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Latent Fluorophore Based on the Trimethyl Lock

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Fluorescent molecules are essential for basic research in the biological sciences, the development of new drugs, the assurance of food safety and environmental quality, and the clinical diagnosis of disease.[1] A wide variety of fluorophores are known.[2] Still, many applications could benefit from stable probes with intense fluorescence that is unmasked by a user-designated chemical reaction.[3] Here, we address this need by introducing a new class of latent fluorophores with desirable attributes.

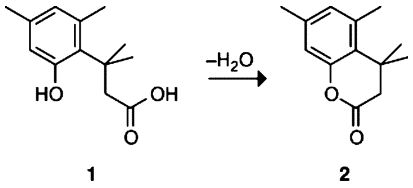
We reasoned that the "trimethyl lock" could provide an attractive means to cloak fluorescence. The trimethyl lock is an *o*-hydroxycinnamic acid derivative (**1**) in which unfavorable steric interactions between three methyl groups encourage rapid lactonization to form a hydrocoumarin (**2**) (Scheme 1).[4] This intramolecular reaction has an effective molarity near 10^{15} M and is a bona fide example of the use of strain to enhance reactivity.[5] We were inspired, in particular, by the utility of the trimethyl lock in pro-drug strategies.[6,7] There, a labile group is attached to the phenolic oxygen, and a drug is condensed with the carboxyl group. Unmasking of the phenolic oxygen leads to rapid lactone formation with concomitant release of the drug.

Xanthenes are perhaps the most widely used fluorophores.[2] Their popularity arises from a high quantum yield ($\Phi \cong 1$) and long emission and excitation wavelengths. Xanthene fluorescence is decreased by modifications that favor the lactone form. For example, acylation of the 3′ and 6′ oxygens of fluorescein eliminates its fluorescence.[8] Fluorescein is, however, an excellent leaving group ($pK_a = 6.32$[9]), which could compromise its use in a latent fluorophore.

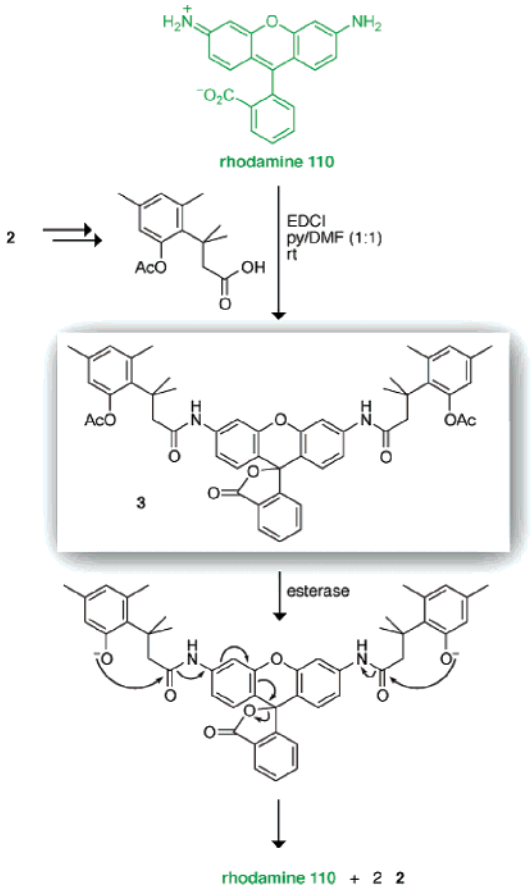
We selected rhodamine 110 as the fluorophore to be masked. The amide groups of *N*,*N*-diacetylated rhodamine 110, which has been used as a protease substrate,[10] are stable in a HeLa cell extract (data not shown). Nonetheless, the trimethyl lock is capable of effecting rapid N → O acyl transfer to liberate an amine upon formation of lactone **2**.[7] Hence, we suspected that the trimethyl lock could link the unmasking of the phenolic oxygen with the production of rhodamine 110 by N → O acyl transfer. We chose esterases as the activating enzyme, due to their high abundance in the mammalian cytosol and known utility in pro-drug strategies.[6] Accordingly, we synthesized pro-fluorophore **3** as a putative esterase substrate by condensation of rhodamine 110 with excess O-acetylated **1** (Scheme 2).

The fluorescence spectrum of pro-fluorophore **3** exhibited near-baseline excitation and emission (Figure 1), even after months of storage in phosphate-buffered saline (PBS). Introduction of pig liver esterase (PLE) to the solution resulted in a large increase in fluorescence with apparent kinetic parameters of $k_{cat}/K_M = 1.9 \times 10^3$ M^{-1} s^{-1} and $K_M = 0.47$ μM.[11] Thus, **3** fulfills several criteria of a useful latent fluorophore, a stable molecule with intense fluorescence that is unmasked by a user-designated chemical reaction.

