Supporting Information

Synthesis and Biological evaluation of histone deactylase inhibitors that are based on FR235222: a cyclic tetrapeptide scaffold.

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Contents:

EXPERIMENTAL PROCEDURE

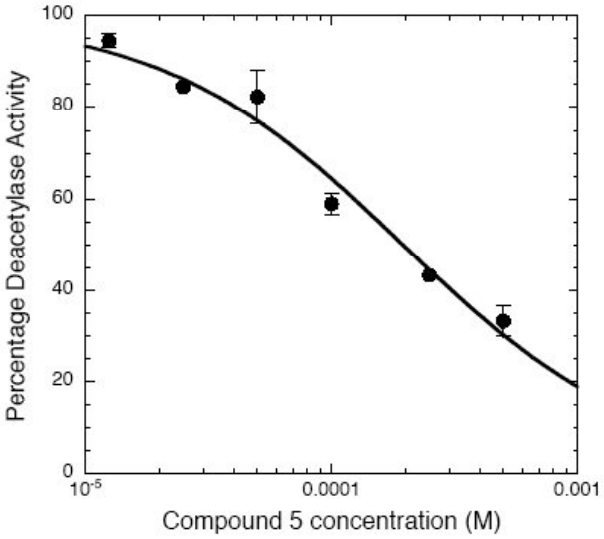
Thymidine Uptake Assays

Proliferation of the HCT-116, HCT-15 colon cancer and WS-1 normal cell lines were tested in the presence and absence of the compounds using ^{3}H-thymidine uptake assays. Cells treated with the compounds were compared to DMSO-treated controls for their ability to proliferate as indicated by the incorporation of ^{3}H-thymidine into their DNA. Cells were cultured in 96 well plates at a concentration of 3,000 cells/well. The media was IMDM for HCT-116, RPMI 1640 for HCT-15 and MEM for WS-1 with L-glutamine, 10% fetal bovine serum and 0.1% penicillin-streptomycin antibiotics. After incubation for approximately 6 hours, the compounds were added. The compounds were dissolved in DMSO at a final concentration of 2 mM and tested at the concentration indicated in the manuscript. The DMSO concentration was held constant in all wells at 1%. After the cells had been incubated with the compounds for 56 hours, 1mCi ^{3}H-thymidine per well was added and the cells were cultured for an additional 16 hours (for the cells to have a total of 72 hours with the drug), at which time the cells were harvested using a PHD cell harvester from Cambridge Technology Incorporated. The samples were then counted in a scintillation counter for 2 minutes each using ScintiVerse universal scintillation fluid from Fisher. Decreases in ^{3}H-thymidine incorporation, as compared to controls, are an indication that the cells are no longer progressing through the cell cycle or synthesizing DNA, as is shown in the studies presented.

HDAC Assays

The HDAC activity was measured using Fluor de Lys™ activity assay (Biomol). Briefly, HeLa lysates (25 μL) were incubated with or without the small molecule inhibitor for 30 min at 30 °C with shaking. Fluor de Lys substrate (25 μL, 100 μM) was added and the reaction mixture was incubated at 37 °C for 45 min with shaking. Fluor de Lys developer (50 μL of 1x) was added and incubated with shaking for 5 min. The fluorescence intensity was determined at 465 nm using a Genios Fluorimeter (Tecan). The deacetylase activity was determined by dividing the fluorescence intensity of the reaction in the presence of FR235222 derivative with the intensity in the absence of inhibitor. At least three determinations were used to calculate the mean and standard error in Figure 3.

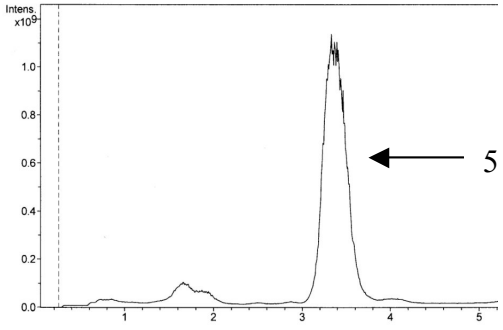
IC_{50} value of compound 5 in deacetylase assay.



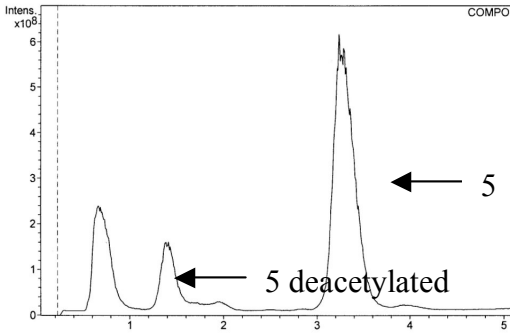
Determined IC_{50} value for this compound was 196 ± 22 μM.

Deacetylase Reaction of HDACs

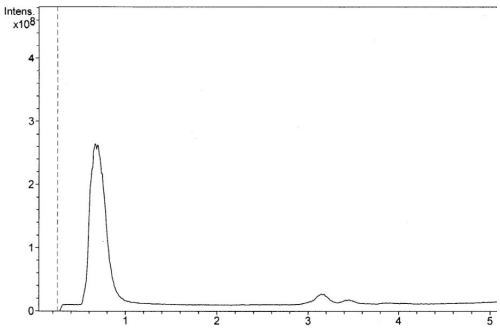
HeLa lysates (25 µL) were incubated with or without compound 5 inhibitor for 60 min at 30 °C with shaking. TSA (1 μM) was then added to the reaction to stop deacetylation. A second identical reaction was incubated for 60 minutes at 30°C with shaking, following by room temperature incubation for ~24 hours before analysis. The amount of deacetylated compound 5 was determined using LC-MS by monitoring the peak intensity of the acetylated compound. The deacetylated compound was found in both lysate containing reactions (c and d in figure below). However, longer incubation does not lead to complete deacetylation (see figure below).



