MAY/JUNE 2002
Volume 13, Number 3

Bioconjugate Chemistry

COMMUNICATIONS

Facile Synthesis of Thiol-Reactive Cy3 and Cy5 Derivatives with Enhanced Water Solubility

Alexei Toutchkine, Perihan Nalbant, and Klaus M. Hahn*

Department of Cell Biology, Scripps Research Institute, 10550, N. Torrey Pines Road, BCC162, La Jolla, California 92037. Received September 25, 2001

The cyanine dyes Cy3 and Cy5 have proven valuable in numerous applications involving conjugation with proteins. Practical syntheses of lysine-selective, succinimidyl ester derivatives of these dyes have been published, and succinimidyl esters are commercially available. However, the published syntheses of cysteine-selective derivatives produce relatively low yields from expensive starting materials, or produce molecules with marginal water solubility for protein labeling. We report here facile syntheses (four steps, >50% overall yield) of iodoacetamide, sulfhydryl-reactive derivatives of the Cy3 and Cy5 fluorophores. These novel derivatives have good water solubility (>2.5 mM) and bear only one reactive side chain, reducing possible protein cross-linking encountered with previous derivatives.

INTRODUCTION

The cyanine dyes Cy3 and Cy5, originally described by Waggoner and co-workers (*1*, *2*), have proven their utility in a wide range of applications. These dyes are bright and photostable, and are well suited to microscopy of living cells. Lysine-reactive succinimidyl ester derivatives of Cy3 and Cy5 for protein labeling can be readily made using published syntheses (*2*, *3*) and are commercially available. Because lysine is relatively abundant in most proteins, lysine-reactive dyes are valuable for introducing several dyes on the same protein (i.e. in antibody labeling for immunofluorescence staining). Lysine-reactive dyes, however, cannot readily be used for precise site-specific dye attachment. Site-specific labeling is usually accomplished through conjugation to cysteine, which is less abundant and can be introduced at the desired site using molecular biology approaches. Site-specific labeling is important in many potentially valuable applications of the cyanine dyes, including fluorescence resonance energy transfer (FRET) microscopy, where dyes must be placed to respond to protein activity without perturbing normal biological behavior (*4*−*6*).

This paper addresses the current inaccessibility of water soluble, cysteine-reactive Cy3 and Cy5 derivatives. Ernst et al. have described cysteine-reactive analogues of these dyes which are substantially less water soluble than the lysine-reactive derivatives and are therefore difficult to use for protein labeling. More recently, Gruber et al. produced water-soluble cysteine-reactive derivatives by linking the succinimidyl ester of the lysine-reactive dyes to chains bearing different thiol-reactive groups (*7*). The dyes used as starting materials for this approach are available only in small quantities and are very expensive. Moreover, Gruber et al. did not prepare any iodoacet-

* To whom correspondence should be addressed. Phone (858) 784-8726, Fax (858) 784-8764, E-mail: khahn@scripps.edu.

10.1021/bc015558q CCC: $22.00
Published on Web 03/27/2002

amido derivatives, which may be advantageous in certain applications.

Here we describe straightforward, high-yielding syntheses of Cy3 and Cy5 derivatives bearing cysteine-selective iodoacetamide reactive groups. The new syntheses place two water-solubilizing sulfonate groups on the dyes, producing reagents with good water solubility for practical protein labeling. Only a single reactive side chain is introduced, minimizing problems with protein cross-linking. The new dyes are referred to as neo-Cy3 and neo-Cy5.

EXPERIMENTAL PROCEDURES

Materials. Analytical grade reagents were purchased from major suppliers.

5-Chloroacetamido-1,3,3-trimethyl-2-methyleneindoline (IV) and 5-chloroacetamidomethyl-1,3,3-trimethyl-2-methyleneindoline (VII) were prepared by the method of Gale (*8*, *9*). The potassium salt of 2,3,3-trimethylindolenine-5-sulfonate was prepared by the method of Waggoner (*2*).

