Latent Blue and Red Fluorophores Based on the Trimethyl Lock

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Fluorescent molecules are indispensable tools in modern biochemical and biological research, being used as labels for biomolecules, indicators for ions, stains for organelles, and substrates for enzymes.[1] The major targets of this last class are hydrolases that catalyze the removal of a masking moiety, thereby modulating fluorescence.[2] A critical property of fluorogenic hydrolase substrates is their chemical stability in aqueous solution, as spontaneous hydrolysis can compete deleteriously with enzymatic activity. New substrate classes that exhibit increased stability while maintaining enzymatic reactivity would be highly desirable.

Our laboratory recently reported on the use of the "trimethyl lock" strategy in the design of a latent fluorophore.[3] This latent fluorophore consists of a trimethyl lock component inserted between a dye and enzyme-reactive group. The trimethyl lock is an o-hydroxycinnamic acid derivative in which unfavorable steric interactions between three methyl groups encourage rapid lactonization to form a hydrocoumarin and release a leaving group.[4, 5] Our initial latent fluorophore exhibited remarkable stability in aqueous solution, but released a xanthene dye (rhodamine 110) upon hydrolytic cleavage by an esterase. Here, we explore the modularity of our design, probing its applicability to unrelated dyes that absorb at short (blue) and long (red) wavelengths.

Coumarin-based compounds comprise an important class of blue dyes with UV or near-UV excitation wavelengths.[6] Acyl esters and acyloxymethyl ethers of 7-hydroxycoumarin (i.e., umbelliferone) can be substrates for esterases.[7–9] 7-Amino-4-methylcoumarin (AMC) is used widely as the basis for protease substrates.[10–12] Upon amidation, the excitation and emission

wavelengths of AMC are shifted to shorter wavelengths with a concomitant reduction in quantum yield.[10]

We reasoned that AMC could be subjected to our latent fluorophore strategy. Accordingly, we condensed AMC with acetylated trimethyl lock 1[13] to give profluorophore 2 (Scheme 1), which displayed the expected hypsochromic shift of excitation and emission spectra relative to free AMC (Figure 1). The hydrolysis of profluorophore 2 was catalyzed by porcine liver esterase (PLE) with $k_{cat}/K_M = 2.5 \times 10^5 \, \text{M}^{-1} \text{s}^{-1}$ and $K_M = 8.2 \, \mu\text{M}$ (Figure 2A). This k_{cat}/K_M value is 100-fold greater than the apparent k_{cat}/K_M value for the latent fluorophore based on rhodamine 110.[3] (We use the term "apparent" because full fluorescence manifestation requires the lactonization of two trimethyl lock moieties.[3])
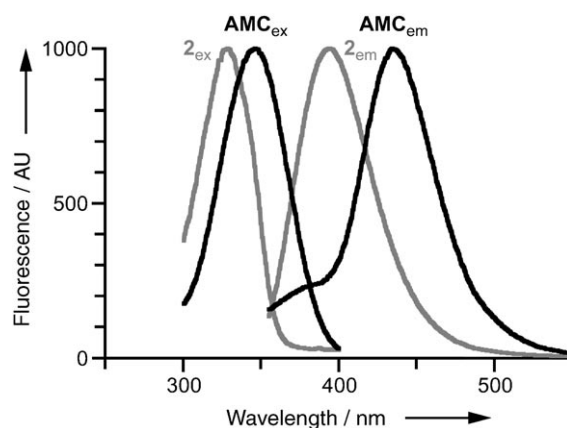
Figure 1. Normalized fluorescent excitation–emission spectra of profluorophore 2 and AMC.

To evaluate the relative stability of profluorophore 2, we monitored the accretion of fluorescence in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA; 1.0 mg mL^{-1}) and either profluorophore 2 or 4-methylumbelliferyl acetate (3, which is a common esterase substrate[9]). Pro-
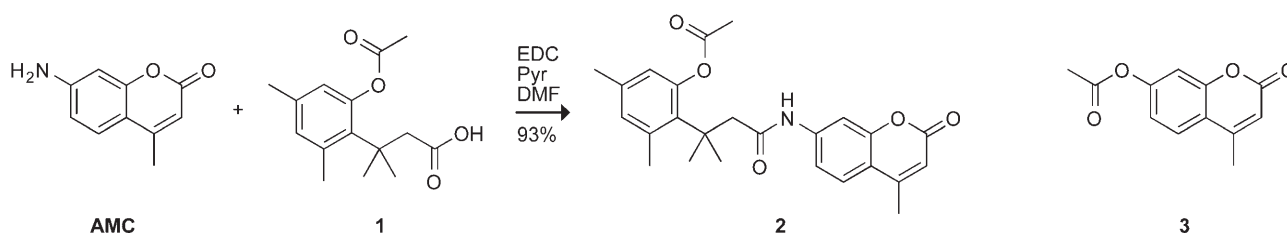
Scheme 1. Route for the synthesis of profluorophore 2, and the chemical structure of 4-methylumbelliferyl acetate (3).