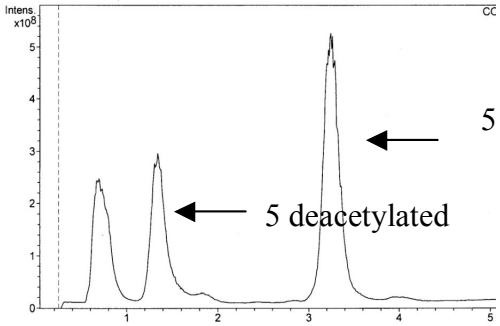
a) compound 5 only



c) compound 5 and lysate incubated 60 mins



b) lysate only



d) compound 5 and lysate incubated 24 hours

a) compound without lysate b) lysate only c) compound with lysate and stopped in 60 minutes with TSA d) compound with lysate run for ~24 hours

General peptide synthesis. All peptide coupling reactions were carried out under argon with dry solvent, using methylene chloride for dipeptide and tripeptide couplings and acetonitrile for tetrapeptide couplings. The amine (1.1 equivalents) and acid (1 equivalent) were weighed into a dry flask along with 3-6 equivalents of DIPEA and 1.2 equivalents of TBTU.* Anhydrous methylene chloride was added to generate a 0.1M solution. The solution was stirred at room temperature and reactions were monitored by TLC. Reactions were run for 1 hour before checking via TLC. If reaction was not complete additional 0.25 equivalents were of HATU and TBTU were added. If reaction was complete then work-up was done by washing with saturated ammonium chloride. (Note: if acetonitrile was used for the reaction, methylene chloride was added to reaction upon workup and the resulting solution was washed with ammonium chloride). After back extraction of aqueous layers with methylene chloride, organic layers were combined, dried over sodium sulfate, filtered and concentrated. Acid/Base wash was used as a primary method of purification. The concentrated product was dissolved in 200mL ethyl acetate, washed with 100mL 10% HCl solution (x2), 100mL saturated sodium bicarbonate solution (x10), and 100mL saturated sodium chloride solution (x2). The product was then dried over sodium sulfate, filtered and concentrated. A secondary method of purification, flash chromatography, was performed using a gradient of ethyl actetate-hexane gave our desired peptide.

* Some coupling reactions would not go to completion using only TBTU and therefore ~0.25 equivalents of HATU, and/or DEPBT were used. In a few cases up to 1.2 equivalents of all three coupling reagents were used.

General Amine deprotection. Amines were deprotected using 20% TFA in methylene chloride (0.1M) with two equivalents of anisole. The reactions were monitored by TLC, where the TLC sample was first worked up in a mini-workup using DI water and methylene chloride to remove TFA. Reactions were allowed to run for 1-2 hours and then concentrated in vacuo.

General Acid deprotection. Acids were deprotected using ~4 equivalents of lithium hydroxide (or until pH=~11) in methanol (0.1 M). The peptide was placed in a flask, along with lithium hydroxide and methanol and stirred for about 2-3 hours for the acid to deprotect. Work-up of reactions involved the acidification of reaction solution using HCl to pH = 1. The aqueous solution was extracted three times with methylene chloride, and the combined organic layer was dried, filtered and concentrated in vacuo.

Macrocyclization procedure (in situ). All tetrapeptides were acid deprotected first using ~8 equivalents of lithium hydroxide (or until pH=11) in methanol (0.1M). The tetrapeptide was placed in a flask, with the lithium hydroxide and methanol stirred overnight. Within 8 hours the acid was usually deprotected. Work-up of reactions involved the acidification of reaction solution using HCl to pH=1. The aqueous solution was extracted three times with methylene chloride and the combined organic layer was dried, filtered and concentrarted in vacuo. Verification of the presence of the free acid was performed via NMR and LCMS. Then, the amine of the tetrapeptide was deprotected using 25% TFA in methylene chloride (0.1M) with two equivalents of anisole. Reaction was monitored by TLC, where the TLC was first worked up in a mini-workup using DI water and methylene chloride to remove TFA. Reactions ran for 1-2 hours and then concentrated in vacuo. Verification of the presence of the free amine and free acid and disappearance of the starting linear protected pentapeptide was performed via LCMS. The crude, dry, double deprotected peptide (free acid and free amine) was then dissolved in a minimum solution of THF: acetonitrile: methylene chloride (2:2:1 ratio). Three coupling agents (DEPBT, HATU, and TBTU) were used at ~0.7 equivalents each. These coupling agents were dissolved in a calculated volume of dry 40% THF, 40% acetonitrile, and 20% methylene chloride that would give a 0.007M overall solution when included in the volume used for the deprotected peptide. The coupling agents were then added to the deprotected peptide solution. DIPEA (6 equivs or more in order to neutralize the pH) were then added to the reaction. The coupling agents are typically not very soluble in acetonitrile, which is why a combination of solvents is used.

After 1 hour, TLC and LCMS (where the LCMS sample was worked up prior to injection) indicated that a product spot was developing. The comparison Rf value in the product spot on TLC was the protected linear tetrapeptide. The reactions were typically complete after 2 hours, and monitoring the starting material deprotected tetrapeptide via LCMS was the easiest method of determining completion. Upon completion, the reaction was worked up by washing with saturated ammonium chloride. After back extraction of aqueous layers with large quantities of methylene chloride, the organic layers were combined, dried, filtered and concentrated. An acid/base wash was performed, with the concentrated product dissolved in 200mL ethyl acetate, washed with 100mL 10% HCl solution (x2), 100mL saturated sodium bicarbonate solution (x10), and 100mL saturated sodium chloride solution (x2). The product was then dried over sodium sulfate, filtered and concentrated. All macrocycles were purified by initially running a crude plug of compound using an ethyl acetate/hexane gradient on silica gel, then running a column on the isolated product. Finally, when necessary reverse phase HPLC was used for additional purification using a gradient of acetonitrile and DI water with 0.1% TFA.

