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Synthesis and Reactivities of 3-Indocyanine-green-acyl-1,3-thiazolidine-2-thione (ICG-ATT) as a New Near-infrared Fluorescent-labeling Reagent

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Abstract—A new near-infrared fluorescent-labeling reagent (ICG-ATT) bearing the 3-acyl-1,3-thiazolidine-2-thione (ATT) moiety with the chemoselective acylation feature and the dye moiety of indocyanine green (ICG) has been developed. Synthesis and reactivities of the ICG-ATT are described.

Introduction

The polymethine dyes absorb and fluoresce in the near-infrared region (600–1200 nm).[1] They show large molar absorptivities and are extremely fluorescent. One of these dyes, indocyanine green (ICG) dye[2] is well known since it has been clinically used to diagnose liver activity. In the near-infrared region, there is minimum interference from absorption scattering and from fluorescence of biological molecules. Therefore, the dye-analogues have been considerable to become a new class of fluorescent labeling reagents. Until now, there have been some reports that the dye alone or its derivatives can link with proteins such as bovine serum albumin (BSA) and immuno gamma globulin (IgG).[3–7]

In these reports, it has been clarified that the ICG-labeled proteins can be exploited for their ultra micro-analysis through the measurement of fluorescence[6] and are capable of being a diagnostic reagent for the minute cancers in stomach.[7] Nagao et al. previously demonstrated some remarkable features in the aminolysis of various 3-acyl-1,3-thiazolidine-2-thione (ATT) derivatives by utilizing the active amide structure.[8,9] In the present article, we describe the synthesis of a new fluorescent labeling reagent, the indocyanine green amide derivative of 1,3-thiazolidine-2-thione (ICG-ATT) and its reactivities to several protected amino acids or proteins in order to investigate the ability of this new compound as a labeling reagent.

Results and Discussion

Preparation of the ICG-ATT

A synthetic method of ICG-ATT is shown in Scheme 1. 3-Nitrogen atom of 1,1,2-trimethylbenz[*e*]indole (**1**) was alkylated with ethyl iodide in acetonitrile under reflux for two days to afford compound (**2**) in 91% yield, which was treated with glutaconaldehyde dianilido hydrochloride in acetic anhydride at 100 °C for 1 h to obtain compound (**4**) in a quantitative yield. *N*-Alkylation of **1** with 6-iodohexanoic acid in acetonitrile (MeCN) under reflux for four days gave compound (**3**) quantitatively. Reaction of **3** with **4** in pyridine at 40 °C furnished an indocyanine green derivative (**5**) bearing a carboxylate group in 77% yield. Finally, **5** was treated with 1,3-thiazolidine-2-thione in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSCD·HCl) and a catalytic amount of dimethylaminopyridine (DMAP)

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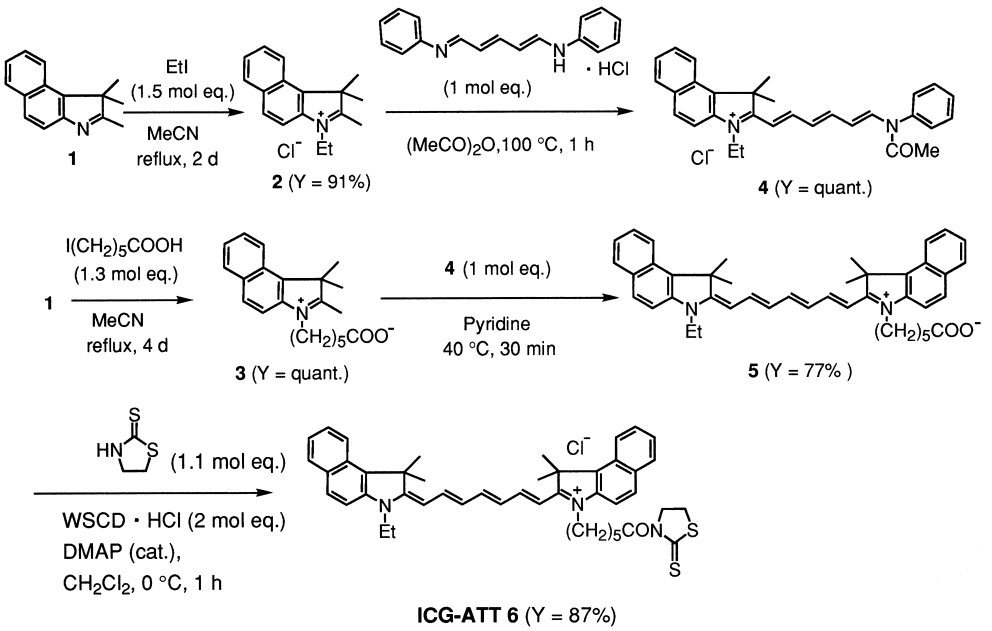


