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Targeted Diazotransfer Reagents Enable Selective Modification of Proteins with Azides

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Abstract

In chemical biology azides are used to chemically manipulate target structures in a bioorthogonal manner for a plethora of applications ranging from target identification to the synthesis of homogeneously modified protein conjugates. While a variety of methods have been established to introduce the azido group into recombinant proteins, a method that directly converts specific amino groups in endogenous proteins is lacking. Here we report the first biotin-tethered diazotransfer reagent DtBio and demonstrate that it selectively modifies the model proteins streptavidin, avidin and the membrane protein BioY on cell surface. The reagent converts amines in the proximity of the binding pocket to azides and leaves the remaining amino groups in streptavidin untouched. Reagents of this novel class will find use in target identification as well as the selective functionalisation and bioorthogonal protection of proteins.

Keywords:

Protein modification, chemical probes, ligand-tethered labelling, vitamin transporters

The discovery that azides react in a truly orthogonal way in complex biological samples opened up the possibility to selectively functionalize biomolecules *in vitro* and *in vivo*.[1–3] Fluorophores, polyethylene glycol groups, carbohydrates, phosphates, proteins and small molecule drugs have been conjugated to azide containing proteins using Staudinger ligation, copper catalysed alkyne-azide cycloaddition (CuAAC) and strain promoted alkyne-azide cycloaddition (SPAAC) reactions.[4–6] The unique reactivity of the relatively small azido group has been exploited for target identification.[7–9] In contrast to affinity handles like biotin, azides have a minimal effect on the biological activity, but they readily enable enrichment of the target after being incorporated. Finally, masking essential amino groups of proteins and peptides as azides provides a means to chemically control biological processes.[10]

The aforementioned applications require the introduction of an azido group onto the protein and over the past decade various methods have been reported that enable this. Both *In situ* metabolic labelling[1,9,11] and chemical modification of surface exposed amines of purified proteins facilitate the global incorporation of azides (Figure 1).[12] These methods are residue specific, but not protein- and site-selective, which limits their applicability. Strategies with increased site-selectivity have been developed to overcome this. Both co-translational incorporation of non-canonical amino acids using techniques like stop codon suppression[13,14] and enzymatic modification of genetically engineered proteins[15,16] have enabled the incorporation of azides with pinpoint precision. By optimizing the reaction conditions, also selective chemical labelling has been achieved. The N-terminal amino group of a protein can be modified selectively at pH 8.5 using 1.75 equivalents of diazotransfer reagent **1**.[17]

Co-translational incorporation of non-canonical amino acids and enzymatic modification methods are also protein-specific and can therefore be employed in the context of more complex surroundings, such as live cells and cell lysates. Disadvantage of both methods is that they are restricted to genetically modified proteins. A suitable method to directly convert an amino group in a target protein to an azide, in complex biological samples, is lacking. Chemical modification with imidazole-1-sulfonyl azide **1** does not require engineering of the protein and it therefore forms an attractive starting point for the development of such a method. However, diazotransfer reagent **1** is not protein specific. Prior work on acylating and alkylating agents shows that targeting the reagent to the protein of interest by tethering it to inhibitors or ligands addresses the issue of specificity.[18–20] We therefore reasoned that diazotransfer reagent **1** could be converted into specific chemical probes in a similar fashion. Linking imidazole-1-sulfonyl azide to a ligand will direct the diazotransfer reagent to amino groups proximal to the binding site of the ligand's respective target and it therefore will increase both the protein specificity and the site-selectivity (Figure 1). We report here the viability of this approach and demonstrate that biotin tethered diazotransfer reagent **2** (DtBio) selectively modifies biotin-binding proteins in complex mixtures. DtBio **2** can be used to modify the previously challenging to target membrane protein BioY, the S-component of the ECF BioY vitamin transporter from *Lactococcus lactis,* on the cell surface with a BODIPY reporter group **3**.[21]

