PATENT ASSIGNMENT

Electronic Version v1.1
Stylesheet Version v1.1

SUBMISSION TYPE:	NEW ASSIGNMENT
NATURE OF CONVEYANCE:	Correction to the spelling of ASSIGNOR name from SOFIA, MICAHEL JOSEPH to SOFIA, MICHAEL JOSEPH for coversheet submitted with assignment previously recorded on Reel 029577 and Frame 0557

CONVEYING PARTY DATA

Name	Execution Date
Michael Joseph Sofia	03/26/2008
Jinfa Du	03/26/2008
Peiyuan Wang	03/26/2008
Dhanapalan Nagarathnam	03/31/2008

RECEIVING PARTY DATA

Name:	PHARMASSET, INC.
Street Address:	303-A College Road East
City:	Princeton
State/Country:	NEW JERSEY
Postal Code:	08540

PROPERTY NUMBERS Total: 1

Property Type	Number
Application Number:	13609614

CORRESPONDENCE DATA

Fax Number: 2122182200
Phone: 212-218-2568
Email: eaustin@fchs.com
Correspondence will be sent to the e-mail address first; if that is unsuccessful, it will be sent via US Mail.
Correspondent Name: Alicia A. Russo

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Address Line 1:	1290 Avenue of the Americas
Address Line 2:	Fitzpatrick, Cella, Harper & Scinto
Address Line 4:	New York, NEW YORK 10104-3800

ATTORNEY DOCKET NUMBER:	03956.053800.2
NAME OF SUBMITTER:	Alicia A. Russo
Signature:	/Alicia A. Russo/
Date:	01/09/2013

Total Attachments: 2
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source=PH3080PC3_OriginalCoverSheet#page1.tif

RECEIPT INFORMATION

EPAS ID:	PAT2227889
Receipt Date:	01/09/2013
Fee Amount:	$40

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Electronic Patent Application Fee Transmittal	
Application Number:	14013237
Filing Date:	29-Aug-2013
Title of Invention:	NUCLEOSIDE PHOSPHORAMIDATE PRODRUGS
First Named Inventor/Applicant Name:	Jinfa Du
Filer:	Neeta C. Thakur/Aubrett Baker
Attorney Docket Number:	37JD-199059-US4
Filed as Large Entity	

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	1252	1	600	600
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
Statutory or Terminal Disclaimer	1814	3	160	480
		Total in USD ($)		**1260**

Electronic Acknowledgement Receipt	
EFS ID:	21602561
Application Number:	14013237
International Application Number:	
Confirmation Number:	7005
Title of Invention:	NUCLEOSIDE PHOSPHORAMIDATE PRODRUGS
First Named Inventor/Applicant Name:	Jinfa Du
Customer Number:	117796
Filer:	Neeta C. Thakur/Aubrett Baker
Filer Authorized By:	Neeta C. Thakur
Attorney Docket Number:	37JD-199059-US4
Receipt Date:	25-FEB-2015
Filing Date:	29-AUG-2013
Time Stamp:	20:08:29
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1260
RAM confirmation Number	7542
Deposit Account	506219
Authorized User	
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1	Foreign Reference	WO9629336.pdf	2332364	no	69
Warnings:					
Information:					
2	Foreign Reference	WO9937753.pdf	4325740	no	111
Warnings:					
Information:					
3	Foreign Reference	WO0107454.pdf	3412426	no	97
Warnings:					
Information:					
4	Foreign Reference	WO2011123645A2.pdf	6575952	no	150
Warnings:					
Information:					
5	Foreign Reference	wo2006035061.pdf	3686907	no	85
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6	Non Patent Literature	PH3080EP_IPS.pdf	2659220	no	35
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7	Non Patent Literature	PH3080EP_Actavis.pdf	2844287	no	38
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8	Non Patent Literature	PH3080EP_Ellis.pdf	1114859 c13b6d09e1fe78a45f89d1111254add7a52bb2d8	no	21
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9	Non Patent Literature	PH3080EP_Fleischer.pdf	7837721 e158586b6237935804f901929da9386e4f9c2e07c	no	47
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10	Non Patent Literature	PH3080EP_MedDuMonde.pdf	1104516 8d900980[illegible]5762	no	27
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11	Non Patent Literature	PH3080EP_Mylan.pdf	462742 [illegible]bf92b	no	11
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16	Non Patent Literature	37JD-199059AU2_EXAM-REPORT.pdf	309809 [illegible]1dd948	no	3
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Information:					

17	Non Patent Literature	Abstract_05-1-11-2006.pdf	514789 8cd0782e6ad95e3457Da560bd59a7385 1a b838e	no	6
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18	Non Patent Literature	PH3080JPD2_2015-01-28_NoticeRejection_IP141173.pdf	73333 ac957408e3c35ab73b28b9aa33d178cc7b 5593e	no	2
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19	Non Patent Literature	P-2327_RESOLUCION_32999.pdf	162805 1284 b0e9 13e1 9b0340 aa644 0a012e7 56da4	no	2
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22	Non Patent Literature	PH3080MXD_2015-02-10_OATranslation_37JD-199059-MX2_P201711MX.pdf	114898 0a0f960b92279ec6f26b676427aae8b3d6c 3431a	no	2
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23	Non Patent Literature	PH3080KRD_2014-11-11_OA_37JD-199059-KR2_2009-FPA-5075D.pdf	76654 40c768d9f265db7af3d01090d6616b1dac fbf5	no	2
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24	Non Patent Literature	PH3080ILD2_2015-01-28_Office-Action_003638ILD1.pdf	149575 8d9171e9b2455cc148cf314df5cf0844449 9afe	no	2
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25	Non Patent Literature	PH3080PEPD3_Rule-70-2_70a-2_2015-12-22_37JD-199059-EP4.pdf	461643 ce4818dc08f4 35d27 7ae1 f3 bea3 63df 16dd5 ce45 3	no	14

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26	Non Patent Literature	2014-18-09_ESR_36JD-199059-EP2.pdf	491552	no	13
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38	Non Patent Literature	Gunic_Cyclic2007.pdf	236191	no	4
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Information:					
39	Non Patent Literature	Sofia_2010.pdf	3126011	no	17
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40	Non Patent Literature	PH3080CND_20141121_OA5EN_37JD-199059-CN.pdf	145614	no	10
Warnings:					
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41	Non Patent Literature	Perrone_Thesis_2007_reduced size.pdf	14119474	no	292
Warnings:					
Information:					
42	Transmittal Letter	2015-02-25_IDS-Tranmittal_37JD-199059-US4.pdf	22441	no	2
Warnings:					
Information:					
43	Transmittal Letter	2015-02-25_SB08_37JD-199059-US4.pdf	130000	no	8

Warnings:					
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44	Non Patent Literature	PH3080IN_I-MAK.pdf	1056597	no	30
Warnings:					
Information:					
45		37JD-199059-US4_AMD_TDS_APPENDICES.pdf	1901320	yes	35

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Specification	2	2
Claims	3	11
Applicant Arguments/Remarks Made in an Amendment	12	14
Terminal Disclaimer Filed	15	23
Appendix to the Specification	24	35

Warnings:					
Information:					
46	Fee Worksheet (SB06)	fee-info.pdf	34246	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	96767783		

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07H 19/10, 19/20, A61K 31/70, C07F 9/6512, 9/6524, A61K 31/675

A1

(11) International Publication Number: **WO 96/29336**

(43) International Publication Date: 26 September 1996 (26.09.96)

(21) International Application Number: PCT/GB96/00580

(22) International Filing Date: 13 March 1996 (13.03.96)

(30) Priority Data:
9505025.8 13 March 1995 (13.03.95) GB

(71) Applicants *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). UNIVERSITY COLLEGE CARDIFF CONSULTANTS LTD. [GB/GB]; P.O. Box 923, Cardiff CF1 3TE (GB). REGA FOUNDATION [BE/BE]; Minderbroedersstraat 10, B-3000 Leuven (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: McGUIGAN, Christopher [GB/GB]; 2 Alfreda Road, Whitchurch, Cardiff CF4 2EH (GB). BALZARINI, Jan [BE/BE]; Kapeldreef 20, B-3001 Heverlee (BE).

(74) Agent: HOWARD, Paul, Nicholas; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CHEMICAL COMPOUNDS

(57) Abstract

The invention relates to masked monophosphate nucleoside analogues, their preparation and their therapeutic use in the treatment of viral infection, including infection by HIV. In particular, the invention relates to aryl phosphoramidate 2',3'-dideoxy and 2',3'-dideoxydidehydro of nucleoside analogues and of PMEA.

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CHEMICAL COMPOUNDS

The present invention relates to a new class of nucleoside analogues and their therapeutic use in the prophylaxis and treatment of viral infection, for example by human immunodeficiency virus (HIV), which is believed to be the aetiological agent in human acquired immunodeficiency syndrome (AIDS).

There has been much interest in the use of nucleoside analogues as inhibitors of HIV. 2',3'-dideoxy-2',3'-didehydrothymidine (d4T) and 3'-azido-3'-deoxythymidine (AZT) are both known inhibitors of HIV [Hitchcock *et al.*, Antiviral Chem. Chemother. (1991), 2, 125; Mansuri *et al.*, Antimicrob. Agents Chemother., (1990), 34, 637.]. The inhibition of HIV by these, and other nucleoside analogues, is conventionally thought to depend upon conversion of the nucleoside analogue *in vivo* to the corresponding 5'-triphosphate by (host-cell) kinase enzymes. However, this absolute dependence upon (host-cell) kinase-mediated activation can lead to poor activity, the emergence of resistance, and clinical toxicity.

In order to reduce the dependence on kinase enzymes the use of masked phosphate pro-drugs of the bioactive nucleotide forms of several chemotherapeutic nucleoside analogues has been suggested [McGuigan *et al.*, Nucleic Acids Res., (1989), 17, 6065; McGuigan *et al.*, Ibid., (1989), 17, 7195; Chawla *et al.*, J. Med. Chem., (1984), 27, 1733; Sergheraert *et al.*, J. Med. Chem. (1993), 36, 826-830.]. In particular, McGuigan et al [J. Med. Chem. 36, 1048-1052 (1993)] have reported the preparation of aryl ester - phosphoramidate derivatives of AZT. In vitro evaluation of these compounds revealed the compounds to have anti-HIV activity. However, in "normal" thymidine kinase rich (TK^+) cells, the activity of such compounds was at least an order of magnitude less than the parent nucleoside AZT. Only in TK-deficient (TK^-) cells, in which the activity of the aryl ester -

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phosphoramidate derivatives was virtually maintained but the activity of AZT was reduced, did the activity of the derivatives exceed that of AZT.

McGuigan et al [Bioorganic & Medical Chemistry Letters, 3,(6), 1203-1206 (1993)] have also reported preparation of triester phosphate derivatives of d4T. Again, in vitro evaluation of these compounds revealed that whilst the compounds have significant anti-HIV activity, the activity is less than that of the parent nucleoside d4T in TK^+ cells.

Abraham and Wagner (Nucleosides and Nucleotides 13(9). 1891-1903 (1994)) have reported the preparation of nucleoside phosphoramidate diesters and triesters but do not report any biological activity.

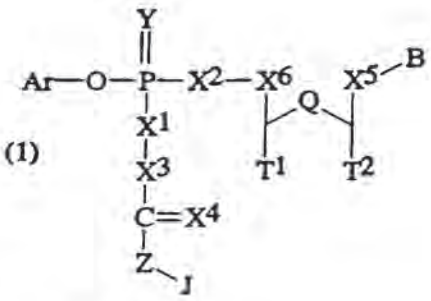
The acyclic nucleoside analogue 9(2-phosphonomethoxyethyl) adenine (PMEA), and analogues thereof, have been demonstrated to show activity against herpes viruses and retroviruses including HIV (Calio et al., Antiviral Res., (1994), 23(1), 77-89; Balzarini et al., AIDS, (1991), 5(1), 21-28).

To date, the approach of providing masked phosphate pro-drugs has failed to enhance the anti-viral activities of the parent nucleoside analogues such as AZT and d4T in TK^+ cells. Furthermore, the emergence of resistance to the nucleoside analogues in their bioactive 5'-triphosphate form has rendered the reported masked phosphate pro-drugs and their parent nucleoside analogues potentially ineffective.

It has now been found that a particular class of masked nucleoside analogues are highly potent viral inhibitors in both TK^- and TK^+ cells, and yet retain activity against nucleoside (e.g. d4T) - resistant virus.

According to the present invention there is provided a compound of the formula (1)

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(1)

wherein Ar is an aryl group;

Y is oxygen or sulphur;

X^1 is selected from O, NR^3, S, CR^3R^4, CR^3W^1 and CW^1W^2 where R^3 and R^4 are independently selected from hydrogen, alkyl and aryl groups; and W^1 and W^2 are heteroatoms;

X^2-X^6 may be absent; or X^6 is CH_2 and X^2 is selected (independently of X^1) from O, NR^3, S, CR^3R^4, CR^3W^1 and CW^1W^2 where R^3 and R^4 are independently selected from hydrogen, alkyl and aryl groups; and W^1 and W^2 are heteroatoms;

X^3 is a C_{1-6} alkyl group;

X^4 is oxygen or CH_2;

X^5 may be absent or is CH_2;

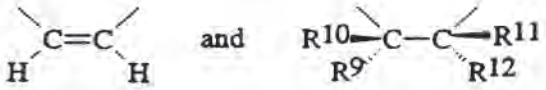
Z is selected from O, NR^5, S, alkyl and aryl groups, where R^5 is selected from hydrogen, alkyl and aryl groups;

J is selected from hydrogen, alkyl, aryl, heterocyclic and polycyclic groups;

Q is selected from O, NR^6, S, CR^6R^7, CR^6W^3 and

CW^3W^4, where R^6 and R^7 are independently selected from hydrogen, alkyl and aryl groups; and W^3 and W^4 are heteroatoms;

T^1 and T^2 are independently selected from hydrogen and CH_2R^8, where R^8 is selected from H, OH and F; or T^1 and T^2 are linked together and together are selected from the groups



where R^9 is selected from H, halogen, CN, NH_2, CO-alkyl and alkyl; and R^{10}, R^{11}, R^{12} are independently selected from H, N_3, halogen, CN, NH_2, CO-alkyl and alkyl;

B is a purine or pyrimidine base;

or a pharmaceutically acceptable derivative or metabolite thereof.

The compounds of the present invention are potent anti-viral agents. In particular, they are highly active against HIV in both TK^- and TK^+ cells. Particularly surprising is the activity of the compounds of the present invention against nucleoside-resistant HIV. These observations indicate that the activity of these compounds is not wholly dependent upon the conventional mode of action (requiring hydrolysis of the phosphate aryl ester and P-X^1 bonds followed by kinase-dependent conversion to the 5'-triphosphate derivative), but arises from an entirely different mode of action. The experimental data presented herein indicates that the compounds and metabolites of the present invention are directly acting as reverse transcriptase (RT) inhibitors via

a previously unrecognised metabolic pathway and mechanism of action.

Reference in the present specification to an alkyl group means a branched or unbranched, cyclic or acyclic, saturated or unsaturated (e.g. alkenyl or alkynyl) hydrocarbyl radical. Where cyclic, the alkyl group is preferably C_3 to C_{12}, more preferably C_5 to C_{10}, more preferably C_5 to C_7. Where acyclic, the alkyl group is preferably C_1 to C_{16}, more preferably C_1 to C_6, more preferably methyl. Reference in the present specification to alkoxy and aryloxy groups means alkyl-O- and aryl-O- groups, respectively. Reference to alkoyl and aryloyl groups means alkyl-CO- and aryl-CO-, respectively.

Reference in the present specification to an aryl group means an aromatic group, such as phenyl or naphthyl, or a heteroaromatic group containing one or more, preferably one, heteroatom, such as pyridyl, pyrrolyl, furanyl and thiophenyl. Preferably, the aryl group comprises phenyl or substituted phenyl.

The alkyl and aryl groups may be substituted or unsubstituted, preferably unsubstituted. Where substituted, there will generally be 1 to 3 substituents present, preferably 1 substituent. Substituents may include halogen atoms and halomethyl groups such as CF_3 and CCl_3; oxygen containing groups such as oxo, hydroxy, carboxy, carboxyalkyl, alkoxy, alkoyl, alkoyloxy, aryloxy, aryloyl and aryloyloxy; nitrogen containing groups such as amino, alkylamino, dialkylamino, cyano, azide and nitro; sulphur containing groups such as thiol, alkylthiol, sulphonyl and sulphoxide; heterocyclic groups which may themselves be substituted; alkyl groups, which may themselves be substituted; and aryl groups, which may themselves be substituted, such as phenyl and substituted phenyl. Alkyl includes substituted and unsubstituted benzyl.

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Reference in the present specification to heterocyclic groups means groups containing one or more, pyrrolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxazolyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, imidazolinyl, pyrazolidinyl, tetrahydrofuranyl, pyranyl, pyronyl, pyridyl, pyrazinyl, pyridazinyl, piperidyl, piperazinyl, morpholinyl, thionaphthyl, benzofuranyl, isobenzofuryl, indolyl, oxyindolyl, isoindolyl, indazolyl, indolinyl, 7-azaindolyl, isoindazolyl, benzopyranyl, coumarinyl, isocoumarinyl, quinolyl, isoquinolyl, naphthridinyl, cinnolinyl, quinazolinyl, pyridopyridyl, benzoxazinyl, quinoxadinyl, chromenyl, chromanyl, isochromanyl and carbolinyl.

References in the present specification to polycyclic groups means a group comprising two or more non-aromatic carbcyclic or heterocyclic rings which may themselves be substituted.

Reference in the present specification to halogen means a fluorine, chlorine, bromine or iodine radical, preferably fluorine or chlorine radical.

The group Ar comprises a substituted or unsubstituted aryl group, wherein the term "aryl group" and the possible substitution of said group is as defined above. Preferably, Ar is a substituted or unsubstituted phenyl group. Particularly preferred substituents are election withdrawing groups such as halogen (preferably chlorine or fluorine), trihalomethyl (preferably trifluoromethyl), cyano and nitro groups. Preferably, Ar is phenyl, 3,5-dichloro-phenyl, p-trifluoromethyl-phenyl, p-cyano-phenyl, or p-nitro-phenyl.

Y may be oxygen or sulphur. Preferably, Y is oxygen.

X^1 is from O, NR^3, S, CR^3R^4, CR^3W^1 and CW^1W^2 where R^3 and R^4 are independently selected from hydrogen, alkyl and aryl groups; and W^1 and W^2 are heteroatoms. Preferably, X^1 is selected from O,S and NR^3. Preferably, X^1 is NR^3. When present, R^3 is preferably H. When present, W^1 and W^2 may independently comprise any heteroatom such as a halogen, preferably

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fluorine.

X^2-X^6 may be absent; or X^6 is CH_2 and X^2 is selected (independently of X^1) from O, NR^3, S, CR^3R^4, CR^3W^1 and CW^1W^2 where R^3 and R^4 are independently selected from H, alkyl and aryl groups; and W^1 and W^2 are heteroatoms. When present, X^2 is preferably oxygen. When present, R^3 is preferably H. When present W^1 and W^2 may independently comprise any heteroatom such as halogen, preferably fluorine.

X^4 is oxygen or CH_2. Preferably, X^4 is oxygen.

X^5 may be absent or is CH_2.

Z may comprise O, NR^5, S, alkyl or aryl groups, where R^5 is selected from H, alkyl and aryl groups. Preferably, Z is O or NR^5. Preferably, R^5 is hydrogen. Most preferably, Z is oxygen.

J is selected from hydrogen, alkyl, aryl, heterocyclic and polycyclic groups. Preferably, J is a substituted or unsubstituted alkyl group. Preferably, J is a substituted or unsubstituted C_{1-6} alkyl group, preferably a benzyl or methyl group.

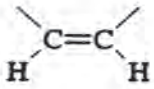
X^3 is a C_{1-6} alkyl group. X^3 may be a C_{1-6} substituted or unsubstituted, branched or unbranched, methylene chain. Preferably, X^3 is a group CR^1R^2 where R^1 and R^2 are independently selected from hydrogen, alkyl and aryl groups. Preferably, at least one of R^1 and R^2 is hydrogen. It will be appreciated that if R^1 and R^2 are different, the carbon atom to which they are bonded is an asymmetric centre. The stereochemistry at this site may be R or S or mixed. When one of R^3 and R^4 is hydrogen, the stereochemistry is preferably S.

Q is selected from O, NR^6, S, CR^6R^7, CR^6W^3 and CW^3W^4, where R^6 and R^7 are independently selected from hydrogen, alkyl and aryl groups; and W^2 and W^3 are heteroactoms such as halogen

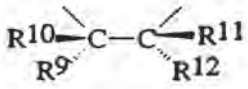
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atoms, preferably fluorine. Preferably, Q is O, S, CH_2 or CF_2. Most preferably, Q is oxygen.

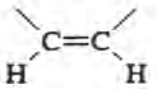
T^1 and T^2 are independently selected from hydrogen and CH_2R^8 where R^8 is selected from H, OH and F; or T^2 and T^2 are linked together and together are selected from the groups:-



and



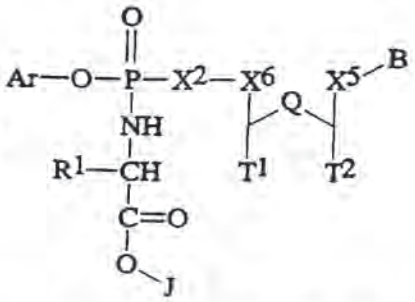
where R^9 is selected from H, halogen, CN, NH_2, CO-alkyl, and alkyl, preferably R^9 is H or F; and R^{10}, R^{11}, and R^{12} are independently selected from H, N_3, halogen, CN, NH_2, CO-alkyl, and alkyl, preferably R^{10}, R^{11} and R^{12} are independently selected from H, F and N_3. It will be appreciated that R^9 corresponds to the 3′ - α position and R^{10} corresponds to the 3′ - β position. Preferably, T^1 and T^2 are linked together and together form the group:-



B comprises a purine or pyrimidine base, such as adenine thymine, uracil, cytosine or guanine and derivatives thereof. Derivatives thereof include substituted purine or pyrimidine bases wherein the substituents are as defined above. Examples of substituted bases include 5-substituted pyrimidine. Preferably, B is adenine or thymine.

Preferably, the present invention provides a compound of formula (2)

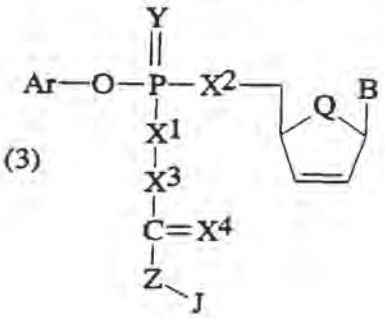
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wherein Ar, R^1, J, X^2, X^5, X^6, Q, T^1, T^2 and B are as defined above; or a pharmaceutically acceptable derivative or metabolite thereof.

It will be appreciated that the group $-NH-CHR^1-CO_2J$ corresponds to a carboxy-protected α-amino acid. Preferably, the group R^1 corresponds to the side chain of a naturally occurring amino acid such as Alanine, Arginine, Asparagine, Aspartic Acid, Cysteine, Cystine, Glycine, Glutamic Acid, Glutamine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, Valine. Preferably, R^1 is Me or $PhCH_2$ corresponding to the side chain of alanine or phenylalanine, respectively. Preferably, the stereochemistry at the asymmetric centre $-CHR^1-$ corresponds to an L-amino acid.

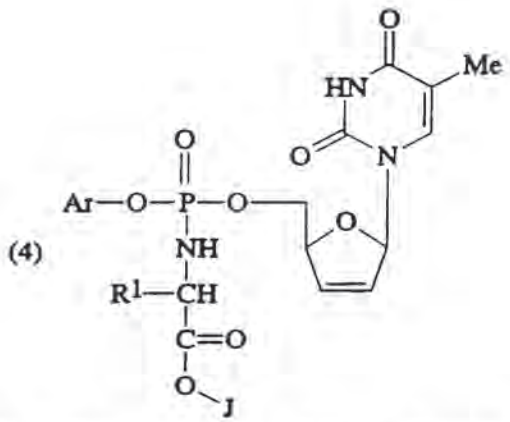
According to one preferred embodiment, the present invention provides a compound of formula (3):-



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wherein Ar, Y, X^1, X^2, X^3, X^4, Z, Q and B are as defined above.

More preferably, the invention provides a compound, according to formula (3), of formula (4):-



wherein Ar, R^1 and J are as defined above; or a pharmaceutically acceptable derivative or metabolite thereof. Preferably, the invention provides a compound of formula (4) in which Ar, R^1 and J are defined in accordance with Table 1.

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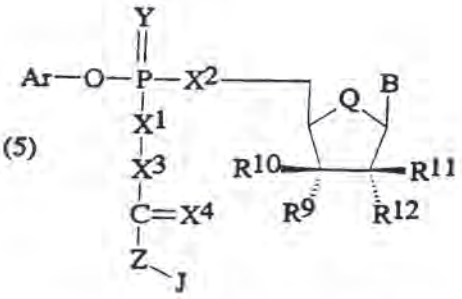
Table 1

Compound Reference	Ar	R^1	J
323	4-EtPh	Me	Me
324	Ph	Me	Me
327	4-FPh	Me	Me
526	3-CF_3Ph	Me	Me
546	3,5-Cl_2Ph	Me	Me
730	Ph	Me	Bzl
776	2,4-Br_2Ph	Me	Me
779	F_5Ph	Me	Me
862	Ph	Me	Hexyl
863	Ph	Bzl	Me
864	Ph	CH_2iPr	Me
865	Ph	iPr	Me
866	Ph	H	Me
867	Ph	$[CH_2]_2$SMe	Me
868	2,4Br_2Ph	Me	Bzl
877	Ph	Bzl	Bzl
878	Ph	Bzl	tBu
892	Ph	Me	Cyclohexyl
893	Ph	Me	tBu
1078	Ph	CH_2CO_2H	Me
1214	Ph	$CH_2CH_2CH_2NHC[NH_2]NH$	Me
1218	Ph	Me	n-Pent
1219	Ph	Me	neo-Pent
1226	Ph	Me	1-Napthyl
1227	Ph	Me	2-Napthyl

According to a further preferred embodiment, the present invention provides a compound of formula (5)

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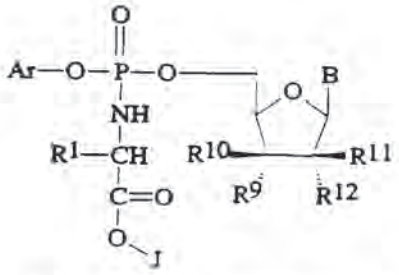
(5)



wherein Ar, Y, X^1, X^2, X^3, X^4, Z, J, R^9, R^{10}, R^{11}, R^{12}, Q and B as defined above.

More preferably, the invention provides a compound, according to formula (5), of the formula (6):-

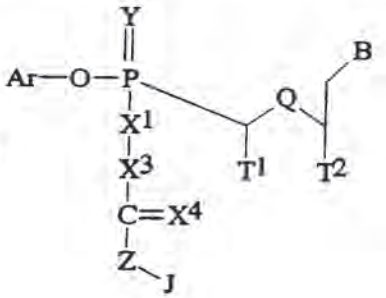
(6)



wherein Ar, R^1, J, R^9, R^{10}, R^{11}, R^{12} and B are as defined above.

According to a further preferred embodiment, the present invention provides a compound of formula (7):-

(7)

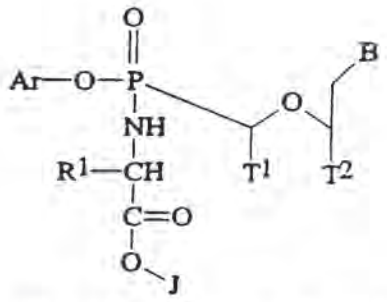


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wherein Ar, Y, X^1, X^3, X^4, Z, J, Q and B are as defined above and T^1 and T^2 are independently selected from H and CH_2R^8 wherein R^8 is as defined above. Preferably, B is a purine base. More preferably, B is adenine. Preferably, T^1 is hydrogen. Preferably, T^2 is CH_2R^8. These compounds are analogues of the acyclic nucleoside analogue 9-(2-phosphonylmethoxyethyl) adenine (PMEA), which has been demonstrated to show activity against herpes viruses and retroviruses (Calio et al., Antiviral Res., (1994), 23(1), 77-89; Balzarini et al., AIDS, (1991), 5(1), 21-28).

More preferably, the invention provides a compound, according to formula (7), of formula (8):-

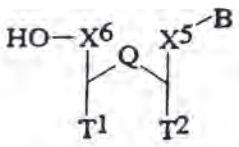
(8)



wherein Ar, R^1, J, T^1, T^2 and B are as defined above.

It is a feature of the aryl ester phosphate compounds (1) of the present invention that they exhibit significantly enhanced anti-viral efficacy, in both in vitro and in vivo tests, in comparison to their corresponding nucleoside analogue (9)

(9)



In addition, the compounds of the present invention exhibit significantly reduced toxicity in comparison to their corresponding analogue (9).

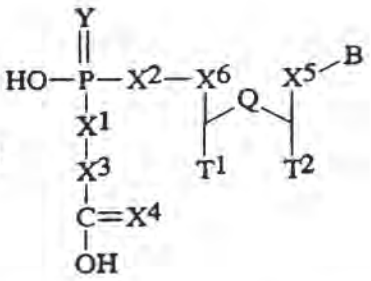
The compounds of the present invention thus exhibit a greatly enhanced selectivity index (ratio of CC_{50} (toxicity):EC_{50} (activity)) in comparison to their corresponding nucleoside analogue.

Experiments with radiolabelled compounds of the present invention have shown that the compounds give enhanced intracellular levels of nucleoside 5'-triphosphate, the enhancement being particularly significant in TK$^-$ cells. Thus, the compounds of the present invention may act in part by the known metabolic pathway.

However, it has been found that the compounds of the present invention show surprising activity against nucleoside resistant strains of HIV. This indicates that the compounds of the present invention are also acting by a pathway independent of a 5'-triphosphate metabolite.

It has been demonstrated that the compounds of the present invention lead to intracellular generation of high levels of a metabolite (10).

(10)

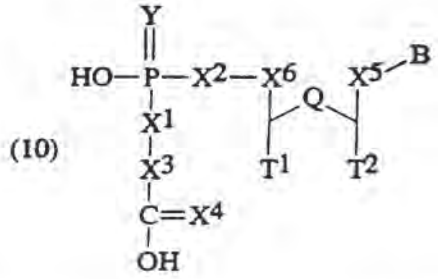


Metabolite (10) may also be prepared by treatment of the corresponding compound according to formula (1) with hog

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liver esterase. Moreover, it has been shown that compounds of formula (10) are direct inhibitors of reverse transcriptase from HIV.

According to a further aspect of the present invention there is provided a compound of formula (10)



(10)

wherein Ar, Y, X^1, X^2, X^3, X^4, X^6, T^1, T^2, Q, X^5 and B are as defined above, or a pharmaceutically acceptable derivative or metabolite thereof.

The intracellular generation of anti-viral metabolites such as (10) is an important feature of the invention for several reasons. Firstly, the direct activity of (10) on RT removes the necessity for further nucleotide-kinase mediated phosphorylation, which may be slow in many cases. In cases where the nucleoside monophosphate is not a substrate for host nucleotide kinases, activation will be poor and anti-viral efficacy low, even if the triphosphate is an excellent RT inhibitor. In such cases, the generation of metabolites such as (10) may lead to a very significant enhancement in antiviral action. Such compounds may be acting directly in their own right or via a rearrangement, decomposition or disproportionation product or via a contaminant. Moreover, the structure of metabolites such as (10) may be further designed to optimise binding to the known structure of RT, and such modified metabolites could be delivered intracellularly using technology herein described, to further enhance the anti-viral effect.

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By "a pharmaceutically acceptable derivative" is meant any pharmaceutically acceptable salt, ester or salt of such ester or any other compound which upon administration to a recipient is capable of providing (directly or indirectly) a compound of formula (1) or (10). By "pharmaceutically acceptable metabolite" is meant a metabolite or residue of a compound of formula (1) or (10) which gives rise to a nucleoside-resistance independent or nucleoside 5'-triphosphate independent mode of reverse transcriptase inhibition exhibited by the compounds of formula (1) or (10).

According to a further aspect of the present invention there is provided a compound according to the present invention for use in a method of treatment, preferably in the prophylaxis or treatment of viral infection.

According to a further aspect of the present invention there is provided use of a compound according to the present invention in the manufacture of a medicament for the prophylaxis or treatment of viral infection.

According to a further aspect of the present invention there is provided a method of prophylaxis or treatment of viral infection comprising administration to a patient in need of such treatment an effective dose of a compound according to the present invention.

The viral infection may comprise any viral infection such as HIV and herpes virus, including HSV 1 and HSV 2, CMV, VZV, EBV, HAV, HBV, HCV, HDV, papilloma, rabies and influenza.

Preferably, the viral infection comprises HIV infection, more preferably HIV-I or HIV-II. It is a feature of the present invention that the compounds exhibit good activity against both HIV-I and HIV-II.

According to a further aspect of the present invention there is provided use of a compound of the present invention in

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the manufacture of a medicament for use in the inhibition of a reverse transcriptase by a nucleoside-resistance independent or nucleoside 5'-triphosphate independent mode of action.

According to a further aspect of the present invention there is provided a pharmaceutical composition comprising a compound of the present invention in combination with a pharmaceutically acceptable excipient.

According to a further aspect of the present invention there is provided a method of preparing a pharmaceutical composition comprising the step of continuing a compound of of the present invention with a pharmaceutically acceptable excipient.

The medicaments employed in the present invention can be administered by oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), rectal, vaginal and topical (including buccal and sublingual) administration.

For oral administration, the compounds of the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredient mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavouring agents, colouring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay

absorption in the gastrointestinal tract.

Capsules for oral use include hard gelatin capsules in which the active ingredient is mixed with a solid diluent, and soft gelatin capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising for example cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

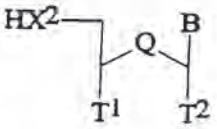
For intramuscular, intraperitoneal, subcutaneous and intravenous use, the compounds of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The compounds of the invention may also be presented as liposome formulations.

In general a suitable dose will be in the range of 0.1 to 300 mg per kilogram body weight of the recipient per day, preferably in the range of 6 to 150 mg per kilogram body weight per day and most preferably in the range 15 to 100 mg per kilogram body weight per day. The desired dose is preferably presented as two, three, four, five or six or

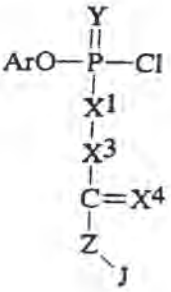
more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing 10 to 1500 mg, preferably 20 to 1000 mg, and most preferably 50 to 700 mg of active ingredient per unit dosage form.

According to a further aspect of the present invention there is provided a process for the preparation of a compound according to the present invention, the process comprising reaction of a compound of formula (11)



with a compound of formula (12)

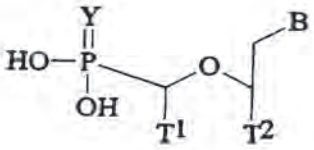
(12)



The reaction may be carried out in the tetranydrofuran in the presence of N-methylimidazole.

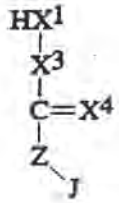
Alternatively, the compounds of the present invention may be prepared by reaction of a compound of formula (13) or a suitable derivative thereof

(13)



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with ArOH and a compound of formula (14) or suitable derivatives thereof



(14)

The invention will now be described with reference to the following Figures and Examples. It will be appreciated that what follows is by way of example only and that modifications to detail may be made whilst still falling within the scope of the invention.

Figure 1 illustrates the in vivo antiviral activity of d4T (comparative) and aryl ester phosphoramidate compound 324 in MSV infected mice. Drug doses are 50[low] or 200[high] mg/kg/day given i.p. for 4 days starting 1 hour before MSV inoculation.

EXPERIMENTAL

All experiments involving water sensitive compounds were conducted under scrupulously dry conditions. Tetrahydofuran was dried by heating under reflux over sodium and benzophenone followed by distallation and storage over active sieves. N-methylimidazole was purified by distillation. Nucleosides were dried at elevated temperature in vacuo over P_2O_5. Proton, carbon and phosphorus Nuclear Magnetic Resonance (^1H, ^{13}C, ^{31}P nmr) spectra were recorded on a Bruker Avance DPX spectrometer

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operating at 300 MHz, 75.5 MHz, and 121.5 MHz respectively. All nmr spectra were recorded in $CDCl_3$ at room temperature (20°C +/-3°C). ^1H and ^{13}C chemical shifts are quoted in parts per million downfield from tetramethyisilane. J values refer to coupling constants and signal splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), multiplet (m) or combinations thereof. ^{31}P chemical shifts are quoted in parts per million relative to an external phosphoric acid standard. Many NMR peaks were further split due to the presence of diastereoisomers at the [chiral] phosphate centre. Chromatography refers to flash column chromotography and was carried out using Merck silica gel 60H (40-60 m, 230-400 mesh) as stationary phase. Thin layer chromotography was performed using Alugram SIL G/UV_{254} aluminium backed silica gel plates.

Mass spectra were recorded by the fast atom bombardment (FAB) mode on a VG 70--250 spectrometer. HPLC data was recorded using an ACS quaternary system with an ODS5 column and an eluent of water/acetonitrile, with 82% water 0-10 mm, and then a linear gradient to 20% water at 30 min, with a flow rate of 2mL/min and detection by UV at 265 nm.

The test compounds were isolated as mixtures of diastereoisomers, with this isomerism arising from mixed stereochemistry at the phosphate centre. The resulting oils did not give useful microanalytical data but were found to be pure by high-field multinuclear NMR spectroscopy and rigorous HPLC analysis.

Preparation of Compounds

The compounds of the present invention were prepared according to the following general procedures.

Preparation of aryl phosphorodichloridates (general procedure)

A solution of the appropriate phenol (30.4 mmol) and

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triethylamine (4.25ml, 30.5 mmol) in dry CH_2Cl_2 (25ml) was added to a solution of freshly distilled $POCl_3$ (10ml, 107mmol) in CH_2Cl_2 (30ml) at -50° and the mixture allowed to stir at ambient temperature overnight. The reaction mixture was filtered and the filtrate evaporated. Ether (20ml) was added and precipitate filtered again. After evaporation the residue was distilled if possible.

Phenyl N-methylalaninyl phosphorochloridate

A solution of triethylamine (1ml-7.17mmol) in 15ml of dry CH_2Cl_2 was added dropwise to a mixture of phenyl phosphorodichloridate (757.4mg, 3.59mmol) and L-alanine methyl ester hydrochloride (500mg, 3.58mmol) in 50ml of dry CH_2Cl_2 at -80°C in one hour. The mixture was then stirred vigorously at -50°C during five hours and CH_2Cl_2 evaporated. 25 ml of dry ether was added and precipitate filtered off under nitrogen. Evaporation of ether gave a colourless oil which was used without further purification for the next step.

Preparation of aryl phosphates of nucleoside analogues (general procedure)

Phenyl N-methylalaninyl phosphorochoridate (250 mg, 0.9 mmol, 2.0equivs) was added to a stirred solution of nucleoside analogue 0.45mmol) and N-methyl imidazole (0.37ml, 143.5µl, 1.8 mmol, 4equivs) in THF (2ml). After 4 hours, the solvent was removed under reduced pressure. The gum was dissolved in chloroform (10ml), and washed with 1M HCl (8ml), sodium bicarbonate (10ml) and water (15ml). The organic phase was dried, and the solvent removed in vacuo. The residue was purified by column chromatography on silica with elution by chloroform-methanol (97:3). Pooling and evaporation of the eluent gave the product as a white solid.

Spectral data

323 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(p-ethylphenyl methoxy alaninyl) phosphoramidate

Yield = 79%

^{31}P ($CDCl_3$) : 3.43 ppm

^{1}H ($CDCl_3$) : 9.25 (0.5H, s, B, NH), 9.23 (0.5H, s, A, NH), 7.34 (0.5H, s, H-6, B), 7.33 (0.5H, s, H-6, A), 7.14 - 7.00 (5H, m, Ph, H-1'), 6.28 (1H, m, H-3'), 5.88 (1H, m, H-2'), 5.00 (1H, m, H-4'), 4.38 - 4.25 (2H, m, H-5'), 3.93 (2H, m, ala-NH, ala-CH), 3.70 (1.5H, s, OMe, A), 3.67 (1.5H, s, OMe, B), 2.60 (2H, q, CH_2CH_3, J=7.5 Hz), 1.84 (1.5H, d, 5-CH_3, J=1.2 Hz), 1.80 (1.5H, d, 5-CH_3, J=1.2 Hz), 1.31 (3H, m, CH_2CH_3), 1.19 (3H, m, ala-CH_3).

^{13}C ($CDCl_3$) : 174.25 (ala-CO, A), 174.12 (ala-CO, B), 164.22 (C-4, B), 164.17 (C-4, A), 151.15 (C-2, B), 151.12 (C-2, A), 148.29 (i-Ph, B), 148.16 (i-Ph, A), 141.24 (p-Ph, A), 141.19 (p-Ph, B), 136.06 (C-6, B), 135.76 (C-6, A), 133.50 (C-3', A), 133.15 (C-3', B), 129.11 (o-Ph, A), 129.05 (o-Ph, B), 127.54 (C-2', A), 127.36 (C-2', B), 120.08 (d, m-Ph, B, J=3.9 Hz), 119.90 (d, m-Ph, A, J=4.9 Hz), 111.51 (C-5, A), 111.40 (C-5, B), 89.83 (C-1', B), 89.60 (C-1', A), 84.88 (d, C-4', B, J=8.8 Hz), 84.70 (d, C-4', A, J=8.8 Hz), 67.11 (d, C-5', A, J=4.9 Hz), 66.48 (d, C-5', B, J=4.9 Hz), 52.65 (OMe), 50.26 (ala-CH, B), 50.13 (ala-CH, A), 28.19 (Ph-CH_2), 20.97 (d, ala-CH_3, B, J=4.9 Hz), 20.90 (d, ala-CH_3, A, J=4.9 Hz), 15.69 (Ph-CH_2CH_3), 12.45 (5-CH_3, A), 12.41 (5-CH_3, B).

MS : $C_{22}H_{29}N_3O_8P$: 494 (MH^+, 5), 368 (MH^+-thymine, 25), 228 (15), 81 (C_5H_5O, base peak) Accurate mass : expected 494.1692; found 494.1693

HPLC : RT = 27.23 and 27.48 min

324 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(phenyl N-methoxy alaninyl) phosphoramidate

Yield = 88%

^{31}P ($CDCl_3$) : 3.20 and 3.86 ppm

^{1}H ($CDCl_3$) : 1.32 and 1.34 (d, 3H, J=6.8Hz, CH_3 ala); 1.81 and 1.84 (d, 3H, 5CH_3); 3.69 and 3.70 (s, 3H, OMe); 3.84-4.00 (m, 2H, CH ala + NH ala); 4.32 (m, 2H, H5'); 5.02 (m, 1H, H4'); 5.88 (m, 1H, H2'); 6.33 (m, 1H, H3'); 7.03 (m, 1H, H1'); 7.15-7.35 (m, 6H, Ar + H6); 9.22 and 9.26 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.52 (5CH_3); 21.02 (CH_3 ala); 50.22-50.35 (CH

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ala); 52.74 (OMe); 66.62-67.29 (C5'); 84.80-84.88 (C4'); 89.69-89.93 (C1'); 111.44-111.57 (C5); 120.13-120.31 (Ar ortho); 125.30 (Ar para); 127.49-127.65 (C2'); 129.87-129.93 (Ar meta); 133.19-133.50 (C3'); 135.77-136.06 (C6); 150.51 (Ar ipso); 151.16 (C2); 164.14 (C4); 174.12 (CO ala)

MS : 466 ($MH^{+\circ}$,7); 340 ($MH^{+\circ}$-base); 200 (17); 136 (47); 89 (25); 81 (C_5H_5O, base peak)

HPLC : RT = 22.48 and 22.87 min

327 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(p-fluorophenyl methoxy alaninyl) phosphoramidate

Yield = 89%

^{31}**P** ($CDCl_3$) : 3.16 ppm

^{1}**H** ($CDCl_3$) : 9.75 (1H, s, NH), 7.24 (0.5H, d, H-6, B, J=1.2 Hz), 7.17 (0.5H, d, H-6, A, J=1.2 Hz), 7.09 (5H, m, Ph, H-1'), 6.22 (1H, m, H-3'), 5.82 (1H, m, H-2'), 4.94 (1H, m, H-4'), 4.30-3.84 (4H, m, ala-NH, ala-CH, H-5'), 3.63 (1.5H, s, OMe, A), 3.62 (1.5H, s, OMe, B), 1.77 (1.5H, d, 5-CH_3, B, J=1.0 Hz), 1.74 (1.5H, d, 5-CH_3, A, J=1.0 Hz), 1.29 (1.5H, d, ala-CH_3, B, J=7.0 Hz), 1.23 (1.5H, d, ala-CH_3, A, J=7.0 Hz).

^{13}**C** ($CDCl_3$) : 174.19 (d, ala-CO, B, J=6.8 Hz), 174.00 (d, ala-CO, A, J=6.8 Hz), 164.25 (C-4, B), 164.20 (C-4, A), 159.77 (d, p-Ph, J=243.6 Hz), 151.14 (C-2), 146.25 (i-Ph), 135.99 (C-6, A), 135.70 (C-6, B), 133.40.(C-3', A), 133.05 (C-3', B), 127.61 (C-2', B), 127.45 (C-2', A), 121.70 (m, o-Ph), 116.37 (d, m-Ph, A, J=23.5 Hz), 116.34 (d, m-Ph, B, J=23.5 Hz), 111.45 (C-5, A), 111.32 (C-5, B), 89.87 (C-1', A), 89.63 (C-1', B), 84.66 (d, C-4', J=5.9 Hz), 67.29 (d, C-5', A, J=4.9 Hz), 66.10 (d, C-5', B, J=4.9 Hz), 52.70 (OMe), 50.26 (ala-CH, A), 50.13 (ala-CH, B), 20.92 (d, ala-CH_3, A, J=4.8 Hz), 20.88 (d, ala-CH_3, B, J=4.8 Hz), 12.45 (5-CH_3, B), 12.41 (5-CH_3, A).

MS : $C_{20}H_{24}N_3O_8PF$: 484 (MH+, 11), 358 (MH^+-thymine, 20), 218 (13), 154 (32), 136 (28), 81 (C_5H_5O, base peak). Accurate mass : expected 484.1285; found 484.1318

HPLC : RT = 25.17 and 25.40 min

526 - 2',3'-dideoxy-2',3'-didehydrothymidine

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5'-(m-trifluromethylphenyl methoxy alaninyl) phosphoramidate
Yield = 80%
^{31}P ($CDCl_3$) : 2.49 and 3.16 ppm
^{1}H ($CDCl_3$) : 9.06 (1H, s, NH), 7.45 (5H, m, H-6, Ph), 7.03 (1H, m, H-1'), 6.31 (1H, m, H-3'), 5.92 (1H, m, H-2'), 5.03 (1H, m, H-4'), 4.32 (2H, m, H-5'), 3.97 (2H, m, ala-NH, ala-CH), 3.71 (1.5H, s, OMe, B), 3.70 (1.5H, s, OMe, A), 1.86 (1.5H, s, 5-CH_3, B), 1.80 (1.5H, d, 5-CH_3, A), 1.36 (3H, m, ala-CH_3).
^{13}C ($CDCl_3$) : 174.06 (d, ala-CO, A, J=6.8 Hz), 173.89 (d, ala-CO, B, J=6.8 Hz), 163.91 (C-4, A), 163.86 (C-4, B), 150.96 (C-2), 150.71 (d, a-Ph, J=5.9 Hz), 135.86 (C-6, A), 135.66 (C-6, B), 133.30 (C-3', A), 133.02 (C-3', B), 132.00 (q, c-Ph, J=32.0 Hz), 130.66 (e-Ph), 127.84 (C-2', B), 127.74 (C-2', A), 123.98 (f-Ph, A), 123.84 (q, CF_3, J=272.0 Hz), 123.79 (f-Ph, B), 122.14 (d-Ph), 117.54 (d, b-Ph, J=3.9 Hz), 111.61 (C-5, B), 111.44 (C-5, A), 90.04 (C-1', B), 89.77 (C-1', A), 84.61 (d, C-4', J=7.8 Hz), 67.60 (d, C-5', B, J=4.9 Hz), 66.89 (d, C-5', A, J=4.9 Hz), 52.87 (OMe), 50.32 (d, ala-CH, A, J=4.8 Hz), 50.26 (d, ala-CH, B, J=4.8 Hz), 21.11 (d, ala-CH_3, B, J=4.9 Hz), 20.99 (d, ala-CH_3, A, J=4.9 Hz), 12.55 (5-CH_3, B), 12.47 (5-CH_3, A).
MS : $C_{21}H_{24}N_3O_8PF_3$: 534 (MH^+, 6), 408 (MH^+-thymine, 8), 268 (10), 149 (10), 81 (C_5H_5O, base peak). Accurate mass : expected 534.1253; found 534.1201
HPLC : RT = 30.56 min

546 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(3,5-dichlorophenyl methoxy alaninyl) phosphoramidate
Yield = 70%
^{31}P ($CDCl_3$) : 2.83 and 3.42 ppm
^{1}H ($CDCl_3$) : 9.74 (1H, s, NH), 7.40 (1H, s, H-6), 7.29 (3H, m, Ph), 7.14 (1H, m, H-1'), 6.44 (1H, m, H-3'), 6.04 (1H, m, H-2'), 5.14 (1H, m, H-4'), 4.48 - 4.07 (5H, m, ala-NH, ala-CH, H-5'), 3.84 (3H, s, OMe), 1.97 (1.5H, s, 5-CH_3, A), 1.92 (1.5H, s, 5-CH_3, B), 1.48 (3H, m, ala-CH_3).
^{13}C ($CDCl_3$) : 173.93 (ala-CO), 164.09 (C-4), 151.27 (i-Ph), 151.06 (C-2), 136.01 (m-Ph), 135.60 (C-6), 133.14 (C-3', B),

132.89 (C-3', A), 127.83 (C-2'), 125.69 (p-Ph), 119.40 (o-Ph), 111.54 (C-5, A), 111.40 (C-5, B), 90.03 (C-1', A), 89.74 (C-1', B), 84.60 (C-4'), 67.68 (C-5', A), 66.98 (C-5', B), 52.85 (OMe), 50.26 (ala-CH), 20.93 (ala-CH_3), 12.51 (5-CH_3).

MS : $C_{20}H_{23}N_3O_8PCl_2$: 534 (MH^+, 8), 408 (MH^+-thymine, 12), 391 (10), 149 (12), 127 (thymineH+, 12), 81 (C_5H_5O, base peak).

Accurate mass : expected 534.0600; found 534.0589

HPLC : RT = 32.19 min

730 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-benzyloxy alaninyl) phosphoramidate

Yield = 92%

^{31}P ($CDCl_3$) : 3.40 and 4.04 ppm

^1H ($CDCl_3$) : 1.24 and 1.26 (d, 3H, J=6.8Hz, CH_3 ala); 1.70 and 1.74 (s, 3H, 5CH_3); 3.86-4.28 (m, 4H, H5'+CH ala+NH); 4.85 (m, 1H, H4'); 5.04 and 5.06 (s, 2H, CH_2Ph); 5.74 (d, 1H, H2'); 6.16 (dd, 1H, H3'); 6.90 (m, 1H, H1'); 7.00-7.30 (m, 11H, Ar + H6); 9.61 (d, 1H, NH)

^{13}C ($CDCl_3$) : 12.52 (5CH_3); 20.98 (CH_3 ala); 50.36-50.52 (CH ala); 66.70-67.18 (C5'); 67.46 (CH_2Ph); 84.63-84.76-84.88 (C4'); 89.68-89.88 (C1'); 111.44-111.55 (C5); 120.18-120.25-120.36-120.43 (Ar ortho, OPh); 125.31 (Ar para, OPh); 127.48-127.61 (C2'); 128.45-128.79-128.83 (Ar, CH_2Ph); 129.87-129.93 (Ar meta, OPh); 133.16-133.45 (C3'); 135.35 (Ar1, CH_2Ph); 135.79-136.07 (C6); 150.44 (Ar1, OPh); 151.18 (C2); 164.21-164.28 (C4); 173.42-173.51-173.65 (CO ala)

HPLC : RT = 34.96 and 35.07 min

MS : $C_{26}H_{28}O_8N_3P$: 542($MH^{+\circ}$; 17); 416 ($MH^{+\circ}$base; 40); 81(100).

Accurate mass : expected 542.1716; found 542.1712

776 - 2',3'-dideoxy-2',3'- didehydrothymidine-5'-(2,4-dibromophenyl N-methylalaninyl) phosphoramidate

Yield = 88%

^{31}P ($CDCl_3$) : 3.07 and 3.62 ppm

^1H ($CDCl_3$) : 1.26 and 1.28 (d, 3H, J=6.8Hz, CH_3 ala); 1.75 and 1.80 (s, 3H, 5CH_3); 2.11 (s, 1H, NH); 3.64 (s, 3H, OMe); 3.92-4.30 (m, 3H, H5'+CHala); 4.98 (m, 1H, H4'); 5.87 (m,

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1H, H2'); 6.26 (m, 1H, H3'); 6.96 (m, 1H, H1'); 7.30-7.60 (m, 4H, Ar + H6); 9.41(bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.51 ($5CH_3$); 21.00 (CH_3 ala); 50.24 (CHala); 52.80 (OMe); 67.37-67.83 (C5'); 84.49-84.61 (C4'); 89.80-89.92 (C1'); 111.60 (C5); 115.49 (Ar2); 118.26 (Ar4); 122.61-122.89 (Ar6); 127.70 (C2'); 131.86 (Ar5); 133.06-133.21 (C3'); 135.64 (Ar3); 135.75-135.88 (C6); 147.01 (Ar1); 151.07 (C2); 164.03 (C4); 173.71-173.82 (COala)

HPLC : RT = 41.17 and 41.30 min

MS : $C_{20}H_{22}O_8N_3PBr_2$: 622,624,626 ($MH^{+\circ}$; 3,6,3); 496,498,500 ($MH^{+\circ}$base; 5,9,5); 81 (100). Accurate mass : expected 621.9516; found 621.9507

779 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(2,3,4,5,6-pentafluorophenyl-N-methylalaninyl)phosphoramidate

Yield = 76%

^{31}P ($CDCl_3$) : 4.74 and 5.66 ppm

^{1}H ($CDCl_3$) : 1.34 and 1.36 (d, 3H, J=6.7Hz, CH_3 ala); 1.75 and 1.81 (s, 3H, $5CH_3$); 3.69 (s, 3H, OMe); 3.92-4.40 (m, 4H, H5'+CH ala+NH); 4.97 (m, 1H, H4'); 5.85 (m, 1H, H2'); 6.29 (m, 1H, H3'); 6.93 (m, 1H, H1'); 7.19 (m, 1H, H6); 9.38 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.23-12.43 ($5CH_3$); 20.83 (CH_3 ala); 50.22-50.34 (CH ala); 52.99 (OMe); 67.75-68.37 (C5'); 84.42-84.52 (C4'); 89.87-90.17 (C1'); 111.75 (C5); 127.69-127.93 (C2'); 132.86-133.13 (C3'); 132-143 (m, Ar); 135.74-135.96 (C6); 151.11 (C2); 164.15 (C4); 173.64-173.76 (COala)

Mass (NOBA matrix) : $C_{20}H_{19}O_8N_3PF_5$: 556 ($MH^{+\circ}$,31); 578 ($M^{\circ+}Na$, 100)

HPLC : RT = 35.90 min

862 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(phenyl N-hexyloxy alaninyl) phosphoramidate

Yield = 88%

^{31}P ($CDCl_3$) : 3.99 and 4.60 ppm

^{1}H ($CDCl_3$) : 0.94 (m, 3H, $C\underline{H}_3CH_2$); 1.28-1.41 (m, 9H, CH_3 ala + 3xCH_2); 1.65 (m, 2H, $CO_2CH_2C\underline{H}_2$); 1.90 and 1.93 (s, 3H,

$5CH_3$); 4.00-4.20 (m, 4H, CH ala + NH ala + $CO_2C\underline{H}_2$); 4.37 (m, 2H, H5'); 5.05 (m, 1H, H4'); 5.94 (m, 1H, H2'); 6.38 (m, 1H, H3'); 7.10 (m, 1H, H1'); 7.15-7.36 (m, 6H, Ar + H6); 9.48 and 9.51 (s, 1H, NH)

^{13}C ($CDCl_3$) : 12.76 ($5CH_3$); 14.39 ($\underline{C}H_3CH_2$); 21.45 (CH_3 ala); 22.88, 25.82, 28.82 and 31.72 (CH_2); 50.63 (CH ala); 66.26 (OCH_2); 66.89-67.43 (C5'); 85.03 (C4'); 89.97 (C1'); 111.68-111.83 (C5); 120.55 (Ar ortho); 125.57 (Ar para); 127.86 (C2'); 130.15 (Ar meta); 133.47-133.70 (C3'); 136.03-136.31 (C6); 150.72 (Ar ipso); 151.37-151.39 (C2); 164.35-164.42 (C4); 174.02 (CO ala)

Mass (NOBA matrix) : $C_{25}H_{34}O_8N_3P$: 536 ($MH^{+\circ}$,24); 558 ($M^{\circ+}Na$, 37)

863 - <u>2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-methoxy-phenylalaninyl) phosphoramidate</u>

Yield = 89%

^{31}P ($CDCl_3$) : 3.96 and 4.35 ppm

^{1}H ($CDCl_3$) : 1.89 (s, 3H, $5CH_3$); 3.00 (m, 2H, CH_2Ph); 3.74 (s, 3H, OMe); 3.80-4.28 (m, 4H, CH ala + NH ala + H5'); 4.94 (m, 1H, H4'); 5.91 (m, 1H, H2'); 6.21-6.30 (m, 1H, H3'); 7.04-7.32 (m, 12H, Ar + H1' + H6); 9.35 (s, 1H, NH)

^{13}C ($CDCl_3$) : 12.54 ($5CH_3$); 40.55 (CH_2Ph); 52.63 (OMe); 55.72-56.01 (CH ala); 66.50-67.10 (C5'); 84.78 (C4'); 89.71-89.95 (C1'); 111.53-111.64 (C5); 120.28 (Ar ortho, OPh); 125.40 (Ar para, OPh); 127.52 (C2'); 128.86, 129.65 and 129.98 (Ar, CH_2Ph); 129.86-129.92 (Ar meta, OPh); 133.18-133.50 (C3'); 135.72 (Ar ipso, CH_2Ph); 135.79-136.06 (C6); 150.46 (Ar ipso, OPh); 151.13-151.17 (C2); 164.12-164.18 (C4); 173.00 (CO ala)

Mass (NOBA matrix) : $C_{26}H_{28}O_8N_3P$: 542 ($MH^{+\circ}$,77); 564 ($M^{\circ+}Na$, 29)

864 - <u>2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-methoxy-leucinyl) phosphoramidate</u>

Yield = 87%

^{31}P ($CDCl_3$) : 4.18 and 4.83 ppm

^{1}H ($CDCl_3$) : 0.91 (m, 6H, $(C\underline{H}_3)_2CH$); 1.42-1.70 (m, 3H, $C\underline{H}_2CH(CH_3)_2$); 1.91 and 1.93 (s, 3H, $5CH_3$); 3.73 (s, 3H, OMe);

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3.76-3.98 (m, 2H, CH ala + NH ala); 4.28-4.46 (m, 2H, H5'); 5.08 (m, 1H, H4'); 5.96 (m, 1H, H2'); 6.36 (m, 1H, H3'); 7.09 (m, 1H, H1'); 7.18-7.35 (m, 6H, Ar + H6); 9.35 (s, 1H, NH)

^{13}C ($CDCl_3$) : 12.76 ($5CH_3$); 22.23-23.01 ($(\underline{C}H_3)_2CH$); 24.75 ($\underline{C}H(CH_3)_2$); 43.86-44.11 ($\underline{C}H_2CH(CH_3)_2$); 52.75 (OMe); 53.42-53.60 (CH ala); 66.92-67.55 (C5'); 85.62 (C4'); 89.92-90.19 (C1'); 111.69-111.83 (C5); 120.37-120.62 (Ar ortho); 125.55-125.58 (Ar para); 127.79 (C2'); 130.12 (Ar meta); 133.51-133.70 (C3'); 136.00-136.36 (C6); 151.05 (Ar ipso); 151.38 (C2); 164.39-164.50 (C4); 174.55-174.88 (CO ala)

Mass (NOBA matrix) : $C_{23}H_{30}O_8N_3P$: 508 ($MH^{+\circ}$,62); 530 ($M^{\circ+}Na$, 59)

865 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-methoxyvalinyl) phosphoramidate

Yield = 86%

^{31}P ($CDCl_3$) : 4.85 and 5.40 ppm

^{1}H ($CDCl_3$) : 0.92 (m, 6H, $(C\underline{H}_3)_2CH$); 1.82 (m, 3H, $C\underline{H}(CH_3)_2$); 1.89 and 1.91 (s, 3H, $5CH_3$); 3.76 (s, 3H, OMe); 3.82 (m, 2H, CH ala + NH ala); 4.30-4.48 (m, 2H, H5'); 5.07 (m, 1H, H4'); 5.96 (m, 1H, H2'); 6.38 (m, 1H, H3'); 7.10 (m, 1H, H1'); 7.18-7.35 (m, 6H, Ar + H6); 9.31 (s, 1H, NH)

^{13}C ($CDCl_3$) : 12.80 ($5CH_3$); 17.77-19.24 ($(\underline{C}H_3)_2CH$); 32.43-32.62 ($\underline{C}H(CH_3)_2$); 52.67 (OMe); 60.32-60.38 (CH ala); 66.92-67.65 (C5'); 85.04 (C4'); 89.98-90.24 (C1'); 111.76-111.87 (C5); 120.45-120.56 (Ar ortho); 125.54-125.59 (Ar para); 127.81-127.86 (C2'); 130.13-130.17 (Ar meta); 133.51-133.72 (C3'); 136.01-136.28 (C6); 150.83 (Ar ipso); 150.87-151.34 (C2); 164.30-164.37 (C4); 173.56-173.65 (CO ala)

Mass : $C_{22}H_{28}O_8N_3P$: 493.6 ($MH^{+\circ}$,100)

866 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-methoxyglycinyl) phosphoramidate

Yield = 90%

^{31}P ($CDCl_3$) : 4.89 and 5.52 ppm

^{1}H ($CDCl_3$) : 1.79 and 1.83 (s, 3H, $5CH_3$); 3.69 (s, 3H, OMe); 3.70-4.05 (m, 4H, CH_2NH + CH ala + NH ala); 4.32 (m, 2H,

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H5'); 4.99 (m, 1H, H4'); 5.92 (m, 1H, H2'); 6.38 (m, 1H, H3'); 6.98 (m, 1H, H1'); 7.05-7.38 (m, 6H, Ar + H6); 9.44 and 9.46 (s, 1H, NH)

^{13}C ($CDCl_3$) : 12.75 ($5CH_3$); 43.15 ($\underline{C}H_2NH$); 52.94 (OMe); 66.78 -67.52 (C5'); 84.98-85.10 (C4'); 89.68-90.16 (C1'); 111.69-111.80 (C5); 120.46-120.59 (Ar ortho); 125.66 (Ar para); 127.66-127.91 (C2'); 130.22 (Ar meta); 133.48-133.87 (C3'); 136.11-136.40 (C6); 150.65 (Ar ipso); 151.45 (C2); 164.46 (C4); 171.41-171.51 (CO ala)

Mass (NOBA matrix) : $C_{19}H_{22}O_8N_3P$: 452 ($MH^{+\bullet}$,74); 474 ($M^{\bullet+}Na$, 46)

867 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-methoxymethioninyl) phosphoramidate

Yield = 81%

^{31}P ($CDCl_3$) : 4.09 and 4.86 ppm

^{1}H ($CDCl_3$) : 1.74 and 1.79 (s, 3H, CH_3S); 1.94 and 1.97 (s, 3H, $5CH_3$); 1.80-2.40 (m, 5H, $C\underline{H}C\underline{H}_2C\underline{H}_2S$); 3.72 and 3.74 (s, 3H, OMe); 3.98-4.32 (m, 4H, H5' + CH ala + NH ala); 4.96 (m, 1H, H4'); 5.84 (m, 1H, H2'); 6.26 (m, 1H, H3'); 6.96 (m, 1H, H1'); 7.05-7.25 (m, 6H, Ar + H6); 9.58 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.80 ($5CH_3$); 15.68 (CH_3S); 29.95 ($\underline{C}H_2SCH_3$); 33.73-33.85 ($\underline{C}H_2CH_2S$);53.06 (OMe); 53.81-54.07 (NH$\underline{C}$H); 67.05-67.70 (C5'); 84.90-85.03 (C4'); 89.98-90.23 (C1'); 111.66-111.86 (C5); 120.39-120.66 (Ar ortho); 125.63 (Ar para); 127.81-127.91 (C2'); 130.18 (Ar meta); 133.44-133.69 (C3'); 136.00-136.38 (C6); 150.72-150.80 (Ar ipso); 151.41 (C2); 164.52 (C4); 173.61-173.94 (CO ala)

Mass (NOBA matrix) : $C_{22}H_{28}O_8N_3PS$: 526 ($MH^{+\bullet}$,46); 548 ($M^{\bullet+}6Na$, 21)

868 - 2',3'-dideoxy-2',3'- didehydrothymidine-5'-(2,4-dibromophenyl N-benzylalaninyl) phosphoramidate

Yield = 82%

^{31}P ($CDCl_3$) : 3.68 and 4.18 ppm

^{1}H ($CDCl_3$) : 1.40 and 1.42 (d, 3H, J=6.7Hz, CH_3 ala); 1.90 and 1.92 (s, 3H, $5CH_3$); 4.04-4.40 (m, 4H, H5'+CHala + NH ala); 4.98 (m, 1H, H4'); 5.20 (s, 2H, CH_2Ph); 5.91 (m, 1H,

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H2'); 6.27 and 6.35 (m, 1H, H3'); 7.06 (bs, 1H, H1'); 7.30-7.70 (m, 9H, Ar + H6); 9.52 (s, 1H, NH)

^{13}C ($CDCl_3$) : 12.86 ($5CH_3$); 21.35 (CH_3 ala); 50.68-50.76 (CHala); 67.67-68.03 (C5'); 67.88 (CH_2Ph); 84.85 (C4'); 90.10-90.20 (C1'); 111.88-111.92 (C5); 115.76-115.91 (Ar2); 118.62-118.72 (Ar4); 122.91-123.22 (Ar6); 127.98 (C2'); 128.75-129.01-129.12 (Ar o,m,p, CH_2Ph); 132.20 (Ar5); 133.38-133.51 (C3'); 135.48 (Ar ipso, CH_2Ph); 135.96 (Ar3); 136.21 (C6); 147.28 (Ar1); 151.39 (C2); 164.34-164.38 (C4); 173.47-173.62 (COala)

Mass (NOBA matrix) : $C_{26}H_{26}O_8N_3PBr_2$: 699-700-701 ($MH^{+\cdot}$, 27-49-29); 721-722-723 ($M^{\cdot+}Na$, 17-21-17)

877 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-methoxyglycinyl) phosphoramidate

Yield = 83%

^{31}P ($CDCl_3$) : 3.91 and 4.33 ppm

^{1}H ($CDCl_3$) : 1.83 and 1.85 (s, 3H, $5CH_3$); 3.01 (m, 2H, $CH\underline{H}_2Ph$); 3.78-4.30 (m, 4H, H5' + $\underline{H}NC\underline{H}$); 4.92 (m, 1H, H4'); 5.89 (m, 1H, H2'); 6.18 and 6.27 (m, 1H, H3'); 7.00-7.40 (m, 17H, Ar + H1' + H6); 9.35 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.62-12.75 ($5CH_3$); 40.65-40.73 ($CH\underline{C}H_2Ph$); 55.95-56.26 ($NHC\underline{H}$); 66.79 -67.27 (C5'); 67.80 ($\underline{C}H_2Ph$); 84.87-85.05 (C4'); 89.92-90.14 (C1'); 111.72-111.82 (C5); 120.45-120.52 (Ar ortho, OPh); 125.60 (Ar para, OPh); 127.73 (C2'); 129.01- 129.07-129.11-129.91- 130.15-130.38-135.29-135.85 (Ar, $2xCH_2Ph$); 130.21 (Ar meta, OPh); 133.36-133.63 (C3'); 136.24 (C6); 150.68-150.77 (Ar ipso, OPh); 151.31-151.35 (C2); 164.28-164.34 (C4); 172.48-172.64 (CO ala)

Mass (NOBA matrix) : $C_{32}H_{32}O_8N_3P$: 618 ($MH^{+\cdot}$, 78); 640 ($M^{\cdot+}Na$, 52)

878 - 2',3'-dideoxy-2',3'-didehydrothymidine-5'-(phenyl N-tert-butylphenylalaninyl) phosphoramidate

Yield = 79%

^{31}P ($CDCl_3$) : 4.27 and 4.50 ppm

^{1}H ($CDCl_3$) : 1.40 and 1.41 (s, 9H, tBu); 1.84 and 1.87 (s, 3H, $5CH_3$); 3.00 (m, 2H, $C\underline{H}_2Ph$); 3.76-4.28 (m, 4H, H5' +

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HNCH); 4.95 (m, 1H, H4'); 5.86 and 5.91 (m, 1H, H2'); 6.26 and 6.30 (m, 1H, H3'); 7.04 (m, 1H, H1'); 7.12-7.25 (m, 11H, Ar + H6); 9.38 and 9.40 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.76-12.79 ($5CH_3$); 28.31 ($(CH_3)_3C$); 40.96-41.04 (CH_2Ph); 56.31-56.65 (NHCH); 66.79 -67.28 (C5'); 82.90-82.92 ($(CH_3)_3C$); 84.94-85.03 (C4'); 89.93-90.11 (C1'); 111.67-111.86 (C5); 120.45 (Ar ortho, OPh); 125.52 (Ar para, OPh); 127.77 (C2'); 127.88-128.83-128.92-136.02 (Ar, CH_2Ph); 130.13 (Ar meta, OPh); 133.54-133.60 (C3'); 136.31 (C6); 150.75-150.84 (Ar ipso, OPh); 151.36 (C2); 164.32-164.37 (C4); 171.89 (CO ala)

Mass (NOBA matrix) : $C_{29}H_{34}O_8N_3P$: 584 ($MH^{+\cdot}$, 26); 606 ($M^{\cdot+}Na$, 41)

892 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(phenyl N-cyclohexyloxy alaninyl) phosphoramidate

Yield = 83%

^{31}P ($CDCl_3$) : 4.11 and 4.71 ppm.

^{1}H ($CDCl_3$) : 1.08-1.82 (m, 16H, CH_3 ala + $5CH_3$ + cyclohexyl); 3.79-4.14 (m, 2H, CH ala + NH ala); 4.27 (m, 2H, H5'); 4.69 (m, CH cyclohexyl); 4.96 (m, 1H, H4'); 5.80 (m, 1H, H2'); 6.24 (m, 1H, H3'); 6.98 (m, 1H, H1'); 7.04-7.32 (m, 6H, Ar + H6); 9.66 and 9.82 (bs, 1H, NH).

^{13}C ($CDCl_3$) : 12.58 ($5CH_3$); 21.18-21.32 (CH_3 ala); 23.73-25.40-31.49-31.58(CH_2 cyclohexyl); 50.47-50.61 (CH ala); 66.69-67.24 (C5'); 74.36(CH cyclohexyl); 84.87 (C4'); 89.72-89.92 (C1'); 111.48-111.63 (C5); 120.26-120.49 (Ar ortho); 125.32-125.37 (Ar para); 127.59-127.73 (C2'); 129.91-129.98 (Ar meta); 133.30-133.51 (C3'); 135.89-136.16 (C6); 150.53 (Ar ipso); 150.67 -151.31(C2); 164.36-164.41 (C4); 173.23 (CO ala).

Mass (NOBA matrix) : $C_{25}H_{32}O_8N_3P$: 534 ($MH^{+\cdot}$, 56); 556 ($M^{\cdot+}Na$, 42)

893 - 2',3'-dideoxy-2',3'-didehydrothymidine 5'-(phenyl N-tButyloxy alaninyl) phosphoramidate

Yield = 79%

^{31}P ($CDCl_3$) : 4.17 and 4.67 ppm.

^{1}H ($CDCl_3$) : 1.34 (m, 3H, CH_3 ala); 1.46 (m, 9H, CH_3 tBu);

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1.87 (d, 3H, 5CH_3); 3.82-4.06 (m, 2H, H5'); 4.29-4.49 (m, 2H, CH ala + NH ala); 5.05 (m, 1H, H4'); 5.91 (m, 1H, H2'); 6.35 (m, 1H, H3'); 7.06 (m, 1H, H1'); 7.15-7.40 (m, 6H, Ar +H6); 9.60 (bs, 1H, NH).

^{13}C ($CDCl_3$) : 12.54 (5CH_3); 21.19-21.35 (CH_3 ala); 28.07 ($C(CH_3)_3$); 50.80-50.89 (CH ala); 66.60-67.18 (C5'); 82.41-82.45($C(Me)_3$); 84.82 (C4'); 89.67-89.87 (C1'); 111.44-111.60 (C5); 120.22-120.41 (Ar ortho); 125.28-125.31 (Ar para); 127.54-127.65 (C2'); 129.88-129.94 (Ar meta); 133.33-133.47 (C3'); 135.84-136.10 (C6); 150.51 (Ar ipso); 150.65-151.20 (C4); 164.19 -164.23 (C2); 172.78-172.93 (CO ala).

Mass (NOBA matrix) : $C_{23}H_{30}O_8N_3P$: 508 ($MH^{+\bullet}$, 82); 530 ($M^{\bullet+}Na$, 48).

2', 3'-Dideoxy-2', 3'-didhydrothymidine-5'-(phenyl methoxy-B-alaninyl) phosphate

Cf 1197

Yield=64%

^{31}P ($CDCl_3$): 6.44, 6.70(1:3)

1H ($CDCl_3$): 1.87* (s, 3H, 5-CH_3), 2.42(t, 2H, CH_2 ala), 3.22* (m, 2H, CH_2 ala), 3.62 (s, 3H, OCH_3), 4.09 (m, 1H, H4'), 4.18-4.39 (m, 2H, H5'), 4.97 (bs, 1H, NH ala), 5.88* (m, 1H, H2'), 6.32* (m, 1H, H3'), 6.99 (m, 1H, H1'), 7.08-7.38 (m, 5H, Ph and H6), 10.01 (bs, 1H, base NH)

^{13}C ($CDCl_3$): 14.52 (5-CH_3), 37.80* (CH_2 ala), 39.28* (CH_2 ala), 53.91* (OCH_3), 68.57* (d, J = 3.92 Hz, C5'), 86.90 (d, J = 8.38 Hz, C4'), 91.68* (C1'), 113.40* (C5), 122.34 (d, J = 4.68 Hz, ortho-Ph), 127.23 (C2'), 129.55* (para-Ph), 131.81* (meta-Ph), 135.45* (C6), 137.99* (C3'), 152.60* (d, J = 5.96 Hz, ipso-Ph), 153.44 (C2), 166.58 (C4), 174.55* (COO)

Mass (NOBA matrix): $C_{20}H_{24}N_3O_8P$ 126 ($thymine^+$,5), 127 ($thymineH^+$,4), 242 ($C_{10}H_{13}PO_4N^+$,9), 243 ($C_{10}H_{14}PO_4N^+$,3), 465 (M^+,4), 466 (MH^+, 8), 467 ($MHNa^+$, 20), 168 ($MHNa^+$, ^{13}O, 5), 187 (MNa^+, 3), 188 ($MHNa^+$, 97), 189 ($MHNa^+$, ^{13}C, 21)

High Resolution MS: found 466.1379 (MH^+), $C_{20}H_{25}N_3O_8P$ requires 466.1379

HPLC: RT = 22.81, 23.27 mins (1:1)

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2',3'-Dideoxy-2',3'-didehydrothymidine-5'-(phenyl methoxy-α-aminobutylryl) phosphate

Cf 1198

Yield = 65%

^{31}P ($CDCl_3$): 6.11, 6.66 (1:2)

^{1}H ($CDCl_3$): 1.78 (m, 2H, CH_2 GABA), 1.85° (s,3H, 5-CH_3), 2.35 (t, 2H, J = 6.95 Hz, CH_2 GABA), 2.97° (m, 2H, CH_2 GABA), 3.68 (s, 3H, OCH_3), 3.93° (m, 1H, H4'), 4.28° (m, 1H, H5'), 4.35° (m, 1H, H5'), 5.02 (bs, 1H, NH GABA), 5.82° (m, 1H, H2'), 6.31 (m, 1H, H3'), 6.98 (m, 1H, H1'), 7.11-7.37 (m, 6H, Ph and H6), 9.91 (bs, 1H, base NH)

^{13}C($CDCl_3$): 12.64 (5-CH_3), 26.72° (CH_2 GABA), 32.25° (CH_2 GABA), 40.98° (CH_2 GABA), 51.94 (OCH_3), 66.93° (C5'), 85.11 (d, J = 8.30 Hz, C4'), 111.40 (C5), 120.46° (d, J = 4.83 Hz, ortho-PH), 125.24 (C2'), 127.59° (para-Ph), 129.88° (meta-Ph), 133.68° (C6), 136.28° (C3'), 150.86° (d, J = 6.45 Hz, ipso-Ph), 151.61 (C2), 164.80 (C4), 173.86° (COO)

Mass (matrix NOBA): $C_{21}H_{26}N_3O_8P$: 127 (thymineH$^+$, 28), 479 (M^+, 3), 480 (MH^+, 59), 481 (MH^+, ^{13}C, 17), 501 (MNa^+, 3), 502 ($MHNa^+$, 59), 503 ($MHNa^+$, ^{13}C, 16)

High Resolution MS: found 480.1486 (MH^+), $C_{21}H_{27}N_3O_8P$ requires 480.1536

HPLC: RT 23.90, 24.33 mins (1:1)

2',3'-Dideoxy-2',3'-didehydrothymidine-5'-(phenyl methoxy-2-aminoisobutylryl) phosphate

Cf 1200

Yield = 36%

^{31}P ($CDCl_3$): 2.38, 3.05 (3:1)

^{1}H ($CDCl_3$): 1.53° (s, 6H, CMe_2), 1.91° (s, 3H, 5-CH_3), 3.71 (s, 3H, OCH_3), 4.31 (m, 2H, H5'), 4.23-4.41 (m, 3H, H4' and H5'), 5.03 (bs, 1H, P-NH) 5.89° (m, 1H, H2'), 6.28° (m, 1H, H3'), 6.99-7.31 (m, 7H, Ph, HO and H1'), 9.09 (bs, 1H, base NH)

^{13}C ($CDCl_3$): 14.27 (5-CH_3), 28.74° ($\underline{C}Me_2$), 54.81° (OCH_3), 58.88° ($\underline{C}Me_2$), 69.03° (d, C', J = 5.58 Hz), 86.57° (d, J = 7.88 Hz, C4'), 91.51° (C1'), 113.24° (C5), 122.01° (d, J = 4.95 Hz, ortho-Ph), 126.88 (C2'), 129.25° (para-Ph), 131.57° (meta-Ph), 135.19° (C6), 137.68° (C3'), 152.52° (d, J = 3.09 Hz, ortho-Ph), 153.05 (C2), 166.12 (C4), 177.69° (COO)

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MS (matrix NOBA): 354 ((MH - thymine)$^+$, base peak), 479 (M^+, 3), 480 (MH^+, 64), 481 (MH^+, ^{13}C, 17), 482 (MH^+, 2 X ^{13}C, 3), 502 (MNa^+, 92), 503 ($MHNa^+$, 24)
High Resolution MS: found 480.1503 (MH^+), $C_{21}H_{27}N_3O_8P$ requires 480.1536
HPLC: RT 24.79, 25.29 mins (1:1)

2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'-(phenylmethoxy-6-aminocaproyl) phosphate

Cf 1199
Yield = 80%
^{31}P ($CDCl_3$): 6.90, 6.30 (1:1)
^1H ($CDCl_3$): 1.28 (s, 2H, CH_2 caproyl), 1.45 (m, 2H, CH_2 caproyl), 1.58 (m, 2H, CH_2 caproyl), 1.82* (s, 3H, 5-CH_3), 2.28 (m, 2H, CH_2 caproyl), 2.87 (m, 2H, CH_2 caproyl), 3.65 (s, 3H, OCH_3), 3.81 (m, 1H, H4'), 4.25 (m, 2H, H5'), 4.95 (bs, 1H, NH caproyl), 5.86* (m, 1H, H2'), 6.31* (m, 1H, H3'), 6.98 (m, 1H H1'), 7.04-7.38* (m, 6H, Ph and H6), 10.12 (bs, 1H, base NH)
^{13}C ($CDCl_3$): 13.47* (5-CH_3), 25.43* (CH_2 caproyl), 27.04* (CH_2 caproyl), 32.15* (CH_2 caproyl), 34.85 (CH_2 caproyl), 42.30* (CH_2 caproyl), 52.61 (OCH_3), 67.92* (C5'), 85.80 (d, J = 8.22 Hz), 90.68* (C4'), 112.25* (C5), 121.17* (d, J = 4.58 Hz, ortho-Ph), 125.99 (C2'), 128.40* (para-Ph), 130.77 (meta-Ph), 134.38* (C6), 137.09* (C3'), 151.69* (d, J = 3.23 Hz, ortho-Ph), 152.26 (C2'), 165.36 (C4'), 175.07 (COO)
MS (matrix Cl): 127 (thymineH$^+$, 42), 508 (MH^+, 18), 509 (MH^+, ^{13}C, 5)
High Resolution MS: found 508.1850 (MH^+), $C_{23}H_{31}N_3O_8P$ requires 508.1849
HPLC: RT 26.33 mins

2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'-(β-alaninyl) phosphate ammonium salt

Cf1216
Yield = 62%
^{31}P (D_2O): 8.84
^1H (D_2O): 1.73 (3H, s, 5-CH_3), 2.18 (2H, m, ala CH_2), 2.65 (m, 2H, ala CH_2), 3.79 (2m, H, H5'), 4.95 (m, 1H, H4'), 5.76 (m,

1H, H2'), 6.35 (m, 1H, H3'), 6.82 (m, 1H, H1'), 7.47 (s, 1H, H6)

^{13}C (D_2O): 11.81 (5-CH_3), 38.51 (ala CH_2), 39.45 (d, ala CH_2, J = 6.64 Hz), 65.41 (d, C5', J = 4.91 Hz), 86.40 (d, J = 9.20 Hz, C4'), 90.20 (C1'), 111.07 (C5), 125.40 (C2'), 134.66 (C3'), 138.54 (C6), 152.53 (C2), 167.00 (C4), 181.04 (COO)

HPLC: RT = 32.74 mins.

2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'(γ - aminobutylryl) phosphate ammonium salt

Cf 1224

Yield = 54%

^{31}P (D_2O): 10.03

^{1}H(D_2O): 1.47 (m, 2H, GABA CH_2), 1.72 (s, 3H, 5-CH_3), 1.98 (m, 2H, GABA CH_2), 2.48 (m, 2H, GABA CH_2), 3.72 (m, 2H, H5'), 4.91 (m, 1H, H4'), 5.72 (m, 1H, H2'), 6.26 (m, 1H, H3'), 6.72 (m, 1H, H1'), 7.45 (s, 1H, H6').

^{13}C (D_2O): 11.79 (5-CH_3), 27.99 (d, J = 7.25 Hz, GABA CH_2), 34.47 (GABA CH_2), 41.17 (GABA CH_2), 65.35 (d, J = 4.68 Hz, C5'), 86.38 (d, J = 9.36 Hz, C4'), 90.27 (C1'), 111.47 (C5'), 125.29 (C2'), 134.70 (C3'), 138.68 (C6), 152.47 (C2), 166.95 (C4), 182.32 (COO)

2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'-(caproyl) phosphate ammonium salt Cf 1217

Yield = 49%

^{31}P (D_2O): 10.18

^{1}H (D_2O): 1.01 (m, 2H, caproyl CH_2), 1.21 (m, 2H, caproyl CH_2), 1.32 (m, 2H, caproyl CH_2), 1.78 (s, 3H, 5-CH_3), 2.05 (m, 2H, caproyl CH_2), 2.58 (m, 2H, caproyl CH_2), 3.78 (m, 2H, H5'), 4.99 (s, 1H, H4'), 6.32 (m, 1H, H3'), 6.82 (m, 1H, H2'), 7.51 (s, 1H, H6)

^{13}C (D_2O): 11.84 (5-CH_3), 25.66 (caproyl CH_2), 26.46 (caproyl CH_2), 31.10 (d, J = 6.82 Hz, caproyl CH_2), 37.06 (caproyl CH_2), 41.47 (caproyl CH_2), 65.37 (d, J = 4.83 Hz, C5'), 86.45 (d, J = 9.74 Hz, C4'), 90.29 (C1'), 111.43 (C5), 125.27 (C2'), 134.80 (C3'), 138.89 (C6), 152.48 (C2), 166.94 (C4), 183.15 (COO).

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2', 3'-dideoxycytidine-5'-(phenyl-N-methoxyalaninyl) phosphoramidate Cf 1221

Yield = 16.6%

^{31}P ($CDCl_3$): 3.94, 4.00

^{1}H ($CDCl_3$): 1.33, 1.35 (2 x d, 3H, CH_3 ala); 1.92, 1.96, 2.41 (1H, 2H, 1H, 3 x m, H2', H3'); 3.66 (s, 3H, OMe); 3.86-4.35 (m, 5H, H4', H5', CH ala, NH ala); 5.63 (2 x d, J = 7.4 Hz, H6), 6.02 (m, 1H, H-1'), 7.12-7.32 (m, 5H, Ar), 7.73 (1H, 2 x d, J = 7.4 Hz, H5)

^{13}C ($CDCl_3$): 20.98 (CH_3 ala); 24.97, 25.11, 32.85 (C2', C3'); 50.12, 50.30 (CH_3 ala); 52.55 (OMe); 67.19, 67.26, 67.50 (C5'); 79.16, 79.27, 79.34 (C4'); 87.29, 87.46 (C1'); 93.48 (C5); 119.99, 120.04, 120.10, 125.05, 125.10, 129.73, 129.77 (CAr); 141.17 (C6); 150.48, 150.57 (C ipso Ar); 155.68 (C2); 165.44 (C4); 173.84, 173.94 (COala)

Mass (ES^+): $C_{19}H_{25}N_4O_7P$: 475 (MNa^+, 100); HPLC: RT = 20.53, 21.22 min

2', 3'-dideoxy-2', 3 -didehydrothymidine 5' -(phenylmethoxysarcosinyl phosphate) Cf 1098

Yield = 65%

^{31}P ($CDCl_3$): 6.80, 7.36 ppm

^{1}H ($CDCl_3$): 1.72 (s, 3H, $5CH_3$); 2.64, 2.67 (s, 3H, NCH_3); 3.62 (s, 3H, OCH_3); 3.40-4.10 (m, 2H, CH_2); 4.20-4.50 (m, 2H, H5'); 4.97 (bs, 1H, H4'), 5.80-5.90 (m, 1H, H2'); 6.30-6.40 (m, 1H, H3'); 6.97 (bs, 1H, H1'); 7.00-7.30 (m, 6H, Ar + H6); 9.59 (bs, 1H, NH)

^{13}C ($CDCl_3$): 12.35 ($5CH_3$); 34.55-34.60-34.65 (NCH_3); 50.67-50.78-50.87 (CH_2); 52.10-52.13 (OCH_3); 62.27-66.77-66.82 (C5'); 84.71-84.84 (C4'); 89.52-89.82 (C1'); 111.16-111.33 (C5); 120-150 (m, Ar); 127.17-127.40 (C2'); 133.25-133.62 (C3'); 135.73-136.11 (C6); 150.85-150.90 (C2); 163.84-163.87 (C4); 170.57-170.60-170.84 ($\underline{C}OOCH_3$)

Mass : $C_{20}H_{24}O_8N_3P$: 488 ((M+Na)+, 100); 466 ($(M+H)^+$, 5)

HPLC : RT = 25.17 and 25.59 min

2', 3'-dideoxy-2', 3'-didehydrothymidine 5' -(phenylethoxysarcosinyl phosphate) Cf 1133

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Yield = 65%

^{31}P ($CDCl_3$): 0.87, 7.41 ppm

^{1}H ($CDCl_3$) : 1.18-1.24 (m, 2H, $CH_3C\underline{H}_2$); 1.80 (s, 3H, $5CH_3$); 2.68, 2.71 (s, 3H, NCH_3); 3.46-3.65 (m, 2H, NCH_2); 3.91-4.45 (m, 2H, H5'); 4.11, 4.13 (s, 3H, $CH_2C\underline{H}_3$); 5.00 (bs, 1H, H4'); 5.82-5.88 (m, 1H, H2'); 6.33-6.37 (m, 1H, H3'); 7.00 (bs, 1H, H1'); 7.10-7.50 (m, 6H, Ar + H6); 8.75 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 12.86-12.89 ($5CH_3$); 14.69 ($CH_2\underline{C}H_3$); 35.06-35.11 (NCH_3); 51.35-51.43-51.51 (NCH_2); 61.77 ($\underline{C}H_2CH_8$); 66.77-67.27-67.33 (C5'); 85.26-85.36 (C4'); 90.01-90.31 (C1'); 111.69-111.86 (C5); 120-151 (m, Ar); 127.73-127.96 (C2'); 133.73-134.10 (C3'); 136.27-136.64 (C6); 151.61 (C2); 164.70 (C4); 170.62-170.66-170.85 ($\underline{C}OOCH_3$)

Mass : $C_{21}H_{26}O_8N_3P$: 502 ($(M+Na)^+$, 100); 480 ($(M+H)^+$, 5)

HPLC : RT = 25.84 and 26.65 min

2', 3'-dideoxy-2', 3'-didehydrothymidine 5'-(methioninyl phosphate) Cf 1156

Yield = 52%

^{31}P ($CDCl_3$) : 7.77 ppm

^{1}H ($CDCl_3$) : 1.75-1.85 (m, 2H, CH_2S); 1.90 (s, 3H, SCH_3); 2.01, 2.10 (s, 3H, $5CH_3$); 2.30-2.50 (m, 2H, $C\underline{H}_2CH_2S$); 3.45-3.60 (m, 1H $C\underline{H}NH$); 3.94 (s, 2H, H5'); 5.05 (bs, 1H, H4'); 5.90-6.00 (m, 1H, H2'); 6.40-6.50 (m, 1H, H3'); 6.93 (bs, 1H, H1'); 7.68 (s, 1H, H6)

^{13}C ($CDCl_3$) : 11.91 ($5CH_3$); 14.46 (SCH_3); 29.58 ($CH_3S\underline{C}H_2CH_2$); 34.69 ($SCH_2CH_2$); 56.42 (CHNH); 65.07-65.13 (C5'); 86.39-86.52 (C4'); 90.14 (C1'); 111.70 (C5); 125.48 (C2'); 134.77 (C3'); 138.91 (C6); 152.61 (C2); 167.18 (C4); 180.84 (COOH)

Mass : $C_{15}H_{22}O_8N_3PS$: 434 ((M-1), 100); 435 ((M), 15)

HPLC: RT = 31.38 min

2', 3'-dideoxy-2', 3'-didehydrothymidine 5'-(glycinyl phosphate) Cf 1163

Yield = 75%

^{31}P ($CDCl_3$) : 11.72 ppm

^{1}H ($CDCl_3$) : 1.83 (s, 3H, $5CH_3$); 3.29 (d, CH_2, J = 7.9Hz); 3.85-3.92 (m, 2H, H5'); 5.00 (s, 1H, H4'); 5.85-5.88 (m, 1H, H2'); 6.38-6.41 (m, 1H, H3'), 6.88-6.90 (bs, 1H H1'); 7.54

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(s, 1H, H6)
^{13}C ($CDCl_3$): 19.09 ($5CH_3$); 52.24 (CH_2); 72.74-72.81 (C5'); 93.61-93.73 (C4'); 97.57 (C1'); 119.08 (C5); 132.80 (C2'); 141.89 (C3'); 145.74 (C6); 159.87 (C2); 174.34 (C4); 186.03-186.15 (COOH)
Mass : $C_{12}H_{16}O_8N_3P$: 360 ((M-1), 100); 361 ((M), 15)
HPLC : RT = 32.57 min

2', 3'-dideoxy-2', 3'-didehydrothymidino 5' -(phenylmethoxyisoleucinyl phosphate) Cf 1186
Yield = 82%
^{31}P ($CDCl_3$) : 4.59, 5.16 ppm
^{1}H ($CDCl_3$) : 0.91-0.99 (m, 6H, CH_3 + CH_3); 1.09-1.26 ($C\underline{H}CH_3$); 1.28-1.56 (m, 2H, CH_2); 1.92, 1.97 (s, 3H, $5CH_3$); 3.60-3.77 (m, 1H, $C\underline{H}NH$); 3.77 (s, 3H, OCH_3); 3.88-3.99 (m, 1H, $N\underline{H}CH$); 4.30-4.52 (m, 2H, H5'); 5.11-5.13 (m, 1H, H4'); 5.95-6.00 (m, 1H, H2'); 6.35-6.45 (m, 1H, H3'); 7.10-.7.13 (m, 1H, H1'); 7.16-7.45 (m, 6H, Ar + H6); 8.68 (bs, 1H, NH)
^{13}C ($CDCl_3$); 11.90-11.92 ($CH_2\underline{C}H_3$); 12.76-12.81 ($5CH_3$); 15.64 ($CH\underline{C}H_3$); 25.06-25.14 ($CH_2\underline{C}HCH_3$); 39.39-39.47-39.52-39.60 (CH_2); 52.61 (OCH_3); 59.38-59.54 (NHCH); 66.94-67.58-67.65 (C5'); 84.91-85.04-85.16 (C4'); 89.94-90.21 (C1'); 111.75-111.87 (C5'); 120-151 (m Ar); 127.82-127.87 (C2'); 133.49-133.69 (C3'); 135.99-136.28 (C6); 151.37 (C2); 164.40 (C4); 173.53-173.59-173.64 ($\underline{C}OOCH_3$)
Mass: $C_{23}H_{30}O_8N_3P$: 529.91 ($(M + Na)^+$, 100)
HPLC : RT = 30.52 and 31.14 min

2', 3'-dideoxy-2', 3'-didehydrothymidine 5'-(phenylalaninyl phosphate) Cf 1187

Yield = 68%
^{31}P ($CDCl_3$) : 7.58 ppm
^{1}H ($CDCl_3$) : 1.70 (s, 3H, $5CH_3$); 2.64-2.80 (m, 2H, $C\underline{H}_2Ph$); 3.57-3.64 (m, 1H, $C\underline{H}NH$); 3.68-3.70 (m, 2H, H5'); 4.85 (s, 1H, H4'); 5.73-5.75 (m, 1H, H2'); 6.26-6.29 (m, 1H, H3'); 6.74-6.75 (m, 1H, H1'); 7.02-7.28 (m, 5H, $CH_2\underline{Ph}$); 7.44 (s, 1H, H6)
^{13}C ($CDCl_3$) : 11.88 ($5CH_3$); 40.92-40.97 (CH_2 ala); 58.27 (CH

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ala); 65.22-65.28 (C5'); 86.36-86.49 (C4'); 90.22 (C1'); 111.63 (C5); 125.38 (C2'); 126-129 (m, Ar); 134.74 (C3');138.31-138.48 (C6); 152.40 (C2); 166.81 (C4); 180.87-180.96 (COOH)

Mass : $C_{19}H_{22}O_8N_3P$: 450 ($(M-1)^-$, 100); 451 ($(M^-$, 20)

HPLC : RT = 32.11 min

2', 3'-dideoxy-2',3'-didehydrothymidine 5'-(valinyl phosphate) Cf 1190

Yield = 67%

^{31}P ($CDCl_3$) : 8.35 ppm

^1H ($CDCl_3$) : 0.72 (t, 6H, $(C\underline{H}_3)_2CH$, J = 7.3 Hz); 1.62-1.73 (m, 1H, $(CH_3)_2C\underline{H}$); 1.77 (s, 3H, $5CH_3$); 3.12 (dd, 1H, $NHC\underline{H}$, J = 5.6 Hz and 9.4 Hz); 3.80 (dd, 2H, H5', J = 3.5 Hz and 4.4 Hz); 4.92 (s, 1H, H4'); 5.76-5.78 (m, 1H, H2'); 6.31-6.35 (m, 1H, H3'); 6.79-6.81 (m, 1H, H1'); 7.53 (s, 1H, H6)

^{13}C ($CDCl_3$) : 11.84 ($5CH_3$); 17.95-18.84 ($(\underline{C}H_3)_2CH$); 32.30-32.38 ($(CH_3)_2\underline{C}H$); 62.43 (CHNH); 65.18-65.24 (C5'); 86.43-86.58 (C4'); 90.25 (C1'); 111.65 (C5); 125.20 (C2'); 134.90 (C3'); 138.73 (C6); 152.52 (C2); 167.05 (C4); 181.27-181.31 (COOH)

Mass : $C_{15}H_{22}O_8N_3P$: 402 ($(M-1)^-$, 100); 403 ($(M)^-$, 30)

HPLC : RT = 31.90 min

2', 3'-dideoxy-2', 3'-didehydrothymidine 5'-(leucinyl phosphate) Cf 1192

Yield = 83%

^{31}P ($CDCl_3$) : 7.98 ppm

^1H ($CDCl_3$) : 0.71 (d, 6H, $(C\underline{H}_3)_2CH$, J = 6.5 Hz); 1.22-1.34 (m, 2H, CH_2); 1.34-1.71 (m, 1H, $(CH_3)_2C\underline{H}$); 1.80 (s, 3H, $5CH_3$); 3.30-3.38 (m, 1H, $C\underline{H}NH$); 3.82-3.85 (m, 2H, H5'); 4.95 (s, 1H, H4'); 5.80-5.82 (m, 1H, H2'); 6.35-6.37 (m, 1H, H3'); 6.81-6.82 (m, 1H, H1'); 7.58 (s, 1H, H6)

^{13}C ($CDCl_3$) : 12.53 ($5CH_3$); 22.88-22.99 ($(\underline{C}H_3)_2CH$); 25.28 (CH_2); 45.27-45.34 ($(CH_3)_2\underline{C}H$); 56.38 (CHNH); 65.74-65.81 (C5'); 87.12-87.25 (C4'); 90.89 (C1'); 112.30 (C5); 125.99 (C2'); 135.49 (C3'); 139.44 (C6); 153.12 (C2); 167.70 (C4); 183.36-183.42 (COOH)

Mass : $C_{16}H_{24}O_8N_3P$: 416 ($(M-1)^-$, 100); 417 ($(M^-$, 20)

HPLC : RT = 35.02 min

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2', 3'-dideoxy-2', 3'-didehydrothymidine 5' -(phenylmethoxyalaninyl phosphate) [fast diastereoisomer] Cf 1193

^{31}P ($CDCl_3$) : 4.51 ppm

^{1}H ($CDCl_3$) : 1.25-1.40 (m, 3H, $CHC\underline{H}_3$); 1.86-1.90 (m, 3H, $5CH_3$); 3.74-3.90 (m, 4H, OCH_3 + CH ala); 4.37-4.47 (m, 2H, H5'); 5.08 (bs, 1H, H4'); 5.91-5.93 (m, 1H, H2'); 6.38-6.41 (m, 1H, H3'); 7.07-7.09 (m, 1H, H1'); 7.20-7.39 (m, 6H, Ar + H6); 9.04 (bs, 1H, NH)

^{13}C ($CDCl_3$) : 10.85 ($5CH_3$); 19.38-19.45 ($CH\underline{C}H_3$); 48.71 ($\underline{C}HCH_3$); 51.14 (OCH_3); 64.91-64.97 (C5'); 83.11-83.22 (C4'); 88.03 (C1'); 109.77 (C5); 118-149 (m, Ar); 125.84 (C2'); 131.88 (C3'); 134.44 (C6); 149.34 (C2); 162.35 (C4); 172.53-172.62 (CO ala)

2', 3'-dideoxy-2',3'-didehydrothymidine 5'-(phenylprolinyl phosphate) Cf 1194

Yield = 41%

^{31}P ($CDCl_3$) : 5.27 ppm

^{1}H ($CDCl_3$) : 1.55 (s, 3H, $5CH_3$); 1.56-2.15 (m, 4H, $CHC\underline{H}_2C\underline{H}_2$); 3.10-3.30 (m, 2H, NCH_2); 3.90-4.00 (m, 1H, NCH); 4.20-4.50 (m, 2H, H5'); 5.11 (s, 1H, H4'); 5.89-5.91 (m, 1H, H2'); 6.41-6.44 (m, 1H, H3'); 6.76-6.78 (m, 1H, H1'); 6.99-7.40 (m, 6H, Ar + H6)

^{13}C ($CDCl_3$) : 11.84 ($5CH_3$); 25.44-25.56 ($\underline{C}H_2CH_2N$); 31.94-32.06 ($\underline{C}H_2CHN$); 47.40-47.46 (NCH_2); 63.31 (CHN); 67.14-67.21 (C5'); 85.56-85.68 (C4'); 90.69 (C1'); 111.00 (C5), 120-150 (m, Ar), 125.07 (C2'), 134.13 (C3'), 138.26 (C6), 152.67 (C2), 166.64 (C4), 181.32 (COOH)

Mass : $C_{21}H_{24}O_8N_3P$: 476 ((M-1)$^-$, 100); 477 ((M)$^-$, 25)

HPLC : RT = 34.16 min

<u>1001 2', 3'-dideoxy-2', 3' didehydroadenosine-5'-(phenyl methoxyalaninyl phosphoramidate:</u>

Yield = 67%

^{1}H (dmso-d6): 8.14 (1H, s, H8), 8.06 (1H, d, H2), 7.07-7.40 (7H, m, Phe-H & NH_2), 6.93 (1H, s, H1'), 6.47 (1H, 2d, H3'), 6.21 (1H, d, H3'), 5.96 (1H, m, NH), 5.11 (1H, m, H4'), 4.10 (2H, m, H5'), 3.5-4.83 (1H, 2m, CH ala), 3.52 (3H, d, MeO),

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1.08 (3H, 2d, CH_3 ala).

