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†Electronic Supplementary Information

Target and identify: triazene linker helps identify azidation sites of labelled proteins via click and cleave strategy

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Content

Supporting Methods

1. General Synthetic Methods

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. 1-(Prop-2-yn-1-yl)piperazine was purchased from Aaron chemistry, 3-azido-7-hydroxy-2H-chromen-2-one was purchased from Carl Roth. DtBio **7** was synthesized according to a published procedure,[1] BODIPY-alkyne **8** was synthesized according to a published procedure.[2] 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, Canada) as the stationary phase. Automated column chromatography was performed on a puriFlash 430evo system (interchim). ^{1}H-, ^{13}C-, APT, COSY, HMQC, HMBC-NMR were recorded on a Varian AMX400 spectrometer (400 and 100 MHz, respectively) or a Bruker Avance (500, 126 Mhz, respectively) using, CD_3OD or DMSO-d_6 as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard (CD_3OD: 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. Infrared (IR) data were recorded on a Perkin Elmer UATR spectrum two FT-IR Spectrometer. Absorbance frequencies are reported in reciprocal centimeters (cm^{-1}). Fluorescence measurements were performed in 96well plate format on a BioTek Synergy H1 multi-mode reader. *WARNING: Diazotransfer reagents and diazonium salts may be shock sensitive and should be handled using appropriate precautions.*

2. Clinker - 3-((4-(prop-2-yn-1-yl)piperazin-1-yl)diazenyl)benzoic acid 1

3-Aminobenzoic acid (137 mg, 1 mmol) was dissolved in 5 mL water and a few drops of methanol were added to facilitate dissolving. The solution was cooled down to 0 °C under constant stirring, and then concentrated hydrochloric acid was added (250 µL, 3 mmol). Amyl nitrite (204 µL, 1.5 mmol) was added portion-wise and the ice bath was removed. The reaction was allowed to warm to room temperature during 1 hour. Meanwhile in a separate round bottom flask, potassium carbonate (500 mg, 3.5 mmol) and 1-(prop-2-yn-1-yl)piperazine (197 mg, 1 mmol) were dissolved in 5 mL water and cooled to 0 °C under stirring. The diazonium salt of 3-aminobenzoic acid was transferred in solution to the second flask in three portions. The ice bath was removed and the reaction was allowed to warm to room temperature during 1 hour. The reaction mixture was loaded directly to a 20 g puriFlash C18 column and the product was eluted with water using MPLC. The fractions containing the product were lyophilized to yield the fluffy, pale yellow solid **1** (193 mg, 79%).

1H NMR (500 MHz, Methanol-d_4) δ 8.04 (t, J = 1.8 Hz, 1H), 7.79 (dt, J = 7.6, 1.4 Hz, 1H), 7.47 (ddd, J = 7.8, 2.1, 1.1 Hz, 1H), 7.34 (t, J = 7.7 Hz, 1H), 3.85 (t, J = 4.4 Hz, 4H), 3.43 (d, J = 2.5 Hz, 2H), 2.78 (t, J = 4.4 Hz, 4H), 2.74 (t, J = 2.5 Hz, 1H).

13C NMR (100 MHz, DMSO-d_6) δ C = 168.40, 148.95, 142.68, 127.30, 126.92, 120.72, 120.64, 78.99, 76.03, 50.38, 45.90. (126 MHz, Methanol-d_4) δ H = 175.29, 151.42, 140.03, 129.12, 128.13, 123.65, 122.57, 78.84, 75.35, 52.13, 47.31.

HR-ESI MS m/z calculated for $[M+H]^+$ 273.1346, found 273.1333 (delta mm -1.30; ppm 4.76)

3. BODIPY-Azide 3

Oxalylchloride (1.2 mL, 14 mmol) was added dropwise to a solution of 6-azidohexanoic acid (1.46 g, 9.3 mmol) in anhydrous toluene (15 mL). A catalytic amount of DMF (0.5 mL) was added and the solution was stirred at room temperature for 3 h. After concentration under reduced pressure, the residue was co-evaporated with toluene (5x) and the resulting crude of 6-azidohexanoyl chloride was used without further purification. A 0.2 M solution of the 6-azidohexanoyl chloride crude was prepared in DCE then 2,4-dimethyl-1H-pyrrole (2 mL, 19.5 mmol) was added. The reaction mixture was stirred at 65°C for 2 h. Diethyl ether (200 mL) was added to the reaction mixture and then it was filtered. The isolated precipitate was dried and used without further purification. This intermediate was dissolved in DCE (0.2 M) and $BF_3{\cdot}OEt_2$ (5.75 mL, 46.5 mmol) was added over 5

min, followed by the dropwise addition of DiPEA (6.48 mL, 37.2 mmol). Nitrogen gas was then bubbled through the solution and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with ethyl acetate (100 mL) and washed with water (50 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and purified with manual flash column chromatography using ethyl acetate:pentane (1:9) as eluent affording BODIPY-N_3 **3** (250 mg, 22%).

^{1}H NMR (400 MHz, $CDCl_3$) 6.05 (s, 2H), 3.31 (t, J = 6.5 Hz, 2H), 3.03 - 2.89 (m, 2H), 2.51 (s, 6H), 2.41 (s, 6H), 1.76 - 1.49 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) 154.10, 146.02, 140.33, 131.54, 121.84, 121.81, 110.16, 51.35, 31.54, 28.85, 28.38, 27.51, 16.56, 14.63, 14.60, 14.57.

4. Clinker Resin 2

Clinker beads were prepared in analogous fashion as reported:[3]

For the preparation of clinker beads, NHS-Sepharose beads (GE Healthcare) were washed with DMSO and reacted with a 4:1 mixture of aminoethanol (9.7 µL/mL beads) and ethylenediamine (2.7 µL/mL beads) for 20 h on an end-over-end shaker at rT in the dark in the presence of triethylamine (15 µL/mL beads) in DMSO (1 vol of DMSO for 1 vol of beads). The beads were then washed with DMSO (3 × 10 mL/mL beads) and DMF (2 × 10 mL/mL beads) and reacted with the clinker **1** (1−4 µmol/mL beads), diisopropylethylamine (3.5 µL/mL beads), trimethylamine (20 µL/mL beads), and PyBrOP (4.7 mg/mL beads) in DMF (1 vol of DMSO for 1 vol of beads) for 20 h at rT in the dark. After being washed with DMSO (3 × 10 mL/mL beads), the beads were reacted with NHS acetate (10 µmol/mL beads) in the presence of triethylamine (20 µL/mL beads) in DMSO (1 vol DMSO for 1 vol beads) overnight at rT. The beads were washed with DMSO (10 mL/mL beads) and ethanol (3 × 10 mL/mL beads) before being stored in ethanol (1 mL/mL beads) at 4 °C. Aliquots of the supernatants before and after coupling were measured by LC−MS to monitor the completion of the coupling reactions.

5. General Biochemical Procedures

Proteins and plasmid

Recombinant expressed Streptavidin (Strp) was purchased from ThermoFisher Scientific. Trypsin was of mass spectrometry grade and purchased from either Roche or Promega. BirA in pET28a (w400-2) was a gift from Eric Campeau (Addgene plasmid # 26624).

SDS-PAGE

Laemmli type SDS-PAGE was performed according to standard literature procedures.[4] 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) or a TruPAGE precast 4-20% gel (SigmaMerck) 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 9500 FLA model (GE Healthcare) using the CY2 settings for BODIPY-alkyne **8** (blue laser excitation at 473 nm and emission filter BPB1). Coomassie staining was carried out with colloidal CBB G250 staining according to the manufactures 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.

Probes and bio-reagents

DtBio was stored at -20 °C as solid. 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 probe exposure to water should be avoided and storage at -20 °C of the stock solutions is advisable. Stock solutions of $CuSO_4$ 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.

6. Clinker Cleavage evaluation with BODIPY-azide

For the initial evaluation of the clinker cleavage condition BODIPY-azide **3** (50 µM, 1 µL of a 2.5 mM stock solution) was dissolved in 25.5 µL of 50 mM HEPES buffer, pH 7.4 and added to 10 µL settled clinker beads (1 µmol/mL -clinker molecule to beads coupling density- in 20 µL volume of ethanol, 1:1 slurry). Then $CuSO_4$ and THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine) were added (1 mM and 2 mM respectively, 1.5 µL of a pre-incubated solution, for prior complex formation, in a ratio of 1:2 from 100 mM stock solutions, each). Finally, ascorbic acid was added to the reaction (4 mM, 2 µL of a 100 mM stock solution). The 0.5 mL low binding plastics microcentrifuge tubes, harbouring the reaction mixtures, were incubated in an end-over-end shaker for 1 h at room temperature under gentle rotation to allow the beads to be evenly dispersed in the reaction solution. Subsequently, the reaction mixture was transferred to a mobicol column (MoBiTec). The flow-through of the reaction, containing unreacted fluorophore and catalyst was collected by centrifugation after placing the mobicol column in a microcentrifuge tube using a table top centrifuge while retaining the now fluorescent beads inside the column. The beads were washed once with 50 µL of 50 mM HEPES buffer, pH 7.4. The column was sealed from the bottom and 50 µL of either of the following solvent (-mixtures) were added to the beads (water; 30% aq. acetonitrile; 0.01, 0.1, 1.0 M formic acid; 0.01, 0.1, 1.0 M hydrochloric acid) followed by top-sealing of the column and 5 min sonication in a water bath. The flow-through of each experiment was collected separately and the procedure was repeated once. The individual flow-through were analysed using a table top UV-light with irradiation at 365 nm.

