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A modular approach for the construction of protein targeting probes: a methodology to synthesise probes based on imine chemistry.

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5.1 INTRODUCTION

Chemical small molecule probes that label a protein of interest (POIs) in biologically relevant settings enable the functional study of this protein in situ. Such probe molecules often consist of a reactive group (i.e. a reactor) tethered to a known binder (i.e. a selector, e.g. a ligand or an inhibitor) of the POI to generate selectivity within the complexity of the experimental setup.\(^1\)–\(^5\) A variety of reactive groups has been used in ligand-directed probes to covalently modify amino acid residues within a protein target.\(^6\)–\(^8\) Acyl imidazoles, activated esters and diazotransfer reagents react upon protein target engagement exclusively with the primary amino functionality of a lysine residue or the N-terminus that are located in the proximity of the probe’s reactor.\(^9\)–\(^12\) Several alternative amino acid side chains can be modified in a chemo-selective way with specific reagents: most prominently, cysteine residues have been targeted with Michael acceptors and alkylating agents.\(^13\),\(^14\) More recently, other reagents with unprecedented chemo- and even site-selectivity have been reported to target alternative, less abundant amino acid side chains; for instance Mannich type reactions for tyrosine,\(^15\) the modification of tryptophan residues with organo-radicals like keto-ABNO,\(^16\) oxaziridine-based reagents for the selective targeting of methionine,\(^17\) or selective targeting of the C-terminus by decarboxylation is feasible in the context of photoredox catalysis.\(^18\) These original targeting strategies hold potential and therefore are likely to be implemented for probe labelling strategies in the future. Finally, photocrosslinkers modify any suitably positioned residue in the proximity of the probe upon activation.\(^19\)–\(^23\) Besides functional studies of proteins via small molecule probes, these reactive groups can also be applied for the target identification of inhibitors, like natural products, with unknown interacting partners, when combined with analytical techniques like mass spectrometry.\(^24\)–\(^26\)

Independent of whether a probe is designed to modify a targeted protein of interest or to identify the targets of a biologically active compound, selecting the appropriate reactive group-ligand combination is key to the success of the probe. Not only may the reactive group alter the biological activity towards a certain interaction partner, but it also determines to a large extend if proteins get labelled at all. When using chemical crosslinkers, the electrophilic trap of the probe should be positioned in the proximity of a nucleophilic residue of the protein to covalently modify the target. To obtain photo-affinity probes that successfully label their targets, it is essential that the crosslinker is not solvent exposed.\(^27\)

While molecular modelling addresses this issue in part, knowledge of the targets is often lacking and a major challenge in the synthesis of affinity probes therefore remains finding the optimal reactive group-ligand combination —a process that can require extensive optimization.\(^28\) As a consequence, sets of probes are synthesized that vary in the position
of the crosslinker and the chemotype of the crosslinker. The synthesis, purification and (biochemical) evaluation of these probes is time consuming, methods that overcome this bottleneck, for instance by speeding up the synthesis of probes, will increase the chances in finding suitable probes for any given protein target within a reasonable time frame. Similar problems are being faced in drug development. Results from this field of research reveal that the synthesis and identification of lead compounds can be drastically simplified by making use of (dynamic-) combinatorial chemistry. In this approach, a mixture of reaction partners is combined to synthesize and screen the biological activity of a compound library in one operation. While dynamic combinatorial approaches have been explored for the synthesis of protein inhibitors and macromolecular sensing tools the exploration of the in situ preparation of protein reactive probes is still in its infancy. In this chapter it is reported that the development of chemical (photo-)affinity probes can benefit from a combinatorial approach in a similar fashion as drug development.

As a proof of concept study for the in situ generation of POI-targeting probes in a combinatorial manner, we opted for the well-established chemistry of hydrazone and oxime formation. These two functional groups are accessible from the condensation reaction between a carbonyl compound, e.g. an aldehyde functionality, and a corresponding α-effect amine nucleophile—a hydrazone or an alkoxyamine—respectively. To establish the protein labelling approach based on combinatorial probe synthesis, two protein model systems were chosen to be functionalized by the probes based on a corresponding ligand or inhibitor: the biotin-binding protein streptavidin (Strp) and the enzyme bovine carbonic anhydrase II (bCAii). Biotin-hydrazide 1 (BtHy) or biotin-alkoxyamine 2 (BtAO) and a benzensulphonamidehydrazide 3 (BSu5Hy) derived from sulphamoylbenzoic acid (SBA) were established as the selectors for the two different proteins. The selector compounds 1-3 were combined with the aldehyde equipped variants of the diazotransfer reagent imidazole-1-sulphonyl azide 4 (DtA), the acyl imidazole-based transfer reagent 5 (AtA), or the photocrosslinker 4-azidobenzaldehyde 6 (PClA) as the protein modifying groups. The in situ generated probes were then used to label streptavidin in the case of the biotin-based selectors or bovine carbonic anhydrase II in the case of the benzenesulphonamide-based selector. The here reported approach allows for rapid screening of different reactive groups and to determine the selectivity profile of a prospective probe in complex mixtures, without the need of going through the full length synthesis and purification of all the individual probes—a quality that will come into its own once the reactive group library is extended and eventually transferred to novel protein targets by introducing new selectors (Figure 1).
Figure 1 Concept of using combinatorial chemistry for in situ probe formation. (A) A protein ligand equipped with an α-effect amine (i.e. hydrazide or alkoxyamine) condenses with an aldehyde bearing protein-reactive group, thereby forming a unique probe left. In a subsequent step, the protein corresponding to the applied selector is labelled with the newly formed probe right. (B) As selectors, the scaffold of the ligand biotin is used to target streptavidin (Strp), the scaffold of the inhibitor sulphamoylbenzoic acid is used to target bovine carbonic anhydrase II (bCAII) top and bottom panel. Aldehyde derivatives of imidazole-1-sulphonyl azide for diazotransfer, acyl imidazole for acyl transfer (in this example an acetylene is introduced into the protein as a bioorthogonal handle) and phenyl azide for photocrosslinking to the protein center panel.
5.2 RESULTS AND DISCUSSION

