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ARTICLES

Cyanine Dye Labeling Reagents: Sulfoindocyanine Succinimidyl Esters

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A series of new fluorescent labeling reagents based on sulfoindocyanine dyes has been developed. We describe the synthesis and properties of these reagents. They contain succinimidyl ester reactive groups and can be readily conjugated to antibodies, avidin, DNA, lipids, polymers, and other amino-group-containing materials. The labeling reagents are water soluble, pH insensitive, and show much reduced dye aggregation under labeling conditions. One of the reagents, Cy3, can be excited with the 488-, 514- and 532-nm laser lines and is optimally excited with the 546-nm mercury arc line. Another, Cy5, can be excited with the 633-nm HeNe and 647-nm Kr laser lines available with many flow cytometers and confocal laser-scanning microscopes. New laser diodes emitting near 650 nm should also be excellent excitation sources for Cy5.

INTRODUCTION

The use of fluorescent labels with antibodies, DNA probes, biochemical analogs, lipids, drugs, cytokines, cells, and polymers has expanded rapidly in recent years. The wider use of fluorescent probes results partly from the evolution of advanced detection instrumentation, particularly electronic imaging microscopes (1) and flow cytometers (2), and partly from the availability of new fluorescent labeling reagents (3, 4). Nevertheless, further progress depends on the availability of fluorescent tags with increased fluorescence brightness, improved photostability, reduced phototoxicity, lower nonspecific binding, and excitation and emission wavelengths better matched

to instrumentation light sources and detectors.

Earlier papers (5–7) from this laboratory introduced a new class of biological labeling reagents based on cyanine dyes with iodoacetamide, isothiocyanate, and succinimidyl ester as reactive groups. These reagents have large extinction coefficients (130 000–250 000 L/mol cm) and fluorescence covering a wide spectral range (500–750 nm). However, their quantum yields and water solubilities are not optimal. In this paper we report the syntheses of amino-reactive cyanine dyes that contain a negatively charged sulfonate group on the aromatic nucleus of the indocyanine fluorophore. These sulfoindocyanine dyes are highly water soluble and can be used to prepare brightly fluorescent materials, which show low nonspecific binding to cellular constituent (8). The different sulfoindocyanines fluoresce in the visible and near-infrared region of the spectrum, well separated from the shorter-wavelength autofluorescence, which is characteristic of many biological specimens.

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EXPERIMENTAL PROCEDURES

Chemicals. 1,3,3-Trimethoxypropene, *p*-hydrazindobenzenesulfonic acid, and iodoethane were purchased from Eastman Kodak Co., Rochester, NY. Disuccinimidyl carbonate (DSC), triethyl orthoformate, 1,4-butanedisulfone, 6-bromohexanoic acid, and *N,N*-diphenylformamidinium were purchased from Aldrich Chemical Co., Milwaukee, WI. Glutaconaldehyde dianil hydrochloride was purchased from Fairmount Chemical Co., Newark, NJ. Malonaldehyde dianil hydrochloride was prepared from malonaldehyde bis(dimethyl acetal) and aniline (9). Sheep γ -globulins (IgG) was purchased from Sigma Chemical Co. Dry solvents, diethyl ether, and ethyl acetate were purchased from Aldrich Chemical Co. All other solvents were purchased from Fisher Scientific, Pittsburgh, PA.

Purification and Spectroscopic Analysis. Purification of dyes was performed on a Spectra-Physics Model SP8700 analytical HPLC unit equipped with a C8-RP column, on a Water associates Model Prep/LC 500 preparative HPLC unit equipped with a C18-RP column. Purification could also be achieved by conventional or flash column chromatography on C18-RP powder (Analtech, Newark, DE). Water-methanol mixtures were used for elution in all experiments. Dyes were recovered from the fractions with a rotary evaporator at 60–70 °C without appreciable loss. Although these purification procedures isolate the fluorophore from various intermediates and precursors, these multi-ionic dyes typically associate with alkali metal cations, halide anions, and water of hydration in nonstoichiometric amounts. Therefore, it is essential to obtain the final products in a form that is chemically well-defined for elemental and mass spectrometric analysis. This was accomplished by passing the dye with unknown counterion composition through a Dowex-50W hydrogen, strongly acidic cation-exchange column that has been previously washed with 0.1 N sulfuric acid and then distilled water. For example, a sample of Cy5.18 contained 1.29% potassium by microanalysis before ion-exchange and 0.00% afterward. The same sample after ion-exchange gave a high-resolution mass spectrum in 3-nitrobenzyl alcohol (CsI-glycerol as a standard) with m/e 743.267 for $C_{37}H_{47}N_2S_2O_{10}$ (calcd, $C_{37}H_{46}N_2S_2O_{10} + H$). Mass spectral determinations were made at the Midwest Center for Mass Spectrometry at the University of Nebraska—Lincoln, Lincoln, NE, with the partial support from NSF Grant #DIR9017262. Elemental analyses (C, H, N, S, and K) were performed by Atlantic Microlab, Inc., Atlanta, GA. Ultraviolet-visible spectra were measured on a Hewlett-Packard HP 8452 diode-array spectrophotometer. The proton NMR spectra were obtained on an IBM 300 FT-NMR spectrometer. NMR signals are described by use of s for singlet, d for doublet, t for triplet, q for quartet, and m for multiplet and are expressed in δ with TMS as internal standard. Fluorescence measurements were performed using a Spex Fluorolog 2 system. Quantum yields were determined as previously described (7). Extinction coefficients were determined from absorbance values of weighed samples of dried material and their estimated molecular weights. Extinction coefficients in Table II were determined from absorbance values of HPLC-purified, ion-exchange-purified (see below), weighed samples of dried material and their molecular weights.

General Synthesis. Seven new cyanine dyes were synthesized using modifications of methods described previously (5–7, 10).

