Site-Selective Three-Component Dual-Functionalization of Peptides and Proteins

*Henrik K. Munch, Jakob E. Rasmussen, Gina Popa, Jørn B. Christensen, Knud J. Jensen**

Supporting information

General

All amino acids, HBTU, HOBt, DIPCDI and TFA were purchased from Iris Biotech (Germany). COMU[2] was supplied by Sigma-Aldrich (Denmark). TentaGel amide resins were purchased from Rapp Polymere (Germany). All other chemicals were purchased from Sigma-Aldrich (Denmark) and used as received without further purification. ^{1}H-, ^{13}C-NMR spectra were recorded on a Bruker Avance 300 spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz), equipped with a 5 mm BBO probe and Bruker Avance III 500 spectrometer (^{1}H at 500 MHz), equipped with a cryoprobe. The chemical shifts are referenced to the residual solvent signal. Microwave heating was performed in a Biotage Initiator microwave instrument. High-resolution mass spectrometry (HR-MS) was performed on a Micromass LCT time-of-flight instrument by direct injection with ionization in positive electrospray mode. LCMS of proteins was performed on a Bruker Daltonics microTOF-Q II/Dionex Ultimate 3000 HPLC. Analytical HPLC: Dionex Ultimate 3000 system with Jupiter C4 (5 μm, 150x4.6 mm) or Gemini-NX C18 (3 μm, 50x4.6 mm) column and a linear gradient flow of CH_3CN-H_2O (0.1% formic acid), thermostated to 42 °C with a column oven, connected to an ESI-MS (MSQ Plus Mass Spectrometer, Dionex). If not otherwise stated, the conversions were determined by integration of HPLC chromatograms at 215 nm. Purifications of peptides were performed on a preparative Dionex Ultimate 3000 HPLC with C4 and C18 columns from FEF chemicals (Denmark) or Phenomenex Gemini Axia. CH_3CN-H_2O (0.1% TFA) was used as eluent with a flow of 10 or 20 ml/min.

Peptides with *N*-terminal serine

The peptides were synthesized employing standard Fmoc solid-phase peptide synthesis using TentaGel S RAM resin (0.23 mmol/g), 4 equiv. of amino acid, 3.9 equiv. HBTU, 4 equiv. HOBt and 7.8 equiv. DIPEA in NMP. The deprotection was carried out with 20% (v/v) piperidine in NMP for 10-15 minutes. The *N*-terminal serine peptides were eventually released from the resin by treatment with TFA-H_2O (19:1) for 2-3 hours. The TFA solution was concentrated by nitrogen flow and precipitated with Et_2O giving a white powder. Purified by preparative RP-HPLC.

H-SQWF-NH_2 (1)
HR-MS (ES): calcd. for $C_{28}H_{35}N_7O_6$ $[M+H]^+$: 566.2722; found: 566.2722 (0 ppm)

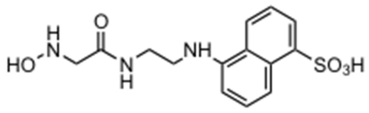
H-SGYGGFL-NH_2 (2):
HR-MS (ES): calcd. for $C_{33}H_{46}N_8O_9$ $[M+H]^+$: 699.3461; found: 699.3472 (1.5 ppm)

H-SEYGGFL-NH_2 (14):
HR-MS (ES): calcd. for $C_{36}H_{50}N_8O_{11}$ $[M+H]^+$: 771.3672; found: 771.3654 (2.3 ppm)

H-SKYGGFL-NH_2 (15):
HR-MS (ES): calcd. for $C_{37}H_{55}N_9O_9$ $[M+Na]^+$: 792.4015; found: 792.4003 (1.5 ppm)

H-SFYGGFL-NH_2 (16):
HR-MS (ES): calcd. for $C_{40}H_{52}N_8O_9$ $[M+H]^+$: 789.3930; found: 789.3910 (2.5 ppm)

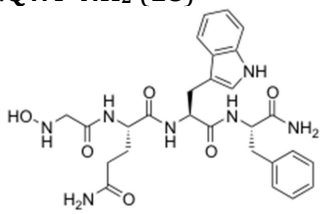
5-((2-(2-(hydroxyamino)acetamido)ethyl)amino)naphthalene-1-sulfonic acid (17)



N-(Iodoacetaminoethyl)-1-naphtylamine-5-sulfonic acid (141.1 mg; 325.1 µmol) was dissolved in 0.5 M $NaHCO_3$ (2 ml) providing a clear yellow solution. 50 wt% hydroxylamine (200 µL; 1612 µmol) was added and the solution was heated in a microwave instrument at 60 °C for 20 min.

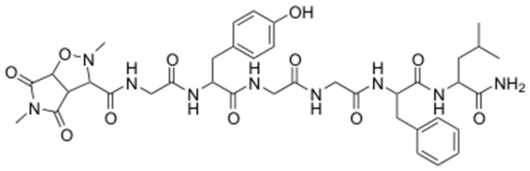
The solution was acidified to pH 2-3 with 37% HCl and purified by preperative RP-HPLC (C-18 Phenomenex Gemini Axia) providing an off-white solid. Yield: 70.5 mg (64%). ^{1}H-NMR (300 MHz; 10 mM Na_2CO_3 in D_2O): δ 8.20 (dt, *J* = 8.6, 1.0, 1H), 8.15 (dd, *J* = 7.3, 1.1, 1H), 8.07 (d, *J* = 8.7, 1H), 7.57 (q, *J* = 8.0, 2H), 6.94 (d, *J* = 7.7, 1H), 3.64 (t, *J* = 6.0, 2H), 3.52-3.48 (m, 4H). ^{13}C NMR (50 mM Na_2CO_3 in D_2O): δ 172.3, 161.1, 143.2, 137.5, 128.4, 127.7, 125.6, 124.4, 123.9, 122.9, 114.5, 106.1, 54.8, 42.4, 37.5.
HR-MS (ES): calcd. for $C_{14}H_{17}N_3O_5S$ $[M+H]^+$: 340.0962; found: 340.0953 (2.6 ppm)

