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A Strain-Promoted [3 + 2] Azide−Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems

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Selective chemical reactions that are orthogonal to the diverse functionality of biological systems are now recognized as important tools in chemical biology.[1] As relative newcomers to the repertoire of synthetic chemistry, these bioorthogonal reactions have inspired new strategies for compound library synthesis,[2] protein engineering,[3] functional proteomics,[4] and chemical remodeling of cell surfaces.[5] The azide has secured a prominent role as a unique chemical handle for bioconjugation. We have made extensive use of the azide as a chemical reporter of glycosylation, employing the Staudinger ligation with phosphines to tag azidosugars metabolically introduced into cellular glycoconjugates.[6] The Staudinger ligation can be performed in living animals without physiological harm,[7] suggesting the potential for applications in noninvasive imaging and therapeutic targeting. Still, the reaction is not without liabilities. The requisite phosphines are susceptible to air oxidation, and their optimization for improved water solubility and increased reaction rate has proven to be synthetically challenging.

The azide has an alternative mode of bioorthogonal reactivity—the [3 + 2] cycloaddition with alkynes described by Huisgen.[8] In its classic form, this reaction has limited applicability in biological systems due to the requirement of elevated temperatures (or pressures) for reasonable reaction rates. Sharpless and co-workers surmounted this obstacle with the development of a copper(I)-catalyzed version, termed "click" chemistry, that proceeds readily at physiological temperatures and in richly functionalized biological environs (Figure 1A). This reaction has enabled the selective modification of virus particles,[3a] nucleic acids,[9] and proteins from complex tissue lysates.[4] Unfortunately, the mandatory copper catalyst is toxic to both bacterial[5b] and mammalian cells,[10] thus precluding applications wherein the cells must remain viable. Catalyst-free Huisgen cycloadditions of alkynes activated by electron-withdrawing substituents have been reported to occur at ambient temperatures.[11] However, these compounds can undergo Michael reaction with biological nucleophiles.

We considered an alternative means of activating alkynes for catalyst-free [3 + 2] cycloaddition with azides: ring strain. In 1961, Wittig and Krebs noted that the reaction between neat cyclooctyne, the smallest of the stable cycloalkynes, and phenyl azide "proceeded like an explosion to give a single product," the triazole.[12] The massive bond angle deformation of the acetylene to 163°[13] accounts for nearly 18 kcal/mol of ring strain.[14] This destabilization of the ground state versus the transition state of the reaction provides a dramatic rate acceleration compared to unstrained alkynes.[15] Here we report that the [3 + 2] cycloaddition of azides and cyclooctyne derivatives (Figure 1B) occurs readily under physiological conditions in the absence of auxiliary reagents. We employed the reaction for the selective chemical modification of living cells without any apparent toxicity.

We synthesized biotinylated cyclooctyne **5** as shown in Scheme 1. Construction of the substituted cyclooctyne core was achieved

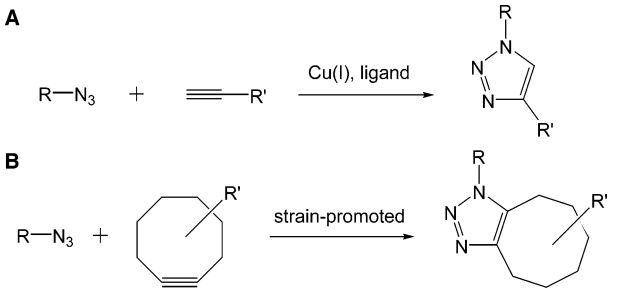
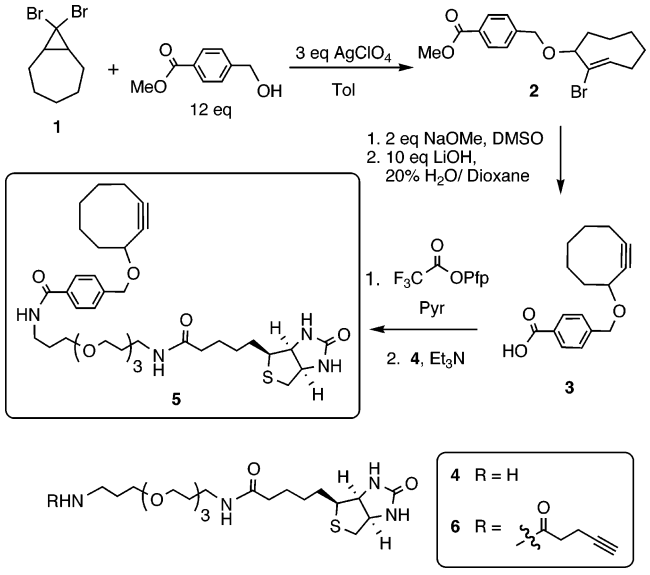


Figure 1. (A) Cu(I)-catalyzed Huisgen cycloaddition ("click" chemistry). (B) Strain-promoted [3 + 2] cycloaddition of azides and cyclooctynes.

Scheme 1



essentially as described by Reese and Shaw.[16] Briefly, compound **1**[17] was treated with silver perchlorate to effect electrocyclic ring opening to the *trans*-allylic cation, which was captured with methyl 4-(hydroxymethyl)-benzoate to afford bromo-*trans*-cyclooctene **2**. Base-mediated elimination of the vinyl bromide followed by saponification yielded versatile intermediate **3**, to which any biological probe can be attached. Finally, compound **3** was coupled to biotin analogue **4**[18] bearing a PEG linker, providing target **5**. Cyclooctyne **3** was stable to mild acid (0.5 N HCl for 30 min), base (0.8 M NaOMe for 30 min), and prolonged exposure to biological nucleophiles such as thiols (120 mM 2-mercaptoethanol for 12 h).