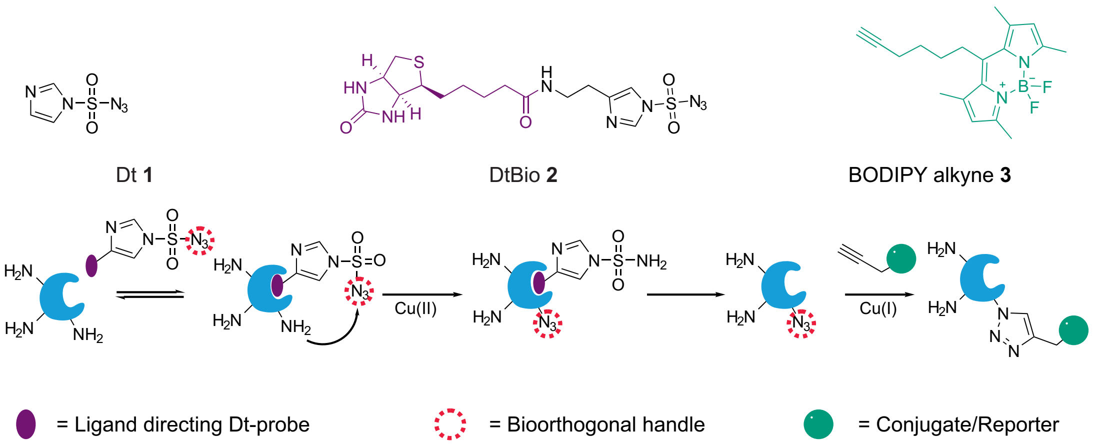
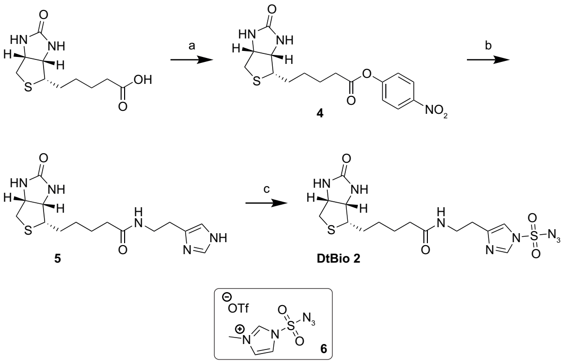


Figure 1. Top: structures of the non-targeted (**1**) and targeted (**2**) diazotransfer reagents and the BODIPY fluorophore (**3**) used as reporter group in the evaluation of the diazotransfer reaction. Bottom: schematic representation of the targeted diazotransfer reagent concept. Ligands bearing the diazotransfer group direct the reagent to the protein of interest. Upon binding an amino group of the protein is transformed into an azide in the presence of Cu(II). The modified protein can be further functionalized with bioorthogonal chemistry.

To assess if targeting diazotransfer reagents to proteins of interest is indeed feasible, we first explored if tethering imidazole-1-sulfonyl azide **1** to D-biotin results in the selective functionalization of streptavidin. A lysine (K121) is located near the biotin-binding site of wild-type core streptavidin.[22] It has been shown that the ε-amine of this residue reacts with ligand-directed reagents and it is therefore conceivable that it will also function as a diazotransfer acceptor, making streptavidin a good model protein.[23] To prepare the DtBio **2** required for these studies, we synthesized *p*-nitrophenyl ester **4** from D-biotin and coupled it to histamine (Scheme 1). Subsequently histamine derivative **5** was transformed into DtBio **2**. Initial attempts to react **5** with *in* *situ* prepared chlorosulfonyl azide, as was described for the synthesis of non-targeted imidazole-1-sulfonyl azide **1**,[24] were unsuccessful. The inherent instability of chlorosulfonyl azide impedes isolation and we therefore explored other sulfonyl azide transfer reagents to synthesize **2**. Culhane and Fokin showed that solid sulfonyl azide transfer reagents can be readily obtained by methylating derivatives of **1** with methyl triflate.[25,26] Gratifyingly, addition of one equivalent of sulfonyl azide transfer reagent **6** to biotin-histamine **5** in DMF gave diazotransfer reagent **2**.



