Design, Synthesis, and Evaluation of Bicyclic Peptides as Ammonium Ionophores

By

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Abstract

A series of bicyclic peptides have been designed and synthesized to provide ammonium ion complexation sites via hydrogen bonding in a tetrahedral geometry. Molecular modeling dynamics and electrostatics studies indicate that target compounds **1d**-**6d** may provide better selectivity for ammonium ions over potassium ions than the ammonium ionophore currently used for blood analysis applications, nonactin. Attempts to synthesize **1d**, cyclo(L-Glu^{1}—D-Val^{2}—L-Ala^{3}—D-Lys^{4}—D-Val^{5}—L-Val^{6})-cyclo-(1γ-4ε), were unsuccessful due to poor solubility of the synthetic intermediates. This led to the design of **2d**-**6d** in which specific amino acid residues were chosen to provide higher solubility. Compound **2d**, cyclo(L-Glu^{1}—D-Ala^{2}—D-Ala^{3}—L-Lys^{4}—D-Ala^{5}—L-Ala^{6})-cyclo-(1γ-4ε), was successfully synthesized, but was also too insoluble for characterization or testing in an ion selective electrode (ISE) sensor format. Compound **6d**, cyclo(L-Glu^{1}—D-Leu^{2}—Aib^{3}—L-Lys^{4}—D-Leu^{5}—D-Ala^{6})-cyclo-(1γ-4ε), was successfully synthesized and characterized. When **6d** was incorporated into an ISE sensor and tested as an ammonium ionophore, results indicated that the bicyclic peptide lacked solubility in the ISE membrane. A ^{13}C-NMR study has been initiated in order to evaluate selectivity of **6d** for ammonium over potassium and sodium cations in solution. Preliminary results with the potassium ionophore valinomycin as a control have been completed.

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1 Introduction

The levels of urea and creatinine in blood are important diagnostic indicators of renal, thyroid, and muscle function.[1] Significant effort has been expended in developing reliable sensors for the detection of these analytes. At the present time, urea and creatinine levels are measured indirectly following enzyme-catalyzed hydrolysis to produce ammonium ions. Typically, measurement of ammonium ion concentrations is achieved by carrier-based ion selective electrodes (ISEs) containing the natural antibiotic nonactin as an ionophore.[2] In ISEs, a highly viscous liquid membrane (such as plasticized PVC) lies between the aqueous sample containing the ion and an internal electrolyte solution. The membrane is doped with a selective ion carrier (nonactin in the case of ammonium) and a lipophilic salt that acts as an ion exchanger. The potential that develops at the membrane/sample interface is proportional to the activity (concentration) of ammonium ions in the aqueous sample.[3]

The two key properties of any ion sensor are sensitivity to the desired ion in the required concentration range and selectivity for one specific ion over all other interfering ions, properties that are primarily imparted by the ionophore. As an ammonium ionophore, nonactin, shown in **Figure 1**, forms four hydrogen bonds from its ethereal oxygens to the ammonium ion.[4] Nonactin-based ISEs show reasonable selectivity for ammonium over sodium ions (log $K_{NH_4^+,Na^+}$ = -2.4)[4], but only modest selectivity over potassium ions (log $K_{NH_4^+,K^+}$ = -1.0)[4]. For this reason, much effort has focused on the development of new

ammonium ionophores, particularly with increased selectivity over potassium ions to improve accuracy of ammonium ion determinations in the presence of potassium ions.[4]

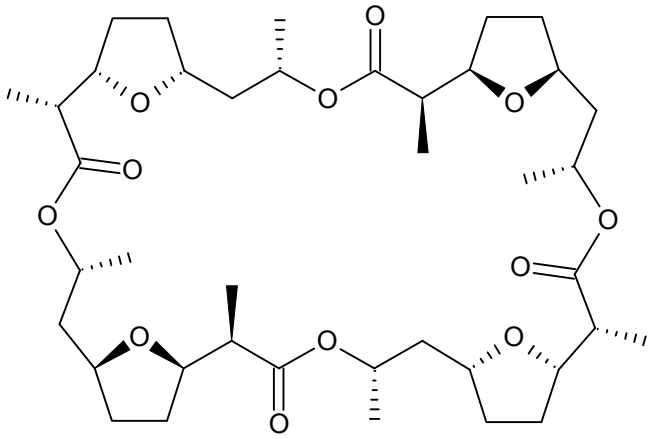


Figure 1: Nonactin, the current industry standard ammonium ionophore

In designing ammonium ionophores, there are three main factors to consider. The first is size-fit requirements. A rigid framework with a cavity appropriately sized for ammonium ion (ionic radius 1.43 Å)[5] is necessary to impart high selectivity over interfering cations of other sizes.[6,7] If the substrate is too flexible, it can change conformations to allow coordination with larger and smaller cations besides the desired ion. In addition, complexation is thermodynamically more favorable when the ionophore is conformationally preorganized into the correct binding geometry in order to minimize the entropic cost of cation binding.[3] Secondly, the ammonium ionophore should exhibit a spatial distribution of lone-pair electrons for effective hydrogen bonding with the tetrahedral ammonium ion.[6] Interfering potassium ions are of similar size (1.33 Å)[5] to ammonium ions, but have spherical symmetry and therefore prefer ionic bonds with coordination numbers of six or higher.[2] For this reason, the coordination geometry is responsible for imparting selectivity for ammonium over potassium ions. Lastly, the

ionophore should be highly lipophilic[6] in order to be compatible with the nonpolar membrane environment[8] of ion selective electrodes and to prevent extraction of the ionophore from the sensor during testing.[3]

One of the earliest ammonium ionophores exhibiting some of these design elements was the spherical macrotricyclic cryptand reported by Lehn et al.[5] (shown in **Figure 2**). This compound exhibited extremely high ammonium over potassium ion selectivity (250 times higher than nonactin) as determined by NMR studies and formed highly stable ammonium complexes (10^5 times higher than nonactin) as calculated by pH metric titration to determine stability constants in aqueous solution. The high selectivity over potassium ion has been attributed to the tetrahedral binding site geometry that favors complexation of the tetrahedral ammonium ion over that of the spherically symmetrical potassium ion. In addition, the macrocyclic nature of the cryptand provides the rigidity necessary to prevent complexation of larger and smaller interfering cations. However, the cryptand is too basic (it exists in its conjugate acid form near neutral blood pH) and hydrophilic (it would leach from the membrane into the aqueous phase during sensor operation) for use as an ionophore in an ISE sensor format.[2] In addition, this system exhibits a very low dissociation constant indicating very slow cation exchange. For this reason, the cryptand is effectively an ammonium ion sink, whereas an ISE sensor application requires reversible ion binding.[8]

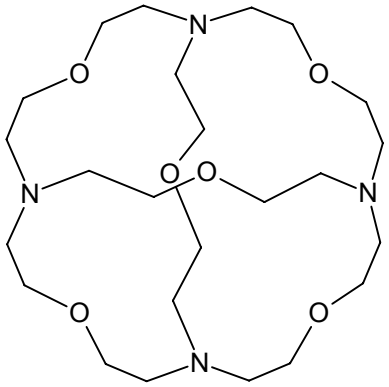


Figure 2: Lehn's macrotricyclic cryptand

Chin et al. reported a rationally designed ammonium receptor, 1,3,5-tris(3,5-dimethylpyrazol-1-ylmethyl)-2,4,6-triethyl benzene (shown in **Figure 3**).[2] This ionophore was designed to have the lone pair electrons on the imine nitrogens preorganized into the correct geometry for binding ammonium ions through hydrogen bonds. The ethyl and methyl groups provide steric interactions to force the receptor into the desired geometry and to block the ligands from binding potassium ions. This ionophore was highly selective (log $K_{NH_4^+,K^+}$ = -2.6 (nonactin –1.0)[4], log $K_{NH_4^+,Na^+}$ = -2.8 (nonactin –2.6)[4]) in an ISE sensor format. However, the ammonium detection limit was 10^{-4} M (100 times higher than nonactin) and therefore the sensor was not sufficiently sensitive.[9] A sensor for blood analysis applications must exhibit sufficient sensitivity to detect lower limits of normal urea and creatinine blood concentrations, which are 1 x 10^{-3} M and 5 x 10^{-5} M respectively.[10]

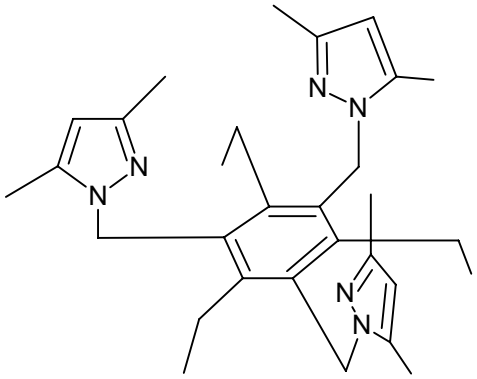


Figure 3: 1,3,5-tris(3,5-dimethylpyrazol-1-ylmethyl)-2,4,6-triethyl benzene ammonium ionophore

Suzuki et al. have synthesized an ammonium ionophore based on a 19-membered crown ether containing three decalino subunits (shown in **Figure 4**).[6] The decalino subunits add rigidity to prevent folding of the receptor to coordinate smaller cations, add bulkiness to block larger interfering cations from entering the cavity, and also increase the lipophilicity of the ionophore. In an ISE sensor format, this ionophore exhibited similar ammonium over potassium ion selectivity (log $K_{NH_4^+,K^+}$ = -1.00 (nonactin –1.0)[4]) and increased ammonium over sodium ion selectivity (log $K_{NH_4^+,Na^+}$ = -3.52 (nonactin –2.6)[4]) compared to nonactin and a nearly Nernstian response (58.1 mV/decade) in the activity range 5 x 10^{-6} – 10^{-1} M ammonium ion. The observation of Nernstian behavior is particularly important. The relation between the potential difference across the sensor membrane and the activity of ammonium ion should follow the well-known Nernst equation (**Equation 1**):

$$E = E° - (2.303RT/nF) \log a \qquad \textbf{(1)}$$

Here, E is the electromotive force (emf) of the cell in volts, E° is the emf cell constant, F is the Faraday constant, *a* is the activity of the analyte, n is the charge of the measured species, R is the gas constant, and T is the temperature. This equation is in the form of y = mx + b and therefore a plot of E versus log *a* will give a straight line with a slope of 2.303RT/nF. For the measurement of monovalent cations at 25°C, n = 1 and the slope becomes 59.16 mV/dec. Thus an ISE that measures ammonium ion and operates according to the Nernst equation should exhibit a slope of 59.16 mV/dec.

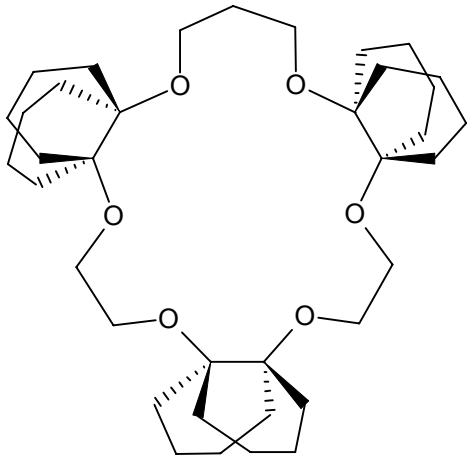


Figure 4: 19-crown-6 ether with decalino subunits

Nam et al. have reported a thiazole containing dibenzo-18-crown-6 derivative (shown in **Figure 5**) as an ammonium ionophore in an ISE sensor format.[9] This design is primarily based on size-fit factors. The aromatic units increase rigidity and the thiazoles provide hydrogen bonding sites. This ionophore exhibited high selectivity (log $K_{NH_4^+,K^+}$ = -1.3 (nonactin –1.0)[4], log $K_{NH_4^+,Na^+}$ = -3.9 (nonactin –2.6)[4]) and a similar detection limit of ~3 x 10^{-6} M compared to nonactin (10^{-6} M)[6] in an ISE sensor format.

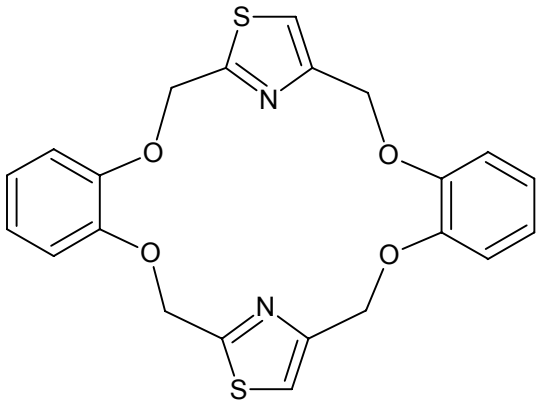


Figure 5: Dibenzo-18-crown-6 ether with thiazole subunits

Kim et al. designed and synthesized an ammonium receptor based on both hydrogen bonding and cation-π interactions, consisting of two benzene rings held rigidly together by three benzocrown ether units as shown in **Figure 6**.[6] This ionophore design involves a rigid cavity of the appropriate size containing a spatial distribution of ether lone pair electrons for tetrahedral coordination to ammonium along with cation-π interactions provided by the top and bottom benzene rings. Compared to nonactin-based sensors, ISEs doped with this ionophore exhibited similar selectivity (log $K_{NH_4^+,K^+}$ = -0.97 (nonactin –1.0)[4], log $K_{NH_4^+,Na^+}$ = -3.00 (nonactin –2.6)[4]) and a similar detection limit of 3.2 x 10^{-6} M (nonactin (10^{-6} M)[6]).

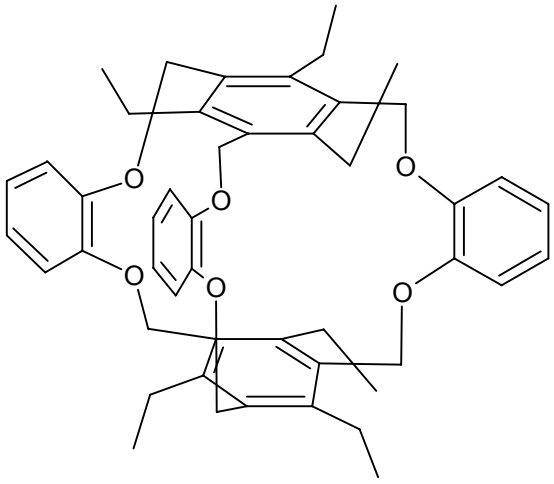


Figure 6: Two benzene rings held together by three benzocrown ether units

McGimpsey et al. have designed and synthesized an ammonium ionophore for an ISE based on a cyclic depsipeptide structure.[8] The design of this ionophore was inspired by valinomycin, the naturally occurring antibiotic having a high selectivity for potassium ions. Valinomycin, a cyclic depsipeptide consisting of alternating amide and ester linkages (12 total) (shown in **Figure 7a**), preorganizes through hydrogen bonding of its amide carbonyl groups to form a pocket with its six ester carbonyl oxygens available for electrostatic stabilization of potassium ions through octahedral-type complexation.[11] The new ionophore (shown in **Figure 7b**) consists of alternating amide and ester units (6 total), which is effectively half of a valinomycin molecule. Unlike valinomycin, this depsipeptide is too rigid to fold upon itself and therefore provides a cavity appropriately sized for ammonium ions, which provides the tetrahedral complexation geometry required for ammonium ion binding, but not the octahedral binding geometry required by potassium ions. ISE sensors incorporating this ionophore exhibited similar selectivity for ammonium over potassium and sodium ions compared to nonactin-based sensors (log

$K_{NH_4^+,K^+}$ = -0.6 (nonactin –1.0)[4], log $K_{NH_4^+,Na^+}$ = -2.1 (nonactin –2.4)[4]) and a nearly Nernstian response (60.1 mV/decade at 37˚C).

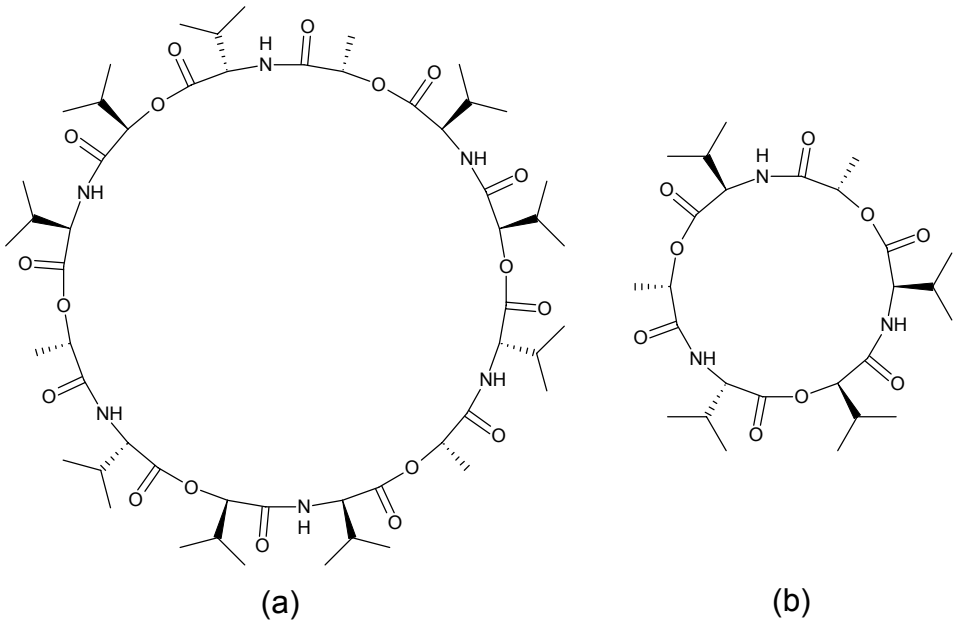


Figure 7: (a) Valinomycin (b) new depsipeptide ammonium ionophore

Cyclic peptides are known to bind and transport metal cations in biological systems.[12] Their ease of synthesis and potential for flexible sequence modification make them good candidates for new ionophores.[13] However, ordinary cyclic peptides are too flexible to bind substrates in a well-defined cavity[14], leading to low selectivity as sensor components. The addition of a second ring in bicyclic peptides should increase cation binding selectivity by increasing rigidity. Andreu et al. have synthesized cyclo(1,5-ε-succinoyl) (Lys-Gly-Gly-Gly)$_2$ (shown in **Figure 8a**) which exhibits a slight preference for binding Sr^{2+} over other cations.[13] Zanotti et al. have reported synthesis, conformation, and calcium-binding studies for the bicyclic nonapeptides cyclo(Glu^1-X^2-

Pro[3]-Gly[4]-Lys[5]-X[6]-Pro[7]-Gly[8])-cyclo-(1γ → 5ε) Gly[9], where X = Ala or Leu (shown in **Figure 8b**).[15,16]

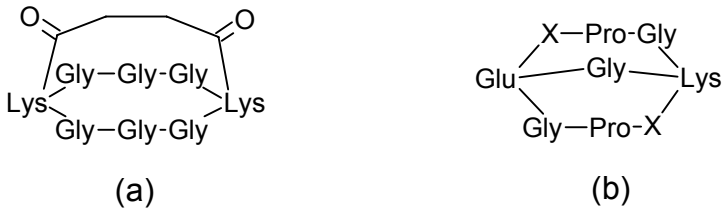


Figure 8: (a) cyclo(1,5- ε-succinoyl) (Lys-Gly-Gly-Gly)$_2$ (b) cyclo(Glu[1]-X[2]-Pro[3]-Gly[4]-Lys[5]-X[6]-Pro[7]-Gly[8])-cyclo-(1γ → 5ε) Gly[9], where X = Ala or Leu

Our research has been directed toward the synthesis and evaluation of new bicyclic peptide ammonium ionophores with predicted ammonium over potassium ion selectivity greater than the industry standard nonactin in an ISE sensor format in order to improve the accuracy of ammonium ion determinations in the presence of potassium ions for clinical evaluation of urea and creatinine levels. The ease of synthesis and demonstrated cation-binding ability make bicyclic peptides promising candidates. Molecular modeling suggests the target compounds **1d**, **2d**, **3d**, **4d**, **5d**, and **6d** shown in **Figure 9** should exhibit tetrahedral coordination geometry in a cavity appropriately sized for ammonium ions.

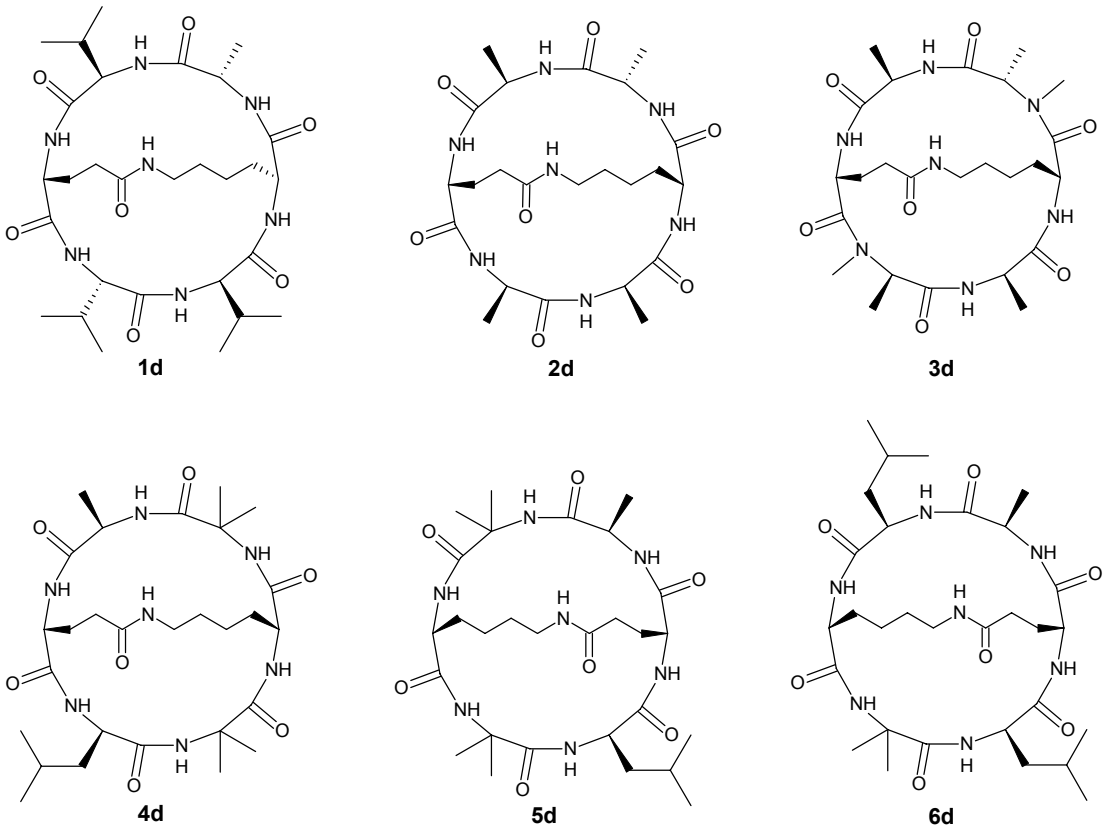


Figure 9: Target bicyclic peptide ammonium ionophores

The naming system for these compounds is based on their preparation from three important synthetic precursors, as shown in **Figure 10** for target **1d** (applicable to all target compounds).

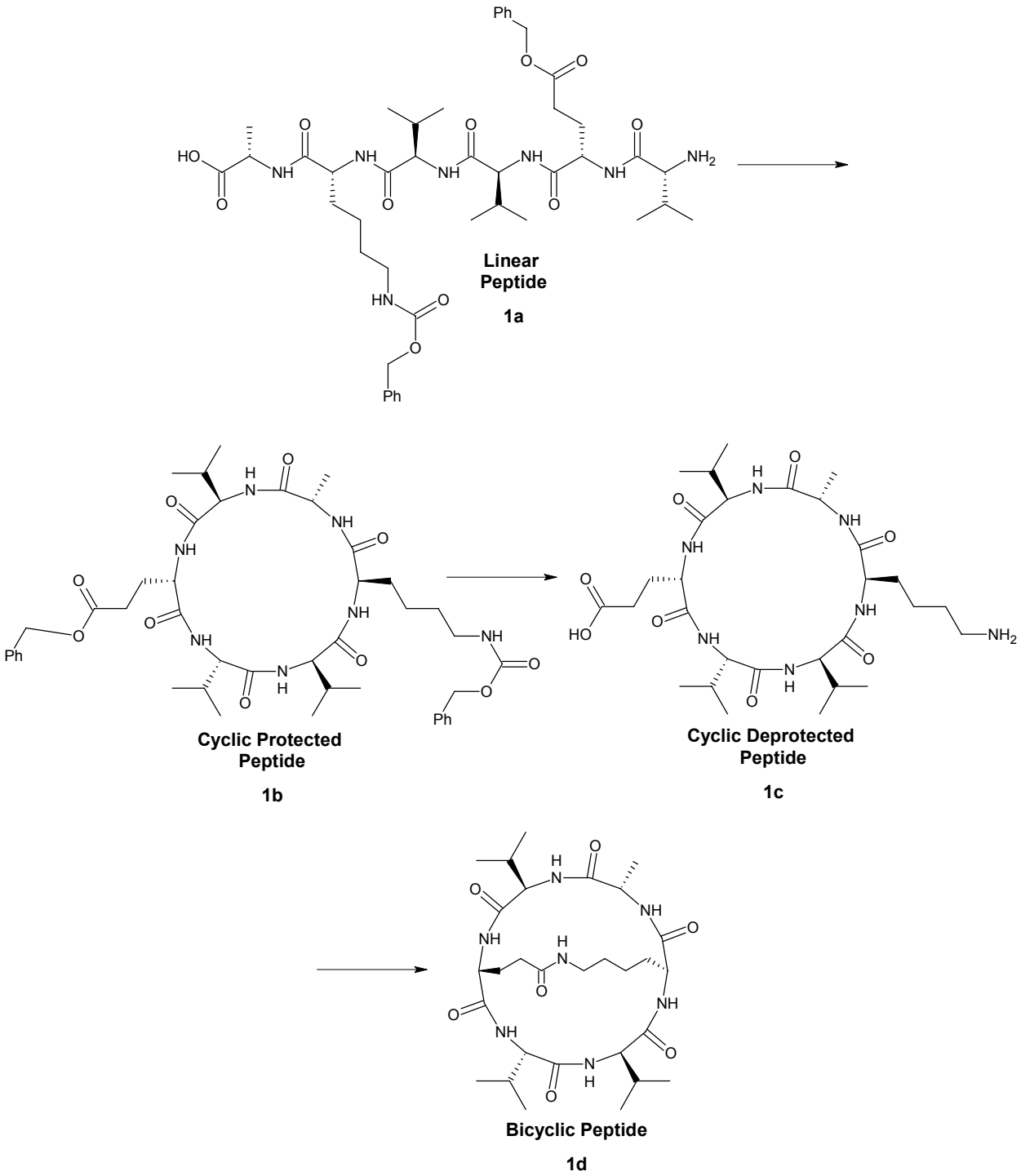


Figure 10: General synthetic scheme for target compound **1d**

2 Experimental

2.1 General Methods and Materials

Mass spectra were performed by Synpep Corp. (Dublin, CA) and Bayer Diagnostics Analytical Department (Medfield, MA). ^{1}H and ^{13}C-NMR were recorded on a Bruker Avance 400. All Fmoc-protected amino acids, all Wang resins, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), and 1-hydroxybenzo-triazole hydrate (HOBT) were purchased from Calbiochem-Novabiochem Corp. All solvents and reagents were analytical reagent grade, purchased from local suppliers, and used as received without further purification. Buffers were prepared with deionized water (18 MΩ·cm).

2.2 Molecular Modeling Calculations

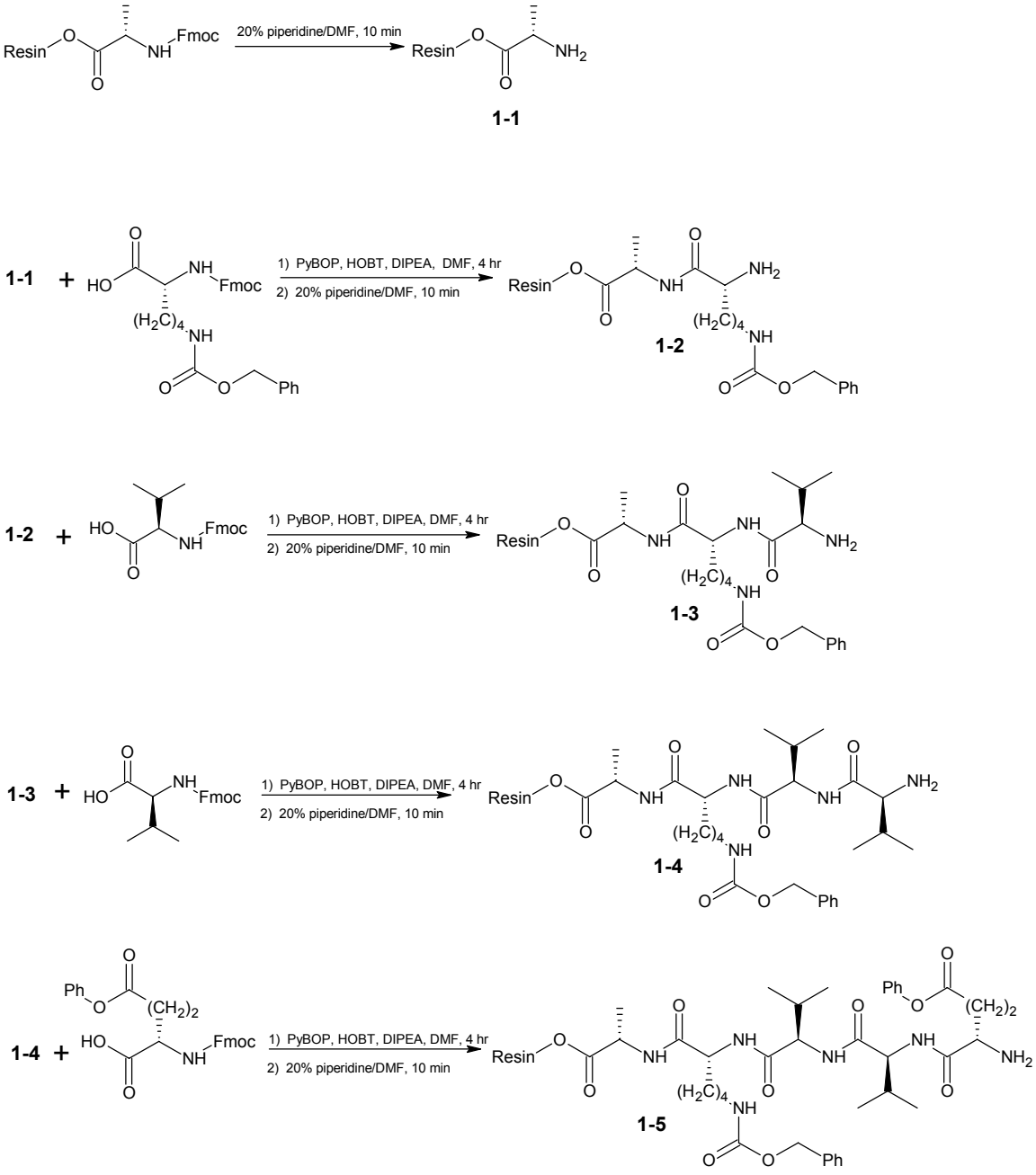
Molecular modeling was performed on an SGI 320 running Windows NT, as previously reported.[8] Calculations were carried out using the Molecular Operating Environment version 2000.02 computing package (Chemical Computing Group Inc., Montreal, PQ, Canada). Structures were minimized first using the AMBER94 potential control under a solvent dielectric of 5. PEF95SAC was used to calculate partial charges. Minimized structures were then subjected to a 30-ps molecular dynamics simulation employing the NVT statistical ensemble. The structures were heated to 400 K, equilibrated at 310 K, and cooled to 290 K in the dynamics thermal cycle at a rate of 10 K/ps. The lowest energy structures obtained from these dynamics calculations were then minimized again.

Using the minimized structures, docking energies of the ammonium and the potassium cations were calculated by employing the default parameters supplied with the program.

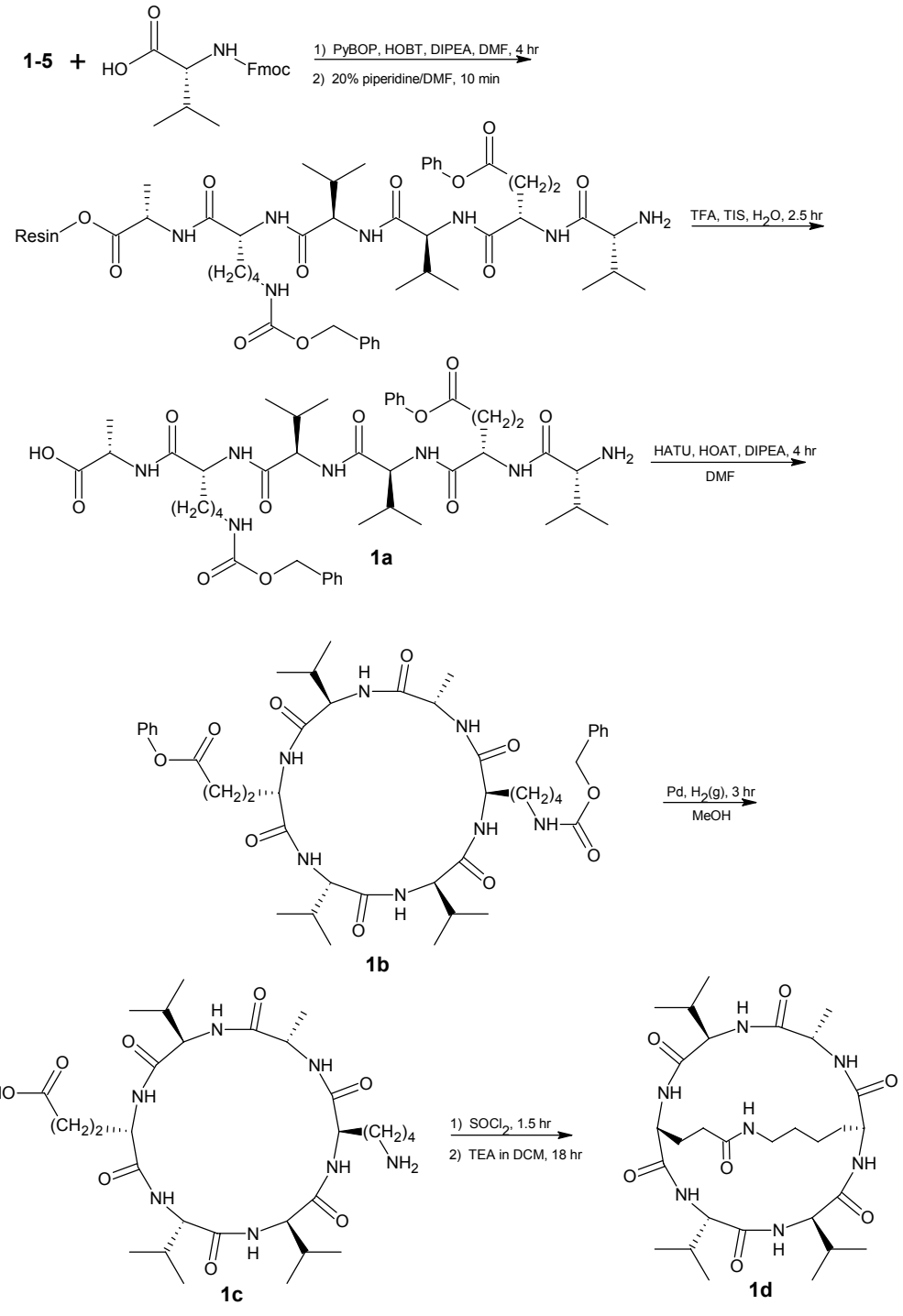
2.3 Synthesis

The synthetic scheme for target compound **1d** is shown in **Scheme 1**.

