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# A Cell-Permeable and Triazole-Forming Fluorescence Probe for Glycoconjugate Imaging in Live Cells<sup>†</sup>

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A new fluorescence-forming probe, coumOCT, designed by fusing cyclooctyne with a coumarin fluorophore was successfully used for the imaging of azido-glycoconjugates in living HeLa cells. This probe is cell-permeable and generates fluorescence after triazole formation, thus minimizing the background signal and enabling the real-time intracellular imaging of glycoconjugate trafficking.

Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC)<sup>1</sup> is an orthogonal reaction that has been widely utilized to label specific biomolecules in complex mixtures, cells and tissues.<sup>2</sup> Further studies have focused on using non- or weakly-fluorescent azides and alkynes to generate highly fluorescent triazole products upon cycloaddition reactions.<sup>3</sup> The distinct fluorescence enhancement induced by the highly efficient CuAAC reaction has given a potentially wide-range of applications in the emerging field of cell biology and functional proteomics.<sup>3a,3d,3j,4</sup> However, the toxicity of Cu(I) has hindered the use of CuAAC in living systems.

To circumvent the cytotoxicity associated with the metal catalyst, ring strain-promoted azide-alkyne cycloadditions (SPAAC) have been developed as an alternative strategy.<sup>5</sup> Cyclooctyne moieties such as difluorinated cyclooctynes (DIFO) and their derivatives are often incorporated as a stem structure into SPAAC reagents to improve the reactivity.<sup>6a,6b</sup> In addition, the cyclooctyne moiety can be fused with other rings such as dibenzocyclooctyne (DIBO),<sup>6c-6f</sup> diarylazacyclooctynone (BARAC),<sup>6g</sup> and bicyclononynes (BCN) to increase the ring strain and reactivity.<sup>6h</sup> Tetramethylthiacycloheptyne (TMTH) bearing a contracted seven-membered ring was also used to increase



**Figure 1.** Structures of SPAAC-based fluorescence-forming probe **1** and two reported probes, CoumBARAC and FI-DIBO.

the reactivity in cycloaddition reactions with azides.<sup>6i-j</sup> To date, two cyclooctyne-based fluorogenic probes, CoumBARAC<sup>7</sup> and FI-DIBO,<sup>8</sup> have been reported by the Bertozzi and the Boons groups, respectively (Fig. 1).

The low fluorescence 7-alkynylcoumarin derivative has been shown to undergo a CuAAC reaction to give the corresponding triazole product with enhanced fluorescence due to the electron-donating property of the triazole ring.<sup>3c</sup> Thus, incorporation of a cyclooctyne to the coumarin moiety 1 may decrease fluorescence, but the SPAAC reaction of 1 with azides is expected to give highly fluorescent triazole products for sensitive detection. This approach was reported by Bertozzi and co-workers using the biarylazacyclooctynone ring fused with a coumarin fluorophore (CoumBARAC).<sup>7</sup> Although the compound undergoes a cycloaddition reaction with 2azidoethanol to give a 10-fold increase in fluorescence intensity, the triazole product exhibited a low quantum yield  $(\Phi_f = 0.04)$  and requires relatively high energy excitation (~300 nm), making it difficult for imaging in living systems. FI-DIBO was not used in live cell imaging and its triazole adduct also gave a relatively low quantum yield. In this study, we report a SPAAC-based fluorescence-forming probe 1 (Fig. 1), namely coumOCT that can be used for the real-time imaging of azidotagged glycans in living cells under no-wash and no-fixation conditions.

Scheme 1 shows the synthesis of **1** using 1-benzosuberone as the starting material. According to the previously reported procedure,<sup>9</sup> 1-benzosuberone was subject to regioselective nitration at the 8-position. The nitro group was then reduced, followed by diazotization and hydroxylation under acid

