Non-Classical Size Dependence of Permeation Defines Bounds for Passive Adsorption of Large Drug Molecules.

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Supporting Information

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Supplementary Table for Pure Compounds:

	Position												PAMPA	LE-MDCK	
cpd.	1	2	3	4	5	6	7	8	9	10	MW	AlogP	P_e (10^{-6} cm/s)	P_e (10^{-6} cm/s)	% recovery
8.1	Leu	Leu	Leu	Leu	Leu	Leu	Pro	Tyr			1037.4	6.8	0.033	<0.1	54.81
8.2	Leu	Ala	Leu	Leu	Leu	Ala	Pro	Tyr			953.3	4.3	19	2.76	26.95
8.3	Ala	Leu	Ala	Leu	Ala	Ala	Pro	Tyr			869.1	1.9	1	0.38	46.00
8.4	Ala	Ala	Ala	Ala	Ala	Ala	Pro	Tyr			785	-0.6	0.023	0.66	78.87
8.5	Ala	Ala	Leu	Leu	Leu	Leu	Pro	Tyr			953.3	4.3	8	10.31	77.84
8.6	Leu	Leu	Ala	Ala	Leu	Leu	Pro	Tyr			953.3	4.3	4	4.47	73.69
8.7	Leu	Leu	Leu	Leu	Ala	Ala	Pro	Tyr			953.3	4.3	6	7.30	73.46
8.8	Ala	Leu	Leu	Leu	Leu	Ala	Pro	Tyr			953.3	4.3	11	8.96	76.68
8.9	Leu	Ala	Leu	Leu	Ala	Leu	Pro	Tyr			953.3	4.3	19	12.72	92.13
8.10	Leu	Ala	Leu	Ala	Leu	Leu	Pro	Tyr			953.3	4.3	24	9.68	82.50
8.11	Abu	Ala	Ala	Nle	Ala	Ala	Pro	Tyr			841	1.5	0.24	0.22	74.01
8.12	Abu	Nle	Abu	Nle	Nle	Ala	Pro	Tyr			911	3.9	3.3	4.02	70.72
8.13	Abu	Nva	Nva	Nva	Nva	Nva	Pro	Tyr			939	4.8	4	7.29	73.92
8.14	Abu	Nle	Nle	Nle	Nle	Nle	Pro	Tyr			1009	7.1	<0.01	<0.1	89.71
9.1	Nva	Abu	Ala	Abu	Ala	Ala	Abu	Pro	Tyr		940	1.7	0.05	0.13	63.23
9.2	Nva	Abu	Ala	Nle	Ala	Nle	Ala	Pro	Tyr		996	3.7	0.8	0.61	77.88
9.3	Nva	Abu	Nva	Nva	Nva	Abu	Nva	Pro	Tyr		1038	5.1	4.5	3.56	67.95
9.4	Nva	Abu	Nle	Nle	Nle	Nle	Nle	Pro	Tyr		1121	7.9	<0.01	<0.1	88.10
10.1	Ala	Nva	Abu	Ala	Abu	Abu	Ala	Abu	Pro	Tyr	1039	1.9	0.12	<0.1	62.86
10.2	Ala	Nva	Abu	Nle	Abu	Ala	Nva	Abu	Pro	Tyr	1095	3.8	1.4	0.58	80.93
10.3	Ala	Nva	Abu	Nle	Abu	Nle	Nle	Abu	Pro	Tyr	1151	5.8	0.8	below LOD	below LOD

SI-Table 1. Pure compound composition and assay data. Red residue names indicate D stereochemistry; black residue names indicate L stereochemistry. Methods for PAMPA and MDCKII-LE are described below. AlogP is the octanol/water partition coefficient as calculated by the Ghose and Crippen atomic contribution model. [1,2]

General Synthetic Procedures

Synthesis of Pure Cyclic Permethylated Peptides

Loading of 2-Chlorotrityl resin

For resin bound for on-resin cyclization, Fmoc-*O*-allyl-Tyr (0.954 g, 0.43 mmol) was added to a flame dried round bottom flask and dried in a vacuum dessicator with phosphorous pentoxide overnight. 2 g of cesium carbonate was added to the flask and it was purged with N_2. 50mL of dry DCM was cannula transferred into the flask followed by 2.5 mL of DIPEA transferred via syringe. After sonication for 10 min, 5 g of 2-chlorotrityl resin was added under a stream of N_2 and allowed to shake for 4 h. The resin was capped with a 15 mL solution of 1:2:17 MeOH:DIPEA:DMF (3 x 15 min). The resin was washed with DMF (3 x 15 mL) followed by DCM (3 x 15 mL). The loading value was calculated by mass increase of dried, loaded resin.

Automated Peptide Synthesis

The linear peptide sequences were synthesized and cyclized using an automated peptide synthesizer (Prelude, Protein Technologies). Fmoc deprotections were carried out with 2% 1,8-diazabicycloundec-7-ene (DBU) and 2% piperidine in DMF for 15 min. Couplings were performed using 4 eq Fmoc-protected amino acid, 3.8 eq O-(azabenzotriazol-1-yl)- N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) and 6 eq N,N-diisopropylethylamine (DIPEA) in N,N-dimethylformamide (DMF, 0.1 M with respect to amino acid) for 1 h. After each coupling and deprotection step, the resin was washed with DMF (3×), dichloromethane (DCM) (3×) and DMF (3×).

On-Resin *O*-allyl and Fmoc deprotection

After the addition of the final residue, deallylation and final Fmoc removal were performed simultaneously with a solution of 1 eq $Pd(Ph_3P)_4$ in THF containing 10% (v/v) piperidine for 3 h. A chelating wash was performed to remove traces of palladium using 5% (w/v) sodium diethyldithiocarbamate andin 5% (v/v) DIPEA in DMF, followed by the previously described DMF-DCM-DMF resin wash sequence.

On-Resin Cyclization

Cyclization was performed with 3 eq (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 3 eq 1-hydroxy-7-azabenzotriazole (HOAt), and 6 eq DIPEA in DMF for 3 h, followed by resin washing with five final DCM washes to remove residual DMF.

On-Resin Permethylation

Following cyclization, cyclic peptides were subjected to an on-resin permethylation. To the resin-bound peptides was added lithium *tert*-butoxide (3 equiv / per theoretical eq NH) in DMSO / THF (1:1) using enough volume to fully cover all resin and dissolve the Li *t*-BuOH resulting in an approximate concentration of ~1M . The resin was agitated for 15 min, after

which methyl iodide was added. After an additional 15 min agitation, the resin was washed exhaustively with water, followed by washings with DMF (3x), MeOH (3x), and DCM (3x).

Peptide Cleavage

Peptides were cleaved with a 5% (v/v) trifluoroacetic acid (TFA) in DCM. The filtrate was concentrated and the crude residue was then purified by reverse phase (Biotage SNAP C18) automated flash chromatography (ACN:H20) and lyophilized to afford white amorphous solids.

Synthesis of Cyclic Peptide Libraries

The permethylated libraries were synthesized using the "split-pool" strategy starting with the allyl ester of N-fluorenylmethyloxycarbonyl (Fmoc)-protected tyrosine linked via the phenolic hydroxyl group to 2-chlorotrityl polystyrene resin (0.4 mmol/g loading value) as described above.

Manual Fmoc Deprotection

After washing the resin with the previously described DMF-DCM-DMF sequence, Fmoc deprotections were carried out with 2% 1,8-diazabicycloundec-7-ene (DBU) and 2% piperidine in DMF. A volume of this deprotection solution approximately twice that of the resin was added to the SPPS tube and agitated on a rotary shaker for 15 min. The solution was then drained and without washing the resin another round of deprotection was added and shaken for an additional 15 min. The resin was then washed using the standard sequence.

Manual Coupling Procedure:

For all couplings, a coupling solution was prepared by dissolving 4 eq of the amino acid and 3.8 eq of HATU in DMF (0.2 M with respect to amino acid). DIPEA (6 eq) was added and the solution was allowed to pre-activate for 30 min, then added to the deprotected, washed resin. The solution was agitated for 45 min on a rotary shaker.

Split-Pool Synthesis:

1 g of resin loaded with Fmoc-L-Tyr-OAllyl was coupled with Fmoc-D-proline as outlined above, then segregated into four separate polypropylene SPPS vessels and deprotected for 30 min. The resin was washed with DMF (3x), MeOH (3x), DCM (3x), and DMF (3x), then coupled with either N-Fmoc-D-norleucine, N-Fmoc-D-norvaline, N-Fmoc-D-aminobutyric acid, or N-Fmoc-D-alanine as outlined above. The resin was washed, pooled into a single vessel, thoroughly mixed, and the split-pool process repeated 4 more times (following the same stereochemistry as compound 8.1-8.4) to generate four sub-libraries of 256 linear heptapeptides. To keep the library size relatively small, all sub-libraries were kept separate and then coupled with N-Fmoc-D-aminobutyric acid.

After the final coupling, each portion of resin was washed and subjected to the simultaneous N/C-terminus deprotection. After deprotection, the resin was washed, cyclized as previously described, washed again, and subjected to the described on-resin permethylation conditions. The resin-bound sub-libraries were cleaved with a 5% solution of TFA in DCM. The collected filtrates were concentrated and the residue redissolved in DMSO at a concentration of

100 mg/mL to give stock solutions of four 256-member sublibraries of permethylated octapeptides.

Libraries of permethylated nonapeptides were generated in a similar manner except after coupling of the final Abu residue, an additional coupling of N-Fmoc-L-norvaline was performed to give four sub-libraries of 256 linear nonapeptides, which were cyclized, permethylated, cleaved, and reconstituted in DMSO as described above using an aliquot of the previously loaded resin.

Libraries of permethylated decapeptides were generated in a similar manner except after the coupling of the final Nva residue (in the preparation of nonapeptides), an additional coupling of N-Fmoc-D-alanine was performed to give four sub-libraries of 256 linear decapeptides, which were cyclized, permethylated, cleaved, and reconstituted in DMSO as described above.

Synthesis of natural products and unrelated pure compounds

Peptides were prepared with the following procedures; Stylissamide G, Cordyheptapeptide B, Scytallidamide B, and Cylindrocyclin were prepared using microwave assisted peptide couplings with installation of N-methyl amino acids followed by solution phase cyclization with COMU. 1NMe3 was prepared as previously described.[3] Cyclosporine A (BMT-to- Leu) was suynthesized using microwave assisted peptide couplings with installation of the appropriate Fmoc-protected N-Me amino acids followed by solution phase cyclization with COMU, with a modified permethylation for the first 4 amino acids. In the case of CSA starting with the Leucine that replaced the BMT residue as the first amino acid on resin the first 3 couplings were done with unmethylated amino acids. The Fmoc was removed, replaced with a Nosyl protecting group and the tetrapeptide was subjected to on resin permethylation. The remaining couplings and amino acids were added as described below.

Loading of 2-Chlorotrityl resin
Fmoc-Xaa (10 mmol) was added to a flame dried round bottom flask and dried in a vacuum dessicator with phosphorous pentoxide overnight. 50mL of dry DCM was cannula transferred into the flask followed by 2.5 mL of DIPEA transferred via syringe. After sonication for 10 min, 5 g of 2-chlorotrityl resin was added under a stream of N_2 and allowed to shake for 4 h. Resin was capped with a 15 mL solution of 1:2:17 MeOH:DIPEA:DMF (3 x 15 min). Resin was washed with DMF (3 x 15 mL) followed by DCM (3 x 15 mL). Loading value was calculated by mass increase of dried, loaded resin.

Microwave Reaction Conditions
Microwave assisted reactions were done in a CEM Discover microwave reactor with an open reaction vessel. Reactions were heated to 50°C with 12 watts of power while temperature was monitored with an IR probe from within the reaction vessel.

Amino Acid Coupling under Microwave Condtions
A solution of 4 eq of Fmoc-Xaa, 4 eq of HBTU, and 4 eq of HOAT, and 6 eq of DIPEA in DMF was allowed to pre-react for 5 min. This solution was added to the deprotected peptide on-resin and allowed to react for 10 min at 50°C with microwave heating. The solution was drained and

resin was washed 3 times with DMF (3 x 3 mL) and DCM (3 x 3 mL). The reaction was monitored by LCMS and repeated until starting material was no longer observed.

Amino Acid Coupling onto N-Methylated Amino Acids under Microwave Conditions
A solution of 4 eq of Fmoc-Xaa, 4 eq of HATU, and 6 eq of DIPEA in DMF was allowed to pre-react for 5 min. This solution was added to the deprotected peptide on-resin and allowed to react for 30 min at 50°C under microwave heating. The solution was drained and resin was washed DMF (3 x 3 mL) and DCM (3 x 3 mL). The reaction was monitored by LCMS and repeated until starting material was no longer observed.

Removal of the *N*-Fmoc Protecting Group Under Microwave Conditions
A solution of 2% piperidine and 2% DBU in DMF was added to the resin. The reaction was allowed to react for 5 min at 50°C under microwave heating then drained. The resin was washed with DMF (3 x 3 mL) and DCM (3 x 3 mL).

Peptide Cleavage
Complete linear peptides were cleaved off resin in 5 resin volumes of 2.5% TFA in DCM for 4 min three times with a DCM wash equivalent to 5 resin volumes in between each step. Solvent was removed under N_2 followed by dissolution in acetone or DCM and evaporation under reduced pressure. Residual TFA was removed *in vacuo* overnight.

Cyclization with COMU
Linear peptides were dissolved in 20 mL of dry acetonitrile with 4 eq of DIPEA and added dropwise to a solution of (final concentration 1 mg crude peptide per mL) 1:1 THF/ACN containing 2 eq of COMU. Reactions were stirred from 0.5 to 24 h until complete cyclization was achieved as monitored by LCMS. The reaction was reduced *in vacuo* for purification via HPLC.

Purification of Peptides
COMU by-products were removed after solution phase cyclization on a Biotage Isolera Prime system equipped with a KP-C18-HS 12g column eluting with H2O/Acetonitrile modified with 0.1% TFA. Peptides were further purified when necessary on a Waters mass-directed prep system equipped with an XBridge BEH130 5µm 19x150 C18 column eluting with H_2O/Acetonitrile modified with 0.1% formic acid.

General Analytical Procedures

HPLC-MS Analysis for reaction and purity assessment:

Purity of individual peptides was performed using HPLC-MS(Waters AutoPure system equipped with a Micromass ZQ200 mass spectrometer). ~1mg/mL samples were prepared in MeOH and 10µL was injected and run using a gradient of H20 → ACN on a Thermo AccuCore C18 column (4.6x50mm). Starting gradient conditions were varied depending on the lipophilicity of the analyte to elute the compound in the middle of the run. Since the compounds ware being assayed for permeability, absolute purity was not crucial as long as the presence of isobaric contaminants were not present that would convolute the assay analysis.

Permeability Analysis of Cyclic Peptide Mixtures by PAMPA:

A 96-well donor plate with 0.45 µ hydrophobic Immobilon-P membrane supports (Millipore) and a 96-well Teflon acceptor plate were used in the PAMPA permeability test. The acceptor plate was prepared by adding 300 µL of 5% DMSO in pH = 7.4 phosphate-buffered saline (PBS) to each well. Donor well solutions of the cyclic peptide libraries were prepared by diluting 10 µL of the DMSO stock solutions prepared above to a final volume of 200 µL with PBS (pH 7.4). The suspensions were centrifuged to remove any insoluble material. A 1% (w/v) solution of lecithin in dodecane was prepared and sonicated before use. 5 µL of the dodecane / lecithin solution was carefully applied to the membrane supports in the wells of the donor plate, with care being taken to not touch the pipet tip to the membrane. Without allowing this solution to evaporate, 150 µL of the peptide solutions were added to the donor wells. The donor plate was then placed on top of the acceptor plate so that the artificial membrane was in contact with the buffer solution below. A lid was placed on the donor well, and the system was covered with a glass evaporating dish and left overnight (~18 h) at room temperature. A wet paper towel was placed on the inside of the chamber to prevent evaporation.

After ~18hr (exact time recorded and used for subsequent calculateions) the donor and acceptor plate were separated and 100µL of each well (donor and acceptor) were transferred to a 96 well plate for quantification. The plate was immediately sealed with a piercable plate cover to prevent any sample evaporation. These solutions were analyzed by UPLC on a C18 Thermo HyperSil column (2.1x30mm, 3µm) with an accurate mass MS detector (Thermo Scientific Orbitrap VelosPro) using +/- 0.02 AMU windows for integration. Permeability (%T) was quantified as the ratio of analyte areas in the acceptor well divided by a theoretical equilibrium ratio based on amounts of combined analyte found in the donor and acceptor wells as follows:

$$\%T = \frac{R_A}{\left(\frac{R_A V_A + R_D V_D}{V_A + V_D}\right)}$$

Where RA and RD are the integrations analyte in the acceptor and donor wells, respectively, and VA and VD are the volumes of the acceptor (300 µL) and donor (150 µL) wells, respectively.

Permeation rates (P_{app}) were calculated from %T by the following equations:

$$P_{app} = -\frac{V_A V_D}{V_A+V_D} \times \frac{\ln(1-\%T)}{At}$$

Where *A* is the active surface area of the filter support (0.24 cm^2), and *t* is time of the incubation period in seconds.

Permeability Analysis of Pure Cyclic Peptides by PAMPA

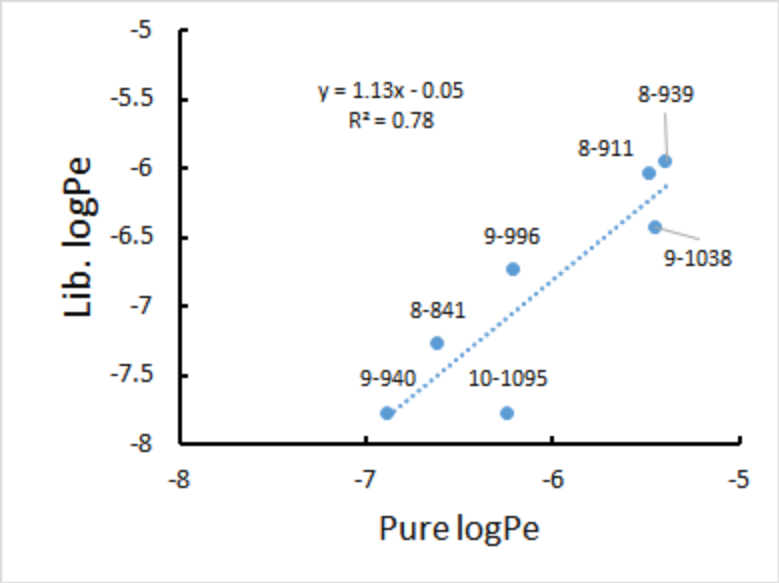
Pure compounds were analyzed via PAMPA as outlined above for mixtures. Donor-well solutions were prepared at 10µM.

Permeability Analysis of Pure Cyclic Peptides by RRCK:

Cell permeability was determined using RRCK cells (Pfizer, Inc. Groton, CT)[1]. RRCK cells were generated in house as a subclone of Madin-Darby Canine Kidney wild-type (MDCK-WT) cells that displayed low expression of endogenous P-glycoprotein (~ 1–2% of MDCK-WT cells, based on mRNA level). Cells were cultured in minimal essential medium α with supplements and passaged when 70–80% confluent. Cell monolayer flux studies were conducted five days after seeding in 24-well transwell inserts, 1.0 µm pore size, (Becton Dickinson, Cowley, UK) at 4.2 x 10^4 cells/cm^2. Donor and acceptor solutions were prepared from HBSS containing HEPES at 20 mM, pH 7.4. Stock solutions of test compounds, prepared at 10 mM in DMSO, were used to prepare donor solutions of 2 µM compound in 0.05% (v/v) DMSO. Apparent permeability (P_{app}) was determined in apical to basolateral (AB) direction in triplicate by incubating with compound for 2 h at 37 °C. Samples of the medium were analyzed by liquid-chromatography tandem mass spectrometry (LC-MS/MS). P_{app} values were calculated according to the equation P_{app} = (Q/t) x 1/C_0 x 1/A, where Q is the sampled concentration in the acceptor compartment, t is the incubation time, C_0 is the initial concentration in the donor compartment and A is the area of the filter of the transwell plate.

Differences in Pure vs Mixture Values:

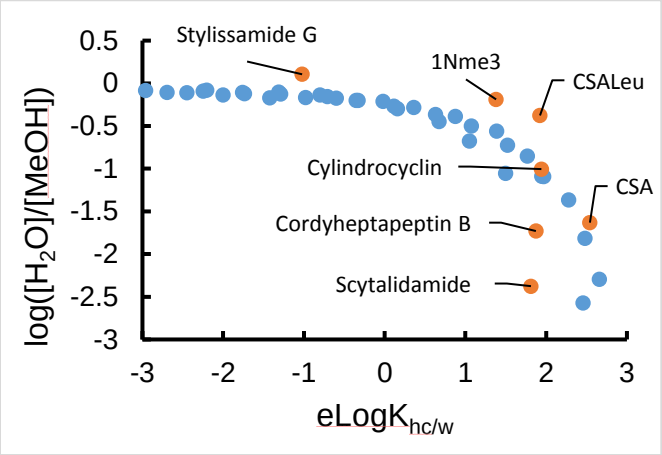
It should be noted that the masses (m/z) used for analysis of the mixtures represent the average for all possible isobaric combinations. The pure compounds however, represent one possible combination. As such they are not directly comparable to the averaged mixtures but the overall trends still hold as demonstrated by SI Figure 1.



SI Figure 1 logSol vs Log $K_{hc/w}$

Filtration Solubility Assay Procedure:

Relative thermodynamic solubilities were assayed by comparing a filtered aqueous solution to a solution prepared from the same stock solution dissolved in MeOH. Aqueous solutions of each sublibrary were prepared the night before by mixing 10 µL of DMSO stock into 1 mL of PBS (pH 7.4) and allowing to incubate for 16 h. Solutions were then centrifuged at 16,000 x g for 5 min. The supernatant was then taken up in a 1 mL syringe and filtered using a 7 mm^2 nylon filter with a pore size of 0.2 µ (Nalgene cat. no. 179-0020). The first half of the filtrate was discarded so as to passivate the filter with analyte. The remainder of the solution was dispensed in to HPLC vial for analysis. MeOH solutions of each sublibrary were prepared immediately before analysis by mixing 10 µL of DMSO stock into 1 mL of HPLC grade MeOH. These solutions were analyzed by UPLC on a C18 Thermo HyperSil column (2.1x30mm, 3µM) with an accurate mass MS detector (Thermo Scientific Orbitrap VelosPro) using +/- 0.02 AMU windows for integration.



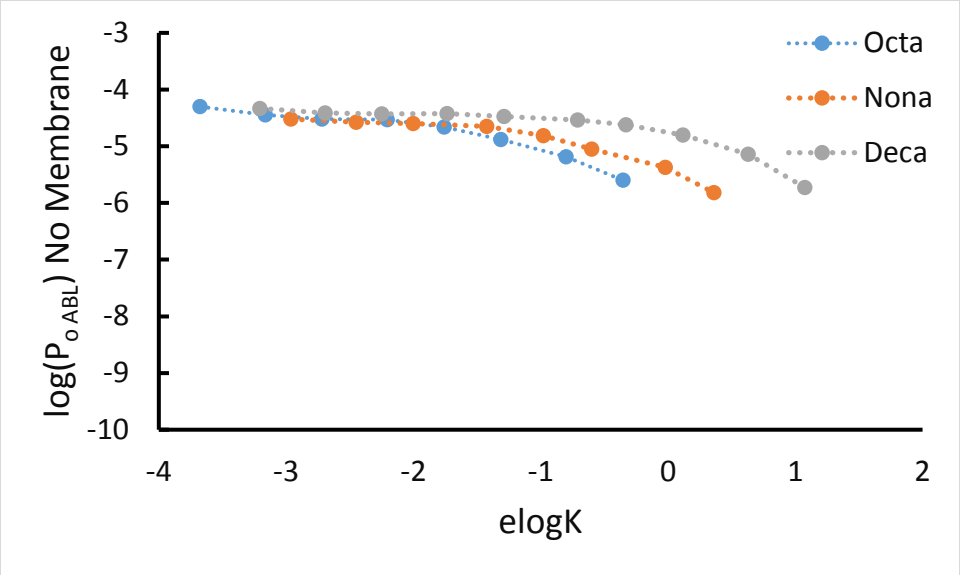
SI Figure 2 logSol vs Log $K_{hc/w}$

Shake Flask Log$K_{hc/w}$ Analysis:

Reagent grade 1,9-decadiene was purchased from Sigma Aldrich and used as is. Prior to use for the assay, 10 mL 1,9-decadiene was saturated with H_2O by shaking with an equal volume of PBS (pH 7.4) and allowing the emulsion to completely separate. 1 µL of a 100mg/mL DMSO stock in the case of the library, or 1 µL of a 10 mM DMSO stock of the pure compounds was added to a 1.5 mL centrifuge tube and then 500 µL of each solvent (1,9-decadiene and H_2O) was added and agitated by vortexing (1 min) and sonicating (30 min). The emulsions were then spun down in a centrifuge for 5 min at 16,000 x g and allowed to sit overnight. 100 µL of each phase was carefully removed with special care taken not to allow contamination from the pipette tip. These solutions were then evaporated overnight under a gentle stream of N_2 and re-suspended in 100 µL of MeOH for quantification via UPLC/MS. These solutions were analyzed by UPLC on a C18 Thermo HyperSil column (2.1 x 30 mm, 3 µ) with an accurate mass MS detector (Thermo Scientific Orbitrap VelosPro) using +/- 0.02 AMU windows for integration. The intensities were then divided (1,9-decadiene/H_2O) to afford the $K_{hc/w}$ value.

Aqueous Boundary Layer PAMPA

In order to determine the effect of the aqueous boundary layer (ABL) in PAMPA with our compounds the assay was run without the dodecane/lipid mixture. Instead, 5 µL of DMSO was used to wet the filter of the 96-well filter plate and the apparatus was assembled as previously described. Since compounds will permeate at their rate of diffusion the assay time was shortened to 2 h to avoid reaching equilibrium. The permeabilities were calculated using the standard P_{app} equation described above and then adjusted using the filtration solubility ratios as previously described to get $P_{o\ ABL}$ values. The values obtained were assumed to be the permeabilities of the compounds either of monomeric species or small (<200-nm) aggregates through the aqueous boundary layer and filter. The downturn at higher lipophilicities is likely due to the presence of small aggregate species which diffuse slower; the P_o's are much greater than those observed in conventional PAMPA suggesting that the effect of the ABL is minimal on our large compounds.



SI-Figure 3. $P_{o\ ABL}$ vs $elogK_{hc/w}$ for the permethylated lipophillicity scanning library.