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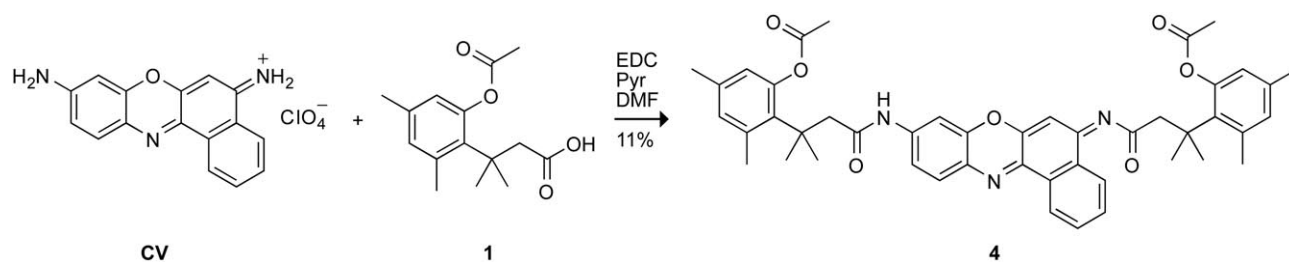   E-mail: raines@biochem.wisc.edu

fluorophore 2 proved to be highly stable compared to 4-methylumbelliferyl acetate (Figure 3). An additional advantage of profluorophore 2 is that the product of its hydrolysis, AMC, shows little change in fluorescence at pH ≥ 5.[1] In contrast, the fluorescence of 4-methylumbelliferone is highly variable due to its pK_a of 7.40[14] being near the physiological pH.
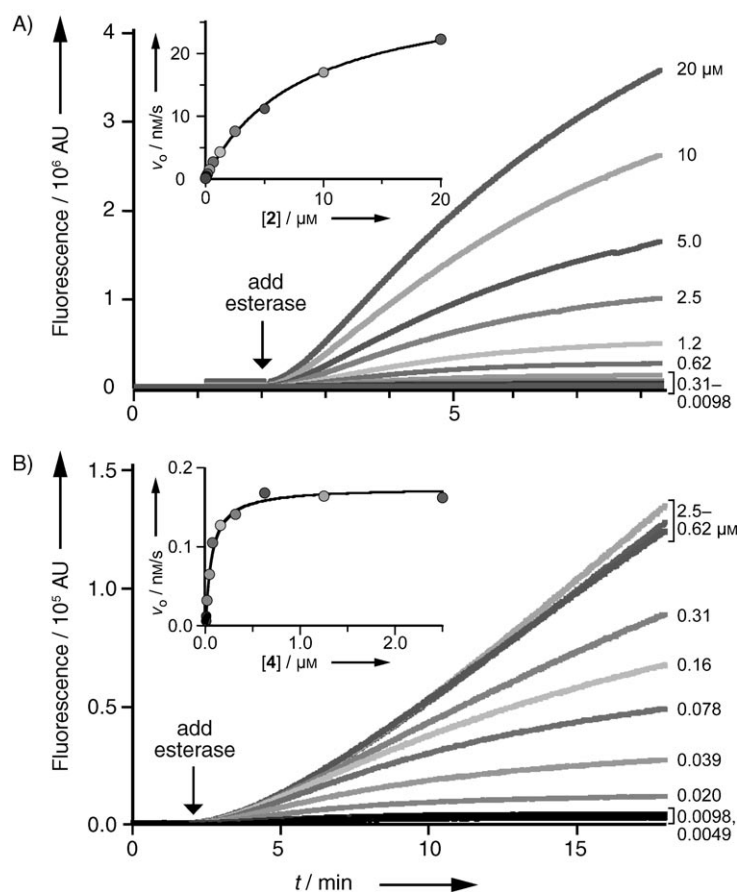
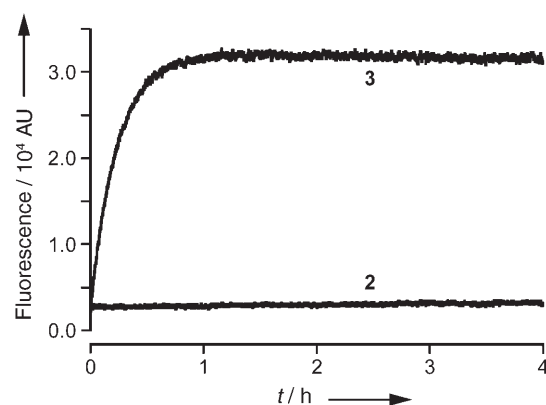**Figure 2.** A) Kinetic traces ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 460$ nm) and Michaelis–Menten plot (inset) for the hydrolysis of profluorophore **2** (20 μM → 9.8 nM) by PLE (2.5 μg mL^{-1}); $k_{cat}/K_M = 2.5 \times 10^5$ M^{-1} s^{-1} and $K_M = 8.2$ μM. B) Kinetic traces ($\lambda_{ex} = 586$ nm, $\lambda_{em} = 620$ nm) and Michaelis–Menten plot (inset) for the hydrolysis of profluorophore **4** (2.5 μM → 4.9 nM)) by PLE (2.5 μg mL^{-1}); apparent $k_{cat}/K_M = 1.2 \times 10^5$ M^{-1} s^{-1} and apparent $K_M = 0.14$ μM.