7. Fluorescence parameters of clicked propynylpiperazine-azidohydroxycoumarin

For the determination of the fluorescence measurement parameters 1-(prop-2-yn-1-yl)piperazine (50 µM, 1 µL of a 2.5 mM stock solution in DMSO) and 3-azido-7-hydroxy-2H-chromen-2-one (50 µM, 1 µL of a 2.5 mM stock solution in DMSO) were dissolved in 44.5 µL of 50 mM HEPES buffer, pH 7.4. Then $CuSO_4$ and THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine) were added (1 mM and 2 mM respectively, 1.5 µL of a pre-incubated solution, for prior complex formation, in a ratio of 1:2 from 100 mM stock solutions, each). Finally, ascorbic acid was added to the reaction (4 mM, 2 µL of a 100 mM stock solution). The reaction mixture was left for 16 h at rT and then diluted in buffer to a concentration of 5 µM (assuming full conversion) in 100 µL total volume to measure in a black 96-well plate designed for fluorescence measurements (ThermoFisher). The wavelength maxima for emission and excitation were determined by measuring fluorescence with either set excitation

wavelength while scanning the emission spectrum, or vice versa, using the study of as reference point.[5] The optimum parameters were determined to be excitation λ_{ex} = 395 nm and emission λ_{em} = 475 nm. The reaction and the measurement was repeated with the optimal parameters in hand measuring concentrations ranging from 1 to 10 µM in triplicate to determine the linear range of the fluorometer at the given parameters.

8. Cleavage evaluation with fluorogenic 3-azido-7-hydroxycoumarin

For a more quantitative evaluation of the clinker cleavage condition 3-azido-7-hydroxy-coumarin (2.5, 5 or 10 µM, 1 µL of a 100, 250 or 500 µM stock solution, respectively) was dissolved in 25.5 µL of 50 mM HEPES buffer, pH 7.4 and added to 10 µL settled clinker beads (1 µmol/mL -clinker molecule to beads coupling density- in 20 µL volume of ethanol, 1:1 slurry). Then $CuSO_4$ and THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) were added (1 mM and 2 mM respectively, 1.5 µL of a pre-incubated solution, for prior complex formation, in a ratio of 1:2 from 100 mM stock solutions, each). Finally, ascorbic acid was added to the reaction (4 mM, 2 µL of a 100 mM stock solution). The 0.5 mL low binding plastics microcentrifuge tubes, harbouring the reaction mixtures, were incubated in an end-over-end shaker for 2 or 16 h at room temperature under gentle rotation to allow the beads to be evenly dispersed in the reaction solution. Subsequently, the reaction mixture was transferred to a mobicol column (MoBiTec). The flow-through of the reaction, containing unreacted coumarin and catalyst was collected by centrifugation after placing the mobicol column in a microcentrifuge tube using a table top centrifuge while retaining the beads inside the column. The beads were washed once with 50 µL of 50 mM HEPES buffer, pH 7.4. The column was sealed from the bottom and 2× 50 µL of the following solutions were added in sequence to the beads (50 mM HEPES buffer, pH 7.4; 0.01 M HCl; buffer; 0.1 M HCl; buffer; 0.1 M HCl; buffer) followed by top-sealing of the column and 5 min sonication in a water bath. The flow-through of each elution step was collected separately and the pH of the solution was adjusted with HEPES buffered sodium hydroxide to reach 7.4 in a total volume of 200 µL.

9. Protein enrichment of azido-streptavidin and on-bead digestion with clinker resin

For the clinker enrichment of azido-streptavidin, recombinant core streptavidin (50 µM; 33.2 µL of a 1 mg/mL stock solution in 10 mM HEPES, pH 7.4) was incubated for 1 h at rT with DtBio **7** (50 µM, 1 µL of a 2.5 stock mM solution) in the presence of Cu(II) (1 mM, 1 µL of a 50 mM solution of $CuSO_4$) in a total volume of 50 µL. Then 25 µL of the diazotransfer reaction mixture were aliquoted and

added to 10 μL settled clinker beads (1 μmol/mL -clinker molecule to beads coupling density- in 20 μL volume of ethanol, 1:1 slurry). THPTA (2.5 mM, 2.5 μL of 50 mM stock solution) and sodium ascorbate (5 mM, 2.5 μL of a 100 mM stock solution) were subsequently added and the reaction was incubated in an end-over-end shaker for 16 h at room temperature under gentle rotation to allow the beads to be evenly dispersed in the reaction solution.

(A) The reaction mixture was then transferred to a mobicol column (MoBiTec) and the flow-through was collected via centrifugation in a microcentrifuge tube. 50 μL of 50 mM HEPES buffer, pH 7.4 were added to the resin and the flow-through was collected in the same microcentrifuge tube. The resin was washed twice with an additional 50 μL of buffer, but the flow-through was collected in a separate microcentrifuge tube. The column was sealed from the bottom and 2× 50 μL of 0.1 M HCl were added to the beads followed by top-sealing of the column and 5 min sonication in a water bath. The flow-through of the two elution steps were collected in one microcentrifuge tube and the pH of the solution was adjusted with HEPES buffered sodium hydroxide to reach approximately 7.4. The three fractions (flow-through, wash and eluate) were lyophilized and the resulting solid was dissolved in 20 μL of 2× reducing sample buffer. The samples were applied to an SDS-PAGE gel, electrophoretically resolved and visualized by coomassie staining.

(B) The reaction mixture was then transferred to a mobicol column (MoBiTec) and the beads were washed with 2× 50 μL 40 mM Tris HCl buffer pH 7.4, then the column was sealed from the bottom and 40 μL 40 mM Tris HCl buffer pH 7.4, 8 M urea were added and the reaction was incubated for 30 min at 50 °C, 700 rpm in a ThermoMixer (Eppendorf) with a sealed top. Once cooled to rT, 250 μL 40 mM Tris HCl buffer pH 7.4 and then 300 ng of trypsin (30 μL of a 10 ng/μL in 50 mM acetic acid) were added to the mixture which was incubated for 16 h at 37 °C, 700 rpm. The beads were washed with 2× 50 μL 40 mM Tris HCl buffer pH 7.4, 10 mL of 30% aq. acetonitrile and 10 mL water. The column was sealed from the bottom and 2× 50 μL of 0.1 M HCl were added to the beads followed by top-sealing of the column and 5 min sonication in a water bath. Subsequently, the beads were washed with 2× 50 μL 30% aq. ACN solution. The combined flow-through of the elution steps was collected in one microcentrifuge tube, the tryptic peptides were dried in a vacuum concentrator (speed-vac) and stored at -20 °C prior to nLC-MS/MS analysis.

10. Protein expression and purification of BirA

E. coli BirA with a C-terminal hexahistidine tag (Addgene plasmid # 26624) was introduced into the BL21 DE3 (RIL) *E. coli* strain by heat-shock transformation. The cells were cultured in Luria Bertani (LB) media supplemented with kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C

until an OD600 of 0.9. BirA expression was induced with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After incubation with shaking at 30 °C for three hours, the cells were harvested by centrifugation and lysed by French press at 4 °C in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl). The lysate was centrifuged to remove debris and unbroken cells and incubated with Ni-NTA agarose (Fisher Scientific). The beads were then washed with lysis buffer and His6-tagged BirA was eluted with lysis buffer containing 330 mM Imidazole. Fractions containing His6-tagged BirA were pooled and subjected to a buffer exchange with a PD-10 desalting column (GE Healthcare) equilibrated in lysis buffer.

11. BirA labelling and clinker pull-down

For the diazotransfer reaction, purified *E.coli* BirA (48 µL of a 0.4 mg/mL stock solution in 50 mM HEPES 150 mM NaCl, pH 7.4) was incubated for 1 h at rT with DtBio **7** (100 µM, 1 µL of a 5 mM stock solution) in the absence or presence of Cu(II) (500 µM, 1 µL of a 25 mM solution of $CuSO_4$) in a total volume of 50 µL.

Then the sample was split:

(A) 10 µL were used for in-gel digest -

The reaction was quenched by adding 2 µL of 5× reducing sample buffer and incubation for 5 min at 95 °C. Subsequently, the sample was subjected to SDS-PAGE for 5 min at 200 V. The gel was stained and the bands were cut and the gel pieces were completely de-stained with 50% acetonitrile (ACN), 50 mM ammonium bicarbonate (ABC), dehydrated with 150 µL acetonitrile, reduced with 10 mM dithiothreitol (30 min at 55°C) and alkylated with 40 mM iodoacetamide (45 min at RT, in the dark) and overnight digested with 10 µL of a 10 ng/µL trypsin (V5111; Promega) at 37°C. Peptides were extracted from the gel pieces by adding, sonicating and collecting sequentially in the same tube: 40 µL 2% trifluoroacetic acid (TFA), 40 µL 33% ACN; 1.7% TFA and 40 µL 67% ACN, 0.7% TFA. The samples were dried under vacuum and reconstituted with 100 µL, 2% ACN. Then, 20 µL 5% (TFA) was added to the sample to reach pH < 3. Solid phase extraction was performed with Pierce C18 tips (87784; Thermo) according to the suppliers manual. The eluate fraction was dried under vacuum and reconstituted with 20 µL 2% ACN, 0.1% formic acid (FA).

