Activity-based proteomics of the endocannabinoid system

Rooden, E.J. van

Chapter 7

Summary and future prospects

In **Chapter 1**, the endocannabinoid system (ECS) and its therapeutic potential are introduced. The endocannabinoid system consists of the cannabinoid CB_1 and CB_2 receptors and their endogenous ligands: 2-arachidonoylglycerol (2-AG) and anandamide (AEA). The enzymes regulating the levels of 2-AG and AEA are interesting targets for the development of small molecule therapeutics, which are currently lacking. Activity-based protein profiling (ABPP) is a powerful method to aid in drug discovery and in particular for the ECS. Activity-based probes are available for the majority of endocannabinoid enzymes: the diacylglycerol lipases (DAGLs), monoacylglycerol lipase (MAGL), several α/β hydrolase domain containing proteins (ABHD4, 6 and 12), phospholipase A2 group IVE (PLA2G4E), fatty acid amide hydrolase (FAAH) and N-acylethanolamine-hydrolyzing acid amidase (NAAA).

The activity-based protein profiling methodology is reviewed in **Chapter 2**. ABPP is a methodology to study a subset of the enzymatically active proteome. ABPP relies on chemical probes that covalently react with active enzymes. The targeted proteins can subsequently be analyzed via a fluorescent or biotin reporter tag of the probe. If the reporter group interferes with target affinity or selectivity, then a two-step probe with a small ligation handle, suited for bioorthogonal chemistry, can be used to introduce a reporter group after the probe has reacted with the target. A diverse set of probes has been developed for many enzyme classes, including serine hydrolases, proteases, deubiquitinases, glycosidases, cytochrome P450s and kinases. Different analytical techniques are currently available to visualize, identify and quantify probe-labeled proteins. Depending on the goal and type of the experiment, available protein amount, throughput and sensitivity requirements, a suitable analytical platform can be chosen. ABPP has well-developed applications in discovering new drug targets and in profiling inhibitors for potency and selectivity. ABPP will, therefore, continue to aid research both in fundamental biology and drug discovery.

ABPP is applied in **Chapter 3** for the search for new drug targets. The endocannabinoid system is considered to be an endogenous protective system in various neurodegenerative diseases. Niemann-Pick Type C is a neurodegenerative lysosomal storage disease in which the role of the endocannabinoid system

has not been studied yet. In **Chapter 3**, serine hydrolase activity in brain proteomes of a Niemann-Pick type C mouse model was measured by ABPP and compared to wild type mice. DAGLα, ABHD4, ABHD6, ABHD12, FAAH and MAGL activities were quantified. Chemical proteomics showed that three lysosomal serine hydrolase activities (retinoid-inducible serine carboxypeptidase, cathepsin A and palmitoyl-protein thioesterase 1) were increased in Niemann-Pick C1 protein (NPC1) knockout mouse brain compared to wild type brain, whereas no difference in endocannabinoid hydrolase activity was observed. These targets might be interesting therapeutic targets for future validation studies. With SDS-PAGE and Western blot, differences in DAGLα protein abundance and activity levels were observed. This proteoform of DAGLα was not measured by the mass spectrometry based method. It was hypothesized that the peptides from this proteoform escape detection in the LC-MS measurement.

In **Chapter 3**, two different mass spectrometry methods were used for relative quantification of probe-labeled enzymes. The first uses dimethyl labeling, in which tryptic peptides are modified with isotopically labeled formaldehyde. These labeled peptides were measured on an Orbitrap mass spectrometer. The second method is label-free, where tryptic peptides are measured unmodified on a Synapt mass spectrometer and alignment software is used to compare the signal of each peptide in different samples. The optimized protocol for this label-free method is described in **Chapter 4**.

Chapter 4 consists of a protocol for identifying the *in vivo* selectivity profile of covalent inhibitors using ABPP with label-free quantitative proteomics as exemplified by experimental data on the diacylglycerol lipase inhibitor DH376 in mouse brain, liver, kidney and testes. The stages of the protocol include tissue lysis, probe incubation, enrichment, sample preparation, liquid chromatography-mass spectrometry measurement, processing and data analysis. The results include evidence of target engagement in a native proteome and the identification of potential off-targets for the inhibitor under investigation. The advantages of label-free quantification methods include high proteome coverage and the ability to analyze multiple samples at the same time.

In the protocol described in **Chapter 4**, enzymes labeled with a biotinylated probe are purified using avidin enrichment. After enrichment and washing steps, trypsin is added to the avidin beads and the resulting peptides are measured. However, the probe-modified peptides will remain on the beads. Identifying these probe-modified peptides is necessary to determine how the probe reacts with its target enzymes. A powerful method to characterize both the probe-labeled proteins and their site of modification is using a cleavable linker.[1–5] Additionally, cleavable linkers can be used for top-down proteomics. To be able to measure the probe-modified peptides or intact proteins, an acid cleavable linker with a mass-tag for enhanced signal intensity in MS was designed (**Fig. 1a**).[6] After bioorthogonal ligation with the probe-labeled enzymes and avidin enrichment, the linker can be cleaved with acid. The liberated