Methods. UV−vis spectra were measured using a Hewlett-Packard 8453 diode array spectrophotometer. Emission and excitation spectra were obtained using a Spex Fluorolog 2 spectrofluorometer at 23 °C. Quantum yields were measured using merocyanine 540 (*10*) or indotricarbocyanine (*11*) as internal standards (*12*). Mass spectra were obtained by MALDI−FT MS techniques on an IonSpec FT MS spectrometer. ^{1}H NMR spectra of 0.5% solutions in CD_3SOCD_3 were recorded on Bruker DRX-400 or DRX-500 spectrometers. The peaks corresponding to the residual protons of CD_3SOCD_3 (2.49 ppm) were used as internal reference. All operations with dyes were performed under dim light. Flasks containing dyes were wrapped with aluminum foil.

2,3,3-Trimethyl-1-(3-sulfonatopropyl)-3*H*-indolinium-5-sulfonate (I). The potassium salt of 2,3,3-trimethylindolenine-5-sulfonate (*2*) (1.49 g, 0.006 mol, vacuum-dried) and 1,3-propane sultone (0.80 g, 0.0066mol) were mixed in 10 mL of dichlorobenzene and heated at 120 °C for 12 h with stirring under nitrogen. The mixture was cooled, 1,2-dichlorobenzene was decanted, and the solid was washed with ether to give practically pure quaternary salt. The yield was 2.20 g (90%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.52 (s, 6H, 2 × CH_3), 2.13 (p, $^3J_{H-H}$ = 7.5 Hz, 2H, $CH_2CH_2CH_2$), 2.65 (t, $^3J_{H-H}$ = 7.5 Hz, 2H, CH_2SO_3), 2.82 (s, 3H, CH_3), 4.61(t, $^3J_{H-H}$ = 7.5 Hz, 2H, CH_2N), 7.70−8.00 (m, 3H, aromatic ring).

2-{(*E*)-2-[Acetyl(phenyl)amino]ethenyl}-3,3-dimethyl-1-(3-sulfonatopropyl)-3*H*-indolinium-5-sulfonate (II). A mixture of the quaternary salt I (2.00 g) and *N*,*N*′-diphenylformamidine (2.00 g) in 10 mL of acetic acid−acetic anhydride (1:2) was heated to reflux for 2 h. The solvent was removed on a rotary evaporator, and the product was washed with acetone until free yellow powder was obtained. The yield was 2.64 g (97%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.71 (s, 6H, 2 × CH_3), 1.84 (p, $^3J_{H-H}$ = 7.5 Hz, 2H, $CH_2CH_2CH_2$), 2.15 (s, 3H, CH_3CO), 2.65 (t, $^3J_{H-H}$ = 7.5 Hz, 2H, CH_2SO_3), 4.21(t, $^3J_{H-H}$ = 7.5 Hz, 2H, CH_2N), 5.40 (d, $^3J_{H-H}$ = 14 Hz, C=C−H), 7.50−8.00 (m, 3H, aromatic ring) 9.05 (d, $^3J_{H-H}$ = 14 Hz, C=C−H).

2-{(1*E*,3*Z*)-3-[5-(Iodoacetylamino)-1,3,3-trimethyl-1,3-dihydro-2*H*-indol-2-ylidene]prop-1-enyl}-1-(3-sulfonatopropyl)-3*H*-indolium-5-sulfonate (Neo-Cy3, VI). A mixture of 1.35 g of anyl II and 0.65 g of 5-chloroacetamido-1,3,3-trimethyl-2-methyleneindoline IV was heated in 20 mL of acetic anhydride at 130 °C with stirring under nitrogen for 45 min. The mixture was cooled to room temperature, and the dye was separated by filtration. The crude dye was chromatographed over silica gel, eluting with acetone−methanol (3:1), to yield 1.15 g (71%) of pure chloroacetamido cyanine V. This dye was refluxed for 3 h in methanol (30 mL) containing 2.0 g of sodium iodide. The solution was cooled, filtered and concentrated in a vacuum to 10 mL. The dye was precipitated by the addition of 100 mL of acetone to give 1.2 g of dye VI (92%). The analytical sample of the dye was purified by HPLC on a Vydac C-18 column (no. 218TP152022, 22 × 250 mm, 15−20 μm), 3 mL/min water−acetonitrile gradient.

