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PII: S0040-4039(13)01939-4
DOI: http://dx.doi.org/10.1016/j.tetlet.2013.11.003
Reference: TETL 43798

To appear in: *Tetrahedron Letters*

Received Date: 5 July 2013
Revised Date: 2 November 2013
Accepted Date: 5 November 2013

Please cite this article as: Chauhan, D.P., Saha, T., Lahiri, M., Talukdar, P., BODIPY based "click on" fluorogenic dyes: Application in live cell imaging, *Tetrahedron Letters* (2013), doi: http://dx.doi.org/10.1016/j.tetlet.2013.11.003

Graphical Abstract

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Tetrahedron Letters
journal homepage: www.elsevier.com

BODIPY based "*click on*" fluorogenic dyes: Application in live cell imaging

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
BODIPY
Azide-Alkyne Click Reaction
Fluorescence
Solvatochromic Properties
Cell Imaging

ABSTRACT

The design, synthesis and photophysical properties of new BODIPY-based fluorogenic "*click on*" dyes are reported. CuAAC reaction of non-fluorescent BODIPY azide with a series of non-fluorescent alkyne molecules resulted in fluorescent triazoles which displayed upto 532-fold enhancement of fluorescence in the red region. Imaging studies confirmed the general trend of cell permeability and a cholesterol linked derivative exhibited selective localization into intracellular membranes.

Traditional small-molecule fluorescent probes used for labelling and sensing of chemical and biological events, exhibit an "*always on*" fluorescence which can mask valuable outcome of the experiments. To overcome this limitation, "*latent*" fluorophores are reacted with the molecules of interest, resulting in "*turn on*" of fluorescence. This phenomenon, also termed as fluorogenic reaction, has been used as a powerful analytical tool in diverse chemical context, *e.g.* detection of phenols,[1] thiophenols[2] and nitrosothiols in water[3] and acetylenes and copper in polymers,[4] etc. Biological applications of fluorogenic transformations are associated with labelling, detection and sensing of biothiols,[5] H_2S,[6] DNA,[7] proteins,[8] single biocatalytic events and bioconjugation processes.[9] Among various fluorogenic bioconjugation methods, copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction or click reaction,[10] and its non-catalytic, strain-promoted versions with either cyclooctynes[11] or oxanorbornadienes[12] have gained burgeoning attention.

The design of CuAAC reaction based fluorogenic probes are focused on the construction of either an alkyne or azide connected to a profluorophore to provide the quenched state. The probe after reacting with a cycloaddition partner provided a triazole compound which displayed strong fluorescence. Often, a photoinduced electron transfer (PET) process from the azide moiety (from nitrogen lone pair) to the profluorophore is responsible for the quenching of fluorescence (Figure 1A). On the other hand, triazole does not permit the PET process (as the lone pair is part of the aromatic system) and induces fluorescence activation.[13] In 2004, Wang and coworkers reported series of 3-azidocoumarins *e.g.* **1** in which the fluorescence is quenched due to the electron-rich α-nitrogen of the azido group.[13a] CuAAC reactions of these alkynes with various non-fluorescent alkynes resulted in the formation of fluorescent triazoles. In 2006, Wong and coworkers reported the 4-azido-1,8-naphthalimide **2** in which represents a non-fluorescent state.[13b] The CuAAC reaction of **2** with alkyne-linked sugars afforded strongly fluorescent triazolo-compounds. In 2008, Xie *et. al.*

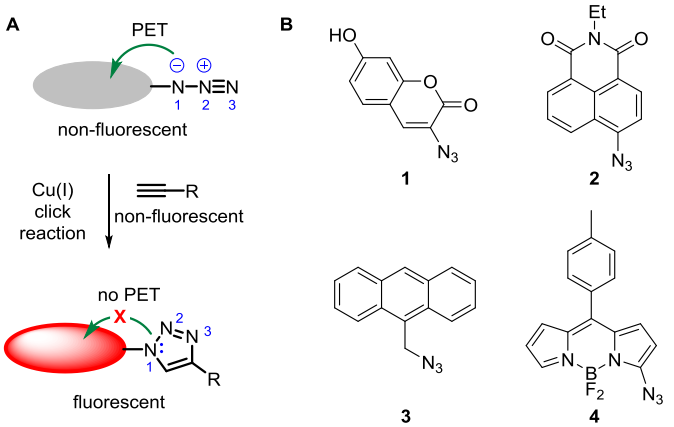

Figure 1. CuAAc based fluorogenic click reaction (**A**). Structures of fluorogenic azides **1** - **4** (**B**).

