, le groupe imino et un groupe alkylimino.
6. Procédé selon la revendication 1, dans lequel Z représente un groupe méthylène.
7. Procédé selon la revendication 1, dans lequel le composé a une configuration de type S pour ce qui est des groupes substituants au niveau du noyau F dans la formule (I) selon la revendication 1.

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221:00, 311:00)

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(22) Date of filing: **10.04.1996**

(54) **Camthothecin derivative with antitumor activity**

Camptothecin-derivat und dessen Verwendung als Antitumormittel

Dérivés de camthothécine et son utilisation comme agent antitumoral

(84) Designated Contracting States:
**AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC
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(56) References cited:
EP-A- 0 296 597 EP-A- 0 495 432

- **PATENT ABSTRACTS OF JAPAN vol. 18, no. 347**
(C-1219), 30 June 1994 & JP-A-06 087746
(DAIICHI SEIYAKU CO LTD), 29 March 1994,

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Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

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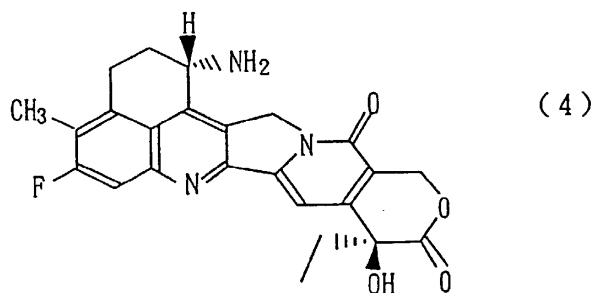
Description

Technical Field

5 [0001] This invention relates to a camptothecin derivative having antitumor activities and an industrially advantageous preparation process therefor.

Background Art

10 [0002] The compound represented by the following formula (4):



25

that is, [(1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-10,13 (9H,15H)-dione] or a salt thereof is a camptothecin derivative which exhibits excellent antitumor activities (Japanese Patent Laid-Open No. 87746/1994).

30

[0003] The conventional hydrochloride, sulfate, hydrobromide, nitrate or the like of the above compound are, however, accompanied by one or more disadvantages such as high hygroscopicity, low filterability and/or low solubility, leading to intractable handling property at industrial preparation.

35

[0004] Further, in order to convert any one of these inorganic salts into a pharmacologically-useful acid addition salt such as the tartrate, malonate or malate, it requires the conversion of the inorganic salt into the sparingly-soluble free compound prior to the formation of the desired salt. Such conventional salts thus require the extra step and in addition, the free compound had low solubility and cannot be handled easily. These conventional salts are therefore disadvantageous from the industrial viewpoint.

40

[0005] Upon preparation of Compound (4) according to the conventional procedure, an unnecessary stereoisomer is formed together with the target stereoisomer theoretically in equal amounts, lowering the yield of the desired compound. And further, to obtain the target stereoisomer, high-performance liquid chromatography should be used. So the target stereoisomer cannot be prepared advantageously in industry by the conventional preparation procedure.

45

[0006] An object of the present invention is therefore to provide a novel acid addition salt of a camptothecin derivative, said salt being free of hygroscopicity, having excellent filterability and solubility and being pharmacologically useful; and also an industrially advantageous preparation process therefor.

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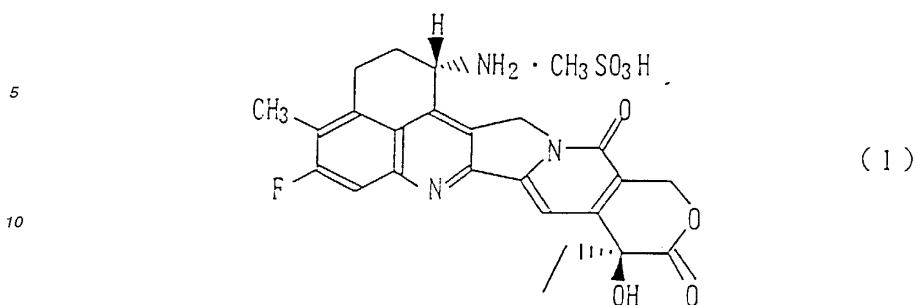
[0007] With the foregoing in view, the present inventors have conducted with an extensive investigation. As a result, it has been found that treatment of the compound represented by the below-described formula (2) with methanesulfonic acid allows isomerization upon removal of the amino-protecting group proceed so that the target isomer can be obtained in an improved yield; that the repetition of the above reaction makes it possible to convert an unnecessary isomer, which has remained after the separation of the necessary isomer, into the desired isomer; that the target product can be selectively collected easily by recrystallization while making use of the difference in solubility between the unnecessary isomer and the desired isomer; and that the methanesulfonate as the target product is useful as a pharmaceutical for the lack of hygroscopicity and good filterability and solubility, leading to the completion of the present invention.

Disclosure of the Invention

55

[0008] The present invention therefore provides the methanesulfonate of a camptothecin derivative, said methanesulfonate being represented by the following formula (1):

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or a hydrate thereof, and also a preparation process thereof.

[0009] The present invention also provides a pharmaceutical composition, preferably an anti-tumor pharmaceutical composition, comprising the Compound (1)

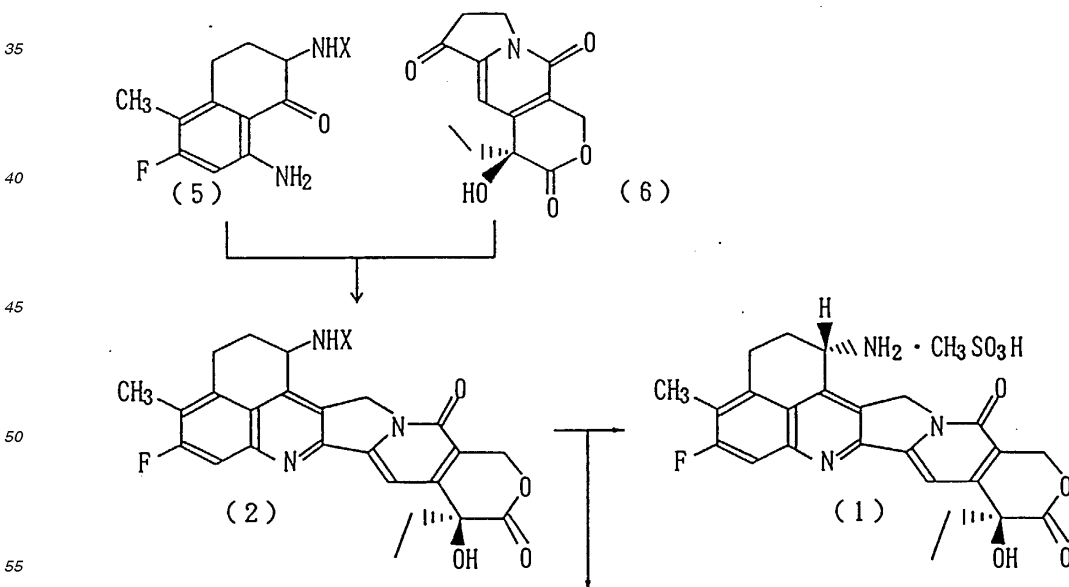
20 Brief Description of the Drawings

[0010] FIG. 1 diagrammatically illustrates weight changes of an invention compound and a control product due to moisture absorption.

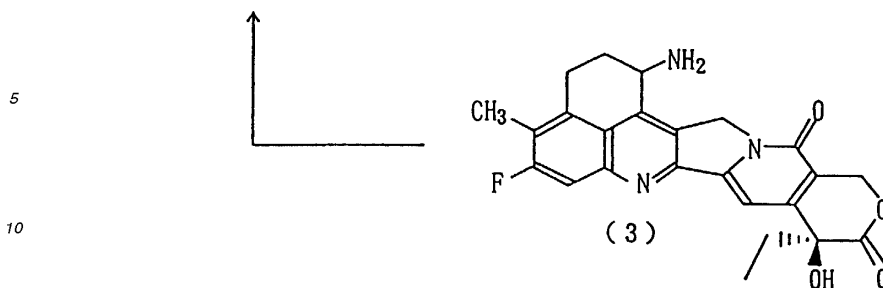
25 Best Modes for Carrying Out the Invention

[0011] The methanesulfonate represented by the formula (1) or a hydrate thereof is in the form of crystals which are free of hygroscopicity, have excellent filterability and solubility and can be easily processed or handled. As the hydrate, the dihydrate of the methanesulfonate is preferred.

[0012] The methanesulfonate (1) of the present invention can be prepared, for example, in accordance with the following reaction scheme.



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wherein X represents a protecting group removable by methanesulfonic acid.

[0013] Described specifically, the methanesulfonate of the camptothecin derivative, said methanesulfonate being represented by the formula (1), or a hydrate thereof can be obtained by reacting Compound (5) with Compound (6) by a known procedure (Japanese Patent Laid-Open No. 87746/1994) to yield Compound (2), treating Compound (2) with methanesulfonic acid and then subjecting the resulting compound to recrystallization.

[0014] The methanesulfonate (1) or its hydrate of the present invention can also be obtained by protecting the amino group of the compound represented by the formula (3) with a protecting group removable by methanesulfonic acid, preferably with an acetyl group, treating the compound with methanesulfonic acid and then subjecting the resulting compound to recrystallization. Even if Compound (3) obtained above is derived from the unnecessary isomer which has remained after separation of Compound (1), it can be converted into Compound (1), so it is still useful.

[0015] Preparation processes for these compounds will hereinafter be described specifically.

[0016] Compound (2) can be obtained by reacting Compound (5) with Compound (6) by a known procedure (the process disclosed in Japanese Patent Laid-Open No. 87746/1994 or the like). In Compound (2), the carbon atom to which the protected amino group is bonded is an asymmetric carbon atom. Compound (2) is generally obtained as a mixture of two isomers, one being an S-form and the other an R-form in the configuration of the protected amino group on the asymmetric carbon atom.

[0017] The methanesulfonate (1) or its hydrate of the present invention can be obtained by treating Compound (2) with methanesulfonic acid and then subjecting the resulting compound to recrystallization.

[0018] As for the amino-protecting group in Compound (2), it is preferred to employ a group removable by the subsequent treatment with methanesulfonic acid. Such a protecting group is selected from the group consisting of acyl groups such as alkanoyl and benzoyl groups which may be substituted by one or more halogen atoms, e.g., acetyl, chloroacetyl, trichloroacetyl and trifluoroacetyl; alkoxy carbonyl groups such as tert-butyloxycarbonyl; alkanoyloxymethyl groups such as pivaloyloxymethyl; a tetrahydropyranyl group; and a formyl group. Among these, acyl groups are preferred, with C₂₋₆ alkanoyl groups, which may be substituted by one or more halogen atoms, being more preferred. An acetyl group is particularly preferred from the viewpoints of the cost and the ease of handling.

[0019] The treatment with methanesulfonic acid is conducted not only for the deprotection of Compound (2) but also for the formation of a salt which is free of hygroscopicity, excellent in filterability and solubility, and stable and therefore easily handled, and for the conversion into the desired isomer.

[0020] Upon deprotection and salt-forming reaction, it is preferred to use methanesulfonic acid in an amount ranging from 5 times to 30 times based on the weight of Compound (2) [volume/weight: the ratio will be designated as 1 times when methanesulfonic acid is used in an amount of 1 ml relative to 1 g of Compound (2)], and about 15 times being particularly preferred (When methanesulfonic acid is used on a weight basis, the amount to be used can be calculated by multiplying its volume by its specific gravity, that is, about 1.5).

[0021] Upon treatment with methanesulfonic acid, it is convenient to use the acid in the form of an aqueous solution. The aqueous solution of methanesulfonic acid is preferred to have a concentration in a range of from 10% to 60%, more preferably about 30%.

[0022] The treatment with methanesulfonic acid may be conducted with an aqueous methanesulfonic acid solution only or in the presence of an organic solvent. Examples of such a solvent include aromatic solvents such as toluene and xylene, alcoholic solvents such as methanol, ethanol and isopropanol, and ether solvents such as dioxane and tetrahydrofuran. When an organic solvent is employed, it is preferred to use the solvent in a range up to about twice as much as the volume of the aqueous methanesulfonic acid solution.

[0023] The treatment may be conducted in a temperature range of from room temperature to the reflux temperature of the solvent employed. A temperature range of from 100°C to 120°C is preferred.

[0024] For the treatment, one hour to several days may be necessary. In general, the treatment for 7 hours or so under stirring is enough for the complete deprotection.

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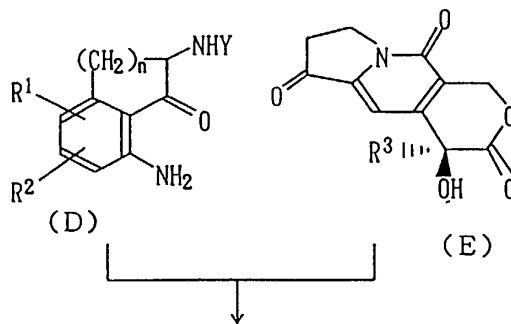
[0025] The above-described treatment conducted using methanesulfonic acid yields a stereoisomer mixture which contains the stereoisomer of the present invention having the desired steric configuration and the unnecessary stereoisomer at a ratio of approximately 1:1. Each of the deprotected compounds is in the form of the methanesulfonate and is dissolved in the aqueous layer. A solid mixture can therefore be obtained by removing water from the aqueous layer.

[0026] The solid isomer mixture so obtained is then recrystallized from a water-alcohol mixture, for example, a mixed solvent of water and an alcoholic solvent such as methanol, ethanol or isopropanol. This surprisingly results in the selective preparation of Compound (1), that is, the methanesulfonate of Compound (4) having S-configurations at the 1-amino group and the 9-position - said Compound (4) being the compound having the target steric configuration from Compound (3). The methanesulfonate of the isomer having the target configuration can be obtained as crystals because its solubility is lower than unnecessary isomer. Thus, the target compound can be selectively collected with ease. The stereoisomer having the unnecessary configuration can hence be obtained in the form of a solution in the recrystallization mother liquid.

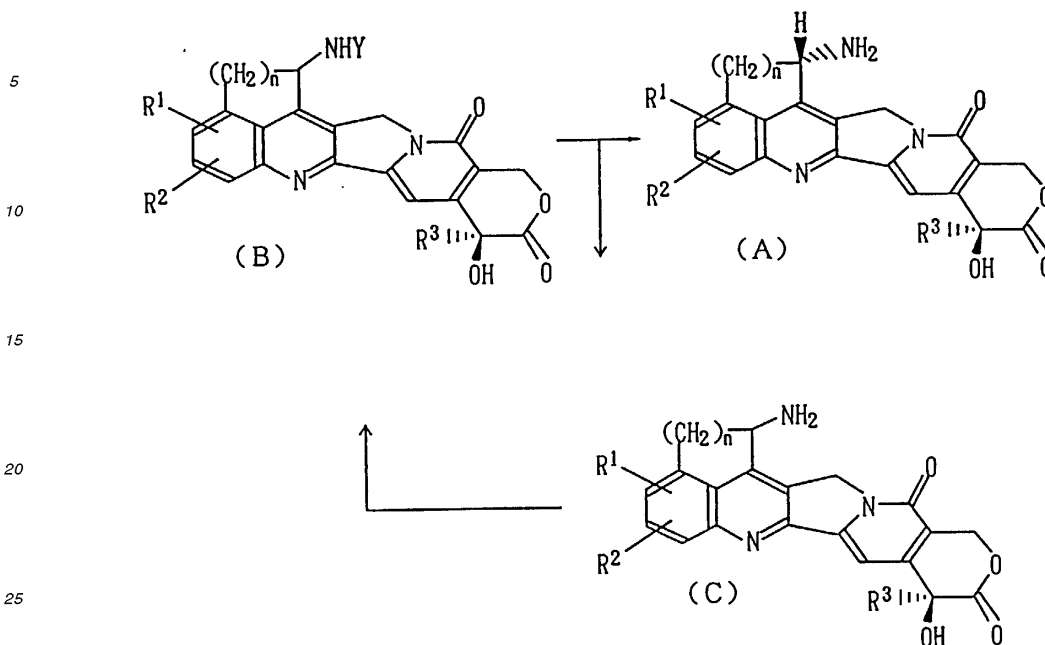
[0027] Incidentally, to obtain a purer target isomer, recrystallization may be repeated for several times.

[0028] The unnecessary stereoisomer within Compound (3), which remains in the recrystallization mother liquid, can be converted into Compound (1) of the target configuration according to the following procedure. Described specifically, a protecting group is introduced into the 1-amino group of the unnecessary stereoisomer, for instance, the compound having an R-configuration around the 1-amino group and an S-configuration at the 9-position, followed by deprotection reaction, whereby concurrently with deprotection, isomerization proceeds on the carbon atom to which the amino group is bonded. The introduction of the protecting group and deprotection reaction thereof are conducted under similar conditions as those described above. By repeating this series of reactions, most of Compounds (3) and Compounds (2) can be converted into Compound (1).

[0029] The process according to the present invention can also be applied as a process to obtain Compounds (A) and (C) as is shown in the following reaction scheme.



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wherein R^1 and R^2 each independently represents a hydrogen atom, a halogen atom, a hydroxyl group or a C_{1-6} alkyl group, R^3 represents a C_{1-6} alkyl group, Y represents a protecting group removable by an acid, and n stands for an integer of 1-3.

[0030] Compound (A) can be obtained by reacting Compound (D) with Compound (E) in a known manner (Japanese Patent Laid-Open No. 87746/1994) to obtain Compound (B), treating the resulting Compound (B) in the presence of an acid and then subjecting the thus-treated compound to recrystallization. Compound (A) can also be obtained by protecting the amino group in Compound (C) with a substituent removable by an acid, treating the protected compound with an acid and then subjecting the resulting compound to recrystallization.

[0031] This process can of course be applied as a process for selectively preparing an isomer of Compound (A) having an S configuration at the position of the substituent "-NHY", or a salt thereof by treating an isomer mixture of Compound (B) containing abundantly an isomer having an R configuration around the "-NHY" position, in the presence of an acid and then subjecting the resulting mixture to recrystallization.

[0032] The methanesulfonate (1) of the camptothecin derivative or the hydrate thereof according to the present invention is free of hygroscopicity, excellent in filterability and solubility and advantageous in handling so that it is useful as a pharmaceutical, particularly as an antitumor agent. When this methanesulfonate (1) of the camptothecin derivative or the hydrate thereof is used as a pharmaceutical, it is desired to use it in the form of a pharmaceutical composition composed of the compound and a pharmaceutically acceptable carrier.

[0033] The preparation process according to the present invention is industrially advantageous, because the target compound can be selectively collected with ease by converting an unnecessary isomer into the target isomer through methanesulfonic acid treatment and then subjecting the so-obtained isomer to recrystallization.

[0034] The present invention will hereinafter be described more specifically by Examples.

Referential Example 1

(4S)-4-Ethyl-7,8-dihydro-4-hydroxy-1H-pyrano[3,4-f]indolizine-3,6,10(4H)-trione

[0035] To 90 ml of 90% trifluoroacetic acid, 3.5 g of (4S)-4-ethyl-6,6-(ethylenedioxy)-7,8-dihydro-4-hydroxy-1H-pyrano[3,4-f]indolizine-3,10(4H)-dione were added at room temperature over 5 minutes, followed by stirring at the same temperature for 30 minutes. After completion of the reaction, the solvent was removed under reduced pressure, followed by the thorough removal of the solvent by a vacuum pump. To the residue so obtained was added 20 ml of

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ethyl acetate. The crystals precipitated were collected by filtration, whereby 2.4 g of the title compound were obtained.

Referential Example 2

5 (9S)-1-Acetylamino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino [1,2-b]quinoline-10,13(9H,15H)-dione

10 **[0036]** To 150 mg of 2-acetylamino-8-amino-6-fluoro-5-methyl-1-tetralone and 158 mg of (4S)-4-ethyl-7,8-dihydro-4-hydroxy-1H-pyrano[3,4-f]indolizine-3,6,10(4H)-trione, 30 mL of toluene and then 150 mg of pyridinium p-toluenesulfonate were added, followed by heating at an external temperature of 120°C under reflux for 28 hours. After completion of the reaction, the solvent was removed under reduced pressure. Acetone was added to the residue and crystals so precipitated were collected by filtration, whereby 169 mg of the title compound were obtained.

15 Melting point: 225-235°C (decomposed)
¹H-NMR(CDCl₃)δ: 1.05(3H,t,J=7.4Hz), 1.88(2H,q,J=7.4Hz), 2.15(3H,s), 2.19-2.43(2H,m), 2.44(3H,s), 3.12-3.17(2H,m), 3.72(1H,s), 5.15-5.33(3H,m), 5.58-5.72(2H,m), 5.98(1H,br-s), 7.57(1H,s), 7.67(1H,d, J=10.9Hz).

Referential Example 3

20 (9S)-1-Acetylamino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino [1,2-b]quinoline-10,13 (9H,15H)-dione

25 **[0037]** To 2.00 g of 2-acetylamino-8-amino-6-fluoro-5-methyl-1-tetralone and 2.10 g of (4S)-4-ethyl-7,8-dihydro-4-hydroxy-1H-pyrano[3,4-f]indolizine-3,6,10(4H)-trione, 250 mL of toluene and then 0.1 g of pyridinium p-toluenesulfonate were added, followed by heating under reflux for 3 hours. The mixture was added with 0.1 g of pyridinium p-toluenesulfonate, followed by heating under reflux for 2.5 hours. The mixture was then added with 0.1 g of pyridinium p-toluenesulfonate, followed by heating under reflux for 38 hours. The reaction mixture was cooled. The solvent was then removed under reduced pressure. To a crystalline residue, acetone was added and the crystals so precipitated were collected by filtration, whereby 3.48 g of the title compound were obtained.

35 Melting point: 225-235°C (decomposed)
¹H-NMR(CDCl₃)δ: 1.05(3H,t,J=7.4Hz), 1.88(2H,q,J=7.4Hz), 2.15(3H,s), 2.19-2.43(2H,m), 2.44(3H,s), 3.12-3.17(2H,m), 3.72(1H,s), 5.15-5.33(2H,m), 5.58-5.72(2H,m), 5.98(1H,br-s), 7.57(1H,s), 7.67(10H,d, J=10.9Hz).

Example 1

40 (1S,9S)-1-Amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]-indolizino [1,2-b]quinoline-10,13(9H,15H)-dione-methanesulfonate-dihydrate

45 **[0038]** To 5.0 g of (9S)-1-acetylamino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione, 150 mL of water were added. To the resulting mixture, 75 mL of methanesulfonic acid and then 150 mL of toluene were added, followed by heating under reflux for 6.5 hours. The reaction mixture was cooled down and the aqueous layer was collected by a separatory funnel. The aqueous layer so collected was filtered through a glass filter. The solvent was removed from the filtrate. To the residue so obtained, 300 mL of methanol and then 200 mL of ethanol were added. The resulting mixture was thereafter cooled. The crystals so precipitated were collected by filtration and washed with ethanol, whereby 2.6 g of crude crystals were obtained together with the mother liquor.

50 **[0039]** The crude crystals so obtained were stirred in a slurry in methanol to yield 2.2 g of the crude crystals. To the resulting crude crystals, 140 mL of water were added, followed by filtration through a membrane filter. After the addition of ethanol to the crystals, the solvent was removed. A 4:1 mixed solvent (165 mL) of ethanol and water was added to the residue so obtained, followed by heating to dissolve the residue in the solvent. The resulting solution was allowed to stand overnight at room temperature. The crystals precipitated were collected by filtration, followed by washing with ethanol, whereby 1.6 g of the title compound were obtained.

55 Melting point: 245-255°C (decomposed)
¹H-NMR(D₂O)δ: 0.73(3H,t,J=7.3Hz), 1.75(2H,q,J=7.3Hz), 2.13(3H,s), 2.50-2.62(2H,m), 2.65(3H,s), 2.83-3.00(1H,

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m), 3.18-3.30(1H,m), 5.16-5.45(5H,m), 7.06(1H,s), 7.10(1H,d,J=10.6Hz).

Elementary analysis for C ₂₄ H ₂₂ FN ₃ O ₄ ·CH ₃ SO ₃ H·2H ₂ O:			
Calculated	C, 52.90;	H, 5.33;	N, 7.40 (%)
Found	C, 52.74;	H, 5.15;	N, 7.35 (%)

IR(KBr)cm⁻¹: 3409, 2936, 1747, 1658, 1589, 1503, 1420, 1252, 1165, 1112, 1044, 884, 773, 554.

Example 2

[0040] The mother liquid obtained in Example 1 was concentrated under reduced pressure. To the residue, 200 ml of methanol were added, followed by the drop-wise addition of 162 ml of triethylamine under ice cooling. After having been confirmed that the mixture was not acidic, 9.9 ml of acetic anhydride were added at room temperature, followed by stirring at the same temperature for one hour. After completion of the reaction, water was added to the reaction mixture. The crystals precipitated were collected by filtration, washed with water and ethanol, and then dried under reduced pressure, whereby 1.9 g of (9S)-1-acetylamino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione were obtained. The product so obtained was de-protected as in Example 1, whereby (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino-[1,2-b]quinoline-10,13(9H,15H)-dione-methanesulfonate was obtained

Test 1

[0041] A hygroscopicity test was conducted on (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13 (9H,15H)-dione-methanesulfonate-dihydrate according to the present invention and (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione-hydrochloride (control product).

[0042] Described specifically, each salt was exposed to high-humidity conditions of 83% and 93% at 25°C. Over a period of 5 days, its weight changes were investigated. The results are shown in FIG. 1.

[0043] As can be seen from the results, the control product shows high hygroscopicity and a fast weight change due to hygroscopicity, while the methanesulfonate according to the present invention shows a gradual and small change, although it has hygroscopicity a little.

[0044] Incidentally, a color change (yellowish brown - brown; originally: yellow) was observed on a sample of the control product when the sample had been stored at a high relative humidity, thereby suggesting the possibility of decomposition by moisture absorption. The degree of a color change was hence studied by a color difference meter. The control product was stored at a relative humidity of 93% and the degree of a color change was measured. The results are shown in Table 1. In the Table, Lab represents a chromaticity (brightness) and ΔE(H) indicates the degree of a color difference. ΔE(H) of 3, 4 or higher indicates that the color difference is noticeable even to naked eye. Samples of the control product showed a ΔE(H) of 7-8 when stored for a month at 25°C and 93% relative humidity of 93% and a ΔE(H) of 15-19 when stored for 2 months under the same conditions. It has hence been concluded that the control product decomposes by moisture absorption.

Table 1

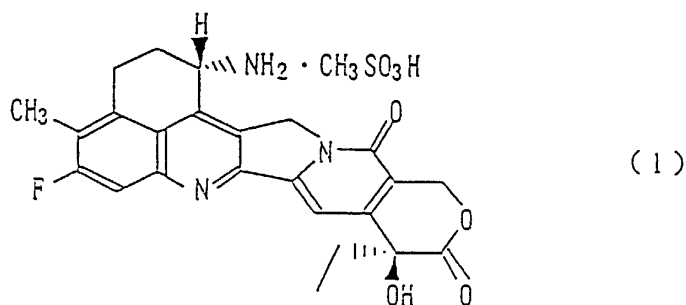
Stored for (month)	Color	Lab	ΔE(H)
0	Yellow	81.68-81.69	-
1	brown-yellow	74.96-75.23	7.16-7.38
2	brown-yellow	62.65-67.43	14.53-19.24

[0045] No color change, on the other hand, was observed on the methanesulfonate according to the present invention.

Claims

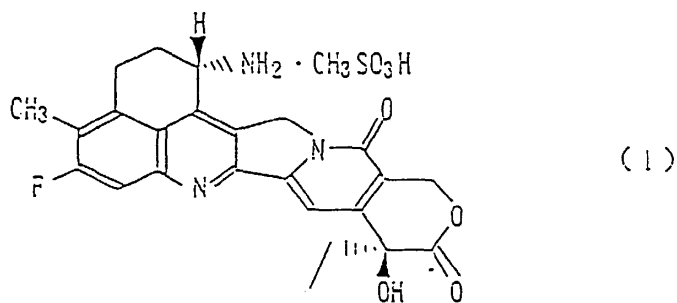
1. A methanesulfonate of a camptothecin derivative, said methanesulfonate being represented by the following formula (1):

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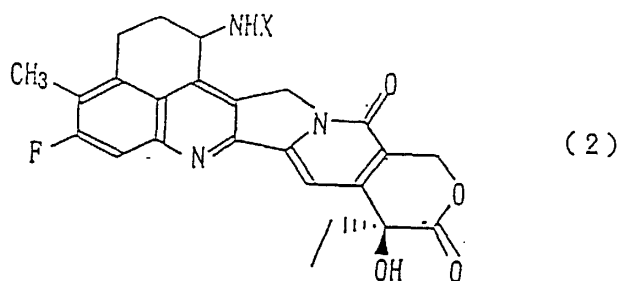


15 or a hydrate thereof.

2. The hydrate of the methanesulfonate of a camptothecin derivative according to claim 1 which is a dihydrate.
3. A pharmaceutical composition comprising the methanesulfonate of a camptothecin derivative or a hydrate thereof as defined in claims 1 or 2 and a pharmaceutically acceptable carrier.
4. The composition according to claim 3, which is an antitumor pharmaceutical composition.
5. A process for the preparation of the methanesulfonate of a camptothecin derivative or a hydrate thereof, said methanesulfonate being represented by the following formula (1) as defined in claim 1



40 which comprises treating, with methanesulfonic acid, a compound represented by the following formula (2):

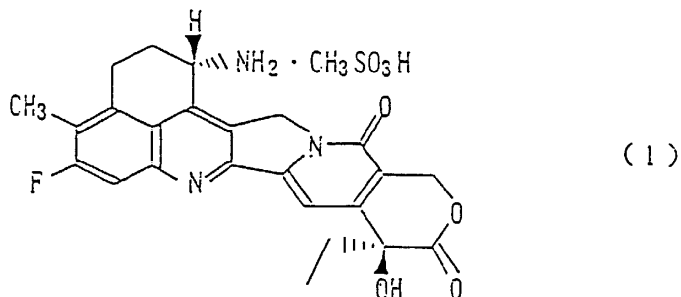


wherein X represents a protective group selected from the group consisting of an acyl group, an alkoxycarboxyl

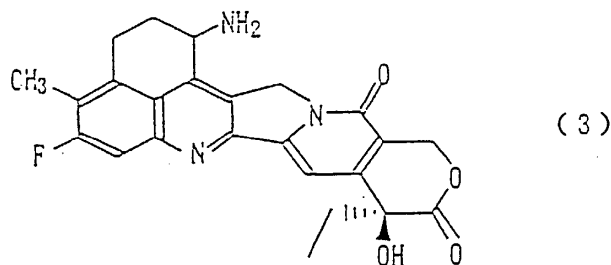
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group, and an alkanoyloxymethyl group and then subjecting the thus-treated compound to recrystallization.

6. A process for the preparation of the methanesulfonate of a camptothecin derivative or a hydrate thereof, said methanesulfonate being represented by the following formula (1):



which comprises protecting the amino group of a compound represented by the following formula (3);



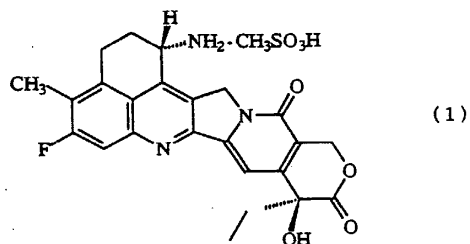
with a protecting group selected from the group consisting of an acyl group, an alkoxycarbonyl group, and an alkanoyloxymethyl group, treating with methanesulfonic acid and then subjecting the thus-treated compound to recrystallization.

7. The process according to claim 5 or 6, characterized in that the protecting group is selected from the group consisting of an acetyl group, a chloroacetyl group, a trichloroacetyl group, a trifluoroacetyl group, a tertbutoxycarbonyl group, a pivaloyloxymethyl group, a tetrahydropyranyl group, and a formyl group
8. The process according to claim 5 or 6, characterized in that the protecting group is an acyl group.
9. The process according to claim 4 or 5, characterized in that the protecting group is a C₂₋₆alkanoyl group.
10. The process according to claim 5 or 6, characterized in that the protecting group is an acetyl group.
11. The process according to any one of claims 5 to 10 wherein a solvent used for recrystallization is a mixture of water and an alcohol.
12. The process according to any one of claims 5 to 11 wherein the hydrate of the methanesulfonate of the camptothecin derivative is a dihydrate.

Patentansprüche

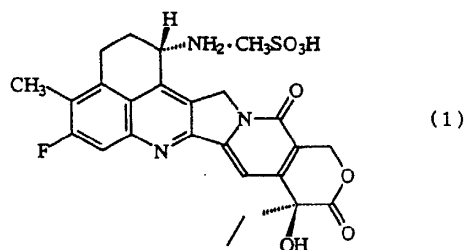
1. Methansulfonat eines Camptothecin-Derivats der folgenden Formel (1)

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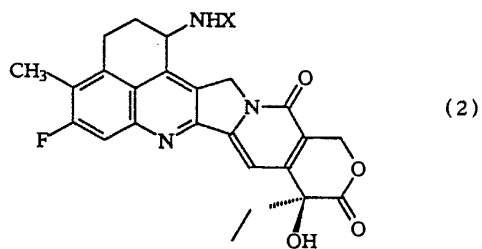


oder eines Hydrates davon.

2. Hydrat des Methansulfonats eines Camptothecin-Derivats nach Anspruch 1, das ein Dihydrat ist.
3. Pharmazeutische Zusammensetzung, umfassend das Methansulfonat eines Camptothecin-Derivats oder ein Hydrat davon nach einem der Ansprüche 1 oder 2 sowie ein pharmazeutisch akzeptablen Träger.
4. Zusammensetzung nach Anspruch 3, die eine pharmazeutische Antitumor-Zusammensetzung ist.
5. Verfahren zum Herstellen des Methansulfonats eines Camptothecin-Derivats der folgenden Formel (1), wie in Anspruch 1 definiert,



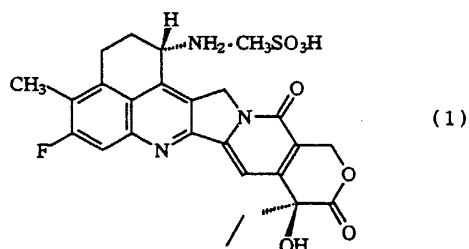
umfassend das Behandeln einer Verbindung der folgenden Formel (2) mit Methansulfonsäure



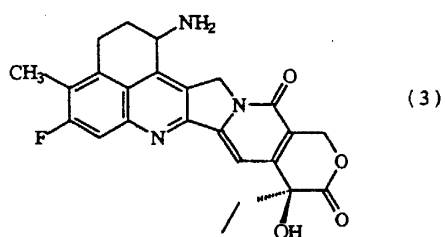
worin X eine Schutzgruppe repräsentiert, ausgewählt aus der Gruppe bestehend aus einer Acylgruppe, einer Alkoxycarboxylgruppe und einer Alkanoyloxymethylgruppe, und Umkristallisieren der so behandelten Verbindung.