Compound 1

Macrocycle 1a-2a-3a-4a (Compound 1) was synthesized following the "Macrocyclization procedure". Utilizing 400 mg (0.51 mmols, 1.0 equivalents) of linear tetrapeptide, 540 uL (6 equivalents) of DIPEA, 116.4 mg (0.36 mmols, 0.7 equivalents) of TBTU, 138 mg (0.36 mmols, 0.7 equivalents) HATU, and 108.4 mg (0.36 mmols, 0.7 equivalents) of DEPBT. The crude reaction was purified by reverse phase-HPLC to yield the macrocycle (2.0 mg, 10% yield).

Rf: 0.5 (EtOAc: Hex 4:1)

^{1}H NMR (400 MHz, CD_3OD): δ 0.8-1.0 (t, 3H), 1.4 (dd, 2H), 1.6-1.7 (m, 2H), 1.8-1.9 (m, 2H), 2.0 (m, 4H), 2.8 (m, 2H), 3.1-3.2 (d, 2H), 3.6-3.7 (m, 2H), 4.1 (m, αH), 4.2 (m, αH), 4.4 (m, αH), 4.6 (m, αH), 6.6 (m, 1H), 6.8 (m, 1H), 7.1-7.3 (m, 5H), 8.1 (m, 1H), 8.3 (m, 1H)

LCMS: m/z calcd for $C_{24}H_{35}N_7O_4$ (M+1) = 486.28, found

Compound 2

Macrocycle 1b-2a-3a-4a (Compound 2) was synthesized following the "Macrocyclization procedure". Utilizing 361mg (0.47 mmols, 1.0 equivalents) of linear tetrapeptide precursor, 480 uL (6 equivalents) of DIPEA, 105 mg (0.33 mmols, 0.7 equivalents) of TBTU, 124 mg (0.33 mmols, 0.7 equivalents) HATU, and 98 mg (0.33 mmols, 0.7 equivalents) of DEPBT. The crude reaction was purified by reverse phase-HPLC to yield the macrocycle (1.5mg, 11% yield).

Rf: 0.5 (EtOAc:Hex 4:1)

^{1}H NMR (400 MHz, CD_3OD): δ 0.8-0.9 (t, 3H), 1.2-1.3 (m, 2H), 1.5-1.6 (m, 2H), 1.8 (m, 2H), 2.0 (m, 2H), 2.2-2.3 (m, 2H), 2.7-2.8 (m, 2H), 3.0-3.1 (d, 2H), 3.5-3.6 (m, 2H), 4.1 (m, αH), 4.3 (m, αH), 4.6 (m, αH), 4.8 (m, αH), 7.1-7.3 (m, 5H)

LCMS: m/z calcd for $C_{24}H_{35}N_7O_4$ (M+1) = 486.28, found 486.28

Compound 3

Macrocycle 1b-2a-3a-4c (Compound 3) was synthesized following the "Macrocyclization procedure". Utilizing 227.5 mg (0..29 mmols, 1.0 equivalents) of linear tetrapeptide, 303 uL (6 equivalents) of DIPEA, 65 mg (0.20 mmols, 0.7 equivalents) of TBTU, 76.9 mg (0.20 mmols, 0.7 equivalents) of HATU, and 60.5 mg

(0.20 mmols, 0.7 equivalents) of DEPBT. The crude reaction was purified by reverse phase-HPLC to yield the macrocycle (1.8 mg, 9% yield).

Rf: 0.5 (EtOAc: Hex 3:1)

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.9-1.0 (t, 3H), 1.3-1.4 (m, 4H), 1.5-1.6 (m, 4H), 1.9-2.0 (m, 4H), 2.8-2.9 (m, 2H), 3.0-3.1 (d, 2H), 3.5-3.6 (t, 2H), 4.2 (br, 2αH), 4.4 (m, 2αH), 6.7 (m, 1H), 7.0 (m, 1H), 7.2-7.3 (m, 5H),

LCMS: m/z calcd for $C_{25}H_{37}N_7O_4$ (M+1) = 500, found 502.5.

Compound 4

Macrocycle 1c-2a-3a-4a (Compound 4) was synthesized following the "Macrocyclization procedure". Utilizing 514 mg (0.65 mmols, 1.0 equivalents) of linear tetrapeptide, 131 uL (4 equivalents) of DIPEA, 105 mg (0.33 mmols, 0.5 equivalents) of TBTU, 125 mg (0.33 mmols, 0.5 equivalents) of HATU, and 98 mg (0.33 mmols, 0.5 equivalents) of DEPBT. The crude reaction was purified by reverse phase-HPLC to yield the macrocycle(2.0mg, 6% yield).

Rf: 0.5 (EtOAc: Hex 1:0)

^{1}H NMR (500 MHz, CD_3OD): δ 0.9-1.0 (t, 3H), 1.3-1.4 (dd, 2H), 1.6 (m, 2H), 1.8 (m, 2H), 2.0-2.1 (m, 2H), 2.2 (m, 2H), 2.7 (t, 2H), 3.1 (m, 2H), 3.5 (t, 2H), 3.8 (m, αH), 4.1 (m, 2αH), 4.2 (s, 2H), 4.7 (m, αH), 7.4-7.5 (m, 4H), 8.1 (m, 1H), 8.2 (m, 1H), 8.3 (m, 1H), 8.4 (m, 1H), 8.8 (m, 2H)

LCMS: m/z calcd for $C_{25}H_{35}N_7O_4$ (M+1) = 498.28, found 540.7*

*We frequently observe a hit for plus two Na^+

Compound 5

Macrocycle 1a-2a-3b-4a (Compound 5) was synthesized following the "Macrocyclization procedure". Utilizing 300 mg (0.59 mmols, 1.0 equivalents) of linear tetrapeptide, 620 uL (6 equivalents) of DIPEA, 94.7 mg (0.29 mmols, 0.5 equivalents) of TBTU, 113 mg (0.29 mmols, 0.5 equivalents) of HATU, and 88.2 mg (0.29 mmols, 0.5 equivalents) of DEPBT. The crude reaction was purified by reverse phase-HPLC to yield the macrocycle(10mg, 3% yield).