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.74 (s, 6H, 2 × CH_3), 1.76 (s, 6H, 2 × CH_3), 2.08 (p, $^3J_{H-H}$ = 7.0 Hz, 2H, $CH_2CH_2CH_2$), 2.70 (t, $^3J_{H-H}$ = 7.0 Hz, 2H, CH_2SO_3), 3.71 (s, 3H, CH_3, N−CH_3), 3.91 (s, 2H, CH_2, CH_2I), 4.30 (t, $^3J_{H-H}$ = 7.0 Hz, 2H, CH_2N), 6.48 (d, $^3J_{H-H}$ = 13.6 Hz, 1H, C=C−H), 6.62 (d, $^3J_{H-H}$ = 13.6 Hz, 1H, C=C−H), 7.48−7.90 (m, 6H, aromatics), 8.38 (t, $^3J_{H-H}$ = 13.6 Hz, C=C−H), 10.59 (s, 1H, NH−CO). MALDI−FTMS: MH^+ found: 750.0746, calculated: 750.0775.

2-[(1*E*,3*E*)-4-Acetyl(phenyl)aminobuta-1,3-dienyl]-3,3-dimethyl-1-(3-sulfonatopropyl)-3*H*-indolium-5-sulfonate (III). A mixture of the quaternary salt I (2.00 g) and malonaldehyde dianilide hydrochloride (0.65 g) in 10 mL of acetic acid−acetic anhydride (1:2) was heated at reflux for 2 h. The solution of anyl was cooled to room temperature and used without further purification in the next step.

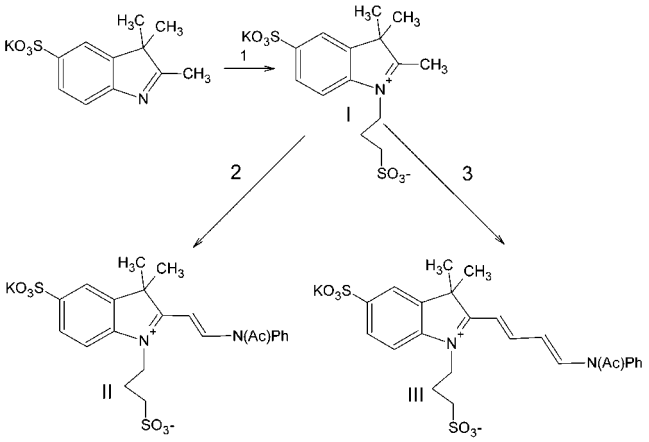
2-[(1*E*,3*E*,5*Z*)-5-(5-(Iodoacetylamino)-1,3,3-trimethyl-1,3-dihydro-2*H*-indol-2-ylidene)penta-1,3-dienyl]-1-(3-sulfonatopropyl)-3*H*-indolium-5-sulfonate (neo-Cy5, VIII). The solution of anyl III was mixed with 1.39 g of 5-chloroacetamidomethyl-1,3,3-trimethyl-2-methyleneindoline VII. The mixture was heated to 120 °C for 20 min and cooled to room temperature. The diethyl ether was added to the mixture to precipitate the dye. The crude dye was chromatographed over silica gel, eluting with acetone−methanol (3:1), to yield 2.14 g (61%) of pure chloroacetamido cyanine (VIII). This dye was refluxed in 30 mL of methanol containing 2.0 g of sodium iodide for 3 h. The solution was cooled, filtered, and concentrated in a vacuum to 10 mL. The dye was precipitated by the addition of 100 mL of acetone to give 2.30 g (95%) of dye IX. The analytical sample of the dye was purified by HPLC on a Vydac C-18 column (the same condition as for neo-Cy3 dye).