6. Verfahren zum Herstellen des Methansulfonats eines Camptothecin-Derivats oder eines Hydrats davon, wobei das Methansulfonat mit folgender Formel darstellbar ist,

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umfassend das Schützen der Aminogruppe einer Verbindung der Formel (3),



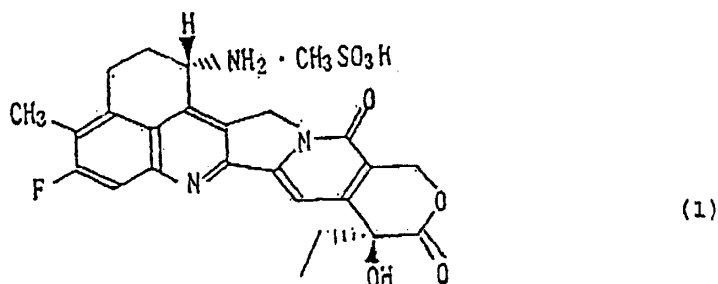
mit einer Schutzgruppe, ausgewählt aus der Gruppe bestehend aus einer Acylgruppe, einer Alkoxycarboxylgruppe und einer Alkanoyloxymethylgruppe, Umsetzen mit Methansulfonsäure und Umkristallisation der derart behandelten Verbindung.

- 30
7. Verfahren nach Anspruch 5 oder 6, dadurch gekennzeichnet, daß die Schutzgruppe ausgewählt ist aus der Gruppe bestehend aus einer Acetylgruppe, einer Chloracetylgruppe, einer Trichloracetylgruppe, einer Trifluoracetylgruppe, einer tert-Butoxycarbonylgruppe, einer Pivaloyloxymethylgruppe, einer Tetrahydropyranolgruppe und einer Formylgruppe.
- 35
8. Verfahren nach Anspruch 5 oder 6, dadurch gekennzeichnet, daß die Schutzgruppe eine Acylgruppe ist.
9. Verfahren nach Anspruch 5 oder 6, dadurch gekennzeichnet, daß die Schutzgruppe eine C₂₋₆-Alkanoylgruppe ist.
- 40
10. Verfahren nach Anspruch 5 oder 6, dadurch gekennzeichnet, daß die Schutzgruppe eine Acetylgruppe ist.
11. Verfahren nach einem der Ansprüche 5 bis 10, worin das zum Umkristallisieren eingesetzte Lösungsmittel eine Mischung von Wasser und einem Alkohol ist.
- 45
12. Verfahren nach einem der Ansprüche 5 bis 11, wobei das Hydrat des Methansulfonats des Camptothecin-Derivats ein Dihydrat ist.

Revendications

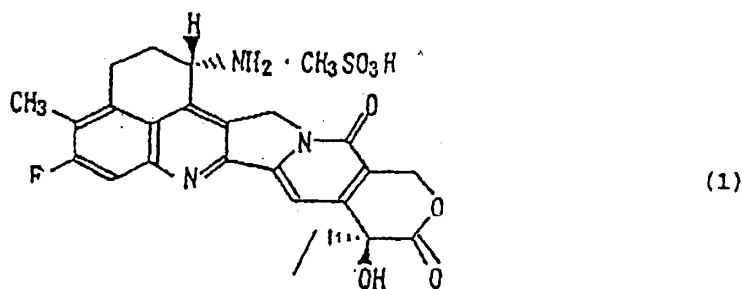
- 50
1. Méthanesulfonate d'un dérivé de camptothécine, ledit méthanesulfonate étant représenté par la formule suivante (1) :
- 55

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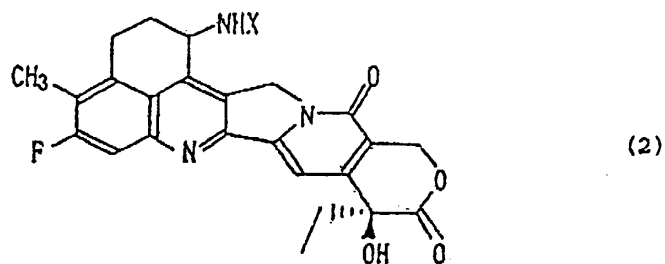


15 ou un hydrate de celui-ci.

2. Hydrate du méthanesulfonate d'un dérivé de camptothécine selon la revendication 1, qui est un dihydrate.
3. Composition pharmaceutique comprenant le méthanesulfonate d'un dérivé de camptothécine, ou un hydrate de celui-ci, tel que définis dans la revendication 1 ou 2 et un véhicule pharmaceutiquement acceptable.
4. Composition selon la revendication 3, qui est une composition pharmaceutique antitumorale.
5. Procédé de préparation du méthanesulfonate d'un dérivé de camptothécine d'un hydrate de celui-ci, ledit méthanesulfonate étant représenté par la formule (1) suivante, telle que définie dans la revendication 1 :



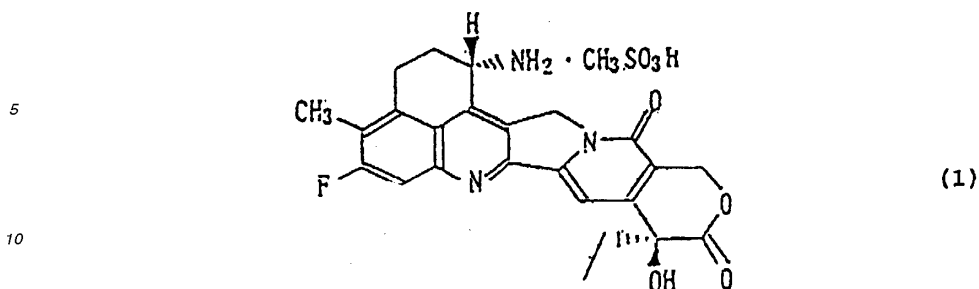
40 lequel procédé comprend les opérations consistant à traiter, par l'acide méthanesulfonique, un composé représenté par la formule (2) suivante :



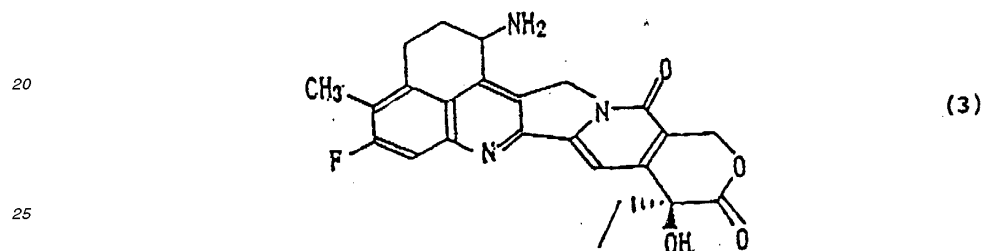
55 dans laquelle X représente un groupe protecteur choisi dans le groupe consistant en un groupe acyle, un groupe alcoxycarboxyle et un groupe alcanoyloxyméthyle ; puis à soumettre le composé ainsi traité à une recristallisation.

6. Procédé de préparation du méthanesulfonate d'un dérivé de camptothécine d'un hydrate de celui-ci, ledit méthanesulfonate étant représenté par la formule (1) suivante :

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15 lequel procédé comprend la protection du groupe amino d'un composé représenté par la formule (3) suivante :



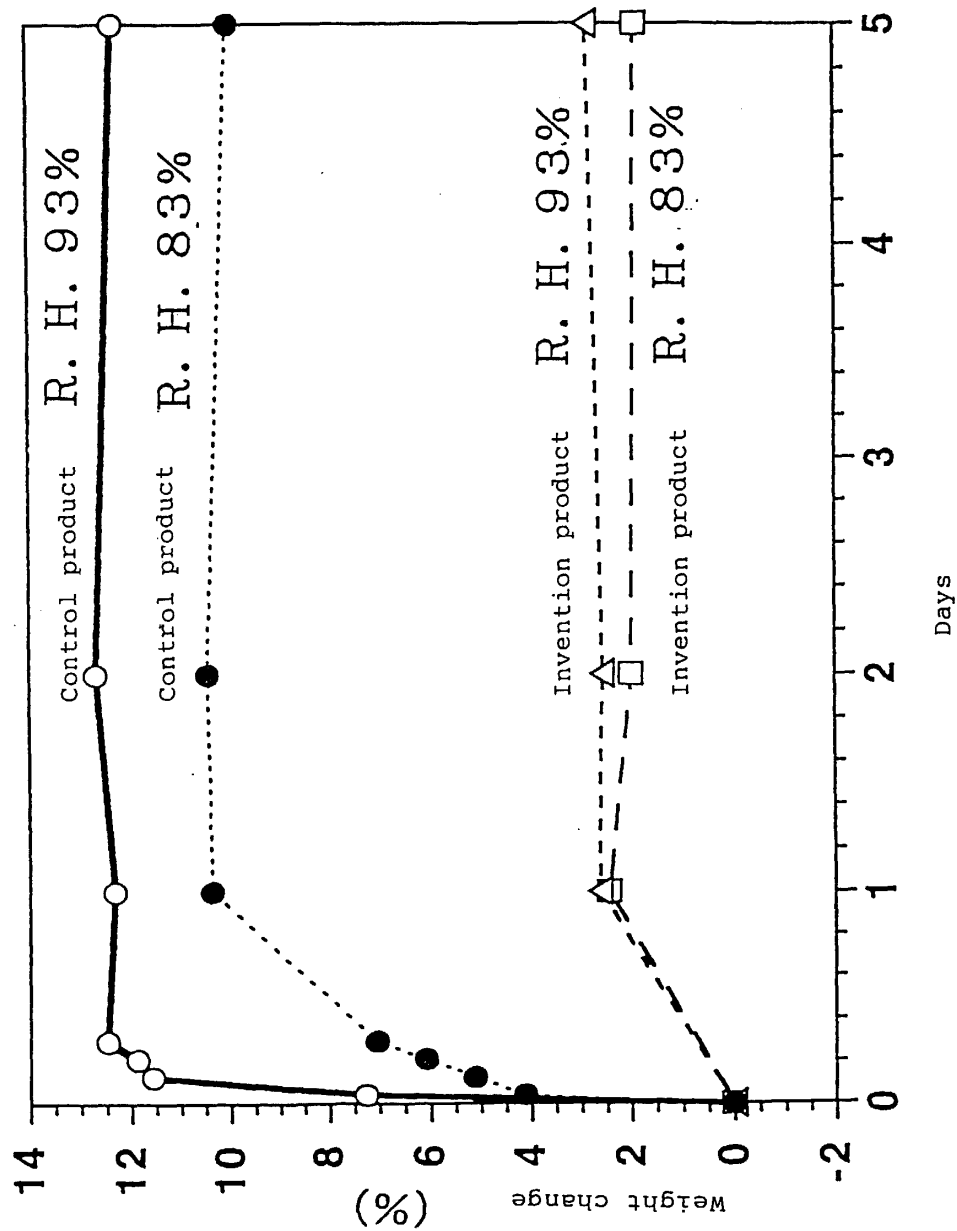
30 avec un groupe protecteur choisi dans le groupe consistant en un groupe acyle, un groupe alcoyloxycarboxyle et un groupe alcanoyloxyméthyle, le traitement par l'acide méthanesulfonique, puis la recristallisation du composé ainsi traité.

- 35
7. Procédé selon la revendication 5 ou 6, caractérisé par le fait que le groupe protecteur est choisi dans le groupe consistant en un groupe acétyle, un groupe chloroacétyle, un groupe trichloroacétyle, un groupe trifluoroacétyle, un groupe tert.-butoxycarbonyle, un groupe pivaloyloxyméthyle, un groupe tétrahydropyrannyle et un groupe formyle.
 8. Procédé selon la revendication 5 ou 6, caractérisé par le fait que le groupe protecteur est un groupe acyle.
 - 40 9. Procédé selon la revendication 5 ou 6, caractérisé par le fait que le groupe protecteur est un groupe alcanoyle en C₂₋₆.
 10. Procédé selon la revendication 5 ou 6, caractérisé par le fait que le groupe protecteur est un groupe acétyle.
 - 45 11. Procédé selon l'une quelconque des revendications 5 à 10, dans lequel le solvant utilisé pour la recristallisation est un mélange d'eau et d'un alcool.
 12. Procédé selon l'une quelconque des revendications 5 à 11 dans lequel l'hydrate du méthanesulfonate du dérivé de camptothécine est un dihydrate.
- 50

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FIG. 1



(11) **EP 0 916 348 A1**

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(54) **DRUG COMPLEXES**

(57) A drug complex characterized in that a residue of a drug compound such as antineoplastic agents and a carboxy(C₁₋₄)alkyldextran polyalcohol obtained by treating a dextran under conditions that enable substantially complete polyalcoholization are bound to each other by means of a spacer comprising an amino acid or a spacer comprising peptide-bonded 2 to 8 amino acids. Said complex is characterized in that it has excellent selectivity to tumorous sites so as to exhibit high antineoplastic activity and also achieves reduced appearance of toxicity.

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Description

Technical Field

5 **[0001]** The present invention relates to a drug complex which is useful as a medicament. More specifically, the present invention relates to a drug complex in which a carboxy(C₁₋₄)alkyldextran polyalcohol that is a polysaccharide derivative and a drug compound such as antineoplastic agents or anti-inflammatory agents are bound to each other via a spacer.

10 Background Art

[0002] Antineoplastic agents, used for treatment of solid cancers such as lung cancer or digestive organ carcinomas and blood cancers such as leukemia, are systemically administered through routes of administration such as intravenous or oral administration, and then, are distributed to specific tumorous sites and inhibit or suppress the proliferation
15 of cancer cells to exhibit their therapeutic efficacy. However, the systemically-administered antineoplastic agents are rapidly taken into livers and reticuloendothelial organs from blood, or rapidly excreted into urine, and accordingly, their blood concentrations may sometimes be lowered to allow the distribution into tumorous sites to be insufficient. In addition, common antineoplastic agents themselves have poor distribution-selectivity to tumorous sites (tumor selectivity), and therefore, the antineoplastic agents are uniformly distributed over various tissues and cells of the whole body, and
20 act as cytotoxins also against normal cells and tissues, which results in problems of the appearance of adverse effects, e.g., emesis, pyrexia, or alopecia at an extremely high rate. Therefore, it has been desired to develop a means of efficiently and selectively distributing antineoplastic agents to tumorous sites.

[0003] As one of such means, a process was proposed in which an antineoplastic agent is bound to a polysaccharide polymer to delay the disappearance of the antineoplastic agent from blood and to enhance selectivity to tumor tissues.
25 For example, Japanese Patent Publication (KOKOKU) No. (Hei) 7-84481/1995 discloses a drug complex in which daunorubicin, doxorubicin, mitomycin C, bleomycin or the like is introduced into a carboxymethylated mannoglucan derivative by means of a Schiff base or an acid amide bond. As the mannoglucan derivative in the invention, carboxymethylated mannoglucan polyalcohols are also used. However, mannoglucan derivatives are too much branched and have complicated structures, and accordingly, it has been difficult to obtain a product with uniform quality
30 suitable for manufacturing medicaments.

[0004] In addition, International Patent Publication WO94/19376 discloses a drug complex in which a peptide chain (the number of amino acid residues: 1 to 8) is bound to a carboxyl group of a polysaccharide having carboxyl groups, and doxorubicin, daunorubicin, mitomycin C, bleomycin or the like is further bound by means of the peptide chain. As the polysaccharide having carboxyl groups, examples are given such as polysaccharides inherently having carboxyl
35 groups in their structures (e.g., hyaluronic acid), and polysaccharides inherently having no carboxyl groups in their structures (e.g., pullulan, dextran, chitin, etc.) in which their hydroxyl groups are modified with carbonyl groups by introducing with carboxy(C₁₋₄)alkyl groups or binding with a polybasic acid such as malonic acid or succinic acid by esterification. The drug complexes are structurally characterized in that a drug such as doxorubicin and the above-mentioned polysaccharide moiety are bound to each other by means of a spacer, and the complexes have higher antineoplastic
40 activity compared to doxorubicin and reduced toxicity and adverse effects.

[0005] As for technologies relating to drug complexes utilizing polyalcoholized polysaccharide derivatives as drug delivery carriers, some reports are available, for example, "Researches on polysaccharide-peptide-doxorubicin complexes-Correlations between stabilities of polysaccharide carriers in blood and their anti-neoplastic activities" (Abstracts of 10th Meeting of the Japan Society of Drug-Delivery System, 279, 1994); "Researches on polysaccharide-peptide-doxorubicin complexes - Pharmacokinetics and anti-neoplastic activity" (Abstracts of 9th Annual Meeting of Japanese
45 Society for the study of xenobiotics, 292, 1994); Abstracts of 19th Seminar of Trends in Research and Development (held by The Organization for Drug A D R Relief, R&D Promotion and Product Review), D-9, 1995; and "Researches on drug delivery to a tumor tissue by polysaccharide carriers" (Abstracts of 12th Colloid and Interface Technology Symposium, The Chemical Society of Japan, 51, 1995).

50 Disclosure of the Invention

[0006] An object of the present invention is to provide a drug complex capable of site-selectively delivering an active ingredient such as antineoplastic agents or anti-inflammatory agents to tumorous sites or the like. More specifically, the
55 object of the present invention is to provide a drug complex which contains a drug compound such as antineoplastic agents or anti-inflammatory agents as a partial structure and can be retained in blood for a long period of time, and furthermore, can site-selectively deliver the drug compound to tumorous sites or inflammatory sites. In addition, another object of the present invention is to provide a method for preparing the drug complexes having the aforementioned fea-

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tures.

[0007] In order to achieve the foregoing object, the present inventors attempted to improve the drug complex disclosed in the International Patent Publication WO94/19376. As a result, they found that, when a dextran derivative obtained by the carboxy(C₁₋₄)alkylation of a polyalcoholized dextran is used as a polysaccharide moiety instead of the polysaccharides having carboxyl groups, high concentration of the medicament was retained for a long period of time after administration, and site-selectivity to tumorous sites or inflammatory sites can significantly be improved. They also found that, in these compounds, the main efficacy such as antineoplastic activity is remarkably enhanced, whereas toxicity is reduced. The present invention was achieved on the basis of these findings.

[0008] The present invention thus provides a drug complex characterized in that a carboxy(C₁₋₄)alkyldextran polyalcohol and a residue of a drug compound are bound to each other by means of a spacer comprising an amino acid or a spacer comprising peptide-bonded 2 to 8 amino acids. According to other embodiments of the present invention, there are provided a medicament comprising the aforementioned drug complex; and a pharmaceutical composition comprising the aforementioned drug complex as an active ingredient, for example, preparations for injection or drip infusion in the form of lyophilized products filled in vials. Furthermore, according to another embodiment of the present invention, a method for preparing the aforementioned drug complex is provided.

[0009] As preferred embodiments of the aforementioned invention, there are provided the above drug complex characterized in that the dextran polyalcohol that constitutes the carboxy(C₁₋₄)alkyldextran polyalcohol is a dextran polyalcohol which is obtained by treating a dextran under conditions that enable substantially complete polyalcoholization; the above drug complex wherein the carboxy(C₁₋₄)alkyldextran polyalcohol is carboxymethyldextran polyalcohol; the above drug complex wherein the drug compound is an antineoplastic agent or an anti-inflammatory agent; the above drug complex wherein the drug compound is an antineoplastic agent which concentration-dependently exhibits antineoplastic activity (an antineoplastic agent exhibiting more potent antineoplastic activity at a higher concentration: sometimes referred to as a concentration-dependent type antineoplastic agent in the present specification); the above drug complex wherein the drug compound is an antineoplastic agent which time-dependently exhibits antineoplastic activity (an antineoplastic agent exhibiting more potent antineoplastic activity at longer working times: sometimes referred to as a time-dependent type antineoplastic agent in the present specification); and the above drug complex wherein the antineoplastic agent is doxorubicin or (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione.

[0010] In addition, as also preferred embodiments, there are provided the above drug complex wherein the spacer is a dipeptide represented by -X-Z- [the symbol "-X-Z-" means a residue which consists of a dipeptide that is formed by peptide bonding of a hydrophobic amino acid (X) and a hydrophilic amino acid (Z) being at the N-terminal side and the C-terminal side, respectively, and whose one hydrogen atom and one hydroxyl group are removed from the amino group at the N-terminus and the carboxyl group at the C-terminus, respectively], or wherein the spacer contains the dipeptide as a partial peptide sequence; the above drug complex wherein the hydrophobic amino acid is phenylalanine and the hydrophilic amino acid is glycine; the above drug complex wherein the spacer is (N-terminus)-Gly-Gly-Phe-Gly-; and the above drug complex wherein an introduced amount of the residue of the antineoplastic agent is in the range of from 1 to 15% by weight, preferably from 3 to 10% by weight, and more preferably from 5 to 6% by weight.

[0011] As particularly preferred embodiments of the present invention, there are provided the above drug complex wherein N-terminus of a peptide represented by H₂N-Gly-Gly-Phe-Gly-COOH is bound to a carboxyl group of carboxymethyldextran polyalcohol by means of an acid-amide bond and C-terminus of the peptide is bound to the 1-amino group of (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione by means of an acid-amide bond; the above drug complex wherein the introduced amount of the (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione residue is in the range of from 2 to 10% by weight; and the above drug complex wherein the carboxy(C₁₋₄)alkyldextran polyalcohol is a carboxymethyldextran polyalcohol having a molecular weight in the range of from 5,000 to 500,000, preferably in the range of from 50,000 to 450,000, and more preferably in the range of from 200,000 to 400,000, and the degree of carboxymethylation per constitutive saccharide residue is in the range of from 0.01 to 2.0, preferably in the range of from 0.1 to 1.0, and more preferably in the range of from 0.3 to 0.5.

[0012] According to another aspect of the present invention, a drug delivery carrier comprising the carboxy(C₁₋₄)alkyldextran polyalcohol is provided. According to preferred embodiments of this aspect of the invention, a molecular weight of the carboxy(C₁₋₄)alkyldextran polyalcohol is in the range of from 5,000 to 500,000, preferably in the range of from 50,000 to 450,000, and more preferably in the range of from 200,000 to 400,000, and the degree of carboxymethylation per constitutive saccharide residue is in the range of from 0.01 to 2.0, preferably in the range of from 0.1 to 1.0, and more preferably in the range of from 0.3 to 0.5. Carboxymethyldextran polyalcohol is provided as the most preferred carrier. From another aspect of the invention, there is provided a use of a carboxy(C₁₋₄)alkyldextran polyalcohol for the manufacture of a drug complex which contains the carboxy(C₁₋₄)alkyldextran polyalcohol bound to the residue of a drug compound.

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[0013] As preferred embodiments of the present invention, there are provided the use of the carboxy(C₁₋₄)alkyldextran polyalcohol for the manufacture of a drug complex in which the residue of a drug compound and the carboxy(C₁₋₄)alkyldextran polyalcohol are bound to each other by means of a spacer; and the use of the carboxy(C₁₋₄)alkyldextran polyalcohol for the manufacture of a drug complex characterized in that the carboxy(C₁₋₄)alkyldextran polyalcohol and the residue of a drug compound are bound to each other by means of a spacer comprising an amino acid or a spacer comprising peptide-bonded 2 to 8 amino acids.

Brief Explanation of the Drawings

[0014]

Fig. 1 shows the GPC chart of the drug complex of the present invention (prepared in Example 8).

Fig. 2 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 8).

Fig. 3 shows the GPC chart of the drug complex of the present invention (prepared in Example 9).

Fig. 4 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 9).

Fig. 5 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 10).

Fig. 6 shows the GPC chart of the drug complex of the present invention (prepared in Example 15).

Fig. 7 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 15).

Fig. 8 shows the GPC chart of the drug complex of the present invention (prepared in Example 28).

Fig. 9 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 28).

Fig. 10 shows the GPC chart of the drug complex of the present invention (prepared in Example 29).

Fig. 11 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 29).

Fig. 12 shows the GPC chart of the drug complex of the present invention (prepared in Example 34).

Fig. 13 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 34).

Fig. 14 shows the GPC chart of the drug complex of the present invention (prepared in Example 39).

Fig. 15 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 39).

Fig. 16 shows the GPC chart of the drug complex of the present invention (prepared in Example 41).

Fig. 17 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 41).

Fig. 18 shows the GPC chart of the drug complex of the present invention (prepared in Example 44).

Fig. 19 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 44).

Fig. 20 shows the GPC chart of the drug complex of the present invention (prepared in Example 47).

Fig. 21 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 47).

Fig. 22 shows the GPC chart of the drug complex of the present invention (prepared in Example 55).

Fig. 23 shows the ultraviolet absorption spectrum of the drug complex of the present invention (prepared in Example 55).

Fig. 24 shows the pharmacokinetics of the drug complex of the present invention (prepared in Example 15). Each point in the figure represents an average value of three experiments.

Best Mode for Carrying out the Invention

[0015] The drug complex of the present invention is characterized in that a carboxy(C₁₋₄)alkyldextran polyalcohol and a residue of a drug compound are bound to each other by means of a spacer comprising an amino acid or a spacer comprising peptide-bonded 2 to 8 amino acids. The residue of a drug compound contained in the drug complex of the present invention is derived from a drug compound used for therapeutic and/or preventive treatment of diseases of mammals including humans as a medicament, for example, an antineoplastic agent, an anti-inflammatory agent, an antibacterial agent or the like, and the residue is composed of a partial structure of the drug compound. However, the drug compound which the residue is derived from is not limited to those mentioned above. In addition, as the drug com-

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compound, any compounds may be used so long as they have one or more reactive functional groups capable of participating in bond formation with a spacer (for example, amino group, carboxyl group, hydroxyl group, thiol group, ester group or the like). The term "drug compound" in the present specification also includes a prodrug compound which contains, as a part thereof, a major structure of a drug compound having pharmacological activity, per se, and can reproduce the compound *in vivo*.

[0016] More specifically, the term "residue of drug compound" in the present specification means a partial structure derived from the drug compound existing in the compound after bond formation, assuming that a bond between the spacer and the residue of a drug compound is formed through a reaction of a reactive functional group of the drug compound and a reactive functional group of the spacer (e.g., dehydration condensation etc.). For example, when the drug compound is represented by D-NH₂, D-COOH, D-COOR, D-OH, D-SH, D-CONH₂, or D-NH-COOR (R is a lower alkyl group or the like), the residue of the drug compound is represented by D-NH- (D-NH-CO-Q etc.), D-CO- (D-CO-NH-Q, D-CO-O-Q, D-CO-S-Q, etc.), D-CO- (D-CO-NH-Q, D-CO-O-Q, D-CO-S-Q, etc.), D-O- (D-O-CO-Q, D-O-Q, etc.), D-S- (D-S-CO-Q, D-S-Q, etc.), D-CONH- (D-CO-NH-CO-Q etc.), and D-NH-CO- (D-NH-CO-O-Q, D-NH-CO-NH-Q, etc.), respectively (the parenthesized represents a bond between the spacer and the residue of the drug compound, wherein Q represents a remaining partial structure of the spacer excluding a reactive functional group). However, the sort of the bond between the spacer and the residue of the drug compound is not limited to those mentioned above. The residue of the drug compound may be bound to the N-terminal amino group or the C-terminal carboxyl group of the spacer, or alternatively, may be bound to a reactive functional group existing in an amino acid that constitutes the spacer.

[0017] As the residue of the drug compound, for example, residues of antineoplastic agents such as doxorubicin, daunorubicin, mitomycin C, bleomycin, cycloctidine, vincristine, vinblastine, methotrexate, platinum antineoplastic agents (cisplatin or derivatives thereof), taxol or derivatives thereof camptothecin or derivatives thereof (antineoplastic agents described in Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 6-87746/1994, preferably (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione disclosed in claim 2, or the like) may preferably be used. In addition, residues of steroidal anti-inflammatory agents such as hydrocortisone succinate and prednisolone succinate and nonsteroidal anti-inflammatory agents such as mefenamic acid, flufenamic acid, diclofenac, ibuprofen, and tinoridine are also preferred.

[0018] As the spacer which binds to the residue of the drug compound, a spacer comprising one amino acid or a spacer comprising 2 to 8 amino acids that are peptide-bonded may be used. More specifically, the spacer has a form of a residue of one amino acid, which means a residue obtained by removing one hydrogen atom and one hydroxyl group from an amino group and a carboxyl group of the amino acid, respectively, or a residue of an oligopeptide comprising peptide-bonded 2 to 8 amino acids, which means a residue obtained by removing one hydrogen atom and one hydroxyl group from the N-terminal amino group and the C-terminal carboxyl group of the oligopeptide, respectively.

[0019] Preferred spacers are residues of oligopeptides comprising 2 to 6 amino acids. The sort of the amino acid constituting the spacer is not particularly limited, and for example, L- or D-amino acids, preferably L- amino acids can be used, and β -alanine, ϵ -aminocaproic acid, γ -aminobutyric acid or the like may also be used as well as α -amino acids. These amino acids other than α -amino acids are preferably located close to the polysaccharide derivative.

[0020] The bonding direction of the spacer is not particularly limited, and generally, the N-terminus of the spacer can be bound to a carboxyl group of the carboxy(C₁₋₄)alkyldextran polyalcohol by means of an acid-amide bond, and the C-terminus of the spacer can be bound to an amino group of the drug compound. Alternatively, for example, where a lysine residue is incorporated as a constitutional unit of the peptide spacer, the α -amino group and the ϵ -amino group of the lysine residue are allowed to form respective acid-amide bonds with carboxyl groups of other amino acids so as to form N-terminuses at both ends of the peptide spacer, which enables bond formation with carboxyl groups of the drug compounds. Moreover, by incorporating one or more residues of diamine compounds or dicarboxylic acid compounds (e.g., residues of diamine compounds such as ethylenediamine or dicarboxylic acid compounds such as succinic acid) in a spacer as constitutional units, a spacer having either N-terminuses or C-terminuses at both ends may be utilized.

[0021] The amino acid sequence of the spacer is not particularly limited. Preferably used spacers include, for example, a spacer being a residue of a dipeptide represented by -X-Z-, wherein X represents a residue of a hydrophobic amino acid and Z represents a residue of a hydrophilic amino acid; and X-Z- means a residue which consists of a dipeptide that is formed by a peptide bond between a hydrophobic amino acid (X) and a hydrophilic amino acid (Z) at the N-terminal side and the C-terminal side, respectively, and whose one hydrogen atom and one hydroxyl group are removed from the amino group at the N-terminus and the carboxyl group at the C-terminus, respectively, and a spacer containing a residue of the dipeptide as a partial peptide sequence. As the hydrophobic amino acid, for example, phenylalanine, tyrosine, leucine or the like can be used, and as the hydrophilic amino acid, for example, glycine, alanine or the like can be used. The spacer may have a repeated sequence of the dipeptide residue (for example, X-Z-X-Z-, -X-Z-X-Z-X-Z-, etc.).

[0022] By using the spacer containing such dipeptide structure, the spacer can be hydrolyzed in tumorous sites or inflammatory sites, which is considered abundant in peptidase, to release the drug compound at high concentration in the sites. The partial structure formed between the spacer containing the above dipeptide and the drug compound by

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binding to each other is a preferred partial structure of the drug complex of the present invention. Where a concentration-dependent antineoplastic agent (e.g., doxorubicin) or the like is used as the residue of the drug compound, for example, a spacer composed of the above dipeptide residue represented by -X-Z- or a spacer containing the above dipeptide residue as a partial peptide sequence may most preferably be used.

5 [0023] In addition, where a time-dependent type antineoplastic agent which requires a retained working time at over certain concentration is used as the residue of the drug compound, enhanced antineoplastic activity may sometimes be obtained by using the above spacer. Examples include the antineoplastic agents disclosed in the Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 6-87746/1994, preferably the antineoplastic agent disclosed in claim 2. Generally, the spacer should not be limited to those mentioned above, and it is necessary to choose an appropriate
10 spacer from viewpoints of the mode of action of the antineoplastic agent, characteristics in pharmacokinetics or appearance of toxicity, releasability *in vivo* of the antineoplastic agent and the like. For carcinomas exhibiting rapid proliferation, it is generally preferred to choose the above spacer capable of releasing the drug compound at high concentration in a short time.

[0024] Specific examples of the spacer are shown in the following table; however, the spacer used for the drug complexes of the present invention is not limited to those mentioned below, and it can be readily understood that one of ordinary skilled in the art can appropriately choose a spacer so as to achieve an optimum releasing rate of a drug compound. In the table, the left ends of peptide sequences are N-terminuses and the residues of drug compounds are bound to C-terminuses. D-Phe represents a D-phenylalanine residue and the other amino acids represent L-amino acids. The degrees of the releasing rates were judged from the degree of appearance of efficacy of drug complexes
15 bound with doxorubicin against Walker 256 tumor-bearing rats, or from the free doxorubicin concentration at the tumorous sites of Walker 256 tumor-bearing rats. Among these spacers, a spacer which can release the drug compound at high concentration in a short time, e.g., (N-terminus)-Gly-Gly-Phe-Gly-, is preferably used for doxorubicin.
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Table 1

5	(a) Spacers having high releasing rate
	-Leu-Gly-
	-Tyr-Gly-
10	-Phe-Gly-
	-Gly-Phe-Gly-
	-Gly-Gly-Phe-Gly-
	-Gly-Phe-Gly-Gly-
15	-Phe-Gly-Gly-Gly-
	-Phe-Phe-Gly-Gly-
	-Gly-Gly-Gly-Phe-Gly-
20	(b) Spacers having relatively high releasing rate
	-Gly-Gly-Phe-Phe-
	-Gly-Gly-Gly-Gly-Gly-Gly-
25	(c) Spacers having relatively low releasing rate
	-Phe-Phe-
	-Ala-Gly-
30	-Pro-Gly-
	-Gly-Gly-Gly-Phe-
35	(d) Spacers having low releasing rate
	-Gly-
	-D-Phe-Gly-
	-Gly-Phe-
40	-Ser-Gly-
	-Gly-Gly-
	-Gly-Gly-Gly-
45	-Gly-Gly-Gly-Gly-

[0025] Although the degree of polyalcoholization of the carboxy(C₁₋₄)alkyldextran polyalcohol, that constitute the moiety of the polysaccharide derivative of the drug complex of the present invention, is not particularly limited, it is preferred that the dextran polyalcohol constituting the carboxy(C₁₋₄)alkyldextran polyalcohol is a dextran polyalcohol obtained by treating a dextran under conditions which enable substantially complete polyalcoholization. For example, a dextran polyalcohol obtained by treating a dextran with large excess amounts of sodium periodate and sodium borohydride successively to achieve substantially complete polyalcoholization can preferably be used as a starting material for manufacturing the drug complex of the present invention. However, the method for the polyalcoholization of adextran is not limited to the method mentioned above, and any methods available to those skilled in the art may be applied.

[0026] The sort of the dextran is not particularly limited, and the dextran may contain α -D-1,6-bonds at any rate. For example, dextrans containing α -D-1,6-bond at the rate of 85% or more, 90% or more, and 95% or more can be used. The molecular weight of the dextran is not particularly limited, and for example, dextrans having a molecular weight of from about 10,000 to about 2,000,000, preferably from about 50,000 to about 800,000 can be used. As the C₁₋₄ alkyl

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group constituting the carboxy(C₁₋₄)alkyl group, a linear or branched C₁₋₄ alkyl group, specifically, methyl group, ethyl group, n-propyl group, isopropyl group, n-butyl group, sec-butyl group or the like can be used, and methyl group can preferably be used. The carboxy(C₁₋₄)alkylation can be carried out, for example, by reacting a halogenated (C₁₋₄)alkyl-carboxylic acid such as chloroacetic acid, bromoacetic acid, α -chloropropionic acid, α -methyl- α -chloropropionic acid, β -chloropropionic acid, α -methyl- β -chloropropionic acid, α -chlorobutyric acid, β -chlorobutyric acid, or γ -chlorobutyric acid, preferably chloroacetic acid, with hydroxyl groups of the dextran polyalcohol to achieve partial or complete carboxy(C₁₋₄)alkylation of the hydroxyl groups.