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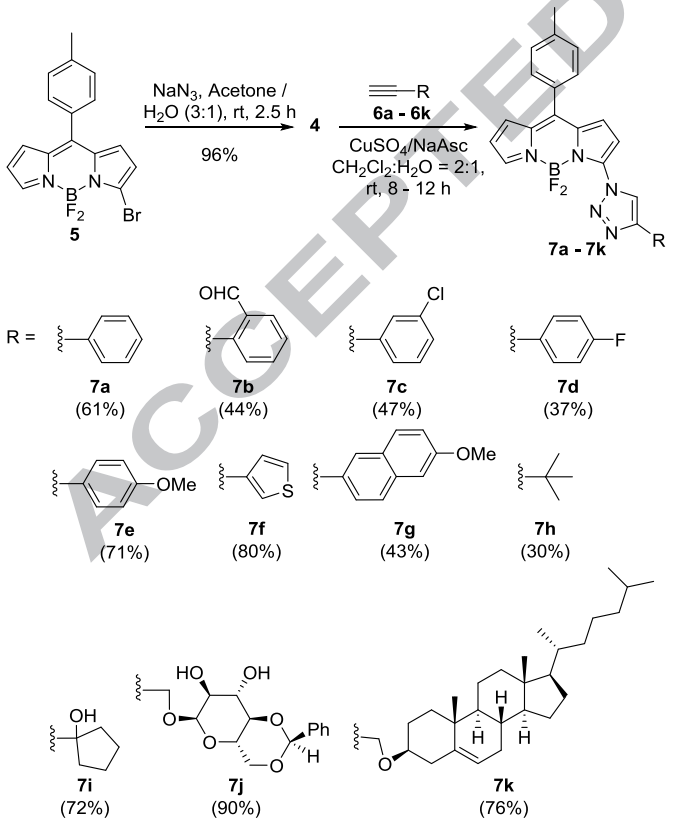
reported azido-anthracene **3** to act as fluorogenic probes via the CuAAC reaction.[13c] Recently, Bertozzi and several other groups applied the concept of copper-free click reaction which has been extensively used for bioconjugation and fluorogenic probe development.[14]

Herein, we report the design and synthesis of a new boron-dipyrromethene (BODIPY) based fluorogenic probe **4** with an azide connected at the 2-position of the BODIPY ring. Advantages due to the intense absorption in visible light, relatively high molar extinction coefficient (ε), biocompatibility, chemical and photochemical stability encouraged us to explore the "*click on*" chemistry with BODIPY fluorophore.[15] Synthesis of the azide **4** was carried out from the 3-bromo-BODIPY **5** by reacting with NaN_3 in acetone/H_2O (1:1) with 84% yield (Scheme 1).[6b] The click reactions were carried out on azide **4** with various alkyne analogues **6a** - **6k**. Selection of these substituents were based on choice of aliphatic (**6h** and **6i**) and aromatic ones (**6a** - **6g**) with additional possibility of further derivatization (**6b**, **6c** and **6i**). Examples also covered a representative protected sugar analogue (**6j**). Considering the importance of BODIPY-cholesterol in sterol trafficking in living cells and organisms,[16] a substituent consisting of cholesterol side chain (**6k**) was also selected. Reactions were carried out in presence of $CuSO_4$ and sodium ascorbate in CH_2Cl_2/H_2O (2:1) at room temperature and stirred for 8 - 12 h to furnish triazoles **7a** - **7k** with yields ranging from 30 to 90%. Relatively lower yields in few cases were possibly due to the reduction of BODIPY-azide **4** to corresponding BODIPY-amine in presence of sodium ascorbate also acting as a reducing agent.[17] This possibility can be rationalized by the electron withdrawing nature of the BODIPY ring which contributes to the reduction of the azide **4** during triazole formation reaction.[18]



Scheme 1. Synthesis of the probe BODIPY-triazole derivatives **7a** - **7k**.

