Precise Installation of Diazo-Tagged Side-Chains on Proteins to Enable In Vitro and In-Cell Site-Specific Labeling

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ABSTRACT: The chemistry of diazo compounds has generated a huge breadth of applications in the field of organic synthesis. Their versatility combined with their tunable reactivity, stability, and chemoselectivity makes diazo compounds desirable reagents for chemical biologists. Here, we describe a method for the precise installation of diazo handles on proteins and antibodies in a mild and specific approach. Subsequent 1,3-cycloaddition reactions with strained alkynes enable both bioimaging through an in-cell “click” reaction and probing of the cysteine proteome in cell lysates. The selectivity and efficiency of these processes makes these suitable reagents for chemical biology studies.

Diazo-containing natural products have been shown to exhibit antitumor and antimicrobial activities at nanomolar–picomolar concentrations, which makes diazo compounds valuable from a biological perspective. Already, diazo compounds have been used to investigate mechanisms of DNA cleavage in vitro, in which the diazo moiety is likely the active pharmacophore directly involved in the damage pathway. Some natural diazo-amino acids isolated from Streptomyces cultures have also found application in medicine. Indeed, the broadly active glutamine antagonist 6-diazo-5-oxo-L-norleucine entered early phase I/II clinical trials based on its activity against carcinomas, lymphomas, and Hodgkin’s disease.

Although commonly observed in small natural products derived from microorganisms, the diazo moiety is nonexistent in higher organisms. This feature enables their use as bioorthogonal probes. The number of functional groups with biocompatibility as chemical reporters is still limited. In this context, azido groups are dominant and widely used in chemical biology because of their chemical stability and excellent reactivity with strained alkynes and nonstrained alkynes when catalyzed by copper. Similarly to azides, the diazo group can undergo uncatalyzed Huisgen addition reactions with strained dipolarophiles, such as terminal alkenes and alkynes. The diazo group has even better characteristics than those of analogous azido groups, such as improved kinetics for cycloaddition reactions and a greater range of reactivity in a variety of thermal, photochemical, and metal-catalyzed reactions. Insertion of diazocarbonyl compounds into C—H and X—H (X = N, O, S, P, Se) bonds, cyclopropanation reactions, dipolar cycloaddition reactions, ylide formation, alkylations, ring expansions, and Wolff rearrangement are examples of these transformations. In fact, functional groups that perform as broad a range of different reactions as diazo groups are relatively rare. Moreover, the diazo group is more electron-rich and has an increased HOMO energy relative to the analogous azido, which displays superior reactivity in normal electron demand cycloaddition reactions with electron-deficient dipolarophiles. However, and as reported by Raines et al., diazoketones, diazoesters, and diazoamides are electron-deficient dipoles and, therefore, typically undergo inverse electron demand cycloadditions. Finally, the tolerance of stabilized diazo groups toward cellular metabolism is also similar to that of azido groups.

The earliest application of diazo reagents for covalent modification of proteins was the direct esterification of carboxylic acids. Diazomethane and stabilized compounds such as α-diazoacetamides were used to label different proteins and nucleic acids to study their structural and functional aspects. New applications of diazo compounds in chemical biology aim to use their unique reactivity to access ligations between biomolecules that cannot be obtained through other
The diazoacylating reagent 1 (Figure 1a) was first reported in 1993 by Badet and co-workers based on N-hydroxysuccinimide (NHS) esters. This reagent was later used to modify several small molecules and biomolecules, which included bovine serum albumin.26 Leeper and co-workers reacted α-diazo NHS ester 1 with lysine residues on lysozyme (6 Lys, no free Cys, 4 disulfides).27 Following the incorporation of the diazo functionality, a fluorescent complex equipped with a tetrathiohydrazobenzocyclooctyne TMDIBO-strained cyclooctyne was reacted with the diazo-protein by means of a [3 + 2] cycloaddition reaction. NHS esters usually react with any exposed primary amine, such as the α-amino group of N-terminal amino acid and ε-amino groups of lysines present in a protein leading to heterogeneous conjugates. More recently, Gaunt and co-workers demonstrated the use of hypervalent iodine reagents 2 (Figure 1a) to target methionine residues to install an electrophilic diazo group on peptides and small proteins.22 Once incorporated into the target biomolecule, the diazo handle was removed by reduction with a Hantzch ester to generate trialkylsulfonium motifs, as exemplified for peptides and small protein thioredoxin. Although remarkable in terms of methionine modification, all the peptides and proteins used had no free Cys (Cys were previously protected with maleimides or were present as nonreactive disulfide bridges). Moreover, the approach uses additives such as thiourea, TEMPO, formic acid, and nonbiological conditions that can limit the scope of therapeutic proteins/antibodies that can be modified.