Scheme 1. Synthesis of diazotransfer reagent DtBio **2** (a) EDC·HCl, p-nitrophenol, DMF, 24 h, rT, 72%, (b) histamine, DMF, 16 h, rT, 83%, (c) sulfonyl azide transfer reagent **6**, DMF, 2 h, 0 °C, 52%.

With DtBio **2** in hand, we evaluated its ability to selectively transfer the diazo group to biotin-binding proteins by incubating a mixture of streptavidin (10 μM), ovalbumin (10 μM) and $CuSO_4$ (1 mM) dissolved in PBS (pH 7.4) for 1 h with reagent **2**. To visualize the proteins that reacted with **2**, we functionalized the introduced azido groups with BODIPY-alkyne **3** employing CuAAC.[27] Upon fluorescence scanning of the SDS PAGE gel, two prominent bands are detected that are absent when DtBio **2** is excluded from the reaction mixture (Figure 2A and Figure S1). The molecular weight of the respective bands match those of monomeric (13 kDa) and tetrameric (52 kDa) core streptavidin (see Figure S1**,** for a gel image that reveals tetramer labelling more clearly than shown in Figure 2A, where a 4–12% bis-tris gradient gel was used, which is not optimal for visualization of the tetramer). Very little fluorescence is detected at the molecular weight of ovalbumin (43 kDa), indicating that DtBio **2** mainly reacts with streptavidin. Treatments that undermine binding of DtBio **2** to streptavidin, like heat-denaturing and pre-incubating with D-biotin, drastically reduce fluorescent labelling (Figure 2A) and further confirm that binding of DtBio **2** to streptavidin is responsible for the observed selectivity. Besides increasing the selectivity, targeting the diazotransfer reagent also enhances the labelling efficiency, as is apparent from the increased fluorescence intensity for samples treated with DtBio **2** compared to those treated with non-targeted reagent **1**[17] (Figure 2A, lanes 6 and 7).

By varying the assay conditions, we studied if the incubation time, the protein to probe ratio and the amount of copper affected the labelling efficiency and selectivity. These experiments revealed that an equimolar amount of probe gives the best signal-to-noise ratios, as judged by comparing the labelling intensity of streptavidin and ovalbumin. Protein to probe ratios above one-to-one result in increased non-specific modification of ovalbumin and stabilize the streptavidin tetramer, while lower probe concentrations lead to less efficient labelling of streptavidin (Figure 2B). With an equimolar amount of probe **2**, saturation of labelling is achieved within approximately 30 minutes. Adding DtBio **2** together with sodium ascorbate, ligand and BODIPY-alkyne **3** to the protein mixture results in minimal labelling, which suggests that diazotransfer is negligible during the copper catalysed click reaction (Figure 2C). Finally, using dibenzocyclooctyne-TAMRA **7**[28] as read-out to exclude that labelling by DtBio **2** originates from traces of copper (II) used in the CuAAC visualisation step revealed that the concentration of the copper catalyst can be lowered to 50 µM without a marked effect. Decreasing the $CuSO_4$ concentration further leads to a reduction in the fluorescence intensity. Interestingly, DtBio **2** even reacts with streptavidin when $CuSO_4$ was omitted during the diazotransfer step, albeit with a decreased efficiency (Figure 2D). These results corroborate those of copper independent protein modification with non-targeted reagent **1**.[17] Competition experiments and heat-inactivation confirm that this particular labelling of tetrameric streptavidin in the complete absence of copper is activity dependent (Figure S5).