Rf: 0.5 (EtOAc: MeOH 49:1)

^{1}H NMR (500 MHz, CD_3OD): δ 0.8-0.9 (t, 3H), 1.2-1.3 (dd, 2H), 1.5-1.6 (dd, 2H), 1.8 (d, 2H), 1.9-2.0 (s, 3H), 2.2-2.3 (m, 4H), 2.7-2.8 (d, 2H), 3.1-3.2 (t, 2H), 3.6-3.7 (t, 2H), 4.1 (m, αH), 4.3 (m, 2αH), 4.4 (m, αH), 4.6 (m, αH), 4.9 (m, 2H), 7.2-7.3 (m, 5H)

LCMS: m/z calcd for $C_{26}H_{37}N_5O_5$ (M+1) = 500.28, found 500.23

Compound 6

Macrocycle 1d-2a-3b-4a (Compound 6) was synthesized following the "Macrocyclization procedure". Utilizing 300 mg (0.56 mmols, 1.0 equivalents) of linear tetrapeptide, 580 uL (6 equivalents) of DIPEA, 91 mg (0.28 mmols, 0.5 equivalents) of TBTU, 107 mg (0.28 mmols, 0.5 equivalents) of HATU, and 84.6 mg (0.28 mmols, 0.5 equivalents) of DEPBT. The crude reaction was purified by reverse phase-HPLC to yield the macrocycle (2mg, 2% yield).

Rf: 0.5 (EtOAc: MeOH 49:1)

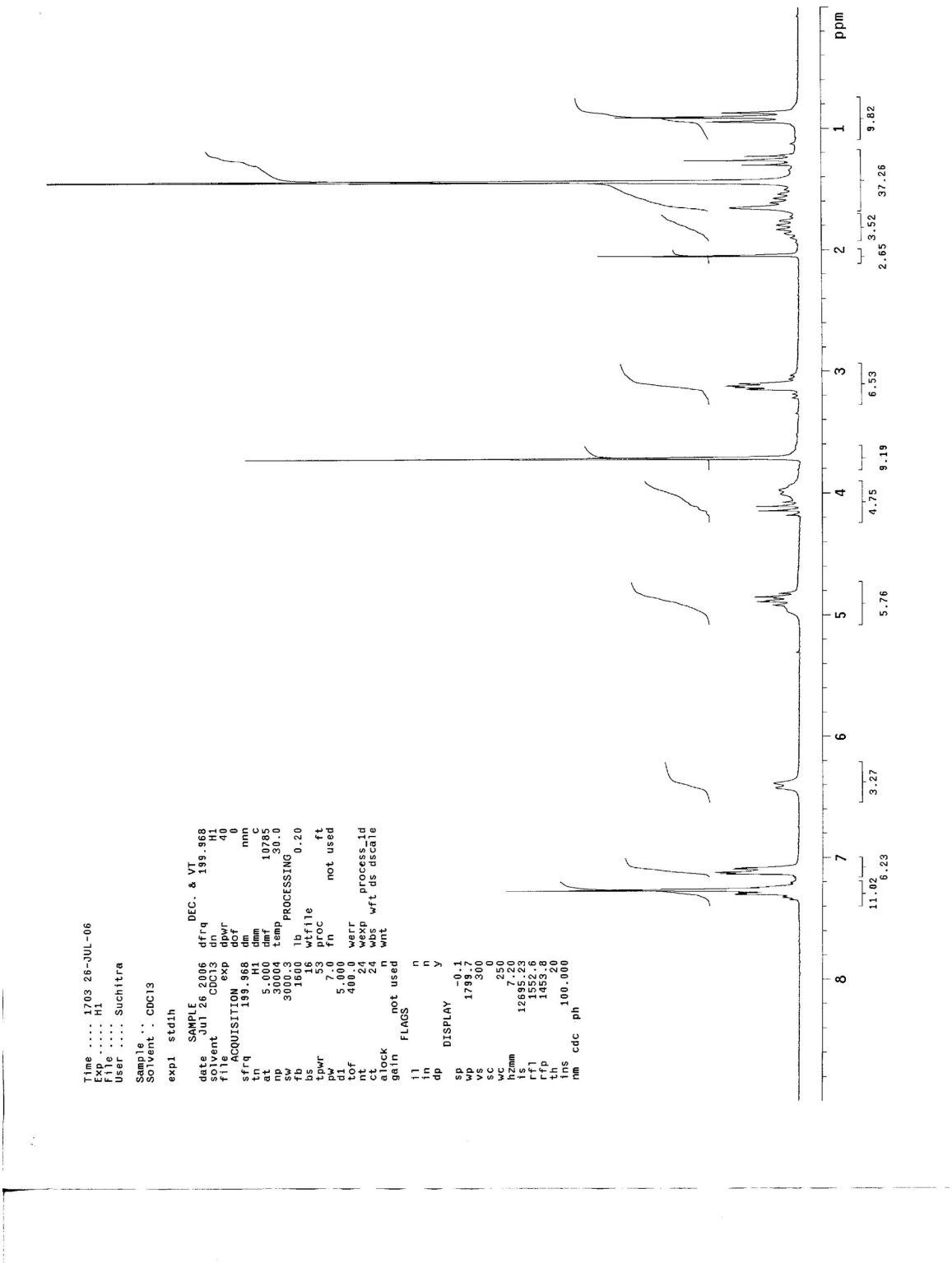
^{1}H NMR (500 MHz, CD_3OD): δ 0.9-1.0 (t, 3H), 1.2-1.3 (m, 2H), 1.4-1.5 (m, 2H), 1.6-1.7 (d, 2H), 1.9-2.0 (m, 5H), 2.2-2.3 (m, 2H), 2.9 (s, 3H), 3.0 (d, 2H), 3.1-3.2 (t, 2H), 3.5 (m, 2H), 3.9 (m 2αH), 4.1 (m, 2αH), 4.5 (m, 2H), 7.2-7.3 (m,5H)