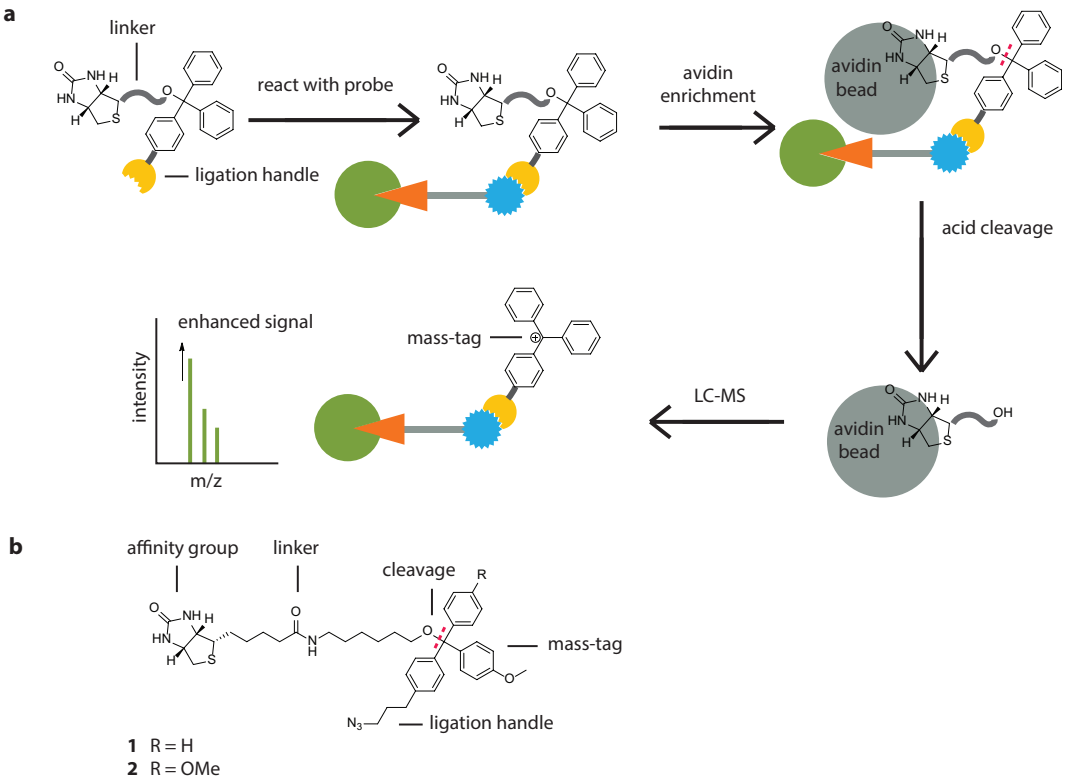
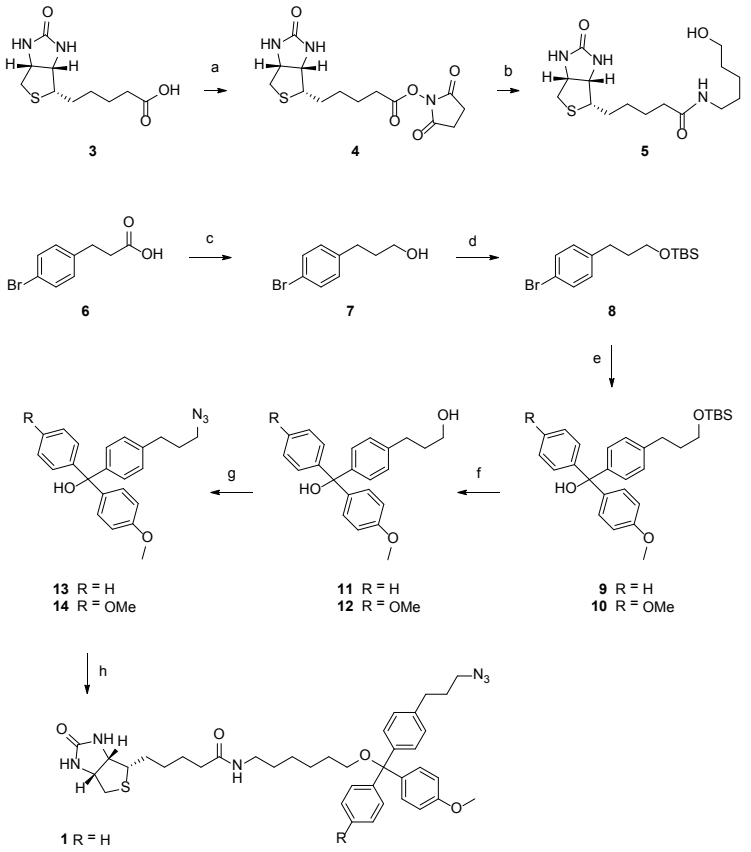


Figure 1. Design of the cleavable trityl-based mass-tag affinity handle. (a) Workflow for the reagent: a trityl moiety as mass-tag and cleavable linker, designed with a biotin moiety and a ligation handle for bioorthogonal chemistry to react with probes. After avidin enrichment, the trityl core is cleaved with acid and the released probe can be measured with LC-MS. The trityl mass-tag will increase the signal intensity. (b) Design of the mass-tag reagents **1** and **2**.

enzymes can either be measured intact or digested with trypsin to provide the probe-labeled peptide. This dual functionality linker is based on the trityl (triarylmethyl) group, which is used extensively in organic chemistry as a protecting group.[7] Para and ortho electron donating groups stabilize the cation and increase signal enhancement in MS. Therefore, two triarylmethyl ethers were designed, monosubstituted (1) or disubstituted (2) with para-methoxys (**Fig. 1b**). An azide was chosen as bioorthogonal ligation handle.

Synthesis. The linker with affinity group was synthesized by conversion of biotin **3** to the activated ester **4**, followed by amide bond formation with an alkyl linker to yield biotin alcohol **5**[8] (**Scheme 1**). The synthesis of the mass-tag started with the reduction of 3-(4-bromophenyl)propionic acid (**6**) with sodium borohydride to alcohol **7**. The hydroxyl group was protected by conversion to the



Scheme 1. Synthesis of the trityl-based mass-tag affinity handles **1** and **2**. Reagents and conditions: (a) NHS, DCC, DMF, 88%; (b) 6-amino-1-hexanol, DMF, 85%; (c) $NaBH_4$, $BF_3{\cdot}Et_2O$, THF, 0 °C > rt, 100%; (d) TBDMS-Cl, imidazole, DMF, 87%; (e) *i*. *n*-BuLi, THF, -78 °C > 0 °C; *ii*. For **9**: 4-methoxybenzophenone, -78 °C > rt, 77%; for **10**: 4,4-dimethoxybenzophenone, -78 °C > rt, 73%; (f) TBAF, THF, 100%; (g) DPPA, DBU, 55 °C, 65% (**13**) and 19% (**14**); (h) **5**, DMF, HCl, molecular sieves 4Å, 14% (**1**) and 20% (**2**).

tert-butyldimethylsilyl ether **8**.[9] The triphenylmethyl core was synthesized from **8** by first forming an organolithium reagent, followed by nucleophilic addition to benzophenone (4-methoxybenzophenone for **9** and 4,4-dimethoxybenzophenone for **10**). The lithium-bromine exchange of **8** did not proceed at -78 °C. However, adding **8** at -78 °C to a solution of *n*-BuLi and allowing the reaction mixture to warm to 0 °C,[10] and subsequently cooling to -78 °C for benzophenone addition, before stirring at rt, resulted in good yields of **9** and **10**.[11] Compounds **9** and **10** were easily visualized on TLC by spraying with H_2SO_4, forming a bright orange spot.[12] Fluorine induced deprotection of **9**[9] was followed by azide substitution of the alcohol **11** by diphenyl phosphoryl azide (DPPA).[13] For **13** a sharp peak at 2093 cm^{-1} was observed in

the IR spectrum, characteristic of azides.[14] The trityl ether **1** was finally formed by mixing the trityl alcohol **13** and biotin alcohol **5** with dry HCl and activated molsieves. Trityl ether **2** was synthesized from **10** with the same procedures, in an overall yield of 4%.

Evaluation. As a model for an acetylene functionalized protein, the free cysteine[15] of bovine serum albumin (BSA) was alkylated with either iodoacetamide or iodoacetamide-alkyne (IAA-alkyne, see Experimental).[16] The resulting functionalized BSA was reacted with different azides using the CuAAC: Cy5-N_3, biotin-N_3, azides **1** and **2** (**Fig. 2**). A clear Cy5 signal is visible at the expected molecular weight of BSA (~69 kDa). To visualize biotinylated protein, a blot with streptavidin-HRP was performed. A chemiluminescent signal is visible for biotin-N_3 at the same height as the fluorescent signal. However, no

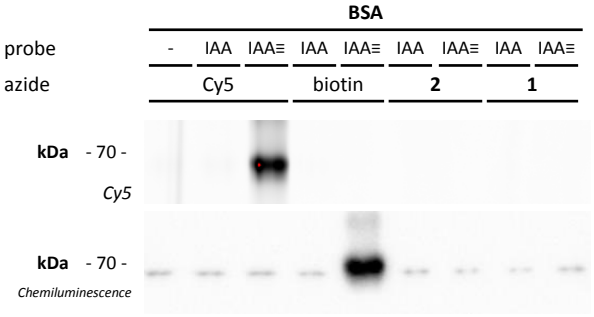
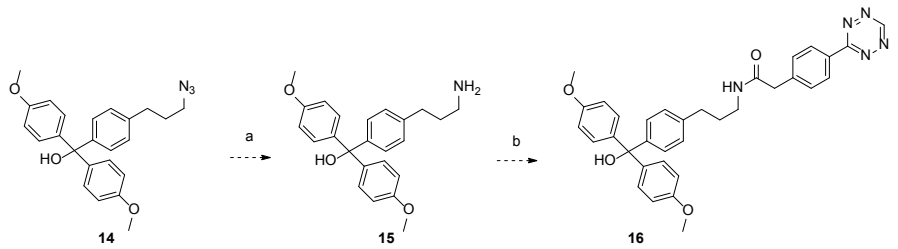


Figure 2. Gel analysis of the CuAAC reaction with BSA-alkyne and different azides. IAA: iodoacetamide. IAA≡: IAA-alkyne.