^{1}H NMR (400 MHz, DMSO-d_6): δ 1.67 (s, 12H, 4xCH_3), 1.98 (p, $^3J_{H-H}$ = 7.3 Hz, 2H, $CH_2CH_2CH_2$), 2.67 (t, $^3J_{H-H}$ = 7.3 Hz, 2H, CH_2SO_3), 3.60 (s, 3H, CH_3, N−CH_3), 3.71 (s, 2H, CH_2, CH_2I), 4.28 (t, $^3J_{H-H}$ = 7.3 Hz, 2H, CH_2N), 4.32 (d, $^3J_{H-H}$ = 5.6 Hz, 2H, CH_2−NH), 6.28 (d, $^3J_{H-H}$ = 14.1 Hz, 1H, C=C−H), 6.40−8.60 (m, 11H). MALDI−FTMS: MH^+found: 790.1078,calculated: 790.1088.

Preparation of Neo-Cy3−Cysteine Conjugate. A 30 mg amount of neo-Cy3 dye was added to 30 mg of cysteine in 2.0 mL of $NaHCO_3$−Na_2CO_3 buffer (pH = 8.0), and the mixture was stirred for 2 h at room temperature. Silica gel TLC (CH_3CN−H_2O: 90−10) showed complete consumption of starting dye. Several drops of hydrochloric acid were added to the mixture until pH = 5, and the conjugate was isolated by preparative HPLC on Vydac C18 column (same condition as for neo-Cy3 dye).

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.74 (s, 6H, 2 × CH_3), 1.76 (s, 6H, 2 × CH_3), 2.08 (p, $^3J_{H-H}$ = 7.0 Hz, 2H, $CH_2CH_2CH_2$), 2.70 (t, $^3J_{H-H}$ = 7.0 Hz, 2H, CH_2SO_3), 3.71

Scheme 1. Synthesis of Acetanilidovinyl Salts[a]

