Simple One-Pot Preparation of Water-Soluble, Cysteine-Reactive Cyanine and Merocyanine Dyes for Biological Imaging

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A simple one-pot procedure for preparation of protein-reactive, water-soluble merocyanine and cyanine dyes has been developed. The 1-(3-ammoniopropyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate bromide (1) was used as a common starting intermediate. The method allows easy preparation of dyes with chloro- and iodoacetamide side chains for covalent attachment to cysteine. By placing a sulfonate group directly on the dye fluorophore system, dyes with high fluorescence quantum yields in water were generated. Both iodo- and chloroacetamido derivatives were shown to be useful in protein labeling. Less reactive chloroacetamides will be preferential for selective labeling of the most reactive cysteines.

INTRODUCTION

Cyanine and merocyanine dyes have been useful as fluorescent labels of proteins in many biological applications, because they have excellent photophysical properties, including high fluorescence quantum yields and extinction coefficients, photostability, and long fluorescence wavelengths that minimally overlap cellular autofluorescence (1). These dyes have been used in single molecule spectroscopy (2, 3), in protein profiling (4), and for quantifying protein conformational changes in living cells (5, 6). Site-specific protein labeling in these applications was accomplished through reaction with cysteine, introduced into proteins at the desired protein sites using molecular biology approaches.

Multiple different approaches have been used to prepare water-soluble, cysteine-reactive cyanine and merocyanine dyes. Gruber et al. produced cysteine-reactive cyanines by linking the succinimimid ester of the lysine-reactive dyes to chains bearing different thiol-reactive groups (7). The dyes used as starting materials for this approach are expensive and available only in small quantities. In methods published by Wagoner et al. (8) and by our group (9), the unstable intermediate chloro- or iodoacetamido-indoline is prepared and then condensed with the appropriate anil to form the final dye (9). Alternately, our published synthesis of cysteine-reactive merocyanine dyes uses Fmoc-protection to mask amino functionality in the intermediate indolium or benzothiozol salts. In the final steps of the synthesis, after assembling the fluorophore structure, the Fmoc group is removed and the amine linked to an iodoacetamido group (6).

Here we report a short synthesis of water-soluble, cysteine-reactive merocyanine and cyanine dyes from the same key intermediate, using a facile, one-pot reaction (Scheme 1). The reaction introduces iodoacetamido side chains into the dyes, which are shown to be cleanly converted into more reactive iodoacetamide derivatives. Novel, highly fluorescent merocyanines and cyanine dyes are produced and their chloroacetamide and iodoacetamide derivatives are used for protein derivatization.

EXPERIMENTAL PROCEDURES

Methods include preparation of the key intermediate 1-(3-ammoniopropyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate and its conversion to merocyanine and cyanine dyes. The reaction of this intermediate with hemicyanine or 3-methoxyprop-2-enyl-1-idene-2-thiobarbituric acid in dimethylformamide in the presence of sodium acetate and chloroacetic acid anhydride (CAA) gave the cyanine or merocyanine dyes, respectively. The pure dyes were obtained by chromatography of crude reaction mixtures on silica gel using acetone–methanol as eluent (Scheme 1). The chloroacetamides were cleanly converted into more reactive iodoacetamides by reaction with sodium iodide in methanol–chloroform.

Materials. Analytical grade reagents were purchased from Sigma-Aldrich Co. Potassium 2,3,3,trimethyl-3H-indole-5-sulfonate (10), 3-[2-{1E,3E}-4-anilinobuta-1,3-dien-1-yl]-1,3-benzothiazol-3-ium-3-ylpropane-1-sulfonate (3) (11), (2Z)-2-{2E}-3-methoxyprop-2-en-1-ylidene]-1-benzothiophen-3(2H)-one 1,1-dioxide (5) (6), and 5-{(2E)-3-methoxyprop-2-en-1-ylidene]-3-dimethyl-2-thioxodyhdropyrimidine-4,6(1H,5H)-dione (6) were prepared as previously described.