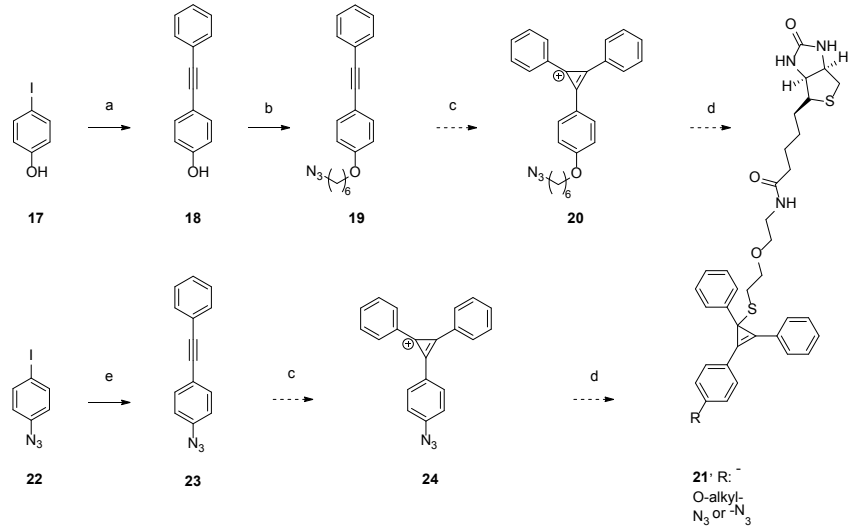
signal is visible for BSA reacted with azides **1** and **2**.

From the SDS-PAGE experiments, it is clear that the CuAAC reaction does not work for these compounds. Different bioorthogonal chemistries could therefore be considered when designing the next generation compounds,[17] such as the inverse electron-demand Diels-Alder reaction with tetrazines and strained alkenes.[18] A potential route for the synthesis of a mass-tag **16** with a tetrazine ligation handle is presented in **Scheme 2**.



Scheme 2. Synthesis of a mass-tag with a tetrazine ligation handle. Reagents and conditions: (a) PPh_3, H_2O; (b) tetrazine-OSu.

A more stable cation used as a mass-tag is the triphenylcyclopropenyl ion.[19] It is the smallest aromatic ring: a three-membered ring containing two electrons.[20] The first derivative of these aromatic rings was synthesized in 1957 by Breslow.[21] Phenyl substituents are not required for the stability of the cation,[22] therefore a smaller mass-tag can be obtained. Two potential synthetic routes to incorporate the tag between an azide and a biotin are shown in **Scheme 3**.



Scheme 3. Proposed synthesis of a mass-tag generating a triphenylcyclopropenyl cation. Reagents and conditions: (a) phenylacetylene, $Pd(OAc)_2$, PPh_3, CuI, Et_3N, THF, 84%; (b) *i*. NaH, DMF *ii*. 1-azido-6-bromohexane (**29**), 87%; (c) *i*. benzene, KOtBu, α,α-dichlorotoluene. *ii*. HBF_4 (aq); (d) biotin-SH; (e) phenylacetylene, MTBE, Et_3N, $Pd(OAc)_2$, PPh_3, CuI, 53%.

Top-down activity-based proteomics. Currently, most proteomics methods use peptide-based bottom-up approaches, where proteins are digested with trypsin before analysis.[26] The activity-based proteomics methods used in **Chapter 3** and **Chapter 4** are examples of a bottom-up approach. The main reason for using this approach is that peptides are more easily separated than intact proteins. Separation of complex proteomes is needed for high proteome coverage. However, bottom-up proteomics suffers from severable draw-backs. For instance, quantification of proteins is inherently problematic due to shared peptides between different proteins (the protein inference problem[27]). Additionally, using peptides for identification of post-translational modifications (PTMs) makes it impossible to assign combinations of PTMs to various proteoforms. A more intuitive approach to proteomics is a top-down approach, where the intact proteins themselves are measured.[28] For example, this method was recently used for characterizing proteoforms of histones with multiple PTMs.[29] Several methods described in this thesis are top-down proteomics techniques, such as SDS-PAGE and Western blotting. Mass spectrometry based top-down proteomics will be standard in the future due to technological advances in intact protein separation. Activity-based proteomics is at an advantage compared to abundance-based proteomics, because biotinylated probes already enable a purification step using avidin chromatography. By using cleavable linkers instead of on-bead digestion to detach the probe-labeled enzymes from avidin beads, the proteins remain intact. For more separating power, reverse-phase chromatography or SDS-PAGE can be used.[30] Top-down activity-based proteomics will enable the identification of active proteoforms and their post-translational modifications.

In **Chapter 5** the synthesis and characterization of the first reported quenched activity-based probes for metabolic serine hydrolases is described. The probes contain a triazole urea electrophilic trap to covalently attach to a catalytic serine. The first probe was active against DAGLα, contains a BODIPY-FL and 2,4-dinitroaniline as fluorophore-quencher pair and was approximately threefold quenched. The second probe contained a Cy5 fluorophore and cAB40 quencher and was more efficiently quenched (± 12-fold). This probe was able to label endogenous ABHD6 *in vitro*, but not did not penetrate the cells. To improve the quenching efficiency of these probes, a possible starting point could be minimalist quenched probes, with only an electrophilic trap, a fluorophore and quencher. Besides the triazole ureas described in **Chapter 5**, another possible electrophilic trap for such minimalist probes is a mixed alkyl aryl phosphonate ester (**Fig. 3a**). This electrophilic trap has been previously used in quenched serine proteases probes.[31] To tune quenching efficiency, various linker lengths and fluorophore-quencher pairs could be synthesized. To decide on a suitable starting point for linker length, computational chemistry can be used. By calculating the lowest energy conformation of the molecule in solution the distance between fluorophore and quencher can be predicted. The optimal distance for FRET energy transfer

for the fluorophore/quencher pair can then be chosen as a starting point for synthesis. To limit the steric bulk of the qABPs, a quencher should function as a leaving group, e.g. dinitrophenol as an aryl on a phosphonate[32] or sulfonate (**Fig. 3b**). Using a successfully quenched minimalist probe as starting point, more selective probes could be designed. For example, fluorescent diaryl phosphonate probes have a small recognition element which provides selectivity for different serine proteases.[33]

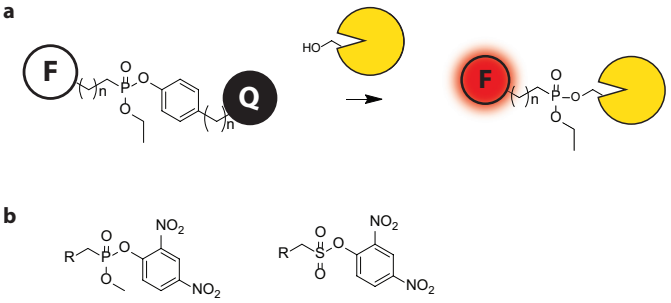


Figure 3. Design of broad-spectrum qABPs for serine hydrolases. (a) General design for mixed alkyl aryl phosphonate esters. F: fluorophore; Q: quencher. (b) 2,4-dinitrophenyl quencher as aryl on phosphonate or sulfonate probes.