^{31}P (dmso-d6): 4.92, 4.78.

^{13}C (dmso-d6): 172.909-172.815 (CO ala), 154.663 (C-2), 152.238 (C-6), 149.524-149.442 (Ar-ipso), 148.782 (C-4), 138.006-137.907 (C-8), 132.286-132.205 (C-2'), 128.621 (Ar-meta), 125.384-125.210 (Ar para), 123.928 (C-3'), 119.067-119.00 (Ar ortho), 118.508 (C-5), 87.311-87.060 (C-1'), 84.485-84.368 (C-4'), 66.093-65.324 (C-5'), 51.477-51.429 (OMe), 49.109-48.989 (C-H ala), 19.903-19.585 (CH_3 ala).

Mass. Calculated MH^+:475.149. Found: 475.151

<u>1093 2', 3'-dideoxy adenosine 5'-(phenyl methoxyalaninyl) phosphoramidate</u>

Yield = 42%

^1H ($CDCl_3$) : 8.32 (1H, s, H-8), 8.12 & 8.11 (1H, 2s, H-2), 7.22 (5H, m, Ar), 6.40 (2H, 2bs, NH_2), 6.30 (1H, t, H-1', J = 5.4 Hz), 4.42 (4H, m, N-H, 2H5' & H4'), 4.00 (1H, 2d, Ala C-H), 3.65 (3H, 2s, OMe), 2.52 (2H, m, H3'), 2.13 (2H, m, H2'), 1.31 (3H, 2d, CH_3 ala, J = 7.3 Hz).

^{31}P ($CDCl_3$) : 4.26, 4.19.

^{13}C nmr ($CDCl_3$) : 174.534, 174.468, 174.441, 174.372 (O-C=O), 156.148 (C-2), 153.331 (C-6), 151.092 & 151.006 (2 Ar ipso), 149.674 & 149.599 (C-4), 139.211 & 139.103 (C-8), 130.040 (Ar meta), 125.325 (Ar para), 120.570 (C-5), 120.508 & 120.327 (Ar ortho), 85.994 & 85.746 (C-1'), 80.105, 79.985 & 79.874 (C-4'), 68.136, 68.067, 67.704 & 67.636 (C-5'), 52.868 (OMe), 50.628 & 50.531 (Ala C-H), 32.712 (C-2'), 26.339 & 26.106 (C-3'), 21.337, 21.264 & 21.190 (CH_3 ala).

Mass: Calculated MH^+: 477.165. Found: 477.164.

<u>1094 2', 3'-dideoxy-2', 3' didehydroadenosine 5'-(phenyl benzylalaninyl) phosphoramidate:</u>

Yield = 65%

^1H ($CDCl_3$): 8.32 (1H, bs, H-8), 7.99 (1H, bs, H-2), 7.21 (11H, m, Ar-H & H1'), 6.34 (1H, m, H3'), 6.07 (1H, m, H2'), 5.81 (2H, 2bs, NH_2), 5.08 (3H, 2bs, Bz-CH_2 & H4'), 4.05 (4H, m, NH, CH, H5'), 1.24 (3H, 2d, methyl ala, J = 6.9 Hz).

^{31}P ($CDCl_3$): 4.21, 3.98

^{13}C ($CDCl_3$): 173.700 & 173.601 (O-C=O), 156.005 (C-2),

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153.728 (C-6), 150.952 & 150.870 (Ar), 150.322 & 150.280 (C-4), 139.484 & 139.368 (C-8), 135.672 (Ar), 133.733 & 133.654 (C-2'), 130.066 (Ar), 129.041, 128.895, 128.635 & 128.601 (Ar), 126.751 & 126.598 (C-3'), 125.375 (Ar), 120.529, 120.463, 120.399, 120.119 & 120.051 (C-5 & Ar), 88.702 & 88.476 (C-1'), 85.907, 85.476, 85.791 & 85.736 (C-4'), 67.632, 67.475 & 67.403 (C-5' and Bz-CH_2), 66.805 & 66.745 (C-5'), 50.677 & 50.542 (Ala C-H), 21.399, 21.335, 21.083 & 21.019 (methyl Ala).

Mass: Calculated MH^+: 551.181. Found: 551.179.

1168 2', 3'-dideoxy-2', 3'-didehydroadenosine 5'-alaninyl phoshoramidate

Yield = 69%

^1H nmr (D_2O): 8.09 (1H, s, H8), 7.88 (1H, s, H2), 6.81 (1H, s, H1'), 6.33 (1H, d, H3'), 6.02 (1H, d, H3'), 5.01 (1H, m, H4'), 4.73 (2H, m, H5'), 3.5-4.83 (1H, 2m, CH ala), 0.89 (3H, 2d, CH_3 ala).

^{31}P (D_2O): 8.34.

^{13}C (D_2O): 183.055 (CO ala), 155.549 (C-2), 152.745 (C-6), 148.643 (C-3), 140.928 (C-8), 134.730 (C-2'), 124.709 (C-3'), 118.527 (C-5), 88.299 (C-1'), 87.199 & 87.073 (C-4'), 65.215-65.149 (C-5'), 52.564 (Alal C-H), 21.435-21.381 (Ala CH_3).

1196 - 2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'-(phenyl dimethoxy glutaminyl phosphoramidate

Yield 33%

^{31}P ($CDCl_2$) 4.14, 4.76

^1H ($CDCl_3$) 1.81, 1.85 ($5CH_3$); 1.91-2.18 (m, 2H, CH_2 Gln); 2.24-2.36 (m, 2H, CH_2 Gln); 3.64 (s, 3H, NMe); 3.69 (s, 3H, OMe); 3.92-4.21 (m, 2H, H5'); 4.23-4.42 (m, 2H, CH Gln, NH Gln); 5.00 (m, 1H, H4'); 5.91 (m, 1H, H2'); 6.31 (m, 1H, H3'); 7.01 (m, 1H, H1'), 7.03-7.34 (m, 6H, Ph, H6); 9.49 (s, 1H, NH)

^{13}C ($CDCl_3$) 12.32-12.36 ($5CH_3$); 29.01-29.42 (CH_2 Gln); 29.46 (NMe); 51.81 (CH Gln); 52.65 (OMe); 53.65-53.92 (CH_2 Gln); 66.63-67.33 (C5'); 84.48-84.71 (C4'); 89.57-89.83 (C1'); 111.29-111.44 (C5); 119.98-120.22 (Ph); 125.21-125.26 (Ph);

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127.39-127.50 (C2'); 129.74-129.78 (Ph); 133.00-133.25 (C3'); 135.60-135.90 (C6); 150.98 (C2); 164.00-164.09 (C4); 172.96-173.23 (CO, CON)
Mass (ES) : $C_{23}H_{29}N_4O_9P$: 536 (M^+, 100); 537 (MH^+, 32)

1214 - 2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'-(phenyl dimethoxy asparaginyl) phosphoramidate
Yield 75%
^{31}P ($CDCl_3$) 1.15, 2.20
^1H ($CDCl_3$) 1.81, 1.86 (s, 3H, $5CH_3$); 2.49-2.92 (m, 2H, CH_2 Asn); 3.64 (s, 3H, NMe); 3.72 (s, 3H, OMe); 4.04-4.26 (m, 2H, H5'); 4.28-4.43 (m, 2H, CH Asn, NH Asn); 5.05 (m, 1H, H4'); 5.89 (m, 1H, H2'); 6.31 (m, 1H, H3'); 7.01 (m, 1H, H1'); 7.14-7.33 (m, 6H, Ph, H6); 8.46 (s, 1H, NH)
^{13}C ($CDCl_3$) 12.28 ($5CH_3$); 51.01 (CH Asn); 52.09 (OMe); 52.94 (CH_2 Asn); 84.75 (C4'); 89.60 (C1'); 111.30 (C5); 125-130 (Ph); 127.32-127.48 (C2'); 133.10-133.41 (C3'); 135.94 (C6)
Mass (ES): $C_{22}H_{27}N_4O_9P$: 522 (M^+, 100); 523 (MH^+, 31)

1215 - 2', 3'-Dideoxy-2', 3'-didehydrothymidine-5'-(phenyl methoxytryptophanyl) phosphoramidate
Yield 100%
^{31}P ($CDCl_3$) 4.15, 4.57
^1H ($CDCl_3$) 1.74 (s, 3H, $5CH_3$); 3.16 (m, 2H, CH_2 Trp); 3.60 (s, 3H, OMe); 3.75-4.05 (m, 2H, H5'); 4.10-4.33 (m, 2H, CH Trp NH Trp); 4.84 (m, 1H, H4'); 5.79 (m, 1H, H2'); 6.15 (m, 1H, H3'); 6.86 (m, 1H, H1'); 6.91 (m, 1H, H6); 7.00-7.49 (m, 10H, Ar); 8.45 (s, 1H, NH Trp); 9.14 (s, 1H, NH)
^{13}C ($CDCl_3$) 14.75 ($5CH_3$); 32.46 (CH_2 Trp); 54.91 (CH Trp); 57.53-57.61 (OMe); 69 (C5'); 87.06 (C4'); 92.03-92.25 (C1'); 111.63 (C5); 127.60 (C2'); 135.45-135.83 (C3'); 138.11-138.62 (C6); 152.78-153.41 (C2); 166.28-166.40 (C4); 175.85 (CO)
Mass (ES): $C_{28}H_{28}N_4O_9P$: 579 (M^+, 100); 580 (M^+, 43)

462 3'-Deoxy-3'- β-azidothymidine 5'-(phenyl methoxylalaninyl)
phosphoramidate
^1H ($CDCl_3$): 1.39 (d, 3H, J = 7.2 Hz, CH_3 ala), 1.94 (s, 3H 5-

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Me), 2.15 (d, 1H, J = 15.5 Hz, H2'), 2.68-2.79 (m, 1H, H2'), 3.72 (s, 3H, OMe), 3.90-4.50 (m, 6H, H3' + H4' + H5' + NH + CHala), 6.18 (dd, 1H, J = 7.5 and 3.1 Hz, H1'), 7.1-7.4 (m, 6H, Ph + H6), 8.82 (bs, 1H, NH).

^{13}C ($CDCl_3$) : 12.67 (5-Me), 20.96, 21.29 (ala-Me), 38.50 (C2'), 50.16, 50.28 (CHala), 52.57 (OMeala), 60.74 (C3'), 64.43 (C5'), 80.17 (C4'), 83.93 (C1'), 111.21 (C5), 120.11 (Ar2), 125.18 (Ar4), 129.73 (Ar3), 135.18 (C6), 159.96 (Ar1), 150.30 (C4), 163.49 (C2), 173.84 (COala).

^{31}P ($CDCl_3$) : 1.55

IR ($CDCl_3$): 3216, 2113, 1685 cm-1.

Mass 509.1543 (MH^+, 40%, calculated 509.1549), 340(12), 250(17), 200(18).

HPLC: RT = 28.48 min.

536 3'-Deoxy-3' β-azidothymidine 5'-(m-trifluoromethylphenyl methoxylalaninyl) phosphoramidate

^1H ($CDCl_3$): 1.39, 1.40 (d, 3H, J = 7.2 Hz, Me-ala), 1.92, 1.93 (s, 3H, 5-CH_3), 2.15 (d, 1H, J = 15.1 Hz, H2'), 2.71-2.80 (m, 1H, H2'), 3.70, 3.71 (s, 3H, OMe), 3.90-4.50 (m, 6H, H3' + H4' + H5' + NH + CHala), 6.19 (dd, 1H, J = 7.7 and 3.3 Hz, H1'), 7.41-7.46 (m, 5H, Ph + H6), 9.52 (bs, 1H, NH).

^{13}C ($CDCl_3$): 12.58 (5-Me), 20.75, 20.83 (CH_3 ala), 38.33, 38.44 (C2'), 50.15, 50.29 (CHala), 52.55 (OMeala), 60.77 (C3'), 64.72 (C5'), 80.05, 80.35 (d, J = 6.8 Hz, C4'), 83.94 (C1'), 111.25 (C5), 117.43 (Ar2), 121.81, 121.86 (Ar4), 123.37 (q, J = 273 Hz, CF_3), 123.74 (Ar6), 130.35 (Ar5), 132.11 (q, J = 33 Hz, Ar3), 135.11 (C6), 150.49 (C4), 150.62 (Ar1), 163.78 (C2), 173.68, 173.87 (d, J = 7.8 Hz, COala).

^{31}P: 2.69

Mass 577 (MH^+, 40%) 340 (13), 268 (14), 250 (12).

HPLC: RT = 30.66 min.

550 3'-Deoxy-3'- β-azidothymidine 5'-(3, 5 -dichlorophenyl methoxylalaninyl) phosphoramidate

^1H ($CDCl_3$): 1.42 (d, 3H, J = 6.8 Hz, Me-ala), 1.94, 1.95 (d, 3H, J = 1.2 Hz, 5-CH_3), 2.17, 2.18 (d, 1H, J = 15.1 Hz, H2'), 2.76-2.85 (m, 1H, H2'), 3.74, 3.75 (s, 3H, OMe), 3.90-4.50 (m, 6H, H3' + H4' + H5' + NH + CHala), 6.20 (dd, 1H, J

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= 7.7 and 3.3 Hz, H1'), 7.19 (m, 2H, Ar2), 7.27 (s, 1H, Ar4), 7.41, 7.42 (s, 1H, H6), 9.04 (bs, 1H, NH).

^{13}C: 12.65 (5-Me), 20.85, 20.91 (CH_3 ala), 38.38, 38.48 (C2'), 50.18, 50.29 (CHala), 52.68 (OMeala), 60.77 (C3'), 64.86, 64.93 (C5'), 79.80, 80.20 (d, J = 8Hz, C4'), 83.97 (C1'), 111.35 (C5), 117.28, 119.38 (d, J = 6Hz, Ar2), 125.58 (Ar4), 135.10 (C6), 135.46, 135.50 (Ar3), 145.35 (Ar1), 150.36 (C4), 163.61 (C2), 173.64, 173.79 (COala).

^{31}P: 2.83

Mass 577, 579, 581 (MH^+ 5:3:1:) 307, 309, 311 (12:8:2) 289 (10)

In vitro Testing

Cells were infected with HIV-1 as previously described [Balzarini et al. AIDS (1991), 5, 21-28]. Briefly, 5 x 10^5 cells per milliliter were infected with HIV-1 or HIV-2 at 100 $CCID_{50}$ (50% cell culture infective dose) per milliliter of cell suspension. Then 100 µL of the infected cell suspension was transferred to microtiter plate wells and mixed with 100 µL of the appropriate dilutions of the test compounds. After 4 days giant cell formation was recorded microscopically in the HIV-infected cell cultures [CEM], and after 5 days the number of viable cells was determined by trypan blue staining of the HIV-infected cell cultures [MT4]. The 50% effective concentration (EC_{50}) and 50% cytoxic concentration (CC_{50}) were defined as the compound concentrations required to reduce by 50% the number of giant cells or viable cells in the virus-infected and mock-infected cell cultures, respectively.

The anti-HIV-1 activities and toxicities of compounds were also assessed in two cell lines:

C8166 cells. Cells were grown in RPMI 1640 with 10% calf serum. 4 x 10^4 cells per microtiter plate well were mixed with 5-fold dilutions of compound prior to addition of 10 $CCID_{50}$ units of III-B strain of HIV-1 and incubated for 5-7 days (Betbeder *et al*. Antiviral Chem. Chemother. 1, 241-247, 1990). Formation of syncytia was examined from 2 days post-infection. Culture fluid was collected at 5-7 days and gp120 antigen production measured by ELISA (Mahmood and Hay, J. Immunol. Meth., 151, 9-13, 1992). The EC_{50} is that concentration of drug [in µM] required to reduce gp120 production by 50%. Cell viability of infected and uninfected cells were assessed by the MTT-Formazen method (Pauwels *et al*. J. Virol. Meth. 20, 309-321, 1988).

JM cells JM cells, which are relatively resistant to the antiviral effects of AZT and a number of its derivatives,

were infected with HIV-1 strains and the antiviral and toxic effects of compounds assessed as for C8166 cells. Both GB8 or IIIB strains of HIV1 were used, with no detectable differences in the end-points noted.

Each assay was carried out in duplicate, on at least two separate occasions, and data quoted are the average of each separate assay.

The compounds of the present invention have been shown to be active against both HIV1 and HIV2 in both TK$^+$ and TK$^-$ cells as illustrated in Table 2.

Table 2

	HIV1 in C8166/JM		HIV2 in CEM TK$^+$/CEM TK$^-$	
Compound	EC_{50} μM C8166	EC_{50} JM	EC_{50} μM CEM TK$^+$	EC_{50} CEM TK$^-$
730	0.0008	0.0008	0.016	0.06
d4T (comparative)	0.08	0.8	1.2	>100

As expected, d4T (comparative) loses activity in the kinase deficient cells (especially CEM TK$^-$), whilst compound 730 of the invention retains good activity in both TK$^+$ and TK$^-$ against both HIV1 and HIV2. Compound 730 of the invention is >1000 times more potent than d4T in TK$^-$ cells. Surprisingly, the compound is 100-fold more potent than d4T in CEM TK$^-$ assays.

The potent activity of the compounds of the invention is further supported by the data in Table 3, which illustrates activity, toxicity and selectivity index of a series of compounds of the present invention.

The enhanced anti-viral potency and reduced cytotoxicity of the phosphate derivatives lead to very large improvements in

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The enhanced anti-viral potency and reduced cytotoxicity of the phosphate derivatives lead to very large improvements in selectivity index [defined as CC_{50}/EC_{50}] evidencing marked improvements in in vivo efficacy compared to d4T (comparative).

Evidence that the compounds of the present invention are acting via a pathway different to that of d4T or AZT is provided by the data of Table 4.

As can be seen, whilst the potency of d4T (comparative) is much reduced in nucleoside resistant strains, the potency of the compounds of the present invention is largely maintained. Thus, it is clear that the compounds of the present invention are not acting primarily via the conventional nucleoside 5'triphosphate derivative.

CEM and MT4 cells (at 400,000 cells/ml) and PBL cells (at 2,000,000 cells/ml) were exposed to different concentrations of [^{3}H] 324 and incubated at 37°C for 24 hours. Then cells were washed twice with cold PBS and to the cell pellet was added 400 µl cold methanol 66%. After standing on ice for 10 min, the cell extract was centrifuged and the supernatent analayzed on HPLC. As shown in Table 5, intracellular D4T-MP (monophosphate) levels increased proportionally in function of the initial concentration of 324 in all three cell lines tested. However, the increase of D4T-TP (triphosphate) levels slowed down at initial 324 concentrations that were higher than 25 µM (for CEM and MT4 cells) or higher than 1.0 µM (for PBL). Surprisingly, a metabolite (designated X) accumulated substantially and predominantly in all three cell types. The accumulation was proportional to the initial 324 concentration, and, again, was lower in PBL than CEM and MT4 cells.

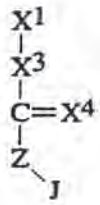
When 1mM 324 was incubated with high concentrations of hog liver esterase at 37°C in Tris-HCl buffer containing 5 mM $MgCl_2$, a time-dependent formation of a metabolite was observed. This metabolites co-eluted with the predominant

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metabolite (X) that was found in the cell extracts after incubation of the intact cells with [^{3}H] 324. metabolite X corresponds to a compound of formula (10), wherein Y is oxygen, X^1 is NH, X^2 is oxygen, B is thymine, R^1 is Me, R^2 is hydrogen.

Data on an expanded range of compounds is presented in Table 6 (d4T analogues) and Table 7 (dideoxy and 3' - β - substituted nucleoside analogues) in which:-

Cpd and Init : refer to the compound reference numbers;
Y : refers to the group:-



Z : refers to the 3' - substituent on a deoxyribose sugar wherein the substituent is in an "α" orientation (R^9) unless designated "up" which refers to a "β" orientation (R^{10});

B : refers to the heterocyclic nucleic acid base, present at C1' in β - orientation; conventional one-letter base codes are used; pyrimidine substituents are at C5.

The data columns are, in order:
HIV1 MT4: EC_{50} in μM for inhibition of HIV-1 in MT4 cells.
HIV2 MT4: EC_{50} in μM for inhibition of HIV-2 in MT4 cells.
CC50 MT4: CC_{50} in μM for toxicity to MT-4 cells.
HIV1 CEM: EC_{50} in μM for inhibition of HIV-1 in CEM cells.
HIV2 CEM: EC_{50} in μM for inhibition of HIV-2 in CEM cells.
HIV2 CEM-TK$^-$: EC_{50} in μM for inhibition of HIV-2 in CEM/TK$^-$ cells.

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CC50 CEM: CC_{50} in μM for toxicity to CEM cells.
EC50 MSV: EC_{50} in μM for inhibition of MSV
MCC MSV: Minimum cytotoxic concentration in MSV assay

Where data of table 6 differs from that presented in Tables 2 to 5, the data of the former relates to the mean result obtained from two or more repeat experiments, whereas the latter relates to individual experimental results.

Table 3

Entry	Ar	R'	J	Activity EC_{50}	Toxicity CC_{50}	Selectivity $CC_{50}/EC_{50} \times 10^3$
323	4-EtPh	Me	Me	0.0032	50	15.6
324	Ph	Me	Me	0.0032	150	46.9
327	4-FPh	Me	Me	0.0032	200	62.5
526	3-CF_3Ph	Me	Me	0.0008	200	250
546	3,5-Cl_2Ph	Me	Me	0.001	100	100
730	Ph	Me	Bzl	0.0008	400	500
776	2,4-Br_2Ph	Me	Me	0.0008	100	125
779	F_5Ph	Me	Me	0.064	80	1.25
862	Ph	Me	Hex	0.0012	500	417
863	Ph	Me	Me	0.016	500	31.2
864	Ph	CH_2iPr	Me	0.016	>1000	>62.5
865	Ph	iPr	Me	0.8	>1000	> 1.25
866	Ph	H	Me	0.8	>1000	> 1.25
867	Ph	$[CH_2]_2$SMe	Me	0.0016	>1000	>62.5
868	2,4Br_2Ph	Me	Bzl	0.0032	500	156
877	Ph	Bzl	Bzl	0.0003	80	267
878	Ph	Bzl	tBu	0.16	150	0.9
892	Ph	Me	Cyclohex	0.0016	500	312
893	Ph	Me	tBu	0.2	>1000	> 5.0
[data are μM for HIV1 in C8166 cells]						
By comparison, similar data for d4T:				0.08	50	0.6
d4T						

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Table 4

Compound	EC_{50} in μM	EC_{50}	SI	EC_{50}	EC_{50}	EC_{50}	EC_{50}
	HIV RT	8166	8166	CEM	CEM TK⁻	HeLa	HeLa
		HIV1	HIV1	HIV1	HIV2	HIV1 d4T-Sensitive	HIV1 d4T-Resistant
d4T	Inactive	0.08	625	0.5	>100	0.86	3.38
324	50	0.0032	62,500	0.18	0.08	n/d	n/d
526	n/d	0.0008	>250,000	0.08	0.06	0.04	0.05
546	n/d	0.001	>200,000	0.06	0.06	0.03	0.04
AZT	Inactive	0.008	>100,000	0.003	>100	n/d	n/d

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Table 5

Metabolism of [^{3}H] 324 after 24 hr incubation in human CEM, MT4 and PBL cells

nmole/10^9 cells												
Metabolite	CEM						MT-4			PBL		
	Initial concentration of [^{3}H] 324 (μM)											
	0.2	1.0	5.0	25	100	500	0.2	25		0.2	1.0	25
324 + D4T	7.6	47.8	228	897	4,333	16,691	7.9	1,255		2.0	12.2	245
D4T-MP	3.9	10.8	54	490	2,259	11,359	29	394		2.4	14.2	355
D4T-DP	1.5	5.1	21.6	75	214	430	2.0	116		0.45	1.8	15.3
D4T-TP	10.3	37.6	177	553	691	938	22.6	535		6.6	27	149
	133	628	3,164	16,193	66,359	204,442	117	14,582		17.6	97.3	1,995

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Cpd	Int	ArO	Y	Z	B	HIV1 M	HIV2 M	CC50 M	HIV1 CEM	HIV2 CEM	2 CEM TK	CC50 CEM	EC50 MSV	MCCMSV
268	AS	-	-	=	T				0 24	1 2	>100			
321	AS(I)	IIexO	IIexO	=	T				>42	>42	>42	36		
322	AS	TCEO	MeValNH	=	T				29	71	59			
323	AS	EtPhO	MeAlaNH	=	T	0 057	0 063	>100	0.07	0.16	0 06	60		
324	AS(I)	PhO	MeAlaNH	=	T	0 081	0 063	>100	0.075	0 075	0 075	100		
325	AS	TCEO	MePheNH	=	T	0 44	0 5	>100	1	2	0 7			
326	AS	PrO	MeAlaNH	=	T	36	84	>250	>230	>230	135	≥250		
345	AS	TCEO	MeMetNH	=	T				8	11	10			
400	AS	TCEO	PrNH	=	T				>40	>40	>40			
401	AS	TCEO	PrNH	=	T				>210	>210	>210			
402	AS	TCEO	BuNH	=	T				118	>204	161			
403	AS	TCEO	EtNH	=	T				>216	>216	>218			
404	AS	TCEO	PrO	=	T				>209	>209	>209			
406	AS	TCEO	MeOCH2CH2N	=	T				>203	>203	>203			
407	AS	TCEO	HO	=	T				0 5	0 5	86			
446	AS	-	-	=	U				>95	>95	>95			
479	ST	IPrO	IPrOSPNH	=	T				>258	>258	>258			
480	ST	BuO	BuOSPNH	=	T				>48	>48	>48			
481	ST	BuO	BuOSPNMe	=	T				>9	>9	>9			
504	AS	TFEO	BuNH	=	T				73	116	≥ 226			
526	AS	mCF3PhO	MeAlaNH	=	T	0 05	0 11	10	0 15	0 15	0 12	30		
546	AS	3 5-Cl2PhO	MeAlaNH	=	T	0 037	0 11	10 5	0 12	0 15	0 12	26 9		
547	AS	mTFMPhO	MeAlaNH	=	U				>3520	>7	>35			
551	AS	EtO	EtO	=	U				>58	>58	>58			
558	AS	PhO	MeAlaNH	=	U				>44	>44	>44			
561	AS	FPhO	MeAlaNH	=	H				85	4	≥ 72			
562	AS	TCEO	TCEO	=	U				>36	>36	>36			
563	AS	EtO	PrNH	=	T				>268	>268	>268			
564	AS	EtO	MeAlaNH	=	T	28 5	62 5	≥ 250	>48	>240	>48	≥250		
730	DC	PhO	BzAlaNH	=	T				0 016	0 016	0 06	25		
740	DC	MeO	MeAlaNH	=	T	25 4	50 9	≥250	20	50	≥250	≥250		
775	DC	HO	HO	=	T				0 8	0 95	33	174		
776	DC	2,4Br2PhO	MeAlaNH	=	T				0 04	0 055	0 025	16		
779	DC	FsPhO	MeAlaNH	=	T	1 72	4 07	82	2 5	3 7	8 5	115		
786	DC	IIexO	H	=	T				0 8	0 5	30	150		
787	DC	MeODigolO	H	=	T				0 65	0 95	45	144		
788	DC	EtO	H	=	T				0 65	0 6	30	115		

TABLE 6

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Cpd	Init	Aro	Y	Z	B	HIV1 M	HIV2 M	CC50 M	HIV1 CEM	HIV2 CEM	2 CEM.TK	CC50 CEM	EC50 MSV	MCCMSV
789	DC	DecO	H	=	T				0.65	0.65	40	64		
790	DC	BuO	H	=	T				0.65	0.9	30	177		
791	DC	OctO	H	=	T				0.4	0.6	23	90		
792	DC	PntO	H	=	T				0.55	0.9	40	137		
793	DC	PrO	H	=	T				0.65	0.65	33	170		
816	DC	C16O	H	=	T				0.85	0.65	15	25		
817	DC	MeO	H	=	T				0.7	0.55	20	146		
828	UC	PhO	C12NH	=	T				>10	>10		14		
829	DC	PhO	C8NH	=	T				>10	>10		23		
830	DC	PhO	HxNH	=	T				140	100	125	≥250		
849	DC	PhO	BzAlaNH	=	U				23	15	10	115		
853	DC	PhO	OH	=	T				0.65	0.7	23	153		
858	DC	PhO	PrNH	=	T				>250	>250	>250	>250		
859	DC	PhO	HxNH	=	T				>50	>50	>50	145		
860	DC	PhO	PntNH	=	T				>250	>250	>250	≥250		
861	DC	PhO	CNEO	=	T				1.2	0.95	17.5	≥250		
862	DC	PhO	HxAlaNH	=	T				0.06	0.055	0.033	48		
863	DC	PhO	MePheNH	=	T	0.2	0.4	34	0.8	1.35	0.33	216		
864	DC	PhO	MeLeuNH	=	T	0.2	0.5	62	1.1	2.23	0.4	≥250		
865	DC	PhO	MeValNH	=	T	3.6	11.2	≥250	12.5	12.5	4	>250		
866	DC	PhO	MeGlyNH	=	T	1.3	6.7	≥250	6	6	7	≥250		
867	DC	PhO	MeMetNH	=	T	0.2	0.4	22	0.6	0.8	0.34	≥250		
868	DC	Br2PhO	BzAlaNH	=	T				0.2	0.2	0.2	50		
870	DC	Br2PhO	BzAlaNH	=	U				>50	>50	12.5	180		
877	DC	PhO	BzPheNH	=	T				0.6	0.6	0.24	44		
878	DC	PhO	tBuPheNH	=	T				2	2	0.65	80		
879	DC	PhO	MeProNH	=	T				>10	>10	>10	42.5		
880	DC	PhO	PhO	=	T				25	25	75	≥250		
881	DC	HO	HO12AlaNH	=	T									
892	GO/D	PhO	ClHxAlaNH	=	T				0.065	0.075	0.09	51.4		
893	GO/D	PhO	tBuAlaNH	=	T				0.85	1.1	0.74	≥250		
932	ASS	PhO	Me D AlaNH	=	T				3	2	2.5	≥250	>100	>100
933	DC	PhO	BzProNH	=	T				35	12.5	>50	92	>100	>100
949	DC	PhO	EtMetNH	=	T				0.8	0.3	0.15	166	52	>100
950	DC	PhO	Et D AlaNH	=	T				250	≥250	>250	>250	>100	>100

TABLE 6 (CONTD.)

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Cpd	Init	ArO	Y	Z	B	HIV1 M	HIV2 M	CC50 M	HIV1 CEM	HIV2 CEM	2.CEM.TK	CC50 CEM	EC50 MSV	MCCMSV
951	DC	PhO	EtAlaNH	=	T				0.1	0.07	0.07	55	25	>100
978	DC	PhO	MeLactO	=	T				40	50	>250	≥ 250	>100	>100
979	DC	PhO	EtLactO	=	T				28	23	160	>250	>100	>100
980	DC	PhO	MeGlycO	=	T				27.5	50	>250	>250	>100	>100
981	DC	PhO	EtGlycO	=	T				12.5	12.5	150	≥ 250	>100	>100
982	DC	PhO	MeMandO	=	T				1.7	0.65	15	94	14	>100
983	DC	M MeEphedIn	heterocycle	=	T				≥ 250	122	>250	>250	>100	>100
1078	SV	PhO	Me2AspNH	=	T				0.55	0.65	0.33	209	31.4	>100
1079	SV	HO	AspNH	=	T				1.8	2.5	70	≥ 250	9.3	>100
1080	SV	HO	MeAspNH[SC]	=	T				3.5	5	110	≥ 250	30.3	>100
1081	ASS	PhO	Me2GLuNH	=	T				8	5.33	1.6	≥ 250	88.8	>100
1083	ASS	HO	GluNH	=	T				8.5	5.5	>250	≥ 250	54.6	>100
1095	ASS	HO	D-AlaNH	=	T				1.3	1.6	10	>250	0.42	>100
1129	SV	HO	MeAlaNH	=	T				2	4.5	50	>250	47.4	>100
1131	SV	OH	OH	=	T				0.4	0.6	50	≥ 250	6.7	>100
1133	LB	PhO	EtOGlyNMe	=	T				75	87.5	>250	≥ 250	>100	>100
1135	SV	MeO	BzAlaNH	=	T				10	15	17.5	>250	>100	>100
1137	SV	OH	BzAlaNH	=	T				0.95	1.6	8	≥ 250	15.7	>100
1139	MW	PhO	OCtOH	=	T				15	15	>50	66.6	>20	>20
1156	LB	HO	MetNH	=	T				1.27	0.7	50	>250	16.2	>100
1163	LB	HO	GlyNH	=	T				2	5	130	>250		
1186	LB	PhO	MeIleNH	=	T				5					
1187	LB	HO	PheNH	=	T				3.5					
1189	YW	PhO	CHxCl2AlaNH	=	T				0.04					
1190	LB	OH	ValNH	=	T				0.7					
1192	LB	OH	LeuNH	=	T				1.4					
1193	LB	PhO	MeAlNH	=	T	[FAST ISOMER]								
1194	LB	PhO	ProNH	=	T				6					
1196	KT	PhO	MeGlutamineNH	=	T				1.2					
1197	HWI	PhO	Me-β-AlaNH	=	T				>250					
1198	HWI	PhO	Me-GABANH	=	T				>250					
1199	HWI	PhO	MeCaproylNH	=	T				>250					
1200	HWI	PhO	MeOCOCMe2Ala	=	T				0.12					
1214	KT	PhO	MeAsparagineN	=	T				0.6					
1215	KT	PhO	MeTrypNH	=	T				4					
1216	HWI	OH	β-AlaNH	=	T				0.7					

TABLE 6 (CONTD.)

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Cpd	Init	ArO	Y	Z	B	HIV1 M	HIV2 M	CC50 M	HIV1 CEM	HIV2 CEM	2.CEM.TK	CC50 CEM	EC50 MSV	MCCMSV
1217	HWT	OH	CaproylNH	=	T				1.4					
1218	PS	PhO	PmtAlaNH	=	T				<0.08					
1219	PS	PhO	neoPmtAlaNH	=	T				<0.08					
1220	PS	PhO	PhenethylAlaN	=	T				0.7					
1224	HWT	OH	Me-GABANH	=	T				1					
1226	PS	PhO	1-NapthMethAla	=	T				<0.08					
1227	PS	PhO	2-NapthMethAla	=	T				<0.08					

TABLE 6 (CONTD.)

Cpd	Init	Aro	Y	Z	B	HIV1 M	HIV2 M	CC50 M	HIV1 CEM	HIV2 CEM	2.CEM.TK	CC50 CEM	EC50 MSV	MCCMSV
462	PB	PhO	MeAlaNH	N3-up	T	3.3	11	121	27.5	40	30			
499	PB	-	-	N3-up	T	0.9	2.3	>250	3	4	>250			
536	PB	mCF3PhO	MeAlaNH	N3-up	T	0.45	0.9	104	1	2	3			
550	PB	3,5Cl2PhO	MeAlaNH	N3-up	T	0.5	1	98	1.4	3	12			
569	PB	-	-	N3-up	U				>400	>400	>400			
571	PB	PhO	MeAlaNH	N3-up	U				>202	>202	117			
657	ASS	PhO	HexNH	N3-up	T				>40	>40	>18			
659	ASS	PhO	BuNH	N3-up	T				>42	>42	>42			
661	ASS	PhO	C12NH	N3-up	T				>7	>7	>7			
687	DC	-	-	N3-up	BzT				2.5	2.8	>100	>100		
731	DC	PhO	BzAlaNH	N3-up	T				0.28	0.7	1.1	88		
739	DC	MeO	MeAlaNH	N3-up	T				10	18	>250	>250		
774	ASS	PhO	MeAlaNH	N3-up	N-OctT				>10	>10		15		
777	DC	2,4-Br2PhO	MeAlaNH	N3-up	T				0.5	0.55	0.19	55		
780	DC	F5PhO	MeAlaNH	N3-up	T				23	33	100	106		
846	ASS	PhO	CNEO	N3-up	T				13	14	>250	>250		
847	ASS	TFEO	CNEO	N3-up	T				12	9	>250	>250		
850	ASS	PhO	OH	N3-up	T				18	9	>250	>250		
855	ASS	TFEO	OH	N3-up	T				17.5	17.5	>250	>250		
856	ASS	HexO	CNEO	N3-up	T				13	25	>250	>250		
857	ASS	HexO	OH	N3-up	T				5	10	>250	>250		
941	ASS	PhO	Me-D-PheNH	N3-up	T				>50	>50		115	>100	>100
1069	OW	-	-	H	A				4	8	17.5	>250	24.3	>100
1071	ASS	HO	HOC(O)AlaNH	N3-up	T				115	100	250	>250	>250	>100
1093	OW	PhO	MeAlaNH	H	A				0.016	0.035	0.055	2.57	1.95	>20
1221	CY	PhO	MeAlaNH	H	C				0.6					
1225	OW	PhO	MeAlaNH	H	T				1.2					

TABLE 7

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In vivo Testing

Inhibitory effects of test compounds on the initiation of MSV-induced tumour formation in NRMI mice and on the survival of MSV innoculated NMRI mice.

Mice infected with Moloney Sarcoma Virus [MSV] were treated daily with either placebo, or d4T [at one of two doses] or with compound 324 at one of the same [equi-molar] doses.

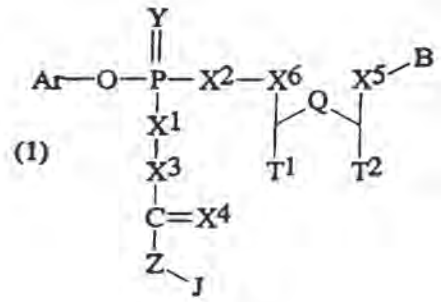
Two- to three-day old NMRI mice (weighing ~ 2 gram) were innoculated subcutaneously (s.c.) in the left hind leg with 50 µl MSV (100 foci forming units, as measured by in vitro determination of the virus-induced transformation of murine C3H embryo fibroblast cells). At 4 to 5 days post-infection, tumours develop and rapidly increase in volume upon further aging of the mice. Within 10 to 12 days post-infection, mice (then weighing ~ 5 to 6 gram) die from the viral infection. Drug treatment started 1 hour prior to infection of the virus, and further compound administration was given daily i.p. for an additional 3 days. The mean day of tumour initiation (± standard deviation) and the mean day of survival of the mice (± standard deviation) was calculated and statistical significance of the average delay of tumour formation and the mean day of survival in the treated groups versus the untreated (control) group was assessed by two-tailed student's t-test.

Whilst d4T failed to give any detectable delay in either tumour appearance or death, a significant effect on both parameters was seen with high-dose compound 324, and an effect on the first disease parameter at low dose [Figure 1].

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CLAIMS:

1. A compound of the formula (1)



(1)

wherein Ar is an aryl group;

Y is oxygen or sulphur;

X^1 is selected from O, NR^3, S, CR^3R^4, CR^3W^1 and CW^1W^2 where R^3 and R^4 are independently selected from hydrogen, alkyl and aryl groups; and W^1 and W^2 are heteroatoms;

X^2-X^6 may be absent; or X^6 is CH_2 and X^2 is selected (independently of X^1) from O, NR^3, S, CR^3R^4, CR^3W^1 and CW^1W^2 where R^3 and R^4 are independently selected from hydrogen, alkyl and aryl groups; and W^1 and W^2 are heteroatoms;

X^3 is a C_{1-6} alkyl group;

X^4 is oxygen or CH_2;

X^5 may be absent or is CH_2;

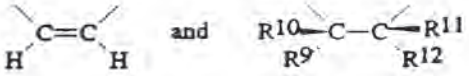
Z is selected from O, NR^5, S, alkyl and aryl groups, where R^5 is selected from hydrogen, alkyl and aryl groups;

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J is selected from hydrogen, alkyl, aryl, heterocyclic and polycyclic groups;

Q is selected from O, NR^6, S, CR^6R^7, CR^6W^3 and CW^3W^4 where R^6 and R^7 are independently selected from hydrogen, alkyl and aryl groups; and W^3 and W^4 are heteroatoms;

T^1 and T^2 are independently selected from hydrogen and CH_2R^8, where R^8 is selected from H, OH and F; or T^1 and T^2 are linked together and together are selected from the groups



where R^9 is selected from H, halogen, CN, NH_2, CO-alkyl and alkyl; and R^{10}, R^{11} and R^{12} are independently selected from H, N_3, halogen, CN, NH_2, CO-alkyl and alkyl;

B is a purine or pyrimidine base;

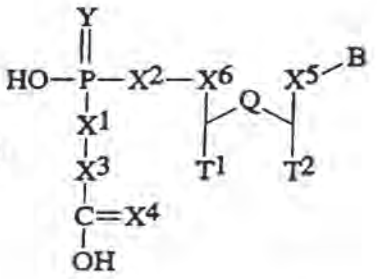
or a pharmaceutically acceptable derivative or metabolite thereof.

2. A compound according to claim 1 wherein

Y is oxygen;
X^1 is NH;
X^3 is CHR^1;
X^4 is oxygen; and
Z is oxygen.

3. A compound of formula (10)

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(10) 

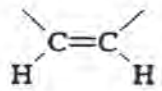
or pharmaceutically acceptable derivative or metabolite thereof.

4. A compound according to claim 3 wherein

Y is oxygen;
X^1 is NH;
X^3 is CHR^1; and
X^4 is oxygen

5. A compound according to any one of claims 1 to 4 wherein

X^2 is oxygen;
X^6 is CH_2;
Q is oxygen;
X^5 is absent; and
T^1 and T^2 together comprise the group:-



6. A compound according to claim 5 wherein B is thymine.

7. A compound according to claim 6 wherein Ar, R^1 and J are defined as follows:-

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Compound Reference	Ar	R^1	J
323	4-EtPh	Me	Me
324	Ph	Me	Me
327	4-FPh	Me	Me
526	3-CF_3Ph	Me	Me
546	3,5-Cl_2Ph	Me	Me
730	Ph	Me	Bzl
776	2,4-Br_2Ph	Me	Me
779	F_5Ph	Me	Me
862	Ph	Me	Hexyl
863	Ph	Bzl	Me
864	Ph	CH_2iPr	Me
865	Ph	iPr	Me
866	Ph	H	Me
867	Ph	$[CH_2]_2$SMe	Me
868	2,4Br_2Ph	Me	Bzl
877	Ph	Bzl	Bzl
878	Ph	Bzl	tBu
892	Ph	Me	Cyclohexyl
893	Ph	Me	tBu
1078	Ph	CH_2CO_2H	Me
1214	Ph	$CH_2CH_2CH_2NHC[NH_2]NH$	Me
1218	Ph	Me	n-Pent
1219	Ph	Me	neo-Pent
1225	Ph	Me	1-Napthyl
1227	Ph	Me	2-Napthyl

8. A compound according to any one of claims 1 to 4 wherein

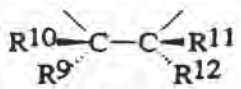
X^2 is oxygen;

X^6 is CH_2;

Q is oxygen;

X^5 is absent; and

T^1 and T^2 together comprise the group:-



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9. A compound according to claim 8 wherein B is adenine or thymine.

10. A compound according to any one of claims 1 to 4 wherein

X^2-X^6 is absent

Q is oxygen;

X^5 is CH_2;

T^1 and T^2 are independently selected from hydrogen and CH_2R^8 wherein R^8 is selected from H, OH and F.

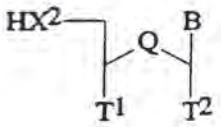
11. A compound according to claim 9 wherein B is adenine.

12. A compound according to any one of claims 1 to 11 for use in a method of treatment, prophylaxis or diagnosis.

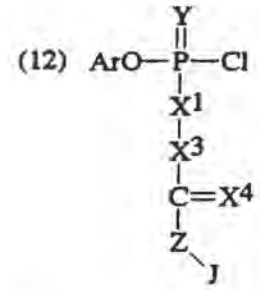
13. Use of a compound according to any one of claims 1 to 11 in the manufacture of a medicament for the treatment or prophylaxis of a viral infection.

14. Use of a compound according to claim 13 wherein the viral infection comprises HIV.

15. A process for the preparation of a compound according to any one of claims 1 to 11 comprising reaction of a compound of formula (11)



with a compound of formula (12)

(12) 

16. A method of prophylaxis or treatment of viral infection comprising administration to a patient in need of such treatment an effective dose of a compound according to any one of claims 1 to 11.

17. Use of a compound according to any one of claims 1 to 11 in the manufacture of a medicament for use in the inhibition of a reverse transcriptase by a nucleoside-resistance independent or nucleoside 5'-triphosphate independent mode of action.

18. A pharmaceutical composition comprising a compound according to any one of claims 1 to 11 in combination with a pharmaceutically acceptable excipient.

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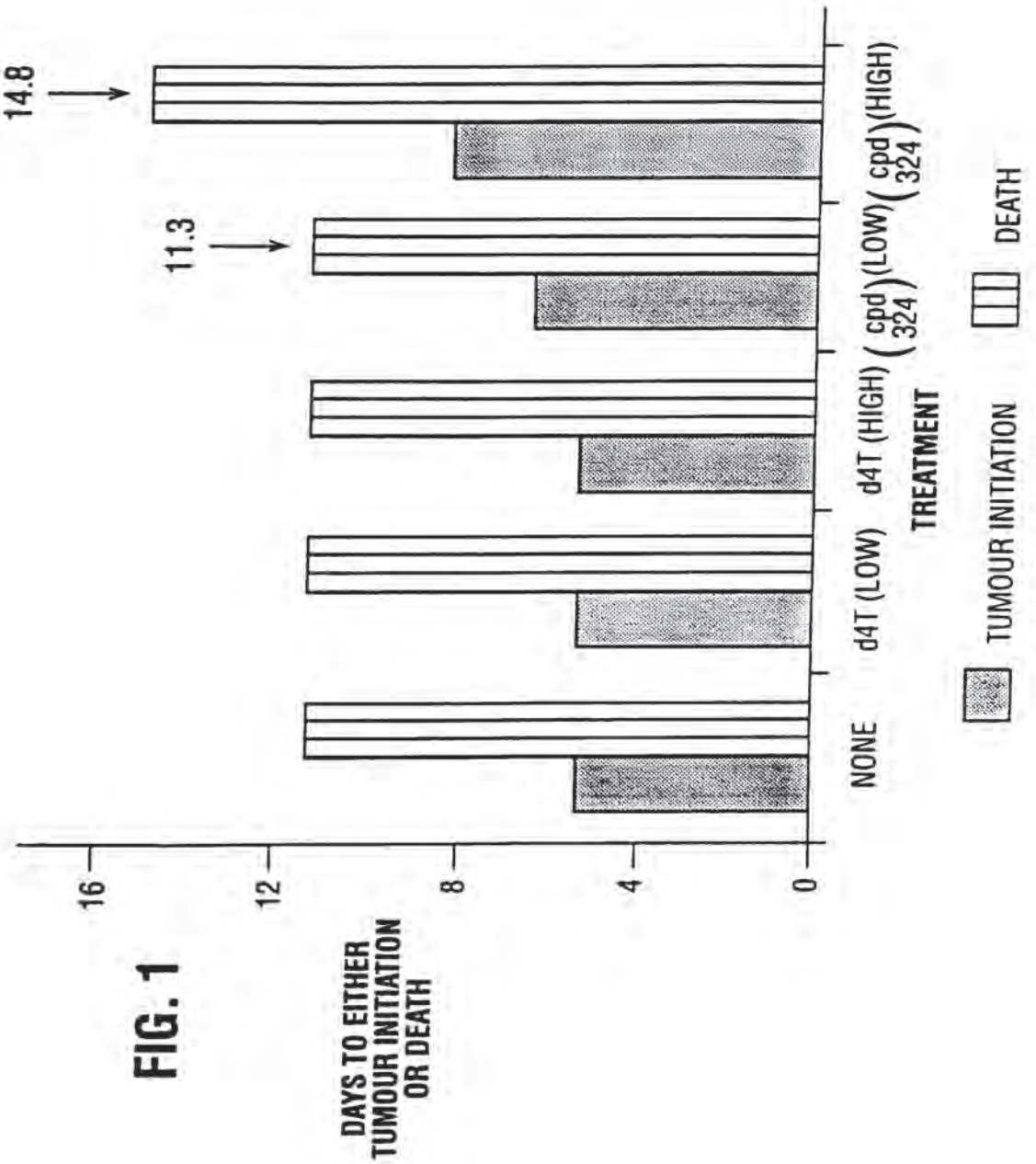

14.8
11.3
16
12
8
4
0
DAYS TO EITHER TUMOUR INITIATION OR DEATH
NONE
d4T (LOW)
d4T (HIGH)
(cpd 324)(LOW)
(cpd 324)(HIGH)
TREATMENT
TUMOUR INITIATION
DEATH

FIG. 1

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 9/10, 9/12, 5/18, C07H 19/04, 19/06, 19/044, A61K 51/00, A01N 43/04

A1

(11) International Publication Number: **WO 99/37753**

(43) International Publication Date: 29 July 1999 (29.07.99)

(21) International Application Number: PCT/US99/01332

(22) International Filing Date: 22 January 1999 (22.01.99)

(30) Priority Data:

60/072,264	23 January 1998 (23.01.98)	US
60/076,950	5 March 1998 (05.03.98)	US
60/108,634	16 November 1998 (16.11.98)	US

(71) Applicant *(for all designated States except US)*: NEWBIOTICS, INC. [US/US]; Suite R, 11760 Sorrento Valley Road, San Diego, CA 92121 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SHEPARD, H., Michael [US/US]; 16825 Via de Santa Fe, Rancho Santa Fe, CA 92067 (US). GROZIAK, Michael, P. [US/US]; 153 Primrose Way, Pala Alto, CA 94303–3047 (US).

(74) Agents: KONSKI, Antoinette, F. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304–1018 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ENZYME CATALYZED THERAPEUTIC AGENTS

(57) Abstract

This invention provides a method for identifying potential therapeutic agents by contacting a target cell with a candidate therapeutic agent which is a selective substrate for an endogenous, intracellular enzyme in the cell which is enhanced in its expression as a result of selection by biologic or chemotherapy. This invention also provides methods and examples of molecules for selectively killing a pathological cell by contacting the cell with a prodrug that is a selective substrate for an endogenous, intracellular enzyme. The prodrug is subsequently converted to a cellular toxin. Further provided by this invention is a method for treating a pathology characterized by pathological, hyperproliferative cells in a subject by administering to the subject a prodrug that is a selective substrate for an endogenous, overexpressed, intracellular enzyme, and converted by the enzyme to a cellular toxin in the hyperproliferative cell.

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ENZYME CATALYZED THERAPEUTIC AGENTS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119 (e) to U.S. Provisional Application Numbers 60/072,264; 60/076,950; and 60/108,634, filed January 23, 1998; March 05, 1998; and November 16, 1998, respectively. The contents of these applications are hereby incorporated by reference into the present disclosure.

TECHNICAL FIELD

The present invention relates to the field of drug discovery and specifically, the design of prodrugs which are substrates for an intracellular enzyme critical to resistance to therapeutics in pathological cells and converted to a cell toxin by the intracellular enzyme.

BACKGROUND

Throughout and within this disclosure, various publications are referenced by first author and date, patent number or publication number. The full bibliographic citation for each reference can be found within the specification or at the end of this application, immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this disclosure to more fully describe the state of the art to which this invention pertains.

Cancer cells are characterized by uncontrolled growth, de-differentiation and genetic instability. The instability expresses itself as aberrant chromosome number, chromosome deletions, rearrangements, loss or duplication beyond the normal diploid

number. Wilson, J.D. et al. (1991). This genomic instability may be caused by several factors. One of the best characterized is the enhanced genomic plasticity which occurs upon loss of tumor suppression gene function (e.g., Almasan, A. et al. (1995)). The genomic plasticity lends itself to adaptability of tumor cells to their changing environment, and may allow for the more frequent mutation, amplification of genes, and the formation of extrachromosomal elements (Smith, K.A. et al. (1995) and Wilson, J.D. et al. (1991)). These characteristics provide for mechanisms resulting in more aggressive malignancy because it allows the tumors to rapidly develop resistance to natural host defense mechanisms, biologic therapies (Wilson, J.D. et al. (1991) and Shepard, H.M. et al. (1988)), as well as to chemotherapeutics. (Almasan, A. et al. (1995) and Wilson, J.D. et al. (1991)).

Cancer is one of the most commonly fatal human diseases worldwide. Treatment with anticancer drugs is an option of steadily increasing importance, especially for systemic malignancies or for metastatic cancers which have passed the state of surgical curability. Unfortunately, the subset of human cancer types that are amenable to curative treatment today is still rather small (Haskell, C.M. eds. (1995), p. 32). Progress in the development of drugs that can cure human cancer is slow. The heterogeneity of malignant tumors with respect to their genetics, biology and biochemistry as well as primary or treatment-induced resistance to therapy mitigate against curative treatment. Moreover, many anticancer drugs display only a low degree of selectivity, causing often severe or even life threatening toxic side effects, thus preventing the application of doses high enough to kill all cancer cells. Searching for anti-neoplastic agents with improved selectivity to treatment-resistant pathological, malignant cells remains therefore a central task for drug development. In addition, widespread resistance to antibiotics is becoming an important, world-wide, health issue. (Segovia, M. (1994) and Snydman, D.R. et al. (1996)).

Classes of Chemotherapeutic Agents

The major classes of agents include the alkylating agents, antitumor antibiotics, plant alkaloids, antimetabolites, hormonal agonists and antagonists, and a variety of miscellaneous agents. See Haskell, C.M., ed., (1995) and Dorr, R.T. and Von Hoff, D.D., eds. (1994).

The classic alkylating agents are highly reactive compounds that have the ability to substitute alkyl groups for the hydrogen atoms of certain organic compounds. Alkylation of nucleic acids, primarily DNA, is the critical cytotoxic action for most of these compounds. The damage they cause interferes with DNA replication and RNA transcription. The classic alkylating agents include mechlorethamine, chlorambucil, melphalan, cyclophosphamide, ifosfamide, thiotepa and busulfan. A number of nonclassic alkylating agents also damage DNA and proteins, but through diverse and complex mechanisms, such as methylation or chloroethylation, that differ from the classic alkylators. The nonclassic alkylating agents include dacarbazine, carmustine, lomustine, cisplatin, carboplatin, procarbazine and altretamine.

Many clinically useful antitumor drugs are natural products of various strains of the soil fungus *Streptomyces*. They produce their tumoricidal effects by one or more mechanisms. All of the antibiotics are capable of binding DNA, usually by intercalation, with subsequent unwinding of the helix. This distortion impairs the ability of the DNA to serve as a template for DNA synthesis, RNA synthesis, or both. These drugs may also damage DNA by the formation of free radicals and the chelation of important metal ions. They may also act as inhibitors of topoisomerase II, an enzyme critical to cell division. Drugs of this class include doxorubicin (Adriamycin), daunorubicin, idarubicin, mitoxantrone, bleomycin, dactinomycin, mitomycin C, plicamycin and streptozocin.

Plants have provided some of the most useful antineoplastic agents. Three groups of agents from this class are the *Vinca* alkaloids (vincristine and vinblastine), the epipodophyllotoxins (etoposide and teniposide) and paclitaxel (Taxol). The *Vinca* alkaloids bind to microtubular proteins found in dividing cells and the nervous system. This binding alters the dynamics of tubulin addition and loss at the ends of mitotic spindles, resulting ultimately in mitotic arrest. Similar proteins make up an important part of nervous tissue; therefore, these agents are neurotoxic. The epipodophyllotoxins inhibit topoisomerase II and therefore have profound effects on cell function. Paclitaxel has complex effects on microtubules.

The antimetabolites are structural analogs of normal metabolites that are required for cell function and replication. They typically work by interacting with cellular enzymes. Among the many antimetabolites that have been developed and clinically tested are methotrexate, 5-fluorouracil (5-FU), floxuridine (FUDR), cytarabine, 6-mercaptopurine (6-

MP), 6-thioguanine, deoxycoformycin, fludarabine, 2-chlorodeoxyadenosine, and hydroxyurea.

Endocrine manipulation is an effective therapy for several forms of neoplastic disease. A wide variety of hormones and hormone antagonists have been developed for potential use in oncology. Examples of available hormonal agents are diethylstilbestrol, tamoxifen, megestrol acetate, dexamethasone, prednisone, aminoglutethimide, leuprolide, goserelin, flutamide, and octreotide acetate.

Drawbacks of Current Chemotherapeutic Agents

Among the problems currently associated with the use of chemotherapeutic agents to treat cancers are the high doses of agent required; toxicity toward normal cells, i.e., lack of selectivity; immunosuppression; second malignancies; and drug resistance.

The majority of the agents that are now used in cancer chemotherapy act by an anti-proliferative mechanism. Toxicity results because many normal cell types (e.g., colon epithelium, hematopoietic cells) have a high proliferative rate. Because of host toxicity, treatment has to be discontinued at dose levels that are well below the dose that would be required to kill all viable tumor cells.

Another side effect associated with present day therapies is the toxic effect of the chemotherapeutic on the normal host tissues that are the most rapidly dividing, such as the bone marrow, gut mucosa and cells of the lymphoid system. The agents also exert a variety of other adverse effects, including neurotoxicity; negative effects on sexuality and gonadal function; and cardiac, pulmonary, pancreatic and hepatic toxicities; vascular and hypersensitivity reactions, and dermatological reactions.

Table 1
Normal and Tumor Breast Epithelial Cells Are
Equally Sensitive to Doxorubicin Chemotherapy

Cell or Tissue	Number of Samples	Average IC_{50}
Normal Breast	13	14.8 ± 8.7ng/ml
Primary Carcinoma (UT)	19	11.4 ± 6.8 ng/ml
Metastatic Carcinoma (UT)	4	36 ± 26.3 ng/ml
Metastatic Carcinoma (Rx)	10	19.8 ± 12.7 ng/ml

From Smith et al. JNCl 74:341-347 (1985).

Hematologic toxicity is the most dangerous form of toxicity for many of the antineoplastic drugs used in clinical practice. Its most common form is neutropenia, with an attendant high risk of infection, although thrombocytopenia and bleeding may also occur and be life threatening. Chemotherapy may also induce qualitative defects in the function of both polymorphonuclear leukocytes and platelets. The hematopoietic growth factors have been developed to address these important side effects. Wilson, J.D. et al. (1991) and Dorr, R.T. and Von Hoff, D.D., eds. (1994).

Most of the commonly used antineoplastic agents are capable of suppressing both cellular and humoral immunity. Infections commonly lead to the death of patients with advanced cancer, and impaired immunity may contribute to such deaths. Chronic, delayed immunosuppression may also result from cancer chemotherapy.

The major forms of neurotoxicity are arachnoiditis; myelopathy or encephalomyelopathy; chronic encephalopathies and the somnolence syndrome; acute encephalopathies; peripheral neuropathies; and acute cerebellar syndromes or ataxia.

Many of the commonly employed antineoplastic agents are mutagenic as well as teratogenic. Some, including procarbazine and the alkylating agents, are clearly carcinogenic. This carcinogenic potential is primarily seen as delayed acute leukemia in patients treated with polyfunctional alkylating agents and inhibitors of topoisomerase II, such as etoposide and the anthracycline antibiotics. Chemotherapy has also been associated with cases of delayed non-Hodgkin's lymphoma and solid tumors. The present invention will minimize these effects since the prodrug will only be activated within tumor cells.

The clinical usefulness of a chemotherapeutic agent may be severely limited by the emergence of malignant cells resistant to that drug. A number of cellular mechanisms are probably involved in drug resistance, e.g., altered metabolism of the drugs, impermeability of the cell to the active compound or accelerated drug elimination from the cell, altered specificity of an inhibited enzyme, increased production of a target molecule, increased repair of cytotoxic lesions, or the bypassing of an inhibited reaction by alternative biochemical pathways. In some cases, resistance to one drug may confer resistance to other, biochemically distinct drugs. An alternative mechanism of resistance to cancer, chemotherapeutics occurs *via* the functional loss of tumor suppressor genes, especially p53, RB and p16. Loss of function of these gene products leads to derepressed expression of enzymes commonly targeted by anti-cancer drugs (e.g., 5FU/thymidylate synthase and methotrexate/dihydrofolate reductase). (Lee, V. et al. (1997), Exp. Cell Res. 234:270-6; Lenz, H.J. et al. (1998), Clinical Cancer Res. 4:1227-34 (1998), Fan, J. and Bertino, J. (1987), Oncogene 14:1191-200). Amplification of certain genes is involved in resistance to biologic and chemotherapy. Amplification of the gene encoding dihydrofolate reductase is related to resistance to methotrexate, while overexpression/amplification of the gene encoding thymidylate synthase is related to resistance to treatment with 5-fluoropyrimidines. Smith (1995). Table 2 summarizes some prominent enzymes in resistance to biologic and chemotherapy.

Table 2
Enzymes Overexpressed in Resistance to Cancer Chemotherapy

Enzyme	Biologic or Chemotherapy	Referenced (Examples)
Thymidylate synthase	Uracil-based Folate-based Quinazoline-based	Lönn, U. et al. *Cancer* 77:107,1996 Kobayashi, H. et al. *Jpn. J. Cancer Res.* 86:1014, 1995 Jackman, AL et al. *Anticancer Drug Des.* 10:573, 1995
Dihydrofolate reductase	Folate-based	Banerjee, D. et al. *Acta Biochem Pol.* 42:457, 1995 Bertino, J.R. et al. *Stem Cells* 14:5, 1996
Tyrosine kinases	TNF-alpha Multidrug resistance	Hudziak, R.M. et al. *PNAS* 85:5102, 1988 Stühlinger, M. et al. *J. Steroid Biochem* 49:39, 1994
MDR-associated proteins (ABC P-gp proteins)	Multidrug resistance	Simon, S.M. and Schindler, M. *PNAS* 91:3497, 1994 Gottesman, M.M. et al. *Annu. Rev. Genet.* 29:607, 1995
CAD*	PALLA**	Smith, K.A. et. al. *Philos. Trans. R. Soc. Lon. B. Biol. Sci.* 347:49, 1995 Dorr, R.T. and Von Hoff, D.D., eds. "Cancer Chemotherapy Handbook" 2nd ed. (Appleton and Lange 1994), pp. 768-773
Topoisomerase I (Colon & Prostate Cancers)	Camptothecin	Husain et al. *Cancer Res.* 54:539, 1994
Ribonucleotide reductase	Hydroxyurea	Wettergren, Y. et al. *Mol. Genet.* 20:267-85, 1994 Yen, Y. et al. *Cancer Res.* 54:3686-91, 1994

* CAD = carbamyl-P synthase, aspartate transcarbamylase, dihydroorotase
** PALA = N-(phosphonacetyl)-L-aspartate

Use of Prodrugs as a Solution to Enhance Selectivity of a Chemotherapeutic Agent

The poor selectivity of anticancer agents has been recognized for a long time and attempts to improve selectivity and allow greater doses to be administered have been numerous. One approach has been the development of prodrugs. Prodrugs are compounds that are toxicologically benign but which may be converted *in vivo* to therapeutically active products. In some cases, the activation occurs through the action of a non-endogenous enzyme delivered to the target cell by antibody ("ADEPT" or antibody-dependent enzyme prodrug therapy (U.S. Patent No. 4,975,278)) or gene targeting ("GDEPT" or gene

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dependent enzyme-prodrug therapy (Melton, R.G. and Sherwood, R.F. (1996)). These technologies have severe limitations with respect to their ability to exit the blood and penetrate tumors. Connors, T.A. and Knox, R.J. (1995).

Accordingly, there is a need for more selective agents which can penetrate the tumor and inhibit the proliferation and/or kill cancer cells that have developed resistance to therapy. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides a method for identifying potential therapeutic agents by contacting a target or test cell with a candidate therapeutic agent or prodrug which is a selective substrate for a target enzyme in the cell. This new approach is named "ECTA", for Enzyme Catalyzed Therapeutic Agents. In one embodiment, the target enzyme is an endogenous, intracellular enzyme which is overexpressed and confers resistance to biologic and chemotherapeutic agents. In a separate embodiment, the activity of the enzyme has been greatly enhanced in a tumor cell as a result of loss of tumor suppressor function (Smith, K.A. et al. (1995) and Li, W. et al. (1995)) and/or selection resulting from previous exposure to chemotherapy, (Melton, R.G. and Sherwood, R.F. (1996) and Lonn, U. et al. (1996)). In a separate embodiment, the target enzyme is an expression product of an infectious agent in the cell.

After the cell is contacted *in vitro* and/or *in vivo* with the candidate prodrug, the cell is assayed for efficacy of the agent by noting if the agent caused a reduction in cellular proliferation or if the agent kills the cell. In one aspect of this invention, the prodrug kills the cell or inhibits the cellular proliferation by the release of a toxic byproduct from the prodrug by the target enzyme. In a further aspect of this invention, one or more "target enzymes" can be used to activate the prodrug so that it releases the toxic byproduct.

Another aspect of this invention includes kits for use in assaying for new prodrugs having the characteristics described herein against target enzymes. The kits comprise the reagents and instructions necessary to complete the assay and analyze the results.

This invention also provides methods and examples of molecules for selectively killing a pathological cell by contacting the cell with a prodrug that is a selective substrate for a target enzyme, e.g., an endogenous, intracellular enzyme as defined above. The substrate is

specifically converted to a cellular toxin by the intracellular target enzyme. In another aspect of this invention, the product of an initial reaction is subsequently fully activated by a common cellular enzyme such as an acylase, phosphatase or other "housekeeping" enzyme (Voet, et al. (1995)) or common cellular constituent (e.g., water) to release the toxic byproduct from the prodrug.

Further provided by this invention is a method for treating a pathology characterized by pathological, hyperproliferative cells in a subject by administering to the subject a prodrug that is a selective substrate for a target enzyme, and selectively converted by the enzyme to a cellular toxin in the hyperproliferative cell. The prodrugs of this invention may be used alone or in combination with other chemotherapeutics or alternative anti-cancer therapies such as radiation.

A further aspect of this invention is the preparation of a medicament for use in treating a pathology characterized by pathological, hyperproliferative cells in a subject by administering to the subject a prodrug that is a selective substrate for a target enzyme, and selectively converted by the enzyme to a cellular toxin in the hyperproliferative cell.

A still further aspect of this invention is a method for identifying the optimal therapeutic for a subject, by isolating cells overexpressing an endogeneous, intracellular enzyme and contacting the cells with at least one of the prodrugs of this invention, and then identifying which of the one or more prodrugs inhibits the proliferation or kills the cells, thereby indentifying the optimal therapeutic for the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the development of resistance to anti-cancer modalities in cells, and the consequences.

Figure 2 schematically shows activation pathways of the prodrugs of this invention.

Figure 3 schematically shows the High Throughput Screen for prodrugs activated by intracellular enzymes important in drug resistance.

Figure 4 schematically shows how to find a lead human thymidylate synthase (TS) prodrug using TS-negative *E. coli* as the cell target.

Figure 5 shows an example of how to use this screen to simultaneously optimize the prodrug for reactivity to

two target enzymes.

Figure 6 shows one embodiment of a multi partitioned substrate for the generation of a thymidylate synthase (TS)-activatable prodrug.

Box 1 represents a masked or absent phosphate group R^7. When masked, it is a phosphoramidate or similar derivative that facilitates cell entry and is processed intracellularly to a monophosphate which can bind to TS. See Fries, K.M. et al. (1995). When absent, R^7 is a hydrogen atom and the prodrug is a substrate for cellular thymidine kinase (TK), which generates the requisite monophosphate *in vivo*.

Box 2 is a 2'-deoxyribofuranose group or other similar sugar, thio-sugar, carbocyclic, or acyclic group which connects the monophosphate to the pyrimidine ring in a manner that supports functional binding of the prodrug to TS and, when R^7 is a hydrogen atom, to TK. This group need not utilize an oxygen atom for attachment to the R^7 group of box 1. Thus, phosphonate analogs of sugar phosphates are acceptable.

Box 3 represents a tether group, wherein n is an integer from 0 to 10, that is a mono- or polyunsaturated electron conduit acting to conduct electrons away from the pyrimidine ring and toward the leaving group R^4 when the prodrug is acted upon by TS. The tether group is comprised of 0 to 10 unsaturated moieties like acyclic vinyl, ethynyl, imine, or azo units or cyclic unsaturated, aromatic, or heteroaromatic ones that can be mixed and matched at will as long as their connectivity provides the requisite electron-conducting conduit.

Box 4 represents a spacer unit X, wherein m is an integer from 0 to 1, that connects the tether to the leaving group R^4. If Box 3, n equals 0, then Box 4, m equals 1. In the preferred form, X is a methylene (CH_2) unit, either bearing substituents or not. Additionally, though, X can be an oxygen, sulfur, nitrogen, or other atoms capable of forming at least two covalent bonds. When X is absent (Box 4, m equals 0), the departure of the leaving group R^4 during the processing of the prodrug by TS leaves behind a pyrimidine nucleotide-based alkylating entity. See Barr, et al. (1983). When X is present (Box 4, m equals 1), the departure of the leaving group R^4 occurs early during the processing of the prodrug by TS.

Box 5 represents a leaving group R^4 that is released by the action of TS on the prodrug. It is itself a toxic antimetabolite or alkylating agent or is an entity that readily produces a toxic antimetabolite or alkylating agent *in vivo*. For example, the leaving group

R^4 can depart as an active metabolite of the anticancer agents Tepa or Thiotepa, a phosphoramide mustard, N-acetylsphingosine (C_2 ceramide, a tumor suppressor lipid) or hydroxyurea (an inhibitor of ribonucleotide reductase) upon release by TS. The leaving group R_1 can also be an α,α-dihalogenated ether, in which case it affords a carboxylic acid group when the α,α-dihalogenated alcohol released by TS undergoes hydrolysis. Thus, the leaving group R^4 can depart as a progenitor to fluoroacetate, fluorocitrate, malonic acid, methylmalonic avid, or 3-nitroproprionic acid, all potent inhibitors of oxidative phosphorylation.

Figure 7 shows TS Western blot of cell lines transfected by plasmid encoding neomycin resistance, with or without the HER-2 protooncogene. Lane (1) MCF7/HER2; (2) MCF7/neo; (3) MDA-MB-435/HER2; (4) MDA-NM-435/neo; (5) BT-20/HER2; (6) BT-20/neo.

Figure 8 shows reaction products of prodrug compounds with the enzyme thymidylate synthase.

DETAILED DESCRIPTION OF THE INVENTION

The invention is achieved by exploiting some of the key genomic and phenotypic changes intimately linked to resistance to biologic and chemotherapy of cancer cells. The invention provides a means for *in vivo* selectively inhibiting the growth and/or killing of cells which have undergone selection by exposure to cancer therapy (including biologic therapy such as tumor necrosis factor (TNF) or chemotherapy). (Refer to Table 2). As a result, certain enzymes which have been activated by mutation or gene amplification are resistant to initial or further therapy by the agent. Unlike prior art therapies directed to creating more potent inhibitors of endogenous, intracellular enzymes, this invention exploits the higher enzyme activity associated with therapy-resistant diseased cells and tissues versus normal cells and tissues and does not rely on inhibiting the enzyme. In one aspect, the tumor cells successfully treated by the prodrugs of this invention are characterized by enhanced target enzyme activity and therefore have a much higher potential to convert the prodrug to its toxic form than do normal cells which do not overexpress the target enzyme. The term "target enzyme" is used herein to define enzymes having one or more of the above noted characteristics.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, *e.g.,* Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)) and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

As used herein, the terms "host cells, "target cells" and "hyperproliferative cells" encompass cells characterized by the activation by genetic mutation or the endogenous overexpression of an intracellular enzyme. In some embodiments, the overexpression of the enzyme is related to loss of tumor suppressor gene product function drug resistance or the genetic instability associated with a pathological phenotype. A number of cellular mechanisms are involved in drug resistance, e.g., altered metabolism of the drug, impermeabilty of the cell with regard to the active compound or accelerated drug elimination from the cell, altered specificity of an inhibited enzyme, increased production of a target molecule, increased repair of cytotoxic lesions, or the bypassing of an inhibited reaction by alternative biochemical pathways. Enzymes activated or overexpressed and related to drug resistance include, but are not limited to thymidylate synthase (TS) (Lönn, U. et al. (1996); Kobayashi, H. et al. (1995); Jackman, A.L. et al. (1995)), dihydrofolate reductase (Banerjee, D. et al. (1995) and Bertino, J.R. et al. (1996)), tyrosine kinases (TNF-α, Hudziak, R.M. et al. (1988)) and multidrug resistance (Stühlinger, M. et al. (1994)); Akdas, A. et al. (1996); and (Tannock, I.F. (1996)); and ATP-dependent multidrug resistance associated proteins (Simon, S.M. and Schindler, M. (1994)) and, in some diseases including colon and prostate cancer, topoisomerase I (Husain et al. (1994)). Alternatively, resistance to one drug may confer resistance to other, biochemically distinct

drugs. While this application is specifically directed to cancer, a similar approach can be applied to enzymes encoded by human and animal pathogens, and in which the inhibitors have failed due to development of resistance.

Amplification of certain genes is involved in resistance to chemotherapy. Amplification of dihydrofolate reductase (DHFR) is related to resistance to methotrexate while amplification of the gene encoding thymidylate synthase is related to resistance to tumor treatment with 5-fluoropyrimidines. Amplification of genes associated with drug resistance can be detected and monitored by a modified polymerase chain reaction (PCR) as described in Kashini-Sabet, et al. (1988), U.S. Patent No. 5,085,983, or the method described herein. Acquired drug resistance can be monitored by the detection of cytogenetic abnormalities, such as homogeneous chromosome staining regions and double minute chromosomes both of which are associated with gene amplification. Alternative assays include direct or indirect enzyme activity assays and both of which are associated with gene amplification (e.g., Carreras & Santi (1995)); other methodologies (e.g. polymerase chain reaction, Houze, T.A. et al. (1997) or immunohistochemistry (Johnson, P.G. et al. (1997)).

Alternatively, the target cell is characterized as having inactivated tumor suppressor function, e.g. loss or inactivation of retinoblastoma (RB) or p53, known to enhance expression of TS (Li, W. et al. (1995)) or DHFR (Bertino, et al. (1996) and Li, W. et al. (1995)).

The prodrugs of this invention are useful to treat or ameliorate any disease wherein the disease-associated enzyme is associated with drug resistance to a chemotherapeutic whether due to loss of tumor suppressor functionality, *in vivo* selection by chemotherapy or a combination. This includes embodiments, where the enzyme is overexpressed, over-accumulated or activated in pathological cells versus normal cells, for example, the TS enzyme. Particularly excluded is the enzyme glutathione-S-transferase which has been shown to be occasionally elevated in some human tumors. Morgan, A.S. et al. (1998). The prodrugs of the subject invention are distinguishable on the basis that the target enzymes of this invention are commonly overexpressed, overaccumulated or activated in pathological cells versus normal cells. The most important principle which distinguishes the current invention from other approaches are:

(1) This invention describes the synthesis of substrates for enzymes like thymidylate synthase. The overexpressed enzyme will generate toxin, preferentially in diseased cells. Previous approaches have relied on an inhibitor. The inhibitors lead to amplified expression of the enzyme, and subsequent resistance to treatment (see, e.g., Lonn, U. et al. (1996).

(2) The current approach is also distinguishable from other "substrate-prodrug" approaches, e.g., the glutathione-S-transferase enzymes (see, e.g., Morgan, A.S. et al. (1998). The enzymes of the GST family are expressed at increased levels in response to toxic insult to the cell. The GST family of enzymes have overlapping substrate specificities, which makes it difficult to design a substrate reactive with only a single species of enzyme with elevated expression in a cancer cell (Morgan, A.S. et al. (1998)). Because each of the enzymes of the current invention (e.g., thymidylate synthase, dihydrofolate reductase and thymidine kinase) is unique with respect to its structure and substrate specificity, it is facile to design unique substrates. Several examples of substrates for thymidylate synthase are provided in the specifications of this application.

(3) In some cases the gene encoding the target enzyme (e.g., thymidylate synthase) may have undergone mutation to give resistance to inhibitors, (Barbour, K.W. et al. (1992) and Dicken, A.P. et al. (1993)) but will still be capable of carrying out reaction with non-inhibitor substrate prodrugs.

(4) A further advantage of this approach is that loss of tumor suppressor function is critical to development of malignancy. The majority of tumor cells have lost one of the p53, RB or p16 tumor suppressor functions. Such a loss results in increased expression of resistance enzymes (*e.g.*, Thymidylate synthase), independent of previous exposure to chemotherapy. The prodrugs described herein will be useful in treating early stages of malignancy, as well as disease previously treated with chemotherapy. Substrates for enzymes like GST require previous exposure of the tumor to chemotherapy in order to achieve sufficient overexpression to offer even the possibility of a modest therapeutic index.

Drug Assay

This invention provides a method for identifying agents which have therapeutic potential for the treatment of hyperproliferative or neoplastic disorders. e.g., cancer. The

method also identifies agents that inhibit the growth of cells or cell cycling of hyperproliferative cells, such as cancer cells. Other cells that are included are bacterial, yeast and parasitic cells which cause disease as a result of inappropriate proliferation in the patient. The agent is considered a potential therapeutic agent if cell proliferation, replication or cell cycling is reduced relative to the cells in a control sample. Most preferably, the cells are killed by the agent. The cells can be procaryotic (bacterial such as *E. coli*) or eucaryotic. The cells can be mammalian or non-mammalian cells, e.g., mouse cells, rat cells, human cells, fungi (e.g., yeast) or parasites (e.g., *Pneumocystis* or *Leishmania*) which cause disease.

As used herein, a "hyperproliferative cell" is intended to include cells that are de-differentiated, immortalized, neoplastic, malignant, metastatic or transformed. Examples of such cells include, but are not limited to a sarcoma cell, a leukemia cell, a carcinoma cell, or an adenocarcinoma cell. More specifically, the cell can be a breast cancer cell, a hepatoma cell, a dectectable cancer cell, pancreatic carcinoma cell, an oesophageal carcinoma cell, a bladder cancer cell, an ovarian cancer cell, a skin cancer cell, a liver carcinoma cell, or a gastric cancer cell. In an alternative embodiment, the target cell can be resistant to a drug or compound used to prevent or kill a cell infected with an infectious agent which is resistant to coventional antibiotics. Infectious agents include bacteria, yeast and parasites, such as trypanosomes.

Specific examples of target enzymes that are the subject matter of this invention are listed in Table 2 (above) or Table 3 (below). These enzymes are involved in resistance to chemotherapy, are endogeneously activated, overexpressed or over-accumulated in a cell characterized by resistance to cancer therapy and associated with a pathological or disease include, but are not limited to enzymes such as a member of the tyrosine kinase superfamily or an ATP-dependent MDR-associated protein, CAD, thymidylate synthase, dihydrofolate reductase, and ribonucleotide reductase. Table 3 provides a list of enzymes which may be targeted by this approach in infectious disease.

Table 3
Enzymes Overexpressed in Infectious Disease, and which Contribute to Drug Resistance

Enzyme	Provides increased Resistance to:
Beta-lactamases	Penicillin and other beta-lactam containing antibiotics
Aminoglycosidase, or aminoglycoside midifying enzymes	Aminoglycoside antibiotics (e.g., streptomycin, gentamycin)
Chloramphenicol transacetylase	Chloramphenicol
Dihydrofolate reductase	Trimethoprim

Reference: Mechanisms of Microbial Disease, 2nd Ed., M. Schaechter, G. Medloff, B.I. Eisenstein, Editor TS Satterfield. Publ. Williams and Wilkins, pp. 973 (1993).

The potentially therapeutic agent identified by the method of this invention is a prodrug that is a substrate for the enzyme and is converted intracellularly to an intracellular toxin. As used herein, a "prodrug" is a precursor or derivative form of a pharmaceutically active agent or substance that is less cytotoxic to target or hyperproliferative cells as compared to the drug metabolite and is capable of being enzymatically activated or converted into the more active form (see Connors, T.A. (1986) and Connors, T.A. (1996)). The toxicity of the agent is directed to cells that are producing the converting enzyme in an amount effective to produce a therapeutic concentration of the cellular toxin in the diseased cell.

This invention also provides a quick and simple screening assay that will enable initial identification of compounds with at least some of the desired characteristics. For purposes of this current invention, the general scheme of one embodiment is shown in Figure 3. This drawing describes how the assay is arranged and the materials necessary for its process. As shown in Figure 3, the assay requires two cell types, the first being a control cell in which the target enzyme is not expressed, or is expressed at a low level. The second cell type is the test cell, in which the target enzyme is expressed at a detectable level, e.g., a high level For example, a procaryotic *E. coli* which does not endogenously express the target enzyme TS is a suitable host cell or target cell. The cell can have a control counterpart (lacking the target enzyme), or in a separate embodiment, a counterpart genetically modified to differentially express the target enzyme, or enzymes (containing the appropriate species of target enzyme). More than one species of enzyme can be used to separately transduce separate host cells, so that the effect of the candidate drug on a target enzyme can be

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simultaneously compared to its effect on another enzyme or a corresponding enzyme from another species.

In another embodiment, a third target cell is used as a control because it receives an effective amount of a compound, such as, for example, the compounds shown below, which have been shown to be potent prodrugs. This embodiment is particularly useful to screen for new agents that are activated by thymidylate synthase.

In another embodiment, transformed cell lines, such as ras-transformed NIH 3T3 cells (ATCC, 10801 University Blvd., Manassas, VA 20110-2209, U.S.A.) are engineered to express variable and increasing quantities of the target enzyme of interest from cloned cDNA coding for the enzyme. Transfection is either transient or permanent using procedures well known in the art and described in Chen, L. et al. (1996), Hudziak, R.M. et al. (1988), or Carter, P. et al. (1992), and in the experimental section below. Suitable vectors for insertion of the cDNA are commercially available from Stratagene, La Jolla, CA and other vendors. The level of expression of enzyme in each transfected cell line can be monitored by immunoblot and enzyme assay in cell lysates, using monoclonal or polyclonal antibody previously raised against the enzyme for immuno-detection. See, e.g., as described by Chen, L. et al. (1996). The amount of expression can be regulated by the number of copies of the expression cassette introduced into the cell or by varying promoter usage. Enzymatic assays to detect the amount of expressed enzyme also can be performed as reviewed by Carreras, C.W. and Santi, D.V. (1995), or the method described in the experimental section below. Tumor cell lines can be selected to express enhanced levels of thymidylate synthase (*e.g.*, colon tumor cells, as described by Copur et al. (1995).

As noted above, cells containing the desired genetic deficiencies may be obtained from Cold Spring Harbor, the Agricultural Research Service Culture Collection, or the American Type Culture Collection. The appropriate strains can also be prepared by inserting into the cell a gene coding for the target enzyme using standard techniques as described in Miller (1992), Sambrook, et al. (1989), and Spector et al. (1998). Growth assays can be performed by standard methods as described by Miller (1992), Sugarman et al. (1985) and Spector et al. (1998).

It should be understood by those skilled in the art that the screen shown in Figure 3 can be applied broadly for the discovery of antibiotics. For example, thymidylate synthase from yeast could be substituted for that of *E. coli* in Figure 4. This would allow the

discovery of specific antifungal antibiotics targeting yeast related pathogens. In addition, other enzymes can be subjected to this treatment. For example, prodrugs which target specifically the dihydrofolate reductase activity of infectious agents, like *Pneumocystis carnii*, could be selected. These agents will be selected for specificity for the target enzyme, and can be shown not to activate the enzyme of the natural host by employing the screening assay described in Figure 3. The control cellular constructs would contain the corresponding normal human enzyme, in order to show lack of toxicity when only the normal human enzyme is present.

For example and as shown in Figure 4, a foreign gene, e.g., a human gene encoding TS, can be inserted into the host cell such that human TS is expressed. This genetically engineered cell is shown as the "test cell" in Figure 3. The "control cell" does not express the target enzyme. In some embodiments it may be necessary to supplement the culture media with the protein product of the target enzyme.

In a separate embodiment, the wild type host cell is deficient or does not express more than one enzyme of interest. As shown in Figure 4, the host cell does not endogenously produce thymidine kinase (TK^-) or thymidylate synthase (TS^-). Genes coding for the human counterpart of these enzymes are introduced into the host cell to obtain the desired level of expression. The level of expression of enzyme in each transfected cell line can be monitored by methods described herein, e.g., by immunoblot and enzyme assay in cell lysates, using monoclonal or polyclonal antibody previously raised against the enzyme for immunodetection. See, e.g., as described by Chen, L. et al. (1996). Enzymatic assays also can be performed as reviewed by Carreras, C.W. and Santi, D.N. (1995) using detectable labeled substituents, e.g. tritium labeled substituents. A possible advantage of the "two enzyme" system is that the requirement for activation by two enzymes preferentially overexpressed in tumor cells will provide increased safety for normal cells.

The test cell is grown in small multi-well plates and is used to detect the biologic activity of test prodrugs. For the purposes of this invention, the successful candidate drug will block the growth or kill the test cell type, but leave the control cell type unharmed.

The candidate prodrug can be directly added to the cell culture media or previously conjugated to a ligand specific to a cell surface receptor and then added to the media. Methods of conjugation for cell specific delivery are well known in the art, see e.g., U.S. Patent Nos. 5,459,127; 5,264,618; and published patent specification WO 91/17424

(published November 14, 1991). The leaving group of the candidate prodrug can be detectably labeled, e.g., with tritium. The target cell or the culture media is then assayed for the amount of label released from the candidate prodrug. Alternatively, cellular uptake may be enhanced by packaging the prodrug into liposomes using the method described in Lasic, D.D. (1996) or combined with cytofectins as described in Lewis, J.G. et al. (1996).

In a separate embodiment, cultured human tumor cells overexpressing the enzyme of interest i.e., target enzyme, are identified as described above. The cells are contacted with the potential therapeutic agent under conditions which favor the incorporation of the agent into the intracellular compartment of the cell. The cells are then assayed for inhibition of cellular proliferation or cell killing.

It should be understood, although not always explicitly stated, each embodiment can be further modified by providing a separate target cell to act as a control by receiving an effective amount of a compound, such as, for example, the compounds shown below, which have been shown to be potent prodrugs.

A high throughput screen to identify biologically active compounds is outlined in Figures 3, 4 and 5. The basis of the test is the ease of genetic manipulation and growth of *E. coli*, and similar single cell organisms (e.g. yeast), see Miller (1992) and Spector et al. (1998). The key step is removing the endogenous enzyme activity corresponding to an enzyme target for prodrug design. This can be done by any of the methods described by Miller (1992), Sambrook, et al. (1989) or Spector et al. (1998). These methods include chemical and biologic (e.g. viral or transponson insertional) mutagenesis, followed by an appropriate selection procedure. The TS negative (TS^-) cell then becomes a negative control for the identification of prodrugs that, when acted upon by thymidylate synthase, become cell toxins. A similar approach can be made with other cell types, e.g. other bacteria, yeast, or other selectable single cell organisms. In the assay, both control and recombinant organisms are compared for sensitivity to the test compounds. As will be understood by those skilled in the art, prodrugs which distinguish between species of enzyme can also be derived from this procedure. For example, otherwise identical cells expressing human and yeast enzymes can be used to detect antibiotic prodrugs which are preferentially toxic only to the cells expressing the yeast enzyme. In this way, novel and specific antibiotics can be discovered.

Example cell lines are ras-transformed NIH 3T3 cells (obtained from the ATCC) and are engineered to express increasing quantities of human thymidylate synthase (Hu TS) from the cloned cDNA. Transfection is done in a transient or permanent basis (see Chen, L. et al. (1996), Hudziak, R.M. et al. (1988), and Carter, P. et al. (1992). NIH-000 (ras-transformed parent cell line); NIH-001 (low expressor of HuTS); NIH-002 (intermediate expressor of Hu TS); NIH-003 (high expressor of HuTS). The level of expression of TS in each cell line is monitored by immunoblot and enzyme assay in cell lysates, using antibody directed versus HuTS protein for immunodetection (e.g., as described in Chen, L. et al. (1996)). Enzymatic assays are performed as reviewed by Carreras and Santi (1995).

Human colorectal and breast tumor cell lines are screened for expression of HuTS enzyme. Cell lines expressing low, moderate and high levels of HuTS will be exposed to drug candidates as described above for the NIH 3T3 cell lines. Growth inhibition and cytotoxicity are monitored as described above. Similar tests can be carried out for each of the enzymes listed in Table 1.

An alternative embodiment for a prodrug taking advantage of TS overexpression in tumor cells is a deoxyuridine phosphoramidate, or other modifications (cited herein) conjugated with a therapeutic radionuclide. An example of a therapeutic radionuclide is rhenium 188. The isotope can be synthesized essentially as described by Callahan, et al. (1989). Alternatively, it can be obtained commercially, for example from Mallicrodt Medical BV, The Netherlands. The therapeutic radionuclide can be conjugated with deoxyuridine, or deoxyuridine 5'-phosphoramidate, or other derivative, by standard methods (for example, as described by Lin, W-Y., et al. (1997)). The radionuclide-containing deoxyuridine phosphoramidate will be preferentially taken up into the DNA of tumor cells overexpressing thymidylate synthase, and cause their death via concentrated emission of beta and gamma radiation. Alternative radionuclides include rhenium-186, and others (Troutner, D.A. (1987)).

In Vivo Administration

The *in vitro* assays are confirmed in animal models bearing human tumors or infected with an antibiotic resistant microorganism to determine *in vivo* efficacy.

Another aspect of this invention is a method for treating a pathology characterized by hyperproliferative cells in a subject comprising administering to the subject a therapeutic amount of a prodrug that is converted to a toxin in a hyperproliferative cell by an endogenous intracellular enzyme as defined herein. In a preferred embodiment, the compound is selected from the compounds defined in the section "Prodrugs," Infra.

When the prodrug is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject.

To determine patients that can be beneficially treated, a tumor sample is removed from the patient and the cells are assayed for the level of expression of the enzyme of interest. If the expression is above that expressed in normal cells so that a toxic amount of the prodrug would cause administered without undesirable side effects, then the tumor or cells are determined to be benefically treated and thus, the patient is suitable for the therapy of this invention. For example, if the target enzyme is expressed at least about 2 times and preferably about 3 times higher than normal cells, the patient is a suitable subject for the therapy method of this invention. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the toxicity of the converted prodrug or cellular toxin.

When delivered to an animal, the method is useful to further confirm efficacy of the prodrug. As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about 10^5 to about 10^9 hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the prodrug is administered, for example, by subcutaneous injection around the tumor. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate. Lovejoy et al. (1997) and Clarke, R. (1996).

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the

dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

The pharmaceutical compositions can be administered orally, intranasally, parenterally or by inhalation therapy, and may take the form of tablets, lozenges, granules, capsules, pills, ampoules, suppositories or aerosol form. They may also take the form of suspensions, solutions and emulsions of the active ingredient in aqueous or nonaqueous diluents, syrups, granulates or powders. In addition to a compound of the present invention, the pharmaceutical compositions can also contain other pharmaceutically active compounds or a plurality of compounds of the invention.

More particularly, a compound of the formula of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

In general, a suitable dose for each of the above-named compounds, is in the range of about 1 to about 100 mg per kilogram body weight of the recipient per day, preferably in the range of about 1 to about 50 mg per kilogram body weight per day and most preferably in the range of about 1 to about 25 mg per kilogram body weight per day. Unless otherwise indicated, all weights of active ingredient are calculated as the parent compound of the formula of the present invention for salts or esters thereof, the weights would be increased proportionately. The desired dose is preferably presented as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing about 1 to about 100 mg, preferably about 1 to above about 25 mg, and most preferably about 5 to above about 25 mg of active ingredient per unit dosage form. It will be appreciated that appropriate dosages of the compounds and compositions of the invention may depend on the type and severity and stage of the disease and can vary from patient to patient.

Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the treatments of the present invention.

Ideally, the prodrug should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the prodrug, optionally in saline, or orally administered, for example, as a tablet, capsule or syrup containing the active ingredient. Desirable blood levels of the prodrug may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component antiviral agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

While it is possible for the prodrug ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Formulations include those suitable for oral, recta, nasal, topical (including transdermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a

water-in-oil liquid emulsion. The active ingredient may also be presented a bolus, electuary or paste.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g., povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Pharmaceutical compositions for topical administration according to the present invention may be formulated as an ointment, cream, suspension, lotion, powder, solution, past, gel, spray, aerosol or oil. Alternatively, a formulation may comprise a patch or a dressing such as a bandage or adhesive plaster impregnated with active ingredients and optionally one or more excipients or diluents.

For diseases of the eye or other external tissues, e.g., mouth and skin, the formulations are preferably applied as a topical ointment or cream containing the active ingredient in an amount of, for example, about 0.075 to about 20% w/w, preferably about 0.2 to about 25% w/w and most preferably about 0.5 to about 10% w/w. When formulated in an ointment, the prodrug may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the prodrug ingredients may be formulated in a cream with an oil-in-water cream base.

If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration of the prodrug ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

The oily phase of the emulsions of this invention may be constituted from known ingredients in an known manner. While this phase may comprise merely an emulsifier (otherwise known as an emulgent), it desirably comprises a mixture of at lease one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier which acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulphate.

The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an

aqueous solvent for the prodrug ingredient. The prodrug ingredient is preferably present in such formulation in a concentration of about 0.5 to about 20%, advantageously about 0.5 to about 10% particularly about 1.5% w/w.

Formulations for ectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as suppositories, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the prodrug ingredient, such carriers as are known in the art to be appropriate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the prodrug ingredient.

Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily subdose, as herein above-recited, or an appropriate fraction thereof, of a prodrug ingredient.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable of oral

administration may include such further agents as sweeteners, thickeners and flavoring agents.

Prodrugs and compositions of the formula of the present invention may also be presented for the use in the form of veterinary formulations, which may be prepared, for example, by methods that are conventional in the art.

Provided below is a brief summary of cells and target enzymes that are useful to activate the prodrugs of this invention.

Tyrosine Kinases

The tyrosine kinase superfamily comprises the EGF receptor (EGFR), the macrophage colony-stimulating factor (CSF-1) receptor (v-fms), and the insulin receptor, which shows 30 to 40% identity with the product of the ros oncogene. More specifically, the members of this superfamily include v-src, c-src, EGFR, HER2, CSF-1 receptor, c-fms, v-ros, insulin receptor, and c-mos. See Figure 8.5 of Burck, K.B. et al., eds. (1988). Overexpression of members of the type 1 receptor tyrosine kinase superfamily has been documented in many types of cancer (Eccles, S.A. et al. (1994-95)). Overexpression of tyrosine kinases is linked to exposure to the α-cancer biologic agent TNF-α (Hudziak, R.M. et al. (1988) and Hudziak, R.M. et al. (1990)) and to chemotherapy (Stühlinger et al. (1994)).

The transforming gene of the Rous sarcoma virus, v-src, encodes an enzyme that phosphorylates tyrosine residues on proteins. The c-src proto-oncogene is found on chromosome 20. Tissues and cell lines derived from tumors of neuroectodermal origin having a neural phenotype express high levels of c-src accompanied by high specific kinase activity.

Several groups of investigators have reported overexpression of c-erbB-2/neu ("HER2") oncogene in cancer cells. Brison (1993) noted that erbB proto-oncogene is amplified in human tumors with resultant overexpression in most cases. Amplification of the c-erbB-2/neu oncogene has been reported in human mammary tumors (Slamon, et al. (1987), van de Vijver et al. (1987), Pupa et al. (1993), and Andersen et al. (1995)) and in bladder tumors (Sauter et al. (1993)), and in every case amplification was accompanied by overexpression. c-erbB-2/neu overexpression also has been reported in ovarian cancer

tissue samples (Slamon, et al. (1989), Meden et al. (1994), and Felip et al. (1995)), and tumors derived from the peripheral nervous system. Sukumar and Barbacid, (1990).

