A Peptide Hydroxamate Library for Enrichment of Metalloproteinases: Towards an Affinity-Based Metalloproteinase Profiling Protocol

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1. Synthetic part

General

Tetrahydrofuran was distilled over $LiAlH_4$ before use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), methanol (MeOH), piperidine, diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. Rink amide MBHA resin (0.64 mmol/g) was purchased at Novabiochem, as well as all appropriately protected amino acids (FmocAlaOH, FmocArg(Pmc)OH, FmocAsp(tBu)OH, FmocGln(Trt)OH, FmocGlu(tBu)OH, FmocHis(Trt)OH, FmocLeuOH, FmocLys(Boc)OH, FmocPheOH, FmocProOH, FmocSer(tBu)OH, FmocThr(tBu)OH, FmocTrp(Boc)OH, FmocTyr(tBu)OH and FmocValOH). *O*-(1H-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 4Å molecular sieves, except methanol and acetonitrile which were stored over 3Å molecular sieves. Molecular sieves were flame dried before use. Unless noted otherwise all reactions were performed under an argon or nitrogen atmosphere.

Column chromatography was performed on Silicycle Silia-P Flash Silica Gel, with a particle size of 40-63 μm. The eluents toluene, ethyl acetate and petroleum ether (40-60 ^oC boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 ml/L) in ethanol, where appropriate, followed by charring at ca. 150 °C.

^1H-, ^{13}C- and ^{19}F-NMR spectra were recorded on a Jeol JNM-FX-200 (200MHz), a Bruker DMX-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (^1H-NMR), $CDCl_3$ (^{13}C-NMR) or TFA (^{19}F NMR) as internal standard.

Mass spectra were recorded on a PE/Sciex API 165 instrument equipped with an Electrospray Interface (ESI) (Perkin-Elmer). High resolution MS (HRMS) spectra were recorded with a Finnigan LTQ-FT (Thermo Electron). IR spectra were recorded on a Shimadzu FTIR-8300 and absorptions are given in cm^{-1}. Optical rotations $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter at room temperature.

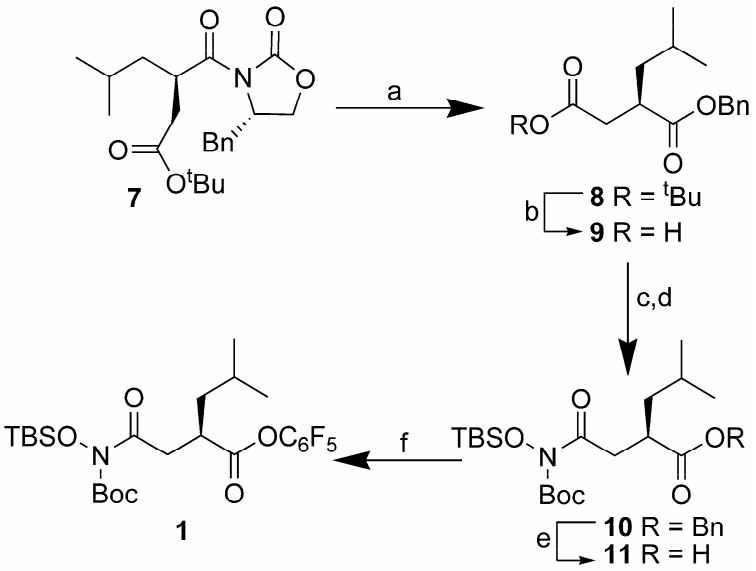
LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3μm C18 50 × 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI. HPLC gradients were 10-90%, 0-50% or 10-50% acetonitrile in 0.1% TFA/water. Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 × 4.6 mm).

The compounds were purified on a Gilson HPLC system coupled to a Phenomenex Gemini 5μm 250×10 mm column and a GX281 fraction collector. The used gradients were either 0-30% or 10-40% acetonitrile in 0.1% TFA/water, depending on the lipophilicity of the product. Appropriate fractions

were pooled, and concentrated in a Christ rotary vacuum concentrator overnight at room temperature at 0.1 mbar.

Compounds

The synthesis of compound **1** (scheme 1) has been reported before.[1]



Scheme 1. a) BnOH, ^{n}BuLi, THF, 0^{o}C, 92%; b) TFA, CH_2Cl_2, 94%; c) $(ClCO)_2$, cat. DMF, CH_2Cl_2; d) TBSONHBoc, DMAP, MeCN, 85% over 2 steps; e) H_2, Pd/C, MeOH; f) C_6F_5OH, EDC, CH_2Cl_2, 55% over 2 steps.

tert-*Butyl 3-(R)-benzyloxycarbonyl-5-methylhexanoate (**8**):* Benzyl alcohol (1.68 ml, 16.36 mmol) was dissolved in THF (40 ml) and cooled to 0 °C. *n*-BuLi (6.14 mL, 1.6M in THF, 9.8 mmol) was added dropwise and the reaction mixture was stirred for 20 minutes. A solution of compound **7**[2] (3.1 g, 8.18 mmol) in THF (5 mL) was added and the reaction was stirred at 0 °C until TLC analysis (10% ethyl acetate/petroleum ether) revealed a complete reaction (about 4 hours). The reaction mixture was quenched with sat. aqueous NH_4Cl and extracted 3 times with ethyl acetate. The combined organic layers were dried over $MgSO_4$ and concentrated. The crude material was purified by column chromatography (3% → 10% ethyl acetate/petroleum ether) giving the product as a colorless oil (yield: 2.4 g, 7.5 mmol, 92%). $[\alpha]_D^{23}$ +1.5 (*c* 1 in $CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$): δ (ppm) 7.40-7.25 (m, 5H), 5.13 (2×d, 2H), 2.98-2.83 (m, 1H), 2.62 (dd, 1H, J_1 = 16.4 Hz, J_2 = 9.1 Hz), 2.35 (dd, 1H, J_1 = 16.1 Hz, J_2 = 5.2 Hz), 1.63-1.15 (m, 3H), 1.40 (s, 9H), 0.92-0.84 (2×d, 6H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ (ppm) 174.92, 170.80, 135.92, 128.31, 127.95, 80.49, 66.05, 40.97, 39.61, 37.70, 27.84, 25.63, 22.51, 22.05. IR (thin film): 2962.5, 1728.1, 1365.5, 1249.8, 1141.8. HRMS: calculated for $C_{19}H_{28}O_4$+H: 321.2060; found: 321.2064.

*3-(R)-Benzyloxycarbonyl-5-methylhexanoic acid (**9**):* Synthesized from **8** as described for its racemic form,[3] however, no spectral data were given for the racemic compound. $[\alpha]_D^{23}$ +10.6 (*c* 1 in $CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$): δ (ppm) 7.36-7.29 (m, 5H), 5.14 (s, 2H), 3.00-2.85 (m, 1H), 2.76 (dd,

1H, J_1 = 16.8 Hz, J_2 = 9.12 Hz), 2.48 (dd, 1H, J_1 = 16.8 Hz, J_2 = 4.9 Hz), 1.65-1.29 (m, 3H), 0.93-0.86 (2×d, 6H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ (ppm) 178.04, 174.86, 135.71, 128.37, 128.01, 127.95, 66.36, 40.91, 39.12, 36.03, 25.60, 22.35, 22.05. IR (thin film): 2954.7, 1705.0, 1450.4, 1388.7, 1157.2. HRMS: calculated for $C_{15}H_{20}O_4$+H: 265.1434; found: 265.1437.

*Verification of the chiral purity of compound **9**:* As a reference compound, the racemic form of **9** was synthesized.[3] NMR data and purities were identical for **9** and its racemate. Chiral HPLC chromatograms are depicted in Figure 1. Based on the relative intensities of the peaks (depicted under the peaks) in chromatogram **B**, the enantiomeric excess of **9** is determined to be >99%.

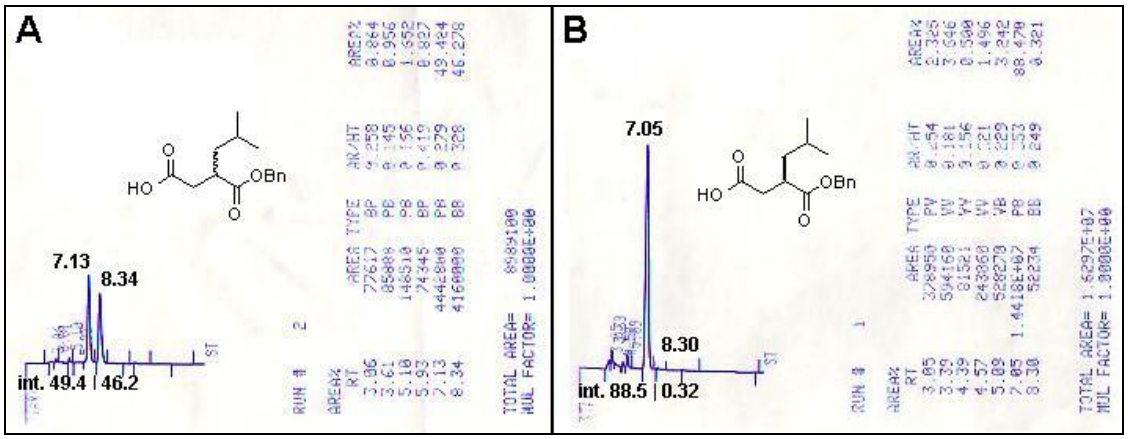


Figure 1. Results from the chiral HPLC analysis of (R)-2-isobutylmonobenzylsuccinate **9** and the racemate synthesized by the method of Chatterjee *et al*.[3] Measurements were done with a Chiral OD column; eluent: hexane:isopropanol = 95:5 + 0,25% AcOH; flow: 1 ml/min.; UV detection at 254 nm. Retention times and relative integrals are given. (**A**) racemate; (**B**) compound **9**.

*Benzyl 2-(R)-isobutyl-3-(N-tert-butoxycarbonyl-N-(tert-butyldimethylsilyloxy)carbamoyl)-propionate (**10**):* Acid **9** (2.11 g, 8.0 mmol) was dissolved in DCM (30 ml) and DMF (2 drops) was added. The solution was cooled to 0°C and oxalyl chloride (40 mmol, 3.4 mL) was added dropwise. The solution was warmed up to room temperature and stirred until gas evolution ceased (about 30 minutes). Toluene (30 ml) was added and the mixture was concentrated. Residual reagent was removed by repeated coevaporation with toluene. The intermediate acid choride was obtained as a yellowish oil and used without further purification. The crude acid chloride was redissolved in MeCN (15 ml) and cooled to 0°C. To this, a mixture of N-Boc-O-TBS-hydroxylamine[4] (8.8 mmol, 2.17 g) and DMAP (16 mmol, 1.95 g) in MeCN (15 ml) was added. The reaction mixture was slowly warmed to room temperature and stirred until TLC (10% ethyl acetate/petroleum ether) revealed a complete reaction (approx. 2 hours). The mixture was diluted with Et_2O (200 mL) and extracted subsequently with 1M HCl and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (5% ethyl acetate/petroleum ether), giving the product as a colourless oil (yield: 3.36 g, 6.8 mmol, 85%). $[\alpha]_D^{23}$ +16.5 (*c* 1 in $CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$): δ (ppm) 7.35-7.29 (m, 5H), 5.18-5.03 (2×d, 2H, J = 12.8 Hz), 3.24 (dd, 1H, J_1 = 17.1 Hz, J_2 = 9.7 Hz), 3.10-3.00 (m, 1H), 2.88 (dd, 1H, J_1 = 17.1 Hz, J_2 = 3.6 Hz), 1.60-1.10 (m, 3H), 1.53 (s,

9H), 0.98 (s, 9H), 0.92-0.83 (2×d, 6H), 0.12 (s, 3H), 0.10 (s, 3H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ (ppm) 175.28, 170.43, 151.93, 136.04, 128.22, 127.79, 84.13, 65.93, 41.06, 39.67, 39.15, 27.78, 25.60, 22.29, 17.96, -5.21. IR (thin film): 2954.7, 1728.1, 1465.8, 1303.8, 1141.8. HRMS: calculated for $C_{26}H_{43}NO_6Si$+H: 494.2932; found: 494.2933.

*2-(R)-Isobutyl-3-(N-tert-butoxycarbonyl-N-(tert-butyl-dimethylsilyloxy)carbamoyl)propionic acid (**11**):* To a solution of compound **10** (3.16 g, 6.4 mmol) in methanol (50 ml) under an argon atmosphere, Pd/C (10%, 150 mg) was added. Hydrogen gas was then bubbled through the mixture until TLC analysis (10% ethyl acetate/petroleum ether) revealed a complete reaction (approx. 1.5 hours). The reaction mixture was filtered over Hyflo and concentrated to obtain the product as a sticky syrup (2.6 g). The compound was used without further purification. $[\alpha]_D^{23}$ +12.4° (*c* 1 in $CHCl_3$). ^{1}H-NMR (200 MHz, $CDCl_3$): δ (ppm) 10.47 (bs, 1H), 3.12 (dd, 1H, J_1 = 16.8 Hz, J_2 = 8.7 Hz), 3.00-2.70 (m, 2H), 1.70-1.15 (m, 3H), 1.47 (s, 9H), 0.91 (s, 9H), 0.88-0.81 (2×d, 6H), 0.06 (s, 3H), 0.04 (s, 3H). ^{13}C-NMR (50 MHz, $CDCl_3$): δ (ppm) 181.62, 170.49, 151.96, 84.28, 40.85, 39.46, 39.06, 27.81, 25.57, 22.20, 17.96, -5.27. IR (thin film): 2954.7, 1705.0, 1465.8, 1249.8, 1145.5. ESI-MS (m/z): 404.2 ($M + H^+$), 426.1 ($M + Na^+$), 442.4 ($M + K^+$), 829.5 ($2M + Na^+$), 304.1 (M – Boc + H^+), 326.0 (M – Boc + Na^+).

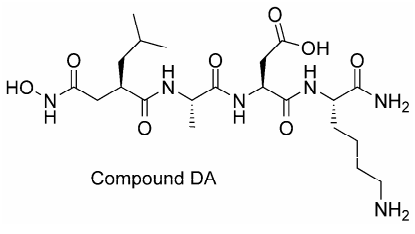
*Pentafluorophenyl 2-(R)-isobutyl-3-(N-tert-butoxycarbonyl-N-(tert-butyl-dimethylsilyloxy)-carbamoyl)propionate (**1**):* A mixture of crude compound **11** (1.15 g), pentafluorophenol (5.6 mmol, 1.03 g) and CH_2Cl_2 (15 ml) was treated with EDC (5.6 mmol, 1.07 g). After stirring the mixture at room temperature overnight, Et_2O (100 ml) was added and the mixture was extracted with 1M HCl and brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (2% ethyl acetate/petroleum ether) and the product was obtained as a colourless oil (Yield: 0.87 g, 1.53 mmol, 55% based on compound **10**). $[\alpha]_D^{23}$ +10.9° (*c* 1 in $CHCl_3$). ^{1}H-NMR (600 MHz, $CDCl_3$): δ (ppm) 3.33-3.28 (m, 2H), 3.09-3.04 (m, 1H), 1.80-1.71 (m, 2H), 1.56 (s, 9H), 1.52-1.47 (m, 1H), 1.00 (d, 3H, *J* = 6.6 Hz), 0.99 (s, 9H), 0.95 (d, 3H, *J* = 6.6 Hz), 0.13, (s, 3H), 0.12 (s, 3H). ^{13}C-NMR (150 MHz, $CDCl_3$): δ (ppm) 171.82, 169.85, 152.03, 141.16 (dd, J_1 = 250 Hz J_2 = 9 Hz), 139.31 (dt, J_1 = 264Hz, J_2 = 13.5 Hz), 137.71 (dt, J_1 = 249 Hz, J_2 = 13.5 Hz), 125.16 (t, J_2 = 12.8 Hz) 84.49, 41.02, 39.91, 39.01, 27.75, 25.67, 25.55, 22.24, 22.01, 18.03, -5.31, -5.39. ^{19}F NMR (376 MHz, $CDCl_3$): δ (ppm) -155.72 (d, 2F, *J* = 17.6 Hz), -162.46 (t, 1F, *J* = 21.6 Hz), -166.77 (dd, 2F, J_1 = 21.4 Hz, J_2 = 17.3 Hz). IR (thin film): 2962.5, 2360.7, 1735.8, 1519.8, 1311.5. HRMS: calculated for $C_{25}H_{36}F_5NO_6Si$+Na: 592.2124; found: 592.2140.