[0027] For example, the dextran polyalcohol is dissolved in an inert solvent which does not participate in the reactions (e.g., water, N,N-dimethylformamide, or dimethyl sulfoxide) and the solution is added with a halogenated (C₁₋₄)alkyl carboxylic acid or a salt thereof in the presence of a base (e.g., sodium hydroxide or potassium hydroxide), and then the mixture is allowed to react for several minutes to several days at a temperature under ice-cooling to about 100°C. The degree of introduction of the carboxy(C₁₋₄)alkyl group may be easily controlled, for example, by suitably choosing the reaction temperature of the carboxy(C₁₋₄)alkylation or the amount of the halogenated (C₁₋₄)alkyl carboxylic acid or bases used as reagents, and these means are well-known to those skilled in the art. The degree of the carboxy(C₁₋₄)alkylation for hydroxyl groups of the dextran polyalcohol is not particularly limited, and for example, the degree may be in the range of from 0.01 to 2.0, preferably from 0.1 to 1.0, and more preferably from 0.3 to 0.5 per residue of the constitutive saccharide. The molecular weight of the carboxy(C₁₋₄)alkyldextran polyalcohol is from about 5,000 to 500,000, preferably from about 50,000 to 450,000, and more preferably from about 200,000 to 400,000 when determined by the gel filtration method.

[0028] The aforementioned carboxy(C₁₋₄)alkyldextran polyalcohol is useful as a drug delivery carrier. Drug complexes in which a drug compound and the carboxy(C₁₋₄)alkyldextran polyalcohol are bound to each other are characterized in, for example, that they have excellent selectivity such as neoplastic selectivity and are capable of maintaining high blood concentration for a long period of time. As for the bond between the drug compound and the carboxy(C₁₋₄)alkyldextran polyalcohol, for example, a method in which both are bound to each other directly by means of an ester bond, or alternatively, a method in which both are bound to each other by means of an appropriate spacer such as those mentioned above can be adopted.

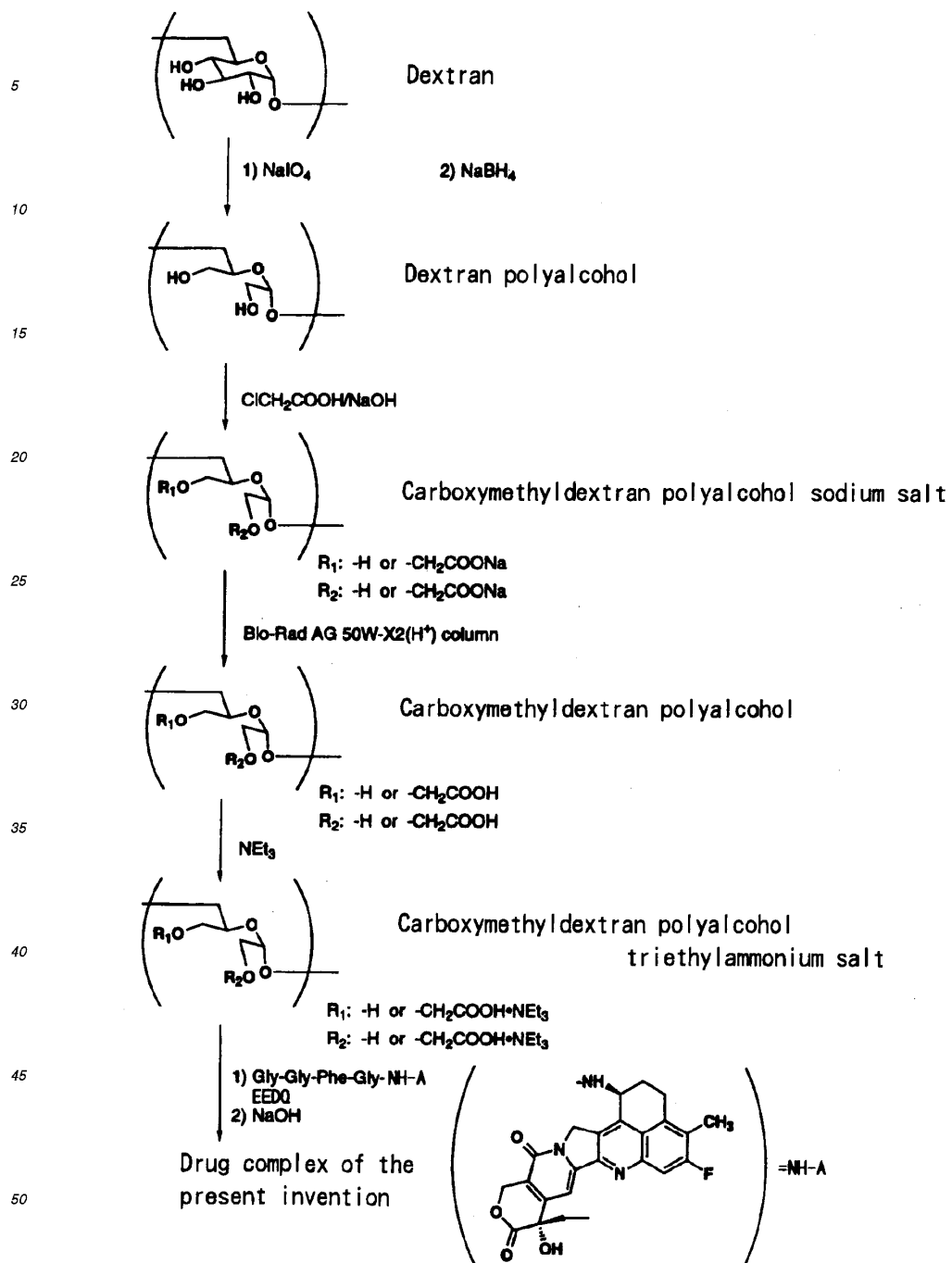
[0029] As for the drug complex bound by means of the spacer, the drug complex of the present invention can be prepared by binding the spacer, that is bound to a residue of a drug compound, to a carboxyl group of the carboxymethyldextran polyalcohol obtained as above. The bond between the spacer and the carboxyl group of the carboxymethyldextran polyalcohol can generally be formed by binding the N-terminal amino group of the spacer to a carboxyl group of the carboxymethyldextran polyalcohol by means of an acid-amide bond. However, the bond between the spacer and the carboxyl group of the carboxymethyldextran polyalcohol is not limited to that described above, and other chemical bonds and bonds utilizing one or more spacers may be used. For example, an acid anhydride may be formed between the C-terminal carboxyl group of the spacer and a carboxyl group of the carboxymethyldextran polyalcohol, or by using a diamine compound such as ethylenediamine as a spacer, each of the carboxyl groups may be bound by means of acid-amide bond to each of the amino groups of the diamine compound.

[0030] When the N-terminal amino group of the spacer is bound to a carboxyl group of the carboxymethyldextran polyalcohol by means of an acid-amide bond, dehydration condensation agents ordinarily used for the synthesis of peptide chain, for example, N,N'-dicycloalkylcarbodiimides such as N,N'-dicyclohexylcarbodiimide (DCC), carbodiimide derivatives such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAPC), benzotriazole derivatives such as 1-hydroxybenzotriazole (HOBt), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (EEDQ) and the like can be used. In addition, the reaction may also be performed by the activated ester method and the acid halide method.

[0031] Although the amount of the residue of the drug compound introduced into the carboxymethyldextran polyalcohol is not particularly limited, the amount should be suitably chosen from the viewpoints of for example, the physico-chemical properties of the drug compound residue, and the pharmacokinetics, efficacy and toxicity of the drug complex of the present invention. Generally, the range of approximately from 0.1 to 30% by weight, preferably approximately from 1 to 15% by weight, and more preferably approximately from 3 to 10% by weight, and most preferably approximately from 5 to 6% by weight may be chosen. The ratio of the residue of the drug compound introduced into the carboxymethyldextran polyalcohol can be easily determined by, for example, the absorption spectrometric analysis.

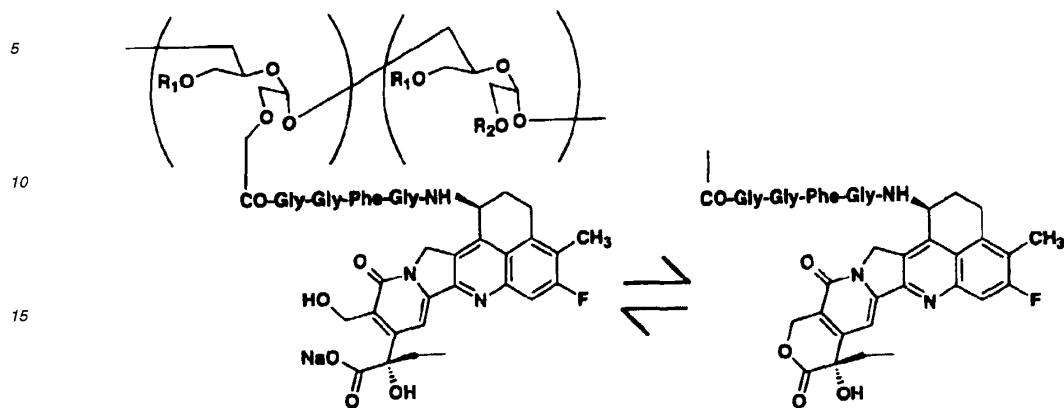
[0032] As an example of the method for manufacturing the drug complex of the present invention, the following scheme shows the preparing process for introducing the residue of the drug compound which is the antineoplastic agent disclosed in claim 2 of the Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 6-87746/1994; however, the drug complexes of the present invention and the methods for manufacturing thereof are not limited to those shown in the scheme. In the scheme below, the introduced amount of the residue of the drug compound is, for example, about from 1 to 15% by weight, preferably about from 2 to 10% by weight. In addition, among the constitutional units of the polyalcohols, only one constitutional unit that is introduced with one or two carboxymethyl groups is exemplified in the scheme below. However, it should be understood that the polysaccharide-derivative moiety of the drug complex of the present invention is not formed by the repetition of the aforementioned constitutional unit.

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Drug complex of the present invention



[0033] It is known that the equilibrium of the aforementioned drug compound lies to the compound whose lactone ring is closed (the ring-closed compound) in an acidic aqueous medium (for example, approximately at pH 3), whereas the equilibrium lies to the compound whose lactone ring is opened (the ring-opened compound) in a basic aqueous medium (for example, approximately at pH 10), and the drug complex introduced with the residue corresponding to the ring-closed or ring-opened compound has similar antineoplastic activity. Accordingly, it should be understood that any of them falls within the scope of the present invention. When a reactant, whose lactone ring is opened, is present in the reaction system, the condensation reaction will progress between the carboxyl group derived from the lactone ring and the amino group derived from the spacer, which results in significant decrease of the reaction yield, and moreover, a desired drug complex cannot sometimes be uniformly obtained. Such side reaction can be avoided by selectively using the ring-closed compound as the reactant.

[0034] That is, the side reaction can be reduced by converting sodium salt of carboxymethyldextran polyalcohol into the triethylammonium salt, and then condensing the N-terminal amino group of the spacer, which is bound to the residue of the drug compound described above, with a carboxyl group of the carboxymethyldextran polyalcohol in a non-aqueous system (in an organic solvent not containing water), which enables an efficient manufacture of the desired product. As the salt of the carboxymethyldextran polyalcohol that can be dissolved in organic solvents, for example, trialkylammonium salt such as triethylammonium salt or trimethylammonium salt, or salt of organic bases such as N-methylpyrrolidine, N-methylmorpholine, or dimethylaminopyridine (DMAP) can be used. As organic solvents, N,N-dimethylformamide, dimethyl sulfoxide or the like can be used.

[0035] The drug complex of the present invention is characterized in that it can specifically exhibit desired pharmacological activity at a local site such as tumorous sites or inflammatory sites depending on the sort of the residue of the drug compound (e.g., the residue of the drug compound such as antineoplastic agents or anti-inflammatory agents), and can reduce toxicity inherent to the drug compound, per se. Although not intended to be bound by any specific theory, the polysaccharide-derivative moiety of the drug complex of the present invention (e.g., carboxymethyldextran polyalcohol) has quite excellent retention in blood and achieves high accumulation into tumorous or inflammatory sites, and hence is useful as a drug delivery carrier and allows the drug complex of the present invention to have neoplastic site-selectivity and inflammatory site-selectivity. Furthermore, it is considered that protease (peptidase) is expressed at tumorous sites or inflammatory sites, and accordingly, the spacer of the drug complex of the present invention is readily hydrolyzed to allow a released drug compound to exhibit its efficacy.

[0036] A medicament containing the drug complex of the present invention may generally be filled in vials or the like in the form of a lyophilized product or other, and provided for clinical use as preparations for parenteral administration such as injections or drip infusions which are dissolved upon use. However, the form of pharmaceutical preparations of the medicament of the present invention is not limited to the aforementioned forms. For the preparation of the above pharmaceutical preparations, pharmaceutical additives available in the field of the art, for example, solubilizers, pH modifiers, stabilizers and the like, can be used. Although the dose of the medicament of the present invention is not particularly limited, it should normally be decided in view of the dose of the drug compound that constitutes the residue of the drug compound, the amount of the residue of the drug compound introduced into the drug complex of the present invention, the condition of a patient, the sort of a disease and the like. For example, where a drug complex of the present

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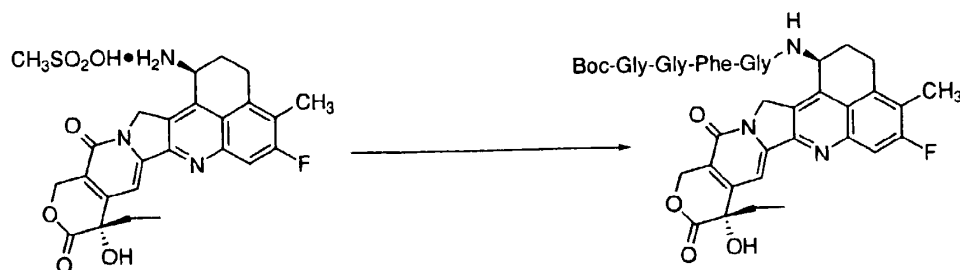
invention is administered parenterally, which is introduced with about 6% by weight of the residue of the antineoplastic agent disclosed in claim 2 of the Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 6-87746/1994, about 1 to 500 mg, preferably about 10 to 100 mg per m² of body surface area per day may generally be administered once a day, and the administration may preferably repeated every 3 to 4 weeks.

Examples

[0037] The present invention will be explained more specifically by examples; however, the scope of the present invention is not limited to the following examples. In the examples, "A-NH-" represents a residue of a drug compound wherein the drug compound has a lactone ring, such as the drug compound disclosed in claim 2 of the Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 6-87746/1994 (sometimes referred to as "DX-8951" in the examples), and the drug compound having a closed lactone ring is represented by A-NH₂. An example includes the group represented by A-NH- in the above-described scheme in which a lactone ring is formed. In addition, A'-NH- represents that the lactone ring of the residue of a drug compound represented by A-NH- is either in the ring-closed form or ring-opened form, or alternatively, a mixture thereof, -DXR represents the residue derived from doxorubicin, and -D51-7059 represents the residue derived from the taxiol derivative shown in Example 55.

[0038] In the examples, otherwise not specifically mentioned, the degree of carboxymethylation in carboxymethyl-dextran polyalcohol (the degree of substitution with carboxymethyl group per constitutive saccharide residue) was determined by converting the sodium salt of the carboxymethyl-dextran polyalcohol into the free acid form, dissolving the resulting acid in an aqueous 0.1N sodium hydroxide solution, and then titrating by using 0.1N hydrochloric acid. An aqueous solution of the sodium salt of the carboxymethyl-dextran polyalcohol was applied to a Bio-Rad AG50W-x 2 (H⁺ form) column and the effluent was lyophilized and then used as a sample. The sample was dissolved in a prescribed excess amount of aqueous 0.1N sodium hydroxide solution and titrated with 0.1N hydrochloric acid using phenolphthalein as an indicator. The degree of carboxymethylation was calculated by using the following equation:
Degree of carboxymethylation = $13.4(a-b)/[s-5.8(a-b)]$ wherein "s" is the weight of the applied sample (mg), "a" is the prescribed excess amount of aqueous 0.1N sodium hydroxide solution (ml), and "b" is the volume of 0.1N hydrochloric acid consumed for the titration (ml). The amount of introduced drug (percent by weight) was determined by the absorption spectroscopic analysis by using characteristic absorptions of the drug compound (approximately 362 nm). The gel filtration was performed under the following conditions: column: TSK gel G4000 PW_{XL}; eluent: 0.1M NaCl; flow rate: 0.8 ml/min; and column temperature: 40°C.

Example 1: 3'-N-(Boc-Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951)



[0039] Boc-Gly-Gly-Phe-Gly (600 mg) and N-hydroxysuccinimide (160 mg) were dissolved in N,N-dimethylformamide (20 ml) and cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (280 mg). To this solution, a solution of methanesulfonate of the drug compound described in claim 2 of the Japanese Patent Unexamined Publication (KOKAI) No. (Hei) 6-87746/1994 (600 mg, the compound described in Example 50 of the above-mentioned patent publication) and triethylamine (0.16 ml) dissolved in N,N-dimethylformamide (30 ml) was added, and the mixture was allowed to react at room temperature for 16 hours with stirring under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 10:1 solution containing 0.5% acetic acid) to give the title compound (1.0 g).

¹H-NMR (DMSO- d₆) δ : 8.40 (d, 1H, J=8.3 Hz), 8.10-8.17 (m, 2H), 7.91-8.01 (m, 1H), 7.78 (d, 1H, J=10.75 Hz), 7.32 (s, 1H), 6.94-6.96 (m, 1H), 6.50 (s, 1H), 5.57 (t, 1H, J=4.5 Hz), 5.43 (s, 2H), 5.23 (s, 2H), 3.77

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(dd, 2H, J=5.85 Hz, J=8.80 Hz), 3.70 (d, 2H, J=4.40 Hz), 3.65 (d, 2H, J=5.35 Hz), 3.56 (d, 2H, J=5.85), 3.15-3.25 (m, 2H), 2.40 (s, 3H), 2.05-2.25 (m, 1H), 1.86 (m, 2H), 1.35 (s, 9H), 0.88 (t, 3H, J=7.35).

Mass (FAB); m/e 854 (M+1)

5 Example 2: Synthesis of 3'-N-(Boc-Gly-Gly-Gly-Phe)-NH-A (A-NH₂=DX-8951)

[0040] Boc-Gly-Gly-Gly-Phe (600 mg) and N-hydroxysuccinimide (160 mg) were dissolved in N,N-dimethylformamide (20 ml) and cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (280 mg). To this solution, a solution of methanesulfonate of DX-8951 (600 mg) and triethylamine (0.16 ml) dissolved in N,N-dimethylformamide (30 ml) was added and the mixture was allowed to react at room temperature for 16 hours with stirring under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 10:1 solution containing 0.5% acetic acid) to give the title compound (700 mg).

15 ¹H-NMR (DMSO-d₆) δ : 8.57 (d, 1H, J=7.8 Hz), 8.19 (d, 1H), 8.05-8.07 (m, 2H), 7.79 (d, 1H, J=11.2 Hz), 7.32 (s, 1H), 7.10 (d, 2H, J=7.8 Hz), 6.93-7.03 (m, 4H), 6.51 (s, 1H), 5.52-5.55 (m, 1H), 5.44 (s, 2H), 5.18 (d, 1H, J=18.5 Hz), 4.84 (d, 1H, J=18.5 Hz), 4.57-4.59 (m, 1H), 3.57-3.71 (m, 6H), 3.15-3.25 (m, 2H), 3.00-3.02 (m, 1H), 2.80-2.90 (m, 1H), 2.40 (s, 3H), 2.05-2.25 (m, 1H), 1.86 (m, 2H), 1.35 (s, 9H), 0.88 (t, 3H, J=7.35 Hz).

Mass (FAB); m/e 854 (M+1)

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Example 3: Synthesis of 3'-N-(Boc-Gly-Gly-Gly-Gly)-NH-A (A-NH₂=DX-8951)

[0041] Boc-Gly-Gly-Gly-Gly (120 mg) and N-hydroxysuccinimide (39 mg) were dissolved in N,N-dimethylformamide (20 ml) and cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (70 mg). To this solution, a solution of methanesulfonate of DX-8951 (150 mg) and triethylamine (0.039 ml) dissolved in N,N-dimethylformamide (10 ml) was added, and the mixture was allowed to react at room temperature for 16 hours with stirring under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 10:1 solution) to obtain the title compound (100 mg).

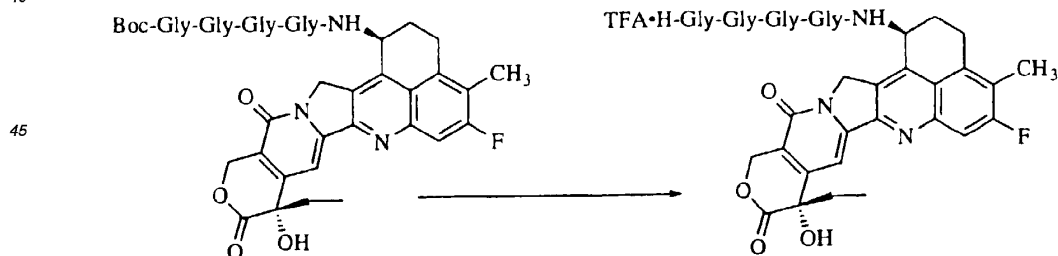
30 ¹H-NMR (DMSO-d₆) δ : 8.40 (d, 1H, J=8.3 Hz), 8.10-8.17 (m, 2H), 7.91-8.01 (m, 1H), 7.78 (d, 1H, J=10.75 Hz), 7.32 (s, 1H), 6.94-6.96 (m, 1H), 6.50 (s, 1H), 5.57 (t, 1H, J=4.5 Hz), 5.43 (s, 2H), 5.23 (s, 2H), 3.77 (dd, 2H, J=5.85 Hz, J=8.80 Hz), 3.70 (d, 2H, J=4.40 Hz), 3.65 (d, 2H, J=5.35 Hz), 3.56 (d, 2H, J=5.85 Hz), 3.15-3.25 (m, 2H), 2.40 (s, 3H), 2.05-2.25 (m, 1H), 1.86 (m, 2H), 1.35 (s, 9H), 0.88 (t, 3H, J=7.35 Hz).

Mass (FAB); m/e 764 (M+1)

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Example 4: Synthesis of 3'-N-(Gly-Gly-Gly-Gly)-NH-A (A-NH₂=DX-8951) trifluoroacetate

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[0042] 3'-N-(Boc-Gly-Gly-Gly-Gly)-NH-A (A-NH₂=DX-8951) (79 mg) was dissolved in trifluoroacetic acid (3 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected to azeotropic distillation twice with methanol (30 ml) and twice with ethanol (30 ml), and then the residue was washed with ether to give the title compound (80 mg).

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¹H-NMR (DMSO-d₆) δ : 8.59-8.61 (m, 1H), 8.50 (d, 1H, J=8.3 Hz), 8.21-8.27 (m, 2H), 7.91-8.01 (br, 3H), 7.81

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(d,1H,J=11.2Hz), 7.32 (s,1H), 6.50-6.52 (br,1H), 5.57-5.59 (m,1H), 5.43 (s,2H), 5.23 (s,2H), 3.80-3.82 (m,3H), 3.70-3.75 (m,3H), 3.15-3.25 (m,2H), 2.41 (s,3H), 2.05-2.25 (m,1H), 1.86-1.88 (m,2H), 0.88 (t,3H,J= 7.35Hz).

Example 5: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0043] Dextran T2000 (10 g, Pharmacia, average molecular weight: 2,000,000) was dissolved in 0.1M acetate buffer (pH 5.5, 1,000 ml) and added with an aqueous solution (1000 ml) of sodium periodate (33.0 g). After stirring at 4°C for 10 days with shielding the light, the mixture was added with ethylene glycol (7.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. Sodium borohydride (14 g) was added and dissolved, and the mixture was then stirred at room temperature overnight. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid, and stirred at 4°C for one hour, and then, adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was subjected to ultrafiltration using a Biomax-30 membrane (Millipore) to remove the low molecular weight fraction. The polymer fraction was lyophilized to obtain dextran polyalcohol. After treating this dextran polyalcohol at pH 3.0 for one hour, the low molecular weight fraction was removed with Biomax-50 membrane, and subsequently, the polymer fraction was removed with Biomax-100 membrane, and the result was lyophilized to give purified dextran polyalcohol (2.0 g). The molecular weight of this substance was 220K (gel filtration, dextran standard).

[0044] This purified dextran polyalcohol (1.8 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (10.5 g) in water (45 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (15 g) was added and dissolved under ice-cooling, and then the mixture was allowed to react at room temperature for 20 hours. After this reaction mixture was adjusted to pH 8 with acetic acid, the low molecular weight fraction was removed by ultrafiltration using a Biomax-10 membrane. The polymer fraction was lyophilized to give sodium salt of carboxymethyldextran polyalcohol (1.8 g). The molecular weight of this substance was 330K (gel filtration, dextran standard) and the degree of carboxymethylation was 0.8.

[0045] The above sodium salt of carboxymethyldextran polyalcohol (300 mg) was dissolve in water, applied to a Bio-Rad AG50W-X2 (200-400 mesh, H⁺ form) column (1.5 × 8.6 cm), and eluted with water. This effluent was added with triethylamine (0.5 ml) and lyophilized to give triethylammonium salt of carboxymethyldextran polyalcohol (380 mg). Portions of the sodium salt of carboxymethyldextran polyalcohol (each 300 mg) were treated with the column as described above to give triethylammonium salt of carboxymethyldextran polyalcohol (380 mg, 400 mg).

Example 6: Synthesis of sodium salt of carboxymethyldextran polyalcohol

[0046] The sodium salt of carboxymethyldextran polyalcohol (0.15 g) obtained in Example 5 above was added to an aqueous solution obtained by dissolving sodium hydroxide (1.05 g) in water (4.5 ml), and then dissolved at room temperature. To this solution, monochloroacetic acid (1.5 g) was added and dissolved under ice-cooling, and the mixture was allowed to react at room temperature for 18 hours. This reaction mixture was adjusted to pH 8 with acetic acid, added dropwise into 90 ml of methanol, and added with 3M aqueous sodium chloride (0.15 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were washed with methanol and then dissolved in water (5 ml), and added with 3M aqueous sodium chloride (0.15 ml). This aqueous solution was filtered through a Millipore filter (0.45 μm), and the filtrate was added dropwise to 35 ml of ethanol and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were washed with ethanol, dissolved in water, and dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight; 6,000-8,000). The inner dialyzate solution was filtered through a Millipore filter (0.22 μm) and lyophilized to give sodium salt of carboxymethyldextran polyalcohol (0.18 g). The degree of carboxymethylation of this substance per saccharide residue was 1.2 (alkalimetry).

Example 7: Synthesis of sodium salt of carboxymethyldextran polyalcohol

[0047] The purified dextran polyalcohol (0.2 g) obtained in Example 5 was added to an aqueous solution obtained by dissolving sodium hydroxide (0.84 g) in water (6 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (1.2 g) was added and dissolved under ice-cooling, and the mixture was allowed to react at room temperature for 18 hours. The reaction mixture was adjusted to pH 8 with acetic acid, added dropwise to 120 ml of methanol, and then added with 3M aqueous sodium chloride (0.2 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were washed with methanol and then dissolved in water (5 ml), and added with 3M aqueous sodium chloride (0.2 ml). This aqueous solution was filtered through a Millipore filter (0.45 μm), and the filtrate was added dropwise to 35 ml of ethanol and the precipitate deposited was collected by centrifugation (3500 rpm, 8 minutes). The precipitates were washed with ethanol, dissolved in water, and dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight; 6,000-8,000). The inner dialyzate solution

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was filtered through a Millipore filter (0.22 μm) and lyophilized to give sodium salt of carboxymethyldextran polyalcohol (0.20 g). The degree of carboxymethylation of this substance per saccharide residue was 0.4 (alkalimetry).

Example 8: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Gly-Phe-NH-A' (A-NH₂=DX-8951)

[0048] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 5 (380 mg, the degree of carboxymethylation: 0.8) was dissolved in N,N-dimethylformamide (30 ml). To this solution, a solution of 3'-N-(Gly-Gly-Gly-Phe)-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt (49 mg) in N,N-dimethylformamide (5 ml), triethylamine (0.017 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (380 mg) were added successively, and then the mixture was allowed to react at room temperature overnight with stirring. This reaction mixture was adjusted to pH 10 with 1M aqueous sodium hydroxide and each of 5 ml portions of the mixture was added dropwise to 25 ml of ethanol. This mixture was added with 3M aqueous sodium chloride (1 ml) and diethyl ether (5 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes).

[0049] The precipitates were dissolved in water and dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight: 6,000-8,000), and the inner dialyzate solution was filtered through a Millipore filter (0.22 μm) and lyophilized. The resulting crude product was dissolved in water (30 ml), adjusted to pH 9 with 0.1M aqueous sodium hydroxide, and treated at 37°C for one hour. This treated solution was dialyzed as described above, and then the inner dialyzate solution was filtered through a Millipore filter (0.22 μm) and lyophilized to give the title compound (289 mg). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min), and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 0.25 mg/ml) are shown in Fig. 1 and Fig. 2, respectively. The content of the drug compound residue in the compound was 5.3% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 9: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0050] The title compound (300 mg) was synthesized according to a similar manner to that of Example 8 by introducing trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A, which had been obtained by removal of the Boc group from 3'-N-(Boc-Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (50 mg) in a manner similar to that of Example 4, into the triethylammonium salt of carboxymethyldextran polyalcohol (380 mg) obtained in Example 5. The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min), and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 0.19 mg/ml) are shown in Fig. 3 and Fig. 4, respectively. The content of the drug compound residue in the compound was 5.3% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 10: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Gly-Gly-NH-A' (A-NH₂=DX-8951)

[0051] The title compound (190 mg) was synthesized according to a similar manner to that of Example 8 by introducing trifluoroacetic acid salt of 3'-N-(Gly-Gly-Gly-Gly)-NH-A, which had been obtained through removal of the Boc group from 3'-N-(Boc-Gly-Gly-Gly-Gly)-NH-A (A-NH₂=DX-8951) (41 mg) in a manner similar to that of Example 4, into the triethylammonium salt of carboxymethyldextran polyalcohol (380 mg) obtained in Example 5. The ultraviolet absorption spectrum of this compound (0.1M Tris buffer solution, pH 9.0, 0.34 mg/ml) is shown in Fig. 5. The content of the drug compound residue in the compound was 5.3% (W/W) when determined based on the absorption at 362 nm in 0.1M Tris buffer solution (pH 9.0).

Example 11: Antitumor activity of the drug complex of the present invention

[0052] Meth A tumor-bearing mice were prepared (7 mice per group) by subcutaneously transplanting 1×10^6 mouse fibrosarcoma Meth A cells into the right inguinal regions of BALB/c male mice (7-week old). On day 7, the drug complex of Example 9 dissolved in distilled water for injection was injected in the tail vein of the Meth A tumor-bearing mice every 4 days 4 times. On day 21 after the transplantation, tumor masses were excised and weighed to calculate the inhibition rate of tumor growth according to the following equation: inhibition rate of tumor growth (%) = $[1 - (\text{average tumor weight of the group administered with the test sample} / \text{average tumor weight of})]$. As a result, it was found that the drug complex of the present invention obtained in Example 9 exhibited remarkably enhanced antitumor activity compared to the drug compound, per se, without the spacer and the polysaccharide derivative, while showing no toxicity (weight loss). The polysaccharide derivative (Example 5), per se, and the drug compound introduced solely with the spacer (trifluoroacetic acid salt of H₂N-Gly-Gly-Phe-Gly-NH-A (A-NH₂=DX-8951) obtained by removing the Boc group from the compound of Example 1 according to the process of Example 4) were

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found to be not effective.

Table 2

Test compound	Dose (mg/kg)	Inhibition rate (%)
Drug compound, per se	7.5×4	76
	1.875×4	46
	0.9375×4	36
Compound of Example 9	$1.4^{1)} \times 4$	94
	$0.7^{1)} \times 4$	59
	$0.35^{1)} \times 4$	41

1) Calculated based on the drug compound

Example 12: Antitumor activity of the drug complex of the present invention

[0053] Meth A tumor-bearing mice (6 mice per group) were prepared according to a similar manner to that of Example 11 and the antitumor activity was compared to that obtained by single administration of the drug complexes of Examples 8 and 9 once on day 7. As a result, the degree of the antitumor activity was as follows: (Polysaccharide derivative)-Gly-Gly-Phe-Gly-NH-A' > (Polysaccharide derivative)-Gly-Gly-Gly-Phe-NH-A' > the drug compound, per se. The compound comprising the residue of the drug compound directly binding to a carboxyl group of the carboxymethyldextran polyalcohol of Example 5 without any spacer (the amount of the drug compound residue introduced: 6.2% by weight) was found to be not effective.

Table 3

Test compound	Dose (mg/kg)	Inhibition rate (%)
Drug compound itself	60	77
	20	59
Compound of Example 8	$10^{1)}$	85
	$5^{1)}$	76
Compound of Example 9	$5^{1)}$	98
	$2.5^{1)}$	87

1) Calculated based on the drug compound

Example 13: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0054] Dextran T500 (10 g, Pharmacia, molecular weight: 500K) was dissolved in 0.1M acetate buffer (pH 5.5, 1000 ml) and added with an aqueous solution (1000 ml) of sodium periodate (33 g). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (7.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide. Sodium borohydride (14 g) was added and dissolved, and then the mixture was stirred overnight. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then adjusted to pH 7.5 with 8M aqueous sodium hydroxide to give Solution 1. Separately, a series of procedures described above was performed using Dextran T500 (10 g, Pharmacia, molecular weight 500K) to obtain Solution 2. Furthermore, a series of procedures described above was repeated by using Dextran T250 (10 g each, Pharmacia, molecular weight 250K) to obtain Solution 3 and Solution 4. These Solutions 1-4 were combined and subjected to ultrafiltration using a Biomax-50 membrane to remove the low molecular weight fraction. The polymer fraction was lyophilized to give dextran polyalcohol (25 g). The molecular weight of this substance was 163K (gel filtration, pullulan standard).

[0055] This dextran polyalcohol (11 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (46.2 g) in water (330 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (66 g) was added

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under ice-cooling and dissolved, and the mixture was allowed to react at room temperature overnight. This reaction mixture was adjusted to pH 9 with acetic acid and desalted by ultrafiltration using a Biomax-30 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (13 g). The molecular weight of this substance was 228K (gel filtration, pullulan standard) and the degree of carboxymethylation was 0.4.

[0056] This sodium salt of carboxymethyldextran polyalcohol (600 mg) was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, H⁺ form) column (diameter: 44 mm, length: 210 mm), and eluted with water. This effluent was added with triethylamine (0.93 ml) and then lyophilized to give the title compound (690 mg).

Example 14: Synthesis of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt

[0057] The 3'-N-(Boc-Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (79 mg) obtained in Example 1 was dissolved in trifluoroacetic acid (3 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected to azeotropic distillation twice with methanol (30 ml) and twice with ethanol (30 ml), and then washed with ether to give the title compound (80 mg).

