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Rapid Cu-Free Click Chemistry with Readily Synthesized Biarylazacyclooctynones

John C. Jewett, Ellen M. Sletten, and Carolyn R. Bertozzi*

Departments of Chemistry and Molecular and Cell Biology and Howard Hughes Medical Institute, University of California, Berkeley, California 94720

Received January 8, 2010; E-mail: crb@berkeley.edu

Bioorthogonal chemical reactions, those that do not interact or interfere with biology, have allowed for exploration of numerous biological processes that were previously difficult to study.[1] A widely used bioorthogonal functional group is the azide, which can be incorporated into myriad biological molecules by feeding cells or organisms azide-functionalized metabolic substrates.[2] The abundance, location, and dynamics of the azide-labeled biomolecules can be monitored by chemical ligation with probes bearing complementary functionality.[3] The copper-catalyzed click reaction between azides and terminal alkynes is ideal for many applications, but copper(I) has the undesirable side effect of being cytotoxic at low concentrations.[4] The reaction of azides with strained alkynes, such as cyclooctynes, relieves that burden by readily forming a triazole product without a toxic catalyst (Figure 1A). Such reactions, in addition to other select cycloadditions, are now being referred to as Cu-free click chemistries.[5]

In our ongoing work toward the design of cyclooctyne probes for live cell and animal imaging, we have focused on two central attributes: kinetic parameters and synthetic facility. Herein we describe a biarylazacyclooctynone (BARAC, **1**) that has exceptional reaction kinetics and whose synthesis is designed to be both modular and scalable (Figure 1B). We employed BARAC for live cell fluorescence imaging of azide-labeled glycans. The high signal-to-background ratio obtained using nanomolar concentrations of BARAC obviated the need for washing steps. Thus, BARAC is a promising reagent for in vivo imaging.

Previously we reported that the addition of electronegative fluorine atoms at the propargylic position of cyclooctyne dramatically increases its rate of reaction with azides (Figure 1B).[6] These difluorinated cyclooctynes (DIFO, **2**) have been employed in applications ranging from synthesis of star dendrimers[7] to labeling live zebrafish embryos.[8] Recently, Boons and co-workers reported on the usage of dibenzocyclooctynes (DIBO, **3a,b**) as detection reagents for the azide.[9] These compounds were remarkably reactive, with rate constants approaching those of the difluorinated cyclooctynes. While the fluorine atoms affected the rate by altering the electronics of the alkyne,[10] the dibenzo system accomplished a similar rate enhancement through increased strain energy.[11] Addition of one more sp^2-like center to the dibenzocyclooctyne ring can have a further rate-enhancing effect, as reported by Debets et al. in their study of an aza-dibenzocyclooctyne bearing an exocyclic amide linkage.[12]

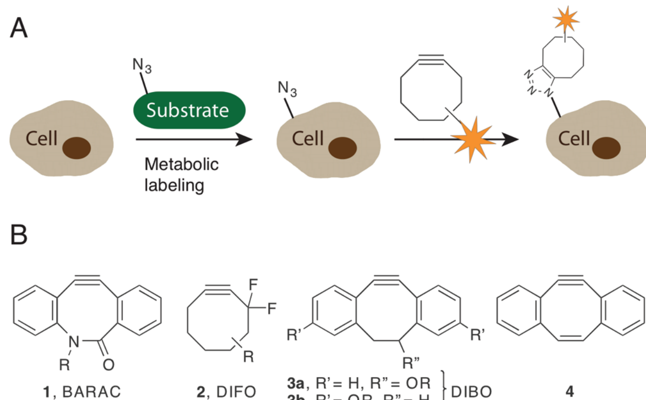
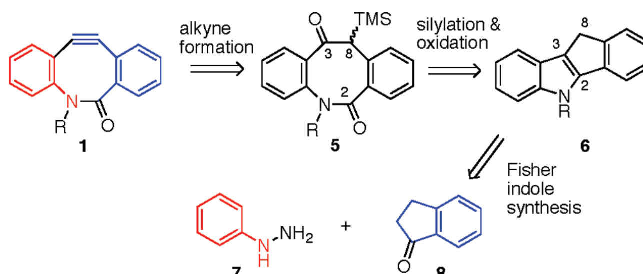


Figure 1. Bioorthogonal reaction of cyclooctyne probes with azide-labeled biomolecules allows their interrogation in cell-based systems. (A) Cells are treated with azide-functionalized metabolic substrates. The azides are then detected with a cyclooctyne-functionalized probe. (B) Cyclooctynes designed for fast Cu-free click chemistry (**1**−**3**) and reactivity studies (**4**). The R-group denotes the location for linkage to a probe moiety.