Membrane Diffusion Calculation:

For the library members (permethyl octa- ,nona- , and deca-peptides) diffusions were calculated accounting for the small effect of the aqueous boundary layer as measured. The equation used was as follows:

$$D_{mem} = \frac{P_o * \partial_{mem}}{K_{hc/w}}$$

The thickness of the membrane, ∂_{mem} , was 0.0125 cm as previously reported

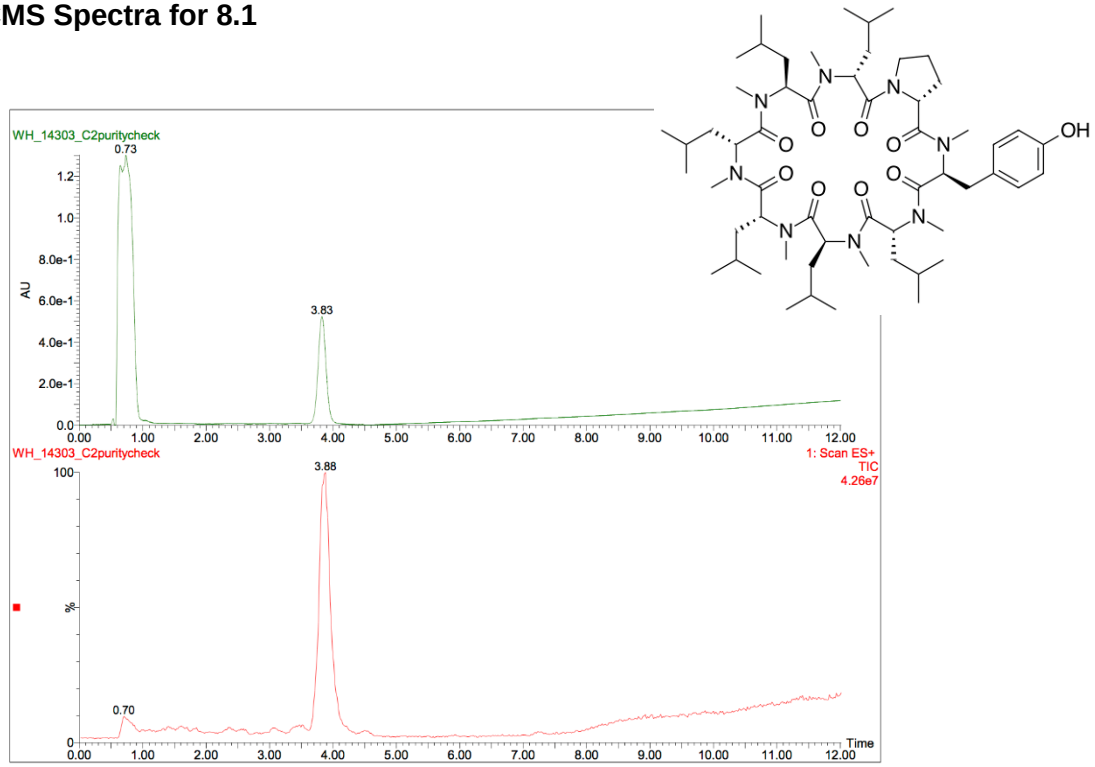
Abbreviations

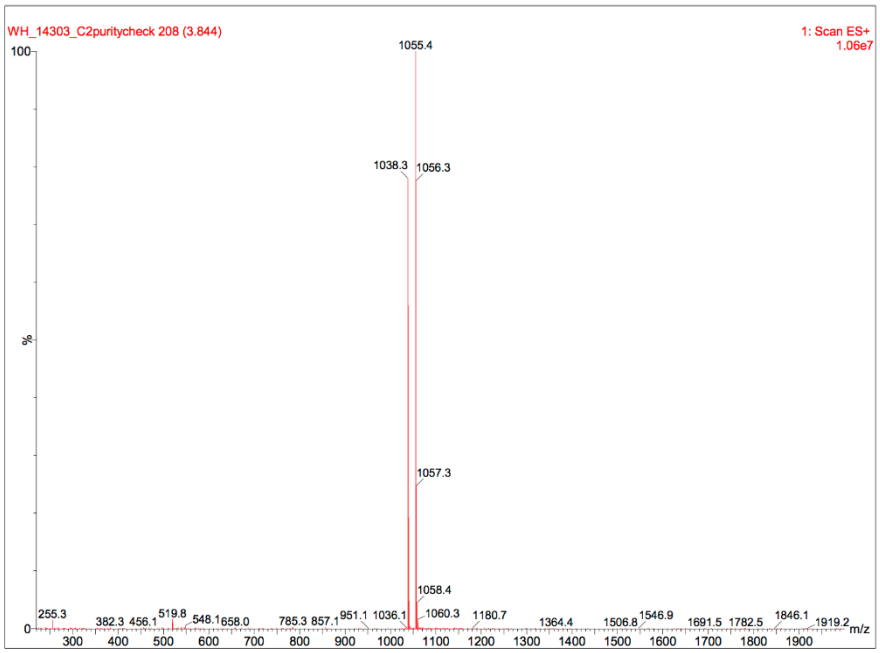
ACN, acetonitrile; COMU, (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate; DBU, 1,8- Diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIAD, diisopropyl azodicarboxylate; DMF, N,N-dimethylformamide; DIC, diisopropylcarbodiimide; DIPEA, diisopropylethylamine, DMSO, dimethylsulfoxide; DVB, divinylbenzene; EtOH, ethanol; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)-uronium hexafluoro-phosphate; HOAt, 1-hydroxy-7-azabenzotriazole; IR, infrared; LCMS, liquid chromatography mass spectrometry; MeOH, methanol; N2, nitrogen; NMP, 1-methyl-2-pyrrolidinone; PTFE, Polytetrafluoroethylene; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; RP, reverse phase; SPPS, solid phase peptide synthesis; *t*-BuOH, *tert*-butoxide; TFA, trifluoroacetic acid; THF, tetrahydrofuran

Characterization of Pure Compounds

Compounds purity was assessed by HPLC-MS as described above.

LCMS Spectra for 8.1





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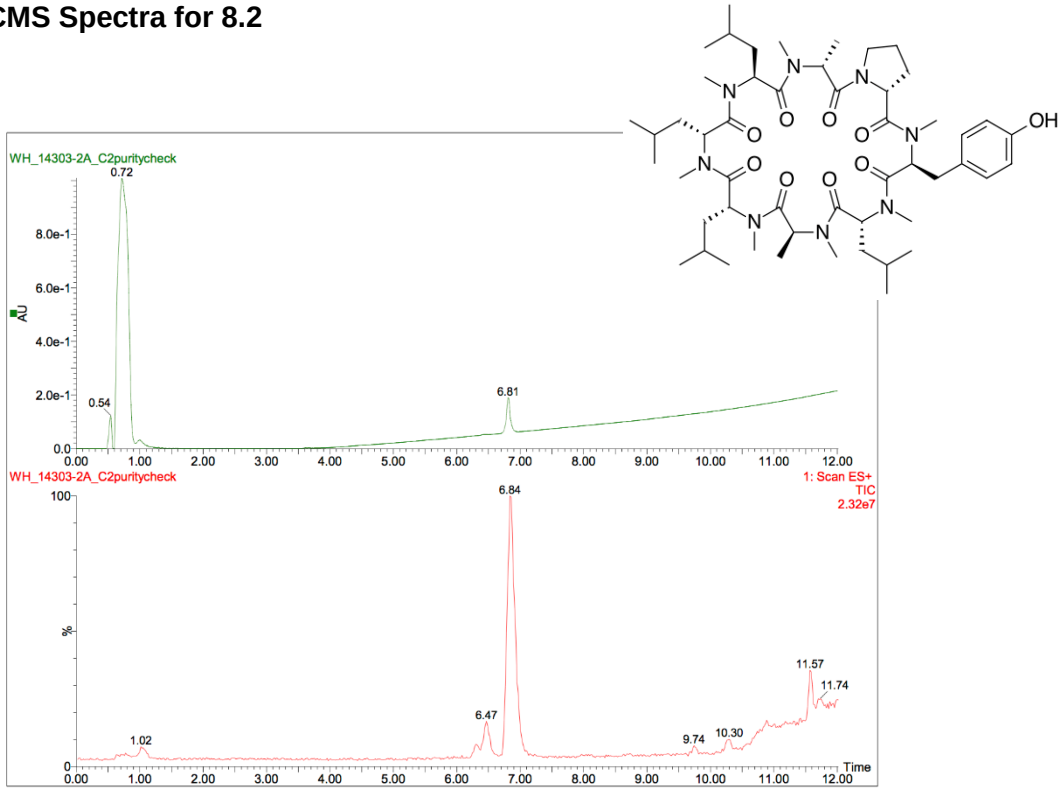
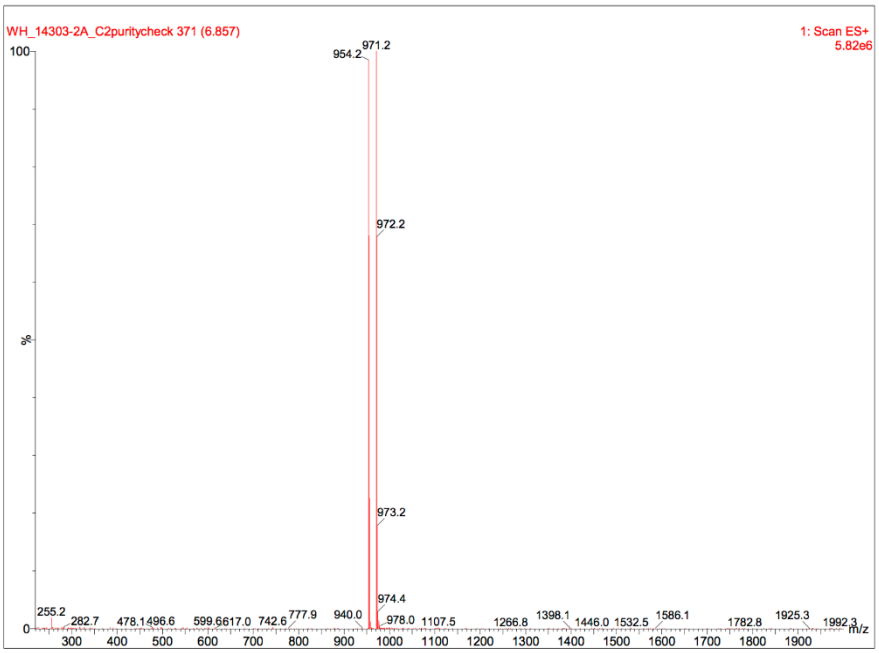
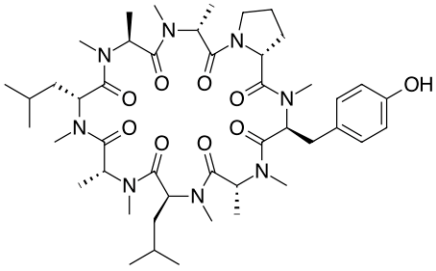
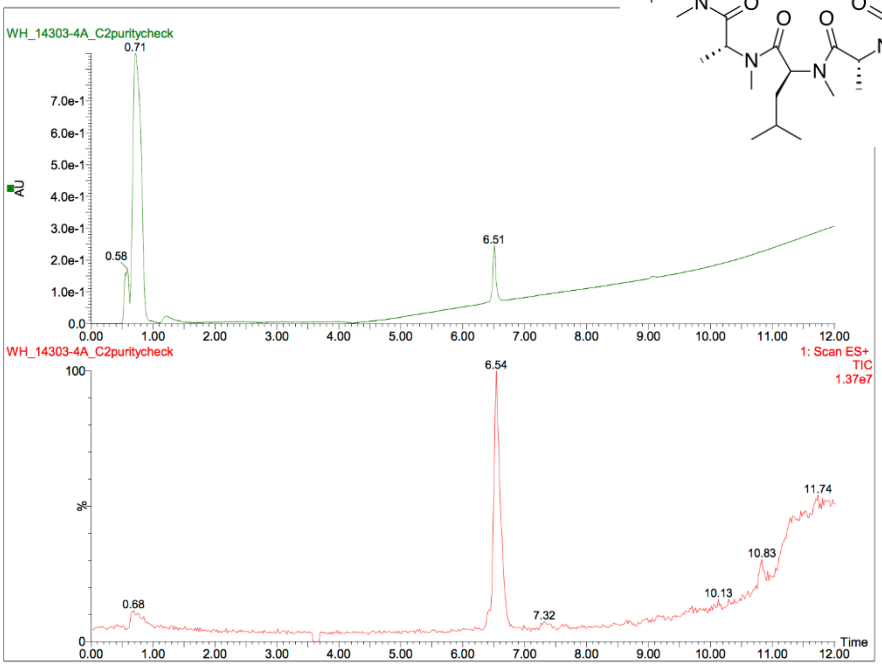
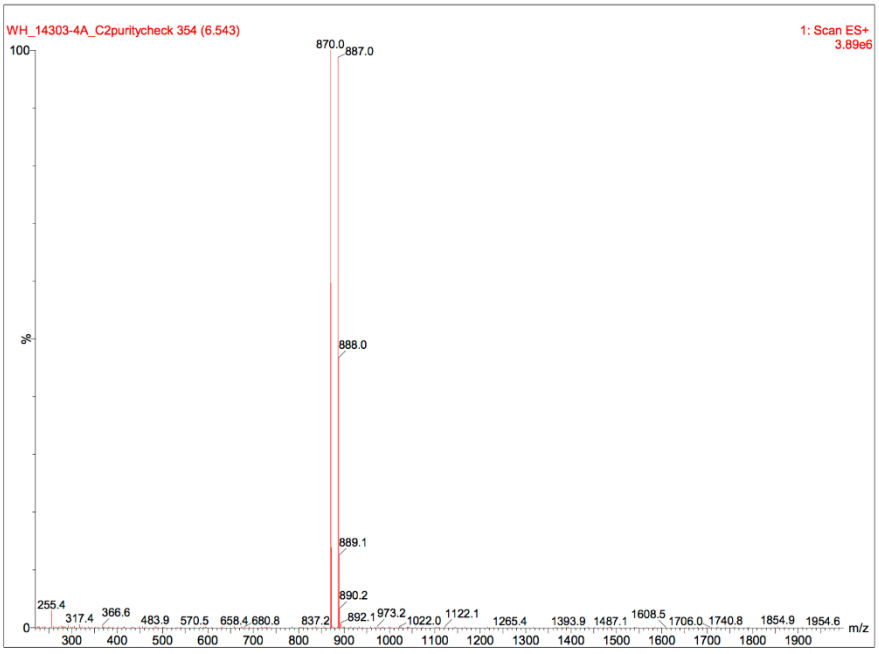