Hydroxylamine peptide HO-GQWF-NH_2 (18)



A TentaGel S RAM resin (800 mg; 0.184 mmol; loading 0.23 mmol/g) was swelled in CH_2Cl_2. The peptide was synthesized using standard Fmoc solid phase peptide synthesis using 4 equiv. of amino acid, 3.9 equiv. HBTU, 4 equiv. HOBt and 7.8 equiv. DIPEA. The deprotection was carried out with 20% (v/v) piperidine in NMP for 15 min.
Fmoc was cleaved after the last coupling and the resin washed with NMP (3x). A solution of α-bromoacetic acid (128 mg; 0.93 mmol) and DIPCDI (145 µl; 0.94 mmol) in NMP was added and coupled for 90 minutes. The Kaiser test was negative indicating a complete reaction. The resin was washed with NMP (3x), CH_2Cl_2 (3x) and NMP (3x). A solution of 50 wt% NH_2OH in H_2O (58 µl; 0.94 mmol) in NMP was added and coupled for 2 hours. The peptide was released from the resin by treatment with TFA-H_2O (19:1) for 3 hours. The TFA solution was concentrated by nitrogen flow and precipitated with Et_2O giving a white powder, which was further purified by preparative RP-HPLC. HR-MS (ES): calcd. for $C_{27}H_{33}N_7O_6$ $[M+H]^+$: 552.2565; found: 552.2523 (7.6 ppm).

Conjugation product 5:

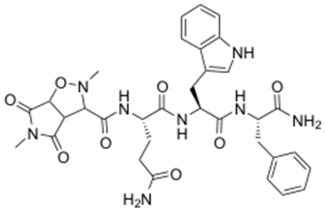


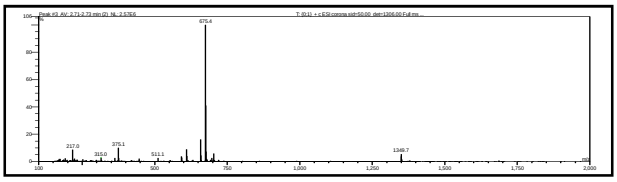
Peptide **3** (9 mg; 12.9 µmol) was dissolved in 200 mM NaOAc buffer pH 4.5/CH_3CN 1:1 (1.2 mL) and treated according to procedure A (see main text). The modified peptide was purified by preparative RP-HPLC and the corresponding fractions freeze dried. Yield: 6 mg (58%)

^{1}H-NMR (500 MHz; D_2O): δ 7.29 (t, *J* = 7.3, 2H, Phe), 7.23 (t, *J* = 7.3, 1H, Phe), 7.19 (d, *J* = 7.5, 2H, Phe), 7.06 (d, *J* = 7.1, 2H, Tyr), 6.76 (d, *J* = 8.1, 2H, Tyr), 4.93 (d, *J* = 7.6, 0.51H, Iso_H5), 4.88 (d, *J* = 7.5, 0.49H, Iso_H5), 4.52 (t, *J* = 7.4, 2H, Tyr+Phe), 4.17 (m, 1H, Ile), 4.12 (d, *J* = 8.1, 1H, Iso_H3), 3.99 (m, 1H, Iso_H4), 3.86 (m, 2H, Gly), 3.79 (m, 4H, Gly), 3.01 (m, 3H, Tyr+Phe), 2.93 (m, 4H, Tyr/Phe+CH_3N), 2.61 (d, *J* = 2.8, 3H, CH_3N), 1.46 (m, 3H, Ile), 0.81 (d, *J* = 5.5, 3H, Ile), 0.75 (d, *J* = 5.3, 3H, Ile). ^{13}C-NMR (126 MHz; D_2O): δ 177.2, 176.9, 173.87, 173.80, 173.0, 171.8, 171.04, 170.86, 154.5, 136.0, 130.56, 130.54, 129.2, 128.8, 127.9, 127.3, 115.4, 55.2, 52.1, 42.6, 42.32, 42.29, 39.7, 36.8, 36.0, 25.2, 24.1, 22.2, 20.47.

HR-MS (ES): calcd. for $C_{38}H_{49}N_9O_{11}$ $[M+H]^+$: 808.3624; found: 808.3607 (2.1 ppm)

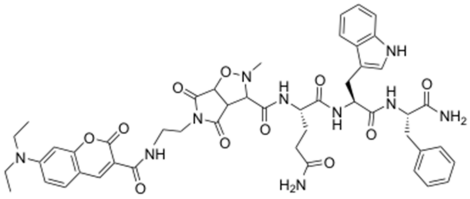
Conjugation product 7:

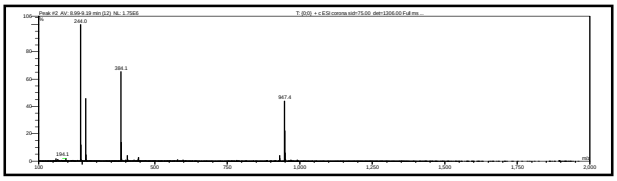




MS (ES): calcd. for $C_{33}H_{38}N_8O_8$ $[M+H]^+$: 675.29; found: 675.4

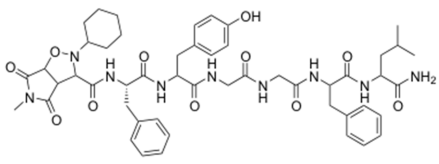
Conjugation product 8:

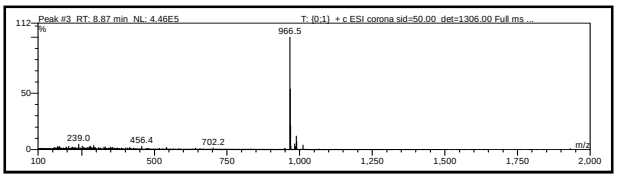




MS (ES): calcd. for $C_{48}H_{54}N_{10}O_{11}$ $[M+H]^+$: 947.40; found: 947.4

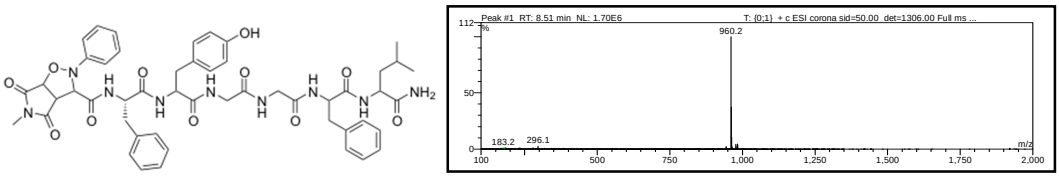
Conjugation product 9





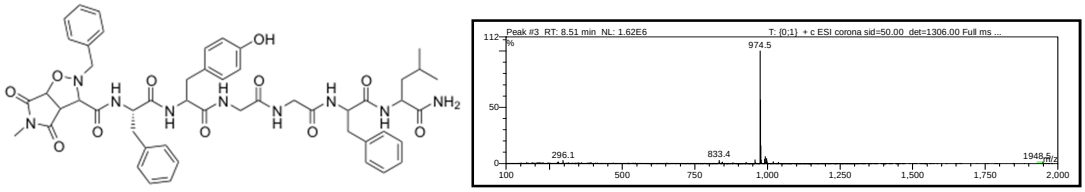
MS (ES): calcd. for $C_{50}H_{63}N_9O_{11}$ $[M+H]^+$: 966.47; found: 966.5

Conjugation product 10



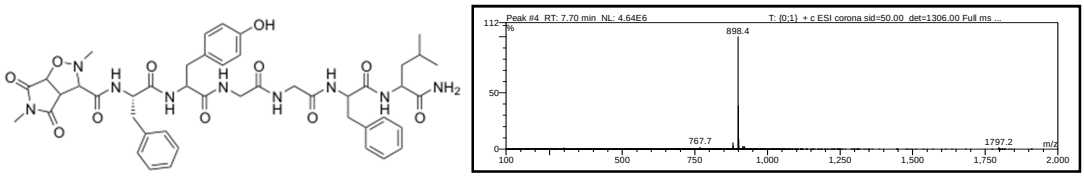
MS (ES): calcd. for $C_{50}H_{57}N_9O_{11}$ $[M+H]^+$: 960.43; found: 960.2.

Conjugation product 11



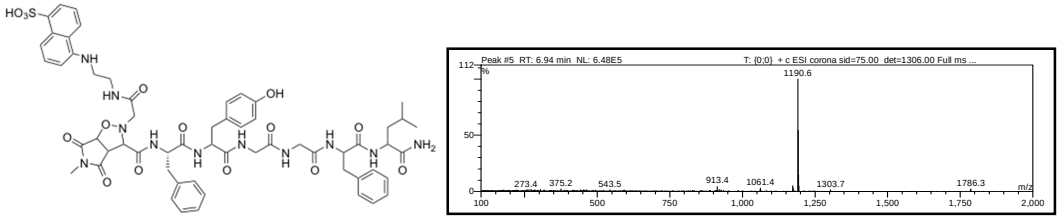
MS (ES): calcd. for $C_{51}H_{59}N_9O_{11}$ $[M+H]^+$: 974.44; found: 974.5.

Conjugation product 5



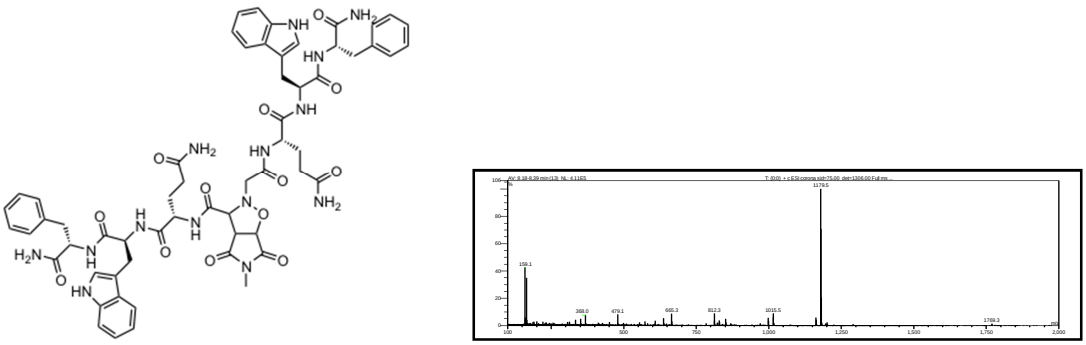
MS (ES): calcd. for $C_{45}H_{55}N_9O_{11}$ $[M+H]^+$: 898.41; found: 898.4.

