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Developing visible fluorogenic 'click-on' dyes for cellular imaging

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Introduction

Fluorescent dyes are a family of optical reporters widely used in studying biomolecules in cells[1] and animals[2]. With an appropriate fluorescent tag, the location and quantity of the target biomolecules or bioprocess can be tracked conveniently. Most dyes fluoresce continuously and show no difference in fluorescence property before and after labels; therefore any leftover unreacted free dye could interfere with the dye attached to the molecules of interest, significantly lowering the signal to background contrast. An alternate way to label target molecules without worrying about the background issue is to use fluorogenic fluorochromes, whose emission wavelength or intensity shift or change after the labeling reaction[3]. Taking advantage of these unique fluorescent properties could minimize the high background issue. Although the benefit of using the fluorogenic fluorochromes is obvious, the approach has not been applied widely for at least two reasons; limited available fluorogenic fluorochromes, and the non-selective reactivity of these dyes. Without specific chemical reactivity, labeling particular molecule remains challenging.

The 'click' reaction between an azide and an alkyne groups has recently been used to label various biomolecules in cells[4-7] and even animals[8,9]. The small size of the azide and alkyne groups enables them to 'trick' some nature synthases, and thus modified amino acids[10], nucleosides[11-13] and monosaccharide[14] with an azide or alkyne reactive group have been reported as the tagging residues for proteins[15], nucleic acids[13] and carbohydrates[16]. Since azide and alkyne reactive groups are not normally expressed in biological systems, the 'click' reaction is specific. Many existing dyes have been engineered with a reactive azide or alkyne functional group for labeling[7,17]; however this modification only solves half of the problem, i.e. specific reactivity. The background signal remains an issue.

A new kind of 'click-on' fluorogenic dyes has lately been reported to overcome the aforementioned problems[18]. The 'click-on' fluorogenic dyes were designed to have low or no baseline fluorescence by masking the core fluorophore with an electron-donating azide group or electron-withdraw alkyne group. After the 'click' reaction, the newly forms triazole conjugating structure delocalizes the local electrons and restore fluorescence. To date, only a handful of successful examples, derived from coumarin (**1, 2**)[18-21], anthracene (**3**)[22] and naphthalimide (**4**, **5**)[23] have been reported (Figure 1). More 'click-on' dyes that fluoresce at different wavelength are needed for various biomolecule labeling and applications.

Recently, we also reported an alkyne-containing benzothiazole 'click-on' dye (**6**), which has extremely low initial fluorescence, and its 'click-on' product shows 158-fold increase in

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Supporting Information. Details of synthesis, characterization and cell culture validation. This material is available free of charge via the Internet at http://pubs.acs.org.

fluorescence[24]. However, its low excitation and emission maxima which are approximately 290 nm and 360 nm, respectively, have restricted its biological application. In order to improve this shortcoming, we hypothesized that an electron-donating group such as dimethylamino or methoxyl group could enhance the effect of conjugating the parental aromatic benzothiazole ring, shifting the excitation and emission spectra into the visible range.

Result and discussion

To add a methoxyl group at the 6-position of benzothiazole, 6-methoxybenzothiazole-2-alkyne **10** was synthesized using **7** as starting material (Scheme 1). Following a previous developed protocol[24], **7** was initially transformed with 86% yield into iodobenzothiazole **8** via Diazotization-Sandmeyer iodination in the presence of *p*-toluenesulfonic acid in acetonitrile[25]. The protected acetylenic benzothiazole **9** was obtained with 68.3% yield after a cross-coupling reaction of iodobenzothiazole **8** and a terminal alkyne with catalytic quantities of $Pd(PPh_3)_2Cl_2$ under a basic condition[26,27]. The prepared **9** was deprotected with KF in MeOH at room temperature for 3h, and then purified by a silica gel column to obtain **10** with 99% yield.

To prepare benzothiazole with dimethylamino at 6-position, 6-dimethylbenzothiazole alkyne **16** was prepared from **11** (Scheme 2). Nitration of **11** was done with a mixture of concentrated sulfuric acid and fuming nitric acid to give **12**. The nitro group at the 6-position was then reduced with iron powder in MeOH under catalytic quantities of acetic acid to afford 6-amino-compound **13**[28]. Reductive amination of **13** with formaldehyde and triacetoxyborohydride in DCM formed 6-dimethyl compound **14**. Compound **16** was prepared, using a similar procedure for **10**, from **14** via cross-coupling and deprotection reaction with 44.5% and 84.6% yield, respectively.