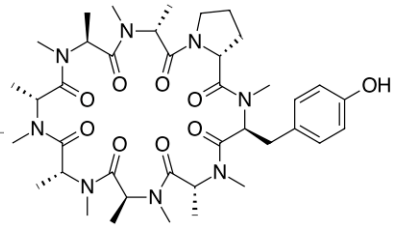
LCMS Spectra for 8.3

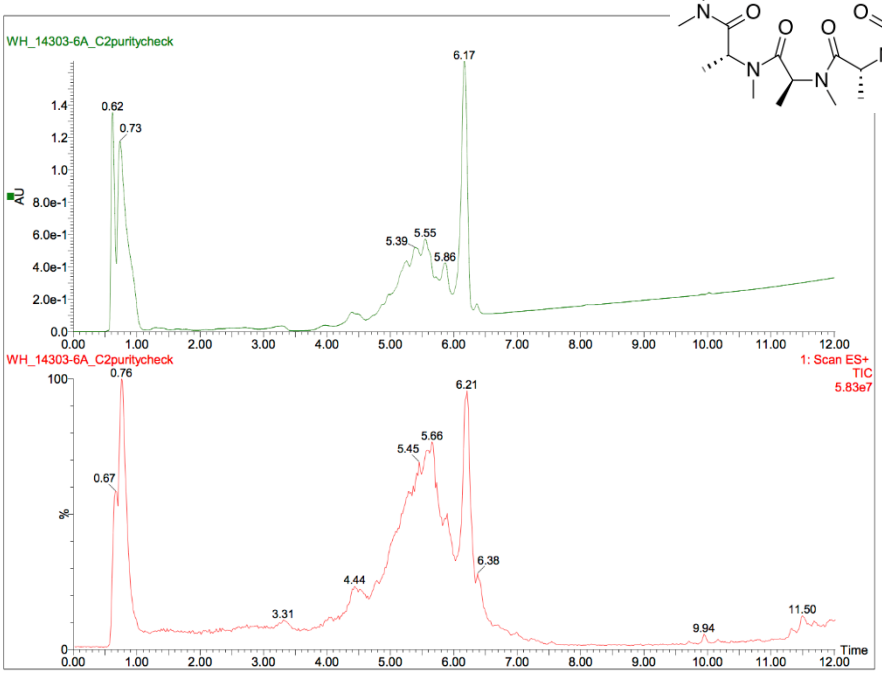


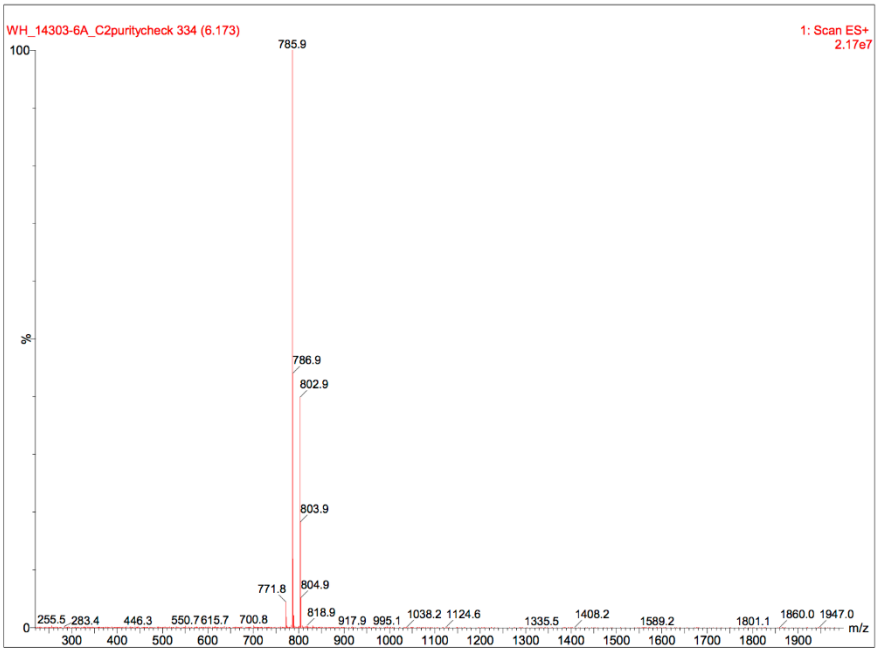




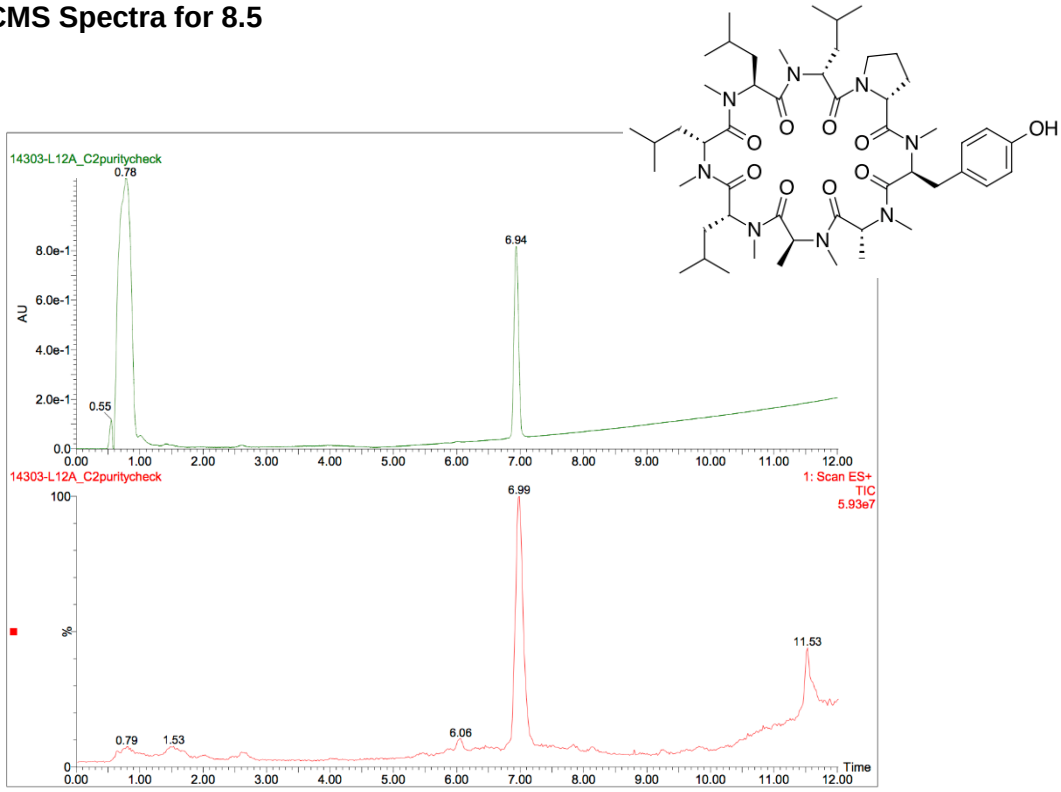
LCMS Spectra for 8.4

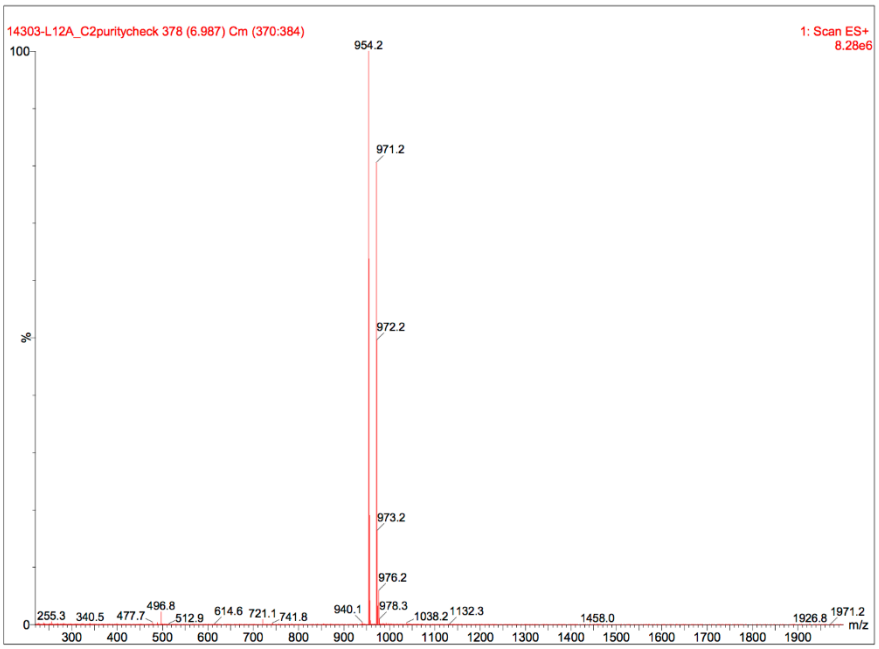




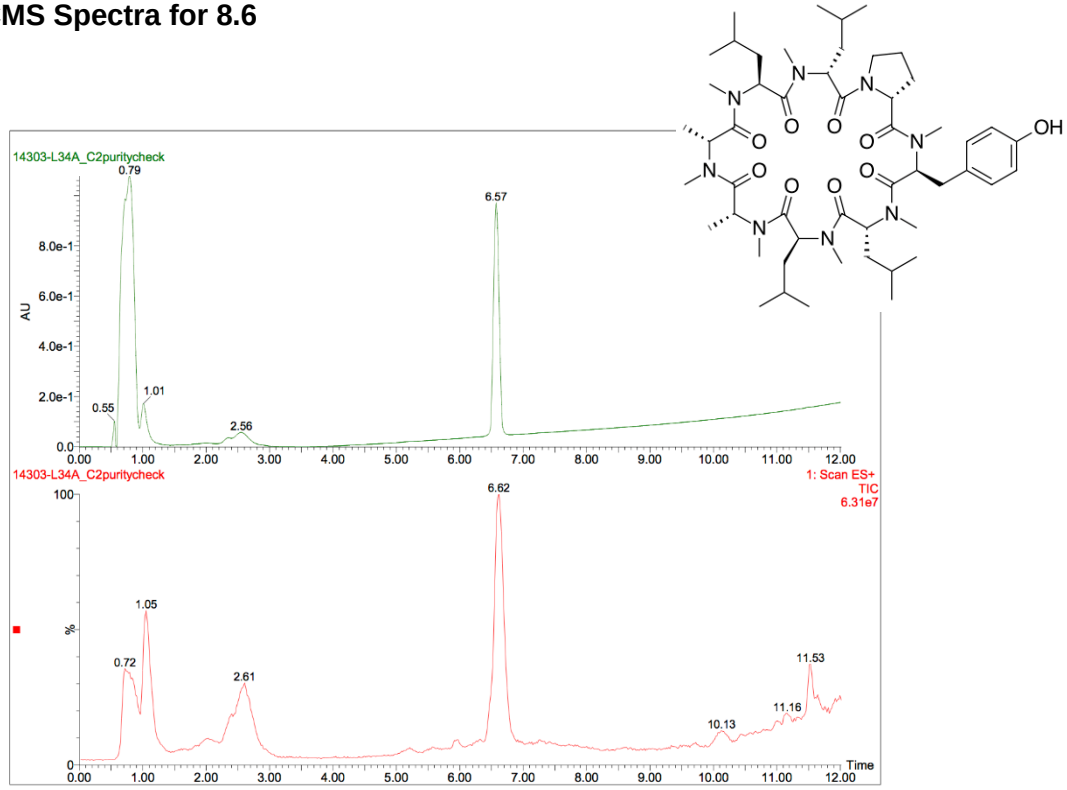


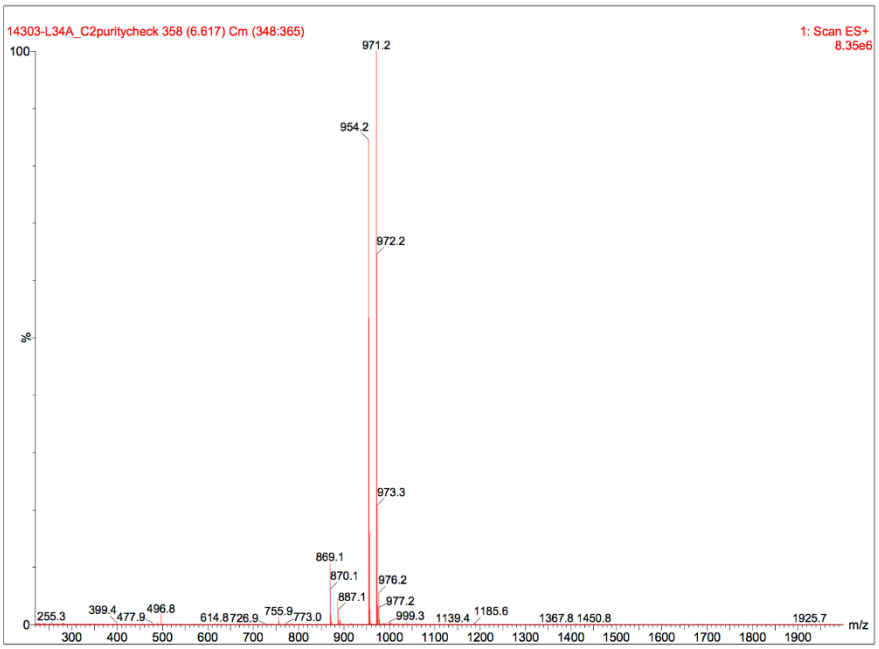
LCMS Spectra for 8.5



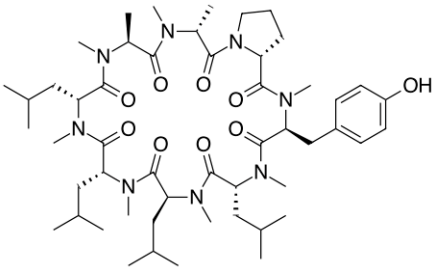


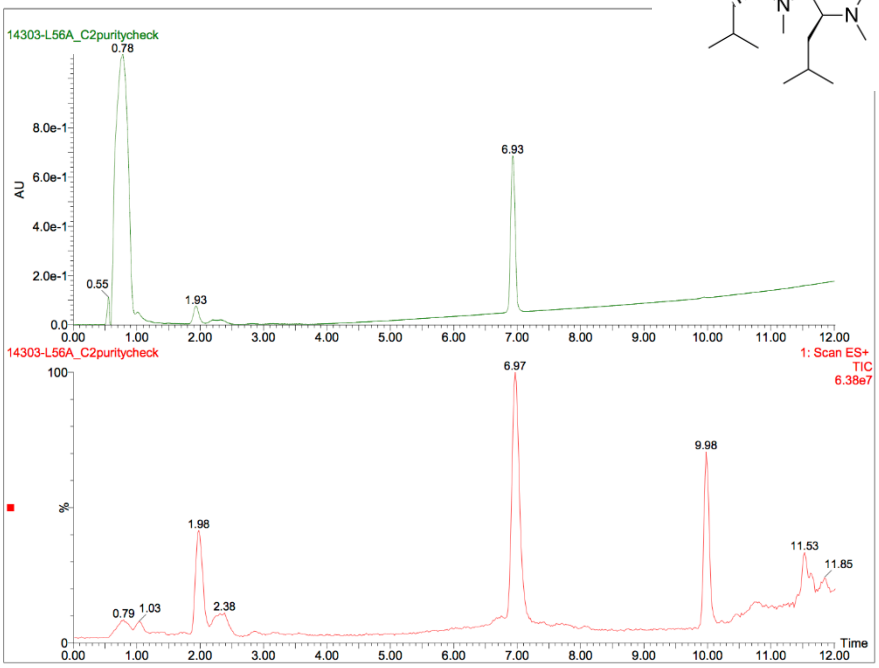
LCMS Spectra for 8.6

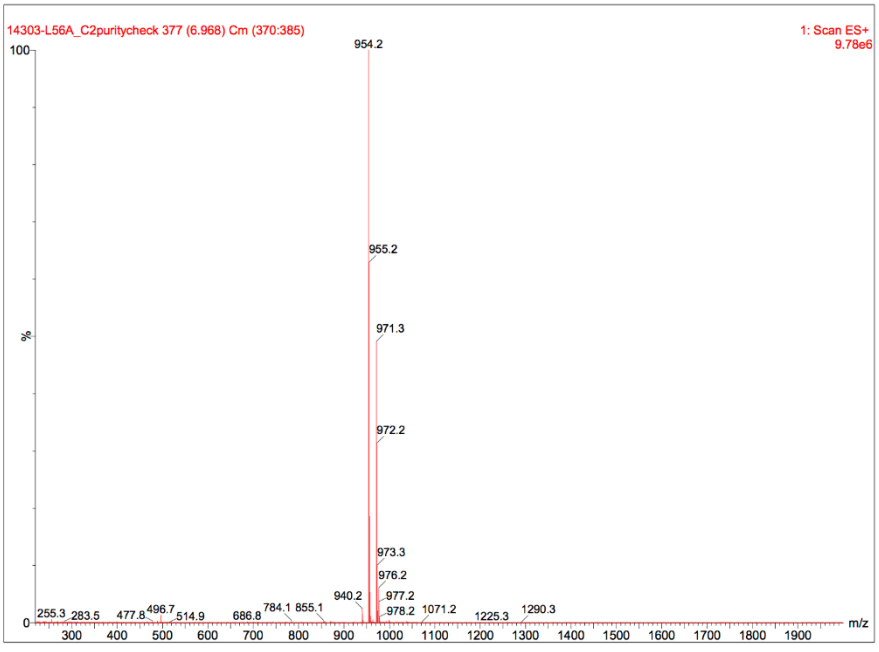




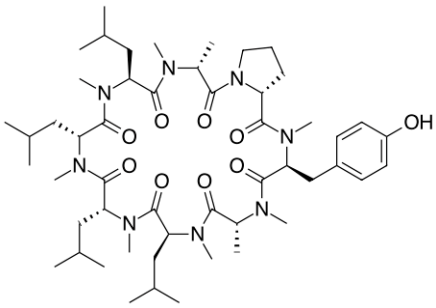
LCMS Spectra for 8.7

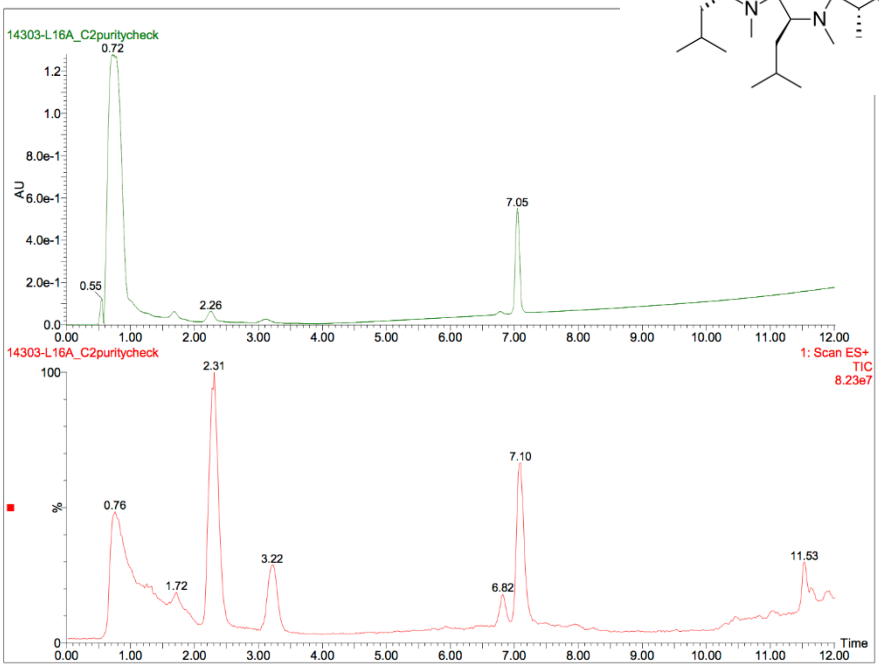


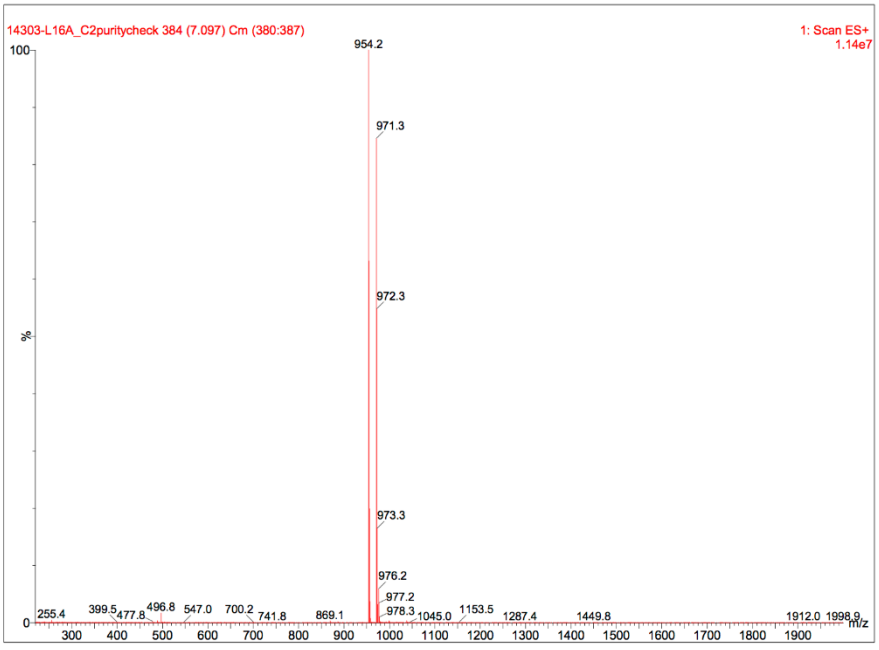




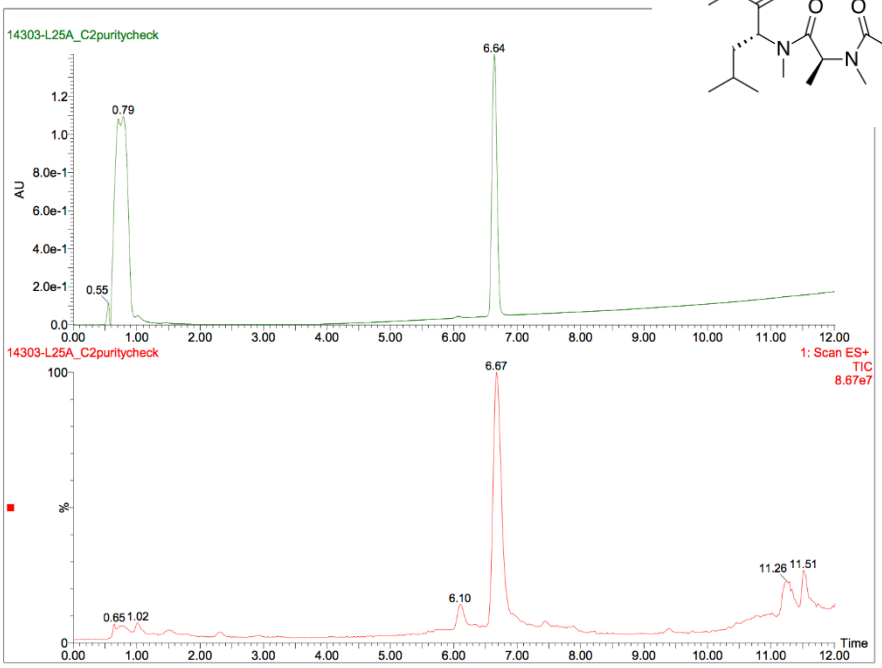
LCMS Spectra for 8.8

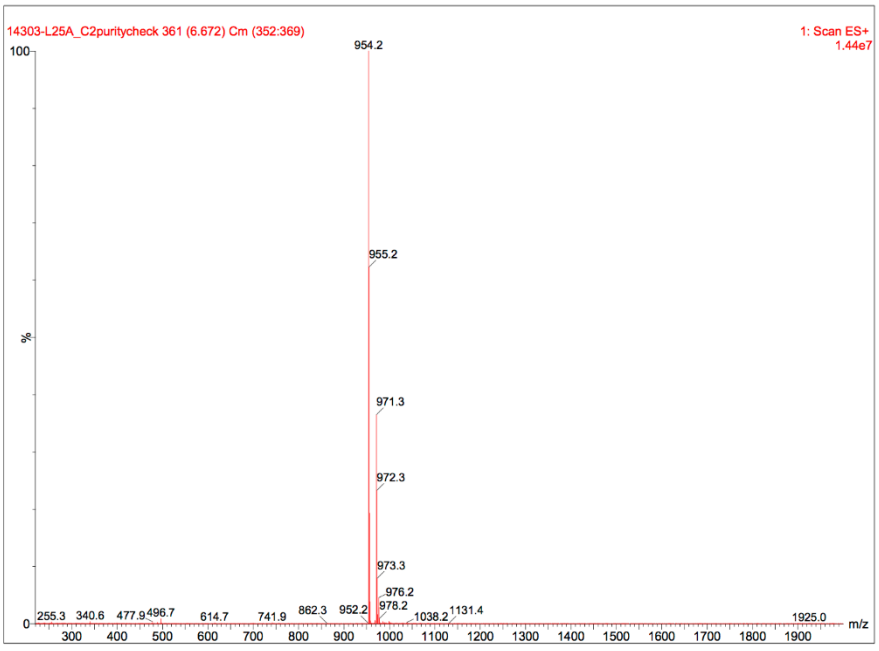




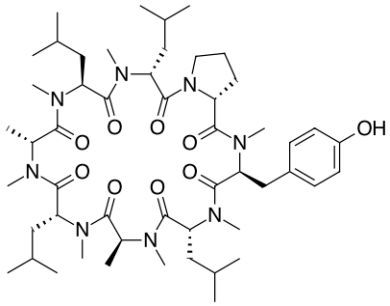


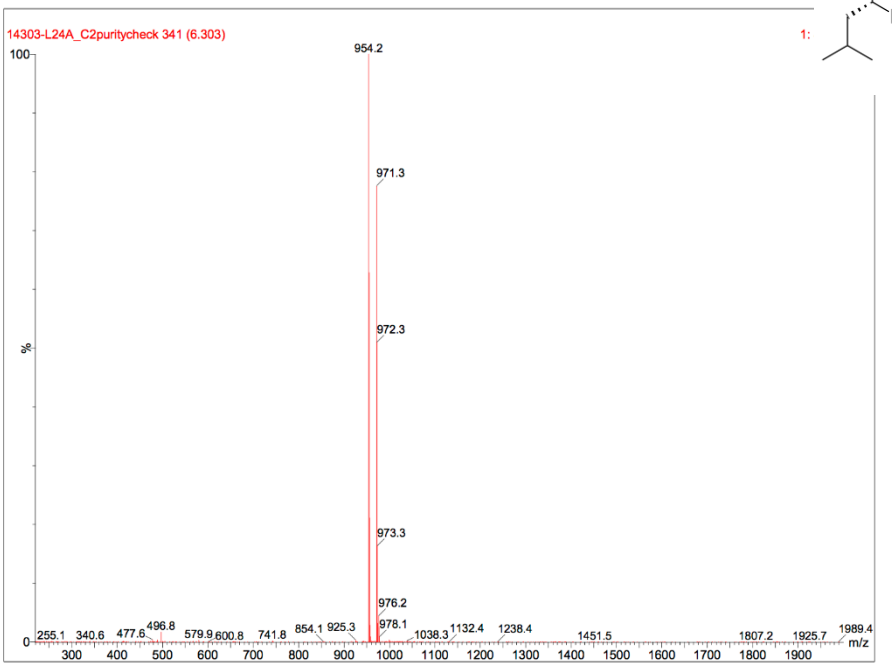
LCMS Spectra for 8.9

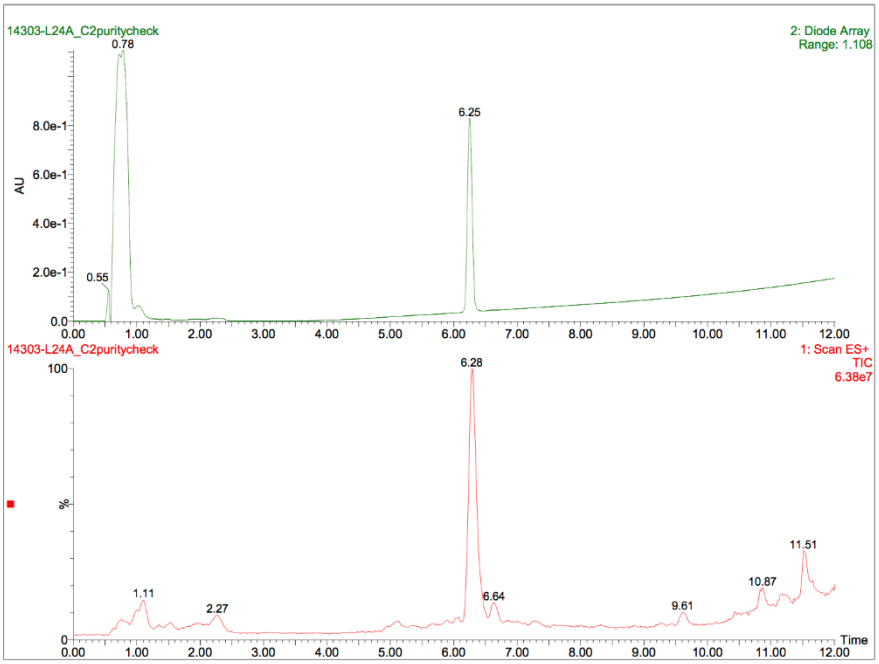




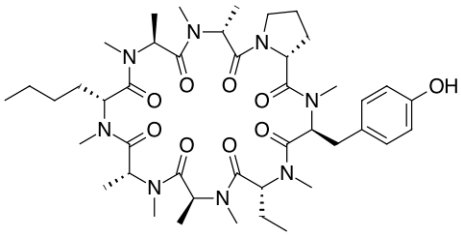
LCMS Spectra for 8.10

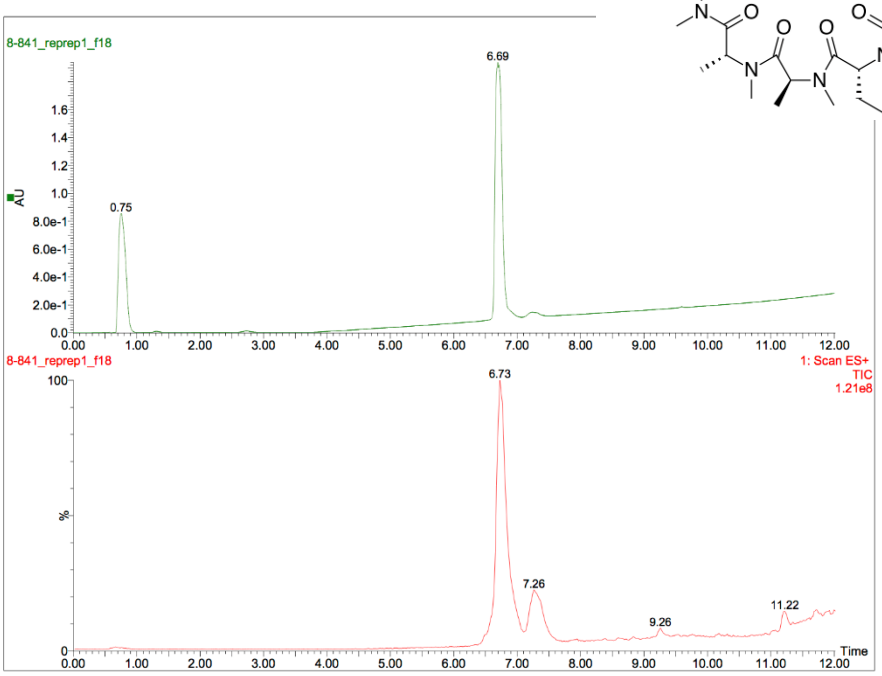




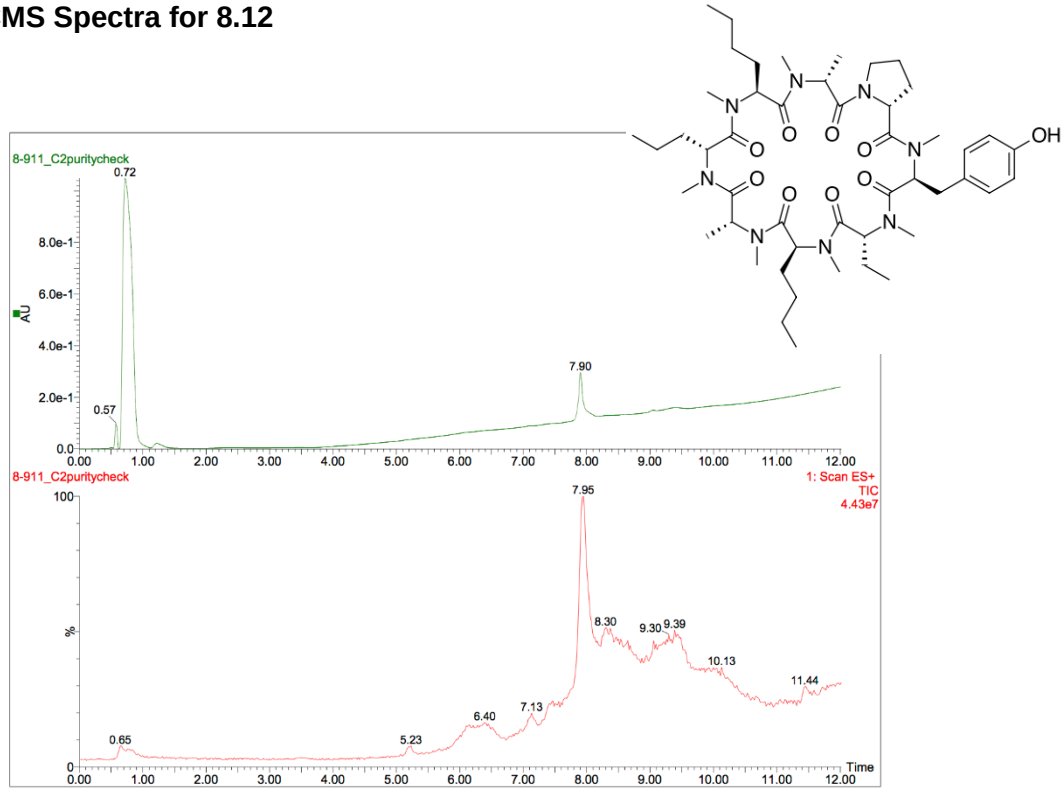


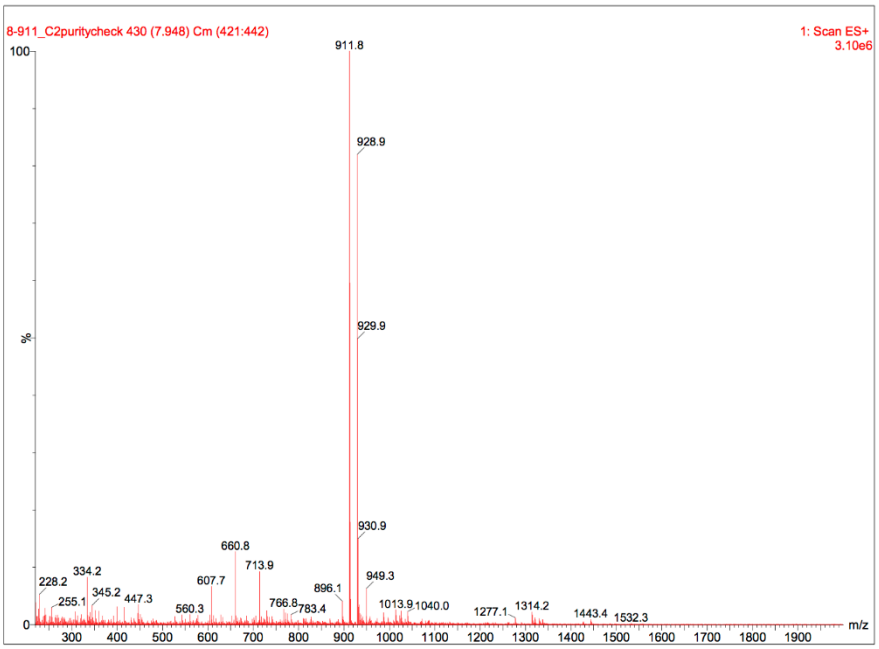
LCMS Spectra for 8.11



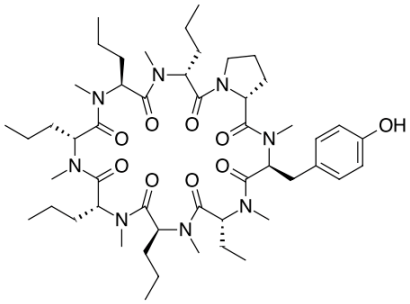


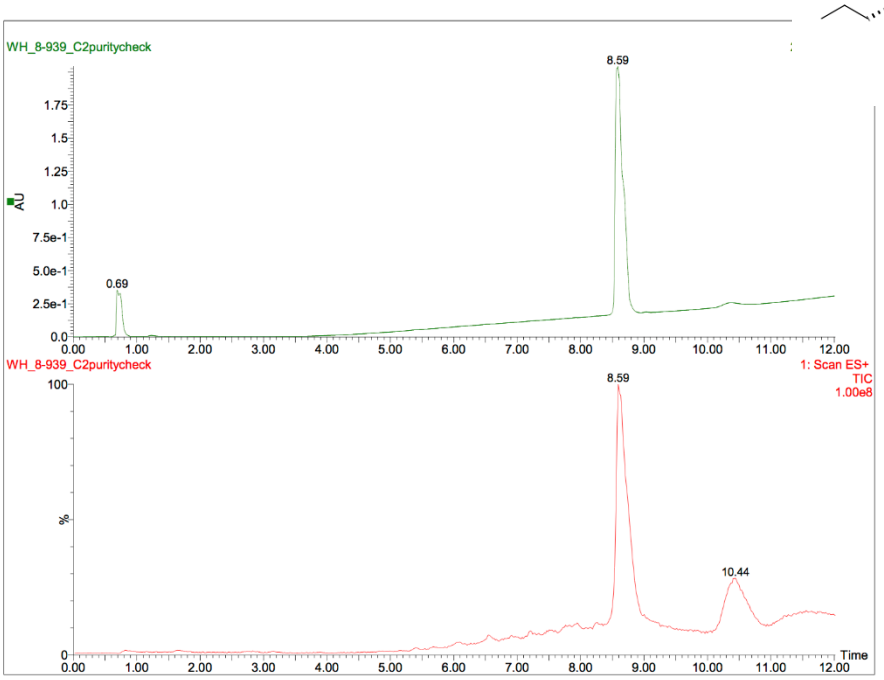
LCMS Spectra for 8.12