Scheme 1: Synthesis of **1d**



Scheme 1: continued



2.3.1 Synthesis of 1a

Solid phase peptide synthesis was carried out on 5 g of Fmoc-Ala-Wang resin (0.41 mmol/g loading). The resin was swelled by adding 30 mL DMF and mixing with N_2 for 30 min., at which point the DMF was removed by aspiration. The resin-bound N-Fmoc protected alanine was deprotected with 20% piperidine in DMF (30 mL, 10 min.). The solution was removed by aspiration and the resin was washed 3x with 30 mL DMF, 3x with 30 mL MeOH, 1x with 30 mL EtOH, and vacuum dried. A Kaiser test was performed by adding 2 drops of each of three solutions to a few resin beads and heating in the oven for 3-4 min. The solutions were prepared by dissolving 5 g of ninhydrin in 100 mL EtOH, dissolving 80 g of phenol in 20 mL EtOH, and adding 2 mL of a 0.001 M aqueous solution of potassium cyanide to 98 mL pyridine.[17] A positive Kaiser test for free amine, as indicated by blue beads, confirmed successful deprotection. The resin was reswelled in 30 mL of DMF mixed with N_2 for 10 min. 2.60 g (2.5 eq) Fmoc-D-Lys(Z), 2.67 g (2.5 eq) PyBOP, 0.69 g (2.5 eq) HOBT, and 1.8 mL (5 eq) diisopropylethylamine (DIPEA) were dissolved in 5 mL DMF and added to the prepared resin. The total volume was increased to 30 mL and mixed with N_2 for 4 hr. at which time the solution was removed by aspiration and the resin was rinsed 3x with DMF, 3x with MeOH, 1x with EtOH, and vacuum dried. A negative Kaiser test confirmed complete coupling. This [deprotection - rinses - Kaiser test - coupling reaction - rinses - Kaiser test] cycle was repeated for the remaining four amino acid residues to give (Resin)—(L-Ala)—(D-Lys(Z))—(D-Val)—(L-Val)—(L-Glu(OBzl))—(D-Val-Fmoc). In the event of incomplete coupling, as indicated by a positive Kaiser test, the coupling reaction was repeated. The terminal valine residue was deprotected and a Kaiser test was performed to

confirm successful deprotection. The resin was rinsed 3x with DMF, 3x with MeOH, 2x with DMF, 2x with MeOH, and vacuum dried.

The linear peptide was cleaved from the resin with 30 mL of TFA/H_20/triisopropylsilane (TIS) 95/2.5/2.5 over 2.5 hr. by mixing with N_2. The peptide solution was removed by aspiration and concentrated down to a few mL's. Cold ether precipitated the linear peptide as a white solid, which was collected by filtration. The peptide was dissolved in MeOH and reprecipitated twice to obtain 1.2 g (67% yield) of a white powder. ^{1}H and ^{13}C-NMR and ESI MS of this and subsequent compounds may be found in **Appendix A**. ^{1}H-NMR (400 MHz, DMSO), δ 0.64-1.02 (m, 18H), 1.13-1.68 (m, 9H), 1.75-2.15 (m, 5H), 2.28-2.51 (m, 2H), 2.88-3.02 (m, 2H), 4.10-4.56 (m, 7H), 5.00 (s, 2H), 5.08 (s, 2H), 7.17-7.51 (m, 12H), 7.65-8.18 (m, 7H), 8.42 (d, J = 7.8 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO), δ 17.7, 17.9, 18.2, 18.2, 18.7, 18.9, 19.7 (CH_3), 22.9, 28.1, 29.4 (CH_2), 30.3 (CH), 30.4 (CH_2), 30.8, 31.1 (CH), 32.2, 40.5 (CH_2), 47.8, 52.2, 52.7, 57.7, 57.7, 58.2 (CH), 65.5, 65.9 (CH_2), 128.1, 128.3, 128.4, 128.7, 128.8 (CH, Ar), 136.5, 137.6 (C, Ar), 156.4, 168.5, 170.9, 171.0, 171.1, 171.4, 172.3, 174.3 (C=O). ESI MS m/z (%) calcd. for $C_{44}H_{66}N_7O_{11}$ ($M+H^+$) 868.5 found 868.2 (100).

2.3.2 Synthesis of 1b

The 1.20 g (1.38 mmol) of linear peptide **1a** was dissolved in approximately 450 mL DMF and 50 mL benzene. 0.682 g (1.3 eq) O-(7-Azabenzotriazole-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), 0.244 g (1.3 eq) 1-Hydroxy-7-

azabenzotriazole (HOAT), and 2.4 mL (10 eq) DIPEA dissolved in 5 mL of DMF was added and the solution was stirred for 24 hr. at room temperature. At this point, the DMF was removed in vacuo, 55-60 ˚C. The residue was dissolved in DCM and extracted 3x with 100 mL of saturated $NaHCO_3$, 3x with 100 mL of 10% citric acid, and dried over Na_2SO_4. The organic solution was concentrated completely, and then dissolved in MeOH. The peptide was precipitated with ether/hexanes at –4 ˚C and collected by filtration to give 170 mg (14% yield) of crude monocyclized peptide. Characterization by ^1H and ^{13}C-NMR and ESI MS was inconclusive.

2.3.3 Synthesis of 1c

The 170 mg (0.200 mmol) of **1b** was combined with 30 mg from a previous synthesis and dissolved in 100 mL of MeOH. The benzyl protecting groups on the glutamic acid and lysine side chains were removed by using 0.2 g of Pd activated carbon (10 wt%), and H_2 at atmospheric pressure while stirring the solution for 3 hr. The spent catalyst was removed by filtration and the solution was concentrated totally in vacuo, 40 ˚C. ^1H and ^{13}C-NMR spectra in DMSO confirmed loss of benzyl groups. The 120 mg (96% yield) of peptide was used without further purification or characterization.

2.3.4 Synthesis of 1d

The 120 mg (0.192 mmol) of **1c** was stirred in 15 mL thionyl chloride for 1.5 hr. at which point the thionyl chloride was removed in vacuo. The residue was dissolved in benzene,

and then concentrated completely to remove residual thionyl chloride. The peptide acid chloride was dissolved in 30 mL DCM, to which 0.27 mL (10 eq) triethylamine (TEA) was added and the solution was stirred for 18 hr. at which point, the solution was concentrated completely, then redissolved in 5% MeOH/DCM. The addition of cold ether precipitated 20 mg of a brown solid which was collected by filtration. Characterization by ESI MS was inconclusive.

2.3.5 Synthesis of 2a

Solid phase synthesis was carried out on 5.035 g of Fmoc-Ala-Wang resin (0.41 mmol/g loading) for the amino acid sequence (L-Ala)—(L-Lys(Z))—(D-Ala)—(D-Ala)—(L-Glu(OBzl))—(D-Ala) using the same general solid phase methods described for compound **1a**.

The linear peptide was cleaved from the resin with 30 mL of TFA/H_20/TIS 95/2.5/2.5 mixed with N_2 for 2.5 hr. The peptide solution was removed by aspiration and concentrated down to a few mL's. Cold ether precipitated the linear peptide as a white solid, which was collected by filtration. The peptide was dissolved in MeOH, precipitated with cold ether, and filtered to obtain 1.24 g (77% yield) of a white powder. R_f(RP) .35 (1.9:1 H_2O/MeCN); ^{1}H-NMR (400 MHz, DMSO), δ 0.90-1.33 (m, 21H), 1.35-1.49 (m, 1H), 1.53-1.77 (m, 2H), 2.05-2.31 (m, 4H), 2.62-2.77 (m, 2H), 3.10-3.26 (m, 1H), 3.53-3.66 (m, 1H), 3.75-4.14 (m, 7H), 4.76 (s, 2H), 4.85 (s, 2H), 7.00 (t, J = 5.6 Hz, 1H), 7.03-7.22 (m, 10H, Ar), 7.67 (d, J = 8.8 Hz, 1H), 7.75 (d, J = 7.1 Hz, 1H), 7.83

(d, J = 7.1 Hz, 1H), 8.19 (d, J = 7.3 Hz, 1H), 8.59 (d, J = 8.1 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO), δ 17.9, 17.9, 18.4, 18.4 (CH_3), 22.8, 27.7, 29.4, 30.2, 32.0, 40.5 (CH_2), 48.4, 48.6, 48.6, 48.8, 52.1, 52.4 (CH), 65.5, 66.0 (CH_2), 128.1, 128.3, 128.4, 128.7, 128.8 (CH, Ar), 136.4, 137.6 (C, Ar), 156.4, 170.4, 170.8, 171.6, 172.1, 172.3, 172.3, 174.7 (C=O). ESI MS m/z (%) calcd. for $C_{38}H_{54}N_7O_{11}$ ($M+H^+$) 784.4 found 784.0 (100).

2.3.6 Synthesis of 2b

1.26 g (1.61 mmol) of **2a** was dissolved in 600 mL of DMF and stirred at 0 ˚C. 0.791 g (1.3 eq) HATU, 0.283 g (1.3 eq) HOAT, and 2.8 mL (10 eq) DIPEA dissolved in 5 mL of DMF was added and the reaction mixture was stirred for 4 hr., at which point the DMF was removed in vacuo, 55-60 ˚C. The residue was dissolved in MeOH and cold ether was added to precipitate a white solid, which was collected by filtration and redissolved in MeOH. A white solid that precipitated out of the MeOH solution was removed by filtration. The solution was concentrated completely and purified by reverse phase (C18) column chromatography (1.2:1 H_2O:MeCN) to obtain 250 mg (20% yield) of a white solid. R_f(RP) .31 (1.2:1 H_2O/MeCN); ^{1}H-NMR (400 MHz, MeOD), δ 1.50-1.82 (m, 18H), 1.91-2.14 (m, 2H), 2.17-2.37 (m, 2H), 2.64-2.79 (m, 2H), 3.31-3.44 (m, 2H), 4.28-4.33 (m, 1H), 4.38-4.44 (m, 1H), 4.51-4.61 (m, 2H), 4.68-4.80 (m, 2H), 5.31 (s, 2H), 5.37 (s, 2H), 7.47-7.71 (m, 10H, Ar); ^{13}C-NMR (100 MHz, MeOD), δ 17.4, 17.9, 18.1, 19.2 (CH_3), 24.4, 27.6, 30.9, 31.5, 32.2, 41.6 (CH_2), 50.0, 50.7, 51.0, 52.0, 55.0, 56.7 (CH), 67.8, 67.9 (CH_2), 129.2, 129.4, 129.6, 129.7, 129.9, 130.0 (CH, Ar), 137.9, 138.8 (C, Ar),

159.4, 174.3, 174.6, 174.6, 174.8, 174.8, 174.9, 175.9 (C=O). ESI MS m/z (%) calcd. for $C_{38}H_{52}N_7O_{10}$ ($M+H^+$) 766.4 found 766.3 (6), calcd. $M+Na^+$ 788.4 found 788.3 (100).

2.3.7 Synthesis of 2c

250 mg of **2b** was combined with 120 mg from a previous synthesis for a total of 370 mg (0.483 mmol) and dissolved in 50 mL of MeOH. The benzyl protecting groups on the glutamic acid and lysine side chains were removed by using 0.2 g of Pd activated carbon (10 wt%), and H_2 at atmospheric pressure while stirring the solution for 3.5 hr. The spent catalyst was removed by filtration and the solution was concentrated completely. Analytical TLC with ninhydrin tests showed a product mixture and therefore the hydrogenation reaction was repeated for 3.5 hr. The product was 160 mg (61% yield) of a white solid. Essentially complete loss of benzyl protecting groups was confirmed by ^1H and ^{13}C-NMR. The peptide was used without further purification or characterization.

2.3.8 Synthesis of 2d

160 mg (0.295 mmol) of **2c** was dissolved in 125 mL of DMF and stirred at 0 ˚C. 0.146 (1.3 eq) HATU, 0.052 g (1.3 eq) HOAT, and 0.52 mL (10 eq) DIPEA dissolved in 5 mL of DMF was added and the reaction mixture was stirred for 4 hr., at which point the DMF was removed in vacuo, 45˚C. The residue was dissolved in MeOH and a white suspension was removed by centrifugation. The solution was concentrated completely and extracted 3x with DCM/H_20. The aqueous portion was vacuum evaporated and

dissolved in 1:1:3 MeOH:EtOAc:DCM. The insoluble portion was removed by filtration. Again, the solution was concentrated completely, dissolved in MeOH, reprecipitated with cold ether, and collected by centrifugation 4x to obtain 45 mg (29% yield) of a yellow solid. This was redissolved in 50 mL of MeOH, cooled to 5 ˚C for 4 hr., and centrifuged to collect 10 mg of an insoluble white precipitate. ESI MS m/z (%) calcd. for $C_{23}H_{37}N_7O_7Na$ ($M+Na^+$) 546.3 found 546.3 (25) in the partially purified product (prior to the final precipitation from MeOH).

2.3.9 Synthesis of 3a

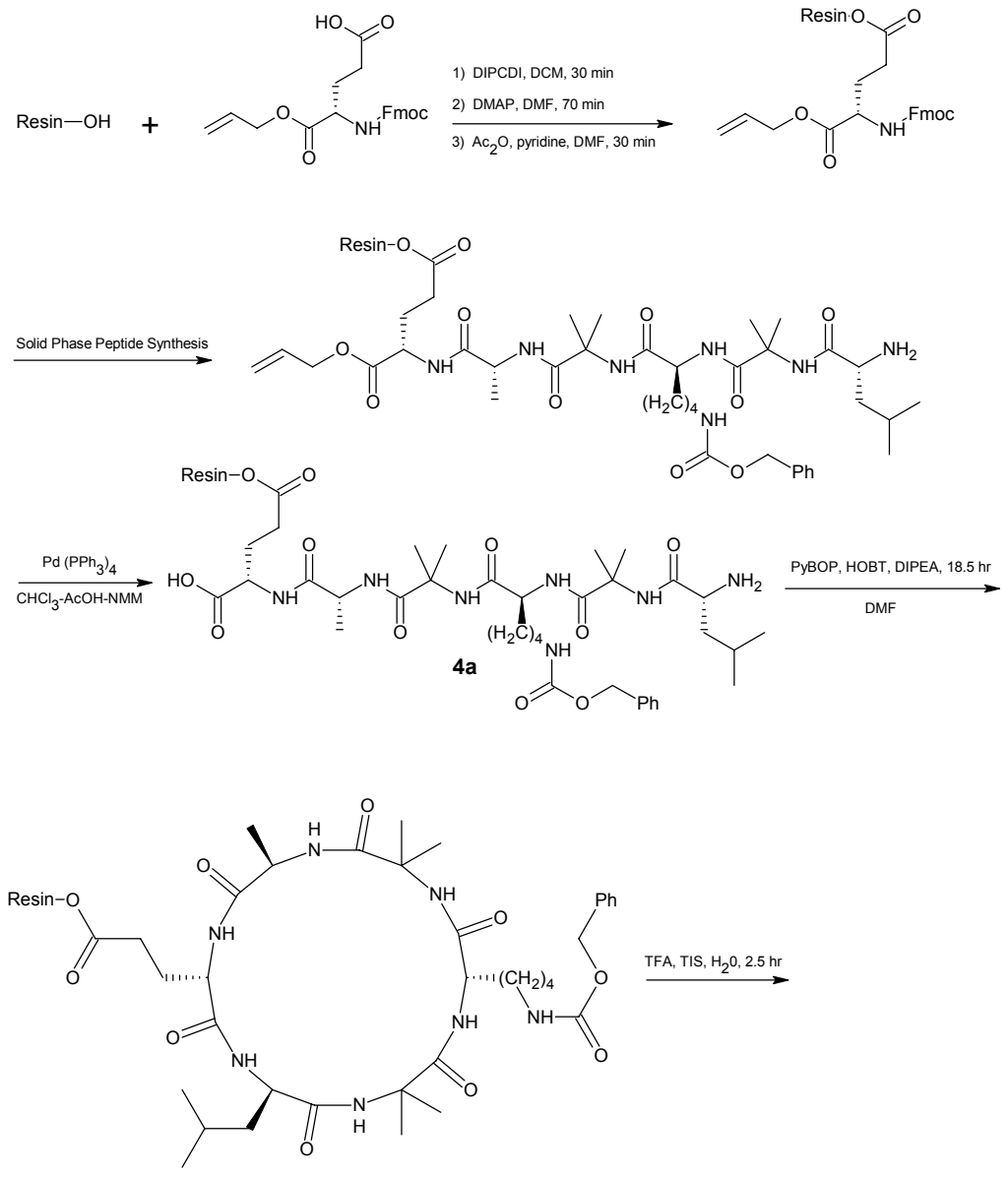
Solid phase synthesis was carried out on Wang resin (1.1 mmol/g loading) for the amino acid sequence (L-MeAla)—(L-Lys(Z))—(D-Ala)—(D-MeAla)—(L-Glu(OBzl))—(D-Ala). 2.803 g Wang resin was swelled in 30 mL DMF mixed with N_2 for 30 min. at which point the DMF was removed by aspiration. 3.006 g (3 eq) of Fmoc-MeAla was dissolved in 50 mL of dry DCM and stirred at 0 ˚C. 1.45 mL diisopropylcarbodiimide (DIPCDI) in 5 mL of DMF was added and the reaction mixture was stirred for 30 min. At this point, the solution was concentrated completely, dissolved in 5 mL DMF, and added to the resin. 0.038 g (0.1 eq) dimethylaminopyridine (DMAP) in DMF was added to the resin and the total volume was increased to 40 mL. The solution was mixed with N_2 for 1.5 hr. covered at room temperature. Loading of the first amino acid to the resin was evaluated by cleaving the Fmoc protection group from a known mass of resin (20 mg) in a 100 mL solution of 20% piperidine in DMF and monitoring the UV absorption at 290 nm. Using a molar extinction coefficient of 4950 a loading of 72% was obtained.

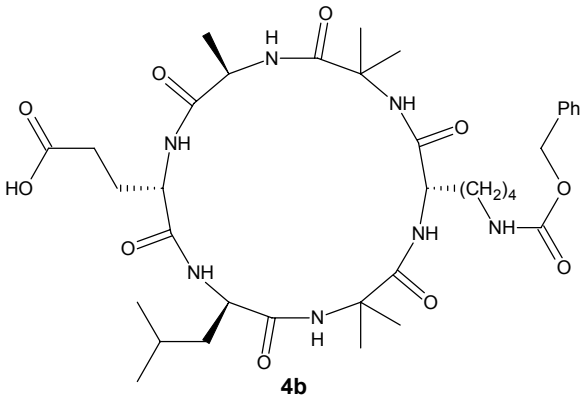
Stepwise addition of the next 5 amino acids was carried out according to the standard solid phase method described for compound **1a**. The Kaiser test was inconclusive for secondary amines, so completeness of reaction was monitored by UV absorption of Fmoc when coupling to resin-bound terminal MeAla residues. In addition, deprotection reactions were allowed longer reaction time (15-20 min.) and repeated.

The linear peptide was cleaved from the resin with 30 mL of TFA/H_20/TIS 95/2.5/2.5 over 2.5 hr. by mixing with N_2. The peptide solution was removed by aspiration and concentrated completely to yield an oil. ESI MS indicated that none of the desired product was formed.

The general scheme for synthesis of the intermediates **4a** and **4b** of target compound **4d** is shown in **Scheme 2**.

Scheme 2: Synthesis of **4a** and **4b**





4b

2.3.10 Synthesis of 4a

Solid phase synthesis was carried out on Wang resin (1.0 mmol/g loading) for the amino acid sequence (L-Glu-OAll)—(D-Ala)—(Aib)—(L-Lys(Z))—(Aib)—(D-Leu). 2.013 g Wang resin was swelled in 30 mL DMF mixed with N_2 for 30 min. at which point the DMF was removed by aspiration. 2.059 g (2.5 eq) of Fmoc-Glu-OAll was dissolved in 50 mL of dry DCM and stirred at 0 ˚C. 0.79 mL DIPCDI in 5 mL of DMF was added and the reaction mixture was stirred for 30 min., at which point the solution was concentrated completely, dissolved in 5 mL DMF, and added to the resin. 0.025 g (0.1 eq) DMAP in DMF was added to the resin and the total volume was increased to 40 mL. The solution was mixed with N_2 for 70 min. at room temperature. Loading of the first amino acid to the resin was evaluated by cleaving the Fmoc protecting group from a known mass of resin (20 mg) in a 100 mL solution of 20% piperidine in DMF and monitoring the UV absorption at 290 nm. Using a molar extinction coefficient of 4950 a loading of 59% was obtained. The resin was capped with 0.22 mL (2 eq to resin-glutamic acid) acetic anhydride and 0.19 mL (2 eq to resin-glutamic acid) pyridine in 30

mL of DMF for 30 min. mixing with N_2. Stepwise addition of the next 5 amino acids was carried out according to the solid phase method described for compound **1a**. The Kaiser test was inconclusive for aminoisobutyric acid free amine, so coupling reactions were repeated twice for 10 hr. for resin-bound terminal Aib residues.

The glutamic acid O-Allyl protecting group was removed according to a modified procedure.[17] A solution of 5.398 g (3 eq) of tetrakis(triphenylphosphine)palladium(0) dissolved in 67.5 mL (15 mL/g of resin) 37:2:1 chloroform/acetic acid/N-methylmorpholine (NMM) was added to the resin and mixed with N_2 for 2 hr. At this point, the solution was filtered off and the resin was subjected to a series of rinses: 30 mL 0.5% DIPEA in DMF, 3x 2 min.; 30 mL 0.5% sodium diethyldithiocarbamate in DMF, 3x 10 min.; 30 mL DMF, 1x 2 hr.; 30 mL DMF, 1x 10 min.; 30 mL 0.5% HOBT in DMF, 3x 5 min.; 30 mL DMF, 3x 1 min.; 30 mL MeOH, 3x 1 min.; 30 mL EtOH, 1x 1 min. The deprotection reaction was repeated and the resin was subjected to similar rinses.

2.3.11 Synthesis of 4b

The resin was swelled in 30 mL DMF mixing with N_2 for 30 min. to prepare for on-resin cyclization. 2.024 g (2.5 eq) PyBOP, 0.526 g (2.5 eq) HOBT, and 1.4 mL (5 eq) DIPEA dissolved in 5 mL DMF was added to the resin. The volume was increased to 50 mL and mixed with N_2 for 18.5 hr. The resin was subjected to the normal solid phase rinses and vacuum dried. The Kaiser test was weakly positive, indicating incomplete cyclization, so

the cyclization reaction was repeated for 23 hr. Again, the Kaiser test was weakly positive, so the reaction was repeated a third time for 6 hr. The resin was then rinsed and vacuum dried.

The peptide was cleaved from the resin with 30 mL of TFA/H_20/TIS 95/2.5/2.5 over 2.5 hr. by mixing with N_2. The peptide solution was removed by aspiration and concentrated down to a few mL's. A white solid precipitated out with the addition of cold ether and was collected by centrifugation. The solid was dissolved in 1 mL MeOH. Added H_2O precipitated a white solid and the yellow solution was decanted off to give 60 mg (5% yield) of a white solid. ESI MS m/z (%) calcd. for $C_{36}H_{56}N_7O_{10}$ ($M+H^+$) 746.4 found 746.2 (4), calcd. M-(Z)+H^+ 612.4 found 611.9 (100).

2.3.12 Synthesis of 5a

Solid phase synthesis was carried out on 5.228 g D-Ala-Wang resin (0.72 mmol/g loading) for the amino acid sequence (D-Ala)—(L-Glu(OBzl))—(D-Leu)—(Aib)—(L-Lys(Z))—(Aib) as described for compound **1a**. The Kaiser test was inconclusive for aminoisobutyric acid free amine so the coupling reaction of the resin-bound terminal Aib residue with Fmoc-Lys(Z) was repeated twice for 20 hr. each time.

The peptide was cleaved from the resin with 30 mL of TFA/H_20/TIS 95/2.5/2.5 over 1.5 hr. by mixing with N_2. The peptide solution was removed by aspiration and concentrated down to a few mL's. The residue was purified by flash column chromatography (19:1

DCM:MeOH then 6.1:1 DCM:MeOH) to give 650 mg of a white solid. Column chromatography (2.6:1 DCM:MeOH) was used to purify additional fractions recovered from the second flash column to give an additional 330 mg for a total of 980 mg (31% yield) of linear peptide. R_f .30 (3:1 DCM/MeOH); ^{1}H-NMR (400 MHz, MeOD), δ 0.58-1.66 (m, 37H), 1.81-1.95 (m, 2H), 2.16-2.30 (m, 1H), 2.36-2.45 (m, 1H), 2.82-2.94 (m, 2H), 3.12 (s, 2H), 3.80-4.06 (m, 4H), 4.75-4.89 (m, 4H), 6.99-7.16 (m, 10H, Ar); ^{13}C-NMR (100 MHz, MeOD), δ 19.3, 21.8, 23.9, 23.9, 24.1 (CH_3), 24.4 (CH_2), 26.0 (CH_3), 26.9 (CH), 27.8 (CH_3), 28.0, 31.1, 32.2, 32.3, 40.7, 41.8 (CH_2), 52.2, 54.6, 55.1, 56.4 (CH), 58.3, 58.4 (C), 67.6, 67.8 (CH_2), 129.2, 129.4, 129.5, 129.9 (CH, Ar), 138.0, 138.8 (C, Ar), 159.4, 173.4, 174.6, 174.7, 174.8, 176.3, 177.6, 180.2 (C=O). ESI MS m/z (%) calcd. for $C_{43}H_{63}N_7O_{11}$ ($M+H^+$) 854.5 found 854.3 (100), calcd. $M+Na^+$ 876.5 found 876.3 (33), calcd. $M+K^+$ 892.5 found 892.3 (23).

2.3.13 Synthesis of 5b

800 mg (0.937 mmol) of linear peptide **5a** was dissolved in 400 mL DMF and stirred at 0 ˚C. 0.464 g (1.3 eq) HATU, 0.166 g (1.3 eq) HOAT, and 2.4 mL (10 eq) DIPEA in 5 mL of DMF was added and the solution was stirred for 4 hr. at which point the DMF was removed in vacuo, 45 ˚C. The residue was taken up in EtOAc and the insoluble white precipitate was removed by filtration. The organic solution was extracted 3x with 30 mL 10% citric acid, 3x with 30 mL saturated $NaHCO_3$, and dried over Na_2SO_4. The solution was purified by flash column chromatography (EtOAc) to give 450 mg (57% yield) of a white solid. R_f .47 (EtOAc); ^{1}H-NMR (400 MHz, $CDCl_3$), δ 0.79-0.98 (m, 6H), 1.09-

2.49 (m, 29H), 2.93-3.36 (m, 2H), 3.73 (t, J = 6.7 Hz, 1H), 4.03-4.21 (m, 1H), 4.41-4.58 (m, 1H), 4.79-4.94 (m, 1H), 4.98-5.18 (m, 4H), 5.62 (t, J = 5.6 Hz, 1H), 6.17 (s, 1H), 6.35-6.57 (m, 1H), 6.97-7.20 (m, 2H), 7.21-7.44 (m, 10H, Ar), 7.61 (s, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$), δ 16.5, 21.4, 21.4 (CH_3), 21.8, 22.5 (CH_2), 22.9, 23.4, 23.8 (CH_3), 24.8 (CH), 26.0, 27.9 (CH_2), 27.9 (CH_3), 30.3, 38.8, 39.5 (CH_2), 50.4, 51.4, 51.5, 53.2 (CH), 56.9, 57.4 (C), 66.4, 66.7 (CH_2), 128.5, 128.6, 128.8, 128.9, 129.1 (CH, Ar), 136.2, 136.9 (C, Ar), 156.8, 172.2, 172.9, 173.5, 174.0, 174.6, 175.9, 176.5 (C=O). ESI MS m/z (%) calcd. for $C_{43}H_{62}N_7O_{10}$ ($M+H^+$) 836.4 found 836.2 (6), calcd. $M+Na^+$ 858.4 found 858.2 (100).

2.3.14 Synthesis of 5c

400 mg (0.478 mmol) of **5b** was dissolved in 100 mL of 1:1 DCM:MeOH. The benzyl protecting groups on the glutamic acid and lysine side chains were removed by using 0.2 g of Pd activated carbon (10 wt%) and H_2 at atmospheric pressure while stirring the solution for 1.5 hr. The spent catalyst was removed by filtration and the solution was concentrated completely in vacuo, 40 ˚C, to give 305 mg (105% yield) of a white solid. ^{1}H and ^{13}C-NMR spectra in MeOD confirmed complete loss of benzyl protecting groups. The peptide was used without further purification or characterization.

2.3.15 Synthesis of 5d

305 mg (0.498 mmol) of **5c** was dissolved in 150 mL of DMF and stirred at 0 ˚C. 0.246 g (1.3 eq) HATU, 0.088 g (1.3 eq) HOAT, and 0.87 mL (10 eq) DIPEA dissolved in 5 mL of DMF was added to the peptide solution for 4 hr. at which point the DMF was removed in vacuo, 45 ˚C. The residue was taken up in EtOAc and the insoluble white precipitate was removed by centrifugation. The organic solution was extracted 3x with 30 mL of 10% citric acid and 3x with 30 mL of saturated $NaHCO_3$ and dried with Na_2SO_4. The solution was concentrated completely and dried in vacuo to give 5 mg of a white solid. Characterization by ESI MS was inconclusive.

2.3.16 Synthesis of 6a

Solid phase synthesis was carried out on 5.642 g D-Ala-Wang resin (0.72 mmol/g loading) for the amino acid sequence (D-Ala)—(L-Glu(OBzl))—(D-Leu)—(Aib)—(L-Lys(Z))—(D-Leu) as described for compound **5a**.

The peptide was cleaved from the resin in two consecutive reactions of 30 mL of TFA/H_20/TIS 95/2.5/2.5 mixed with N_2 for 30 min. each. The peptide solutions were removed by aspiration, combined, and concentrated to a few mL's. The residue was purified by column chromatography (4.6:1 DCM:MeOH) and then flash column chromatography (19:1 DCM:MeOH) to give 1.40 g (39% yield) of a light yellow solid. R_f .30 (4.6:1 DCM/MeOH); ^{1}H-NMR (400 MHz, MeOD), δ 0.61-0.79 (m, 14H), 1.01-1.35 (m, 16H), 1.37-1.63 (m, 9H), 1.82-1.99 (m, 2H), 2.17-2.30 (m, 1H), 2.32-2.43 (m,

1H), 2.89 (t, *J* = 6.7 Hz, 2H), 3.36-3.48 (m, 2H), 3.67-3.75 (m, 1H), 3.82-3.91 (m, 2H), 4.01-4.11 (m, 2H), 4.80-4.91 (m, 4H), 7.03-7.16 (m, 10H, Ar); ^{13}C-NMR (100 MHz, MeOD), δ 18.9, 21.7, 22.8, 23.5, 24.0, 24.1 (CH_3), 24.5 (CH_2), 26.0, 26.9 (CH), 27.7 (CH_3), 28.4, 31.0, 32.0, 32.6, 40.6, 41.8, 41.8 (CH_2), 52.5, 53.3, 54.4, 54.7, 56.1 (CH), 58.3 (C), 67.6, 67.8 (CH_2), 129.2, 129.4, 129.5, 129.9 (CH, Ar), 138.0, 138.8 (C, Ar), 159.4, 173.7, 174.9, 174.9, 175.1, 175.9, 177.5, 180.6 (C=O). ESI MS m/z (%) calcd. for $C_{45}H_{67}N_7O_{11}$ ($M+H^+$) 882.5 found 882.6 (15), calcd. $M+Na^+$ 904.5 found 904.6 (30), calcd. $M+K^+$ 920.5 found 920.6 (18).

2.3.17 Synthesis of 6b

1.361 g (1.544 mmol) of linear peptide **6a** was dissolved in 680 mL DMF and stirred at 0 ˚C. 0.764 g (1.3 eq) HATU, 0.274 g (1.3 eq) HOAT, and 2.7 mL (10 eq) DIPEA dissolved in 5 mL of DMF was added and the solution was stirred for 4 hr. at which point the DMF was removed in vacuo, 45 ˚C. The residue was taken up in EtOAc and the insoluble white precipitate was removed by filtration. The organic solution was extracted 3x with 30 mL 10% citric acid, 3x with 30 mL saturated $NaHCO_3$, and dried over Na_2SO_4. The solution was purified by flash column chromatography (EtOAc) to give 600 mg (45% yield) of a white solid. R_f .40 (EtOAc); ^{1}H-NMR (400 MHz, $CDCl_3$), δ 0.80-0.99 (m, 12H), 1.19-1.91 (m, 21H), 2.01-2.24 (m, 3H), 2.39-2.48 (m, 2H), 3.10-3.26 (m, 2H), 3.98-4.15 (m, 2H), 4.18-4.28 (m, 1H), 4.28-4.40 (m, 1H), 4.48-4.58 (m, 1H), 5.02-5.12 (m, 4H, Ar), 5.44 (t, *J* = 5.9 Hz, 1H), 6.58 (d, *J* = 7.8 Hz, 1H), 6.64 (d, *J* = 8.6 Hz, 1H), 6.94 (d, *J* = 5.06 Hz, 1H), 7.06 (s, 1H), 7.28-7.36 (m, 10H, Ar), 7.41-7.46 (m,

1H), 7.56 (d, J = 7.1 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$), δ 17.3, 21.9, 22.1 (CH_3), 22.5 (CH_2), 23.6, 23.6 (CH_3), 23.7 (CH_3), 24.4 (CH_2), 25.4, 25.4 (CH), 28.0 (CH_3), 28.1, 29.2, 30.5, 39.7, 40.3, 40.4 (CH_2), 51.5, 51.7, 52.4, 52.6, 54.1 (CH), 57.0 (C), 66.9, 67.0 (CH_2), 128.2, 128.3, 128.4, 128.7, 128.7 (CH, Ar), 135.9, 136.8 (C, Ar), 156.8, 172.0, 172.9, 173.0, 173.0, 173.3, 174.1, 174.7 (C=O). ESI MS m/z (%) calcd. for $C_{45}H_{65}N_7O_{10}Na$ ($M+Na^+$) 886.5 found 886.6 (100).