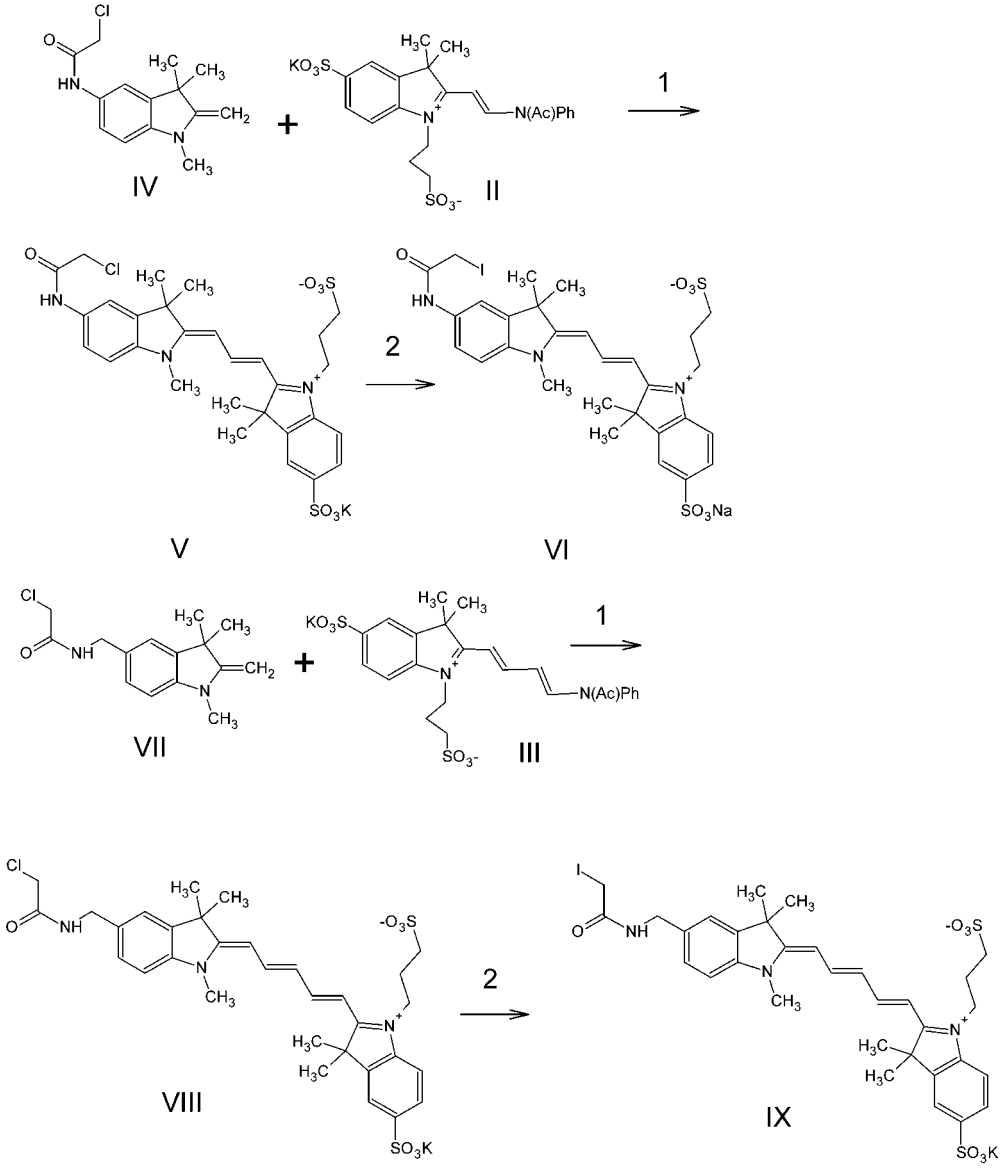


[a] Reagents and conditions: (1) propane sultone, 120 °C; (2) diphenylformamidine, Ac_2O; (3) malonaldehyde dianyl monochloride, $AcOH-Ac_2O$.

(s, 3H, CH_3, $N-CH_3$), 3.91 (s, 2H, CH_2, CH_2I), 4.30 (t, $^3J_{H-H}$ = 7.0 Hz, 2H, CH_2N), 6.48 (d, $^3J_{H-H}$ = 13.6 Hz, 1H, C=C−H), 6.62 (d, $^3J_{H-H}$ = 13.6 Hz, 1H, C=C−H), 7.48−7.90(m, 6H, aromatics), 8.38 (t, $^3J_{H-H}$ = 13.6 Hz, C=C−H), 10.59 (s, 1H, NH−CO). MALDI−FTMS: MH^+ found:743.1868, calculated: 743.185.

Protein Labeling. A fragment of Wiskott Aldrich Syndrome Protein (WASP, residues 201−320), mutated to contain a single cysteine (F271C), was labeled with Cy3 and separately with Cy5 dye. A stock solution of the dye in DMSO (10−20 mM) was added to 200 μL of protein solution (200 μM in sodium phosphate buffer, pH = 7.5), to produce a final dye concentration of 1−2 mM. The reaction mixture was incubated for 4 h at room temperature and then quenched by addition of 1 μL mercaptoethanol. The reaction mixture was spun at 12000 rpm for 2 min to remove any precipitates, which might have formed during the reaction, and the supernatant was purified using G25 sepharose gel filtration. The dye−protein adduct was clearly separated from free dye during gel-filtration. Purity of the conjugates was confirmed by SDS−PAGE electrophoresis. No free dye was seen in purified protein conjugates. Control samples of free dye were clearly visible on the gel at lower MW than protein. Conjugates formed single, highly colored fluorescent

Scheme 2. Synthesis of the Dyes[a]



[a] Reagents and conditions: (1) AcONa, Ac_2O, 120 °C; (2) NaI, MeOH, 65 °C.