Conjugation product 12



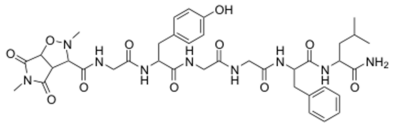
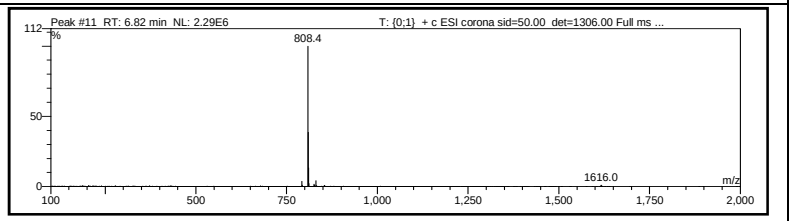
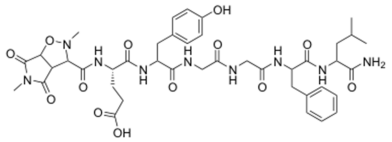
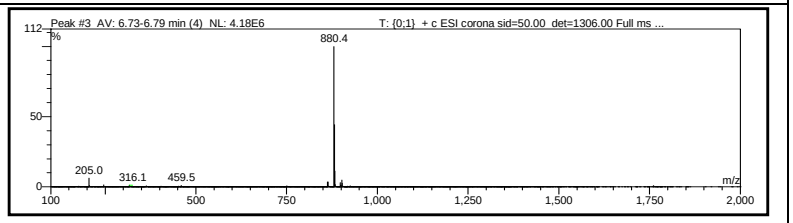
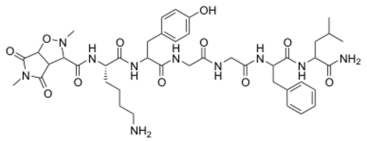
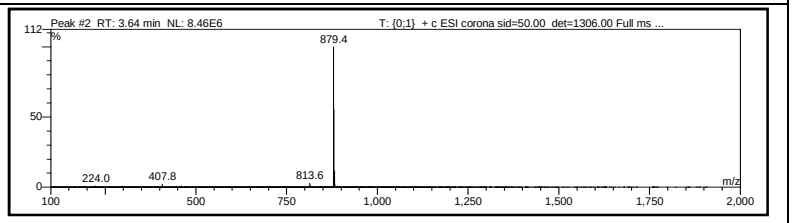
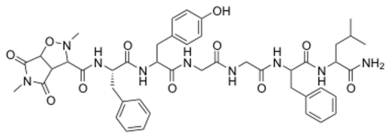
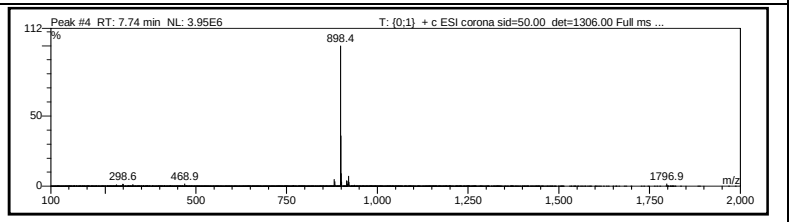
MS (ES): calcd. for $C_{58}H_{67}N_{11}O_{15}S$ $[M+H]^+$: 1190.46; found: 1190.6.

Conjugation product 13



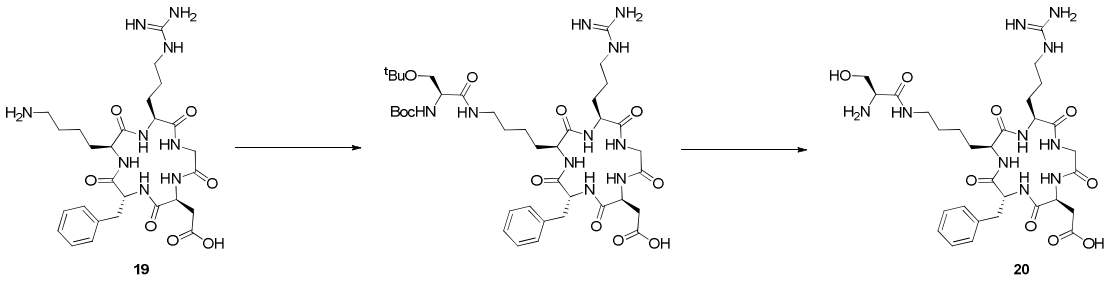
MS (ES): calcd. for $C_{61}H_{74}N_{14}O_{13}$ $[M+H]^+$: 1179.50; found: 1179.5

Table S1: One-pot modification of peptides with varying amino acids adjacent to the *N*-terminal serine

Peptide	Product	Mass calcd. $[M+H]^+$	Mass found $[M+H]^+$	Conv	
2		808.36	808.4	92[a]	
14		880.38	880.4	87[a]	
15		879.44	879.4	84	
16		898.41	898.4	84	

[a]The reaction mixture was analyzed on both C18 and C4 analytical LCMS as the Michael adduct between aniline and *N*-methylmaleimide (added in excess) has the same retention time as either the nitrone or the isoxazolidine product signal, respectively. Combining the data from both chromatograms enable the determination of the conversion.

Synthesis of cRGDfK(*S*) (20):



