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Rapid Synthesis, Screening, and Identification of Xanthone- and Xanthene-Based Fluorophores Using Click Chemistry

Junqi Li,[†] Mingyu Hu,[†] and Shao Q. Yao*[,†,‡]

Departments of Chemistry and Biological Sciences, NUS MedChem Program of the Life Sciences Institute, National University of Singapore, Singapore 117543

chmyaosq@nus.edu.sg

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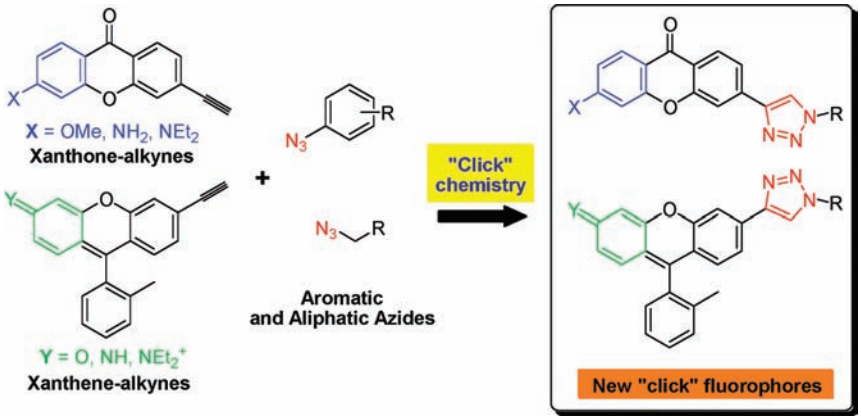
ABSTRACT



A panel of new fluorophores with emission wavelengths from blue to yellow regions using the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction of alkyne-functionalized xanthones and xanthenes with various azides have been synthesized. Screening of the "click" products led to the identification of "hit" fluorophores which showed a fluorescence increase upon triazole formation. These novel "click" fluorophores could potentially be used for bioconjugation and bioimaging purposes.

Small organic dyes and fluorescent probes are well-established labeling agents and sensors in both chemical and biological systems.[1] Despite their wide-ranging applications and popularity, the underlining photophysical properties of some of these dyes in relation to their molecular structures are not yet well understood. Consequently, the rational design of novel fluorophores possessing highly predictable and desirable properties has remained elusive. The lack of well-defined rules to govern fluorophore design has driven combinatorial efforts in fluorophore discovery in recent years.[2] The most straightforward synthetic approach in the combinatorial fluorophore synthesis is the modification of core structures from known fluorophores to furnish analogues of the parental molecules. This has thus far led to the discovery of new fluorescent molecules with a range of spectroscopic properties.[3]

We are particularly interested in the use of the Cu(I)-catalyzed azide−alkyne cycloaddition,[4] the representative

[†] Department of Chemistry.
[‡] Department of Biological Sciences.

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reaction in "click" chemistry,[4b] for the rapid assembly of fluorophores. The Wang and Fahrni groups independently introduced the concept of fluorogenic "click" reactions in which a weakly fluorescent azido- or alkynylcoumarin is converted into a fluorescent molecule by triazole formation using "click" chemistry.[3f,5] This unique feature, coupled with the bioothorgonal nature of the cycloaddition reaction, has found useful applications in the fluorescence labeling and visualization of glycans,[6] newly synthesized proteins,[7] and lipids.[8] In addition, the combinatorial discovery of new fluorescent dyes is facilitated by the modular and highly efficient characteristics of the "click" reaction. At present, only the coumarins,[3f,5] carbostyrils,[3g] anthracenes,[9] naphthalimides,[6a] and pyridyloxazole mimics[3h] comprise the family of "click" fluorophores in which "click" chemistry has been used as a fluorogenic reaction and/or for diversification to generate analogues of the parental fluorophore. One major drawback of the current "click" fluorophores is that all of them are UV-excited dyes, making them undesirable choices for bioimaging applications where cells or tissues are used. The key aim in the current work is to extend the "click" chemistry-mediated discovery of fluorescent dyes to previously unexplored fluorophore scaffolds, especially those with excitation wavelengths in the visible range.