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Scheme 1. Synthesis of the SPAAC-based fluorescence-forming probe 1 and the corresponding triazoles 9 and 10. Reagents and conditions: (a) conc.  $H_2SO_4$ ,  $KNO_3$ , 0 °C, 1.5 h, 72%; (b) Sn, conc. HCl,  $C_2H_5OH$ , reflux, 50 min, 82%; (c) 10% aq.  $H_2SO_4$ ,  $NaNO_2$ , 0 °C to rt, 72 h, 76%; (d) BnBr,  $K_2CO_3$ , DMF, rt, 24 h, 98%; (e) TMSCHN<sub>2</sub>, BF<sub>3</sub>·OEt<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C, 12 h, 73%; (f) NaBH<sub>4</sub>, CH<sub>3</sub>OH, 0 °C, 1 h; (g) TIPSOTf, 2,6-lutidine, CH<sub>2</sub>Cl<sub>2</sub>, rt, 1 h, 96% for two steps; (h) H<sub>2</sub>, Pd/C, CH<sub>3</sub>OH, EtOAc, 1 h; (i) paraformaldehyde, MgCl<sub>2</sub>, Et<sub>3</sub>N, CH<sub>3</sub>CN, reflux, 12 h, 87% for two steps; (j) Ph<sub>3</sub>P=C=C=O, toluene, 90 °C, 1.5 h, 83%; (k) TBAF, THF, 0 °C to rt, 1 h; (l) (COCl)<sub>2</sub>, DMSO, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, -78 °C to rt, 1 h, 78% for two steps; (m) NaHMDS, Tf<sub>2</sub>NPh, -78 °C to rt, 2 h, 32%; (n) BnN<sub>3</sub> (1.5 equiv.), CH<sub>3</sub>CN, rt, 2 h, 95%; (o) *N*-azidoacetylmannosamine (1.5 equiv.), MeOH, H<sub>2</sub>O, rt, 2 h, 92%.

hydroxyl group as a benzyl ether, the cyclic ketone **3** underwent a ring expansion by treatment with TMSdiazomethane in the presence of  $BF_3 \cdot OEt_2$  to afford the cyclooctanone product **4** in 73% yield. Reduction of the carbonyl group with NaBH<sub>4</sub>, followed by silylation, gave the silyl ether **5** in 96% yield. The benzyl group in **5** was removed by hydrogenation, and the phenol intermediate was treated with excess of paraformaldehyde in the presence of  $Et_3N$  and MgCl<sub>2</sub> to form the salicylaldehyde **6**. The coumarin scaffold in **7** was constructed by treatment of **6** with freshly prepared ketenylidenetriphenylphosphorane. After desilylation and oxidation, ketone **8** was obtained in 78% yield. The carbonyl group in **8** was converted to enol triflate, which was subsequently treated with a strong base NaHMDS to render an elimination reaction, giving the coumOCT probe **1**.

To evaluate the feasibility of probe **1** as a reagent for the SPAAC-based fluorescence labeling, we first studied its reaction scope and kinetics using benzyl azide (BnN<sub>3</sub>) as a model substrate. The SPAAC reaction of **1** with BnN<sub>3</sub> in acetonitrile was completed in 2 h at room temperature to give triazole **9** in 95% yield (Scheme 1). The reactivity of **1** with benzyl azide (1:1) in CD<sub>3</sub>CN was determined by integration of multiple chemical shifts in the <sup>1</sup>H-NMR spectrum to yield a second-order rate constant of 0.012  $M^{-1}s^{-1}at 25$  °C. We also

observed the cycloaddition reaction between **1** and *N*-azidoacetylmannosamine (ManNAz), proceeding similarly to give the triazole **10** with a second-order rate constant of 0.010  $M^{-1}s^{-1}$  at 25 °C in a solution of CD<sub>3</sub>OD–D<sub>2</sub>O (5:1, v/v) (Fig. S1 and S2, ESI†).

Table S1 (ESI<sup>†</sup>) shows the absorption and fluorescence data of **1**, **9** and **10** recorded under simulated physiological conditions (PBS buffer containing 10% DMSO, pH 7.4). As expected, formation of triazoles **9** and **10** was accompanied by a significant increase in fluorescence intensity with a large Stokes shift into a standard range for coumarin emission (Fig. S3a, ESI<sup>†</sup>). Upon excitation at 330 nm, probe **1** produced a weak emission band centered at 405 nm with a low quantum yield ( $\Phi_f = 0.011$ ), whereas both triazoles **9** and **10** exhibited a strong fluorescence at 435 nm with a quantum yield of 0.23 and 0.21, respectively.