Figure 3. Time course of the generation of fluorescence ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 460$ nm) from profluorophore **2** (50 nM) or methylumbelliferyl acetate **3** (50 nM) in PBS containing BSA (1 mg mL^{-1}).

To access longer wavelengths, we turned to the oxazine class of red dyes. One such dye, cresyl violet (CV) has been used for decades to stain tissues[15,16] and as a laser dye.[17] Although CV has a maximal absorbance at 586 nm, its fused benzo ring broadens its absorption spectrum[18] and thereby allows excitation with a variety of light sources. Diamide derivatives of CV are virtually nonfluorescent and thus useful in the production of fluorogenic protease substrates.[19–21]

The attributes of CV make this dye an attractive candidate for our latent fluorophore strategy. Accordingly, we condensed CV with acetylated trimethyl lock **1**[13] to give profluorophore **4** (Scheme 2). Although profluorophore **4** was stable in PBS containing BSA (data not shown), its hydrolysis was catalyzed by PLE with apparent kinetic parameters of $k_{cat}/K_M = 1.2 \times 10^5$ M^{-1} s^{-1} and $K_M = 0.14$ μM (Figure 2B). The k_{cat}/K_M value for the hydrolysis of profluorophore **4** is similar to that of profluorophore **2**; the K_M value is, however, ten times lower than that of profluorophore **2**.

Profluorophore **4** serves as a probe for esterase activity in live mammalian cells. The broad excitation peak allowed confocal microscopy experiments with excitation at 543 nm and emission at 605 nm. Profluorophore **4** was hydrolyzed by esterases endogenous in the cells of a rodent (Figure 4A) or human (Figure 4B) to give a red-stained cytosol within minutes. The somewhat enhanced fluorescence observed in the human cells could be indicative of more efficient internalization or hydrolysis of the latent fluorophore. After longer incubation, the liberated CV became further localized in subcellular compartments (data not shown); this is consistent with previous reports.[21] To date, there have been few reports of long-wavelength esterase substrates that are useful in cell biology.[1] Its evident stability, optical properties, and fast intracellular eduction make profluorophore **4** useful in a wide variety of biological applications, especially in assays involving fluorophores of different wavelengths.

