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Supplementary Information (SI)

DNA-Encoded Chemical Libraries enable the discovery of potent PSMA-ligands with substantially reduced affinity towards the GCPIII anti-target

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1 Materials and Instrumentations

1.1 Reagents, solvents, purification methods and software

Solvents and chemicals were used as purchased from ABCR GmbH, Sigma-Aldrich, Merck, VWR, Iris Biotech, Combi-Blocks, Apollo scientific, Enamine, MedChemExpress, TCI, Acros and Alfa Aesar. Milli-Q® water (mQ H_2O) was obtained from a Millipore Milli-Q system (Merck). Oligonucleotides were purchased from LGC Biosearch Technologies or Eurofins and quantified with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). Selections were performed using KingFisher (Thermo Fisher Scientific) magnetic particle processor. Materials for ligations and for polymerase-chain reactions were purchased from New England Biolabs. PCR purification and gel extraction kits were purchased from Qiagen.

Purification of oligonucleotides

Preparative reversed-phase high-performance liquid chromatography (RP-HPLC) for the purification of oligonucleotides was performed with an Agilent 1200 Series as well as with a Waters Alliance HT coupled with a PDA UV detector that have been equipped with a XTerra Shield RP18 preparative column (125 Å, 10 x 150 mm). Gradients of eluent C (100 mM triethylammonium acetate (TEAA) pH 7 in mQ H_2O) and eluent D (100 mM TEAA pH 7 in 80% acetonitrile) were applied at a flow rate of 4 mL/min. After purification fractions were combined, dried under reduced pressure in a SpeedVac vacuum concentrator (RVC 2-25CDplus, Martin Christ) and re-dissolved for ethanol (EtOH) precipitation.

Purification methods and gradients:

- Purification of library pools after splint ligation was performed using the following method: flow rate: 4 mL/min, 60°C column temperature, gradient: 0-1 min 95% C, 1-12.8 min 95% to 84% C, 12.8-13.3 min 84% to 30% C, 13.3-18 min 30% C, 18-20 min 30% to 0% C, 20-25 min 0% C, 25-25.1 min 0% to 95% C, 25.1-26 min 95% C).

Purification of library pools after coupling of the HITs (no splint ligation) was performed using the following methods:

- ASM DELs (derived from PRA DEL and MB pool 3) were purified using Agilent 1200 Series: (% of eluent C) 0-2 min 90% C, 2-10 min 90% to 50% C, 10-13 min 50% to 0% C, 13-17 min 0% C, 17-17.1 min 0& to 90% C, 17.1-18 min 90% C. Flow 4 mL/min. Column temperature: 30 °C.
- The other ASM DELs were purified using Waters Alliance HT (% of eluent C) 0-1 min 95% C, 1-5 min 95% to 85% C, 5-7 min 85% to 20% C, 7-15 min 20% C, 15-15.1 min 20% to 0% C, 15.1-18 min 0% C, 18-18.1 min 0% to 95% C, 18.1-20 min 95% C. Flow 4 mL/min. Column temperature: 30 °C.

Characterization of oligonucleotides

Liquid Chromatography-Mass Spectrometry (LC-MS) spectra were recorded on an Agilent 1260 Series LC equipped with an ACQUITY UPLC Oligonucleotide BEH C18 column (130 Å, 1.7 µm, 2.1 x 50 mm) coupled to an Agilent 6100 Series Single Quadrupole MS. The following gradient of eluent A (15 mM TEA, 400 mM HFIP in mQ H_2O) and eluent B (methanol) was applied at a flow rate of 0.4 mL/min and 60°C column temperature: 0-0.2 min 95% A, 0.2-8.2 min 95% to 5% A, 8.2-8.7 min 5% A, 8.7-9.2 min 5 to 95% A, 9.2-13 min 95% A.

Liquid Chromatography-Mass Spectrometry (LC-MS) spectra were recorded on a Waters Acquity UPLC H-Class system coupled to an ESI-TOF-MS (Waters Xevo G2XS Q-TOF) which was equipped with a Waters Acquity BEH C18 column (2.1 x 50 mm, 130 Å, 1.7 µm). A gradient of mQ water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B) was applied at 40°C column temperature and a flow rate of 0.6 mL/min (5% to 80% B in 6 min).

Purifications and analytical methods for small molecules

Direct-phase medium-pressure liquid chromatography was performed using a Combi*Flash* NextGen 300+ (TELEDYNE ISCO) on RediSep® Silver Flash column (size from 4 grams to 40 grams). Gradient and solvent mixtures are specified for every purification.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Reversed-phase high-performance liquid chromatography of small amounts of product was performed on a semi preparative Agilent 1200 Series RP-HPLC with a PDA UV detector. The system was equipped with a Synergi 4 µm, Polar-RP 80 Å 10 × 150 mm C18 column using flow rate of 5 mL/min with the following gradient of eluent A (mQ H_2O 0.1% TFA) and eluent B (acetonitrile 0.1% TFA): 0-13 min 90% to 0% A, 13-14 min 0% A, 14-15 min 0% to 90% A, 15-16 min 90% A. Product fractions were combined and lyophilized overnight.

Reversed-phase high-performance liquid chromatography of big amounts of product was performed on a preparative Thermo Fisher UltiMate 3000 with PDA UV detector, using a Hypersil Prep HS phenyl column 5 um, 150 nm at a flow rate of 30 mL/min with linear gradients of solvents A and B (A= Millipore water with 0.1% TFA, B = ACN with 0.1% TFA): 0-1 min 90% A, 1-20 min 90%to 0% A, 20-22 min 0% A, 22-23 min 0% to 90% A, 23-24 min 90% A. Product fractions were combined and lyophilized overnight.

Compounds that were not visible at 260 nm wavelength were detected at 210 nm wavelength.

High-Resolution mass spectrometry (HR-MS)

HR-MS were performed on a Q Exactive Mass Spectrometer (Thermo Fisher Scientific). The analyte was injected directly into the MS at a flow rate of 4 µL/min. Both MS1 and MS2 spectra were recorded. MS1 spectra were obtained with a resolution of 70000. MS2 spectra were obtained by inducing fragmentation of the molecule with a NCE (normalized collision energy) = 25 and with a resolution of 70000.

HR-MS analysis were performed with analytical purpose to confirm compound identity but not to analyze the purity.

1H NMR and 13C NMR spectra

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were measured at 298 K on a Bruker AV400 (400 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) with deuterated solvents as reference signal. The coupling constants (*J*) are given in Hz using the following multiplicity abbreviations: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, quintet = qu, sextet = sext, doublet of doublets = dd, doublet of triplets = dt, triplet of doublets =td, doublet of doublet of doublets = ddd, m = multiplet.

Software

Databases were organized with InstantJChem (ChemAxon) and ChemInventory. Fingerprints have been visualized and evaluated with MATLAB R2019b (The MathWorks, Inc.) and DataWarrior (OpenMolecules). Data fitting and statistical analysis were performed with GraphPad Prism (version 8, Graphpad Software, Inc.). Flow cytometry data was analyzed using FlowJo (version 10, Tree Star). MS data were analyzed with MassLynx (Waters) and ChemStation (Agilent). NMR were processed with ACD/Lab and Mnova version 14 Mestrelab Research.

Statistical analysis

Data reported in this article have been generated as independent replicates. Values deriving from replicate measurements are given as mean ± standard error. Error bars have been calculated based on standard deviation of two or three replicates.

2 Library Synthesis

Different on-DNA amide coupling conditions were screened on a set of Fmoc amino acids in order to construct DNA-Encoded Chemical Libraries. The efficiency of the coupling has been tested on a model of amine terminal DNA strand. Unreactive amino acids (threshold 75% yield by LC-MS) have been discarded and not used for library construction.

2.1 LL (Linear Library) Synthesis

Fmoc amino acids used for LL library construction were coupled to the DNA following the three procedures described below.

2.1.1 General procedures for on-DNA amide formation

General procedure (I) for amide coupling using EDC/HOAt/NMM

Fmoc-amino acids (200 mM in dimethyl sulfoxide (DMSO), 26 eq.) were diluted with DMSO (56 mM) and a freshly prepared stock solution of EDC:HOAt:NMM (100:20:100 mM in DMSO, 12:2.4:12 eq.) was added. After 15 min at rt, the pre-activated acid was added to the free amino oligonucleotide (1 eq., 1.6 mM), previously dissolved in MOPS buffer (100 mM MOPS, 1 M NaCl, pH 8). The reaction was shaken for 15 h at 37°C and then quenched with a 500 mM NH_4OAc solution. The mixture was shaken for 30 min at 30 °C followed by EtOH precipitation, centrifugation and drying under reduced pressure.

General procedure (II) for amide coupling using EDC/s-NHS

Pre-activation of Fmoc-amino acids (200 mM in DMSO, 50 eq.) was performed by diluting the original stock with DMSO (56 mM). To this solution was added 1- ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 100 mM in DMSO, 48 eq.) and N-

hydroxysulfosuccinimide (s-NHS, 333 mM in DMSO:water 2:1, 133 eq.). After 30 min at 30°C, the pre-activated acid was added to the free amino oligonucleotide (1 eq., 1.6 mM), previously dissolved in MOPS buffer (100 mM MOPS, 1 M NaCl, pH 8). The reaction proceeded for 15 h at rt. The DNA was then EtOH precipitated, centrifuged and dried under reduced pressure.

General procedure (III) for amide coupling using DMT-MM

Fmoc-amino acids (200 mM in DMSO, 200 eq.) were diluted with DMSO (56 mM) and DMT-MM (200 mM in water, 150 eq.) was added. The pre-activation proceeded for 15 min at rt and the acid was added to the free amino-oligonucleotide (1 eq., 1.6 mM), previously dissolved in MOPS buffer (100 mM MOPS, 1 M NaCl, pH 8). The reaction was shaken for 15 h at 37°C. The DNA was then EtOH precipitated, centrifuged and dried under reduced pressure.

2.1.2 LL library synthesis - Step 1

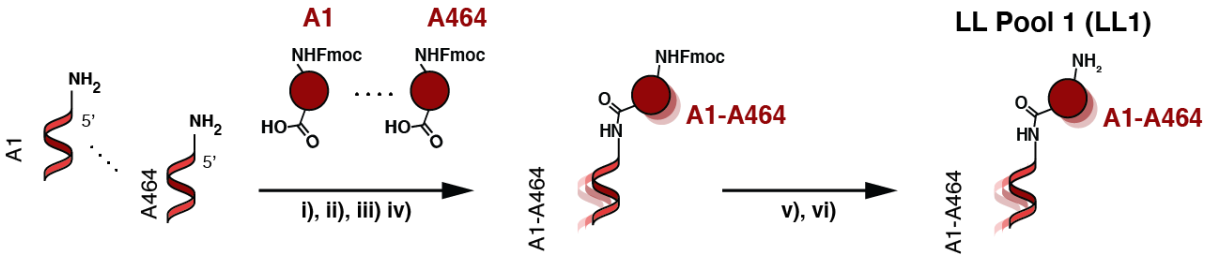


Figure S1. Schematic illustration of the synthesis of **LL-DEL Pool 1**. i) Amide coupling of 464 Fmoc-amino acids to individual Code A (A1-A464); ii) EtOH precipitation; iii) HPLC purification of individual derivatized oligonucleotides; iv) Pooling of the 464 derivatized oligonucleotides to generate Pool 1; v) Fmoc deprotection; vi) EtOH precipitation.

Amide coupling of 464 Fmoc-amino acids

Amide couplings of the 464 Fmoc-amino acids with the corresponding 464 codes A were performed following the coupling procedures (**I**), (**II**) and (**III**). The Fmoc-amino acid was pre-

activated and then added to a solution of 50 nmol of code A (5'-amino-C6-GGAGCTTCTGAATTCTGTGTGCTGXXXXXXCGAGTCCCATGGCGC-3' where XXXXXX are the bases used as coding region) in MOPS buffer (100 mM MOPS, 1 M NaCl, pH 8). The reaction was stopped by EtOH precipitation, centrifuged and the pellet was dried under reduced pressure using SpeedVac. Then, the crude was dissolved in 0.1 M TEAA. The DNA-conjugates were individually purified by RP-HPLC. After lyophilization, the DNA pellet was re-dissolved in water (200 µL) analysed by LC-MS and quantified by UV absorbance.

Pool 1 and Fmoc deprotection

The 464 DNA-conjugates (2 nmol each) were combined to generate the pool 1 without further purification. To an aqueous solution of the pool 1, TEA (10% v/v) were added, and the reaction was shaken for 2 h at rt. Subsequently, the DNA was EtOH precipitated, centrifuged and dried under reduced pressure using a SpeedVac. Afterwards, the dry DNA pellet was dissolved in water and the derived **LL pool 1** was used as crude for the elongation with Code B in the next step.

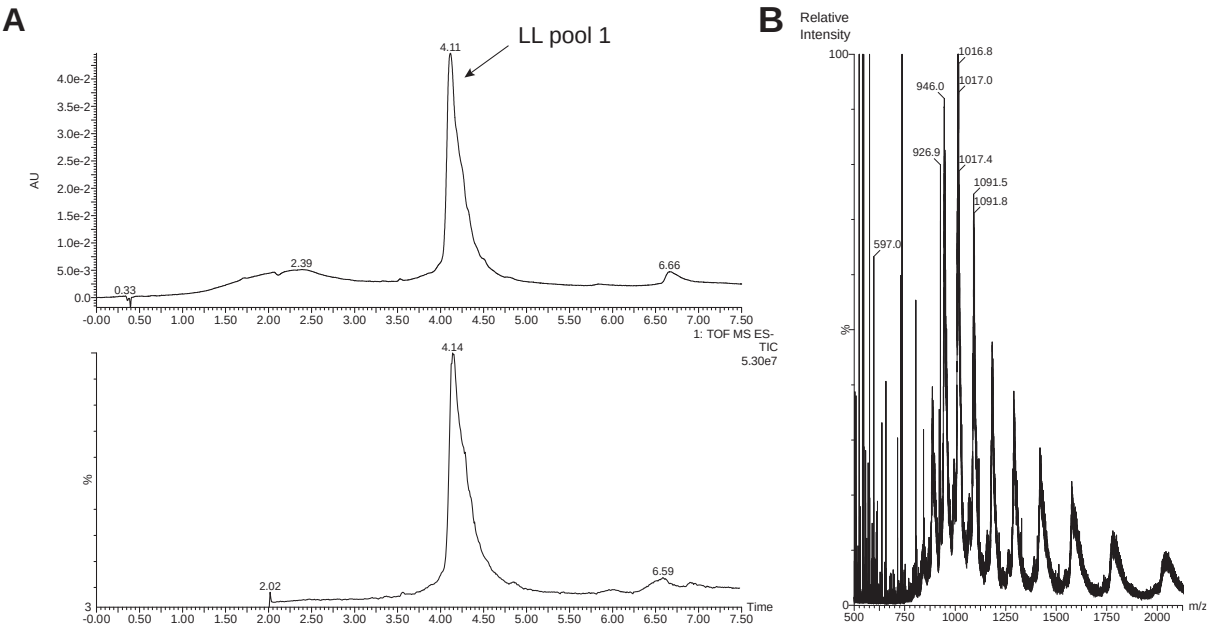


Figure S2. LC-MS analysis of **LL pool 1** after Fmoc deprotection and EtOH precipitation**. A**. LC chromatogram of **LL pool 1** registered at 260 nm; **B**. Non-deconvoluted mass spectra of **LL pool 1**.

2.1.2 LL library synthesis - Step 2

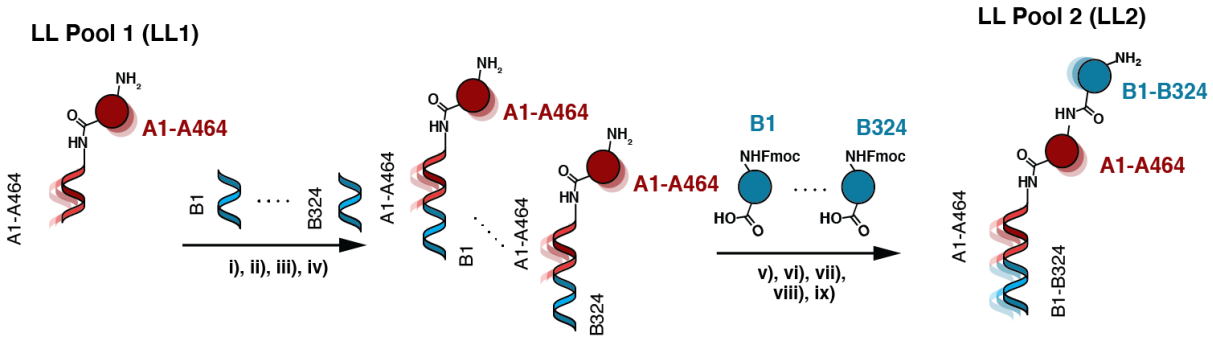


Figure S3. Schematic illustration of the synthesis of **LL-DEL Pool 2**. i) Splitting of Pool 1 in 324 wells; ii) Splint Ligation of 324 individual Code B (B1-B324); iii) EtOH precipitation; iv) Amide coupling of 324 Fmoc-amino acids to the respective derivatized Code B (B1-B324); v) Pooling of the 324 derivatized oligonucleotides to generate Pool 2; vi) EtOH precipitation; vii) Fmoc deprotection; viii) EtOH precipitation; ix) HPLC purification.

Splint ligation with CodeB

The pool 1 was aliquoted in 324 wells (2.67 nmol each.) using 96-well plates. To each aliquot, 324 unmodified oligonucleotides were added a code B (5'-Phosp-CGGATCGACGYYYYYYYGCGTCAGGCAGC-3', 4.0 nmol, 1.5 eq. where YYYYYYY are the bases used for the coding region), the Adaptor 1 (5'-CGTCGATCCGGCGCCATGGG-3', 4.1 nmol, 1.6 eq.) and T4 DNA ligase buffer (21 µL, New England BioLabs, Catalog: B0202S). The mixture was heated for 5 min at 70 °C. The solution was then cooled down to rt for 30 min, and T4 DNA Ligase (6.5 µL, 2600 U, New England BioLabs, Catalog: M0202S) was added to each well. After 2h at rt the reactions were quenched by heating for 10 min at 65°C. The successful encoding was evaluated by electrophoresis analysis (Invitrogen™ Novex™ TBE-Urea Gels, 15%, Catalog: EC68855BOX). The DNA pellets were EtOH precipitated, centrifuged and dried under reduced pressure.

Amide coupling of 324 Fmoc-amino acids, Pool 2, Fmoc deprotection and Purification

The DNA pellets previously encoded with Codes B, were singularly coupled to 324 Fmoc-amino acids following the coupling procedures (**I**), (**II**) and (**III**). The Fmoc-amino acid was

pre-activated, and the solution was subsequently added to the DNA pellets (2.67 nmol, 1 eq.) already dissolved in MOPS buffer (100 mM MOPS, 1 M NaCl, pH 8). At completion of the coupling, the 324 reactions were pooled together (pool 2) and EtOH precipitated. Fmoc deprotection of the pool was performed by adding water (1.5 mL), and TEA (150 µL, 10% v/v). The reaction proceeded for 3 h at 37 °C. The DNA pellet was EtOH precipitated twice and then was purified by RP HPLC at 70 °C to remove the adaptor and the residual code B. The successful encoding and removal of adaptor was determined by electrophoresis gel and LC-MS (**Figures S4-S5)** After lyophilization of combined fractions, the DNA pellet was re-dissolved in water (4 mL) and quantified by UV absorbance (120 nmol of LL pool 2).

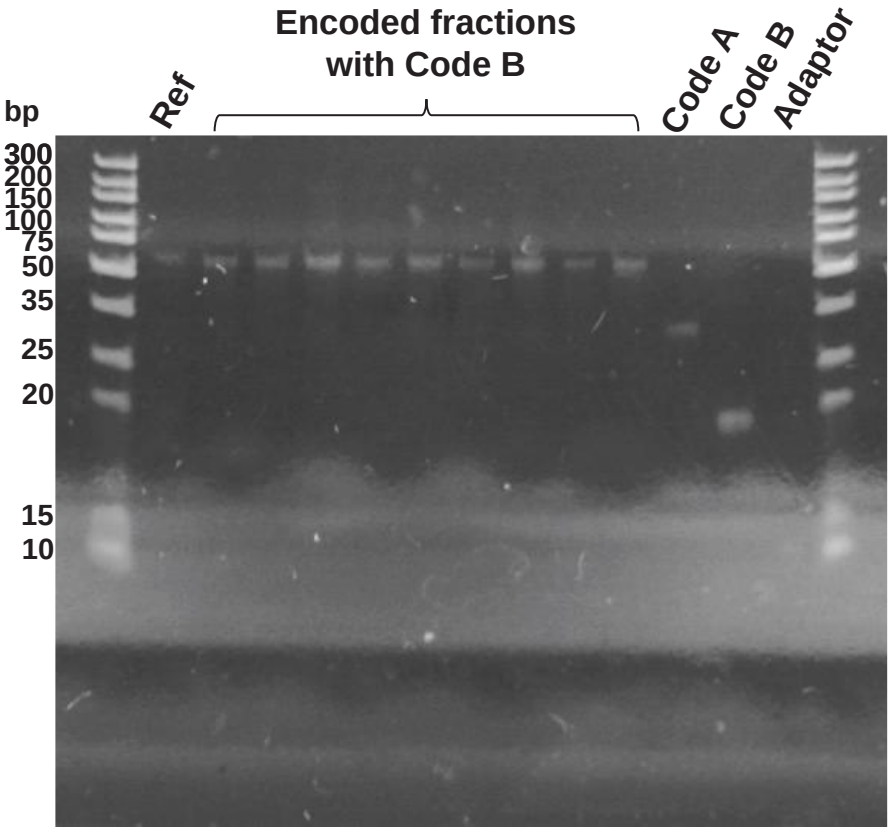


Figure S4. TBE Urea 15% gel of LL pool 2 fractions obtained after HPLC purification.

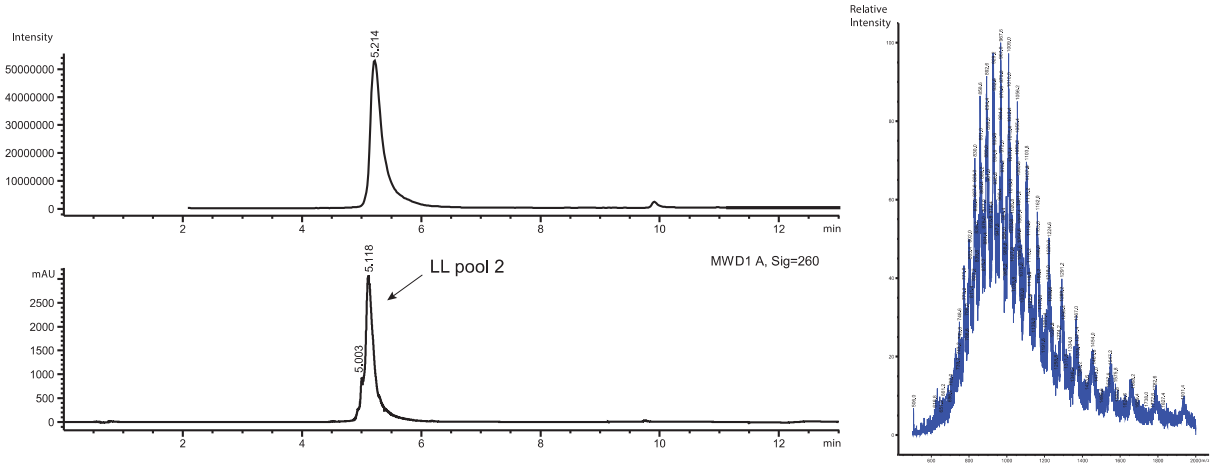


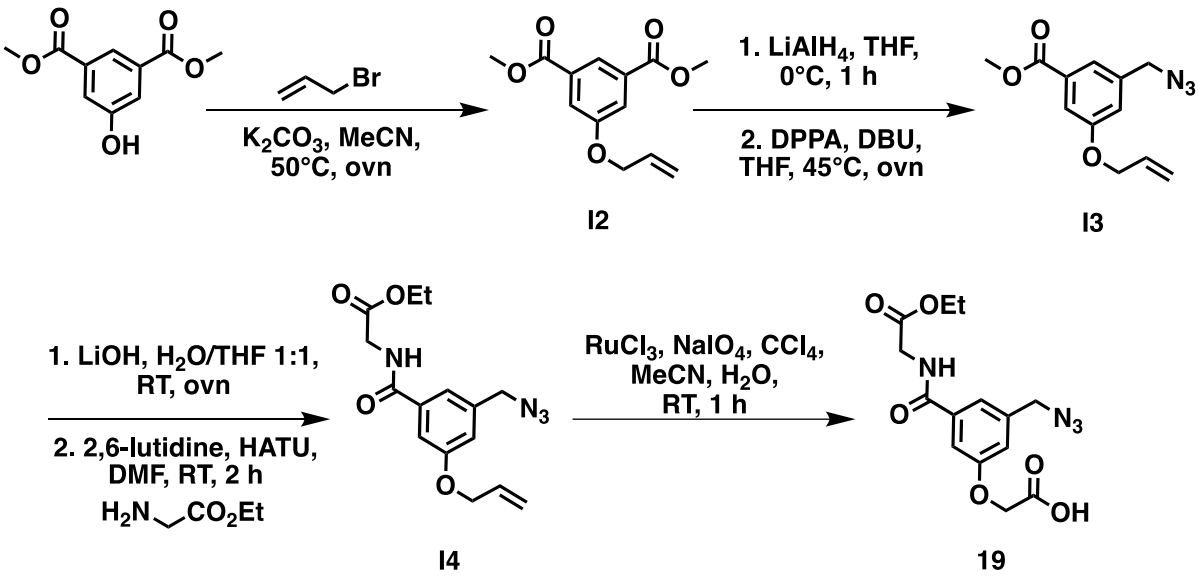
Figure S5. LC-MS analysis of **LL pool 2** after Fmoc deprotection and RP-HPLC purification**. A**. LC chromatogram of **LL pool 2** registered at 260 nm. **B**. Non-deconvoluted mass spectra of **LL pool 2**.

2.2 MB Library Synthesis

General remark for synthetic procedures.

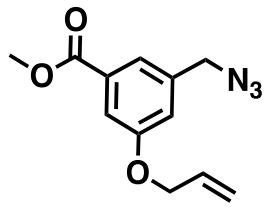
Solution-phase intermediates are reported as **In** (n = 1, 2, …).

2.2.1 MB scaffold synthesis (compound 19)



Scheme S1. Schematic illustration of the synthesis of compound **19.**

Synthesis of methyl 3-(allyloxy)-5-(azidomethyl)benzoate (I3)



Commercially available dimethyl-5-hydroxyisophatalate (4.00 g, 19.3 mmol, 1 eq.) was dissolved in Acetonitrile (MeCN or ACN, 50 mL) and K_2CO_3 (6.7 g, 48.2 mmol, 2.5 eq.) was added. Afterwards, allyl bromide (2.0 mL, 23.1 mmol, 1.2 eq.) was added dropwise to the reaction mixture. The reaction was stirred overnight at 50 °C and monitored by Thin Layer Chromatography (TLC), then ethyl acetate (EtOAc) was added and washed with water (x 2), the organic layer was washed with brine (x 1) and then dried with Na_2SO_4. The solvent was removed under reduced pressure to obtain compound **I2** as white solid. The crude product was used for the next step without further purification. Compound **I2** (2.55 g, 10.2 mmol, 1 eq.) was dissolved in anhydrous tetrahydrofuran (THF, 34 mL). $LiAlH_4$ 1 M in THF (5.1 mL, 5.1 mmol, 0.5 eq.) was added dropwise in 20 min at 0 °C. The mixture was stirred for 1 h at 0 °C and monitored by TLC. Water (3 mL) was added to quench the $LiAlH_4$ in excess and the reaction was neutralized with 2 M HCl. The aqueous phase was extracted with EtOAc (x 3), the combined organic phases were washed with brine (x 1) and then dried with Na_2SO_4 and evaporated. The crude was purified by flash chromatography on silica gel (Hexane:EtOAc, elution gradient from 20% to 40% of EtOAc) to afford the reduced derivative of compound **I2** (1.83 g) that was not isolated. The benzylic alcohol derivative of **I2** was dissolved in 80 mL of anhydrous THF and DPPA (2.65 mL, 12.3 mmol, 1.5 eq) and DBU (1.84 mL, 12.3 mmol, 1.5 eq.) were added. The mixture was stirred overnight at 45 °C. The solvent was evaporated, and the crude was extracted with EtOAc, washed with water (x 2), brine (x 1) and then dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude was purified by flash chromatography on silica gel (Hexane:EtOAc, elution gradient from 10% to 30% of EtOAc)

to obtain compound **I3** (2.28 g, yield = 90%). HR-MS (ESI+) *m/z* calculated for $C_{12}H_{13}N_3O_3$ $[M+H]^+$ 248.10297; detected 248.10306; accuracy = 0.36 ppm. ^1H NMR (400 MHz, DMSO-d6): δ (ppm) = 7.57 (t, *J* = 1.4 Hz, 1 H), 7.45 (dd, *J* = 2.5, 1.3 Hz, 1 H), 7.26 (dd, *J* = 2.4, 1.6 Hz, 1 H), 6.01 – 6.08 (m, 1 H), 5.42 (dd, *J* = 17.4 Hz, 1.6 Hz, 1 H), 5.29 (dd, *J* = 10.5, 1.6 Hz, 1 H), 4.66 (dt, *J* = 5.1, 1.5 Hz, 2 H), 4.53 (s, 2 H), 3.87 (s, 3 H).

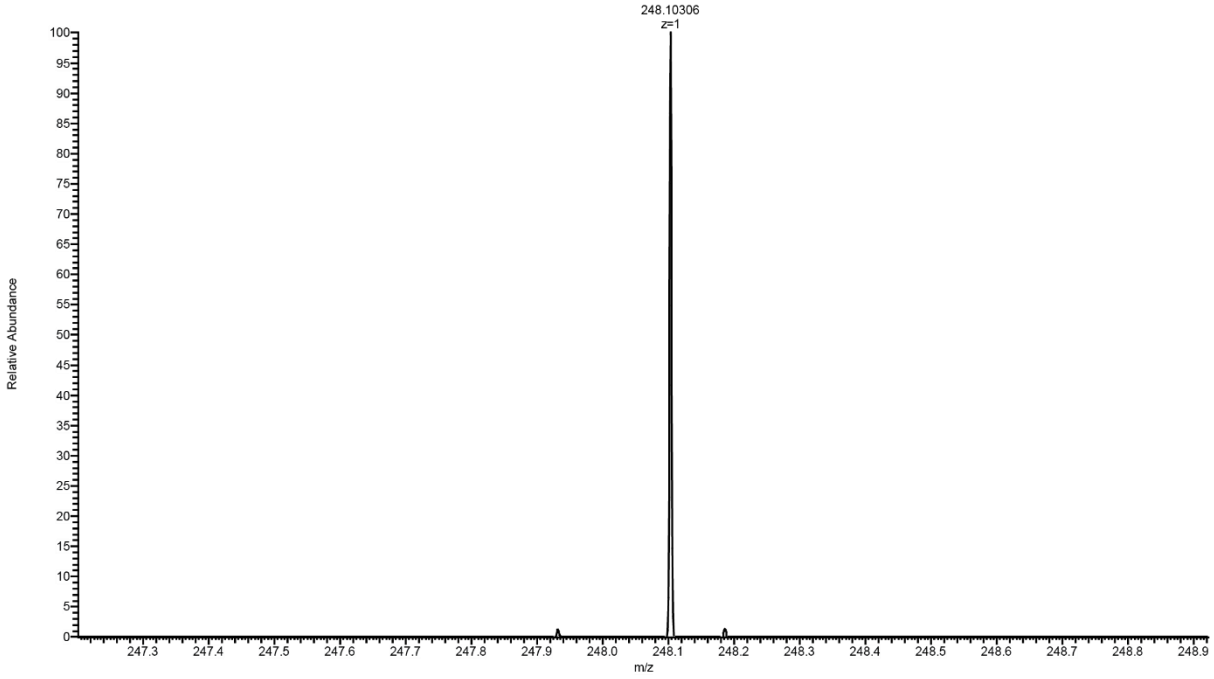
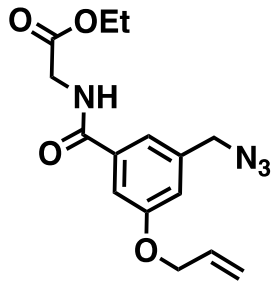


Figure S6. HR-MS of intermediate **I3**

Synthesis of ethyl (3-(allyloxy)-5-(azidomethyl)benzoyl)glycinate (I4)



Compound **I3** (2.03 g, 8.21 mmol, 1 eq.) was dissolved in 26 mL of 1 M LiOH in THF and the reaction was stirred overnight at rt. A 5% Na_2CO_3 aqueous solution was added, and the mixture was extracted with EtOAc (x 1). The aqueous layer was acidified with 2 M HCl (to pH = 2)

and was extracted with dichloromethane (DCM, x 3). The combined organic phases were dried over Na_2SO_4 and evaporated to obtain the free acid as white solid (1.00 g). The crude compound (0.83 g, 3.58 mmol, 1 eq.) was dissolved in dimethylformamide (DMF, 12 mL), and glycine ethyl ester hydrochloride (0.50 g, 3.58 mmol, 1 eq.), 2,6-lutidine (0.83 mL, 7.16 mmol, 2 eq.) and HATU (1.64 g, 4.30 mmol, 1.2 eq.) were added. The mixture was stirred for 2 h at room temperature. Reaction solvent was removed under reduced pressure, and the crude was dissolved in EtOAc, washed with water, the combined organic phases were dried over Na_2SO_4 and evaporated. The crude was purified by flash chromatography on silica gel (Hexane:EtOAc, elution gradient from 20% to 50% of EtOAc) to obtain compound **I4** (0.933 g, 82%). HR-MS (ESI+) *m/z* calculated for $C_{15}H_{18}N_4O_4$ $[M+H]^+$ 319.14008; detected: 319.13966; accuracy = 1.32 ppm. ^{1}H NMR (400 MHz, DMSO-d6,): δ (ppm) = 8.98 (t, *J*=5.8 Hz, 1 H), 7.43 – 7.47 (m, 2 H), 7.16 (s, 1 H), 6.05 – 6.11 (m, 1 H), 5.44 – 5.45 (m, 1 H), 5.30 – 5.41 (m, 1 H), 4.65 (dt, *J*= 5.3, 1.3 Hz, 2 H), 4.50 (s, 2 H), 4.13 (q, *J* = 7.1 Hz, 2 H), 4.00 (d, *J* = 5.8 Hz, 2 H), 1.21 (t, *J* = 7.1 Hz, 3 H).

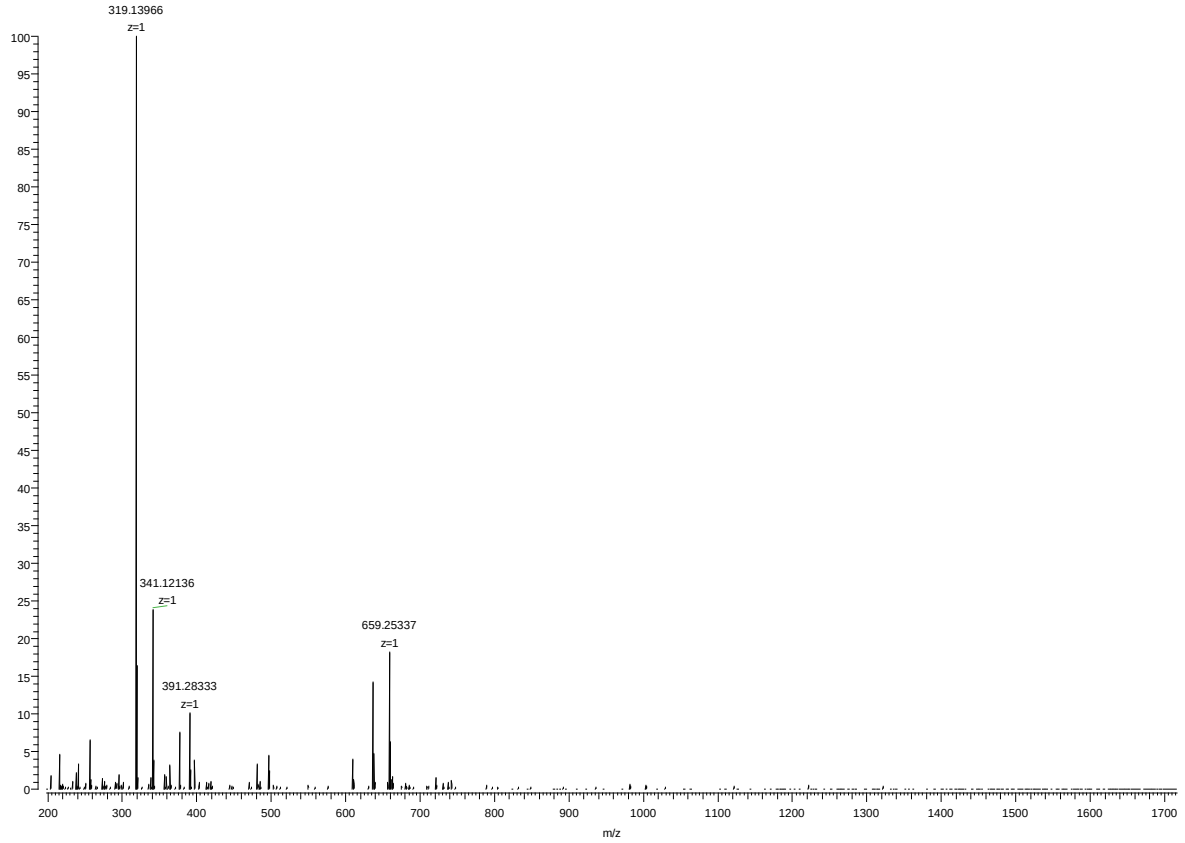
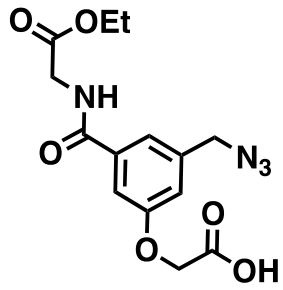


Figure S7. HR-MS of intermediate **I4**

Synthesis of 2-(3-(azidomethyl)-5-((2-ethoxy-2-oxoethyl)carbamoyl)phenoxy)acetic acid (19; MB library scaffold)



To a solution of compound **I4** (0.933 g, 2.73 mmol, 1 eq.) in MeCN:CCl_4:water (3:3:4, 100 mL), $RuCl_3$•H_2O (0.0186 g, 2% w/w) and $NaIO_4$ (5.84 g, 27.3 mmol, 10 eq.) were added. The mixture was stirred for 1 h at rt. After evaporation of the solvent, water was added and the mixture was extracted with DCM (x 3) and with DCM:MeOH (9:1, x 2). the combined organic phases were dried over Na_2SO_4 and evaporated. The crude was purified by flash

chromatography on silica gel (DCM:MeOH, 5% of MeOH) to obtain the final scaffold **19** (0.67 g, 73%). HR-MS (ESI+) *m/z* calculated for $C_{14}H_{16}N_4O_6$ $[M+H]^+$ 337.11426; detected: 337.11354; accuracy = 2.14 ppm. ^{1}H NMR (400 MHz, DMSO-d6): δ (ppm) = 13.09 (bs, 1 H), 8.99 (t, *J* = 6.3 Hz, 1 H), 7.45 - 7.53 (m, 1 H), 7.31 - 7.44 (m, 1 H), 7.04 - 7.20 (m, 1 H), 4.77 (s, 2 H), 4.51 (s, 2 H), 4.13 (q, *J* = 7.1 Hz, 2 H), 4.00 (d, *J* = 6.3 Hz, 2 H), 1.21 (t, *J* = 7.1 Hz, 3 H). ^{13}C NMR (100 MHz, DMSO-d6): δ (ppm) =170.38, 170.26, 166.33, 158.22, 138.04,135.78, 120.42, 118.19, 1128.98, 112.94, 65.06, 60.94, 14.57.

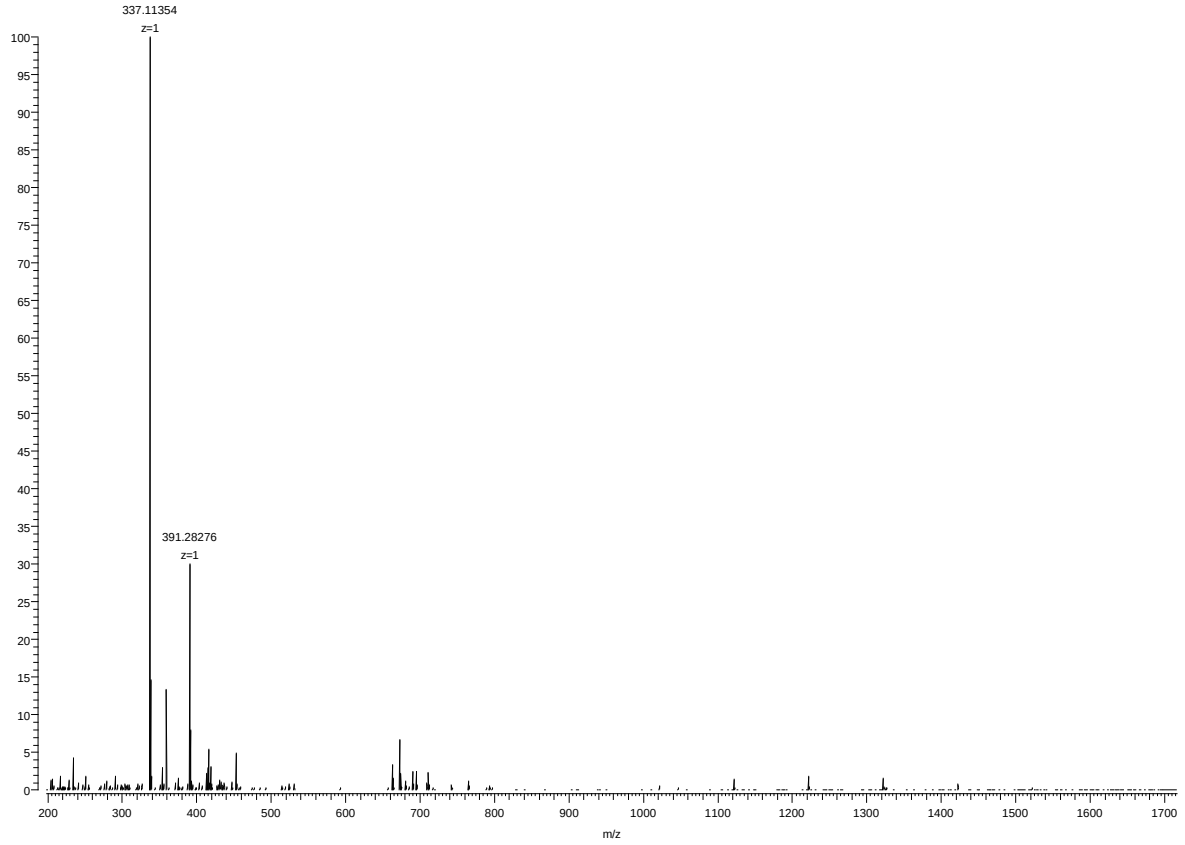


Figure S8. HR-MS of compound **19**

2.2.2 MB library synthesis - Step 1

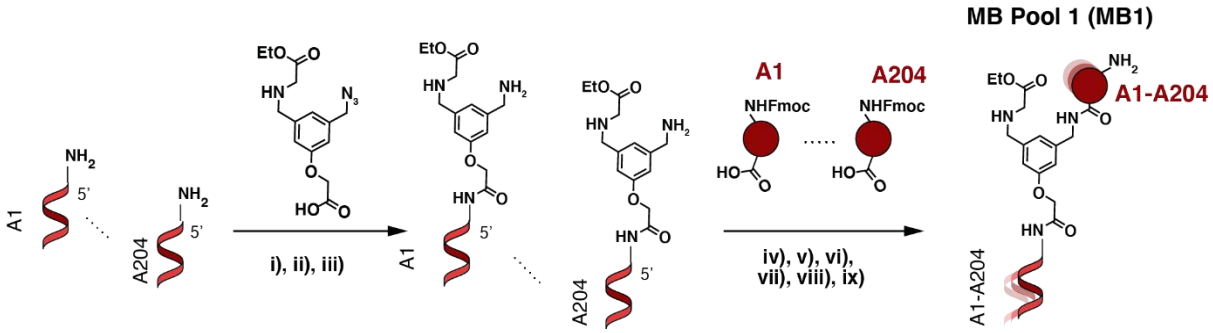


Figure S9. Schematic illustration of the synthesis of **MB-DEL Pool 1**. i) Coupling of the scaffold (compound **19**) to 204 individual Codes A (A1-A204); ii) Staudinger reaction; iii) EtOH precipitation; iv) Coupling of 204 Fmoc-amino acids to the derivatized Codes A (A1-A204); v) EtOH precipitation; vi) HPLC purification of individual derivatized oligonucleotides; vii) Pooling of the 204 derivatized oligonucleotides to generate Pool 1; viii) Fmoc deprotection; ix) EtOH precipitation.

Coupling of the Scaffold

Each of 204 amino modified oligonucleotide bearing a unique sequence (code A: 5'-amino-C6-GGAGCTTCTGAATTCTGTGTGCTGXXXXXXCGAGTCCCATGGCGC-3', where XXXXXX are the bases used as coding region) were derivatized with the scaffold (**19**) following the reported protocol.

A 500 mM stock solution of compound **19** in DMSO (5 µL, 2.50 µmol, 50 eq.) was diluted with DMSO (235 µL) in a 2 mL Eppendorf tube. A 333 mM stock solution of sulfo-NHS in DMSO:water 2:1 (20 µL, 6.7 µmol, 133 eq.) was added, followed by a 100 mM DMSO solution of EDC (24 µL, 2.4 µmol, 48 eq.). After 20 min at rt, the pre-activation mixture was added to 50 nmol of the amino-modified oligonucleotide pre-dissolved in 25 µL of water and 50 µL of TEA buffer (500 mM TEA•HCl, pH 10) and the reaction was carried out for 16 h at 37 °C. The DNA was precipitated from the aqueous phase by adding 10% v/v of 5 M NaCl and 3 volumes of cold EtOH and was left overnight at -20 °C. The mixture was then centrifuged for 1 h at 4 °C at 14'000 rpm, the supernatant was discarded, and the DNA pellet was dried under reduced pressure using a SpeedVac. Afterwards, the crude DNA pellet was dissolved in 200 µL MOPS

buffer (100 mM MOPS, pH 7.5) and 100 mM TCEP was added (20 µL, 2.0 µmol, 40 eq.). The resulting mixture was shaken overnight at 30°C. The DNA pellet was EtOH precipitated, centrifuged and dried under reduced pressure using a SpeedVac as described above.

Amide coupling of 204 Fmoc-amino acids

The singularly encoded oligonucleotides bearing the deprotected scaffold were distributed in 204 wells, the solvent was removed, and the crude DNA pellet (50 nmol) was dissolved in 10 µL of water and 10 µL of MOPS buffer (100 mM MOPS, 1M NaCl, pH 8). Each of the 204 Fmoc-amino acids (6.5 µL, 200 mM in DMSO, 1.3 µmol, 26 eq.) were diluted with DMSO (17.5 µL) and 6 µL of a freshly prepared stock solution of EDC:HOAt:NMM (100:20:100 mM in DMSO, 12:2.4:12 eq) were added. The resulting mixture was shaken for 10 min at 25 °C. Successively, the pre-activation mixture was added to the DNA solution and the reaction was shaken at 37 °C. After 1 hour a second addition of Fmoc-amino acid (6 µL, 200 mM in DMSO, 1.3 µmol, 26 eq) pre-activated with EDC:HOAt:NMM (6 µL, 100:20:100 mM in DMSO, 12:2.4:12 eq.) was performed and the reaction mixture was carried out at 37 °C for an additional hour. The reaction was stopped by EtOH precipitation After centrifugation, the pellet was dried under reduced pressure and the crude was dissolved in 0.1 M TEA Acetate (pH=7). The DNA-conjugates were individually purified by RP-HPLC, then for each code 1 conjugate the eluted fractions were lyophilized, re-dissolved in water, analysed by LC-MS and quantified (yields around 40-90 %).

Pool 1 and Fmoc deprotection

All the 204 DNA-conjugates (2 nmol each) were combined to generate the pool 1. Fmoc deprotection was performed in water (700 µL) and DMSO (1000 µL) with the addition of triethylamine (51 µL, 3% v/v). The mixture was shaken for 3 h at 37 °C. The DNA was EtOH

precipitated, centrifuged and dried. Afterwards, the DNA was dissolved in water to obtain the Fmoc deprotected **MB pool 1** (MB pool 1 final concentration 30 µM)**.**

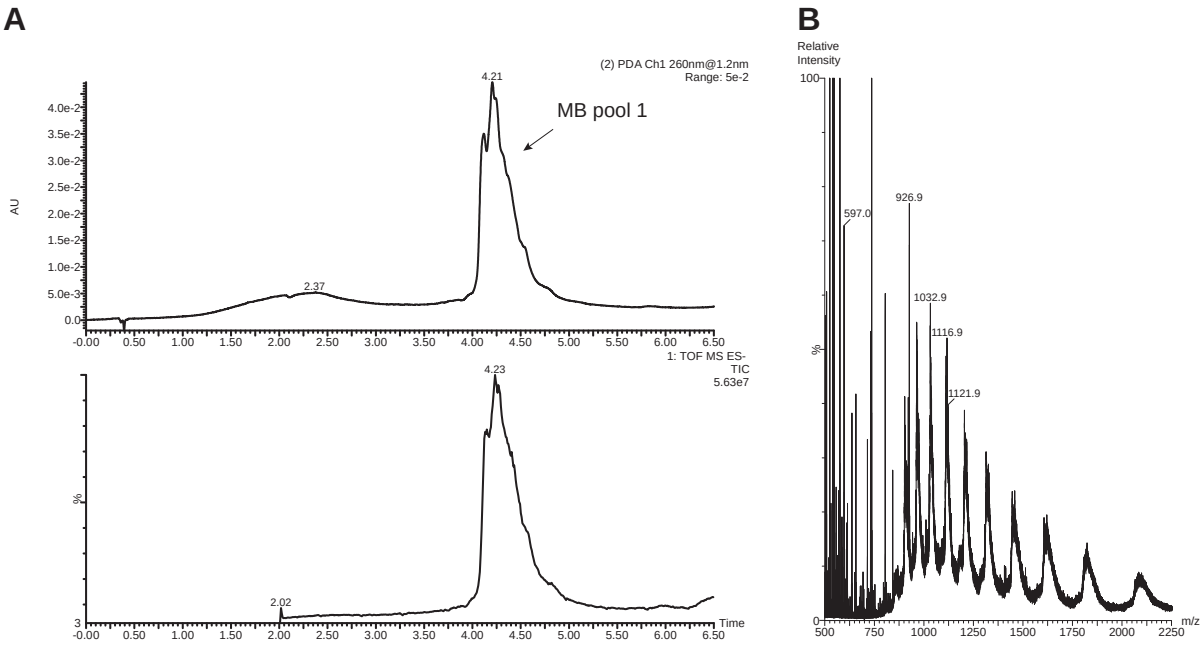


Figure S10. LC-MS analysis of **MB pool 1** after Fmoc deprotection and EtOH precipitation**. A**. LC chromatogram of **MB pool 1** registered at 260 nm; **B**. Non-deconvoluted mass spectra of **MB pool 1**.

2.2.3 MB library synthesis - Step 2

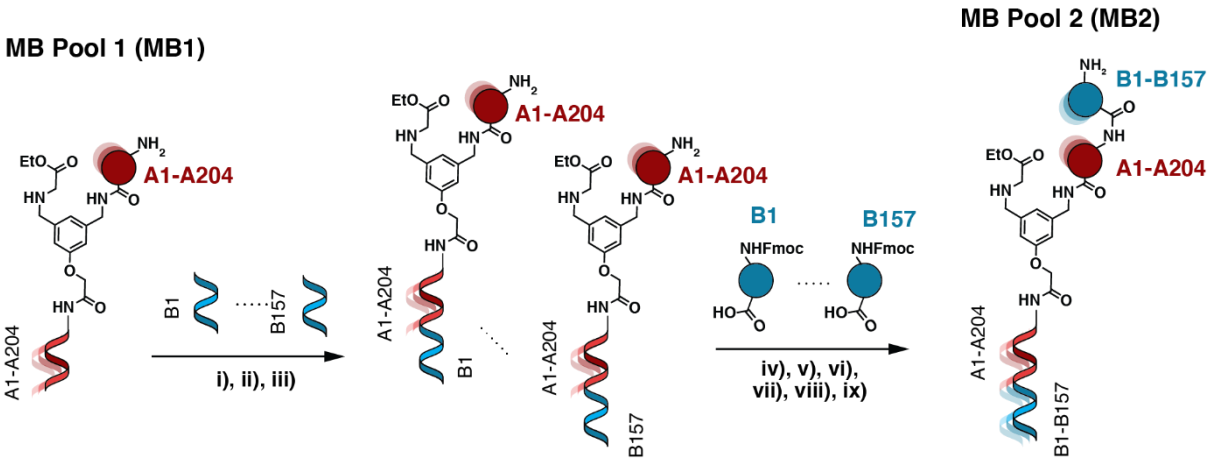


Figure S11. Schematic illustration of the synthesis of **MB-DEL Pool 2**. i) Splitting of the Pool 1 in 157 wells; ii) Splint Ligation of 157 individual Codes B (B1-B157); iii) EtOH precipitation; iv) Amide coupling of 157 Fmoc-amino acids to the respective derivatized Codes B (B1-B157); v) Pooling of the 157 derivatized oligonucleotides to generate Pool 2; vi) EtOH precipitation; vii) HPLC purification; viii) Fmoc deprotection; ix) EtOH precipitation.

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Splint ligation with CodeB

The pool 1 was aliquoted in 157 wells (2.6 nmol, 86 µL, 1 eq.) using 96-well plates. To each aliquot, one of 157 unmodified oligonucleotides (code B: 5'-Phosp-CGGATCGACGYYYYYYYGCGTCAGGCAGC-3', 4.0 nmol, 1.5 eq., where YYYYYYY are the bases used as coding region), was added. Then, Adaptor 1 (5'-CGTCGATCCGGCGCCATGGG-3', 4.1 nmol, 1.6 eq.) and T4 DNA ligase buffer (21 µL, New England BioLabs, Catalog: B0202S) were distributed in each well. The mixtures were heated for 5 min at 70 °C. The solutions were cooled down to rt in 30 min, and T4 DNA Ligase (6.5 µL, 2600 U, New England BioLabs, Catalog: M0202S) was added to each well. After 2h at rt the reactions were quenched by heating for 10 min at 65°C. The successful encoding was evaluated by electrophoresis analysis (Invitrogen™ Novex™ TBE-Urea Gels, 15%, Catalog: EC68855BOX) **Figure S12**. The DNA pellets were EtOH precipitated, centrifuged, and dried under reduced pressure.

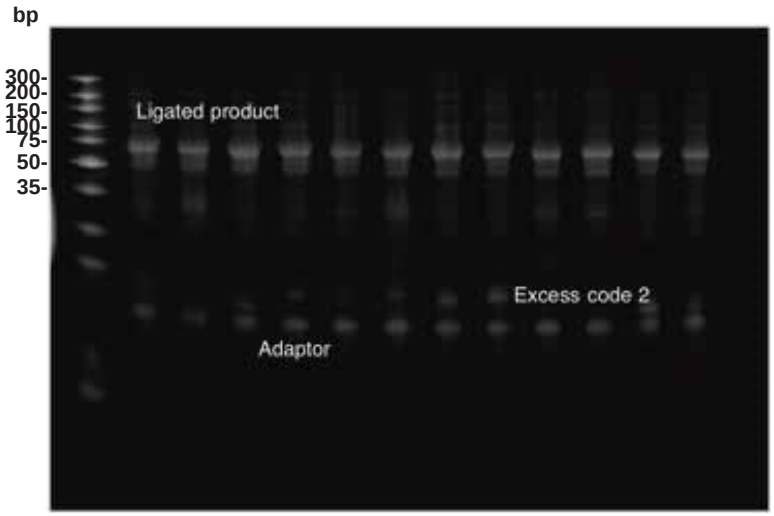


Figure S12. TBE-Urea 15% gel of MB pool 1 fractions after encoding with Code B.

Amide coupling of 157 Fmoc-amino acids

The DNA pellets previously encoded with Codes B were singularly coupled to 157 Fmoc-amino acids following the same procedure already described for the amide coupling in Step 1. After two hours at 37 °C, the reaction was quenched by adding 25 µL of a 500 mM NH_4OAc solution and the mixture was shaken for 30 min at 30 °C. All the 157 reactions were pooled together to generate the pool 2 followed by two EtOH precipitations. The recovered pellet was purified by RP-HPLC at 70 °C to remove the adaptor and excess of code 2. The purified pool 2 was dried by lyophilization, re-dissolved in water (4.5 mL), and quantified (MB Pool 2: 219 nmol).

Fmoc deprotection of Pool 2

Pool 2 was dried and re-dissolved in water (360 µL) and DMSO (540 µL). Triethylamine (27 µL, 3% v/v) was added to the pool 2 solution and the reaction was carried out for 3 h at 37 °C. The DNA pellet was EtOH precipitated, centrifuged and dried under reduced pressure. Afterwards, the DNA was re-dissolved in water analysed by LC-MS (**Figure S13**), to afford the final Fmoc deprotected pool 2 (MB pool 2 final concentration 21 µM).

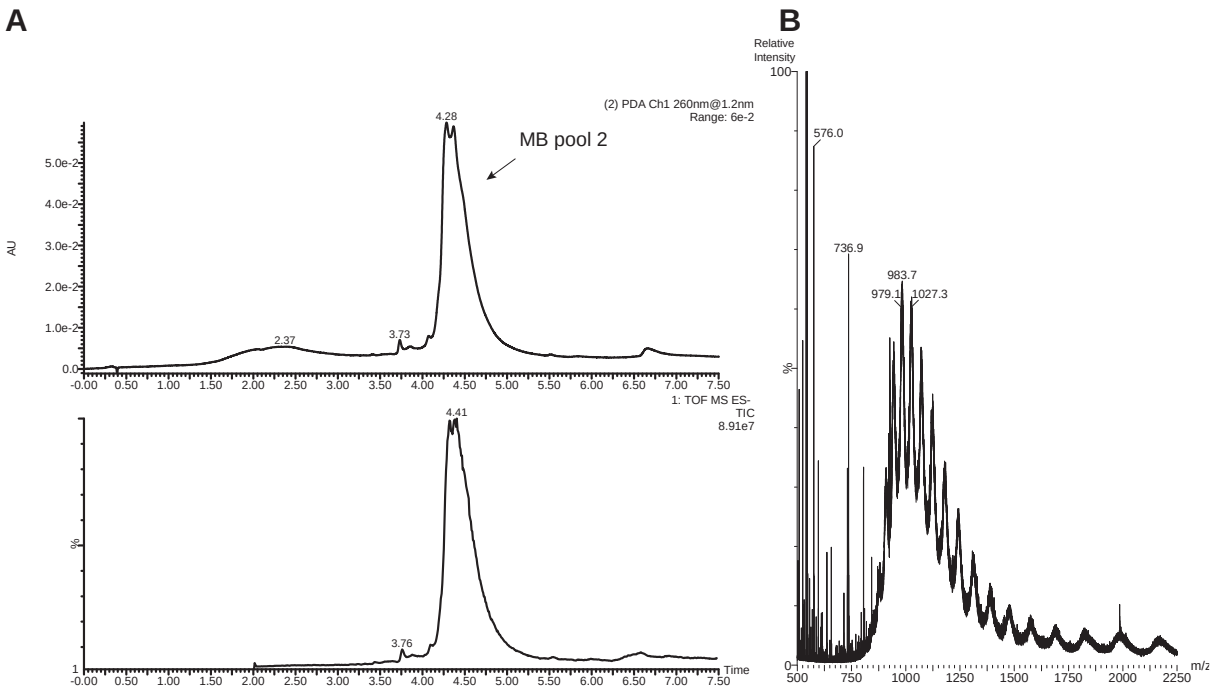


Figure S13. LC-MS analysis of **MB pool 2** after Fmoc deprotection and EtOH precipitation. **A**. LC chromatogram of **MB pool 2** registered at 260 nm. **B**. Non-deconvoluted mass spectra of **MB pool 2**.

2.2.4 MB library synthesis - Step 3

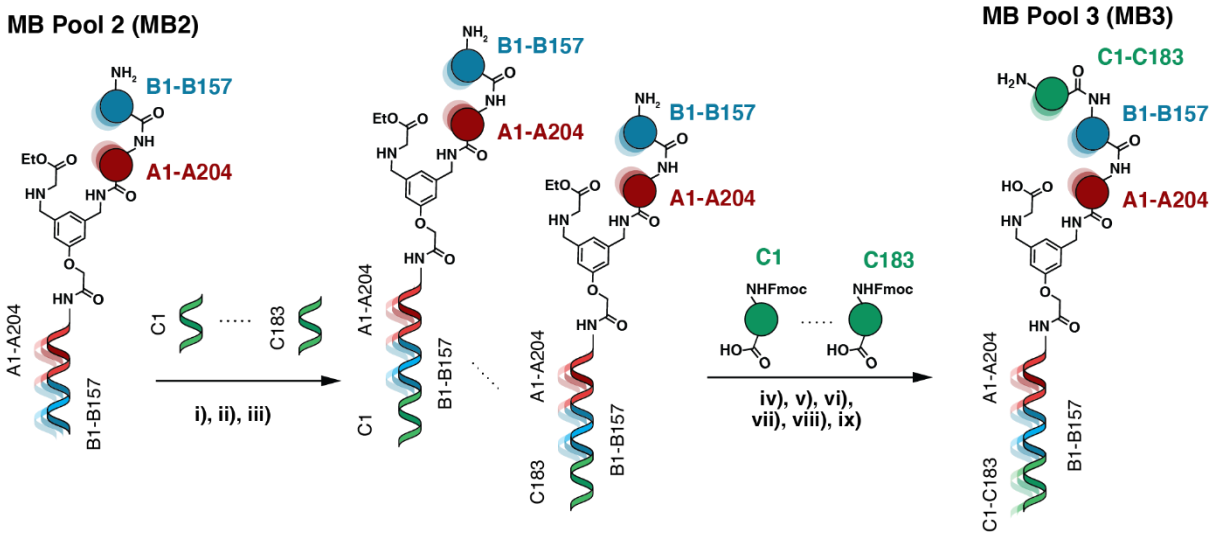


Figure S14. Schematic illustration of the synthesis of **MB-DEL Pool 3**. i) Splitting of the Pool 2 in 183 wells; ii) Encoding by enzymatic polymerization of 183 individual Codes C (C1-C183); iii) EtOH precipitation; iv) Amide coupling of 183 Fmoc-amino acids to the respective derivatized Codes C (C1-C183); v) Pooling of the 183 derivatized oligonucleotides to generate Pool 3; vi) EtOH precipitation; vii) HPLC purification; viii) Fmoc deprotection and ethyl ester hydrolysis; ix) EtOH precipitation.

Encoding by enzymatic polymerization

An aliquot of pool 2 (91.5 nmol) was split in 183 wells (0.5 nmol, 23.5 µL) and 183 unmodified oligonucleotides (code C: 5'- GCTCTGCAC GGTCGCZZZZZZZGCTGCCTGACGC-3', 1 nmol, 2 eq. where ZZZZZZZ are the bases used as coding region) were added, followed with addition of NEB Buffer 2 (10 µL, Thermo Fisher, Catalog: EP0052) and kept for 15 min at 60 °C. After the system was cooled down to room temperature, a solution of 2'-deoxynucleotide triphosphates (5 mM, 10 µL, 100 eq) and Klenow Fragment (2.5 µL, 50 U, Thermo Fisher, Catalog: EP0052) were added. The polymerase extension reaction stood for 1 h at 25°C. The successful encoding was evaluated by electrophoresis analysis (Invitrogen™ Novex™ TBE

Gels, 20%, Catalog: EC63155BOX), **Figure S15**. The DNA was EtOH precipitated, centrifuged and dried under reduced pressure.

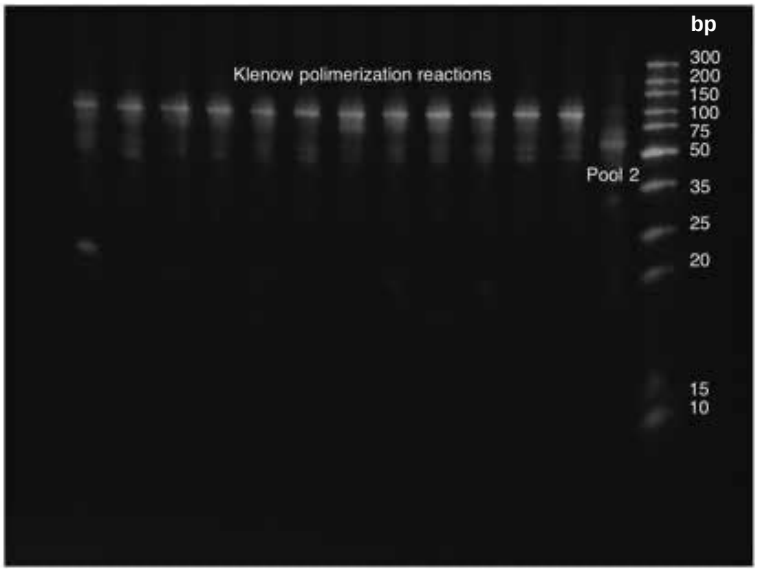


Figure S15. TBE-Urea 15% gel of MB pool 2 fractions after Klenow polimerization.

Amide coupling of 183 Fmoc-amino acids

The DNA pellets previously encoded with Codes C were coupled to 183 Fmoc-amino acids following the same procedure already described for the amide coupling in Step 1 and 2. After two hours at 37 °C, the reaction was quenched by adding 25 µL of a 500 mM NH_4OAc solution and the mixture was shaken for 30 min at 30 °C. All the 183 reactions were pooled together to generate the pool 3 followed by two EtOH precipitations. The recovered pellet was purified by RP-HPLC at 25 °C to remove the excess of code 3. The purified pool 3 was dried by lyophilization, re-dissolved in water (1.5 mL), and quantified (MB pool 3 37.4 nmol).

Fmoc deprotection and ethyl ester hydrolysis

Pool 3 was further dried and re-dissolved in water (250 µL) and 1 M piperidine (50 µL) was added. After 1h at 37°C, the DNA pellet was EtOH precipitated, centrifuged and dried under

reduced pressure as previously described. Consecutively, the pellet was re-dissolved in 200 µL of water, to perform a second EtOH precipitation and 16.1 nmol of MB pool 3 were obtained and analysed by LC-MS and by electrophoresis gel (**Figures S16-S17**).

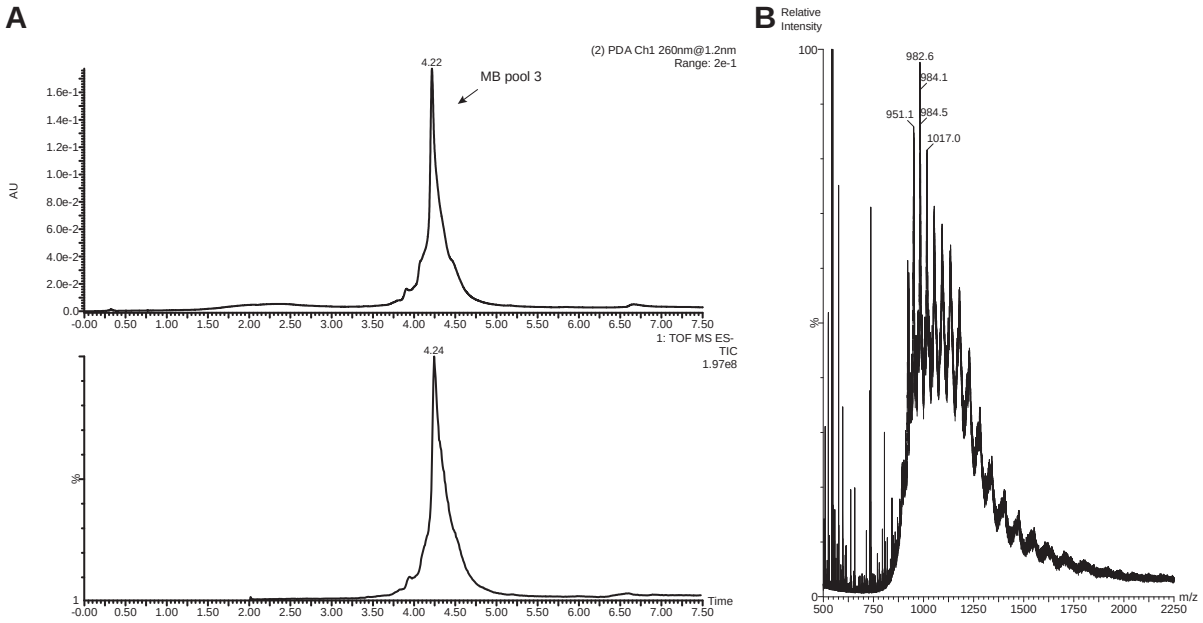


Figure S16. LC-MS analysis of **MB pool 3** after Fmoc deprotection, ethyl ester hydrolysis and EtOH precipitation. **A**. LC chromatogram of **MB pool 3** registered at 260 nm. **B**. Non-deconvoluted mass spectra of **MB pool 3**.

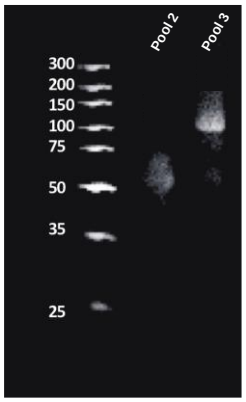


Figure S17. TBE Urea 15% gel of final MB pool 2 and MB pool 3.

2.3 PRA Library Synthesis

PRA DNA-Encoded chemical Library synthesis has been already described in Puglioli et al[1]. The synthetic scheme is shown in **Figure S18**.

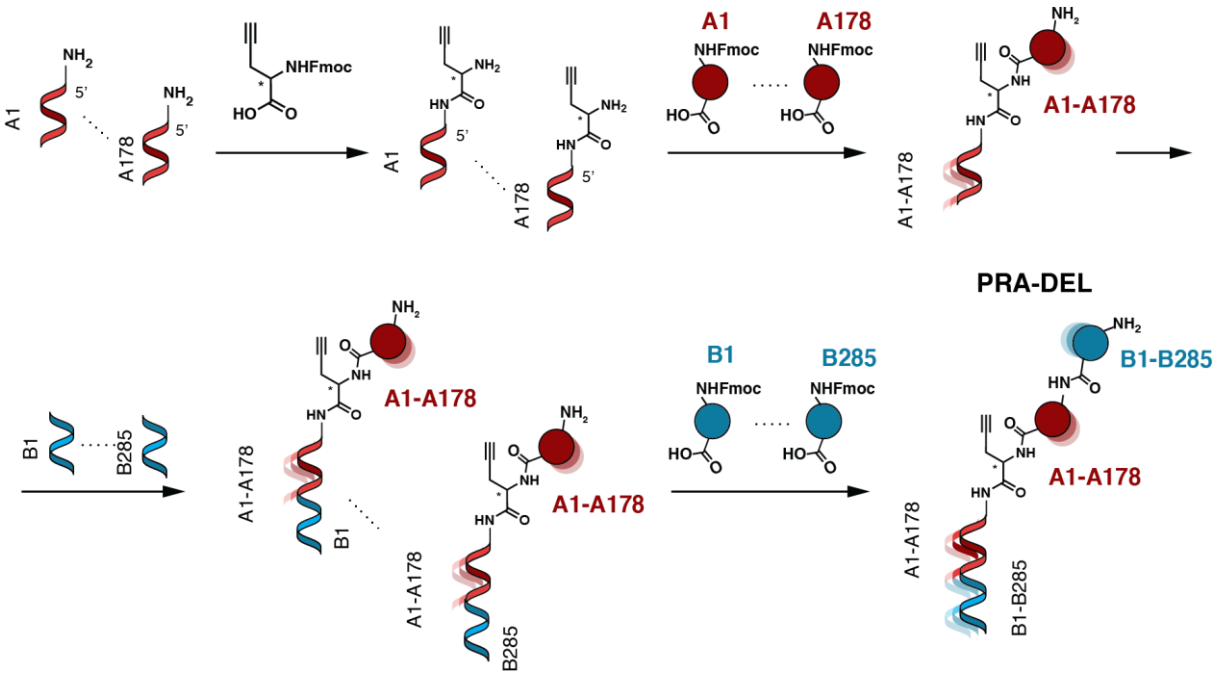


Figure S18. Schematic illustration of the synthesis of **PRA-DEL**.

3 Affinity and Selectivity Maturation (ASM) DELs

Preparation of affinity and selectivity maturation DNA-Encoded Chemical Libraries (ASM-DELs)

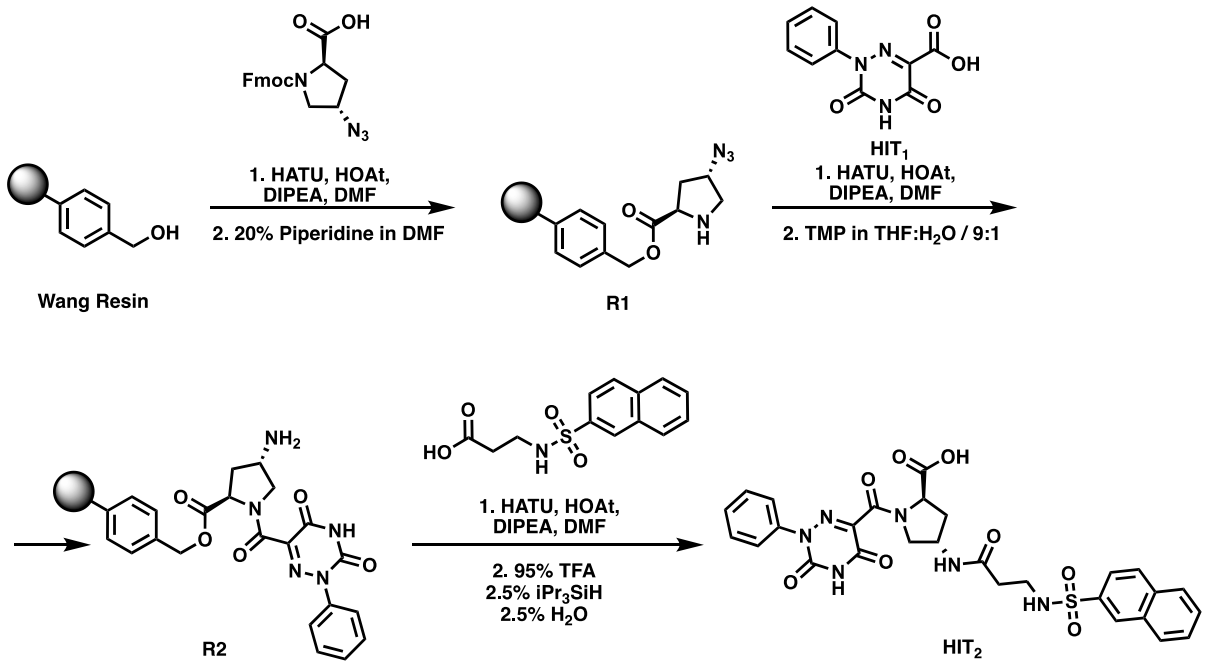
ASM-DELs were synthesized as free amino-terminal DNA conjugate pools, and subsequently derivatized in parallel by amide bond formation with HIT_1, HIT_2, and HIT_3 to obtain the final ASM-DEL: MB1-HITx, MB2-HITx, MB3-HITx, LL1-HITx. LL2-HITx, PRA-HITx (**Manuscript**, **Figure 1**).

3.1 Synthesis and Validation of the HITs

3.1.1 Synthesis of the HITs for the generation of ASM-DELs

3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylic acid (HIT_1) was commercially available and was provided by Enamine (CAS 4315-71-3).

Synthesis of (2*R*,4*S*)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxylic acid (HIT_2). **HIT_2** was synthesized by solid-phase synthesis using Wang resin (loading 1.1 mmol/g). The resin was swollen in DMF for 30 min prior to every reaction. Resin intermediates are presented as **Rn** (**n** = **1**, **2**, …), and have not been isolated.



Scheme S2. Schematic illustration of the synthesis of **HIT_2**.

Commercially available (2*R*,4*S*)-1-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-4-azidopyrrolidine-2-carboxylic acid (250 mg , 0.66 mmol, 2 eq.), HATU (240 mg, 0.63 mmol, 1.9 eq.), HOAt (23 mg, 0.17 mmol, 0.5 eq.) were dissolved in DMF (2 mL) and DIPEA (0.23 mL, 1.32 mmol, 4 eq.) was added. After 5 min of acid pre-activation, the solution was added to 0.3 g of Wang

resin (0.33 mmol, 1 eq.) and the reaction was incubated for 2 h at rt. The resin was first washed 5 times with DMF followed by incubation with 20% piperidine in DMF (1 x 30 min and 2 x 10 min) to perform Fmoc deprotection and afford compound **R1**. To remove residual piperidine, the resin was washed 10 times with DMF. Afterward, compound 3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylic acid (**HIT$_1$**, 154 mg, 0.66 mmol, 2 eq.), HATU (240 mg, 0.63 mmol, 1.9 eq.), HOAt (23 mg, 0.17 mmol, 0.5 eq.) and DIPEA (0.23 mL, 1.32 mmol, 4 eq.) were dissolved in DMF and after 5 min of activation, the solution was added to **R1** (0.33 mmol, 1 eq.). The reaction was incubated for 2 h at rt and washed 5 times with DMF. Subsequently, 1.32 mL of trimethylposphine (1M TMP in THF, 1.32 mmol, 4 eq.) were diluted in 2 mL of THF:H_2O (9:1), then added to the resin and kept at rt for 2 h. The resin was washed 3 times with a solution of THF:H_2O (9:1) and 10 times with DMF to obtain the desired free-NH_2 derivative **R2**. Commercially available 3-(naphthalene-2-sulfonamido)propanoic acid (184 mg, 0.66 mmol, 2 eq.), was pre-activated with HATU (240 mg, 0.63 mmol, 1.9 eq.), HOAt (23 mg, 0.17 mmol, 0.5 eq.) and DIPEA (0.23 mL, 1.32 mmol, 4 eq.) in DMF for 5 min. The pre-activation solution was added to the compound **R2** (0.33 mmol, 1 eq.) and the reaction was incubated for 2 h at rt. Afterwards, the resin was washed 5 times with DMF, 5 times with DCM and was dried under vacuum. When the resin was completely dry, a cleavage solution consisting of 95% TFA, 2.5% water, 2.5% iPr_3SiH was added to the resin and incubated for 1 h at rt. The cleavage step was repeated two times (2 x 10 min), and the cleaved fractions were collected in one vial. TFA was removed under reduced pressure and the crude was purified by RP-HPLC to afford **HIT$_2$** as a white solid (29 mg, overall yield = 15%). HR-MS (ESI+) *m/z* calculated for $C_{28}H_{26}N_6O_8S$ $[M+H]^+$ 607.16056; detected: 607.15914; accuracy = 2.34 ppm. ^{1}H NMR (400 MHz, MeOD): δ (ppm) = 8.39- 8.41 (m, 1H), 8.00 – 8.04 (m, 2H), 7.94-7.97 (m, 1H), 7.80 – 7.84 (m, 1H), 7.59-7.67 (m, 2H), 7.35 – 7.53 (m, 5H), 4.67 – 4.78 (m, 1H), 4.38 – 4.45 (m, 1H), 3.83 – 3.89 (m, 1H), 3.57 – 3.73 (m,

1H), 3.11 – 3.17 (m, 2H), 2.17 – 2.40 (m, 4H). ^{13}C NMR (100 MHz, MeOD): δ (ppm) = 172.93, 171.98, 161.04, 154.87, 147.99, 139.73, 138.45, 137.35, 134.83, 132.26, 129.23, 128.92, 128.65, 128.56, 128.48, 128.72, 127.72, 127.31, 125.39, 122.04, 57.87, 53.55, 49.00, 39.05, 35.84, 34.13.

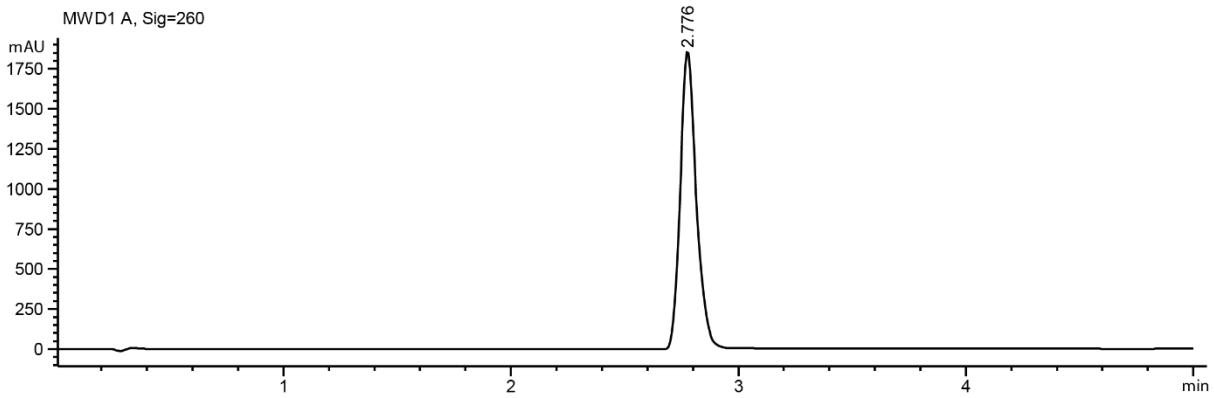


Figure S19. HPLC chromatogram of **HIT$_2$** recorded at λ = 260 nm.

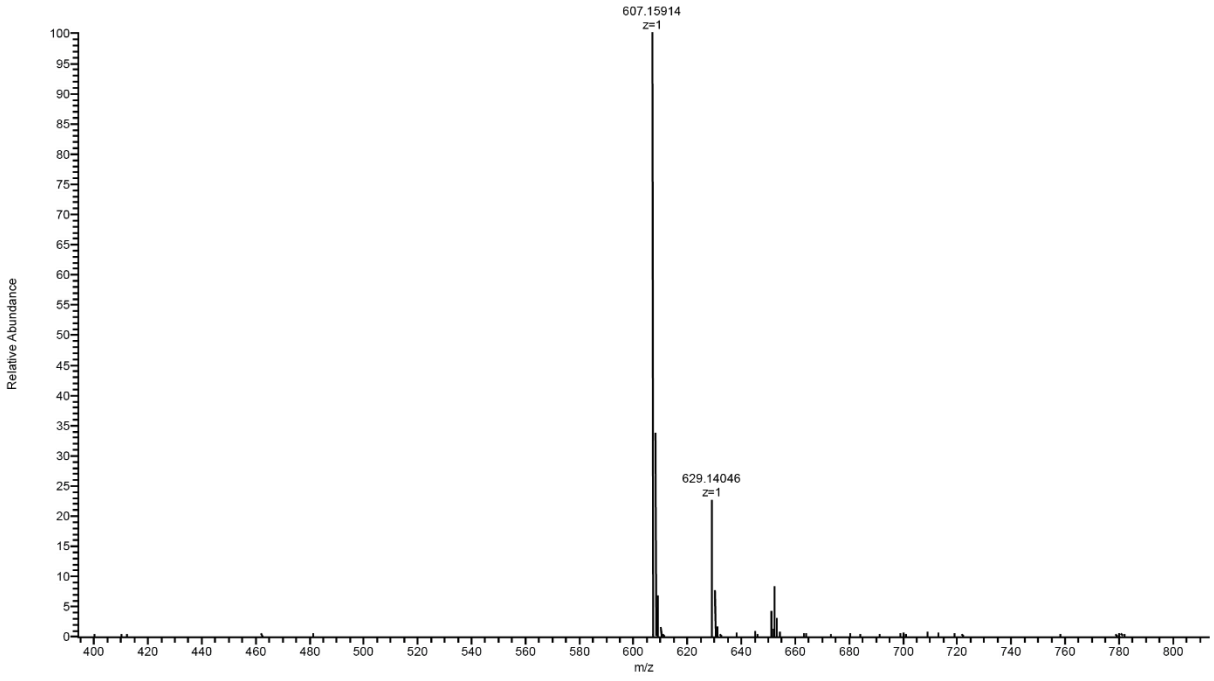
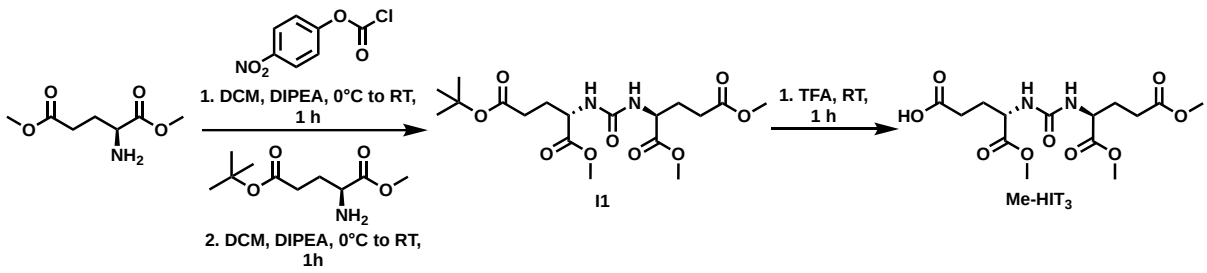


Figure S20. HR-MS of **HIT$_2$**

Synthesis of (*S*)-4-(3-((*S*)-1,5-dimethoxy-1,5-dioxopentan-2-yl)ureido)-5-methoxy-5-oxopentanoic acid (Me-HIT$_3$)

HIT$_3$ was synthesized and coupled to the libraries as a precursor reported here with the name of **Me-HIT$_3$**. **Me-HIT$_3$** presents one free carboxylic function and three methyl esters. Methyl groups were removed after library conjugation to afford the final on-DNA **HIT$_3$**.

Me-HIT$_3$ was synthesized using solution-phase synthesis. Solution-synthesis intermediates are named **In** (**n = 1**, **2**, …).



Scheme S3. Schematic illustration of the synthesis of Methyl protected HIT$_3$ (**Me-HIT$_3$**).

