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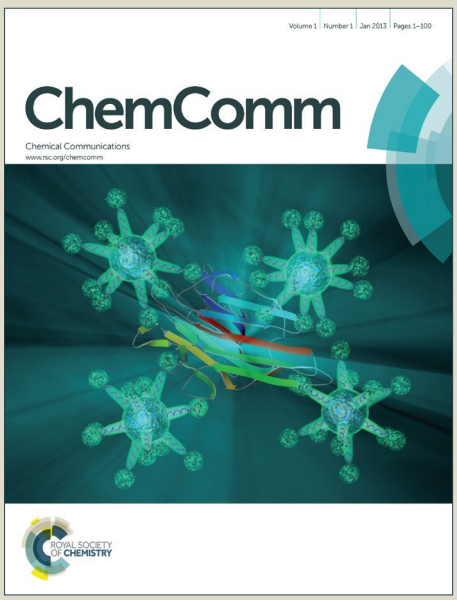

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Synthesis of Photoactivatable Azido-acyl Caged Oxazine Fluorophores for Live-cell Imaging

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Andrew V. Anzalone[a,b], Zhixing Chen[a,c] and Virginia W. Cornish[a,b]

We report the design and synthesis of a photoactivatable azido-acyl oxazine fluorophore. Photoactivation is achieved cleanly and rapidly with UV light, producing a single fluorescent oxazine photoproduct. We demonstrate the utility of azido-acyl caged oxazines for protein specific labeling in living mammalian cells using the TMP-tag technology.

Fluorescence-based methods have emerged as powerful tools to study basic molecular processes within living cells. With the continuing development of new microscopy techniques comes the demand for brighter, multifunctional fluorescent probes[1,2]. Great strides have been made over the past several decades in devising chemical tools for protein specific labeling[3–8] and intracellular molecular sensing[9] using fluorescent reporters. Likewise, there has been significant progress in the development of single molecule super-resolution imaging technologies[10,11] for localizing biomolecules in cells with unprecedented precision. For live-cell imaging, these methods require probes with high overall brightness and photostability, preferably with absorbance and emission of red or near-IR light, cellular permeability, and amenable cellular properties (i.e. devoid of non-specific staining)[1]. Several other useful features, such as fluorogenicity[12] and photoactivatability[13], are also exploited in various applications. Thus, while the microscopy toolkit expands to meet new challenges in the imaging arena, so too should our repertoire of small molecule fluorophores in order to maximize the potential of emerging technologies.

Oxazines comprise a class of live-cell fluorescent imaging reagents exemplified by the popular commercial dye Atto 655. Their remarkable photostability, notably higher in comparison to the cyanine fluorophores, and emission in the red/far-red region of the visible spectrum, notably redder than the rhodamine dye class, make these fluorophores well-suited to cellular and single molecule-based techniques. Oxazines also undergo photoswitching[14], which has been exploited for super-resolution dSTORM imaging[15]. However, one drawback for oxazines relates to their unreliable behavior within the living cell context, either due to low permeability or non-specific staining[16] leading to high background. Also, to date, the derivatization of oxazine scaffolds has not been extensively explored, potentially because of synthetic challenges. Therefore, probe development would be accelerated by modernized synthetic methods that enable the diversification of the scaffold with new chemical features, either to improve cellular behavior or to install useful functionality.

Previously, other fluorophore scaffolds have been derivatized to overcome challenges of solubility and cell-permeability[17–19], and brightness[20], and to introduce functionality such as photoactivatability[21–23]. Representative examples include fluorescein diacetate, which is cell permeable and cleaved by intracellular esterases[24] to generate a fluorescent product, and the photoactivatable fluorophores $NVOC_2$-Rhodamine[25] and azido-DCDHF[26] (Fig. 1). While NVOC is commonly used as a photocleavable group, it incorporates unwanted bulk and hydrophobicity into the probe. Also, in the case of $NVOC_2$-Rhodamine, full uncaging of the fluorophore requires two photochemical cleavage events. Azido-DCDHF contains a smaller photoactivation motif that minimally perturbs its overall structure and requires just a single uncaging event; however, the photoreaction gives rise to a mixture of products.

