New glycoconjugated cyanine dyes as fluorescent labeling reagents

Mark V. Reddington

Center for Light Microscope Imaging and Biotechnology, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA

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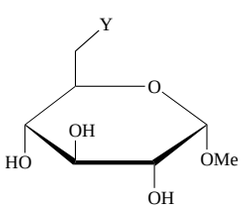
New highly water soluble cyanine dyes incorporating methyl glucopyranoside residues and reactive functionality are synthesised. The new dyes are conjugated to aminodextrans and to antibodies. The absorption and fluorescence properties of the new dyes and of the labeled substrates are discussed.

Introduction

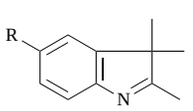
Fluorescence detection techniques are dependent on the physical characteristics of the dyes that they employ.[1–5] Many dyes that have excellent photophysical properties (high extinction coefficients and high quantum yields) have limited utility because other properties such as solubility and photostability are poor.[6] In particular, for most biological applications good aqueous solubility for the fluorophore is crucial. A common problem with many commercially available fluorescent labeling reagents is the need for them to be dissolved in organic solvents, such as DMF, prior to substrate labeling in aqueous media. Such solvents can have a deleterious effect upon sensitive substrates. The solubility of organic dyes affects the degree to which they interact with themselves in solution and when conjugated to substrates and therefore directly influences their light absorption and emission properties.[6,7] The problem of non-specific staining of cellular matter by the dye, which reduces signal to noise during observation, is also a function of the dye's hydrophobicity and of the polarity of its appended functional groups.[6] In addition, the dye should not cause precipitation of the substrate once labeling has occurred. There is a need for fluorescent reagents that are bright, that have good aqueous solubility, and that give low non-specific staining. In recent years, a series of reagents based on sulfocyanine dyes has become commercially available.[8–10] These dyes, which are highly polar due to the presence of two sulfonic acid residues, have excellent photophysical properties combined with good aqueous solubility. To determine if further improvements could be achieved for the properties of cyanine dye based fluorescent reagents, the sulfonic acid groups were replaced with carbohydrate residues to create water soluble cyanine dyes with reduced polarity. The synthesis, spectral properties and antibody labeling capabilities of the new dyes are reported here.

Results and discussion

Methyl glucopyranoside **1** was chosen to be the water solubilising sugar residue because its glycosidic linkage is fixed and