Scheme 1.

in CH_2Cl_2 at 0 °C to give the desired ICG-ATT (**6**) in 87 % yield.

Reaction of the ICG-ATT with amino acid derivatives

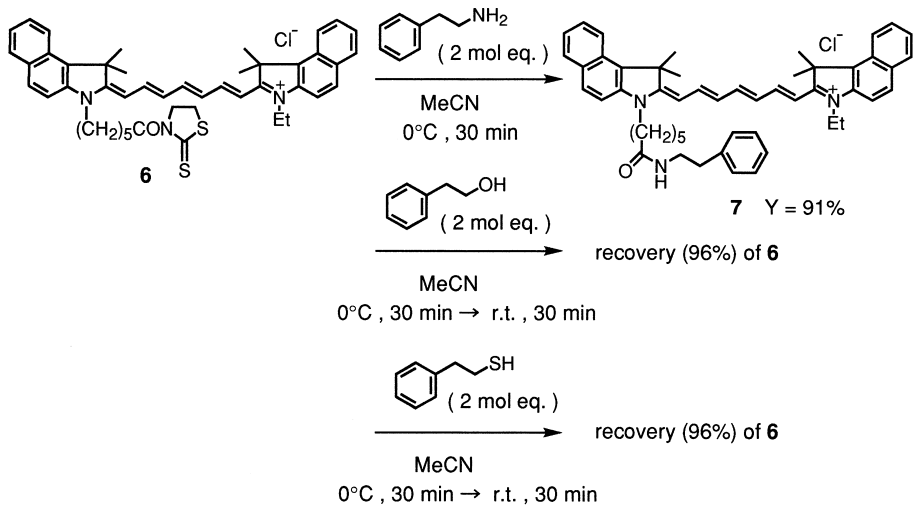
In general, the protein-labeling reagent must efficiently react with the functional groups such as NH_2-, SH-, and OH- groups of the corresponding amino acid residues in the proteins. In the case of ICG-ATT (**6**), a chemoselective reaction with the amino groups seems to be expected on the basis of the reactivity of the ATT moiety.[8] Specifically, the ε-amino group of the lysine residue must be a key target functional one. Thus, a series of coupling reactions of **6** with 1-amino-, 1-hydroxy-, and 1-mercapto-2-phenylethyl derivatives and some protected amino acid (lysine and glycine) derivatives were examined. All results are summarized in Scheme 2 and Table 1. As shown in Scheme 2, the ICG-ATT (**6**) reacted with 2-phenylethylamine in MeCN at 0 °C to give the amide compound (**7**) in 91% yield. However, similar treatment of **6** with 2-phenylethylalcohol or 2-pheneyethylthiol resulted in recovery (96%) of **6**, respectively as anticipated. Thus, the ICG-ATT (**6**) should be a chemoselective labeling reagent for the amino group as well as the usual ATT.[9]

Furthermore, it was clarified that the amino group of the protected lysine and glycine derivatives were promptly acylated by **6** under the mild conditions to give the corresponding amides (**8–10**) in good yields as shown in Table 1. The acylation reactions smoothly proceeded even in an aqueous acetonitrile or tetrahydrofuran solution at 0 °C or room temperature. Namely, these results suggested that this labeling reagent (**6**) could react with proteins in an aqueous solution to form the amide bond.

