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Diazo Groups Endure Metabolism and Enable Chemoselectivity in Cellulo

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Supporting Information

ABSTRACT: We introduce a stabilized diazo group as a reporter for chemical biology. ManDiaz, which is a diazo derivative of *N*-acetylmannosamine, is found to endure cellular metabolism and label the surface of a mammalian cell. There its diazo group can undergo a 1,3-dipolar cycloaddition with a strained alkyne, providing a signal comparable to that from the azido congener, ManNAz. The chemoselectivity of diazo and alkynyl groups enables dual labeling of cells that is not possible with azido and alkynyl groups. Thus, the diazo group, which is approximately half the size of an azido group, provides unique opportunities for orthogonal labeling of cellular components.

Appreciation[1] of the broad utility of the Huisgen azide–alkyne 1,3-dipolar cycloaddition[2] has had a profound impact on chemical biology.[3] The diazo group shares this reactivity with the azido group while conferring additional versatility. For example, cycloaddition with a diazo compound can be tuned to be much faster or much slower than that with its azide analogue.[4] Diazo compounds offer other useful modes of reactivity, including the *O*-alkylation of carboxylic acids in water to form esters.[5] Indeed, the utility of diazo compounds in chemical synthesis was established long ago[6] and likely exceeds that of azides.[7] Nevertheless, rightful concern about their toxicity and high, even explosive, reactivity[8] has deterred the application of diazo compounds in chemical biology. While recent work has demonstrated their utility in biomolecular transformations in vitro,[4] we are unaware of any application of diazo compounds in cellulo or in vivo.

A diazo group has attributes of an ideal reporter for chemical biologists. Smaller than an azido group (Figure 1A), a diazo group has the same number of atoms as a methyl group (RCH_3 versus $RCHN_2$) or a methylene group ($R^1R^2CH_2$ versus $R^1R^2CN_2$). Moreover, a diazo compound can be prepared readily from its parent azide by simple deimidogenation, that is, loss of "NH" (Figure 1B).[9] This deimidogenation reaction allows access to diazo compounds in aqueous solution via abstraction of an α-proton from an incipient acyl triazene with a mild base such as bicarbonate. The requisite acidity of that α-proton requires conjugation of the anion with, for example, an amidic carbonyl group.[10] We hypothesized that such mitigation of reactivity could allow diazo compounds to endure physiological conditions.

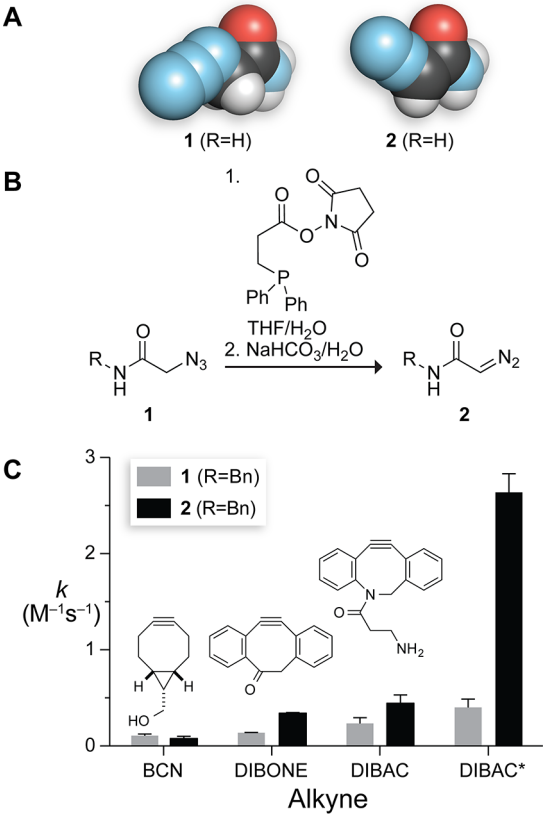

Figure 1. (A) Space-filling models of diazo and azido derivatives of acetamide. Diazo and azido groups add 15.4 and 31.6 Å^2 of solvent-accessible surface area, respectively (see the Supporting Information). (B) Scheme for the deimidogenation of an azide to form a diazo compound.[9] (C) Bar graph of the rate constants for the reactions of an azide (**1**, R = Bn) and a diazo compound (**2**, R = Bn) with strained cyclooctynes in CD_3CN or *1:1 CD_3CN/H_2O as determined with ^1H NMR spectroscopy.