1 Y = OH
2 Y = Br

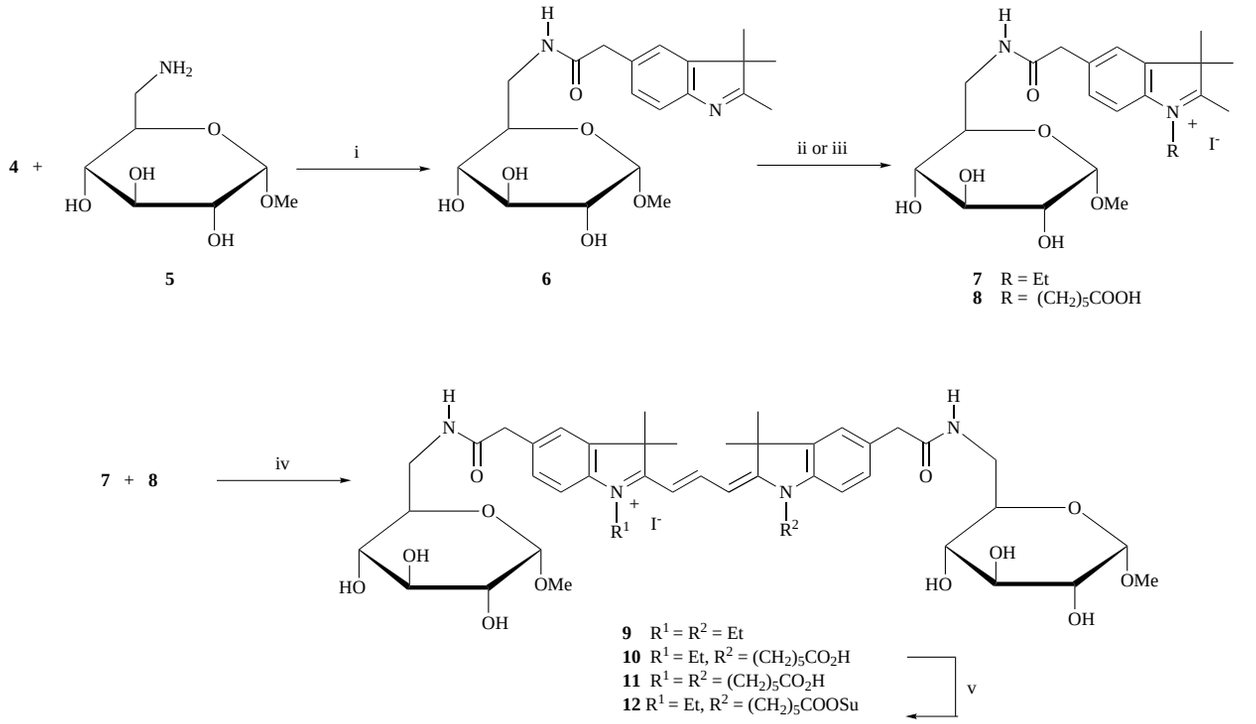


3 R = H
4 R = CH_2CO_2H

because of its ease of derivatisation without recourse to any protecting group chemistry. Alkylation of the heterocyclic precursors to cyanine dyes using a halogenated or a sulfonate ester derivative of **1** offers the simplest method for introduction of the sugar and creates a link that will not be cleaved readily by enzymes in biological environments, thus ensuring a stable labeling reagent. Methyl 6-bromo-6-deoxy-α-glucopyranoside **2** was prepared using *N*-bromosuccinimide (NBS) and PPh_3,[11] but treatment of trimethyl-3*H*-indole **3** or carboxymethyltrimethyl-3*H*-indole[8] **4** with **2** proved unsatisfactory as a method to introduce the carbohydrate residue, due to low yields. Consequently, an alternative route involving the formation of an amide link was used.

Methyl 6-azido-6-deoxy-α-glucopyranoside was prepared from **1** in a two step one-pot process using NBS and PPh_3 followed by treatment with sodium azide,[12] and the azide was then reduced to methyl 6-amino-6-deoxy-α-glucopyranoside **5** using PPh_3 and concentrated aqueous ammonia.[13] Slight modifications to the literature procedures were made to allow more rapid attainment of the amine **5** and are described in the Experimental section. Coupling[14] of carboxymethyl-3*H*-indole **4** with amine **5** gave the key 3*H*-indole-substituted methyl glycoside intermediate **6** in 92% yield (Scheme 1). Alkylation of **6** allows for the introduction of a variety of functional groups (*e.g.* CO_2H, OH, SH, NH_2) prior to dye formation. Treatment of **6** with iodoethane in acetonitrile or with iodohexanoic acid in nitromethane afforded the hygroscopic quaternary indolium dye precursors **7** and **8** in 86 and 80% yields, respectively. Purification of precursor **8** by either silica gel or C_{18} reversed phase silica gel chromatography is made difficult by the compound's poor stability. Collected fractions of **8** turned pink/red after only a few minutes standing open to the atmosphere. Once the solvents were removed from these fractions the solid residues continued to darken. Dye precursor **7** showed similar instability. Consequently, the crude compounds were used in the following reactions. Condensation of equimolar portions of **7** and **8** with triethyl orthoformate in pyridine afforded a mixture of three indocyanine dyes **9**, **10** and **11**. The unsymmetrical dye **10** was isolated by reversed phase chromatography, in 24% yield. The symmetrical dyes **9** and **11** are more easily obtained by self-condensation of **7** and **8**, respectively. Reaction of dye **10** with tetramethyl(succinimido)uronium tetrafluoroborate (TSTU)[15] in DMF afforded the succinimidyl active ester **12**, which can be used to label amine containing substrates.

The long term stability of these active ester reagents is of concern due to the possibility of ester formation between the active ester functionality and the hydroxy groups of the sugar. No noticeable deterioration of the reagent was observed when it was stored at room temperature under anhydrous conditions for a period of five months. The active ester **12** was readily soluble in water and did not show appreciable hydrolysis at neutral pH, or self-condensation, over a period of 16 h. Thus, no organic solvents are required for labeling of biological substrates with **12**, in aqueous media. Active ester **12** was conjugated to aminodextrans and to sheep immuno-γ-globulin (IgG)[9] to provide a range of labeling densities on the antibody. A labeling efficiency of greater than 40% was observed using



Scheme 1 *Reagents and conditions*: i, DCC, hydroxysuccinimide (HOSu), Et_3N, DMF; ii, EtI, MeCN; iii, $I(CH_2)_5CO_2H$, $MeNO_2$; iv, $HC(OEt)_3$, Py; v, TSTU, Pr^i_2EtN, DMF

Table 1 Absorption and fluorescence data of dyes **9**, **10**, **11** and Cy3 and labeled substrates

Dye	Solvent	Absorption λ_{max}/nm	Emission λ_{max}/nm	φ_f
9	Water	550	564	0.04
	Ethanol	560	576	0.10
	DMSO	564	581	0.20
10	Water	552	576	0.06
	PBS	552	—	0.06
	Ethanol	560	577	0.12
	DMSO	566	582	0.23
11	Water	552	569	0.08
	Ethanol	562	577	0.16
	DMSO	566	583	0.28
10–Dextran	Water	554	571	0.10
Cy3[9]	PBS	550	565	0.05
Cy3–lgG[9]	PBS	—	—	0.10

PBS: phosphate buffered saline.

standard procedures[9] in carbonate–hydrogen carbonate buffer at pH 9.5. Precipitation of the protein was observed only when greater than 6–7 dyes were attached to it.