2,3,3-Trimethylindoleninium-5-sulfonate (I). This intermediate was synthesized by conventional Fisher indole synthesis (11, 12). To a 2-L three-necked flask

equipped with mechanical stirrer and reflux condenser were added acetic acid (300 mL), 3-methyl-2-butanone (168 mL, 1.6 mol), and *p*-hydrazinobenzenesulfonic acid (100 g, 0.53 mol). The mixture was heated to reflux for 3 h and then cooled for several hours when a pink solid separated (93 g, mp 290–95 °C, yield 73%). ^1H NMR (D_2O) δ 7.13 (s, 1 H, aromatic 4-H), 7.1 (dd, 1 H, $J = 7.0$, 1.2 Hz, aromatic 6-H), 6.5 (d, 1 H, $J = 7$ Hz, aromatic 7-H), 1.4 (s, 6 H, $C(CH_3)_2$). Singlet for 2-methyl did not appear in D_2O , but appeared when NMR was recorded in DMSO- d_6 . Product was converted to the potassium salt by stirring its solution in methanol with a saturated solution of potassium hydroxide in 2-propanol. The alkaline solution turns yellow and the potassium salt of the sulfoindole precipitated as yellow solid almost quantitatively: ^1H NMR (D_2O) δ 7.13 (s, 1 H, aromatic 4-H), 7.1 (dd, 1 H, $J = 7.0$, 1.2 Hz, aromatic 6-H), 6.5 (d, 1 H, $J = 7$ Hz, aromatic 7-H), 2.2 (s, 3 H, CCH_3), 1.4 (s, 6 H, $C(CH_3)_2$); $R_f = 0.5$ (RP-C18, water).

1-Ethyl-2,3,3-trimethylindoleninium-5-sulfonate (II). A mixture of potassium salt of indoleninium sulfonate I (11 g, 0.04 mol) and a large excess of ethyl iodide (40 mL) was heated to boiling for 24 h. The reaction mixture was cooled, excess ethyl iodide was decanted, and the light purple color solid was triturated with acetone (3 $\times$ 50 mL) to remove potassium iodide. The light purple powder was used in further experiments without additional purification: λ_{max} (water) = 228, 274 nm; ^1H NMR (D_2O) δ 8.13 (s, 1 H, 4-H), 8.03 (dd, 1 H, $J = 9.0$, 1.1 Hz, 6-H), 7.2 (d, 1 H, $J = 9$ Hz, 7-H), 4.51 (q, 2 H, $J = 7.5$ Hz, α -CH₂), 1.59 (t, 3 H, β -CH₃), 1.61 (s, 6 H, $(CH_3)_2$); $R_f = 0.16$ (RP-C18, water).

1-(ϵ -Carboxypentynyl)-2,3,3-trimethylindoleninium-5-sulfonate (III). The potassium salt of 2,3,3-trimethylindoleninium-5-sulfonate (I) (11 g, 0.04 mol) and 6-bromohexanoic acid (9.8 g, 0.05 mol) were mixed in 1,2-dichlorobenzene (100 mL) and heated at 110 °C for 12 h under nitrogen. The mixture was cooled, 1,2-dichlorobenzene was decanted, and the solid was triturated with 2-propanol until free powder was obtained: λ_{max} (water) = 274 nm; ^1H NMR (D_2O) δ 8.13 (s, 1 H, 4-H), 8.03 (dd, 1 H, $J = 9.0$, 1.1 Hz, 6-H), 7.2 (d, 1 H, $J = 9$ Hz, 7-H), 4.51 (t, 2 H, $J = 7.5$ Hz, α -CH₂), 2.25 (t, 2 H, $J = 7.5$ Hz, ϵ -CH₂), 1.99 (m, 2 H, β -CH₂), 1.35–1.66 (m, 4 H, γ - and δ -CH₂), 1.61 (s, 6 H, $(CH_3)_2$); $R_f = 0.27$ (RP-C18, water).

1-(δ -Sulfonatobutyl)-2,3,3-trimethylindoleninium-5-sulfonate (IV). The potassium salt of 2,3,3-trimethylindoleninium-5-sulfonate (I) (11 g, 0.04 mol) and 1,4-butanedisulfone (6.5 g, 0.048 mol) were mixed in 1,2-dichlorobenzene (50 mL) and heated at 110 °C for 12 h. The mixture was cooled, 1,2-dichlorobenzene was decanted, and the solid was triturated with 2-propanol until free powder was obtained: λ_{max} (water) = 274 nm; ^1H NMR (D_2O) δ 8.13 (s, 1 H, 4-H), 8.03 (dd, 1 H, $J = 9$, 1.1 Hz, 6-H), 7.2 (d, 1 H, $J = 9$ Hz, 7-H), 4.51 (t, 2 H, $J = 7.5$ Hz, α -CH₂), 2.25 (t, 2 H, $J = 7.5$ Hz, δ -CH₂), 1.99 (m, 2 H, β -CH₂), 1.35–1.66 (m, 2 H, γ -CH₂), 1.61 (s, 6 H, $(CH_3)_2$); $R_f = 0.58$ (RP-C18, water-methanol 1:1).

