Xanthamide Fluorescent Dyes

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Two derivatives of fluorescein, termed “xanthamides,” were prepared from fluorescein, an inexpensive dye. Relative to fluorescein, which contains a 6-phenolic OH and a 2'-carboxyl, the first derivative (5) contains a carboxymethyl ether at the 6-position and a secondary amide of dimethylamine at the 2'-position. The second derivative (8) contains a corresponding 6-methyl ether and a secondary amide of isonpiceptic acid at the 2'-position. Thus, both derivatives contain a single carboxyl group, making them monofunctional. Especially 8 is much more photostable (about 10 times) than either fluorescein or BODIPY FL dye when exposed to ordinary light (tungsten light bulb). When tested for relative response in a capillary electrophoresis instrument fitted with an argon ion laser detector (488 nm) and a broad band emission filter, 8 was found to be 4-fold less bright than fluorescein. Both xanthamides, consistent with prior literature on 6-O-alkylated fluorescein, exhibit relatively pH-independent fluorescence (pH 4–10 was tested here). Because they possess two absorbance maximums, the xanthamides can be excited over a broader wavelength range than fluorescein or BODIPY. These collective properties of the xanthamides will make them advantageous over fluorescein and BODIPY dyes for some applications.

Fluorescent dyes are widely employed in both qualitative and quantitative chemical and biological analysis and in other areas as well. A great diversity of such dyes is used because the physicochemical properties of the dyes vary widely, and different combinations of properties (e.g., absorption and emission maximums, functional groups, polarity, microenvironmental dependence of the fluorescence) suit different applications. One property that is nearly always beneficial is high stability, both chemically and physically, including photostability.

For the application of fluorescent-tagging trace analytes to improve detection sensitivity, the overall stability of the dye becomes particularly important. This is largely because an excess of dye is necessary to label a trace analyte efficiently, and breakdown products of the dye can create chemical noise, which in turn compromises sensitivity.

We have been exploring functionalized BODIPY dyes as tags for labeling and detection of DNA adducts,12 but the limited stability of these dyes has impeded our work.3,4 They are also quite expensive. Accordingly, we have sought an alternative dye. It is well-known that the stability of some chemicals can be increased via steric protection, so we decided to explore derivatives of fluorescein, a low-cost dye with good chemical stability. Ester (e.g., refs 5–8) and primary amide9–11 derivatives of the carboxyl group of fluorescein have been reported, sometimes in combination with alkylation of the 6-phenolic OH group.5–7 It was encouraging that the quantum yield of fluorescein can be fairly maintained (18–31% relative to that of fluorescein) even when its 6-hydroxy group is alkylated.5,6 However, the photostability of these derivatives of fluorescein has not been reported. We decided to combine 6-O-alkylation with conversion of the carboxyl group to a secondary amide to provide steric protection. Relatively small steric groups were chosen, nevertheless, to maintain good dye solubility. As reported here, such derivatives, which we have termed “xanthamides,” indeed are relatively photostable.

EXPERIMENTAL SECTION

Materials. All chemicals were from Aldrich (Milwaukee, WI) unless indicated otherwise. Fluorescein for the photostability study was purified by silica flash chromatography with ethyl acetate/hexane, 1:1, v/v. 1H and 13C NMR were recorded on a Varian 300 MHz Mercury-300 spectrometer; UV spectra, in a 1-cm cuvette on a Hewlett-Packard 8453 spectrophotometer; and fluorescence emission spectra, on a Perkin-Elmer LS-50B luminescence spectrometer.

Photostability Study. This was conducted by irradiating a 50 nM sample in 50 mM aqueous sodium borate, pH 10, using a Hotspot lamp (Cheltenham, PA) with a 200 W soft white bulb (General Electric, Canada) positioned 10 cm from the sample flask. The latter flask was cooled by flowing room-temperature water. Fluorescence spectra were recorded on aliquots taken at different exposure times. Oxygen was not removed from these samples prior to irradiation. BODIPY FL dye (D-2183), or “BODIPY” for this study, was from Molecular Probes (Eugene, OR).


10.1021/ac020368+ CCC: $22.00 © 2002 American Chemical Society Published on Web 11/08/2002

Analytical Chemistry, Vol. 74, No. 24, December 15, 2002 6397
Capillary Electrophoresis-Laser Induced Fluorescence (CE-LIF) System. The home-built CE-LIF containing an argon ion laser, which provides excitation at 488 nm, was described before, but the prior emission filters between the capillary and the photomultiplier tube were replaced with two broadband interference filters (center wavelength at 550 nm with a fwhm of 70 nm from CVI Laser Corporation, Albuquerque, NM). This was done to capture the broad emission from the xanthamid dyes better than the prior 10-nm fwhm filter.