Recently, our group introduced a new fluorophore, Singapore Green,[10] a structural hybrid of Tokyo Green (a fluorescein analogue),[11] and Rhodamine 110 with emission and excitation properties similar to both (Figure 1). We reasoned that replacement of the oxygen electron donor at the 6′ position with an alkyne in both Singapore Green and Tokyo Green will significantly decrease the fluorescence output of their xanthene core. We further extended this design to Rhodamine B by similarly substituting the diethylamino group at the 6′-position with an alkyne, as well as replacing the carboxylic acid moiety in Rhodamine B with a methyl group at the 2-position to lock the xanthene core in the conjugated quinol−iminium form. We anticipate that the

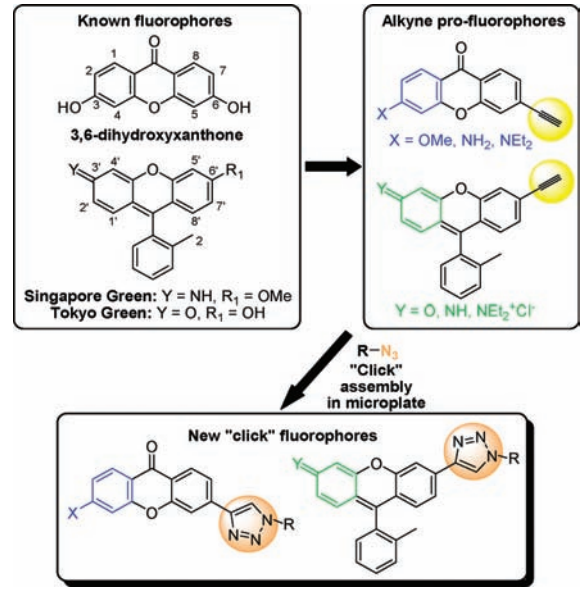

Figure 1. Design of xanthone- and xanthene-based fluorophores from known fluorophores.

formation of a triazole ring at this position using "click" chemistry will result in a fluorescence change in these xanthene−alkynes through an extended π-conjugated system and that this change can be tuned by the use of azides with different electronic properties. In the interest of extending the emission range of our "click" fluorophores from the blue to the yellow region, we also used the blue-light emitting xanthones which are synthetic precursors of our xanthenes (Figure 1). Similar to the design of our xanthene−alkynes, we replaced the heteroatom at the 6-position with an alkyne to yield the xanthone−alkyne for "click" modification. We noted that while there are several reports on the synthesis and spectroscopic characterizations of rosamine dyes from 3,6-disubstituted xanthones,[3b,12] to the best of our knowledge there are no detailed studies on xanthone-based fluorophores and their fluorescence properties. In this paper, we describe the synthesis of 6 xanthone− and xanthene−alkynes, the rapid microplate-based assembly of the "click" fluorophores, and the subsequent characterization and identification of "hit" fluorophores with varying fluorescence characteristics.

As shown in Scheme 1, the general synthetic strategy for the alkynes **A**, **B**, **D**, and **E** involves the desymmetrization of the common starting material 3,6-dihydroxyxanthone **1** to give the appropriate substituent at the 6-position, leaving the other phenolic group for conversion into a triflate which serves as the substrate for Sonogashira coupling with trimethylsilylacetylene. Deprotection of the TMS group affords alkynes **A** and **B**, while Grignard addition to the xanthone followed by removal of the protecting groups gave alkynes **D** and **E** (see the Supporting Information for full synthetic details). The synthesis of alkynes **C** and **F** followed the same strategy. Starting from 3-nitro-6-hydroxyxanthone