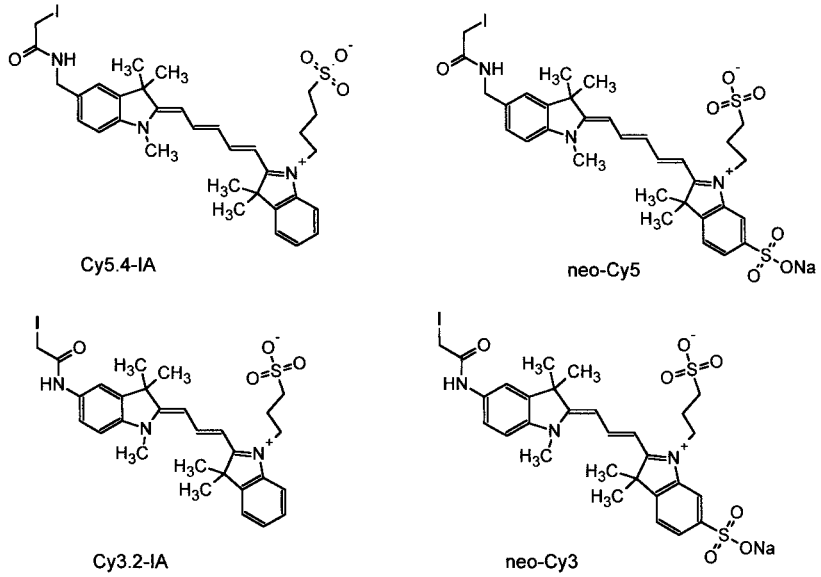


Figure 1. Structure of cysteine-reactive neo-Cy3 and neo-Cy5 dyes and previously described (*1*) Cy3.2-IA and Cy5.4-IA dye.

Table 1. Absorption and Fluorescence Data for Neo-Cy3, Neo-Cy5, and Labeled Substrates

dye	solvent	absorption, λ_{max}/nm (ϵ^a/M^{-1})	emission, λ_{max}/nm	ϕ^b
neo-Cy3, VI	PBS[c]	551 (98 000)	573	0.02
	MeOH	563 (95 000)	583	0.03
	DMSO	573 (97 000)	595	0.06
neo-Cy5, IX	PBS	643 (170 000)	660	0.10
	MeOH	648 (200 000)	665	0.22
	DMSO	656 (195 000)	675	0.25
neo-Cy3-cysteine	PBS	552 (107 000)	573	0.02
neo-Cy3-WASP fragment	PBS	551 (100 000)	572	0.02
neo-Cy5-WASP fragment	PBS	647 (180 000)	664	0.13
Cy3.2-IA[d]	EtOH	565 (116 000)	590	0.07
Cy5.4-IA[d]	EtOH	652 (128 000)	669	0.27

[a] Extinction coefficient, error is ±5%. [b] Quantum yield of fluorescence, error is ±10%. [c] Phosphate-buffered saline. [d] Reference 1.

protein bands with molecular weights corresponding to the WASP fragment. The dye-to-protein ratio was calculated by measuring protein and dye concentrations using absorbance spectroscopy as previously described (*13*). In each case this ratio was between 0.9 and 1.0.

RESULTS AND DISCUSSION

The most widely used thiol-reactive groups for protein modification are maleimide and iodoacetamide. Maleimide adducts have disadvantages in that they can hydrolyze, thereby causing significant changes in the fluorescence of protein conjugates, and they can undergo a nucleophilic ring-opening reaction with amines to yield cross-linked protein products (*13*). We therefore chose the iodoacetamide group as the reactive side chain for the new dyes. They contain one reactive side chain rather than the two found in some earlier derivatives. This abrogates problems of protein cross-linking and inhomogeneous conjugation sometimes seen during labeling.