Here, we report the design and synthesis of new oxazine derivatives and demonstrate feasibility for their use in live-cell imaging. First, we have addressed the synthetic chemistry challenges by employing recent advances in transition metal catalysis to synthesize acyl oxazine intermediates, providing an avenue for facile functionalization of the oxazine scaffold. Second, we prepared azido-acyl oxazines and demonstrated that they undergo rapid and clean photoconversion to the oxazine fluorophores through cleavage of the acyl moiety accompanied by the concomitant release of molecular nitrogen. Lastly, using the TMP chemical tagging technology, we show that azido-acyl oxazines are useful reagents for live mammalian cell imaging, especially when compared to the native uncaged oxazine fluorophores.

[a] Department of Chemistry, Columbia University, New York, New York, 10027, USA.
[b] Department of Systems Biology, Columbia University, New York, New York 10032, USA
[c] Current address: Department of Chemistry, Stanford University, Stanford, CA 94305, USA.

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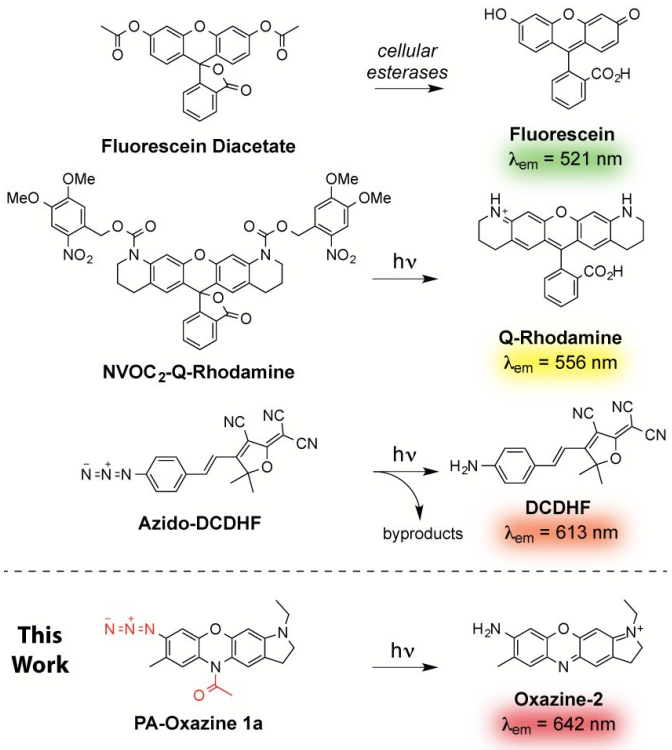


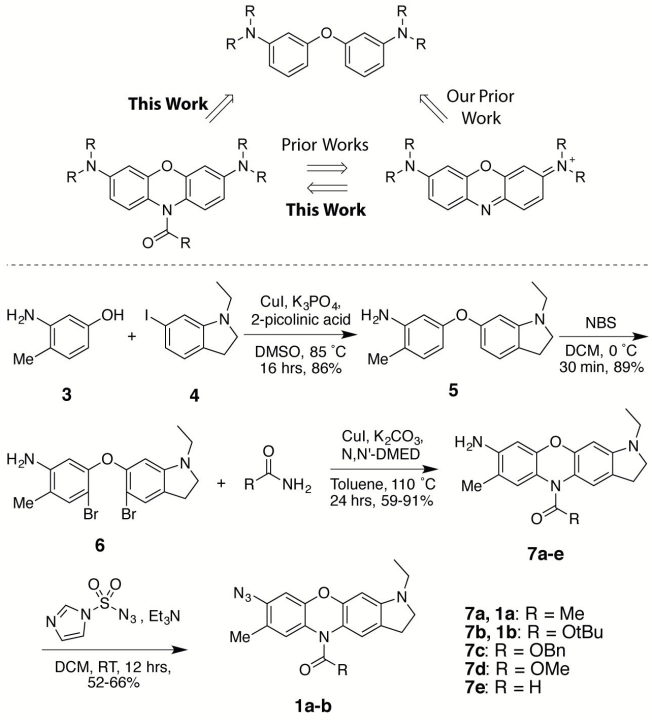
Fig. 1 Strategies for caging fluorescent dyes. Approaches include trapping molecules in non-fluorescent states with enzymatically cleavable or photocleavable capping groups. Some pro-fluorescent molecules are intrinsically photoactivatable.