The digest was split in half and analysed directly by nLC-MS/MS (method see below) or subjected to a clinkerPD: 10 µL of the digest were added to 10 µL settled clinker beads (1 µmol/mL -clinker molecule to beads coupling density- in 20 µL volume of ethanol, 1:1 slurry). THPTA (2.5 mM, 2.5 µL of 50 mM stock solution) and sodium ascorbate (5 mM, 2.5 µL of a 100 mM stock solution) were subsequently added in a total volume of 50 µL 50 mM HEPES buffer pH 7.4 and the reaction was incubated in an end-over-end shaker for 16 h at room temperature under gentle rotation to allow

the beads to be evenly dispersed in the reaction solution. The reaction mixture was then transferred to a mobicol column (MoBiTec) and the beads were washed with 2× 50 µL 50 mM HEPES buffer pH 7.4, 10 mL of 30% aq. acetonitrile and 10 mL water. The column was sealed from the bottom and 2× 50 µL of 0.1 M HCl were added to the beads followed by top-sealing of the column and 5 min sonication in a water bath. Subsequently, the beads were washed with 2× 50 µL 30% aq. ACN solution. The combined flow-through of the elution steps was collected in one microcentrifuge tube, released peptides were dried in a vacuum concentrator (speed-vac) and stored at -20 °C prior to nLC-MS/MS analysis.

(B) 20 µL were used for conjugation and in-gel fluorescence measurement -

To the solution BODIPY-alkyne **8** (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 reaction was quenched by adding 5 µL of 5× sample buffer and denaturing for 5 min at 95 °C. 10 µL of the reaction mixture were used for in-gel fluorescence measurements.

12. LC-MS/MS analysis: Streptavidin

Nanoflow liquid chromatography electrospray ionisation tandem mass spectrometry (nLC-MS/MS) was performed with an Eksigent nanoLC-Ultra 1D+ system (Eksigent, Dublin, CA) coupled to an Orbitrap Velos instrument (Thermo Scientific). The peptides were delivered to a trap column (100 µm × 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 × 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.

13. LC-MS/MS analysis: BirA

Nanoflow liquid chromatography electrospray ionisation tandem mass spectrometry (nLC-MS/MS) was performed with an Easy-nLC II (Thermo) coupled to an LTQ XL / LTQ Orbitrap XL instrument (Thermo Scientific).

The peptides were delivered to a trap column (100 µm × 2 cm, pre-packed with Silica Spherical Fully Porous C18-AQ 5 µm resin (Thermo)) at a flow rate of 5 µL/min in 100% solvent A (0.1% formic acid (FA), 2% acetonitrile (ACN), in HPLC grade water). After loading and washing, peptides were transferred to an analytical column (PicoFrit Self-Pack Column 75 µm ID x 20cm, 10 µm tip packed in-house with 3 µm 120A Reprosil-PUR C18-AQ µm resin, Dr. Maisch) and separated at a flow rate of 200 nL/min using a 45 min gradient ranging from 2% to 28% solvent B (solvent B: 0.1% FA in

ACN). The eluent was sprayed via the pico frit tip of 1.3 kV and a heated capillary temperature of 200 °C. The LTQ XL / LTQ Orbitrap XL 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 300−1650) were acquired in the Orbitrap at a resolution of 30000. Ions for MS/MS spectra of up to 8 precursor ions were generated in the multipole collision cell by using higher energy collision-induced dissociation (HCD) normalized collision energy of 35% and analysed in the LTQ XL. Precursor ion isolation width was set to 3.0 m/z, the maximum injection time for MS/MS was 30 ms, the precursor ion count for triggering an MS/MS event was set at 500 and dynamic exclusion was set to 45 s.

14. MS Data Analysis

Data analysis was performed using MaxQuant v1.5.8.3 with the integrated search engine Andromeda.[6,7] For peptide and protein identification, raw files were searched against the FASTA files for core Streptavidin (P22629-1, Fig. S2) and *E.coli* BirA (P06709-1, Fig. S4) obtained from UniProtKB (http://www.uniprot.org), with oxidation of methionine, N-terminal protein acetylation and Dt->Lys and Dt->N-term (Modification of lysine, except on C-terminus of peptide, or modification on protein N-terminus: H(-2)N(+2)); 25.9905 Da mass gain) for diazotransfer, and ClinkerFragment->DtLys and ClinkerFragment->DtN-term (Modification of lysine, except on C-terminus of peptide, or modification on protein N-terminus: C(+7)H(+10)N(+4)); 150.0905 Da mass gain) for clinker pull-down fragments as variable modifications; and carbamidomethylation of cysteines as fixed modification.

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.

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Supporting Schemes and Figures

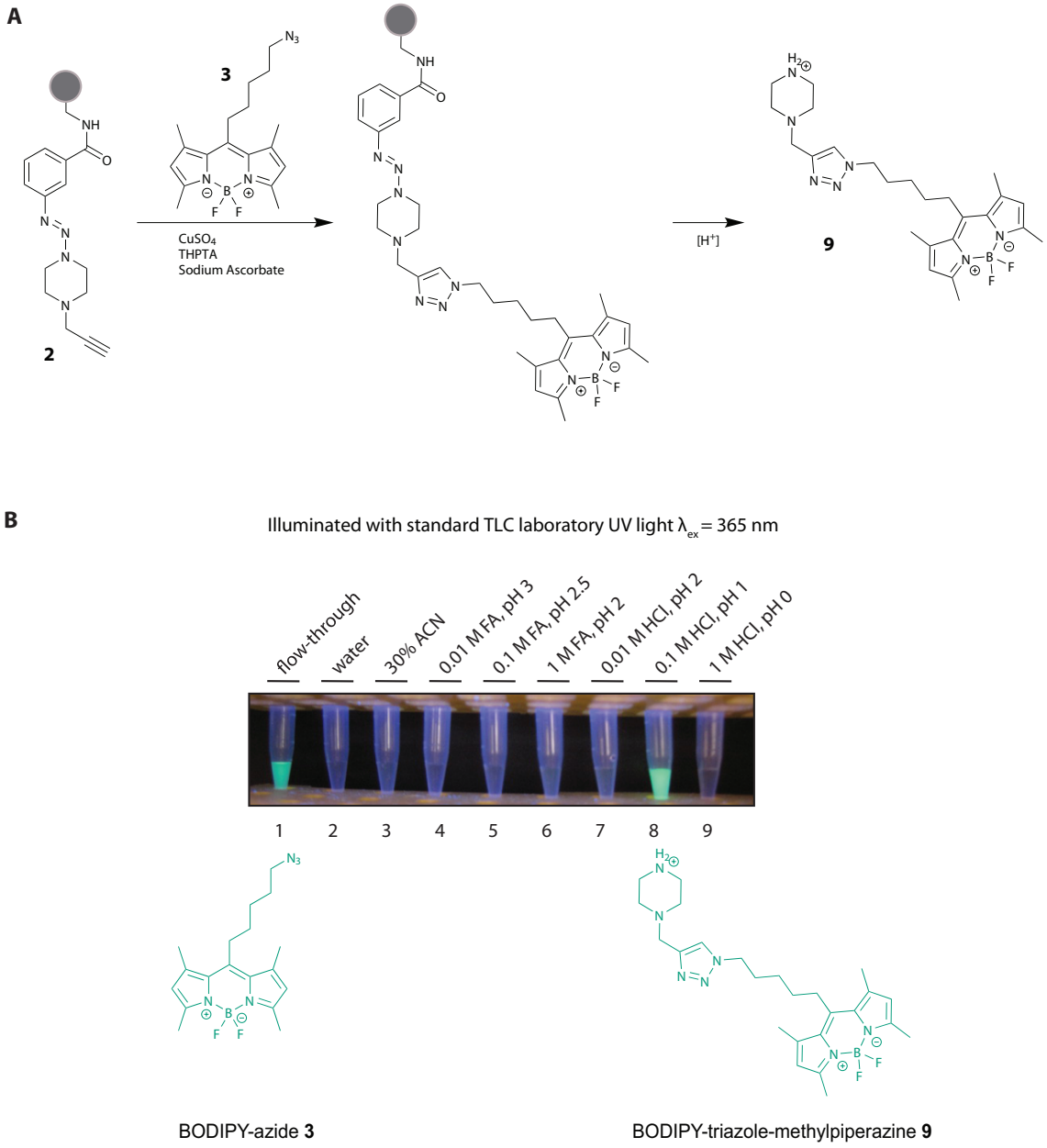


Fig. S1 **Click-and-release of BODIPY-azide 3 under acidic conditions employing clinker resin 2.** (A) in the first step, the clinker resin **2** is functionalised with the inherently fluorescent BODIPY-azide **3** by means of copper catalysed alkyne-azide cycloaddition click chemistry. In the second step the fluorophore **3**, now bearing the clinker fragment via a triazole group **9**, is released by acidifying the resin to a pH of 1. (B) The resin coupled to **3** was incubated with the indicated solutions (lane 1 to 9). The eluate was collected in a microcentrifuge tube and illuminated at a wavelength of 365 nm. Lane 1 shows the flow-through of unbound **3**. Sharp elution of **9** is observed upon an acidification to a pH of 1 (lane 8). Further acidification to a pH of 0 leads to disintegration of the resin material and an obliteration of fluorescence (lane 9).