All of the reagents, both the selectors and the reactive groups, are readily accessible either directly from a commercial source or from commercially available building blocks in one to three steps: 1 was synthesised from the methyl ester of (+)-Biotin and hydrazine hydrate, 2 was purchased, 3 was synthesized according to the same strategy reported in chapter 4, where the 4-nitrophenyl activated ester was substituted with hydrazine to form the hydrazide, 4 was synthesised from 4-imidazolecarboxaldehyde according to the strategy reported in chapters 2 and 4, 5 was synthesised from propargyl chloroformate and 4-imidazolecarboxaldehyde and 6 was synthesised from 4-formylphenylboronic acid and sodium azide (Scheme 1).

To assess whether probe formation was feasible according to the condensation strategy between the selector part bearing the α-effect amine nucleophile and the aldehyde bearing reactive group we incubated biotin-hydrazide 1 with the diazotransfer reagent 4 and acyl imidazole 5 overnight in DMSO and analysed the mixture with UPLC-MS. We observed the formation of the hydrazone products with small amount of side products, confirming that the probes can be prepared in situ (Figure 2). The analysis of the chromatograms reveals further that in case of the probe DtHy-DtA 7 both starting materials (reactor and selector) are completely consumed (both are detectable individually in their unreacted form by this method, data not shown) after the overnight reaction and that the following side products have formed: the two major compounds identified are biotin and biotin-hydrazone-imidazole, suggesting that the diazotransfer probe is less stable towards hydrolysis than the probe DtBio as discussed in chapter 2. In case of the second probe DtHy-AtA 8 the starting materials were not completely consumed after the overnight reaction but at the same time less hydrolysis product was observed. These findings suggest that probe formation times need to be optimized for each probe individually, to find the ideal point in time that yields maximum conversion, i.e. probe formation, and at the same time minimal probe hydrolysis.
Figure 2 In situ probe formation monitored with LC-MS. Selectors and Reactors were mixed from
stock solutions in DMSO 1:1 overnight. The samples were diluted in water prior to chromatographic analysis. (A) Biotin-hydrazide and Dt-aldehyde were incubated to yield 7 and analysed with LC-MS. (B) Biotin-hydrazide and At-aldehyde were incubated to yield 8 and analysed with LC-MS. Note: top panel – base peak chromatogram, centre panel – mass range selected for product ion [M+H]+, bottom panel – mass spectrum of selected ion peak. Peaks towards the end of the gradient are not sample-borne but rather due to column/instrument contamination and therefore excluded form the images.

