

## Catch-enrich-release approach for amine-containing natural products

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## 1 General Procedure

Unless otherwise stated, all chemicals were of reagent grade and purchased from *Sigma-Aldrich*, *Merck*, *Fluorochem*, *Acros*, *Alfa Aesar* or *Honeywell*. All reactions were carried out in flame-dried glassware under protecting gas (N<sub>2</sub> or Ar) unless aqueous reagents were used. Solvents for reactions were either anhydrous or HPLC grade. Solvents for work-up and purification were distilled from technical grade. Evaporation of solvents *in vacuo* was carried out on a rotary evaporator at 40 °C bath temperature and appropriate pressure. Reactions were monitored by thin layer chromatography (TLC) or UHPLC-MS. **TLC** was performed on Merck TLC Silica gel 60 F<sub>254</sub> plates (aluminium sheets) pre-coated with a fluorescent indicator and visualized using UV or staining (CAM, KMnO<sub>4</sub>). **Melting Points** were determined on a Büchi Melting Point B-545. **Specific optical rotation**  $[\alpha]_D^T$ : *Jasco P-2000 Polarimeter*; measured at the indicated temperature *T*. All given values for  $[\alpha]_D^T$  have the dimension ° mL dm<sup>-1</sup> g<sup>-1</sup>. **Infrared spectra** (IR): *SpectrumTwo FT-IR Spectrometer (Perkin-Elmer)* equipped with a *Specac Golden Gate<sup>TM</sup> ATR* (attenuated total reflection) accessory; applied as neat samples or as films; 1/λ in cm<sup>-1</sup>. **Nuclear magnetic resonance spectra** (NMR): <sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub>, CD<sub>3</sub>OD or DMSO-*d*<sub>6</sub> on the instruments *Bruker AV-500* (500 MHz); chemical shift δ in ppm relative to solvent signals (δ = 7.26 ppm for CDCl<sub>3</sub>, 3.31 ppm for CD<sub>3</sub>OD, 2.50 ppm for DMSO-*d*<sub>6</sub>), coupling constant *J* is given in Hz. <sup>13</sup>C NMR spectra were recorded in CDCl<sub>3</sub>, CD<sub>3</sub>OD or DMSO-*d*<sub>6</sub> on the instruments *Bruker AV-500* (101 MHz); chemical shift in ppm relative to solvent signals (δ = 77.16 ppm for CDCl<sub>3</sub>, 49.00 ppm for CD<sub>3</sub>OD, 39.53 ppm for DMSO-*d*<sub>6</sub>). **Solid phase peptide synthesis**: The peptide synthesizer CS136XT (CS Bio) was used. **Freeze-dryer**: Lyophilization was performed using a Cryst Freeze dryer Alpha 1-4 LD plus. **Plate reader**: The microplate reader used for the experiments was the Synergy H1 apparatus from BioTek. **Preparative high-performance liquid chromatography**: Preparative HPLC separations were performed on a Shimadzu HPLC system (LC-20AP dual pump, CBM-20A Communication Bus Module, SPP-20, A UV/VIS Detector, FRC-10A Fraction collector) using reverse-phase (RP) columns Gemini-NX C18 (150 mm x 4.6 mm; 3 μm, 10 Å). **Ultra high-performance liquid chromatography coupled to mass spectrometry** (UHPLC-MS): *Ultimate 3000 LC* instrument (*Thermo Fisher Scientific*) coupled to a triple quadrupole *Quantum Ultra EMR MS* (*Thermo Fisher Scientific*) using a reversed-phase column (*Kinetex<sup>®</sup> EVO C18*; 1.7 μm;

100 Å, 50 x 2.1 mm; *Phenomenex*). The LC was equipped with an *HPG-3400RS* pump, a *WPS-3000TRS* autosampler, a *TCC-3000RS* column oven and a *Vanquish DAD* detector (all *Thermo Fisher Scientific*). The following solvents were applied: H<sub>2</sub>O + 0.1% HCOOH (A), CH<sub>3</sub>CN + 0.1% HCOOH (B). Samples were prepared using HPLC grade solvents (CH<sub>3</sub>CN, CH<sub>3</sub>OH, H<sub>2</sub>O) and filtered over a 4 mm syringe filter, PTFE (hydrophilic), pore size: 0.22 µm obtained from BGB Analytik AG. The MS was equipped with an H-ESI II ion source. The source temperature was 250 °C, the capillary temperature 270 °C and capillary voltage 3500 V, and datasets were acquired at resolution 0.7 on Q3 in centroid mode. **High-resolution electrospray ionization mass spectra (HRMS-ESI):** *On flow injection:* High-resolution mass spectra were acquired on a *Qexactive* instrument (*ThermoFisher Scientific*, Bremen, Germany) equipped with a heated electrospray (ESI) ionization source and connected to a *Dionex Ultimate 3000* UHPLC system (*ThermoFischer Scientifics*, Germering, Germany). The samples were dissolved in CH<sub>3</sub>OH, CH<sub>3</sub>CN or H<sub>2</sub>O at a concentration of ca. 50 µg mL<sup>-1</sup> thereof 1 µL was injected on-flow with a XRS auto-sampler (*CTC*, Zwingen, Switzerland). The mobile phase (120 µL mL<sup>-1</sup> flow rate) consisting of CH<sub>3</sub>OH + 0.1% HCOOH or CH<sub>3</sub>CN /H<sub>2</sub>O 2:8 + 0.1% HCOOH was chosen according to the solubility. Ion source parameters were set as follow: spray voltage 3.0 kV; capillary temperature 280 °C; sheath gas 30 L min<sup>-1</sup>; aux gas 8; 30 L min<sup>-1</sup>; s-lens RF level 55.0; and aux gas temperature 250 °C. Full scan MS were acquired in the alternating (+)/(-)-ESI mode and over the ranges  $m/z = 80\text{'1}200$ ,  $133\text{'2}000$ , or  $200\text{'3}000$  at 70'000 resolution (full width half-maximum) and with automatic gain control (AGC) target of 3.00E+06. The maximum allowed ion transfer time (IT) was 30 ms. Masses were calibrated below 2 ppm accuracy between  $m/z = 130.06619$  and  $1621.96509$  in the positive and between  $265.14790$  and  $1779.96528$  in the negative ESI mode using the Pierce<sup>®</sup> ESI calibration solutions (*ThermoFisher Scientific*, Rockford, USA). Additionally, contaminations of erucamide ( $m/z = 338.34174$ , (+)-ESI) and palmitic acid ( $m/z = 255.23295$ , (-)-ESI) were used as lock masses in (+)-and (-)-ESI, respectively. The UHPLC-HRMS measurements were also done using a *ThermoFisher Scientific* UHPLC *Vanquish Horizon* system (*Diode Array Detector*, *Split Sampler HT*, *Binary Pump H*, *Column Compartment H*) connected to *ThermoFisher scientific* mass spectrometer (*Orbitrap Exploris 240*). The separation was performed using *A Kinetex™ EVO C<sub>18</sub>* column (50 x 2.1 mm, 1.7 µm, *Phenomenex*, USA) was used for separation, the column oven was kept at 40 °C, the flow was set to 0.4 mL/min, and the volume of injection was 1 µL. The solvent system was composed of A (H<sub>2</sub>O + 0.1 % FA), B (MeCN + 0.1 % FA) and the gradient started

from 5 % B. The gradient was kept for 0.5 min at 5% B, was increased to 100 % B over 3 min, and kept for 2 min at 100% B.

### 1.1 Protocol for enzymatic cleavage analysis with probe 6

A modified version of a reported procedure was used for this experiment.<sup>1</sup> For the enzymatic release experiments, two buffer solutions were used:

**Assay buffer:** 50 mM MES, 250 mM NaCl, pH = 5

**Activation buffer:** acetate buffer solution 50 mM, 100 mM NaCl, pH = 4.0

Legumain was diluted to obtain a concentration of 100 ng/ $\mu$ L (1.26  $\mu$ L of enzyme mixed in 28.74  $\mu$ L of activation buffer). The solution was shaken for 2h at 37°C. The solution of the enzyme was diluted to 15 ng/ $\mu$ L by adding assay buffer (170  $\mu$ L). In the well plate, solutions of AMC were added at a concentration of 0.1, 1, 10, 50, and 100  $\mu$ M in assay buffer to build the calibration curve. A solution of 15  $\mu$ L of the probe 6 (100  $\mu$ M in assay buffer) was added to the 96 well plate (black) and a solution of 15  $\mu$ L of the substrate Z-Ala-Ala-Asn-AMC (100  $\mu$ M in assay buffer) was used at the positive control. A solution of legumain (15  $\mu$ L, 15 ng/ $\mu$ L in assay buffer) was then added to the wells and negative controls were performed by adding only the assay buffer. The 96 well plate was analyzed with the plate reader with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The data were recorded for 180 min with a fluorescent reading mode from the top of the plate. The concentration of the released substrate was determined using the calibration curve and the efficacy of the process was calculated based on an initial concentration of the substrate of 100  $\mu$ M. The experiments were performed in triplicate.

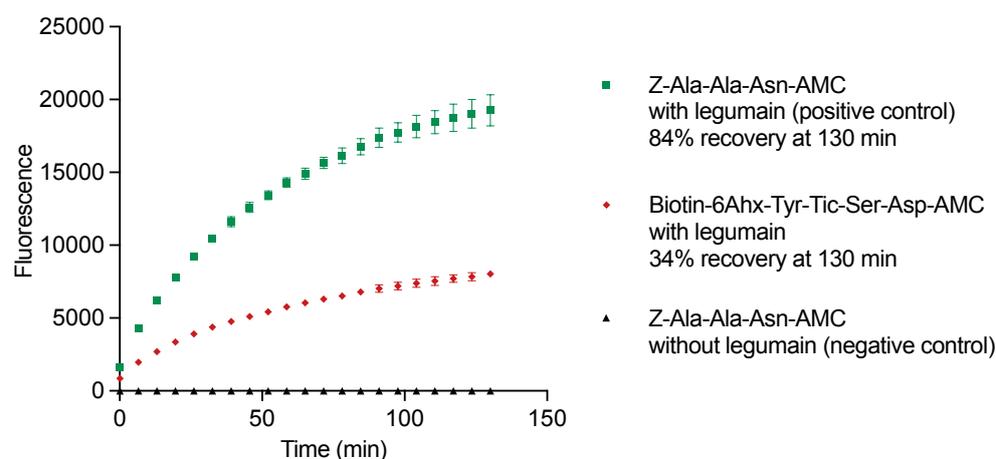


Fig. S1: Enzymatic release of biotin-6Ahx-Tyr-Tic-Ser-Asp-AMC (probe 6) with legumain (15 ng/mL) in red diamond, positive control using Z-Ala-Ala-Asn-AMC with legumain (15 ng/mL) in green square, and negative control using Z-Ala-Ala-Asn-AMC without legumain in black triangle.

## 1.2 Protocol for release from solid support

### 1.2.1 Phenyl/chloroform procedure

The procedure is adapted from the literature with modification.<sup>2</sup>

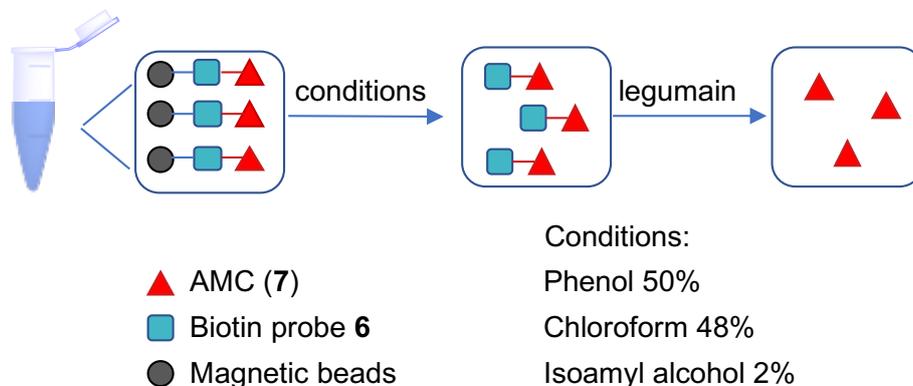


Fig. **S2**: Schematic representation of the release from the solid support using a phenol chloroform mixture.

The streptavidin magnetic beads (50  $\mu$ L, 5 mg/mL, Sigma-Aldrich) were transferred into an Eppendorf tube (0.5 mL o. 0.2 mL, DNA low binding). After putting the samples on a magnet (30 s), the supernatant was removed and the magnetic beads were washed with PBS (3 x 50  $\mu$ L, 0.003 M, 0.15 NaCl, pH = 7.2, 0.1% BSA). PBS (15  $\mu$ L, 0.003 M, 0.15 NaCl, pH = 7.2, 0.2% BSA) and the probe 6 (15  $\mu$ L, 100  $\mu$ M in PBS) were incubated at RT or 37  $^{\circ}$ C for 2 h with the magnetic beads on the shaker (500 rcf). After incubation, the samples were washed with PBS (3 x 50  $\mu$ L). For the release, the beads were gently mixed with a solution of phenol/chloroform/isoamyl alcohol (50  $\mu$ L, 25:24:1). The samples were then put on a magnet (30 s) and part of the organic phase (45  $\mu$ L) was transferred in a vial. After chloroform (50  $\mu$ L) was added, the samples were then put on a magnet (30 s) and part of the organic phase (45  $\mu$ L) was transferred in a vial. The combined organic phases were centrifuged (1 min at 20000 g), 85  $\mu$ L were transferred to a vial, the samples were dried under a gentle flow of nitrogen. The compounds were resuspended in the assay buffer (30  $\mu$ L) and the samples were used for the enzymatic release. The experiment was performed in triplicate. The enzymatic release was performed as described in the section **1.1**

## 1.2.2 Peptides analogues with biotin derivatives

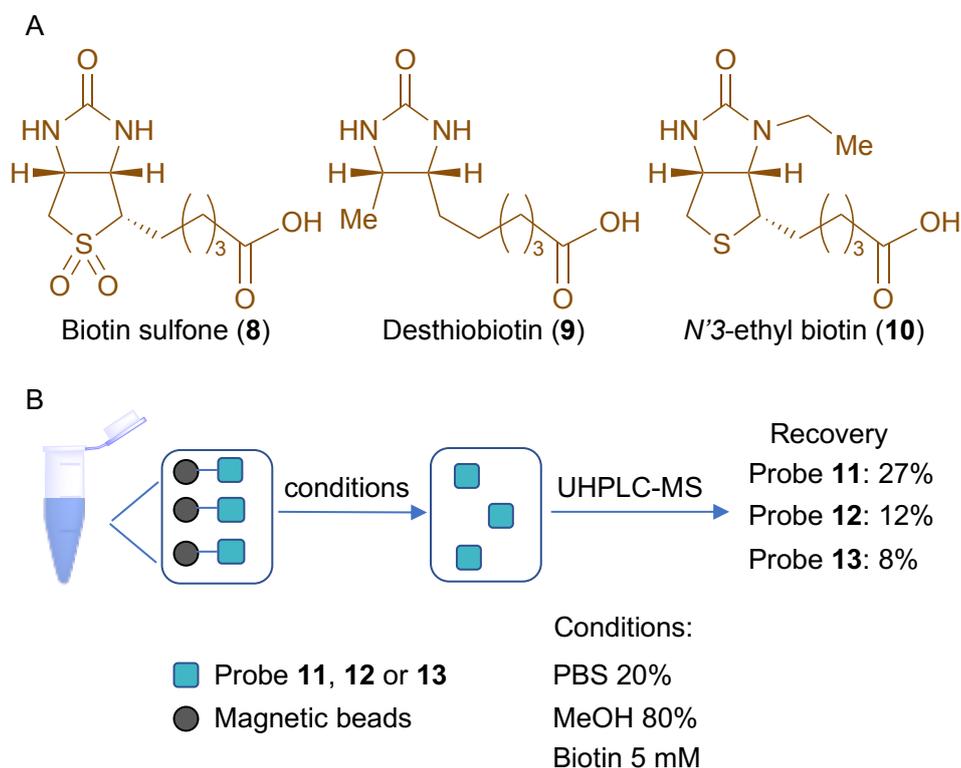


Fig. **S3**: A) Schematic representation of the release from the solid support using the biotin-sulfone-based probe **11**, desthiobiotin-based probe **12**, and the *N*'3-ethyl biotin probe **13**. B) Structures of biotin sulfone (**8**), desthiobiotin (**9**), and *N*'3-ethyl biotin (**10**).

The streptavidin magnetic beads (50  $\mu$ L, 5 mg/mL, Sigma-Aldrich) were transferred into an Eppendorf tube. After putting the samples on a magnet (30 s), the supernatant was removed and the magnetic beads were washed with PBS (3 x 50  $\mu$ L, 0.003 M, 0.15 NaCl, pH = 7.2, 0.1% BSA). PBS (15  $\mu$ L, 0.003 M, 0.15 NaCl, pH = 7.2, 0.2% BSA) and the probe **11** (15  $\mu$ L, 100  $\mu$ M in PBS) for biotin sulfone (**8**), the probe **12** for desthiobiotin (**9**), and the probe **13** for *N*'3-ethylbiotin (**10**) were incubated at 30  $^{\circ}$ C (10 min) with the magnetic beads on the shaker (500 rcf). For the release, the beads were washed with a PBS:MeOH mixture (3 x 50  $\mu$ L, 2:8). The beads were incubated at 37  $^{\circ}$ C (500 rcf, 5 min) with a PBS:MeOH mixture (3 x 50  $\mu$ L, 2:8 and 5 mM biotin). The samples were put on a magnet (30 s) and the supernatant was collected. The procedure was repeated twice, and the supernatants were combined (150  $\mu$ L). The solvent was removed under a gentle flow of nitrogen. The vials were cooled at -20  $^{\circ}$ C (20 min) and CH<sub>3</sub>OH (60  $\mu$ L) was added to the samples, part of the solvent (45  $\mu$ L) was transferred in Eppendorf, centrifuged (5 min, 20000 g), the supernatant (30  $\mu$ L) was filtered

and transferred into HPLC vials, and the samples were analyzed by UHPLC-MS with a calibration curve of the respective peptide (150, 100, 50, 20, 1,0.1  $\mu$ M). The release was tested on the following peptides: biotin sulfone-based probe **11** (27%), desthiobiotin-based probe **12** (12%) and *N*'3-ethyl biotin-based probe **13** (8%). The experiment was performed in triplicate

### 1.3 Complete procedure using probe 11 with pseudoephedrine (2)

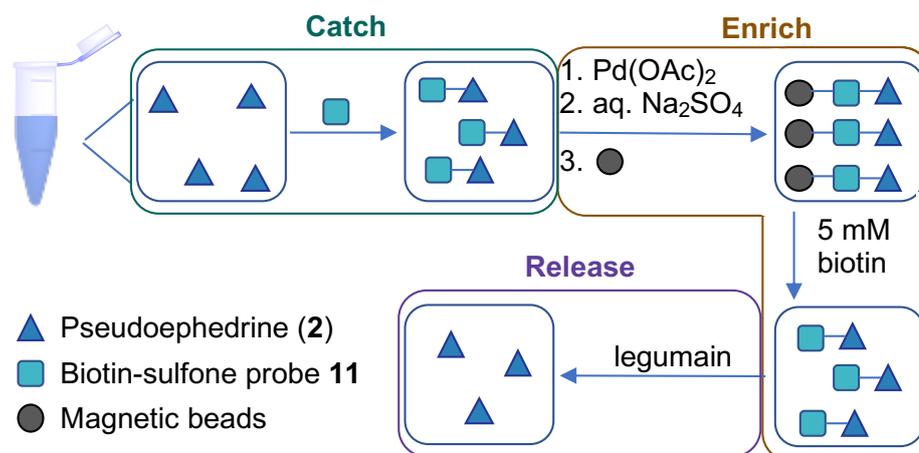


Fig. S4: Schematic representation of the complete procedure using the probe **11** and with pseudoephedrine (**2**).