The absorption and fluorescent properties of dyes **9**, **10** and **11** and the labeled dextran are shown in Table 1. Attachment of the carbohydrate residues does not appreciably change the basic indocyanine absorption and fluorescence properties compared to the commercial dye Cy3 **13**. The wavelengths of the absorption and fluorescence bands are relatively solvent insensitive. Increasing solvent viscosity reduces conformational mobility of the excited dye and lowers non-radiative energy loss.[9,16] Thus,

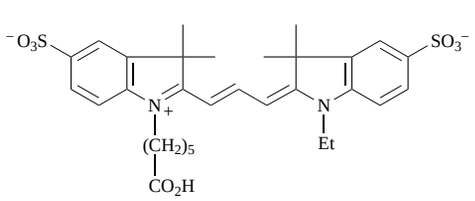


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Table 2 Absorption and fluorescence data for **10**–IgG conjugates in PBS

(Dye/protein)	A_{552}/A_{522}	φ_f	AB/1000
0.64	1.55	0.19	15
1.0	1.50	0.18	24
2.1	1.40	0.15	40
2.9	1.33	0.12	45
3.8	1.27	0.09	45
4.7	1.25	0.09	52
5.2	1.20	0.08	52
5.7	1.18	0.06	47
6.1	1.17	0.06	50

AB: Antibody brightness/$dm^3\ mol^{-1}\ cm^{-1}$.

there is an increase in quantum yields of fluorescence (φ_f) along the series water < ethanol < DMSO. Reduced conformational mobility with increasing size of the side chains on the chromophore is responsible for the increase in φ_f along the series **9** < **10** < **11** < labeled dextran. The absorption spectra of the dyes are nearly identical to their fluorescence excitation spectra indicating that ground state dye–dye interactions are insignificant. The carbohydrate residues provide sufficient aqueous solubility to prevent aggregation of the organic fluorophores in aqueous solution. The labeled dextran showed similar properties to dye **10**; however, the ratio of the absorption maximum at 552 nm to the shoulder at 522 nm is decreased slightly compared to that of dye **10**.

When conjugated to antibody the photophysical properties of the dye are dependent on its labeling density (Table 2 and Fig. 1). At low dye to protein ratios the absorption properties resemble those of dye **10** and of the labeled dextran, but the quantum yields are higher than that of **10** because of reduced conformational mobility when the dye is attached to the surface of the protein.[9] As the labeling density increases the ratio of the absorbance maximum at 552 nm to that at 522 decreases in accord with increasing formation of intramolecular dye dimers and aggregates on the protein surface. These aggregates have greatly reduced quantum yields compared to the monomeric dye.[7] Thus, the quantum yield of the labeled protein decreases

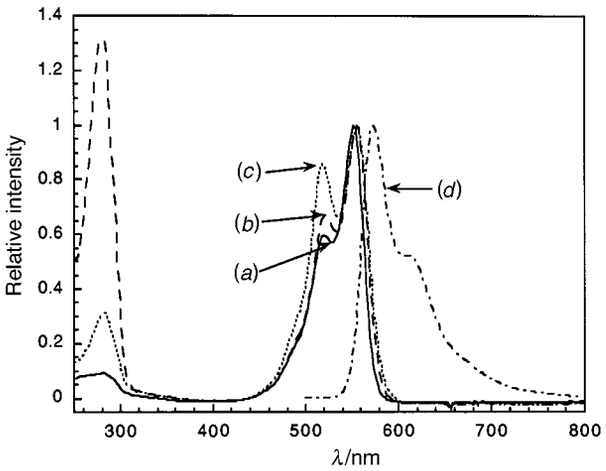

Fig. 1 Absorption spectra of (*a*) dye **10**, (*b*) **10**–IgG ([dye]/[protein] = 1), (*c*) **10**–IgG ([dye]/[protein] = 6), and (d) fluorescence emission spectrum of **10**–IgG ([dye]/[protein] = 1) in PBS

as the labeling density increases. A maximum antibody brightness (AB = no. of dyes × φ_f × ε/dm^3 mol^{-1} cm^{-1})[17] is achieved with a labeling density of 4–5 dyes per antibody. At low labeling densities antibody labeled with dye **10** has a higher quantum yield and is brighter than antibody labeled with Cy3. Inactivation of antibodies upon multiple labeling sometimes occurs. It can happen with almost any labeling reagent and is a function of the individual antibody, the particular fluorophore and the number of fluorophores.[18,19] There is no reason to think that the new dyes described here would cause problems beyond those for any other fluorescent label.

In summary, the new cyanine dyes are highly water soluble, easily synthesised and have spectral properties similar to their non-glycoconjugated analogs. They can be conjugated to amine containing substrates such as antibodies and dextrans with relatively high efficiencies to give brightly labeled substrates.