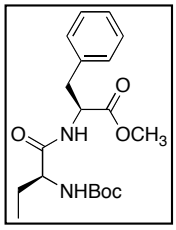
LCMS: m/z calcd for $C_{27}H_{39}N_5O_5$ (M+1) = 514.3, found 514.2

TABLE OF COMPOUNDS WITH TEXT DESCRIBING STRUCTURAL VARIATIONS

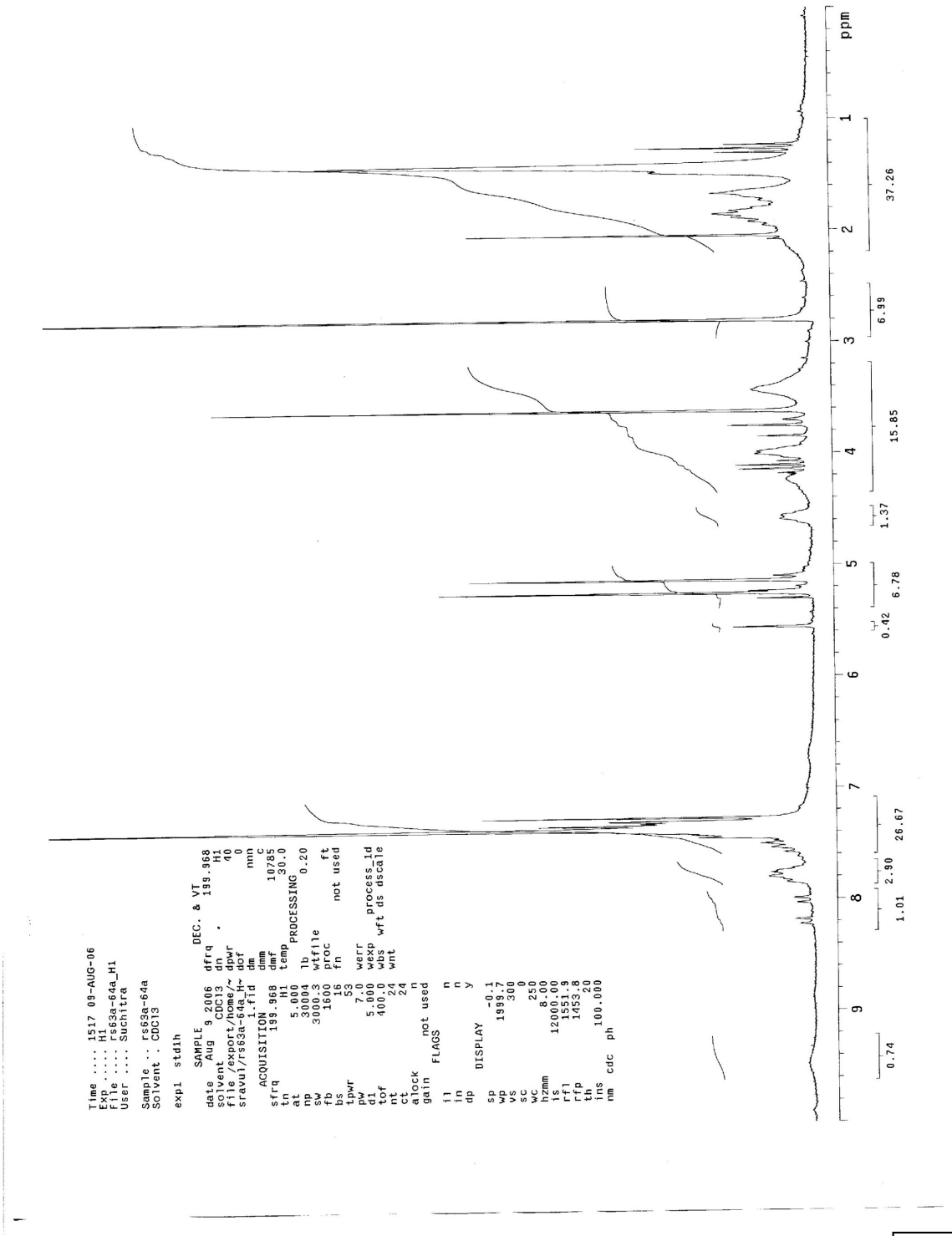
Comp. #	aa_1	aa_2	aa_3	aa_4	ClogP
1	Phe	Abu	Arg	D-Pro	**0.79**
2	D-Phe	Abu	Arg	D-Pro	**0.79**
3	D-Phe	Abu	Arg	D-Pip	**0.37**
4	Thq	Abu	Arg	D-Pro	**0.85**
5	Phe	Abu	Lys-Ac	D-Pro	**0.58**
6	N-Me-Phe	Abu	Lys-Ac	D-Pro	**0.34**