In **Chapter 6**, triazole urea probes for diacylglycerol lipase (DAGL) were designed with different bio-orthogonal handles: an alkyne, alkene or norbornene. Two regioisomers of each triazole urea were generated and characterized using NMR analysis supported by DFT calculations. The selectivity of the probes was assessed with activity-based protein profiling and norbornene probe **4** was selected as the most selective DAGL-probe. This probe was potent against endogenous DAGLα (IC_{50} = 5 nM) and was successfully applied as a two-step activity-based probe for labeling of DAGLα using a bioorthogonal ligation in living cells. The inverse electron-demand Diels-Alder reaction has several advantages to surpass the CuAAC reaction as the "go-to" reaction for bioorthogonal chemistry, but improvements are required to make more compact reactants than trans-cyclooctene and aromatic substituted tetrazines. Cyclopropene as a strained alkene is promising in this regard, with possibilities to modify the ring making the reaction faster. Tetrazine chemistry is currently being actively explored.[34] Additionally, minimalist linkers containing the bioorthogonal handles will become more synthetically accessible and commercially available.

To conclude, this thesis described several new strategies and tools for activity-based protein profiling to improve the drug discovery process. A new label-free quantification method for activity-based proteomics was developed. This method allowed the comparison of multiple samples within the same experiment, as exemplified by mapping the *in vivo* off-target interaction landscape of the diacylglycerol lipase inhibitor DH376 in various tissues. Furthermore, this method resulted in the identification of three potential drug targets for Niemann-Pick Type C disease. Additionally, new quenched and two-step probes were developed for cellular profiling of diacylglycerol lipases. It is anticipated that these novel tools and strategies will help to bring forward new treatment opportunities for diseases in which aberrant 2-arachidonoylglycerol signaling plays a role.

Experimental

Synthesis

General remarks. Reagents were purchased from Sigma Aldrich, Acros or Merck and used without further purification unless noted otherwise. Some reactions were performed using oven or flame-dried glassware and dry solvents. All moisture sensitive reactions were performed under an argon or nitrogen atmosphere. Traces of water were removed from starting compounds by co-evaporation with toluene. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV 400 MHz spectrometer at 400 (^{1}H) and 100 (^{13}C) MHz using $CDCl_3$, CD_3OD or $(CD_3)_2SO$ as solvent, unless stated otherwise. Spectra were analyzed using MestReNova 11.0.3. Chemical shift values are reported in ppm with tetramethylsilane or solvent resonance as the internal standard ($CDCl_3$, δ 7.26 for ^{1}H, δ 77.16 for ^{13}C; CD_3OD, δ 3.31 for ^{1}H, δ 49.00 for ^{13}C; $(CD_3)_2SO$, δ 2.50 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, td = triple doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constants J (Hz), and integration. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemmi C18 50x4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Adantage Max mass spectrometer with ESI. The applied buffers were H_2O, MeCN and 1.0% TFA in H_2O (0.1% TFA end concentration). High resolution mass spectra (HRMS) were recorded by direct injection on a q-TOF mass spectrometer (Synapt G2-Si) equipped with an electrospray ion source in positive mode with leu-enkephalin (*m/z* = 556.2771) as an internal lock mass. The instrument was calibrated prior to measurement using the MS/MS spectrum of Glu-1-fibrinopeptide B. HPLC purification was performed on a preparative LC-MS system (Agilent 1200 series) with an Agilent 6130 Quadruple MS detector. IR spectra were recorded on a Shimadzu FTIR-8300 and are reported in cm^{-1}. Flash chromatography was performed using SiliCycle silica gel type SilicaFlash P60 (230 – 400 mesh). TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using UV-irradiation, a KMnO4 stain (K_2CO_3 (40 g), $KMnO_4$ (6 g), H_2O (600 mL) and 10% NaOH (5 mL)) or a H_2SO4 stain (20% H_2SO_4 in EtOH). Molecules shown are drawn using Chemdraw v16.0.

2,5-Dioxopyrrolidin-1-yl-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl) pentanoate (4). To a solution of biotin (0.224 g, 1 mmol) and N-hydroxysuccinimide (0.12 g, 1.0 mmol) in DMF (7.3 mL) was added N,N'-dicyclohexylcarbodiimide (0.27 g, 1.3 mmol). The reaction mixture was stirred o/n, during which a white precipitate formed. The mixture was filtered and the filtrate was

concentrated. The white powder that was obtained was triturated with Et_2O, then filtered and left to dry to the air. The final product (0.30 g, 0.88 mmol, 88% yield) was a white powder, contaminated with dicyclohexylurea. ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 6.41 (d, J = 18.9 Hz, 2H), 4.30 (dd, J = 7.7, 4.9 Hz, 1H), 4.20 – 4.09 (m, 1H), 3.09 (dd, J = 7.8, 4.7 Hz, 1H), 2.92 – 2.81 (m, 1H), 2.73 – 2.52 (m, 3H), 1.76 – 1.50 (m, 4H), 1.54 – 1.34 (m, 2H), 1.34 – 1.10 (m, 1H), 1.14 – 0.94 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.32, 168.98, 162.71, 61.01, 59.19, 55.27, 39.95, 30.02, 27.87, 25.48, 25.36, 24.51.

N-(6-Hydroxyhexyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanamide (5). To a solution of **4** (0.30 g, 0.88 mmol) in DMF (8.8 mL) was added 6-amino-1-hexanol (0.12 g, 1.1 mmol) and the reaction mixture was stirred o/n and concentrated. Purification of the residue by silica gel column chromatography (10% MeOH in DCM) yielded the title compound (0.26 g, 0.75 mmol, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.49 (dd, J = 7.9, 4.9 Hz, 1H), 4.30 (dd, J = 7.9, 4.4 Hz, 1H), 3.54 (t, J = 6.5 Hz, 2H), 3.35 (s, 2H), 3.26 – 3.12 (m, 3H), 3.03 – 2.82 (m, 1H), 2.75 – 2.63 (m, 1H), 2.20 (t, J = 7.3 Hz, 2H), 1.82 – 1.27 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.95, 63.38, 62.87, 61.62, 57.02, 41.04, 40.31, 36.82, 33.57, 30.43, 29.79, 29.51, 27.84, 26.96, 26.64.