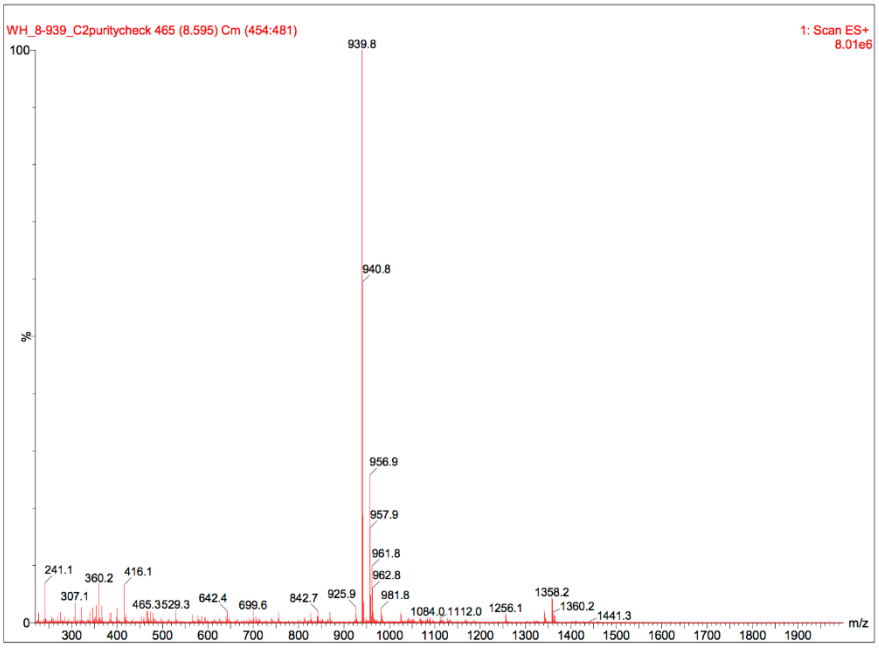




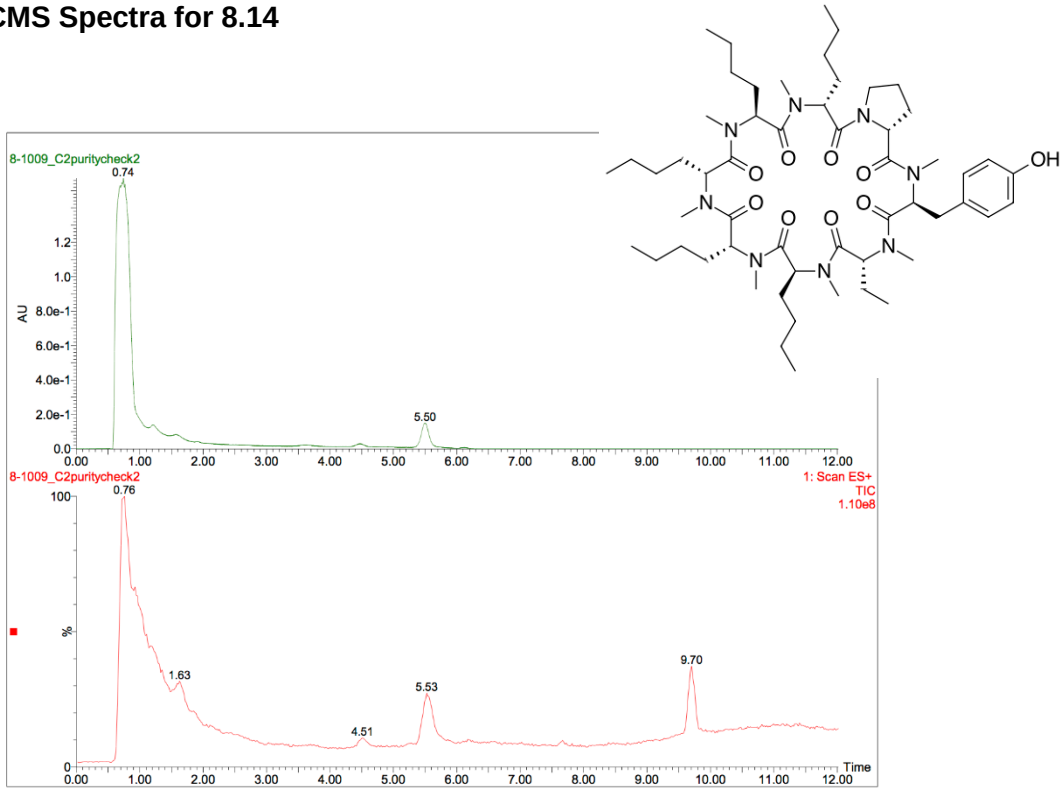
LCMS Spectra for 8.13

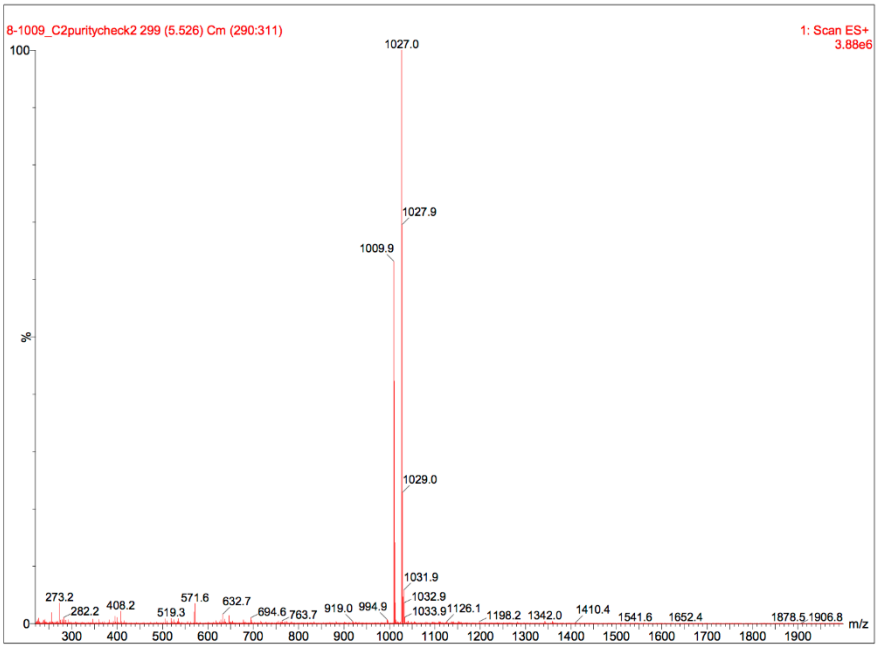




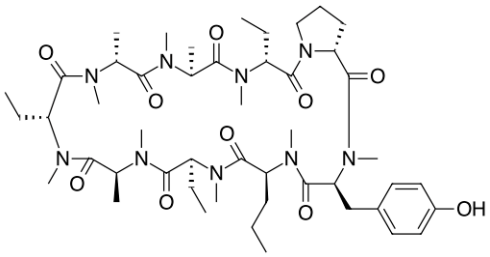


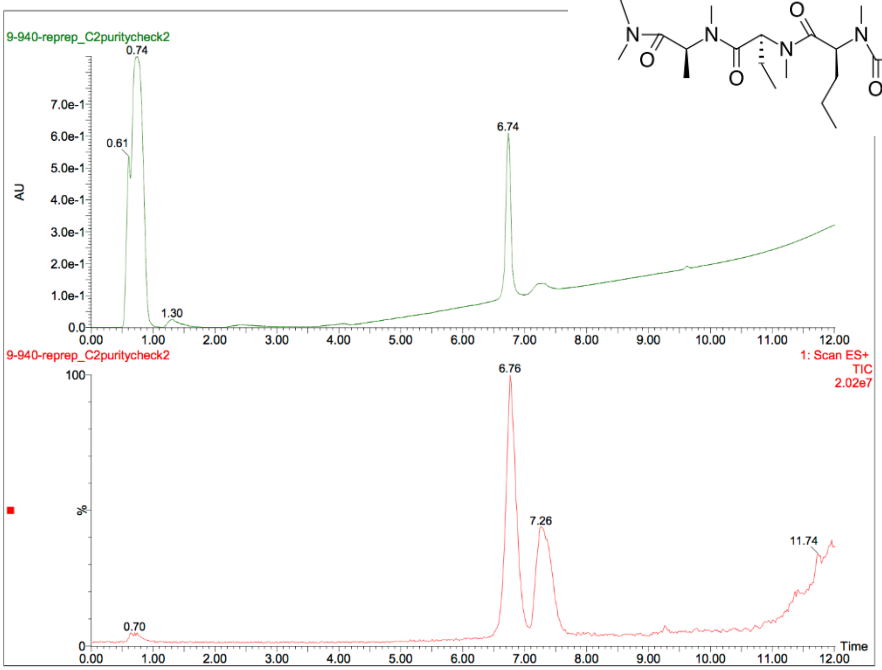
LCMS Spectra for 8.14

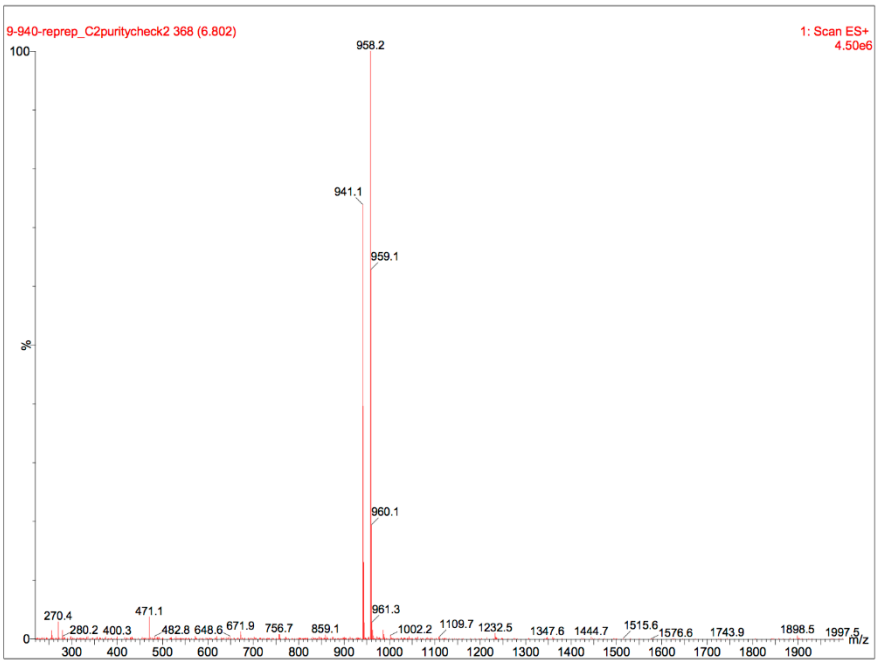




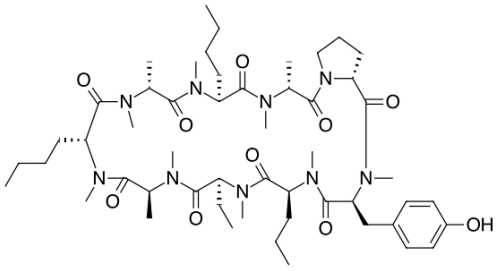
LCMS Spectra for 9.1

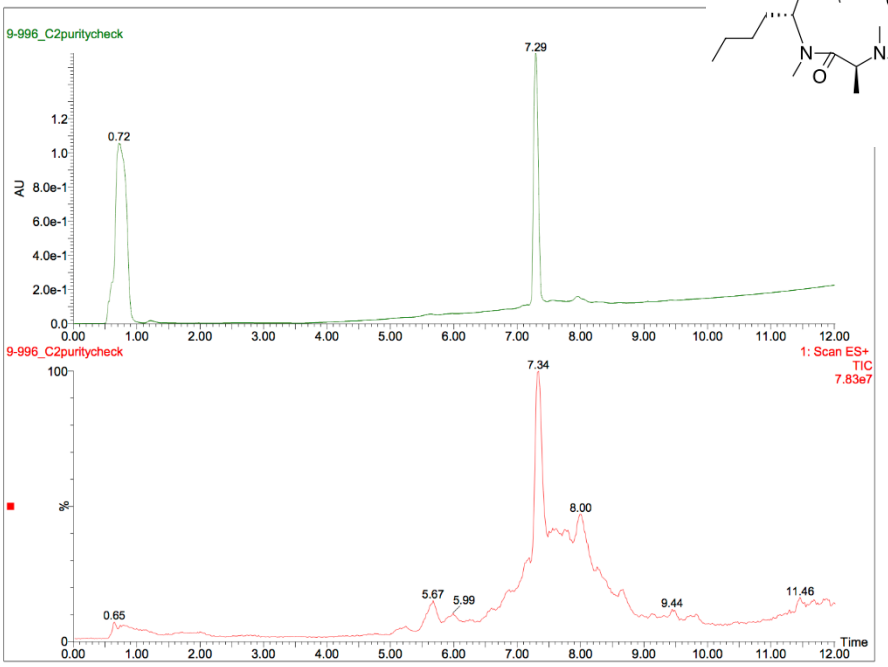


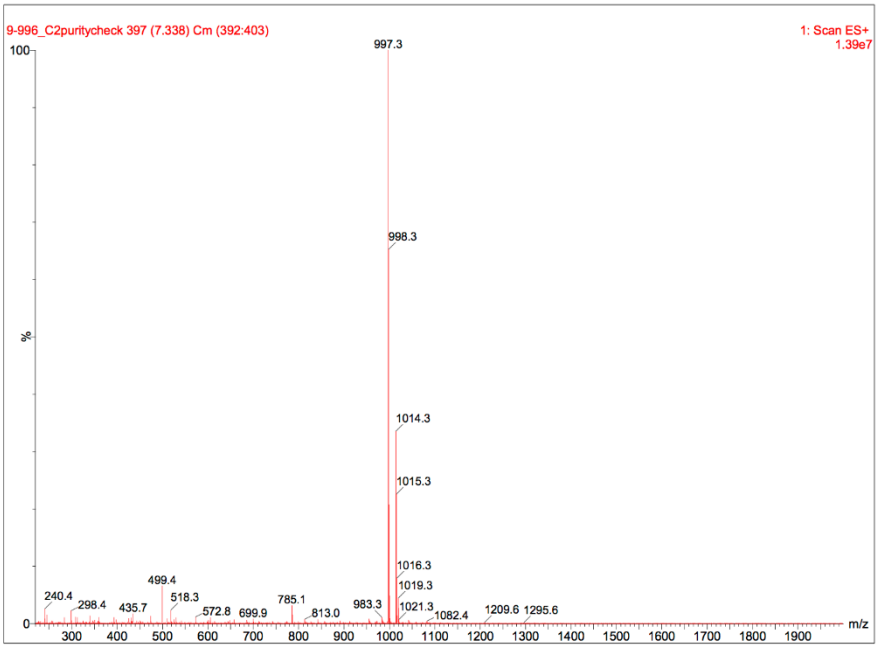




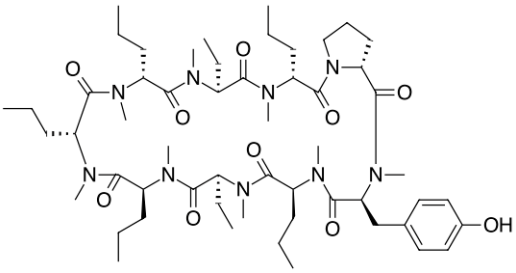
LCMS Spectra for 9.2

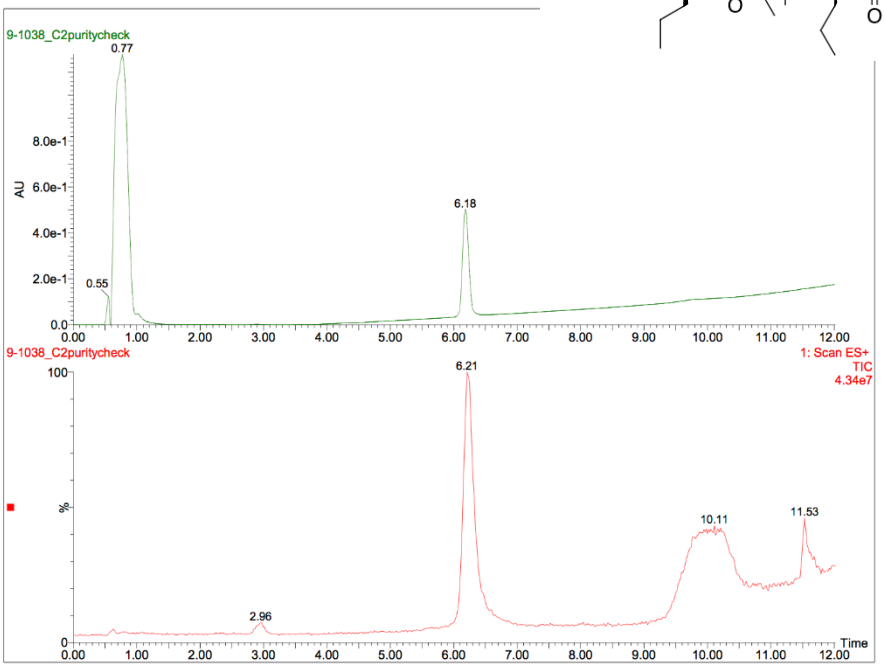


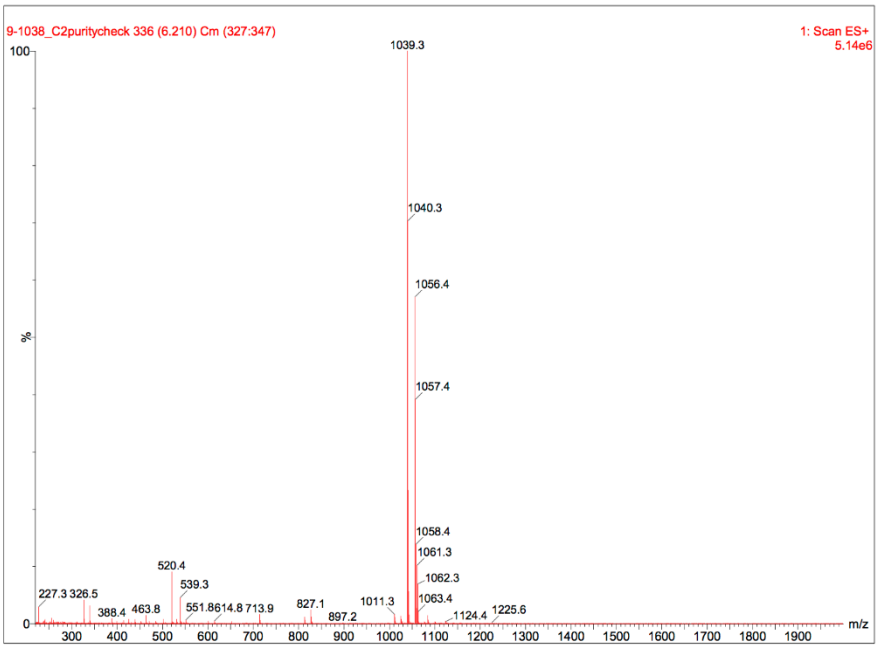




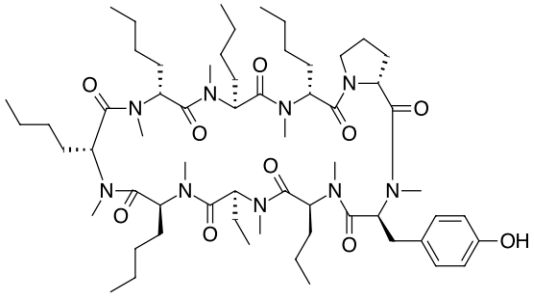
LCMS Spectra for 9.3

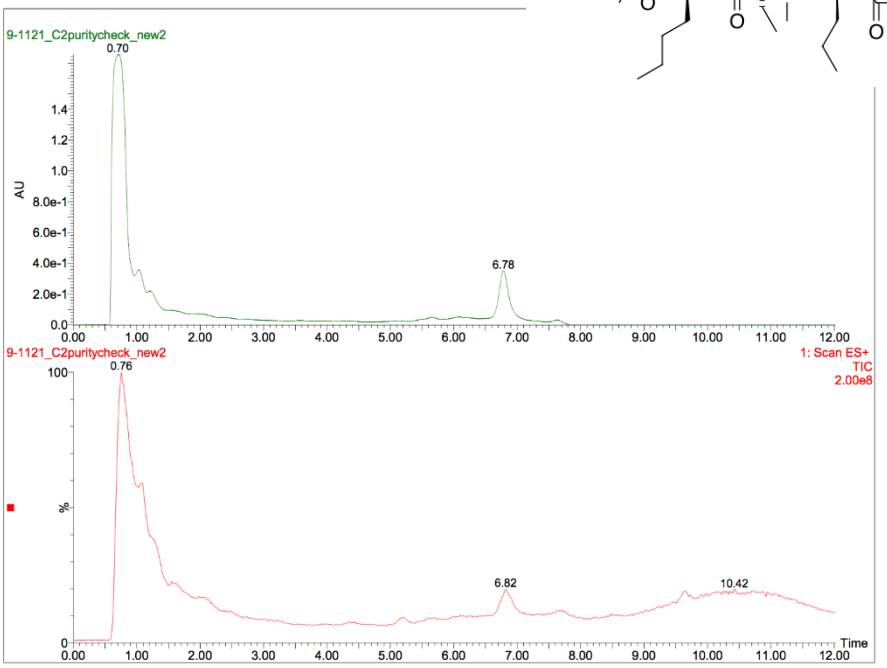


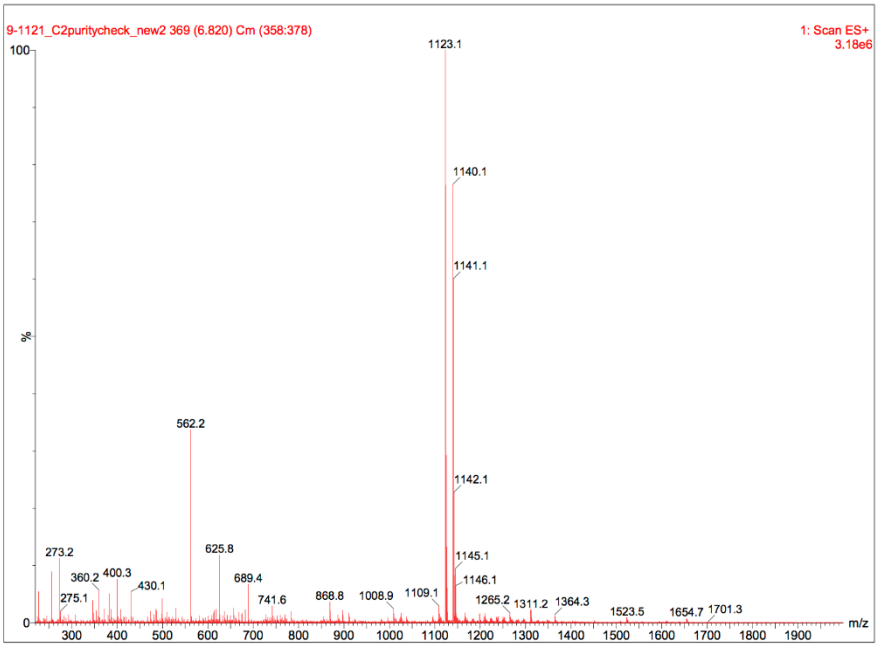




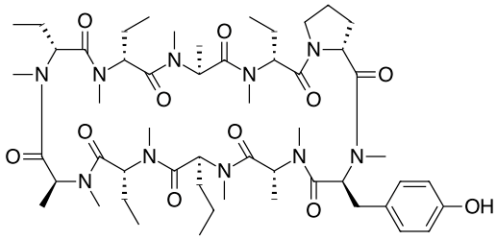
LCMS Spectra for 9.4

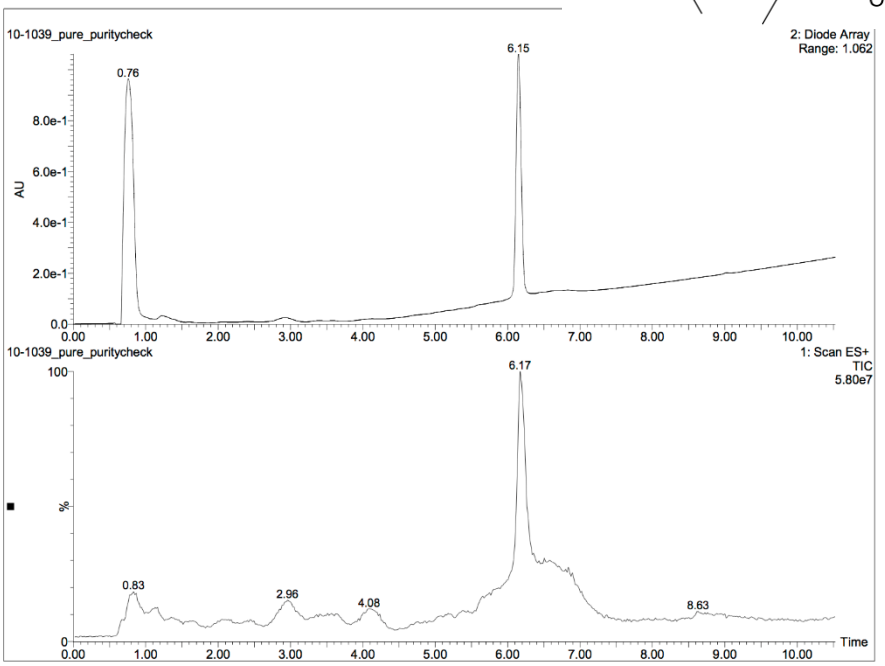


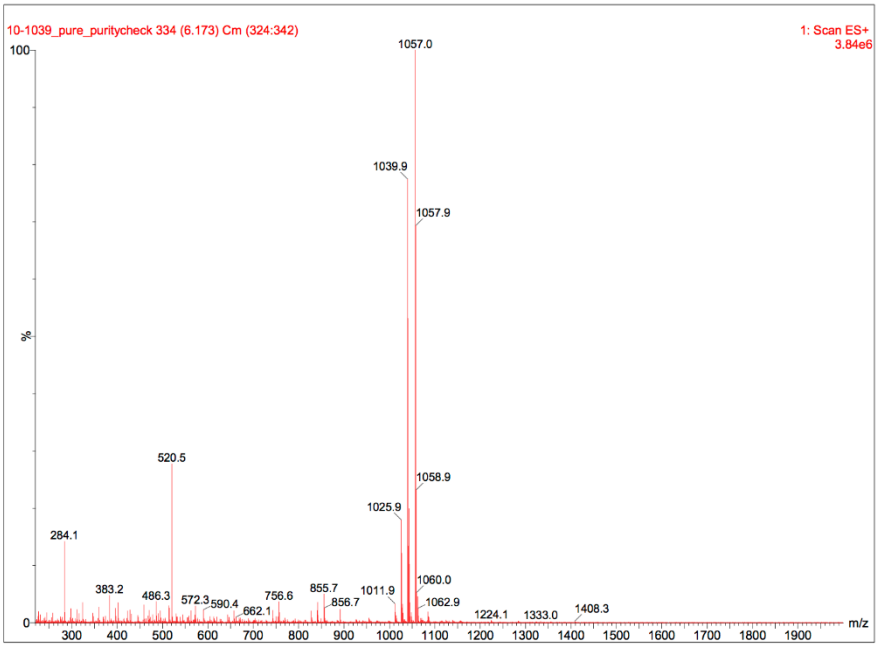




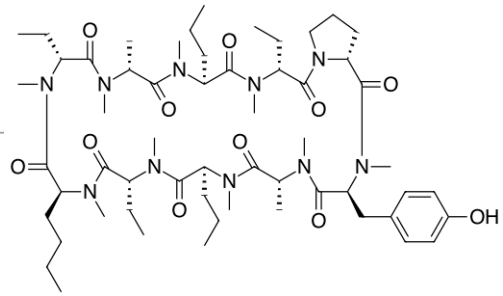
LCMS Spectra for 10.1

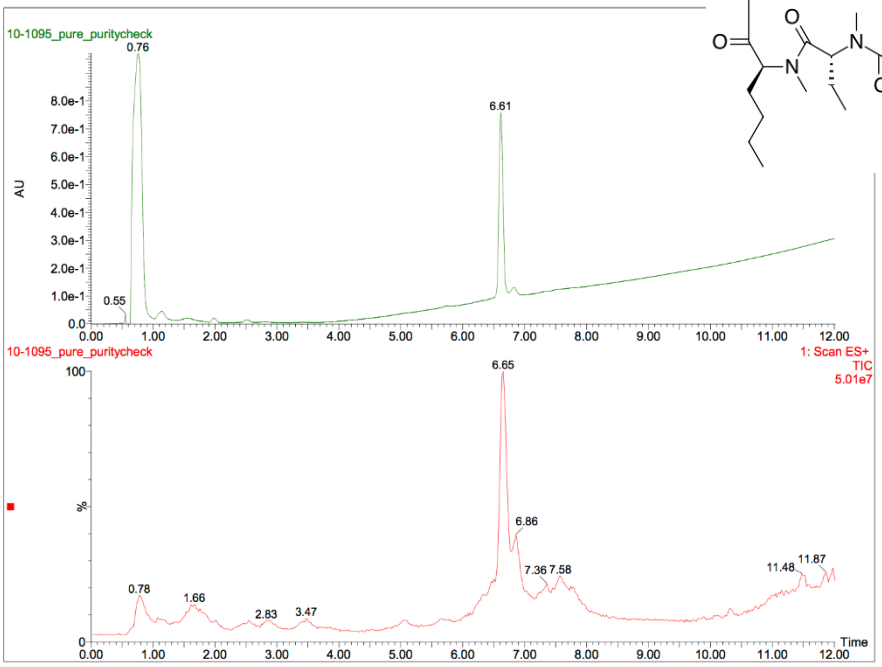


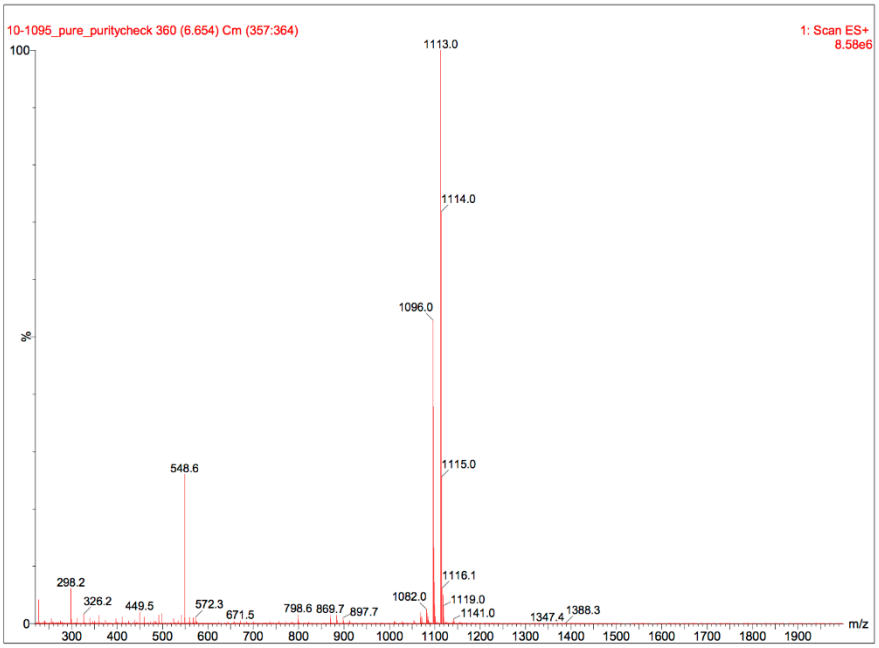




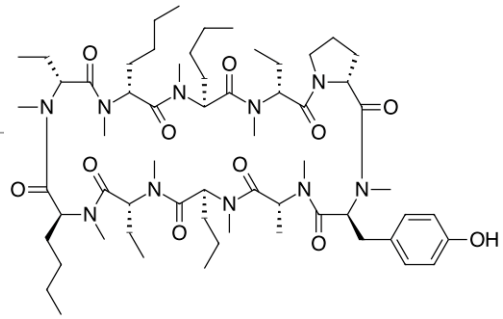
LCMS Spectra for 10.2

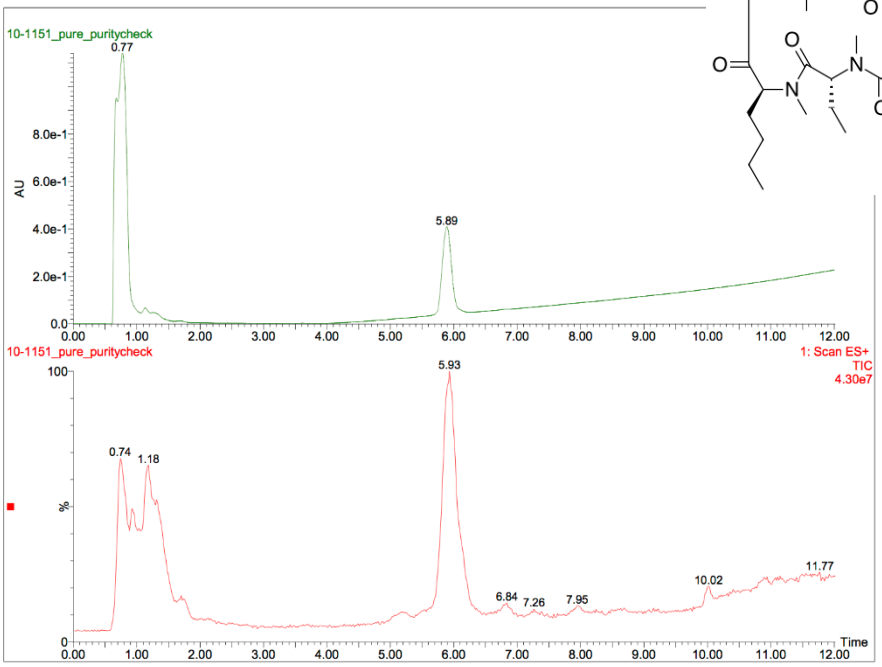


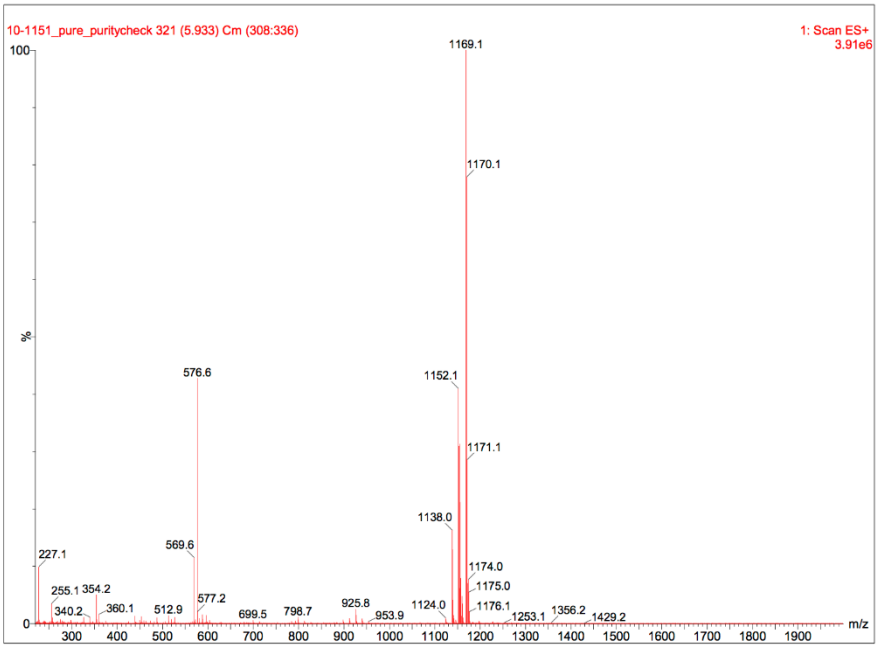




LCMS Spectra for 10.3

