



Development of benzothiazole ‘click-on’ fluorogenic dyes

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ABSTRACT

‘Click-on’ fluorogenic reaction: a non-fluorescent benzothiazole with an electron-deficient alkyne group at 2-position reacts with azide containing molecules could form fluorescent adducts.

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To study specific molecules in cells, precise labeling is the key. Ideally, the fluorescent reporter should only tag the interested targets. Fluorescent proteins co-expressed with targeted molecules have been widely used in studying cellular events, and the fluorescent signal makes the real time microscopic visualization of process possible.^{1,2} However, the fluorescent proteins are large proteins, which could be too big for many applications. Small peptide tags have also been introduced into protein expressing systems for site-specific labeling.^{3,4} For instance, a peptide tag recognized by Texas-red dye has been expressed in cells for fluorescent calcium sensing.⁵ Tetracysteine (–Cys-Cys-Pro-Gly-Cys–) containing proteins, which could be labeled by fluorogenic biarsenical probes, have been developed for cell labels.^{6,7} Yet these methods are still protein based approaches, which cannot be applied to investigate non-protein molecules. In order to study other biomolecules, flexible non-protein based labeling approaches are required.

Theoretically, chemical approaches with millions of diversities should be handy for intracellular labeling of biomolecules. Nevertheless, site-specific chemical labeling of biomolecules is not an easy task. Reactions that require non-physiological conditions, such as heat, extreme pH, or organic solvents, cannot be applied to normal cells. Commonly used conjugating groups, including activated ester which react with amino groups and maleimide which react with sulfhydryl groups, these groups are not practical for the site-specific labeling, due to high numbers of amino and sulfhydryl groups in cells. A special chemoselective ligation reaction has to be designed for each type of target. A few chemical ligation approaches using ketone, azide, and alkyne as reactive groups

have been reported for cell labels as well.⁸ The typical procedure is started by introducing a small percentage of chemically modified building units together with normal building units, such as amino acids, monosaccharides, or nucleosides, into cell culture. If the appended chemical group was small enough and could be tolerated by natural synthase, the biosynthetic machinery would assemble the biomacromolecules containing those unusual building units. The introduced chemical reactive groups presented on biomacromolecules then would serve as the linking points for subsequent fluorescent tagging.

In recent years, ‘click reaction’ has become a handy tool used in drug discovery,^{9–11} combinatorial chemistry,¹² material science,¹³ and tagging of biomolecules.¹⁴ The 1,2,3-triazole formation from an azide and an alkyne can be done in aqueous condition, therefore this approach has been applied in cells^{15,16} and, most recently, in animals.¹⁷ Fluorescent reporters have been clicked onto almost all kinds of biomolecules, such as nucleic acids, proteins, carbohydrates, and lipids. However, most fluorescent reporters used in click chemistry are prepared from existing fluorochromes which are functionalized with an alkyne or azide group through a linker. Although the azide/alkyne click reaction is specific, excess fluorochromes are always added to the reaction to ensure good conversion. Since those reporters fluoresce at all time, high background fluorescence signals prohibit real time visualization. Recently, few fluorogenic dyes, such as coumarin **1** and **2**,^{12,18} anthracene **3**¹⁹ and naphthalimide **4** derivatives,²⁰ were reported to have no-fluorescent property until the click reaction occurred. These ‘click-on’ fluorogenic dyes will not have the aforementioned high background issue, because they are designed to be non-fluorescent in their initial structure (Fig. 1). They only fluoresce after the completion of click reaction; therefore, the excess unreacted dyes will not interfere with fluorescence signal.

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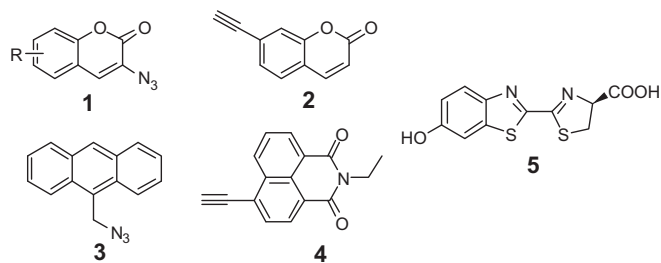


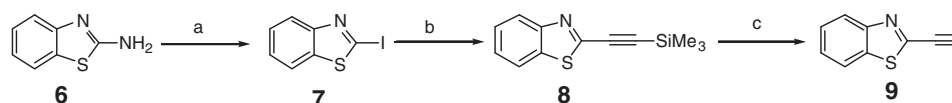
Figure 1. Examples for existing fluorogenic dyes and luciferin **5**.