¹H-NMR (DMSO-d₆) δ : 8.53 (d, 1H, J=8.3Hz), 8.40-8.48 (m, 2H), 8.28 (d, 1H, J=8.3Hz), 7.95-8.07 (br, 3H), 7.81 (d, 1H, J=10.2Hz), 7.30-7.37 (m, 2H), 7.15-7.30 (m, 5H), 6.50-6.55 (br, 1H), 5.50-5.57 (m, 1H), 5.41 (d, 2H, J=7.82Hz), 5.25 (s, 2H), 4.55-4.62 (m, 1H), 3.55-3.92 (m, 6H), 3.15-3.25 (br, 2H), 2.98-3.03 (m, 1H), 2.73-2.82 (m, 1H), 2.40 (s, 3H), 2.05-2.25 (m, 1H), 1.84-1.92 (m, 2H), 0.88 (t, 3H, J=7.35Hz).

Example 15: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0058] The sodium salt of carboxymethyldextran polyalcohol obtained in Example 13 (400 mg) was converted into the triethylammonium salt (470 mg) and dissolved in N,N-dimethylformamide (30 ml). To this solution, a solution of trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) obtained in Example 14 (62 mg) in N,N-dimethylformamide (5 ml), triethylamine (0.02 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (470 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring and shielding the light. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. To the mixture, 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml) were added, and the precipitates deposited were collected by centrifugation. The precipitates were dissolved in 0.5M aqueous sodium chloride, adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling and then dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight; 6,000-8,000). The inner dialyzate solution was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (600 mg). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh; solvent: 0.1 M NaCl; flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 0.1 mg/ml) are shown in Fig. 6 and Fig. 7, respectively. The content of the drug compound residue in the compound was 5.8% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 16: Synthesis of 3'-N-(Gly-Gly-Gly-Phe)-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt

[0059] The 3'-N-(Boc-Gly-Gly-Gly-Phe)-NH-A (A-NH₂=DX-8951) (79mg) obtained in Example 2 was dissolved in trifluoroacetic acid (3 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected to azeotropic distillation twice with methanol (30 ml) and twice with ethanol (30 ml), and then the residue was washed with ether to give the title compound (80 mg).

¹H-NMR (DMSO-d₆) δ : 8.62-8.66 (m, 2H), 8.23 (d, 1H, J=8.3Hz), 8.18-8.20 (m, 1H), 7.98-8.10 (br, 2H), 7.79 (d, 1H, J=10.7Hz), 7.32 (s, 1H), 7.09 (d, 2H, J=7.3Hz), 6.93-7.03 (m, 4H), 6.50-6.60 (br, 1H), 5.52-5.55 (m, 1H), 5.44 (s, 2H), 5.18 (d, 1H, J=18.5Hz), 4.80 (d, 1H, J=18.5Hz), 4.57-4.59 (m, 1H), 3.57-3.71 (m, 6H), 3.15-3.25 (m, 2H), 3.00-3.02 (m, 1H), 2.80-2.90 (m, 1H), 2.50 (s, 3H), 2.05-2.25 (m, 1H), 1.86-2.00 (m, 2H), 0.88 (t, 3H, J=7.35Hz).

Example 17: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Gly-Phe-NH-A' (A-NH₂=DX-8951)

[0060] The sodium salt of carboxymethyldextran polyalcohol obtained in Example 13 (1.0 g) was converted into the triethylammonium salt (1.2 g) and dissolved in N,N-dimethylformamide (90 ml). To this solution, a solution of trifluoroacetic acid salt of 3'-N-(Gly-Gly-Gly-Phe)-NH-A (A-NH₂=DX-8951) obtained in Example 16 (158 mg) in N,N-dimethylformamide (15 ml), triethylamine (0.05 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (1.2 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring and shielding the light.

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Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and then the precipitates deposited were collected by centrifugation. The precipitates were dissolved in 0.5M aqueous sodium chloride, adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling and dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight; 6,000-8,000). The inner dialyzate solution was filtered through a Millipore filter (0.22 μ m) and then lyophilized to obtain the title compound (1.4 g). The content of the drug compound residue in this compound was 5.2% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 18: Synthesis of Boc-Gly-Phe-Leu-OH

[0061] H-Gly-Phe-Leu-OH (3.0 g) was added to 50% aqueous dioxane (48 ml) and ice-cooled. To this solution, 1N aqueous sodium hydroxide (9.45 ml) and a dioxane solution (24 ml) containing (Boc)₂O (2.27 g) were added, and the mixture was stirred overnight. 1N hydrochloric acid (9.45 ml) was added to the reaction mixture and the solvent was evaporated. The resulting residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 5:1 solution) to obtain the title compound (2.5 g).

Example 19: Synthesis of Boc-Gly-Phe-Leu-Gly-OBzl

[0062] The Boc-Gly-Phe-Leu-OH obtained in Example 18 (2.4 g) and N-hydroxysuccinimide (656 mg) were dissolved in N,N-dimethylformamide (50 ml), cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (1.17 g) and stirred for 2 hours. To this solution, a N,N-dimethylformamide solution (40 ml), in which tosylate of H-Gly-OBzl (1.9 g) and triethylamine (0.79 ml) had been dissolved, was added and the mixture was allowed to react with stirring at room temperature for 16 hours. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 50:1 solution) to give the title compound (2.0 g).

¹H-NMR (DMSO-d₆) δ : 8.20-8.30 (m, 1H), 8.12 (d, 1H, J = 8.3Hz), 7.83 (d, 1H, J = 8.3Hz), 7.32-7.37 (m, 5H), 6.89-6.95 (m, 1H), 5.12 (s, 1H), 4.52-4.59 (br, 1H), 4.34 (dd, 1H, J = 7.3Hz, J = 15.1Hz), 3.93 (dd, 1H, J = 5.5Hz, J = 17.2Hz), 3.84 (dd, 1H, J = 5.5Hz, J = 17.2Hz), 3.54 (dd, 1H, J = 5.9Hz, J = 16.7Hz), 3.42 (dd, J = 5.9Hz, J = 16.7Hz), 3.00 (dd, 1H, J = 4.4Hz, 13.7Hz), 2.78 (dd, 1H, J = 8.8Hz, J = 13.2Hz), 1.50-1.65 (m, 1H), 1.45 (t, 2H, J = 7.3Hz), 1.36 (s, 9H), 0.86 (d, 3H, J = 6.4Hz), 0.82 (d, 3H, J = 6.4Hz).

Example 20: Synthesis of Boc-Gly-Phe-Leu-Gly-OH

[0063] The Boc-Gly-Phe-Leu-OBzl (1.7 g) obtained in Example 19 was dissolved in a mixed solution of ethyl acetate (30 ml) and methanol (30 ml), and added with 5% Pd-C (1.5 g) to perform catalytic reduction. The reaction mixture was filtered and the filtrate was evaporated to dryness under reduced pressure to give the title compound (1.15 g).

Example 21: Synthesis of 3'-N-(Boc-Gly-Phe-Leu-Gly)-NH-A (A-NH₂=DX-8951)

[0064] The Boc-Gly-Phe-Leu-Gly-OH obtained in Example 20 (200 mg) and N-hydroxysuccinimide (58 mg) were dissolved in N,N-dimethylformamide (5 ml). After cooling at 4°C, N,N'-dicyclohexylcarbodiimide (104 mg) was added to the solution and dissolved. To this solution, a N,N-dimethylformamide solution (5 ml), in which methanesulfonate of DX-8951 (224 mg) and triethylamine (0.059 ml) had been dissolved, was added and the mixture was allowed to react with stirring at room temperature for 16 hours under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 10:1 solution containing 0.5% acetic acid) to give the title compound (200 mg).

¹H-NMR (DMSO-d₆) δ : 8.35 (d, 1H, J = 7.8Hz), 8.08-8.18 (m, 2H), 7.75-7.85 (m, 2H), 7.32 (s, 1H), 7.10 (d, 2H, J = 6.8Hz), 7.08-7.13 (m, 3H), 6.85-6.95 (br, 1H), 6.40-6.65 (br, 1H), 5.52-5.55 (m, 1H), 5.46 (d, 1H, J = 18.5Hz), 5.37 (d, 1H, J = 18.5Hz), 5.24 (s, 2H), 4.44-4.52 (m, 1H), 4.15-4.25 (m, 1H), 3.68-3.72 (m, 2H), 3.40-3.52 (m, 2H), 3.15-3.25 (br, 2H), 2.85-2.95 (m, 1H), 2.65-2.75 (m, 1H), 2.40 (s, 3H), 2.05-2.25 (m, 1H), 1.80-1.91 (m, 2H), 1.50-1.65 (m, 1H), 1.45 (t, 2H, J = 7.3Hz), 1.35 (s, 9H), 0.88 (t, 3H, J = 7.4), 0.86 (d, 3H, J = 6.4Hz), 0.82 (d, 3H, J = 6.4Hz).

Example 22: Synthesis of 3'-N-(Gly-Phe-Leu-Gly)-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt

[0065] The 3'-N-(Boc-Gly-Phe-Leu-Gly)-NH-A (A-NH₂=DX-8951) (97 mg) obtained in Example 21 was dissolved in trifluoroacetic acid (3 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected

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to azeotropic distillation twice with methanol (30 ml) and twice with ethanol (30 ml), and then washed with ether to obtain the title compound (95 mg).

¹H-NMR (DMSO-d₆) δ : 8.57 (d, 1H, J =8.3Hz), 8.47 (d, 1H, J =8.3Hz), 8.32 (d, 1H, J =7.8Hz), 8.17 (t, 1H, J =5.5Hz), 7.81-7.91 (br, 3H), 7.79 (d, 1H, J =10.7Hz), 7.32 (s, 1H), 7.21-7.23 (m, 5H), 7.12-7.17 (m, 1H), 6.45-6.55 (br, 1H), 5.57 (q, 1H, J =4.4Hz), 5.43 (d, 1H, J =16.1Hz), 5.34 (d, 1H, J =16.1Hz), 5.23 (s, 2H), 4.67 (dt, 1H, J =4.0 Hz, J =9.0 Hz), 4.31 (dd, 1H, J =8.5Hz, J =15.0 Hz), 4.0-4.4 (br, 1H), 3.74-3.76 (m, 2H), 3.56 (dd, 1H, J =6.0 Hz, J =16.0 Hz), 3.41 (dd, 1H, J =6.0 Hz, J =16.0 Hz), 3.17-3.19 (br, 2H), 3.02 (dd, 1H, J =4.0 Hz, J =14.0 Hz), 2.70 (dd, 1H, J =10.0 Hz, J =14.0 Hz), 2.40 (s, 3H), 2.05-2.15 (m, 1H), 1.85 (dt, 2H, J =7.0 Hz, J =14.0 Hz), 1.50-1.55 (m, 1H), 1.45 (t, 2H, J =6.0 Hz), 1.35 (s, 9H), 0.88 (t, 3H, J =7.4), 0.85 (d, 3H, J =6.4Hz), 0.80 (d, 3H, J =6.4Hz).

Example 23: Synthesis of carboxymethyldextran polyalcohol-Gly-Phe-Leu-Gly-NH-A' (A-NH₂=DX-8951)

[0066] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 13 (690 mg) was dissolved in N,N-dimethylformamide (50 ml). To this solution, a solution of the trifluoroacetic acid salt of 3'-N-(Gly-Phe-Leu-Gly)-NH-A (A-NH₂=DX-8951) (95 mg) obtained in Example 22 in N,N-dimethylformamide (10 ml), triethylamine (0.03 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (690 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation. The precipitates were dissolved in 0.5M aqueous sodium chloride, adjusted to pH 9 with 0.1M aqueous sodium hydroxide, and dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight; 6,000-8,000). The inner dialyzate solution was filtered through a Millipore filter (0.22 μm), and then the filtrate was lyophilized to obtain the title compound (600 mg). The content of the drug compound residue in this compound was 4.8% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 24: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0067] Dextran T500 (50 g, Pharmacia, molecular weight: 500K) was dissolved in 0.1M acetate buffer (pH 5.5, 5000 ml), and an aqueous solution (5000 ml) of sodium periodate (165.0 g) was added. After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (35.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide. Sodium borohydride (70 g) was added and dissolved, and then the mixture was stirred overnight. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then adjusted to pH 7.5 with 8M aqueous sodium hydroxide. The resulting solution was subjected to ultrafiltration using a Biomax-50 membrane to remove the low molecular weight fraction. The polymer fraction was lyophilized to obtain dextran polyalcohol (27.1 g). The molecular weight of this substance was 140K (gel filtration, pullulan standard).

[0068] This dextran polyalcohol (5 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (21 g) in water (150 ml), and dissolved at room temperature. To this solution, monochloroacetic acid (30 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature overnight. This reaction mixture was adjusted to pH 8 with acetic acid and then desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (5.6 g). The molecular weight of this substance was 263K (gel filtration, pullulan standard) and the degree of carboxymethylation was 0.4.

[0069] This sodium salt of carboxymethyldextran polyalcohol (2.0 g) was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, H⁺ form) column (diameter: 44 mm, length: 210 mm), and eluted with water. This effluent was added with triethylamine (4 ml) and lyophilized to obtain the title compound (2.2 g).

Example 25: Synthesis of trimethylammonium salt of carboxymethyldextran polyalcohol

[0070] The sodium salt of carboxymethyldextran polyalcohol (1.0 g) obtained in Example 24 was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, Me₃N H⁺ form) column, and eluted with water. This effluent was lyophilized to obtain the title compound (950 mg).

Example 26: Synthesis of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) hydrochloride

[0071] In a manner similar to that of Example 14, 3'-N-(Gly-Gly-Phe-Gly)-NH-A trifluoroacetic acid salt obtained from 3'-N-(Boc-Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (400mg) was dissolved in water/MeOH (1:4), applied to a Bio-

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Rad AG 1-X8 (200-400 mesh, Cl⁻ form) column (1.5 cm × 8.6 cm), and eluted with the above solvent. This effluent was concentrated and then lyophilized to obtain the title compound (310 mg).

¹H-NMR (DMSO-d₆) δ : 8.53 (d, 1H, J=8.5Hz), 8.46-8.48 (m, 1H), 8.37-8.39 (m, 1H), 7.95 (d, 1H, J=8.0Hz), 7.80 (s, 3H), 7.78 (d, 1H, J=11.1Hz), 7.34 (s, 1H), 7.14-7.24 (m, 5H), 6.50 (s, 1H), 5.56-5.60 (m, 1H), 5.35-5.40 (m, 2H), 5.24 (s, 2H), 4.51-4.56 (m, 1H), 3.86 (dd, J=4.8, 13.5Hz, 1H), 3.68-3.79 (m, 3H), 3.54 (s, 2H), 3.15-3.22 (m, 2H), 3.01 (dd, J=5.6, 13.5Hz, 1H), 2.78 (dd, J=9.6, 3.5Hz, 1H), 2.41 (s, 3H), 2.12-2.23 (m, 2H), 1.81-1.89 (m, 2H), 0.88 (t, 3H, J=7.2Hz).

Mass (FAB): m/e 753 (M+1)

Example 27: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0072] The trimethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 25 (0.1 g) was dissolved in N,N-dimethylformamide (6 ml). To this solution, a solution of the hydrochloride of 3'-N-(Gly-Gly-Phe-Gly)-NH-A' (A-NH₂=DX-8951) (24 mg) obtained in Example 26 in N,N-dimethylformamide (10 ml), triethylamine (5 μl), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (0.1 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-30 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to give the title compound (90 mg). The content of the drug compound residue in this compound was 11% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 28: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0073] The trimethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 25 (0.1 g) was dissolved in N,N-dimethylformamide (6 ml). To this solution, a solution of the 3'-N-(Gly-Gly-Phe-Gly)-NH-A' (A-NH₂=DX-8951) hydrochloride (36 mg) obtained in Example 26 in N,N-dimethylformamide (10 ml), triethylamine (8 μl), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (0.1 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the precipitate deposited were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 12 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-30 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to give the title compound (80 mg). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min), and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 36 μg/ml) are shown in Fig. 8 and Fig. 9, respectively. The content of the drug compound residue in the compound was 15% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 29: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-NH-A' (A-NH₂=DX-8951)

[0074] Dextran T250 (20 g, EXTRASYNTHESSE, average molecular weight: 250K) was dissolved in 0.1M acetic acid buffer (pH 5.5, 2000 ml) and added with an aqueous solution (2000 ml) of sodium periodate (66.0 g). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (14.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. Sodium borohydride (28 g) was added and dissolved, and then the mixture was stirred overnight at room temperature. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then, adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. The low molecular weight fraction was removed from the resulting aqueous solution by ultrafiltration using a Biomax-30 membrane to obtain Retained solution 1 that did not pass through the membrane. Separately, Dextran T250 (50 g, EXTRASYNTHESSE, average molecular weight: 250K) was dissolved in 0.1M acetate buffer (pH 5.5, 5000 ml) and added with an aqueous solution (5000 ml) of sodium periodate (165 g). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (35.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. Sodium borohydride (70 g) was added and dissolved, and then the mixture was stirred overnight at room temperature. The reaction

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mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then, adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. The low molecular weight fraction was removed from the resulting aqueous solution by ultrafiltration using a Biomax-30 membrane to obtain Retained solution 2 that did not pass through the membrane. Retained solutions 1 and 2 were combined, subjected to ultrafiltration using a Biomax-30 membrane to remove the low molecular weight fraction from the fraction, that had passed through Biomax-50 membrane, and lyophilized to obtain dextran polyalcohol (25.7 g). The molecular weight of this substance was 47K (gel filtration, pullulan standard).

[0075] This dextran polyalcohol (5 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (35 g) in water (150 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (50 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 18 hours. This reaction mixture was adjusted to pH 8 with acetic acid and desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (7.2 g). The molecular weight of this substance was 127K (gel filtration, pullulan standard) and the degree of carboxymethylation was 0.8. This sodium salt of carboxymethyldextran polyalcohol (2.2 g) was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, H⁺ form) column (diameter: 44 mm, length: 210 mm) and eluted with water. This effluent was added with triethylamine (4 ml) and then lyophilized to give triethylammonium salt of carboxymethyldextran polyalcohol (2.69 g).

[0076] This triethylammonium salt of carboxymethyldextran polyalcohol (2.67 g) was dissolved in N,N-dimethylformamide (200 ml). To this solution, a solution obtained by dissolving the trifluoroacetic acid salt of 3'-N-(Gly-Gly-Gly-Phe)-NH-A, which had been obtained by removing the Boc group according to the method similar to that of Example 16 from 3'-N-(Boc-Gly-Gly-Gly-Phe)-NH-A (A-NH₂=DX-8951) (350 mg) prepared in a similar manner to that of Example 2, and triethylamine (0.116 ml) in N,N-dimethylformamide (10 ml), and a solution obtained by dissolving 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (2.67 g) in N,N-dimethylformamide (10 ml) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. This reaction mixture was added with 3M aqueous sodium chloride (100 ml) and each of 8 ml portions of the mixture was added dropwise to each 30 ml of ethanol. To each mixture, 3M aqueous sodium chloride (1 ml) and diethyl ether (5 ml) were added, and the precipitates deposited were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were washed with acetone and then dissolved in water, and added with 3M aqueous sodium chloride (10 ml), and then adjusted to pH 9 with 0.1M aqueous sodium hydroxide, and further treated at 37°C for 1 hour. The treated solution was desalted by ultrafiltration using a Biomax-10 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 µm) and then lyophilized to give the title compound (2.30 g). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 0.20 mg/ml) are shown in Fig. 10 and Fig. 11, respectively. The content of the drug compound residue in the compound was 5.8% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 30: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0077] To a solution (2000 ml) of Dextran T10 (20 g, Pharmacia, average molecular weight: 10K) in 0.1M acetic acid buffer (pH 5.5) was added an aqueous solution (2000 ml) of sodium periodate (66.0 g). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (14.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. Sodium borohydride (28 g) was added and dissolved, and the mixture was stirred overnight at room temperature. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then, adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was subjected to ultrafiltration using a Biomax-5 membrane (Millipore) to remove the low molecular weight fraction, and the remaining solution that had not passed the membrane was passed through a Biomax-30 membrane. The resulting filtrate was lyophilized to obtain dextran polyalcohol (8.0 g). The molecular weight of this substance was 13K (gel filtration, pullulan standard).

[0078] This dextran polyalcohol (3.7 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (25.9 g) in water (111 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (37 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 20 hours. This reaction mixture was adjusted to pH 8 with acetic acid and desalted by ultrafiltration using a Biomax-5 membrane. The remaining solution that had not passed through the membrane was lyophilized to give sodium salt of carboxymethyldextran polyalcohol (6.2 g). The molecular weight of this substance was 37K (gel filtration, pullulan standard) and the degree of carboxymethylation was 0.9.

[0079] This sodium salt of carboxymethyldextran polyalcohol (6.0 g) was dissolved in water, applied to a Bio-Rad AG50W-X2 (200-400 mesh, H⁺ form) column, and then eluted with water. This effluent was added with triethylamine (9.3 ml) and then lyophilized to obtain the title compound (7.2 g).

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Example 31: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0080] The dextran polyalcohol (3.9 g) obtained in Example 30 was added to an aqueous solution obtained by dissolving sodium hydroxide (16.3 g) in water (117 ml), and dissolved at room temperature. To this solution, monochloroacetic acid (23.4 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 18 hours. This reaction mixture was adjusted to pH 8 with acetic acid and desalted by ultrafiltration using a Biomax-5 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (5.0 g). The molecular weight of this substance was 28K (gel filtration, pullulan standard) and the degree of carboxymethylation was 0.5. This sodium salt of carboxymethyldextran polyalcohol (4.8 mg) was converted into the triethylammonium salt in a similar manner to that of Example 30 to obtain the title compound (5.6 g).

Example 32: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0081] An aqueous solution (2000 ml) of sodium periodate (66.0 g) was added to a solution (2000 ml) of Dextran 4 (20 g, Funakoshi, average molecular weight: 4K-6K) in 0.1M acetic acid buffer (pH 5.5). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (14.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. Sodium borohydride (28 g) was added and dissolved, and the mixture was stirred overnight at room temperature. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then, adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was subjected to ultrafiltration using a Biomax-3 membrane (Millipore) to remove the low molecular weight fraction. The filtrate obtained was lyophilized to give dextran polyalcohol (6.0 g). The molecular weight of this substance was 9K (gel filtration, pullulan standard). This dextran polyalcohol (2.7 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (18.9 g) in water (81 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (27 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 20 hours. This reaction mixture was adjusted to pH 8 with acetic acid and desalted by ultrafiltration using a Biomax-5 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (4.2 g). The molecular weight of this substance was 20K (gel filtration, pullulan standard), and the degree of carboxymethylation was 0.9.

[0082] This sodium salt of carboxymethyldextran polyalcohol (4.0 g) was converted into the triethylammonium salt in a similar manner to that of Example 30 to obtain the title compound (4.8 g).

Example 33: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0083] The dextran polyalcohol (2.7 g) obtained in Example 32 was added to an aqueous solution obtained by dissolving sodium hydroxide (11.3 g) in water (81 ml), and dissolved at room temperature. To this solution, monochloroacetic acid (16.2 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 18 hours. This reaction mixture was adjusted to pH 8 with acetic acid and desalted by ultrafiltration using a Biomax-5 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (2.7 g). The molecular weight of this substance was 16K (gel filtration, pullulan standard) and the degree of carboxymethylation was 0.5. This sodium salt of carboxymethyldextran polyalcohol (2.7 g) was converted into the triethylammonium salt in a similar manner to that of Example 30 to obtain the title compound (3.1 g).

Example 34: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0084] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 30 (1.5 g) was dissolved in N,N-dimethylformamide (90 ml). To this solution, a solution of triethylamine (0.07 ml) and trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A' (A-NH₂=DX-8951) (210 mg) in N,N-dimethylformamide (40 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (1.5 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. Each was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and then the precipitates deposited were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-3 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (1.3 g). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel

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PW-4000XL, Tosoh, solvent: 0.1M NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 65 µg/ml) are shown in Fig. 12 and Fig. 13, respectively. The content of the drug compound residue in the compound was 6.4% (W/W) when determined based on the absorption at 362 nm in 0.1M Tris buffer solution (pH 9.0).

Example 35: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0085] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 31 (1.2 g) was dissolved in N,N-dimethylformamide (90 ml). To this solution, a solution of triethylamine (0.056 ml) and trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (168 mg) in N,N-dimethylformamide (30 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (1.2 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. Each was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-3 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 µm) and then lyophilized to obtain the title compound (1.0 g). The content of the drug compound residue in this compound was 4.8% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 36: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0086] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 32 (1.2 g) was dissolved in N,N-dimethylformamide (90 ml). To this solution, a solution of triethylamine (0.056 ml) and trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (168 mg) in N,N-dimethylformamide (30 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (1.2 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. Each was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-3 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 µm) and then lyophilized to obtain the title compound (1.0 g). The content of the drug compound residue in this compound was 5.9% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

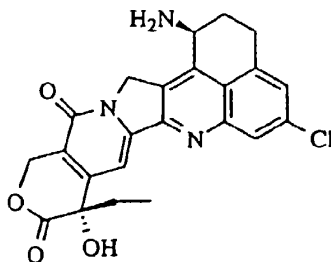
Example 37: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0087] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 33 (1.5 g) was dissolved in N,N-dimethylformamide (90 ml). To this solution, a solution of triethylamine (0.07 ml) and trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (210 mg) in N,N-dimethylformamide (40 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (1.5 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. Each was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-3 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 µm) and then lyophilized to obtain the title compound (1.3 g). The content of the drug compound residue in this compound was 4.6% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 38: Synthesis of Boc-Gly-Gly-Phe-Gly-NH-A (A-NH₂=DW-8286)

[0088] Boc-Gly-Gly-Phe-Gly (42 mg) and N-hydroxysuccinimide (12 mg) were dissolved in N,N-dimethylformamide (2 ml), and cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (22 mg). To this solution, a N,N-dimethylformamide solution (6 ml), in which hydrochloride of the compound represented by the following formula:

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[(1s,9s)-1-amino-5-chloro-9-ethyl-2,3-dihydro-9-hydroxy-1H,12H-benzo[de]pyrano-[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione: DW-8286] (50 mg) and triethylamine (0.01 ml) were dissolved was added and the mixture was allowed to react with stirring and shielding the light at room temperature for 16 hours. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 10:1 solution containing 0.5% acetic acid) to obtain the title compound (27 mg).

¹H-NMR (CDCl₃) δ : 8.10-8.20 (br, 1H), 7.95-8.05 (br, 1H), 7.70-7.80 (br, 2H), 7.50-7.60 (br, 1H), 7.40-7.50 (br, 1H), 7.10-7.25 (m, 5H), 7.05-7.15 (br, 1H), 5.85-5.95 (br, 1H), 5.50-5.60 (br, 1H), 5.40-5.50 (m, 1H), 5.25-5.35 (m, 1H), 5.05-5.15 (m, 1H), 4.90-5.00 (m, 1H), 4.70-4.80 (br, 1H), 4.10-4.25 (br, 2H), 3.60-3.90 (m, 4H), 3.10-3.40 (m, 3H), 2.95-3.05 (br, 1H), 2.15-2.30 (br, 1H), 1.75-1.90 (br, 2H), 1.39 (s, 9H), 0.80-1.00 (m, 3H).

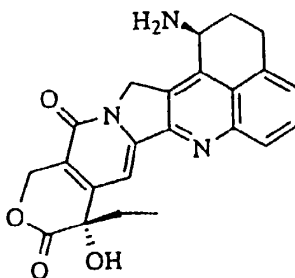
Example 39: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DW-8286)

[0089] The triethylammonium salt of carboxymethyldextran polyalcohol (175 mg) obtained in Example 24 was dissolved in N,N-dimethylformamide (20 ml). To this solution, a solution of trifluoroacetic acid salt of 3'-N-(Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DW-8286) (29 mg), which had been obtained from 3'-N-(Boc-Gly-Gly-Phe-Gly)-NH-A (27 mg) prepared in Example 38 by removing the Boc group in a similar manner to that of Example 4, and triethylamine (9 μl) in N,N-dimethylformamide (5 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (175 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-30 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (135 mg). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 99 μg/ml) are shown in Fig. 14 and Fig. 15, respectively. The content of the drug compound residue in the compound was 6.1% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 40: Synthesis of 3'-N-(Boc-Gly-Gly-Phe-Gly)-NH-A (A-NH₂=DW-8089)

[0090] Boc-Gly-Gly-Phe-Gly (163 mg) and N-hydroxysuccinimide (45 mg) were dissolved in N,N-dimethylformamide (10 ml), cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (79 mg). To this solution, a N,N-dimethylformamide solution (30 ml), in which tosylate of the compound represented by the following formula:

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15 [(1*s*,9*s*)-1-amino-9-ethyl-2,3-dihydro-9-hydroxy-1*H*,12*H*-benzo[*de*]pyrano[3',4':6,7]-indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione: DW-8089] (170 mg) and triethylamine (0.054 ml) were dissolved, was added and the mixture was allowed to react with stirring at room temperature overnight under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 94:6 solution containing 0.5% acetic acid) to obtain the title compound (100 mg).

20 ¹H-NMR (DMSO-*d*₆) δ : 8.51 (d, 1*H*, *J*=8.5Hz), 8.41 (t, 1*H*, *J*=5.6Hz), 8.29 (s, 1*H*), 8.17 (d, 1*H*, *J*=8.0Hz), 8.03 (d, 1*H*, *J*=8.0Hz), 7.90 (dd, 1*H*, *J*=4.8, 5.6Hz), 7.79 (t, 1*H*, *J*=5.6Hz), 7.53 (d, 1*H*, *J*=7.2Hz), 7.36 (s, 1*H*), 7.13-7.25 (m, 5*H*), 6.94-6.95 (m, 1*H*), 5.60-5.63 (m, 1*H*), 5.36-5.47 (m, 2*H*), 5.21-5.30 (m, 2*H*), 4.42-4.47 (m, 1*H*), 3.63-3.96 (m, 3*H*), 3.51-3.59 (m, 3*H*), 3.31-3.40 (m, 1*H*), 3.09-3.21 (m, 1*H*), 3.02 (dd, 1*H*, *J*=4.8, 13.5Hz), 2.76-2.81 (m, 1*H*),
25 2.13-2.17 (m, 2*H*), 1.85-1.90 (m, 2*H*), 1.37 (s, 9*H*), 0.89 (t, 3*H*, *J*=8.0Hz).
Mass (FAB); *m/e* 822 (*M*+1)

Example 41: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-NH-A' (A-NH₂=DW-8089)

30 [0091] The triethylammonium salt of carboxymethyldextran polyalcohol (1.6 g) obtained in Example 24 was dissolved in *N,N*-dimethylformamide (60 ml). To this solution, a solution obtained by dissolving trifluoroacetic acid salt of 3'-*N*-(Gly-Gly-Phe-Gly)-NH-A' (A-NH₂=DW-8089), which had been obtained from 3'-*N*-(Boc-Gly-Gly-Phe-Gly)-NH-A' (200 mg) prepared in Example 40 by removing the Boc group in a similar manner to that of Example 4, and triethylamine (0.07 ml) in *N,N*-dimethylformamide (20 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (1.6 g) were added
35 successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. Each was added with 3*M* aqueous sodium chloride (2.5 ml) and diethyl ether (25 ml), and the deposited precipitates were collected by centrifugation (2500 rpm, 8 minutes). The precipitates were washed with ethanol, then dissolved in water, added with 3*M* aqueous sodium chloride (20 ml), and adjusted to pH 9 with 0.1*M* aqueous sodium hydroxide. This solution was desalted by ultrafiltration using a Biomax-
40 10 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (1.20 g). The result obtained by GPC analysis after dissolving the compound in 0.1*M* aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 *M* NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1*M* Tris buffer solution, pH 9.0, 0.26 mg/ml) are shown in Fig. 16 and Fig. 17, respectively. The content of the drug compound residue in the compound was
45 5.0% (W/W) when determined based on the absorption at 362 nm in 0.1*M* Tris buffer solution (pH 9.0).

Example 42: Synthesis of Trt-Gly-Gly-Phe-Gly-OH

50 [0092] Trt-Gly-Gly-Phe-Gly-OBzl (670 mg), 10% Pd-C (100 mg), and ammonium formate (200 mg) were added to DMF (5 ml) and stirred for three hours. The reaction mixture was filtered, the filtrate was evaporated to dryness under reduced pressure, and then the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 8: 1 solution) to obtain the title compound (300 mg).

55 ¹H-NMR (CD₃OD) δ : 7.16-7.45 (m, 20*H*), 4.66 (dd, 1*H*, *J* =9.8,5.4Hz), 3.93 (d, 1*H*, *J* =16.6Hz), 3.80 (d, 1*H*, *J* =17.6Hz), 3.78 (d, 1*H*, *J* =16.6Hz), 3.68 (d, 1*H*, *J* =17.1Hz), 3.23 (dd, 1*H*, *J* =14.2,5.4Hz), 2.90 (d, 1*H*, *J* =13.7Hz), 2.90 (s, 1*H*).

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Example 43: Synthesis of 3'-N-(Gly-Gly-Phe-Gly)-DXR hydrochloride

[0093] Trt-Gly-Gly-Phe-Gly-OH (100 mg) and N-hydroxysuccinimide (22 mg) were dissolved in DMF (4 ml), and the mixture was added with N,N'-dicyclohexylcarbodiimide (40 mg) under ice-cooling and stirred at 4°C for 2 hours. To this solution, a solution of N-methylmorpholine (0.019 ml) and doxorubicin (DXR) hydrochloride (92 mg) dissolved in DMF (20 ml) was added, and the mixture was stirred at 4°C for 16 hours. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 20:1 solution). The resulting compound was dissolved in 75% acetic acid (1 ml) and stirred for 1 hour. Water (20 ml) was added, and the precipitated solid mass was removed by filtration, and then the filtrate was lyophilized and the resulting powder was dissolved in water (5 ml). This solution was passed through a AG-1X8 (Cl⁻ form) column and eluted with water, and then the effluent was washed with dichloromethane, and the aqueous layer was lyophilized to obtain the title compound (40 mg).

¹H-NMR (CD₃OD) δ : 7.95 (d, 1H, J = 7.3Hz), 7.82 (t, 1H, J = 7.8Hz), 7.54 (d, 1H, J = 8.3Hz), 7.16-7.26 (m, 5H), 5.43 (d, 1H, J = 3.4Hz), 5.14 (br, 1H), 4.72 (s, 2H), 4.42 (dd, 1H, J = 8.3, 6.8Hz), 4.30 (q, 1H, J = 6.8Hz), 4.14-4.18 (m, 1H), 4.03 (d, 1H, J = 16.6Hz), 4.02 (s, 3H), 3.86 (d, 1H, J = 18.5Hz), 3.83 (d, 1H, J = 17.1Hz), 3.75 (d, 1H, J = 16.1Hz), 3.73 (d, 1H, J = 16.1Hz), 3.62 (br, 1H), 3.58 (d, 1H, J = 16.6Hz), 3.10-3.15 (m, 2H) 3.00 (d, 1H, J = 18.6Hz), 2.94 (dd, 1H, J = 14.2, 8.8Hz), 2.38 (d, 1H, J = 14.2Hz), 2.18 (dd, 1H, J = 14.2, 4.4Hz), 1.94-2.00 (m, 1H), 1.71 (dd, 1H, J = 12.7, 4.4Hz), 1.28 (d, 3H, J = 6.3Hz).