2.3.18 Synthesis of 6c

571 mg (0.661 mmol) of **6b** was dissolved in 150 mL of 1:1 DCM:MeOH. The benzyl protecting groups on the glutamic acid and lysine side chains were removed by using 0.2 g of Pd activated carbon (10 wt%) and H_2 at atmospheric pressure while stirring the solution for 1.5 hr. The spent catalyst was removed by filtration and the solution was concentrated completely in vacuo, 40 ˚C, to give 400 mg (94% yield) of a white solid. ^{1}H and ^{13}C-NMR spectra in MeOD confirmed complete loss of benzyl groups. The peptide was used without further purification or characterization.

2.3.19 Synthesis of 6d

202 mg (0.316 mmol) of **6c** was dissolved in 100 mL DMF and stirred at 0 ˚C. 0.156 g (1.3 eq) HATU, 0.056 g (1.3 eq) HOAT, and 0.55 mL (10 eq) DIPEA dissolved in 5 mL DMF was added to the peptide solution and stirred for 4 hr. at which point the DMF was removed in vacuo, 45 ˚C. The residue was taken up in EtOAc and the insoluble white

precipitate was removed by filtration. The organic solution was extracted 3x with 30 mL 10% citric acid, 3x with 30 mL saturated $NaHCO_3$, dried over Na_2SO_4, and concentrated completely to give 26 mg of a whitish yellow solid.

The cyclization reaction and workup described above were repeated on the remaining 190 mg of **6c** to give an additional 33 mg for a total of 60 mg (16% yield) of whitish yellow solid. ^{1}H-NMR (400 MHz, MeOD), δ 0.84-1.00 (m, 12H), 1.25-1.89 (m, 24H), 1.99-2.21 (m, 4H), 4.09-4.42 (m, 5H); ^{13}C-NMR (100 MHz, MeOD), δ 17.4, 22.0, 22.7, 23.8, 23.9 (CH_3), 24.4 (CH_2), 24.8, 26.4 (CH_3), 26.6, 26.6 (CH), 27.7, 29.0, 31.7, 31.9, 40.2, 41.7, 41.8 (CH_2), 54.3, 54.3, 54.0, 54.0, 52.8 (CH), 58.2 (C), 174.2, 174.5, 174.9, 175.3, 175.4, 175.6, 176.9 (C=O). ESI MS m/z (%) calcd. for $C_{30}H_{52}N_7O_7$ ($M+H^+$) 622.4 found 622.3 (100).

2.4 ISE Membrane and Electrode Preparation

This work was performed by Dr. John Benco. Six membrane cocktails were prepared to test **6d**. The specific formulations are as follows: M1, 69/30/1 wt % of nitrophenyl octyl ether (NPOE)/PVC/**6d**; M2, same as M1 with 10 mol % of potassium tetrakis (4-chlorophenyl)borate (KtpClPB) to **6d**; M3, same as M1 with 50 mol % of KtpClPB to **6d**; M4, 69/30/1 wt % of dioctyl phthalate (DOP)/PVC/**6d**; M5, same as M4 with 10 mol % of KtpClPB to **6d**; M6, same as M4 with 50 mol % of KtpClPB to **6d**. Membrane cocktails were prepared as 10 wt % solutions in THF. The base electrodes were constructed in a thick-film planar format using a polymeric internal electrolyte layer as

previously reported.[8] Two wafers composed of 100 individual electrode elements each were used for the sensor construction. The polymer, methacrylamidopropylmethyl-ammonium chloride (MAPTAC), for the internal electrolyte was prepared as a 10 wt % solution in EtOH, spun on to the planar wafers at 750 rpm for 30 s, and allowed to dry for 1 h before membrane deposition. Internal electrolyte thickness was ~3.5 μm. The wafers were then quartered, giving wafers of 25 sensors each. Membrane cocktails were deposited (0.9 mL) onto the wafers and allowed to cure for 24 h before use, giving a membrane thickness of ~105 μm. The planar wafers were singulated by hand, giving 25 sensors for each formulation.

2.5 ISE Testing

This work was carried out by Dr. John Benco. The sensors were housed in the proprietary flow-through cell used with the Bayer Diagnostics Rapidpoint 400 critical care system as previously reported.[8] This system uses a saturated Ag/AgCl reference cell. Two flow cells were constructed, which contained 1-2 sensors of each formulation for a total of 14 tested sensors. Each cell was tested individually on the Rapidpoint system that maintains a 37˚C temperature for the cell. The sensors were tested using solutions containing NH_4Cl (0.5-100 mM), 100 mM Tris buffer (pH 7.2), and 0.05 g/L Brij 700. Selectivity testing was based on the separate solution method (SSM)[18], where $i = j = 0.1$ M.

2.6 ^{13}C-NMR Study of Valinomycin binding Potassium Cations

This study was based on a modified procedure.[19] A solution was made by dissolving 20 mg of commercially available valinomycin in 0.50 mL of 1:1 (v:v) MeOD:$CDCl_3$. A ^{13}C-NMR spectrum was recorded after each 10 μL addition of stock solutions of potassium thiocyanate in 1:1 (v:v) MeOH:$CHCl_3$. **Table 1** shows the KSCN concentrations for each experiment. ^{13}C-NMR spectra were recorded by locking on MeOD with 512—9000 scans and the spectra were calibrated with respect to MeOD.

Table 1: ^{13}C-NMR experiments of valinomycin with increasing amounts of KSCN

Spectrum	KSCN (mmol)	Total Volume (mL)	[KSCN] (mM)
1	0.0	0.50	0.0
2	0.2	0.51	0.4
3	0.4	0.52	0.8
4	0.6	0.53	1.1
5	0.8	0.54	1.5
6	1.2	0.55	2.2
7	1.6	0.56	2.9
8	2.0	0.57	3.5
9	2.4	0.58	4.1
10	2.8	0.59	4.7
11	3.2	0.60	5.3
12	3.6	0.61	5.9
13	4.5	0.62	7.3
14	5.4	0.63	8.6
15	6.3	0.64	9.8
16	7.2	0.65	11.1
17	8.1	0.66	12.3
18	9.0	0.67	13.4
19	9.9	0.68	14.6
20	10.8	0.69	15.7
21	11.7	0.70	16.7
22	12.6	0.71	17.7
23	13.5	0.72	18.8
24	14.4	0.73	19.7
25	15.3	0.74	20.7
26	16.2	0.75	21.6
27	17.1	0.76	22.5

3 Results and Discussion

3.1 Selection of Synthetic Methods

The decision to utilize a solid phase Fmoc-protection strategy on a Wang resin was based on the demonstrated success of that strategy in our working group as well as by others.[17,20-23] These methods are well understood and give good yields. The mechanisms of Fmoc deprotection and PyBOP/HOBT-mediated amino acid coupling are shown below in **Figures 11** and **12**. The benzyl protecting groups on the glutamic acid and lysine side chains were chosen because the protected residues are available commercially and because they are known to be stable to Fmoc deprotection conditions (basic) and resin cleavage conditions (acidic). These groups are easily removed by hydrogenation.

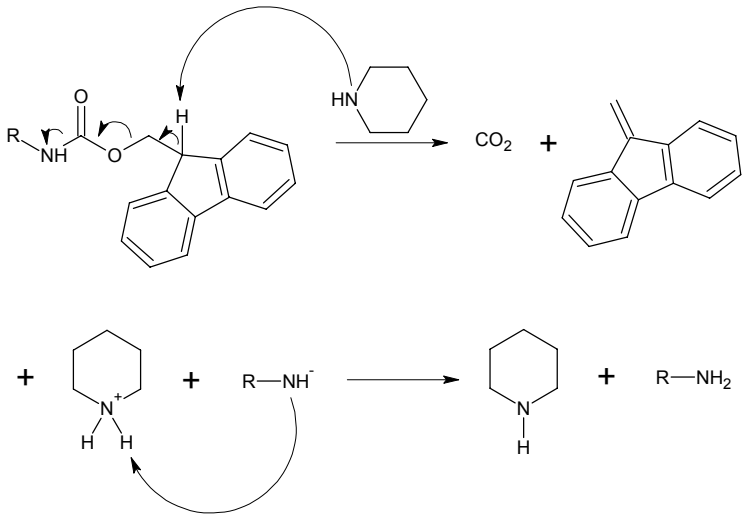


Figure 11: Mechanism of Fmoc deprotection

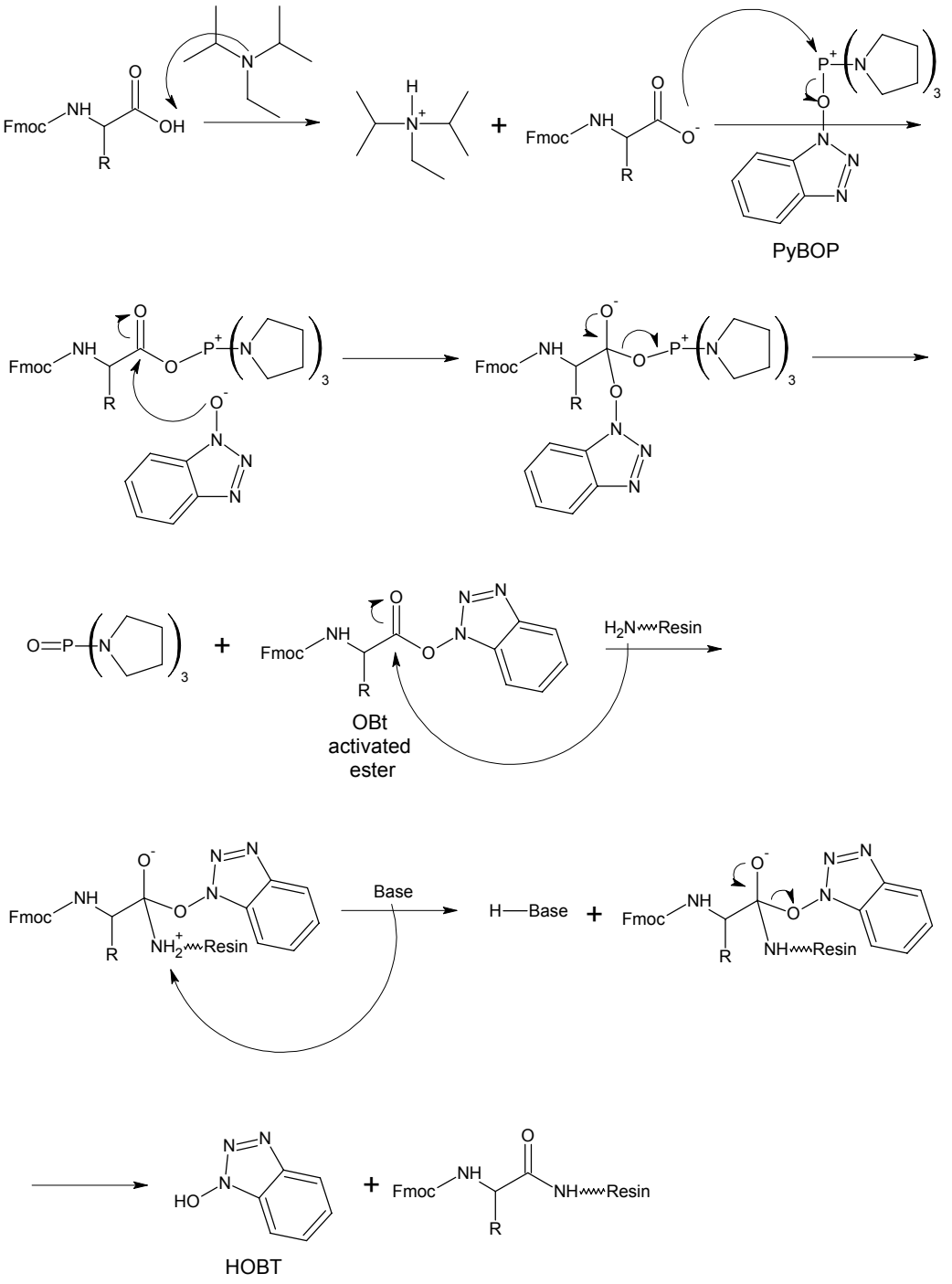


Figure 12: Mechanism of PyBOP/HOBT-mediated coupling reaction

The use of the activating agents HATU and HOAT in cyclization of the linear peptide was based on the experience of both Ghadiri's working group[24] as well as that of our own. The reaction is carried out in DMF under dilute (2 mg/ mL) conditions in order to favor intramolecular cyclization over intermolecular coupling. The mechanism of HATU/HOAT-mediated coupling is very similar to that shown for PyBOP in **Figure 12**. Thionyl chloride was employed for the second cyclization step, based on the success our group has had with the reagent in the cyclization of a depsipeptide ammonium ionophore.[8]

3.2 Design and Synthesis

3.2.1.1 Design of 1d

The design of bicyclic peptide target compound **1d** has elements that are similar to the cyclic depsipeptide ammonium ionophore[8] reported recently by our group (shown in **Figure 7b**). Target **1d** is the bicyclic and all-amide equivalent of this depsipeptide, both of which are shown in **Figure 13**. The stereochemical pattern, L—D—D—L—L—D, of both is based on the potassium ionophore valinomycin $(L—D—D—L)_3$ and was expected, based on modeling, to prevent formation of helices that would hinder cyclization.

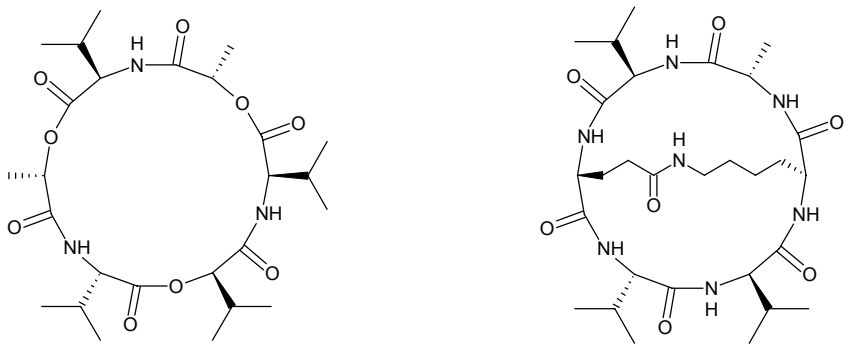


Figure 13: Depsipeptide ammonium ionophore (left) and target compound **1d** (right)

Molecular modeling of **1d** indicated that it may offer enhanced ammonium over potassium selectivity compared to nonactin, the industry standard ammonium ionophore. Whereas nonactin has a flexible crown-ether backbone that allows wrapping-type complexes with both ammonium and potassium ions, bicyclic peptide **1d** has a more rigid structure that provides the tetrahedral coordination required for complexation of ammonium ions, but cannot effectively exhibit the octahedral coordination required for complexation of potassium ions. **Figure 14** shows minimized structures of **1d** with both ammonium and potassium ions. Ammonium ion sits deeply within the cavity and forms at least four hydrogen bonds to **1d**. On the other hand, potassium does not sit as deeply within the pocket, indicating less favorable complexation.

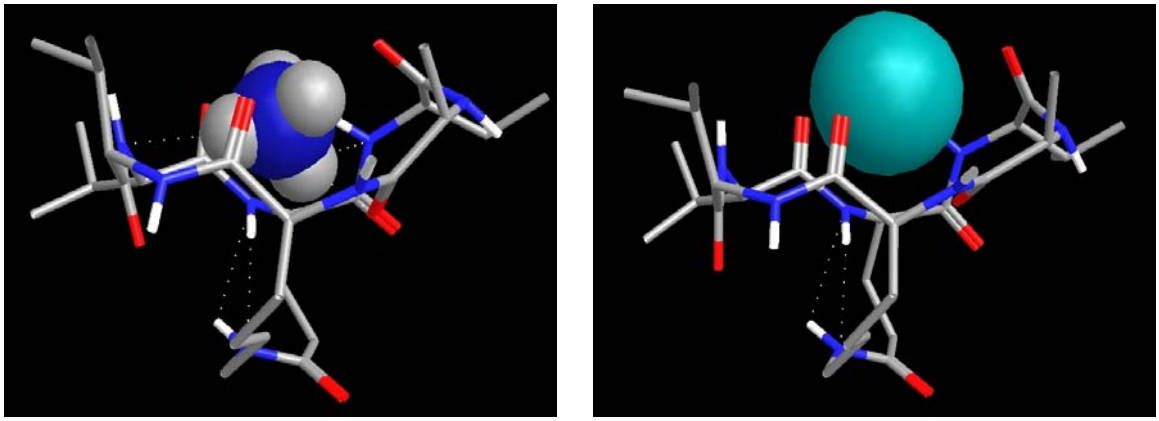


Figure 14: Complexation of 1d with ammonium (left) and potassium (right) cations

To estimate the selectivity of **1d** for binding ammonium over potassium ions compared to nonactin, docking energies were obtained for the ion/ionophore complex in each case, as previously reported.[8] The difference in docking energies between ammonium and potassium ion for **1d** was 12 kcal/mol more negative than that calculated for nonactin. While these calculations give relative values only, they indicate qualitatively that **1d** may show increased ammonium over potassium selectivity compared to nonactin.

3.2.1.2 Attempted Synthesis of 1d

The linear peptide **1a** was synthesized and characterized in good (67%) yield. However, **1a** exhibited poor solubility and was very difficult to dissolve in a variety of solvents, including DMSO and DMF. After the first cyclization reaction to give **1b**, the solvent extraction process created an emulsion between the DCM and aqueous layers, probably due to the limited solubility of the peptide in either phase. Based on the low yield of **1b**

(14 %), the decision was made to start a new synthesis of **1a** in order to scale up the amount of the intermediate **1b**. In an attempt to increase the yield during the cyclization of the second batch of **1a**, the reaction was carried out in 500 mL of DMF at 0 ˚C for 4 hr in order to favor intramolecular cyclization over intermolecular coupling by decreasing the rate of collisions by lowering the temperature. The product of the second synthesis was purified by multiple precipitations from MeOH/ether only, since **1b** did not exhibit sufficient solubility to purify by column chromatography and we believed much of the desired product of the first synthetic attempt was lost during the solvent extraction in the emulsion that formed. Intermediate **1b** was never unambiguously characterized due to the large amount of impurities present, ultimately caused by the poor solubility that prevented the use of effective purification techniques. The ^{1}H and ^{13}C-NMR spectra showed the expected signals for peptide **1b**, but clearly contained considerable impurities. The MS showed no **1b**, but this can potentially be explained by the poor solubility of **1b** (Synpep Corp., the contractor who provided the MS analysis, used only acetonitrile as a matrix). The final cyclization with thionyl chloride appeared completely unsuccessful, resulting in a brown solid that contained no **1d** by MS. In fact, activation of the carboxyl group by acid chloride formation is rarely done by peptide practitioners due to its reputation of being "overactivated" and therefore prone to numerous side reactions.[25]

3.2.2.1 Design of 2d

The attempted synthesis of target compound **1d** highlighted the importance of solubility in the synthesis of peptides. Target compound **2d** was designed in an attempt to increase solubility of intermediate and final compounds by decreasing aggregation through the replacement of bulky valine residues by smaller alanine residues. Additionally, it was realized that the incorporation of D-lysine and L-glutamic acid in target compound **1d** would result in two different atropoisomers depending on which side of the cyclic peptide the second ring is formed. As shown in **Figure 15**, one isomer has the bridgehead hydrogen on glutamic acid pointing outside the cavity and the bridgehead hydrogen on lysine pointing in towards the cavity and the other has the bridgehead hydrogen on glutamic acid pointing in towards the cavity and the bridgehead hydrogen on lysine pointing outside the cavity. In an attempt to favor the formation of just one atropoisomer, the stereochemistry of the bridgehead residues were both designed as L. As shown in **Figure 15**, the two possible atropoisomers of **2d** can have both of the bridgehead hydrogens pointing in toward the central cavity or both pointing out away from the central cavity. Based on the work of Paolillo et al.[16], the isomer with both hydrogens pointing out away from the central cavity should be favored by steric interactions during formation of the second ring. The stereochemistry assignment, L—L—D—D—L—D, was designed to give the two most expensive amino acids, the benzyl protected lysine and glutamic acid residues, the naturally occurring L configuration in order to minimize overall cost of the synthesis while still retaining the stereochemical pattern seen in valinomycin and the previous bicyclic target compound **1d**. Molecular modeling calculations to estimate the selectivity of target compound **2d** with ammonium and

potassium ions gave a difference in docking energies similar to that calculated for target compound **1d**, indicating that the change in amino acid residues and stereochemistry should not significantly affect complexation behavior.

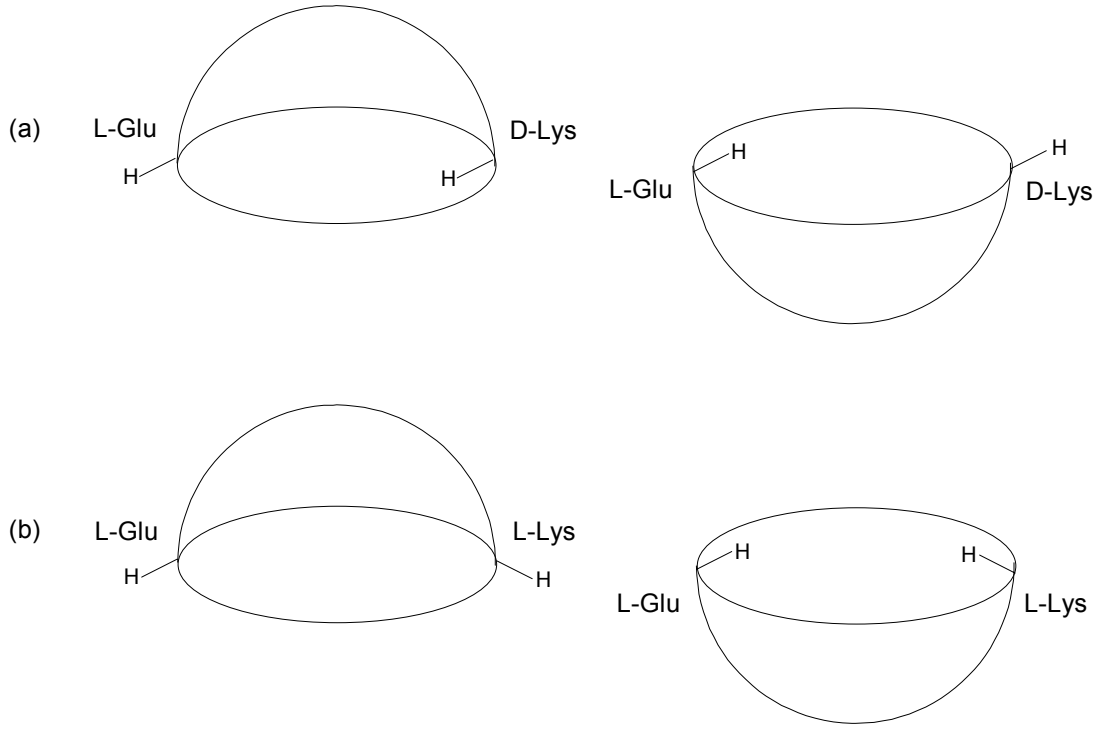


Figure 15: The two possible atropoisomers of (a) target compound **1d** (b) target compound **2d**

3.2.2.2 Synthesis of 2d

In order to evaluate the most effective cyclization conditions, the preliminary synthesis product **2a** was divided. One portion was subjected to cyclization using HATU/HOAT activation and the other portion was activated with thionyl chloride. The product of the HATU/HOAT cyclization **2b** was characterized by MS and ^{1}H and ^{13}C-NMR, but no **2b** was visible in the MS of the product of the thionyl chloride reaction. Acid chlorides of benzyloxycarbonyl-protected peptides such as **2a** are known to undergo the serious side

reaction of spontaneous decomposition to the corresponding Leuchs anhydride with loss of benzyl chloride.[25] Both the linear peptide **2a** and the monocyclized intermediate **2b** showed limited solubility. However, solubility was sufficiently high in acetonitrile/water solvent systems to successfully purify **2b** through reverse phase column chromatography. The success of the HATU/HOAT method in the first cyclization led to the decision to employ this method for the final cyclization. Compound **2d** was characterized by MS in the crude product mixture and was subsequently purified by a series of precipitations from MeOH/ether in which a marked decrease in solubility was observed. The final precipitated **2d** was essentially insoluble and therefore it was impossible to characterize by MS or NMR or to test its utility as an ammonium ionophore in an ISE sensor format.

3.2.3.1 Design of 3d

Target compound **3d** was designed to exhibit increased solubility by preventing the intermolecular hydrogen bonding that is generally believed to be responsible for the poor solubility of peptides[26] such as **2a**-**2d**. We predicted, based on investigations by Narita[26], that the replacement of two of the alanine residues in **2d** with N-methyl alanine residues in **3d** would increase solubility by disrupting hydrogen bonding between molecules. The amino acid sequence was designed to prevent cyclization at a hindered N-methylated amine. The stereochemistry was unchanged from bicyclic peptide **2d** to bicyclic peptide target **3d**.

3.2.3.2 Attempted Synthesis of 3d

Solid phase synthesis of **3a** was complicated by the incorporation of the two N-methylated residues because the Kaiser test used to monitor deprotection and coupling reactions is inconclusive for secondary amines. The Novabiochem catalog contains a method for detecting the free secondary amine of proline residues called a chloranil test.[17] However, this technique was inconclusive, usually giving orange or brown resin beads, when a blue color is indicative of a positive test, in both the presence and absence of secondary amine. For this reason, the extent of coupling was instead estimated by cleaving the Fmoc protecting group from a known mass of resin (20 mg) in a 100 mL solution of 20% piperidine in DMF and monitoring the UV absorption at 290 nm. Since the extinction coefficient of the Fmoc group is known at this wavelength to be 4950 $M^{-1}cm^{-1}$, the UV absorption of the free Fmoc moiety can be used to calculate the concentration of the Fmoc group in solution, which can be related to the amount of peptide on the known mass of resin. However, this technique has limited sensitivity. The MS of the crude mixture after the resin cleavage reaction showed that the linear peptide **3a** was not present, but various deletion peptides were present. This was strong evidence that N-terminal coupling reactions of the N-methylated residues were not effective.

3.2.4.1 Design of 4d

Like target compound **3d**, **4d** was designed to have increased solubility by preventing intermolecular hydrogen bonding. However, due to the limited success of coupling reactions involving N-methylated residues in the synthesis of **3d**, the decision was made

to incorporate two aminoisobutyric acid residues, which are also known to increase solubility by disrupting β-sheet formation[26], instead of the N-methylated alanine residues. Additionally, we replaced one alanine residue with a leucine residue in an attempt to increase the solubility of the peptides by increasing their lipophilicity.

The synthetic strategy for target **4d** was developed after a literature search of on-resin cyclization methods.[27-37] On-resin cyclization methods are desirable because, in theory, intermolecular reactions to form polymers are impossible and therefore yields of the intramolecular reaction should be increased dramatically compared to solution-phase cyclization reactions. In addition, this strategy would eliminate one step from the synthesis. The method involves anchoring the side chain of glutamic acid to the resin and building the linear peptide using standard solid phase techniques using an allyl protecting group on the carboxy terminus during chain elongation. This is followed by solution-phase deprotection of the allyl protecting group prior to on-resin head-to-tail peptide cyclization.[37] The allyl-protecting group strategy was chosen over other on-resin cyclization methods because it required the smallest overall change in strategy. This synthetic scheme still allowed the use of Fmoc protecting groups on the Wang resin and benzyl protection for the lysine residue.

3.2.4.2 Attempted synthesis of 4d

The allyl-protecting group on-resin cyclization method gave only 5% yield of crude peptide **4b** based on an estimated resin loading of 59%. This is too low for a practical

synthesis of **4d**. In addition, MS showed that ~97% of the crude peptide **4b** had been deprotected (benzyloxycarbonyl protecting group) at the lysine residue leading to the conclusion that the benzyl group was removed during the allyl deprotection reaction. Since deprotection occurred prior to the cyclization reaction, the deprotected and monocyclized peptide formed was most likely a mixture of head-to-tail cyclized and head-to-side chain cyclized products since both the N-terminus and the lysine side chain amine were available for participation in the cyclization reaction.

3.2.5.1 Design of 5d

Based on the low yield (5% to resin loading) obtained by this on-resin cyclization method, the decision was made to return to our previous synthetic strategy of solid phase synthesis to produce the linear peptide followed by two solution-phase cyclization reactions. Target compound **5d** is the analogue of **4d** produced by this synthetic strategy. The amino acid sequence was reversed in order to make the solid phase synthesis easier by starting with an alanine residue, which is commercially available preloaded on the resin.

3.2.5.2 Attempted synthesis of 5d

The first attempt to make **5a** via solid phase synthesis resulted in a low yield of an oil. This oil was found to contain primarily **5a** that had been deprotected (the benzyloxycarbonyl protecting group had been lost from the lysine side chain according to

the MS). We suspected that the benzyloxycarbonyl protecting group was lost during the 6 hr. cleavage reaction. The work of Erickson et al. confirmed that benzyloxycarbonyl protecting groups are approximately 40 to 60 times less stable than benzyl ester protecting groups, and that they are removed under the acidic conditions (95% TFA) of the cleavage reaction.[38] They observed 0.5% deprotection of Lys(Z) after 20 min. exposure to 50% TFA in CH_2Cl_2. In the second solid phase synthesis of **5a**, the cleavage reaction time was shortened to 1.5 hr. and repeated twice in order to test which reaction time produced optimum yields of the linear peptide. MS of the two cleavage fractions showed a good yield of **5a** in the first fraction, but essentially none in the second cleavage fraction. This supported the conclusion that longer cleavage times lowered the yield of linear peptide **5a** through loss of the benzyloxycarbonyl protecting group.

Both the linear peptide **5a** and the monocyclized intermediate **5b** exhibited dramatically increased solubility in organic solvents compared to intermediates **1a**, **2a**, **1b**, and **2b**. The linear peptide **5a** was completely soluble in pure DCM, despite the free acid and free amine functions that may be expected to prevent solubility in such a nonpolar solvent. The great solubility of the intermediates **5a** and **5b** made purification by column chromatography highly successful.

The final cyclization reaction and subsequent work-up were performed exactly the same as the first cyclization reaction, but gave only 5 mg of a white solid after the solvent extraction. Characterization by MS was inconclusive, since only very low intensity signals of the correct mass were observed.

3.2.6.1 Design of 6d

After study structures generated by molecular modeling and physical models of target peptide **5d**, we concluded that the bulky aminoisubutyric acid residues were causing the ring to be too rigid to allow for the second cyclization to occur. Specifically, we hypothesized that the methyl groups on the aminoisobutyric acid residue between the alanine and lysine residues were initiating an unfavorable steric interaction with the alanine carbonyl group as the lysine and glutamic acid side chains began to come together to form the second ring.

Target bicyclic peptide **6d** was designed on the basis of this hypothesis. The interfering aminoisobutyric acid residue was replaced by a lipophilic leucine residue for the purpose of decreasing steric hindrance while attempting to retain the good solubility observed in the intermediates of **5d**. Both computer and physical models led us to believe that a D configuration for the alanine and leucine residues would be most favorable for promoting the second cyclization reaction through steric interactions forcing the L-glutamic acid and L-lysine side chains onto the same side of the ring.

3.2.6.2 Synthesis of 6d

Intermediates **6a** and **6b** were synthesized and purified using the same methods that proved successful for the second synthesis of **5a** and **5b**. Both intermediates also exhibited the good solubility seen in **5a** and **5b**. In this case, the second cyclization reaction was successful in producing bicyclic peptide **6d** in 16% yield, lending support to

our hypothesis of harmful steric interactions caused by the aminoisobutyric acid residue preventing cyclization of **5c**.

Multiple precipitations were performed on **6d** from THF/ether, DCM/hexane, and MeOH/ether/hexane solvent systems in attempts to purify the bicyclic peptide (purification by chromatography was not possible because **6d** was not visible on TLC plates by UV absorption, iodine, or p-anisaldehyde visualization methods). Unfortunately, none of these precipitations purified the bicyclic peptide based on ^{1}H and ^{13}C-NMR (**6d** seemed partially soluble in all of the tried solvent systems).

3.3 Sensor Fabrication and Testing

Bicyclic peptide **6d** was incorporated into a planar ISE structure employing a polymeric solid contact material and tested in a commercially available point-of-care clinical diagnostic system, as previously reported.[8] Six membrane formulations were tested in order to determine which environment would yield the best potentiometric response. Each sensor membrane consisted of plasticized PVC. Formulations differed as to the type of plasticizer used (NPOE or DOP) and the amount of the lipophilic salt KtpClPB present (0, 10, or 50 mol% to **6d**).

Figures 16 and **17** show the potentiometric responses of the six membrane formulations to increasing concentrations of aqueous NH_4Cl. The membrane containing the plasticizer NPOE with 50 mol% KtpClPB to **6d** exhibited the largest potential increases with

increasing ammonium concentration (highest slope (60.7 mV/dec), closest to the ideal Nernstian response (see **Table 2**)). The membranes containing the plasticizer NPOE in the absence of KtpClPB and all formulations containing the plasticizer DOP exhibited sub Nernstian behavior.