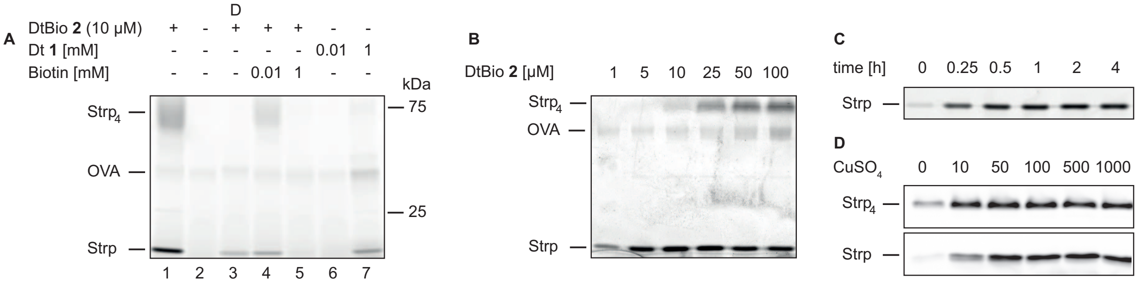


Figure 2. Biochemical evaluation of diazotransfer probe DtBio **2** with streptavidin (Strp) as target protein. (A) A mixture of Strp and OVA labelled with indicated amount DtBio **2** (lane 1) or Dt **1** (lane 6,7) and clicked to BODIPY-alkyne **3** subsequent to diazotransfer is resolved on a bis-tris 4-12% gradient gel and visualized by fluorescence scanning. Heat-inactivation with 1% SDS prior to incubation (lane 3) and competition with D-biotin (lanes 4,5) were used as controls. Note: biotin and derivatives thereof (such as DtBio **2**) stabilize tetrameric Strp. (B) A mixture of streptavidin and OVA

was incubated with indicated amount of DtBio **2** after which the modified proteins were visualized as described above (resolved on a 15% Laemmli type SDS-PAGE gel). (C) Crop showing the labelling intensity of monomeric streptavidin incubated with DtBio **2** (10 μM) for the indicated time after which the modified proteins were visualized as described above. (D) Crops showing the labelling intensity of tetrameric and monomeric streptavidin incubated with DtBio **2** (10 μM) in the presence of the indicated amount of $CuSO_4$ after which the modified proteins were visualized with DBCO-TAMRA **7**. For uncropped images and coomassie brilliant blue (*CM*) staining of the gels see Figures S1-4.

The biochemical assays on purified protein revealed that streptavidin gets labelled at low concentrations of DtBio **2**, only when the probe can bind to its target. Binding of **2** should position the diazotransfer moiety in the proximity of the amino group of lysine K121 (Figure S6). Therefore, if labelling is dependent on binding, DtBio **2** should exclusively modify this residue, while leaving the N-terminal amine and lysines K80, K132 and K134, which are not in proximity of the reactive group, untouched. To validate if this is indeed the case, we identified the modification sites by digesting streptavidin that was priorly incubated with DtBio **2** with trypsin and analysing the tryptic peptides with nano LC-MS/MS. We searched the raw MS data for modified peptides, taking into account that converting the ε-amine of a lysine side chain into an azide obliterates the trypsin cleavage site at the carboxy terminal end of the residue. The only modified residue identified was lysine K121 (all other residues were identified only in their unmodified version, see SI excel file “Mass spectrometry”), which demonstrates that the probe (1) binds to streptavidin and (2) only reacts with nearby amino groups. These results indicate that in essence any biotin-binding protein should be amenable for labelling with DtBio **2** as long as it contains a suitably positioned lysine group or N-terminus. Avidin and its deglycosylated variant NeutrAvidin are structurally related to streptavidin and contain a lysine residue (K111) oriented in a similar position in the extended loop between strands seven and eight of the prominent β-barrel structure of the proteins.[20] Indeed these proteins are also modified by DtBio **2**. Even though labelling is less efficient, presumably due to the suboptimal linker length in the probe molecule, it demonstrates that the use of DtBio **2** is not limited to streptavidin (Figure S7).