Synthesis of the library

Rink amide resin was rinsed with DCM, MeOH and DMF (2× each), then deprotected by shaking with 20% piperidine in DMF for 10 minutes (2×), and rinsed with DMF and DCM (2× each). Loading of the resin was effected by shaking with FmocLys(Boc)OH (5 equiv relative to the stated loading), HCTU (5 equiv) and 0.45 M DIPEA (10 equiv) in NMP for 2 h. The resin was filtered, then rinsed with DMF and DCM (2× each). Any non-reacted amines were capped by shaking the resin with Ac_2O (5 equiv) in 0.45 M DIPEA (10 equiv) in NMP for 5 minutes. After rinsing the resin with DMF, DCM and Et_2O (2× each) and drying *in vacuo*, Fmoc determination (UV measurement at 300 nm) gave a loading of 0.47 mmol/g.

Twelve portions of 80 μmol (170 mg resin) were rinsed with DCM and DMF (2× each), deprotected using 20% piperidine in DMF (10 min) and shaken with preactivated solutions of the appropriate Fmoc-amino acids Aa^1 (400 μmol), HCTU (400 μmol, 165 mg) and DIPEA (800 μmol, 1.8 mL 0.45 M in NMP) for 1 h. After rinsing with DMF, DCM and Et_2O (2× each), and drying *in vacuo*, each portion was in turn divided into eight equal portions. Every portion (~ 10 μmol) was respectively reacted with preactivated solutions of the eight amino acids Aa^2 (50 μmol each), HCTU (50 μmol) and DIPEA (100 μmol as a 0.45 M solution in NMP) and shaken for 1 h. After rinsing the resins with DMF and DCM (2× each), all portions were deprotected using 20% piperidine in DMF for 10 min, then filtered and rinsed with DMF and DCM (2× each). Finally, to all 96 resins was added compound **1** (250 μL 0.2 M in NMP) and DIPEA (44 μL 0.45 M in NMP). After shaking for 2 h, the resins were filtered, rinsed with DMF, MeOH and DCM (2× each) and treated with 95% TFA/water (0.5 mL) for 1 h (with the exception of resins containing Arg(Pmc), these were reacted 2.5 h). The filtrates, as well as small portions of 95% TFA/water used for rinsing the resins, were collected in tubes containing chilled Et_2O/petroleum ether (1/1, ~ 5 mL) and left at -20 °C overnight. The tubes were then centrifuged, and the filtrates were decanted. The crude products were analysed by LC-MS, then purified by semi-preparative HPLC.

The table below shows the yields of each of the 96 compounds after HPLC purification relative to the initial capacity of the Fmoc-Lys-Rink resin. The first letter corresponds to Aa^1, the second to Aa^2. For example, compound DA refers to the compound at which first Asp and then Ala was coupled:



Compound DA

Compound code	mol. wt.	mg	µmol	Yield %
DA	503	1.18	2.35	23.5
DD	547	0.33	0.6	6
DF	579	0.59	1.0	10
DH	569	0.92	1.6	16
DL	545	0.68	1.2	12
DV	531	0.54	1.0	10
DW	618	0.97	1.6	16
DY	595	0.88	1.5	15
EA	517	1	1.93	19.3
ED	561	0.44	0.78	7.8
EF	593	0.93	1.6	16
EH	583	0.94	1.6	16
EL	559	1.05	1.88	18.8
EV	545	1.32	2.42	24.2
EW	632	0.92	1.5	15
EY	609	1.12	1.84	18.4
FA	535	1.07	2.0	20
FD	579	0.35	0.6	6
FF	611	0.49	0.8	8
FH	601	1.1	1.83	18.3
FL	577	0.41	0.71	7.1
FV	563	0.88	1.6	16
FW	650	0.47	0.72	7.2
FY	627	0.79	1.3	13
HA	525	0.72	1.4	14
HD	569	0.24	0.42	4.2
HF	601	0.48	0.8	8
HH	591	0.97	1.6	16
HL	567	0.69	1.2	12
HV	553	0.46	0.83	8.3
HW	640	0.33	0.52	5.2
HY	617	0.7	1.1	11
LA	501	0.89	1.8	18
LD	545	0.17	0.31	3.1
LF	577	0.38	0.66	6.6
LH	567	1.23	2.17	21.7
LL	543	0.61	1.1	11
LV	529	0.97	1.8	18
LW	616	0.2	0.32	3.2
LY	593	0.53	0.89	8.9
PA	485	0.87	1.8	18
PD	529	0.59	1.1	11
PF	561	0.8	1.4	14
PH	551	1.04	1.89	18.9
PL	527	0.56	1.1	11
PV	513	0.88	1.7	17
PW	600	0.59	0.98	9.8
PY	577	1.27	2.2	22

Compound code	mol. wt.	mg	µmol	Yield %
QA	516	0.44	0.85	8.5
QD	560	0.18	0.32	3.2
QF	592	0.45	0.76	7.6
QH	582	1.56	2.68	26.8
QL	558	0.9	1.6	16
QV	544	0.56	1.0	10
QW	631	0.52	0.82	8.2
QY	608	0.17	0.28	2.8
RA	544	0.44	0.81	8.1
RD	588	0.81	1.38	13.8
RF	620	0.3	0.48	4.8
RH	610	1.21	1.98	19.8
RL	586	1.03	1.76	17.6
RV	572	0.68	1.2	12
RW	659	0.38	0.58	5.8
RY	636	0.42	0.66	6.6
SA	475	0.73	1.5	15
SD	519	0.21	0.4	4
SF	551	0.55	1.0	10
SH	541	1.3	2.4	24
SL	517	1.08	2.09	20.9
SV	503	0.72	1.4	14
SW	590	0.61	1.0	10
SY	567	0.55	0.97	9.7
TA	489	2.05	4.19	41.9
TD	532	0.36	0.68	6.8
TF	565	0.78	1.4	14
TH	555	1.09	1.96	19.6
TL	531	0.32	0.6	6
TV	517	1.3	2.51	25.1
TW	604	0.44	0.73	7.3
TY	581	1.67	2.87	28.7
WA	574	0.62	1.1	11
WD	618	0.15	0.24	2.4
WF	650	0.2	0.31	3.1
WH	640	0.65	1.0	10
WL	616	0.35	0.57	5.7
WV	602	0.36	0.6	6
WW	689	0.32	0.46	4.6
WY	666	0.22	0.33	3.3
YA	551	1.29	2.34	23.4
YD	595	0.31	0.52	5.2
YF	627	0.43	0.69	6.9
YH	617	1.7	2.76	27.6
YL	593	0.91	1.5	15
YV	579	0.64	1.1	11
YW	666	0.45	0.68	6.8
YY	627	0.42	0.67	6.7

2. Biochemical part

Materials

Recombinant human MMP-12 catalytic domain and MMP-9 catalytic domain (without fibronectin type II inserts) were a gift from AstraZeneca R&D (Lund & Moelndal, Sweden) and were produced in *E. coli* (Parkar 2000, Shipley 1996). Recombinant human ADAM-17 ectodomain was from R&D systems.

The fluorogenic MMP substrate Mca-PLGL-Dpa-AR-NH_2 (where Mca = (7-methoxycoumarin-4-yl)acetyl and Dpa = dinitrophenyl)-L-2,3-diaminopropionyl) was from Bachem, the ADAM substrate Mca-PLAQAV-Dpa-RSSSR-NH_2 was from R&D systems.

N-hydroxysuccinimide (NHS)-Sepharose was from Amersham Bioscience.

Acetonitrile (gradient grade) was from Biosolve, ultra-pure water was produced in-house by an Elga water purifying system and used for all mobile phase and buffer preparations.

Other chemicals were purchased from Sigma.

Determination of inhibitor efficacy

The efficacy of the novel inhibitors was tested by evaluating their ability to inhibit proteolytic conversion of a fluorogenic substrate by recombinant metalloproteases. A fixed concentration of each inhibitor (final concentration 100 nM) was incubated with 5 ng of either MMP-9 catalytic domain, MMP-12 catalytic domain or ADAM-17 ectodomain in assay buffer (for MMP-9 and -12: 50 mM Tris pH 7.4, 0,1 M NaCl, 10 mM $CaCl_2$, 0.05 % w/v Brij-35; for ADAM-17: 25 mM Tris pH 9.0, 2.5 µM $ZnCl_2$, 0.005% w/v Brij-35) in 96-well plates (Costar White). The appropriate fluorogenic substrate was added to a final concentration of 2 µM and proteolysis rates were determined by measuring fluorescence ($\lambda_{ex,em}$ = 320, 440 nm) increase using a Fluostar Optima plate reader (BMG Labtech) at 28°C. The remaining catalytic activity was calculated by comparing with proteolysis rates of 5 ng uninhibited enzyme (see table below) and plotted in heat maps generated in Matlab (thanks to Peter Horvatovich, University of Groningen).

Inhibitor	MMP9	MMP12	ADAM17	Inhibitor	MMP9	MMP12	ADAM17	Inhibitor	MMP9	MMP12	ADAM17
DA	97,3	25,3	90,0	**LA**	98,7	36,2	73,5	**SA**	69,2	29,1	42,2
DD	102,3	51,4	105,7	**LD**	98,2	55,2	92,1	**SD**	66,2	42,5	96,2
DF	55,8	4,3	92,5	**LF**	57,0	2,1	25,4	**SF**	8,6	7,2	11,2
DH	90,8	11,6	97,6	**LH**	92,5	13,7	69,0	**SH**	27,9	14,7	33,6
DL	95,2	7,4	98,7	**LL**	98,3	4,3	41,6	**SL**	28,4	9,0	11,8
DV	106,0	11,0	100,6	**LV**	85,6	8,6	30,0	**SV**	30,1	9,6	8,4
DW	70,4	8,5	108,0	**LW**	42,6	2,3	36,5	**SW**	3,4	3,1	14,1
DY	65,3	9,0	101,8	**LY**	53,7	3,6	32,8	**SY**	3,7	5,0	8,9
EA	101,2	45,0	92,3	**PA**	101,8	83,9	64,8	**TA**	99,3	74,2	86,0
ED	103,2	85,5	91,4	**PD**	97,8	101,2	94,0	**TD**	95,4	62,1	94,8
EF	77,8	7,6	86,8	**PF**	72,9	63,2	49,7	**TF**	50,8	15,9	41,2
EH	98,2	11,4	99,5	**PH**	84,9	68,2	50,3	**TH**	73,2	22,9	66,0
EL	106,2	18,1	92,2	**PL**	100,2	77,5	43,0	**TL**	52,7	8,8	26,7
EV	106,8	23,4	96,7	**PV**	97,4	50,3	48,5	**TV**	70,0	19,4	26,0
EW	103,3	33,0	103,3	**PW**	73,1	36,9	63,7	**TW**	21,2	7,1	35,8
EY	86,8	10,2	99,7	**PY**	72,5	45,3	63,3	**TY**	32,7	13,5	33,7
FA	96,7	24,4	47,6	**QA**	100,0	36,0	68,0	**WA**	84,6	16,3	56,3
FD	91,8	43,3	92,8	**QD**	96,6	59,0	90,7	**WD**	85,0	45,6	96,7
FF	24,6	0,6	16,4	**QF**	73,3	8,7	57,8	**WF**	21,6	4,3	23,0
FH	81,4	63,1	67,9	**QH**	90,8	61,4	87,0	**WH**	48,5	7,2	58,6
FL	79,7	3,7	33,3	**QL**	90,0	7,7	53,9	**WL**	33,6	2,9	21,2
FV	82,6	3,4	19,4	**QV**	97,1	9,5	53,9	**WV**	64,6	6,3	20,4
FW	10,4	0,4	40,2	**QW**	42,7	2,8	58,8	**WW**	18,5	4,6	48,3
FY	49,8	9,0	55,6	**QY**	12,8	1,9	16,1	**WY**	8,1	4,2	13,3
HA	95,1	44,6	78,8	**RA**	97,6	25,8	23,2	**YA**	88,4	17,9	60,8
HD	93,8	82,7	89,8	**RD**	96,2	81,5	87,6	**YD**	70,5	32,6	81,3
HF	31,3	4,1	35,4	**RF**	66,5	4,6	6,5	**YF**	13,2	1,8	16,3
HH	72,2	17,4	67,0	**RH**	88,1	14,0	14,7	**YH**	52,7	12,2	64,0
HL	72,1	7,4	43,7	**RL**	93,8	12,8	8,8	**YL**	52,1	5,2	25,0
HV	86,4	15,0	49,6	**RV**	99,5	9,5	5,0	**YV**	54,8	4,3	17,4
HW	21,0	3,2	53,4	**RW**	54,1	4,4	9,5	**YW**	4,9	3,2	28,1
HY	29,2	7,6	56,1	**RY**	42,8	4,0	7,2	**YY**	8,1	1,1	18,3

Remaining enzyme activity (%) after inhibition.

Determination of IC_{50} values

The IC_{50} values of eight selected novel inhibitors were determined in a competitive enzyme activity assay monitoring conversion of the same fluorogenic substrates by recombinant metalloproteinases in presence of increasing concentrations inhibitor. Measurements were performed in 96-well plates (Costar white), where each well contained 5 ng of either MMP-9 catalytic domain, MMP-12 catalytic domain or ADAM-17 and a final concentration of 2 μM of the appropriate substrate in a final volume of 100 μl assay buffer. Proteolysis rates were determined by measuring fluorescence increase like above. Seven-point inhibition curves (0-10 μM) were plotted in Origin 7.0 (Micronal) and IC_{50} values were determined by sigmoidal fitting.

Immobilization of inhibitors on Sepharose beads

The eight selected inhibitors were linked to Sepharose like described earlier.[5] Briefly NHS-activated Sepharose beads were washed at 4ºC with several volumes of 1 mM HCl and several volumes of coupling buffer (0.1 M K_2HPO_4, pH 7.5). The washed beads were then incubated with an equal volume of a 5 mM solution of inhibitor in coupling buffer for 2h in a shaking incubator (Eppendorf thermomixer, 1200 rpm, 25°C). After removal of the supernatant the unreacted NHS-groups were quenched by incubation with 10 bead volumes of blocking buffer (0.5 M ethanolamine in coupling buffer).

The immobilization was monitored by HPLC analysis of each inhibitor-containing coupling buffer before and after the procedure. Analysis was performed on a Merck-Hitachi system fitted with a UV detector at 214 nm. Samples were separated on a Zorbax Eclipse XDB-C8 column (4.6 x 150 mm, 5 μm particles, Agilent technologies). Mobile phase A was 0.1 % v/v TFA in water, mobile phase B was 0.1 % TFA in acetonitrile. Samples were diluted ten times in mobile phase A and 10 μL was injected. A linear gradient separation was performed by increasing the percentage B from 0 to 60 % in 30 minutes. The theoretical ligand density for each immobilized inhibitor was calculated by evaluation of the peak-area for the free inhibitor before and after the immobilization (see table below).

	[immobilized inhibitor] (mM)
DV	5.00[a]
FF	4.58
FW	4.98
PD	4.88
PL	3.23
QY	4.48
SF	3.73
YW	4.75

[a] No detectable inhibitor remained after immobilization.

Determination of extraction yield with the immobilized inhibitors

The efficacy of the immobilized inhibitors for use as activity-based affinity ligands in solid phase extraction (SPE) of MMPs and ADAMs was determined by performing column-based extractions on each material. Each batch of inhibitor-beads was slurry packed into Prospekt SPE cartridges fitted on one side with a 0.2 µm stainless steel frit (Spark Holland, 2 mm ID x 10 mm) in MMP assay buffer. The packed cartridges were placed in a clamp and attached to a syringe pump (KD scientific). Extractions were performed in the assay buffers described above. Before extraction the cartridges were conditioned by flushing two times 250 µL of the appropriate assay buffer. All steps were performed at 50 µL/min.

Samples containing recombinant enzyme (200 µL of 0.5 or 5 nM solution) were applied to the cartridges and the cartridges were washed with four times 200 µL assay buffer to determine breakthrough. All eluting fractions were collected separately and immediately placed on ice. Between extractions the cartridges were regenerated by eluting bound enzyme with 10 mM EDTA in assay buffer and reconditioning with two times 200 µL assay buffer.