2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic Acid 2,5-Dioxo-pyrrolidin-1-yl Ester, 2. Fluorescein 1 (3.32 g, 10 mmol), N-hydroxysuccinimide (1.17 g, 10 mmol), and dicyclohexylcarbodiimide (2.10 g, 10 mmol) in 15 mL of dry dimethylformamide (DMF) were heated to 70–80 °C under nitrogen for 1 h. After ice-bath cooling, dicyclohexylurea was filtered off. The DMF solution was subject to flash chromatography using first ethyl acetate/ hexane (50:50, v/v) and then acetone, 1.33 g of product was obtained (31%).

1H NMR (DMSO-d6, ppm): 7.76 (m, 1H), 7.51 (d, 2H, J = 9 Hz), 7.35–7.45 (m, 1H), 7.17 (d, 2H, J = 9.6 Hz), 6.75–6.80 (m, 2H, 4H), 2.97 (s, 3H).

13C NMR (CD3OD, ppm): 187.20 (broad), 170.72, 166.31, 161.28, 156.32, 153.35, 137.38, 133.31, 132.40, 131.73, 131.57, 130.92, 130.87, 129.02, 128.80, 118.72, 115.82, 115.77, 105.53, 102.30, 69.15 (broad), 39.96, 35.12.

1-(2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-benzoyl)-piperidine-4-carboxylic Acid tert-Butyl Ester, 6. Compound 2 (430 mg, 1 mmol) was dissolved in 10 mL of DMF followed by tert-butyl isononopetate 11 (186 mg, 1 mmol) and triethylamine (418 μL, 3 mmol). The reaction mixture was stirred for 1 h at room temperature, neutralized with 300 μL of acetic acid, and isolated by flash chromatography using ethyl acetate/methanol (80:20, v/v), giving 384 mg (77%).

1H NMR (CD3OD, ppm): 7.78–7.67 (m, 1H), 7.55–7.45 (m, 1H), 7.43–7.44 (m, 2H), 6.80–6.64 (m, 4H), 4.12–3.95 (m, 1H), 3.71–3.54 (m, 1H), 3.09–2.91 (m, 1H), 2.79–2.55 (m, 1H), 2.50–2.28 (m, 1H), 1.85–1.58 (m, 2H), 1.40 (s, 9H), 1.33–1.02 (m, 2H).

1-(2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)-benzoyl)-piperidine-4-carboxylic Acid tert-Butyl Ester, 7. Compound 6 (65 mg, 0.13 mmol) in 3 mL of DMF, potassium carbonate (90 mg, 0.65 mmol), and 81 μL of methyl iodide were mixed. After 60 °C for 90 min under nitrogen, the mixture was cooled and subject to flash chromatography using ethyl acetate/methanol (90:10, v/v), giving 42.3 mg (63%).

1H NMR (CD3OD, ppm): 7.78–7.67 (m, 1H), 7.66–7.58 (m, 1H), 7.55–7.45 (m, 1H), 7.32–7.16 (m, 3H), 7.04–6.93 (m, 1H), 6.71–6.59 (m, 1H), 6.56–6.47 (m, 1H), 4.15–3.90 (m, 1H), 3.99 (s, 3H), 3.76–3.56 (m, 1H), 3.16–2.90 (m, 1H), 2.81–2.58 (m, 1H), 2.47–2.27 (m, 1H), 1.86–1.60 (m, 2H), 1.40 (s, 9H), 1.35–1.13 (m, 2H).

1-(2-(6-Methoxy-3-oxo-3H-xanthen-9-yl)-benzoyl)-piperidine-4-carboxylic Acid, 8. Compound 7 (30 mg, 0.058 mmol) was dissolved and stirred in 3 mL of trifluoroacetic acid at room temperature for 2 h. After evaporation, the reaction mixture was subject to flash chromatography using ethyl acetate/methanol (50:50, v/v), giving 20 mg (75%).

1H NMR (CD3OD, ppm): 7.74–7.69 (m, 2H), 7.66–7.60 (m, 1H), 7.50–7.45 (m, 1H), 7.32–7.16 (m, 3H), 7.09–6.94 (m, 1H), 6.66–6.57 (m, 1H), 6.48–6.44 (m, 1H), 4.31–4.01 (m, 1H), 3.97 (s, 3H), 3.77–3.62 (m, 1H), 3.02–2.81 (m, 1H), 2.71–2.52 (m, 1H), 2.30–2.17 (m, 1H), 1.82–1.67 (m, 2H), 1.60–1.36 (m, 2H).