Scheme 2. Route for the synthesis of profluorophore **4**.
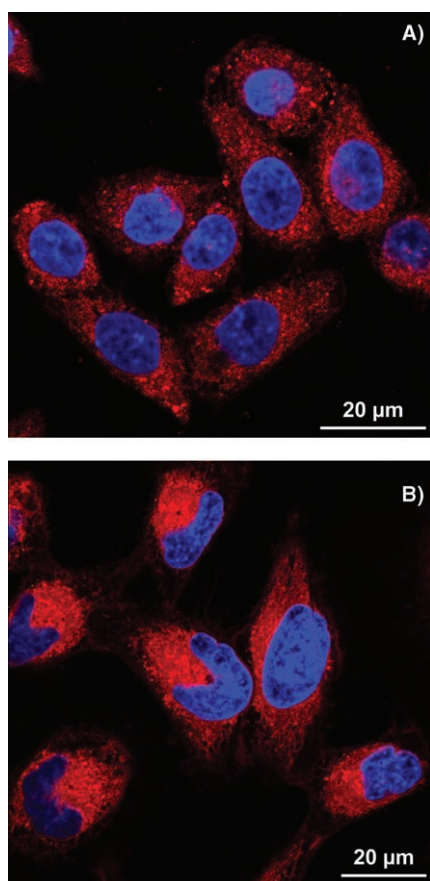
Figure 4. Unwashed mammalian cells incubated for 15 min with profluorophore **4** (10 μM) at 37 °C in DMEM and counter-stained with Hoechst 33342 (5 % v/v CO_2(g), 100 % humidity). A) CHO K1 cells. B) HeLa cells.

In conclusion, we have established that one component of our latent fluorophores—the dye—is modular (Figure 5). Specifically, we have now prepared useful latent fluorophores from three dyes (blue, green,[3] and red), all linked by a trimethyl lock moiety to an esterase-reactive group. In future work, we shall explore the modularity of the other component—the enzyme-reactive group. We anticipate that the end result will be a broad spectrum of stable latent fluorophores with numerous applications in biochemical and biological research.
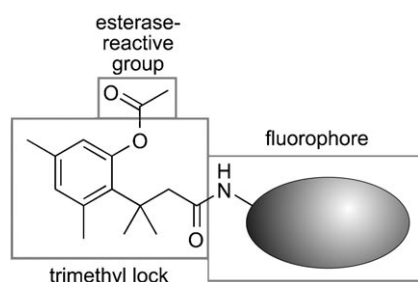
Figure 5. Modules in the latent fluorophores described in this work.

Experimental Section

AMC (i.e., coumarin 440) and CV·$HClO_4$ (i.e., cresyl violet 670 perchlorate) were from Exciton (Dayton, OH). Dimethylformamide was drawn from a Baker CYCLE-TAINER solvent-delivery system. All other reagents were from Aldrich (Milwaukee, WI) or Fisher Scientific (Hanover Park, IL) and used without further purification.

PBS contained KCl (0.20 g), KH_2PO_4 (0.20 g), NaCl (8.0 g), and Na_2HPO_4·$7H_2O$ (2.16 g) in 1.00 L at pH 7.4.

Thin-layer chromatography was performed by using aluminum-backed plates coated with silica gel containing F_{254} phosphor and visualized by UV illumination or developed with I_2, ceric ammonium molybdate, or phosphomolybdic acid stain. Flash chromatography was performed on open columns with silica gel-60 (230–400 mesh), or on a FlashMaster Solo system (Argonaut, Redwood City, CA) with Isolute® Flash Si II columns (International Sorbent Technology, Hengoed, Mid Glamorgan, UK).

NMR spectra were obtained with a Bruker DMX-400 Avance spectrometer at the National Magnetic Resonance Facility at Madison (NMRFAM). Mass spectrometry was performed with a Micromass LCT (electrospray ionization, ESI) mass spectrometer at the Mass Spectrometry Facility in the Department of Chemistry.

7-Amino-4-methylcoumarin trimethyl lock (2): AMC (53 mg, 0.304 mmol) was dissolved in anhydrous DMF (2.0 mL) and anhydrous pyridine (1.0 mL). 3-(2′-Acetoxy-4′,6′-dimethylphenyl)-3,3-dimethylpropanoic acid[13] (**1**; 100 mg, 0.378 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 117 mg, 0.609 mmol) were added, and the reaction mixture was stirred at ambient temperature for 48 h. Solvent was removed under reduced pressure, and the residue was purified by column chromatography (silica gel, 0–40 % v/v gradient of EtOAc in hexanes). Compound **2** was isolated as an off-white crystalline solid (119 mg, 93 %). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 7.66$ (br s, 1H), 7.39 (d, $J = 8.6$ Hz, 1H), 7.21 (m, 2H), 6.76 (s, 1H), 6.65 (s, 1H), 6.15 (d, $J = 1.3$ Hz, 1H), 2.55 (s, 2H), 2.41 (s, 3H), 2.39 (s, 3H), 2.36 (d, $J = 1.2$ Hz, 3H), 2.25 (s, 3H), 1.71 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 172.51$, 169.96, 161.13, 154.08, 152.17, 150.16, 141.56, 139.13, 137.40, 133.15, 132.82, 124.82, 123.46, 115.68, 115.27, 113.06, 106.65, 51.11, 40.53, 32.25 (2C), 25.49, 21.95, 20.15, 18.51 ppm; HRMS (ESI): m/z 444.1797 [M+Na]$^+$ ([$C_{25}H_{27}NO_5Na$] = 444.1787).