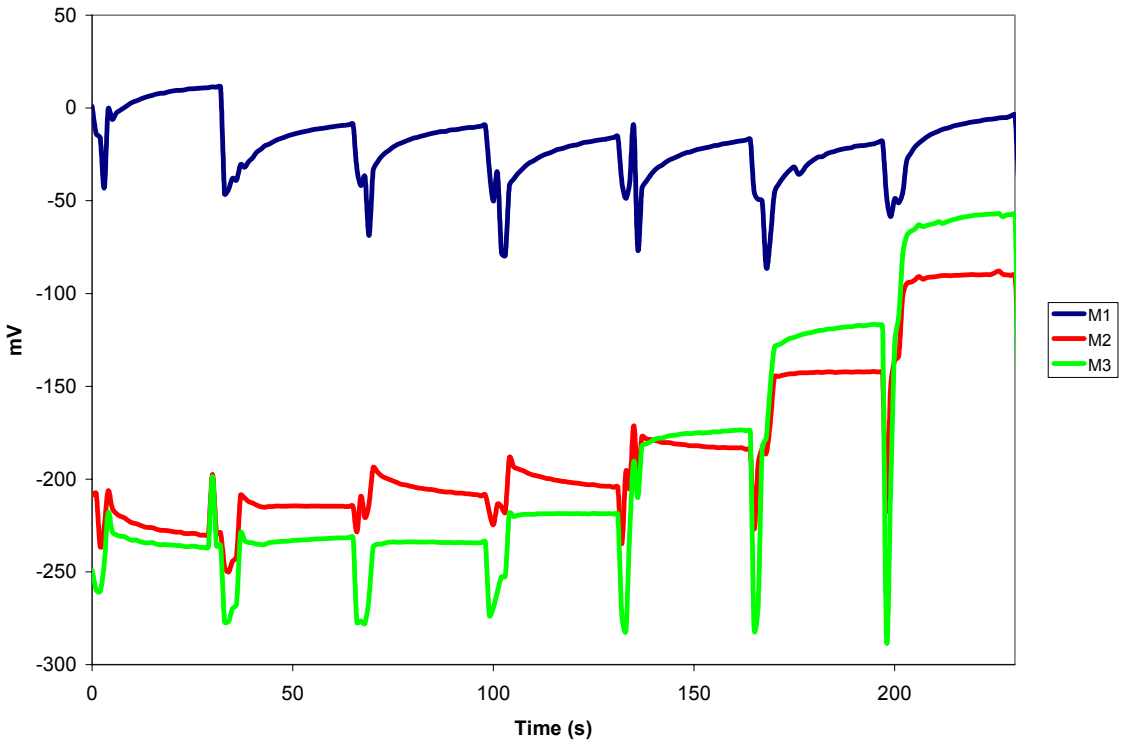


Figure 16: Potentiometric responses of planar ISEs to NH_4^+ (10^{-4} – 10^{-1} M) for membranes 1-3 based on **6d**

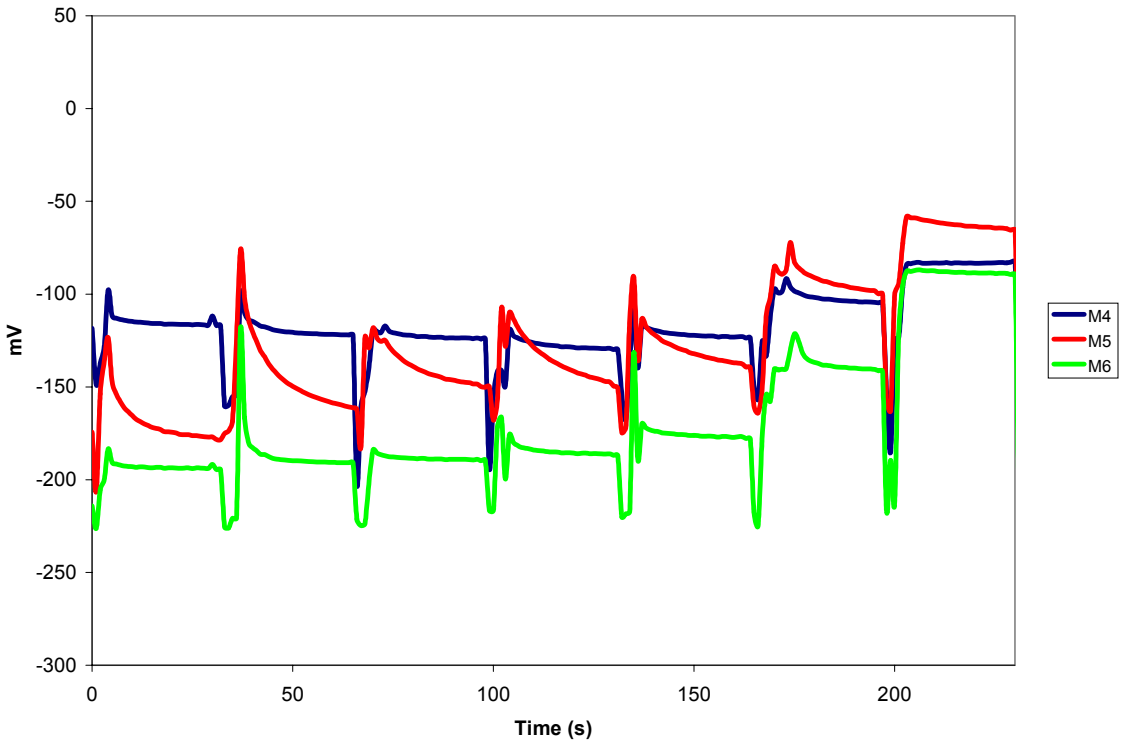


Figure 17: Potentiometric responses of planar ISEs to NH_4^+ (10^{-4} – 10^{-1} M) for membranes 4-6 based on **6d**

In the case of membrane formulations utilizing NPOE (M1-M3), it can be seen that as the mole ratio of lipophilic salt to **6d** increases there is a corresponding increase in the slope of the sensors as a function of ammonium concentration. In particular, it is observed that in the absence of lipophilic salt (i.e. formulation M1) the sensors essentially exhibit no response to ammonium ions. This general trend is also observed in the case of the membrane formulations utilizing DOP (M4-M6). These results suggest that the response of the sensors is not determined by the ionophore (and is therefore independent of the ionophore), but rather is a function of the lipophilic salt concentration.

Table 2 shows the results of selectivity studies that were carried out on the six membrane formulations using the separate solution method. M3, the formulation showing the best response for ammonium cation, was in fact the most selective for potassium, followed by ammonium, sodium, calcium, magnesium, and lithium cations ($K^+ > NH_4^+ > Na^+ > Ca^{2+} > Mg^{2+} > Li^+$). The other membrane formulations (with the exception of M1, which was least selective for ammonium) showed a similar selectivity pattern. This sequence of cation selectivity shows a good correlation to the Hofmeister series ($K^+ > NH_4^+ > Na^+ > Li^+ \sim Ca^{2+} > Mg^{2+}$), which has been observed for ISE membranes incorporating the plasticizers NPOE and DOP without any ionophore component.[18] The sequence of cation selectivities (in the absence of ionophore) is determined by the difference between the standard free energies of solvation for the ions in the aqueous and organic phases respectively. Taken alone, the results of the selectivity studies indicate that **6d** is more selective for potassium ions than for ammonium ions. However, the potentiometric results (as shown in **Figures 16** and **17**) in combination with the selectivity data provide evidence that the potentiometric response of the sensors is independent of the ionophore and is instead dependent only on the lipophilic salt likely acting as a non-specific ion exchanger. In light of these results, we suggest that the ionophore is not soluble in the membrane phase and is therefore incapable of forming complexes with ions. This suggestion is supported by the low solubility of **6d** observed in nonpolar solvents such as THF and $CHCl_3$. These solvents have dielectric constants (ε = 7.6 and 4.8 respectively) on the same order of NPOE and DOP plasticized PVC membranes (ε_{mem} = 14 and 4.8 respectively)[18] and thus model the polymeric matrix of the membrane phase. In addition,

^{1}H-NMR spectra of **6d** in $CDCl_3$ and THF-d_8 show broad structureless resonances for all peaks, indicative of poor solubility.

Table 2: Selectivity of **6d** for Ammonium over Other Cations

	Selectivity coefficients, log $K^{POT}_{NH_4^+,j}$					
Membrane[a]	Li^+	Na^+	K^+	Ca^{2+}	Mg^{2+}	Slope[b]
M1	0.5	0.7	1.2	0.6	0.4	6.8
M2	-1.8	-1.2	-0.3	-1.6	-1.7	48.6
M3	-2.3	-1.0	0.1	-1.5	-1.8	60.7
M4	-1.0	-0.7	0.0	-0.9	-1.1	20.7
M5	-0.7	-0.3	0.3	-0.8	-1.0	37.5
M6	-1.4	-0.8	0.0	-2.6	-2.6	45.8
Nonactin[d]	-3.5[4]	-2.4[4]	-1.0[4]	-3.8[7]	-4.0[7]	59.3[c]

[a] M1, 69/30/1 wt % of NPOE/PVC/**6d**; M2, same as M1 with 10 mol % of KtpClPB to **6d**; M3, same as M1 with 50 mol % of KtpClPB to **6d**; M4, 69/30/1 wt % of DOP/PVC/**6d**; M5, same as M4 with 10 mol % of KtpClPB to **6d**; M6, same as M4 with 50 mol % of KtpClPB to **6d**. [b]Determined between 10^{-3} and 10^{-1} M cation at 37ºC. [c]At 25ºC. [d]Data for nonactin taken from references indicated.

3.4 ^{13}C-NMR Study of Valinomycin binding Potassium Cations

Due to the apparent lack of solubility of **6d** in the ISE membranes, the decision was made to employ solution NMR methods in an attempt to evaluate the selectivity of **6d** for ammonium over other interfering cations. Selectivities of ionophores in solution are calculated from the ratios of the equilibrium dissociation constants for the metal-ligand complexes of the various cations. The dissociation constant (K_d) of a ligand with a given metal cation may be calculated by fitting a sigmoidal plot of experimental data to **Equation 2** to give K_d:

$$y = \frac{B_{max} x}{K_d + x} \qquad \textbf{(2)}$$

Here, x is the concentration of free ligand, y is the complex fraction, and B_{max} is the maximum number of binding sites. Based on molecular modeling, it appears that the carbonyl groups of **6d** are primarily responsible for forming the hydrogen bonds to ammonium cations (or ion-dipole interactions with interfering cations), although some amide nitrogen atoms also seem to contribute. The carbonyl groups should give the best measure of complexation because they should undergo the greatest change in electronic environment upon cation binding. Carbonyl participation in hydrogen bonding (or ion-dipole interactions) causes an increase in electron density toward the oxygen atom with a concurrent decrease in electron density away from the carbon atom. For this reason, the deshielded carbon atoms of the carbonyls of **6d** should show a downfield shift in the ^{13}C-NMR spectra upon complexation. Comparison of the integration of the free versus complexed carbonyl signals in the ^{13}C-NMR spectra of **6d** should allow determination of the extent of complexation occurring in a given solution.

In order to establish test conditions and validate the method, a control study of valinomycin was performed. Valinomycin (shown in **Figure 7a**) is the standard potassium ionophore used commercially in biosensors. A cyclic depsipeptide consisting of alternating amide and ester linkages (12 total), valinomycin preorganizes through hydrogen bonding of its amide carbonyl groups to form a pocket with its six ester carbonyl oxygens available for electrostatic stabilization of potassium ions through octahedral-type complexation. Due to the symmetry of the molecule, free (uncomplexed)

valinomycin only shows four carbonyl signals, two ester and two amide, in its ^{13}C-NMR spectrum. The ion-dipole interaction which takes place upon binding of potassium by valinomycin induces a shift of electron density on the carbonyl bonds towards the oxygen atoms resulting in an observed downfield shift of these signals.[19]

The decision was made to employ a 1:1 $CDCl_3$:MeOD solvent system in the NMR study since cation complexation is most favorable in nonpolar solvents, but the polar solvent MeOD is necessary for solubility of **6d**. 20 mg of valinomycin was dissolved in 0.50 mL of 1:1 $CDCl_3$:MeOD (3.6 mM solution) and the initial spectrum was attained. Spectra were performed on the same solution after every 10 μL addition of stock solutions of KSCN in 1:1 $CHCl_3$:MeOH (with an increasing number of scans as the solvent peaks grew in intensity). The downfield (complexed) carbonyl signals began to appear at a 1:1.6 mole ratio of valinomycin to KSCN and gradually increased in comparison to the free valinomycin signals (see **Figure 18**) until the free signals were indistinguishable from the noise (complexation ~100%) at a 1:5.75 mole ratio of valinomycin to KSCN.

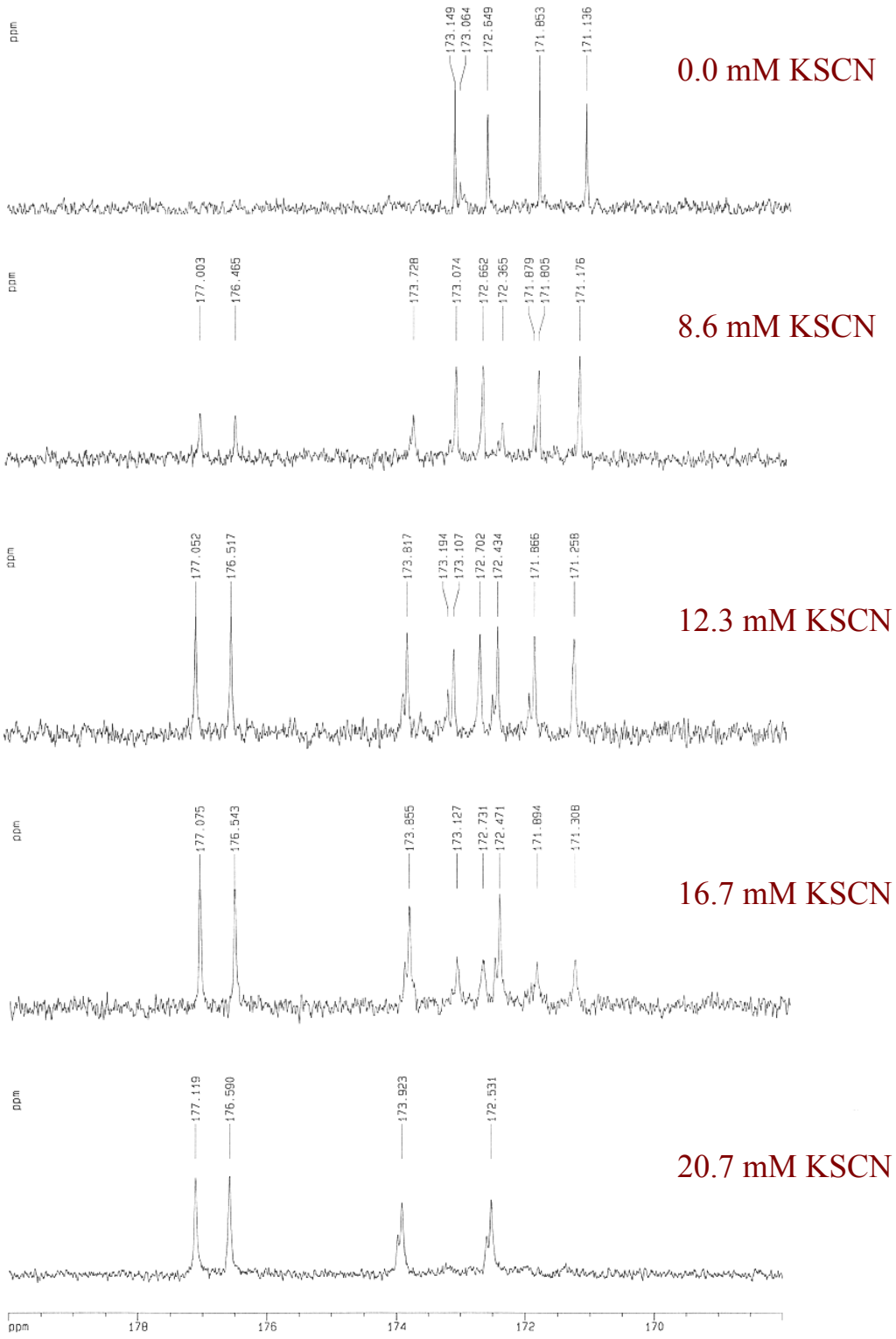


Figure 18: ^{13}C-NMR carbonyl signals of valinomycin as a function of [KSCN]

The extent of complexation of each solution was estimated from comparison of the integration (by peak height) of the farthest upfield (~171 ppm) and farthest downfield (~177 ppm) signals, which were selected because no splitting of these peaks was observed. The slight signal splitting observed is probably due to the loss of symmetry (and therefore signal equivalence) caused by the change in conformation upon binding the potassium cation. The plot of complexation versus KSCN concentration is shown in **Figure 19**. This data was imported into SigmaPlot 8.02 and the program settings for simple ligand binding for one site saturation were used to fit the curve to Equation 2, resulting in $K_d = 10^{-2}$ M. This is two orders of magnitude higher than the known value for the binding of potassium by valinomycin in MeOH ($K_d = 10^{-4}$ M).[18] However, the difference could be due to water contamination of the sample ($K_d = 0.4$ M in H_2O)[18], which could increase the dissociation constant significantly.

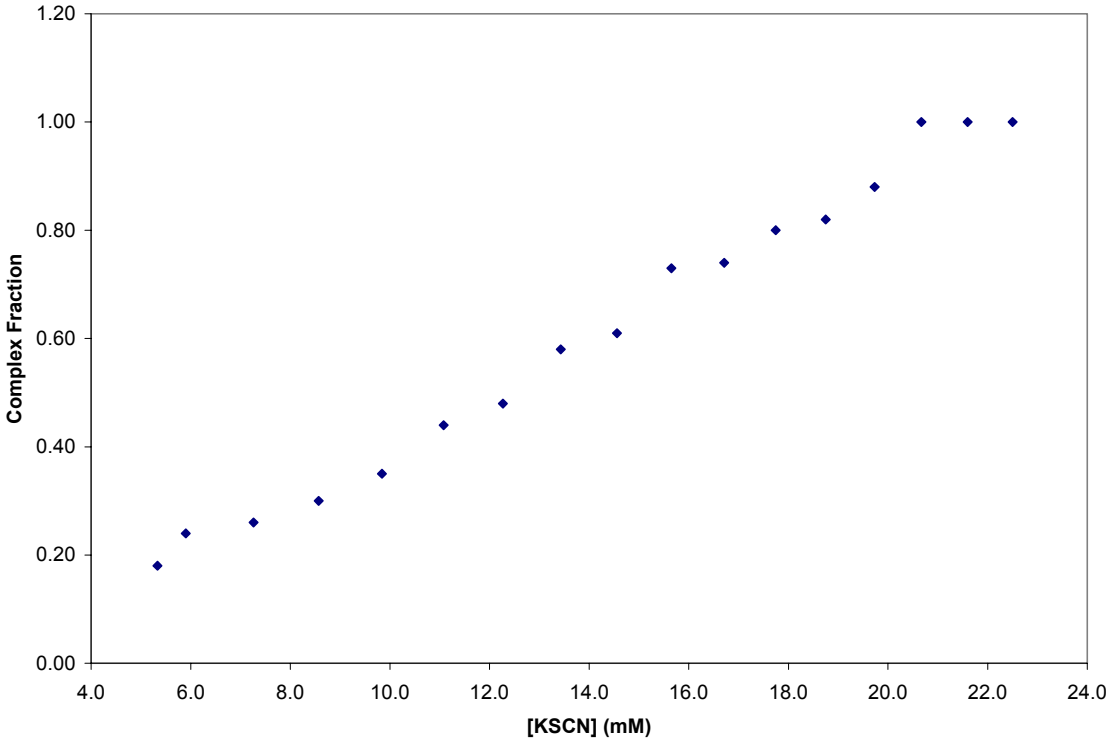


Figure 19: Valinomycin-potassium complex as a function of potassium concentration

4 Conclusions

A series of bicyclic peptides have been designed for complexation of ammonium cations through hydrogen bonding. Molecular modeling suggested these compounds could exhibit better ammonium over potassium cation selectivity than nonactin, the industry standard ammonium ionophore, in an ISE sensor format by exhibiting the tetrahedral coordination geometry required for ammonium binding, but not the octahedral coordination geometry required for potassium binding.

Bicyclic peptide **2d** was synthesized, but lacked the solubility necessary for characterization or evaluation in an ISE sensor. We were successful in enhancing solubility of the peptide intermediates of **5d** in organic solvents through the incorporation of two aminoisobutyric acid residues. However, the second cyclization reaction was unsuccessful, presumably due to an unfavorable steric interaction between one of the aminoisobutyric acid residues and the carbonyl group of the neighboring alanine residue. Replacement of this aminoisobutyric acid residue in **5d** with a leucine residue led to the successful synthesis and characterization of bicyclic peptide **6d**. **6d** was incorporated into an ISE sensor and tested as an ammonium ionophore, but lacked solubility in the ISE membrane.

Future work will involve a solution ^{13}C-NMR study of ammonium, potassium, and sodium ion binding with **6d**. Preliminary results of a control ^{13}C-NMR potassium-binding study of valinomycin suggest this method should be successful in evaluating selectivity of 6d for ammonium over interfering cations.

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Appendix: ^{1}H, ^{13}C, and DEPT135 NMR Spectra and ESI MS

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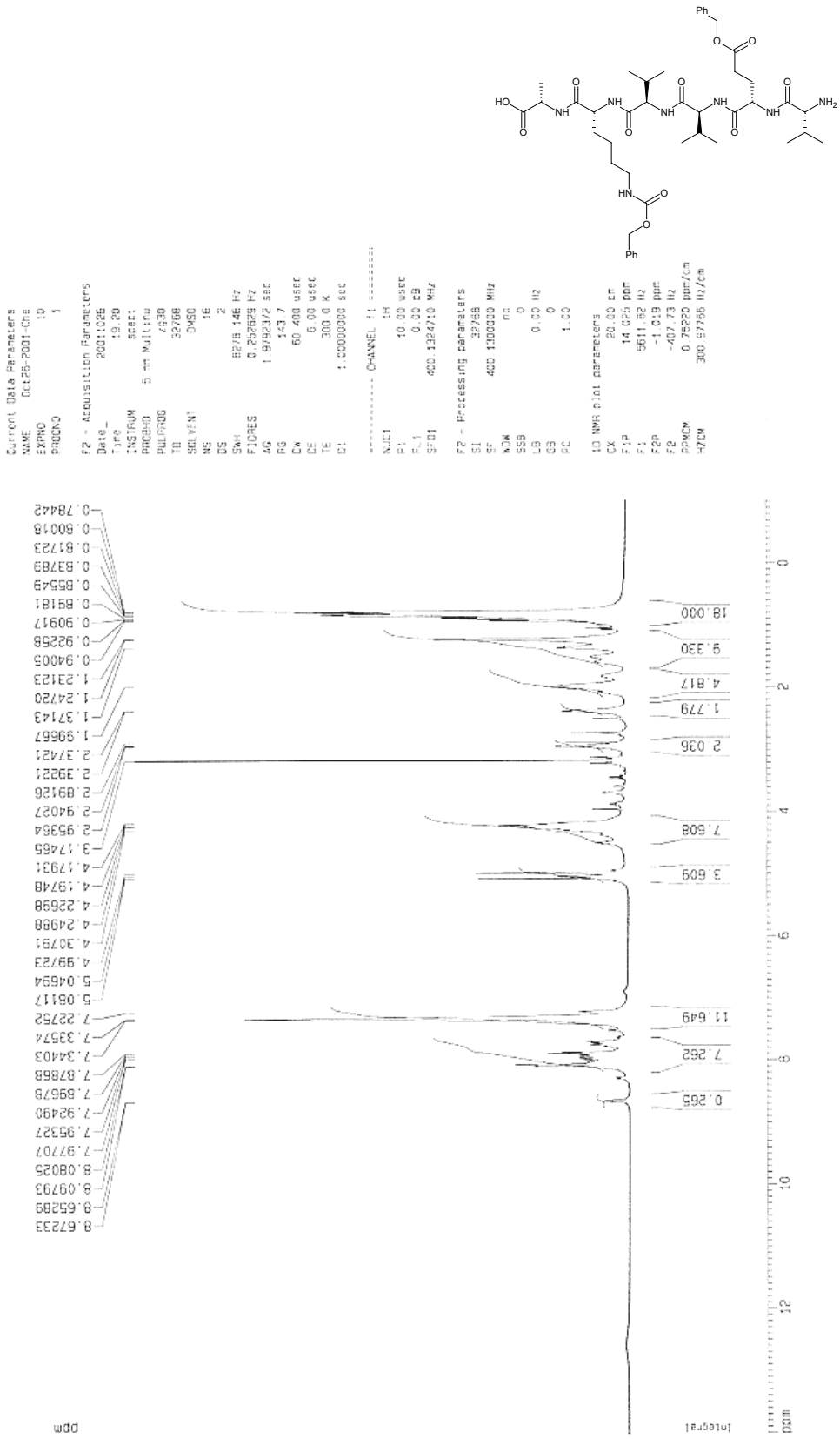
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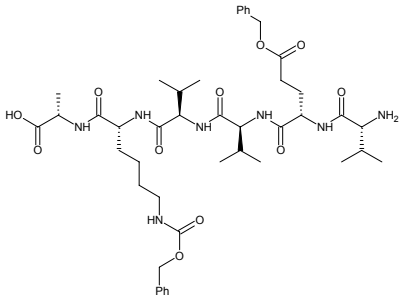

