Targeted diazotransfer to proteins
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A proof-of-concept study for targeted diazotransfer probes: modifying biotin binding proteins.

This chapter has been adapted from the original publication:

2.1 INTRODUCTION

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-azole cycloaddition (CuAAC) and strain promoted alkyne-azole 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 labelling1,9,11 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 suppression13,14 and enzymatic modification of genetically engineered proteins15,16 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
Targeted Diazotransfer Probes

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 Targeted Diazotransfer 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.
2.2 RESULTS AND DISCUSSION

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 sulfonyl azide transfer reagents can be readily obtained by methylating derivatives of 1 with methyl triflate, yielding the salts as an easier-to-handle and more stable solid.\(^{25}\) Gratifyingly, addition of one equivalent of sulfonyl azide transfer reagent 6 to biotin-histamine 5 in DMF gave diazotransfer reagent 2.

![Scheme 1](image)

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 mM), ovalbumin (10 mM) and CuSO₄ (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. 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). The molecular weight of the respective bands match those of monomeric (13 kDa) and tetrameric (52 kDa) core streptavidin. 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 (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. Probe to protein ratios above one-to-one result in an 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 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 CuSO₄ concentration further leads to a reduction in the fluorescence intensity. Interestingly, DtBio 2 even reacts with streptavidin when CuSO₄ 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.\textsuperscript{17} Competition experiments and heat-inactivation confirm that this particular labelling of tetrameric streptavidin in the complete absence of copper is activity dependent (Figure 2E).

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 of gel image showing the labelling intensity of monomeric streptavidin incubated with DtBio 2 (10 mM) for the indicated time after which the modified proteins were visualized as described above. (D) Crop of gel image showing the labelling intensity of tetrameric and monomeric streptavidin incubated with DtBio 2 (10 mM) in the presence of the indicated amount of CuSO\textsubscript{4} after which the modified proteins were visualized with DBCO-TAMRA 7. (E) Copper independent labelling of streptavidin. A mixture of streptavidin (Strp) and ovalbumin (OVA) was incubated with indicated amount of DtBio 2. The modified proteins were clicked to DBCO-TAMRA 7 subsequent to diazotransfer and resolved on a 15% SDS-PAGE Laemmli type gel. panel).
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, according to a qualitative molecular docking study (Figure 3A). 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 protein 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, Figure 4), which demonstrates that the probe (1) binds to streptavidin and (2) only reacts with nearby amino groups.
Figure 3 **Diazotransfer between DtBio 2 and streptavidin:** (A) DtBio 2 binding streptavidin; image was generated with the biotin bound homo-dimer of Strp (PDB ID: 3RY2; lower subunit depicted as cartoon in firebrick, binding biotin as sticks in magenta, upper subunit as cartoon in slate binding 2 depicted as sticks in colours according to elemental composition, lysine residues and N-terminus.}

- **Signal peptide**
- **Core streptavidin**

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**Chapter 2**

64
highlighted in yellow) using the molecular design software Moloc (http://www.moloc.ch) and PyMOL (http://www.pymol.org). The zoom into the section where the diazotransfer moiety of 2 is brought into proximity of the primary amine of lysine K121 (K145 in full length streptavidin). (B) Primary sequence of streptavidin in the one letter code retrieved from the uniprot entry P22629. The sequence of commercially available core streptavidin is highlighted in slate. (C) MS² spectrum with a top score (169, calculated by MaxQuant/Andromeda) of the typtic Strp peptide including the identified lysine modification at K121 (highlighted by a star). Spectrum was generated with MaxQuant viewer.²⁸ Red and blue bars above and below the sequence indicate identified fragment ions.

Colors indicate five identified peptides - full sequence coverage of core Streptavidin

Figure 4 The LC-MS/MS analysis identified the entire sequence length of core streptavidin. (A). While peptides 2 – 3 were identified on MS2 level, peptide 1 was identified on MS1 level only. (B) Shows the spectrum of the triply charged peptide 1. Due to low abundance it was not picked for MS2 analysis.
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. 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 5).

![Figure 5](Images showing labelling of Streptavidin, NeutrAvidin and Avidin (1 μg each) in the presence of no-saturating (1 μM, A) and supersaturating (10 μM, B) DtBio 2 concentrations. At saturating concentrations, the tetramer is stabilized by binding of the probe, as has also been observed for biotin. Up to 10 min of boiling of the protein in SDS containing sample buffer is not sufficient to denature all protein when using saturating conditions. Top panel: fluorescence scan (FL) of the SDS-PAGE gel. Bottom panel: silver staining (SL) of the same gel.)
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 6A). 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 6B).