The dibenzocyclooctynes are stable, but one extra degree of unsaturation across the ring renders an ene-yne (**4**) that is highly reactive but also unstable (Figure 1B).[13] In designing new cyclooctyne probes we sought to brush against the line between stability and reactivity without crossing it. In looking for functional groups that have varying degrees of double-bond character we arrived at the amide. Amides have a significant resonance structure wherein the lone pair of electrons on the nitrogen atom is delocalized between the nitrogen and carbonyl. There are no reported cyclooctynes that have an amide within the ring, as in BARAC; thus their balance of stability and reactivity is an intriguing unanswered question. Further, BARAC derivatives may have solubility and pharmacokinetic properties that are superior to their parent all-carbon cyclooctynes.[14,15]

In planning a synthesis of BARAC, we designed a modular route that would allow for future modifications to examine the relationship of structure and activity within biological systems. When synthesizing cyclooctynes, it is essential to build strain after ring formation; accordingly the amide, functionalized with a linking moiety, was not an ideal bond disconnection to pursue. The reactions previously employed to form the cyclooctyne's strained triple bond have undermined efficient syntheses by imposing long reaction times and low yields.[6,14,16] To overcome this hurdle, we sought to install the alkyne from α-trimethylsilyl ketone **5** (Scheme 1). Although unprecedented in the synthesis of cyclooctynes,

Scheme 1. Retrosynthesis of BARAC (**1**)



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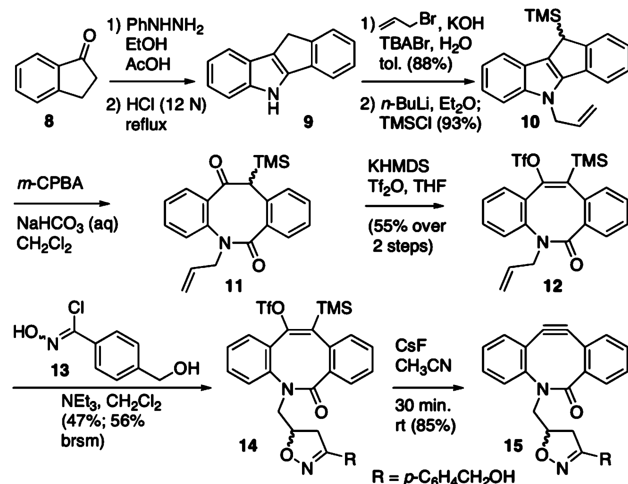
Scheme 2. Synthesis of BARAC (**15**)



formation of the enol triflate from this ketone could allow for strained alkyne formation using mild conditions and short reaction times.[17] However, such α-silyl ketones are synthetically challenging. Selective carbon−silicon bond formation under enolate-forming conditions is problematic due to the oxophilic nature of silicon coupled with the electronegativity of oxygen.[18]

We envisioned introducing the silyl group prior to the carbonyl, and toward this end, we harnessed the susceptibility of indoles to oxidative C3−C4 bond cleavage.[19] Silylation of the C8-position of indole **6** should be facile due to its relatively acidic protons.[20] The indole, formed from a Fisher indole synthesis between phenyl hydrazine (**7**) and 1-indanone (**8**), was an ideal early precursor because of the wide variety of aryl hydrazines and indanones that are either commercially available or readily synthesized in large quantities.

As shown in Scheme 2, Fisher indole synthesis gave commercially available indole **9**.[21] The indole nitrogen was alkylated with allyl bromide using phase transfer conditions,[22] thereby protecting this sensitive functionality and also introducing a functional handle for future probe attachment. This initial sequence is amenable to large-scale preparation with only one purification step at the end of the sequence. The TMS group was then installed in a straightforward manner to form **10**.

Oxidation of indole **10** with excess *m*-CPBA cracked open the central rings to unveil cyclic keto-amide **11**, which was unstable to silica-gel purification. Interestingly, these conditions left the terminal alkene untouched. Treatment of the potassium enolate of ketone **11** with trifluoromethanesulfonyl anhydride gave **12**. Selective reaction of the terminal alkene with a nitrile oxide generated *in situ* from chlorooxime **13** installed a linker for conjugation to a probe molecule.[23] Reaction of **14** with CsF introduced the strained alkyne in excellent yield in under 30 min at rt. In summary, the synthesis of BARAC (**15**) from commercial intermediate **9** was accomplished in 6 steps in 18% overall yield. Importantly, BARAC was stable to traditional chromatography and on the benchtop, unlike its prototype **4**.