Our group has recently reported the design of a new class of Michael-acceptor reagents, carbonylacrylic derivatives, that undergo very rapid, chemoselective thiol addition with Cys residues on proteins and antibodies.23,24 In an attempt to provide a robust reagent capable of site-selectively introducing a diazo handle on protein and antibodies, we developed carbonylacrylic derivative αβ-unsaturated diazoketone 3 (Figure 1c),15 which reacts in high yields with Cys under biological conditions at near-neutral pH. The resulting diazoconjugates showed resistance to degradation in aqueous media and did not react with natural thiols, such as glutathione (GSH), which provides opportunities for further bioorthogonal functionalization in chemical biology.

**RESULTS AND DISCUSSION**

We started our work by preparing diazocarbonylacrylic reagent 3 by means of a Horner–Wadsworth–Emmons (HWE) olefination reaction between diazophosphonate 425 and the commercially available aldehyde phenylglyoxal 5. Different HWE conditions and bases were evaluated for the formation of αβ-unsaturated diazoketone 3 (Table S1). By using NaH, the desired E-olefin was obtained in low to moderate yields (Table S1, Entries 2, 3, 6, 7, and 16). The best results were obtained with N,N-diisopropylethylamine (DIPEA) as base and lithium chloride as additive. In this case, the isolated yield was 78% with 3:1 selectivity for the desired E isomer that can be easily separated by flash chromatography (Table S1, Entry 17). Importantly, diazo 3 can be stored at −20 °C for 12 months in the absence of light without degrading. Next, we evaluated the reaction between the tert-butylxoxycarbonyl (Boc)/benzoxycarbonyl (Cbz)-protected amino acid Cys-methyl ester with stoichiometric amounts of diazocarbonylacrylic 3. The reaction proceeded smoothly in DMF and was complete in <10 min at room temperature to give adducts 7a/7b in 90% yield. The same result was obtained with a 30% DMF solution in sodium phosphate buffer (NaPi pH 8.0, 50 mM) as solvent in a reaction open to the air (Figure 2). The stability of the Cys-adduct in DMF/NaPi buffer (pH 7.4 and 8.0, 50 mM) was evaluated by liquid chromatography–mass spectrometry (LC-MS) analysis after 24 h at room temperature. No reaction was observed after incubation with 1.5 equiv of GSH (Figure 2c and Figure S1a–d). Importantly, this result shows that the diazo group remained intact in the presence of reduced GSH, which corroborates previous reports in the literature.13 The reactivity of diazoketones 7b and 10 with equimolar amounts of commercially available alkyne (1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (8) was then confirmed (Figure 2d,e). The cycloaddition was complete after 24 h at room temperature with a mixture of DMF/NaPi buffer (pH 8.0, 50 mM). Pyrazoles 9 and 11 were obtained in 70% and 99% yield, respectively. In the case of diazoacetophenone 10, the starting material was fully converted (>99%) into cycloaddition product 11, and no byproducts were observed (Figure 2f and Figure S2).