All the synthesized triazole derivatives were characterized by NMR and Mass spectrometric analysis. Triazoles **7a** - **7c**, **7f** and **7i** were re-crystallized from CH_2Cl_2/hexane system and single crystal X-ray diffraction data also confirmed assigned structures of triazoles **7a** (Figure S1), **7b** (Figure S2), **7c** (Figure 2A), **7f** (Figure 2B) and **7i** (Figure S5).

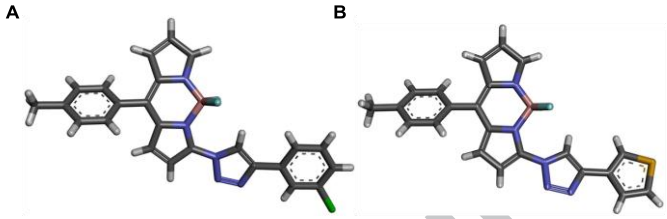


Figure 2. Crystal structures of (**A**) **7c** and (**B**) **7f**.

All BODIPY derivatives displayed appreciable solubility in water and ranges of organic solvents. Photophysical properties of the azide **4** (10 μM) and triazoles **7a** - **7k** (10 μM each) were recorded in HEPES buffer (10 mM, pH = 7.4). In solution, all the compounds showed a strong $S_0 \rightarrow S_1$ ($\pi \rightarrow \pi^*$) transition between 502 - 540 nm (Figure S6 - S17), unambiguously assigned to the boradiazaindacene chromophore.[19] At higher energy, a weaker band around 370 - 390 nm, can be attributed to the $S_0 \rightarrow S_2$ ($\pi \rightarrow \pi^*$) transition of the BODIPY (Figure S6-S17). Although, high molar absorption coefficient, ε = 23190 M^{-1} cm^{-1} was calculated for the azide **4**, it did not exhibit any significant fluorescence when excited at its λ_{max}^{2} value. The *off*-fluorescence can be corroborated to the PET mechanism from the azide to the BODIPY core.[13a] On the other hand, trizoles **7i** and **7j** exhibited λ_{em} = 530 and 528 nm, respectively when excited at respective λ_{max}^{2} values. Other triazoles displayed relatively higher λ_{em} in the range of 574 - 650 nm (Table 1). All triazoles when excited at respective λ_{max}^{1} values, exhibited emission spectra identical to those observed during λ_{max}^{2} excitations (Figure S6 - S17). The turn-*on* fluorescence of **7a** - **7k** confirms the deactivation of the PET pathway due to conjugation of nitrogen (N_1) lone pair in triazole ring (Figure 1A). In HEPES (10 mM, pH = 7.4) buffer, azide **4** displayed quantum yield, Φ = ~ 0 (standard: rhodamine G in water, Φ = 0.76). On the other hand, appreciable high Φ = 0.013, 0.025 and 0.021 were determined for **7c**, **7i**, and **7j**, respectively (in HEPES buffer, 10 mM, pH = 7.4). Other triazole derivatives provided Φ values ranging from 0.02 - 0.005 (Table 1).

Table 1. Photophysical properties of azide **4** and triazoles **7a**-**7k** in HEPES buffer (10 mM, pH = 7.4).