Example 44: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-DXR

[0094] The sodium salt of carboxymethyldextran polyalcohol (1.5 g) obtained in Example 24 was converted into the trimethylammonium salt (1.2 g) in a manner similar to that of Example 25, and then 400 mg of this salt was dissolved in N,N-dimethylformamide (24 ml). To this solution, a solution of 3'-N-(Gly-Gly-Phe-Gly)-DXR hydrochloride (76 mg) in N,N-dimethylformamide (24 ml), triethylamine (24 μl) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (400 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.5 ml) and diethyl ether (20 ml), and the deposited precipitates were collected by centrifugation (3,500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and desalted by ultrafiltration using a Biomax-30 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (40 mg). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 7.4, 36 μl) are shown in Fig. 18 and Fig. 19, respectively. The content of the drug compound residue in the compound was 6.0% (W/W) when determined based on the absorption at 480 nm in PBS (pH 7.4).

Example 45: Synthesis of triethylammonium salt of carboxymethyldextran polyalcohol

[0095] Dextran T150 (20 g, Pharmacia, average molecular weight: 150K) was dissolved in 0.1M acetate buffer (pH 5.5, 2000 ml) and added with an aqueous solution (2000 ml) of sodium periodate (66.0 g). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (14.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7.5 with 8M aqueous sodium hydroxide under ice-cooling. Sodium borohydride (28 g) was added and dissolved, and then the mixture was stirred at room temperature overnight. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid, and stirred at 4°C for 1 hour. The pH of the mixture was adjusted to 7.5 with 8M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was concentrated to 500 ml by ultrafiltration using a Biomax-5 membrane (Millipore) to obtain Solution 1. Separately, a series of procedures described above was performed using Dextran T110 (20 g) to obtain Solution 2. Solution 1 and Solution 2 were combined, and the combined solution was adjusted to pH 3.0 and incubated at 40°C for 4 hours, and then adjusted to pH 7 to obtain a solution containing the dextran polyalcohol with lowered molecular weight. The solution was passed through a Biomax-30 membrane and desalted by ultrafiltration using a Biomax-5 membrane, and then lyophilized to obtain dextran polyalcohol (4.6 g). The molecular weight of this substance was 17K (gel filtration, pullulan standard).

[0096] This dextran polyalcohol (2.5 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (17.5 g) in water (75 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (25 g) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 20 hours. This reaction mixture was adjusted to pH 9 with acetic acid and then desalted by ultrafiltration using a Biomax-5 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyldextran polyalcohol (4.0 g). The molecular weight of this substance was 45K (gel filtration, pullulan standard), and the

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degree of carboxymethylation was 0.9.

[0097] This sodium salt of carboxymethyl dextran polyalcohol (3.7 g) was dissolved in water, applied to a Bio-Rad AG50W-X2 (200-400 mesh, H⁺ form) column, and eluted with water. This effluent was added with triethylamine (5.8 ml) and then lyophilized to obtain the title compound (4.4 g).

Example 46: Synthesis of carboxymethyl dextran polyalcohol-Gly-Gly-Gly-Phe-NH-A' (A-NH₂=DX-8951)

[0098] The triethylammonium salt of carboxymethyl dextran polyalcohol obtained in Example 45 (4.4 g) was dissolved in N,N-dimethylformamide (300 ml). To this solution, a solution of triethylamine (0.19 ml) and trifluoroacetic acid salt of 3'-N-(Gly-Gly-Gly-Phe)-NH-A' (A-NH₂=DX-8951) (580 mg) in N,N-dimethylformamide (45 ml) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (4.4 g) were added successively, and the mixture was allowed to react at room temperature overnight with stirring and shielding the light. This reaction mixture was adjusted to pH 10 with 1M aqueous sodium hydroxide, and then each of 5 ml portions of the mixture was added dropwise to each 25 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (1 ml) and diethyl ether (5 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in water and dialyzed against purified water using a dialysis membrane (Spectrapore 1, cut-off molecular weight; 6,000-8,000), and the inner dialyzate solution was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (3.4 g). The content of the residue of the drug compound in this compound was 4.6% (W/W) when determined based on the absorption at 362 nm in 0.1M Tris buffer solution (pH 9.0).

Example 47: Synthesis of α-methylcarboxymethyl dextran polyalcohol-Gly-Gly-Gly-Phe-NH-A' (A-NH₂=DX-8951)

[0099] The dextran polyalcohol (2 g) obtained in Example 45 was added to an aqueous solution obtained by dissolving sodium hydroxide (14 g) in water (60 ml) and dissolved at room temperature. To this solution, α-bromopropionic acid (19 ml) was added under ice-cooling and dissolved, and then the mixture was allowed to react at room temperature for 18 hours. The reaction mixture was adjusted to pH 8 with acetic acid and desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of α-methylcarboxymethyl dextran polyalcohol (2.95 g). The molecular weight of this substance was 45K (gel filtration, pululan standard). The degree of α-methylcarboxymethylation per saccharide residue was obtained according to the cases of carboxymethyl dextran polyalcohol as follows. An aqueous solution of the sodium salt of α-methylcarboxymethyl dextran polyalcohol was applied to a Bio-Rad AG 50W-X2 (H⁺ form) column, and the effluent was lyophilized and used as a sample. This sample was dissolved in a prescribed excess amount of 0.1N aqueous solution of sodium hydroxide and titrated with 0.1N hydrochloric acid using phenolphthalein as an indicator. The degree of α-methylcarboxymethylation was obtained according to the equation: the degree of α-methylcarboxymethylation = 13.4(a-b)/[s-7.2(a-b)] wherein "s" is the amount of sample used (mg), "a" is the prescribed excess amount of 0.1N aqueous solution of sodium hydroxide (ml), and "b" is the amount of 0.1N hydrochloric acid consumed for the titration (ml). As a result, the degree of α-methylcarboxymethylation was found to be 0.8.

[0100] This sodium salt of α-methylcarboxymethyl dextran polyalcohol (2.2 g) was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, H⁺ form) column (diameter: 44 mm, length: 210 mm), and then eluted with water. This effluent was added with triethylamine (4 ml) and then lyophilized to give triethylammonium salt of α-methylcarboxymethyl dextran polyalcohol (2.69 g).

[0101] This triethylammonium salt of α-methylcarboxymethyl dextran polyalcohol (2.68 g) was dissolved in N,N-dimethylformamide (60 ml). To this solution, a solution obtained by dissolving trifluoroacetic acid salt of 3'-N-(Gly-Gly-Gly-Phe)-NH-A' (A-NH₂=DX-8951), which had been obtained in a similar manner to that of Example 16 by removing the Boc group from 3'-N-(Boc-Gly-Gly-Gly-Phe)-NH-A' (350 mg) synthesized similarly to that of Example 2, and triethylamine (0.116 ml) in N,N-dimethylformamide (10 ml), and a solution obtained by dissolving 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (2.68 g) in N,N-dimethylformamide (10 ml) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. This reaction mixture was added with 3M aqueous sodium chloride (40 ml), and each of 6 ml portions of the mixture was added dropwise to each 30 ml of ethanol. Each was added with 3M aqueous sodium chloride (1 ml) and diethyl ether (5 ml), and the precipitate deposited was collected by centrifugation (3500 rpm, 8 minutes). This precipitate was washed with acetone, then dissolved in water, added with 3M aqueous sodium chloride (10 ml), adjusted to pH 9 with 0.1M aqueous sodium hydroxide, and treated at 37°C for 1 hour. This treated solution was desalted by ultrafiltration using a Biomax-10 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (2.15 g). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (0.1M Tris buffer solution, pH 9.0, 0.21 mg/ml) are shown in Fig. 20 and Fig. 21, respectively. The content of the drug compound residue in the resulting product was 5.9% (W/W) when determined based on the

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absorption at 362 nm in 0.1M Tris buffer solution (pH 9.0).

Example 48: Synthesis of 3'-N-(Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt

5 **[0102]** A mixture of p-toluenesulfonic acid salt of Phe-Gly-OBzl (3.06 g), Boc-Gly-OH (1.10 g), N-hydroxysuccinimide (941 mg), N-methylmorpholine (0.725 ml), and N,N-dimethylformamide (40 ml) was cooled to 4°C, and added with N,N'-dicyclohexylcarbodiimide (1.56 g). The mixture was allowed to react overnight at room temperature with stirring, and then evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 98:2 solution) to give Boc-Gly-Phe-Gly-OBzl (1.93 g).

10 ¹H-NMR (DMSO-d₆) δ : 8.52 (dd, 1H, J=5.6, 6.4Hz), 7.97 (d, 1H, J=8.8Hz), 7.30-7.39 (m, 5H), 7.15-7.26 (m, 5H), 6.83 (t, 1H, J=5.6Hz), 5.14 (s, 1H), 4.52-4.57 (m, 1H), 3.87-3.96 (m, 2H), 3.57 (dd, 1H, J=5.6, 16.7Hz), 3.43 (dd, 1H, J=5.6, 16.7Hz), 3.01 (dd, 1H, J=4.8, 14.3Hz), 2.77 (dd, 1H, J=5.6, 14.3Hz), 1.37 (s, 9H).

15 **[0103]** The resulting Boc-Gly-Phe-Gly-OBzl (1.78 g) was dissolved in ethyl acetate (60 ml) and subjected to catalytic reduction for 24 hours in the presence of 5%-Pd-C (1.8 g). The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to obtain Boc-Gly-Phe-Gly-OH (1.41 g).

20 ¹H-NMR (DMSO-d₆) δ : 8.35 (t, 1H, J=5.6Hz), 7.94 (d, 1H, J=8.8Hz), 7.15-7.26 (m, 5H), 6.85 (dd, 1H, J=5.6, 6.4Hz), 4.52-4.58 (m, 1H), 3.76 (d, 2H, J=5.6Hz), 3.56 (dd, 1H, J=6.4, 16.7Hz), 3.43 (dd, 1H, J=5.6, 16.7Hz), 3.03 (dd, 1H, J=5.0, 13.5Hz), 2.79 (dd, 1H, J=9.5, 13.5Hz), 1.37 (s, 9H).

25 **[0104]** The Boc-Gly-Phe-Gly-OH (500 mg) obtained above and N-hydroxysuccinimide (161 mg) were dissolved in N,N-dimethylformamide (10 ml). To this solution, a N,N-dimethylformamide (50 ml) solution, in which methanesulfonate of DX-8951 (530 mg) and triethylamine (0.146 ml) were dissolved, was added. The mixture was cooled to 4 °C, added with N,N'-dicyclohexylcarbodiimide (268 mg), and allowed to react overnight with stirring at room temperature under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol =96:4 solution) to obtain 3'-N-(Boc-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (100 mg).

30 ¹H-NMR (DMSO-d₆) δ : 8.39 (d, 1H, J=8.0Hz), 8.34 (t, 1H, J=5.6Hz), 7.98 (d, 1H, J=7.2Hz), 7.78 (d, 1H, J=10.3Hz), 7.33 (s, 1H), 7.13-7.24 (m, 5H), 6.80 (dd, 1H, J=5.6, 6.4Hz), 5.55-5.61 (m, 1H), 5.44 (d, 1H, J=16.0Hz), 5.41 (d, 1H, J=16.0Hz), 5.25 (s, 2H), 4.43-4.46 (m, 1H), 3.69-3.79 (m, 2H), 3.50 (dd, 1H, J=5.6, 16.7Hz), 3.41 (dd, 1H, J=5.6, 16.7Hz), 3.16-3.19 (m, 2H), 2.98 (dd, 1H, J=4.8, 14.3Hz), 2.79 (dd, 1H, J=9.5, 14.3Hz), 2.41 (s, 3H), 2.19-2.25 (m, 1H), 2.10-2.15 (m, 1H), 1.82-1.90 (m, 2H), 1.35 (s, 9H), 0.88 (t, 3H, J=8.0Hz).
Mass (FAB); m/e 797 (M+1)

35 **[0105]** The resulting 3'-N-(Boc-Gly-Phe-Gly)-NH-A (A-NH₂=DX-8951) (100 mg) was dissolved in trifluoroacetic acid (3 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected to azeotropic distillations twice with methanol (30 ml) and twice with ethanol (30 ml), and then washed with ether to obtain the title compound (80 mg).

40 ¹H-NMR (DMSO-d₆) δ : 8.52-8.62 (m, 1H), 7.94 (s, 3H), 7.79 (t, 1H, J=11.1Hz), 7.34 (s, 1H), 7.15-7.27 (m, 5H), 6.52 (s, 1H), 5.57-5.61 (m, 1H), 5.36-5.46 (m, 2H), 5.24 (s, 2H), 4.66-4.70 (m, 1H), 3.69-3.81 (m, 2H), 3.61-3.68 (m, 1H), 3.40-3.47 (m, 1H), 3.15-3.23 (m, 1H), 3.01 (dd, 1H, J=4.0, 13.5Hz), 2.77 (dd, 1H, J=9.5, 13.5Hz), 2.12-2.23 (m, 2H), 1.81-1.91 (m, 2H), 0.89 (t, 3H, J=7.2Hz).
Mass (FAB); m/e 697 (M+1)

Example 49: Synthesis of 3'-N-(Phe-Gly)-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt

50 **[0106]** Boc-Phe-Gly (771 mg) and N-hydroxysuccinimide (300 mg) were dissolved in N,N-dimethylformamide (10 ml). To this solution, a N,N-dimethylformamide (50 ml) solution, in which methanesulfonate of DX-8951 (1058 mg) and triethylamine (0.293 ml) were dissolved, was added. The mixture was cooled to 4°C, and then added with N,N'-dicyclohexylcarbodiimide (494 mg) and allowed to react with stirring at room temperature overnight under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol =98:2 solution) to obtain 3'-N-(Boc-Phe-Gly)-NH-A (A-NH₂=DX-8951) (1.20 g).

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¹H-NMR (DMSO-d₆) δ : 8.29 (d, 1H, J=8.0Hz), 8.21 (t, 1H, J=4.8Hz), 7.76 (d, 1H, J=10.3Hz), 7.32 (s, 1H), 7.13-7.25 (m, 5H), 6.92 (d, 1H, J=7.2Hz), 6.49 (s, 1H), 5.56-5.61 (m, 1H), 5.44 (d, 1H, J=15.9Hz), 5.38 (d, 1H, J=15.9Hz), 5.25 (s, 2H), 4.08-4.12 (m, 1H), 3.78 (d, 1H, J=4.8Hz), 3.16-3.25 (m, 2H), 2.99 (dd, 1H, J=4.0, 13.5Hz), 2.72 (dd, 1H, J=10.3, 13.5Hz), 2.40 (s, 3H), 2.09-2.35 (m, 2H), 1.80-1.91 (m, 2H), 1.16 (s, 9H), 0.88 (t, 3H, J=8.0Hz).

Mass (FAB); m/e 741 (M+1)

[0107] The 3'-N-(Boc-Phe-Gly)-NH-A (170 mg) obtained above was dissolved in trifluoroacetic acid (4 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected to azeotropic distillations twice with methanol (10 ml) and twice with ethanol (10 ml), and then washed with ether to obtain the title compound (100 mg).

¹H-NMR (DMSO-d₆) δ : 8.88 (t, 1H, J=4.8Hz), 8.68 (d, 1H, J=8.7Hz), 8.05-8.15 (m, 3H), 7.79 (d, 1H, J=11.1Hz), 7.26-7.36 (m, 5H), 6.52 (d, 1H, J=7.2Hz), 5.57-5.62 (m, 1H), 5.43 (d, 1H, J=15.9Hz), 5.38 (d, 1H, J=15.9Hz), 5.19-5.28 (m, 1H), 4.10-4.18 (m, 1H), 3.93 (dd, 1H, J=4.8, 16.7Hz), 3.82 (dd, 1H, J=4.8, 16.7Hz), 3.17-3.24 (m, 2H), 3.14 (dd, 1H, J=4.8, 13.5Hz), 2.95 (dd, 1H, J=8.0, 13.5Hz), 2.42 (s, 3H), 2.14-2.25 (m, 2H), 1.83-1.91 (m, 2H), 0.89 (t, 3H, J=8.0Hz).

Mass (FAB); m/e 640 (M+1)

Example 50: Synthesis of 3'-N-Gly-NH-A (A-NH₂=DX-8951) trifluoroacetic acid salt

[0108] Methanesulfonate of DX-8951 (530 mg) and triethylamine (0.28 ml) were dissolved in N,N-dimethylformamide (10 ml), cooled to 4°C, and added with N-hydroxysuccinimide ester of Boc-Gly (327 mg). The mixture was allowed to react with stirring at room temperature overnight under light-shielded conditions. This reaction mixture was evaporated to dryness under reduced pressure, and then the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol =98:2 solution) to obtain 3'-N-(Boc-Gly)-NH-A (A-NH₂=DX-8951) (500 mg).

¹H-NMR (DMSO-d₆) δ : 8.38 (d, 1H, J=8.3Hz), 7.77 (d, 1H, J=10.7Hz), 7.31 (s, 1H), 6.89-6.91 (m, 1H), 6.49 (s, 1H), 5.55-5.59 (m, 1H), 5.45 (d, 1H, J=16.1Hz), 5.38 (d, 1H, J=16.1Hz), 5.27 (d, 1H, J=19.0Hz), 5.18 (d, 1H, J=19.0Hz), 3.50-3.62 (m, 2H), 3.15-3.19 (m, 2H), 2.41 (s, 3H), 2.18-2.24 (m, 1H), 2.08-2.12 (m, 1H), 1.81-1.91 (m, 2H), 1.31 (s, 9H), 0.87 (t, 3H, J=8.0Hz).

Mass (FAB); m/e 593 (M+1)

[0109] The 3'-N-(Boc-Gly)-NH-A (100 mg) obtained above was dissolved in trifluoroacetic acid (2 ml) and allowed to stand for one hour. The solvent was evaporated, and the residue was subjected to azeotropic distillations twice with methanol (10 ml) and twice with ethanol (10 ml), and then washed with ether to obtain the title compound (70 mg).

¹H-NMR (DMSO-d₆) δ : 8.88 (d, 1H, J=8.8Hz), 8.08 (s, 3H), 7.81 (d, 1H, J=11.2Hz), 7.34 (s, 1H), 6.52 (s, 1H), 5.63-5.67 (m, 1H), 5.45 (d, 1H, J=16.7Hz), 5.40 (d, 1H, J=16.7Hz), 5.36 (d, 1H, J=19.1Hz), 5.25 (d, 1H, J=19.1Hz), 3.56 (s, 2H), 3.11-3.19 (m, 2H), 2.43 (s, 3H), 2.23-2.28 (m, 1H), 2.11-2.19 (m, 1H), 1.81-1.91 (m, 2H), 0.88 (t, 3H, J=8.0Hz).

Mass (FAB); m/e 493 (M+1)

Example 51: Synthesis of trimethylammonium salt of carboxymethyldextran polyalcohol

[0110] Dextran T500 (50 g, Pharmacia, molecular weight: 500K) was dissolved in 0.1M acetate buffer (pH 5.5, 5000 ml) and added with an aqueous solution (5000 ml) of sodium periodate (165.0 g). After stirring at 4°C for ten days with shielding the light, the mixture was added with ethylene glycol (35.0 ml) and stirred overnight. The reaction mixture was adjusted to pH 7 with 8M aqueous sodium hydroxide. Sodium borohydride (70 g) was added and dissolved, and the mixture was stirred overnight. The reaction mixture was ice-cooled, adjusted to pH 5.5 with acetic acid and stirred at 4°C for one hour, and then, adjusted to pH 7.5 with 8M aqueous sodium hydroxide. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain dextran polyalcohol (20.2 g). The molecular weight of this substance was 159K (gel filtration, pullulan standard).

[0111] This dextran polyalcohol (7.5 g) was added to an aqueous solution obtained by dissolving sodium hydroxide (31.5 g) in water (225 ml) and dissolved at room temperature. To this solution, monochloroacetic acid (45 g) was added under ice-cooling and dissolved, and the mixture was allowed to react at room temperature overnight. This reaction mixture was adjusted to pH 8 with acetic acid and then desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was lyophilized to obtain sodium salt of carboxymethyl-

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dextran polyalcohol (8.5 g). The molecular weight of this substance was 274K (gel filtration, pullulan standard), and the degree of carboxymethylation was 0.4. This sodium salt of carboxymethyldextran polyalcohol (2.0 g) was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, H⁺ form) column (diameter: 44 mm, length: 210 mm), and eluted with water. This effluent was added with triethylamine (4 ml) and then lyophilized to obtain the title compound (2.2 g).

Example 52: Synthesis of carboxymethyldextran polyalcohol-Gly-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0112] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 51 (200 mg) was dissolved in N,N-dimethylformamide (7 ml). To this solution, a solution of the trifluoroacetic acid salt of 3'-N-(Gly-Phe-Gly)-NH-A' (A-NH₂=DX-8951) obtained in Example 48 (41 mg) in N,N-dimethylformamide (5 ml), triethylamine (0.014 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (100 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.0 ml) and diethyl ether (25 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (190 mg). The content of the drug compound residue in this compound was 4.5% (W/W) when determined based on the absorption at 362 nm in 0.1M Tris buffer (pH 9.0).

Example 53: Synthesis of carboxymethyldextran polyalcohol-Phe-Gly-NH-A' (A-NH₂=DX-8951)

[0113] The sodium salt of carboxymethyldextran polyalcohol obtained in Example 24 (2.5 g) was dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, Et₃N H⁺ form) column, and eluted with water. This effluent was lyophilized to give triethylammonium salt of carboxymethyldextran polyalcohol (2.5 g).

[0114] This triethylammonium salt of carboxymethyldextran polyalcohol (200 mg) was dissolved in N,N-dimethylformamide (12 ml). To this solution, a solution of the trifluoroacetic acid salt of 3'-N-(Phe-Gly)-NH-A' (A-NH₂=DX-8951) (42 mg) obtained in Example 49 and triethylamine (0.016 ml) in N,N-dimethylformamide (5 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (200 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring and shielding the light. This reaction mixture was added with water (300 ml) and subjected to ultrafiltration using a ultrafiltration membrane 10K (Filtron). The remaining solution that had not passed through the membrane was adjusted to pH 10 with 0.1N aqueous sodium hydroxide, and passed through a filtration membrane (0.16 μm, Filtron). The filtrate was desalted by ultrafiltration using a Biomax-50 membrane, and then filtered through a Millipore filter (0.22 μm) and lyophilized to obtain the title compound (180 mg). The content of the drug compound residue in this compound was 6.1% (W/W) when determined based on the absorption at 362 nm in 0.1 M Tris buffer solution (pH 9.0).

Example 54: Synthesis of carboxymethyldextran polyalcohol-Gly-NH-A' (A-NH₂=DX-8951)

[0115] The triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 51 (370 mg) was dissolved in N,N-dimethylformamide (10 ml). To this solution, a solution of the trifluoroacetic acid salt of 3'-N-Gly-NH-A' (A-NH₂=DX-8951) obtained in Example 50 (57 mg) in N,N-dimethylformamide (3 ml), triethylamine (0.027 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (185 mg) were added successively, and then the mixture was allowed to react at room temperature overnight with stirring. Each of 5 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. The mixture was added with 3M aqueous sodium chloride (2.0 ml) and diethyl ether (25 ml), and the deposited precipitates were collected by centrifugation (3500 rpm, 8 minutes). The precipitates were dissolved in 0.5M aqueous sodium chloride and adjusted to pH 9 with 0.1M aqueous sodium hydroxide under ice-cooling. The resulting aqueous solution was desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 μm) and then lyophilized to obtain the title compound (290 mg). The content of the drug compound residue in this compound was 0.5% (W/W) when determined based on the absorption at 362 nm in 0.1M Tris buffer (pH 9.0).

Example 55: Synthesis of carboxymethyldextran polyalcohol-Gly-Gly-Phe-Gly-D51-7059

[0116] Boc-Gly-Gly-Phe-Gly-OH (200 mg) was dissolved in trifluoroacetic acid (4 ml) and stirred for 1.5 hours. The solvent was evaporated, and the residue was subjected to azeotropic distillations twice with methanol (10 ml) and twice with ethanol (10 ml) and washed with ether to obtain trifluoroacetic acid salt of Gly-Gly-Phe-Gly-OH (225 mg).

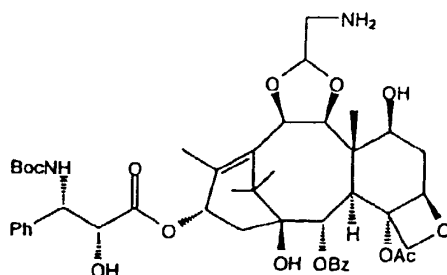
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¹H-NMR (DMSO-d₆) δ : 8.48 (dd, 1H, J=5.6, 5.6Hz), 8.59 (dd, 1H, J=5.6, 6.4Hz), 8.29 (d, 1H, J=4.8Hz), 7.23-7.26 (m, 4H), 7.16-7.20 (m, 1H), 4.58 (ddd, 1H, J=4.8, 4.8, 10.4Hz), 3.89 (dd, 1H, J=5.6, 16.7Hz), 3.76-3.79 (m, 2H), 3.67 (dd, 1H, J=5.6, 16.7Hz), 3.56 (s, 2H).

[0117] The trifluoroacetic acid salt of Gly-Gly-Phe-Gly-OH (200 mg) obtained above was dissolved in water (10 ml), added with triethylamine to adjust the pH to 9.0, then added with a solution of 9-fluorenylmethyl N-hydroxysuccinimidyl-carbonate (200 mg) in acetonitrile (5 ml), and stirred at room temperature for four hours while maintaining the pH in the range of 8.0 through 8.5 by using triethylamine. The reaction mixture was added with 1.5N hydrochloric acid (50 ml), and the deposited precipitates were collected by filtration, washed with water, and purified by silica gel column chromatography (eluent: dichloromethane:methanol = 4:1 solution) to obtain Fmoc-Gly-Gly-Phe-Gly-OH (151 mg).

¹H-NMR (DMSO-d₆) δ : 8.28-8.32 (m, 1H), 8.08-8.12 (m, 1H), 7.85-7.89 (m, 2H), 7.68-7.72 (m, 2H), 7.57-7.65 (m, 1H), 7.38-7.43 (m, 2H), 7.29-7.34 (m, 2H), 7.20-7.25 (m, 4H), 7.14-7.17 (m, 1H), 4.45-4.52 (m, 1H), 4.26-4.30 (m, 2H), 4.19-4.24 (m, 1H), 3.77 (dd, 1H, J=5.6, 16.7Hz), 3.58-3.69 (m, 4H), 3.42-3.52 (m, 1H), 3.06 (dd, 1H, J=4.0, 13.5Hz), 2.78 (dd, 1H, J=4.0, 13.5Hz).

[0118] The Fmoc-Gly-Gly-Phe-Gly-OH obtained above (24 mg), the taxol derivative represented by the following formula:



[D51-7059: 9,10-O-(2-aminoethylidene)-13-O-[3-(tert-butoxycarbonylamino)-2-hydroxy-3-phenyl]-propanoyl-10-deacetyl-9-dihydrobaccatin III] (20 mg), and N-hydroxysuccinimide (7 mg) were dissolved in N,N-dimethylformamide (1 ml). This solution was cooled to 4°C and then added with N,N'-dicyclohexylcarbodiimide (9 mg), and the mixture was allowed to react at room temperature overnight with stirring. This reaction mixture was evaporated to dryness under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 96:4 solution) to obtain Fmoc-Gly-Gly-Phe-Gly-D51-7059 (21 mg).

¹H-NMR (CDCl₃) δ : 8.06 (d, 2H, J=8.1Hz), 7.75 (d, 2H, J=8.1Hz), 7.18-7.61 (m, 23H), 7.62 (dd, 1H, J=7.2, 8.0Hz), 6.07 (dd, 1H, J=7.9, 8.8Hz), 5.98 (d, 1H, J=4.8Hz), 5.63 (d, 1H, J=8.8Hz), 5.00-5.40 (m, 4H), 4.92 (s, 1H), 4.60-4.69 (m, 2H), 4.41 (d, 2H, J=6.4Hz), 4.35 (d, 1H, J=8.0Hz), 4.29 (d, 1H, J=8.0Hz), 4.21 (t, 1H, J=7.5Hz), 3.96-4.07 (m, 3H), 3.73-3.86 (m, 4H), 3.37-3.41 (m, 1H), 3.19-3.23 (m, 1H), 3.00 (dd, 1H, J=8.0, 13.5Hz), 2.85-2.89 (m, 3H), 2.29 (s, 3H), 2.05-2.40 (m, 4H), 1.57 (s, 3H), 1.56 (s, 3H), 1.53 (s, 3H), 1.40 (s, 9H), 1.22 (s, 3H).

Mass (FAB); m/e 1413 (M+Na)

[0119] The Fmoc-Gly-Gly-Phe-Gly-D51-7059 obtained above (21 mg) was dissolved in dichloromethane (1.8 ml) and added with piperazine (0.2 ml), and then the mixture was allowed to react at room temperature for 1 hour. This reaction mixture was purified by silica gel column chromatography (eluent: dichloromethane:methanol = 94:6 solution) to obtain Gly-Gly-Phe-Gly-D51-7059 (16 mg).

¹H-NMR (CDCl₃) δ : 8.10 (d, 2H, J=8.1Hz), 7.89-7.94 (m, 1H), 7.62 (dd, 1H, J=7.2, 8.0Hz), 7.45-7.50 (m, 2H), 7.17-7.42 (m, 12H), 7.10-7.16 (m, 1H), 6.97 (dd, 1H, J=5.6, 6.4Hz), 6.08 (dd, 1H, J=8.0, 8.7Hz), 6.02 (d, 1H, J=4.8Hz), 5.62 (d, 1H, J=11.1Hz), 5.23-5.30 (m, 1H), 5.23 (d, 1H, J=7.2Hz), 5.10 (s, 1H), 4.98-5.00 (m, 1H), 4.60-4.63 (m, 1H), 4.38 (d, 1H, J=8.8Hz), 4.33 (d, 1H, J=8.8Hz), 4.13 (s, 1H), 4.04 (dd, 1H, J=5.6, 16.7Hz), 3.93 (dd, 1H, J=5.6, 16.7Hz), 3.82 (d, 1H, J=7.2Hz), 3.73-3.82 (m, 2H), 3.43-3.49 (m, 1H), 3.30-3.38 (m, 2H), 3.24 (dd, 1H, J=6.4, 14.3Hz), 3.04 (dd, 1H, J=8.0, 14.3Hz), 2.89-3.07 (m, 3H), 2.30 (s, 3H), 2.01-2.50 (m, 4H), 1.70 (s, 3H), 1.62 (s, 3H), 1.61 (s, 3H), 1.40 (s, 9H), 1.26 (s, 3H).

Mass (FAB); m/e 1169 (M+1)

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[0120] The Gly-Gly-Phe-Gly-D51-7059 synthesized according to the above method (33 mg) was dissolved in N,N-dimethylformamide (0.5 ml). To this solution, a solution of the triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 24 (180 mg) in N,N-dimethylformamide (7 ml), and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (180 mg) were added successively, and the mixture was allowed to react at room temperature overnight with stirring. Each of 4 ml portions of this reaction mixture was added dropwise to each 10 ml of ethanol. Each was added with 3M aqueous sodium chloride (2.0 ml) and diethyl ether (25 ml), and the deposited precipitates were collected by centrifugation (2500 rpm, 8 minutes). The precipitates were washed with ethanol, then dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, Na⁺ form) column (diameter: 15 mm, length: 85 mm), and eluted with water to obtain Solution 1. Separately, Gly-Gly-Phe-Gly-D51-7059 (10 mg) was dissolved in N,N-dimethylformamide (0.5 ml), and then added successively with a solution of the triethylammonium salt of carboxymethyldextran polyalcohol obtained in Example 24 (60 mg) in N,N-dimethylformamide (5 ml) and a solution of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (60 mg) in N,N-dimethylformamide (0.25 ml), and the mixture was allowed to react at room temperature overnight with stirring. This reaction mixture was added dropwise to 10 ml of ethanol, and then the resulting mixture was added with 3M aqueous sodium chloride (2.0 ml) and diethyl ether (25 ml), and the precipitates deposited were collected by centrifugation (2500 rpm, 8 minutes). The precipitates were washed with ethanol, then dissolved in water, applied to a Bio-Rad AG 50W-X2 (200-400 mesh, Na⁺ form) column (diameter: 15 mm, length: 85 mm), and eluted with water to obtain Solution 2. Solution 1 and Solution 2 were combined and then desalted by ultrafiltration using a Biomax-50 membrane. The remaining solution that had not passed through the membrane was filtered through a Millipore filter (0.22 µm) and then lyophilized to obtain the title compound (208 mg). The result obtained by GPC analysis after dissolving this compound in 0.1M aqueous sodium chloride (column: TSK Gel PW-4000XL, Tosoh, solvent: 0.1 M NaCl, flow rate: 0.8 ml/min) and the ultraviolet absorption spectrum of the compound (methanol:water = 10:1 solution, 1.69 mg/ml) are shown in Fig. 22 and Fig. 23, respectively. The content of the drug compound residue in the compound was 5.3% (W/W) when determined based on the absorption at 240 nm in a methanol:water = 10:1 solution.

Example 56: Antitumor activity of the drug complex of the present invention

[0121] Meth A tumor-bearing mice (6 mice per group) were prepared according to a similar manner to that of Example 11 and the antitumor activity of the drug complex of Example 15 was examined by single administration in a similar manner to that of Example 12. As a result, the drug complex of Example 15 exhibited remarkably enhanced antitumor activity and broader effective dose-range compared to the drug compound itself of Example 12.

Test compound	Dose (mg/kg) ¹⁾	Inhibition rate (%)
Compound of Example 15	10	100
	5	99
	2.5	95
	1.25	83

1) Calculated based on the drug compound

Example 57: Antitumor activity of the drug complex of the present invention

[0122] SC-6 tumor-bearing nude mice (5 mice per group) were prepared by subcutaneously transplanting a piece of SC-6 human gastric tumor block into the right inguinal regions of nude mice (BALB/c-nu/nu, male). On day 27 after the transplantation, the drug complex of Example 15 dissolved in distilled water for injection was given as a single intravenous administration and its antitumor activity was compared to that of the drug compound, per se. As a result, the drug complex of Example 15 exhibited higher antitumor activity compared to the drug compound, per se, whereas there was no death due to toxicity.

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5	Test compound	Dose (mg/kg)	Inhibition rate (%)	Numbers of died mice/mice used
	Drug compound, per se	60	98	2/5
		15	61	0/5
10	Compound of Example 15	8 ¹⁾	100	0/5
		2 ¹⁾	71	0/5

1) Calculated based on the drug compound

15 Example 58: Antitumor activity of the drug complex of the present invention

[0123] Human lung cancer QG-90-bearing nude mice (5 mice per group) were prepared according to a similar manner to that of Example 57. On day 16 after the transplantation, the drug complex of Example 15 dissolved in distilled water for injection was given as a single intravenous administration and its antitumor activity was compared to that of the drug compound, per se. As a result, the drug complex of Example 15 exhibited remarkably enhanced antitumor activity and broader effective dose-range compared to the drug compound, per se.

25	Test compound	Dose (mg/kg)	Inhibition rate (%)	Numbers of died mice/mice used
	Drug compound, per se	50	65	0/5
		12.5	51	0/5
30	Compound of Example 15	7 ¹⁾	98	0/5
		1.75 ¹⁾	97	0/5

1) Calculated based on the drug compound

35 Example 59: Antitumor activity of the drug complex of the present invention

[0124] Meth A tumor-bearing mice (6 mice per group) were prepared according to a similar manner to that of Example 11, and the antitumor activity of the drug complex of Example 41 was examined in the cases of single administration in a similar manner to that of Example 12, and its antitumor activity was compared to that of the drug compound, per se. As a result, the drug complex of Example 41 exhibited remarkably enhanced antitumor activity and broader effective dose-range compared to the drug compound, per se.