The reactivity and ‘click-on’ fluorogenic properties of the prepared compounds **10** and **16** were tested by performing a [3+2] cycloaddition, also known as ‘click’ reaction, with model analogs **17(a~c)**, representative analogs of nucleoside, amino acids and linker (Scheme 3). Cu^{2+}/TBTA (Tris[(1 - benzyl - 1H - 1,2,3 - triazol - 4 - yl)methyl] amine) was used as the catalyst in DMF/H_2O for 1h at room temperature. Near quantitative conversion was obtained for all reactions (Supporting material).

As expected, a red-shift was observed as an electron-donating group was added to the 6-position of benzothiazol. In aqueous buffer, the emission maxima of 6-methoxyl dye **10** and 6-dimethylamino dye **16** was approximately 390 nm and 500 nm, respectively. More red-shift was achieved with dimethylamino dye **16**. The emission maximum of the parent benzothiazole **6** is approximately 360 nm. Importantly, both dyes **10** and **16** were ‘clickable’ fluorogenic dyes whose ‘clicked’ products had a significant increase in fluorescence intensity (Figure 2). The quantum yield of **18b** was 0.36, but that of its parent compound **10** is only 0.05 in EtOH. The fluorescence intensity of **18b** (10μM) in pH=7.0 PBS buffer was 7.9-fold stronger than that of the parent dye **10** under the same condition. The quantum yield of **19b** and its parent compound **16** in EtOH were 0.65 and 0.49, respectively. The fluorescence intensity of **19b** (10μM) in pH=7.0 BPS buffer was 3.5-fold stronger than that of the parent dye **16**. Interestingly, the click reaction had little effect on wavelength.

Dimethylamino series, compounds **16** and **19**, had a long Stoke shift, 140-150 nm, in PBS buffer, while the Stoke shift of methoxyl series, compounds **10** and **18**, was approximately 70 nm. Solvent effect was also more significant for the dimethylamino series (Table 1). The Stoke shift for compounds **19** in methanol was shortened to 100 -110 nm. In contrast, the 6-methoxy series only showed approximately 10 nm difference between the two solvents.

The fluorescent property of compounds **10** and **16**, and their corresponding 'clicked' dyes **18b** and **19b** was further investigated in different pH condition. As seen in Figure 3, compounds **10** and **18b** were relatively pH stable, while compounds **16** and **19b** showed clear pH-dependent. From Figure 3b, the fluorescence intensities of compound **16** and **19b** were down to zero at pH lower than 2. The fluorescence intensity of compound **16** and **19b** increased with increasing pH, reaching the maxima at pH 6-8. The fluorescence intensity of **19b** dropped considerably on pH increase to 10; but the fluorescence intensity of **16** decreased only slightly. This clear pH dependence could be due to protonation of the dimethylamino group. In contrast, the methoxyl group cannot be protonated as easy as the amino group, thus dyes **10** and **18b** are more pH stable.

The potential application of the developed 'click' fluorogenic dye was then validated in cell culture. For a better fluorescent contrast in biological application, dye **16** which has a long emission maximum and Stoke shift was selected for the study. The azide group was introduced into HeLa cells by incubating with AZT **17a** (2μM) for 16 hrs. AZT is a nucleoside analog which is known to be incorporated into DNA. After a thorough washing, cells were incubated with dye **16** and $CuSO_4$ for 3 h, followed by washes, and then reducing agent NaAsc was added to initiate the 'click' reaction. Thirty minutes later, the cells were washed and monitored under microscope. After 'click' reaction, an approximately 2-fold high fluorescent signal was observed in the AZT treated cells using the common filter set for DAPI (4′, 6-diamidino-2-phenylindole) stain (Figure 4). The fluorescent signal clearly highlight the nucleus, indicating that incorporated AZT reacted with dye **16** and the 'click' reaction is able to brighten the fluorescent signal in cells. In a separate experiment, the cells were treated with AZT **17a** and dye **16**, but $CuSO_4$. The fluorescent signal level of these cells is compatible to it of the cells without AZT **17a**.