#### 1.3.1 Catching step

##### *Amide coupling*

In an Eppendorf (0.5 mL low DNA binding), HATU (15  $\mu$ L, 200 mM in dry DMF, 2.0 equiv.), pseudoephedrine (**2**, 15  $\mu$ L, 330 mM in dry DMF, 3.3 equiv.) and probe **11** (15  $\mu$ L, 100 mM in dry DMF, 1 equiv.) were mixed gently and incubated on the shaker at 37  $^{\circ}$ C (500 rcf) for 21 h. The reaction mixture was then transferred to a vial and the solvent was removed under a flow of nitrogen. After resuspension in degassed H<sub>2</sub>O (1.5 mL), the amide reaction product was used for the deprotection.

##### *Deprotection*

In an Eppendorf, TPPTS in degassed H<sub>2</sub>O (10  $\mu$ L, 15 mM, 100 equiv.) and Pd(OAc)<sub>2</sub> in degassed H<sub>2</sub>O (5  $\mu$ L, 30 mM, 100 equiv.) were mixed gently. MgCl<sub>2</sub> in degassed H<sub>2</sub>O (10  $\mu$ L, 6 mM, 40 equiv.) and morpholine in degassed H<sub>2</sub>O (5  $\mu$ L, 30 mM, 100 equiv.) were added and the mixture was vortex (30 s). The amide reaction product in degassed H<sub>2</sub>O (15  $\mu$ L, 100  $\mu$ M, 1 equiv.) was added and the suspension was incubated on the shaker at RT (500 rcf, 2 h). A sat. aqueous solution of Na<sub>2</sub>SO<sub>4</sub> (100  $\mu$ L), was added to the reaction mixture. After vortexing the Eppendorf tube (30 s), the suspension was diluted with MeCN (0.5 mL) and filtered through a PTFE syringe filter (0.22  $\mu$ m) and the solvent was removed under a flow of nitrogen.

### 1.3.2 Enrichment step

The streptavidin magnetic beads (50  $\mu\text{L}$ , 5 mg/mL, Sigma-Aldrich) were transferred into an Eppendorf tube. After putting the samples on a magnet (30 s), the supernatant was removed and the magnetic beads were washed with PBS (3 x 50  $\mu\text{L}$ , 0.003 M, 0.15 NaCl, pH = 7.2, 0.1% BSA). PBS (15  $\mu\text{L}$ , 0.003 M, 0.15 NaCl, pH = 7.2, 0.2% BSA) and the resuspended mixture (15  $\mu\text{L}$ , 100  $\mu\text{M}$  in PBS (0.003 M, 0.15 NaCl, pH = 7.2, 0.2% BSA)) was incubated at 30 °C (10 min) with the magnetic beads on the shaker (500 rcf). The beads were washed with a PBS: MeOH mixture (3 x 50  $\mu\text{L}$ , 2:8). The beads were incubated at 37 °C (500 rcf, 5 min) with a PBS/MeOH mixture (3 x 50  $\mu\text{L}$ , 2:8 and 5 mM biotin). The samples were put on a magnet (30 s) and the supernatant was collected. The procedure was repeated twice, and the supernatants were combined (150  $\mu\text{L}$ ). The solvent was removed under a gentle flow of nitrogen. The samples were resuspended in MeCN (500  $\mu\text{L}$ ). After sonication, the suspension was centrifuged in an Eppendorf tube (5 min, 20000 g). The supernatant was transferred in a vial and the solvent was removed under a flow of nitrogen and the vials were cooled at -20 °C (20 min).

### 1.3.3 Enzymatic releasing step

**Assay buffer:** 50 mM MES, 250 mM NaCl, pH = 5

**Activation buffer:** acetate buffer solution 50 mM, 100 mM NaCl, pH = 4.0

Legumain was diluted to obtain a concentration of 100 ng/ $\mu\text{L}$  (1.26  $\mu\text{L}$  of enzyme mixed in 28.74  $\mu\text{L}$  of activation buffer). The solution was shaken for 2h at 37°C. The solution of the enzyme was diluted to 15 ng/ $\mu\text{L}$  by adding assay buffer (170  $\mu\text{L}$ ). In an Eppendorf, the resuspended samples (30  $\mu\text{L}$ , assay buffer) and legumain (30  $\mu\text{L}$ ) were added and incubated at 37 °C (500 rcf, 3 h). Afterwards the samples were centrifuged (5 min, 20000 g), and analyzed by UHPLC with the corresponding pseudoephedrine (**2**) calibration curve (50, 40, 30, 20, 10, 1, 0.1  $\mu\text{M}$ ). The experiment was performed in triplicate.

The same procedure was repeated with a complex matrix containing an initial concentration of 66 mM of serotonin (**1**), pseudoephedrine (**2**), 1-deoxynojirimycin (**3**), histamine, acarbose, vancomycin, isopropanol, prop-2-en-1-ol, and 4-methylbenzenethiol. Pseudoephedrine (**2**) was recovered with 9% yield, serotonin (**1**) with 3% and 1-deoxynojirimycin (**3**) was observed and the concentration was lower than 1%.

The procedure was used with a biomass extract from a cyanobacterial mat sample collected in the river Areuse in Neuchâtel (Switzerland) by Sami Zhioua from the group of Professor Pilar Junier (University of Neuchatel, Switzerland). The sample (50 mg) was extracted with H<sub>2</sub>O (500 µL, 0.1% formic acid) by 3 cycles of sonication (3 min) followed by centrifugation (21000 x g, 5 min). Part of the sample (450 µL) was transferred in a brown vial and dried under a gentle flow of nitrogen. The sample was resuspended in 20 µL of dry DMF and used in the catching procedure. A few modifications have been done to the general protocol such as the use of 2 x 15 µL of dry DMF for the addition of the extract in the amide coupling step, the use of 3 x 15 µL of the coupled probe in its addition to the deprotection step, and the beads were washed with PBS (50 µL) before being washed with PBS: MeOH mixture after their incubation with the deprotected probe.

The UHPLC-HRMS measurements were performed with a UHPLC system (Vanquish Horizon, ThermoFischer) equipped with a Diode Array Detector, Split Sampler HT, Binary Pump H, Column Compartment H connected to an HRMS (Exploris 240, ThermoFischer). For the analysis, the HILIC column Aquity BEH amide (Waters, 2.1 x 100 mm, 1.7µm) was used, 1 µL was injected, the flow rate was 400 µLmin<sup>-1</sup> and the solvent was composed of A: H<sub>2</sub>O + 0.015% NH<sub>4</sub>OAc and B: MeCN + 0.1 HCOOH. The running gradient started with 90% B for 0.5 min, decreased to 60% B over 1 min, stayed at 60% B for 5 min, decreased to 20% B over 2 min, and stayed at 20% B for 1 min. The ion source parameters of the HRMS in positive mode were set as follows: spray voltage 3.4 kV; capillary temperature 325 °C; sheath gas 50 L min<sup>-1</sup>; aux gas 10 L min<sup>-1</sup>; sweep gas 1 L min<sup>-1</sup>; and vaporizer temperature 330 °C. The EASY-IC™ internal calibration system was used at the beginning of each run. A full scan (res: 60000, scan range: 100-1500, RF lens: 70%, positive mode) was performed with data-dependent MS2 (isolation window of 0.4 *m/z*, isolation offset of 0.1 *m/z*, collision energy normalized, orbitrap resolution of 30000, and auto scan range mode) with filters (intensity threshold: 50000, dynamic exclusion after 5 times if occurs in 30s with an exclusion duration of 2.5 s).

The samples were analyzed using Compound Discoverer (ThermoFischer, Version 3.3.0.550) by checking compounds present in the original extract and as products of the chemoselective approach, and by selecting compounds possessing HRMS/HRMS spectra. A list of 50 compounds was obtained (table S1, Fig. S5) and the comparison of those compounds with the

CyanoMetDB<sup>3</sup> led to the identification of anatoxin-a (calc: 166.12264 *m/z*, obs: 166.12249) and dihydroanatoxin-a (calc: 168.13829 *m/z*, obs: 168.13809). The structure of both compounds was confirmed by comparing their HRMS/HRMS with those reported (Figs. S6 and S7 ).<sup>4</sup>

Table **S1**: List of compounds present in the extract and in the sample released from the probe by legumain. The compounds 11 and 12 correspond to anatoxin-a (**3**) and dihydroanatoxin-a, respectively.

Compounds	<i>m/z</i>	RT [min]	Compounds	<i>m/z</i>	RT [min]
1	107.05231	2.003	26	328.1751	0.902
2	113.03445	1.194	27	340.25892	1.275
3	130.10856	2.032	28	340.25905	1.017
4	144.04759	0.833	29	340.25914	1.194
5	146.11747	1.211	30	360.14966	2.844
6	151.06127	1.26	31	365.10487	2.735
7	153.04053	2.072	32	377.31588	0.847
8	156.04768	1.167	33	380.20684	0.85
9	160.07553	0.852	34	394.30605	1.117
10	164.09292	1.067	35	396.27398	0.85
<b>11</b>	<b>166.12249</b>	<b>0.942</b>	36	412.14159	0.816
<b>12</b>	<b>168.13809</b>	<b>1.116</b>	37	412.269	0.85
13	192.08769	1.171	38	448.16532	2.746
14	204.12279	1.175	39	475.32486	1.271
15	217.0638	0.837	40	492.25228	2.084
16	220.11771	0.883	41	505.17852	2.032
17	220.1178	1.104	42	507.38982	1.182
18	241.15434	2.087	43	522.20225	2.979
19	245.0765	1.955	44	538.294	1.282
20	270.20197	1.236	45	538.29417	2.032
21	276.10746	1.093	46	556.44244	1.255
22	296.13507	1.188	47	566.42691	2.018
23	296.18521	1.047	48	579.25926	0.826
24	312.12992	2.085	49	579.25972	0.992
25	326.23225	0.851	50	834.53893	0.865

A

	Tags	Name	Formula	Annot. ΔMass [ppm]	Calc. MW	m/z	RT [min]	MS2	Reference Ion	Group Areas		
										Blank	Extract	Probe
1		anatoxin-a	C10 H15 N O	-0.94	165.11521	166.12249	0.942		[M+H] <sup>+</sup> 1	5.89e4	5.49e7	4.13e6
2		dihydroanatoxin-a	C10 H17 N O	-1.19	167.13082	168.13809	1.116		[M+H] <sup>+</sup> 1	4.24e4	2.38e9	1.83e6

B

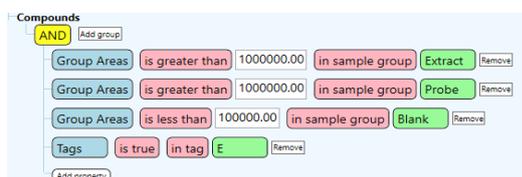


Fig. S5: A) Results obtained after the statistical comparison between the original extract (Extract) and the compounds obtained after the enzymatic release (Probe). B) The parameters of the filter used during the analysis of those samples, for clarity a tag was added to display only anatoxin-a and dihydroanatoxin-a.

HRMS/HRMS spectrum of anatoxin-a (3)

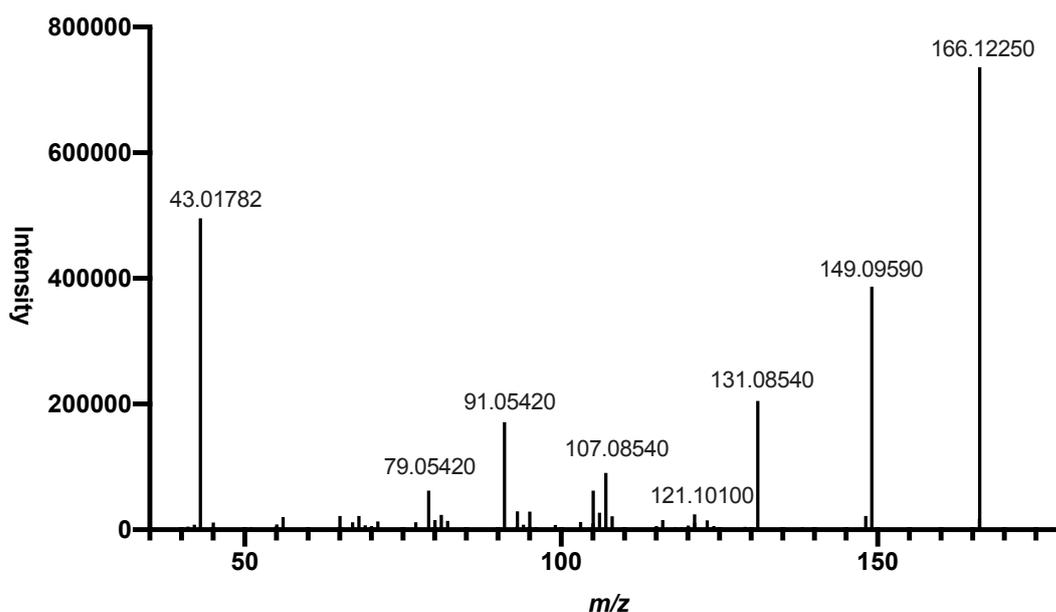


Fig. S6: HRMS/HRMS spectrum of anatoxin-a (3) from the analysis of the cyanobacteria mats extract.

HRMS/HRMS spectrum of dihydroanatoxin-a

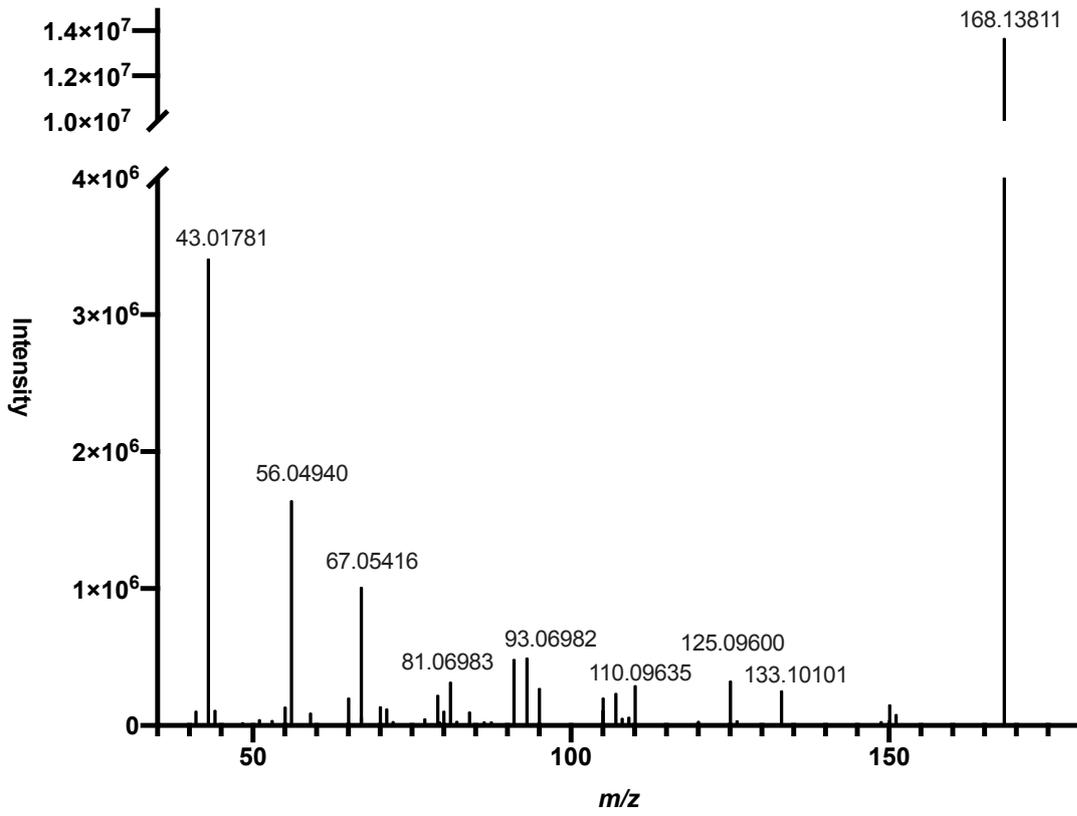
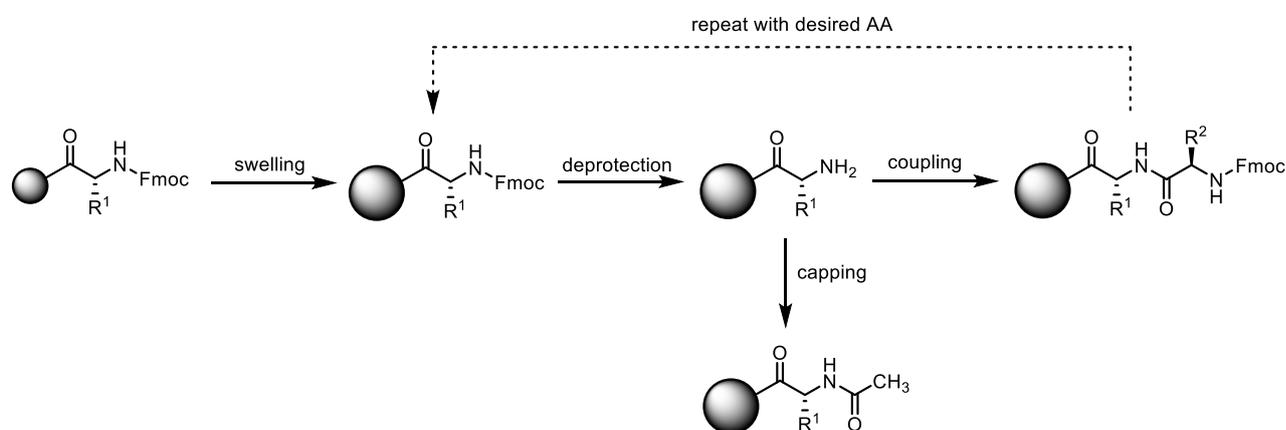


Fig. S7: HRMS/HRMS spectrum of dihydroanatoxin-a from the analysis of the cyanobacteria mats extract.

## 2 Synthesis of Peptides

The peptides were synthesized using a peptide synthesizer.

### 2.1 General Procedure / Peptide Synthesizer Program



#### Swelling (only for the first preloaded resin):

The preloaded resin (1.00 g, 1.0 equiv.) was placed into the reaction vessel and allowed to swell in CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (4:1, 20 mL) for 1 h while gently mixing by turning the reaction vessel.

#### Fmoc-deprotection:

After removal of the solvent and washing with R1 (DMF, 2 x 15 mL), the Fmoc-protecting group was removed by addition of R9 (20% piperidine in DMF, 10 mL) and R3 (DMF, 5 mL), followed by mixing for 5 min. After washing with R1 (DMF, 15 mL), the deprotection was repeated with mixing for 10 min, followed by washing with R1 (DMF, 3 x 15 mL) and R2 (*i*-PrOH, 2 x 15 mL).

#### Coupling:

A solution of AA (5 mL, 3.0 equiv.) was pre-mixed with a solution of R4 (HATU, 5 mL, 3.0 equiv.), a solution of R5 (DIPEA, 5 mL, 3 equiv.) by bubbling air for 6 min and the resulting mixture was then diluted with R3 (DMF, 5 mL). This mixture was added to the resin in the reaction vessel and mixed for 2 h. After completion of the coupling step, the reaction mixture was removed, and the resin was washed with R1 (DMF, 3 x 15 mL) and R2 (*i*-PrOH, 2 x 15 mL).

#### Capping

A solution of R3 (DMF, 5 mL), R6 (Ac<sub>2</sub>O, 5 mL, 50 equiv.), R7 (DIPEA, 5 mL, 50 equiv.) were added to the resin in the reaction vessel and mixed for 30 min. After completion of the

capping step, the reaction mixture was removed, and the resin was washed with R1 (DMF, 3 x 15 mL) and R2 (*i*-PrOH, 2 x 15 mL).

## 2.2 Procedure for amino acid loading

### 2.2.1 Resin Loading Determination:

**Blank:** To a 10 mL volumetric flask, piperidine in DMF (20%, 200  $\mu$ L) was added and the volume was made up to 10 mL with EtOH.

