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A Bioorthogonal Ligation Enabled by Click Cycloaddition of *o*-Quinolinone Quinone Methide and Vinyl Thioether

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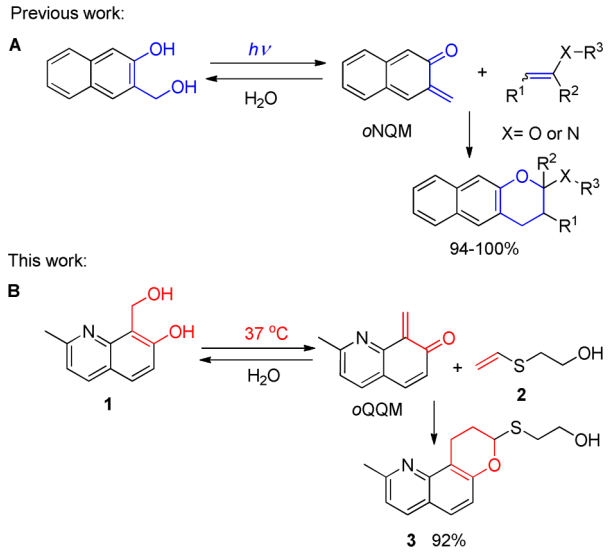
Supporting Information

ABSTRACT: There is an increasing interest in the use of bioorthogonal ligation to advance biomedical research through selective labeling of biomolecules in living systems. Accordingly, discovering new reactions to expand the toolbox of bioorthogonal chemistry is of particular interest to chemical biologists. Herein we report a new bioorthogonal ligation enabled by click hetero-Diels–Alder (HDA) cycloaddition of in situ-generated *o*-quinolinone quinone methides and vinyl thioethers. This reaction is highly selective and proceeds smoothly under aqueous conditions. The functionalized vinyl thioethers are small and chemically stable in vivo, making them suitable for use as bioorthogonal chemical reporters that can be effectively coupled to various biomolecules. We utilized this bioorthogonal ligation for site-specific labeling of proteins as well as imaging of bioactive small molecules inside live cells.

Bioorthogonal ligations have been widely used to facilitate studies of diverse and complex biological processes.[1] As such reactions should meet the stringent requirements of click chemistry,[2] including high reactivity and selectivity of functional groups, good stability in aqueous solutions, and excellent biocompatibility and high reaction rate under physiological conditions, only a handful of bioorthogonal ligations have been developed and broadly utilized to date. The select remarkable works include nucleophilic addition of hydrazine or alkoxyamine to ketone or aldehyde,[3] Staudinger ligation,[4] Cu(I)-catalyzed azide–alkyne cycloaddition (AAC),[5] Cu-free strain-promoted AAC (SPAAC),[6] reactions involving alkenes such as 1,3-dipolar[7] and Diels–Alder cycloadditions,[8−11] quadricyclane ligation,[12] and Pictet–Spengler ligation.[13] Although we have witnessed the significant advance of this field over the past decade, the discovery of new reactions, especially ones compatible with AAC, which is still the gold standard for bioorthogonal ligations,[1b] in order to expand the toolbox of bioorthogonal chemistry remains a pressing task, yet it is challenging because of the aforementioned stringent requirements for ideal bioorthogonal ligations.

o-Quinone methides (*o*QMs) are highly reactive and versatile synthetic intermediates that can undergo rapid and selective hetero-Diels–Alder (HDA) cycloadditions with electron-rich dienophiles.[14] The traditional methods for generation of *o*QMs include oxidation, high temperature, photoirradiation, and the use of strong acid or base. *o*QMs have been broadly utilized in natural product synthesis[15] but rarely applied to bioorthogonal ligation to date, presumably because of the required harsh reaction conditions, which would be detrimental to the cell or organism. Arumugam and Popik[16] have pioneered the development of photochemically generated *o*-naphthoquinone methides (*o*NQMs), which undergo facile cycloadditions with dienophiles such as vinyl ethers or enamines (Scheme 1A). However, the requirement of UV light might hinder their applications to living systems.