In order to apply the approaches displayed in Figure 1 to the oxazine class of fluorophores, we chose to build upon the report that acyl oxazines undergo UV-induced cleavage of the N-acyl bond to generate oxazine dyes[27]. However, because acyl oxazines uncage relatively slowly, the extent of UV exposure required for efficient photoactivation is incompatible with living cells. Moreover, competitive Photo-Fries rearrangement products are obtained in addition to the desired oxazines. Inspired by azido-DCDHF, we set out to synthesize azido-acyl oxazines (Fig. 1, PA-oxazine **1a**) in an attempt to enhance photoactivation. Because these caged oxazines would exist in the same oxidation state as their fluorophore products, the net reaction does not require oxidation and therefore would likely result in fewer side reactions and undesirable byproducts. We anticipated that the release of molecular nitrogen would provide a strong thermodynamic driving force for irreversibility of acyl bond cleavage, favoring the forward reaction and resulting in fewer radical recombination byproducts.

Previously, acyl oxazines have been prepared from their parent oxazine fluorophores by in situ reduction of the oxazine to its *leuco* form in the presence of an acylating agent (Scheme 1, Prior Works). However, this procedure is limited in its compatibility with other functionality, and is circuitous from a synthetic standpoint. To circumvent these challenges, we sought to take advantage of our previously reported diaryl ether scaffold[28] to prepare acyl oxazines directly without passing through the oxazine dye. Given the electronically activated nature of the diaryl ether system, we reasoned that bromination followed by coupling with primary amides or carbamates would furnish acyl oxazine products that could then be carried forward for facile derivitization (Scheme 1).

This synthetic strategy was initiated by synthesis of the diaryl ether **5** according to our previously reported strategy, utilizing a key copper(I) promoted reaction between aminophenol **3** and iodoindoline **4** (Scheme S1). Dibromination of **5** was achieved using N-bromosuccinimide, proceeding exclusively with the desired regioselectivity to afford **6** in high yield. Then, a second key copper(I) promoted reaction[29] was implemented to construct the oxazine core, where the initial coupling between the amide or carbamate and **6** is followed by a second intramolecular coupling resulting in cyclized acyl oxazine products **7a-e**. Importantly, this reaction occurs in the presence of non-protected primary anilines, which are orthogonal under the conditions employed.

Coupling/cyclization products bearing various acyl substituents were prepared in good to excellent yields, and several of these derivatives became colorful upon exposure to UV light. Of note, acyl oxazines can be deprotected under the appropriate conditions to yield leuco dyes, which upon exposure to air spontaneously oxidize to furnish the fluorescent oxazines. Alternatively, further chemical modification of the fluorophore can be carried out prior to removal of the acyl substitutent. Thus, this approach provides a general route to the construction of the oxazine core allowing for functionalization of the scaffold before deprotection of the fluorophore's sensitive conjugated cationic system.



Scheme 1 Synthesis of azido-acyl oxazines via acyl oxazine intermediates. Prior synthesis of acyl oxazines was achieved through the oxazine dye, whereas our approach directly synthesizes acyl oxazines from diaryl ether intermediates. N,N'-DMED = N,N'-dimethylethylenediamine. NBS = N-bromosuccinimide.

From acyl oxazines, synthesis of the target azido-acyl oxazines **1a** and **1b** was achieved by diazotransfer from imidazole sulfonyl azide. As anticipated, azido-acyl oxazines were extremely sensitive to UV light and readily converted to fluorescent photoproducts after brief exposures (Fig. 2). In the UV-Vis absorbance and fluorescence

traces of representative compound **1b**, increasing exposure to UV light leads to an absorbance band that steadily arises in the visible region with a λ_{max} of 612 nm as well as a fluorescence band with λ_{max} centered at 642 nm. Closer inspection of the UV region within Figure 2b displays several isosbestic points for the photochemical process (Fig. S1, ESI), indicative of a clean reaction devoid of accumulating intermediates or byproducts.