45	Test compound	Dose (mg/kg)	Inhibition rate (%)
	Drug compound, per se	100	64
		50	56
50		25	34
	Compound of Example 41	25 ¹⁾	99
		12.5 ¹⁾	95
55		6.25 ¹⁾	81
		3.125 ¹⁾	61

1) Calculated based on the drug compound

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Example 60: Antitumor activity of the drug complex of the present invention

[0125] Meth A tumor-bearing mice (6 mice per group) were prepared according to a similar manner to that of Example 11, and the antitumor activity was examined according to a similar method to that in Example 12 by single administration of the drug complexes of Examples 29, 46, and 47, respectively. As a result, all of the drug complexes exhibited high antitumor activity and broader effective dose-range.

Test compound	Dose (mg/kg) ¹⁾	Inhibition rate (%)
Compound of Example 29	30	99
	20	99
	10	89
	5	79
Compound of Example 46	100	94
	80	92
	40	82
	20	75
Compound of Example 47	100	96
	80	94
	40	97
	20	75

1) Calculated based on the drug compound

Example 61: Antitumor activity of the drug complex of the present invention

[0126] Meth A tumor-bearing mice (6 mice per group) were prepared according to a similar manner to that of Example 11, and antitumor activity of the drug complex of Example 44 was examined according to a similar method to that of Example 12 by single administration, and its antitumor activity was compared to that of the drug compound (Doxorubicin), per se. As a result, the drug complex of Example 44 exhibited remarkably enhanced antitumor activity and broader effective dose-range compared to the drug compound, per se.

Test compound	Dose (mg/kg)	Inhibition rate (%)	Numbers of died mice/mice used
Drug compound, per se	20	-	6/6
	10	64	0/6
	5	39	0/6
Compound of Example 44	40 ¹⁾	96	0/6
	20 ¹⁾	96	0/6
	10 ¹⁾	87	0/6
	5 ¹⁾	76	0/6

1) Calculated based on the drug compound

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Example 62: Pharmacokinetics of the drug complex of the present invention

[0127] Meth A tumor-bearing mice were prepared according to a similar manner to that of Example 11, the drug complex of Example 15 was given as single administration in a similar manner to that of Example 12 (10 mg/kg: calculated as the drug compound), and the change of the drug complex concentration in various tissues was determined. As a result, the drug complex of Example 15 was found to have extremely long retention of blood level, high distribution in tumor tissues, and high tumor selectivity against liver and small intestine. The results are shown in Fig. 24.

Industrial Applicability

[0128] The drug complex of the present invention that is introduced with a residue of a drug compound such as anti-neoplastic agents is characterized in that it has excellent selectivity to tumorous sites so as to exhibit high antineoplastic activity and also achieves reduced appearance of toxicity.

Claims

1. A drug complex characterized in that a carboxy(C₁₋₄)alkyldextran polyalcohol and a residue of a drug compound are bound to each other by means of a spacer comprising an amino acid or a spacer comprising peptide-bonded 2 to 8 amino acids.
2. The drug complex according to claim 1, characterized in that the dextran polyalcohol that constitutes the carboxy(C₁₋₄)alkyldextran polyalcohol is a dextran polyalcohol which is obtained by treating a dextran under a condition that enables substantially complete polyalcoholization.
3. The drug complex according to claim 1 or claim 2, wherein the carboxy(C₁₋₄)alkyldextran polyalcohol is carboxymethyldextran polyalcohol.
4. The drug complex according to any one of claims 1 to 3, wherein the drug compound is an antineoplastic agent or an anti-inflammatory agent.
5. The drug complex according to claim 4, wherein the drug compound is an antineoplastic agent which concentration-dependently exhibits antineoplastic activity.
6. The drug complex according to claim 4, wherein the drug compound is an antineoplastic agent which time-dependently exhibits antineoplastic activity.
7. The drug complex according to claim 4, wherein the antineoplastic agent is doxorubicin or (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13(9H,15H)-dione.
8. The drug complex according to any one of claims 5 to 7, wherein the spacer is a dipeptide represented by -X-Z- wherein "-X-Z-" represents a residue which consists of a dipeptide that is formed by peptide bonding of a hydrophobic amino acid (X) and a hydrophilic amino acid (Z) being at the N-terminal side and the C-terminal side, respectively, and whose one hydrogen atom and one hydroxyl group are removed from the amino group at the N-terminus and the carboxyl group at the C-terminus, respectively, or wherein the spacer contains the dipeptide as a partial peptide sequence.
9. The drug complex according to claim 8, wherein the hydrophobic amino acid is phenylalanine and the hydrophilic amino acid is glycine.
10. The drug complex according to claim 9, wherein the spacer is (N-terminus)-Gly-Gly-Phe-Gly-.
11. The drug complex according to any one of claims 5 to 10, wherein an introduced amount of the residue of the antineoplastic agent is in the range of from 1 to 15% by weight.
12. The drug complex according to any one of claims 5 to 10, wherein an introduced amount of the residue of the antineoplastic agent is in the range of from 3 to 10% by weight.

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13. The drug complex according to any one of claims 5 to 10, wherein an introduced amount of the residue of the anti-neoplastic agent is in the range of from 5 to 6% by weight.
14. The drug complex according to claim 1, wherein the N-terminus of a peptide represented by $H_2N-Gly-Gly-Phe-Gly-COOH$ is bound to a carboxyl group of a carboxymethyldextran polyalcohol by means of an acid-amide bond and C-terminus of the peptide is bound to the 1-amino group of (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione by means of an acid-amide bond.
15. The drug complex according to claim 14, wherein the introduced amount of (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione residue is in the range of from 2 to 10% by weight.
16. The drug complex according to claim 14 or claim 15, wherein the carboxy(C_{1-4})alkyldextran polyalcohol is a carboxymethyldextran polyalcohol having a molecular weight in the range of from 5,000 to 500,000 and the degree of carboxymethylation is in the range of from 0.01 to 2.0.
17. The drug complex according to claim 14 or claim 15, wherein the carboxy(C_{1-4})alkyldextran polyalcohol is a carboxymethyldextran polyalcohol having a molecular weight in the range of from 50,000 to 450,000 and the degree of carboxymethylation is in the range of from 0.1 to 1.0.
18. The drug complex according to claim 14 or claim 15, wherein the carboxy(C_{1-4})alkyldextran polyalcohol is a carboxymethyldextran polyalcohol having a molecular weight in the range of from 200,000 to 400,000 and the degree of carboxymethylation is in the range of from 0.3 to 0.5.
19. The drug complex according to claim 14, wherein the introduced amount of (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]-quinoline-10,13(9H,15H)-dione residue is in the range of from 5 to 6% by weight, the molecular weight of carboxy(C_{1-4})alkyldextran polyalcohol is about 228,000, and the degree of carboxymethylation is about 0.4.
20. A drug delivery carrier for binding a drug compound which comprises a carboxy(C_{1-4})alkyldextran polyalcohol.
21. The drug delivery carrier according to claim 20, wherein the molecular weight of the carboxy(C_{1-4})alkyldextran polyalcohol is in the range of from 5,000 to 500,000, and the degree of carboxymethylation is in the range of from 0.01 to 2.0.
22. The drug delivery carrier according to claim 20, wherein the molecular weight of the carboxy(C_{1-4})alkyldextran polyalcohol is in the range of from 50,000 to 450,000, and the degree of carboxymethylation is in the range of from 0.1 to 1.0.
23. The drug delivery carrier according to claim 20, wherein the molecular weight of the carboxy(C_{1-4})alkyldextran polyalcohol is in the range of from 200,000 to 400,000, and the degree of carboxymethylation is in the range of from 0.3 to 0.5.
24. The drug delivery carrier according to any one of claims 20 to 23, wherein the carboxy(C_{1-4})alkyldextran polyalcohol is a carboxymethyldextran polyalcohol.
25. A use of a carboxy(C_{1-4})alkyldextran polyalcohol for a manufacture of a drug complex containing the carboxy(C_{1-4})alkyldextran polyalcohol bound to a residue of a drug compound.
26. A use of a carboxy(C_{1-4})alkyldextran polyalcohol for a manufacture of a drug complex in which a residue of a drug compound and the carboxy(C_{1-4})alkyldextran polyalcohol are bound to each other by means of a spacer or without a spacer.

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Fig. 1

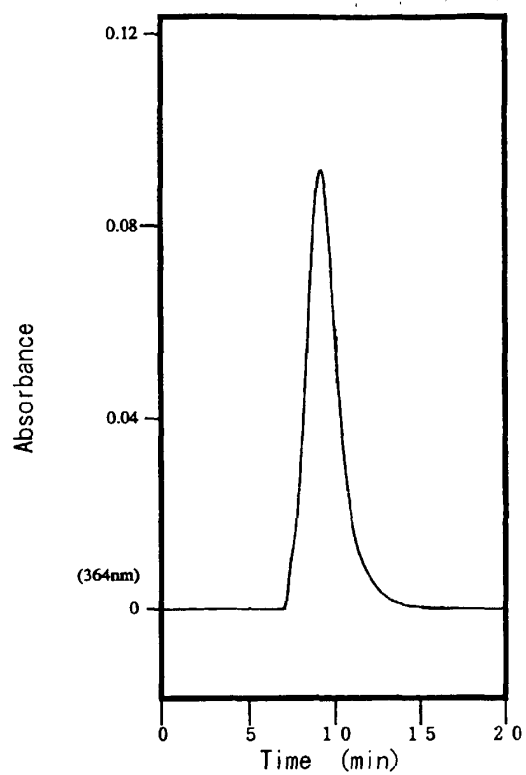


Fig. 2

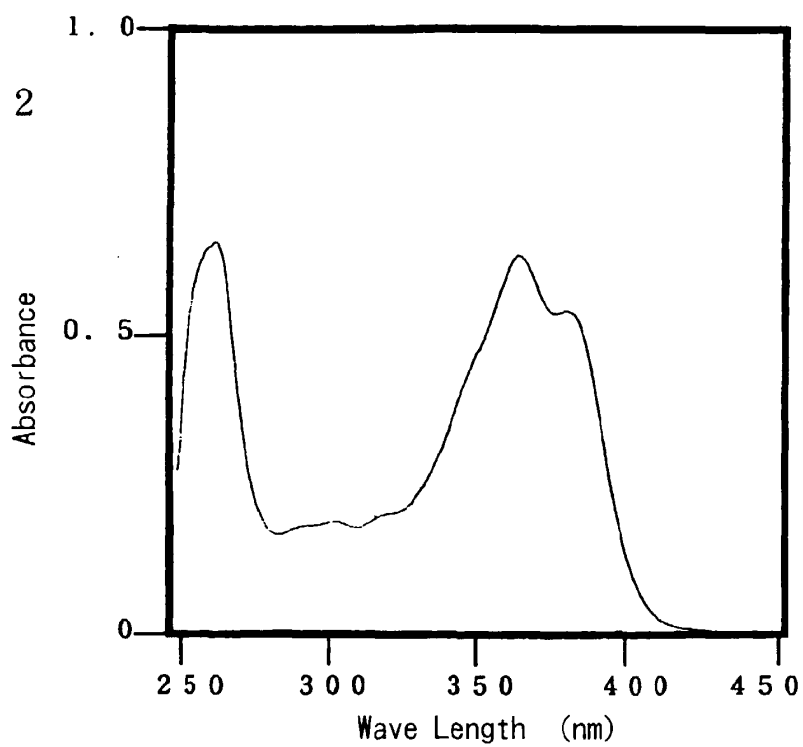


Fig. 3

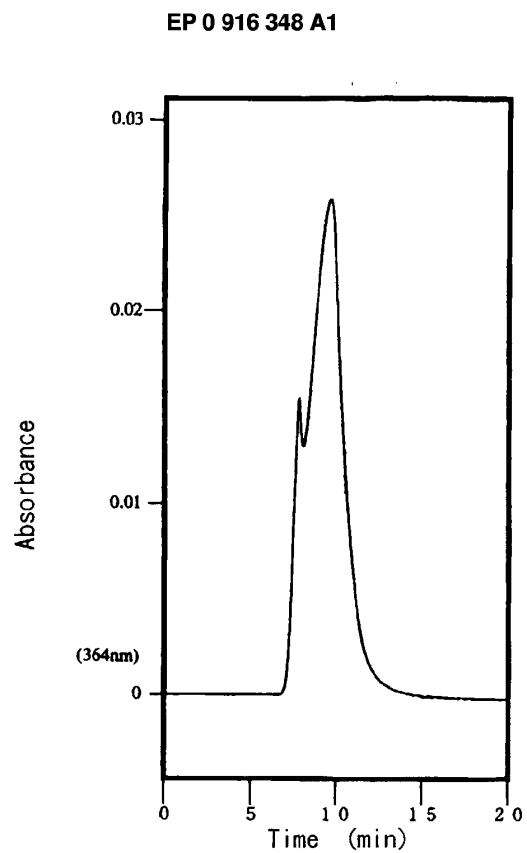
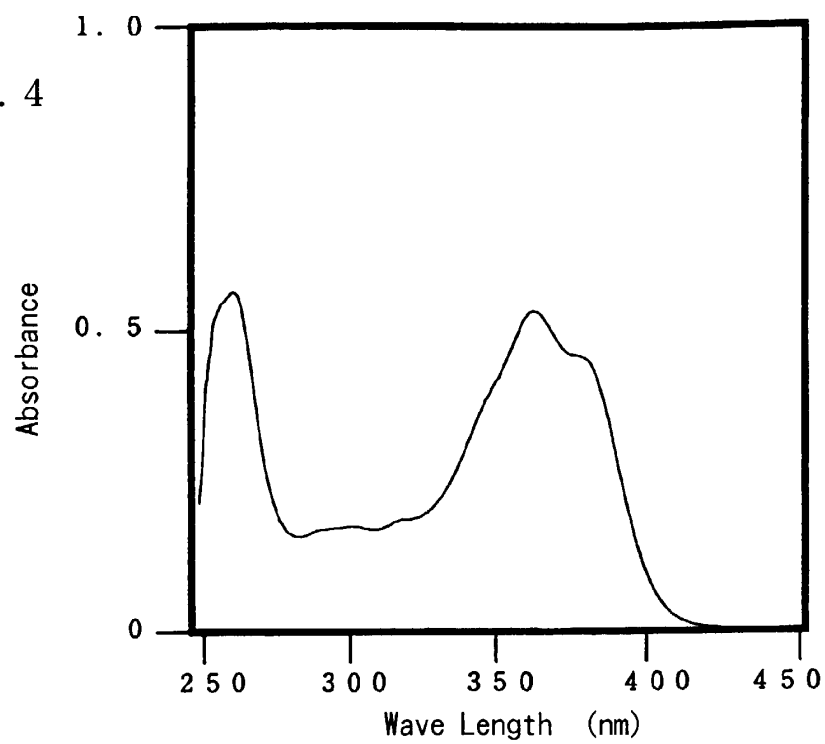
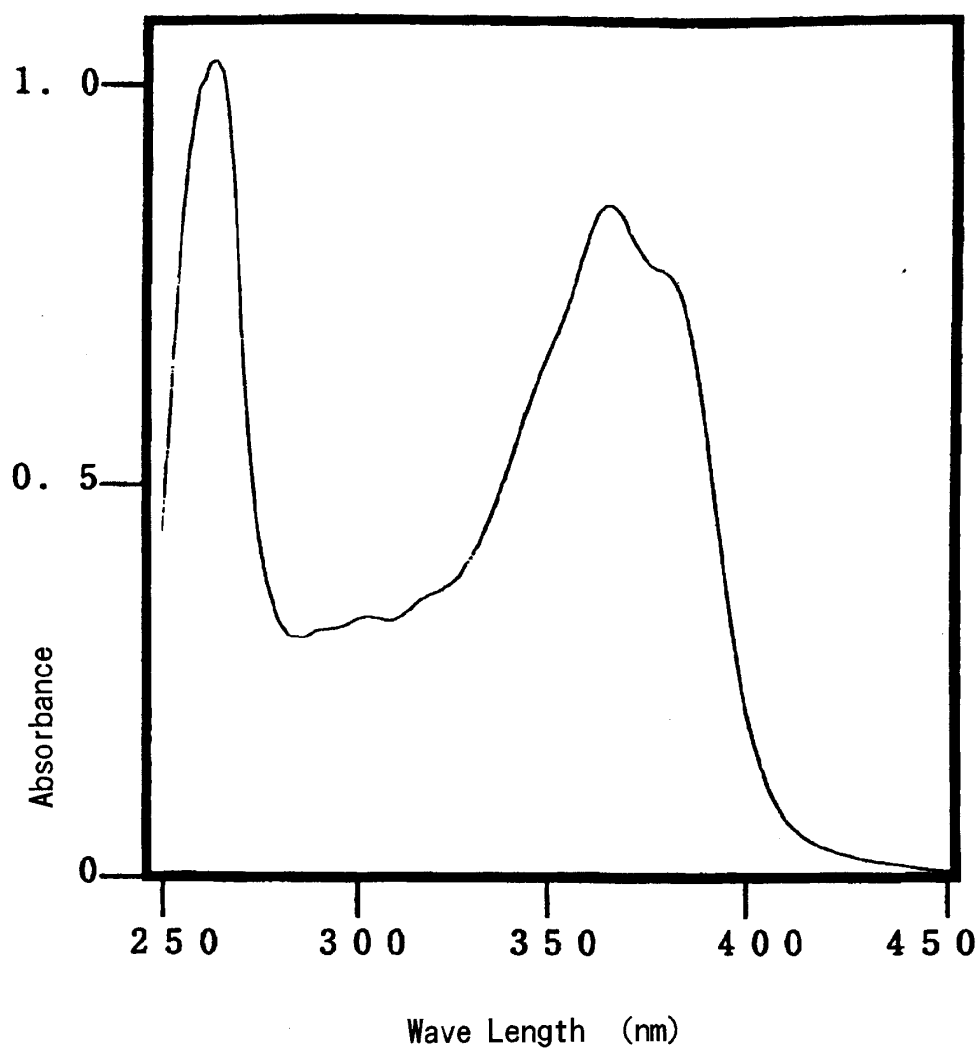


Fig. 4



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Fig. 5



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Fig. 6

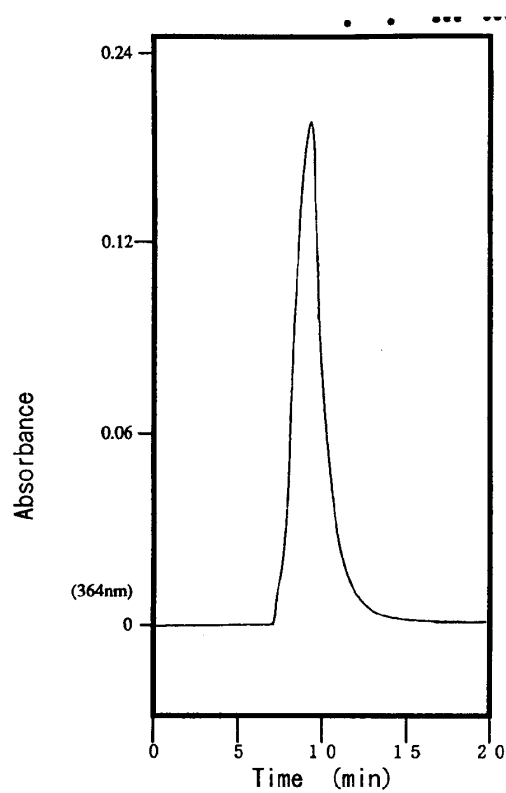
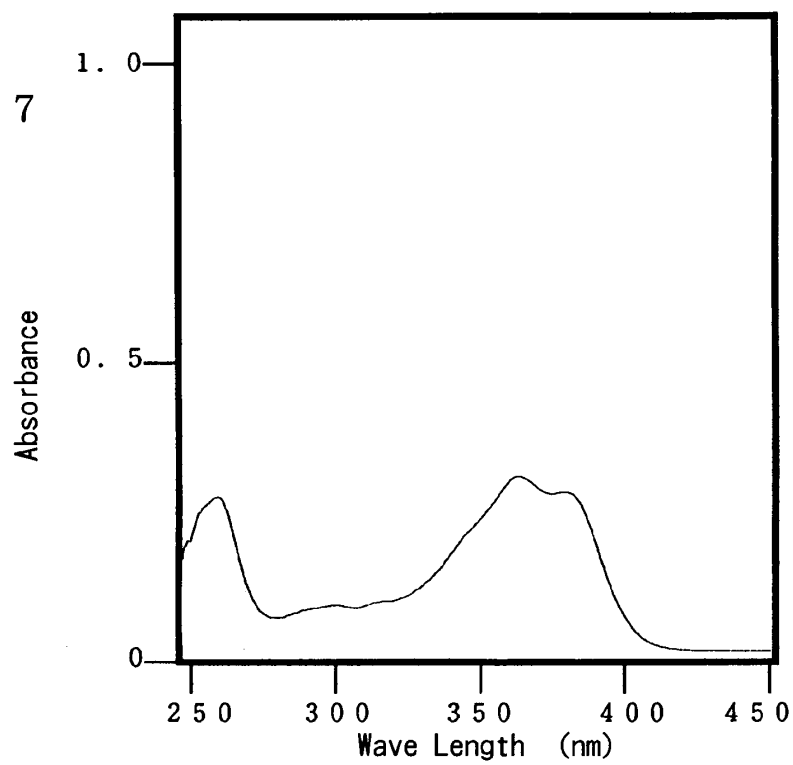


Fig. 7



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Fig. 8

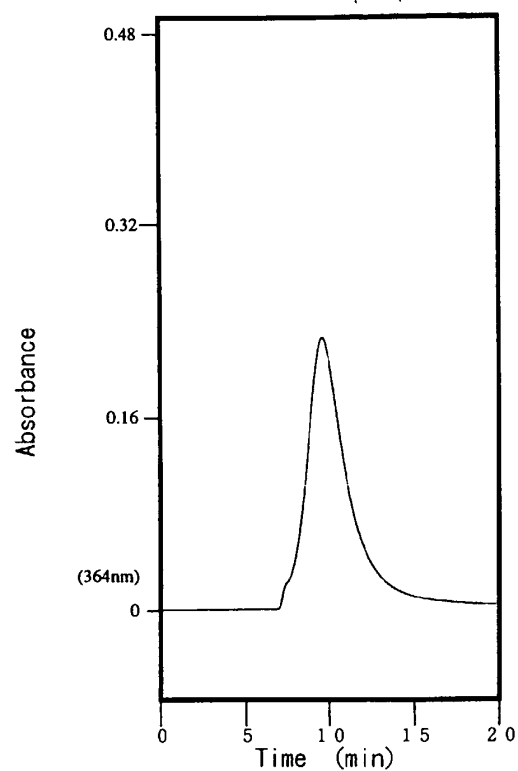
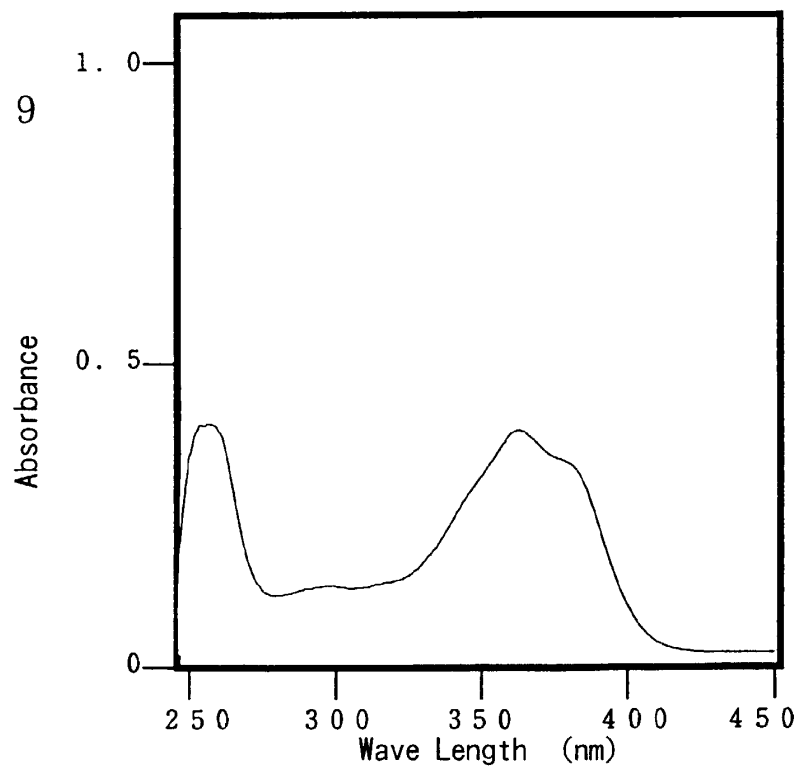


Fig. 9



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Fig. 10

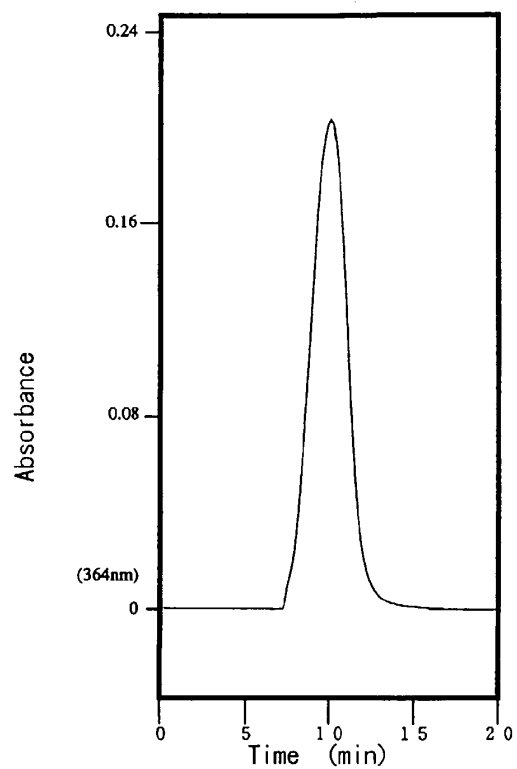
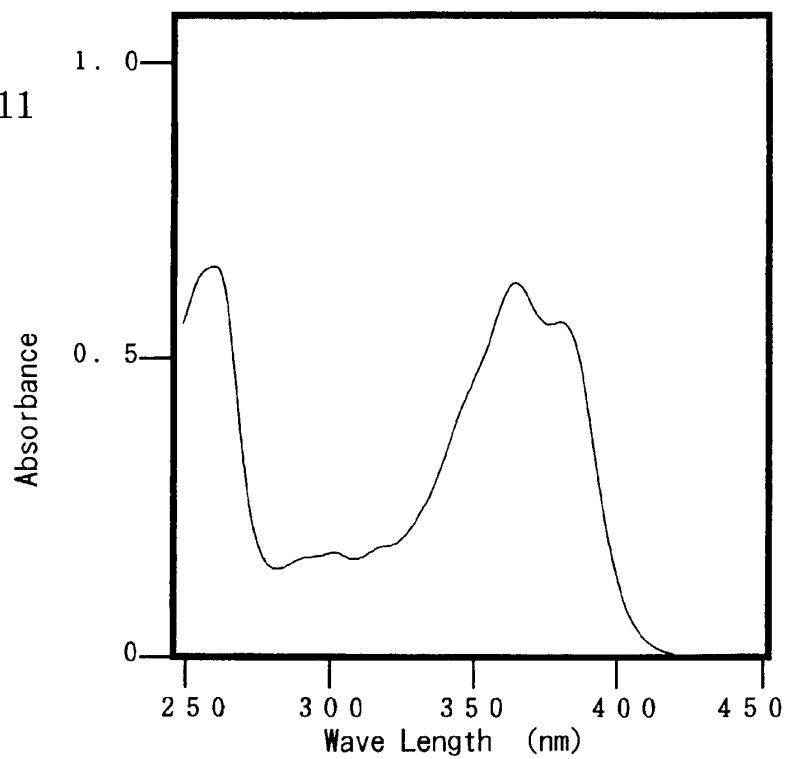


Fig. 11



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Fig. 12

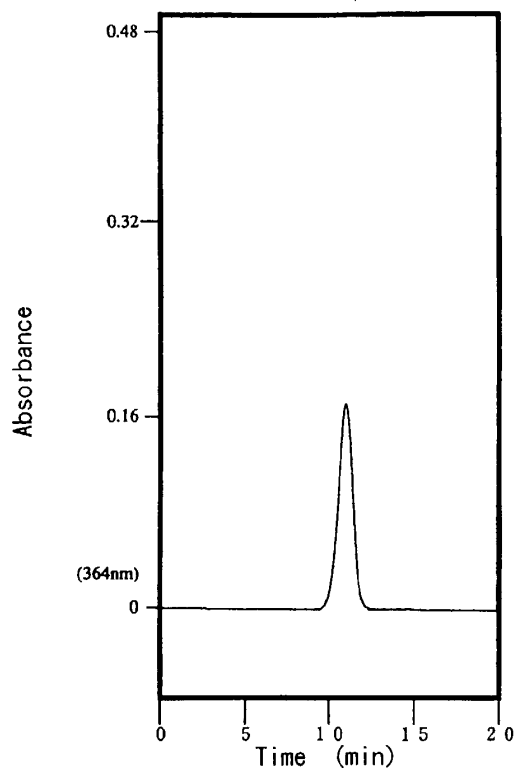
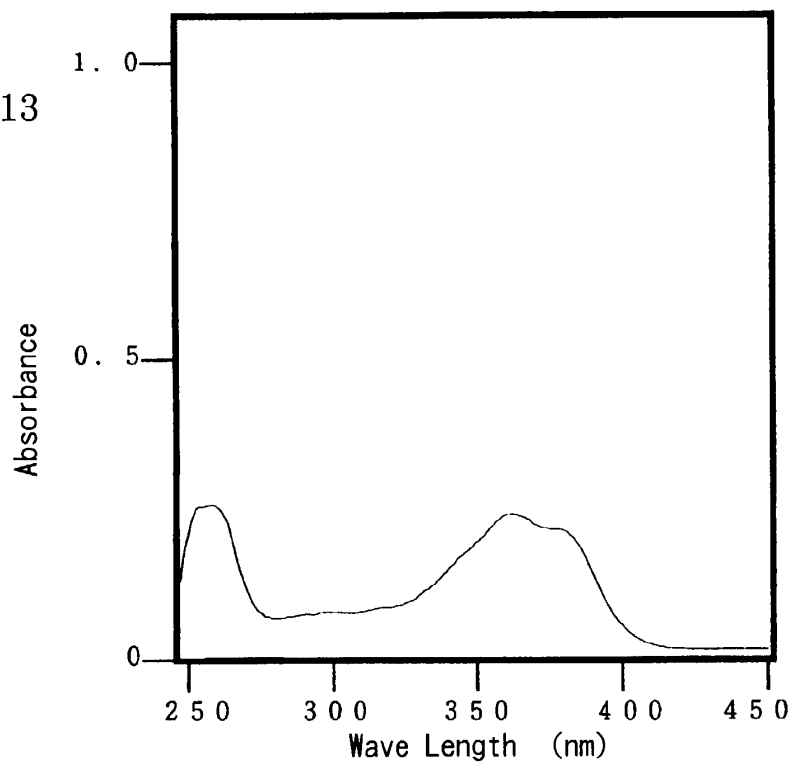


Fig. 13



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Fig. 14

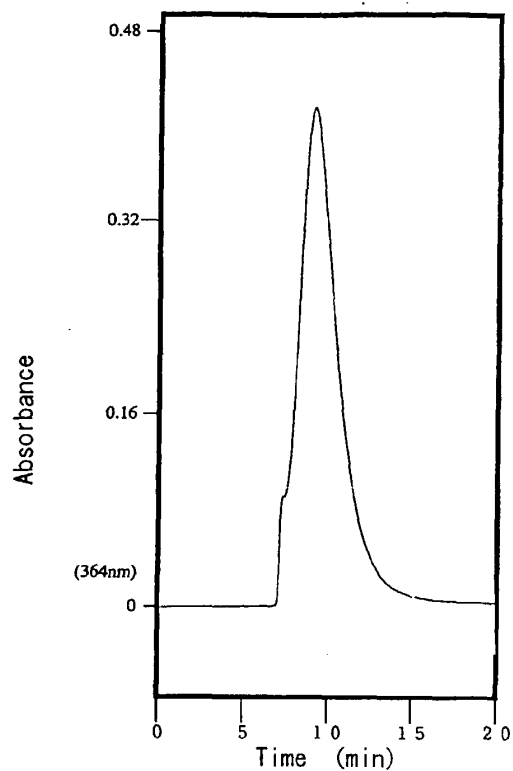
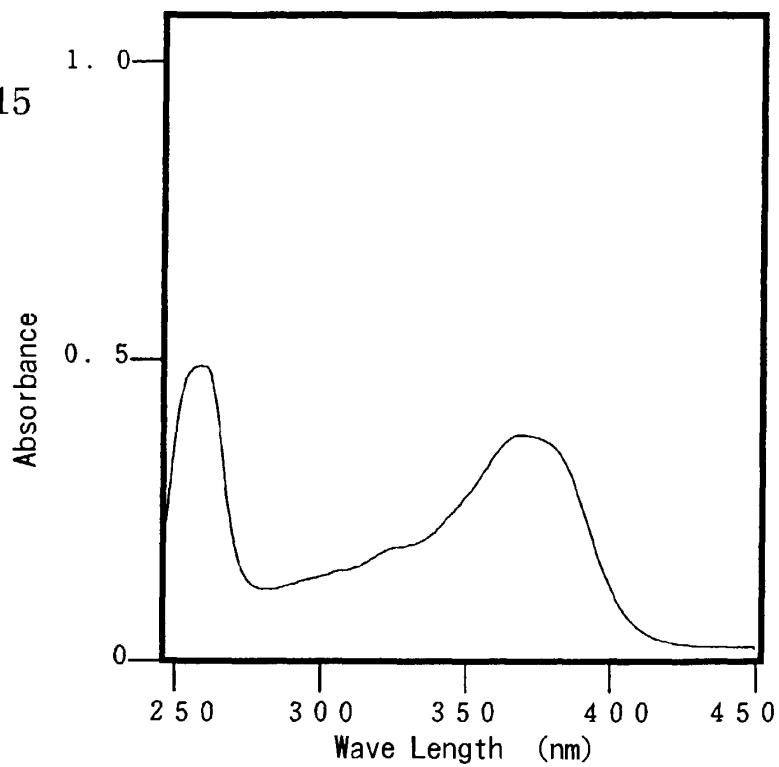