Having established that DtBio **2** labels purified biotin-binding proteins site-selectively in an activity dependent manner, we extended its use to more biologically relevant settings. We first determined the selectivity by reacting *E. coli* cell lysates containing spiked in streptavidin with 10 μM of DtBio **2** (Figure 3A). Also under these conditions, specific labelling of streptavidin with minimal background is observed. Again labelling could be abolished by heat-inactivating or pre-incubating with a competitor. Extending on this finding we tested common hen egg white which contains 0.05% (weight/dry weight) native avidin. In order to probe the functionality of DtBio **2** in avidin's natural environment, we incubated a dilute solution of egg white with **2**. Indeed the probe labels avidin at a protein dilution of 1:1000 in HEPES buffer. Labelling could be abolished by heat-denaturing or pre-incubating with the competitor biotin (Figure S8). These experiments prompted us to explore if DtBio **2** could be employed on live cells to label BioY[29], the biotin specific S-component of the energy-coupling factor transporter protein complex ECF BioY, which transports biotin from the extracellular space into the cytosol of the Gram-positive bacterium *Lactococcus lactis.* Analysis of the crystal structure of BioY revealed the absence of primary amines in the proximity of the biotin-binding site. For the purpose of labelling this membrane protein on the cell surface, we introduced a lysine residue near the binding pocket by exchanging either asparagine 79, located in a flexible loop between beta strands three and four, or arginine 93, which is buried deeper inside the biotin binding pocket of the protein (N79K and R93K mutants, respectively). To assure that these mutations do not affect biotin binding, we determined the binding affinities of wild-type BioY and its mutants for biotin using isothermal titration calorimetry. Both mutants bind biotin with affinities similar to the affinity of wild-type BioY, and DtBio **2** binds to these mutants with ~5-fold and ~12-fold decreased affinities for R93K and N79K, respectively. We used *Lactococcus lactis* strains expressing these variants of BioY for the cell surface labelling experiments. After on-cell labelling and cell disruption, the lysate was analysed by fluorescence scanning of the SDS-PAGE gel. N79K-BioY showed concentration dependent labelling with DtBio **2** (Figure S9). We validated that the labelled protein corresponds to BioY using higher probe concentrations. As expected, the mutant could be visualized with BODIPY alkyne **3** after diazotransfer of DtBio using CuAAC click chemistry, while only background labelling was observed for wt-BioY and untreated samples. HisTag purification of labelled N79K-BioY and a subsequent western blot directed against the HisTag confirmed labelling of the target (Figure 3B)**.**

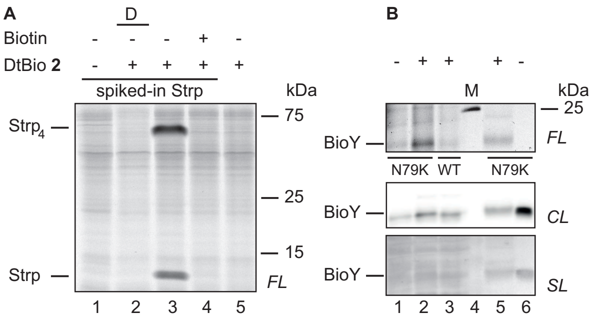


Figure 3. Labelling of streptavidin in *E.coli* lysates (A). A mixture of Strp (0.5 μg) and *E.coli* lysate (2.0 mg/mL) was incubated with DtBio **2** (10 μM). The modified proteins are visualized by clicking them to BODIPY-alkyne **3** subsequent to diazotransfer and resolving them on a 15% SDS-PAGE gel. (B) Cell surface labelling of N79K-BioY and wt-BioY. Cells expressing either form of BioY were treated with DtBio **2** (250 μM) in the presence of 1 mM $CuSO_4$ for 1h, after which the modified proteins were clicked to **3**. Labelling of BioY was visualized with fluorescence scanning (*FL*) of the lysate subsequent to cell disruption (top panel); detection of BioY subsequent to western blotting of the same gel using HisProbe-HRP and chemiluminescence (*CL*) as read-out (middle); and silver staining of the same gel post-transfer (lower panel) (lanes 1-3). To further confirm the labelling signal from N79K-BioY, the same sample as in lane 2 (B) was used for Ni-NTA bead purification of N79K-BioY and the eluate is compared to untreated purified N79K-BioY (lanes 5,6).