Experimental

General

All reagents and solvents were obtained from either Aldrich, Sigma, Kodak or Fisher, and were used as received unless otherwise stated. Solvents were dried according to standard literature procedures. NMR spectra were recorded on an IBM 300 MHz spectrometer. ^{1}H NMR spectra were referenced using the residual solvent signal (D_2O: 4.65). Addition of 0.1% v/v methanol to D_2O provided an internal reference signal (49.1 ppm) for ^{13}C spectra. Coupling constants (*J*) are given in Hz. Signals designated with a ′ refer to the aromatic ring. Absorption spectra were recorded on a Hewlett-Packard HP8452 diode array spectrophotometer and fluorescence spectra were recorded on a SPEX Fluorolog 2 spectrometer. Quantum yields were determined using rhodamine 6G (φ_f = 0.95 in ethanol) as a standard.[9] IR spectra were recorded on a Mattson Infinity 60Ar FTIR spectrometer as KBr discs. Melting points were recorded on an Electrothermal apparatus and are uncorrected. Flash chromatography refers to the procedure of Still *et al.*[20] and was performed on either silica gel 60 (Baker) or C_{18} bonded silica gel (Analtech). TLC was performed on either silica gel 60 (Merck) or C_{18} impregnated silica gel (Analtech) glass plates. Ether refers to diethyl ether.

Methyl 6-deoxy-6-amino-α-glucopyranoside 5[21]

To a dry 500 cm^3 flask, under argon, were added a stirring bar, compound **1** (10.2 g, 52.6 mmol), PPh_3 (27.6 g, 105 mmol) and dry DMF (250 cm^3). The mixture was cooled in an ice bath. NBS (18.9 g, 106 mmol) was added and the mixture was stirred at 0 °C for 20 min. The ice bath was replaced with an oil bath and the mixture was heated at 55 °C for 3 h. Methanol (10 cm^3) was added and the mixture was stirred for a further 10 min. Sodium azide (20.4 g, 314 mmol) was added and the mixture was heated at 85 °C for 4 h. The solvent was removed under high vacuum and the residue was partitioned between water (250 cm^3) and methylene chloride (200 cm^3). The aqueous phase was washed twice more with methylene chloride (2 × 200 cm^3) and was filtered. The aqueous solution was passed through a column of AG 501 mixed bed ion exchange resin (350 g). The resin was washed with water (400 cm^3). The combined aqueous solutions were evaporated *in vacuo* and the residue was dried under high vacuum over P_2O_5 overnight, in a 500 cm^3 flask. PPh_3 (39.0 g, 148 mmol) and dry DMF (200 cm^3) were added to the flask and the mixture was stirred at room temperature, under argon, for 2 h. Concentrated aqueous ammonia (40 cm^3) was added to the reaction mixture and stirring was continued for 20 h. The volatile components were removed under high vacuum and the residue was partitioned between water (300 cm^3) and methylene chloride (300 cm^3). The aqueous phase was washed twice more with methylene chloride (2 × 200 cm^3) and was filtered. The aqueous solution was passed through a column of Dowex 50 (8X) strongly acidic cation exchange resin (H-form) (4 × 12 cm). The resin was washed with water (400 cm^3), methanol (1000 cm^3), water (400 cm^3) and 2 M aqueous ammonia (500 cm^3). The ammonia containing eluent was collected and evaporated *in vacuo* to afford amine **5** as an off-white hygroscopic solid (6.23 g, 62%); $\delta_H(D_2O)$ 2.60 (1H, dd, *J* 6.7 and 13.7, 6-H), 2.85 (1H, dd, *J* 2.6 and 13.7, 6-H), 3.14 (1H, dd, *J* 9.8 and 8.8, 3-H), 3.27 (3H, s, OCH_3), 3.38–3.53 (3H, m, 2-H, 3-H and 4-H); δ_C 41.6, 55.1, 71.4, 72.0, 73.2, 99.3; *m/z* (FAB) 216.0 (M + Na, 100%), 238.0 (M + 2Na, 45%), 198.9, 172.8 (Found: M + Na, 216.0845. $C_7H_{15}NO_5$ requires *M* + Na, 216.0848).

N-(3,4,5-Trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)-2-(2,3,3-trimethyl-3*H*-indol-5-yl)acetamide 6