Figure 6 **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) Image showing labelling of Avidin from diluted (1:1000 in 50 mM HEPES buffer pH 7.5) chicken egg white modified with indicated amount of DtBio 2 and BODIPY alkyne 3 as visualisation agent. ΔT indicates denaturing of protein sample prior to addition of 2. Lane 5 shows pre labelled commercial avidin with 1 μM of 2. Top panel: fluorescence scan (*FL*) of the SDS-PAGE gel. Bottom panel: either coomassie staining (CM), or silver staining (*SL*) of the same gel.
These experiments prompted us to explore if DtBio 2 could be employed on live cells to label BioY\textsuperscript{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 \textit{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 \textit{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 7). 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 7D).
Figure 7 **Labelling of BioY and BioY mutants with DtBio 2.** (A) Biotin binding to wt-BioY (PDB ID: 4DVE, cartoon depiction in firebrick, biotin as spheres in magenta, lysine residues highlighted as sticks in arginine, R93

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Figure 7 Labelling of BioY and BioY mutants with DtBio 2. (A) Biotin binding to wt-BioY (PDB ID: 4DVE, cartoon depiction in firebrick, biotin as spheres in magenta, lysine residues highlighted as sticks in arginine, R93.
lime) (B) BioY depicted as cartoon in firebrick with arginine 93 depicted as sticks in lime. (C) The mutant N79K-BioY as binding pose of DtBio 2 (protein as cartoon in firebrick and probe as spheres in magenta, the mutated residue as sticks in limon) prepared with the molecular design software Moloc (http://www.moloc.ch) and PyMOL (http://www.pymol.org) (D) 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). (E) Binding affinities of the different mutants for biotin and DtBio 2 determined with ITC. This measurement was performed once, the error represents the standard deviation from three measurements, the error represents the standard deviation from two measurements.

2.3 CONCLUSION

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 mM 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.
2.4 EXPERIMENTAL
2.4.1 CHEMISTRY
2.4.1.1 GENERAL PROCEDURES

All solvents used for reaction, extraction, filtration and chromatography were of commercial grade, and used without further purification. Reagents were purchased from Sigma-Aldrich, TCI, or fluorochem, unless otherwise noted, and were used without further purification. Non-targeted diazotransfer reagent 1H-imidazole-1-sulfonyl azide hydrochloride $1^{2,30}$ and BODIPY-alkyne $3^{31}$ were synthesized according to a published procedure. TLC was performed on Merck silica gel 60 F254, 0.25 mm plates and visualization was done by UV light, iodine ($I_2$ crystals in silica) and ninhydrin staining (solution of ninhydrin (0.3 g) in $n$-butanol (100 mL) and acetic acid (3 mL)). Manual flash column chromatography was performed using silica (SilicaFlash P60, 230-400 mesh, Silicycle) as the stationary phase. $^1$H-, $^{13}$C-, APT-NMR were recorded on a Varian AMX400 spectrometer (400 and 100 MHz, respectively) using CDCl$_3$, CD$_3$OD or DMSO-$d_6$ as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard (CD$_3$OD: δ3.31 for $^1$H, δ 49.15 for $^{13}$C; DMSO-$d_6$: δ3.33 for $^1$H δ 39.52 for $^{13}$C). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, t = triplet, q = quartet, p = quintet, m = multiplet, apparent quartet = app q, apparent quintet = app p), coupling constants $J$ (Hz), and integration. LCMS was performed on an LCQ Fleet mass spectrometer coupled to a Vanquish UHPLC system. High resolution mass measurements were performed using a ThermoScientific LTQ OrbitrapXL spectrometer. Infrared (IR) data were recorded on a Perkin Elmer UATR spectrum two FT-IR Spectrometer. Absorbance frequencies are reported in reciprocal centimeters (cm$^{-1}$). Mass accuracy is reported in delta Thomson (Th (m/z)) and parts per million of deviation of the calculated mass. WARNING: Diazotransfer reagents may be shock sensitive and should be handled using appropriate precautions.