Recognizing the usefulness of the switchable dyes, we have developed a new benzothiazole based fluorogenic ‘click-on’ dye and demonstrated its potential usages in nucleic acid and protein labeling.

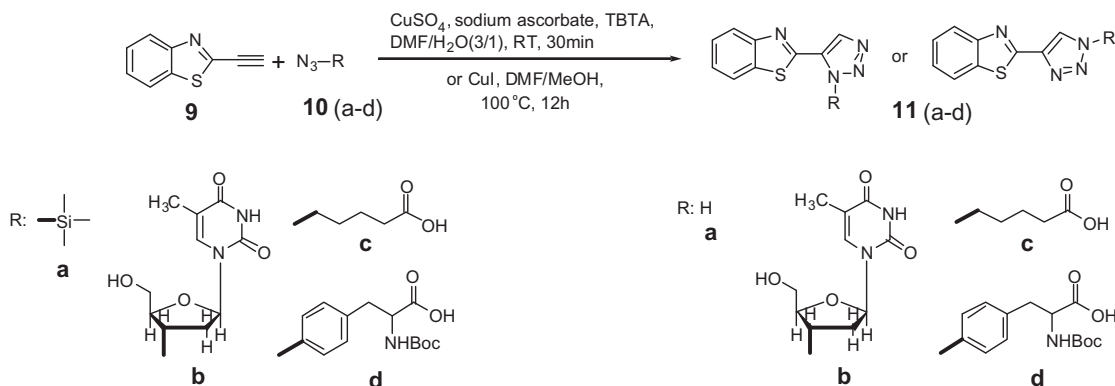
Benzothiazole alkyne **9** was chosen as the profluorophore since its analog luciferin **5** has been shown to have fluorescent

property.^{21–24} Based on the chemical structure of benzothiazole ring, it was hypothesized that an electron-deficient alkyne or electron-rich azide groups at the 2-position would diminish the fluorescence property. However, a click reaction which adds a conjugated triazole ring onto the parent benzothiazole ring will convert the chromophore into a fluorophore.

Benzothiazole alkyne **9** was prepared using **6** as the starting material (Scheme 1). Following the protocol developed by Elena et al., **6** was transformed into iodobenzothiazole **7** by a diazotization–iodination in the presence of *p*-toluenesulfonic acid in acetonitrile.²⁵ The protected acetylenic benzothiazole **8** was obtained by the cross-coupling reaction of iodobenzothiazole **7** with a terminal alkyne using catalytic quantities of $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ and CuI under basic conditions.²⁶ The prepared **8** was then applied to a silica gel column for further purification; whereas it was found that the protecting trimethylsilyl group came off automatically during chromatographic purification, resulting in the desired benzothiazole alkyne **9**. As expected, **9** whose absorbance maximum is 286 nm (Fig. S1) is non-fluorescent.



Scheme 1. Reagents and condition: (a) *p*- $\text{MeC}_6\text{H}_4\text{SO}_3\text{H}$, MeCN, rt to 10 °C, KI, NaNO_2 , H_2O , 10 min, 10–15 °C; (b) (1) CuI , $\text{PdCl}_2(\text{PPh}_3)_2$, NEt_3 , DCM, (2) NaHCO_3 , H_2O ; (c) acid.



Scheme 2. Click reaction.

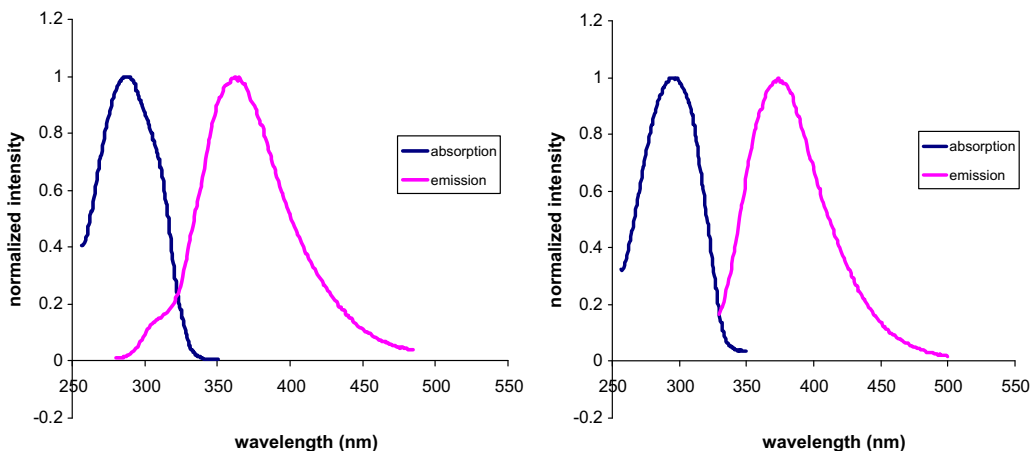


Figure 2. Absorption and fluorescence of dye **16** (left) and **11a** (right) in MeOH.