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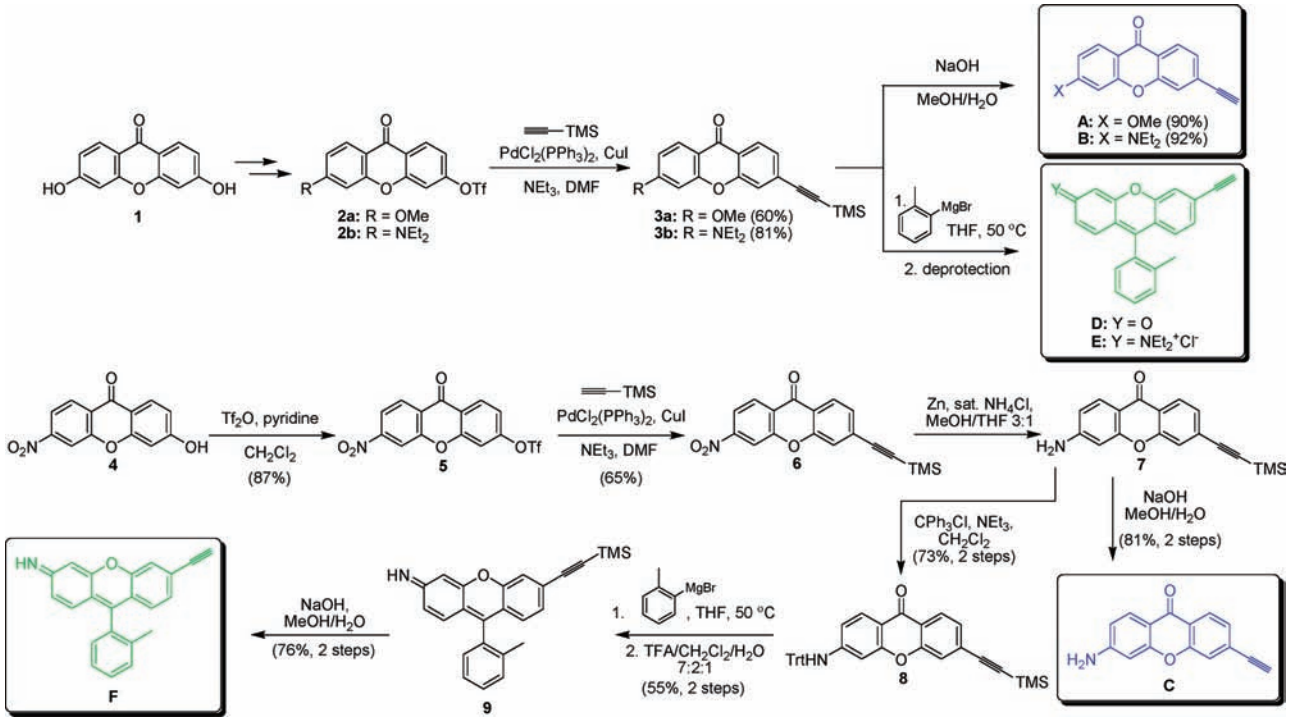
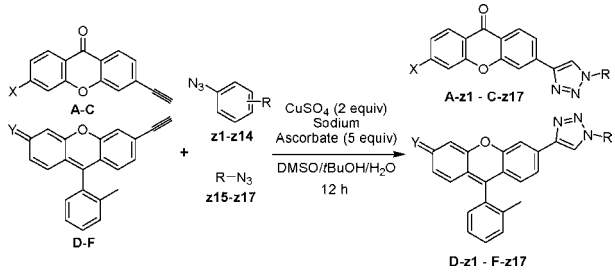
Scheme 1. Synthesis of Alkynes A−F



4, it was converted to triflate **5** which underwent Sonogashira coupling to give the TMS-protected nitroxanthone **6**. The nitro group was reduced under mild conditions to aniline **7** with zinc in MeOH/THF buffered at pH 5. Aniline **7** was protected with a trityl protecting group, followed by Grignard reaction to give the trityl-protected xanthene **9**. Subsequent deprotection of the trityl and TMS groups furnished the final alkyne **F**. Alkyne **C** was obtained after deprotection of the TMS group in **7**.