Having established that hydrazones can be formed from reagents 4 and 5, in combination with biotin-hydrazide 1, we applied these chemicals in the synthesis and biochemical evaluation of ligand-directed protein probes. Prior to the labelling experiment, we mixed BtHy 1, BtAO 2 or BSu5Hy 3 (25 mM) with an equimolar amount of aldehyde 4, which installs an azide via diazotransfer onto the protein, or aldehyde 5, which installs an acetylene function via acyl transfer onto the protein. After incubating the two probe components overnight, we obtained reagents 7 (BtHy-DtA), 8 (BtHy-AtA), 9 (BtAO-DtA), 10 (BtAO-AtA), 11 (BSu5Hy-DtA) and 12 (BSu5Hy-AtA). Probes 7-10 target streptavidin and probes 11 and 12 target bovine carbonic anhydrase II. To control for non-specific background labelling (i.e. not enhanced by the proximity effect upon selective target binding), we also prepared the corresponding control reagents using acetohydrazide 13 as non-targeting selector group. Mixing of 13 with the two reactors 4 and 5 resulted in the control probes 14 (AcHy-DtA) and 15 (AcHyAtA). The thus obtained probes were incubated with a mixture of Strp, bCAII and ovalbumin (OVA, as an additional background labelling indicator) in HEPES buffer, pH 7.4, for 1 hour. Subsequently, the bioorthogonal tag that was introduced to the protein via the probe was reacted with a fluorophore reporter group bearing the cognate reactive partner for click chemistry (BODIPY-alkyne (16) for probes 7, 9 and 11 and BODIPY-azide (17) for probes 8, 10 and 12). Making use of the copper-catalyzed azide-alkyne cycloaddition reaction enabled the formation of the protein-fluorophore bioconjugate via the stable and biocompatible triazole group (CuAAC, Figure 3A). SDS-PAGE of the reacted protein samples and subsequent fluorescent scanning of the gel revealed that the selective labelling of both streptavidin and bCAII in a protein mixture is indeed feasible with this strategy. While the background signal (Figure 3B, band intensities for bCAII and OVA) for all four biotin based probes (lanes 1,4,5,8) is at a similarly low level compared to the control compound (lanes 3 and 7) the signal intensity for labelled streptavidin is most intense for the two component probe 9 (BtAO-DtA, lane 4). Furthermore, carbonic anhydrase was solely labelled when the aldehydes 4 and 5 were reacted with benzenesulphonamidehydrazide 3, which underlines
that the proteins only get labelled in the presence of the targeting moiety. For this protein the two component combination between selector 3 and the acyl transfer group 5 as reactor gives the most intense signal. Comparing the fluorescence intensity of the labelled proteins indicates that the in situ formed diazotransfer and acyl transfer probes label streptavidin and carbonic anhydrase II with a similar efficiency.

Figure 3 **Protein labelling with two-component probes.** (A) The protein streptavidin is incubated with the priorly formed probes 7 (for diazotransfer), or 8 (for acyl-acetylene transfer). Subsequent to the introduction of the bioorthogonal handle azide or acetylene, respectively, click chemistry is used to introduce the fluorophore BODIPY into the protein. (B) Fluorescence scan of an SDS-PAGE gel resolving the labelled protein mixture. Band intensity corresponds to labelling efficiency left. Control compounds used in these experiments to monitor Selector effect on labelling efficiency right.
We then aimed to perform the same experiments with the aldehyde bearing photocrosslinker 6 (PClA) as the reactor of the probe. Compared to the other two reactive groups 4 and 5 that install a bioorthogonal handle covalently onto the protein, which allows for the direct protein functionalization with click chemistry in a subsequent step, the photocrosslinker does not possess these group-transferring properties but forms a covalent adduct with the protein upon irradiation triggered nitrene formation and insertion.\textsuperscript{39,40} The so formed adduct is not amenable for click chemistry and thus this modification is not detectable by means of a fluorescent read-out with this strategy. However, the imine bond that was formed when the selector hydrazide condensed with a reactor aldehyde is amenable for imine exchange if an excess of an alternative α-effect amine nucleophile is presented to the hydrazone linkage. And thus, by adding a fluorophore bearing a hydroxylamine moiety (the cyanine based fluorophore Alexa 647 hydroxylamine\textsuperscript{41}) subsequent to probe-protein incubation and irradiation, the protein-photocrosslinker adduct can undergo the transimination reaction with the fluorophore. With this strategy, in-gel fluorescence detection of the modified proteins will be feasible (Figure 4A).

The ligand-directed photocrosslinker probe 18 (BtHy-PClA) was again obtained by mixing equimolar amounts of selector 1 and reactor 6, allowing imine formation overnight. The in situ formed probe 18 was incubated with a mixture of streptavidin and ovalbumin in PBS buffer, pH 7.4, for 0.5 hour. The sample was then irradiated under UV light, 312 nm, for two minutes while keeping the samples on ice. After one hour at room temperature the hydroxylamine bearing fluorophore AlexaFluor 647 was added in a 10X molar excess. The transimination reaction was allowed to take place overnight.

Here, SDS-PAGE resolving of the reacted protein samples and subsequent fluorescent scanning of the gel indicate that a) the photocrosslinker probe 18 reacted with its target protein Strp and b) that imine exchange between the probe-protein adduct and the hydroxylamine fluorophore is indeed feasible. The fluorescence band intensity is attenuated when a competitor (biotin in 10X molar excess) is added to the reaction mixture before the probe was added (Figure 4B, compare lane 1 with 2) demonstrating that protein modification takes place more efficiently upon probe binding to the protein. Adding the control compound probe 19 (obtained from reacting leucinehydrazide, LeuHy, as a nonspecific selector and 6) to the protein shows even lower labelling intensity but still not as low as when the protein mixture is incubated with the fluorophore but neither of the two probe (compare lanes 3 and 4).