Labeling experiments of proteins with the ICG-ATT

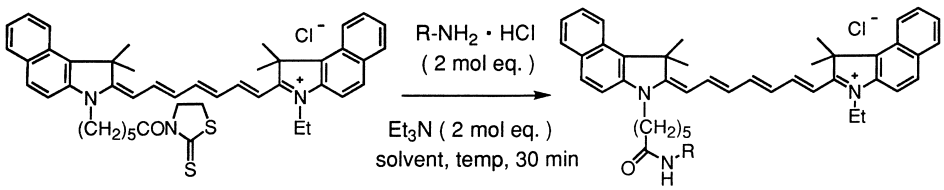
Labeling reactions of proteins were performed by treatment of bovine serum albumin (BSA) or bovine immuno gamma globulin (IgG) with an excess amount of **6** in a mixture of buffer [0.1 M phosphate buffer (pH 7.5) for BSA or 0.1 M carbonate buffer (pH 9.5) for IgG] and DMF solutions for 30 min at room temperature as schematically depicted in Scheme 3. Then the unbounded dye was removed by gel permeation (Sephadex LH-20) column chromatography. The ICG-to-protein ratio for each ICG-labeled protein (**12**) was determined as follows. The protein concentrations in **12** (BSA or IgG) were determined by using the protein-assay kit (Bio-Rad Inc., CA). The concentration of the total of ICG in **12** was obtained from the absorbance (at 789 nm) of the DMF solution and the ε values of the ICG derivative. Thus, the ratio (ICG/protein) was estimated to be 21.5 ± 3.8 (mean ± S.E.M.:n = 6) (BSA) or 23.6 ± 2.7 (n = 5) (IgG). It was clearly confirmed that the ICG-ATT could easily react with these proteins.

However, the ICG derivatives are well known to bind non-covalently to proteins. Therefore, in order to be sure of a meaningful ICG/protein ratio for the covalently



Scheme 2.

Table 1. Reaction of the ICG-ATT (**6**) with protected amino acids



8 : R = -$(CH_2)_4$CH(NHAc)COOMe

9 : R = -$(CH_2)_4$CH(NHZ)COOMe

10: R = -CH_2COOEt

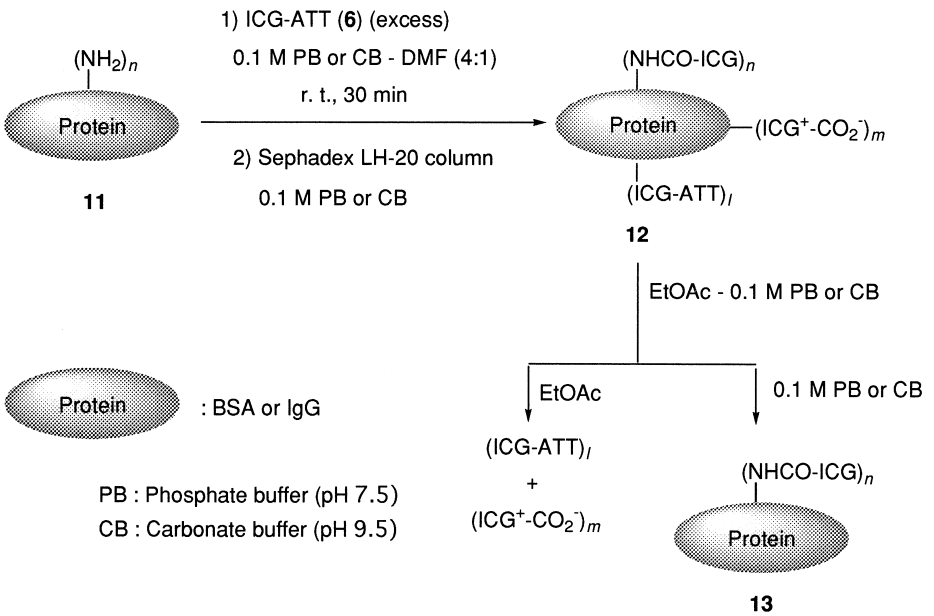
R-NH_2·HCl	Product	Solvent	Temperature	Yield (%)[a]
Ac-Lys-OMe·HCl	**8**	MeCN-H_2O (4:1)	rt	81
Ac-Lys-OMe·HCl	**8**	MeCN-H_2O (4:1)	0°C	86
Ac-Lys-OMe·HCl	**8**	MeCN-H_2O (4:1)	−10°C	47
Ac-Lys-OMe·HCl	**8**	THF-H_2O (4:1)	rt	80
Z-Lys-OMe·HCl	**9**	MeCN	rt	85
Z-Lys-OMe·HCl	**9**	MeCN	0°C	89
Gly-OEt·HCl	**10**	MeCN-H_2O (4:1)	rt	82
Gly-OEt·HCl	**10**	MeCN-H_2O (4:1)	0°C	36[b]