Methods. Absorption spectra were recorded on a Hewlett-Packard UV–vis spectrophotometer, and fluorescence measurements were taken on a Spex Fluorolog 2 spectrofluorometer. NMR spectra were obtained on a Bruker 500 MHz DRX 500 spectrometer. Mass spectra were obtained on a Hewlett-Packard 5890 gas chromatograph equipped with a 5971A mass selective detector (MS-El). Quantum yields were measured using merocyanine 540 (12) or Cy5 (7) as an internal standards (13).

Synthesis of Intermediate 1: 1-(3-Ammoniopropyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate Bromide (1). To a suspension of 2.77 g of potassium 2,3,3-trimethyl-3H-indole-5-sulfonate in dichlorobenzene was added 2.18 g of 3-bromopropylamine hydrobromide. The mixture was stirred at 130 °C under nitrogen for 12 h. It was then cooled and the solid was filtered. The solid was stirred with 50 mL of hot methanol for 10 min, filtered, and dried. The yield was 2.45 g (65%). NMR (400 MHz, DClO–DMSO-d6) 1.48 (s, 6H, 2 × CH3), 2.13 (p, JH–H = 6.2 Hz, 2H, CH2–CH2NH3+), 4.45 (t, JH–H = 6.2 Hz, 2H, CH2–CH2NH3+), 7.81–7.87 (m, 2H), 7.94 (s, 1H). ESI-MS: 297 (M+, positive ion detection).

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Scheme 1. Synthesis of Water-Soluble Dyes with Chloro- or Iodoacetamido Reactive Groups

**General Method for the Preparation of the Chloroacetoamide-Substituted Dyes.** To a stirred suspension of 377 mg (1.00 mmol) of 1 in 10 mL of DMF were added 425 mg (2.50 mmol) chloroacetic anhydride (CAA), 1.20 mmol of acceptor intermediate (2, 3, 4, or 5), and 200 mg (2.50 mmol) of sodium acetate. The mixture was stirred at room temperature for 1 h. The DMF was removed under vacuum, and the residue was purified by column chromatography on silica gel using acetone–acetate. The mixture was stirred at room temperature for 1 h.

Reagents and conditions: A. Chloroacetic anhydride, sodium acetate, DMF, rt, 1 h. B. NaI, MeOH–CHCl₃, reflux, 24 h.

Sodium (2-((1E,3E,5E)-5-(1-(3-(2-Chloroacetamido)propyl)-3,3-dimethyl-2-thioxo-2-thioxotetrahydrothiazolo[3,2-a]thiazol-3-ium-6-sulfonate) or Cy5S-CAA (9a). The yield was 308 mg (51%). NMR (500 MHz, DMSO-d₆): δ 1.64 (s, 6H, 2 × CH₃), 1.82 (p, 3J_H–H = 6.8 Hz, 2H, CH₂CH₂CH₂), 3.24 (q, 3J_H–H = 6.8 Hz, 2H, CH₂CH₂NH), 3.61 (s, 3H, NCH₃), 4.08 (m, 4H, CH₂N and CH₂Cl), 6.27 (m, 2H), 6.54 (t, 3J_H–H = 12.3 Hz, 1H), 7.30 (d, 3J_H–H = 8.4 Hz, 1H), 7.33 (d, 3J_H–H = 8.4 Hz, 1H), 7.60–7.66 (m, 2H), 7.89 (d, 3J_H–H = 1.5 Hz, 1H), 8.10–8.41 (m, 3H). ESI-MS: 660 ((M–Na)⁻, negative ion detection).