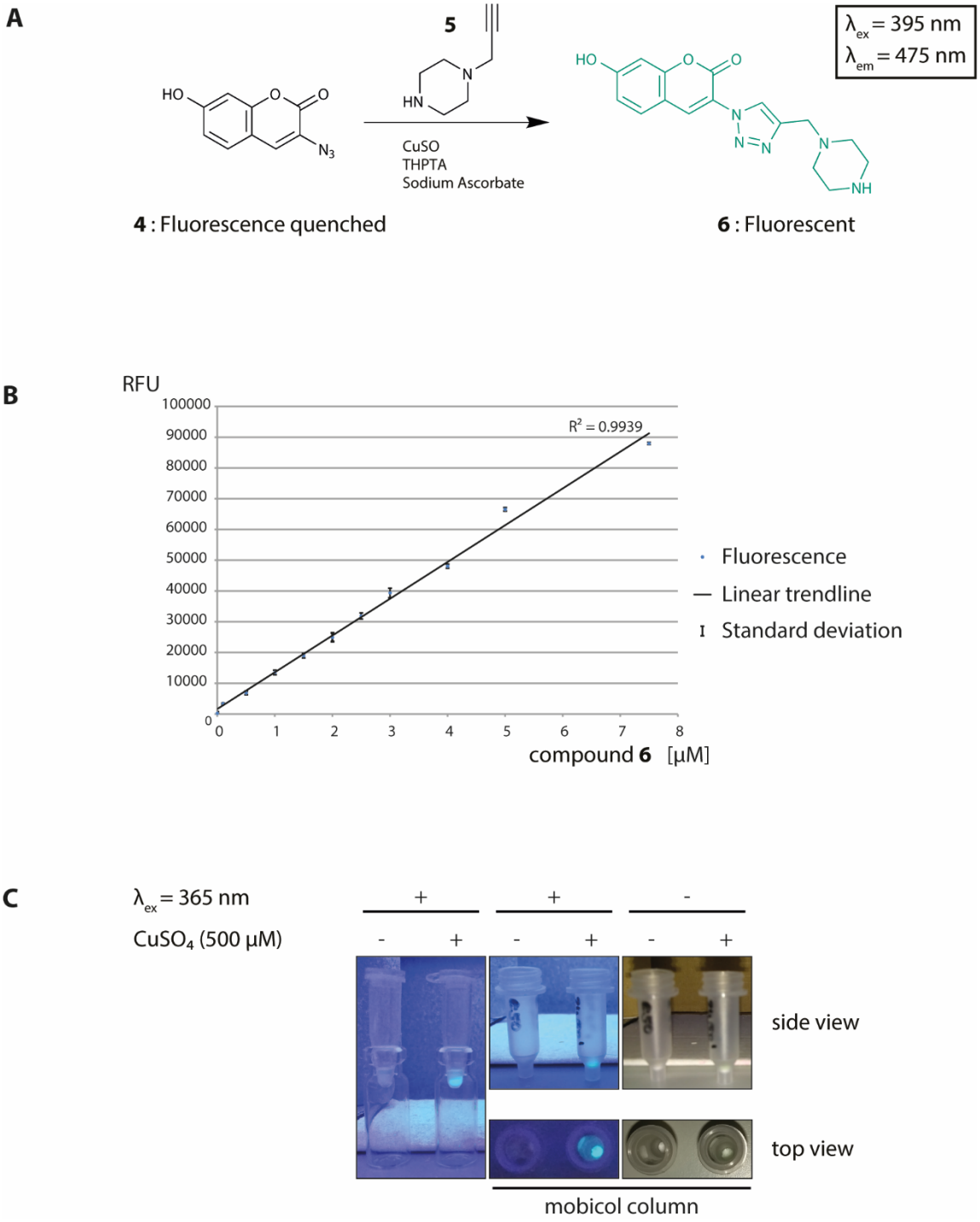


Fig. S2 **Coumarin 4 enables quantification of click-and-release efficiency of clinker resin 2.** (A) Compound **4** is coupled to **5** by means of copper catalysed alkyne-azide cycloaddition click chemistry to yield the fluorescent compound **6**, the cleavage product of clicking **4** to clinker resin **2**. **6** was used to determine the optimal parameters for excitation and emission of the cleavage product. (B) **6** was then used to determine the concentration range within the linear range of the fluorometer, error bars indicate standard deviation, points measured in triplicates. (C) Images depicting the experimental set-up of a clinker pull down with **4**. **6** is not formed by omitting copper from the reaction mixture in the control experiment, and thus no fluorescence is observed upon irradiation at a wavelength of 365 nm.

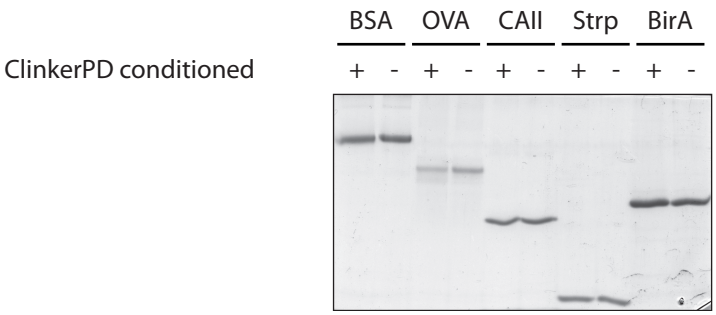


Fig. S3 **No significant degradation of proteins is observed applying clinker pull-down conditions.** Five different proteins (BSA – Bovine Serum Albumin, 69.3 kDa; OVA – Chicken Egg Albumin, 42.9 kDa; CAII – Bovine Carbonic Anydrase II, 29.1 kDa; Strp – core Streptavidin, 13.3 kDa; BirA – Bifunctional ligase/repressor 35.2 kDa) were subjected to conditions applied during a clinkerPD protocol (sonication for 30 min in a solution of 0.1 M aq. HCl, pH 1) and subsequently resolved with SDS-PAGE. Image shows commassie stain of the gel, comparing the treated protein with the same amount of protein in buffer.

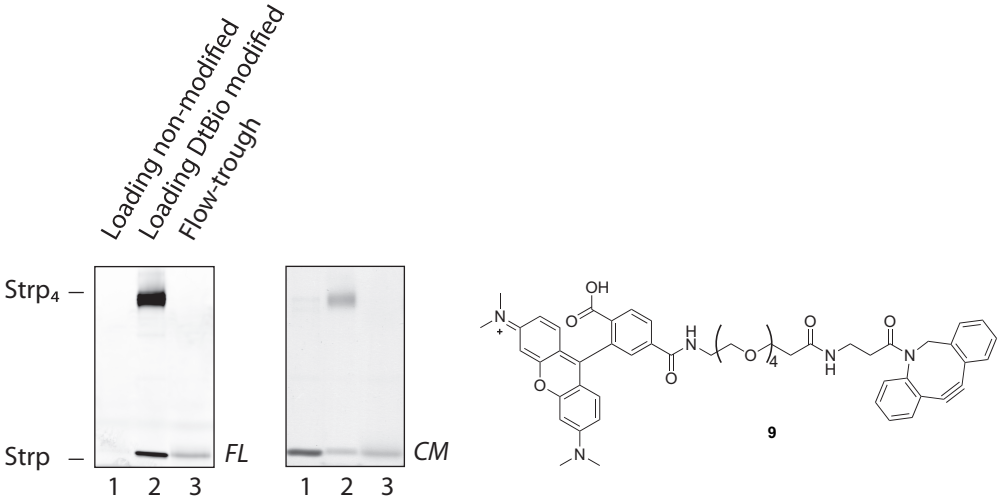
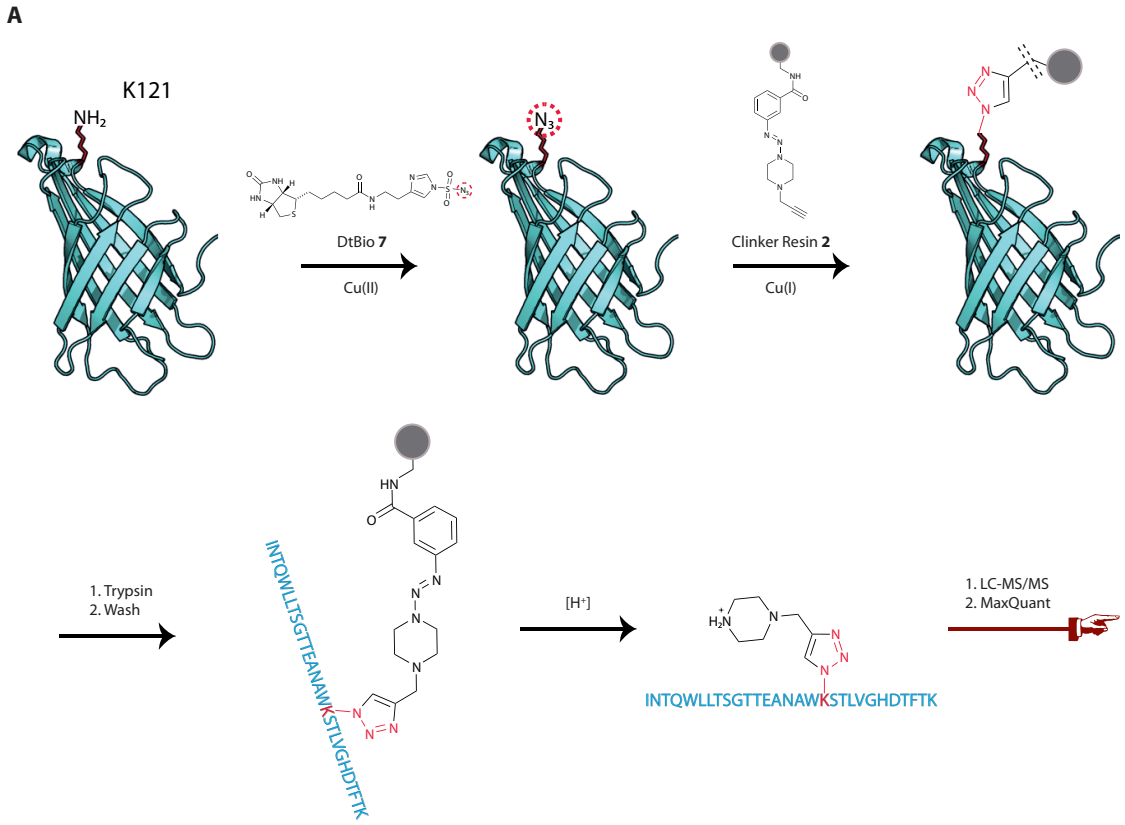


Fig. S4 **Clinker beads loading control of DtBio 7 modified streptavidin. Probe labelling efficiency determines protein loading.** Fluorescence scan (left) and commassie stain (right) of the SDS-PAGE gel post modification with DtBio and subsequent labelling with the strain promoted alkyne DBCO-TAMRA **9**. The weak fluorescent signal in lane 3 suggests that some residual azide bearing protein did not react with the clinker resin. The incomplete capture of the protein, however, is mainly directed by the amount of protein that was modified by the DtBio probe.