BODIPY	λ_{max}^{1} (nm)	λ_{max}^{2} (nm)	ε^{a} (M^{-1} cm^{-1})	$\lambda_{em}^{2\,b}$ (nm)	Φ
4	370	515	23190	0	0
7a	390	538	34500	580	0.012
7b	390	534	30560	575	0.005
7c	390	538	27200	580	0.013
7d	390	538	22700	580	0.013
7e	380	540	15880	594	0.008
7f	390	535	9410	582	0.018
7g	377	540	21290	650	0.002
7h	380	530	24830	574	0.019
7i	383	502	12250	530	0.025
7j	380	502	13830	528	0.021
7k	380	535	7500	578	0.002

[a] Calculated at corresponding λ_{max}^{2} value. [b] Fluorescence spectra of compounds were recorded at 10 μM concentrations in HEPES buffer (with 0.5% DMSO) at pH = 7.4.

The quantitative fluorescence intensity enhancement for triazoles **7a** - **7k** (10 μM each) were determined relative to the azide **4** (10 μM) at 580 nm in HEPES buffer (10 mM, pH = 7.4). This data indicated a 532-fold fluorescence enhancement for

triazole **7i** (Figure 3). Similarly, 334- and 332-fold enhancements in fluorescence were observed for triazoles **7a** and **7c**, respectively. For other triazoles, the increase in fluorescence intensity varied from 15- to 300-fold.

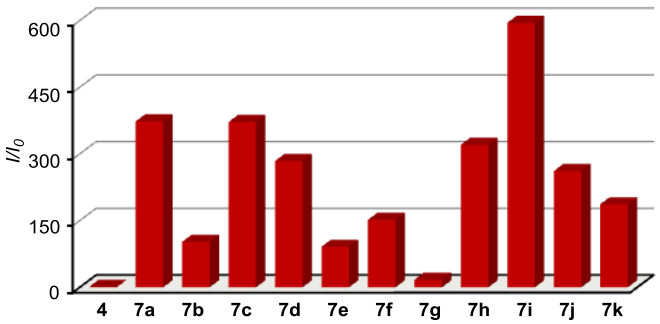


Figure 3. Comparison of fluorescence intensities (at λ = 580 nm with excitation at respective λ_{max} values) of BODIPY triazoles **7a** - **7k** (10 μM each) relative to the azide **4** (10 μM).

Naked eye detection of fluorogenic click reactions was studied in the next stage. Briefly, first 12-wells of a 24-well plate were used in which each well represents either azide **4** or a single triazole product (50 μM) in HEPES (10 mM, pH = 7.4). All BODIPY compounds **4** and **7a** - **7k** displayed pink color under ambient light (Figure 4A). When placed under the hand-held UV lamp (λ_{ex} = 365 nm), the well containing the azide **4** remained dark (Figure 4B). Significantly, low fluorescence illuminations were observed for **7e** and **7g** as expected from the Figure 3. Triazoles **7i** and **7j** exhibited green fluorescence which corroborates to their observed λ_{em} values. Other triazoles (**7a**-**7d**, **7f**, **7h** and **7k**) exhibited fluorescence in the red region.

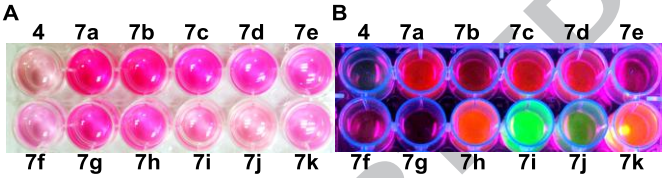


Figure 4. Naked eye detection of change in color (**A**) and fluorescence (**B**) of **7a** - **7k** (50 μM each) compared to **4** (50 μM). Photographs were taken either under ambient light (**A**) or under a hand-held UV lamp (**B**) with λ_{ex} = 365 nm.