Enzyme activity in the collected fractions was determined by the activity assay described above and related to the activity in the original sample.

Extraction of active ADAM-17 from lung carcinoma

Human alveolar carcinoma cell line A549 (ATCC nr. CCL-185) was grown to 90% confluency in RPMI-1640 with l-glutamine (Cambrex) supplemented with 10% (v/v) fetal bovine serum (Cambrex) and 20 µg/mL gentamycin (Centafarm) in 25 cm^2 culture plates at 37°C, 5% CO_2. The cells were serum starved for 24 h and stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for different time periods.

After stimulation, the cells were harvested in 1 mL ice-cold lysis buffer (25 mM Tris pH 9.0, 2.5 µM $ZnCl_2$, 1 % v/v Triton X-100). After cell lysis (1h on ice) the lysates were centrifuged in a cooled (4°C) Eppendorf centrifuge at 20.000 x *g* for 15 minutes to remove cell debris. The supernatant was kept on ice until the extraction.

Spark cartridges (2 mm ID x 10 mm) packed with either Sepharose immobilized inhibitor FF or control ethanolamine Sepharose were equilibrated with lysis buffer (2 x 250 µL pumped at 50 µL/min). The lysates were applied to the cartridge (250 µL, 25 µL/min) and the flowthrough was collected. The cartridges were washed once with lysis buffer (250 µL, 50 µL/min) and three times with wash buffer (25 mM Tris pH 9.0, 2.5 µM $ZnCl_2$, 0.005 % v/v Brij-35). Elution was carried out by competitive elution with a competitive inhibitor in wash buffer (100 µM inhibitor SF, 3 x 60 µL, 10 µL/min) and a non-specific elution step with 1% SDS in wash buffer (100 µL, 20 µL/min).

60 µL of each sample was diluted with 20 µl non-reducing sample buffer (Bio-Rad) and after heating (5 min at 95°C) separated by discontinuous SDS-PAGE according to Laemmli [Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-

685, (1970)] on 0.75 mm thick slabs (8 % polyacrylamide). Electrophoresis was carried out in a Mini-Protean III cell (Bio-Rad).

The proteins were transferred to Immun-blot PVDF membranes (Bio-Rad) by wet Western blotting in a mini Trans-blot cell (Bio-Rad) at 350 mA for 60 minutes in 25 mM Tris, 190 mM glycine with 20% v/v methanol. The membranes were blocked overnight in TBS-T (10 mM Tris pH 7.4, 137 mM NaCl, 0.05% (v/v) Tween-20) supplemented with 5 % w/v non-fat dried milk (Protifar, Nutricia) at 4°C. Incubation with the primary anti-ADAM17 ectodomain monoclonal antibody (R&D systems, clone 111636) was performed at room temperature for 2 h at 0.5 µg/mL in TBS-T with 1 % w/v non-fat milk, followed by 1.5 h exposure to a secondary rabbit anti-mouse antibody conjugated with alkaline phosphatase (Sigma-Aldrich) at a 1:15,000 dilution. Bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Duchefa).

References

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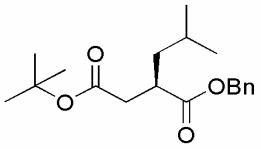
3 S. Chatterjee, M. Iqbal, S. Mallya, S. E. Senadhi, T. M. O'Kane, B. A. McKenna, D. Bozyczko-Coyne, J. C. Kauer, R. Siman and J. P. Mallamo, *Bioorg. Med. Chem.*, 1998, **41**, 509.

4 J. M. Altenburger, C. Mioskowski, H. d'Orchymont, D. Schirlin, C. Schalk and C. Tarnus, *Tetrahedron Lett.*, 1992, **33**, 5055.

5 J. R. Freije, T. Klein, J. A. Ooms, J. P. Franke and R. Bischoff, *Prot. Res.*, 2006, **5**, 1186.

Compound 4



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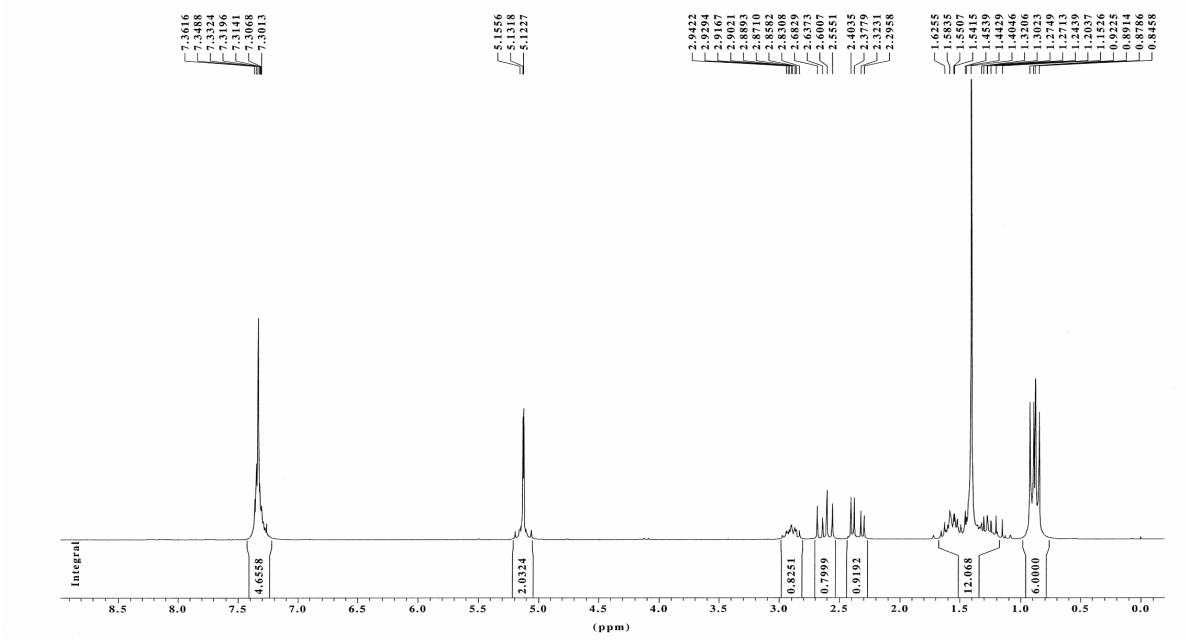

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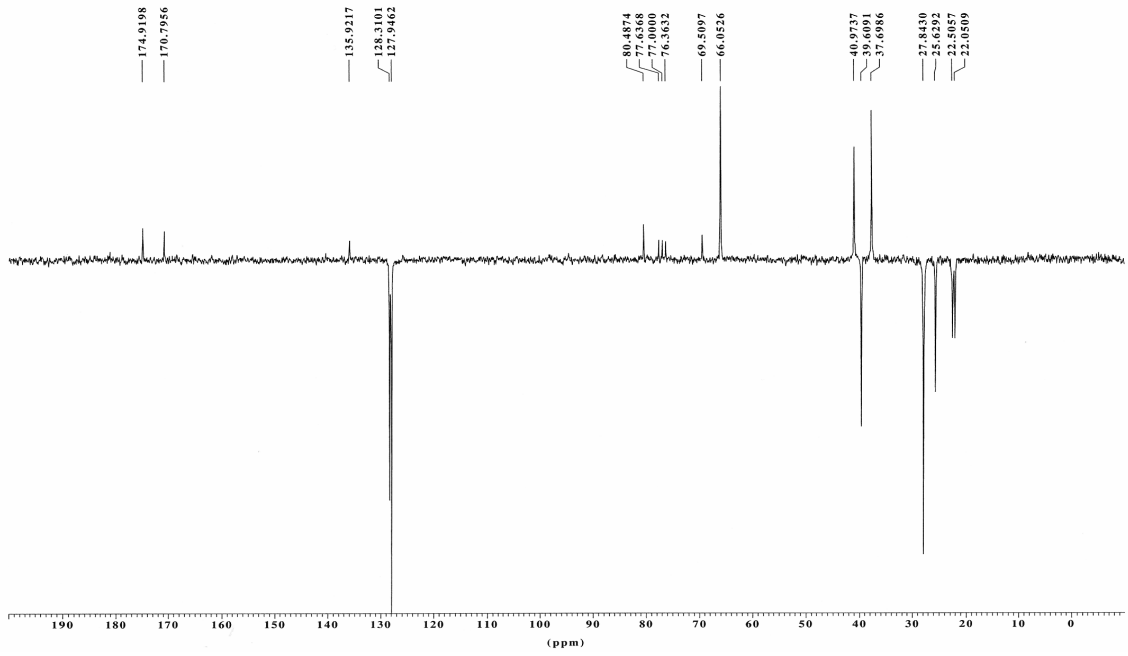

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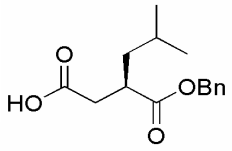

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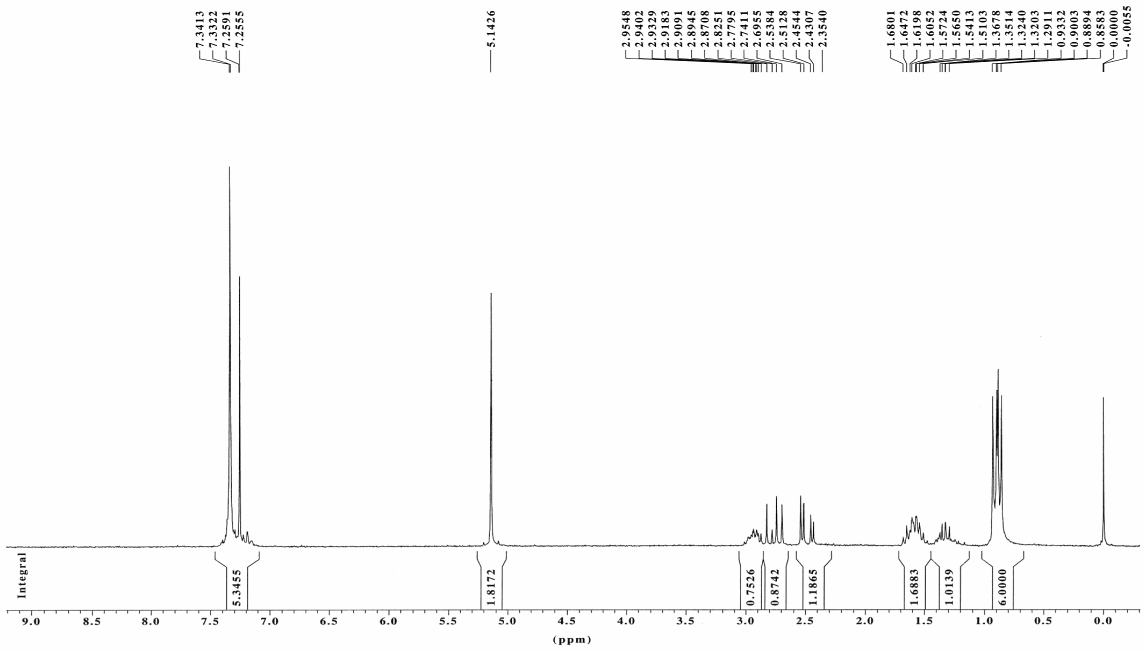

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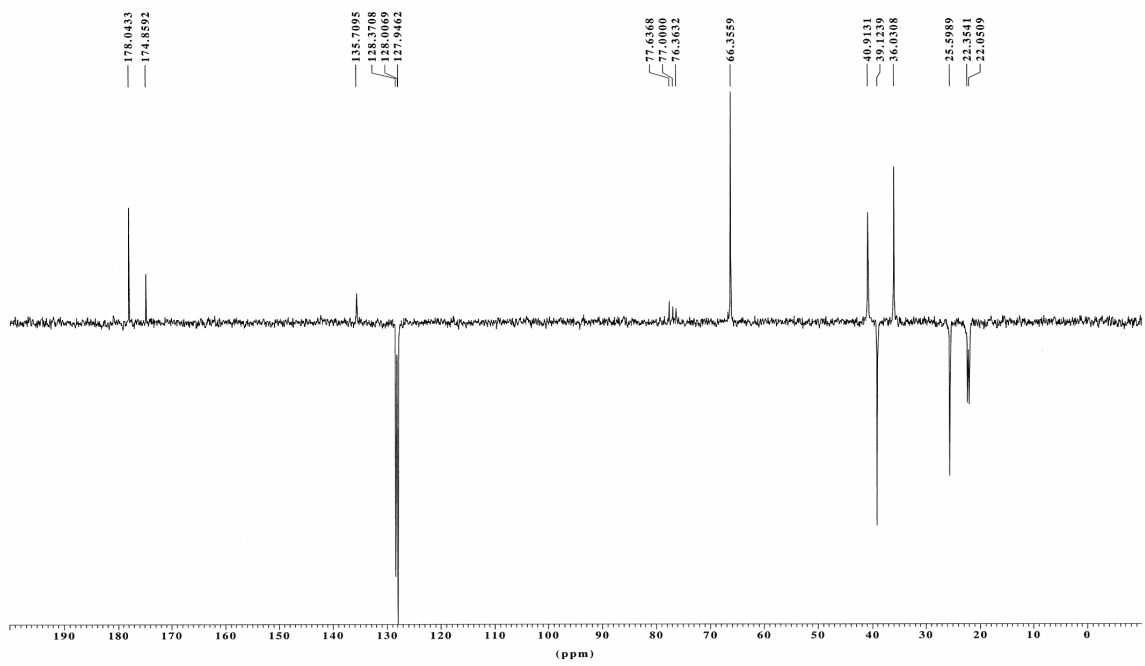

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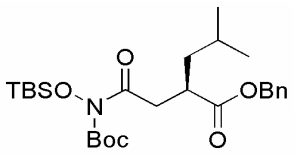

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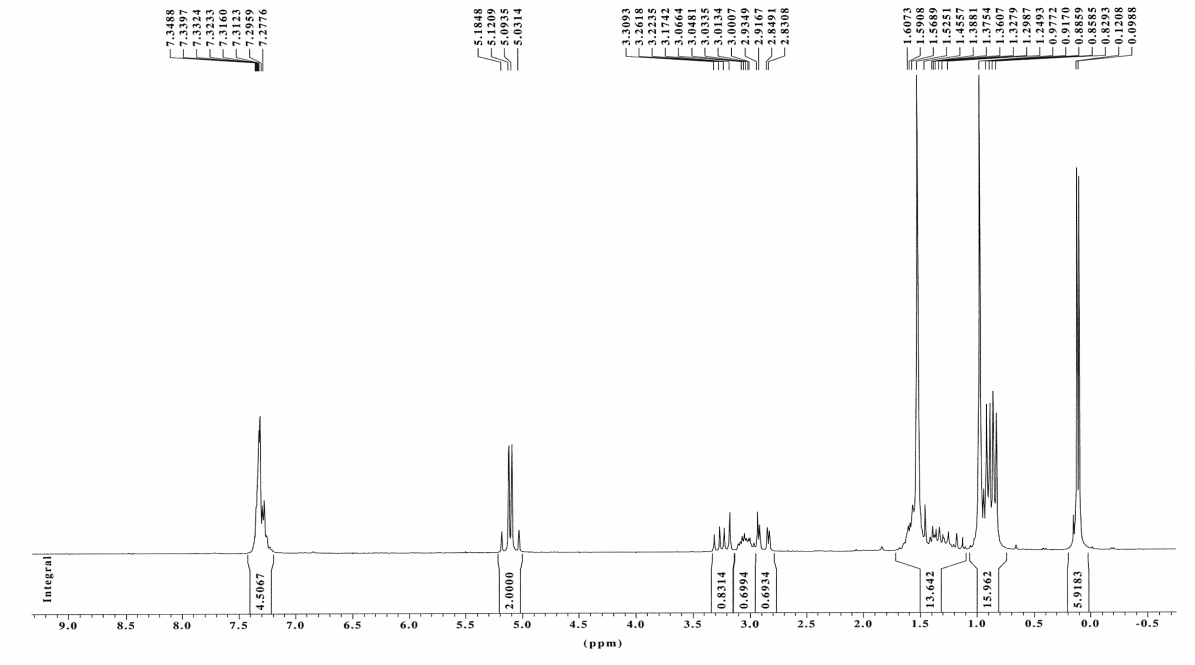