We sought to assess the resilience of a diazo group in a meaningful context. To do so, we chose metabolic trafficking, which is more demanding than the mere demonstration of chemoselective reactivity in a biomolecular milieu. The labeling of cell-surface glycans is an ideal theater for this test because of the high tolerance demanded by cellular biosynthetic machinery,[11] the rigid constraint on size,[12] and the precedent

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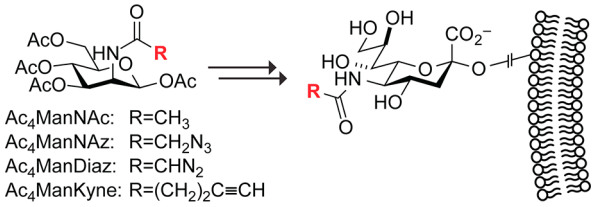
for this labeling established by Reutter, Bertozzi, and others with derivatives of *N*-acetylmannosamine (ManNAc) (Scheme 1).[13]

Scheme 1



We began by examining the in vitro reactivity of a relevant diazo compound with strained functionalizable alkynes. Three cyclooctynes were reacted with a representative azide (**1**, R = Bn) and diazo compound (**2**, R = Bn). In acetonitrile, the reactions with the diazo compound were as fast or faster than those with the azide (Figure 1C). Notably, the addition of water augmented both the rates and their differential, likely as a result of stabilization of the especially polar transition state for cycloaddition with the diazo compound.[4b,14] On the basis of these data, we chose DIBAC as an optimal functionalizable alkyne for our experiments.

Next, we sought to confirm the chemoselectivity of the diazo group in a biological context. First, we stirred a solution of *N*-benzyl-2-diazoacetamide (**2**, R = Bn) and glutathione for 24 h and did not observe a reaction (Figure S1 in the Supporting Information). Then we linked a diazoacetamide to biotin to probe for nonspecific labeling in cellulo. CHO K1 cells treated with the diazo–biotin conjugate or its parent azide showed no labeling on an immunoblot, in contrast to an analogous acrylate (Figure 2A). Likewise, nonspecific labeling with the diazo group was not detectable with flow cytometry or confocal microscopy at 24 h (Figure 2B,C).

Next, we asked whether a diazo compound would be accepted by an endogenous biosynthetic pathway alongside extant biomolecules. To answer this question, we synthesized the stabilized diazo compound Ac_4ManDiaz (Scheme 1) by deimidogenation of Ac_4ManNAz. The four acetyl groups enhance cell permeability and are hydrolyzed by intracellular esterases.[15] We added Ac_4ManDiaz or Ac_4ManNAz to medium containing live CHO K1 cells. After 2 days, any extracellular diazo or azido groups were reacted with a DIBAC–biotin conjugate and labeled with avidin–Alexa Fluor 488, which is green. Super-resolution images of cells exposed to Ac_4ManDiaz and Ac_4ManNAz were indistinguishable (Figure 3A). Quantification with flow cytometry revealed that labeling with Ac_4ManDiaz was slightly less efficient (Figure 3B), despite its higher reaction rate (Figure 1C). The differential labeling is likely related to the instability of a diazo group at low pH. Most peracylated sugars are taken up by passive diffusion across the outer membrane, but some are taken up by endocytosis.[15] The low pH of endosomes[16] likely leads to C-protonation and hydrolysis of the ensuing diazonium salt.[5] Finally, we showed that Ac_4ManDiaz is metabolized and displayed on the surface of three other cell types, though at different rates (Figure 3C).