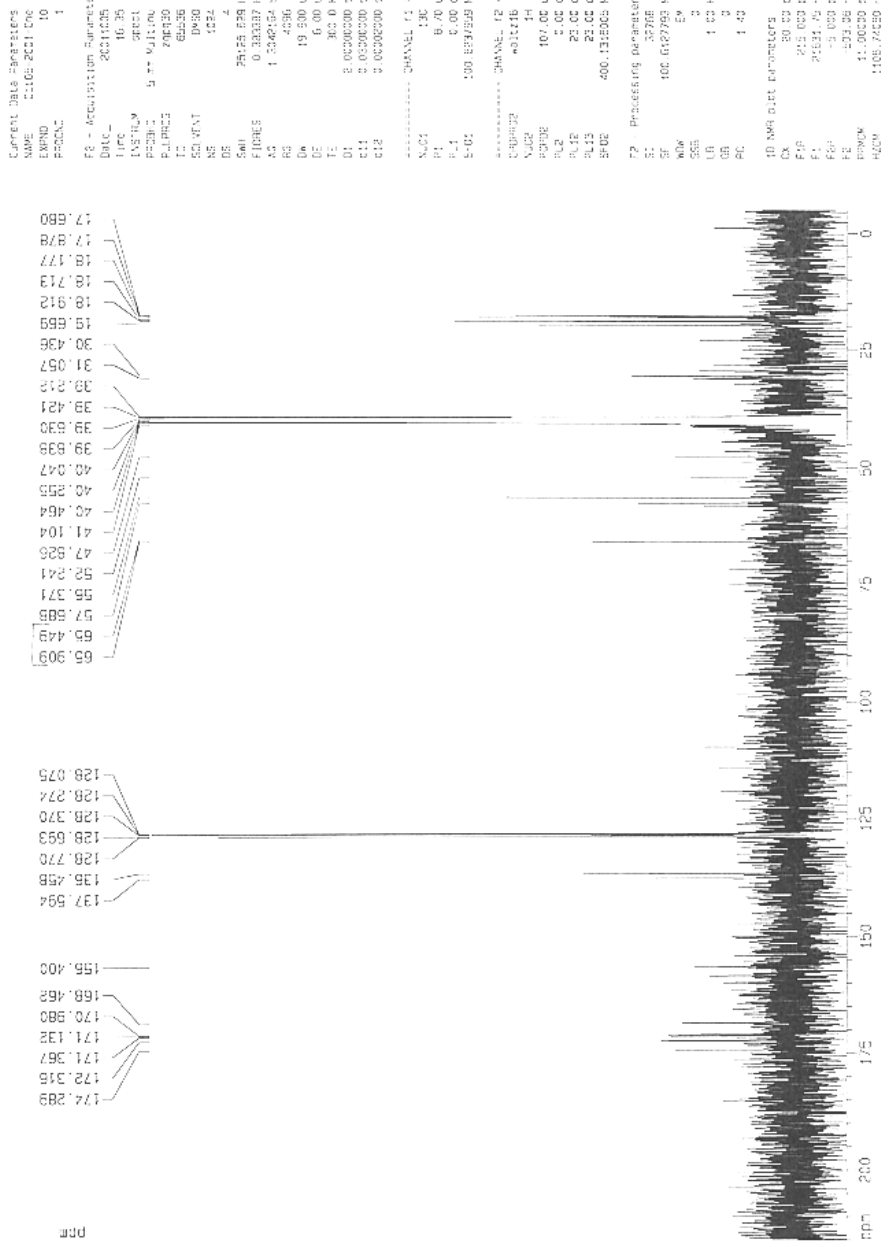

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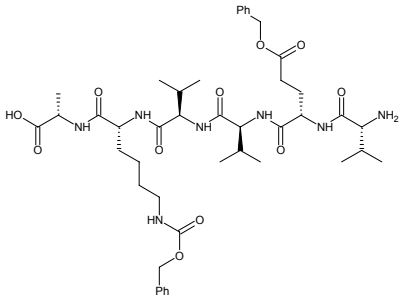
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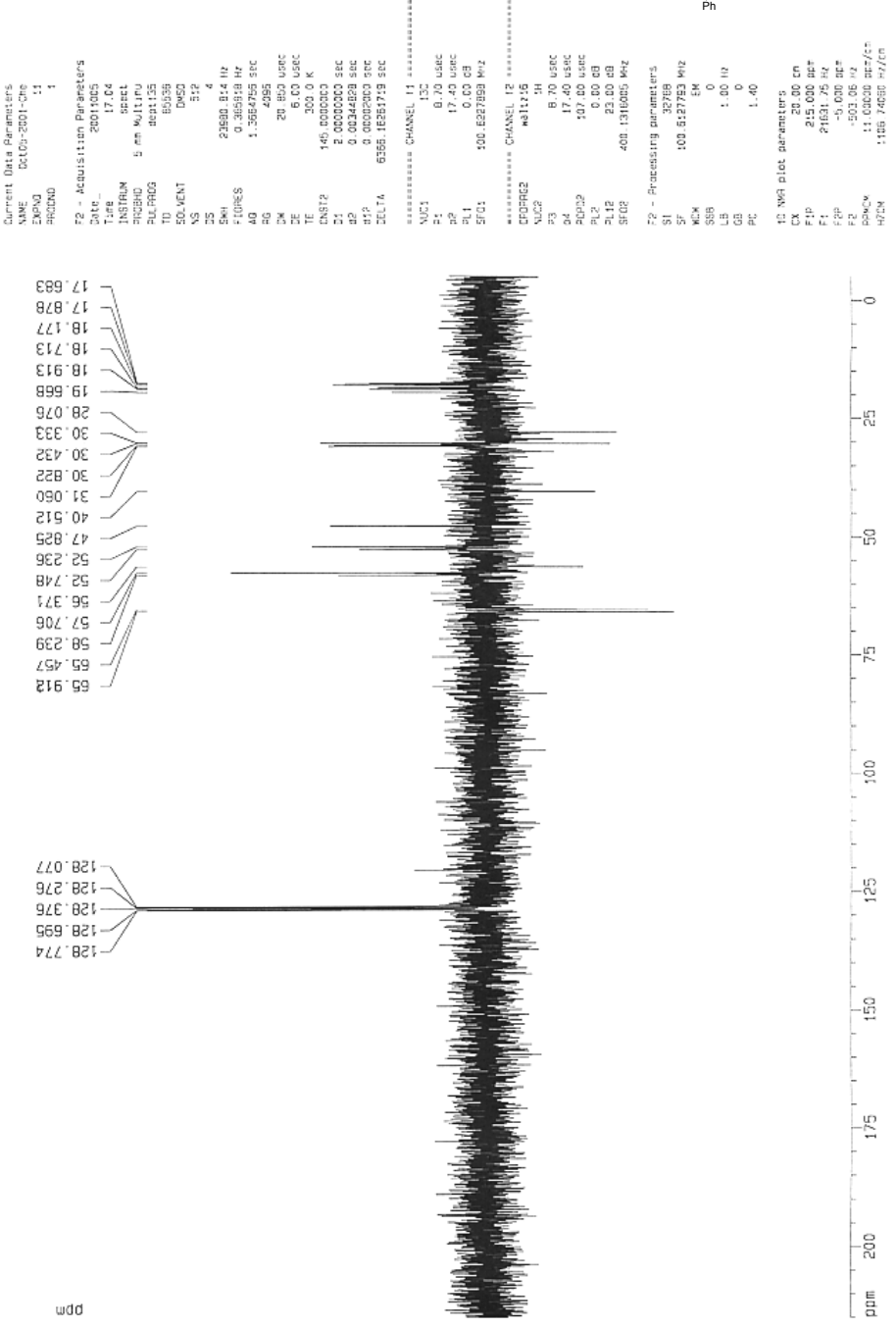
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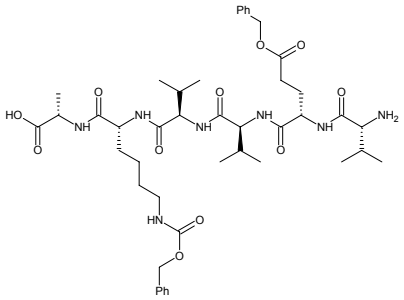
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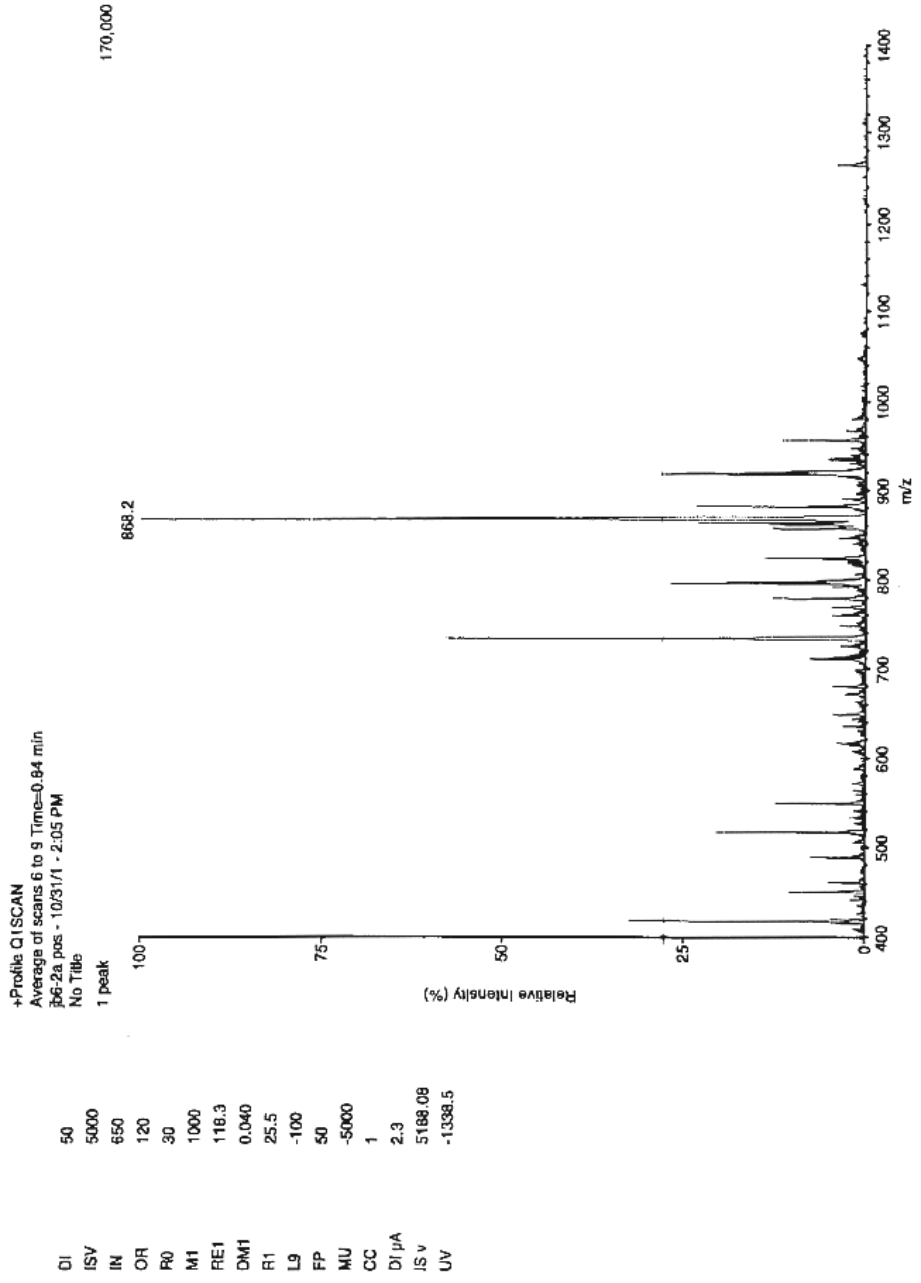
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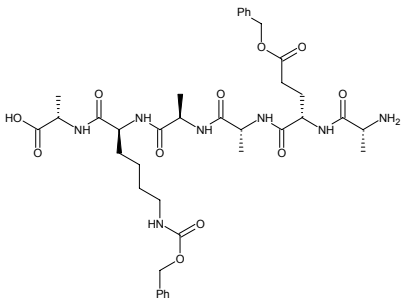
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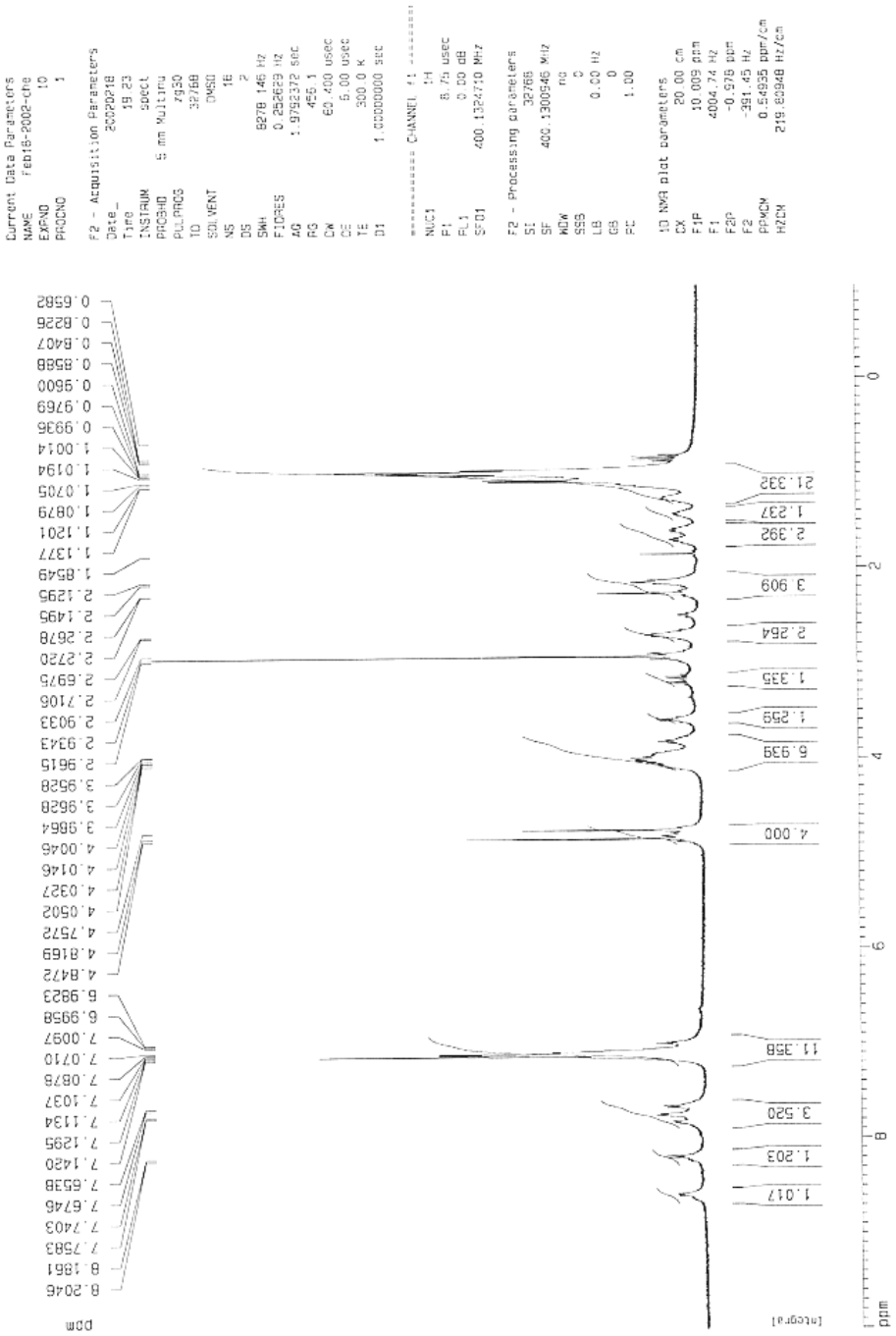
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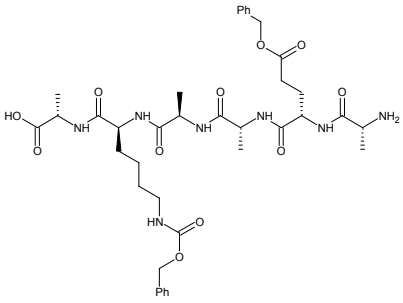
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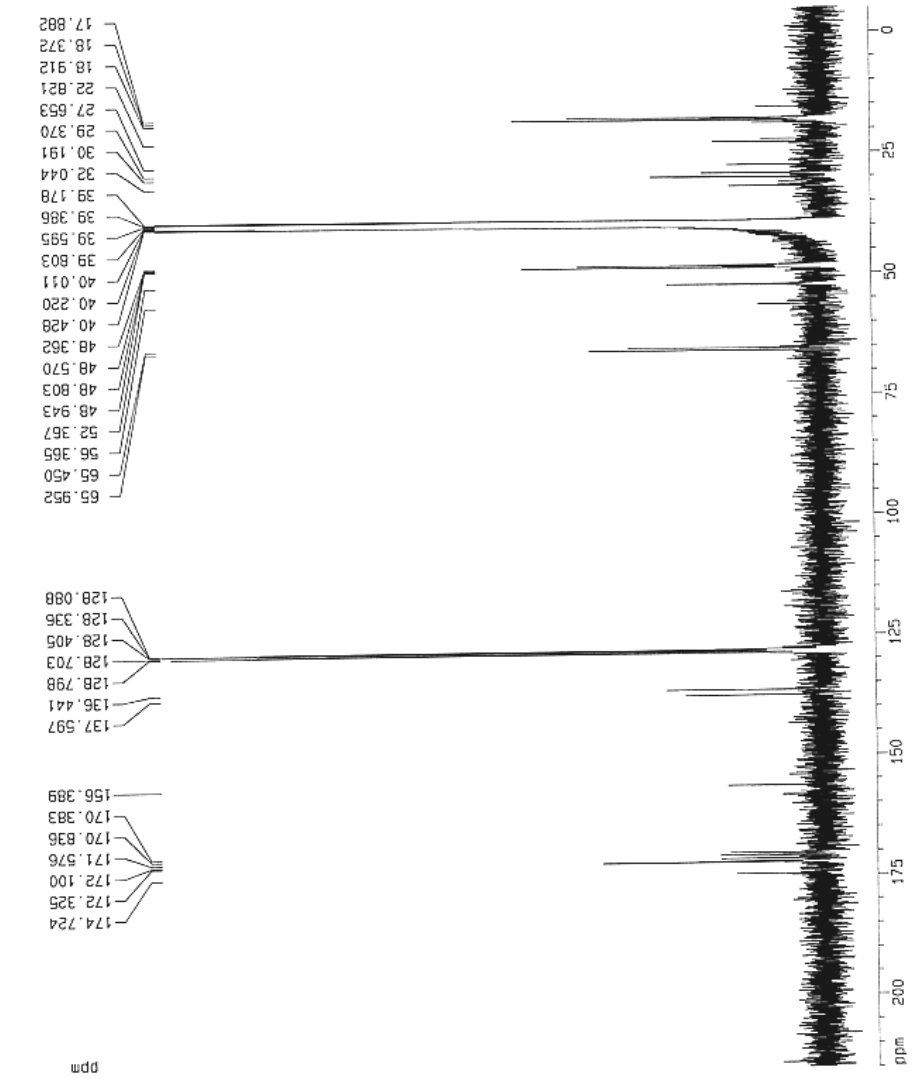
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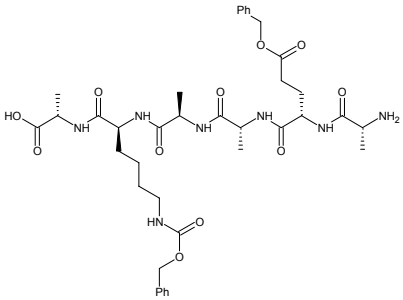
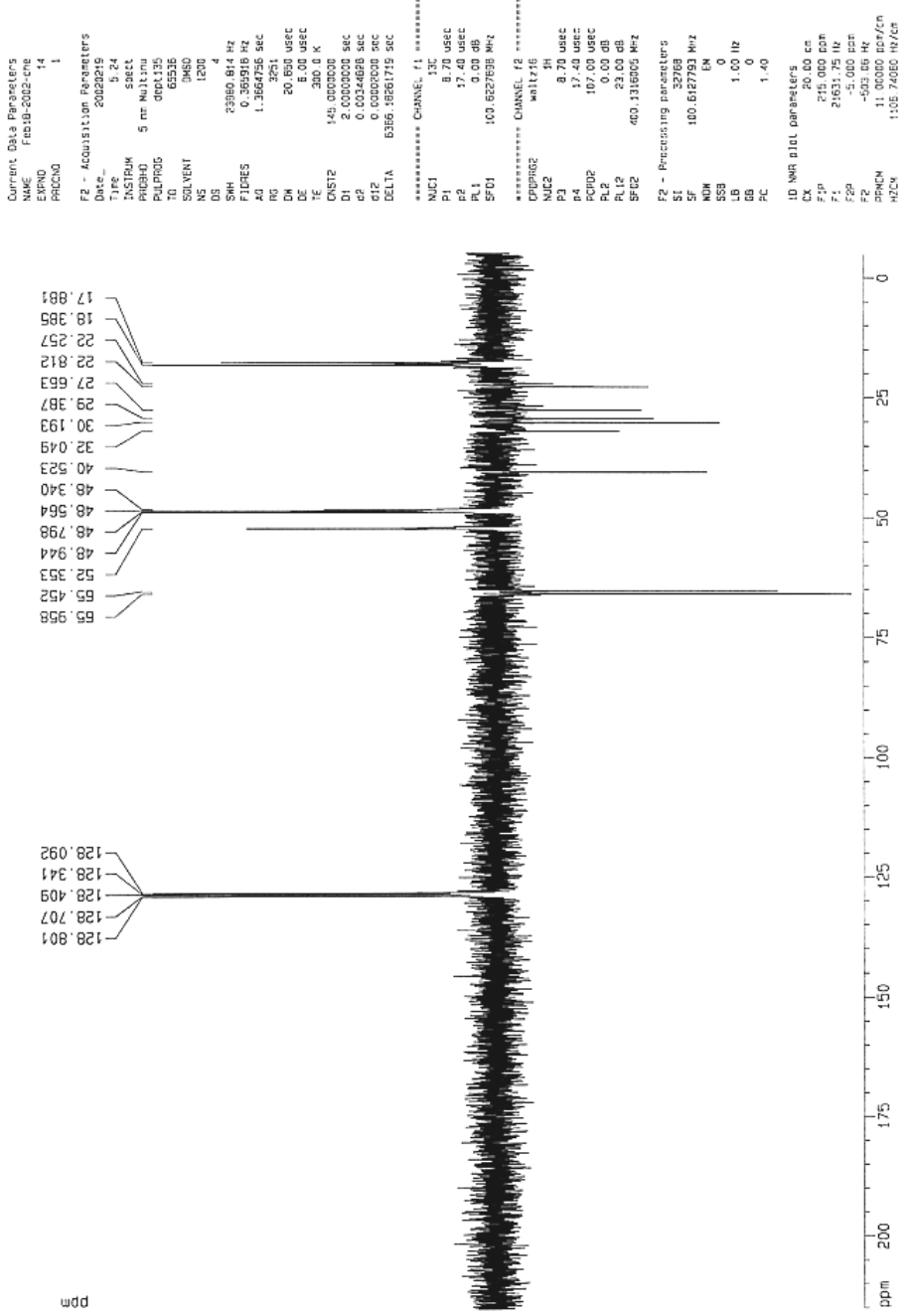
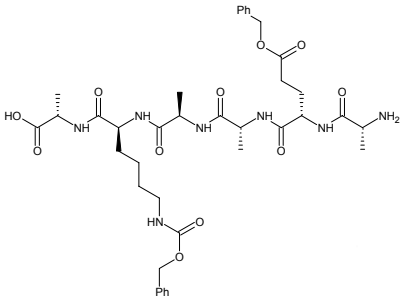
d11 0.03000000 sec
d12 0.00002000 sec
========== CHANNEL f1 ==========
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SFO1 100.6237959 MHz
========== CHANNEL f2 ==========
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127793 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.75 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.74060 Hz/cm
17.882
18.372
18.912
22.821
27.653
29.370
30.191
32.044
39.178
39.386
39.595
39.603
40.011
40.220
40.428
48.362
48.570
48.803
48.943
52.367
56.365
65.450
65.952
128.088
128.336
128.405
128.703
128.798
136.441
137.597
156.389
170.383
170.836
171.576
172.100
172.325
174.724
ppm
0
25
50
75
100
125
150
175
200

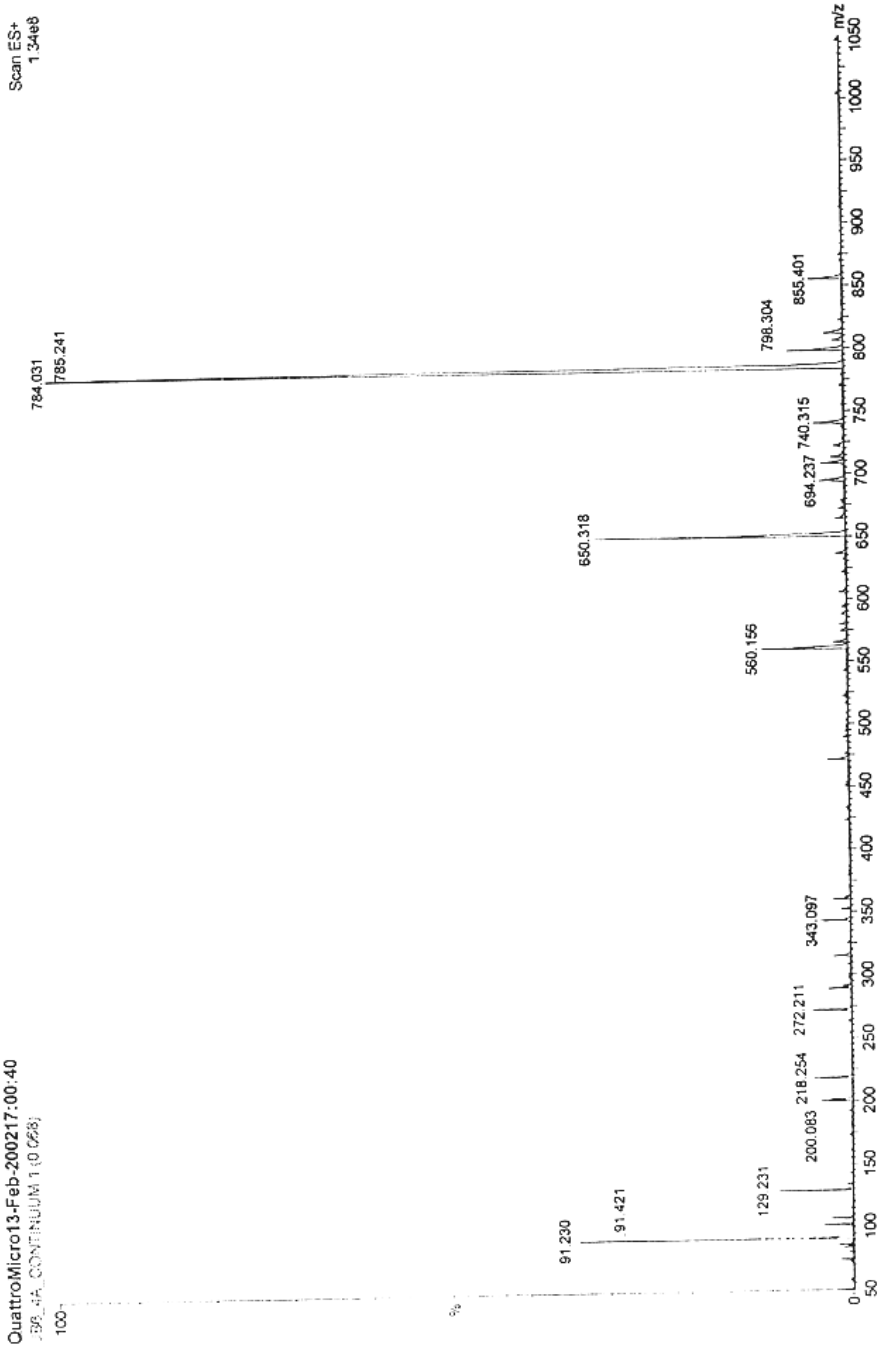
2a


Ph
O
O
HO
NH
NH
NH
NH
NH
NH2
HN
O
O
Ph

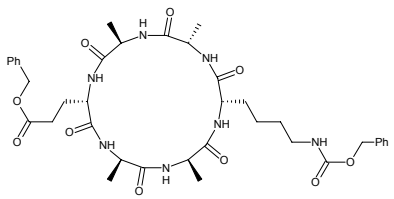

Current Data Parameters
NAME Feb19-2002-cne
EXPNO 14
PROCNO 1
F2 - Acquisition Parameters
Date_ 20020219
Time 5.24
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG dept135
TD 65536
SOLVENT DMSO
NS 1200
DS 4
SWH 23980.814 Hz
FIDRES 0.365916 Hz
AQ 1.36647936 sec
RG 3251
DW 20.850 usec
DE 6.00 usec
TE 300.0 K
CNST12 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 6356.18261719 sec
========== CHANNEL f1 ==========
NUC1 13C
P1 8.70 usec
P2 17.40 usec
PL1 0.00 dB
SFO1 100.6227698 MHz
========== CHANNEL f2 ==========
CPDPRG2 waltz16
NUC2 1H
P3 8.70 usec
P4 17.40 usec
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127793 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.75 Hz
F2P -5.000 ppm
F2 -503.66 Hz
PPMCM 11.00000 ppm/cm
HZCM 1108.74080 Hz/cm
17.881
18.385
22.257
22.812
27.653
29.387
30.193
32.049
40.523
48.340
48.564
48.798
48.944
52.353
55.452
55.958
128.092
128.341
128.409
128.707
128.801
ppm
0
25
50
75
100
125
150
175
200

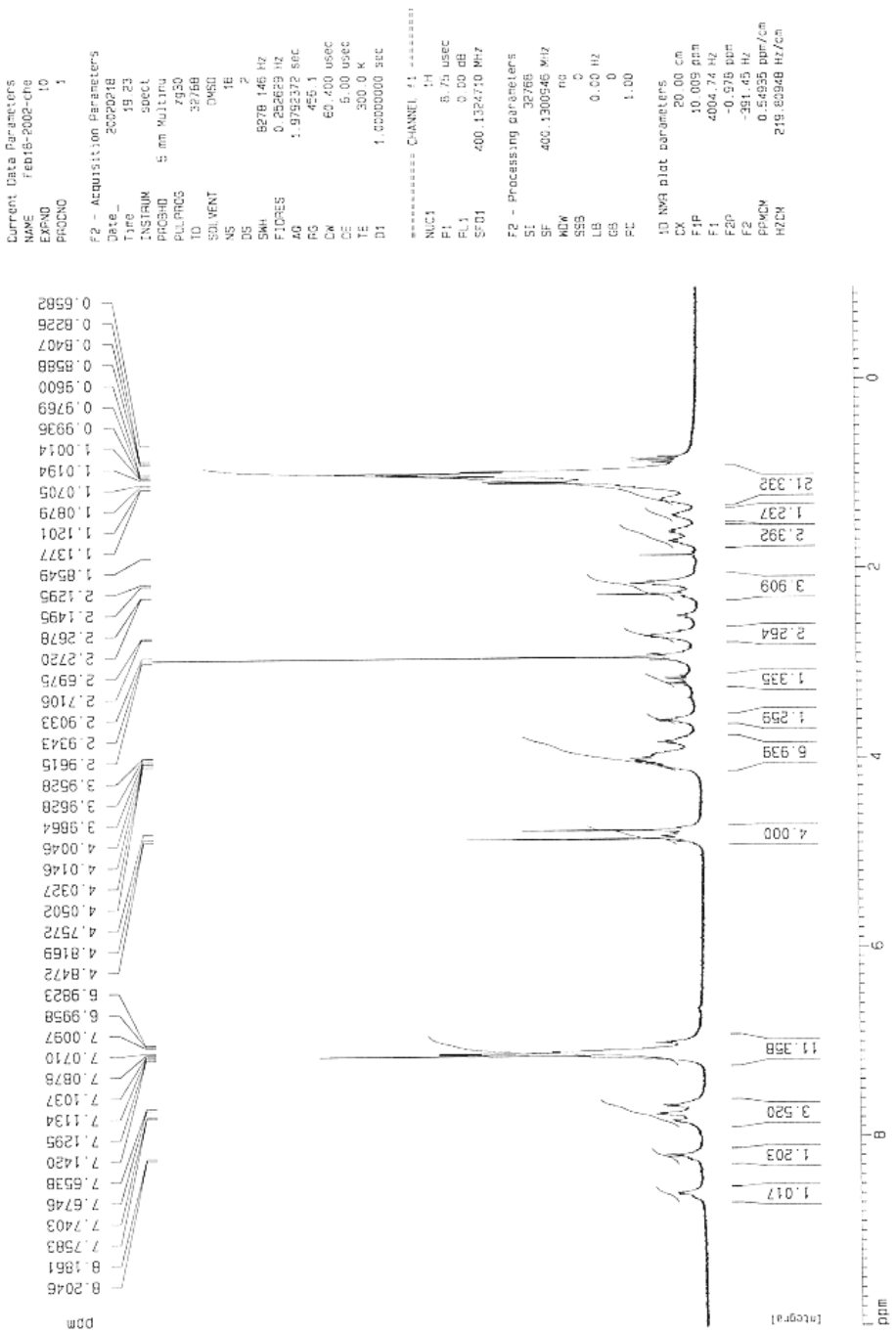
2a


Ph
O
O
HO
NH
NH
NH
NH
NH
NH2
HN
O
O
Ph

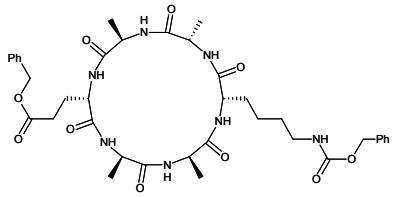

Scan ES+
1.34e8
m/z
QuattroMicro13-Feb-200217:00:40
100
%
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050
784.031
785.241
798.304
855.401
694.237
740.315
650.318
560.156
343.097
272.211
218.254
200.083
129.231
91.421
91.230

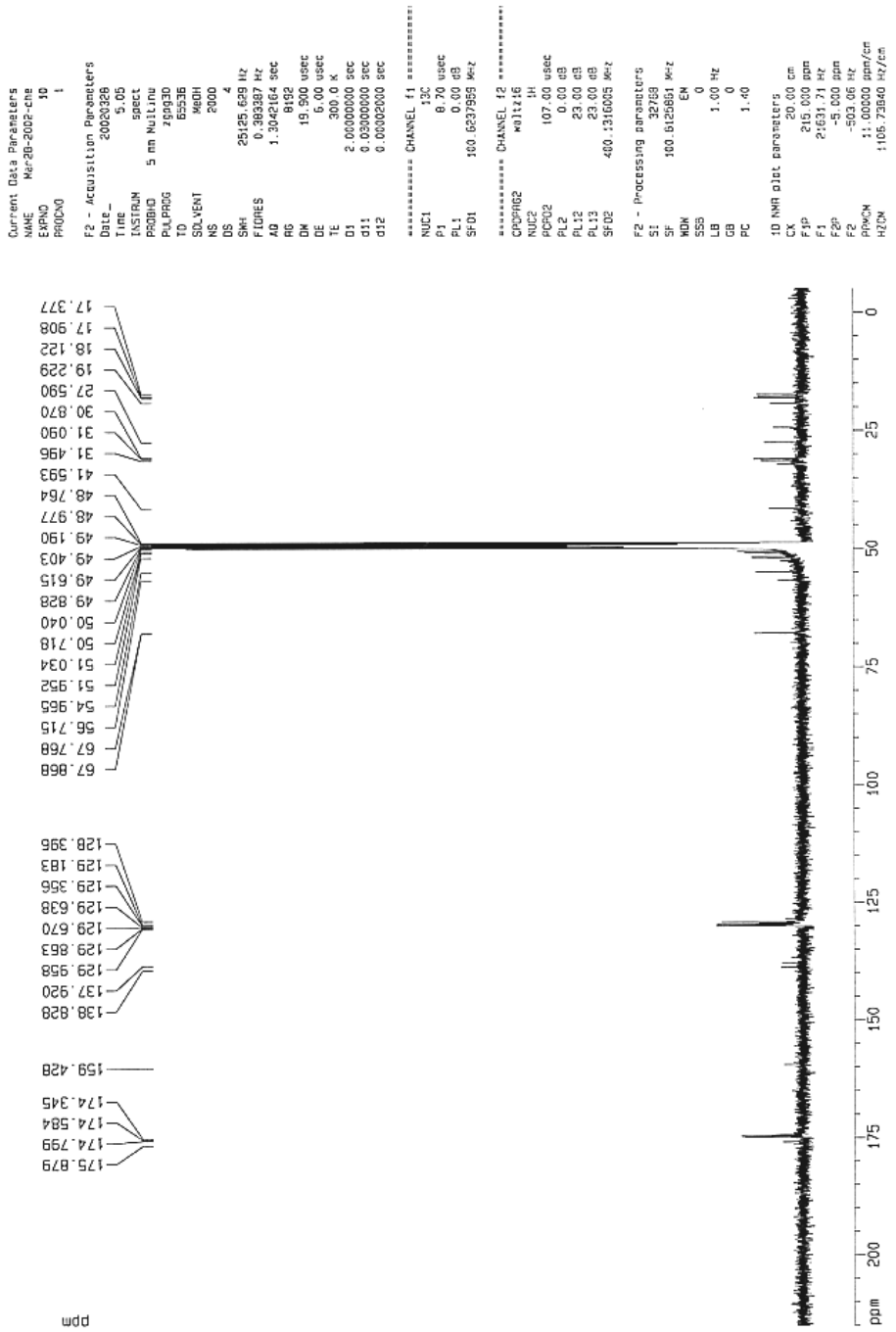
2b



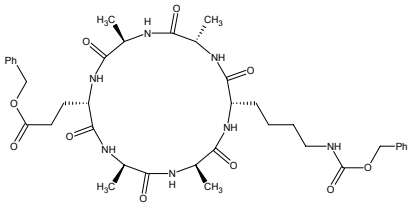

Current Data Parameters
NAME FEB18-2002-cha
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20020218
Time 19.23
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zg30
TD 32768
SOLVENT CH3OD
NS 16
DS 2
SWH 8278.146 Hz
FIDRES 0.252629 Hz
AQ 1.9782372 sec
RG 456.1
DW 60.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 8.75 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300546 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00
10 NMR plot parameters
CX 20.00 cm
F1P 10.009 ppm
F1 4004.74 Hz
F2P -0.976 ppm
F2 -391.45 Hz
PPMCM 0.54935 ppm/cm
HZCM 219.89948 Hz/cm
ppm
integral
21.332
1.237
2.392
3.909
2.264
1.335
1.259
6.939
4.000
11.358
3.520
1.203
1.017

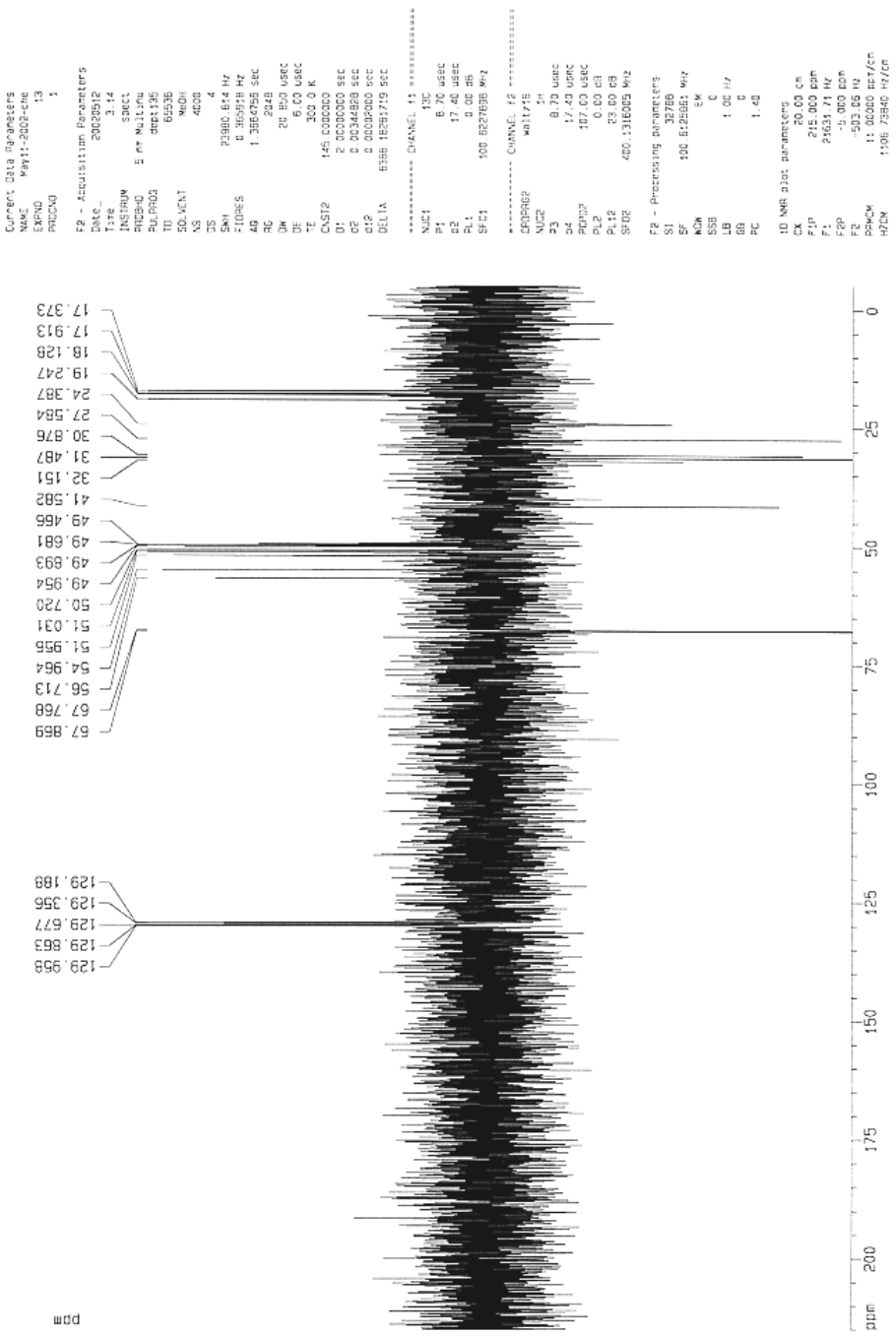
2b



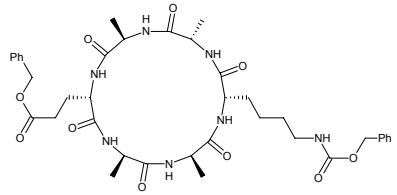

Current Data Parameters
NAME Mar20-2002-che
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20020320
Time 5.05
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zgpg30
TD 65536
SOLVENT MeOH
NS 2000
DS 4
SWH 25125.629 Hz
FIDRES 0.383387 Hz
AQ 1.3042164 sec
RG 8192
DW 19.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
d11 0.03000000 sec
d12 0.00002000 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SFO1 100.6237959 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6125861 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73640 Hz/cm
17.377
17.908
18.122
19.229
27.590
30.870
31.090
31.496
41.593
48.764
48.977
49.190
49.403
49.615
49.828
50.040
50.718
51.034
51.952
54.965
56.715
67.768
67.868
128.396
129.183
129.356
129.638
129.670
129.863
129.958
137.920
138.828
159.428
174.345
174.584
174.799
175.879
ppm
0
25
50
75
100
125
150
175
200