To probe the SPAAC reaction under conditions that would be more typical for biomolecule labeling, we investigated the fluorescence response and time course for the reaction of **1** with ManNAz. The experiments indicated that more than 90% of ManNAz was consumed in 40 min, and the fluorescence intensity reached a plateau in less than 1 h (Fig. S3b, ESI<sup>†</sup>).

Aberrant glycosylation on the surface of malignant cells is often observed in pathological conditions, such as inflammation and cancer metastasis. HeLa cell is the most widely used model system for studying human cellular and molecular biology as well as cancer biology.We sought to evaluate the performance of **1** in live cell imaging.HeLa cells were cultured in the presence of peracetylated *N*azidoacetylmannosamine (Ac<sub>4</sub>ManNAz) for 3 days to metabolically produce the azido-sialic acid experessing cells. As



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**Figure 2.** (a) Fluorescence images of sialylated glycoconjugate using coumOCT (1) for SPAAC in live cells by confocal microscopy; (b) Localization of probe-labeled sialylated glycoconjugates in HeLa cells under confocal microscopy. The labeled cells were stained with anti-GM130 followed by Cy3-conjugated antibody (for Golgi, red). (Scale bar:  $10 \,\mu$ m)

a negative control, HeLa cells were grown in the presence of peracetylated *N*-acetylmannosamine

(Ac<sub>4</sub>ManNAc). The cells were incubated with **1** for 30 min at 37 <sup>o</sup>C under no-wash conditions. The intense fluorescence labeling was observed for Ac<sub>4</sub>ManNAz-treated cells relative to the background with control (Ac<sub>4</sub>ManNAc) cells (Fig. 2a and Fig. S4, ESI<sup>+</sup>). Furthermore, the location of azido-containing glycoconjugates in living cells was visualized by confocal microscopy. The cells labeled by probe **1** were subsequently fixed and stained with anti-GM130 followed by Cy3-conjugated antibody for marking Golgi apparatus.<sup>10</sup> The blue fluorescent signal derived from the coumarin probe was apparent in Ac<sub>4</sub>ManNAz-treated cells but not in Ac<sub>4</sub>ManNAc-treated cells. The images indicated that coumOCT was labeled to azido-sialic acid bearing glycoconjugates inside the living cell as well as on the cell surface, and significantly overlapped with the Golgi staining (red) (Fig. 2b and Fig. S5, ESI<sup>+</sup>).

Interestingly, the fluorescence signals were observed not only on the cell surface but also inside the cells in the living cell-imaging experiment. We therefore examined whether coumOCT is a cell-permeable probe that could be utilized for direct intracellular labeling in live cells. In order to obtain fluorescence images of azido-bearing glycoconjugates reacting with coumOCT inside the living cell, HeLa cells were incubated with Ac<sub>4</sub>ManNAz for 1 h and subsequently the unbound Ac<sub>4</sub>ManNAz was removed. We expected that the cellpermeable Ac<sub>4</sub>ManNAz was firstly taken up into the cytosol before incorporation into glycoconjugates in the Golgi apparatus and the endoplasmic reticulum (ER).<sup>11</sup> After treatment with natural or azido sugar in living cells and labeled by coumOCT, we then performed imaging to monitor the trafficking of the azido-bearing glycoconjugates. The time course experiment was conducted by exposing the cells at 30min intervals under no-wash and no-fixation conditions (Fig. 3 and Fig. S6, ESI<sup>+</sup>). The Ac<sub>4</sub>ManNAz-treated cells showed a time-dependent increase in fluorescence intensity inside the living cell and then reached saturation after 1.5 h incubation. The punctate staining in Ac<sub>4</sub>ManNAz-treated cell may be related to transportation of sialic acid-containing glycoconjugates during incubation with probe 1. These results indicate that coumOCT possesses good cell-membrane permeability and specific for the azido-sialic acid glycoconjugates labeling in live cells.

Peracetylated *N*-azidoacetylgalactosamine (Ac<sub>4</sub>GalNAz) and *N*-azidoacetylglucosamine (Ac<sub>4</sub>GlcNAz) have been used for metabolic labeling of glycans in living cell.<sup>12</sup> We further examined the use of coumOCT for glycan labeling with different azido-sugars in living cells (Fig. 4 and Fig. S7, ESI<sup>+</sup>).