Table 1UV absorption and emission spectra of compounds **11a–d** and **16**

	11a	11b	11c	11d	16
$\lambda_{\text{max(abs)}} [\text{nm}]$	286	278	290	294	297
$\lambda_{\text{max(em)}} [\text{nm}]$	363	351	353	354	374

Condition: in MeOH, excitation wavelength: 285 nm.

To confirm that the proposed ‘click-on’ reaction can create a fluorophore, the benzothiazole alkyne **9** was first reacted with trimethylsilylazide **10a** (according to method by Jin et al.)²⁷ in DMF/MeOH using CuI as catalyst, at 100 °C for 12 h (Scheme 2). The reaction conversion was only around 20%. But importantly, the clicked product **11a** was fluorescent, having excitation and emission at 285 nm and 363 nm, respectively, in methanol (Fig. 2 (right)). Later, refer to method by Chan et al.,²⁸ TBTA (tris-(benzyltriazolylmethyl)amine) was used as the catalyst under room temperature for 1 h, the same reactions were tested with other azide containing analogs, including nucleoside AZT (azidothymidine) (**10b**), 6-azido-hexanoic acid (**10c**) and Boc-*p*-azido-Phe-OH (**10d**).

Near quantitative conversion was obtained for all reactions (Table S1), and all clicked products are fluorescent as compound **11a**. In general, linking different groups to the 2-position of the 1,2,3-triazole ring has little effect on the absorption and emission maxima (Table 1). All ‘click-on’ products (**11a–d** and **16**) showed absorption maximum at around 278–297 nm, emission maximum at around 351–374 nm and Stokes’ shift around 70–80 nm in methanol. The fluorescence change of the ‘click-on’ reaction was monitored in real time by reacting compound **9** ($\phi = 0.14\%$ in water, using L-tryptophen as a reference) with **10d** ($\phi = 3.5\%$, in water) catalyzed in an aqueous solution of sodium ascorbate and CuSO₄ at rt. The broad emission band was observed from approximately 310 nm to 500 nm, which is characteristic of **11d** (Fig. S2), once the CuSO₄ was added to the mixture solution of **9** and **10d** in 1 mM NaAsc aqueous solution. As the reaction proceeds, the fluorescence emission increases strongly. The fluorescent signal increased from 1.6 a.u. to 254 a.u. within 24 h, representing a 158-fold increase in fluorescence signal (Fig. 3).

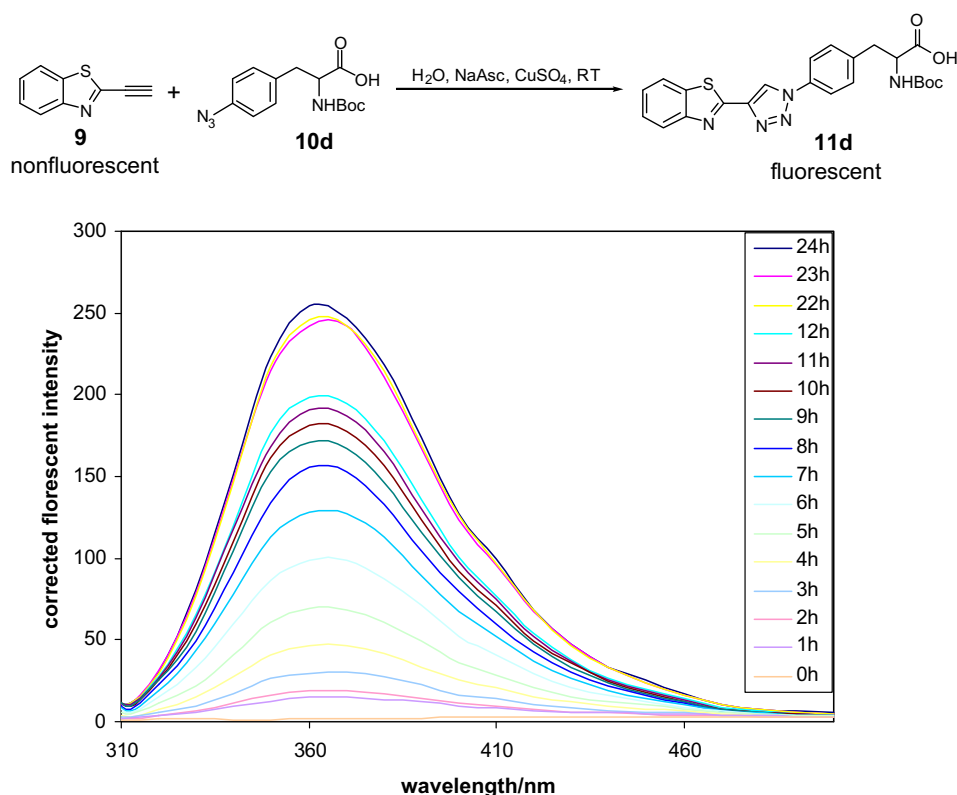
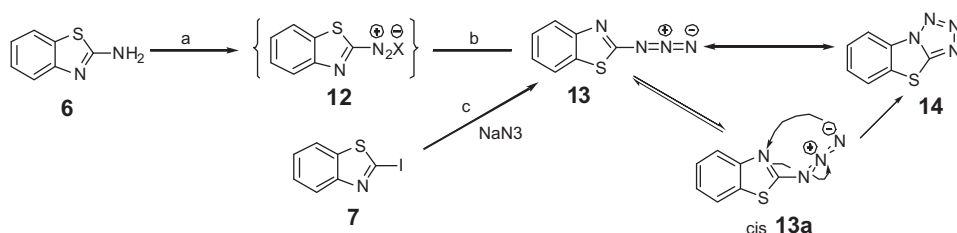
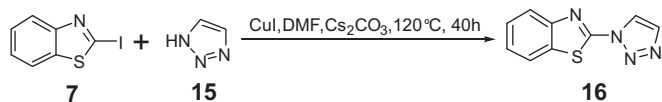