This well-known enhanced reactivity of strained alkynes26 was corroborated by quantum mechanical calculations (Figure 3). Thus, the lower activation barriers calculated for the cycloadditions between small bicycloolone (8′) and dipole diazoacetone (7′) and diazoacetophenone (10) (ΔG‡ = 27.0 and 26.5 kcal mol−1, respectively) revealed a much higher reactivity in comparison to the same reaction with unstrained dimethylacetylene (A) (ΔG‡ = 41.3 and 41.5 kcal mol−1, respectively, see SI). Analogously, cycloadditions between 7′ and 10 with strained dibenzocyclooctyne (DBCO) 12′ (see experiments below) showed much lower calculated activation barriers (ΔG‡ = 25.8 and 25.9 kcal mol−1, respectively) than those for the reactions with linear diphenylacetylene (B) (ΔG‡ = 39.7 and 39.0 kcal mol−1, respectively, see SI). Such billion- to nearly trillion-fold acceleration makes the reaction proceed at room temperature within 24 h, as corroborated experimentally.

Based on the calculated frontier molecular orbitals (FMO, see SI), diazoketones are electron-deficient reagents and undergo inverse-electron demand (IED) cycloadditions with either linear or strained alkynes. The same reactivity was previously reported for the cycloaddition of diazoacetophe-
none and strained cyclooctyne DIBONE. However, despite the LUMO \textsubscript{diazocompound}−HOMO \textsubscript{alkyne} energy gap being the smallest calculated one, the more appropriate symmetry of the LUMO+1 and LUMO+2 orbitals of diazocetone (i.e., having bigger coefficients at the diazo carbon atom), which lead to effectively larger HOMO−LUMO energy gaps, suggests that diazoketones are ambiphilic dipoles with no clear preference for NED (normal electronic demand) or IED (inverse electron demand) pathways. The inadequacy of simple FMO energies to quantitatively predict the outcome of these strain-promoted cycloadditions is reflected by the fact that the \( \Delta E_{\text{IED}} \) (LUMO \textsubscript{diazocompound}−HOMO \textsubscript{alkyne}) energy gaps calculated for the very slow reactions between \( 7' \) and \( 10 \) with linear diphenylacetylene (B) are smaller (\( \Delta E_{\text{IED}} = 156.8 \) and \( 144.8 \) kcal mol\(^{-1} \)) than those calculated for the much faster reactions between the same dipoles and the analogous strained alkyne 12' (\( \Delta E_{\text{IED}} = 158.9 \) and \( 146.9 \) kcal mol\(^{-1} \)).

On the other hand, it is widely accepted that the enormous acceleration observed for strain-promoted cycloadditions lies in the much lower energy needed to distort the strained alkynes into the geometry of the transition state (i.e., reduced distortion energies) compared to the linear alkyne analogues (SI for the calculated values). After this new class of Cys-specific diazo reagents had been prepared, we explored their use in bioconjugation on different proteins (Figure 4a). Diazocarbonylacrylic 3 was initially evaluated for labeling the engineered version of ubiquitin Ub-K63C.28 This protein contains a solvent-exposed Cys residue at position 63. Complete conversion (>95%) into the expected product with 25 equiv of 3 at room temperature after shaking for 3 h was confirmed by LC-MS analysis (Figure 4b). With the engineered variant of the C2A domain of synaptotagmin-I (C2Am), a protein used as an apoptosis imaging agent that contains a free Cys residue 95 on C2Am, the diazo modification at the Cys residue 95 on C2Am. CD of albumin and albumin-3.
A protein was converted completely into the expected product (95%) with 50 equiv of 3 after 18 h at room temperature (Figure 4d). Chemical controls that involved blocking annexin V-Cys 316 with Ellman’s reagent prior to reaction with 3 showed complete selectivity for Cys and no cross-reactivity with Lys (Figures S13–S15). These results are in complete agreement with the previous data obtained for carbonylreactive Cys. In the case of an engineered human albumin variant V0354, a protein used for drug delivery applications, 30,31 5 equiv was used to obtain full conversion into the expected product after 2 h at room temperature (Figure 3e). These results indicate that the required amount of 3 is dependent on the reactivity, chemical microenvironment, and local availability of the Cys residues. The Cys-selectivity of diazocarbonylacrylic reagent 3 was corroborated for C2Am by using trypsin digestion followed by peptide mapping with mass spectrometry (Figure 4f and Figure S16). In addition, circular dichroism (CD) analysis of albumin and modified albumin-3 showed no alterations in the secondary structural content (Figure 4g and Figure S17), which reflects the mildness and efficiency of the conjugation process (for CD analysis of C2Am and C2Am-3, see Figure S18).