B

MRKIVVAAIA VSLTTVSITA SASADPSKDS KAQVSAAEAG ITGTWYNQLG STFIVTAGAD

GALTGTYESA VGNAESRYVL TGRYDSAPAT DGSGTALGWT VAWKNNYRNA HSATTWSGQY

121

VGGAEARINT QWLLTSGTTE ANAWKSTLVG HDTFTKVKPS AASIDAAKKA GVNNGNPLDA VQQ

Fig. S5 **Streptavidin: identification of modification site by tandem mass spectrometry subsequent to clinker pull-down and on-bead digestion.** (A) Model depicting tertiary structure of monomeric streptavidin to visualise the clinker pull-down work-flow: the probe DtBio **7** binds to streptavidin and modifies it by diazotransfer to lysine K121. The protein is then clicked to the clinker resin **2** and digested with trypsin while covalently attached to the beads. Acidification to a pH of 1 releases the covalently bound peptide bearing the original modification site now marked with the clinker fragment. (PDB code: 3RY2, PyMOL used for ray tracing). (B) Uniprot sequence for entry P22629-1, streptavidin. Purple backdrop indicates the sequence of the commercially available core streptavidin. Red letters/squares indicate primary amines in the sequence. Blue backdrop indicates the peptide identified by tandem mass spectrometry bearing the clinker fragment modification (charge: +3, *m/z*: 1124.2441 Th, mass: 3369.7 Da, mass gain compared to unmodified peptide: 150.1 Da, Andromeda score of peptide: 61.5).

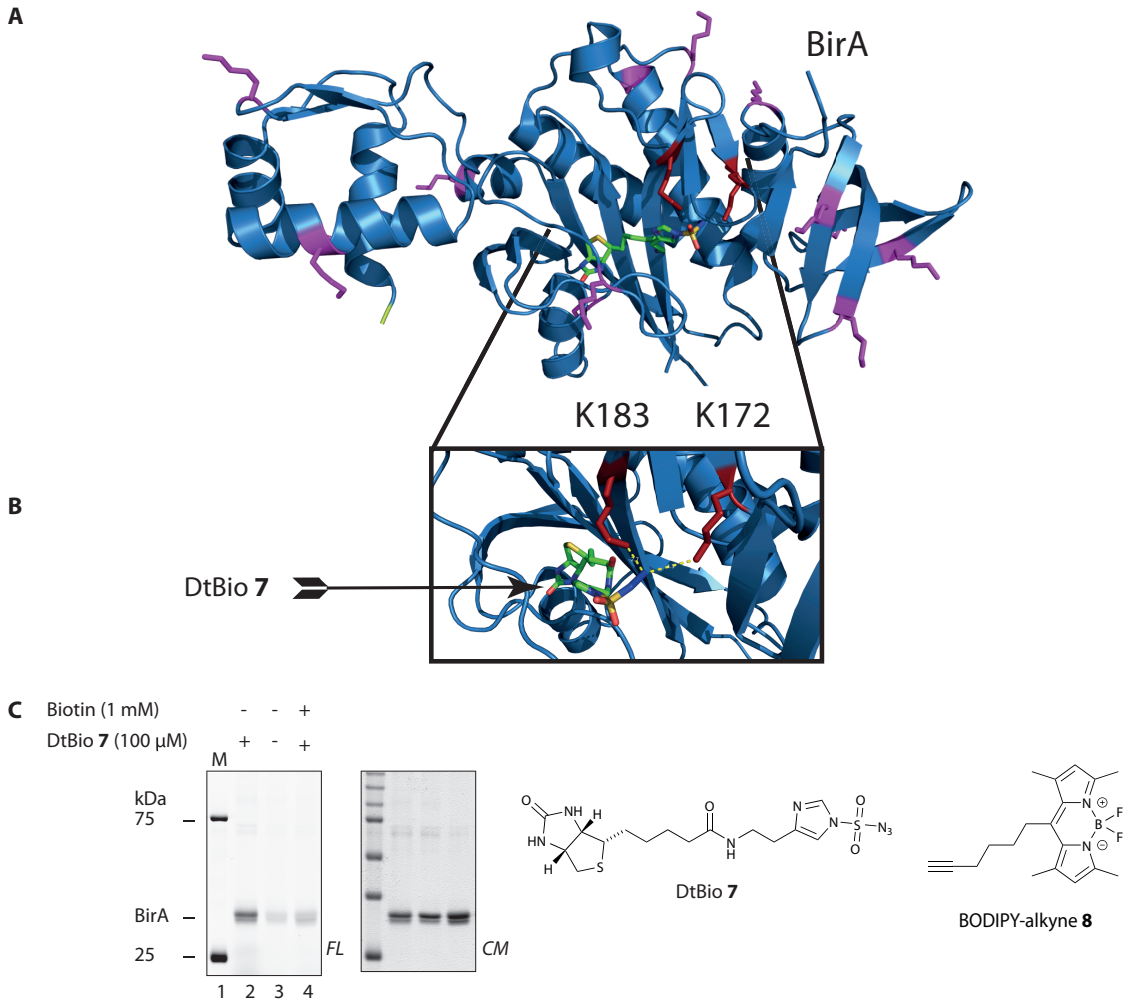


Fig. S6 **DtBio 7 binds to the active site of BirA and modifies lysine K183 selectively by diazotransfer.** (A) Model depicting DtBio **7** (shown as sticks, coloured by element) bound to the active site of BirA (PDB code: 4WF2, shown as cartoon, coloured in blue; lysine as sticks, coloured in purple (distant lysines) or red (proximal lysines). Note: crystal structure does not contain the full protein sequence. (PyMOL used for ray tracing; SeeSAR and LeadIT (https://www.biosolveit.de) used for generation of pose). (B) Close up of DtBio **7** bound to the BirA active site. The probe modifies lysine K183 exclusively amongst 19 possible diazotransfer acceptors within BirA (18 lysine side chains and the N-terminus). (C) BirA is diazotised by probe DtBio 7 and subsequently modified with BODIPY-alkyne 8 for in-gel fluorescence visualisation.*FL* depicts fluorescent scan of the SDS-PAGE gel of BirA modified with BODIPY-alkyne **8** via copper catalysed alkyne-azide cycloaddition

click chemistry (CY2 settings), *CM* indicates the coomassie stain of the same gel. Lane 3 indicates the background signal for unspecific labelling of BirA and lane 4 indicates competition of labelling by adding 10 × of biotin to the reaction mixture prior to adding probe **7** to the protein. (D) Uniprot sequence for entry P06709-1, BirA, *E.coli* (sequence coverage of full digest is 95%). Lysine residues are coloured purple and highlighted with a purple square (non-modified) or red dotted circle (modified). Orange backdrop indicates the peptide identified by tandem mass spectrometry bearing the clinker fragment modification (charge: +3, *m/z*: 464.6293 Th, mass: 1390.8 Da, mass gain compared to unmodified peptide: 150.1 Da, Andromeda score of peptide: 90.6). (E) MS/MS spectrum of the identified peptide.