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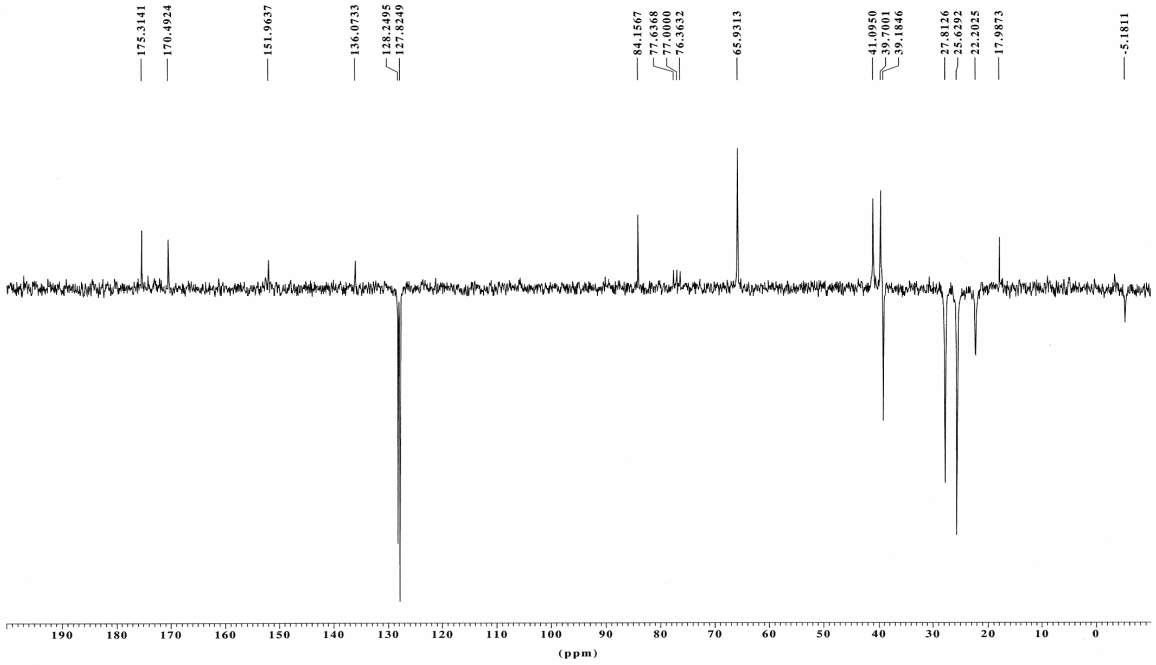

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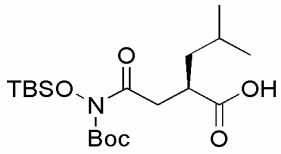
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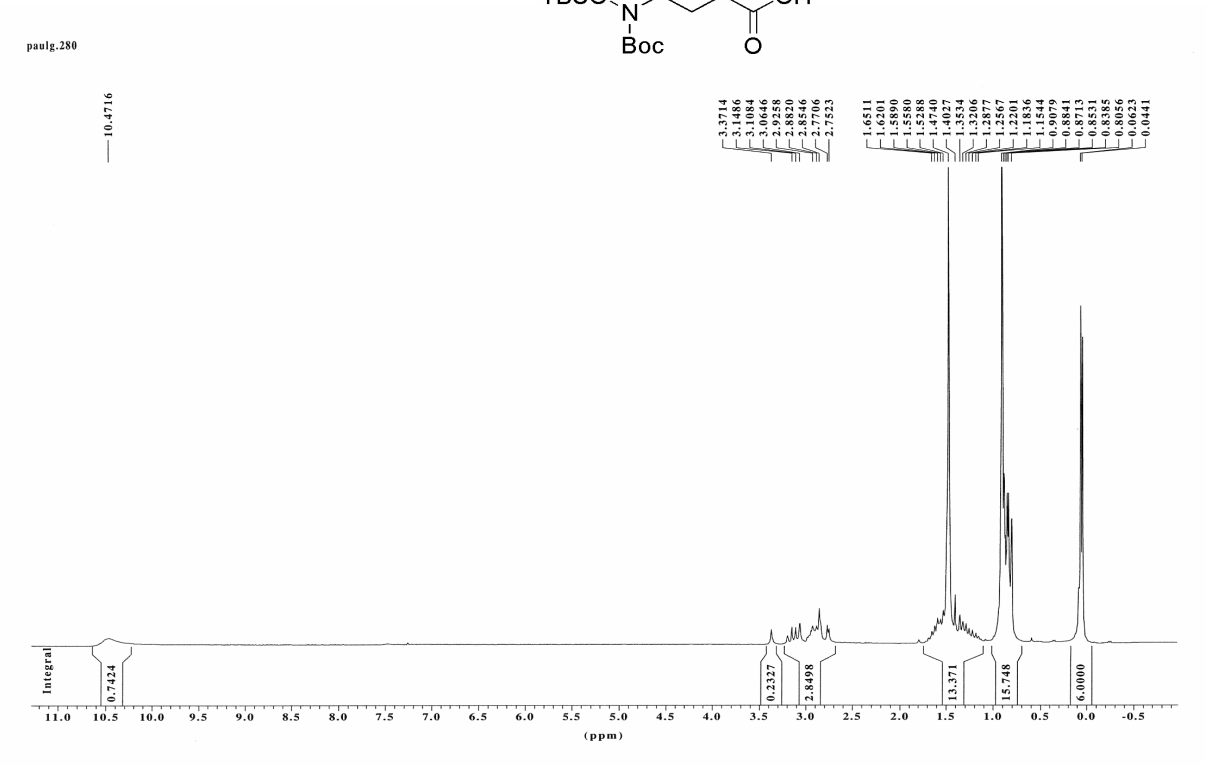
Boc
O
O
OH
O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2.8546
2.7706
2.7523
1.6511
1.6201
1.5890
1.5580
1.5288
1.4740
1.4027
1.3534
1.3206
1.2877
1.2567
1.2201
1.1836
1.1544
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0.8841
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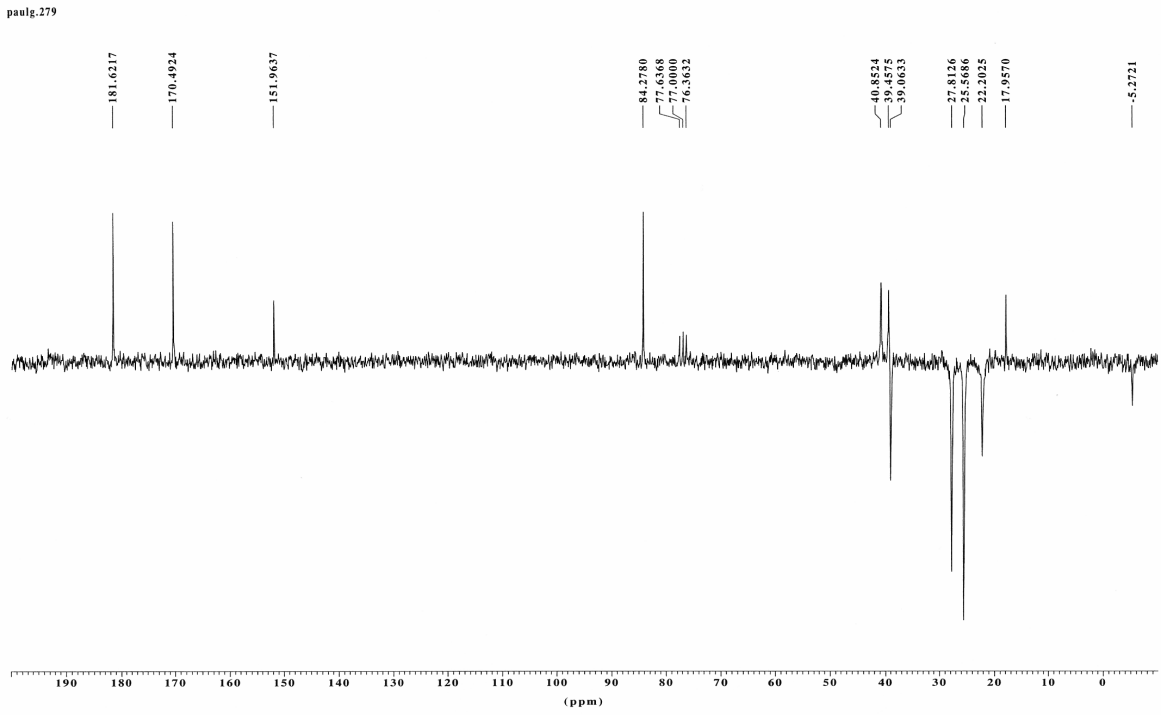
0.8531
0.8385
0.8056
0.0623
0.0441
Integral
0.7424
0.2327
2.8498
13.371
15.748
6.0000
11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
(ppm)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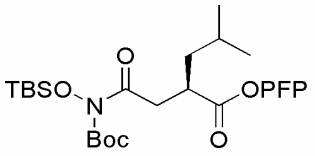
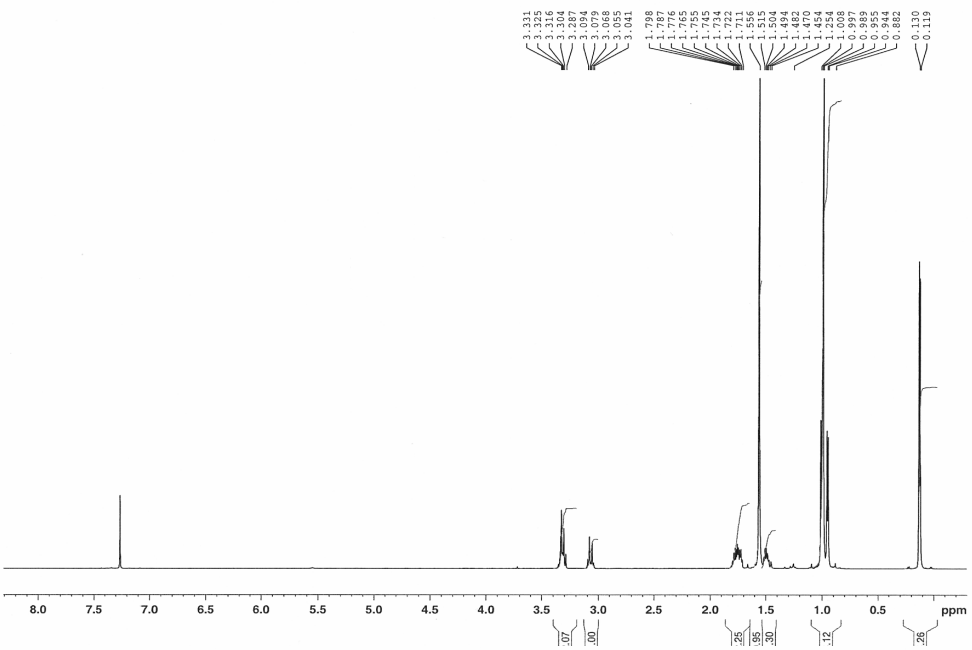
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
(ppm)

Compound 1



1H NMR dmx-600

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PROCNO                   1

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PULPROG                zg30
TD                    65536
SOLVENT               CD2Cl2
NS                        17
DS                         4
SWH                8992.806 Hz
FIDRES             0.137219 Hz
AQ                 3.6438515 sec
RG                        64
DW                    55.600 usec
DE                     9.50 usec
TE                    298.0 K
D1                 2.00000000 sec
TD0                       1

======== CHANNEL f1 =======
NUC1                      1H
P1                     8.30 usec
PL1                   -6.00 dB
SFO1           600.1339010 MHz

F2 - Processing parameters
SI                        32768
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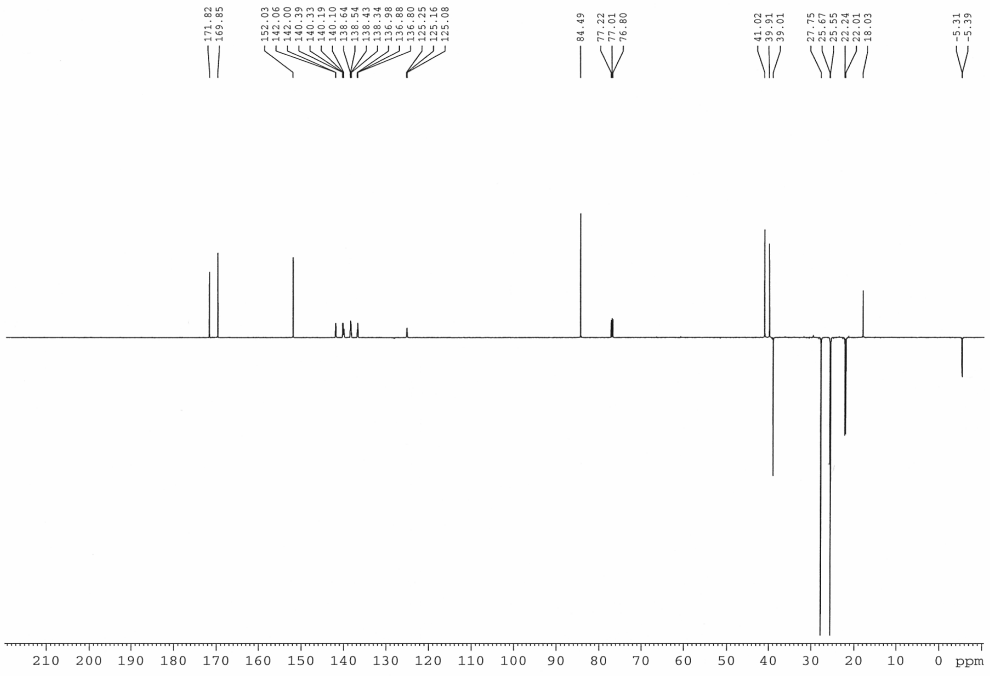
13C APT dmx-600 PG01 in CDCL3

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SWH               34722.223 Hz
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DE                     8.50 us
TE                    298.0 K
CNST2             155.0000000
CNST11              1.0000000
D1                  1.50000000 se
d20                 0.00645161 se
DELTA               0.00001592 se
TD0                      16

======== CHANNEL f1 ======
NUC1                     13C
P1                    12.50 us
p2                    25.00 us
PL1                   -6.00 dB
SFO1           150.9185940 MH

======== CHANNEL f2 ======
CPDPRG2              waltz16
NUC2                      1H
PCPD2                 80.00 us
PL2                   -6.00 dB
PL12                  13.68 dB
SFO2           600.1324000 MH