The synthesis of both new cyanine dyes was accomplished using the same general method (*14*), in which Fischer's bases IV and VII were condensed with acetanilidovinyl salts II and III (Scheme 2). These straightforward syntheses produced the dyes in four steps with overall yields of 57% (neo-Cy3) and 50% (neo-Cy5). The reactivity of these iodoacetamido dyes was verified by facile formation of conjugates with cysteine and with purified protein (see Materials and Methods).

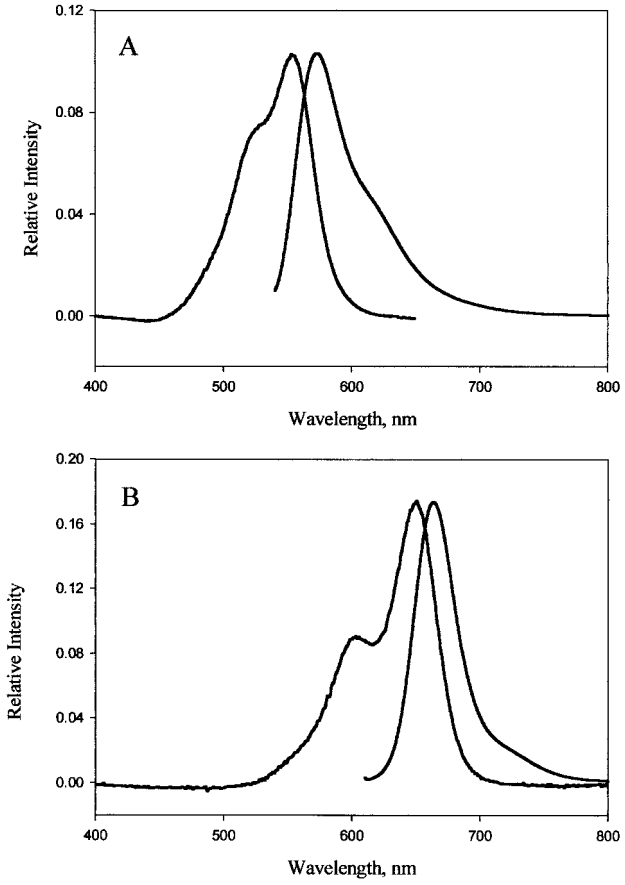


Figure 2. Normalized absorbance and fluorescence spectra of neo-Cy3-WASP (A) and neo-Cy5-WASP (B) conjugates in PBS (pH = 7.4), concentrations 1 μM.

Importantly, the neo-Cy dyes bear two sulfonate groups (Figure 1), and this leads to excellent water solubility (neo-Cy3, 3 mM; neo-Cy5, 2.5 mM), bringing accessible concentrations into the range typically used for protein labeling. The fluorescence properties of the neo-Cy dyes are given in Table 1. The quantum yields, extinction coefficients, and fluorescence maxima of the neo-Cy dyes deviate little from those reported for Cy3 and Cy5. As

with Cy3 and Cy5, the absorbance and emission maxima of the new dyes show little solvent sensitivity, while the quantum yields are greater in more hydrophobic (methanol) or more viscous (dimethyl sulfoxide) solvents (Table 1) as was previously reported for many polymethine dyes (*15*). The main nonfluorescent deactivation path for the exited state of cyanine dyes is photoisomerization around the central double bond, and the rate of this photoisomerization becomes smaller as hydrophobicity or viscosity of the solvent increases (*15*).

Attachment of the dyes to cysteine or to a protein (a fragment of WASP, see Materials and Methods) did not alter the dyes' favorable fluorescence properties (Table 1, Figure 2).

Both the absorbance and fluorescence spectra are similar to spectra of free dyes, indicating that there is no quenching of fluorescence after attachment of dyes to protein.