2.4.1.2 SYNTHESIS

**Biotin p-nitrophenol Ester (4)**

$\delta$-Biotin (2.44 g, 10.0 mmol) was suspended in anhydrous DMF (50 mL) and $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride (EDC-HCl, 2.30 g, 12.0 mmol) was added. The mixture was stirred for one hour at room temperature. Then $p$-nitrophenol (1.53 g, 11.0 mmol) was added and the reaction was left at room temperature under stirring until $\delta$-biotin was consumed according to LCMS (24 h). The reaction volume was reduced to about one-tenth concentrating in vacuo. Then 1 M HCl (100 ml) was added to the concentrate under
continuous stirring. The resulting white precipitate was filtered off and successively washed with 1 M HCl (50 mL), water (50 mL) and ether (50 mL). The resulting white solid was dried under high vacuum, then suspended in ethanol and heated to a boil. After all the material had dissolved the solution was cooled to room temperature and further in an ice-water bath. The crystals that separated were collected and dried under high vacuum to yield 3 (2.63 g, 72%).

\[ \text{1H NMR (400 MHz, Methanol-}d_4\text{)} \delta_H = 8.28 \text{ (m, 2H), 7.33 \text{ (m, 2H), 4.50 (ddd, } J=8.0, 5.0, 1.0, 1H), 4.32 \text{ (dd, } J=7.9, 4.5, 1H), 3.22 \text{ (ddd, } J=9.1, 5.6, 4.5 \text{ Hz, 1H), 2.93 (dd, } J=12.8, 5.0, 1H), 2.73 \text{ (d, } J=12.7, 0.8, 1H), 2.66 \text{ (t, } J=7.3, 7.3, 2H), 1.79 \text{ (m, 3H), 1.67 (m, 1H), 1.57 (m, 2H) }\]

\[ \text{13C NMR (101 MHz, DMSO-}d_6\text{)} \delta_C = 170.8, 162.3, 155.0, 150.8, 144.6, 124.9, 122.8, 60.6, 58.8, 54.9, 39.5, 32.9, 27.6, 27.5, 23.8 \]

\[ \text{HRMS (ESI-orbitrap) } m/z \text{ calculated for [M+H]^+ 366.1113, found 366.1118 (delta mTh 0.4; ppm 1.2) }\]

**Biotin histamine (5)**

p-Nitrophenyl ester 4 (1.27 g, 3.5 mmol) was dissolved in anhydrous DMF (35 mL) and histamine (0.35 g, 3.18 mmol) was added. The reaction was left at room temperature under stirring until histamine was consumed according to TLC (overnight). The resulting precipitate was filtered off, washed with ether and dried under high vacuum. The mother liquor was reduced to about one-tenth of its original volume and ether was stirred into the concentrate. The resulting precipitate was filtered off, washed with ether and dried under high vacuum. The combined yellow solid was recrystallized from ethanol. The resulting pale yellow crystals were collected and dried under high vacuum to yield 5 (0.89 g, 83%).

\[ \text{1H NMR (400 MHz, Methanol-}d_4\text{)} \delta_H = 7.60 \text{ (s, 1H), 6.83 \text{ (s, 1H), 4.46 (ddd, } J=8.0, 5.0, 1.0, 1H), 4.26 \text{ (dd, } J=7.9, 4.5, 1H), 3.39 \text{ (t, } J=7.1, 7.1, 2H), 3.15 \text{ (ddd, } J=8.9, 5.8, 4.4, 1H), 2.89 \text{ (dd, } J=12.7, 5.0, 1H), 2.74 \text{ (t, } J=7.4, 2H), 2.67 \text{ (d, } J=12.7, 1H), 2.14 \text{ (t, } J=7.4, 7.4, 2H), 1.60 \text{ (m, 4H), 1.37 (q, } J=7.7, 7.4, 7.4, 2H) }\]

\[ \text{13C NMR (101 MHz, Methanol-}d_4\text{)} \delta_C = 176.1, 166.1, 135.7, 127.1, 116.5, 63.4, 61.6, 57.0, 41.0, 40.2, 36.7, 29.7, 29.5, 27.7, 26.9 \]

\[ \text{HRMS (ESI-orbitrap) } m/z \text{ calculated for [M+H]^+ 338.1645, found 338.1644 (delta mTh 0.1; ppm 0.3) }\]
DtBio (2)
A solution of biotin-histamine 5 (51 mg, 0.15 mmol) in anhydrous DMF (1 mL) was slowly added to a stirred solution of 6 (51 mg, 0.15 mmol) in anhydrous DMF (2 mL) at 0 °C. The reaction was stirred for 4 hours and subsequently adsorbed onto celite. The product was purified by flash column chromatography using silica as stationary phase and a mobile phase of 10% methanol in DCM. The isolated white solid was recrystallized from ethyl acetate to yield DtBio 2 (34 mg, 52%). **WARNING: Diazotransfer reagents may be shock and heat sensitive. Reactions should only be carried out on small scale and the compounds should be handled using appropriate precautions.**