F2 - Processing parameters
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WDW                      EM
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GB                        0
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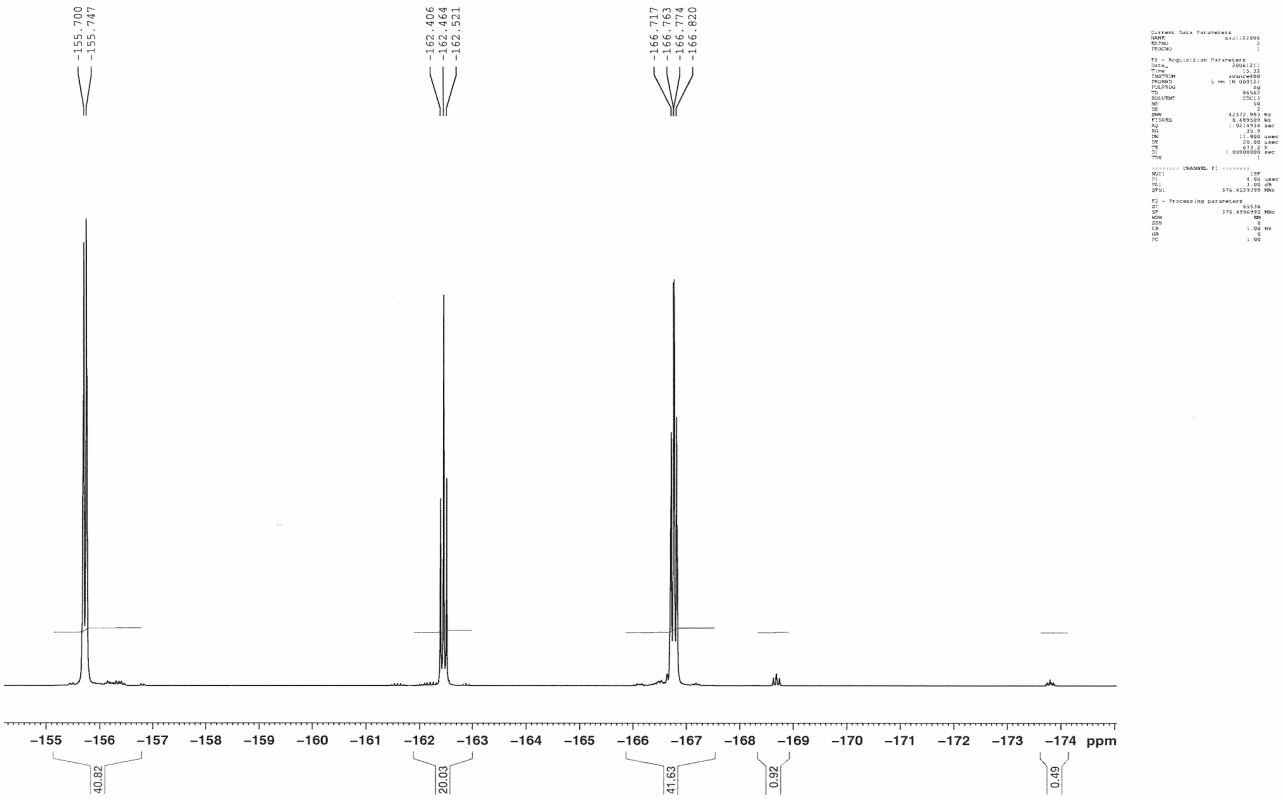
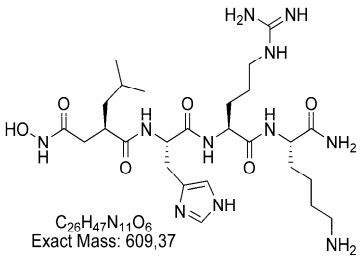
Pg01 in CDCL3 19Fluor nmr av400 ref= cf3cooh in CDCL3 = -79.59 ppm



Compound RH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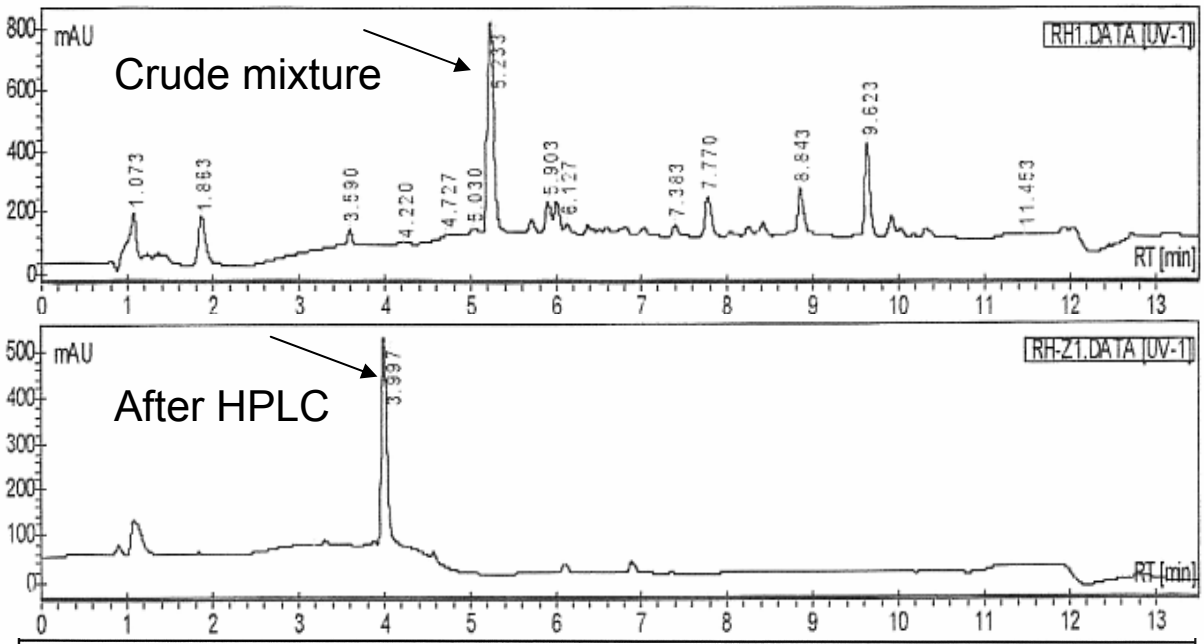
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Crude mixture
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4.220
4.727
5.030
5.233
5.903
6.127
7.383
7.770
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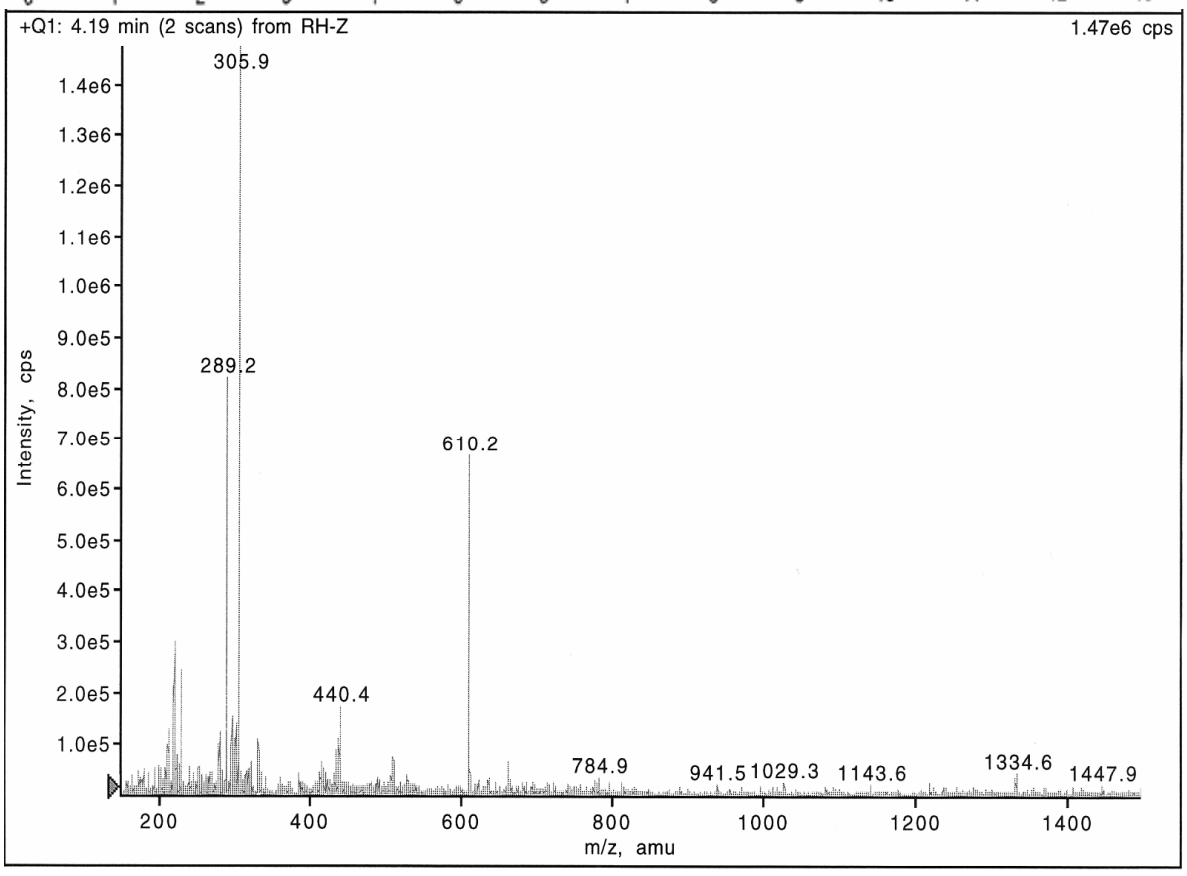
9.623
11.453
RT [min]
mAU
After HPLC
RH-Z1.DATA [UV-1]
3.997
RT [min]


+Q1: 4.19 min (2 scans) from RH-Z
1.47e6 cps
305.9
289.2
610.2
440.4
784.9
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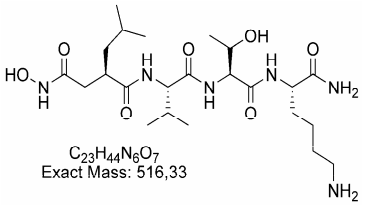
1029.3
1143.6
1334.6
1447.9
Intensity, cps
m/z, amu

Compound TV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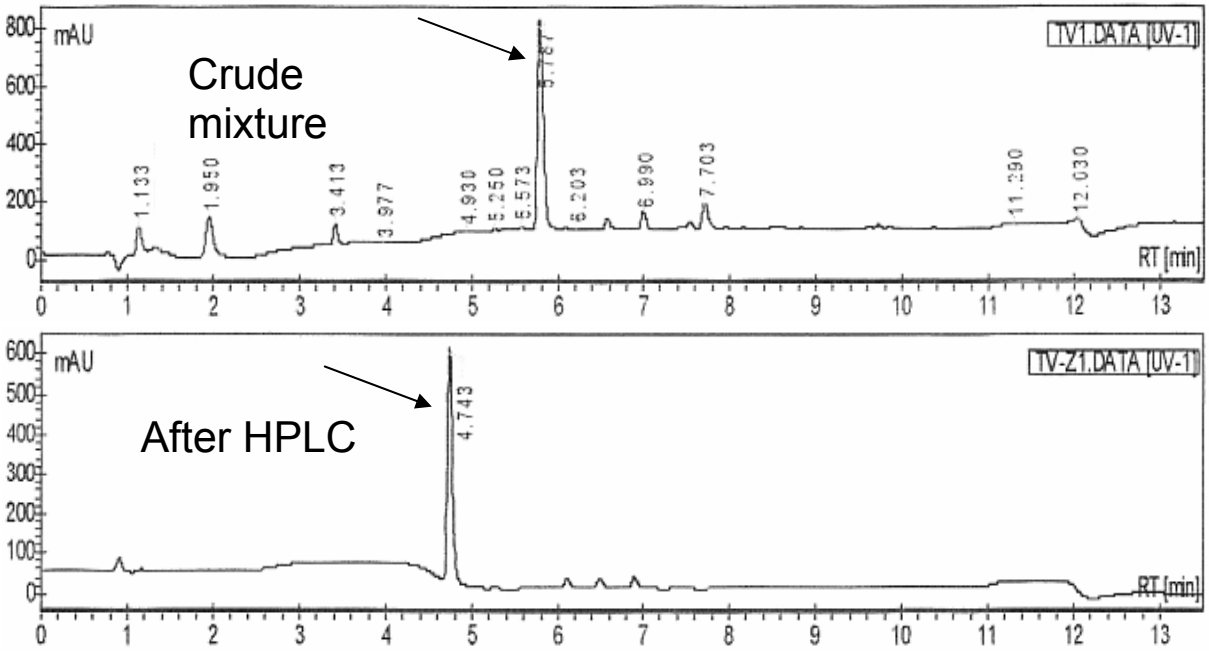
Exact Mass: 516,33


mAU
Crude mixture
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5.250
5.573
5.787
6.203
6.990
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mAU
After HPLC
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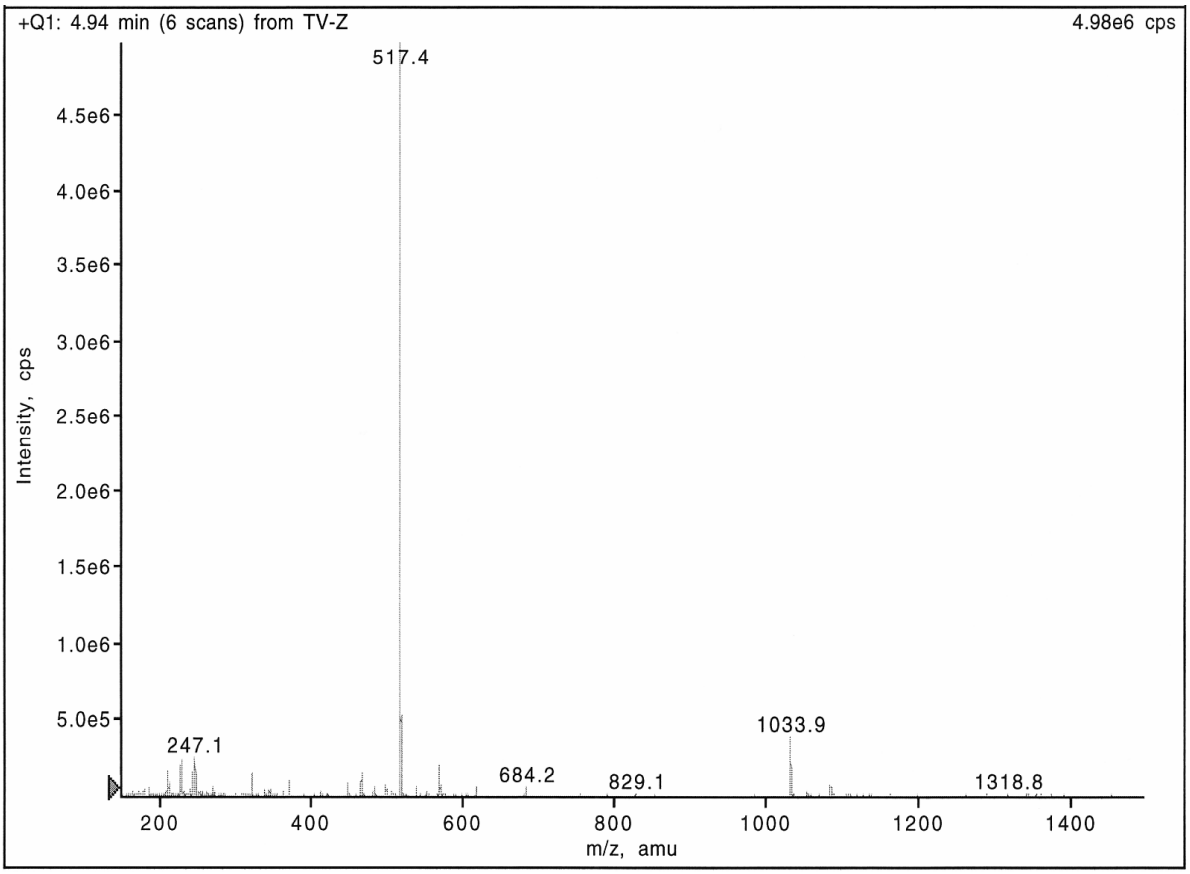
RT [min]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4.98e6 cps
517.4
Intensity, cps
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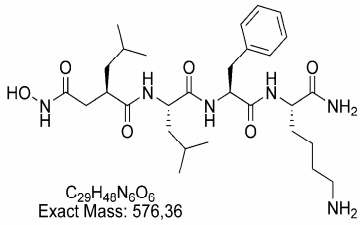
684.2
829.1
1033.9
1318.8
m/z, amu