**Analyte:** An Eppendorf vial containing the loaded resin (10.866 mg) and piperidine in DMF (20%, 200  $\mu$ L), were rotated for 10 min. After settling of the resin, the supernatant was transferred to a volumetric flask (20 mL) and the process was repeated. The suspension was diluted with EtOH and filtered over a PTFE syringe filter (0.22  $\mu$ m) into the 20 mL volumetric flask. The volume was made up to 20 mL with EtOH and 0.5 mL of this solution was diluted with the blank solution (0.5 mL) and filled into a cuvette for analysis.

**Standard:** An Eppendorf vial containing Fmoc-Asp(OAll)-OH (2.599 mg, 0.657  $\mu$ mol) was rinsed with piperidine in DMF (20%, 400  $\mu$ L) into a volumetric flask (20 mL). The volume was made up to 20 mL with EtOH. This solution (0.5 mL) was diluted with the blank solution (0.5 mL) and filled into a cuvette for analysis.

The UV absorbance of the analyte as well as the standard solution was measured at 301 nm against the blank solution. According to Lambert-Beer's Law:

$$c(\text{analyte}_{dil}) = \frac{A(\text{analyte}_{dil}) \times c(\text{standard}_{dil})}{A(\text{standard}_{dil})} = \frac{0.7825 \times 0.149 \text{ mM}}{0.8668} = 0.165 \text{ mM}$$

$$c(\text{analyte}) = 2 \times c(\text{analyte}_{dil}) = 0.329 \text{ mM}$$

$$n(\text{analyte}) = c(\text{analyte}) \times V = 0.329 \text{ mM} \times 0.02 \text{ L} = 0.00659 \text{ mmol}$$

$$\text{Loading} = \frac{n(\text{analyte})}{m(\text{resin})} = \frac{0.00659 \text{ mmol}}{0.010677 \text{ g}} = 0.62 \text{ mmol/g}$$

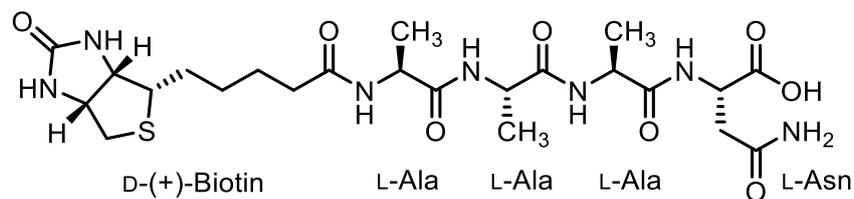
### 2.2.2 Resin Loading:

Wang resin (1.02 g, 1.0 equiv., 200-400 mesh, extent of labeling: 0.5-1.0 mmol/g OH loading, 1 % cross-linked with divinylbenzene) and  $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$  (4:1, 20 mL) were added to a fritted syringe (20 mL) connected a two-necked round bottle flask. The mixture was bubbled under nitrogen flow for 1 h.  $\text{CH}_2\text{Cl}_2$  was added twice to compensate for the loss of solvent. The solvent was then removed by filtration and the resin was washed with DMF (2 x 15 mL) followed by the addition of Fmoc-Asp(OAll)-OH (1.22 g, 3.09 mmol, 3.1 equiv.), HATU (1.15 g, 3.02 mol, 3.0 equiv.), DIPEA (0.52 mL, 3.00 mmol, 3.0 equiv.), and DMF (5.5 mL). The mixture was bubbled for 16 h. The suspension was filtered and washed with DMF (2 x 15 mL) as well as  $\text{CH}_2\text{Cl}_2$  (2 x 15 mL). The loaded resin was then dried *in vacuo* for 24 h.



**Fig. S8.** Reaction set-up for the resin loading using a syringe equipped with a frit and a flow of nitrogen.

#### Biotin-Ala-Ala-Ala-Asn-OH (4)



The peptide synthesizer was charged with:

Vessel: Preloaded Fmoc-Asn(Trt)-Wang resin (200-400 mesh, 0.58 mmol/g)

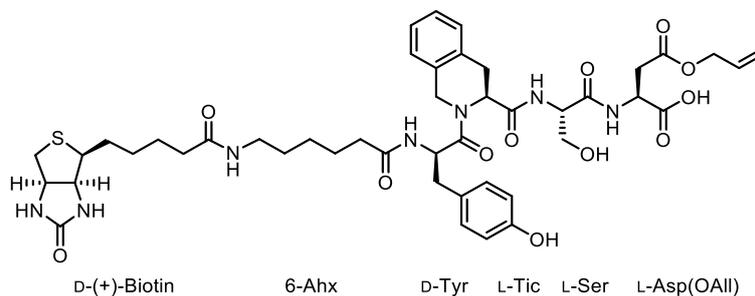
- R1:                      DMF (1000 mL)  
R2:                      *i*-PrOH (600 mL)  
R3:                      DMF (120 mL)  
R4:                      HATU (2.91 g) + DMF (22 mL)  
R5:                      DIPEA (1.3 mL) + DMF (20.7 mL)  
R6:                      Ac<sub>2</sub>O (22.1 mL) + DMF (9.9 mL)  
R7:                      DIPEA (22 mL)  
R9:                      Piperidine (22 mL) + DMF (88 mL)  
AA1:                      Fmoc-Ala-OH (1.89 g) + DMF (16.5 mL)  
XX1:                      (D)-(+)-Biotin (1.74 g) + DMF (5.5 mL)

(considering an 10% excess to compensate for errors of the instrument) and the General Procedure for Peptide Synthesis was followed (page **S14**).

After the peptide synthesizer completed the program, the resin was transferred into a 50 mL flask and TFA/TES/H<sub>2</sub>O (90:5:5, 20 mL) was added and stirred for 2 h to cleave the peptide from the resin. The resin was removed by filtration and washed with DMF (3 x 10 mL). The solvents were then removed under a stream of nitrogen to give a slightly yellow liquid. Et<sub>2</sub>O was added to precipitate the peptide. The obtained white suspension was transferred to centrifuge tubes and centrifuged (2000 rcf, 3 min, rt). The supernatant was separated from the sedimented peptide and evaporated under reduced pressure. To the residue was again added Et<sub>2</sub>O, centrifuged and the supernatant was discarded. The combined solid phases were dried *in vacuo* to give the peptide (0.58 mmol, **quant.**) a colorless solid. The solid was insoluble in most solvent and slightly soluble in DMSO. The compound was obtained as a TFA salt.

**FT-IR**  $\tilde{\nu}$  (film) 3275m, 3063w, 2971w, 2930w, 2802w, 2490w, 1668s, 1626s, 1546s, 1471m, 1426m, 1323w, 1196s, 1125s, 1024m, 961w, 894w, 834m, 798m, 721s, 688m, 610m, 574m, 518m, 457m  $\text{cm}^{-1}$ ;  **$^1\text{H}$  NMR** (500 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.94 – 7.77 (m, 4H), 7.29 (s, 1H), 6.81 (s, 1H), 6.32 (s, 1H), 6.26 (s, 1H), 4.42 – 4.30 (m, 1H), 4.22 – 4.08 (m, 4H), 4.03 (s, 1H), 3.02 – 2.95 (m, 1H), 2.72 (dd,  $J = 12.4, 5.0$  Hz, 1H), 2.49 – 2.36 (m, 3H), 2.00 (td,  $J = 7.3, 2.3$  Hz, 2H), 1.54 – 1.45 (m, 1H), 1.44 – 1.29 (m, 4H), 1.25 – 1.14 (m, 1H), 1.13 – 1.02 (m, 9H) ppm;  **$^{13}\text{C}$  NMR** (126 MHz,  $\text{DMSO-d}_6$ )  $\delta$  172.6, 172.2, 172.0, 171.8, 171.7, 171.2, 162.8, 158.1 (q,  $J = 31.2$  Hz, C(TFA)), 117.2 (q,  $J = 300$  Hz, C(TFA)), 61.0, 59.2, 55.4, 48.6, 47.99, 47.97, 47.8, 40.4, 36.6, 34.9, 28.1, 28.0, 25.2, 18.2, 18.10, 18.07 ppm; **HRMS** ESI(+), (MeCN) calculated for  $\text{C}_{23}\text{H}_{37}\text{N}_7\text{O}_8\text{SNa}^+$   $[\text{M}+\text{Na}]^+$ : 594.23165, found: 594.23023.

## 2.3 Biotin-6-Ahx-D-Tyr-Tic-Ser-Asp(OAll)-OH (5)<sup>[2]</sup>



### Peptide Synthesis:

The peptide synthesizer was charged with:

Vessel: Manual loaded Fmoc-Asp(OAll)-Wang resin (200-400 mesh, 0.58 mmol/g)

- R1:            DMF (1000 mL)
- R2:            *i*-PrOH (600 mL)
- R3:            DMF (120 mL)
- R4:            HATU (2.91 g) + DMF (22 mL)
- R5:            DIPEA (1.3 mL) + DMF (20.7 mL)
- R6:            Ac<sub>2</sub>O (22.1 mL) + DMF (9.9 mL)
- R7:            DIPEA (22 mL)
- R9:            Piperidine (22 mL) + DMF (88 mL)
- AAX:          Fmoc-Ser-OH (1.89 g) + DMF (16.5 mL)
- AAX:          Fmoc-Tic-OH
- AAX:          Fmoc-D-Tyr(*t*-Bu)-OH
- XX1:          (D)-(+)-Biotin (1.74 g) + DMF (5.5 mL)

(considering an 10% excess to compensate for errors of the instrument)

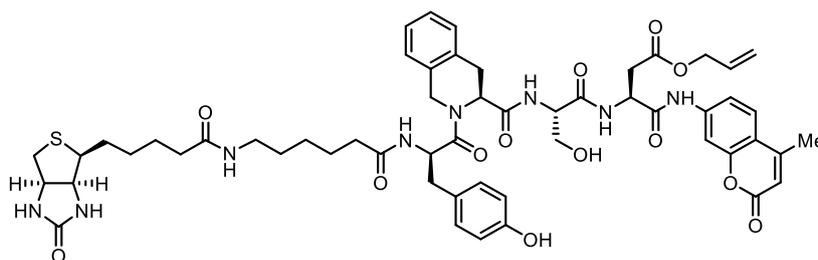
and the General Procedure for Peptide Synthesis was followed (page **S14**).

After the peptide synthesizer completed the program, the resin was transferred into a 50 mL flask and TFA/TES/H<sub>2</sub>O (90:5:5, 20 mL) was added and stirred for 2 h to cleave the peptide from the resin. The resin was removed by filtration and washed with DMF (3 x 10 mL). The solvent was evaporated on a rotary evaporator and the residual liquid was further evaporated under a stream of nitrogen to give a slightly yellow liquid. Et<sub>2</sub>O was added to precipitate the peptide. The obtained white suspension was transferred to centrifuge tubes and centrifuged (2000 rcf, 3 min, rt). The supernatant was separated from the sedimented peptide and

evaporated under reduced pressure. To the residue was again added Et<sub>2</sub>O, centrifuged and the supernatant was discarded. The residue was purified by preparative RP-HPLC [Gemini NX, C18, 5 μ, 110 Å, 250 mm × 21.2 mm, solvent A: H<sub>2</sub>O + 0.1% HCOOH, solvent B: MeCN + 0.1% HCOOH, 20 mL/min; LC time program (min –% B): 5 min – 5%, 30 min – 95%, 30.5 min – 100%] to afford, after lyophilization, peptide **5** (t<sub>R</sub> = 17.5 min, 158 mg, 171 μmol, **41%**) as a slightly yellow solid.

**Optical rotation:**  $[\alpha]_D^{23} = -20.1^\circ$  (c 1.1, CH<sub>3</sub>OH); **FTIR:**  $\tilde{\nu} = 3346, 2975, 2932, 1777, 1714, 1597, 1579, 1530, 1487, 1468, 1426, 1385, 1366, 1327, 1269, 1249, 1170, 1102, 1072, 1059, 915, 878, 827, 792, 720, 684, 647, 592, 530 \text{ cm}^{-1}$ ; **<sup>1</sup>H NMR** (500 MHz, CD<sub>3</sub>OD)  $\delta = 7.17\text{--}7.08$  (m, 4H), 7.05–7.03 (m, 2H), 6.64–6.59 (m, 2H), 6.00–5.85 (m, 1H), 5.31 (dq, *J* = 17.2, 1.8 Hz, 1H), 5.21 (dq, *J* = 10.5, 1.6 Hz, 1H), 5.10 (dd, *J* = 5.6, 3.8 Hz, 1H), 4.97–4.91 (m, 1H), 4.79–4.70 (m, 2H), 4.59 (dt, *J* = 5.6, 1.5 Hz, 2H), 4.52–4.45 (m, 1H), 4.33–4.28 (m, 1H), 4.19–4.12 (m, 1H), 4.10 (d, *J* = 15.1 Hz, 1H), 3.72 (dd, *J* = 11.6, 6.2 Hz, 1H), 3.63 (dd, *J* = 11.6, 4.5 Hz, 1H), 3.24–3.15 (m, 4H), 2.99–2.79 (m, 6H), 2.73–2.68 (m, 1H), 2.27 (t, *J* = 7.5 Hz, 2H), 2.20 (t, *J* = 7.5 Hz, 2H), 1.75–1.56 (m, 6H), 1.53–1.41 (m, 4H), 1.38–1.30 (m, 2H); **<sup>13</sup>C-NMR** (101 MHz, CD<sub>3</sub>OD)  $\delta = 176.7, 176.1, 174.9, 173.6, 172.7, 171.8, 166.1, 157.7, 134.3, 133.6, 133.2, 131.5, 129.1, 128.4, 127.9, 127.7, 127.1, 118.5, 116.3, 66.6, 63.4, 62.9, 61.6, 57.3, 57.0, 55.3, 53.7, 50.3, 46.9, 41.1, 40.3, 37.6, 37.1, 36.9, 36.2, 31.2, 30.0, 29.8, 29.5, 27.5, 26.9, 26.5$ ; **ESI-HRMS** (CH<sub>3</sub>OH): *m/z* 922.40125 (C<sub>45</sub>H<sub>60</sub>N<sub>7</sub>O<sub>12</sub>S<sup>+</sup>; [M+H]<sup>+</sup>, calc. 922.40152) ; **Melting point** = 115 – 120°C

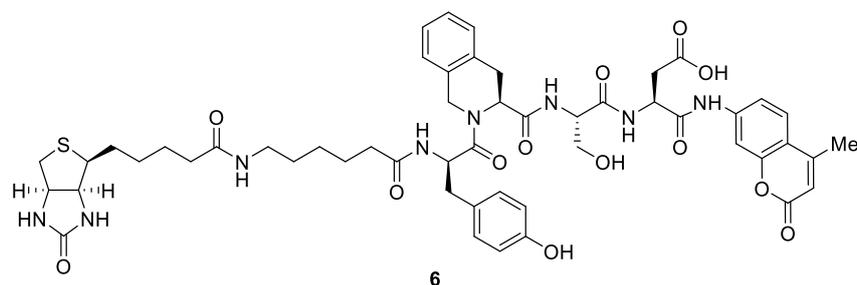
## 2.4 Biotin-6-Ahx-D-Tyr-Tic-Ser-Asp-AMC-OAll



In a microwave vial, peptide **5** (40.0 mg, 43.4  $\mu\text{mol}$ , 1.0 equiv.), 7-Amino-4-methylcoumarin (19.0 mg, 108  $\mu\text{mol}$ , 2.0 equiv.) and HATU (41.2 mg, 108  $\mu\text{mol}$ , 2.0 equiv.) were dissolved in dry DMF (540  $\mu\text{L}$ ). The vial was capped and the reaction was stirred at 40  $^{\circ}\text{C}$  for 3 days. The DMF was removed under a flow of nitrogen and the residue was purified by preparative RP-HPLC [Gemini NX, C18, 5  $\mu$ , 110  $\text{\AA}$ , 250 mm  $\times$  21.2 mm, solvent A:  $\text{H}_2\text{O}$  + 0.1%  $\text{HCOOH}$ , solvent B:  $\text{MeCN}$  + 0.1%  $\text{HCOOH}$ , 20 mL/min; LC time program (min – % B): 5 min – 5%, 30 min – 95%, 30.5 min – 100%] to afford, after lyophilization, biotin-6-Ahx-D-Tyr-Tic-Ser-Asp-AMC-OAll ( $t_{\text{R}}$  = 19.5 min, 22.0 mg, 20.3  $\mu\text{mol}$ , **38%**) as a slightly yellow solid.

**Optical rotation:**  $[\alpha]_{\text{D}}^{23} = -36.8^{\circ}$  ( $c$  1.0,  $\text{CH}_3\text{OH}$ ); **FTIR:**  $\tilde{\nu} = 3301, 3074, 2929, 2860, 1701, 1643, 1617, 1575, 1516, 1417, 1390, 1365, 1332, 1266, 1223, 1172, 1070, 1017, 989, 925, 850, 752, 686, 576, 557, 530 \text{ cm}^{-1}$ ;  **$^1\text{H NMR}$**  (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 7.82$  (d,  $J = 2.0$ , Hz, 1H), 7.69 (d,  $J = 8.7$  Hz, 1H), 7.63 (dd,  $J = 8.7, 2.0$  Hz, 1H), 7.17–7.10 (m, 3H), 7.05–7.00 (m, 3H), 6.60 (d,  $J = 8.5$  Hz, 2H), 6.23 (d,  $J = 1.3$  Hz, 1H), 5.97–5.87 (m, 1H), 5.31 (dq,  $J = 17.2, 1.6$  Hz, 1H), 5.20 (dq,  $J = 10.5, 1.4$  Hz, 1H), 5.06 (dd,  $J = 5.6, 3.8$  Hz, 1H), 4.94–4.88 (m, 2H), 4.78 (d,  $J = 15.2$  Hz, 1H), 4.60 (dq,  $J = 5.7, 1.5$  Hz, 2H), 4.48 (dd,  $J = 7.9, 5.0$  Hz, 1H), 4.29 (dd,  $J = 7.9, 4.4$  Hz, 1H), 4.10 (dd,  $J = 5.6, 4.2$  Hz, 1H), 4.05 (d,  $J = 15.1$  Hz, 1H), 3.81 (dd,  $J = 11.7, 5.7$  Hz, 1H), 3.65 (dd,  $J = 11.7, 4.2$  Hz, 1H), 3.26 (dd,  $J = 15.4, 3.8$  Hz, 1H), 3.19–3.06 (m, 3H), 2.98–2.87 (m, 3H), 2.78–2.66 (m, 2H), 2.45 (d,  $J = 1.2$  Hz, 3H), 2.25 (t,  $J = 7.4$  Hz, 2H), 2.18 (t,  $J = 7.6$  Hz, 2H), 1.77–1.49 (m, 6H), 1.49–1.36 (m, 4H), 1.35–1.23 (m, 2H);  **$^{13}\text{C-NMR}$**  (101 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 176.8, 176.0, 175.1, 173.5, 172.2, 171.8, 171.1, 166.1, 163.2, 157.7, 155.2, 143.2, 134.3, 133.5, 133.2, 131.5, 129.1, 128.5, 128.0, 127.6, 127.2, 126.7, 118.6, 117.4, 116.3, 113.7, 108.2, 66.7, 63.4, 62.9, 61.6, 58.2, 57.0, 55.7, 53.9, 52.2, 46.9, 41.1, 40.2, 37.6, 36.8, 36.7, 36.3, 31.3, 30.0, 29.8, 29.5, 27.5, 26.9, 26.5, 18.6$ ; ; **ESI-HRMS** ( $\text{CD}_3\text{OH}$ ):  $m/z$  1079.45512 ( $\text{C}_{55}\text{H}_{67}\text{N}_8\text{O}_{13}\text{S}^+$ ;  $[\text{M}+\text{H}]^+$ , calc. 1079.45428)

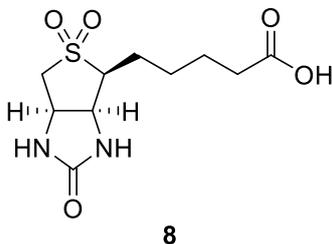
## 2.5 Biotin-6-Ahx-D-Tyr-Tic-Ser-Asp-AMC (6)



Biotin-6-Ahx-D-Tyr-Tic-Ser-Asp-AMC-OAll (6.0 mg, 5.6  $\mu\text{mol}$ , 1.0 equiv.) and  $\text{Pd}(\text{PPh}_3)_4$  (1.3 mg, 1.1  $\mu\text{mol}$ , 0.2 equiv.) were dissolved in dry DMF (360  $\mu\text{L}$ ). Morpholine (0.4  $\mu\text{L}$ , 36.2  $\mu\text{mol}$ , 2.0 equiv.) was added and the reaction was stirred for 1 h at RT. Upon completion of the reaction as indicated by UHPLC-MS, DMF was removed under a flow of nitrogen and the residue was purified by preparative RP-HPLC (Gemini NX, C18, 5  $\mu\text{m}$ , 110  $\text{\AA}$ , 250 mm  $\times$  21.2 mm, solvent A:  $\text{H}_2\text{O}$  + 0.1%  $\text{HCOOH}$ , solvent B:  $\text{CH}_3\text{CN}$  + 0.1%  $\text{HCOOH}$ , 20 mL/min; LC time program (time – % B): 5 min – 5%, 30 min – 95%, 30.5 min – 100%) to afford, after lyophilization, peptide **6** (2.2 mg, 2.0  $\mu\text{mol}$ , **38%**,  $t_{\text{R}}$  = 16.1 min) as a white solid.