3-(4-Bromophenyl)propan-1-ol (7). To a cooled (0 °C) suspension of 3-(4-bromophenyl)propionic acid (13.32 g , 58 mmol) in dry THF (120 mL) was added $NaBH_4$ (4.4 g, 116 mmol), followed by dropwise addition of $BF_3{\cdot}Et_2O$ (14.6 mL, 116 mmol). The reaction mixture was warmed to rt and stirred o/n. The reaction was cooled to 0 °C and carefully quenched by dropwise addition of 1 M HCl. The aqueous layer was extracted with Et_2O (3x). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (20 > 50% EtOAc in pentane) afforded the title compound as a clear oil (12.8 g, 58 mmol, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.3 Hz, 2H), 3.66 (t, J = 6.4 Hz, 2H), 2.74 – 2.57 (m, 2H), 2.23 (s, 1H), 1.86 (dt, J = 13.9, 6.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.81, 131.53, 130.30, 119.68, 62.10, 34.03, 31.52.

(3-(4-Bromophenyl)propoxy)(*tert*-butyl)dimethylsilane (8). To a solution of **7** (12.8 g, 58 mmol) in dry DMF (250 mL) were added TBDMS-Cl (10.5 g, 69.6 mmol) and imidazole (9.86 g, 145 mmol) and the mixture was stirred o/n. H_2O (400 mL) was added and the aqueous layer was extracted with Et_2O (3 x 250 mL). The combined organic layers were washed with H_2O (200 mL), brine (200 mL), dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1:99 EtOAc:pentane) afforded the title compound (16.6 g, 50.4 mmol, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, J = 8.3 Hz, 2H), 7.07 (d, J = 8.3 Hz, 2H), 3.61 (t, J = 6.2 Hz, 2H), 2.69 – 2.59 (m, 2H), 1.86 – 1.72 (m, 2H), 0.91 (s, 9H), 0.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.34, 131.43, 130.40, 119.50, 62.19, 34.39, 31.64, 26.09, 18.47, -5.14.

(4-(3-((*tert*-Butyldimethylsilyl)oxy)propyl)phenyl)(4-methoxyphenyl)(phenyl)methanol (9). To a cooled (-78 °C) solution of *n*-BuLi (1.6 M in hexanes, 7.5 mL, 12 mmol) was added dropwise a solution of **8** (3.29 g, 10 mmol) in Et_2O (80 mL) in 20 min. The reaction mixture was allowed to warm to 0 °C and stirred for 1 h. The reaction mixture was cooled to -78 °C before dropwise addition of a solution of 4-methoxybenzophenone (2.12 g, 10 mmol) in THF (40 mL) in 15 min. The reaction mixture was allowed to gradually warm up to rt and stirred for 30 min. The reaction was quenched with H_2O and diluted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$, H_2O, brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1% Et_3N, 2 > 10% EtOAc in pentane) afforded the title compound (3.6 g, 7.7 mmol, 77%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 – 7.13 (m, 11H), 6.88 (d, J = 8.8 Hz, 2H), 3.83 (s, 3H), 3.72 (t, J = 6.3 Hz, 2H), 3.00 (s, 1H),

2.85 – 2.66 (m, 2H), 1.92 (dt, J = 13.7, 6.4 Hz, 2H), 1.00 (s, 9H), 0.14 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.62, 147.35, 144.66, 141.18, 139.48, 129.28, 128.04, 127.92, 127.88, 127.11, 125.48, 113.20, 81.65, 62.47, 55.25, 34.38, 31.73, 26.08, 18.42, -5.14.

(4-(3-((*tert*-Butyldimethylsilyl)oxy)propyl)phenyl)*bis*(4-methoxyphenyl)methanol (10). To a cooled (-78 °C) solution of *n*-BuLi (1.6 M in hexanes, 7.5 mL, 12 mmol) was added dropwise a solution of **8** (3.29 g, 10 mmol) in Et_2O (90 mL) in 20 min. The reaction mixture was allowed to warm to 0 °C and stirred for 25 min. The reaction mixture was cooled to -78 °C before dropwise addition of a solution of 4,4'-dimethoxybenzophenone (2.42 g, 10 mmol) in THF (40 mL) in 15 min. The reaction mixture was allowed to gradually warm up to rt and stirred o/n. The reaction was quenched with H_2O and diluted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$, H_2O, brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1% Et_3N, 5 > 10% EtOAc in pentane) afforded the title compound (3.2 g, 7.3 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 – 7.09 (m, 8H), 6.86 – 6.80 (m, 4H), 3.80 (s, 6H), 3.63 (t, J = 6.4 Hz, 2H), 2.67 (m, 3H), 1.89 – 1.76 (m, 2H), 0.90 (s, 9H), 0.04 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.68, 144.88, 141.25, 139.72, 129.22, 128.09, 127.85, 113.24, 81.46, 62.55, 55.39, 34.44, 31.78, 26.11, 18.49, -5.11.

3-(4-(Hydroxy(4-methoxyphenyl)(phenyl)methyl)phenyl)propan-1-ol (11). To a solution of **9** (3.6 g, 7.7 mmol) in THF (7.7 mL) was added TBAF (1.0 M in THF, 23 mL, 23 mmol). The reaction mixture was stirred over the weekend, diluted with EtOAc and washed with H_2O, brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1% Et_3N, 20 > 70% EtOAc in pentane) afforded the title compound (2.7 g, 7.7 mmol, 100%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.21 (m, 6H), 7.23 – 7.01 (m, 6H), 6.90 – 6.74 (m, 2H), 3.79 (s, 3H), 3.66 (t, J = 6.4 Hz, 2H), 2.82 (s, 1H), 2.77 – 2.64 (m, 2H), 1.95 – 1.81 (m, 2H), 1.36 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.73, 147.29, 144.86, 140.84, 139.43, 129.29, 128.07, 128.04, 127.98, 127.92, 127.23, 81.70, 62.42, 55.38, 34.20, 31.73.

3-(4-(Hydroxybis(4-methoxyphenyl)methyl)phenyl)propan-1-ol (12). To a solution of **10** (0.32 g, 0.73 mmol) in THF (3 mL) was added TBAF (1.0 M in THF, 1.5 mL, 1.5 mmol). The reaction mixture was stirred for 2 h, diluted with EtOAc and washed with H_2O, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1% Et_3N, 50 > 70% EtOAc in pentane) afforded the title compound (0.24 g, 0.73 mmol, 100%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.13 (m, 8H), 6.81 (m, 4H), 3.77 (s, 6H), 3.63 (t, J = 6.5 Hz, 2H), 2.96 (s, 1H), 2.77 – 2.57 (m, 2H), 1.95 – 1.76 (m, 2H), 1.64 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.60, 145.07, 140.69, 139.67, 129.19, 127.97, 127.94, 113.20, 81.37, 62.32, 55.33, 34.14, 31.69.

(4-(3-Azidopropyl)phenyl)(4-methoxyphenyl)(phenyl)methanol (13). To a solution of **11** (697 mg, 2 mmol) in DMF (4 mL) was added DPPA (517 µL, 2.4 mmol), DBU (359 µL, 2.4 mmol) and NaN_3 (195 mg, 3 mmol). The reaction mixture was stirred o/n at 55 °C. H_2O and Et_2O were added, the organic layer was seperated and washed with brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (20% EtOAc, 1% Et_3N in pentane) afforded the title compound (0.48 g, 1.3 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.08 (m, 16H), 6.86 – 6.78 (m, 2H), 3.79 (s, 3H), 3.28 (t, J = 6.8 Hz, 2H), 2.79 (s, 1H), 2.68 (t, J = 8.4, 6.8 Hz, 2H), 1.90 (dq, J = 8.7, 6.9 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.75, 147.23, 145.14, 139.85, 139.36, 129.98, 129.28, 128.13, 128.08, 127.99, 127.90, 127.26, 125.72, 120.22, 113.30, 81.68, 55.36, 50.76, 32.43, 30.42. IR: sharp peak at 2093 cm^{-1} (N_3).