Sodium (E)-1-(3-(2-Chloroacetamido)propyl)-3,3-dimethyl-4,6-dioxo-2-thioxothiazolo[3,2-a]thiazol-5(6H)-ylidene)but-2-enyldene)-3,3-dimethylindoline-5-sulfonate or I-TBA-CAA (8). The yield was 313 mg (63%). NMR (300 MHz, DMSO-d₆): δ 1.64 (s, 6H, 2 × CH₃), 1.85 (p, 3J_H–H = 6.8 Hz, 2H, CH₂CH₂CH₂), 2.03 (p, 3J_H–H = 6.8 Hz, 2H, CH₂CH₂CH₂), 2.60 (t, 3J_H–H = 6.8 Hz, 2H, CH₂SO₃), 3.25 (q, 3J_H–H = 6.8 Hz, 2H, CH₂CH₂NH), 3.96 (t, 3J_H–H = 6.8 Hz, 2H, CH₂N), 4.06 (2H, CH₂CH₂CH₂), 4.67 (t, 3J_H–H = 6.8 Hz, 2H, CH₂N), 6.07 (d, 3J_H–H = 13.0 Hz, 1H), 6.51 (t, 3J_H–H = 13.0 Hz, 1H), 6.98 (d, 3J_H–H = 13.0 Hz, 1H), 7.15 (d, 3J_H–H = 8.1 Hz, 1H), 7.50–7.70 (m, 4H), 7.98–8.15 (m, 4H), 8.42 (t, 3J_H–H = 6.0 Hz, 1H, NH). ESI-MS: 678 ((M–Na)⁻, negative ion detection).
RESULTS AND DISCUSSION

Synthesis. As shown in Scheme 1, cyanine and merocyanine dyes were prepared using a facile one-pot procedure from the key intermediate, 1-(3-ammoniopropyl)-2,3,3-trimethyl-3H-indolium-5-sulfonate (intermediate 1). This intermediate was prepared by reaction of potassium 2,3,3-trimethyl-3H-indole-5-sulfonate with 3-bromopropylamine hydrobromide in dichloromethane at 130 °C. The intermediate precipitates during the reaction and can be easily purified from unreacted starting compounds by simple washing with hot methanol, as the intermediate is practically insoluble in that solvent. The reaction of intermediate 1 with 3-methoxyprop-2-ethyl-1-ide-2-thiobarbituric acid or with 3-methoxyprop-2-enyl-1-ide-2-hemicyanine produced the cyanine dyes bearing chloroacetamido reactive groups. Use of the same reaction conditions with hemicyanines 2 or 3 produced the cyanine dyes shown. The dyes were purified by chromatography of crude reaction mixtures on silica gel using acetonemethanol as an eluent. Iodoacetamides were prepared by the reaction of chloroacetamides with sodium iodide in methanol—chloroform mixture. The yields of both merocyanine and cyanine chloroacetamido dyes were in the range of 50—65%, and the substitution of iodide for chloride occurred with 3-methoxyprop-2-enylethylene1,1-dioxido-2-thiobenzylideneindoline-5-sulfonate or I-SO-IAA (9b).

The degree of labeling D/P (dye-to-protein ratio) was calculated using the following formula:

\[ \text{D/P} = \frac{A_{\text{dye}} / \epsilon_{\text{dye}}}{A_{\text{prot}} / \epsilon_{\text{prot}}} \]

where \( A_{\text{dye}} \) is the absorbance at the absorption maximum of the dye, \( A_{\text{prot}} \) is the absorbance at the absorption maximum of EGFP (490 nm), \( \epsilon_{\text{dye}} \) is the extinction coefficient of the dye in H₂O, and \( \epsilon_{\text{prot}} \) is the extinction coefficient of EGFP (61000).
The enamine then reacts with 3-methoxyprop-2-enyl-1-iden-2-benzothiophen-3-one 1,1-dioxide 4 to give amino-substituted dye 11. The dye 11 is not stable, and its solution rapidly loses color, as Michael addition of the free amino group to the polymethylene chain destroys the dye fluorophore system. In the presence of chloroaacetanhydride, the free amine is quickly trapped as the chloroacetamide (Scheme 2). This mechanism is supported by trapping of unstable dye intermediate 10 with amino-reactive compounds, including acetic anhydride or acetic acid succinimidyl ester.