**Optical rotation:**  $[\alpha]_{\text{D}}^{23} = -44.2^\circ$  ( $c$  1.2,  $\text{CH}_3\text{OH}$ ); **FTIR:**  $\tilde{\nu} = 3306, 2930, 1700, 1576, 1517, 1417, 1391, 1365, 1262, 1225, 1070, 1018, 800, 752, 530 \text{ cm}^{-1}$ ;  **$^1\text{H NMR}$**  (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 7.85$  (d,  $J = 2.0$  Hz, 1H), 7.72 (d,  $J = 8.7$  Hz, 1H), 7.66 (dd,  $J = 8.7, 2.0$  Hz, 1H), 7.17 – 7.12 (m, 3H), 7.05 – 7.02 (m, 3H), 6.60 (d,  $J = 8.5$  Hz, 2H), 6.25 (d,  $J = 7.7$  Hz, 1H), 5.04 (dd,  $J = 5.5, 3.9$  Hz, 1H), 4.92 (dd,  $J = 10.0, 6.0$  Hz, 1H), 4.84 – 4.77 (m, 4H), 4.48 (dd,  $J = 7.9, 5.0$  Hz, 1H), 4.30 (dd,  $J = 7.9, 4.4$  Hz, 1H), 4.12 – 4.08 (m, 1H), 4.04 (d,  $J = 15.0$  Hz, 1H), 3.82 (dd,  $J = 11.7, 5.7$  Hz, 1H), 3.65 (dd,  $J = 11.6, 4.3$  Hz, 1H), 3.26 (dd,  $J = 15.3, 3.8$  Hz, 1H), 3.21 – 3.04 (m, 3H), 2.99 – 2.88 (m, 3H), 2.78 – 2.66 (m, 2H), 2.47 (s, 3H), 2.25 (t,  $J = 7.3$  Hz, 2H), 2.19 (t,  $J = 7.4$  Hz, 2H), 1.77 – 1.49 (m, 6H), 1.49 – 1.37 (m, 4H), 1.34 – 1.22 (m, 2H);  **$^{13}\text{C-NMR}$**  (101 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 176.9, 176.1, 175.1, 174.2, 173.6, 172.1, 171.5, 166.1, 163.3, 157.8, 155.3, 143.3, 134.3, 133.2, 131.5, 129.0, 128.5, 128.0, 127.6, 127.2, 126.7, 117.4, 116.3, 113.7, 108.2, 63.4, 62.9, 61.6, 58.2, 57.0, 55.8, 53.9, 52.4, 47.0, 41.1, 40.2, 37.6, 36.9, 36.5, 36.2, 31.3, 30.0, 29.8, 29.5, 27.4, 26.9, 26.5, 18.6$ ; **ESI-HRMS** ( $\text{CH}_3\text{OH}$ ):  $m/z$  1039.42311 ( $\text{C}_{52}\text{H}_{63}\text{N}_8\text{O}_{13}\text{S}^+$ ;  $[\text{M}+\text{H}]^+$ , calc. 1039.42298

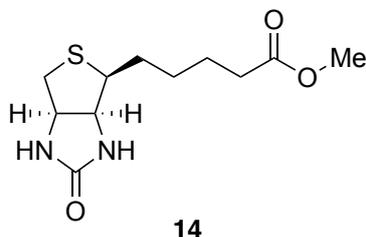
## 2.6 Biotin Sulfone (8)



The procedure was adapted from the literature.<sup>5</sup> Biotin (5.20 g, 21.3 mmol, 1 equiv.) was suspended in CH<sub>3</sub>COOH (60 mL) in a round bottom flask (250 mL). After addition of H<sub>2</sub>O<sub>2</sub> (30% in H<sub>2</sub>O, 20.4 mL), the suspension was stirred at RT. After 10 min a clear solution was observed, and the mixture was stirred for additional 24 h. The resulting white suspension was cooled to 0°C, filtered and rinsed with ice cold Et<sub>2</sub>O (500 mL). Drying *in vacuo* resulted in the desired biotin sulfone **8** (5.36 g, 19.4 mmol, 91%) as a white solid. The data were in agreement with those reported.<sup>5</sup>

**Optical rotation:**  $[\alpha]_D^{23} = 146.3^\circ$  (*c* 1.1, H<sub>2</sub>O); **FTIR:**  $\tilde{\nu} = 3395, 3269, 3033, 2934, 1923, 1693, 1644, 1485, 1465, 1442, 1412, 1398, 1364, 1326, 1303, 1290, 1269, 1241, 1227, 1204, 1194, 1162, 1127, 1077, 997, 903, 822, 758, 747, 729, 702, 671, 636, 614, 577, 561, 530, 501, 485$  cm<sup>-1</sup>; **<sup>1</sup>H NMR** (500 MHz, DMSO-*d*<sub>6</sub>)  $\delta = 12.05$  (br. s, 1H), 6.69 (s, 1H), 6.59 (s, 1H), 4.45–4.34 (m, 2H), 3.36–3.29 (m, 1H), 3.17 (q, *J* = 6.9 Hz, 1H), 3.02 (d, *J* = 14.2 Hz, 1H), 2.22 (t, *J* = 7.5 Hz, 2H), 1.72–1.59 (m, 2H), 1.58–1.50 (m, 2H), 1.46–1.37 (m, 2H); **<sup>13</sup>C-NMR** (101 MHz, DMSO-*d*<sub>6</sub>)  $\delta = 174.0, 161.2, 59.9, 53.8, 53.1, 48.6, 33.1, 25.2, 24.1, 20.8$ . **ESI-HRMS** (H<sub>2</sub>O): *m/z* 277.08522 (C<sub>10</sub>H<sub>17</sub>N<sub>5</sub>O<sub>2</sub>S; [M+H]<sup>+</sup>; calc. 277.08527); **Melting point** = 275 – 278°C

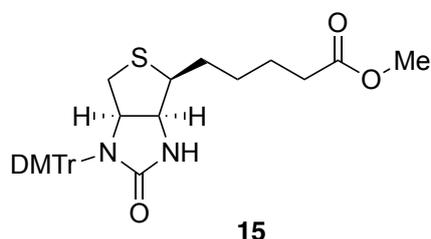
## 2.7 methyl 5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**14**)



The procedure was adapted from the literature.<sup>6</sup> Biotin (10.11 g, 41.4 mmol, 1 equiv.) was suspended in dry CH<sub>3</sub>OH (200 mL). HCl (2 mL, 2 M in CH<sub>3</sub>OH) was added and the reaction mixture was stirred under nitrogen at RT for 18 h. UHPLC showed still starting material so 2 mL of HCl (3 M in CH<sub>3</sub>OH) were added. After 3 h, full conversion was observed. The mixture was concentrated and recrystallized in CH<sub>3</sub>OH to afford the biotin methylester **14** (7.0 g, 41.4 mmol, 66% yield).

**R<sub>f</sub>** = 0.4 (CH<sub>2</sub>Cl<sub>2</sub>: 10% CH<sub>3</sub>OH); **Optical rotation**:  $[\alpha]_D^{23} = +58.4^\circ$  (*c* 1.0, CHCl<sub>3</sub>); **FTIR**:  $\tilde{\nu} = 3277, 2923, 1745, 1707, 1465, 1432, 1306, 1288, 1266, 1249, 1238, 1218, 1205, 1171, 1137, 1118, 1101, 1039, 1016, 1001, 861, 851, 761, 744, 703, 655, 609, 574, 558, 546 \text{ cm}^{-1}$ ; **<sup>1</sup>H NMR** (500 MHz, DMSO-*d*<sub>6</sub>)  $\delta = 6.42$  (s, 1H), 6.35 (s, 1H), 4.33–4.28 (m, 1H), 4.13 (ddd, *J* = 7.7, 4.4, 1.9 Hz, 1H), 3.58 (s, 3H), 3.10 (ddd, *J* = 8.5, 6.2, 4.4 Hz, 1H), 2.82 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.57 (d, *J* = 12.4 Hz, 1H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.68–1.22 (m, 6H); **<sup>13</sup>C-NMR** (101 MHz, DMSO-*d*<sub>6</sub>)  $\delta = 173.0, 162.7, 61.0, 59.2, 55.3, 51.2, 33.1, 28.0, 24.5$ ; **ESI-HRMS** (H<sub>2</sub>O): *m/z* 259.11103 (C<sub>11</sub>H<sub>19</sub>N<sub>2</sub>O<sub>3</sub>S<sup>+</sup>; [M+H]<sup>+</sup>, calc. 259.11109); **Melting point** = 161 – 162°C

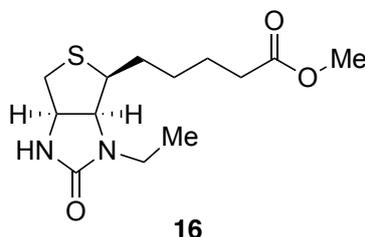
## 2.8 methyl 5-((3a*S*,4*S*,6a*R*)-1-(bis(4-methoxyphenyl)(phenyl)methyl)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**15**)



The procedure was adapted from the literature.<sup>6</sup> Biotin methylester **14** (1.71 g, 6.63 mmol, 1 equiv.) in a 100 mL round flask was dried under high vacuum pump overnight, and then dissolved in dry pyridine (40 mL) and stirred under nitrogen atmosphere at RT. DMTrCl (4.49 g, 13.3 mmol, 2 equiv.) was added at once, followed by the addition of DMAP (81 mg, 0.66 mmol, 0.1 equiv.) at once. The reaction mixture was stirred under nitrogen at RT for 24 h. Excess of DMTrCl was quenched by the addition of MeOH (4 mL) and the reaction mixture was stirred for an additional 5 min. The mixture was concentrated, extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 50 mL), washed with aq. NaHCO<sub>3</sub> (3 x 50 mL), brine (3 x 50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo*. The crude mixture was purified by column chromatography on SiO<sub>2</sub> (CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH; 99:1 to 97:3) to afford *N*1'-DMTr biotin methylester **15** (3.2 g, 5.76 mmol, 87% yield) as a yellow solid.

**R<sub>f</sub>** = 0.2 (CH<sub>2</sub>Cl<sub>2</sub>:CH<sub>3</sub>OH; 97:3); **Optical rotation**:  $[\alpha]_D^{23} = +70.1^\circ$  (*c* 1.1, CHCl<sub>3</sub>); **FTIR**:  $\tilde{\nu} = 3219, 3088, 3000, 2949, 2930, 2856, 2838, 1733, 1699, 1607, 1583, 1509, 1462, 1441, 1416, 1365, 1338, 1298, 1252, 1217, 1180, 1118, 1034, 908, 86, 792, 757, 729, 703, 586 \text{ cm}^{-1}$ ; **<sup>1</sup>H NMR** (500 MHz, CDCl<sub>3</sub>)  $\delta = 7.32 - 7.25$  (m, 5H), 7.21 - 7.12 (m, 4H), 6.85 - 6.78 (m, 4H), 4.60 (d, *J* = 1.7 Hz, 1H), 4.42 - 4.37 (m, 1H), 4.37 - 4.32 (m, 1H), 3.79 (s, 6H), 3.67 (s, 3H), 3.16 - 3.10 (m, 1H), 2.46 (dd, *J* = 13.0, 2.1 Hz, 1H), 2.35 - 2.25 (m, 3H), 1.72 - 1.57 (m, 5H), 1.54 - 1.32 (m, 1H); **<sup>13</sup>C-NMR** (101 MHz, CDCl<sub>3</sub>)  $\delta = 174.0, 161.0, 158.6, 143.8, 135.9, 135.8, 131.4, 129.9, 127.8, 127.1, 113.0, 72.9, 65.7, 59.8, 55.4, 54.1, 51.8, 39.2, 33.8, 29.0, 28.4, 24.8$ ; **ESI-HRMS** (CH<sub>3</sub>OH:CHCl<sub>3</sub>; 3:2): *m/z* 561.24261 (C<sub>32</sub>H<sub>36</sub>N<sub>2</sub>O<sub>5</sub>S<sup>+</sup>; [M+H]<sup>+</sup>; calc. 561.24177); **Melting point** = 67 - 71°C

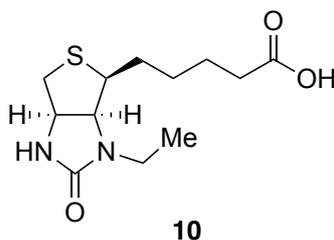
## 2.9 methyl 5-((3a*S*,4*S*,6a*R*)-3-ethyl-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**16**)



The procedure was adapted from the literature.<sup>6</sup> *N*1'-DMTr biotin methylester **15** (358 mg, 0.64 mmol, 1 equiv.) and NaH (51 mg, 1.28 mmol, 60 wt%, 2 equiv.) were dissolved in 4 mL dry DMF and stirred under nitrogen at RT. After stirring for 10 min, CH<sub>3</sub>CH<sub>2</sub>I (102 μL, 1.28 mmol, 2 equiv.) was added. The reaction mixture was stirred for 3 h. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (40 mL), washed with H<sub>2</sub>O (3 x 30 mL), brine (3 x 30 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo*. Toluene was used as azeotrope to help removing DMF. The yellow foam was taken further as crude without further purification. The crude was dissolved in CH<sub>3</sub>COOH (60 mL, 80%). The reaction mixture was stirred RT for 20 min. The mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 40 mL), washed with H<sub>2</sub>O (3 x 30 mL), brine (3 x 30 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo*. The crude mixture was purified by column chromatography on SiO<sub>2</sub>(CH<sub>2</sub>Cl<sub>2</sub>: CH<sub>3</sub>OH, 100:5) to give the product **16** (785.6 mg, 3.7 mmol, 73% yield) over two steps.

**R<sub>f</sub>** = 0.3 (CH<sub>2</sub>Cl<sub>2</sub>: CH<sub>3</sub>OH, 100:5); **Optical rotation**:  $[\alpha]_D^{23} = +91.1^\circ$  (*c* 1.1, CHCl<sub>3</sub>); **FTIR**:  $\tilde{\nu} = 3259, 2924, 2854, 1735, 1691, 1459, 1435, 1357, 1174, 1071, 1017, 862, 800, 761, 699, 610, 495$  cm<sup>-1</sup>; **<sup>1</sup>H NMR** (500 MHz, CDCl<sub>3</sub>)  $\delta = 4.88 - 4.79$  (m, 1H), 4.67 (dd, *J* = 9.2, 5.4 Hz, 1H), 4.54 (q, *J* = 7.2 Hz, 1H), 4.13 - 4.04 (m, 3H), 3.67 - 3.60 (m, 1H), 3.37 (ddd, *J* = 26.5, 13.3, 6.7 Hz, 2H), 3.23 (dd, *J* = 12.5, 3.6 Hz, 1H), 2.82 - 2.69 (m, 2H), 2.11 - 2.03 (m, 2H), 2.04 - 1.88 (m, 1H), 1.88 - 1.77 (m, 1H), 1.68 (t, *J* = 7.1 Hz, 1H), 1.56 (t, *J* = 7.3 Hz, 3H); **<sup>13</sup>C-NMR** (101 MHz, CDCl<sub>3</sub>)  $\delta = 173.1, 162.1, 64.1, 59.5, 57.2, 53.9, 50.7, 37.4, 33.1, 27.9, 33.7, 23.9, 11.7$ ; **ESI-HRMS** (H<sub>2</sub>O): *m/z* 287.14211 (C<sub>12</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub>S<sup>+</sup>; [M+H]<sup>+</sup>, calc. 287.14239); **Melting point** = 75 - 78°C

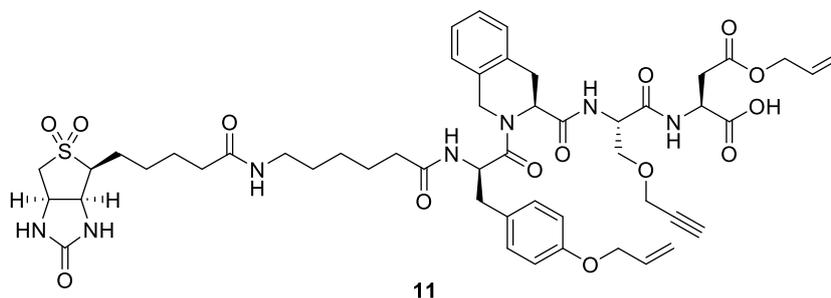
## 2.10 N<sup>3</sup>-ethyl Biotin (10)



The procedure was adapted from the literature.<sup>6</sup> The white solid **16** (786.00 mg, 2.7 mmol) was dissolved in CH<sub>3</sub>OH (12 mL) in a round bottom flask (100 mL). NaOH (1 M, 14 mL) was added. The reaction mixture was stirred at RT for 1 h. The mixture was concentrated *in vacuo* and acidified with HCl (2 M, 6 mL) to pH 3.0. The mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 30 mL). The combined organic layer was washed with brine (3 x 30 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to afford **10** (556.20 mg, 2.0 mmol, 74%) as a white solid.

**Optical rotation:**  $[\alpha]_D^{23} = 59.6^\circ$  (*c* 1.0, H<sub>2</sub>O); **FTIR:**  $\tilde{\nu} = 3277, 2932, 2863, 2555, 2255, 2126, 1995, 1690, 1461, 1430, 1378, 1345, 1305, 1253, 1222, 1210, 1181, 1050, 1023, 1004, 919, 861, 823, 759, 686, 630, 611, 583, 531, 466 \text{ cm}^{-1}$ ; **<sup>1</sup>H NMR** (500 MHz, DMSO-*d*<sub>6</sub>)  $\delta = 12.00$  (s, 1H), 6.60 (s, 1H), 4.28 – 4.20 (m, 1H), 4.16 (dd, *J* = 8.6, 4.9 Hz, 1H), 3.42 (q, *J* = 7.6 Hz, 1H), 3.22 (dt, *J* = 10.3, 4.2 Hz, 1H), 2.88 – 2.78 (m, 2H), 2.60 (dd, *J* = 12.3, 2.5 Hz, 1H), 2.21 (t, *J* = 6.5 Hz, 2H), 1.73 – 1.22 (m, 6H), 1.01 (t, *J* = 7.1 Hz, 3H); **<sup>13</sup>C-NMR** (101 MHz, DMSO-*d*<sub>6</sub>)  $\delta = 174.4, 161.6, 63.6, 56.9, 55.1, 37.7, 33.4, 28.5, 27.9, 24.2, 12.0$ .; **ESI-HRMS** (H<sub>2</sub>O): *m/z* 273.12660 (C<sub>12</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub>S<sup>+</sup>; [M+H]<sup>+</sup>, calc. 273.12674); **Melting point** = 105 – 110°C

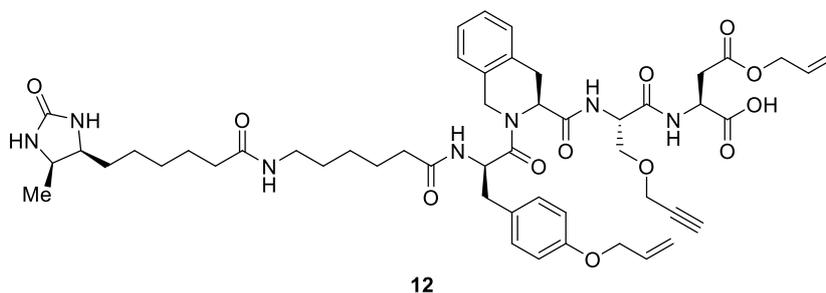
## 2.11 Biotin Sulfone-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (11)



Prepared using the protocol described for the preparation of the peptide **5** (page **S19**) with Fmoc-L-Asp(OAll)-Wang resin (1.0 g, 200-400 mesh, 0.44 mmol/g), (D)-(+)-biotin sulfone (**8**, 0.42 g, 1.5 mmol), Fmoc-D-Tyr(OAll)-OH (0.67 g, 1.5 mmol) and Fmoc-L-Ser(OPPG)-OH (0.74 g, 2.0 mmol) to afford peptide **11** (0.31 g, 0.29 mmol, 68%,  $t_R = 21.7$  min) as a white solid.