CY3.18.OH (IX). Intermediate III (10 g, 0.028 mol) was dissolved in pyridine (50 mL) in a 250-mL round-bottom flask equipped with a reflux condenser. The solution was heated to reflux. Triethyl orthoformate (8.3 mL, 0.05 mol) was added slowly (1 mL at a time, every 15 min). The solution was heated for an additional 2 h. A 4-fold excess of triethyl orthoformate was essential for completion of the reaction. The mixture was cooled and diluted with several volumes of diethyl ether. A product separated in the form of a red powder from which the

supernatant fluid was removed by decantation. It was dissolved in methanol (40 mL) and reprecipitated with addition of 2-propanol. The product was collected on filter paper and dried (17.5 g, yield 80%). Cy3.18.OH made by this method showed two spots on TLC [RP-C18, methanol-water 25:75; R_f = 0.8 (major spot) and 0.21 (minor spot)]. Crude dye (5.0 g) was dissolved in water (30 mL) and chromatographed on a reversed-phase C18 column (70 g). Pure dye obtained as the pyridinium salt (2.5 g, yield 40%) was converted into its potassium salt: $^1\text{H NMR}$ (D_2O) δ 8.45 (t, 1 H, J = 14 Hz, β proton of the bridge), 7.9 (s, 2 H, 4-H, 4'-H), 7.8 (d, 2 H, J = 7.7 Hz, 6-H, 6'-H), 7.4 (d, 2 H, J = 7.7 Hz, 7-H, 7'-H), 6.45 (d, 2 H, J = 14 Hz, α , α' protons of the bridge), 4.05 (t, 4 H, J = 7.5 Hz, α -, α' -CH₂), 2.25 (t, 4 H, J = 7.5 Hz, ϵ -, ϵ' -CH₂), 1.99 (m, 4 H, β -, β' -CH₂), 1.35–1.66 (m, 8 H, γ -, γ' -, δ -, δ' -CH₂), 1.71 (s, 12 H, s (CH₃)₂); R_f = 0.24 (RP C18, methanol-water 1.7:8.3).

Cy3.29.OH (XI). This unsymmetrical indocarbocyanine was synthesized in two steps.

(a) *Intermediate 415 (V).* A mixture of the quaternary salt of indolenine II (4 g, 0.015 mol) and *N,N'*-diphenylformamidinium (3.3 g, 0.017 mol) in acetic acid (20 mL) was heated to reflux. The completion of the reaction was monitored by absorption spectra in methanol, which showed that as the reaction progressed the absorption maximum at 286 nm declined almost to the vanishing point and the absorption at 415 nm rose correspondingly (about 3–4 h). Extended heating produced some symmetrical dye (<5%). Acetic acid was removed on a rotary evaporator and the product was triturated with a mixture of ethyl acetate and water (50:50). Excess *N,N'*-diphenylformamidinium was dissolved in ethyl acetate while water removed any symmetrical dye and unreacted intermediate II. The crude orange powder (1.8 g, 30%) thus obtained was immediately used in the next reaction: λ_{max} (water) = 415 nm; $^1\text{H NMR}$ (D_2O) δ 8.15 (s, 1 H, 4-H), 8.0 (d, 2 H, J = 7.0 Hz, 6-H), 7.9 (d, 1 H, J = 7 Hz, 7-H), 7.1–7.55 (m, 7 H, two vinyl and five aromatic protons of aniline), 4.51 (q, 2 H, J = 7.5 Hz, α -CH₂), 1.35–1.6 (s merged in t, 9 H, β -CH₃ and (CH₃)₂); R_f = 0.81 (RP-C18, methanol-water 3.7:6.3).

(b) *Preparation of Dye.* The intermediate V (4.7 g, 0.013 mol) was redissolved in an acetic anhydride (25 mL) and pyridine (25 mL) mixture. A UV/vis spectrum showed that the absorption at 415 nm disappeared almost immediately and a new band appeared at 385 nm. Indolenine salt III (5.3 g, 0.015 mol) was added and the mixture was heated at 110 °C until the absorption maxima at 385 nm almost vanished. The mixture was cooled and the dye precipitated with ethyl ether. After washing with 2-propanol (3 $\times$ 10 mL) the solid was recovered by filtration and purified by HPLC on reverse-phase C18 using a water-methanol mixture as eluent (2.3 g, yield 25%): $^1\text{H NMR}$ (D_2O) δ 8.4 (t, 1 H, J = 14 Hz, β proton of the bridge), 7.8 (s, 2 H, 4-H, 4'-H), 7.6 (d, 2 H, J = 12 Hz, 6-H, 6'-H), 7.3 (m, 2 H, 7-H, 7'-H), 6.3–6.1 (m, 2 H, α , α' protons of the bridge), 4.1 (m, 4 H, α -, α' -CH₂), 2.9 (t, 2 H, J = 7 Hz, CH₂COOH), 1.4–2.0 (m, 21 H, 3 CH₂, 1 CH₃, and 2 (CH₃)₂); R_f = 0.51 (RP-C18, methanol-water 2.5:7.5).

Cy3.19.OH (X). This unsymmetrical dye was prepared by condensation of intermediates VI and III following the procedure of Cy3.29.OH (yield 5%): $^1\text{H NMR}$ (D_2O) δ 8.4 (t, 1 H, J = 13 Hz, β proton of the bridge), 7.75 (s, 2 H, 4-H, 4'-H), 7.6 (d, 2 H, J = 12 Hz, 6-H, 6'-H), 7.3 (m, 2 H, 7-H, 7'-H), 6.3 and 6.2 (2 d of 1 H each, J = 13 Hz, α , α' protons of the bridge), 4.1 (m, 4 H, α -, α' -CH₂), 2.9 (t, 2 H, J = 7 Hz, CH₂SO₃), 2.5 (t, 2 H, J = 7.5 Hz, CH₂COOH),

1.4–2.0 (m, 22 H, 5 CH₂ groups, with s at 1.6 for 2 (CH₃)₂); R_f = 0.23 (RP C18, water-methanol 9:1).