CONCLUSION

The many published applications of the original Cy3 and Cy5 dyes attest to their value. We hope that the straightforward syntheses presented here will make cysteine-reactive derivatives accessible to more researchers.

ACKNOWLEDGMENT

We are grateful to Velda Comstock for expert secretarial assistance. This work was supported by NIH grant GM57464 to K.M.H. and by a Leukemia & Lymphoma Society fellowship (3427-02) to A.T.

LITERATURE CITED

(1) Ernst, L. A., Gupta, R. K., Mujumdar, R. B., and Waggoner, A. S. (1989) Cyanine dye labeling reagents for sulfhydryl groups. *Cytometry 10*, 3−10.

(2) Mujumdar, R. B., Ernst, L. A., Mujumdar, S. R., Lewis, C. J., and Waggoner, A. S. (1993) Cyanine dye labeling reagents: sulfoindocyanines succinimidyl esters. *Bioconjugate Chem. 4*, 105−111.

(3) Reddington, M. V. (1998) New glycoconjugated cyanine dyes as fluorescent labeling reagents. *J. Chem. Soc., Perkin Trans. 1* 143−147.

(4) Kraynov, V. S., Chamberlain, C. E., Bokoch, G. M., Schwartz, M. A., Slabaugh, S., and Hahn, K. M. (2000) Localized Rac activation dynamics visualized in living cells. *Science 290*, 333−337.

(5) Kenworthy, A. K. (2001) Imaging protein−protein interactions using fluorescence resonance energy transfer microscopy. *Methods 24*, 289−296.

(6) Mi, Z., Mai, J., Lu, X., and Robbins, P. D. (2000) Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. *Mol. Ther. 2*, 339−347.

(7) Gruber, H. J., Kada, G., Pragl, B., Riener, C., Hahn, C. D., Harms, G. S., Ahrer, W., Dax, T. G., Hohenthanner, K., and Knaus, H.-G. (2000) Preparation of Thiol-Reactive Cy5 Derivatives from Commercial Cy5 Succinimidyl Ester. *Bioconjugate Chem. 11*, 161−166.

(8) Gale, D. J., and Wilshire, J. F. K. (1974) Fibre-Reactive Basic Dyes. I−Polymethine Dyes Containing the N−Chloroacetyl Group. *J. Soc. Dyers Colour. 90*, 97−100.

(9) Gale, D. J., Lin, J., and Wilshire, J. F. K. (1977) The Amidomethylation and Bromination of Fisher's Base. The Preparation of Some New Polymethine Dyes. *Aust. J. Chem. 30*, 689−694.

(10) Onganer, Y., Yin, M., Bessire, D. R., Quitevis, E. L. (1993) Dynamic Solvatation Effects on the Cis−Trans Isomerization Reaction: Photoisomerization of Merocyanine 540 in Polar Solvents. *J. Phys. Chem. 97*, 2344−2354.

(11) Sims, P. J., Waggoner, A. S., Wang, C. H., and Hoffman, J. F. (1974) Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry 13*, 3315−3330.

(12) Demas, J. N., and Crosby, G. A. (1971) The Measurement of Photoluminescence Quantum Yields. A Review. *J. Phys. Chem. 75*, 991−1024.

(13) Haugland, R. P. (1996) *Handbook of Fluorescent Probes and Research Chemicals*; Molecular Probes Inc., Eugene, OR.

(14) Hamer, F. M. (1964) The Cyanine Dyes and Related Compounds, in *The Chemistry of Heterocyclic Compounds* (A. Wiessberger, Ed.) Interscience, New York.

(15) Mandal, D., Pal, S. K., Sukul, D., and Bhattacharyya, B. (1999) Photophysical Processes of Merocyanine 540 in Solutions and in Organized Media. *J. Phys. Chem. A 103*, 8156−8159.

BC015558Q