Commercially available dimethyl *L*-glutamate hydrochloride (500 mg, 2.36 mmol, 1 eq.) was dissolved in dry DCM (3 mL) and DIPEA (1.2 mL, 3 eq.) was added. An anhydrous solution of 4-nitrophenyl chloroformate (524 mg, 2.60 mmol, 1.1 eq.) in DCM (2 mL) was added dropwise at 0°C and the reaction was stirred at rt. After 30 min 5-(*tert*-butyl) 1-methyl *L*-glutamate hydrochloride (599 mg, 2.36 mmol, 1 eq.) and 1 additional eq. of DIPEA (0.4 mL, 1 eq.) were added to the solution. The reaction was stirred for 1 h at rt and checked by LC-MS analysis. The solvent was evaporated under reduced pressure and a brown oil was obtained. A partial purification of the crude was achieved using flash chromatography on silica gel (DCM:MeOH, elution gradient from 0% to 20% of MeOH) in order to remove major by-products and unreacted compounds. The afforded 5-(*tert*-butyl) 1-methyl (((*S*)-1,5-dimethoxy-1,5-dioxopentan-2-yl)carbamoyl)-*L*-glutamate (compound **I1**) was not isolated and was used

as crude product for the next step. Tert-butyl deprotection of compound **I1** (197 mg, 0.47 mmol, 1 eq.) was performed by the addition of neat TFA (4 mL). The reaction was stirred for 1 h at rt. TFA was removed under reduced pressure and the crude was dissolved in MeCN: H_2O (1:1) to be purified by RP-HPLC affording **Me-HIT$_3$** as white powder (120 mg, yield = 70%). HR-MS (ESI+) *m/z* calculated for $C_{14}H_{22}N_2O_9$ $[M+H]^+$ 363.13981; detected: 363.13996; accuracy = 0.41 ppm. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 6.35 – 6.49 (m, 2H), 4.09 – 4.16 (m, 2H), 3.61 (s, 6H), 3.57 (s, 3H), 2.30- 2.37 (m, 2H), 2.21-2.28 (m, 2H), 1.88 – 1.99 (m, 2H), 1.71 – 1.82 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 173.96, 173.64, 173.16, 172.63, 157.19, 53.60, 51.83, 51.40, 41.85, 29.75, 29.53, 27.08, 18.08, 16.74.

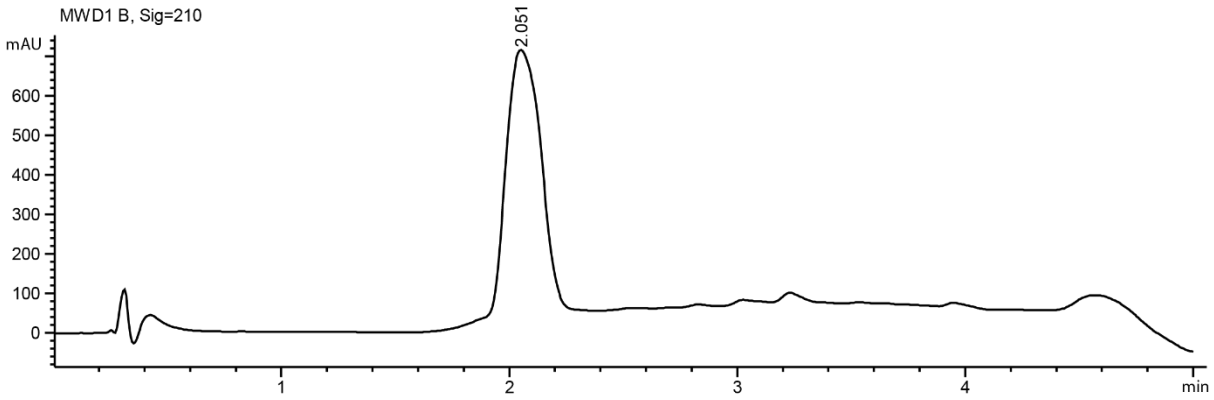


Figure S21. HPLC chromatogram of **Me-HIT$_3$** recorded at λ = 210 nm.

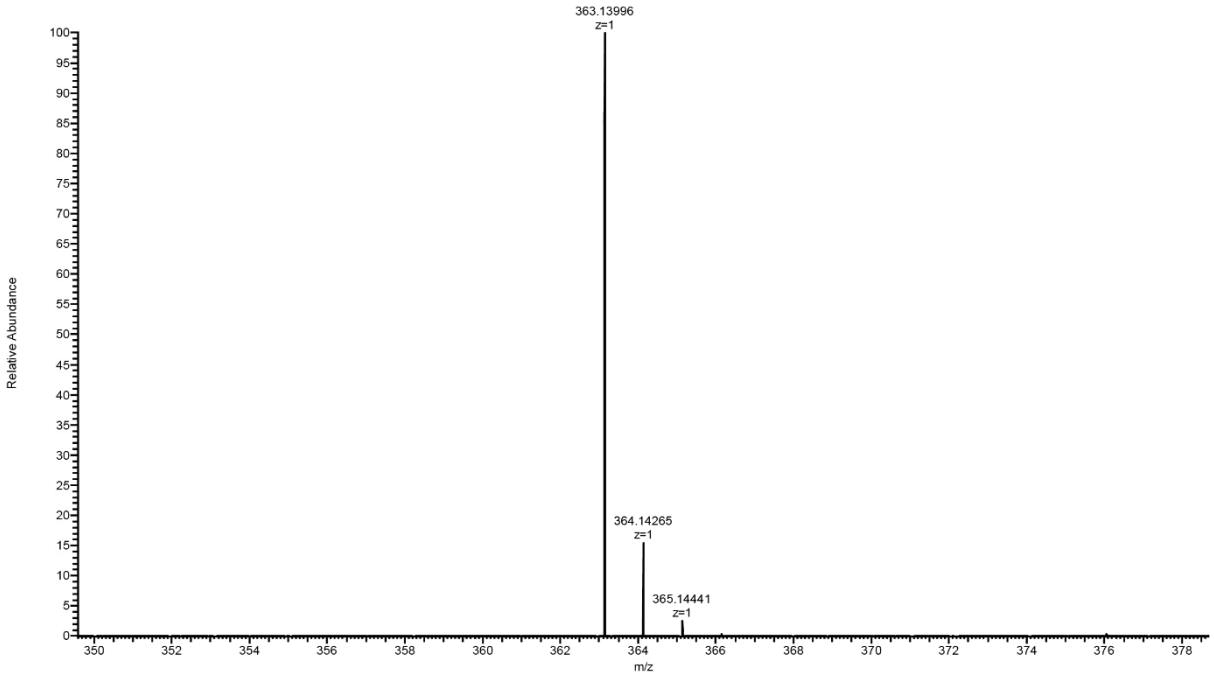
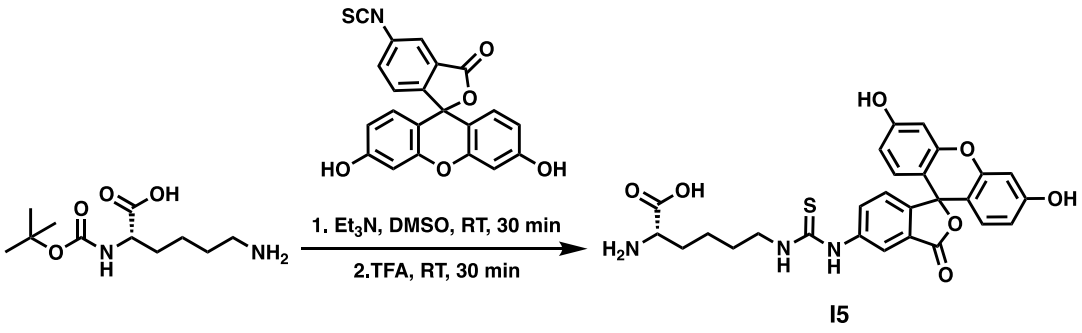


Figure S22. HR-MS of compound **Me-HIT$_3$**

3.1.2 Synthesis of FITC derivatives of the HITs (2, 6, 12)

The HIT$_1$, HIT$_2$ and HIT$_3$ were synthesized as fluorescent derivatives (Compounds **2**, **6**, **12**) to perform initial validation before coupling with the libraries.

Synthesis of N^6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-L-lysine (I5 = Linker 1, L$_1$)



Scheme S4. Schematic illustration of the synthesis of intermediate **I5**.

Commercially available (tert-butoxycarbonyl)-L-lysine (100 mg, 0.41 mmol, 1 eq.) was dissolved in 2 mL dimethyl sulfoxide (DMSO) and 5-FITC (160 mg, 0.41 mmol, 1 eq.) and Et_3N (229 µL, 1.64 mmol, 4 eq.) were added. The reaction was stirred for 30 min at room temperature. The solvent was evaporated under reduced pressure and trifluoroacetic acid (TFA, 2 mL) was added to the residue. After 30 min at room temperature, TFA was evaporated, and the crude was re-dissolved in a solution of DMF:ACN:H_2O 1:1:1 and purified by RP-HPLC to obtain intermediate **I5** (**I5 = L_1**) as orange solid (68 mg, 31% yield). HR-MS (ESI+) *m/z* calculated for $C_{27}H_{25}N_3O_7S$ $[M+H]^+$ 536.14860; detected: 536.14810; accuracy = 0.93 ppm

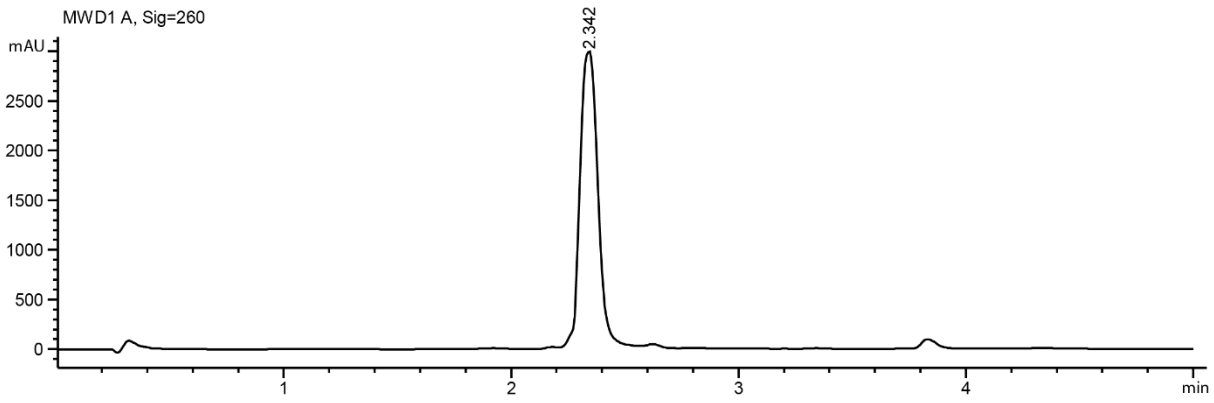


Figure S23. HPLC chromatogram of intermediate **I5** recorded at λ = 260 nm.

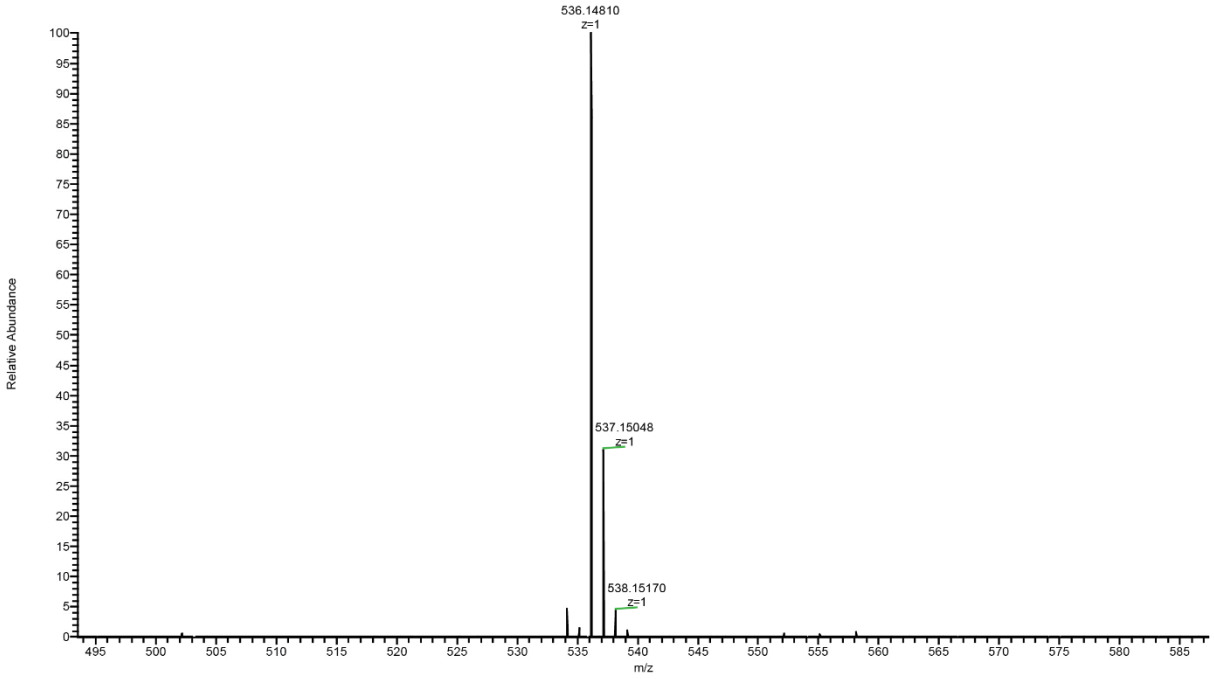
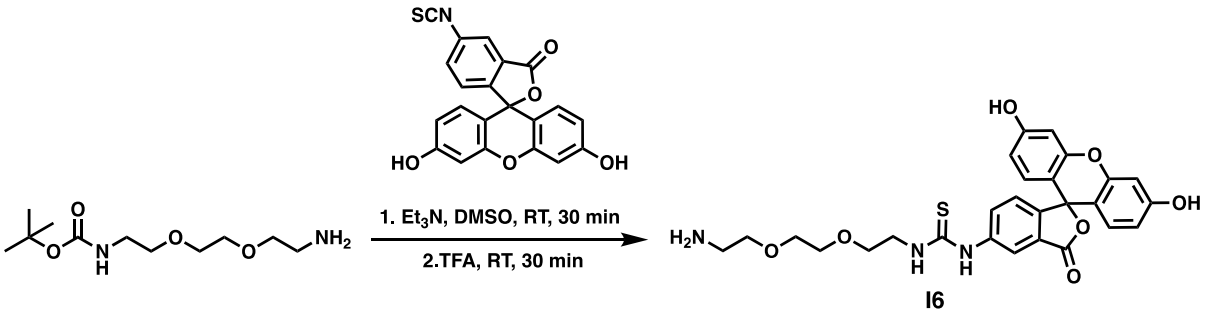


Figure S24. HR-MS of intermediate **I5**.

Synthesis of 1-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-3-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thiourea (I6 = Linker 2, L_2)



Scheme S5. Schematic illustration of the synthesis of intermediate **I6**.

Tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (100 mg, 0.40 mmol, 1 eq.) was dissolved in 2 mL DMSO and 5-FITC (156 mg, 0.40 mmol, 1 eq.) and triethylamine (Et_3N or TEA, 223 µL, 1.60 mmol, 4 eq.) were added. The reaction was stirred for 30 min at room temperature. The solvent was evaporated under reduced pressure and TFA (2 mL) was added to the residue. After 30 min at room temperature, TFA was evaporated, and the crude was re-dissolved in a solution of DMF:ACN:H_2O 1:1:1 and purified by RP-HPLC to obtain

intermediate **I6** (**I6 = L_2**) as orange solid (77 mg, 36% yield). HR-MS (ESI+) *m/z* calculated for $C_{27}H_{27}N_3O_7S$ $[M+H]^+$ 538.16425; detected: 538.16408; accuracy = 0.32 ppm

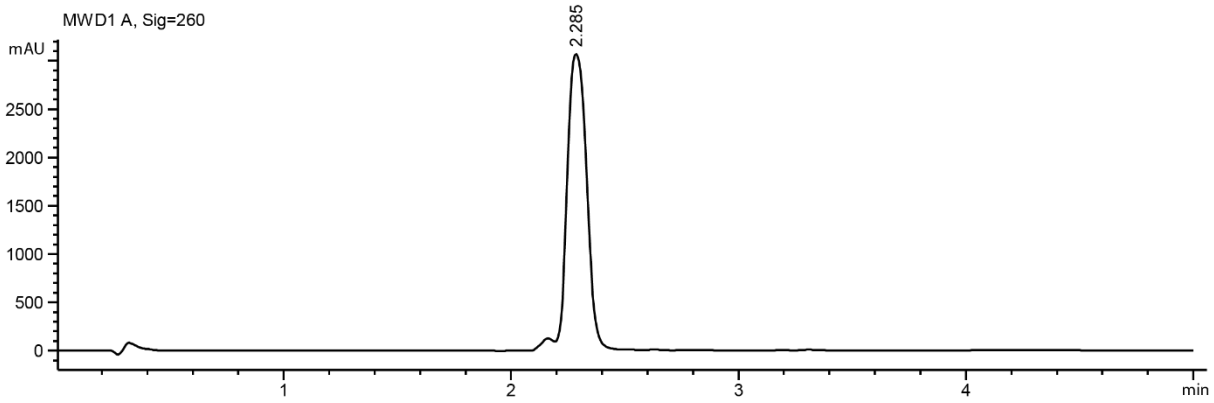


Figure S25. HPLC chromatogram of intermediate **I6** recorded at λ = 260 nm.

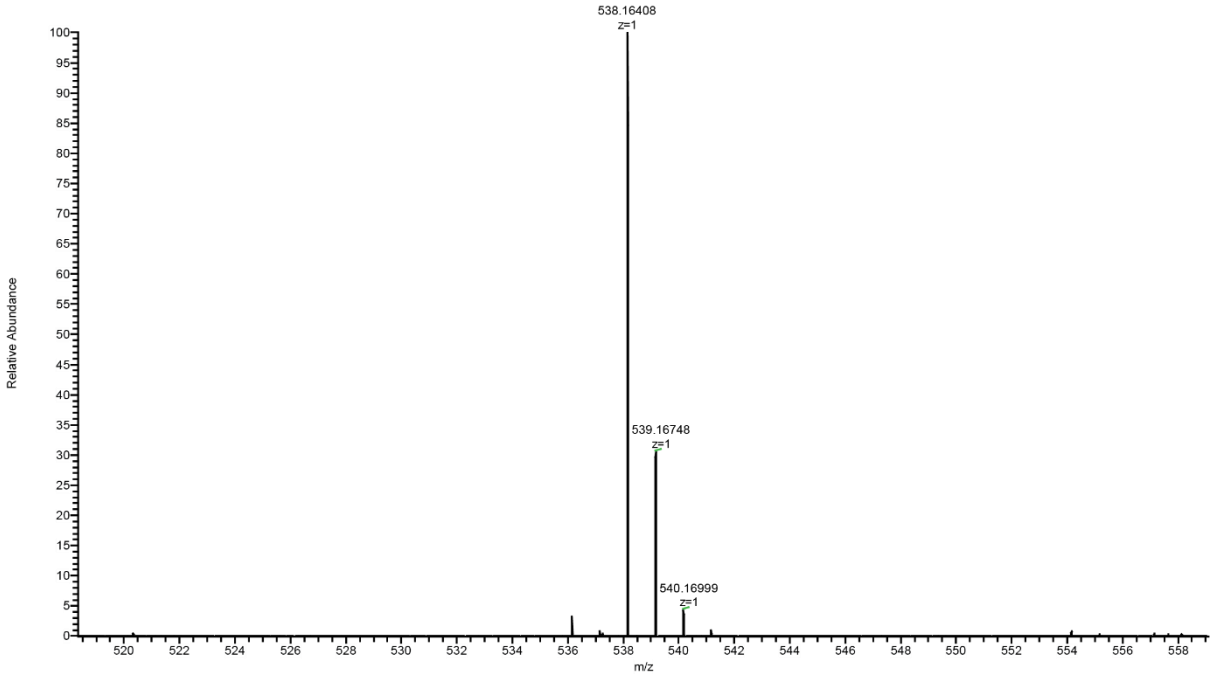
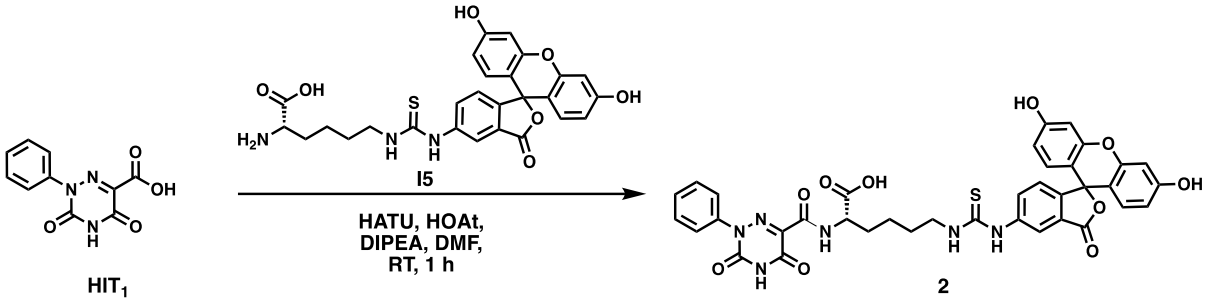


Figure S26. HR-MS of intermediate **I6**.

Synthesis of N6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-N2-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-L-lysine (2)



Scheme S6. Schematic illustration of the synthesis of compound **2**.

HIT$_1$ (10 mg, 0.040 mmol, 1.1 eq.) was dissolved in DMF (500 µL), and HATU (14 mg, 0.036 mmol, 1 eq.), HOAt (2.5 mg, 0.018 mmol, 0.5 eq.) and DIPEA (25 µL, 0.144 mmol, 4 eq.) were added. The acid was activated for 5 min at room temperature and **I5** (19 mg, 0.036 mmol, 1 eq.). After 1 hour at room temperature, the solvent was removed under reduced pressure, the crude was re-dissolved in DMF:ACN:H_2O 1:1:1 and purified by RP-HPLC to obtain compound **2** as an orange solid (9.5 mg, yield = 35%). HR-MS (ESI+) *m/z* calculated $C_{37}H_{30}N_6O_{10}S$ $[M+H]^+$ 751.18169; detected: 751.18116; accuracy = 0.71 ppm

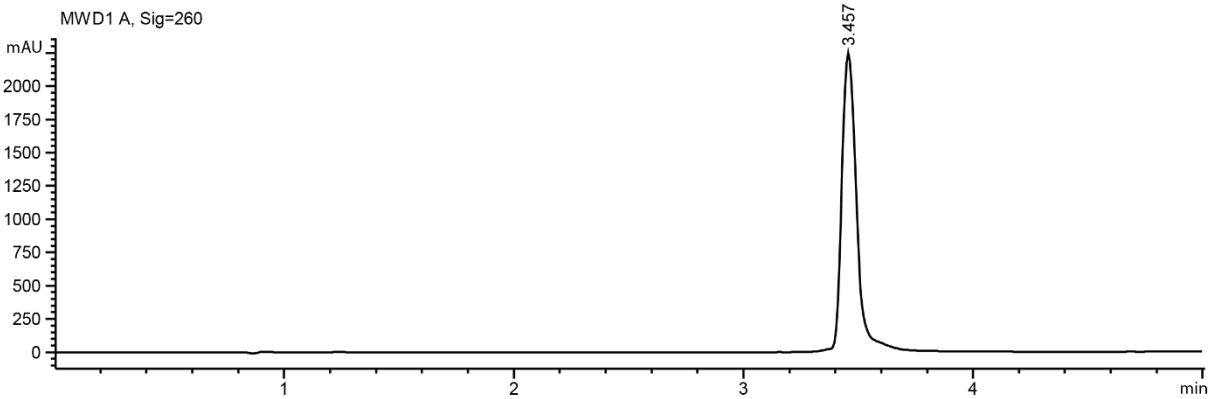


Figure S27. HPLC chromatogram of compound **2** recorded at λ = 260 nm.

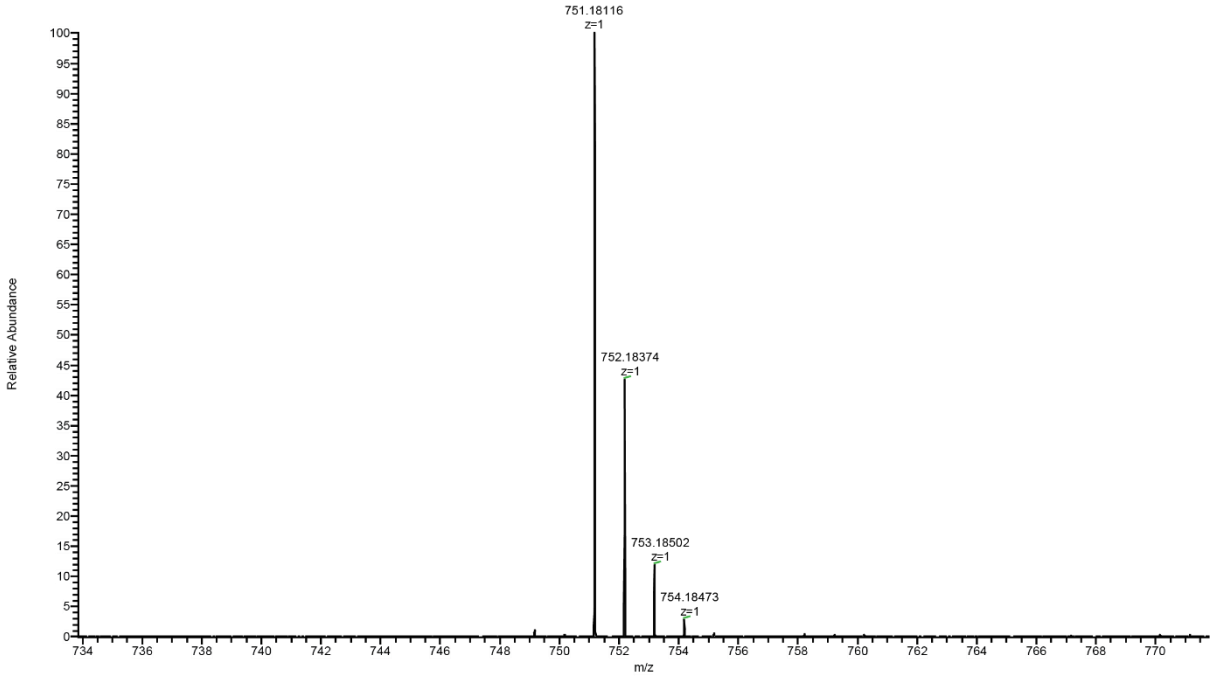
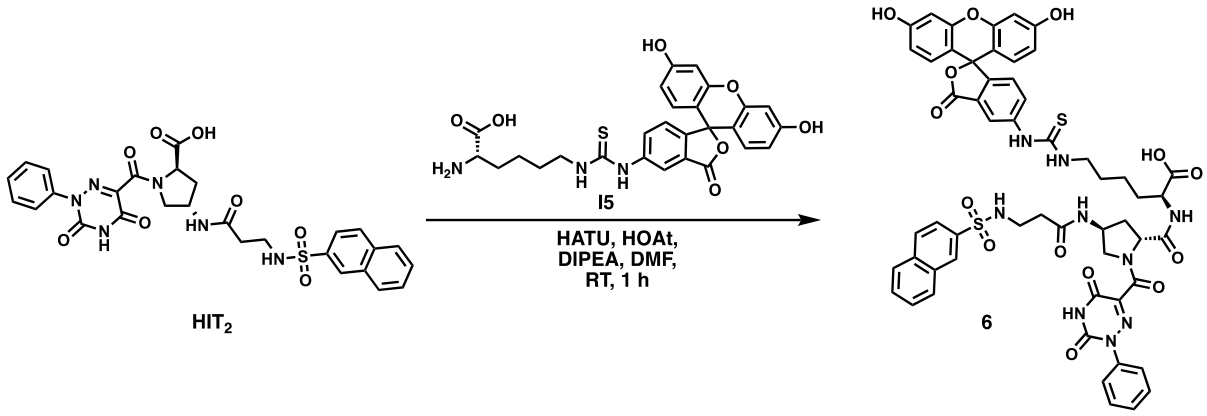


Figure S28. HR-MS of compound **2**.

Synthesis of N6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-N2-((2R,4S)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carbonyl)-L-lysine (6)



Scheme S7. Schematic illustration of the synthesis of compound **6**.

HIT$_2$ (7 mg, 0.011 mmol, 1.1 eq.) was dissolved in DMF (500 µL), and HATU (4 mg, 0.010 mmol, 1 eq.), HOAt (0.7 mg, 0.005 mmol, 0.5 eq.) and DIPEA (7 µL, 0.040 mmol, 4 eq.) were added. The acid was activated for 5 min at room temperature and **I5** (5.4 mg, 0.010 mmol, 1 eq.). After 1 hour at room temperature, the solvent was removed under reduced pressure, the crude was re-dissolved in DMF:ACN:H_2O 1:1:1 and purified by RP-HPLC to obtain compound **6** as an orange solid (4 mg, yield = 36%). HR-MS (ESI+) *m/z* calculated $C_{55}H_{49}N_9O_{14}S_2$ $[M+H]^+$ 1124.29132; detected:1124.29116; accuracy = 0.14 ppm

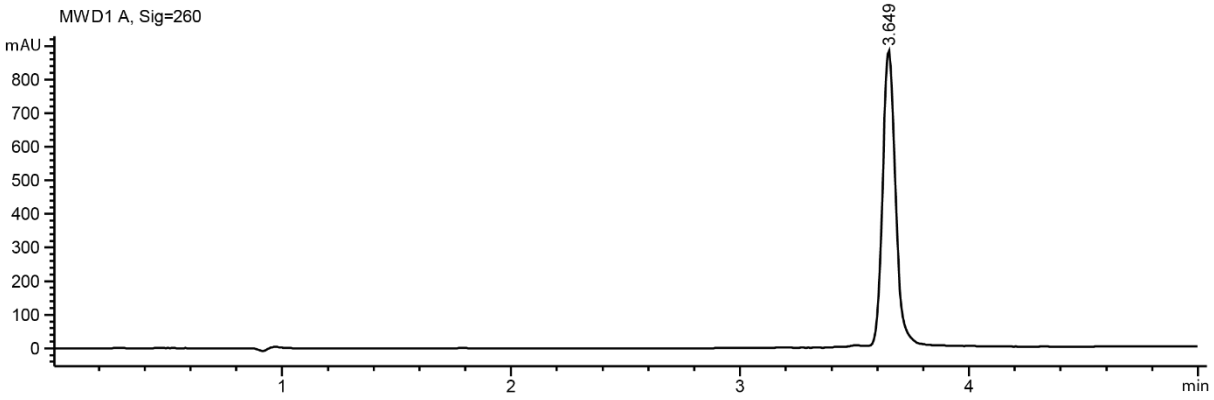


Figure S29. HPLC chromatogram of compound **6** recorded at λ = 260 nm.

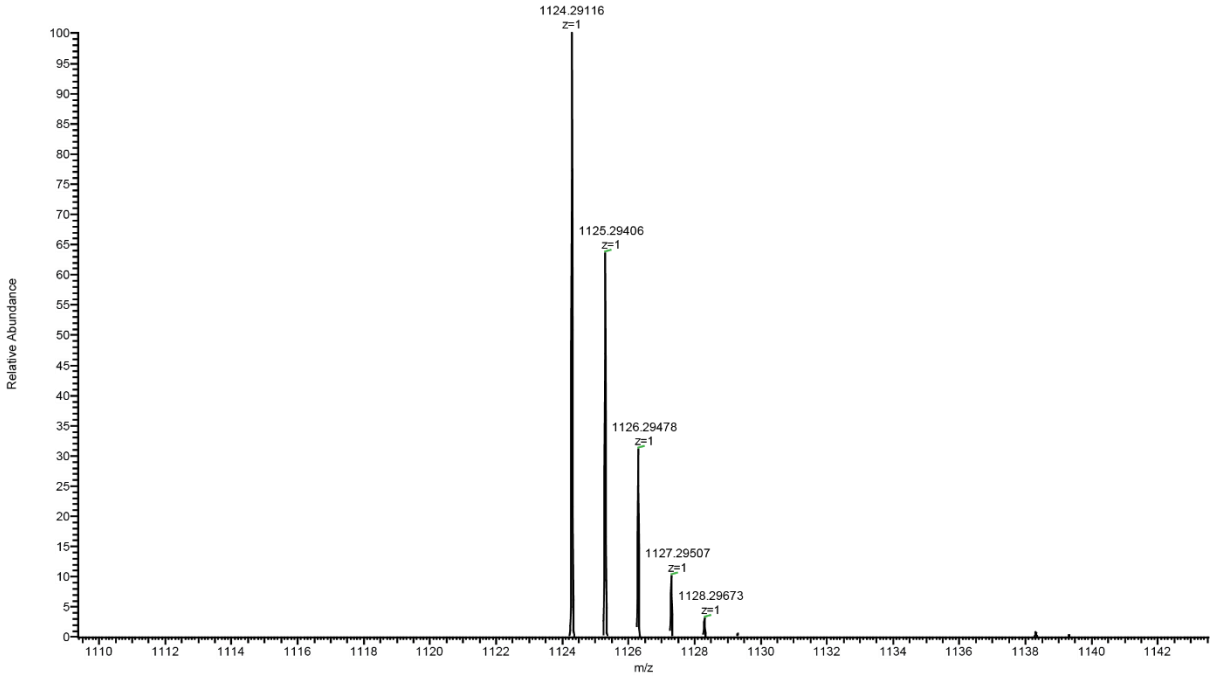
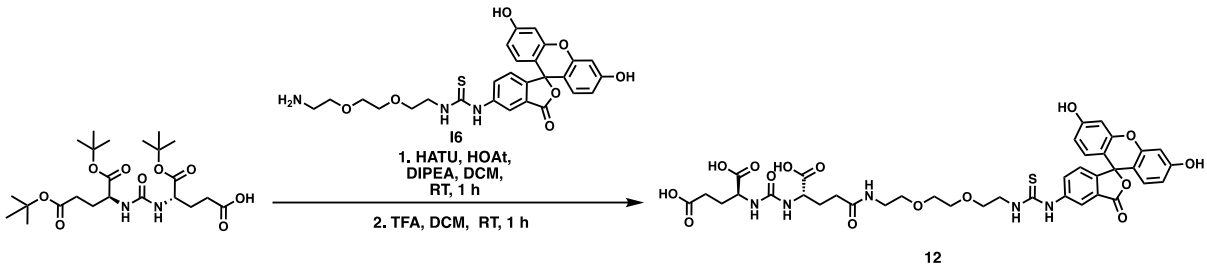


Figure S30. HR-MS of compound **6**.

Synthesis of (15S,19S)-1-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12,17-dioxo-1-thioxo-5,8-dioxa-2,11,16,18-tetraazahenicosane-15,19,21-tricarboxylic acid (12)



Scheme S8. Schematic illustration of the synthesis of compound **12**.

Commercially available (S)-5-(tert-butoxy)-4-(3-((S)-1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoic acid (10 mg, 0.021 mmol, 1.1 eq.) was dissolved in DCM (500 µL), and HATU (7 mg, 0.019 mmol, 1 eq.), HOAt (1.4 mg, 0.010 mmol, 0.5 eq.) and DIPEA (13 µL, 0.076 mmol, 4 eq.) were added. The acid was activated for 5 min at room temperature and added to compound **I6** (10 mg, 0.019 mmol, 1 eq.). The reaction was stirred at room temperature for 1 h followed by the addition of TFA (500 µL). After 1 hour at room temperature, solvent and TFA were removed under reduced pressure, the crude was re-dissolved in DMF:H_2O 1:1 and purified by RP-HPLC to obtain compound **12** as an orange solid (2 mg, yield = 13%). HR-MS (ESI+) *m/z* calculated for $C_{38}H_{41}N_5O_{15}S$ $[M+H]^+$ 840.23926; detected:840.23872; accuracy = 0.64 ppm

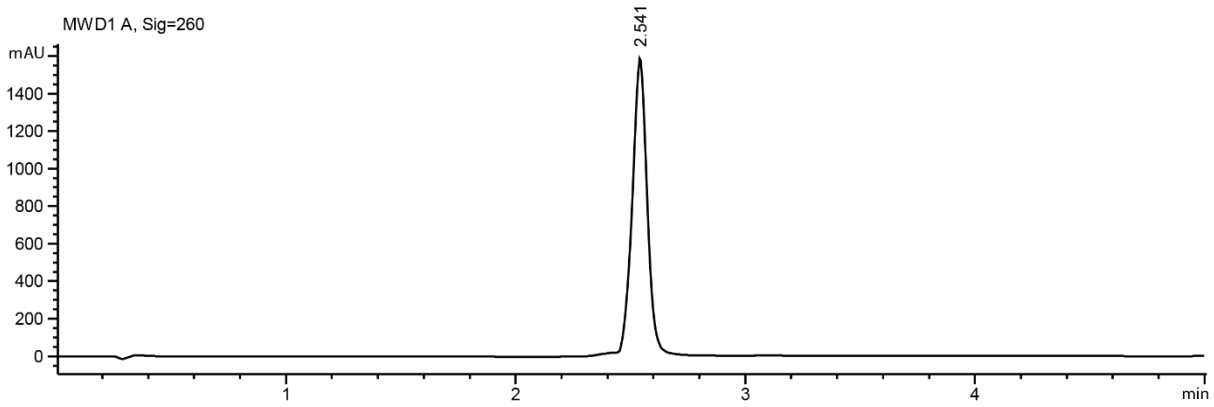


Figure S31. HPLC chromatogram of compound **12** recorded at λ = 260 nm.

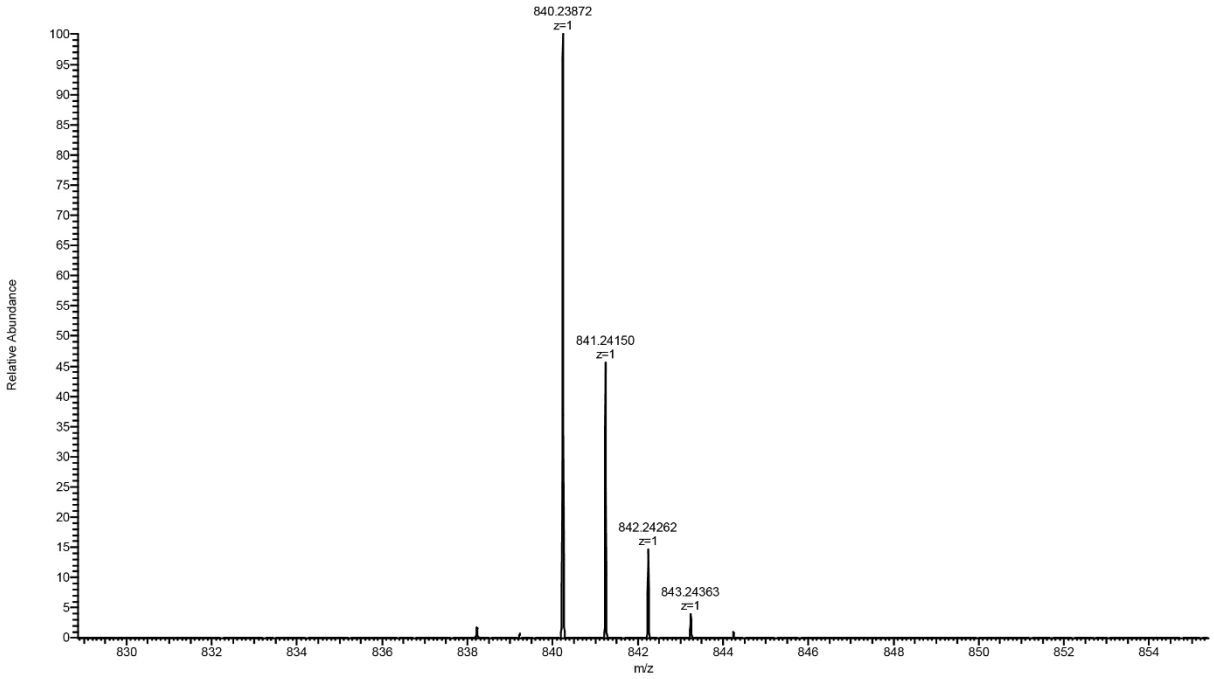


Figure S32. HR-MS of compound **12**.

3.1.3 Validation of the HITs by Fluorescence Polarization against PSMA and GCPIII

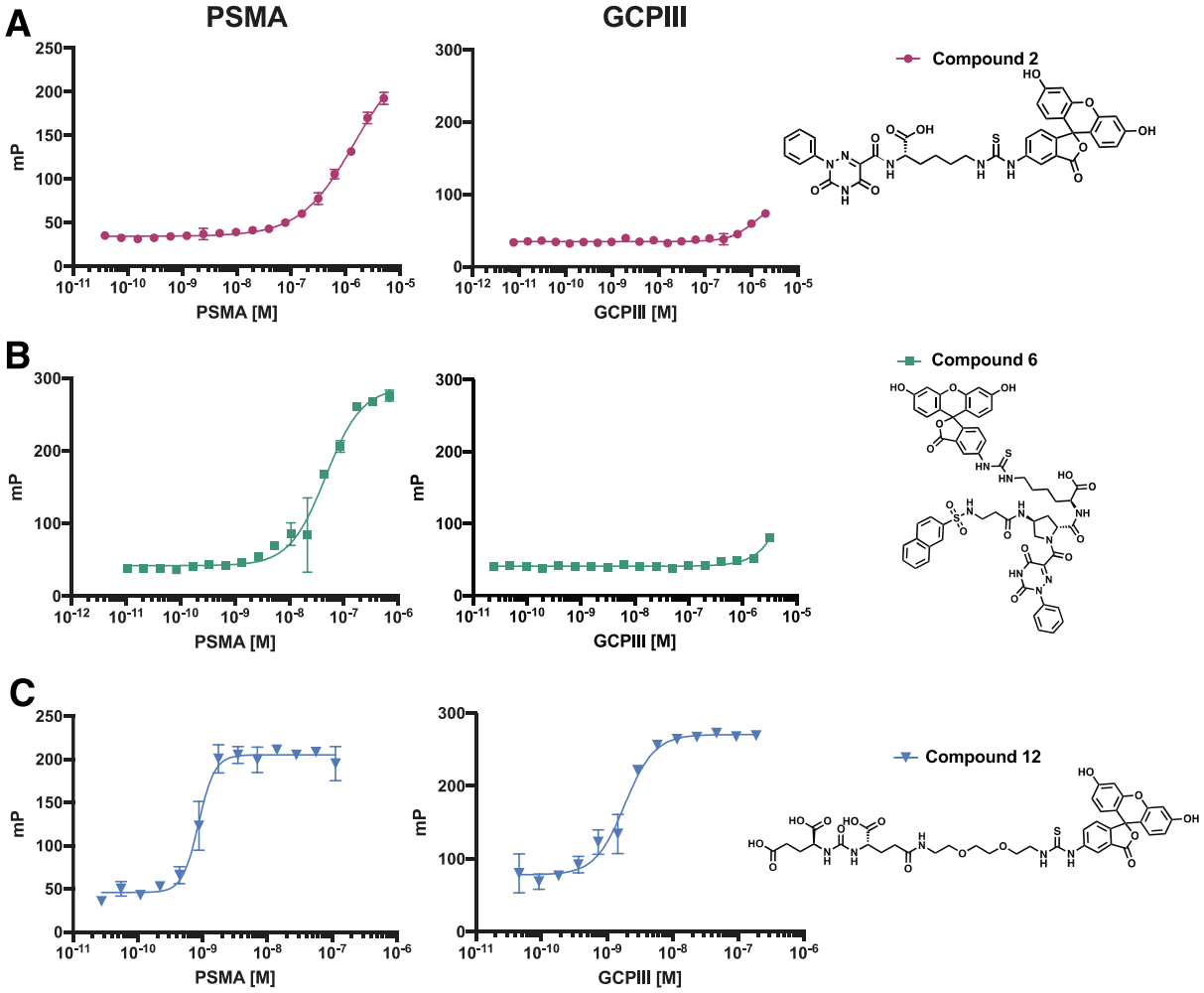


Figure S33. Fluorescence Polarization curves against PSMA (left) and GCPIII (right) for compound **2** (**A**, $K_{D,PSMA}$ = 1.4 ± 0.7 µM; $K_{D,GCPIII}$ = ND), compound **6** (**B**, $K_{D,PSMA}$ = 47 ± 1.2 nM; $K_{D,GCPIII}$ = ND) and compound **12** (**C**, $K_{D,PSMA}$ = 0.9 ± 1.2 nM; $K_{D,GCPIII}$ = 1.9 ± 0.4 nM).

3.2 Synthesis and characterization of ASM-DELs

3.2.1 Preparation of affinity and selectivity maturation DNA-Encoded Chemical Libraries (ASM-DELs)

ASM-DELs were synthesized as free amino-terminal DNA conjugate pools, and subsequently derivatized in parallel by amide bond formation with HIT_1, HIT_2, and HIT_3 to obtain the final ASM-DEL: MB1-HITx, MB2-HITx, MB3-HITx, LL1-HITx. LL2-HITx, PRA-HITx (**Manuscript**, **Figure 1**). Schematic representation of ASM DELs synthesis is reported below.

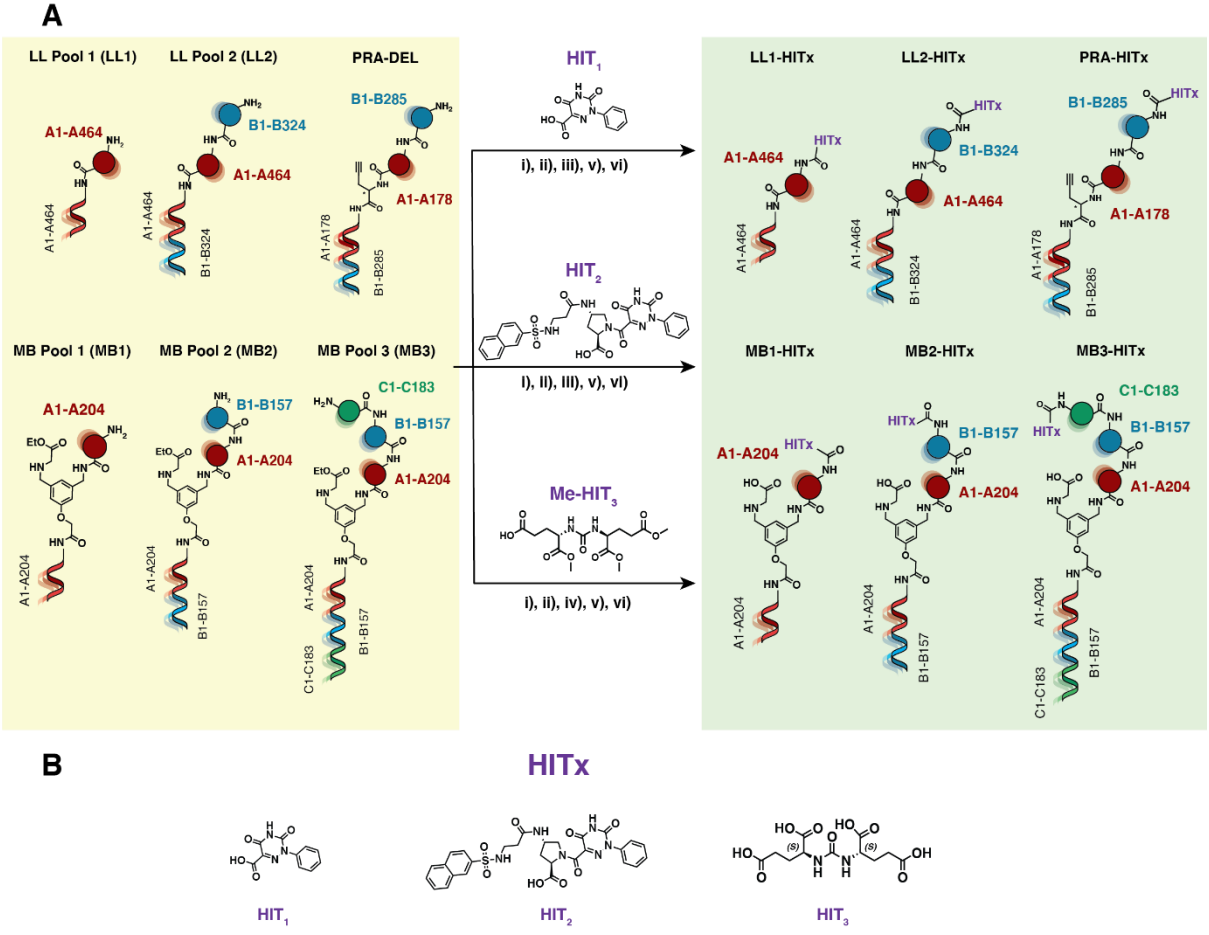


Figure S34. (**A**) General representation of ASM-DEL synthesis, via coupling HIT_1, HIT_2, Methylate HIT_3 to the six library pools. i) Amide coupling of the HITs with the amino terminal group of the library pools; ii) EtOH precipitation; iii) only for MB library pools: hydrolysis of ethyl ester group of MB scaffold and EtOH precipitation; iv) Hydrolysis of methyl ester groups of Me-HIT_3 and ethyl ester group of MB scaffold for MB pools, followed by EtOH purification; v) only for derivatized pool 1

libraries (LL1, MB1): elongation via splint ligation with a dummy Code B and EtOH precipitation; vi) Final HPLC purification. (**B**) Molecular structures of the final HIT_1, HIT_2, HIT_3.

HIT_1, **HIT_2**, **Me-HIT_3** were all synthesized as free carboxylic acids as shown in **Figure S34**. The three HITs were coupled to the free amino-terminal DNA conjugate pools using three different amide coupling protocols, specific to each HIT. The coupling procedures are reported below. The free amino DNA pools used for the construction of ASM DELs were the following: **MB pool 1** (5 nmol), **MB pool 2** (2 nmol), **MB pool 3** (1 nmol), **LL pool 1** (5 nmol), **LL pool 2** (5 nmol) and **PRA DEL** (0.5 nmol). The amount of water and buffer used to dissolve the DNA was calculated to have a DNA concentration between 0.2 and 0.5 mM. The amount of DMSO added was calculated to have a final ratio DMSO:water 1.5:1.

Coupling of HIT_1. HIT_1 was coupled to the free amino groups of the library pools using EDC and s-NHS as coupling reagents. HIT_1 (200 mM in DMSO, 50 eq.) was diluted in DMSO and EDC (100 mM in DMSO, 48 eq.) and s-NHS (333 mM in DMSO:water 2:1, 133 eq.) were added. After 30 min at rt, the pre-activated acid was added to a solution of each free amino DEL in MOPS buffer (100 mM MOPS, 1 M NaCl, pH 8). The reactions were carried out at 37°C overnight and subsequently a second addition of the HIT_1 (200 mM in DMSO, 50 eq.) pre-activated with EDC and s-NHS was performed. The reactions proceeded for further 4 h and then were stopped by adding 10% v/v of 5 M NaCl and 3 volumes of cold EtOH. The DNA was EtOH precipitated at -20°C overnight then centrifuged for 1h at 4°C, at 14’000 rpm, the supernatant was discarded, and the pellet was dried under reduced pressure using a SpeedVacuum.

Coupling of HIT_2 and Me-HIT_3. The two HITs were coupled to the free amino groups of the library pools using EDC and s-NHS as coupling reagents. HITs (200 mM in DMSO, 85 eq.) were diluted in DMSO and EDC (100 mM in DMSO, 84 eq.) and s-NHS (100 mM in DMSO:water 2:1, 40 eq.) were added. The acids were activated for 30 min at rt and then added to the DNA pools, pre-dissolved in a 1:1 water:TEA HCl (100 mM TEA HCl, pH 10) solution. The reactions were stirred overnight at rt and then the DNA was EtOH precipitated as previously described, centrifuged for 1h at 4°C, at 14'000 rpm, the supernatant was discarded, and the pellet was dried under reduced pressure using a SpeedVacuum. After coupling, all Me-HIT_3-bearing libraries and MB1-HIT_1, MB1-HIT_2, MB2-HIT_1, MB2-HIT_2 libraries went through methyl and ethyl esters hydrolysis to obtain the free carboxylic groups.

Me-HIT_3-bearing ASM DELs - Methyl esters hydrolysis. A 200 mM aqueous solution of LiOH (1000 eq.) was added to the DNA pellet (1 eq.), previously dissolved in water ($LiOH_{aq.sol.}$:water 1:1) and the reaction was carried out for 1 h at rt. The DNA was precipitated with 10% v/v of Acetate Buffer (3M Acetate, pH 5) and 3 volumes of cold EtOH and was kept at -20°C overnight. The pellets were centrifuged for 1h at 4°C, at 14'000 rpm, the supernatant was discarded, and the DNA was dried under reduced pressure using a SpeedVacuum.

MB pool 1 and pool 2 ASM-DELs - Ethyl ester hydrolysis. MB1-HIT_1, MB1-HIT_2, MB2-HIT_1, MB2-HIT_2 (5 nmol) were dissolved in water (50 µL each) and TEA (5 µL each) was added. After 1h at rt, the DNA pellets were precipitated with 10% v/v of 5 M NaCl and 3 volumes of cold EtOH and were kept at -20°C overnight. The pellets were centrifuged for 1h at 4°C, at 14'000 rpm and dried under reduced pressure.

The ASM-DEL were purified by RP-HPLC to remove the excess of small molecule and coupling by-products and analysed by LCMS (From **Figure S35B** to **Figure S52)**.

Elongation of Pool 1 libraries and final purification

MB1 and LL1 derivatized with the HITs were elongated with a dummy code in order to allow the screening and sequencing processes. Six different code B were used in order to differentiate the combination DEL-HITx To the dry DNA pools (5 nmol, 1 eq.), 140 µL of code B (0.05 mM, 7 nmol, 1.5 eq., 5'- GGATCGACGXXXXXXXGCGTCAGGCAGC-3'), 25 µL of Adaptor1 (0.4 mM, 10 nmol, 2 eq., 5'- CGTCGATCCGGCGCCATGG-3'), 240 µL of water and 45 µL of 10x T4 DNA Ligase Reaction Buffer (1x final, 500 mM Tris-HCl, 100 mM MgCl2, 100 mM dithiothreitol, 10 mM ATP, pH 7.5 New England Biolabs, #B0202S) were added. The reactions were heated for 5 min at 70°C and cooled down to rt. Afterwards, 6 µL of T4 DNA ligase (New England Biolabs, #M0202L, 400'000 units/mL, 2'500 units per reaction) were added. The ligations were kept for 2 h at room temperature and quenched by heating at 65°C for 10 min. The reactions were analyzed by LC-MS and EtOH precipitated. The DNA pellets were re-dissolved in 0.1 M TEAA, and the pools were purified by RP-HPLC (**Figure S35A, S36A, S37A, S41A, S42A, S43A**) at 70 °C to remove the adaptor and excess of code B.

All the derivatized pools were purified by RP-HPLC. Fractions of final pools were confirmed via Agarose gel electrophoresis, combined and analysed by LC-MS (from **Figure S35B** to **Figure S52**). Additionally, the final generated ASM-DELs were analysed via 15% TBE- Urea gel (**Figure S53**).

RP-HPLC and LC-MS analysis of ASM DELs

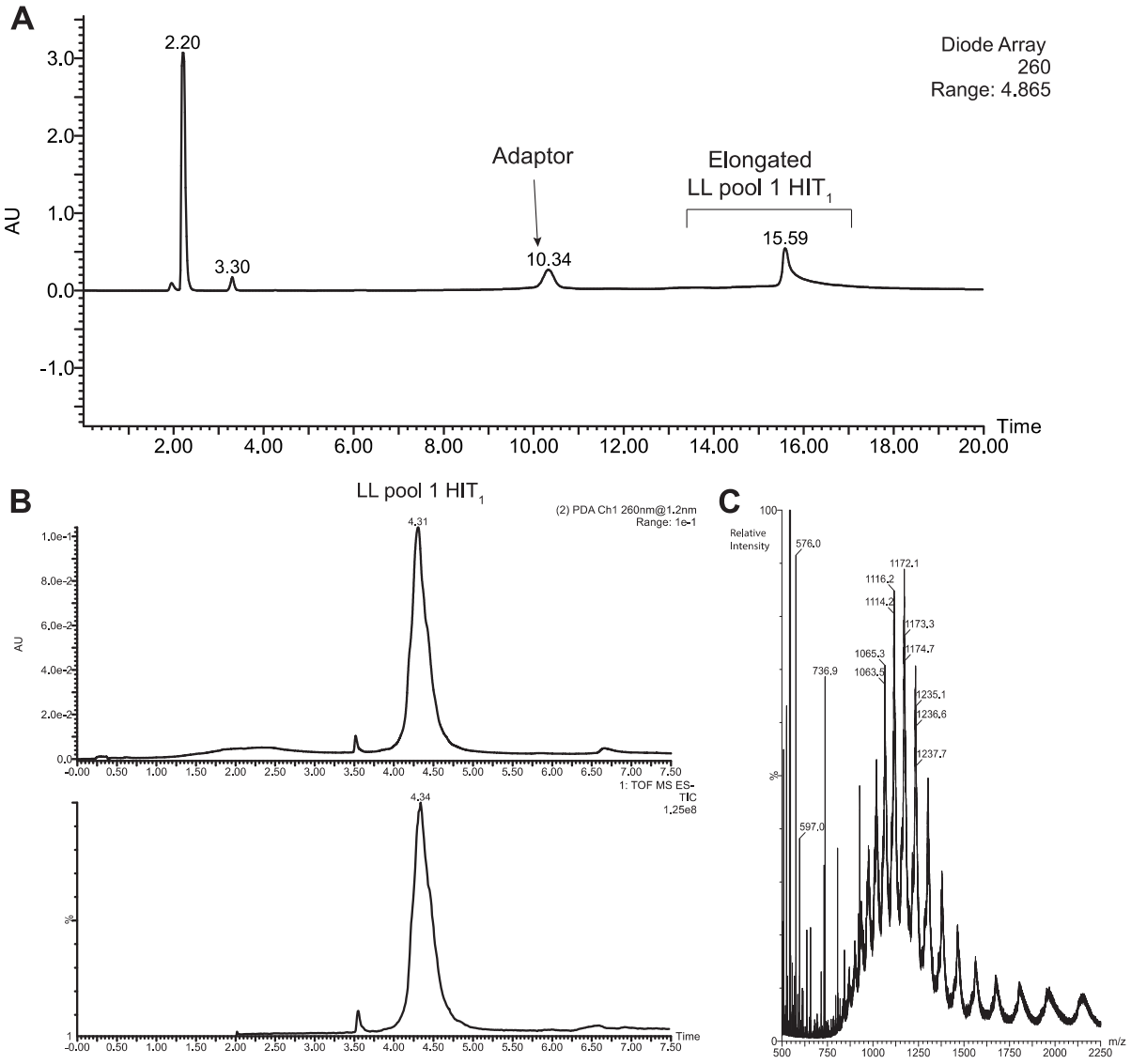


Figure S35. Analysis of **LL pool 1** after coupling with the **HIT$_1$** and encoding with dummy Code B. **A.** RP-HPLC chromatogram of **LL1 HIT$_1$** registered at 260 nm. **B.** LC chromatogram of **LL1 HIT$_1$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **LL1 HIT$_1$**.

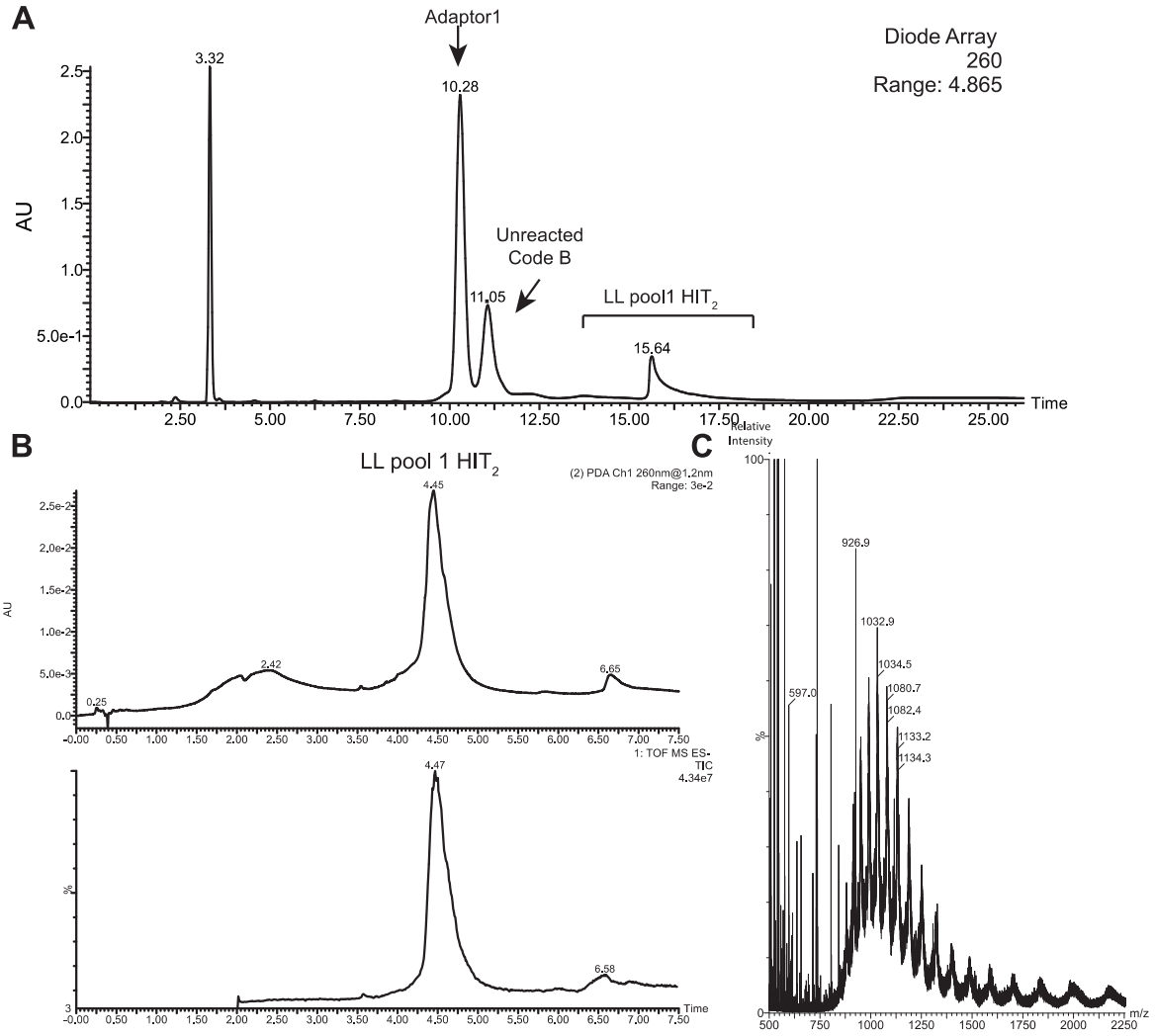


Figure S36. Analysis of **LL pool 1** after coupling with the **HIT$_2$** and encoding with dummy Code B. **A.** RP-HPLC chromatogram of **LL1 HIT$_2$** registered at 260 nm. **B.** LC chromatogram of **LL1 HIT$_2$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **LL1 HIT$_2$**.

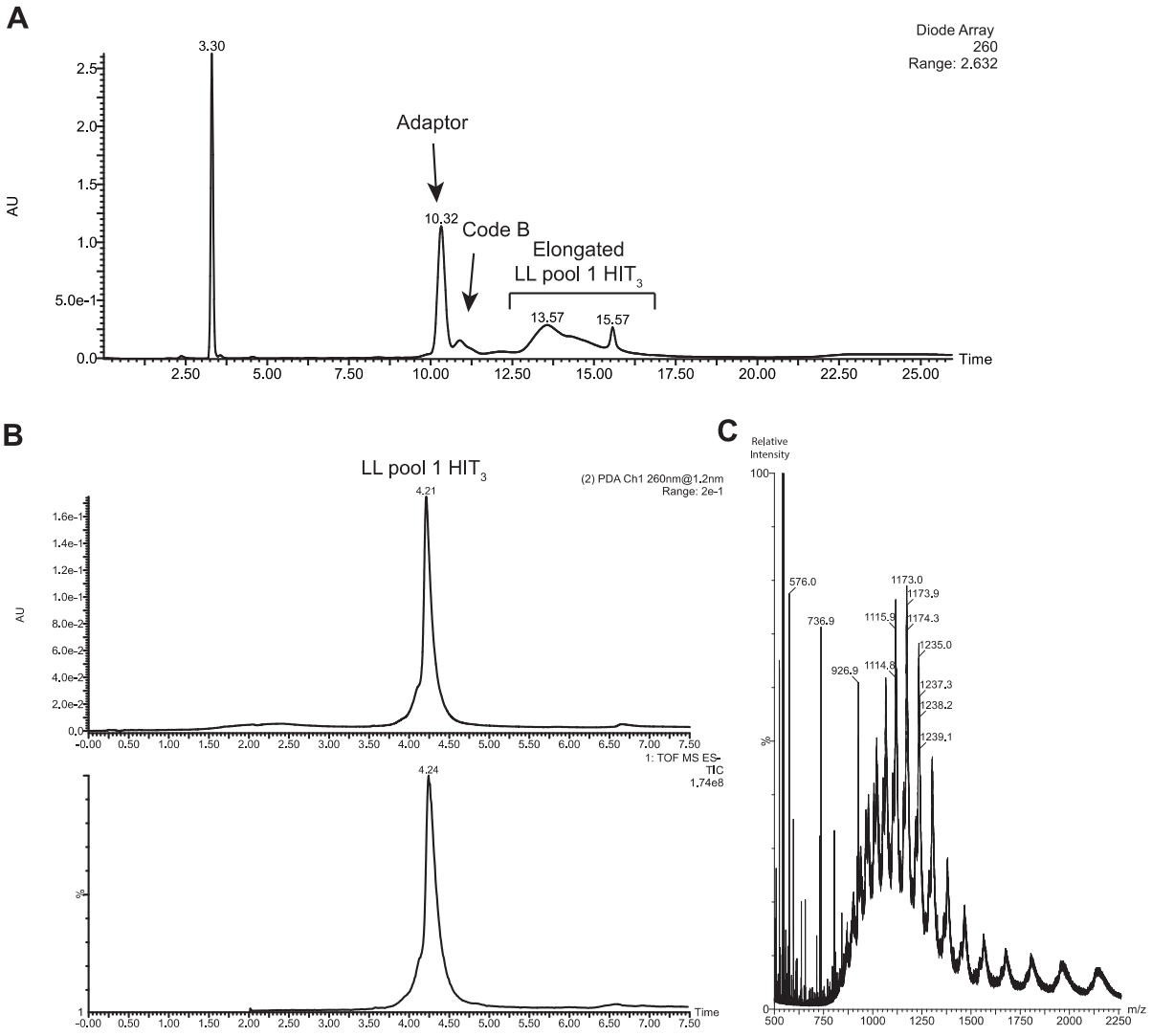


Figure S37. Analysis of **LL pool 1** after coupling with the **HIT$_3$**, methyl ester hydrolysis and encoding with dummy Code B. **A.** RP-HPLC chromatogram of **LL1 HIT$_3$** registered at 260 nm. **B.** LC chromatogram of **LL1 HIT$_3$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **LL1 HIT$_3$**.

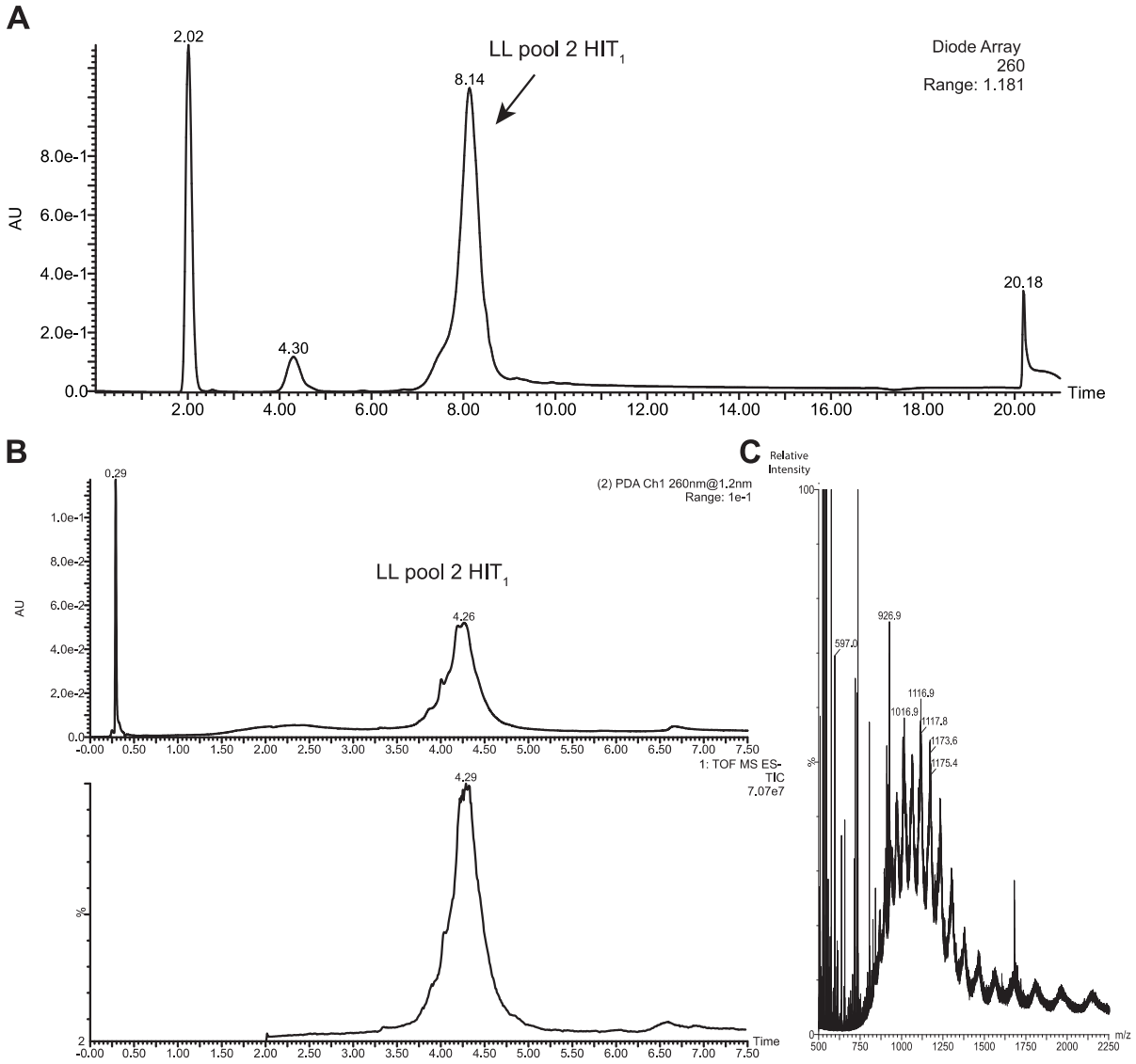


Figure S38. Analysis of **LL pool 2** after coupling with the **HIT$_1$**. **A.** RP-HPLC chromatogram of **LL2 HIT$_1$** registered at 260 nm. **B.** LC chromatogram of **LL2 HIT$_1$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **LL2 HIT$_1$**.

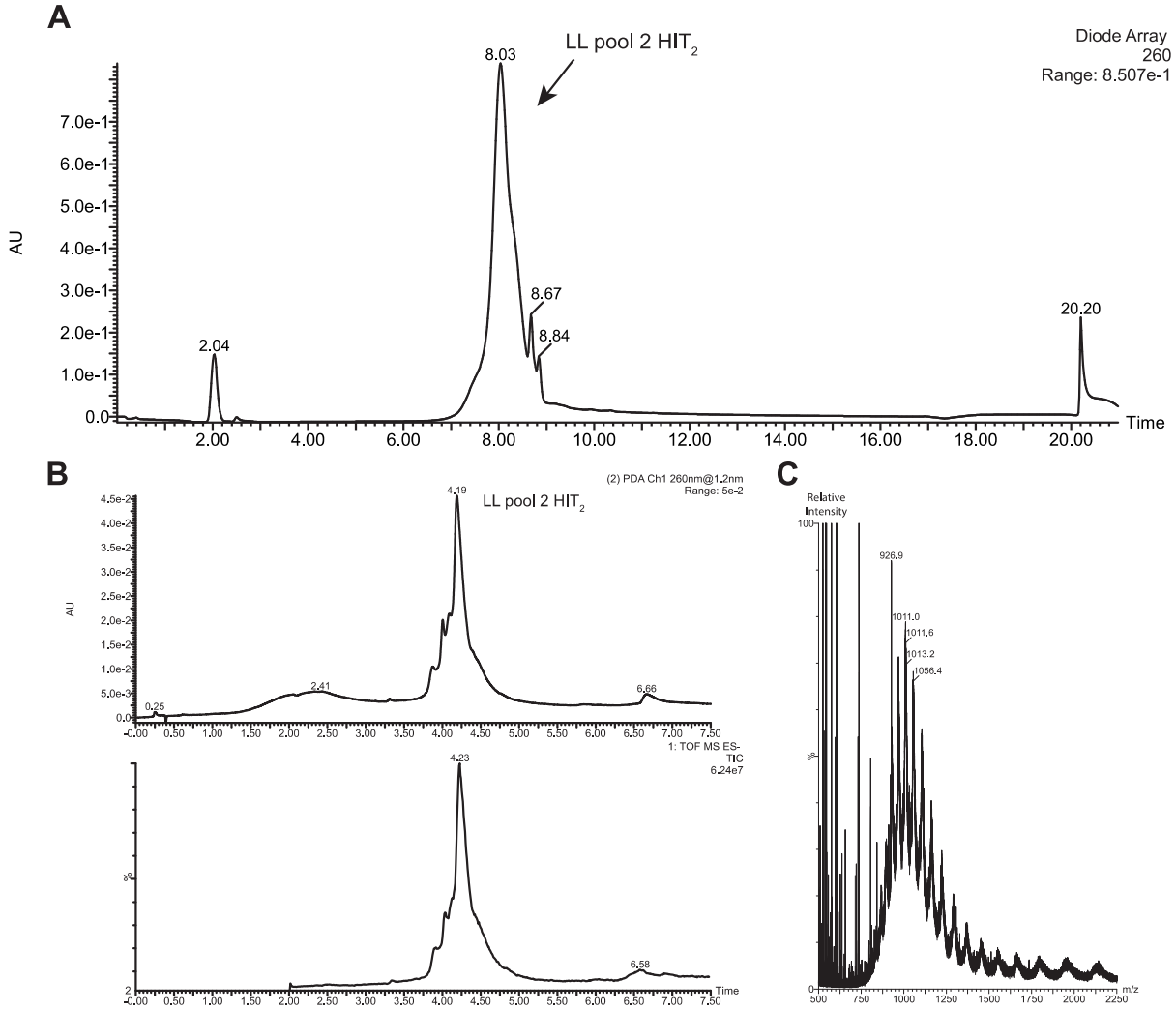


Figure S39. Analysis of **LL pool 2** after coupling with the **HIT_2**. **A.** RP-HPLC chromatogram of **LL2 HIT_2** registered at 260 nm. **B.** LC chromatogram of **LL2 HIT_2** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **LL2 HIT_2**.

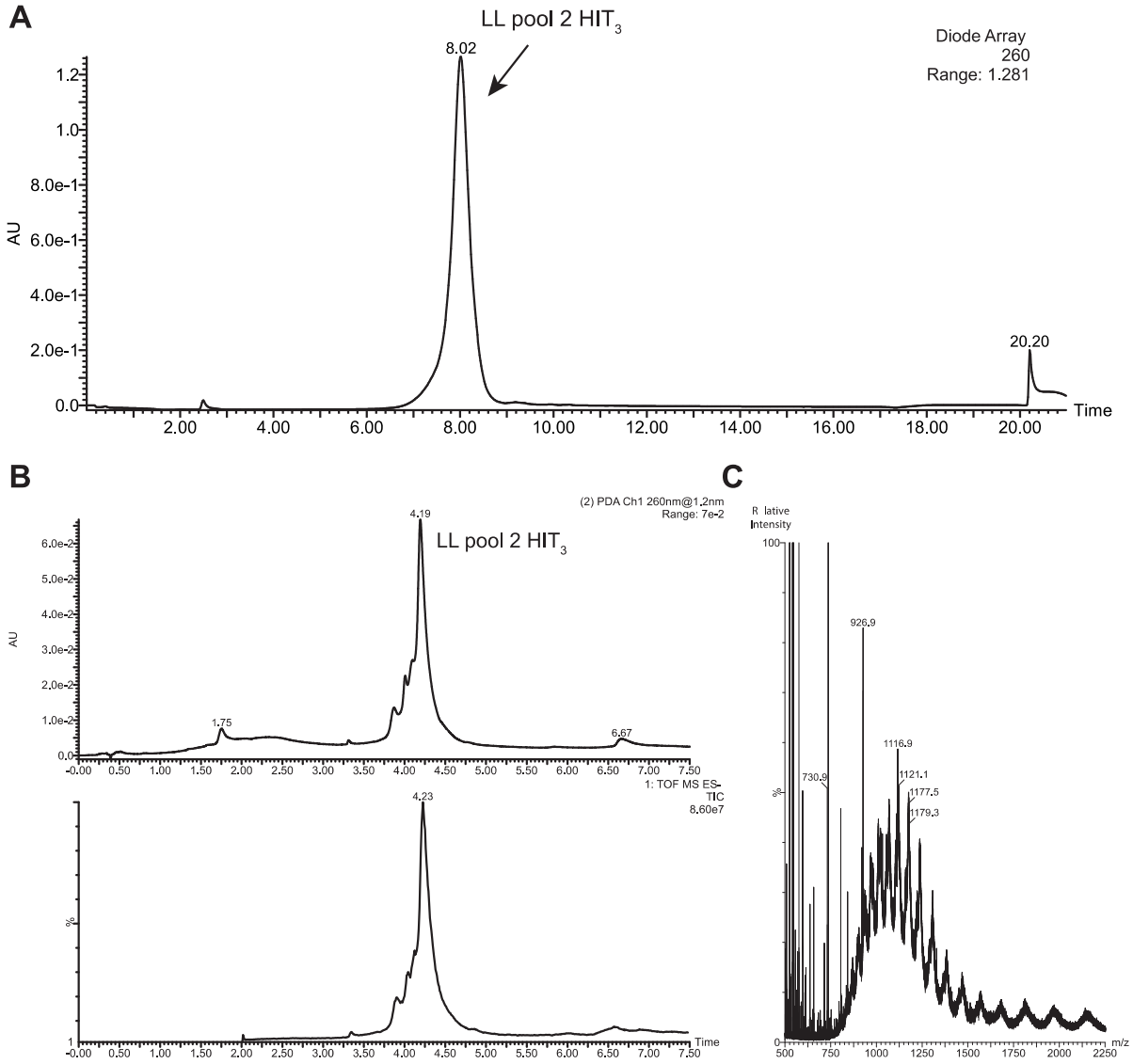


Figure S40. Analysis of **LL pool 2** after coupling with the **HIT$_3$** and methyl ester hydrolysis. **A.** RP-HPLC chromatogram of **LL2 HIT$_3$** registered at 260 nm. **B.** LC chromatogram of **LL2 HIT$_3$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **LL2 HIT$_3$**.

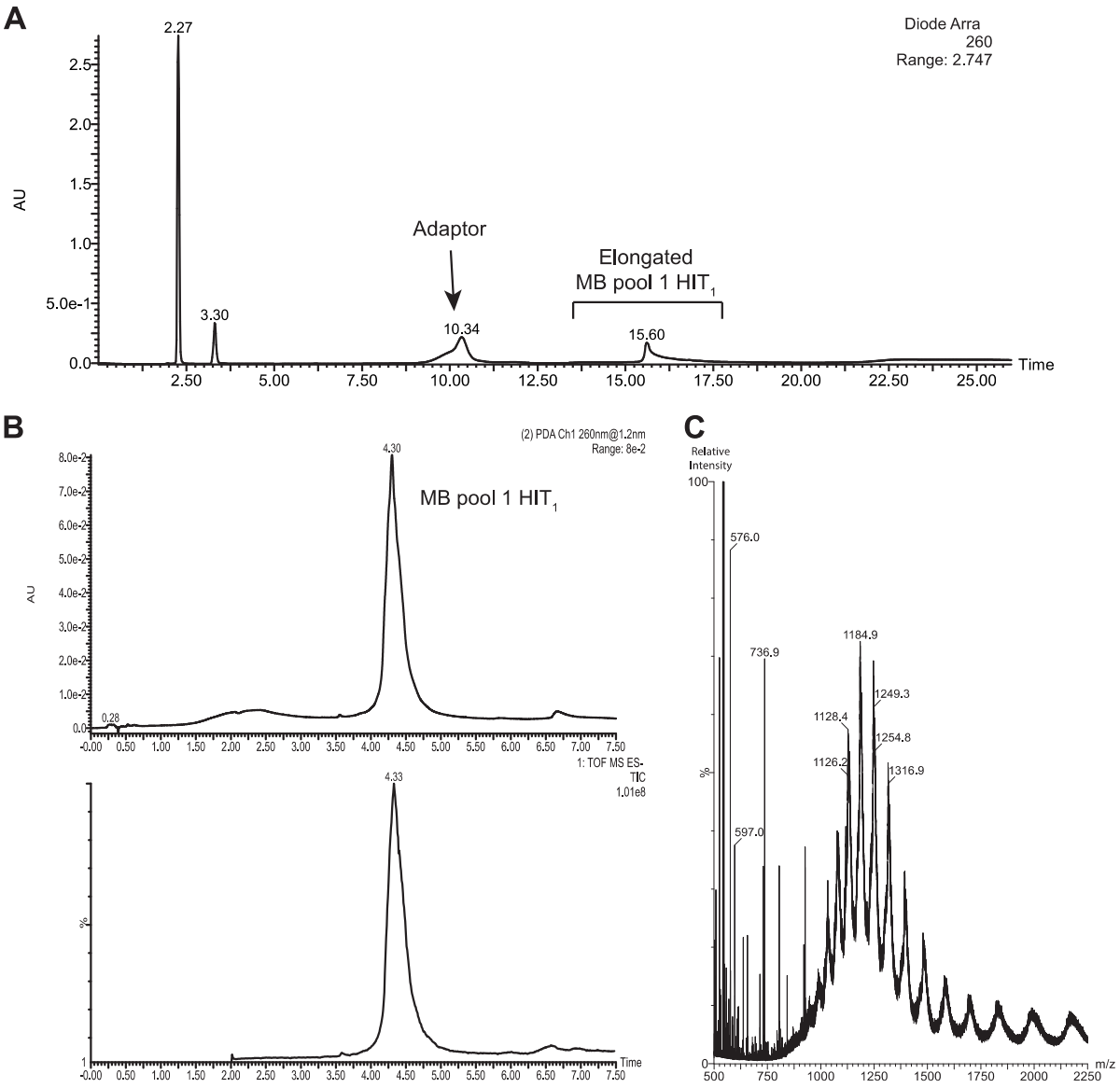


Figure S41. Analysis of **MB pool 1** after coupling with the **HIT$_1$**, ethyl ester hydrolysis and encoding with dummy Code B. **A.** RP-HPLC chromatogram of **MB1 HIT$_1$** registered at 260 nm. **B.** LC chromatogram of **MB1 HIT$_1$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB1 HIT$_1$**.

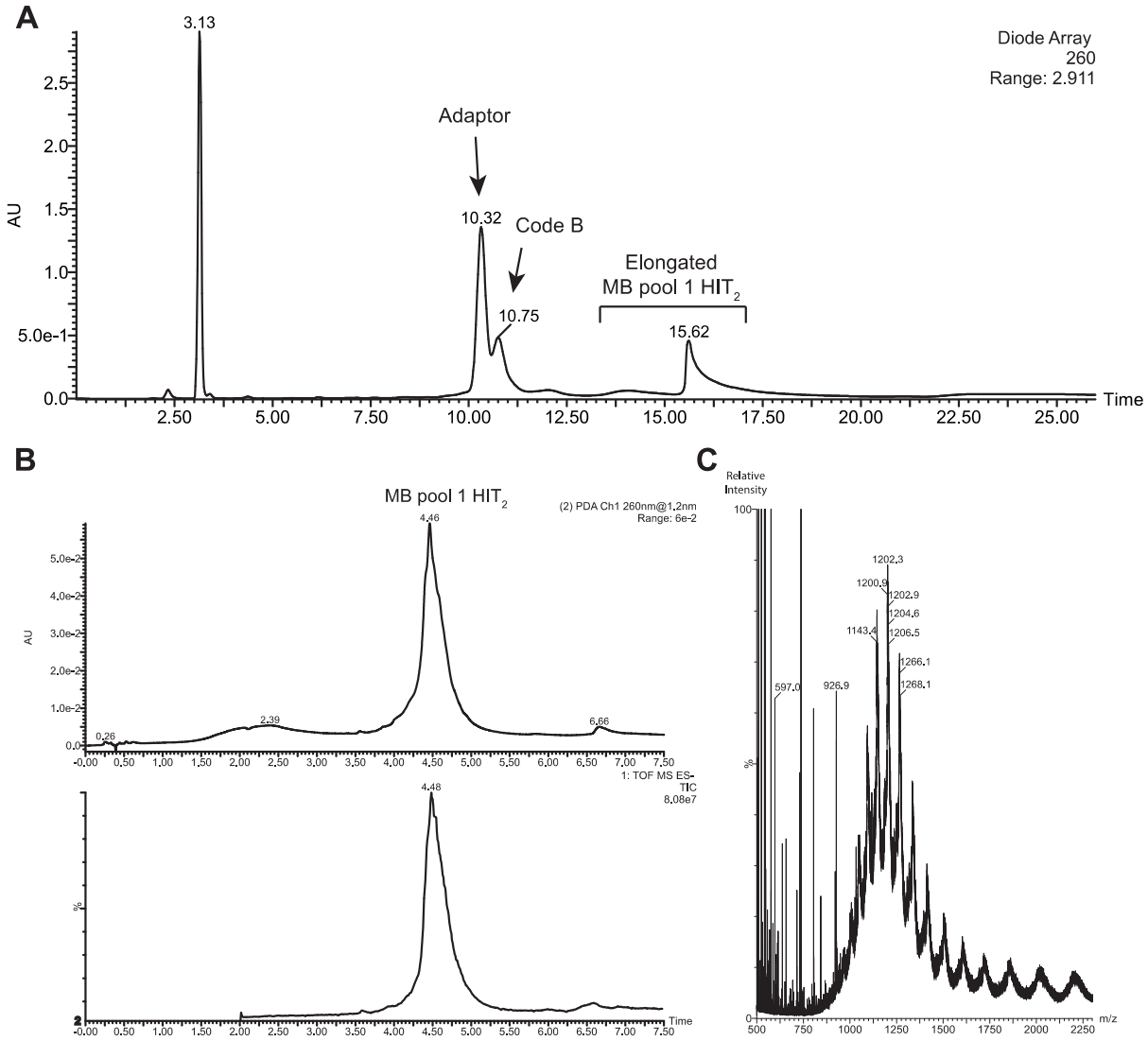


Figure S42. Analysis of **MB pool 1** after coupling with the **HIT$_2$**, ethyl ester hydrolysis and encoding with dummy Code B. **A.** RP-HPLC chromatogram of **MB1 HIT$_2$** registered at 260 nm. **B.** LC chromatogram of **MB1 HIT$_2$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB1 HIT$_2$**.