To 2-chlorotrityl chloride polystyrene resin (500 mg; 1.3 mmol/g) pre-swollen in CH_2Cl_2 was added a solution of Fmoc-Asp-OAll (771 mg; 1.95 mmol) in anhydrous CH_2Cl_2 (6 mL) and DIPEA (679 µL; 3.9 mmol). The suspension was gently agitated for 3 h. The Fmoc group was removed by treatment with 20% piperidine in NMP (5+20 min) and the peptide was elongated with Fmoc-Gly-OH (773 mg; 2.6 mmol), Fmoc-Arg(Pbf)-OH (1.67 g; 2.6 mmol), Fmoc-Lys(Boc)-OH (1.22 g; 2.6 mmol), and Fmoc-D-Phe-OH (1.01 g; 2.6 mmol) by standard Fmoc-based SPPS using COMU (1.03 g; 2.4 mmol) and DIPEA (906 µL; 5.2 mmol) in NMP (6 mL) for each coupling. The resin was treated with $(CH_3)_2NH{\cdot}BH_3$ (766 mg; 13 mmol) and $Pd(PPh_3)_4$ (75 mg; 65 µmol) dissolved in degassed anhydrous CH_2Cl_2 (6 ml) under argon for 30 min. Presumably due to poor swelling of the resin in NMP at this point, Fmoc-removal with 40% piperidine in NMP was sluggish. To ensure complete removal of the N-terminal Fmoc-protecting group it was necessary to treat the resin with 40% piperidine in CH_2Cl_2 for 3x20 min. The peptide was cyclized with PyBOP (1.01 g; 1.95 mmol) and DIPEA (680 µL; 3.9 mmol) in CH_2Cl_2 (10 mL) for 16 h. The peptide was cleaved from the support with 20% HFIP in CH_2Cl_2 (25 mL), and precipitated and washed three times with Et_2O. Purification by prep-HPLC (H_2O/CH_3CN/0.1% TFA, Phenomenex Gemini Axia C_{18} column) yielded 50 mg (12%) of the sidechain protected peptide. The sidechain protecting groups were removed by treatment with TFA/H_2O/TES 90:5:5 (10 mL) for 1h, followed by precipitation and washing with Et_2O, giving peptide **19**.

A solution of Boc-Ser(tBu)-OH (103 mg; 232 µmol) and COMU (74 mg; 174 µmol) dissolved in NMP (0.6 mL) and DIPEA (81 µl; 464 µmol) was added to the deprotected c(RGDfK) peptide **19** (35 mg; 58 µmol) dissolved in NMP (200 µl) and shaken at room temperature. LCMS of the crude mixture showed full conversion after 15 min. Only a small amount of doubly acylated peptide was detected in the TIC (but not by UV). The solution was diluted with water (ca. 6 ml) and CH_3CN (ca. 2 ml) and pH neutralized with AcOH. A subsequent prep-HPLC purification (C_{18} Axia column, H_2O/CH_3CN/TFA, 10-40% gradient) and lyophilization gave 32 mg (40%) of the desired peptide, the major peptide byproduct corresponding to a loss of water.

The Boc and tBu protecting groups were removed by treatment with 95% TFA/H_2O for 1 h, followed by evaporation of the solvent and washing with Et_2O, yielding 30 mg of peptide **20**.

HR-MS (ES): calcd. for $C_{30}H_{46}N_{10}O_9$ $[M+H]^+$: 691.3522; found: 691.3515 (1.0 ppm).

C(RGDfK(*S*)) Conjugation product (21)

Peptide **20** (3 mg; 4.3 µmol) was dissolved in 200 mM acetate buffer (pH 4.5; 300 µL). $NaIO_4$ (1.1 mg; 5 µmol) dissolved in 16 µl buffer was added. KI (5.2 mg; 31 µmol) and sodium ascorbate (4.3 mg; 19 µmol) dissolved in buffer (33 µL) was added and the solution shaken for 5 min. Aniline hydrochloride (1.1 mg; 8.7 µmol) dissolved in buffer

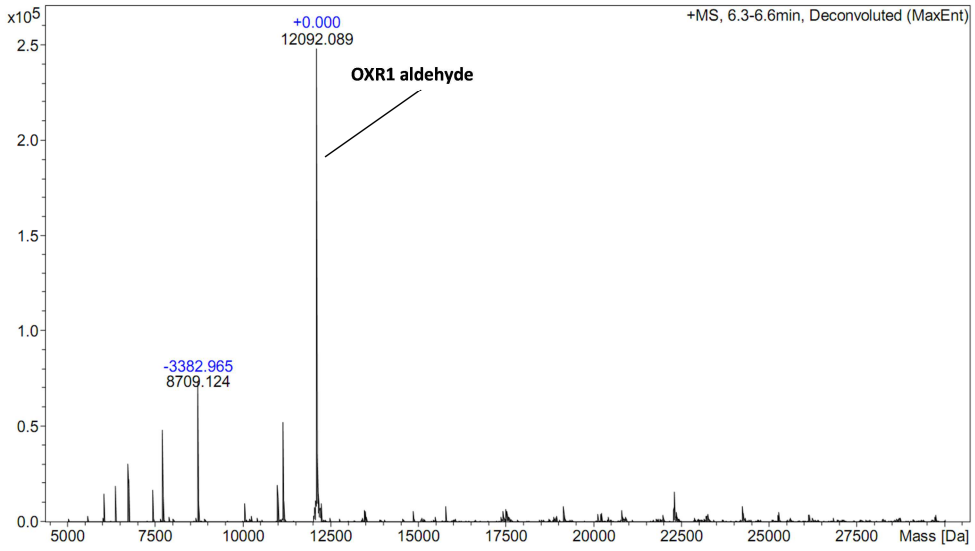
(10 μL) was added. N-hydroxylamine-AEDANS (5 mg; 13.0 μmol) was partially dissolved in 25 mM Na_2CO_3 (100 μL), and added to the peptide solution. After 2h, DOTA-maleimide dissolved in acetate buffer (20 μL) was added to the peptide solution which was gently shaken at 37 °C overnight. The reaction mixture was purified by preparative HPLC to afford the 2 diastereomers in a ratio of 58:42 (combined yield of 5.2 mg; 79%). HR-MS (ES): calcd. for $C_{65}H_{92}N_{18}O_{22}$ $[M+2H]^{2+}$: 754.3172; found: 754.3180 (1.0 ppm).