Figure 3. Fluorescence emission spectra increase of a 3 ml H₂O solution containing **9** (10 μM), **10d** (10 μM), NaAsc (1 mM) and CuSO₄ (10 μM) ($\lambda_{\text{ex}} = 285 \text{ nm}$) within 24 h at rt.



Scheme 3. Reagents and condition: (a) *p*-MeC₆H₄SO₃H, MeCN, 0–10 °C, NaN₃, NaNO₂, H₂O, 10 min, 10–15 °C; (b) NaN₃; (c) NaN₃, MeCN.



Scheme 4. Preparation of compound 16.

The above results showed that the electron deficient alkyne at position 2 of the benzothiazole ring is critical to the ‘click-on’ reaction. Nevertheless, the ‘click-on’ reaction is bi-directional, it is interesting to see if an electron-rich azide group at the same position could have a similar ‘click-on’ effect. Photoinduced electron transfer (PET) effect by lone pair electrons has been widely documented in various fluorochromes.²⁹ To synthesize benzothiazole azide **13**, two different approaches, direct diazotization of the compound **6** and substitution of an iodol group on benzothiazole **7** with sodium azide, were tried. However none of them could lead to the desired compound **13**. The compound **13** seemed not stable,²⁰ but forming the byproduct **14** through a spontaneous cyclization as previously suggested (Scheme 3).³⁰ Since the chemical property didn’t allow isolating the ‘click-on’ compound **13**, the expected final clicked product was prepared instead to validate the hypothesis. The clicked product **16** was synthesized by reacting iodobenzothiazole (**7**) with 1,2,3-triazole (**15**) referencing a reported protocol (Scheme 4).²⁴ Interestingly the compound **16** excites at 297 nm and emits at 374 nm (Fig. 2 (left)). Comparing the structures of the compounds **16** and **11a**, the substitution positions on the 1,2,3-triazole ring are different, but the conjugating double bonds inside of 1,2,3-triazole ring of both compounds are extended over the benzothiazole ring, resulting in similar fluorescent property. Although the ‘click-on’ compound **13** was not obtained in this preparation, the compound **16** has suggested that the fluorescent property of an electron rich azide group containing dye could also be activated by an alkyne group through a ‘click-on’ reaction.

In conclusion, we describe the first development of benzothiazole ‘click-on’ fluorogenic dyes. The click-on dye could have an electron-rich azide group or an electron-deficient alkyne group at the 2-position. The key requisition to achieve the ‘click-on’ fluorescent property is that the newly formed double bonds inside of the 1,2,3-triazole ring have to form conjugate double bonds with the parent fluorophore. Potentially, the same principle could be extended to design other fluorescence dyes. The demonstrated model reactions with functionalized nucleoside and amino acid have indicated that the developed click-on dye could be applied to label various biomolecules, such as nucleic acids, proteins and other molecules.

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Supplementary data

Supplementary data associated with (experimental details of synthesis and characterization of the products) this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.009.

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