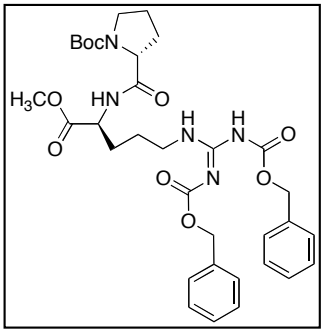
Phe = Phenylalanine
Thq = Tetrahydroquinoline
Abu = Aminobutyric acid
Arg = Arginine
Lys-Ac = Acetyl lysine
Pro = Proline
Pip = Piperdinyl



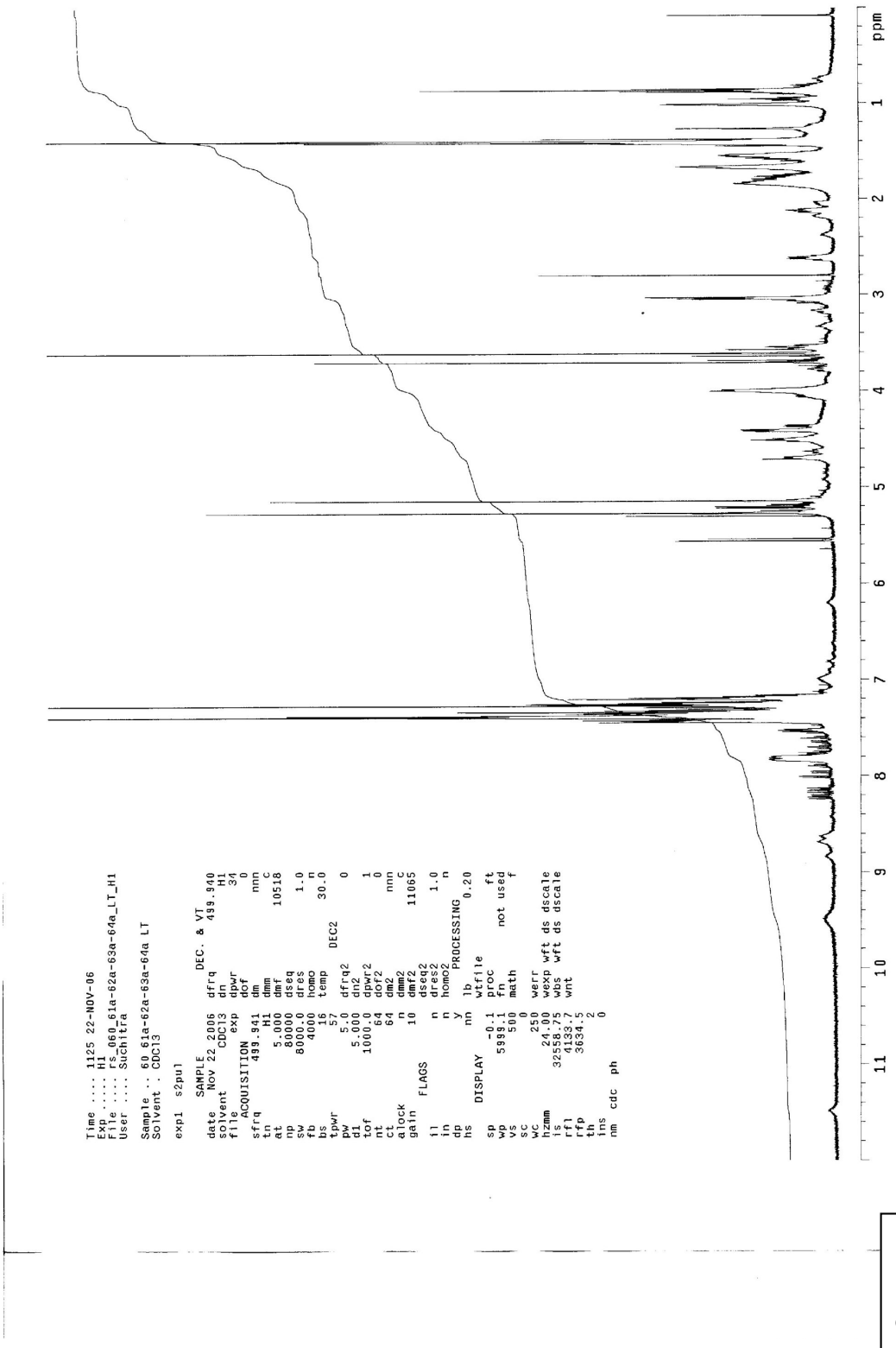