Although the reactions indicate that photocrosslinking and subsequently hydrazone-oxime exchange is feasible, the inefficient transimination reaction still hampered the reliable detection of the protein-probe adduct in the case of bovine carbonic anhydrase II (data not
Figure 4 **Protein labelling with the two-component photocrosslinker 16.** (A) The protein of interest, POI, is incubated with a priorly-formed, photocrosslinker-equipped affinity probe. Subsequent to light-triggered covalent bond formation between probe and protein the transimination reaction takes place. By exchanging the hydrazide bearing selector for an alkoxyamine bearing fluorophore an oxime bond is formed and the protein is decorated with a fluorophore. (B) Fluorescence scan of an SDS-PAGE gel resolving the labelled protein mixture. Band intensity corresponds to labelling efficiency left. Targeted-probe and control compounds used in these experiments to validate this labelling approach right.
Exchange of reactive groups through transimination between probes during the protein labelling step could limit their application and we therefore monitored whether exchange occurred by adding carbonic anhydrase II targeting probes equipped with diazotransfer reagent 4 (i.e. probe BSu5Hy-DtA 11) or acyl imidazole 5 (i.e. probe BSu5Hy-AtA 12) and the corresponding streptavidin probes (i.e. probes BtAO-DtA 9 and BtAO-AtA 10; Figure 5A) to a mixture of the three proteins ovalbumin, bovine carbonic anhydrase II and streptavidin. Since these two reagents install a bioorthogonal group onto the protein, an azide in case of the diazotransfer probes and a propargyl group in the case of the acyl transfer probes, they can be read-out simultaneously if a step-wise bioconjugation approach is chosen, where the azide is reacted first with strain promoted azide-alkyne cycloaddition (SPAAC) and then the acetylene group with copper catalysed acetylene-azide cycloaddition chemistry (CuAAC).

Addition of a catalytic amount of copper (II) is required to efficiently label proteins with targeted diazotransfer reagents. However, the added catalyst also might affect labelling by acyl imidazole based probes and we therefore first determined the metal catalyst’s effect on the labelling efficiency and selectivity of the acyl transfer group. Copper did not seem to enhance labelling by hydrazones of reagent 5, but it did result in a pronounced off-target labelling, specifically that of bCAII through the streptavidin-directed probe BtHy-AtA 8 (compare Figure 5B, lane 2 with Figure 3B lane 5). Fortunately, the oxime-based probe was considerably more selective in the presence of copper and biotin alkoxyamine 2 (probe BtAO-AtA 10) and it also resulted in an increase in the labelling efficiency (compare Figure 5B, lane 5 with Figure 3B lane 8). Therefore probe 10 was used for the exchange studies.

Incubating the protein mixture simultaneously with the biotin coupled diazotransfer reagent 9 and the benzenesulphonamide hydrazone coupled acyl imidazole reagent 12 resulted in the selective functionalization of streptavidin with an azido group and carbonic anhydrase with a propargyl group, as evidenced by the subsequent and sequential application of the SPAAC reaction using DBCO-CY5 and the CuAAC reaction with followed in-gel fluorescence measurement (Figure 3A, lane 1). Similarly, carbonic anhydrase and streptavidin could be equipped with an azido group and a propargyl group, respectively, by reversing the reagent combination (i.e. by applying the probes BSu5Hy-DtA 11 and BtAO-AtA 10, lane 2). Furthermore, treating the proteins with a mixture of probes targeted to a single protein resulted in dual labelling of the respective protein (lanes 3 and 4) and both proteins could be targeted with the same reactive group (azide lane 5, acetylene lane 6).

The outcome of this experiment clearly demonstrates that the exchange between probes is minimal, suggesting that the combinatorial mix-and-match approach is feasible even when several probes targeting different proteins and equipped with different reporter groups are applied simultaneously.
Figure 5 Simultaneous labelling of two proteins with two distinct bioorthogonal groups. (A) A mixture of the three proteins OVA (43 kDa), bCAii (29 kDa) and Strp (13 kDa) was incubated with different combinations of the probes 9-12 and subsequently reacted with a fluorophore, corresponding to the introduced bioorthogonal chemical handle (CuAAC with BODIPY-azide 17, in case of the acyl transfer probes 10 and 12, or SPAAC with DBCO-CY5 20, in case of the diazotransfer probes 9 and 11). Note: colours are chosen to correspond to the signals collected by the two different excitation wavelengths defined by the gel fluorescence scanner. (B) Acyl transfer reaction dependent on the presence of a species of copper(II), compare Figure 3B.
Finally, the methodology was applied to study the effect of the linker length between selector and reactor of the probe on the labelling efficiency to carbonic anhydrase. A series of benzenesulphonamide hydrazides was prepared containing a linker that ranges from zero to eight carbon atoms (BSuHy 21, BSu2Hy 22, BSu3Hy 23, BSu4Hy 24, BSu5Hy 3, BSu7Hy 25) and these hydrazides were reacted with diazotransfer reagent 4, or acyl imidazole reagent 5. The labelling experiments with the resulting probes (BSuHy-DtA/AtA 26/27, BSu2Hy-DtA/AtA 28/29, BSu3Hy-DtA/AtA 30/31, BSu4Hy-DtA/AtA 32/33, BSu5Hy-DtA/AtA 11/12, BSu7Hy-DtA/AtA 34/35) demonstrate that the optimal linker length for the diazotransfer reagent and the acyl imidazoles is approximately two to three carbon atoms. Both shortening and extending the linker resulted in decreased labelling efficiency (Figure 6B).