To a dry 25 cm^3 flask, under argon, were added a stirring bar, acid **4**[8] (111 mg, 0.51 mmol), dicyclohexylcarbodiimide (159 mg, 0.77 mmol) and *N*-hydroxysuccinimide (118 mg, 1.0 mmol). The flask was cooled in an ice bath for 5 min and then dry DMF (2 cm^3) was added. The mixture was stirred at 0 °C for 8 h, and then at 4 °C (in refrigerator) for 72 h. The solid was filtered off and was washed with DMF (1 cm^3). The combined DMF solutions were evaporated under high vacuum. The residue was taken up in acetonitrile (1.5 cm^3) and was filtered. The acetonitrile was removed *in vacuo* to afford the succinimidyl ester of **4**, which was used without further purification. The active ester was dissolved in dry DMF (0.5 cm^3) and was added to a mixture of amine **5** (150 mg, 0.75 mmol) and triethylamine (0.10 cm^3) in dry DMF (1.0 cm^3). The mixture was stirred at 40 °C overnight. The volatile components were removed under high vacuum to afford a pale orange oil, which was subjected to flash chromatography on silica gel with dichloromethane–methanol (9 : 1) as eluent. Evaporation of the solvent *in vacuo* afforded **6** as a white hygroscopic solid (186 mg, 92%); mp > 165 °C (decomp.); R_f 0.23 [dichloromethane–methanol (9 : 1)]; ν_{max}/cm^{-1} 2924, 2852, 1643, 1400, 1049; $\delta_H(D_2O)$ 1.16 (6H, s, 2 × Ar-CH_3), 2.14 (3H, s, Ar-CH_3), 2.90 (3H, s, OCH_3), 3.02 (1H, dd, *J* 9.0 and 9.8, 3-H), 3.17 (1H, dd, *J* 7.6 and 14.4, 5-H), 3.30 (1H, dd, *J* 3.9 and 9.8, 2-H), 3.35–3.56 (6H, m, 4-H, 6-H, and Ar-CH_2), 4.48 (1H, dd, *J* 9.0 and 9.8, 3-H), 7.14 (1H, dd, *J* 1.6 and 7.8, 6′-H), 7.56 (1H, d, *J* 1.6, 4′-H), 7.65 (1H, d, *J* 7.8, 7′-H); δ_C 14.6, 22.2, 40.3, 42.6, 53.9, 54.7, 69.9, 71.4, 71.5, 73.0, 99.1, 119.1, 122.8, 128.7, 132.6, 146.5, 151.1, 174.7, 191.8; *m/z* (FAB) 415.1 (M + Na, 100%), 393.2 (M + H, 22%), 437.1 (22), 325.9 (20), 199.0 (17), 176.0 (45) (Found: M + Na, 415.1854. $C_{20}H_{28}N_2O_6$ requires *M* + Na, 415.1845).

1-Ethyl-2,3,3-trimethyl-5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl) carbamoyl]methyl}-3*H*-indolium iodide 7

To a 25 cm^3 flask, under argon, were added a stirring bar, amide

6 (150 mg, 0.38 mmol), acetonitrile (3 cm^3) and ethyl iodide (2.5 cm^3). The mixture was heated at reflux for 16 h and was then cooled to room temperature. The volatile components were removed under high vacuum to give a pale brown residue, which was triturated with acetone (2 × 5 cm^3). The solid was dissolved in methanol (4 cm^3) and the solution was filtered. The methanol was removed *in vacuo* to afford **7** as a hygroscopic pale brown solid (180 mg, 86%); R_f 0.26 [dichloromethane–methanol (8:2)]; ν_{max}/cm^{-1} 2973, 2931, 1649, 1544, 1479, 1438, 1369, 1338, 1049, 823, 467; $\delta_H(D_2O)$ 1.41 (3H, t, *J* 7.5, CH_2CH_3), 1.44 (6H, s, 2 × Ar-CH_3), 3.05 (3H, s, OCH_3), 3.10 (1H, dd, *J* 8.9 and 9.6, 3-H), 3.21–3.30 (1H, m, 5-H), 3.36 (1H, dd, *J* 3.8 and 9.8, 2-H), 3.43–3.56 (3H, m, 4-H and 6-H), 3.64 (2H, s, Ar-CH_2), 4.36 (2H, q, *J* 7.4, N-CH_2-), 4.57 (1H, d, *J* 3.7, 1-H), 7.44 (1H, dd, *J* 8.3 and 1.5, 6′-H), 7.56 (1H, d, *J* 1.5, 4′-H), 7.65 (1H, d, *J* 8.3, 7′-H); δ_C 12.4, 21.8, 40.3, 42.2, 42.3, 43.6, 54.5, 54.7, 69.9, 71.4, 73.0, 99.2, 115.3, 124.3, 130.1, 137.5, 139.9, 142.6, 173.9, 195.8; *m/z* (FAB) 422.2 (M − I + H, 100%), 421.2 (M − I, 97%), 186.1 (13) (Found: M − I, 421.2319. $C_{22}H_{33}N_2O_6$ requires M − I, 421.2339)

1-(5-Carboxypentyl)-2,3,3-trimethyl-5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-3*H*-indolium iodide 8