To perform the drug screening assay, tumor cell lines will be assayed for expression of the oncogene or will be engineered to express varying levels of tyrosine kinase. Selected cell lines are cultured and candidate drugs are added in varying concentrations. The cells are assayed for cell killing or inhibition of cellular proliferation, as described in Hudziak, R.M. et al. (1988) and Hudziak, R.M. et al. (1990).

Dihydrofolate Reductase

Methotrexate is a potent inhibitor of dihydrofolate reductase, an enzyme necessary for intracellular folate metabolism. Dihydrofolate reductase functions to regenerate tetrahydrofolate from dihydrofolate, a product of the thymidylate synthase reaction (Voet, et al. eds. (1995), p. 813). It is well established that an important mechanism of resistance of cells to methotrexate is an increase in DHFR activity due to amplification of the DHFR gene. Banerjee, D. et al. (1995), Schimke, R.T. et al. (1988). Lönn, U. et al. (1996) reported that amplification of the DHFR gene occurred in breast cancer patients who previously received adjuvant chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil [CMF]) after surgery. Lack of the retinoblastoma (Rb) may also lead to enhanced MTX resistance as a consequence of an increase in DHFR mRNA expression activity without gene amplification. Li, W.W. et al. (1995). Cell lines with mutated p53 have been shown to undergo gene amplification, and the resistant cells are selected by chemotherapy. Banerjee, D. et al. (1995), Yin, Y. et al. (1992) and Livingston, L.R. et al. (1992). For the purposes of performing the assay of this invention, Schimke, R.T. et al. (1988) describes several mouse, hamster and human cell lines. Alternatively, the PCR method of Lönn U. et al. (1996) is used to assay DHFR gene amplification and identify cells that are useful in the method of identifying therapeutic agents as described herein. The nucleotide sequence of the cDNA coding for the human dihydrofolate reductase is provided in Masters, J.N. and Attardi, G. (1983) and cells can be engineered to express varying levels of the enzyme as noted herein. Dicken, A.P. et al. (1993) describes a mutant DHFR gene selected by chemotherapy. Purification of DHFR and assays related to enzyme function are described in Nakano, T. et al. (1994). Alternatively, cDNA encoding

DHFR is transfected into NIH 3T3 cells. Candidate drugs are added in varying concentrations and cell killing and inhibition of proliferation are assayed.

Antimetabolites dependent on dihydrofolate reductase activity can be synthesized by the attachment of, for example, an alkylating group to either the N5 or the C6 position of dihydrofolate. Reduction of the N5-C6 bond by DHFR will result in the release of the alkylating agent. In addition to the alkylating groups, any moiety whose release by DHFR results in the production of a toxin or an antimetabolite will be useful in the practice of the invention. These compounds can be further modified by the addition of a phosphatese or phosphoramidate moiety.

Multidrug Resistant Tumors

Multidrug resistance (MDR) is a generic term for the variety of strategies tumor cells use to evade the cytotoxic effects of anticancer drugs. MDR is characterized by a decreased sensitivity of tumor cells not only to the drug employed for chemotherapy but also to a broad spectrum of drugs with neither obvious structural homology nor common targets. This pleiotropic resistance is one of the major obstacles to the successful treatment of tumors. MDR may result from structural or functional changes at the plasma membrane or within the cytoplasm, cellular compartments, or nucleus. Molecular mechanisms of MDR are discussed in terms of modifications in detoxification and DNA repair pathways, changes in cellular sites of drug sequestration, decreases in drug-target affinity, synthesis of specific drug inhibitors within cells, altered or inappropriate targeting of proteins, and accelerated removal or secretion of drugs.

One of the mechanisms implicated in MDR results from amplification and over-expression of a gene known as the ATP-dependent multidrug resistant associated protein (MRP) in drug selected cell lines. For a review of the mechanisms of MDR, see Gottesman, M.M. et al. (1995) and Noder et al. (1996).

To establish MDR cell lines, drug selections are conducted in either a single step or in multiple steps as described in Gottesman, M.M. et al. (1995) and Simon, S.M. and Schindler, M. (1994), and references cited therein. The isolation of DNA sequences coding for MDR from various mammalian species is described in Gros, P. et al. (1986), Gudkov, A.V. et al. (1987), and Roninson, I.B. et al. (1984), and reviewed in Gottesman, M.M. et al. (1995), and cells can be engineered to express varying levels of this enzyme as

described above. The prodrug targeting MDR will be based upon the ATPase activity of this transporter.

Ribonucleotide Reductase

The enzyme ribonucleotide reductase reduces ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates. The enzyme is a tetramer made up of two α-subunits and two β-subunits. Hydroxyurea specifically blocks this reaction by interacting with the tyrosyl free radical (Tyr-122) of the β_2-substrate complex. Voet et al. (1995). The goal in targeting this reaction is to allow the accumulation of the free radical product O_2^-, which is highly cytotoxic.

Application of Technology to Other Diseases

While the primary focus of this application is directed to cancer, it should be recognized that the technology is broadly applicable to other diseases, especially antibiotic resistant bacterial infections. The β-lactam antibiotics encounter resistance in bacteria as the result of overexpression of β-lactamases. Hamilton-Miller, J.M.T. and Smith, J.T. eds. (1979) p. 443. Other enzymes, such as the aminoglycoside phosphotransferese Type III, are induced and selected for following treatment with aminoglycoside antibiotics, such as kanamycin. McKay, G.A. et al. (1994). For the purpose of this application, prodrug substrates derived from known substrates will be prepared that will not block enzyme activity, but will instead take advantage of the high enzyme activity to generate intracellular toxins to the infectious agents.

Thymidylate Synthase

The overexpression of thymidylate synthase is associated with colon cancer, breast cancer, gastric cancer, head and neck cancer, liver cancer and pancreatic cancer. These diseases are currently treated by antimetabolite drugs (uracil-based, folate-based, or quinazoline-based, (see Table 1)). In each of these cases it is likely that tumor suppressor loss and/or 5-fluorouracil therapy can lead to amplified activity of TS, or select for drug resistant forms of the enzyme, and thereby lead to drug-resistance of the disease relapse. Lönn, U. et al. (1996) reported that amplification of the TS gene occurred in breast cancer patients who previously received adjuvant chemotherapy (cyclophosphamide,

methotrexate, 5-fluorouracil [CMF]) after surgery. This enhanced TS expression is in addition to the basic increase of TS which results from loss of tumor suppressor function. The principal reaction normally performed by TS is the synthesis of deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF) from deoxyuridine monophosphate (dUMP) and N(5),N(10)-methylene-tetrahydrofolate (THF). In one embodiment, a derivative of uracil or THF is provided to cells expressing TS. For purposes of this invention, "uracil" (base only) and "uridine" (base and sugar) are used interchangeably and synonomously. Table 4 (below) summarized the many cancer types impacted by elevated TS expression.

Table 4
Thymidylate Synthase Overexpression Impacts Survival of Cancer Patients
Median Survival Time (Months)

Cancer	TS (Low)	TS (High)	References
Breast	84	54	Pestalozzi et al., 1997
NSCLC	46	10	Volm and Mattern, 1992
Colon	13.6	8.2	Leichman et al., 1997
Rectal	> 60	24	Johnston et al., 1994
Head and Neck	> 72	24	Johnston et al., 1997
Stomach	43	6	Lenz et al., 1995
	Average > 53.1	Average 21	

Fold difference between averages > 2.5

The derivative or "prodrug" is converted by the enzyme into highly cytotoxic metabolites. The low level of TS expressed in normal cells will not produce a toxic amount of the converted toxin. High levels of TS expressed in disease tissues generate more toxin and thereby lead to an inhibition of cell proliferation and/or cell death. For example, current therapy utilizes 5-fluorodeoxyuridylate to inhibit TS activity. During the reaction with substrate, the fluorine atom irreversibly becomes attached to the TS enzyme and inhibits it. In one embodiment, the proposed new therapeutic allows TS to complete the reaction but generates a modified product that, when incorporated into DNA, causes a toxic effect. The enzyme product may also block other critical cellular functions (e.g. protein synthesis or energy metabolism). Conversion of the prodrug also can release a metabolite, such as CN^- which is toxic to the cell. Derivatives of uracil/dUMP and N(5)(10)-THF can be synthesized, all of which have the potential of generating toxic product after metabolic transformation by TS.

Primary sequences show that TS is one of the most highly conserved enzymes. Perry, K. et al. (1990). Crystal structures of TS from several procaryotic species, *Lactobacillus casei* (Hardy, L.W. et al. (1987); Finer-Moore, J. et al. (1993)) and *Escherichia coli* (Perry, K. et al. (1990)); an eukaryote *Leishmania major* (Knighton, E.R. et al. (1994)); and T4 phage (Finer-Moore, J.S. et al., (1994)) have been determined

and indicate that tertiary structure also is very well conserved. The sequence alignment of the species of TS whose three dimensional structures have been determined and is shown in Schiffer, C.A. et al. (1995). From these amino acid sequences, the DNA sequences can be deduced or isolated using methods well known to those of skill in the art. Sambrook, et al. (1989). Alternatively, some 29 TS sequences from different organisms have been cloned and deposited into the DNA databases as described in Carreras, C.W. and Santi, D.V. (1995). The sequence of human thymidylate synthase gene, its cloning, expression and purification is provided in Takeishi, K. et al. (1985), Davisson, V.J. et al. (1989) and Davisson, V.J. et al. (1994). Genes encoding the TS protein and containing the necessary regulatory sequences, are constructed using methods well known to those of skill in the art. The gene encoding TS is introduced to target cells by electroporation, transformation or transfection procedures. Sambrook, et al. (1989). Alternatively, the gene is inserted into an appropriate expression vector by methods well known in the art, e.g., as described in Carreras, C.W. and Santi, D.V. (1995), Miller (1992) and Spector et al. (1998). The expression vector inserts the TS gene into the cells. The cells are then grown under conditions which favor the expression and production of TS protein.

Human gastric cancer cell lines, MKN-74, MKN-45, MKN-28 and KATO-III can be used in the assay described above to identify potential therapeutic agents which are selective substrates for TS. MKN-74 and MKN-45 are established from well and poorly differentiated adenocarcinomas, respectively. These cell lines and culture conditions are described in Osaki, M. et al. (1997) and references cited therein. Alternatively, tumor cell lines such as those described by Copur, S. et al. (1995), which have been selected by 5-FU to overexpress thymidylate synthase may be used.

Quantitation of TS can be performed using enzymatic biochemical assays that are well known to those with skill in the art. To quantify the level of TS protein and TS gene expression from human tumor tissue samples, the methods as reported by Johnston, P.G. et al. (1991) and Horikoshi, T. et al. (1992) provide sensitive assays. Alternatively, the PCR method of Lönn, U. et al. (1996) is used to assay TS gene amplification and identify cells that are useful in the method of identifying therapeutic agents as described herein.

As is apparent to one skilled in the art, control cell culture systems without drug and separately with a reference drug such as the compounds exemplified below, also are assayed. A lead compound is one which preferentially kills target cells with about 2-fold and

preferably about 3-fold or greater activity than normal cells. This invention also provides the agents identified by the methods described herein.

In another aspect, this invention provides a method for inhibiting the proliferation of a hyperproliferative cell, by first conducting the above assay. A prodrug identified by this assay is contacted with the cell and converted to a toxic metabolite in the cell by an endogenous intracellular enzyme as described above. Growth or cytotoxicity of bacteria, yeast and other cell types can be monitored as described by Miller et al. (1992), Sugarman et al. (1985) and Spector et al. (1998).

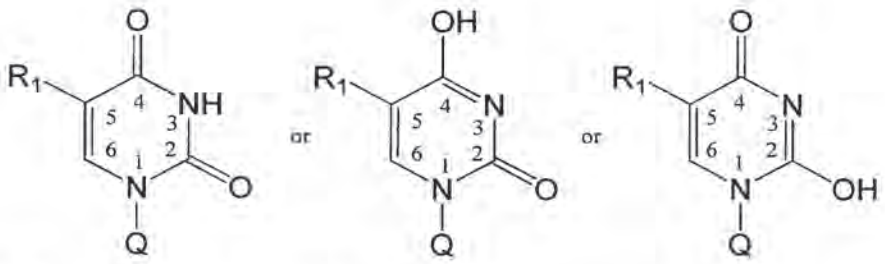
TS Prodrugs

In a preferred embodiment, the present invention involves two classes of compounds activated by TS, each a derivative of 5-substituted deoxyuridine monophosphate or a protected 5-substituted uracil or deoxyuridine monophosphate. Protected 5-substituted deoxyuridine monophosphate derivatives are those in which the phosphate moiety has been blocked through the attachment of suitable chemical protecting groups. Protection of 5-substituted deoxyuridine monophosphate derivatives can improve solubility, facilitate cellular penetration, facilitate passage across the blood-brain barrier, and prevent action of cellular or extracellular phosphatases, which might otherwise result in loss of the phosphate group. In another embodiment, 5-substituted uracil or uridine derivatives are administered to cells containing nucleoside kinase activity, wherein the 5-substituted uracil/uridine derivative is converted to a 5-substituted uridine monophosphate derivative. Uridine derivatives may also be modified to increase their solubility, cell penetration, and/or ability to cross the blood-brain barrier.

Action of thymidylate synthase upon 5-substituted uridine monophosphate derivatives can release the substituent attached to the 5-position ("leaving group") of the pyrimidine ring. The released substituent is then capable, either inherently or following reaction with another cellular component, of acting as a toxin or an inhibitor of cellular proliferation.

General Synthesis of Compounds of Class I

The L and D isomers of the compounds of Class I have the structure:



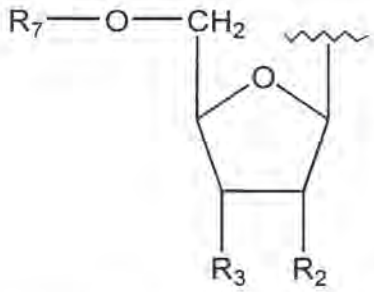
In the above formulae, R_1 (at the 5-position) is or contains a leaving group which is a chemical entity that has a molecular dimension and electrophilicity compatible with extraction from the pyrimidine ring by thymidylate synthase, and which upon release from the pyrimidine ring by thymidylate synthase, has the ability to inhibit the proliferation of the cell or kill the cell.

In the above formulae, Q is a phosphate or phosphoramidate derivative containing a chemical entity selected from the group consisting of sugar groups, thio-sugar groups, carbocyclic groups, and derivatives thereof. Examples of sugar groups include, but are not limted to, monosaccharide cyclic sugar groups such as those derived from oxetanes (4-membered ring sugars), furanoses (5-membered ring sugars), and pyranoses (6-membered ring sugars). Examples of furanoses include threo-furanosyl (from threose, a four-carbon sugar); erythro-furanosyl (from erythrose, a four-carbon sugar); ribo-furanosyl (from ribose, a five-carbon sugar); ara-furanosyl (also often referred to as arabino-furanosyl; from arabinose, a five-carbon sugar); xylo-furanosyl (from xylose, a five-carbon sugar); and lyxo-furanosyl (from lyxose, a five-carbon sugar). Examples of sugar group derivatives include "deoxy", "keto", and "dehydro" derivatives as well as substituted derivatives. Examples of thio sugar groups include the sulfur analogs of the above sugar groups, in which the ring oxygen has been replaced with a sulfur atom. Examples of carbocyclic groups include C_4 carbocyclic groups, C_5 carbocyclic groups, and C_6 carbocyclic groups which may further have one or more subsituents, such as -OH groups.

In one embodiment, Q is a β-D-ribofuranosyl group of the formula:

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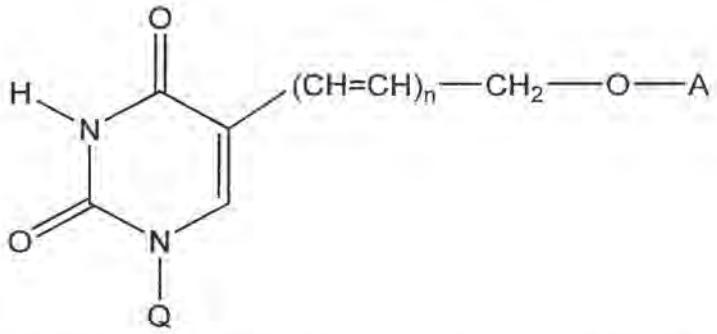
wherein R_7 is selected from the group consisting of phosphoryl, phosphoramidate and derivatives thereof, and wherein R_2 and R_3 are the same or different and are independently -H or -OH.

In some embodiments, R_1 is an alkenyl group, i.e., $(-CH=CH)_n-R_4$, wherein n is an integer from 0 to 10, and R_4 is a halogen such as is I^- or Br^-, CN^- or mercury; wherein R_2 is H and R_3 is -OH; wherein R_2 is OH and R_3 is H; wherein R_2 and R_3 are H; or wherein R_2 and R_3 are OH.

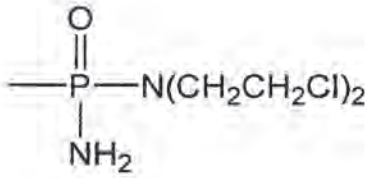
In another aspect, R_1 is an alkenyl group, i.e., $(-CH=CH)_n-R_4$, wherein n is an integer from 0 to 10, and R_4 is or contains a group selected from the group consisting of H, a halogen, alkyl, alkene, alkyne, hydroxy, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, a cyanide, cyanate and thiocyanate halovinyl group, a halomercuric group, -S-heteroaryl, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, -NHOH, -NHO-alkyl, $NH_2CONHO-$, and $NHNH_2$. In these embodiments, further aspects include: wherein R_2 and R_3 are H; wherein R_2 is OH and R_3 is H; herein R_2 is H and R_3 is OH; or wherein R_2 and R_3 are OH.

A preferred embodiment for the substituent in the R_1 position is one that could undergo an allylic interchange as shown in Figure 8.

In a still further aspect, the candidate therapeutic agent is a compound of the formula:

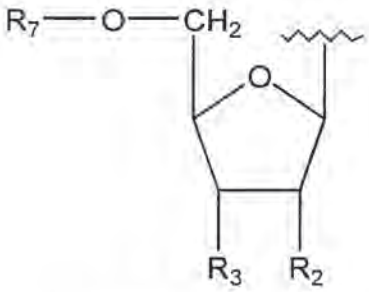


wherein n is an integer from 0 to 10; wherein A is a phosphoramide derivative, or a compound of the formula:



and wherein Q is selected from the group consisting of H, an unsubstituted or substituted sugar as defined above and a substituted or unsubstituted carbocyclic as defined above.

In a further embodiment, the compounds described above, are modified by Q having the structure:

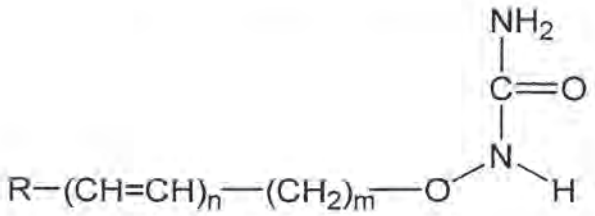


wherein R_7 is selected from the group consisting of H, phosphoryl, phosphoramidate and derivatives thereof, and wherein R_2 and R_3 are the same or different and are independently -H or -OH. In one embodiment, R^7 is not H. For these embodiments, R_1 also can have the structure $-(CH=CH)_n-R_4$, wherein n is an integer from 0 to 10, and R_4 is selected

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from the group consisting of H, a halogen, alkyl, alkene, alkyne, hydroxy, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, -S-heteroaryl, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, a cyanide, cyanate and thiocyanate halovinyl compound, a halomercuric compound, -NHOH, -NHO-alkyl, $NHNH_2$, and NH_2CONHO-.

Additionally, in a further aspect, the candidate therapeutic agent is a compound of the formula:



where R = 2'-deoxy-5-uridyl, m is 0 or 1, and n is an integer from 0 to 10.

Where appropriate, the compounds can be in any of their enantiomeric, diasteriomeric, or stereoisomeric forms, including, for example, D- or L-forms, and can be in any stereochemical configuration, including, for example, α- or β-anomeric form.

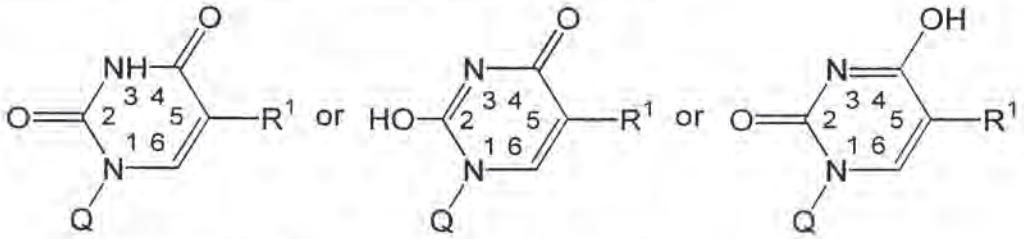
Synthesis of the above noted 5-substituted pyrimidine nucleosides and 5-substituted pyrimidine nucleoside monophosphates can be accomplished by methods that are well-known in the art. For example, treatment of 5-chloromercuri-2'-deoxyuridine with haloalkyl compounds, haloacetates or haloalkenes in the presence of Li_2PdCl_4 results in the formation, through an organopalladium intermediate, of the 5-alkyl, 5-acetyl or 5-alkene derivative, respectively. Wataya, et al. (1979) and Bergstrom, et al. (1981). Another example of C5-modification of pyrimidine nucleosides and nucleotides is the formation of C5-*trans*-styryl derivatives by treatment of unprotected nucleotide with mercuric acetate followed by addition of styrene or ring-substituted styrenes in the presence of Li_2PdCl_4. Bigge, et al. (1980). Pyrimidine deoxyribonucleoside triphosphates were derivatized with mercury at the 5 position of the pyrimidine ring by treatment with mercuric acetate in acetate buffer at 50° for 3 hours. Dale, et al. (1973). Such treatment would also be expected to be effective for modification of monophosphates; alternatively, a modified triphosphate could be converted enzymatically to a modified monophosphate, for example, by controlled treatment with alkaline phosphatase followed by purification of monophosphate. Other moieties, organic or nonorganic, with molecular properties similar

to mercury but with preferred pharmacological properties could be substituted. For general methods for synthesis of substituted pyrimidines, for example, U.S. Patent Nos. 4,247,544; 4,267,171; and 4,948,882; and Bergstrom et al. (1981). The above methods would also be applicable to the synthesis of derivatives of 5-substituted pyrimidine nucleosides and nucleotides containing sugars other than ribose or 2'-deoxyribose, for example 2'-3'-dideoxyribose, arabinose, furanose, lyxose, pentose, hexose, heptose, and pyranose. An example of a 5-position substituent is the halovinyl group, e.g. E-5-(2-bromovinyl)-2'-deoxyuridylate. Barr, P.J. et al. (1983). This compound is synthesized as follows as described in the experimental section.

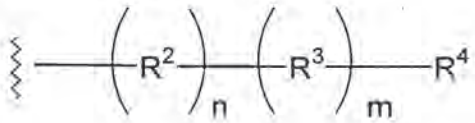
Alternatively, 5-bromodeoxyuridine, 5-iododeoxyuridine, and their monophosphate derivatives are available commercially from Glen Research, Sterling, VA (USA), Sigma-Aldrich Corporation, St. Louis, MO (USA), Moravek Biochemicals, Inc., Brea, CA (USA), ICN, Costa Mesa, CA (USA) and New England Nuclear, Boston, MA (USA). Commercially-available 5-bromodeoxyuridine and 5-iododeoxyuridine can be converted to their monophosphates either chemically or enzymatically, though the action of a kinase enzyme using commercial available reagents from Glen Research, Sterling, VA (USA) and ICN, Costa Mesa, CA (USA). These halogen derivatives could be combined with other substituents to create novel and more potent antimetabolites.

General Synthesis of Compounds of Class II

In a further aspect, the prodrug contacted with the cell overexpressing thymidylate synthase is an L or D isomer of a compound of the formula:

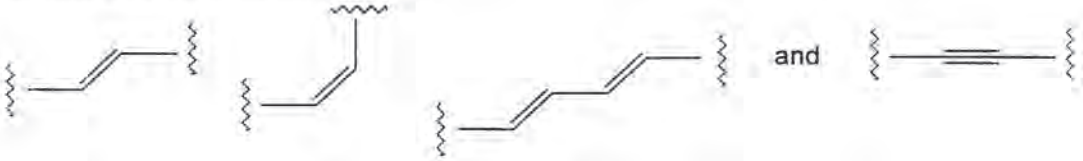


In the above formulae, R^1 is a moiety of the formula:

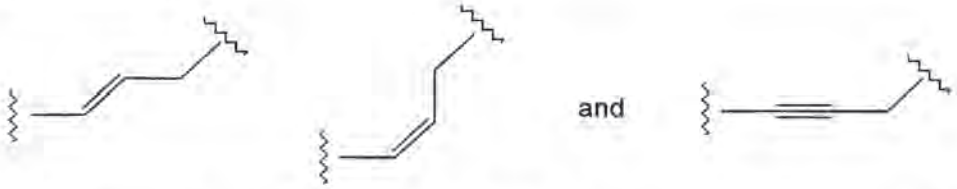


In the above formulae, R^2 is or contains a divalent electron conduit moiety. In one embodiment, R^2 is or contains a mono- or polyunsaturated electron conduit acting to conduct electrons away from the pyrimidine ring and toward the leaving group R^1. In one embodiment, R^2 is selected from the group consisting of: an unsaturated hydrocarbyl group; an aromatic hydrocarbyl group comprising one or more unsaturated hydrocarbyl groups; and, a heteroaromatic group comprising one or more unsaturated hydrocarbyl groups.

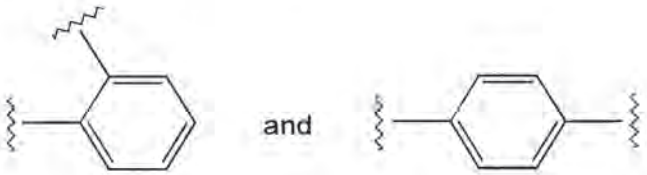
In one embodiment, R^2 is an unsaturated hydrocarbyl group having a structure selected from the group consisting of:



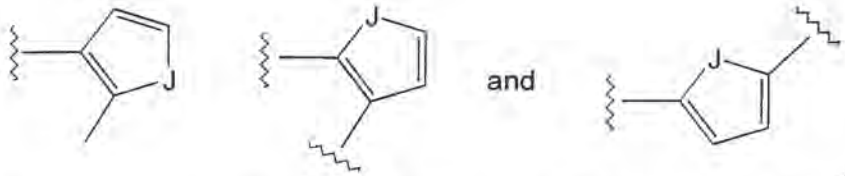
In one embodiment, R^2 and R^3, taken together form a structure selected from the group consisting of:



In one embodiment, R^2 is an aromatic hydrocarbyl group having a structure selected from the group consisting of:

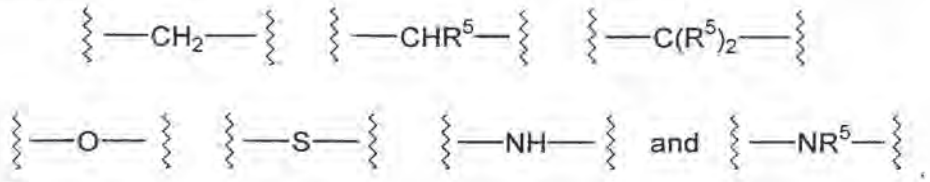


In one embodiment, R^2 is a heteroaromatic group having a structure selected from the group consisting of:



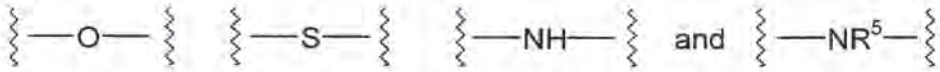
wherein J is a heteroatom, such as -O-, -S-, or -Se-, or a heteroatom group, such as -NH- or $-NR^{ALK}-$, where R^{ALK} is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

In the above formulae, R^3 is a divalent spacer moiety, also referred to as a spacer unit. In one embodiment, R^3 is a divalent spacer moiety having a structure selected from the group consisting of:



wherein R^5 is the same or different and is independently a linear or branched alkyl group having from 1 to 10 carbon atoms, or a cycloalkyl group having from 3 to 10 carbon atoms.

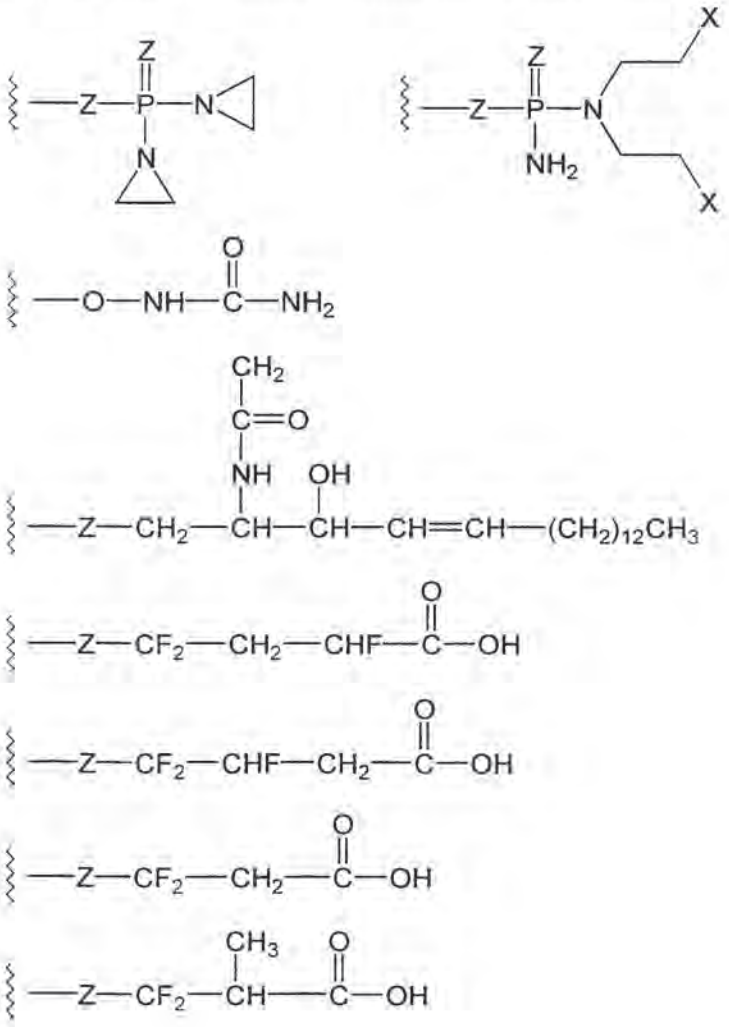
In one embodiment, R^3 is a divalent spacer moiety having a structure selected from the group consisting of:

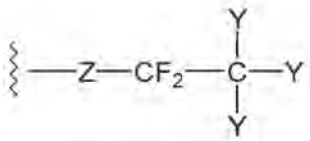


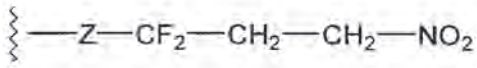
In the above formula, n is an integer from 0 to 10 and, m is 0 or 1. In one embodiment, n is an integer from 0 to 10 and, m is 1. In one embodiment, n is 0 and m is 0. In one embodiment, when R^7 is -H, then n is not zero. In one embodiment, when R^7 is -H, then m is not zero. In one embodiment, when R^7 is -H, then n is not zero and m is not zero. In one embodiment, when R^7 is -H, then R^4 is not a halogen (i.e., -F, -Cl, -Br, -I). In one embodiment, when R^7 is -H, and m is zero, then R^4 is not a halogen (i.e., -F, -Cl, -Br, -I). In one embodiment, when R^7 is -H, and m is zero and n is zero, then R^4 is not a halogen (i.e., -F, -Cl, -Br, -I).

In the above formula, R^4 is a toxophore moiety. As used herein, the term "toxophore" shall mean a moiety which is or contains a leaving group which is a chemical entity that has a molecular dimension and electrophilicity compatible with extraction from the pyrimidine ring by thymidylate synthase, and which upon release from the pyrimidine ring by thymidylate synthase, has the ability to inhibit the proliferation of the cell or kill the cell.

In one embodiment, the toxophore is or contains a leaving group that is activated or released by an intracellular enzyme overexpressed in the cell. In one embodiment, R^4 is or contains a group having a structure selected from the group consisting of:





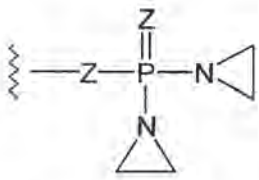


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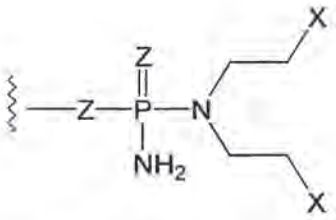


wherein X is -Cl, -Br, -I, or other potent leaving group (including, but not limited to, -CN. -OCN, and -SCN); Y is the same or different, and is independently -H or -F; and Z is the same or different and is independently -O- or -S-.

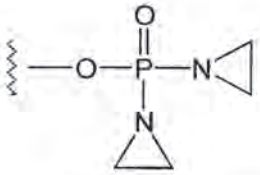
In one embodiment, R^4 is or contains a group having a structure selected from the group consisting of:



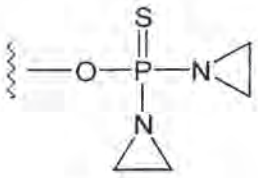
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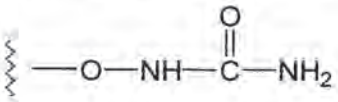
In one embodiment, R^4 is or contains a group having a structure selected from the group consisting of:



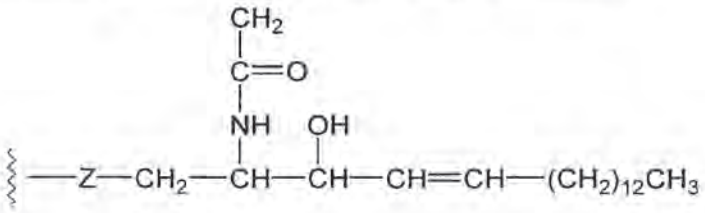
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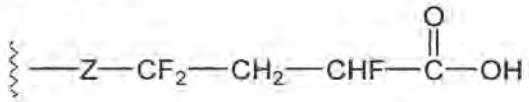
In one embodiment, R^4 is or contains a group having the structure:

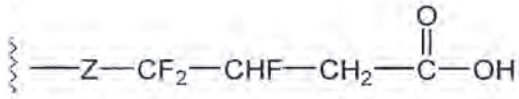


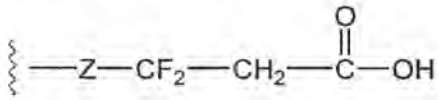
In one embodiment, R^4 is or contains a group having the structure:

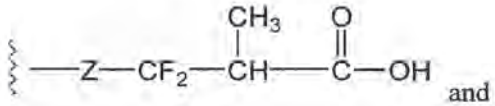


In one embodiment, R^4 is or contains a group having a structure selected from the group consisting of:

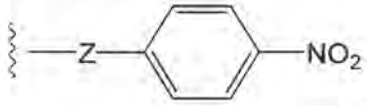




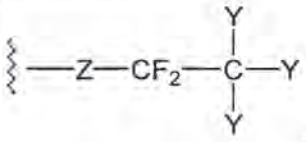




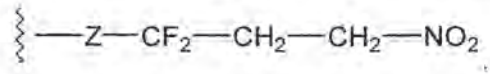
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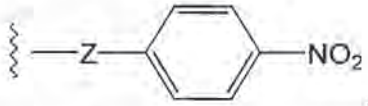
In one embodiment, R^4 is or contains a group having the structure:



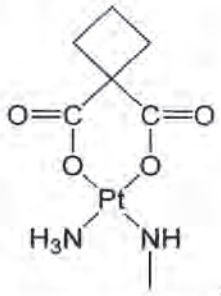
In one embodiment, R^4 is or contains a group having the structure:



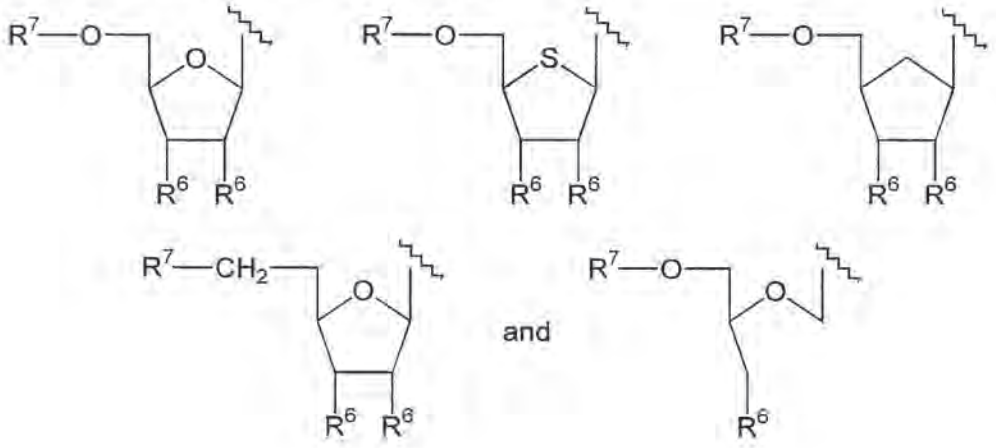
In one embodiment, R^4 is or contains a group having the structure:



In one embodiment, R^4 is or contains a chemical entity selected from the group consisting of: -Br, -I, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, -S-heteroaryl, -CN, -OCN, -SCN, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, -NHOH, -NHO-alkyl, NH_2CONHO-, $NHNH_2$, $-N_3$, and a derivative of cis-platin, such as:

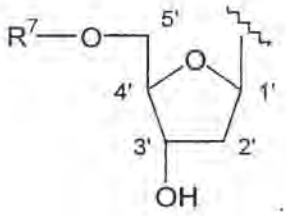


In the above formulae, Q is or contains a sugar moiety or a similar moiety which supports functional binding of the prodrug to the enzyme, *e.g.*, TS or TK. In one embodiment, Q is selected from the group consisting of:

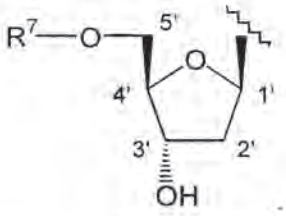


wherein R^6 is the same or different and is independently -H, -OH, $-OC(=O)CH_3$, or other protected hydroxyl group (including, but not limited to, benzoyl, $-COC_6H_5$, and toluoyl, $-COC_6H_4CH_3$); and, R^7 is hydrogen , a phosphate group, a phosphoramidate group, or other phosphorus containing group.

In one embodiment, Q is:

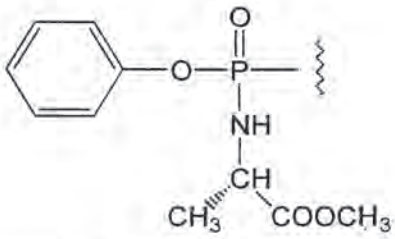


In one embodiment, Q is:



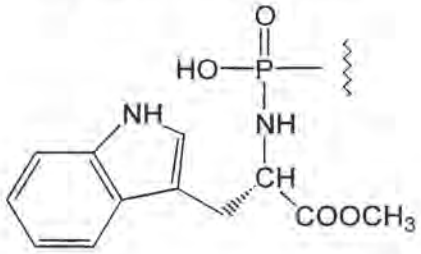
In one embodiment, R^7 is hydrogen. In one embodiment, R^7 is not hydrogen. In one embodiment, R^7 is a phosphate group or a phosphoramidate group. In one embodiment, R^7 is a phosphate group or a phosphoramidate group. In one embodiment, R^7 is a phosphoramidate group.

In one embodiment, R^7 is a phosphoramidate group derived from an amino acid, including, for example, the twenty naturally occuring amino acids. In one embodiment, R^7 is a phosphoramidate group derived from alanine. In one embodiment, R^7 is or contains a group having the structure:



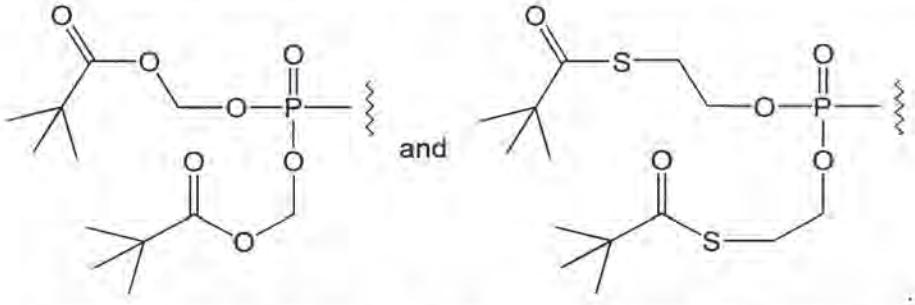
The above group, and methods for its preparation, are described in McGuigan et al. (1993), and McGuigan et al. (1996).

In one embodiment, R^7 is a phosphoramidate group derived from tryptophan. In one embodiment, R^7 is or contains a group having the structure:



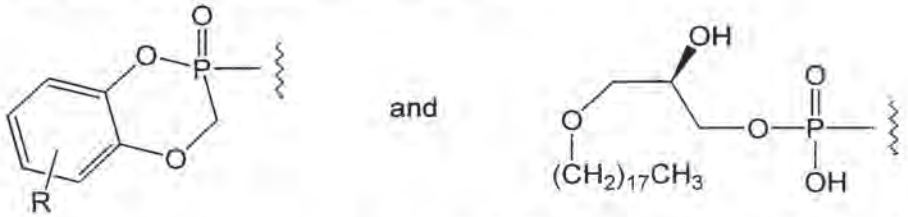
The above group, and methods for its preparation, are described in Abraham et al., (1996).

In one embodiment, R^7 is a phosphate group. In one embodiment, R^7 is or contains a group having a structure selected from the group consisting of:



The first of the two above groups, and methods for its preparation, are described in Freed et al. (1989); Sastry et al., (1992); Farquhar et al. (1994), and Farquhar et al. (1995). The second of the two above groups, and methods for its preparation, are described in Valette et al. (1996); and Benzaria et al. (1996).

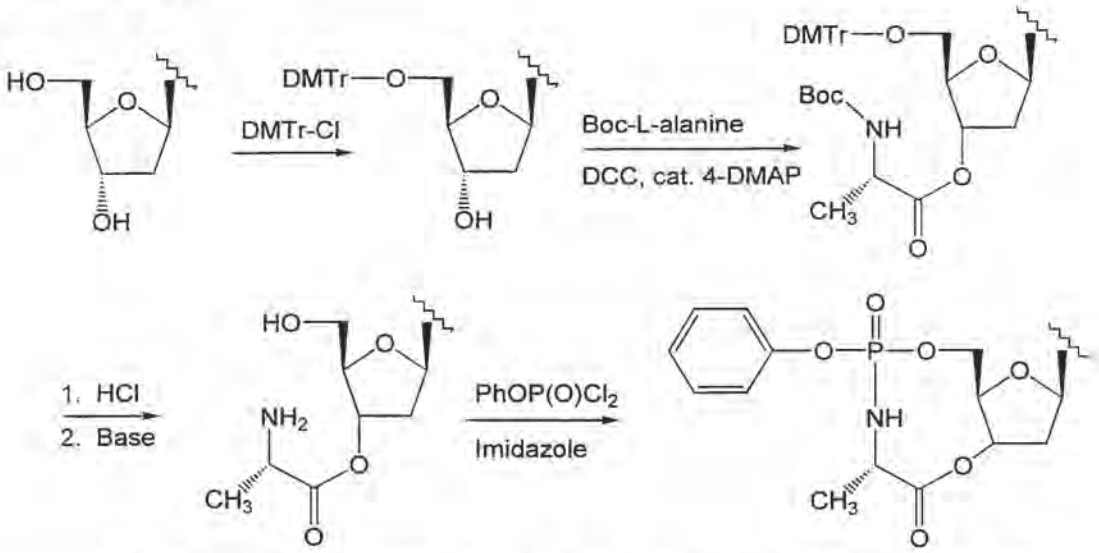
In one embodiment, R^7 is or contains a group having a structure selected from the group consisting of (where R is an aromatic substituent):



The first of the two above groups, and methods for its preparation, are described in Meier et al. (1997); Meier et al., (1997); and Meier et al., (1997). The second of the two

above groups, and methods for its preparation, are described in Hostetler et al. (1997); and Hostetler et al., published International Patent Application No. WO 96/40088 (1996).

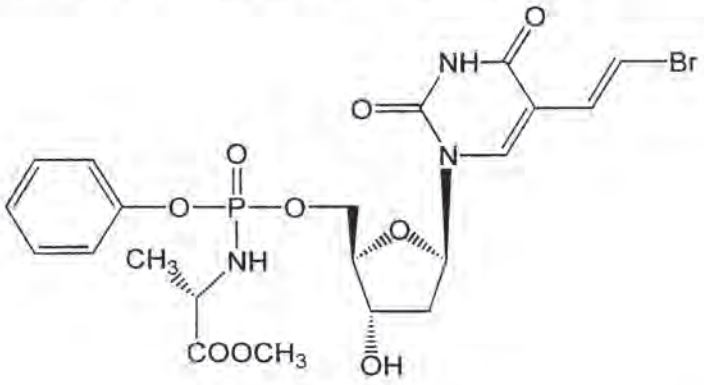
In one embodiment, the R^7 forms a cyclic group within Q. One such embodiment, and a method for its preparation, is shown below (where DMTr is 4,4'-dimethoxytrityl, Boc is t-butyloxycarbonyl, DCC is 1,3-dicyclohexylcarbodiimide, and 4-DMAP is 4-dimethylaminopyridine):



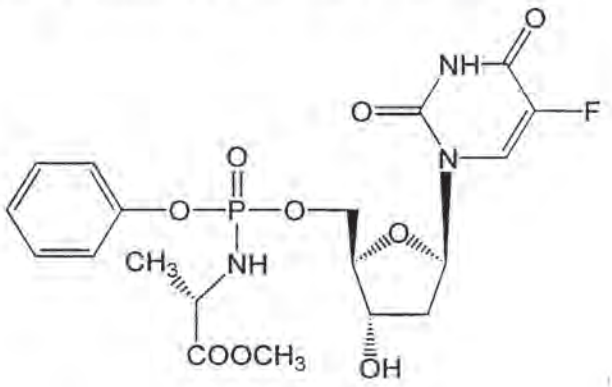
In one embodiment, the compound may be in any enantiomeric, diasteriomeric, or stereoisomeric form, including, D-form, L-form, α-anomeric form, and β-anomeric form.

In one embodiment, the compound may be in a salt form, or in a protected or prodrug form, or a combination thereof, for example, as a salt, an ether, or an ester.

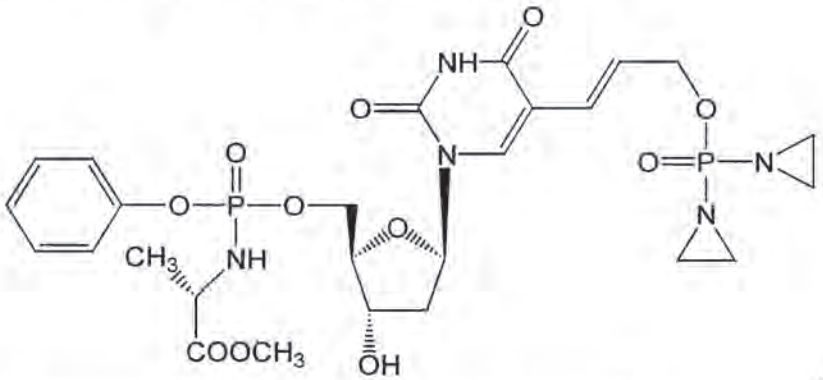
In one embodiment, the prodrug is a compound of the formula:



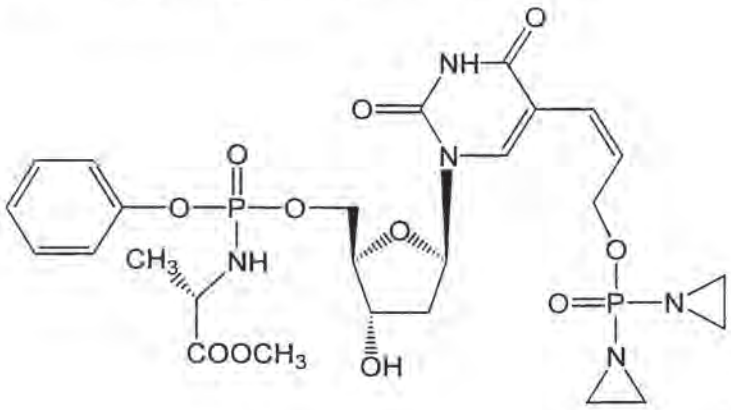
In one embodiment, the prodrug is a compound of the formula:



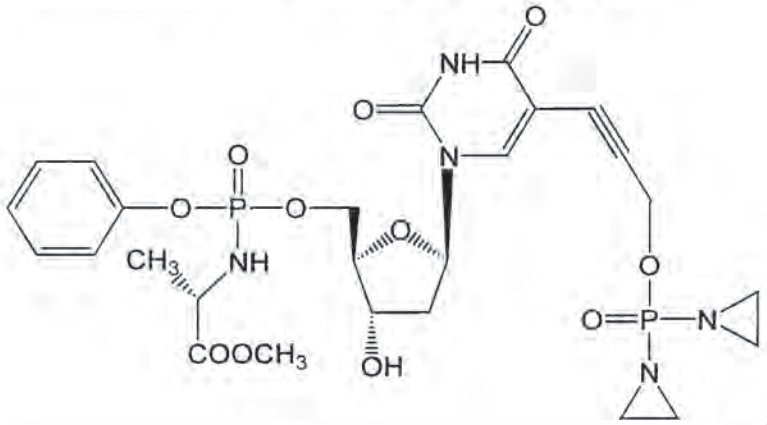
In one embodiment, the prodrug is a compound of the formula:



In one embodiment, the prodrug is a compound of the formula:



In one embodiment, the prodrug is a compound of the formula:



In a separate embodiment, the above structures are further modified to possess thiophosphodiaziridine instead of phosphodiaziridine groups, using the methods described below.

General Synthesis Strategy for Compounds of Class II

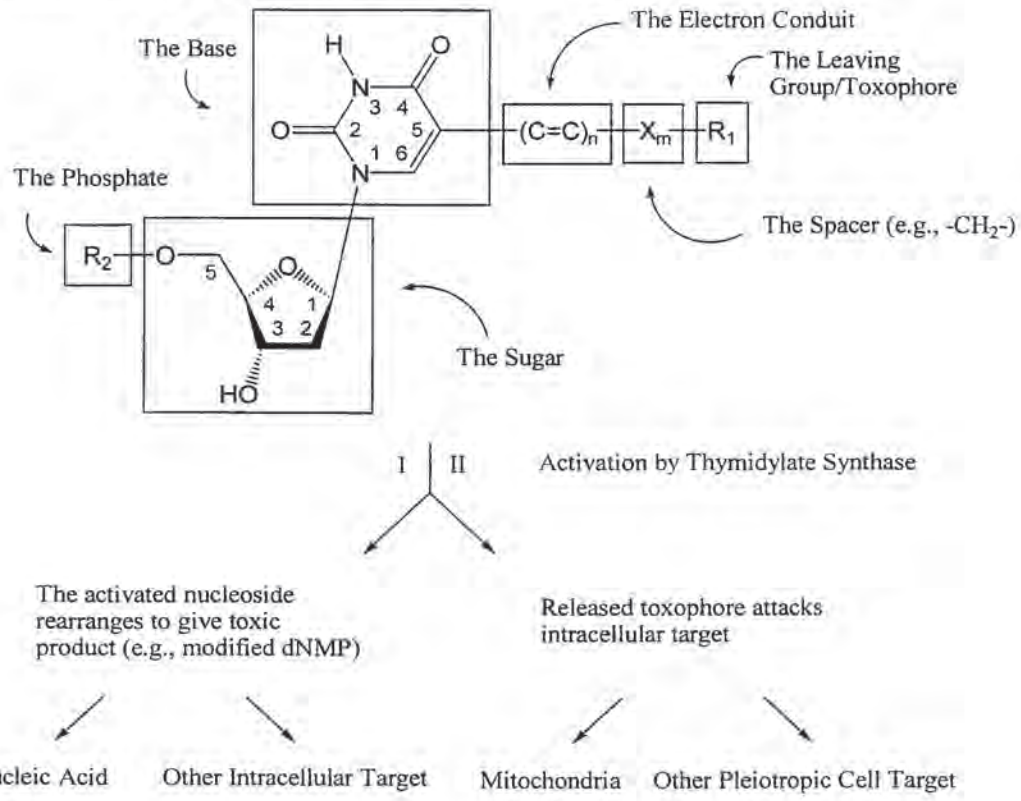
The structures at the 5-position of uracil are referred to as the tethers because they connect the proposed leaving group (toxophore) to the heterocycle. Upon activation of the heterocycle by reaction with Cys-195 in human TS, a negative charge is conducted from the 6-position of uracil into the tether. This mechanism has been described for the 5'-monophosphorylated versions of (*E*)-5-(bromovinyl)-2'-deoxyuridine (BVDU) by Barr et al. (1983) and of (*E*)-5-(3,3,3-trifluoro-l-propenyl)-2'-deoxyuridine (TFPe-dUrd) by Wataya et al. (1979), Santi (1980), and Bergstrom et al. (1984).

The tether "spacer" between the toxin and dNMP must be unsaturated so that it can conduct the toxin-labilizing negative charge supplied by the TS-Cys-sulfhydryl attack. Of the many unsaturated organic functionalities available for this purpose, the vinyl, allyl, and propargyl units are simple, small, and readily accessible synthetically. The vinyl and allyl units have the advantage that they can be prepared in either of two non-interconvertible geometric isomeric forms. Thus, they can be used as "probes" of prodrug accommodation by the TS active site. On the other hand, the propargyl unit has the advantage of being cylindrically symmetrical, so that TS-catalyzed toxin release from this type of tether does

not depend upon its orientation with respect to dUMP's uracil ring, as is the case with the vinyl and allyl molecules.

Two distinct approaches have been taken to design the nucleotide-based prodrugs of this invention. One is based on the structure of BVDU monophosphate and features a leaving group/toxin directly attached to the terminus of a (poly)vinyl substituent at C5 of dUMP. This is the vinyl tether approach. These compounds are defined in part as "Class I" compounds. The other is based on the structure of TFPe-dUMP and is similar to the first but has a methylene unit separating the leaving group/toxin and the unsaturated unit and thus contains an allyl or propargyl unit, described below as "Class II". This is the allyl tether approach.

Structures for 5'-phosphoramidate versions of each type are shown below:



The mechanism of activation of a propargyl version of the allyl tether approach has a precedent in the interaction of both 5-ethynyl-2'-deoxyuridine 5'-monophosphate (EdUMP) and 5-(3-hydroxy-l-propynyl)-2'deoxyuridine 5'-monophosphate (HOPdUMP)

with TS (Barr et al. 1981, Barr and Robins 1983). EdUMP is a potent inhibitor of TS (Ki = 0.1 μM), and likely forms an allene-based species at the active site. HOPdUMP (Ki = 3.0 μM) shows unusual inhibition kinetics, which might be due to formation of a cumulene-based species at the active site.

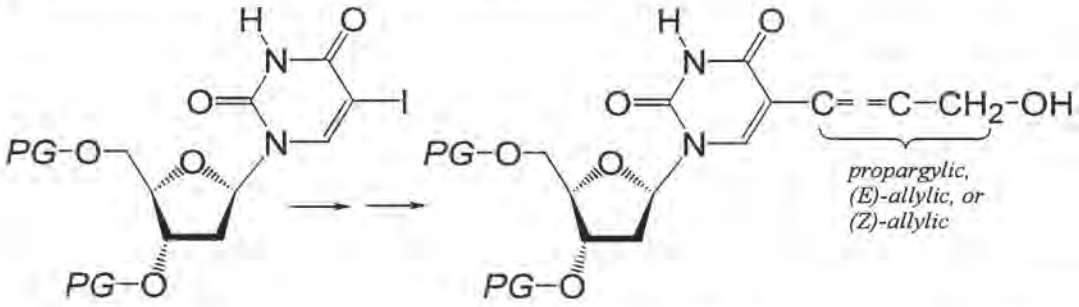
5-Alkylidenated 5,6-dihydrouracils similar in structure to the intermediate common to both the vinyl and allyl tether approach mechanisms have been synthesized recently (Anglada et al. 1996). These were shown to be highly electrophilic. Their ready reaction with ethanol to generate 5-(ethoxymethyl)uracils is a precedent for the water addition that regenerates catalytically competent TS in the mechanisms shown in Figure 8. Even more recently, the existence of the long-elusive C5 methylene intermediate produced by TS was demonstrated by trapping studies (Barrett et al. (1998)).

The synthesis of C5 propargylic and allylic alcohol-equipped 2'-deoxyuridines is straightforward. Many of these and their close derivatives are reported in the literature, and some have even been studied in connection with TS. For example, 5-alkynyl-dUMPs including the 5-(3-methoxy-l-propynyl) and 5-(3-hydroxy-l-propynyl) ones have been examined as TS inhibitors (Barr et al. (1981)) and some of these have been shown to become incorporated into the DNA of TS-deficient cancer cells (Balzarini et al. (1985)).

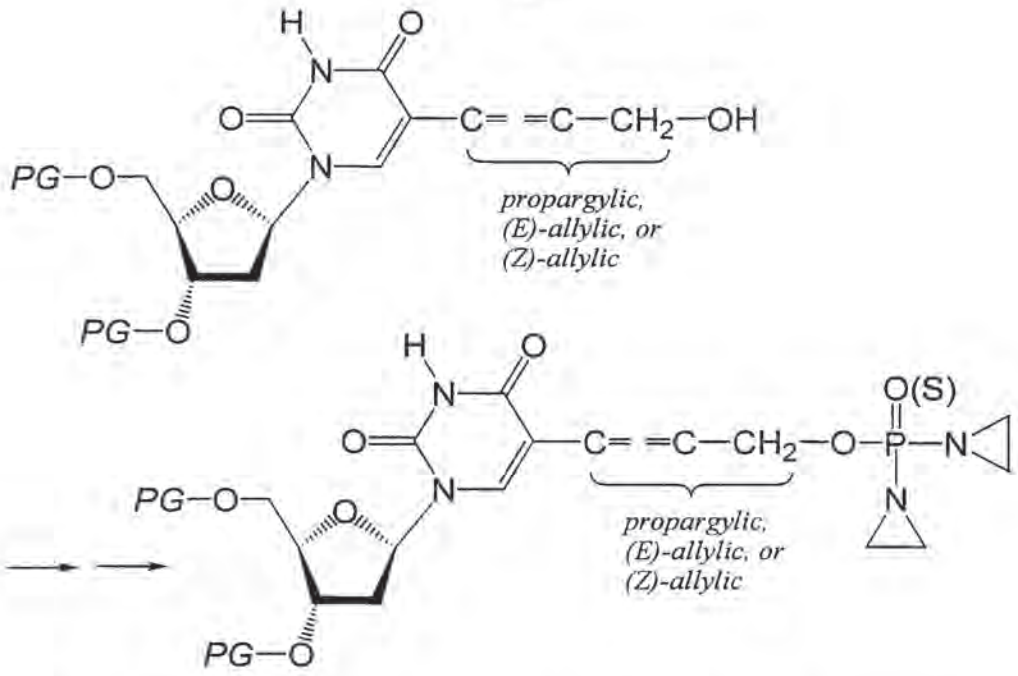
Both 5-mercuri- (Ruth et al. (1978)) and 5-iodouridines (Robins et al. (1981)) readily condense with alkenes and alkynes in the presence of a palladium catalyst to afford C5 tether-equipped uridines. The latter route is the more often employed (Robins et al. (1982)), Asakura and Robins (1988) and (1990)). High-yielding condensations of protected 5-iodo-2'-deoxyuridines with t-butyidimethylsilyl propargyl ether (Graham et al. (1998) De Clercq et al. (1983), methyl propargyl ether (Tolstikov et al. (1997)) and even propargyl alcohol itself (Chaudhuri et al. (1995) Goodwin et al. (1993)) have been achieved. The 3-hydroxy-l-propynyl substituent introduced by the latter reaction can also be accessed by DIBAL-H reduction of a methacrylate group (Cho et al. (1994)), itself arising from the same Heck reaction used in the synthesis of BVDU. These palladium-catalyzed reactions are so versatile that they can used to condense very long and elaborately-functionalized propargyl-based tethers to 5-iodo-2'-deoxyuridines (Livak et al. (1992) Hobbs (1989)). (Z)-Allyl-based tethers are generated by the partial hydrogenation of a propargylic precursor over Undiar catalyst (Robins and Barr (1983)) whereas the (E)-

allyl-based ones are best prepared by Heck coupling of an (E)-tributylstannylated ethylene (Crisp (1989)).

Closely following the literature procedures, a t-butyldimethylsilyl propargyl ether-equipped 3', 5'-di-O-protected 2'-deoxyuridine (Graham et al. (1998), De Clercq et al. (1983)) is prepared and a portion of it converted to the corresponding (Z)-allyl ether (Robins and Barr (1983)) is reduced. Because the TBAF-mediated removal of a TBDMS group generates an oxyanion that ran be functionalized *in situ*, these TBDMS-protected propargyl- and (Z)-allytic-tethered nucleosides will serve as convenient precursors to some of the toxophore-equipped targets. For the (E)-allyl alcohol equipped nucleoside. the known O-tetrahydropyranyl ether derivative is prepared by the literature Heck coupling of an (E)-tributylstannylated ethylene (Crisp (1989)).

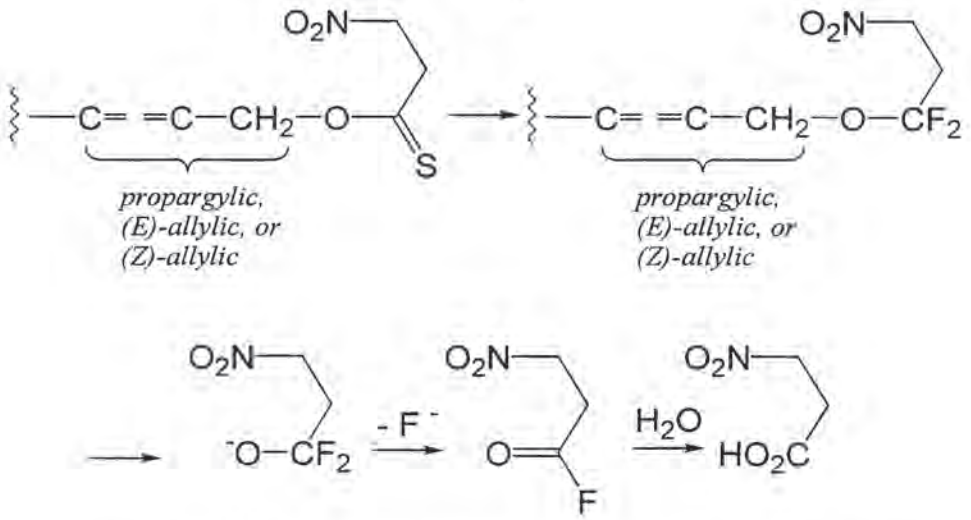


Using a two step literature protocol (Phelps et al. (1980) Hsiao and Bardos (1981)), the propargylic and (E) and (Z)-allylic alcohols are converted to their corresponding bis-aziridinyl phosphoramidates or thiophosphoramidates so that TS processing of the 5'-mononucleotide versions will release an active metabolite of the cytostatic drugs TEPA or ThioTEPA (Dirven et al. (1995)), respectively.

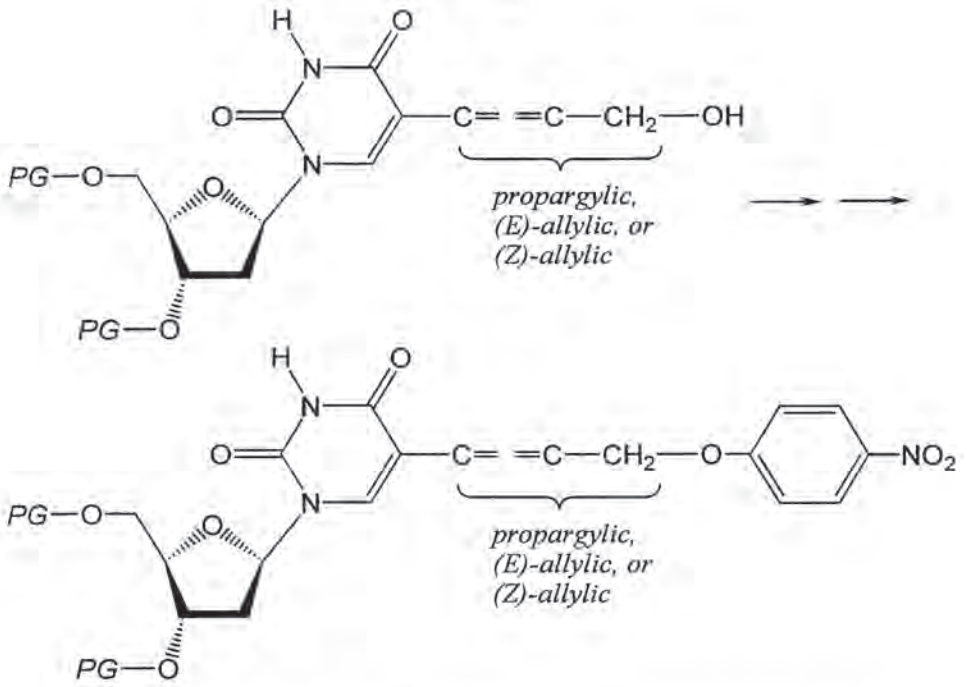


To avoid the potential hydrolytic lability of esters derived from the propargylic or allylic alcohols, the carboxylic acid moiety of toxophores like 3-nitroproprionic acid is "masked" by attaching them to the alcohol as α,α-difluoro ethers. To do this, a propargyl or allyl thioester tether is first prepared by treating the normal ester with Lawesson's reagent (Cava and Levinson (1985)), and then converting the thioester to the α,α-difluoro ether in an established fashion with tetrabutylammonium dihydrogentrifluoride (Kuroboshi and Hiyama (1991) and (1994)). The α,α-difluoro ether-based tether is then condensed with a protected 5-iodo-2'-deoxyuridine as usual. As an α,α-difluoro ether, the masked toxophore will be stable until TS releases it as a latent reactive acyl fluoride. The ensuing rapid hydrolysis will generate the carboxylic acid-based toxophore *in situ*.

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p-Nitrophenyl ether derivatives of the CS propargylic and (E) and (Z)-allylic alcohol-equipped 2'-deoxyuridines provide good reagents for *in vitro* TS enzyme assays, because the spectrophotometric, kinetic determination of released p-nitrophenol will identify those tethered dUMP platforms that bind to TS in a manner that permits facile catalytic release of a leaving group from the end of the tether. The needed p-nitrophenyl ethers will be obtained either directly from the alcohols by a base-catalyzed condensation with 4-fluoronitrobenzene or *de novo* by a Heck coupling of an appropriate p-nitrophenyl propargyl or allyl ether. The 5'-monophosphates needed for the TS assay are generated from the nucleosides either enzymatically or chemically according to a well-established regioselective 5'-monophosphorylation protocol (Imai et al. (1969)).



Many 5-substituted 2'-deoxyuridines are not substrates for human TK, but interestingly 5-(4-hydroxy-l-butynyl)-2'-deoxyuridine was found to be an exception (Barr et al. (1981)). Thus, it is expected that some of the toxophore equipped nucleosides will also possess propitious TK substrate activity, and so they will be examined for activity against tumor cell growth. Still, the preparation of the 5'-phosphoramidates is now a simple matter according to this newly developed regioselective procedure, and so these pro-prodrug versions are prepared and tested as well.

Cominatorial Synthesis

A rapid way to achieve a lead compound is through the use of combinatorial chemistry methodologies. Because a great deal is known about the mechanism of action of the thymidylate synthase enzyme (Schiffer, et al. (1995)), the following issues are anticipated: cellular entry by the prodrug, phosphorylation of the prodrug by thymidine kinase, and conversion from prodrug (uridine derivative) to active drug by thymidylate synthase. With respect to cellular entry, modifications of a phosphorylated nucleotide may be employed (see, for example, U.S. Patent Nos. 5,233,031 and 5,627,165). In addition, preferential phosphorylation of the prodrug in tumor cells is facilitated as a result of

overexpression of thymidine kinase in most tumor cells as a result of tumor suppressor gene loss (Hengstschlager et al. (1996)). Similarly, preferential activation of the phosphorylated prodrug or phosphoramidate derivative will occur in tumor cells because of the overexpression of thymidylate synthase accompanying tumor suppressor loss (Li, W. et al. (1995)) and chemotherapy (Peters, G.J. et al. (1995)). Combinatorial chemistry targeting the 5-position of the uridine ring (from which the cell toxin may be generated), or the 5'-position of the pentose sugar (to facilitate binding to thymidylate synthase), will greatly expedite the discovery of lead compounds by optimizing the structure of the leaving group (cell toxin) from the uridine ring, as well as facilitating optimization of a phosphorylation competent group at the 5'-position of the pentose sugar. For purposes of the screen shown in Figures 3, 4 and 5, the chemical entity being tested will be generated by either a single site directed chemistry (Figure 4), or simultaneously at two sites on the molecule (Figure 5). Together with the screen shown in Figure 3, a powerful system for discovery of prodrugs targeting thymidylate synthase has been developed. The combinatorial chemistry approach used will be similar to that described in Lam, K.S. (1997). In one embodiment, prodrugs that provide toxic leaving groups are shown in Figure 6. The uridine substrates shown in Figure 6 take advantage of phosphoramidase (present in all cells) and elevated thymidylate synthase (TS) in tumor cells. As is understood by those of skill in the art, this rationalized drug design can be broadly applied to the synthesis of other prodrugs as defined herein.

It should be understood by those skilled in the art that the screen shown in Figure 3 can be applied broadly for the discovery of antibiotics. For example, thymidylate synthase from yeast could be substituted for that of *E. coli* in Figure 4. This would allow the discovery of specific antifungal antibiotics targeting yeast related pathogens. In addition, other enzymes can be subjected to this treatment. For example, prodrugs which target specifically the dihydrofolate reductase activity of infectious agents, like *Pneumocystis carnii*, could be selected. These agents will be selected for specificity for the target enzyme, and can be shown not to activate the enzyme of the natural host by employing the screening assay described in Figure 3. The control cellular constructs would contain the corresponding normal human enzyme, in order to show lack of toxicity when only the normal human enzyme is present.

The drugs or agents of the invention or as described herein can be made more permeable, for example across cell membranes and across the blood-brain barrier, by means of various chemical modifications. These would include attachment, to their phosphate moiety, of various functional groups which improve membrane permeability. Such functional groups include, but are not limited to, those described in U.S. Patent Nos. 5,233,031 and 5,627,165; Fries, K.M., et al. (1995) and McGuigan, C., et al. (1984). Certain phosphoramidate derivatives of dideoxyuridine (ddU) are active against HIV and successfully bypass thymidine kinase. McGuigan, C., et al. (1984). Such chemical modifications can also serve to protect substituted pyrimidine monophosphate derivatives from the action of cellular and extracellular phosphatases. While the mercury or halogen derivatives may be effective, it is expected that modifications that release alkylating or antimetabolic compounds will be preferred. A preferred embodiment is shown in Figure 6.

Derivatives of the Compounds of Class I and II

Salts, esters, and ethers of the above compounds disclosed herein are also within the scope of this invention. Salts of the prodrugs of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicyclic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic and benzenesulfonic acids. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, can be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts. Examples of bases include alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and compounds of formula NW_4^+, wherein W is C_{1-4} alkyl.

Examples of salts include: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate,

phenylproprionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+, NH_4^+, and NW_4^+ (wherein W is a C_{1-4} alkyl group).

For therapeutic use, salts of the compounds of the present invention will be pharmaceutically acceptable. However, salts of acids and bases which are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

Esters of the prodrugs or compounds identified by the method of this invention include carboxylic acid esters (i.e., -O-C(=O)R) obtained by esterification of the 2'-, 3'- and/or 5'-hydroxy groups, in which R is selected from (1) straight or branched chain alkyl (for example, n-propyl, t-butyl, or n-butyl), alkoxyalkyl (for example, methoxymethyl), aralkyl (for example, benzyl), aryloxyalkyl (for example, phenoxymethyl), aryl (for example, phenyl optionally substituted by, for example, halogen, C_{1-4}alkyl, or C_{1-4}alkoxy or amino); (2) sulfonate esters, such as alkylsulfonyl (for example, methanesulfonyl) or aralkylsulfonyl; (3) amino acid esters (for example, L-valyl or L-isoleucyl); (4) phosphonate esters and (5) mono-, di- or triphosphate esters. The phosphate esters may be further esterified by, for example, a C_{1-20} alcohol or reactive derivative thereof, or by a 2,3-di-(C_{6-24})acyl glycerol. In such esters, unless otherwise specified, any alkyl moiety present advantageously-contains from 1 to 18 carbon atoms, particularly from 1 to 6 carbon atoms, more particularly from 1 to 4 carbon atoms. Any cycloalkyl moiety present in such esters advantageously contains from 3 to 6 carbon atoms. Any aryl moiety present in such esters advantageously comprises a phenyl group. Examples of lyxo-furanosyl prodrug derivatives of the present invention include, for example, those with chemically protected hydroxyl groups (e.g., with O-acetyl groups), such as 2'-*O*-acetyl-lyxo-furanosyl; 3'-*O*-acetyl-lyxo-furanosyl; 5'-*O*-acetyl-lyxo-furanosyl; 2',3'-di-*O*-acetyl-lyxo-furanosyl and 2',3',5'-tri-*O*-acetyl-lyxo-furanosyl.

Ethers of the compounds of the present invention include methyl, ethyl, propyl, butyl, isobutyl, and sec-butyl ethers.

In a further embodiment, the substrate may not be chemically related to pyrimidines or folates, but rather synthesized based upon known parameters of rational drug design. See Dunn, W.J. et al. (1996).

Chemical assays for products, for example, where a reaction product is an anti-metabolite of the bromovinyl-derivatives of dUMP, are described in the Examples provided below or by Barr, P.J. et al. (1983).

Diagnostic Application

Another aspect of the present invention pertains to the methods for screening for a therapeutic agent, comprising contacting a target cell with a prodrug compound this invention.

In the embodiment, the prodrug is a compound to the group of Class II, as described below, wherein R^4 is:



which target cell favor the incorporation of the compound into the target cell, for the diagnostic purpose of detecting intracellular levels of thymidylate synthase.

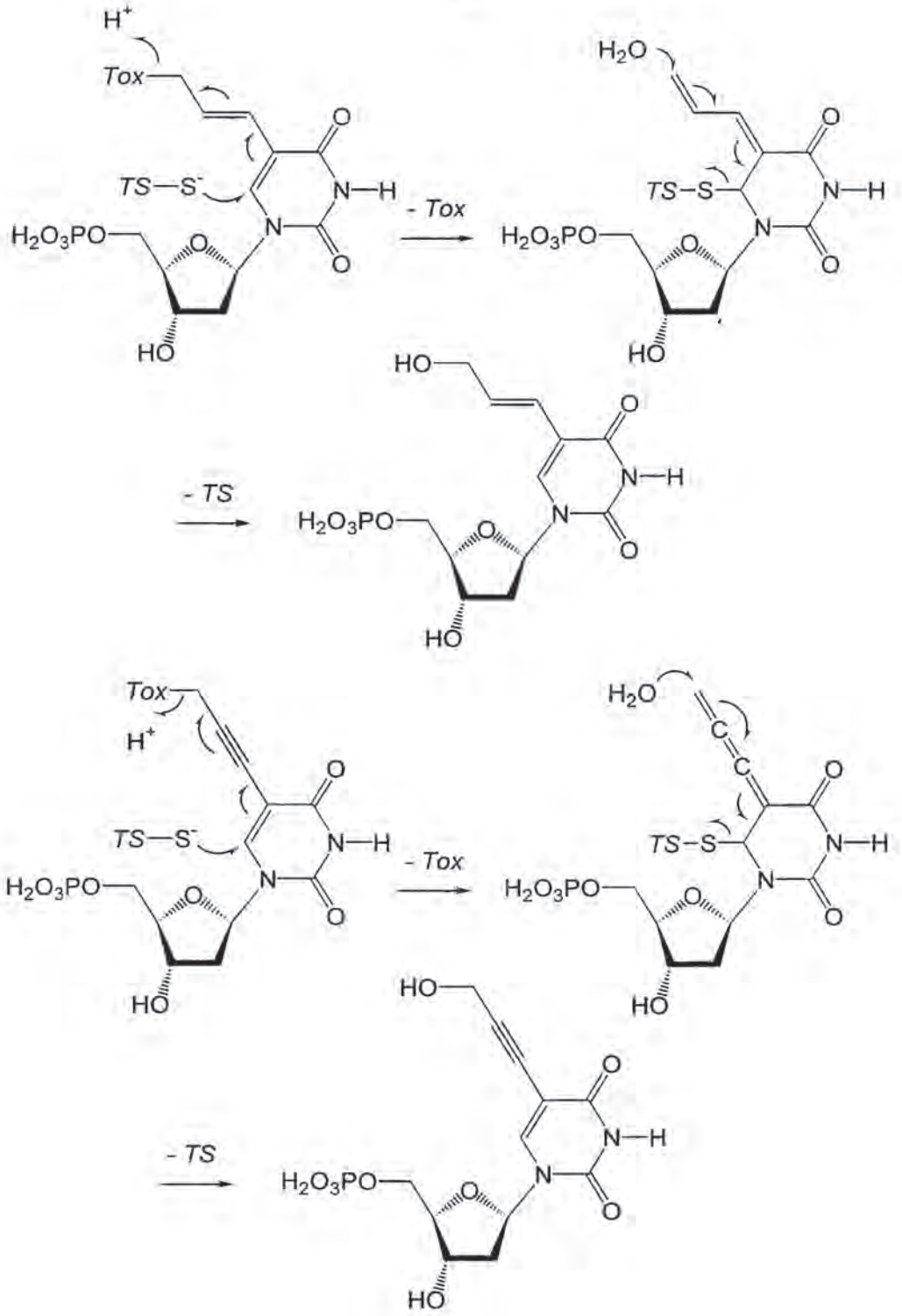
EXAMPLES

The following examples are specifically directed to the target enzyme TS. It is apparent to those skilled in the art that the following methods can be modified for the discovery of other prodrugs to target enzymes as defined herein, or used as a controlin the discovery of other prodrugs for TS and another intracellular enzymes.

Synthesis of Prodrugs

A phosphoramidate derivative of 5-fluoro-2'-deoxyuridine (5-FUdR), and (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and a phosphoramidate derivative of BVDU were synthisized.

BVDU was prepared from 2'-deoxyuridine according to a literature procedure (Dyer, et al. (1991)). According to standard methods, it was first protected at the O5' position with a dimethoxytrityl (DMTr) group and then at the O3' position with a tertbutyldimethylsilyl (TBDMS) group, and then the 5'-O-DMTr group was removed by treatment with acid (see below).


H+
Tox
TS—S-
H2O3PO
HO
- Tox
H2O
- TS
Tox
H+
TS—S-
H2O3PO
HO
- Tox
H2O
- TS
H2O3PO
HO

The 3'-O-TBDMS-protected nucleoside thus obtained reacted with phenyl L-methoxyalaninyl phosphorochloridate (PMPC) McGuigan, et al. (1992)) to give the 3'-O-TBDMS-protected BVDU-PA. However, unexpectedly, the phosphoramidate functionality was found to be labile to the very mild desilylation conditions of tetrabutylammonium fluoride (TBAF) on silica gel, by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. This observation of a sensitivity to mildly basic conditions provides one possible explanation why, save for one arabinonucleoside (McGuigan, et al. (1998)), all of the nucleoside 5'-phosphoramidate derivatives reported to date have lacked a 3'-hydroxy group functionality (see e.g., McGuigan, et al. (1996); McGuigan, et al. (1993); McGuigan, et al. (1994), Balzarini, et al. (1997), Balzarini, et al. (1996)). A novel synthetic method was developed to solve this problem.

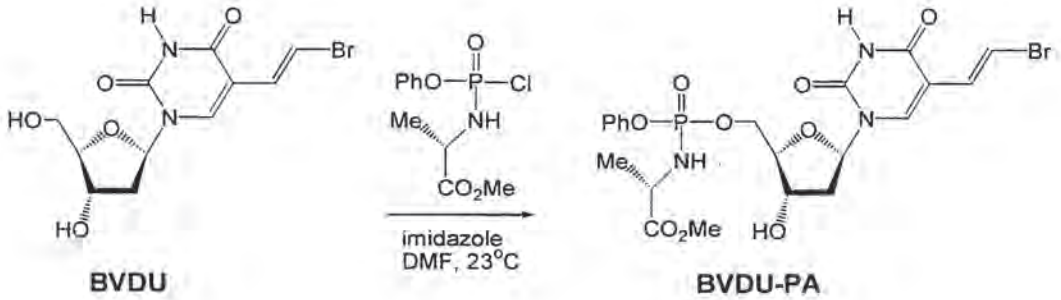
To synthesize (E)-5-(Bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate (BVDU-PA), a solution of BVDU (420 mg, 1.26 mmol) in 2 mL of anhydrous DMF under argon was treated with imidazole (103 mg, 1.51 mmol) and then dropwise with phenyl L-methoxyalaninyl phosphorochloridate (350 mg, 1.26 mmol (McGuigan, et al. (1992)). The reaction mixture was stirred at 23 °C under argon for 24 hours. By TLC on silica gel using 10% methanol in dichloromethane as eluent, the generation of BVDU-PA (R_f 0.70) from BVDU (R_f 0.53) had occurred, but only to a partial extent (ca. 15%), and so additional imidazole (52 mg, 0.75 mmol) and PMPC reagent (175 mg, 0.63 mmol) was added and the mixture stirred again at 23 °C under argon for 12 hours. By TLC, the progress of the reaction had increased somewhat (ca. 30% complete). The solution was reduced in volume to 0.75 mL by rotary evaporation and then was diluted with an equal volume of dichloromethane and was applied directly to a dry 4 mm silica gel Chromatotron plate. Radial chromatography using 250 mL of dichloromethane to elute residual DMF followed by 10% methanol in dichloromethane to elute product and starting material gave 144 mg (20%) of BVDU-PA and 294 mg of unreacted BVDU, for a 67% yield of BVDU-PA based on unrecovered starting material. If the ^{1}H NMR spectrum of product revealed the presence of imidazole (δ 7.65 and 7.01) or DMF (δ 7.95, 2.89, and 2.73), an additional radial chromatographic purification was performed. In this way, BVDU-PA with a purity of at least 98% by TLC and ^{1}H NMR was obtained as an oil or a foam-powder. ^{1}H NMR ($(CD_3)_2SO$) δ 11.4 (bs, exchanges with D_2O, 1, 3'OH), 8.28 (t, 1, H6), 7.35 (m, 2, Ph), 7.31 (d, 1, vinyl 1H), 7.20 (m, 3, Ph), 6.89 (d, 1, vinyl 2H), 6.19

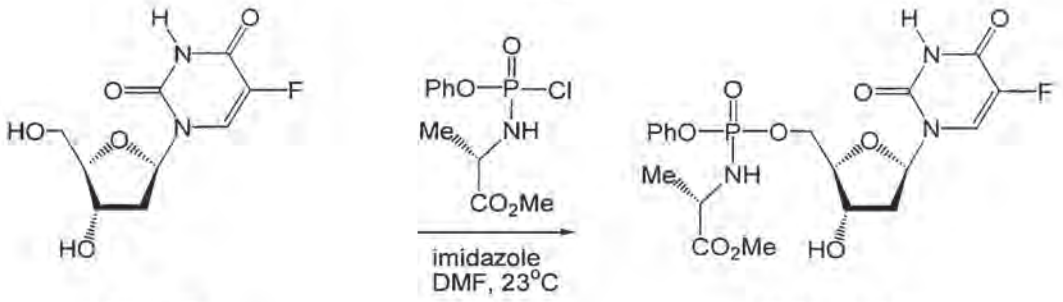
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(pseudo-t, 1, H1'), 6.08 (t, exchanges with D_2O, 1, alaninyl NH), 5.45 (bs, exchanges with D_2O, 1, 3'OH), 4.32 (m, 1, H4'), 4.22 (m, 2, 5'CH_2), 3.97 (m, 1, H3'), 3.86 (t, 1, alaninyl CH), 3.58 (s, 3, CO_2Me), 2.15 (m, 2, 2'CH_2), 1.23 (t, 3, alaninyl CH_3). $J_{\text{vinyl CH-vinyl CH}} = 13.5$, $J_{H1'-H2'} \sim 6.8$, $J_{H2'-H3'} \sim 5$, $J_{H3'-H4'} \sim 0$, alaCH-Ala-NH_3 NH ~ 6 Hz. $^1H/^1H$ COSY 2D NMR spectroscopy provided confirmation of spectral assignments.

In a similar fashion, 5-Fluoro-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate (5FUdR-PA), was obtained in a purity of at least 98%, by TLC and ^1H NMR. ^1H NMR ($(CD_3)_2SO$) δ 11.9 (bs, exchanges with D_2O, 1, N3H), 7.88 (t, 1, H6), 7.36 (m, 2, Ph), 7.19 (m, 3, Ph), 6.15 (pseudo-t, 1, H1'), 6.07 (t, exchanges with D_2O, 1, alaninyl NH), 5.42 (bs, exchanges with D_2O, 1, 3'OH), 4.21 (m, 3, H4' and 5'CH2), 3.98 (m, 1, H3'), 3.84 (t, 1, alaninyl CH), 3.58 (s, 3, CO_2Me), 2.08 (m, 2, 2'CH_2), 1.22 (t, 3, alaninyl CH3). $J_{H6-5F} = 7.1$, $J_{H1'-H2'} \sim 5.2$, $J_{H2'-H3'} \sim 2$, $J_{H3'-H4'} \sim 0$, $J_{\text{alaninyl CH-alaninyl NH}} \sim 6$ Hz. $^1H/^1H$ COSY 2D NMR spectroscopy provided confirmation of spectral assignments. Low-resolution mass spectrum (DCI-NH3), m/z 505 (MNH_4+), 488 (MH+).

It was reasoned that the direct condensation of an unprotected 2'-deoxyribonucleoside with PMPC might very well proceed to give the desired phosphoramidate and in a 5'-O-regioselective manner if it were conducted in the absence of base but the presence of a scavenger for the HCl produced. Both BVDU and 5FUdR condensed with the McGuigan reagent in the presence of imidazole in anhydrous DMF solution to give the desired BVDU-PA and 5FUdR-PA, respectively (see below). The reaction conditions have not been optimized, and although the reactions do not proceed to completion, the readily separable product mixture in both cases consists largely of only desired product and starting material, by thin layer chromatography (TLC). The synthetic scheme is summarized below.





5FUdR **5FUdR-PA**

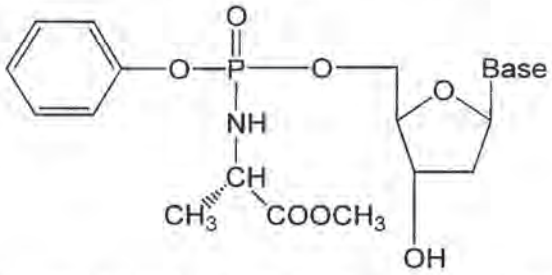
(E)-5-(Bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate (BVDU-PA).

TLC monitoring (10% methanol in dichloromethane as eluent) revealed the production of BVDU-PA (R_f 0.70) from BVDU (R_f 0.53). In this way, BVDU-PA was obtained in a purity of at least 98%, by TLC and ^{1}H NMR. ^{1}H NMR ($(CD_3)_2SO$). A ^{1}H/^{1}H COSY 2D NMR spectrum confirmed the spectral assignments. Low-resolution mass spectrum by direct chemical ionization (DCI) using NH_3, *m/z* 593/591 ($M•NH_4^+$), 576/574 ($M•H^+$).

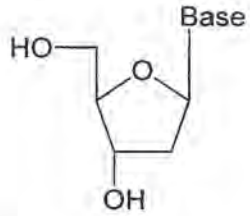
5-Fluoro-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate (5FUdR-PA). In a similar fashion, 5FUdR-PA was obtained in a purity of at least 98%, by TLC and ^{1}H NMR. ^{1}H NMR ($(CD_3)_2SO$). A ^{1}H/^{1}H COSY 2D NMR spectrum confirmed the spectral assignments. Low-resolution mass spectrum (DCI-NH_3), *m/z* 505 ($M•NH_4^+$), 488 ($M•H^+$).

Thus, one aspect of the invention pertains to methods of preparing a 2'-deoxy, 3'-hydroxy, 5'-phosphoramidate of a furanosyl nucleoside, which methods involve reacting an unprotected furanosyl nucleoside which is 2'-deoxy, 3'-hydroxy, and 5'-hydroxy, with a phosphochloridate in the presence of an HCl scavenger. In one embodiment, the phosphochloridate comprises a phosphorus substituent which is derived from an amino acid, such as alanine. In one embodiment, the phosphochloridate is phenyl-

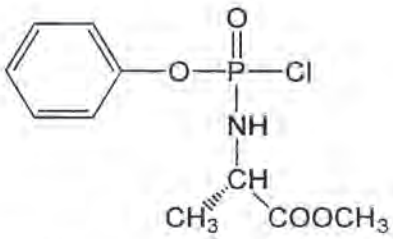
L-methoxyalanine phosphorochloridate. Thus, in a further embodiment, the present invention pertains to a method of forming a compound of the formula:



wherein "Base" denotes a nucleic acid base (such as uracil, thymine, cytosine, adenine, or guanine, but preferably uracil, or a derivative thereof); which method comprises the step of reacting a compound of the formula:



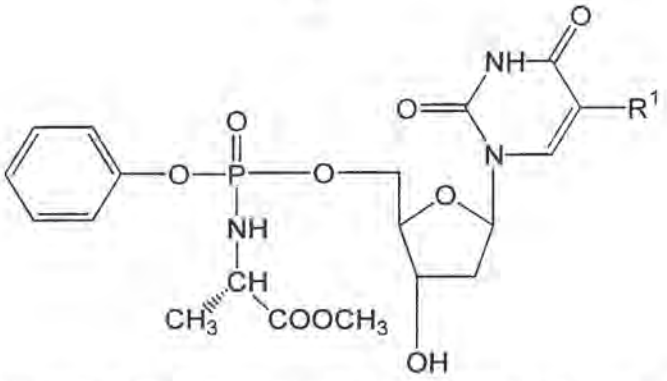
with a compound of the formula:



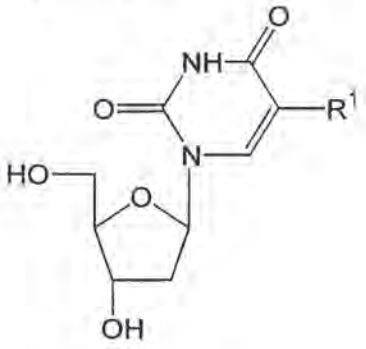
in the presence of an HCl scavenger.

In one embodiment, the furanosyl nucleoside is a uridine nucleoside. In another embodiment, the furanosyl nucleoside is a uridine ribofuranosyl nucleoside. In another embodiment, the furanosyl nucleoside is a uridine β-D-ribofuranosyl nucleoside. Thus, in one embodiment, the present invention pertains to a method of forming a compound of the formula:

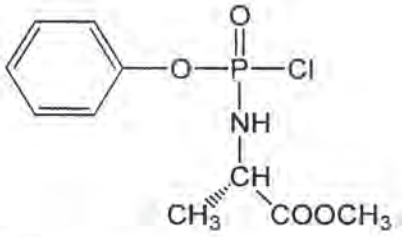
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wherein R^1 is a substituent (including, for example, those described above for R^1); which method comprises the step of reacting a compound of the formula:



with a compound of the formula:



in the presence of an HCl scavenger.

In one embodiment, the reaction is performed in the absence of base, but in the presence of an HCl scavenger, such as imidazole. In an alternate embodiment, the reaction is carried out in a non-aqueous medium. In another embodiment, the reaction is carried out in a non-aqueous medium comprising anhydrous dimethylformamide (DMF).

Chemical and Cell-Based Assays

Two cell lines, H630R10 (Copur, et al. (1995)) and normal colon epithelial cells, CCD18co (ATCC) were used in these assays. The H630P (Copur et al. (1995)) cell line was selected for resistance to 10μM 5-FU to give rise to H630R10. The characterization of these cell lines which expresses an elevated level of the TS enzyme. A normal colon epithelial cell type CCD18co (available from the ATCC) was used for comparison with H630R10 for sensitivity to test compounds. Cell lines expressing p185 - HER2 or neomycin marker alone were prepared as described by Pegram et al. (1997) shows that H630R and H630R10 expresses 10 fold increased thymidylate synthase enzyme as compared with CCD18co as determined by Western blot analysis.

The ability of the test compounds to block proliferation of cells was determined by the crystal violet procedure (Sugarman et al. (1985) and Antelman et al. (1995)).

Compounds were dissolved in dimethyl sulfoxide to a concentration of 1M. They were further diluted as necessary into DMEM cell culture medium, and subsequently into the first wells of the 96 Well microtiter plate. Each concentration was tested in triplicate on the target cell line. Compound concentrations from 1 μM to 3000 μM were tested. Cells were incubated with compound for 72 hours, the plates were washed, and the cells fixed with methanol and stained with crystal violet as described in Sugarman et al. (1995) and Antelman, et al. (1995).

Western Blot Analysis of TS Levels in Cell Lines

Western blot experiments were performed with the human normal colon epithelium cell type CCD18co (obtained from ATCC, Manassas, VA), colon adenocarcinoma cell line H630R10 (obtained from Dr. S. Copur, Yale University), and HER2-transfected breast cancer cell lines (Pegram et al. (1997)). Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5,150 mM NaCl, 0.5% Triton X-100, 0.1% SDS and 0.5% Deoxycholic acid, sodium salt and protease inhibitors). Protein concentrations were determined by using BCA-200 protein assay kit (obtained from Pierce, Rockford, IL). 10 μg of proteins from each cell line were resolved by 12% SDS-PAGE. The separated proteins were transferred onto PVDF membrane (obtained from Amersham, England), followed by immunoblot with human thymidylate synthase monoclonal primary antibody and anti-tubulin monoclonal antibody (manufactured by NeoMarkers, Fremont, CA). Horseradish peroxidase linked

sheep anti-mouse Ig was used as secondary antibody (Amersham). The ECL plus kit (Amersham) was used for detection of immunoreactivity. The bands corresponding to thymidylate synthase were quantified and normalized to that of tubulin by image analysis (Molecular Dynamics Storm).

RT-PCR Analysis of TS mRNA in Cell Lines

Expression level of human thymidylate synthase transcripts in different cell lines were quantified by using RT-PCR. Oligonucleotide primers for amplification of the human thymidylate synthase and B-actin were designed as follows: Thymidylate synthase sense primer 5'-GGGCAGATCCAACACATCC-3' (corresponding to bases 208-226 of thymidylate synthase cDNA sequence, Genbank Accession No. X02308), antisense primer 5'-GGTCAACTCCCTGTCCTGAA-3' (corresponding to bases 564-583), β-actin sense primer 5'-GCCAACACAGTGCTGTCTG-3' (corresponding to bases 2643-2661 of β-actin gene sequence, Genbank accession no. M10277) and antisense primer 5'-CTCCTGCTTGCTGATCCAC-3' (corresponding to bases 2937-2955).

Total RNAs were isolated from CCD18co and H630R10 cells, using Rneasy mini kit (obtained from Qiagen, Valencia, CA). To monitor for possible DNA contamination, the primers for amplification of β-actin were designed to span the exon4/intron5/exon5 junction. Genomic DNA template leads to a 313 bp β-actin fragment, and cDNA template generates a 210 bp product.

Reverse transcription reactions were performed, using SuperScript preamplification system (Gibco/BRL, Gaithersburg, MD). 3 μg total RNA was applied in a volume of 20 μl buffer to conduct reverse transcription reaction, followed manufacturer's protocol. PCR reactions were performed in a volume of 48 μl, containing 3 μl of cDNA mixture from reverse transcription reaction, 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-Cl, pH 8.4, 0.2 mM of each dNTP, 0.4 μM of thymidylate synthase sense and antisense primers and 3 units of Tag DNA polymerase (obtained from Promega, Madison, WI). The reaction mixtures were incubated at 94°C for 3 min, followed by 10 cycles of 1 min incubation at 94°C, 1 min incubation at 58°C , and then 1 min incubation at 72°C. After 10 cycles, human β-actin primers in 2 μl were added to achieve a final concentration of 0.2 μM, bringing the final reaction volume to 50 μl. PCR reaction was continued to a total of 28 cycles, followed by a 7 min incubation at 72°C.

5 μL of PCR products were resolved by electrophoresis in 2% agarose gel, followed by staining with SYBR Gold nucleic acid gel stain (obtained from Molecular Probes, Eugene, OR). The DNA bands corresponding to thymidylate synthase were quantified and normalized to that of β-actin by Molecular Dynamics Storm.

Northern Blot Analysis

Northern blots were obtained from Invitrogen (Carlsbad, CA) and hybridized to a cloned TS cDNA probe. Hybridization signals were normalized vs. ribosomal protein S9 as a housekeeping transcript. A tumor was considered to overexpress TS mRNA if the normalized signal was enhanced at least 2-fold as compared to the normal tissue control.

Cloning, Expression and Purification of Human Thymidylate Synthase

An *E. coli* plasmid expression vector for human thymidylate synthase (TS) was constructed using a modified human TS cDNA in pGCHTS. This clone, obtained from Dr. Dan Santi, contains the complete open reading frame for human TS.

For expression in *E. coli* the complete human TS ORF was subcloned into the T7 promoter expression vector pET28a (Novagen Inc.). The resulting plasmid vector encodes the synthesis of human TS as a recombinant fusion protein with a six histidine followed by a thrombin cleavage site. This fusion protein adds a total of 20 extra amino acids to the amino terminus of human TS. To produce recombinant protein, the human TS expression vector was introduced into the *E. coli* strain BL21(DE3), a strain with the T7 RNA polymerase gene inserted into the chromosome under the control of the lac operator.

The human TS-poly-His fusion protein was purified using a metal chelating affinity resin (Novagen Inc.). Protein purification was followed by electrophoresis on 10% SDS polyacrylamide gels. Protein concentrations were determined using the Pierce BCA protein assay. Approximately 10 mg of human TS fusion protein was recovered from a 500 ml culture of *E. coli*. When visualized with silver stain only the band corresponding to human TS was apparent. The identity of the human TS band was confirmed by performing a Western blot with the anti-TS monoclonal antibody TS106 (NeoMarkers).