Expression and purification of OXR1

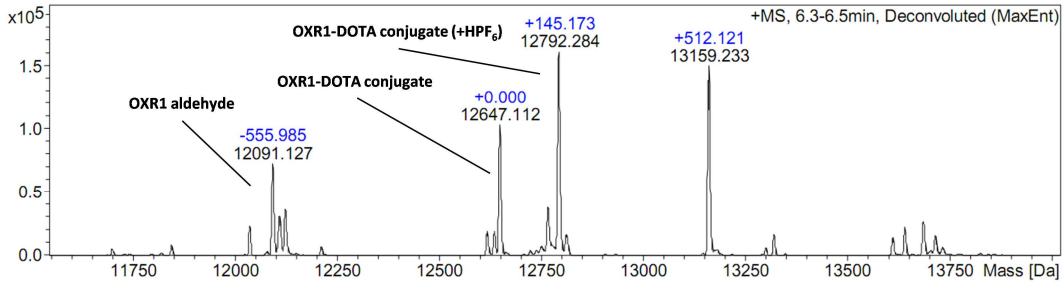
The recombinant variant of OXR1 displaying the amino acid sequence 8-118 (SSGTIEYTVESRDSLNSIALKFDTTPNELVQLNKLFSRAVVTGQVLYVPDPEYVSSVESSPSLSP VSPLSPTSSEAEFDKTTNPDVHPTEATPSSTFTGIRPARVVSSTSEEE) of human OXR1 was expressed using the pNIC28-SS vector in E. coli BL21(DE3) (Merck).[1] Expression was performed in TB supplemented with kanamycin (50 μg/ml) and inoculated with 1% of overnight growth culture, in shaking flasks. The cultures were grown at 37 °C and 200 rpm until the optical density reached 1.2, after which the flasks were moved to 18 °C and 180 rpm for 60 min, when protein expression was induced by adding 0.3 mM IPTG (Sigma-Aldrich). The bacteria were cultivated for another 18 h at 18 °C and then harvested by centrifugation at 4,000 g for 10 min. Cell pellets were resuspended in lysis buffer (50 mM phosphate, 300 mM NaCl, 10 mM imidazole, 10 % glycerol, 0.5 mM TCEP, pH 7.5) supplemented with 1x Complete EDTA-free protease inhibitor (Roche), and 50 units/ml Benzonase (Sigma-Aldrich) and passed three times through a homogenizer. The cell free extract was obtained after centrifugation at 11,000 g, 30 min, 4 °C and it was filtered through a 0.22 μm PES bottle top filter (Sartorius) and analyzed by SDS-PAGE. The over-expressed protein was purified on ÄKTA Xpress system at 4 °C via affinity chromatography followed by size-exclusion chromatography. In brief, the HisTrap FF crude column (GE Healthcare) was equilibrated with lysis buffer containing 10 mM imidazole before loading the cell free extract, and the bound protein was eluted in lysis buffer containing 300 mM imidazole. The buffer was exchanged to gel filtration buffer (50 mM phosphate, 150 mM NaCl, pH 7.50) by dialysis. His-tag removal was performed by adding 1 μl of Tobacco Etch Virus (TEV) protease (ProTEV, Promega) per 20 μg of purified protein, in presence of 1mM DTT and the reaction was incubated at 4 °C overnight. Hereafter, the released protein was purified using a Ni^{2+} HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with gel filtration buffer and the protein was eluted using a linear gradient of imidazole in gel filtration buffer. The buffer was changed again to gel filtration buffer by using a PD10 desalting column (GE Healthcare). After tag-removal, the purification was completed by size-exclusion chromatography, using a HiLoad 16/60 Superdex 75 preparative grade column. The purified protein was analyzed by SDS-PAGE and LC-MS.

DOTA-modification of OXR1 (22)

OXR1 (0.7 mM, 900 μL) in 50 mM phosphate, 150 mM NaCl, pH 7.5 was treated with $NaIO_4$ (10 equiv.) at 4 °C for 10 min. The buffer was exchanged into fresh 50 mM phosphate, 150 mM NaCl, pH 7.5 to remove excess periodate using a PD10 desalter (GE Healthcare). The protein solution was concentrated to 0.7 mM and *p*-anisidine (3 equiv.) dissolved in Milli-Q water (40 μL) was added. MeNHOH·HCl (5 equiv.) dissolved in Milli-Q water (10 μl) was added and the solution left at 4 °C for 2 h. DOTA-maleimide (25 equiv.) was added directly to the protein solution which was left at 4 °C for 4 d. The reaction was monitored by ESI-LCMS.



ESI-MS of OXR1 (MW 12,123.2) after oxidation with $NaIO_4$.



ESI-MS of OXR1 reaction mixture after DOTA conjugation.

List of abbreviation:

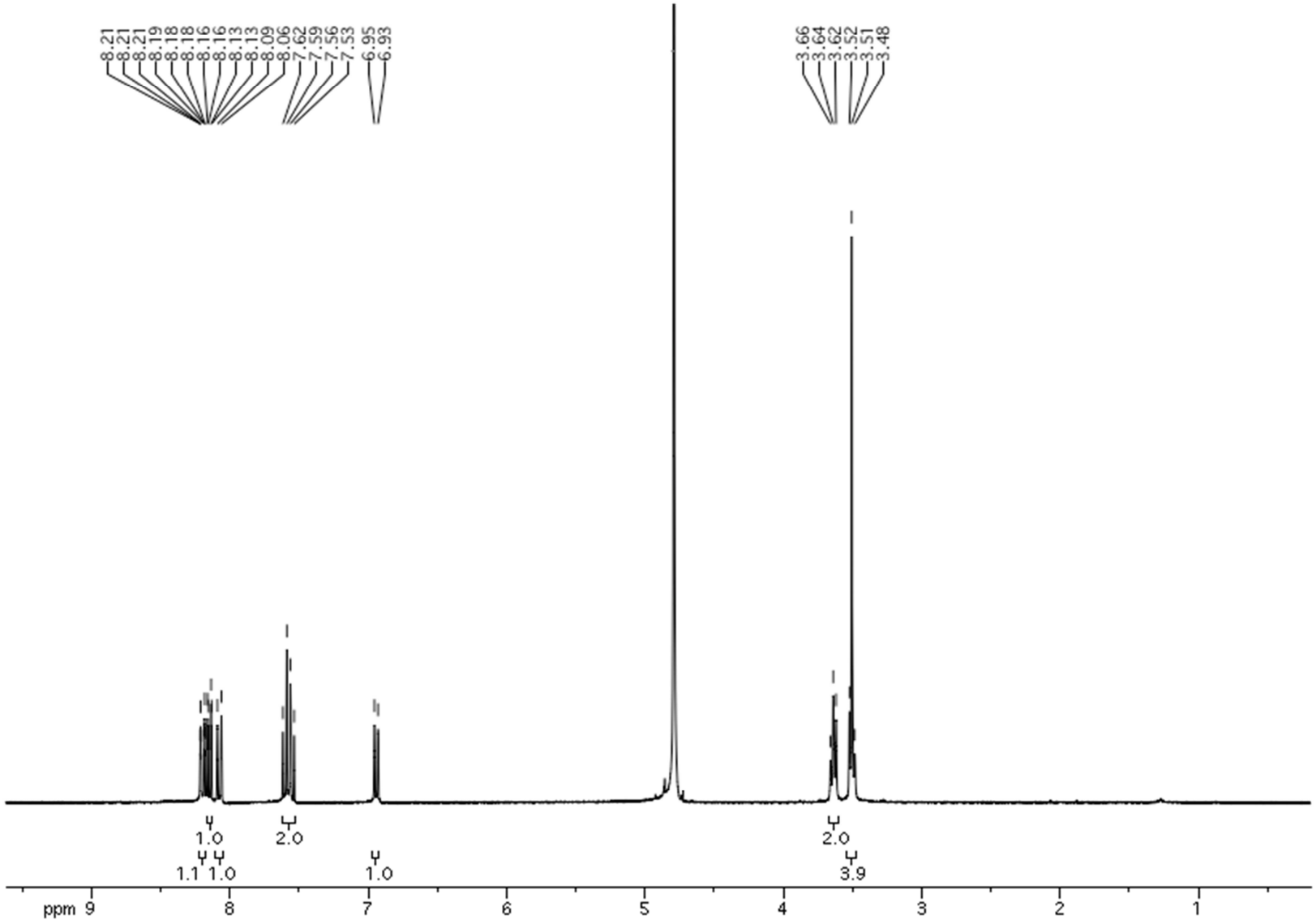
Ac:	acetyl
APT:	attached proton test
Boc:	*tert*-butoxycarbonyl
calcd:	calculated
COMU:	(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
DIPEA:	diisopropylethylamine
DIPCDI:	*N*,*N*'-diisopropylcarbodiimide
Equiv:	equivalents
HBTU:	*N*-[1H-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate
HFIP:	hexafluoro-2-propanol
HOBt:	1-hydroxybenzotriazol
NMP:	*N*-methylpyrrolidone
PyBOP:	benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate
TES:	triethylsilane
TFA:	trifluoroacetic acid

References

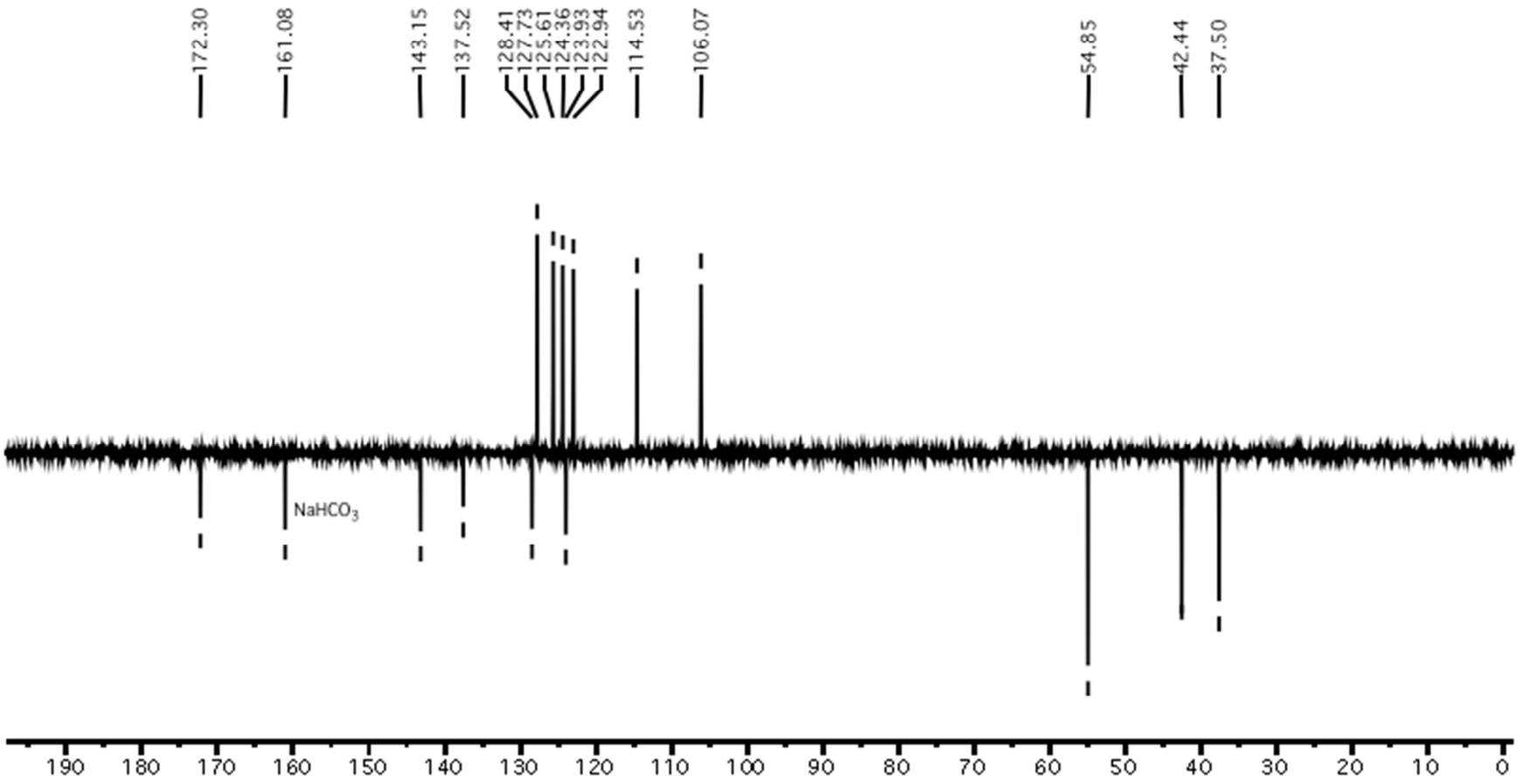
[1] E. Vernet, J. Sauer, A. Andersen, K. J. Jensen, B. Voldborg, *Anal. Biochem.* 2011, **414**, 312–314.