To a 25 cm^3 flask, under argon, were added a stirring bar, amide **6** (75 mg, 0.19 mmol), iodohexanoic acid (926 mg, 3.8 mmol) and nitromethane (2.5 cm^3). The mixture was heated at reflux for 16 h and was then cooled to room temperature. The solvent was removed *in vacuo* and the dark brown residue was partitioned between dichloromethane (30 cm^3) and water (50 cm^3). The aqueous layer was washed twice more with dichloromethane (2 × 30 cm^3) and was filtered. Removal of the water *in vacuo* gave a pale brown solid residue that was triturated with acetone (2 × 5 cm^3). The solid was dissolved in methanol (4 cm^3) and the solution was filtered. The methanol was removed *in vacuo* to afford **8** as a hygroscopic pale brown solid (98 mg, 81%); R_f 0.18 [dichloromethane–methanol (6:4)]; ν_{max}/cm^{-1} 2929, 1649, 1577, 1465, 1430, 1261, 1065, 825; $\delta_H(D_2O)$ 1.24–1.37 (2H, m, central CH_2), 1.42 (6H, s, 2 × Ar-CH_3), 1.45–1.57 (2H, m, $CH_2CH_2CO_2H$), 1.77–1.90 (2H, m, N-CH_2CH_2), 2.20–2.29 (2H, m, CH_2CO_2H), 3.00 (3H, s, OCH_3), 3.03–3.10 (H, m, 3-H), 3.21–3.27 (1H, m, 5-H), 3.34 (1H, dd, *J* 3.8 and 9.7, 2-H), 3.41–3.56 (3H, m, H-4 and 6-H), 3.62 (2H, s, Ar-CH_2), 4.33 (2H, t, *J* 7.5, N-CH_2-), 4.54 (1H, d, *J* 3.8, 1-H), 7.41 (1H, dd, *J* 1.5 and 8.4, 7′-H), 7.53 (1H, d, *J* 1.5 and 8.4, 4′-H), 7.61 (H, d, *J* 8.4, 7′-H); δ_C 13.0, 21.9, 23.9, 25.4, 27.0, 33.7, 40.2, 42.3, 48.0, 54.5, 54.7, 61.6, 69.9, 71.3, 73.0, 99.1, 115.5, 124.2, 130.1, 137.6, 140.1, 142.5, 173.8, 178.6, 196.3; *m/z* (FAB) 507.3 (M − I, 100%), 529.3 (33), 272.2 (14), 176.0 (35) (Found: M − I, 507.2693. $C_{26}H_{39}N_2O_8$ requires M − I, 507.2706).

5-{[*N*-(3,4,5-Trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-2-[3-(5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-1-ethyl-3,3-dimethyl-2,3-dihydro-1*H*-indol-2-ylidene)propenyl]-1-ethyl-3,3-dimethyl-3*H*-indolium iodide 9

To a dry 10 cm^3 flask, under argon, were added a stirring bar, iodide **7** (19 mg, 3.5 μmol), pyridine (1 cm^3) and triethyl orthoformate (0.1 cm^3). The mixture was stirred at room temperature for 5 min and was then heated at reflux for 90 min. The mixture was cooled to room temperature and the volatile components were removed under high vacuum. The dark purple residue was dissolved in the minimum amount of methanol and this solution was poured into stirred ether (100 cm^3). The solid was filtered off and was subjected to reversed phase flash chromatography (50–80% methanol in water). The dye containing fractions were evaporated *in vacuo* and the residue was dissolved in methanol (2 cm^3). The solution was filtered and the methanol was removed *in vacuo* to afford **9** as a dark red solid (11.2 mg, 66%); mp 163–180 °C (decomp.); R_f 0.08 [$C_{18}SiO_2$, water–methanol (1:1)]; ν_{max}/cm^{-1} 2968, 2929, 1645, 1560, 1450, 1427, 1367, 1342, 1255, 1199, 1120, 1041, 817, 688, 565, 449; $\delta_H(D_2O)$ 1.22 (6H, t, *J* 7.1, 2 × CH_2CH_3), 1.57 (12H, s, 4 × Ar-CH_3), 2.95 (6H, s, OCH_3), 3.04 (2H, dd, *J* 8.9 and 9.7, 3-H), 3.22 (2H, dd, *J* 7.7 and 14.1, 5-H), 3.32 (2H, dd, *J* 3.7 and 9.8, 2-H), 3.39–3.53 (10 H, m, 4-H, 6-H and Ar-CH_2), 3.94 (4H, q, *J* 7.4, N-CH_2), 4.51 (2H, d, *J* 3.7, 1-H), 6.14 (2H, d, *J* 13.6 , Ar-C*H*), 7.12 (2H, d, *J* 8.3, 7′-H), 7.22 (2H, dd, *J* 1.5 and 8.3, 6′-H), 7.32 (2H, d, *J* 1.5, 4′-H), 8.23 (1H, t, *J* 13.5, Ar-CHC*H*); δ_C 12.1, 27.3, 39.4, 40.3, 42.2, 54.8, 69.9, 71.4, 71.5, 73.0, 99.2, 102.2, 111.1, 123.2, 129.6, 132.7, 140.5, 141.3, 173.7, 174.0; *m/z* (FAB) 851.3 (M − I, 33%), 550.5 (17), 307 (37), 176 (60) (Found: M − I, 851.4417. $C_{45}H_{63}N_4O_{12}I$ requires M − I, 851.4442).