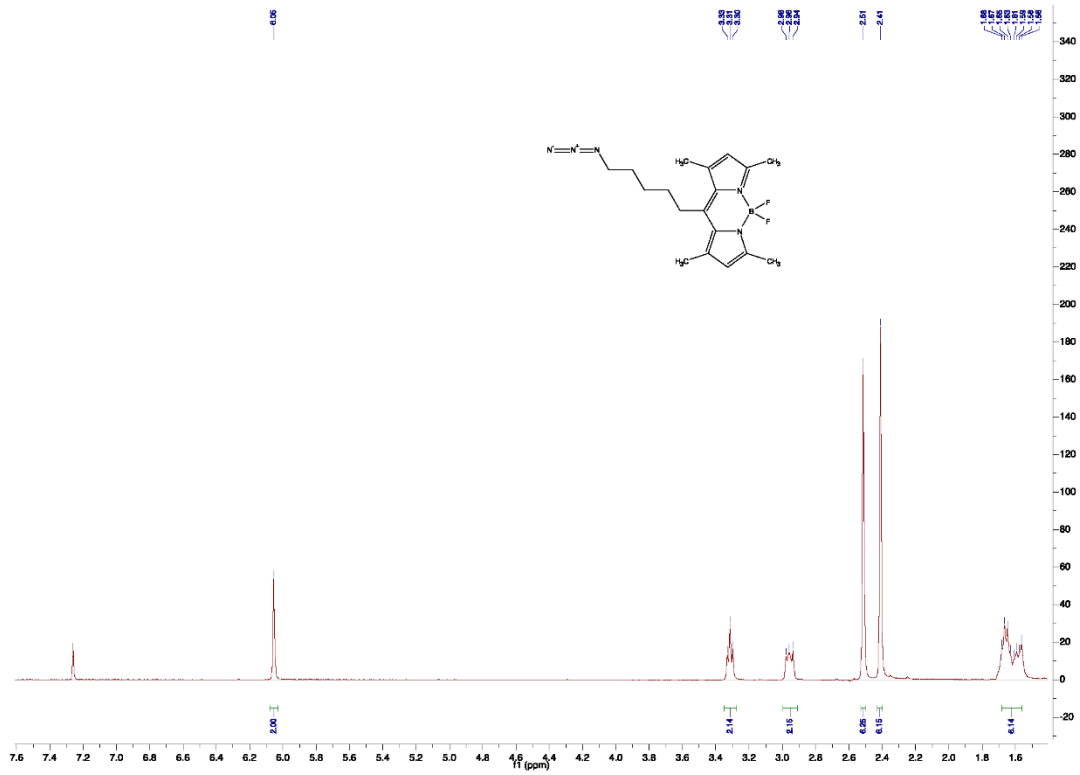


Fig. S7 **^{1}H-NMR of compound BODIPY-azide 3 in $CDCl_3$**

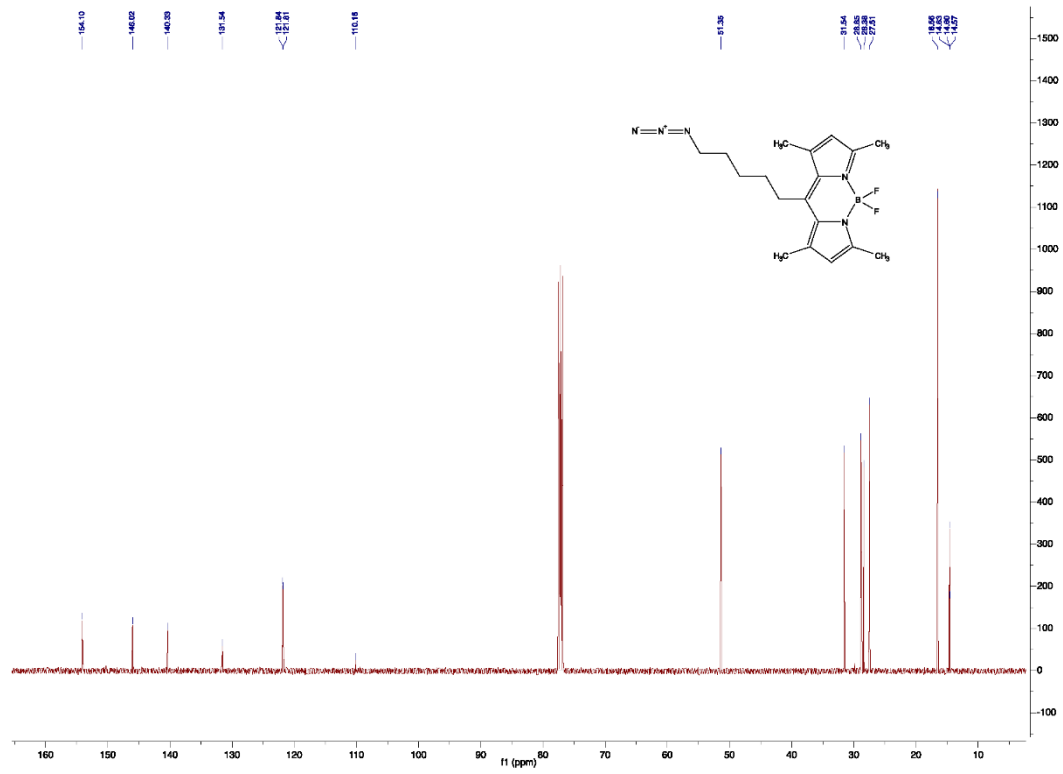


Fig. S8 **^{13}C-NMR of compound BODIPY-azide 3 in $CDCl_3$**

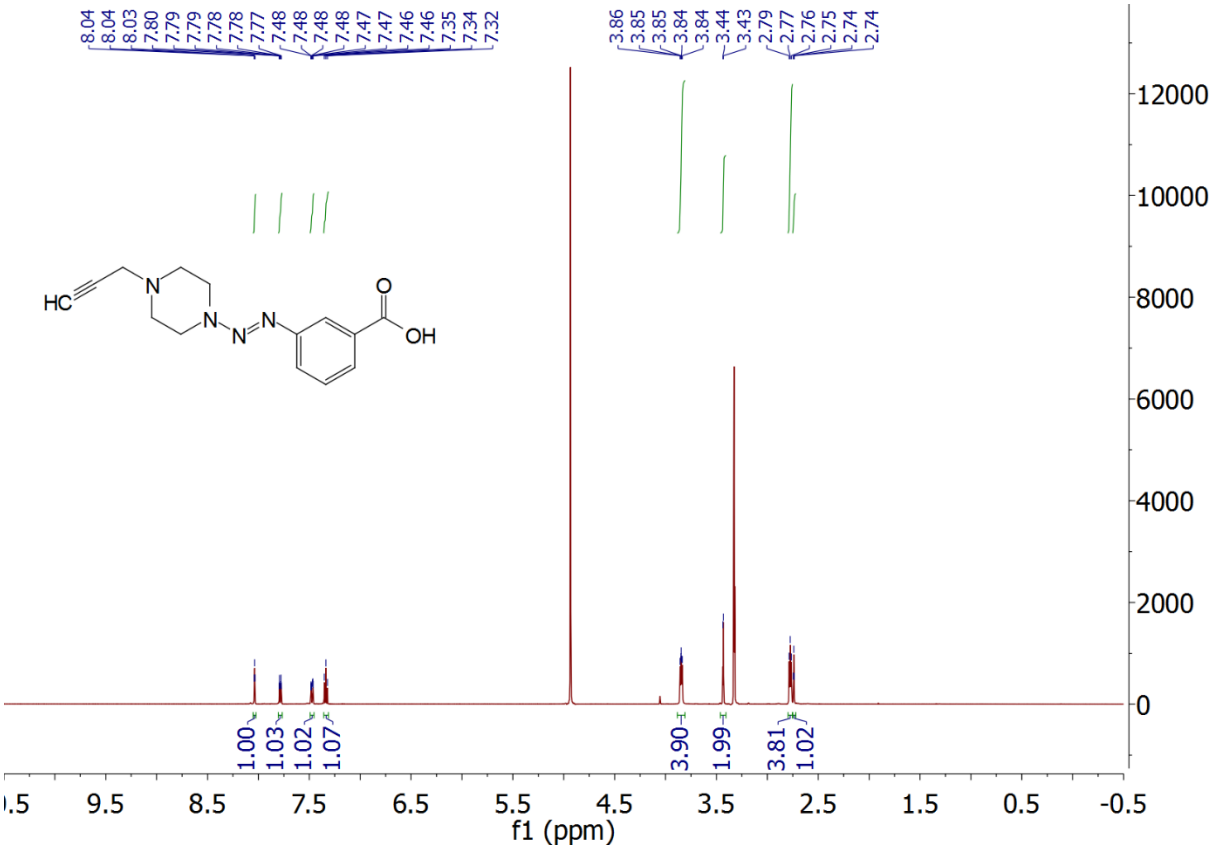


Fig. S9 **^{1}H-NMR of compound clinker 1 in CD_3OD**

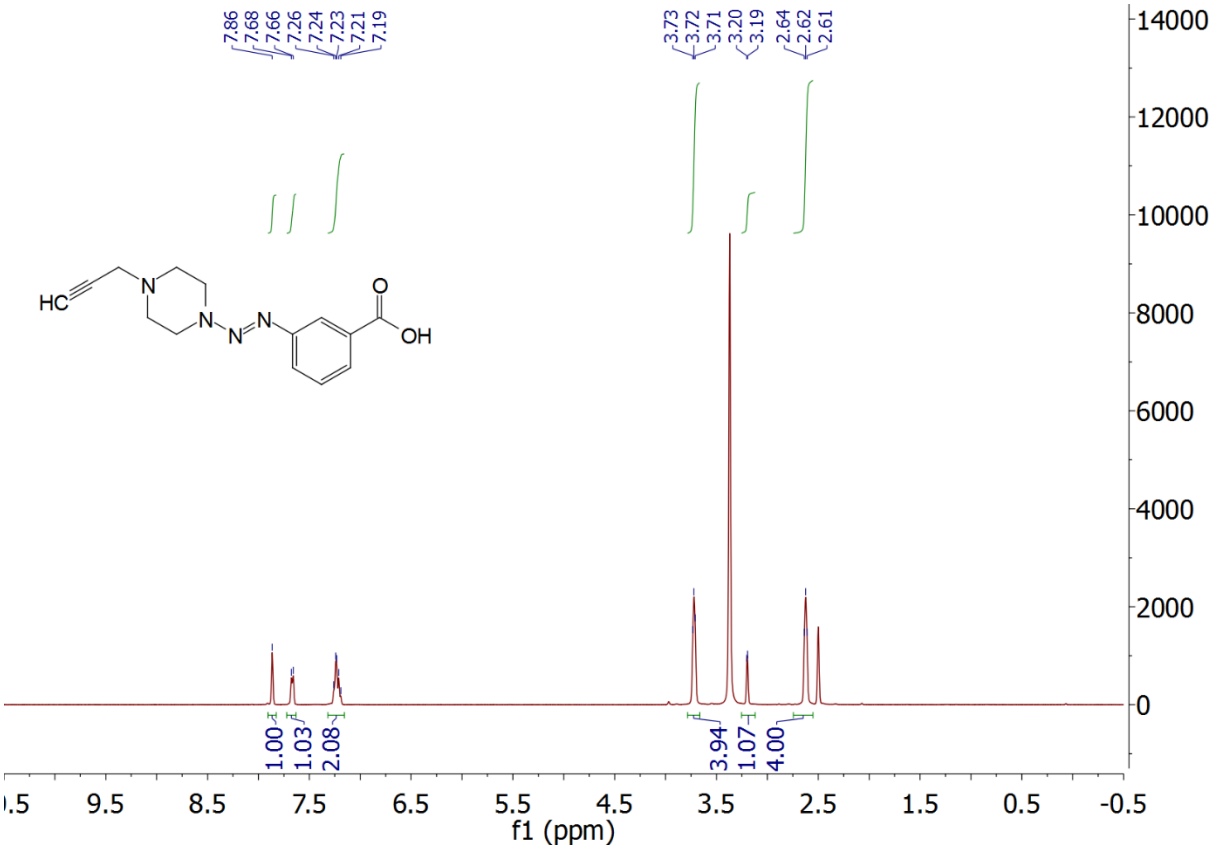