[a] Isolated yield.
[b] Determined by ^{1}H-NMR.

ICG-labeled protein (**13**), it is advisable to test the ICG-labelled protein (**12**) by subjecting it to extraction with ethyl acetate from the buffer solution. Namely, the covalently ICG-linked proteins are soluble in the buffer solution while the ICG derivative(s) which have been non-covalently linked can be extracted with ethyl acetate as shown in Scheme 3. The measurement of the absorbance (at 789 nm) in the buffer solution of **13** can lead us to assess the real degree of the covalently-linked ICG. Thus, the ratio of remaining ICG in the aqueous solution to the extracted ICG with ethylacetate was 44.3 ± 2.9 (n = 3)% in BSA or 69.2 ± 4.4 (n = 5)% in IgG which indicated that the ICG/protein ratio of **13** resulted in 9.5 (BSA) or 16.3 (IgG), respectively.

UV-vis and fluorescent spectral properties

The absorption and fluorescent properties of ICG-ATT (**6**), ICG-BSA (**12** and **13**), and ICG-IgG (**12** and **13**) are summarized in Table 2. The spectra of each compound



Scheme 3.

show that their λmax, λex, and λem exist in the near-infrared region. The spectral properties were reflected to the structure of the original ICG-dye moiety, and confirmed the potency of the ICG-ATT (**6**) as a near-infrared labeling reagent.

In conclusion, the ICG-ATT (**6**) newly prepared here proved to be a near-infrared fluorescent-labeling reagent useful for proteins and amino acid compounds. This reagent may also label other compounds such as nucleic acids, biologically important molecules, and drugs bearing the amino groups.

Experimental

All melting points were determined on a Yanagimoto micro apparatus and are uncorrected. The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL-FX-200 or a JEOL-GSX-400 spectrometer. Chemical shifts are given in δ values (ppm) using tetramethylsilane as an internal standard. Positive ion fast-atom bombardment (FAB) or electron impact (EI) mass spectra (MS) were obtained on a JEOL-SX-102A instrument. Elementary combustion analyses were within ±0.4% of theoretical values. UV-vis spectra were obtained using a Beckman 650-40 spectrometer. Fluorescence spectra were recorded on a Hitachi 650-40 luminescence spectrometer. Column chromatography was performed using Merck silica gel 60 (70–230 mesh). 1,1,2-Trimethylbenz[*e*]indole (**1**) was supplied by Daiichi Chemical Co. Ltd.

Table 2. UV-vis and fluorescent spectral data[a]

Compound	UV-vis abs (λmax)	Fluorescent	
		λex	λem
ICG-ATT (**6**)	789 nm	765 nm	830 nm
ICG-BSA (**12**, **13**)	789 nm	766 nm	828 nm
ICG-IgG (**12**, **13**)	789 nm	765 nm	826 nm

[a] All spectra were measured in a DMF solution.