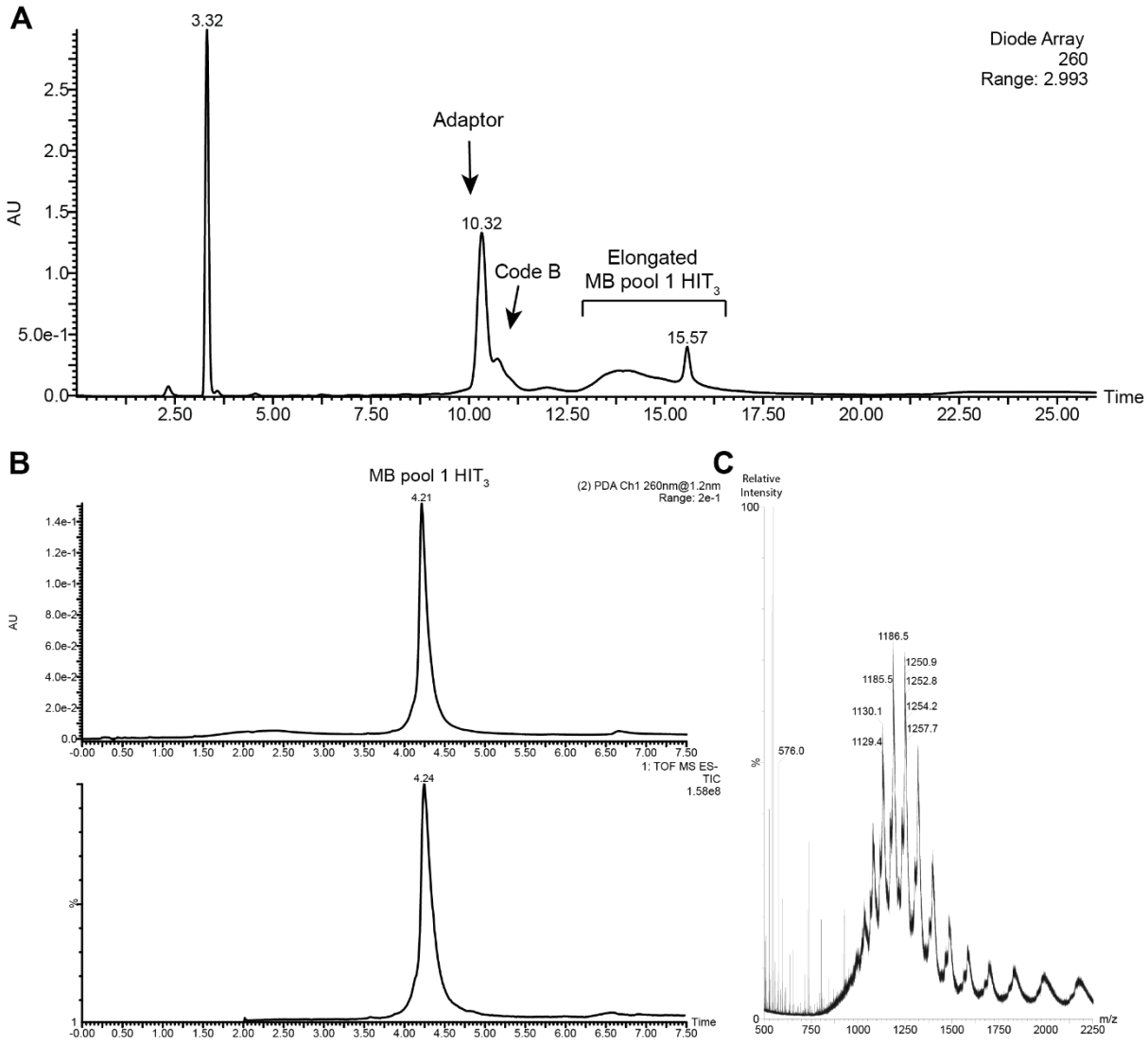


Figure S43. Analysis of **MB pool 1** after coupling with the **HIT$_3$**, ethyl and methyl ester hydrolysis and encoding with dummy Code B. **A.** RP-HPLC chromatogram of **MB1 HIT$_3$** registered at 260 nm. **B.** LC chromatogram of **MB1 HIT$_3$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB1 HIT$_3$**.

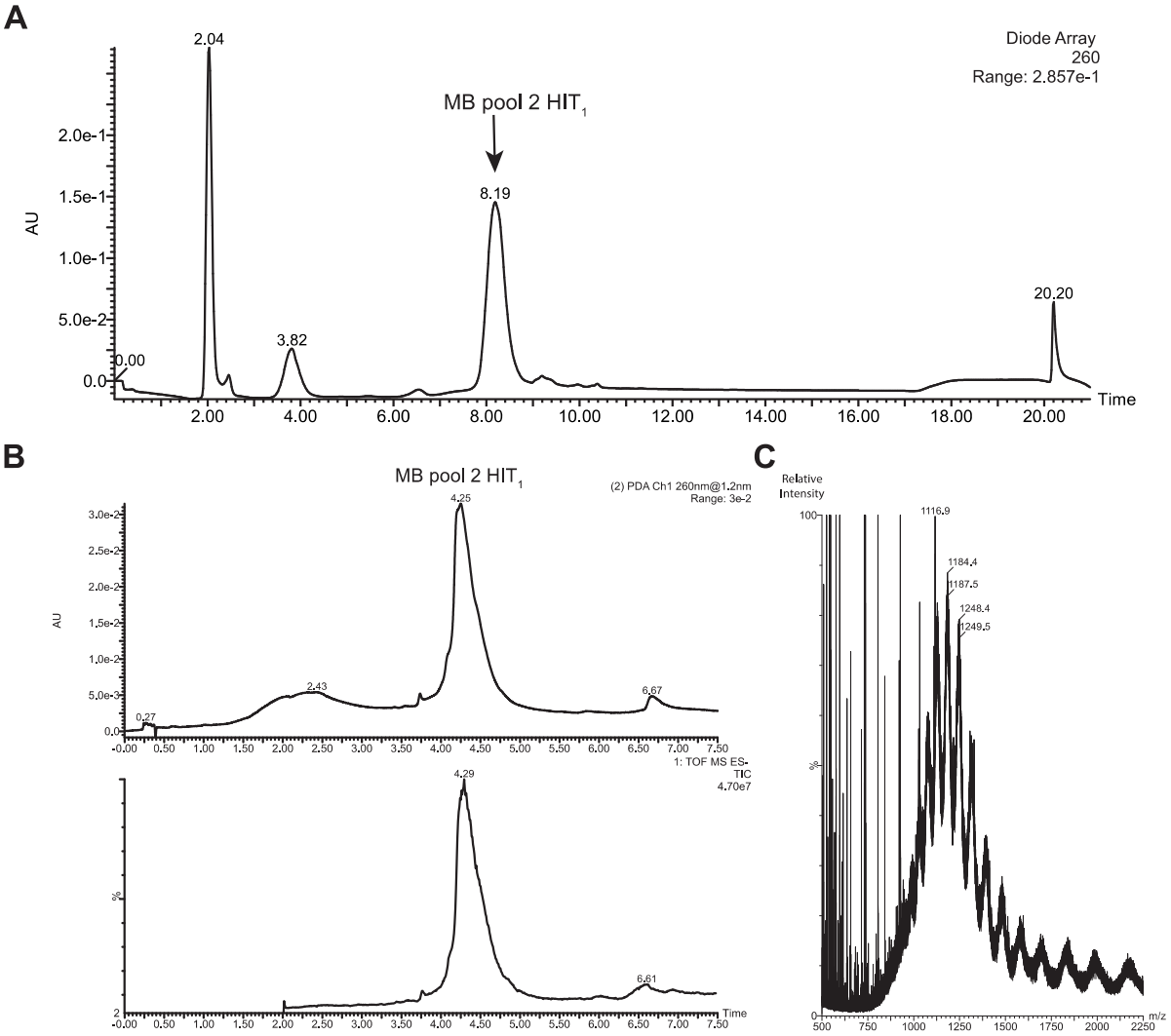


Figure S44. Analysis of **MB pool 2** after coupling with the **HIT$_1$** and ethyl ester hydrolysis. **A.** RP-HPLC chromatogram of **MB2 HIT$_1$** registered at 260 nm. **B.** LC chromatogram of **MB2 HIT$_1$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB2 HIT$_1$**.

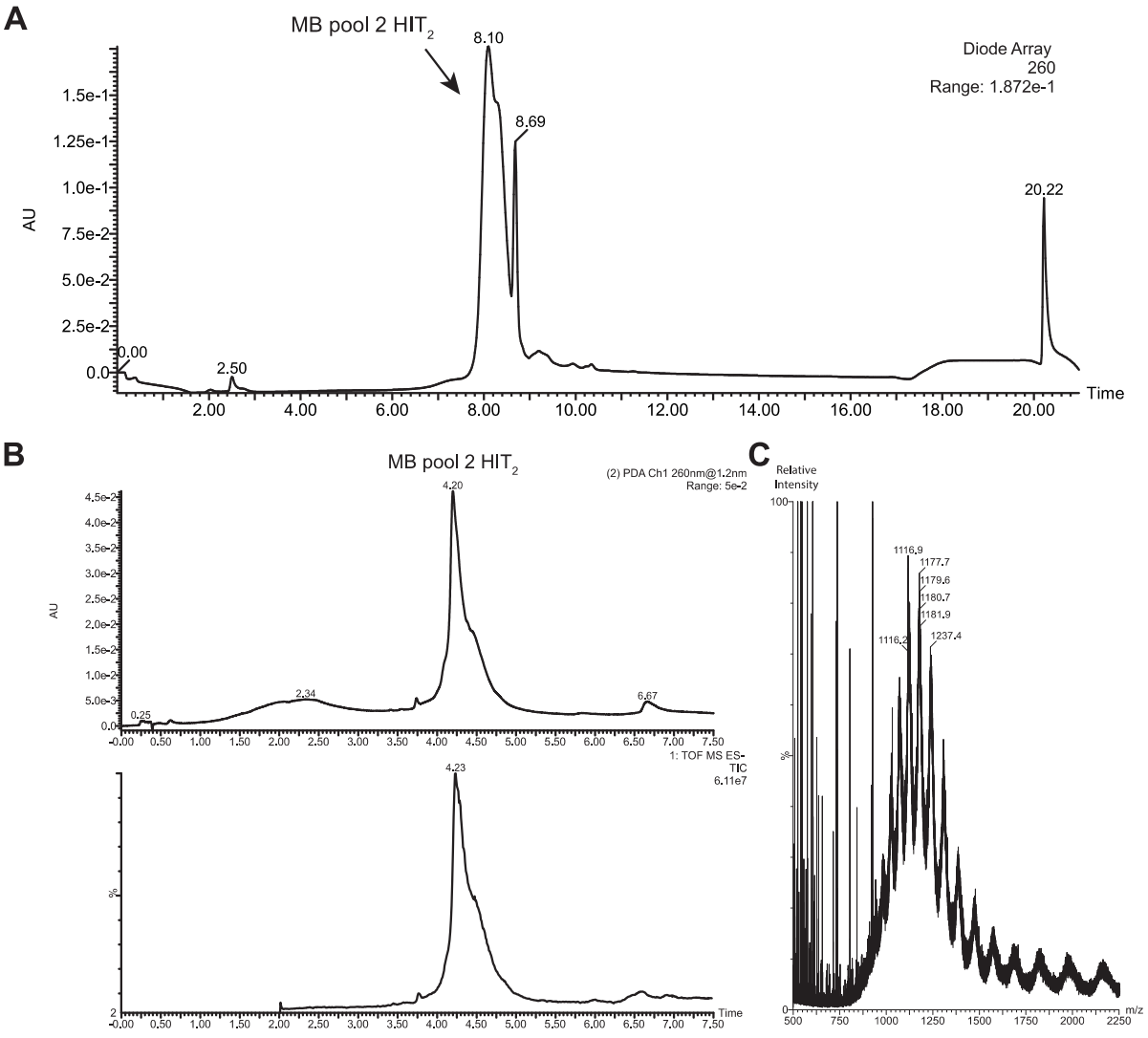


Figure S45. Analysis of **MB pool 2** after coupling with the **HIT$_2$** and ethyl ester hydrolysis. **A.** RP-HPLC chromatogram of **MB2 HIT$_2$** registered at 260 nm. **B.** LC chromatogram of **MB2 HIT$_2$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB2 HIT$_2$**.

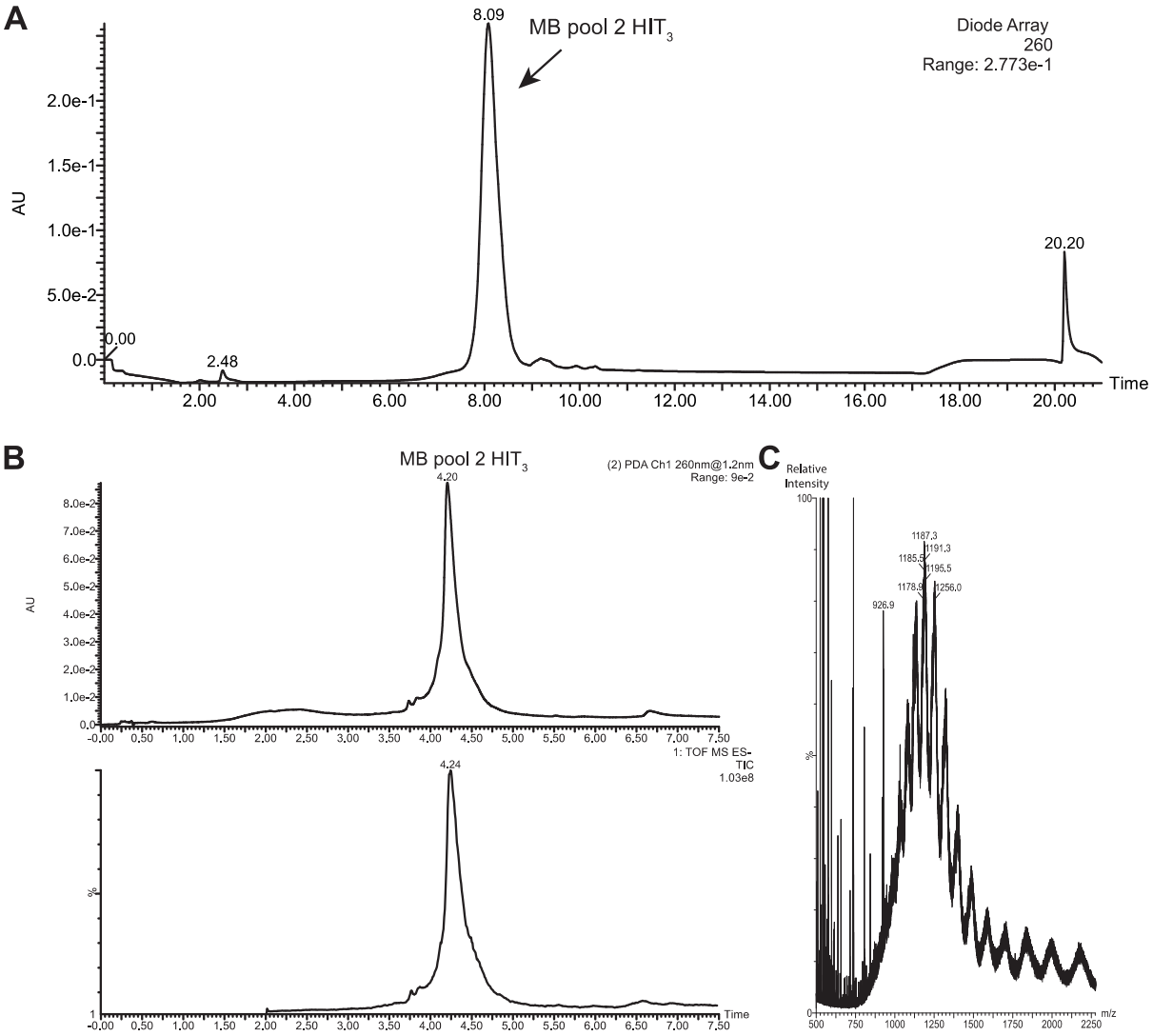


Figure S46. Analysis of **MB pool 2** after coupling with the **HIT$_3$** followed by methyl and ethyl ester hydrolysis. **A.** RP-HPLC chromatogram of **MB2 HIT$_3$** registered at 260 nm. **B.** LC chromatogram of **MB2 HIT$_3$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB2 HIT$_3$**.

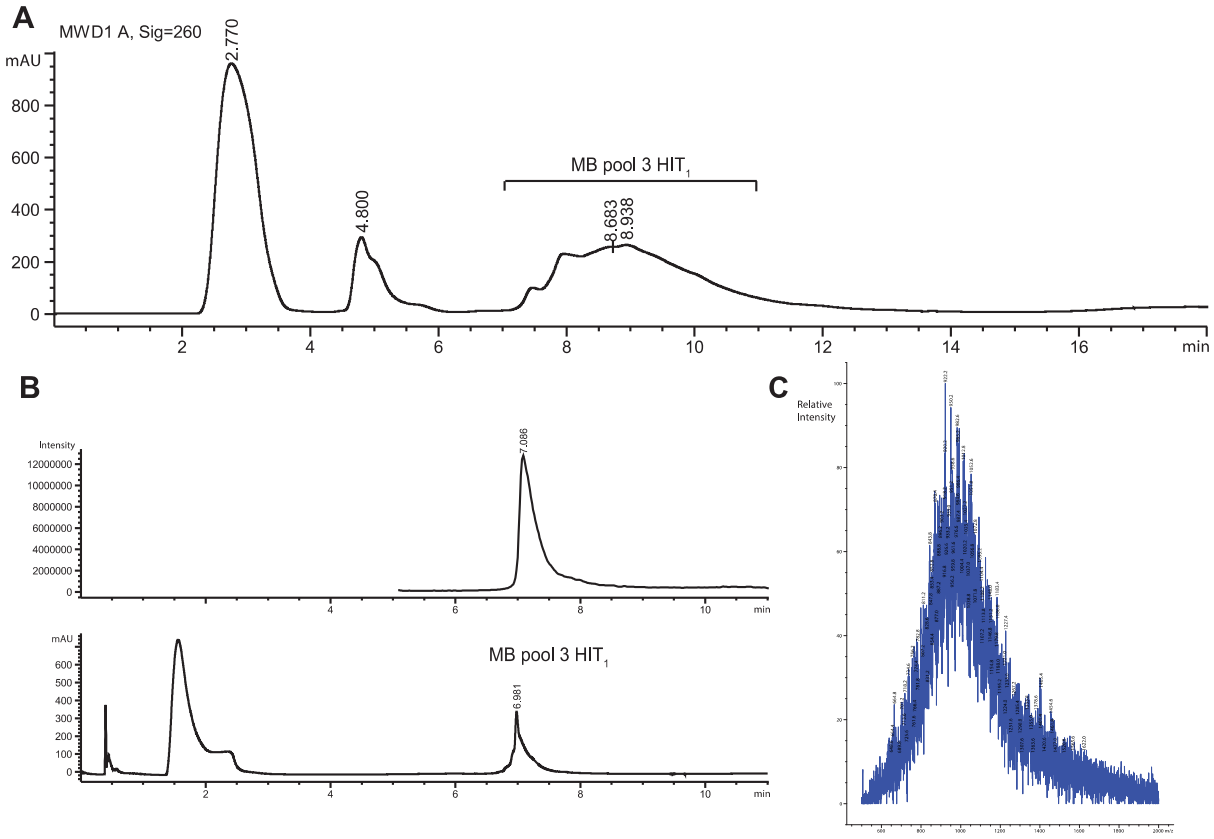


Figure S47. Analysis of **MB pool 3** after coupling with the **HIT$_1$**. **A.** RP-HPLC chromatogram of **MB3 HIT$_1$** registered at 260 nm. (Due to the low amount of library available, the UV signal resulted relatively low). **B.** LC chromatogram of **MB3 HIT$_1$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB3 HIT$_1$**.

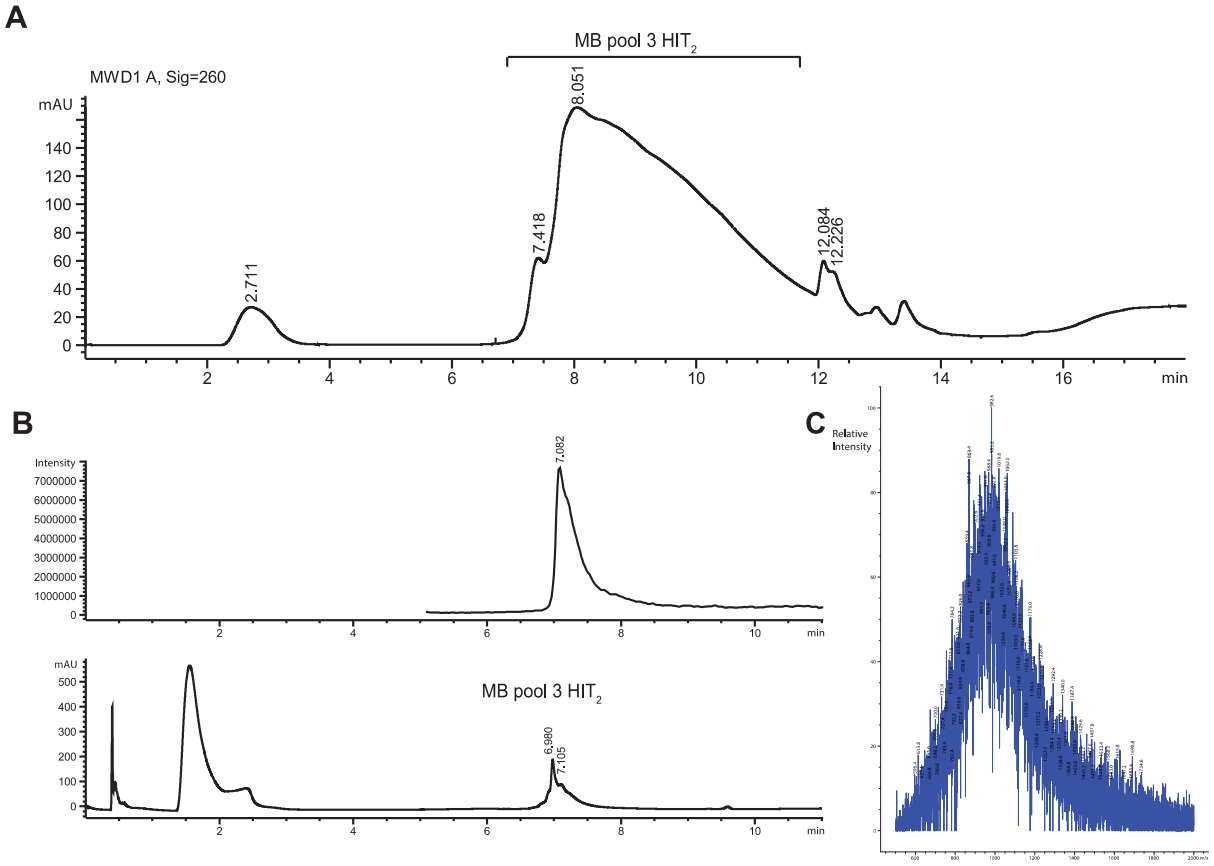


Figure S48. Analysis of **MB pool 3** after coupling with the **HIT$_2$**. **A.** RP-HPLC chromatogram of **MB3 HIT$_2$** registered at 260 nm. (Due to the low amount of library available, the UV signal resulted relatively low). **B.** LC chromatogram of **MB3 HIT$_2$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB3 HIT$_2$**.

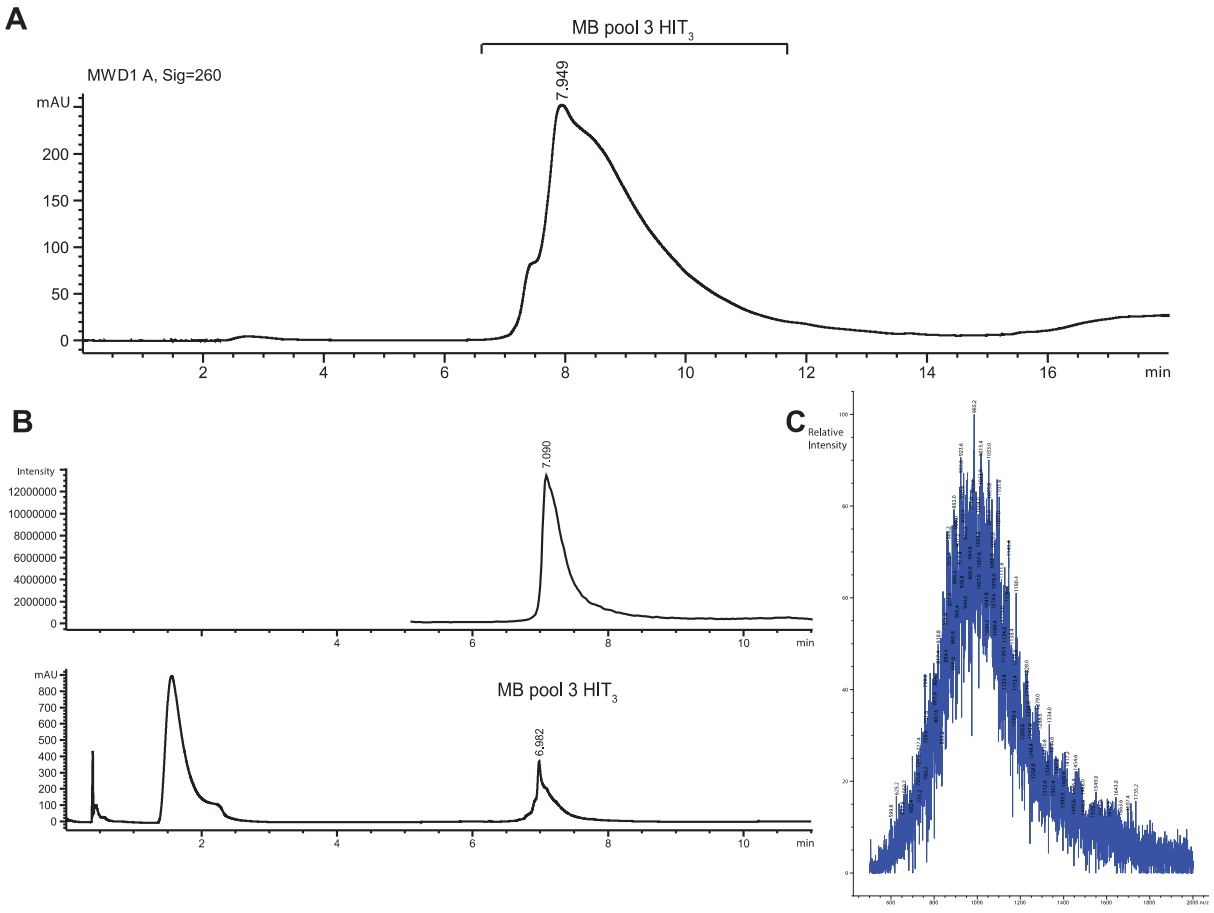


Figure S49. Analysis of **MB pool 3** after coupling with the **HIT$_3$** and methyl ester hydrolysis. **A.** RP-HPLC chromatogram of **MB3 HIT$_3$** registered at 260 nm. (Due to the low amount of library available, the UV signal resulted relatively low). **B.** LC chromatogram of **MB3 HIT$_3$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **MB3 HIT$_3$**.

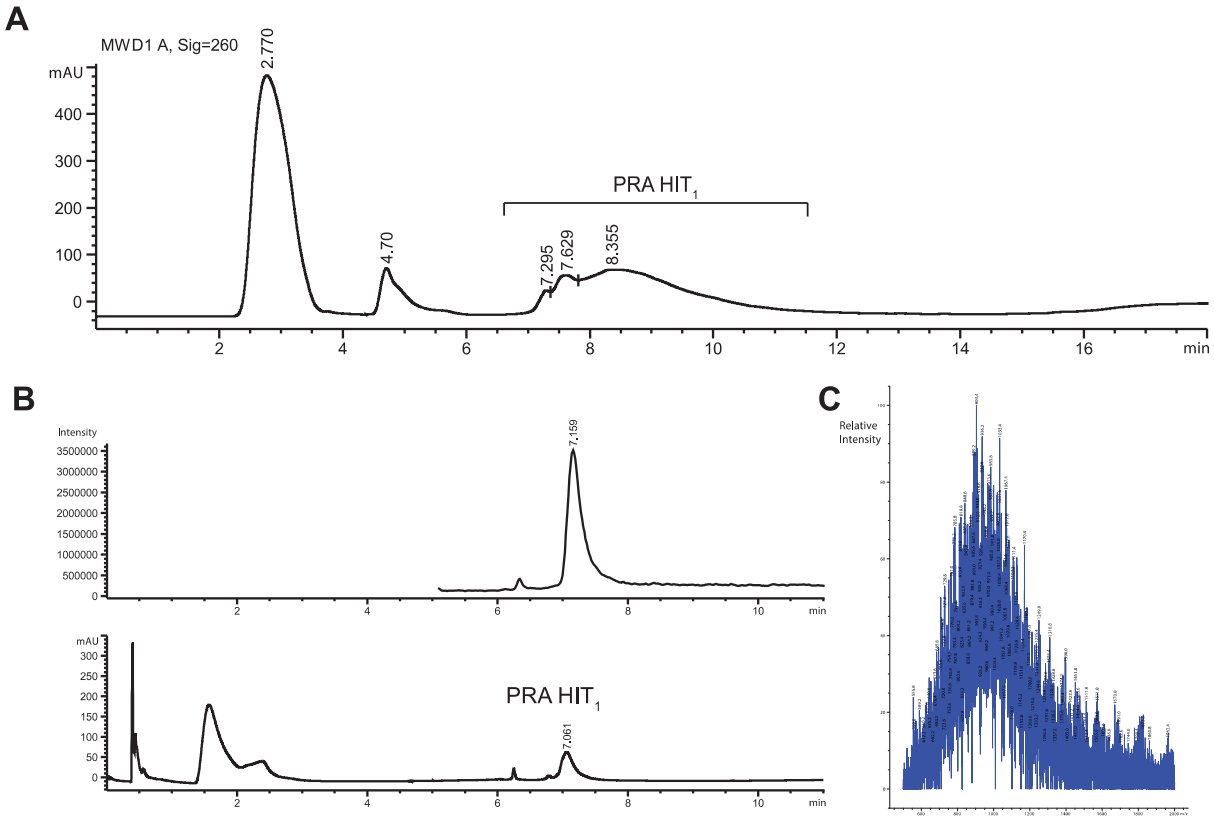


Figure S50. Analysis of **PRA DEL** after coupling with the **HIT$_1$ A.** RP-HPLC chromatogram of **PRA HIT$_1$** registered at 260 nm. (Due to the low amount of library available, the UV signal resulted relatively low). **B.** LC chromatogram of **PRA HIT$_1$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **PRA HIT$_1$**.

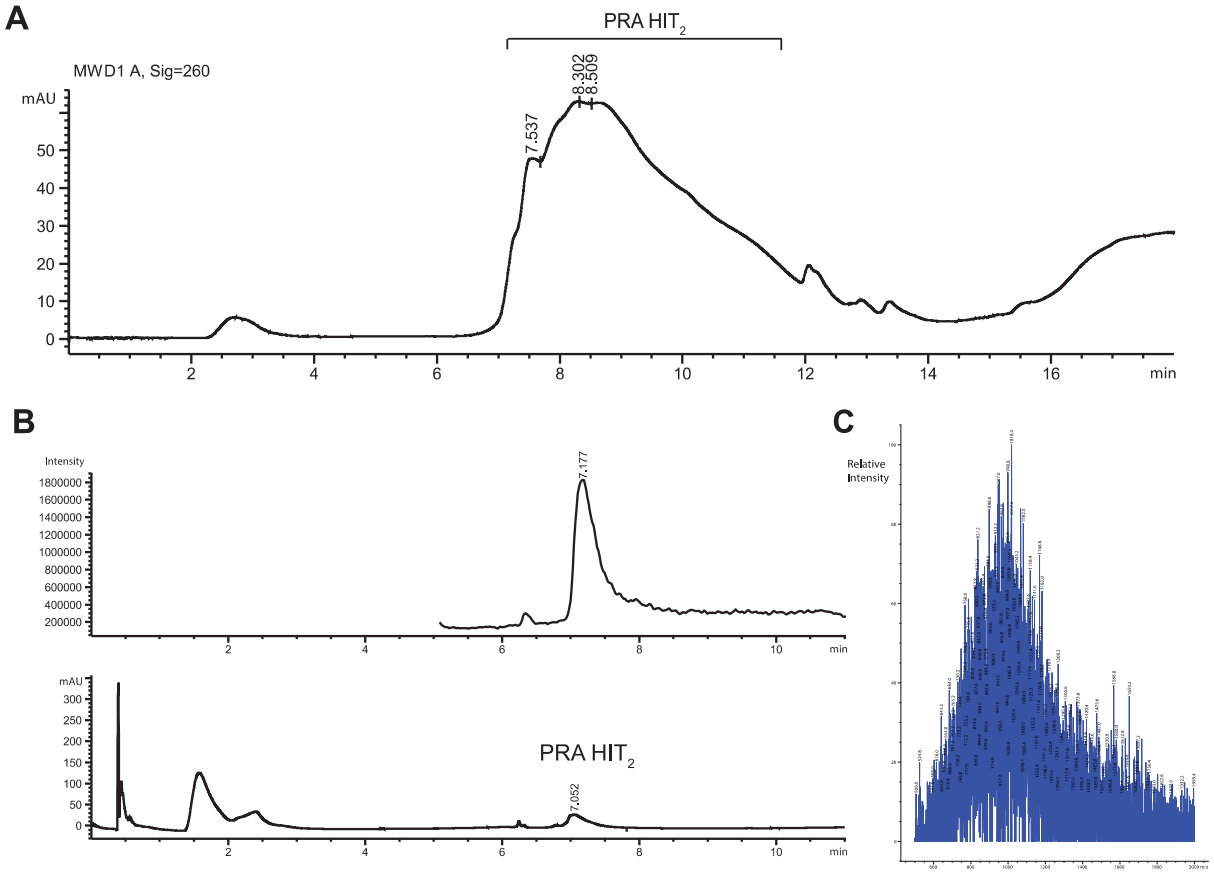


Figure S51. Analysis of **PRA DEL** after coupling with the **HIT$_2$**. **A.** RP-HPLC chromatogram of **PRA HIT$_2$** registered at 260 nm. (Due to the low amount of library available, the UV signal resulted relatively low). **B.** LC chromatogram of **PRA HIT$_2$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **PRA HIT$_2$**.

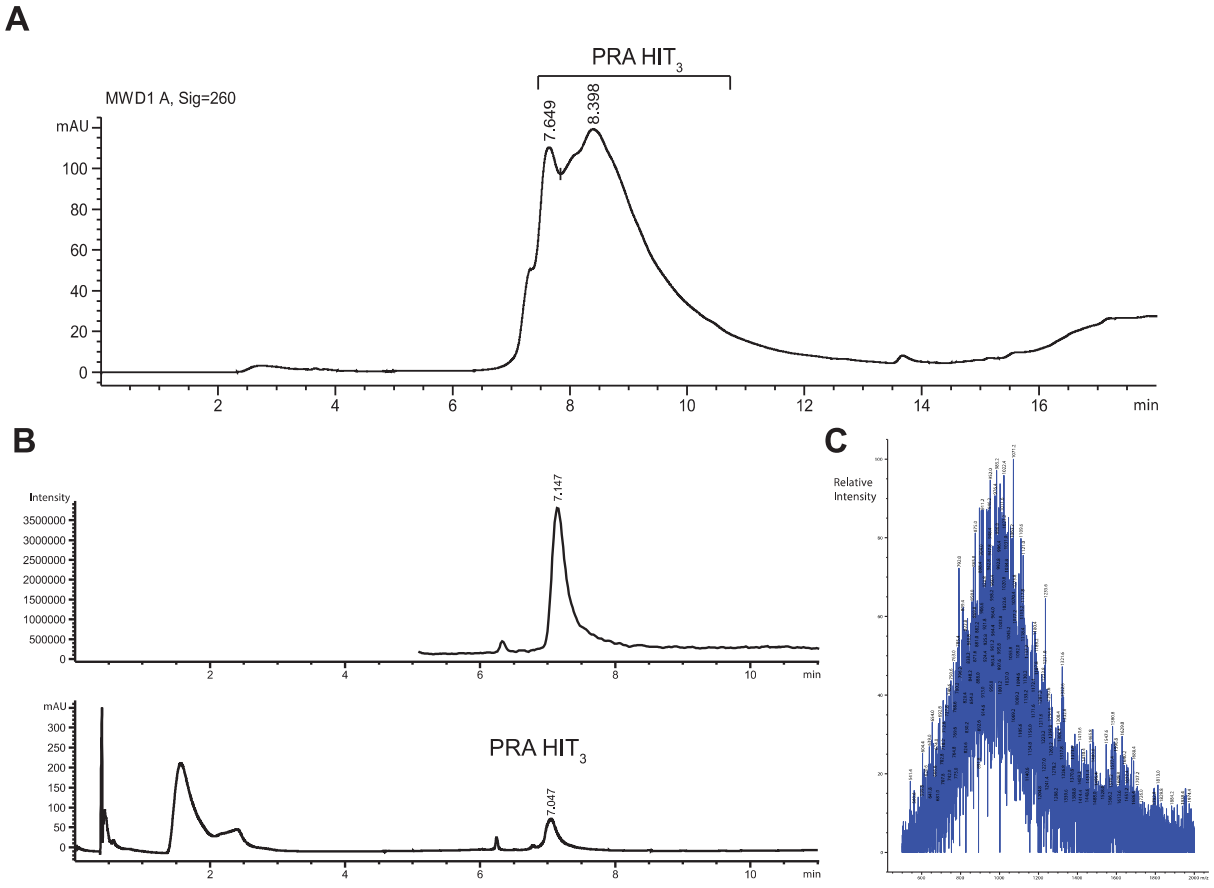


Figure S52. Analysis of **PRA DEL** after coupling with the **HIT$_3$** and methyl ester hydrolysis. **A.** RP-HPLC chromatogram of **PRA HIT$_3$** registered at 260 nm. (Due to the low amount of library available, the UV signal resulted relatively low). **B.** LC chromatogram of **PRA HIT$_3$** after purification recorded at 260 nm. **C**. Non-deconvoluted mass spectra of **PRA HIT$_3$**.

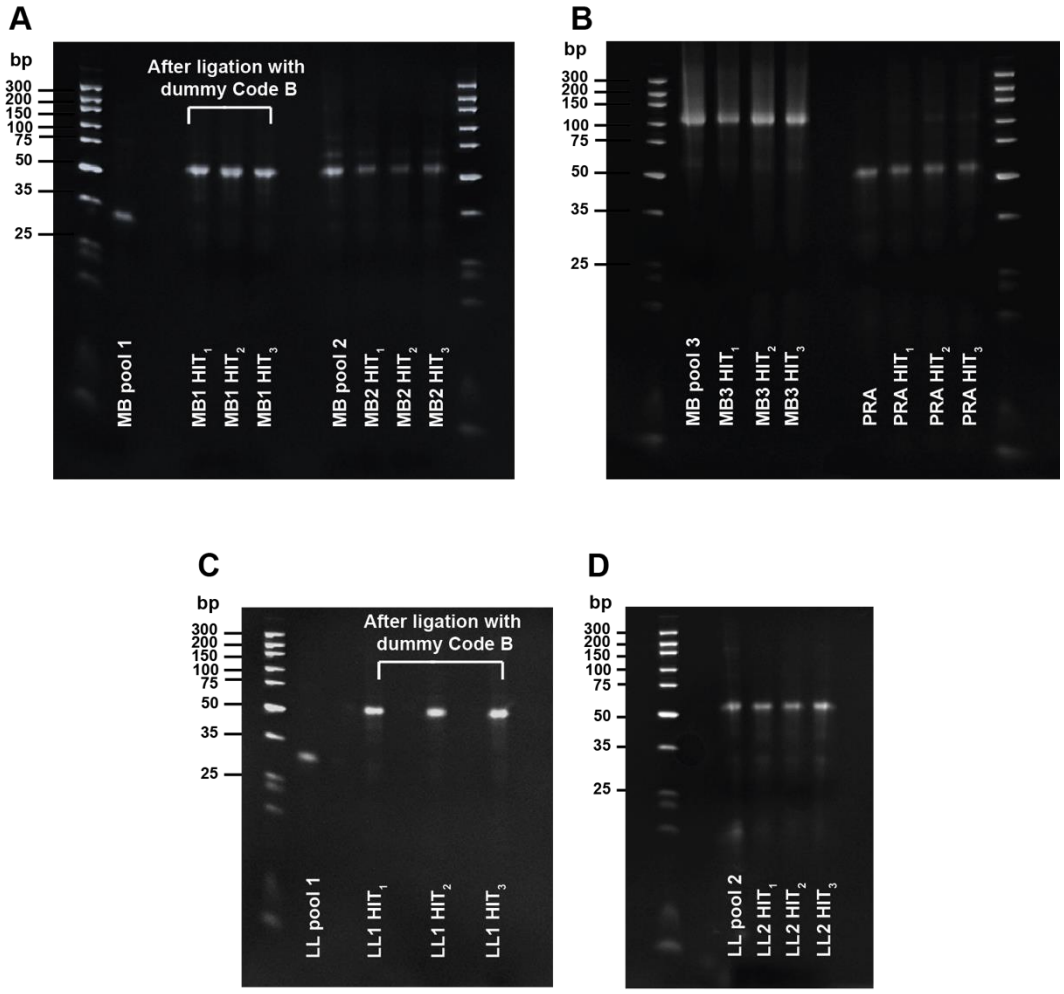


Figure S53. 15% TBE-UREA gel of final ASM DELs. **A. MB pool 1** starting material compared to **MB pool 1** derivatized with **HIT$_1$**, **HIT$_2$** and **HIT$_3$** encoded with dummy code B. **MB pool 2** starting material compared to **MB pool 2** derivatized with **HIT$_1$**, **HIT$_2$** and **HIT$_3$**. **B. MB pool 3** starting material compared to **MB pool 3** derivatized with **HIT$_1$**, **HIT$_2$** and **HIT$_3$**; **PRA DEL** starting material compared to **PRA DEL** derivatized with **HIT$_1$**, **HIT$_2$** and **HIT$_3$**. **C. LL pool 1** starting material compared to **LL pool 1** derivatized with **HIT$_1$**, **HIT$_2$** and **HIT$_3$** encoded with dummy code B. **D. LL pool 2** starting material compared to **LL pool 2** derivatized with **HIT$_1$**, **HIT$_2$** and **HIT$_3$**.

4 Protein Production, Biotinylation and Characterization

4.1 Protein Production and Biotinylation

hPSMA and hGCPIII were recombinantly produced from CHO-S cells and purified by Ni-NTA Agarose affinity chromatography following previously reported protocols [2].

The two enzymes bear an Avi-tag, which allowed enzymatic biotinylation using BirA. Protein characterization and analytical data are provided in **Figures S54, and S55**. Protein buffer was exchanged to BirA buffer (100 mM Tris pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$) using PD-10 columns. The protein (5 mg, in 6 mL BirA buffer, 0.9 mg/mL), was mixed with 350 µg MBP-BirA, 625 µL of 500 mM ATP in 1 M Tris HCl pH 9.5, 500 µL of 20 mM biotin in DMSO and half a tablet of protease inhibitor cocktail (cOmplete™ Protease Inhibitor Cocktail, Roche) previously dissolved in 750 µL of mQ water. The enzymatic reaction was gently shaken in a Hula Mixer overnight, in the dark at room temperature. The protein was then concentrated using Vivaspin® 20 centrifugal concentrators (30'000 Da molecular weight cutoff, Sartorius, #VS2012) and then purified by size-exclusion chromatography.

4.2 Protein Characterization

Size-Exclusion Chromatography (SEC)

Protein purifications were performed on an Äkta Pure FPLC system (GE Healthcare) that was equipped with a Superdex S200 10/300 column (GE Healthcare), using a flow rate of 0.75 mL/min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Precast SurePAGE Bis-Tris 4-12% gels (GenScript, #M00653) were used with MOPS running buffer following the supplier's procedure (180 V, 110 mA, 45 min-1 h). Proteins (5 µg) were mixed with Laemmli buffer (protein/buffer 5:1) and loaded into the gel. Precision Plus Protein™ All Blue Prestained Protein Standards (4 µL, Bio-Rad, #1610373) was used as size reference. Proteins were revealed by Coomassie staining (**Figure S54 B** and **S55 B**).

Band shift assay

Band shift assay using SDS-PAGE was performed to check biotinylation success. Parallelly, avidin (5 μg), biotinylated protein (5 μg) and a mixture of avidin with the respective biotinylated protein (5 μg + 5 μg) were loaded into the gel to visualize complex formation as band shift. (**Figure S54 C** and **S55 C**).

PSMA and GCPIII activity assay

PSMA was diluted in assay buffer (50 mM HEPES, 100 mM NaCl pH 7.4) to a concentration of 2 nM. A 10 mM stock of the substrate (Ac-Asp-Glu, Merck #A5930) in 40 mM NaOH was prepared and diluted in assay buffer to a concentration of 40 μM. Protein (12.5 μL, 2 nM in assay buffer) and substrate (12.5 μL, 40 μM in assay buffer) were mixed into a Greiner 384-well plate (PS, F-bottom, Black, non-binding, Merck, #781900). The plate was sealed, and the reaction was incubated for 1h at 37°C. The reaction was stopped by heating at 95°C for 5 min. Parallelly, OPA (ortho-phthaldialdehyde, Merck, #P1378) was dissolved in DMSO (0.37 M) and then diluted in 200 mM NaOH 0.1% β-mercaptoethanol to reach a concentration of 15 mM (OPA solution). 12.5 μL of OPA solution were added to each well, followed by incubation for 10 min at room temperature in the dark. Fluorescence signal was detected and quantified using Tecan Spark ($\lambda_{excitation}$ = 330 nm, $\lambda_{emission}$ = 450 nm). The same procedure was applied for GCPIII, but, supplementing the assay buffer with 1 mM $MnCl_2$ and performing substrate incubation overnight at room temperature. Heat inactivated PSMA (ΔPSMA) and GCPIII (ΔGCPIII) served as negative controls. Each experiment was performed in duplicates (**Figure S54 D and S55 D**).

Table S1. Protein parameters.

Protein	**MW (KDa)**	**Extinction coefficient (280 nm) $M^{-1}cm^{-1}$**	**Buffer**	**Biontinylation**

PSMA	82.5 (monomer)	118.735	HEPES 50 mM NaCl 100 mM 1 mM $CaCl_2$ 0.5 mM $MgCl_2$ pH 7.4	Avi-TAG
GCPIII	83 (monomer)	120.225	HEPES 50 mM NaCl 100 mM pH 7.4	Avi-TAG

Biotinylated and non biotinylated PSMA characterization

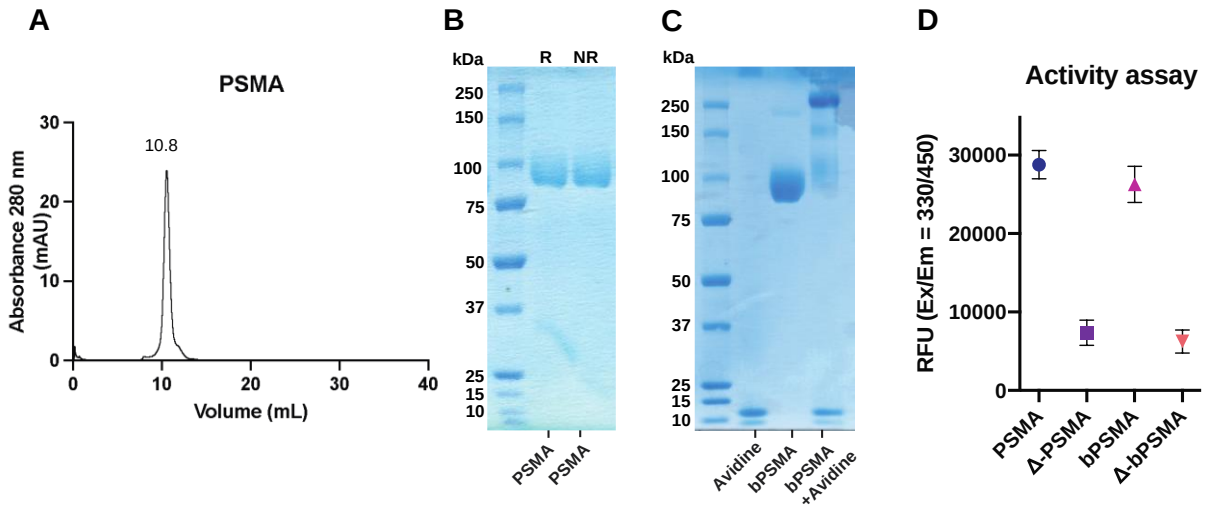


Figure S54. (**A**) SEC of PSMA in dimeric format. **B**) SDS-PAGE of PSMA using reducing and non-reducing buffer. **C**) Band shift assay of enzymatically biotinylated PSMA. **D**) Enzymatic activity assay of biotinylated (bPSMA), non-biotinylated PSMA, and heat-inactivated enzymes (Δb-/PSMA) as negative controls. Data are presented as mean values ± standard deviations.

Biotinylated and non biotinylated GCPIII characterization

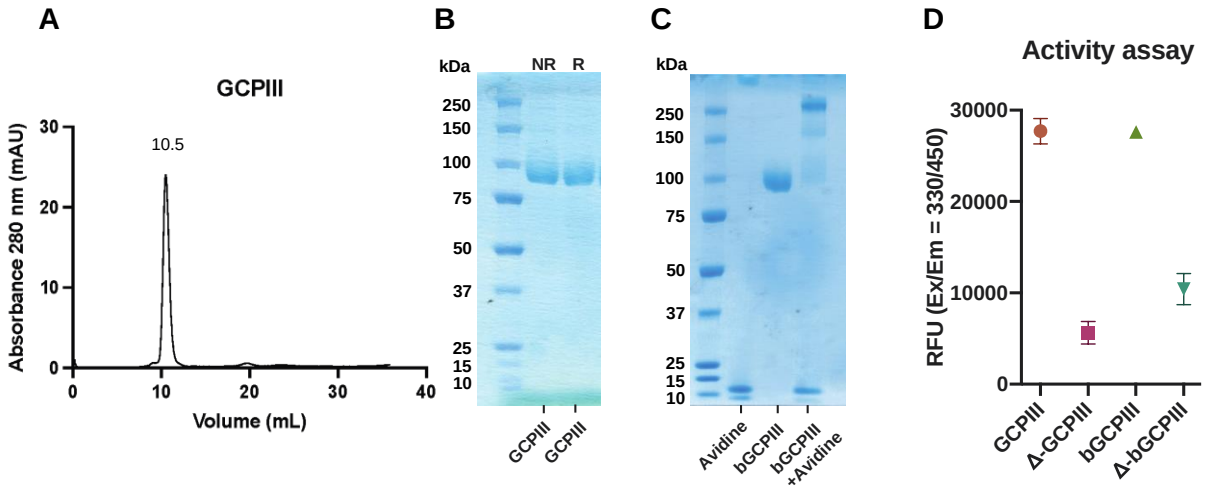


Figure S55. (**A**) SEC of GCPIII in dimeric format. **B**) SDS-PAGE of GCPIII using non-reducing and reducing buffer. **C**) Band shift assay of enzymatically biotinylated GCPIII. **D**) Enzymatic activity assay of biotinylated (bPSMA), non-biotinylated PSMA, and heat-inactivated enzymes (Δb-/PSMA) as negative controls. Data are presented as mean values ± standard deviations.

5 Solid-Phase Affinity Selections

Affinity selections were performed using 10^9 copies per library member per selection. Affinity selections were performed in parallel using Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific, #65001) with an automatic KingFisher (Thermo Fisher Scientific) magnetic particle processor following the methods reported in literature[3]. All the selections were performed in duplicate against biotinylated PSMA and biotinylated GCPIII to isolate enriched compounds selectively for PSMA (**Appendix 13.1**). Enrichment Factors (EFs) for a given library member (AyBzCwHITx combination) were calculated dividing the sequence counts (Counts) of that library member with the average count (AC) of the respective selection experiment (**Table S2**). Next-generation sequencing raw data were analyzed using a C++ program, and the results were evaluated using a dedicated software MATLAB R2023a (The MathWorks).

5.1 PCR amplification, sequencing and fingerprints

PCR amplification and sequencing

The selection eluates were amplified by two consecutive amplification steps (PCR1 and PCR2), as previously reported[3].

PCR primers

XXXXXX = variable region to encode the selection

YYYYYY = variable region to encode the selection

PCR1-a (forward):
5' TACACGACGCTCTTCCGATCT**XXXXXX**GGAGCTTCTGAATTCTGTGTG

PCR1-b (backward):
5' CAGACGTGTGCTCTTCCGATC**YYYYYY**GCTGCCTGACGC

For MB pools derivatized libraries the following PCR1 primers were used:

PCR1-a:
5'd TACACGACGCTCTTCCGATCT**XXXXXX**GGAGCTTCTGAATTCTGTGTG

PCR1-b:
5'd CAGACGTGTGCTCTTCCGATC**YYYYYY**GCTCTGCACGGTCGC

PCR2 is performed on each PCR1 product singularly in order to enable Illumina sequencing.

PCR2-a:
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

PCR2-b:
5'CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTC
CGATC

PCR2 pools were sequenced by Illumina high-throughput sequencing (MiSeq and NovaSeq) to process and analyze the data as reported[3]

Selection fingerprints

Selections were performed in duplicates. The selection fingerprints are reported in the **Appendix 13.1**

Table S2. Detailed analysis of selections. Average counts (AC) are calculated as total counts divided by library size.

Library	Library size (n of building blocks)	Target	Total counts	Average Counts	Figure Ref. Fingerprint
LL1 HIT1	464	näive	345075	743.7	**S128D**
		näive	385117	830.0	**S128D**
		PSMA	21770	46.9	**S128A**
		PSMA	2682	5.8	**S128A**
		GCPIII	3924	8.5	**S128B**
		GCPIII	2512	5.4	**S128B**
		no protein	2490	5.4	**S128C**
		no protein	2371	5.1	**S128C**
LL1 HIT2	464	näive	399937	861.9	**S129D**
		näive	334902	721.8	**S129D**
		PSMA	47058	101.4	**S129A**
		PSMA	41254	88.9	**S129A**
		GCPIII	13810	29.8	**S129B**
		GCPIII	4518	9.7	**S129B**
		no protein	296	0.6	**S129C**
		no protein	637	1.4	**S129C**
LL1 HIT3	464	näive	400681	863.5	**S130D**
		näive	434503	936.4	**S130D**
		PSMA	14427	31.1	**S130A**
		PSMA	6052	13.0	**S130A**
		GCPIII	6529	14.1	**S130B**
		GCPIII	5135	11.1	**S130B**
		no protein	475	1.0	**S130C**
		no protein	437	0.9	**S130C**
LL2 de novo	150336	PSMA	156866	1.0	**S131A**
		PSMA	149764	1.0	**S131A**
		GCPIII	81500	0.5	**S131B**
		GCPIII	115380	0.8	**S131B**
		no protein	26286	0.2	**S131C**
		no protein	20159	0.1	**S131C**

Library	Library size (n of building blocks)	Target	Total counts	Average Counts	Figure Ref. Fingerprint
LL2 HIT$_1$	150336	näive	995488	6.6	**S132D**
		PSMA	165368	1.1	**S132A**
		PSMA	153363	1.0	**S132A**
		GCPIII	52616	0.3	**S132B**
		GCPIII	54230	0.4	**S132B**
		no protein	22893	0.2	**S132C**
		no protein	11942	0.1	**S132C**
LL2 HIT$_2$	150336	näive	808587	5.4	**S133D**
		PSMA	150707	1.0	**S133A**
		PSMA	160442	1.1	**S133A**
		GCPIII	151062	1.0	**S133B**
		GCPIII	155041	1.0	**S133B**
		no protein	170048	1.1	**S133C**
		no protein	150615	1.0	**S133C**
LL2 HIT$_3$	150336	näive	856250	5.7	**S134D**
		PSMA	588839	3.9	**S134A**
		PSMA	701671	4.7	**S134A**
		GCPIII	598043	4.0	**S134B**
		GCPIII	660044	4.4	**S134B**
		no protein	68726	0.5	**S134C**
		no protein	65140	0.4	**S134C**
MB1 HIT$_1$	204	näive	167183	819.5	**S135D**
		näive	165598	811.8	**S135D**
		PSMA	16752	82.1	**S135A**
		PSMA	17252	84.6	**S135A**
		GCPIII	7075	34.7	**S135B**
		GCPIII	4715	23.1	**S135B**
		no protein	1646	8.1	**S135C**
		no protein	9483	46.5	**S135C**

Library	Library size (n of building blocks)	Target	Total counts	Average Counts	Figure Ref. Fingerprint
MB1 HIT$_2$	204	näive	128681	630.8	**S136D**
		näive	132689	650.4	**S136D**
		PSMA	44440	217.8	**S136A**
		PSMA	58491	286.7	**S136A**
		GCPIII	22409	109.8	**S136B**
		GCPIII	9197	45.1	**S136B**
		no protein	279	1.4	**S136C**
		no protein	684	3.4	**S136C**
MB1 HIT$_3$	204	näive	144178	706.8	**S137D**
		näive	203770	998.9	**S137D**
		PSMA	3863	18.9	**S137A**
		PSMA	3649	17.9	**S137A**
		GCPIII	2814	13.8	**S137B**
		GCPIII	2695	13.2	**S137B**
		no protein	141	0.7	**S137C**
		no protein	139	0.7	**S137C**
MB2 de novo	32028	PSMA	162180	5.1	**S138A**
		PSMA	232494	7.3	**S138A**
		GCPIII	37294	1.2	**S138B**
		GCPIII	41509	1.3	**S138B**
		no protein	11434	0.4	**S138C**
		no protein	18952	0.6	**S138C**
MB2 HIT$_1$	32028	näive	422788	13.2	**S139D**
		näive	402528	12.6	**S139D**
		PSMA	35972	1.1	**S139A**
		PSMA	27706	0.9	**S139A**
		GCPIII	6843	0.2	**S139B**
		GCPIII	10372	0.3	**S139B**
		no protein	2266	0.1	**S139C**
		no protein	5455	0.2	**S139C**
MB2 HIT$_2$	32028	näive	508464	15.9	**S140D**
		näive	128070	4.0	**S140D**
		PSMA	191049	6.0	**S140A**
		PSMA	189342	5.9	**S140A**
		GCPIII	94061	2.9	**S140B**
		GCPIII	96115	3.0	**S140B**
		no protein	5421	0.2	**S140C**
		no protein	7922	0.2	**S140C**

Library	Library size (n. of building blocks)	Target	Total counts	Average Counts (AC)	Figure Ref. Fingerprint
MB2 HIT$_3$	32028	näive	609204	19.0	**S141D**
		näive	486108	15.2	**S141D**
		PSMA	129570	4.0	**S141A**
		PSMA	130897	4.1	**S141A**
		GCPIII	116305	3.6	**S141B**
		GCPIII	122677	3.8	**S141B**
		no protein	12028	0.4	**S141C**
		no protein	9719	0.3	**S141C**
MB3 de novo	5861124	PSMA	1668148	0.3	**S142A**
		PSMA	1950583	0.3	**S142A**
		GCPIII	1822597	0.3	**S142B**
		GCPIII	1756397	0.3	**S142B**
		no protein	1440768	0.2	**S142C**
		no protein	1248734	0.2	**S142C**
MB3 HIT$_1$	5861124	näive	3078286	0.5	**S143D**
		näive	2633135	0.4	**S143D**
		PSMA	2515611	0.4	**S143A**
		PSMA	2382743	0.4	**S143A**
		GCPIII	1700176	0.3	**S143B**
		GCPIII	1731274	0.3	**S143B**
		no protein	2798104	0.5	**S143C**
		no protein	2250884	0.4	**S143C**
MB3 HIT$_2$	5861124	näive	1039151	0.2	**S144D**
		näive	2928753	0.5	**S144D**
		PSMA	1952538	0.3	**S144A**
		PSMA	1333370	0.2	**S144A**
		GCPIII	1288592	0.2	**S144B**
		GCPIII	1478304	0.3	**S144B**
		no protein	1193615	0.2	**S144C**
		no protein	1046844	0.2	**S144C**
MB3 HIT$_3$	5861124	näive	30849907	5.3	**S145D**
		näive	2642992	0.5	**S145D**
		PSMA	5715120	1.0	**S145A**
		PSMA	6453068	1.1	**S145A**
		GCPIII	6115868	1.0	**S145B**
		GCPIII	5174715	0.9	**S145B**
		no protein	3657577	0.6	**S145C**
		no protein	5133182	0.9	**S145C**
PRA de novo	50730	PSMA	429259	8.5	**S146A**
		PSMA	505437	10.0	**S146A**
		GCPIII	168250	3.3	**S146B**
		GCPIII	89760	1.8	**S146B**
		no protein	104092	2.1	**S146C**
		no protein	142738	2.8	**S146C**

Library	Library size (n of building blocks)	Target	Total counts	Average Counts	Figure Ref. Fingerprint
PRA HIT$_1$	50730	näive	756920	14.9	**S147D**
		näive	702819	13.9	**S147D**
		PSMA	18250	0.4	**S147A**
		PSMA	59621	1.2	**S147A**
		GCPIII	22474	0.4	**S147B**
		GCPIII	22831	0.5	**S147B**
		no protein	1742	0.0	**S147C**
		no protein	1166	0.0	**S147C**
PRA HIT$_2$	50730	näive	389826	7.7	**S148D**
		näive	392307	7.7	**S148D**
		PSMA	11295	0.2	**S148A**
		PSMA	32082	0.6	**S148A**
		GCPIII	16013	0.3	**S148B**
		GCPIII	15844	0.3	**S148B**
		no protein	27274	0.5	**S148C**
		no protein	3039	0.1	**S148C**
PRA HIT$_3$	50730	näive	561471	11.1	**S149D**
		näive	658245	13.0	**S149D**
		PSMA	110413	2.2	**S149A**
		PSMA	266299	5.2	**S149A**
		GCPIII	245291	4.8	**S149B**
		GCPIII	259858	5.1	**S149B**
		no protein	5690	0.1	**S149C**
		no protein	2157	0.0	**S149C**

The enrichment factors (EF) for each building block combination were calculated using the reported general equations specified for a combination of 2 building blocks (BB_A and BB_B). AC is the average number of counts and TC is the total number of counts for a specific selection.

$$AC(sel) = \frac{TC\ (sel)}{library\ size}$$

$$EF\ (BB_A; BB_B) = \frac{counts\ (BB_A; BB_B)}{AC(sel)}$$

6 Compound Resynthesis

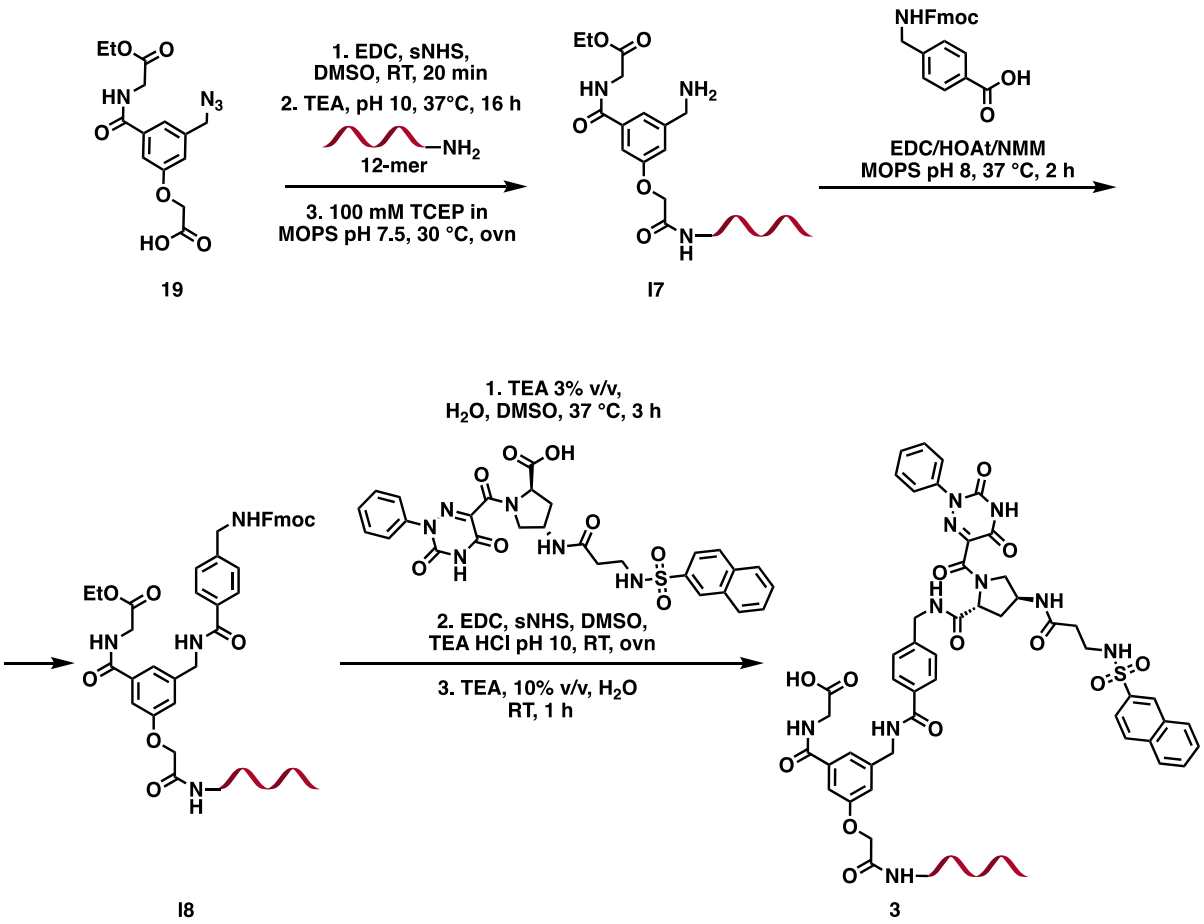
Synthesis off-DNA of PSMA-specific compounds isolated by ASM-DEL selections

Compounds **1-22** were synthesized either by solid phase synthesis or in solution.

6.1 On-DNA compound resynthesis

6.1.1 Synthesis scheme for compound 3

On-DNA hit resynthesis was performed with a short 5'-amino modified 12-mer oligonucleotide (5' C_6-amino-TAGTAGCCATCC). Synthesis of compound **3** was performed following the conditions described for MB library construction (**Supplementary Information 2.2**) and coupling of HIT_2 for ASM-DEL construction (see Manuscript, **Material and Methods**).



Scheme S9. Schematic illustration of on-DNA synthesis of compound **3** using 12-mer oligonucleotide. LCMS data of Intermediates are reported in **Appendix 15.2**. 12-mer = 3784 Da

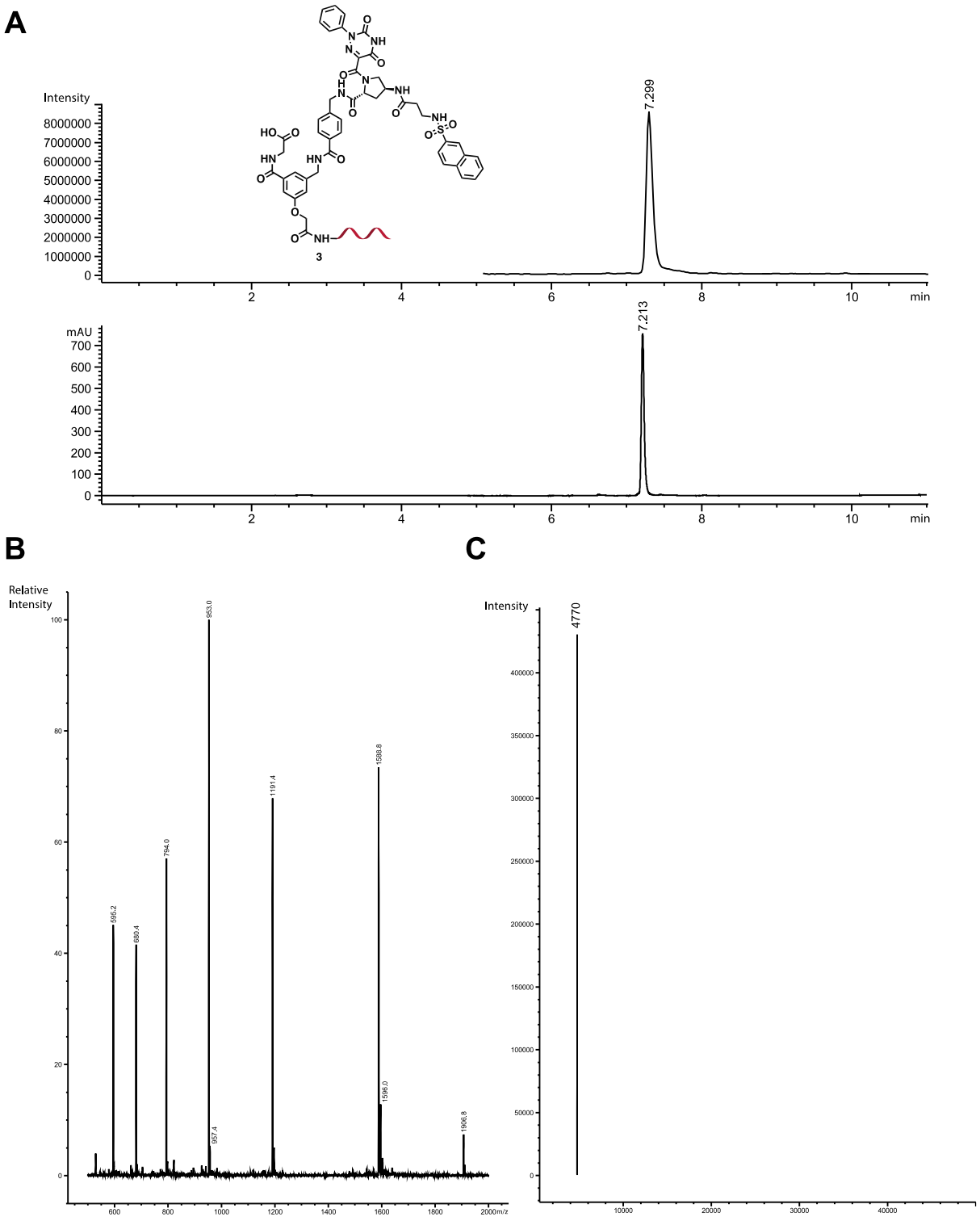
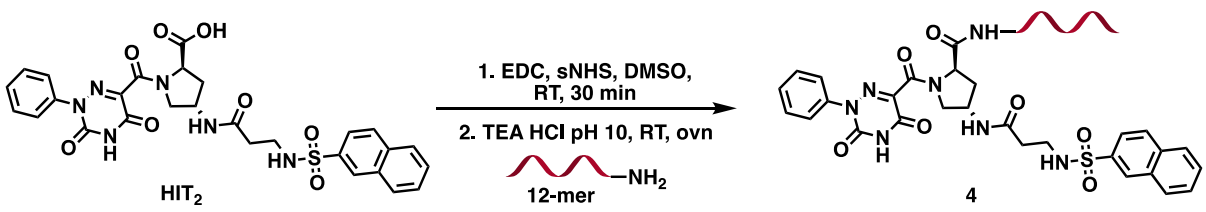


Figure S56. (**A**) MS trace and LC chromatogram of compound **3**. (**B**) Raw mass spectrum of compound **3** and its deconvolution (**C**) resulted in an observed mass of 4770 Da (calculated mass: 4769.3 Da).

6.1.2 Synthesis scheme for compound 4

Synthesis of compound **4** was performed following the conditions described for coupling of HIT_2 for ASM-DEL construction and using 12-mer oligonucleotide (see Manuscript, **Material and Methods**)



Scheme S10. Schematic illustration of on-DNA synthesis of compound **4** using 12-mer oligonucleotide. 12-mer = 3784 Da

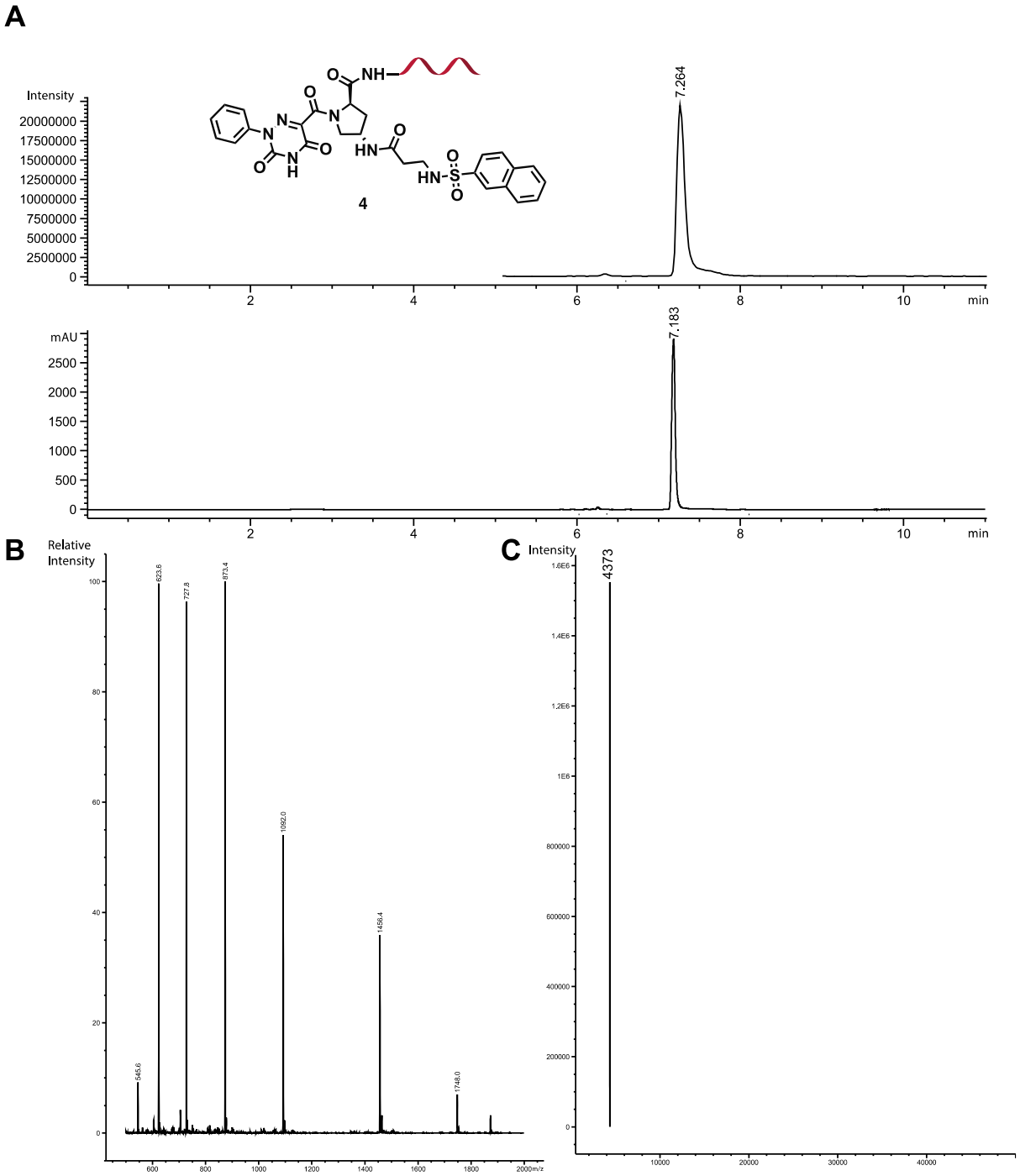


Figure S57. (**A**) MS trace and LC chromatogram of compound **4**. (**B**) Raw mass spectrum of compound **4** and its deconvolution (**C**) resulted in an observed mass of 4373 Da (calculated mass: 4372.2 Da).

6.2 Off-DNA compound resynthesis

6.2.1 General solid-phase synthesis procedures

Solid-phase synthesis was performed using Fmoc-Lys(Boc)-Wang resin (220-400 mesh, 0.5mmol/g; Novabiochem, #8560130001) and Wang resin (100-200 mesh, 1.1 mmol/g; Merck, #13609,)

Before every reaction, resin was swollen for 30 min in DMF. Reactions were performed in reaction columns (Carl Roth) and shaked on a rotator mixer (Reax 2, Heidolph Instruments GmbH & Co. KG) at rt.

Fmoc deprotection

Resin was incubated with 20% piperidine in DMF for (1 x 30 min and 2 x 10 min). After Fmoc deprotection the resin was washed 5-10 times with DMF to remove the residual piperidine. The deprotection efficiency was confirmed by TNMB test.

Amide coupling

A solution of the acid (2 eq.), HATU (1.9 eq.), HOAt (0.5 eq.) and DIPEA (4 eq.) in DMF was added to the peptide resin bearing a free amino group. The reaction was incubated for 4 h and then quenched by washing the resin 7 times with DMF.

Mini Cleavage for LC-MS monitoring analysis

A little amount of resin was picked up and transferred to an Eppendorf tube. 20 µL of TFA were added to the beads and after incubation for 10 min at rt, the reaction was quenched with 100 µL of ACN. The suspension is filtered and LC-MS analysis were performed.

Azide reduction

A solution of trimethylposphine (4 eq) in THF:H_2O (9:1) is added to the resin. The reaction was incubated for 2h. Afterwards the resin was washed 3 times with a solution of THF:water (9:1) and 10 times with DMF.

TNBS Test

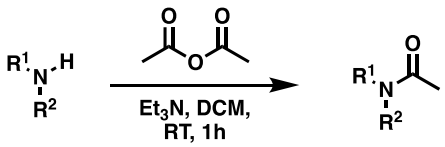
The TNBS (2,4,6-trinitrobenzenesulfonic acid) is used for the detection of primary amino groups. A small amount of beads is taken and poured into a solution of 1M aqueous TNBS (2 µL) in DMF:DIPEA (9:1, 20 µL) and incubated for 5 minutes. In presence of a free primary amino group, the beads turn orange/red.

Resin cleavage and purification

Before performing cleavage of the resin, the beads were always washed 5 times with DCM. Cleavage solution was prepared with 95% TFA, 2.5% water, 2.5% TIPS and was added to the resin. The cleavage step was performed three times (1 h each at rt). Cleavage fractions were collected and dried under reduced pressure, dissolved in a solution of ACN:water (1:1) and purified by reversed-phase HPLC.

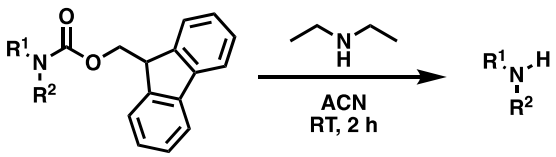
6.2.2 General solution-phase synthesis procedures

Acetylation



The amine was dissolved in DCM and acetic anhydride (1 eq.) and DMAP (2 eq.) were added. The reaction was stirred generally for 1 h at rt to afford the acetylated amine with full conversion.

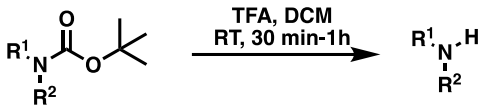
Fmoc deprotection



The Fluorenylmethyloxycarbonyl group was removed by adding diethylamine (5 eq.) to a solution of the protected amine in ACN. Typically, the reaction was stirred for 2 h at rt and

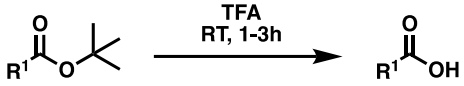
then dried under reduced pressure. Afterwards, a solution of ACN:water (9:1) was added to the crude. The mixture was washed 3 times with pentane to remove the fluorenyl group produced by deprotection.

Boc deprotection



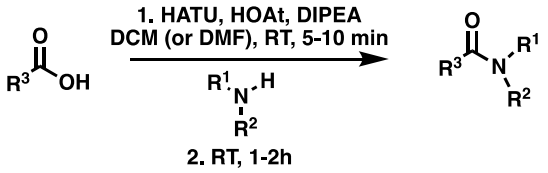
The Boc protected amine was dissolved in DCM and TFA (or directly neat TFA) was added to the solution. The reaction was stirred for 30 min up to 1 h at rt. TFA was removed under reduced pressure. Subsequently, the product was either dissolved in ACN:water (1: 1) and purified by RP-HPLC or further dried by lyophilization and used as crude for the next step reaction.

Tert-Butyl deprotection



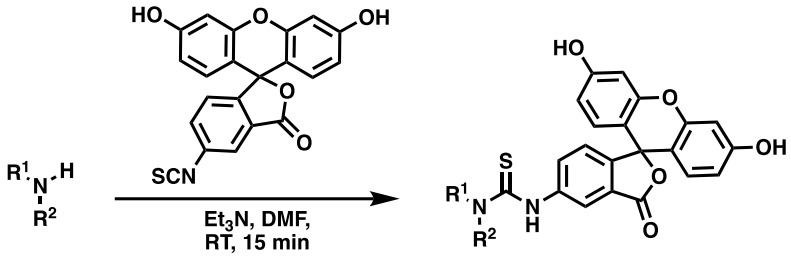
The Tert-butyl group was removed directly by adding TFA to the dried protected acid. The deprotection was stirred for 1 h up to 3 h at rt. After removal of TFA under reduced pressure, the products were either dissolved in ACN:water (1: 1) and purified by RP-HPLC or further dried by lyophilization and used as crude for the next step reaction.

Amide coupling



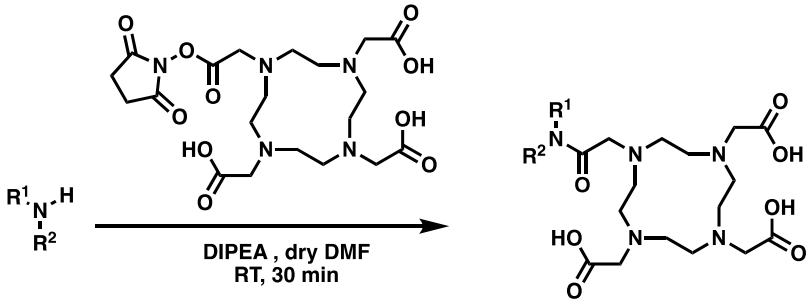
The acid (1.1 eq.) was pre-activated with HATU (1 eq.), HOAt (0.5 eq.) and DIPEA (4 eq.) in DMF for 5 min at rt. Afterwards, the amine (1 eq.) was added and the reaction mixture was stirred for 1 h up to 2 h at rt.

Fluoresceination with 5-FITC



To a solution of the amine (1 eq) in DMF, fluorescein-5-isothiocyanate (5-FITC, 1 eq.) and triethylamine (4 eq.) were added and the reaction was stirred for 15 min at rt in the dark. The crude products were directly purified by RP-HPLC.

DOTA conjugation

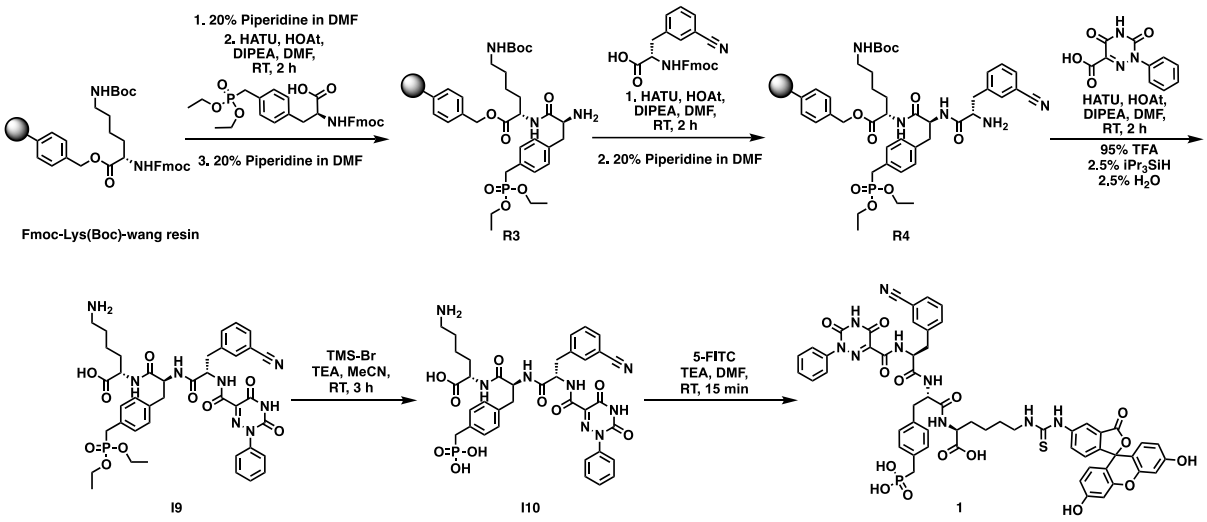


To a solution of the amine (1 eq) in dry DMF, DOTA-NHS ester (1.5 eq.) and DIPEA (4 eq.) were added and the reaction was stirred for 30 min at rt. The crude products were then purified by RP-HPLC.

The compounds isolated from the target selections and screening were re-synthesized off-DNA using a Lys or PEG_2 linker to allow the labelling with a fluorophore (5-fluorescence isothiocyanate, FITC) or a chelating cage for radioisotopes (DOTA). All compounds were constructed following standard solid-phase peptide synthetic procedures or solution phase protocols. Intermediates on resin are reported as **Rn** (n = 1, 2, …) and have not been isolated. In solution-synthesis intermediates are named **In** (n = 1, 2, …).