**Optical rotation:**  $[\alpha]_D^{23} = -20.0^\circ$  ( $c$  1.1,  $\text{CH}_3\text{OH}$ ); **FT-IR:**  $\tilde{\nu}$  (film) 3289, 2935, 2835, 1705, 1640, 1537, 1442, 1360, 1305, 1224, 1178, 1135, 1111, 1021, 994, 839, 749, 695, 633, 558, 530  $\text{cm}^{-1}$ ;  **$^1\text{H NMR}$**  (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 7.26 - 7.14$  (m, 1H), 7.14 - 7.09 (m, 4H), 7.03 - 6.97 (m, 1H), 6.72 (d,  $J = 8.4$  Hz, 2H), 5.99 - 5.85 (m, 2H), 5.35 - 5.19 (m, 3H), 5.12 (dd,  $J = 10.6, 1.7$  Hz, 1H), 5.08 (dd,  $J = 5.8, 3.7$  Hz, 1H), 4.95 (dd,  $J = 9.6, 6.1$  Hz, 1H), 4.75 (d,  $J = 15.1$  Hz, 1H), 4.72 - 4.65 (m, 1H), 4.61 - 4.57 (m, 4H), 4.38 - 4.33 (m, 1H), 4.32 (t,  $J = 4.9$  Hz, 2H), 4.08 - 4.02 (m, 1H), 3.97 (d,  $J = 2.3$  Hz, 2H), 3.68 (d,  $J = 6.2$  Hz, 2H), 3.36 (dd,  $J = 14.5, 6.3$  Hz, 1H), 3.24 - 3.12 (m, 6H), 2.98 (qd,  $J = 13.0, 7.8$  Hz, 2H), 2.93 - 2.72 (m, 4H), 2.27 - 2.21 (m, 4H), 1.90 (dq,  $J = 9.1, 6.8$  Hz, 1H), 1.77 (dq,  $J = 15.0, 7.9, 7.3$  Hz, 1H), 1.73 - 1.64 (m, 2H), 1.63 - 1.56 (m, 2H), 1.55 - 1.50 (m, 3H), 1.39 - 1.27 (m, 2H);  **$^{13}\text{C-NMR}$**  (101 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 174.6, 174.4, 173.4, 172.0, 171.5, 171.4, 170.4, 169.9, 169.8, 163.4, 157.8, 133.5, 132.8, 132.1, 132.0, 130.1, 127.9, 127.7, 127.0, 126.4, 125.8, 117.2, 116.0, 114.5, 78.9, 75.0, 68.2, 68.2, 65.3, 60.5, 57.6, 54.4, 53.9, 53.3, 53.2, 52.1, 49.8, 48.9, 45.4, 39.0, 36.4, 35.6, 35.3, 34.8, 29.9, 28.7, 26.2, 25.8, 25.3, 25.1, 21.3$ ; **ESI-HRMS** ( $\text{CH}_3\text{OH}$ ):  $m/z$  1032.43836 ( $\text{C}_{51}\text{H}_{66}\text{N}_7\text{O}_{14}^+$ ;  $[\text{M}+\text{H}]^+$ , calc. 1032.43830)

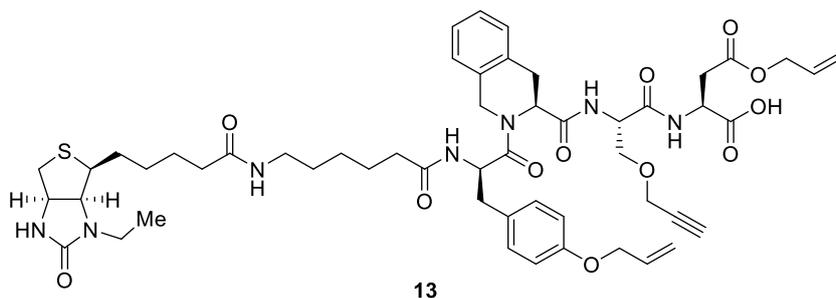
## 2.12 Desthiobiotin-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (12)



Prepared using the protocol described for the preparation of the peptide **11** (page **S28**) with Fmoc-L-Asp(OAll)-Wang resin (1.0 g, 200-400 mesh, 0.43 mmol/g), (D)-(+)-desthiobiotin (**9**, 0.31 g, 1.4 mmol), Fmoc-D-Tyr(OAll)-OH (0.63 g, 1.4 mmol) and Fmoc-L-Ser(OPPG)-OH (0.53 g, 1.5 mmol) to afford peptide **12** (0.07 g, 0.07 mmol, 17%,  $t_R = 23.0$  min) as a white solid.

**UHPLC MS:**  $t_R = 3.37$  min; **Optical rotation:**  $[\alpha]_D^{23} = -33.7^\circ$  ( $c$  1.1,  $\text{CH}_3\text{OH}$ ); **FTIR:**  $\tilde{\nu} = 3290, 2934, 2861, 1732, 1639, 1539, 1511, 1435, 1359, 1223, 1178, 1105, 1021, 993, 929, 840, 748, 654, 531 \text{ cm}^{-1}$ ;  **$^1\text{H NMR}$**  (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 7.24 - 7.08$  (m, 5H), 7.03 - 6.97 (m, 1H), 6.72 (d,  $J = 8.6$  Hz, 2H), 5.98 - 5.86 (m, 2H), 5.35 - 5.16 (m, 3H), 5.12 (dd,  $J = 10.5, 1.9$  Hz, 1H), 5.08 (dd,  $J = 5.7, 3.7$  Hz, 1H), 4.98 - 4.92 (m, 2H), 4.79 - 4.64 (m, 2H), 4.64 - 4.48 (m, 3H), 4.40 - 4.26 (m, 3H), 4.08 - 4.00 (m, 1H), 4.01 - 3.88 (m, 2H), 3.85 - 3.76 (m, 1H), 3.69 (p,  $J = 6.0, 5.1$  Hz, 3H), 3.26 - 3.04 (m, 3H), 3.04 - 2.85 (m, 3H), 2.84 - 2.72 (m, 3H), 2.26 (t,  $J = 7.4$  Hz, 2H), 2.19 (t,  $J = 7.5$  Hz, 2H), 1.61 (dq,  $J = 10.2, 7.3$  Hz, 4H), 1.55 - 1.19 (m, 8H), 1.10 (d,  $J = 6.4$  Hz, 3H);  **$^{13}\text{C-NMR}$**  (101 MHz,  $\text{CD}_3\text{OD}$ )  $\delta = 176.1, 175.9, 174.7, 173.3, 173.3, 172.8, 172.7, 171.7, 171.2, 166.1, 159.1, 134.8, 134.1, 133.5, 133.3, 131.4, 129.2, 129.0, 128.3, 127.8, 127.2, 118.5, 117.4, 115.8, 80.2, 76.4, 69.6, 66.6, 58.9, 57.3, 55.3, 55.2, 54.6, 53.5, 52.6, 50.2, 46.7, 40.3, 37.7, 37.0, 37.0, 36.1, 31.2, 30.7, 30.2, 30.1, 27.5, 27.1, 26.9, 26.4, 15.7$ ; **ESI-HRMS** ( $\text{CH}_3\text{OH}$ ):  $m/z$  970.49227 ( $\text{C}_{51}\text{H}_{68}\text{N}_7\text{O}_{12}^+$ ;  $[\text{M}+\text{H}]^+$ , calc. 970.49205)

### 2.13 N<sup>3</sup>-Ethyl Biotin-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (13)

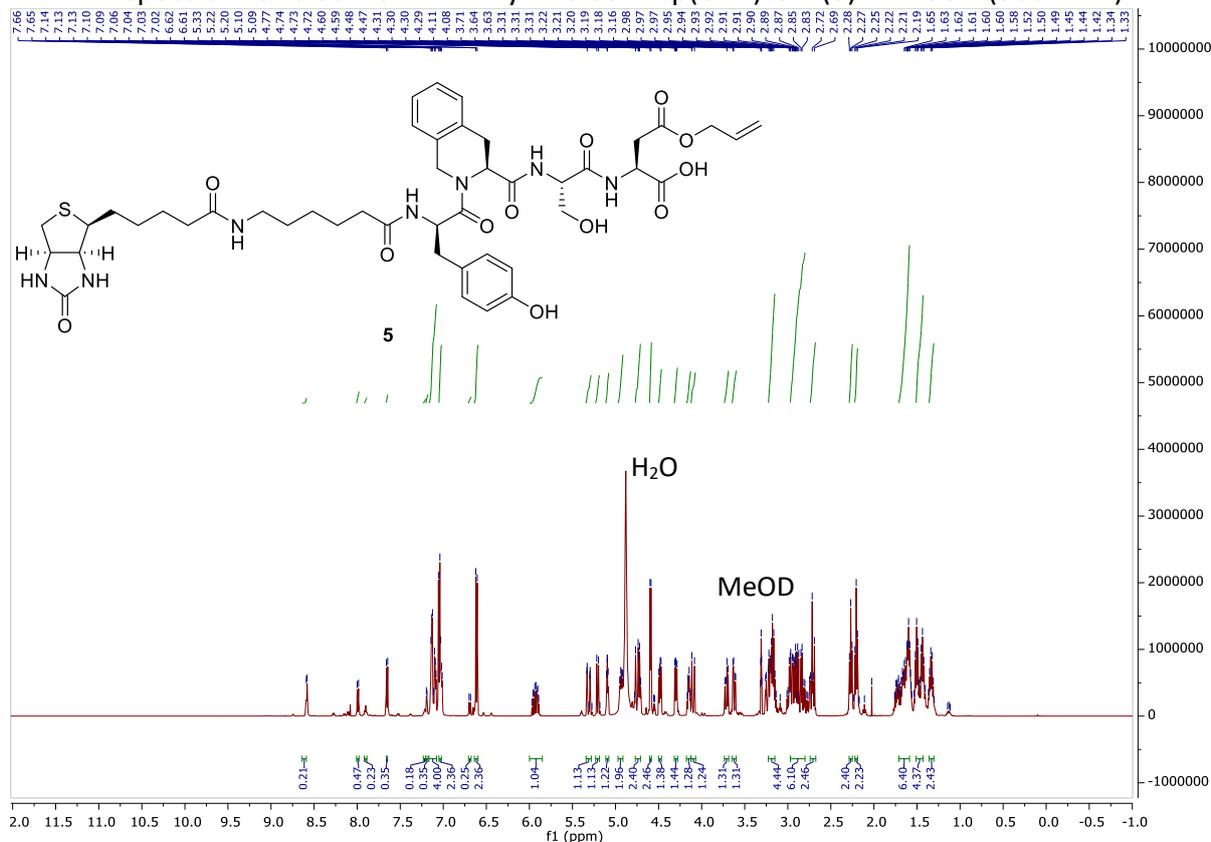


Prepared using the protocol described for the preparation of the peptide **11** (page **S28**) with Fmoc-L-Asp(OAll)-Wang resin (1.0 g, 200-400 mesh, 0.51 mmol/g), N<sup>3</sup>-Ethyl biotin (**10**, 0.25 g, 1.0 mmol), Fmoc-D-Tyr(OAll)-OH (0.79 g, 1.8 mmol) and Fmoc-L-Ser(OPropargyl)-OH (0.64 g, 1.8 mmol) to afford peptide **13** (0.13 g, 0.13 mmol, 25%,  $t_R = 23.3$  min) as a white solid.

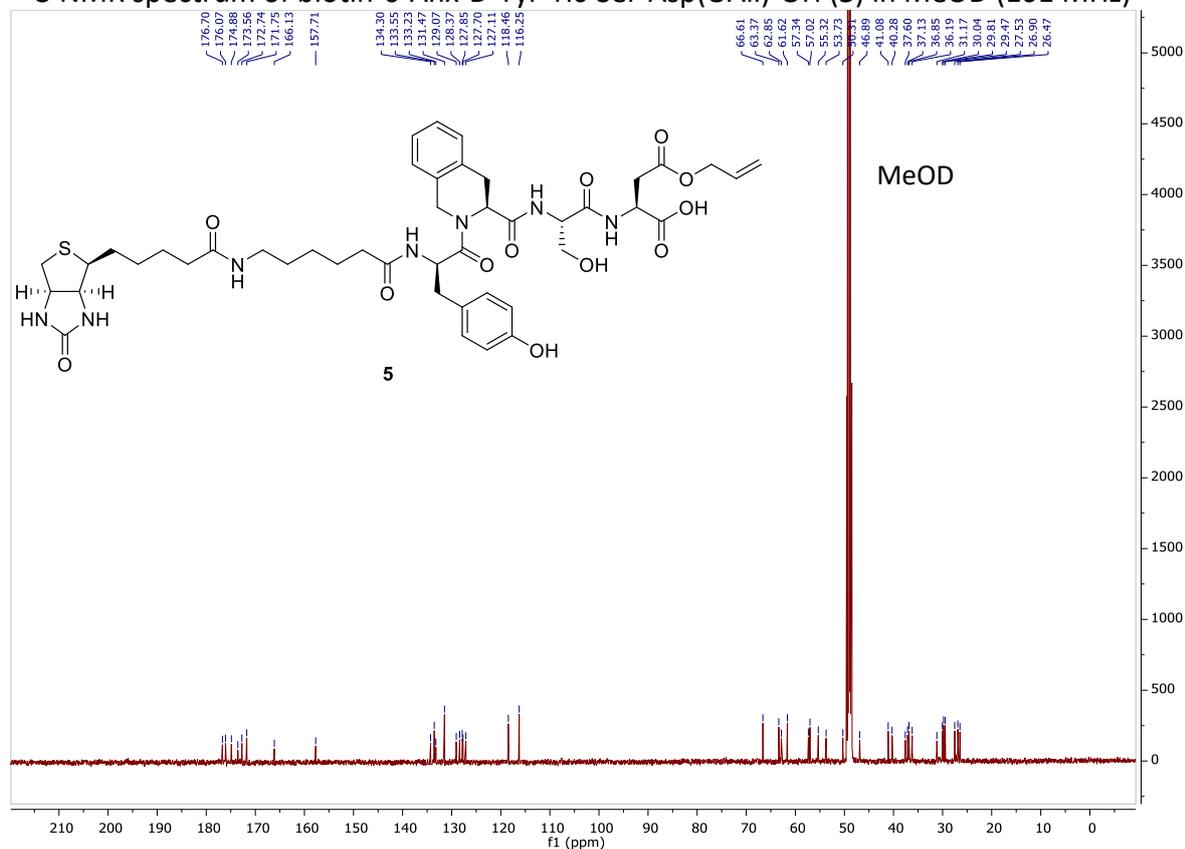
**UHPLC MS:**  $t_R = 3.05$  min; **Optical rotation:**  $[\alpha]_D^{23} = -55.6^\circ$  ( $c$  1.1, CH<sub>3</sub>OH); **FTIR:**  $\tilde{\nu} = 3292, 3071, 2932, 2862, 1736, 1601, 1535, 1511, 1439, 1379, 1356, 1241, 1225, 1178, 1108, 1048, 1022, 994, 929, 841, 751, 685, 612, 570$  cm<sup>-1</sup>; **<sup>1</sup>H NMR** (500 MHz, CD<sub>3</sub>OD)  $\delta = 7.24 - 7.18$  (m, 1H), 7.15 - 7.10 (m, 4H), 7.03 - 6.98 (m, 1H), 6.72 (d,  $J = 8.4$  Hz, 2H), 5.98 - 5.84 (m, 2H), 5.34 - 5.17 (m, 4H), 5.12 (d,  $J = 10.8$  Hz, 1H), 5.08 (dd,  $J = 5.7, 3.8$  Hz, 1H), 4.79 - 4.72 (m, 1H), 4.72 - 4.66 (m, 1H), 4.59 (d,  $J = 5.7$  Hz, 2H), 4.52 (dd,  $J = 28.9, 5.5$  Hz, 1H), 4.41 - 4.34 (m, 2H), 4.33 - 4.27 (m, 3H), 4.09 - 4.00 (m, 1H), 4.00 - 3.89 (m, 2H), 3.69 (d,  $J = 6.3$  Hz, 2H), 3.58 (dq,  $J = 14.4, 7.2$  Hz, 1H), 3.28 - 3.24 (m, 1H), 3.22 - 3.11 (m, 3H), 3.04 - 2.85 (m, 5H), 2.85 - 2.81 (m, 2H), 2.81 - 2.70 (m, 3H), 2.26 (t,  $J = 7.4$  Hz, 2H), 2.23 - 2.15 (m, 2H), 1.78 - 1.65 (m, 2H), 1.65 - 1.56 (m, 2H), 1.56 - 1.45 (m, 3H), 1.45 - 1.29 (m, 3H), 1.11 (t,  $J = 14.3, 1.5$  Hz, 3H); **<sup>13</sup>C-NMR** (101 MHz, CD<sub>3</sub>OD)  $\delta = 175.9, 175.8, 174.6, 173.2, 172.8, 172.7, 171.6, 171.1, 164.7, 159.1, 134.7, 134.1, 133.4, 133.3, 131.4, 129.2, 129.0, 128.3, 127.8, 127.1, 118.5, 117.4, 115.7, 115.6, 80.2, 76.4, 69.5, 69.5, 66.6, 66.1, 59.4, 58.9, 56.7, 55.2, 54.6, 53.4, 50.2, 46.7, 40.2, 39.4, 39.1, 37.7, 36.9, 36.9, 36.1, 31.2, 30.1, 30.1, 29.7, 27.5, 26.8, 26.4, 12.5$ ; **ESI-HRMS** (CH<sub>3</sub>OH):  $m/z$  1028.47885 (C<sub>53</sub>H<sub>70</sub>N<sub>7</sub>O<sub>12</sub>S<sup>+</sup>; [M+H]<sup>+</sup>, calc. 1028.47977)