CY5.18.OH (XII). A mixture of indoleniniumsulfonate II (10 g, 0.028 mol), potassium acetate (1.4 g, 0.014 mol), and methanol (60 mL) was refluxed at 60 °C for 2.5 h. 1,3,3-Trimethoxypropene (2 mL) was added every 30 min for 2 h (total 8 mL). At the end of the reaction, the mixture was filtered to remove the potassium acetate. The filtrate was kept at 0–4 °C for 15–18 h. The crystalline dye obtained was filtered and washed with 2-propanol (3 $\times$ 50 mL) and dried under vacuum (8.1 g, yield 75%): $^1\text{H NMR}$ (D_2O) δ 7.9 (t, 2 H, J = 14 Hz, β , β' protons of the bridge), 7.7 (s, 2 H, 4-H, 4'-H), 7.6 (d, 2 H, J = 8 Hz, 6-H, 6'-H), 7.4 (d, 2 H, J = 7.6 Hz, 7-H, 7'-H), 6.4 (t, 1 H, J = 15 Hz, γ proton of the bridge), 6.0 (d, 2 H, J = 15 Hz, α , α' protons of the bridge), 3.9 (t, 4 H, J = 7.5 Hz, α -, α' -CH₂), 2.15 (t, 4 H, J = 7.5 Hz, ϵ -, ϵ' -CH₂), 1.7 (m, 4 H, β -, β' -CH₂), 1.3–1.66 (m, 8 H, γ -, γ' -, δ -, δ' -CH₂), 1.51 (s, 12 H, 2 (CH₃)₂); R_f = 0.58 (RP-C18, methanol-water 2.5:7.5).

Cy5.29.OH (XIV). (a) *Intermediate 445 (VII).* A solution of indolenine II (4 g, 0.015 mol) and malonaldehyde dianil hydrochloride (4.4 g, 0.017 mol) in a mixture of acetic acid (20 mL) and acetic anhydride (20 mL) was heated to reflux. The completion of the reaction was monitored by absorption spectra in methanol, which showed that as the reaction progressed the absorption maximum at 286 nm declined almost to the vanishing point and the absorption at 445 nm rose correspondingly (approximately 4 h). Some symmetrical dye (<5%) was always formed if heating was continued for a long time. Acetic acid was removed with a rotary evaporator and the product was washed with ethyl acetate to remove excess malonaldehyde dianil hydrochloride. The crude product thus obtained was used in the next reaction. The intermediate decomposed on standing (UV/vis in water—additional bands at 485 and 515 nm). However this hydrolyzed product can be used in the next reaction: $^1\text{H NMR}$ (D_2O) δ 8.15 (s, 1 H, 4-H), 8.0 (d, 2 H, J = 7.0 Hz, 6-H), 7.9 (d, 1 H, J = 7.0 Hz), 7.1–7.55 (m, 7 H, 2 vinyl and 5 aromatic protons of aniline), 4.51 (q, 2 H, J = 7.5 Hz, α -CH₂), 1.35–1.6 (s, m, 9 H, β -CH₃ and (CH₃)₂); R_f = 0.48 (RP-C18, water-methanol 1:1).

(b) *Preparation of Dye.* The intermediate VII was dissolved in an acetic anhydride and pyridine mixture. The UV spectra of the mixture was recorded to ensure an absorbance at 445 nm for the acetate form of the intermediate. Indolenine III (5.3 g, 0.015 mol) was added and reaction mixture was heated at 110 °C until the absorption maxima at 445 nm almost vanished. The mixture was cooled and the dye precipitated with ethyl acetate. The gummy product was washed with 2-propanol (3 $\times$ 10 mL). The crude solid was reverse-phase (C18) chromatographed using a water-methanol mixture as eluent: $^1\text{H NMR}$ (D_2O) δ 9.1 (d, 2 H), 8.7 (t, 1 H), and 8.4 (t, 2 H) (pyridine protons); 7.9–8.2 (m, 6 H, 4-H, 4'-H, 6-H, 6'-H, β , β' protons of the bridge), 7.5 (m, 2 H, 7-H, 7'-H), 6.7 (t, 1 H, J = 13 Hz, γ proton of the bridge), 6.2 (m, 2 H, α , α' protons of the bridge), 4.0–4.2 (m, 4 H, α -, α' -CH₂), 2.9 (t, 2 H, J = 7 Hz, CH₂COOH), 1.4–2.0 (m, 21 H, 3 CH₂ groups, 1 CH₃ with s at 1.6 for 2 (CH₃)₂); R_f = 0.56 (RP C18, methanol-water 3.7:6.3).

Cy5.19.OH (XIII). This unsymmetrical dye was prepared by condensation of intermediates VIII and III following the procedure of Cy5.29 (yield 5%): $^1\text{H NMR}$ (D_2O) δ 7.95 (t, 2 H, J = 13.2 Hz, β , β' protons of bridge), 7.8 (s, 2 H, 4 and 4'-H), 7.65 (d, 2 H, J = 7.7 Hz, 6-H, 6'-H), 7.3–7.4 (2 d, 2 H, 6-H, 6'-H), 6.5 (t, 1 H, J = 13 Hz, γ -H), 6.2 (2 d, 2 H, J = 13 Hz, α -H, α' -H), 4.1 (m, 4 H, α -, α' -

CH₃), 2.9 (t, 2 H, $J = 7$ Hz, CH₂SO₄), 2.45 (t, 2 H, $J = 7.5$ Hz, CH₂COOH), 1.4–2.0 (m, 22 H, 5 CH₂ groups, with s at 1.6 for 2 (CH₃)₂); $R_f = 0.75$ (RP C18 water–methanol 7.5:2.5).

Cy7.18.OH (XV). Glutaconaldehyde dianil hydrochloride (143 mg, 0.5 mmol) was dissolved in a heated mixture of acetic anhydride (4 mL) and pyridine (1 mL). Intermediate III (353 mg, 1 mmol) was added and mixture was heated to reflux for additional 10 min and then cooled. The dye was precipitated with diethyl ether. The supernatant liquid was separated. Solid was redissolved in a minimum volume of methanol and reprecipitated with 2-propanol. The bright purple solid was collected on filter paper and dried (164 mg, yield 20%). It was converted into its potassium salt and then purified by HPLC (RP-C18) with a water–methanol mixture as eluent: $\lambda_{\max} = 750$ nm; ¹H NMR (D₂O) δ 7.75 (m, 6 H, 4'-H, 6-H, 6'-H, and γ , γ' protons of the bridge), 7.4 (t, 1 H, $J = 13$ Hz, δ proton of the bridge), 7.2 (d, 2 H, $J = 7.7$ Hz, 7-H, 7'-H), 6.35 (t, 2 H, $J = 13$ Hz, β , β' protons of the bridge), 6.15 (d, 2 H, $J = 7$ Hz, α -, α' -H), 4.1 (br, 4 H, α -, α' -CH₂), 2.2 (t, 2 H, $J = 7.5$ Hz, CH₂COOH), 1.2–1.7 (m, 24 H, 6 CH₂ groups, with s at 1.5 for 2 (CH₃)₂); $R_f = 0.25$ (RP-C18, water–methanol 7.5:2.5).