$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta_H = 8.26$ (s, 1H), 7.49 (s, 1H), 4.49 (dd, $J=7.9$, 4.9, 1H), 4.30 (dd, $J=8.0$, 4.5, 1H), 3.49 (app q, $J=6.5$, 2H), 3.20 (dt, $J=9.6$, 5.1, 1H), 2.93 (dd, $J=12.7$, 4.9, 1H), 2.79 (t, $J=6.8$, 2H), 2.71 (d, $J=12.7$, 1H), 2.18 (t, $J=7.3$ 2H), 1.66 (m, 4H), 1.42 (p, $J=7.4$, 2H)

$^{13}$C NMR (101 MHz, Methanol-$d_4$) $\delta_C = 176.1$, 143.9, 123.3, 120.2, 116.0, 63.4, 61.6, 57.0, 41.0, 39.4, 36.8, 29.7, 29.5, 28.8, 26.9

HRMS (ESI-orbitrap) $m/z$ calculated for [M+H]$^+$ 443.1278, found 443.1271 (delta mTh 0.7, ppm 1.6)

IR 3294, 2148, 1676, 1420, 1184

1-(azidosulfonyl)-3-methyl-1H-imidazol-3-ium trifluoromethanesulfonate (Sulfonyl Azide Transfer Reagent 6)
Synthesis according to published procedure:25 Methyl triflate (120 μL, 1.06 mmol) was added drop-wise to the stirred free base of sulfuryl imidazolium salt 1 (209 mg, 1 mmol) in diethyl ether (20 mL) at 0 °C. After 4 hours the reaction was complete according to TLC. The white crystalline precipitate was vacuum filtered and washed with dry diethyl ether to yield (253 mg, 75%). **WARNING: The stability of this sulfonyl azide transfer reagent has not been determined and it may be shock and heat sensitive. Reactions should only be carried out on small scale and the compounds should be handled using appropriate precautions. Mother liquors should not be concentrated.**

$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta_H = 9.96$ (1 H, s), 8.27 (1 H, s), 7.87 (1 H, s), 4.05 (3 H, s)

$^{13}$C NMR (101 MHz, Methanol-$d_4$) $\delta_C = 140.7$, 127.0, 121.9, 37.7
2.4.2 BIOCHEMISTRY

2.4.2.1 GENERAL PROCEDURES

Proteins
Recombinant expressed Streptavidin (Strp), Avidin and Neutravidin were purchased from ThermoFisher Scientific. Chicken egg ovalbumin (OVA) was purchased from Sigma.

SDS-PAGE and western blot analysis
Laemmli type SDS-PAGE was performed according to standard literature procedures. Gels were prepared using acrylamide-bis ready-to-use solution 40% (37.5:1) (Merck Millipore) and separated on a Mini-PROTEAN Tetra cell (Bio-Rad). Alternatively, proteins were separated on a NuPAGE Novex 4-12% bis-tris protein gel (Invitrogen) using an X Cell SureLock Mini-Cell system using MOPS buffer (ThermoFisher Scientific) where indicated. Fluorescence scanning of SDS-PAGE gels was performed on a typhoon gel and blot imager 9400, or trio+, or 9500 FLA model (GE Healthcare) using the CY2 settings for BODIPY-alkyne 3 (blue laser excitation at 488 nm and emission filter at 520 nm) and the CY3 settings for DBCO-TAMRA 7 (green laser excitation at 532 nm and emission filter at 580 nm). Coomassie staining was carried out with Coomassie Brilliant Blue (CBB) R250 staining (AMRESCO) according to literature procedures or with colloidal CBB G250 staining according to the manufacturers protocol (Roti-Blue, Carl Roth). Silver staining was carried out using standard protocols with a 0.1% silver nitrate aqueous solution and 0.04% formaldehyde in a 2% sodium carbonate aqueous solution as developing agent.

For western blot analysis, proteins were transferred to a PVDF membrane for visualization via ECL subsequent to separation by gel electrophoresis. To this end the proteins were blotted onto a PVDF membrane (GE Healthcare) using a Bio-Rad (Hercules) Mini Trans-Blot system for wet blotting according to the manufacturer’s protocol using Tobin buffer without methanol. Electroblotting was followed by visualisation with SuperSignal West HisProbe Kit according to the manufacture’s protocol (ThermoFisher Scientific). BioRad precision plus protein standards dual color was used as molecular weight marker.