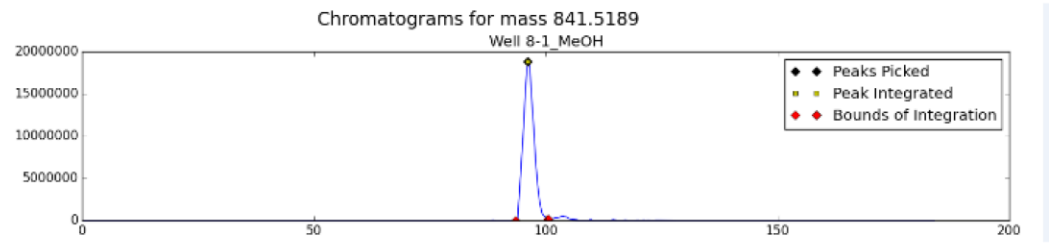


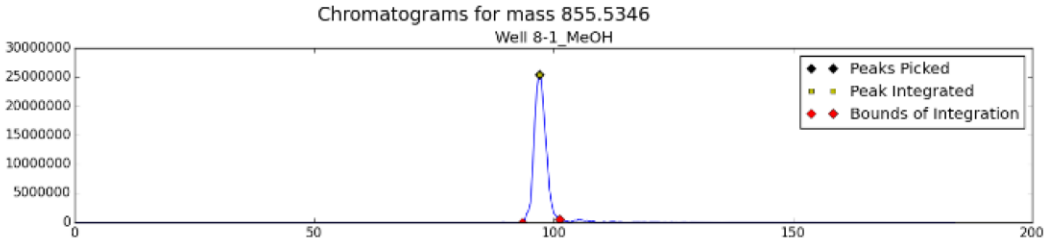


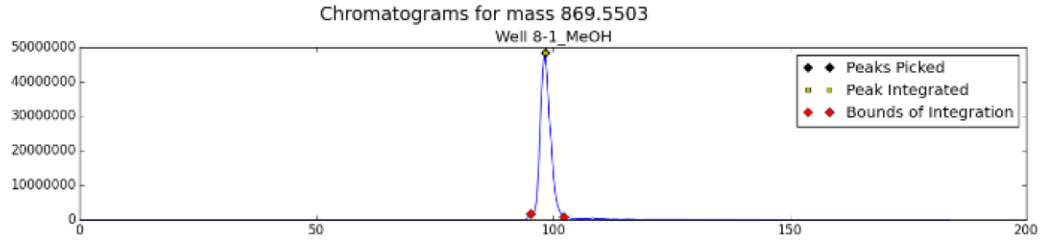


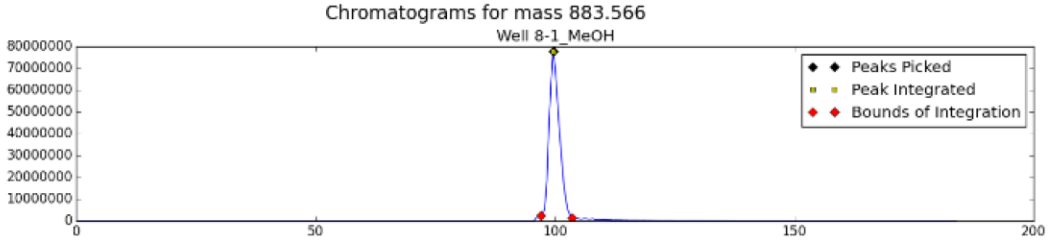
Extracted Ion Chromatograms for Lipophillicity Scanning Mixtures:

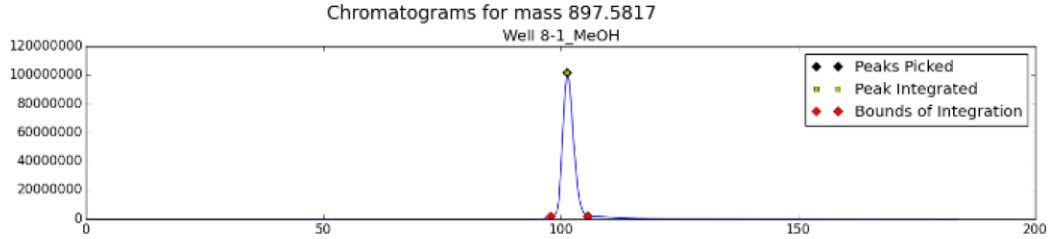
Octapeptide Extracted Ion Chromatograms

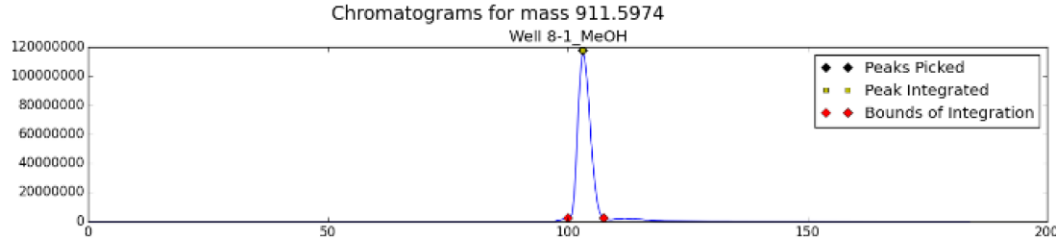








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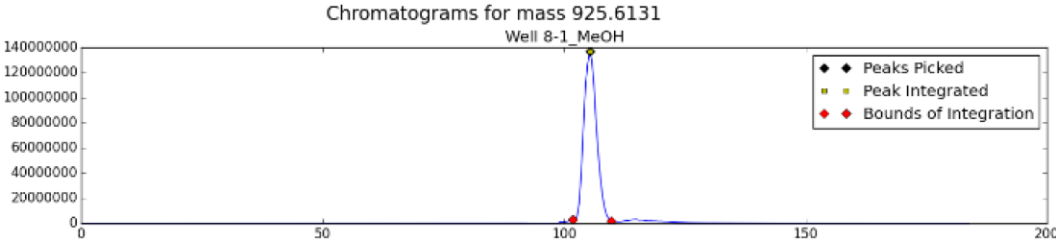
Well 8-1_MeOH
Peaks Picked
Peak Integrated
Bounds of Integration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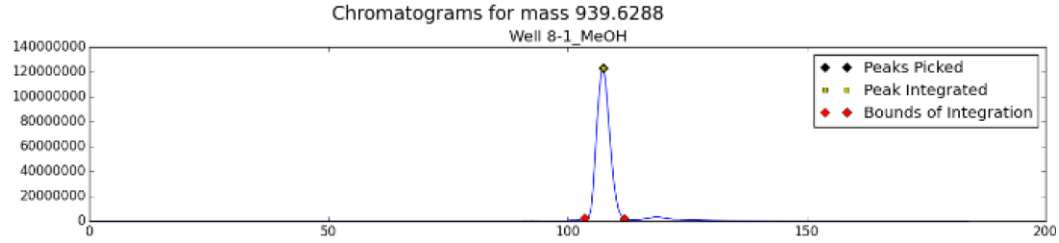
Peaks Picked
Peak Integrated
Bounds of Integration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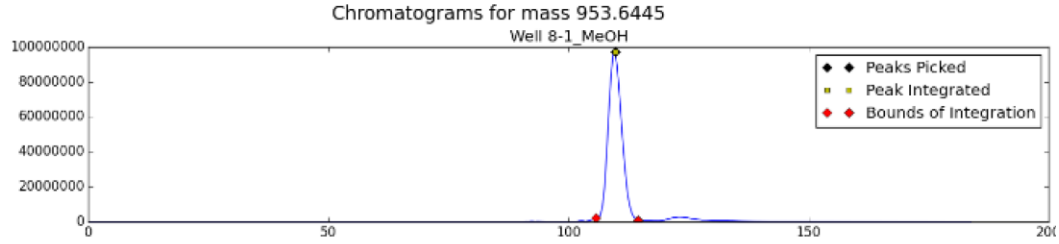
Bounds of Integration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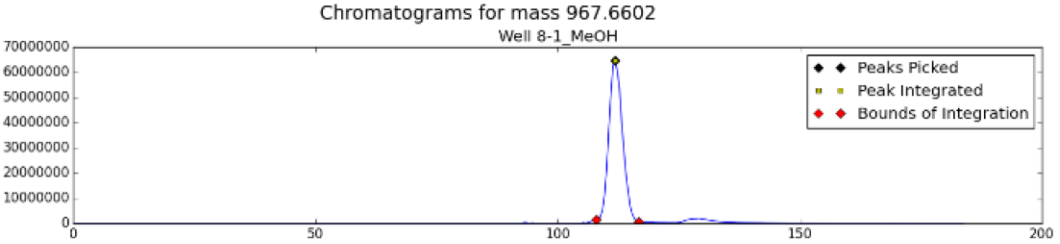
Bounds of Integration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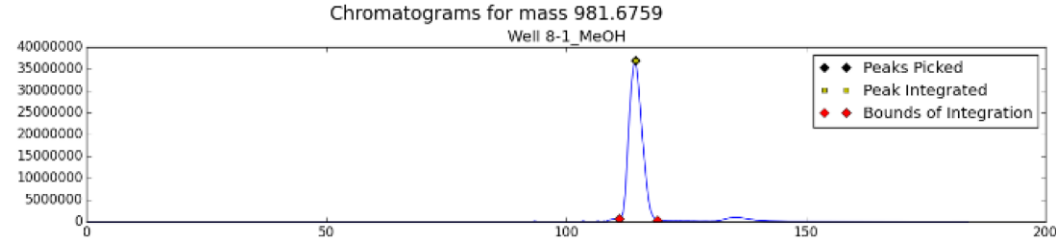
Peak Integrated
Bounds of Integration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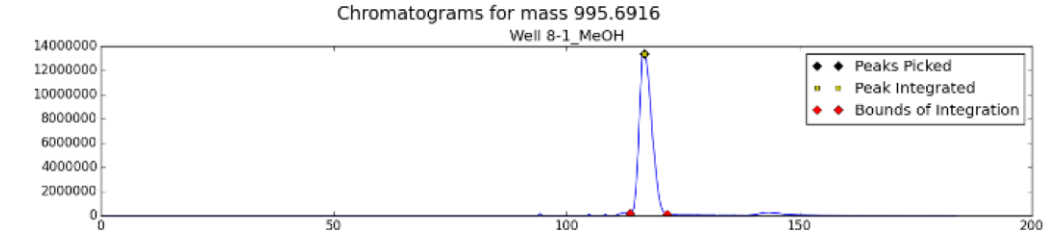
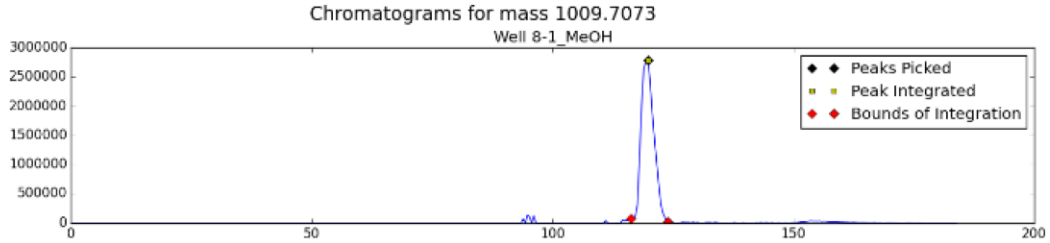
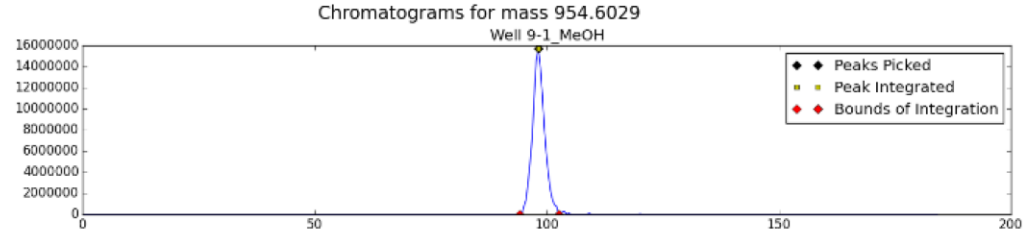
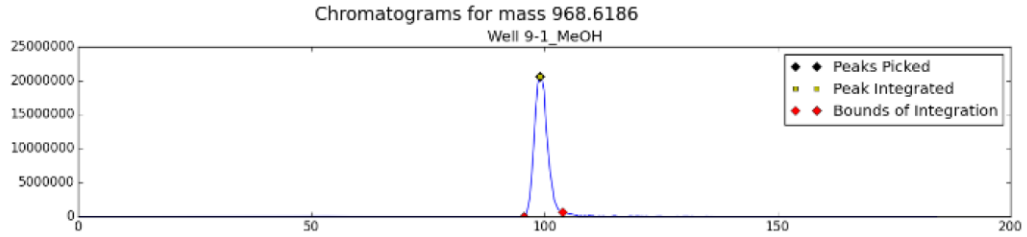
Bounds of Integration