[2] A. El-Faham, R. S. Funosas, R. Prohens, F. Albericio, *Chem. Eur. J.* 2009, **15**, 9404-9416.

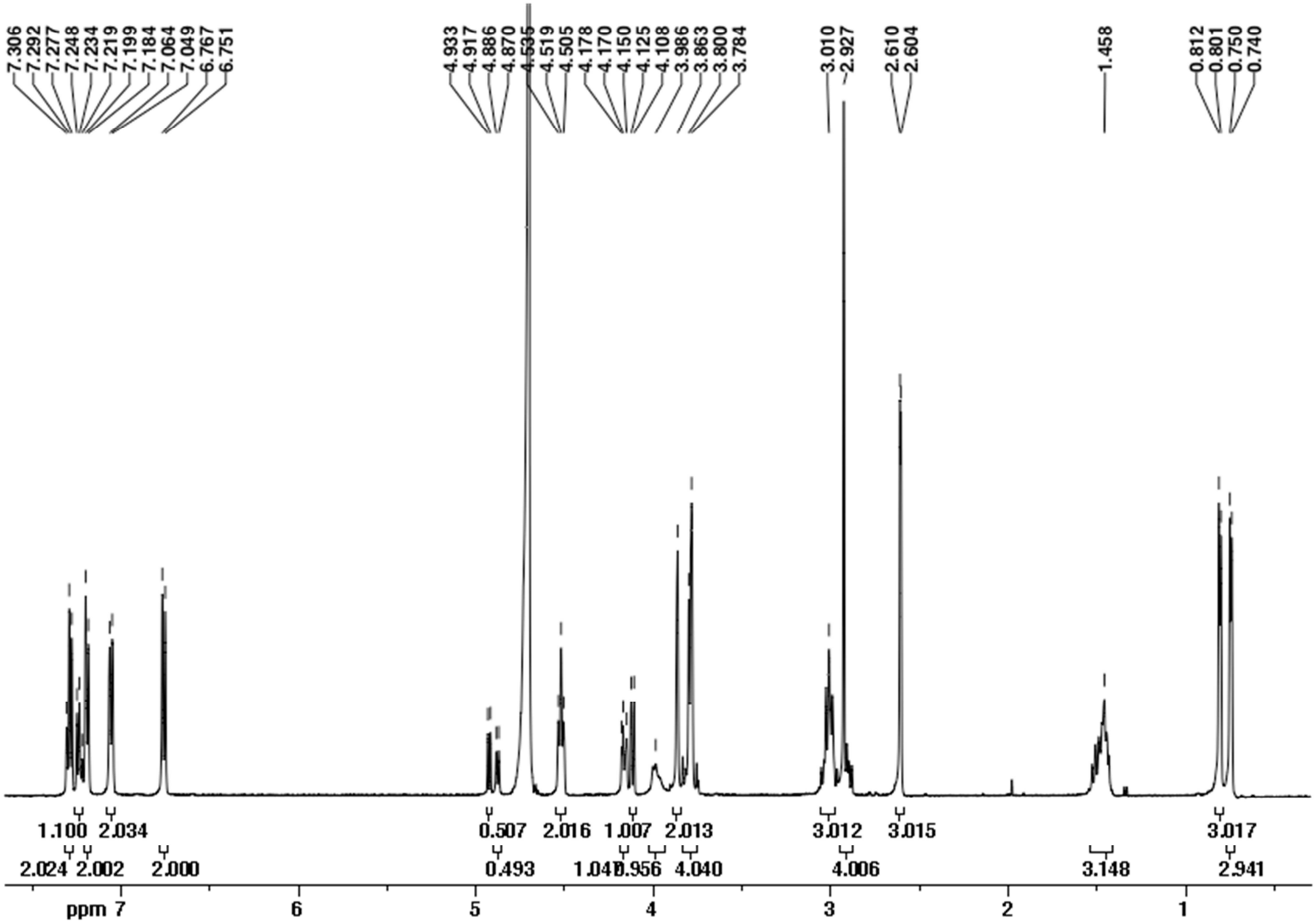
^{1}H-NMR of 1,5-hydroxylamino AEDANS 17 (10mM Na_2CO_3 in D_2O)



^{13}C-APT of 1,5-hydroxylamino AEDANS 17 (50mM Na_2CO_3 in D_2O)



^{1}H-NMR of conjugation product 5



^{13}C-NMR of conjugation product 5