To confirm that Ac_4ManDiaz was trafficked into sialic acid in the same manner as Ac_4ManNAz, we grew CHO K1 cells in each peracylated sugar at 250 μM for 3 days and labeled them with DIBAC–biotin as described above. We then treated the cells with either neuraminidase (sialidase) or peptide-*N*-glycosidase F (PNGase F). Both the azido and diazo sugars showed high levels of labeling with DIBAC–biotin in the absence of enzyme, with diazo labeling being (86 ± 3)% of the azido labeling. Exogenous addition of either enzyme decreased the labeling levels sharply (Figure S2), indicating that labeling

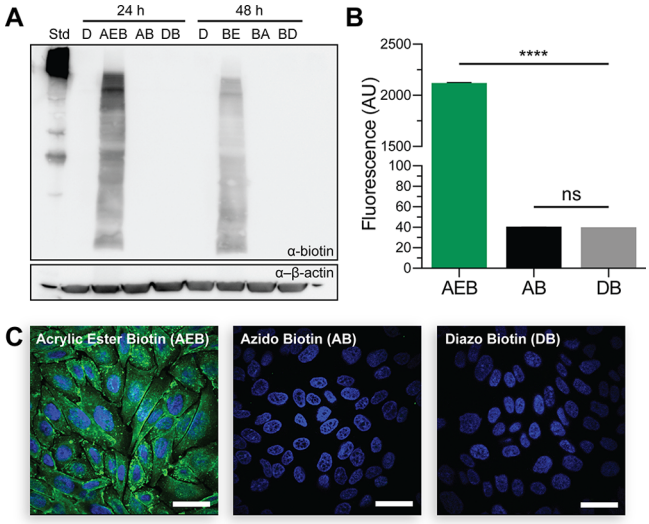


Figure 2. Chemoselectivity of diazo and azido groups. (A) Immunoblot of the biotin signals of cell lysates grown in either DMSO (D), acrylic ester–biotin (AEB) (1 mM), azido–biotin (AB) (25 μM), or diazo–biotin (DB) (25 μM) for 24 or 48 h. (B) Flow cytometry analysis of cells grown as in (A) for 24 h ($p < 0.0001$). (C) Microscopy of fixed and permeabilized cells grown as in (A) for 24 h. Cells were stained with avidin–Alexa Fluor 488 and Hoechst 33342. Scale bars: 25 μm.

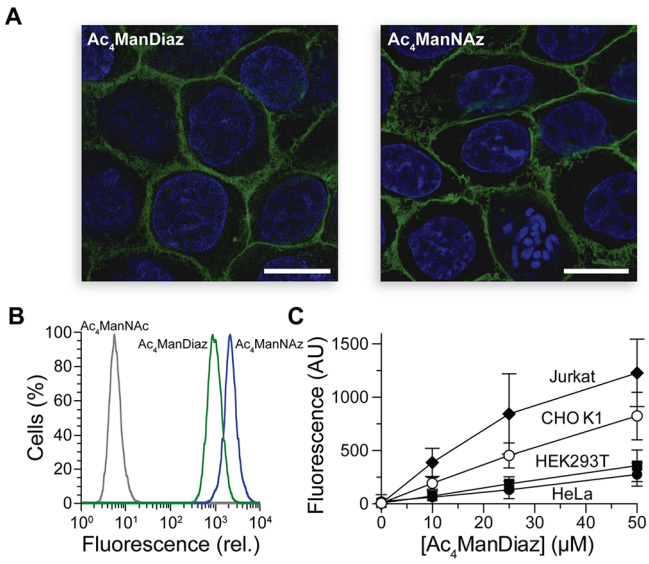


Figure 3. Trafficking of diazo and azido sugars in mammalian cells. (A) Super-resolution images of CHO K1 cells grown in medium containing Ac_4ManDiaz or Ac_4ManNAz (25 μM) for 2 days, washed, treated with DIBAC–biotin (10 μM) for 60 min and then avidin–Alexa Fluor 488 and Hoechst 33342, and fixed. Scale bars: 10 μm. (B) Histogram of CHO K1 cells grown in medium containing Ac_4ManNAc, Ac_4ManDiaz, or Ac_4ManNAz (25 μM) as in (A). (C) Graph of the concentration-dependent fluorescence of Jurkat, CHO K1, HEK293T, and HeLa cells grown in medium containing Ac_4ManDiaz (0–50 μM) as in (A). The data in (B) and (C) were acquired by flow cytometry.