The absorbance and fluorescence traces of the photoproducts overlay nearly identically with those of the independently synthesized oxazine **2**, which is the expected product of the photoreaction (Fig. S2, ESI). Additionally, HPLC analysis of the crude photoreaction indicated formation of just a single major product, identified as the anticipated oxazine by co-elution with the independently synthesized authentic marker **2** (Fig. S3, ESI). Together, these results demonstrate that the photoreaction proceeds cleanly to a single oxazine product **2** generated by cleavage of the acyl group and release of molecular nitrogen.

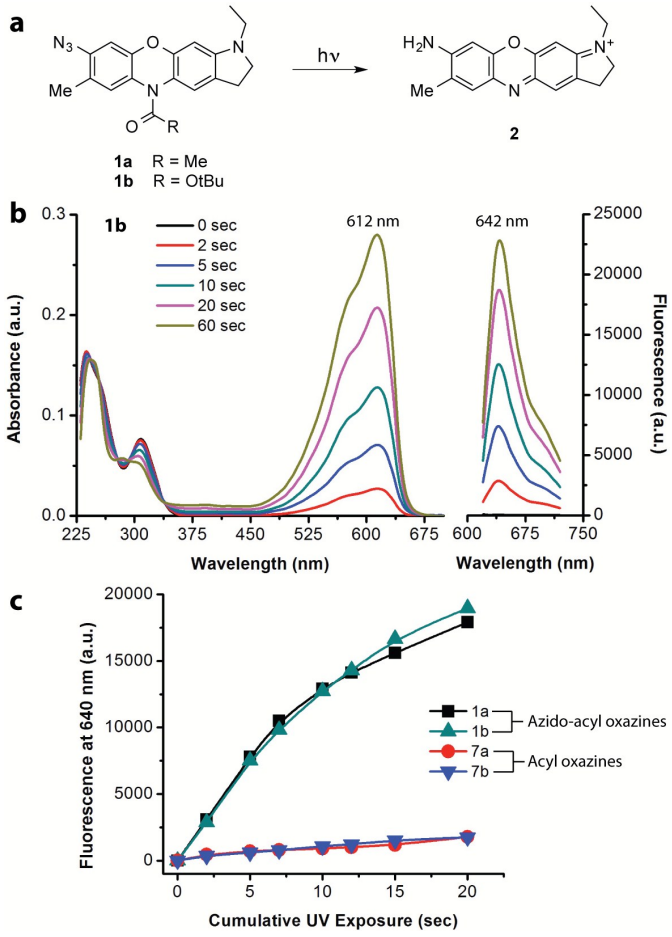


Fig. 2 The azido-acyl oxazine uncaging photoreaction. (a) Photochemical reaction of azido-acyl oxazines. (b) Absorbance and fluorescence spectra (excitation = 594 nm) of **1b** as a function of total exposure to 254 nm light. (c) Comparison of photoactivation rates for the azido-acyl oxazines **1a** and **1b** vs. the corresponding acyl oxazines **7a** and **7b** in 100% ethanol.

In comparison to the acyl oxazines **7a** and **7b**, the corresponding azido-acyl oxazine derivatives **1a** and **1b** undergo more rapid uncaging to the fluorophore in response to UV light, with rate accelerations of 20-30 fold (Fig. 2c). To assess whether the photoactivatable oxazines were viable reagents for live-cell imaging, we first tested the azido-acetyl oxazine derivative **1a** for permeability and photoactivatability in living cells. HeLa cells incubated with 2 μM of **1a** were irradiated with 365 nm light for 1s, resulting in a dramatic increase in the fluorescence signal (Fig. S4, ESI). This demonstrated that the photoactivatable oxazine is cell permeable and capable of undergoing photoactivation within the cellular environment.