Scheme 1. Development of Bioorthogonal Ligations Using *o*QMs



To improve the efficiency and robustness of *o*QM chemistry in order to make it more suitable for bioorthogonal ligation, we envisioned that both reaction partners for the HDA cycloaddition should be optimized. Conceivably, the introduction of more electronegative heteroatoms such as nitrogen into the *o*QM precursor should improve its reactivity for the HDA cycloaddition as well as its hydrophilicity. We also considered that vinyl thioethers (VTs), which are small, chemically stable, and able to act as more electron-rich dienophiles, should be

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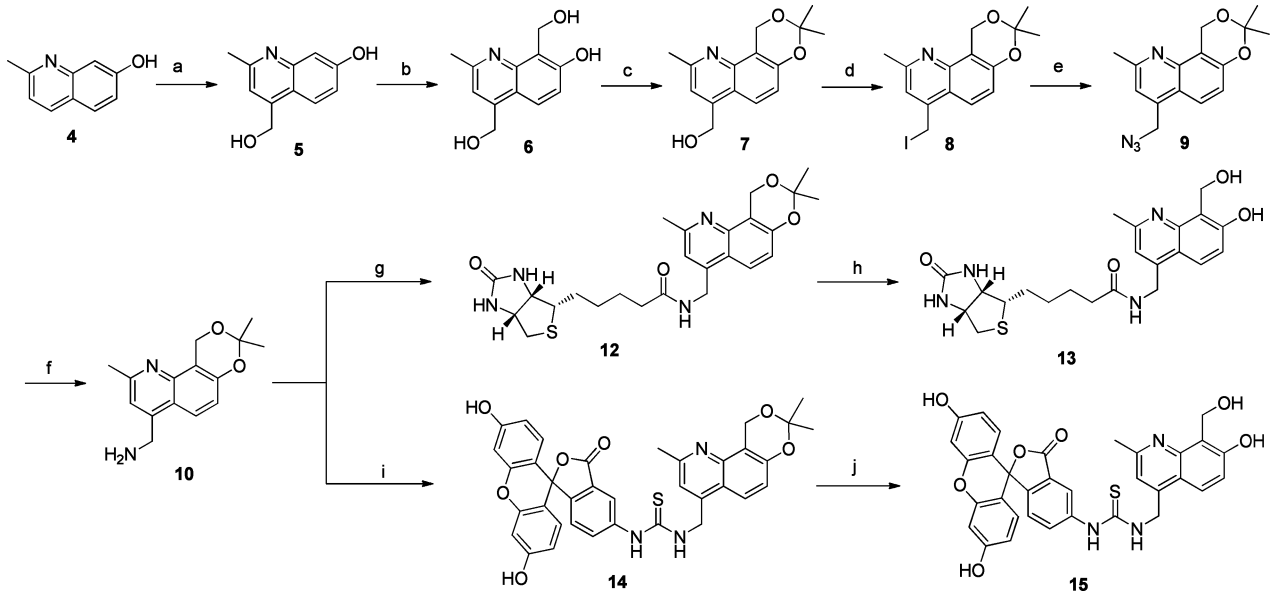
Scheme 2. Synthesis of Biotinylated and Fluoresceinated *o*QQM Precursors[a]



[a]Reagents and conditions: (a) $FeSO_4 \cdot 7H_2O/H_2O_2$, CH_3OH, conc. H_2SO_4, 50 °C, 3 h, 70% brsm. (b) 37% HCHO(aq), 2 M NaOH, 1.5 h. (c) 2,2-Dimethoxypropane, PPTS (cat.), DMF, 80 °C, 5.5 h, 32% over two steps. (d) I_2, imidazole, PPh_3, DCM, 3 h, 95%. (e) NaN_3, DMF, 12 h, quant. (f) 5% Pd/C, H_2, MeOH, 12 h, quant. (g) **11**, TEA, DMF, 2 h, 88%. (h) 0.5 M HCl/THF, 48 h, 95%. (i) FITC isomer, DIPA, DMF, 16 h, 95%. (j) 0.8 M HCl/THF, 48 h, 90%.