HeLa cells were incubated in the presence of Ac<sub>4</sub>GalNAz and Ac<sub>4</sub>GlcNAz for three days, respectively. Peracetylated *N*-acetylgalactosamine (Ac<sub>4</sub>GalNAc) and *N*-acetylglucosamine (Ac<sub>4</sub>GlcNAc) were

Ac₄ManNAz				Ac₄ManNAc
00:00	00:30	01:00	01:30	01:30
а	d	g	j	m 🦉
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ь —	e	h	k 	n
с 	f	-	-	• *

**Figure 3.** Time-lapse intracellular fluorescence imaging of sialylated glycoconjugate trafficking using coumOCT (1) for SPAAC in live cells by confocal microscopy. The labeled cells were stained with DRAQ5<sup>TM</sup> for nucleus staining (red). (Scale bar: 10  $\mu$ m)

employed as the corresponding control sugars. The cells treated with Ac<sub>4</sub>GlcNAz and Ac<sub>4</sub>GalNAz showed distinct patterns in fluorescence channels (Fig. 4g, m), whereas those treated with control sugar showed almost no labeling in the corresponding channels (Fig. 4j, p). These images indicated that coumOCT can be used to specifically label azido-GalNAc and azido-GlcNAc containing glycoconjugates inside the living cell and on the cell surface. Next, we compared the fluorescence cell imaging upon treatment with different azidosugars using coumOCT. The coumOCT-labeled cells with different azido-sugars show cell surface staining and distinct labeling of intracellular Golgi apparatus. The phenomenon suggested that these azido-sugar incorporated glycoconjugates were first produced in the Golgi apparatus through metabolic processing, then transported to the cell surface. Moreover, although coumOCT also labels the cells treated with Ac<sub>4</sub>GalNAz, the relatively weak fluorescence output is attributed to the low efficiency of Ac<sub>4</sub>GalNAz-mediated metabolic processes in HeLa cells.<sup>13</sup> The experiments indicated that coumOCT can be employed as a fluorescence-forming probe for detection of azido-bearing glycoconjugates in living cell by SPAAC-based triazole formation chemistry.

A potentially problematic side reaction of cyclooctynes is the addition reaction with the endogenous thiol group to form the corresponding vinyl sulfide. For example, three cyclooctynes (DIBO, DIBAC and BCN) are susceptible to thiolyne addition with peptidylcysteines.<sup>14</sup> On the other hand, FI-DIBO is a cyclooctyne-based fluorogenic probe, but the triazole adduct will react with thiol-containing molecules such as glutathione and DTT to quench fluorescence by addition of thiol to the cyclopropenone moiety of triazole.<sup>8</sup> To test

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whether there is non-specific staining of **1** and the corresponding triazole **9** due to the reaction of proteinic or endogenous thiol groups with the triple bond or double bond, an experiment was performed by incubation of **1** and **9** with 3-mercaptopropionic acid in  $CDCl_3$  (Fig. S8–S10, ESI<sup>+</sup>). The NMR spectral analyses suggested that **1** and **9** were inactive to the thiol-yne or thiol-ene addition with 3-mercaptopropionic acid. These results show that the application of **1** as a fluorescence-forming probe allows



**Figure 4.** A comparison of fluorescence images upon treatment with different azido-sugars in live cells using coumOCT (1) for SPAAC by confocal microscopy. (Scale bar:  $10 \mu m$ )

detection of metabolically incorporated glycoconjugates in living cells without non-specific staining by thiol-yne addition, and the formed triazoles would not undergo thiol-ene addition to quench fluorescence.

In conclusion, we have developed a new SPAAC-based fluorescence-forming probe coumOCT (1) for real-time imaging in living cells under no-wash and no-fixation conditions. Without side-by-side comparison, probe 1 and FI-DIBO appear to have a similar reactivity toward benzyl azide, but probe 1 given a higher quantum yield and more stable triazole product. Furthermore, our study establishes that probe 1 is a fluorescence turn-on probe for imaging azido-containing glycoconjugates in living cells. The SPAAC reaction is spontaneous and no washing steps are needed. Moreover, coumOCT is cell-permeable with no problem of background labeling, leading to very low background fluorescence, and thereby facilitating specific target imaging in living cells. Our current study represents a significant advance in cell imaging, and should be potentially applicable to the real-time detection of biochemical events in vivo.

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