Probes and bio-reagents
Probes were stored at -20 °C as solids. Stock solutions were prepared at 100 mM in anhydrous DMSO, stored at -20 °C and found to be stable (only little hydrolysis was observed according to LC-MS) under these conditions over the course of more than a year. Aliquots from the stock solutions were taken to prepare solutions with the appropriate concentrations according to the experimental set-up in anhydrous DMSO. To increase the shelf life of the
probes exposure to water should be avoided and storage at -20 °C of the stock solutions is advisable. Stock solutions of CuSO₄ and THPTA were prepared in water and stored at rT. The solutions were used over the course of one month and then prepared freshly. Solutions of sodium ascorbate in water were always prepared fresh from the salt.

2.4.2.2 BIOCHEMICAL EVALUATION OF DTBIO

*Biochemical activity of DtBio* 2 was tested on Streptavidin (10 μM, 2.65 μL of a 1 mg/mL stock solution in 10 mM PBS, pH 7.4). The protein was incubated for 1 h at rT with DtBio 2 (10 or 1 μM, 0.5 μL of a 0.4 or 0.04 mM solution) in the presence of Cu(II) (1 mM, 1 μL of a 20 mM solution of CuSO₄) and 5 μg ovalbumin (OVA) in PBS (10 mM, pH 7.4, total volume: 20 μL). For thermal stability Streptavidin, Avidin and Neutravidin 1.0 μL of a 1 mg/mL stock solution in 10 mM PBS, pH 7.4 were used under the same conditions. Conventional egg white was diluted 1000X in HEPES 50 mM pH 7.5 and tested as above. *Reactions should be mixed well after addition of the probe because the DMSO settles on the reaction vessel bottom. This ensures even distribution of the probe in the reaction mixture. A gentle way to accomplish this is by stirring the solution several times with a pipette tip.* Subsequently, BODIPY-alkyne 3 (25 μM, 1 μL of a 0.575 mM solution), THPTA (1 mM, 1 μL of a 23 mM solution) and sodium ascorbate (2 mM, 1 μL of a 46 mM solution) were added. After thorough mixing the reaction was allowed to stand for 2 h at rT in the dark. The reactions were quenched by adding 5 μl of 5X sample buffer³² and denaturing for 5 min at 95 °C.

*The non-targeted diazotransfer experiments* were conducted with reagent Dt 1 identical to those with the targeted probe (10 μM or 1 mM, 0.5 μL of a 0.4 mM or a 40 mM solution, respectively).

*For the competition experiments,* d-biotin was added to the protein solution (10 μM or 1 mM, 0.5 μL of a 0.4 mM or 40 mM solution, respectively) and incubated for 30 min at rT prior to addition of the probe. For heat denaturing experiments 1% SDS was added to the protein solution (1 μL of a 20% solution in water w/v). Then the sample was heated for 10 min at 95 °C. Once the reaction mixture had cooled to room temperature the probe and CuSO₄ were added.

*For the probe concentration dependent experiments,* the protein solution was incubated with increasing concentration of probe (0.5 μL of a 40X stock) for 1h and subsequently clicked to the BODIPY-alkyne 3 as described above.

*For the time course experiments,* the protein solution was pre-incubated with the appropriate probe for 15 minutes, 30 minutes, 1 hour, 2 hours or 4 hours before being
reacted with BODIPY-alkyne 3 as described above. For the 0 h time point, the probe was added to the protein solution and immediately BODIPY-alkyne 3 (25 μM, 1 μL of a 0.575 mM solution), THPTA (1mM, 1 μL of a 23 mM solution) and sodium ascorbate (2 mM, 1 μL of a 46 mM solution) were added.

For copper concentration dependent experiments, the protein solution was incubated for 1 h at rT with DtBio 2 (10 μM, 0.5 μL of a 0.4 mM solution) in the presence of a decreasing amount of CuSO₄ (1 μL of a 20X stock). Before the click reaction, EDTA (5 mM final concentration) and glycine (5 mM final concentration) were added then DBCO-TAMRA 7 (25 μM, 1 μL of a 0.575 mM solution) was added. After thorough mixing the reaction was allowed to stand for 2 h at rT in the dark.