better bioorthogonal chemical reporters. Accordingly, we prepared 8-(hydroxymethyl)-2-methylquinolin-7-ol (**1**)[17] as an *o*-quinolinone quinone methide (*o*QQM) precursor and 2-(vinylthio)ethanol (**2**)[18] as a dienophile, which enabled us to investigate the HDA cycloaddition further (Scheme 1B). To our delight, we observed that under physiological conditions (H_2O, 37 °C, 24 h), **1** and **2** could undergo a click HDA cycloaddition to furnish **3** in 92% isolated yield. Interestingly, the *o*QQM could be generated smoothly without the need for a catalyst or UV light. The second-order rate constant for this reaction was estimated to be $(1.5 \pm 0.1) \times 10^{-3}$ M^{-1} s^{-1} (Figure S1 in the Supporting Information),[17] which is comparable to those for the widely used Staudinger ligation ($k = 2.5 \times 10^{-3}$ M^{-1} s^{-1})[19] and first-generation SPAAC ($k = 1.2 \times 10^{-3}$ M^{-1} s^{-1}).[6f]

The stability of the VT reporter under physiological conditions, especially in the presence of oxidants or reactive amino acids, was examined next (Figures S2 and S3). As a result, **2** was shown to be inert toward various oxidants such as O_2 and H_2O_2 (200 nM) as well as either cysteine (20 mM) or lysine (20 mM) in D_2O at 37 °C for 24 h.[17] Notably, we also found that **2** was stable at pH 4−7.4 (Figure S4). Concerned that the *o*QQM would undergo Michael addition with the free thiol functionality of cysteine residues, we also investigated the reaction of **1** and **2** in the presence of cysteine (Figure S5). Gratifyingly, because the Michael addition with the thiol group is reversible and the HDA cycloaddition with the VT is irreversible, the HDA cycloadduct **3** was observed exclusively in H_2O at 37 °C after 24 h.[17] The reversibility of the thiol Michael addition was supported by an experiment in which the thiol Michael addition product further reacted with **2** to generate the HDA cycloadduct **3** (Figure S6). Ultimately, we tested the stability of thioacetal **3** under acidic conditions and found it to be quite stable at pH 4.0−7.4 (Figure S7).

Next, we aimed to evaluate whether the *o*QQM−VT ligation is compatible with the widely used SPAAC reaction. When **1** and **2** were combined with (azidomethyl)benzene (**19**) and 5,6-didehydro-11,12-dihydrodibenzo[*a*,*e*]cyclooctene (**20**) in H_2O/CH_3CN at 37 °C, we observed that the two ligations proceeded simultaneously without interfering with each other (Figure S8).[17]

Having established the model reaction for the *o*QQM−VT ligation, we sought to prepare both biotin and fluorescein-labeled *o*QQM precursors for further applications to biological systems (Scheme 2). The synthesis commenced with direct, regioselective C−H functionalization of readily available 2-methylquinolin-7-ol (**4**) to afford **5**. Under basic conditions, **5** reacted with formaldehyde to generate **6**, which was subsequently protected with 2,2-dimethoxypropane, affording **7** in 32% yield over two steps. Primary alcohol **7** was transformed to iodide **8**, which was further substituted with an azido group to furnish **9**. Azide **9** was reduced with H_2 in the presence of Pd/C (5%) to afford a quantitative yield of primary amine **10**, which could be further coupled with different tags. Treatment of **10** with the readily available biotin tetrafluorophenyl ester (biotin-TFP, **11**) smoothly generated amide **12**, which was further deprotected under acidic conditions (HCl/THF) to obtain the desired biotinylated *o*QQM precursor **13**. In addition, coupling of **10** with commercially available fluorescein isothiocyanate (FITC) isomer followed by deprotection provided the fluoresceinated compound **15**.