Conclusion

We describe the preparation of two 2-ethynyl-benzothiazole derivatized 'click-on' fluorogenic dyes. The two derivatives with an electron-donating dimethylamino or methoxyl group at the 6-position showed a 30 to 130 nm red-shift in fluorescence wavelength, compared to the parent benzothiazole fluorogenic dye. The model reactions with functionalized nucleoside and amino acid indicated that the developed 'click-on' dye could label various biomolecules, such as nucleic acids, proteins and other molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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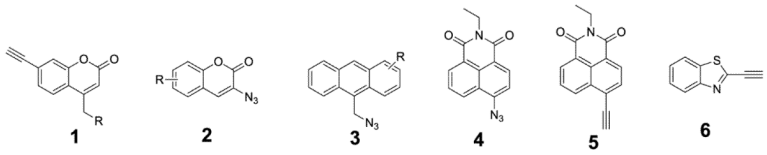


Figure 1.
Example for existing fluorogenic dyes

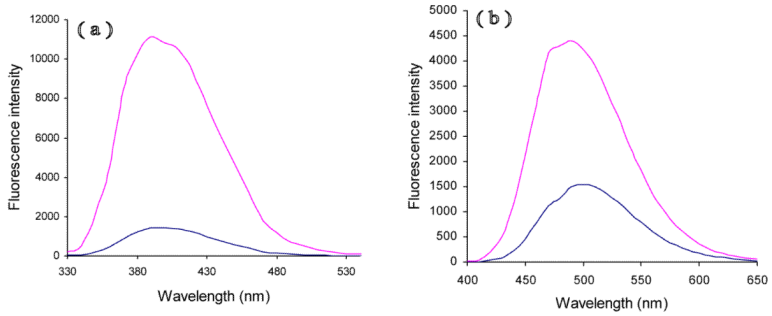


Figure 2.

Emission spectra of benzothiazole derivatives (10μM) in 3ml pH=7 buffer solution containing: (a) Compound **10** (blue) and **18b** (red) (λex=316nm); (b) Compound **16** (blue) and **19b** (red) (λex=375nm).

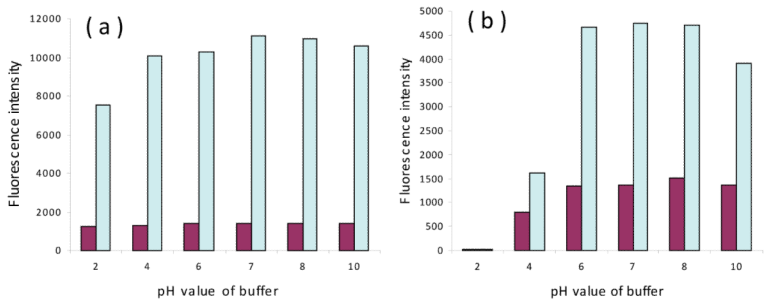


Figure 3.

Effect of pH on fluorescence intensity ratio of benzothiazole derivatives in different pH PBS buffer. Condition: (a) compound **10** (Brown) and **18b** (Cyan), λex: 316nm, 10μM. (b) compound **16** (Brown) and **19b** (Cyan), λex: 375nm, 10μM.

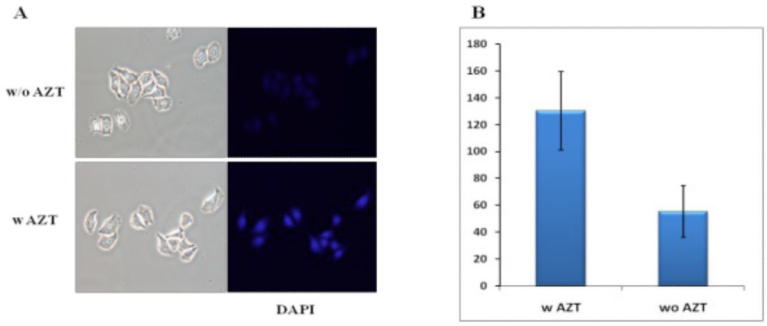
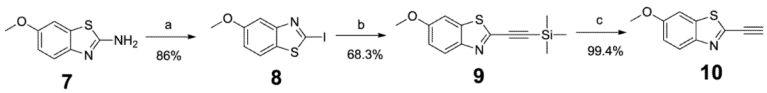


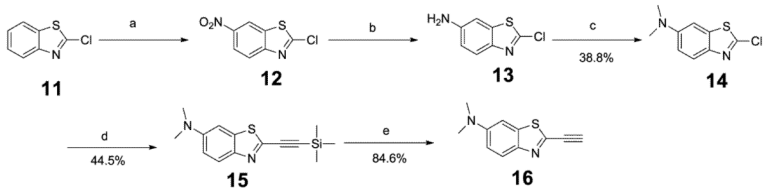
Figure 4.