## 2.15 NMR Spectra

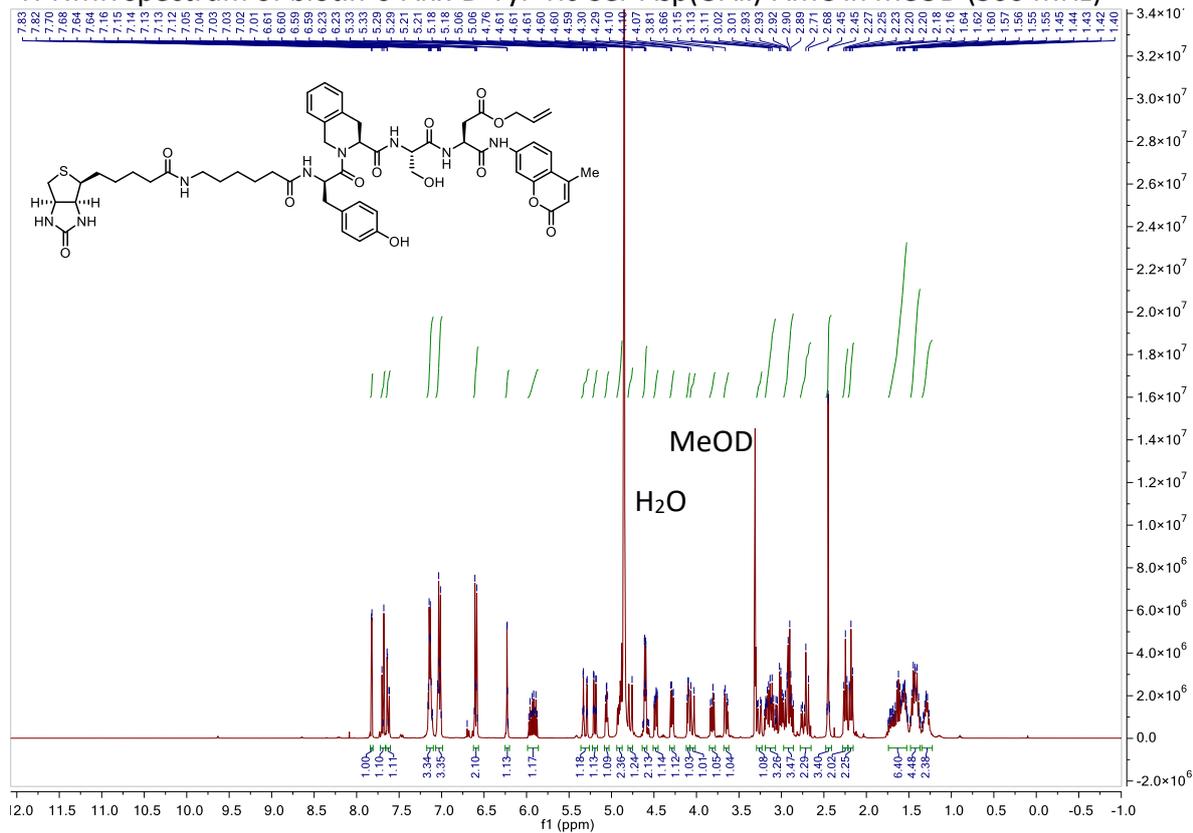
<sup>1</sup>H-NMR spectrum of biotin-6-Ahx-D-Tyr-Tic-Ser-Asp(OAll)-OH (**5**) in MeOD (500 MHz)



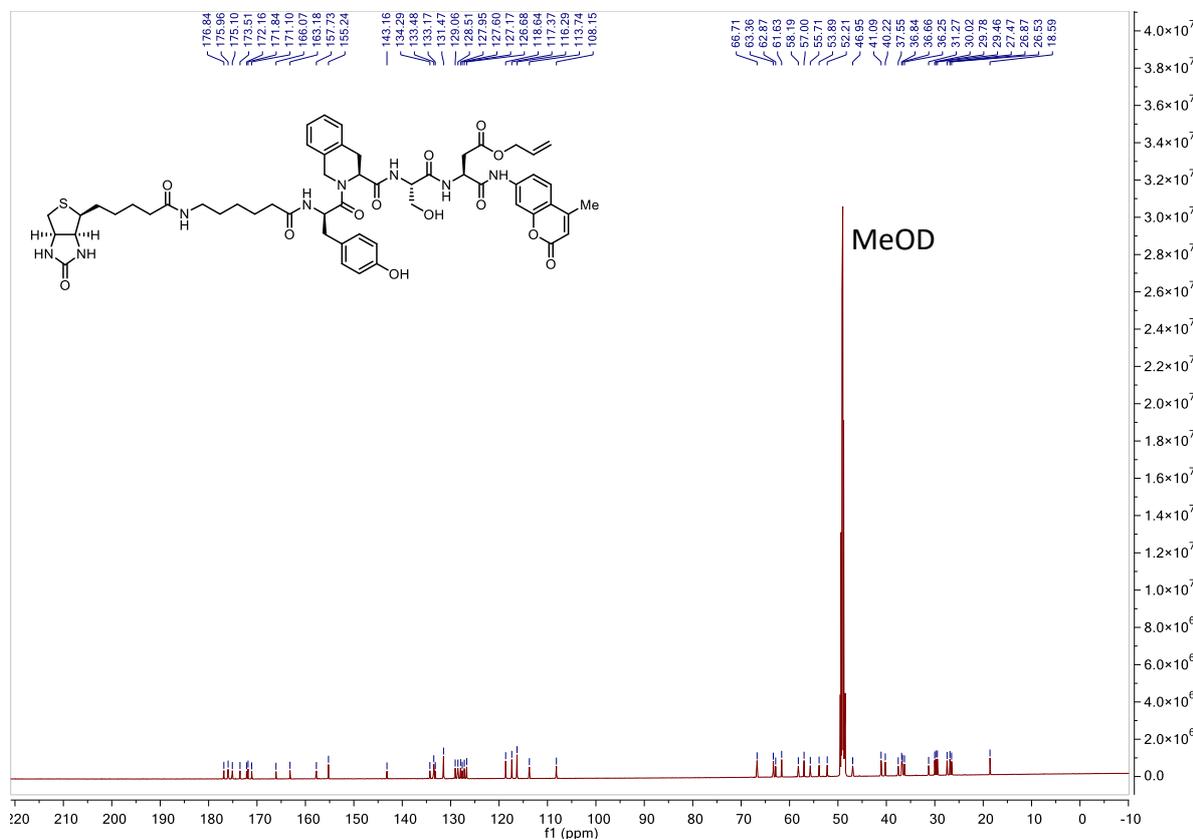
<sup>13</sup>C-NMR spectrum of biotin-6-Ahx-D-Tyr-Tic-Ser-Asp(OAll)-OH (**5**) in MeOD (101 MHz)



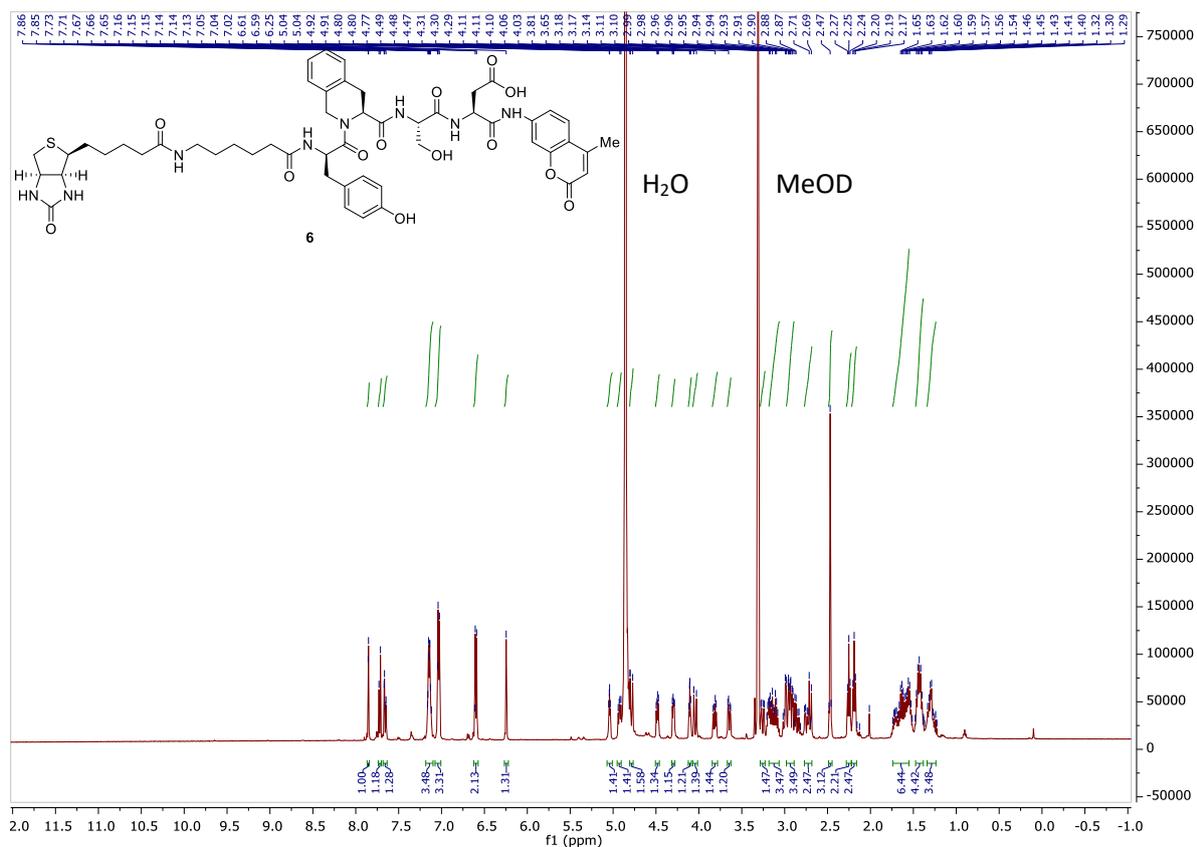
<sup>1</sup>H-NMR spectrum of biotin-6-Ahx-D-Tyr-Tic-Ser-Asp(OAll)-AMC in MeOD (500 MHz)



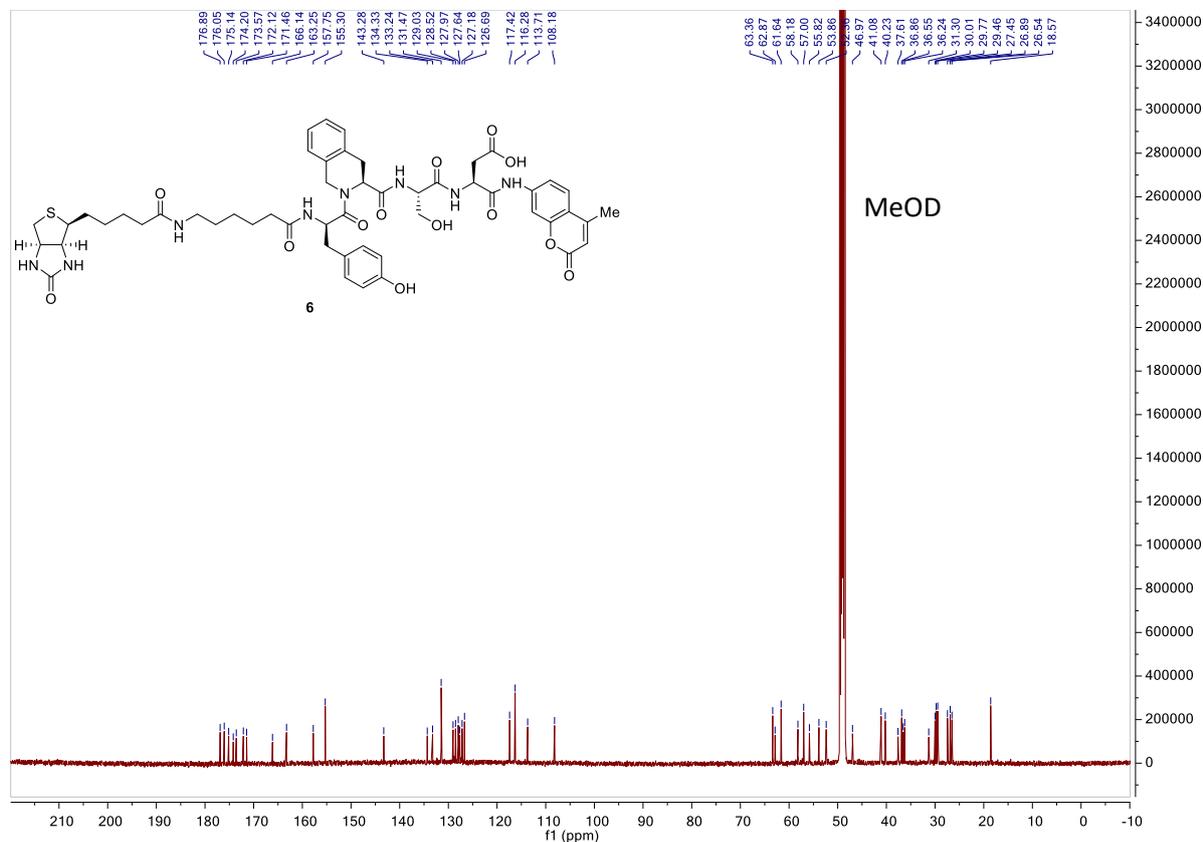
<sup>13</sup>C-NMR spectrum of biotin-6-Ahx-D-Tyr-Tic-Ser-Asp(OAll)-AMC in MeOD (101 MHz)



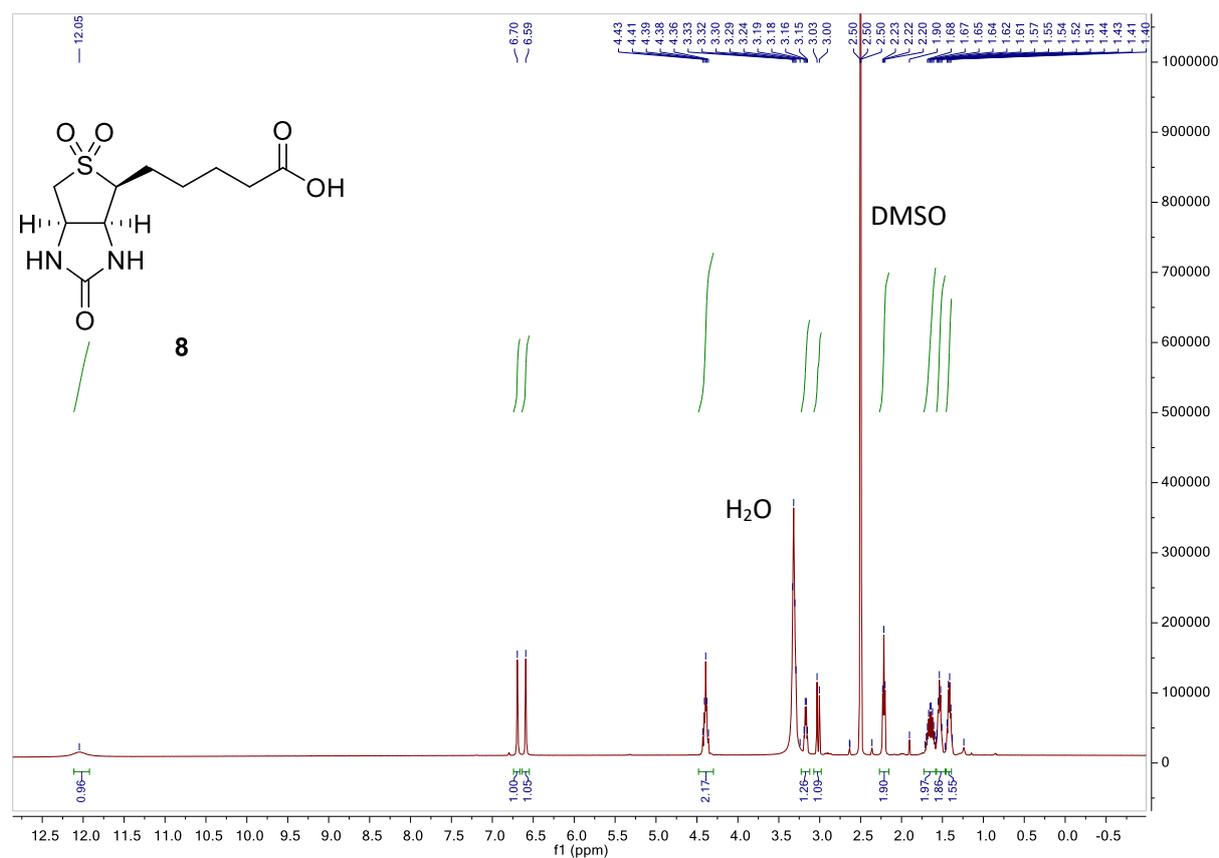
<sup>1</sup>H-NMR spectrum of biotin-6-Ahx-D-Tyr-Tic-Ser-Asp-AMC (6) in MeOD (500 MHz)



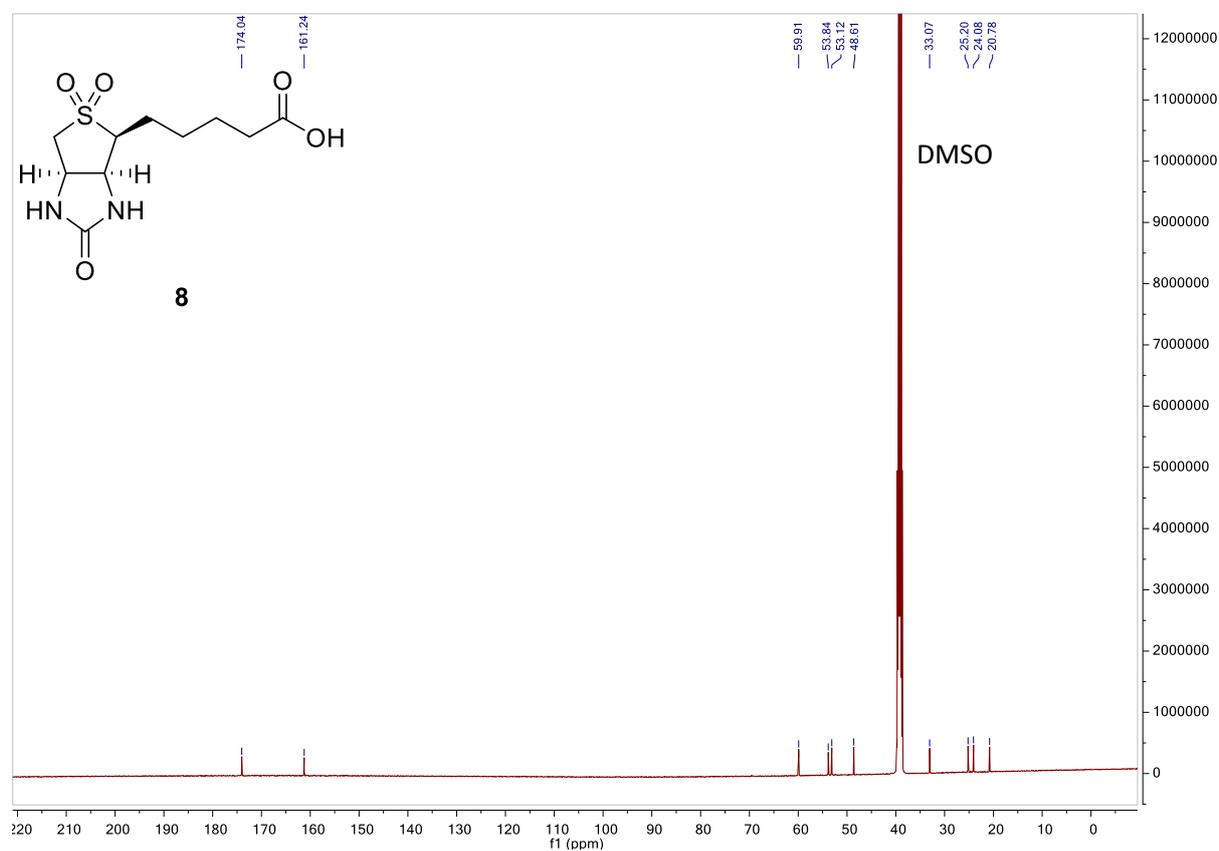
<sup>13</sup>C-NMR spectrum of biotin-6-Ahx-D-Tyr-Tic-Ser-Asp-AMC (6) in MeOD (101 MHz)