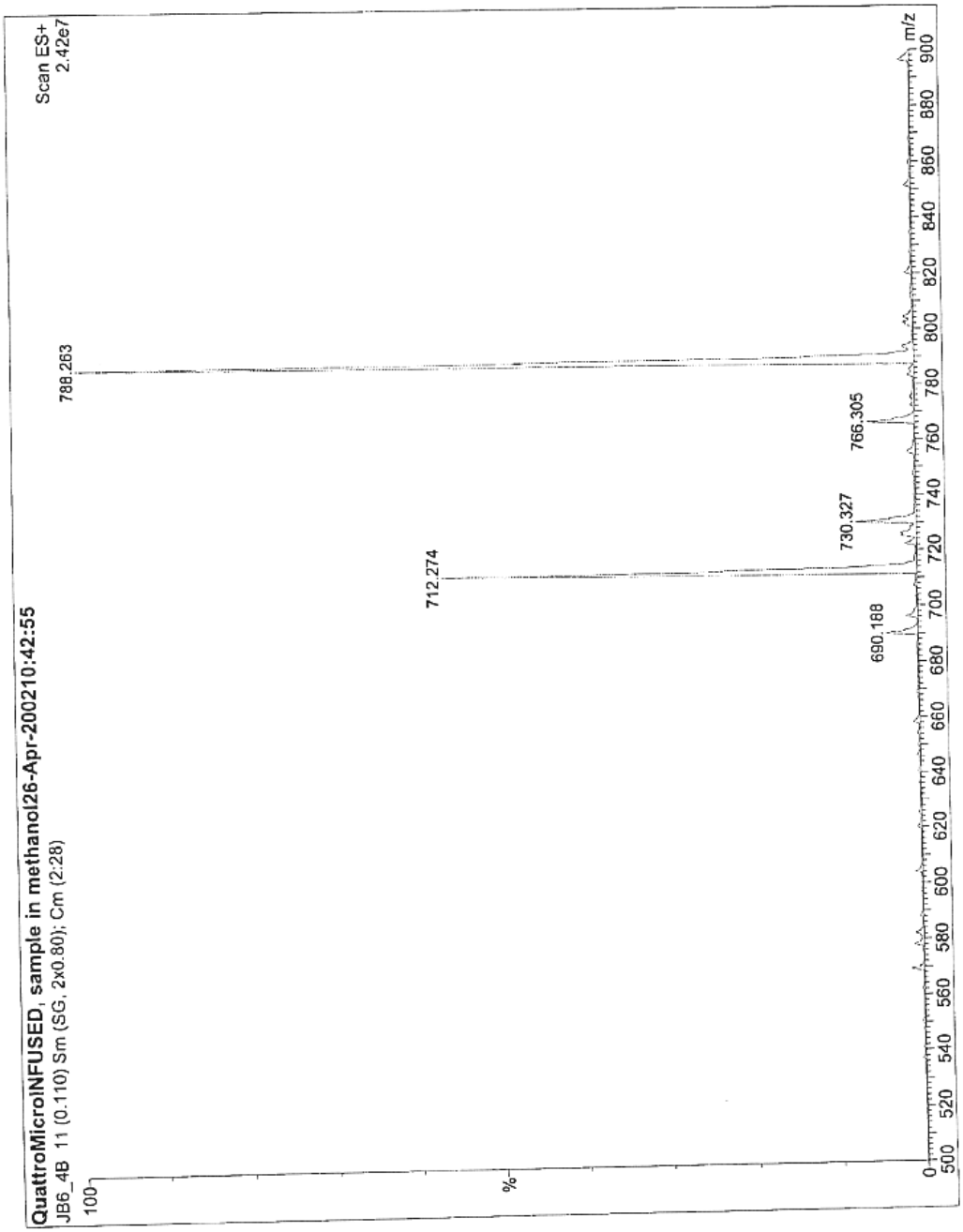
2b



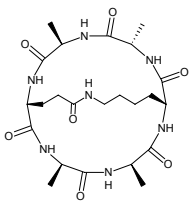

Current Data Parameters
NAME May11-2008-che
EXPNO 13
PROCNO 1
F2 - Acquisition Parameters
Date_ 20080512
Time 3.14
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG dept135
TD 65536
SOLVENT MeOH
NS 4020
DS 4
SWH 29880.614 Hz
FIDRES 0.365918 Hz
AQ 1.3966756 sec
RG 2048
DW 20.850 usec
DE 6.00 usec
TE 300.0 K
CNST2 145.0000000
D1 2.00000000 sec
D2 0.00344828 sec
d12 0.00002000 sec
DELTA 0.5586 1E281719 sec
========== CHANNEL f1 ==========
NUC1 13C
P1 8.70 usec
p2 17.40 usec
PL1 0.00 dB
SFO1 100.6227638 MHz
========== CHANNEL f2 ==========
CPDPRG2 waltz16
NUC2 1H
P3 8.70 usec
P4 17.40 usec
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6256861 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73840 Hz/cm
17.373
17.913
18.128
19.247
24.387
27.584
30.876
31.487
32.151
41.582
49.466
49.681
49.893
49.954
50.720
51.031
51.956
54.964
56.713
67.768
67.869
129.188
129.356
129.677
129.863
129.958
ppm
0
25
50
75
100
125
150
175
200

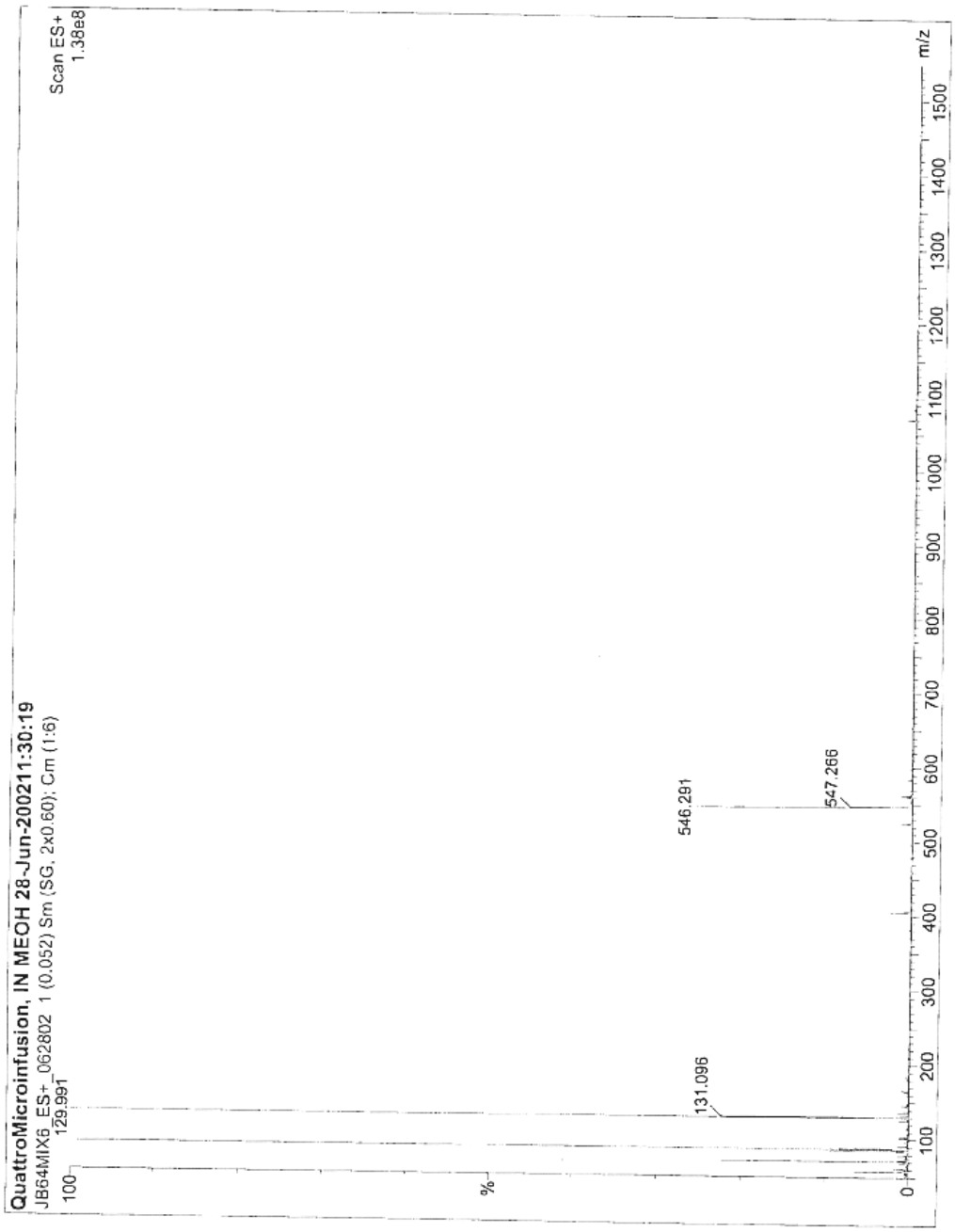
2b



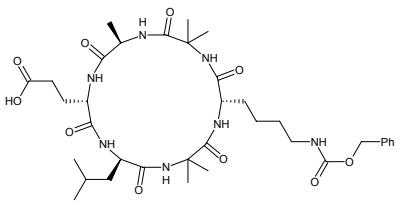

QuattroMicroINFUSED, sample in methanol26-Apr-200210:42:55
JB6_4B 11 (0.110) Sm (SG, 2x0.80); Cm (2:28)
Scan ES+
2.42e7
788.263
766.305
730.327
712.274
690.188
100
%
0
m/z
500
520
540
560
580
600
620
640
660
680
700
720
740
760
780
800
820
840
860
880
900

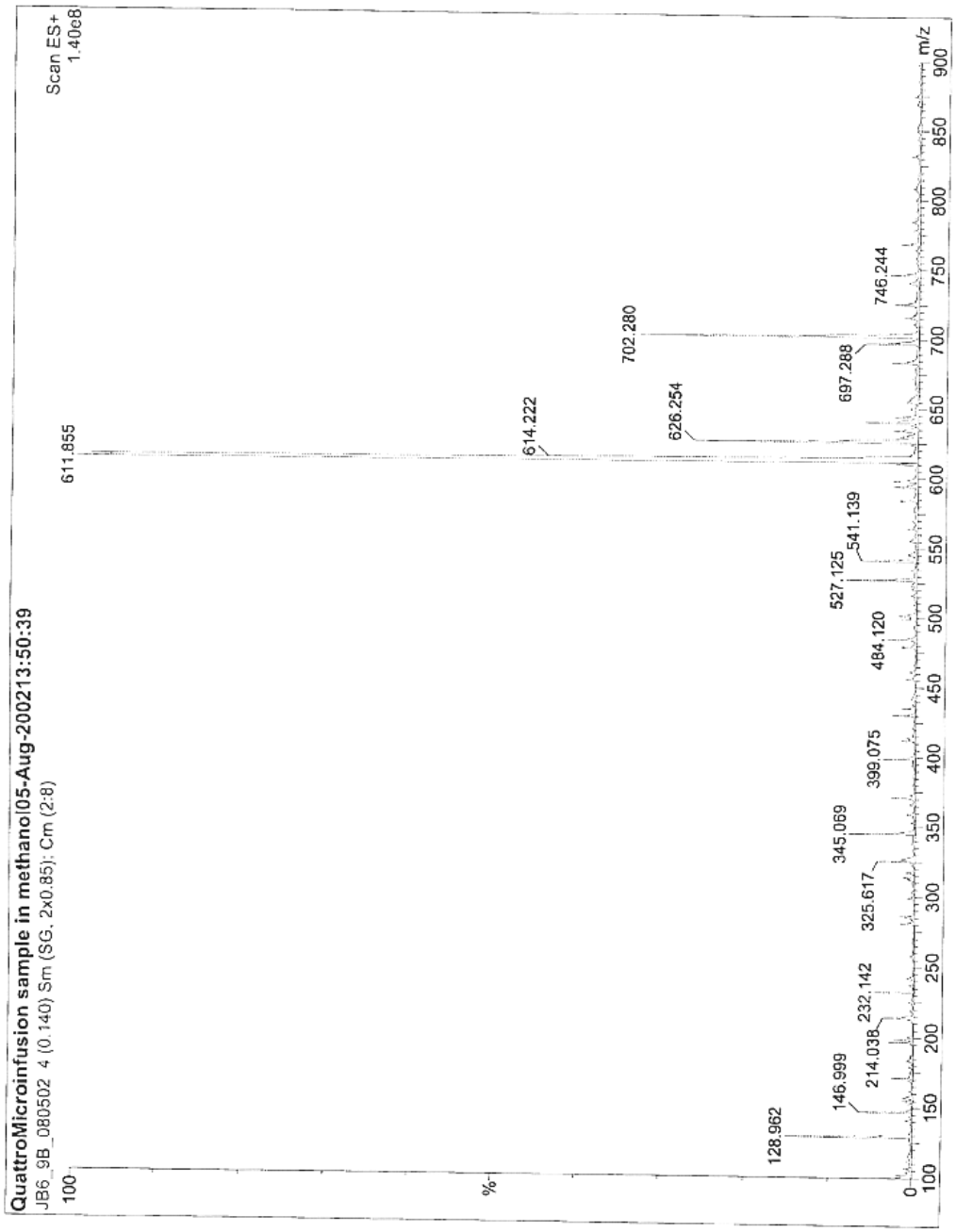
2d



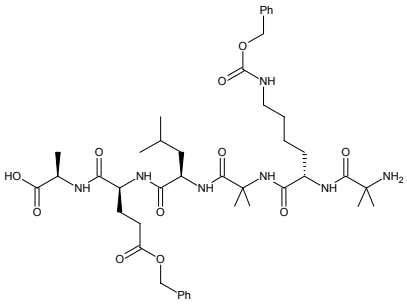

QuattroMicroinfusion, IN MEOH 28-Jun-200211:30:19
JB64MIX6 ES+ 062802 1 (0.052) Sm (SG, 2x0.60); Cm (1:6)
Scan ES+
1.38e8
129.991
131.096
546.291
547.266
%
m/z

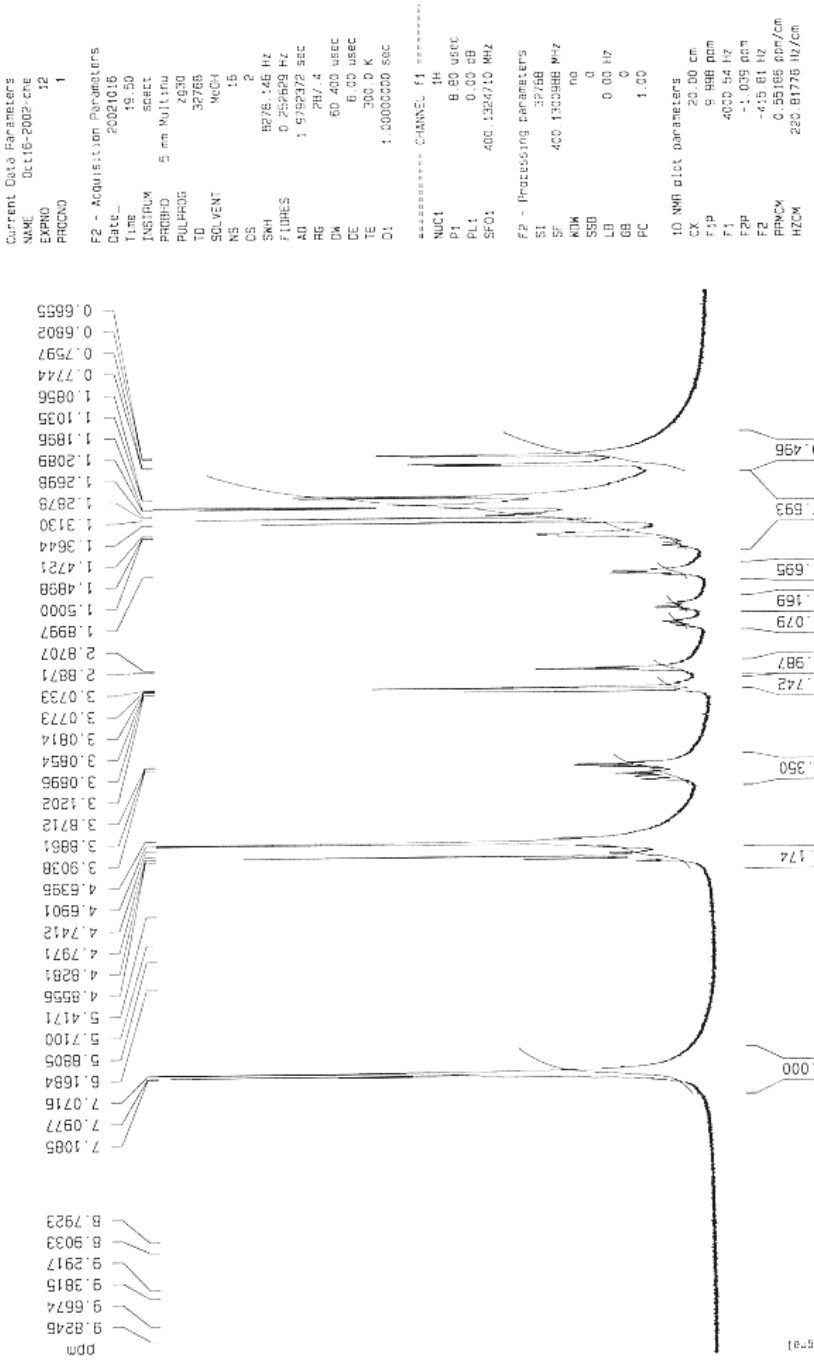
4b



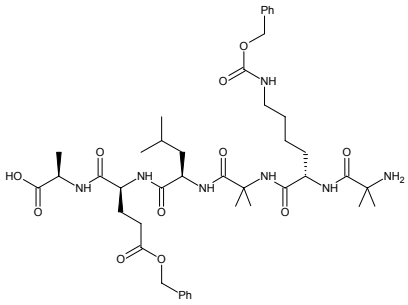

QuattroMicroinfusion sample in methanol05-Aug-200213:50:39
JB6_9B_080502 4 (0.140) Sm (SG, 2x0.85); Cm (2:8)
Scan ES+
1.40e8
100
%
0
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
m/z
611.855
614.222
626.254
697.288
702.280
746.244
527.125
541.139
484.120
399.075
345.069
325.617
232.142
214.038
146.999
128.962

5a


Ph
O
NH
HO
O
NH
O
NH
O
NH
O
NH
O
NH
NH2
O
O
Ph

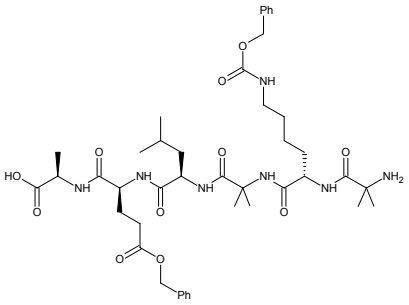

Current Data Parameters
NAME Oct16-2002-che
EXPNO 12
PROCNO 1
F2 - Acquisition Parameters
Date_ 20021016
Time 19.50
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zg30
TD 32768
SOLVENT MeOH
NS 16
DS 2
SWH 8278.146 Hz
FIDRES 0.252689 Hz
AQ 1.9762432 sec
RG 287.4
DW 60.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 8.80 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00
1D NMR plot parameters
CX 20.00 cm
F1P 9.998 ppm
F1 4000.54 Hz
F2P -1.039 ppm
F2 -415.81 Hz
PPMCM 0.55166 ppm/cm
HZCM 220.81778 Hz/cm
0.6655
0.6802
0.7597
0.7744
1.0856
1.1035
1.1856
1.2089
1.2698
1.2878
1.3130
1.3644
1.4721
1.4898
1.5000
1.8997
2.8707
2.8871
3.0733
3.0773
3.0814
3.0854
3.0896
3.1202
3.8712
3.8861
3.9038
4.6356
4.6901
4.7412
4.7971
4.8281
4.8556
5.4171
5.7100
5.8805
6.1684
7.0716
7.0977
7.1085
8.7923
8.9033
9.2917
9.3815
9.6674
9.8246
ppm
10.456
27.693
1.695
1.169
1.079
1.987
1.742
4.350
4.174
10.000
integral
0
2
4
6
8
ppm

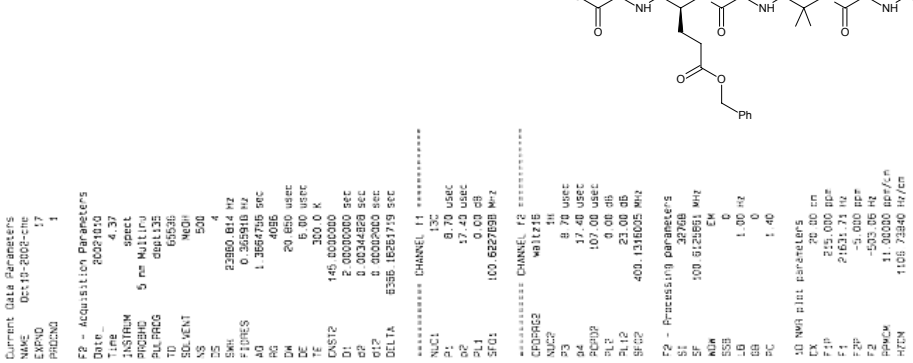
5a


Ph
O
NH
HO
NH
NH
NH
NH
NH
NH2
O
Ph

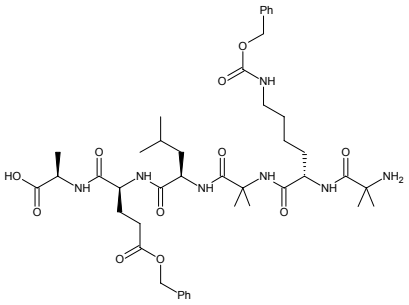
Current Data Parameters
NAME Oct14-2002-che
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20021015
Time 2.24
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zgpg30
TD 65536
SOLVENT MeOH
NS 3000
DS 4
SWH 25125.629 Hz
FIDRES 0.383397 Hz
AQ 1.3042154 sec
RG 4096
DW 19.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
d12 0.00002000 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SFO1 100.6237959 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6125851 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 236.279 ppm
F1 23772.60 Hz
F2P -13.448 ppm
F2 -1353.03 Hz
PPMCM 12.48633 ppm/cm
HZCM 1256.28149 Hz/cm
19.071
21.828
23.931
24.031
24.388
25.964
26.964
27.783
31.132
32.314
41.746
48.801
49.014
49.228
49.441
49.654
49.868
50.081
54.497
58.337
58.422
67.601
67.777
129.180
129.418
129.562
129.910
129.957
138.006
138.804
159.429
173.366
174.651
174.839
176.344
177.581
ppm
0
25
50
75
100
125
150
175
200
ppm

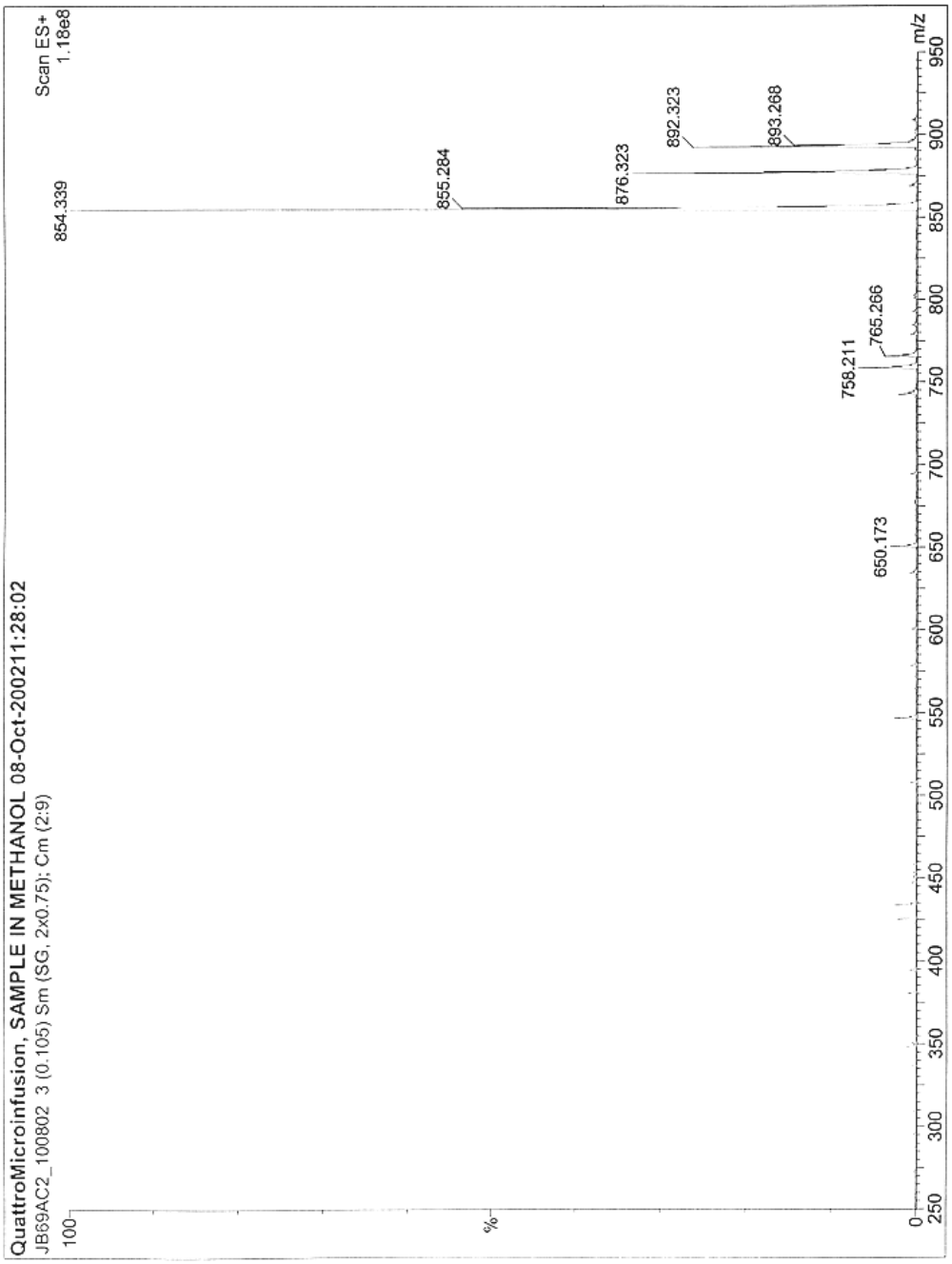
5a


Ph
O
NH
HO
NH
NH
NH
NH
NH
NH2
O
Ph

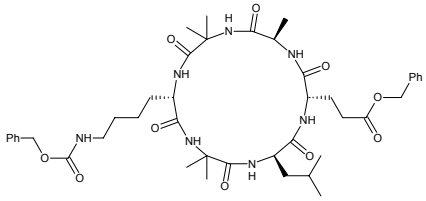

Current Data Parameters
NAME 0219-2008-ene
EXPNO 17
PROCNO 1
F2 - Acquisition Parameters
Date_ 20021210
Time 4.37
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG dept135
TD 65536
SOLVENT MeOH
NS 500
DS 4
SWH 23980.814 Hz
FIDRES 0.365910 Hz
AQ 1.3664736 sec
RG 4096
DW 20.850 usec
DE 6.00 usec
TE 300.0 K
CNST2 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00002000 sec
DELTA 8336.16261719 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
p2 17.40 usec
PL1 0.00 dB
SFO1 100.6227698 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
P3 8.70 usec
P4 17.40 usec
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6128991 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73840 Hz/cm
19.255
21.831
23.869
24.151
24.423
25.960
26.911
27.753
28.025
31.092
32.211
40.710
41.753
52.204
54.577
55.112
56.396
67.588
67.761
129.929
129.894
129.534
129.443
129.172
ppm
0
25
50
75
100
125
150
175
200

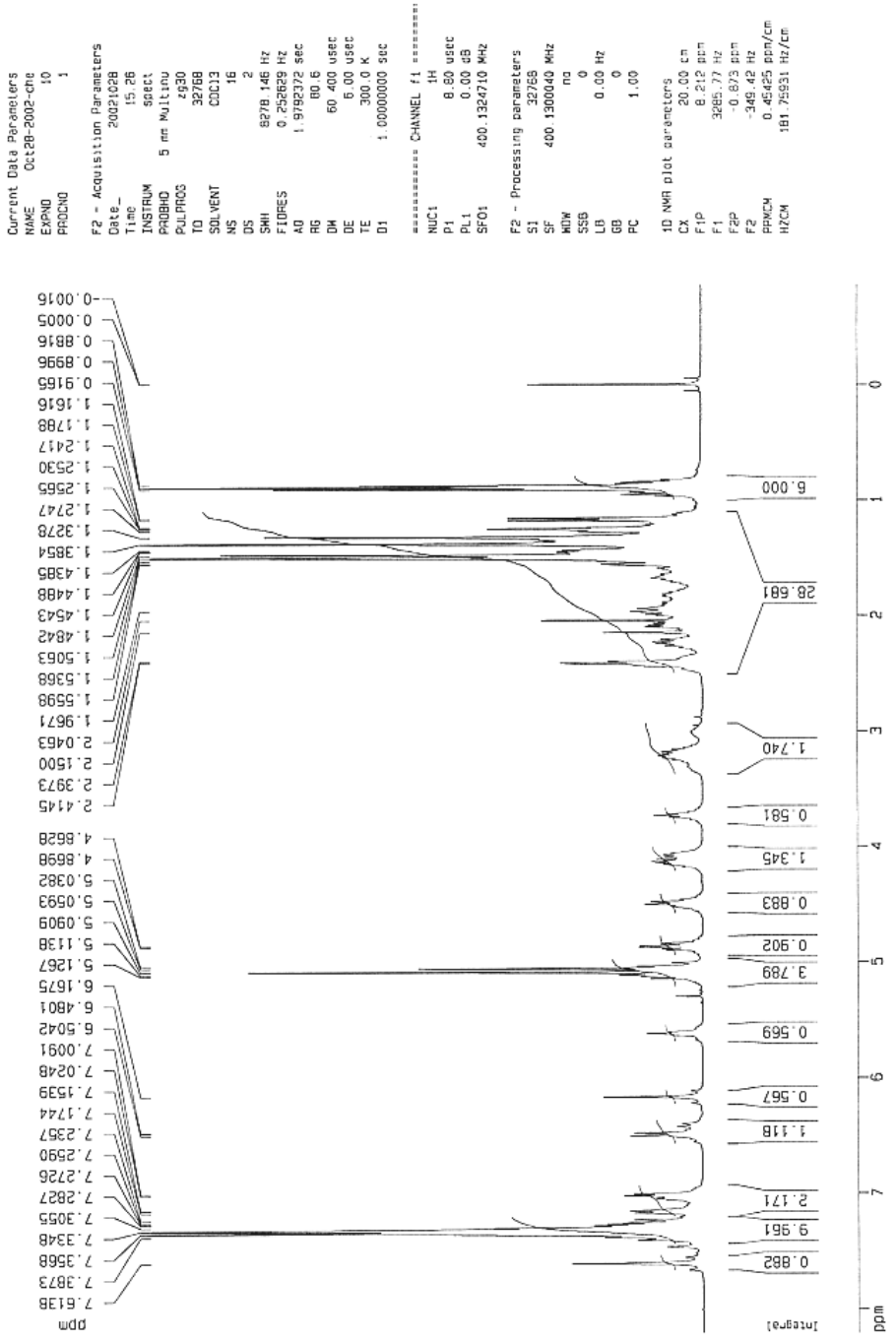
5a


Ph
O
O
NH
HO
O
NH
O
NH
O
NH
O
NH
O
NH
NH2
O
O
O
Ph

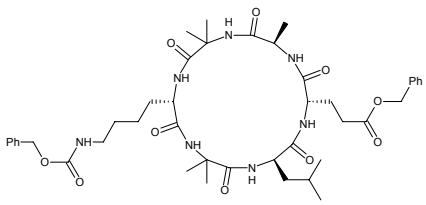

QuattroMicroinfusion, SAMPLE IN METHANOL 08-Oct-200211:28:02
JB69AC2_100802 3 (0.105) Sm (SG, 2x0.75); Cm (2:9)
Scan ES+
1.18e8
100
%
0
854.339
855.284
876.323
892.323
893.268
758.211
765.266
650.173
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
m/z

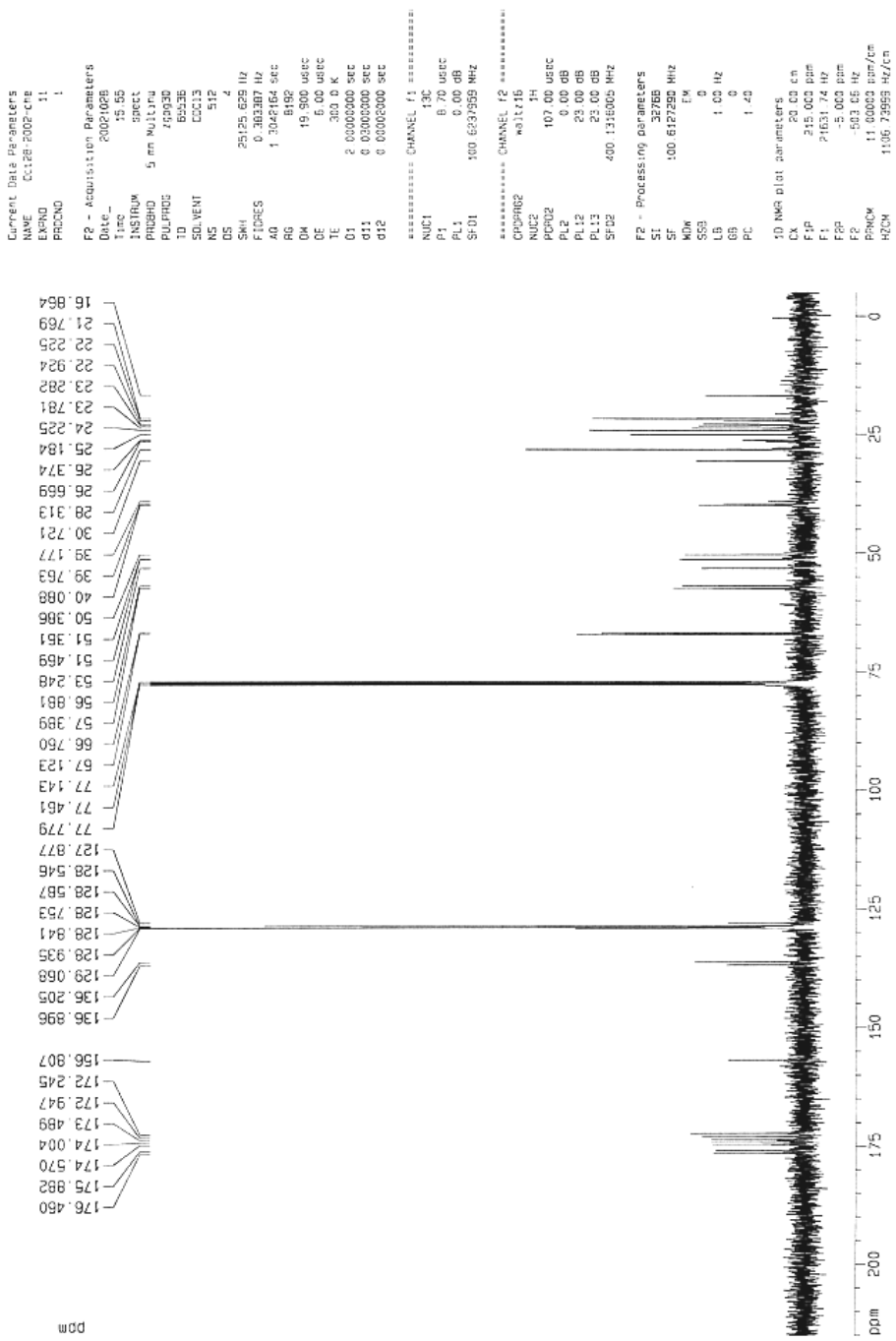
5b



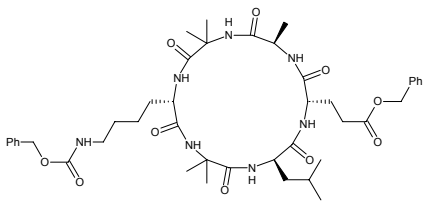

Current Data Parameters
NAME Oct28-2002-che
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20021028
Time 15.26
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 16
DS 2
SWH 8278.146 Hz
FIDRES 0.252629 Hz
AQ 1.9782372 sec
RG 80.6
DW 60.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
========== CHANNEL f1 ==========
NUC1 1H
P1 8.80 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300040 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00
1D NMR plot parameters
CX 20.00 cm
F1P 8.212 ppm
F1 3285.77 Hz
F2P -0.873 ppm
F2 -349.42 Hz
PPMCM 0.45425 ppm/cm
HZCM 181.75931 Hz/cm
ppm
Integral
6.000
28.681
1.740
0.581
1.345
0.883
0.902
3.789
0.569
0.567
1.118
2.171
9.961
0.882