1-(Trifluoroacetyl)piperidine-4-carboxylic Acid, 9. To isononopetic acid (2.6 g, 20 mmol) was added trifluoroacetic anhydride (8.5 mL, 60 mmol) with stirring over 5 min at room temperature, followed after 2 h by evaporation and then drying in a vacuum desiccator over phosphorus pentoxide. The solid was suspended in 100 mL of water, stirred for 1 h at room temperature, filtered, and dried, giving 2.93 g (65%).

1H NMR (CD3OD, ppm): 4.35–4.20 (m, 1H), 4.03–3.86 (m, 1H), 3.41–3.28 (m, 1H), 3.18–2.99 (m, 1H), 2.78–2.60 (m, 1H), 2.12–1.95 (m, 2H), 1.80–1.56 (m, 2H).

tert-Butyl 1-(trifluoroacetyl)piperidine-4-carboxylate, 10. Compound 9 (1.15 g, 5 mmol) was suspended in 8 mL of CHCl3 in a pressure tube (Z40041-6 from Aldrich), followed by cooling in a dry ice acetone bath. Isobutylene (~15 mL) was condensed into the tube, and sulfuric acid (0.1 mL) was added. The sealed reaction mixture was magnetically stirred at 40 °C until the solution was homogeneous (3 d). After the tube was cooled in an ice bath, the mixture was transferred to a round bottom flask and concentrated by rotary evaporation. Ethyl acetate (100 mL) was

added, and the organic phase was washed with pH 9.5 carbonate buffer (3 x 50 mL) and saturated aqueous NaCl. After drying with MgSO4, rotary evaporation gave 1.04 g (74%) of a colorless oil. 

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\begin{align*}
\text{H NMR (CDCl}_3, \text{ppm): } & 4.36 - 4.17 (m, 1H), 4.01 - 3.82 (m, 1H), 3.37 - 3.14 (m, 1H), 3.14 - 2.97 (m, 1H), 2.59 - 2.42 (m, 1H), 2.10 - 1.87 (m, 2H), 1.84 - 1.59 (m, 2H), 1.44 (s, 9H).
\end{align*}
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\text{tert-Butyl Piperidine-4-carboxylate, 11. Compound 10 (1.41 g, 5 mmol) was suspended in 20 mL of methanol/water (70:30, v/v). After potassium carbonate (2.07 g, 15 mmol) was added, the mixture was stirred at room temperature for 4 h, concentrated by rotary evaporation, and extracted with ethyl acetate (3 x 50 mL). The organic phase was washed with brine, dried with MgSO4, and concentrated by rotary evaporation, giving a light oil that solidified after standing overnight in a desiccator, yielding 670 mg (72%). H NMR (acetone-\text{d}_6, \text{ppm): } 3.06 - 2.89 (m, 2H), 2.63 - 2.44 (m, 2H), 2.31 - 2.16 (m, 1H), 1.79 - 1.67 (m, 2H), 1.55 - 1.34 (m, 2H), 1.42 (s, 9H).

**RESULTS AND DISCUSSION**

A shown in Figure 1, relatively simple chemical reactions yield the targeted xanthamide dyes 5 and 8. Commercially available reagents were used throughout, except that we prepared the tert-butyl piperidine-4-carboxylate (11) that was used in reaction e. Compound 11 was prepared from isonipecotic acid via protection of the amino group with trifluoroacetic anhydride, as described in the Experimental Section. Yields were not optimized, but the overall preparative yields of 5 and 8 at the milligram level were 13 and 11% respectively. At an earlier stage in our project, isonipecotic acid methyl ester was employed instead of 11 in reaction e, but overall, it gave a lower yield of final product as a result of some hydrolysis of the amide during deprotection of the methyl ester with NaOH/methanol. Although 8 was stable for at least 3 days at room temperature in pH 10 aqueous buffer, the amide group hydrolyzed (monitored by a wavelength shift in the absorbance spectrum) after overnight storage in 0.01 N aqueous sodium hydroxide. As seen, both 5 and 8 contain a carboxyl group for additional conjugation reactions.