Figure 6 Evaluating the ideal linker length with the two component probe approach. (A) Benzenesulphonamide hydrazides with different linker lengths are condensed to the reactive groups 3 and 4. (B) In-gel fluorescence signal intensity indicates the best linker-reactive group combination to find the best probe for bovine carbonic anhydrase.
5.3 CONCLUSION

In conclusion, we here describe a novel combinatorial approach to prepare ligand-directed chemical probes. Readily available aldehyde functionalized protein labelling reagents can be reacted with hydrazides and alkoxyamines protein-selecting groups to form targeted probes in situ. The resulting probes modify their corresponding targets, the protein of interest, selectively within a mixture of proteins. The described methodology allows straightforward screening of reactive group-ligand combinations. Mixing benzenesulphonamide hydrazides with different reagents enabled identifying the optimal linker lengths for different reactive groups. Hydrazides are readily prepared from ester containing compounds, making our mix-and-match approach accessible for a large amount of proteins.
5.4 EXPERIMENTAL
5.4.1 CHEMISTRY
5.4.1.1 GENERAL CHEMICAL 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. 4-azidobenzaldehyde 6, 48 BODIPY-alkyne 16, 49 BODIPY-N$_3$ 17, 57 sulphonyl azide transfer reagent 36, 50 and the PNP-esters 37-42 (Chapter 4) 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. Automated column chromatography was performed on REVELERIS Purification Systems (Buchi). 1H-, 13C- and APT-NMR spectra were recorded on a Varian AMX400 spectrometer (400 and 101 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 (CDCl$_3$: δ7.26 for $^1$H, δ 77 for $^{13}$C; CD$_3$OD: δ3.31 for $^1$H, δ 49.15 for $^{13}$C; DMSO-d$_6$: δ2.50 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), 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. 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.
Scheme 1 *Synthesis of the selectors and reactors not directly available from commercial vendors.*

Procedures described in the text below.
5.4.1.2 SYNTHESIS OF SELECTORS

General procedure for the synthesis of hydrazides 3 and 21-25

Before the chemicals were added, the round bottom flask was covered with a septum and the air inside was exchanged by nitrogen. Subsequently, the indicated amounts of ethanol and hydrazine hydrate (50-60%) were added. The starting material was dissolved in 1 mL DMF and added to the hydrazine solution via a syringe pump. The reactions were stirred at room temperature overnight and under nitrogen atmosphere. On the next day, product formation was verified by TLC, after which precipitation of the product was induced by adding approximately 20-30 mL diethyl ether under stirring. After stirring for an additional two hours, the precipitated product was isolated by vacuum filtration. The white or pale yellow solids were washed two to three times with a small amount of diethyl ether and one time with a small amount of ethanol. After these purification steps the solid product was dried under vacuum for an additional three hours (first crop). The volume of the mother liquor was reduced in vacuo. Then diethyl ether was quickly added again to obtain a second crop of the compound, which was again filtered, washed and vacuum dried.

4-(hydrazinecarbonyl)benzenesulphonamide (BSuHy 21)
PNP ester 37 (320 mg, 1 mmol) was converted into the hydrazide by adding it to ethanol (10 mL) containing hydrazine (0.56 mL, 10 mmol) as described in the general procedure. After work-up, the product 21 was obtained as an off-white solid in (89 mg, 41%)
\[
{^1}H \text{ NMR} \ (400 \text{ MHz, DMSO-}d_6) \delta 9.95 \ (s, 1H), \ 7.96 \ (d, \ J = 8.2 \text{ Hz}, \ 2H), \ 7.87 \ (d, \ J = 8.5 \text{ Hz}, \ 2H), \ 7.46 \ (s, \ 2H), \ 4.56 \ (s, \ 2H)
\]
\[
{^{13}}C \text{ NMR} \ (101 \text{ MHz, DMSO-}d_6) \delta 164.7, \ 146.1, \ 136.2, \ 127.6, \ 125.6
\]
HRMS (ESI-orbitrap) m/z calculated for [M+Na]+ 238.0257, found 238.0267 (delta mTh 1.0, 4.3 ppm)