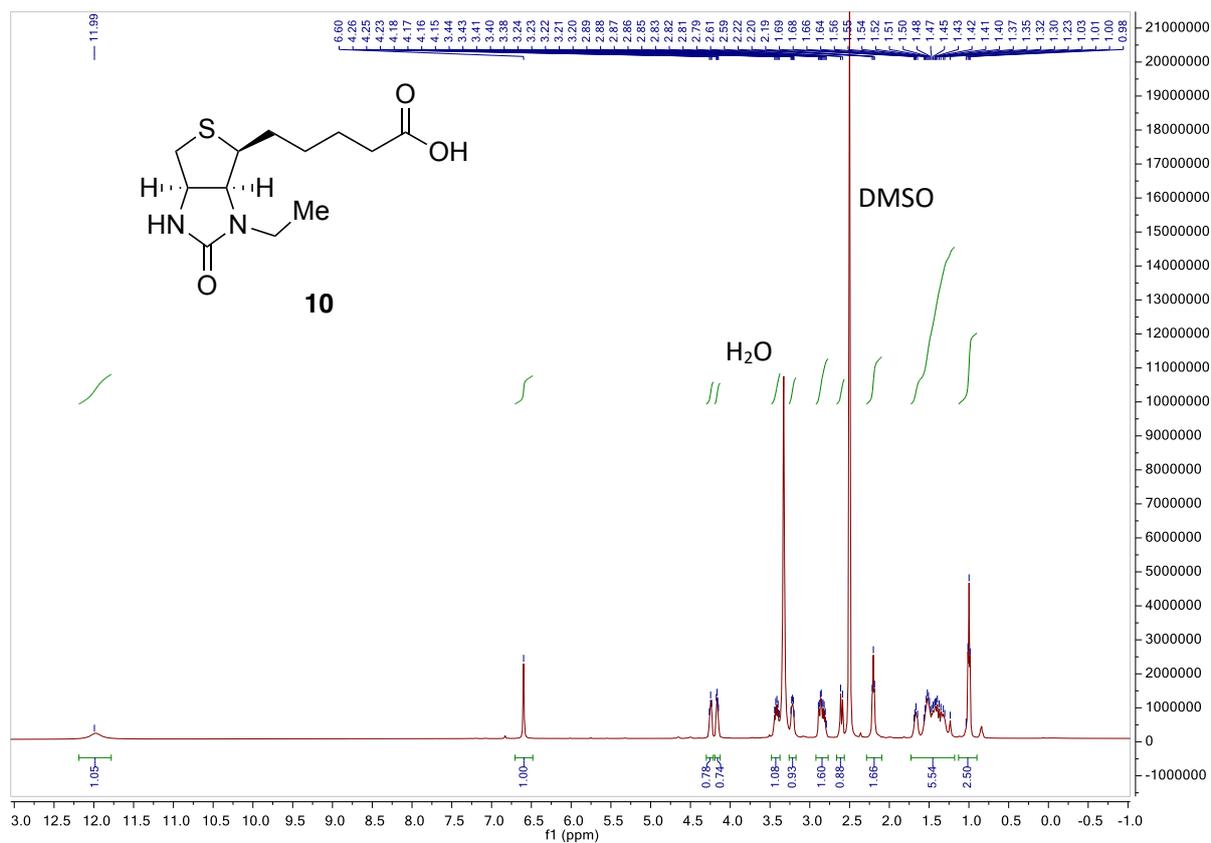
<sup>1</sup>H-NMR spectrum of biotin sulfone (**8**) in DMSO (500 MHz)



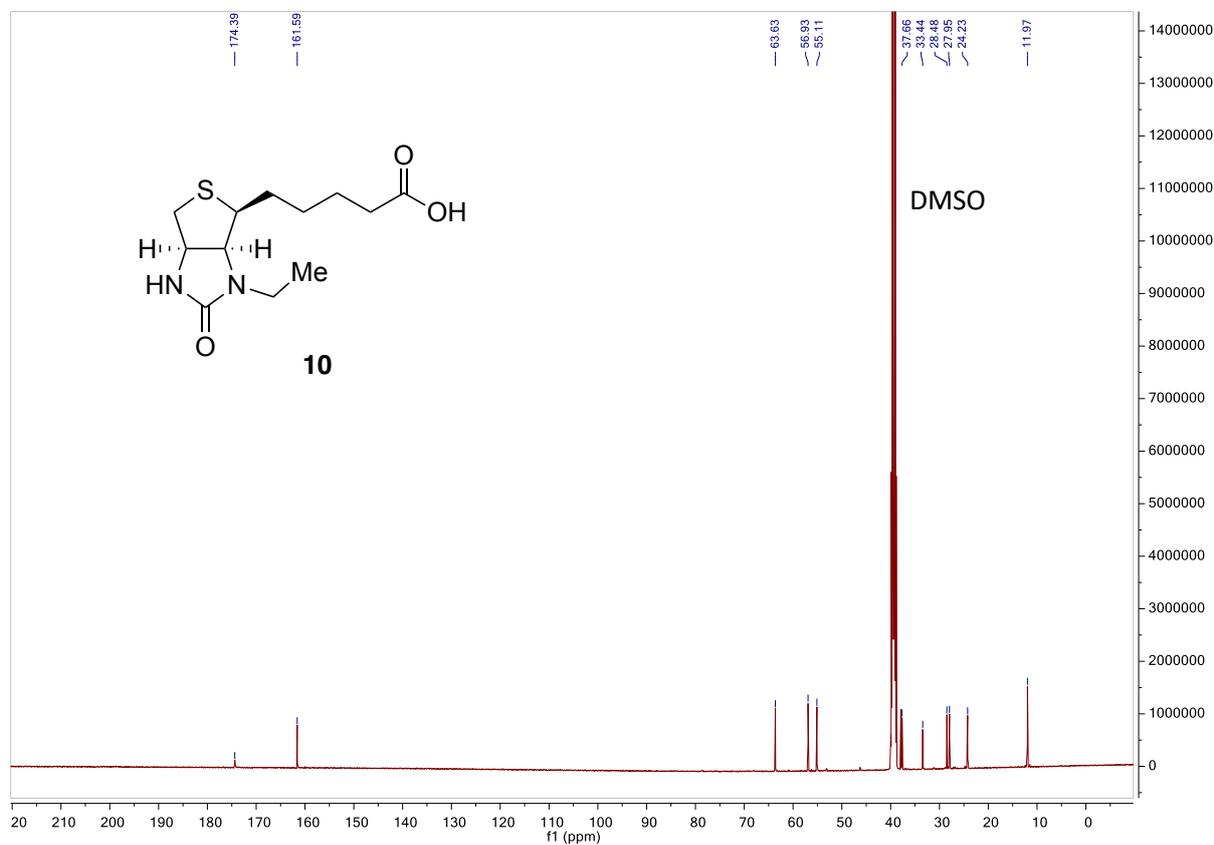
<sup>13</sup>C-NMR spectrum of biotin sulfone (**8**) in DMSO (101 MHz)



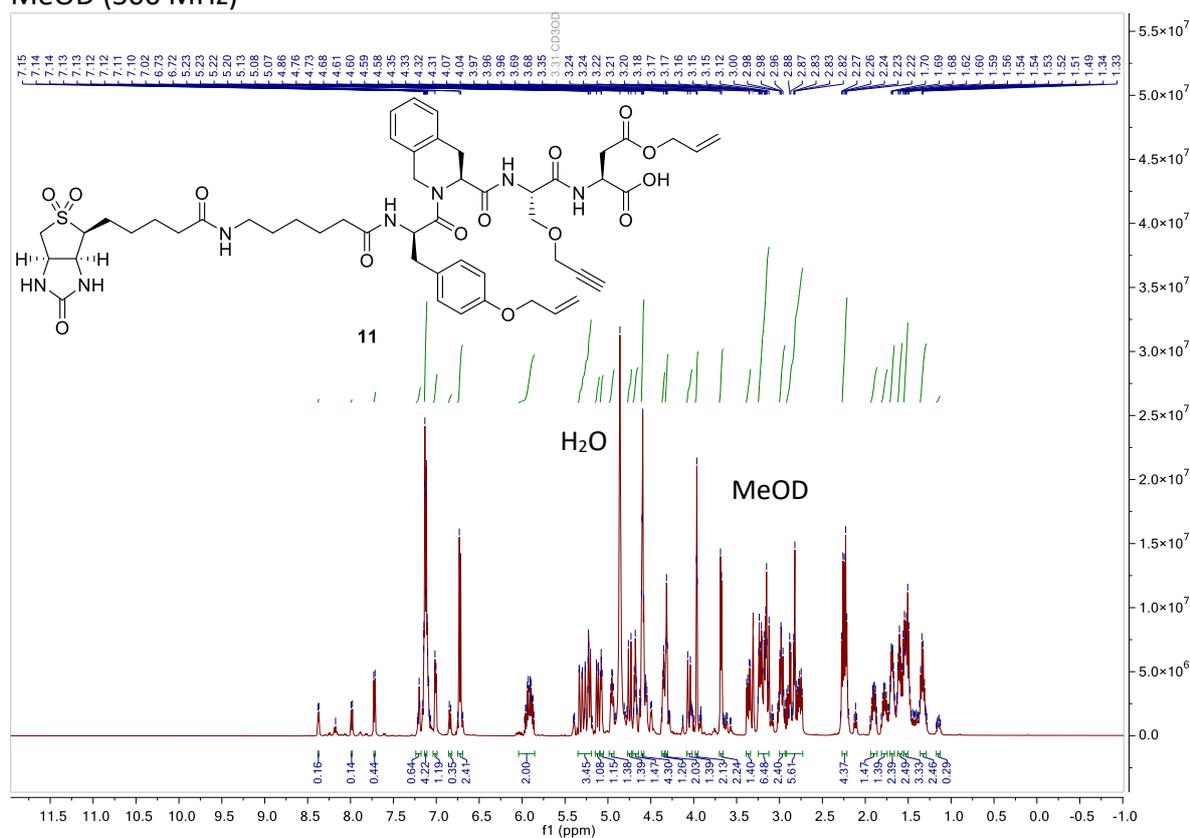
<sup>1</sup>H-NMR spectrum of *N'*3-ethyl biotin (**10**) in DMSO (500 MHz)



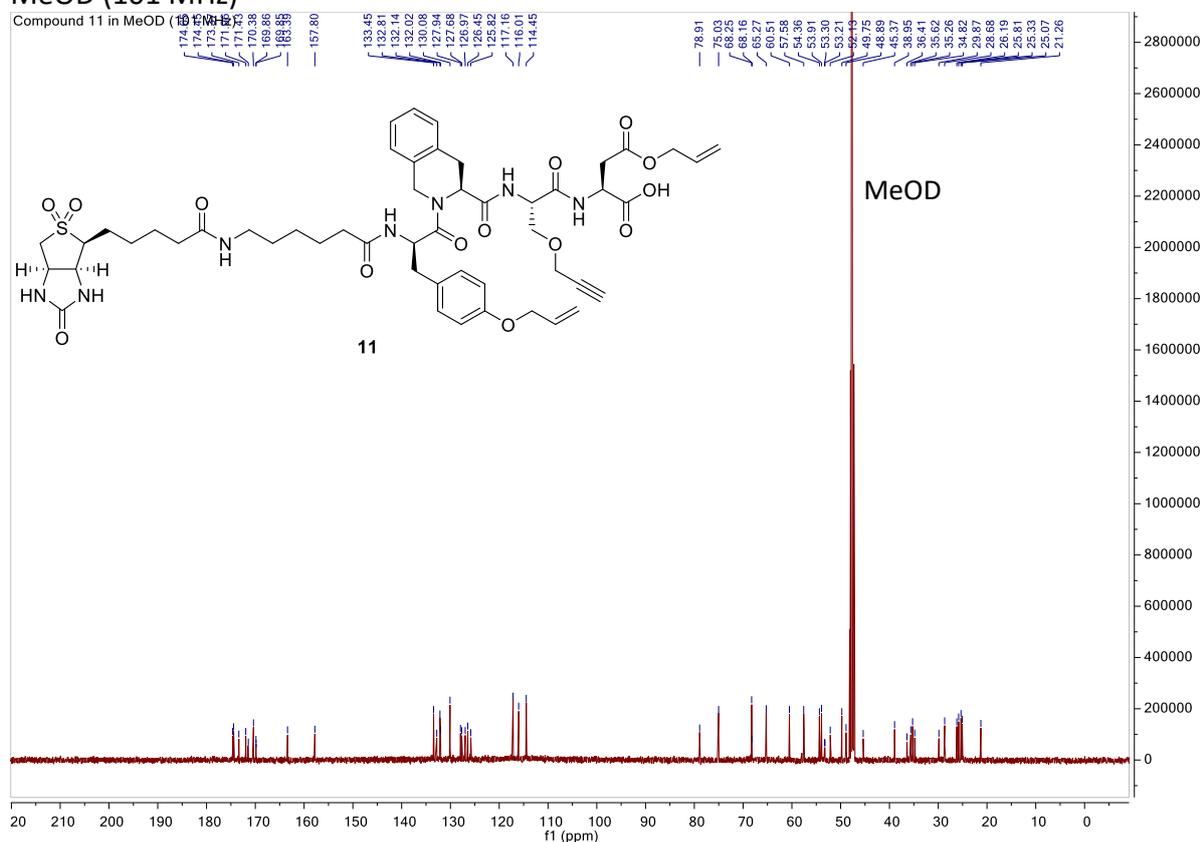
<sup>13</sup>C-NMR spectrum of *N'*3-ethyl biotin (**10**) in DMSO (101 MHz)



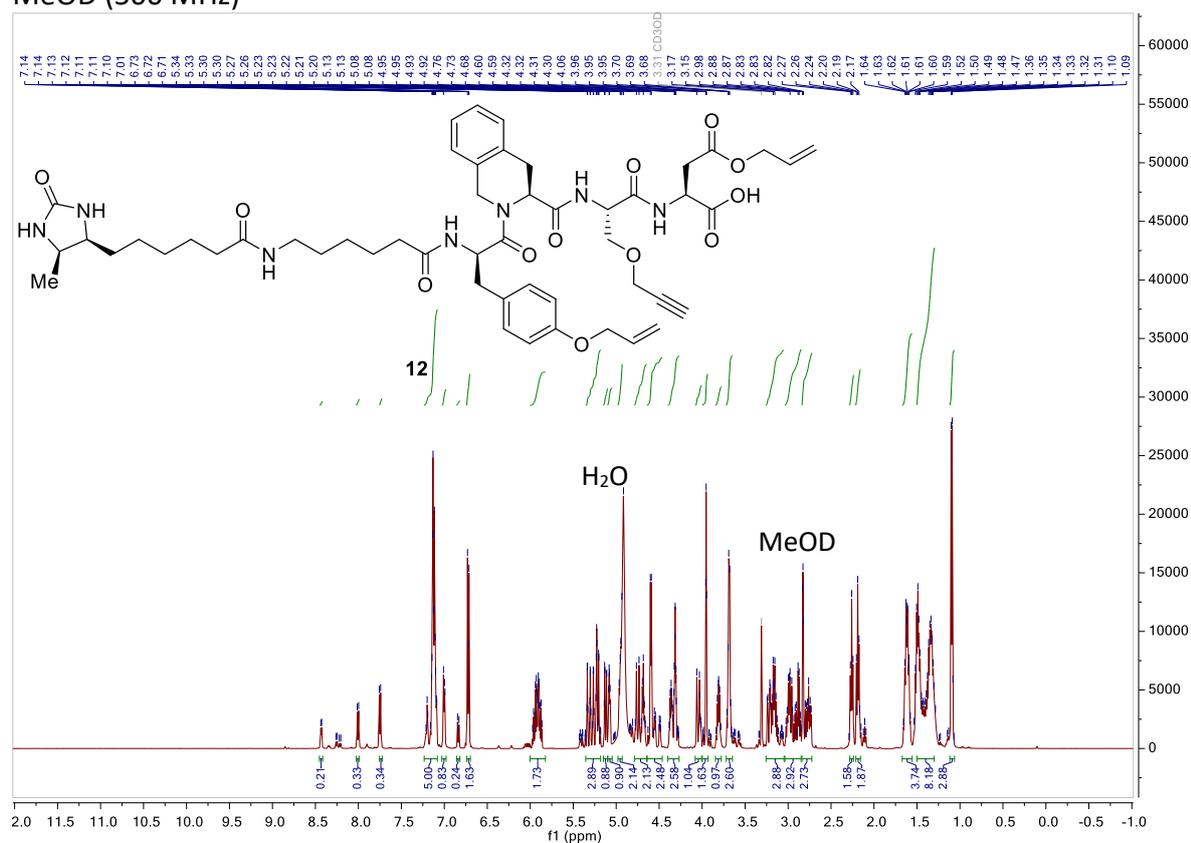
<sup>1</sup>H-NMR spectrum of biotin sulfone-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (**11**) in MeOD (500 MHz)



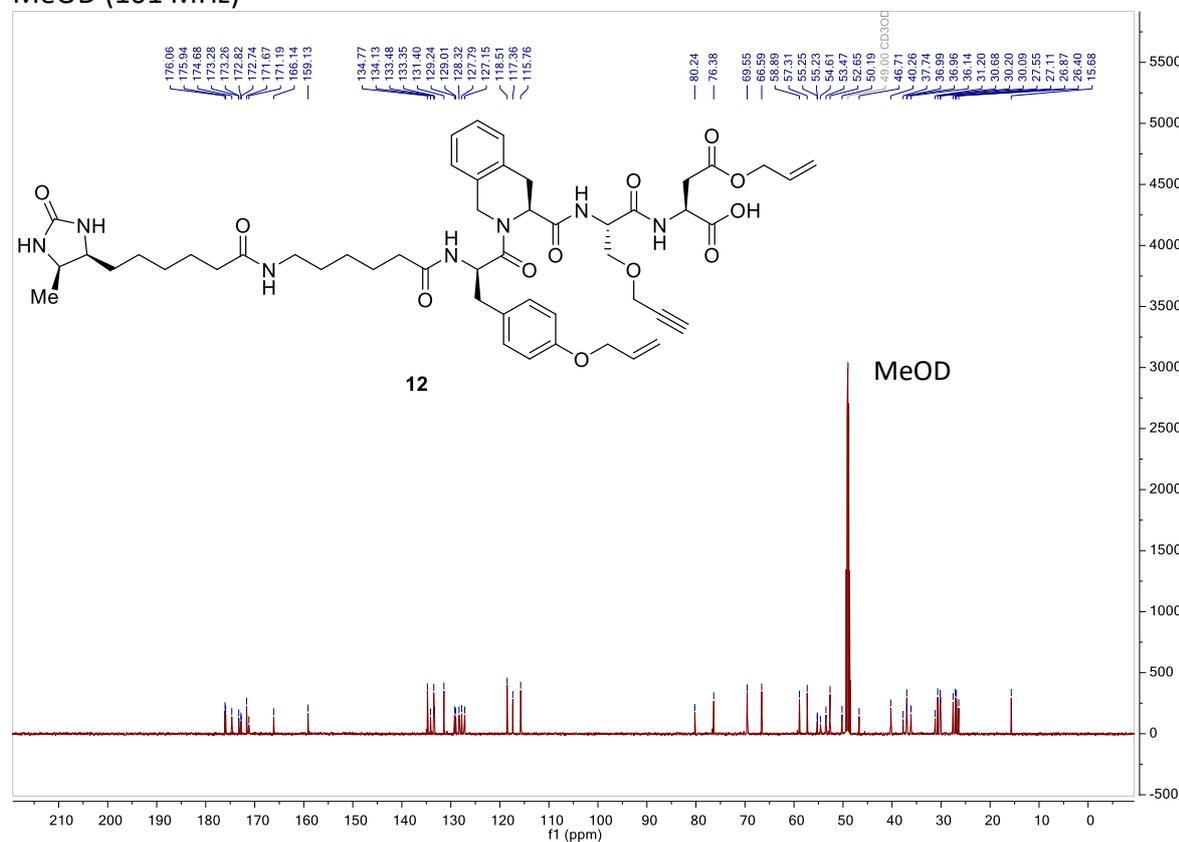
<sup>13</sup>C-NMR spectrum of biotin sulfone-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (**11**) in MeOD (101 MHz)