Thymidylate Synthase Enzyme Activity Assay

TS activity was measured using the spectrophotometric assay of Wahba and Friedkin (1961). In this assay, TS activity is monitored by measuring the increase in absorbency at 340 nm that occurs when the co-factor 5,10 methylene tetrahydrofolte is oxidized to dihydrofolate as dUTP is converted to dTTP. Enzyme prepared by this method has a specific activity of 0.5 - 0.65 units/mg protein. One unit is defined as the production of one micromole of dTNP per minute. This value is similar to that reported by Pedersen-Lane et al. (1997).

Experimental Results

These results establish the feasibility of utilizing thymidylate synthase as a cytotoxin generator in tumor cells. In this assay, CCD18co cells (normal colon epithelium), and H630R10, a derivative of H630P which expresses several-fold more thymidylate synthase (Copur et al. (1995)) were employed. A western blot analysis of TS protein levels in each cell type was performed and normalized between samples by comparison with antibody directed vs. human tubulin (Table 5). A rank order of H630R10 (13x) and CCD18co (lx) was obtained.

Comparison of mRNA levels between cell lines as determined by RT-PCR was also performed. See Table 5. Similarly to the western blot analysis, the rank order was H630R10 (24 x) and CCD18co (lx), when normalized vs. a β-actin RT-PCR standard.

Table 5
Analysis of TS Protein and mRNA Expression in CCD18co and H630R10 cells

	TS Expression	
Cell Lines	Relative mRNA Level	Relative Protein Level
CCD18co	1	1
H630R10	24	13

HER2 Protooncogene Expression Increases TS Levels in Tumor Cells

TS expression levels in neomycin and HER2/neu-transfected human breast carcinoma cells were assessed using immunoblotting techniques. The results (see Figure 7) demonstrate an increase in TS expression following transfection and overexpression of human HER2/neu cDNA. Similar results were obtained with HER-2/neu-transfected

human ovarian carcinoma cells. The MCF7/HER2 and MCF7/neo cell lines are target cells for assays of this invention because induction of TS is most pronounced (about 20-fold) in these cell types.

The fluoropyrimidine, 5-FUdR, inhibits cell growth via mechanisms similar to 5-FU. This includes cytotoxic effects resulting from incorporation into nucleic acid, as well as inhibition of TS enzyme activity (Goodman and Gilman (1996)). It is therefore expected that cells expressing higher levels of TS will often be more resistant (have a higher IC_{50}) than cells which express lower amounts of the enzyme. Table 6 (Line 1) demonstrates that this is the result obtained. Furthermore, as expected from earlier work, BVDU has little activity in the assay (Table 6; line 2). This presumably results from the inability of BVDU to be monophosphorylated by human thymidine kinase, a requirement for binding to thymidylate synthase (Balzarini et al. (1987), Balzarini et al. (1993), Carreras and Santi (1995)). There is no significant difference in sensitivity of the two cell lines to BVDU. Activation of BVDU occurs with phosphoramidation of the 5'-hydroxyl of the ribose moiety. BVDU-PA (Table 6, line 3) has more than 10-fold greater activity on the higher expressing H630R10 cell line than on CCD18co cells.

Table 6
Higher Levels of TS Expression Predict Greater Sensitivity to BVDU-PA

	$IC_{50}(\mu M)$[1]	
Compound	CCD18co	H630R10
1. 5-FUdR	9.5M ± 0.42	169.8 ± 2.4
2. BVDU	2540 ± 58.2	2876.2 ± 54.5
3. BVDU-PA	2810.2 ± 75.1	216.7 ± 15.7

[1] Standard error less than 20%

These two cell lines were then compared with respect to sensitivity to 5FUdR, and the phosphoramidate derivative of BVDU (BVDU-PA). Because 5FUdR inhibits cell growth via the same mechanism as 5FU, it is expected that CCD18co will be more sensitive (have a lower IC-50) than H630R10, since the latter cell line has a higher intracellular level of thymidylate synthase. The data presented in Table 7 demonstrated that this is the result obtained, using the crystal violet assay, described above. This result

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also illustrates one of the key problems with current cancer chemotherapeutics, *i.e.*, that they are often more toxic to normal cells than to cancer cells. The data shown in Table 7 show that normal colon epithelial cells (CCD18co; IC-50=9.5μM) are approximately 18-fold more sensitive to 5FUdR than are the H630-R10 colon tumor cells (IC-50 = 169.8). The prodrug substrate, BVDU-PA, however, reverses this relationship. BVDU-PA, which is converted by TS into cytotoxic moieities, is approximately 13-fold more potent on the high TS expression H630R10 cell (IC_{50} = 216.7) than on the normal colon cell type (IC_{50} = 2810.2).

Table 7

TS Prodrug BVDU-PA has Greater Activity on a Thymidylate Synthase Overexpressing Tumor Cell Line than on Normal Cells (1, 2).

Compound	IC_{50}	
	CCD18co (normal colon epithelium)	H630R10 (colon cancer cell line)
5-FUdR (floxuridine)	9.5μM	169.8μM
BVDU-PA	2810.2μM	216.7μM

1. Representative data from several assays.
2. Assay performed with triplicate wells at each concentration. Standard error is less than 20%.

This result was confirmed using the Alamar Blue Assay (commercially available from ACCUMED Int., West Lake, OH). The data in Table 8, below show that even 5-FU has non-selective properties that make it toxic to normal and tumor cells.

Table 8

Normal Cells Are At Least as Sensitive to 5-Fluorouracil as Tumor Cells

Normal Cells	IC_{50}	Tumor Cells	IC_{50}
Colon Epithelium (CCD18co)	0.2	Breast (MCF7)	0.61
Skin Fibroblast (DET551)	1.1	Breast (MDA 468)	0.40
Lung Epithelium (W138)	0.4	Sarcoma (Saos 2)	11.30
	$\overline{X}$ = 0.57 μM		$\overline{X}$ = 4.10 μM

An additional assay of this invention requires candidate drugs to be screened in reaction mixtures containing human thymidylate synthase with and without N5N10-methylenetetrahydrofolate, and the candidate prodrug. The leaving group of the candidate prodrug (e.g., at the pyrimidine 5 position) is labeled, for example, with tritium using methods well known in the art. The control substrate is similarly labeled (e.g. 5-^{3}H) dUMP, under the same reaction conditions. The assays are done similarly to the description provided in Carreras, C.W. and Santi, D.V. (1995), and references cited therein. The human thymidine synthase can be purified from *E. coli* containing the expressed human thymidylate synthase. See Davisson, V.J. et al. (1989) and Davisson, V.J. et al. (1994). This approach provides a scaleable assay capable of screening large numbers of candidate compounds.

Determination of Intracellular Products of TS Prodrug Metabolism

It is important to determine the intracellular products of TS prodrug metabolism in order to substantiate proposed mechanism of activation and action and to define agents that are candidate thereapeutics. One acceptable view of intracellular metabolism of aryl phosphodiester amidates involves enzymatic conversion into a carboxylic acid, intramolecular rearrangement of the phosphomonoester amidate into to a 5'-monophosphoryl nucleoside (Valette et al. (1996)). However, this mechanism is unlikely to describe intracellular processing of all phosphoramidate-based pronucleotides. For example, a different mechanism was proposed for aryl phosphomonoester amidate processing, one involving the simple direct conversion of the phosphoramidate to the monophosphate species by a phosphoramidate hydrolates (McIntee et al. (1997) and Fries et al. (1995)). Regardless of the mechanism for unmasking the nucleoside monophosphate, this assay will detect products of TS conversion of the intracellular monophosphate to cytotoxic compounds within the cell.

The proposed reaction products of prodrug compounds with TS are shown in Figure 8. To accomplish this task, cells are incubated with an amount of prodrug compounds that induces 50% growth inhibition of high TS expressing cell lines (in the 72H assay supra.). Both low and high TS expresser cells are used (eg., CCD18co vs. H630R10). Time course studies are performed in which treated cells are processed according to the method described by McIntee et al. (1997). Cells are lysed with 60%

methanol in water at -20°C, and particulate residue removed by centrifugation. The supernatants are dried and stored at -20°C. The aliquots are evaluated initially by RP-HPLC and then by LC-MS to document the intracellular conversation of the phosphoramidate to the monophosphate and also the ensuring transformation of the monophosphate by thymidylate synthase.

Documentation of Substrate Activity Utilizing Purified Thymidylate Synthase

This assay determines the specific activities of future TS enzyme preparations, to assure the activity of such preparations over time, and to determine whether compounds screened for suitable activity do inactivate the TS enzyme under *in vitro* reaction conditions.

To determine what reaction products are produced when prodrug compounds are incubated together with purified recombinant TS enzyme, reaction conditions similar to those employed with the TS activity assay are used to generate reaction products *in vitro*. These reaction products will are then analyzed by GC-mass spectrometry to identify the actual molecules formed.

In Vivo Efficacy Studies

1. Cell Lines and Cell Culture

MCF7/NEO and MCF7/HER-2-transfected breast cell lines were obtained from Dr. Dennis Slamon (UCLA). MCF7/HER-2-transfected human breast carcinoma cells as well as other HER-2/neu-transfected cell lines demonstrate an increase in TS expression (Pegram et al. (1997)). Thus, in additional to CCD18co and H630R10, these lines are suitable for testing differential response to prodrug. The differential responses are first confirmed *in vitro* for MCF7/NEO and MCF7/HER-2 cells to 5-FUdR and BVDU-PA using the same *in vitro* experimental approach described above. The HT1080 tumor cell lines from Dr. Bertino (Banerjee et al. (1998)) are similarly characterized.

2. Xenograft Models for Testing of Prodrug Compounds

MCF7/HER-2 breast cells form xenografts in athymic mice with high efficiency and their growth properties have been thoroughly characterized (Pegram et al. (1997)).

The growth properties of this xenograft model are so uniform that a computer model, which can predict the growth trajectory of these tumors following treatment with different drug combinations, has been developed by Dr. Angela Lopez and Dr. Elliot Landau in the Department of Biomathematics at the UCLA School of Medicine (Lopez, et al. pending submission). Because of uniformity and reproducibility of this model, it has become very useful in prectinical testing of novel experimental therapeutics. For example, this model formed the basis for the preliminary *in vivo* analysis of the drug Herceptin® which has recently been approved by the US Food and Drug Administration for the treatment of metastatic breast cancer (Pegram, et al. (1998)). The colon tumor cell lines (HT1080/NEO and HT1080/E2F1.1) have demonstrated tumorigenicity (Banerjee et al. (1998)).

Ras-transformed NIH 3T3 cell lines are transplanted subcutaneously into immunodeficient mice. Initial therapy may be direct intratumoral injection. The expected result is that increased level of expression of human TS or a target enzyme leads to enhanced antitumor activity by the drug candidates. Similar studies are performed with human tumors expressing increasing levels of human TS or a target enzyme, and demonstrating that efficacy in response to drug correlates with their level of human TS expression or target enzyme. Optionally, experiments are be performed as above except the drug will be administered intravenously into the animals to address issues related to efficacy, toxicity and pharmacobiology of the drug candidates. In a further embodiment, control animals will receive an effective amount of a compound identified above under the section entitled "Prodrugs," *supra*.

More specifically, the in vivo studies can be conducted by using two different xenograft models (See Table 9).

Table 9
Tumorigenic Cell Lines Used For *In Vivo* Efficacy Testing

Xenograft	Relative TS Expression	Cell of Origin	Source
MCF7/NEO MCF7/HER2	low (1 X) high (20 X)	human breast carcinoma	Dr. Slamon
H630R10	high (13X)	human colon carcinoma	Copur et al. (1995)
HT1080/NEO HT1080/E2F1.1	low (1 X) high (14 X)	human colon carcinoma	Dr. Bertino Banerjee et al. (1998).

Test cells with defined expression of TS (indicated in Table 9) are injected subcutaneously at 5 X 10^6/site in the flank region of 4-6 week old (20-25 gram) CD-1 (nu/nu) athymic mice (Charles River Laboratories, Wilmington, MA) as described (Pegram, et al. (1997)). Eight mice (four animals per cage) are used for each treatment group (vehicle control solution or prodrug compounds). Two xenografts are established per mouse both reduce the number of animals needed for each experiment and improve the statistical power to detect differences in response between groups. Individual mice are identified by tagging the ears so that xenograft trajectories are calculated for individual mice in addition to the analysis of group means. All mice used in the MCF-7 breast xenograft model are female and, prior to cell injection, are primed with 17B-estradiol (Innovative Research of America, Toledo, OH) applied subcutaneously in a biodegradable carrier binder (1.7 mg estradiol/pellet) to promote tumor cell growth. Prospective tumor volumes, calculated as (width^2 X length)/2 are monitored twice weekly by serial micrometer measurements by a single blinded observer. Animals are assigned to each treatment group such that the mean starting tumor volumes will be the same in each group. Statistical tests are performed (single-factor ANOVA) to assure uniformity in starting tumor volumes between treatment groups. The prodrugs or isovolumetric vehicle control solution are administered by single I.P. injection in the first set of experiments. If efficacy is identified in the initial experiments, additional studies will be performed using a daily subcutaneous dosing schedule X 5 days with total prodrug dose equal to the IC_{10} defined in the single I.P. dosing studies.

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For these efficacy experiments prodrug dosing will commence when the means xenograft volumes in each group reach 5OMM^3. Mean tumor volumes of prodrug-treated relative to control-treated animals will be plotted using descriptive statistics with graphical analysis. Statistical tests (single factor ANOVA) will be applied to compare xenograft volume differences between treatment groups. Log-transformation will be applied for ANOVA computations, when indicated, to stabilize variance in xenograft volume data sets. Non-parametric statistical tests can be applied if necessary depending on xenograft volume data distribution. Differences in efficacy between xenograft with high TS expression will be compared to those with low TS expression using prodrug-treated/control-treated tumor volume ratios (T/C ratios) using statistics as described (Pegram, et al. (1997)). This methodology can accommodate differences in intrinsic growth rates between high TS-expressing xenografts and low TS-expressing xenografts.

In vivo studies also can be conducted as described by Harris, MP et al. (1996) and Antelman, D. et al. (1995).

3. Dose Finding Studies in Non-Tumor-Bearing Athymic Mice to Define the Maximum Tolerated Dose (MTD)

Groups of 6 CD-1 nu/nu athymic mice (Charles River Laboratories) (3 male, 3 female) are injected with a single dose of prodrug via the intraperitoneal (I.P.) route. Dosing will begin empirically with the initial dose defined by the amount of a prodrug to achieve a serum concentration equal to the IC_{50} *in vitro*, assuming the volume of distribution of the prodrug to be restricted to the total body water (TBW) compartment of the mouse (0.6 X body mass in grams TBW_{mouse}). If no toxicity is observed at this level, then dose escalation proceeds in groups of 6 mice at half-log intervals. If toxicity is encountered at the empiric dose level, then repeat dose escalation experiments beginning at 10% of the toxic dose will proceed likewise. Mice are observed daily for mobility, grooming behavior, and ability to take food and water. Mouse weights will be assessed weekly. The MTD will be defined as the dose resulting in 10% or less loss in body weight during the observation period, or a dose = 90% of the LD_{10}. If lethality is encountered at a particular dose level then repeat experiment (assuming no toxicity is observed at this level). The observation period is about 60 days. Dose escalation will proceed in

prospective cohorts at 3 week intervals if no toxicity is encountered at a particular dose level.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

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Hostetler et al., Patent Application No. WO 96/40088 (1996)

CLAIMS

What is claimed is:

1. A method for identifying potential therapeutic agents, comprising:

(a) contacting a target cell with a candidate therapeutic phosphoryl or phosphoramidate prodrug that is a selective substrate for a target enzyme, under conditions that favor the incorporation of the agent into the intracellular compartment of the target cell;

(b) assaying the target cell for inhibition of cellular proliferation or cell killing.

2. The method of claim 1, wherein the prodrug is a phosphoramidate derivative of 2'-deoxyuridine.

3. A method for identifying potential therapeutic agents, comprising:

(a) contacting a target cell with a candidate therapeutic phosphoryl or phophoramidate prodrug having a detectably labeled toxic leaving group and that is a selective substrate for a target enzyme, under conditions that favor the incorporation of the agent into the intracellular compartment of the target cell;

(b) assaying the culture media for the amount of label released and comparing it to the amount of label released.

4. The method of claim 3, wherein the prodrug is a phosphoramidate derivative of 2'-deoxyuridine.

5. The method of any of claims 1 to 5, wherein the target cell is characterized as resistant to a chemotherapeutic drug.

6. The method of any of claims 1 to 5, wherein the target enzyme is amplified as a result of selection *in vivo* by chemotherapy.

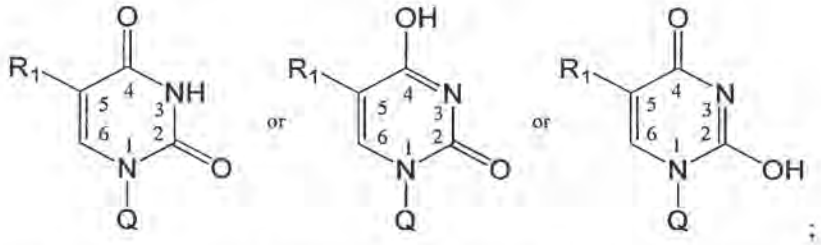
7. The method of any of claims 1 to 5, wherein the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell.

8. The method of any of claims 1 to 5, wherein the target enzyme is thymidylate synthase.

9. A method for inhibiting the proliferation of a hyperproliferative cell, comprising contacting the cell with a phosphoryl or phorphoramidate prodrug that is selectively converted to a toxin in the cell by an endogenous, intracellular enzyme.

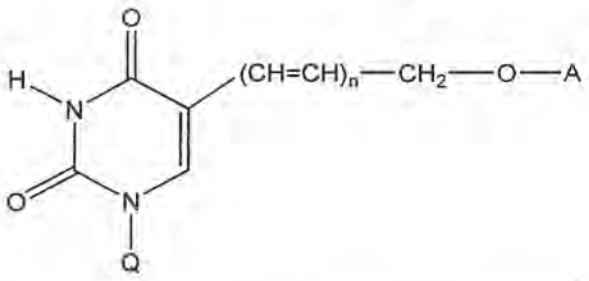
10. A method for treating a pathology characterized by hyperproliferative cells in a subject comprising administering to the subject a phosphoryl or phosphoramidate prodrug that is converted to a toxin in a hyperproliferative cell by an intracellular enzyme that is endogenously overexpressed or over-accumulated in the cell.

11. The method of any of claims 9 or 10, wherein the candidate therapeutic agent is an L- or D- compound of the formula:



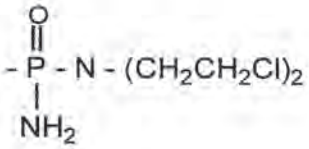
;

wherein R_1 is or contains a chemical entity that has a molecular dimension and electrophilicity compatible with extraction from the pyrimidine ring by thymidylate synthase, and which upon release from the pyrimidine ring by thymidylate synthase has the ability to inhibit the proliferation of the cell or kill the cell; or a compound of the formula:



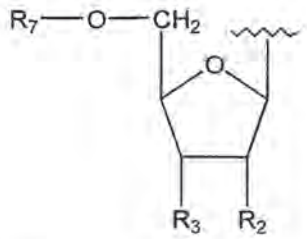
;

wherein n is an integer from 0 to 10; wherein A is a phosphoryl or phosphoramide derivative, or a compound of the formula:



wherein Q is a phosphoryl or phosphoramidate derivative containing a chemical entity selected from the group consisting of sugar groups, thio-sugar groups, carbocyclic groups, and derivatives thereof.

12. The method of claim 11, wherein Q has the formula:

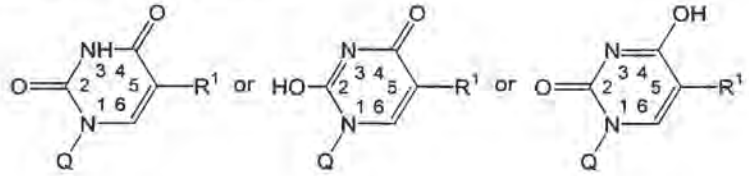


wherein R_7 is selected from the group consisting of phosphoryl, phosphoramidate and derivatives thereof, and wherein R_2 and R_3 are the same or different and are independently -H or -OH.

13. The method of claim 11, wherein R_1 is a halogen.

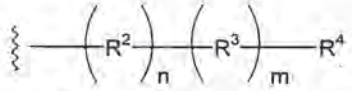
14. The method of claim 11, wherein R_1 is an alkyl group, i.e., $(-CH=CH)_n-R_4$, wherein n is an integer from 0 to 10, and R_4 is selected from the group consisting of H, a halogen, alkyl, alkene, alkyne, hydroxy, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, -S-heteroaryl, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, a cyanide, cyanate and thiocyanate cyanide, cyanate and thiocyanate halovinyl compound, a halomercuric compound, -NHOH, -NHO-alkyl, and $NHNH_2$.

15. A compound of the formula:



wherein:

R^1 is a moiety of the formula:



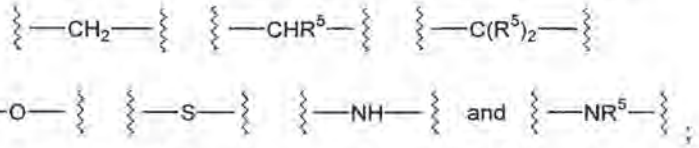
R^2 is a divalent electron conduit moiety selected from the group consisting of:

an unsaturated hydrocarbyl group;

an aromatic hydrocarbyl group comprising one or more unsaturated hydrocarbyl groups; and,

a heteroaromatic group comprising one or more unsaturated hydrocarbyl groups;

R^3 is a divalent spacer moiety selected from the group consisting of:

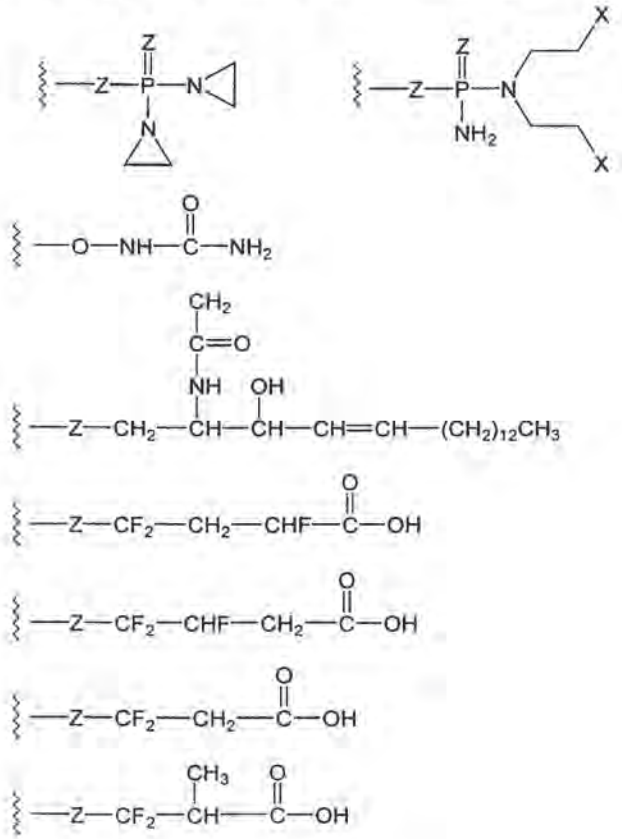


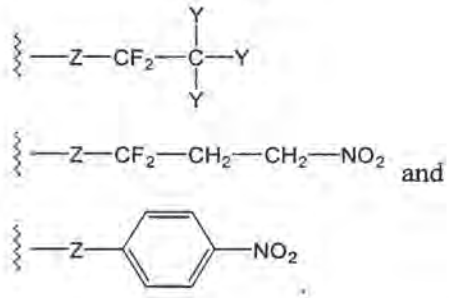
R^5 may be the same or different and is independently a linear or branched alkyl group having from 1 to 10 carbon atoms, or a cycloalkyl group having from 3 to 10 carbon atoms;

n is an integer from 0 to 10;

m is 0 or 1;

R^4 is a toxophore moiety selected from the group consisting of:





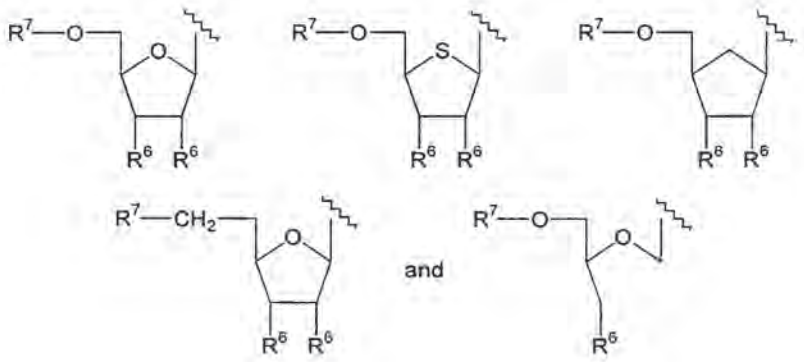
and

X is -Cl, -Br, -I, or other potent leaving group, with the provisio that when R^7 is -H, and M is zero, then R^4 is not a halogen or when m is zero and n is zero, then R^4 is not a halogen;

Y is independently -H or -F;

Z is independently -O- or -S-;

Q is a sugar moiety selected from the group consisting of:



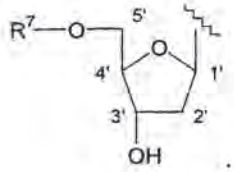
R^6 is independently -H, -OH, $-OC(=O)CH_3$, or other protected hydroxyl group; and,

R^7 is hydrogen, a phosphate group, or a phosphoramidate group;

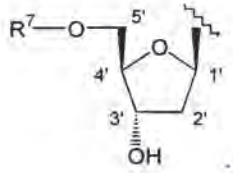
and wherein said compound may be in any enantiomeric, diasteriomeric, or stereoisomeric form, including, D-form, L-form, α-anomeric form, and β-anomeric form.

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16. A compound according to claim 15, wherein Q is:



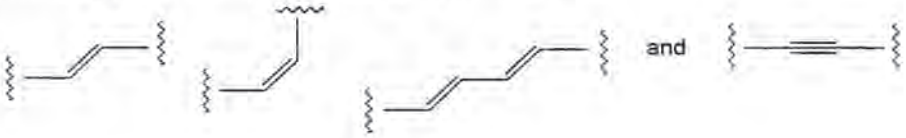
17. A compound according to claim 15, wherein Q is:



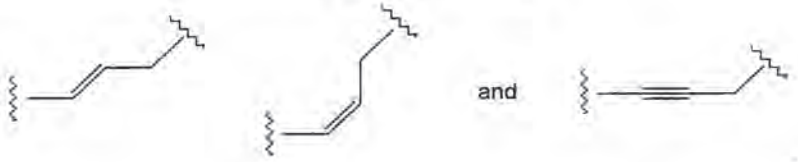
18. A compound according to any one of claims 15 to 17, wherein R^3 is a divalent spacer moiety selected from the group consisting of:



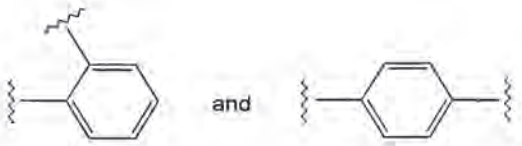
19. A compound according to any one of claims 15 to 17, wherein R^2 is an unsaturated hydrocarbyl group selected from the group consisting of:



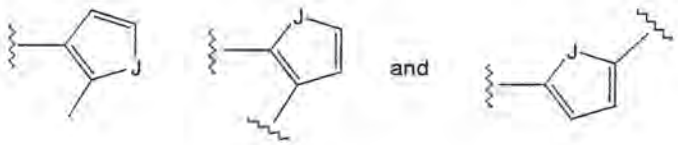
20. A compound according to any one of claims 15 to 17, wherein R^2 and R^3, taken together form a structure selected from the group consisting of:



21. A compound according to any one of claims 15 to 17, wherein R^2 is an aromatic hydrocarbyl group selected from the group consisting of:

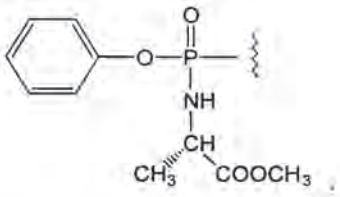


22. A compound according to any one of claims 15 to 17, wherein R^2 is a heteroaromatic group selected from the group consisting of:

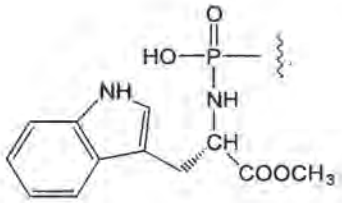


wherein J is -O-, -S-, -Se-, -NH-, or $-NR^{ALK}-$, wherein R^{ALK} is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

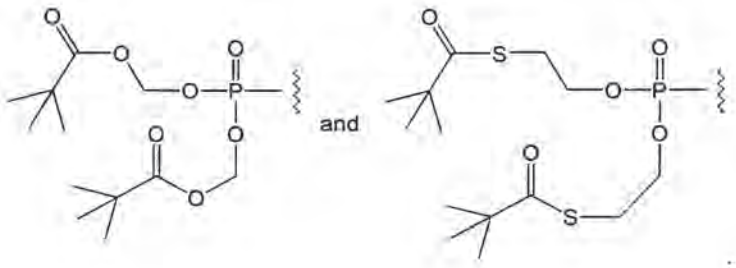
23. A compound according to any one of claims 15 to 22, wherein R^7 is selected from the group consisting of:



24. A compound according to any one of claims 15 to 22, wherein R^7 is selected from the group consisting of:

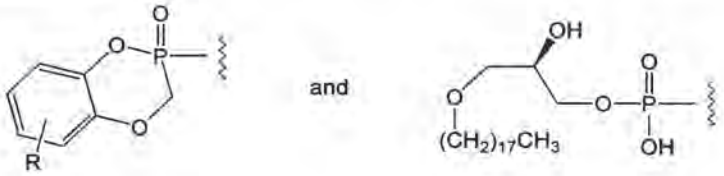


25. A compound according to any one of claims 15 to 22, wherein R^7 is selected from the group consisting of:



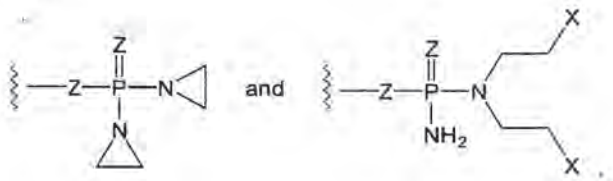
and

26. A compound according to any one of claims 15 to 22, wherein R^7 is selected from the group consisting of:



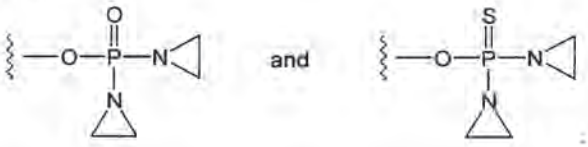
and

27. A compound according to any one of claims 15 to 22, wherein R^4 is selected from the group consisting of:



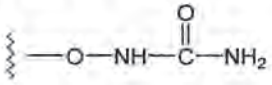
and

28. A compound according to any one of claims 15 to 27, wherein R^4 is selected from the group consisting of:

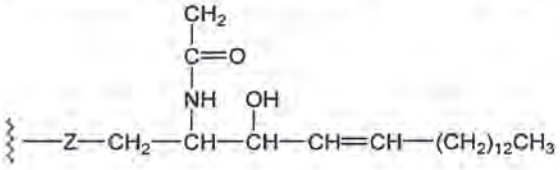


and

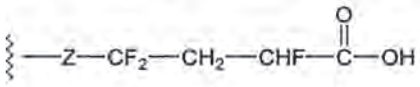
29. A compound according to any one of claims 15 to 27, wherein R^4 is:

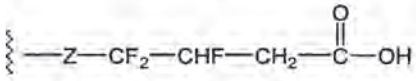


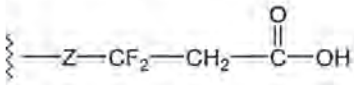
30. A compound according to any one of claims 15 to 27, wherein R^4 is:



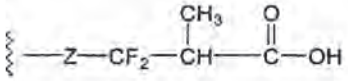
31. A compound according to any one of claims 15 to 27, wherein R^4 is:



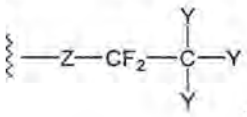




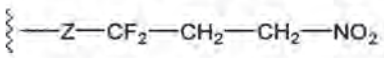
or



32. A compound according to any one of claims 15 to 27, wherein R^4 is:



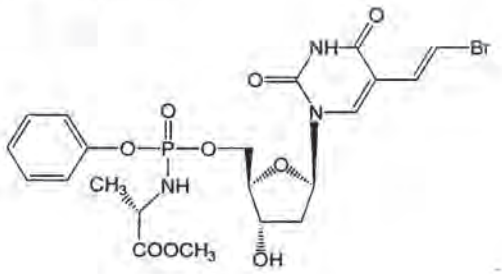
33. A compound according to any one of claims 15 to 27, wherein R^4 is:



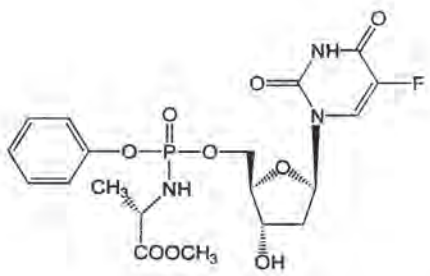
34. A compound according to any one of claims 15 to 27, wherein R^4 is:



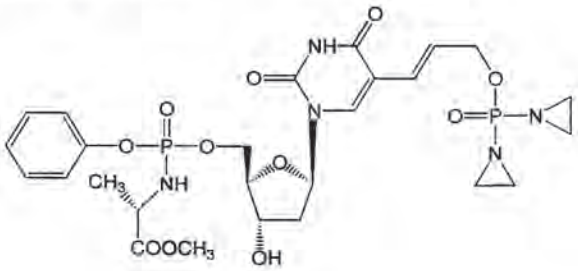
35. A compound of the formula:



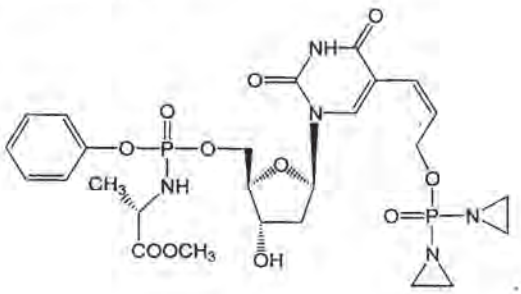
36. A compound of the formula:



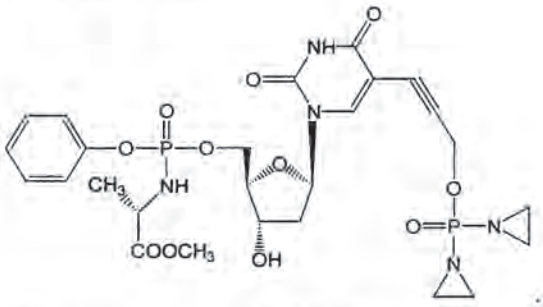
37. A compound of the formula:



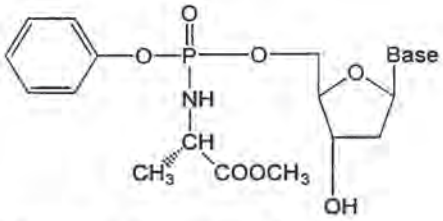
38. A compound of the formula:



39. A compound of the formula:

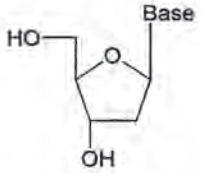


40. A method of forming a compound of the formula:

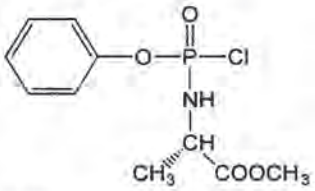


wherein "Base" denotes a nucleic acid base;

which method comprises the step of reacting a compound of the formula:

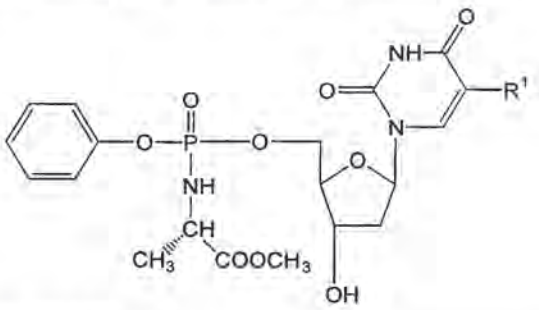


with a compound of the formula:



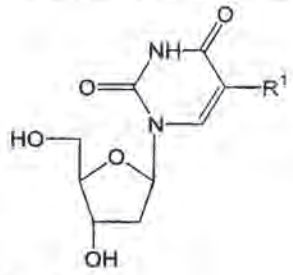
in the presence of an HCl scavenger.

41. A method of forming a compound of the formula:

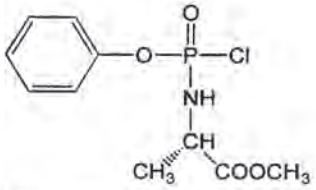


wherein R^1 is a substituent;

which method comprises the step of reacting a compound of the formula:



with a compound of the formula:



in the presence of an HCl scavenger.

42. The method according to claim 40 or 41, wherein said HCl scavenger is imidazole.

43. The method according to any one of claims 40 to 42, wherein said reaction is performed in a non-aqueous solvent comprising dimethylformamide.

44. A method for screening for a therapeutic agent, comprising:

(a) contacting a first target cell with a compound of any of claims 15 or 37 to 39, under conditions that favor the incorporation of the compound into the intracellular

compartment of the target cell and a second target cell with a potential therapeutic agent, under conditions that favor the incorporation of the compound into the intracellular compartment of the target cell; and

(b) assaying the second target cell for inhibition of cellular proliferation or cell killing.

45. The method of claim 44, wherein the target cell is characterized as resistant to a chemotherapeutic drug.

46. The method of claim 44, wherein the target cell is characterized as expressing a target enzyme that is amplified as a result of selection *in vivo* by chemotherapy.

47. The method of claim 46, wherein the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell.

48. The method of claim 47, wherein the endogeneous overexpression of an intracellular enzyme is the result of amplification of the gene coding for the enzyme.

49. The method of claim 47, wherein the enzyme is thymidylate synthase.

50. Use of a compound of any of any of claims 15 or 37 to 39, for the preparation of a medicament to treat inhibit the proliferation of a cell.

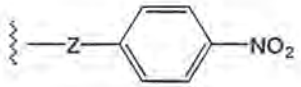
51. A method for inhibiting the proliferation of a hyperproliferative cell, comprising contacting the cell with an effective amount of a compound of any of claims 15 or 37 to 39.

52. The method of claim 66, wherein the hyperproliferative cell is characterized by the endogenous overexpression of an intracellular enzyme.

53. The method of claim 52, wherein the enzyme is thymidylate synthase.

54. A method for treating a pathology characterized by hyperproliferative cells in a subject comprising administering to the subject a compound of any of claims 15 or 37 to 39.

55. A method for screening for a therapeutic agent, comprising contacting a target cell with a compound of any of claims 15 or 37 to 39, wherein R^4 is:



which target cell favor the incorporation of the compound into the target cell, for the diagnostic purpose of detecting intracellular levels of thymidylate synthase.

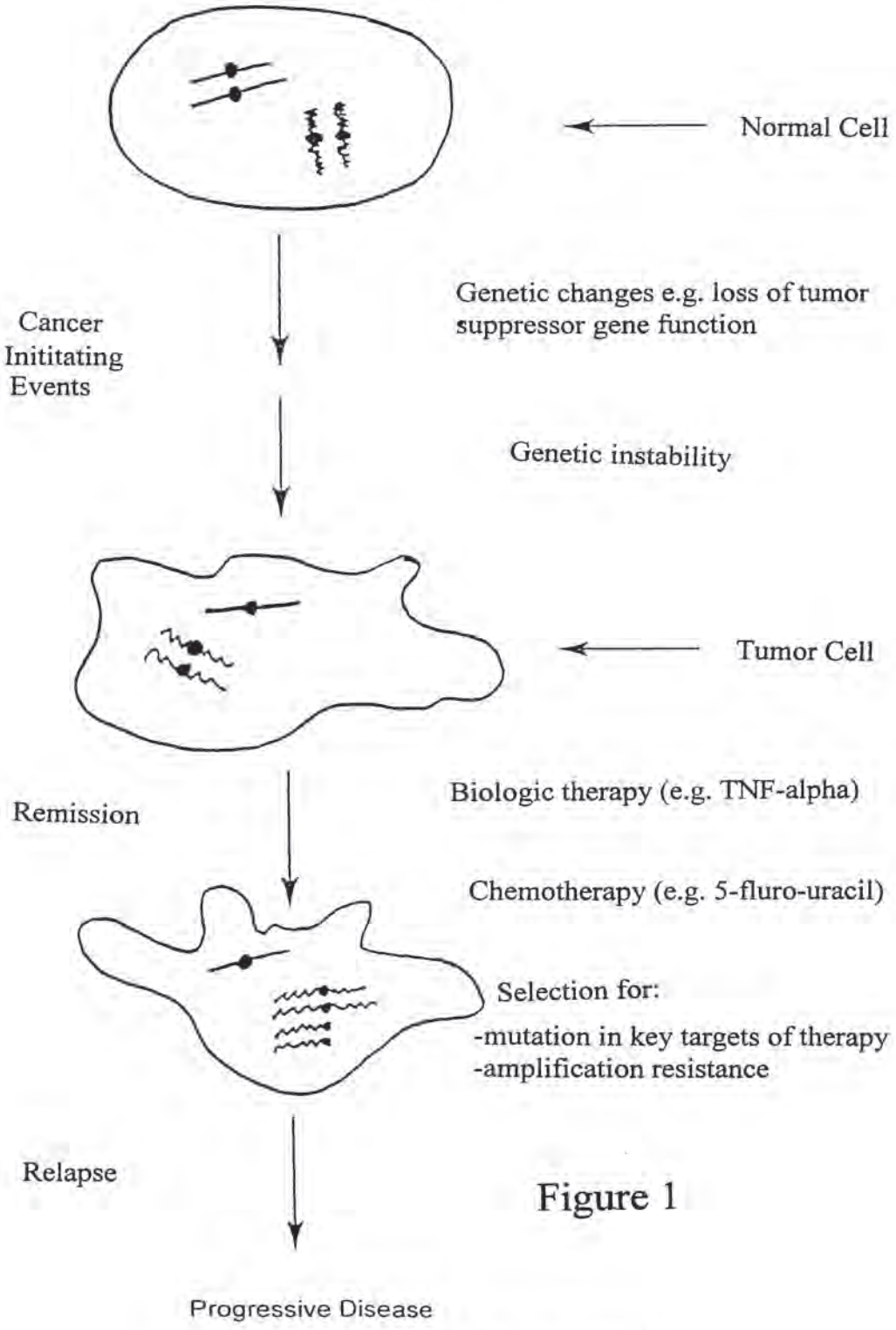


Figure 1

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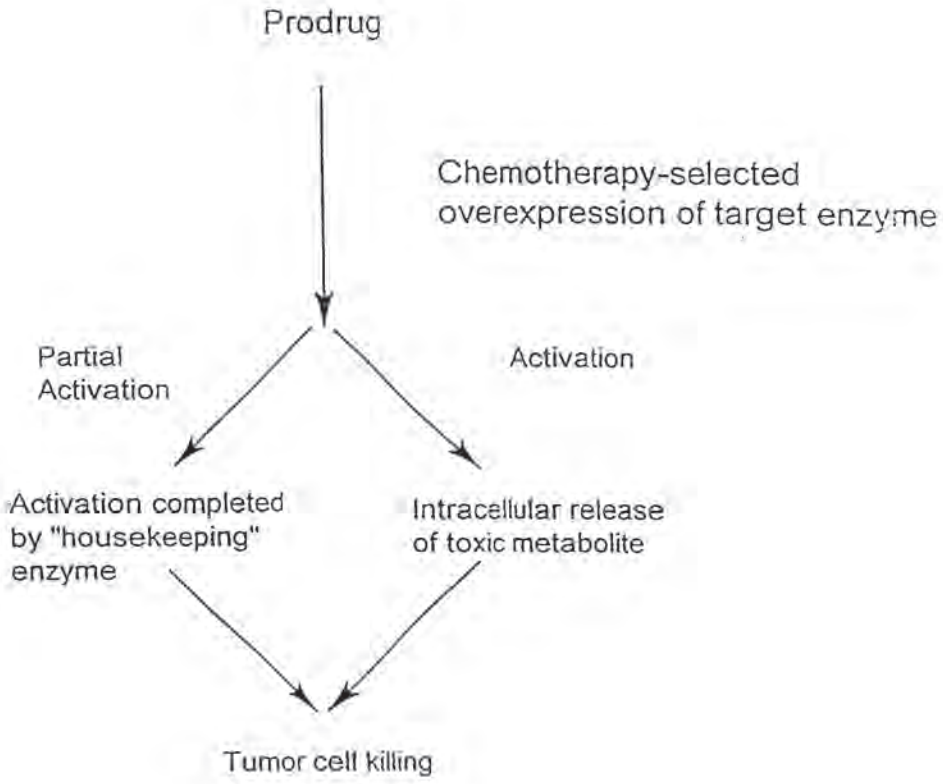


Figure 2

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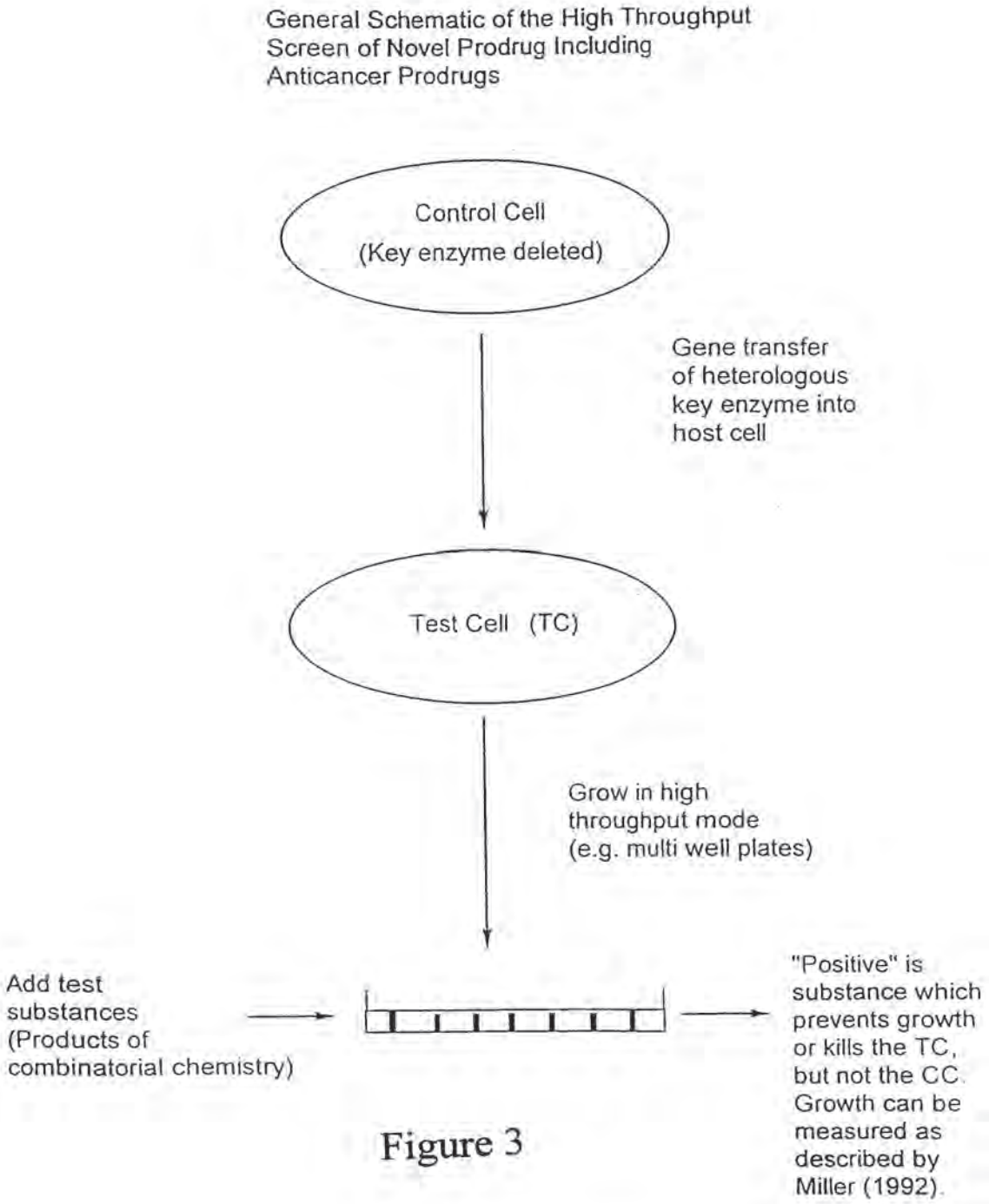


Figure 3

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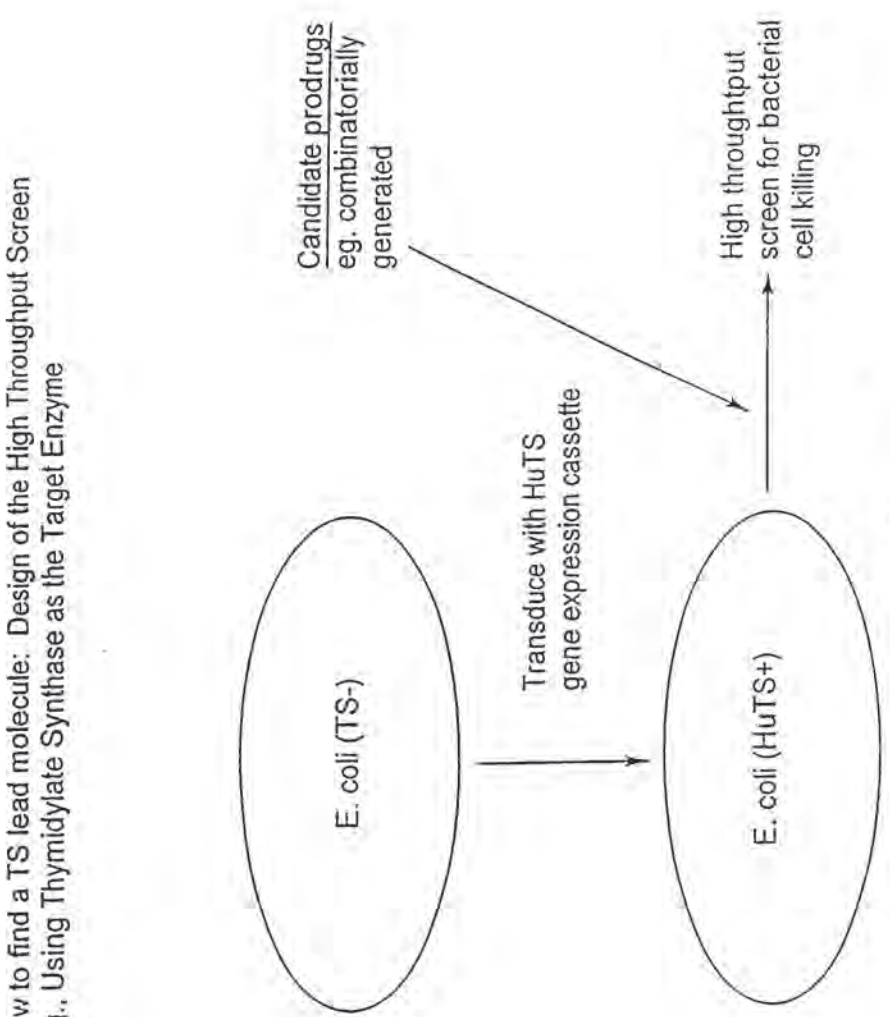


Figure 4

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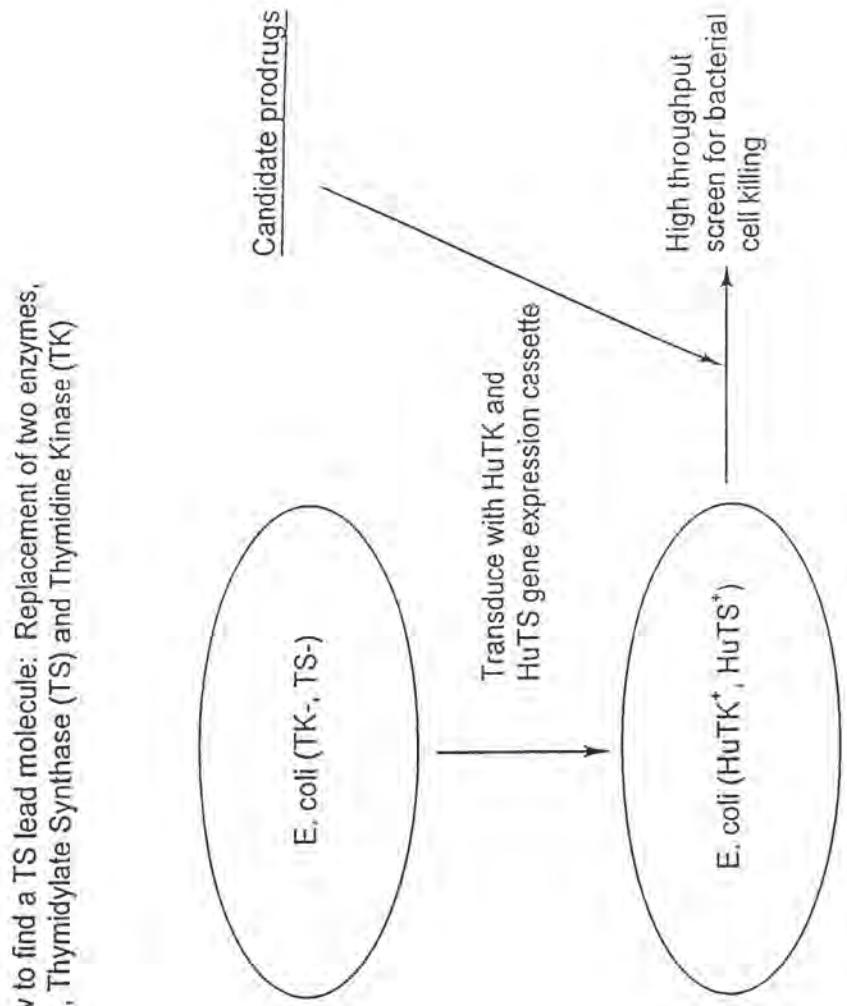


Figure 5

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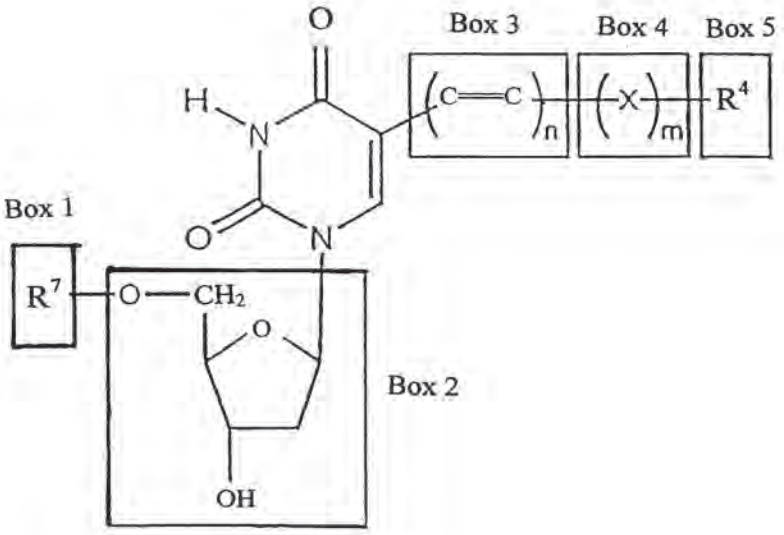


Figure 6

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Figure 7

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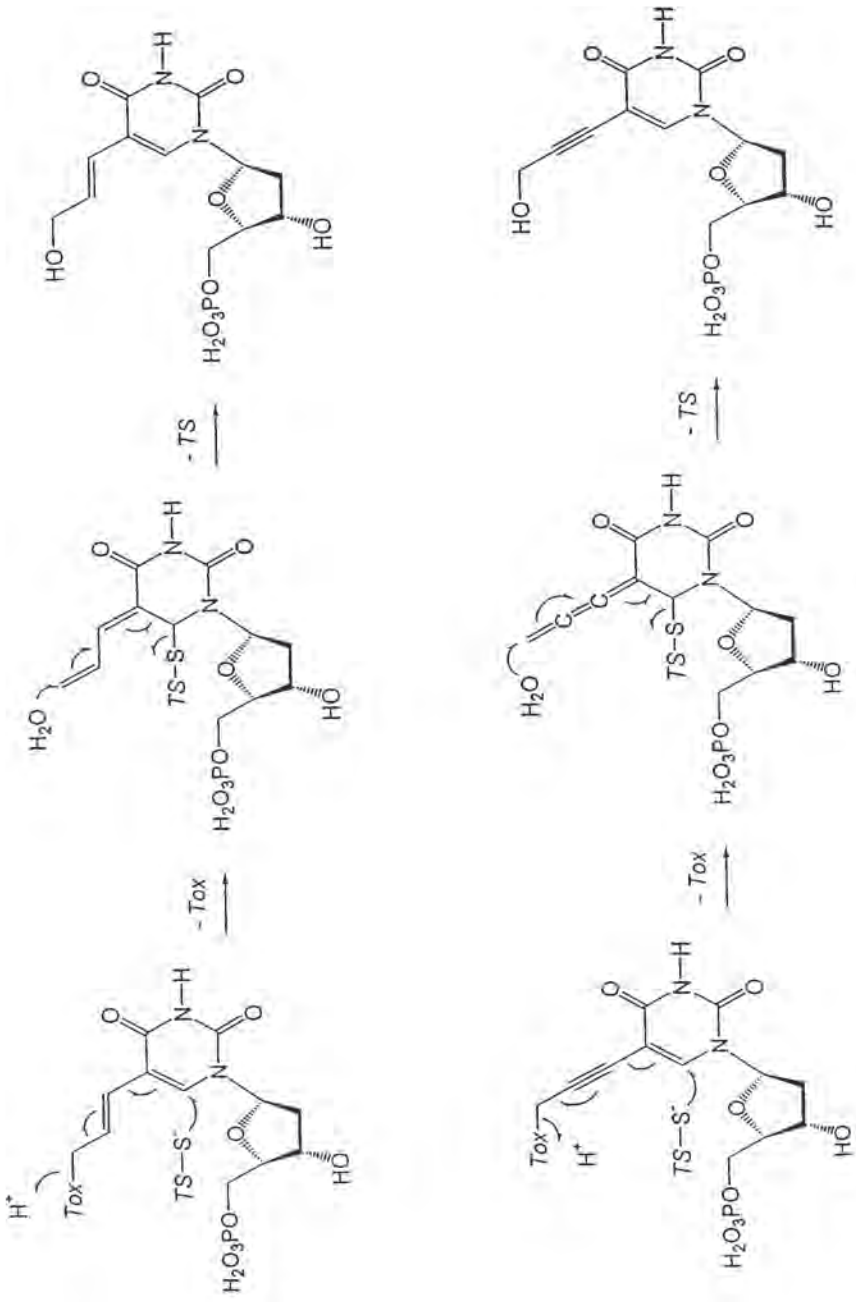


Figure 8

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number
WO 01/07454 A1

(51) International Patent Classification[7]: C07H 19/04, C12N 9/10, A61K 31/706, A61P 35/00

(21) International Application Number: PCT/US00/20008

(22) International Filing Date: 21 July 2000 (21.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/145,356	22 July 1999 (22.07.1999)	US
60/145,437	23 July 1999 (23.07.1999)	US
60/191,315	21 March 2000 (21.03.2000)	US

(71) Applicant *(for all designated States except US)*: **NEWBIOTICS, INC.** [US/US]; 11760-R Sorrento Valley Road, San Diego, CA 92121 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHEPARD, H., Michael** [US/US]; 16825 Via de Santa Fe, Rancho Santa Fe, CA 92067 (US). **CHAN, Ming, Fai** [US/US]; 1642 Orchard Wood Road, Encinitas, CA 92024 (US). **GROZIAK, Michael, P.** [US/US]; 153 Primrose Way, Palo Alto, CA 94303 (US).

(74) Agents: **KONSKI, Antoinette, F.** et al.; Baker & McKenzie, 660 Hansen Way, Palo Alto, CA 94304 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: ENZYME CATALYZED THERAPEUTIC ACTIVATION

(57) Abstract: This invention provides novel substrate compounds that selectively inhibit the proliferation of pathological cells, for example, pathological cells that endogenously overexpress a target enzyme that confers resistance to biologic and chemotherapeutic agents. The enzyme acts on a substrate compound to 1) convert it to a cellular toxin and/or 2) release a toxic byproduct. In one embodiment, the activity of the target enzyme has been greatly enhanced in a target cell as a result of loss of tumor suppressor function and/or selection resulting from previous exposure to chemotherapy. In another embodiment, the pathological cell contains a target enzyme that is an expression product of an infectious agent in the cell. Further provided by this invention is a method for treating a subject by delivering to the subject a prodrug as described herein. The prodrugs of this invention may be used alone or in combination with other chemotherapeutics or alternative anti-cancer therapies such as radiation.

ENZYME CATALYZED THERAPEUTIC ACTIVATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to the following U.S. provisional applications, U.S. Serial Nos.: 60/145,356; 60/145,437; and 60/191,315, filed July 22, 1999; July 23, 1999 and March 21, 2000, respectively, the contents of which are hereby incorporated by reference into the present disclosure.

TECHNICAL FIELD

The present invention relates to the field of drug discovery and specifically, the design of prodrugs that are substrates for endogenous intracellular enzymes that are overexpressed in pathological cells.

BACKGROUND OF THE INVENTION

Throughout and within this disclosure, various publications are referenced by first author and date, patent number or publication number. The full bibliographic citation for each reference can be found within the specification or at the end of this application, immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this disclosure to more fully describe the state of the art to which this invention pertains.

Cancer is one of the most commonly fatal human diseases worldwide. Treatment with anticancer drugs is an option of steadily increasing importance, especially for systemic malignancies or for metastatic cancers that have passed the state of surgical curability. Unfortunately, the subset of human cancer types that are amenable to curative treatment today is still rather small (Haskell, C.M. (1995)) resulting in about 600,000 deaths per year. See, Cancer Facts & Figures, 1999 American Cancer Society. Progress in the development of drugs that can cure human cancer is slow, with success limited to a few hematological malignancies and fewer solid tumor types (Dorr and Van Hoff (1994)). Progress in discovering therapies that

are based upon disease mechanism offers opportunities for future success. (Cobleigh, M.A. et al. (1999); and Roth, J.A. et al. (1999)).

The heterogeneity of malignant tumors with respect to their genetics, biology and biochemistry as well as primary or treatment-induced resistance to therapy mitigate against curative treatment. Moreover, many anticancer drugs display only a low degree of selectivity, causing often severe or even life threatening toxic side effects, thus preventing the application of doses high enough to kill all cancer cells. Searching for anti-neoplastic agents with improved selectivity to treatment-resistant pathological, malignant cells remains therefore a central task for drug development.

Cancer cells are characterized by uncontrolled growth, de-differentiation and genetic instability. The instability expresses itself as aberrant chromosome number, chromosome deletions, rearrangements, loss or duplication beyond the normal diploid number. (Wilson, J.D. et al. (1991)). This genomic instability may be caused by several factors. One of the best characterized is the enhanced genomic plasticity which occurs upon loss of tumor suppressor gene function (e.g., Almasan, A. et al. (1995a) and Almasan, A. et al. (1995b)). The genomic plasticity lends itself to adaptability of tumor cells to their changing environment, and may allow for the more frequent mutation, amplification of genes, and the formation of extrachromosomal elements (Smith, K.A. et al. (1995) and Wilson, J.D. et al. (1991)). These characteristics provide for mechanisms resulting in more aggressive malignancy because it allows the tumors to rapidly develop resistance to natural host defense mechanisms, biologic therapies (see, Wilson, J.D. et al. (1991) and Shepard, H.M. et al. (1988)), as well as to chemotherapeutics (see, Almasan, A. et al. (1995a); and Almasan, A. et al. (1995b)).

In addition, the clinical usefulness of a chemotherapeutic agent may be severely limited by the emergence of malignant cells resistant to that drug. A number of cellular mechanisms are probably involved in drug resistance, e.g., altered metabolism of the drugs, impermeability of the cell to the active compound or accelerated drug elimination from the cell, altered specificity of an inhibited enzyme, increased production of a target molecule, increased repair of cytotoxic lesions, or the bypassing of an inhibited reaction by alternative biochemical pathways. In some cases, resistance to one drug may confer resistance to other, biochemically distinct drugs. An alternative mechanism of resistance to cancer chemotherapeutics occurs *via* the functional loss of tumor suppressor genes. The best characterized of these are p53, RB and p16. (Funk,

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J.O. 1999; and Teh, B.T. (1999)). Loss of function of these gene products leads to depressed expression of enzymes commonly targeted by anti-cancer drugs (e.g., 5-fluorouridyl ("5FU")/thymidylate synthase and methotrexate/dihydrofolate reductase). (Lee, V. et al. (1997); Lenz, H.J.et al. (1998); and Fan, J. and Bertino, J. (1987)). Amplification of certain genes is involved in resistance to biologic and chemotherapy. Amplification of the gene encoding dihydrofolate reductase is related to resistance to methotrexate, while overexpression/amplification of the gene encoding thymidylate synthase is related to resistance to treatment with 5-fluoropyrimidines. (Smith, K.A. et al. (1995)). Table 1 summarizes some prominent enzymes in resistance to biologic and chemotherapy.

Table 1

Enzymes Overexpressed in Resistance to Cancer Chemotherapy

Enzyme	Biologic or Chemotherapy	Referenced (Examples)
Thymidylate synthase	Uracil-based Folate-based Quinazoline-based	Lönn, U. et al. (1996) Kobayashi, H. et al. (1995) Jackman, A.L. et al. (1995a)
Dihydrofolate reductase	Folate-based	Banerjee, D. et al. (1995) Bertino, J.R. et al. (1996)
Tyrosine kinases	TNF-alpha Multidrug resistance	Hudziak, R.M. et al. (1988) Stühlinger, M. et al. (1994)
MDR-associated proteins (ABC P-gp proteins)	Multidrug resistance	Simon, S.M. and Schindler, M. (1994) Gottesman, M.M. et al. (1995)
CAD*	PALLA**	Smith, K.A. et. Al. (1995) Dorr, R.T. and Von Hoff, D.D., eds. (1994)
Topoisomerase I (Colon & Prostate Cancers)	Camptothecin	Husain et al. (1994)
Ribonucleotide reductase	Hydroxyurea	Wettergren, Y. et al. (1994) Yen, Y. et al. (1994)

* CAD = carbamyl-P synthase, aspartate transcarbamylase, dihydroorotase
** PALA = N-(phosphonacetyl)-L-aspartate

The poor selectivity of anticancer agents has been recognized for a long time and attempts to improve selectivity and allow greater doses to be administered have been numerous. One approach has been the development of prodrugs. Prodrugs are compounds that are toxicologically benign but which may be converted *in vivo* to therapeutically active products. In some cases, the activation occurs through the action of a non-endogenous enzyme delivered to the target cell by antibody ("ADEPT" or antibody-dependent enzyme prodrug therapy (U.S. Patent No.4,975,278)) or gene

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targeting ("GDEPT" or gene dependent enzyme-prodrug therapy (Melton, R.G. and Sherwood, R.E. (1996)). These technologies have severe limitations with respect to their ability to exit the blood and penetrate tumors. See, Connors, T.A. and Knox, R.J. (1995).

A number of nucleotide and nucleoside analogs have been developed and tested as both anti-tumor and anti-viral agents. For example 5-flurouracil (5FU) and 5-fluoro-deoxyuridine (5FUdR) have been widely used as chemotherapeutic agents based on their ability to be converted intracellularly into inhibitors of the thymidylate synthase (TS) enzyme. As with many other enzyme inhibitory chemotherapeutic agents, cancer therapy using 5FU frequently leads to selection for aggressive drug resistant tumor cells which are refractory to further treatment with this drug. (Aschele, C. et al. (1994); Mader, R.M. et al. (1998); Lönn, U. et al. (1996); Paradiso, A. et al. (2000); and Edler, D. et al. (2000)).

Particular attention has also been paid to halogenated nucleoside analogs such as (E)-5-(2-Bromovinyl)-2'-deoxyuridine (BVdU). These types of compounds were originally developed as anti-viral agents based on the observation that they required phosphorylation to the monophosphate nucleotide form in order to elicit cytotoxic responses and this phosphorylation was preferentially accomplished by herpes virus thymidine kinase (TK) enzymes. BVdU and related compounds have consistently shown limited toxicity to normal mammalian cells, while they have been effective at killing virally infected cells. (DeClerq, E. et al. (1997)).

Throughout the extensive testing of nucleoside analogs such as BVDU as anti-viral and anti-cancer agents, they have consistently been reported to have minimal toxicity to both normal and cancer cells. These observations have been supported by results demonstrating the preferential phosphorylation of such compounds by viral thymidine kinase enzymes, which explains the low toxicity of these compounds for mammalian cells. Thus, BVdU and related nucleoside analogs have not been developed as anti-cancer therapeutic agents. (De Clerq, E. et al. (1997)).

DISCLOSURE OF THE INVENTION

This invention provides novel compounds that selectively inhibit the proliferation of pathological cells, for example, pathological cells that endogenously overexpress a target enzyme that confers resistance to biologic and chemotherapeutic agents. The

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enzyme acts on a prodrug compound of this invention to 1) convert it to a cellular toxin and/or 2) release a toxic byproduct. In another aspect of this invention, the product of an initial reaction with a resistance enzyme, is subsequently fully activated by a common cellular enzyme such as an acylase, phosphatase or other "housekeeping" enzyme (Voet, et al. (1995)) or common cellular constituent (e.g., water) to release the toxic byproduct from the prodrug.

In one embodiment, the activity of the target enzyme has been greatly enhanced in a target cell as a result of loss of tumor suppressor function and/or selection resulting from previous exposure to chemotherapy. In another embodiment, the pathological cell contains a target enzyme that is an expression product of an infectious agent in the cell. The expression product can confer antibiotic resistance.

Another aspect of this invention includes assays for screening for new prodrugs that can be activated by target enzymes. Kits to perform such assays containing the reagents and instructions necessary to complete the assay and analyze the results are also provided by this invention.

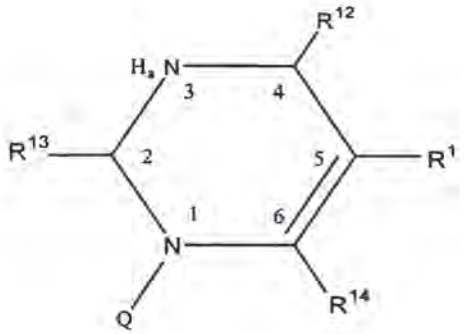
Further provided by this invention is a method for treating a subject by delivering to the subject a prodrug as described herein. The prodrugs of this invention may be used alone or in combination with other chemotherapeutics or alternative anti-cancer therapies such as radiation.

A further aspect of this invention is the preparation of a medicament for use in treating a subject suffering from a pathology characterized by cells expressing a target enzyme.

A still further aspect of this invention is a method for identifying the optimal therapeutic for a subject, by isolating cells expressing a target enzyme and contacting the cells with at least one of the prodrugs of this invention, and then identifying which of the one or more prodrugs inhibits the proliferation or kills the cells, thereby identifying the optimal therapeutic for the subject.

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The prodrugs of this invention have the following structure:



or tautomers thereof;

wherein R^{12} or R^{13} may be the same or different and are selected from the group consisting of oxo, OH or $NHNH_2$;

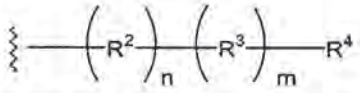
wherein if a is 0 or 1, providing that if a is 0 and R^{13} is oxo, then a double bond exits between position 3 and 4 and R^{12} is $NHNH_2$ or

if a is 0 and R^{12} is oxo, then a double bond exists between position 2 and 3 and R^{13} is $NHNH_2$; or

if a is 1, then R^{12} and R^{13} are both oxo;

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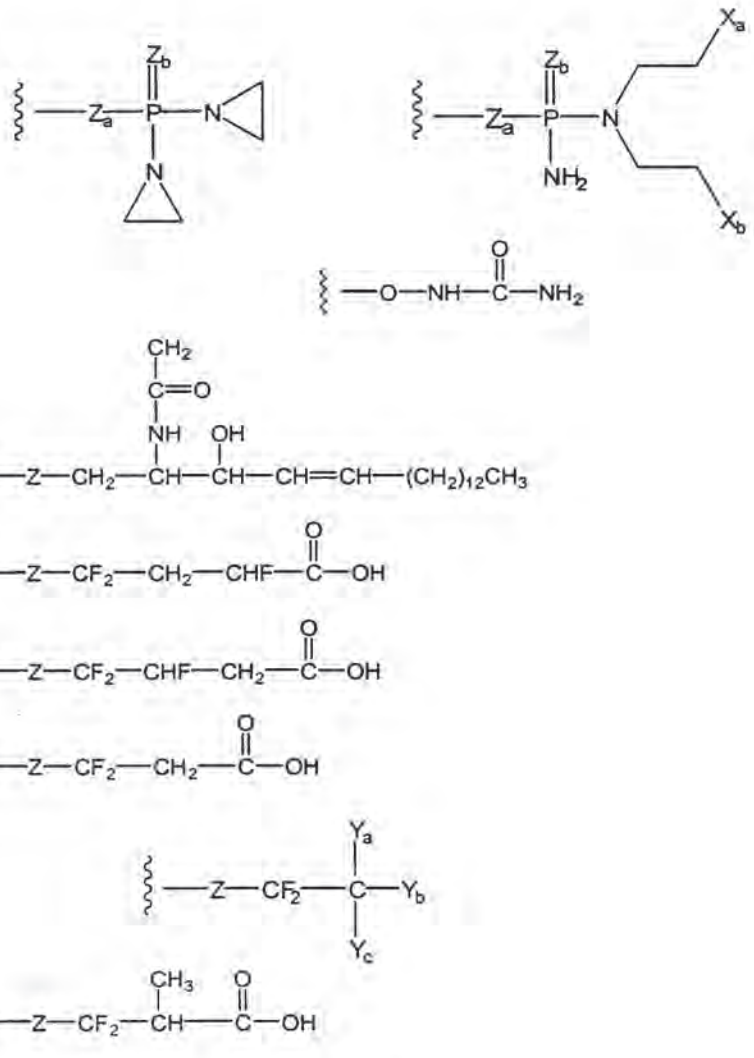
wherein R^1 is a substituent having the formula:



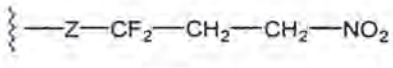
wherein R^2 and R^3 are independently an unsaturated or a saturated hydrocarbyl group;

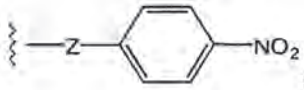
wherein n is 0 or an integer from 1 to 10 and m is 0 or 1; and

wherein R^4 is selected from the group consisting of F, Cl, Br, I, CN, SO_3H, CO_2H, $CO_2CH_2CH_3$, CO_2CH_3, $SI(CH_3)_3$, CHO, NO_2, CF_3, CCl_3, $CH=C(R^{15})_2$ or a substituent having the structure:



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and


;

wherein X_a and X_b are independently the same or different and are selected from the group consisting of Cl, Br, I, and a potent leaving group;

wherein Y_a, Y_b or Y_c are independently the same or different and are H or F;

wherein Z, Z_a and Z_b are independently the same or different and are O or S; and

wherein R^{14} is H or F, providing if R^{14} is F, then a is 1 and R^{12} and R^{13} are both oxo; and

wherein Q is selected from the group consisting of a sugar, a carbocyclic, and an acyclic compound, or a masked phosphate derivative or phosphoramidate derivative thereof.

BRIEF DESCRIPTION OF THE FIGURES

The concepts described herein are displayed graphically in Figure 1A and 1B using the example of the thymidylate synthase enzyme and the compound NB1011™. Figure 1A shows how higher levels of TS in tumor cells can lead to preferential generation of toxin. Figure 1B shows the conversion of NB1011™ to BVdUMP, and subsequent interaction with TS to generate nucleotide toxin.

Figures 2A and 2B are flow charts illustrating a high throughput screen of this invention.

Figure 3A shows relative TS levels in various human tissues. Figure 3B shows a comparison of average TS mRNA levels between normal and human colon tumor tissues.

Figure 4 shows the synthetic scheme for the furo[2,3-d]pyrimidinone nucleosides of this invention. The synthetic protocol is described in detail, *infra*.

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MODES FOR CARRYING OUT THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, organic chemistry, medicinal chemistry and recombinant DNA, which are within the skill of the art. See, *e.g.*, Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, F.M. Ausubel et al. eds., (1987); the series METHODS IN ENZYMOLOGY, Academic Press, Inc.; PCR 2: A PRACTICAL APPROACH, M.J. MacPherson et al., eds. (1995); Spector, D.L. et al. (1998) CELLS: A LABORATORY MANUAL, Vols I to III, Cold Spring Harbor Press; and ANIMAL CELL CULTURE, R.I. Freshney, ed. (1987).

As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

The term "overexpression" shall mean at least 2 fold, preferably 3 fold, and more preferably 4 fold and most preferably 5 fold or more expression over normal levels or levels measured from normal or non-pathological cells.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

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A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

"Target" or "pathological" cells include hyperproliferative cells that are de-differentiated, immortalized, neoplastic, malignant, metastatic or transformed. Examples include, but are not limited to cancer cells such as sarcoma cells, leukemia cells, carcinoma cells, or adenocarcinoma cells. Specified cancers include, but are not limited to breast cancer cells, hepatoma cells, liver cancer cells, pancreatic carcinoma cells, oesophageal carcinoma cells, bladder cancer cells, gastrointestinal cancer cells, ovarian cancer cells, skin cancer cells, prostate cancer cells, and gastric cancer cells.

Target or pathological cells overexpress an intracellular enzyme that is related to any of a loss of tumor suppressor gene product function, drug resistance or genetic instability. Alternatively, resistance to one drug may confer resistance to other, biochemically distinct drugs. Unlike prior art therapies directed to creating more potent inhibitors of endogenous, intracellular enzymes, this invention exploits the higher enzyme activity associated with therapy-resistant diseased cells and tissues versus normal cells and tissues and does not rely on inhibiting the enzyme. The term "target enzyme" is used herein to define enzymes having one or more of the above noted characteristics.

Gene products activated or overexpressed and related to drug resistance include, but are not limited to thymidylate synthase (TS) (Lönn, U. et al. (1996); Kobayashi, H. et al. (1995); and Jackman, A. L. et al. (1995b)), dihydrofolate reductase (Banerjee, D. et al. (1995) and Bertino, J. R. et al. (1996)), tyrosine kinases (TNF-α, Hudziak, R. M. et al. (1988)) and multidrug resistance (Stühlinger, M. et al. (1994)); Akdas, A. et al.

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(1996); and (Tannock, I.F. (1996)); and ATP-dependent multidrug resistance associated proteins (Simon, S.M. and Schindler, M. (1994)) and, in some diseases including colon and prostate cancer, topoisomerase I (Husain et al. (1994)).

Amplification of dihydrofolate reductase (DHFR) is related to resistance to methotrexate while amplification of the gene encoding thymidylate synthase is related to resistance to tumor treatment with 5-fluoropyrimidine. Amplification of genes associated with drug resistance can be detected and monitored by a modified polymerase chain reaction (PCR) as described in Kashini-Sabet, et al. (1988), U.S.Patent No. 5,085,983, or the method described herein. Acquired drug resistance can be monitored by the detection of cytogenetic abnormalities, such as homogeneous chromosome staining regions and double minute chromosomes both of which are associated with gene amplification. Alternative assays include direct or indirect enzyme activity assays and each of which are associated with gene amplification (e.g., Carreras and Santi (1995)); other methodologies (e.g. polymerase chain reaction, Houze, T. A. et al. (1997) or immunohistochemistry (Johnson, P. G. et al. (1997)).

The enzyme glutathione-S-transferase has been shown to be occasionally elevated in some human tumors (Morgan, A. S. et al. (1998)), but nevertheless is excluded from "target enzyme" as used herein because it is a member of a gene family encoding enzymes with overlapping specificities.

In sum, the prodrugs of the subject invention are distinguishable from conventional therapeutics on the basis that the target enzymes of this invention are commonly overexpressed, over-accumulated or activated in pathological cells versus normal cells. The most important principles that distinguishes the current invention from other approaches are:

(1) This invention describes the synthesis of substrates for enzymes like thymidylate synthase. The overexpressed enzyme will convert prodrugs to toxin, preferentially in diseased cells. Previous approaches have mostly relied on inhibitors of these enzymes. The inhibitors lead to amplified expression of the enzyme, and subsequent resistance to treatment (see, e.g., Lönn, U. et al. (1996); Paradiso, A. et al. (2000); and Edler, D. (2000)).

(2) The current approach is also distinguishable from other "substrate-prodrug" approaches, e.g., the glutathione-S-transferase enzymes (see, e.g., Morgan, A.S. et al. (1998)). The enzymes of the GST family are expressed at increased levels in

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response to toxic insult to the cell. The GST family of enzymes has overlapping substrate specificities, which makes it difficult to design a substrate reactive with only a single species of enzyme with elevated expression in a cancer cell (Morgan, A.S. et al. (1998)). Because the target enzymes of the current invention (e.g., thymidylate synthase, dihydrofolate reductase and thymidine kinase) are unique with respect to its structure and substrate specificity, it is facile to design unique substrates. Several examples of substrates for thymidylate synthase are provided infra.

(3) In some cases the gene encoding the target enzyme (e.g., thymidylate synthase) may have undergone mutation to give resistance to inhibitors, (Barbour, K.W. et al. (1992) and Dicken, A.P. et al. (1993)) but will still be capable of carrying out reaction with non-inhibitor substrate prodrugs.

(4) An advantage of this approach is that loss of tumor suppressor function is critical to development of malignancy. The majority of tumor cells have lost one of the p53, RB or p16 tumor suppressor functions. (Funk, J.O. (1999); Banerjee, D. (1998); and Teh, B.T. et al. (1999)). Such a loss results in increased expression of resistance enzymes (*e.g.*, thymidylate synthase), independent of previous exposure to chemotherapy. The prodrugs described herein will be useful in treating early stages of malignancy, as well as disease previously treated with chemotherapy. Substrates for enzymes like GST include many compounds unrelated to chemotherapy. (Whalen and Boyer (1998)).

The concepts described above are displayed graphically in Figure 1A and 1B using the example of the thymidylate synthase enzyme and the compound NB1011™.

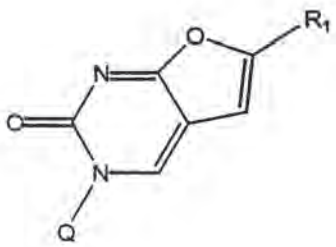
In an alternative embodiment, the target cell is defined by its resistance to a drug or compound unrelated to cancer. In this case, the cell is infected with a microorganism that confers resistance to an antibiotic, e.g. beta-lactamase. Organisms include bacteria, yeast and parasites, such as trypanosomes. Table 2 provides a list of enzymes targeted by this approach in infectious disease.

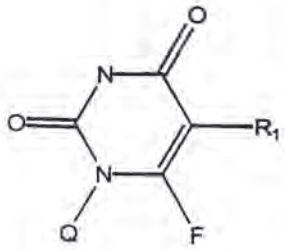
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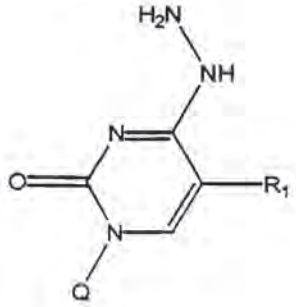
Table 2 Enzymes Overexpressed in Infectious Disease, and which Contribute to Drug Resistance	
Enzyme	Provides increased Resistance to:
Beta-lactamases	Penicillin and other beta-lactam containing antibiotics
Aminoglycosidase, or aminoglycoside midifying enzymes	Aminoglycoside antibiotics (e.g., streptomycin, gentamycin)
Chloramphenicol transacetylase	Chloramphenicol
Dihydrofolate reductase	Trimethoprim
Reference: Mechanisms of Microbial Disease, 2nd Ed., M. Schaechter, G. Medloff, B.I. Eisenstein, Editor TS Satterfield. Publ. Williams and Wilkins, p. 973 (1993).	

The prodrugs of this invention are selected from the group consisting of the L and D isomers of compounds having the structures:

I. or II. or III.

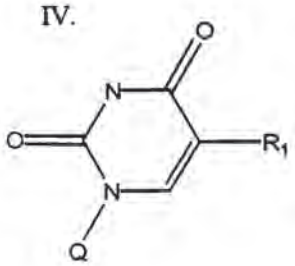






or

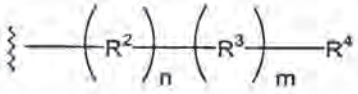
IV.



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wherein:

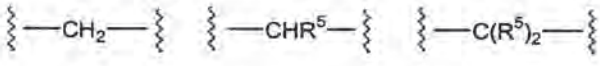
R^1 is a moiety of the formula:

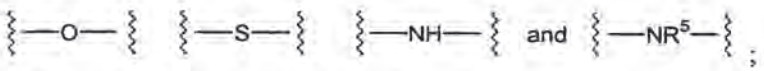


with the proviso that in compound I, n can be 0.

R^2 is a divalent electron conduit moiety selected from the group consisting of an unsaturated hydrocarbyl group; an aromatic hydrocarbyl group comprising one or more unsaturated hydrocarbyl groups; and, a heteroaromatic group comprising one or more unsaturated hydrocarbyl groups;

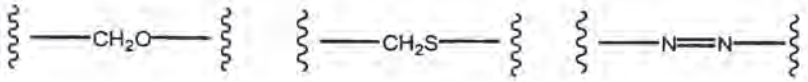
R^3 is selected from the group consisting of:

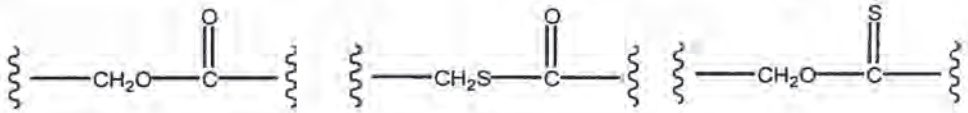


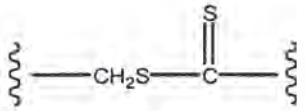


R^5 may be the same or different and is independently a linear or branched alkyl group having from 1 to 10 carbon atoms, or a cycloalkyl group having from 3 to 10 carbon atoms, or a halogen (e.g., F, Cl, Br, I);

or the group:

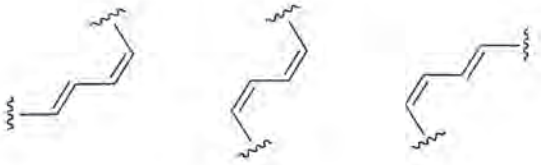






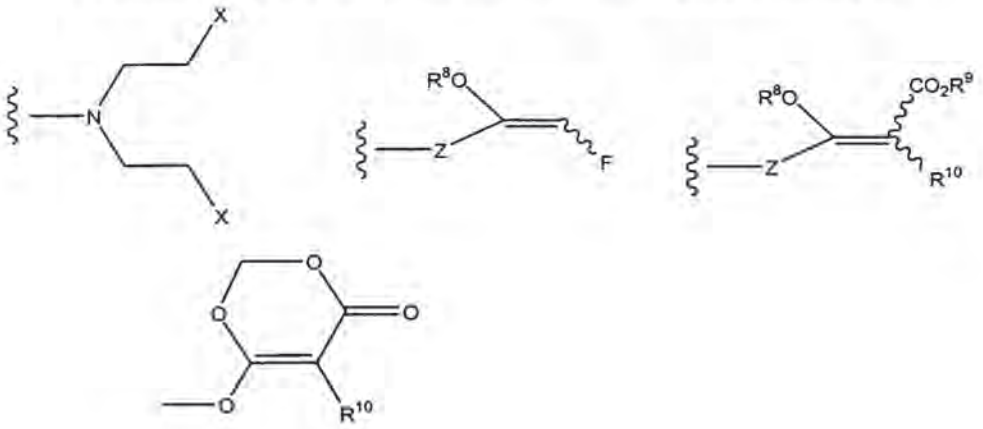
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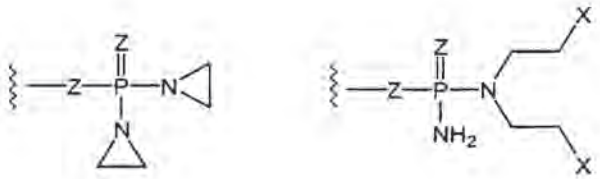
or the group:

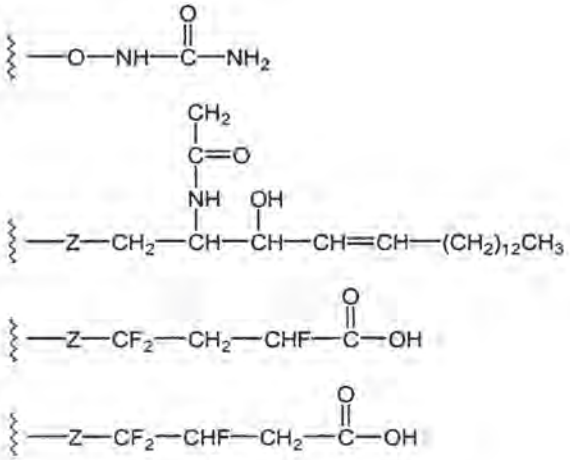


wherein n is 0 or an integer from 1 to 10 and m is 0 or 1;

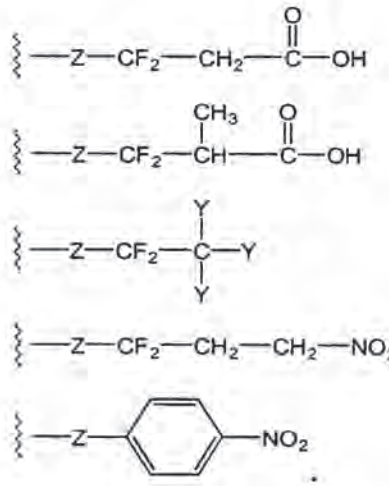
wherein R^4 can be a substituent selected from the group consisting of







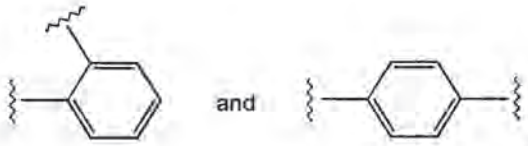
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wherein R^8 and R^9 are lower alkyls and R^{10} is H or CH_3 and each X substituent is independently the same or different from the other and is -Cl, -Br, -I, or other potent leaving group, with the proviso that when R^7 is -H, and m is zero, then R^4 is not a halogen or when m is zero and n is zero, then R^4 is not a halogen;

wherein each Y substituent is independently the same or different from the other and is independently -H or –F and each Z substituent is independently the same or different from the other and is -O- or -S-.

In one aspect, m is 0 and R^2 is an aromatic hydrocarbyl group selected from the group consisting of:



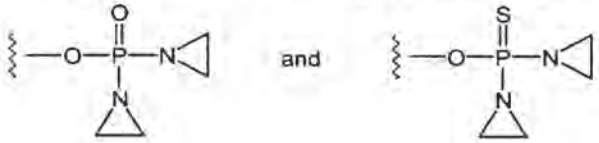
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In an alternative aspect, m is 0 and R^2 is a heteroaromatic group selected from the group consisting of:

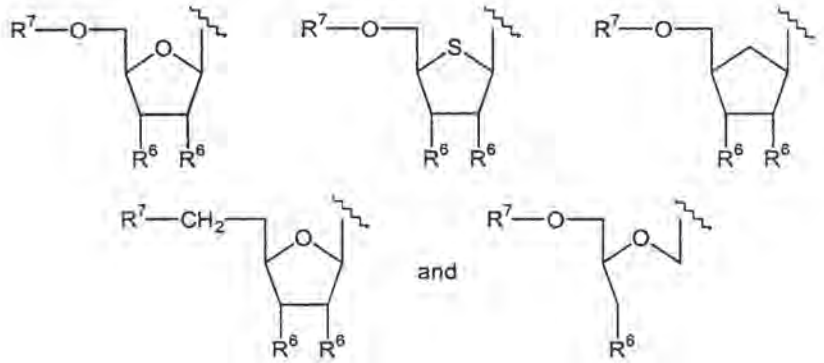


wherein J is selected from the group consisting of -O-, -S-, -Se-, -NH-, and -NR^{ALK}- and wherein R^{ALK} is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

In one specific aspect, R^4 is selected from the group consisting of:



Q is a selected from the group consisting of a sugar, a carbocylic, an acyclic compound and masked phosphate or phosphoramidate derivatives thereof. The masked phosphate or phosphoramidate derivation is attached to Q at the 5' position of the compound, shown in more detail below. For example, Q is a moiety selected from the group consisting of:



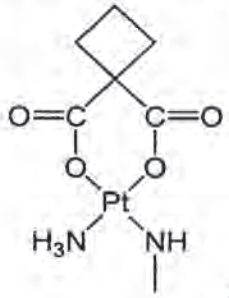
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wherein each R^6 is independently the same or different from the other and is selected from the group consisting of -H, -OH, $-OC(=O)CH_3$, F, and other protected hydroxyl group; and,

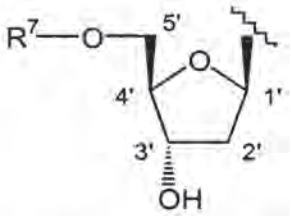
R^7 is hydrogen, a phosphate group, a phosphodiester group, or a phosphoramidate group attached to Q at the 5' position.

It should be understood, although not explicitely stated that any of the prodrugs of this invention may be in any enantiomeric, diasteriomeric, or stereoisomeric form, including, D-form, L-form, α-anomeric form, and β-anomeric form.

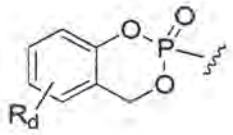
In one embodiment, R^4 is or contains a chemical entity selected from the group consisting of: -Br, -I, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, -S-heteroaryl, -CN, -OCN, -SCN, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, -NHOH, -NHO-alkyl, $NH_2CONHO-$, $NHNH_2$, $-N_3$, and a derivative of cis-platin, such as:



In one embodiment, Q is:

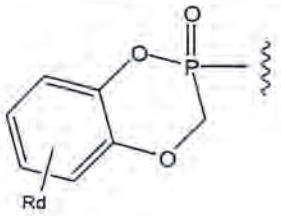


wherein R^7 is as described above, or more specifically, a structure such as:

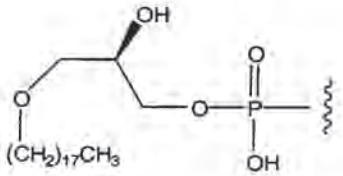


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or a compound having the structure:

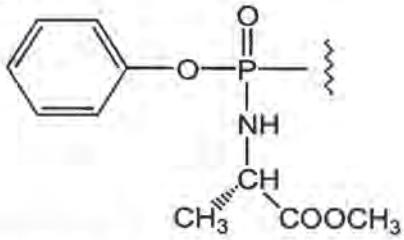


and



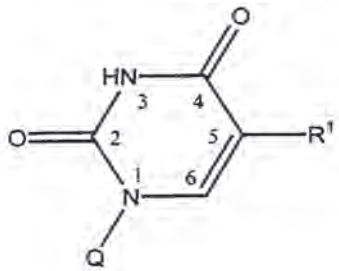
wherein R_d is an aromatic substituent.

In an alternative embodiment, R^7 is a phosphoramidate group derived from an amino acid, including, for example, the twenty naturally occurring amino acids. In one embodiment, R^7 is a phosphoramidate group derived from alanine. In one embodiment, R^7 is or contains a group having the structure:



The above group, and methods for its preparation, are described in McGuigan, et al. (1993), and McGuigan, et al. (1996).

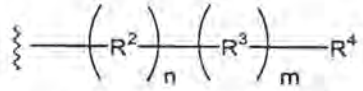
This invention also provides an L-or D- isomer of the compound having the structure:



or tautomers thereof,

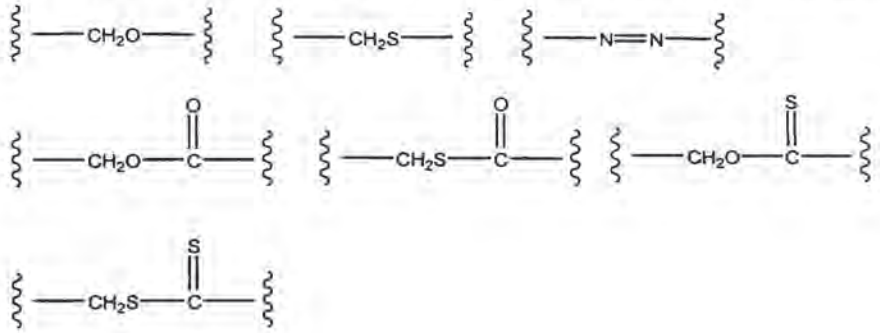
wherein R^1 is a substitutent having the formula:

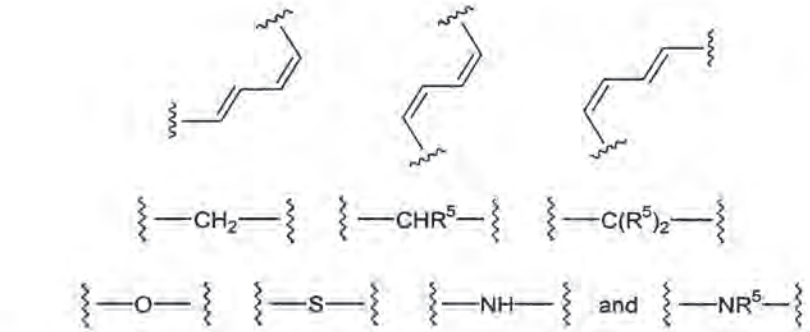
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wherein R^2 and R^3 are the same or different and are independently an unsaturated or a saturated hydrocarbyl group; wherein R^4 is selected from the group consisting of F, Cl, Br, I, CN, SO_3H, CO_2H, $CO_2CH_2CH_3$, $SI(CH_3)_3$, CHO, NO_2, CF_3, CCl_3, $C(R^5)_2$; wherein R^5 is selected from the group consisting of F, Cl, Br, and I; wherein n is 0 or an integer from 1 to 10 and m is 0 or 1; and Q is as defined above.