Succinimidyl Esters of (Carboxyalkyl)cyanine Dyes. A procedure for making succinimidyl active esters using disuccinimidyl carbonate (DSC) has been described by Ogura et al. (13). It was modified for sulfocyanines. In a typical experiment (carboxyalkyl)sulfocyanine was dissolved in a mixture of dry DMF (2 mL/100 mg of dye) and dry pyridine (0.1 mL/100 mg of dye). Disuccinimidyl carbonate (DSC) (1.5 equiv/carboxyl group) was added and the mixture was stirred at 55–60 °C for 90 min under nitrogen. After diluting the mixture with dry ethyl acetate, the supernatant was decanted. The product was either washed repeatedly with solvent or dissolved in DMF and reprecipitated. Nearly quantitative yields of cyanine active esters were obtained.

The formation of the active ester was confirmed by its reaction with benzylamine in DMF or its reaction with taurine in a pH 9.4 bicarbonate buffer. Reversed-phase C18 TLC plates were spotted with the conjugate and the OSu and hydrolyzed carboxylate product for comparison and developed with a water–methanol mixture. Since activation was sometimes incomplete, reverse-phase HPLC was also used to determine the percentage of fluorochrome in the active ester form. The sample was eluted through an Alltech Econosphere 250 mm $\times$ 4.6 mm C18 RP column using a mixture of 25% acetonitrile and 75% water containing 0.1% trifluoroacetic acid. The percentages of activated and unactivated cyanine fluorophore were determined by integration of the absorbance signals from a Varian UV/vis detector.

Stability of the Active Ester in Aqueous Buffers. Hydrolysis rates of Cy5.29.OSu (XIX) in aqueous buffers were determined as follows. Reactions were started by adding 200 μ L of aqueous phosphate buffer (at pH 7.0, 7.5, 8.0, 8.5 and 9.4) to tubes containing 1 mg of Cy5.29.OSu. At each time interval following initiation of the reaction, 25 μ L of the reaction solution was loaded as a small strip on a C18-reversed-phase, 5 $\times$ 10 cm TLC plate. The plate was developed using acetonitrile–water 2.5:7.5 containing 0.1% trifluoroacetic acid. The band containing dye in the carboxylic acid form ($R_f = 0.88$) and the band containing unhydrolyzed succinimidyl ester ($R_f = 0.2$) were scraped from the TLC plate and extracted in methanol (5 mL). The absorbance of each extract was measured at

650 nm and the fraction of hydrolyzed dye was calculated from the relative absorbances of the two extracts.

General Protein Labeling Procedures. Stock solutions of the sulfoindocyanine succinimidyl active esters were made in dry DMF (1 mg/100 μ L) and are stable for days when stored at 4 °C in a desiccator. The active esters are also stable in distilled water for several hours provided the pH of the solution is not basic. Aqueous solutions of the dyes can be used for labeling antibodies if use of DMF is not suitable for certain antibodies. The concentration of the reactive dye in the stock solution was determined by measuring the absorbance of an aliquot of the appropriately diluted stock solution in phosphate-buffered saline (PBS) and using the extinction coefficient of the dye (Table II). Generally, antibody labeling was carried out in 0.1 M carbonate–bicarbonate buffer (pH 9.4) for 15 min at room temperature. One milligram of sheep IgG (6.45 mM) was dissolved in 0.25–1 mL of buffer solution and the desired amount of dye (e.g. 20 μ L of the 1 mg/100 μ L stock solution) was added during vigorous vortex mixing. Unconjugated dye was separated from the labeled protein by gel permeation chromatography (0.7 $\times$ 20 cm column of Sephadex G-50) using pH 7 buffer solution as eluent (8).

Protein Labeling Rates at Different pH Values. A series of labeling reactions were set up as described in the general protein labeling procedure above, except that the reactions were carried out at pH 7.0, 7.5, 8.0, 8.5, and 9.4 with 1 mg of antibody per 250 μ L of buffer. Aliquots of the reactions were removed at time points and labeled antibody was separated from unreacted dye as described above. The dye-to-protein ratio for each sample was determined as described below.

Determination of Dye-to-Antibody Ratios. A simple method for estimating dye/protein (D/P) ratios involves direct measurement of the protein absorbance at 280 nm and dye absorbance at the absorption maximum. Specifically, the dye/protein ratio is calculated using the equation below with measured values of the absorbance of the

$$D/P = \frac{A_{\text{dye}} E_{\text{prot}}}{(A_{280} - 0.05 A_{\text{dye}}) E_{\text{dye}}}$$

labeled dye (Cy3 at 550 nm or Cy5 at 650 nm) and the absorbance of protein at 280 nm. The extinction coefficient of the dye is obtained from Table II and the extinction coefficient of the IgG antibody at 280 nm was taken to be 170 000 L/mol cm. The factor of 0.05 (used for both Cy3 and Cy5) in the denominator accounts for dye absorption at 280 nm, which is 5% of the absorption of the dye at its maximum absorption (A_{dye}).