We studied the reactivity of BARAC derivative **15** using benzyl azide as a model substrate. Gratifyingly, the second-order rate constant in acetonitrile at rt was 0.96 M^{-1} s^{-1} (Figures S1 and S2, Supporting Information), over 12-fold higher than the rate constant for DIFO under identical conditions and over 450 times higher than that for an unactivated cyclooctyne.[11,24]

We next sought to evaluate BARAC's performance in live cell labeling experiments. Using a carbamate linkage, we conjugated BARAC to biotin, affording BARAC-biotin (**16**), or to fluorescein, affording BARAC-Fluor (**17**, Figure 2A) (Scheme S1 and Figure S7, Supporting Information). Azides were introduced into cell surface glycans using a strategy we employ routinely.[25] We treated cultured Jurkat cells with peracetylated *N*-azidoacetyl mannosamine (Ac_4ManNAz) to introduce the corresponding azido sialic acids (SiaNAz) into their cell-surface glycoproteins and lipids. Then, the cells were labeled with BARAC-biotin at a concentration of 1 μM for 1 h. Staining with fluorescein isothiocyanate (FITC-avidin) and flow cytometry analysis produced the data shown in Figure 2B. The cells showed robust azide-specific labeling with no significant background labeling compared to cells treated with FITC-avidin alone (Figures S3 and S4, Supporting Information). Even at a concentration of 50 nM, BARAC-biotin still showed significant cell labeling in 1 h (Figures S3 and S4, Supporting Information).

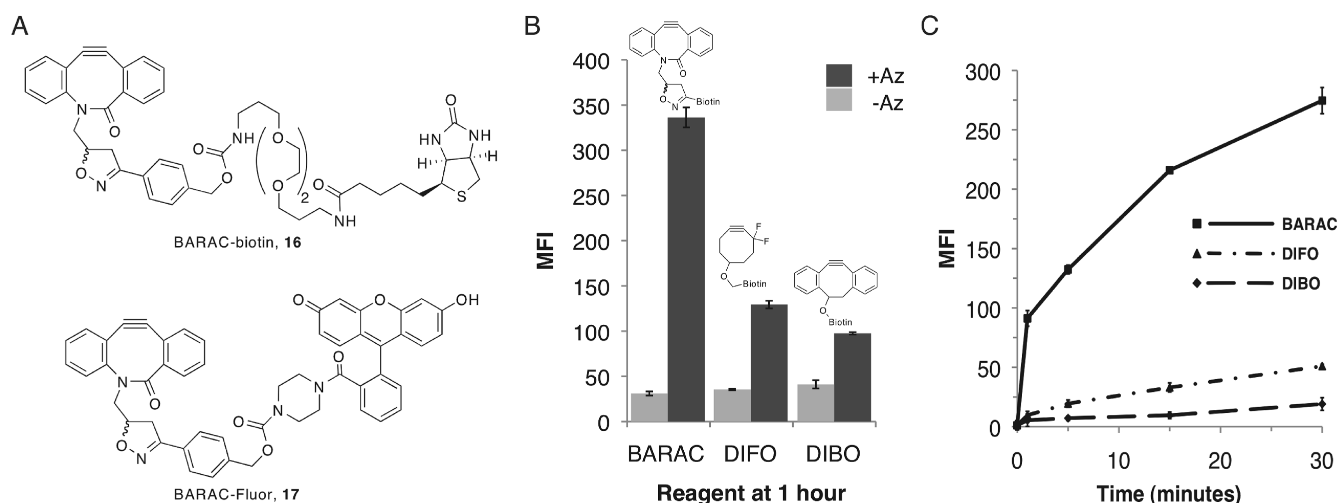


Figure 2. BARAC-probe conjugates label live cells with superior sensitivity compared to DIFO and DIBO reagents. (A) Structures of BARAC-biotin (**16**) and BARAC-Fluor (**17**). (B−C) Flow cytometry plots of live cell labeling with BARAC-biotin. Jurkat cells were incubated with (+Az) or without (−Az) 25 μM Ac_4ManNAz for 3 days. The cells were labeled with 1 μM cyclooctyne-biotin for various times and then treated with FITC-avidin. Cyclooctyne-biotin probes used were DIBO-biotin, BARAC-biotin, or DIFO-biotin. The degree of labeling was quantified by flow cytometry. The level of fluorescence is reported in mean fluorescence intensity (MFI, arbitrary unit). Error bars represent the standard deviation of three replicate experiments. (B) Comparison of the efficiencies of labeling of different cyclooctyne reagents after 1 h. (C) Time-dependent labeling of cyclooctyne-biotin probes. MFI reported as difference between signal of cells +Az and signal of cells −Az.