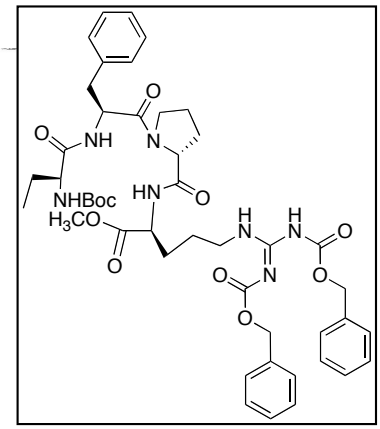
Compound 1 – NMR Dipeptide 1a2a



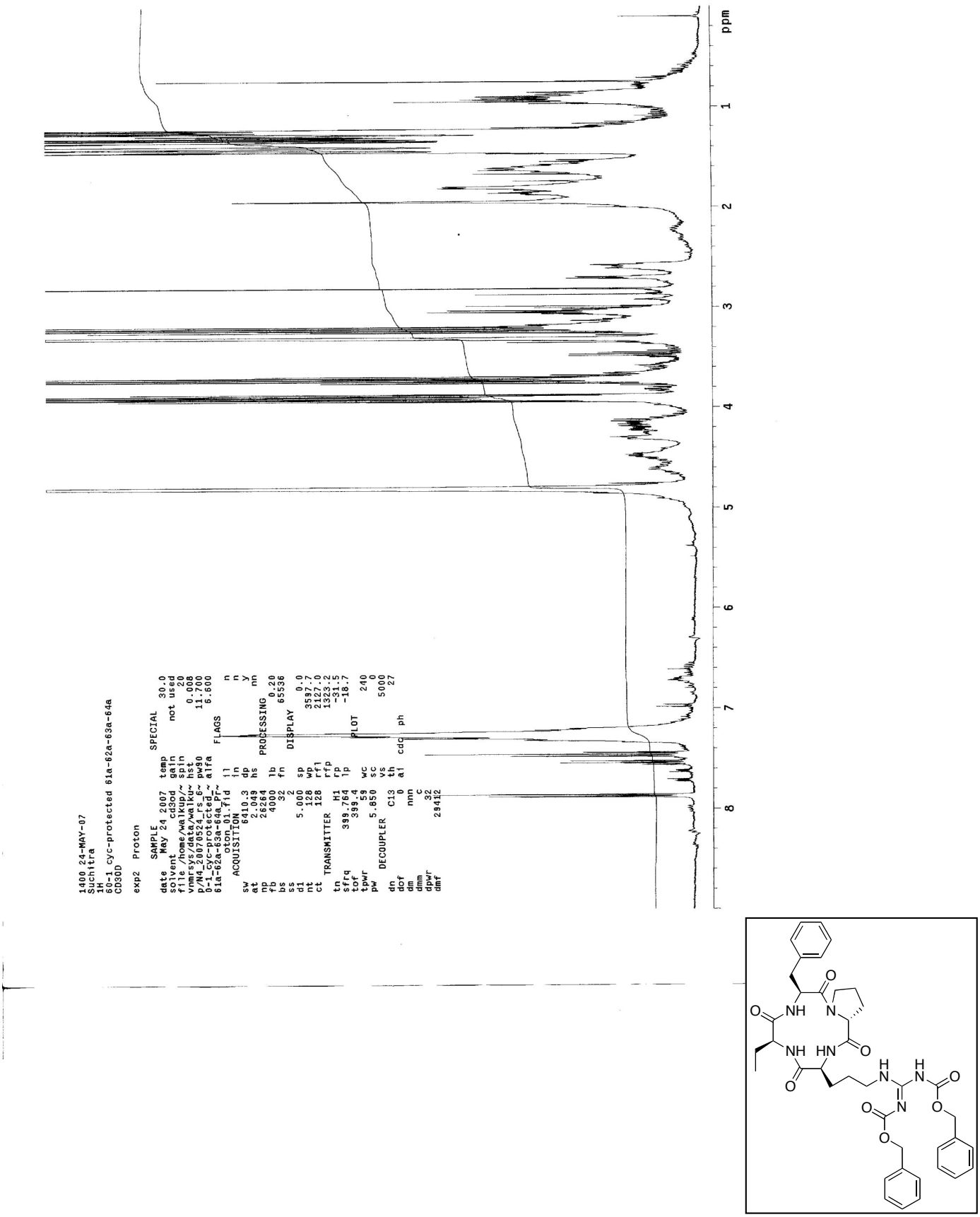


Compound 1 – NMR dipeptide 3a-4a

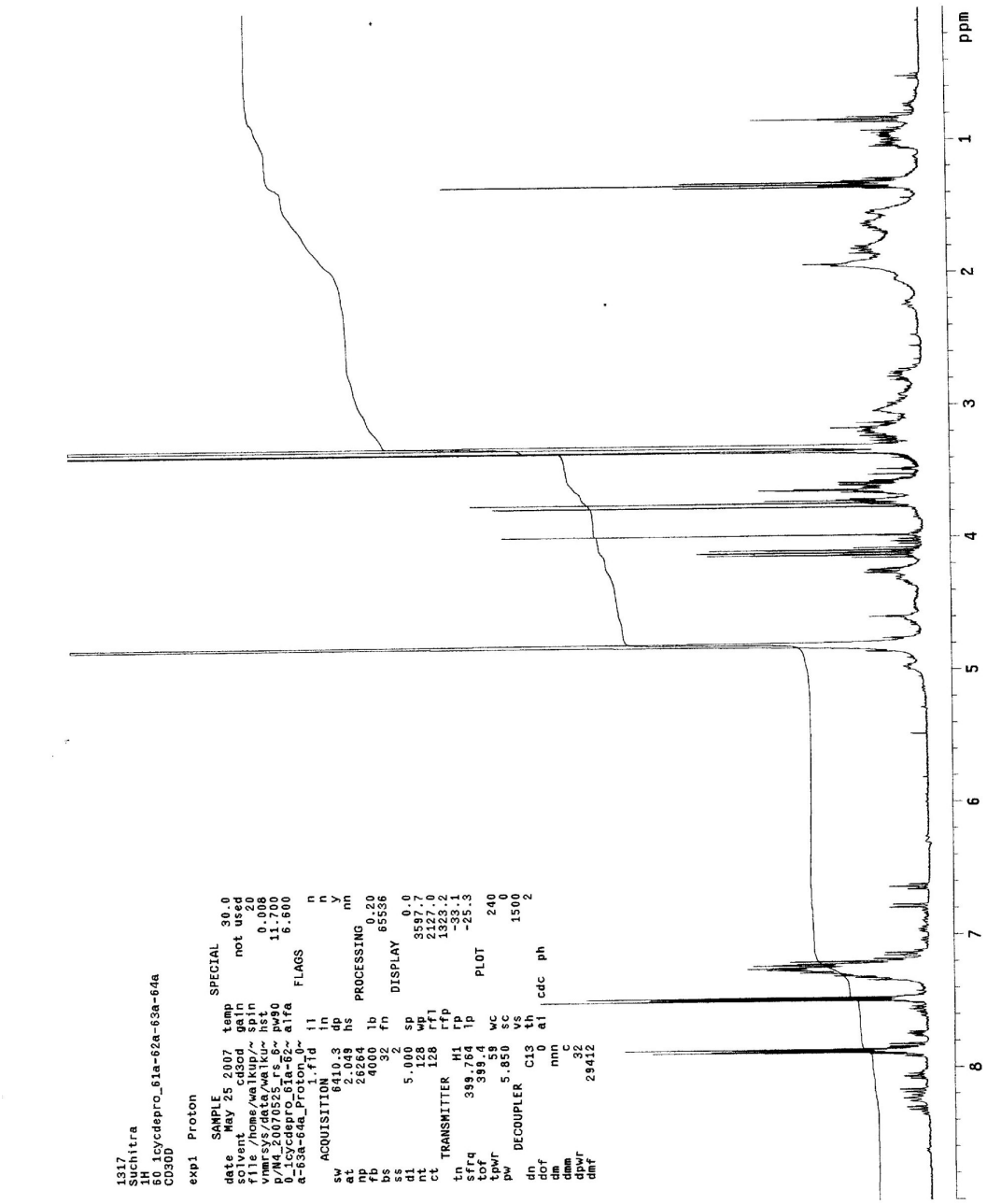