Fig. S10 **^{1}H-NMR of compound clinker 1 in DMSO-*d6***

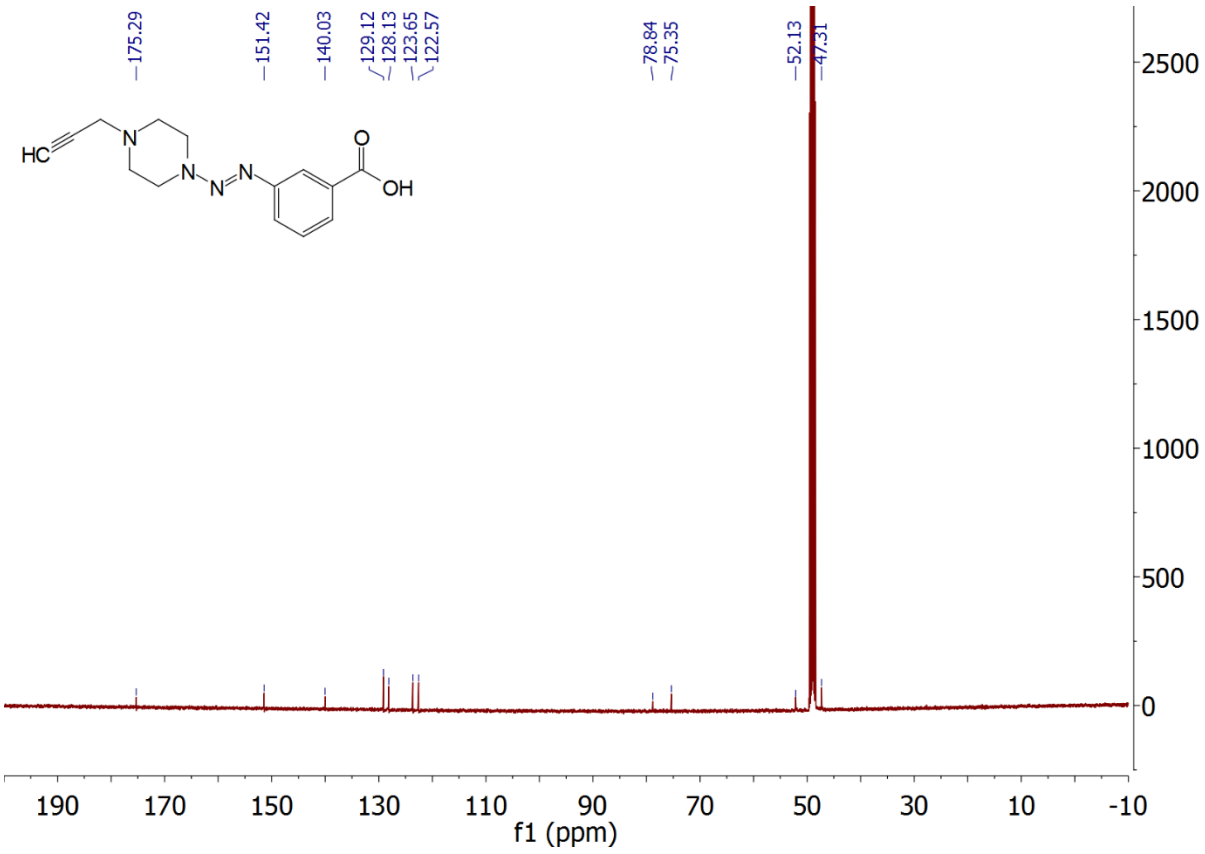


Fig. S11 **^{13}C-NMR of compound clinker 1 in CD_3OD**

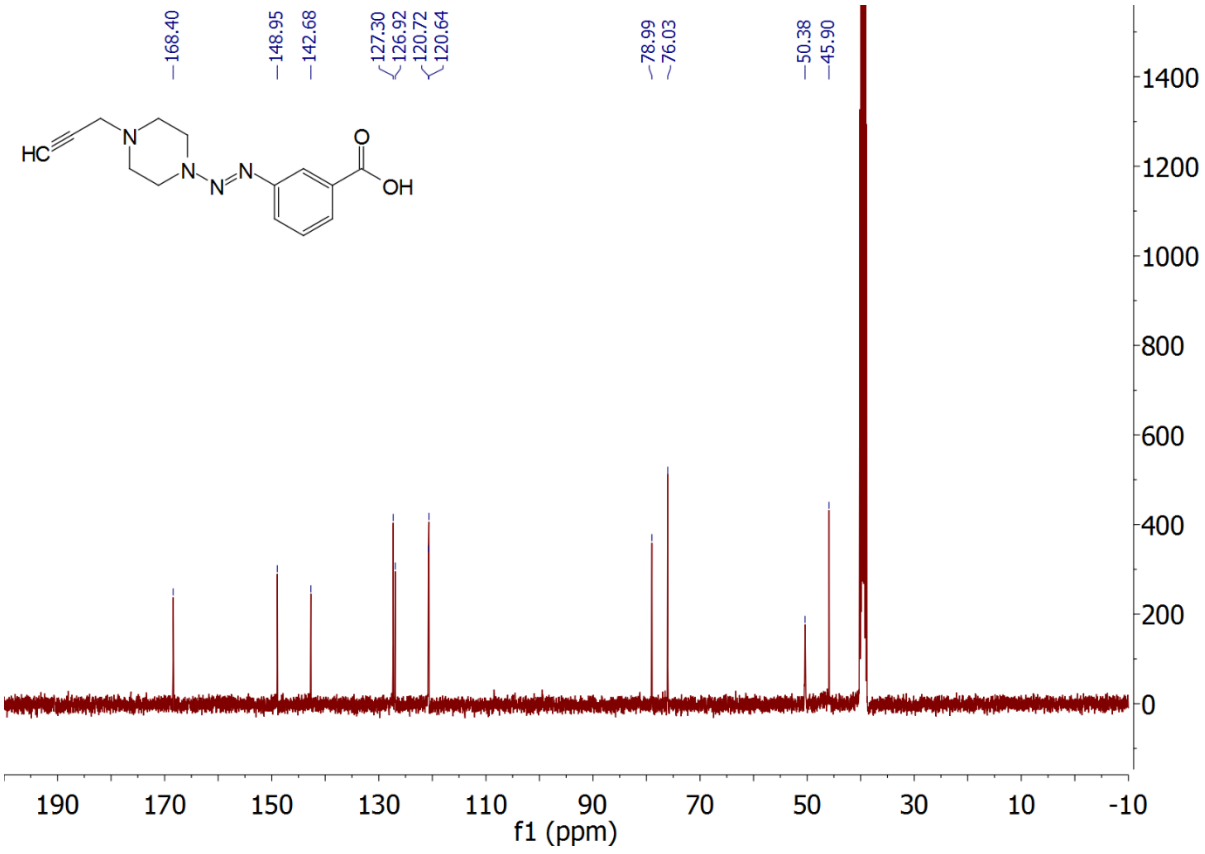


Fig. S12 **^{13}C-NMR of compound clinker 1 in DMSO-*d6***

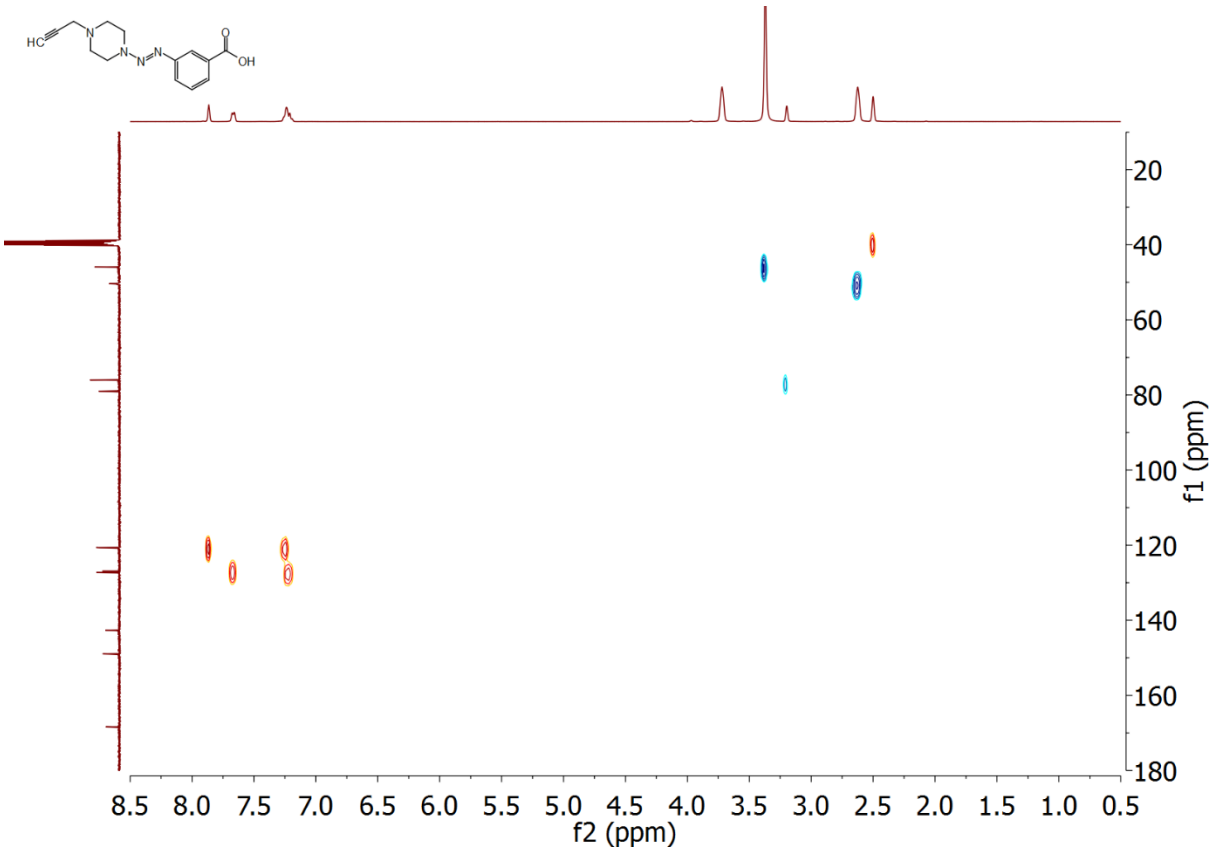


Fig. S13 **HMQC of compound clinker 1**