We performed model reactions with compound **3** and 2-azidoethanol, benzyl azide, or *N*-butyl α-azidoacetamide (see Supporting Information for details). In all cases, the only products observed were the two regioisomeric triazoles formed in approximately equal amounts. We next applied the reaction for covalent labeling of

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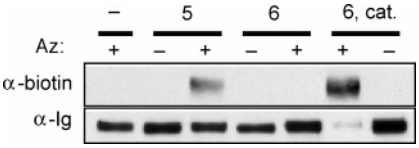

Figure 2. Labeling of azide-modified GlyCAM-Ig with alkyne probes. Purified GlyCAM-Ig was treated with buffer (−), 250 μM **5**, or 250 μM **6** alone or in the presence (cat) of $CuSO_4$, TCEP, and a triazolyl ligand, overnight at room temperature. Reaction mixtures were quenched with 2-azidoethanol and analyzed by Western blot probing with HRP−α-biotin (upper panel). The blot was then stripped and reprobed with HRP−α-Ig (lower panel).

biomolecules. The recombinant glycoprotein GlyCAM-Ig[19] was expressed in CHO cells in the presence of peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz), leading to metabolic incorporation of the corresponding *N*-azidoacetyl sialic acid (SiaNAz) into its glycans.[20] Control samples of GlyCAM-Ig were expressed in the absence of azido sugar. The purified GlyCAM-Ig samples were incubated with 250 μM **5** overnight, the unreacted cyclooctyne was quenched with excess 2-azidoethanol, and the samples were analyzed by Western blot probing with HRP−α-biotin (Figure 2). Robust biotinylation was observed for GlyCAM-Ig modified with SiaNAz. Native GlyCAM-Ig lacking azides showed no background labeling, underscoring the exquisite selectivity of the strain-promoted cycloaddition.

As a point of comparison, we performed similar reactions with biotin-modified terminal alkyne **6** (Scheme 1). In the absence of reagents for copper catalysis, no glycoprotein labeling was observed (Figure 2). As expected on the basis of reports from Sharpless, Finn, and Cravatt,[3a,4] addition of $CuSO_4$, TCEP, and a triazolyl ligand resulted in facile labeling of the azide-modified glycoprotein. The blot was stripped and reprobed with HRP−α-IgG to confirm equal protein loading. Interestingly, we consistently observed diminished α-IgG immunoreactivity for azide-modified GlyCAM-Ig labeled with **6** in the presence of copper. It is possible that the combination of triazole products and copper damages the epitope recognized by HRP−α-IgG.

Finally, we investigated the utility of the strain-promoted reaction for live cell labeling. Jurkat cells were incubated with 25 μM Ac_4-ManNAz for 3 d to introduce SiaNAz residues into their cell-surface glycoproteins.[5a,21] The cells were reacted with various concentrations of **5** for 1.5 h at room temperature and then stained with FITC−avidin and analyzed by flow cytometry. As shown in Figure 3A, cells displaying azides showed a dose-dependent increase in fluorescence upon treatment with the cyclooctyne probe. No detectable labeling of cells lacking azides was observed. The cell-surface reaction was also dependent on duration of incubation with **5** (Figure 3B) and the density of cell-surface azides (Supporting Information). No negative effects on cell viability were observed. Curious as to how the strain-promoted cycloaddition compares to the Staudinger ligation, we reacted azide-labeled cells with either compound **5** or a previously reported phosphine−biotin probe.[6b] As shown in Figure 3C, the cell-surface reactions were comparable, with the Staudinger ligation proceeding about twice as efficiently.

In summary, the strain-promoted [3 + 2] azide−alkyne cycloaddition can be used for selective modification of biomolecules and living cells without apparent physiological harm. An important next step will be its extension to living animals. In the future, ring strain might be more thoroughly explored as a means to promote otherwise reticent reactions with potential bioorthogonality.

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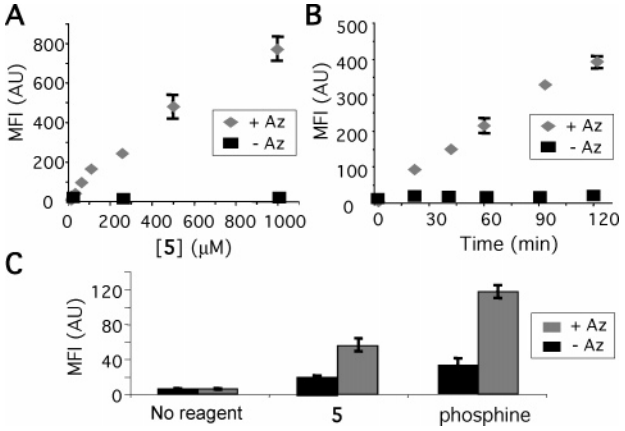

Figure 3. Cell-surface labeling with compound **5**. Jurkat cells were incubated in the presence (+Az) or absence (−Az) of 25 μM Ac_4ManNAz for 3 d. (A) The cells were reacted with various concentrations of **5** for 1 h at room temperature and treated with FITC−avidin; mean fluorescence intensity (MFI) was determined by flow cytometry. (B) Cells were incubated with 250 μM **5** at room temperature and analyzed as in A. (C) Cells were incubated with 100 μM probe for 1 h at room temperature and analyzed as in A. Error bars represent the standard deviation from three replicates. AU = arbitrary fluorescence units.

Supporting Information Available: Synthetic procedures and spectral data, and protocols for kinetic analyses and biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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