Compound FL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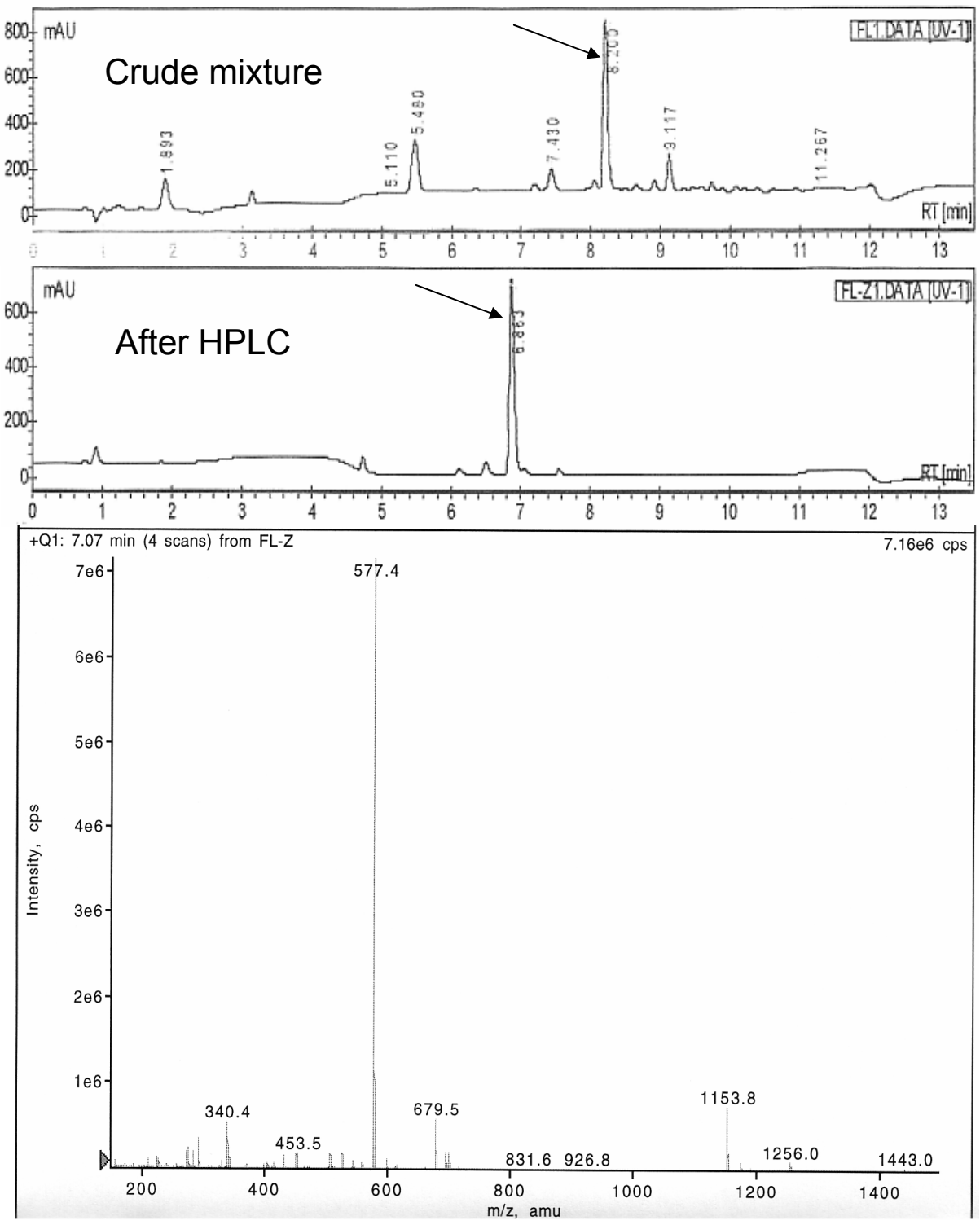
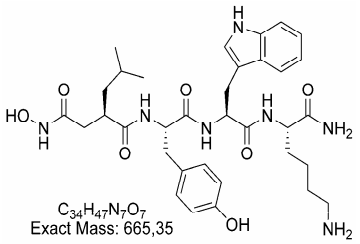
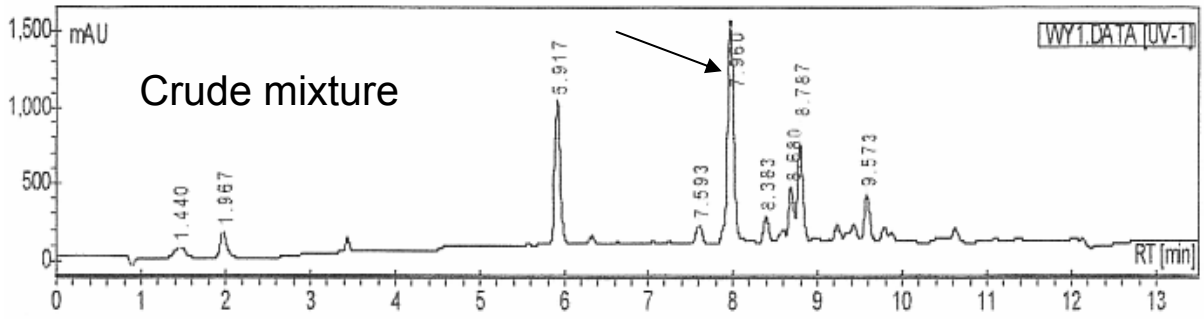
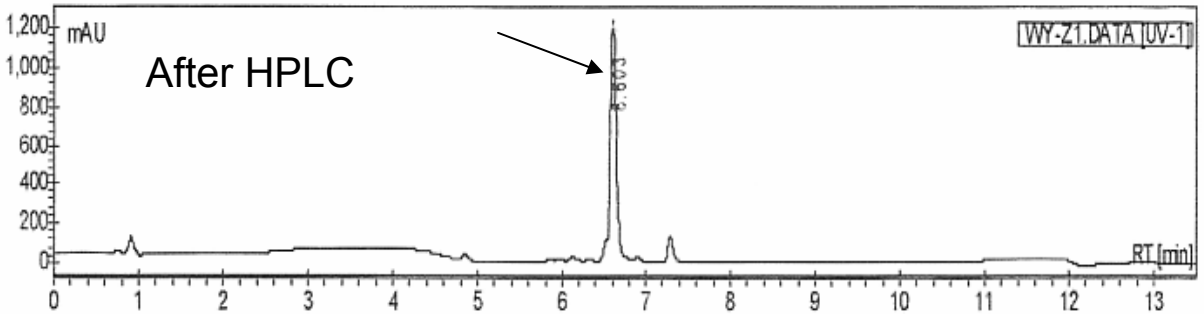
O
O
N
H
O
O
N
H
O
NH2
NH2
C29H46N6O6
Exact Mass: 576,36

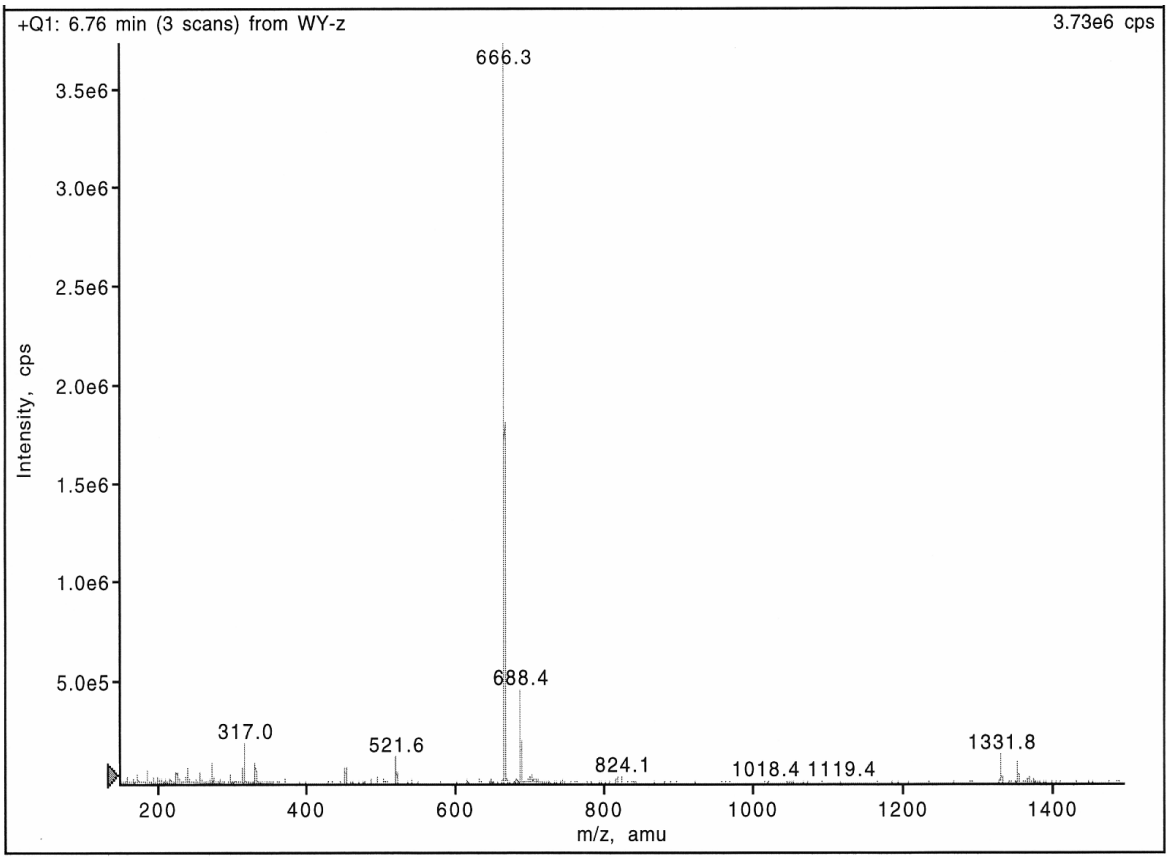

Crude mixture
mAU
FL1.DATA [UV-1]
1.893
5.110
5.480
7.430
8.205
9.117
11.267
RT [min]
After HPLC
mAU
FL-Z1.DATA [UV-1]
6.863
RT [min]
+Q1: 7.07 min (4 scans) from FL-Z
7.16e6 cps
Intensity, cps
577.4
340.4
453.5
679.5
831.6
926.8
1153.8
1256.0
1443.0
m/z, amu

Compound WY

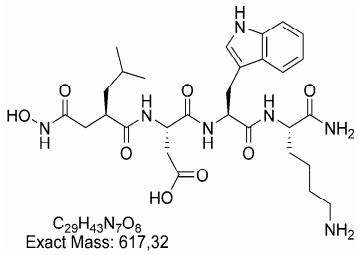

$C_{34}H_{47}N_7O_7$
Exact Mass: 665,35

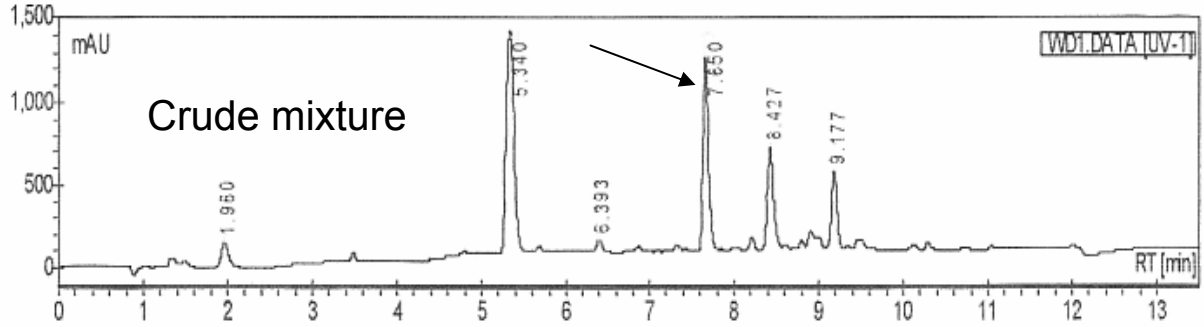

Crude mixture
mAU
WY1.DATA [UV-1]
RT [min]
1.440
1.967
5.917
7.593
7.960
8.383
8.580
8.787
9.573

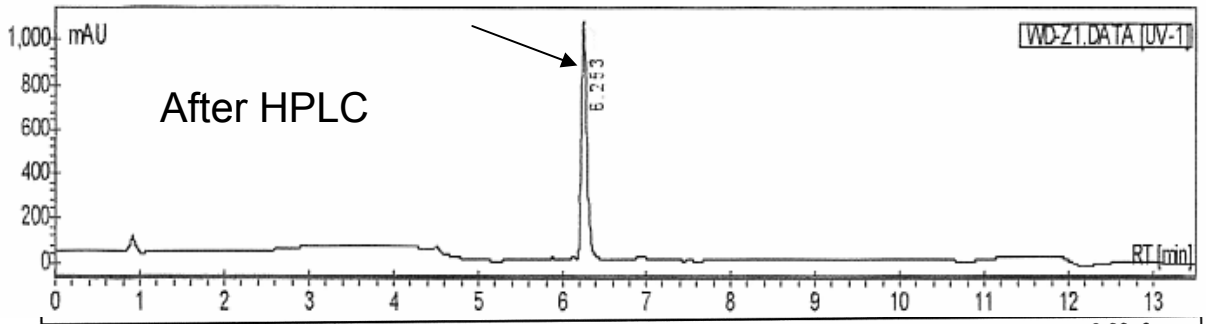

After HPLC
mAU
WY-Z1.DATA [UV-1]
RT [min]
6.603

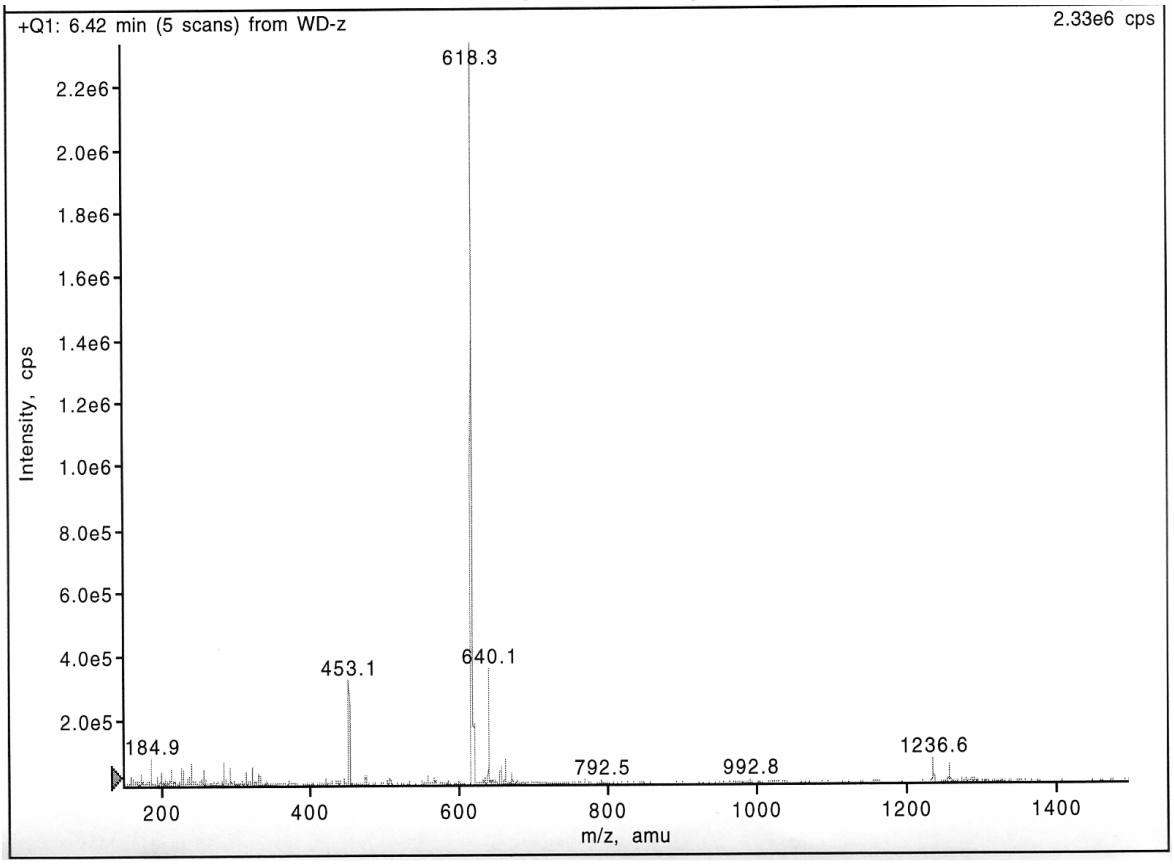

+Q1: 6.76 min (3 scans) from WY-z
3.73e6 cps
Intensity, cps
m/z, amu
666.3
688.4
317.0
521.6
824.1
1018.4 1119.4
1331.8