1,1,2-Trimethyl-3-ethylbenz[*e*]indolium iodide salt (2). To a solution of 1,1,2-trimethylbenz[*e*]indole (**1**) (1.0 g, 4.8 mmol) in MeCN (40 mL) was added ethyl iodide (1.1 g, 7.2 mmol) and then the mixture was heated under reflux for two days. The reaction mixture was concentrated in vacuo and to the residue was added ether (80 mL). The resulting solids were repeatedly washed with ether to obtain the title compound (**2**) as dark purple solids (1.6 g, 91%). mp 213–218 °C (decomp); ^{1}H NMR (200 MHz, CD_3OD) δ 1.7 (t, J = 7.3 Hz, 2H), 1.9 (s, 6H), 3.2 (s, 3H), 4.9 (q, J = 7.3 Hz, 2H), 7.6–8.2 (m, 6H); HRFAB-MS m/z 238.1594 (calcd for $C_{17}H_{20}N$ 238.1596) M^+-HI + H; Anal. Calcd for $C_{17}H_{20}NI$: C, 55.90; H, 3.83. Found: C, 55.67; H, 3.65.

1,1,2-Trimethyl-3-(6-carboxylatohexyl)benz[*e*]indolium iodide salt (3). To a solution of 1,1,2-trimethylbenzindole (**1**) (5.7 g, 27.2 mmol) in MeCN (240 mL) was added 6-iodohexanoic acid (8.6 g, 35.4 mmol) and then the mixture was heated under reflux for 4 days. The

reaction mixture was concentrated in vacuo and to the residue was added ether (500 mL). The resulting solids were repeatedly washed with ether to obtain the title compound (**3**) as dark purple solids (9.8 g, quant.). mp 220–224 °C (decomp); ^{1}H NMR (200 MHz, CD_3OD) δ 1.6 (m, 2H), 1.7 (m, 2H), 1.8 (s, 6H), 2.1 (m, 2H), 2.4 (t, J = 6.8 Hz, 2H), 3.3 (s, 3H), 4.6 (t, J = 7.6 Hz, 2H), 7.7–7.9 (m, 2H), 8.0 (d, J = 9.0 Hz, 1H), 8.1–8.4 (m, 3H); HRFAB-MS m/z 324.1950 (calcd for $C_{21}H_{26}NO_2$ 324.1964) $M^+ + H$.

2-[6-(*N*-Phenyl-*N*-acetylamino)-1,3,5-heptatrienyl]-1,1-dimethyl-3-ethyl-1H-benz[*e*]indolium chloride salt (4). A suspension of compound (**2**) (8.0 g, 22.0 mmol) and glutaconaldehyde dianil hydrochloride (6.3 g, 22.0 mmol) in acetic anhydride (160 mL) was heated at 100 °C for 1 h. After cooling, the reaction mixture was poured into water (900 mL). The resulting solids were repeatedly washed with water to obtain the title compound (**4**) as dark red solids (11.0 g, quant.). mp 165–168 °C (decomp); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.6 (t, J = 7.1 Hz, 3H), 1.97 (s, 3H), 2.00 (s, 6H), 4.8 (m, 2H), 5.4 (t, J = 12.5 Hz, 1H), 6.9 (dd, J = 9.8, 10.0 Hz, 1H), 7.1–8.3 (m, 15H); HRFAB-MS m/z 435.2428 (calcd for $C_{43}H_{31}N_2O_2$ 435.2436) M^+-HCl + H.