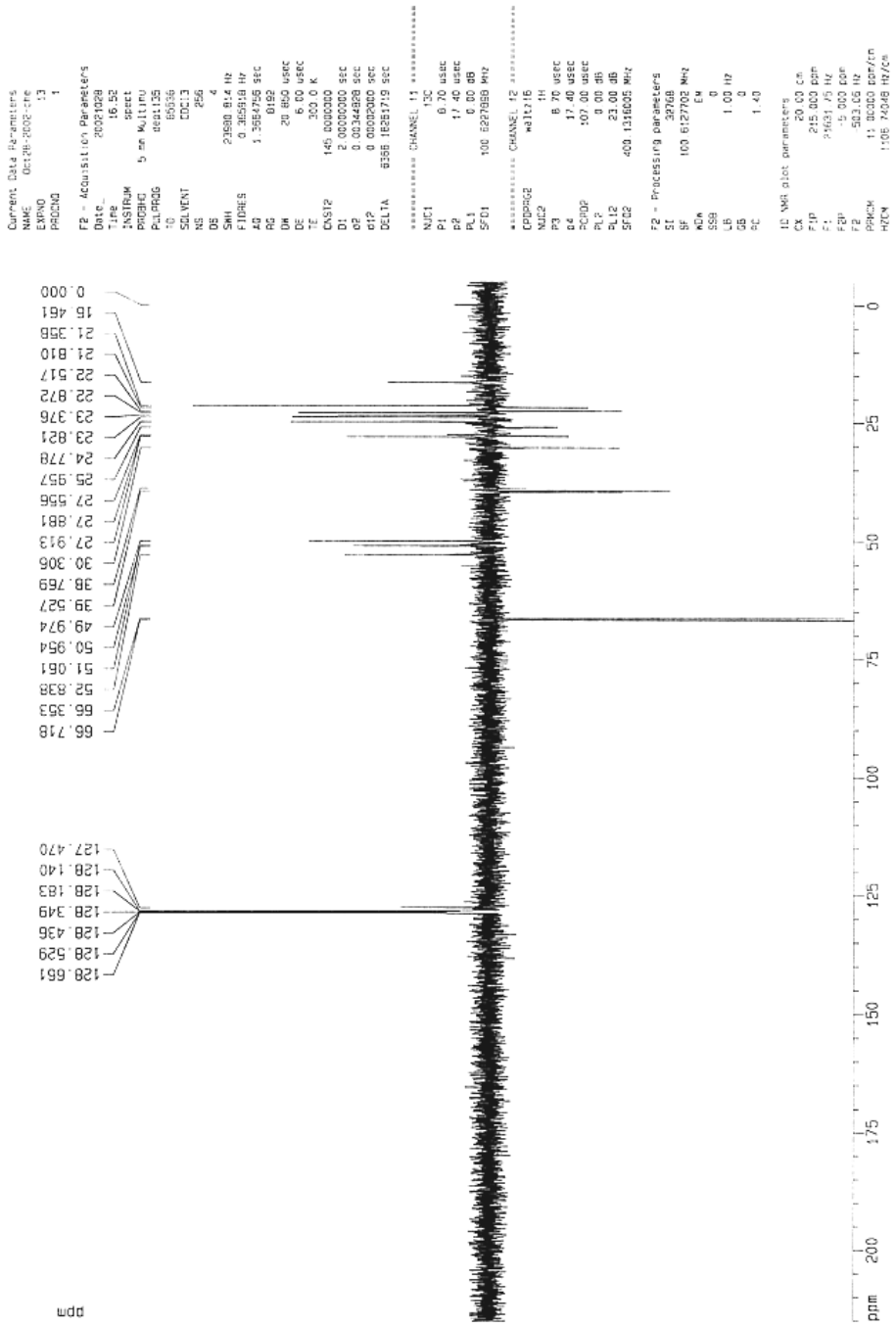
5b



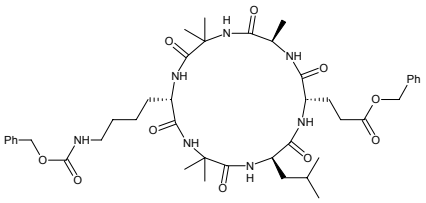

Current Data Parameters
NAME Oct28-2002-cne
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date_ 20021028
Time 15.55
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 512
DS 4
SWH 25125.629 Hz
FIDRES 0.383387 Hz
AQ 1.3042164 sec
RG 8192
DW 19.900 usec
DE 6.00 usec
TE 302.0 K
D1 2.00000000 sec
d11 0.03000000 sec
d12 0.00002000 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SF01 100.6237959 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SF02 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127290 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.74 Hz
F2P -5.000 ppm
F2 -503.66 Hz
PPMCM 11.00003 ppm/cm
HZCM 1106.73959 Hz/cm
16.864
21.769
22.225
22.924
23.282
23.781
24.225
25.184
26.374
26.669
28.313
30.721
39.177
39.763
40.088
50.386
51.361
51.469
53.248
56.881
57.389
66.760
67.123
77.143
77.461
77.779
127.877
128.546
128.587
128.753
128.841
128.935
129.068
136.205
136.896
156.807
172.245
172.947
173.489
174.004
174.570
175.882
176.460
ppm
0
25
50
75
100
125
150
175
200

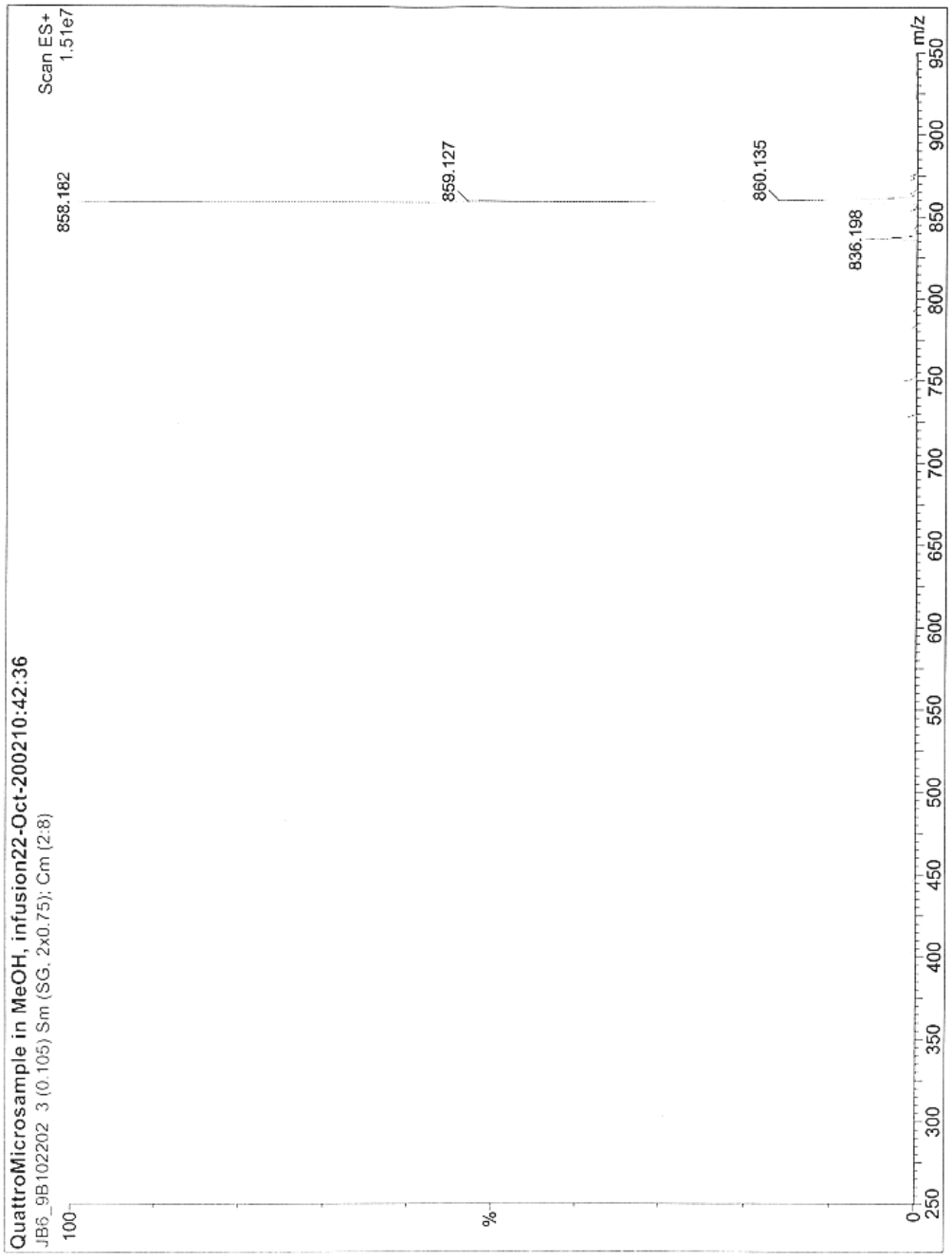
5b



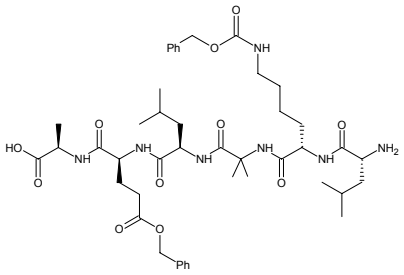


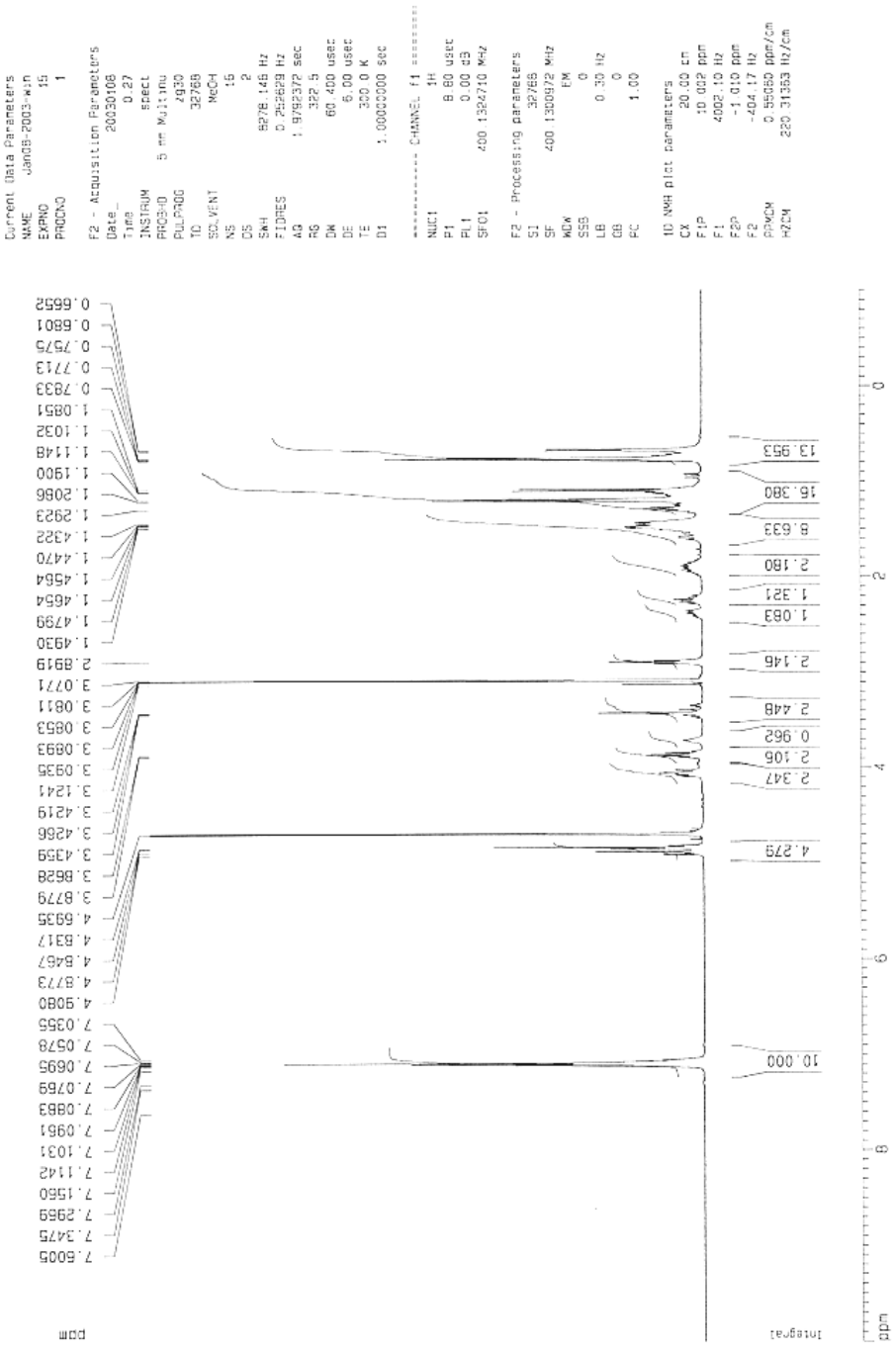
5b



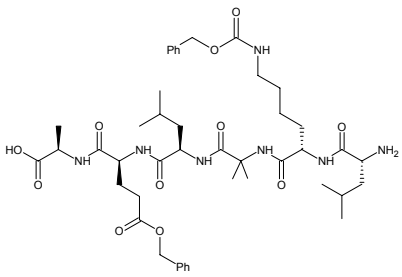

QuattroMicrosample in MeOH, infusion22-Oct-200210:42:36
JB6_9B102202 3 (0.105) Sm (SG, 2x0.75); Cm (2:8)
Scan ES+
1.51e7
858.182
859.127
860.135
836.198
100
%
0
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
m/z

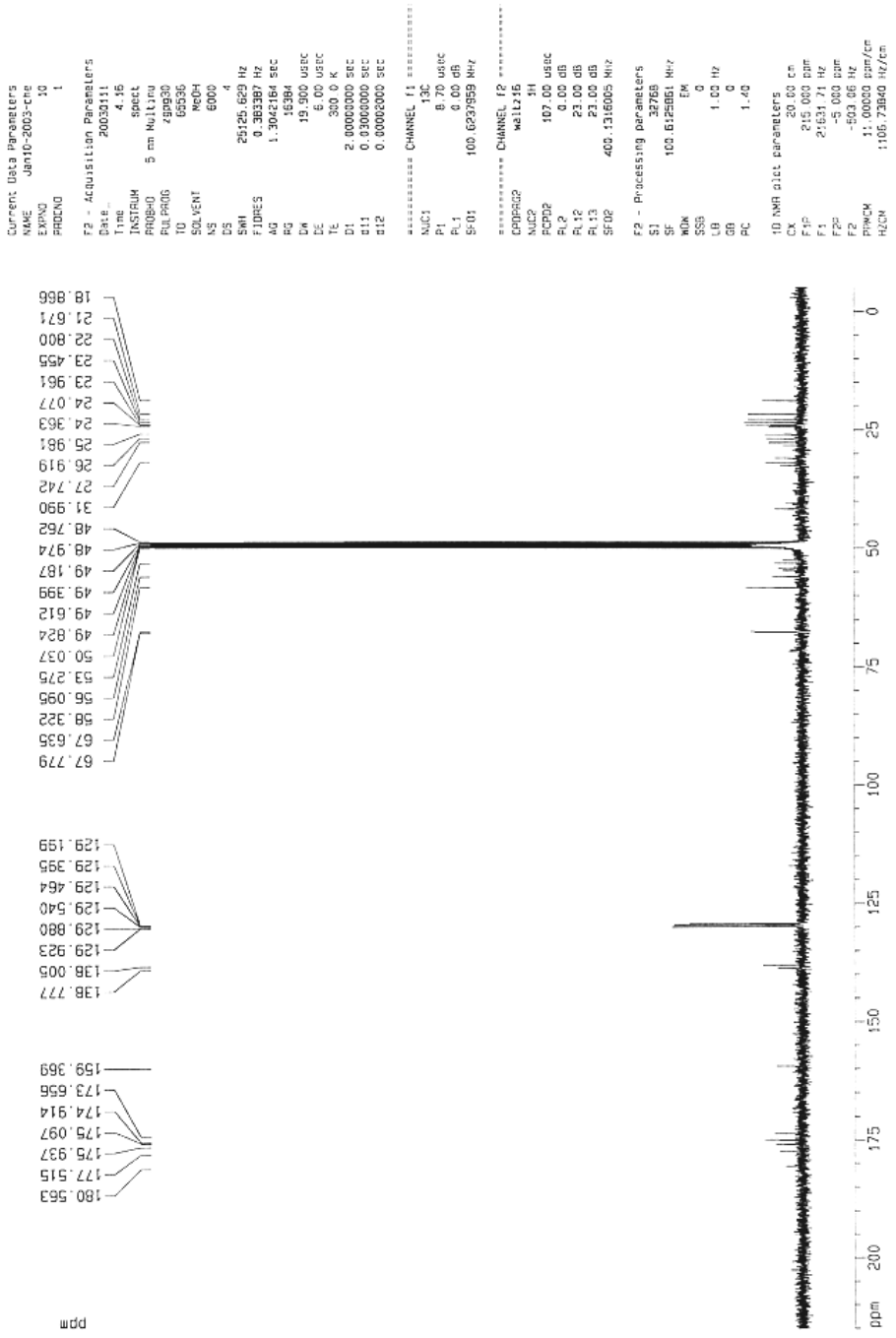
6a


Ph
O
NH
HO
NH
NH
NH
NH
NH
NH2
Ph

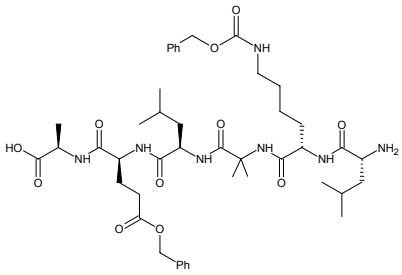

Current Data Parameters
NAME JanO8-2003-wan
EXPNO 15
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030108
Time 0.27
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zg30
TD 32768
SOLVENT MeOH
NS 16
DS 2
SWH 8278.146 Hz
FIDRES 0.252829 Hz
AQ 1.979372 sec
RG 322.5
DW 60.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
========== CHANNEL f1 ==========
NUC1 1H
P1 8.80 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300972 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
1D NMR plot parameters
CX 20.00 cm
F1P 10.002 ppm
F1 4002.10 Hz
F2P -1.010 ppm
F2 -404.17 Hz
PPMCM 0.95050 ppm/cm
HZCM 220.31353 Hz/cm
7.6005
7.3475
7.2969
7.1560
7.1142
7.1031
7.0961
7.0883
7.0769
7.0695
7.0578
7.0355
4.9080
4.8773
4.8467
4.8317
4.6935
3.8779
3.8628
3.4359
3.4266
3.4219
3.1241
3.0935
3.0893
3.0853
3.0811
3.0771
2.8919
1.4930
1.4799
1.4654
1.4564
1.4470
1.4322
1.2923
1.2086
1.1900
1.1148
1.1032
1.0851
0.7833
0.7713
0.7575
0.6801
0.6652
ppm
13.953
16.380
8.633
2.180
1.321
1.083
2.146
2.448
0.962
2.106
2.347
4.279
10.000
integral
ppm

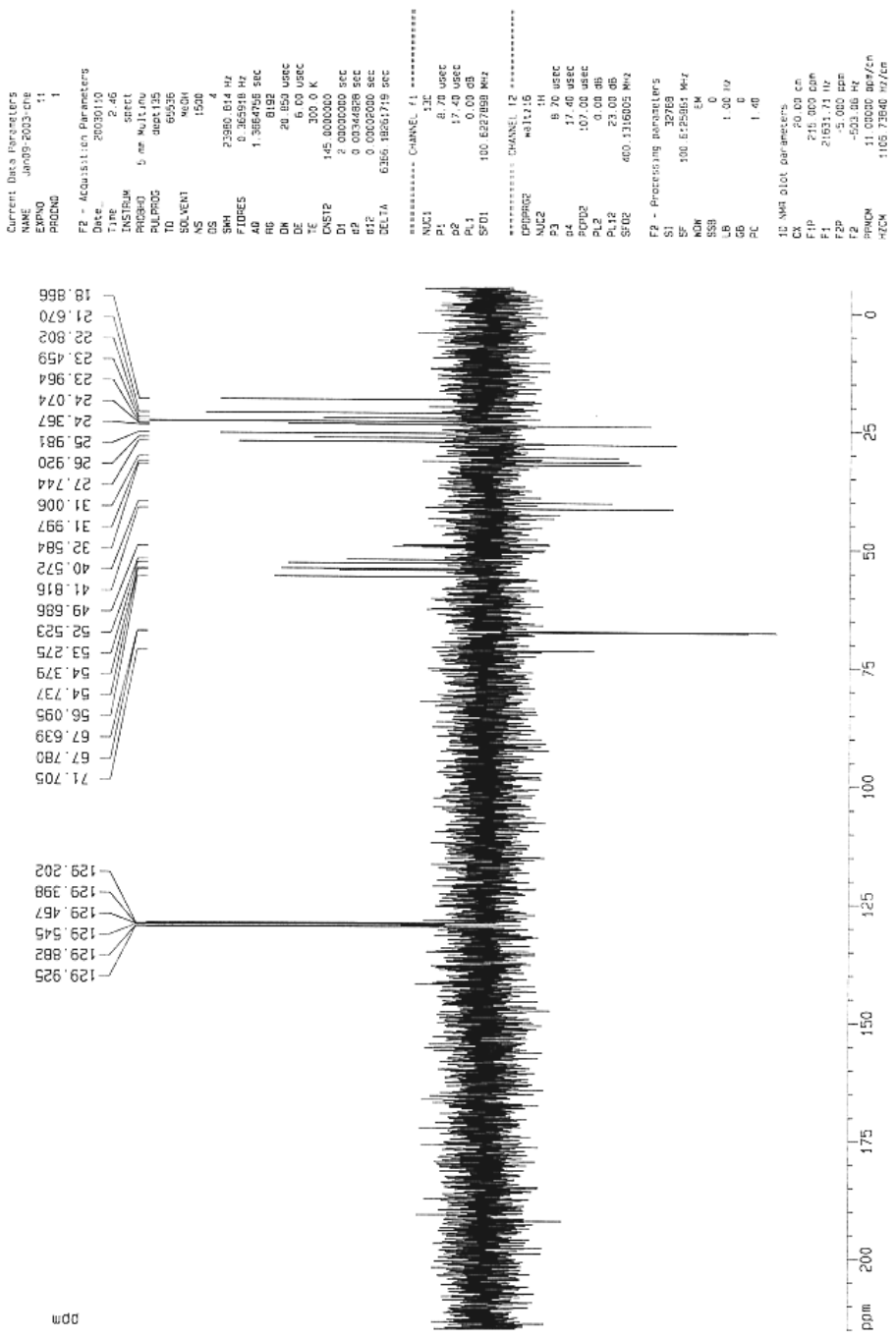
6a



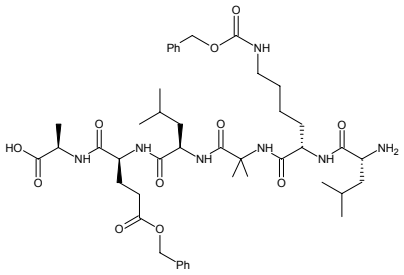

Current Data Parameters
NAME Jan10-2003-cne
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030111
Time 4.16
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zgpg30
TD 65536
SOLVENT MeOH
NS 6000
DS 4
SWH 25125.629 Hz
FIDRES 0.383387 Hz
AQ 1.3042164 sec
RG 16384
DW 19.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
========== CHANNEL f1 ==========
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SF01 100.6237959 MHz
========== CHANNEL f2 ==========
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SF02 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6125861 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73040 Hz/cm
18.866
21.671
22.800
23.455
23.961
24.077
24.363
25.981
26.919
27.742
31.990
48.762
48.974
49.187
49.399
49.612
49.824
50.037
53.275
56.095
58.322
67.635
67.779
129.159
129.395
129.464
129.540
129.880
129.923
138.005
138.777
159.369
173.656
174.914
175.097
175.937
177.515
180.563
ppm

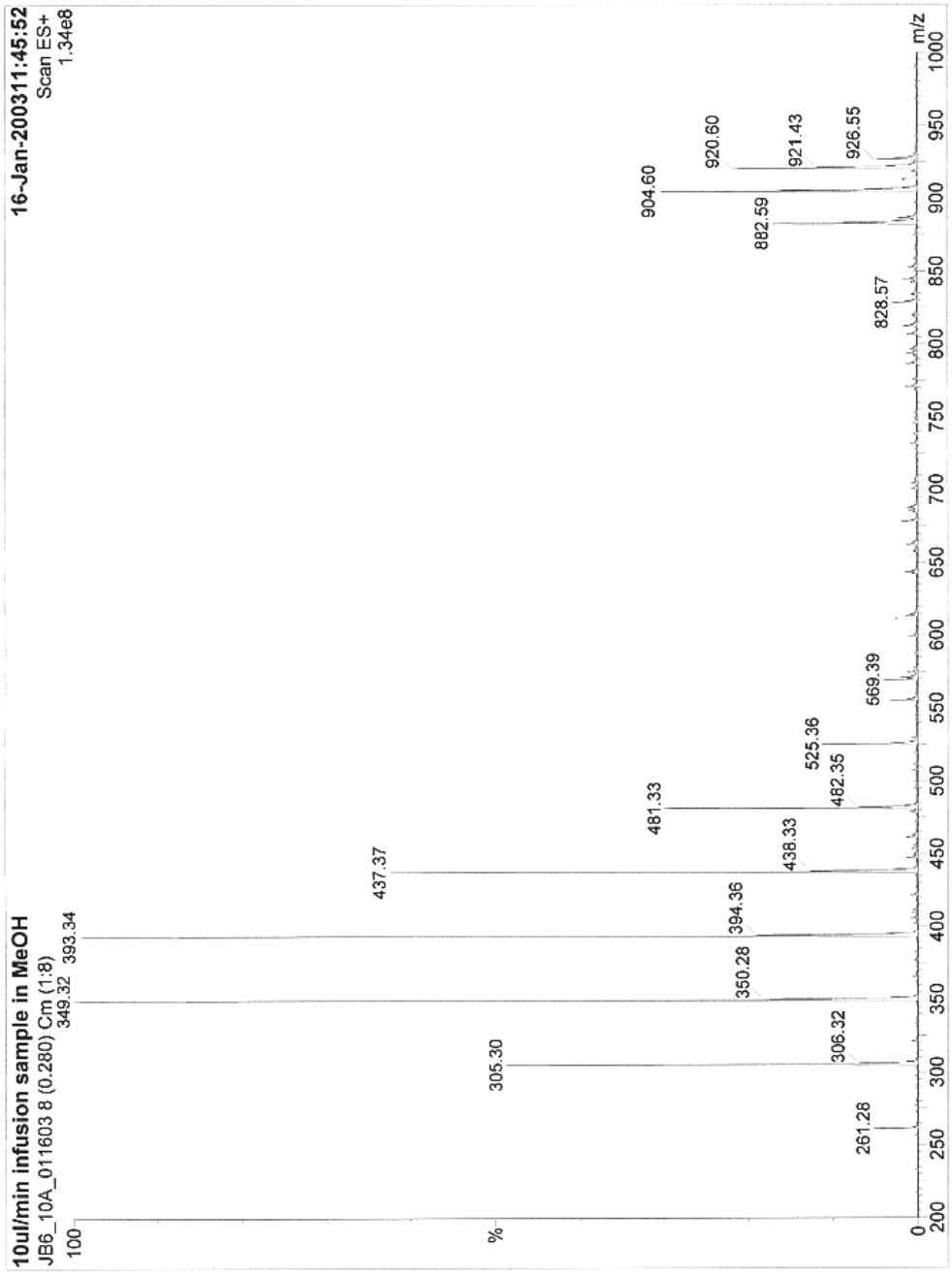
6a



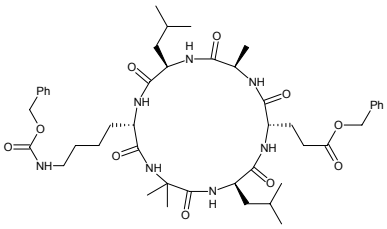

Current Data Parameters
NAME Jan09-2003-cfe
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030110
Time 2.46
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG dept135
TD 65536
SOLVENT MeOH
NS 1500
DS 4
SWH 23980.814 Hz
FIDRES 0.365918 Hz
AQ 1.36864756 sec
RG 8192
DW 20.850 usec
DE 6.00 usec
TE 300.0 K
CNST2 145.0000000
D1 2.00000000 sec
d2 0.00344828 sec
d12 0.00020000 sec
DELTA 0.36618719 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
p2 17.40 usec
PL1 0.00 dB
SFO1 100.6227898 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
P3 8.70 usec
p4 17.40 usec
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6228861 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1105.73840 Hz/cm
ppm
18.866
21.670
22.802
23.459
23.964
24.074
24.367
25.981
26.920
27.744
31.006
31.997
32.584
40.572
41.816
49.686
52.523
53.275
54.379
54.737
56.095
67.639
67.780
71.705
129.202
129.398
129.467
129.545
129.882
129.925
0
25
50
75
100
125
150
175
200

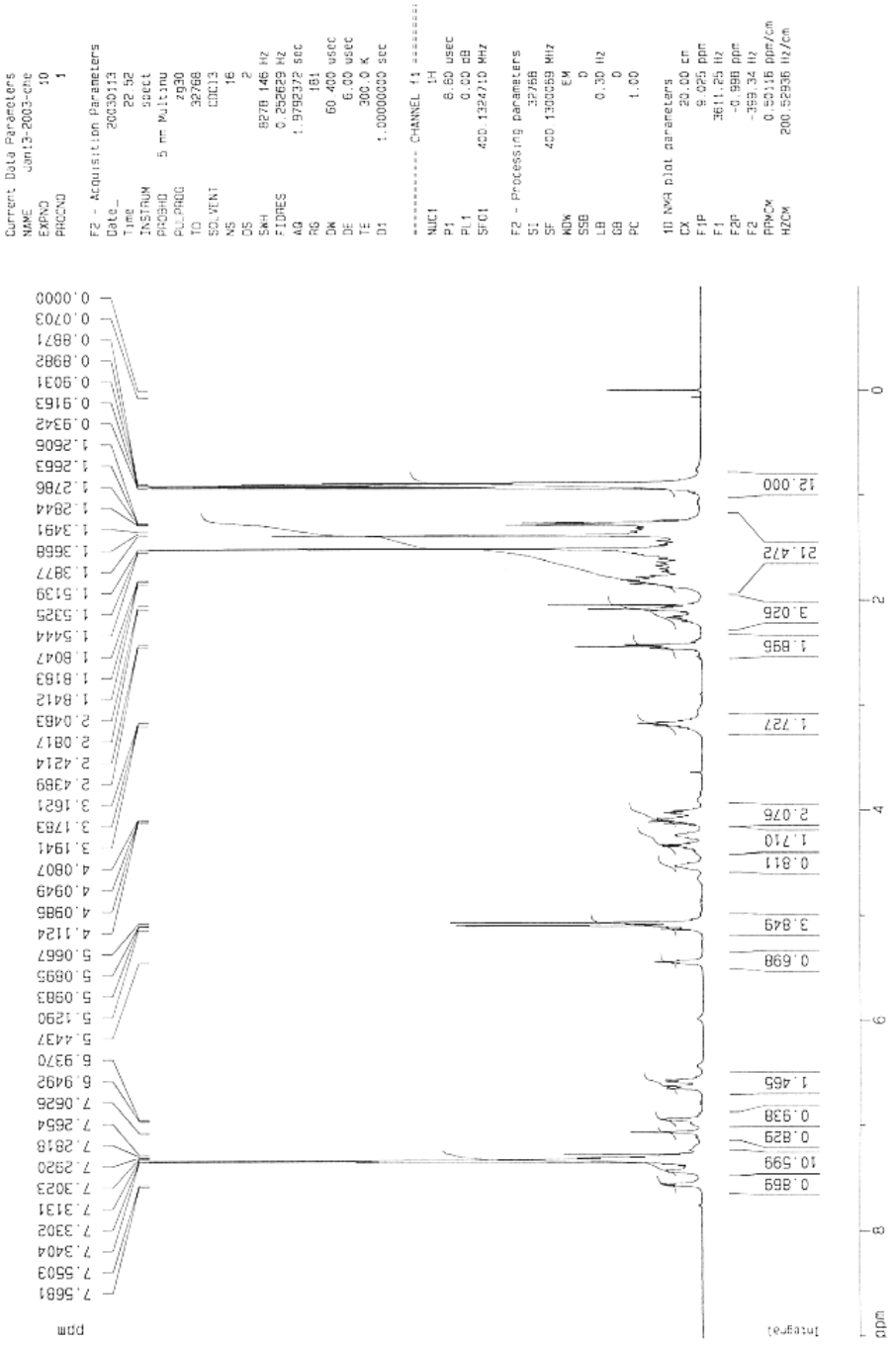
6a



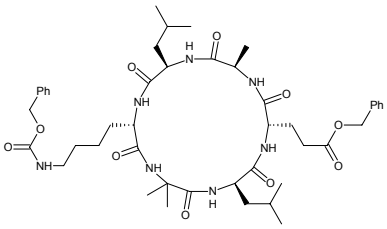

16-Jan-200311:45:52
Scan ES+
1.34e8
10ul/min infusion sample in MeOH
JB6_10A_011603 8 (0.280) Cm (1:8)
100
%
0
m/z
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
261.28
305.30
306.32
349.32
350.28
393.34
394.36
437.37
438.33
481.33
482.35
525.36
569.39
828.57
882.59
904.60
920.60
921.43
926.55