Once introduced into target biomolecules, the diazo group was confirmed by using highly selective strain-promoted [3 + 2] cycloaddition reactions (Figure 5a). Depending on the protein, 100–700 equiv of strained alkyne (1R,8S,9S)-bicyclo-[6.1.0]jnon-4-yn-9-ylmethanol (8) was applied. Full conversion into the expected pyrazole was observed after 3–18 h. In the case of diazo-ubiquitin, 700 equiv of 8 was used and the reaction was complete after 18 h at room temperature in sodium phosphate buffer, pH 8.0, 50 mM (Figure 5b). The same conditions were applied to C2Am to obtain >95% conversion after reaction for 6 h (Figure 5c). Diazoc-Annexin V can be fully converted with a reduced amount of 8 (100 equiv) after reaction for 18 h at room temperature (Figure 5d). With 700 equiv, full conversion of annexin V can be obtained after just 3 h (Figure S23). For albumin, the best conditions were 100 equiv and 18 h at room temperature. It is important to mention that all of these reactions can be done in one pot, which obviates the need to purify the diazo-protein before cycloaddition with the cyclooctyne. For instance, C2Am can be first reduced with TCEP (20 equiv, RT, 30 min), conjugated with 3 (25 equiv, RT, 1–2 h), and then reacted with cyclooctyne 8 (700 equiv, RT, 18 h) without any purification step (the same results were obtained by purifying each step with a spin desalting column or buffer exchange columns).

Encouraged by the simplicity of the method and the stability of the protein diazo-conjugates, we evaluated the potential application of diazocarbonylacrylic reagent 3 to modify Cys-tagged antibodies, specifically the smaller recombinant fragment nanobody 2Rb17c, an internalizing antibody with one engineered Cys targeting the Her2 antigen. 32 Initial treatment with TCEP (20–50 equiv, 1 h, room temperature) afforded the free surface-exposed Cys (Figure 5a) in >95% conversion. Complete conversion into the Cys-tagged diazo-nanobody was then obtained with 10 equiv of 3 after 3 h at room temperature in sodium phosphate buffer (pH 8.0, 50 mM NaCl) in a reaction open to the air (Figure 6a). Deconvoluted mass spectra of the site-selectively modified antibody is shown in Figure 6b and Figure S25, and indicates that the starting material was converted into the expected pyrazole product.

![Figure 5. Confirming the diazo on protein. (a) Schematic of the cycloaddition reaction with 8. (b–e) ESI−MS spectra of conjugates (b) ubiquitin-8 (700 equiv, 18 h), (c) C2Am-8 (700 equiv, 6 h), (d) Annexin V−8 (100 equiv, 18 h or 700 equiv, 3 h), and (e) Albumin-8 (100 equiv, 18 h). Full spectra and details are presented in Figures S19–S23.](https://dx.doi.org/10.1021/acs.bioconjchem.0c00232)

![Figure 6. Production of diazo-antibodies for click chemistry. (a) ESI−MS spectra of diazo-conjugate. Full spectra and details are presented in the SI. (c) Diazo-nanobody 1,3-dipolar cycloaddition reaction with DBCO-Cy5. (d) ESI−MS spectra of fluorescent nanobody-conjugate that shows all the starting material was converted into the expected pyrazole product.](https://dx.doi.org/10.1021/acs.bioconjchem.0c00232)
were immediately imaged with a Leica SP5 inverted confocal microscope. Fluorescence intensity appeared greater inside the cells pretreated with 2Rb17c-3 than cells treated with dye alone. The same results were observed when analyzing the images of low-expressing MCF-7 cells; i.e., no fluorescence staining was detected. This experiment demonstrates the retained capability of the modified nanobody to bind to the HER2 receptor and the effectiveness of the diazo group for in-cell “click” reactions in an analogous manner to the commonly used azide functionality. Further experiments also suggested that the click reaction occurred inside the cell upon nanobody internalization (Figures S29 and 30).