For copper free labelling with DBCO-TAMRA, the protein solution was incubated for 1 h or 16 h at rT with the probe (10 μM, 0.5 μL of a 0.4 mM solution) and subsequently incubated with DBCO-TAMRA 7 (25 μM, 1 μL of a 0.575 mM solution). Non-targeted diazotransfer reactions were performed by incubating the protein mixture with 1 (10 μM or 1 mM, 0.5 μL of a 0.4 mM or a 40 mM solution, respectively) in the absence of copper; competition experiments as well as time-dependent labelling experiments were performed as described for the copper catalysed reactions.

For labelling of streptavidin in spiked-in lysates, 25 μg of E.coli lysate were spiked with 0.5 μg of Strp. This mixture was incubated for 1 h at rT with DtBio 2 (10 μM, 0.5 μL of a 0.4 mM solution) in the presence of Cu(II) (50 μM, 1 μL of a 1 mM solution of CuSO₄) in Tris (10 mM, pH 7.6, total volume: 20 μL). Competition (10X compared to probe concentration), denaturing and non-targeted (1X compared to probe concentration) experiments were conducted as described above. Subsequently, BODIPY-alkyne 3 (25 μM, 1 μL of a 0.575 mM solution), THPTA (100 μM, 1 μL of a 2.3 mM solution) and sodium ascorbate (1 mM, 1 μL of a 23 mM solution) were added. After thorough mixing the reaction was allowed to stand for 1 h at rT in the dark.

2.4.2.3 TESTING OF BIOY

Mutagenesis of BioY

For the expression of wild-type BioY, the original pNZnHis₈-BioY was used. For mutagenesis, the gene encoding BioY was placed in the pREnHis plasmid equipped with an N-terminal His₈-tag. Mutations were introduced by quick change PCR using the primers given in Table 1. After verification of the DNA sequence (GATC, Germany), the mutated pREnHis plasmids were converted into mutated pNZnHis plasmids using the vector backbone exchange protocol, in order to obtain plasmids that could be used as expression vectors.
in *L. lactis*.

**Table 1. Primers for mutagenesis of wild-type BioY.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>N79K</td>
<td>cctgttcgacaggagaagtggctcgctagtttc</td>
<td>gaaactagccgacacccctccctgtcagaacagg</td>
</tr>
<tr>
<td>R93K</td>
<td>ggccttcaggttaataagatagcctgctttaac</td>
<td>gttaagagccaggctactttatatccacctgaagccc</td>
</tr>
</tbody>
</table>

**Expression of wild-type BioY and the mutants**

The expression of wild-type BioY and the N79K- and R93K-mutants was performed as described previously with some modifications.29,35 Briefly, *L. lactis* NZ9000 cells36 carrying the pNZnHis-BioY or mutated plasmids were grown semi-anaerobically in chemically defined medium35 without biotin and supplemented with 2.0 % (w/v) of glucose and 5 μg/mL of chloramphenicol in a 3 L bioreactor at 30°C and pH 6.5. When an OD$_{600}$ of roughly 1.5 was reached, expression of wild-type BioY or its mutants was induced by addition of 0.1 % (v/v) of culture supernatant from a nisin-A producing strain. Protein expression was allowed for three hours, after which the cells were harvested by centrifugation (15 min, 7,446 x g, 4°C). After one round of washing the cells with Buffer A (50 mM KPi, pH 8.0), the cells were resuspended in the same buffer, frozen in liquid nitrogen and stored at -80°C.

**Preparation of membrane vesicles and protein purification**

In order to purify wild-type BioY and its mutants for affinity determinations by ITC, membrane vesicles were prepared from the cells that were stored at -80°C. After these cells had thawed, 5 mM of MgSO$_4$ and a spatula tip of DNAse were added and the cells were lysed by high-pressure disruption (Constant Cell Disruption Systems Ltd, UK; twofold passage at 269 MPa and 5°C). Subsequently, 1 mM of PMSF was added and the cell debris was separated from the membrane vesicle by low-speed centrifugation (30 min, 27,167 x g, 4°C). Next, the membrane vesicles were harvested by high-speed centrifugation (120 min, 185,677 x g, 4°C) and resuspended in Buffer A to a final volume of 5 mL per 1 L of cell culture. Then the membrane vesicles were divided in aliquots of 5 mL, frozen in liquid nitrogen and stored at -80°C.