2-[7-(1,3-Dihydro-1,1-dimethyl-3-ethylbenz[*e*]indolin-2-ylidene)-1,3,5-heptatrienyl]-1,1-dimethyl-3-(6-carboxylatohexyl)-1H-benz[*e*]indolium inner salt (5). A solution of compounds (**3**) (0.10 g, 0.31 mmol) and (**4**) (0.15 g, 0.31 mmol) in pyridine (2 mL) was stirred at 40 °C for 30 min. After removal of the solvent in vacuo, the residue was purified by silica gel chromatography eluting with $CHCl_3$-MeOH (100:1–10:1) to give the title compound (**5**) (0.15 g, 77%) as dark green solids. mp 179–183 °C (decomp); ^{1}H NMR(200 MHz, $CDCl_3$) δ 1.4 (t, J = 6.8 Hz, 3H), 1.5–2.0 (m, 6H), 1.9 (s, 12H), 2.4 (t, J = 6.6 Hz, 2H), 4.2 (m, 4H), 6.1 (d, J = 13.4 Hz, 1H), 6.3 (d, J = 13.7 Hz, 1H), 6.7 (t, J = 12.7 Hz, 2H), 7.3–8.2 (m, 15H).; HRFAB-MS m/z 623.3641 (calcd for $C_{43}H_{47}N_2O_2$ 623.3638) $M^+ + H$.

2-[7-(1,3-Dihydro-1,1-dimethyl-3-ethylbenz[*e*]indolin-2-ylidene)-1,3,5-heptatrienyl]-1,1-dimethyl-3-[[6-[(1,3-thiazolidine-2-thion)-1-yl]-6-oxo]hexyl]-1H-benz[*e*]indolium chloride salt (6). To a solution of compound (**5**) (200 mg, 0.321 mmol) in CH_2Cl_2 (4 mL) were added 1,3-thiazolidine-2-thione (42 mg, 0.353 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (123 mg, 0.642 mmol), and 4-dimethylaminopyridine (4 mg, 0.032 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h followed by addition of 0.1 N HCl (10 mL), and then extracted with $CHCl_3$ (10 mL×3). The $CHCl_3$ extract was washed with water and concentrated in vacuo. The oily residue was purified by silica gel chromatography eluting with $CHCl_3$-MeCN (30:1-4:1) to afford the title compound (**6**) as dark green solids (212 mg, 87%). mp 158–161 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.5 (t, J = 7.1Hz, 3H), 1.2–2.1 (m, 6H), 2.0 (s, 12H), 3.28 (t, J = 7.3 Hz, 2H), 3.34 (dd, J = 7.3, 7.8 Hz, 2H), 4.1–4.4 (m, 4H), 4.6 (dd, J = 7.3, 7.8 Hz, 2H), 6.3 (dd, J = 12.7, 13.2 Hz, 2H), 6.7 (m, 2H), 7.3–8.2 (m, 15H); HRFAB-MS m/z 724.3408 (calcd for $C_{46}H_{50}N_3OS_2$ 724.3395) M^+-HCl + H λex = 765 nm, λem = 830 nm (DMF).

2-[7-(1,3-Dihydro-1,1-dimethyl-3-ethylbenz[*e*]indolin-2-ylidene)-1,3,5-heptatrienyl]-1,1-dimethyl-3-[5-[*N*-(2-phenylethyl)aminocarbonyl]pentyl]-1H-benz[*e*]indolium chloride salt (7). To a solution of compound (**6**) (94 mg, 0.124 mmol) in MeCN (2.5 mL) was added 2-phenylethylamine (30 mg, 0.247 mmol) at 0 °C. After being stirred at 0 °C for 30 min, the reaction mixture was poured into 1N HCl and then extracted with $CHCl_3$ (10 mL×3). The $CHCl_3$ extract was washed with water (10 mL). After removal of the solvent in vacuo, the residue was purified by gel filtration chromatography on a Sephadex LH-20 (1.5×50 cm) column eluting with MeOH to afford the title compound (**7**) as dark green solids (85 mg, 91%). mp 132–135 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.5 (t, J = 7.1 Hz, 3H), 1.2–2.2 (m, 18H), 2.4 (t, J = 7.1 Hz, 2H), 2.9 (dd, J = 7.1, 8.3 Hz, 2H), 3.5 (dd, J = 6.6, 8.3 Hz, 2H), 4.0–4.3 (m, 4H), 6.1 (d, J = 13.4 Hz, 1H), 6.3 (d, J = 13.7 Hz, 1H), 6.5–6.8 (m, 2H), 7.1–8.2 (m, 20H); HRFAB-MS m/z 726.4408 (calcd for $C_{51}H_{56}N_2O$ 726.4423) M^+-HCl + H.