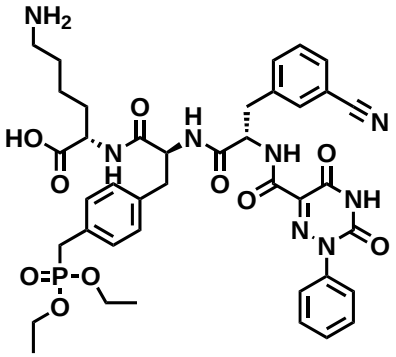
6.2.3 Synthesis scheme for compound 1

Synthesis of **1** was performed starting from Fmoc-Lys (Boc)-Wang Resin and proceeded in solution for the last three steps.



Scheme S11. Schematic illustration of the synthesis of compound **1**.

Synthesis of ((S)-2-((S)-3-(3-cyanophenyl)-2-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamido)propanamido)-3-(4-((diethoxyphosphoryl)methyl)phenyl)propanoyl)-L-lysine (I9)



Fmoc-Lys (Boc)-Wang Resin (80 mg, 0.050 mmol, 1 eq.) was Fmoc deprotected by adding 20% piperidine in DMF (1 x 30 min and 2 x 10 min) and washed 10 times with DMF to remove residual piperidine. Afterwards, commercially available (*S*)-2-((((9*H*-fluoren-9-

yl)methoxy)carbonyl)amino)-3-(4-((diethoxyphosphoryl)methyl)phenyl)propanoic acid (54 mg , 0.100 mmol, 2 eq., CAS 160253-13-4), HATU (36 mg, 0.095 mmol, 1.9 eq.), HOAt (3 mg, 0.025 mmol, 0.5 eq.) were dissolved in DMF (1 mL) and DIPEA (35 µL, 0.200 mmol, 4 eq.) was added. After 5 min of acid activation, the solution was added to the resin (80 mg, 0.05 mmol, 1 eq.) and the reaction was incubated for 2 h at rt. The resin was first washed 5 times with DMF followed by incubation with 20% piperidine in DMF (1 x 30 min and 2 x 10 min) and then washed again 10 times with DMF to get the Fmoc deprotected compound **R3**. To the resin **R3,** a solution of S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(3-cyanophenyl)propanoic acid (41 mg , 0.100 mmol, 2 eq.), pre-activated for 5 min with HATU (36 mg, 0.095 mmol, 1.9 eq.), HOAt (3 mg, 0.025 mmol, 0.5 eq.) and DIPEA (35 µL, 0.200 mmol, 4 eq.) in DMF (1 mL) was added. After 2 h of incubation at rt, the resin was washed 5 times with DMF, and 20% piperidine in DMF was added (1 x 30 min and 2 x 10 min). Subsequently the beads were washed 10 times and compound **R4** was obtained. Commercially available **HIT$_1$** (23 mg, 0.100 mmol, 2 eq.), HATU (36 mg, 0.095 mmol, 1.9 eq.), HOAt (3 mg, 0.025 mmol, 0.5 eq.) were dissolved in DMF (1 mL) and DIPEA (35 µL, 0.200 mmol, 4 eq.) was added. The acid was activated for 5 min and then the solution was added to the resin **R4**. The coupling proceeded for 2 h at room temperature and the reaction was stopped by washing with DMF (x 5). A cleavage solution of 95% TFA, 2.5% water, 2.5% iPr_3SiH was freshly prepared and added to **R4**. The resin was incubated for 1 h at rt and the cleavage step was repeated two further times (2 x 10 min), the cleaved fractions were collected, dried under reduced pressure to remove TFA and the crude was the purified by RP-HPLC to obtain the **I9** as white solid (15 mg, yield = 36%). HR-MS (ESI+) *m/z* calculated $C_{40}H_{47}N_8O_{10}P$ $[M+H]^+$ 831.32255; detected: 831.31934; accuracy = 3.86 ppm. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 12.69 (s, 1H), 8.89 (d, *J*=8.1 Hz, 1H), 8.44 (dd, *J* = 22.4, 8.0 Hz, 2H), 7.66 – 7.73 (m, 3H), 7.63-7.65 (m, 2H), 7.48 – 7.54 (m, 5H), 7.38-7.47 (m, 2H), 7.23 (d, *J* = 8.0, 2H), 7.16 (dd,

J = 8.3, 2.4 Hz, 2H), 4.79 (td, *J* = 7.7, 4.5 Hz, 1H), 4.57 – 4.62 (m, 1H), 4.26 (td, *J* = 8.3, 5.2 Hz, 1H), 3.84 – 3.93 (m, 4H), 3.04 – 3.16 (m, 4H), 2.94 – 2.99 (m, 1H), 2.75 – 2.81 (m, 3H), 1.73 – 1.82 (m, 1H), 1.61 – 1.69 (m, 1H), 1.52 – 1.59 (m, 2H), 1.35 – 1.43 (m, 2H), 1.12 (td, *J* = 7.1, 1.4 Hz, 6H).

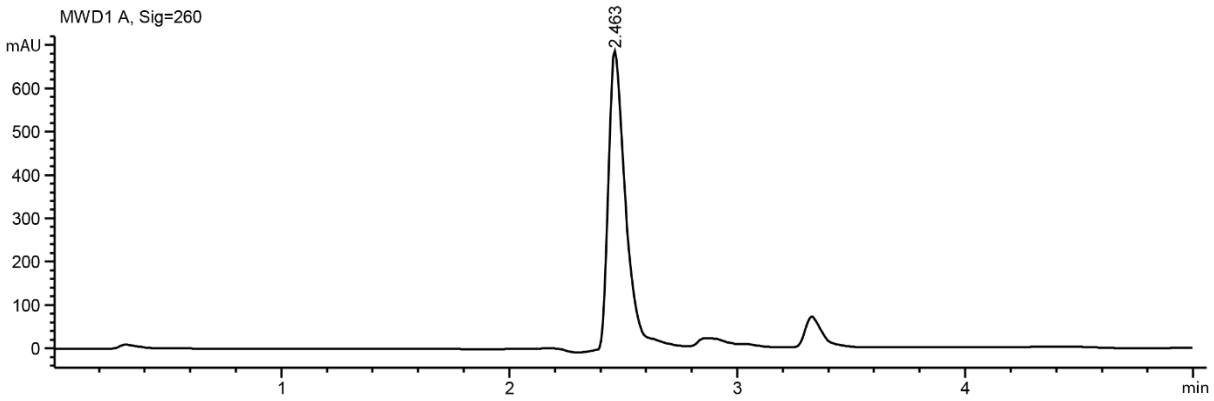


Figure S58. HPLC chromatogram of intermediate **I9** recorded at λ = 260 nm.

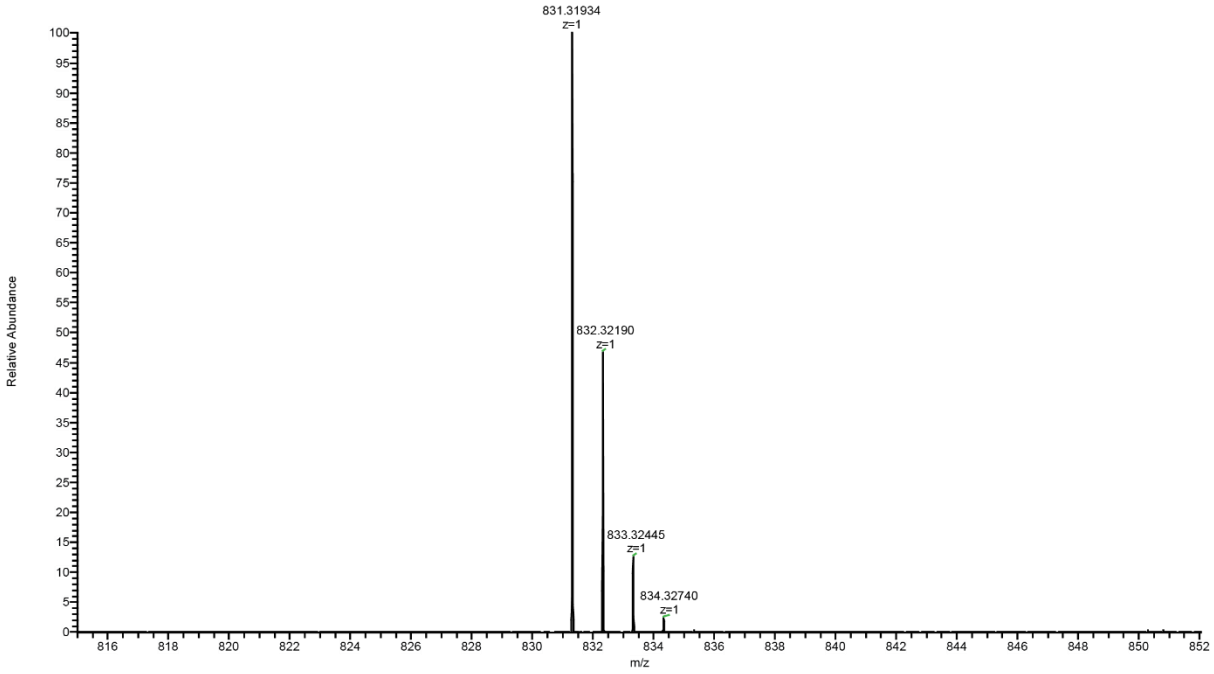
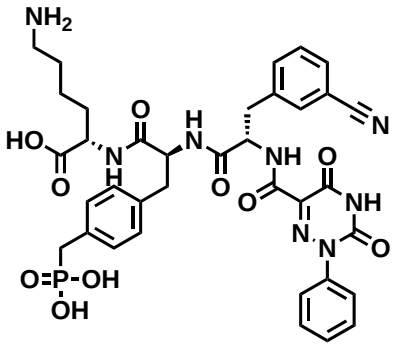


Figure S59. HR-MS of intermediate **I9**.

Synthesis of ((S)-2-((S)-3-(3-cyanophenyl)-2-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamido)propanamido)-3-(4-(phosphonomethyl)phenyl)propanoyl)-L-lysine (I10)



Under inert atmosphere, **I9** (15 mg, 0.018 mmol, 1 eq.) and TEA dry (25 µL, 0.180 mmol, 10 eq.) were dissolved in anhydrous ACN (1 mL). Bromotrimethylsilane (TMSBr, 48 µL 0.360 mmol 20 eq.) was added and the reaction was stirred for 3 h at room temperature. The reaction was quenched with a mixture of water:MeCN 1:1, the solvents were evaporated and the crude was purified by RP-HPLC affording compound **I10** as white solid (1 mg, yield = 6%). HR-MS (ESI+) *m/z* calculated for $C_{36}H_{39}N_8O_{10}P$ $[M+H]^+$ 775.25995; detected: 775.25786; accuracy = 2.70 ppm.

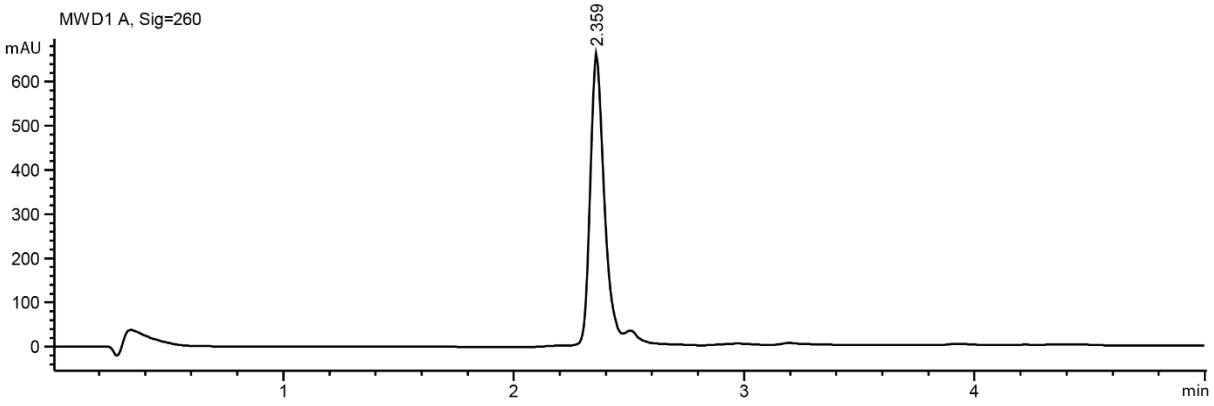


Figure S60. HPLC chromatogram of intermediate **I10** recorded at λ = 260 nm

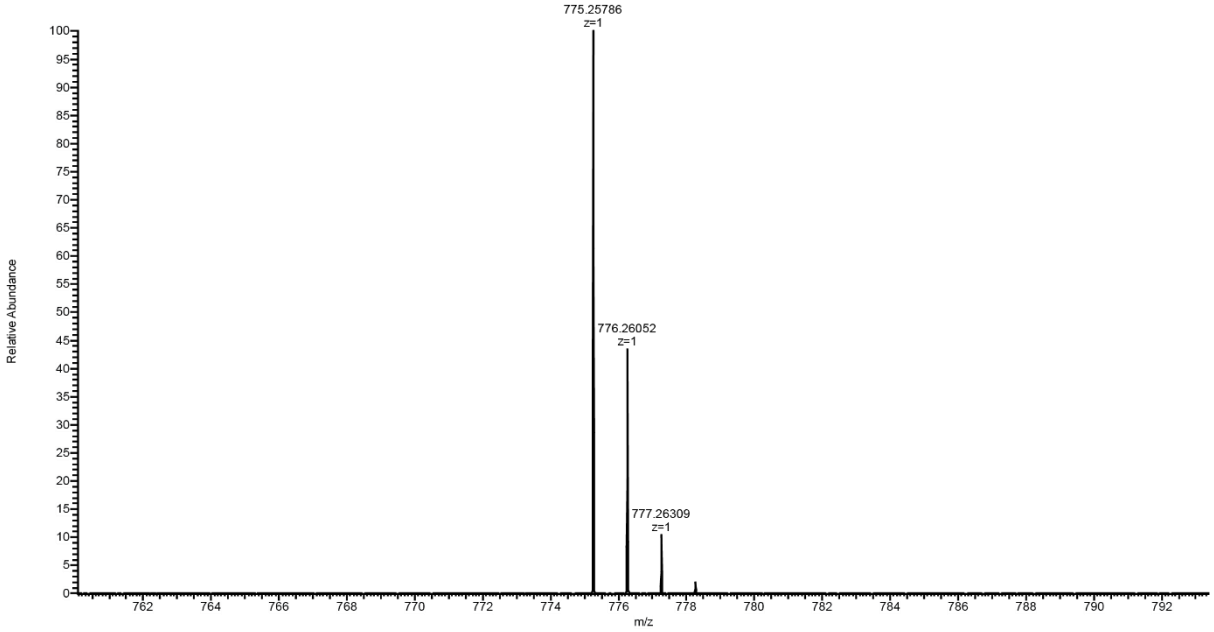
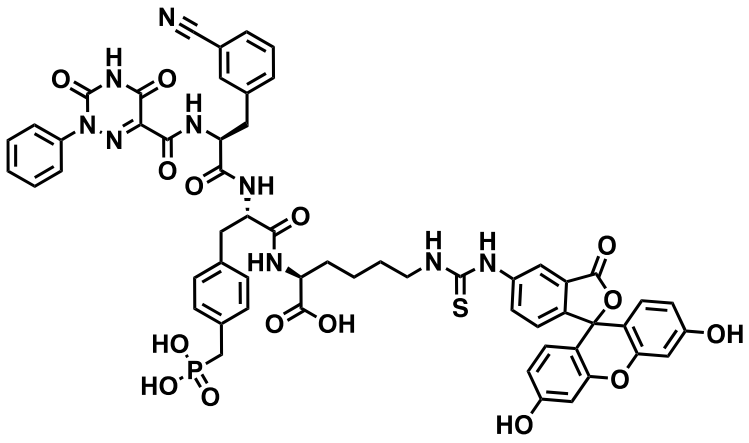


Figure S61. HR-MS of intermediate **I10**.

Synthesis of N^2-((S)-2-((S)-3-(3-cyanophenyl)-2-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamido)propanamido)-3-(4-(phosphonomethyl)phenyl)propanoyl)-N6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-L-lysine (1)



In a 1.5 mL Eppendorf Tube, **I10** (1 mg, 1.3 µmol, 1 eq.) was dissolved in DMF (30 µL), and fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 6.5 µL, 1.3 µmol, 1 eq.) and TEA (1 M in DMF, 5.2 µL, 5.2 µmol, 4 eq.) were added. The reaction was stirred for 15 min at room temperature in the dark and the product formation was monitored by LC-MS. The crude was

diluted with a solution MeCN:H_2O 1:1 and purified by RP-HPLC to obtain compound **1** as orange solid (0.8 mg, yield = 68%). HR-MS (ESI+) *m/z* calculated for $C_{57}H_{50}N_9O_{15}PS$ $[M+H]^+$ 1164.29575; detected: 1164.29725; accuracy = 1.29 ppm

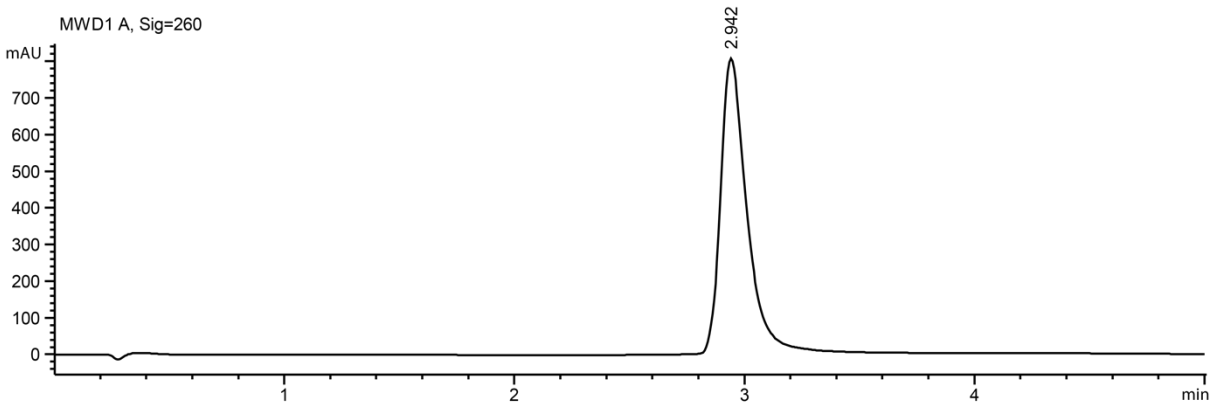


Figure S62. HPLC chromatogram of compound **1** recorded at λ = 260 nm

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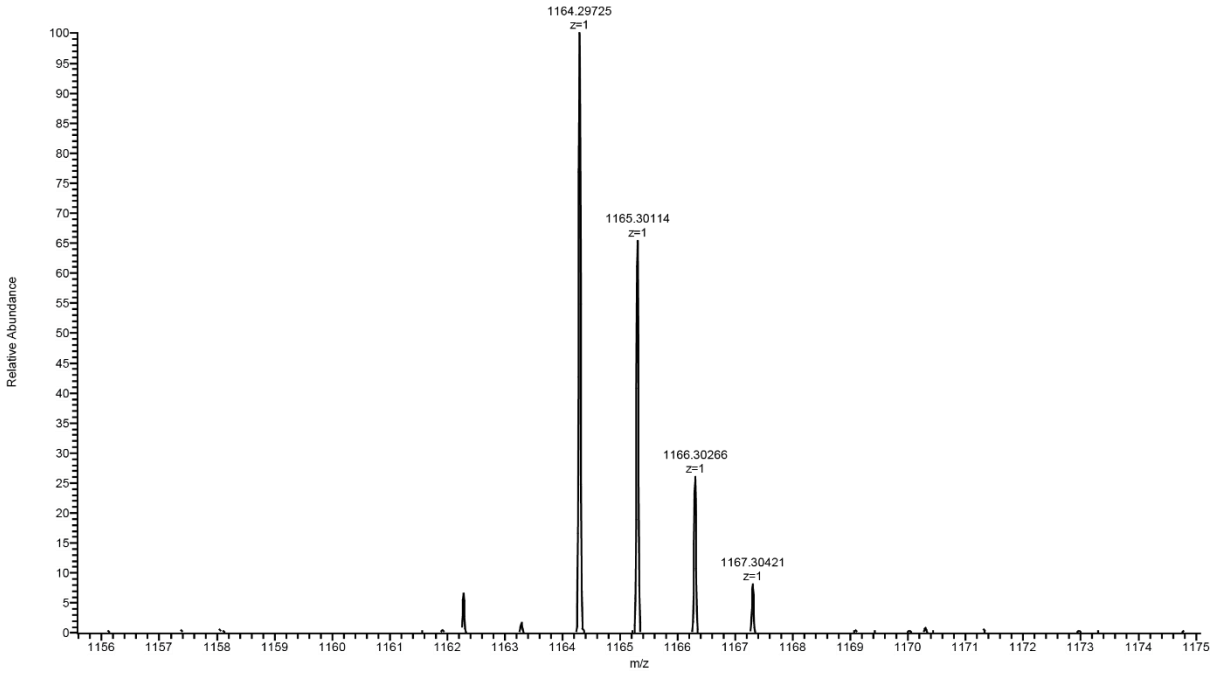
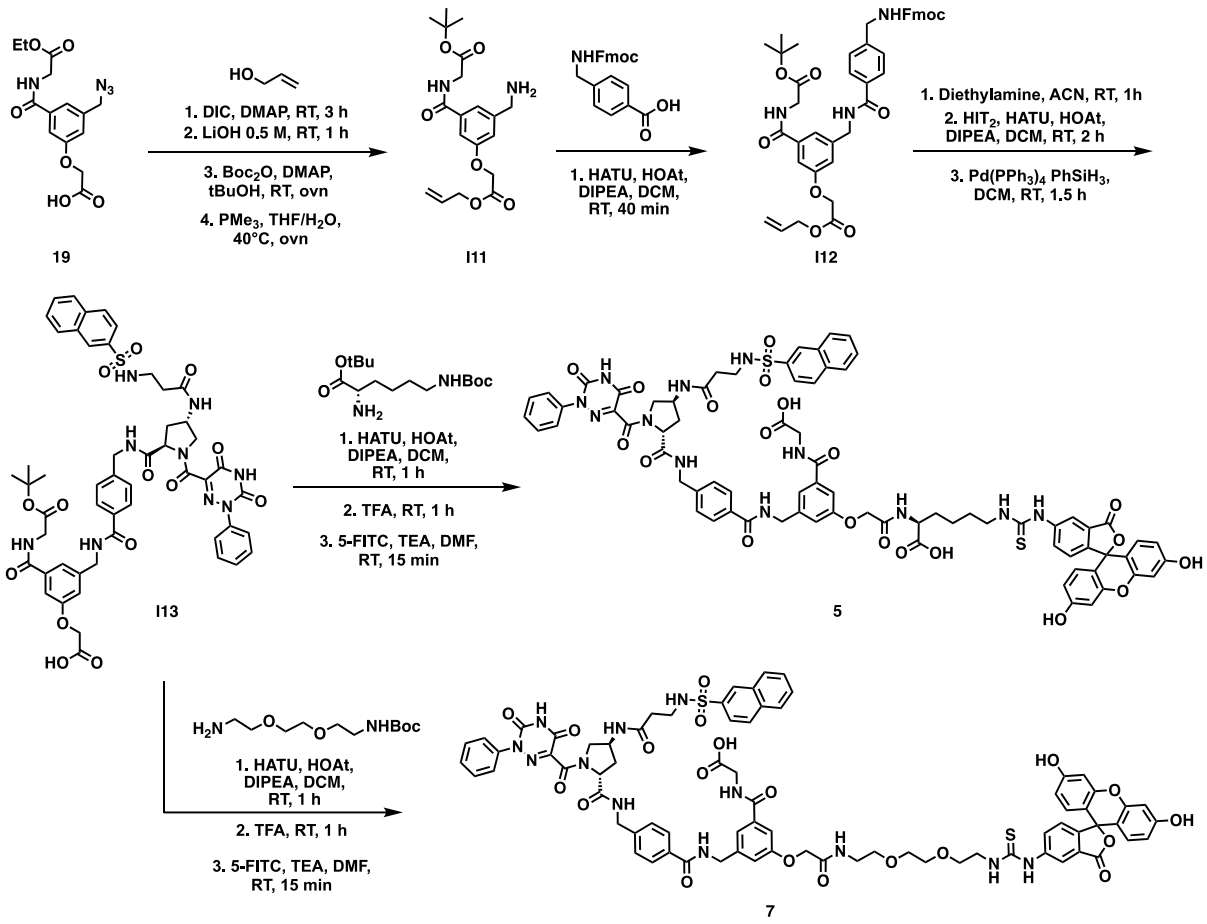


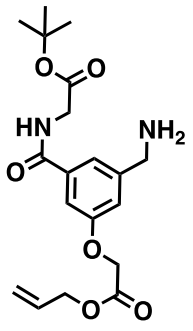
Figure S63. HR-MS of compound **1**.

6.2.4 Synthesis scheme for compounds 5 and 7.



Scheme S12. Schematic illustration of off-DNA synthesis of compounds **5** and **7**.

Synthesis of allyl 2-(3-(aminomethyl)-5-((2-(tert-butoxy)-2-oxoethyl)carbamoyl)phenoxy)acetate (I11)



To a 0°C cooled solution of compound **19** (200 mg, 0.60 mmol, 1 eq.) in DCM (5 mL), DIC (186 µL, 1.20 mmol, 2 eq.), DMAP (14 µL, 0.06 mmol, 0.1 eq.) and HOBt (89 mg, 0.66 mmol, 1.1 eq.) were added. After a slow addition of Prop-2-en-1-ol (49 µL, 0.72 mmol, 1.2 eq.), the mixture was warmed up to room temperature and stirred for 3 h. The solid was filtered and the filtrate was washed with Brine (x 3) and the solvent was evaporated under reduced pressure. The crude was then dissolved in THF (2 mL) and a 1 M solution of LiOH in H_2O (2.4 mL, 2.4 mmol, 4 eq.) was added. After 1 h at room temperature, water was added, and the mixture was washed with DCM. The aqueous layer was then neutralized and extracted using a mixture of DCM:EtOH 8:2 (x 3), dried over Na_2SO_4 and solvents were evaporated. The crude was not purified and directly used for the next step. Boc anhydride (327 mg, 1.5 mmol, 2.5 eq.) and DMAP (42 µL, 0.18 mmol, 0.3 eq.) were added to a solution of the carboxylic acid in tBuOH (3 mL). The reaction was stirred overnight at room temperature and dried under high vacuum. EtOAc was added and the product was extracted thrice using $NaHCO_3$ s.s. The organic layer was washed with Brine and EtOAc was removed under reduced pressure. Staudinger reduction was performed as follows. The crude product was dissolved in a mixture of THF: H_2O 9:1 and 1M PMe_3 in THF (0.9 mL, 0.9 mmol, 1.5 eq.) was added. The reaction was stirred overnight at 40 °C. The crude was dried under reduced pressure and purified by RP-HPLC to obtain compound **I11** as a white solid. (30 mg, overall yield = 13%). HR-MS (ESI+) *m/z* calculated $C_{19}H_{26}N_2O_6$ $[M+H]^+$ 378.18636; detected: 378.18613; accuracy = 0.61 ppm. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.89 (t, *J* = 5.9 Hz, 1H), 8.34 (s, 2H), 7.61 (t, *J* = 1.5 Hz, 1H), 7.46 (dd, *J* = 2.5, 1.4 Hz, 1H), 7.28 (dd, *J* = 2.5, 1.5 Hz, 1H), 5.94 (ddt, *J* = 17.3, 10.7, 5.4 Hz,, 1H), 5.34 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.24 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.93 (s, 2H), 4.67 (dt, *J* = 5.5, 1.5 Hz, 2H), 4.06 (s, 2H), 3.91 (d, *J* = 5.8 Hz, 2H), 1.42 (s, 9H).

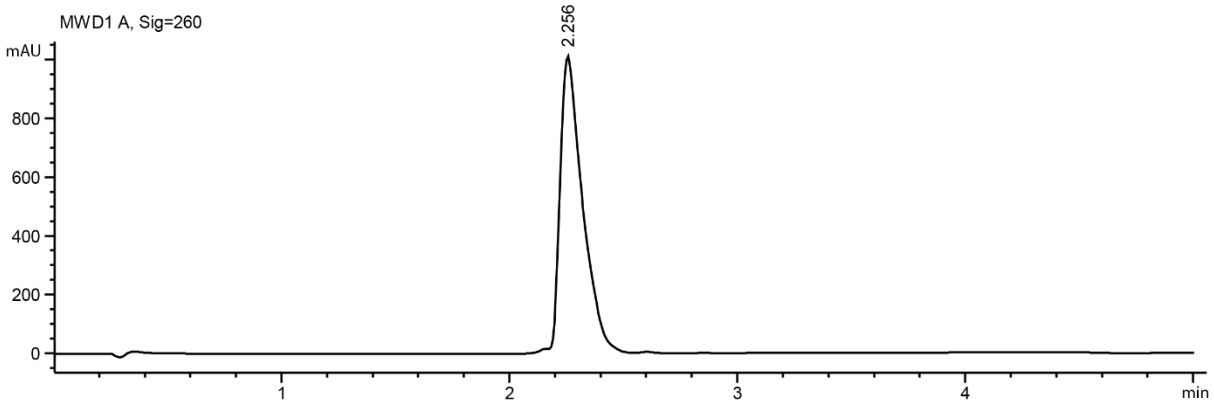


Figure S64. HPLC chromatogram of intermediate **I11** recorded at λ = 260 nm

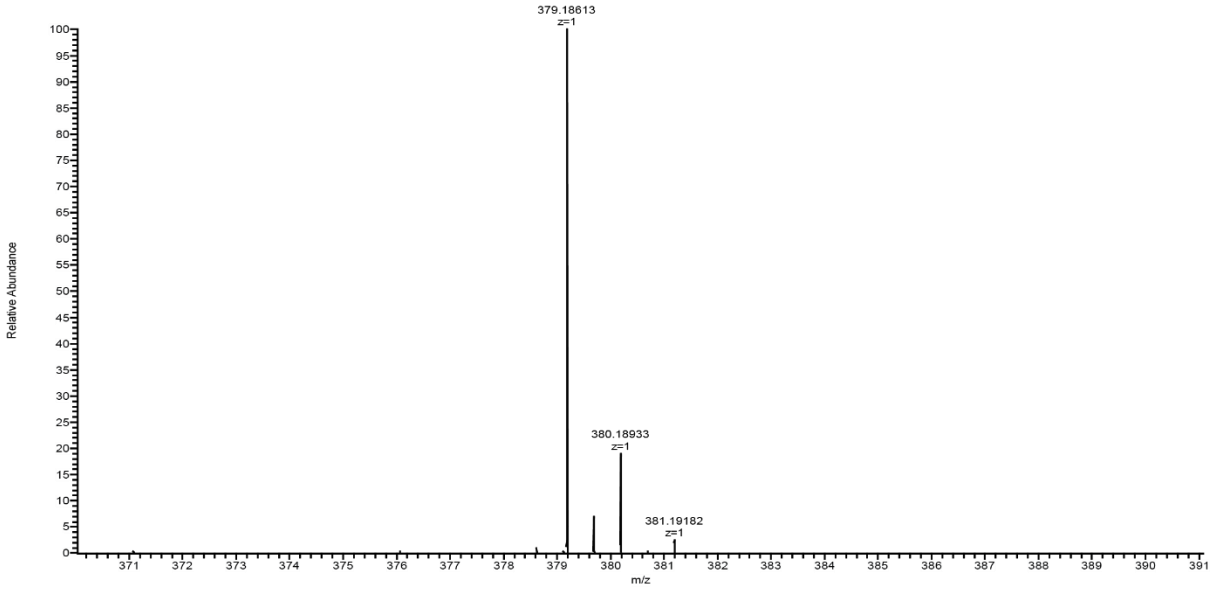
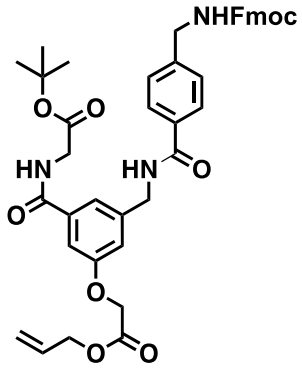


Figure S65. HR-MS of intermediate **I11**.

Synthesis of allyl 2-(3-((4-(((((9H-fluoren-9-yl)methoxy)carbonyl)amino)methyl)benzamido)methyl)-5-((2-(tert-butoxy)-2-oxoethyl)carbamoyl)phenoxy)acetate (I12)



4-(((((9H-fluoren-9-yl)methoxy)carbonyl)amino)methyl)benzoic acid (29.5 mg, 0.079 mmol, 1.2 eq.) was dissolved in DCM (1 mL) and HATU (28 mg, 0.073 mmol, 1.1 eq.), HOAt (4.5 mg, 0.033 mmol, 0.5 eq.) and DIPEA (35 µL, 0.198 mmol, 3 eq.) were added. After 5 min, intermediate **I11** (25 mg, 0.066 mmol, 1 eq.) was added to the mixture and the coupling was stirred for 40 min at room temperature. The reaction was quenched with water and extracted with DCM (x 3), washed with brine (x 1) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the crude was purified by silica gel flash chromatography (elution gradient Hexane: EtOAc, from 0% to 100% of EtOAc in 20 min) to obtain intermediate **I12** as a white solid. (34 mg, yield = 70%). HR-MS (ESI+) *m/z* calculated $C_{42}H_{43}N_3O_9$ $[M+H]^+$ 734.30721; detected: 734.30657; accuracy = 0.87 ppm. ^{1}H NMR (400 MHz, Aceton-d_6): δ (ppm) = 7.91 (d, J = 8.2 Hz, 2H), 7.86 (d, J = 7.3 Hz, 2H), 7.67 – 7.73 (m, 2H), 7.54 (s, 1H), 7.38 – 7.43 (m, 4H), 7.35 – 7.36 (m, 1H), 7.30 – 7.34 (m, 2H), 7.16 (s, 1H), 5.93 (ddt, J = 17.2, 10.4, 5.6 Hz, 1H), 5.32 (dq, J = 17.2, 1.6 Hz, 1H), 5.20 (dq, J = 10.5, 1.4 Hz, 1H), 4.83 (s, 2H), 4.62 – 4.66 (m, 4H), 4.39 – 4.41 (m, 4H), 4.24 (t, J = 8 Hz, 1H), 3.99 – 4.00 (m, 2H), 1.44 (s, 9H).

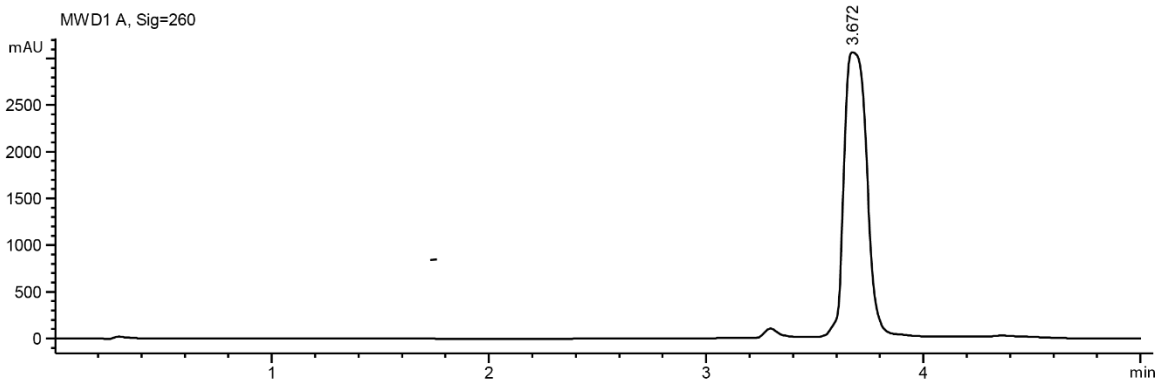


Figure S66. HPLC chromatogram of intermediate **I12** recorded at λ = 260 nm.

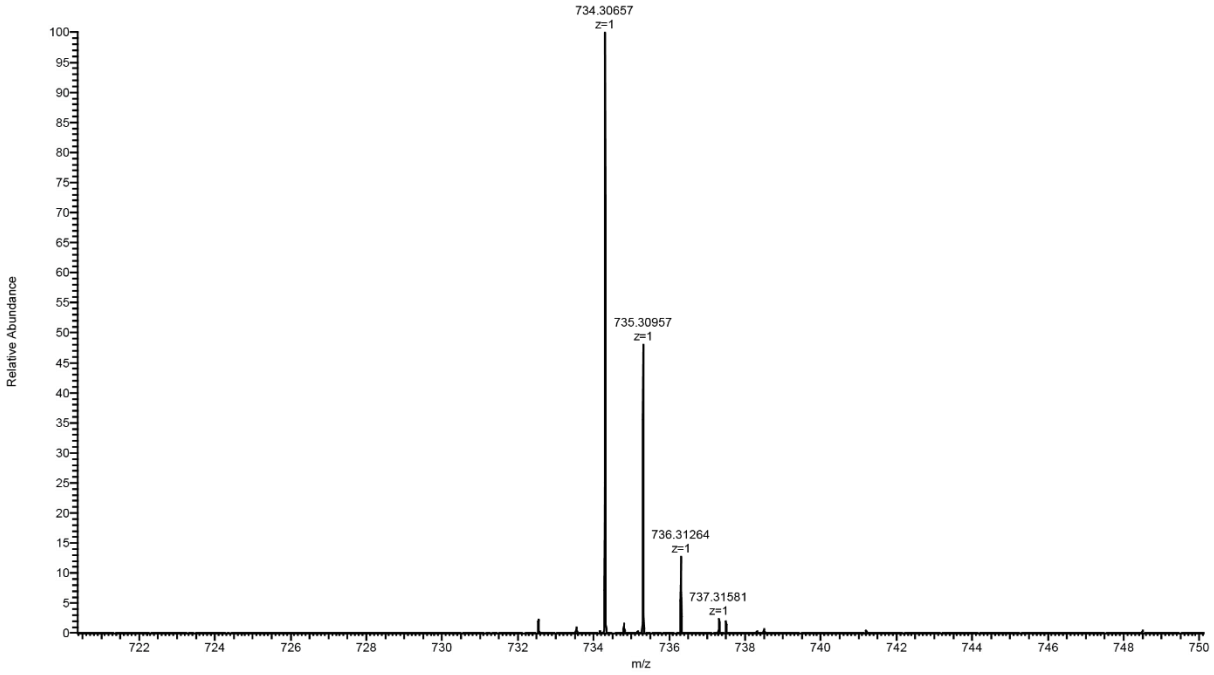
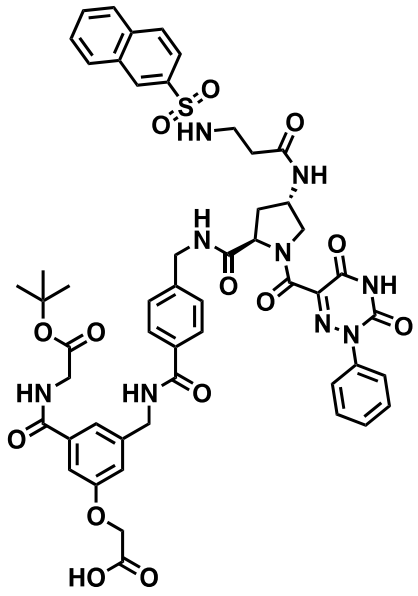


Figure S67. HR-MS of intermediate **I12**.

Synthesis of 2-(3-((2-(tert-butoxy)-2-oxoethyl)carbamoyl)-5-((4-(((2R,4S)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxamido)methyl)benzamido)methyl)phenoxy)acetic acid (I13)



Intermediate **I12** (30 mg, 0.041 mmol, 1 eq.) was dissolved in ACN (1 mL) and diethylamine (42 µL, 0.410 mmol, 10 eq.) was added. The Fmoc deprotection was stirred for 1 h at rt. The solvent was removed under reduced pressure and the crude was dissolved in a mixture of MeCN:H_2O (9:1, 10 mL). The mixture was washed with pentane (x 3) and dried under reduced pressure. The crude was then redissolved in MeCN:H_2O and dried overnight using the lyophilizer, 17 mg of crude compound were afforded. The compound was not isolated and amide coupling was performed. **HIT_2** (24 mg, 0.040 mmol, 1.2 eq.), was dissolved in DCM (1 mL) and HATU (14 mg, 0.036 mmol, 1.1 eq.) and DIPEA (23 µL, 0.132 mmol, 4 eq.) were added and the acid was activated for 5 min. The Fmoc deprotected amine (17 mg, 0.033 mmol, 1 eq.) was added to the mixture and the reaction was stirred for 2h at room temperature. The coupling was stopped with water and extracted with DCM (x 3), washed with brine (x 1) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the crude underwent Alloc deprotection. The reaction was carried out under dry conditions, starting material (1 eq.) was dissolved in 1 mL of anhydrous DCM, $PhSiH_3$ (41 µL, 0.330, 10 eq.) and $Pd(PPh_3)_4$ (20 mg, 0.017 mmol, 0.5 eq.) were added. The mixture was stirred for 1.5 h at room temperature and subsequently filtered over celite and washed with DCM. The solvent was

evaporated under reduced pressure and the crude was purified by RP-HPLC to afford compound **I13** (6 mg, overall yield = 17%). HR-MS (ESI+) *m/z* calculated $C_{52}H_{53}N_9O_{14}S$ $[M+H]^+$ 1060.35054; detected: 1060.34902; accuracy = 1.43 ppm. ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) = 12.54 (d, *J* = 8.1 Hz, 1H), 9.01 (t, *J* = 5.8 Hz 1H), 8.82 (t, , *J* = 6.0 Hz, 1H), 8.55 – 8.58 (m, 1H), 8.42 (s, 1H), 8.12 – 8.18 (m, 3H), 8.04 (d, *J* = 7.9 Hz, 1H), 7.65 – 7.84 (m, 6H), 7.35 – 7.49 (m, 7H), 7.27 (s, 1H), 7.18 – 7.21 (m, 1H), 7.04 (s, 1H), 6.52 (s, 1H), 4.71 (s, 1H), 4.53 – 4.57 (m, 1H), 4.47 (d, *J* = 5.7 Hz, 2H), 4.33 – 4.41 (m, 3H), 3.87 (d, *J* = 5.8 Hz, 2H), 3.78 – 3.82 (m, 1H), 3.51(s, 1H), 2.95 – 2.98 (m, 2H), 2.20 – 2.26 (m, 2H), 2.03 – 2.08 (m, 2H), 1.40 (S, 9H).

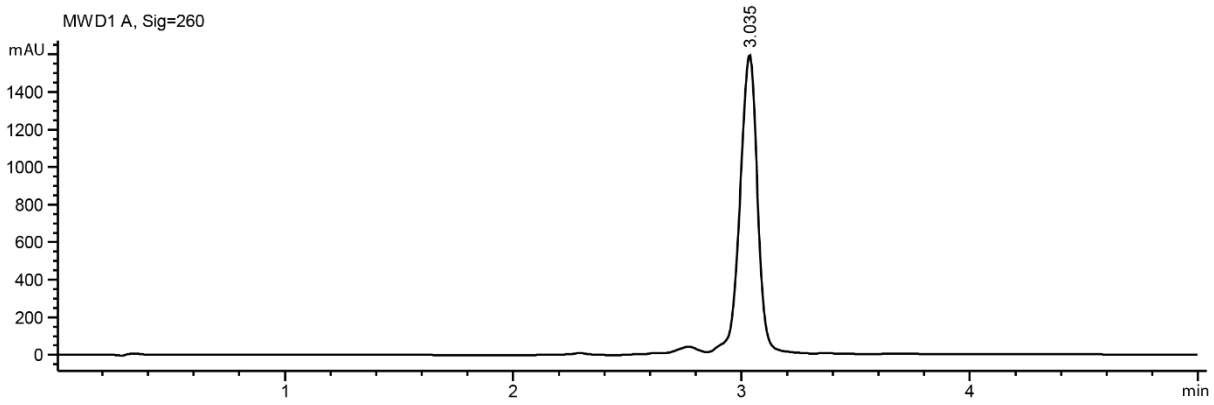


Figure S68. HPLC chromatogram of intermediate **I13** recorded at λ = 260 nm.

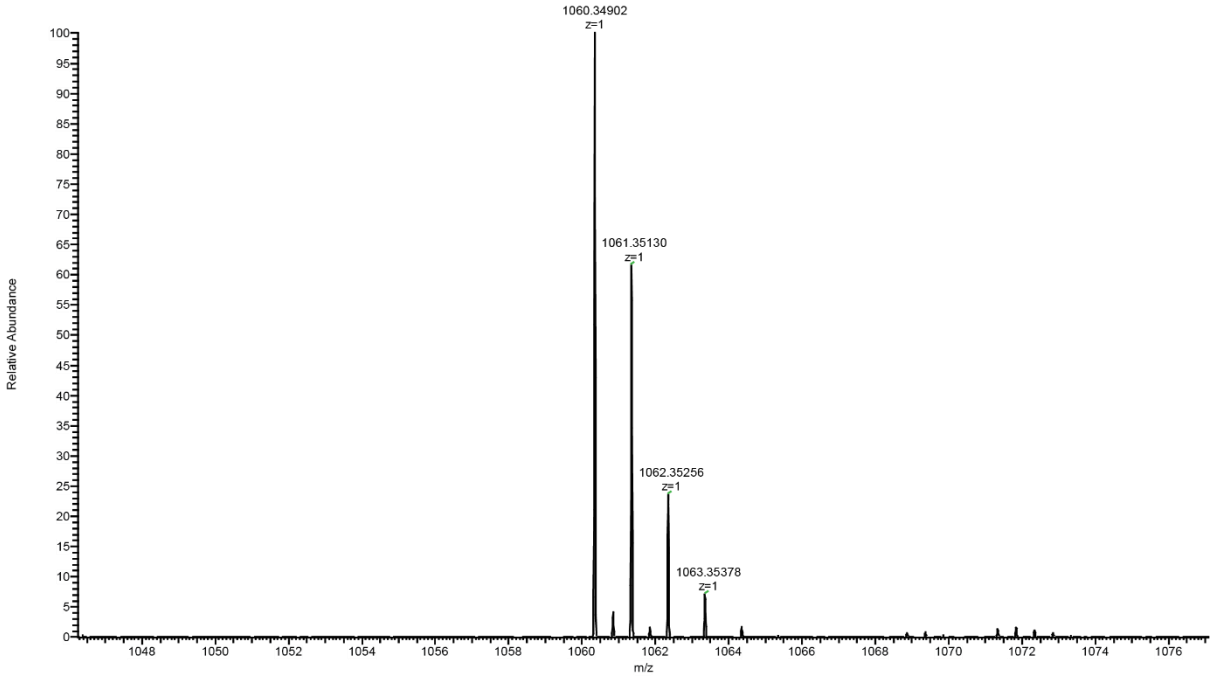
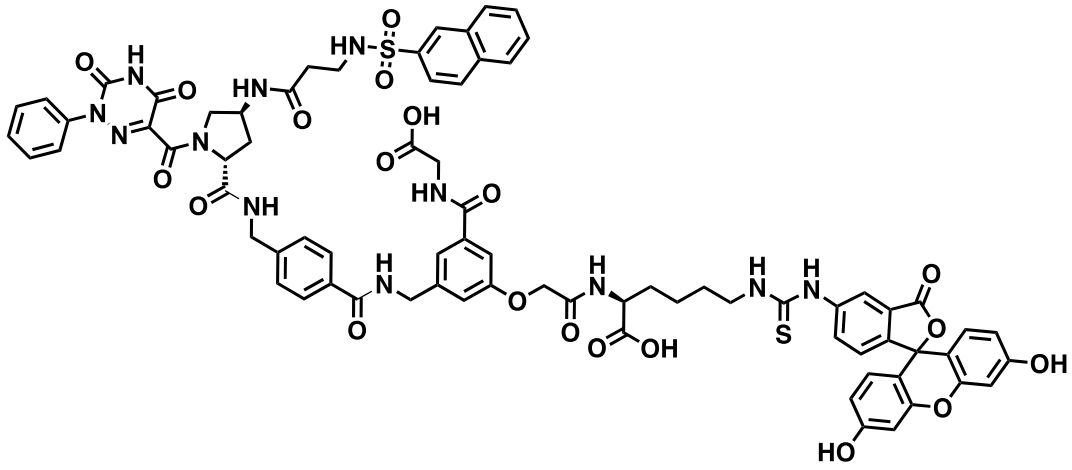


Figure S69. HR-MS of intermediate **I13**.

Synthesis of N^2-(2-(3-((carboxymethyl)carbamoyl)-5-((4-(((2R,4S)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxamido)methyl)benzamido)methyl)phenoxy)acetyl)-N6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-L-lysine (5)



To a solution of intermediate **I13** (3 mg, 0.003 mmol, 1 eq.) in DCM (500 µL), HATU (1.1 mg, 0.003 mmol, 1 eq.) and DIPEA (2 µL, 0.012 mmol, 4 eq.) were added. After 5 min at rt,

tert-butyl N6-(tert-butoxycarbonyl)-L-lysinate (0.91 mg, 0.003 mmol, 1 eq.) was added to the mixture and the coupling proceeded for 1 h at room temperature. The solvents were evaporated and 3 mL of neat TFA were added to the crude to perform Boc and Tert-butyl deprotection. After 1 hour at rt, the reaction was completed and TFA was removed under reduced pressure and the crude was used for the next step. The crude product (1 eq.) was dissolved in DMF (30 µL), and TEA (1.7 µL, 0.012 mmol, 4 eq.) was added, followed by fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 15 µL, 0.003 mmol, 1 eq.) The reaction was stirred for 15 min at room temperature in the dark. The product was purified by RP-HPLC to get the final compound **5** as orange solid (0.3 mg, overall yield = 6%). HR-MS (ESI+) *m/z* calculated $C_{75}H_{68}N_{12}O_{20}S_2$ $[M+2H]^{2+}$ 761.21299; detected: 761.21130; accuracy = 2.22 ppm

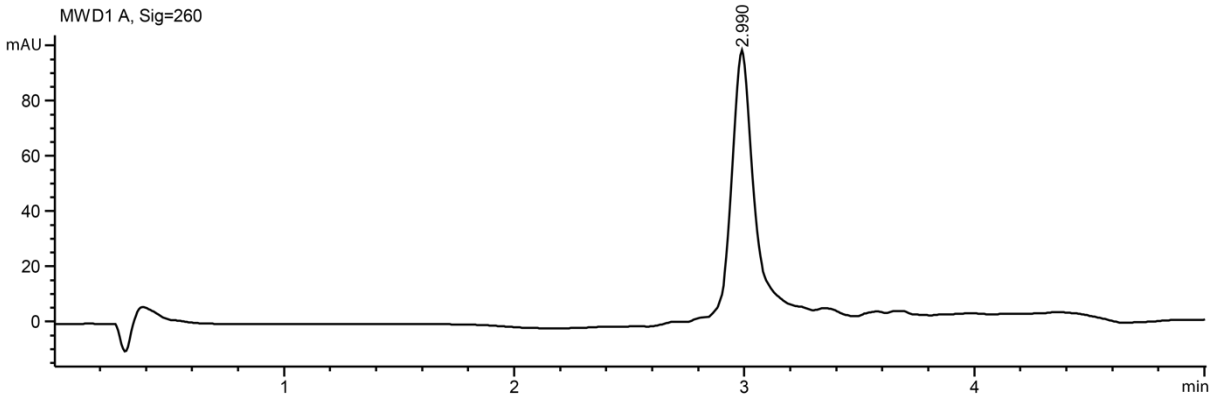


Figure S70. HPLC chromatogram of compound **5** recorded at λ = 260 nm.

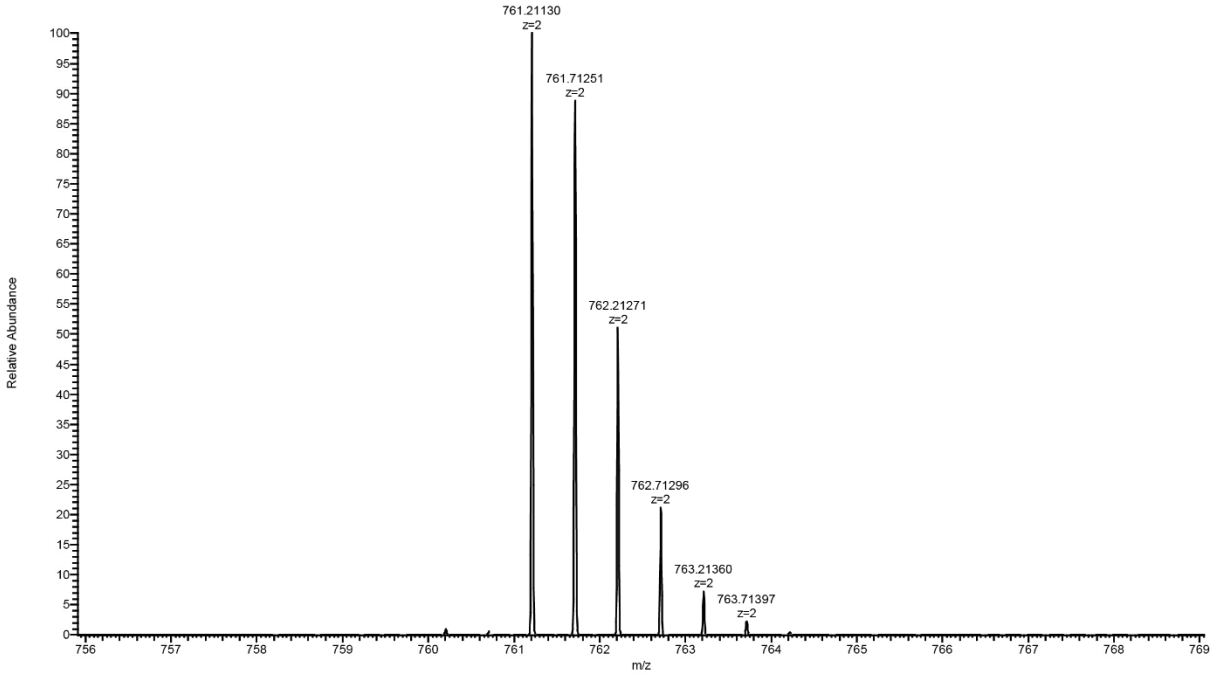
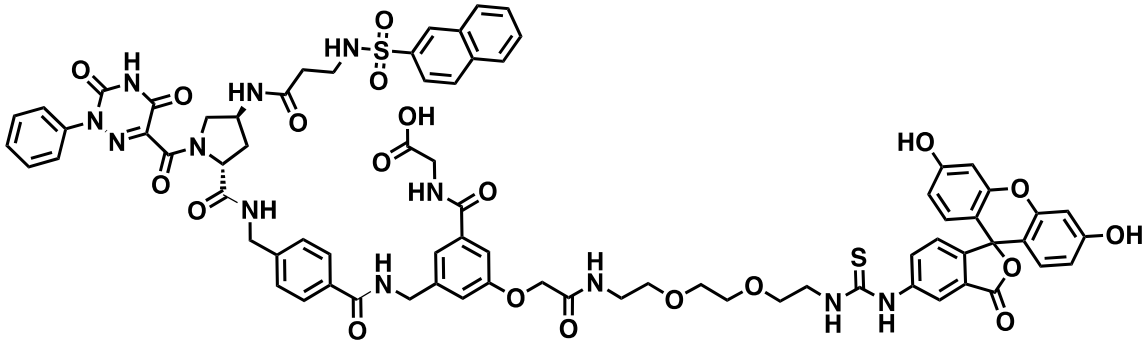


Figure S71. HR-MS of compound **5**.

Synthesis of (3-((1-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-diazatridecan-13-yl)oxy)-5-((4-(((2R,4S)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxamido)methyl)benzamido)methyl)benzoyl)glycine (7)



To a solution of intermediate **I13** (3 mg, 0.003 mmol, 1 eq.) in DCM (500 µL), HATU (1.1 mg, 0.003 mmol, 1 eq.) and DIPEA (2 µL, 0.012 mmol, 4 eq.) were added. After 5 min at rt, tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (200 mM in DCM, 15 µL, 0.003 mmol, 1 eq.) was added to the mixture and the coupling was carried out for 1 h at room

temperature. The solvents were evaporated and 1 mL of neat TFA were added to the crude to perform Boc and Tert-butyl deprotection. After 1 hour at rt, the reaction was completed and TFA was removed under reduced pressure and the crude was used for the next step. The crude product (1 eq.) was dissolved in DMF (30 µL), and TEA (1.7 µL, 0.012 mmol, 4 eq.) was added, followed by fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 15 µL, 0.003 mmol, 1 eq.) The reaction was stirred for 15 min at room temperature in the dark. The product was purified by RP-HPLC to get the final compound **7** as orange solid (0.7 mg, overall yield = 15%). HR-MS (ESI+) *m/z* calculated $C_{75}H_{70}N_{12}O_{20}S_2$ $[M+H]^+$ 1523.43435; detected:1523.43545; accuracy = 0.72 ppm

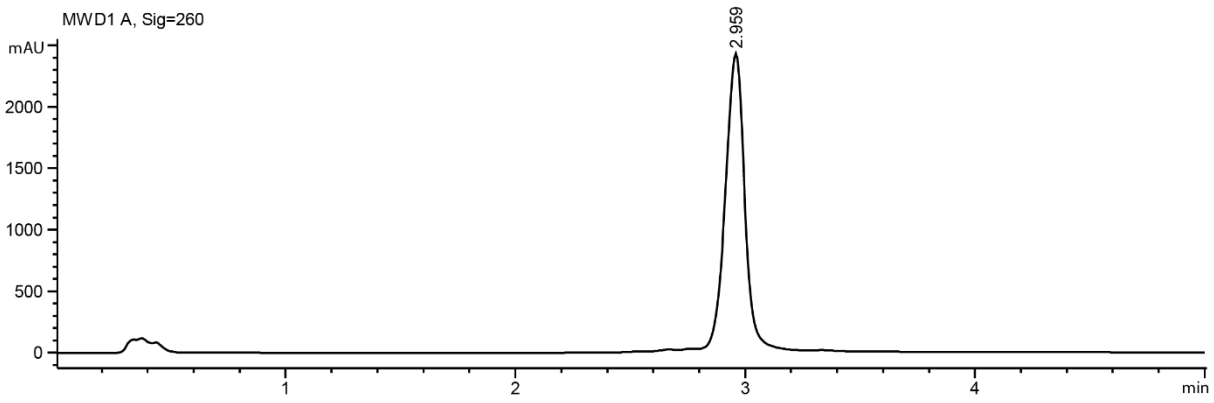


Figure S72. HPLC chromatogram of compound **7** recorded at λ = 260 nm.

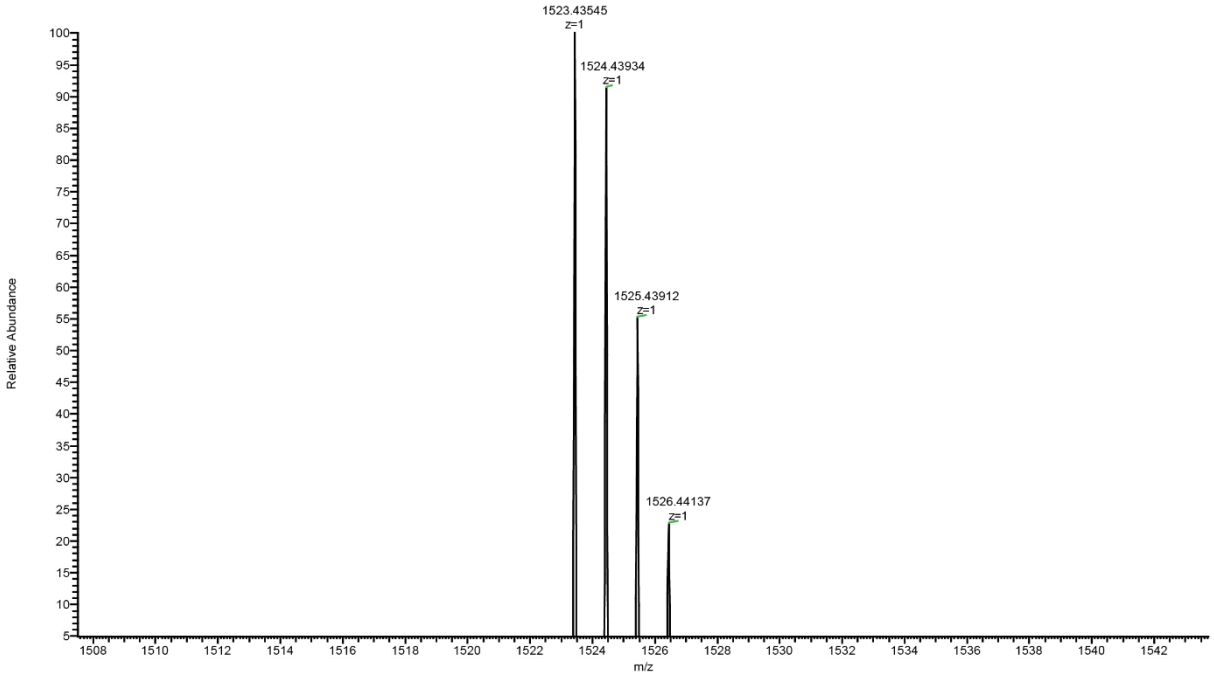
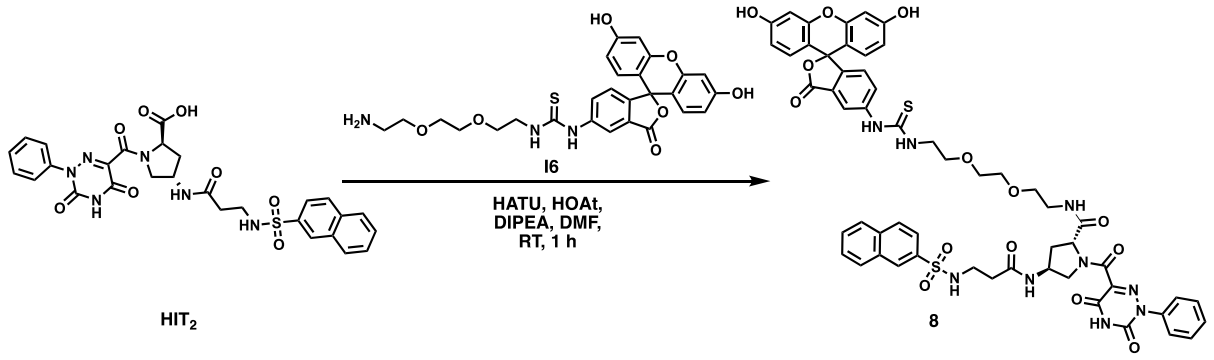


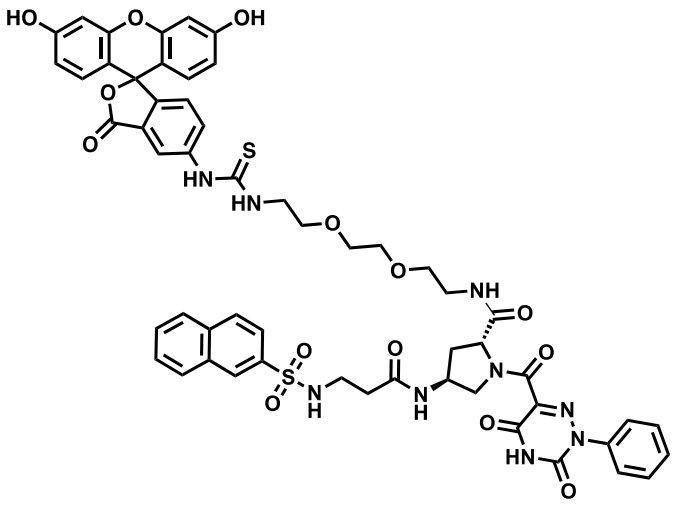
Figure S73. HR-MS of compound **7**.

6.2.5 Synthesis scheme for compound 8



Scheme S13. Schematic illustration of off-DNA synthesis of compound **8**.

Synthesis of (2R,4S)-N-(2-(2-(2-(3-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)ethoxy)ethoxy)ethyl)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxamide (8)



HIT$_2$ (7 mg, 0.011 mmol, 1.1 eq.) was dissolved in DMF (500 µL), and HATU (4 mg, 0.010 mmol, 1 eq.), HOAt (0.7 mg, 0.005 mmol, 0.5 eq.) and DIPEA (7 µL, 0.040 mmol, 4 eq.) were added. The acid was activated for 5 min at room temperature and **I6** (5.4 mg, 0.010 mmol, 1 eq.). After 1 hour at room temperature, the solvent was removed under reduced pressure, the crude was re-dissolved in DMF:ACN:H_2O 1:1:1 and purified by RP-HPLC to obtain compound **8** as an orange solid (6 mg, yield = 53%). HR-MS (ESI+) *m/z* calculated $C_{55}H_{51}N_9O_{14}S_2$ $[M+H]^+$ 1126.30697; detected:1126.30661; accuracy = 0.32 ppm

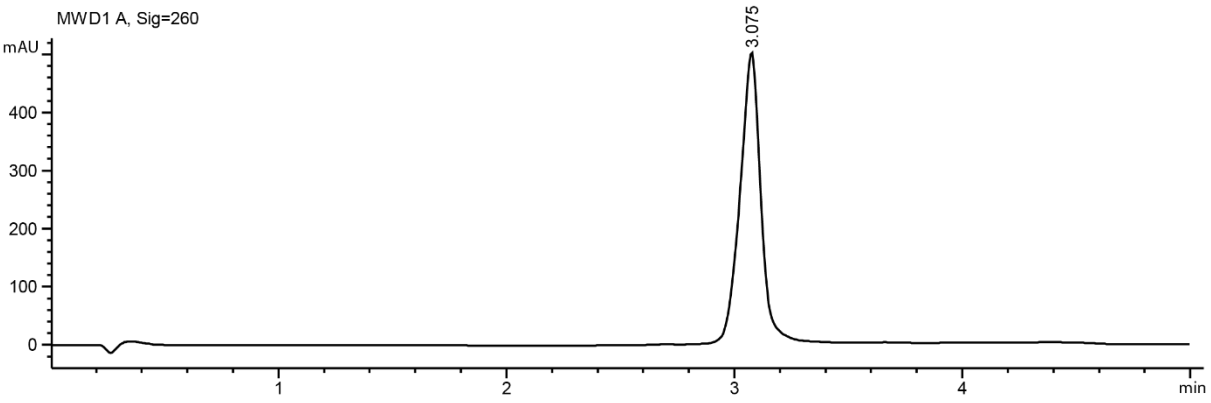


Figure S74. HPLC chromatogram of compound **8** recorded at λ = 260 nm.

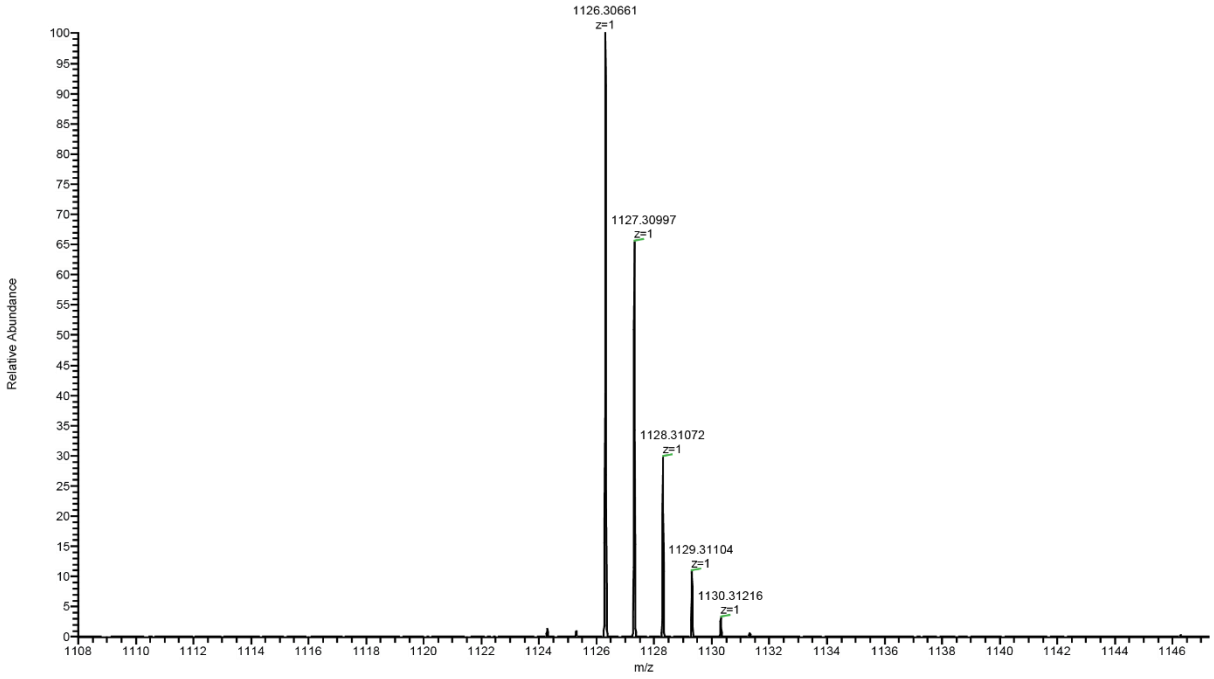
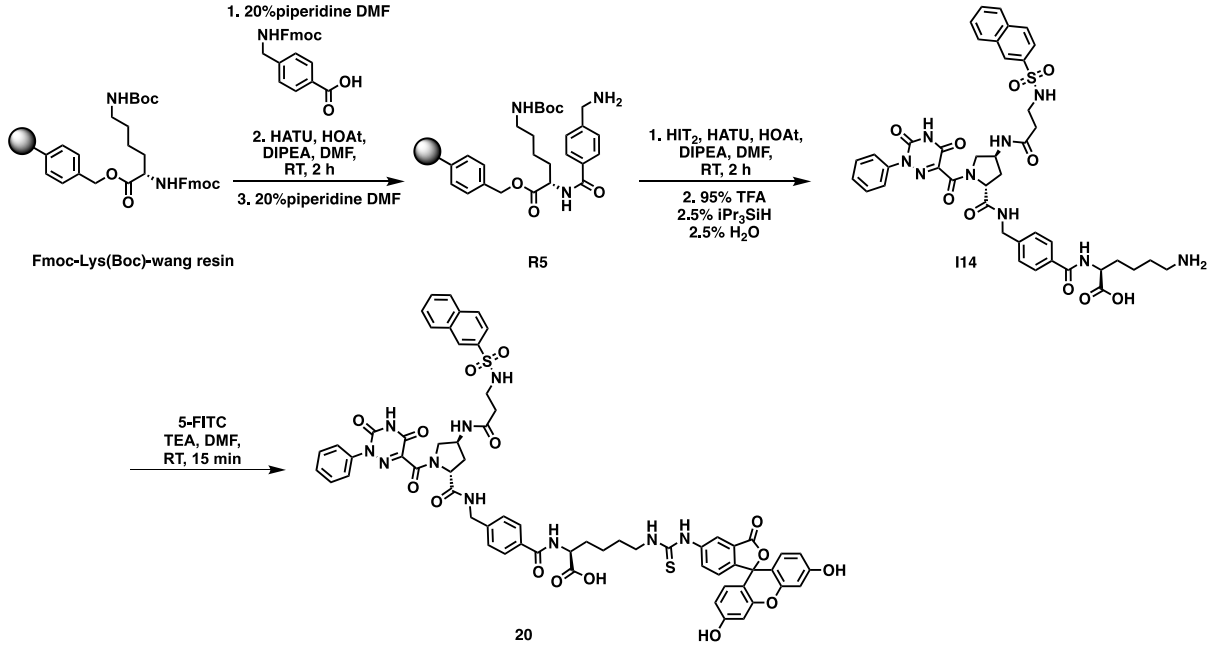


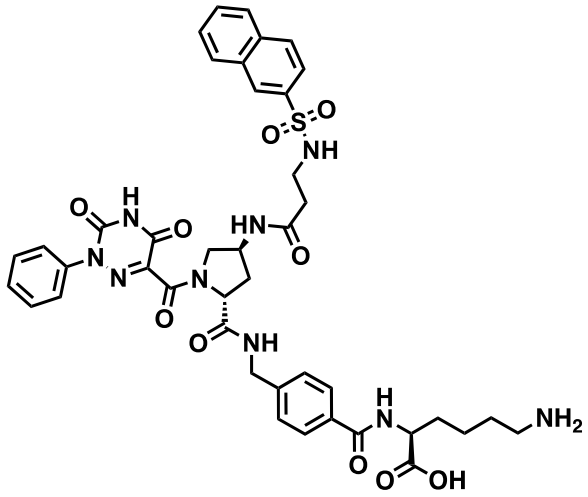
Figure S75. HR-MS of compound **8**.

6.2.6 Synthesis scheme for compound 20



Scheme S14. Schematic illustration of off-DNA synthesis of compound **20**.

Synthesis of (4-(((2R,4S)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxamido)methyl)benzoyl)-L-lysine (I14)



Fmoc-Lys (Boc)-Wang Resin (100 mg, 0.060 mmol, 1 eq.) was Fmoc deprotected by adding 20% piperidine in DMF (1 x 30 min and 2 x 10 min) and washed 10 times with DMF to remove residual piperidine. Afterwards, commercially available 4-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)methyl)benzoic acid (45 mg , 0.120 mmol, 2 eq.), HATU (43 mg, 0.114 mmol, 1.9 eq.), HOAt (4 mg, 0.030 mmol, 0.5 eq.) were dissolved in DMF (2 mL) and DIPEA (42 µL, 0.240 mmol, 4 eq.) was added. After 5 min of acid activation, the solution was added to the resin (0.060 mmol, 1 eq.) and the reaction was incubated for 2 h at rt. The resin was first washed 5 times with DMF followed by incubation with 20% piperidine in DMF (1 x 30 min and 2 x 10 min) and then washed again 10 times with DMF to get the Fmoc deprotected compound **R5**. A solution of HIT_2 (73 mg, 0.120 mmol, 2 eq.), HATU (43 mg, 0.114 mmol, 1.9 eq.), HOAt (4 mg, 0.030 mmol, 0.5 eq.) and DIPEA (42 µL, 0.240 mmol, 4 eq.) in DMF (3 mL) was added to the resin **R5**. After 2 h of incubation at rt, the resin was washed 5 times with DMF and then 5 times with DCM. Subsequently, a cleavage solution of 95% TFA, 2.5% water, 2.5% iPr_3SiH was freshly prepared and added to **R5**. The resin was incubated for 1 h at rt and the cleavage step was repeated two further times (2 x 10 min), the cleaved fractions were

collected, dried under reduced pressure to remove TFA and the crude was then purified by RP-HPLC to obtain **I14** as white solid (9 mg, yield = 17%). HR-MS (ESI+) *m/z* calculated $C_{42}H_{45}N_9O_{10}S$ $[M+H]^+$ 868.30829; detected: 868.30609; accuracy = 2.53 ppm

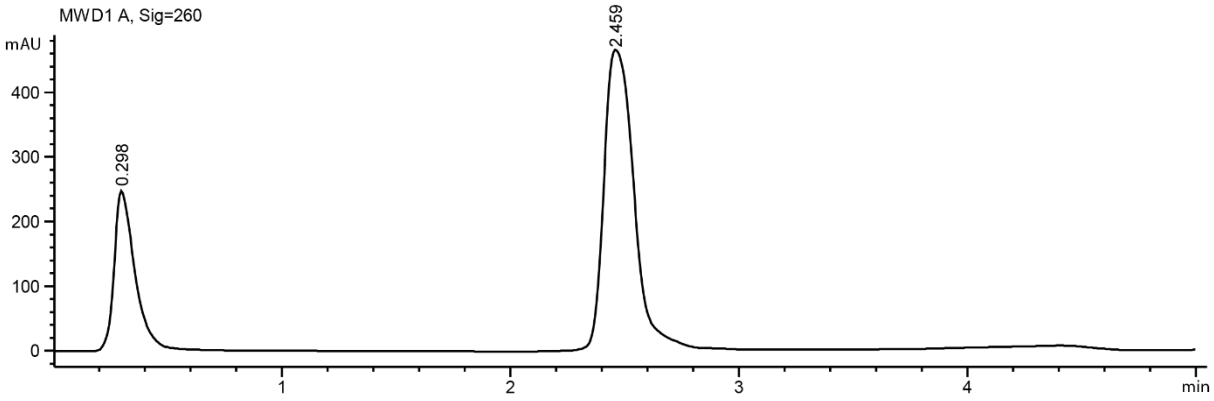


Figure S76. HPLC chromatogram of intermediate **I14** recorded at λ = 260 nm.

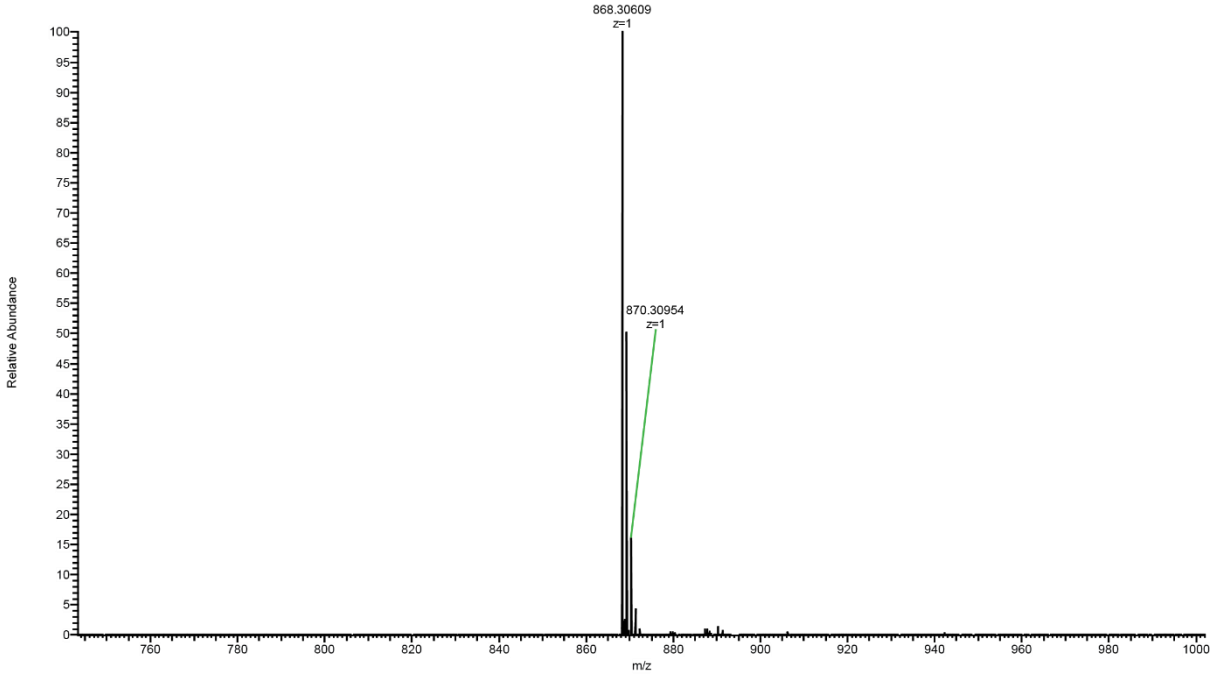
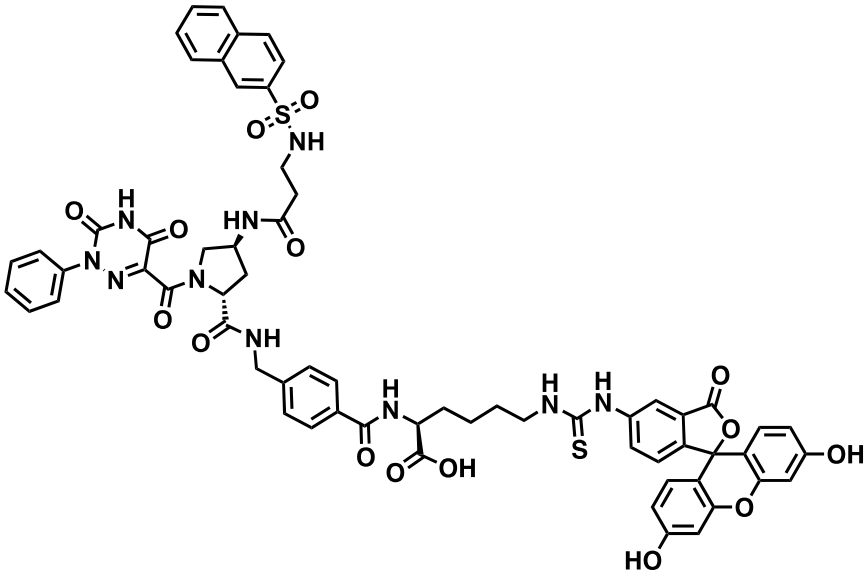


Figure S77. HR-MS of intermediate **I14**.

Synthesis of N^6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)-N2-(4-(((2R,4S)-1-(3,5-dioxo-2-phenyl-2,3,4,5-tetrahydro-1,2,4-

triazine-6-carbonyl)-4-(3-(naphthalene-2-sulfonamido)propanamido)pyrrolidine-2-carboxamido)methyl)benzoyl)-L-lysine (20)



In a 1.5 mL Eppendorf Tube, **I14** (1 mg, 1.2 µmol, 1 eq.) was dissolved in DMF (30 µL), and fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 6 µL, 1.2 µmol, 1 eq.) and TEA (1 M in DMF, 4.8 µL, 4.8 µmol, 4 eq.) were added. The reaction was stirred for 15 min at room temperature in the dark and the product formation was monitored by LC-MS. The crude was diluted with a solution ACN:H_2O 1:1 and purified by RP-HPLC to obtain compound **20** as orange powder (0.7 mg, yield = 50%). HR-MS (ESI+) *m/z* calculated $C_{63}H_{56}N_{10}O_{15}S_2$ $[M+2H]^{2+}$ 629.17568; detected: 629.17402; accuracy = 2.64 ppm

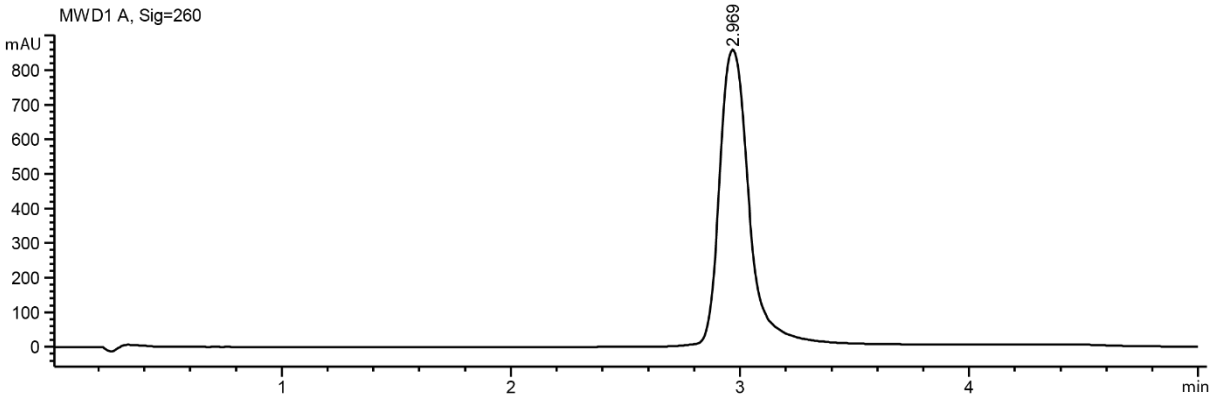


Figure S78. HPLC chromatogram of compound **20** recorded at λ = 260 nm.

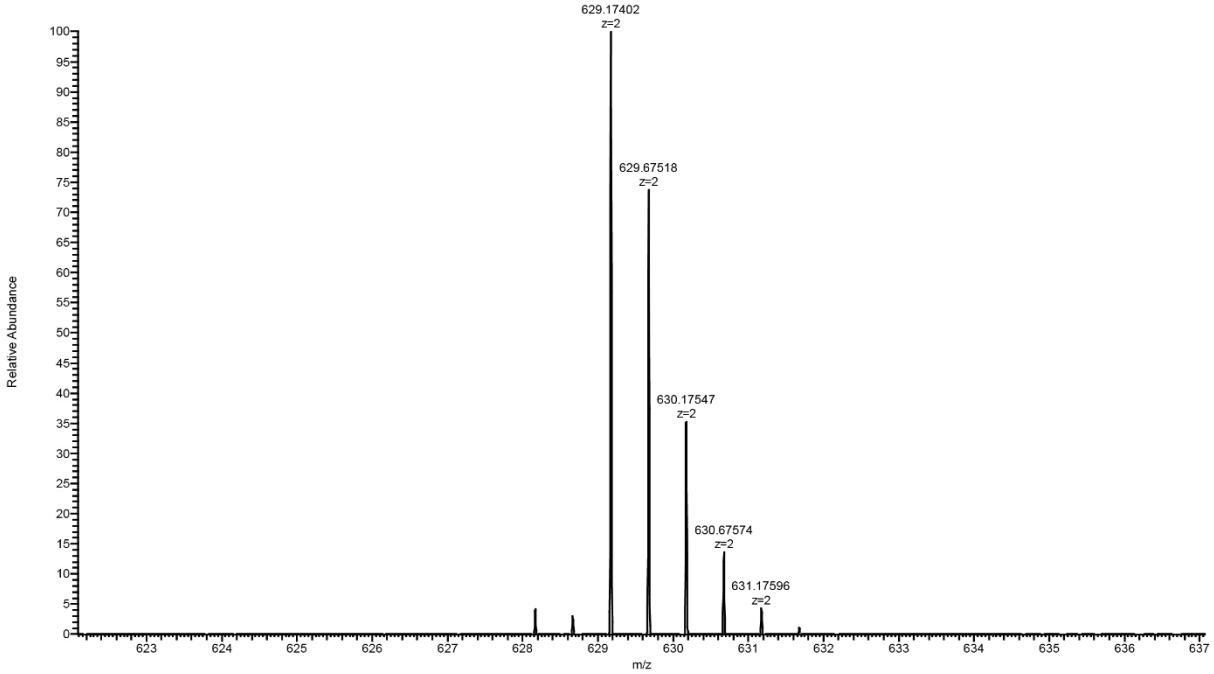
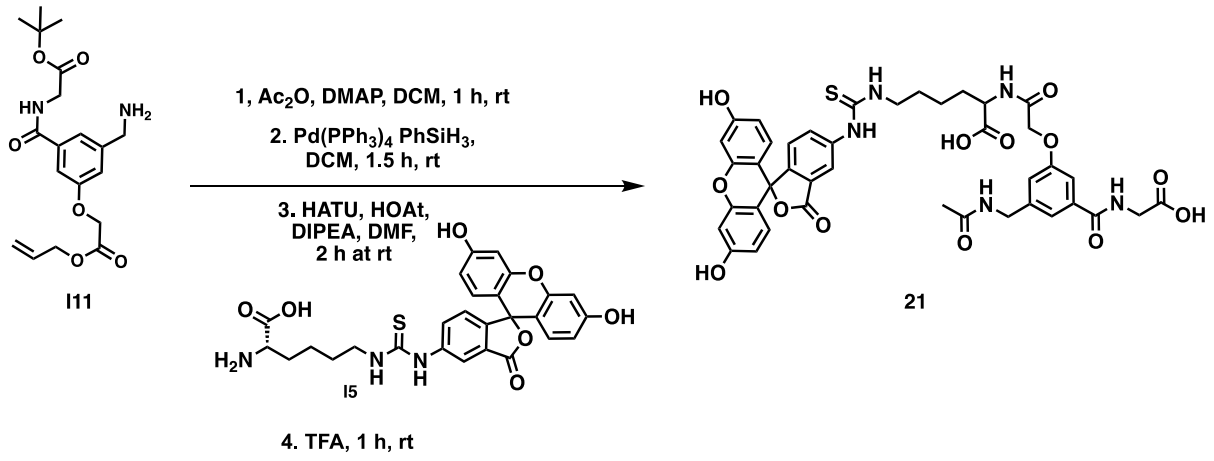


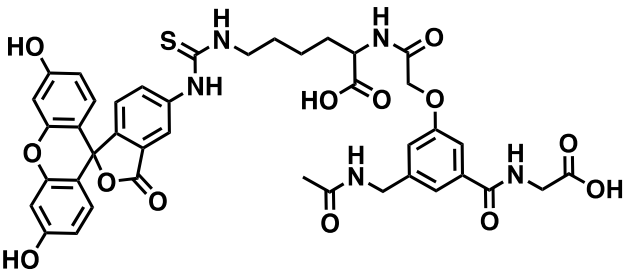
Figure S79. HR-MS of compound **20**.