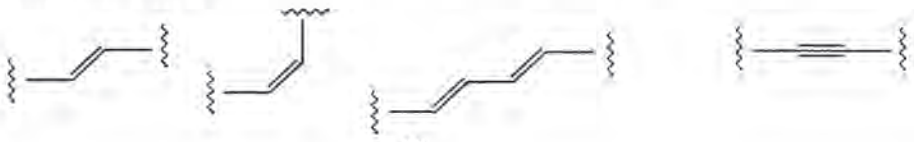
In one aspect, m is 0 and R^2 is selected from the group consisting of:

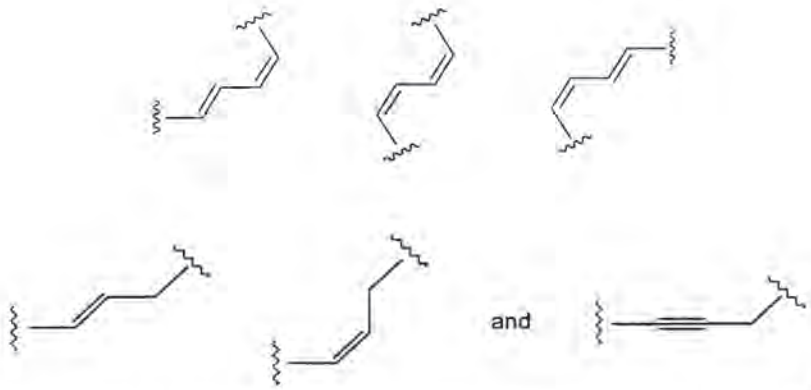




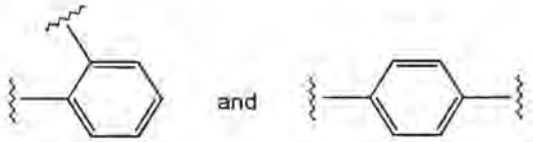
wherein R^5 is independently the same or different and is selected from the group consisting of a linear or branched alkyl group having from 1 to 10 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, CN and a halogen.

In another aspect, R^2 and R^3 taken together is an alkenyl or alkynyl and is selected from the group consisting of:





In another aspect, m is 0 and R^2 is an aromatic hydrocarbyl group selected from the group consisting of:



In a further aspect, m is 0 and R^2 is a heteroaromatic group selected from the group consisting of:

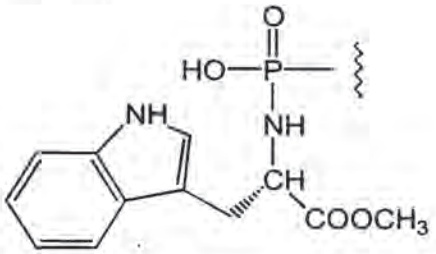


wherein J is selected from the group consisting of -O-, -S-, -Se-, -NH-, and -NR^{ALK}- and wherein R^{ALK} is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

For these embodiment, R^7 is as described above.

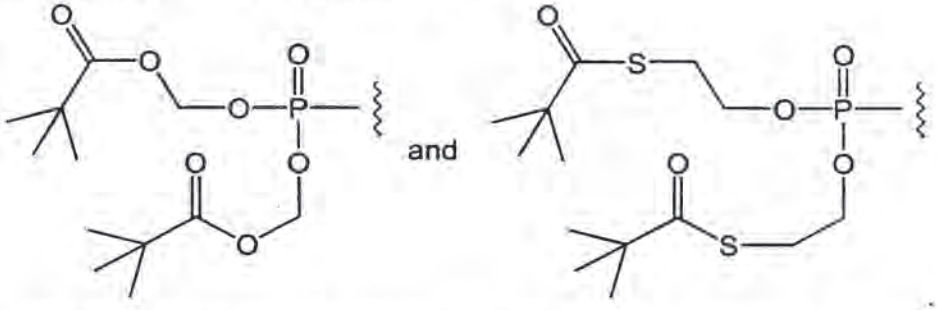
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In a specific embodiment, R^7 is:



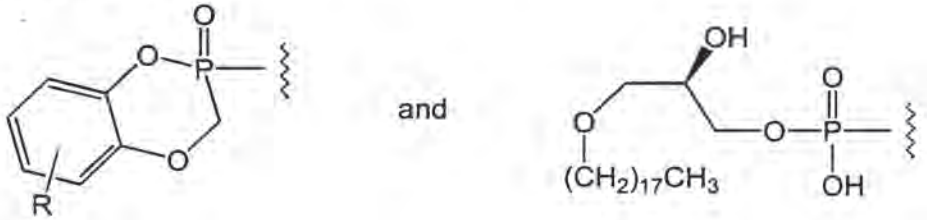
The above group, and methods for its preparation, are described in Abraham, et al. (1996).

In one embodiment, R^7 is a phosphate group or is or contains a group having a structure selected from the group consisting of:



The first of the two above groups, and methods for its preparation, are described in Freed, et al. (1989); Sastry, et al., (1992); Farquhar, et al. (1994), and Farquhar, et al. (1995). The second of the two above groups, and methods for its preparation, are described in Valette, et al. (1996); and Benzaria, et al. (1996).

In one embodiment, R^7 is or contains a group having a structure selected from the group consisting of:

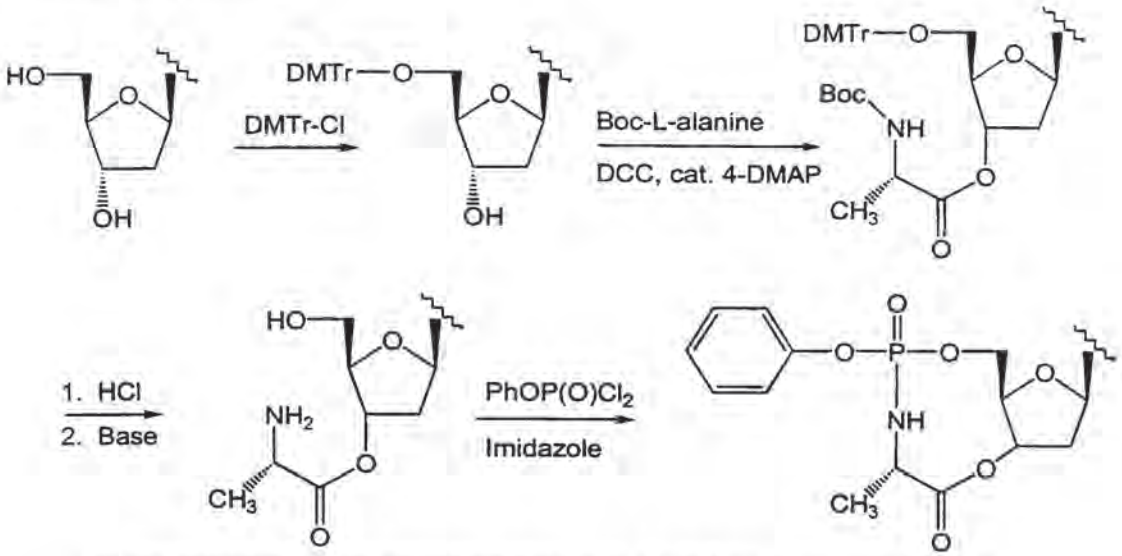


where R is an aromatic substituent the first of the two above groups, and methods for its preparation, are described in Meier, et al. (1997). The second of the two above

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groups, and methods for its preparation, are described in Hostetler, et al. (1997); and Hostetler, et al., published International Patent Application No. WO 96/40088 (1996).

In one embodiment, the R^7 forms a cyclic group within Q. One such embodiment, and a method for its preparation, is shown below (where DMTr is 4,4'-dimethoxytrityl, Boc is t-butyloxycarbonyl, DCC is 1,3-dicyclohexylcarbodiimide, and 4-DMAP is 4-dimethylaminopyridine):



In one embodiment, the compound may be in a salt form, or in a protected or prodrug form, or a combination thereof, for example, as a salt, an ether, or an ester.

In a separate embodiment, the above structures are further modified to possess thiophosphodiaziridine instead of phosphodiaziridine groups, using the methods described below.

Synthesis of the above noted 5-substituted pyrimidine derivatives can be accomplished by methods that are well-known in the art. For example, treatment of 5-chloromercuri-2'-deoxyuridine with haloalkyl compounds, haloacetates or haloalkenes in the presence of Li_2PdCl_4 results in the formation, through an organopalladium intermediate, of the 5-alkyl, 5-acetyl or 5-alkene derivative, respectively. Wataya, et al. (1979) and Bergstrom, et al. (1981). Another example of C5-modification of pyrimidine nucleosides and nucleotides is the formation of C5-*trans*-styryl derivatives by treatment of unprotected nucleotide with mercuric acetate followed by addition of styrene or ring-substituted styrenes in the presence of Li_2PdCl_4. Bigge, et al. (1980).

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Pyrimidine deoxyribonucleoside triphosphates were derivatized with mercury at the 5 position of the pyrimidine ring by treatment with mercuric acetate in acetate buffer at 50° for 3 hours. Dale, et al. (1973). Such treatment also would be expected to be effective for modification of monophosphates; alternatively, a modified triphosphate could be converted enzymatically to a modified monophosphate, for example, by controlled treatment with alkaline phosphatase followed by purification of monophosphate. Other moieties, organic or nonorganic, with molecular properties similar to mercury but with preferred pharmacological properties could be substituted. For general methods for synthesis of substituted pyrimidines, see for example, U.S. Patent Nos. 4,247,544; 4,267,171; and 4,948,882; and Bergstrom, et al. (1981). The above methods would also be applicable to the synthesis of derivatives of 5-substituted pyrimidine nucleosides and nucleotides containing sugars other than ribose or 2'-deoxyribose, for example 2'-3'-dideoxyribose, arabinose, furanose, lyxose, pentose, hexose, heptose, and pyranose. An example of a 5-position substituent is the halovinyl group, e.g. E-5-(2-bromovinyl)-2'-deoxyuridylate. Barr, P.J. et al. (1983).

Alternatively, 5-bromodeoxyuridine, 5-iododeoxyuridine, and their monophosphate derivatives are available commercially from Glen Research, Sterling, VA (USA), Sigma-Aldrich Corporation, St. Louis, MO (USA), Moravek Biochemicals, Inc., Brea, CA (USA), ICN, Costa Mesa, CA (USA) and New England Nuclear, Boston, MA (USA). Commercially-available 5-bromodeoxyuridine and 5-iododeoxyuridine can be converted to their monophosphates either chemically or enzymatically, though the action of a kinase enzyme using commercial available reagents from Glen Research, Sterling, VA (USA) and ICN, Costa Mesa, CA (USA). These halogen derivatives could be combined with other substituents to create novel and more potent antimetabolites.

The structures at the 5-position of uracil are referred to as the tethers because they connect the proposed leaving group (toxophore) to the heterocycle. Upon activation of the heterocycle by reaction with the Cys residue in the active site of a human enz, TS, for example, a negative charge is conducted from the 6-position of uracil into the tether. This mechanism has been described for the 5'-monophosphorylated versions of (*E*)-5-(bromovinyl)-2'-deoxyuridine (BVdU) by Barr, P. J. et al. (1983) and of (*E*)-5-(3,3,3-trifluoro-l-propenyl)-2'-deoxyuridine (TFPe-dUrd) by Wataya, et al. (1979), Santi (1980); and Bergstrom, et al. (1984).

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The tether "spacer" between the toxin and dNMP must be unsaturated so that it can conduct the toxin-labilizing negative charge supplied by the TS-Cys-sulfhydryl attack. Of the many unsaturated organic functionalities available for this purpose, the vinyl, allyl, and propargyl units are simple, small, and readily accessible synthetically. The vinyl and allyl units have the advantage that they can be prepared in either of two non-interconvertible geometric isomeric forms. Thus, they can be used as "probes" of prodrug accommodation by the enzyme active site. On the other hand, the propargyl unit has the advantage of being cylindrically symmetrical, so that enz-catalyzed toxin release from this type of tether does not depend upon its orientation with respect to dUMP's uracil ring, as is the case with the vinyl and allyl molecules.

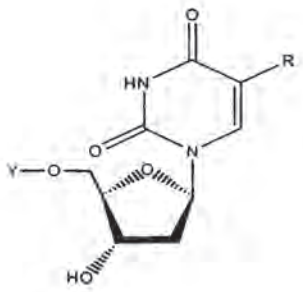
Two distinct approaches have been taken to design of some of the nucleotide-based prodrugs of this invention. One is based on the structure of BVdU monophosphate and features a leaving group/toxin directly attached to the terminus of a (poly)vinyl substituent at C5 of dUMP. This is the vinyl tether approach. The other is based on the structure of TFPe-dUMP and is similar to the first but has a methylene unit separating the leaving group/toxin and the unsaturated unit and thus contains an allyl or propargyl unit. This is the allyl tether approach.

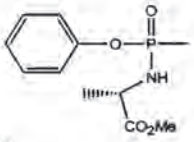
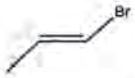
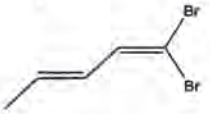
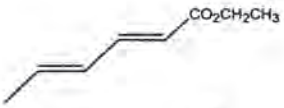
The mechanism of activation of a propargyl version of the allyl tether approach has a precedent in the interaction of both 5-ethynyl-2'-deoxyuridine 5'-monophosphate (EdUMP) and 5-(3-hydroxy-l-propynyl)-2'deoxyuridine 5'-monophosphate (HOPdUMP) with TS (Barr, et al. (1981); Barr and Robins 1983). EdUMP is a potent inhibitor of TS (Ki = 0.1 TM), and likely forms an allene-based species at the active site. HOPdUMP (Ki = 3.0 TM) shows unusual inhibition kinetics, which might be due to formation of a cumulene-based species at the active site.

5-Alkylidenated 5,6-dihydrouracils similar in structure to the intermediate common to both the vinyl and allyl tether approach mechanisms have been synthesized recently (Anglada et al. 1996). These were shown to be highly electrophilic. Their ready reaction with ethanol to generate 5-(ethoxymethyl)uracil is a precedent for the water addition that regenerates catalytically competent TS. Even more recently, the existence of the long-elusive C5 methylene intermediate produced by TS was demonstrated by trapping studies (Barrett, et al. (1998)).

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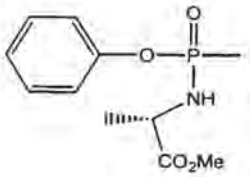
Particular embodiments include the compounds having the L or D structures shown below. Compounds are identified by structure and a numerical designation.



R	Y = 	Y = H
	NB1011	NB1015 (BVdU)
	NB1012	—
	NB1013	NB1020
	NB1014	NB1027
	NB1016	NB1021
	NB1017	NB1024

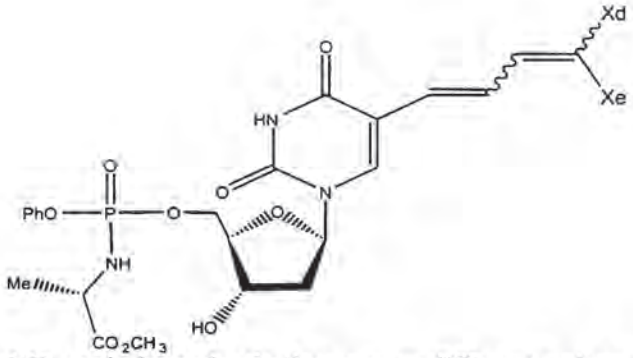
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(Continued from Previous Page)

R	Y = 	Y = H
	NB1018	NB1022
	NB1019	NB1023
	–	NB102
-H	NB1025	NB1026
$-C_8H_{17}$	–	NB102

Preferred embodiments are shown below:

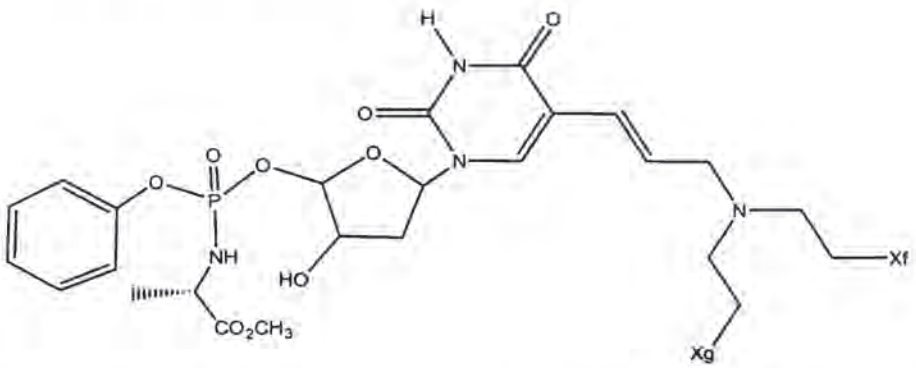
A compound having the structure:



wherein X_d and X_e are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN or the nucleoside analogs thereof. In a more preferred aspect, X_d is Cl or Br and X_e is H.

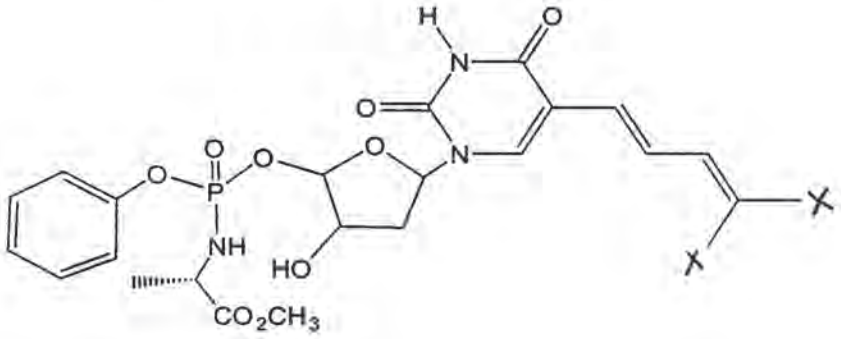
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A compound having the structure:



wherein X_f and X_g are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN or the nucleoside analogs thereof. In a preferred embodiment, X_f and X_g are the same and are each is Cl or Br.

A compound having the structure of the formula:

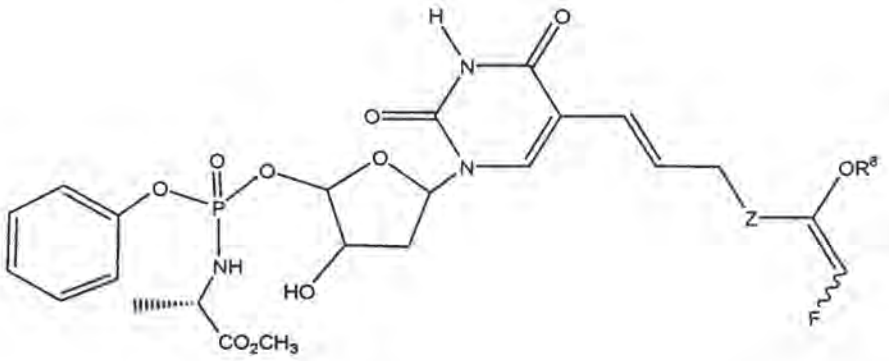


wherein each X is selected from the group consisting of Cl, Br, I, and CN;

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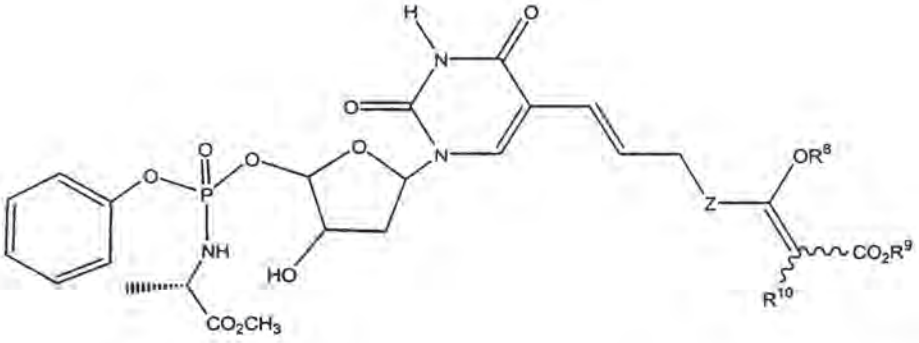
or the nucleoside analogs thereof. In a preferred embodiment, X is Cl or Br and in a more preferred embodiment, X is Br.

A compound having the structure:



wherein R^8 is a lower straight or brainched chain alkyl, or the nucleoside analogs thereof.

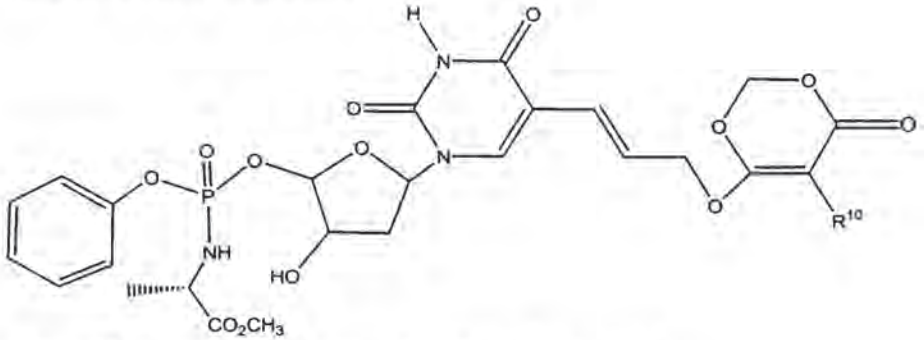
A compound having the structure:



wherein R^8 and R^9 are lower straight or branched chain alkyls and R^{10} is H or CH_3 or the nucleoside analogs thereof.

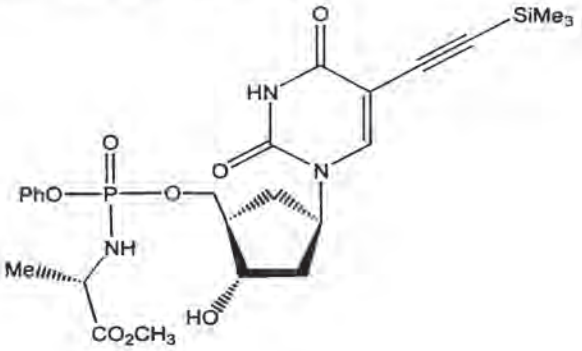
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A compound having the structure:

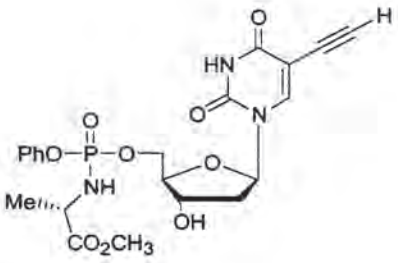


wherein R^{10} is H or CH_3;or the nucleoside analog thereof.

A compound having the structure:



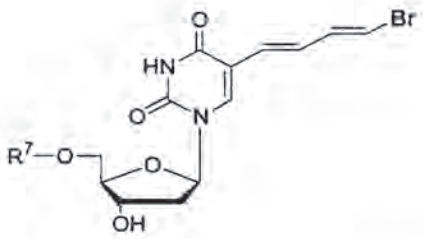
A compound having the structure:



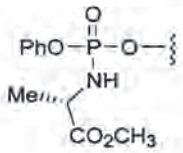
or the nucleoside analog thereof

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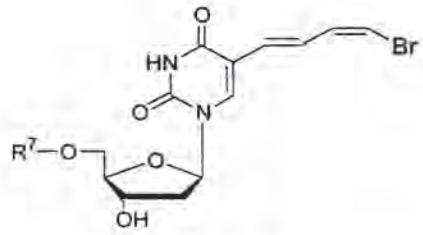
A compound as described herein, wherein the compound has the structure:



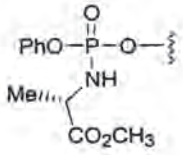
In one aspect, R^7 = H. In another aspect, R^7 is a substituent having the structure:



A compound as described herein wherein the compound has the structure:

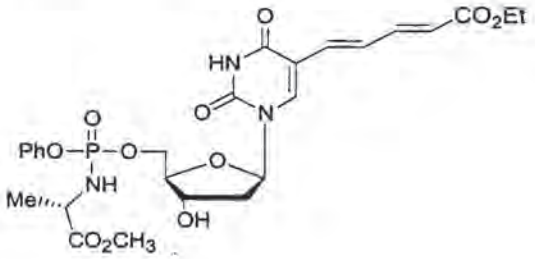


wherein R^7 = H or R^7 is a substituent having the structure

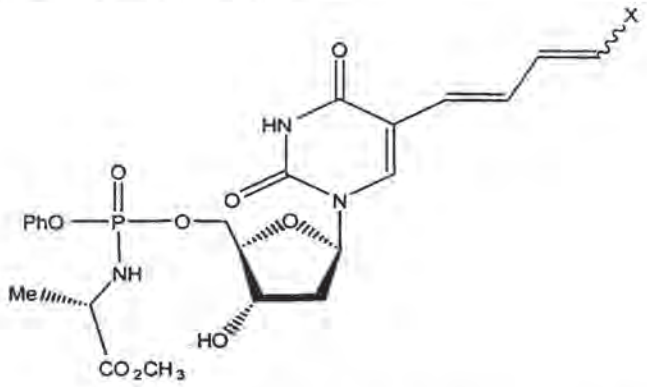


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A compound having the structure:

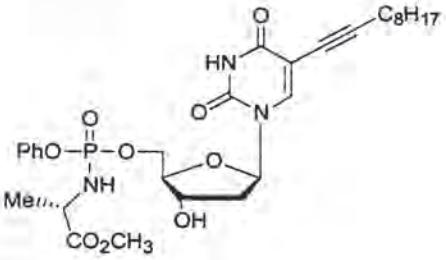


A compound having the structure:



wherein each X is selected from the group consisting of CO_2Et, Cl, and Br; or the nucleoside analog thereof.

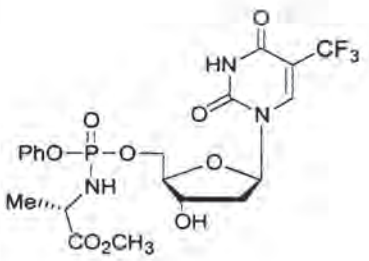
A compound having the structure:



or the nucleoside analog thereof.

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A compound having the structure:



or the nucleoside analog thereof.

The prodrugs can be combined with a carrier, such as a pharmaceutically acceptable carrier, for use *in vitro* and *in vivo*.

This invention also provides a quick and simple screening assay that will enable initial identification of novel compounds with at least some of the desired characteristics (see Figure 2A and 2B). The assay requires at least two cell types, the first being a control cell in which the target enzyme is not expressed or is expressed at a low level, e.g., a normal cell. The second cell type is the test cell in which the target enzyme is expressed at a detectable level, e.g., a high level. This cell can be a tumor cell line that is selected for enhanced levels of target enzymes. Alternatively, a cell genetically modified to differentially express the target enzyme or enzymes (containing the appropriate species of target enzyme) can be used. Transfection of host cells with polynucleotides encoding the target enzyme is either transient or permanent using procedures well known in the art and described in Chen, L. et al. (1996); Hudziak, R.M. et al. (1988); or Carter, P. et al. (1992), and in the experimental section below. The cells can be procaryotic (bacterial such as *E. coli*) or eucaryotic. The cells can be mammalian or non-mammalian cells, e.g., mouse cells, rat cells, human cells, fungi (e.g., yeast) or parasites (e.g., *Pneumocystis* or *Leishmania*) which cause disease.

Suitable vectors for insertion of the cDNA are commercially available from Stratagene, La Jolla, CA and other vendors. The amount of expression can be regulated by the number of copies of the expression cassette introduced into the cell or by varying promoter usage. The level of expression of enzyme in each transfected cell line can be monitored by immunoblot and enzyme assay in cell lysates, using monoclonal or polyclonal antibody previously raised against the enzyme for immuno-detection.

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(Chen, L. et al. (1996)). Enzymatic assays to detect the amount of expressed enzyme also can be performed as reviewed by Carreras, C.W. and Santi, D.V. (1995), or the method described in the experimental section below.

In a further aspect, more than one species of enzyme can be used to separately transduce separate host cells, so that the effect of the candidate drug on a target enzyme can be simultaneously compared to its effect on another enzyme or a corresponding enzyme from another species.

In another embodiment, a third target cell is used as a control because it receives an effective amount of a prodrug compound of this invention. This embodiment is particularly useful to screen for new agents that are activated by thymidylate synthase. In yet a further aspect, at least one additional test cell system is set up to test the synergistic potential of the test therapeutic in combination with a known therapy or agent.

For the purposes of this invention, the successful candidate drug will block the growth or kill the test cell type, but leave the control cell type unharmed. Growth assays can be performed by standard methods as described by Miller (1992); Sugarman, B. J. et al. (1985) and Spector, D. L. et al. (1998), or using the methods described in the experimental section below.

It will be understood by those skilled in the art that the screen can be applied broadly for the discovery of antibiotics. For example, thymidylate synthase from yeast could be substituted for that of human or *E. coli* forms of the enzyme. This would allow the discovery of specific antifungal antibiotics targeting yeast related pathogens. In addition, other enzymes can be subjected to this treatment. For example, prodrugs that target specifically the dihydrofolate reductase activity of infectious agents, like *Pneumocystis carnii*, could be selected. These agents will be selected for specificity for the target enzyme, and can be shown not to activate the enzyme of the natural host by employing the screening assay described herein. The control cellular constructs would contain the corresponding normal human enzyme, in order to show lack of toxicity when only the normal human enzyme is present.

Figures 2A and 2B show how one may conduct at least one embodiment of this aspect of the invention. A foreign gene, e.g., a human gene encoding TS, is inserted into the host cell such that human TS is expressed. The "control cell" does not express the

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target enzyme. In some embodiments it may be necessary to supplement the culture media with the protein product of the target enzyme.

The candidate prodrug can be directly added to the cell culture media and the target cell or the culture media is then assayed for the amount of label released from the candidate prodrug if the prodrug contains a detectable label. Alternatively, cellular uptake may be enhanced by packaging the prodrug into liposomes using the method described in Lasic, D.D. (1996) or combined with cytofectins as described in Lewis, J.G. et al. (1996).

An alternative embodiment for a prodrug taking advantage of TS overexpression in tumor cells is a deoxyuridine phosphoramidate, or other modifications (cited herein) conjugated with a therapeutic radionuclide. An example of a therapeutic radionuclide is rhenium 188. The isotope can be synthesized essentially as described by Callahan, et al. (1989). Alternatively, it can be obtained commercially, for example from Mallicrodt Medical BV, The Netherlands. The therapeutic radionuclide can be conjugated with deoxyuridine, or deoxyuridine 5'-phosphoramidate, or other derivative, by standard methods for example, as described by Lin, W.Y. et al. (1997). The radionuclide-containing deoxyuridine phosphoramidate will be preferentially taken up into the DNA of tumor cells overexpressing thymidylate synthase and cause their death via concentrated emission of beta and gamma radiation. Alternative radionuclides include rhenium-186, and others. (Troutner, D.A. (1987)).

The compounds are useful to predict whether a subject will be suitably treated by a prodrug of this invention by delivering to a sample containing the cell to be treated a prodrug and assaying for cell death or inhibition of cell proliferation. Applicants provide kits for determining whether a pathological cell or a patient will be suitably treated by this therapy by providing at least one prodrug of this invention and instructions for use.

This invention also provides a method for inhibiting the proliferation of a pathological or target cell *in vitro* or *in vivo* by delivering to the cell an effective amount of a prodrug of this invention. When practiced *in vivo*, the method is useful to treat a pathology characterized by target cells in a subject by delivering to the subject an effective amount of a prodrug of this invention.

When the target cell is target cell is resistant to a chemotherapeutic drug, the method can be further modified by contacting or administering to the cell or patient an effective amount of the drug to which the cell has developed resistance. Because the prodrugs of this invention can reverse resistance to the prior therapy, subsequent to

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successful treatment with a prodrug of this invention, administration of the previous therapy can again inhibit growth or metastasis of tumors. Examples where this may occur include, but are not limited to when the target cell expresses an enzyme that is amplified as a result of selection *in vivo* by chemotherapy or when the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell. An example of such an enzyme is thymidylate synthase which has been shown to be overexpressed as a result of prior chemotherapy and confers a drug resistant phenotype on the cell to the prior drug.

The prodrugs of this invention can also be combined with other known therapies to enhance or synergize the therapeutic effects of either or both prior therapies or the therapeutic effect of the prodrug. Such prior therapies include, but are not limited to cancer chemotherapy, radiation therapy and surgery.

When delivered to an animal, the method also is useful to further confirm efficacy of the prodrug. As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about 10^5 to about 10^9 hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the prodrug is administered, for example, by intraperitoneal or intravenous routes. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate. (Lovejoy et al. (1997); Clarke, R. (1996); and Pegram, M. D. et al. (1997)).

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The prodrug, combinations of drugs, or compositions containing either can be used in the manufacture for medicaments for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

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The pharmaceutical compositions can be administered orally, intranasally, parenterally or by inhalation therapy, and may take the form of tablets, lozenges, granules, capsules, pills, ampoules, suppositories or aerosol form. They may also take the form of suspensions, solutions and emulsions of the active ingredient in aqueous or nonaqueous diluents, syrups, granulates or powders. In addition to a compound of the present invention, the pharmaceutical compositions can also contain other pharmaceutically active compounds or a plurality of compounds of the invention.

More particularly, a compound of the formula of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

In general, a suitable dose for each of the above-named compounds, is in the range of about 1 to about 100 mg per kilogram body weight of the recipient per day, preferably in the range of about 1 to about 50 mg per kilogram body weight per day and most preferably in the range of about 1 to about 25 mg per kilogram body weight per day. Unless otherwise indicated, all weights of active ingredient are calculated as the parent compound of the formula of the present invention for salts or esters thereof, the weights would be increased proportionately. The desired dose is preferably presented as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing about 1 to about 100 mg, preferably about 1 to above about 25 mg, and most preferably about 5 to above about 25 mg of active ingredient per unit dosage form. It will be appreciated that appropriate dosages of the compounds and compositions of the invention may depend on the type and severity and stage of the disease and can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the treatments of the present invention.

Ideally, the prodrug should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the prodrug, optionally in saline, or orally administered, for

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example, as a tablet, capsule or syrup containing the active ingredient. Desirable blood levels of the prodrug may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component antiviral agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

While it is possible for the prodrug ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented a bolus, electuary or paste.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g., povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

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Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Pharmaceutical compositions for topical administration according to the present invention may be formulated as an ointment, cream, suspension, lotion, powder, solution, past, gel, spray, aerosol or oil. Alternatively, a formulation may comprise a patch or a dressing such as a bandage or adhesive plaster impregnated with active ingredients and optionally one or more excipients or diluents.

For diseases of the eye or other external tissues, e.g., mouth and skin, the formulations are preferably applied as a topical ointment or cream containing the active ingredient in an amount of, for example, about 0.075 to about 20% w/w, preferably about 0.2 to about 25% w/w and most preferably about 0.5 to about 10% w/w. When formulated in an ointment, the prodrug may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the prodrug ingredients may be formulated in a cream with an oil-in-water cream base.

If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound that enhances absorption or penetration of the prodrug ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

The oily phase of the emulsions of this invention may be constituted from known ingredients in a known manner. While this phase may comprise merely an emulsifier (otherwise known as an emulgent), it desirably comprises a mixture of at lease one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier that acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

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Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulfate.

The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent for the prodrug ingredient. The prodrug ingredient is preferably present in such formulation in a concentration of about 0.5 to about 20%, advantageously about 0.5 to about 10% particularly about 1.5% w/w.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as suppositories, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the prodrug ingredient, such carriers as are known in the art to be appropriate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the prodrug ingredient.

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Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable of oral administration may include such further agents as sweeteners, thickeners and flavoring agents.

Prodrugs and compositions of the formula of the present invention may also be presented for the use in the form of veterinary formulations, which may be prepared by methods that are conventional in the art.

The following examples are intended to illustrate, but not limit the invention.

Materials and Methods

Synthesis of Nucleoside ECTA Compounds

Synthesis of the above noted 5-substituted pyrimidine nucleosides can be accomplished by methods that are well-known in the art and briefly described above.

In one embodiment, the present invention involves four classes of compounds. Each class is defined by the structure of the uricil base, or modified uricil base present. These classes are ECTA compounds where: 1) the base is a furano-pyrimidinone derivative of uracil; 2) the base is 6-fluoro uracil; 3) the base is 4-hydrazone substituted uracil derivative; 4) the base is uracil. The uracil or modified uracil derived base is used to synthesize compounds substituted with toxic leaving groups at the 5 position,

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attached by an electron conduit tether at this 5 position, and including an appropriate spacer moiety between the electron conduit and the toxic leaving group. The ECTA compounds can be unphosphorylated, 5' monophosphate, 5' phosphodiester, or 5' protected ("masked") deoxyuridines or comparable derivatives of alternative carbohydrate moieties, as described below. Protected 5-substituted deoxyuridine monophosphate derivatives are those in which the phosphate moiety has been blocked through the attachment of suitable chemical protecting groups. Protection of 5-substituted deoxyuridine monophosphate derivatives can improve solubility, facilitate cellular penetration, facilitate passage across the blood-brain barrier, and prevent action of cellular or extracellular phosphatases, which might otherwise result in loss of the phosphate group. In another embodiment, 5-substituted uracil or uridine derivatives are administered to cells containing nucleoside kinase activity, wherein the 5-substituted uracil/uridine derivative is converted to a 5-substituted uridine monophosphate derivative. Uridine derivatives may also be modified to increase their solubility, cell penetration, and/or ability to cross the blood-brain barrier.

Action of thymidylate synthase upon 5-substituted uridine monophosphate derivatives can release the substituent attached to the 5-position ("leaving group") of the pyrimidine ring. The released substituent is then capable, either inherently or following reaction with another cellular component, of acting as a toxin or an inhibitor of cellular proliferation.

Synthesis of ECTA compounds with propargyl tethers

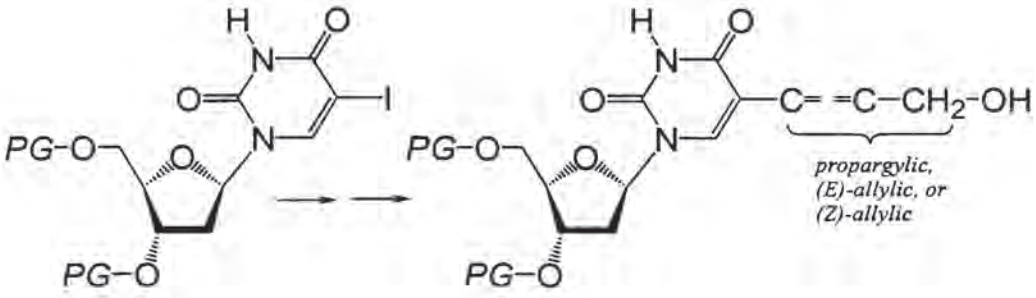
The synthesis of propargylic and allylic alcohol-equipped 2'-deoxyuridines is straightforward. Many of these and their close derivatives are reported in the literature, and some have even been studied in connection with TS. For example, 5-alkynyl-dUMPs including the 5-(3-methoxy-1-propynyl) and 5-(3-hydroxy-1-propynyl) ones have been examined as TS inhibitors (Barr, and Robins (1981)) and some of these have been shown to become incorporated into the DNA of TS-deficient cancer cells (Balzarini, J. et al. (1985)).

Both 5-mercuri- (Ruth, J. L. et al. (1978)) and 5-iodouridines (Robins, M. J. et al. (1981)) readily condense with alkenes and alkynes in the presence of a palladium catalyst to afford C5 tether-equipped uridines. The latter route is the more often employed (Robins, M. J. et al. (1982)), Asakura, J. et al. (1988) and (1990)). High-

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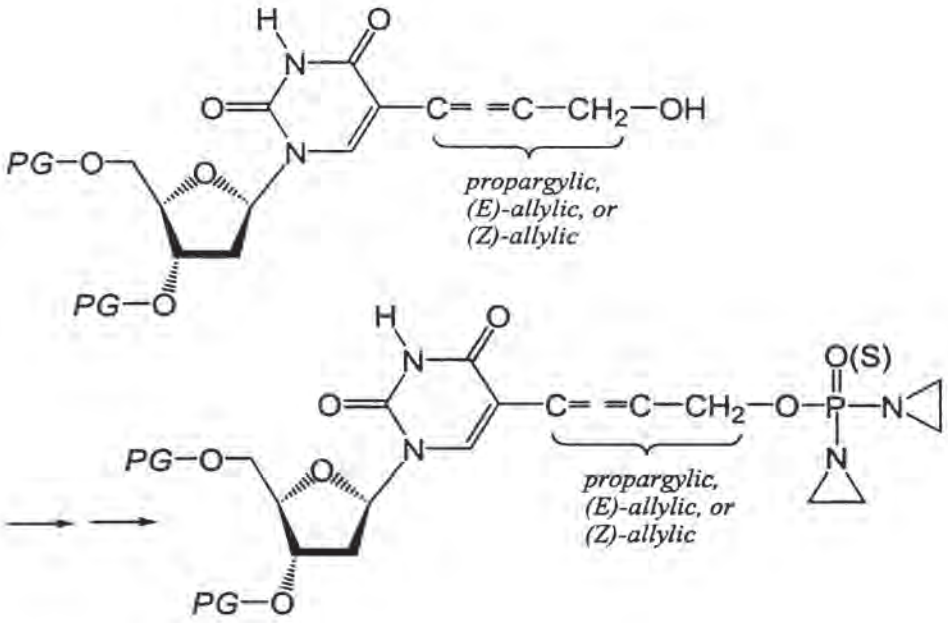
yielding condensations of protected 5-iodo-2'-deoxyuridines with t-butyidimethylsilyl propargyl ether (Graham, et al. (1998); De Clercq, et al. (1983), methyl propargyl ether (Tolstikov, et al. (1997)) and even propargyl alcohol itself (Chaudhuri, et al. (1995) and Goodwin, et al. (1993)) have been achieved. The 3-hydroxy-l-propynyl substituent introduced by the latter reaction can also be accessed by DIBAL-H reduction of a methacrylate group (Cho, Y. M. et al. (1994)), itself arising from the same Heck reaction used in the synthesis of BVdU. These palladium-catalyzed reactions are so versatile that they can used to condense very long and elaborately-functionalized propargyl-based tethers to 5-iodo-2'-deoxyuridines. (Livak, et al. (1992) and Hobbs (1989)). (Z)-Allyl-based tethers are generated by the partial hydrogenation of a propargylic precursor over Undiar catalyst (Robins, M. J. et al. (1983)) whereas the (E)-allyl-based ones are best prepared by Heck coupling of an (E)-tributylstannylated ethylene (Crisp, (1989)).

Closely following the literature procedures, a t-butyldimethylsilyl propargyl ether-equipped 3', 5'-di-O-protected 2'-deoxyuridine (Graham, et al. (1998), and De Clercq, et al. (1983)) is prepared and a portion of it, converted to the corresponding (Z)-allyl ether, (Robins and Barr (1983)) is reduced. Because the TBAF-mediated removal of a TBDMS group generates an oxyanion that can be functionalized *in situ*, these TBDMS-protected propargyl- and (Z)-allytic-tethered nucleosides will serve as convenient precursors to some of the toxophore-equipped targets. For the (E)-allyl alcohol equipped nucleoside, the known O-tetrahydropyranyl ether derivative is prepared by the literature Heck coupling of an (E)-tributylstannylated ethylene (Crisp (1989)).



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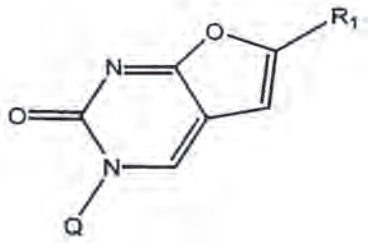
Using a two step literature protocol (Phelps et al. (1980) and Hsiao and Bardos (1981)), the propargylic and (E) and (Z)-allylic alcohols are converted to their corresponding bis-aziridinyl phosphoramidates or thiophosphoramidates so that TS processing of the 5'-mononucleotide versions will release an active metabolite of the cytostatic drugs TEPA or ThioTEPA (Dirven, et al. (1995)), respectively.



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Synthesis of furano-pyrimidinones

Synthesis of furano-pyrimidinones begins with synthesis of a C5 propargylic -alcohol-equipped 2'-deoxyuridine. Furano-pyrimidinone compounds are then be formed from the O-tetrahydropyranyl ether derivative described above. Synthesis proceeds by reaction of the second carbon of the propargyl bond with the oxygen attached to the C4 position of the pyrimidine ring to yield a fluorescent furano-pyrimidinone which can be readily separated from the reaction mix. Such compounds provide an additional basis for synthesis of ECTA compounds through various combinations of specific electron conduits, spacers and toxic leaving groups.



The furo[2,3-d]pyrimidinone nucleosides were prepared by condensing 2',3'-di-O-p-toluoyl or 2',3'-di-O-acetyl-5-iodo-2'-deoxyuridine with 1-(tetrahydropyranyloxy)-2-propyne ("New methods of synthesis of δ-aminoethylpyrazoles" Jones, R. G. and Mann, M. J. (1953)) under conditions known to promote the formation of these fluorescent compounds (Robins, M. J. et al.(1983)). Base-catalyzed removal of the carbohydrate protecting groups gave the 6-(tetrahydropyran-2-yloxymethyl)-substituted bicyclic nucleoside which was either subjected to standard acidic THP group hydrolysis (TFA in CH_2Cl_2) or was regioselectively 5'-phosphoramidated by the same procedure used to prepare BVdU-PA and 5FUdR-PA. After the phosphoramidation, the THP group could be removed by acidic hydrolysis, as shown in Figure 4.

TS ECTA compounds based on furano-pyrimidinones

Toxic R^4 leaving groups can be attached to the furan-2 methyl alcohol using methods similar to those employed to attach toxic leaving groups to the hydroxyl on the C5 propargyl uridine compound, as explained with the synthesis of the TEPA and

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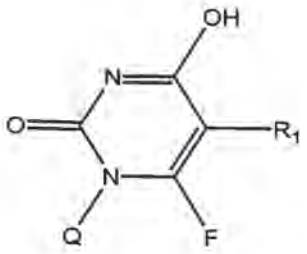
ThioTEPA derivatives described above. A variety of alternative toxic leaving groups, apparent to one skilled in the art, are envisioned. In addition, modifications to the length and composition of the R^2 electron conduit component and of the composition of the R^3 spacer element are also envisioned.

TS ECTA compounds based on furano-pyrimidinones can also consist of variously modified "Q" moeities. Many 5-substituted 2'-deoxyuridines are not substrates for human TK, but interestingly 5-(4-hydroxy-l-butynyl)-2'-deoxyuridine was found to be an exception (Barr, P. J. et al. (1981)). The ECTA compounds can have a free 5' hydroxyl, a 5' monophosphate, or a 5' phosphoramidate group attached to alternative carbohydrate groups. A novel method for synthesis of such phosphoramidate compounds is accomplished by reacting a 2-deoxy 3'-hydroxy, 5'-hydroxy unprotected nucleotide with a phosphochloridate in the presence of an HCl scavenger. In a preferred embodiment, the phosphochloridate comprises a phosphorus substituent which is derived from an amino acid such as alanine. For example, the phosphochloridate can be phenyl-L-methoxyalanine phosphorochloridate.

C6 Fluoro uridine and C4 hydozone based compounds

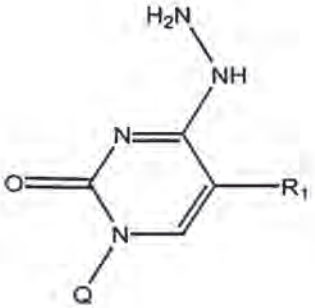
The neutral thiol addition to the pyrimidine C5-C6 double bond proceeds as an exothermic reaction (3-9 kcal per mol; see review by Les, A. et al. (1998) in the normal TS reaction with dUMP. Alternative substituents to the TS reactive hydrogen at the 6 position that can facilitate the formation of the sulfydryl bond with the enzyme, via the active human TS cysteine (homologous with cys-198 of *L. casei*), include fluorine. Such substituents at other positions in the pyrimidine ring can also facilitate the reaction between the substrate and TS. For instance, a 4-hydrazone substitution on the uracil (as described by Les, A. et al. (1998) facilitates formation of the thiol with TS. It is important that the resulting nucleotide-thiol (TS) intermediate rearranges in such a way as to release the altered nucleotide which can be accomplished passively via hydrolysis.

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The introduction of fluorine at the C6 position has not been previously reported, but it can be synthesized by following the synthetic descriptions of Krajewskas and Shugar (1982), who describe the synthesis of a number of 6 substituted uracil and uridine analogues.

Chemistry facilitating substitutions at the C4 position of the pyrimidine base are well known by those skilled in the art. Examples of literature descriptions include Wallis et al. (1999); Negishi, et al. (1996), Barbato et al. (1991), Barbato, et al. (1989) and Holy et al. (1999). These synthetic techniques also enable combinations of substitutions, for instance at the C4 and C5 positions of the pyrimidine ring (Pluta, et al. 1999) or the C2 and C4 positions of the pyrimidine ring (Zeid, et al. (1999)).



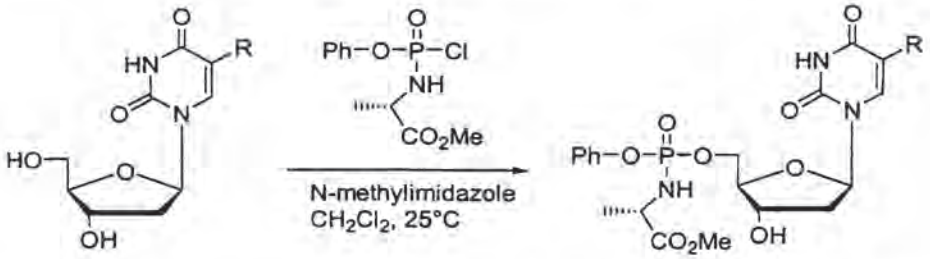
In another embodiment of the invention, ECTA compounds are synthesized by addition of alternative electron conduits, spacer moieties and toxic leaving groups to either the C6 fluoro-uridine base or the C4 hydrazone modified pyrimidine. Methods described above for synthesis of 2, deoxyuridine based ECTA compounds can again be employed for synthesis of such molecules.

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Synthesis of Nucleoside Phenyl Methoxyalaninyl Phosphoramidates

The use of phosphoramidates as phosphate prodrugs for nucleotides was first reported by McGuigan, C. et al. (1993) and McGuigan, C. et al. (1994). These authors showed that phosphoramidate derivatives of antiviral 2',3'-dideoxynucleoside derivatives such as d4T retain their antiviral activities in thymidine-kinase deficient cells. Further studies showed that the phosphoramidate group was hydrolyzed to the phosphate group inside cells (McGuigan, C. et al. (1996); Balzarini, J. et al. (1996); and Saboulard, et al. (1999)). The phospharamidates were synthesized by reacting 2',3'-dideoxynucleosides with phenyl methoxyalaninyl phosphorochloridate (PMPC).

Since only one hydroxyl group is present, these reactions usually proceed smoothly. In compounds where more than one hydroxyl group is present, the appropriately protected nucleoside might be required. Since the 5'-OH group of 2'-deoxynucleosides is much less hindered than the 3'-OH group, selective phosphoramidation with PMPC is possible under carefully controlled conditions. Both BVdU and 5FUdR condensed with PMPC in the presence of N-methylimidazole in anhydrous CH_2Cl_2 to give the corresponding phosphoramidates. In both cases, the desired product was readily separable from the starting material using column chromatography on silica gel. The synthetic scheme is summarized below.



The following examples are intended to illustrate, but not limit the invention.

Examples 1 and 2

Synthesis of ECTA compounds with propargyl tethers

Using the general synthetic procedure described *supra*, **bis-aziridin-1-yl-phosphinic acid 3-[2-deoxyuridin-5-yl]-prop-2-ynyl ester** was synthesized and analyzed by ^{1}H NMR to yield the following result: ^{1}H NMR ($(CD_3)_2SO$) complicated

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due to noise. Salient features: δ 8.28 (d, 1, H6), 6.10 (pseudo-t, 1, H1'), 5.26 (m, exchanges with D_2O, 1, 3'-OH), 5.13 (m, exchanges with D_2O, 1, 5'-OH), 4.81 (q or dd, 2, propargyl-CH_2), 4.24 (m, 1, H3'), 3.57 (m, 2, 5'-CH_2), 2.15-2.0 (m, 8, aziridine-CH_2).

Bis-aziridin-1-yl-phosphinothioic acid 3-[2-deoxyuridin-5-yl]-prop-2-ynyl ester was also synthesized and analyzed by ^1H NMR to yield the following result: ^1H NMR ($(CD_3)_2SO$) complicated due to noise. Salient features: δ 8.29 (d, 1, H6), 6.10 (pseudo-t, 1, H1'), 5.22 (m, exchanges with D_2O, 1, 3'-OH), 5.10 (m, exchanges with D_2O, 1, 5'-OH), 4.88 (q or dd, 2, propargyl-CH_2), 4.31 (m, 1, H3'), 3.52 (m, 2, 5'-CH_2), 2.15-2.0 (m, 8, aziridine-CH_2).

Examples 3 to 8

Synthesis of furano-pyrimidinones

Using the general synthetic procedure described *supra*, the following compounds were prepared.

Example 3

3-(2-Deoxy-β-D-ribofuranosyl)-6-(tetrahydropyran-2-yloxymethyl)furo[2,3-*d*]pyrimidin-2(3H)-one. ^1H NMR ($(CD_3)_2SO$) δ 8.80 (s, 1, H4), 6.74 (s, 1, H5), 6.16 (pseudo-t, 1, H1'), 5.27 (d, exchanges with D_2O, 1, 3'-OH), 5.12 (t, exchanges with D_2O, 1, 5'-OH), 4.72 (m, 1, THP-H2), 4.56 (q, 2, CH_2OTHP), 3.92 (m, 1, H4'), 3.64 (m, 2, 5'-CH_2), 2.40 (m, 1, H2'a), 2.03 (m, 1, H2'b), 1.68 and 1.50 (m, 8, THP). Low-resolution mass spectrum (DCI-NH_3) on bis-TMS derivative, *m/z* 323 ($B+TMS+H^+$), 511 (MH^+), 583 ($M+TMS^+$).

Example 4

3-(2-Deoxy-β-D-ribofuranosyl)-6-(hydroxymethyl)furo[2,3-*d*]pyrimidin-2(3H)-one. ^1H NMR ($(CD_3)_2SO$) δ 12.0 (bs, 1, OH), 8.24 (s, 1, H4), 6.53 (s, 1, H5), 5.51 (pseudo-t, 1, H1'), 4.42 (m, 2, CH_2OH). Low-resolution mass spectrum (DCI-NH_3), *m/z* 167 ($B+2H^+$), 184 ($B+NH4^+$).

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Example 5

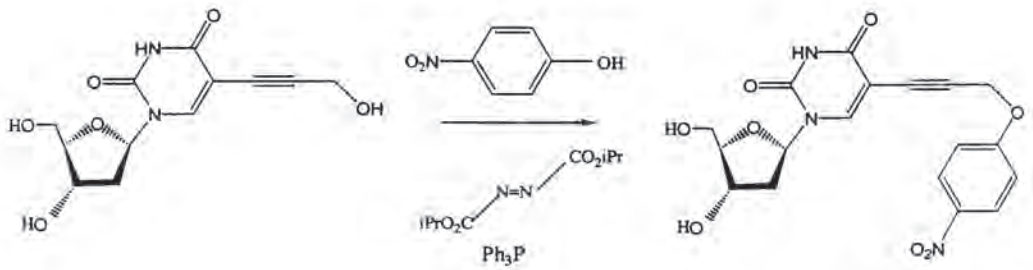
1-[6-(Tetrahydropyran-2-yloxymethyl)furo[2,3-d]pyrimidin-2(3H)-on-3-yl]-2-deoxy-β-D-ribofuranos-5-yl phenyl methoxy-L-alaninylphosphoramidate. 1H NMR ((CD3)2SO) complicated due to presence of diastereomers. Salient features: δ 8.62 and 8.59 (each s, each 1, H4), 7.4-7.1 (m, 5, PhO), 6.61 and 6.60 (each s, each 1, H5), 6.25 (m, 1, H1'), 4.56 (q, 2, propargyl-CH2), 3.56 and 3.54 (each s, each 3, CO2Me), 2.0 (m, 1, H2'b), 1.22 (m, 3, alaninyl-α-Me). Low-resolution mass spectrum (DCI-NH3), m/z 167 ($B+2H^+$), 184 ($B+H^++NH4+-THP$).

Example 6

1-[6-(Hydroxymethyl)furo[2,3-*d*]pyrimidin-2(3H)-on-3-yl]-2-deoxy-β-D-ribofuranos-5-yl phenyl methoxy-L-alaninylphosphoramidate. ^1H NMR ($CDCl_3$) complicated due to presence of diastereomers. Salient features: δ 8.5 (s, 1, H4), 7.4-7.1 (m, 5, PhO), 6.36 and 6.30 (each s, each 1, H5), 6.23 (m, 1, H1'), 3.67 and 3.65 (each s, each 3, CO_2Me), 2.69 (m, 1, H2'a), 2.10 (m, 1, H2'b), 1.35 (m, 3, alaninyl-α-Me). Low-resolution mass spectrum (DCI-NH_3), *m/z* 525 (MH^+), 595 (MNH_4^+).

Example 7

The 4-nitrophenyl ether derivative of 5-(3-hydroxy-1-propynyl)-2'-deoxyuridine was prepared according to standard ether synthesis as shown below.



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Example 8

5-[3-(4-Nitrophenoxy)-1-propynyl]-2'-deoxyuridine. A solution of pre-dried 5-(3-hydroxy-1-propynyl)-2'-deoxyuridine Robins, M. J. et al., (1983) (565 mg, 2 mmol) in 40 mL of anhydrous THF under argon was treated with 4-nitrophenol (696 mg, 5 mmol), triphenylphosphine (787 mg, 3 mmol), and diisopropyl azodicarboxylate (590 L, 3 mmol), and the reaction mixture heated at 60 °C until the solution was clear, and then 1 h longer. The mixture was allowed to cool to 23 °C and then it was evaporated onto SiO_2 and purified by chromatography using $MeOH/CH_2Cl_2$ as eluent to afford 107 mg (13%) of the desired ether product: mp 112-118 °C. ^1H NMR ($(CD_3)_2SO$) δ 11.65 (s, exchanges with D_2O, 1, NH), 8.29 (s, 1, H6), 8.24 (d, $J = 9.3$ Hz, 2, *m*-ArH), 7.23 (d, $J = 9.3$ Hz, 2, *o*-ArH), 6.09 (pseudo-t, 1, H1'), 5.17 (s, 2, propargyl-CH_2), 4.22 (m, 1, H3'), 3.80 (m, 1, H4'), 3.59 (m, 2, 5'-CH_2), 2.13 (pseudo-t, 2, 2'-CH_2). Low-resolution mass spectrum (DCI-NH_3) on *per*-trimethylsilyated material, *m/z* 547 [$M(TMS)_2H^+$], 565 [$M(TMS)_2NH_4^+$], 620 [$M(TMS)_3H^+$].

Example 9

5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine

(a) 5-(Carbomethoxyvinyl)-2'-deoxyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (I)

A slurry of 5-(carbomethoxyvinyl)-2'-deoxyuridine (3.0 g, 9.6 mmol), 3,4-dihydro-2*H*-pyran (22 mL, 21.3 mmol) and pyridinium *p*-toluenesulfonate (PPTS, 0.242 g, 0.96 mmol) in dimethylformamide (DMF, 5 mL) was stirred at 50°C for 18 h. The resulting solution was concentrated *in vacuo* (bath temperature 45°C) to give a thick, pale yellow oil. The oil was dissolved in EtOAc and the solid was filtered. The solution was again concentrated. The oil obtained was purified by column chromatography on silica gel using 50-75% EtOAc/hexane as eluent to give 3.81 g (85%) of pure product as a colorless oil.

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(b) 5-(3-Hydroxyprop-1-enyl)-2'-deoxyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (II)

A solution of (I) (3.5 g, 7.27 mmol) in CH_2Cl_2 (14 mL) was cooled to -78°C in a dry ice/acetone bath. Diisobutylaluminum hydride (DIBAL-H) in toluene (1.0 M, 24 mL, 24.0 mmol) was added dropwise over 2 h while the temperature was maintained at -78°C. The solution was stirred at -78°C for an additional 2 h and MeOH (2.5 mL) was added dropwise to destroy any excess DIBAL-H. The reaction mixture was cannulated into a mixture of 30% citric acid solution (50 mL), ice (25 g) and EtOAc (30 mL) over ca. 20 min. The phases were separated and the aqueous phase was extracted with EtOAc (2 × 25 mL). The combined organic phase was washed with saturated $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$ and concentrated to give 3.288 g (100%) of colorless oil

(c) 5-(3-Oxoprop-1-enyl)-2'-dexoyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (III)

To a solution of crude (II) obtained from above (1.988 g, 4.4 mmol) in CH_2Cl_2 (9 mL) was added solid pyridinium dichromate (PDC; 1.82 g, 4.8 mmol) with water cooling. The suspension was stirred while acetic acid (0.4 mL) was added dropwise. The water bath was removed and the reaction was stirred at room temperature for 1 h. The crude product was filtered through a pad of florisil (2 × 2.5 cm) and the florisil washed with 35 mL EtOAc. The brown solution obtained was filtered through another column of florisil (3.5 cm diam × 2.5 cm height). The filtrate was concentrated to give 1.273 g (64% yield) of very light brown oil.

(d) 5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (IV)

(Carbethoxymethylene)triphenylphosphorane (0.32 mg, 0.92 mmol) was added to a solution of the crude aldehyde (III) (0.344 g, 0.77 mmol). The solution darkened and turned rust color. After 1 h, (III) was completely consumed as judged by thin layer chromatography. The solvent was evaporated and the crude product was purified by

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column chromatography on silica gel using 35-45% EtOAc/hexane as eluent. The pure product (0.310 g, 78% yield) was obtained as colorless oil.

(e) 5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine (V)

5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (IV) (0.637 g, 1.22 mmol) was dissolved in MeOH (1.5 mL) and PPTS (0.049 g, 0.16 mmol) was added. The solution was stirred at 50°C for 7.5 h and left at room temperature overnight. A white precipitate was formed. The reaction mixture was cooled to 0°C and filtered to give pure (V) as a white solid (0.188 g). The filtrate was concentrated and chromatographed on silica gel using 50-100% EtOAc/hexane as eluent to give a further 0.180 g product. The total yield of the product was 0.368 g (86%).

^{1}H NMR (DMSO-d_6): 1.22 (3H, t, J = 7 Hz), 2.17 (2H, br t, J = 5.5 Hz), 3.55-3.75 (2H, m), 3.81 (1H, m), 4.12 (2H, q, J = 7 Hz), 4.25-4.28 (1H, m), 5.19 (1H, t, J = 4.8 Hz), 5.27 (1H, d, J = 4.1 Hz), 5.98 (1H, d, J = 14.5 Hz), 6.14 (1H, t, J = 6.3 Hz), 6.75 (1H, d, J = 14.5 Hz), 7.18-7.30 (2H, m), 8.30 (1H, s), 11.56 (1H, s).

Example 10

5-(4-Carbomethoxy-1,3-butadienyl)-2'-dexoyuridine (Va)

A solution of triethylamine (3.9 mL, 28.2 mmol) in dioxane (12 mL) was deareated by bubbling nitrogen through for 15 min. Palladium acetate (0.60 g, 0.26 mmol) and triphenylphosphine (0.183 g, 0.70 mmol) were added and the solution was heated at 70°C for 20 min to give a dark brown solution. 5-Iodo-3'-deoxyuridine (5.0 g, 14.1 mmol) and methyl 2,4-pentadienoate (2.5 g, 22.3 mmol) was added and the mixture was heated under reflux for 15h. The solvent and volatile components were evaporated in vacuo and the residue was partitioned between water (15 mL) and EtOAc (15 mL). The phases were separated and the aqueous phase was extracted twice with EtOAc (10 mL each). The combined organic phase was washed with brine and concentrated. The residue was dissolved in MeOH (15 mL) and allowed to cool to room temperature. The solid formed was collected by filtration, washed with a small quantity of MeOH and dried in vacuo to give 0.38 g brown powder.

^{1}H NMR (DMSO-d_6): 2.17 (2H, t, J = 6.4 Hz), 3.55-3.70 (2H, m), 3.66 (3H, s), 3.82 (1H, q, J = 3.6 Hz), 4.27 (1H, m), 5.18 (1H, t, J = 4.9 Hz), 5.26 (1H, d, J = 4.5 Hz),

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5.99 (1H, d, J = 14.4 Hz), 6.14 (1H, d, J = 6.4 Hz), 6.74 (1H, d, J = 14.8 Hz), 7.20-7.35 (2H, m), 8.30 (1H, s), 11.56 (1H, s).

The filtrate from above was concentrated and chromatographed on silica gel using 60-100% EtOAc/hexanes as eluent to give another 0.70 g of product as a brown foam. The combined yield was 1.08 g (22.6%).

Example 11

5-(4-Carboxy-1,3-butadienyl)-2'-dexoyuridine (VI)

Method I

5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine (V, from Example 1) (0.449 g, 1.28 mmol) was dissolved in 2N NaOH (3 mL) and stirred at 25°C. After 20 min, a precipitate was formed and TLC showed that the starting material was completely consumed. The mixture was cooled to 0°C and acidified to pH 1 with 2N HCl. The resulting off-white solid was filtered off, washed with water and dried *in vacuo* to give 0.225 g (54%) product.

^{1}H NMR (DMSO-d_6): 2.12-2.19 (2H, m), 3.50-3.70 (2H, m), 3.75-3.85 (1H, m), 4.24-4.29 (1H, m), 5.19 (1H, t, J = 4.8 Hz), 5.27 (1H, d, J = 4.2 Hz), 5.80-5.95 (1H, m), 6.14 (1H, t, J = 6.4 Hz), 6.60-6.75 (1H, m), 7.15-7.25 (2H, m), 8.26 (1H, s), 11.56 (1H, s), 12.16 (1H, br s).

The filtrate and washings were combined and evaporated to dryness. The resulting sticky yellow solid was dissolved in MeOH from which a white precipitate was formed. The solid was filtered off to give an additional 0.200 g of product.

Method II

The title compound can also be prepared from 5-(4-carbomethoxy-1,3-butadienyl)-2'-dexoyuridine (**Va**) (prepared according to the method in Example 2) in comparable yield as mentioned above.

Example 12

5-(4-Bromo-1*E*,3*E*-butadienyl)-2'-dexoyuridine (VIIa) and

5-(4-Bromo-1*E*,3*Z*-butadienyl)-2'-dexoyuridine (VIIb)

To a solution of 5-(4-carboxy-1,3-butadienyl)-2'-dexoyuridine (VI) (0.200 g, 0.62 mmol) in DMF (1 mL) was added $KHCO_3$ (0.185 g, 1.84 mmol) and the mixture

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was stirred for 20 min at 25°C. A solution of *N*-bromosuccinimide (0.117 g, 0.65 mmol) in DMF (0.3 mL) was added dropwise. Smooth gas evolution (CO_2) occurred throughout the addition. The resulting brown suspension was stirred for 2 h at 25°C at which time TLC showed that (**VI**) was completely consumed. Water (10 mL) was added to the suspension and the resulting solution was extracted with EtOAc (2 × 15 mL). The extract was dried over $MgSO_4$ and the solvent was evaporated *in vacuo* to give a yellow solid (178 mg, 80% yield) consisting of a mixture of two isomers as shown by ^{1}H NMR. The crude product was separated by semi-preparative HPLC (reversed phase C18 column) using 20% acetonitrile in water as the mobile phase to give the following isomers:

5-(4-Bromo-1*E*,3*Z*-butadienyl)-2'-dexoyuridine: retention time 10.5 minutes; ^{1}H NMR: (DMSO-d_6): 2.11-2.18 (2H, m), 3.50-3.70 (2H, m), 3.80 (1H, distorted q, J = 3.5 Hz), 4.25 (1H, br s), 5.08 (1H, br s), 5.25 (1H, br s), 6.15 (1H, t, J = 6.5 Hz), 6.40 (1H, d, J = 7 Hz), 6.53 (1H, d, J = 15.6 Hz), 6.83 (1H, dd, J = 7, 10 Hz), 7.39 (1H, dd, J = 10, 15.6 Hz).

5-(4-Bromo-1*E*,3*E*-butadienyl)-2'-dexoyuridine: retention time 15.1 minutes; ^{1}H NMR (DMSO-d_6): 2.12-2.16 (2H, m), 3.50-3.70 (2H, m), 3.80 (1H, q, J = 3.2 Hz), 4.26 (1H, m), 5.13 (1H, br s), 5.25 (1H, br s), 6.14 (1H, t, J = 6.5 Hz), 6.36 (1H, d, J = 15.6 Hz), 6.67 (1H, d, J = 13.1 Hz), 6.84 (1H, dd, J = 11, 13.1 Hz), 7.04 (1H, dd, J = 11, 15.6 Hz).

Example 13

Using the procedures mentioned in Example 11, Method II, the following compounds can be obtained in a similar fashion: 5-(4-chloro-1,3-butadienyl)-2'-dexoyuridine (using *N*-chlorosuccinimide in place of *N*-bromosuccinimide in Step B); 5-(4-iodo-1,3-butadienyl)-2'-dexoyuridine (using iodine in sodium idodide in place of *N*-bromosuccinimide).

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Example 14

5-(2-Bromovinyl)-2'-Deoxyuridine Phenyl *N*-Methoxy-L-alaninyl Phosphoramidate

Phenyl *N*-Methoxy-*L*-alaninyl Phosphorochloridate

L-alanine methyl ester hydrochloride (245.8 g; 1.76 mol) was placed in a 12 L three-neck round bottom flask (equipped with a mechanical stirrer and thermometer) followed by 4.0 L of dichloromethane. The mixture was stirred for 15 minutes at room temperature. Phenyl phosphodichloridate (370.0 g; 1.76 mol) was added to the mixture and stirring was continued for 15 min at room temperature. The flask was placed in the bath with dry ice and the stirring was continued for 20 minutes until a uniform suspension was formed.

Freshly distilled tri-n-butylamine (626.5 g; 3.38 mol) was added dropwise (~90 min) with vigorous stirring to the reaction mixture so that the temperature inside the flask was held at ~0°C. The bath was removed and the stirring was continued for 6 hours at room temperature. The solution was concentrated to ~2.84 L by evaporating several portions of the mixture on a rotary evaporator and the mixture was sealed under argon and stored at -20°C. The product was 85% pure by phosphorus NMR to give an estimated concentration of phenylmethoxyalaninyl phosphochloridate of ~0.5 M.

Example 15

5-(2-Bromovinyl)-2'-Deoxyuridine Phenyl *N*-Methoxy-*L*-alaninyl Phosphoramidate (NB1011)

The reaction was performed under argon atmosphere. 5-(2-bromovinyl)-2'-deoxyuridine (BVdU) (204 g; 612 mmol) was placed in three-neck 3 L round bottom flask equipped with mechanical stirrer. The flask was placed in ice-water bath and 1600 mL (~ 800 mmol) of phenylmethoxyalaninyl phosphochloridate reagent were added using an addition funnel over 15 min with vigorous stirring of the reaction mixture, followed by the addition of 100 mL of N-methylimidazole over 5 minutes using syringe. After 5 minutes the mixture became clear and after 10 minutes the ice-water bath was removed to allow the mixture to warm up to room temperature while stirring was continued. The reaction was monitored by reversed phase HPLC and was complete in 3 hours. The reaction was quenched by the addition of 100 mL of methanol and the mixture was evaporated to an oil, re-dissolved in 6 L of dichloromethane and

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passed through 800 g of silica gel. The major portion of BVdU-PA referred to herein as NB1011, was passed through the column during the loading and finally the elution of NB1011 was completed by passing 5 L of 5% methanol in dichloromethane. All fractions containing NB1011 were combined and evaporated to an oil, the residue was dissolved in 4 L of ethyl acetate and the mixture was extracted with water (2 x 2 L). The organic layer was dried with sodium sulfate, filtered, and washed with ethyl acetate (3 x 300 mL). The combined filtrate and washings were evaporated to produce a lightly colored white foam; total weight ~540 g.

The crude product was purified by two silica gel chromatography using 0-5% MeOH in CH_2Cl_2 and 10% MeOH in CH_2Cl_2, respectively, as eluent. The yield of product (>98% pure) was 64 g.

Example 16

Using the methods described in Example 15, the phenyl *N*-methoxy-*L*-alanyl phosphoramidates of the following nucleosides were prepared:

1. 5-(4,4-dibromo-1,3-butadienyl)-2'-deoxyuridine,
2. 5-(2-chlorovinyl)-2'-deoxyuridine,
3. 5-trifluoromethyl-2'-deoxyuridine,
4. 5-(4-carbethoxy-1,3-butadienyl)-2'-deoxyuridine,
5. 5-(4-carbomethoxy-1,3-butadienyl)-2'-dexoyuridine,
6. 5-(4-bromo-1*E*,3*E*-butadienyl)-2'-deoxyuridine,
7. 5-(4-bromo-1*E*,3*Z*-butadienyl)-2'-deoxyuridine,
8. 5-(trimethylsilylethynyl)-2'-deoxyuridine,
9. 5-(ethynyl)-2'-deoxyuridine,
10. 5-(1-decynyl)-2'-deoxyuridine,
11. 3-(2'-deoxy-*β*-*D*-ribofuranosyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one
12. 3-(2'-deoxy-*β*-*D*-ribofuranosyl)-6-octyl-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one.

Derivatives

Salts, esters, and ethers of the above compounds disclosed herein are also within the scope of this invention. Salts of the prodrugs of the present invention may be

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derived from inorganic or organic acids and bases. Examples of acids include hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicyclic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic and benzenesulfonic acids. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, can be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts. Examples of bases include alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and compounds of formula NW_4^+, wherein W is C_{1-4} alkyl.

Examples of salts include: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylproprionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+, NH_4^+, and NW_4^+ (wherein W is a C_{1-4} alkyl group).

For therapeutic use, salts of the compounds of the present invention will be pharmaceutically acceptable. However, salts of acids and bases which are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

Esters of the prodrugs or compounds identified by the method of this invention include carboxylic acid esters (i.e., -O-C(=O)R) obtained by esterification of the 2'-, 3'- and/or 5'-hydroxy groups, in which R is selected from (1) straight or branched chain alkyl (for example, n-propyl, t-butyl, or n-butyl), alkoxyalkyl (for example, methoxymethyl), aralkyl (for example, benzyl), aryloxyalkyl (for example, phenoxymethyl), aryl (for example, phenyl optionally substituted by, for example, halogen, C_{1-4}alkyl, or C_{1-4}alkoxy or amino); (2) sulfonate esters, such as alkylsulfonyl (for example, methanesulfonyl) or aralkylsulfonyl; (3) amino acid esters (for example, L-valyl or L-isoleucyl); (4) phosphonate esters and (5) mono-, di- or triphosphate esters.

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The phosphate esters may be further esterified by, for example, a C_{1-20} alcohol or reactive derivative thereof, or by a 2,3-di-(C_{6-24})acyl glycerol. In such esters, unless otherwise specified, any alkyl moiety present advantageously-contains from 1 to 18 carbon atoms, particularly from 1 to 6 carbon atoms, more particularly from 1 to 4 carbon atoms. Any cycloalkyl moiety present in such esters advantageously contains from 3 to 6 carbon atoms. Any aryl moiety present in such esters advantageously comprises a phenyl group. Examples of lyxo-furanosyl prodrug derivatives of the present invention include, for example, those with chemically protected hydroxyl groups (e.g., with O-acetyl groups), such as 2'-*O*-acetyl-lyxo-furanosyl; 3'-*O*-acetyl-lyxo-furanosyl; 5'-*O*-acetyl-lyxo-furanosyl; 2',3'-di-*O*-acetyl-lyxo-furanosyl and 2',3',5'-tri-*O*-acetyl-lyxo-furanosyl.

Ethers of the compounds of the present invention include methyl, ethyl, propyl, butyl, isobutyl, and sec-butyl ethers.

In a further embodiment, the substrate may not be chemically related to pyrimidines or folates, but rather synthesized based upon known parameters of rational drug design. See Dunn, W.J. et al. (1996).

Chemical assays for products, for example, where a reaction product is an anti-metabolite of the bromovinyl-derivatives of dUMP, are described in the Examples provided below or by Barr, P.J. et al. (1983).

Assays for Amount of Nucleoside and Monophosphate In Cells

This assay was performed with the compound NB 1011. However, it understood to those of skill in the art that the below method is easily modifed for application or use with the prodrugs of this invention.

The cell lines MCF7 (breast cancer cell line); MCF7TDX (Tomudex resistant breast cancer cell line) and H630R10 (5-FU resistant colon cancer cell line obtained from Dr. S. Copur, Yale University, see Copur, S. et al. (1995)) in RPMI medium supplemented with 10% FCS and antibiotics were plated in 100 mm Petri dishes at the amount of 750,000 cells per dish and were incubated overnight at 37°C. 100 μM of BVdU was added to each dish and the cells continued to grow for 2 days at 37°C. After that, the medium was aspirated and the cells were washed twice with phosphate buffered saline (PBS). Then 1 ml of PBS per dish was added and the dishes were

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placed in a -80°C freezer. The cells were disrupted via freezing/thawing procedure, repeated twice. The cell debris in the resulting suspension were spun down (13,000 rpm, 10 minutes) and the supernatant was deproteinized by passing though a Centrifugal Filter Device (Centrifree, 30,000 cut-off, Amicon) by centrifugation at 5,000 rpm for 30 minutes on Sorvall Super T21 centrifuge (Rotor SL-50-T, 1,912 g) at 20°C. The pass-through was concentrated to the volume of 100 µl, and injected onto an HPLC column (Adsorbosphere HS, C18, 5 µm, 4.6 mm x 150 mm, Alltech). The samples were analyzed using acetonitrile gradient in water containing trifluoroacetic acid. The peaks were detected at 300 nm, the maximum of absorption of BVdU-derivatives, and the amounts of compounds were expressed as peak area at 300 nm. The identification of compounds was done by comparison of their retention times and spectra with those of authentic standards.

AlamarBlue Cell Proliferation Assay

This assay was performed with the compound NB1011 and the prodrugs of this invention. Cells growing exponentially were transferred to 384-well flat bottom tissue culture plates. All cell types were plated at a density of 500 cells per well in 25 µL of complete medium (RPMI 1640 + 10% fetal bovine serum + antibiotics/antimycotics). After 24 hours (day 0), 25 µL of complete medium containing the compounds over the dose range of 10^{-3} to 10^{-10} M were added in triplicate. Drug exposure time was 120 hours (day 5), after which growth inhibition was assayed. 5 µL of the redox indicator, alamarBlue, was added to each well (10% v/v). After 4 hours incubation at 37°C, fluorescence was monitored at 535 nm excitation and 595 nm emission.

Crystal Violet Cell Proliferation Inhibition Assay

This assay was performed with the compound NB1011 and the prodrugs of this invention. The ability of the test compounds to block proliferation of cells was determined by the crystal violet procedure. (Sugarman, B. J. et al. (1985); and Antelman, D. et al. (1995)).

Compounds were dissolved in dimethyl sulfoxide to a concentration of 1M. They were further diluted as necessary into DMEM cell culture medium, and subsequently into the first wells of the 96-well microtiter plate. Each concentration was

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tested in triplicate on the target cell line. Compound concentrations from 1 μM to 3000 μM were tested. Cells were incubated with compound for 72 hours, the plates were washed, and the cells fixed with methanol and stained with crystal violet as described in Sugarman, B. J. et al. (1985); and Antelman, D. et al. (1995).

Western Blot Analysis of TK and TS Levels in Cell Lines

Western blot experiments were performed using human normal colon epithelium cell type CCD18co (obtained from ATCC, Manassas, VA), colon adenocarcinoma cell line H630R10 resistant to 5-FU (obtained from Dr. S. Copur, Yale University, see Copur, S. et al. (1995), and a HER2-transfected breast cancer cell lines (Pegram, M. D. et al. (1997)). Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5,150 mM NaCl, 0.5% Triton X-100, 0.1% SDS and 0.5% deoxycholic acid, sodium salt and protease inhibitors). Protein concentrations were determined by using BCA-200 protein assay kit (obtained from Pierce, Rockford, IL). 10 μg of proteins from each cell line were resolved by 12% SDS-PAGE. The separated proteins were transferred onto PVDF membrane (obtained from Amersham, England), followed by immunoblot with human thymidylate synthase monoclonal primary antibody and anti-tubulin monoclonal antibody (manufactured by NeoMarkers, Fremont, CA). Horseradish peroxidase linked sheep anti-mouse Ig was used as secondary antibody (Amersham). The ECL plus kit (Amersham) was used for detection of immunoreactivity. The bands corresponding to thymidylate synthase were quantified and normalized to that of tubulin by image analysis (Molecular Dynamics Storm).

RT-PCR Analysis of TS mRNA in Cell Lines

Expression level of human thymidylate synthase transcripts in different cell lines were quantified by using RT-PCR. Oligonucleotide primers for amplification of the human thymidylate synthase and B-actin were designed as follows: Thymidylate synthase sense primer (SEQ ID NO:1) 5'-GGGCAGATCCAACACATCC-3' (corresponding to bases 208-226 of thymidylate synthase cDNA sequence, Genbank Accession No. X02308), antisense primer (SEQ ID NO:2) 5'-GGTCAACTCCCTGTCCTGAA-3' (corresponding to bases 564-583), β-actin sense primer (SEQ ID NO:3) 5'-GCCAACACAGTGCTGTCTG-3' (corresponding to bases

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2643-2661 of β-actin gene sequence, Genbank Accession No. M10277) and antisense primer (SEQ ID NO:4) 5'-CTCCTGCTTGCTGATCCAC-3' (corresponding to bases 2937-2955).

Total RNAs were isolated from cells using Rneasy mini kit (obtained from Qiagen, Valencia, CA). To monitor for possible DNA contamination, the primers for amplification of β-actin were designed to span the exon4/intron5/exon5 junction. Genomic DNA template leads to a 313 bp β-actin fragment, and cDNA template generates a 210 bp product.

Reverse transcription reactions were performed, using SuperScript preamplification system (Gibco/BRL, Gaithersburg, MD). 3 μg total RNA was applied in a volume of 20 μl buffer to conduct reverse transcription reaction, followed manufacturer's protocol.

PCR reactions were performed in a volume of 48 μl, containing 3 μl of cDNA mixture from reverse transcription reaction, 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-Cl, pH 8.4, 0.2 mM of each dNTP, 0.4 TM of thymidylate synthase sense and antisense primers and 3 units of Taq DNA polymerase (obtained from Promega, Madison, WI). The reaction mixtures were incubated at 94°C for 3 min, followed by 10 cycles of 1 min incubation at 94°C, 1 min incubation at 58°C, and then 1 min incubation at 72°C. After 10 cycles, human β-actin primers in 2 μl were added to achieve a final concentration of 0.2 μM, bringing the final reaction volume to 50 μl. PCR reaction was continued to a total of 28 cycles, followed by a 7 min incubation at 72°C.

5 μL of PCR products were resolved by electrophoresis in 2% agarose gel, followed by staining with SYBR Gold nucleic acid gel stain (obtained from Molecular Probes, Eugene, Oreg.). The DNA bands corresponding to thymidylate synthase were quantified and normalized to that of β-actin by Molecular Dynamics Storm.

Northern Blot Analysis

Northern blots were obtained from Invitrogen (Carlsbad, CA) and hybridized to a cloned TS cDNA probe. Hybridization signals were normalized vs. ribosomal protein S9 as a housekeeping transcript. A tumor was considered to overexpress TS mRNA if the normalized signal was enhanced at least 2-fold as compared to the normal tissue control.

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Cloning, Expression and Purification of Human Thymidylate Synthase

For expression in *E. coli* the complete human TS ORF was subcloned into the T7 promoter expression vector pET28a (Novagen, Inc.). The resulting plasmid vector encodes the synthesis of human TS as a recombinant fusion protein with a six histidine followed by a thrombin cleavage site. This fusion protein adds a total of 20 extra amino acids to the amino terminus of human TS. To produce recombinant protein, the human TS expression vector was introduced into the *E. coli* strain BL21(DE3), a strain with the T7 RNA polymerase gene inserted into the chromosome under the control of the lac operator.

The human TS-poly-His fusion protein was purified using a metal chelating affinity resin (Novagen, Inc.). Protein purification was followed by electrophoresis on 10% SDS polyacrylamide gels. Protein concentrations were determined using the Pierce BCA protein assay. Approximately 10 mg of human TS fusion protein was recovered from a 500 ml culture of *E. coli*. When visualized with silver stain only the band corresponding to human TS was apparent. The identity of the human TS band was confirmed by performing a Western blot with the anti-TS monoclonal antibody TS106 (NeoMarkers).

Thymidylate Synthase (TS) Enzyme Activity Assay

TS activity was measured using the spectrophotometric assay of Wahba, et al. (1961). In this assay, TS activity is monitored by measuring the increase in absorbency at 340 nm that occurs when the co-factor 5,10 methylene tetrahydrofolte is oxidized to dihydrofolate as dUTP is converted to dTTP. Enzyme prepared by this method has a specific activity of 0.5 - 0.65 units/mg protein. One unit is defined as the production of one micromole of dTMP per minute. This value is similar to that reported by Pedersen-Lane, J. et al. (1997).

Determination of Intracellular Products of TS Prodrug Metabolism

It is important to determine the intracellular products of TS prodrug metabolism in order to substantiate proposed mechanism of activation and action and to define agents that are candidate therapeutics. One acceptable view of intracellular metabolism of aryl phosphodiester amidates involves enzymatic conversion into a carboxylic acid,

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intramolecular rearrangement of the phosphomonoester amidate into to a 5'-monophosphoryl nucleoside. See, Valette et al. (1996). However, this mechanism is unlikely to describe intracellular processing of all phosphoramidate-based pronucleotides. For example, a different mechanism was proposed for aryl phosphomonoester amidate processing, one involving the simple direct conversion of the phosphoramidate to the monophosphate species by a phosphoramidate hydrolates. See, McIntee et al. (1997) and Fries et al. (1995). Regardless of the mechanism for unmasking the nucleoside monophosphate, this assay will detect products of TS conversion of the intracellular monophosphate to cytotoxic compounds within the cell.

Cells are incubated with an amount of prodrug compounds that induces 50% growth inhibition of high TS expressing cell lines (in the 72H assay, *supra*). Both low and high TS expresser cells are used (e.g., CCD18co vs. H630R10). Time course studies are performed in which treated cells are processed according to the method described by McIntee, E. J. et al. (1997). Cells are lysed with 60% methanol in water at -20°C, and particulate residue removed by centrifugation. The supernatants are dried and stored at -20°C. The aliquots are evaluated initially by RP-HPLC and then by LC-MS to document the intracellular conversation of the phosphoramidate to the monophosphate and also the ensuring transformation of the monophosphate by thymidylate synthase.

Documentation of Substrate Activity Utilizing Purified Thymidylate Synthase

This assay determines the specific activities of future TS enzyme preparations, to assure the activity of such preparations over time, and to determine whether compounds screened for suitable activity do inactivate the TS enzyme under *in vitro* reaction conditions.

To determine what reaction products are produced when prodrug compounds are incubated together with purified recombinant TS enzyme, reaction conditions similar to those employed with the TS activity assay are used to generate reaction products *in vitro*. These reaction products will are then analyzed by GC-mass spectrometry to identify the actual molecules formed.

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Results

TS is highly expressed in several tumor cell lines and primary tumors

Thymidylate synthase expression levels in normal human brain, heart, kidney, spleen, liver, colon, lung, small intestine, stomach muscle, testis, ovary, uterus, prostate, thyroid gland, salivary gland, adrenal gland, skin, peripheral blood lymphocytes, bone marrow and from human colon and matched human normal tissues were determined using qualitative RT-PCR, as described above. Primers for β-actin were designed to span the exon4/intron5/exon5 junction to monitor for possible DNA contamination. Therefore, results reported in Figures 3 are shown as relative TS mRNA levels. Figure 3A shows relative TS mRNA in the multiple normal human tissues. Figure 3B is a comparison of average TS mRNA between normal and tumor human colon tissues.

Thymidylate synthase (TS) protein levels in various cultured normal and tumor cell lines were determined following procedures described above. Protein levels were measured by Western blot analysis; the results are shown below.

Table 3.
TS protein level in human normal and tumor cell types

Cell strains	Description	TS level[1]
NHOST	Bone, osteoblast	51
NPRSC	Prostate, stroma	98
CCD18co	Colon epithelium, fibroblast	100
WI38	Lung, embryonic	150
DET551	Skin, fibroblast-like, embryonic	177
MRC9	Lung, fibroblast-like, embryonic	211
NHDF	Skin, fibroblast	224
NHLF	Lung, fibroblast	236
Average		156±24[2]

1. Thymidylate Synthase (TS) - TS levels were determined by using Western blot analysis, the quantified expression levels were expressed as values relative to that of cell strain CCD 18co.
2. Standard error

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Tumor Cells (≥ 4X TS)

Cell lines	Description	TS level[1]
H630/TDX	Colon, carcinoma, TDX[2] resistant	671
HCTC(+)	Colon, carcinoma, increased TS	1276
MCF7/TDX	Breast, adenocarcinoma, TDX[2] resistant	1980
H630R10	Colon, carcinoma, 5-FU[3]resistant	2305
Average		1558±354[4]

1. Thymidylate Synthase (TS) - TS levels were determined by using Western blot analysis, the quantified expression levels were expressed as values relative to that of cell strain CCD 18co.
2. Tomudex
3. 5-Fluorouracil
4. Standard Error

The Amounts of BVdUMP and BVdU Measured In Cells

The amounts of BVdUMP or BVdU present in lysates cell lines MCF7, MCF7TDX, and H630R10 each treated with BVdU, are expressed as an area of a corresponding HPLC peak at 300 nm. Note that extinction coefficients of these compounds at 300 nm are the same, therefore the comparison of the peak areas allows direct comparison of the amounts formed.

Table 4, below, shows the level of BVdUMP and BVdU measured inside the cells after 2 days treatment. Cells were incubated in complete media with 100 μm BVdU. Lysates were prepared and BVdUMP and BVdU levels were determined.

Table 4.

Cell Lines	BVdUMP	BVdU	BvdUMP/(BVdUMP + BVdU) x100
MCF7	**63.7**	**90.8**	**44.2**
MCF7TDX	**10.9**	**39.0**	**21.2**
H630R10	**< 1**	**26.3**	**< 4**

The table shows that BVdU can penetrate all three cell types, but appears as BVdUMP most prominently in MCF7 and MCF7TDX cell lines. Little conversion occurs in the H630R10 cell line.

In a separate experiment, MCF7TDX and H630R10 cell lysates were used to catalyze BVdU phosphorylation in a cell free system with ATP added as a phosphate

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donor method. This confirmed the much higher rate of this reaction in MCF7TDX cell lyzate as compared to H630R10 cell lysate. Tumor lysates prepared as described (Look, K.Y. et al. (1997)) can be prepared and used in this assay with BVdU (or other candidate nucleoside) to determine whether a given patient is a candidate for nucleoside versus phosphoramidate therapy.

These results show that in MCF7TDX cells BVdU can be phosphorylated to yield the corresponding monophosphate, a substrate for subsequent TS reaction whereas in H630R10 cells such phosphorylation goes on significantly slower than in MCF7TDX. It is important to mention that MCF7TDX is a breast tumor cell line. It has been reported that in breast tumor cells a TK with properties different from human TK from other tissue is found. (Madec, A. et al. (1988)). This TK might be responsible for BVdU phosphorylation. Based on the results presented above it is expected that nucleosides, such as BVdU, might be especially useful for treatment of certain types of cancer, including breast cancer, where BVdU can be phosphorylated. Moreover, the experimental procedure described above, allows *in vitro* determination of feasibility of using BVdU or other nucleosides for a given cancer type using the following approach. If a tissue sample is available the cells may be treated *in vitro* with BVdU and possible formation of BVdUMP can be monitored. Should BVdUMP formation be registered the tumor type must be considered as a candidate for successful nucleoside treatment as opposed to phosphoramidate derivatives.

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Table 5

Alamar Blue Cytotoxicity Assay of Normal and Tumor Cells

	MCF7TDX	IC50 (μM) H630R10	HT1080	Mean Tumor	IC50 (μM) CCd18co	Det551	Mean Normal
NB1011	2	82	182	88.7	414	398	406
BVDU	0.02	201	719	306.7	1000	ND	1000
NB1012	127	82	-	104.5	ND	110	110
NB1013	26	92	ND	59.0	ND	570	570
NB1020	0.48	326	1000	442.2	1000	ND	1000
NB1014	396	287	ND	341.5	ND	239	239
NB1016	877	337	ND	607.0	ND	338	338
NB1021	637	141	1000	592.7	1000	ND	1000
NB1017	55	52	ND	53.5	ND	137	137
NB1024	4	14	258	92.0	1000	ND	1000
NB1018	115	164	ND	139.5	ND	412	412
NB1022	8	0.03	3	3.7	9	ND	9
NB1019	251	95	ND	173.0	ND	ND	-
NB1023	5	0.11	2	2.4	11	ND	11
NB1026	1000	1000	1000	1000.0	1000	ND	1000
NB1025	49	192	454	231.7	378	ND	378

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Table 6.

Crystal Violet Assay of Normal and Tumor Cells

	IC50 (uM)		Mean	IC50 (uM)		Mean
	H630R10	HT1080 #12	Tumor	CCD18co	Det551	Normal
NB1011	130	1.2	65.6	408	356	382
BVDU	405	7	206.0	1000	625	812.5
NB1017	111	17	64.0	206	253	229.5
NB1024	92	3.3	47.7	784	460	622
NB1018	248	20	134.0	254	431	342.5
NB1022	3.8	0.3	2.1	0.7	3.6	2.15
NB1019	220	24	122.0	162	824	493
NB1023	2.7	0.2	1.5	0.9	3.3	2.1

Numbering in Tables 5 and 6 refer to the structures, shown infra. Results from testing using the alamarBlue assay are shown in Table 5, and results from crystal violet based assays are shown in Table 6. MCF7-TDX was derived from MCF7 breast tumor cells via selection in cell culture in the presence of Tomudex, a direct inhibitor of TS. Such selection often results in high intracellular levels of TS (Freemantle, S. J. et al. (1995)). Similarly, H630R10 is a 5FU (fluoropyrimidine) resistant colon cancer epithelial cell line derived from H630 colon cancer cells. See, Copur, S. et al. (1995). HT1080, #12, is a fibrosarcoma tumor cell line that expresses high levels of TS via a transgene introduced into HT1080 tumor cells. Normal cell lines used in these assays include CCD18co (normal colon epithelium) and Det551 (normal skin).

The data in both Tables 5 and 6 indicate that most compounds are active as phosphoramidates and nucleosides. One exception is NB1026™, which has little detectable activity as a phosphoramidate, but is active as a nucleoside (NB1025™). This result indicates that NB1026™ may not be activated similar to NB1011™. Cytotoxicity results with the nucleosides, especially BVdU, NB1020™ (ClVdU) and NB1024™, are surprising since the literature teaches that 5- substituted compounds like BVdU are not efficiently monophosphorylated by human cells unless they express viral

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thymidine kinase. See, Balzarini, J. et al. (1985) and De Clerq, E. et al. (1997). These authors have proposed that once phosphorylated by the herpesvirus-encoded TK, the 5-substituted nucleotide derivative can bind to the TS enzyme and inactivate it. The results in Tables 4-6 demonstrate, for the first time, that at least some tumor cells may have an unusual thymidine kinase activity capable of phosphorylating 5-substituted uridine molecules similar to BVdU, and the other nucleosides shown herein. Alternatively, some tumor cells may have an unusually high amount, or form the normal TK enzyme (Suki, S. et al. (1995); and Romain, S. et al. (1995)), leading to a low, but sufficient activation of nucleosides to their corresponding monophosphates.

The compound NB1024™ as tested above consists of a mixture of two isomers, 5-(4-Bromo-1*E*,3*E*-butadienyl)-2'-dexoyuridine (VIIa), (Isomer 2), and 5-(4-Bromo-1*E*,3*Z*-butadienyl) -2'-dexoyuridine (VIIb), (Isomer 1). These two compounds were separated by semi-preparative HPLC (reversed phase C18 column) using 20% acetonitrile in water as the mobile phase as described above (Example 12). The ability of each isolated compound to block cell proliferation was then determined by the crystal violet procedure. Results are shown in Table 7. The two isomers display markedly different activities with the Z isomer, showing significantly greater growth inhibiting activity in comparison with the E isomer.

Table 7. Cytotoxic Activity of NB1024 Isomers

	TS HT1080 #12	MCF7TDX	CCD18co	Det551
NB1011	4.3	4.2	461	263
BVdU	ND	<0.8	>1000	>1000
NB1024 Mixture	10.7	6.4	>300	>300
NB1024 Isomer 1	10.1	9.8	>300	>300
NB1024 Isomer 2	>300	>300	>300	>300

The first two cell lines, TS HT1080 #12 and MCF7TDX express high levels of TS, while the other two, CCD18co and Det551, are normal cells.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes

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and modifications can be made therein without departing from the spirit and scope thereof.