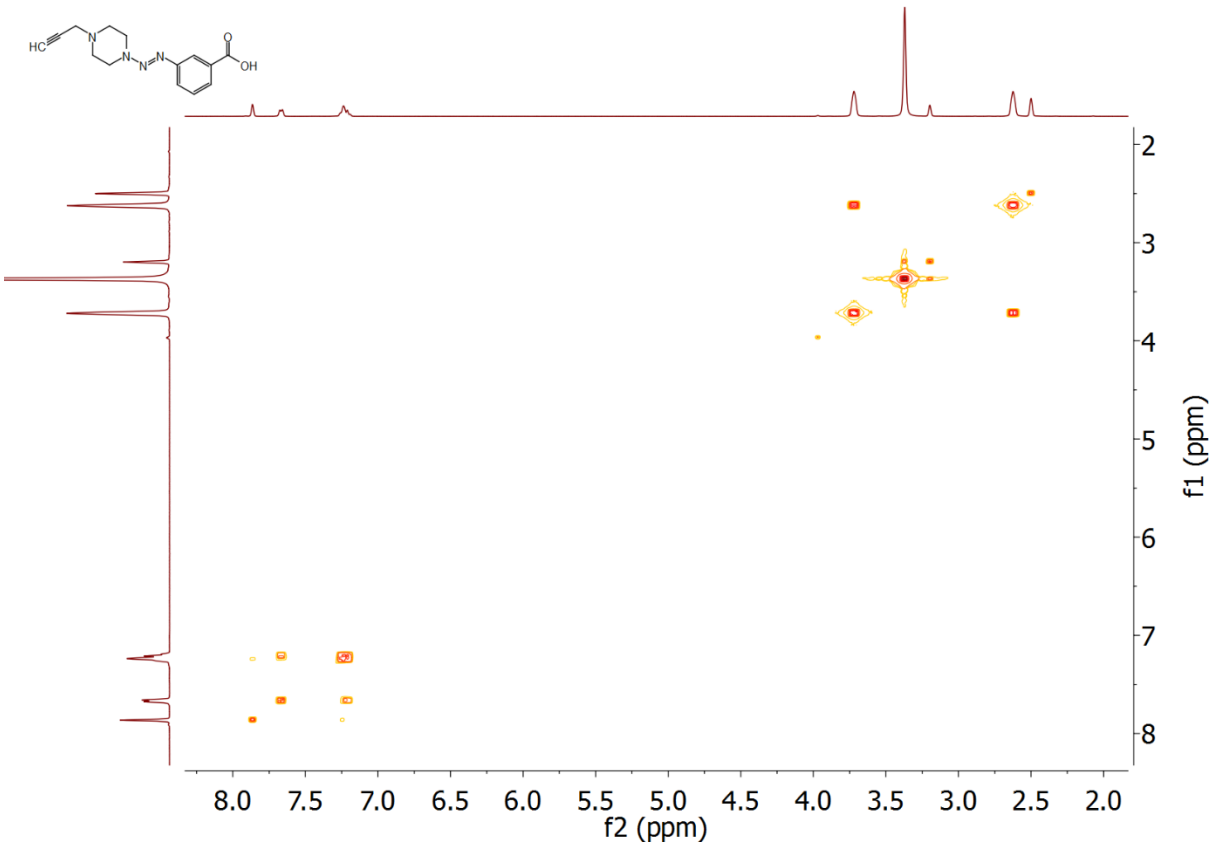


Fig. S14 **COSY of compound clinker 1**

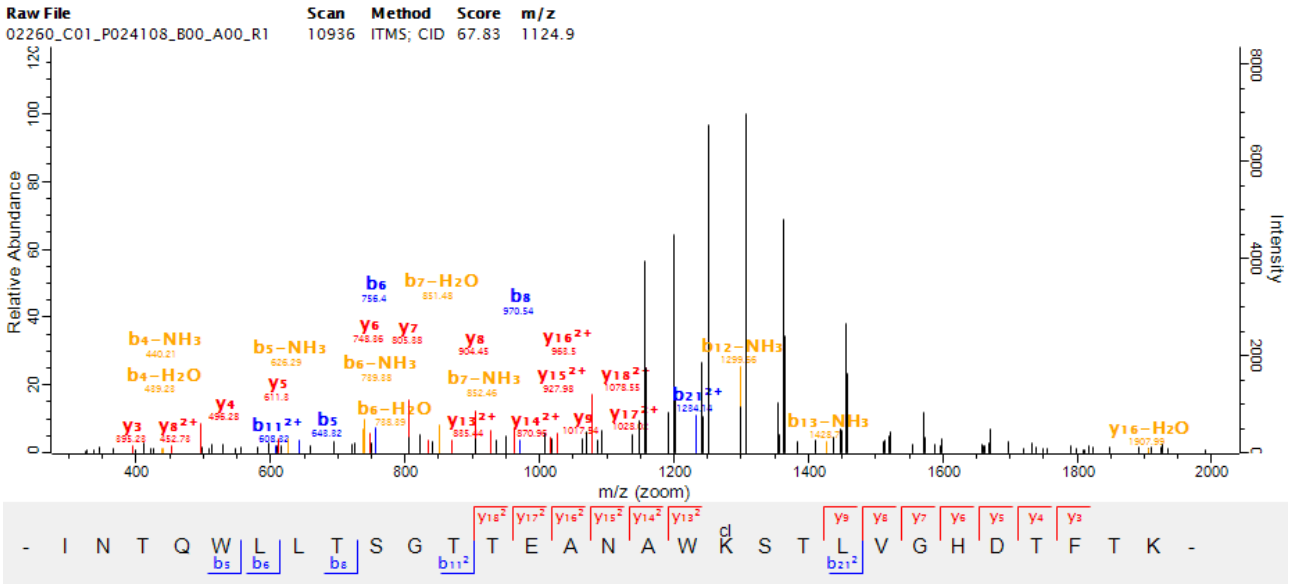


Fig. S15 **MS^2 spectrum of the clinker fragment bearing streptavidin peptide.**

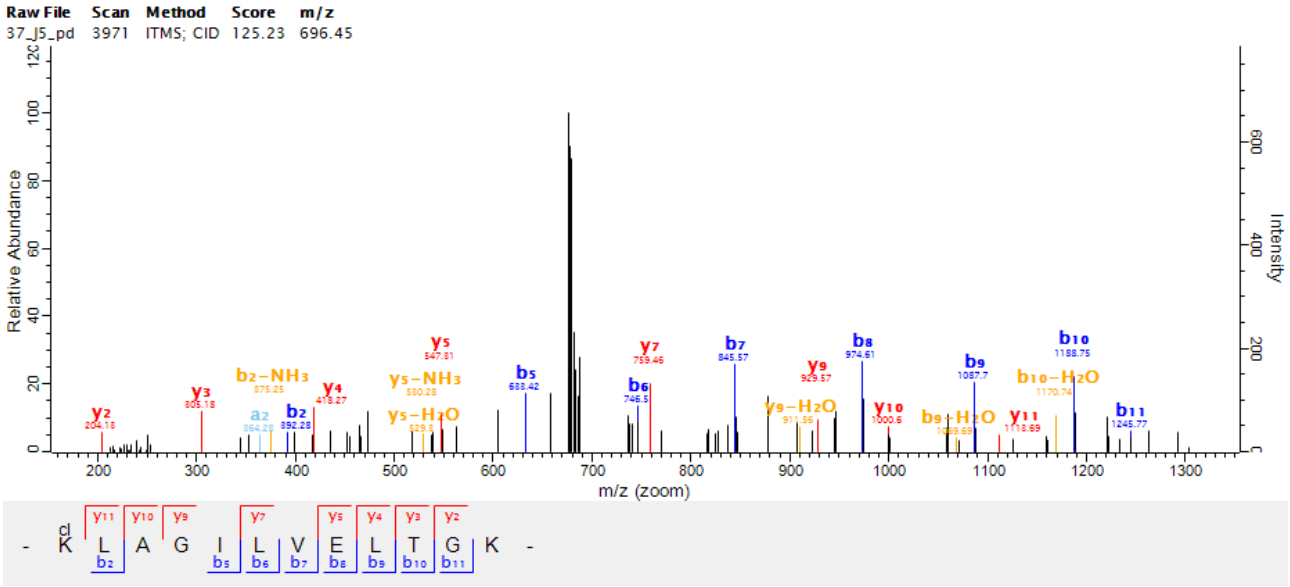


Fig. S16 **MS^2 spectrum of the clinker fragment bearing BirA peptide.**