Treatment of ICG-ATT (6) with 2-phenylethylalcohol or 2-phenylethylthiol. To a solution of compound (**6**) (29 mg, 0.038 mmol) in MeCN (2.5 mL) was added 2-phenylethylalcohol (9.6 mg, 0.077 mmol) at 0 °C. After being stirred at 0 °C for 30 min and then at room temperature for 30 min, the reaction mixture was poured into 1N HCl and then extracted with $CHCl_3$ (10mL×3). The $CHCl_3$ extract was washed with water (10 mL) followed by evaporation in vacuo to give an oily residue. The residue was chromatographed on a silica gel colunm eluting with $CHCl_3$-MeOH (100:1-4:1) to give the compound (**6**) as dark green solids in 96% recovery. Similar treatment of **6** (30 mg, 0.040 mmol) with 2-phenylethylthiol (11.1 mg, 0.079 mmol) afforded the starting compound (**6**) (29 mg) in 96% recovery.

***N*-ε-[6-[2-[7-(1,3-Dihydro-1,1-dimethyl-3-ethylbenz[*e*]indolin-2-ylidene)-1,3,5-heptatrienyl]-1,1-dimethyl-1H-benz-[*e*]indolium-3-yl]hexanamide]-*N*-α-acetyl-L-lysine methylester chloride salt (8).** To a solution of compound (**6**) (56 mg, 0.074 mmol) in MeCN (20 mL) were added a solution of *N*-α-acetyl-L-lysine methylester hydrochloride (35 mg, 0.147 mmol) in water (0.5 mL) and triethylamine (15 mg, 0.147 mmol) at 0 °C. After being stirred at 0 °C for 30 min, the reaction mixture was

poured into 1N HCl (10 mL) and then extracted with $CHCl_3$ (10 mL×3). The $CHCl_3$ extract was washed with water (10 mL). After removal of the solvent in vacuo, the residue was purified by gel filtration chromatography on a Sephadex LH-20 (2.0×50 cm) column eluting with MeOH to afford the title compound (**8**) as dark green solids (54 mg, 86%). mp 126–129 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.8–2.0 (m, 27H), 2.1 (s, 3H), 2.5 (t, J = 6.7 Hz, 2H), 3.2–3.4 (m, 2H), 3.7 (s, 3H), 4.1–4.3 (m, 4H), 4.4–4.5 (m, 1H), 6.1 (d, J = 13.4 Hz, 1H), 6.4 (d, J = 13.7 Hz, 1H), 6.6 (dd, J = 12.2, 12.7 Hz, 1H), 6.9 (dd, J = 10.3, 13.2 Hz, 1H), 7.1–8.2 (m, 15H); HRFAB-MS m/z 807.4827 (calcd for $C_{52}H_{63}N_4O_4$ 807.4849) M^+-HCl + H.

***N*-ε-[6-[2-[7-(1,3-Dihydro-1,1-dimethyl-3-ethylbenz[*e*]indolin-2-ylidene)-1,3,5-heptatrienyl]-1,1-dimethyl-1H-benz[*e*]-indolium-3-yl]hexanamide]-*N*-α-benzyloxycarbonyl-L-lysine methylester chloride salt (9).** The title compound (**9**) was prepared by the similar method described above and obtained as dark green solids (89%). mp 142–144 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.2–2.2 (m, 27H), 2.4 (t, J = 6.7 Hz, 2H), 3.2 (m, 2H), 3.7 (s, 3H), 4.0–4.4 (m, 5H), 5.1 (s, 2H), 5.7 (d, J = 7.6 Hz, 1H), 6.1 (d, J = 13.4 Hz, 1H), 6.4 (d, J = 13.2 Hz, 1H), 6.5–6.9 (m, 2H), 7.1–8.2 (m, 19H); HRFAB-MS m/z 899.5142 (calcd for $C_{58}H_{67}N_4O_5$ 899.5111) M^+-HCl + H.