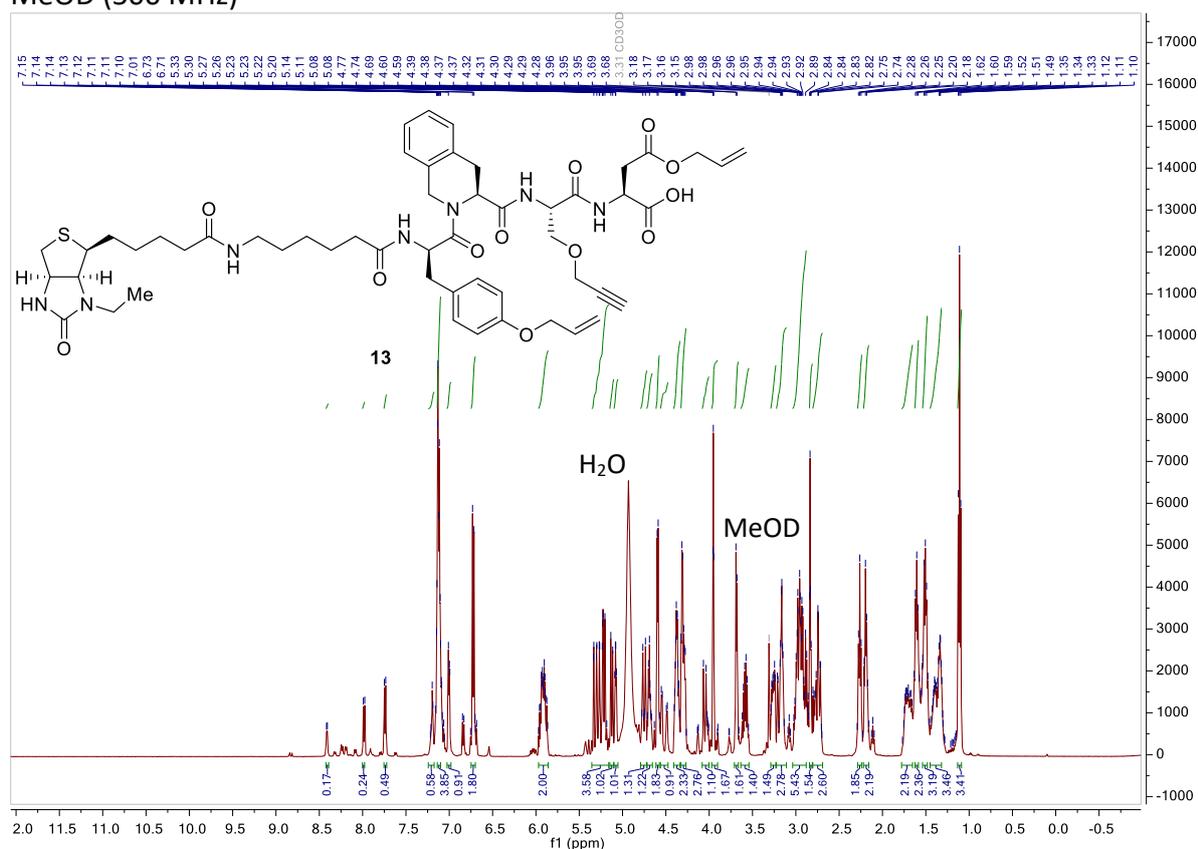
<sup>1</sup>H-NMR spectrum of desthiobiotin-6-Ahx-D-Tyr(OAlI)-Tic-Ser(OPPG)-Asp(OAlI)-OH (**12**) in MeOD (500 MHz)



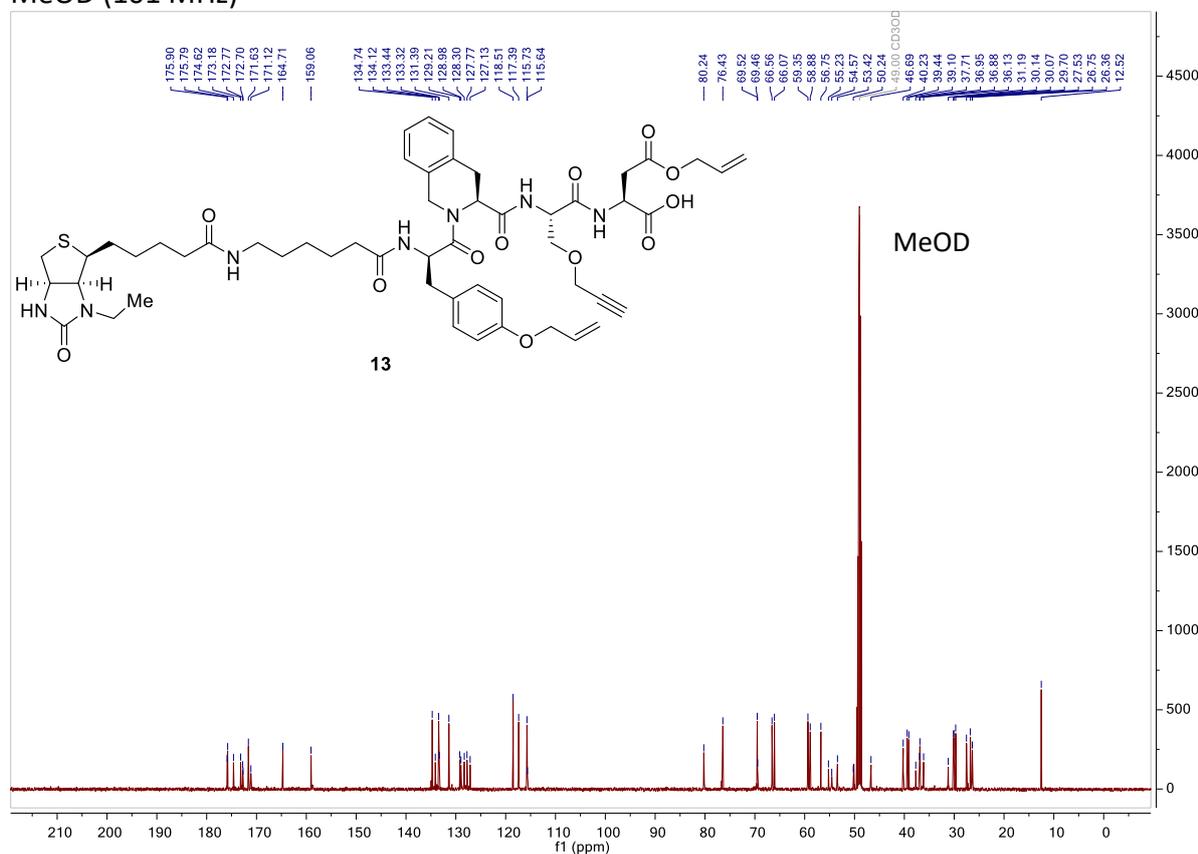
<sup>13</sup>C-NMR spectrum of desthiobiotin-6-Ahx-D-Tyr(OAlI)-Tic-Ser(OPPG)-Asp(OAlI)-OH (**12**) in MeOD (101 MHz)



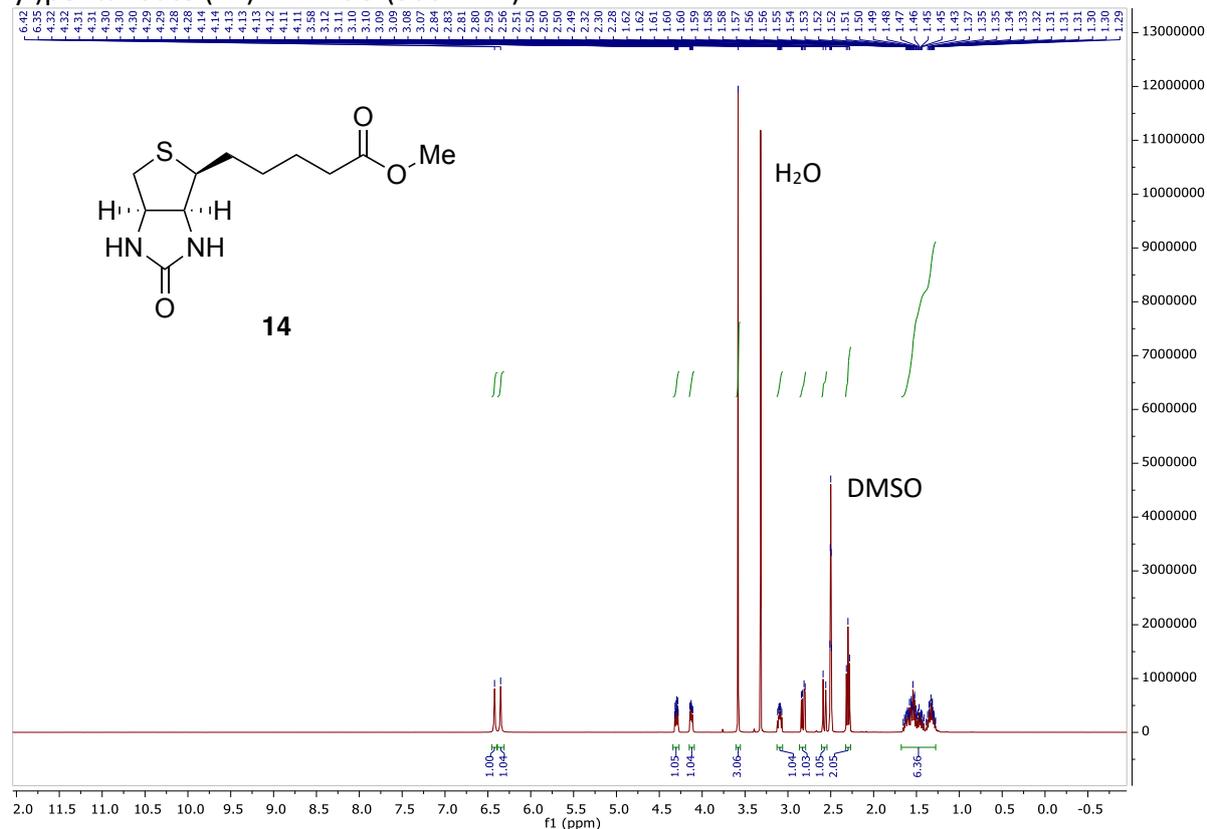
<sup>1</sup>H-NMR spectrum of *N*'3-ethyl biotin-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (**13**) in MeOD (500 MHz)



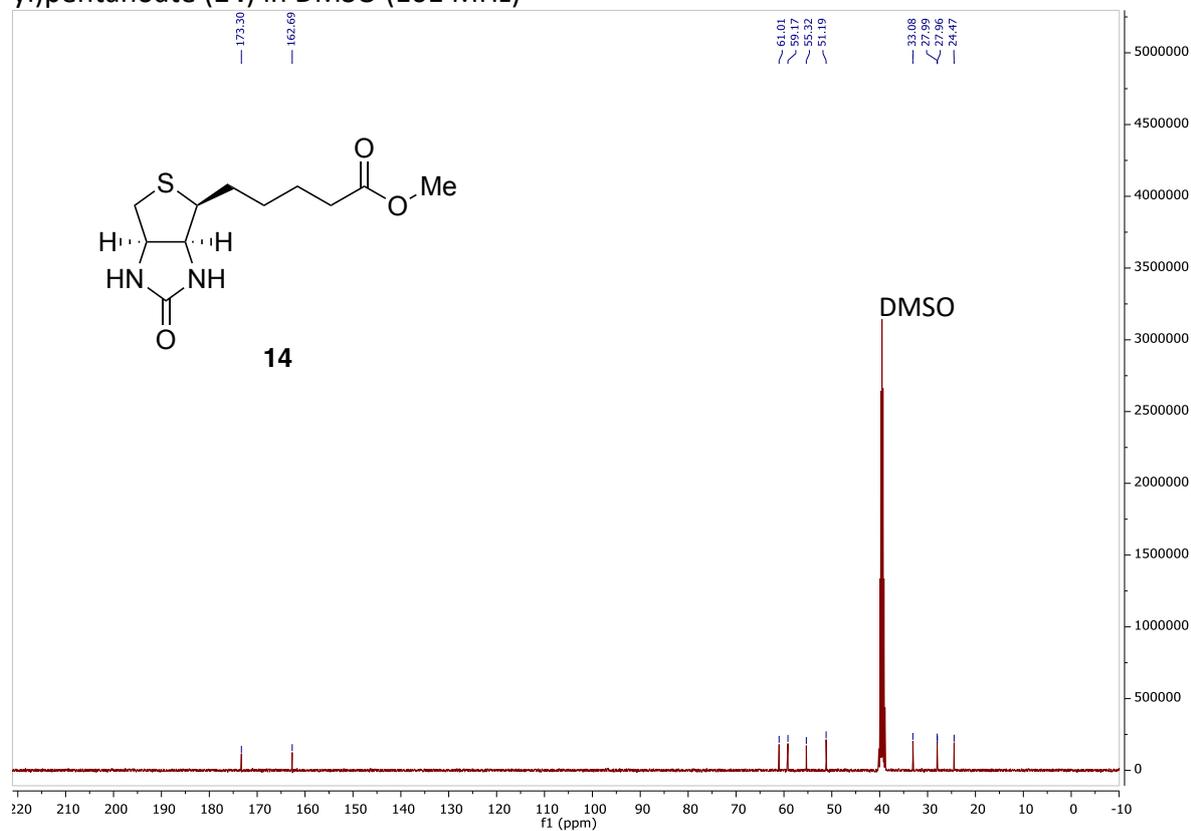
<sup>13</sup>C-NMR spectrum of *N*'3-ethyl biotin-6-Ahx-D-Tyr(OAll)-Tic-Ser(OPPG)-Asp(OAll)-OH (**13**) in MeOD (101 MHz)



<sup>1</sup>H-NMR spectrum of methyl 5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**14**) in DMSO (500 MHz)

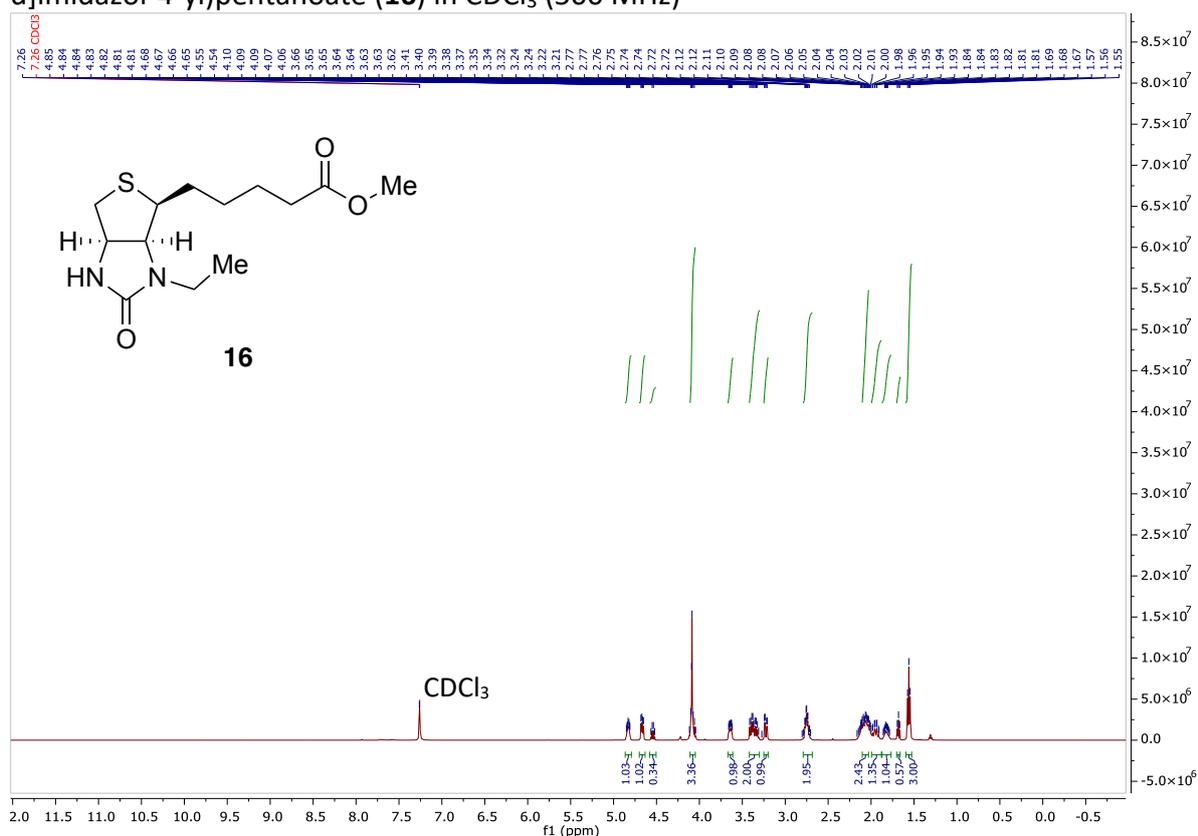


<sup>13</sup>C-NMR spectrum of methyl 5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**14**) in DMSO (101 MHz)

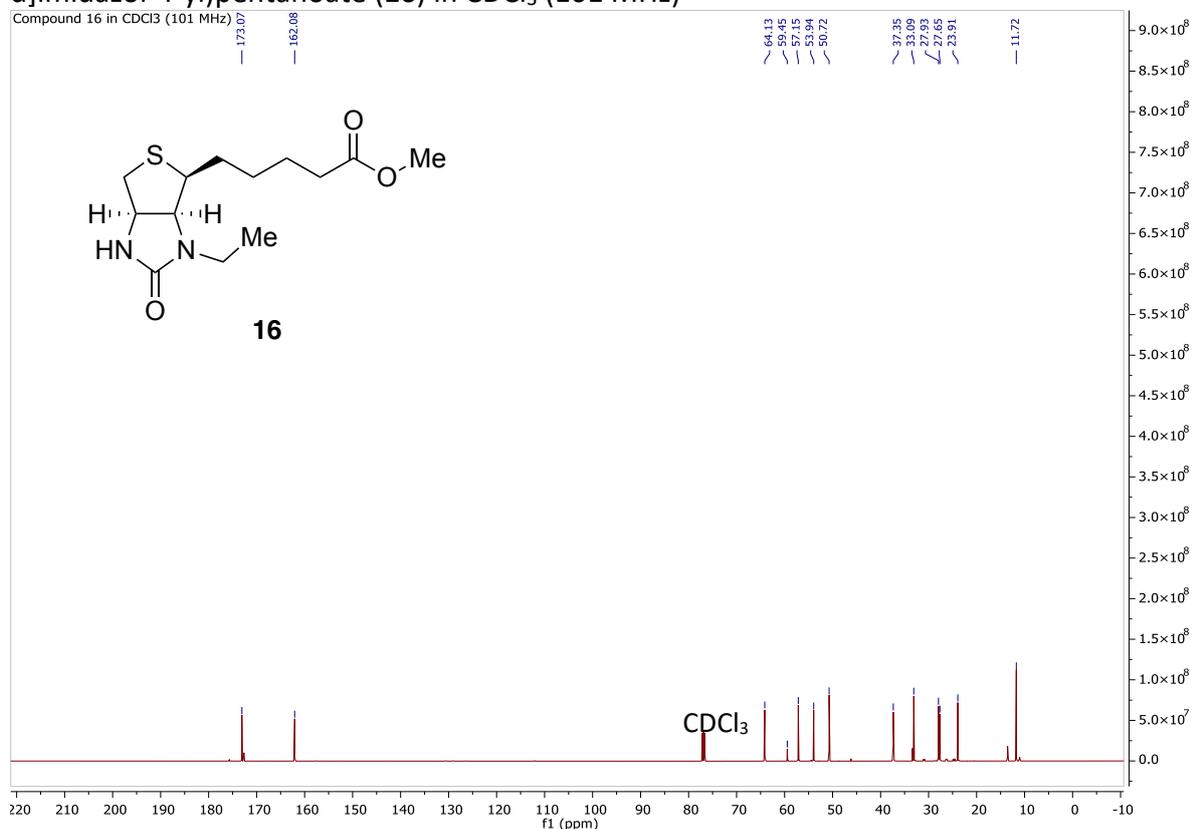




<sup>1</sup>H-NMR spectrum of methyl 5-((3*a*S,4*S*,6*a*R)-3-ethyl-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**16**) in CDCl<sub>3</sub> (500 MHz)



<sup>13</sup>C-NMR spectrum of methyl 5-((3*a*S,4*S*,6*a*R)-3-ethyl-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (**16**) in CDCl<sub>3</sub> (101 MHz)



### 3 References

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