For comparative purposes, we performed similar experiments using DIFO-biotin[6] and DIBO-biotin[16c] (Figure 2B). BARAC-biotin showed the best labeling after 1 h. To quantify the differences in reaction kinetics among the reagents in the context of cell surface labeling, we measured cell surface fluorescence at various time points during a 30-min incubation with the cyclooctynes (Figure 2C). After 1 min, BARAC-biotin gave a 10-fold higher signal than either DIFO-biotin or DIBO-biotin, consistent with BARAC's ~12-fold higher rate constant. The high level of reactivity of BARAC-biotin was not accompanied by any cytotoxicity compared to cells treated with no cyclooctyne reagent (Figures S5 and S6, Supporting Information).

We next evaluated BARAC-Fluor (**17**) as a reagent for direct fluorescence imaging of live Chinese hamster ovary (CHO) cells. CHO cells grown in either the presence or absence of 50 μM $Ac_4ManNAz$ were treated with 5 μM BARAC-Fluor (**11**) for 5 min at rt, washed, and then imaged (Figure 3A−H). The azide-labeled CHO cells showed robust cell surface fluorescence.

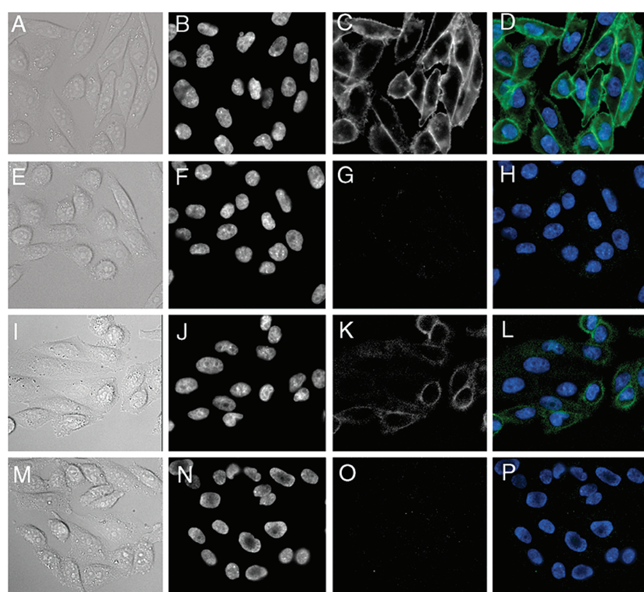


Figure 3. Imaging of azide-labeled glycans on live cells using BARAC-Fluor (**17**). (A−P) CHO cells were incubated with (A−D, I−L) or without (E−H, M−P) 50 μM $Ac_4ManNAz$ for 3 days. (A−H) The cells were subsequently labeled with 5 μM BARAC-Fluor and Hoechst-33342 for 5 min and then washed and imaged. (I−P) The cells were subsequently labeled with 250 nM BARAC-Fluor for 30 min and Hoechst-33342 and then imaged without washing. Channels shown are differential interference contrast bright-field (A, E, I, M), the blue DAPI channel (B, F, J, N), the green FITC channel (C, G, K, O), and the DAPI/FITC channels merged (D, H, L, P).

In some situations, washing away unbound fluorescent labeling reagents is difficult or impossible. Such cases include whole animal imaging experiments, real-time imaging of cultured cells, and situations in which the biomolecular targets are intracellular. We reasoned that BARAC's superior sensitivity, the consequence of its kinetics and low nonspecific background reactivity, would allow for use of low labeling concentrations that eliminate the need for washing steps. Cells were cultured as before but labeled with 250 nM BARAC-Fluor for 30 min at rt. Without washing away excess reagent we imaged the cells (Figure 3I−P) and observed clear labeling above background. BARAC is therefore a promising reagent for real-time and in vivo imaging of azide-labeled biomolecules.