With the six alkynes in hand, we selected a total of 17 aromatic and aliphatic azides with different electronic properties for the "click" chemistry reaction (Scheme 2; see

Scheme 2. "Click" Assembly of Fluorophores



the Supporting Information for the azides used). A 102-membered fluorophore library was assembled in a 384-deep well block by mixing each of the alkynes with a different azide in DMSO/*t*-BuOH/H_2O to give a unique pair of alkyne and azide in each well. The reaction was initiated by adding a solution of $CuSO_4$ (2 equiv) and sodium ascorbate (5 equiv). The "click" reaction for the xanthene−alkynes **D**−**F** proved to be highly efficient, with the alkynes consumed within 12 h to give the products in high purity as monitored by LC−MS. The xanthone−alkynes **A**−**C** were considerably less reactive with some incomplete reaction after 12 h (see the Supporting Information). The desired product was obtained for all compounds with the exception of products with azides **z13** and **z14**, which did not give the desired products for all the alkynes and were thus omitted from subsequent studies. For preliminary evaluation of the fluorescence properties of the library, the crude reaction mixture was diluted to 400 μM in DMSO, which was further diluted to 20 μM for fluorescence screening. Since the quantum yield of fluorophores can be significantly influenced by solvent effects, we chose four different solvents—H_2O (aqueous, with DMSO as cosolvent to prevent precipitation), DMSO (polar aprotic), EtOH (polar protic), and DCE (apolar aprotic)—to record the excitation and emission spectra of all the fluorophores in the library (see the Supporting Information for details).

The results are summarized in the form of a heat map displaying the fluorescence intensities of each fluorophore relative to its alkyne precursor (that is, the xanthone or xanthene core prior to click chemistry) (Figure 2). Several observations can be made from the heat map. As anticipated, the majority of the "click" products registered an increase in fluorescence intensity (red squares) over the parent alkyne, but a considerable number also led to a fluorescence decrease

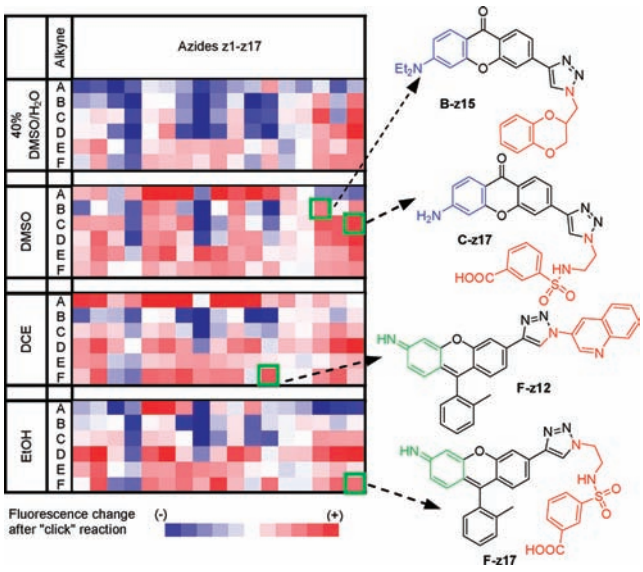


Figure 2. Heat map showing relative fluorescence intensities and structures of the four "hit" fluorophores selected for further studies. See the Supporting Information for details on the generation of the heat map.