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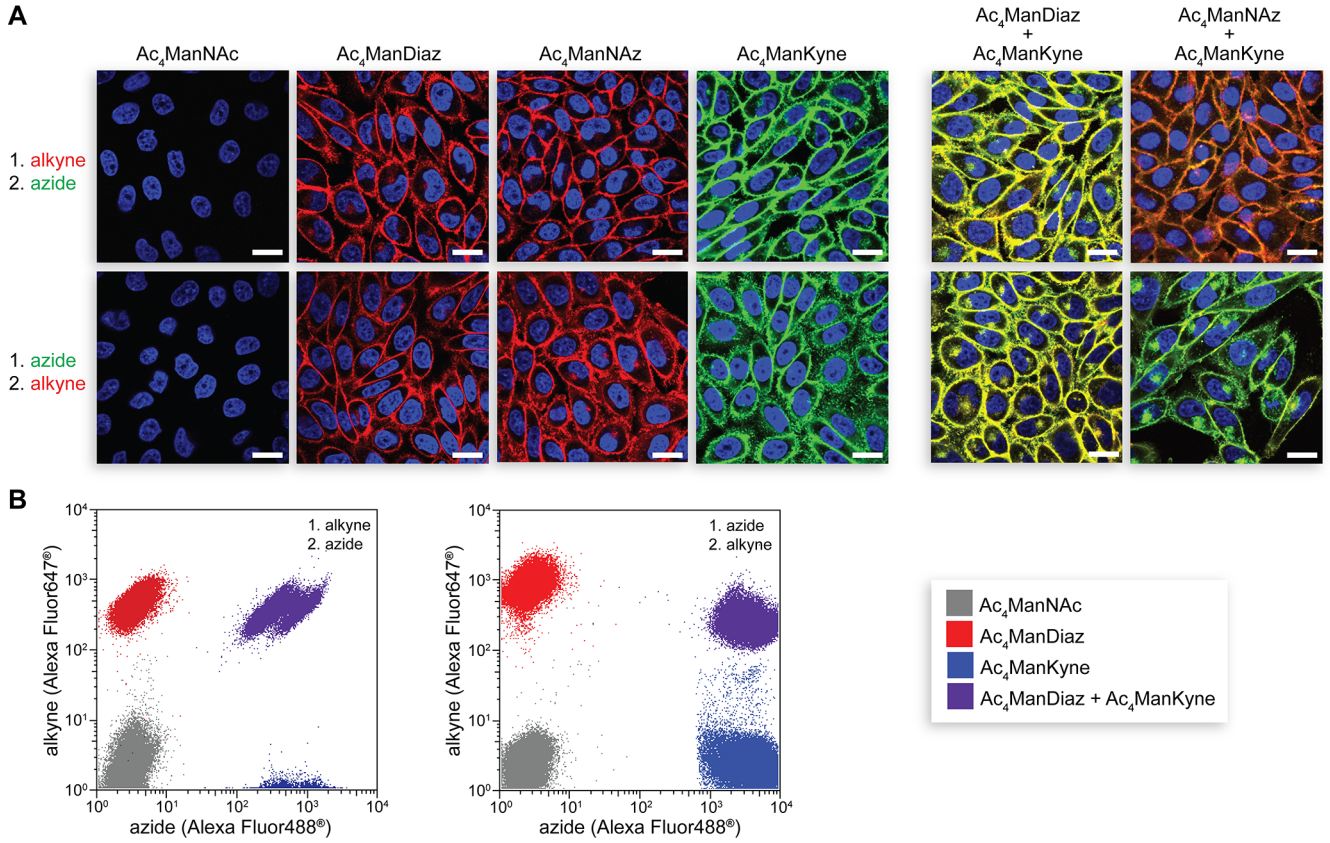

Figure 4. Dual labeling of mammalian cells with diazo and alkynyl sugars. (A) Images of CHO K1 cells grown in medium containing derivatives of *N*-acetylmannosamine for 2 days, labeled by cycloaddition with an alkyne (red) and azide (green), and visualized with confocal microscopy. Scale bars: 10 μm. (B) Plots demonstrating the dual labeling of cells that had metabolized Ac_4ManDiaz and Ac_4ManKyne. Data were acquired by flow cytometry.

was due to incorporation of the sugars.[17] In addition, using the pendant biotin of the DIBAC conjugate, we isolated the cellular metabolites generated from the azido and diazo precursors and observed the expected sialic acid conjugates by mass spectrometry (Figure S3).