A more accurate method is needed when the labeling reagent shows significant spectral changes when bound to the antibody molecule. For example, this approach is needed when both dimers and monomers of dye, which have different absorption peaks and different extinction coefficients, are present on the protein at higher D/P ratios. In this case, the labeled protein is dissolved in formamide for absorption spectroscopy, and the extinction coefficients of the dye are determined independently for the calculation.

RESULTS

Names of the New Labeling Reagents. The basic structure of the sulfoindocyanine dyes developed in this work is shown in Figure 1. Following the naming scheme for reactive polymethine dyes used in the previous papers (5–7) of this series, these dyes will be referenced as Cy3.18.OSu (XVI) or as Cy5.29.OSu (XIX). In these

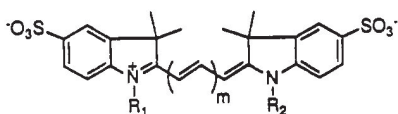
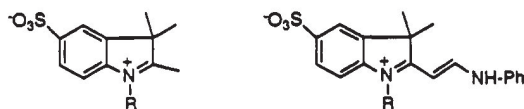
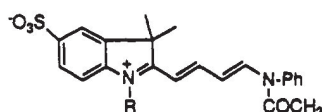


Figure 1. Structure of cyanine dye.



- I, R = H
 II, R = CH₂CH₃
 III, R = CH₂(CH₂)₄COOH
 IV, R = CH₂(CH₂)₃SO₃⁻
 V, R = CH₂CH₃
 VI, R = CH₂(CH₂)₃SO₃⁻



- VII, R = CH₂CH₃
 VIII, R = CH₂(CH₂)₃SO₃⁻

Figure 2. Structures of key intermediates used in the synthesis of symmetrical and unsymmetrical sulfoindocyanines.

Table I. Structures of Sulfoindocyanine Dyes

dye		m	R ₁	R ₂
no.	designation			
IX	Cy3.18.OH	1	(CH ₂) ₅ COOH	(CH ₂) ₅ COOH
X	Cy3.19.OH	1	(CH ₂) ₅ COOH	(CH ₂) ₄ SO ₃
XI	Cy3.29.OH	1	(CH ₂) ₅ COOH	CH ₂ CH ₃
XII	Cy5.18.OH	2	(CH ₂) ₅ COOH	(CH ₂) ₅ COOH
XIII	Cy5.19.OH	2	(CH ₂) ₅ COOH	(CH ₂) ₄ SO ₃
XIV	Cy5.29.OH	2	(CH ₂) ₅ COOH	CH ₂ CH ₃
XV	Cy7.18.OH	3	(CH ₂) ₅ COOH	(CH ₂) ₅ COOH
XVI	Cy3.18.OSu	1	(CH ₂) ₅ COOSu	(CH ₂) ₅ COOSu
XVII	Cy3.29.OSu	1	(CH ₂) ₅ COOSu	CH ₂ CH ₃
XVIII	Cy5.18.OSu	2	(CH ₂) ₅ COOSu	(CH ₂) ₅ COOSu
XIX	Cy5.29.OSu	2	(CH ₂) ₅ COOSu	CH ₂ CH ₃

names, the "Cy" portion indicates that they are cyanine dyes while OSu indicates that the compounds have succinimidyl ester reactive groups. The number immediately after the "Cy" indicates the number of bridge carbons (i.e. Cy3, $m = 1$; Cy5, $m = 2$). The number following the decimal point indicates a unique dye structure, which is determined by the substituents (R₁, R₂) on the structure.

Syntheses of Sulfoindocyanine Labeling Reagents.

The sulfoindocyanine dyes developed in this work are shown in Table I. The reactivity, the charges, and the solubility properties of basic indolenine dye can be readily controlled by placing appropriate substituents at R₁, R₂, and benzene rings of the nucleus. The sulfoindocyanines have SO₃⁻ groups at the 5 and 5' positions to increase water solubility. The R₁ and R₂ substituents consist of one or two carboxyl alkyl groups that can be activated to form succinimidylesters. Succinimidylesters readily react with primary amines at basic pH, and less readily with hydroxyl-containing materials. The number of double bonds in the polymethine chain between the heterocyclic nuclei allows control of the color of the fluorochrome.

The synthesis schemes using the intermediate structures of Figure 2 are analogous to those described in earlier papers from this laboratory (5-7). Generally, a fluorescent cyanine dye is formed when a quaternized indolenine nucleus, which contains a carboxyl alkyl group for later activation of the fluorophore, is linked to a second identical

(or different) indolenine nucleus through a polymethine chain of three, five, or seven conjugated methine carbon atoms. After purification of the resulting fluorophore, the carboxyl group(s) is(are) activated to produce the cyanine dye labeling reagent.

The key intermediate, indoleninium-5-sulfonate required to produce the new group of labeling reagents is prepared from *p*-hydrazinobenzenesulfonic acid and 3-methyl-2-butanone by conventional Fisher indole synthesis (11). Indolenine thus obtained is converted into a potassium salt before quaternization. We attempted to increase the water solubility of the reagents by incorporating fewer methyl groups between the OSu group and the indole ring nitrogen. However, quaternization of the sulfoindole nucleus with bromoacetic acid proved difficult and sulfoindocyanines bearing a propionic acid group produced low yields of active esters. Ultimately, we found that sulfoindocyanines derived from nuclei bearing a five-carbon carboxylic acid (hexanoic acid) attached to the nitrogen could be prepared without significant difficulty, and a number of the useful labeling reagents reported in this paper have this group as a linker for formation of the active ester.

Monoreactive Cy3.19.OH (X) and Cy5.19.OH (XIII) dyes bearing a sulfonatobutyl group [R₁ = (CH₂)₄SO₃⁻ and R₂ = (CH₂)₅COOH] were obtained in poor yields and were difficult to activate. When the sulfonatobutyl group is replaced by an ethyl group (R₁ = C₂H₅), the resulting dyes Cy3.29.OH (XI) and Cy5.29.OH (XIV), were obtained in good yield and provided excellent protein labeling. This study reveals that various combinations of charged groups and length of alkyl chains affect the preparation and labeling efficiency of succinimidyl esters.