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Supporting Information Available: Experimental procedures, spectral data, and assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974–6998.
(2) Prescher, J. A.; Bertozzi, C. R. *Nat. Chem. Biol.* **2005**, *1*, 13–21.
(3) Kele, P.; Li, X.; Link, M.; Nagy, K.; Herner, A.; Lorincz, K.; Beni, S.; Wolfbeis, O. S. *Org. Biomol. Chem.* **2009**, *7*, 3486–3490.
(4) Wolbers, F.; ter Braak, P.; Le Gac, S.; Luttge, R.; Andersson, H.; Vermes, I.; van den Berg, A. *Electrophoresis* **2006**, *27*, 5073.
(5) For a recent review of all bioorthogonal Cu-free click cycloadditions, see: Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, in press DOI:10.1039/B901970G.
(6) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793–16797.
(7) Johnson, J. A.; Baskin, J. M.; Bertozzi, C. R.; Koberstein, J. T.; Turro, N. J. *Chem. Commun.* **2008**, 3064–3066.
(8) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664–667.
(9) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253–2255.
(10) Ess, D. H.; Jones, G. O.; Houk, K. N. *Org. Lett.* **2008**, *10*, 1633–1636.
(11) Rate constants for these reactions have been measured in different solvents, thus a direct comparison cannot be made on the basis of published data. However, DIBO has been reported to react with benzyl azide in methanol with a second-order rate constant of 0.06 M^{-1} s^{-1} (ref 16c), whereas DIFO reacts with the same substrate in acetonitrile with a second-order rate constant of 0.08 M^{-1} s^{-1} (ref 6).
(12) Debets, M. F.; van Berkel, S. S.; Schoffelen, S.; Rutjes, F. P. J. T.; van Hest, J. C. M.; van Delft, F. L. *Chem. Commun.* **2010**, *46*, 97–99.
(13) (a) Wong, H. N. C.; Garratt, P. J.; Sondheimer, F. *J. Am. Chem. Soc.* **1974**, *96*, 5604–5605. (b) Gugel, H.; Meier, H. *Chem. Ber.* **1980**, *113*, 1431–1443.
(14) Sletten, E. M.; Bertozzi, C. R. *Org. Lett.* **2008**, *10*, 3097–3099.
(15) Chang, P. V.; Prescher, J. A.; Sletten, E. M.; Baskin, J. M.; Miller, I. A.; Agard, N. J.; Lo, A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1821–1826.
(16) (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046–1504. (b) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486–11493. (c) For a recent paper that addresses this problem, see: Poloukhtine, A. A.; Mbua, N. E.; Wolfert, M. A.; Boons, G.-J.; Popik, V. V. *J. Am. Chem. Soc.* **2009**, *131*, 15769–15776.
(17) Atanes, N.; Escudero, S.; Pérez, D.; Guitián, E.; Castedo, L. *Tetrahedron Lett.* **1998**, *39*, 3039–3040.
(18) Sampson, P.; Wiemer, D. F. *J. Chem. Soc., Chem. Commun.* **1985**, 1746–1747.
(19) Letcher, R. M.; Kwok, N.-C.; Lo, W.-H.; Ng, K.-W. *J. Chem. Soc., Perkin Trans. 1* **1998**, 1715–1719.
(20) (a) Abraham, T. *Monat. Für Chemie* **1989**, *120*, 117–126. (b) Grandini, C.; Camurati, I.; Guidotti, S.; Mascellani, N.; Resconi, L.; Nifant'ev, I. E.; Kashulin, I. A.; Ivchenko, P. V.; Mercandelli, P.; Sironi, A. *Organometallics* **2004**, *23*, 344–360.
(21) Okamoto, T. A.; Kobayashi, S. M.; Yamamoto, H. N. German Pat. DE1952019 1970.
(22) Nifant'ev, I. E.; Kashulin, I. A.; Bagrov, V. V.; Abilev, S. K.; Lyubimova, I. K. *Russ. Chem. Bull.* **2001**, *50*, 1439–1445.
(23) (a) For an early review on this type of reaction, see: Huisgen, R. *Angew. Chem., Int. Ed.* **1963**, *2*, 565–598. (b) For a recent application of this chemistry, see: Gutsmiedl, K.; Wirges, C. T.; Ehmke, V.; Carell, T. *Org. Lett.* **2009**, *11*, 2405–2408.
(24) The half-life for the reaction of equimolar amounts of BARAC with benzyl azide at 10 mM is ~45 s at rt. The half-life of the potential background reaction of 2 mM BARAC with 5 mM glutathione in CD_3CN/D_2O (1:2) was 24 h at rt.
(25) Laughlin, S. T.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 12–17.

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