6.2.7 Synthesis scheme for compound 21



Scheme S15. Schematic illustration of off-DNA synthesis of compound **21**.

Synthesis of N^2-(2-(3-(acetamidomethyl)-5-((carboxymethyl)carbamoyl)phenoxy)acetyl)-N6-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)carbamothioyl)lysine (21)



Compound **I11** (10 mg, 0.026 mmol, 1 eq.) was dissolved in DCM (500 µL) and acetic anhydride (2.5 µL, 0.026, 1 eq.) and DMAP (12 µL, 0.052 mmol, 2 eq.) were added. The reaction was stirred for 1 h at room temperature. The solvent was evaporated and the crude was dissolved in 1 mL of anhydrous DCM, $PhSiH_3$ (32 µL, 0.260, 10 eq.) and $Pd(PPh_3)_4$ (15 mg, 0.013 mmol, 0.5 eq.) were added. The mixture was stirred for 1.5 h at room temperature and subsequently filtered over celite and washed with DCM. The compound was dried under reduced pressure and was used without purification for the next reaction. The crude product (0.026 mmol, 1.1 eq.) was dissolved in DMF (500 µL), and HATU (10 mg, 0.026 mmol, 1 eq.), HOAt (1.8 mg, 0.013 mmol, 0.5 eq.) and DIPEA (18 µL, 0.104 mmol, 4 eq.) were added. After 5 min at room temperature, **I5** (14 mg, 0.026 mmol, 1 eq.) was added and the reaction was stirred for 1 hour at room temperature. DMF was evaporated under high vacuum and TFA (500 µL) was added. After 1 hour at room temperature, TFA was removed under reduced pressure, the crude was re-dissolved in DMF:H_2O 1:1 and purified by RP-HPLC to obtain compound **21** as an orange solid (1.3 mg, yield = 6%). HR-MS (ESI+) *m/z* calculated $C_{41}H_{39}N_5O_{13}S$ $[M+H]^+$ 842.23378; detected:842.23184; accuracy = 2.30 ppm.

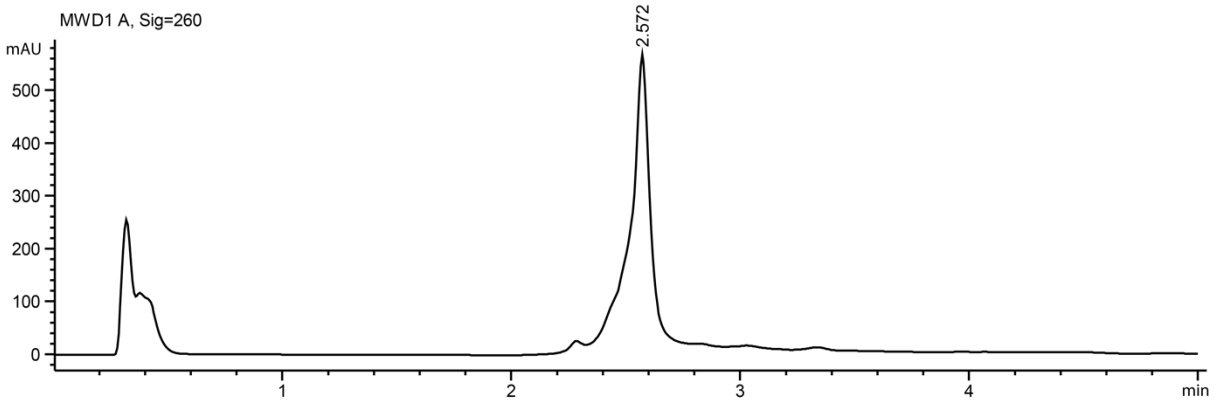


Figure S80. HPLC chromatogram of compound **21** recorded at λ = 260 nm.

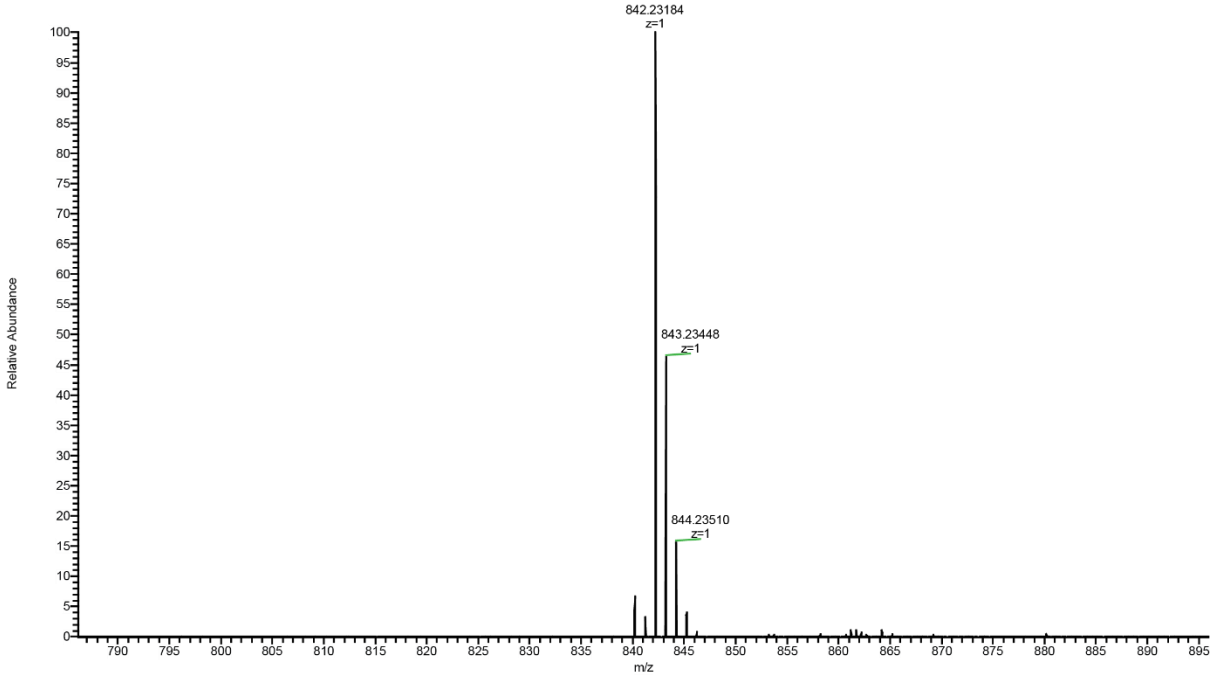
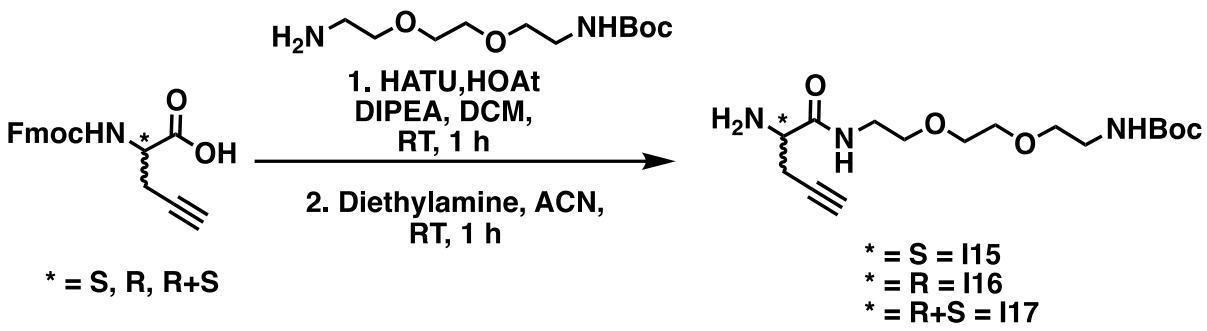


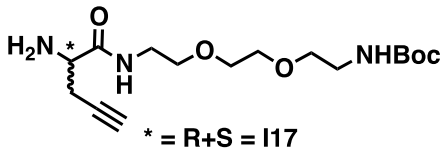
Figure S81. HR-MS of compound **21**.

6.2.8 Synthesis scheme for intermediate I15, I16, I17



Scheme S16. Schematic illustration of synthesis of intermediates **I15**, **I16** and **I17**.

Synthesis of tert-butyl (2-(2-(2-(2-aminopent-4-ynamido)ethoxy)ethoxy)ethyl)carbamate (I17)



A racemate mixture of 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)pent-4-ynoic acid (100 mg, 0.298 mmol, 1 eq.) was dissolved in 5 mL of DCM and HATU (113 mg, 0.298 mmol, 1 eq.), HOAt (20 mg, 0.149 mmol, 0.5 eq.) and DIPEA (208 µL, 1.192 mmol, 4 eq.) were added to the solution. The acid was activated for 5 min at rt and tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (74 mg, 0.298 mmol. 1 eq.) was added to the reaction. The amide coupling was stirred for 1 h at room temperature and the reaction was monitored by LC-MS. Afterwards, the solvent was removed under reduced pressure and the crude was dissolved in ACN (3 mL) and Fmoc deprotection was performed (1 h). The crude was purified by RP-HPLC and a waxy solid (compound **I17**) was obtained (60 mg, yield = 59%). HR-MS (ESI+) *m/z* calculated $C_{16}H_{29}N_3O_5$ $[M+H]^+$ 344.21800; detected: 344.21786; accuracy = 0.41 ppm. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.55 (t, J = 5.5 Hz, 1H), 8.28 (s, 2H), 6.76 (t, J = 5.8 Hz, 1H), 3.90 (s, 1H), 3.56 – 3.60 (m, 1H), 3.51 (s, 3H), 3.43 – 3.46 (m, 2H), 3.37 (t, J

= 6.3 Hz, 2H), 3.22 – 3.34 (m, 2H), 3.11 – 3.12 (m, 1H), 3.06 (q, *J* = 6.1 Hz, 2H), 2.67 – 2.72 (m, 2H), 1.37 (s, 9H).

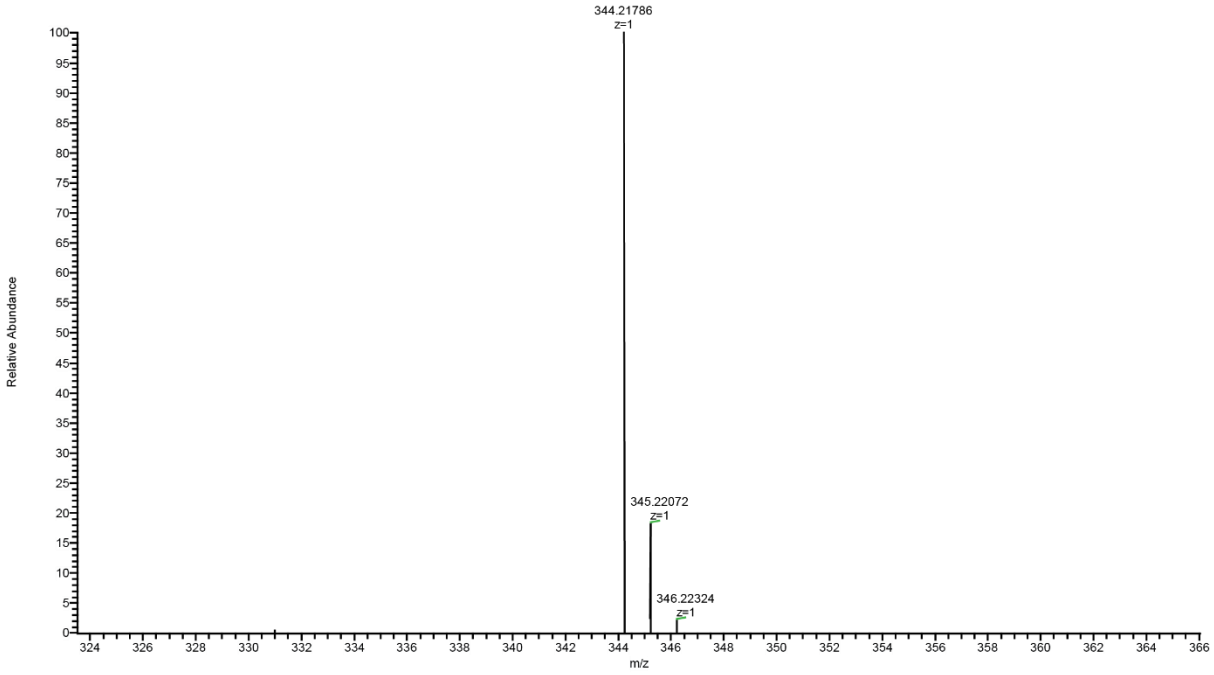
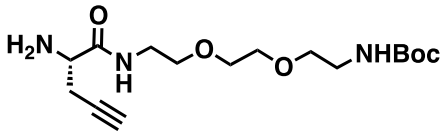


Figure S82. HR-MS of intermediate **I17**.

Synthesis of *tert*-butyl (*S*)-(2-(2-(2-(2-aminopent-4-ynamido)ethoxy)ethoxy)ethyl)carbamate (I15)



Synthesis of **I15** was performed following the same synthetic procedure reported for **I17**.

I15 = (46 mg, yield = 45%), HR-MS (ESI+) *m/z* calculated $C_{16}H_{29}N_3O_5$ $[M+H]^+$ 344.21800; detected: 344.21796; accuracy = 0.12 ppm.

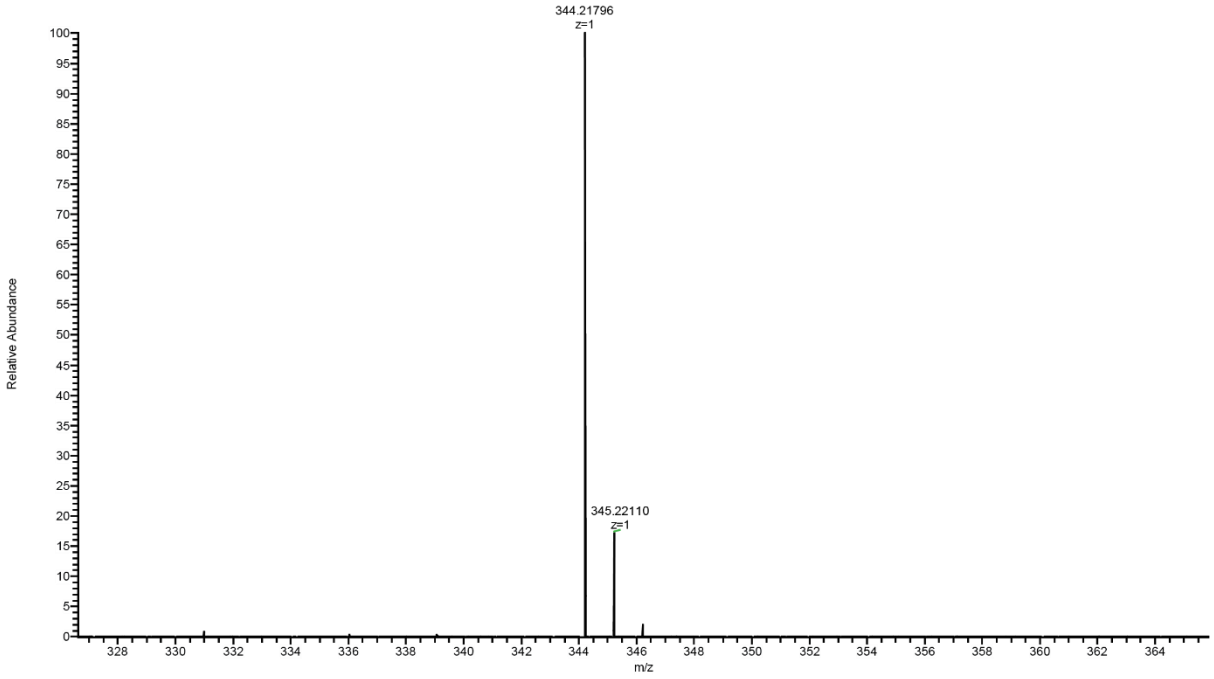
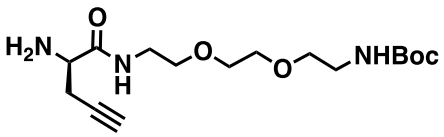


Figure S83. HR-MS of intermediate **I15**.

Synthesis of *tert*-butyl (*R*)-(2-(2-(2-(2-aminopent-4-ynamido)ethoxy)ethoxy)ethyl)carbamate (I16).



Synthesis of **I16** was performed following the same synthetic procedure reported for **I17**.

I16 = (40 mg, yield = 39%), HR-MS (ESI+) *m/z* calculated $C_{16}H_{29}N_3O_5$ $[M+H]^+$ 344.21800; detected: 344.21800; accuracy = 0 ppm

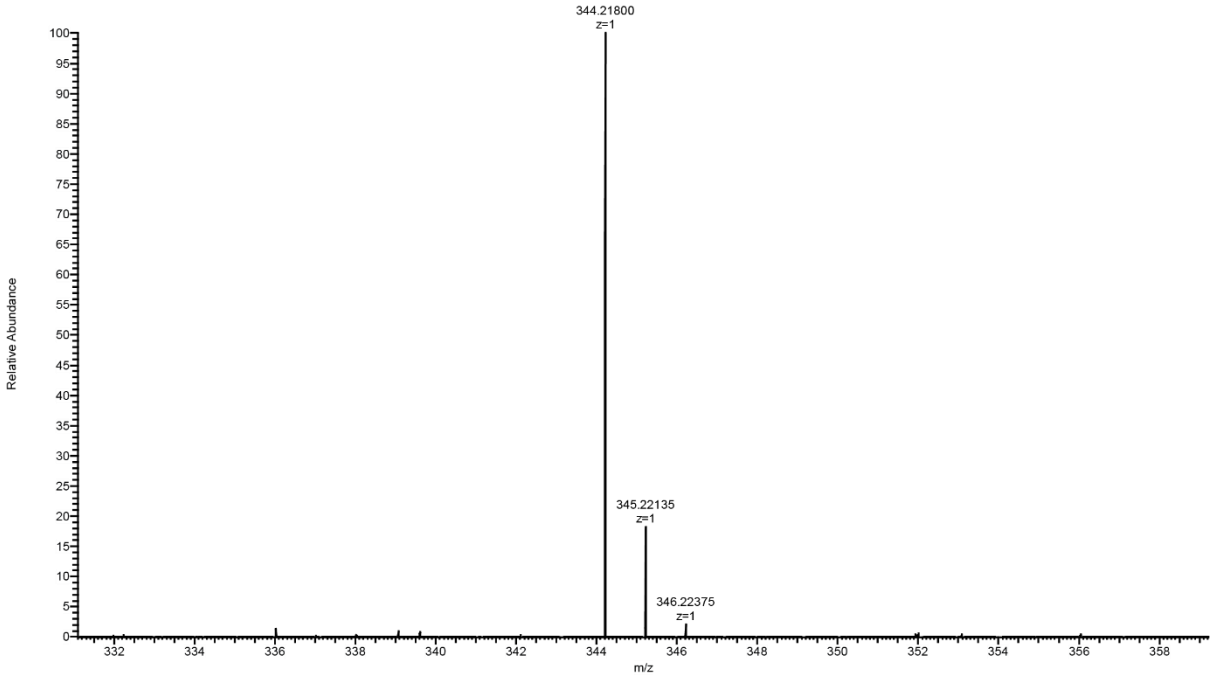
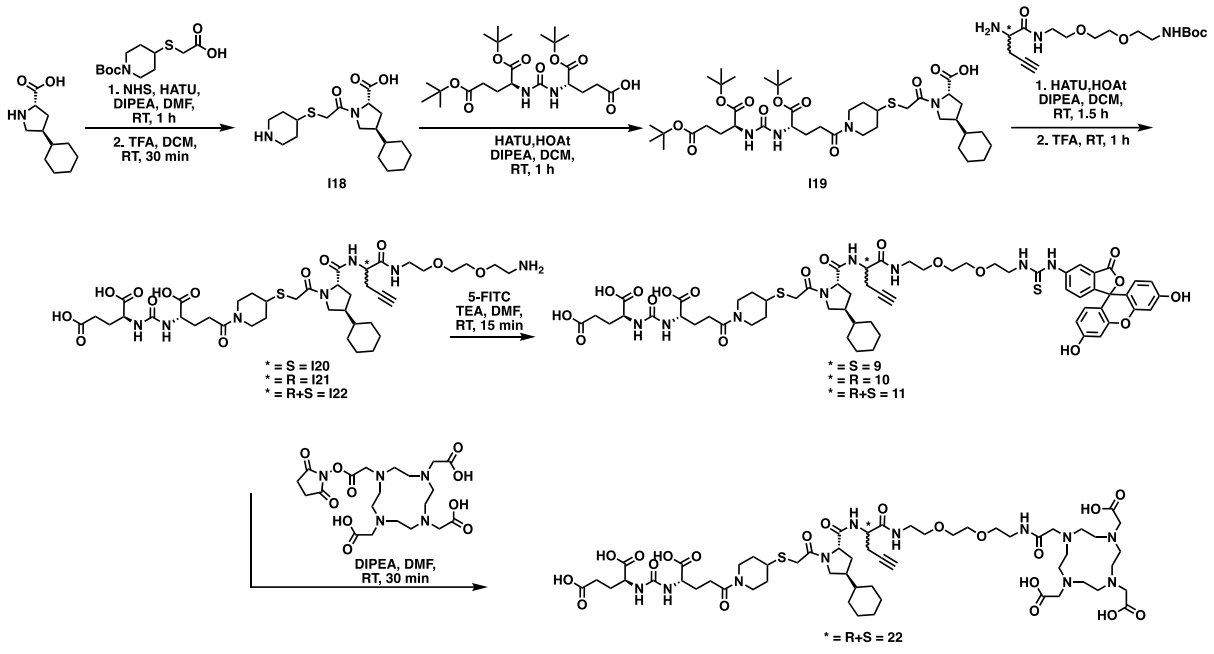


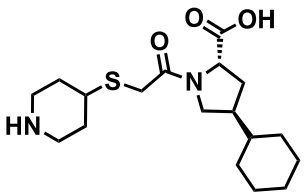
Figure S84. HR-MS of intermediate **I16**.

6.2.9 Synthesis scheme for compounds 9, 10, 11 and 22



Scheme S17. Schematic illustration of synthesis of compounds **9**, **10**, **11** and **22**.

Synthesis of (2*S*,4*S*)-4-cyclohexyl-1-(2-(piperidin-4-ylthio)acetyl)pyrrolidine-2-carboxylic acid (I18)



Commercially available 2-((1-(tert-butoxycarbonyl)piperidin-4-yl)thio)acetic acid (110 mg, 0.399 mmol, 1.1 eq.), HATU (138 mg, 0.363 mmol, 1 eq.) and N-hydroxysuccinimide (NHS, 42 mg, 0.363 mmol, 1 eq.) were dissolved in dry DMF (3 mL) and DIPEA (253 µL, 1.452 mmol, 4 eq.) was added. The activation of the acid as NHS ester, was stirred for 20 min at room temperature and subsequently, (2S,4S)-4-cyclohexylpyrrolidine-2-carboxylic acid (72 mg, 0.363 mmol, 1 eq.) was added to the solution. The reaction proceeded for 1 h at rt and then DMF was removed under reduced pressure. The crude product was dissolved in DCM (2 mL) and 2 mL of TFA were added. The Boc deprotection proceeded for 30 min at room temperature followed by removal of TFA under reduced pressure, the product was purified by RP-HPLC and compound **I18** was obtained as a white solid (82 mg, overall yield = 64%). HR-MS (ESI+) *m/z* calculated $C_{18}H_{30}N_2O_3S$ $[M+H]^+$ 355.20499; detected: 355.20502; accuracy = 0.08 ppm. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.63 – 8.79 (m, 2H), 7.41 (s, 2H), 4.47 (d, *J* = 9.0 Hz, 1H), 3.91 (t, *J* = 8.4 Hz, 1H), 3.37 - 3.40 (m, 3H), 3.02 – 3.17 (m, 3H), 2.12 – 2.24 (m, 3H), 1.82 – 1.88 (m, 3H), 1.61 – 1.73 (m, 5H), 1.13 – 1.26 (m, 4H), 0.91 – 1.04 (m, 2H).

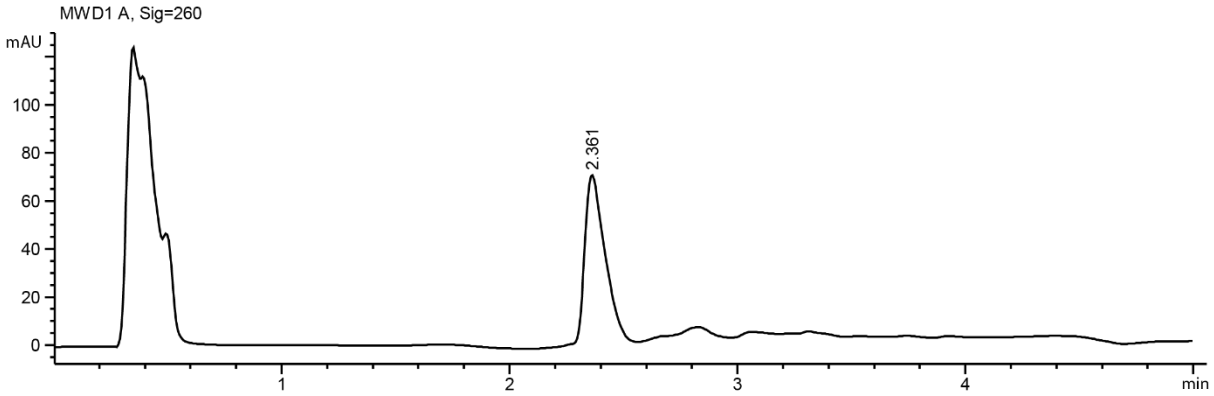


Figure S85. HPLC chromatogram of intermediate **I18** recorded at λ = 260 nm.

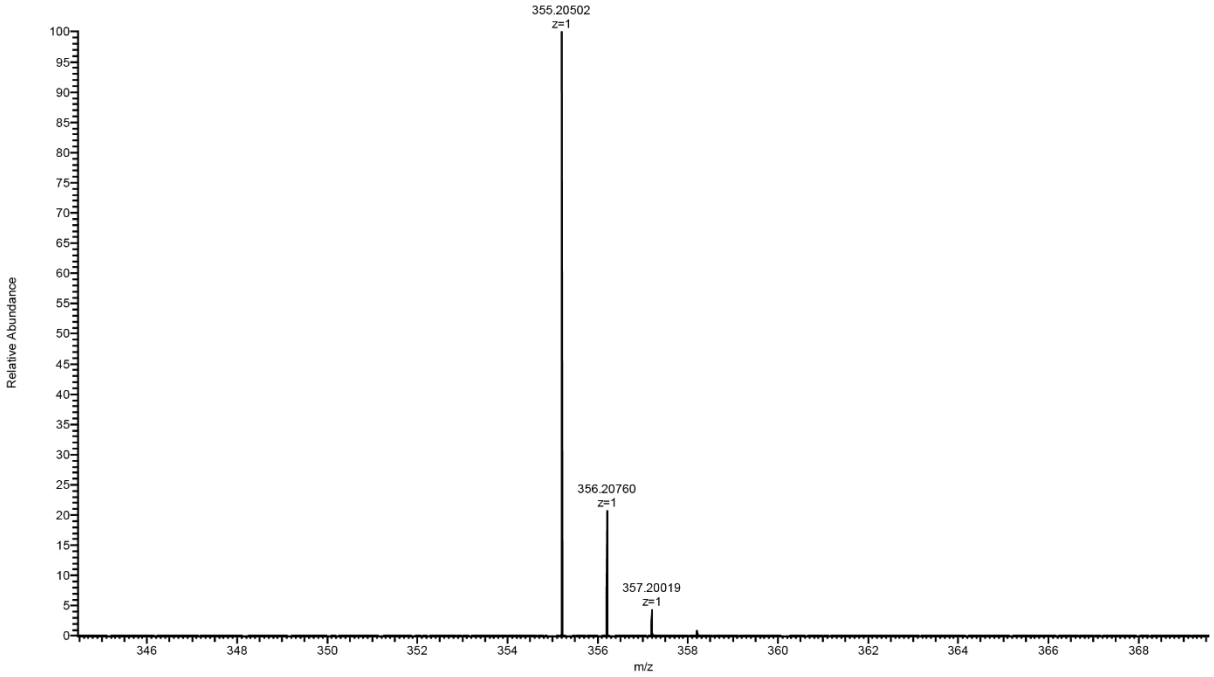
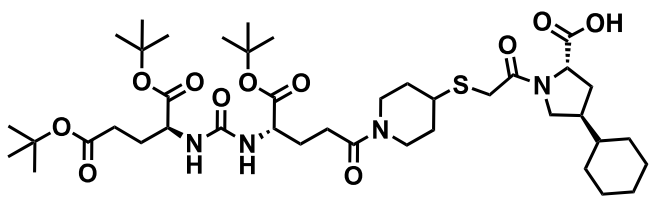


Figure S86. HR-MS of intermediate **I18**.

Synthesis of (2*S*,4*S*)-1-(2-((1-((*S*)-5-(*tert*-butoxy)-4-(3-((*S*)-1,5-di-*tert*-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoyl)piperidin-4-yl)thio)acetyl)-4-cyclohexylpyrrolidine-2-carboxylic acid (I19)



To a solution of commercially available (S)-5-(tert-butoxy)-4-(3-((S)-1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoic acid (91 mg, 0.186 mmol, 1.1 eq.) in DCM (3 mL), HATU (64 mg, 0.169 mmol, 1 eq.), HOAt (12 mg, 0.085 mmol, 0.5 eq.) and DIPEA (118 µL, 0.676 mmol, 4 eq.) were added. The acid was pre-activated for 10 min at room temperature, followed by the addition of compound **I18** (60 mg, 0.169 mmol, 1 eq.). The amide coupling was stirred at room temperature for 1 h and solvent was evaporated under reduced pressure. The crude was re-dissolved in a mixture of DMF:H_2O:MeCN (1:1:1) and purified by RP-HPLC to obtain compound **I19** as a white solid (101 mg, yield = 72%). HR-MS (ESI+) *m/z* calculated $C_{41}H_{68}N_4O_{11}S$ $[M+H]^+$ 825.46781; detected: 825.46572; accuracy = 2.53 ppm. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.55 (s, 3H), 4.60 (t, *J* = 7.9 Hz, 1H), 4.25 – 4.29 (m, 3H), 3.69 – 3.98 (m, 1H), 3.77 – 3.82 (m, 1H), 3.28 – 3.40 (m, 2H), 3.08 – 3.21 (m, 3H), 2.93 – 2.98 (m, 1H), 2.44 – 2.58 (m, 2H), 2.30 – 2.37 (m, 3H), 1.85 – 2.25 (m, 7H), 1.55 – 1.82 (m, 7H), 1.45 (s, 18H), 1.43 (s. 9H), 1.29 – 1.38 (m, 1H), 1.18- 1.26 (m, 4H), 0.96 – 1.05 (m, 2H).

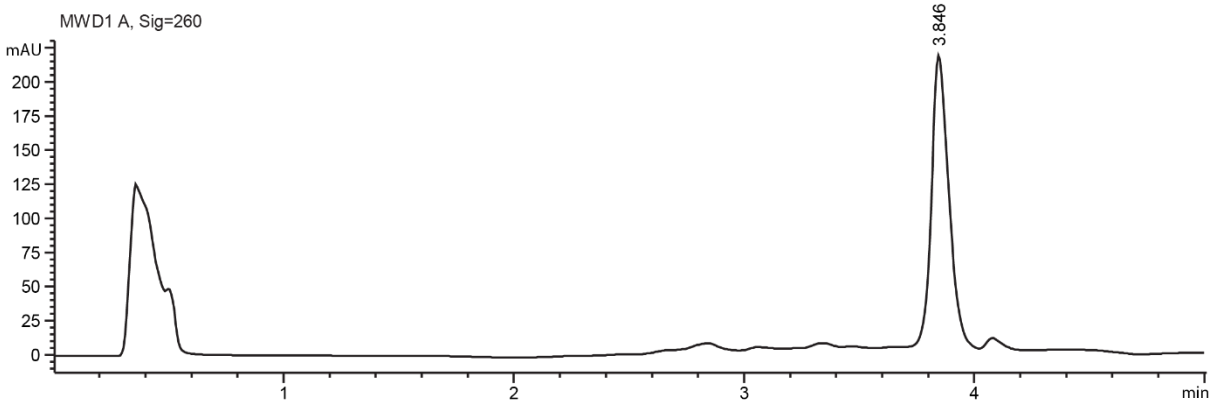


Figure S87. HPLC chromatogram of intermediate **I19** recorded at λ = 260 nm.

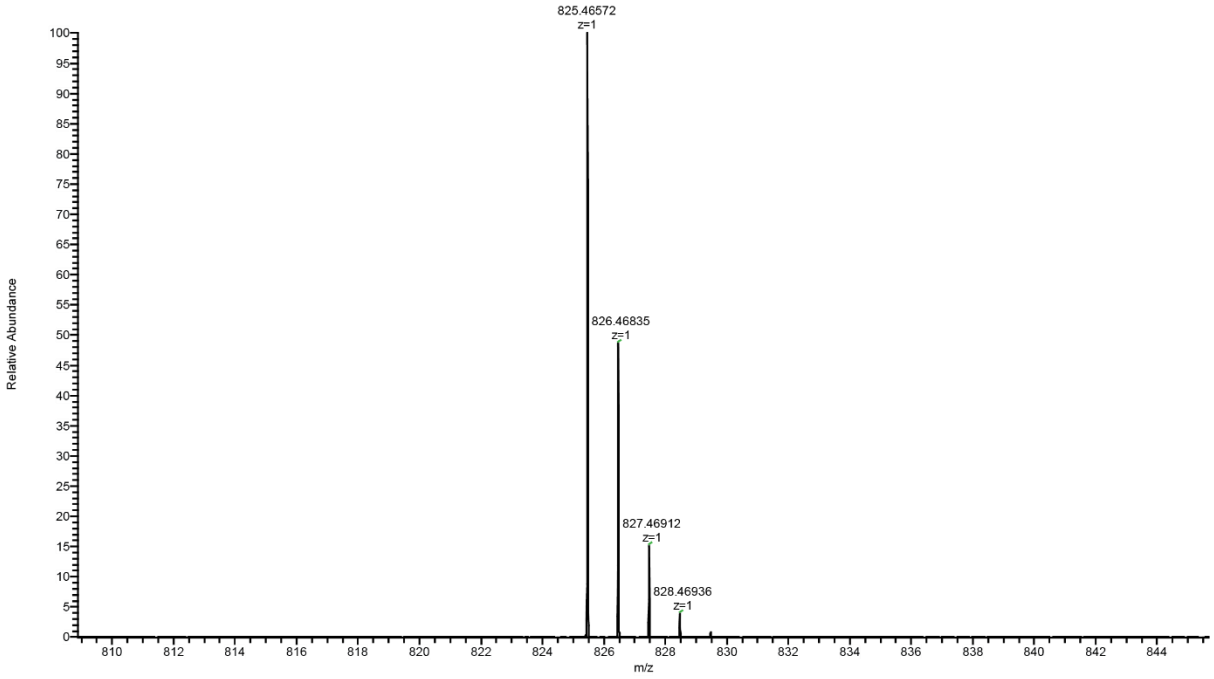
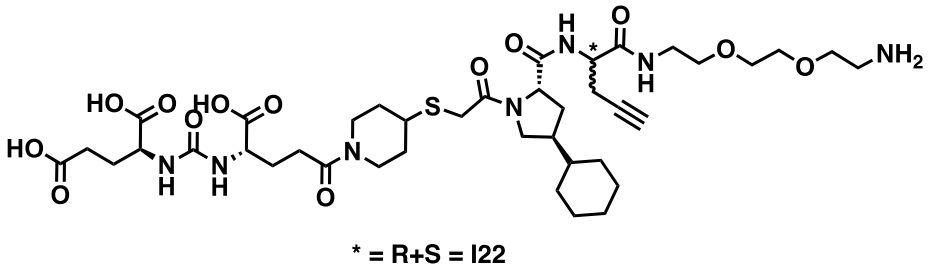


Figure S88. HR-MS of intermediate **I19**.

Synthesis of (((1*S*)-4-(4-((2-((2*S*,4*S*)-2-((1-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-1-oxopent-4-yn-2-yl)carbamoyl)-4-cyclohexylpyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-1-carboxy-4-oxobutyl)carbamoyl)-*L*-glutamic acid (I22)



*** = R+S = I22**

Compound **I17** and compound **I19** were reacted together to synthesize compound **I22**. Compound **I19** (40 mg, 0.049 mmol, 1.1 eq), HATU (17 mg, 0.045 mmol, 1 eq.), HOAt (3 mg, 0.023 mmol, 0.5 eq.) and DIPEA (31 µL, 0.180 mmol, 4 eq.) were dissolved in DCM (1 mL) and the mixture was stirred for 5 min at rt. Afterwards, compound **I17** (15.5 mg, 0.045, 1 eq.) was added to the mixture and the coupling proceeded for 1.5 h at rt. The solvent was evaporated under reduced pressure, and neat TFA (3 mL) was added to the reaction crude. The Boc and Tert-butyl deprotection were performed at rt for 1 h followed by removal of TFA under high

vacuum. The product was re-dissolved in a mixture of DMF:H_2O:MeCN (1:1:1) and purified by RP-HPLC. Compound **I22** was obtained as a waxy solid (7 mg, overall yield = 18%). HR-MS (ESI+) *m/z* calculated $C_{40}H_{63}N_7O_{13}S$ $[M+H]^+$ 882.42773; detected:882.42525; accuracy = 2.81 ppm. ^1H NMR (400 MHz, DMSO-d_6, diastereomeric mixture): δ (ppm) 12.48 (bs, 2H), 8.31 – 8.43 (m, 1H), 7.94 – 8.09 (m, 1H), 7.76 – 7.89 (m, 2H), 6.34 (t, J = 7.1 Hz, 1H), 4.30 – 4.43 (m, 2H), 4.06 – 4.17 (m, 2H), 3.39 – 3.83 (m, 22H), 3.16 – 3.29 (m, 3H), 2.95 – 3.10 (m, 3H), 2.75 – 2.89 (m, 2H), 2.54 – 2.63 (m, 1H), 2.18 – 2.47 (m, 4H), 2.02 – 2.12 (m, 1H), 1.85 – 1.98 (m, 4H), 1.58 – 1.75 (m, 5H), 1.11 – 1.43 (m, 5H), 0.85 – 0.96 (m, 2H).

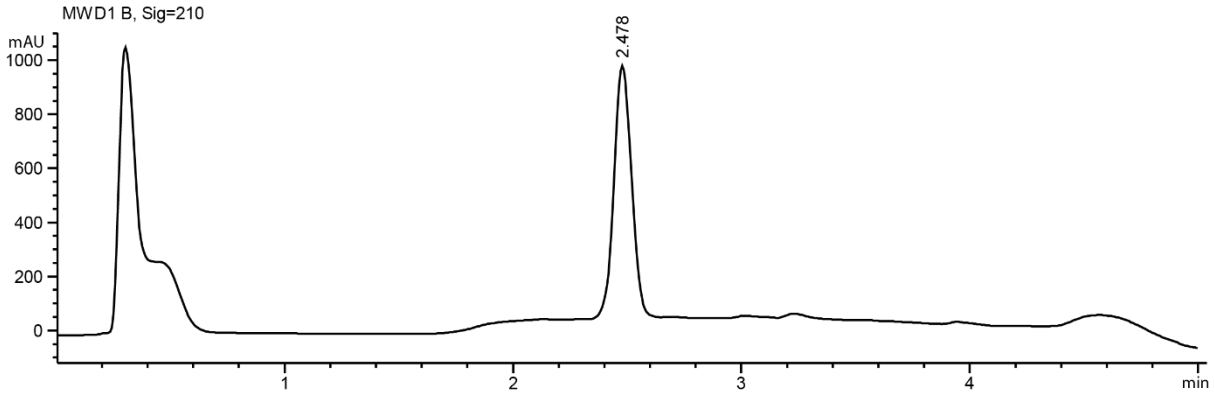


Figure S89. HPLC chromatogram of intermediate **I22** recorded at λ = 210 nm.

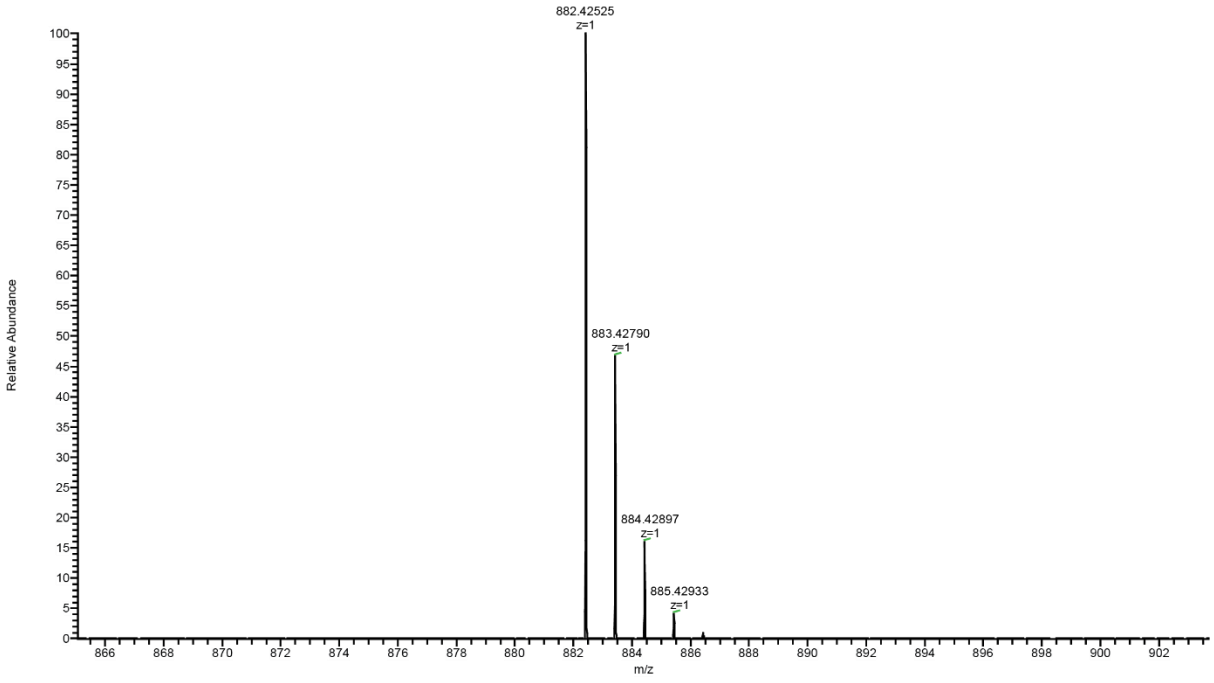
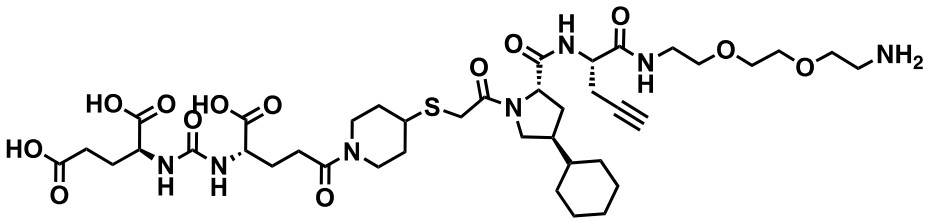


Figure S90. HR-MS of intermediate **I22**.

Synthesis of (((*S*)-4-(4-((2-((2*S*,4*S*)-2-(((*S*)-1-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-1-oxopent-4-yn-2-yl)carbamoyl)-4-cyclohexylpyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-1-carboxy-4-oxobutyl)carbamoyl)-*L*-glutamic acid (I20)



Synthesis of **I20** was performed following the same synthetic procedure reported for **I22**. **I20** was synthesized starting from **I15** and **I19** (8 mg, overall yield = 20%). HR-MS (ESI+) *m/z* calculated $C_{40}H_{63}N_7O_{13}S$ $[M+H]^+$ 882.42773; detected: 882.42700; accuracy = 0.83 ppm. ^1H NMR (400 MHz, DMSO-d_6,): δ (ppm): 12.55 (bs, 2H), 7.94 – 8.01 (m, 1H), 7.75 – 7.76 (m, 2H), 6.34 (t, J = 7.2 Hz, 1H), 4.30 – 4.43 (m, 2H), 4.06 – 4.17 (m, 2H), 3.37 – 3.83 (m, 33 H), 3.15 - 3.29 (m, 3H), 2.93 – 3.10 (m, 4H), 2.73 – 2.84 (m, 2H), 2.53 – 2.63 (m, 1H), 2.18 – 2.47 (m, 4H), 1.83 – 2.04 (m, 5H), 1.60 – 1.75 (m, 6H), 1.11 -1.29 (m, 5H), 0.84 – 0.95 (m, 2H).

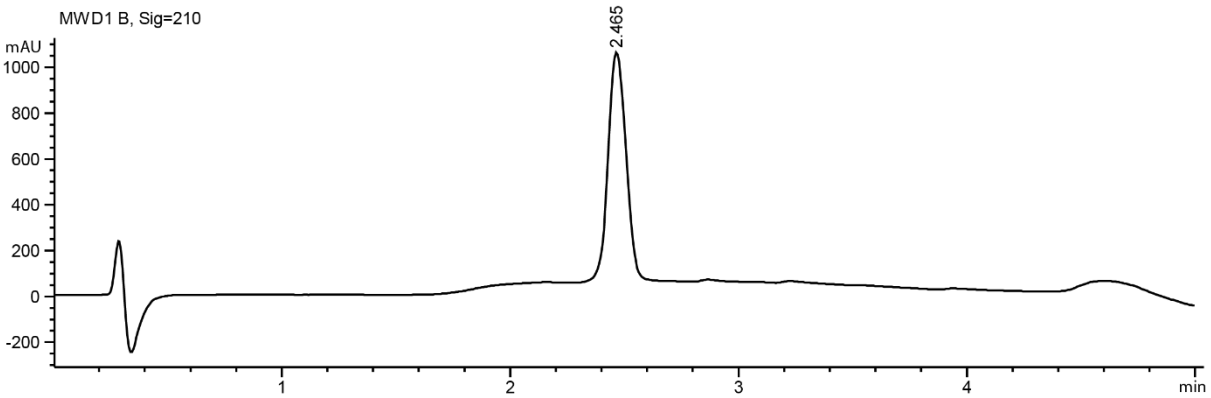


Figure S91. HPLC chromatogram of intermediate **I20** recorded at λ = 210 nm.

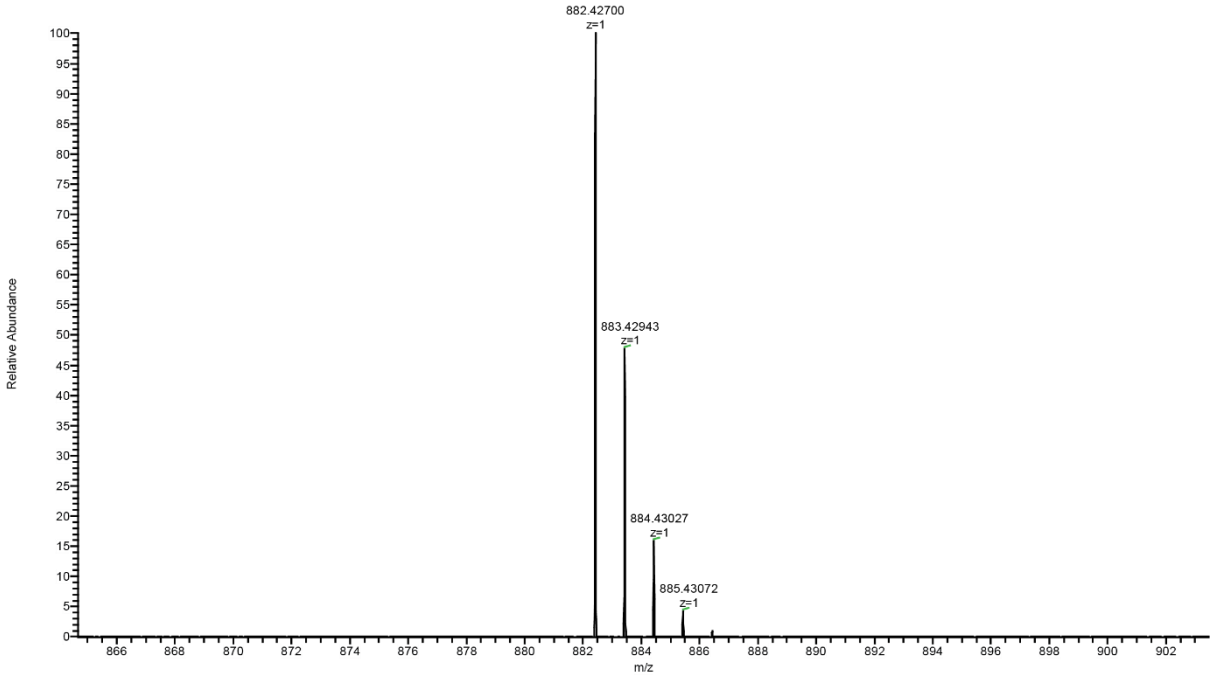
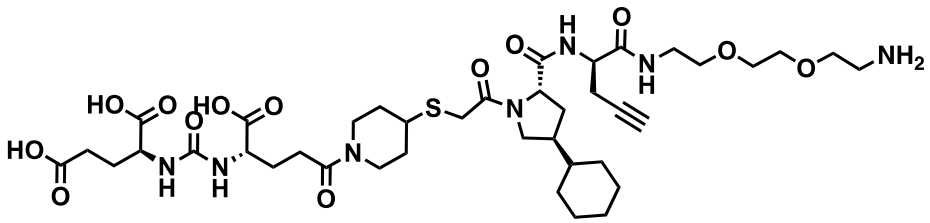


Figure S92. HR-MS of intermediate **I20**.

Synthesis of (((*S*)-4-(4-((2-((2*S*,4*S*)-2-(((*R*)-1-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-1-oxopent-4-yn-2-yl)carbamoyl)-4-cyclohexylpyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-1-carboxy-4-oxobutyl)carbamoyl)-*L*-glutamic acid (I21)



Synthesis of **I21** was performed following the same synthetic procedure reported for **I22**.

I21 was synthesized starting from **I16** and **I19** (7 mg, overall yield = 18%). HR-MS (ESI+) *m/z* calculated $C_{40}H_{63}N_7O_{13}S$ $[M+H]^+$ 882.42773; detected: 882.42600; accuracy = 1.96 ppm

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm): 12.54 (bs, 2H), 8.31-8.43 (m, 1H), 7.74 – 7.90 (m, 2H), 6.31 – 6.35 (m, 1H), 4.30 – 4.42 (m, 2H), 4.06 – 4.15 (m, 2H), 3.40 – 3.84 (m, 33H), 3.16 -3.24 (m, 2H), 2.94 – 3.12 (m, 3H), 2.66 – 2.86 (m, 2H), 2.54 – 2.63 (m, 1H), 2.18 – 2.47 (m,

3H), 2.07 – 2.14 (m, 1H), 1.87 – 1.97 (m, 3H), 1.60 – 1.77 (m, 5H), 1.10 – 1.38 (m, 4H), 0.85 – 0.99 (m, 1H).

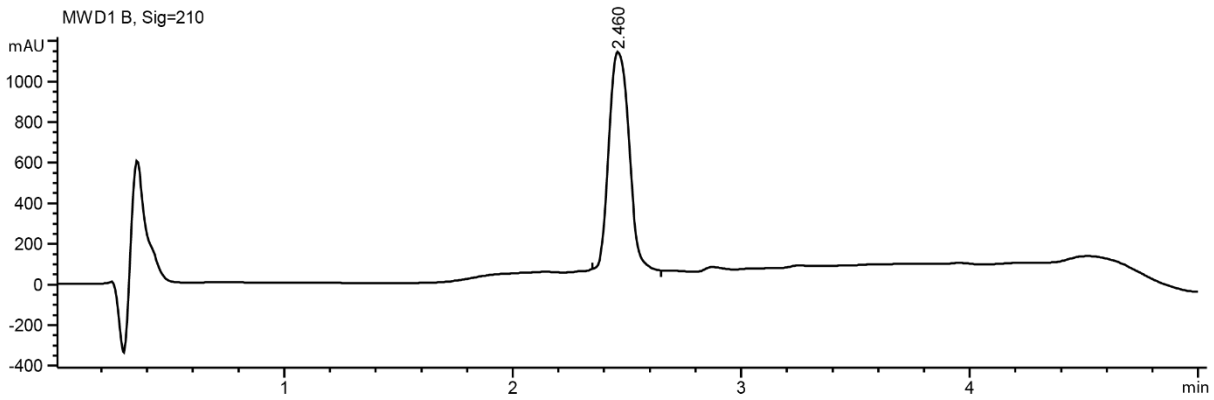


Figure S93. HPLC chromatogram of intermediate **I21** recorded at λ = 210 nm.

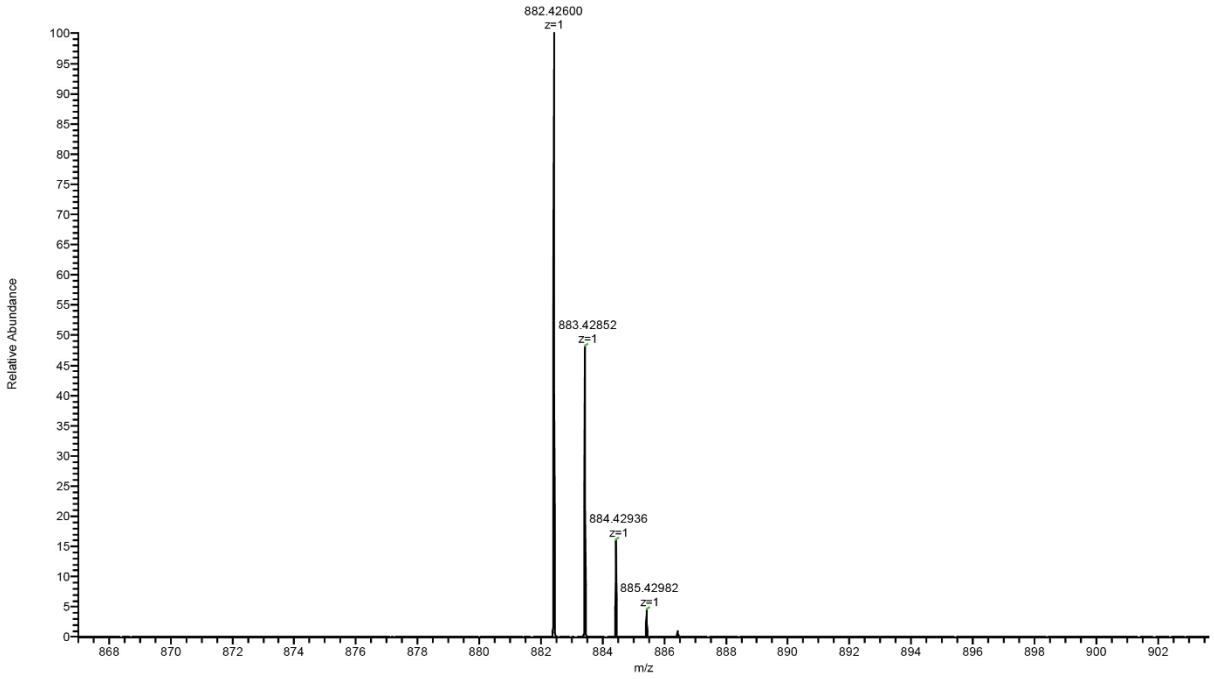
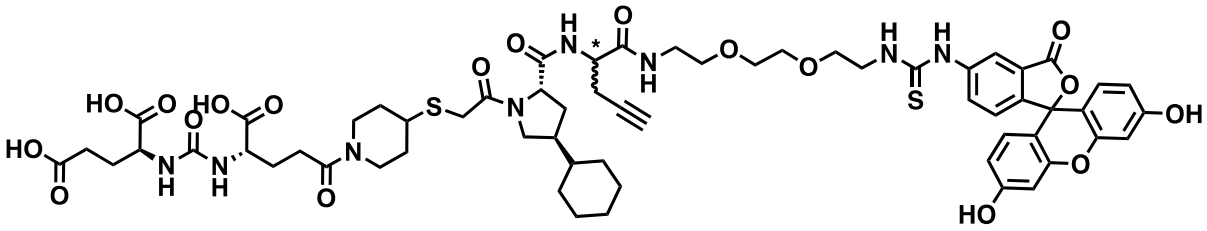


Figure S94. HR-MS of intermediate **I21**.

Synthesis of (((1*S*)-1-carboxy-4-(4-((2-((2*S*,4*S*)-4-cyclohexyl-2-((1-((3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-diazahexadec-15-yn-13-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-4-

oxobutyl)carbamoyl)-*L*-glutamic acid (11)



*** = R+S = 11**

To a solution of compound **I22** (1 mg, 1.13 µmol, 1 eq.) and fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 5.6 µL, 1.13 µmol, 1 eq.) in DMF (50 µL), TEA (1 M in DMF, 4.5µL, 4.52 µmol, 4 eq.) was added. The reaction was stirred for 15 min at room temperature in the dark. The crude was diluted with a mixture of MeCN:H_2O (1:1) and purified by RP-HPLC to obtain the final compound **11** as orange solid (0.8 mg, yield = 59%). HR-MS (ESI+) *m/z* calculated $C_{61}H_{74}N_8O_{18}S_2$ $[M+H]^+$ 1271.46353; detected: 1271.46314; accuracy = 0.31 ppm

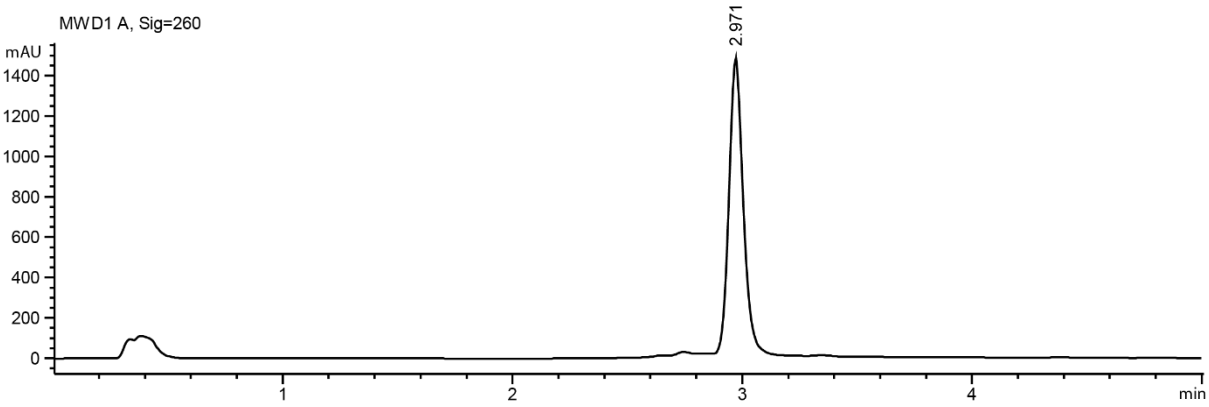


Figure S95. HPLC chromatogram of compound **11** recorded at λ = 260 nm.

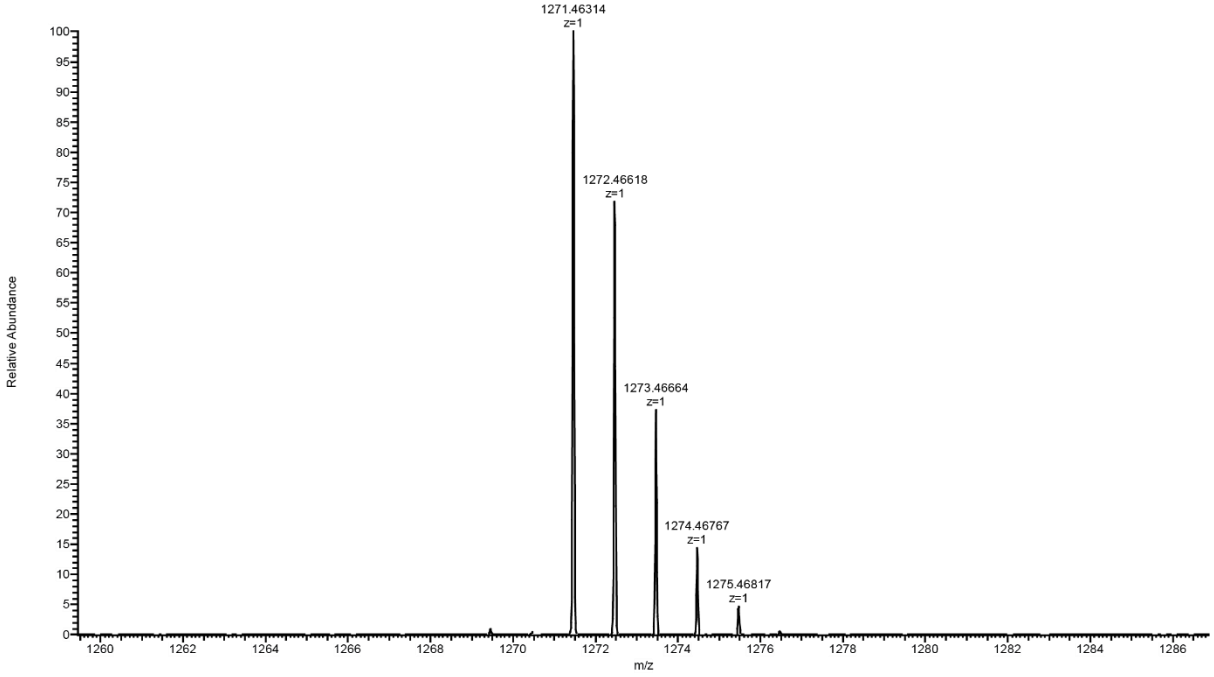
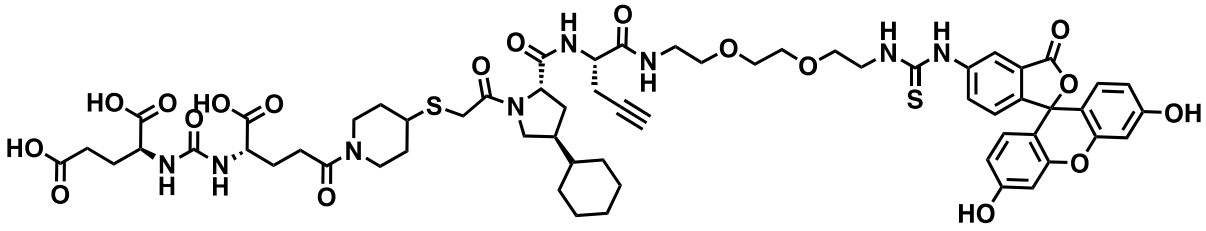


Figure S96. HR-MS of compound **11**.

Synthesis of (((*S*)-1-carboxy-4-(4-((2-((2*S*,4*S*)-4-cyclohexyl-2-(((*S*)-1-((3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-diazahexadec-15-yn-13-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (9)



Synthesis of **9** was performed following the same synthetic procedure reported for compound **11**. Compound **9** was synthesized starting from **I20** (0.5 mg, yield = 35%). HR-MS (ESI+) *m/z* calculated $C_{61}H_{74}N_8O_{18}S_2$ $[M+H]^+$ 1271.46353; detected: 1271.46108; accuracy = 1.93 ppm

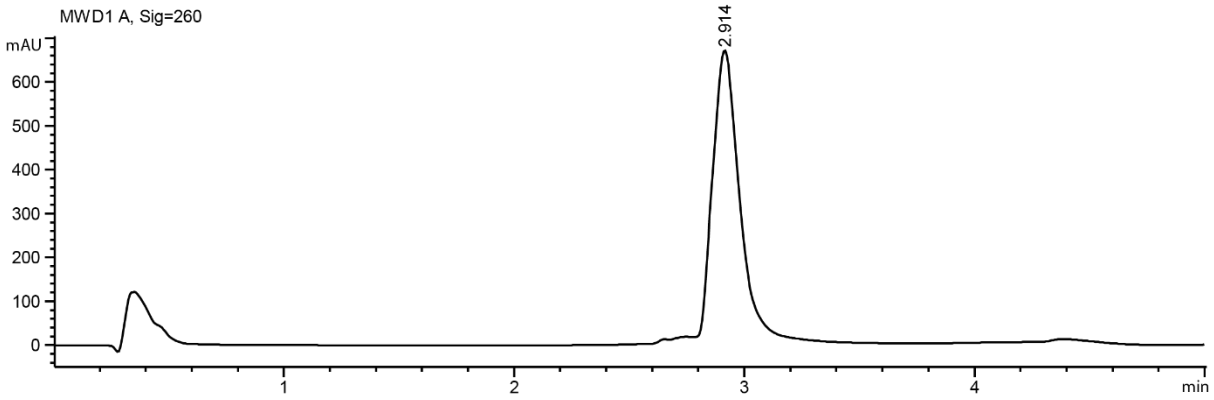


Figure S97. HPLC chromatogram of compound **9** recorded at λ = 260 nm.

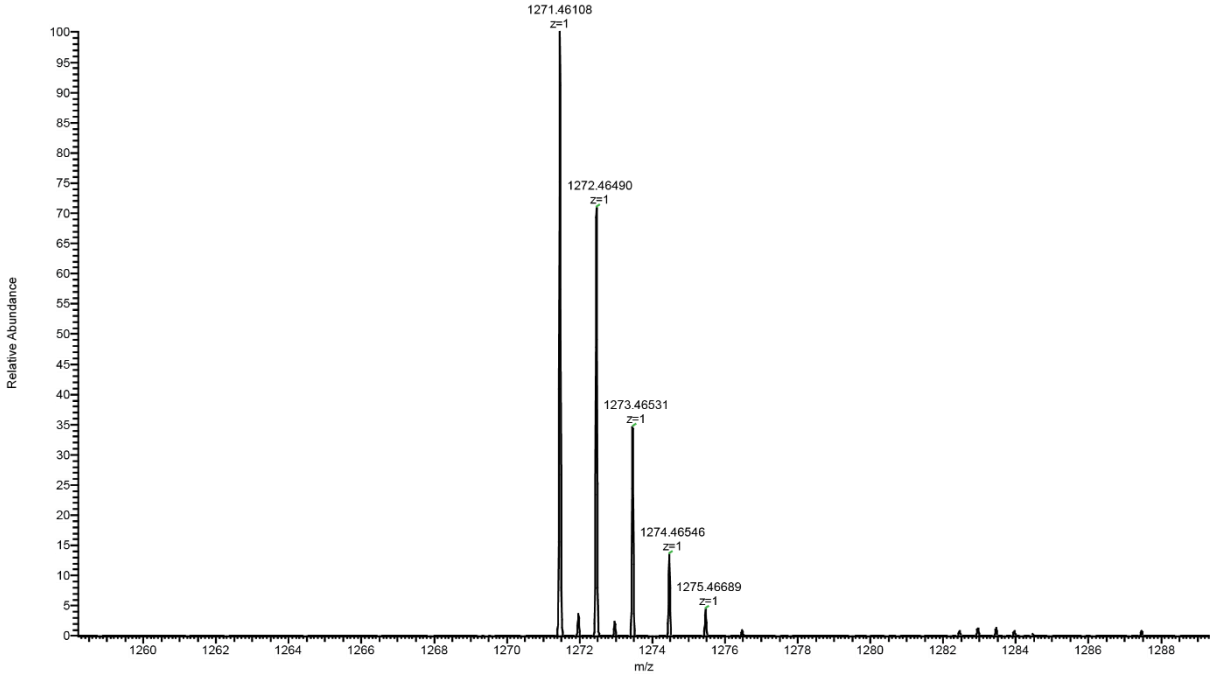
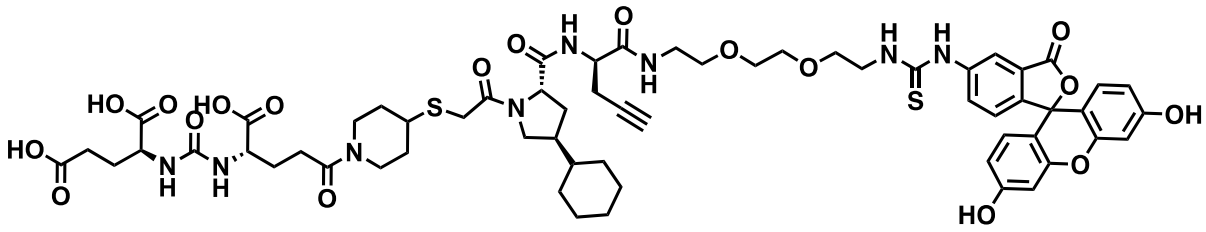


Figure S98. HR-MS of compound **9**.

Synthesis of (((*S*)-1-carboxy-4-(4-((2-((2*S*,4*S*)-4-cyclohexyl-2-(((*R*)-1-((3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-diazahexadec-15-yn-13-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (10)



Synthesis of **10** was performed following the same synthetic procedure reported for compound **11**. Compound **10** was synthesized starting from **I21** (0.6 mg, yield = 35%). HR-MS (ESI+) *m/z* calculated $C_{61}H_{74}N_8O_{18}S_2$ $[M+H]^+$ 1271.46353; detected: 1271.46068; accuracy = 2.24 ppm

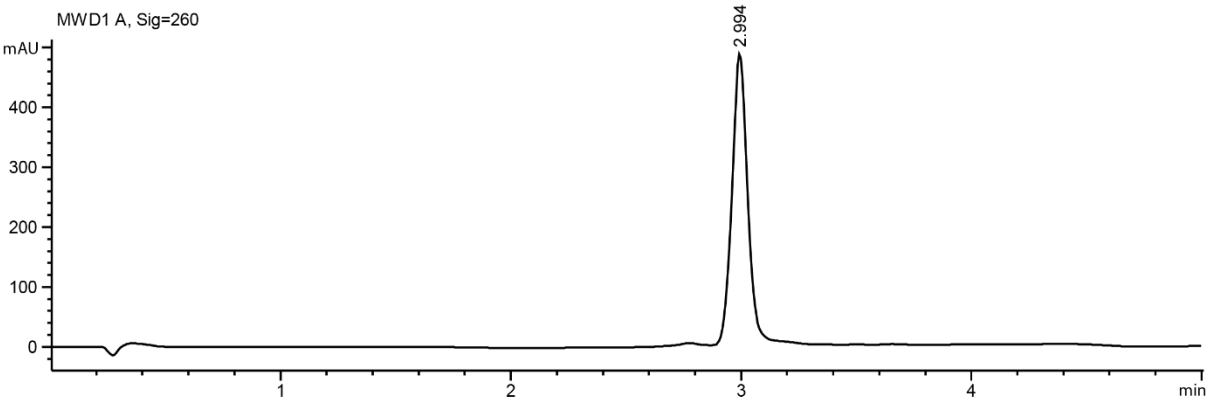


Figure S99. HPLC chromatogram of compound **10** recorded at λ = 260 nm.

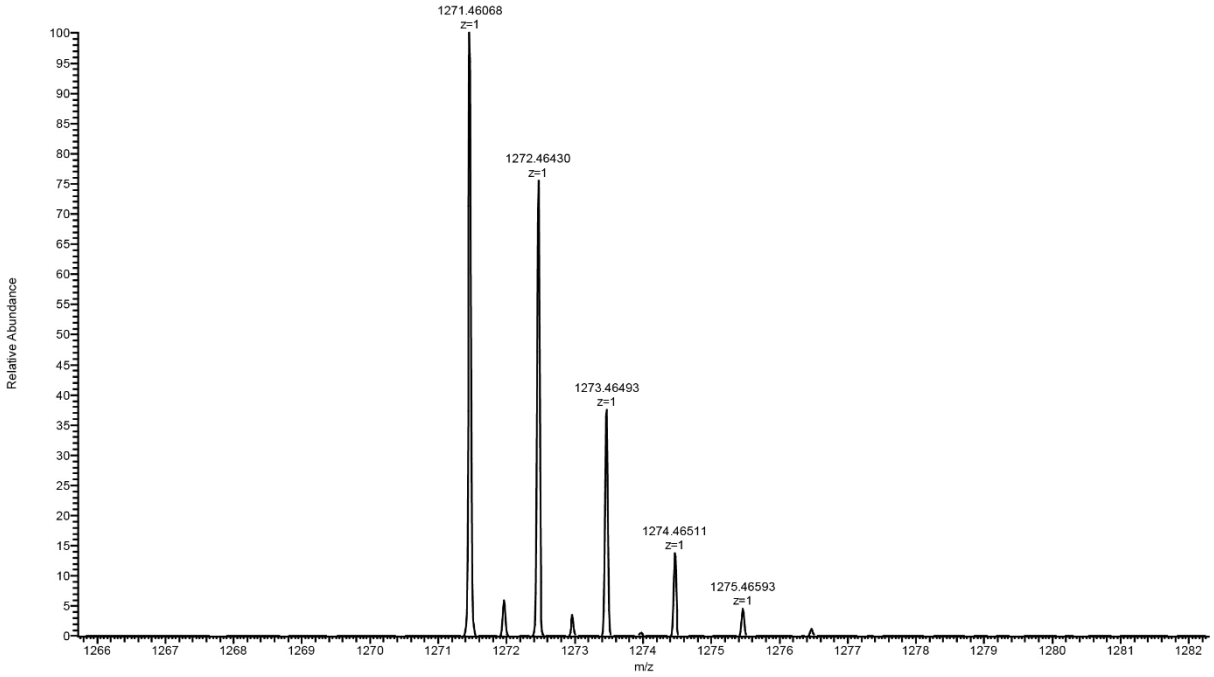
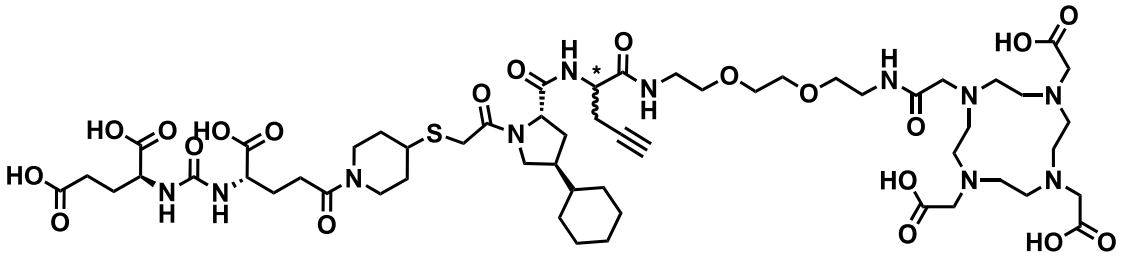


Figure S100. HR-MS of compound **10**.

Synthesis of (((1*S*)-1-carboxy-4-(4-((2-((2*S*,4*S*)-4-cyclohexyl-2-((2,13-dioxo-1-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-6,9-dioxa-3,12-diazaheptadec-16-yn-14-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (22)



Compound **I22** (10 mg, 0.011 mmol, 1 eq.) and DIPEA (8 µL, 0.044 mmol, 4 eq.) were dissolved in anhydrous DMF (200 µL) and 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester (DOTA-NHS ester, 10 mg, 0.013 mmol, 1.2 eq.) was added to the solution. The reaction was stirred at room temperature and after 30 min, the solution was diluted with a mixture of MeCN:H_2O (1:1) and the crude was purified

by RP-HPLC to get the final compound **22** as waxy solid (6 mg, yield = 43%). HR-MS (ESI+) *m/z* calculated $C_{56}H_{89}N_{11}O_{20}S$ $[M+2H]^{2+}$ 634.80758; detected: 634.80788; accuracy = 0.47 ppm. ^{1}H NMR (400 MHz, DMSO-d_6, diastereomeric mixture): δ (ppm): 12.67 (bs, 3H), 8.54 (s, 1H), 8.29 – 8.41 (m, 1H), 7.93 – 8.09 (m, 1H), 7.76 – 7.91 (m, 1H), 6.34 (t, *J* = 7.0 Hz, 1H), 4.30 – 4.42 (m, 2H), 4.06 – 4.16 (m, 4H), 3.07 – 3.89 (m, 56H), 2.73 – 2.89 (m, 2H), 2.56 – 2.62 (m, 1H), 2.18 – 2.47 (m, 4H), 2.02 – 2.14 (m, 1H), 1.88 – 1.98 (m, 4H), 1.59 – 1.76 (m, 6H), 1.10 – 1.41 (m, 6H), 0.83 – 0.99 (m, 2H).

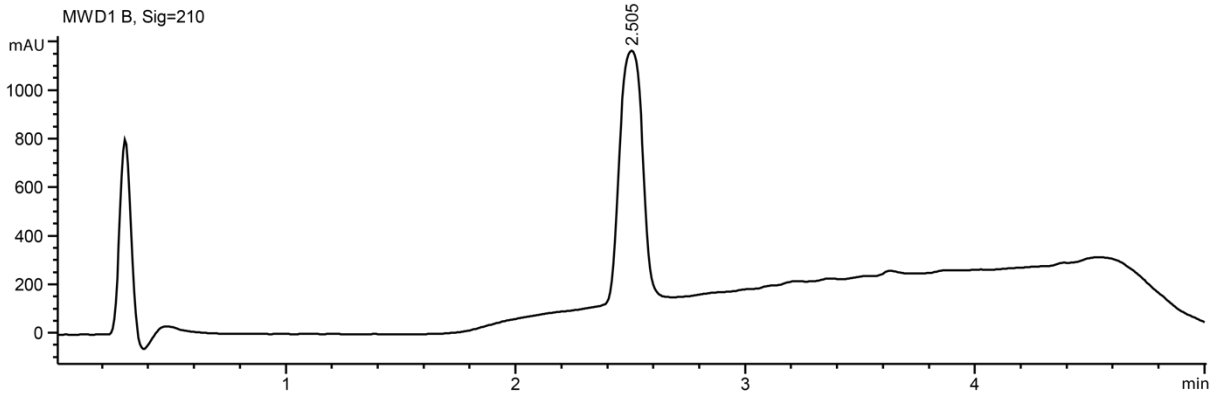


Figure S101. HPLC chromatogram of compound **22** recorded at λ = 210 nm.

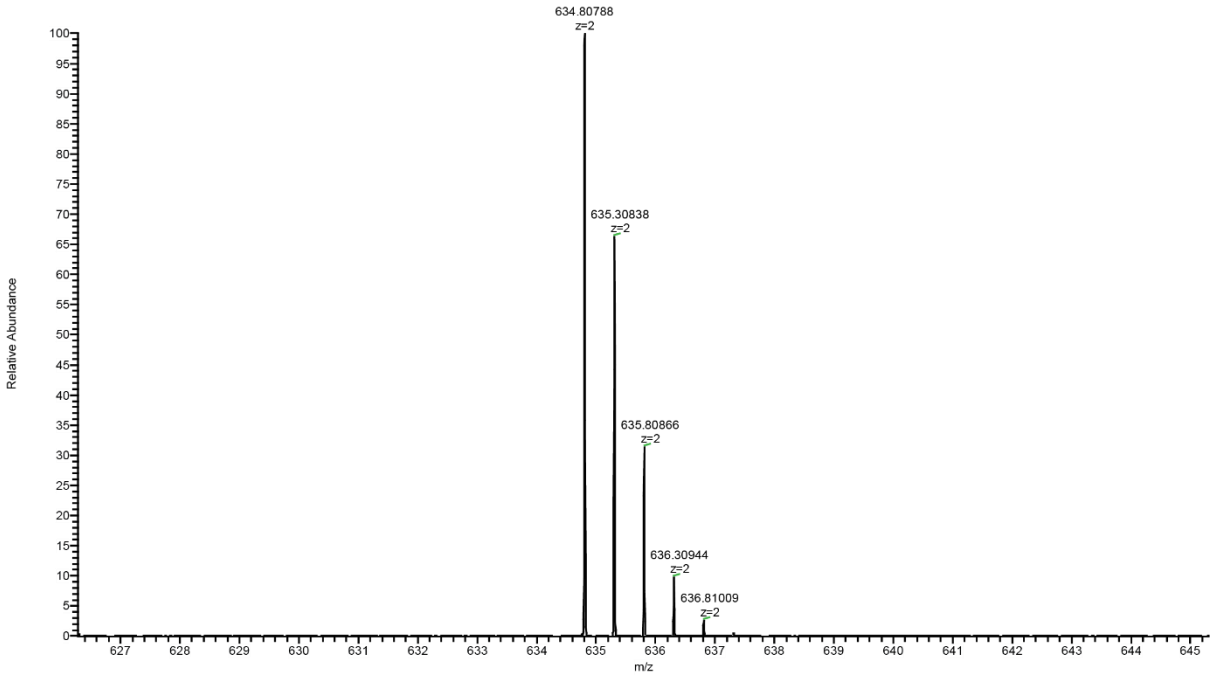
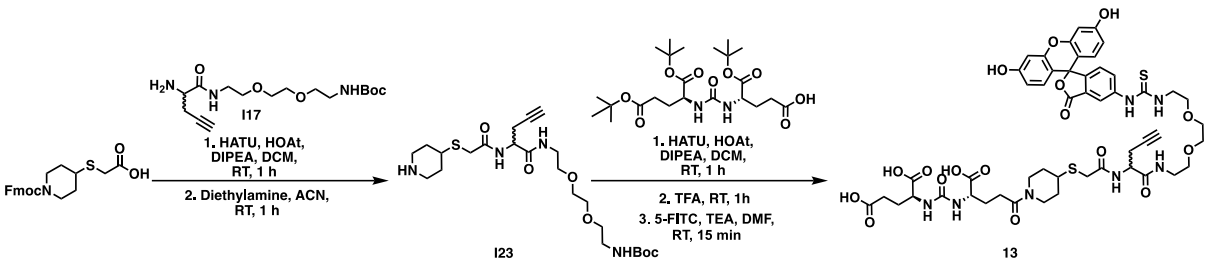


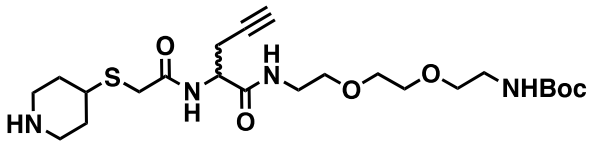
Figure S102. HR-MS of compound **22**.

6.2.10 Synthesis scheme for compound 13



Scheme S18. Schematic illustration of synthesis of compound **13**.