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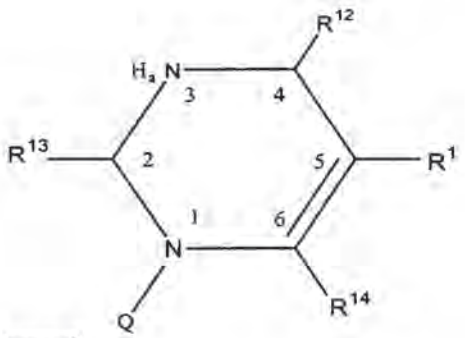
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What is claimed is:

1. A compound having the structure:



or tautomers thereof;

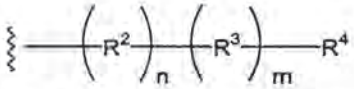
wherein R^{12} or R^{13} may be the same or different and are selected from the group consisting of oxo, OH or $NHNH_2$;

wherein if a is 0 or 1, providing that if a is 0 and R^{13} is oxo, then a double bond exits between position 3 and 4 and R^{12} is $NHNH_2$ or

if a is 0 and R^{12} is oxo, then a double bond exists between position 2 and 3 and R^{13} is $NHNH_2$; or

if a is 1, then R^{12} and R^{13} are both oxo;

wherein R^1 is a substituent having the formula:



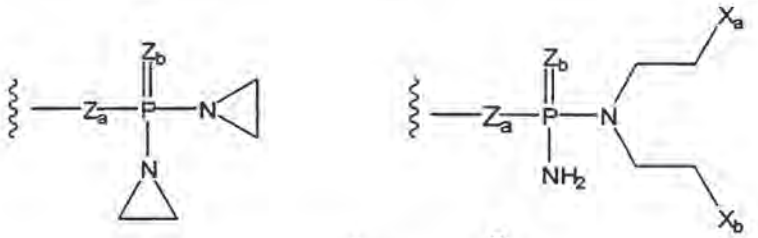
wherein R^2 and R^3 are independently an unsaturated or a saturated hydrocarbyl group;

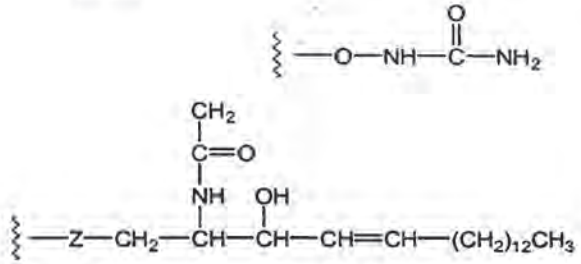
wherein n is 0 or an integer from 1 to 10 and m is 0 or 1; and

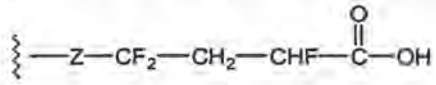
wherein R^4 is selected from the group consisting of F, Cl, Br, I, CN, SO_3H, CO_2H, $CO_2CH_2CH_3$, CO_2CH_3, $SI(CH_3)_3$, CHO, NO_2, CF_3, CCl_3,

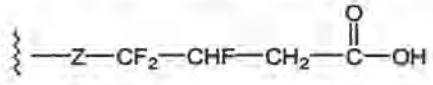
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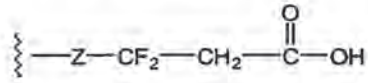
$CH=C(R^{15})_2$ or a substituent having the structure:

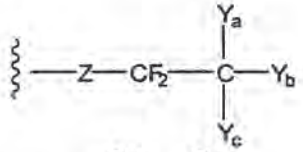


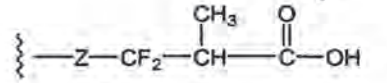


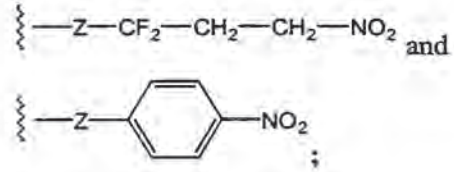












and

;

wherein X_a and X_b are independently the same or different and are selected from the group consisting of Cl, Br, I, and a potent leaving group;

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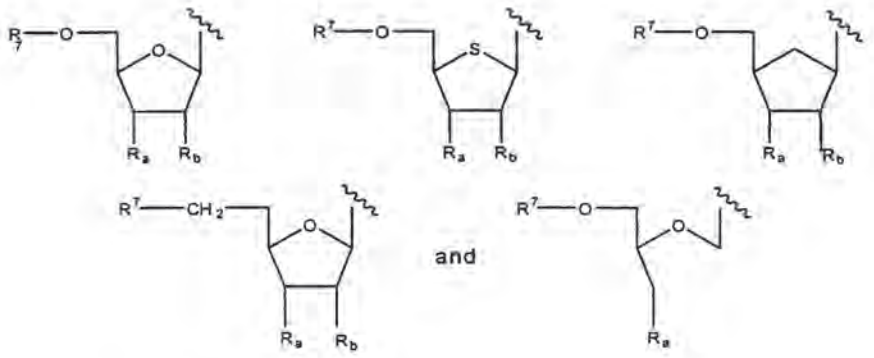
wherein Y_a, Y_b or Y_c are independently the same or different and are H or F;

wherein Z, Z_a and Z_b are independently the same or different and are selected from the group consisting of O and S; and

wherein R^{14} is H or F, providing if R^{14} is F, then a is 1 and R^{12} and R^{13} are both oxo; and

wherein Q is selected from the group consisting of a sugar, a carbocyclic, and an acyclic compound, or a masked phosphate derivative or phosphoramidate derivative thereof.

2. The compound of claim 1, wherein Q is selected from the group of substituents having the structure:



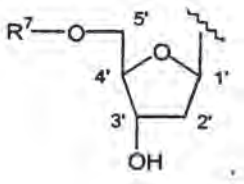
wherein R_a and R_b are independently the same or different and are selected from the group consisting of Br, Cl, F, I, H, OH, $OC(=O)CH_3$, and a protected hydroxyl group; and

wherein R^7 is attached to Q at the 5' position of Q and is selected from the group consisting of a hydrogen, a phosphate group, a phosphodiester group or a phosphoramidate group;

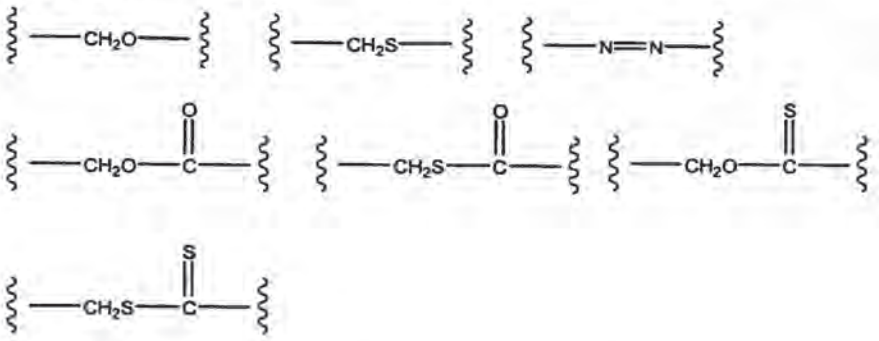
and wherein the compound may be in any enantiomeric, diasteriomeric, or stereoisomeric form, including D-form, L-form, α-anomeric form, and β-anomeric form.

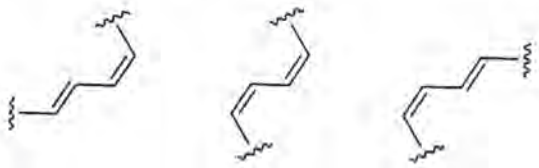
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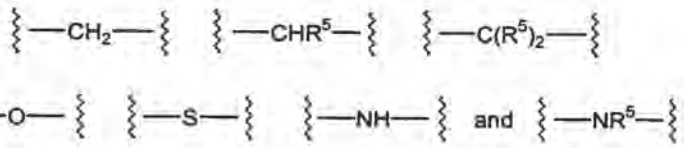
3. The compound of claim 2, wherein Q is:



4. The compound of claim 1, wherein m is 0 and R^2 is selected from the group consisting of:



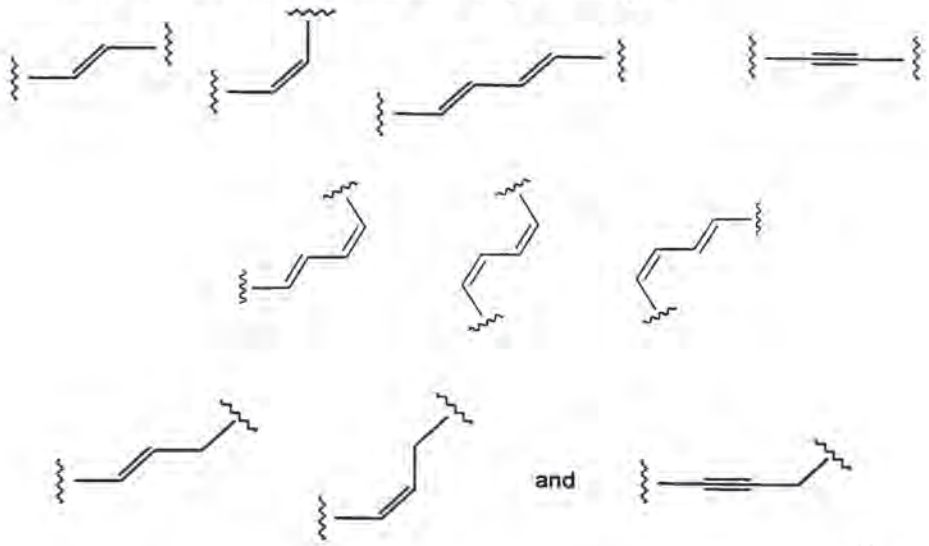




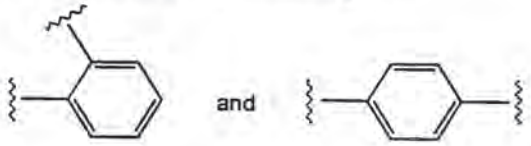
wherein R^5 is independently the same or different and is selected from the group consisting of a linear or branched alkyl group having from 1 to 10 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, CN and a halogen.

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5. A compound of claim 1, wherein R^2 and R^3 taken together is an alkenyl or alkynyl and is selected from the group consisting of:



6. A compound of claim 1, wherein m is 0 and R^2 is an aromatic hydrocarbyl group selected from the group consisting of:



7. A compound of claim 1, wherein m is 0 and R^2 is a heteroaromatic group selected from the group consisting of:

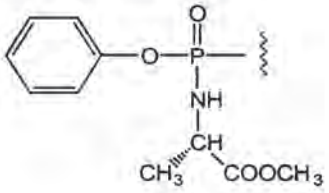


wherein J is selected from the group consisting of -O-, -S-, -Se-, -NH-, and -NR^{ALK}-.

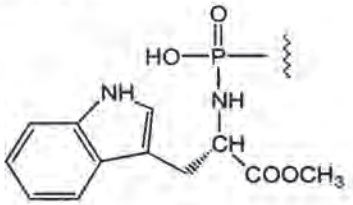
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and wherein R^{ALK} is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

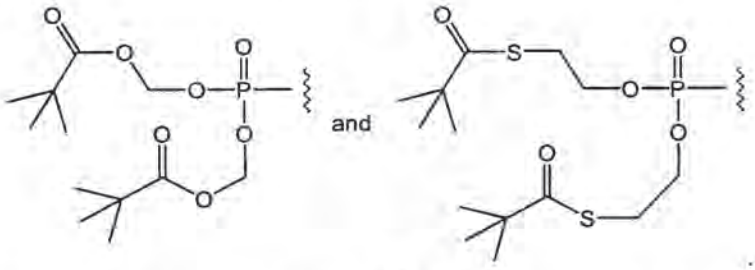
8. A compound of claim 2, wherein R^7 is:



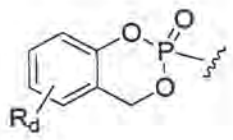
9. A compound of claim 2, wherein R^7 is:



10. A compound of claim 2, wherein R^7 is selected from the group consisting of:

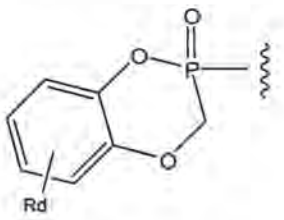


11. A compound of claim 2, wherein R^7 is selected from the group consisting of:

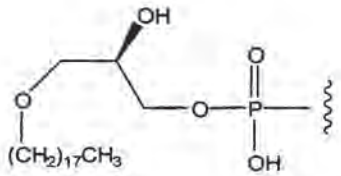


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or a compound having the structure:

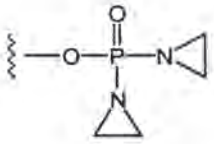


and

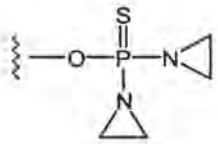


wherein R_d is an aromatic substituent.

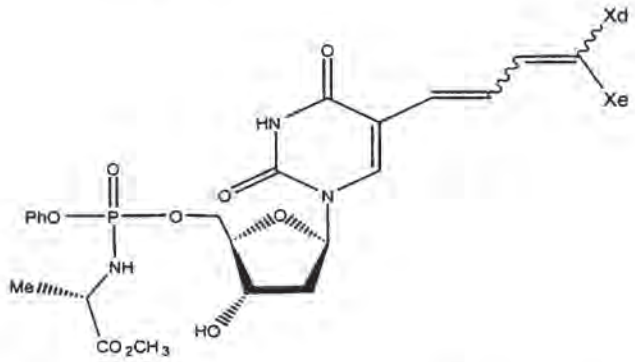
12. A compound of claim 1, wherein R^4 is selected from the group consisting of:



and



13. A compound having the structure:



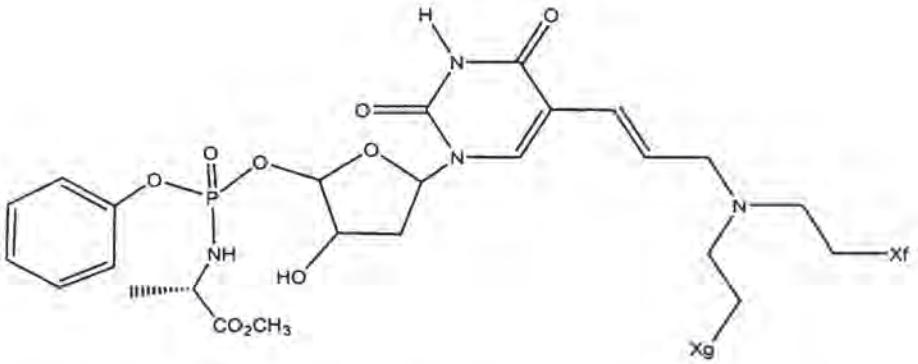
wherein X_d and X_e are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN;

or the nucleoside analogs thereof.

14. The compound of claim 13, wherein X_d is Cl or Br and X_e is H.

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15. A compound having the structure:



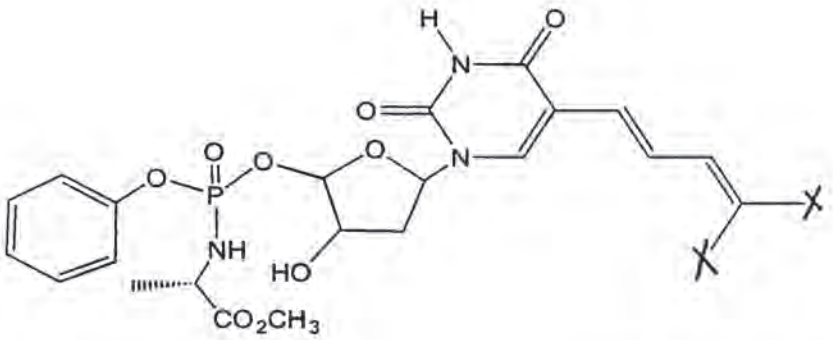
wherein X_f and X_g are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN;

or the nucleoside analogs thereof.

16. The compound of claim 15, wherein X_f and X_g are the same and are each is Cl or Br.

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17. A compound having the structure of the formula:

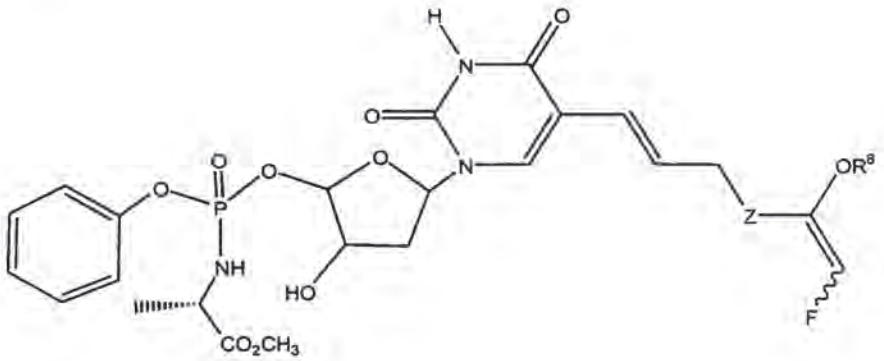


wherein each X is selected from the group consisting of Cl, Br, I, and CN; or the nucleoside analogs thereof.

18. The compound of claim 17, wherein X is Cl or Br.

19. The compound of claim 18, wherein X is Br.

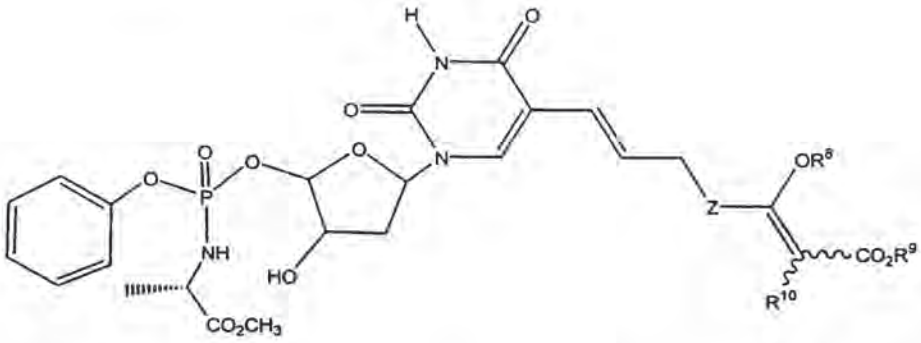
20. A compound having the structure:



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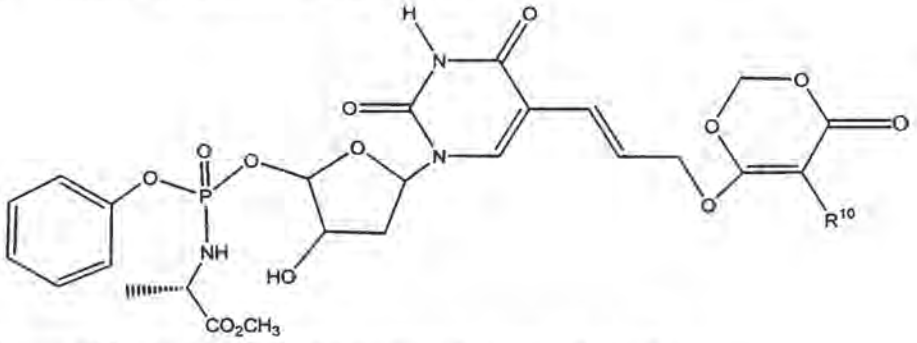
wherein R^8 is a lower straight or brainched chain alkyl;
or the nucleoside analogs thereof.

21. A compound having the structure:



wherein R^8 and R^9 are lower straight or branched chain alkyls and R^{10} is H or CH_3;
or the nucleoside analogs thereof.

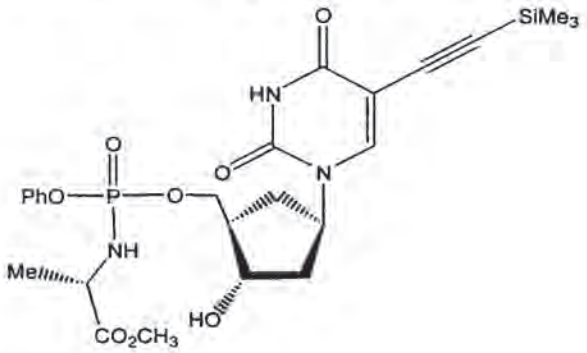
22. A compound having the structure:



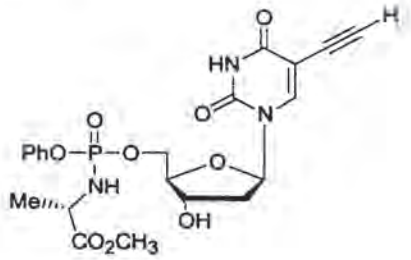
wherein R^{10} is H or CH_3;or the nucleoside analog thereof.

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23. A compound having the structure:

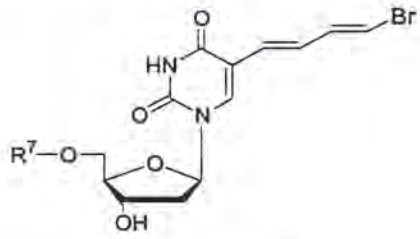


24. A compound having the structure:



or the nucleoside analog thereof.

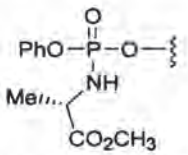
25. The compound of claim 1, wherein the compound has the structure:



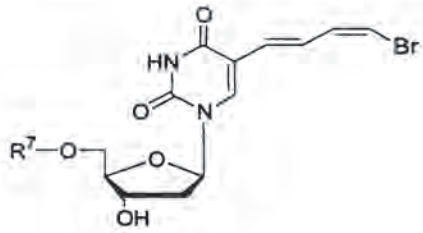
26. The compound of claim 25, wherein $R^7 = H$.

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27. The compound of claim 25, wherein R^7 is a substituent having the structure:

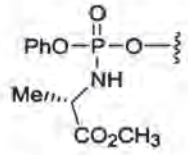


28. The compound of claim 1, wherein the compound has the structure:

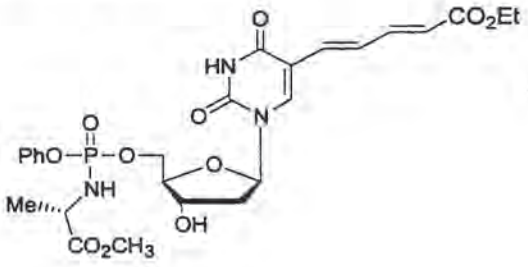


29. The compound of claim 28, wherein $R^7 = H$.

30. The compound of claim 28 wherein R^7 is a substituent having the structure

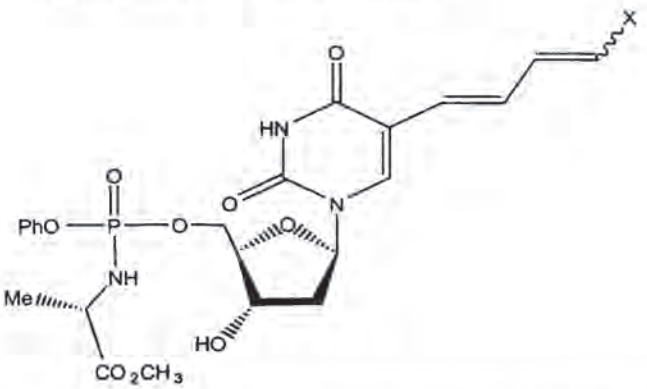


31. A compound having the structure:



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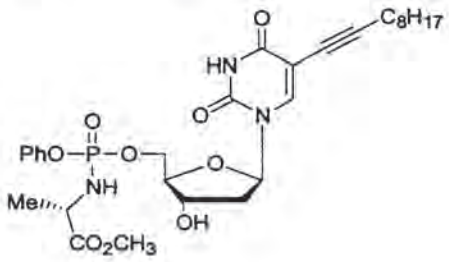
32. A compound having the structure:



wherein X is selected from the group consisting of CO_2Et, Cl, and Br;

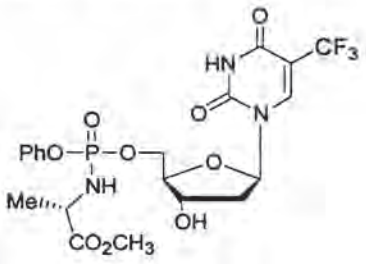
or the nucleoside analog thereof.

33. A compound having the structure:



or the nucleoside analog thereof.

34. A compound having the structure:



or the nucleoside analog thereof.

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35. A composition comprising the compounds of claims 1 to 34, and a carrier.

36. The composition of claim 35, wherein the carrier is a pharmaceutically acceptable carrier.

37. A method for screening for a therapeutic agent, comprising:
(a) contacting a sample containing a target cell with any of the compounds of claims 13 to 34;
(b) contacting a separate sample of the target cell with a potential therapeutic agent; and
(c) comparing the samples for inhibition of cellular proliferation or cell killing.

38. The method of claim 37, wherein the target cell is characterized as resistant to a chemotherapeutic drug.

39. The method of claim 37, wherein the target cell is characterized as expressing a target enzyme that is amplified as a result of selection *in vivo* by chemotherapy.

40. The method of claim 37, wherein the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell.

41. Use of any of the compounds of claims 13 to 34 for the preparation of a medicament to treat or inhibit the proliferation of a pathological cell.

42. A method for inhibiting the proliferation of a pathological cell, comprising contacting the cell with an effective amount of any of the compounds of claims 13 to 34.

43. The method of claim 42, wherein the pathological cell is resistant to a chemotherapeutic drug.

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44. The method of claim 42, wherein the pathological cell expresses a target enzyme that is amplified as a result of selection *in vivo* by chemotherapy.

45. The method of claim 44, wherein the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell.

46. The method of claim 45, wherein the enzyme is thymidylate synthase.

47. A method for treating a pathology characterized by the proliferation of target cells in a subject comprising administering to the subject any of the compounds of claims 13 to 44.

48. The method of claim 47, wherein the target cell is resistant to a chemotherapeutic drug.

49. The method of claim 47, wherein the target cell expresses an enzyme that is amplified as a result of selection *in vivo* by chemotherapy.

50. The method of claim 49, wherein the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell.

51. The method of claim 50, wherein the enzyme is thymidylate synthase.

52. The method of claim 48, further comprising contacting the cell with the prior chemotherapy after resistance to the prior chemotherapy has been reversed.

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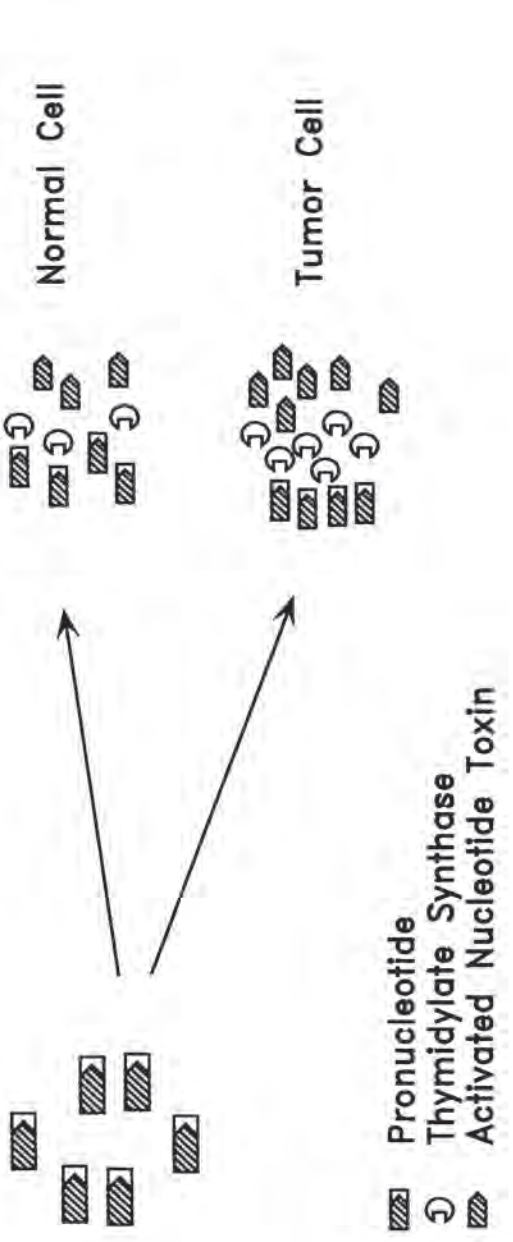


Fig. 1A

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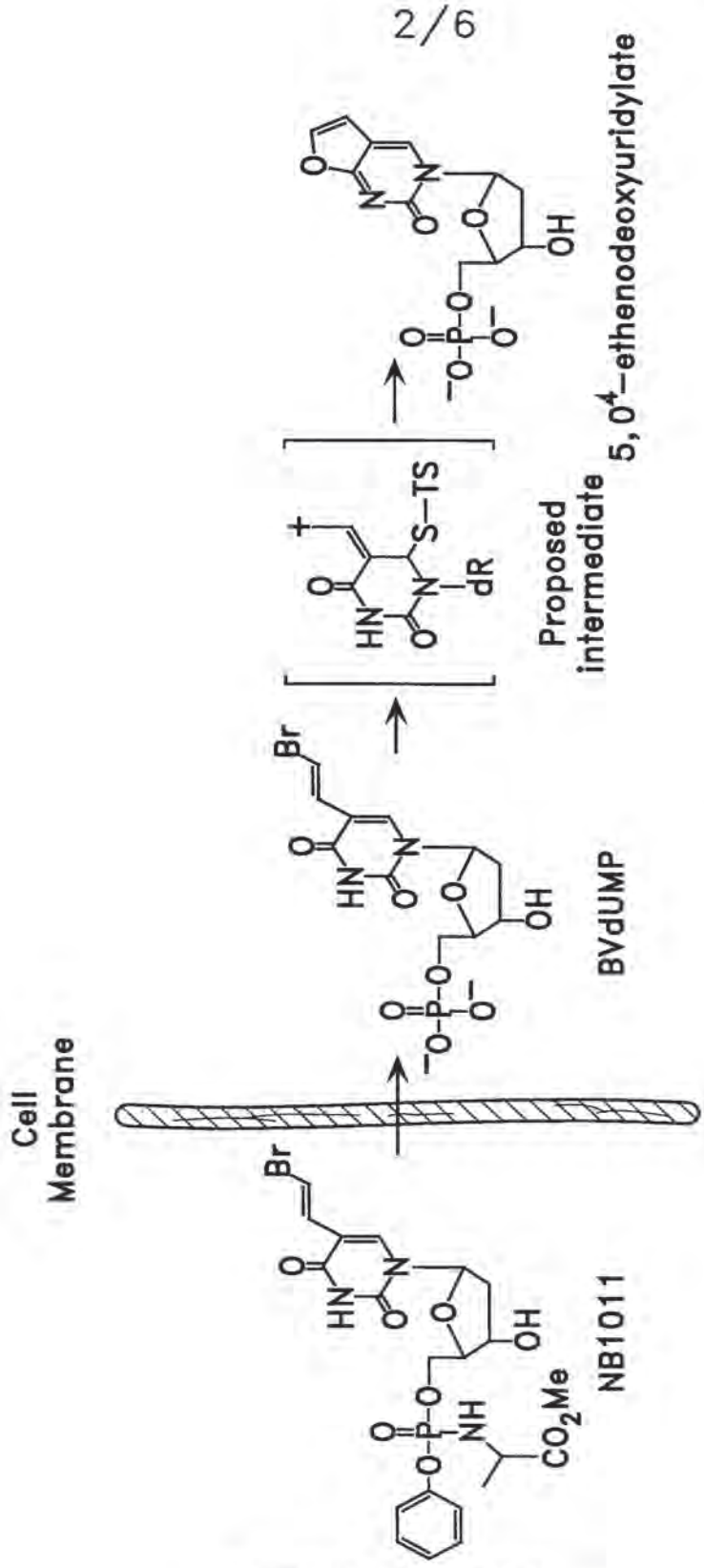


Fig. 1B

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How to find a TS lead molecule:
Design of the High Throughput Screen
e.g., Using Thymidylate Synthase as
the Target Enzyme

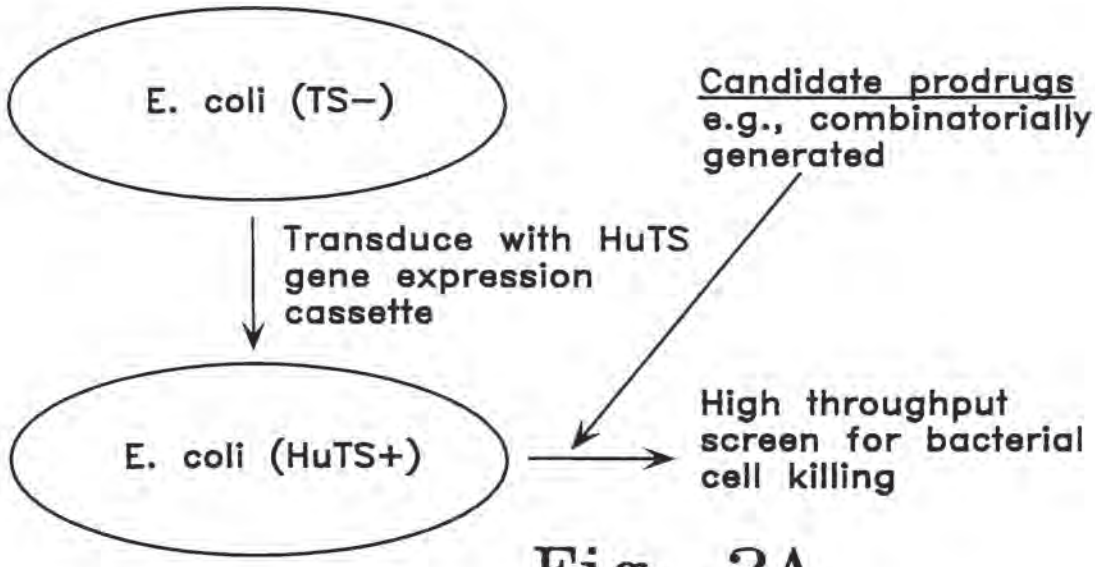


Fig. 2A

How to find a TS lead molecule:
Replacement of two enzymes,
e.g., Thymidylate Synthase (TS)
and Thymidine Kinase (TK)

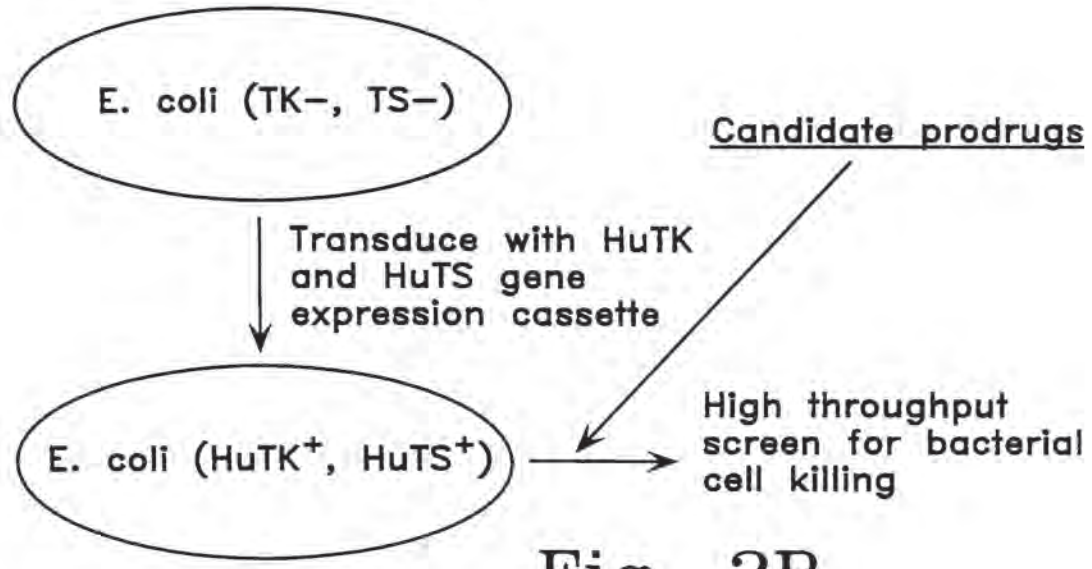


Fig. 2B

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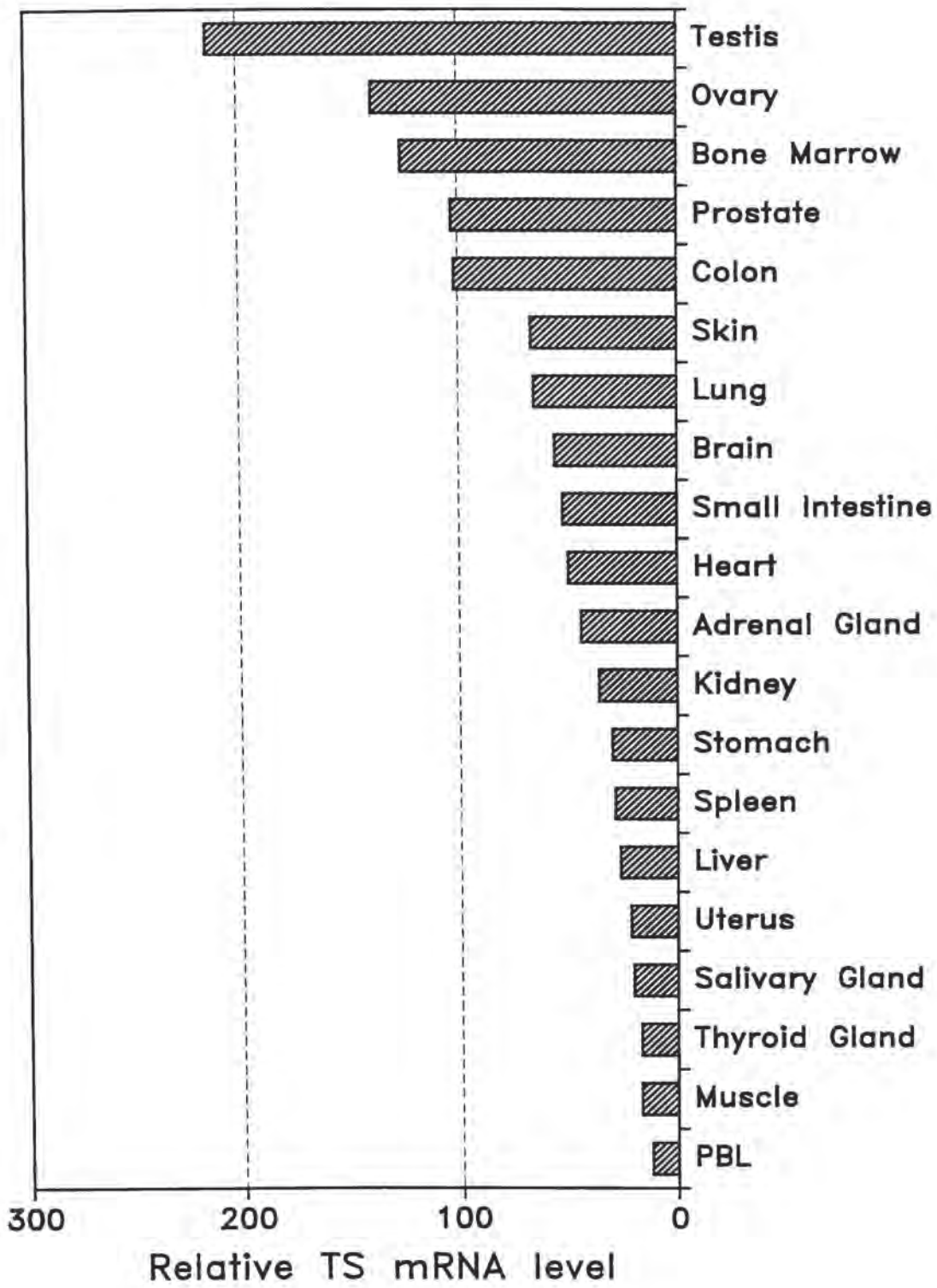


Fig. 3A

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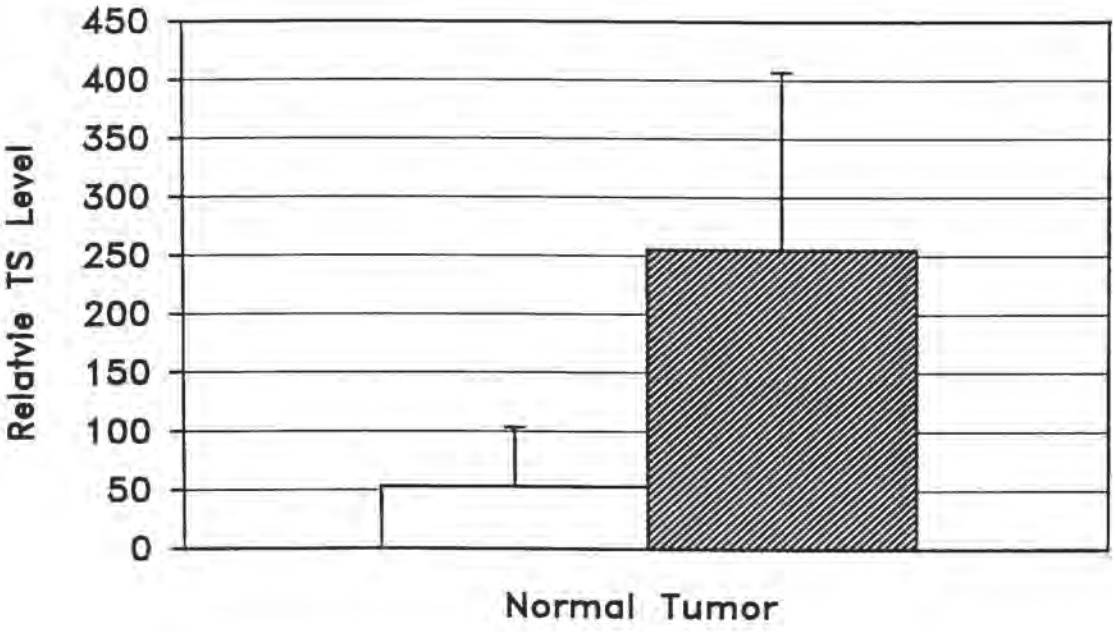


Fig. 3B

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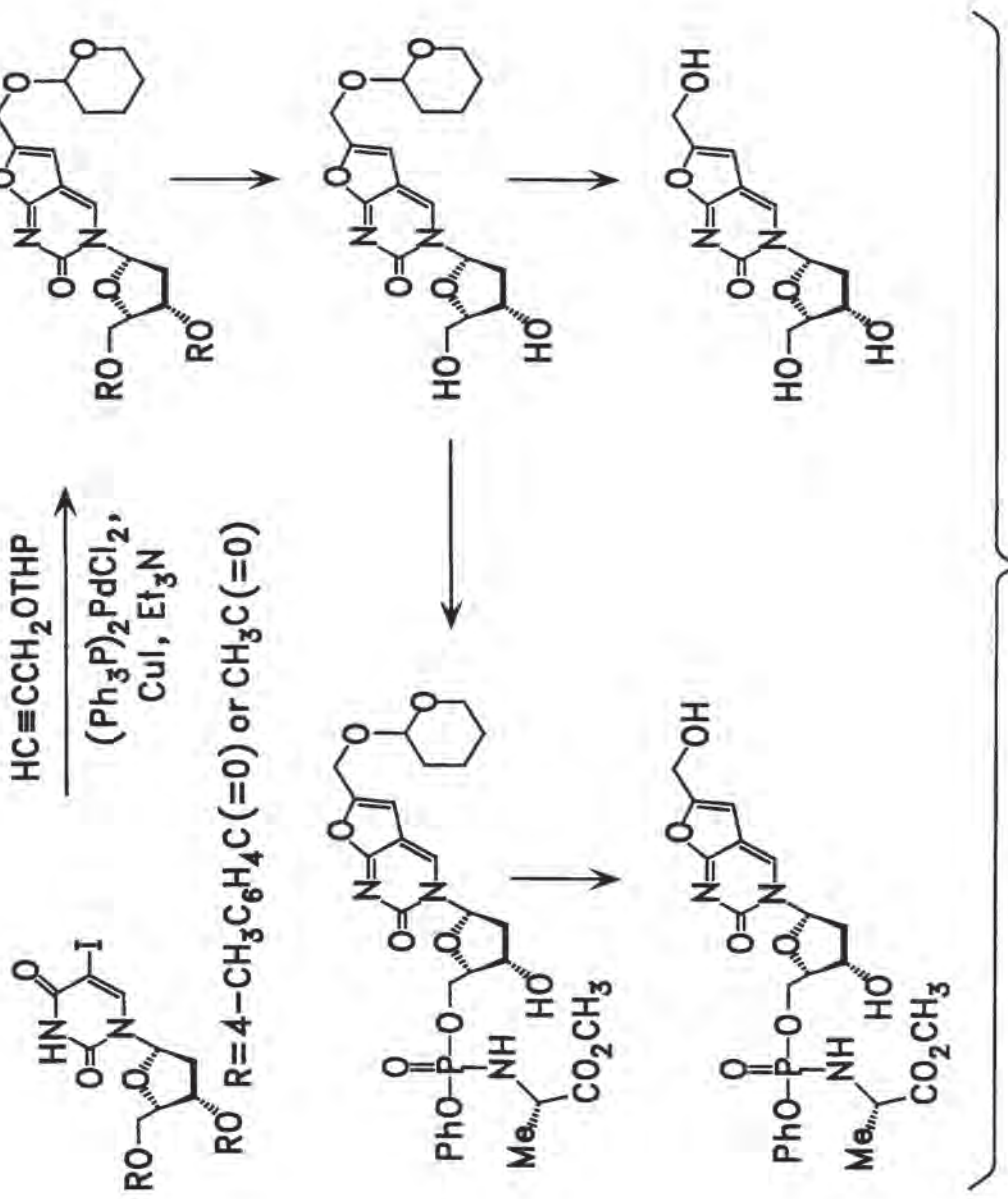


Fig. 4

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 October 2011 (06.10.2011)

PCT

(10) International Publication Number
WO 2011/123645 A2

(51) International Patent Classification:
C07H 19/06 (2006.01) *C07F 9/26* (2006.01)
C07H 19/207 (2006.01) *A61P 31/14* (2006.01)
A61K 31/7072 (2006.01)

(21) International Application Number:
PCT/US2011/030725

(22) International Filing Date:
31 March 2011 (31.03.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/319,548	31 March 2010 (31.03.2010)	US
61/319,513	31 March 2010 (31.03.2010)	US
12/783,680	20 May 2010 (20.05.2010)	US

(71) Applicant *(for all designated States except US)*: **PHARMASSET, INC.** [US/US]; 303a College Road East, Princeton, NJ 08540 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROSS, Bruce, S.** [US/US]; 8 Keppel Road, Plainsboro, NJ 08536 (US). **SOFIA, Michael, Joseph** [US/US]; 3066 Antler Drive, Doylestown, PA 18901 (US). **PAMULAPATI, Ganapati, Reddy** [IN/US]; 4608 Hunters Glen Drive, Plainsboro, NJ 08536 (US). **RACHAKONDA, Suguna** [IN/US]; 1272 Sharonbrook Drive, Twinsburg, OH 44087 (US). **ZHANG, Hai-Ren** [US/US]; 8680 Manahan Drive, Ellicott City, MD 21043 (US). **CHUN, Byoung-Kwon** [KR/US]; 135 Heritage Street, Robbinsville, NJ 08691 (US). **WANG, Peiyuan** [CN/US]; 20 Radburn Road, Glen Rock, NJ 07452 (US).

(74) Agents: **KOWALCHYK, Katherine, M.** et al.; Merchant & Gould P.C., P.O.Box 2903, Minneapolis, MN 55402-0903 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) Title: NUCLEOSIDE PHOSPHORAMIDATES

(57) Abstract: Disclosed herein are nucleoside phosphoramidates and their use as agents for treating viral diseases. These compounds are inhibitors of RNA-dependent RNA viral replication and are useful as inhibitors of HCV NS5B polymerase, as inhibitors of HCV replication and for treatment of hepatitis C infection in mammals.

WO 2011/123645 A2

NUCLEOSIDE PHOSPHORAMIDATES

Priority

This application is claims priority to U.S. Patent Application No. 61/319,513, filed March 31, 2010, U.S. Patent Application No. 61/319,548, filed March 31, 2010, and U.S. Patent Application No. 12/783,680, filed May 20, 2010, the subject matter of which are incorporated by reference in their entirety.

Field of the Invention

Disclosed herein are nucleoside phosphoramidates and their use as agents for treating viral diseases. These compounds are inhibitors of RNA-dependent RNA viral replication and are useful as inhibitors of HCV NS5B polymerase, as inhibitors of HCV replication and for treatment of hepatitis C infection in mammals.

Background

Hepatitis C virus (HCV) infection is a major health problem that leads to chronic liver disease, such as cirrhosis and hepatocellular carcinoma, in a substantial number of infected individuals, estimated to be 2-15% of the world's population. There are an estimated 4.5 million infected people in the United States alone, according to the U.S. Center for Disease Control. According to the World Health Organization, there are more than 200 million infected individuals worldwide, with at least 3 to 4 million people being infected each year. Once infected, about 20% of people clear the virus, but the rest can harbor HCV the rest of their lives. Ten to twenty percent of chronically infected individuals eventually develop liver-destroying cirrhosis or cancer. The viral disease is transmitted parenterally by contaminated blood and blood products, contaminated needles, or sexually and vertically from infected mothers or carrier mothers to their offspring. Current treatments for HCV infection, which are restricted to immunotherapy with recombinant interferon-α alone or in combination with the nucleoside analog ribavirin, are of limited clinical benefit. Moreover, there is no established vaccine for HCV. Consequently, there is an urgent need for improved therapeutic agents that effectively combat chronic HCV infection.

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The HCV virion is an enveloped positive-strand RNA virus with a single oligoribonucleotide genomic sequence of about 9600 bases which encodes a polyprotein of about 3,010 amino acids. The protein products of the HCV gene consist of the structural proteins C, E1, and E2, and the non-structural proteins NS2, NS3, NS4A and NS4B, and NS5A and NS5B. The nonstructural (NS) proteins are believed to provide the catalytic machinery for viral replication. The NS3 protease releases NS5B, the RNA-dependent RNA polymerase from the polyprotein chain. HCV NS5B polymerase is required for the synthesis of a double-stranded RNA from a single-stranded viral RNA that serves as a template in the replication cycle of HCV. Therefore, NS5B polymerase is considered to be an essential component in the HCV replication complex (K. Ishi, et al, *Heptology*, 1999, 29: 1227-1235; V. Lohmann, et al., *Virology*, 1998, 249: 108-118). Inhibition of HCV NS5B polymerase prevents formation of the double-stranded HCV RNA and therefore constitutes an attractive approach to the development of HCV-specific antiviral therapies.

HCV belongs to a much larger family of viruses that share many common features.

Flaviviridae Viruses

The Flaviviridae family of viruses comprises at least three distinct genera: *pestiviruses*, which cause disease in cattle and pigs; *flavivruses*, which are the primary cause of diseases such as dengue fever and yellow fever; and *hepaciviruses*, whose sole member is HCV. The flavivirus genus includes more than 68 members separated into groups on the basis of serological relatedness (Calisher et al., *J. Gen. Virol*, 1993,70,37-43). Clinical symptoms vary and include fever, encephalitis and hemorrhagic fever (*Fields Virology*, Editors: Fields, B. N., Knipe, D. M., and Howley, P. M., Lippincott-Raven Publishers, Philadelphia, PA, 1996, Chapter 31, 931-959). Flaviviruses of global concern that are associated with human disease include the Dengue Hemorrhagic Fever viruses (DHF), yellow fever virus, shock syndrome and Japanese encephalitis virus (Halstead, S. B., *Rev. Infect. Dis.*, 1984, 6, 251-264; Halstead, S. B., *Science*, 239:476-481, 1988; Monath, T. P., *New Eng. J. Med*, 1988, 319, 64 1-643).

The pestivirus genus includes bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV, also called hog cholera virus) and border disease virus (BDV) of sheep (Moennig, V. et al. *Adv. Vir. Res.* 1992, 41, 53-98). Pestivirus

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infections of domesticated livestock (cattle, pigs and sheep) cause significant economic losses worldwide. BVDV causes mucosal disease in cattle and is of significant economic importance to the livestock industry (Meyers, G. and Thiel, H.J., Advances in Virus Research, 1996, 47, 53-118; Moennig V., et al, Adv. Vir. Res. 1992, 41, 53-98). Human pestiviruses have not been as extensively characterized as the animal pestiviruses. However, serological surveys indicate considerable pestivirus exposure in humans.

Pestiviruses and hepaciviruses are closely related virus groups within the Flaviviridae family. Other closely related viruses in this family include the GB virus A, GB virus A-like agents, GB virus-B and GB virus-C (also called hepatitis G virus, HGV). The hepacivirus group (hepatitis C virus; HCV) consists of a number of closely related but genotypically distinguishable viruses that infect humans. There are at least 6 HCV genotypes and more than 50 subtypes. Due to the similarities between pestiviruses and hepaciviruses, combined with the poor ability of hepaciviruses to grow efficiently in cell culture, bovine viral diarrhea virus (BVDV) is often used as a surrogate to study the HCV virus.

The genetic organization of pestiviruses and hepaciviruses is very similar. These positive stranded RNA viruses possess a single large open reading frame (ORF) encoding all the viral proteins necessary for virus replication. These proteins are expressed as a polyprotein that is co- and post-translationally processed by both cellular and virus-encoded proteinases to yield the mature viral proteins. The viral proteins responsible for the replication of the viral genome RNA are located within approximately the carboxy-terminal. Two-thirds of the ORF are termed nonstructural (NS) proteins. The genetic organization and polyprotein processing of the nonstructural protein portion of the ORF for pestiviruses and hepaciviruses is very similar. For both the pestiviruses and hepaciviruses, the mature nonstructural (NS) proteins, in sequential order from the amino-terminus of the nonstructural protein coding region to the carboxy-terminus of the ORF, consist of p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

The NS proteins of pestiviruses and hepaciviruses share sequence domains that are characteristic of specific protein functions. For example, the NS3 proteins of viruses in both groups possess amino acid sequence motifs characteristic of serine proteinases and of helicases (Gorbalenya et al., *Nature*, 1988, 333, 22; Bazan and Fletterick *Virology*, 1989, 171, 637-639; Gorbalenya et al., *Nucleic Acid Res.*, 1989,

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17, 3889-3897). Similarly, the NS5B proteins of pestiviruses and hepaciviruses have the motifs characteristic of RNA-directed RNA polymerases (Koonin, E.V. and Dolja, V.V., *Crit. Rev. Biochem. Molec. Biol.* 1993, 28, 375-430).

The actual roles and functions of the NS proteins of pestiviruses and hepaciviruses in the lifecycle of the viruses are directly analogous. In both cases, the NS3 serine proteinase is responsible for all proteolytic processing of polyprotein precursors downstream of its position in the ORF (Wiskerchen and Collett, *Virology*, 1991, 184, 341-350; Bartenschlager et al., *J. Virol.* 1993, 67, 3835-3844; Eckart et al. *Biochem. Biophys. Res. Comm.* 1993,192, 399-406; Grakoui et al., *J. Virol.* 1993, 67, 2832-2843; Grakoui et al., *Proc. Natl. Acad Sci. USA* 1993, 90, 10583-10587; Hijikata et al., *J. Virol.* 1993, 67, 4665-4675; Tome et al., *J. Virol.*, 1993, 67, 4017-4026). The NS4A protein, in both cases, acts as a cofactor with the NS3 serine protease (Bartenschlager et al., *J. Virol.* 1994, 68, 5045-5055; Failla et al., *J. Virol.* 1994, 68, 3753-3760; Xu et al., *J. Virol.*, 1997, 71:53 12-5322). The NS3 protein of both viruses also functions as a helicase (Kim et al., *Biochem. Biophys. Res. Comm.*, 1995, 215, 160-166; Jin and Peterson, *Arch. Biochem. Biophys.*, 1995, 323, 47-53; Warrener and Collett, *J. Virol.* 1995, 69,1720-1726). Finally, the NS5B proteins of pestiviruses and hepaciviruses have the predicted RNA-directed RNA polymerases activity (Behrens et al., EMBO, 1996, 15, 12-22; Lechmann et al., *J. Virol.*, 1997, 71, 8416-8428; Yuan et al., *Biochem. Biophys. Res. Comm.* 1997, 232, 231-235; Hagedorn, PCT WO 97/12033; Zhong et al, *J. Virol.*, 1998, 72, 9365-9369).

Currently, there are limited treatment options for individuals infected with hepatitis C virus. The current approved therapeutic option is the use of immunotherapy with recombinant interferon-α alone or in combination with the nucleoside analog ribavirin. This therapy is limited in its clinical effectiveness and only 50% of treated patients respond to therapy. Therefore, there is significant need for more effective and novel therapies to address the unmet medical need posed by HCV infection.

A number of potential molecular targets for drug development of direct acting antivirals as anti -HCV therapeutics have now been identified including, but not limited to, the NS2-NS3 autoprotease, the N3 protease, the N3 helicase and the NS5B polymerase. The RNA-dependent RNA polymerase is absolutely essential for

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replication of the single-stranded, positive sense, RNA genome and this enzyme has elicited significant interest among medicinal chemists.

Inhibitors of HCV NS5B as potential therapies for HCV infection have been reviewed: Tan, S.-L., et al., *Nature Rev. Drug Discov.*, 2002, 1, 867-881; Walker, M.P. et al., *Exp. Opin. Investigational Drugs*, 2003, 12, 1269-1280; Ni, Z-J., et al., *Current Opinion in Drug Discovery and Development*, 2004, 7, 446-459; Beaulieu, P. L., et al., *Current Opinion in Investigational Drugs*, 2004, 5, 838-850; Wu, J., et al., *Current Drug Targets-Infectious Disorders*, 2003, 3, 207-219; Griffith, R.C., et al, *Annual Reports in Medicinal Chemistry*, 2004, 39, 223-237; Carrol, S., et al., *Infectious Disorders-Drug Targets*, 2006, 6, 17-29. The potential for the emergence of resistant HCV strains and the need to identify agents with broad genotype coverage supports the need for continuing efforts to identify novel and more effective nucleosides as HCV NS5B inhibitors.

Nucleoside inhibitors of NS5B polymerase can act either as a non-natural substrate that results in chain termination or as a competitive inhibitor which competes with nucleotide binding to the polymerase. To function as a chain terminator the nucleoside analog must be taken up by the cell and converted *in vivo* to a triphosphate to compete for the polymerase nucleotide binding site. This conversion to the triphosphate is commonly mediated by cellular kinases which imparts additional structural requirements on a potential nucleoside polymerase inhibitor. Unfortunately, this limits the direct evaluation of nucleosides as inhibitors of HCV replication to cell-based assays capable of *in situ* phosphorylation.

In some cases, the biological activity of a nucleoside is hampered by its poor substrate characteristics for one or more of the kinases needed to convert it to the active triphosphate form. Formation of the monophosphate by a nucleoside kinase is generally viewed as the rate limiting step of the three phosphorylation events. To circumvent the need for the initial phosphorylation step in the metabolism of a nucleoside to the active triphosphate analog, the preparation of stable phosphate prodrugs has been reported. Nucleoside phosphoramidate prodrugs have been shown to be precursors of the active nucleoside triphosphate and to inhibit viral replication when administered to viral infected whole cells (McGuigan, C., et al., *J. Med. Chem.*, 1996, 39, 1748-1753; Valette, G., et al., *J. Med. Chem.*, 1996, 39, 1981-1990; Balzarini, J., et al., *Proc. National Acad Sci USA*, 1996, 93, 7295-7299; Siddiqui, A. Q., et al., *J. Med. Chem.*, 1999, 42, 4122-4128; Eisenberg, E. J., et al.,

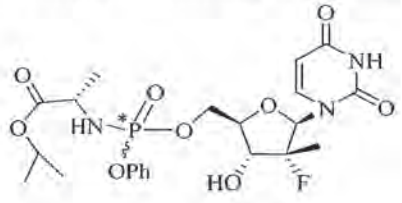
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Nucleosides, Nucleotides and Nucleic Acids, 2001, 20, 1091-1098; Lee, W.A., et al., *Antimicrobial Agents and Chemotherapy*, 2005, 49, 1898); US 2006/0241064; and WO 2007/095269.

Also limiting the utility of nucleosides as viable therapeutic agents is their sometimes poor physicochemical and pharmacokinetic properties. These poor properties can limit the intestinal absorption of an agent and limit uptake into the target tissue or cell. To improve on their properties prodrugs of nucleosides have been employed. It has been demonstrated that preparation of nucleoside phosphoramidates improves the systemic absorption of a nucleoside and furthermore, the phosphoramidate moiety of these "pronucleotides" is masked with neutral lipophilic groups to obtain a suitable partition coefficient to optimize uptake and transport into the cell dramatically enhancing the intracellular concentration of the nucleoside monophosphate analog relative to administering the parent nucleoside alone. Enzyme-mediated hydrolysis of the phosphate ester moiety produces a nucleoside monophosphate wherein the rate limiting initial phosphorylation is unnecessary. To this end, U.S. Patent Application 12/053,015, which corresponds to WO 2008/121634 and US 2010/0016251, discloses a number of phosphoramidate nucleoside prodrugs, many of which show activity in an HCV assay. Several compounds disclosed in US 2010/0016251 were tested as a potential clinical candidate for approval by the FDA.

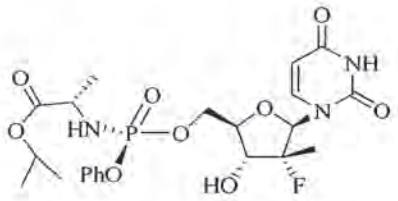
Summary of the Invention

Disclosed herein is a compound represented by formula **4** and its respective phosphorus-based diastereomers represented by formulas S_P-**4** and R_P-**4**.

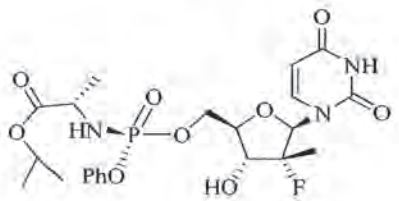


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S_P-4



R_P-4

Brief Description of the Drawings

Figure 1. High resolution XRD diffractogram of **4**.

Figure 2. High resolution XRD diffractogram of R_P-**4**.

Figure 3. High resolution XRD diffractogram of S_P-**4** (Form 1).

Figure 4. High resolution XRD diffractogram of S_P-**4** (Form 1).

Figure 5. High resolution XRD diffractogram of S_P-**4**·CH_2Cl_2 (Form 2).

Figure 6. High resolution XRD diffractogram of S_P-**4**·$CHCl_3$ (Form 3).

Figure 7. High resolution XRD diffractogram of S_P-**4** (Form 4).

Figure 8. High resolution XRD diffractogram of S_P-**4** (Form 5).

Figure 9. High resolution XRD diffractogram of S_P-**4** (amorphous).

Figure 10. X-Ray Crystal Structure for S_P-**4** (Form 1)

Figure 11. X-Ray Crystal (Isotropic) Structure for S_P-**4**·CH_2Cl_2 (Form 2)

Figure 12. X-Ray Crystal (Anisotropic) Structure for S_P-**4**·CH_2Cl_2 (Form 2)

Figure 13. X-Ray Crystal Structure for S_P-**4**·$CHCl_3$ (Form 3)

Figure 14. FT-IR spectrum of **4**.

Figure 15. FT-IR spectrum of R_P-**4**.

Figure 16. FT-IR spectrum of S_P-**4**

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Figure 17. TGA and DSC analysis of **4**.

Figure 18. TGA and DSC analysis of R_P-**4**.

Figure 19. TGA and DSC analysis of S_P-**4**.

Figure 20A. X-Ray Crystal Structure for **8** (S_P-isomer) (molecule no. 1 of the asymmetric unit).

Figure 20B. X-Ray Crystal Structure for **8** (S_P-isomer) (molecule no. 2 of the asymmetric unit).

Figure 21._ High resolution XRD diffractogram of S_P-4 (Form 6).

Figure 22A. X-Ray Crystal Structure for (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate (molecule no. 1 of the asymmetric unit).

Figure 22B. X-Ray Crystal Structure for (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate (molecule no. 2 of the asymmetric unit).

Detailed Description of the Invention

Definitions

The phrase "a" or "an" entity as used herein refers to one or more of that entity; for example, a compound refers to one or more compounds or at least one compound. As such, the terms "a" (or "an"), "one or more", and "at least one" can be used interchangeably herein.

The terms "optional" or "optionally" as used herein means that a subsequently described event or circumstance may but need not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, "optional bond" means that the bond may or may not be present, and that the description includes single, double, or triple bonds.

The term "P*" means that the phosphorus atom is chiral and that it has a corresponding Cahn-Ingold-Prelog designation of "R" or "S" which have their accepted plain meanings.

The term "purified," as described herein, refers to the purity of a given compound. For example, a compound is "purified" when the given compound is a major component of the composition, i.e., at least 50% w/w pure. Thus, "purified"

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embraces at least 50% w/w purity, at least 60% w/w purity, at least 70% purity, at least 80% purity, at least 85% purity, at least 90% purity, at least 92% purity, at least 94% purity, at least 96% purity, at least 97% purity, at least 98% purity, at least 99% purity, at least 99.5% purity, and at least 99.9% purity, wherein "substantially pure" embraces at least 97% purity, at least 98% purity, at least 99% purity, at least 99.5% purity, and at least 99.9% purity

The term "metabolite," as described herein, refers to a compound produced in vivo after administration to a subject in need thereof.

The term "about" (also represented by ~) means that the recited numerical value is part of a range that varies within standard experimental error.

The expression "substantially as shown in..." a specified XRPD pattern means that the peak positions shown in the XRPD pattern are substantially the same, within visual inspection or resort to selected peak listings (± 0.2 °2θ). One of ordinary skill understands that the intensities can vary depending on the sample.

The term "substantially anhydrous" means that a substance contains at most 10% by weight of water, preferably at most 1% by weight of water, more preferably at most 0.5% by weight of water, and most preferably at most 0.1% by weight of water.

A solvent or anti-solvent (as used in reactions, crystallization, etc. or lattice and/or adsorbed solvents) includes at least one of a C_1 to C_8 alcohol, a C_2 to C_8 ether, a C_3 to C_7 ketone, a C_3 to C_7 ester, a C_1 to C_2 chlorocarbon, a C_2 to C_7 nitrile, a miscellaneous solvent, a C_5 to C_{12} saturated hydrocarbon, and a C_6 to C_{12} aromatic hydrocarbon.

The C_1 to C_8 alcohol refers to a straight/branched and/or cyclic/acyclic alcohol having such number of carbons. The C_1 to C_8 alcohol includes, but is not limited to, methanol, ethanol, n-propanol, isopropanol, isobutanol, hexanol, and cyclohexanol.

The C_2 to C_8 ether refers to a straight/branched and/or cyclic/acyclic ether having such number of carbons. The C_2 to C_8 ether includes, but is not limited to, dimethyl ether, diethyl ether, di-isopropyl ether, di-n-butyl ether, methyl-t-butyl ether (MTBE), tetrahydrofuran, and dioxane

The C_3 to C_7 ketone refers to a straight/branched and/or cyclic/acyclic ketone having such number of carbons. The C_3 to C_7 ketone includes, but is not limited to,

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acetone, methyl ethyl ketone, propanone, butanone, methyl isobutyl ketone, methyl butyl ketone, and cyclohexanone.

The C_3 to C_7 ester refers to a straight/branched and/or cyclic/acyclic ester having such number of carbons. The C_3 to C_7 ester includes, but is not limited to, ethyl acetate, propyl acetate, n-butyl acetate, etc.

The C_1 to C_2 chlorocarbon refers to a chlorocarbon having such number of carbons. The C_1 to C_2 chlorocarbon includes, but is not limited to, chloroform, methylene chloride (DCM), carbon tetrachloride, 1,2-dichloroethane, and tetrachloroethane.

A C_2 to C_7 nitrile refers to a nitrile have such number of carbons. The C_2 to C_7 nitrile includes, but is not limited to, acetonitrile, propionitrile, etc.

A miscellaneous solvent refers to a solvent commonly employed in organic chemistry, which includes, but is not limited to, diethylene glycol, diglyme (diethylene glycol dimethyl ether), 1,2-dimethoxy-ethane, dimethylformamide, dimethylsulfoxide, ethylene glycol, glycerin, hexamethylphsphoramide, hexamethylphosphorous triame, N-methyl-2-pyrrolidinone, nitromethane, pyridine, triethyl amine, and acetic acid.

The term C_5 to C_{12} saturated hydrocarbon refers to a straight/branched and/or cyclic/acyclic hydrocarbon. The C_5 to C_{12} saturated hydrocarbon includes, but is not limited to, n-pentane, petroleum ether (ligroine), n-hexane, n-heptane, cyclohexane, and cycloheptane.

The term C_6 to C_{12} aromatic refers to substituted and unsubstituted hydrocarbons having a phenyl group as their backbone. Preferred hydrocarbons include benzene, xylene, toluene, chlorobenzene, o-xylene, m-xylene, p-xylene, xylenes, with toluene being more preferred.

The term "halo" or "halogen" as used herein, includes chloro, bromo, iodo and fluoro.

The term "blocking group" refers to a chemical group which exhibits the following characteristics. The "group" is derived from a "protecting compound." Groups that are selective for primary hydroxyls over secondary hydroxyls that can be put on under conditions consistent with the stability of the phosphoramidate (pH 2-8) and impart on the resulting product substantially different physical properties allowing for an easier separation of the 3'-phosphoramidate-5'-new group product from the unreacted desired compound. The group must react selectively in good

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yield to give a protected substrate that is stable to the projected reactions (see Protective Groups in Organic Synthesis, 3rd ed. T. W. Greene and P. G. M. Wuts, John Wiley & Sons, New York, N.Y., 1999). Examples of groups include, but are not limited to: benzoyl, acetyl, phenyl-substituted benzoyl, tetrahydropyranyl, trityl, DMT (4,4'-dimethoxytrityl), MMT (4-monomethoxytrityl), trimethoxytrityl, pixyl (9-phenylxanthen-9-yl) group, thiopixyl (9-phenylthioxanthen-9-yl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX), etc.; C(O)-alkyl, C(O)Ph, C(O)aryl, CH_2O-alkyl, CH_2O-aryl, SO_2-alkyl, SO_2-aryl, *tert*-butyldimethylsilyl, *tert*-butyldiphenylsilyl. Acetals, such as MOM or THP and the like are considered possible groups. Fluorinated compounds are also contemplated in so far that they can be attached to the compound and can be selectively removed by passing through a fluorous solid phase extraction media (FluoroFlash®). A specific example includes a fluorinated trityl analog, trityl analog 1-[4-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)phenyl)-1,1-diphenylmethanol. Other fluorinated analogs of trityl, BOC, FMOC, CBz, etc. are also contemplated. Sulfonyl chlorides like p-toluenesulfonyl chloride can react selectively on the 5' position. Esters could be formed selectively such as acetates and benzoates. Dicarboxylic anhydrides such as succinic anhydride and its derivatives can be used to generate an ester linkage with a free carboxylic acid, such examples include, but are not limited to oxalyl, malonyl, succinyl, glutaryl, adipyl, pimelyl, superyl, azelayl, sebacyl, phthalyl, isophthalyl, terephthalyl, etc. The free carboxylic acid increases the polarity dramatically and can also be used as a handle to extract the reaction product into mildy basic aqueous phases such as sodium bicarbonate solutions. The phosphoramidate group is relatively stable in acidic media, so groups requiring acidic reaction conditions, such as, tetrahydropyranyl, could also be used.

The term "protecting group" which is derived from a "protecting compound," has its plain and ordinary meaning, i.e., at least one protecting or blocking group is bound to at least one functional group (e.g., –OH, $–NH_2$, etc.) that allows chemical modification of at least one other functional group. Examples of protecting groups, include, but are not limited to, benzoyl, acetyl, phenyl-substituted benzoyl, tetrahydropyranyl, trityl, DMT (4,4'-dimethoxytrityl), MMT (4-monomethoxytrityl), trimethoxytrityl, pixyl (9-phenylxanthen-9-yl) group, thiopixyl (9-phenylthioxanthen-9-yl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX), etc.; C(O)-alkyl, C(O)Ph, C(O)aryl, C(O)O(lower alkyl), C(O)O(lower alkylene)aryl (e.g., –

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C(O)OCH$_2$Ph), C(O)Oaryl, CH$_2$O-alkyl, CH$_2$O-aryl, SO$_2$-alkyl, SO$_2$-aryl, a protecting group comprising at least one silicon atom, such as, *tert*-butyldimethylsilyl, *tert*-butyldiphenylsilyl, Si(lower alkyl)$_2$OSi(lower alkyl)$_2$OH (such as, –Si(^{i}Pr)$_2$OSi(^{i}Pr)$_2$OH.

The term "protecting compound," as used herein and unless otherwise defined, refers to a compound that contains a "protecting group" and that is capable of reacting with a compound that contains functional groups that are capable of being protected.

The term "leaving group", as used herein, has the same meaning to the skilled artisan (Advanced Organic Chemistry: reactions, mechanisms and structure– Fourth Edition by Jerry March, John Wiley and Sons Ed.; 1992 pages 351-357) and represents a group which is part of and attached to a substrate molecule; in a reaction where the substrate molecule undergoes a displacement reaction (with for example a nucleophile), the leaving group is then displaced. Examples of leaving groups include, but are not limited to: halogen (F, Cl, Br, and I), preferably Cl, Br, or I; tosylate, mesylate, triflate, acetate, camphorsulfonate, aryloxide, and aryloxide substituted with at least one electron withdrawing group (e.g., p-nitrophenoxide, 2-chlorophenoxide, 4-chlorophenoxide, 2,4-dinitrophenoxide, pentafluorophenoxide, etc.), etc. The term "electron withdrawing group" is accorded its plain meaning here. Examples of electron withdrawing groups include, but are not limited to, a halogen, –NO2, –C(O)(lower alkyl), –C(O)(aryl), –C(O)O(lower alkyl), –C(O)O(aryl), etc.

The term "basic reagent", as used herein, means a compound that is capable of deprotonating a hydroxyl group. Examples of basic reagents include, but are not limited to, a (lower alk)oxide ((lower alkyl)OM) in combination with an alcoholic solvent, where (lower alk)oxides include, but are not limited to, MeO$^-$, EtO$^-$, ^{n}PrO$^-$, ^{i}PrO$^-$, ^{t}BuO$^-$, ^{i}AmO- (iso-amyloxide), etc., and where M is an alkali metal cation, such as Li$^+$, Na$^+$, K$^+$, etc. Alcoholic solvents include (lower alkyl)OH,, such as, for example, MeOH, EtOH, ^{n}PrOH, ^{i}PrOH, ^{t}BuOH, ^{i}AmOH, etc. Non-alkoxy bases can also be used such as sodium hydride, sodium hexamethyldisilazane, lithium hexamethyldisilazane, lithium diisopropylamide, calcium hydride, sodium carbonate, potassium carbonate, cesium carbonate, DBU, DBN, Grignard reagents, such as (lower alkyl)Mg(halogen), which include but are not limited to MeMgCl, MeMgBr, ^{t}BuMgCl, ^{t}BuMgBr, etc.

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The term "base" embraces the term "basic reagent" and is meant to be a compound that is capable of deprotonating a proton containing compound, i.e., a Bronsted base. In addition to the examples recited above, further examples of a base include, but are not limited to pyridine, collidine, 2,6-(loweralkyl)-pyridine, dimethyl-aniline, imidazole, N-methyl-imidazole, pyrazole, N-methyl-pyrazole, triethylamine, di-isopropylethylamine, etc.

The term "non-nucleophilic base" means a compound that is capable of acting as a Brønsted base, but has low nucleophilicity. Examples of non-nucleophilic bases include, but are not limited to, potassium carbonate, cesium carbonate, di-isopropylamine, di-isopropylethylamine, triethylamine, quinuclidine, naphthalene-1,8-diamine, 2,2,6,6-tetramethylpiperidine, 1,8-diazabicycloundec-7-ene, 4-dimethylamino-pyridine, pyridine, a 2,6-di-C1-6-alkyl-pyridine, a 2,4,6-tri-C1-6-alkyl-pyridine, 1,5-diazabicyclo[4.3.0]non-5-ene, and 1,4-diazabicyclo[2.2.2]octane.

The term "electron withdrawing group" is accorded its plain meaning. Examples of electron withdrawing groups include, but are not limited to, a halogen (F, Cl, Br, or I), $-NO_2$, –C(O)(lower alkyl), –C(O)(aryl), –C(O)O(lower alkyl), –C(O)O(aryl), etc.

The term "co-crystallates" include co-crystallates of **4**, R_P-**4**, or S_P-**4** in combination with salts, which embraces pharmaceutically acceptable salts.

The term "salts," as described herein, refers to a compound comprising a cation and an anion, which can produced by the protonation of a proton-accepting moiety and/or deprotonation of a proton-donating moiety. It should be noted that protonation of the proton-accepting moiety results in the formation of a cationic species in which the charge is balanced by the presence of a physiological anion, whereas deprotonation of the proton-donating moiety results in the formation of an anionic species in which the charge is balanced by the presence of a physiological cation.

The phrase "pharmaceutically acceptable salt" means a salt that is pharmaceutically acceptable. Examples of pharmaceutically acceptable salts include, but are not limited to: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as glycolic acid, pyruvic acid, lactic acid, malonic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric

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acid, 3-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethane-disulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 4-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, 4-toluenesulfonic acid, camphorsulfonic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, salicylic acid, muconic acid, and the like or (2) basic addition salts formed with the conjugate bases of any of the inorganic acids listed above, wherein the conjugate bases comprise a cationic component selected from among Na^+, K^+, Mg^{2+}, Ca^{2+}, $NH_gR'''_{4-g}{}^+$, in which R''' is a C_{1-3} alkyl and g is a number selected from among 0, 1, 2, 3, or 4. It should be understood that all references to pharmaceutically acceptable salts include solvent addition forms (solvates) or crystal forms (polymorphs) as defined herein, of the same acid addition salt.

The term "alkyl" refers to an unbranched or branched chain, saturated, monovalent hydrocarbon residue containing 1 to 30 carbon atoms. The term "C_{1-M} alkyl" refers to an alkyl comprising 1 to M carbon atoms, where M is an integer having the following values: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30. The term "C_{1-4} alkyl" refers to an alkyl containing 1 to 4 carbon atoms. The term "lower alkyl" denotes a straight or branched chain hydrocarbon residue comprising 1 to 6 carbon atoms. "C_{1-20} alkyl" as used herein refers to an alkyl comprising 1 to 20 carbon atoms. "C_{1-10} alkyl" as used herein refers to an alkyl comprising 1 to 10 carbons. Examples of alkyl groups include, but are not limited to, lower alkyl groups include methyl, ethyl, propyl, *i*-propyl, *n*-butyl, *i*-butyl, *t*-butyl or pentyl, isopentyl, neopentyl, hexyl, heptyl, and octyl. The term (ar)alkyl or (heteroaryl)alkyl indicate the alkyl group is optionally substituted by an aryl or a heteroaryl group respectively.

The term "alkenyl" refers to an unsubstituted hydrocarbon chain radical having from 2 to 10 carbon atoms having one or two olefinic double bonds, preferably one olefinic double bond. The term "C_{2-N} alkenyl" refers to an alkenyl comprising 2 to N carbon atoms, where N is an integer having the following values: 3, 4, 5, 6, 7, 8, 9, or 10. The term "C_{2-10} alkenyl" refers to an alkenyl comprising 2 to 10 carbon atoms. The term "C_{2-4} alkenyl" refers to an alkenyl comprising 2 to 4 carbon atoms. Examples include, but are not limited to, vinyl, 1-propenyl, 2-propenyl (allyl) or 2-butenyl (crotyl).

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The term "aryl," as used herein, and unless otherwise specified, refers to substituted or unsubstituted phenyl (Ph), biphenyl, or naphthyl, preferably the term aryl refers to substituted or unsubstituted phenyl. The aryl group can be substituted with one or more moieties selected from among hydroxyl, F, Cl, Br, I, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, and phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in T.W. Greene and P.G. M. Wuts, "Protective Groups in Organic Synthesis," 3rd ed., John Wiley & Sons, 1999.

The term "aryloxide," as used herein, and unless otherwise specified, refers to substituted or unsubstituted phenoxide (PhO–), p-phenyl-phenoxide (p-Ph-PhO–), or naphthoxide, preferably the term aryloxide refers to substituted or unsubstituted phenoxide. The aryloxide group can be substituted with one or more moieties selected from among hydroxyl, F, Cl, Br, I, –C(O)(lower alkyl), –C(O)O(lower alkyl), amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, and phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in T.W. Greene and P.G. M. Wuts, "Protective Groups in Organic Synthesis," 3rd ed., John Wiley & Sons, 1999.

The term "preparation" or "dosage form" is intended to include both solid and liquid formulations of the active compound and one skilled in the art will appreciate that an active ingredient can exist in different preparations depending on the desired dose and pharmacokinetic parameters.

The term "excipient" as used herein refers to a compound that is used to prepare a pharmaceutical composition, and is generally safe, non-toxic and neither biologically nor otherwise undesirable, and includes excipients that are acceptable for veterinary use as well as human pharmaceutical use.

The term "crystalline" refers to a situation where a solid sample of either S_P-**4** or R_P-**4** has crystalline characteristics when determined by X-ray powder diffraction or a single crystal X-ray technique.

The term "crystal-like" refers to a situation where a solid sample of either S_P-**4** or R_P-**4** has crystalline characteristics when determined by one means, e.g., visually or by optical or polarizing microscopy, but does not have crystalline characteristics when determined by another means, e.g., x-ray powder diffraction.

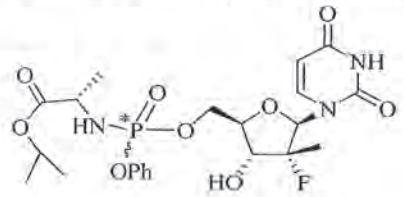
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Methods of visually determining the crystallinity of a solid sample by visual or by optical or by polarizing microscopy are disclosed in USP <695> and <776>, both of which are incorporated by reference. A solid sample of either S_P-**4** or R_P-**4** that is "crystal-like" may be crystalline under certain conditions but may become non-crystalline when subjected to other conditions.

The term "amorphous" refers to a situation where a solid sample of either S_P-**4** or R_P-**4** is neither crystalline nor crystal-like.

Embodiments

A first embodiment is directed to a compound represented by formula **4**:

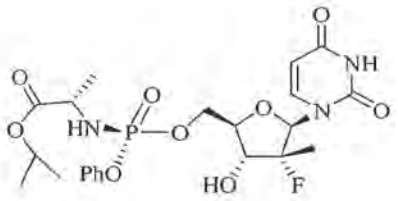


4

wherein P* represents a chiral phosphorus atom. Due to the chiral phosphorus atom, the compound represented by formula **4** comprises two diastereomers designated as R_P-**4** and S_P-**4**. The compound represented by formula **4** can also be part of a solvate, a hydrate, or a mixed solvate/hydrate. The solvate is designated as **4**·n*S*, while the hydrate is designated as **4**·mH_2O, where *S* is a lattice solvent, n varies by an integer or non-integer amount from about 0 to about 3 and m varies by an integer or non-integer amount from about 0 to about 5. Finally, the compound represented by formula **4** might not exist as a solvate or hydrate, but have a certain advantageous amount of adsorbed solvent (*S*) or water. In which case, the amount of *S* or water can vary from about 0 wt. % to about 10 wt.% based on the weight of the compound represented by formula **4**. The compound represented by formula **4** and its solvates and hydrates thereof is crystalline, crystal-like, or amorphous.

A second embodiment is directed to a compound represented by formula R_P-**4**:

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R_P-4

The compound represented by formula R_P-**4** can also be part of a solvate, a hydrate, or a mixed solvate/hydrate. The solvate is designated as R_P-**4**·n*S*, while the hydrate is designated as S_P-**4**·mH_2O, where *S* is a lattice solvent, n varies by an integer or non-integer amount from about 0 to about 3 and m varies by an integer or non-integer amount from about 0 to about 5. Finally, the compound represented by formula R_P-**4** might not exist as a solvate, hydrate, or mixed solvate/hydrate, but have a certain advantageous amount of adsorbed solvent (*S*), water, or both *S* and water. In which case, the amount of *S* or water can vary from about 0 wt. % to about 10 wt.% based on the weight of the compound represented by formula R_P-**4**. The compound represented by formula R_P-**4** and its solvates and hydrates thereof is crystalline, crystal-like, or amorphous.

A first aspect of the second embodiment is directed to crystalline R_P-**4**.

A second aspect of the second embodiment is directed to crystalline R_P-**4** having XRPD 2θ-reflections (°) at about: 6.6, 7.1, 9.0, 11.6, 17.9, 20.7, 24.1, 24.4, and 26.2.

A third aspect of the second embodiment is directed to a crystalline R_P-**4** having XRPD 2θ-reflections (°) at about: 6.6, 7.1, 9.0, 11.0, 11.6, 12.0, 16.0, 17.9, 19.6, 20.7, 21.0, 21.7, 21.9, 22.2, 23.1, 24.1, 24.4, 26.1, 27.3, 27.7, and 28.2.

A fourth aspect of the second embodiment is directed to crystalline R_P-**4** having an XRPD diffraction pattern substantially as that shown in Fig. 2.

A fifth aspect of the second embodiment is directed to R_P-**4** having the following FT-IR peaks (cm^{-1}): 1742, 1713, 1679, 1460, 1377, 1259, 1157, and 1079.

A sixth aspect of the second embodiment is directed to R_P-**4** having an FT-IR spectrum substantially as that shown in Fig. 15.

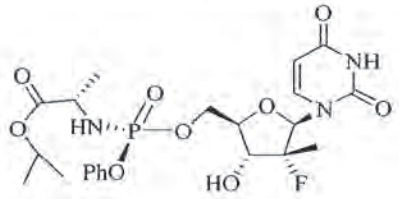
A seventh aspect of the second embodiment is directed to substantially pure R_P-**4**.

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An eighth aspect of the second embodiment is directed to substantially pure crystalline R_P-**4**.

A ninth aspect of the second embodiment is directed to substantially pure amorphous R_P-**4**.

A third embodiment is directed to a compound represented by formula S_P-**4**:



S_P-**4**

The compound represented by formula S_P-**4** can also be part of a solvate, a hydrate, or a mixed solvate/hydrate. The solvate is designated as S_P-**4**·n*S*, while the hydrate is designated as S_P-**4**·mH_2O, where *S* is a lattice solvent, n varies in an integer or non-integer amount from about 0 to about 3 and m varies in an integer or non-integer amount from about 0 to about 5. Finally, the compound represented by formula S_P-**4** might not exist as a solvate or hydrate, but have a certain advantageous amount of adsorbed solvent (*S*) or water. In which case, the amount of *S* or water can vary from about 0 wt. % to about 10 wt.% based on the weight of the compound represented by formula S_P-**4**. The compound represented by formula S_P-**4** and its solvates and hydrates thereof is crystalline, crystal-like, or amorphous.

A first aspect of the third embodiment is directed to crystalline S_P-**4**.

A second aspect of the third embodiment is directed to a monoclinic crystalline S_P-**4**, preferably having the following unit cell parameters a ~ 12.88 Å, b ~ 6.17 Å, c ~ 17.73 Å, and β ~ 92.05°.

A third aspect of the third embodiment is directed to a monoclinic crystalline S_P-**4**, preferably having the following unit cell parameters a ~ 20.09 Å, b ~ 6.10 Å, c ~ 23.01 Å, and β ~ 112.29°.

A fourth aspect of the third embodiment is directed to a monoclinic crystalline S_P-**4**, preferably having the following unit cell parameters a ~ 12.83 Å, b ~ 6.15 Å, c ~ 17.63 Å, and β ~ 91.75°.

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A fifth aspect of the third embodiment is directed to a monoclinic crystalline *S*P-**4**, preferably having the following unit cell parameters a ~ 12.93 Å, b ~ 6.18 Å, c ~ 18.01 Å, and β ~ 96.40°.

A sixth aspect of the third embodiment is directed to a crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 5.2, 7.5, 9.6, 16.7, 18.3, 22.2.

A seventh aspect of the third embodiment is directed to a crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 5.0, 7.3, 9.4, and 18.1.

An eighth aspect of the third embodiment is directed to a crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 4.9, 6.9, 9.8, 19.8, 20.6, 24.7, and 26.1.

A ninth aspect of the third embodiment is directed to a crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 6.9, 9.8, 19.7, 20.6, and 24.6.

A ninth aspect of the third embodiment is directed to a crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 5.0, 6.8, 19.9, 20.6, 20.9, and 24.9.

A tenth aspect of the third embodiment is directed to a crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 5.2, 6.6, 7.1, 15.7, 19.1, and 25.0.

An eleventh aspect of the third embodiment is directed to crystalline *S*P-**4** having XRPD 2θ-reflections (°) at about: 6.1, 8.2, 10.4, 12.7, 17.2, 17.7, 18.0, 18.8, 19.4, 19.8, 20.1, 20.8, 21.8, and 23.3.

A twelfth aspect of the third embodiment is directed to crystalline *S*P-**4** having an XRPD diffraction pattern substantially as that shown in any one of Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8, and Fig 21.

A thirteenth aspect of the third embodiment is directed to *S*P-**4** having the following FT-IR peaks (cm^{-1}) at about: 1743, 1713, 1688, 1454, 1378, 1208, and 1082.

A fourteenth aspect of the third embodiment is directed to *S*P-**4** having an FT-IR spectrum substantially as that shown in Fig. 7.

A fifteenth aspect of the third embodiment is directed to substantially pure *S*P-**4**.

A sixteenth aspect of the third embodiment is directed to substantially pure crystalline *S*P-**4**.

A seventeenth aspect of the third embodiment is directed to substantially pure amorphous *S*P-**4**.

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Dosage, Administration, and Use

A fourth embodiment is directed to a composition for the treatment and/or prophylaxis of any of the viral agents using any of compounds **4**, R_P-**4**, or S_P-**4**. Possible viral agents include, but are not limited to: hepatitis C virus, hepatitis B virus, Hepatitis A virus, West Nile virus, yellow fever virus, dengue virus, rhinovirus, polio virus, bovine viral diarrhea virus, Japanese encephalitis virus, or those viruses belonging to the groups of Pestiviruses, hepaciviruses, or flavaviruses.

An aspect of this embodiment is directed to a composition for the treatment of any of the viral agents disclosed herein said composition comprising a pharmaceutically acceptable medium selected from among an excipient, carrier, diluent, and equivalent medium and any of compounds **4**, R_P-**4**, or S_P-**4**, that is intended to include its hydrates, solvates, and any crystalline forms of any of compounds **4**, R_P-**4**, or S_P-**4** or its hydrates and solvates thereof.

The compounds **4**, R_P-**4**, or S_P-**4** may be independently formulated in a wide variety of oral administration dosage forms and carriers. Oral administration can be in the form of tablets, coated tablets, hard and soft gelatin capsules, solutions, emulsions, syrups, or suspensions. The compounds **4**, R_P-**4**, or S_P-**4** are efficacious when administered by suppository administration, among other routes of administration. The most convenient manner of administration is generally oral using a convenient daily dosing regimen which can be adjusted according to the severity of the disease and the patient's response to the antiviral medication.

The compounds **4**, R_P-**4**, or S_P-**4** together with one or more conventional excipients, carriers, or diluents, may be placed into the form of pharmaceutical compositions and unit dosages. The pharmaceutical compositions and unit dosage forms may be comprised of conventional ingredients in conventional proportions, with or without additional active compounds and the unit dosage forms may contain any suitable effective amount of the active ingredient commensurate with the intended daily dosage range to be employed. The pharmaceutical compositions may be employed as solids, such as tablets or filled capsules, semisolids, powders, sustained release formulations, or liquids such as suspensions, emulsions, or filled capsules for oral use; or in the form of suppositories for rectal or vaginal administration. A typical preparation will contain from about 5% to about 95% active compound or compounds (w/w).

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The compounds **4**, R_P-**4**, or S_P-**4** can be administered alone but will generally be administered in admixture with one or more suitable pharmaceutical excipients, diluents or carriers selected with regard to the intended route of administration and standard pharmaceutical practice.

Solid form preparations include, for example, powders, tablets, pills, capsules, suppositories, and dispersible granules. A solid carrier may be one or more substances which may also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. In powders, the carrier generally is a finely divided solid which is a mixture with the finely divided active component. In tablets, the active component generally is mixed with the carrier having the necessary binding capacity in suitable proportions and compacted in the shape and size desired. Suitable carriers include but are not limited to magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. Solid form preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like. Examples of solid formulations are exemplified in EP 0524579; US 2002/0142050; US 2004/0224917; US 2005/0048116; US 2005/0058710; US 2006/0034937; US 2006/0057196; US 2006/0188570; US 2007/0026073; US 2007/0059360; US 2007/0077295; US 2007/0099902; US 2008/0014228; US 6,267,985; US 6,294,192; US 6,383,471; US 6,395,300; US 6,569,463; US 6,635,278; US 6,645,528; US 6,923,988; US 6,932,983; US 7,060,294; and US 7,462,608, each of which is incorporated by reference.

Liquid formulations also are suitable for oral administration include liquid formulation including emulsions, syrups, elixirs and aqueous suspensions. These include solid form preparations which are intended to be converted to liquid form preparations shortly before use. Examples of liquid formulation are exemplified in U.S. Patent Nos. 3,994,974; 5,695,784; and 6,977,257. Emulsions may be prepared in solutions, for example, in aqueous propylene glycol solutions or may contain emulsifying agents such as lecithin, sorbitan monooleate, or acacia. Aqueous suspensions can be prepared by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins,

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methylcellulose, sodium carboxymethylcellulose, and other well known suspending agents.

The compounds **4**, *R*P-**4**, or *S*P-**4** may be independently formulated for administration as suppositories. A low melting wax, such as a mixture of fatty acid glycerides or cocoa butter is first melted and the active component is dispersed homogeneously, for example, by stirring. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool, and to solidify.

The compounds **4**, *R*P-**4**, or *S*P-**4** may be independently formulated for vaginal administration. Pessaries, tampons, creams, gels, pastes, foams or sprays containing in addition to the active ingredient such carriers as are known in the art to be appropriate. Certain of these formulations may also be used in conjunction with a condom with or without a spermicidal agent.

Suitable formulations along with pharmaceutical carriers, diluents and excipients are described in *Remington: The Science and Practice of Pharmacy* **1995,** edited by E. W. Martin, Mack Publishing Company, 19th edition, Easton, Pennsylvania, which is hereby incorporated by reference. A skilled formulation scientist may modify the formulations within the teachings of the specification to provide numerous formulations for a particular route of administration without rendering compositions containing the compounds contemplated herein unstable or compromising their therapeutic activity.

Additionally, the purified compounds **4**, *R*P-**4**, or *S*P-**4** may be independently formulated in conjunction with liposomes or micelles. As to liposomes, it is contemplated that the purified compounds can be formulated in a manner as disclosed in U.S. Patent Nos. 4,797,285; 5,013,556; 5,077,056; 5,077,057; 5,154,930; 5,192,549; 5,213,804; 5,225,212; 5,277,914; 5,316,771; 5,376,380; 5,549,910; 5,567,434; 5,736,155; 5,827,533; 5,882,679; 5,891,468; 6,060,080; 6,132,763; 6,143,321; 6,180,134; 6,200,598; 6,214,375; 6,224,903; 6,296,870; 6,653,455; 6,680,068; 6,726,925; 7,060,689; and 7,070,801, each of which is incorporated by reference. As to micelles, it is contemplated that the purified compounds can be formulated in a manner as disclosed in U.S. Patent Nos. 5,145,684 and 5,091,188, both of which are incorporated by reference.

The fifth embodiment is directed to a use of any of compounds **4**, *R*P-**4**, or *S*P-**4** in the manufacture of a medicament for the treatment of any condition the result of an infection by any one of the following viral agents: hepatitis C virus,

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West Nile virus, yellow fever virus, degue virus, rhinovirus, polio virus, hepatitis A virus, bovine viral diarrhea virus and Japanese encephalitis virus.

The term "medicament" means a substance used in a method of treatment and/or prophylaxis of a subject in need thereof, wherein the substance includes, but is not limited to, a composition, a formulation, a dosage form, and the like, comprising any of compounds **4**, R_P-**4**, or S_P-**4**. It is contemplated that the use of any of compounds **4**, R_P-**4**, or S_P-**4** in the manufacture of a medicament, for the treatment of any of the antiviral conditions disclosed herein, either alone or in combination with another compound disclosed herein. A medicament includes, but is not limited to, any one of the compositions contemplated by the fourth embodiment disclosed herein.

A sixth embodiment is directed to a method of treatment and/or prophylaxis in a subject in need thereof said method comprises administering a therapeutically effective amount of any of compounds **4**, R_P-**4**, or S_P-**4** to the subject.

It is intended that a subject in need thereof is one that has any condition the result of an infection by any of the viral agents disclosed herein, which includes, but is not limited to, hepatitis C virus, West Nile virus, yellow fever virus, degue virus, rhinovirus, polio virus, hepatitis A virus, bovine viral diarrhea virus or Japanese encephalitis virus, flaviviridae viruses or pestiviruses or hepaciviruses or a viral agent causing symptoms equivalent or comparable to any of the above-listed viruses.

The term "subject" means a mammal, which includes, but is not limited to, cattle, pigs, sheep, chicken, turkey, buffalo, llama, ostrich, dogs, cats, and humans, preferably the subject is a human. It is contemplated that in the method of treating a subject thereof of the ninth embodiment can be any of the compounds contemplated herein, either alone or in combination with another compound disclosed herein.

The term "therapeutically effective amount" as used herein means an amount required to reduce symptoms of the disease in an individual. The dose will be adjusted to the individual requirements in each particular case. That dosage can vary within wide limits depending upon numerous factors such as the severity of the disease to be treated, the age and general health condition of the patient, other medicaments with which the patient is being treated, the route and form of administration and the preferences and experience of the medical practitioner involved. For oral administration, a daily dosage of between about 0.001 and about 10 g, including all values in between, such as 0.001, 0.0025, 0.005, 0.0075, 0.01,

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0.025, 0.050, 0.075, 0.1, 0.125, 0.150, 0.175, 0.2, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, and 9.5, per day should be appropriate in monotherapy and/or in combination therapy. A particular daily dosage is between about 0.01 and about 1 g per day, including all incremental values of 0.01 g (i.e., 10 mg) in between, a preferred daily dosage about 0.01 and about 0.8 g per day, more preferably about 0.01 and about 0.6 g per day, and most preferably about 0.01 and about 0.25 g per day, each of which including all incremental values of 0.01 g in between. Generally, treatment is initiated with a large initial "loading dose" to rapidly reduce or eliminate the virus following by a decreasing the dose to a level sufficient to prevent resurgence of the infection. One of ordinary skill in treating diseases described herein will be able, without undue experimentation and in reliance on knowledge, experience and the disclosures of this application, to ascertain a therapeutically effective amount of the compound disclosed herein for a given disease and patient.

Therapeutic efficacy can be ascertained from tests of liver function including, but not limited to protein levels such as serum proteins (e.g., albumin, clotting factors, alkaline phosphatase, aminotransferases (e.g., alanine transaminase, aspartate transaminase), 5'-nucleosidase, γ-glutaminyltranspeptidase, etc.), synthesis of bilirubin, synthesis of cholesterol, and synthesis of bile acids; a liver metabolic function, including, but not limited to, carbohydrate metabolism, amino acid and ammonia metabolism. Alternatively the therapeutic effectiveness may be monitored by measuring HCV-RNA. The results of these tests will allow the dose to be optimized.

A first aspect of the sixth embodiment is directed to a method of treatment and/or prophylaxis in a subject in need thereof said method comprises administering to the subject a therapeutically effective amount of a compound represented by any of compounds **4**, R_P-**4**, or S_P-**4** and a therapeutically effective amount of another antiviral agent; wherein the administration is concurrent or alternative. It is understood that the time between alternative administration can range between 1-24 hours, which includes any sub-range in between including, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, and 23 hours.