The fluorescence spectrum of xanthamide 8 is shown in Figure 2, along with those of fluorescein and a BODIPY dye for comparison. The latter two dyes are widely employed, and many instruments that detect them have been set up. We see similar emission maximums for all three dyes, but 8 exhibits a broader emission on the high wavelength side. The peak at 488 nm in...
spectrum C is due to scattered excitation light. The inset in the figure shows the absorbance spectrum of 8. The broadness of its absorption spectrum matches the broadness of its emission spectrum, as one would expect. Dye 8 has absorbance maximums especially at 459 and 483 nm (459 and 485 nm for 5; data not shown), which means that its fluorescence can be stimulated not only by the same lasers that are commonly employed to excite fluorescein and BODIPY dyes, but also by lasers providing somewhat lower wavelengths. These absorption maximums are close to the values of the corresponding maximums at 456 and 481 nm for corresponding 6-O-alkyl fluorescein-2′-methyl esters reported by Adamczyk et al.,6 who used a somewhat different solvent (40 mM sodium phosphate, 20% methanol, pH 7.4). Zhao et al.7 reported absorption maximums of 450 and 475 nm (and showed spectra) in neutral to alkaline buffers of corresponding 6-O-ethyl fluorescein-2′-ethyl ester.

We next turned our attention to the property of 5 and 8 that was of special interest in this study, namely photostability upon exposure to ordinary light. To assess this, we subjected a solution of each dye at constant temperature to the directed radiation (metal-framed desk lamp) from a conventional 200-W soft white light bulb. Aliquots were removed as a function of time for measurement of fluorescence. For comparison, fluorescein and BODIPY were tested in the same way. As seen in Figure 3, both xanthamides 5 and 8, and especially the latter compound, are significantly more photostable (about 10-fold for 8) than fluorescein and BODIPY under these conditions. Hirschfeld has reported the photostability of fluorescein upon exposure to 488-nm light.13 To estimate the relative contributions of the alkyl and secondary amide groups to the photostability of 8, we prepared the corresponding phenolic compound, 12 (structure not shown), in which the ethereal CH3 of 8 is replaced with H. Compound 12 has 40% residual fluorescence after 8 h of light exposure (data not shown). Thus, both the methyl and isonicotinic acid moieties of 8 are important for the relatively high photostability of 8.

A preliminary effort to explore the relative photostability of a corresponding primary amide derivative of 8 was unsuccessful.

Although reaction of 2 with 6-aminocaproic acid gave a product (9) possessing the expected mass (by electrospray mass spectrometry) and an NMR spectrum that seemed to fit the expected product, this compound showed no absorbance above 300 nm. Without studying this product further, we assumed that the desired primary amide had formed but then continued via its N or O atom to attack the C9 of the xanthene moiety, giving a product analogous to the nonfluorescent lactone form of fluorescein. Consistent with this assumption, corresponding colorless hydrazide derivatives of fluorescein have been reported.14 This prompted us to attempt to prepare the primary amide derivative of fluorescein reported by Papadopoulos et al.11 In their reaction,
fluorescein and N-decylamine were reacted at an elevated temperature. However, we were unable to reproduce their results. When we acidified the reaction mixture at its conclusion (as a modification of their procedure), we recovered unreacted fluorescein. We did not attempt to prepare the primary amide derivatives of fluorescein that have been reported by others.9,10

As expected from prior work on 6-O-alkyl derivatives of fluorescein,5,6 5 and 8 exhibit fluorescence that is relatively independent of pH throughout the range of ~4–10 that we tested, as shown in Figure 4. As is well-known,5,6 it is the alkylation at the 6-OH that establishes this behavior. Unlike fluorescein, 8, therefore, can be employed as a fluorescent tag in acidic environments, as pointed out before by Lohse et al.5 for 6-O-alkyl fluorescein esters.

We evaluated the brightness of 8 relative to fluorescein by subjecting each to capillary electrophoresis with laser-induced fluorescence detection. The equipment for this experiment is the same as what we used before,12 except that we changed the emission filters to encompass more of the broad emission of 8, and thereby favor the detection of 8 relative to fluorescein. This gave the electropherograms shown in Figure 5. Taking into account the difference in the concentrations of the injected dyes, we learn that 8 is about 25% as bright as fluorescein under these conditions.

CONCLUSION

We believe that xanthamide dyes, especially 8 and its future derivatives, will replace fluorescein or BODIPY dyes in some applications. When exposed to ordinary light, dye 8 is ~10 times more photostable than these latter dyes. Unlike fluorescein, it offers relatively pH-independent fluorescence, and it avoids a reactive phenolic site that can complicate some conjugation reactions. Unlike BODIPY, 8 can be obtained via a simple, low-cost synthetic pathway. Further, many dyes with a fluorescein core that offer a wide variety of absorption and emission maximums along with other physicochemical characteristics are available. Potentially, a similar family of xanthamide-core dyes can emerge.

ACKNOWLEDGMENT

This work was supported by Contract 340811E3119 from the Massachusetts Department of Public Health, and NIEHS Grant ES10539. Contribution No. 820 from the Barnett Institute.

Received for review June 3, 2002. Accepted September 26, 2002.
AC020368+