2-[3-(1-{5-Carboxypentyl}-5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-3,3-dimethyl-2,3-dihydro-1*H*-indol-2-ylidene)propenyl]-1-ethyl-5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-3,3-dimethyl-3*H*-indolium iodide 10

To a dry 25 cm^3 flask, under argon, were added a stirring bar, acid **8** (35.5 mg, 56 μmol), iodide **7** (32 mg, 55 μmol), pyridine (1 cm^3) and triethyl orthoformate (0.25 cm^3). The mixture was stirred at room temperature for 5 min and was then heated at reflux for 75 min. The mixture was cooled to room temperature and the volatile components were removed under high vacuum. The dark purple residue was subjected to reversed phase flash chromatography (0–80% methanol in water). The unsymmetrical dye containing fractions were evaporated *in vacuo* to afford a dark red residue, which was dissolved in water (0.5 cm^3) and subjected to size exclusion chromatography on sephadex G15 (25 × 1 cm) with water as eluent. The dye containing fractions were evaporated *in vacuo* and the residue was dissolved in methanol (2 cm^3). The solution was filtered and the methanol was removed *in vacuo* to afford **10** as a dark red solid (14 mg, 24%); mp 166–179 °C (decomp.); R_f 0.25 [$C_{18}SiO_2$, water–methanol (1:1)]; ν_{max}/cm^{-1} 2919, 2850, 1603, 1560, 1504, 1466, 1427, 1368, 1190, 1160, 1054, 801, 739, 689, 467; $\lambda_{max}(H_2O)/nm$ 552 ($\varepsilon/dm^3\ mol^{-1}\ cm^{-1}$ 135 000), 520 (90 000); $\delta_H(D_2O)$ 1.14 (3H, t, *J* 6.8, CH_2CH_3), 1.17–1.26 (2H, br quintet, central CH_2), 1.39–1.50 (14H, m, 4 × Ar-CH_3 and $CH_2CH_2CO_2H$), 1.53–1.66 (2H, br quintet, N-CH_2CH_2), 2.09 (2H, t, *J* 7.2, CH_2CO_2H), 2.95 (6H, s, OCH_3), 3.04 (2H, t, *J* 9.2, 3-H), 3.18 (2H, dd, *J* 7.5 and 14.1, 5-H), 3.33 (2H, dd, *J* 3.8 and 9.3, 2-H), 3.38–3.52 (10H, m, 4-H, 6-H, and Ar-CH_2), 3.78–3.92 (4H, br m, N-CH_2), 4.50 (2H, d, *J* 3.7, 1-H), 6.04 and 6.07 (2H, overlapping d, *J* 13.3 , Ar-C*H*), 7.03–7.09 (2H, m, 7′-H), 7.11–7.17 (2H, m, 6′-H), 7.24 (2H, s, 4′-H), 8.19 (1H, t, *J* 13.3, Ar-CHC*H*); δ_C 11.9, 25.9, 26.3, 26.9, 27.3, 27.4, 37.7, 39.4, 40.4, 42.4, 44.1, 54.8, 70.1, 71.5, 71.6, 73.1, 99.3, 102.2, 102.4, 111.3, 111.5, 123.2, 129.7, 132.8, 140.7, 141.2, 141.5, 141.7, 150.5, 174.1, 174.4, 174.5, 183.2; *m/z* (FAB) 937.5 (M − I, 60%), 391 (100%) (Found: M − I, 937.4799. $C_{49}H_{69}N_4O_{14}I$ requires M − I, 937.4810).

1-(5-Carboxypentyl)-2-[3-(1-{5-carboxypentyl}-5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-3,3-dimethyl-2,3-dihydro-1*H*-indol-2-ylidene)propenyl]-5-{[*N*-(3,4,5-trihydroxy-6-methoxytetrahydropyran-2-ylmethyl)carbamoyl]methyl}-3,3-dimethyl-3*H*-indolium iodide 11

To a dry 25 cm^3 flask, under argon, were added a stirring bar, acid **8** (100 mg, 160 μmol), pyridine (1 cm^3) and triethyl orthoformate (0.150 cm^3). The mixture was stirred at room temperature for 5 min and was then heated at reflux for 90 min. The mixture was cooled to room temperature and the volatile components were removed under high vacuum. The dark purple residue was subjected to reversed phase flash chromatography (0–50% methanol in water). The dye containing fractions were evaporated *in vacuo* to afford a dark red residue, which was dissolved in water (0.5 cm^3) and subjected to size exclusion