Examples of "another antiviral agent" include, but are not limited to: HCV NS3 protease inhibitors (see EP 1881001, US 2003187018, US 2005267018, WO 2003006490, WO 200364456, WO 2004094452, WO 2005028502, WO

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2005037214, WO 2005095403, WO 2007014920, WO 2007014921, WO 2007014922, WO 2007014925, WO 2007014926, WO 2007015824, WO 2008010921, and WO 2008010921); HCV NS5B Inhibitors (see US 2004229840, US 2005154056, US 2005-98125, US 20060194749, US 20060241064, US 20060293306, US 2006040890, US 2006040927, US 2006166964, US 2007275947, US 6784166, US20072759300, WO 2002057287, WO 2002057425, WO 2003010141, WO 2003037895, WO 2003105770, WO 2004000858, WO 2004002940, WO 2004002944, WO 2004002977, WO 2004003138, WO 2004041201, WO 2004065367, WO 2004096210, WO 2005021568, WO 2005103045, WO 2005123087, WO 2006012078, WO 2006020082, WO 2006065335, WO 2006065590, WO 2006093801, WO 200702602, WO 2007039142, WO 2007039145, WO 2007076034, WO 2007088148, WO 2007092000, and WO2007095269); HCV NS4 Inhibitors (see WO 2005067900 and WO 2007070556); HCV NS5a Inhibitors (see US 2006276511, WO 2006035061, WO 2006100310, WO 2006120251, and WO 2006120252); Toll-like receptor agonists (see WO 2007093901); and other inhibitors (see WO 2000006529, WO 2003101993, WO 2004009020, WO 2004014313, WO 2004014852, and WO 2004035571); and compounds disclosed in U.S. Patent Application No. 12/053,015, filed March 21, 2008 (US 2010/0016251) (the contents of which are incorporated by reference), interferon-α, interferon-β, pegylated interferon-α, ribavirin, levovirin, viramidine, another nucleoside HCV polymerase inhibitor, a HCV non-nucleoside polymerase inhibitor, a HCV protease inhibitor, a HCV helicase inhibitor or a HCV fusion inhibitor.

When any of compounds **4**, R_P-**4**, or S_P-**4** are administered in combination with another antiviral agent the activity may be increased over the parent compound. When the treatment is combination therapy, such administration may be concurrent or sequential with respect to that of the nucleoside derivatives. "Concurrent administration" as used herein thus includes administration of the agents at the same time or at different times. Administration of two or more agents at the same time can be achieved by a single formulation containing two or more active ingredients or by substantially simultaneous administration of two or more dosage forms with a single active agent.

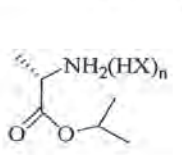
It will be understood that references herein to treatment extend to prophylaxis as well as to the treatment of existing conditions. Furthermore, the term

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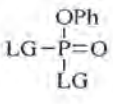
"treatment" of a HCV infection, as used herein, also includes treatment or prophylaxis of a disease or a condition associated with or mediated by HCV infection, or the clinical symptoms thereof.

Preparation

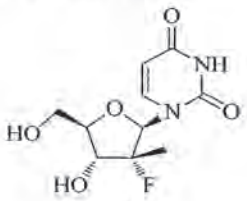
A seventh embodiment is directed to a process for preparing any one of compounds **4**, R_P-**4**, or S_P-**4**, which comprises: a) reacting an isopropyl-alanate, **A**, a di-LG-phenylphosphate, **B**, 2'-deoxy-2'-fluoro-2'-C-methyluridine, **3**, and a base to obtain a first mixture comprising at least one of S_P-**4** and R_P-**4**



A



B



3

wherein X is a conjugate base of an acid, n is 0 or 1, and LG is a leaving group; b) reacting the first mixture with a protecting compound to obtain a second mixture comprising at least one of protected S_P-**4** and protected R_P-**4**; and c) optionally subjecting the second mixture to crystallization, chromatography, or extraction in order to obtain **4**, S_P-**4**, or R_P-**4**.

In a first aspect of the seventh embodiment, the isopropyl alanate is present as its hydrochloric acid salt, which is preferably, substantially anhydrous.

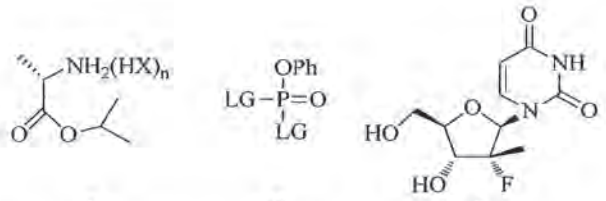
In a second aspect of the seventh embodiment, the base is N-methylimidazole.

In a third aspect of the seventh embodiment, the mole ratio of **A**-to-**B**-to-**3** is about 1.6-to-1.3-to-1.

In a fourth aspect of the seventh embodiment, the protecting compound is t-butyl-dimethyl-silyl-chloride.

An eighth embodiment is directed to a process for preparing S_P-**4** or R_P-**4**, which comprises: a) reacting an isopropyl-alanate, **A**, a di-LG-phenylphosphate, **B**, 2'-deoxy-2'-fluoro-2'-C-methyluridine, **3**, and a base to obtain a first mixture comprising at least one of S_P-**4** and R_P-**4**

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A B 3

wherein X is a conjugate base of an acid, n is 0 or 1, and LG is a leaving group; and b) optionally subjecting the second mixture to crystallization, chromatography, or extraction in order to obtain purified S_P-**4** or R_P-**4**.

A first aspect of the eighth embodiment for preparing R_P-**4** additionally includes further purifying the second mixture or the purified R_P-**4** by dissolving or suspending the second mixture or the purified R_P-**4** mixture in a solvent; optionally followed by seeding with crystalline R_P-**4**; and adding sufficient anti-solvent to obtain crystalline R_P-**4**.

A second aspect of the eighth embodiment for preparing S_P-**4** additionally includes further purifying the second mixture or the purified S_P-**4** by d) dissolving or suspending the second mixture or the purified S_P-**4** in a solvent followed by seeding with crystalline S_P-**4** at about room temperature; collecting a first solid the majority of which comprises S_P-**4**; dissolving the first solid in a solvent at its reflux temperature; and cooling or adding an anti-solvent to obtain a second solid.

A third aspect of the eighth embodiment for the preparation of S_P-**4**, additionally includes further purifying S_P-**4** by d) dissolving or suspending the second mixture or the purified S_P-**4** mixture in a first solvent followed by adding an anti-solvent so as to obtain a first composition in which the residual solvent/anti-solvent is removed by decanting to obtain a residue; treating the residue with a solution containing the first solvent and anti-solvent to yield a second composition whereby upon reducing the pressure affords a first solid; dissolving or suspending the first solid using a second solvent so as to obtain a third composition; adding seed crystals of S_P-**4** to the third composition; collecting a second solid; dissolving or suspending the second solid in a third solvent, optionally heated to the reflux temperature of the third solvent to obtain a fourth composition, and , if necessary, cooling the fourth composition to obtain a third solid comprising S_P-**4** which is collected by filtration.

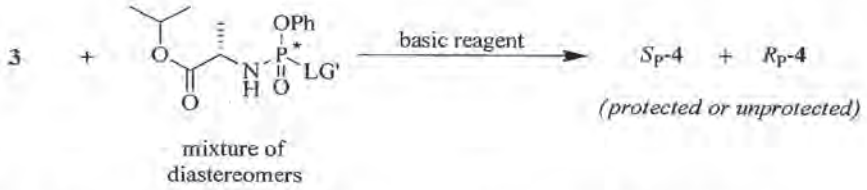
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In a fourth aspect of the eighth embodiment for the preparation of S_P-**4**, S_P-**4** is further purified by the second mixture or the purified S_P-**4** by d) adding silica gel to the second mixture or the purified S_P-**4** followed by solvent evaporation to afford a dry slurry; stirring the dry slurry in a first solvent/anti-solvent combination to obtain a first wet slurry; decanting the first solvent/anti-solvent combination from the first wet slurry to obtain a second wet slurry and a first composition; adding to the second wet slurry a second solvent/anti-solvent combination followed by stirring; decanting the second solvent/anti-solvent combination from the second wet slurry to obtain a third wet slurry and a second composition; optionally repeating steps g)–h) on the third wet slurry or additional wet slurries; evaporating the solvent from the second composition, and optionally any additional composition obtained from optional step i) to obtain a first solid; dissolving or suspending the first solid in a solution containing a third solvent and optionally a fourth solvent to obtain a third composition; optionally adding seed crystals of S_P-**4** to the third composition; obtaining from the third composition a second solid comprising S_P-**4**; and optionally recrystallizing the second solid using a third solvent to obtain a third solid comprising S_P-**4**.

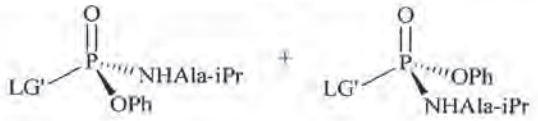
One of ordinary skill will appreciate that the compounds can be separated by traditional extraction, traditional crystallization or traditional chromatographic techniques. Traditional chromatographic techniques include, but are not limited to, chromatography on silica gel (using, e.g., 3-5% methanol in DCM or 4-6% isopropanol in DCM) to produce enhanced levels of one isomer (50-100%) and then crystallize it. Alternatively, one could use reversed phase chromatography (using, e.g., 1-30% acetonitrile-aqueous mobile phase). Furthermore the compounds can be isolated by supercritical fluid chromatography SFC with carbon dioxide as the main solvent and alcohols such as methanol as a modifier, preferably using the appropriate chiral media, such as, Daicel Chiralpack IA. Alternatively, SMB chromatography may be employed using the appropriate chiral media, such as, Daicel ChiralPack IA, using a mixture of solvents such as hexanes/isopropanol or single solvents such as ethyl acetate.

A ninth embodiment is directed to a process for preparing S_P-**4**, which comprises: a) reacting an isopropyl-alanyl-phosphoramidate with a 3'-O-protected or unprotected **3**, and a basic reagent to obtain a composition comprising protected or unprotected S_P-**4**

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wherein the isopropyl-alanyl-phosphoramidate is comprised of a mixture of diastereomers represented by the following structures:



C C'

wherein the ratio of **C**:**C'** is about 1:1.

In a first aspect, the basic reagent is t-butylmagnesium chloride and the ratio of **C**:**C'** is greater than or equal to about 1:1.

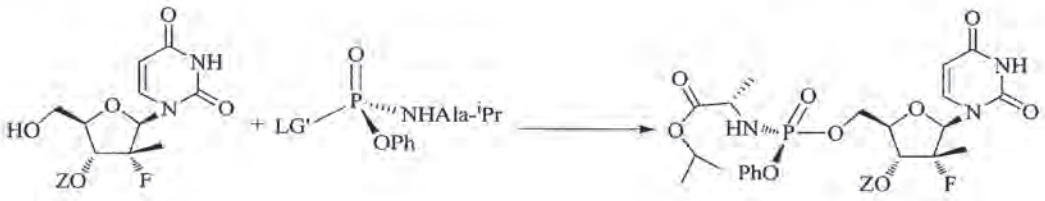
In a second aspect, the basic reagent is t-butylmagnesium chloride and the ratio of **C**:**C'**; is greater than about 1:1.

In a third aspect, the basic reagent is t-butylmagnesium chloride and the ratio of **C**:**C'** is at least about 1.5:1, about 2.3:1, about 4:1, about 5.7:1, about 9:1, about 19:1, about 32.3:1, about 49:1, or about 99:1.

A fourth aspect the LG' is selected from among, 2,4-dinitrophenoxide, 4-nitrophenoxide, 2-nitrophenoxide, 2-chloro-4-nitrophenoxide, 2,4-dichlorophenoxide, and pentafluorophenoxide, the basic reagent is t-butylmagnesium chloride, and the ratio of **C**:**C'** is at least about 1.5:1, about 2.3:1, about 4:1, about 5.7:1, about 9:1, about 19:1, about 32.3:1, about 49:1, or about 99:1.

A fifth aspect for preparing S_P-**4**, comprises: a) reacting an isopropyl-alanyl-phosphoramidate (**C**) with a 3'-O-protected or unprotected **3**, and a basic reagent to obtain a composition comprising protected or unprotected S_P-**4**

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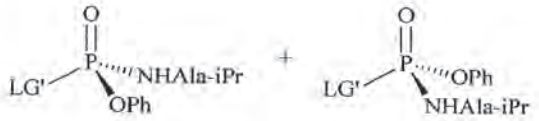


3 C

wherein Z is a protecting group or hydrogen; LG' is a leaving group; and b) optionally subjecting the obtained protected or unprotected S_P-**4** to chromatography, extraction, or crystallization in order to obtain purified protected or unprotected S_P-**4**. In a sub-embodiment, LG' is tosylate, camphorsulfonate, or an aryloxide substituted with at least one electron withdrawing group; more preferably, LG' is selected from among 2,4-dinitrophenoxide, 4-nitrophenoxide, 2-nitrophenoxide, 2-chloro-4-nitrophenoxide, 2,4-dichlorophenoxide, or pentafluorophenoxide. In a further sub-embodiment, when S_P-**4** is protected, i.e., Z is not hydrogen, the process of the ninth embodiment is further directed to deprotecting protected S_P-**4**. In a further sub-embodiment, the reaction is conducted in a polar aprotic solvent, such as, tetrahydrofuran or another etheral solvent either one being alone or in combination with each other or with a C_2 to C_7 nitrile, such as acetonitrile.

The process of the ninth embodiment further comprises 1) reacting $(LG')P(O)(LG)_2$, wherein LG, independent of LG', is a leaving group, with (i) isopropyl-alanate and a first base to obtain (LG')P(O)(LG)(NHAla-^{i}Pr) followed by reacting (LG')P(O)(LG)(NHAla-^{i}Pr) with phenol and a second base to obtain a mixture comprising **C** and **C'**, (ii) phenol and a first base to obtain (LG')P(O)(LG)(OPh) followed by reacting (LG')P(O)(LG)(OPh) with isopropyl-alanate and a second base to obtain a mixture comprising **C** and **C'**, or (iii) combining isopropyl-alanate, phenol, and at least one base to obtain a mixture comprising **C** and **C'**; or 2) reacting $(PhO)P(O)(LG)_2$, wherein LG is a leaving group, with (i) isopropyl-alanate and a first base to obtain (PhO)P(O)(LG)(NHAla-^{i}Pr) followed by reacting (PhO)P(O)(LG)(NHAla-^{i}Pr) with a leaving group precursor (LG'H) and a second base to obtain a mixture comprising **C** and **C'**,

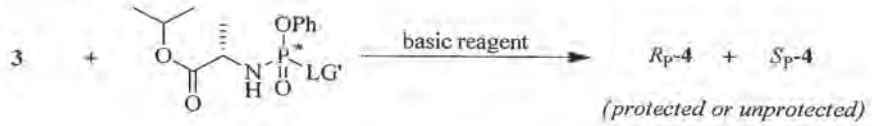
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C C'

and subjecting the mixture to chromatography or crystallizing the mixture to obtain **C**. In an aspect of the ninth embodiment, the isopropyl alanate is present as its hydrochloric acid salt, which is preferably, substantially anhydrous.

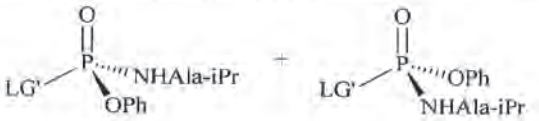
A tenth embodiment is directed to a process for preparing R_P-**4**, which comprises: a) reacting an isopropyl-alanyl-phosphoramidate with a 3'-O-protected or unprotected **3**, and a basic reagent to obtain a composition comprising protected or unprotected R_P-**4**



mixture of diastereomers

(protected or unprotected)

wherein the isopropyl-alanyl-phosphoramidate is comprised of a mixture of diastereomers represented by the following structures:



C C'

wherein the ratio of **C'**:**C** is about 1:1.

In a first aspect, the basic reagent is t-butylmagnesium chloride and the ratio of **C'**:**C** is greater than or equal to about 1:1.

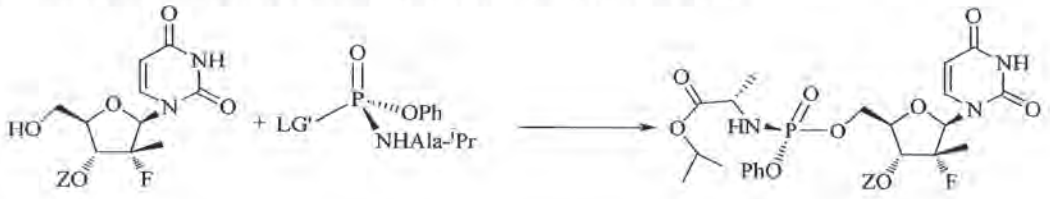
In a second aspect, the basic reagent is t-butylmagnesium chloride and the ratio of **C'**:**C**; is greater than about 1:1.

In a third aspect, the basic reagent is t-butylmagnesium chloride and the ratio of **C'**:**C** is at least about 1.5:1, about 2.3:1, about 4:1, about 5.7:1, about 9:1, about 19:1, about 32.3:1, about 49:1, or about 99:1.

A fourth aspect the LG' is p-nitrophenoxide, the basic reagent is t-butylmagnesium chloride, and the ratio of **C'**:**C** is at least about 1.5:1, about 2.3:1, about 4:1, about 5.7:1, about 9:1, about 19:1, about 32.3:1, about 49:1, or about 99:1.

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A fifth aspect for preparing R_P-**4**, comprises: a) reacting an isopropyl-alanyl-phosphoramidate (**C'**) with a 3'-O-protected or unprotected **3**, and a basic reagent to obtain a composition comprising protected or unprotected R_P-**4**



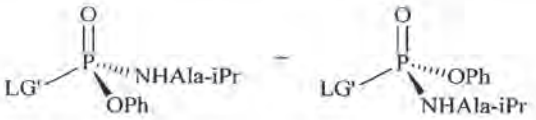
3 **C'**

wherein Z is a protecting group or hydrogen; LG' is a leaving group; and b) optionally subjecting the obtained protected or unprotected R_P-**4** to chromatography, extraction, or crystallization in order to obtain purified protected or unprotected R_P-**4**. In a sub-embodiment, LG' is tosylate, camphorsulfonate, or an aryloxide substituted with at least one electron withdrawing group; more preferably, LG' is selected from among p-nitrophenoxide, 2,4-dinitrophenoxide, and pentafluorophenoxide. In a further sub-embodiment, when R_P-**4** is protected, i.e., Z is not hydrogen, the process of the ninth embodiment is further directed to deprotecting protected R_P-**4**. In a further sub-embodiment, the reaction is conducted in a polar aprotic solvent, such as, tetrahydrofuran or another etheral solvent either one being alone or in combination with each other or with a C_2 to C_7 nitrile, such as acetonitrile.

The process of the tenth embodiment further comprises 1) reacting $(LG')P(O)(LG)_2$, wherein LG, independent of LG', is a leaving group, with (i) isopropyl-alanate and a first base to obtain (LG')P(O)(LG)(NHAla-^{i}Pr) followed by reacting (LG')P(O)(LG)(NHAla-^{i}Pr) with phenol and a second base to obtain a mixture comprising **C** and **C'**, (ii) phenol and a first base to obtain (LG')P(O)(LG)(OPh) followed by reacting (LG')P(O)(LG)(OPh) with isopropyl-alanate and a second base to obtain a mixture comprising **C** and **C'**, or (iii) combining isopropyl-alanate, phenol, and at least one base to obtain a mixture comprising **C** and **C'**; or 2) reacting $(PhO)P(O)(LG)_2$, wherein LG', independent of LG, is a leaving group, with (i) isopropyl-alanate and a first base to obtain (PhO)P(O)(LG)(NHAla-^{i}Pr) followed by reacting (PhO)P(O)(LG)(NHAla-^{i}Pr) with

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a leaving group precursor and a second base to obtain a mixture comprising **C** and **C'**,

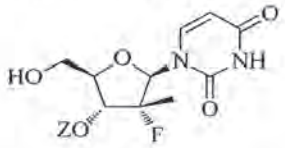


C C'

and subjecting the mixture to chromatography or crystallizing the mixture to obtain **C'**. In an aspect of the ninth embodiment, the isopropyl alanate is present as its hydrochloric acid salt, which is preferably, substantially anhydrous.

An eleventh embodiment is directed to a composition obtained by the processes recited in the seventh embodiment, the eighth embodiment, the ninth embodiment or the tenth embodiment as well as their respective aspects. An aspect of the eleventh embodiment is directed to a composition obtained by any one of the exemplified embodiments disclosed below. The so obtained composition can be crystalline, crystal-like, amorphous, or a combination thereof.

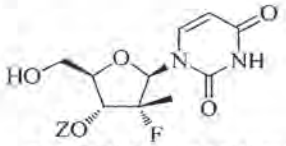
A twelfth embodiment is directed to a compound **3**



3

wherein Z is a protecting group or hydrogen; which is useful for the preparation of R_P-**4** or S_P-**4**.

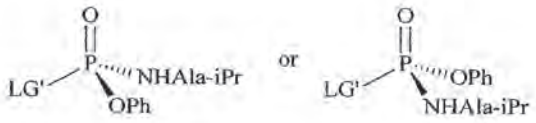
A first aspect of the twelfth embodiment is selected from among a compound having the following structure



3a: Z = -C(O)$CH_2CH_2C(O)CH_3$
3b: Z = -C(O)OCH_2Ph
3c: Z = -Si(Me)$_2$^{t}Bu
3d: Z = -Si(^{i}Pr)$_2$OSi(^{i}Pr)$_2$OH

A thirteenth embodiment is directed to a compound, its salt, hydrate, solvate, or combination thereof, represented by the following structures

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C C'

where LG' is a leaving group, which is useful for the preparation of R_P-**4** or S_P-**4**.

In a first aspect of the thirteenth embodiment, LG' is tosylate, camphorsulfonate, an aryloxide, or an aryloxide substituted with at least one electron withdrawing group.

In a second aspect of the thirteenth embodiment, LG' is selected from among 2,4-dinitrophenoxide, 4-nitrophenoxide, 2-nitrophenoxide, 2-chloro-4-nitrophenoxide, 2,4-dichlorophenoxide, or pentafluorophenoxide.

In a third aspect of the thirteenth embodiment, LG' is pentafluorophenoxide or 4-nitro-phenoxide.

A fourth aspect of the thirteenth embodiment is directed to compound **C**, wherein LG' is 2,4-dinitrophenoxide, 4-nitrophenoxide, 2-nitrophenoxide, 2-chloro-4-nitrophenoxide, 2,4-dichlorophenoxide, or pentafluorophenoxide.

A fifth aspect of the thirteenth embodiment is directed to compound C, wherein LG' is 4-nitrophenoxide or pentafluorophenoxide.

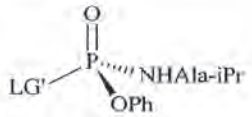
A sixth aspect of the thirteenth embodiment is directed to compound C, wherein LG' is 4-nitrophenoxide.

A seventh aspect of the thirteenth embodiment is directed to crystalline compound C, wherein LG' is 4-nitrophenoxide.

An eighth aspect of the thirteenth embodiment is directed to compound C, wherein LG' is pentafluorophenoxide.

A ninth aspect of the thirteenth embodiment is directed to crystalline compound C, wherein LG' is pentafluorophenoxide.

A fourteenth embodiment is directed to a process for preparing a compound represented by the structural formula



C

crystallizing the compound from a composition, comprising

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a) a first composition;

b) a second leaving group precursor;

c) a non-nucleophilic base; and

d) a liquid composition;

wherein the first composition comprises the compound and its corresponding P-based diastereomer.

In a first aspect of the fourteenth embodiment, the mole amount of the compound and the mole amount of its P-based diastereomer are the same or different.

In a second aspect of the fourteenth embodiment, the mole amount of the compound is greater than the mole amount of its corresponding P-based diastereomer or vice versa.

In a third aspect of the fourteenth embodiment, the second leaving group precursor is 2,4-dinitrophenol, 4-nitrophenol, 2-nitrophenol, 2-chloro-4-nitrophenol, 2,4-dichlorophenol, or pentafluorophenol.

In a fourth aspect of the fourteenth embodiment, LG' is pentafluorophenoxide. In a first sub-aspect, the second leaving group precursor is pentafluorophenol. In a second sub-aspect, the amount of pentafluorophenol ranges from about 0.01 mole equivalents to about 10 mole equivalents relative to the mole amount of the compound and its P-based diastereomer and all mole equivalents in between. In a third sub-aspect, the amount of pentafluorophenol ranges from about 0.1 mole equivalents to about 1 mole equivalents relative to the mole amount of the compound and its P-based diastereomer and all mole equivalents in between.

In a fifth aspect of the fourteenth embodiment, the crystallizing occurs at a temperature that ranges from about -10°C to about +40°C and all temperature values in between. In a first sub-aspect, the crystallizing occurs at about room temperature.

In a sixth aspect of the fourteenth embodiment, the non-nucleophilic base is selected from among potassium carbonate, cesium carbonate, di-isopropylamine, di-isopropylethylamine, triethylamine, quinuclidine, naphthalene-1,8-diamine, 2,2,6,6-tetramethylpiperidine, 1,8-diazabicycloundec-7-ene, 4-dimethylamino-pyridine, pyridine, a 2,6-di-C_{1-6}-alkyl-pyridine, a 2,4,6-tri-C_{1-6}-alkyl-pyridine, and mixtures thereof. In a first sub-aspect, the non-nucleophilic base is triethylamine or 1,8-diazabicycloundec-7-ene. In a second sub-aspect, the non-nucleophilic base is triethylamine.

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In a seventh aspect of the fourteenth embodiment, the non-nucleophilic base is present in an amount that ranges from about 0.01 equivalents mol to about 10 mol equivalents, and all mole equivalents in between, relative to the total mole amount of the compound and its P-based diastereomer. In a first sub-aspect, the non-nucleophilic base is present in an amount that ranges from about 0.1 mol equivalents to about 1 mol equivalents, and all mole equivalents in between, relative to the total mole amount of the compound and its P-based diastereomer .

In an eighth aspect of the fourteenth embodiment, the solubility of the compound is less than the solubility of its corresponding P-based diastereomer in the liquid composition or vice versa.

In a ninth aspect of the fourteenth embodiment, the liquid composition comprises at least one of a solvent and an anti-solvent. In a first sub-aspect, the liquid composition comprises at least one of a C_1 to C_8 alcohol, a C_2 to C_8 ether, a C_3 to C_7 ketone, a C_3 to C_7 ester, a C_1 to C_2 chlorocarbon, a C_2 to C_7 nitrile, a C_5 to C_{12} saturated hydrocarbon, and a C_6 to C_{12} aromatic hydrocarbon. In a second sub-aspect, the liquid composition comprises at least one of a C_2 to C_8 ether, a C_3 to C_7 ester, a C_5 to C_{12} saturated hydrocarbon, and a C_6 to C_{12} aromatic hydrocarbon. In a third sub-aspect, the liquid composition comprises at least one of a C_2 to C_8 ether, a C_3 to C_7 ester, and a C_5 to C_{12} saturated hydrocarbon. In a fourth sub-aspect, the liquid composition comprises at least one of ethyl acetate, t-butyl-methylether, and hexane. In a fifth sub-aspect, the liquid composition comprises ethyl acetate and hexane. In a sixth sub-aspect, the liquid composition comprises t-butyl-methylether and hexane.

In a tenth aspect of the fourteenth embodiment, the amount of liquid composition ranges from about 1 mL to about 10 mL for every gram of the first composition and all mL/g values in between.

An eleventh aspect of the fourteenth embodiment further comprises adding crystalline compound to the composition. A first sub-aspect further comprises adding about 0.1 to about 1 wt. %, and all wt. % values in between, of crystalline compound to the first composition.

A twelfth aspect of the fourteenth embodiment further comprises

a) reacting $PhOP(O)(LG)_2$ and ^{i}Pr-Ala-NH_2•HCl in the presence of a first base to obtain (PhO)P(O)(LG)(NHAla-^{i}Pr);

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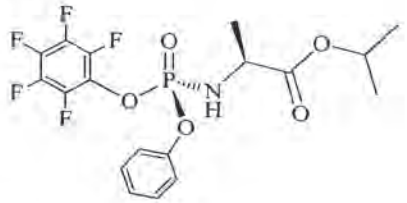
b) reacting (PhO)P(O)(LG)(NHAla-^{i}Pr) with a first leaving group precursor (LG'H) in the presence of a second base to obtain the composition comprising the compound and its P-based diastereomer;

wherein LG and LG', independent of each other, are leaving groups;

wherein the first leaving group precursor and the second leaving group precursor are the same or different; and

wherein the first base and the second base are the same or different.

A fifteenth embodiment is directed to a process for preparing crystalline (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate having the following structure,



which comprises:

crystallizing (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate from a second composition comprising

a) a first composition;

b) pentafluorophenol;

c) a non-nucleophilic base; and

d) a liquid composition;

wherein the second composition comprises (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate and (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate.

In a first aspect of the fifteenth embodiment, the mole amount of the (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate and the mole amount of the (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate are the same or different.

In a second aspect of the fifteenth embodiment, the mole amount of the (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate is

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greater than the mole amount of the (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate.

In a third aspect of the fifteenth embodiment, the amount of pentafluorophenol ranges from about 0.01 mole equivalents to about 10 mole equivalents (and all mole equivalent values in between) relative to the mole amount of (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate and (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate. In a first sub-aspect, the amount of pentafluorophenol ranges from about 0.1 mole equivalents to about 1 mole equivalents (and all mole equivalent values in between) relative to the mole amount of (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate and (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate.

In a fourth aspect of the fifteenth embodiment, the crystallizing occurs at a temperature that ranges from about -10°C to about +40°C and all temperature values in between. In a first sub-aspect, the crystallizing occurs at about room temperature.

In a fifth aspect of the fifteenth embodiment, the non-nucleophilic base is selected from among potassium carbonate, cesium carbonate, di-isopropylamine, di-isopropylethylamine, triethylamine, quinuclidine, naphthalene-1,8-diamine, 2,2,6,6-tetramethylpiperidine, 1,8-diazabicycloundec-7-ene, 4-dimethylamino-pyridine, pyridine, a 2,6-di-C_{1-6}-alkyl-pyridine, a 2,4,6-tri-C_{1-6}-alkyl-pyridine, and mixtures thereof. In a first sub-aspect, the non-nucleophilic base is triethylamine or 1,8-diazabicycloundec-7-ene. In a second sub-aspect, the non-nucleophilic base is triethylamine.

In a sixth aspect of the fifteenth embodiment, the non-nucleophilic base is present in an amount that ranges from about 0.1 to about 1 mol equivalents (and all mole equivalent values in between) relative to the total mole amount of (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate and (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate.

In a seventh aspect of the fifteenth embodiment, the solubility of (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate is less than the solubility of (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate in the liquid composition.

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In an eighth aspect of the fifteenth embodiment, the liquid composition comprises at least one of a solvent and an anti-solvent. In a first sub-aspect, the liquid composition comprises at least one of a C_1 to C_8 alcohol, a C_2 to C_8 ether, a C_3 to C_7 ketone, a C_3 to C_7 ester, a C_1 to C_2 chlorocarbon, a C_2 to C_7 nitrile, a C_5 to C_{12} saturated hydrocarbon, and a C_6 to C_{12} aromatic hydrocarbon. In a second sub-aspect, the liquid composition comprises at least one of a C_2 to C_8 ether, a C_3 to C_7 ester, a C_5 to C_{12} saturated hydrocarbon, and a C_6 to C_{12} aromatic hydrocarbon. In a third sub-aspect, the liquid composition comprises at least one of a C_2 to C_8 ether, a C_3 to C_7 ester, and a C_5 to C_{12} saturated hydrocarbon. In a fourth sub-aspect, the liquid composition comprises at least one of ethyl acetate, t-butyl-methylether, and hexane. In a fifth sub-aspect, the liquid composition comprises ethyl acetate and hexane. In a sixth sub-aspect, the liquid composition comprises t-butyl-methylether and hexane.

In a ninth aspect of the fifteenth embodiment, the amount of liquid composition ranges from about 1 to about 10 mL for every gram (and all mL/g values in between) of the first composition.

A tenth aspect of the fifteenth embodiment further comprises adding crystalline (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate to the second composition.

An eleventh aspect of the fifteenth embodiment further comprises adding about 0.1 to about 1 wt. % (and all wt.% values in between) of crystalline (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate based on the total weight of (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate in the first composition.

A sixteenth embodiment is directed to crystalline (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate obtained by the process of the fifteenth embodiment.

A seventeenth embodiment is directed to a process for preparing (S)-isopropyl 2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate, which comprises:

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crystallizing (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate from a second composition comprising

a) a first composition;

b) pentafluorophenol;

c) a non-nucleophilic base; and

d) a liquid composition;

wherein the first composition comprises (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate and (S)-isopropyl 2-(((R)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate.

A first aspect of the seventeenth embodiment is directed to a process for preparing (S)-isopropyl 2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate, which comprises:

contacting (S)-isopropyl 2-(((S)-(perfluorophenoxy)(phenoxy)phosphoryl)amino)propanoate with a product obtained by reacting a t-butylmagnesium halide with 1-((2R,3R,4R,5R)-3-fluoro-4-hydroxy-5-(hydroxymethyl)-3-methyltetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione with a t-butylmagnesium halide.

In a second aspect of the seventeenth embodiment, the contacting occurs in a medium having a temperature that ranges from about 0°C to about 40°C and all temperature values in between.

In a third aspect of the seventeenth embodiment, the contacting occurs in a medium having a temperature that ranges from about 0°C to about 30°C and all temperature values in between.

In a fourth aspect of the seventeenth embodiment, the mole ratio of t-butylmagnesium halide to 1-((2R,3R,4R,5R)-3-fluoro-4-hydroxy-5-(hydroxymethyl)-3-methyltetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione ranges from about 2 to about 2.2. In a first sub-aspect, the mole ratio of t-butylmagnesium halide to 1-((2R,3R,4R,5R)-3-fluoro-4-hydroxy-5-(hydroxymethyl)-3-methyltetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione is about 2.1.

In a fifth aspect of the seventeenth embodiment, the t-butylmagnesium halide is t-butylmagnesium chloride.

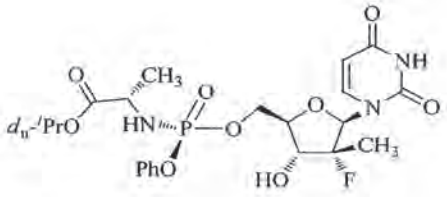
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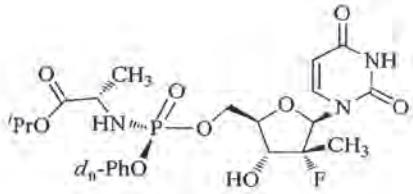
An eighteenth embodiment is directed to a process for preparing substantially pure (S)-isopropyl 2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate, which comprises:

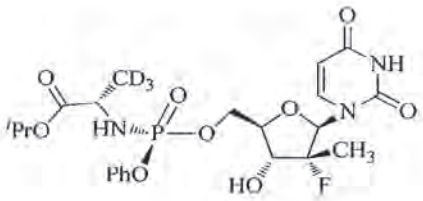
obtaining (S)-isopropyl 2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate according to any of the relevant embodiments disclosed herein, and

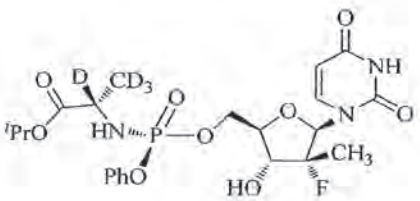
crystallizing the so-formed (S)-isopropyl 2-(((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)amino)propanoate.

A nineteenth embodiment is directed to an isotopically-labeled analog of R_P-**4** or S_P-**4**. The term "isotopically-labeled" analog refers to an analog of R_P-**4** or S_P-**4** that is a "deuterated analog", a "^{13}C-labeled analog," or a "deuterated/^{13}-C-labeled analog." The term "deuterated analog" means a compound described herein, whereby a ^{1}H-isotope, i.e., hydrogen (H), is substituted by a ^{2}H-isotope, i.e., deuterium (D). Deuterium substitution can be partial or complete. Partial deuterium substitution means that at least one hydrogen is substituted by at least one deuterium. For instance, for R_P-**4** or S_P-**4**, one of ordinary skill can contemplate at least the following partial deuterated analogs (where "d_n" represents n-number of deuterium atoms, such as, for an isopropyl group n = 1-7, while for a phenyl group, n = 1-5), as well as those depicted below.

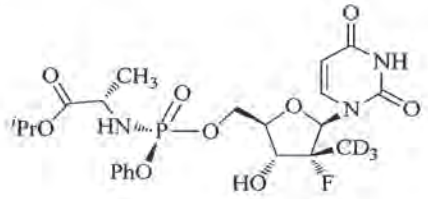


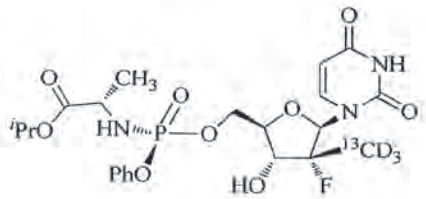






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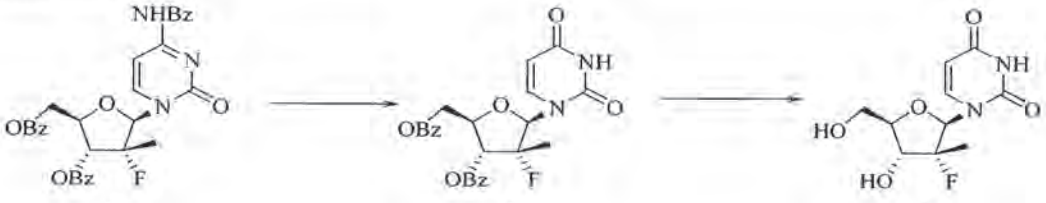
Although the methyl groups depicted above are shown as being completely deuterated, one will recognize that partial-deuterated variations are also possible, such as, $-CDH_2$ and $-CD_2H$. Isotopic labels on the furanose and base are also contemplated. Likewise, the terms "^{13}C-labeled analog" and "deuterated/^{13}-C-labeled analog" refers to a compound described herein, whereby carbon atom is enriched with a ^{13}C-isotope meaning that the extent of enrichment exceeds the usual natural abundance of about 1.1%.

Examples

Not to be limited by way of example, the following examples serve to facilitate a better understanding of the disclosure.

Synthetic Aspects

In order to prepare the uridine nucleoside, one could take advantage of an advanced tribenzoylated cytidine intermediate in the synthesis of certain 3',5'-diacylated analogs of **3** (see below) already produced efficiently on a pilot plant scale (see WO 2006/031725 or US 2006/0122146, both of which are incorporated by reference in their entirety). The following method was found to be scalable and cost-efficient.



1 **2** **3**

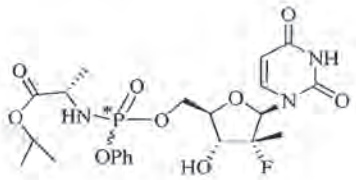
3',5'-O-dibenozyl-2'-deoxy-2'-fluoro-2'-C-methyl-N^4-benzoylcytidine (**1**) is obtained by a method disclosed in WO 2006/031725 and WO 2008/045419 both of

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which are hereby incorporated by reference in its entirety. **1** is treated with 70% aqueous acetic acid to form 3',5'-O-dibenozyl-2'-deoxy-2'-fluoro-2'-C-methyl-uridine (**2**). The benzoyl esters can be hydrolyzed by a number of methods as well, e.g., alkoxides in alcoholic solvent, such as sodium methoxide in methanol, potassium carbonate in methanol, or ethanol analogs, alkylamines such as methylamine in methanol, butylamine etc. Methanolic ammonia was chosen for the larger scale work. The uridine product (**3**) can be purified by crystallization to afford a 70% yield from the tribenzoylated cytidine (**1**).

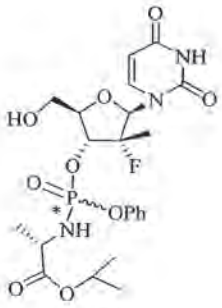
Numerous literature procedures detail different routes and conditions to make phosphoramidates using several fold equivalents of reagents. See, for example, McGuigan et al. *J. Med. Chem.* 2005, 48, 3504-3515 and McGuigan et al. *J. Med. Chem.* 2006, 49, 7215. For process scale work, there is only one presently known example, which is disclosed in Lehsten et al., *Org. Process Res. Dev.* 2002, 6, 819-822 ("Lehsten"). In this reference, the authors introduce the concept of a "one-pot procedure" in which an amino acid hydrochloride salt and phenyl dichlorophosphate are reacted together with N-methylimidazole in dichloromethane. Later the nucleoside is added to form the desired 5'-O-phosphoramidate product, which in the present case would yield a compound represented by formula **4**. Unfortunately, the Lehsten procedure suffered from drawbacks. For example, the Lehsten procedure utilized a far larger excess of reagents than was necessary which added to the cost and difficulty of chromatographic purification. Furthermore, Lehsten suggested that one could control the reaction selectivity on the 5'-hydroxyl over the 3'-hydroxyl compared to a literature reference through using lower temperatures and slow addition of the nucleoside.

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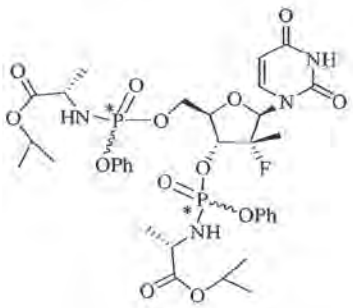
4

5'-O-phosphoramidate
(2 diastereomers)



5

3'-O-phoshoramidate
(2 diastereomers)



6

3',5'-bis-O-phosphoramidate
(4 diastereomers)

Using the Lehsten procedure for the compounds disclosed herein provided for about 1-5% of mono-substituted 3'-O-phosphoramidate diastereomers (**5**) and about 10-30% of the bis-substituted product (**6**). As the polarity of the 3'-diastereomers was very similar to the desired 5'-diastereomers (**4**), chromatographic separation was very challenging. Scaling up the process was nearly impossible without discarding a substantial portion of the less polar 5'-diastereomers (**4**) or accepting a higher level of contamination of the 3'-diastereomers (**5**). In an initial 50 g scale-up, the resultant product contained a 3'-diastereomer (**5**) contamination of about 3%, which co-eluted with the less polar of the 5'-diastereromer (**4**).

Disclosed herein are reaction conditions which use lesser amounts of reagents and a method to selectively remove the impurity 3'-O-phosphoramidate diastereomers (**5**) with an easier chromatographic separation thereby affording the desired 5'-O-phosphoramidate diastereomers in much higher purity (**4**).

For the reagent stoichiometry, a study was made in which the stoichiometry of the reagents was systematically changed and the results were monitored by phosphorus NMR of the crude reaction as Lehsten had reported. In the more successful runs, the isolated yield and purity of the desired product were compared. It was observed that the primary 5'-hydroxyl reacts at a faster rate than the secondary 3'-hydroxyl. This creates a competing situation between the reaction progress of consuming all the starting nucleoside and converting 5'- and 3'-monosubstituted products (**4** and **5**) to the 5',3'-bis substituted products (**6**). The 3'-monosubstituted product converts to the bis product at a faster rate than the 5'-monosubstituted product, so it is possible to reduce the 3'-diastereomer contamination level by

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pushing the reaction more to the bis-substituted products. However, with an effective way to remove the 3'-diastereomers, the reaction can be optimized to produce more of the desired 5'-diastereomer without having to sacrifice as much of the 5'-diastereomer being converted to the bis-substituted (**6**). It was also observed that the amino acid hydrochloride is very hygroscopic. As any water present would consume an equivalent amount of the phenyl dichlorophosphate reagent, care must be taken to keep the amino acid substantially anhydrous or it should be made substantially anhydrous prior to use. In short, Lehsten had reported that the optimum ratio of amino acid to phenyl dichlorophosphate to nucleoside was 3.5:2.5:1 respectively. It was found that the optimum ratio of amino acid to phenyl dichlorophosphate to nucleoside of about 1.6 to about 1.3 to about 1 is optimal under conditions in which the 3'-diastereomer can be efficiently removed and when the amino acid hydrochloride is substantially anhydrous. By using a smaller amount of the reagents, a cost savings is realized coupled with a simplification of the chromatographic separation of the desired product from reagent by-products and from the reduced level of bis diastereomers.

In one alternative procedure, a 3'-hydroxy-blocked derivative of **3** was prepared using a t-butyldimethylsilyl blocking group in two steps. This was then converted to its 5'-phosphoramidate derivative. The desire being that the silyl group could then be removed and there would be no 3' isomers (**5**) or 3',5'-bis phosphoramidates (**6**). A similar approach was demonstrated by Borch and Fries (U.S. Patent 5,233,031) in a low overall yield on an alkyl phosphoramidate.

Another alternative approach was to use the direct synthesis and then use chemistry to help differentiate the 3'-diastereomer impurities **5** from the desired 5'-diastereomers **4** to help the separation. A group was desired that would selectively react with the free primary hydroxyl of the 3'-O-phosphoramidate impurity **5** over the free secondary hydroxyl of the desired 5'-O-phosphoramidate **4**. It was also desired that the blocking group significantly change the polarity of the resulting 5'-O-blocked 3'-O-phoshoramidate product from the desired 5'-O-phosphoramidate **4**. There would be no extra step needed to remove the blocking group as the desired 5'-diastereomers **4** would not be changed. The chemically altered 3'-diastereomers would then allow easier chromatographic separation or separation by special scavenging supports or by extractions.

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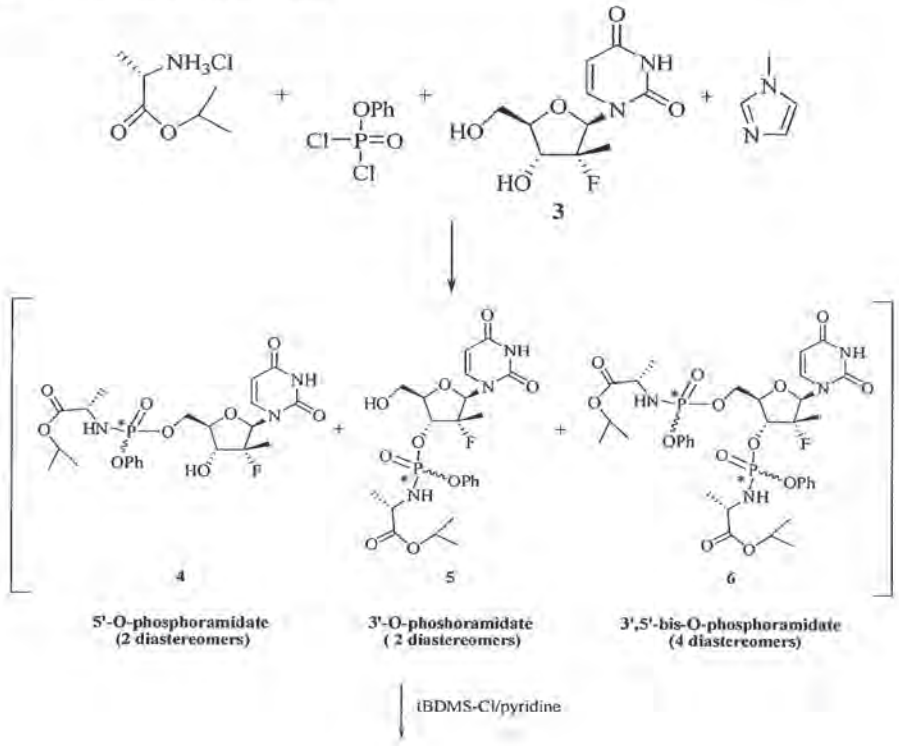
Specifically, the blocking group tert-butyldimethylsilyl (tBDMS) met these criteria and was the first one to be demonstrated and subsequently used on a multi-kilogram scale. Under certain conditions such as in pyridine as solvent and base, the tBDMS group reacts with high selectively at the primary hydroxyl position over the 3' secondary hydroxyl position. The phosphoramidate reaction uses N-methylimidazole (NMI) as a base. In the presence of NMI, the silylation is less selective. Preferably, the amount of NMI should be reduced. This can be accomplished easily after the phosphoramidate reaction by washing the reaction solution with 1 N hydrochloric acid. The NMI and the remaining starting nucleoside are removed, leaving a crude mixture of mono and bis substituted products and reagent by-products. This is then dissolved in pyridine and treated with tert-butyldimethylsilyl chloride. The 3'-monosubstituted product **5** is converted in a few hours or less to the 5'-O-tBDMS-3'-O-phosphoramidate **7**. The reaction progress can be monitored by HPLC. The polarity of this silylated product **7** is less than the bis-phosphoramidate **6** and is readily removed by chromatography. Using this method, it was possible to reduce the level of 3'-monophosphoramidate **5** to less than 0.1% of the 5'-product **4** compared to 1-3% without the silyl treatment. Similarly, treatment with dimethoxytriphenylmethyl chloride (DMT-Cl) under the same conditions worked just as well. It was also easier to identify the DMT reaction product by TLC as DMT containing molecules stain bright orange on heating or exposure to acid. One can also envision many other blocking groups, as noted above.

Both the reaction conditions and the scavenging of the 3'-impurity are general methods and could be applied to most nucleoside phosphoramidates with a free 3' hydroxyl. The phosphoramidate moiety could be any combination of amino acid ester and aromatic alcohol. The nucleoside moiety could be any nucleoside in which a 5' phosphoramidate would lead to a 5'-monophosphate and could be further metabolized to the 5'-triphosphate form.

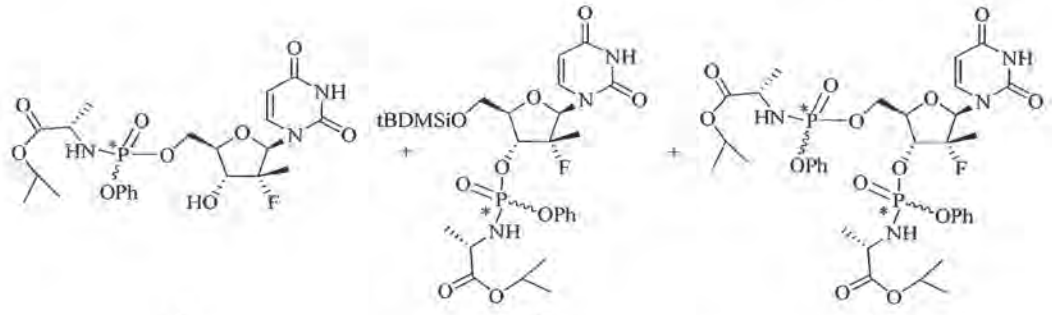
The following scheme is the main reaction scheme illustrated for making isopropyl L-alanate phenyl phosphoramidate of 2'-deoxy-2'-fluoro-2'-C-methyluridine with the major product as the desired 5'-O-phosphoramidate (**4**, two diastereomers) and the minor product as the 3'-O-phosphoramidate (**5**, two diastereomers) and the 3',5' -bis-O-phosphoramidate (**6**, four diastereomers). The reagents are added in the stoichiometric ratios as described in the method of

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preparation section. The reaction is allowed to proceed until about 5% of the starting material remains as judged by UV visualization on thin layer chromatography (TLC). Also UPLC/MS showed approximately 10% of the 3',5' bis-phosphoramidate **6** had formed compared to the desired 5'-product. After quenching and an acidic aqueous workup, the crude residue from the organic layer was prepared for the silylation. Under the described reaction conditions, the silyl group preferentially reacted with the free 5'-hydroxyl of the 3'-O-phosphoramidate to form **7**. The reaction was continued until the 3'-O-phosphoramidate was no longer detectable by UPLC/MS.



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4 7 6

3'-O-phoshoramidate-5'-O-tBDMS
(2 diastereomers)

After working up the silylation reaction, the desired product is subjected to chromatography on silica gel and is eluted with a gradient of methanol in dichloromethane (1-4%). The desired 5'-monophosphoramidate **4** elutes last.

Method of Preparation

Example 1. Preparation of 2'-deoxy-2'-fluoro-2'-C-methyluridine (3)

In a 10 L flask, was added 3', 5'-O-dibenozyl-2'-deoxy-2'-fluoro-2'-C-methyl-N4-benzoylcytidine (500 g, 0.874 mol) and 70% aqueous acetic acid (7.5 L). The solution was heated to reflux (110°C) for 20 h. TLC indicated a complete reaction (Rf 0.6 in 5% methanol in dichloromethane (DCM)). The mixture was cooled to ambient temperature and diluted with water (2 L). After stirring for 2 h, the resulting precipitate was collected by filtration and the solid was rinsed with water (5 L) and dried in the atmosphere at ambient temperature for 12 h to afford 360 g (88%). This dibenzoyluridine intermediate was used directly in the next step by adding it all to freshly prepared methanolic ammonia (5.4 L, ca 25%) at 0°C. This temperature was maintained for 3 h and then allowed to warm to 15°C for 24 h. TLC indicated a complete reaction (Rf 0.4 in 10% methanol in DCM). The reaction mixture was filtered through a Celite bed and concentrated under reduced pressure to give the crude product (216 g). The crude product was stirred with ethyl acetate (325 mL) for 3 h at ambient temperature. The resulting solid was collected by filtration and washed with ethyl acetate (216 mL). The solid was dried under vacuum at ambient temperature for 4h to afford 160 g (78%) of the desired product

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in 98.7% HPLC purity. ^{1}H-NMR (DMSO-d_6) δ 11.44 (br s, 1H, NH), 7.95 (d, 1H, C-6H), 5.97 (d, 1H, C-1'H), 5.64 (d, 1H, C-5H), 3.84-3.77 (m, 3H, C-5'-Ha, C-3'H. C-4'H), 3.63-3.60 (m, 1H, C5'-Hb), 1.23 (d, 3H, C-2'-CH_3). ES-MS M-1 259.

Example 2. Preparation of (S)-2-{[(1R,4R,5R)-5-(2,4-Dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-(R)-fluoro-3-hydroxy-4-methyl-tetrahydro-furan-2-ylmethoxy]-phenoxy-phosphorylamino}-propionic acid isopropyl ester (4)

Synonym: 5'-O-(Isopropyl-L-alanate, phenyl phosphoramidyl)-2'-deoxy-2'-fluoro-2'-C-methyl-uridine diastereomeric mixture.

A 5 L 3-necked flask was fitted with a mechanical stirrer, brine ice bath, internal thermometer, and a nitrogen atmosphere. The flask was charged with L-alanine isopropyl ester hydrochloride (82.0 g, 0.490 moles) and anhydrous dichloromethane (0.80 L). While this was stirring, phenyl dichlorophosphate (85.0 g, 0.40 moles) was added in one lot and stirred. While maintaining the internal temperature between -5 to 5°C, a solution of N-methylimidazole (NMI, 250 g, 3.07 moles) in dichloromethane (250 mL) was added over a period of a half hour. The solution was allowed to stir for 1 h in this temperature range. 2'-Deoxy-2'-fluoro-2'-C-methyl-uridine (**3**, 80.0 g, 0.307 moles) was added at 0°C in one portion and then the reaction flask was allowed to warm up slowly in the brine bath. At 1 h, the internal temperature was up to -2°C. TLC (5% Methanol in DCM) at 1 h showed that more than 50% of nucleoside was consumed. The bath was removed and the reaction flask reached ambient temperature over 1 h more. TLC after 3 h and at 5 h total showed 95% of the starting nucleoside was consumed. The reaction mixture was quenched by adding methanol (100 mL) and stirring the reaction for 5 minutes.

The reaction mixture was washed with 1N HCl (2 X 500 mL) followed by saturated sodium bicarbonate solution (2 X 500 mL). The separated organic layer was dried over anhydrous sodium sulfate (50 g) and filtered. The solution was evaporated under reduced pressure and then under high vacuum to dryness to give the crude product as a viscous oil (170 g). NMRs of the crude product (^{31}P and ^{1}H) were taken. The ^{31}P-NMR indicated about 1% of the total phosphorus integration was due to the presence of the 3' isomer **5**.

To the crude product was added anhydrous pyridine (1700 mL). The solvent was evaporated under reduced pressure and then under high vacuum in order to

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reduce the water content of the crude mixture through co-evaporation. The resulting oil was re-dissolved in anhydrous pyridine (500 ml) and then was added excess t-butyldimethylsilyl chloride (9.0 g, 60mM). The reaction was stirred at ambient temperature. Reaction progress was monitored by UPLC/MS. After 3 hours, the 3' impurity **5** could no longer be detected and the reaction was quenched by the addition of methanol (50 mL).

The reaction was evaporated under reduced pressure to an oil. The residue was dissolved in ethyl acetate (1.5 L) and washed with 1N HCl (2X 500 mL), followed by saturated sodium bicarbonate solution (2 X 500 mL). The organic layer was dried over anhydrous sodium sulfate (50 g), filtered and evaporated under reduced pressure to give the crude product as a pale yellow oil.

The crude oil was diluted with the same volume of dichloromethane and loaded onto a 2.5 Kg silica gel cartridge in a radial compression module at 100 psi of air pressure. Using a gradient pump at 60 psi and a flow rate of 400 mL/min, the cartridge was washed with methylene chloride (4L) followed by a gradient 1-4% methanol in methylene chloride (48 L). Most of the major impurities (di-(isopropylalanyl)phenyl phosphate, 3',5'-bis phosphoramidate (**6**), 3'-phosphoramidate-5'-TBDMS adduct (**7**)) eluted with ~3% gradient. The desired product eluted between 3 and 4% methanol. The product containing fractions were sorted into two lots. The first contained small amounts of upper impurities and the latter was pure product. The first set of fractions contained small amounts of less polar impurities (upper impurities) such as the 3',5'-bis phosphoramidate and the di-alanylphenyl phosphate and a mostly the Rp diastereomer and required a second column purification. (The relative terminology, upper vs. lower refers to the elution on normal-phase silica-gel chromatography, where the "upper isomer" means the first eluting isomer.) The second set of fractions did not have a significant amount of impurities –just the remaining R_P and mostly the S_P diastereomers. It was later recombined with the twice-columned fractions. The solvent was evaporated under reduced pressure and the resulting white foam was further dried (0.20 mmHg) for 1 h to give 42 g of the impure lot (4:1 upper vs lower isomer based of ^{31}P-NMR) and 38 g of the pure lot (1:3 upper vs lower isomer). The impure lot was recolumned in a similar manner to give 3.8 g of 97% pure upper isomer (fraction set aside) and 36 g of pure product in a 4:1 ratio. The two main lots were dissolved in DCM, combined, evaporated under reduced pressure and dried (50°C, 0.2 mmHg, 24 h) to

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get 74 g (45.7%) of pure product **4** with a diastereomeric ratio of 48: 51, as a white foam, mp about 75-85°C.

In order to produce an amorphous solid of the diastereomeric mixture, 74 g of the white foam was stirred in with t-butyl methyl ether (750 mL) resulting in a partial solution and a gummy solid residue. While stirring, heptanes (750 mL) was added slowly and the suspension was mechanically stirred for 1 hour until most of the gum was converted to a white solid. The solid was scraped up with a spatula and the resulting slurry was filtered. The solid was washed with heptanes (4 X 50 mL) and dried under vacuum (50°C, 0.2 mmHg, 24 h) to give a white, amorphous powder (64 g) with a broad melting range of ca 70-80°C. ^{1}H and ^{31}P NMR conformed to structure and HPLC showed a purity of 99.8% with a diastereomeric ratio of 46:54 (also confirmed by ^{31}P NMR).

Alternative method to make solid mixture of **4**. After chromatography, the residue was co-evaporated with dichloromethane twice (5 mL/g) and dried for 24 h at 35-40°C at 35-45 mTorr. The foam residue was sieved through a 250 micron screen and further dried under the same conditions until the residual dichloromethane fell below 400 ppm as measured by headspace GC. The resulting fine off-white to white amorphous powder has a glass transition temperature range of 53.7 to 63.5°C.

Characterization of the mixture of isomers (**4**): ^{1}H-NMR ($CDCl_3$) δ 10.05 (br s, 1H, NH, S_P), 10.00 (br s, 1H, NH, R_P), 7.49 (d, 1H, C6-H, S_P), 7.36 (m, 5H, C6-H, R_P, aromatic), 7.23-7.14 (m, 6H, R_P/S_P, aromatic), 6.18 (br d, 2H, C1'-H, R_P/S_P), 5.63 (d, 1H, C5-H, S_P), 5.58 (d, 1H, C5-H, R_P), 5.01 (m, 2H, C*H*-$(CH_3)_2$, R_P/S_P), 4.46-4.33 (m, 8H, C-5'-H_2, ala-NH, C3'-OH, R_P/S_P), 4.12 (m, 2 H, ala-C*H*-CH_3, R_P/S_P), 4.01-3.85 (m, 4H, C3'-H, C4'-H, R_P/S_P), 1.39-1.22 (m, 12H, all CH_3, R_P/S_P).

^{31}P-NMR ($CDCl_3$) δ 3.60 (R_P), 3.20 Sp relative to triphenylphosphate at -17.80 ppm. ES-MS M+1 530.2. Elemental Analysis: Calculated % (including 0.29% water as found by Karl Fisher analysis) C, 49.75; H, 5.54; N, 7.90, F, 3.58, P, 5.84. Found %: C, 49.50; H, 5.44; N, 7.85; F, 3.62; P, 6.05.

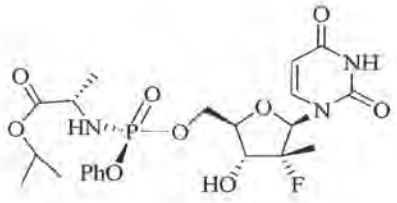
Discussion on separation of isomers

Compound **4** due to the chirality at phosphorus is comprised of two diastereomers, which are designated as S_P-**4** and R_P-**4**. The stereochemical

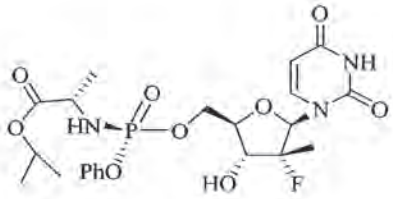
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assignment was made based on single crystal X-ray analysis of S_P-**4**. Both R_P-**4** andS_P-**4** gave crystalline product.

The procedures for crystallization are outlined below.



S_P-**4**



R_P-**4**

Example 3. Crystallization of the R_P-**4** isomer. The chromatographed fraction of containing the first eluting, less polar R_P-**4** isomer (3.8 g, 97% pure) was dissolved in isopropanol (36 g) and diluted with heptanes until cloudy (72 g). The solution was seeded and stirred at ambient temperature for 5 h. The resulting solid was collected by vacuum filtration, washed with heptanes (2x20 mL) and dried (50°C, 0.2 mm, 24 h) to 2.3 g of very small white needles mp 136.2-137.8°C. HPLC purity of the resultant material was found to be 99.02%.

R_P-**4**: ^{1}H-NMR ($CDCl_3$) δ 9.10 (br s, 1H, NH), 7.36 (m, 2H, *o*-aromatic), 7.26-7.16 (m, 4 H, C6-H, *m,p*-aromatic), 6.16 (br d, 1H, C1'-H), 5.58 (d, 1H, C5-H), 5.01 (sept, 1H, C*H*-(CH_3)$_2$), 4.52-4.47 (m, 2H, C-5'-H_2), 4.10 (d, 1H, C3'-H), 4.02-3.76 (m, 4H, ala-NH, C3'-OH, C4'-H, ala-C*H*-CH_3), 1.37-1.20 (m, 12H, all CH_3).

Example 4. Preparation and crystallization of S_P-4.

Method 1: Direct precipitation from crude **4**: To a stirred solution of L-alanine isopropyl ester hydrochloride (10.5g, 61.5mmol, azeotropically dried, two times, with 50 mL of toluene each time) in dichloromethane (100 mL) was added phenydichlorophosphate (7.5 mL, 50 mmol) at room temperature. The mixture was cooled to -10°C and then was added a solution of NMI (30.5 mL, 384.3mmol) in 30 mL of dichloromethane over a period of 30 min. After completion of the addition, the mixture was stirred between -10 and -15°C for 1h. To the above mixture was added 2'-deoxy-2'-fluoro-2'-C-methyluridine (**3**) (10g, 38.4mmol) in one lot and the mixture was stirred below -10°C for 3h and then slowly allowed to warm to 20°C (6h). The mixture was stirred at this temperature over night (15h) and then quenched

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with 10 mL of methanol. The solvent was evaporated and the residue was re-dissolved in EtOAc (200 mL). The EtOAc layer was washed with water (100mL), 1N HCl (3x75 mL), 2 % aqueous $NaHCO_3$ solution (50 mL) and brine (50mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was dried under high vacuum for 2h to give white foam (22 g).

The above foam was dissolved in 33 mL of DCM and then was added 65 mL of IPE (isopropyl ether) to give a saturated solution. The solution was filtered though a small pad of Celite and the filtrate was stirred with S_P-**4** seeds for 72h at ambient temperature (about 22°C –note that cooling the suspension to 0°C led to oiling out the crude product). The white solid was filtered, washed with IPE (20mL) and dried to give 4.58g (~85:15 mixture of S_P-**4**:R_P-**4** respectively as determined by ^{31}P NMR) of a white powder. The above solid was suspended in 23 mL of DCM and then refluxed for 3h. The mixture was cooled to room temperature and stirred for 15h. The white solid was filtered, washed with 4.5 mL of cold DCM and dried under high vacuum at 45°C to give pure S_P-**4**, mp 93.9-104.7°C, HPLC purity 99.74% (3.11g, 15.2 % from the uridine nucleoside).

S_P-**4** ^{1}H-NMR ($CDCl_3$) δ 8.63 (br s, 1H, NH), 7.47 (d, 1H, C6-H), 7.30 (m, 2H, *o*-aromatic), 7.26-7.18 (m, 3H, *m,p*-aromatic), 6.18 (br d, 1H, C1'-H), 5.70 (d, 1H, C5-H), 5.02 (sept, C*H*-$(CH_3)_2$), 4.53 (m, 2H, C-5'-H_2), 4.11 (d, 1H, C3'-H), 3.97 (m, 3H, C3'-OH, C4'-H, ala-C*H*-CH_3), 3.77 (br s, 1H, ala-NH), 1.39 (d, 3H,C2'-CH_3), 1.37 (d, 3H, ala-CH_3), 1.24 (d, 6H, CH-$(CH_3)_2$).

Method 2: Oiling out from crude **4**: To a stirred solution of L-alanine isopropyl ester hydrochloride (20.6g, 123mmol, azeotropically dried, two times, with 75 mL of toluene each time) in dichloromethane (200 mL) was added phenydichlorophosphate (14.9 mL, 100mmol) at room temperature. The mixture was cooled to -10°C and then was added a solution of NMI (61.3 mL, 769mmol) in 60 mL of dichloromethane over a period of 30 min. After completion of the addition, the mixture was stirred between -10°C and -15°C for 1h. To the above mixture was added 2'-deoxy-2'-fluoro-2'-C-methyluridine (**3**) (20g, 76.9mmol) in one lot and the mixture was stirred below -10°C for 3h and then slowly allowed to warm to 20°C (6h). The mixture was stirred at this temperature over night (15h) and then quenched with 10 mL of methanol. The solvent was evaporated and the residue was re-dissolved in EtOAc (400 mL). The EtOAc layer was washed with water (200mL), 1N HCl (3x100 mL), 2 %

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aqueous $NaHCO_3$ solution (100 mL) and brine (50mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was dried under high vacuum for 2h to give white foam (43 g). The above foam was dissolved in 86 mL of EtOAc in a two neck round bottom flask equipped with a mechanical stirrer. While stirring, 100 mL of heptane was added slowly and the suspension was stirred for 1h. The top layer was decanted and the residue was again stirred with 50 mL of 2:3 EtOAc/heptane solutions for 10 min and then decanted. The residue was dried under high vacuum to give white foam (31g).

The above foam was dissolved in 46 mL of DCM and then was added 95 mL of IPE to give a saturated solution. The solution was filtered though a small pad of Celite and the filtrate was stirred with S_P-**4** seeds for 72h at ambient temperature. The white solid was filtered, washed with IPE (30mL) and dried to give 7.33g (~85:15 mixture of S_P-**4** : R_P-**4** respectively as determined by ^{31}P NMR) of white powder. The above solid was suspended in 36 mL of DCM and then refluxed for 3h. The mixture was cooled to room temperature and stirred for 15h. The white solid was filtered, washed with 7.5 mL of cold DCM and dried under high vacuum at 45°C to give >99% pure S_P-**4**, (4.78g, 11.6 % from the uridine nucleoside).

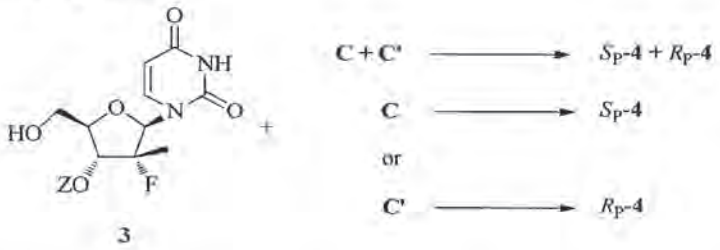
Method 3: Silica gel loading of crude **4**: 5.0 g of crude **4** was produced as in the same manner as the mixture of diastereomers just before the column chromatography step starting with approximately 2.5 g of 2'-deoxy-2'-fluoro-2'-C-methyluridine (**3**). The crude was dissolved in 10 mL of DCM and 10 g of silica gel was added to the solution. The solvent was evaporated to give dry slurry. The slurry was stirred with 40 mL of 50% EtOAc/hexanes for 15 min and then filtered. The silica gel was washed with additional 10 mL of 50% EtOAc/hexanes. The silica gel was then washed with 15% MeOH/DCM (100 mL) and collected separately. The solvent was evaporated and dried under high vacuum to give 4.0 g of residue (foam). The residue was dissolved in DCM (6mL) and then was added ~9mL of IPE to make a saturated solution. The mixture was then gently stirred overnight with S_P-**4** seeds at ambient temperature. The white solid was filtered and washed with IPE (5 mL) to give 1.28 g of product. ^{31}P NMR revealed that the above product contains 77:23 mixture of S_P-**4** : R_P-**4** respectively. This was recrystallized from 20 mL of DCM to obtain 0.75 g of >99% pure S_P-**4** (about 12% from the uridine nucleoside). This preparation of S_P-**4** does not require the silylation step as done for the mixture,

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so the entire reaction procedure is shown above. Aspects of single crystalline and polymorphic forms of S_P-**4** are presented below.

Method 4: 40.0 g of 1:1 mixture of **4** was dissolved in 90 mL of dichloromethane. Diisopropylether (70 mL) was added to the above solution to give a saturated solution. (The quantity of diisopropyl ether may vary based on the purity of the product.) The solution was seeded with pure S_P-**4** (> 99%) and the mixture was gently stirred with a stir bar at room temperature for 20h (formation of solid was observed after 2h). The solid was filtered, washed with 40 mL of the mixture of diisopropylether/dichloromethane (1:1) and dried to give white solid (16.6 g, 89.35 % pure S_P-**4** by NMR). This solid was suspended in 83 mL dichloromethane and refluxed for 3h. The suspension was cooled to room temperature and stirred over night. The solid was filtered and washed with 10mL of cold DCM. The solid was dried under vacuum to give S_P-**4** (13.1 g, 99.48 % pure by HPLC). 11g of this solid was redissolved in 330 mL of DCM under hot conditions. The solution was cooled to room temperature and left at this temperature over night. The crystalline product was filtered and dried to give 10.5 g of S_P-**4** (99.74 % by HPLC).

Compounds S_P-**4** and R_P-**4** may alternatively be prepared, in accordance with the ninth or tenth embodiment, by reacting nucleoside (protected or unprotected) **3** with an isopropyl-alanyl-phosphoramidate (mixture of **C** and **C'**, **C** or **C'**), as shown in the following equation.

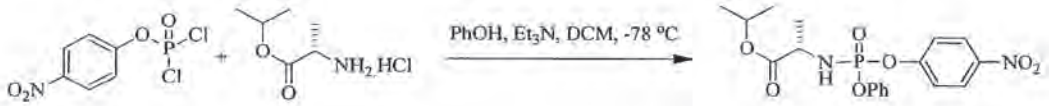


P.D. Howes et al. *Nucleosides, Nucleotides & Nucleic Acids* 2003, Vol. 22, Nos. 5-8, pp. 687-689 ("Howes") discloses 2'- and 5'-phosphoramidates obtained by a reaction with t-butylmagnesium chloride. There, Howes discloses that when a 3'-deoxy-cytidine nucleoside is reacted with (S)-2-[chloro-phenoxy-phosphorylamino] propionic acid methyl ester in the presence of 1.2 equivalents of t-butylmagnesium chloride, selective phosphorylation on the 2'-position occurred, but that with an additional equivalent of t-butylmagnesium chloride selective phosphorylation on the

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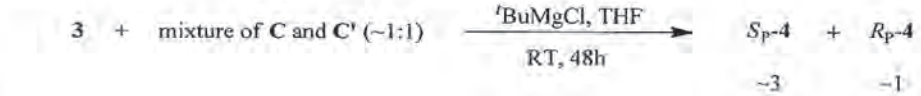
5'-position occurred. This disclosure should be contrasted to that which is disclosed in Howes' Scheme 1.

Example 5-1. Preparation of (S)-2-[(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester



To a stirred solution of 4-nitrophenyl phoshorodichloridate 12.8g, 50 mmol) in dichloromethane (100 mL) was added a solution of phenol and triethylamine (7.7 mL, 55 mmol) in dichloromethane (100 mL) at -78°C over a period of 20min. The mixture was stirred at this temperature for 30min and then transferred to another round bottom flask containing L-alanine isopropyl ester hydrochloride (8.38g, 50mmol) in dichloromethane (100 mL) at 0°C. To the mixture was added second portion of triethylamine (14.6 mL, 105 mmol) over a period of 15min. The mixture was stirred at 0°C for 1h and then the solvent was evaporated. The residue was triturated with ethyl acetate (150 mL) and the white solid was filtered off. The filtrate was concentrated under reduced pressure to give pale yellow oil. The crude compound was chromatographed using 0-20% ethyl acetate/hexanes gradient to give product (17g, 83 %yield) as a mixture of diastereomers in about 1:1 ratio. **^{31}P NMR (162 MHz, DMSO-d6):** δ -0.31, -0.47; **^{1}H NMR (400 MHz, DMSO-d6)**: δ 8.31-8.27 (m, 2H), 7.51-7.37(m, 4H), 7.27-7.19(m, 3H), 6.70-6.63(m, 1H), 4.85-4.78(m, 1H), 3.97-3.86(m, 1H), 1.21-1.19(m, 3H), 1.11-1.09 (m, 6H); **MS (ESI)** *m/z* 407 $(M-1)^+$. **^{31}P NMR (162 MHz, $CDCl_3$):** δ -2.05, -2.10; **^{1}H NMR (400 MHz, $CDCl_3$)**: δ 8.22 (d, J = 9.2Hz, 2H), 7.41-7.33(m, 4H), 7.26-7.18(m, 3H), 5.05-4.96(m, 1H), 4.14-4.05(m, 1H), 3.93-3.88(m, 1H), 1.38(d, J = 6.8Hz, 3H), 1.22 (dd, J = 6.2 & 3.0Hz, 6H); **MS (ESI)** *m/z* 407 (M-1)+.

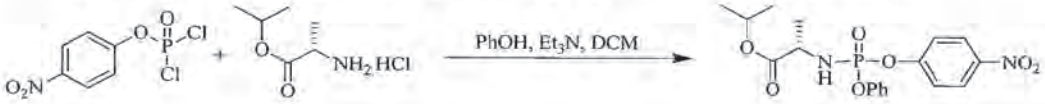
Example 5-2. Preparation of S_P-4/R_P-4.



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To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl)-1H-pyrimidine-2,4-dione (130mg, 0.5mmol) in dry THF (1.5mL) was added a 1.0M solution of tert-butylmagnesium chloride (1.05mL, 1.05mmol, 2.1 equiv)) at room temperature over a period of 5min. After 30min, a solution of (S)-2-[(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (1:1 mixture of isomers, 408mg, 1mmol) in THF (1.5mL) was added drop-wise over a period of 5min. The mixture was allowed to stir at room temperature for 48h and then quenched with saturated aqueous NH_4Cl (20mL). The mixture was partitioned between ethyl acetate (50mL) and water (20mL). The combined organic extract was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to give a pale yellow residue. Column chromatography of the residue using 0-2% MeOH/dichloromethane gradient gave a white foamy solid (125mg, 47% yield, mixture of ***S*P-4/*R*P-4** in about 3.05:1.0 ratio).

Example 6. Preparation and non-chromatographic isolation of (S)-2-[(S)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester



L-alanine isopropyl ester hydrochloride (330 g, 1.97 mol) was pre-dried by co-evaporation with toluene (2 x 400 mL) under reduced pressure and then dried in a vacuum oven (50°C, 0.2 mmHg, 17 h). To a stirred solution of 4-nitrophenyl phosphorodichloridate (500.0 g, 1.953 mol) in anhydrous dichloromethane (3.0 L) was added a solution of phenol (183.8 g, 1.953 mol) and triethylamine (300 mL, 2.15 mol) in dichloromethane (900 mL) at -60°C internal temperature over a period of 3 hours. The mixture was stirred at this temperature for additional 30 min and then allowed to warm up to -5°C over 2.5 hours. The pre-dried amino acid ester was added at -5~0°C under an atmosphere of nitrogen over 10 mins. The residue of aminoester salt in the addition flask was transferred to the reaction mixture via rinsing with dichloromethane (2 x 100 mL). The mixture was stirred at 0°C for 40 mins and a second portion of triethylamine (571 mL, 4.10 mol) was added over a period of 40 mins at 0°C. The mixture was stirred at 0~10°C for 3 h and then the

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white solid (triethylamine hydrochloride) was filtered off and rinsed with dichloromethane (3 x 300 mL). The filtrate was concentrated under reduced pressure and the residue was triturated with methyl t-butyl ether (MTBE, 4 L). The additional solid salt thus formed was filtered off and rinsed with MTBE (3 x 150 mL). The filtrate was concentrated under reduced pressure to give clear light brown color oil. The residue was co-evaporated with hexanes (2 x 140 mL) to remove any residual MTBE and further dried under vacuum at 40°C for 2 hours. The dry residue was mixed with diisopropyl ether (IPE, 1.1 L) and stirred at 5 °C in an ice-water bath. Small amount of crystal seeds of the desired S_P-isomer of the product was added to the solution and the mixture was stirred at 5°C for over 22 h to form a medium thick slurry. This was allowed to stand in a freezer (-10°C) for 44 h. The precipitated product was collected via filtration and rinsed with pre-cooled mixed solvents of IPE and hexanes (1:1, 3 x 190 mL). The solid was dried under vacuum (0.5 mm Hg) at ambient temperature until a constant weight was obtained to give 227.23 g (yield: 28.5%) as a white powder solid. The ratio of two diastereomers S_P:R_P was 9.65/1 based on ^{31}P NMR (162 MHz, DMSO-d_6, δ -0.31 (S_P), -0.47). The product was recrystallized by dissolving in IPE (840 mL) while heating in a 60°C bath. The above solution was stirred at room temperature for 1 h and then a small amount of crystal Sp isomer seeds was added. White powder solid was formed within 2 hours and the flask was stored in a freezer (-10°C) for 16 hours. A white and fine crystalline solid obtained was filtered, washed with pre-cooled IPE (3x 50 mL) and dried under vacuum (ambient, 0.5 mm Hg) to a constant weight to give white fluffy solid (177.7 g, 22% overall yield or 44% overall yield based on theoretical yield of the S_P isomer) with diastereomeric ratio of 48/1 based on P-NMR. Mp 62-66°C.

^{31}P NMR (162 MHz, DMSO-d6): δ -0.31; **^{1}H NMR (400 MHz, DMSO-d6)**: δ 8.30-8.27 (m, 2H), 7.49(d, J=8.8Hz, 2H), 7.41-7.37(m, 2H), 7.23-7.19 (m, 3H), 6.66 (dd, J=13.6, 10.0Hz, 1H), 4.86-4.78 (m, 1H), 3.97-3.86 (m, 1H), 1.19 (d, J=7.2Hz, 3H), 1.10(d, J=6.4Hz, 6H);

^{31}P NMR (162 MHz, $CDCl_3$): δ -2.05; (162 MHz, DMSO-d6): δ -0.31; **^{1}H NMR (400 MHz, $CDCl_3$):** δ 8.22 (d, J=9.2Hz, 2H), 7.41-7.33(m, 4H), 7.26-7.18(m, 3H), 5.05-4.96(m, 1H), 4.14-4.05(m, 1H), 3.93-3.88(m, 1H), 1.38(d, J=6.8Hz, 3H), 1.22 (dd, J=6.2 & 3.0Hz, 6H); ^{1}H NMR (400 MHz, DMSO-d6): δ 8.30-8.27 (m, 2H), 7.49(d, J=8.8Hz, 2H), 7.41-7.37(m, 2H), 7.23-7.19 (m, 3H), 6.66 (dd, J=13.6,

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10.0Hz, 1H), 4.86-4.78 (m, 1H), 3.97-3.86 (m, 1H), 1.19 (d, J=7.2Hz, 3H), 1.10(d, J=6.4Hz, 6H)

MS (ESI) *m/z* 407 $(M-1)^+$.

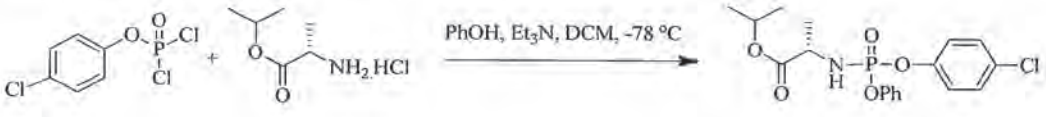
The stereochemistry of **8** (S_P-isomer) has been confirmed by single crystal X-ray crystallography, see details provided below.

Example 7. Separation of the diastereomeric mixture (S)-2-[(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester by SFC

A sample of the mixture of diastereomers (4.8 g) enriched with the R_P-isomer was subjected to SFC using a ChiralPak AD-H (2x15 cm) column and eluted with 35% isopropanol in carbon dioxide at 100 bar. An injection loading of 4 mL of sample at a concentration of 17 mg/mL of methanol was used. The R_P-isomer [(S)-2-[(R)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester] eluted first. The appropriate fractions of the multiple runs were combined and concentrated under reduced pressure to give 2.9 g of the R_P-isomer [(S)-2-[(R)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester] as a light yellow viscous oil and 1.9 g of the S_P-isomer [(S)-2-[(S)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester] as a white solid. Analytical data of R_P-isomer is similar to the product isolated by the above crystallization method.

Analytical Data for (S)-2-[(R)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (8, R_P-isomer): ^{31}P NMR (162 MHz, DMSO-d6): δ -0.47; ^{1}H NMR (400 MHz, DMSO-d6): δ 8.30-8.27 (m, 2H), 7.46-7.38 (m, 4H), 7.27-7.20 (m, 3H), 6.68 (dd, J=13.8, 10.2Hz, 1H), 4.86-4.77 (m, 1H), 3.97-3.86 (m, 1H), 1.20 (d, J=7.2Hz, 3H), 1.10(dd, J=6.2, 2.2Hz, 6H); **MS (ESI)** *m/z* 407 $(M-1)^+$.

Example 8-1. Preparation of racemic 2-[(4-chloro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (±):



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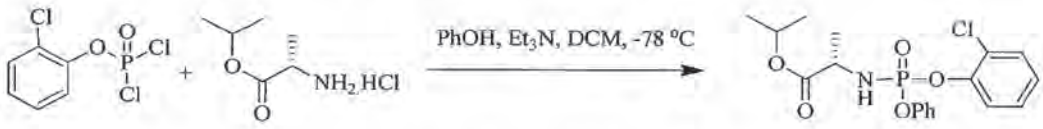
To a stirred solution of 4-chloro-phenyl phoshorodichloridate (2.45g, 10.0 mmol) in dichloromethane (20 mL) was added a solution of phenol (0.94 g, 10 mmol) and triethylamine (1.56 mL, 11 mmol) in dichloromethane (20mL) at -78°C over a period of 20 min. The mixture was stirred at this temperature for 30min and then transferred to another round bottom flask containing L-alanine isopropyl ester hydrochloride (1.67 g, 10 mmol) in dichloromethane (50 mL) at 0°C. To the mixture was added second lot of triethylamine (2.92 mL, 21 mmol) over a period of 15min. The mixture was stirred at 0°C for 1h and then the solvent was evaporated. The residue was triturated with ethyl acetate (30mL) and the white solid was filtered off. The filtrate was concentrated under reduced pressure to give pale yellow oil. The crude compound was chromatographed using 10-20% ethyl acetate/hexanes gradient to give product (2.0 g, 50% yield) as a mixture of diastereomers in about 1:1 ratio. **^{31}P NMR (162 MHz, $CDCl_3$):** δ -1.58, -1.62; **^{1}H NMR (400 MHz, $CDCl_3$)**: δ 7.06-7.51 (m, 8H), 7.15-7.28 (m, 2H), 7.29-7.47(m, 2H), 4.0-4.10(m, 1H), 3.82-3.88 (m, 3H), 1.35-1.36 (dd, 6H); 1.19-1.22 (m, 3H). **MS (ESI)** *m/z* 398 $(M-1)^+$. The resultant product is purified by extraction, crystallization, or chromatography, as noted above.

Example 8-2. Preparation of (S)-Isopropyl 2-((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2 yl)methoxy)(phenoxy)-phosphorylamino)propanoate (4).

To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl)-1H-pyrimidine-2,4-dione (**3**, 2.6 g, 10 mmol) in dry THF (50 mL) was added a 1.7 M solution of tert-butylmagnesium chloride (12.4 mL, 21 mmol, 2.1 equiv)) at room temperature over a period of 15 min. After 30 min, a solution of racemic **(2-[(4-chloro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester** (4.08g, 10mmol) in THF (15mL) was added drop wise over a period of 10min. The mixture was allowed to stir at room temperature for 72. TLC co-spot with authentic product showed around 5% of the desired product had formed compared to the starting nucleoside.

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Example 9-1. Preparation of racemic 2-[(2-chloro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (±).



To a stirred solution of 2-chloro-phenyl phoshorodichloridate (9.8 g, 40 mmol) in dichloromethane (80 mL) was added a solution of phenol (3.76 g, 40 mmol) and triethylamine (6.16 mL, 44 mmol) in dichloromethane (80 mL) at -78°C over a period of 20 min. The mixture was stirred at this temperature for 30 min and then transferred to another round bottom flask containing L-alanine isopropyl ester hydrochloride (6.7 g, 40 mmol) in dichloromethane (150 mL) at 0°C. To the mixture was added second portion of triethylamine (11.6 mL, 84 mmol) over a period of 15 min. The mixture was stirred at 0°C for 1h and then the solvent was evaporated. The residue was triturated with ethyl acetate (100mL) and the white solid was filtered off. The filtrate was concentrated under reduced pressure to give pale yellow oil. The crude compound was chromatographed using 10-20% ethyl acetate/hexanes gradient to give product (11.3g, 72 % yield) as a mixture of diastereomers in about 1:1 ratio. **^{31}P NMR (162 MHz, $CDCl_3$):** δ -1.58, -1.61; **^{1}H NMR (400 MHz,$CDCl_3$)**: δ 7.06-7.51 (m, 8H), 5.02-5.94 (m, 1H), 4.10-4.16(m, 1H), 3.31-3.94(m, 1H), 1.18-1.35(m, 3H), 1.38-1.40 (dd, 6H); **MS (ESI)** *m/z* 398 $(M-1)^+$. The resultant product is purified by extraction, crystallization, or chromatography, as noted above.

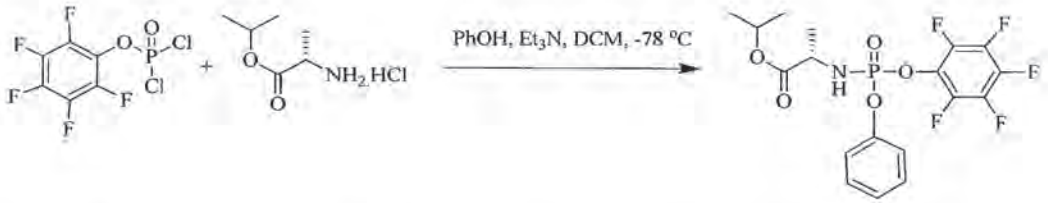
Example 9-2. Preparation of (S)-isopropyl 2-((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2 yl)methoxy)(phenoxy)- phosphorylamino)propanoate.

To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl)-1H-pyrimidine-2,4-dione (**3**, 2.6 g, 10 mmol) in dry THF (50 mL) was added a 1.7 M solution of tert-butylmagnesium chloride (12.4 mL, 21 mmol, 2.1 equiv)) at room temperature over a period of 15 min. After 30 min, a solution of **(2-[(2-chloro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester** (racemic 4.08 g, 10 mmol) in

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THF (15mL) was added drop wise over a period of 10min. The mixture was allowed to stir at room temperature for 72h. TLC co-spot with authentic product showed around 5-10% of the desired product had formed compared to the starting nucleoside.

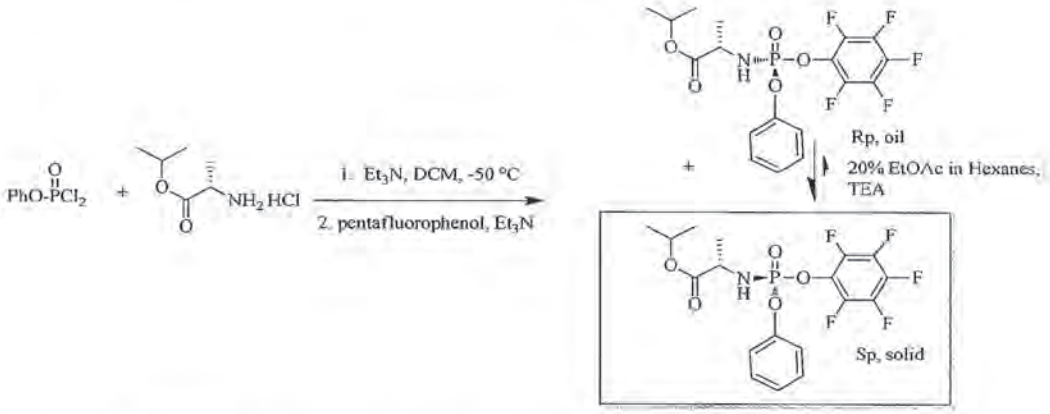
Example 10-1. Preparation of racemic 2-[(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (±).



To a stirred solution of pentafluorophenyl phoshorodichloridate (6.0 g, 20 mmol) in dichloromethane (40 mL) was added a solution of phenol and triethylamine (3.08 mL, 22 mmol) in dichloromethane (40 mL) at -78°C over a period of 20 min. The mixture was stirred at this temperature for 30 min and then transferred to another round bottom flask containing L-alanine isopropyl ester hydrochloride (3.35 g, 20 mmol) in dichloromethane (100 mL) at 0 °C. To the mixture was added second lot of triethylamine (5.84 mL, 42 mmol) over a period of 15 min. The mixture was stirred at 0°C for 1h and then the solvent was evaporated. The residue was triturated with ethyl acetate (60mL) and the white solid was filtered off. The filtrate was concentrated under reduced pressure to give pale yellow oil as a mixture of diastereomers in about 1:1 ratio. **^{31}P NMR (162 MHz, $CDCl_3$):** δ -0.49, -0.58. The resultant product is purified by extraction, crystallization, or chromatography, as noted above.

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Example 10-2. Preparation of diastereomeric mixture (S)-2-[(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester and isolation of single diastereomer (S)-2-[(S)-(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester via crystallization-induced dynamic resolution with multiple crops.



To a 2 L of three-necked round bottom flask fitted with a mechanic stirrer and low temperature thermometer were added 60 g (284 mmol) of phenyl dichlorophosphate and 300 mL of anhydrous dichloromethane. The solution was cooled to 0°C under nitrogen atmosphere and *iso*-propyl alanate hydrochloride salt (oven dried *in vacuo*, 47.7 g, 284 mmol) was added quickly as a solid. The mixture was stirred and cooled to -55°C in a dry ice-acetone bath. A solution of 60.32 g of triethylamine (596 mmol) in 300 mL of dichloromethane was added through an addition funnel over 70 minutes. The white cloudy mixture was stirred at -55°C for half hour and then the temperature was raised to -5°C slowly over 1.5 h. A pre-cooled (room temperature) mixture of pentafluorophenol (52.42 g, 284 mmol) and triethylamine (32.11 g, 317 mmol) in 180 mL of dichloromethane was added to the mixture via an addition funnel over 1 h at 0°C and the resulting mixture was stirred at 0°C for 4 hours. The white precipitate (TEA•HCl) was filtered out and rinsed with dichloromethane (3x 50 mL). The filtrate was concentrated under reduced pressure and white solid residue was triturated in 880 mL of *t*-butyl methyl ether (TBME) at room temperature for one hour. White suspension was filtered and solid was rinsed with TBME (3x 150 mL). Solid was distributed in a mixture of ethyl acetate (600 mL) and water (150 mL).

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Organic layer was separated and washed with water (3 x 100 mL). Organic layer was dried over $MgSO_4$ and concentrated to afford 29.92 g (66 mmol) of product (S_P-isomer, as confirmed by X-ray crystallography, *infra*) as a white feather solid.

The filtrate from above TBME trituration was concentrated under reduced pressure to a white solid residue and the solid was triturated in 450 mL of mixed ethyl acetate and hexanes (20:80, v/v) at room temperature for 75 minutes. Solid (solid 1) was collected by filtration and rinsed with 20% ethyl acetate in hexanes (75 mL, 2x 30 mL). The mother liquor was concentrated to furnish an off-white solid that was triturated in 20% ethyl acetate in hexanes (185 mL) at room temperature for 17 hours. A white solid (solid 2) was collected by filtration and rinsed with 20% ethyl acetate in hexanes (2 x 10 mL). Solid 1 and solid 2 were combined and dissolved in 1.2 L of ethyl acetate. The solution was washed with water (3x 150 mL), brine (50 mL) and dried over $MgSO_4$. The solution was concentrated under reduced pressure to afford 72.8 g (161 mmol) of pure product. Total amount of product was 102.72 g (226 mmol, 80%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.38-7.33 (m, 2 H), 7.27-7.24 (m, 2 H), 7.23-7.19 (m, 1 H), 5.04 (hept, 1 H), 4.18-4.09 (m, 1 H), 4.01-3.96 (m, 1 H), 1.45 (d, 3 H), 1.25 (dd, 6 H). ^{31}P NMR ($CDCl_3$, 162 MHz) δ; -0.50.

Example 10-3: Preparation of diastereomeric mixture (S)-2-[(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester and isolation of single diastereomer (S)-2-[(S)-(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester via crystallization-induced dynamic resolution in a single crop.

To a 1 L of dry three-necked flask fitted with a low-temperature thermometer and a mechanical stirrer was loaded phenyl phosphorodichloridate (25 g, 118.5 mmol). Anhydrous dichloromethane (125 mL) was added and the solution was cooled to 0°C. The alanine ester salt (oven dried) (19.86 g, 1 eq) was added quickly under N_2 while agitated. The solution was cooled to ca -50°C (internal temperature (in an acetone/dry ice bath under N_2). A solution of triethylamine (25.2 g, 2.1 eq) in DCM (125 mL) was added dropwise via an addition funnel over 0.5 h at -50°C and the resulting white slurry was stirred at about -50°C for 0.5 h. The mixture was allowed to warm up to 0°C over 1.5 h and then a pre-mixed cooled solution of

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pentafluorophenol (21.82 g, 1 eq) and TEA (13.2 g, 1.1 eq) (caution: heat released while mixing pentafluorophenol and TEA) in 75 mL of DCM was added over 0.5 h at 0°C via an addition funnel. The mixture was stirred at 0°C for additional 4 h.

The mixture was filtered through a Buchner funnel and the collected solid triethylamine hydrochloride was rinsed with DCM (3 x 40 mL). The filtrate was checked by ^{31}P-NMR (ratio ca 1.14:1 favored the S_P-diastereomer – downfield peak) and was divided into two parts of equal weight. One of them was concentrated under reduced pressure. The white solid residue (31 g) was triturated in a mixture of EtOAc and hexanes (150 mL, 20:80, v/v) at RT for 17 h allowing time for dynamic resolution of the less soluble S_P isomer. The white slurry was filtered and solid was rinsed with 20% EtOAc in hexanes (2x 25 mL). The solid (22.58 g) was checked by ^{1}H-NMR and ^{31}P-NMR and it contained product as one isomer contaminated with triethylamine hydrochloride salt. The solid was dissolved and partitioned in 310 mL of EtOAc and 100 mL of water. After separation of the organic layer, the aqueous layer was back-extracted with EtOAc (50 mL). The combined organic layer was washed with water (3x 80 mL), brine (50 mL) and dried over $MgSO_4$. The solution was concentrated under reduced pressure and then dried under high vacuum at RT to a constant weight to furnish 17.36 g of product as a white solid from the one half of the reaction.. The yield is 64%. The mother liquor from above was concentrated to a gummy residue (7.89 g) that contained the reagents with a ratio of 1:1.2 (desired/undesired) based on ^{31}P-NMR.

Example 10-4. Preparation of (S)-2-[(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester

DCM (11.5 L) was charged into the clean and dry glass reactor. Phenyldichlorophosphate (2.3 kg, 10.9 mol) was charged to the reactor under nitrogen. The solution was then cooled to 0°C. L-Alanine isopropylester hydrochloride (1.83 kg , 10.9 mol) was then added in one portion and continued stirring for 30 min. The reaction mass was cooled to -50°C internal temperature by using a dry ice/acetone bath. A mixture of TEA (2.1 eq, 3.17 L) in DCM (11.5 L) was added to the above reaction solution slowly over a period of 8 h to keep the internal temperature between -40 to -50°C. After completion of addition, the

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reaction was maintained at the same temperature range for about 1h. The mixture was allowed to warm to 0°C over about 4h.

In the meantime, in another reactor, DCM (6.9 L) was charged, and then pentaflurophenol (2.0 Kg, 10.9 mol) was added under nitrogen. The solution was cooled to 0°C, then TEA (1.1 eq, 1.65 L) was added to the pentafluorophenol solution (exothermic) over a period of about 2 h. The resulting solution in turn was slowly added to the first solution containing the phenyl dichlorophosphate and amino acid ester while keeping the temperature between 0 to 5°C over a period of about 7 h. After the completion of the addition, stirring was continued at that temperature range for about 4 h. The reaction progress was monitored by HPLC. When less than 5% of pentafluorophenol remained, the reaction was stopped. Note that chiral HPLC indicated an even mixture of diastereomers of the product at this point.