*For protein purification*, the membrane vesicle were thawed and diluted in Buffer B (50 mM KPi, pH 7.5, 300 mM NaCl, 10 % (v/v) glycerol, 1 % (v/v) maltose-neopentyl glycol 3 (MNG-3)). After solubilization of the membrane vesicles for 60 min at 4°C while gently rocking, the unsolubilized material was removed by centrifugation (20 min, 442,906 x g, 4°C) and the supernatant was incubated for 60 min at 4°C while gently rocking with Ni$^{2+}$-sepharose resin (column volume (CV) = 0.5 mL), which had been equilibrated with Buffer C.
Chapter 2

(50 mM KPi, pH 7.5, 300 mM NaCl, 10 % (v/v) glycerol, 50 mM imidazole, 0.03 % (v/v) MNG-3). Subsequently, the column material was washed with 30 CVs of Buffer C, after which the protein eluted from the column material in three fractions of 500, 750 and 500 μL of Buffer D (50 mM KPi, pH 7.5, 300 mM NaCl, 10 % (v/v) glycerol, 500 mM imidazole, 0.03 % (v/v) MNG-3), respectively. To the second elution fraction of 700 μL, 1 mM of EDTA was added and the fraction was purified by size-exclusion chromatography using a Superdexx 200 10/300 gel filtration column (GE Healthcare), which had been equilibrated with buffer E (50 mM KPi, pH 7.5, 150 mM NaCl, 0.03 % (v/v) MNG-3). After size-exclusion chromatography, the elution fraction containing wild-type BioY or its mutants were combined and used for affinity determination by ITC.

Affinity determination by ITC

The binding affinities of wild-type BioY or its mutants for biotin and DtBio 2 were determined by isothermal titration calorimetry (ITC) using a MicroCal iTC200 apparatus (GE Healthcare) with a cell volume of 200 μL. The measurements were performed at 25°C with a protein concentration of 8.37 - 21.4 mM in the sample cell. The ligands were added in steps of 1 μL. Analysis of the data was done using the MicroCal LLC iTC200 software.

Cell surface labelling of BioY.

Lactococcus lactis cells obtained from the fermenter cultivation recombinantly expressing BioY were pelleted (6000 rcf, 15 min, 4 °C) and resuspended to a final OD600 of 50 in 1 mL total volume of KPi buffer (50 mM, pH 8.0). The cell suspension was incubated for 1 h at rT with DtBio 2 (10, 100, 250 μM: 10 μL of a 1, 10, 25 mM stock solution) in the presence of Cu(II) (1 mM; 10 μL of a 100 mM solution of CuSO₄) on an orbital shaker. Then the cells were pelleted (5000 rcf, 1 min, rT), the supernatant was removed and the cells were resuspended in 1 mL total volume of KPi buffer (50 mM, pH 8.0) and washed once. Subsequently, BODIPY-alkyne 3 (50 μM, 5 μL of a 10 mM solution), Cu(II) (1 mM, 10 μL of a 100 mM solution), THPTA (1 mM, 10 μL of a 100 mM solution) and sodium ascorbate (2 mM, 20 μL of a 100 mM solution) were added to the suspension which it was left for 2 h at rT on an orbital shaker. Then the cells were pelleted (5000 rcf, 1 min, rT), the supernatant was removed and the cells were resuspended in 1 mL total volume of KPi buffer (50 mM, pH 8.0) and washed once. Subsequently, BODIPY-alkyne 3 (50 μM, 5 μL of a 10 mM solution), Cu(II) (1 mM, 10 μL of a 100 mM solution), THPTA (1 mM, 10 μL of a 100 mM solution) and sodium ascorbate (2 mM, 20 μL of a 100 mM solution) were added to the suspension which it was left for 2 h at rT on an orbital shaker. Then the cells were pelleted (5000 rcf, 1 min, rT), the supernatant was removed and the cells were resuspended in 1 mL total volume of KPi buffer (50 mM, pH 8.0). Glass beads (250 to 300 mg, Ø 0.1 mm; Sigma) were added and the cells were ruptured using a tissue lyser (5 min at 50 Hz under cooling, Qiagen, Venlo, Netherlands). The glass beads and cellular debris were removed by centrifugation (12,000 rcf, 5 min, 4 °C) and PMSF (1 mM final concentration) was added to the supernatant. The supernatant was isolated and stored at -80 °C. For subsequent SDS-PAGE fluorescence gel analysis an aliquot of 200
μL of the supernatant was used. The protein was precipitated from the cell lysate using the method described by Wessel and Fluegge\textsuperscript{37} and subsequently redissolved in 20 μL of sample buffer.