Synthesis of *tert*-butyl (2,5-dioxo-1-(piperidin-4-ylthio)-4-(prop-2-yn-1-yl)-9,12-dioxa-3,6-diazatetradecan-14-yl)carbamate (I23)



Commercially available 2-((1-(((9H-fluoren-9-yl)methoxy)carbonyl)piperidin-4-yl)thio)acetic acid (13 mg, 0.032 mmol, 1.1 eq.) was dissolved in DCM (1 mL) and HATU (11 mg, 0.029 mmol, 1 eq.), HOAt (2 mg, 0.015 mmol, 0.5 eq.) and DIPEA (20 µL, 0.116 mmol, 4 eq.) were added. After 5 min at room temperature, compound **I17** (10 mg, 0.029 mmol, 1 eq.) was added. The reaction was stirred at room temperature for 1 h followed by solvent evaporation under reduced pressure. The crude was re-dissolved in ACN (1 mL) and diethylamine (15 µL, 0.145 mmol, 5 eq.) was added to perform Fmoc deprotection as reported in the general procedures for solution synthesis (6.2.2). The crude was purified by RP-HPLC to obtain compound **I23** as a waxy solid (6 mg, yield = 41%). HR-MS (ESI+) *m/z* calculated $C_{23}H_{40}N_4O_6S$ $[M+H]^+$ 501.27413; detected: 501.27370; accuracy = 0.86 ppm

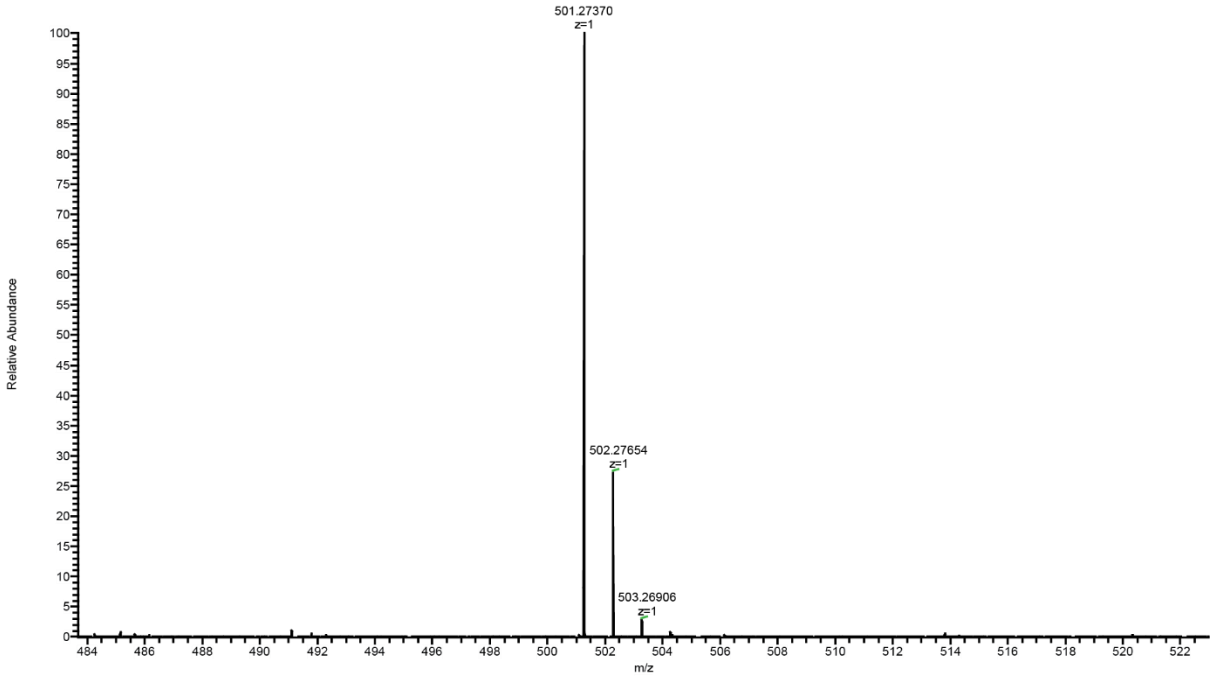
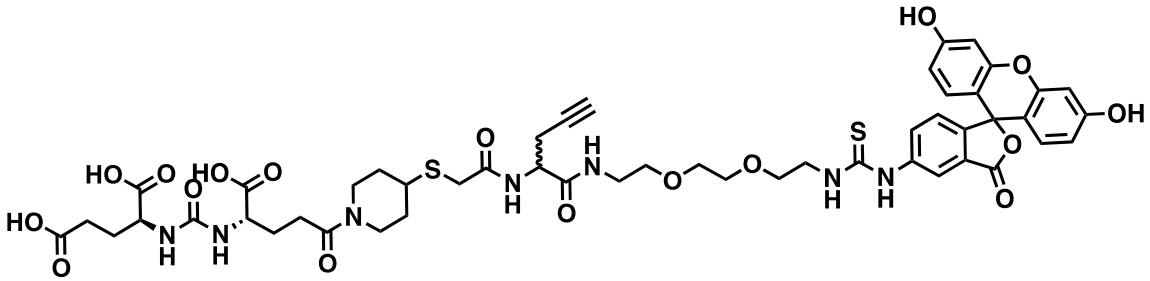


Figure S103. HR-MS of intermediate **I23**.

Synthesis of (((1*S*)-1-carboxy-4-(4-((2-((1-((3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-diazahexadec-15-yn-13-yl)amino)-2-oxoethyl)thio)piperidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (13)



To a solution of (S)-5-(tert-butoxy)-4-(3-((S)-1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoic acid (5.4 mg, 0.011 mmol, 1.1 eq.) in DCM (500 µL), HATU (4 mg, 0.010 mmol, 1 eq.), HOAt (0.7 mg, 0.005 mmol, 0.5 eq.) and DIPEA (7 µL, 0.04 mmol, 4 eq.) were added. After 5 min, compound **I23** (5 mg, 0.010 mmol, 1 eq.) was added to the mixture. The reaction was stirred for 1 h at room temperature followed by solvent evaporation under reduced

pressure. Neat TFA (1 mL) was added to the crude and deprotection was stirred for 1 hour at room temperature. Afterwards, TFA was removed under reduced pressure and the crude re-dissolved in DMF. TEA (14 µL, 0.100 mmol, 10 eq.) and fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 50 µL, 0.010 mmol, 1 eq.) were added to the solution. The reaction was stirred for 15 min at rt and the product was purified by RP-HPLC to obtain compound **13** as an orange solid (0.9 mg, yield = 8%). HR-MS (ESI+) *m/z* calculated $C_{50}H_{57}N_7O_{17}S_2$ $[M+H]^+$ 1092.33251; detected: 1092.33043; accuracy = 1.90 ppm

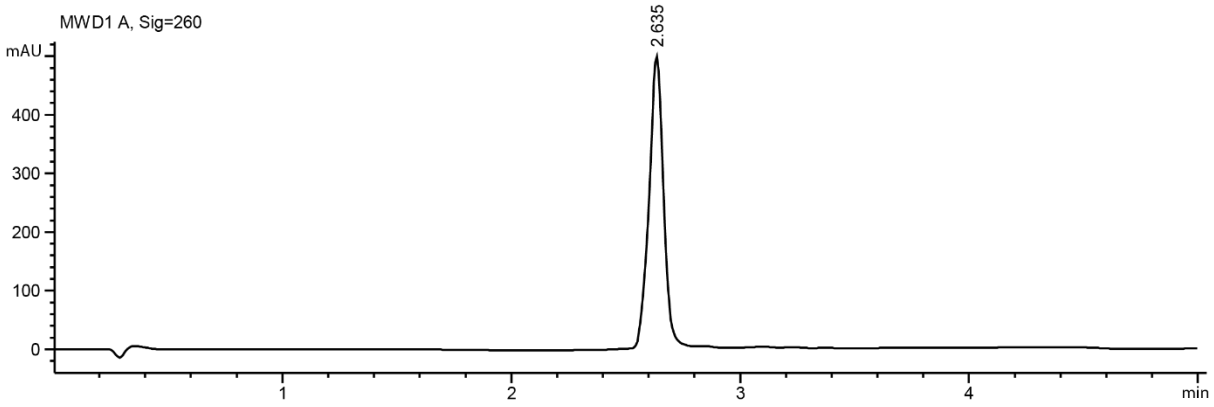


Figure S104. HPLC chromatogram of compound **13** recorded at λ = 260 nm.

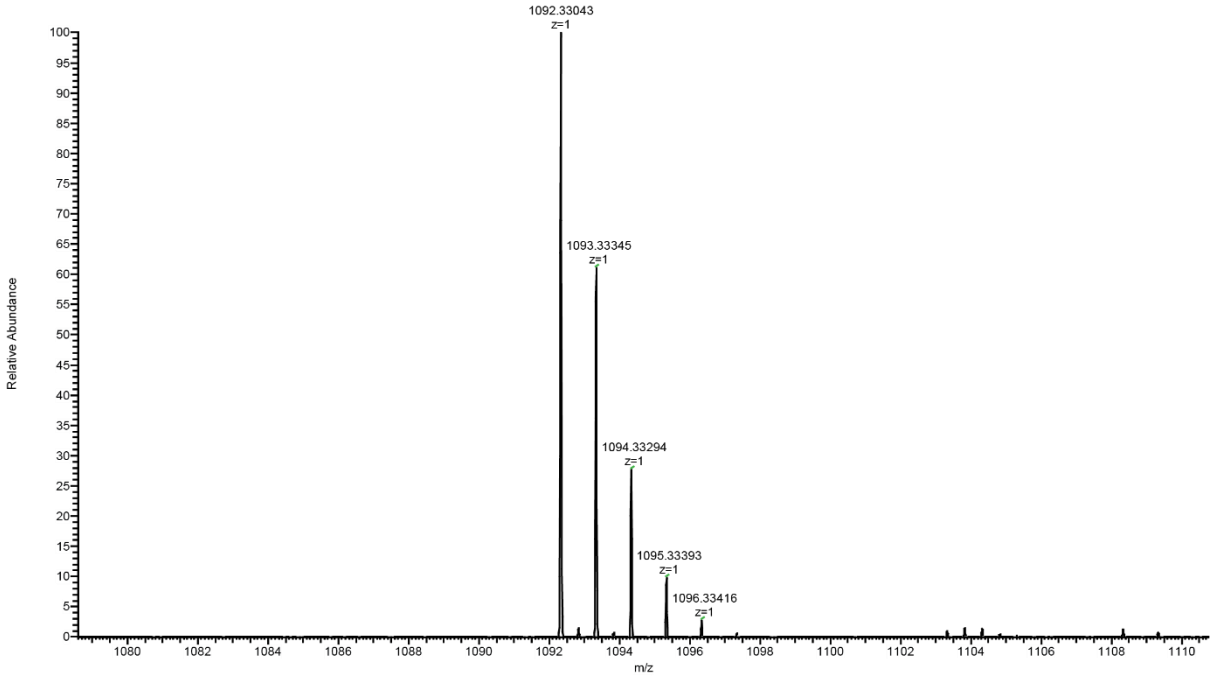
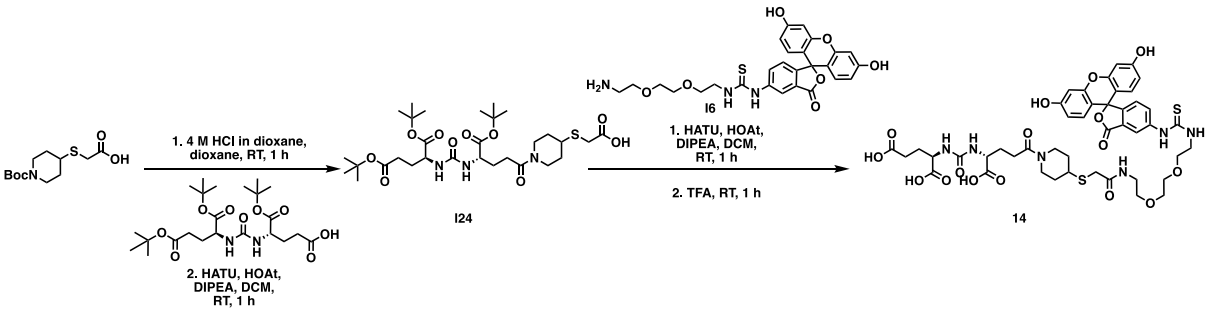


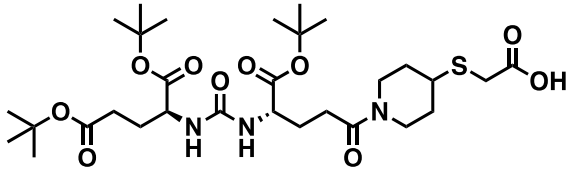
Figure S105. HR-MS of compound **13**.

6.2.11 Synthesis scheme for compound 14



Scheme S19. Schematic illustration of synthesis of compound **14**.

Synthesis of 2-((1-((*S*)-5-(*tert*-butoxy)-4-(3-((*S*)-1,5-di-*tert*-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoyl)piperidin-4-yl)thio)acetic acid (I24)



2-((1-(tert-butoxycarbonyl)piperidin-4-yl)thio)acetic acid (10 mg, 0.036 mmol, 1 eq.) was dissolved in dioxane (500 µL) and 4M HCl in dioxane (36 µL, 0.144 mmol, 4 eq.) was added at 0°C and the reaction was warmed up to room temperature and stirred for 1h. The crude was dried under reduced pressure and was used for the next step. Compound (S)-5-(tert-butoxy)-4-(3-((S)-1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoic acid (15 mg, 0.040 mmol, 1.1 eq.) was dissolved in DCM (500 µL), and HATU (13.7 mg, 0.036 mmol, 1 eq.), HOAt (2.5 mg, 0.018 mmol, 0.5 eq.) and DIPEA (44 µL, 0.252 mmol, 7 eq.) were added. The acid was activated for 5 min at room temperature and added to the previously Boc-deprotected compound. The reaction was stirred at room temperature for 1 h followed by solvent evaporation under reduced pressure. The crude was purified by RP-HPLC and compound **I24** was obtained as a white waxy solid (5 mg, overall yield = 20%). HR-MS (ESI+) *m/z* calculated $C_{30}H_{51}N_3O_{10}S$ $[M+H]^+$ 646.33679; detected: 646.33559; accuracy = 1.86 ppm

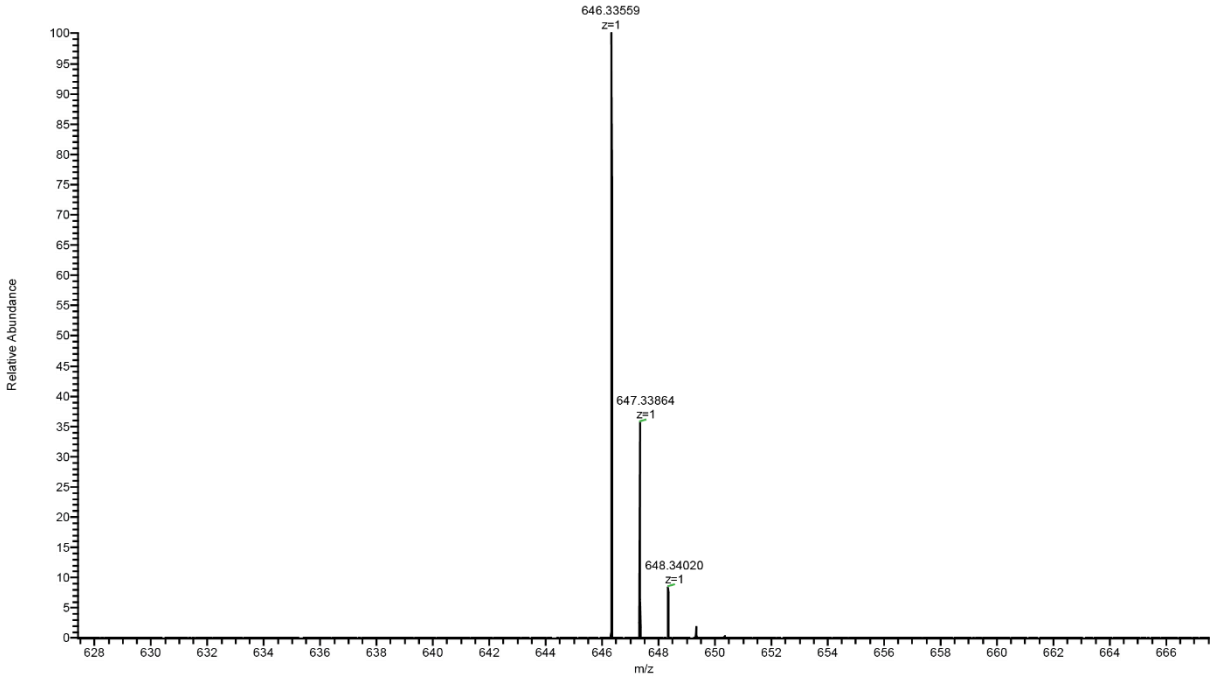
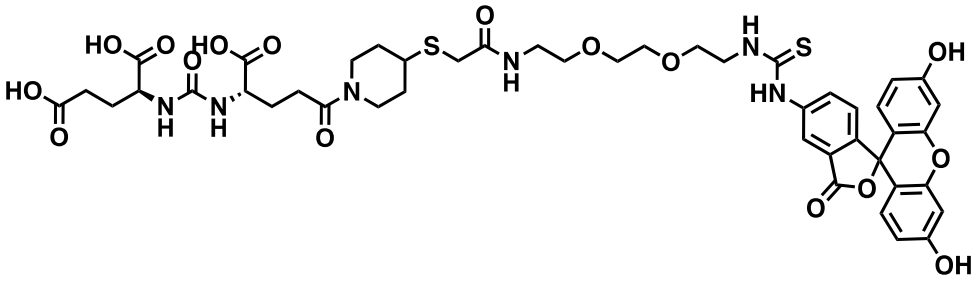


Figure S106. HR-MS of intermediate **I24**.

Synthesis of (((*R*)-1-carboxy-4-(4-((1-((3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-diazatridecan-13-yl)thio)piperidin-1-yl)-4-oxobutyl)carbamoyl)-*D*-glutamic acid (14)



To a solution of compound **I24** (5 mg, 0.008 mmol, 1 eq.) in DCM (500 µL), HATU (3 mg, 0.008 mmol, 1 eq.), HOAt (0.5 mg, 0.004 mmol, 0.5 eq.) and DIPEA (6 µL, 0.036 mmol, 4 eq.) were added. After 5 min, compound **I6** (4.3 mg, 0.008 mmol, 1 eq.) was added to the mixture. The reaction was stirred for 1 h at room temperature followed by solvent evaporation under reduced pressure. Neat TFA (1 mL) was added to the crude and deprotection was stirred for 1 hour at room temperature. Subsequently, solvent and TFA were removed under reduced pressure, the crude was re-dissolved in DMF and purified by RP-HPLC to obtain compound **14** as an orange solid (0.5 mg, yield = 6%). HR-MS (ESI+) *m/z* calculated $C_{45}H_{52}N_6O_{16}S_2$ $[M+H]^+$ 997.29540; detected 997.29290; accuracy = 2.51 ppm

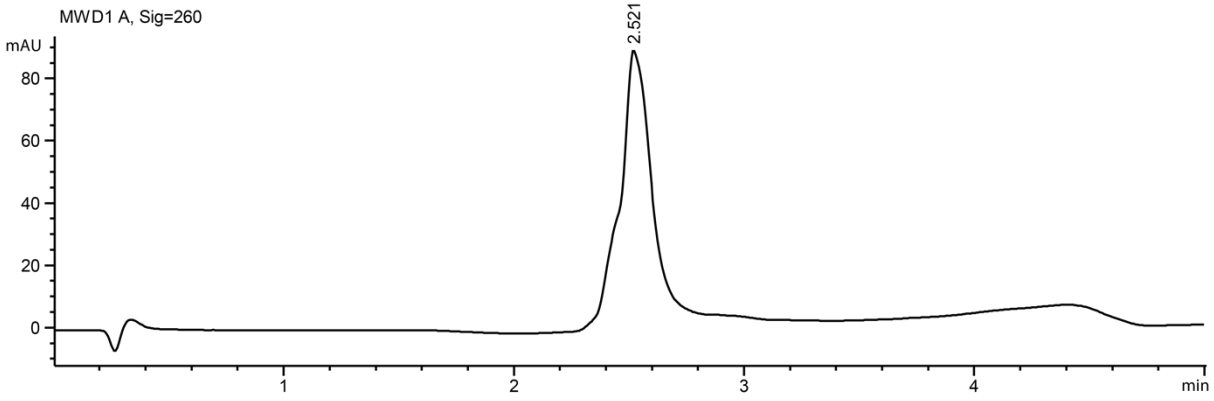


Figure S107. HPLC chromatogram of compound **14** recorded at λ = 260 nm.

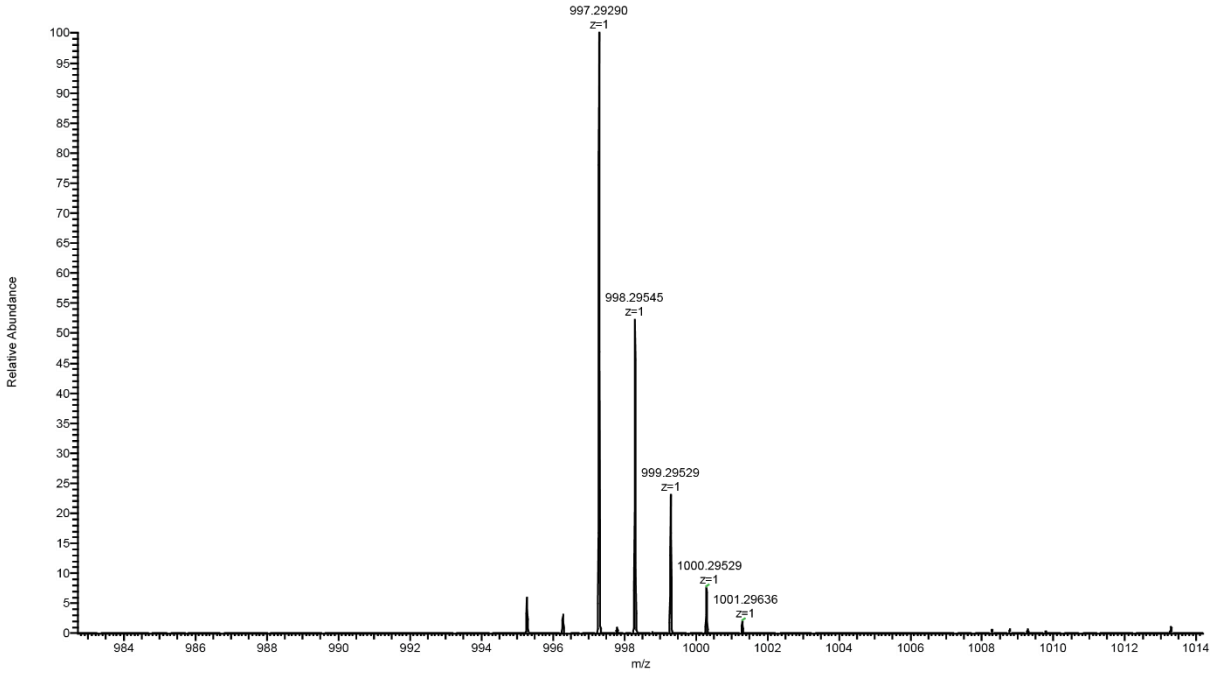
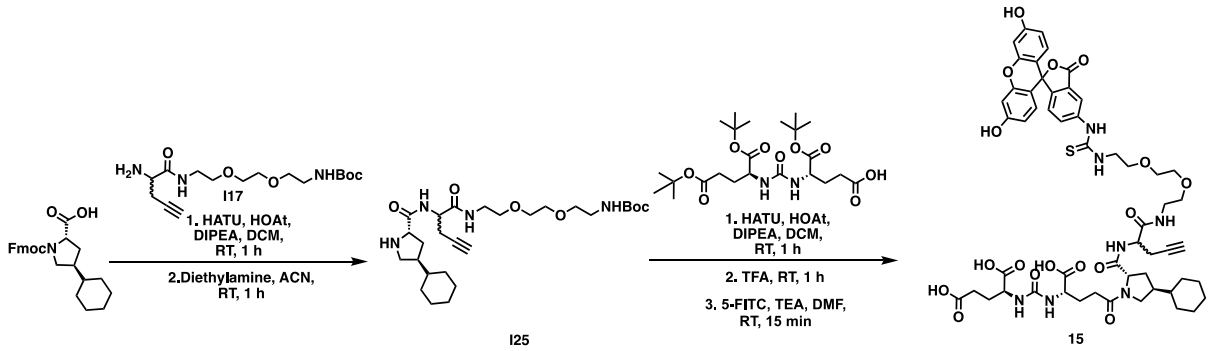


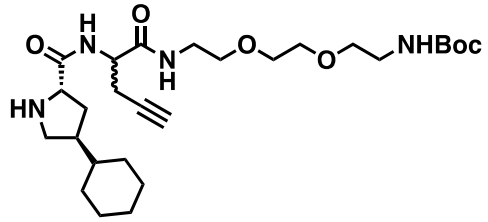
Figure S108. HR-MS of compound **14**.

6.2.12 Synthesis scheme for compound 15



Scheme S20. Schematic illustration of synthesis of compound **15**.

Synthesis of *tert*-butyl (1-((2*S*,4*S*)-4-cyclohexylpyrrolidin-2-yl)-1,4-dioxo-3-(prop-2-yn-1-yl)-8,11-dioxa-2,5-diazatridecan-13-yl)carbamate (I25)



Commercially available (2*S*,4*S*)-1-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-4-cyclohexylpyrrolidine-2-carboxylic acid (15 mg, 0.036 mmol, 1.1 eq.) was dissolved in DCM (500 µL) and HATU (12.5 mg, 0.033 mmol, 1 eq.), HOAt (2 mg, 0.017 mmol, 0.5 eq.) and DIPEA (23 µL, 0.132 mmol, 4 eq.) were added. After 5 min at room temperature, compound **I17** (11.3 mg, 0.033 mmol, 1 eq.) was added. The reaction was stirred at room temperature for 1 h followed by solvent evaporation under reduced pressure. The crude was re-dissolved in ACN (500 µL) and diethylamine (17 µL, 0.165 mmol, 5 eq.) was added to perform Fmoc deprotection as reported in the general procedures (6.2.2). The crude was purified by RP-HPLC to obtain compound **I25** as a waxy solid (5 mg, yield = 31%). HR-MS (ESI+) *m/z* calculated $C_{27}H_{46}N_4O_6$ $[M+H]^+$ 523.34901; detected 523.34765; accuracy = 2.6 ppm

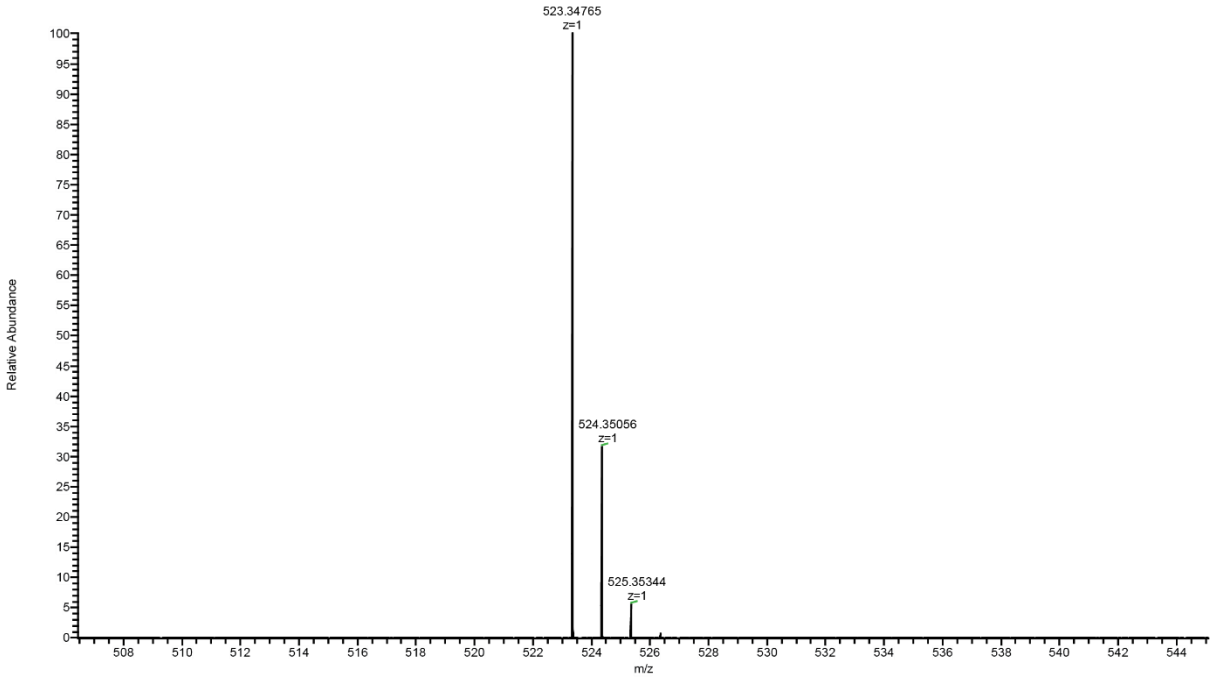
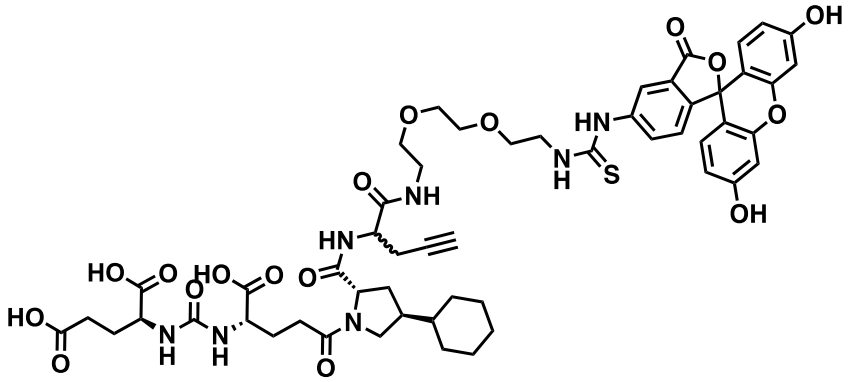


Figure S109. HR-MS of intermediate **I25**.

Synthesis of (((1*S*)-1-carboxy-4-((2*S*,4*S*)-4-cyclohexyl-2-((1-((3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-12-oxo-1-thioxo-5,8-dioxa-2,11-

diazahexadec-15-yn-13-yl)carbamoyl)pyrrolidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (15)



To a solution of (S)-5-(tert-butoxy)-4-(3-((S)-1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoic acid (5 mg, 0.010 mmol, 1 eq.) in DCM (0.5 mL), HATU (4 mg, 0.010 mmol, 1 eq.), HOAt (0.7 mg, 0.005 mmol, 0.5 eq.) and DIPEA (7 µL, 0.04 mmol, 4 eq.) were added. After 5 min at room temperature, compound **I25** (5 mg, 0.010 mmol, 1 eq.) was added to the mixture. The reaction was stirred at room temperature for 1 h followed by solvent evaporation under reduced pressure. Neat TFA (1 mL) was added to the crude and deprotection was stirred for 1 hour at room temperature. Subsequently TFA was removed under reduced pressure and the crude was dissolved in DMF, TEA was added to the solution (14 µL, 0.10 mmol, 10 eq.) followed by fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 50 µL, 0.010 mmol, 1 eq.). The reaction was stirred for 15 min and the compound was directly purified by RP-HPLC to obtain compound **15** as an orange powder (0.9 mg, overall yield = 8%). HR-MS (ESI+) *m/z* calculated $C_{54}H_{63}N_7O_{17}S$ $[M+H]^+$ 1114.40739 ; detected 1114.40626; accuracy = 1.01 ppm

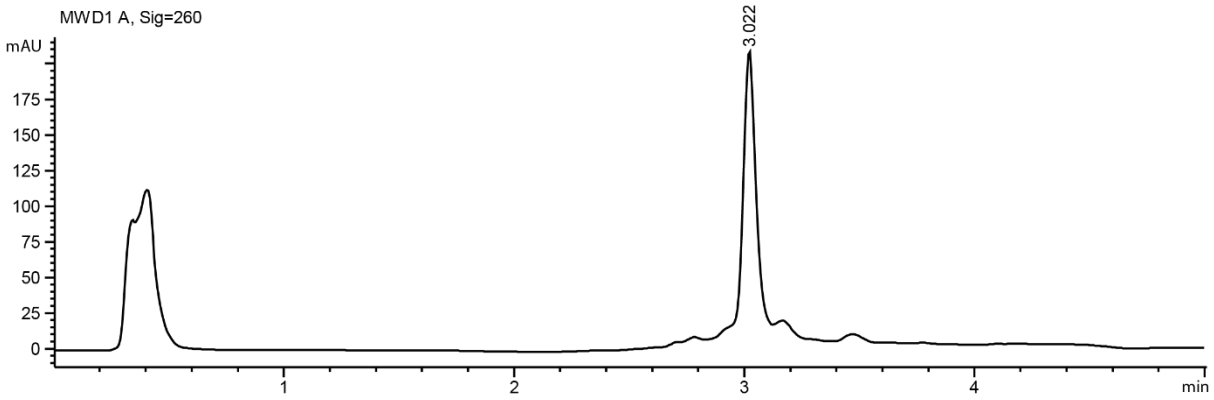


Figure S110. HPLC chromatogram of compound **15** recorded at λ = 260 nm.

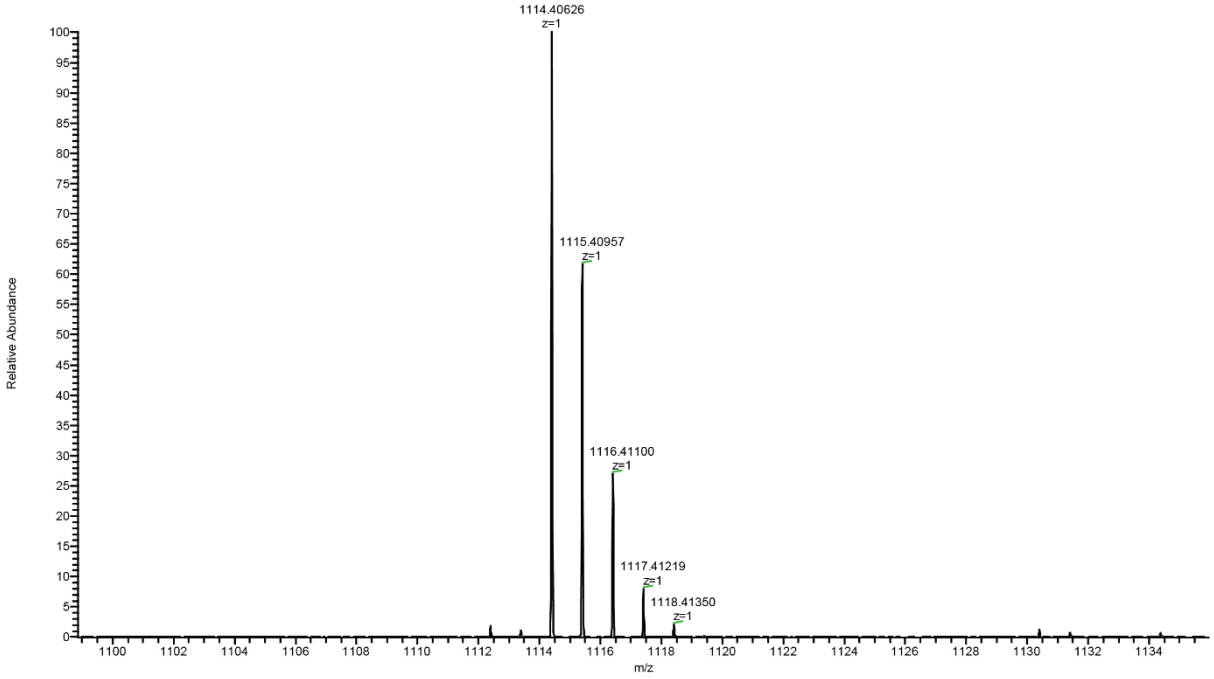
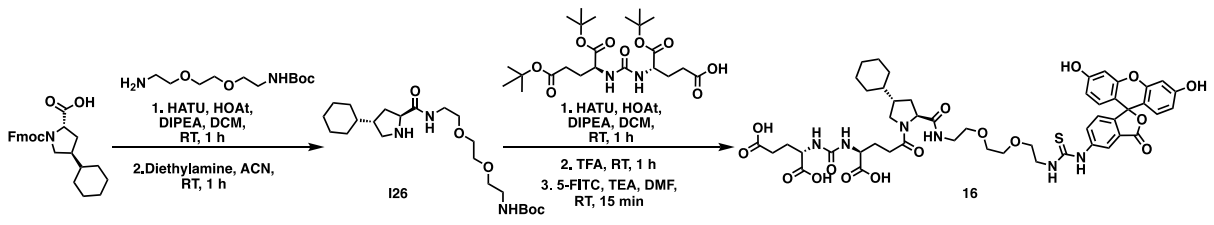


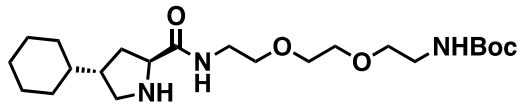
Figure S111. HR-MS of compound **15**.

6.2.13 Synthesis scheme for compound 16



Scheme S21. Schematic illustration of synthesis of compound **16**.

Synthesis of *tert*-butyl (2-(2-(2-((2*S*,4*S*)-4-cyclohexylpyrrolidine-2-carboxamido)ethoxy)ethoxy)ethyl)carbamate (I26)



Commercially available (2*S*,4*S*)-1-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-4-cyclohexylpyrrolidine-2-carboxylic acid (18.5 mg, 0.044 mmol, 1.1 eq.) was dissolved in DCM (500 µL) and HATU (15 mg, 0.040 mmol, 1 eq.), HOAt (3 mg, 0.02 mmol, 0.5 eq.) and DIPEA (28 µL, 0.160 mmol, 4 eq.) were added. After 5 min at room temperature, compound tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (10 mg, 0.040 mmol, 1 eq.) was added. The reaction was stirred at room temperature for 1 h followed by solvent evaporation under reduced pressure. The crude was re-dissolved in ACN (500 µL) and diethylamine (21 µL, 0.200 mmol, 5 eq.) was added to perform Fmoc deprotection as reported in the general procedures (6.2.2). The crude was purified by RP-HPLC to obtain compound **I26** as a waxy solid (5.5 mg, overall yield = 33%). HR-MS (ESI+) *m/z* calculated $C_{22}H_{41}N_3O_5$ $[M+H]^+$ 428.31190 ; detected 428.31068; accuracy = 2.85 ppm

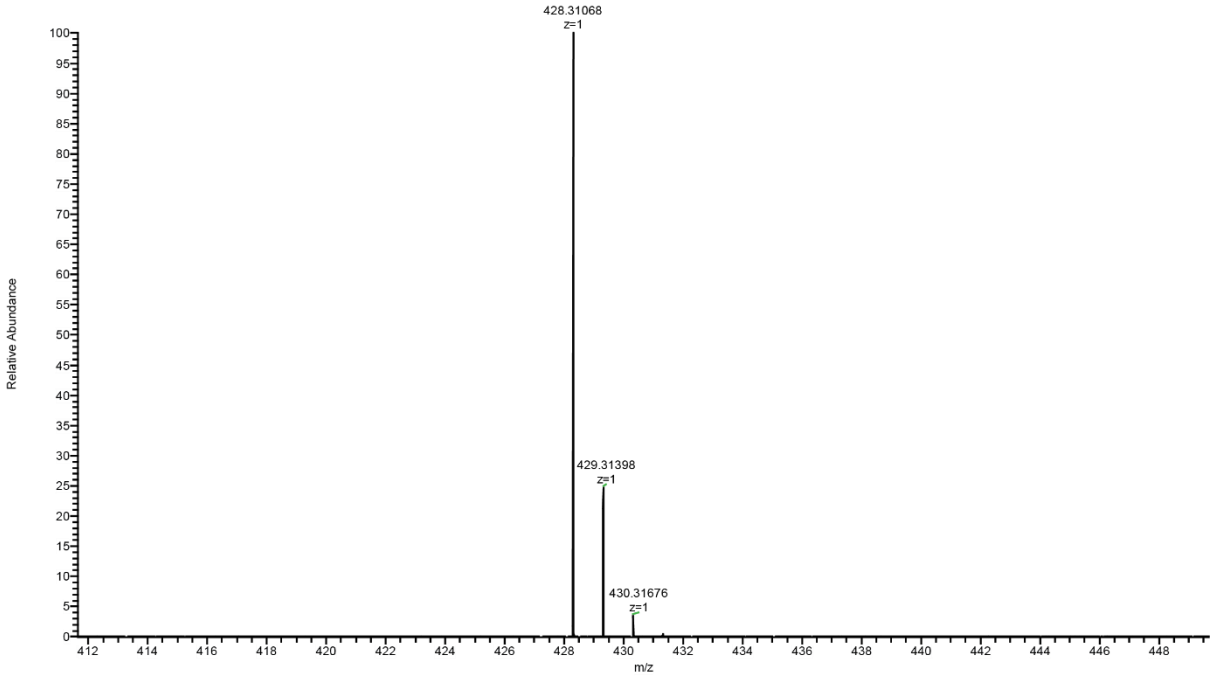
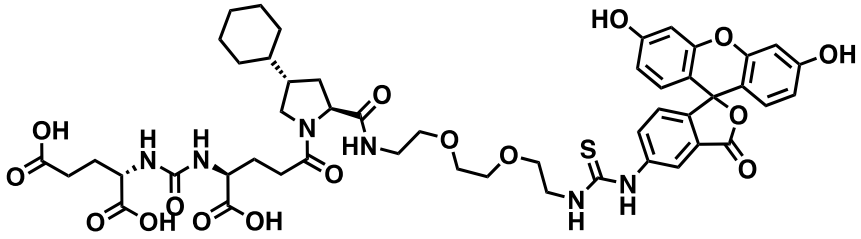


Figure S112. HR-MS of intermediate **I26**.

Synthesis of (((*S*)-1-carboxy-4-((2*S*,4*S*)-4-cyclohexyl-2-((2-(2-(2-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)ethoxy)ethoxy)ethyl)carbamoyl)pyrrolidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (16)



To a solution of (S)-5-(tert-butoxy)-4-(3-((S)-1,5-di-tert-butoxy-1,5-dioxopentan-2-yl)ureido)-5-oxopentanoic acid (6 mg, 0.012 mmol, 1 eq.) in DCM (0.5 mL), HATU (4.6 mg, 0.012 mmol, 1 eq.), HOAt (0.8 mg, 0.006 mmol, 0.5 eq.) and DIPEA (8 µL, 0.048 mmol, 4 eq.) were added. After 5 min at room temperature, compound **I26** (5 mg, 0.012 mmol, 1 eq.) was added to the mixture. The reaction was stirred at room temperature for 1 h followed by solvent evaporation under reduced pressure. Neat TFA (1 mL) was added to the crude and deprotection was stirred

for 1 hour at room temperature. Subsequently TFA was removed under reduced pressure and the crude was dissolved in DMF, TEA was added to the solution (17 µL, 0.120 mmol, 10 eq.) followed by fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 60 µL, 0.012 mmol, 1 eq.). The reaction was stirred for 15 min and purified by RP-HPLC to obtain compound **16** as an orange powder (1.1 mg, overall yield = 9%). HR-MS (ESI+) *m/z* calculated $C_{49}H_{58}N_6O_{16}S$ $[M+H]^+$ 1019.37028 ; detected 1019.36838; accuracy = 1.86 ppm

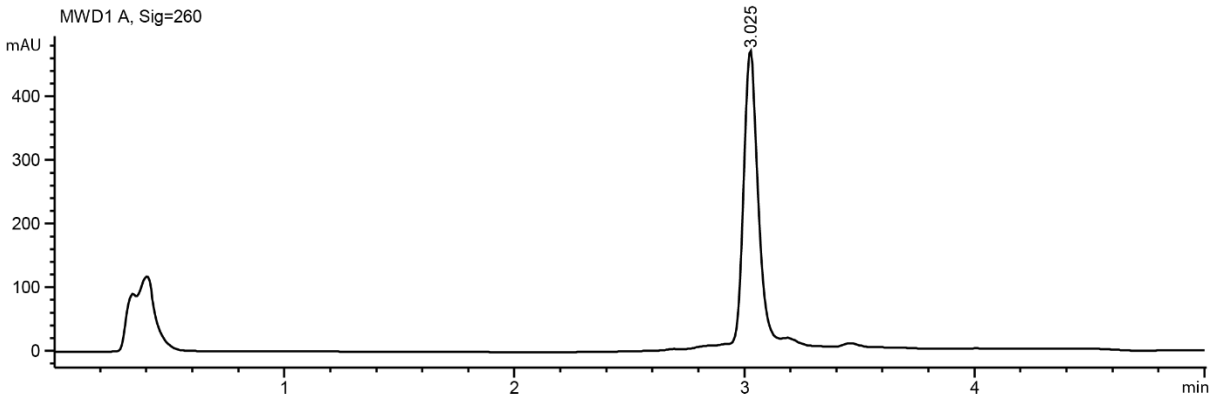


Figure S113. HPLC chromatogram of compound **16** recorded at λ = 260 nm.

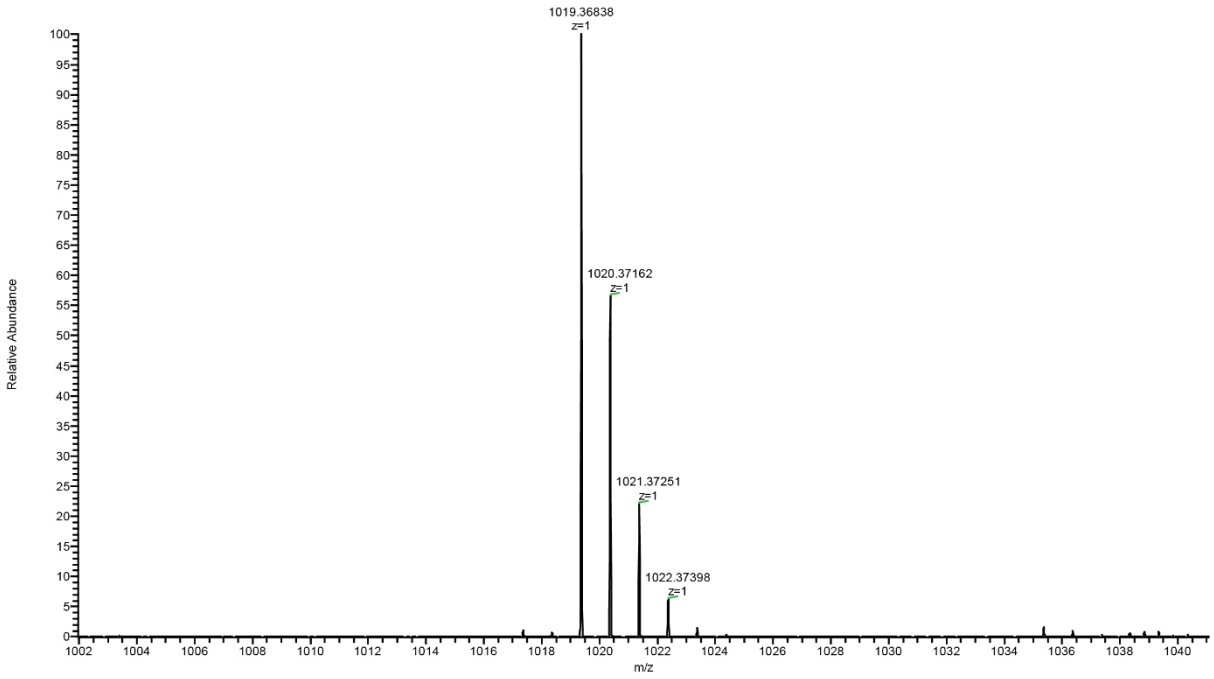
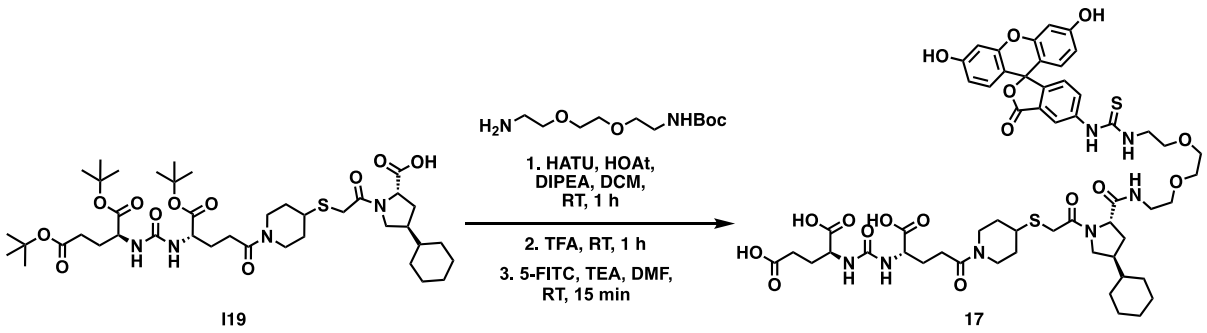


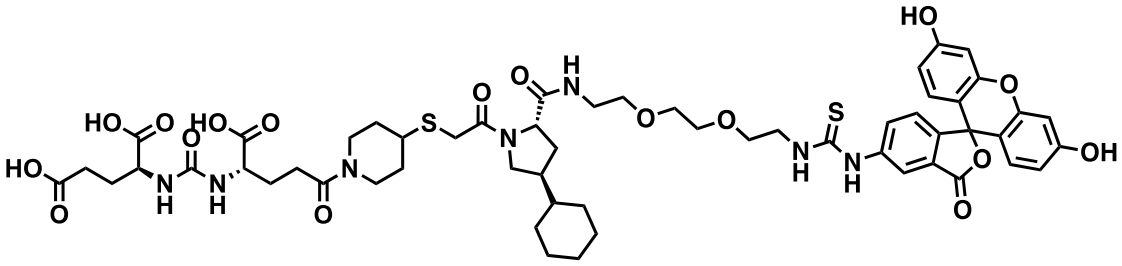
Figure S114. HR-MS of compound **16**.

6.2.14 Synthesis scheme for compound 17



Scheme S22. Schematic illustration of synthesis of compound **17**.

Synthesis of (((*S*)-1-carboxy-4-(4-((2-((2*S*,4*S*)-4-cyclohexyl-2-((2-(2-(2-(3-(3',6'-dihydroxy-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)ethoxy)ethoxy)ethyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)thio)piperidin-1-yl)-4-oxobutyl)carbamoyl)-*L*-glutamic acid (17)



Compound **I19** (10 mg, 0.012 mmol, 1.1 eq.) was dissolved in DCM (1 mL) and HATU (4 mg, 0.011 mmol, 1 eq.), HOAt (0.75 mg, 0.006 mmol, 0.5 eq.) and DIPEA (8 µL, 0.044 mmol, 4 eq.) were added. After 5 min, compound tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (3 mg, 0.011 mmol, 1 eq.) was added to the mixture. The reaction was stirred for 1 h at room temperature and subsequently solvent was evaporated under reduced pressure. The crude was re-dissolved in neat TFA (1 mL), and the reaction was stirred for 1 hour at room temperature. Afterwards, TFA was removed under reduced pressure and the

crude was dissolved in DMF, TEA (15 µL, 0.11 mmol, 10 eq.) and fluorescein-5-isothiocyanate (5-FITC, 200 mM in DMF, 55 µL, 0.011 mmol, 1 eq.) were added to the solution. The reaction was stirred for 15 min at rt and the product was purified by RP-HPLC to obtain compound **17** as an orange solid (1.4 mg, yield = 11%). HR-MS (ESI+) *m/z* calculated $C_{56}H_{69}N_7O_{17}S_2$ $[M+H]^+$ 1176.42641; detected 1176.42439; accuracy = 1.72 ppm

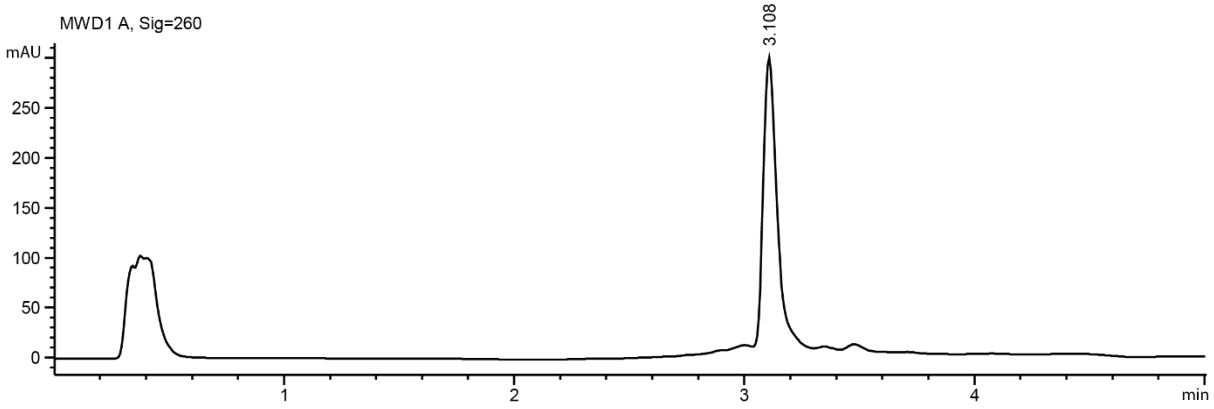


Figure S115. HPLC chromatogram of compound **17** recorded at λ = 260 nm.

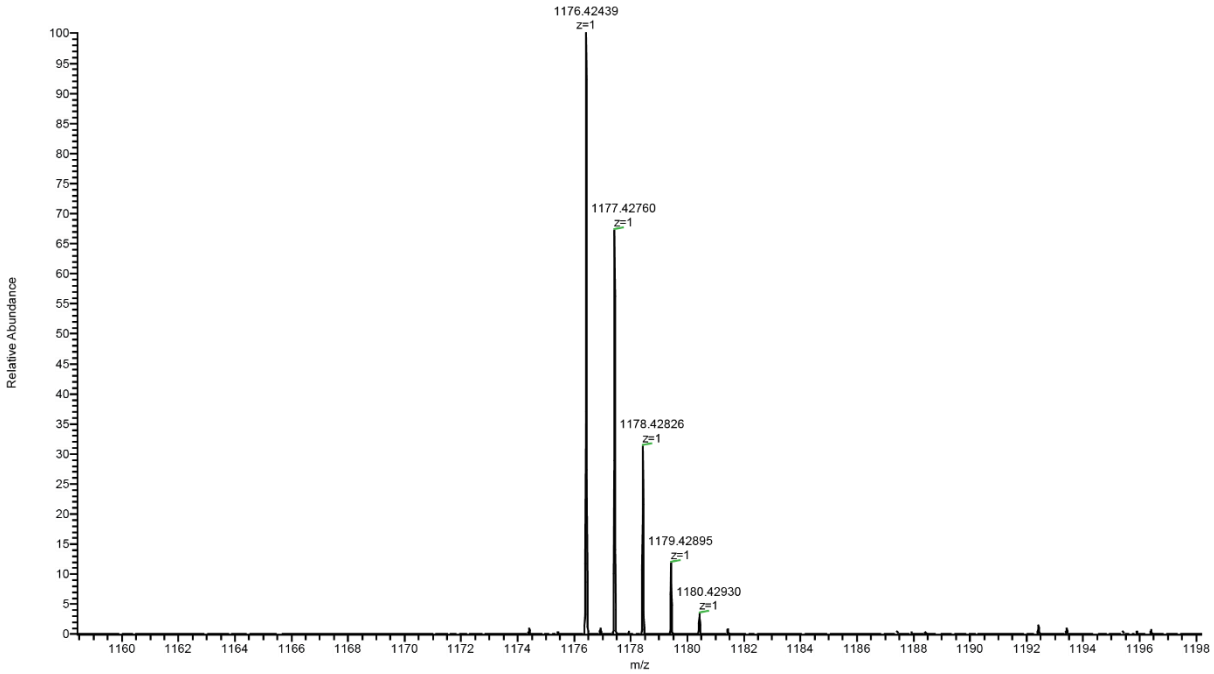
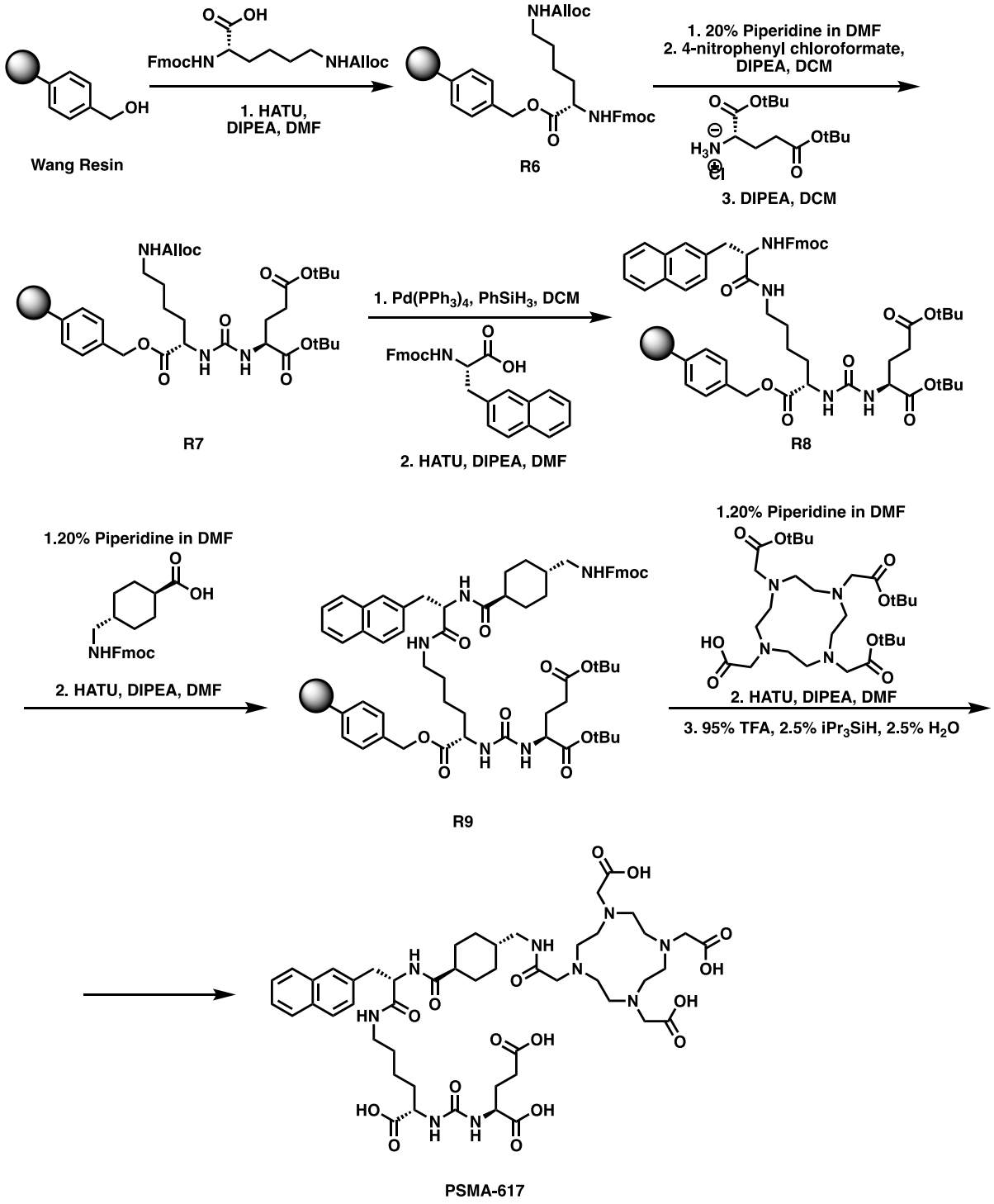


Figure S116. HR-MS of compound **17**.

6.2.15 Synthesis scheme for PSMA-617



Scheme S23. Schematic illustration of synthesis of **PSMA-617**.

Synthesis of (((*S*)-1-carboxy-5-((*S*)-3-(naphthalen-2-yl)-2-((1*r*,4*S*)-4-((2-(4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetamido)methyl)cyclohexane-1-carboxamido)propanamido)pentyl)carbamoyl)-*L*-glutamic acid (PSMA-617)

Commercially available Wang resin (230 mg, 0.25 mmol, 1 eq.) was coupled to Fmoc-Lys (Alloc)-OH (283 mg, 0.625 mmol, 2.5 eq.) using HATU (228 mg, 0.600 mmol, 2.4 eq.) and DIPEA (218 µL, 1.25 mmol, 5 eq.) and the resin was incubated overnight at room temperature. After Fmoc deprotection (as described in general procedure) of **R6**, the resin was swollen in DCM (10 min) and the solvent removed. Consecutively, a solution containing 4-Nitrophenyl chloroformate (101 mg, 0.5 mmol, 2 eq.) and DIPEA (174 µL, 1 mmol, 4 eq.) in anhydrous DCM (3 mL) was added to the resin. One hour after reaction, the resin was washed five times with DCM. A solution of L-Glutamic acid di-*tert*-butyl ester hydrochloride (148 mg, 0.5 mmol, 2.0 eq.) and DIPEA (174 µL, 1 mmol, 4 eq.) in anhydrous DCM (3 mL) was added to the resin and the mixture was incubated for 1h to obtain the urea **R7**. Afterwards, the Alloc group was deprotected by the addition of $Pd(PPh_3)_4$ (66.4 mg, 0.058 mmol, 0.23 eq.) and $PhSiH_3$ (308 µL, 2.5 mmol, 10 eq.) in anhydrous DCM (3 mL). The mixture was incubated for 1 hour at room temperature. The solvent was removed and the resin was washed with DCM (x5) and DMF (x5) to remove residual reagents. Fmoc-3-(2-naphthyl)-L-alanine (219 mg, 0.5 mmol, 2 eq.) was coupled to the resin by using HATU (190 mg, 0.5 mmol, 2 eq.) and DIPEA (174 µL, 1 mmol, 4 eq.), and the resin was incubated overnight at room temperature to afford intermediate **R8**. **R8** was Fmoc deprotected and reacted with trans-4-(Fmoc-aminomethyl) cyclohexanecarboxylic acid (237 mg, 0.625 mmol, 2.5 eq.) which was pre-activated with HATU (228 mg, 0.600 mmol, 2.4 eq.) and DIPEA (218 µL, 1.25 mmol, 5 eq.) and the reaction was carried out overnight at room temperature to afford intermediate **R9**. The resin was split in 0.05 mmol aliquots before proceeding with further steps. After Fmoc removal, 2-(4,7,10-tris(2-(tert-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid (85.9 mg, 0.15

mmol, 3.00 eq.) was amide-coupled to the resin using HATU (53 mg, 0.14 mmol, 2.8 eq.) and 6 equiv. DIPEA (52 µL, 0.3 mmol, 6 eq.) and the reaction was incubated for 3 hours at rt. After washings, the resin was cleaved using a solution of 95:2.5:2.5 TFA:H_2O:TIPS. Cleavage fractions were combined and dried under reduced pressure for subsequent purification by RP-HPLC. Compound **PSMA-617** was obtained as a white solid (5 mg, 10% yield). HR-MS (ESI+) *m/z* calculated $C_{49}H_{71}N_9O_{16}$ $[M+2H]^{2+}$ 521.75822; detected 521.75729; accuracy = 1.78 ppm

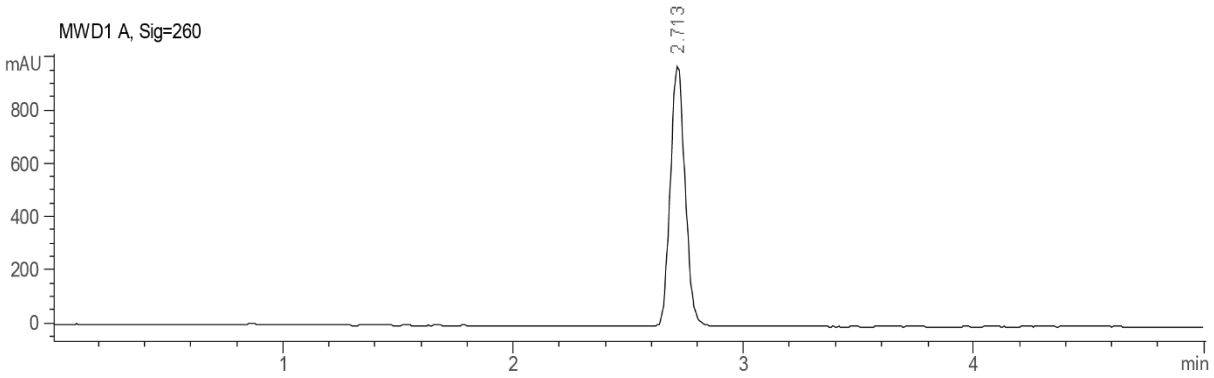


Figure S117. HPLC chromatogram of **PSMA-617** recorded at λ = 260 nm.

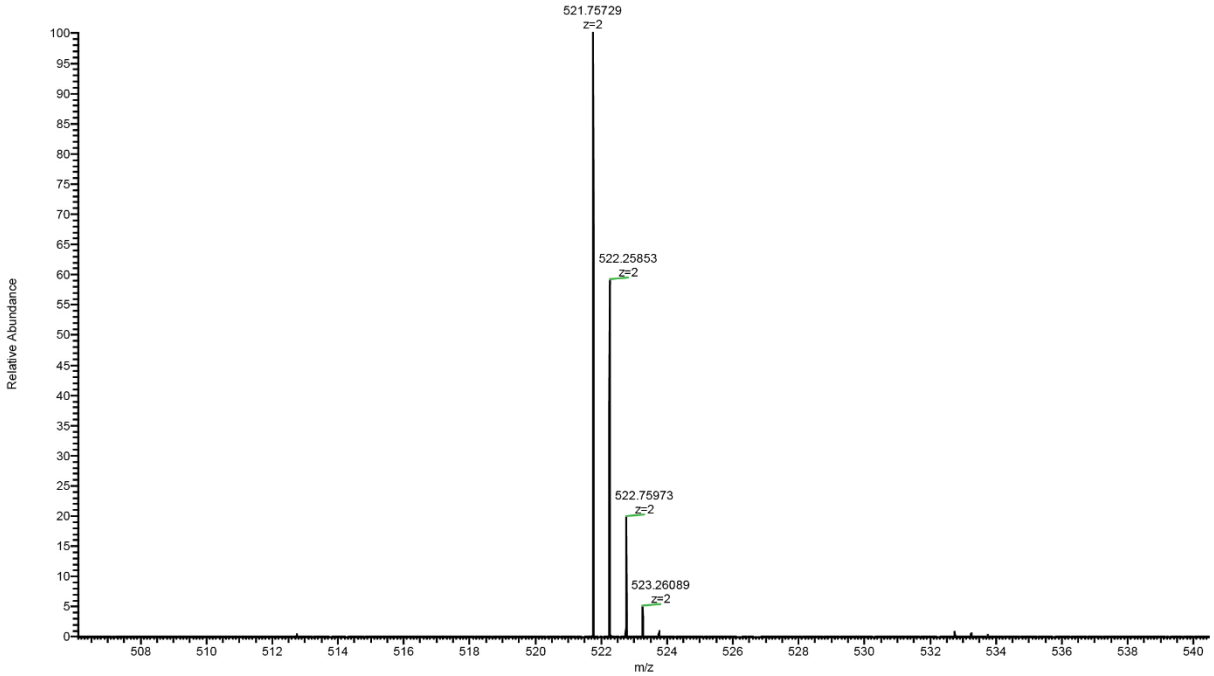
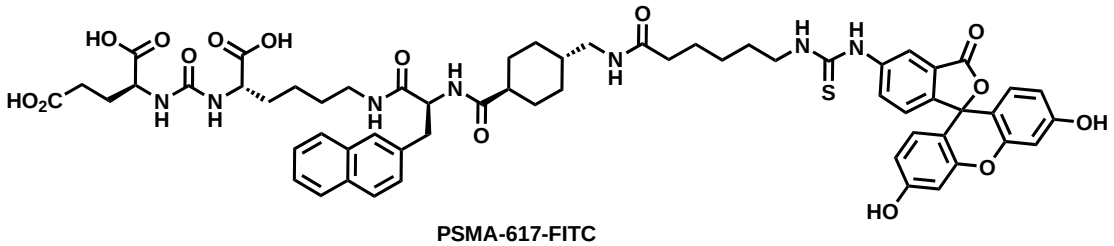


Figure S118. HR-MS of **PSMA-617**.

6.2.16 Synthesis of PSMA-617-FITC



PSMA-617-FITC

Synthesis of PSMA-617-FITC has been already described and reported[2,4]

PSMA-617-FITC was used as fluorescent derivative of PSMA-617 to perform flow cytometry analysis to assest successful transduction of HT1080.wt cells with hPSMA lentiviral vector to develop HT1080.hPSMA cell line (**Supplementary Information Chapter 9**). The compound was also used to perform interlization studies by confocal microscopy experiment.

7 Fluorescence Polarization

Fluorescence Polarization was used to perform validation of binding affinities of compounds. Curves and K_D values of the main compounds can be found in the Manuscript.

FP was performed using 384-well microplates (Greiner Bio-One, #784076). The proteins were serially diluted in the respective buffer with a 1:1 dilution at a final volume of 5 µL per well. The off-DNA Fluorescent labelled compounds were dissolved in DMSO and diluted in the protein buffer to reach a final concentration of 10 nM (1% DMSO). The concentration of each compound was determined by measuring the absorbance at λ=498 nm (ε=73,000 cm^{-1} M^{-1}, l=0.1 cm) with a Thermofisher nanodrop 2000. 5 µL of the FITC-labelled compounds were added to each well and after a short centrifugation of the plate, the fluorescence polarization was measured using a Tecan Spark Multimode Microplate Reader (FITC: $\lambda_{Excitation}$ = 485 ± 20

nm, $\lambda_{Emission}$ = 535 ± 25 nm for Fluorescence conjugates). For On-DNA validation, the HIT-bearing oligonucleotide (25 µL, 2 µM in phosphate-buffered saline, PBS) was annealed to the complementary 3′-amino modified 8-mer LNA (5′ GGCTACTA-C_6-amino 3′) previously conjugated to BODIPY-TMR-X (25 µL, 2 µM in PBS)[5] (Thermo Fisher Scientific, #D6117) by mixing the two solutions in a 1:1 ratio. The mixture was heated to 70°C for 5 min and cooled down to room temperature for 30 min. The generated heteroduplex was then diluted to a final concentration of 50-100 nM in the respective protein buffer and 5 µL were added to each well where the protein was already diluted. FP was measured in a Tecan Spark Multimode Microplate Reader (BODIPY: $\lambda_{Excitation}$ = 535 ± 25 nm, $\lambda_{Emission}$ = 595 ± 35 nm).

Additional validation data are reported here.

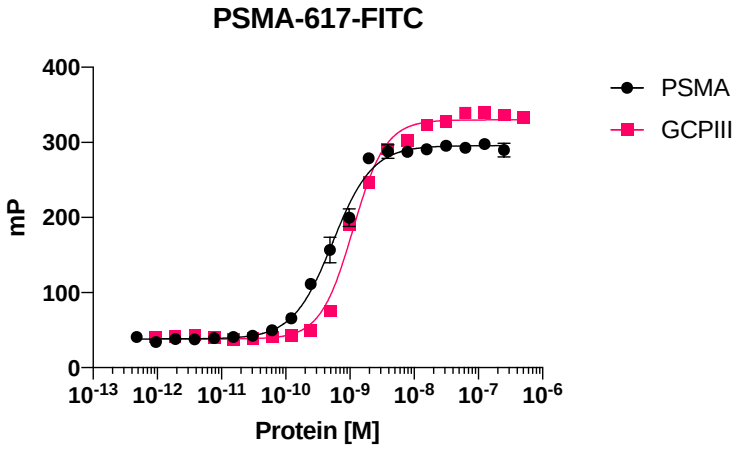


Figure S119. Fluorescence Polarization of compound PSMA-617-FITC tested against PSMA ($K_{D, PSMA}$ = 0.53 ± 0.9 nM, black curve) and GCPIII ($K_{D, GCPIII}$ = 1.12 ± 0.2 nM, pink curve)

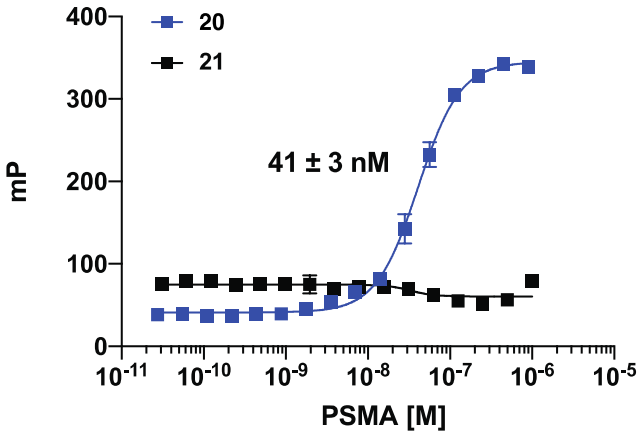


Figure S120. Fluorescence Polarization of compound **20** ($K_{D,PSMA}$ = 41 ± 3 nM) and **21** against PSMA. The experiment was performed to assess the binding affinity of fragments of the compound **3**.

8 Cell Culture

HT1080.hPSMA, HT1080.wt cells were grown in Dulbecco's modified Eagle medium (Gibco, #41966) and LNCaP cells in RPMI-1640 (Gibco, #21875034) supplemented with 10% foetal bovine serum (Gibco, #10270106) and 1× antibiotic-antimycoticum (Gibco, #15240062) at 37 °C, 5% CO_2. Cell passaging was performed every two days for HT1080.hPSMA, HT1080.wt and every three days for LNCaP. Cells were detached using trypsin-EDTA 0.25% (Thermo Fisher Scientific, #25200072).

9 HT1080.hPSMA Cell line development

hPSMA Lentiviral transfer vector and amino acid sequence

The strategy to generate the lentiviral transfer vector with **hPSMA** is shown below, together with the amino acid sequence.

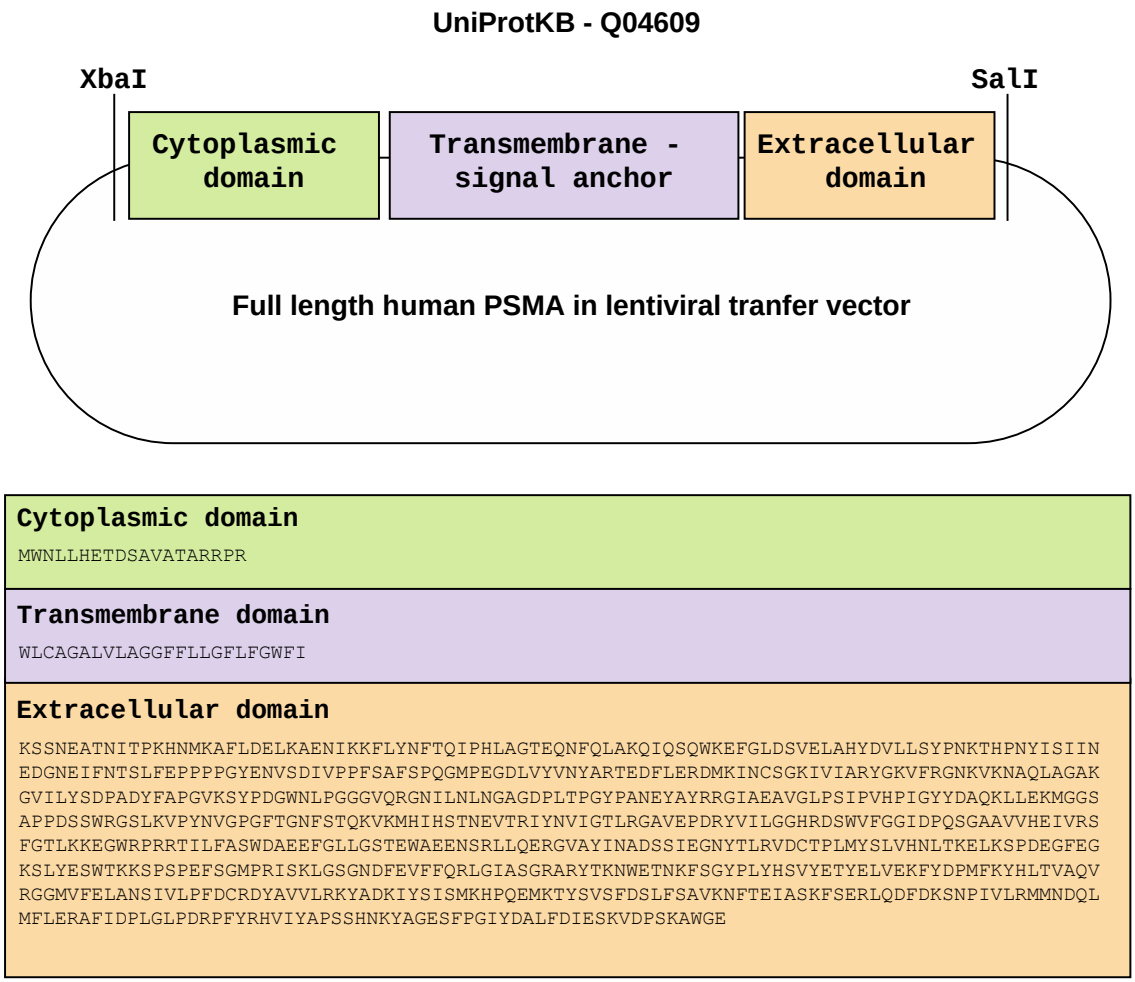


Figure S121. Graphical representation of full length human PSMA lentiviral transfer vector used for cell transduction. Aminoacidic sequence of full length human PSMA is reported.

Viral transduction of HT1080.wt cells to generate HT1080.hPSMA stable cell line.

Development of stable cell line expressing human PSMA was performed as previously described[6,7]. HT1080wt cells were seeded at a density of 0.1 million cells/mL in a 24 well-plate (50'000 cells/well). Next day, Polybrene (Santa Cruz Biotechnology, #134220) was added to the wells reaching a final concentration of 8 µg/ mL and 15 µL of the virus were added to the cells. The plate was then centrifuged at 1,000 x g for 90 minutes at 37 °C, followed by overnight incubation of the cells (37°C, 5% CO_2). The following day, the media was exchanged, and cells were kept growing and expanding for two weeks according to the general cell culture procedures. Positivity of the cells was tested by flow cytometry analysis, and subsequently

positive cells were sorted using BD FACS Aria III sorter, and two different cell populations were afforded (high and low antigen expression cell lines). Positive staining was performed using PSMA-617-FITC (50 nM) and HT1080wt were used as negative control. Experiments were performed in duplicates.

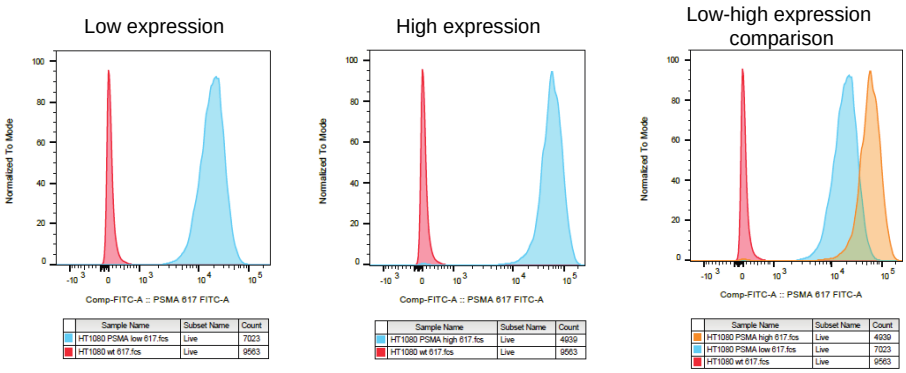


Figure S122. Flow cytometry analysis performed on HT1080.hPSMA and HT1080.wt (negative control). Low antigen-expression and high antigen-expression HT1080.hPSMA populations were sorted and stained with PSMA-617-FITC (structure reported in **Supplementary Information 6.2.16**).

10 Flow Cytometry

Cells (LNCaP, HT1080.wt, HT1080.hPSMA) were grown to confluency and were detached using Accutase cell detachment solution (Merck, #SCR005), counted and washed once with fluorescence-activated cell sorting (FACS) buffer (0.5% bovine serum albumin (BSA), 2 mM EDTA in PBS pH 7.4, filtered). The cells were then resuspended in FACS buffer at a density of 5'000'000 cells mL^{-1}. Cell Strainer Snap Caps with Corning Falcon test tube (Corning, #352235) were used to disperse cells and generate a single-cell suspension. Cells were then transferred into a 96-well U-bottom plate (Greiner Bio-One, #650180) at 100 µL (500'000 cells) per well. All the following steps were performed by keeping the cells on ice. Cells were incubated in the plate for 20 min for blocking and parallelly, a staining solution of fluorescein-labelled compounds (50-250 nM in FACS buffer) was prepared. Consecutively, the cells were

centrifuged for 3 min at 1100 rpm, and 4 °C, the supernatant was discarded, and the cell pellet was resuspended in the staining solution (200 µL per well). Cells were incubated in the dark for 1 h, centrifuged and washed once with PBS (100 µL per well). Afterwards, 100 µL of near-infrared (NIR) dye (Zombie NIR, Fixable Viability Kit, BioLegend, #423105, diluted 1:1'000 in PBS) were added to each well. The live/dead staining was performed for 30 min in the dark and subsequently the cells were washed once with PBS. The cell pellet was taken up in 200 µL PBS buffer and fluorescence signal was analyzed with a flow cytometer (BD LSRFortessa™ Cell Analyzer, Biosciences). Results were evaluated using FlowJo v10 software.

11 Confocal Microscopy

Internalization studies of compound **11** and PSMA-617-FITC were performed by confocal microscopy.

LNCaP, HT1080.hPSMA and HT1080.hFAP cells were seeded into 4-well coverslip chamber plates (Sarstedt, #94.6190.402) at a density of 10^4 cells per well in the respective medium (RPMI for LNCaP cells, Gibco™ #11875093, DMEM for HT1080 cells, Gibco™ #11520416) supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotic-antimycotic, and HEPES (10 mM, pH 7.5) and allowed to grow for 24 h under standard culture conditions. Afterwards, the culture medium was discarded and 400 µL of a solution of the fluorescein-conjugate compound (**11**, PSMA-617-FITC, 100 nM) together with Hoechst 33342 nuclear dye (1 µM Invitrogen #H3570) in fresh medium, was added to the wells. Randomly selected colonies were imaged at 1 h, 8 h and 24 h after incubation with the compound on a SP8 confocal microscope equipped with an AOBS device (Leica Microsystems).

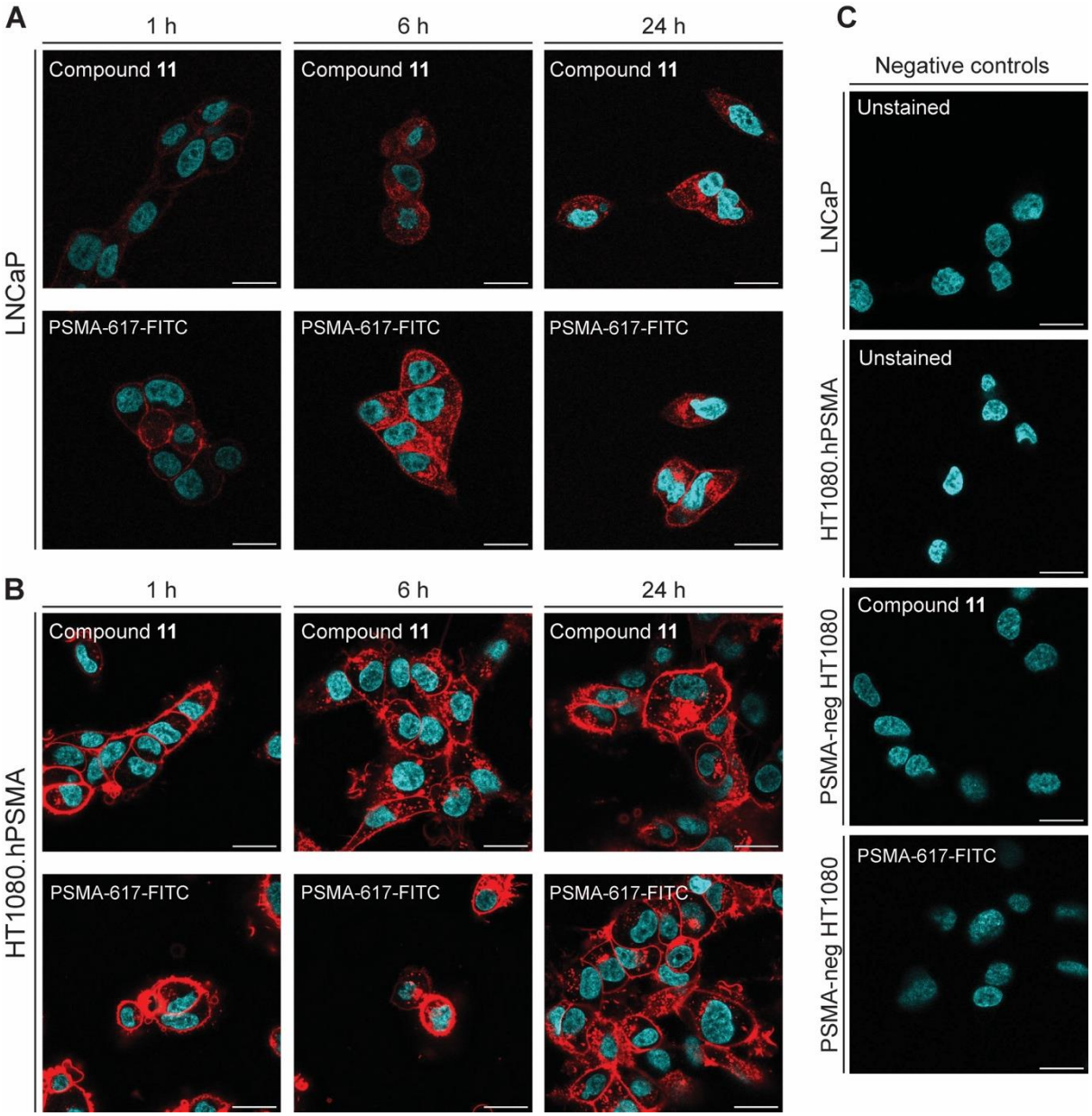


Figure S123. Confocal microscopy images after incubation of compound **11** (A70-B104-FITC, 100 nM) and PSMA-617-FITC (100 nM) with PSMA-positive cells (**A** LNCaP, **B** HT1080.hPSMA), at 1 h, 6 h and 24 h. No interaction with PSMA-negative HT1080 and no fluorescent signal in the unstained cells were observed (**C** negative controls). Red=fluorescein derivatives staining; blue=Hoechst 33342 staining. Scale bar, 20 μm.

12 *In Vivo* Biodistribution

12.1 Animal License

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number ZH006/2021 granted by the Veterinäramt des Kantons Zürich.

12.2 In vivo Biodistribution studies

HT1080.hPSMA tumor cells were used for *in vivo* biodistribution experiments. HT1080.hPSMA cells were generated performing a stable lentiviral transduction of HT1080.wt following the procedures described in **Supplementary Information section 9**. Tumor cells were grown in DMEM (Gibco, #11965092). with 10% FBS and 1% antibiotic-antimycotic and detached with Trypsin-EDTA 0.05%. Cells were then resuspended in Hanks' Balanced Salt Solution medium (Gibco, #14170112). and aliquots of 5,000,000 cells / 100 µL were injected in the right flank of 6 female athymic BALB/cAnN-*Foxn1*$^{nu/nu}$/Rj mice (6-8 week of age, Janvier). After 12 days, when the subcutaneous tumor had grown to an average volume of ~180 mm^3, mice were randomized in 2 groups (3 mice per group) and [^{177}Lu]-A70-B104-DOTA (100 µL, 1 nmol/mouse, 1MBq; 50 MBq/kg, 50 nmol/kg) and [^{177}Lu]Lu-PSMA-617 (100 µL, 0.5 nmol/mouse, 1MBq; 50 MBq/kg, 25 nmol/kg) were intravenously administered.

12.3 Radiolabeling Protocol

Radiolabeling of compound A70-B104-DOTA and PSMA-617 to perform biodistribution experiment.