Absorption and Fluorescence Properties. These reactions generated novel, bright merocyanine and cyanine dyes (Table 1). They had high extinction coefficients, in the range of 120,000–215,000 and moderate fluorescence quantum yields of 0.1–0.4. The absorption characteristics of dyes 6 and 9a were very similar to those of the published analogues neoCy5 (9) and I-SO-IAA (6). Substitution with a sulfonato group (–SO3) at the aromatic ring in the new merocyanine dye 9a and in the new cyanine dye 6 significantly increased the fluorescence quantum yields in polar solvents (H₂O, MeOH) compared to I-SO-IAA and neo-Cy5. For the cyanine, the quantum yield in water more than doubled, and for the merocyanine it increased 5-fold. Similar increases in dye fluorescence upon ring-substitution with sulfonato-groups have been reported for rhodamines (14) and cyanines (15). Previously, it was thought that dye aggregation is the main reason for the significant drop in dye fluorescence quantum yields in aqueous solutions (14, 15). The I-SO-IAA dye does not form H-aggregates (6), and its fluorescence is still significantly lower in water than the fluorescence of the dye 9a. Although the mechanism of fluorescence quenching of I-SO-IAA in water is not clear, it likely includes either dynamic quenching of excited dye molecules through collision with dye in the ground state (16) or static quenching through association (other than H-aggregation) of ground state dye molecules (17). In both cases placement of negatively charged sulfonato groups directly on the dye fluorophore system (dyes 6, 9a in present study) rather than on alkyl side chains (I-SO-IAA and neoCy5) will reduce dye–dye interactions in water because of Coulombic repulsion and will lead to higher fluorescence quantum yields. Brightness in polar solvents is especially valuable for imaging applications, where dyes are conjugated to proteins and exposed to water.

Table 1. Absorption and Fluorescence Data for Merocyanine and Cyanine Dyes

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* Extinction coefficient. † Absorption maximum. ‡ Fluorescence maximum. § Fluorescence quantum yield. From ref 6. ′ From ref 9. ′′ Phosphate-buffered saline.

Protein Labeling. The reactivity of the chloroaacetamido and iodoacetamido derivatized was compared by covalent derivatization of EGFP-Erk2 with the chloro- and iodoacetamido derivatized of I-SO dye (9a and 9b). Chloroaacetamido-reactive groups are not used in protein labeling because of their much lower reactivity toward thiols. In previous studies, chloroaacetamides have been about 300 times less reactive than iodoacetamides in reaction with mercaptoacetic acid (18). When EGFP-Erk2 was labeled with 9a and 9b under identical conditions (10:1 dye to protein ratio, 2 h, room temperature), the iodoacetamide 9b produced conjugates with an average dye/protein (D/P) of 1.25, while labeling with chloroaacetamide 9a produced conjugates with D/P of 0.2 (Figure 1).

**Figure 1.** Labeling of EGFP-Erk2 with dyes 9a and 9b.

This protein has a total of nine free cysteines (two in EGFP and seven in Erk2) that can potentially react with halogen-acetamido groups. The relatively low D/P produced by iodoacetamide labeling implies that there is only one reactive cysteine in EGFP-Erk2. Surprisingly, the reactivity of chloroaacetamido-substituted dye was only six times lower than that of the iodoacetamido dye. The diminished difference between the two derivatives may be explained by a two-step reaction mechanism, in which slow formation of the dye–protein complex is followed by rapid reaction with a thiol group. In such a process the reactivity of the substrate will be determined by the rate of dye–protein complex formation, which should be similar for dyes with chloroaacetamido and iodoacetamido side chains.

**SUMMARY**

A simple one-pot procedure for preparation of protein-reactive, water-soluble merocyanine and cyanine dyes has been developed. The method allows easy preparation of dyes with chloro- and iodoacetamide side chains for covalent attachment to cysteine. Novel bright dyes were generated, and both iodo-
and chloroacetamido derivatives were shown to be useful in protein labeling.

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LITERATURE CITED


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