To ascertain the optimal conditions for labeling of CHO K1 cells that had metabolized Ac_4ManDiaz, we exposed these cells to 0−20 μM DIBAC−biotin for 60 min or 10 μM DIBAC−biotin for 0−120 min. Although labeling continued to increase with increasing levels of DIBAC−biotin, concentrations in excess of 10 μM or times beyond 60 min began to elicit cytotoxicity (Figures S4 and S5). Accordingly, we chose labeling at 10 μM for 60 min as a compromise between high labeling efficiency and cell viability. Notably, the cytotoxic activities of Ac_4ManDiaz and Ac_4ManNAz were similar (LD_{50} ∼ 1 mM; Figure S6).

Next, we sought to perform chemoselective dual labeling on the cell surface.[18] Unlike azide **1** (R = Bn), diazo compound **2** (R = Bn) is a poor substrate for Cu(I)-catalyzed cycloaddition with a terminal alkyne in aqueous solution (data not shown). Hence, we reasoned that a cell surface displaying both diazo and terminal alkynyl groups could provide opportunities for orthogonal reactivity.

To test this hypothesis, we allowed cells to metabolize Ac_4ManDiaz, Ac_4ManKyne, or both. We then probed for diazo groups by using strain-promoted cycloaddition with DIBAC−biotin and labeling with avidin−Alexa Fluor 594 (microscopy) or avidin−Alexa Fluor 647 (flow cytometry), which are red; we probed for alkynyl groups by using Cu(I)-catalyzed cycloaddition with picolyl azide−Alexa Fluor 488,[19] which is green. Both microscopy and flow cytometry indicated that the two cycloaddition reactions could be performed in either order without interfering cross-reactivity (Figure 4). In marked contrast, performing the same experiment with Ac_4ManNAz and Ac_4ManKyne resulted in diminished cell-surface labeling, especially when Cu(I)-catalyzed cycloaddition was performed first. We suspect that the labeling was diminished by the cross-linking of cell-surface azido and alkynyl groups, as these two functional groups are not orthogonal in the presence of Cu(I). The evident clumping of cells displaying both azido and alkynyl groups also suggests that the glycocalyx of two cells can be in such close proximity that their sialic acid residues react to form covalent cross-links. The data in Figure 4 indicate that diazo compounds, unlike azides, are orthogonal to terminal alkynes in a cellular context, and this orthogonality enables novel dual-labeling experiments.

To provide another example of the chemoselectivity of the diazo group, we performed dual labeling with Ac_4ManDiaz and Ac_4GalKyne, which is a peracetylated galactosamine functionalized with an alkyne. Metabolism positions the galactosamine moiety in the core of mucin-type O-linked glycoproteins.[20] Again, we probed for diazo groups by using strain-promoted cycloaddition with DIBAC−biotin and subsequent labeling with avidin−Alexa Fluor 594 (microscopy) or avidin−Alexa Fluor 647 (flow cytometry), and we probed for alkynyl groups by using Cu(I)-catalyzed cycloaddition with picolyl azide−Alexa Fluor 488.[19] Both microscopy and flow cytometry data showed an orthogonal labeling pattern that colocalized at the

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cell surface (Figure S7A,C). Because of the lower abundance of the alkynyl sugar (Figure S7B), the deleterious cross-reactivity with the Ac_4ManNAz metabolite was even more apparent in this experiment. Labeling of the alkyne was diminished substantially in the presence of the azido sugar (Figure S7D). In contrast, labeling of the alkyne was not affected by the presence of the diazo group. These data highlight the importance of orthogonal labeling methods for the simultaneous analysis of more than one metabolite and how diazo and alkynyl groups can provide the requisite chemoselectivity.

We conclude that stabilized diazo groups can rival azido groups as probes in chemical biology. The diazo group is smaller than an azido group and has overlapping but distinct reactivity. Remarkably, an α-diazo amide is able to survive complex metabolic transformations in a mammalian cell. Although other systems exist for dual labeling,[18] we conclude that none provide the small size, metabolic stability, and chemoselective reactivity of the diazo group. Our findings encourage the development of new biocompatible reactions for stabilized diazo compounds that could further manifest their potential.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic methods and analytical data along with cell biological methods and additional microscopy and flow cytometry data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(10) **Caution!** The deimidogenation procedure (ref 9) cannot be used to produce an unstabilized diazo compound (e.g., a primary diazoalkane). Such unstabilized diazo compounds are dangerous (ref 8), and their use should never be attempted in the context of chemical biology. See the Supporting Information for more safety information.

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