Counterions also play a role in the synthesis of the ester. All (carboxyethyl)- and (carboxypentyl)sulfoindocyanines were obtained as monopyridinium salts where pyridine was used as solvent. It was observed that these pyridinium salts do not form the active esters readily and had to be converted into monopotassium salts before activation was successful.

Absorption and Fluorescence of the Basic Fluorophores. Direct attachment of the sulfonic acid group to the aromatic system of the chromophore does not appreciably change the basic indocyanine dye absorption and emission wavelengths. The absorption spectra of carboxylic acid-containing cyanines and their active esters have narrow absorption and emission bands and low vibronic shoulders on the short-wavelength sides of the absorption peaks (Figure 3). With the introduction of the two sulfonate groups, these dyes dissolve easily in water, even at millimolar concentrations, with no evidence of new absorption bands that are indicative of dimers and other dye-dye interactions (14).

Use of the Sulfoindole Reagent To Label Amino-Containing Materials. Sulfoindocyanine succinimidyl esters react easily with material containing neutral primary amino groups (pH > pK_a ~ 9-10, typically). Figure 4b shows that antibody labeling is very fast at pH greater than 8. Interestingly, the final D/P ratio reached in less than 1 h is higher at high pH, possibly because the ε-aminolysine groups in acidic environments of the antibody are not deprotonated until a higher pH is reached, or alternatively, because there may be a pH-dependent conformational change that exposes new amino groups at high pH. It is also clear from these experiments that adequate labeling can take place at neutral pH, provided that the labeling reaction is given a few hours. Figure 4a

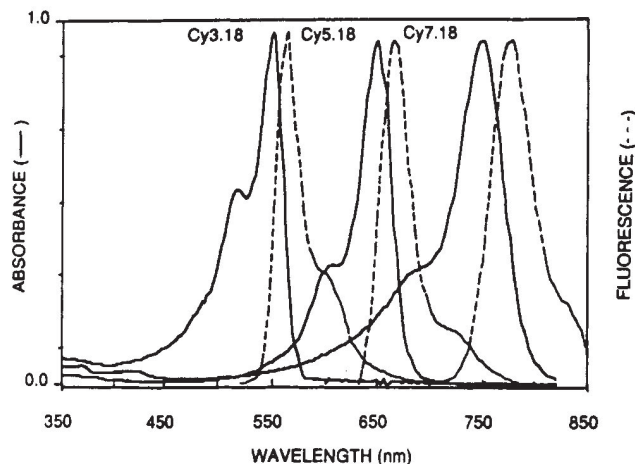


Figure 3. Absorption (—) and emission (---) spectra of Cy3.18, Cy5.18, and Cy7.18 in PBS.

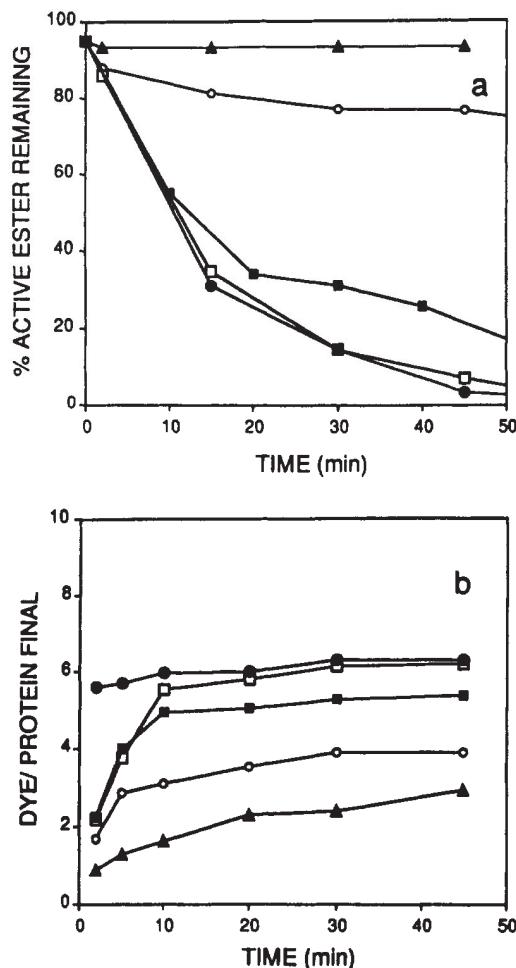


Figure 4. (a) Stability of succinimidyl active ester over time in buffer solutions (see methods) at pH 7.0 ($\blacktriangle$), pH 7.5 ($\circ$), pH 8.0 ($\blacksquare$), pH 8.5 ($\square$), and pH 9.4 ($\bullet$). (b) Extent of labeling of sheep IgG with Cy5.29.OSu over time in buffer solutions at pH 7.0 ($\blacktriangle$), pH 7.5 ($\circ$), pH 8.0 ($\blacksquare$), pH 8.5 ($\square$), and pH 9.4 ($\bullet$).

clearly shows that, for aqueous buffers in the pH range 7–9.4, the rate of protein labeling (at 1 mg of protein/250 μ L) is at least 10 times faster than the rate of hydrolysis of the succinimidyl ester.