Scheme 1



Scheme 2



We next investigated the biological utility of pro-fluorophore **3**. To be useful, a latent fluorophore must remain nonfluorescent in a biological environment. Accordingly, we monitored the accumulation of fluorescence in Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) containing either fluorescein diacetate (which is a widely used pro-fluorophore[8]) or **3**. Solutions containing fluorescein diacetate became fluorescent in minutes (Figure 2A). In contrast, solutions containing **3** lacked

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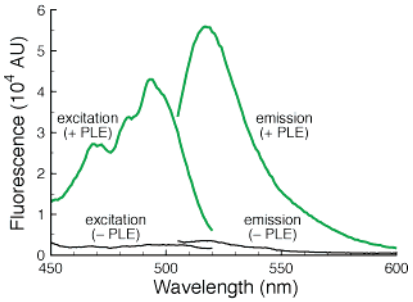

Figure 1. Excitation−emission spectra of pro-fluorophore **3** (5 μM) incubated for 4 h in PBS without or with PLE (0.25 mg/mL).

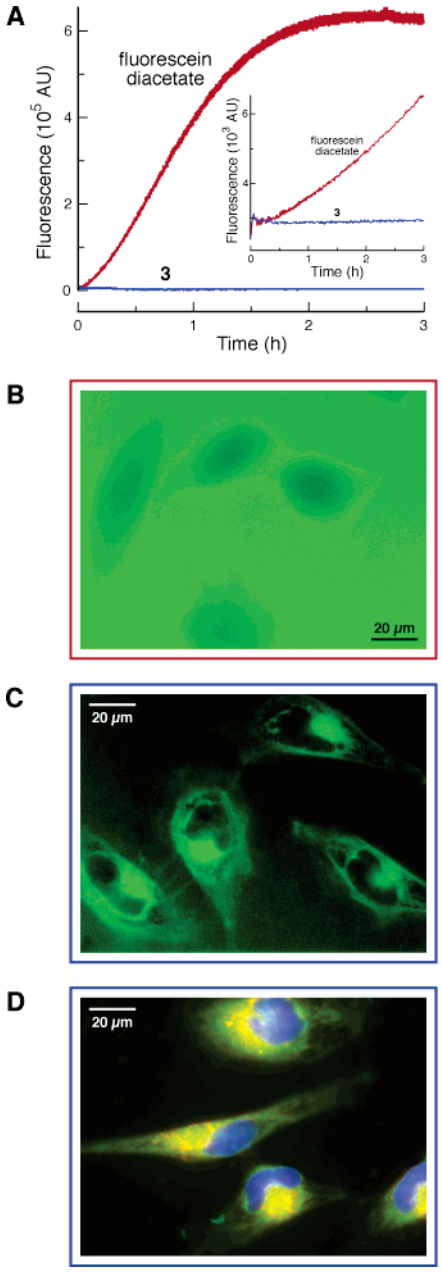

Figure 2. (A) Time course of the generation of fluorescence (λ_{ex} = 492 nm, λ_{em} = 520 nm) by fluorescein diacetate (red; 5 μM) and pro-fluorophore **3** (blue; 5 μM) in DMEM or PBS (inset). (B and C) Unwashed HeLa cells incubated for 2 h with fluorescein diacetate (panel B; 10 μM) or **3** (panel C; 10 μM) at 37 °C in DMEM (5% CO_2, 100% humidity). (D) Washed HeLa cells incubated for 2 h with **3** (10 μM) and counter-stained with LysoTracker Red and Hoescht 33342.

fluorescence after 3 h, indicative of a high stability for **3** in a biological environment.

Finally, we used fluorescence microscopy to determine the fate of fluorescein diacetate and pro-fluorophore **3** in DMEM containing HeLa cells. The manifestation of fluorescence from fluorescein diacetate was much greater in the medium than within the HeLa cells (Figure 2B). In contrast, HeLa cells incubated with **3** displayed marked fluorescence, while extracellular regions remained non-fluorescent (Figure 2C). Intracellular fluorescence was absent in the nucleus but strong in the cytosol and lysosomes (Figure 2D), indicative of high esterase activity in these subcellular compartments.

We conclude that latent fluorophores based on the trimethyl lock possess a unique combination of chemical stability and biological utility. We anticipate that replacing the acetyl groups in pro-fluorophore **3** with other functional groups (phosphoryl, sulfuryl, glycosyl, etc.) would yield an ensemble of latent fluorophores that are stable and nonfluorescent until undergoing a user-designated chemical reaction. The development of latent fluorophores (xanthene-based and otherwise) with such alternative triggers as well as handles for biomolecular conjugation is underway in our laboratory.

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Supporting Information Available: Procedures and additional data for syntheses and analyses reported herein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Data were obtained in PBS containing PLE (2.5 μg/mL). Unmasking of the second amino group leads to the manifestation of ca. 90% of the fluorescence (ref 10).

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