Compound **22** (A70-B104-DOTA, 2 µL, 2.5 mM solution in mQ Water, 4% DMSO, 5 nmol) was diluted with 69.5 µL of NaOAc buffer (1 M, pH 4.5). Then, 2.5 µL of [^{177}Lu]$LuCl_3$ (5 MBq, 2MBq/µL) were added and the mixture was heated at 95°C for 10 minutes under stirring to obtain compound **18**. The labelling efficiency was investigated by HPLC using a radiometric detector as represented in **Figure S124A**.

PSMA-617 (10 mL, 1 mM solution in mQ Water, 2% DMSO, 10 nmol) was diluted with 1M NaOAc buffer (pH 4.5, 170 mL). $^{177}LuCl_3$ (50 MBq, 2.5 MBq/mL) was added and the mixture was heated at 95°C for 10 minutes under stirring and then cooled down at room temperature to obtain Pluvicto^{TM}. The labelling efficiency was investigated by HPLC using a radiometric detector as represented in **Figure S124B**.

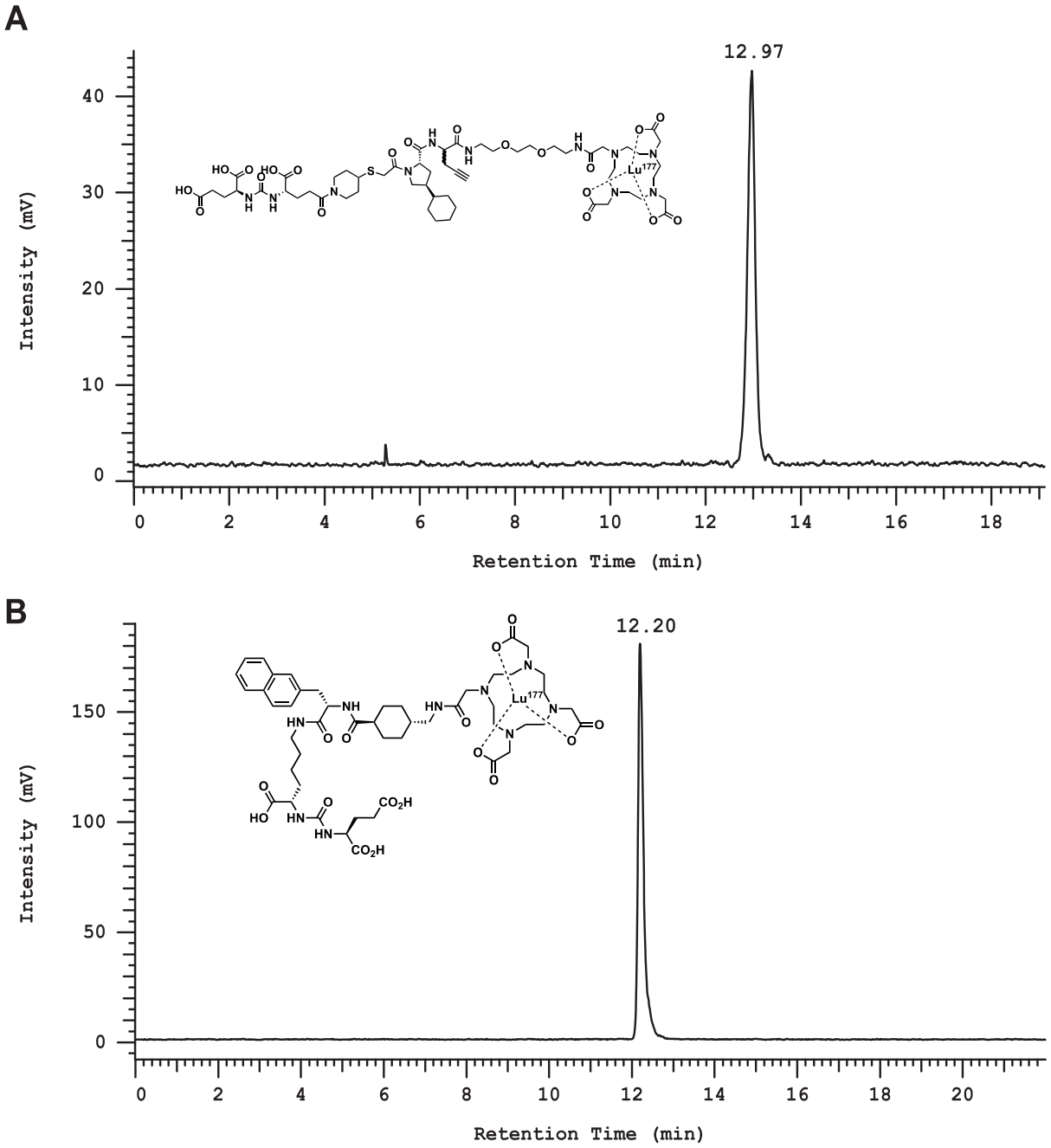


Figure S124. Radio HPLC chromatogram of compound **18** ([^{177}Lu]Lu-A70-B104-DOTA) (**A**) and **Pluvicto**^{TM} ([^{177}Lu]Lu-PSMA-617) (**B**), which were radiolabelled for biodistribution experiment.

12.4 Biodistribution Data

12.4.1 %ID/g data

The %ID/g value for each organ and tumors were calculated with the following equation:

$$\%\,ID/g\ \ (organ) = \frac{organ\ radioactivity}{organ\ weight} \cdot \frac{1}{total\ injected\ radioactivity} \cdot 100\%$$

Table S3. Quantitative in vivo biodistribution of compound **18** (^{177}Lu-A70-B104-DOTA) and **Pluvicto™** at 2 h time point after intravenous administration (50 MBq/kg, 50 nmol/kg; 50 MBq/kg, 25 nmol/kg, respectively) in mice bearing HT1080.hPSMA tumors. Data are reported as average %ID/g ± standard deviation (n = 3).

	%ID/g	
Organs	**Compound 18** (3 mice)	**Pluvicto™** (3 mice)
Tumor (HT1080.hPSMA)	7.38 ± 2.48	32.43 ± 5.49
Heart	0.04 ± 0.01	0.03 ± 0.01
Lung	0.13 ± 0.06	0.07 ± 0.04
Liver	0.08 ± 0.01	0.06 ± 0.01
Spleen	0.08 ± 0.02	0.12 ± 0.01
Intestine	0.09 ± 0.07	0.03 ± 0.02
Kidney	2.22 ± 0.46	2.16 ± 0.26
Blood	0.05 ± 0.01	0.02 ± 0.01
Muscle	0.14 ± 0.18	0.03 ± 0.02
Tail	0.03 ± 0.03	0.10 ± 0.05

12.4.2 Tumor-to-organ ratio data

Table S4. Tumor-to-organ ratios of compound **18** (^{177}Lu-A70-B104-DOTA) and **Pluvicto™** at 2 h time point after intravenous administration (50 MBq/kg, 50 nmol/kg; 50 MBq/kg, 25 nmol/kg, respectively) in mice bearing HT1080.hPSMA tumors. Data are reported tumor:organ ratio ± standard deviation (n = 3).

Tumor-to-organ ratio		
Organs	**Compound 18** (3 mice)	**Pluvicto™** (3 mice)
Heart	202.75 ± 9.86	970.90 ± 556.68
Lung	56.97 ± 52.68	436.47 ± 183.75
Liver	94.91 ± 21.30	518.78 ± 68.15
Spleen	92.82± 20.82	260.06 ± 63.13
Intestine	83.11± 109.95	1045.98 ± 757.75
Kidney	3.33± 1.06	15.00 ± 2.25
Blood	147.30± 27.20	2079.63 ± 1727.26
Muscle	53.64± 96.78	957.85 ± 621.73
Tail	212.37 ± 1064.31	310.89 ± 178.23

13 Autoradiography Experiment

OCT-embedded frozen tissue sections (10 μm) were defrosted and fixed in ice-cold acetone (-20 °C) for 15 min. Successively, the sections were washed twice with PBS (pH 7.4) and the tissues were circled with a Dako Pen (Agilent, #S200230-2). After drying, the sections were blocked with a solution of 20% fetal calf serum (FCS, Invitrogen) and 3% bovine serum albumin (BSA) in PBS (pH 7.4) for 30 min. After washing three times with PBS (pH 7.4), 500 μL of the labelled [^{177}Lu]Lu-PSMA-617 and [^{177}Lu]-A70-B104-DOTA (5 nM, 47 kBq; 50 nM, 455 kBq, respectively, in PBS with 1% BSA, pH 7.4) were added. The tissues were incubated with the radioactive staining for 1 h at room temperature, and after the solution was discarded, the slides were washed three times in PBS. The dry slides were placed on a phosphor screen and left 1h before measuring with the Phosphor-Imager (CR-35 Bio HR Elysia Raytest).

13.1 Radiolabeling Protocol

Radiolabeling of compound A70-B104-DOTA and PSMA-617 to perform autoradiography experiment.

PSMA-617 (200 µM in 1 M NaOAc buffer, pH 4.5, 2.5 µL) was diluted with acetate buffer (1 M, pH 4.5, 7.5 µL) and [^{177}Lu]$LuCl_3$ (10 µL, 2 MBq/ µL) was added. The reaction mixture was stirred at 90 °C for 10 min and subsequently cooled down passively to obtain the radiolabeled [^{177}Lu]Lu-PSMA-617 (Pluvicto™, 1 MBq/ µL) and diluted with PBS . The labelling efficiency was investigated by HPLC as represented in **Figure S125B**. The same procedure was followed for the radiolabelling of compound **22** (A70-B104-DOTA) to afford compound **18**. (**Figure S125A**)

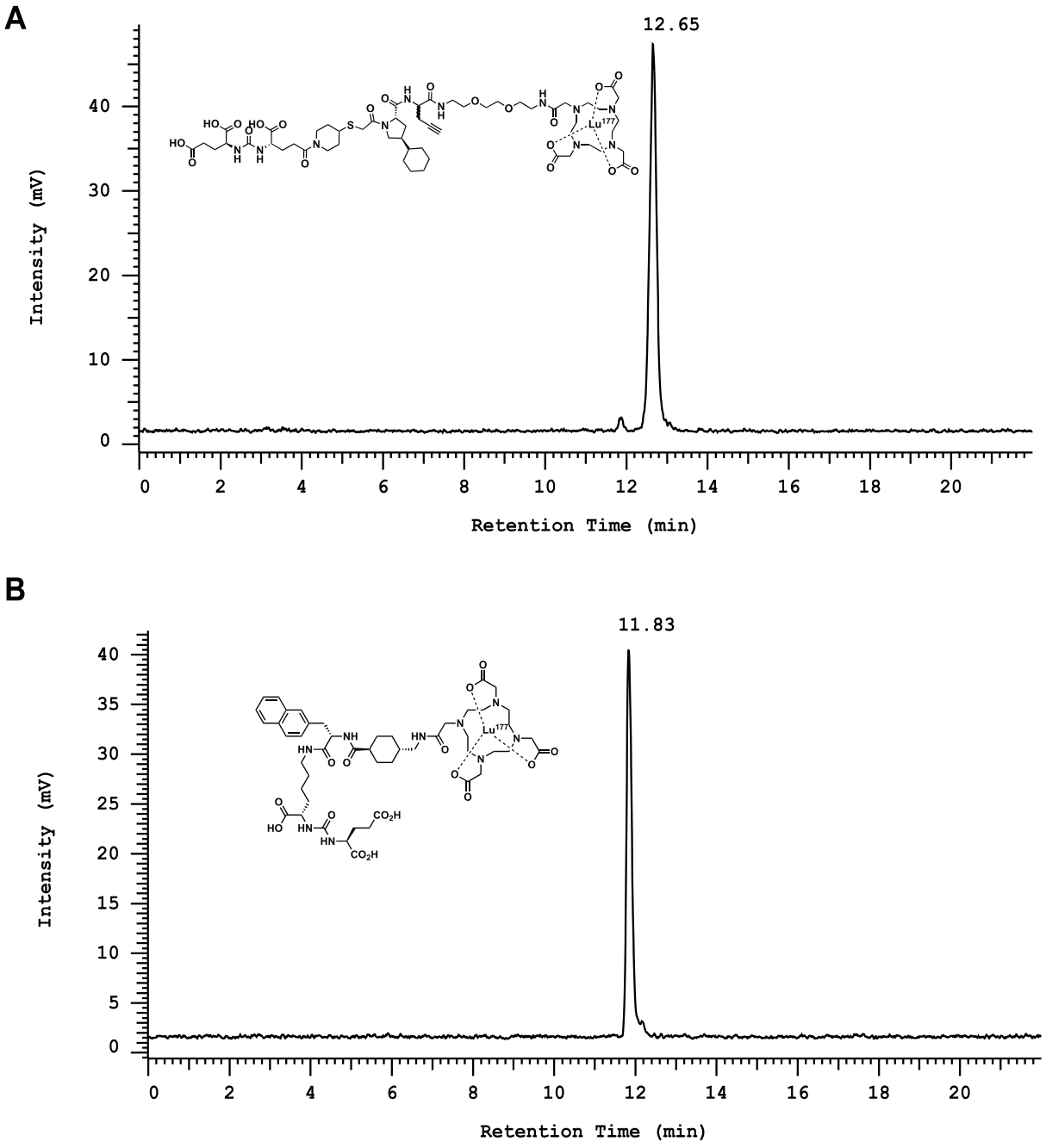


Figure S125. Radio HPLC chromatogram of compound **18** ([^{177}Lu]Lu-A70-B104-DOTA) (**A**) and **Pluvicto**^{TM} ([^{177}Lu]Lu-PSMA-617) (**B**), which were radiolabelled for autoradiography experiment.

13.2 Autoradiography on Human Salivary Glands

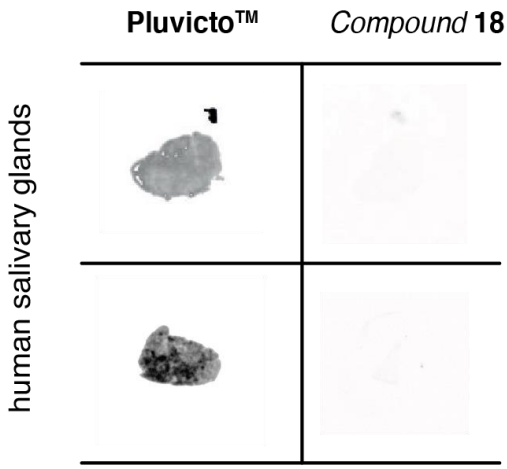


Figure S126. Replicates of autoradiography staining of human salivary glands performed with Pluvicto™ (5 nM, 60 kBq) and compound **18** (5 nM, 60 kBq), after overnight incubation of the slices on a phophor screen.

13.3 Autoradiography on Minipig Tissues

Autoradiography protocol is described in **Material and Methods** (Manuscript).

Human salivary glands have been purchased from Amsbio. Minipig salivary glands have been provided by Ellegaard Göttingen Minipigs A/S.

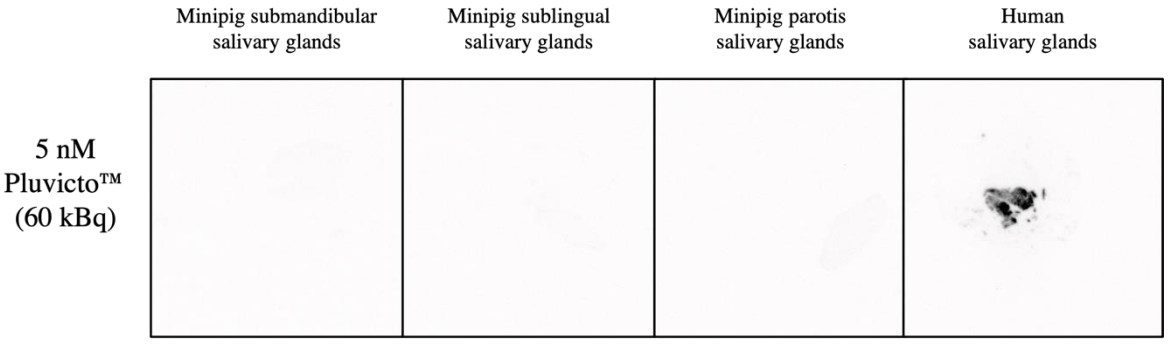


Figure S127. Autoradiography staining of Minipig salivary glands (submandibular, sublingual and parotis, from left to right) compared to human salivary glands, performed with Pluvicto™ (5 nM, 60 kBq

14 References

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15 Appendix

15.1 Selection Fingerprints

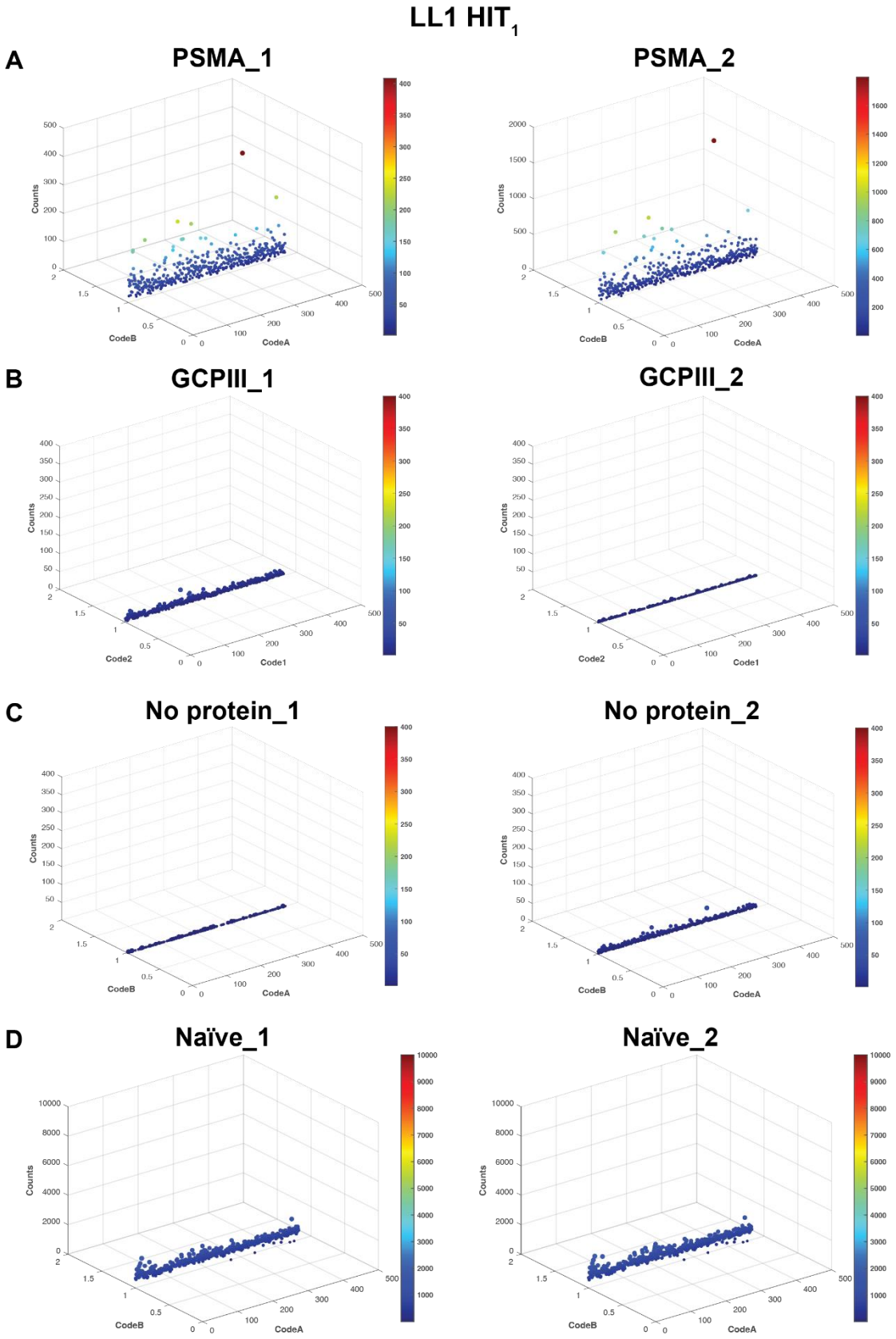

Figure S128. Fingerprints of LL1 HIT_1 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. LL1 HIT_1 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

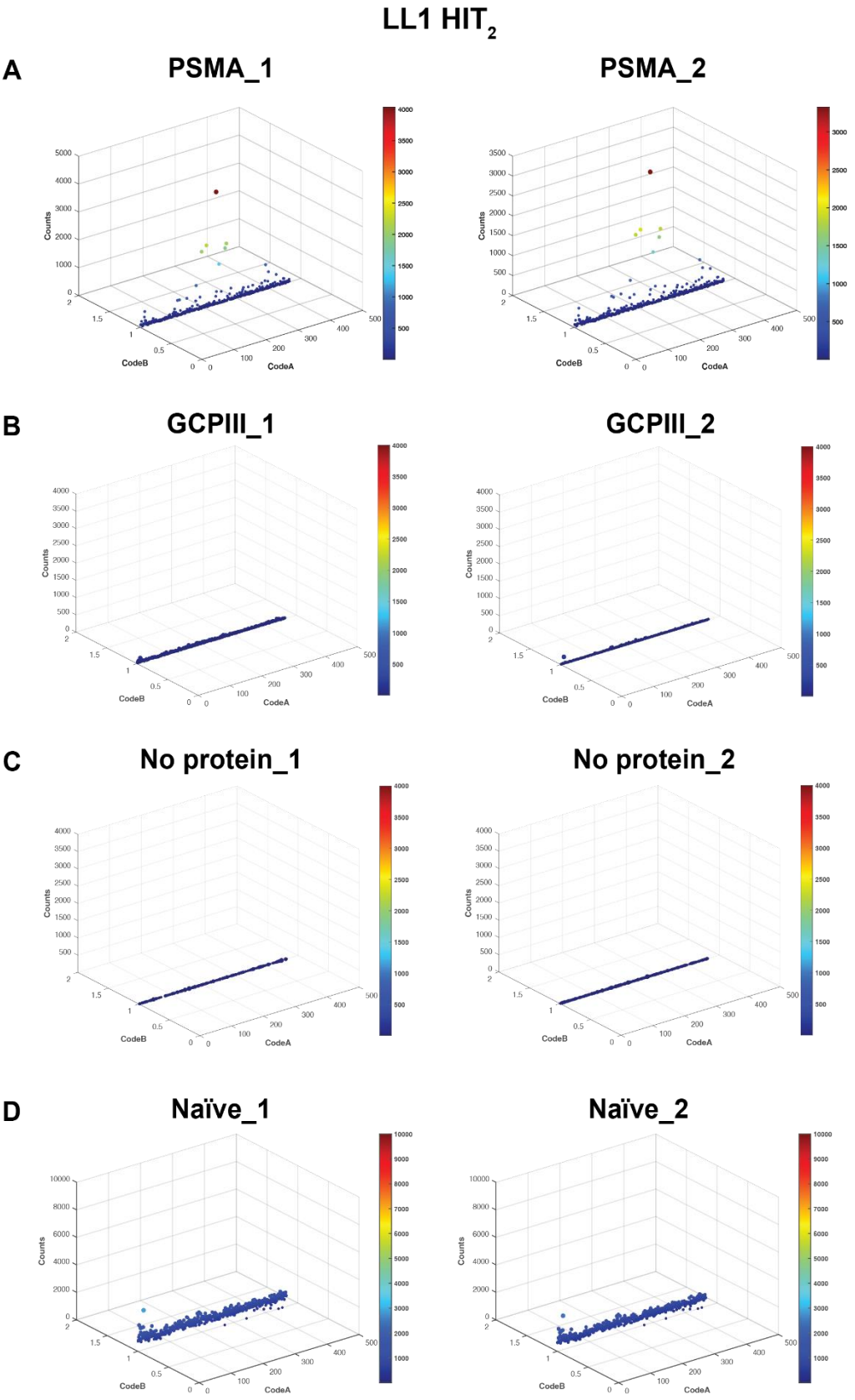

Figure S129. Fingerprints of LL1 HIT_2 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. LL1 HIT_2 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

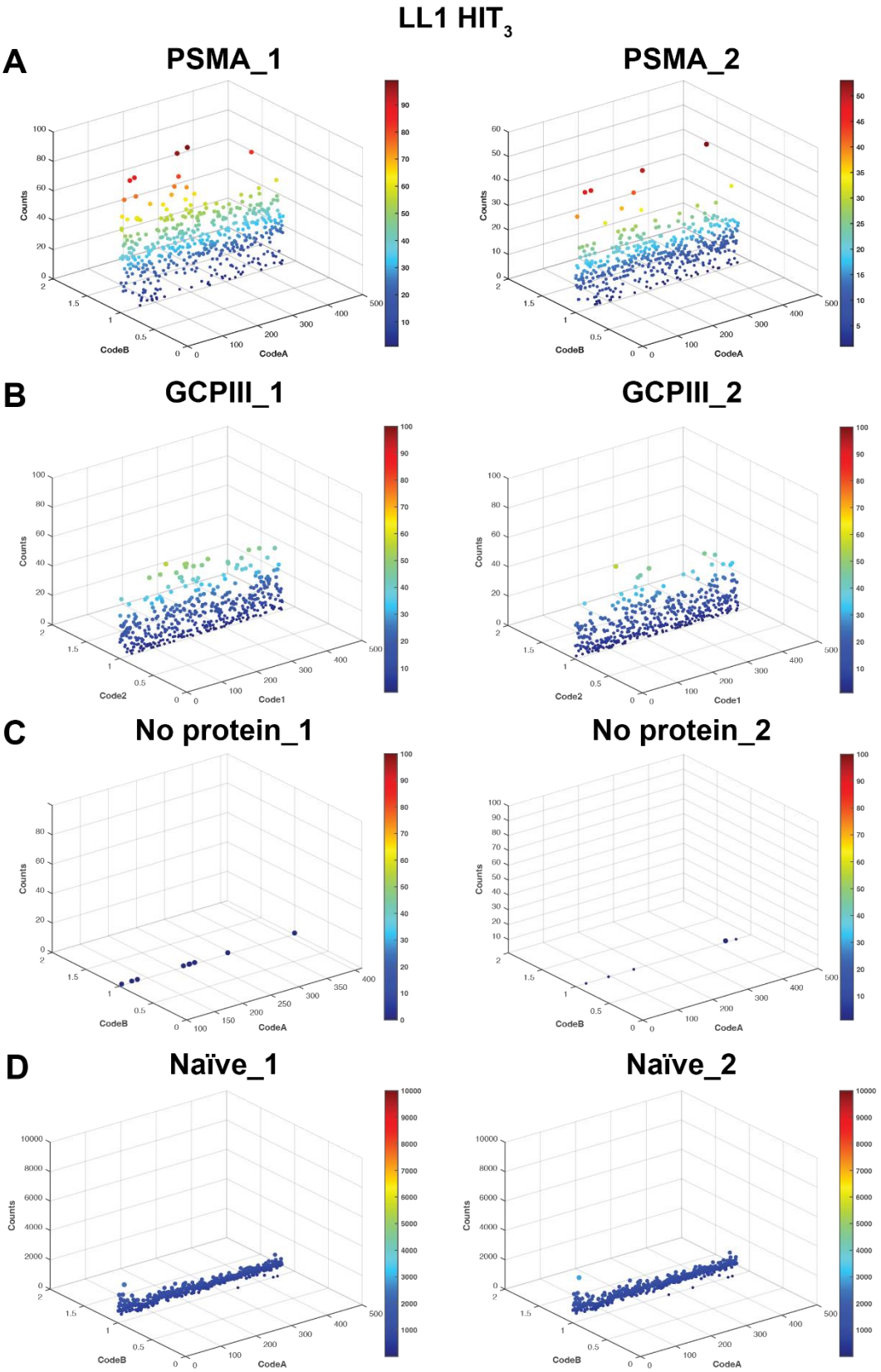

Figure S130 Fingerprints of LL1 HIT_3 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. LL1 HIT_3 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

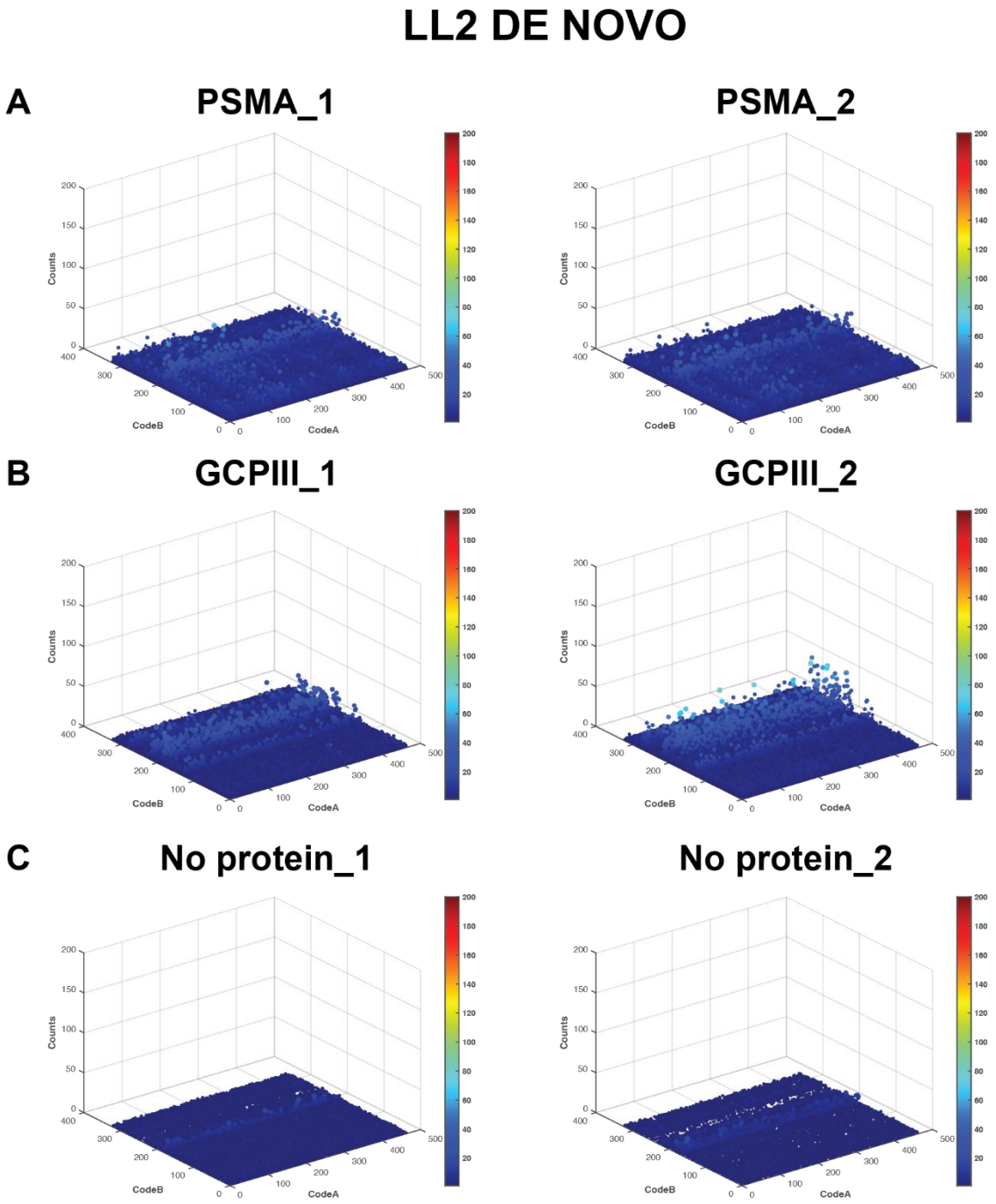

Figure S131. Fingerprints of LL2 de novo (not derivatized with HITs) after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. Normalized sequence counts are color-coded as indicated by the color bar.

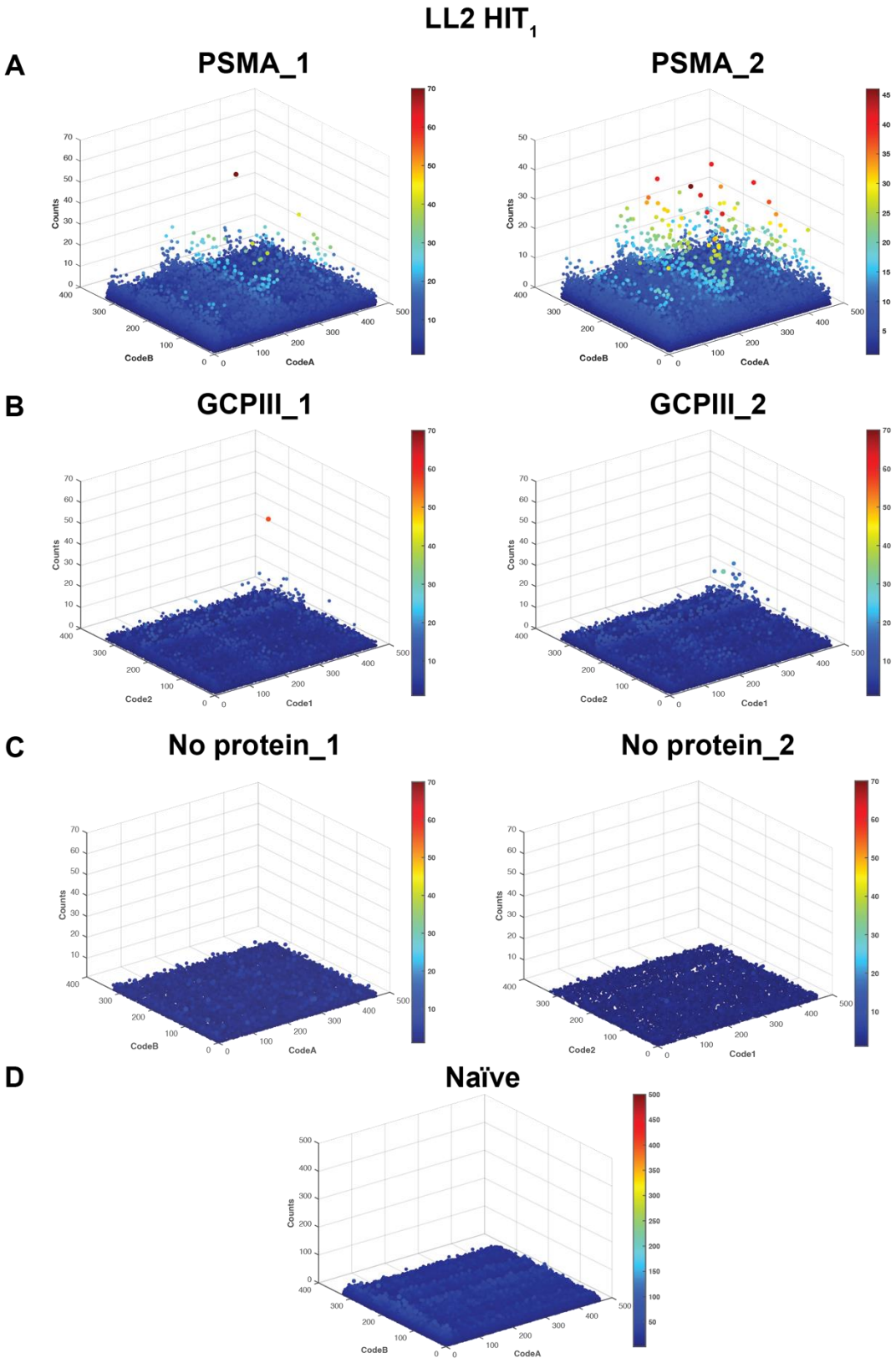

Figure S132. Fingerprints of LL2 HIT_1 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. LL2 HIT_1 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

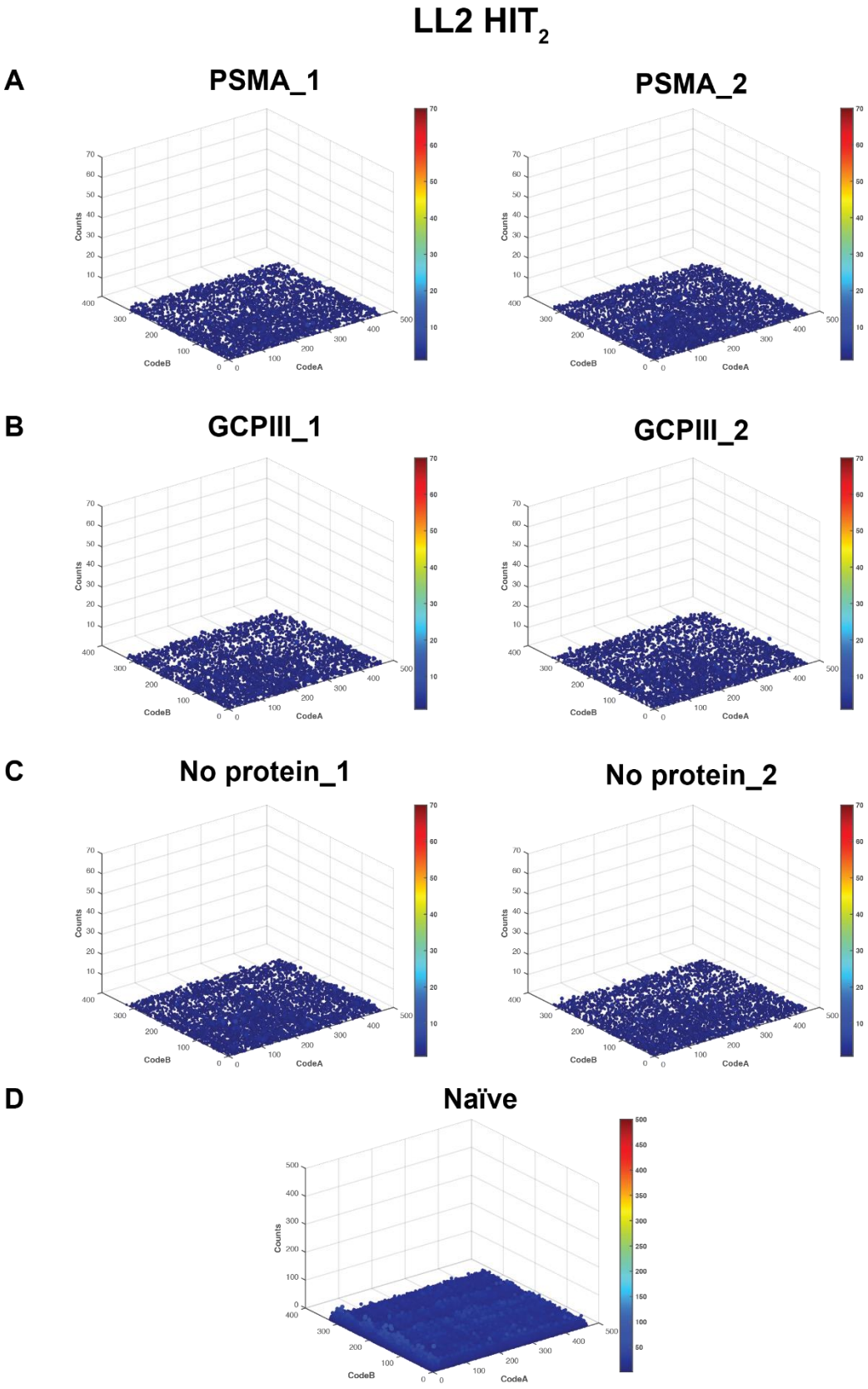

Figure S133. Fingerprints of LL2 HIT_2 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. LL2 HIT_2 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

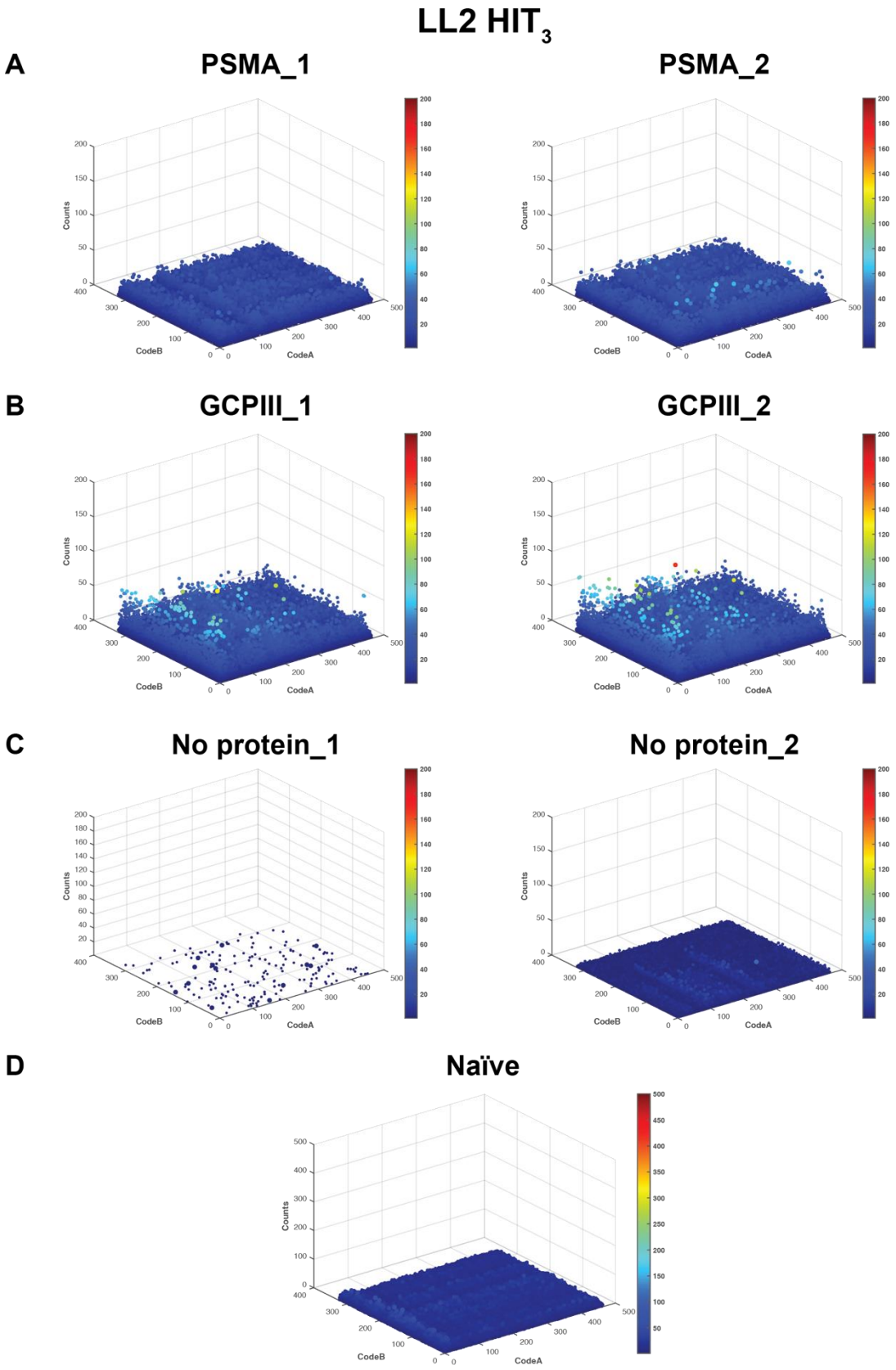

Figure S134. Fingerprints of LL2 HIT_3 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. LL2 HIT_3 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

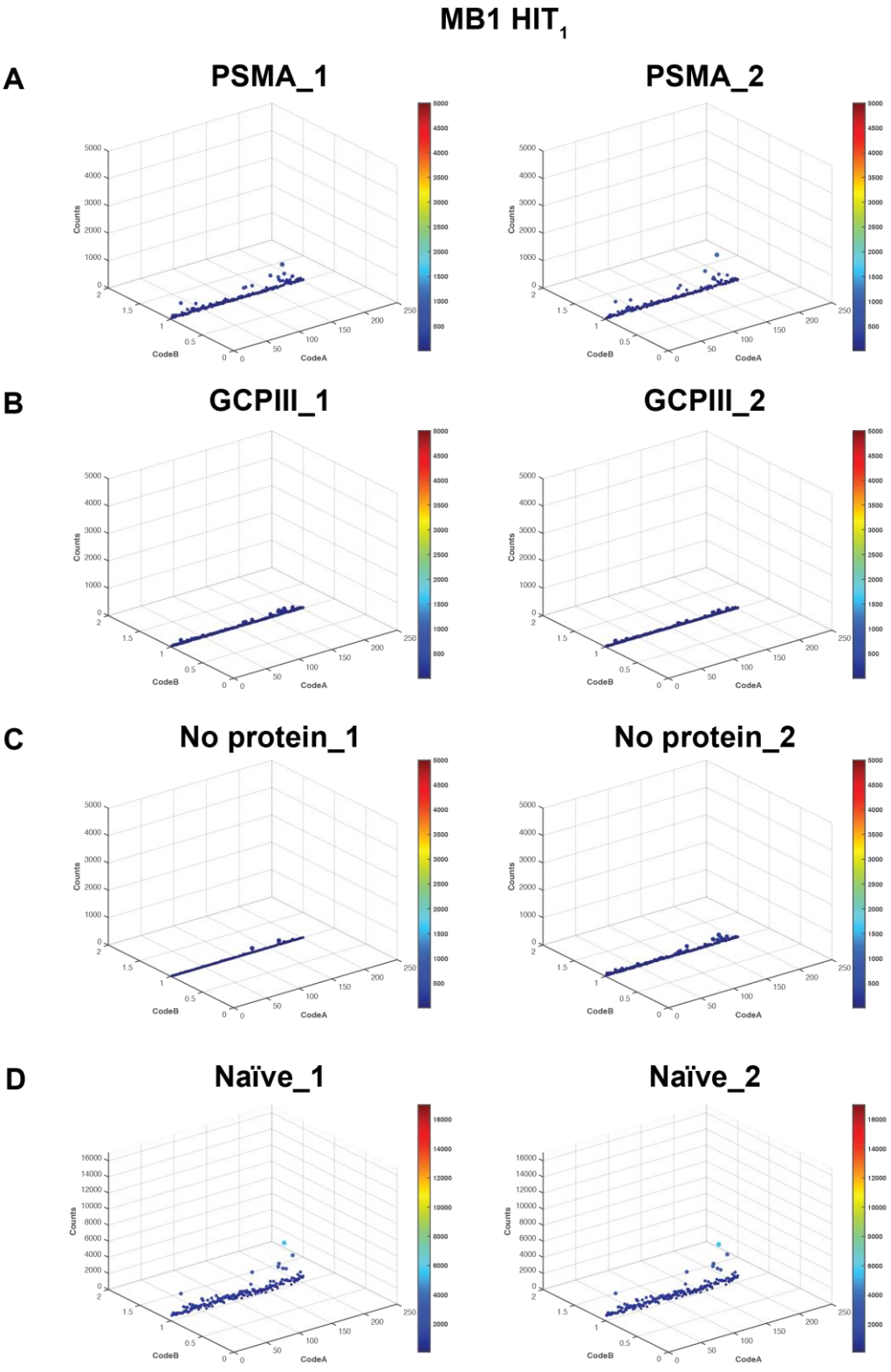

Figure S135. Fingerprints of MB1 HIT_1 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB1 HIT_1 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

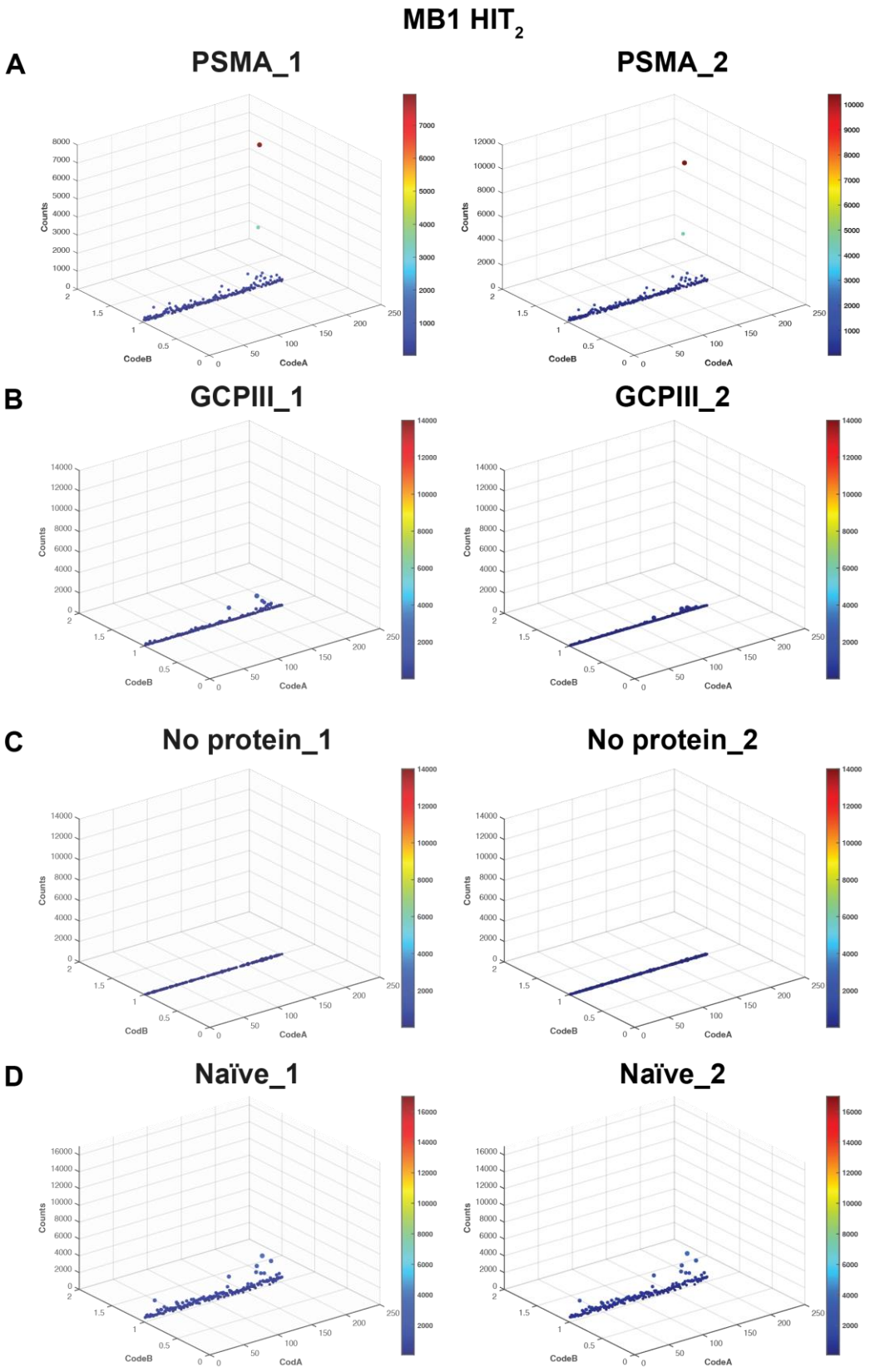

Figure S136. Fingerprints of MB1 HIT_2 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB1 HIT_2 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

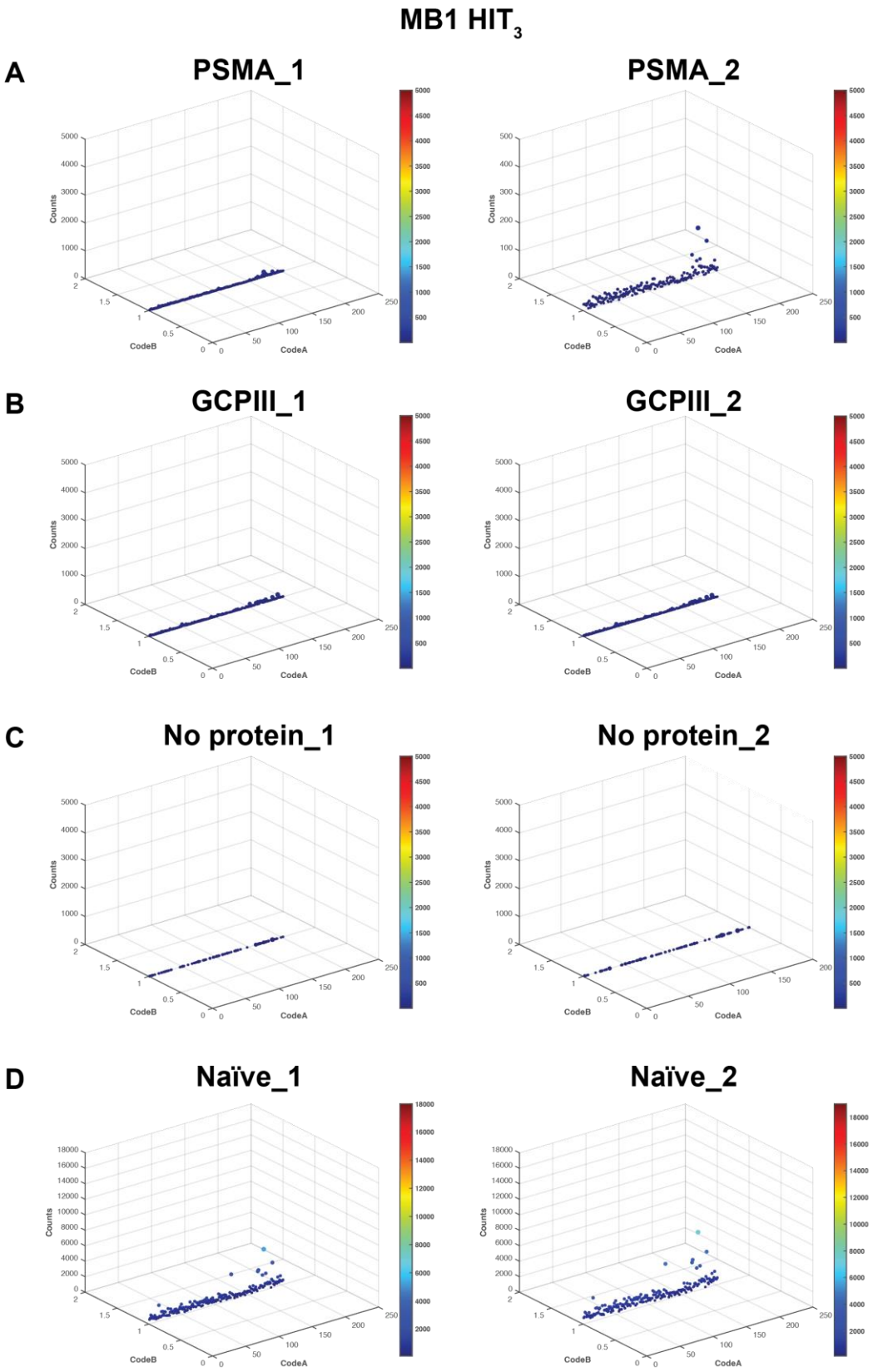

Figure S137. Fingerprints of MB1 HIT_3 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB1 HIT_3 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

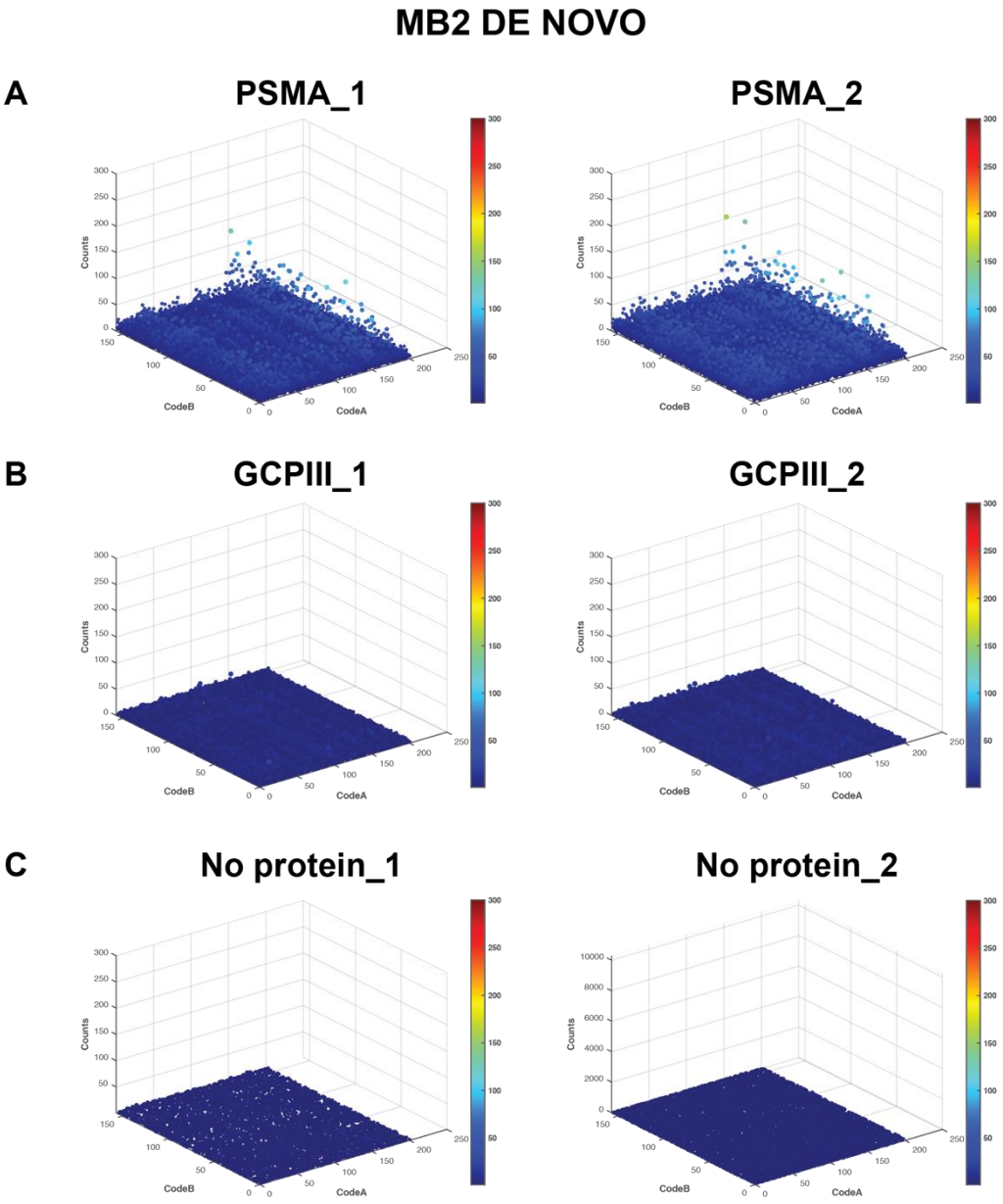

Figure S138. Fingerprints of MB2 de novo after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. Normalized sequence counts are color-coded as indicated by the color bar.

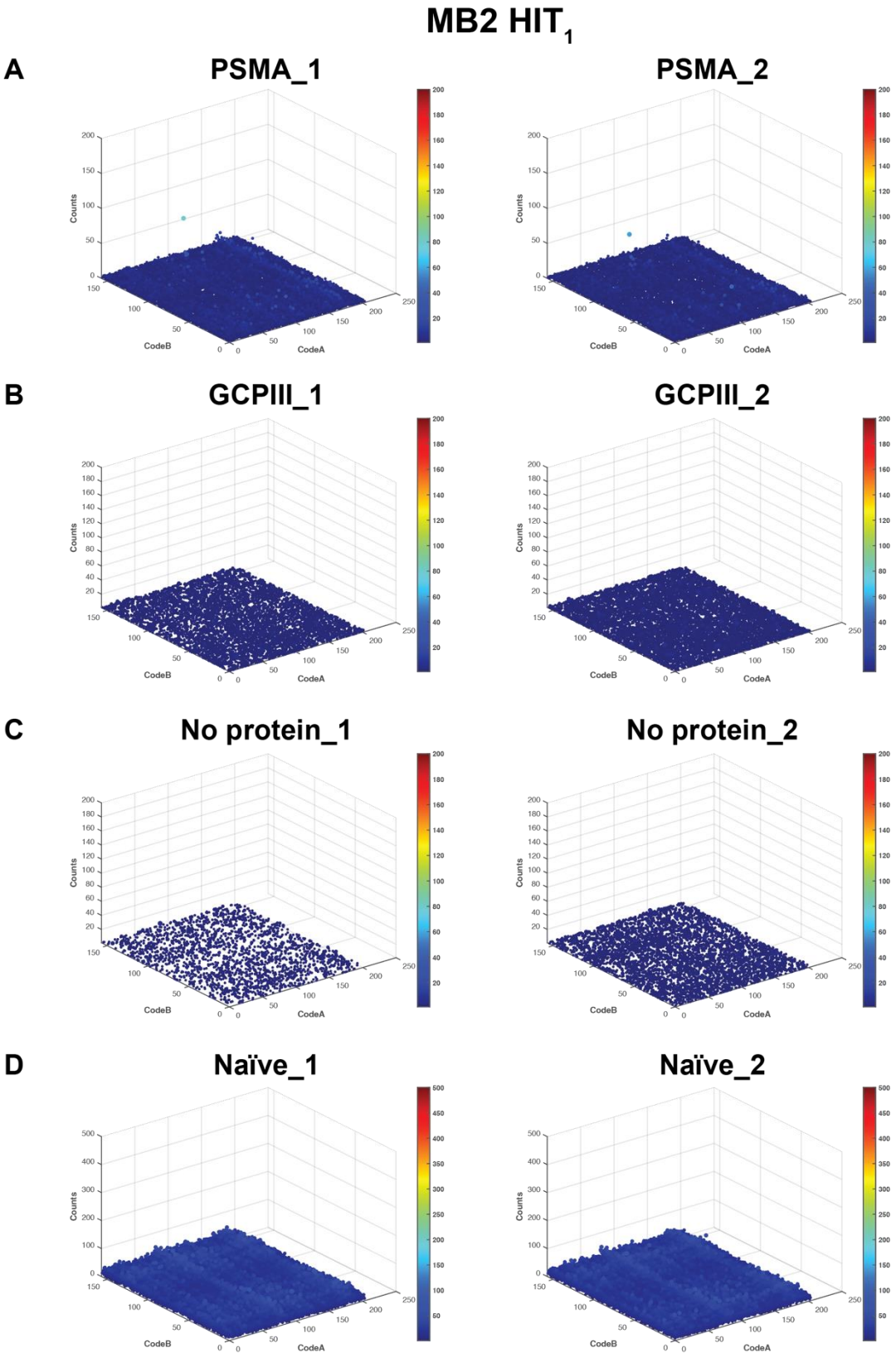

Figure S139. Fingerprints of MB2 HIT_1 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB2 HIT_1 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

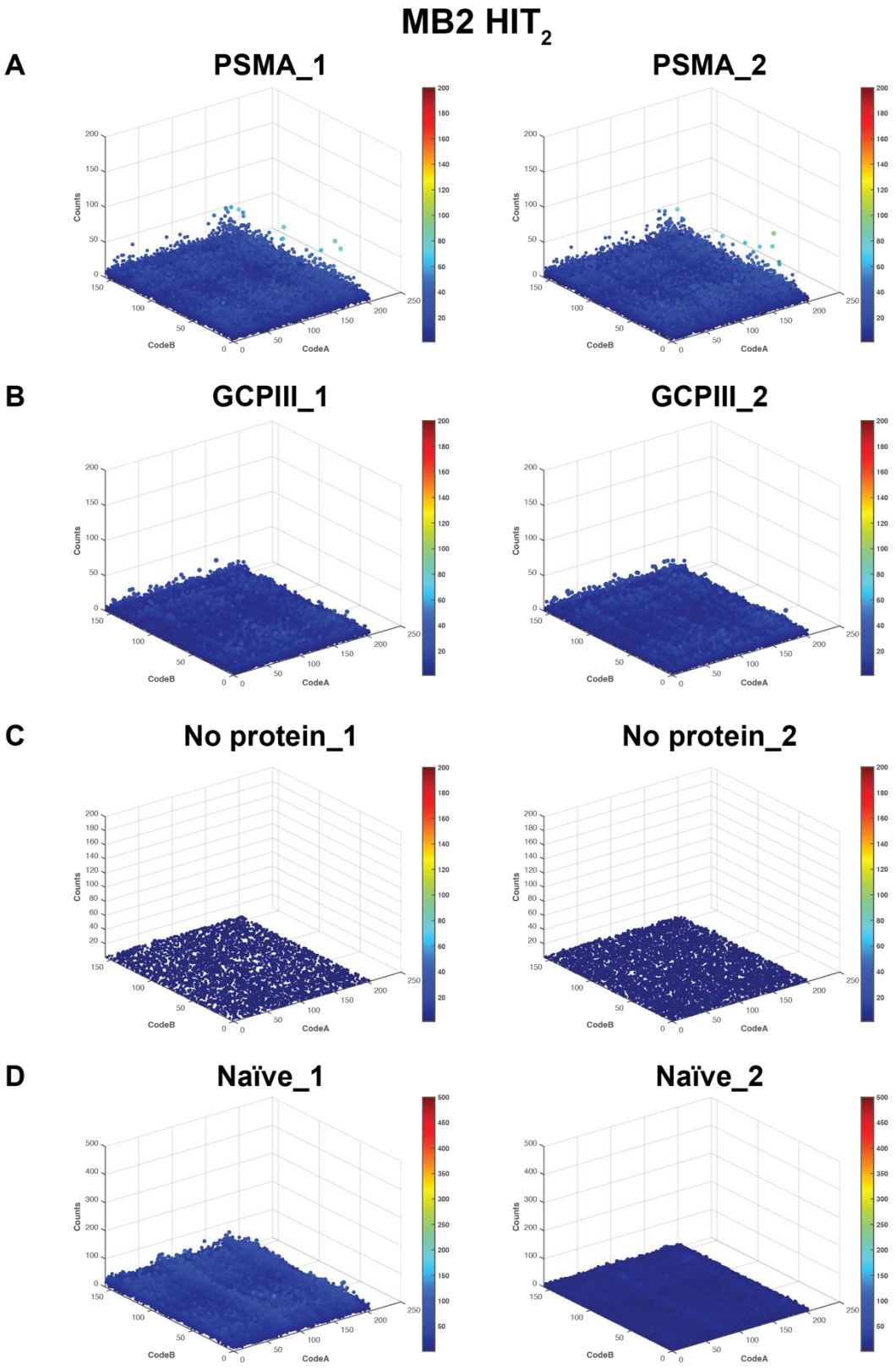

Figure S140. Fingerprints of MB2 HIT_2 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB2 HIT_2 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

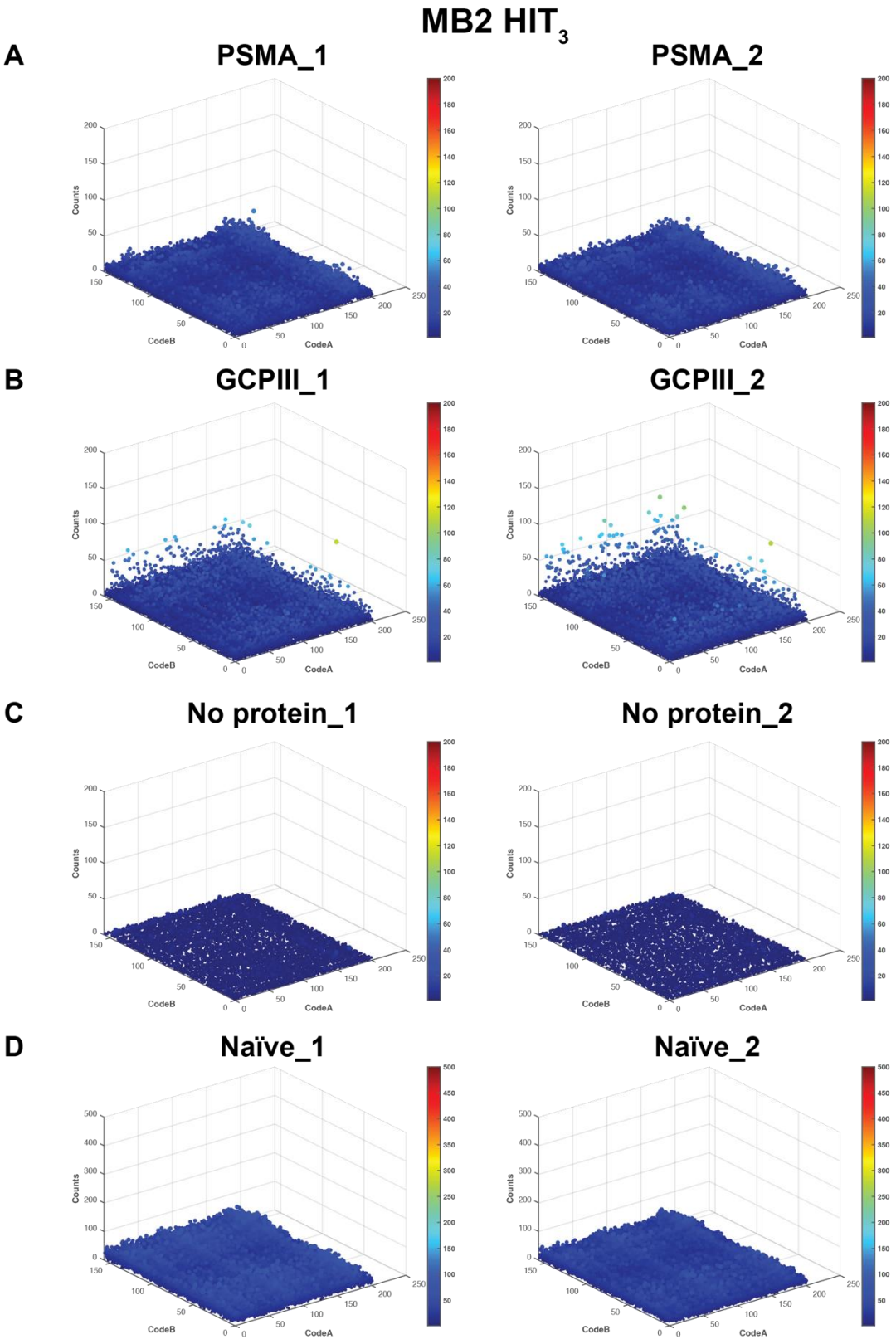

Figure S141. Fingerprints of MB2 HIT_3 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB2 HIT_3 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

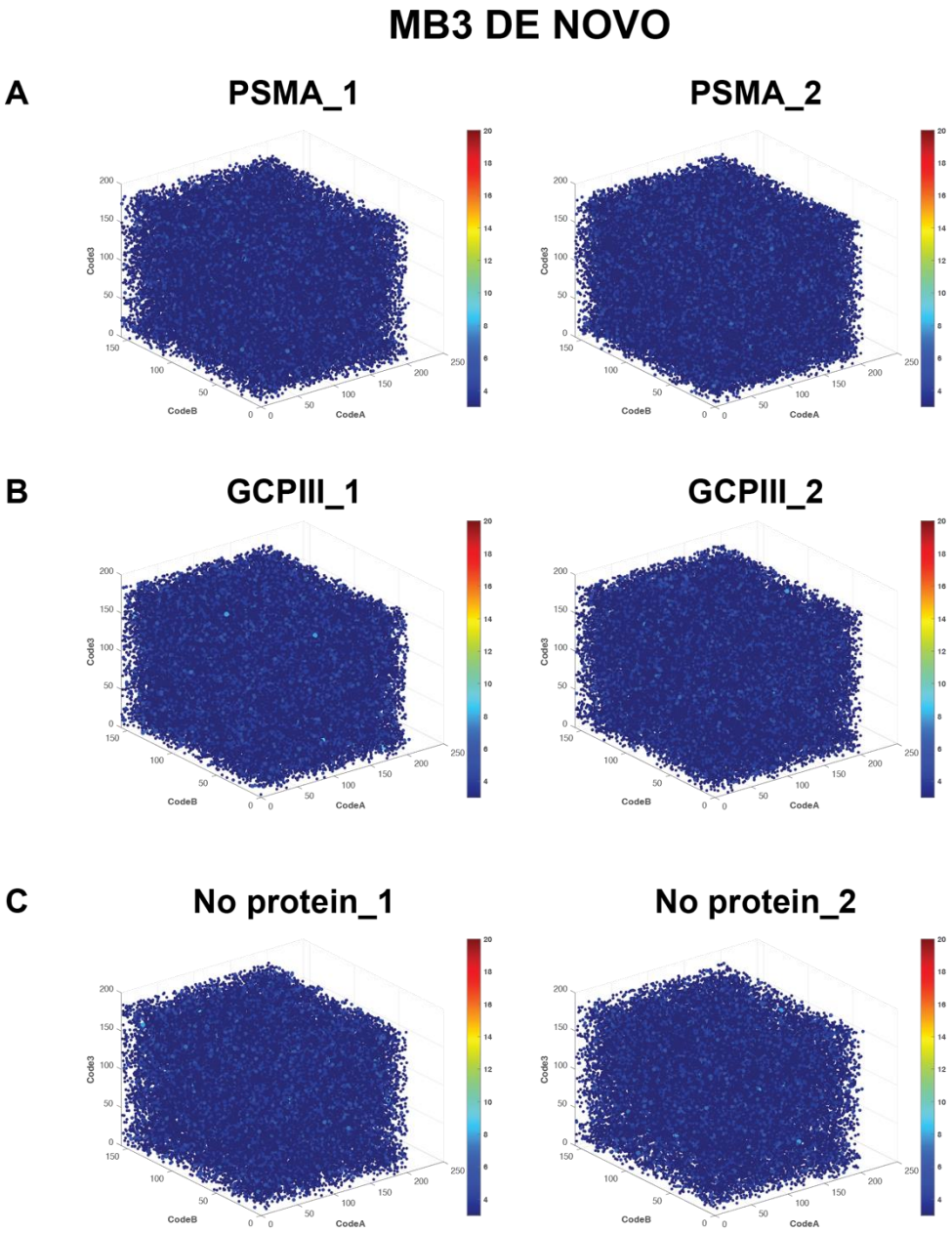

Figure S142. Fingerprints of MB3 de novo after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. Normalized sequence counts are color-coded as indicated by the color bar.

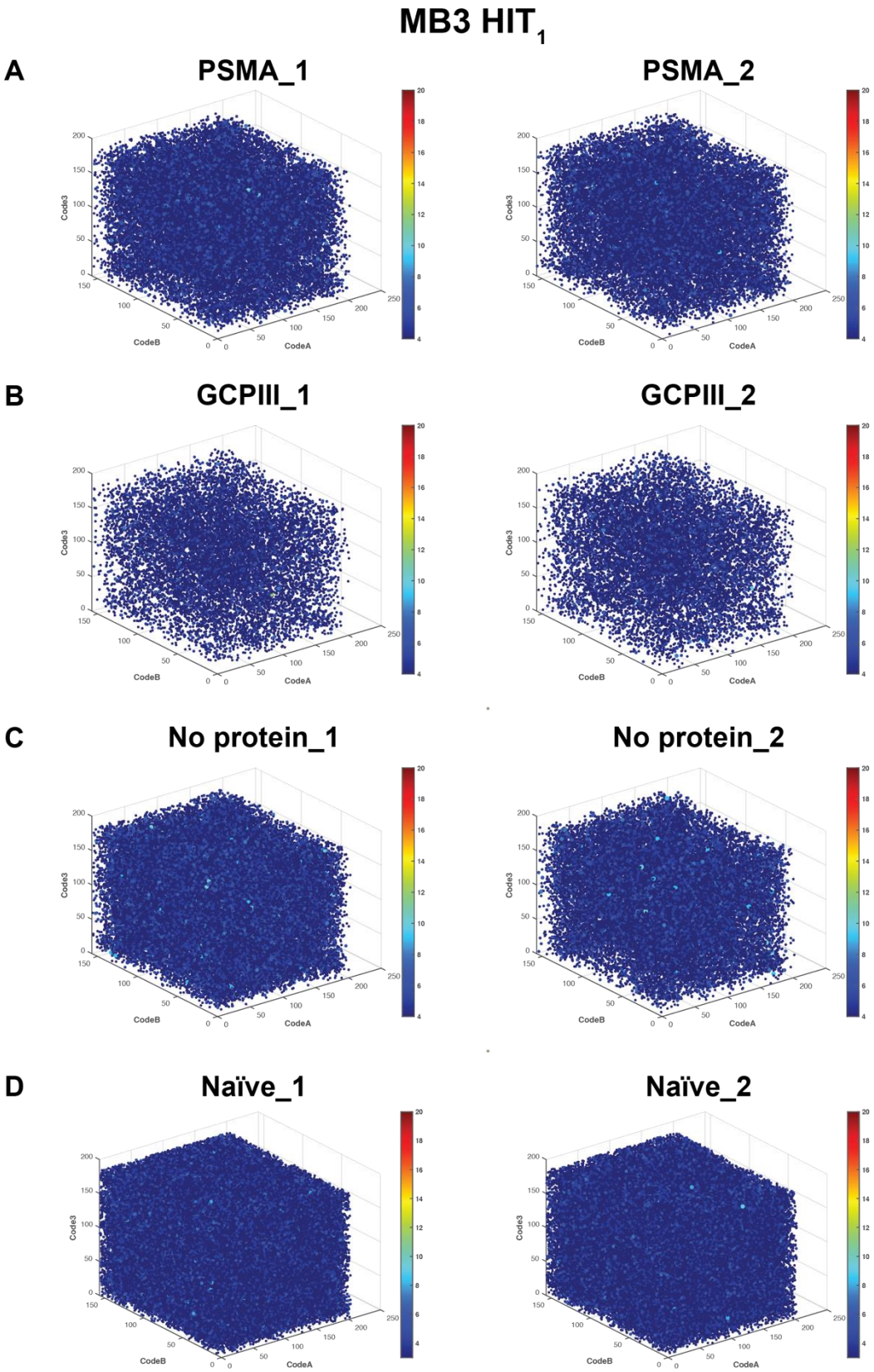

Figure S143. Fingerprints of MB3 HIT_1 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB3 HIT_1 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

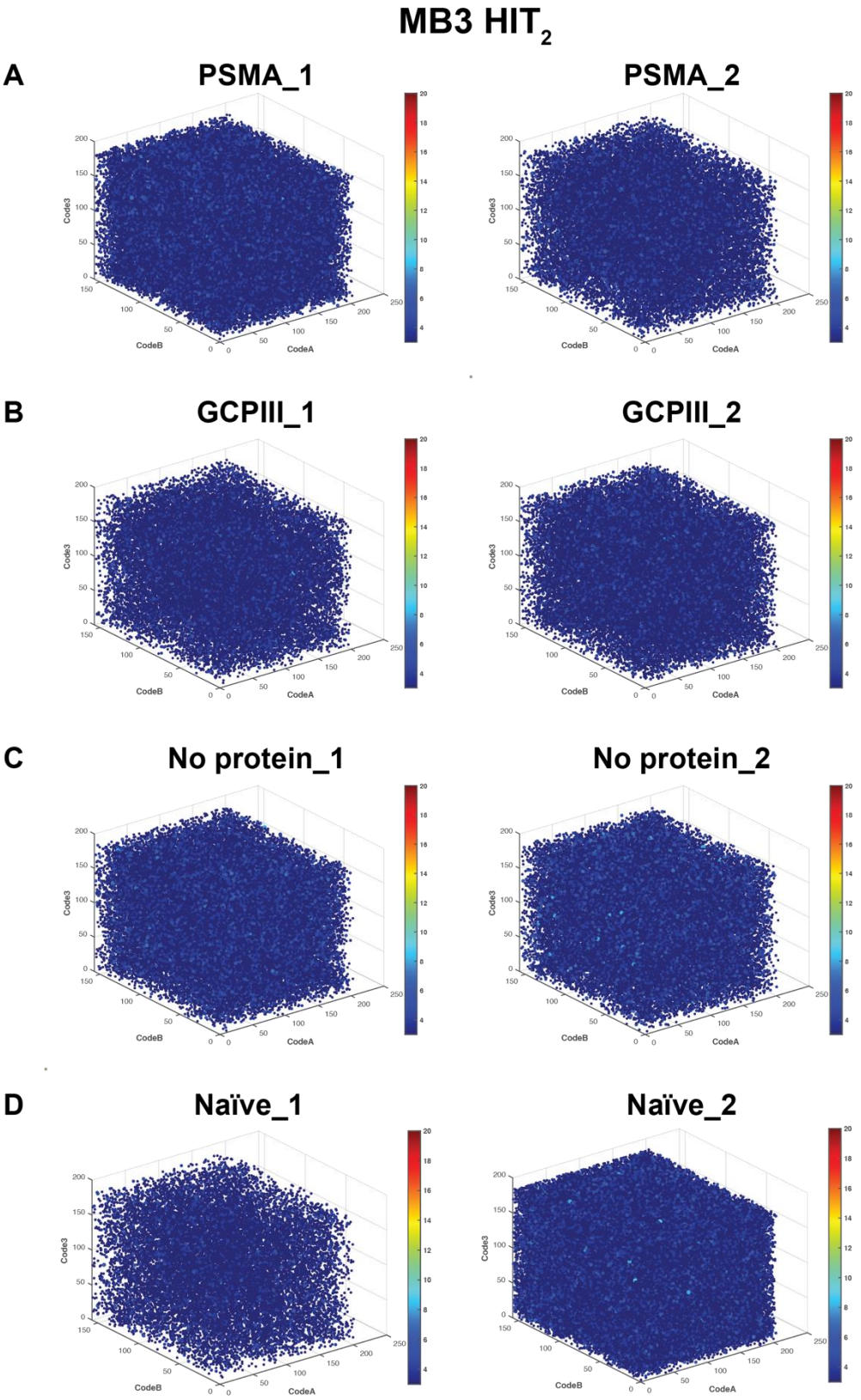

Figure S144. Fingerprints of MB3 HIT_2 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB3 HIT_2 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

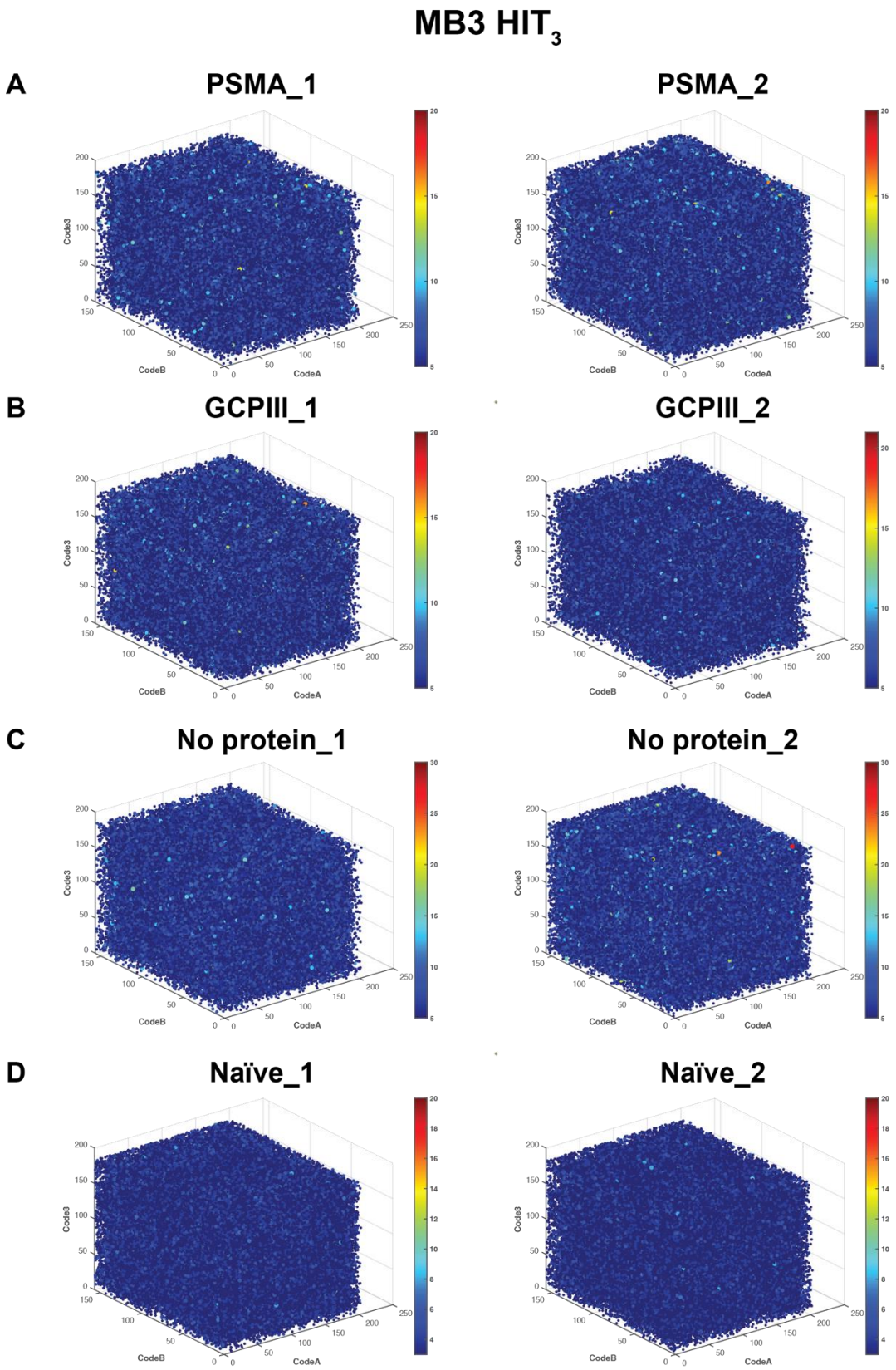

Figure S145. Fingerprints of MB3 HIT_3 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. MB3 HIT_3 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

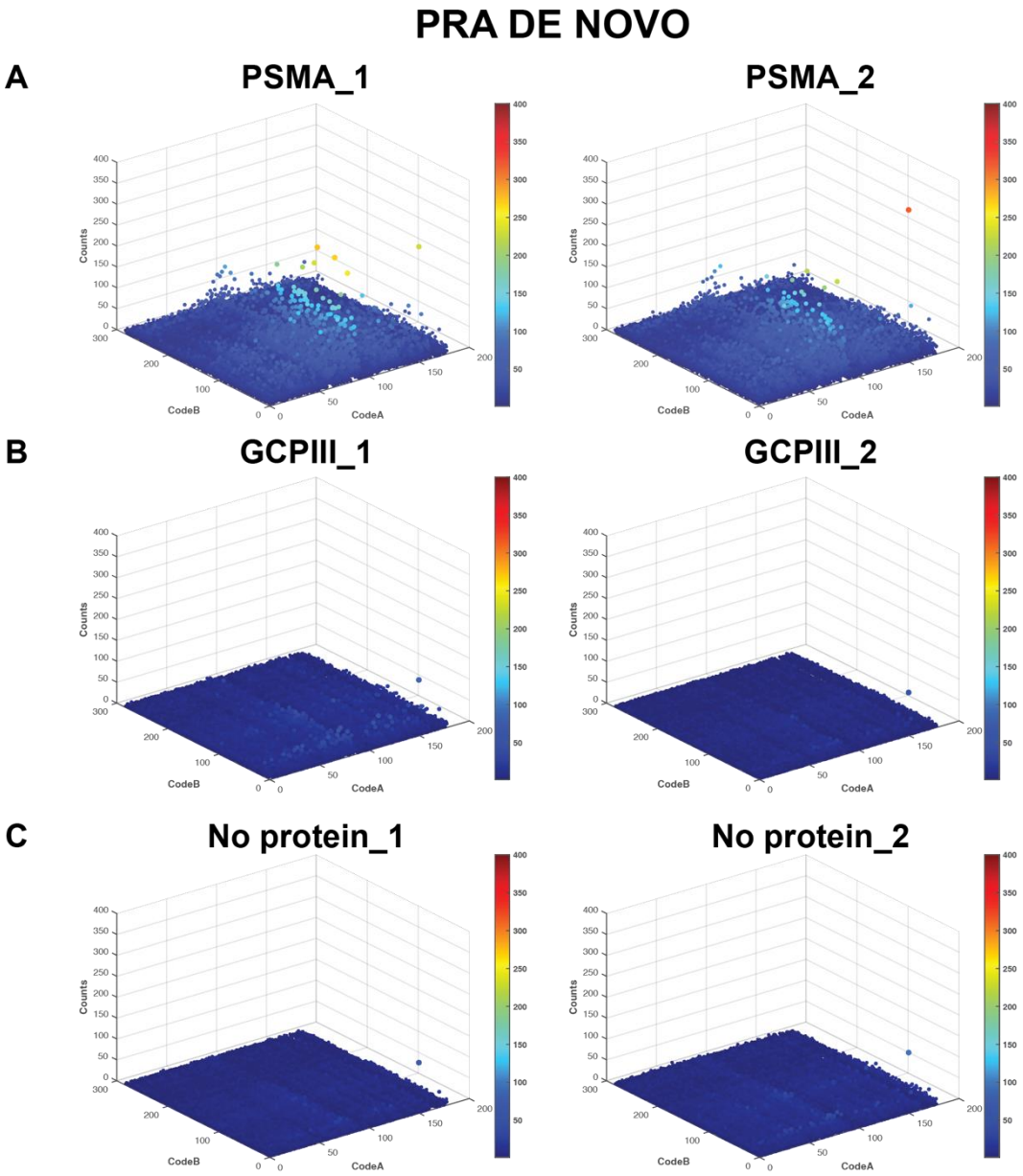

Figure S146. Fingerprints of PRA de novo after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. Normalized sequence counts are color-coded as indicated by the color bar.