Cresyl violet bis(trimethyl lock) (4): CV·$HClO_4$ (250 mg, 0.691 mmol) was dissolved in anhydrous DMF (2.0 mL) and anhydrous pyridine (2.0 mL). Compound **1**[13] (394 mg, 1.38 mmol) and EDC (265 mg, 1.38 mmol) were added, and the reaction mixture was stirred at ambient temperature for 24 h. The solvent was removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 and 5 % v/v HCl(aq). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with H_2O and saturated brine, and dried over anhydrous $MgSO_4$. Removal of the solvent gave a brown solid that was purified by column chromatography (silica gel, 30 % v/v EtOAc in hexanes). Compound **4** was isolated as a red-brown solid (55 mg, 11 %). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 8.57$ (dd, $J = 7.9$, 1.1 Hz, 1H), 8.12 (dd, $J = 8.0$, 1.2 Hz, 1H), 7.72 (br s, 1H), 7.65 (ddd, $J = 8.0$, 7.2, 1.4 Hz, 1H), 7.59 (ddd, $J = 7.9$, 7.2, 1.4 Hz, 1H), 7.50 (d, $J = 8.6$ Hz, 1H), 7.46 (d, $J = 2.1$ Hz, 1H), 6.85 (dd, 8.6, 2.4 Hz, 1H), 6.77 (m, 1H), 6.66 (m, 2H), 6.47 (m, 1H), 5.98 (s, 1H), 3.19 (s, 2H), 2.54 (s, 2H), 2.51 (s, 3H), 2.43 (s, 3H), 2.41 (s, 3H), 2.26 (s, 3H), 2.25 (s, 3H), 2.05 (s, 3H), 1.73 (s, 6H), 1.66 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 188.04$, 172.79, 169.95, 169.91, 154.02, 150.21, 149.40, 147.92, 145.84, 144.77, 140.86, 139.24,

138.20, 137.37, 136.13, 133.48, 133.23, 132.88, 132.47, 132.13, 131.07, 130.77, 130.74, 129.49, 129.40, 125.56, 124.24, 123.41, 123.12, 115.63, 105.51, 101.12, 52.88, 51.15, 40.64, 39.34, 32.28 (2 C), 32.06 (2 C), 25.55, 25.33, 21.99, 21.88, 20.17, 20.10 ppm; HRMS (ESI): m/z: 754.3471 $[M+H]^+$ ($C_{46}H_{48}N_3O_7 = 754.3492$).

Fluorescence spectroscopy: Fluorometric measurements were made with a QuantaMaster1 photon-counting spectrofluorometer from Photon Technology International (South Brunswick, NJ) equipped with sample stirring, and fluorescence-grade quartz or glass cuvettes from Starna Cells (Atascadero, CA). All measurements were recorded at ambient temperature ($23 \pm 2\,^\circ$C). Compounds were prepared as stock solutions in DMSO and diluted so that the DMSO concentration did not exceed 1% v/v. PLE ($M_W = 163$ kDa[22]) was from Sigma (St. Louis, MO; product number E2884) as a suspension in $(NH_4)_2SO_4$ (3.2 M), and was diluted to appropriate concentrations in PBS before use. Kinetic parameters were calculated with Microsoft Excel 2003 and GraphPad Prism 4.

Cell preparation and imaging: CHO K1 or HeLa cells were plated on Nunc Lab-Tek II 8-well chamber coverglass (Fisher Scientific) and grown to 70% confluence at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing fetal bovine serum (10% v/v). Cells were then incubated with profluorophore **4** (10 μM) for 15 min at 37 °C and imaged immediately. Nuclear staining was accomplished by addition of Hoechst 33342 (2 μg mL^{-1}) for the final 5 min of incubation.

Cells were imaged with a Nikon Eclipse TE2000-U confocal microscope equipped with a Zeiss AxioCam digital camera. Excitation at 408 nm was provided by a blue-diode laser, and emission light was passed though a filter centered at 450 nm with a 35 nm bandpass. Excitation at 543 nm was provided by a HeNe laser, and emission light was passed through a filter centered at 605 nm with a 75 nm band-pass.

Acknowledgements

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