In conclusion, we here report the first ligand tethered-diazotransfer reagent and show that this reagent selectively modifies biotin-binding proteins in protein mixtures, cell lysates and on living cells. The reagents rapidly label the target proteins in an activity-dependent manner. 50 μM of copper (II) sulfate is sufficient to efficiently catalyse the diazotransfer reaction, but labelling even occurs in the absence of catalyst. Mass spectrometry analysis of tryptic digests of labelled streptavidin revealed that targeting the reagent not only enhances the protein-specificity, but also the site-selectivity. We showed that DtBio **2** can be used to label N79K BioY and further optimization of the probe may lead to cell surface labelling methods that can be used to study uptake of biotin by BioY. Finally, the reported method provides a starting point for the design of other targeted diazotransfer reagents that modify different proteins in complex samples and may find use in target identification strategies.

ASSOCIATED CONTENT

Supporting information

The supporting information is available free of charge on the ACS Publications website. Labelling of streptavidin resolved on a 15% SDS-PAGE gel; Coomassie brilliant blue stains of the gels in Figure 2; Uncropped images of the gels in Figure 2C and 2D; Docking pose of DtBio **2** bound to streptavidin; MS^2 spectrum of the modified peptide in streptavidin; Labelling of streptavidin, neutravidin and avidin at saturating and subsaturating probe concentrations; Labelling of avidin in egg white; Docking poses, binding affinities and labelling of BioY and BioY mutants and ITC data; Procedures for the synthesis of DtBio **2**, labelling of proteins with DtBio **2**, generation of BioY mutants, purification of BioY and BioY mutants, determination of binding affinities, cell surface labelling of BioY and MS analysis; ^{1}H-NMR and ^{13}C-NMR spectra; LC-MS data of DtBio **2**. Excel table “Mass spectrometry” including the MaxQuant search results for trypsin digested, diazotised streptavidin and spectrum for N-terminal peptide.

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Notes

The authors declare no competing interests

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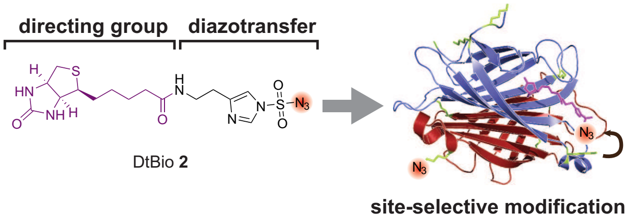
directing group
diazotransfer
HN
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N_3
DtBio 2
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N_3
site-selective modification

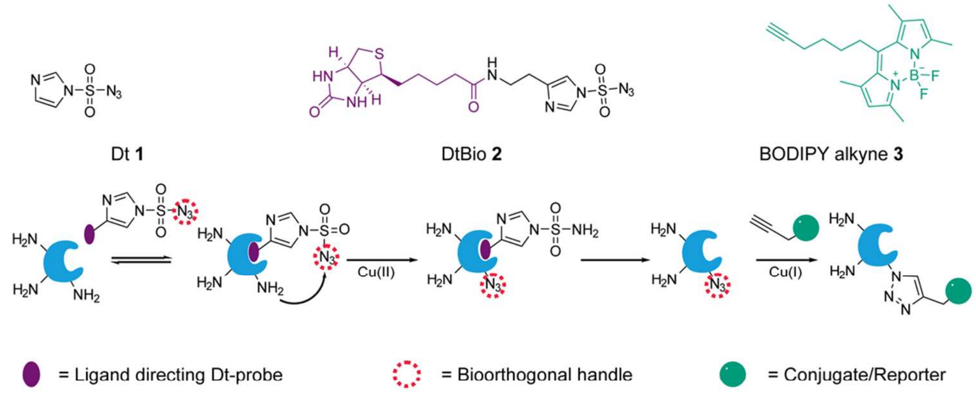

Figure 1. Top: structures of the non-targeted (1) and targeted (2) diazotransfer reagents and the BODIPY fluorophore (3) used as reporter group in the evaluation of the diazotransfer reaction. Bottom: schematic representation of the targeted diazotransfer reagent concept. Ligands bearing the diazotransfer group direct the reagent to the protein of interest. Upon binding an amino group of the protein is transformed into an azide in the presence of Cu(II). The modified protein can be further functionalized with bioorthogonal chemistry.