With **13** and **15** in hand, we first conducted selective protein labeling studies in vitro (Figure 1). Using *N*-succinimidyl 2-(vinylthio)ethyl carbonate (**16**), we coupled the free lysine residues of bovine serum albumin (BSA) with the required VT reporter (Figure 1A). The VT-modified BSA protein was subsequently incubated with either biotinylated **13** or fluoresceinated **15**, and the resulting products were then assayed by Western blots using biotin antibody or in-gel fluorescence measurements, respectively. Robust biotinylation was observed for BSA modified with the VT reporter (Figure 1B). In addition, we were delighted to find that fluorescein

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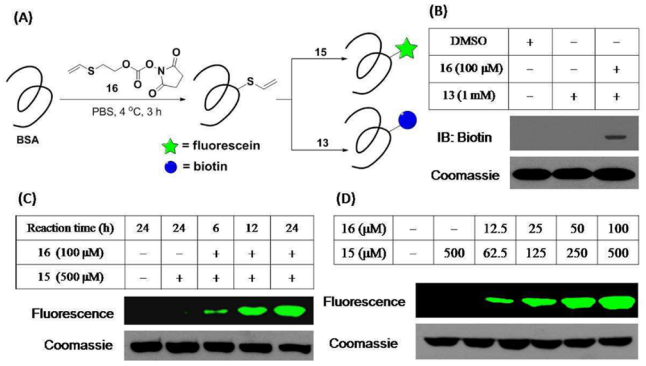


Figure 1. Labeling of VT-modified BSA with **13** or **15**. (A) Modification of free lysine residues on BSA with **16** and subsequent labeling with **13** or **15**. (B) Western blot analysis of biotin-labeled BSA using biotin antibody. (C, D) Western blot analyses of (C) time-dependent and (D) dose-dependent fluorescein labeling of BSA. In B–D, protein loading was assessed by Coomassie staining.

labeling of VT-modified BSA proceeded effectively in an excellent time- and dose-dependent manner with very little background labeling of unmodified BSA (Figure 1C,D).

Encouraged by these results, we then investigated the utility of *o*QQM–VT ligation for live-cell imaging. To evaluate whether the fluoresceinated *o*QQM precursor **15** would be useful for intracellular studies, we examined its cell-membrane permeability and cytotoxicity. Hela cells were first incubated with **15** at different concentrations for 24 h, and the cells were then washed thoroughly to ensure that little **15** was left around the cell surface. We observed homogeneous fluorescence staining inside the Hela cells using a confocal microscope (Figure 2A). Furthermore, although compounds that sponta-

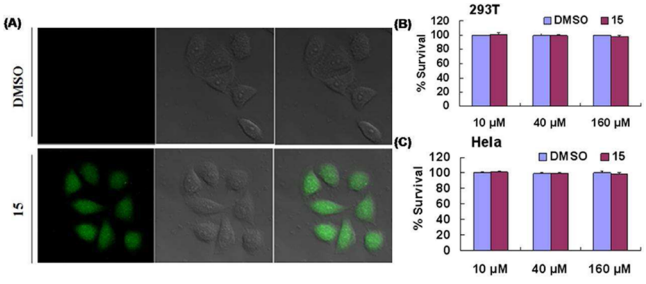
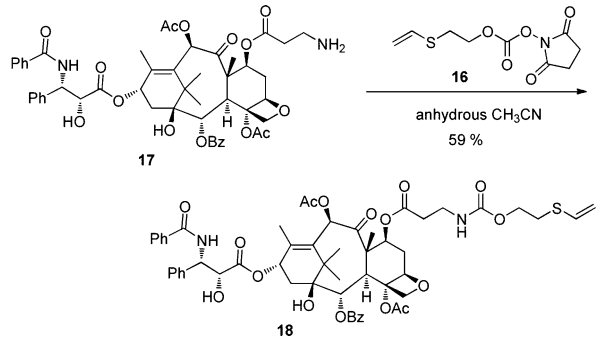


Figure 2. Studies of cell-membrane permeability and cytotoxicity of fluoresceinated *o*QQM precursor **15**. (A) Hela cells were treated with dimethyl sulfoxide (DMSO) or 20 μM **15** for 24 h and then visualized with green fluorescence. (B) 293T cells and (C) Hela cells were treated with either DMSO or **15** at three different concentrations (10, 40, and 160 μM) for 24 h. The cell viability was determined by MTT assay. The reported results are representative of three independent experiments.

neously produce reactive *o*QMs might be cytotoxic,[20] no detectable cytotoxicity of **15** was observed in either 293T or Hela cells at high concentrations of up to 160 μM (Figure 2B,C).