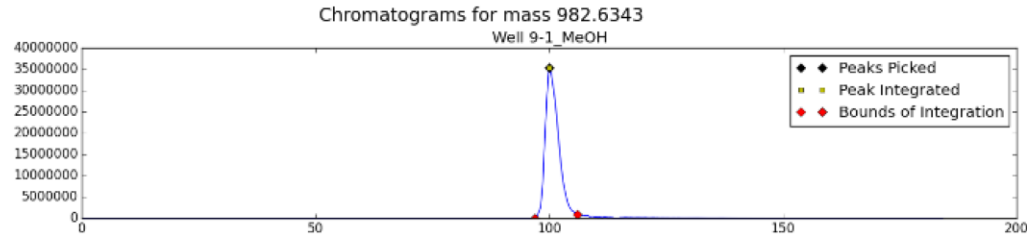


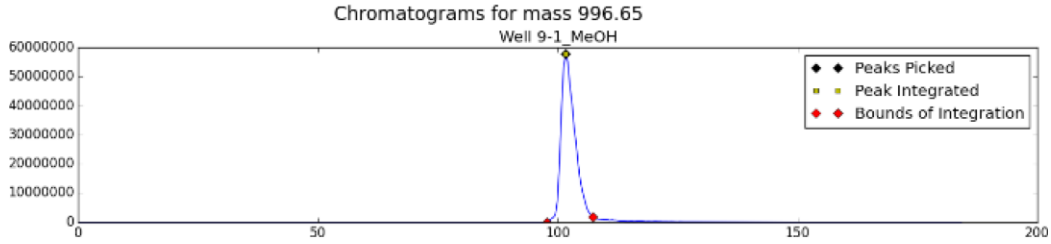


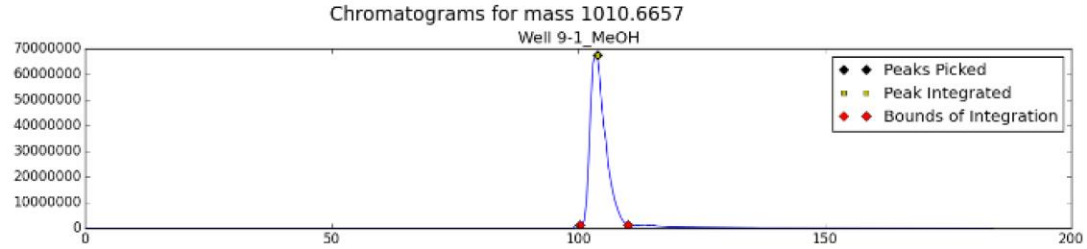
Nonapeptide Extracted Ion Chromatograms

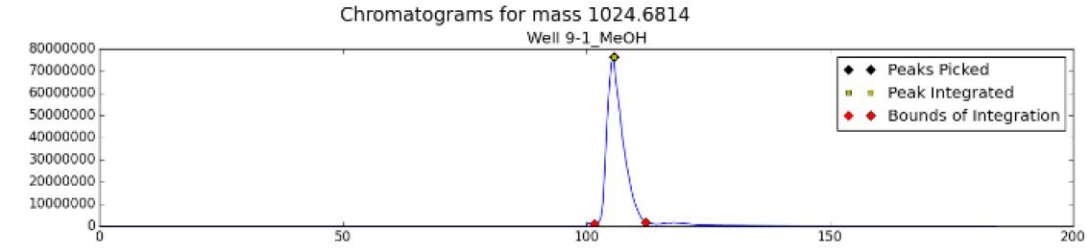









Chromatograms for mass 1010.6657
Well 9-1_MeOH
Peaks Picked
Peak Integrated
Bounds of Integration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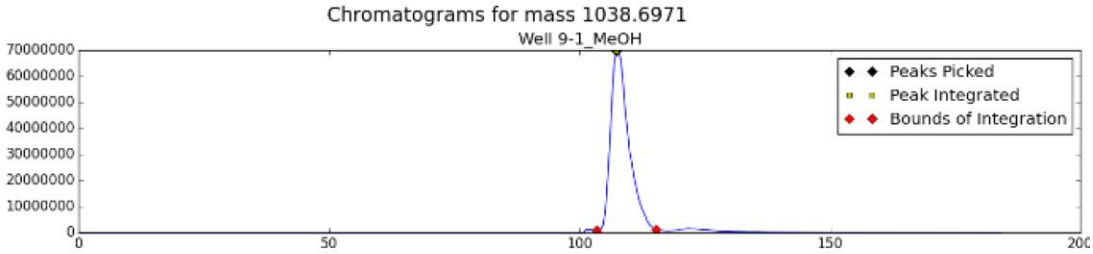
Peaks Picked
Peak Integrated
Bounds of Integration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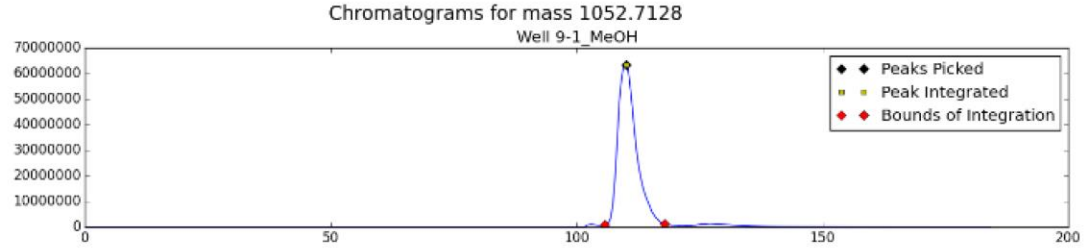
Peaks Picked
Peak Integrated
Bounds of Integration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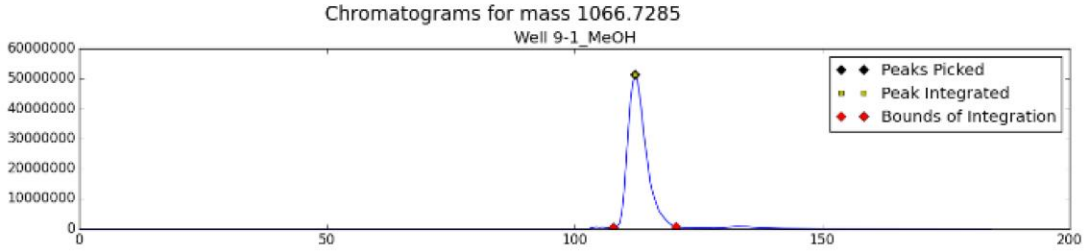
Peaks Picked
Peak Integrated
Bounds of Integration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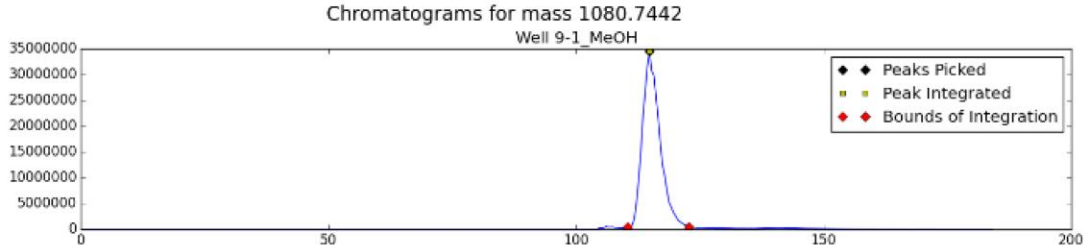
Peaks Picked
Peak Integrated
Bounds of Integration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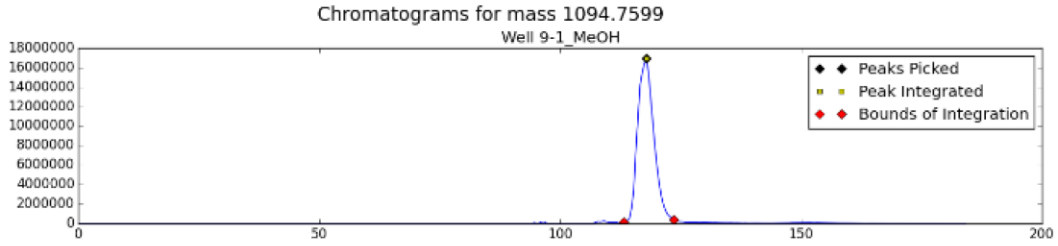
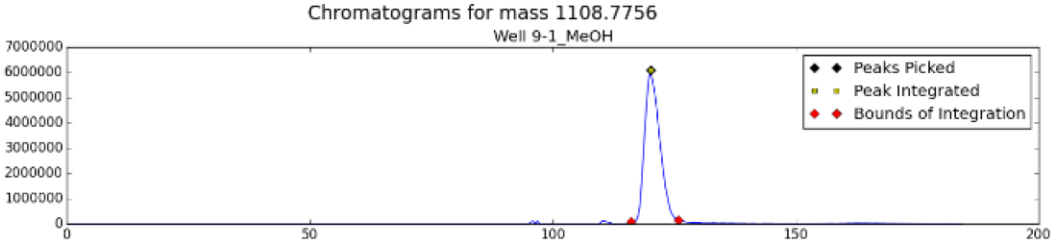
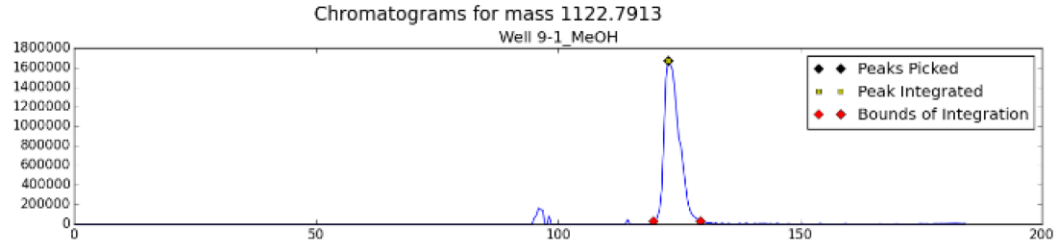
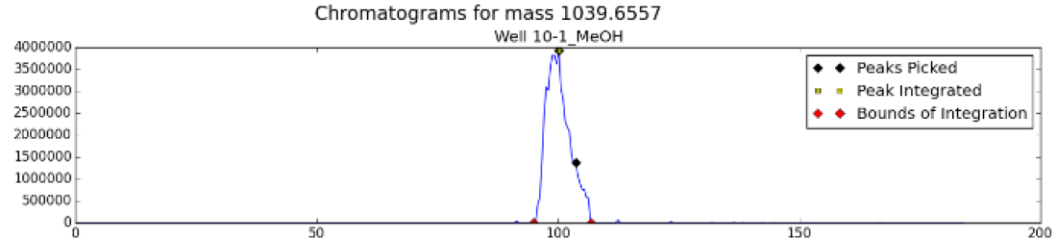
Peaks Picked
Peak Integrated
Bounds of Integration

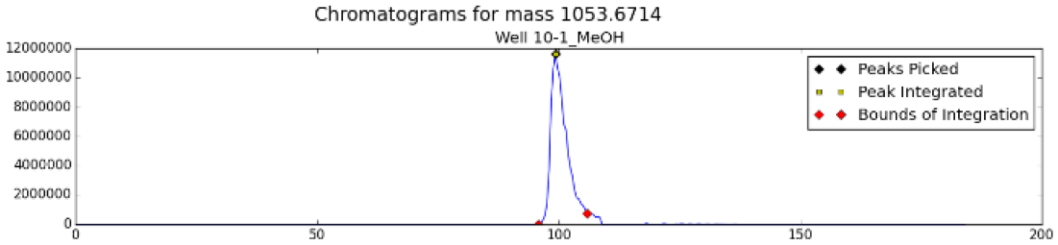


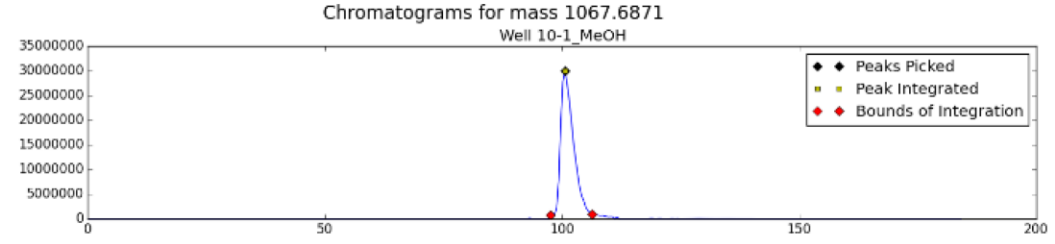


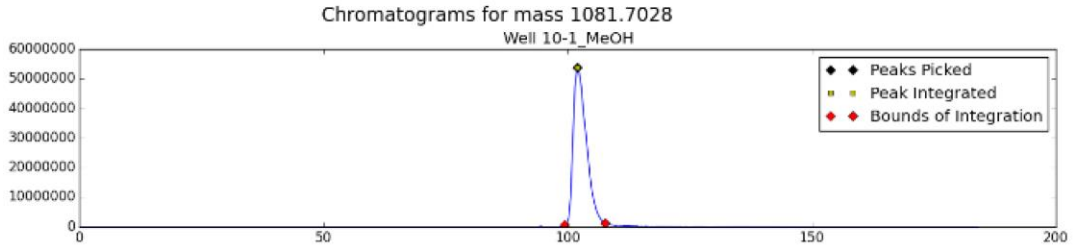


Decapeptide Extracted Ion Chromatograms





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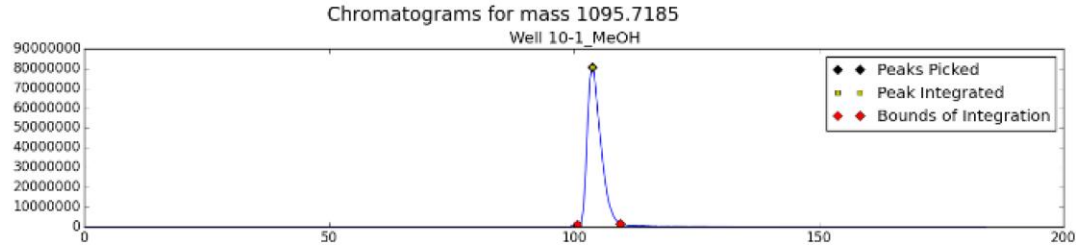
Well 10-1_MeOH
Peaks Picked
Peak Integrated
Bounds of Integration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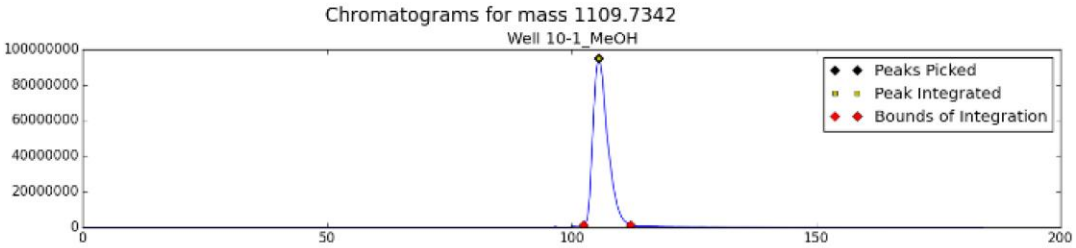
Peak Integrated
Bounds of Integration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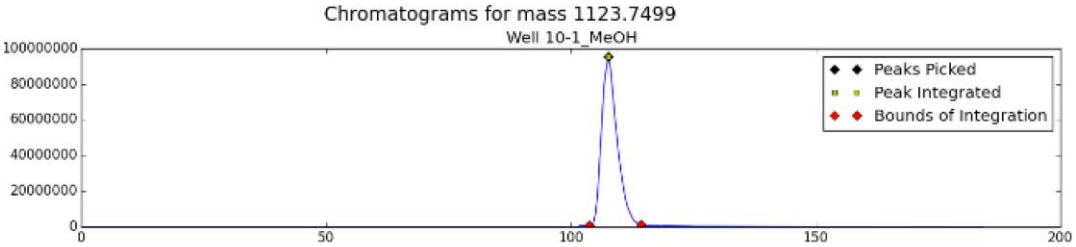
Bounds of Integration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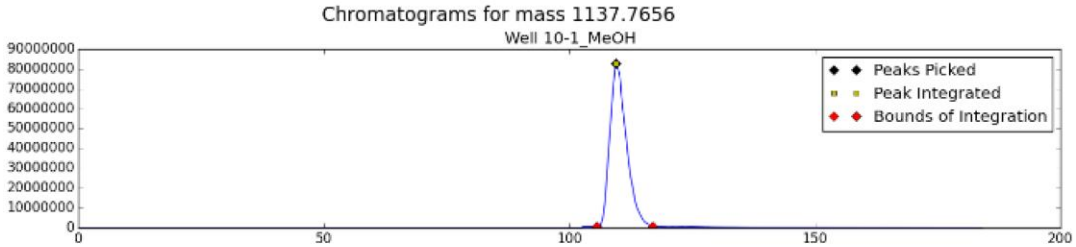
Peaks Picked
Peak Integrated
Bounds of Integration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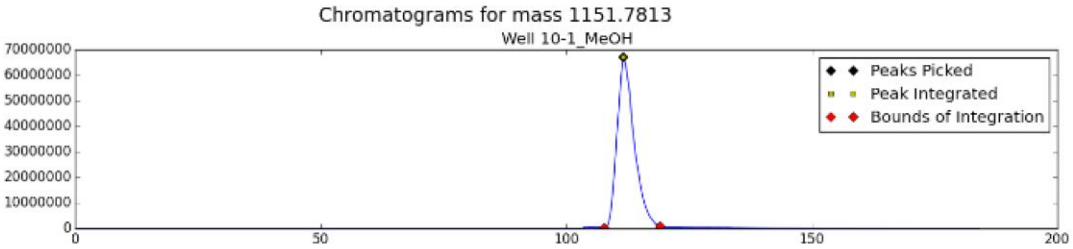
Peaks Picked
Peak Integrated
Bounds of Integration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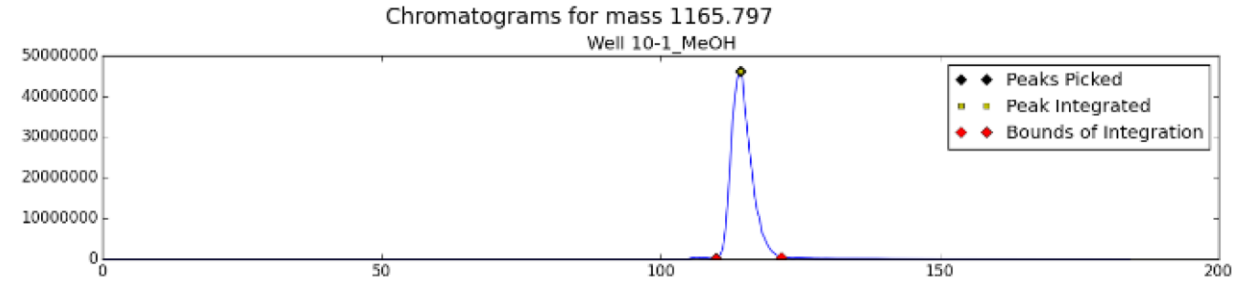
Peak Integrated
Bounds of Integration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Bounds of Integration
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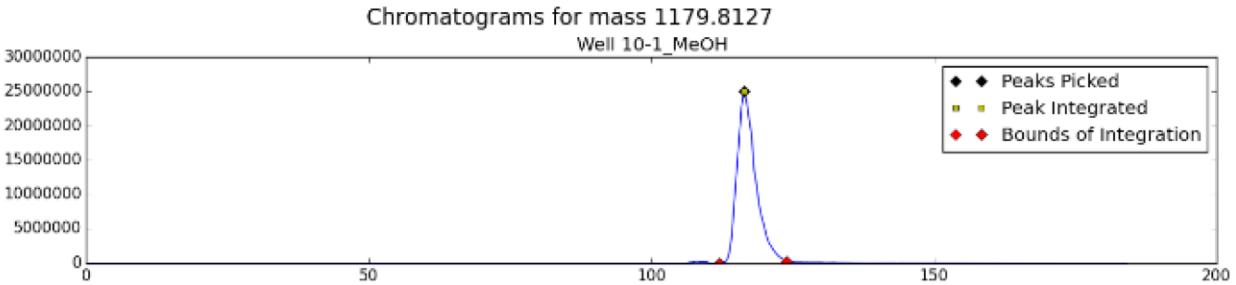
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Bounds of Integration
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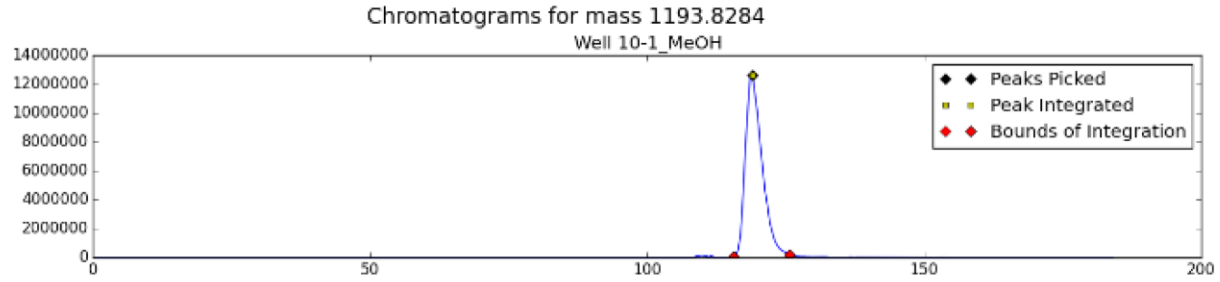
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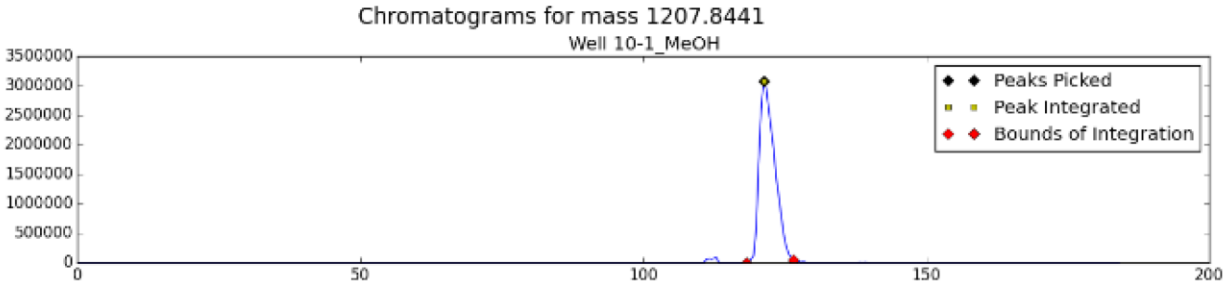
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Peaks Picked
Peak Integrated
Bounds of Integration
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500000
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0
50
100
150
200

Synthesis of natural products and Analogs

Peptides were prepared with the following procedures; Stylissamide G[4], Cordyheptapeptide B[5], Scytallidamide B[6], and Cylindrocyclin[7] were prepared using microwave assisted peptide couplings with installation of pre-methylated amino acids followed by solution phase cyclization with COMU. 1NMe3 was prepared as previously described.[3] Cyclosporin-BMT to leucine was using microwave assisted peptide couplings with installation of pre-methylated amino acids followed by solution phase cyclization with COMU, with a modified permethylation for the first 4 amino acids. In the case of CSA starting with the Leucine that replaced the BMT residue as the first amino acid on resin the first 3 couplings were done with unmethylated amino acids. The Fmoc was removed, replaced with a Nosyl protecting group and the tetrapeptide was subjected to on resin permethylation. The remaining couplings and amino acids were added as described below.

Loading of 2-Chlorotrityl resin
Fmoc-Xaa (10 mmol) was added to a flame dried round bottom flask and dried in a vacuum dessicator with phosphorous pentoxide overnight. 50mL of dry DCM was cannula transferred into the flask followed by 2.5 mL of DIPEA transferred via syringe. After sonication for 10 min, 5 g of 2-chlorotrityl resin was added under a stream of N_2 and allowed to shake for 4 h. Resin was capped with a 15 mL solution of 1:2:17 MeOH:DIPEA:DMF (3 x 15 min). Resin was washed with DMF (3 x 15 mL) followed by DCM (3 x 15 mL). Loading value was calculated by mass increase of dried, loaded resin.

Microwave Reaction Conditions
Microwave assisted reactions were done in a CEM Discover microwave reactor with an open reaction vessel. Reactions were heated to 50°C with 12 watts of power while temperature was monitored with an IR probe from within the reaction vessel.

Amino Acid Coupling under Microwave Condtions
A solution of 4 eq of Fmoc-Xaa, 4 eq of HBTU, and 4 eq of HoAT, and 6 eq of DIPEA in DMF was allowed to pre-react for 5 min. This solution was added to the deprotected peptide on-resin and allowed to react for 10 min at 50°C under microwave heating. The solution was drained and resin was washed 3 times with DMF (3 x 3 mL) and DCM (3 x 3 mL). The reaction was monitored by LCMS and repeated until starting material was no longer observed.

Amino Acid Coupling onto N-Methylated Amino Acids under Microwave Conditions
A solution of 4 eq of Fmoc-Xaa, 4 eq of HATU, and 6 eq of DIPEA in DMF was allowed to pre-react for 5 min. This solution was added to the deprotected peptide on-resin and allowed to react for 30 min at 50°C under microwave heating. The solution was drained and resin was washed DMF (3 x 3 mL) and DCM (3 x 3 mL). The reaction was monitored by LCMS and repeated until starting material was no longer observed.

Difficult Amino Acid Coupling with BTC

A solution of 1.33 eq of BTC, 4 eq of Fmoc-Xaa in dry THF was allowed to pre-react for 1 min. A solution of 8 eq of DIPEA in a minimum volume of THF was added to the resin that had been washed 3x with dry THF. To the solution of BTC and Fmoc-Xaa was added 8 eq of collidine. After prereaction for 1 minute this solution was added to the resin and the reaction was allowed to proceed for 6 hours under an atmosphere of dry Ar. The solution was drained and resin was washed DMF (3 x 3 mL) and DCM (3 x 3 mL). The reaction was monitored by LCMS and repeated until starting material was no longer observed.

Removal of the *N*-Fmoc Protecting Group Under Microwave Conditions
A solution of 2% piperidine and 2% DBU in DMF was added to the resin. The reaction was allowed to react for 5 min at 50°C under microwave heating then drained. The resin was washed with DMF (3 x 3 mL) and DCM (3 x 3 mL).

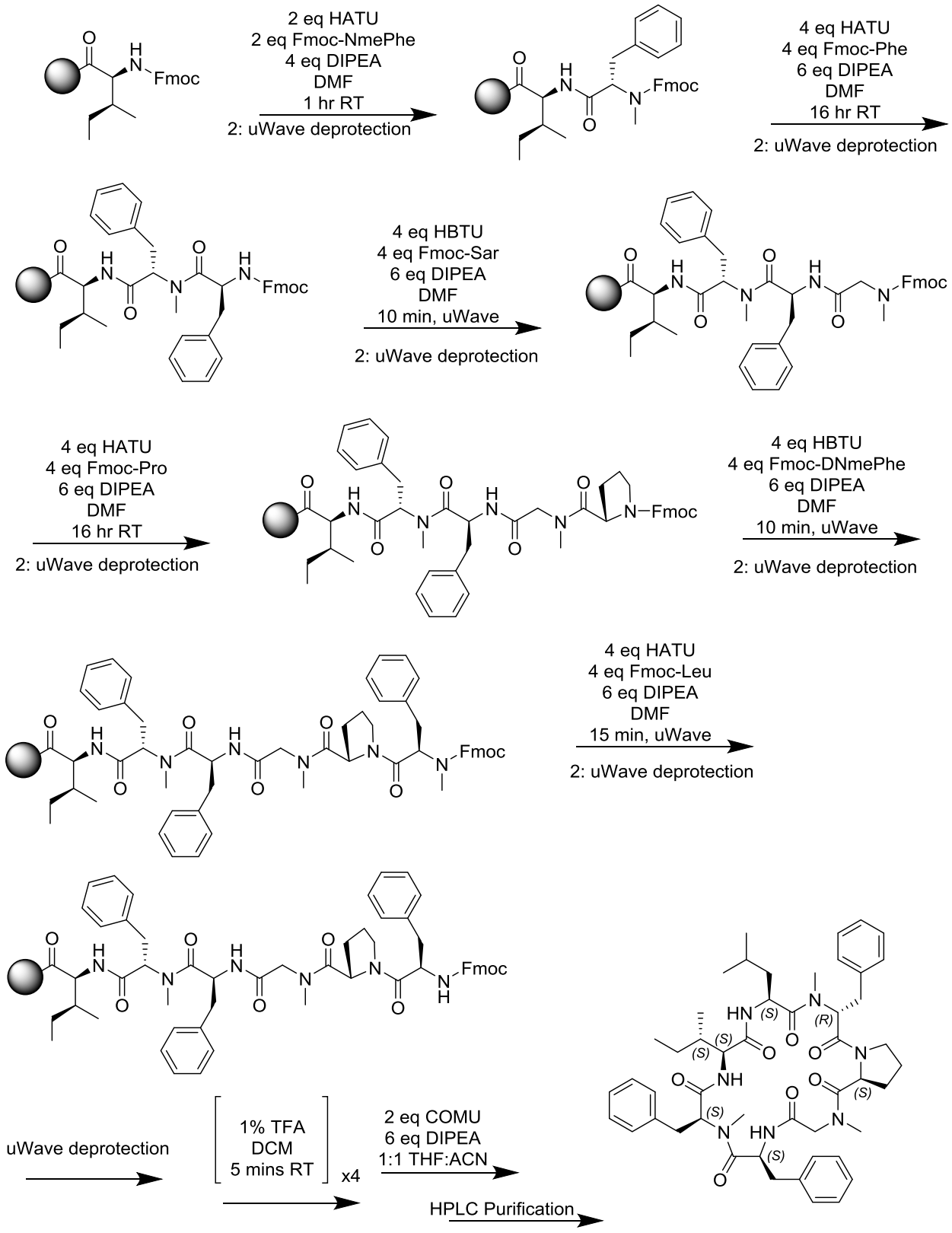
Peptide Cleavage
Complete linear peptides were cleaved off resin in 5 resin volumes of 2.5% TFA in DCM for 4 minutes three times with a 5 resin volume DCM wash in between each step. Solvent was removed under N_2 followed by dissolution in acetone or DCM and evaporation under reduced pressure. Residual TFA was removed *in vacuo* overnight.

Cyclization with COMU
Linear peptides were dissolved in 20 mL of dry acetonitrile with 4 eq of DIPEA and added dropwise to a solution of (final concentration 1 mg crude peptide per mL) 1:1 THF/ACN containing 2 eq of COMU. Reactions were stirred from 0.5 to 24 h until complete cyclization was achieved as monitored by LCMS. The reaction was reduced *in vacuo* for purification via HPLC.

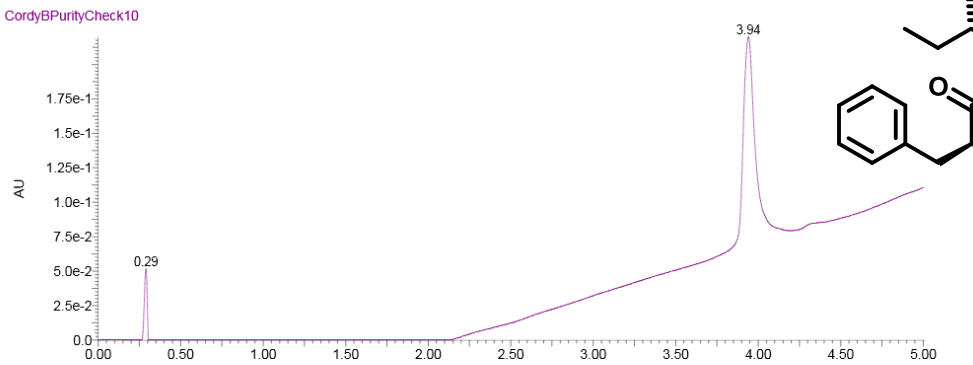
Purification of Peptides
COMU by-products were removed after solution phase cyclization on a Biotage Isolera Prime system equipped with a KP-C18-HS 12g column eluting with H2O/Acetonitrile modified with 0.1% TFA. Peptides were further purified when necessary on a Waters mass-directed prep system equipped with an XBridge BEH130 5µm 19x150 C18 column eluting with H_2O/Acetonitrile modified with 0.1% formic acid.

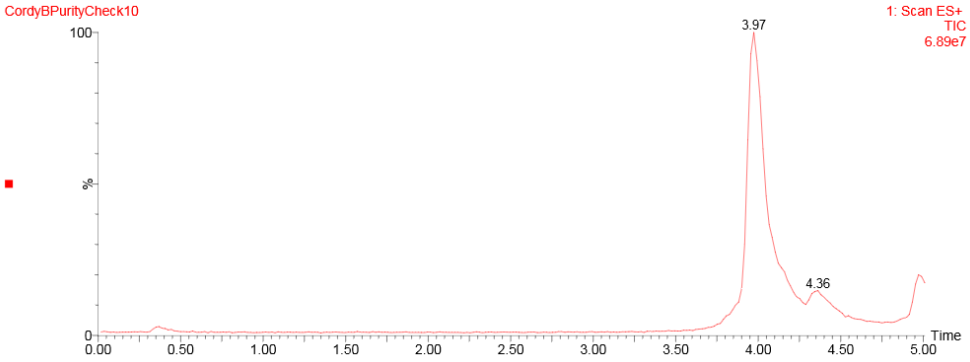
NMR Analysis and Assignment
Spectra were acquired on either a Varian/Agilent 600 MHz NMR with a Unity Inova console with a 5 mm triple resonances cold probe or on an Agilent/Varian 500 MHz NMR with Unity Plus console and a 5 mm broadband probe or a 5 mm HX indirect detection probe.