Fig. 15



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Fig. 16

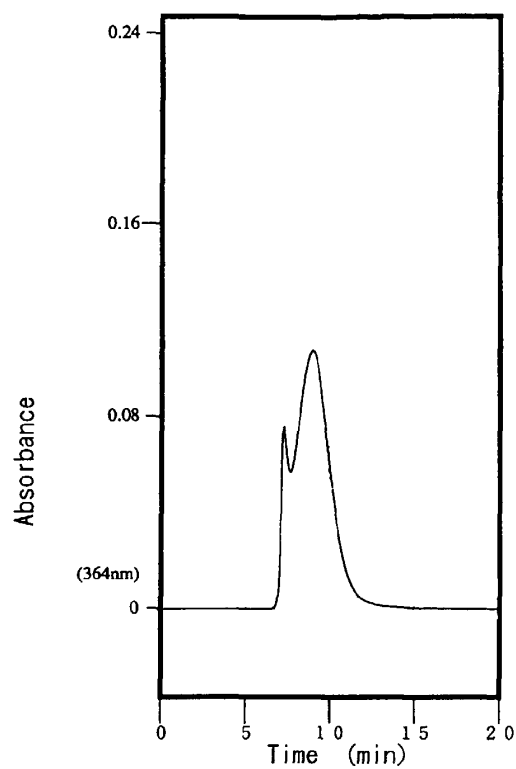
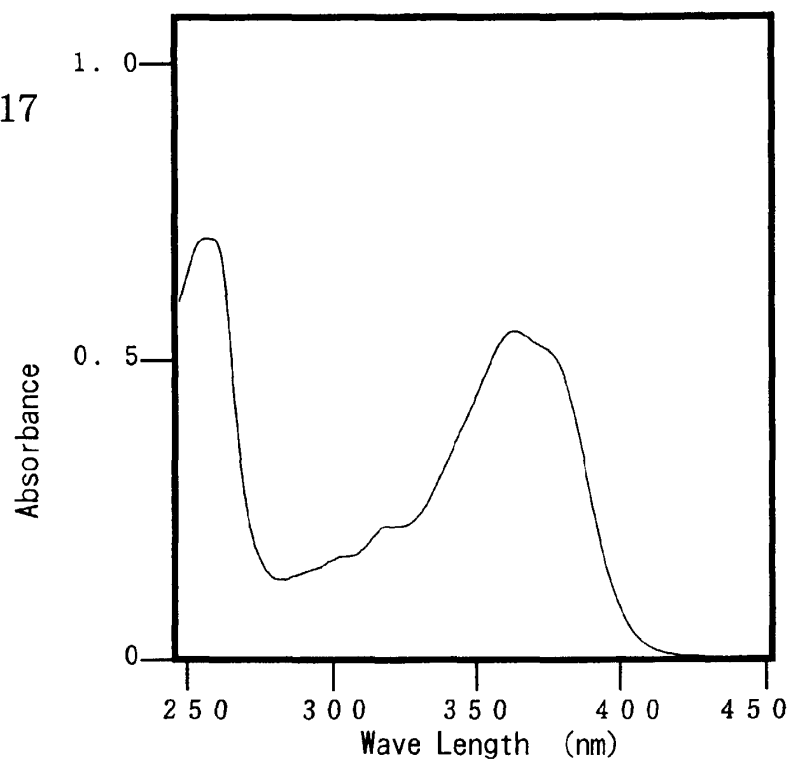


Fig. 17



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Fig. 18

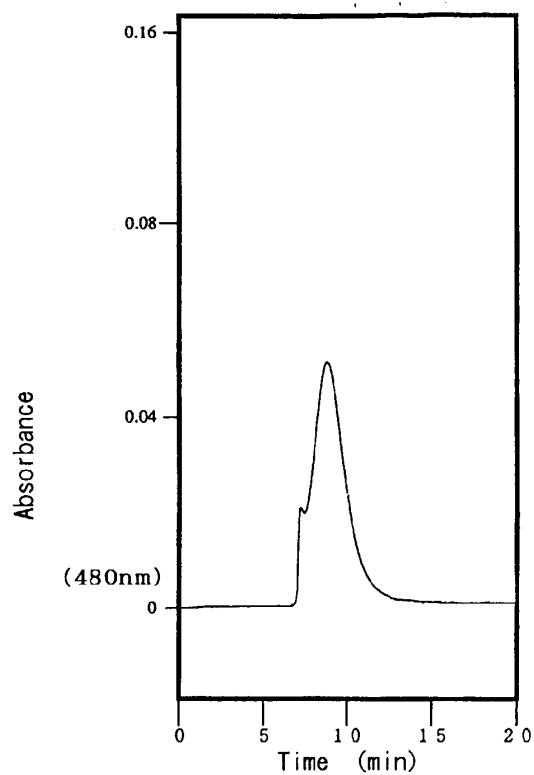
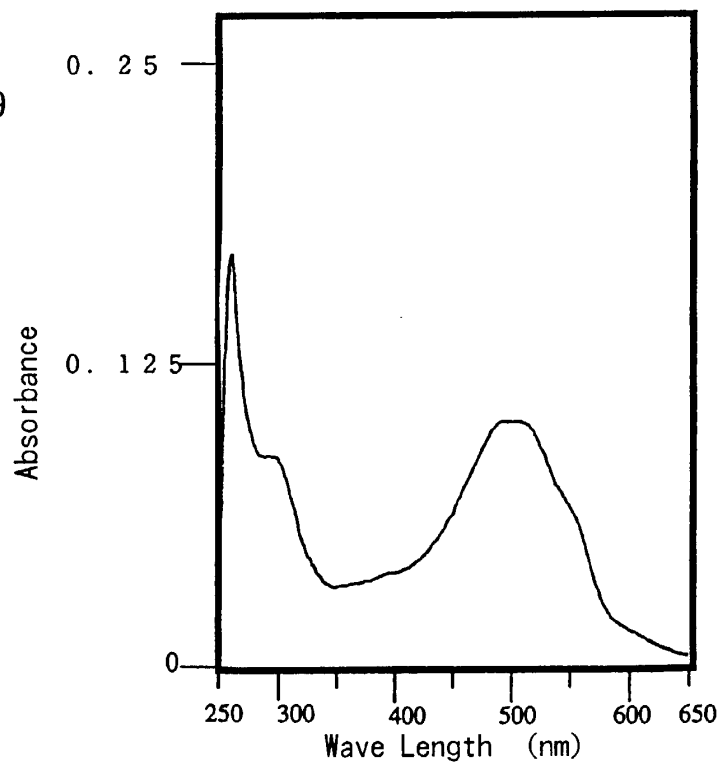


Fig. 19



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Fig. 20

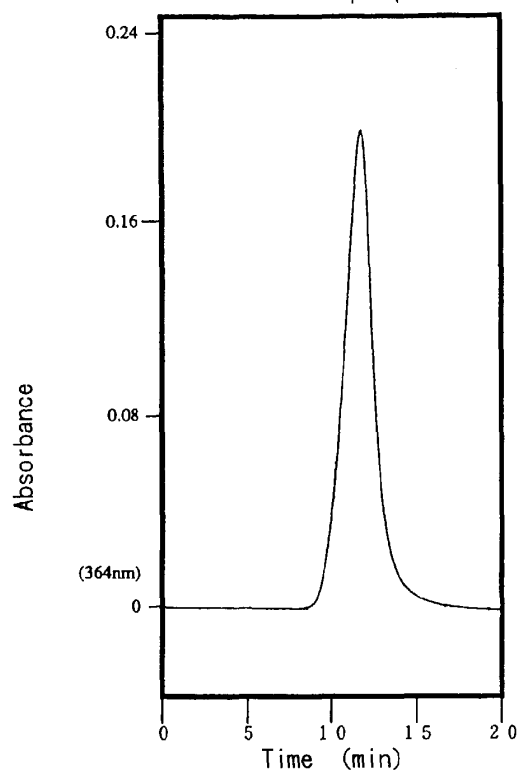
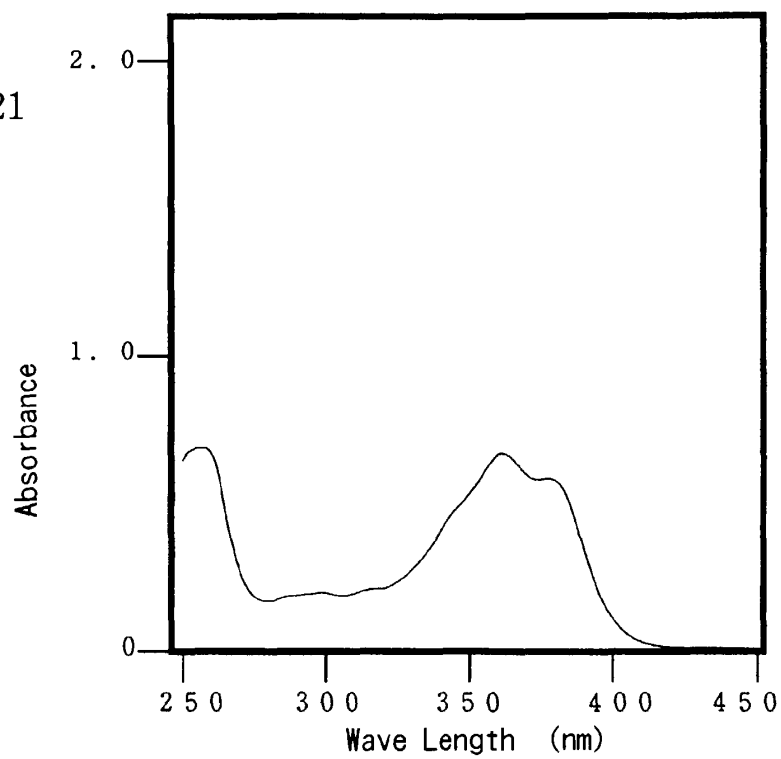


Fig. 21



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Fig. 22

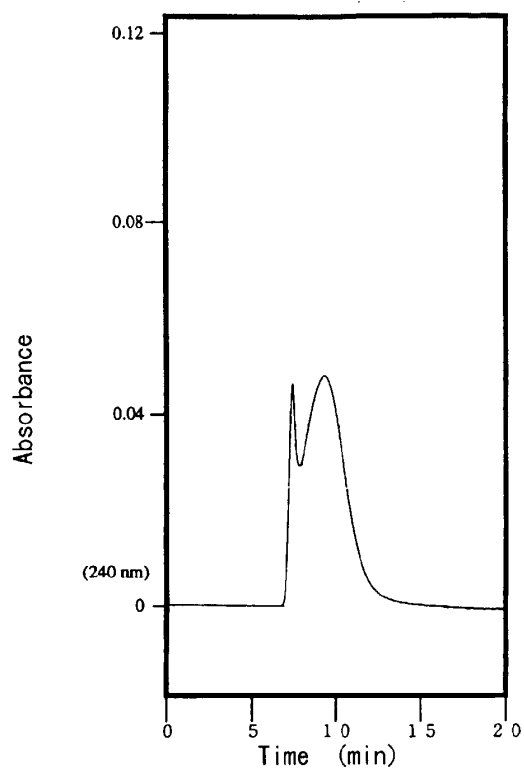
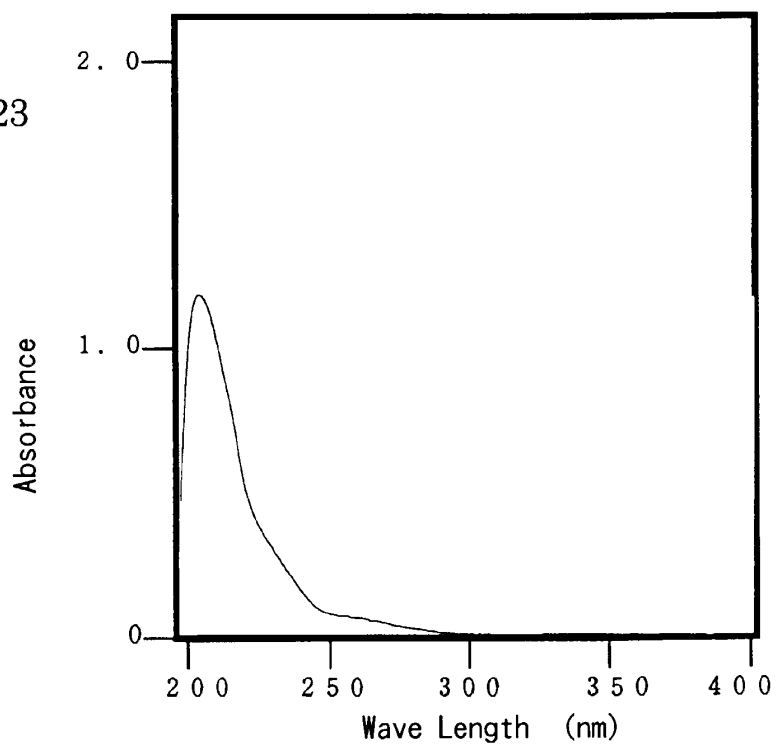
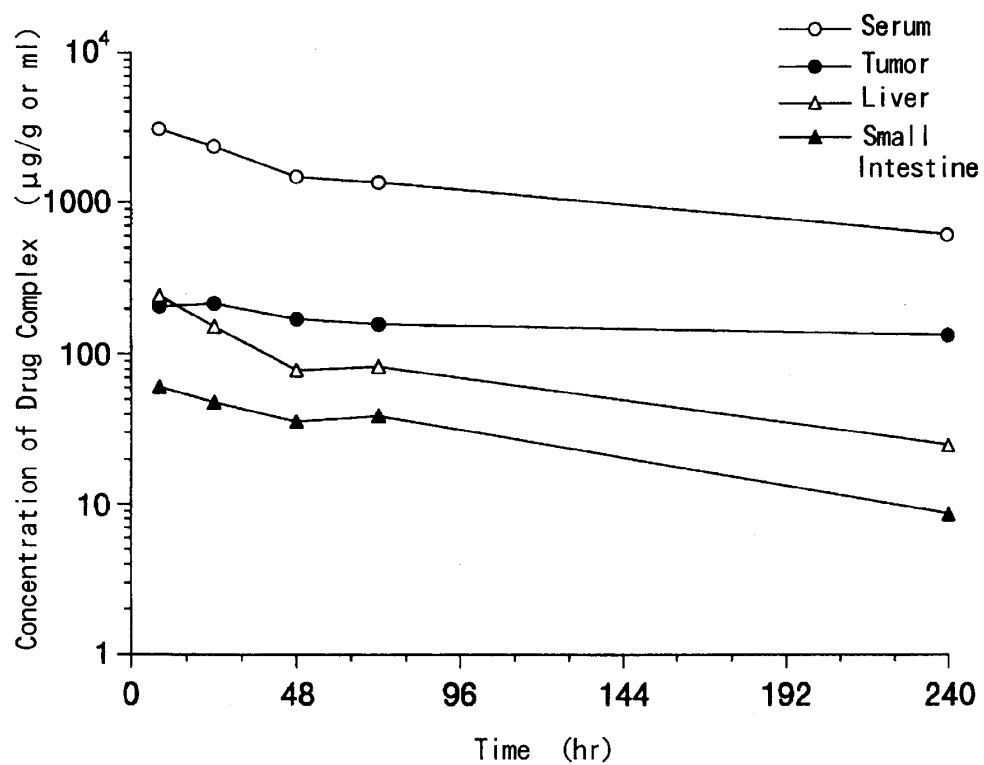


Fig. 23



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Fig. 24



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP97/01914

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl ⁶ A61K47/48		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 5-39306, A (Drug Delivery System Institute, Ltd.), February 19, 1993 (19. 02. 93) (Family: none)	1 - 26
A	JP, 59-220197, A (Snow Brand Milk Products Co., Ltd.), December 11, 1984 (11. 12. 84) (Family: none)	1 - 26
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search August 11, 1997 (11. 08. 97)		Date of mailing of the international search report August 19, 1997 (19. 08. 97)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.

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47/36				47/36	
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(21) 出願番号		特願2001-80188 (P2001-80188)		(71) 出願人 000002831 第一製薬株式会社 東京都中央区日本橋 3 丁目14番10号	
(22) 出願日		平成13年 3 月21日 (2001. 3. 21)		(72) 発明者 大薄 悟 東京都江戸川区北葛西 1 丁目16番13号 第一製薬株式会社東京研究開発センター内	
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(32) 優先日		平成12年 3 月22日 (2000. 3. 22)		(74) 代理人 100096219 弁理士 今村 正純 (外 2 名)	
(33) 優先権主張国		日本 (J P)			
最終頁に続く					

(54) 【発明の名称】 水酸基を有する薬物を含むDDS化合物

(57) 【要約】

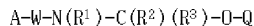
【課題】 血清中での安定性に優れ、かつ有効成分である医薬化合物を腫瘍部位などに対して部位選択的に移行させることができるDDS化合物を提供する。

【解決手段】 式(1)：A-W-N(R¹)-C(R²)(R³)-O-Q (Aは薬物担体である高分子を示し；Wは式中の窒素原子にC末端で結合する1個のアミノ酸残基を含むスペーサー又は該窒素原子にC末端で結合するペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーを示し；R¹、R²、及びR³は水素原子、アルキル基、アリール基、カルボキシル基、又はアルコキシカルボニル基を示し、R¹、R²、及びR³のうちいずれか2つが互いに結合して4から8員環を形成してもよく；O-Qは水酸基を有する医薬化合物の残基を示す)で表されるDDS化合物。

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【特許請求の範囲】

【請求項1】 下記の式(I)：



(式中、Aは薬物担体である高分子を示し；Wは式(I)中の窒素原子にC末端で結合する1個のアミノ酸残基を含むスペーサー又は式(I)中の窒素原子にC末端で結合するペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーを示し；R¹、R²、及びR³はそれぞれ独立に水素原子、置換基を有することもあるアルキル基、置換基を有することもあるアリール基、カルボキシル基、又はアルコキシカルボニル基を示し、R¹、R²、及びR³のうちいずれか2つが互いに結合して4から8員環を形成してもよく；O-Qは水酸基を有する医薬化合物の残基を示す)で表されるDDS化合物。

【請求項2】 薬物担体がカルボキシル基を有する多糖誘導体である請求項1に記載のDDS化合物。

【請求項3】 Wがペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーである請求項1又は2に記載のDDS化合物。

【請求項4】 R¹、R²、及びR³が水素原子である請求項1から3のいずれか1項に記載のDDS化合物。

【請求項5】 カルボキシル基を有する多糖誘導体がカルボキシC₁₋₄アルキルデキストランポリアルコールである請求項2から4のいずれか1項に記載のDDS化合物。

【請求項6】 カルボキシC₁₋₄アルキルデキストランポリアルコールを構成するデキストランポリアルコールが、実質的に完全にポリアルコール化可能な条件下でデキストランを処理して得られたデキストランポリアルコールである請求項5に記載のDDS化合物。

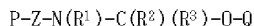
【請求項7】 カルボキシC₁₋₄アルキルデキストランポリアルコールがカルボキシメチルデキストランポリアルコールである請求項5又は6に記載のDDS化合物。

【請求項8】 医薬化合物が抗腫瘍剤又は抗炎症剤である請求項1から7のいずれか1項に記載のDDS化合物。

【請求項9】 医薬化合物が1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジノールである請求項8に記載のDDS化合物。

【請求項10】 Wが-Gly-Gly-Phe-Gly-である請求項9に記載のDDS化合物。

【請求項11】 下記の式(II)：



(式中、Pは水素原子又はアミノ基の保護基を示し；Zは式(II)中の窒素原子にC末端で結合する1個のアミノ酸残基又は式(II)中の窒素原子にC末端で結合するペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を示し；R¹、R²、及びR³はそれぞれ

独立に水素原子、置換基を有することもあるアルキル基、置換基を有することもあるアリール基、カルボキシル基、又はアルコキシカルボニル基を示し、R¹、R²、及びR³のうちいずれか2つが互いに結合して4から8員環を形成してもよく；O-Qは水酸基を有する医薬化合物の残基を示す)で表される化合物。

【請求項12】 R¹、R²、及びR³が水素原子であり、医薬化合物が1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジノールであり、Zが-Gly-Gly-Phe-Gly-である請求項11に記載の化合物。

【請求項13】 請求項1ないし10のいずれか1項に記載のDDS化合物の製造に用いる請求項11又は12に記載の化合物。

【発明の詳細な説明】

【0001】

【発明の属する技術分野】本発明は、多糖誘導体などの薬物担体と抗腫瘍剤などの医薬化合物とをスペーサーを介して結合させたドラッグデリバリーシステム(DDS)化合物に関するものである。

【0002】

【従来の技術】肺癌や消化器癌などの固形癌や白血病などの血液癌の治療に際して用いられる抗腫瘍剤は、静脈内投与や経口投与などの投与経路により全身的に投与された後、特定の腫瘍部位に移行して癌細胞の増殖を阻害しない抑制することにより治療効果を発揮する。しかしながら、全身投与された抗腫瘍剤は、血中から肝臓、細網内皮系臓器に速やかに取り込まれたり、あるいは速やかに尿中排泄されるために、血中濃度が低下して腫瘍部位への移行が制限される場合がある。また、通常の抗腫瘍剤自体の腫瘍部位への移行選択性(腫瘍選択性)が低いために、抗腫瘍剤が全身の様々な細胞や組織に広く分布してしまい、正常な細胞や組織に対しても細胞毒として作用し、下痢、発熱、嘔吐、あるいは脱毛などの副作用をさわめて高率に発生させるという問題がある。従って、抗腫瘍剤を効率的かつ選択的に腫瘍部位に移行させる手段の開発が求められている。

【0003】このような手段の一つとして、多糖誘導体を薬物担体として用い、該多糖誘導体に対して抗腫瘍剤を結合させて抗腫瘍剤の血中における消失を遅延させるとともに、癌組織への指向性を高める方法が提案されている。カルボキシル基を有する多糖のカルボキシル基にペプチド鎖を介して薬剤を結合させたもの(国際公開W0094/19376号)、カルボキシルメチル化されたマンノグルカン誘導体にシッフ塩基やアミド結合を介して薬剤を導入したもの(特公平7-84481号)、ポリアルコール化多糖誘導体を薬物担体として用い、ペプチド鎖を介して、さらにはペプチド鎖およびパラアミノベンジルオキシカルボニル基を介して薬剤を結合させ

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たもの(国際公開WO99/61061号)等が開示されている。

【0004】しかしながら、これらの手段は、薬物担体等に薬剤を結合させる際、主に医薬化合物のアミノ基を介して結合させることが志向されたものであったため、医薬化合物の水酸基を介して上記担体等への結合を行った場合、DDS化合物本来の部位選択性が発揮されない場合があった。すなわち、医薬化合物の水酸基を介して上記薬物担体等への結合を行った場合、該DDS化合物の血清中での安定性が著しく低く、血中で医薬化合物が遊離してしまうため、部位選択的な薬物移行が達成されないという問題が生じていた。

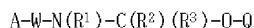
【0005】

【発明が解決しようとする課題】本発明の課題は、抗腫瘍剤や抗炎症剤など水酸基を有する医薬化合物と薬物担体とを1から8個のアミノ酸を含むスペーサーを介して結合させたDDS化合物であって、血清中での安定性に優れ、かつ有効成分である医薬化合物を腫瘍部位などに対して部位選択的に移行させることができるDDS化合物を提供し、さらには水酸基を有する抗腫瘍剤等の副作用の低減を可能とすることにある。

【0006】

【課題を解決するための手段】本発明者らは上記の課題を解決すべく鋭意努力した結果、抗腫瘍剤や抗炎症剤などの水酸基を有する医薬化合物と薬物担体とを1から8個のアミノ酸を含むスペーサーを介して結合させたDDS化合物において、該スペーサーと医薬化合物との間にさらに置換又は無置換のアミノメチレンリンカーを挿入すると、血清中での安定性に優れ、かつ医薬化合物を腫瘍部位などに対して確実かつ部位選択的に移行させることができるDDS化合物を提供できることを見出した。さらに、本発明のDDS化合物は、医薬化合物単独で用いた場合に比べ、下痢等の副作用が低減されることを見出した。本発明はこれらの知見を基にして完成されたものである。

【0007】すなわち、本発明は、下記の式(I)：



(式中、Aは薬物担体である高分子を示し；Wは式(I)中の窒素原子にC末端で結合する1個のアミノ酸残基を含むスペーサー又は式(I)中の窒素原子にC末端で結合するペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーを示し； R^1 、 R^2 、及び R^3 はそれぞれ独立に水素原子、置換基を有することもあるアルキル基、置換基を有することもあるアリール基、カルボキシル基、又はアルコキシカルボニル基を示し、 R^1 、 R^2 、及び R^3 のうちいずれか2つが互いに結合して4から8員環を形成してもよく；O-Qは水酸基を有する医薬化合物の残基を示す)で表されるDDS化合物を提供するものである。

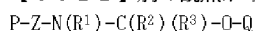
【0008】上記発明の好ましい態様によれば、薬物担

体がカルボキシル基を有する多糖誘導体である上記のDDS化合物；Wがペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーである上記のDDS化合物； R^1 、 R^2 、及び R^3 が水素原子である上記のDDS化合物；カルボキシル基を有する多糖誘導体がカルボキシ C_{1-4} アルキルデキストランポリアルコールである上記のDDS化合物；カルボキシ C_{1-4} アルキルデキストランポリアルコールを構成するデキストランポリアルコールが、実質的に完全にポリアルコール化可能な条件下でデキストランを処理して得られたデキストランポリアルコールである上記のDDS化合物；及びカルボキシ C_{1-4} アルキルデキストランポリアルコールがカルボキシメチルデキストランポリアルコールである上記のDDS化合物が提供される。

【0009】さらに好ましい態様によれば、医薬化合物が抗腫瘍剤又は抗炎症剤である上記のDDS化合物；医薬化合物が1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジノールである上記のDDS化合物；及びWが-Gly-Gly-Phe-Glyである上記のDDS化合物が提供される。

【0010】別の観点からは、上記式(I)で表されるDDS化合物を含む医薬が本発明により提供される。この医薬は、医薬化合物の種類に応じて、腫瘍の治療や炎症性疾患の予防及び／又は治療などに用いることができる。さらに本発明により、腫瘍の治療方法であって、上記式(I)で表されるDDS化合物の治療有効量をヒトを含む哺乳類動物に投与する工程を含む方法が提供される。また、炎症性疾患の予防及び／又は治療方法であって、上記式(I)で表されるDDS化合物の予防及び／又は治療有効量をヒトを含む哺乳類動物に投与する工程を含む方法が提供される。

【0011】別の観点からは、下記の式(II)：



(式中、Pは水素原子又はアミノ基の保護基を示し；Zは式(II)中の窒素原子にC末端で結合する1個のアミノ酸残基又は式(II)中の窒素原子にC末端で結合するペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を示し； R^1 、 R^2 、及び R^3 はそれぞれ独立に水素原子、置換基を有することもあるアルキル基、置換基を有することもあるアリール基、カルボキシル基、又はアルコキシカルボニル基を示し、 R^1 、 R^2 、及び R^3 のうちいずれか2つが互いに結合して4から8員環を形成してもよく；O-Qは水酸基を有する医薬化合物の残基を示す)で表される化合物が提供される。

【0012】この化合物の好ましい態様によれば、 R^1 、 R^2 、及び R^3 が水素原子であり、医薬化合物が1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジノールであり、Zが-G

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ly-Gly-Phe-Gly- である上記の化合物が提供される。式(II)で表される化合物は、上記式(I)で表されるDDS化合物の製造に用いることができる。

【0013】

【発明の実施の形態】本発明により提供されるDDS化合物は、式(I)： $A-W-N(R^1)-C(R^2)(R^3)-O-Q$ で表される。式中、 $O-Q$ は水酸基を有する医薬化合物の残基を示す（式中のOは該水酸基を構成する酸素原子を示す）が、本発明のDDS化合物に含まれる医薬化合物の残基は、例えば、抗腫瘍剤、抗炎症剤、抗菌剤などの医薬としてヒトを含む哺乳類の病気の治療及び／又は予防に用いられる医薬化合物の主要な部分構造を意味している。もっとも、該医薬化合物の用途は上記のものに限定されることはなく、医薬化合物としては、1又は2以上の水酸基を有するものであればいかなるものを用いてもよい。本明細書において医薬化合物という場合には、それ自体が医薬作用を有する化合物の主要構造をその部分構造として含み生体内で該化合物を再生することができるプロドラッグ化合物も含まれる。

【0014】本明細書において、医薬化合物の残基とは、1又は2以上の水酸基を有する医薬化合物の1つの水酸基から水素原子を除いて得られる残基を意味している。すなわち、本明細書において、医薬化合物を $H-O-Q$ （式中の $H-O$ は医薬化合物が有する1又は2以上の水酸基のうちの1つの水酸基を示す）で表し、医薬化合物の残基を $O-Q$ で表す。

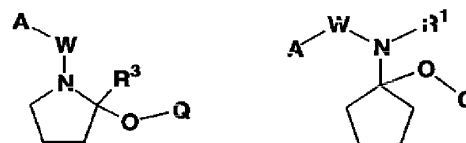
【0015】医薬化合物の種類は特に限定されないが、例えば、抗腫瘍剤または抗炎症薬等が好適である。抗腫瘍剤としては、例えば、5'-デオキシ-5-フルオロウリジン（ドキシフルリジン）、5'-デオキシ-5-フルオロシチジン等の5-フルオロウリジン、5-フルオロシチジン誘導体、エトボシド等のボドフィロトキシン誘導体、7-エチル-9-ヒドロキシカンプトテシン等の水酸基を有するカンプトテシン誘導体、タキソールもしくはその誘導体、チャートリュウシン（Chartreusin）もしくはその誘導体、9-ヒドロキシエリプチシン等の水酸基を有するエリプチシン誘導体、ラジシコル（radicicol）またはオキシミジン I（oximidine I）等のフェノール性水酸基を有する制癌性マクロライド等を用いることができる。抗炎症薬としては、例えば、ヒドロコルチゾン、プレドニゾロンなどのステロイド系抗炎症剤を用いることができる。

【0016】式(I)及び式(II)において、 R^1 、 R^2 、及び R^3 はそれぞれ独立に水素原子、置換基を有することもあるアルキル基、置換基を有することもあるアリール基、カルボキシル基、又はアルコキシカルボニル基を示す。 R^1 、 R^2 、及び R^3 のうちいずれか2つが互いに結合して4から8員環を形成してもよい。 R^1 、 R^2 、及び R^3 が示すアルキル基は、直鎖状、分枝鎖状、環状、又はそれらの組み合わせのいずれでもよく、例えば、炭素

数1〜10個、好ましくは炭素数1〜6個、より好ましくは炭素数1〜4個のアルキル基を用いることができる。アルキル基として、例えば、メチル基、エチル基、n-プロピル基、イソプロピル基、シクロプロピル基、n-ブチル基、sec-ブチル基、tert-ブチル基、イソブチル基、シクロブチル基、シクロプロピルメチル基、n-ペンチル基、n-ヘキシル基などを挙げることができる。 R^1 、 R^2 、及び R^3 のうちいずれか2つが互いに結合して4から8員環を形成してもよく、該環上には1又は2個以上のアルキル基が置換していてもよい。 R^1 及び R^2 が結合して環を形成する場合、及び R^2 及び R^3 が結合して環を形成する場合の例としては、それぞれ下記の例を挙げることができる。

【0017】

【化1】



【0018】上記アルキル基が置換基を有する場合、置換基の種類、並びに置換基の個数及び置換位置は特に限定されず、2個以上の置換基が存在する場合には、それらは同一でも異なってもよい。アルキル基上に存在可能な置換基の例としては、例えば、水酸基、ハロゲン原子（フッ素原子、塩素原子、臭素原子、又はヨウ素原子のいずれでもよい）、アルコキシ基（メトキシ基、エトキシ基、n-プロポキシ基など）、置換若しくは無置換のアリールオキシ基（フェノキシ基など）、アミノ基、モノアルキルアミノ基（メチルアミノ基、エチルアミノ基など）、ジアルキルアミノ基（ジメチルアミノ基、ジエチルアミノ基、エチルメチルアミノ基など）、メルカプト基、アルキルチオ基（メチルチオ基、エチルチオ基など）、アミジノ基、グアニジノ基、ウレイド基、カルボキシル基、アルコキシカルボニル基、アリール基（置換若しくは無置換のフェニル基など）を挙げることができる。

【0019】 R^1 、 R^2 、及び R^3 が示すアリール基が置換基を有する場合、置換基の種類、並びに置換基の個数及び置換位置は特に限定されず、2個以上の置換基が存在する場合には、それらは同一でも異なってもよい。置換基としては、上記のアルキル基について例示した置換基を用いることができる。

【0020】また、アルキル基について例示した上記の置換基において、アリール環が置換基を有する場合、置換基の種類、並びに置換基の個数及び置換位置は特に限定されず、2個以上の置換基が存在する場合には、それらは同一でも異なってもよい。アリール環上の置換基としては、例えば、アルキル基、ハロゲン化アルキル

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基、ヒドロキシアルキル基、アルコキシル基（メトキシ基、エトキシ基など）、アルケニル基（ビニル基、アリル基など）、アルキニル基（プロパルギル基など）、水酸基、ハロゲン原子（フッ素原子、塩素原子、臭素原子、又はヨウ素原子のいずれでもよい）、カルボキシル基、アルコキシカルボニル基（メトキシカルボニル基など）、アルカノイル基（アセチル基など）、ハロアルカノイル基（トリフルオロアセチル基など）、アリール基（フェニル基、ナフチル基など）、アラールキル基（ベンジル基など）、アリールオキシ基（フェノキシ基など）、アラールキルオキシ基（ベンジルオキシ基など）、アロイル基（ベンゾイル基など）、ヘテロアリール基（ピリジル基、キノリル基など）、アミノ基、モノアルキルアミノ基、ジアルキルアミノ基、カルバモイル基、ニトロ基、シアノ基、アミジノ基、グアニジノ基、ウレイド基、アルキルスルフィニル基、アルキルスルホニル基、チオール基、又はアルキルチオ基などを挙げることができるが、これらに限定されることはない。

【0021】Wで表されるスペーサーとしては、1個のアミノ酸残基を含むスペーサー又はペプチド結合した2から8個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーを用いることができる。該スペーサーは、1個のアミノ酸の残基（アミノ酸のアミノ基及びカルボキシル基からそれぞれ1個の水素原子及び1個の水酸基を除いた残基を意味する）、又はペプチド結合した2ないし8個のアミノ酸残基を含むオリゴペプチドの残基（N末端のアミノ基及びC末端のカルボキシル基からそれぞれ1個の水素原子及び1個の水酸基を除いた残基を意味する）の形態を有しており、C末端（1個のアミノ酸を含むスペーサーの場合にはカルボキシル基）で $-N(R^1)-C(R^2)(R^3)-O-Q$ で表される基の窒素原子にペプチド結合するように配置される。

【0022】一般的には、Aで表される薬物担体とスペーサーとの結合は、薬物担体のカルボキシル基とスペーサーのN末端（1個のアミノ酸を含むスペーサーの場合にはアミノ基）とのペプチド結合により形成される。もっとも、スペーサーと薬物担体との結合は上記のペプチド結合に限定されることなく、他の化学結合や1又は2以上のスペーサーを利用した結合であってもよい。例えば、スペーサー中に1個又は2個以上のジカルボン酸化合物の残基（例えばコハク酸などのジカルボン酸の残基など）を構成単位として含めて両末端がカルボキシル基となるようなスペーサーとした場合には、薬物担体中の反応性アミノ基などを結合に利用できる。

【0023】好ましいスペーサーは2から6個のアミノ酸残基で構成されるオリゴペプチド残基を含むスペーサーである。スペーサーを構成するアミノ酸の種類は特に限定されないが、例えば、L-又はD-アミノ酸、好ましくはL-アミノ酸を用いることができ、 α -アミノ酸のほか、 β -アラニン、 ϵ -アミノカプロン酸、 γ -アミノ酪酸