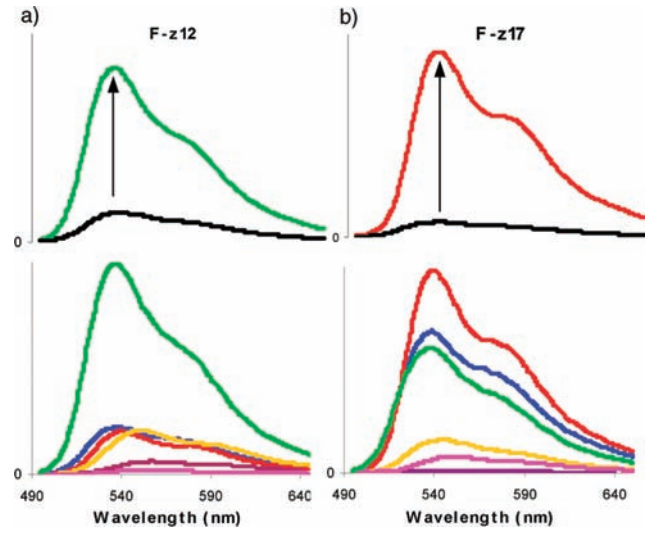


Figure 3. Emission spectra of (a) F-z12 and (b) F-z17 in different solvents (bottom graphs). Top graphs: emission spectra of F-z12 (in DCE; green line) and F-z17 (in EtOH; red line) were compared against the alkyne precursor F (in each corresponding solvent; black line). Black arrows indicate the increase in fluorescence after "click" reaction. See the Supporting Information for full spectra.

(blue squares). In particular, a morpholino substituent in the azide (**z4**, column 4) led to a consistent diminution of fluorescence throughout the alkyne series, possibly due to photoinduced-electron-transfer (PET) quenching from the nitrogen atom, and a strongly electron-withdrawing nitro substituent (**z8**, column 8) also effectively quenched the fluorescence in alkynes series **A**−**D**. Notably, the "click" reaction between aliphatic azides (column 15−17) and the xanthene−alkynes was fluorogenic, implying that triazole formation is itself sufficient for fluorescence activation. This feature is particularly important when searching for green light-emitting "click" fluorophores for use in labeling azide-modified biomolecules for bioimaging purposes.[5−7]

To examine in detail the fluorescence properties of our "click" fluorophores, we picked a few "hits" from each of the six alkyne series for scale-up, purification, and characterization. These "hits" were selected on the basis of the following points: (i) they showed significantly higher fluorescence intensity than their alkyne precursors; (ii) the "hits" give a good representation of aromatic and aliphatic azides of different properties; and (iii) their fluorescence intensities showed some solvent sensitivity (**F-z12** and **F-z17**). The spectroscopic properties of four of the brightest "hits" (structures shown in Figure 2) and their corresponding alkyne precursors were further evaluated (see the Supporting Information for full details). It was found that triazole formation led to an increase in both molar absorptivity and quantum yield, leading to an overall increase in brightness ($\varepsilon\Phi_f$) of 2−3-fold for **B-z15** and **C-z17** in DMSO, and ∼10-fold for **F-z12** in DCE and **F-z17** in EtOH (Figure 3). **F-z12** and **F-z17** also displayed different solvent sensitivity despite being derivatives of the same alkyne, with **F-z12** fluorescing most brightly in DCE, while in other solvents it is considerably less bright. **F-z17** is the brightest in EtOH and has varying intensities in other solvents. There is, however, no direct trend between the fluorescence intensities and solvent dielectric constants, suggesting that the observed solvent effects are specific to the molecular structure of the fluorophore. Because these effects shown in both our microplate screening and detailed analysis are not easily predicted, the combinatorial approach to various analogues is an advantage in searching for the desired fluorophore properties as demonstrated in this report.

In conclusion, we have successfully designed and synthesized two new classes of "click" fluorophores based on the xanthone and xanthene scaffolds. The rapid assembly of these fluorophores enabled by "click" chemistry gave easy access to xanthene analogues, which are traditionally difficult to synthesize. We have also identified two fluorophores, **F-z12** and **F-z17**, in which triazole formation resulted in a significant fluorescence increase. These fluorophores could potentially be used as green light-emitting substitutes for the existing "click" fluorophores in bioconjugation and bioimaging applications.[5−7] Their utilities in live cell imaging are in progress.

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Supporting Information Available: Experimental procedures, characterization of new compounds, and fluorescence spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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