The reaction suspension was filtered through a Nutsche filter to remove most of the suspended triethylamine hydrochloride salt. The salt cake was washed with excess amount of DCM (9 L) and this wash was added to the main filtrate. The filtrate was concentrated under reduced pressure at 35°C to give a solid residue. The solid residue was co-evaporated with hexane (4 L) to further reduce levels of residual DCM. To this residual solid was added 20% MTBE/Hexane 6 L and the suspension was stirred for about 17 hr at ambient temperature and monitored by HPLC. The pH of the solution remained basic due to residual TEA. During this time, a dynamic resolution took place in which the precipitated solid was the desired S_P-**4** isomer and the supernatant remained in equilibrium between S_P-**4** and R_P-**4**.

The suspension was passed through a Nutsche filter and the desired product solid, still contaminated with TEA hydrochloride, was washed with 5% MTBE/Hexane (1 L). The solid was dissolved in ethyl acetate (35 L) and the solution was washed with water (3 x 35 L) and brine (10 L) and then the solution was dried over solid sodium sulfate, filtered concentrated under reduced pressure keeping the reactor temp below 44°C. The solid residue was co-evaporated with hexane (4 L). The reactor was brought to ambient temperature and 5% MTBE/Hexane (5 L) was added. The thick suspension was stirred for 15 min and then the solid was collected by filtration. The collected solid was washed with of hexane (2.5 L)and dried under high vacuum at ambient temperature to a constant

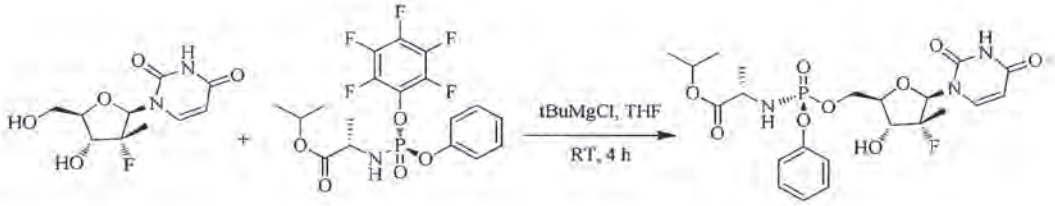
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weight to give the final product (S_P-**4**) as a white solid, 2.6 kg (53%); 99.5% pure by HPLC, 0.4% of other R_P-**4**.

Example 10-5. Preparation of (S)-isopropyl 2-((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2 yl)methoxy)(phenoxy)- phosphorylamino)propanoate.

To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl)-1H-pyrimidine-2,4-dione (**3**, 2.6g, 10mmol) in dry THF (50mL) was added a 1.7M solution of tert-butylmagnesium chloride (12.4mL, 21mmol, 2.1 equiv)) at room temperature over a period of 15min. After 30min, a solution of crude racemic (**2-[(2,3,4,5,6-pentafluoro phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester** (4.08g, 10mmol) in THF (15mL) was added drop wise over a period of 10min. The mixture was allowed to stir at room temperature for 72h. TLC co-spot with authentic product showed around 40-50% of the desired product had formed compared to the starting nucleoside.

Examle 10-6. Preparation of (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (S_P-4) using (S)-2-[(S)-(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester and purification by crystallization only.



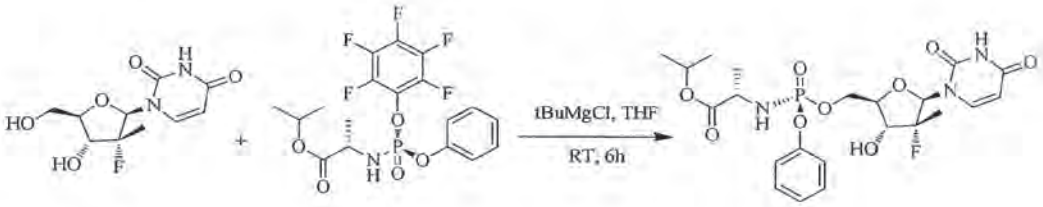
To a stirred solution of **3** (10g, 38.46mmol, dried under vacuum at 50°C for 20h) in dry THF (165mL) was added a 1.7M solution of *tert*-butylmagnesium chloride in THF (47.5 mL, 80.77 mmol) over a period of 20min while keeping the

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flask in a cold water bath (5°C) under a nitrogen atmosphere. After completion of the addition, the cold bath was removed and the white suspension was stirred at room temperature (20°C) for 30 min. A solution of (*S*)-2-[(*S*)-(2,3,4,5,6-pentafluorophenoxy)-phenoxyphosphorylamino] propionic acid isopropyl ester (20.9 g, 46.11 mmol) in anhydrous THF (165 mL) was then added to the reaction mixture over a period of 30 min. The mixture was stirred at room temperature (20°C) for 3.5 h. Stirring was continued for additional 1.5 h, at which stage TLC indicated >95% conversion and no significant difference in 3',5'-*bis*-phosphoramidate impurity intensity from 2h. The reaction mixture was quenched with saturated aq. NH_4Cl (10 mL) and then the solvent was evaporated at 25°C. The residue was partitioned between ethyl acetate (400 mL) and saturated ammonium chloride (60 mL)/water (20 mL) mixture. The organic layer was separated, washed with saturated ammonium chloride (80 mL) and water (3 x 60 mL). The aqueous layer up to this point was kept separately. The organic layer was washed with 5% aq. sodium carbonate (3 x 50 mL) and water (2 x 60 mL). The first aqueous layer was extracted with additional ethyl acetate (100 mL), washed with water (2 x 20 mL) and then the aqueous layer obtained from the sodium carbonate washings was extracted with the same ethyl acetate extract. The organic layer was washed with water (2 x 20 mL) and combined with the main lot. The combined organic layers were dried over anhydrous sodium sulfate, filtered and concentrated to give a foamy solid (19.32 g). The residue was dissolved in 60 mL of dichloromethane (a white solid was precipitated out and a cake was formed in about five minutes) and then was added 25 mL of IPE. The suspension was stirred at room temperature for 2 h. The white solid was filtered, washed with 1:1 mixture of cold (0°C) IPE/dichloromethane (20 mL) and dried to give 11.77 g (58% yield) of the product as an amorphous white solid. The above solid was re-dissolved in dichloromethane (350 mL), filtered and evaporated under atmospheric pressure (45°C bath temperature) to a volume of ~120 mL. The solution was allowed to stand at room temperature (21°C) for 20 h. The white crystalline solid (dichloromethane solvate) was collected by filtration, washed with cold (0°C) dichloromethane (10 mL) and dried under high vacuum for 4 h at ambient temperature to give pure non-solvated product (10.62 g, 52% yield) as white needles. HPLC purity 99.8%. Spectral properties match those values reported herein.

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Example 10-7. Preparation of (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate (S_P-4) using (S)-2-[(S)-(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester, modified reaction conditions and workup and purification by crystallization only.

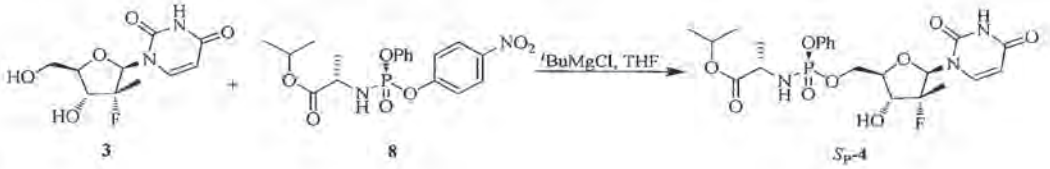


To a stirred suspension of 1-((2R,3R,4R,5R)-3-fluoro-4-hydroxy-5-(hydroxymethyl)-3-methyltetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (**3**, 5.0 g, 19.1 mmol, dried under vacuum at 50°C for 20 h) in dry THF (75 mL) was added a 1.7 M solution of tert-butylmagnesium chloride in THF (23.7 mL, 40.35 mmol) using an addition funnel over a period of 30 min at -5°C. The white suspension was stirred at this temperature for 30 min and then warmed to ambient temperature (20°C) at which temperature it was stirred for additional 30 min. The reaction mixture was cooled to 5°C and then was added a solution of (*S*)-2-[(*S*)-(2,3,4,5,6-pentafluorophenoxy)-phenoxyphosphorylamino] propionic acid isopropyl ester (10.45 g, 23.06 mmol) in THF (50 mL) over a period of 30 min. The mixture was stirred at 5°C for 18 h, cooled to -5°C and then quenched with 2N HCl (25 mL). Toluene (100 mL) was added to the mixture and warmed to room temperature. After 20 min the layers were separated. The organic layer was washed with 1N HCl (2 x 10mL), water (10 mL), 5% aqueous Na_2CO_3 (4 x 10mL), water (2 x 10mL) and brine (10 mL). All the aqueous layers were re-extracted with toluene (20 mL), washed with 5% aqueous Na_2CO_3 (2 x 5 mL), water (10 mL) and brine (5 mL). The combined organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to an approximate volume of 20 mL. Dichloromethane (20 mL) was added to the solution and the mixture was stirred at room temperature for 18 h. The solid was filtered, washed with 1:1 MTBE/DCM mixture (2 x 10mL) and dried under high vacuum to give white solid (7.7 g). HPLC of the solid at this indicated

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98.21% S_P-**4**, 0.18% of un-reacted **3** and 0.67% of 3',5'-bis-phosphoramidate impurity. The above solid of S_P-**4** was re-dissolved in dichloromethane (77 mL, heated in a pressure vessel at 55°C) and allowed to stand at room temperature for 20 h. The crystalline solid was filtered washed with cold dichloromethane (5 mL, 0°C) and dried under high vacuum to give pure product as a white solid (6.9 g, 68% yield, 99.79% pure by HPLC).

The preparation and purification of **C** or **C'** provides for direct access to either S_P-**4** or R_P-**4**, as illustrated in the following examples.



Example 11. Preparation of S_P-**4** (32 mg-scale): To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl) -1H-pyrimidine-2,4-dione **3** (32 mg, 0.12 mmol) in dry THF (1mL) was added a 1M solution of tButylmagnesium chloride (0.26 mL, 0.26 mmol, 2.1 equiv)) at room temperature over a period of 3 min. After 30 min, a solution of (S)-2-[(S)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (**8**, S_P-isomer) in THF (0.5mL) was added drop wise over a period of 3min. The mixture was allowed to stir at room temperature for 42h and then quenched with saturated aqueous NH_4Cl (10mL). The mixture was partitioned between ethyl acetate and water. The combined organic extract was dried over anhydrous sodium sulfate and concentrated. The residue was chromatographed using 0-4% methanol/dichloromethane gradient to give S_P-**4** as foamy solid (29mg, 44.5%yield). ^1H and ^{31}P NMR agree to that which is disclosed herein.

Example 12. Preparation of S_P-**4** (2.6 g-scale, without chromatography): To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl)-1H-pyrimidine-2,4-dione (2.6 g, 10 mmol) in dry THF (50 mL) was added a 1.7 M solution of tert-butylmagnesium chloride (12.4 mL, 21 mmol, 2.1 equiv)) at room temperature over a period of 15 min. After 30

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min, a solution of (S)-2-[(S)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (**8**, S_P-isomer, 4.08g, 10mmol) in THF (15 mL) was added drop wise over a period of 10min. The mixture was allowed to stir at room temperature for 6 0h and then quenched with saturated aqueous NH_4Cl (20mL). The mixture was partitioned between ethyl acetate (150 mL) and sequentially, 10% aqueous Na_2CO_3 (3 x 20 mL) and water (20 mL). The combined organic extract was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to give a pale yellow residue (3.8 g). The residue was dissolved in dichloromethane (7.6 mL) and then stirred for 20h at room temperature. The white solid was filtered, washed with 1:1 IPE/dichloromethane (5 mL) and dried under vacuum to give pure product as white solid (1.85 g, 35%yield).

Example 13. Preparation of S_P-4 using NaHMDS: To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl) -1H-pyrimidine-2,4-dione (71 mg, 0.27 mmol) in dry THF (2.0 mL) was added a 2.0 M solution of sodium bis(trimethylsilyl)amide (NaHMDS) in THF (270 µL, 0.54 mmol) at
-78°C over a period of 2 min. After 30 min, a solution of (S)-2-[(S)-(4-Nitro-phenoxy)-phenoxy-phosphorylamino]-propionic acid isopropyl ester (**8**, S_P-isomer, 111 mg, 0.27 mmol) in THF (1 mL) was added to the mixture. The reaction mixture was allowed stir at this temperature for 2h and then warmed to -20°C at which temperature it was stirred for additional 20h. TLC indicated ~30% of unreacted nucleoside starting material. Hence, additional 0.5 equivalents of the reagent (55 mg, 0.14 mmol) in THF (0.5 mL) was added to the reaction mixture and stirred for another 6 h. The reaction mixture was quenched with saturated aqueous ammonium chloride solution and then partitioned between ethyl acetate and water. The combined organic extract was dried over anhydrous sodium sulfate and concentrated to give a light brown residue. Column chromatography of the crude product using 0-5% methanol/dichloromethane gradient gave S_P-**4** (22 mg, 15% yield), 3'-phoshoramidate (**5**, S_P-isomer, 11.5 mg, 16% yield) and bis phosphoramidate (**6**, S_P, S_P-isomer, 12.6mg).

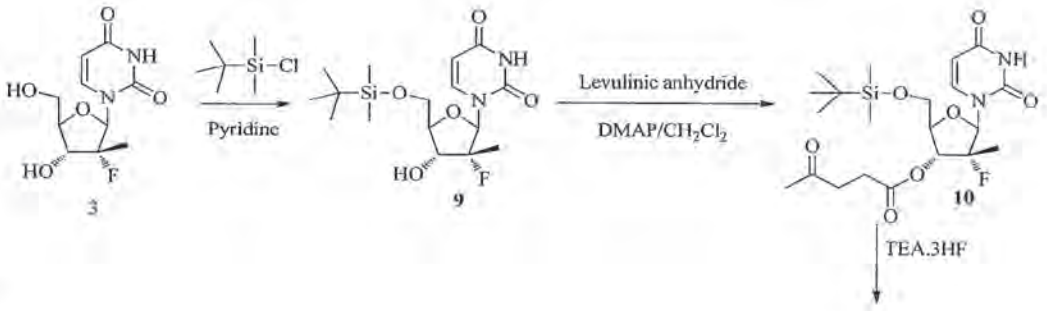
Example 14. Preparation of R_P-4 (260 mg-scale): To a stirred solution of 1-((2R,3R,4R,5R)-3-Fluoro-4-hydroxy-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-

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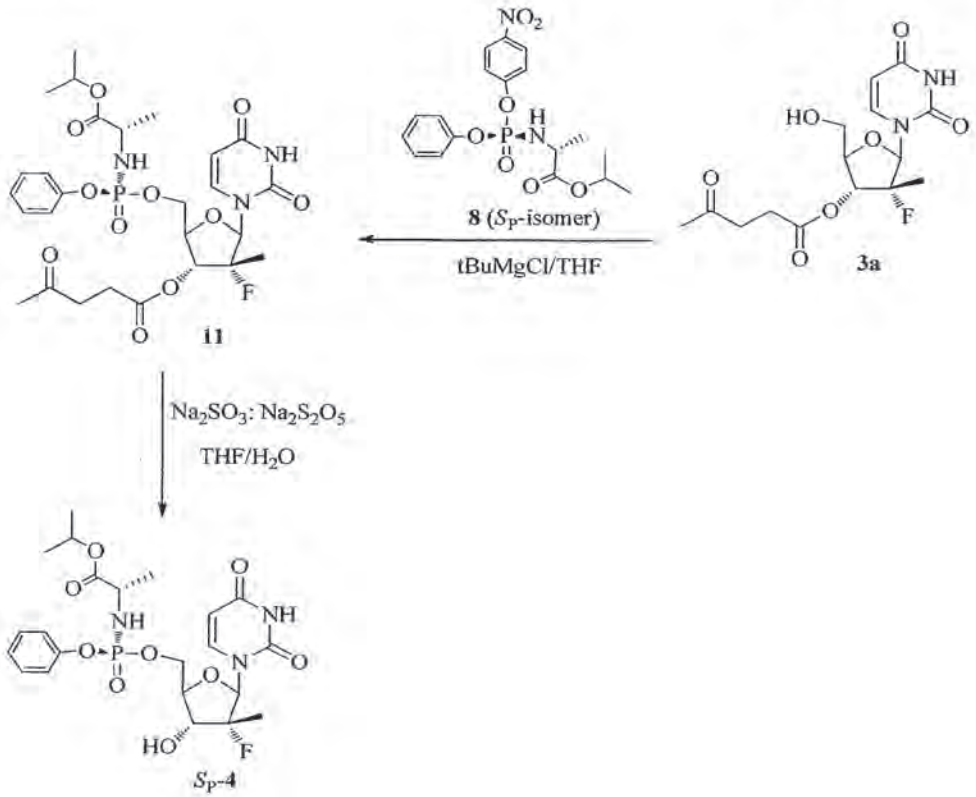
yl)-1H-pyrimidine-2,4-dione (260 mg, 1 mmol) in dry THF (6 mL) was added a 1.7 M solution of tert-butylmagnesium chloride (1.23 mL, 2.1 mmol, 2.1 equiv)) at room temperature over a period of 5 min. After 30 min, a solution of (S)-2-[(R)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (**8**, R_P-isomer) in THF (3 mL) was added drop wise over a period of 3 min. The mixture was allowed to stir at room temperature for 96 h and then quenched with saturated aqueous NH_4Cl (10 mL). The mixture was partitioned between ethyl acetate (50 mL) and water (2 x 20 mL). The combined organic extract was dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure to give a pale yellow residue (490 mg). The residue was chromatographed using 0-5% methanol/dichloromethane gradient to give product as a white solid (160 mg, 30% yield).

The preparation of S_P-**4** or R_P-**4** may also be achieved by reacting 3'-protected 3 with the appropriate reagent **C** or **C'** or a mixture containing **C** and **C'**, as illustrated in the following examples.

Example 15. Preparation of S_P-4 with 3a as a Synthetic Intermediate



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Example 15-1. Synthesis of 5'-*O*-*tert*-Butyldimethylsilyl-2'-deoxy-2'-fluoro-2'-*C*-methyluridine (9):

To a stirred solution of 2'-deoxy-2'-fluoro-2'-*C*-methyluridine (**3**, 81.1 g, 312 mmol) in dry pyridine (750 mL) was added drop-wise a solution of TBDMSCl (103.19 g, 685.6 mmol) in dry pyridine (500 mL) over a period of 45 min at ambient temperature. The reaction was allowed to stir at ambient temperature for 24 h. Methanol (85 mL) was added to the reaction mixture and it was allowed to stir for 10 min and then the solvents were distilled off under reduced pressure. Hot water (45°C) (1 L) was added to the reaction mass and the mixture extracted with ethyl acetate (2 x 500 mL), washed with water (1 x 500 mL). The organic layer was dried over anhydrous sodium sulfate. Ethyl acetate was distilled off and the residue obtained was co-evaporated with toluene (2 x 500 mL) to give crude **9** as a white foam. Yield = 116.9 g (quantitative). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.1 (s,6H), 0.91 (s, 9H), 1.22 (d, 3H, J = 21 Hz), 2.50 (s, 2H), 3.75-4.05 (m,4H), 5.54 (d, 1H, J

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= 9 Hz), 5.73 (s, 1H), 6.0 (d, 1H, J = 18 Hz), 7.81 (d, 1H, J = 9 Hz), 8.57 (br, s, 1H), 11.1 (s, 1H).

Example 15-2. Synthesis of 5'-*O* -(*tert* –Butyldimethylsilyl)-3'-*O*-levulinyl-2'-deoxy-2'-fluoro 2'-*C*-methyl-uridine (10):

To a stirred solution of nucleoside **9** (116.9 g, 312.1 mmol) in DCM (1 L) was added DMAP (30.5 g, 249.7 mmol) and this was allowed to stir at RT for 20 min. A soln. of levulinic anhydride (133.6 g, 642.3 mmol) in DCM (200 mL) was added to the mixture and allowed to stir for 24 h. TLC of the mixture indicated completion of reaction. Cold water (500 mL) was added and the mixture stirred for 20 min. Layers were separated and the organic layer was washed with sat. sodium bicarbonate solution (2 x 250 mL), dried over anhydrous sodium sulfate and then the solvent was distilled under reduced pressure to give yellow oil. Crude yield: 197.6 g (135 %). The material was used as is for the next step. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.11 (s, 6H), 0.94 (s, 9H), 1.34 (d, 3H, J = 21 Hz), 2.22 (s, 3H), 2.6-2.89 (m, 4H), 3.72 (m, 1H), 4.01 (d, 1H, J = 12 Hz), 4.23 (d, 1H, J = 9 Hz), 5.33 (dd, 1H, J = 15 Hz), 5.73 (d, 1H, J = 6 Hz), 6.26 (d, 1H, J = 15 Hz), 8.12 (d, 1H, J = 12 Hz), 8.72 (br, s, 1H).

Example 15-3. Synthesis of 3'-*O*-levulinyl-2'-deoxy-2'-fluoro 2'-*C*-methyl-uridine (3a):

Crude **10** (197.6 g, ~312.1 mmol) was dissolved in DCM (1 L) to which was added TEA.3HF (50.3 g, 312.1 mmol) and allowed to stir overnight at ambient temperature. TLC of the mixture indicated about 50 % completion of reaction. Another equivalent of TEA.3HF (50.3 g, 312.1 mmol) was added and the reaction mixture was allowed to stir for 6 h. TLC at this point indicated about 10 % of unreacted starting material. Another 0.25 eq of TEA.3HF (12.5 g, 78.0 mmol) was added and the reaction mixture was allowed to stir overnight. Reaction mixture was concentrated to dryness to give yellow oil. Crude from all the batches was purified by column chromatography on silica gel (0-2% MeOH in DCM) to give 124.1 g of 3'-levulinate as a white foam solid (90% purified yield over three steps from2'-deoxy-2'-fluoro-2'-C-methyluridine). ^{1}H NMR: ($CDCl_3$, 400 MHz) δ 1.55 (d, 3H, CH3, J = 20 Hz), 2.36 (s, 3H, CH3), 2.8-3.03 (m, 5H, CH2CH3), 3.91-3.96 (dd, 1H,

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CH''), 4.2-4.25 (m, 1H, CH'), 4.34 (dd, 1H, CH, J = 8 Hz), 5.25 (dd, 1H, J = 16 Hz), 5.93 (d, 1H, J = 8 Hz), 8.20 (d, 1H, J = 8 Hz), 9.18 (s, 1H).

Example 15-4. Stereoselective synthesis of (S)-2-{[(1R,4R,5R)-5-(2,4-Dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-(R)-fluoro-3-(4-oxopentanoyl)-4-methyl-tetrahydro-furan-2-ylmethoxy]-phenoxy-phosphorylamino}-propionic acid (S)-isopropyl ester (11):

To a solution of the nucleoside (**3a**, 1.00 mmol, 358 mg) in 5 ml anhydrous THF that was cooled to 0°C was added tBuMgCl (1.7 M in THF, 2 eq) and allowed it to warm to ambient temperature and stirred for half hour. To this mixture was added the reagent (ca. 97% chiral purity) (S)-2-[(S)-(4-nitro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (**8**, S_P-isomer) (408 mg, 1.00 mmol, 1.00 eq.) in one lot and allowed it to stir at rt. After 16 h, there was ~30% starting material left. The reaction mixture was quenched with saturated NH_4Cl solution 10 ml, and the aqueous phase was extracted with ethyl acetate (3 x25 ml). The combined organic layer was washed with brine and dried over anhydrous sodium sulfate and evaporated to dryness to give a pale yellow foam (500 mg). This was purified by silica gel chromatography using 2-5% methanol in methylene chloride to give the product as a white foam (275 mg) of about 97% P chiral purity and unreacted starting material (162 mg). Based on consumed starting material, the yield was 76%. ^{31}P NMR ($CDCl_3$, 162 MHz): 3.7 ppm; ^1H NMR ($CDCl_3$, 400 MHz): δ 1.22 (dd, 6H, J = 6.4 Hz), 1.37 (s, 3H), 1.58 (s, 3H), 2.18 (s, 3H), 2.63-2.9 (m, 4H), 4.0 (d, 1H, J = 8 Hz), 4.2-4.33 (m, 1H), 4.57 (d, 1H, J = 8Hz), 4.96-5.00 (sept, 1H), 5.2 (dd, 1H, J = 9 Hz), 5.42 (d, 1H, J = 8Hz), 6.19 (d, 1H, J = 18Hz), 7.15-7.35 (m, 5H), 7.5 (d, 1H, J = 5.6 Hz), 8.2 (br, s, 1H).

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Example 15-5. Synthesis of (S)-2-{[(1R,4R,5R)-5-(2,4-Dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-(R)-fluoro-3-hydroxy-4-methyl-tetrahydro-furan-2-ylmethoxy]-phenoxy-phosphorylamino}-propionic acid (S)-isopropyl ester (S_P-4)

A solution of sodium sulfite was prepared by adding $Na_2S_2O_3$ (1.51 g) and $Na_2S_2O_5$ (0.57 g) in water (25 mL). To a solution of the levulinate (**11**, 250 mg, 0.40 mmol) in anhydrous THF (2.5 mL) was added 1.0 ml of the sodium sulfite solution. This was allowed to stir at room temperature for 4 h. The reaction mixture was poured in to water (15 mL) and extracted with ethyl acetate (3x25 mL) dried and evaporated to give quantitatively a white solid product with about 97% P chiral purity which matched the physical and spectral properties of S_P-**4** produced directly from the unprotected nucleoside.

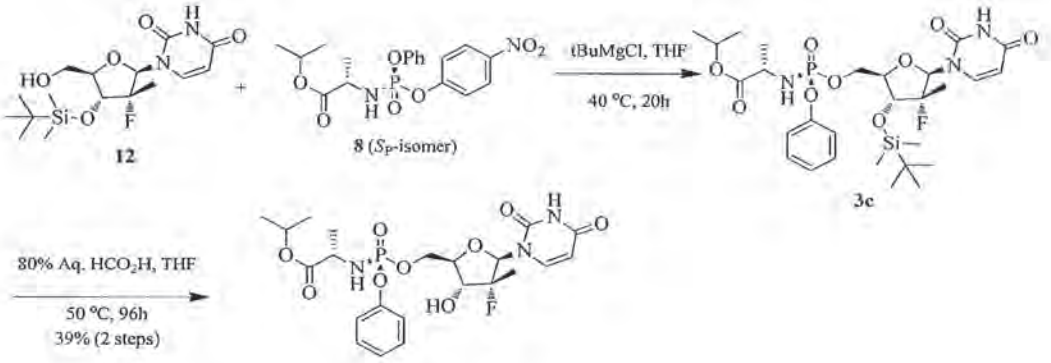
Example 16. Alternative procedure for preparing S_P-4 from 3a.

To a stirred solution of 4-oxo-pentanoic acid (2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-fluoro-2-hydroxymethyl-4-methyl-tetrahydro-furan-3-yl ester (**3a**, 210 mg, 0.59 mmol) in dry THF (1.5 mL) was added a 1.7 M solution of tert-butylmagnesium chloride (1.07 mL, 1.82 mmol) at room temperature over a period of 2 min. Initially, a white precipitate was observed and after 10 min the reaction mixture turned to dark yellow solution. After 30min, a solution of (S)-2-[(S)-(4-nitrophenoxy)-phenoxy-phosphorylamino]-propionic acid isopropyl ester (**8** (S_P-isomer), 382mg, 0.94mmol) in THF (1.5mL) was added drop wise over a period of 3min. The mixture was heated at 40°C for 5h at which time TLC and ^{1}H NMR indicated less than 2% of unreacted starting material. The reaction was quenched with saturated aqueous ammonium chloride and then partitioned between ethyl acetate and water. The combined organic layer was washed with 10% aqueous Na_2CO_3 solution (3 x 10 mL), followed by water. The organic layer was dried over anhydrous sodium sulfate and concentrated to give brown color residue (410 mg). The crude product was dissolved in tetrahydrofuran (1.0 mL) and then was added an aqueous solution of the mixture of sodium sulfite (37 mg, 0.295 mmol) and sodium metabisulfite (224 mg, 1.18 mmol) in 1mL of water. The mixture was heated at 45°C for 20 h at which stage only about 10% conversion was observed by TLC, hence the additional sodium sulfite (74 mg) and sodium metabisulfite (448 mg) was

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added and the heating was continued for additional 52 h. At this time, about 40% conversion observed by TLC. The reaction mixture was partitioned between water and ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate and concentrated to give a brown residue (210 mg). Column chromatography of the residue using 0-5% MeOH/DCM gradient gave unreacted starting material (89mg) and S_P-**4** (57mg, 18% yield, 24% based on recovered starting material).

Example 17. Preparation of S_P-4 with 3c as a Synthetic Intermediate



S_P-4

Example 17-1. Preparation of of 1-[(2R,3R,4R,5R)-4-(tert-butyldimethylsilanyloxy)-3-fluoro-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl]-1H-pyrimidine-2,4-dione, 12.

To a solution of **3** (10.0g, 38.43 mmol) in pyridine (50 mL) were added dichloromethane (50 mL). The solution was cooled to 0°C. To the solution was added 4,4'-dimethoxytrityl chloride (14.32 g, 42.27 mmol) and the solution was stirred at 0°C for 5 h. Methanol (5 mL) was added to quench the reaction. The solution was concentrated to dryness under reduced pressure and the residue was partitioned between ethyl acetate (500 mL) and water (50 mL). The organic solution was washed with brine (50 mL) and dried (sodium sulfate, 4 g). The solvent was removed under reduced pressure and the residue was dissolved in dichloromethane (100 mL). To the solution were added imidazole (7.83 g, 115 mmol) and t-butyldimethylsilyl chloride (8.68 g, 57.6 mmol). The solution was stirred at ambient

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temperature for 16 h. Methanol was added to quench the reaction (5 mL) and the solvent was removed under reduced pressure and the residue was partitioned between ethyl acetate (500 mL) and water (50 mL). The organic solution was dried (sodium sulfate, 4 g) and evaporated under reduced pressure. The residue was purifed by column chromatography (10-40% EtOAc in Hexane) to give 5'-O-DMT-3'-O-tBDMS intermediate product. This is turn was treated with 1% trifluoroacetic acid in dichloromethane (200 mL). The solution was stirred at ambient temperature for 1h. Water (20 mL) was added and the solution was stirred at ambient for another 1h. Methanol (5 mL) was slowly added and the solution was stirred at ambient for another 1h. Ammonium hydroxide was added to adjust the solution pH to 7. The organic solution was separated, dried (sodium sulfate, 4 g) and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (1-5% methanol in dichloromethane) to give **12** as a white solid 7.5g in 50% yield over the three steps. ^{1}H NMR (DMSO-d6) δ (ppm) 11.48 (br s, 1H, N*H*), 7.94 (d, 1H, H-6), 6.00 (d, 1H, H-1'), 5.69 (d, 1H, H-5), 4.06 (dd, 1H, 3'-H), 3.85 (m, 2H, H-5'a, H-4'), 3.58 (br d, 1H, H-5'b), 1.27 (d, 3 H, 2-CH_3), 0.89 (s, 9H, C(CH_3)$_3$), 0.12 (s, 6H, Si(CH_3)$_2$).

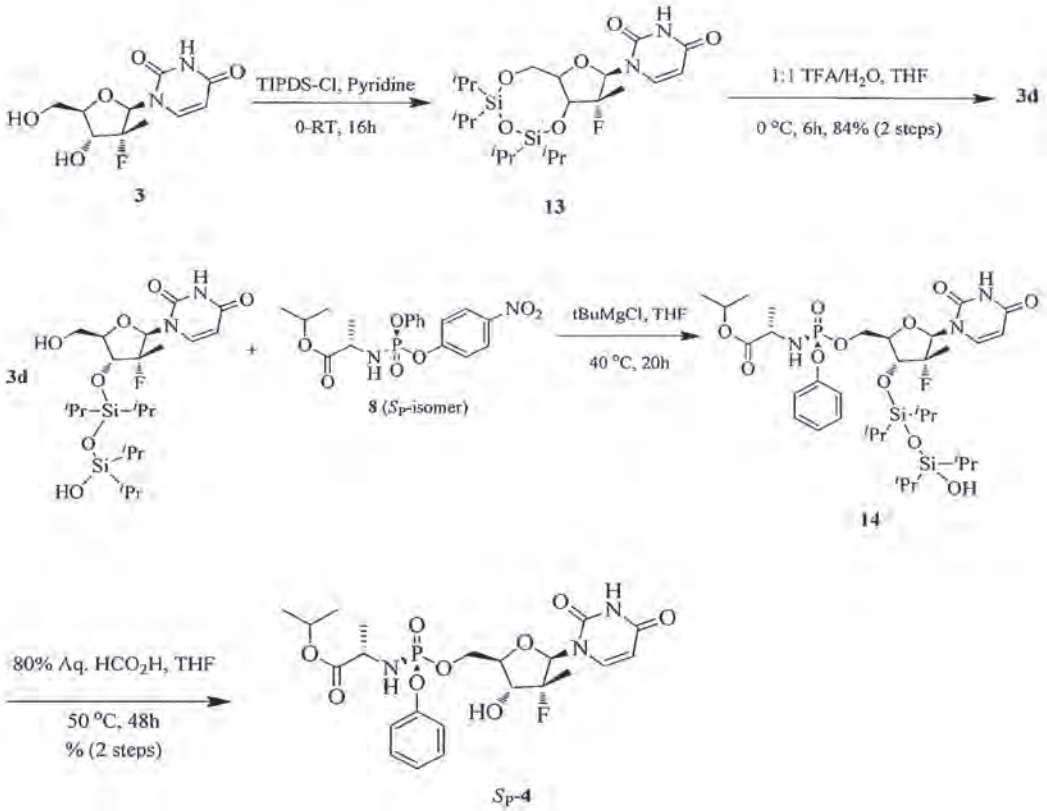
Example 17-2. Preparation of S_P-4 using 1-[(2R,3R,4R,5R)-4-(tert-butyldimethylsilanyloxy)-3-fluoro-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl]-1H-pyrimidine-2,4-dione (3c).

To a stirred solution of 1-[(2R,3R,4R,5R)-4-(tert-butyldimethylsilanyloxy)-3-fluoro-5-hydroxymethyl-3-methyl-tetrahydro-furan-2-yl]-1H-pyrimidine-2,4-dione (**12**, 374 mg, 1 mmol) in dry THF (3 mL) was added a 1.7 M solution of tert-butylmagnesium chloride (1.8 mL, 3.1 mmol)) at room temperature over a period of 2 min. Initially, a white precipitate was observed and after 10min the reaction mixture turned to clear dark yellow solution. After 30 min, a solution of (S)-2-[(S)-(4-nitrophenoxy)-phenoxy-phosphorylamino]-propionic acid isopropyl ester (**8**, S_P-isomer, 653 mg, 1.6 mmol) in THF (2.5 mL) was added drop wise over a period of 3 min. The mixture was heated at 40°C for 20 h at which time TLC and ^{1}H NMR indicated less than 5% of unreacted starting material. The reaction mixture was quenched with saturated aqueous ammonium chloride and then partitioned between ethyl acetate and water. The organic layer was washed with 10% aqueous Na_2CO_3

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solution (3 x 10 mL), followed by water (20 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to give brown residue containing **3c** (850 mg). The crude product was dissolved in tetrahydrofuran (2 mL) and was added 0.8 mL of 80% aqueous formic acid at room temperature. The reaction mixture was heated at 50°C for 96h. About 70% conversion was observed by TLC. The reaction mixture was poured into cold saturated aqueous sodium bicarbonate and then partitioned between ethyl acetate and water. The combined organic layer was dried over anhydrous sodium sulfate and concentrated to give brown residue (220 mg). Column chromatography of the residue using 0-5% MeOH/DCM gradient gave unreacted starting material (21 mg) and S_P-**4** (77 mg, 35% yield, 39% yield based on recovered starting material).

Example 18. Preparation of S_P-4 with 3d as a Synthetic Intermediate



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Example 18-1. Preparation of 3d

To a stirred solution of **3** in pyridine (20 mL) at 0°C was added TIPDS-Cl drop-wise over a period of 15 min. The mixture was slowly allowed to warm to room temperature at which temperature it was stirred for 16 h. The pyridine was evaporated and the residue was co-evaporated with toluene (50 mL). The residue was then triturated with hexanes and the white precipitate was filtered off using a pad of Celite. The filtrate was concentrated under reduced pressure to give a foamy solid (12.97 g). The crude product (**13**) was redissolved in tetrahydrofuran (75mL) and was added an aqueous solution of TFA (75mL, 1:1 TFA/water) at 0°C over a period of 20 min. The mixture was stirred at this temperature for 6 h. TLC indicated ~5% of starting material. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ until pH 8 and then extracted with ethyl acetate. The combined organic extract was washed with water, dried and concentrated to give white crystalline solid. Further trituration of the solid with hexanes (30 mL) gave white solid which was filtered and dried under high vacuum to give **3d** (10.1 g, 84 % yield over 2 steps). **^{1}H NMR (400 MHz, $CDCl_3$)**: δ 8.83 (bs, 1H), 7.94 (bd, J=6.0Hz, 1H), 6.10(bd, J=18.4Hz, 1H), 5.71 (d, J=8.2Hz, 1H), 4.43 (bs, 1H), 4.36 (dd, J=22.6, 9.0Hz, 1H), 4.27 (bs, 1H), 4.10(d, J=13.2Hz, 1H), 4.03 (d, J=9.2Hz, 1H), 3.92 (d, J=13.2Hz, 1H), 1.39 (d, J=22.0Hz, 3H), 1.11-0.92 (m, 28H).

Example 18-2. Preparation of S_P-4

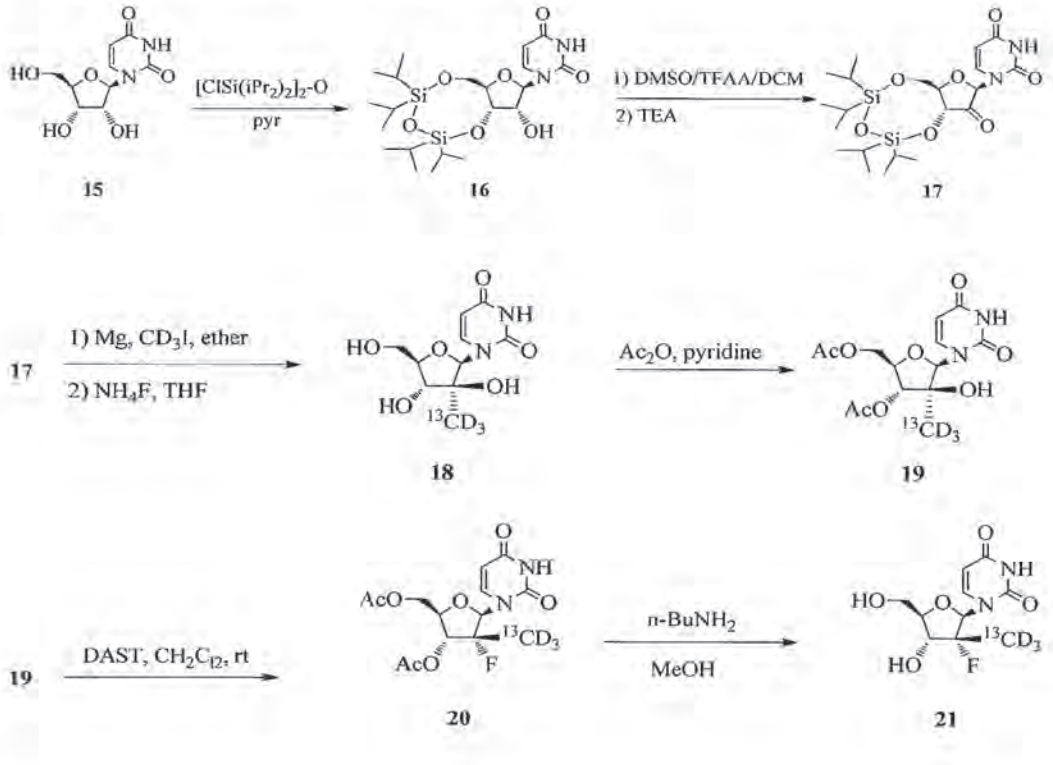
To a stirred solution of **3d** (520 mg, 1 mmol) in dry THF (5 mL) was added a 1.7M solution of tert-butylmagnesium chloride (1.8 mL, 3.1 mmol, 3.1 equiv)) at room temperature over a period of 15 min. After 30 min, a solution of (S)-2-[(S)-(4-nitro-phenoxy)-phenoxyphosphorylamino] propionic acid isopropyl ester (**8**, S_P-isomer, 653 mg, 1.6 mmol) in THF (1 mL) was added drop wise over a period of 3 min. The mixture was allowed to stir at room temperature for 60 h. ^{1}H and ^{31}P NMR of the crude sample indicated mixture of diastereomers in about 1:0.76. The reaction mixture was quenched with saturated aqueous NH_4Cl (20 mL). The mixture was partitioned between ethyl acetate (150 mL) and sequentially, 10% aqueous Na_2CO_3 (3 x 20mL) and water (20 mL). The combined organic extract was dried over

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anhydrous sodium sulfate, filtered and concentrated under reduced pressure to give a pale yellow residue (**14**, 878 mg).

The above compound, **14**, was redissolved in tetrahydrofuran (3 mL) and then was added 80% aqueous formic acid. The mixture was heated at 55°C for 20h. The reaction mixture was cooled to 0°C, and then quenched with saturated aqueous sodium bicarbonate (pH 7.0). The reaction mixture was then partitioned between ethyl acetate and water. The combined organic layer was dried over sodium sulfate and concentrated to give 560mg of the residue. The residue was chromatographed using 0-5% methanol/dichloromethane gradient to give unreacted starting material (**14**, 242mg) and S_P-**4** (80mg, 15% yield) as a white solid.

Example 19. Preparation of Isotopically Labeled S_P-4



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Example 19-1. Preparation of 1-((6aR,8R,9R,9aS)-9-hydroxy-2,2,4,4-tetraisopropyltetrahydro-6H-furo[3,2-f][1,3,5,2,4]trioxadisilocin-8-yl)pyrimidine-2,4(1H,3H)-dione, 16

Uridine (**15**, 100.0 g, 409.5 mmol) was co-evaporated to dryness with anhydrous pyridine (600 mL) and re-suspended in anhydrous pyridine (700 mL). To this stirred fine suspension was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (135.7 g, 482.5 mmol) over 60 min at ambient temperature. After stirring the fine suspension for 17 h at ambient temperature, the reaction was quenched by adding methanol (20 mL) and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate (1.5 L) and water (2 L). The organic layer was further washed with 5% hydrochloric acid (2 x 1 L), brine (500 mL), dried over solid sodium sulfate (50 g), filtered and concentrated under reduced pressure to the crude product, ca 250 g. The residue was subjected to a filtration column using silica gel (1.75 kg) and a gradient of ethyl acetate in hexanes 20-65%. The pure product fractions as judged by a homogenous TLC (Rf 0.55 in 1:1 hexanes-ethyl acetate) were combined and concentrated under reduced pressure and dried (40°C, 0.2 mm Hg, 24 h) to afford 145.5 g (76%) of **16** as a white foam solid. An additional fraction (35 g) of slightly impure **16** was also collected. ^{1}H NMR (DMSO-d_6) δ (ppm) 11.35 (s, 1H, N*H*), 7.66 (d, 1H, J = 7.6 Hz, *H*-6), 5.57 (d, 1H, J = 4.8 Hz, 2'-O*H*), 5.50-5.49 (m, 2H, 1'-*H* and *H*-5), 4.14-4.18 (m, 3H, 2', 3', 4'-*H*), 3.97-3.87 (m, 2H, 5'-*H*a and *H*b), 1.02-0.95 (m, 28H, C*H*(CH_3)$_2$).

Example 19-2. Preparation of 1-((6aR,8R,9aR)-2,2,4,4-tetraisopropyl-9-oxotetrahydro-6H-furo[3,2-f][1,3,5,2,4]trioxadisilocin-8-yl)pyrimidine-2,4(1H,3H)-dione, 17

To a dry three-necked round flask were added anhydrous DCM (600 mL) and DMSO (30.82 g, 394.5 mmol). The solution was cooled to -78°C in a dry ice/acetone bath under an atmosphere of nitrogen. Trifluoroacetic anhydride (neat, 77.7 g, 369.8 mmol) was added via a syringe over 40 mins and afforded a cloudy mixture. To the mixture a solution of uridine derivative **16** in DCM (600 mL) was added dropwise over 75 mins at -78°C via an addition funnel. The heterogeneous mixture was stirred for 2 h at -78~-65°C and then anhydrous triethylamine (92 mL)

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was added via a syringe quickly to form a clear light yellow solution. After 1 h at low temperature, the reaction was complete as shown by TLC (30% EtOAc in hexanes). The cooling bath was removed and the reaction mixture was warmed up slowly to ambient temperature over 1 h. The reaction was quenched by addition of sat. NH_4Cl (180 mL). Water (200 mL) was added and organic layer was separated. The aqueous layer was extracted again with DCM (300 mL). The combined organic layer was washed with water (3x 400 mL), brine (150 mL), and dried over Na_2SO_4. Removal of solvent afforded a sticky brown residue.

The crude oil residue (contained trace of DCM) was stored overnight in the freezer. After overnight, some crystal solid was observed in the oil. The oil was dissolved in 500 ml hexanes at ambient temperature. The solution was stored in the freezer for 24 hours and more solid was formed. Solid was collected via filtration and rinsed with cold 10% DCM in hexanes (1 L) to remove most of the orange color. The solid (**17**) was dried under vacuum for 2 h and then air dried for 24 h. The solid weighed 21 g after dried at 50 °C under vacuum. The filtrate was concentrated and the residue was purified via column chromatography (10-70% ethyl acetate in hexanes) to afford an additional 37 g (combined yield of 97%) of **17** as a light orange solid.

Example 19-3. Preparation of 1-((2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)-3-^{13}C-perdeuteriomethyltetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione, 18

Magnesium (3.53 g, 147 mmol), washed with 5% aqueous hydrochloric acid and dried (50°C, 0.2mm Hg, 24 h), was put into a two neck round bottomed flask equipped with a magnetic stirrer and a condensor. The flask was filled with argon gas and then anhydrous ether (80 mL) was added. To the magnesium in ether was added slowly perdeuterio-^{13}C methyl iodide (15.06 g, 110.3 mmol), which generated an exothermic reaction. After the reaction mixture was cooled down, the supernatant was transferred to a solution of dried compound **17** (50°C, 0.2mm Hg, 15 h) (10.0 g, 20.63 mmol) in anhydrous THF (1 L) at -50°C over 20 min. The temperature was allowed to rise to
-40°C and the mixture was stirred at between -40 to -25°C for 4h. Upon completion of reaction, the mixture was diluted with EtOAc (1L) at -50 °C and then brine (300

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mL) was added slowly. The organic layer was separated and then washed with sat'd ammonium chloride solution (300 mL x 2) and dried with sodium sulfate. After filtration and concentration under reduced pressure, the residue was dissolved in MeOH (250 mL). Ammonium fluoride (12 g) and TBAF (400 mg) were added. The resulting mixture was stirred at 90°C for 7h and then concentrated with silica gel (20 g) under reduced pressure. After thorough vacuum drying, the obtained residue was purified by flash silica gel column chromatography (MeOH:CH_2Cl_2 = 1:20 to 1:10) give compound **18** (5 g, 46%) as a white solid. ^{1}H NMR (DMSO-d_6) δ (ppm) 11.26 (s, 1H, NH), 7.65 (d, 1H, J = 8.4 Hz, H-6), 5.77 (d, 1H, J = 2.4 Hz, H-1'), 5.57 (d, 1H, J = 8.0 Hz, H-5), 5.46 (d, 1H, J = 5.2 Hz, HO-3'), 5.24 (d, 1H, J = 2.4 Hz, HO-2'), 5.14 (t, 1H, J = 5.6 Hz, HO-5'), 3.74-3.56 (m, 4H, H-3', 4', 5', 5'').

Example 19-4. Preparation of ((2R,3R,4S,5R)-3-acetoxy-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-hydroxy-4-^{13}C-perdeuteriomethyltetrahydrofuran-2-yl)methyl acetate, 19

To a solution of compound **18** (5.00 g, 19.1 mmol) in anhydrous pyridine (100 mL) was added acetic anhydride (3 mL) at ambient temperature. The resulting mixture was stirred at amibient temperature for 15h, diluted with EtOAc (250 mL), washed with water (50 mL x 3), and dried with sodium sulfate. After filtration and concentration, the residue was purified by flash column chromatography (MeOH 0 to 5% in CH_2Cl_2) to give compound **19** (4.0 g, 68%) as a gray solid.

Example 19-5. Preparation of ((2R,3R,4R,5R)-3-acetoxy-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-4-^{13}C-perdeuteriomethyltetrahydrofuran-2-yl)methyl acetate, 20

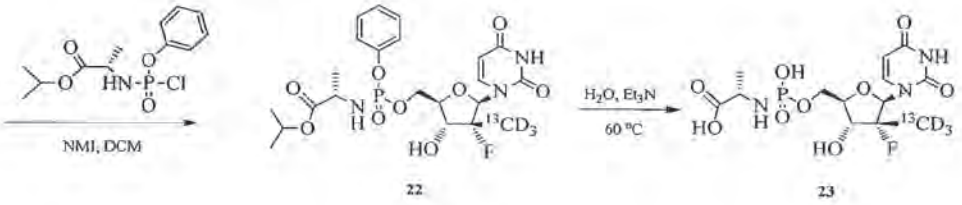
To a solution of compound **19** (2.33 g, 6.73 mmol) in anhydrous CH_2Cl_2 (60 mL) was added DAST (1.33 mL, 10.1 mmol) at -78°C slowly. The resulting mixture was stirred for 30 min after exposed to ambient temperature. An additional two 2.33 g scale reactions and one 1.00 g scale reaction were conducted exactly the same way. All four reaction mixtures were combined, diluted with CH_2Cl_2 (300 mL), and washed with ice-water (100 mL x 2) and then cold aqueous $NaHCO_3$ solution (100 mL x 2). After drying, filtration, and concentration, the residue was

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purified by flash silica gel column chromatography (EtOAc 0% to 50% in hexanes, compound came out at around 48%) to give compound **20** (2.0 g, 24% from total 7.99 g of compound **19**) as a white solid. ^{1}H NMR ($CDCl_3$) δ (ppm) 8.27 (s, 1H, NH), 7.55 (d, 1H, J = 8.4 Hz, H-6), 6.17 (d, 1H, J = 18.8 Hz, H-1'), 5.78 (dd, 1H, J = 1.2, 8.4 Hz, H-5), 5.12 (dd, 1H, J = 9.6, 21.6 Hz, H-3'), 4.40-4.31 (m, 3H, H-4', 5', 5''), 2.19 (s, 3H, CH_3), 2.15 (s, 3H, CH_3).

Example 19-6. Preparation of 1-((2R,3R,4R,5R)-3-fluoro-4-hydroxy-5-(hydroxymethyl)-3-^{13}C-perdeuteriomethyltetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione, 21

To a solution of compound **20** (2 g, 5.74 mmol) in methanol (20 mL) was added n-butylamine (6 mL). The resulting mixture was stirred at rt for 15h and concentrated with silica gel *in vacuo*. The obtained residue was purified by flash silica gel column chromatography (MeOH 0 to 10% in CH_2Cl_2) to give compound **21** (1.3 g, 85%) as a white solid. ^{1}H NMR (CD_3OD) δ (ppm) 8.08 (d, 1H, J = 8.0 Hz, H-6), 6.13 (d, 1H, J = 18.4 Hz, H-1'), 5.70 (d, 1H, J = 8.0 Hz, H-5), 3.99 (d, 1H, J = 13.6 Hz, H-5'), 3.97-3.91 (m, 2H, H-3', 4'), 3.80 (dd, 1H, J = 2.0, 12.8 Hz, H-5''), ESMS (M+1) estimated 265, observed 265.



Example 19-7. Preparation of (S)-Isopropyl 2-((((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-^{13}C-perdeuteriomethyltetrahydrofuran-2-yl)methoxy)(phenoxy)phosphorylamino)propanoate, 22

To a solution of the unprotected nucleoside **21** (207 mg, 0.783 mmol) and *N*-methylimidazole (0.4 ml, 5 mmol) in THF (4 mL) was added the pre-made phosphorochloridate in THF (1.0 M, 2.35 ml, 2.35 mmol) at 0°C dropwise. The reaction was slowly warmed to ambient temperature over 1 h and then water (1 mL)

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and EtOAc (5 mL) were added. The organic solution was washed with sat. aq. mono basic sodium citrate (2 x 2 ml), sat. aq. $NaHCO_3$ (1 x 2 ml), dried ($MgSO_4$) and concentrated under reduced pressure. The crude was purified by silica column chromatography using 0 to 5% ^{i}PrOH in CH_2Cl_2 as eluents to give the phosphoramidate, **22** (216 mg, 52%, 1:1 mixture of P-diastereomers) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.54 (s, 1H), 7.56 (d, J = 6.8 Hz, 1H), 7.40-7.35 (m, 2H), 7.23-7.18 (m, 3 H), 6.14-5.96 (m, 2H), 5.89 (dd, J = 5.6, 25.6 Hz, 1H), 5.55 (t, J = 8.4 Hz, 1H), 4.85 (dq, J = 1.6, 6.0 Hz, 1H), 4.44-4.32 (m, 1H), 4.25 (m, 1H), 4.06-3.98 (m, 1H), 3.86-3.70 (m, 2H), 1.30-1.08 (m, 9H); ^{31}P NMR (162 MHz, DMSO-d_6) δ 4.90, 4.77; LRMS (ESI) $[M + H]^+$ calculated for $C_{21}{}^{13}CH_{27}D_3FN_3O_9P$ 534.5, found 534.4.

Example 19-8. Preparation of (2S)-2-(((((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-^{13}C-perdeuteriomethyltetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)amino)propanoic acid, 23

Phosphoramidate **22** (147 mg, 0.276 mmol) was suspended in triethylamine (2 mL) and water (0.5 mL), and heated at 60°C for 30 h. Then the volatile components were evaporated under reduced pressure. The crude was purified by silica column chromatography by eluting with 50-70% ^{i}PrOH in CH_2Cl_2 and then, 0 to 20% NH_4OH in ^{i}PrOH to give **23** as a white solid (95 mg, 83%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.00 (d, J = 8.4 Hz, 1H), 5.98 (d, J = 19.2 Hz, 1H), 5.52 (d, J = 8.4 Hz, 1H), 4.02-3.81 (m, 4H), 1.10 (d, J = 6.8 Hz, 3H); ^{31}P NMR (162 MHz, DMSO-d_6) δ 8.12; LRMS (ESI) $[M + H]^+$ calculated for $C_{12}{}^{13}CH_{17}D_3FN_3O_9P$ 416.3, found 416.4.

Properties of Samples of R_P-4, 4, and S_P-4

Samples of R_P-**4**, **4**, and S_P-**4** were analyzed by X-Ray Powder Diffraction (XRPD), Nuclear Magnetic Resonance (NMR) spectrometry, Fourier Transform Infrared (FT-IR) spectroscopy, Differential Scanning Calorimetry (DSC), Thermal Gravimetric Analysis (TGA), Gravimetric Vapor Sorption (GVS), Thermodynamic Aqueous Solubility, and High Performance Liquid Chromatography (HPLC).

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Example 20. X-Ray Powder Diffraction

Samples of R_P-**4**, **4**, and S_P-**4** were analyzed by X-Ray Powder Diffraction (XRPD) under the following regimen.

a. Bruker AXS/Siemens D5000

X-Ray Powder Diffraction patterns were collected on a Siemens D5000 diffractometer using Cu Kα radiation (40kV, 40mA), θ-θ goniometer, divergence of V20 and receiving slits, a graphite secondary monochromator and a scintillation counter. The instrument is performance checked using a certified Corundum standard (NIST 1976). The software used for data collection was Diffrac Plus XRPD Commander v2.3.1 and the data were analyzed and presented using Diffrac *Plus* EVA v 11.0.0.2 or v 13.0.0.2.

Ambient conditions

Samples run under ambient conditions were prepared as flat plate specimens using powder as received. Approximately 35 mg of the sample was gently packed into a cavity cut into polished, zero-background (510) silicon wafer. The sample was rotated in its own plane during analysis. The details of the data collection are: angular range: 2 to 42°2θ; step size: 0.05°2θ; and collection time: 4 s.step^{-1}.

b. Bruker AXS C2 GADDS

X-Ray Powder Diffraction patterns were collected on a Bruker AXS C2 GADDS diffractometer using Cu Kα radiation (40 kV, 40 mA), automated XYZ stage, laser video microscope for auto-sample positioning and a HiStar 2-dimensional area detector. X-ray optics consists of a single Göbel multilayer mirror coupled with a pinhole collimator of 0.3 mm.

The beam divergence, i.e. the effective size of the X-ray beam on the sample, was approximately 4 mm. A θ-θ continuous scan mode was employed with a sample - detector distance of 20 cm which gives an effective 2θ range of 3.2°– 29.7°. Typically the sample would be exposed to the X-ray beam for 120 seconds. The

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software used for data collection was GADDS for WNT 4.1.16 and the data were analyzed and presented using Diffrac *Plus* EVA v 9.0.0.2 or v 13.0.0.2.

Ambient conditions

Samples run under ambient conditions were prepared as flat plate specimens using powder as received without grinding. Approximately 1-2 mg of the sample was lightly pressed on a glass slide to obtain a flat surface.

X-Ray Powder Diffraction (XRPD)

4 was found by XRPD to be amorphous (see **Fig. 1**). High resolution XRPD analysis of R_P-**4** prepared according to Example 3 confirmed a crystalline solid exhibiting a different powder pattern to that of S_P-**4** (prepared according to Example 4, Method 4), which was also confirmed to be a crystalline solid. The XRPD results table for R_P-**4** and S_P-**4** are shown in **Table 1** with all peaks exhibiting an intensity of ≤5 % (R_P-**4**) and ≤3% (S_P-**4**) excluded.

Table 1. XRPD Data for R_P-4 and S_P-4.

XRPD data for R_P-**4**		XRPD data for S_P-**4**(Form 1)	
Angle 2-Theta °	**Intensity %**	**Angle 2-Theta °**	**Intensity %**
6.616	51.1	4.900	6.8
7.106	40.5	5.190	19.8
8.980	30.0	7.501	100.0
11.020	21.7	8.355	4.1
11.559	77.1	8.965	7.7
11.950	12.8	9.619	21.2
13.023	5.2	10.145	3.6
14.099	6.2	14.393	4.9
15.121	5.7	16.300	7.0
15.624	5.4	16.688	10.6
16.003	17.8	17.408	5.5
17.882	100.0	17.820	8.2
18.567	8.8	18.262	31.5
19.564	22.7	18.600	6.3
20.280	5.6	18.900	7.3
20.728	42.5	19.150	6.1
21.047	19.9	19.696	4.8
21.671	22.0	20.398	4.4
21.943	23.3	20.710	6.9
22.214	18.9	21.950	6.1

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XRPD data for R_P-4		XRPD data for S_P-4(Form 1)	
Angle 2-Theta °	**Intensity %**	**Angle 2-Theta °**	**Intensity %**
23.074	28.5	22.175	12.2
24.145	30.3	22.511	5.6
24.355	39.1	22.924	3.1
25.366	7.6	23.360	6.5
26.146	36.2	23.538	7.1
27.000	9.0	23.910	7.4
27.313	15.6	24.873	3.7
27.677	22.7	25.123	4.9
28.219	12.8	25.649	4.2
28.661	6.2	26.748	5.2
29.450	6.8	27.339	3.7
29.735	9.4	27.646	3.5
31.372	8.2	28.066	3.1
31.967	10.9	29.050	3.0
32.295	6.4	29.541	3.6
33.001	11.4	30.178	3.8
33.774	11.8	31.648	3.1
34.385	6.6	32.721	3.5
34.734	6.5	33.154	3.0
35.600	7.3	33.923	3.5
35.965	13.1	34.341	3.1
36.409	14.7	35.465	3.5
36.880	7.0	36.923	3.1
37.509	5.9	37.760	3.4
37.870	6.0	38.404	3.3
38.313	5.8	40.416	3.1
38.943	8.4		
40.093	6.6		
40.511	7.8		
41.429	6.5		

A sample of S_P-**4** was ground with a pestle and mortar, and then successively passed through 500 and 250 μm sieves to yield the sample as a fine powder. This sample was reanalyzed by high resolution XRPD, confirming no form change had occurred.

Example 21. Crystallization Studies for S_P-4.

Crystalline S_P-**4** exhibits polymorphism. Thus, an aspect is directed to crystalline S_P-**4** and its individual polymorphic forms. S_P-**4** can exist in at least five polymorphic forms, designated as Forms 1-5. Furthermore, amorphous S_P-**4** can also be prepared. A typical crystallization provides for dissolving about 100 mg of

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S_P-**4** in an appropriate volume of crystallization solvent (acetonitrile (5 vol), chloroform (5 vol), n-butyl acetate (7 vol), dichloromethane (50 vol), anisole (7 vol), and 1:1 MTBE/heptane (50 vol)) and then allowing for evaporation of the solution at 5 °C. Various crystalline forms were obtained, but each form, upon filtration and/or drying, afforded Form 1.

Forms 1, 2 and 3 are a non-solvated form, 1:1 DCM solvate and 1:1 chloroform solvate, respectively, as was confirmed by singe crystal X-ray and XRPD analysis. Forms 4 and 5 were obtained from crystallization of S_P-**4** from solutions of acetonitrile and anisole, respectively. Sufficient data could not be collected to determine whether Forms 4 and 5 are unsolvated, hydrated or solvated since single crystals of sufficient quality were not obtained. Forms 4 and 5 transform to Form 1 on filtration. Two additional crystalline forms are obtained upon crystallization of S_P-**4** from n-butyl acetate (^{n}BuAc) and a solution containing methyl-^{t}butyl ether (MTBE) and heptane; upon filtration both of these crystalline forms convert to Form 1. Forms 2 and 3 also transform to Form 1 on isolation. Form 1 is a non-solvated form that exhibits a broad melting endotherm with an onset temperature of 94.3°C and ΔH_{fus} of 24.0 kJ mol^{-1}. An additional XRPD pattern of S_P-**4** Form 1 is depicted in **Figure 4**.

Conversion of S_P-4, Form 1 to S_P-4, Form 6

Form 1 can be converted to Form 2 in at least two ways.

First, by allowing fine crystals of Form 1 to be exposed to atmospheric humidity for several days, a monohydrate of Form 1 is produced with an appearance of a solidified gum. After grinding the monohydrate solid to a fine powder, the XRPD pattern remains consistent with Form 1. Upon standing in an open vessel for 6-10 weeks, the ground material slowly changes to Form 6 as an anhydrous solid. Form 1 is stable for at least 2 years in a sealed container.

Alternatively, Form 1 can be suspended in water 5-50 mg/mL at ambient temperature and over a few hours be transformed into Form 6. The water transformation process efficiency can be improved by heating the water to a point to dissolve more Form 1 and to increase the fluidity of the immiscible portion of S_P-**4** from a stiff gum to a suspended oil at about or above. Over time, Form 6 can begin to crystallize out at 50°C. Further cooling the suspension to 0-10°C leads to a higher

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recovery of solid. Crystallization from water also removes more polar trace impurities, leading to improved overall purity.

Redissolving Form 6 into as organic solvent such as dichloromethane or acetonitrile followed by crystallization provides Form 1, even when seeding with crystalline Form 6.

To a dry 100 mL one-neck round bottom flask equipped with an rubber septum and magnetic stir bar was charged 1.04 grams of S_P-**4**, Form 1. HPLC purity 99.7%. Charged 40 mL of DI water. Began stirring suspension vigorously while heating to 50°C. Once the temperature reached 50°C, the mostly homogenous solution was held for 60 mins, during which time solids began to precipitate from solution, forming a thin slurry. The slurry was cooled to 20°C over 90 mins and held for 16 hours at 20°C, followed by cooling further to 0-5°C over 30 mins and held at 0-5°C for 2.5 hours. The slurry was filtered on a medium-porosity glass fritted funnel and washed with 10 mL of ice-chilled water. The wet cake was suction dried for 2 hours before drying in a vacuum oven overnight for 23 hours at 50°C. Isolated 0.88 g (84.6% recovery) of S_P-**4**, Form 6.

Form 6 has an observed melting point of about 124.5-126°C.

Example 21-1. S_P-4 Form 1

A peak listing of S_P-**4** Form 1 is presented in **Table 2.**

Angle 2-Theta °	Intensity % %
5.0	74.8
7.3	100.0
7.8	2.2
8.2	6.8
8.8	9.3
9.4	23.5
10.0	8.4
11.4	4.2
13.3	3.0
14.2	6.1
14.9	3.5
16.1	7.9
16.6	13.2

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Angle 2-Theta °	Intensity % %
17.3	15.4
17.7	10.1
18.1	42.6
18.4	7.6
18.7	11.4
18.9	5.7
19.3	5.0
19.6	2.9
20.2	8.5
20.5	11.5
20.8	3.6
21.8	7.2
22.0	14.5
22.4	9.6
23.2	5.3
23.4	5.8
23.5	4.6
23.8	7.4
24.0	3.1
24.7	2.5
25.0	13.0
25.5	3.1
26.6	4.5
27.2	3.2
27.5	2.2
28.1	2.9
30.0	3.2

Example 21-2. *S*P-4 Form 2

An XRPD pattern of *S*P-**4** Form 2 is depicted in **Figure 5**.

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A peak listing of *S*P-**4** Form 2 is presented in **Table 3**.

Angle 2-Theta °	Intensity % %
4.9	44.1
5.1	19.1

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Angle 2-Theta °	Intensity % %
6.9	62.1
8.7	6.8
9.8	28.6
10.1	10.4
13.7	7.0
16.7	3.1
19.5	8.9
19.8	45.5
20.1	18.6
20.4	3.6
20.6	25.6
20.9	15.9
21.1	10.9
22.1	3.4
24.6	38.7
24.7	100.0
25.1	61.2
26.1	53.3
39.0	6.3

Example 21-3. *S*P-4 Form 3

An XRPD pattern of *S*P-**4** Form 3 is depicted in **Figure 6**.

A peak listing of *S*P-**4** Form 3 is presented in **Table 4**.

Angle 2-Theta °	Intensity % %
5.0	10.0
6.9	23.3
9.8	22.6
19.7	34.8
20.6	100.0
21.8	10.5
24.6	65.3
34.7	4.1

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Example 21-4. S_P-5 Form 4

An XRPD pattern of S_P-**4** Form 4 is depicted in **Figure 7**.

A peak listing of S_P-**4** Form 4 is presented in **Table 5**.

Angle 2-Theta °	Intensity % %
5.0	29.8
6.8	100.0
8.2	4.8
8.7	5.2
9.9	3.8
13.7	1.7
14.9	4.8
19.9	22.5
20.4	2.1
20.6	20.0
20.9	20.0
24.7	3.4
24.9	29.9
25.1	1.5
36.8	3.1

Example 21-5. S_P-4 Form 5

An XRPD pattern of S_P-**4** Form 5 is depicted in **Figure 8**.

A peak listing of S_P-**4** Form 5 is presented in **Table 6**.

Angle 2-Theta °	Intensity % %
5.2	52.9
6.6	100.0
7.1	25.9
9.7	12.1
10.4	16.4
13.4	11.4

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Angle 2-Theta °	Intensity % %
15.7	25.8
19.1	31.1
19.9	12.9
20.0	9.0
21.3	3.5
25.0	22.3
25.6	2.3
26.3	5.9
26.9	2.0
31.7	2.1

Example 21-5. S_P-4 Form 6

An XRPD pattern of S_P-4 Form 6 is depicted in Figure 21.

A peak listing of S_P-4 Form 6 is presented in following table.

Angle 2-Theta °	d-spacing Å	Intensity %
6.08	14.51	66.7
8.2	10.77	62.1
10.38	8.52	29.8
10.85	8.14	10.4
12.17	7.26	12.0
12.7	6.96	66.4
13.73	6.44	14.9
14.1	6.27	13.8
15.91	5.57	3.1
16.83	5.26	8.7
17.17	5.16	19.7
17.66	5.01	56.2
17.95	4.93	37.7
18.79	4.72	59.0
19.1	4.64	14.3
19.41	4.57	37.2
19.8	4.48	46.0
20.11	4.41	68.8
20.82	4.26	100.0
21.81	4.07	36.8
22.03	4.03	7.4
23.03	3.86	14.2

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Angle 2-Theta °	d-spacing Å	Intensity %
23.26	3.82	21.6
23.64	3.76	6.3
23.89	3.72	7.0
24.73	3.6	3.3

Example 21-7. S_P-4 (Amorphous)

An XRPD pattern for amorphous S_P-**4** is depicted in **Figure 9.**

Example 22. Single Crystal X-ray Crystallography of S_P-4 and its solvates

Example 22-1. Single Crystal X-Ray Crystallography of S_P-4 (Form 1)

Figure 10 shows an X-ray crystal structure for S_P-**4** Form 1. There, the figure shows a view of molecules of Form 1 from the crystal structure showing the numbering scheme employed. Anisotropic atomic displacement ellipsoids for the non-hydrogen atoms are shown at the 50% probability level. Hydrogen atoms are displayed with an arbitrarily small radius.

The structure solution was obtained by direct methods, full-matrix least-squares refinement on F^2 with weighting $w^{-1} = \sigma^2(F_o^2) + (0.0592P)^2 + (0.6950P)$, where $P = (F_o^2+2F_c^2)/3$, anisotropic displacement parameters, empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm. Final $wR^2 = \{\Sigma[w(F_o^2-F_c^2)^2]/\Sigma[w(F_o^2)^2]^{1/2}\} = 0.0871$ for all data, conventional $R_1 = 0.0329$ on F values of 7090 reflections with $F_o > 4\sigma(F_o)$, S = 1.016 for all data and 870 parameters. Final Δ/σ(max) 0.001, Δ/σ(mean), 0.000. Final difference map between +0.534 and -0.36 e Å^{-3}.

Table 7. Single Crystal Parameters of Form 1

Molecular formula	$C_{22}H_{29}F_1N_3O_9P_1$				
Molecular weight	529.45				
Crystal system	Monoclinic				
Space group	$P2_1$	a	20.0898(5)Å,	α	90°,
		b	6.10290(10)Å,	β	112.290(3)°,
		c	23.0138(6)Å,	γ	90°
V	2610.79(10)Å^3				

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Z	4
D_c	1.347g.cm^{-1}
μ	1.475mm^{-1}
Source, λ	Cu Kα, 1.54178Å
F(000)	1112
T	100(1)K
Crystal	Colorless plate, 0.12 x 0.09 x 0.03mm
Data truncated to	0.80 Å
θ_{max}	74.48°
Completeness	99.4%
Reflections	14854
Unique reflections	7513
R_{int}	0.0217

Example 22-2. Single Crystal X-Ray Crystallography of *S*P-4 (Form 2)

Figure 11 shows an X-ray crystal structure for *S*P-4 Form 2. There, this figure shows a view of molecules of Form 2 from the crystal structure showing the numbering scheme employed. The heteroatoms were resolved isotropically due to very weak data. Hydrogen atoms are not displayed.

The structure solution was obtained by direct methods, full-matrix least-squares refinement on F^2 with weighting $w^{-1} = \sigma^2(F_o^2) + (0.0975P)^2 + (10.6969P)$, where $P = (F_o^2+2F_c^2)/3$, anisotropic displacement parameters, empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm. Final $wR^2 = \{\Sigma[w(F_o^2-F_c^2)^2]/\Sigma[w(F_o^2)^2]^{1/2}\} = 0.1883$ for all data, conventional $R_1 = 0.0741$ on F values of 2525 reflections with $F_o > 4\sigma(F_o)$, S = 1.05 for all data and 158 parameters. Final Δ/σ(max) 0.000, Δ/σ(mean), 0.000. Final difference map between +1.388 and -0.967 e Å^{-3}.

Table 8. Single Crystal Parameters of Form 2

Molecular formula	$C_{23}H_{31}Cl_2FN_3O_9P$				
Molecular weight	614.38				
Crystal system	Monoclinic				
Space group	*P2$_1$*	*a*	12.8315(3)Å,	α	90°,
		b	6.14530(10)Å,	β	91.752(2)°,
		c	17.6250(4)Å,	γ	90°
V	1389.14(5)Å^3				
Z	2				

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D_c	1.469g.cm^{-1}
μ	3.196mm^{-1}
Source, λ	Cu-K, 1.54178Å
F(000)	640
T	293(2)K
Data truncated to	0.80 Å
θ_{max}	62.23°
Completeness	91.1%
Reflections	3528
Unique reflections	2562
R_{int}	0.0227

Example 22-3. Single Crystal X-Ray Crystallography of S_P-4 (Form 2)

Figure 12 depicts an X-ray Crystal Structure (ORTEP – anisotropic) S_P-**4** (Form 2). A crystal structure of the methylene chloride solvate of S_P-**4** (Form 2), $C_{23}H_{31}N_3PO_9FCl_2$, yields a monoclinic space group $P2_1$ (systematic absences 0k0: k=odd) with a=12.8822(14) Å, b=6.1690(7) Å, c=17.733(2) Å, β=92.045(3)°, V=1408.4(3)Å^3, Z=2 and d_{calc}=1.449 g/cm^3. X-ray intensity data were collected on a Rigaku Mercury CCD area detector employing graphite-monochromated Mo-K_α radiation (λ=0.71073 Å) at a temperature of 143K. Preliminary indexing was performed from a series of twelve 0.5° rotation images with exposures of 30 seconds. A total of 648 rotation images were collected with a crystal to detector distance of 35 mm, a 2θswing angle of -12°, rotation widths of 0.5° and exposures of 30 seconds: scan no. 1 was a ϕ-scan from 315° to 525° at ω= 10° and χ = 20°; scan no. 2 was an ω-scan from -20° to 5° at χ = -90° and ϕ = 315°; scan no. 3 was an ω-scan from -20° to 4° at χ = -90° and ϕ = 135°; scan no. 4 was an ω-scan from -20° to 5° at χ= -90° and ϕ = 225°; scan no. 5 was an ω-scan from -20° to 20° at χ = -90° and ϕ = 45°. Rotation images were processed using CrystalClear (CrystalClear: Rigaku Corporation, 1999), producing a listing of unaveraged F^2 and σ(F^2) values which were then passed to the CrystalStructure (CrystalStructure: Crystal Structure Analysis Package, Rigaku Corp. Rigaku/MSC (2002)) program package for further processing and structure solution on a Dell Pentium III computer. A total of 7707 reflections were measured over the ranges $5.48 \leq 2\theta \leq 50.04$ °, $-14 \leq h \leq 15$, $-7 \leq k \leq 6$, $-19 \leq l \leq 21$ yielding 4253 unique reflections (R_{int} = 0.0180). The intensity data

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were corrected for Lorentz and polarization effects and for absorption using REQAB (minimum and maximum transmission 0.824, 1.000).

The structure was solved by direct methods (SIR97, SIR97: Altomare, A., M. Burla, M. Camalli, G. Cascarano, C. Giacovazzo, A. Guagliardi, A. Moliterni, G. Polidori & R. Spagna (1999). *J. Appl. Cryst.*, **32**, 115-119). Refinement was by full-matrix least squares based on F^2 using SHELXL-97 (SHELXL-97: Sheldrick, G.M. (2008) *Acta Cryst.*, **A64**, 112-122). All reflections were used during refinement. The weighting scheme used was $w=1/[\sigma^2(F_o^2)+ 0.0472P^2 + 0.4960P]$ where $P = (F_o^2 + 2F_c^2)/3$. Non-hydrogen atoms were refined anisotropically and hydrogen atoms were refined using a "riding" model. Refinement converged to R_1=0.0328 and wR_2=0.0817 for 4046 reflections for which $F > 4\sigma(F)$ and R_1=0.0348, wR_2=0.0838 and GOF = 1.056 for all 4253 unique, non-zero reflections and 358 variables ($R_1 = \sum ||F_o| - |F_c|| / \sum |F_o|$; $wR_2 = \{ \sum w (F_o^2 - F_c^2)^2 / \sum w(F_o^2)^2\}^{1/2}$; $GOF = \{ \sum w (F_o^2 - F_c^2)^2 / (n - p)\}^{1/2}$; where n = the number of reflections and p = the number of parameters refined). The maximum Δ/σ in the final cycle of least squares was 0.000 and the two most prominent peaks in the final difference Fourier were +0.312 and -0.389 e/Å^3. The Flack absolute structure parameter refined to -0.06(6) thus corroborating the stereochemistry of the title compound.

Table 1 lists cell information, data collection parameters, and refinement data. Final positional and equivalent isotropic thermal parameters are given in Table 2. Anisotropic thermal parameters are in Table 3. ("ORTEP-II: A Fortran Thermal Ellipsoid Plot Program for Crystal Structure Illustrations". C.K. Johnson (1976) ORNL-5138.) representation of the molecule with 30% probability thermal ellipsoids displayed.

Table 9. Summary of Structure Determination of Compound S_P-4·CH_2Cl_2.

Formula:	$C_{23}H_{31}N_3PO_9FCl_2$
Formula weight:	614.38
Crystal class:	monoclinic
Space group:	$P2_1$ (#4)
Z	2
Cell constants:	
a	12.8822(14)Å

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b	6.1690(7) Å
c	17.733(2) Å
β	92.045(3)°
V	1408.4(3) Å ^{3}
μ	3.48 cm^{-1}
crystal size, mm	0.42 x 0.12 x 0.10
D_{calc}	1.449 g/cm^3
F(000)	640
Radiation:	Mo-K_α(λ=0.71073Å)
2θ range	5.48 – 50.04 °
hkl collected:	$-14 \leq h \leq 15$; $-7 \leq k \leq 6$; $-19 \leq l \leq 21$
No. reflections measured:	7707
No. unique reflections:	4253 (R_{int}=0.0180)
No. observed reflections	4046 (F>4σ)
No. reflections used in refinement	4253
No. parameters	358
R indices (F>4σ)	R_1=0.0328
	wR_2=0.0817
R indices (all data	R_1=0.0348
	wR_2=0.0838
GOF:	1.056
Final Difference Peaks, e/Å^3	+0.312, -0.389

Example 22-4. Single Crystal X-Ray Crystallography of *S*P-4 (Form 3)

Figure 13 shows an X-ray crystal structure for *S*P-**4** Form 3. There, this figure shows a view of molecules of Form 3 from the crystal structure showing the numbering scheme employed. Anisotropic atomic displacement ellipsoids for the non-hydrogen atoms are shown at the 50% probability level. Hydrogen atoms are displayed with an arbitrarily small radius.

The structure solution was obtained by direct methods, full-matrix least-squares refinement on F^2 with weighting $w^{-1} = \sigma^2(F_o^2) + (0.0512P)^2 + (0.6810P)$, where $P = (F_o^2+2F_c^2)/3$, anisotropic displacement parameters, empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm. Final $wR^2 = \{\Sigma[w(F_o^2-F_c^2)^2]/\Sigma[w(F_o^2)^2]^{1/2}\} = 0.0796$ for all data, conventional $R_1 = 0.0294$ on F values of 2486 reflections with $F_o > 4\sigma(F_o)$, S = 1.068 for all data and 377 parameters. Final Δ/σ(max) 0.001, Δ/σ(mean), 0.000. Final difference map between +0.211 and -0.334 e Å^{-3}.

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Table 10. Single Crystal Parameters of Form 3

Molecular formula	$C_{23}H_{30}Cl_3F_1N_3O_9P_1$				
Molecular weight	648.82				
Crystal system	Monoclinic				
Space group	*P21*	*a*	12.9257(4)Å,	α	90°,
		b	6.18080(10)Å,	β	96.399(2)°,
		c	18.0134(4)Å,	γ	90°
V	1430.15(6)Å^3				
Z	2				
D_c	1.507g.cm^{-1}				
μ	3.977mm^{-1}				
Source, λ	Cu Kα, 1.54178Å				
F(000)	672				
T	100(1)K				
Crystal	Colorless needle, 0.22 x 0.03 x 0.02mm				
Data truncated to	0.80 Å				
θ_{max}	74.41°				
Completeness	69.1%				
Reflections	3062				
Unique reflections	2607				
R_{int}	0.0198				

Example 23. Stability at elevated temperatures and relative humidity

A sample of R_P-**4** was stored in a humidity chamber at 40°C and 75% relative humidity for one week, and the sample was reanalyzed by XRPD. The powder pattern obtained for R_P-**4** showed no substantial change during the course of the experiment, meaning that no change in solid form was observed. This should be contrasted to a sample of **4**, which deliquesced within about 16 hours upon storage at 40°C and 75 % relative humidity. Indeed, an illustration of the deliquescent nature of **4** is illustrated by the following. A sample of **4** was passed through a 250 μm sieve then samples were stored at 40°C / 75 % RH and 25°C / 53 % relative humidity and visual observations were taken at regular intervals. The results are given in **Table 4**.

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Table 11. Stability of **4** to elevated relative humidity.

Conditions	t = 1.5 h	t = 4.5 h	t = 6.5 h	t = 8.5 h	t = 73 h
40°C / 75%RH	Deliquescence	-	-	-	-
25°C / 53%RH	No deliquescence	Sticky solid	Partial deliquescence	Almost complete deliquescence	Deliquescence

Upon storage at 40 °C and 75 % relative humidity a sample of S_P-**4** deliquesced inside 16 hours. For instance, a sample of S_P-**4** was ground with a pestle and mortar, and then successively passed through 500 and 250 μm sieves to yield the sample as a fine powder. Samples of this material were stored at 40°C and 75% relative humidity and 25°C and 53 % RH and visual observations were taken at regular intervals. The results are given in **Table 5.**

Table 12. Stability of S_P-**4** to elevated relative humidity.

Conditions	t = 1.5 h	t = 4.5 h	t = 104 h
40 °C / 75 % RH	No deliquescence	Deliquescence	-
25 °C / 53 % RH	No deliquescence	No deliquescence	No deliquescence

XRPD analysis of the sample after storage at 25°C and 53% RH for 104 hours showed no significant changes in the diffractograms produced indicating that no form change had occurred.

Example 24. Fourier Transform – Infrared (FT-IR) Spectrometry

Data were collected on a Perkin-Elmer Spectrum One fitted with a universal Attenuated Total Reflectance (ATR) sampling accessory. The data were collected and analyzed using Spectrum v5.0.1 software.

The IR spectrum obtained for **4**, R_P-**4**, and S_P-**4** are shown in Figs. 5–7, respectively. Selected peaks, in wavenumbers (cm^{-1}) are recited below:

4: ~1680, ~1454, ~1376, ~1205, ~1092, ~1023 (Fig. 14);

R_P-**4**: ~1742, ~1713, ~1679, ~1460, ~1377, ~1259, ~1157, ~1079 (Fig. 15); and

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*S*P-**4 (Form 1)**: ~1743, ~1713, ~1688, ~1454, ~1378, ~1208, ~1082 (Fig. 16).

Example 25. Differential Scanning Calorimetry (DSC) Thermo-Gravimetric Analysis (TGA)

DSC data were collected on a TA Instruments Q2000 equipped with a 50 position auto-sampler. The calibration for thermal capacity was carried out using sapphire and the calibration for energy and temperature was carried out using certified indium.

Modulated temperature DSC was carried out on typically 0.8-1.2 mg of each sample, in a pin-holed aluminum pan, using an underlying heating rate of 2°C.min^{-1} and temperature modulation parameters of ± 0.2 °C.min^{-1} and 40 seconds. A purge of dry nitrogen at 50 ml.min^{-1} was maintained over the sample.

The instrument control software was Advantage for Q Series v2.8.0.392 and Thermal Advantage v4.8.3 and the data were analyzed using Universal Analysis v4.3A.

DSC data were collected on a Mettler DSC 823e equipped with a 34 position auto-sampler. The instrument was calibrated for energy and temperature using certified indium. Typically 0.8-1.2 mg of each sample, in a pin-holed aluminum pan, was heated at 10°C.min^{-1} from 25°C to 250°C. A nitrogen purge at 50 ml.min^{-1} was maintained over the sample. The instrument control and data analysis software was STARe v9.20.

DSC data for *S*P-**4** (Form 6) were collected using a DSC instrument (TA Q2000), using a heating rate of 10 °C/ min under a continuous flow of dry nitrogen gas (100 ml/min). Approximately 2.2 mg of sample was accurately weighed and heated in a non-hermetically sealed, 'Tzero' pan with a loose-fitting lid. The instrument was calibrated (enthalpy and temperature) with an indium standard and (heat capacity) with a sapphire standard. Uncertainties are estimated to be ±0.1 °C for temperatures and ±5 % for measured enthalpies. TA Universal Analysis software was employed to measure onset temperatures.

TGA data were collected on a Mettler TGA/SDTA 851e equipped with a 34 position auto-sampler. The instrument was temperature calibrated using certified indium. Typically 8-12 mg of each sample was loaded onto a pre-weighed aluminum

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crucible and was heated at 10°C.min^{-1} from ambient temperature to 350°C. A nitrogen purge at 50 ml.min^{-1} was maintained over the sample. The instrument control and data analysis software was STARe v9.20.

DSC analysis of **4** showed a single broad endotherm with an onset of 58.7°C (ΔH 14 J.g^{-1}) confirmed to be due to molecular relaxation during the glass transition by further modulated DSC analysis (Fig. 17). TGA analysis of **4** showed no weight loss before decomposition above 240°C, confirming the material to be non-solvated. As the XRPD analysis of **4** confirmed the material to be amorphous, modulated DSC analysis was undertaken in an attempt to calculate the glass transition temperature, which was found to be 57°C.

DSC analysis showed a single sharp endotherm with an onset of 136.2 °C (ΔH 76 J.g^{-1}) confirmed to be a melt by hot stage microscopy. See Fig. 18. TGA analysis of R_P-**4** showed no weight loss before decomposition above 240 °C, confirming the material to be non-solvated.

DSC analysis of S_P-**4** showed a single broad endotherm with an onset of 93.9°C (ΔH 43 J.g^{-1}) confirmed to a melt by hot stage microscopy. See Fig. 19. TGA analysis of S_P-**4** showed no weight loss before decomposition above 240°C, confirming the material to be non-solvated.

DSC analysis of S_P-**4** (Form 6) showed a broad endotherm with an onset of 120.7°C (ΔH 79 J.g-1).

Example 26. Gravimetric Vapour Sorption (GVS)

SMS DVS Intrinsic

Sorption isotherms were obtained using a SMS DVS Intrinsic moisture sorption analyzer, controlled by SMS Analysis Suite software. The sample temperature was maintained at 25 °C by the instrument controls. The humidity was controlled by mixing streams of dry and wet nitrogen, with a total flow rate of 200 ml.min^{-1}. The relative humidity was measured by a calibrated Rotronic probe (dynamic range of 1.0-100 %RH), located near the sample. The weight change, (mass relaxation) of the sample as a function of %RH was constantly monitored by the microbalance (accuracy ±0.005 mg).

Typically 5-20 mg of sample was placed in a tared mesh stainless steel basket under ambient conditions. The sample was loaded and unloaded at 40 %RH

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and 25°C (typical room conditions). A moisture sorption isotherm was performed as outlined below (2 scans giving 1 complete cycle). The standard isotherm was performed at 25°C at 10 %RH intervals over a 0.5-90 %RH range.

Table 13. Method Parameters for SMS DVS Intrinsic Experiments

Parameters	Values
Adsorption - Scan 1	40 - 90
Desorption / Adsorption - Scan 2	90 - 0, 0 - 40
Intervals (%RH)	10
Number of Scans	2
Flow rate ($ml.min^{-1}$)	200
Temperature (°C)	25
Stability ($°C.min^{-1}$)	0.2
Sorption Time (hours)	6 hour time out

The sample was recovered after completion of the isotherm and re-analyzed by XRPD.

GVS analysis showed R_P-**4** to be non-hygroscopic exhibiting reversible uptake of approximately 0.2 wt % of water from 0 to 90 % relative humidity. Re-analysis of the sample by XRPD after the GVS experiment showed no change in form.

A sample of S_P-**4** was ground with a pestle and mortar, and then successively passed through 500 and 250 μm sieves to yield the sample as a fine powder that was then analyzed using a modified single cycle method. The sample was taken from 40 % RH (approximately ambient) to 60 % RH, instead of 90 % for the standard method, and then cycled to 0 % and back to 40 % RH. This analysis showed S_P-**4** to be non-hygroscopic up to 60 % RH, with reversible uptake of ~0.2 % by weight of water from 0 to 60 % RH.

Example 27. Thermodynamic Aqueous Solubility

Aqueous solubility was determined by suspending a sufficient amount of compound in water to give a maximum final concentration of ≥10 $mg.ml^{-1}$ of the parent free-form of the compound. The suspension was equilibrated at 25 °C for 24 hours then the pH was measured. The suspension was then filtered through a glass fiber C filter into a 96 well plate. The filtrate was then diluted by a factor of 101. Quantitation was by HPLC with reference to a standard solution of approximately

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0.1 mg.ml^{-1} in DMSO. Different volumes of the standard, diluted and undiluted sample solutions were injected. The solubility was calculated using the peak areas determined by integration of the peak found at the same retention time as the principal peak in the standard injection.

Table 14. HPLC Method Parameters for Solubility Measurements

Type of method:	Reverse phase with gradient elution		
Column:	Phenomenex Luna, C18 (2) 5µm 50 x 4.6 mm		
Column Temperature (°C):	25		
Standard Injections (µl):	1, 2, 3, 5, 7, 10		
Test Injections (µl):	1, 2, 3, 10, 20, 50		
Detection: Wavelength, Bandwidth (nm) :	260,80		
Flow Rate (ml.min^{-1}):	2		
Phase A:	0.1% TFA in water		
Phase B:	0.085% TFA in acetonitrile		
Timetable:	Time (min)	% Phase A	% Phase B
	0.0	95	5
	1.0	80	20
	2.3	5	95
	3.3	5	95
	3.5	95	5
	4.4	95	5

Analysis was performed under the above-noted conditions on an Agilent HP1100 series system equipped with a diode array detector and using ChemStation software vB.02.01-SR1.

Table 15. Aqueous solubility result for R_P-4, 4, and S_P-4.

Sample ID	pH of Unfiltered mixture	Solubility / mg.ml^{-1}	Comments
R_P-4	7.12	1.58	Suspension
4	7.03	6.11	Residual solid
S_P-4	6.88	5.65	Residual solid

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Example 28. Chemical Purity Determination by HPLC

Various HPLC conditions can be used to determine the chemical purity of the compounds disclosed herein. One such example is disclosed above in relation to the thermodynamic aqueous solubility studies. Another example is disclosed below.

HPLC Conditions:

LC:	Waters Alliance 2695 Separations Module, Waters 2996 PDA detector and Waters Empower 2 Software (Version 6.00)	
Column:	Phenomenex Luna C18(2); 4.6 x 50mm; 3μm	
Flow rate:	1.2 mL/min	
Injection Volume:	10μL	
Mobile phase:	Solvent A: 95% Water with 5% Methanol and 10mM Ammonium Acetate; pH~5.3	
	Solvent B: MeOH with 10mM Ammonium Acetate	
Gradient:	hold at 0%B	3 min
	0-47%B	3-4 min
	hold at 47 %B	4-10 min
	47%-74%B	10-11min
	hold at 74%B	11-13.5min
	return to 0%B	13.5-13.6 min
	hold at 0%B	13.6-15.5 min

Under these conditions, the purity of **4**, R_P-**4**, and S_P-**4** was determined to be ~99.6, ~99%, and ~99.5%, respectively. It is noted that higher purities can be realized by optimizing the methods disclosed above.

Inspection of the XRPD diffractograms shows that the two crystalline single diastereoisomers gave clearly different XRPD patterns. Additionally, there was a clear difference in the melting point of the two crystalline diastereoisomers, with R_P-**4** having a considerably higher onset than S_P-**4** (136°C vs. 94°C).

Example 29. Additional Separation Methods

The following SFC separation (conditions listed below) yielded adequate separation of a mixture of the diastereomers, R_P-**4** and S_P-**4**.

Preparative Method:	Analytical Method:
Chiralpak AS-H (2 x 25 cm) SN# 07-8656	Chiralpak AS-H (25 x 0.46cm)
20% methanol/CO_2 (100 bar)	20% methanol/CO_2 (100 bar)
50 ml/min, 220 nm.	3 ml/min, 220 nm.
Conc.: 260mg/30ml methanol, inj vol.: 1.5ml	

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The following SFC separation (conditions listed below) yielded adequate separation of a mixture of the diastereomers, R_P-**4** and S_P-**4**.

Preparative Method:	Analytical Method:
Chiralpak IA(2 x 15 cm) 802091	Chiralpak IA(15 x 0.46 cm)
30% isopropanol(0.1% DEA)/CO_2, 100 bar	40% methanol(DEA)/CO_2, 100 bar
60 mL/min, 220 nm.	3 mL/min, 220 nm.
inj vol.: 2 mL, 20 mg/mL methanol	

Table 16. Summary of results from the batch characterization of R_P-4, 4, and S_P-4.

Analysis	R_P-4	4	S_P-4
Proton NMR	Single diastereoisomer	1:1 Mixture of diastereoisomers	Single diastereoisomer
XRPD	Crystalline – different from S_P-4	Amorphous	Crystalline – different from R_P-4
DSC	Endotherm; melt - 136°C	Endotherm; 59°C	Endotherm; melt - 94°C
TGA	No wt loss, decomposition >240°C	No wt loss, decomposition >240°C	No wt loss, decomposition >240°C
IR	See above	See above	See above
Aq Solubility ($mg.ml^{-1}$)	1.58	6.11	5.65
HPLC Purity	96.9 %	99.6 %	99.5 %
40°C / 75 % RH	No form change	Deliquescence inside 1.5 h	Deliquescence inside 4.5 h
25°C / 53 % RH	-	Deliquescence	No form change
GVS	Non-hygroscopic up to 90 % RH	-	Non-hygroscopic up to 60 % RH

Example 30. X-Ray Crystallography of 8 (S_P-isomer)

Compound **8** (S_P-isomer), $C_{18}H_{21}N_2PO_7$, crystallizes in the monoclinic space group $P2_1$ (systematic absences 0k0: k=odd) with a=5.3312(4)Å, b=15.3388(8)Å, c=23.7807(13)Å, β=92.891(3)°, V=1942.2(2)Å^3, Z=4, and d_{calc}=1.397 g/cm^3. X-ray intensity data were collected on a Bruker APEXII CCD area detector employing graphite-monochromated Mo-Kα radiation (λ=0.71073 Å) at a temperature of 100(1)K. Figures 20A and 20B show molecules numbered 1 and 2, respectively, of the asymmetric unit.

Preliminary indexing was performed from a series of thirty-six 0.5° rotation frames with exposures of 30 seconds. A total of 3608 frames were collected with a crystal to detector distance of 70.00 mm, rotation widths of 0.5° and exposures of 20 seconds:

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scan type	2θ	ω	φ	χ	frames
φ	-35.50	279.40	27.32	48.96	725
φ	24.50	22.31	35.56	69.08	692
ω	-13.00	321.68	247.79	69.08	95
φ	34.50	204.08	28.21	-92.80	293
φ	-30.50	310.60	214.10	54.21	361
φ	32.00	304.67	24.47	50.72	722
φ	-35.50	122.14	316.59	-78.84	720

Rotation frames were integrated using SAINT (Bruker (2009) SAINT. Bruker AXS Inc., Madison, Wisconsin, USA.) producing a listing of unaveraged F^2 and $\sigma(F^2)$ values which were then passed to the SHELXTL (Bruker (2009) SHELXTL. Bruker AXS Inc., Madison, Wisconsin, USA.) program package for further processing and structure solution on a Dell Pentium 4 computer. A total of 6909 reflections were measured over the ranges $1.58 \leq \theta \leq 25.09°$, $-6 \leq h \leq 6$, $-18 \leq k \leq 18$, $-28 \leq l \leq 28$ yielding 6909 unique reflections (Rint = 0.0581). The intensity data were corrected for Lorentz and polarization effects and for absorption using SADABS (Sheldrick, G.M. (2007) SADABS. University of Gottingen, Germany.) (minimum and maximum transmission 0.6093, 0.7452).

The structure was solved by direct methods (SHELXS-97 (Sheldrick, G.M. (2008) Acta Cryst. A64,112-122.)). Refinement was by full-matrix least squares based on F^2 using SHELXL-97 (Sheldrick, G.M. (2008) Acta Cryst. A64, 112-122.). All reflections were used during refinement. The weighting scheme used was $w=1/[\sigma^2(F_o^2)+ (0.0000P)^2 + 14.0738P]$ where $P = (F_o^2 + 2F_c^2)/3$. Non-hydrogen atoms were refined anisotropically and hydrogen atoms were refined using a riding model. Refinement converged to R1 = 0.0847 and wR2 = 0.1899 for 6173 observed reflections for which $F > 4\sigma(F)$ and R1=0.0963 and wR2=0.1963 and GOF =1.119 for all 6909 unique, non-zero reflections and 512 variables ($R1 = \Sigma||F_o| - |F_c|| / \Sigma |F_o|$; $wR2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma w(F_o^2)^2]^{1/2}$; $GOF = [\Sigma w(F_o^2 - F_c^2)^2/(n - p)]^{1/2}$; where n = the number of reflections and p = the number of parameters refined). The maximum Δ/σ in the final cycle of least squares was 0.000 and the two most prominent peaks in the final difference Fourier were +0.402 and -0.559 e/Å^3. **Figs 20A** and **20B** are ORTEP (30% probability thermal ellipsoids) of molecules 1 and 2 of the asymmetric unit.

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Table 17. Summary of Structure Determination of Compound 8 (S_P-isomer)

Empirical formula	$C_{18}H_{21}N_2PO_7$
Formula weight	408.34
Temperature	100(1) K
Wavelength	0.71073 Å
Crystal system	monoclinic
Space group	$P2_1$
Cell constants:	
a	5.3312(4) Å
b	15.3388(8) Å
c	23.7807(13) Å
β	92.891(3)°
Volume	1942.2(2) $Å^3$
Z	4
Density (calculated)	1.397 Mg/m^3
Absorption coefficient	0.185 mm^{-1}
F(000)	856
Crystal size	0.40 x 0.10 x 0.08 mm^3
Theta range for data collection	1.58 to 25.09°
Index ranges	$-6 \leq h \leq 6$, $-18 \leq k \leq 18$, $-28 \leq l \leq 28$
Reflections collected	6909
Independent reflections	6909 [R(int) = 0.0581]
Completeness to theta = 25.09°	99.6 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7452 and 0.6093
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	6909 / 1 / 512
Goodness-of-fit on F^2	1.119
Final R indices [I>2sigma(I)]	R1 = 0.0847, wR2 = 0.1899
R indices (all data)	R1 = 0.0963, wR2 = 0.1963
Absolute structure parameter	0.1(2)
Largest diff. peak and hole	0.402 and -0.559 $e.Å^{-3}$

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