などを用いてもよい。このような α -アミノ酸以外のアミノ酸は、スペーサー中で薬物担体に近接した位置に配置されることが好ましい。

【0024】オリゴペプチド残基を含むスペーサーを用いる場合のアミノ酸配列は特に限定されないが、例えば、スペーサーが-S-T-で表されるジペプチドの残基（Sは疎水性アミノ酸の残基を示し、Tは親水性アミノ酸の残基を示し、-S-T-は疎水性アミノ酸(S)と親水性アミノ酸(T)とがそれぞれN末端側及びC末端側となってペプチド結合したジペプチドのN末端のアミノ基及びC末端のカルボキシル基からそれぞれ1個の水素原子及び1個の水酸基を除いた残基を意味する）であるか、又は該ジペプチドの残基を部分ペプチド配列として含むスペーサーを好適に用いることができる。疎水性アミノ酸としては、例えば、フェニルアラニン、チロシン、ロイシンなどを用いることができ、親水性アミノ酸としては、例えば、グリシン、アラニンなどを用いることができる。スペーサーがこのようなジペプチド残基の繰り返し配列（例えば-S-T-S-T-, -S-T-S-T-S-T-など）を有していてもよい。

【0025】このようなジペプチド構造を含むスペーサーを用いると、スペーサーがペプチダーゼが豊富であると考えられる腫瘍部位や炎症部位で加水分解され、当該部位において短時間に高濃度の医薬化合物が遊離するので、上記ジペプチドを含むスペーサーと医薬化合物とが結合して形成される部分構造は、本発明のDDS化合物の好ましい部分構造である。医薬化合物の残基として、濃度に依存した抗腫瘍作用を発現する抗腫瘍剤（より高い濃度でより強い抗腫瘍作用を発現する抗腫瘍剤：濃度依存型の抗腫瘍剤、例えば、ドキソルビシンなど）の残基を用いる場合には、-X-Z-で示される上記のジペプチド残基からなるスペーサー又は該ジペプチド残基を部分ペプチド配列として含むスペーサーを用いることが好ましい。

【0026】一方、医薬化合物の残基として、一定の濃度以上で作用時間の持続を必要とする抗腫瘍剤（例えば、メトトレキサートなど）を用いる場合にも、上記のスペーサーを用いることによって高い抗腫瘍効果を達成できる場合があるが、一般的には、上記のスペーサーに限定されることなく、抗腫瘍剤の体内動態の特徴や毒性などの観点から好ましいスペーサーを選択する必要がある。なお、一般的に、増殖の速い癌種に対しては、短時間に高濃度の医薬化合物を遊離することができる上記のスペーサーを選択することが好ましい。特に、本発明のDDS化合物における医薬化合物の遊離速度は、スペーサー部分の酵素的分解が実質的な律速段階になっているので、適宜のスペーサーを酵素的分解速度の見地から選択することによって医薬化合物の所望の遊離速度を容易に達成できる。

【0027】スペーサーとして利用可能なオリゴペプチ

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ド残基の具体例を以下の表に示すが、本発明のDD S化合物に用いられるスペーサーは以下のものに限定されることはなく、スペーサー種類の選択は、医薬化合物の至適な遊離速度を与えるように当業者が適宜なしうることはいうまでもない（表中、ペプチド配列は左側がN末端であり、C末端側に-N(R¹)-C(R²)(R³)-O-Q で表される基の窒素原子がペプチド結合する。D-Phe はD-フェニルアラニン残基を示し、その他のアミノ酸はL-アミノ酸を示す。なお、遊離速度の大小はドキソルビシンを結合したDD S化合物のWalker 256 担癌ラットに対する薬効の発現の程度、または Walker 256 担癌ラットの腫瘍部位における遊離ドキソルビシン濃度によって判定した。））。例えば、ドキソルビシンに対しては（N 末端）-Gly-Gly-Phe-Gly-等の短時間に高濃度の医薬化合物を遊離することができるスペーサーを用いることが好ましい。

【0028】

【表1】

(a) 遊離速度が大きいスペーサー

-Leu-Gly-

-Tyr-Gly-

-Phe-Gly-

-Gly-Phe-Gly-

-Gly-Gly-Phe-Gly-

-Gly-Phe-Gly-Gly-

-Phe-Gly-Gly-Gly-

-Phe-Phe-Gly-Gly-

-Gly-Gly-Gly-Phe-Gly-

(b) 遊離速度が比較的大きいスペーサー

-Gly-Gly-Phe-Phe-

-Gly-Gly-Gly-Gly-Gly-Gly-

(c) 遊離速度が比較的小さいスペーサー

-Phe-Phe-

-Ala-Gly-

-Pro-Gly-

-Gly-Gly-Gly-Phe-

(d) 遊離速度が小さいスペーサー

-Gly-

-D-Phe-Gly-

-Gly-Phe-

-Ser-Gly-

-Gly-Gly-

-Gly-Gly-Gly-

-Gly-Gly-Gly-Gly-

【0029】上記式(II)で表される化合物におけるZとしては、上記のWで表されるスペーサーについて説明したアミノ酸残基又はオリゴペプチド残基を用いることができる。Pは水素原子又はアミノ酸の保護基を示す。アミノ基の保護基の種類は特に限定されないが、例えば、プロテクティブ・グループス・イン・オーガニック・シンセシス (Protective Groups in Organic Synthesis)、グリーン (T. W. Greene) 著、ジョン・ワイリー・アンド・サンズ・インコーポレイテッド (John Wiley & Sons Inc.) (1981 年) に具体的に記載されている

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ものを好適に用いることができる。

【0030】Aで表される薬物担体としては、多糖誘導体のほか、合成高分子などを用いることができる。多糖誘導体及び合成高分子としては、生体に対して実質的に毒性を示さず、薬物担体として作用できるものであればいかなるものを用いてもよい。例えば、DDS化合物の製造に従来より用いられている多糖誘導体及び合成高分子は本発明のDDS化合物にいずれも利用可能である。例えば、カルボキシル基を有する多糖誘導体は本発明のDDS化合物に好適に使用でき、ポリアルコール化多糖誘導体は特に好適である。また、合成高分子としては、例えば、ポリエチレングリコール類；ポリグルタミン酸、ポリアスパラギン酸、若しくはポリリジンなどのポリアミノ酸類；またはN-(2-ヒドロキシプロピル)メタクリルアミド誘導体などのポリビニル化合物の誘導体を用いることができる。

【0031】例えば、デキストラン、イヌリン等の多糖類、カルボキシメチルデキストラン、カルボキシメチルマンノグルカン等のカルボキシルアルキル基が導入された多糖誘導体、W097/46260に開示されるカルボキシ C_{1-4} アルキルデキストランポリアルコール誘導体等の多糖から誘導されるポリアルコールのカルボキシルアルキル化された誘導体、N-(2-ヒドロキシプロピル)メタクリルアミドとアクリル酸の共重合高分子（HPMA）等の共重合高分子、ポリ(L-リジン)、ポリ(L-アスパラギン酸)等の合成ポリ(アミノ酸)、ヒト血清アルブミン等の蛋白質、ポリエチレングリコール等の末端に水酸基を有するポリエーテル等を用いることができるがこれらに限定されることはない。

【0032】より具体的には、カルボキシル基を有する多糖誘導体としては、例えば、多糖類又はそれらを化学的若しくは生物学的に修飾した誘導体であって分子中にカルボキシル基を有するものであればいかなるものを用いてもよい。例えば、ヒアルロン酸、ヘクチン酸、アルギン酸、コンドロイチン、ヘパリンなどの多糖類のほか、プルラン、デキストラン、マンナン、キチン、イヌリン、レバン、キシラン、アラバン、マンノグルカン、キトサン、プルランなどの多糖の一部又は全部の水酸基に対してカルボキシル基を有する官能基を導入したものなどを用いることができる。

【0033】例えば、水酸基をカルボキシ C_{1-4} アルキル化したものや、水酸基が多塩基酸の1つのカルボキシル基をエステル結合させたものなどを好適に用いることができる。また、上記の多糖類をポリアルコール化した後に、カルボキシル基を有する官能基を導入したものを用いてもよい。本明細書において用いられる多糖誘導体という用語には、糖を構成要素として含む多糖化合物のほか、多糖化合物の環状糖部分を部分的又は完全に開環して得られる化合物（例えばポリアルコール化合物など）を含めて、最も広義に解釈する必要がある。これらの多

糖誘導体のうち、カルボキシ C_{1-4} アルキルデキストランポリアルコールやカルボキシ C_{1-4} アルキルプルランポリアルコールなどを用いることが好ましい。以下、本発明のDDS化合物の製造にカルボキシ C_{1-4} アルキルデキストランポリアルコールを利用する場合についてより具体的に説明するが、本発明のDDS化合物における薬物担体はカルボキシ C_{1-4} アルキルデキストランポリアルコールに限定されることはない。

【0034】本発明の薬物担体の製造に用いるカルボキシ C_{1-4} アルキルデキストランポリアルコールのポリアルコール化度は特に限定されないが、カルボキシ C_{1-4} アルキルデキストランポリアルコールを構成するデキストランポリアルコールが、実質的に完全にポリアルコール化可能な条件下においてデキストランを処理して得られたデキストランポリアルコールであることが好ましい。

【0035】カルボキシ C_{1-4} アルキルデキストランポリアルコールを製造するために用いるデキストランの種類は特に限定されず、 α -D-1,6-結合を任意の割合で含んでいてもよい。例えば、 α -D-1,6-結合の割合が85%以上、90%以上、又は95%以上のデキストランなどを用いることができる。デキストランの分子量は特に限定されないが、例えば10,000程度から2,000,000程度のもの、好ましくは50,000程度から800,000程度のものを用いることができる。カルボキシ C_{1-4} アルキルデキストランポリアルコールのカルボキシ C_{1-4} アルキル基を構成する C_{1-4} アルキルとしては、直鎖又は分枝鎖の C_{1-4} アルキル、具体的にはメチル基、エチル基、n-プロピル基、イソプロピル基、n-ブチル基、sec-ブチル基などを用いることができるが、好ましくはメチル基を用いることができる。

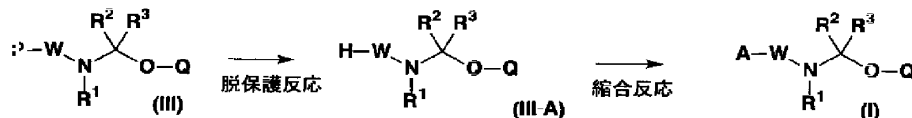
【0036】出発原料としてデキストランを用いる場合には、デキストランに大過剰の過ヨウ素酸ナトリウムと水素化ホウ素ナトリウムとを順次作用させてデキストランを実質的に完全にポリアルコール化したデキストランポリアルコールを製造することができる。もっとも、デキストランのポリアルコール化の方法は上記のものに限定されることはなく、当業者に利用可能なものであればいかなる方法を採用してもよい。カルボキシ C_{1-4} アルキル化は、例えば、デキストランポリアルコールの水酸基に対してクロル酢酸、ブロム酢酸、 α -クロロプロピオン酸、 α -メチル α -クロロプロピオン酸、 β -クロロプロピオン酸、 α -メチル β -クロロプロピオン酸、 α -クロロ酪酸、 β -クロロ酪酸、 γ -クロロ酪酸などのハロゲン化 C_{1-4} アルキルカルボン酸、好ましくはクロル酢酸を反応させて水酸基を部分的又は完全にカルボキシ C_{1-4} アルキル化することにより行うことができる。

【0037】例えば、デキストランポリアルコールを反応に関与しない不活性溶媒（例えば、水、N,N-ジメチルホルムアミド、ジメチルスルホキシド等）に溶解し、塩

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基(例えば、水酸化ナトリウムや水酸化カリウム等)の存在下にハロゲン化 C_{1-4} アルキルカルボン酸またはその塩を添加し、氷冷下ないし100℃程度の温度範囲で数分ないし数日間反応させればよい。カルボキシ C_{1-4} アルキル基の導入の程度は、例えば、カルボキシ C_{1-4} アルキル化の反応温度や試薬として用いるハロゲン化 C_{1-4} アルキルカルボン酸及び塩基の量を適宜選択することにより容易に調節可能であり、そのような手段は当業者に周知である。デキストランポリアルコールの水酸基に対するカルボキシ C_{1-4} アルキル化の程度は特に限定されないが、例えば、構成糖残基あたり0.01~2.0の範囲、好ましくは0.1~1.0の範囲である。このようにして、カルボキシ C_{1-4} アルキルデキストランポリアルコールのナトリウム塩又はカリウム塩などのアルカリ金属塩の形態の水溶液を調製することができる。

【0038】上記のようにして調製されるアルカリ金属塩の形態の多糖誘導体のほか、有機アミンの塩の形態の多糖誘導体を薬物担体の原料として用いてもよい。有機アミンの塩の形態の多糖誘導体は、実質的に水を含まない有機溶媒中に高濃度に溶解できるので、この塩を用いると反応を非水系で行うことができ、反応効率を顕著に高めることができる場合がある。有機アミンの塩としては、例えば、トリエチルアミン、トリメチルアミン、トリエタノールアミンなどの脂肪族アミン類の塩のほか、N-メチルピロリジン、N-メチルピペリジン、N-メチルモルホリン、ジメチルアミノピリジンなどの脂環式又は芳香族アミン類の塩、塩化テトラメチルアンモニウム、塩化テトラエチルアンモニウムなどの四級アンモニウム塩などを用いることができる。



【0042】多糖誘導体を用いた反応を非水系で行う場合には、実質的に水を含まない有機溶媒であって、反応種(多糖誘導体の有機アミンの塩及び遊離又は塩の形態の式(III-A)の化合物)を溶解することができるものならばいかなるものを用いてもよい。例えば、N,N-ジメチルホルムアミド、ジメチルスルホキシド、アセトアミド、N-メチルピロリドン、スルホランなどを用いることが好適である。薬物担体に導入される医薬化合物残基の量は特に限定されないが、医薬化合物残基の種類、並びにDDS化合物の体内動態、薬効、及び毒性などの観点から適宜選択すべきである。一般的には、0.1~30重量%、好ましくは2~15重量%程度の範囲を選択することができる。薬物担体に導入された医薬化合物残基の割合は、例えば、吸光度分析などにより容易に決定することが可能である。

【0043】上記式(III)で表される化合物は、例え

【0039】多糖誘導体のナトリウム塩から有機アミンの塩への変換は、イオン交換樹脂などを用いて行うことができる。例えば、カルボキシメチルデキストランポリアルコールのナトリウム塩を水に溶解し、Bio-Rad AG50 W-X2 (200-400 メッシュ、H⁺型)樹脂を充填したカラムに付して水で溶出した後、トリエチルアミンなどの有機アミンを添加して凍結乾燥することができる。また、カルボキシメチルデキストランポリアルコールのナトリウム塩を水に溶解し、トリエチルアンモニウム型の樹脂を通過させることによって一工程で変換を行うことも可能である。

【0040】本発明の式(I)で表されるDDS化合物は、例えば、下記のスキームに従って製造することができる。式(III)で表されるアミノ酸残基又はオリゴペプチド残基のN末端の保護基を適当な脱保護反応により除去して式(III-A)の化合物を得た後、薬物担体のカルボキシル基を酸アミド結合により結合させる方法により製造することができる。酸アミド結合の形成には、ペプチド鎖の合成に用いる通常の脱水縮合剤、例えば、N,N'-ジシクロヘキシルカルボジイミド(DCC)のようなN,N'-ジシクロアルキルカルボジイミド類、1-エチル-3-(3-ジメチルアミノプロピル)カルボジイミド(EDAPC)等のカルボジイミド誘導体、1-ヒドロキシベンゾトリアゾール(HOBT)のようなベンゾトリアゾール誘導体のほか、1-エトキシカルボニル-2-エトキシ-1,2-ジヒドロキシキノリン(EDQ)などを用いることができる。また、活性エステル法や酸ハライド法などにより反応を行ってもよい。

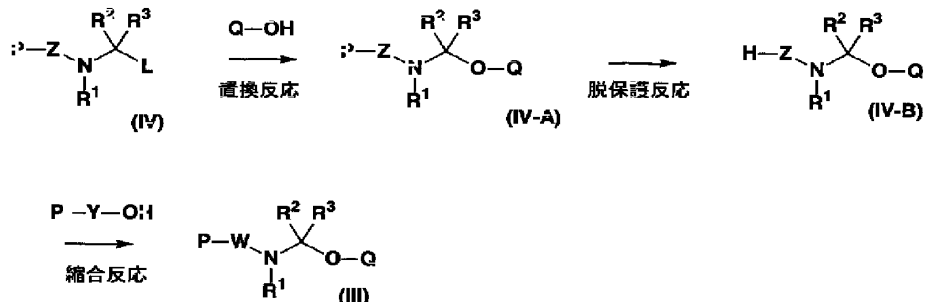
【0041】

【化2】

ば、下記のスキームに示す方法で製造することができる。式(IV)で表される化合物(式中、Lはアセトキシ基又は水酸基などを示す)と水酸基を有する医薬化合物とを、塩基性条件下又は酸触媒条件下で置換反応に付することにより式(IV-A)で表される化合物を得ることができる。式(IV-A)で表される化合物のN末端アミノ基の保護基を適当な条件下で脱保護した後、必要に応じてペプチド鎖を伸長することにより式(III)で表される化合物を得ることができる。式(IV)で表される化合物は、例えば、N末端アミノ基が保護されたオリゴペプチドを四酢酸鉛と適当な溶媒中で処理することにより得ることができる(A. P. Gledhill et. al., J. Org. Chem., 51, 3196-3201, 1986)。または、末端アミノ基が保護されたオリゴペプチドの酸アミドまたはアミノ基が保護されたアミノ酸の酸アミドをアルデヒド又はケトンと処理することにより得ることができる。

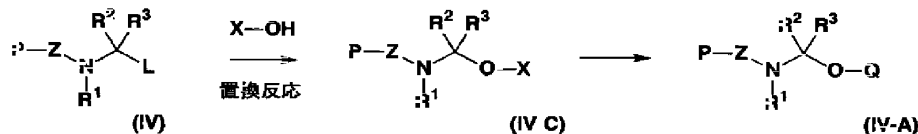
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【0044】



(スキーム中、Y はアミノ基が保護された1～8個のアミノ酸からなるオリゴペプチドを示す。)

【0045】化合物(IV-A)は、例えば、下記のスキームに示すように式(IV)で表される化合物を水酸基を有する医薬化合物の合成中間体X-OHとの置換反応に付して式(I



【0047】なお、本発明のDDS化合物の製造方法のさらに具体的な例を実施例に示した。当業者は、上記の一般的な説明及び実施例の具体的な説明を基にして、出発原料や反応試薬を適宜選択することにより、また必要に応じて反応条件や工程に適宜の修飾ないし改変を加えることにより、上記一般式(I)に包含される本発明のDDS化合物及び式(II)に包含される化合物を製造することができる。

【0048】本発明のDDS化合物は、医薬化合物の残基の種類(例えば、抗腫瘍剤または抗炎症剤などの医薬化合物の残基)に応じて、所望の医薬活性を腫瘍部位や炎症部位などの局所において特異的に発現させることができ、かつ、医薬化合物自体の有する毒性を低減できるという特徴を有する。特に、本発明のDDS化合物は、水酸基を有する医薬化合物の部位選択性向上およびそれに伴う副作用低減に有用である。アミノメチレンリンカーを介して水酸基を有する医薬化合物を結合した場合、該DDS化合物は血清中での安定性に優れ、血中での医薬化合物の遊離が著しく低減される。その結果、腫瘍部位や炎症部位などの局所での医薬化合物の選択的な遊離が実現でき、医薬化合物の薬効を保持したまま、副作用を低減できる。低減される副作用としては下痢、嘔吐、発熱、脱毛等が挙げられる。この中でも、急性に発生する下痢の抑制という点においても顕著な効果が見られた。

【0049】本発明のDDS化合物を含む医薬は、通常、凍結乾燥品などの形態でバイアル等に充填することができ、用時溶解型の注射用または点滴用製剤等の非経

V-C)で表される化合物とした後、適当な変換反応を経由して製造することもできる。

【0046】

【化4】

口投与用製剤として臨床に提供されるが、このような医薬の製剤形態は上記態様に限定されることはない。上記製剤の製造には、例えば、溶解補助剤、pH調節剤、安定化剤など当業界で利用可能な製剤用添加物を用いることができる。本発明のDDS化合物の投与量は特に限定されないが、通常は、医薬化合物残基を構成する医薬化合物の投与量、DDS化合物中に導入された医薬化合物の残基の量、患者の状態や疾患の種類などを勘案して決定すべきである。例えば、医薬化合物として抗腫瘍剤である1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジノールの残基が約5重量%程度の割合で導入されたDDS化合物を投与する場合には、非経口投与の場合には、一般に一日あたり体表面積1m²につき約0.1~100mg程度、好ましくは約1~30mgの範囲で一回投与し、3~4週毎に繰り返すことが好ましい。

【0050】

【実施例】以下、実施例により本発明をさらに具体的に説明するが、本発明の範囲は下記の実施例に限定されることはない。以下の実施例中、Boc: tert-ブトキシカルボニル基、AM: アミノメチレンリンカー、Dex: デキストラン、PA: ポリアルコール、Fmoc: 9-フルオレニルメトキシカルボニル基を表し、薬物(1)は1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジノールを表す。

【0051】例1

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tert-ブチル2-[[[(1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロベニル]-5-メチル-1H-ピラゾール-1-イル)-4-ビリミジニル]-3-アゼチジニル]オキシ)メチル]アミノ]-2-オキソエチルカルバメート {Boc-Gly-AM-薬物(1)}

例1-1

[[2-[(tert-ブトキシカルボニル)アミノ]アセチル]アミノ]メチル アセテートBoc-グリシルグリシン (以下、実施例において「Boc-Gly-Gly-OH」と表記し、類似物質についても同様である。) 4.64 g、テトラヒドロフラン (THF) 120 ml、およびトルエン40 mlからなる混合物にピリジン1.62 mlおよび四酢酸鉛 {Pb(OAc)₄} 9.75 gを加え、4時間加熱還流した。反応液を室温まで冷却した後、不溶物をセライト汙過により除き、減圧下で濃縮した。残渣を酢酸エチルに溶解させ、水、次いで飽和食塩水で洗浄した後、有機層を無水硫酸ナトリウムで乾燥した。溶媒を減圧下で留去した後、残渣をシリカゲルカラムクロマトグラフィーに付し、酢酸エチル-ヘキサン (1:1) 混合溶媒で展開して、目的物を含む画分を濃縮することにより標記化合物2.80 gを無色シロップとして得た。¹H-NMR (CDCl₃) δ: 1.46 (s, 9 H), 2.07 (s, 3 H), 3.23 (d, 2 H, J = 5.6 Hz), 5.1-5.2 (m, 1 H), 5.26 (d, 2 H, J = 7.3 Hz), 7.25-7.35 (m, 1 H).

【0052】例1-2

tert-ブチル2-[[[(1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロベニル]-5-メチル-1H-ピラゾール-1-イル)-4-ビリミジニル]-3-アゼチジニル]オキシ)メチル]アミノ]-2-オキソエチルカルバメート {Boc-Gly-AM-薬物(1)}
1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロベニル]-1H-ピラゾール-1-イル)-4-ビリミジニル]-3-アゼチジノール {薬物(1)} 48 mgのTHF 5 ml懸濁液を0℃に冷却した後、ナトリウムヘキサメチルジシラジド (NaHMDS) の1 M THF溶液0.12 mlを加え、10分間攪拌した後、例1-1で得た化合物30 mgのTHF 2 ml溶液を加えた。反応液を0℃で45分攪拌した後、室温でさらに1時間攪拌した。反応液に水を加えた後、クロロホルムで抽出し、有機層を無水硫酸ナトリウムで乾燥した後、溶媒を減圧下で留去した。残渣をプレパラティブTLCに付し、メタノール-クロロホルム (5:95) 混合溶媒で展開し、目的物を含む画分から標記化合物28 mgを得た。

¹H-NMR (CDCl₃) δ: 1.47 (s, 9 H), 2.64 (t, 4 H, J = 5 Hz), 2.65 (s, 3 H), 3.19 (d, 2 H, J = 7 Hz), 3.23 (t, 4 H, J = 5 Hz), 3.83 (d, 2 H, J = 6 Hz), 3.90-4.00 (m, 2 H), 4.20-4.30 (m, 2 H), 4.55-4.60 (m, 1 H), 4.78 (d, 2 H, J = 7 Hz), 4.82 (s, 2 H), 5.15-5.25 (m, 1 H), 6.03 (dt, 1 H, J = 16 Hz, 7 Hz), 6.11 (s, 1 H), 6.25 (tt, 1 H, J = 9 Hz, 2 Hz), 6.37 (dd, 2 H, J = 11 Hz, 2 Hz), 6.38 (d, 1 H, J =

16 Hz), 6.98 (t, 1 H, J = 7 Hz), 7.74 (s, 1 H).

【0053】例1-3 (別途合成法)

tert-ブチル2-[[[(1-ベンズヒドリル-3-アゼチジニル]オキシ)メチル]アミノ]-2-オキソエチルカルバメート 1-ベンズヒドリル-3-アゼチジノール2.72 gおよび例1-1で得た化合物2.80 gをTHF 28 mlに溶解させ、4規定水酸化ナトリウム水溶液2.9 mlを加えた後、室温で4時間攪拌した。反応液に水50 mlを加え、1時間攪拌した後、飽和食塩水を加え、クロロホルムで3回抽出した。有機層を無水硫酸ナトリウムで乾燥させた後、減圧下で濃縮し、残渣をシリカゲルカラムクロマトグラフィーに付し、ヘキサン:酢酸エチル (2:3) 混合溶媒で展開し、目的物を含む画分を濃縮することにより、標記化合物1.11 gを無色シロップとして得た。

¹H-NMR (CDCl₃) δ: 1.43 (s, 9 H), 2.90-2.95 (m, 2 H), 3.45-3.50 (m, 2 H), 3.76 (d, 2 H, J = 6 Hz), 4.26 (m, 1 H), 4.33 (s, 1 H), 4.69 (d, 2 H, J = 7 Hz), 5.00-5.15 (br, 1 H), 6.70-6.85 (m, 1 H), 7.10-7.50 (m, 10 H).

【0054】例1-4 (別途合成法)

tert-ブチル2-[[[(1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロベニル]-5-メチル-1H-ピラゾール-1-イル)-4-ビリミジニル]-3-アゼチジニル]オキシ)メチル]アミノ]-2-オキソエチルカルバメート {Boc-Gly-AM-薬物(1)}

例1-3で得た化合物790 mgをエタノール25 mlに溶解させた後、10%パラジウム-炭素 (50%含水) 500 mgを加え、加圧下 (5 kg/cm²) で20時間接触水素添加を行った。不溶物を汙過により除き、汙液を濃縮した後、残渣をクロロホルムに溶解させ、無水硫酸ナトリウムで乾燥させた。溶媒を減圧下で留去した後、残渣をジメチルホルムアミド40 mlに溶解させ、4-クロロ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロベニル]-5-メチル-1H-ピラゾール-1-イル)-2-ビリミジニルアミン603 mgおよびトリエチルアミン0.177 mlを加えた後、80℃で24時間攪拌した。反応液を減圧下で濃縮した後、残渣をクロロホルムに溶解させ、飽和炭酸水素ナトリウム水溶液、次いで飽和食塩水で洗浄した。有機層を無水硫酸ナトリウムで乾燥させ、減圧下で濃縮した後、残渣をシリカゲルカラムクロマトグラフィーに付し、メタノール:クロロホルム (2:98~4:96) 混合溶媒で展開し、目的物を含む画分を濃縮することにより、標記化合物729 mgを淡黄色アモルファスとして得た。NMRスペクトルは例1-2で合成した化合物と一致した。

【0055】例2

2-アミノ-N-[[[(1-[2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロベニル]-1H-ピラゾール-1-イル)-4-ビリミジニル]-3-アゼチジニル]オキシ)メチル]アセトアミド {H-Gly-AM-薬物(1)}

例1-2で得た化合物28 mgを乾燥ジクロロメタン3 ml

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に溶解させ、0°Cに冷却した後、トリフルオロ酢酸（以下TFAと略）0.3 mlを加え、室温で1時間攪拌した。反応液を減圧下で濃縮した後、残渣をクロロホルムに溶解させ、1規定水酸化ナトリウム水溶液で洗浄した。水層をクロロホルムで抽出した後、有機層を合わせ、無水硫酸ナトリウムで乾燥させた後、溶媒を減圧下で留去した。残渣をプレパラティブTLCに付し、水-メタノール-クロロホルム（7:3:1）分配有機層で展開し、目的物を含む画分から標記化合物12 mgを得た。

¹H-NMR (CDCl₃) δ: 2.6-2.70 (m, 4 H), 2.63 (s, 3 H), 3.18 (d, 2 H, J = 7 Hz), 3.22 (t, 4 H, J = 5 Hz), 3.45 (brs, 2 H), 3.90-4.00 (m, 2 H), 4.20-4.30 (m, 2 H), 4.55-4.65 (m, 1 H), 4.79 (d, 2 H, J = 7 Hz), 4.83 (brs, 2H), 6.02 (dt, 1 H, J = 16 Hz, 7 Hz), 6.09 (s, 1 H), 6.24 (tt, 1 H, J = 9 Hz, 2 Hz), 6.35 (dd, 2 H, J = 11 Hz, 2 Hz), 6.36 (d, 1 H, J = 16 Hz), 7.72 (s, 1 H), 8.11 (brs, 1 H)。

【0056】例3

tert-ブチル13-([1-(2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-5-メチル-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジニル]オキシ)-7-ベンジル-2,5,8,11-テトラオキソ-3,6,9,12-テトラアザトリ-1-デシルカルバメート {Boc-Gly-Gly-Phe-Gly-AM-薬物(1)}

例2で得た化合物12 mgのジメチルホルムアミド(以下DMF2ml)溶液にBoc-Gly-Gly-Phe-OH 33 mg、N-ヒドロキシスクシイミド10 mg、およびジシクロヘキシルカルボジイミド18 mgを0°Cに加え、室温で5時間攪拌した。反応液を減圧下で濃縮した後に、残渣をクロロホルムに溶解させ、1規定水酸化ナトリウム水溶液で洗浄した。水層をクロロホルムで抽出した後、有機層を合わせ、無水硫酸ナトリウムで乾燥させた。溶媒を減圧下で留去して得られた残渣をプレパラティブTLCに付し、メタノール-クロロホルム（1:9）混合溶媒で展開し、目的物を含む画分から標記化合物16 mgを得た。

¹H-NMR (CDCl₃) δ: 1.43 (s, 9 H), 2.60-2.70 (m, 4 H), 2.62 (s, 3 H), 3.05 (dd, 1 H, J = 14 Hz, 8 Hz), 3.18 (d, 2 H, J = 7 Hz), 3.22 (t, 4 H, J = 5 Hz), 3.7-4.0 (m, 9 H), 4.15-4.25 (m, 2 H), 4.50-4.55 (m, 1 H), 4.59 (dd, 1 H, J = 14 Hz, 7 Hz), 4.70 (dd, 1 H, J = 11 Hz, 6 Hz), 4.81 (dd, 1H, J = 11 Hz, 7 Hz), 4.99 (s, 2 H), 5.65-5.75 (m, 1 H), 6.02 (dt, 1 H, J = 16 Hz, 7 Hz), 6.07 (s, 1 H), 6.25 (t, 1 H, J = 9 Hz, 2 Hz), 6.36 (dd, 2 H, J = 11 Hz, 2 Hz), 6.37 (d, 1 H, J = 16 Hz), 7.15-7.30 (m, 5 H), 7.35-7.50 (m, 2 H), 7.55-7.65 (m, 1 H), 7.65-7.70 (m, 1 H), 7.70 (s, 1 H)。

【0057】例4

9H-フルオレン-9-イルメチル 2-[(2-[[[1-(2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジ

ニル]-1-プロペニル]-5-メチル-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジニル]オキシ)メチル]アミノ]-2-オキシエチル]アミノ]-1-ベンジル-2-オキソエチルカルバメート {Fmoc-Phe-Gly-AM-薬物(1)}

例1-2で得た化合物65 mg、N-[(9H-フルオレン-9-イルメトキシ)カルボニル]フェニルアラニン (Fmoc-Phe-OHと標記) 72 mg、およびN-ヒドロキシコハク酸イミド22 mgのDMF 5 ml溶液に、氷冷下DCC 110 mgを加え、徐々に室温に戻しながら24時間攪拌した。反応液を酢酸エチルで希釈した後、飽和炭酸水素ナトリウム水溶液、水、および飽和食塩水で順次洗浄し、無水硫酸ナトリウムで乾燥した後、溶媒を留去した。得られた残渣をシリカゲルカラムクロマトグラフィーに付し、メタノール:クロロホルム（1:9）混合溶媒で展開し、目的物を含む画分を濃縮することにより、標記化合物119 mgを得た。

¹H NMR (CDCl₃) δ: 2.5-2.6 (m, 4 H), 2.64 (s, 3 H), 3.0-3.1 (m, 1 H), 3.18 (d, 2 H, J = 7.8 Hz), 3.2-3.3 (m, 5 H), 3.8-3.9 (m, 1 H), 4.1-4.2 (m, 5 H), 4.3-4.4 (m, 2 H), 4.4-4.5 (m, 2 H), 4.6-4.7 (m, 2 H), 4.7-4.8 (m, 1 H), 6.03 (dt, 1 H, J = 15.9, 6.6 Hz), 6.09 (s, 1 H), 6.25 (t, 1 H, J = 8.3 Hz), 6.36 (d, 2 H, J = 8.3 Hz), 6.38 (d, 1 H, J = 5.9 Hz), 7.1-7.3 (m, 7 H), 7.36 (t, 2 H, J = 7.3 Hz), 7.48 (d, 2 H, J = 7.3 Hz), 7.71 (s, 1 H), 7.72 (d, 2 H, J = 7.3 Hz), 8.01 (s, 1 H)。

【0058】例5

2-アミノ-N-(2-[[[1-(2-アミノ-6-(4-[(E)-3-[4-(3,5-ジフルオロフェニル)-1-ピペラジニル]-1-プロペニル]-5-メチル-1H-ピラゾール-1-イル)-4-ピリミジニル]-3-アゼチジニル]オキシ)メチル]アミノ]-2-オキソエチル)-3-フェニルプロパナミド {H-Phe-Gly-AM-薬物(1)}

例4で得た化合物 119 mgの1,4-ジオキサン（4.0 ml）溶液に、ピペリジン400 (l)を加え2時間攪拌した。反応液を濃縮し、得られた残渣をシリカゲルカラムクロマトグラフィーに付し、メタノール:クロロホルム（1:9）混合溶媒で展開し、目的物を含む画分を濃縮することにより、標記化合物63 mgを得た。

¹H NMR (CDCl₃) δ: 2.5-2.6 (m, 4 H), 2.65 (s, 3 H), 2.81 (dd, 1 H, J = 13.6, 9.0 Hz), 3.18 (d, 2 H, J = 6.8 Hz), 3.2-3.3 (m, 5 H), 3.72 (dd, 1 H, J = 9.0, 4.4 Hz), 3.9-4.0 (m, 4 H), 4.2-4.3 (m, 2 H), 4.5-4.6 (m, 1 H), 4.7-4.8 (m, 3 H), 6.05 (dt, 1 H, J = 15.9, 6.6 Hz), 6.11 (s, 1 H), 6.25 (t, 1 H, J = 8.3 Hz), 6.36 (d, 2 H, J = 8.3 Hz), 6.38 (d, 1 H, J = 15.9 Hz), 6.9-7.0 (m, 1 H), 7.1-7.3 (m, 5 H), 7.70 (s, 1 H), 7.9-8.0 (m, 1H)。

【0059】例6

CM-Dex-PA-Phe-Gly-AM-薬物(1)

【化5】