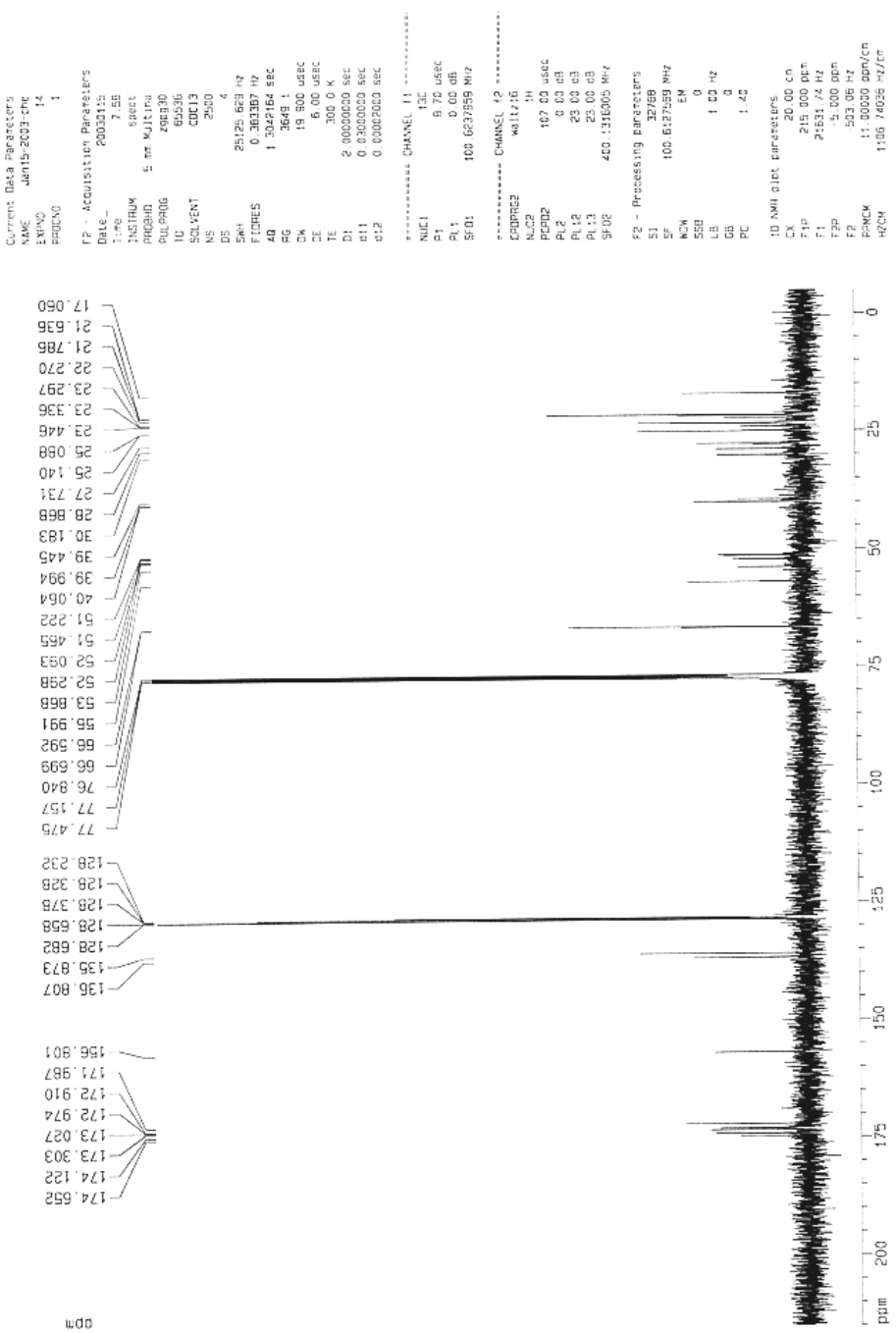
6b



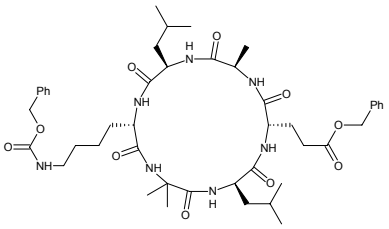

Current Data Parameters
NAME Jan13-2003-che
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030113
Time 22.52
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 16
DS 2
SWH 8278.146 Hz
FIDRES 0.252629 Hz
AQ 1.9792372 sec
RG 181
DW 60.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 8.60 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300059 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
10 NM-1 plot parameters
CX 20.00 cm
F1P 9.025 ppm
F1 3611.25 Hz
F2P -0.998 ppm
F2 -399.34 Hz
PPMCM 0.45116 ppm/cm
HZCM 200.52936 Hz/cm
ppm
Integral
12.000
21.472
3.026
1.856
1.727
2.076
1.710
0.811
3.849
0.698
1.465
0.938
0.829
10.599
0.869

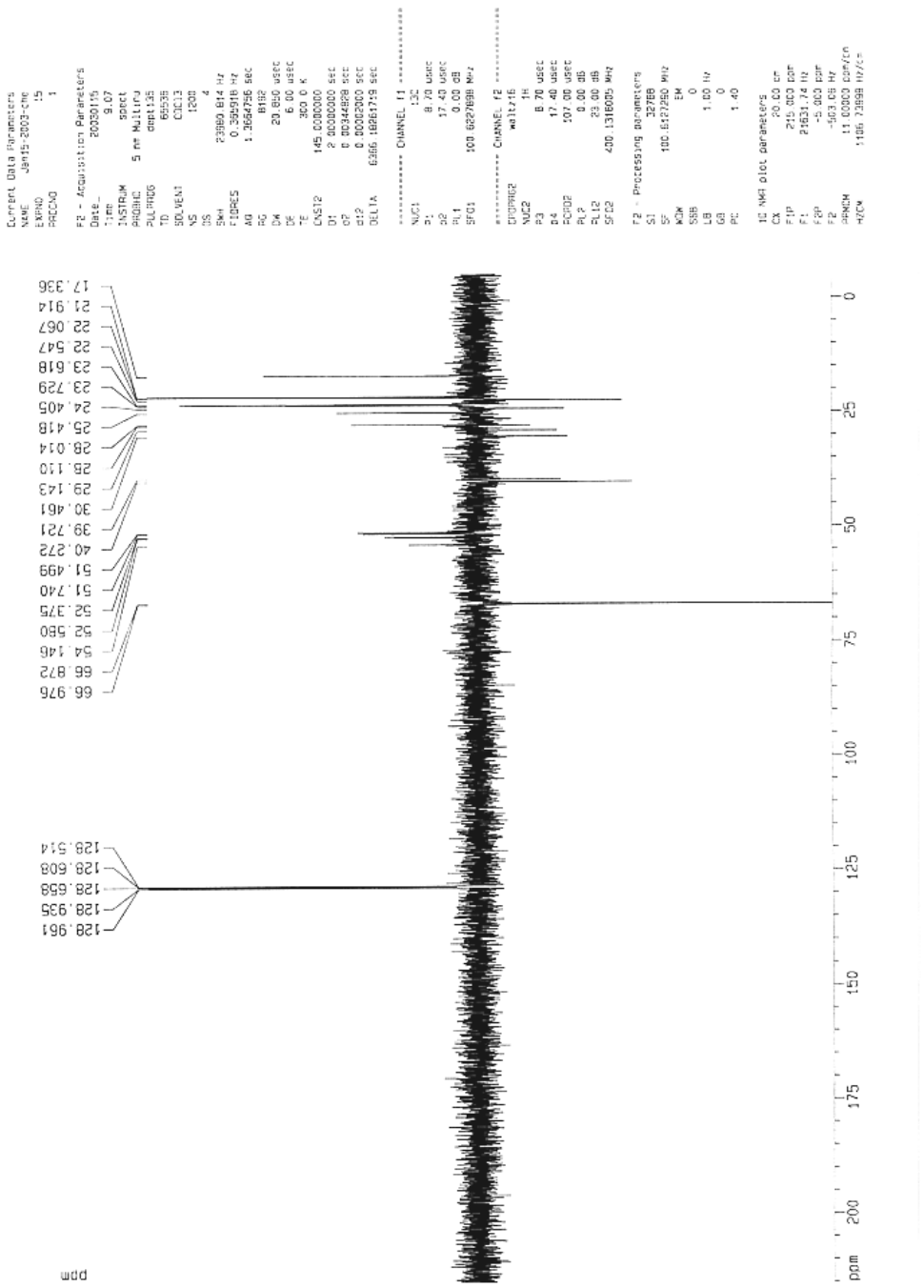
6b


Ph
O
NH
O
O
NH
O
NH
O
NH
O
NH
O
N
H
O
O
NH
O
O
Ph

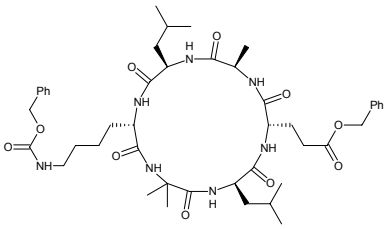

Current Data Parameters
NAME Jan15-2003-cne
EXPNO 14
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030115
Time 7.58
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 2500
DS 4
SWH 25125.628 Hz
FIDRES 0.383367 Hz
AQ 1.3042164 sec
RG 3649.1
DW 19.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SF01 100.6237859 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SF02 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127559 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.74 Hz
F2P -5.000 ppm
F2 503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.74036 Hz/cm
17.060
21.636
21.786
22.270
23.297
23.336
23.446
25.088
25.140
27.731
28.868
30.183
39.445
39.994
40.064
51.222
51.465
52.093
52.298
53.868
56.991
66.592
66.699
76.840
77.157
77.475
128.232
128.328
128.378
128.658
128.682
135.873
136.807
156.801
171.987
172.910
172.974
173.027
173.303
174.122
174.652
ppm
0
25
50
75
100
125
150
175
200

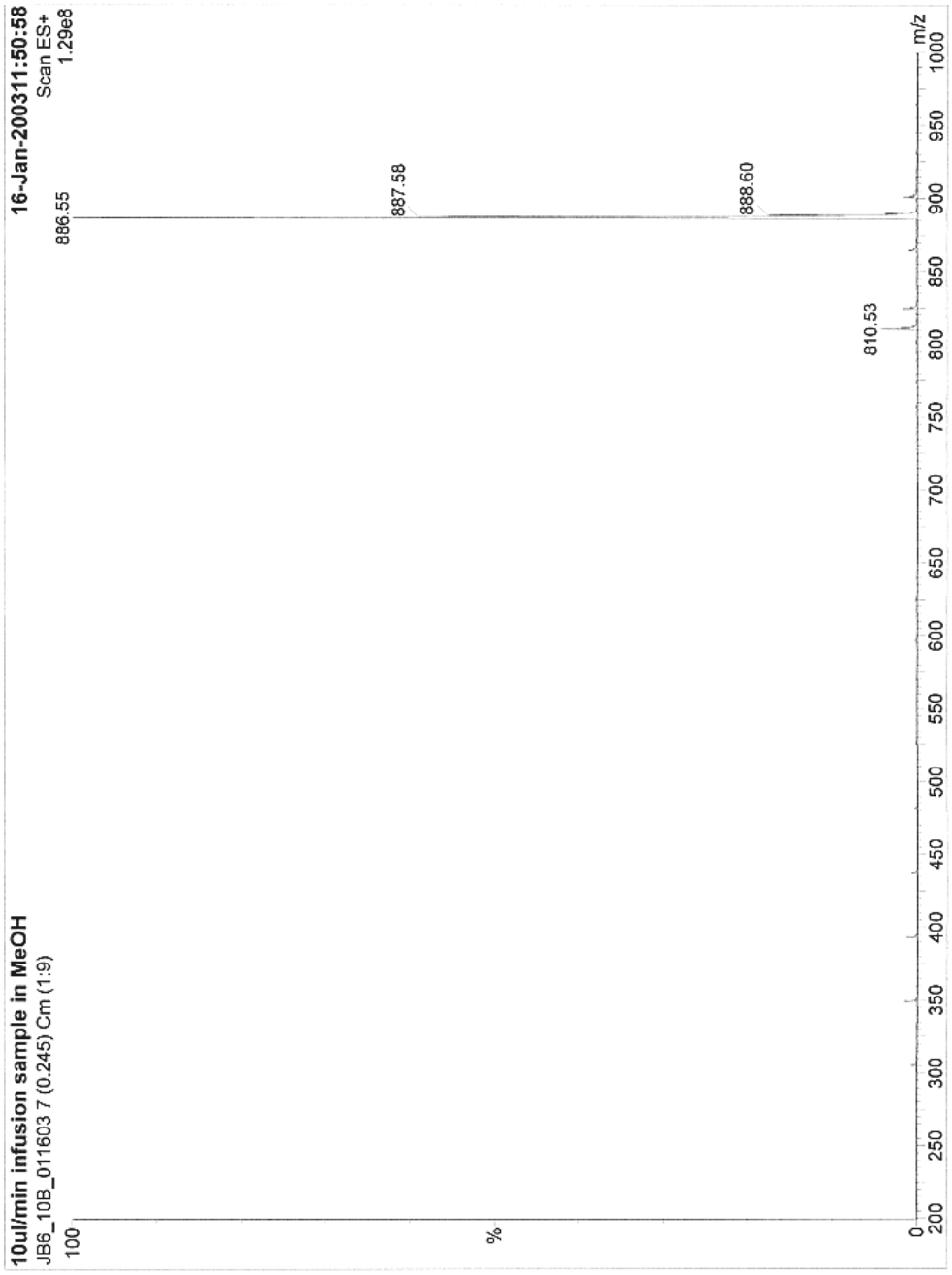
6b



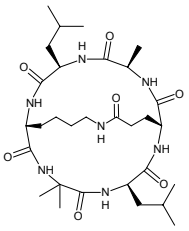

Current Data Parameters
NAME Jan15-2003-che
EXPNO 15
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030115
Time 9.07
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zgpg135
TD 65536
SOLVENT CDCl3
NS 1200
DS 4
SWH 23980.814 Hz
FIDRES 0.365918 Hz
AQ 1.36647456 sec
RG 8192
DW 20.850 usec
DE 6.00 usec
TE 300.0 K
CNST12 145.0000000
D1 2.00000000 sec
D2 0.00344828 sec
D12 0.00002000 sec
DELTA 0.866-1.66E1719 sec
======== CHANNEL f1 ========
NUC1 13C
P1 8.70 usec
P2 17.40 usec
PL1 0.00 dB
SFO1 100.6227698 MHz
======== CHANNEL f2 ========
DECPRG waltz16
NUC2 1H
P3 8.70 usec
P4 17.40 usec
PCPOF 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
SFO2 400.1318005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127260 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.74 Hz
F2P -5.000 ppm
F2 -563.65 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73998 Hz/cm
17.336
21.914
22.067
22.547
23.618
23.729
24.405
25.418
28.014
28.110
29.143
30.461
39.721
40.272
51.499
51.740
52.375
52.580
54.146
66.872
66.976
128.514
128.608
128.658
128.935
128.961
ppm

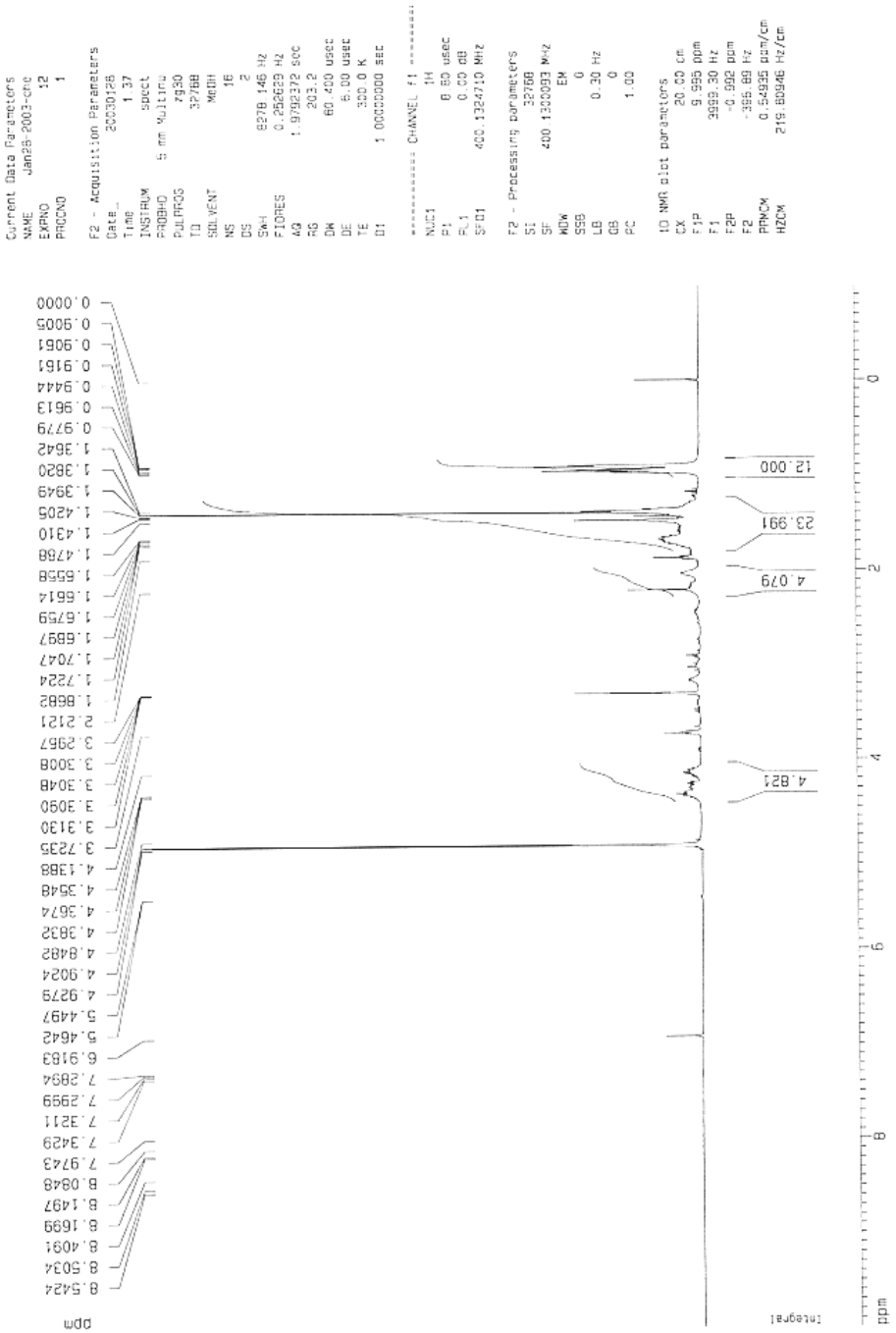
6b



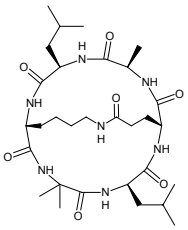

16-Jan-200311:50:58
Scan ES+
1.29e8
886.55
887.58
888.60
810.53
10ul/min infusion sample in MeOH
JB6_10B_011603 7 (0.245) Cm (1:9)
100
%
0
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z

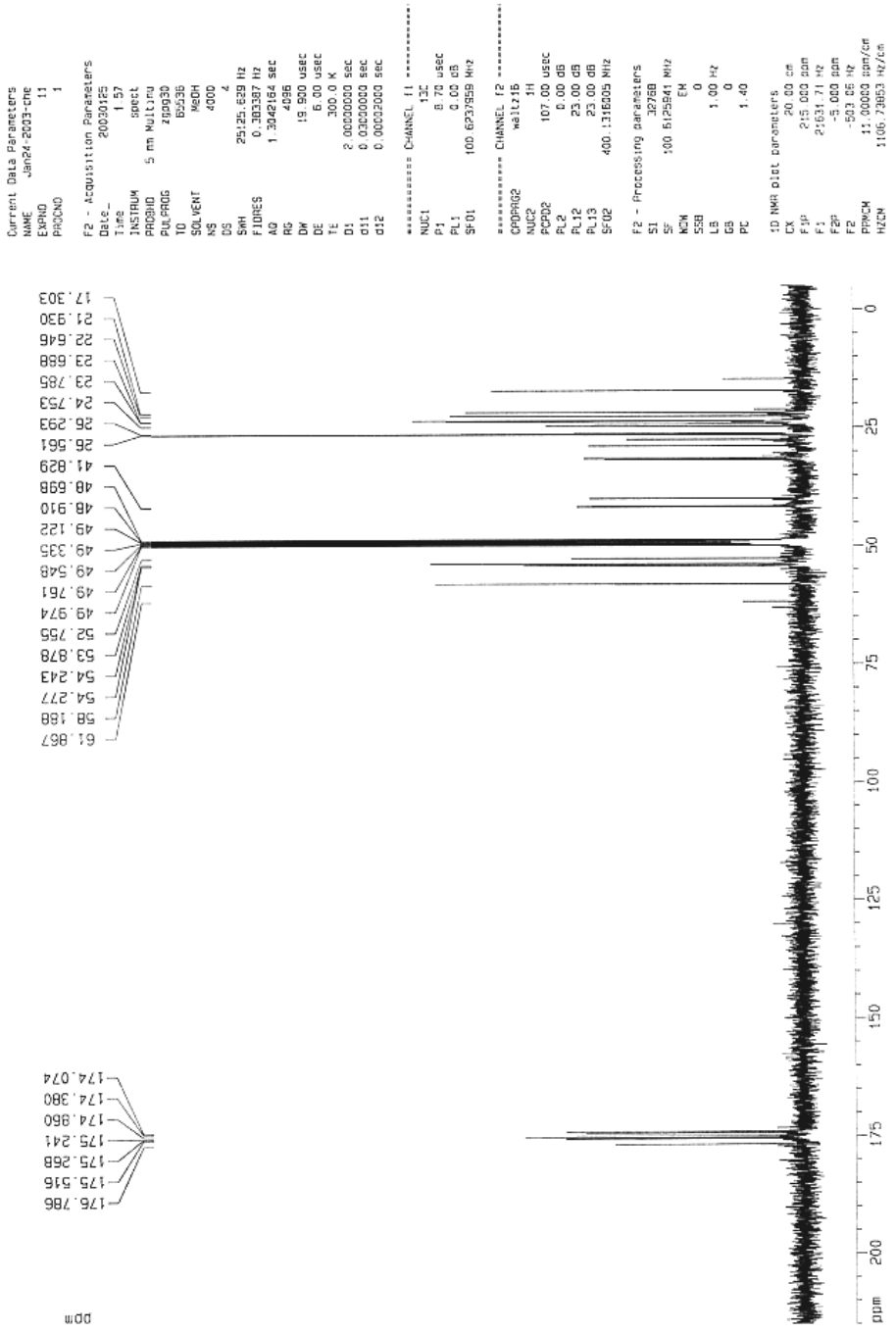
6d



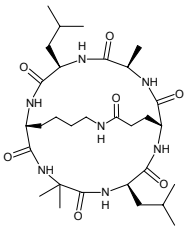

Current Data Parameters
NAME Jan26-2003-cmc
EXPNO 12
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030126
Time 1.37
INSTRUM spect
PROBHD 5 mm Multino
PULPROG zg30
TD 32768
SOLVENT MeOH
NS 16
DS 2
SWH 8278.146 Hz
FIDRES 0.252629 Hz
AQ 1.9792372 sec
RG 203.2
DW 60.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
CHANNEL f1
NUC1 1H
P1 8.60 usec
PL1 0.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300093 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
1D NMR plot parameters
CX 20.00 cm
F1P 9.995 ppm
F1 3999.30 Hz
F2P -0.992 ppm
F2 -396.89 Hz
PPMCM 0.54935 ppm/cm
HZCM 219.80946 Hz/cm
12.000
23.991
4.079
4.821
integral
ppm

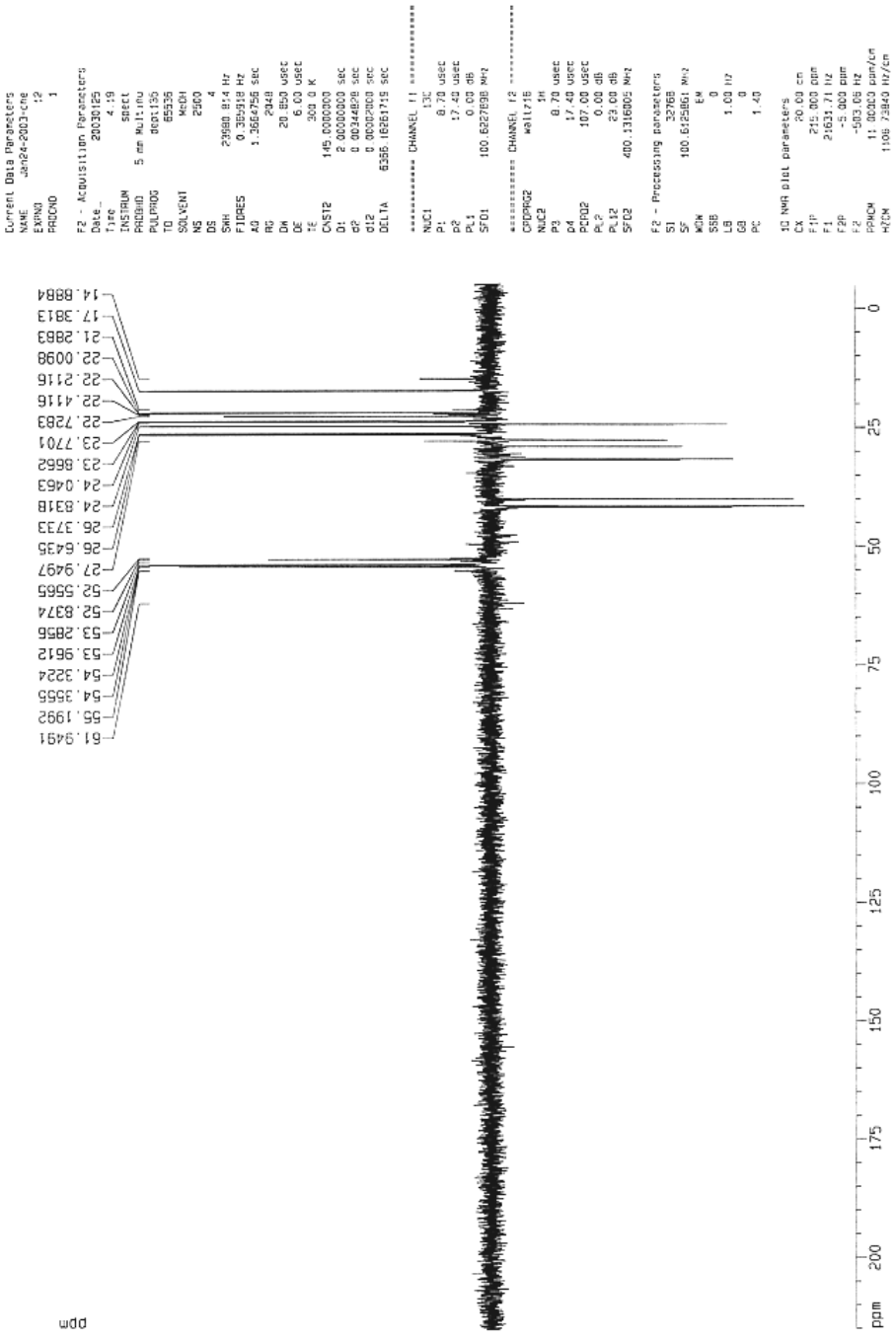
6d



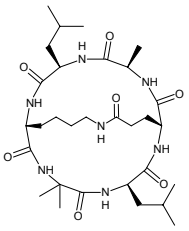

Current Data Parameters
NAME Jan24-2003-che
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030125
Time 1.57
INSTRUM spect
PROBHD 5 mm Multinu
PULPROG zgpg30
TD 65536
SOLVENT MeOH
NS 4000
DS 4
SWH 25125.629 Hz
FIDRES 0.383387 Hz
AQ 1.3042164 sec
RG 4096
DW 19.900 usec
DE 6.00 usec
TE 300.0 K
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
CHANNEL f1
NUC1 13C
P1 8.70 usec
PL1 0.00 dB
SF01 100.6233959 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
PL13 23.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6125841 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.05 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73853 Hz/cm
ppm
17.303
21.930
22.646
23.688
23.785
24.753
26.293
26.561
41.829
48.698
48.910
49.122
49.335
49.548
49.761
49.974
52.755
53.878
54.243
54.277
58.188
61.867
174.074
174.380
174.860
175.241
175.268
175.516
176.786
0
25
50
75
100
125
150
175
200

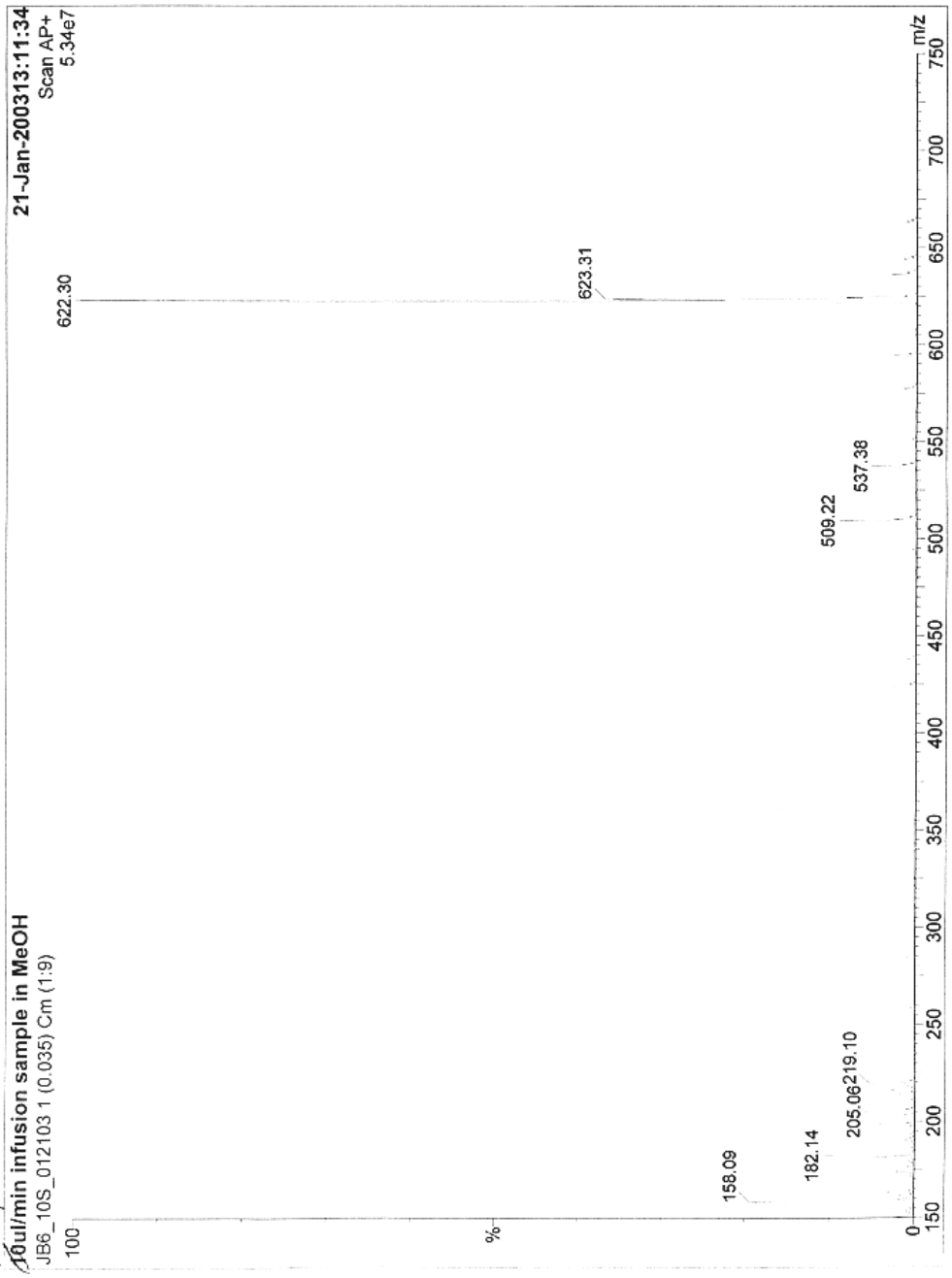
6d




Current Data Parameters
NAME jan24-2003-cne
EXPNO 12
PROCNO 1
F2 - Acquisition Parameters
Date_ 20030125
Time 4.19
INSTRUM spect
PROBHD 5 mm multinu
PULPROG deptl35
TD 65536
SOLVENT MeOH
NS 2560
DS 4
SWH 23980.814 Hz
FIDRES 0.365931 Hz
AQ 1.3664755 sec
RG 2448
DW 20.850 usec
DE 6.00 usec
TE 300.0 K
CNST12 145.0000000
D1 2.0000000 sec
d2 0.0034489 sec
d12 0.0000200 sec
DELTA 6356.1626171 sec
======== CHANNEL f1 ========
NUC1 13C
P1 8.70 usec
p2 17.40 usec
PL1 0.00 dB
SFO1 100.6227690 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
P3 8.70 usec
P4 17.40 usec
PCPD2 107.00 usec
PL2 0.00 dB
PL12 23.00 dB
SFO2 400.1316002 MHz
F2 - Processing parameters
SI 32768
SF 100.6125861 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
1D NMR plot parameters
CX 20.00 cm
F1P 215.000 ppm
F1 21631.71 Hz
F2P -5.000 ppm
F2 -503.06 Hz
PPMCM 11.00000 ppm/cm
HZCM 1106.73840 Hz/cm
61.9491
55.1992
54.3555
54.3224
53.9612
53.2856
52.8374
52.5565
27.9497
26.6435
26.3733
24.8318
24.0463
23.8662
23.7701
22.7283
22.4116
22.2116
22.0098
21.2883
17.3813
14.8884
ppm
0
25
50
75
100
125
150
175
200
ppm

6d




21-Jan-200313:11:34
Scan AP+
5.34e7
10ul/min infusion sample in MeOH
JB6_10S_012103 1 (0.035) Cm (1:9)
100
%
0
622.30
623.31
509.22
537.38
158.09
182.14
205.06219.10
150
200
250
300
350
400
450
500
550
600
650
700
750
m/z