(4-(3-Azidopropyl)phenyl)bis(4-methoxyphenyl)methanol (14). To a solution of **12** (757 mg, 2 mmol) in DMF (4 mL) was added DPPA (517 µL, 2.4 mmol), DBU (359 µL, 2.4 mmol) and NaN_3 (195 mg, 3 mmol). The reaction mixture was stirred o/n at 55 °C. H_2O and Et_2O were added, the organic layer was washed with brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (10 > 20% EtOAc, 1% Et_3N in pentane) afforded the title compound (0.15 g, 0.38 mmol, 19%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 – 7.07 (m, 8H), 6.81 (m, 4H), 3.77 (s, 6H), 3.27 (t, J = 6.8 Hz, 2H), 2.82 (s, 1H), 2.68 (t, J = 7.6 Hz, 2H), 1.89 (p, J = 6.9 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.64, 145.36, 139.70, 139.59, 129.17, 128.03, 128.00, 113.22, 81.35, 55.32, 50.73, 32.40, 30.39. IR: sharp peak at 2093 (-N_3).

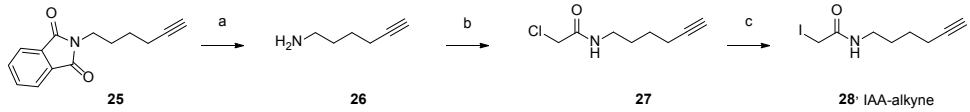
N-(6-((4-(3-Azidopropyl)phenyl)(4-methoxyphenyl)(phenyl)methoxy)hexyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanamide (1). To a solution of **5** (34 mg, 0.10 mmol) in DMF (1 mL) were added activated molecular sieves (4 Å, 180 mg), **13** (75 mg, 0.2 mmol) in 2 x 0.2 mL DMF and HCl (4 M in dioxane, 250 µL). The reaction mixture was stirred over the weekend. Et_3N (0.3 mL) was added to the reaction mixture. Purification of the residue by silica gel column chromatography (2 > 5 % (1:9 NH_4OH (30% aq.) : MeOH) in DCM) afforded the title compound (10 mg, 14 µmol, 14%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.46 – 7.08 (m, 11H), 6.84 (d, J = 8.9 Hz, 2H), 4.44 (dd, J = 7.8, 4.6 Hz, 1H), 4.26 (dd, J = 7.9, 4.4 Hz, 1H), 3.77 (s, 3H), 3.33 – 3.25 (m, 6H), 3.15 (ddt, J = 9.2, 6.8, 3.0 Hz, 3H), 3.04 (t, J = 6.4 Hz, 2H), 2.99 (s, 1H), 2.92 – 2.84 (m, 2H), 2.71 – 2.62 (m, 3H), 2.18 (t, J = 7.3 Hz, 2H), 1.93 – 1.23 (m, 20H).^{13}C NMR (100 MHz, CD_3OD) δ 175.92, 166.08, 160.04, 146.55, 143.98, 141.05, 137.45, 131.39, 129.82, 129.45, 128.79, 128.67, 127.74, 113.94, 87.20, 64.37, 63.35, 61.59, 57.03, 55.70, 51.79, 41.05, 40.32, 36.81, 33.32, 31.64, 31.01, 30.36, 29.78, 29.50, 27.82, 27.19, 26.95. HRMS *m/z* calculated for $C_{39}H_{50}N_6O_4S$ $[M + Na]^+$ 721.3506, found 721.3506.

N-(6-((4-(3-Azidopropyl)phenyl)*bis*(4-methoxyphenyl)methoxy)hexyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanamide (2). To a solution of **5** (68 mg, 0.20 mmol) in DMF (2 mL) were added activated molecular sieves (4 Å, 150 mg), **14** (162 mg, 0.4 mmol) in 2 x 0.4 mL DMF and HCl (4 M in dioxane, 50 µL). The reaction mixture was stirred o/n. Et_3N (0.1 mL) was added to the reaction mixture. Purification of the residue by silica gel column chromatography (2 > 5 % (1:9 NH_4OH (30% aq.) : MeOH) in DCM) afforded the title compound (30 mg, 41 µmol, 20%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.36 – 7.23 (m, 6H), 7.10 (d, J = 8.3 Hz, 2H), 6.86 – 6.76 (m, 4H), 4.42 (dd, J = 7.8, 4.4 Hz, 1H), 4.25 (dd, J = 7.9, 4.4 Hz, 1H), 3.74 (s, 6H), 3.26 (t, J = 6.7 Hz, 2H), 3.19 – 3.09 (m, 3H), 3.03 (t, J = 6.4 Hz, 2H), 2.98 (s, 1H), 2.90 – 2.82 (m, 2H), 2.65 (dd, J = 14.8, 8.1 Hz, 3H), 2.18 (t, J = 7.3 Hz, 2H), 1.84 (dt, J = 14.2, 6.8 Hz, 2H), 1.76 – 1.22 (m, 16H).^{13}C NMR (100 MHz, CD_3OD) δ 175.86, 165.86, 159.88, 144.51, 140.81, 138.00, 131.08, 129.55, 128.76, 113.93, 86.90, 64.29, 63.33, 61.56, 57.01, 55.69, 51.78, 41.06, 40.32, 36.81, 33.31, 31.62, 31.05, 30.37, 29.77, 29.49, 27.84, 27.20, 26.95. HRMS *m/z* calculated for $C_{40}H_{52}N_6O_5S$ $[M+Na]^+$ 751.3612, found 751.3614.

4-(Phenylethynyl)phenol (18). This procedure was adapted from literature procedures.[23] A mixture of PPh_3 (52 mg, 0.2 mmol), CuI (10 mg, 0.05 mmol), 4-iodophenol (2.2 g, 10 mmol) and phenylacetylene (1.3 mL, 12 mmol), THF (10 mL) and triethylamine (6.9 mL) was sonicated with nitrogen bubbling though. $Pd(OAc)_2$ (22 mg, 0.1 mmol) was added and after stirring for 1 h, the reaction mixture was diluted with Et_2O, washed with sat. aq. NH_4Cl, H_2O, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (5 > 10% EtOAc in pentane) yielded the product (1.6 g, 8.4 mmol, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.48 (m, 2H), 7.45 – 7.40 (m, 2H), 7.38 – 7.28 (m, 3H), 6.88 – 6.72 (m, 2H), 4.90 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.68, 133.42, 131.59, 128.46, 128.13, 123.61, 115.82, 115.63, 89.30, 88.21.

1-((6-Azidohexyl)oxy)-4-(phenylethynyl)benzene (19). To a solution of **18** (0.49 g, 2.5 mmol) in DMF (25 mL) was added NaH (60 % dispersion in mineral oil, 100 mg, 2.5 mmol). After stirring for 1 h, 1-azido-6-bromohexane (**29**, 0.42 g, 2.1 mmol) was added and the reaction mixture was stirred o/n, cooled to 0 °C, quenched with H_2O, extracted with Et_2O, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (5% Et_2O in pentane) yielded the product (0.48 g, 1.5 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 – 7.43 (m, 4H), 7.40 – 7.28 (m, 3H), 6.99 – 6.81 (m, 2H), 3.96 (t, J = 6.4 Hz, 2H), 3.28 (t, J = 6.9 Hz, 2H), 1.80 (dq, J = 8.0, 6.4 Hz, 2H), 1.63 (p, J = 7.0 Hz, 2H), 1.58 – 1.38 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.21, 133.16, 131.55, 128.42, 128.03, 123.72, 115.31, 114.61, 89.55, 88.14, 67.88, 51.49, 29.19, 28.92, 26.63, 25.80. IR: 2089 ($-N_3$).