N-(3-hydrazineyl-3-oxopropyl)-4-sulphamoylbenzamide (BSu2Hy 22)
PNP ester 38 (100 mg, 0.25 mmol) was converted into the hydrazide by adding it to ethanol (3.1 mL) containing hydrazine (0.14 mL, 2.5 mmol) as described in the general procedure. After work-up, the product 22 was obtained as an off-white solid in (40 mg, 55%).
1H NMR (400 MHz, DMSO-d6) δ 9.04 (s, 1H), 8.70 (t, J = 5.6 Hz, 1H), 7.97 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 8.4 Hz, 2H), 7.39 (s, 2H), 4.19 (s, 2H), 3.47 (td, J = 7.3, 5.6 Hz, 2H), 2.33 (t, J = 7.2 Hz, 2H)
13C NMR (101 MHz, DMSO-d6) δ 169.6, 165.1, 146.2, 137.3, 127.8, 125.6, 36.2, 33.4
HRMS (ESI-orbitrap) m/z calculated for [M+H]+ 287.0809, found 287.0818 (delta mTh 0.9, 3.2 ppm)

N-(4-hydrazineyl-4-oxobutyl)-4-sulphamoylbenzamide (BSu3Hy 23)
PNP ester 39 (100 mg, 0.25 mmol) was converted into the hydrazide by adding it to ethanol (3.1 mL) containing hydrazine (0.14 mL, 2.5 mmol) as described in the general procedure. After work-up, the product 23 was obtained as an off-white solid in (29 mg, 40%).
1H NMR (400 MHz, DMSO-d6) δ 8.96 (s, 1H), 8.66 (t, J = 5.5 Hz, 1H), 7.98 (d, J = 8.2 Hz, 2H), 7.89 (d, J = 8.1 Hz, 2H), 7.41 (s, 2H), 4.16 (s, 2H), 3.26 (app q, J = 6.6 Hz, 2H), 2.08 (t, J = 7.5 Hz, 2H), 1.75 (p, J = 7.3 Hz, 2H)
13C NMR (101 MHz, DMSO-d6) δ 171.3, 165.1, 146.1, 137.5, 127.8, 125.6, 31.0, 25.2
HRMS (ESI-orbitrap) m/z calculated for [M+H]+ 301.0965, found 301.0976 (delta mTh 1.1, 3.7 ppm)

N-(5-hydrazineyl-5-oxopentyl)-4-sulphamoylbenzamide (BSu4Hy 24)
PNP ester 40 (100 mg, 0.24 mmol) was converted into the hydrazide by adding it to ethanol (3.1 mL) containing hydrazine (0.13 mL, 2.4 mmol) as described in the general procedure. After work-up, the product 24 was obtained as an off-white solid in (59 mg, 81%).
1H NMR (400 MHz, DMSO-d6) δ 8.93 (s, 1H), 8.63 (t, J = 5.6 Hz, 1H), 7.98 (d, J = 8.5, 2H), 7.88 (d, J = 8.4, 2H), 7.46 (s, 2H), 4.14 (d, J = 3.6 Hz, 2H), 3.25 (app q, J = 6.3 Hz, 2H), 2.04 (t, J = 6.9 Hz, 2H), 1.52 (m, 4H)
13C NMR (101 MHz, DMSO-d6) δ 171.4, 165.0, 146.1, 137.5, 127.8, 125.6, 33.1, 28.7, 22.8
HRMS (ESI-orbitrap) m/z calculated for [M+H]+ 315.1122, found 315.1133 (delta mTh 1.2, 3.9 ppm)

N-(6-hydrazineyl-6-oxohexyl)-4-sulphamoylbenzamide (BSu5Hy 3)
PNP ester 41 (100 mg, 0.23 mmol) was converted into the hydrazide by adding it to ethanol (3.1 mL) containing hydrazine (0.13 mL, 2.4 mmol) as described in the general procedure. After work-up, the product 3 was obtained as an off-white solid in (23 mg, 31%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.90 (s, 1H), 8.61 (t, J = 6.0 Hz, 1H), 7.97 (d, J = 8.1 Hz, 2H), 7.88 (d, J = 8.1 Hz, 2H), 7.46 (s, 2H), 4.13 (d, J = 4.1 Hz, 2H), 3.25 (q, J = 6.4 Hz, 2H), 2.01 (t, J = 7.5 Hz, 2H), 1.52 (m, 4H), 1.28 (m, 2H)

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 171.5, 165.0, 146.1, 137.6, 127.8, 125.6, 33.4, 28.9, 28.8, 26.2, 25.0

HRMS (ESI-orbitrap) m/z calculated for [M+H]$^+$ 329.1278, found 329.1288 (delta mTh 1.0, 2.9 ppm)

$N$-(8-hydrazineyl-8-oxooctyl)-4-sulphamoylbenzamide (BSu7Hy 25)

PNP ester 42 (100 mg, 0.22 mmol) was converted into the hydrazide by adding it to ethanol (3.1 mL) containing hydrazine (0.12 mL, 2.4 mmol) as described in the general procedure. After work-up, the product 25 was obtained as an off-white solid in (47 mg 62%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.89 (s, 1H), 8.61 (t, J = 5.6 Hz, 1H), 7.97 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 8.4 Hz, 2H), 7.46 (s, 2H), 4.13 (s, 2H), 3.25 (app q, J = 6.7 Hz, 2H), 1.99 (m, 2H), 1.50 (m, 4H), 1.29–1.23 (m, 6H)