**Purification of wild-type BioY and N79K-BioY after cell surface labelling**

For purification of the modified N79K-BioY 800 μL of labelled lysate was taken, diluted in Buffer B and solubilization was allowed for 60 min at 4°C while gently rocking. Subsequently, the unsolubilized material was removed by centrifugation and the proteins were purified by Ni\textsuperscript{2+}-sepharose affinity chromatography as described above, now using Ni\textsuperscript{2+}-sepharose resin with a column volume of only 0.25 mL and eluting the proteins in three fractions of 250 μL of Buffer D.

2.4.3 MASS SPECTROMETRY

**Sample preparation for mass spectrometry analysis**

For the diazotransfer reaction, streptavidin (50 μM; 33.2 μL of a 1 mg/mL stock solution in 10 mM PBS, pH 7.4 was incubated for 1 h at rT with DtBio 2 (50 μM, 1 μL of a 2.5 stock solution) in the presence of Cu(II) (1 mM, 1 μL of a 50 mM solution of CuSO\textsubscript{4}) in a total volume of 50 μL. The reaction was quenched by adding 15 μL of LDS sample buffer (4X, 50 mM DTT) and incubating the solution at room temperature for one hour. Subsequently, the samples were split in half and the resulting samples (2X30 μL) were loaded onto a 4−12% Bis-Tris NuPAGE gel. The samples were run about 1 cm into the gels, after which they were stained with colloidal Coomassie blue staining solution, and the bands were excised and subjected to in-gel tryptic digestion (Promega) according to standard procedures\textsuperscript{38}, but omitting an alkylation step since streptavidin does not contain any cysteine residues. The tryptic peptides were dried in a vacuum concentrator (speed-vac) and stored at -20 °C prior to direct nLC-MS/MS analysis.

**LC-MS/MS analysis**

Nanoflow liquid chromatography electrospray ionisation tandem mass spectrometry (nLC-MS/MS) was performed with an Eksigent nanoLC-Ultra 1D+ system (Eksigent) coupled to an Orbitrap Velos instrument (Thermo Scientific). The peptides were delivered to a trap column (100 μm X 2 cm, packed in-house with Reprosil-Pur C18-AQ 5 μm resin, Dr. Maisch) at a flow rate of 5 μL/min in 100% solvent A (0.1% formic acid, FA, in HPLC grade water). After 10 min of loading and washing, peptides were transferred to an analytical column (75 μm X 40 cm, packed in-house with Reprosil-Gold C18, 3 μm resin, Dr. Maisch) and separated
at a flow rate of 300 nL/min using a 60 min gradient ranging from 2% to 32% solvent C in B (solvent B: 0.1% FA and 5% DMSO in HPLC grade water, solvent C: 0.1% FA and 5% DMSO in acetonitrile). The eluent was sprayed via stainless steel emitters (Thermo) at a spray voltage of 2.2 kV and a heated capillary temperature of 275 °C. The Orbitrap Velos mass spectrometer was operated in positive ion mode and programmed to acquire in data-dependent mode, automatically switching between MS and MS/MS. Full scan MS spectra (m/z 360−1300) were acquired in the Orbitrap at a resolution of 30 000 (m/z 400) using an automatic gain control (AGC) target value of 1e6 charges. Ions for MS/MS spectra of up to 10 precursor ions were generated in the multipole collision cell by using higher energy collision-induced dissociation (HCD, AGC target value 4e4, normalized collision energy of 30%) and analysed in the Orbitrap at a resolution of 7 500. Precursor ion isolation width was set to 2.0 Th, the maximum injection time for MS/MS was 100 ms, the precursor ion count for triggering an MS/MS event was set at 500 and dynamic exclusion was set to 20 s. Internal calibration was enabled for MS mode using the ion signal of a dimethyl sulfoxide cluster (m/z 401.922720) as a lock mass.

Data analysis was performed using MaxQuant v1.5.3.30 with the integrated search engine Andromeda.\textsuperscript{39,40} For peptide and protein identification, raw files were searched against the fasta file generated from the uniprot entry P22629 with oxidation of methionine and Lys_N3 and N-term_N3 for diazotransfer as variable modifications.

Default search parameters were used and trypsin/P was selected as the proteolytic enzyme, with up to 3 missed cleavage sites allowed. Precursor ion tolerance was set to 20 ppm for the first search and a tolerance of 4.5 ppm was allowed for the main search. The fragment ion tolerance was set to 0.5 Th. Peptide identifications required a minimal length of seven amino acids, and all data sets were adjusted to 1% PSM and 1% protein FDR.