1-Azido-4-(phenylethynyl)benzene (23). A mixture of 1-azido-4-iodobenzene (0.5 M in MTBE, 5 mL, 2.5 mmol), phenylacetylene (0.33 mL, 3 mmol) and triethylamine (1.7 mL) was sonicated with nitrogen bubbling though. $Pd(OAc)_2$ (5.6 mg, 0.025 mmol), PPh_3 (13 mg, 0.05 mmol) and CuI (2.4 mg, 0.013 mmol) were added and after 1.5 h the reaction mixture was diluted with Et_2O, washed with sat. aq. NH_4Cl, H_2O, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (0 > 1% Et_2O in pentane) yielded the product (0.29 g, 1.3 mmol, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 – 7.47 (m, 4H), 7.40 – 7.29 (m, 3H), 7.02 – 6.94 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.04, 133.20, 131.68, 128.50, 128.47, 123.20, 119.94, 119.19, 89.83, 88.81. IR: 2126 ($-N_3$).



Scheme 4. Synthesis of IAA-alkyne (**28**). Reagents and conditions: (a) hydrazine hydrate, EtOH, 98%; (b) chloroacetic anhydride, N-methyl morpholine, 29%; (c) NaI, acetone, 56%.

5-Hexynylamine (26). To a suspension of N-(5-hexynyl)phtalimide (**25**, 1.1 g, 5.0 mmol) in EtOH (20 mL) was added hydrazine hydrate (1.2 mL, 20 mmol). The reaction mixture was refluxed for 1 h and cooled to rt. Sat. aq. $NaHCO_3$ was added and the pH of the aqueous layer was adjusted to 10 with NaOH (10 M), before being extracted with EtOAc (5 x 50 mL). The combined organic layers were acidified with 1 M HCl and concentrated to yield the title compound as a HCl salt (0.66 g, 4.9 mmol, 98%). ^{1}H NMR (400 MHz, MeOD) δ 2.98 (t, 2H), 2.32 - 2.28 (m, 3H), 1.90 - 1.77 (m, 2H), 1.67 - 1.59 (m, 2H).

2-Chloro-N-(hex-5-yn-1-yl)acetamide (27). To a cooled (0 °C) solution of **26** (654 mg, 4.9 mmol) in DCM (25 mL) was added N-methyl morpholine (2.2 mL, 20 mmol), followed by portionwise addition of chloroacetic anhydride (1.7 g, 10 mmol). The reaction was stirred o/n at rt, diluted with Et_2O (250 mL), washed with HCl (3 M), NaOH (1 M, 3x), brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (10 > 50% EtOAc in petroleum ether) yielded the title compound (0.24 g, 1.4 mmol, 29%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.69 (s, 1H), 4.01 (s, 2H), 3.29 (q, J = 6.8 Hz, 2H), 2.19 (td, J = 6.8, 2.6 Hz, 2H), 1.94 (t, J = 2.6 Hz, 1H), 1.75 – 1.42 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.96, 83.86, 68.93, 42.70, 39.33, 28.39, 25.57, 18.07.

N-(Hex-5-yn-1-yl)-2-iodoacetamide (28, IAA-alkyne). To a solution of **27** (0.24 g, 1.4 mmol) in acetone (5.6 mL) was added NaI (0.43 g, 2.9 mmol) and the reaction mixture was stirred o/n.

The solvent was removed and purification of the residue by silica gel column chromatography (3:7 EtOAc:PE) afforded the title compound (210 mg, 0.79 mmol, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (s, 1H), 3.73 (s, 2H), 3.35 – 3.25 (m, 2H), 2.24 (td, J = 6.9, 2.7 Hz, 2H), 1.99 (t, J = 2.6 Hz, 1H), 1.73 – 1.54 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.51, 83.99, 68.95, 39.90, 28.30, 25.61, 18.14, -0.13.

1-Azido-6-bromohexane (29). To a heated (50 °C) solution of 1,6-dibromohexane (2.3 mL, 15 mmol) in DMF (20 mL) was added NaN_3 (0.65 g, 10 mmol). The reaction mixture was stirred at 55 °C o/n, cooled to rt, diluted with Et_2O and washed with NaOH (1 M), brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (0 > 3% Et_2O in PE) yielded the product (0.42 g, 2.1 mmol, 21%). NMR ($CDCl_3$) corresponded to literature.[24]

Biochemical methods

General. Cy5-N_3 was synthesized according to previously published procedures[36] and biotin-N_3 was purchased from Bio-Connect Life Sciences. All buffers and solutions were prepared using Millipore water (deionized using a MilliQ A10 Biocel, with a 0.22 µm filter) and analytical grade reagents and solvents. Bio-Rad Imagelab v5.2.1 was used for gel analysis.

Click conditions.[15] To a solution of bovine serum albumin (0.1 g/L in 100 mM phosphate buffer (pH 8), 16.25 µL, 1.6 µg) was added IAA-alkyne **29** (200 µM in DMSO, 1 µL) or IAA (200 µM in DMSO, 1 µL) and the reaction mixture was incubated for 1 h at rt. Subsequently, to each sample, one of the different azides was added: Cy5-N_3, biotin-N_3, **1** or **2** (0.8 mM, 1 µL), followed by 0.75 µL of a premixed CuSO4/THPTA solution: CuSO4 (8 mM, 5 µL) and THPTA (20 mM, 10 µL). Finally, sodium ascorbate (100 mM, 1 µL) was added and the mixture was incubated for 1 h at 37 °C.
SDS-PAGE. The samples were denatured with Laemmli buffer (final concentrations: 60 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 1.25% (v/v) β-mercaptoethanol, 0.01% (v/v) bromophenol blue) and resolved on a 10% acrylamide SDS-PAGE gel (180 V, 75 min). The Cy3/Cy5 channels were scanned on the ChemiDoc (Bio-Rad) before proteins were transferred to a 0.2 µm polyvinylidene difluoride membrane by Trans-Blot Turbo™ Transfer system (Bio-Rad). The membrane was washed with TBS (50 mM Tris, 150 mM NaCl) and TBST (TBS, 0.05% Tween 20) before blocking with 5% milk (w/v, Elk magere melkpoeder, FrieslandCampina) in TBST for 1 h, washed with TBST (3x), incubated with Streptavidin-HRP (Invitrogen), diluted 1:4000 in 5% BSA (TBST), washed with TBST (3x), washed with TBS and developed in the dark with 10 mL luminol, 100 µL ECL enhancer and 3 µL H_2O_2 (30%). The signal was detected on the ChemiDoc (Bio-Rad) using standard chemiluminescence settings.