$^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 171.6, 165.0, 146.1, 137.6, 127.8, 125.6, 40.1, 39.9, 39.7, 33.4, 29.0, 28.6, 26.4, 25.3, 25.2

HRMS (ESI-orbitrap) m/z calculated for [M+H]$^+$ 359.1591, found 359.1602 (delta mTh 1.1, 3.0 ppm)

5.4.1.3 SYNTHESIS OF REACTORS

4-formyl-1H-imidazole-1-sulphonyl azide (DtA 3)

A solution of sulphonyl azide transfer reagent 39 (386 mg, 1.1 mmol) in dry DMF (2 mL) was added drop-wise to an ice water-bath-cooled solution of 4-imidazolecarboxaldehyde (96 mg, 1.0 mmol) in dry acetonitrile (8 mL) over one hour under nitrogen atmosphere. After stirring at rT for 4h, the solution was concentrated and dry-loaded onto celite. Subsequently, the crude was purified by automated column chromatography (1% methanol in DCM to 10% methanol in DCM) to yield 4 (51 mg, 25%) as a white solid.
\[ ^1\text{H} \text{NMR} \ (400 \text{ MHz, CD}_3\text{OD}) \ \delta \ 9.87 \ (s, \ 1\text{H}), \ 8.52 \ (s, \ 1\text{H}), \ 8.46 \ (s, \ 2\text{H}) \]

\[ ^{13}\text{C} \text{NMR} \ (101 \text{ MHz, CD}_3\text{OD}) \ \delta \ 185.7, \ 146.5, \ 138.6, \ 126.5 \]

HRMS (ESI-orbitrap) \( m/z \) calculated for [M+H]\(^+\) 202.0032, found 202.0094 (delta mTh 0.3, 1.4 ppm)

**prop-2-yn-1-yl 4-formyl-1H-imidazole-1-carboxylate (AtA 4)**

Propargyl chloroformate (0.11 mL, 1.1 mmol) was added drop-wise, to an ice water-bath-cooled suspension of 4-imidazolecarboxaldehyde (221 mg, 2.3 mmol) in dry ether (5 mL) under nitrogen atmosphere. After stirring at rT for 4h, the suspension was purified by column chromatography (100% pentane to 60% ether/pentane, \( R_f \) 0.39; the crude was loaded directly from the flask onto the column) to yield 5 (57 mg, 28%) as a white solid.

\[ ^1\text{H} \text{NMR} \ (400 \text{ MHz, CDCl}_3): \ \delta = 9.95 \ (s, \ 1\text{H}), \ 8.23 \ (s, \ 1\text{H}), \ 8.10 \ (s, \ 1\text{H}), \ 5.05 \ (m, \ 2\text{H}), \ 2.68 \ (m, \ 1\text{H}) \]

\[ ^{13}\text{C} \text{NMR} \ (100 \text{ MHz, CDCl}_3): \ \delta = 185.8, \ 142.9, \ 138.2, \ 122.0, \ 77.8, \ 75.3, \ 56.4 \]

HRMS (ESI-orbitrap) \( m/z \) calculated for [M+H]\(^+\) 179.0454, found 179.0451 (delta mTh 0.3, 1.6 ppm)
5.4.2 BIOCHEMISTRY
5.4.2.1 GENERAL BIOCHEMICAL PROCEEDURES

Proteins
Recombinant expressed Streptavidin (Strp) was purchased from ThermoFisher Scientific. Bovine Carbonic Anhydrase II (bCAII) was purchased from Serva (extracted) and Chicken Egg Ovalbumin (OVA) was purchased from Sigma.

SDS-PAGE
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). Electrophoresis was conducted either with Tris-tricine type SDS-PAGE,$^51$ performed according to standard literature procedures or Laemmli-type SDS-PAGE.$^52$ Fluorescence scanning of SDS-PAGE gels was performed on a typhoon gel and blot imager 9500 FLA model (GE Healthcare) using the CY2 settings for the BODIPY (blue laser excitation at 473 nm and emission filter BPB1) and the CY5 settings for DBCO-Cy5. After fluorescent scanning of the gel, the proteins were stained with a coomassie brilliant blue R250 solution according to standard protocols.