Compound WD

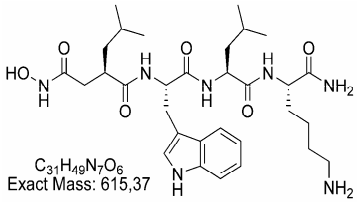

$C_{29}H_{43}N_7O_8$
Exact Mass: 617,32

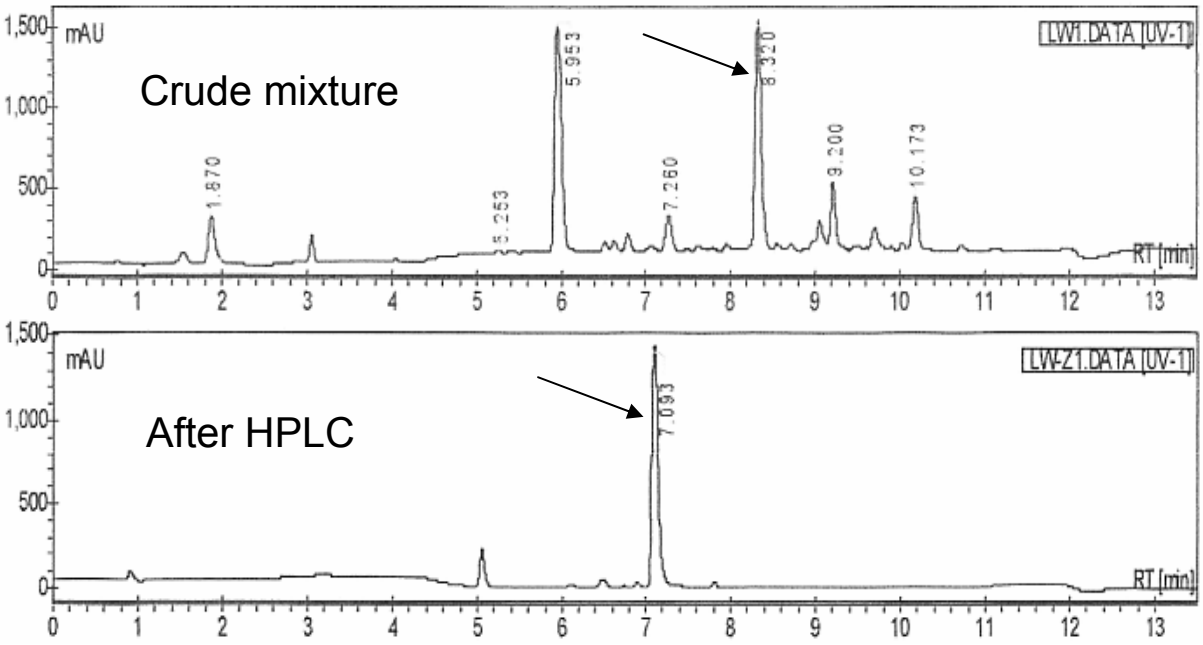

Crude mixture
mAU
WD1.DATA [UV-1]
1.950
5.340
6.393
7.650
8.427
9.177
RT [min]

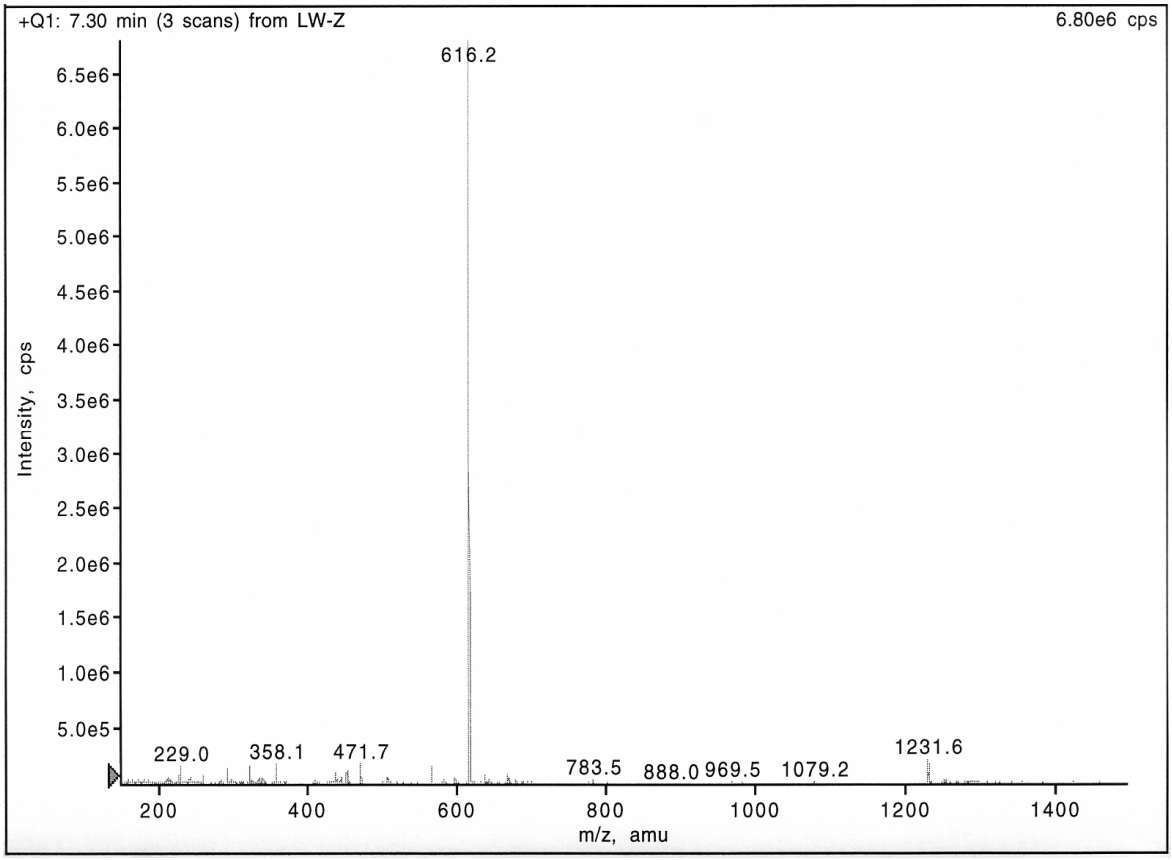

After HPLC
mAU
WD-Z1.DATA [UV-1]
6.253
RT [min]


+Q1: 6.42 min (5 scans) from WD-z
2.33e6 cps
618.3
453.1
640.1
184.9
792.5
992.8
1236.6
Intensity, cps
m/z, amu

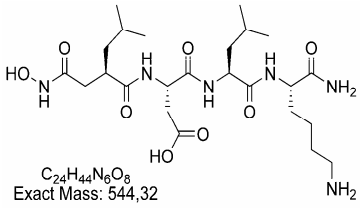
Compound LW

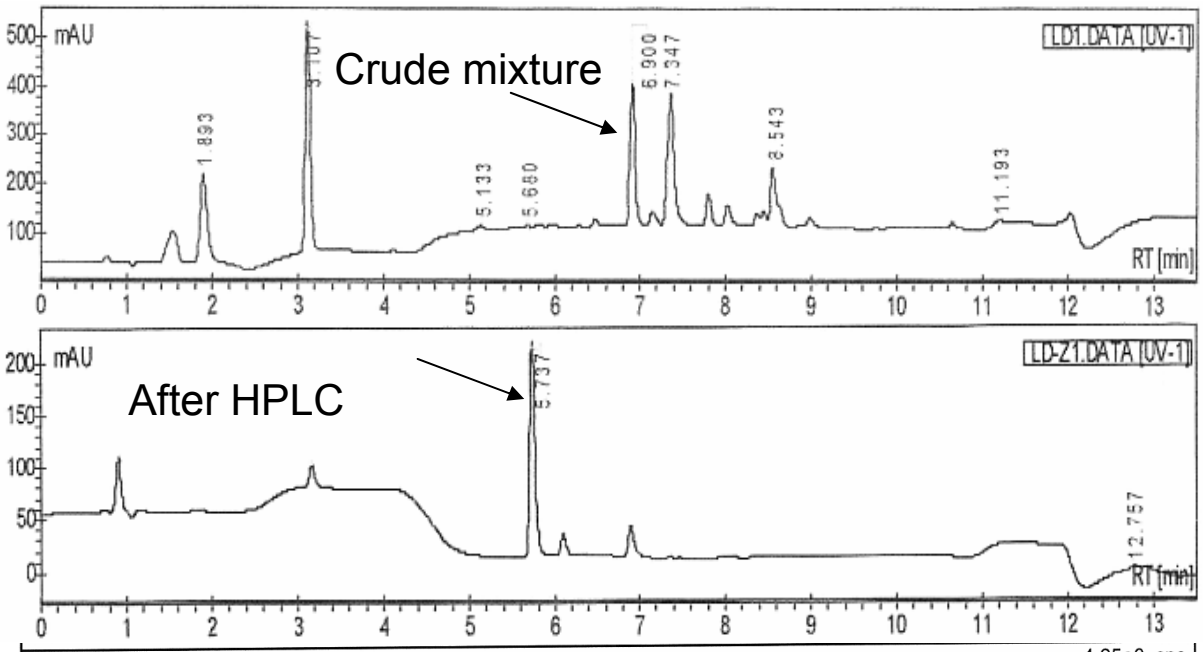

C31H49N7O6
Exact Mass: 615,37

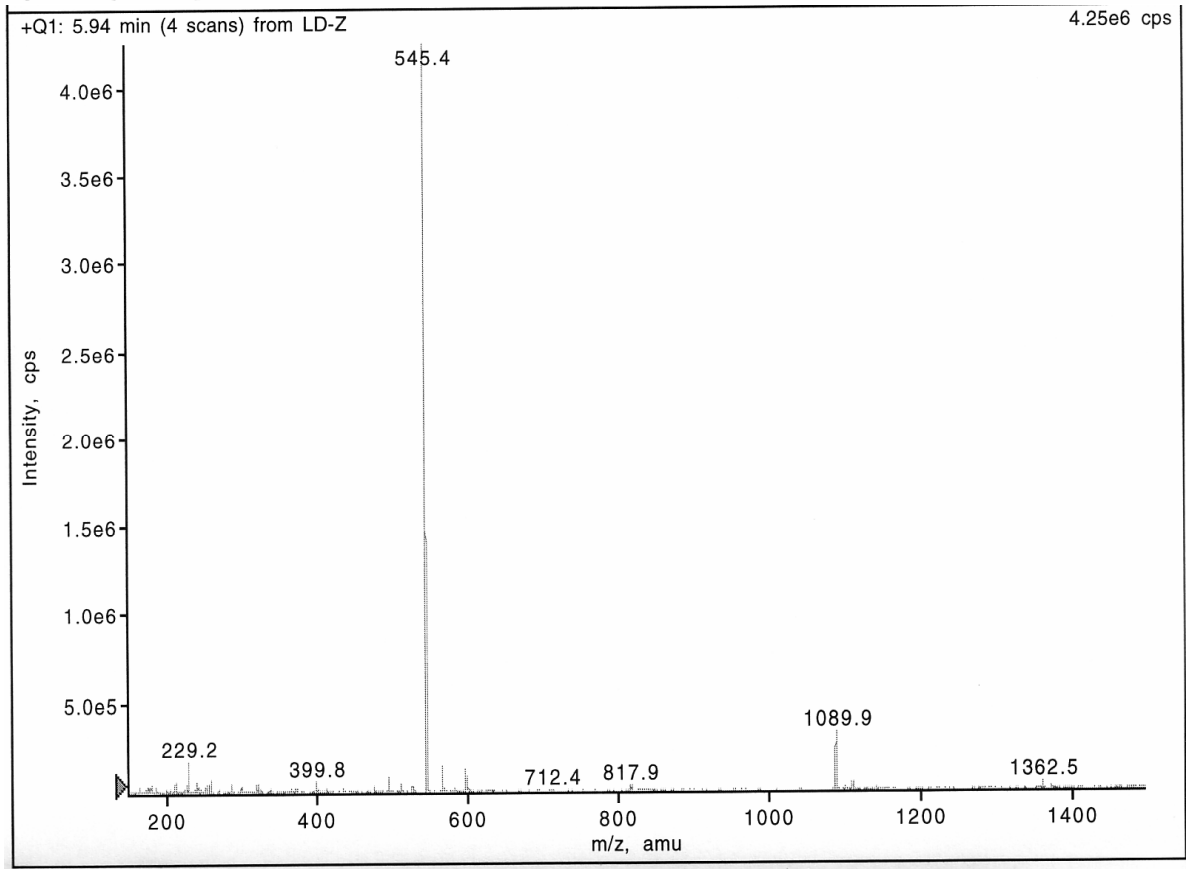

Crude mixture
mAU
LW1.DATA [UV-1]
1.870
5.253
5.953
7.260
8.320
9.200
10.173
RT [min]
After HPLC
mAU
LW-Z1.DATA [UV-1]
7.093
RT [min]


+Q1: 7.30 min (3 scans) from LW-Z
6.80e6 cps
616.2
Intensity, cps
229.0
358.1
471.7
783.5
888.0
969.5
1079.2
1231.6
m/z, amu

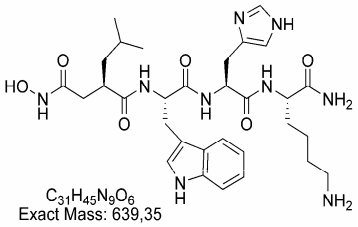
Compound LD

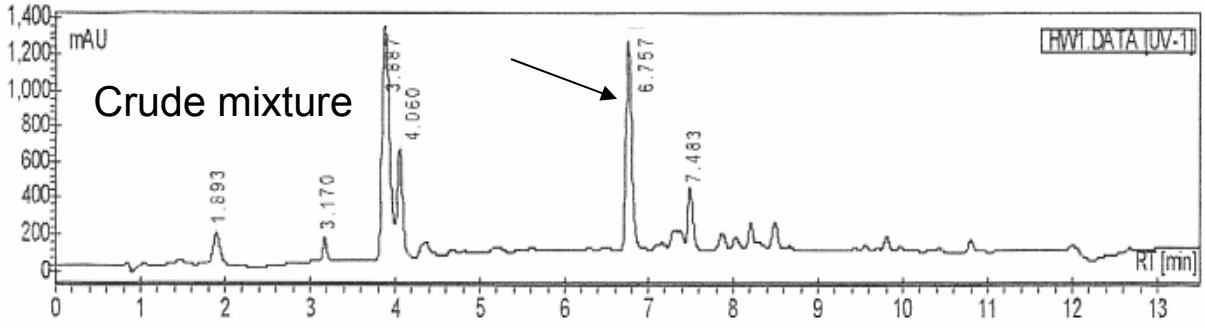

$C_{24}H_{44}N_6O_8$
Exact Mass: 544,32

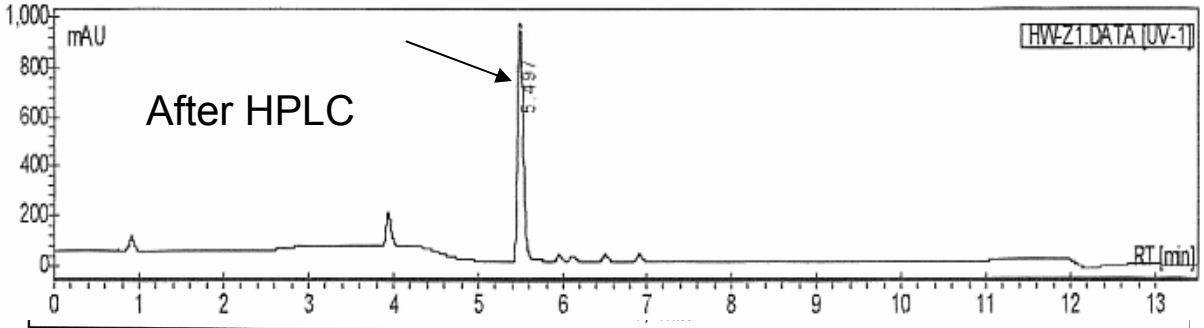

mAU
LD1.DATA [UV-1]
Crude mixture
1.893
3.107
5.133
5.680
6.900
7.347
8.543
11.193
RT [min]
mAU
LD-Z1.DATA [UV-1]
After HPLC
5.757
12.757
RT [min]