chromatography on Sephadex G15 (25 × 1 cm) with water as eluent. The dye containing fractions were evaporated *in vacuo* and the residue was dissolved in methanol (2 cm^3). The solution was filtered and the methanol was removed *in vacuo* to afford **11** as a dark red solid (21 mg, 23%); mp 181–198 °C (decomp.); R_f 0.55 [$C_{18}SiO_2$, water–methanol (1:1)]; ν_{max}/cm^{-1} 2962, 2920, 2852, 1645, 1556, 1450, 1425, 1132, 1097, 1051, 804, 619, 459; $\delta_H(D_2O)$ 1.17–1.26 (4H, m, central CH_2), 1.40–1.52 (16H, m, 4 × Ar-CH_3 and CH_2CH$_2$CO$_2$H), 1.57–1.68 (4H, m, N-CH$_2$CH_2), 2.09 (4H, t, *J* 7.3, CH_2CO$_2$H), 2.95 (6H, s, OCH_3), 3.03 (2H, t, *J* 9.3, 3-H), 3.17 (2H, dd, *J* 7.7 and 14.4, 5-H), 3.31 (2H, dd, *J* 3.8 and 9.7, 2-H), 3.37–3.52 (10H, m, 4-H, 6-H, and Ar-CH_2), 3.87 (4H, br t, N-CH_2), 4.50 (2H, d, *J* 3.7, 1-H), 6.04 (d, *J* 13.4, Ar-C*H*), 7.06 (2H, d, *J* 8.3, 7′-H), 7.17 (2H, dd, *J* 0.8 and 8.3, 6′-H), 7.26 (2H, d, *J* 0.8, 4′-H), 8.23 (2H, t, *J* 13.5, Ar-CHC*H*); δ_C 25.3, 26.0, 26.7, 27.3, 36.4, 40.3, 42.3, 44.0, 49.2, 54.7, 69.9, 71.4, 71.5, 73.0, 99.2, 102.3, 111.5, 123.1, 129.6, 132.7, 141.1, 141.4, 150.4, 174.2, 174.5, *m/z* (FAB) 1023.5 (M − I, 100%), 1045 (17), 391 (12) (Found: M − I, 1023.5173. $C_{53}H_{75}N_4O_{16}I$ requires M − I, 1023.5178).

Active ester 12

To a dry 1 cm^3 microscale reaction vial was added dye **10** (11.8 mg, 12.1 μmol), tetramethyl(succinimido)uronium tetrafluoroborate (11.2 mg, 37.2 μmol), dry DMF (0.120 cm^3), and Pr^i_2-EtN (0.010 cm^3). The vial was sealed and the mixture was stirred at room temperature for 1 hour. The mixture was transferred to a 25 cm^3 round bottomed flask using acetonitrile (0.40 cm^3) and the volatile components were removed under high vacuum. The residue was treated with acetonitrile (0.20 cm^3) and ether (10 cm^3) was added to precipitate the dye components. The supernatant liquid was removed *via* a filter tipped cannulating needle. Acetonitrile was added again and the dye components were precipitated by addition of ether. The supernatant liquid was removed as before. The solid residue was washed twice with methylene chloride (2 × 3 cm^3), was dried under high vacuum for 30 min, and was then treated with water (0.30 cm^3). The mixture was filtered through a cotton wool plug and was lyophilized to afford the active ester (12.2 mg, 95%), R_f 0.12 [$C_{18}SiO_2$, water–methanol (1:1)].

Dextran labeling

To a solution of aminodextran (*M*r 40000, approx. 7.2 amino groups per dextran) (8.5 mg, 0.2 μmol) in 0.02 M carbonate–hydrogen carbonate buffer pH 9.5 (1.0 cm^3) was added a solution of active ester **12** (2.3 mg, 2 μmol) in DMF (0.050 cm^3). After mixing, the solution was allowed to stand overnight, and was then filtered. The solution was loaded onto a column of Sephadex G50 (3.5 × 15 cm) and the column was eluted with buffer (10 mM Tris, 50 mM NaCl, 1 mM NaN_3, pH 7.5). The labeled dextran was collected ahead of the hydrolysed labeling reagent. The solution was dialysed against water (3 × 800 cm^3) and the aqueous solution was lyophilized to afford the labeled dextran as a pink–red solid.

Protein labeling[9]

A stock solution of sheep IgG (*M*r 155000) was prepared at a concentration of 4 mg cm^{-3} (approx. 2.6 μM) in a carbonate–hydrogen carbonate buffer (0.02 M, pH 9.5.) Aliquots (0.25 cm^3) of the stock were dispensed into vials. A stock solution of dye active ester **12** was prepared at a concentration of 5 mM (approx. 1 mg per 0.20 cm^3) in water. Aliquots of the dye stock solution were added to those of the protein to afford a series of starting dye to protein ratios in the range 1.5:1 to 20:1. The solutions were mixed well and left to stand at room temperature overnight. The samples were filtered and then were loaded onto a column of sephadex G50 (0.7 × 12 cm) which was eluted with PBS (pH 7.5). The labeled protein was collected ahead of the hydrolysed dye and was studied without further purification. Calculation of final dye to protein (D/P) ratios and antibody brightness (AB) was performed as described in ref. 9.

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