Probes and bio-reagents
All probes were prepared as 50 mM stock solutions in anhydrous DMSO and stored at -20 °C. Aliquots from the stock solutions were taken to prepare solutions with the appropriate concentrations according to the experimental set-up in anhydrous DMSO. Stock solutions of CuSO₄ (100 mM) and THPTA (100 mM) 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.
5.4.2.2 BIOCHEMICAL EVALUATION

Protein labelling with the reactors 4 and 5: For in situ probe formation, 1 μL of hydrazides 1 or 3, alkoxyamine 2 or acethydrazide (stocks of 50 mM in DMSO) was added to 1 μL of one of the reagents 4 and 5 (stocks of 50 mM in DMSO). The resulting mixture was incubated overnight at room temperature. For probe formation monitoring via LCMS, the solution was then diluted to a final concentration of 50 μM in water. For the protein labelling experiment, the solution was diluted with 248 μL of DMSO to obtain a 200 μM probe stock. Recombinant core streptavidin (210 μL of a 100 μM stock solution in 10 mM HEPES, pH 7.4), bovine carbonic anhydrase II (42.5 μL of a 100 μM stock solution in 10 mM HEPES, pH 7.4) and ovalbumin (26.5 μL of a 400 μM stock solution in 10 mM HEPES, pH 7.4) were added to a buffered solution (151 μL, 10 mM HEPES, pH 7.4). The resulting protein mixture was divided over re-sealable plastic reaction cups (8 μL of the protein mixture per cup). To the protein solution was added 1 μL of the probe stock solution and, where indicated, 1 μL of copper sulphate (1 mM) was added (otherwise 1 μL of buffer was added). The reaction mixture was incubated for 1 hour (for reagents derived from 4 and 5). For the subsequent click reaction (CuAAC), BODIPY-alkyne or BODIPY-azide (8 μL of a 5 mM stock), DMSO (25 μL), water (10 μL), THPTA/CuSO_4 (1/1, 10 μL, 20 mM) and sodium ascorbate (10 μL, 20 mM) were mixed. Of this mixture, 5 μL was added to the labelling reactions and the resulting solutions were incubated for an additional hour. Subsequently, 17 μL of 2X reducing sample buffer was added and the samples were boiled for 15 min. Half of the sample was loaded on a 12% Tris-Tricine or Laemmli type PAGE gel, electrophoretically resolved and analysed by in-gel fluorescence scanning.

Protein labelling with the photocrosslinker 16: Four individual reactions were set up in 10 mM KH₂PO₄ PBS buffer, pH 7.4, with a total volume of 20 μL, all containing 2.66 μg core streptavidin (10 μM). The first reaction was pre-incubated with 1 mM biotin (final concentration, f.c.) for approximately 10 minutes. Then, compound 16, 10 μM (f.c.), was added. The second reaction was treated equally, except that pre-incubation with the competitor biotin was omitted. The third reaction was incubated with control compound 19, 10 μM (f.c.), instead of 16. The forth sample (the mock control) was prepared by adding DMSO, void of any compound, to the reaction. The four reaction mixtures were left at room temperature for 30 min, after which they were exposed to light of a wavelength of 312 nm for 2 minutes. After one hour of the irradiation step, Alexa-647 hydroxylamine,^{53} 100 μM (f.c.), was added. The reaction was incubated at room temperature overnight. The next day, protein loading buffer was added to all samples, and subsequently the samples were boiled
for 10 minutes (96 °C) followed by SDS-PAGE using a 15% Laemmli type gel and visualisation via in-gel fluorescence.

**Dual labelling experiments.** To determine if imine exchange occurred between probes during protein labelling, the above described triple protein solution mixture (8 μL) was incubated with diazotransfer probes 7 and/or 11 (1 μL of 200 μM stock) and/or acyl imidazole transfer probes 8 and/or 12 (1 μL of 200 μM stock), as indicated in figure 5, for 1 hour. Subsequently, DBCO-CY5 (1 μL of 200 μM stock) was added and the mixture was incubated for an additional 1 h. Finally, the above-described BODIPY-azide click mix (5 μL) was added and the mixture was incubated for another hour. Subsequently, 2X reducing sample buffer (17 μL) was added and the samples were boiled for 15 min. Half of the sample was loaded on a 12% Tris-Tricine PAGE gel, electrophoretically resolved and analysed by in-gel fluorescence scanning using the CY2 and the CY5 settings.

**Screening of the linker length:** 1 μL of the hydrazides 3, 21-25 (50 mM) was added to 1 μL of reagents 4 or 5 (50 mM). The resulting mixture was incubated overnight at room temperature, after which it was diluted with 248 μL of DMSO to obtain a 200 μM probe stock solution. Bovine carbonic anhydrase II (33 μL of a 100 μM stock solution in 10 mM HEPES, pH 7.4) and ovalbumin (30 μL of a 400 μM stock solution in 10 mM HEPES, pH 7.4) were added to HEPES (465 μL, 10 mM HEPES, pH 7.4). Of this protein solution, 8 μL was added to re-sealable plastic reaction cups and subsequently 1 μL of probe stock solution was added. For reagents derived from 4, 1 μL of copper sulphate (1 mM) was added to the labelling mixture. Subsequently, the reaction was incubated for 1 hour, before being reacted with 5 μL of the appropriate click mix for 1 hour (containing the cognate BODIPY fluorophore). The proteins were denatured by adding 4X reducing sample buffer (5 μL) and boiling for 5 min, resolved electrophoretically on a 12% Laemmli type SDS-PAGE gel and visualized by fluorescent scanning of the gel using the CY2 scanner settings.
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