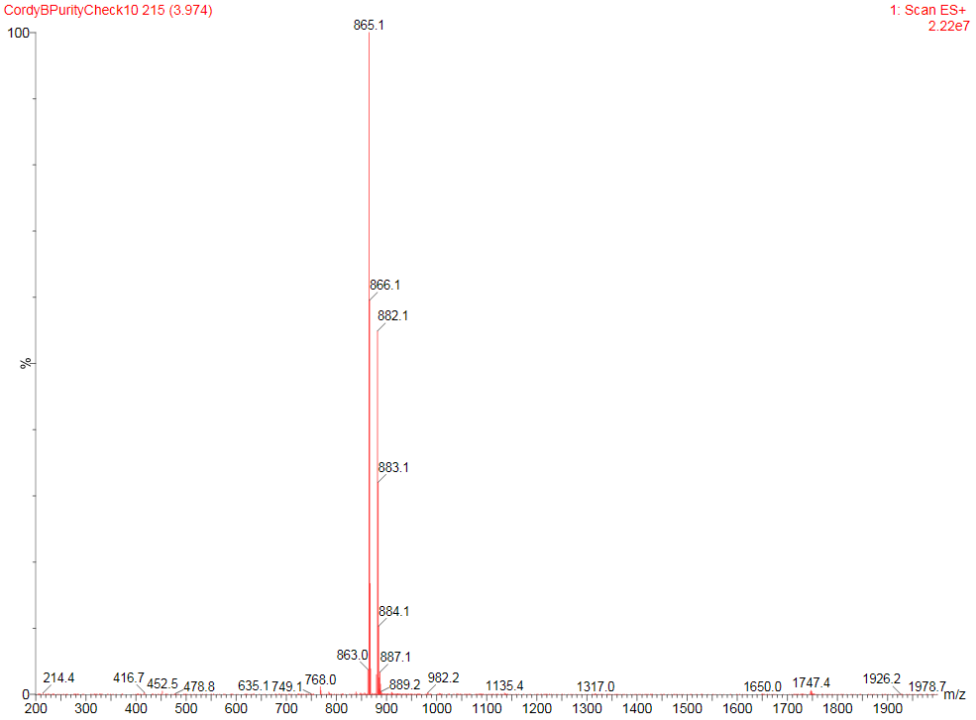
Synthesis of Cordyheptapeptide B



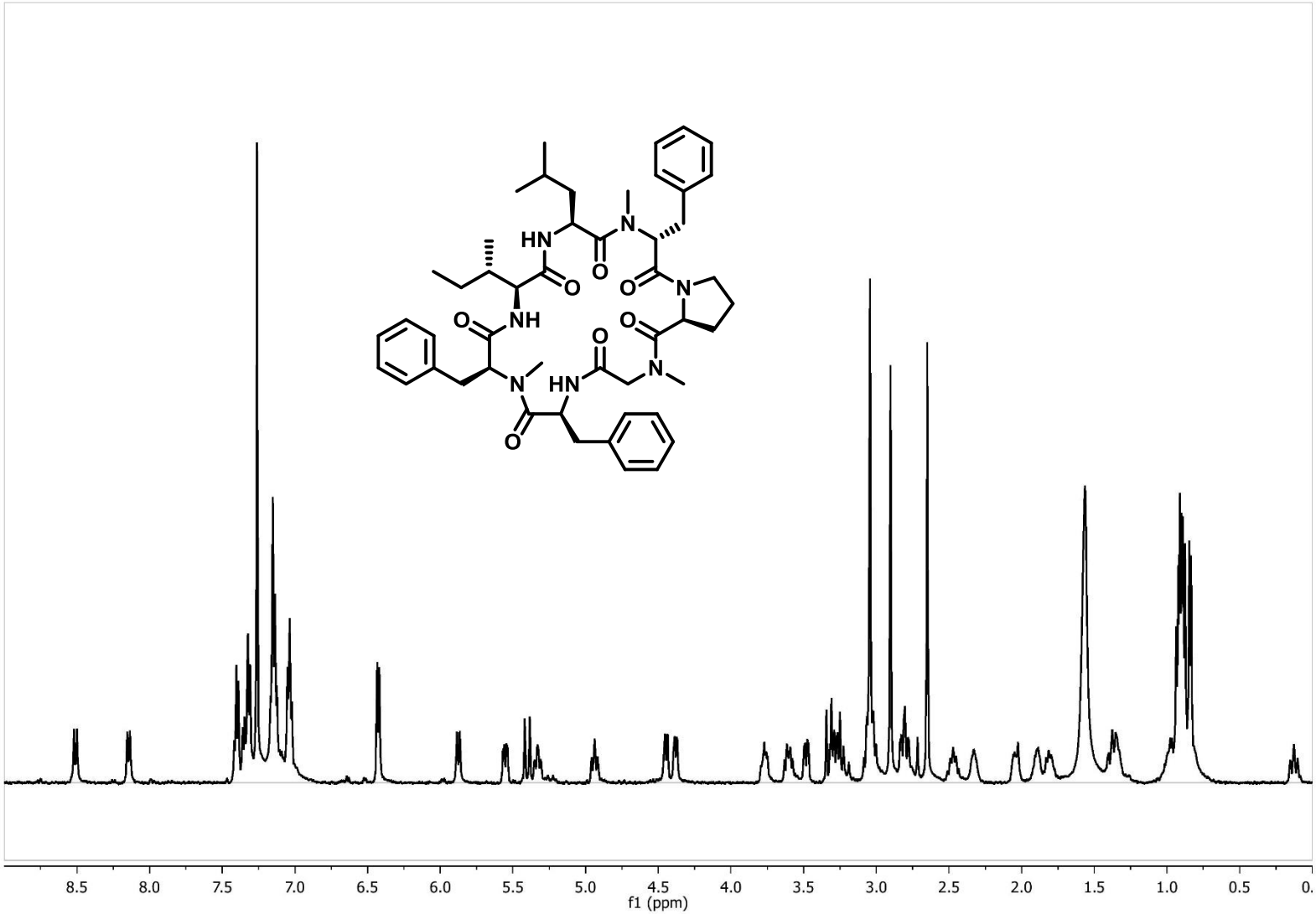
LCMS Spectra for Cordyheptapeptide B





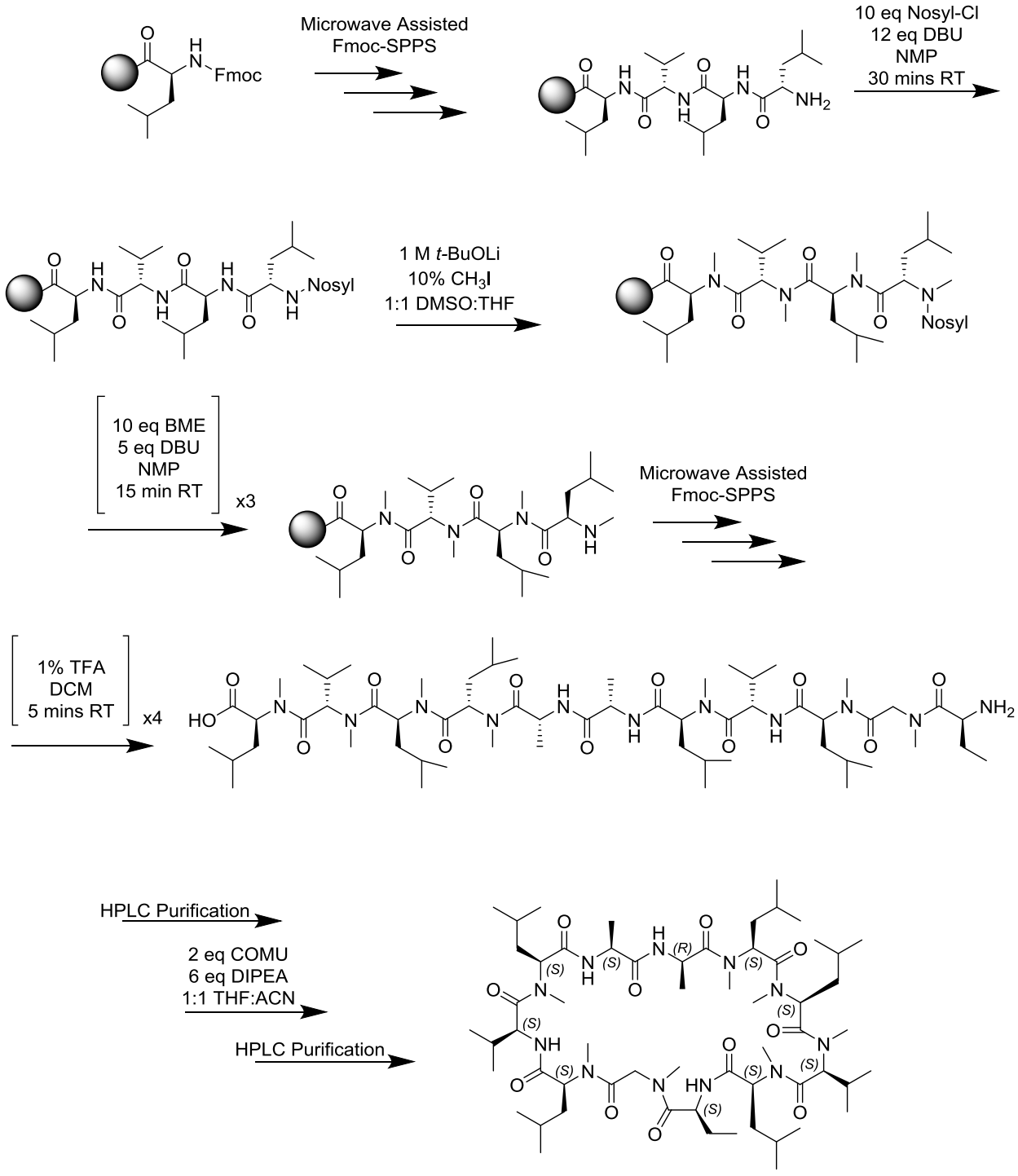


NMR Spectra for Cordyheptapeptide B in $CDCl_3$

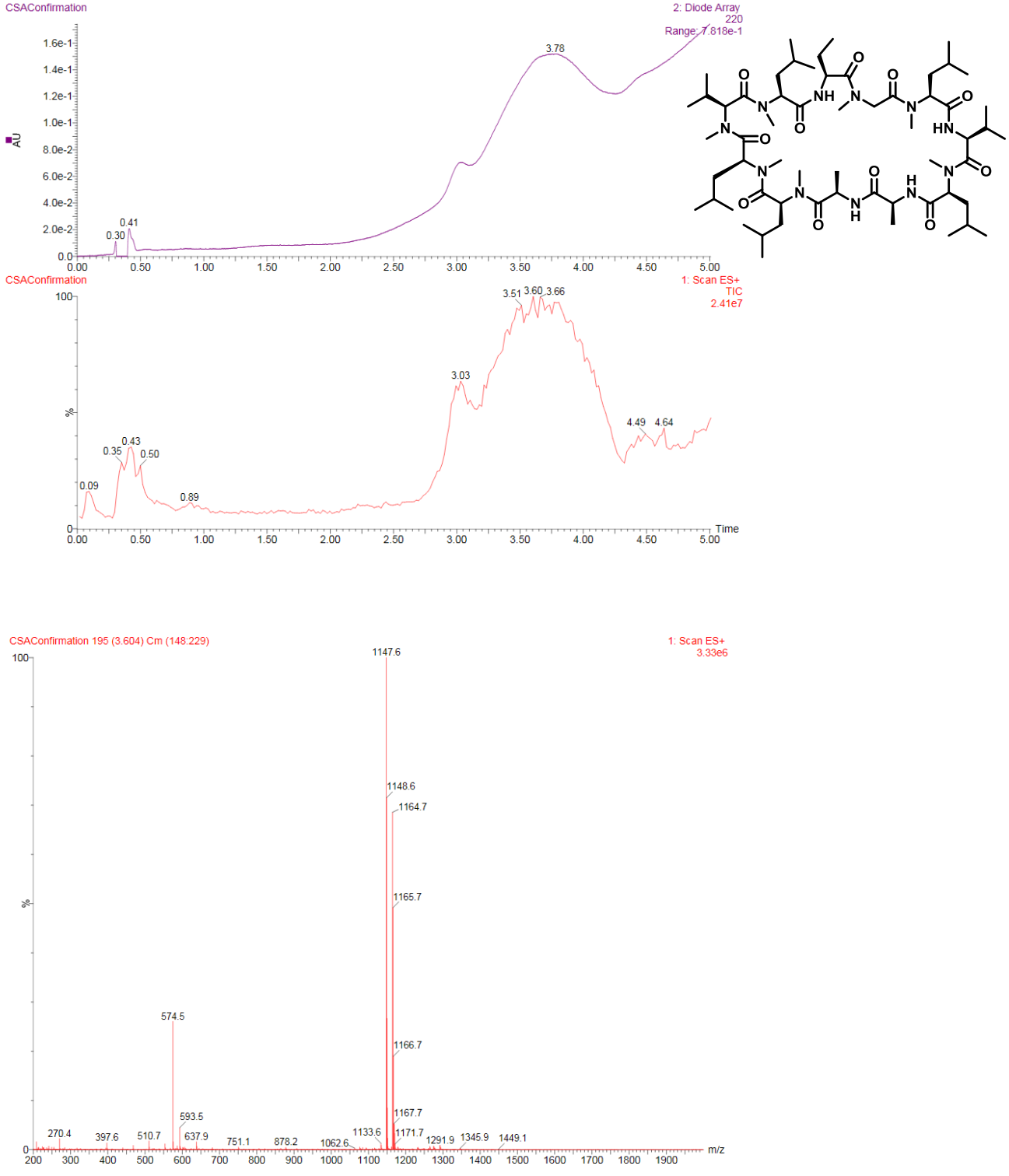


Synthesis of Cyclosporine A BMT to Leucine

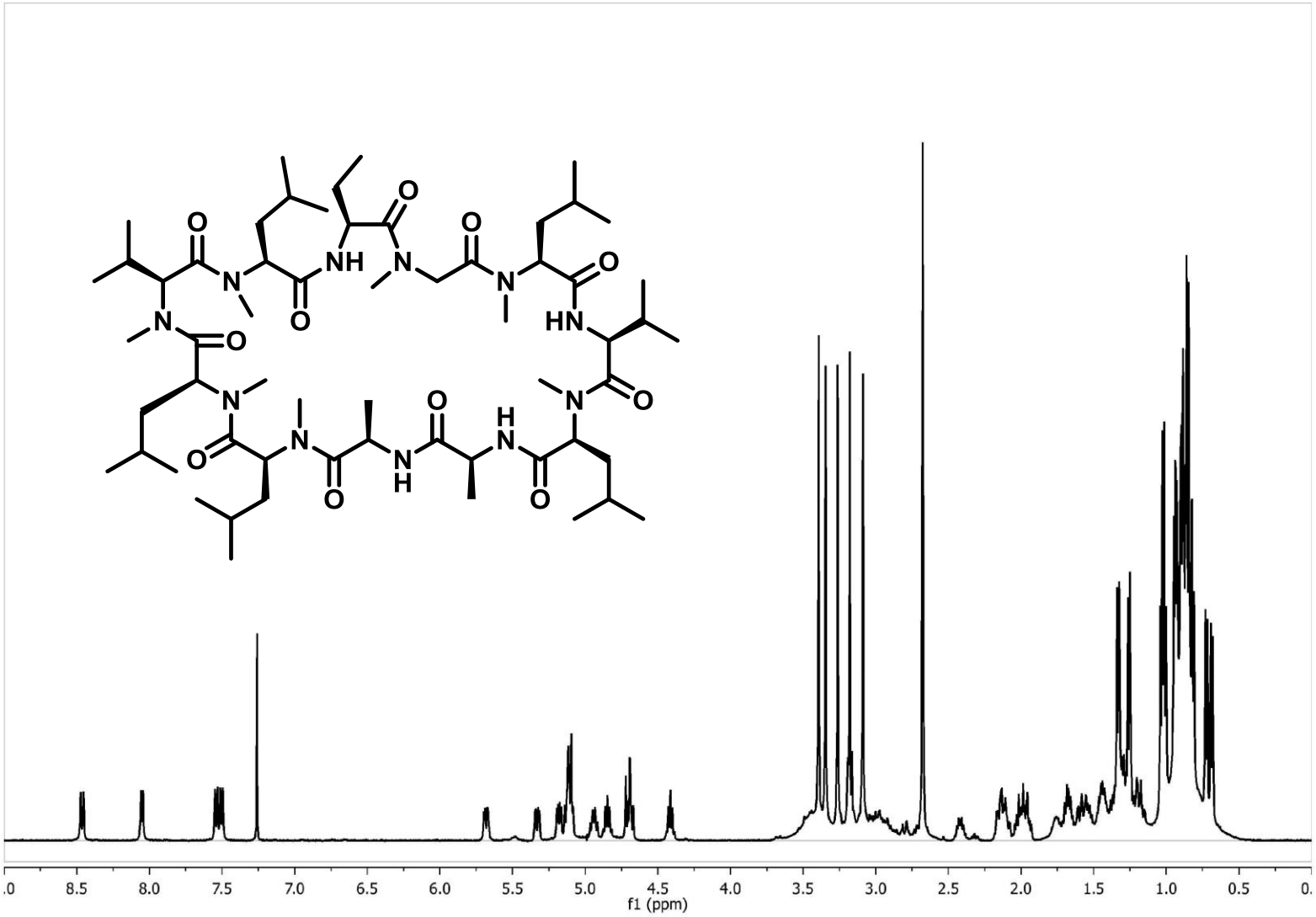
Sequence: Leu - Val - Leu - Leu - DAla - LAla - LNmeLeu - LVal - LNmeLeu - Sarc - LAbu



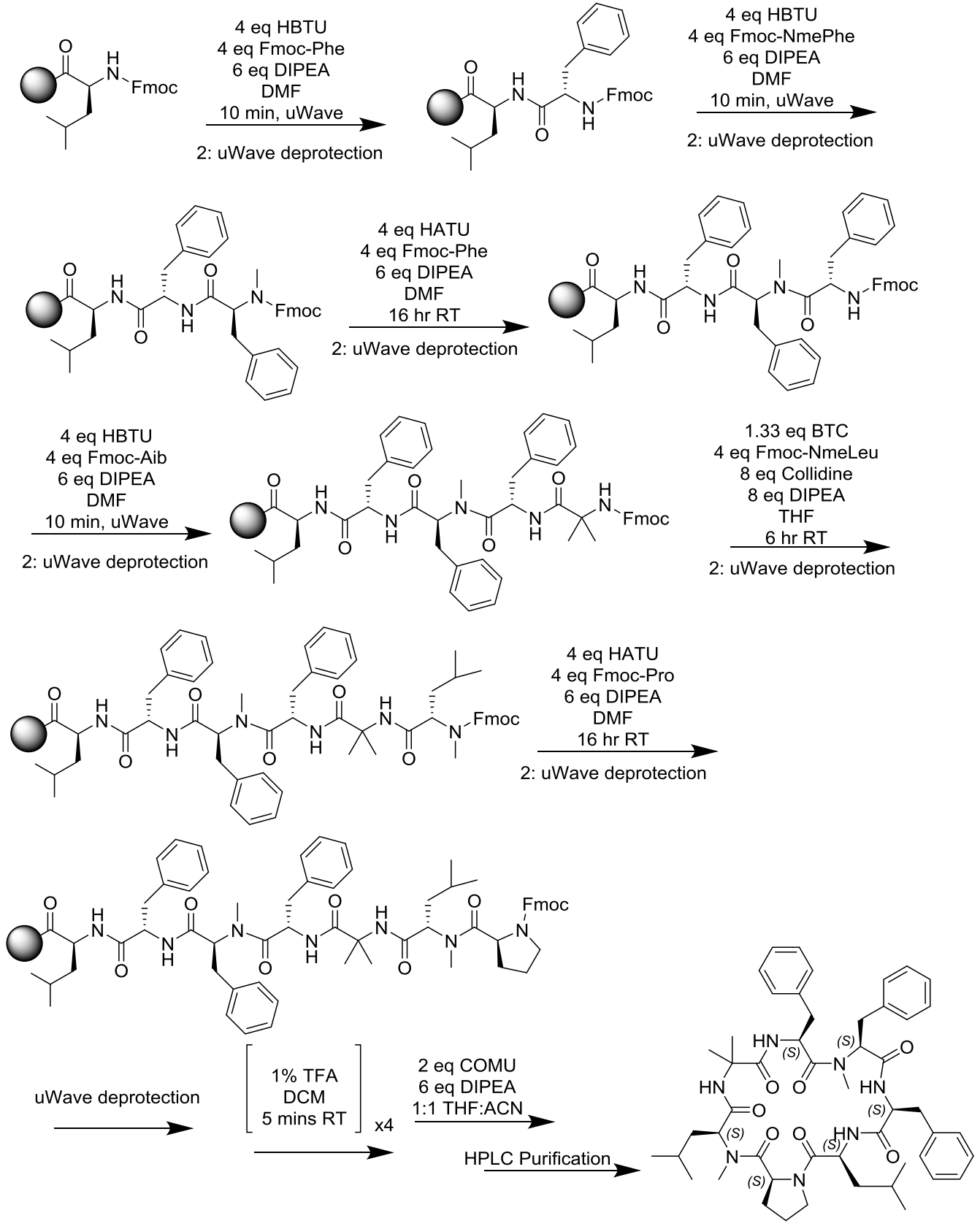
LCMS Spectra for Cyclosporine A BMT to Leucine



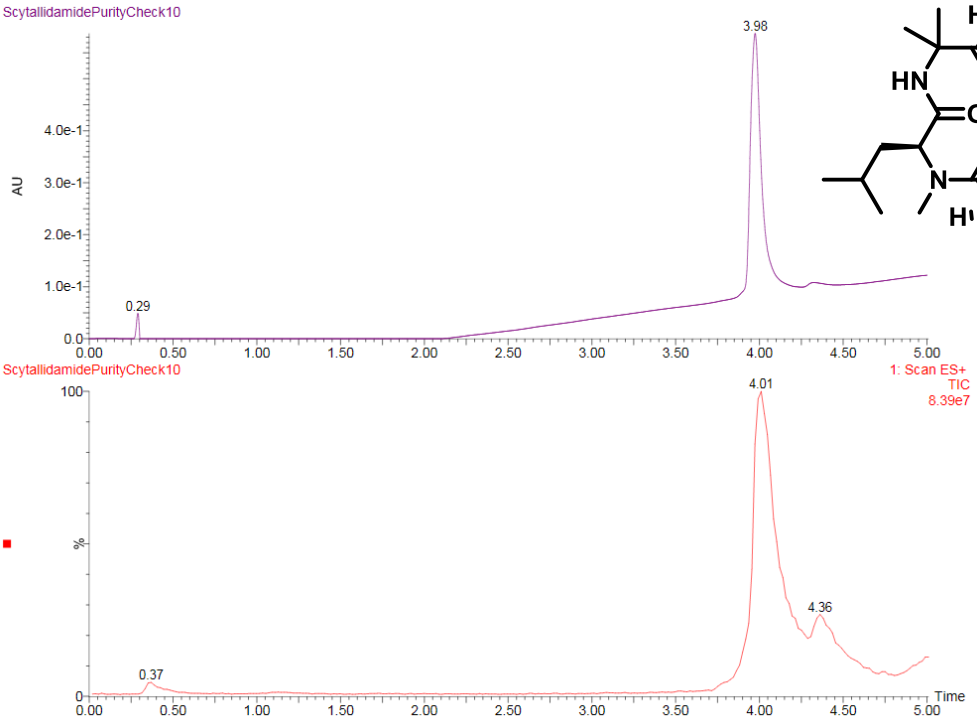
NMR Spectra for Cyclosporine A BMT to Leucine in $CDCl_3$