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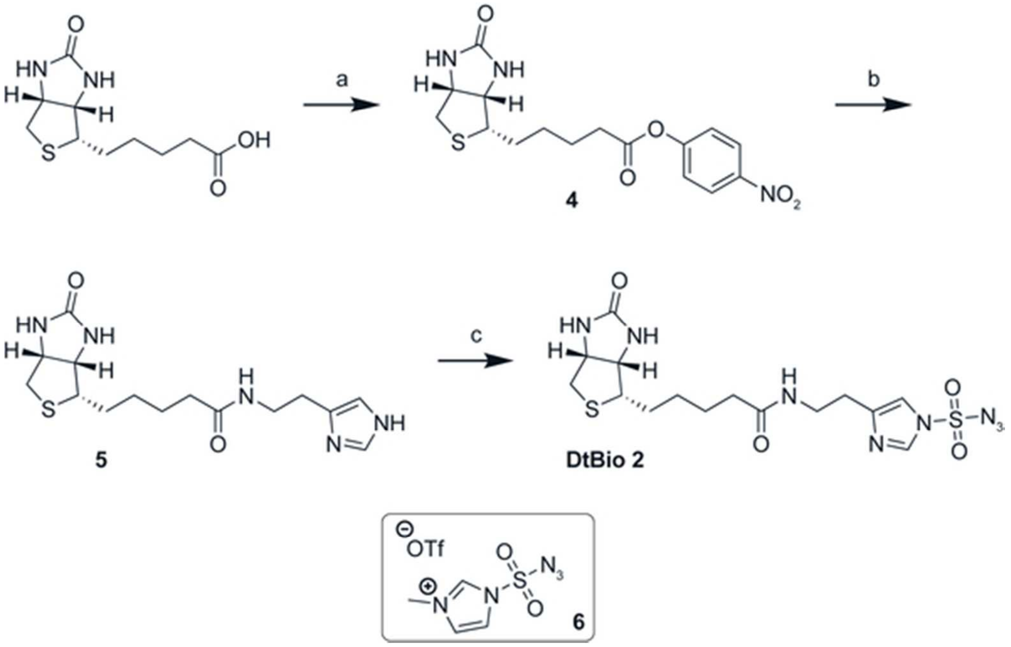

Scheme 1. Synthesis of diazotransfer reagent DtBio 2 (a) EDC·HCl, p-nitrophenol, DMF, 24 h, rT, 72%, (b) histamine, DMF, 16 h, rT, 83%, (c) sulfonyl azide transfer reagent 6, DMF, 2 h, 0 °C, 52%.

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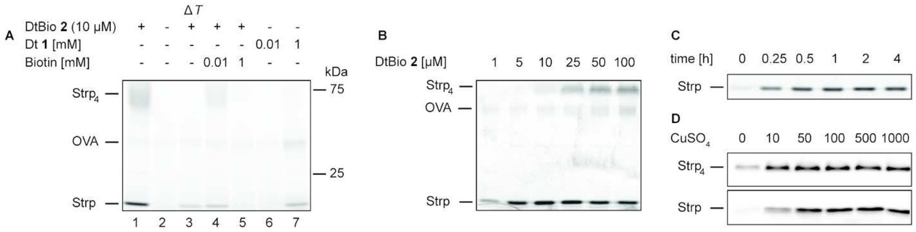

Figure 2. Biochemical evaluation of diazotransfer probe DtBio 2 with streptavidin (Strp) as target protein. (A) A mixture of Strp and OVA labelled with indicated amount DtBio 2 (lane 1) or Dt 1 (lane 6,7) and clicked to BODIPY-alkyne 3 subsequent to diazotransfer is resolved on a bis-tris 4-12% gradient gel and visualized by fluorescence scanning. Heat-inactivation with 1% SDS prior to incubation (lane 3) and competition with D-biotin (lanes 4,5) were used as controls. Note: biotin and derivatives thereof (such as DtBio 2) stabilize tetrameric Strp. (B) A mixture of streptavidin and OVA was incubated with indicated amount of DtBio 2 after which the modified proteins were visualized as described above (resolved on a 15% Laemmli type SDS-PAGE gel). (C) Crop showing the labelling intensity of monomeric streptavidin incubated with DtBio 2 (10 μM) for the indicated time after which the modified proteins were visualized as described above. (D) Crops showing the labelling intensity of tetrameric and monomeric streptavidin incubated with DtBio 2 (10 μM) in the presence of the indicated amount of $CuSO_4$ after which the modified proteins were visualized with DBCO-TAMRA 7. For uncropped images and coomassie brilliant blue (CM) staining of the gels see Figures S1-4.