Given these promising results, we then tested the trimethoprim (TMP) conjugate of the photoactivatable oxazine for labeling *E. coli* dihydrofolate reductase (eDHFR) in HEK293T cells (Fig. 3). TMP binds to eDHFR with nanomolar affinity, allowing specific labeling of a protein of interest (POI) when it is fused to eDHFR by staining with TMP-fluorophore conjugates. We prepared the TMP conjugate of the photoactivatable acetyl-azido oxazine (**TMP-1a**) as well as the native oxazine fluorophore (**TMP-2**) as a point of comparison (Fig. 3a). Labeling of nuclear localized H2B-eDHFR with **TMP-1a** followed by a brief UV excitation resulted in bright fluorescent labeling of the target protein (Fig. 3b). This signal co-localizes well with co-transfected H2B-EGFP. Interestingly, when **TMP-2** was imaged, very weak staining was observed, likely on account of poor cell permeability. We also observed clean labeling of a plasma membrane (PM) localized eDHFR with the photoactivatable **TMP-1a**, whereas **TMP-2** showed only weak staining of the intracellular DHFR (Fig. 3c). Of note, we have observed similarly poor cellular behavior with other oxazine analogs (data not shown). These results indicate that the caged azido-acetyl oxazine is a superior reagent for live-cell applications when compared to its corresponding oxazine fluorophore.

In conclusion, we present a new synthetic route to the synthesis and derivatization of oxazine dyes and demonstrate the utility of this approach through the design and synthesis of a photoactivatable azido-acyl motif for oxazine fluorophores. We demonstrate that azido-acyl oxazines undergo rapid and clean photoconversion to a single major fluorescent product, and further apply these probes for live-cell imaging. Importantly, caged oxazines were found to be useful alternatives to native oxazine dyes on account of their improved staining specificity. The comprehensive photophysical characterizations of this dye are currently ongoing and will be reported in due course. This new class of reagents should expand our toolkit for probing molecular mechanisms of biology in living cells.

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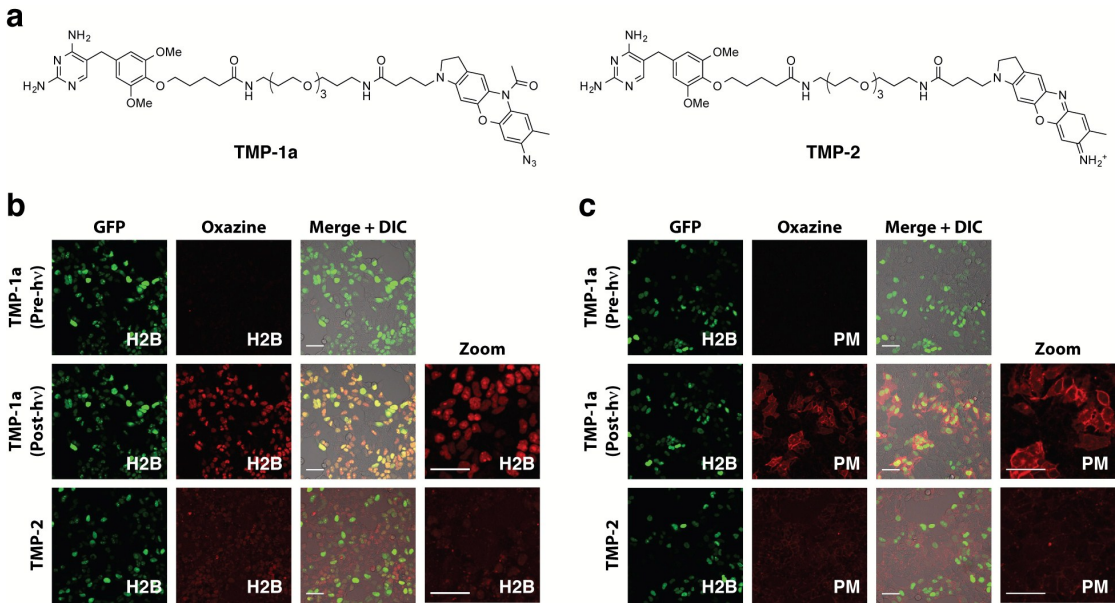

Fig. 3 Protein-specific labeling in HEK293T cells with TMP-oxazine conjugates. (**a**) Structures of the photoactivatable **TMP-1a** and the oxazine fluorophore **TMP-2**. (**b**) Labeling of a nuclear localized H2B-eDHFR fusion and (**c**) a plasma membrane (PM) localized eDHFR in HEK293T cells, co-tranfected with H2B-EGFP. Scale bar = 50 μm. Photoactivation conducted with 365nm light.

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