With the strategy validated on protein/antibody models in vitro and in live cells, we next evaluated the capability of the diazo compound as probe for the detection of cysteines in cell lysates. For this, we tested the diazoketone 3 as well as the relatively similar diazoester 13 for comparison (Figure 8). The activation barriers calculated for the cycloadditions between model strained bicyclononyne (8') and DBCO (12') and methyl phenyl diazoacetate (13) (ΔG‡ = 25.6 and 24.1 kcal mol⁻¹) were very similar to those of the diazoketones used in the previous assays, which recommended their use as efficient labeling reagents in proteins and cell lysates (SI). In the protein level, these compounds showed equivalent kinetics (Figures S31–36), stability in the presence of GSH (Figures S37–S43), and reactivity for cycloaddition reactions with DBCO and BCN reagents (Figures S44–S49). To determine optimal labeling conditions in the lysates, the concentrations of probes 3 and 13 were systematically varied. Accordingly, HeLa cell lysates were treated with the diazo probes 3 and 13 (5–50 mM) for 2 h at room temperature. Samples were analyzed by SDS-PAGE and Western blotting after ligation of the diazo to an alkynetagged biotin derivative prior to SDS-PAGE. After the incubation with the diazo probes for 2 h, the lysates were treated with biotin-PEG-DBCO (100 mM) and biotin-PEG-alkyne (100 mM) in the presence or absence of CuSO₄. Western blots were developed using an Alexa Fluor 555 streptavidin conjugate. (a) Assessment of diazoketone 3 (2.5 mM) with DBCO-biotin (5–100 mM) and alkyn-biotin (5–100 mM), (b) Assessment of diazoester 13 (2.5 mM) with DBCO-biotin (5–100 mM) and alkyn-biotin (5–100 mM), (c,d) Variation in the concentration of 3 and 13 (5, 10, and 50 mM) with fixed amounts of DBCO-biotin (100 mM) and alkyn-biotin (100 mM); see Supporting Information for detailed conditions and protocols.

### CONCLUSIONS

In summary, we have developed a rapid and site-specific strategy for the installation of a diazo functionality on proteins and antibodies. The reaction proceeds through a thiol-Michael addition reaction of Cys to diazocarboxylic reagent 3. We applied this mild method to modify various Cys-containing proteins and antibodies. The use of this method allows for the specific labeling of cysteines in various biological samples and provides a versatile tool for bioorthogonal chemistry applications.
proteins, such as ubiquitin, C2Am, Annexin V, and albumin variant V0354 with full conversion, and showed that these modified proteins remained stable and retained their secondary structure. Antibodies, such as nanobody 2Rb17c, were also modified successfully. We confirmed the presence of the diazo moiety through a 1,3-cycloaddition reaction with strained alkynes to give homogeneous conjugates in high yields. Cell microscopy studies proved the effectiveness of the diazo group for in-cell “click” reactions in an analogous manner to the commonly used azide functionality. We also showed the diazoketone 3, together with the diazoester 13, can be used as probes to label cysteines in cell lysates using strained and nonstrained alkynes. This is the first example of installation of a diazo handle on proteins and antibodies in a site-selective manner and, as such, provides opportunities to explore new biorthogonal reactions in chemical biology.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00232. Detailed methods, characterization data, and additional imaging, spectra, and structures (PDF)

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**Notes**

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### REFERENCES


(11) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. J. Org. Chem. 67 (9), 3057–3064.