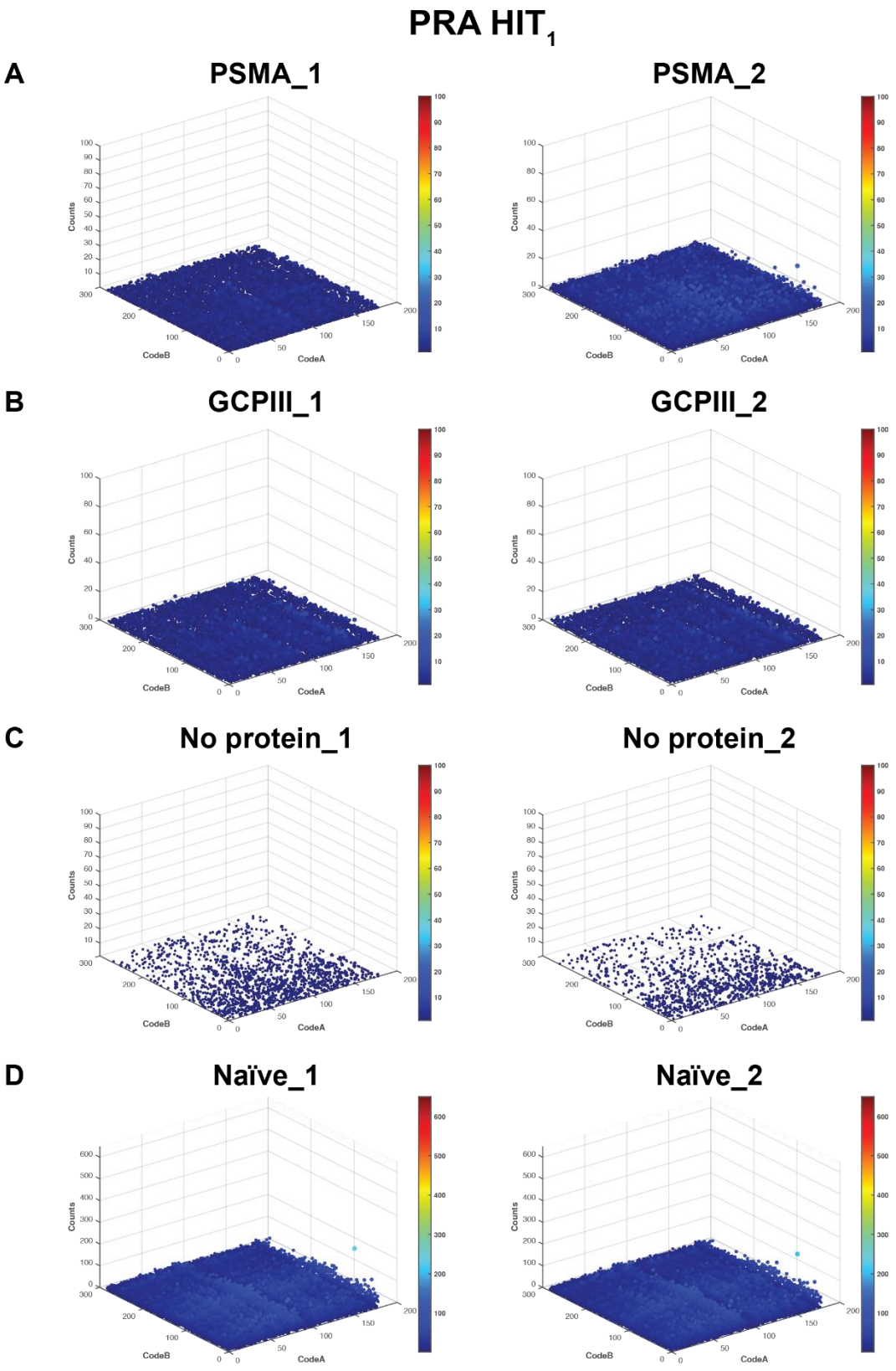

Figure S147. Fingerprints of PRA HIT_1 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. PRA HIT_1 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

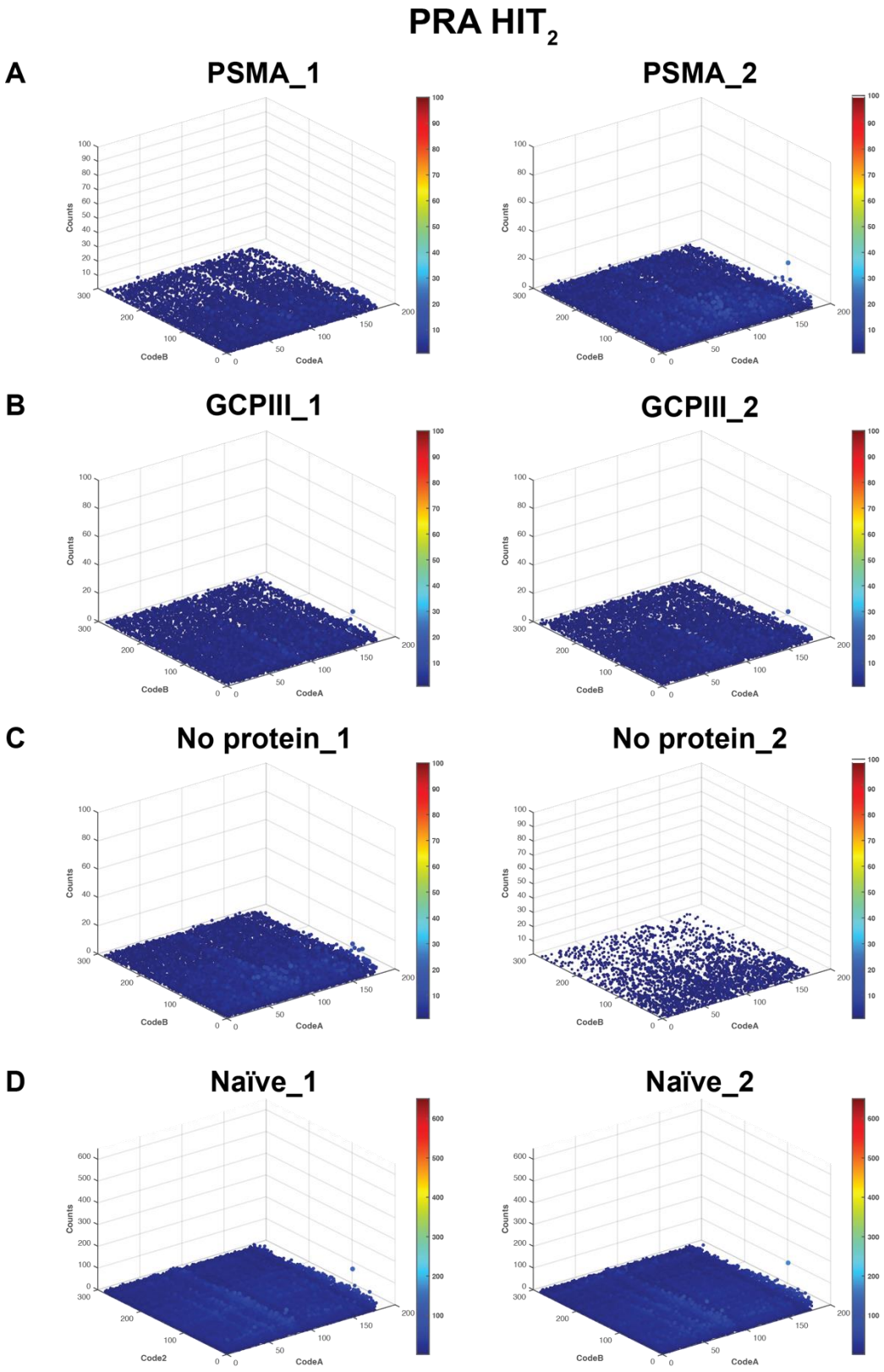

Figure S148. Fingerprints of PRA HIT_2 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. PRA HIT_2 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

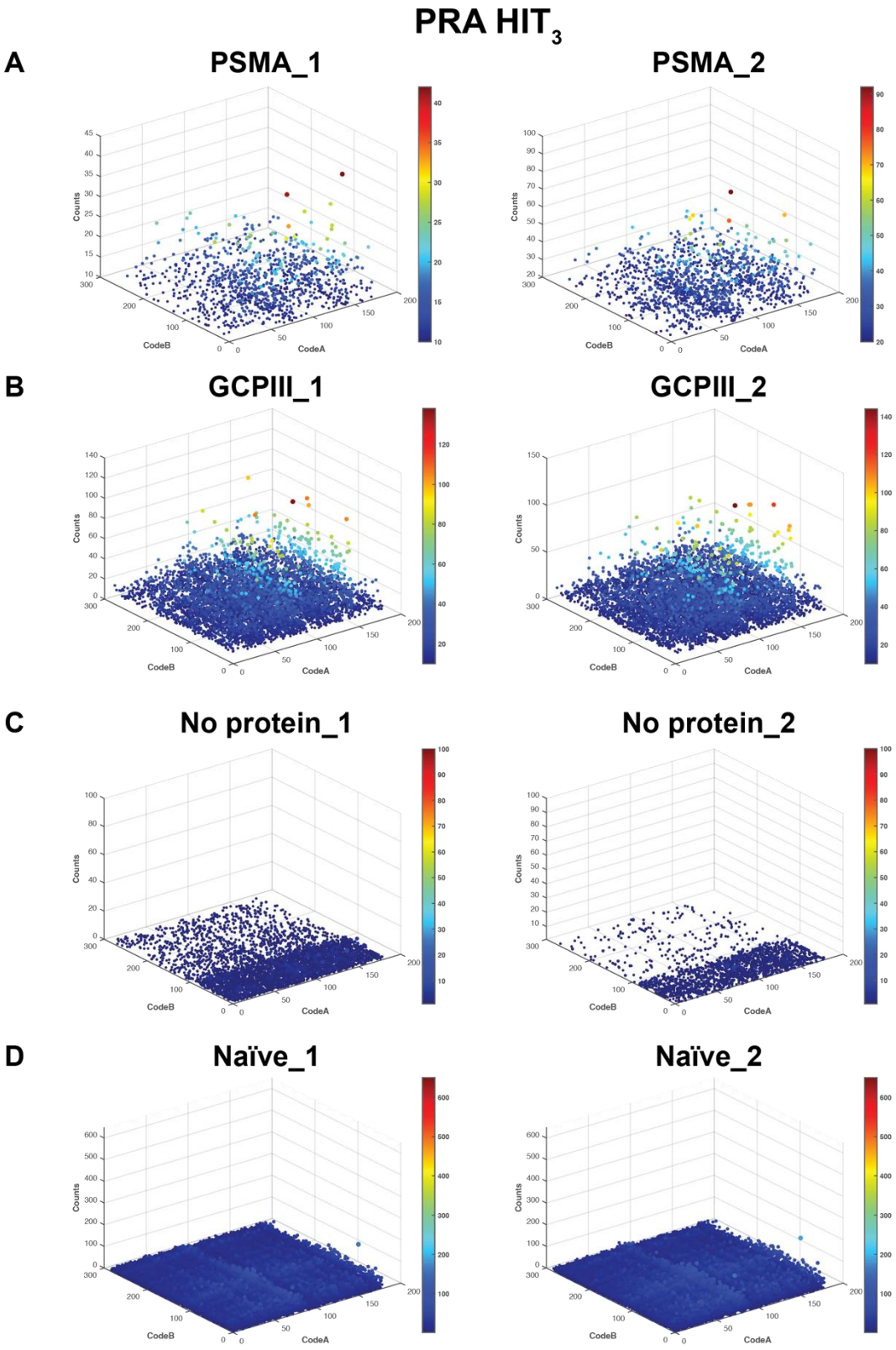

Figure S149. Fingerprints of PRA HIT_3 after performing affinity selections against PSMA (**A**), GCPIII (**B**) and no protein (**C**) in duplicates. PRA HIT_3 before performing selections (**D**) Normalized sequence counts are color-coded as indicated by the color bar.

15.2 ON-DNA LC-MS of Intermediates

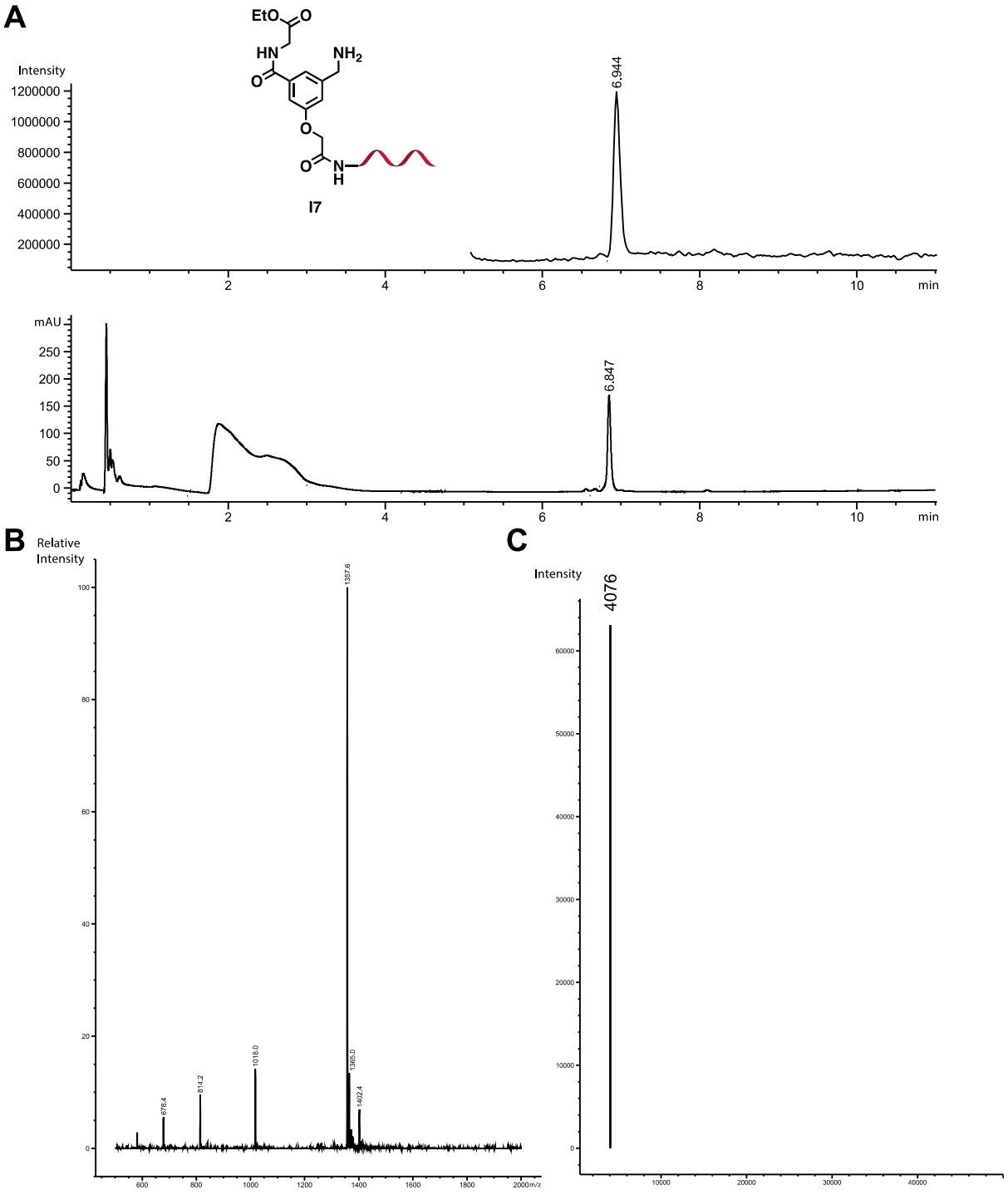

Figure S150. (**A**) MS trace and LC chromatogram of compound **I7**. (**B**) Raw mass spectrum of compound **I7** and its deconvolution (**C**) resulted in an observed mass of 4076 Da (calculated mass: 4076.1 Da).

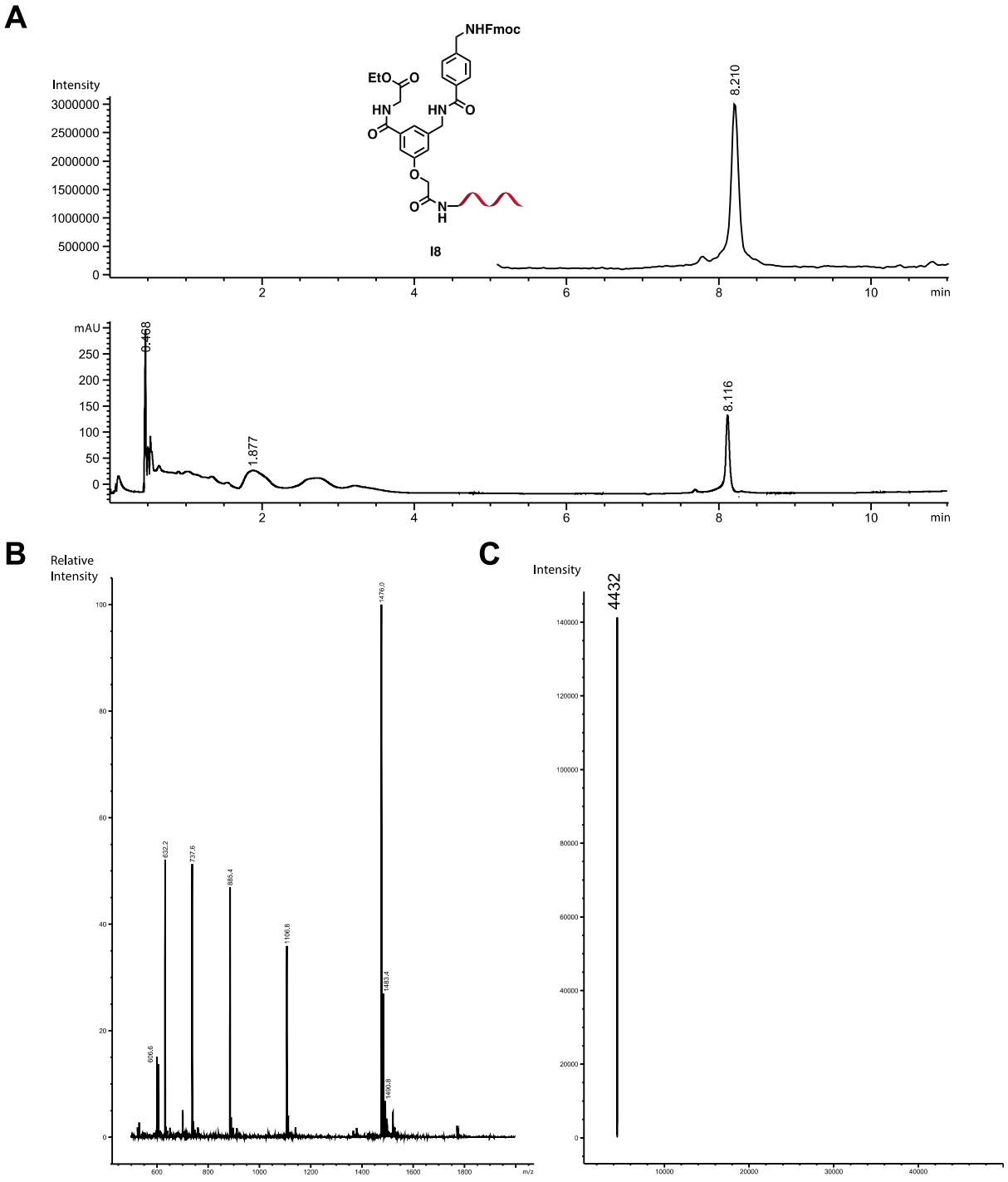

Figure S151. (**A**) MS trace and LC chromatogram of compound **I8**. (**B**) Raw mass spectrum of compound **I8** and its deconvolution (**C**) resulted in an observed mass of 4432 Da (calculated mass: 4431.2 Da).

15.3 NMR data

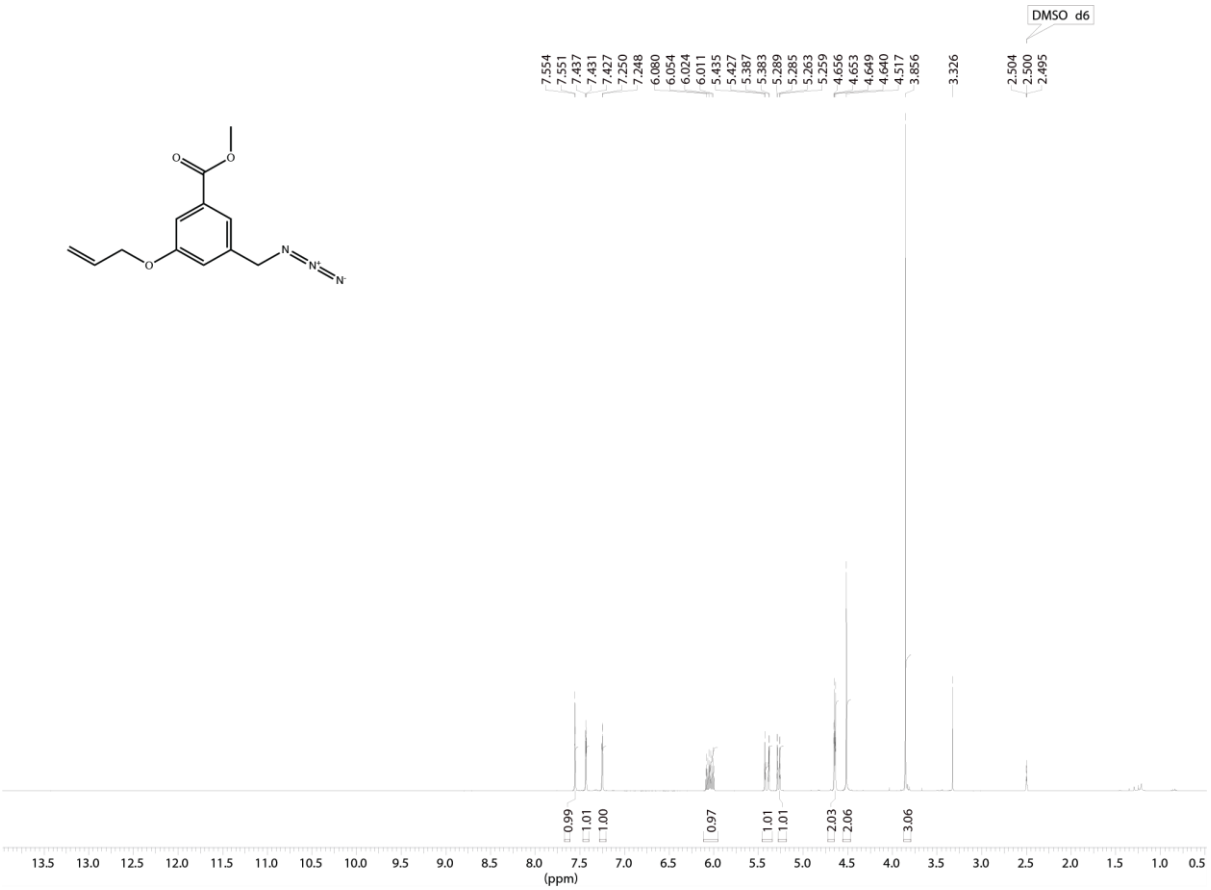

Figure S152. ^{1}H NMR of compound **I3**.

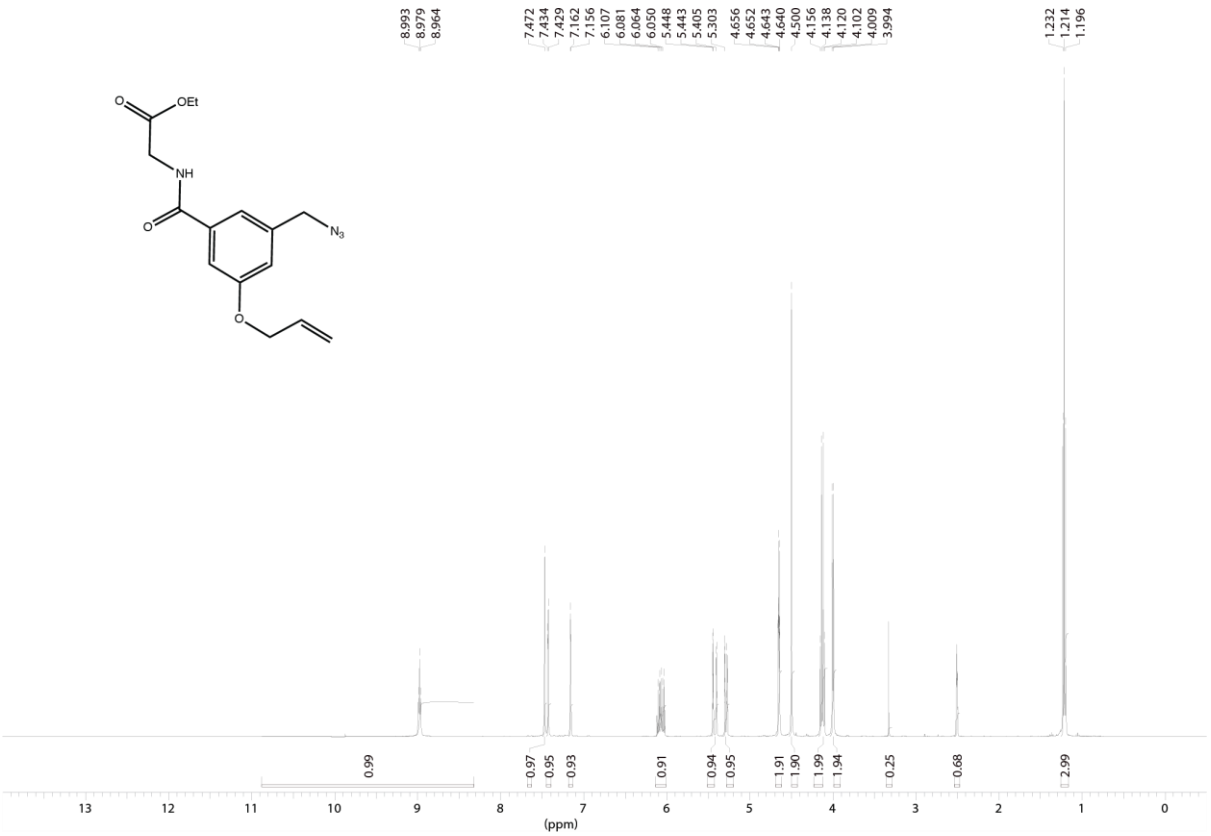

Figure S153. ^{1}H NMR of compound **I4**.

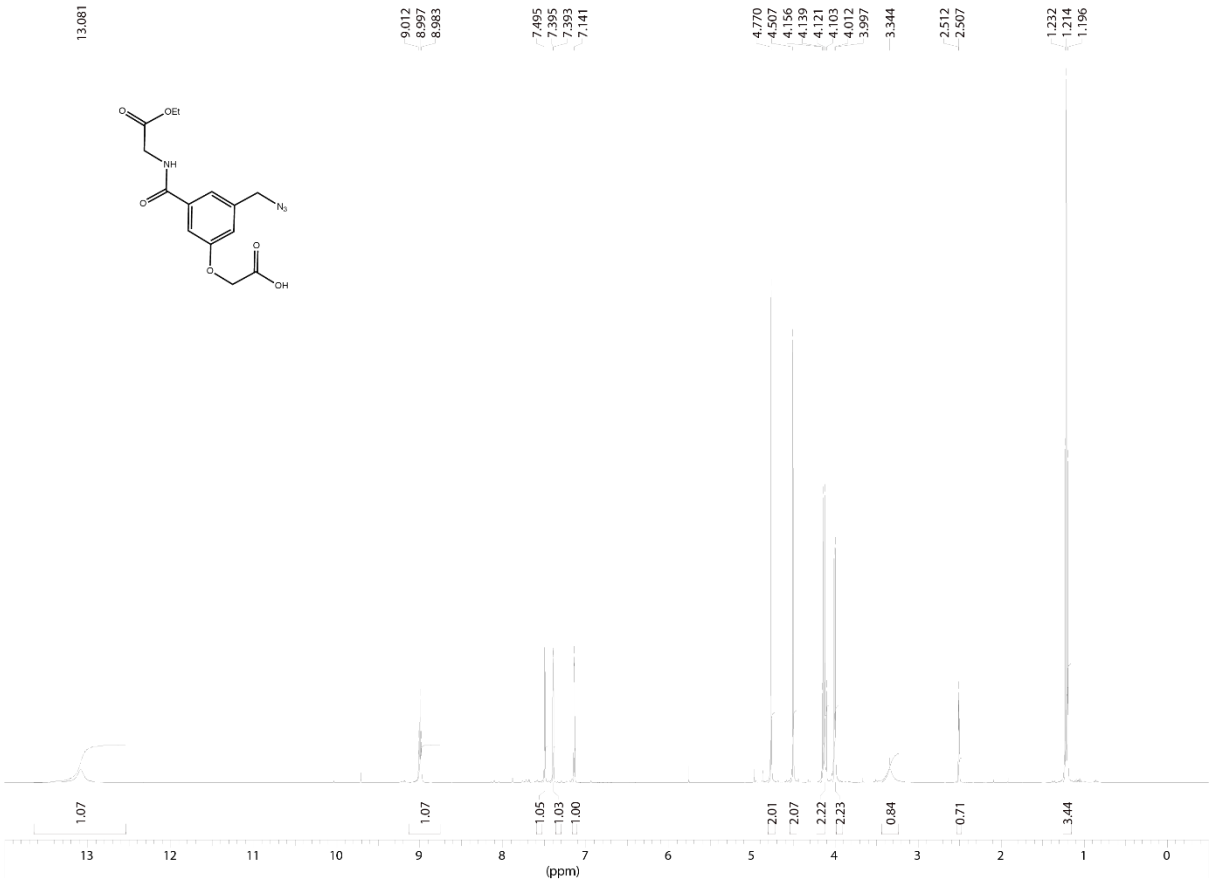

Figure S154. ^{1}H NMR of compound **19**.

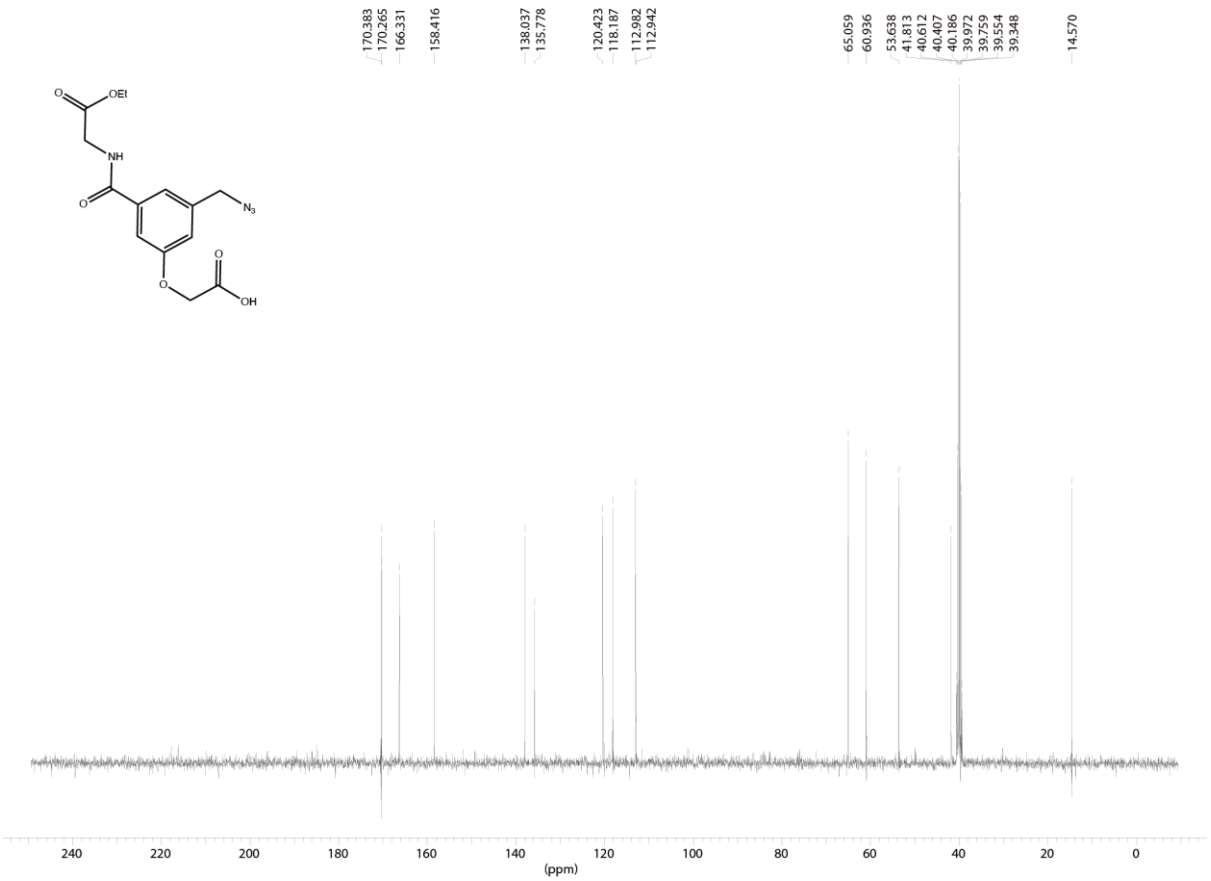

Figure S155. ^{13}C NMR of compound **19**.

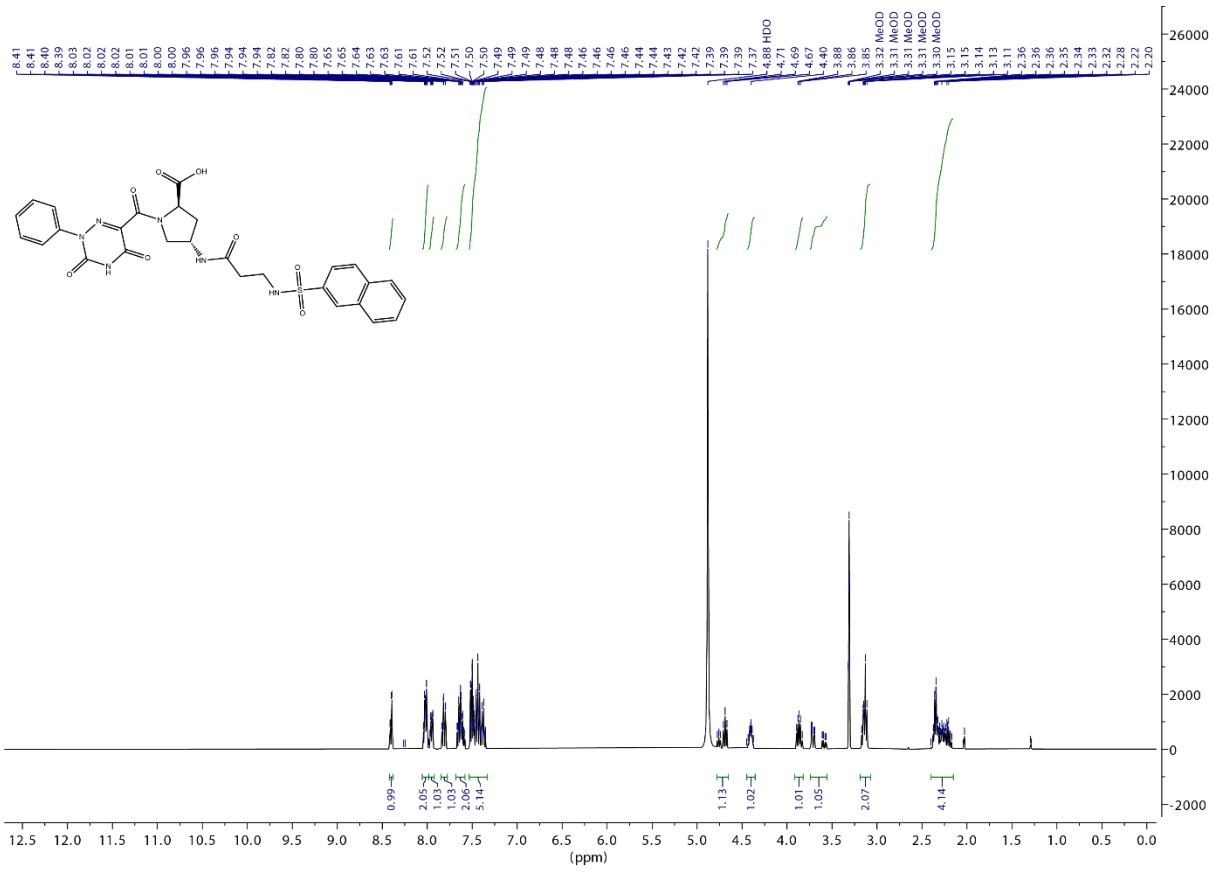

Figure S156. ^{1}H NMR of compound **HIT$_2$**.

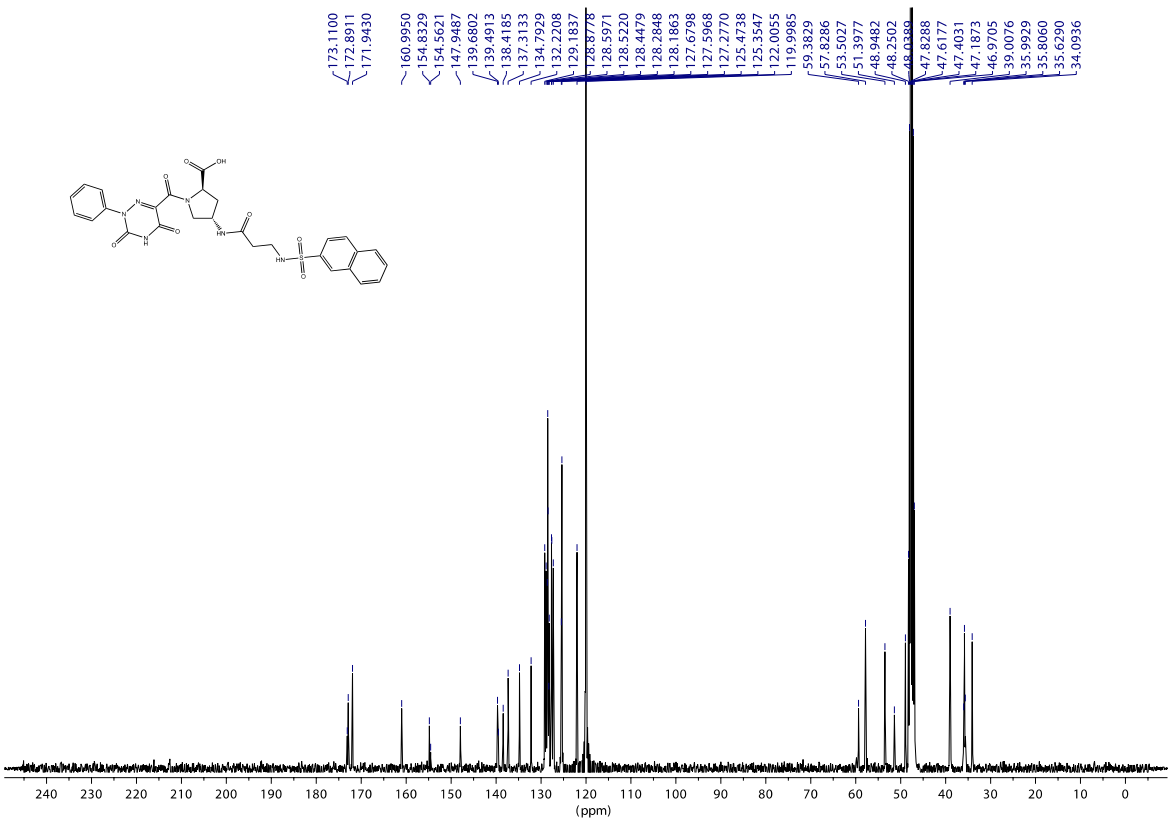

Figure S157. ^{13}C NMR of compound **HIT$_2$**.

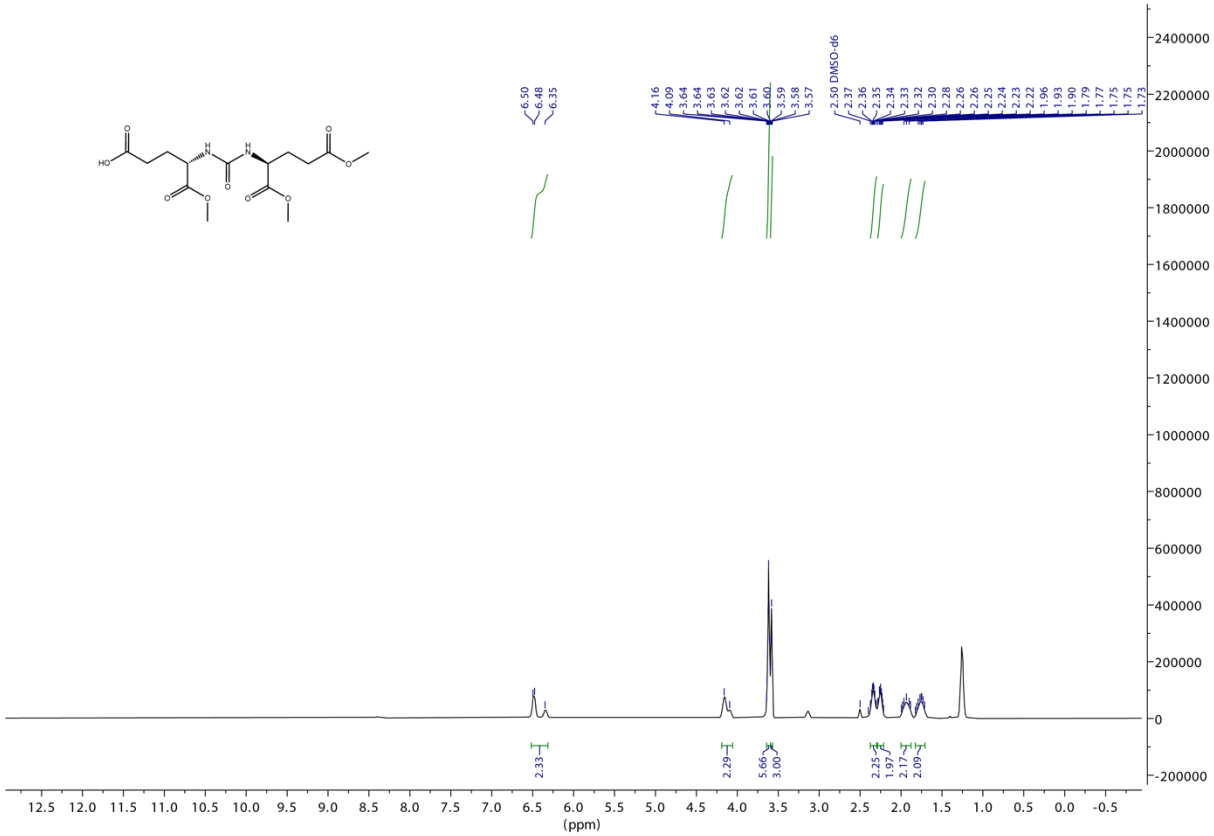

Figure S158. ^{1}H NMR of compound **Me-HIT3**.

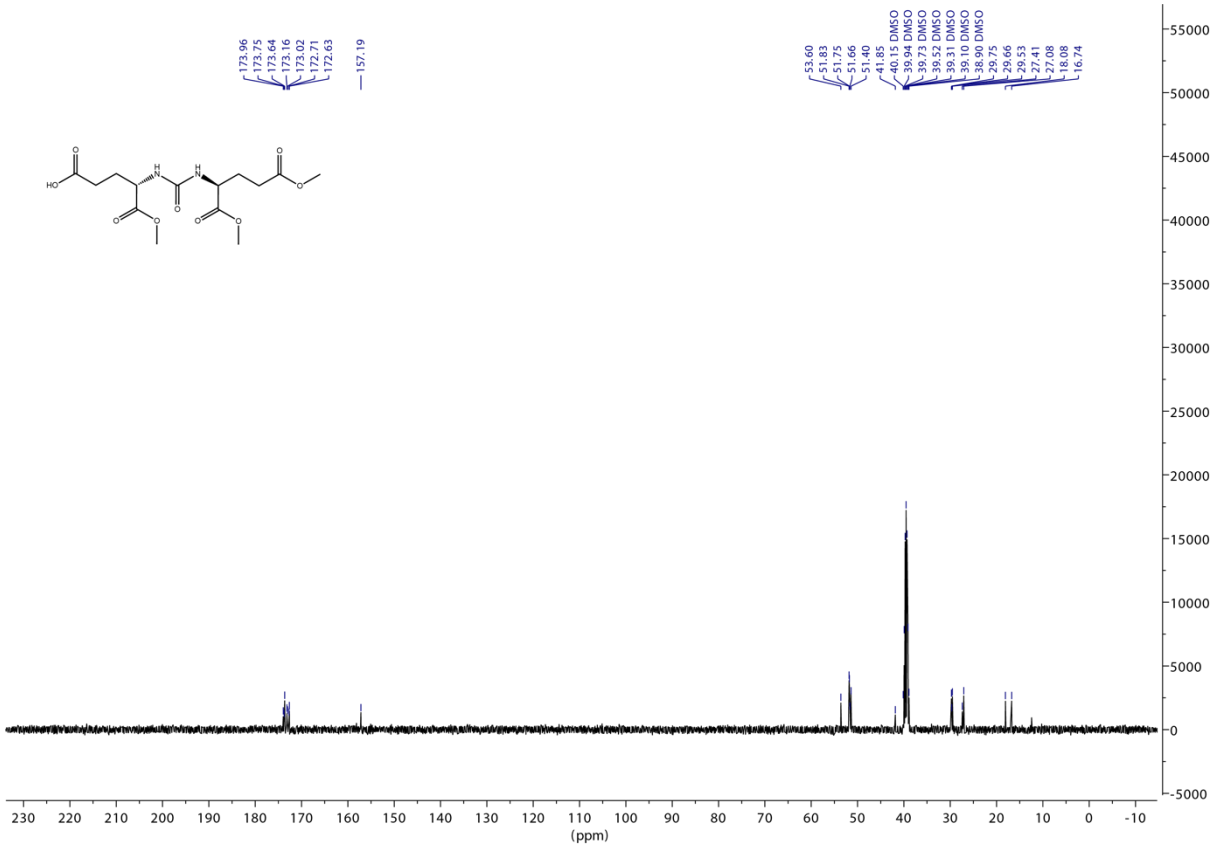

Figure S159. ^{13}C NMR of compound **Me-HIT$_3$**.

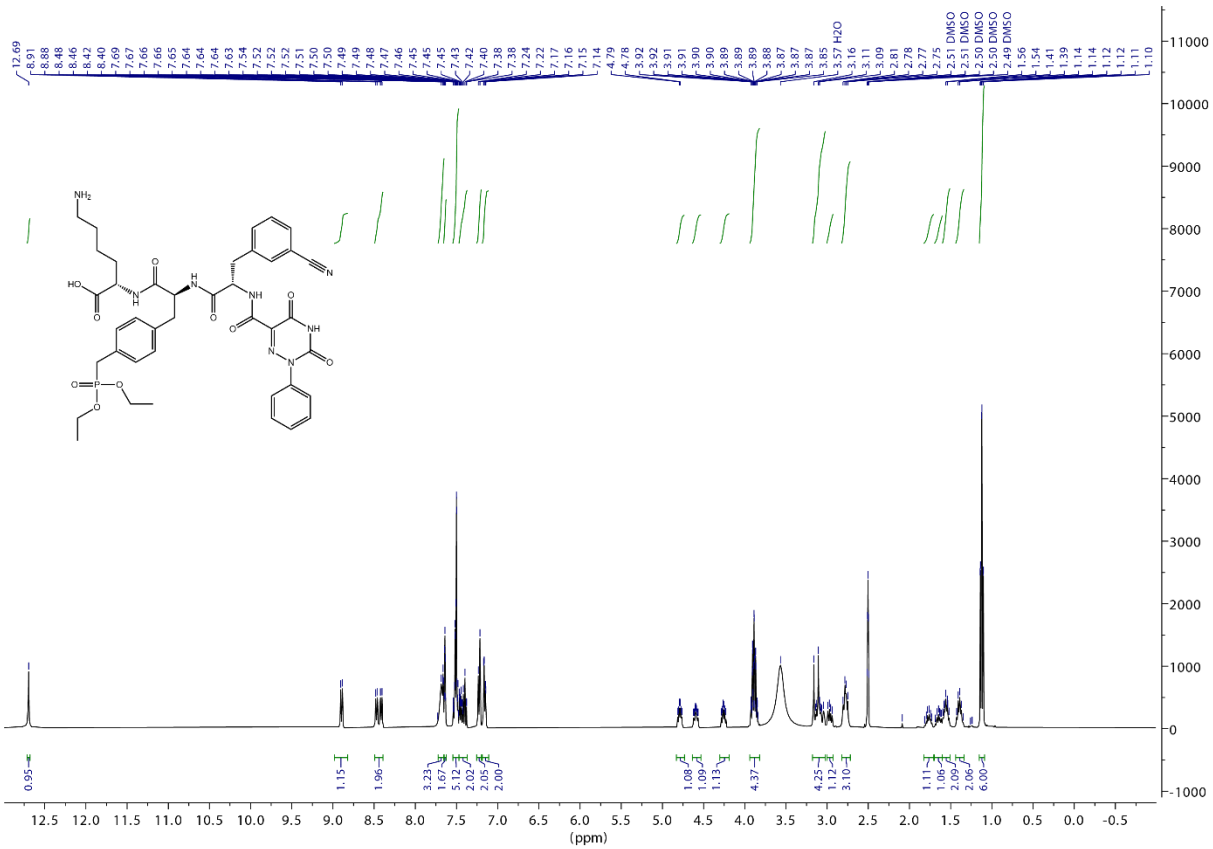

Figure S160. ^{1}H NMR of compound **I9**.

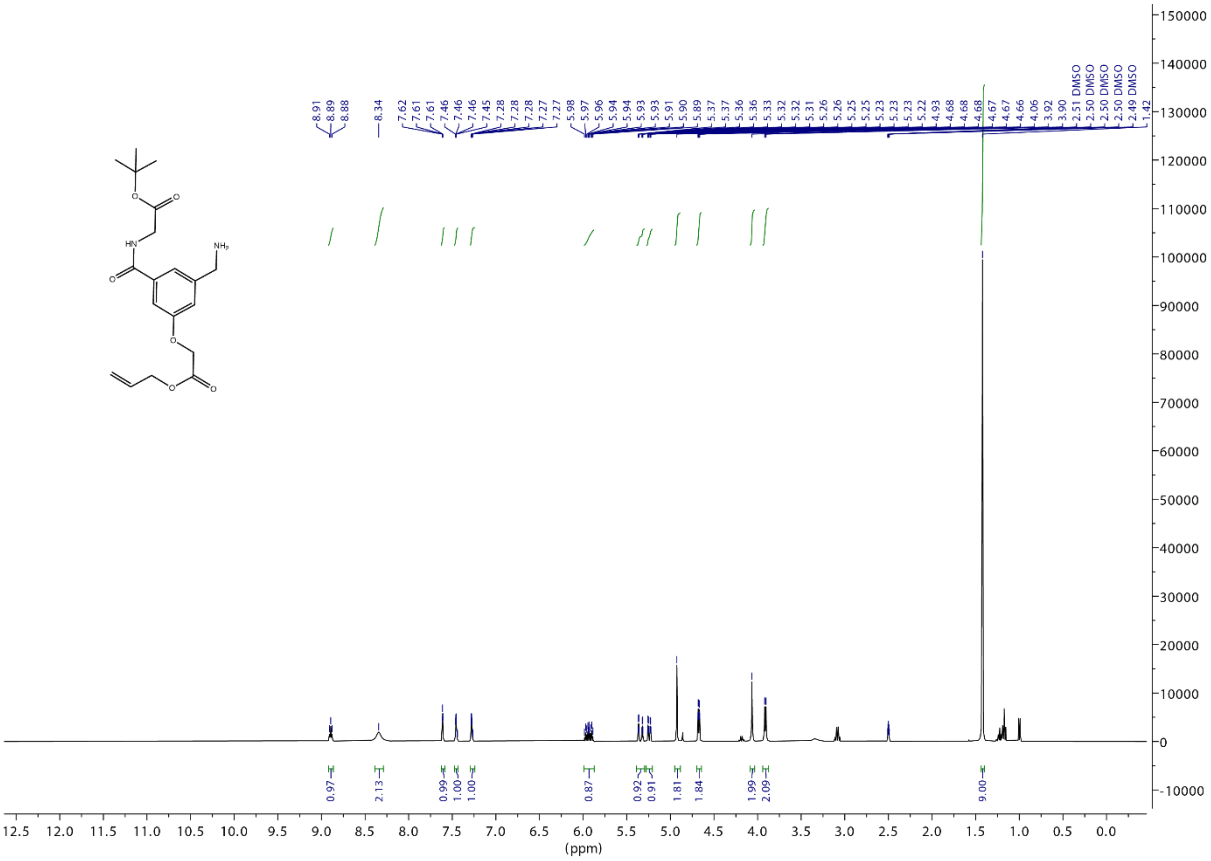

Figure S161. ^{1}H NMR of compound **I11**.

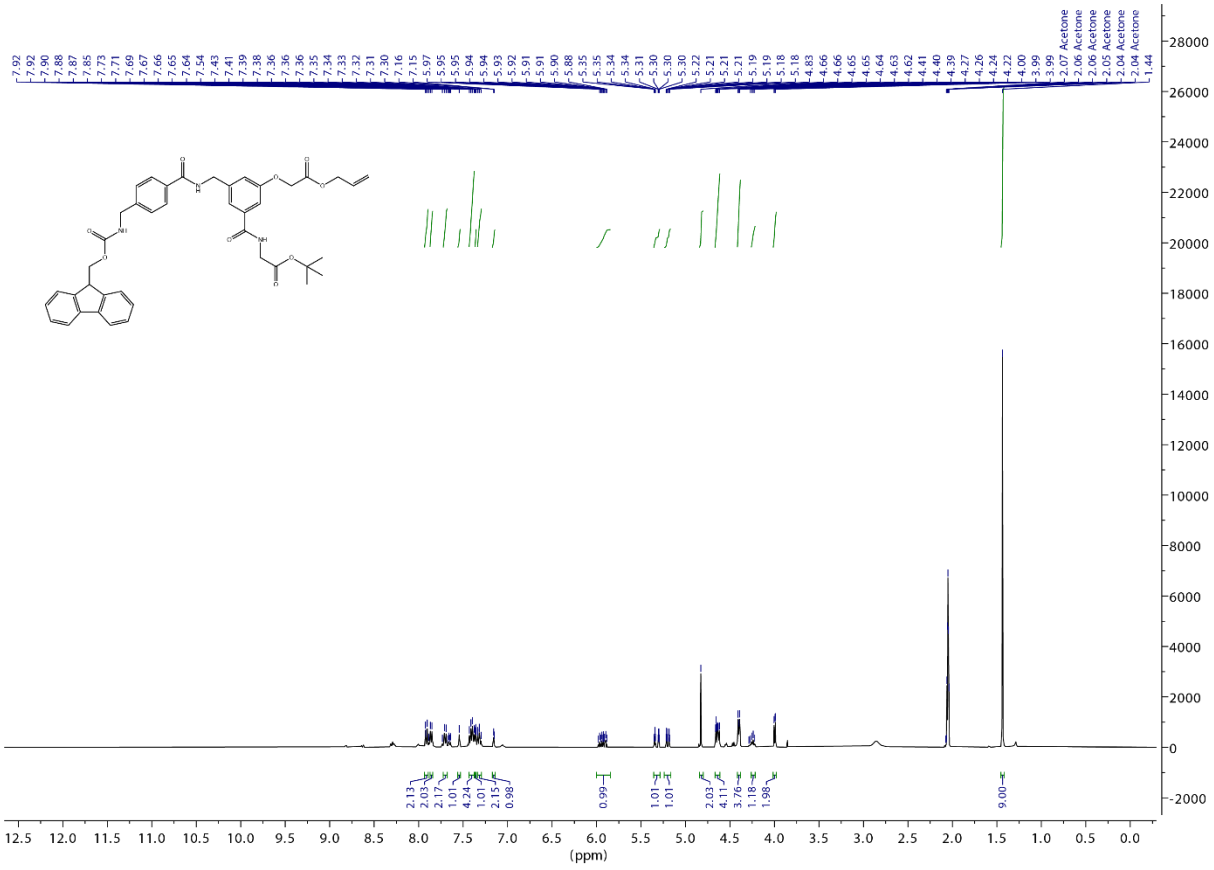

Figure S162. ^{1}H NMR of compound **I12**.

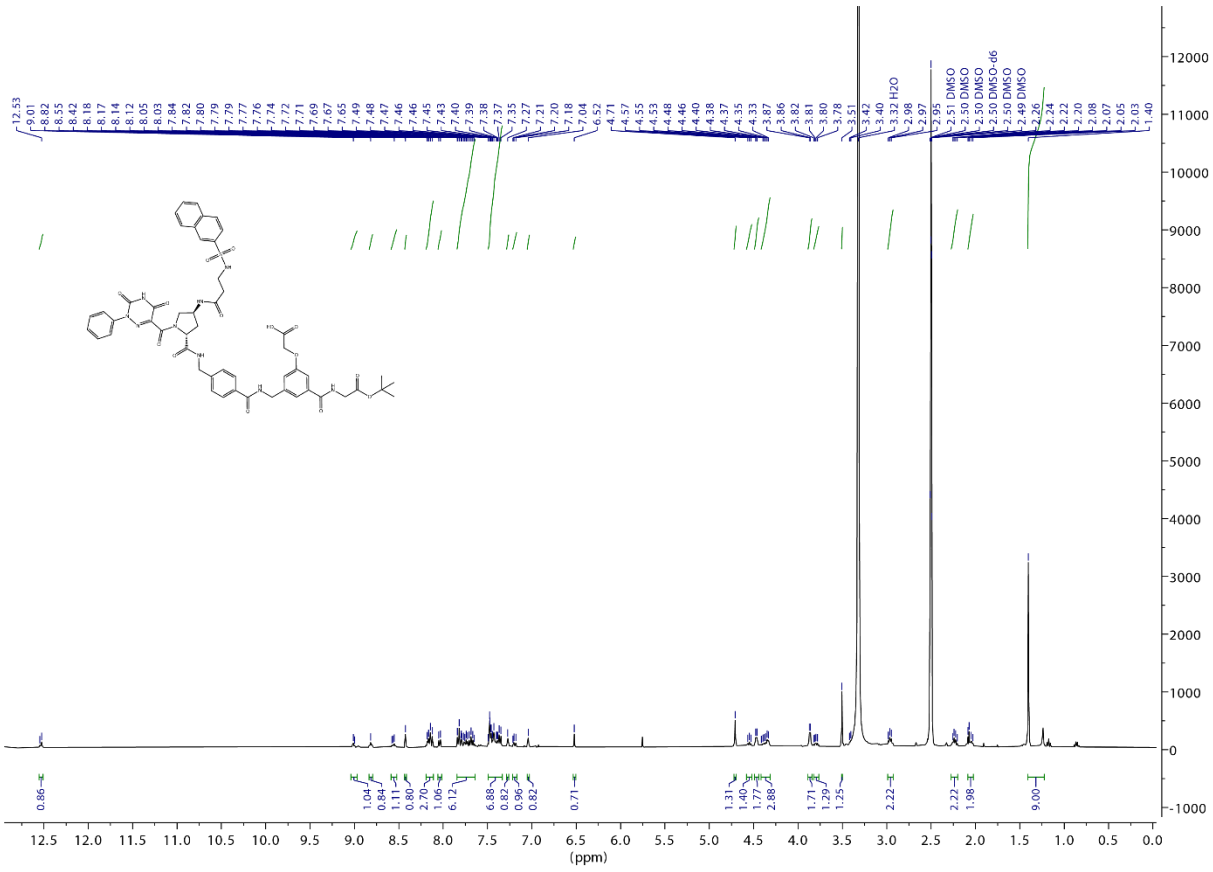

Figure S163. ^{1}H NMR of compound **I13**.

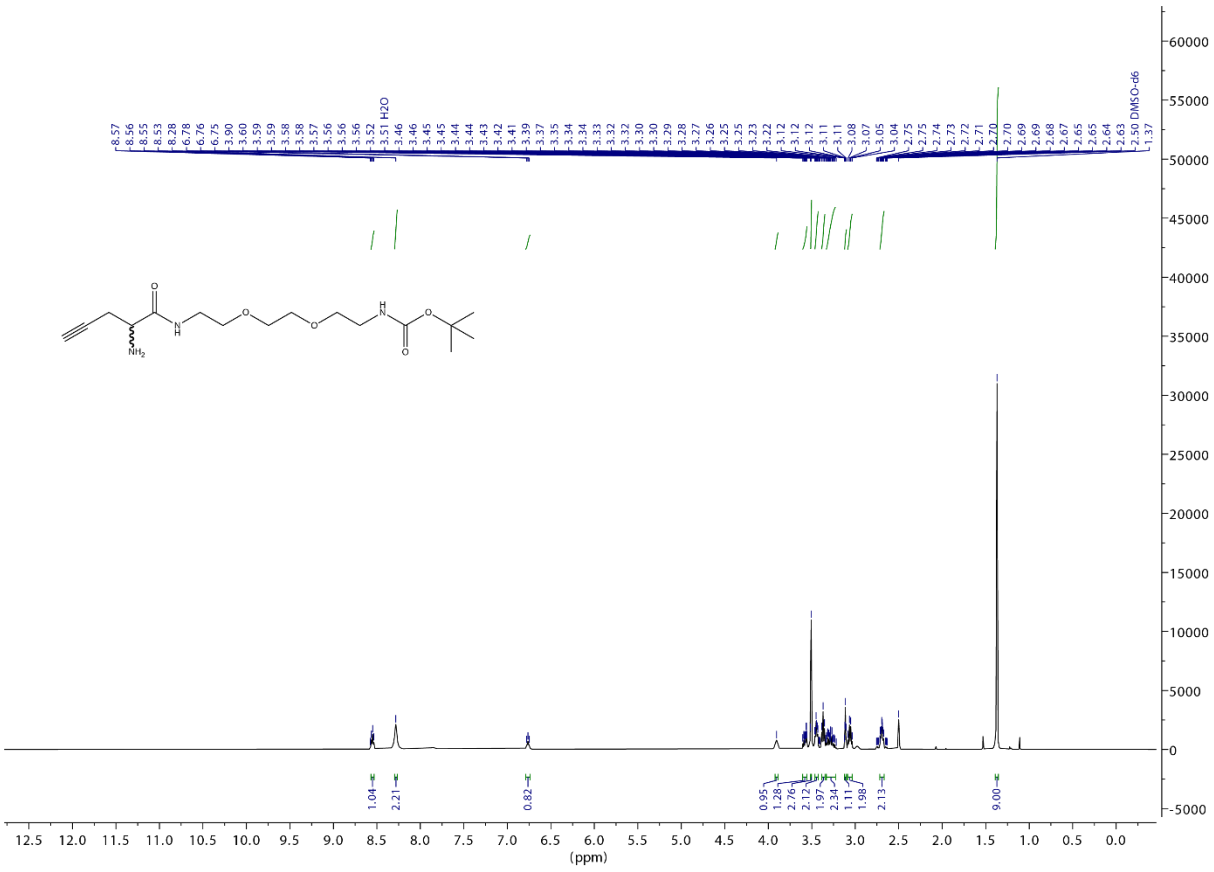

Figure S164. ^{1}H NMR of compound **I17**.

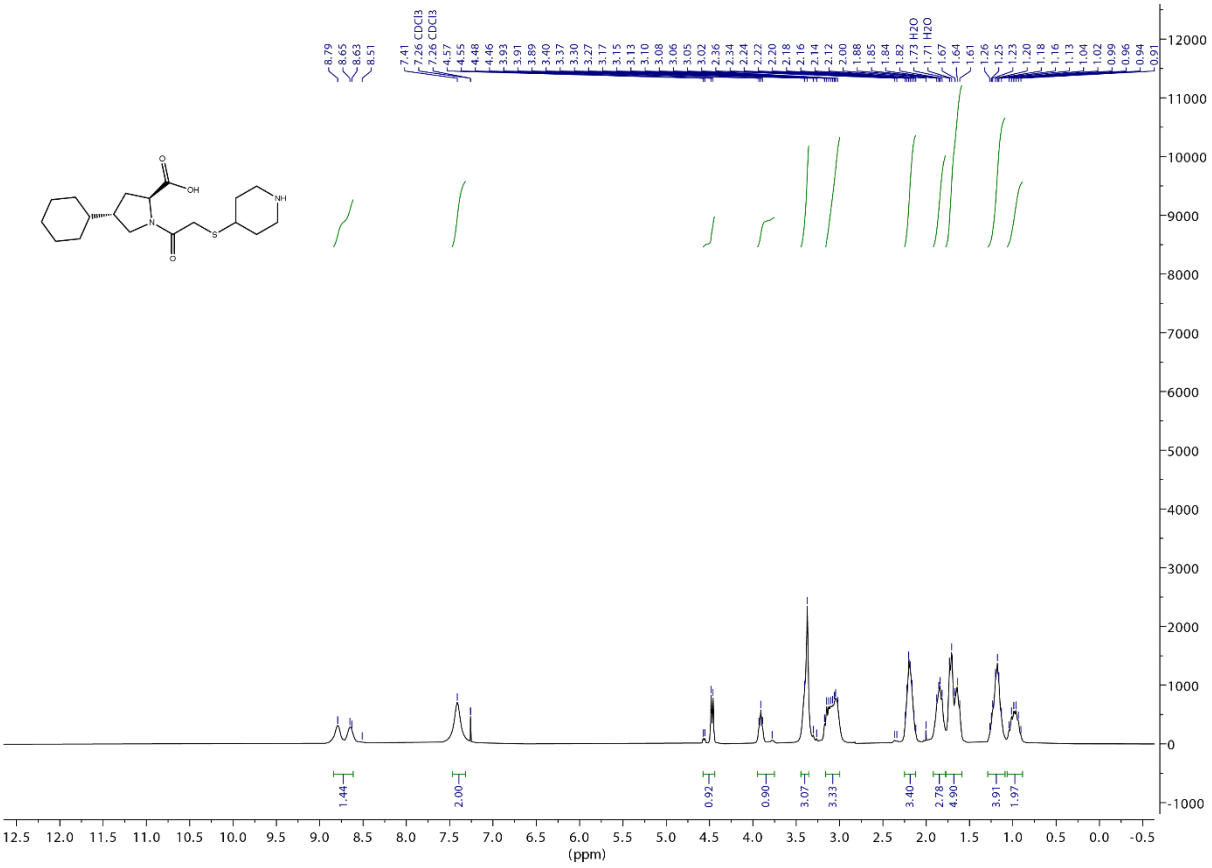

Figure S165. 1H NMR of compound **I18**.

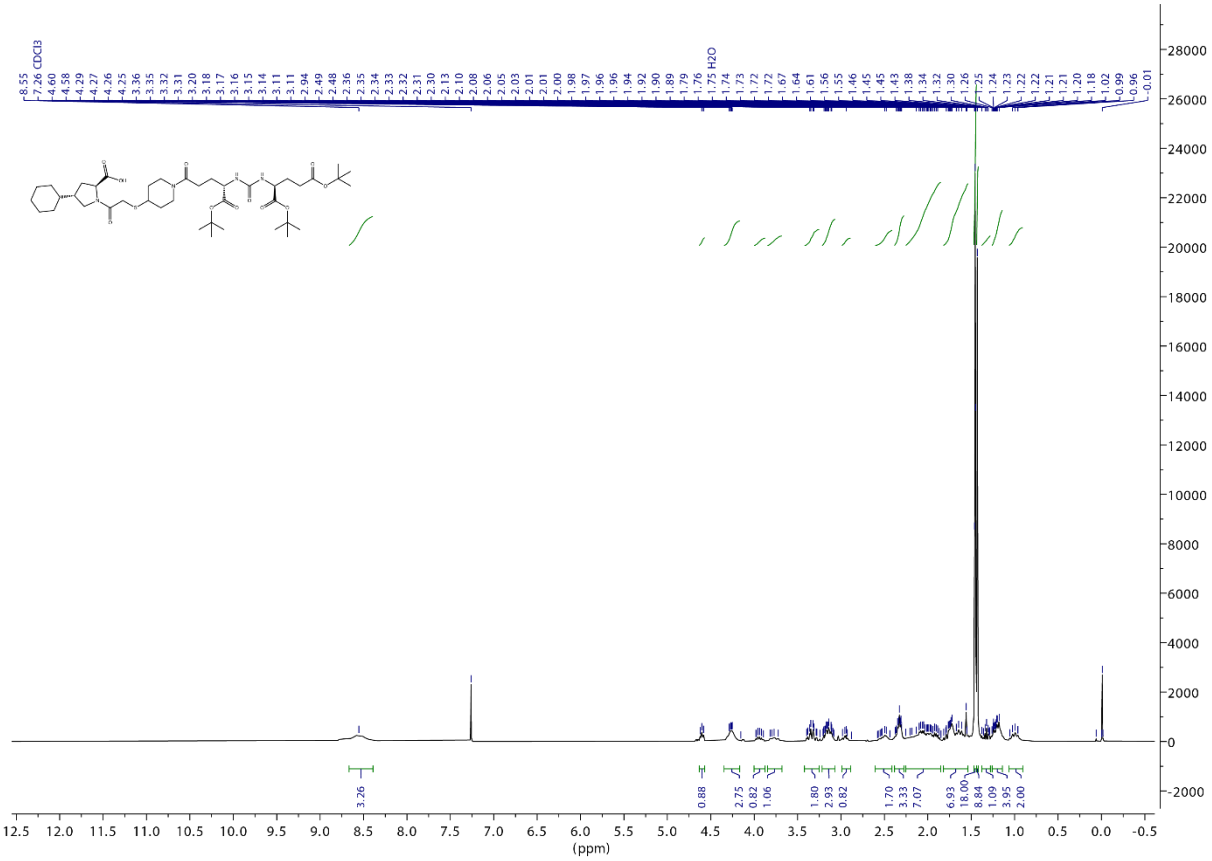

Figure S166. ^{1}H NMR of compound **I19**.

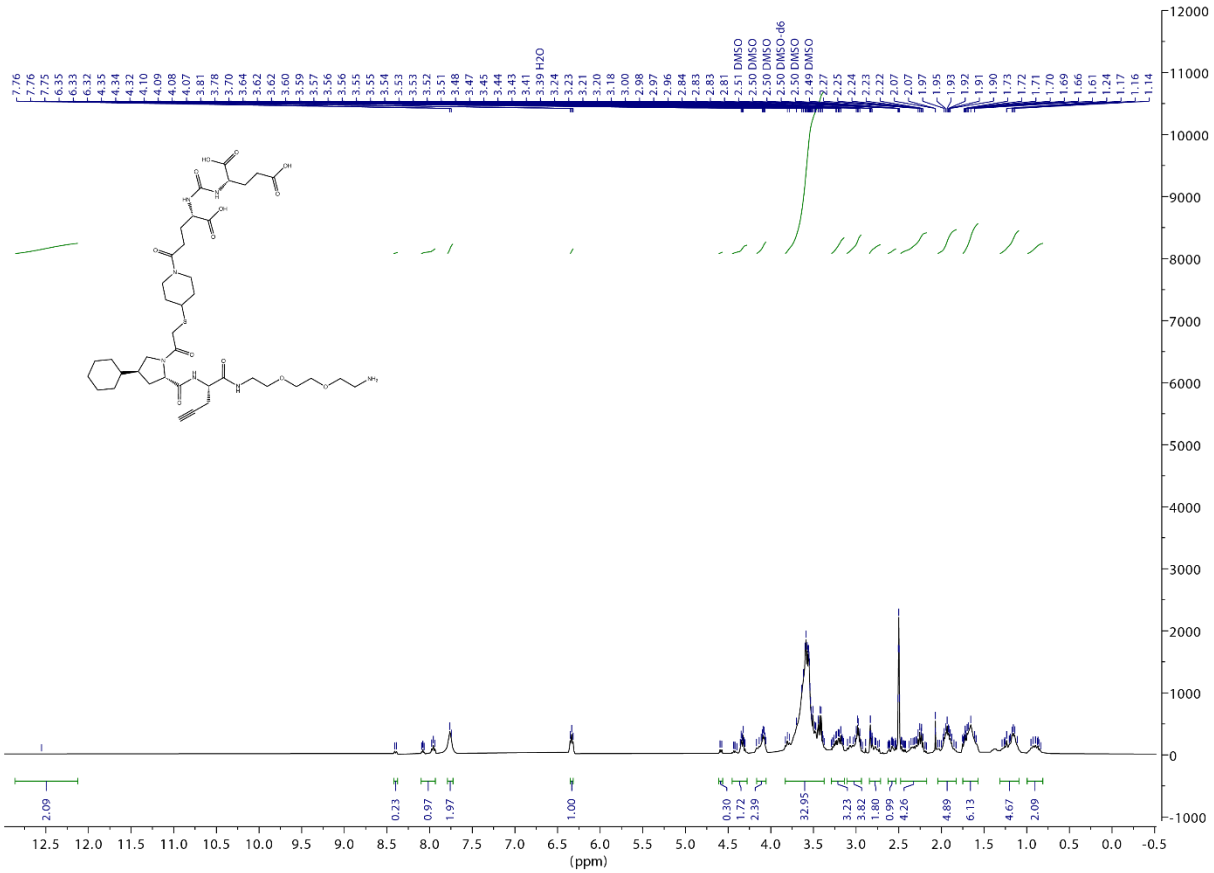

Figure S167. ^{1}H NMR of compound **I20**.

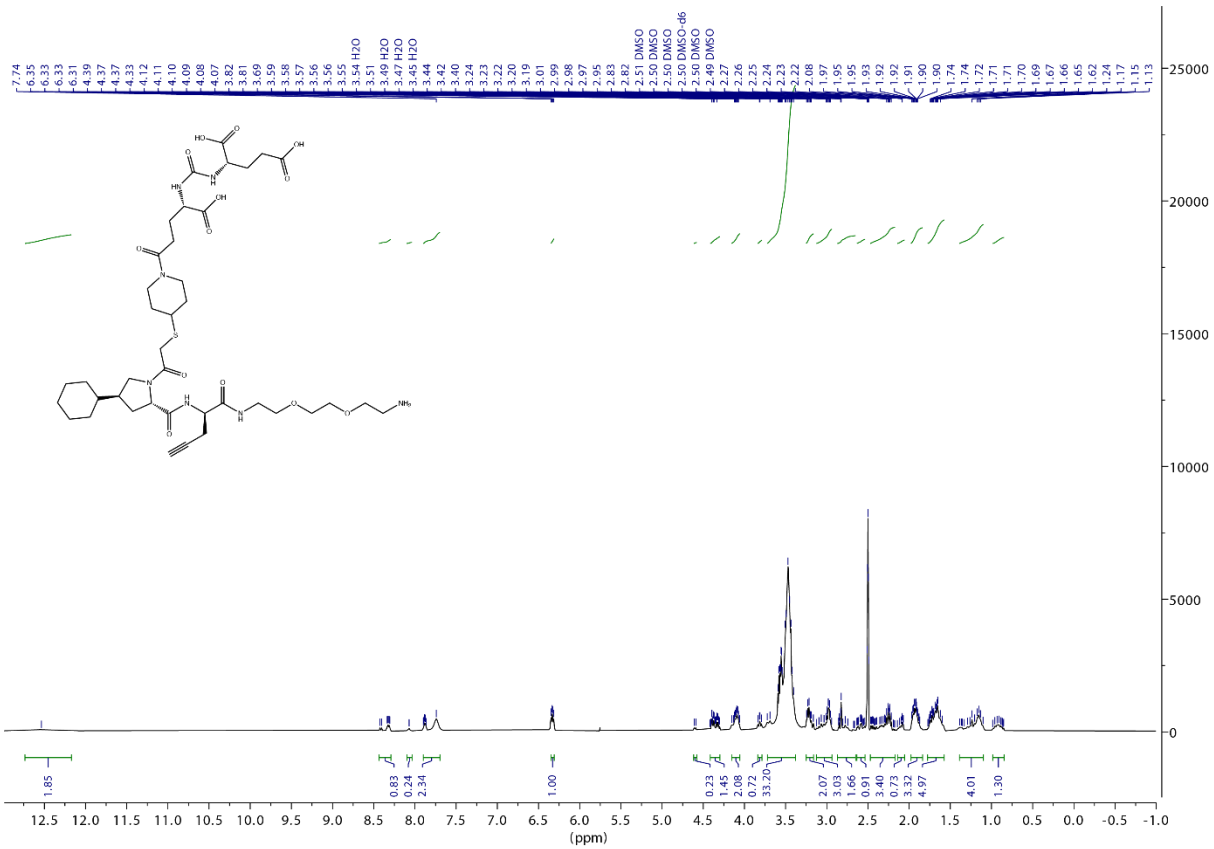

Figure S168. ^{1}H NMR of compound **I21**.

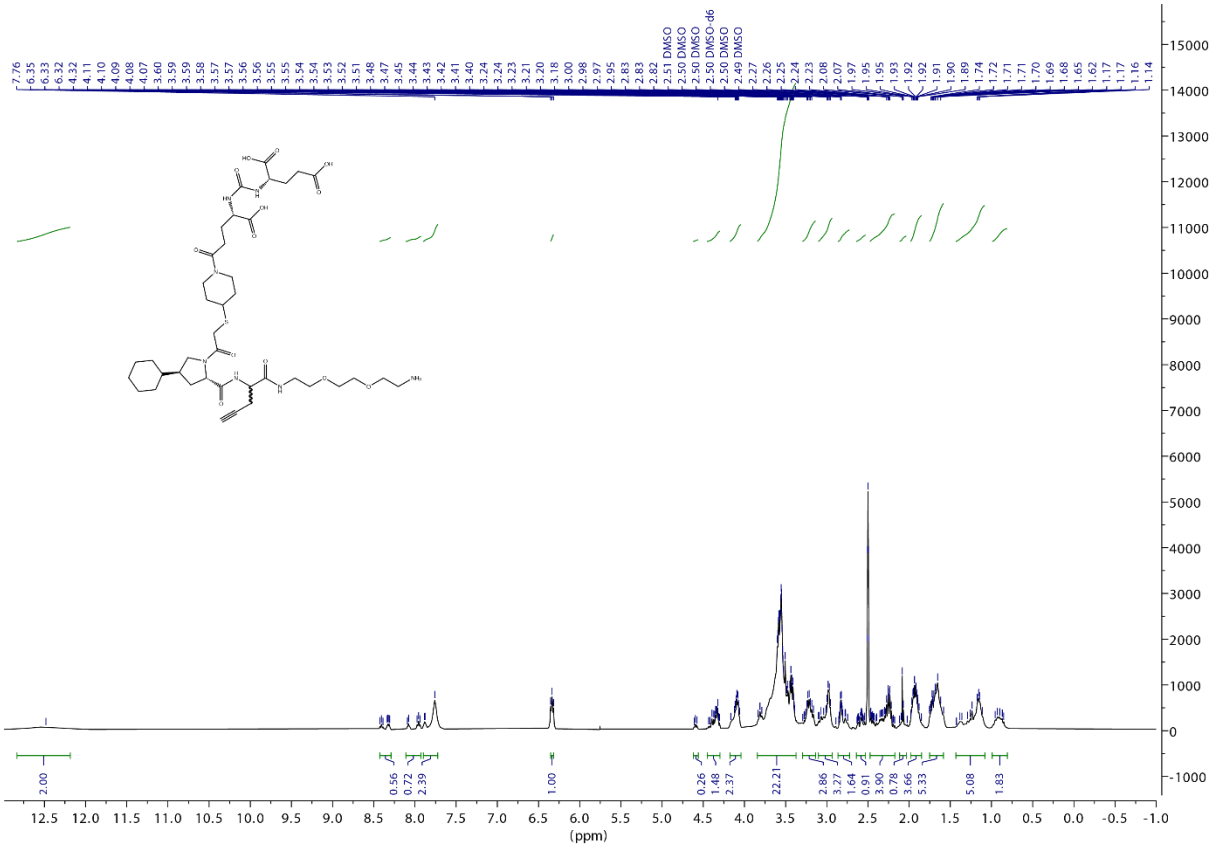

Figure S169. ^{1}H NMR of compound **I22**.

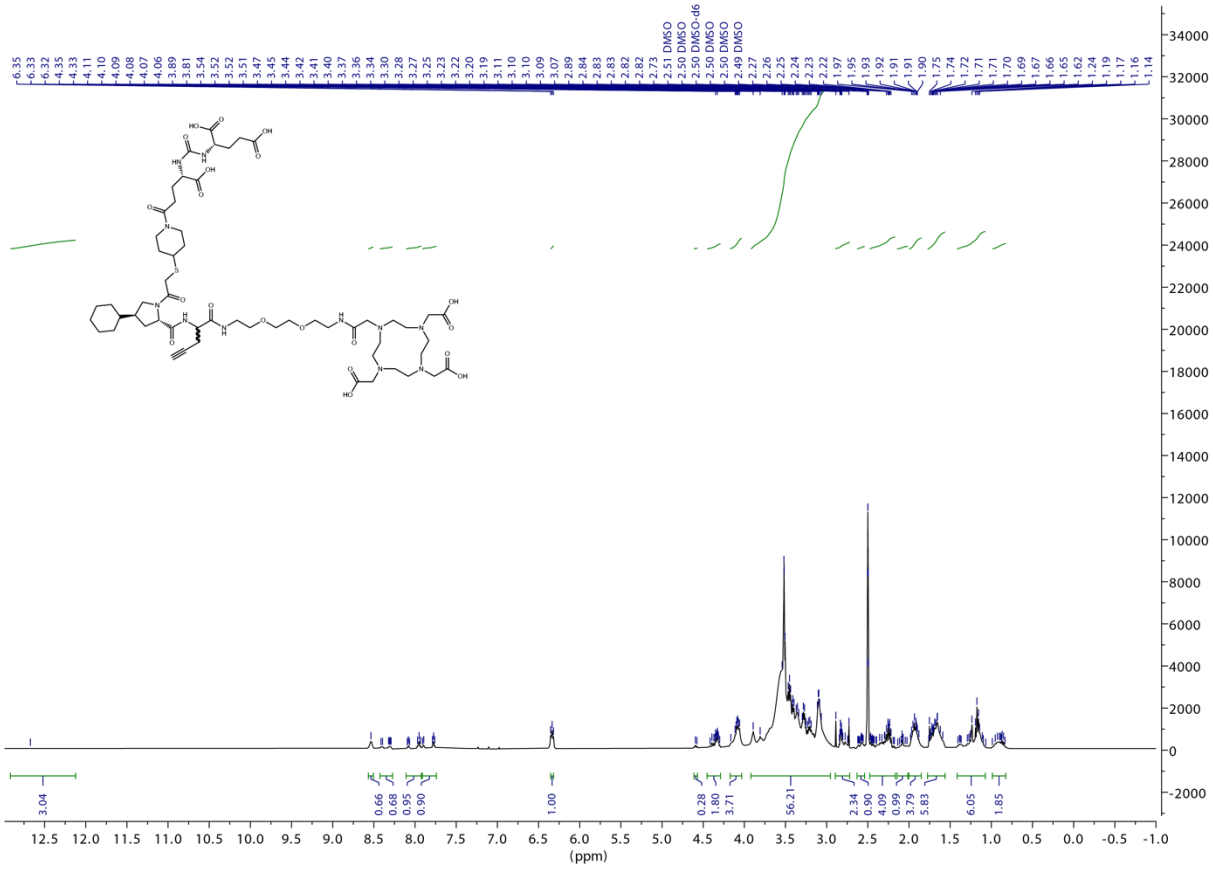

Figure S170. ^{1}H NMR of compound **22**.