Effective imaging of bioactive small molecules inside live cells could significantly facilitate forward chemical genetics.[21] We therefore sought to demonstrate the capability of *o*QQM–VT ligation for in vivo imaging of VT-labeled small molecules. Taxol was selected as a showcase for this study because it has been widely used in cancer chemotherapy through selective targeting of microtubules, which could provide us a well-defined intracellular structure to image.[22] VT-labeled taxol (**18**) was prepared smoothly through coupling of **16** to the previously reported taxol derivative 7-β-alanyltaxol (**17**)[23] (Scheme 3). Initial biological evaluations also confirmed that **18** retained taxol's antitumor activity in Hela cells (Figure S9).[17]

Scheme 3. Synthesis of VT-Labeled Taxol (18)



For live-cell imaging studies, Hela cells were first treated with 500 nM **18** for 2 h at 37 °C. After washing with medium three times, the cells were exposed to medium containing 20 μM **15** for 12 h in an incubator, washed, and imaged on a confocal microscope. We observed clearly stained intracellular structures of tubule networks in the group treated with **18** (Figure 3).

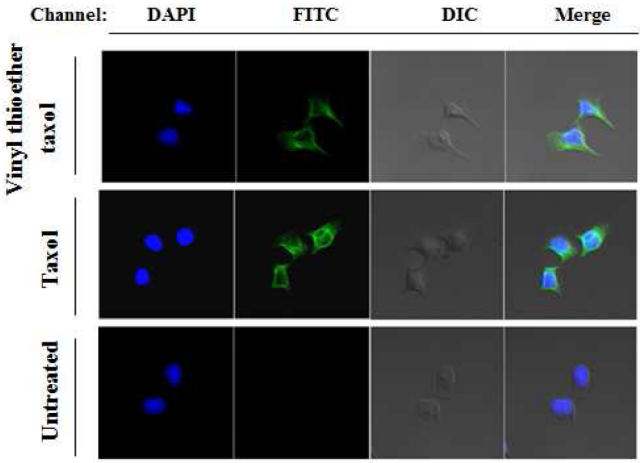


Figure 3. Imaging of VT-labeled taxol in live cells. Group treated with VT-labeled taxol: live HeLa cells were stained with **15** (20 μM) at 37 °C for 12 h after treatment with 500 nM **18** for 2 h. Group treated with taxol: live HeLa cells were stained by immunostaining with antitubulin after treatment with 500 nM taxol for 2 h. Untreated group: live HeLa cells were treated with **15** (20 μM) at 37 °C for 12 h after treatment with DMSO for 2 h. All of the cells were treated with the nuclear stain 4-amidinophenyl-6-indolecarbamidine dihydrochloride (DAPI) before being imaged on a confocal microscope.

This staining pattern was comparable to standard immunostaining using antitubulin in the taxol-treated group. Control experiments using **15** alone generated extremely low level fluorescence background signals, providing additional evidence that the fluorescence imaging was indeed generated by the specific HDA cycloaddition. Collectively, these findings indicate that *o*QQM–VT ligation is suitable for in vivo labeling experiments and will be useful for chemical genetic strategies.

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In conclusion, we have developed a new bioorthogonal ligation that is enabled by click hetero-Diels–Alder cycloaddition of *o*-quinolinone quinone methide (*o*QQM) and vinyl thioether (VT). We have also demonstrated that this ligation is efficient and robust under physiological conditions and is compatible with the widely used strain-promoted azide–alkyne cycloaddition. Furthermore, we have successfully utilized this bioorthogonal ligation for site-specific labeling of proteins as well as imaging of a taxol derivative inside live cells. Hence, we can expect that the *o*QQM–VT ligation will become a promising addition to the bioorthogonal chemistry compendium and facilitate studies of complex biological processes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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‖Q.L. and T.D. contributed equally.

Notes

The authors declare no competing financial interest.

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