***N*-[6-[2-[7-(1,3-Dihydro-1,1-dimethyl-3-ethylbenz[*e*]indolin-2-ylidene)-1,3,5-heptatrienyl]-1,1-dimethyl-1H-benz[*e*]indolium-3-yl]hexanamide]-glycine ethylester chloride salt (10).** The title compound (**10**) was prepared by the similar method (at room temperature) described above and obtained as dark green solids (82%). mp 145–150 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.3 (t, J = 7.1 Hz, 3H), 1.4–2.2 (m, 21H), 2.4 (t, J = 6.7 Hz, 2H), 4.0–4.4 (m, 8H), 6.1–7.1 (m, 4H), 7.2–8.2 (m, 15H); HRFAB-MS m/z 704.4165 (calcd for $C_{47}H_{54}N_3O_3$ 708.4177) M^+-HCl + H.

Treatment of protein (BSA or IgG) with ICG-ATT (6). A DMF solution (250 μL) of ICG-ATT (**6**) (3.7 μmol/mL) was added to a solution of BSA (2 mg) in 0.1 M phosphate buffer solution (pH 7.5, 1 mL). After being stood on for 30 min, the reaction mixture was submitted to Sephadex LH-20 column chromatography (1.5 cm ×50 cm) eluting with 0.1 M phosphate buffer solution (pH 7.5) to give an ICG-labeled protein fraction (**12**). The concentrations of protein and ICG-moiety in the eluted protein fraction (**12**) were determined by the method described in the text. In the case of bovine IgG (2 mg), the labeling reaction with a DMF solution (250 μl) of ICG-ATT (**6**) (3.7 g mol/mL) was carried out in the same manner as mentioned above using 0.1 M carbonate buffer (pH 9.5) instead of 0.1 M phosphate buffier (pH 7.5). The labeling reactions were repeated several times. The data are recorded as mean ± S.E.M. in the text.

Treatment of the buffer solution of ICG-labeled protein (BSA or IgG) (12) with ethylacetate. The 0.1 M phosphate or carbonate buffer solution (1 mL) including the ICG-labeled protein (BSA or IgG) (**12**), obtained by the Sephadex LH-20 column chromatography as described above, was added to ethylacetate (1 mL). The mixture was stirred vigorously for 30 min and then the water layer including **13** was submitted to the UV-vis and fluorescent analyses.

References and Notes

1. Patonay, G.; Antoine, M. D. *Anal. Chem*., **1991**, *63*, 321A.
2. U.S. Patent 2, 895, 955 (1959).
3. Southwick, P. L.; Ernst, L. A.; Tauriello, E. W.; Parker, S. R.; Mujumdar, R. B.; Mujumdar, S. R.; Clever, H. A.; Waggoner A. S. *Cytometry*, **1990**, *11*, 418.
4. Ernst L. A.; Gupta, R. K.; Mujumdar, R. B.; Waggoner A. S. *Cytometry*, **1989**, *10*, 3.
5. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Waggoner, A. S. *Cytometry*, **1989**, *10*, 11.
6. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. L; Waggoner, A. S. *Bioconjugate Chem*., **1933**, *4*, 105.
7. Ito, S.; Muguruma, N.; Kakehashi, Y.; Hayashi S.; Okamura, S.; Shibata, H.; Okahisa, T.; Kanamori, M.; Shibamura, S.; Takesako, K.; Nozawa, M.; Ishida, K.; Shiga, M. *Bioorg. Med. Chem. Let*., **1995**, *5*, 2689.
8. Nagao, Y.; Miyasaka, T.; Seno, K.; Fujita, E. *J. Chem. Soc., Perkin Trans. 1*, **1984**, 2439.
9. Nagao, Y.; Fujita, E. *Heterocycles*, **1982**, *17*, 537.