References

1. Speers, A. E. & Cravatt, B. F. A tandem orthogonal proteolysis strategy for high-content chemical proteomics. *J. Am. Chem. Soc.* **127**, 10018–10019 (2005).
2. Fonović, M., Verhelst, S. H. L., Sorum, M. T. & Bogyo, M. Proteomics evaluation of chemically cleavable activity-based probes. *Mol. Cell. Proteomics* **6**, 1761–1770 (2007).
3. Leriche, G., Chisholm, L. & Wagner, A. Cleavable linkers in chemical biology. *Bioorganic Med. Chem.* **20**, 571–582 (2012).
4. Rudolf, G. C., Heydenreuter, W. & Sieber, S. A. Chemical proteomics: Ligation and cleavage of protein modifications. *Curr. Opin. Chem. Biol.* **17**, 110–117 (2013).
5. Szychowski, J. *et al.* Cleavable biotin probes for labeling of biomolecules via azide-alkyne cycloaddition. *J. Am. Chem. Soc.* **132**, 18351–18360 (2010).
6. Li, Y. *et al.* Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry for biomedical analysis. *Biomed. Chromatogr.* **20**, 597–604 (2006).
7. Wuts, P. G. M. & Greene, T. W. *Greene's Protective Groups in Organic Synthesis, Fourth Edition*, 152. (2007).
8. Susumu, K. *et al.* Enhancing the stability and biological functionalities of quantum dots via compact multifunctional ligands. *J. Am. Chem. Soc.* **129**, 13987–13996 (2007).
9. Corey, E. J. & Venkateswarlu, A. Protection of Hydroxyl Groups as tert-Butyldimethylsilyl Derivatives. *J. Am. Chem. Soc.* **94**, 6190–6191 (1972).
10. Bailey, W. F., Luderer, M. R. & Jordan, K. P. Effect of solvent on the lithium-bromine exchange of aryl bromides: Reactions of n-butyllithium and tert-butyllithium with 1-bromo-4-tert-butylbenzene at 0 °C. *J. Org. Chem.* **71**, 2825–2828 (2006).
11. Geurink, P. P. *et al.* Design of peptide hydroxamate-based photoreactive activity-based probes of zinc-dependent metalloproteases. *Eur. J. Org. Chem.* 2100–2112 (2010).
12. Deno, N. C., Jaruzelski, J. J. & Schriesheim, A. Carbonium Ions. I. An Acidity Function (C0) Derived from Arylcarbonium Ion Equilibria. *J. Am. Chem. Soc.* **77**, 3044–3051 (1955).
13. Thompson, A. S., Humphrey, G. R., DeMarco, A. M., Mathre, D. J. & Grabowski, E. J. J. Direct conversion of activated alcohols to azides using diphenyl phosphorazidate. A practical alternative to Mitsunobu conditions. *J. Org. Chem.* **58**, 5886–5888 (1993).
14. Lieber, E., Rao, C. N. R., Chao, T. S. & Hoffman, C. W. W. Infrared Spectra of Organic Azides. *Anal. Chem.* **29**, 916–918 (1957).
15. Hong, V., Presolski, S. I., Ma, C. & Finn, M. G. Analysis and optimization of copper-catalyzed azide-alkyne cycloaddition for bioconjugation. *Angew. Chem. Int. Ed.* **48**, 9879–9883 (2009).
16. Backus, K. M. *et al.* Proteome-wide covalent ligand discovery in native biological systems. *Nature* **534**, 570–4 (2016).
17. Patterson, D. M., Nazarova, L. A. & Prescher, J. A. Finding the Right (Bioorthogonal) Chemistry. *ACS Chem. Biol.* **9**, 592–605 (2014).
18. Elliott, T. S., Bianco, A., Townsley, F. M., Fried, S. D. & Chin, J. W. Tagging and Enriching Proteins Enables Cell-Specific Proteomics. *Cell Chem. Biol.* **23**, 805–815 (2016).
19. Topolyan, A. P. *et al.* A triphenylcyclopropenylium mass tag: synthesis and application to ultrasensitive LC/MS analysis of amines. *Analyst* **141**, 3289–3295 (2016).
20. Breslow, R. & Yuan, C. The sym-Triphenylcyclopropenyl Cation, a Novel Aromatic System. *J. Am. Chem. Soc.* **80**, 5991–5994 (1958).
21. Breslow, R. Synthesis of the s-Triphenylcyclopropenyl Cation. *J. Am. Chem. Soc.* **79**, 5318 (1957).
22. Breslow, R., Lockhart, J. & Chang, H. W. The Diphenylcyclopropenyl Cation. Synthesis and Stability. *J. Am. Chem. Soc.* **83**, 2375–2379 (1961).
23. Phetrak, N. *et al.* Regioselectivity of Larock Heteroannulation: A Contribution from Electronic Properties

of Diarylacetylenes. *J. Org. Chem.* **78**, 12703 (2013).
24. Coutrot, F. & Busseron, E. Controlling the chair conformation of a mannopyranose in a large-amplitude [2] rotaxane molecular machine. *Chem. - A Eur. J.* **15**, 5186–5190 (2009).
25. Xu, R. & Breslow, R. 1,2,3-Triphenylcyclopropenium Bromide. *Org. Synth.* **74**, 72 (1997).
26. Aebersold, R. & Mann, M. Mass spectrometry-based proteomics. *Nature* **422**, 198–207 (2003).
27. Nesvizhskii, A. I. & Aebersold, R. Interpretation of Shotgun Proteomic Data. *Mol. Cell. Proteomics* **4**, 1419–1440 (2005).
28. Catherman, A. D., Skinner, O. S. & Kelleher, N. L. Top Down Proteomics: Facts and Perspectives. *Biochem. Biophys. Res. Commun.* **445**, 683–693 (2014).
29. Zhou, M., Paša-Tolić, L. & Stenoien, D. L. Profiling of Histone Post-Translational Modifications in Mouse Brain with High-Resolution Top-Down Mass Spectrometry. *J. Proteome Res.* **16**, 599–608 (2017).
30. Takemori, N. *et al.* Top-down/Bottom-up Mass Spectrometry Workflow Using Dissolvable Polyacrylamide Gels. *Anal. Chem.* **89**, 8244-8250 (2017).
31. Serim, S., Baer, P. & Verhelst, S. H. L. Mixed alkyl aryl phosphonate esters as quenched fluorescent activity-based probes for serine proteases. *Org. Biomol. Chem.* **13**, 2293–9 (2015).
32. Cevasco, G. & Thea, S. Kinetic Study on the Alkaline Hydrolysis of Some Tetracoordinate P″ Esters of 2,4-Dinitrophenol. J. Chem. Soc. PerkinTrans. 2. 1103–1105 (1994).
33. Edgington-Mitchell, L. E. *et al.* Fluorescent diphenylphosphonate-based probes for detection of serine protease activity during inflammation. *Bioorganic Med. Chem. Lett.* **27,** 254–260 (2017).
34. Wieczorek, A., Werther, P., Euchner, J. & Wombacher, R. Green- to far-red-emitting fluorogenic tetrazine probes – synthetic access and no-wash protein imaging inside living cells. *Chem. Sci.* **8**, 1506–1510 (2017).
35. Patterson, D. M., Nazarova, L. A. & Prescher, J. A. Finding the Right (Bioorthogonal) Chemistry. *ACS Chem. Biol.* **9**, 592–605 (2014).
36. Kvach, M. V. *et al.* A convenient synthesis of cyanine dyes: Reagents for the labeling of biomolecules. *Eur. J. Org. Chem.* 2107–2117 (2008).