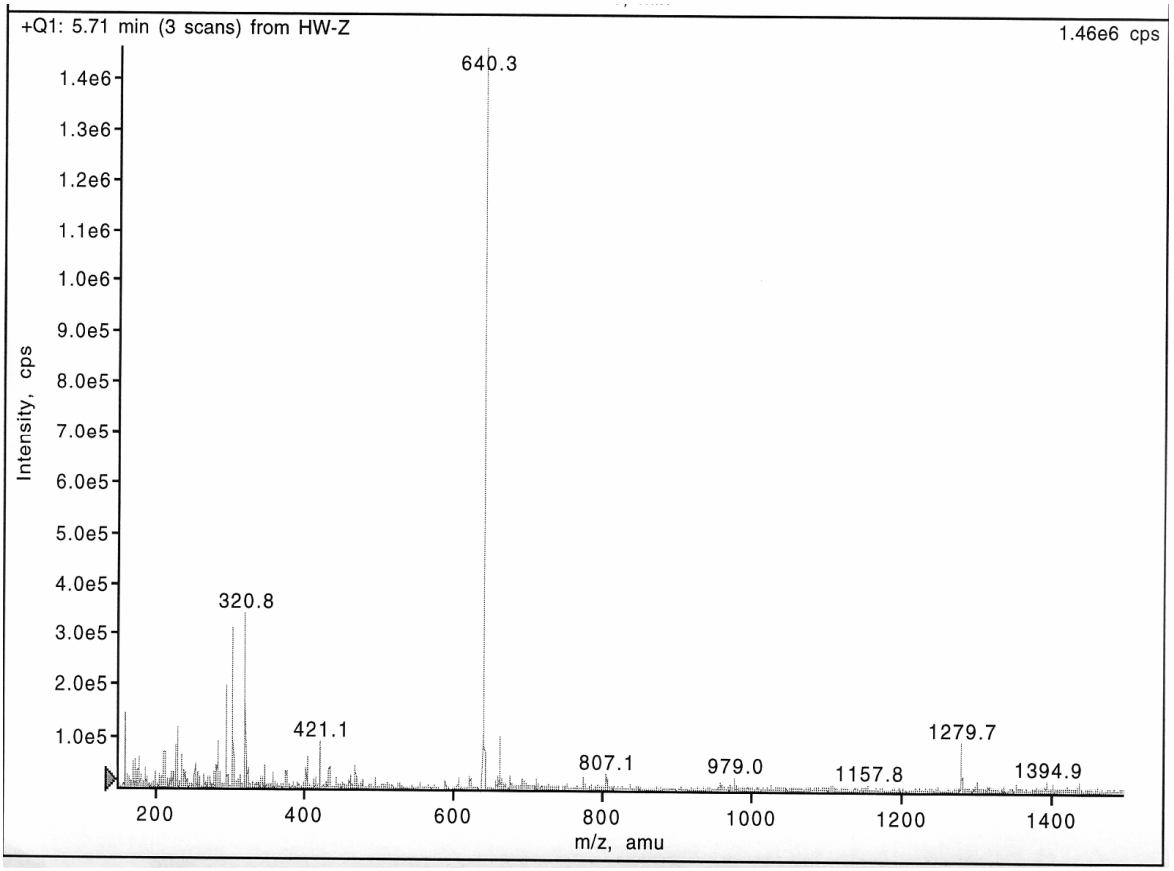

+Q1: 5.94 min (4 scans) from LD-Z
4.25e6 cps
545.4
Intensity, cps
229.2
399.8
712.4
817.9
1089.9
1362.5
m/z, amu

Compound HW

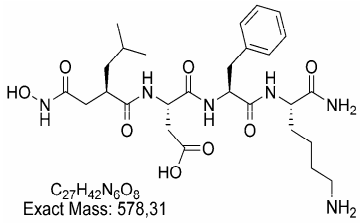

$C_{31}H_{45}N_9O_6$
Exact Mass: 639,35


Crude mixture
mAU
HW1.DATA [UV-1]
1.693
3.170
3.887
4.060
6.757
7.483
RT [min]

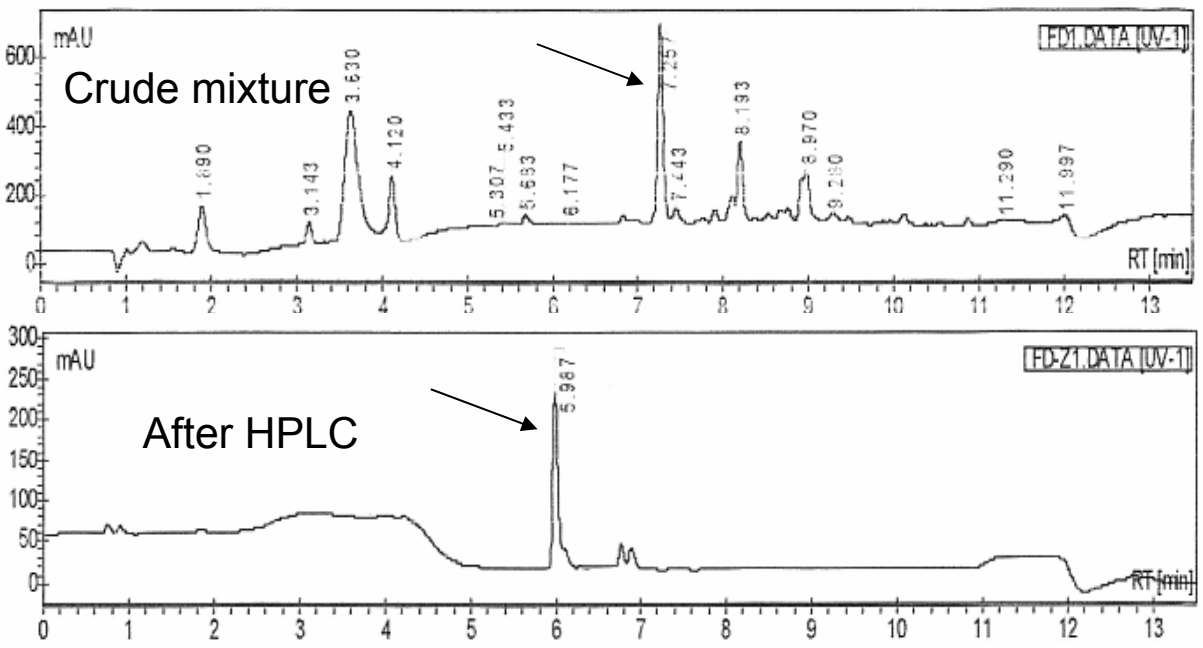

After HPLC
mAU
HW-Z1.DATA [UV-1]
5.497
RT [min]

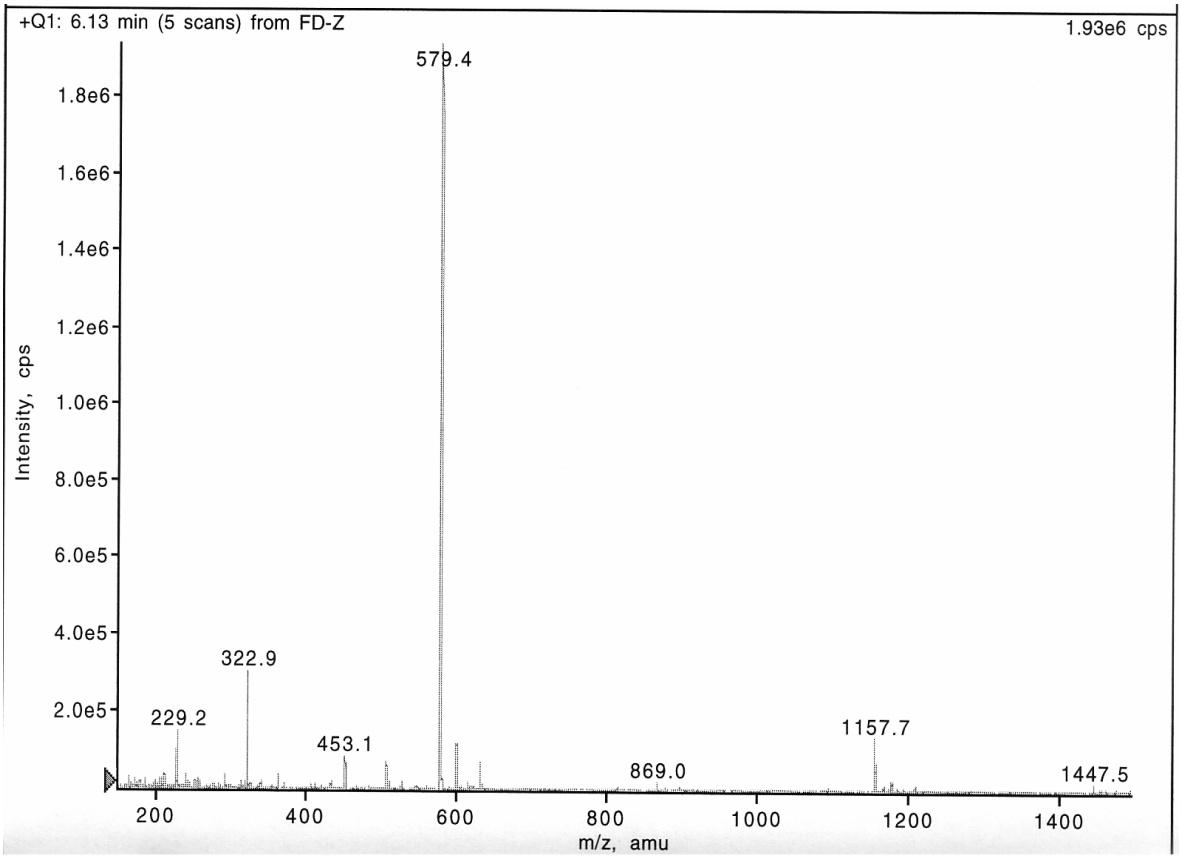

+Q1: 5.71 min (3 scans) from HW-Z
1.46e6 cps
Intensity, cps
m/z, amu
640.3
320.8
421.1
807.1
979.0
1157.8
1279.7
1394.9

Compound FD

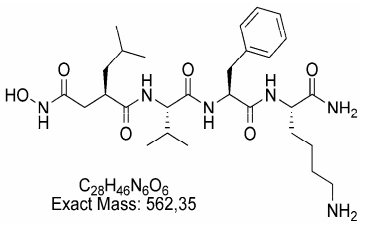


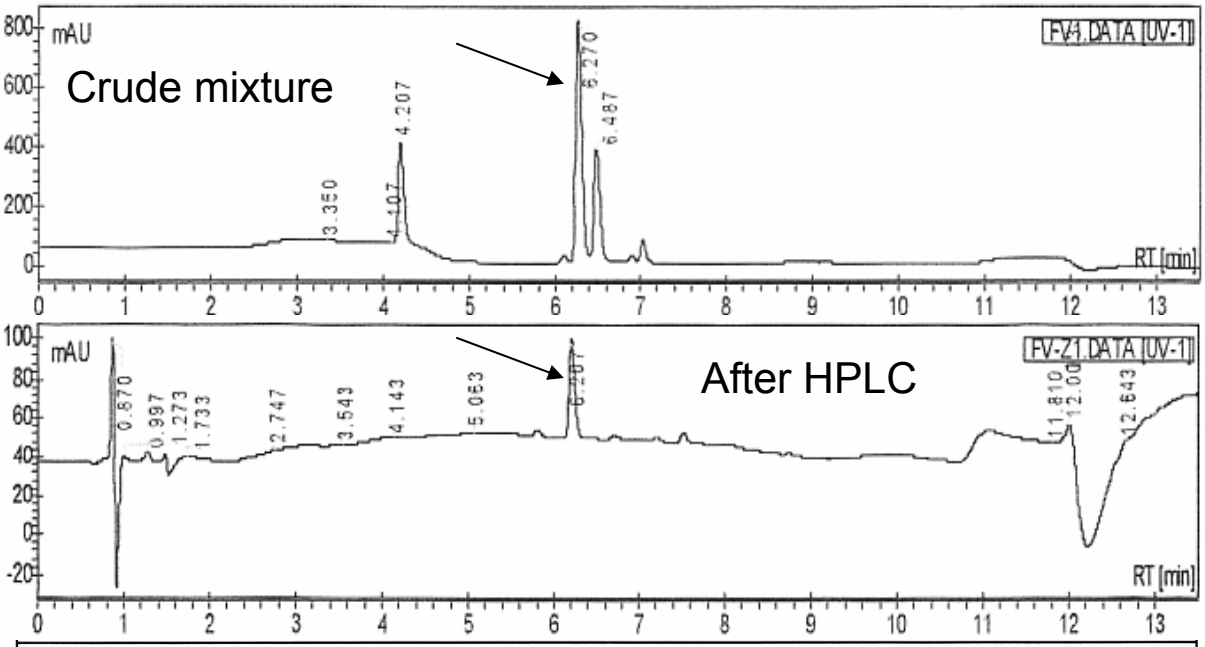
$C_{27}H_{42}N_6O_8$
Exact Mass: 578,31

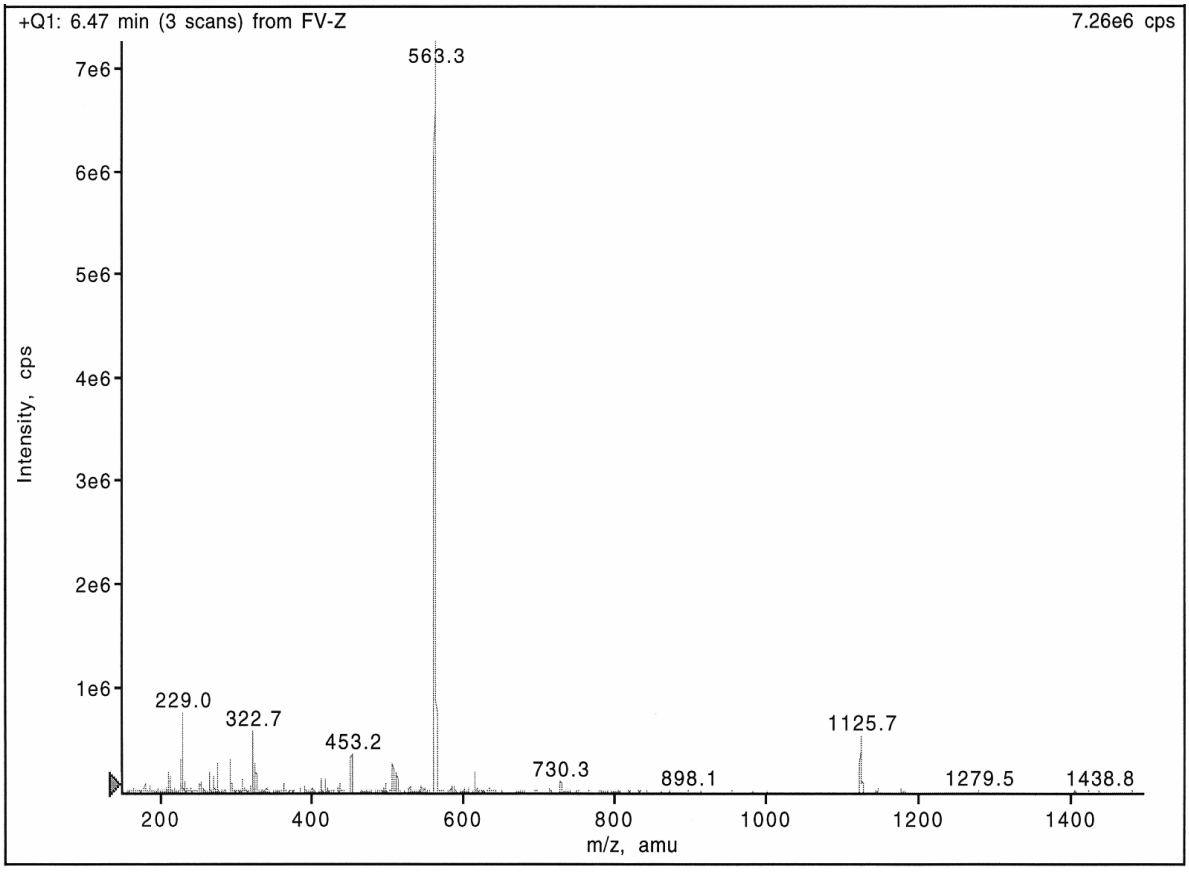




Compound FV


$C_{28}H_{46}N_6O_6$
Exact Mass: 562,35


Crude mixture
mAU
FV1.DATA [UV-1]
3.350
4.107
4.207
6.270
6.487
RT [min]
After HPLC
mAU
FV-Z1.DATA [UV-1]
0.870
0.997
1.273
1.733
2.747
3.543
4.143
5.063
6.467
11.810
12.643
RT [min]


+Q1: 6.47 min (3 scans) from FV-Z
7.26e6 cps
563.3
Intensity, cps
m/z, amu
229.0
322.7
453.2
730.3
898.1
1125.7
1279.5
1438.8