Further we studied the solvatochromism properties of BODIPY triazole **7c** (10 μM). This derivative was selected because of its relatively high ε and Φ values. Absorption and fluorescence spectra of **7c** were recorded in various solvents including polar, nonpolar, protic and aprotic ones (Table 2, Figure S18). For the triazole, a decreasing order of λ_{max} was observed with the increase in solvent polarity (*i.e.* λ_{max} = 531 nm in cyclohexane and λ_{max} = 517 nm in DMF). However, in H_2O a significantly high λ_{max} = 535 nm was observed. BODIPY **7c** displayed comparable λ_{em} values (537 - 545 nm) in all solvent, except in H_2O (λ_{em} = 578 nm). For a symmetrical BODIPY derivative, two identical zwitterionic structures **I** and **II** (ignoring the triazole ring) are possible (Figure 5). However, introduction of triazole ring contributes an additional canonical form **III** with relatively higher dipole moment. The observed trend in λ_{max} value (from cyclohexane to DMF) infers a better stabilization of the ground state in polar solvent compared to the excited state leading to decrease in λ_{max}. Therefore, the observed increasing trend Stoke shift, $\Delta\nu$ (*i.e.* $\Delta\nu$ = 483 cm^{-1} in cyclohexane and $\Delta\nu$ = 892 cm^{-1} in DMF) can also be corroborated to the stabilization of the ground state.[20] However, contribution from hydrogen bonding between the excited state of **7c** and H_2O may be responsible for the stabilization of the state compared to that in other solvents. As a result, bathochromic shifts in both λ_{max} and λ_{em} were observed in the solvent leading to a large $\Delta\nu$ = 1390 cm^{-1}.

Table 2. Solvatochromic properties of **7a** (10 μM) in various solvents.

Solvent	P' [a]	λ_{ex} (nm)	ν_{ex} (cm^{-1})	λ_{em} (nm)	ν_{em} (cm^{-1})	$\Delta\lambda$ (nm)	$\Delta\nu$ (cm^{-1})
Cyclohexane	0.2	531	18832	545	18348	14	483
CCl_4	1.6	532	18796	548	18248	16	548
2-Propanol	3.9	524	19083	540	18518	16	565
Chloroform	4.1	528	18939	545	18348	17	590
Methanol	5.1	518	19305	537	18621	19	683
Acetonitrile	5.8	516	19379	538	18587	22	792
DMF	6.4	517	19342	542	18450	25	892
Water	10.2	535	18691	578	17301	43	1390

[a] Solvent polarity index.

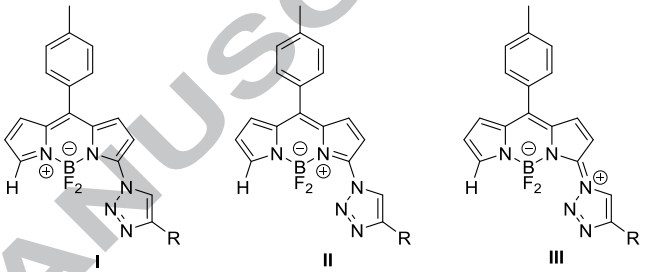


Figure 5. Major contributing resonance structures of BODIPY-triazole.

Red shifted excitation wavelength and low cytotoxicity of BODIPY inspired us to evaluate applicability of synthesized analogs as fluorescent markers of specific cellular organism. Initial screening of cell permeability was conducted with **7a** (Figure S19A). Based on the higher fluorescence intensity and Φ values BODIPY-triazoles **7c**, **7f** and **7i** were selected for live cell imaging studies. On the other hand, **7k** bearing a cholesterol unit with BODIPY-triazole core was selected to evaluate its membrane accumulation. When each of triazoles **7c**, **7f** and **7i** (500 nM in 1:1000 DMSO/DMEM v/v, pH = 7.4) was separately incubated with the HeLa cells at 37 °C for 30 min followed by washing with PBS, good permeability of these compounds into the cell and significant fluorescence was observed (Figure 5 and Figure S19).

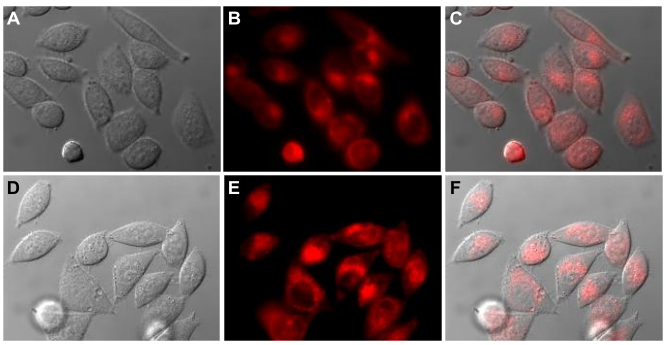


Figure 6. Images of HeLa cells: brightfield (**A**), fluorescence (**B**), and overlay (**C**), incubated with probe **7c** (500 nM) for 30 min. (**D**-**F**) are the respective brightfield, fluorescence and overlay image of HeLa cells incubated with probe **7f** (500 nM) for 30 min.