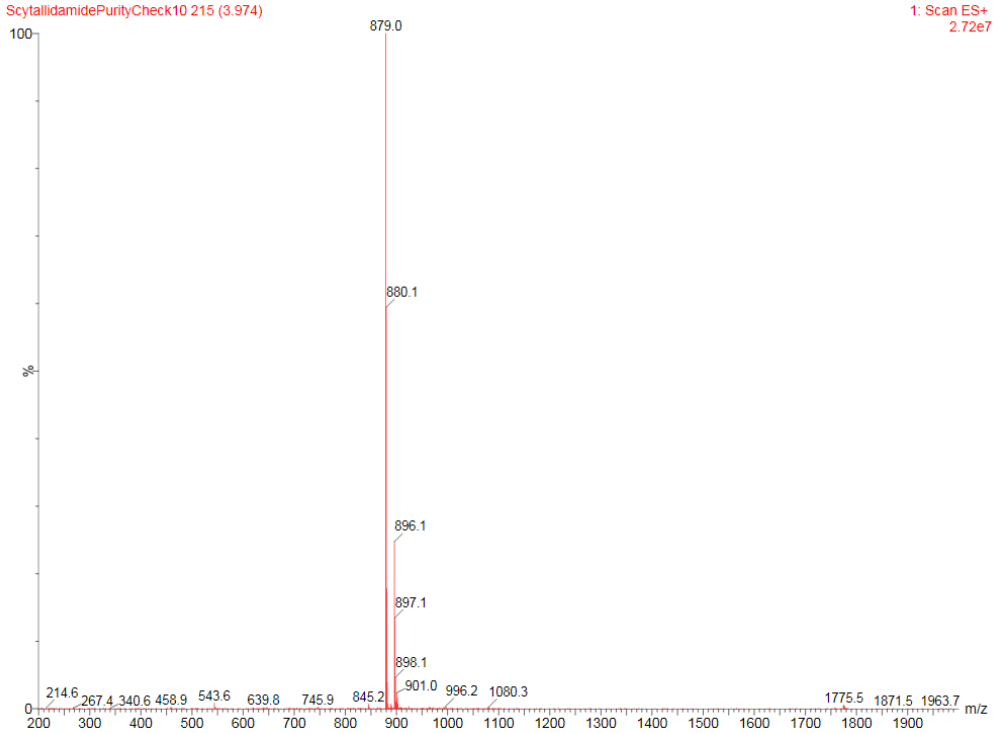


Synthesis of Scytallidamide B

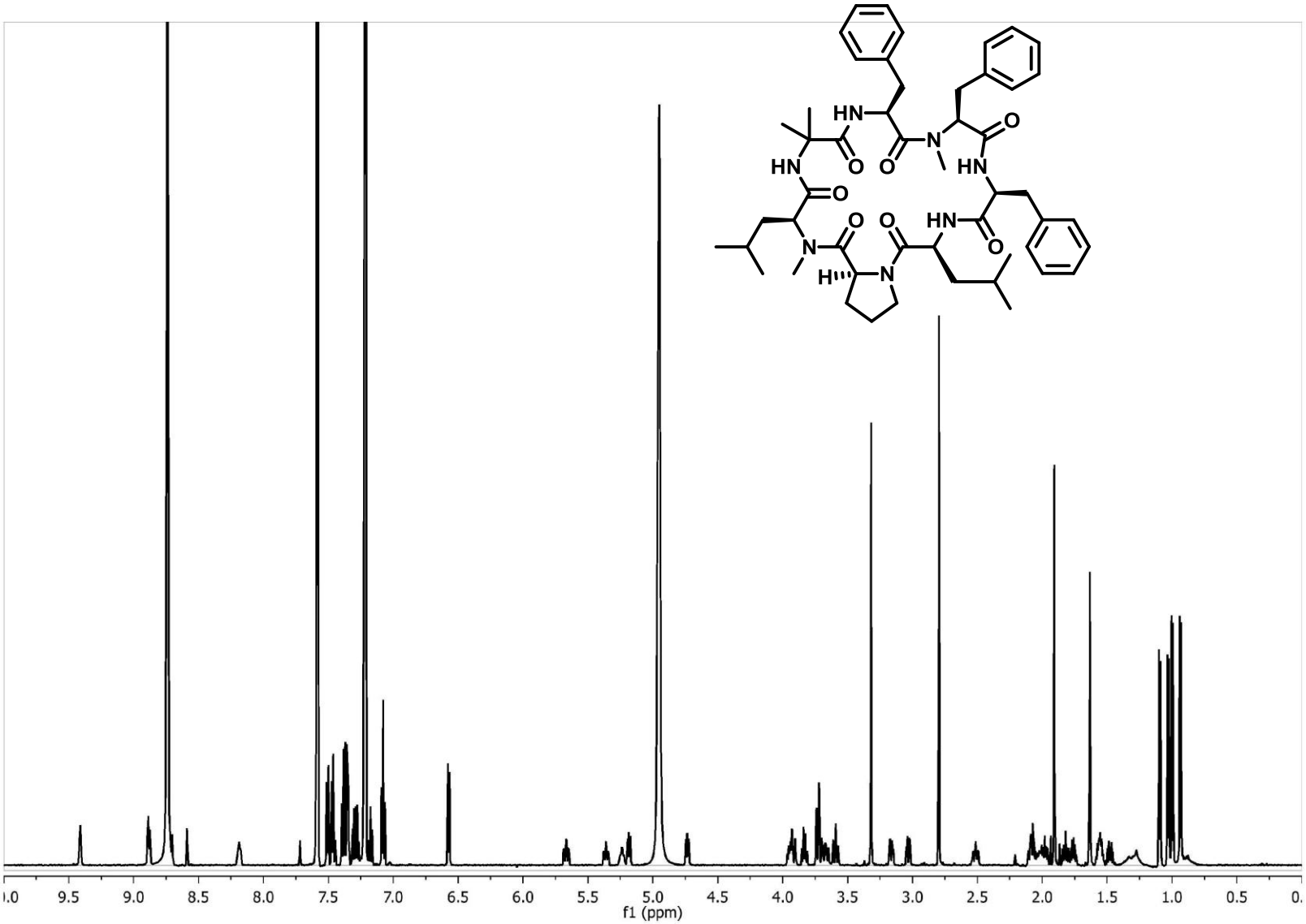


LCMS Spectra for Scytallidamide B

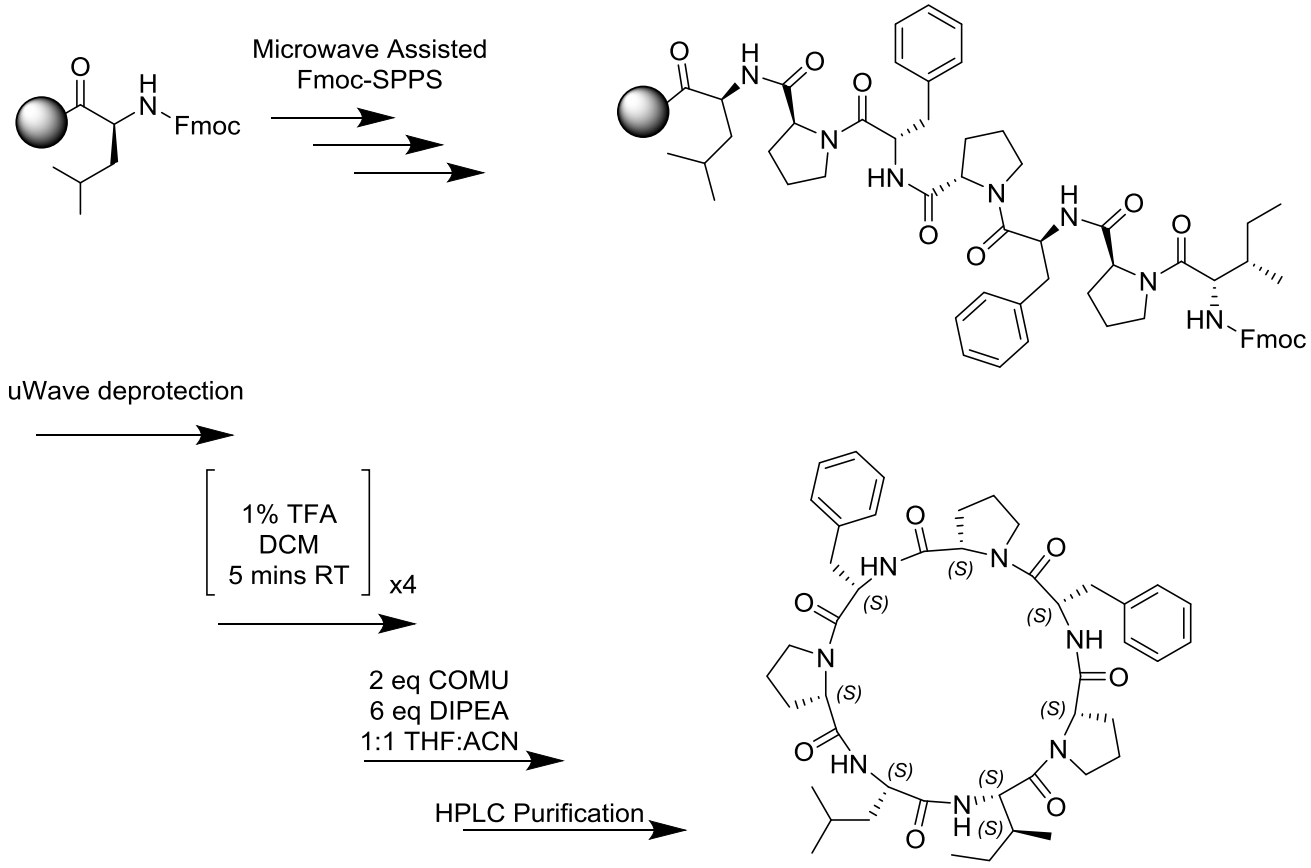




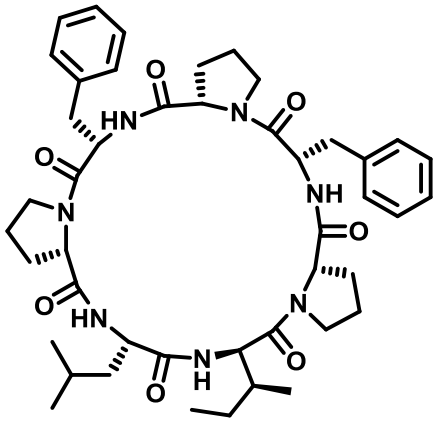
NMR Spectra for Scytallidamide B in Pyridine *d*5

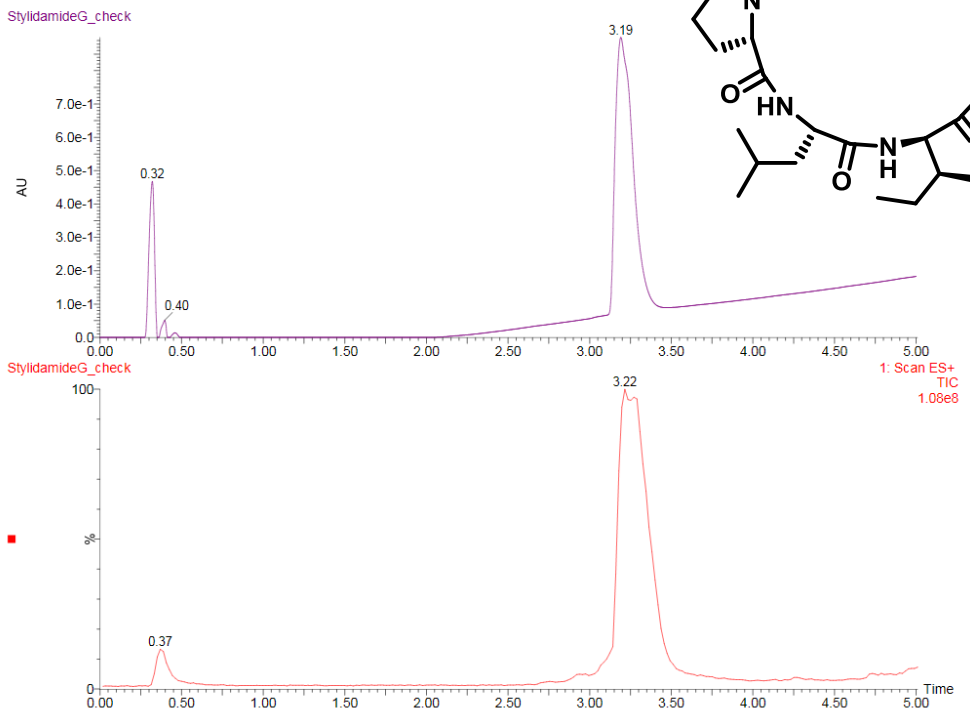


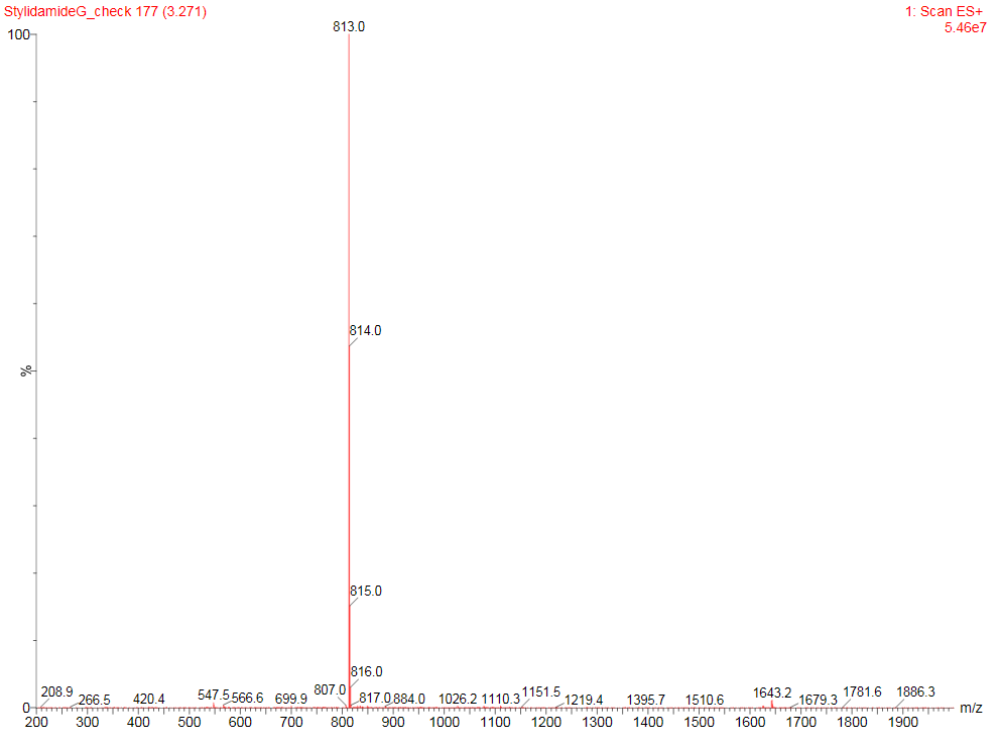
Synthesis of Styllisamide G



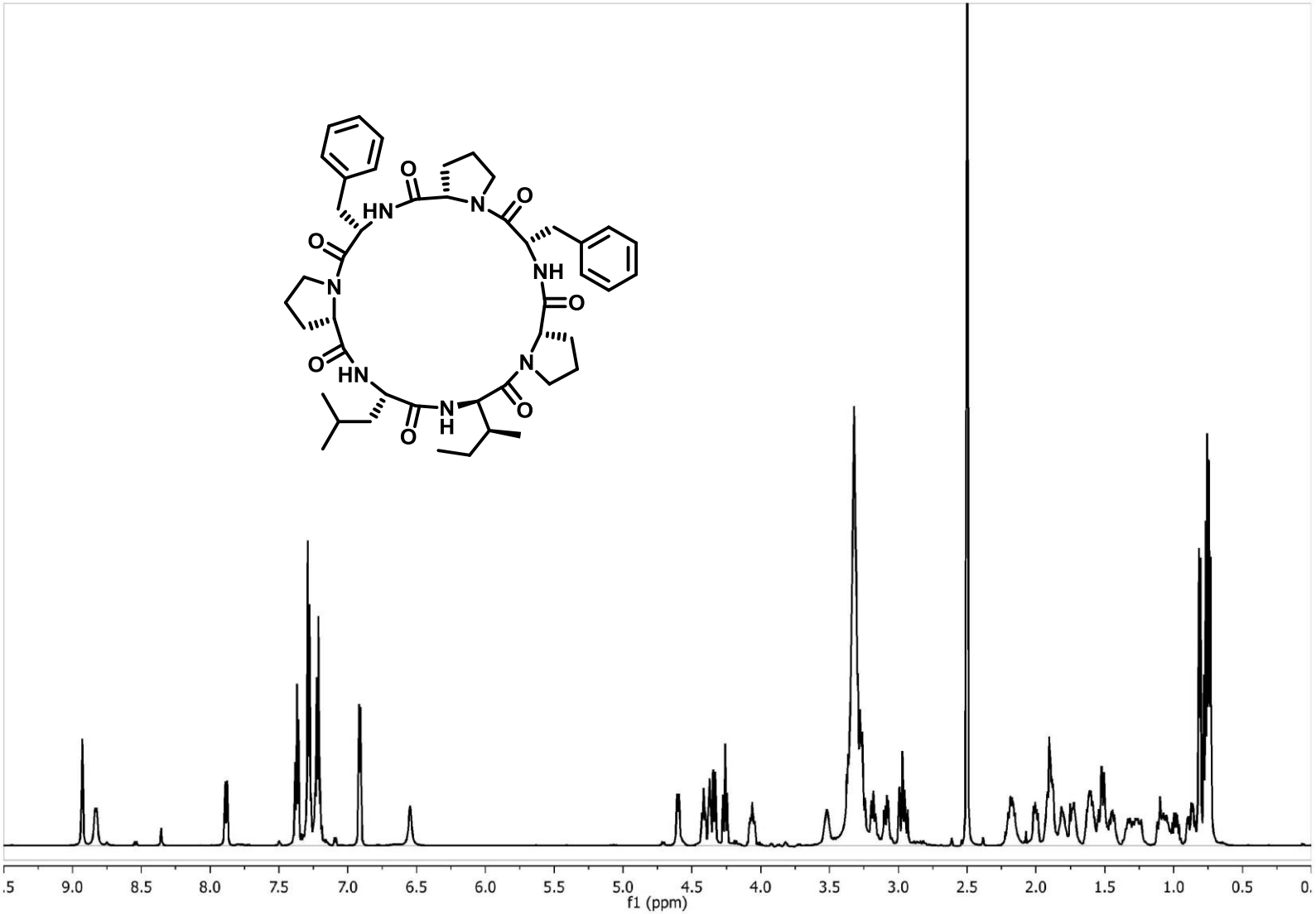
LCMS Spectra for Styllisamide G



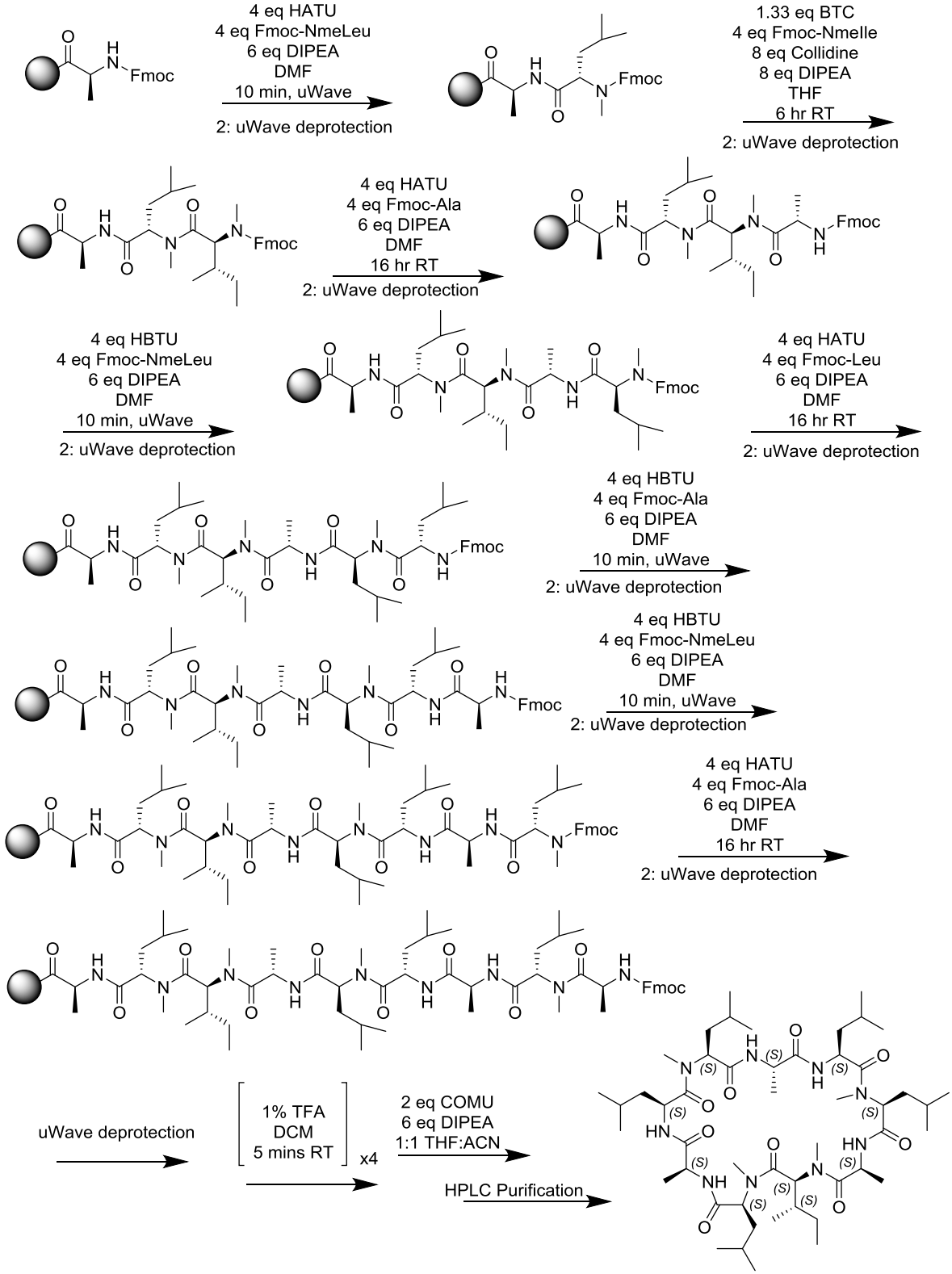




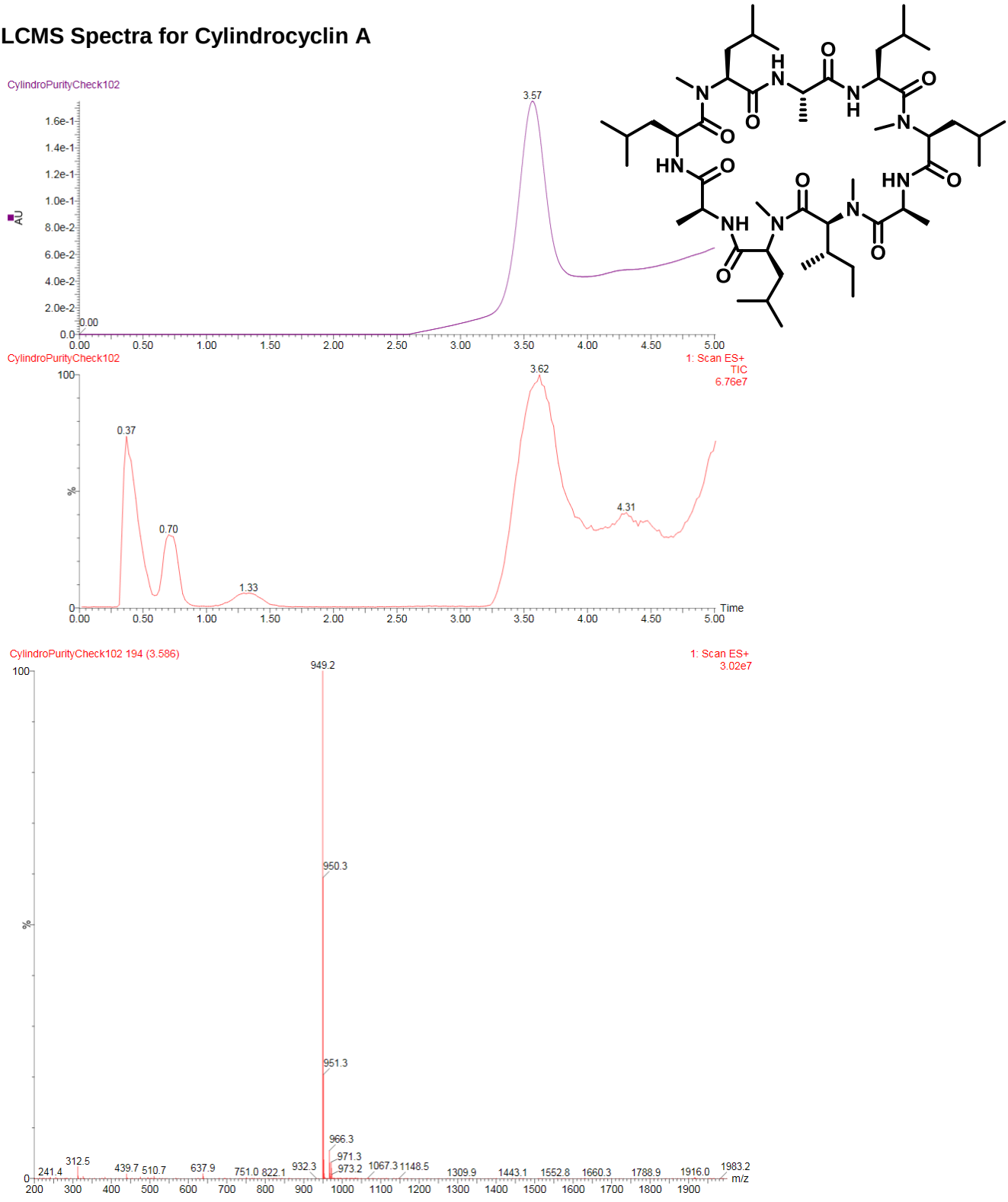
NMR Spectra for Styllisamide G in DMSO *d*6



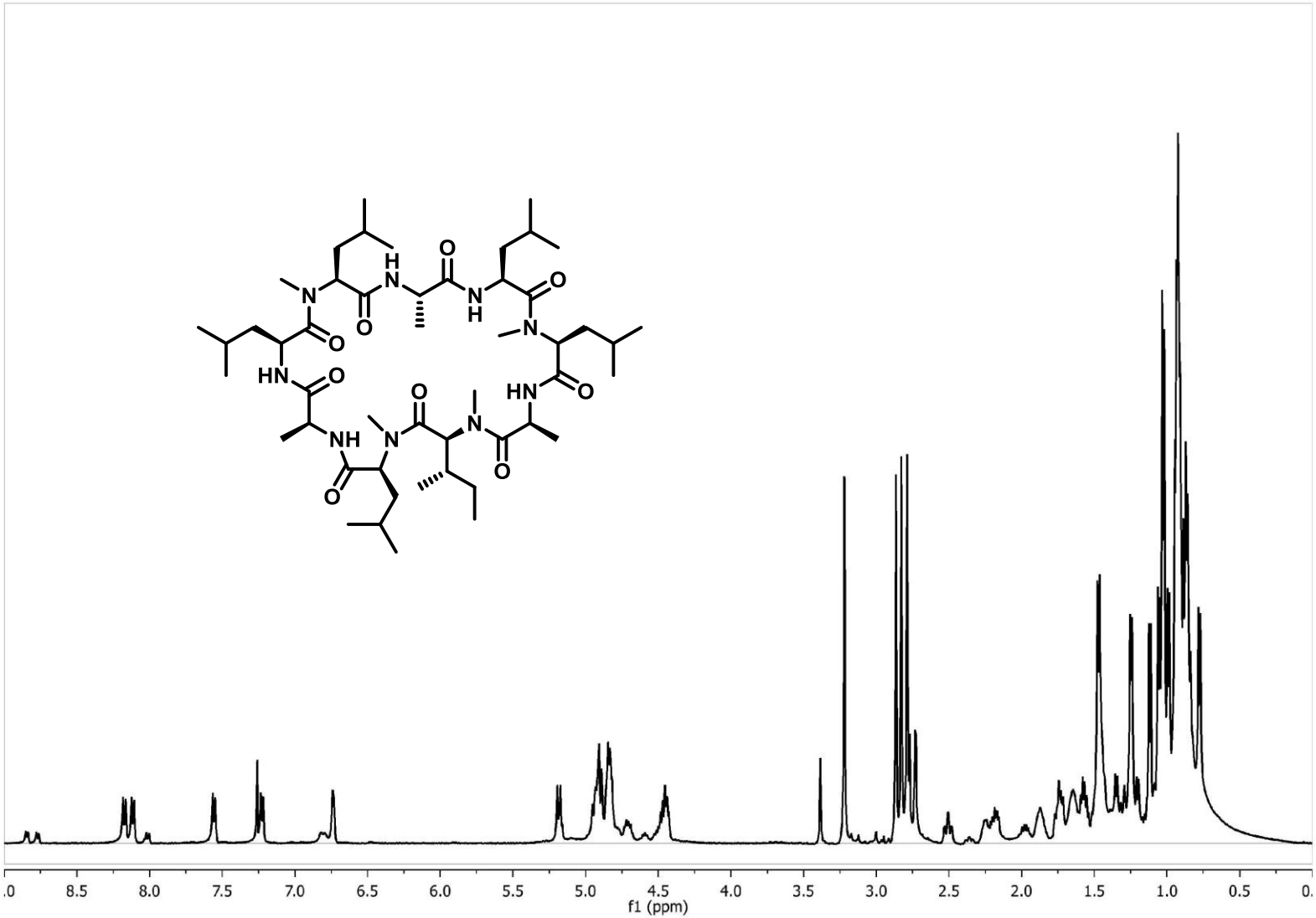
Synthesis of Cylindrocyclin A



LCMS Spectra for Cylindrocyclin A



NMR Spectra for Cylindrocyclin A in $CDCl_3$



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