Absorption and Fluorescence of Labeled Amines. Table II shows absorption and emission properties of the cyanine succinimidyl esters that have been reacted with low molecular weight alkylamines. There is minimal dependence of solvent on the absorption and emission

Table II. Spectral Properties of Key Sulfoindocyanine Dyes

dye	solvent	abs max	em max	ϕ	ϵ^d (L/mol cm)
Cy3.18	ethanol	560	575	0.09 ^a	150 000
	formamide	558			
	PBS ^c	550	565	0.04	150 000
	glycerol (25 °C)	558	571	0.52	
	glycerol (8 °C)	558	569	0.51	
Cy5.18	ethanol	658	677	0.40 ^b	250 000
	formamide	656			
	PBS	650	667	0.27	250 000
	glycerol (25 °C)	656	674	0.39	
	glycerol (8 °C)	658	672	0.43	
Cy5.29	ethanol	658	677	0.30	
	formamide	658			
	PBS	650	666	0.20	
Cy5.29-taurine	PBS (pH 7)	650	665	0.20	
	PBS (pH 9.4)	650	665	0.22	
Cy7.18	ethanol	758	789	—	200 000
	formamide	764	—		
	PBS	750	777	—	200 000

^a Based on rhodamine-6G quantum yield in ethanol as standard.

^b Based on Di-S-C2-(5) as standard (22). ^c Phosphate-buffered saline.

^d Estimated error range error = +5%.

wavelengths. However, the quantum yields of Cy3 and Cy5 dyes are somewhat higher in organic solvents. The quantum yields of Cy5 derivatives, and especially the Cy3-labeled amines, depend strongly on solvent viscosity. For example, Table II shows that Cy3 is ~10 times more fluorescent in glycerol at 8 °C than it is in water at room temperature.

DISCUSSION

We developed a series of fluorescent labeling reagents that contain negatively charged sulfonate groups directly attached to the indocyanine nuclei. These dyes are intrinsically much more water soluble than cyanines without aryl sulfonate groups (7). This property makes these labeling reagents very easy to use because they dissolve rapidly into aqueous buffers with no aggregation under normal labeling conditions. Presumably, because of the location of the sulfonic acid groups on the ring structure of the fluorophore, these labeling reagents have less affinity for one another on the protein surface, and as a result, sulfoindocyanines activated with succinimidyl ester groups can be used to tag antibodies and other proteins with significantly less dimer formation and much greater fluorescence (8). Sulfoindocyanines have been used to produce highly fluorescent antibodies (15, 16), phycoerythrin (17), transferrin (18), dextrans (14), and 5'-amino-modified oligonucleotides (19).

The speed of the labeling reaction depends on the concentration of the reactive dye, the concentration of the target, and the pH of the reaction medium. Generally, procedures for the labeling proteins with isothiocyanates and succinimidyl esters call for 1–24 h of incubation in aqueous buffer at pH 8.5–9.5 (20). We find that less than 10 min is sufficient at pH 8.5–9.4 with the succinimidyl esters of sulfoindocyanines. At the higher pH value, the labeling produces a greater D/P ratio for antibody labeling (Figure 4b). Since the sulfoindocyanine OSu reagents hydrolyze only slowly in aqueous buffers at pH 7, it is also possible to label pH-sensitive materials at neutral pH by increasing the incubation time to several hours. In labeling reactions, the buffering agent should not contain primary amino groups, or other good nucleophiles.

The absorption maximum, extinction coefficient, quantum yield, and fluorescence wavelength of Cy3.18 and Cy5.18 bound to low molecular weight primary amines

are essentially the same as for the corresponding carboxylic acid forms of Cy3.18 and Cy5.18. The quantum yields are somewhat higher in organic solvents but do not depend on pH over the range of 4–10. Highly viscous solvents (e.g., cold glycerol, Table II) that reduce the conformational flexibility of Cy3 increase its quantum yield (21). The fluorescence-enhancing effect of a rigid microenvironment may explain the remarkable increase in quantum yield of Cy3 when it is conjugated to antibodies. It is plausible that the use of polymeric microscope slide mounting media might produce a higher Cy3 fluorescence. Cy5-labeled material, on the other hand, shows only a minimal dependence of quantum yield on environmental rigidity.

The reactive cyanines have one or two OSu groups. Symmetrical dyes with two OSu groups are easier to synthesize but may cross-link different amino groups on the same target or on different targets. We have found that labeling of antibody at 1 mg/mL produces almost no cross-linking (7). Cross-linking between amino groups on different proteins can occur, however, when both protein and dye concentrations are high.

The emission maxima for members of the series range from 575 to 780 nm. The carbocyanine reagent Cy3.18 can be excited with the 488-, 514-, and 532-nm laser lines and is optimally excited with the 546-nm mercury arc line. Because the extinction coefficient of Cy3.18 on the blue shoulder at 488 nm is still about 25% (22 000 L/mol cm) of its maximum at 550 nm, it is even possible to perform two-color flow cytometry using Cy3.18-labeled antibodies with fluorescein-labeled antibodies. Our preliminary observations indicate that the photostability of Cy3 is comparable to that of rhodamines (16) and significantly greater than that of fluorescein and also that Cy3.18 and Cy5.18 produce conjugated antibodies with less nonspecific binding than those obtained using TRITC and other nonsulfonated cyanines. Because of its brightness, water solubility, photostability, and low nonspecific binding, Cy3 may be considered an ideal substitute for tetramethylrhodamine isothiocyanate (TRITC).

The red-fluorescing Cy5.18 can be excited with the 633-nm HeNe laser line and the 647-nm line of a Kr laser. Excitation with these long-wavelength laser lines produces less autofluorescence than with short-wavelength excitation, and as a result, Cy5.18-labeled antibodies can be detected even on macrophages by flow cytometry. In most cases adequate signals can be obtained in flow cytometry and laser confocal scanning microscopes using inexpensive HeNe lasers. The photostability of Cy5 derivatives is comparable to, or somewhat better than that of fluorescein. The labeling reagent Cy5.18.OSu may be considered as a low molecular weight, easy to use alternative to allophycocyanine (8).

The near-IR fluorescing Cy7.18.OH can be excited in the 700–750-nm range and emits maximally near 780 nm. Antibodies labeled with Cy7.18 are not as bright and photostable as those generated with Cy3.18 and Cy5.18, but useable images can be obtained when the antigen density is sufficiently high and a cooled CCD camera is used to acquire the images.

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