'Click on' fluorescence images of cells (A), and quantitation of fluorescent signal (B) after reacting with compound **16** (10 μM) in HeLa cells (x20). The fluorescent signal was imaged using the convention DAPI filter set.



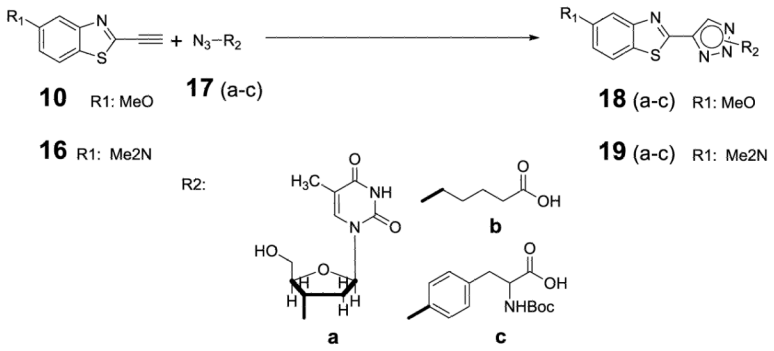
Scheme 1.

Reagents and conditions: (a) 1. p-$MeC_6H_4SO_3H$, MeCN, RT-10°C, 2. $NaNO_2$, H_2O, 10min, 10-15°C. 3 KI, RT, overnight. (b) 1.ethynyltrimethylsilane, CuI, $PdCl_2(PPh_3)_2$, NEt_3, DCM, 2. $NaHCO_3$, H_2O. (c) KF, MeOH, RT, 3h.



Scheme 2.

Reagents and conditions: (a) HNO_3, H_2SO_4. (b) Fe, EtOH, AcOH, 55°C, 3h. (c) 36% formaldehyde, DCM, $NaBHAc_3$, HOAc, RT, 2h. (d) 1.ethynyltrimethylsilane, CuI, $PdCl_2(PPh_3)_2$, NEt_3, DCM, 2. $NaHCO_3$, H_2O. (e) KF, MeOH, RT, 3h.



Scheme 3.

Model 'Click' reactions Reagents and conditions: $CuSO_4$, sodium ascorbate, TBTA, DMF/H_2O(3/1), RT, 30min

Table 1

UV absorption and emission spectra of compounds **10**, **16**, **18**(**a-c**) and **19**(**a-c**)

	$\lambda_{max(abs)\ (nm)}$	$\lambda_{max(em)\ (nm)}$	Φ	Stoke shift (nm)
10	316(MeOH) 316 (H_2O)	379 (MeOH) 393 (H_2O)	0.05*	63 77
18a	316(MeOH) 316 (H_2O)	381 (MeOH) 392 (H_2O)		65 76
18b	315(MeOH) 315(H_2O)	379 (MeOH) 391 (H_2O)	0.36*	64 76
18c	314(MeOH) 314 (H_2O)	381 (MeOH) 390 (H_2O)		67 76
16	364(MeOH) 343(H_2O)	460 (MeOH) 500 (H_2O)	0.49**	96 157
19a	356(MeOH) 350(H_2O)	466 (MeOH) 492 (H_2O)		110 142
19b	355(MeOH) 335(H_2O)	459 (MeOH) 493 (H_2O)	0.65**	104 158
19c	352(MeOH) 332(H_2O)	459 (MeOH) 491 (H_2O)	0.63**	107 159

Quantum yield condition:

* Solvent: EtOH, λex: 330 nm; slit: 4 nm, λem: 345-550 nm; slit: 4 nm, Inc.: 1 nm; reference compound: 9,10- diphenylanthracence, Φ = 0.90

** Solvent: EtOH, λex: 360 nm, slit: 2 nm, λem: 375-650 nm; slit: 2 nm, Inc.: 1 nm; reference compound: 7-diethylamino-4-methylcoumarin, Φ = 0.73.