When permeability of the cholesterol linked BODIPY-triazole **7k** (500 nM) was tested in HeLa cells after 30 minutes of incubation, its predominant accumulation at the nuclear membrane was observed (Figure 7A, B). This was further

confirmed (Figure 7D) by marking the location of the nucleus with Hoechst stain (Figure 6C, blue emission) and overlaying with cell image of probe **7k** (Figure 7D, red emission). However, localization of the BODIPY at other intracellular membranes cannot be ruled out. No significant accumulation of **7k** in the plasma membrane was detected. This inference was achieved by comparing the DIC and fluorescence image for a particular cell. This is probably due to preferential adherence of cholesterol into the double lipid bilayer of nuclear membrane over single lipid bilayer of the cellular membrane.

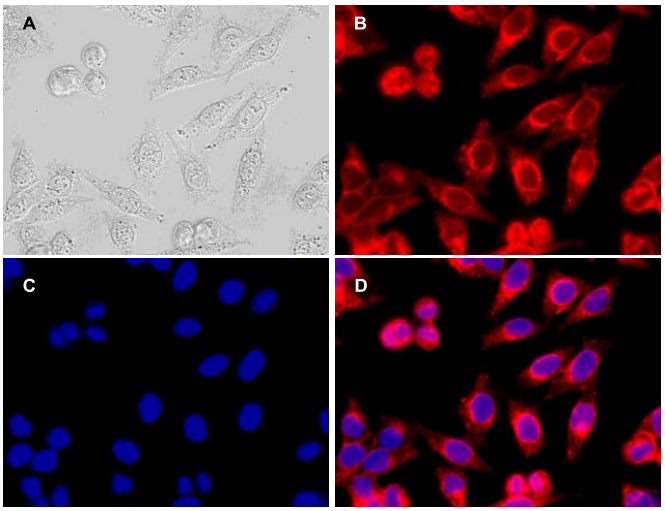


Figure 7. Images of HeLa cells incubated with 500 nM of **7k** for 30 minutes and 2 μM of Hoechst for 15 minutes respectively (1:1000 DMSO/DMEM v/v, pH = 7.4). Bright field (**A**), fluorescence at red channel (**B**), fluorescence at blue channel (**C**) and overlay of both fluorescence image (**D**).

In summary, we have developed BODIPY-based fluorogenic dyes having red shifted absorption and emission wavelengths. BODIPY azide **4** was non-fluorescent while CuAAC reaction resulted in BODIPY triazoles **7a** - **7k** displaying up to 532-fold enhancement in red/green fluorescence. Solvatochromism studies for the triazole **7c** showed increase in Stoke shift with the increase in solvent polarity. Live cell imaging studies for **7a**, **7c**, **7f** and **7i** confirmed the permeability of these dyes into the cytoplasm of Hela cells. Interestingly, the cholesterol-linked dye **7k** displayed its preferential accumulation at the intracellular membranes over plasma membrane. Simple synthesis and intracellular membrane incorporation of the fluorescent sterol derivative offers its potential application for cholesterol trafficking in living cells and organisms. A systematic study in this line is in progress.

Acknowledgments

P.T. is grateful to the Director, IISER Pune, and DAE (Grant No. 2010/20/37C/6/BRNS/2480) for financial support. D.P.C. and T.S. thank CSIR and UGC for research fellowships.

Supplementary data

Supplementary data (complete experimental procedures, and spectral data for all new compounds, crystal structure parameters of newly reported crystals and details of photophysical characterization data for **4**, and **7a-7k**) associated with this article can be found, in the online version, at

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