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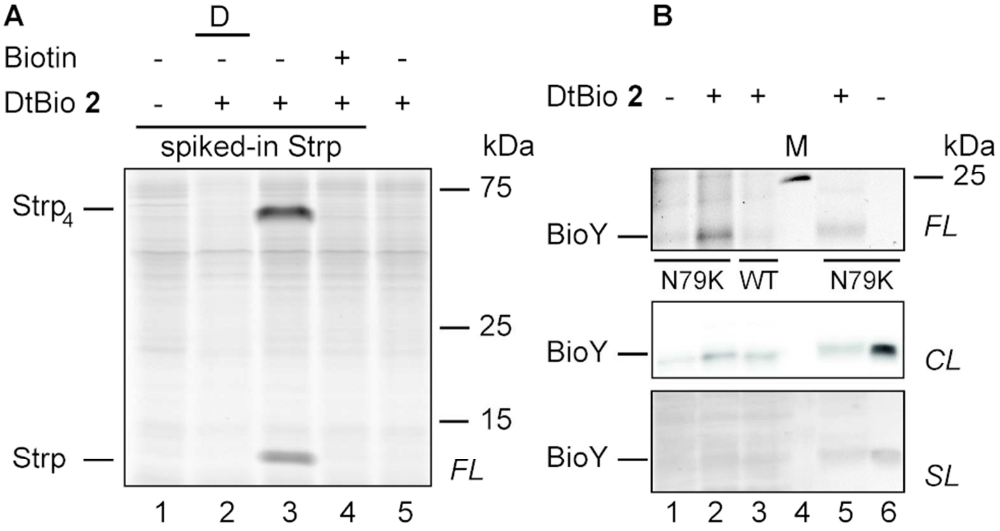

Figure 3. Labelling of streptavidin in E.coli lysates (A). A mixture of Strp (0.5 µg) and E.coli lysate (2.0 mg/mL) was incubated with DtBio 2 (10 µM). The modified proteins are visualized by clicking them to BODIPY-alkyne 3 subsequent to diazotransfer and resolving them on a 15% SDS-PAGE gel. (B) Cell surface labelling of N79K-BioY and wt-BioY. Cells expressing either form of BioY were treated with DtBio 2 (250 µM) in the presence of 1 mM CuSO4 for 1h, after which the modified proteins were clicked to 3. Labelling of BioY was visualized with fluorescence scanning (FL) of the lysate subsequent to cell disruption (top panel); detection of BioY subsequent to western blotting of the same gel using HisProbe-HRP and chemiluminescence (CL) as read-out (middle); and silver staining of the same gel post-transfer (lower panel) (lanes 1-3). To further confirm the labelling signal from N79K-BioY, the same sample as in lane 2 (B) was used for Ni-NTA bead purification of N79K-BioY and the eluate is compared to untreated purified N79K-BioY (lanes 5,6).

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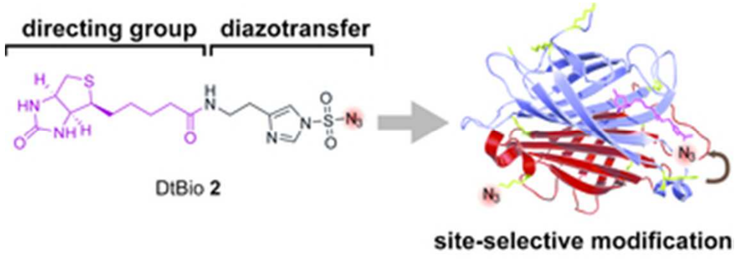

Table of contents: Biotin-tethered diazotransfer reagents enable site-selective modification of biotin-binding proteins

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