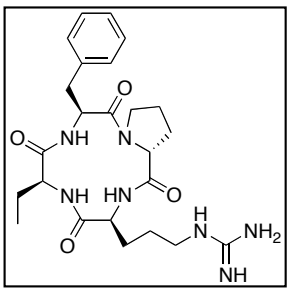


Compound 1 – NMR linear tetrapeptide 1a-2a-3a-4a

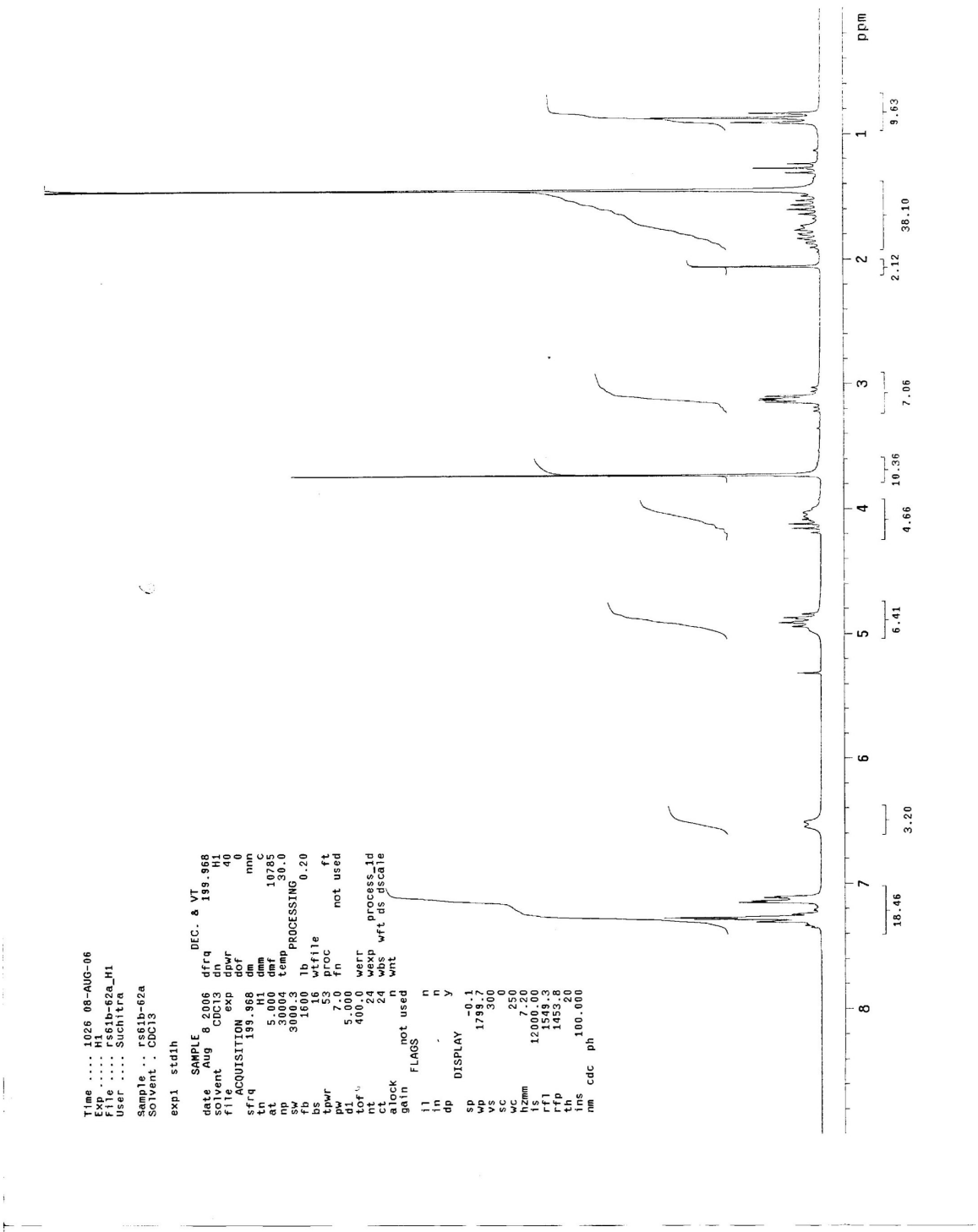


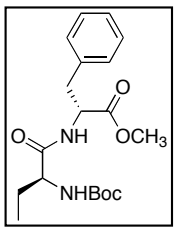
Compound 1 – NMR cyclized protected tetrapeptide 1a-2a-3a-4a



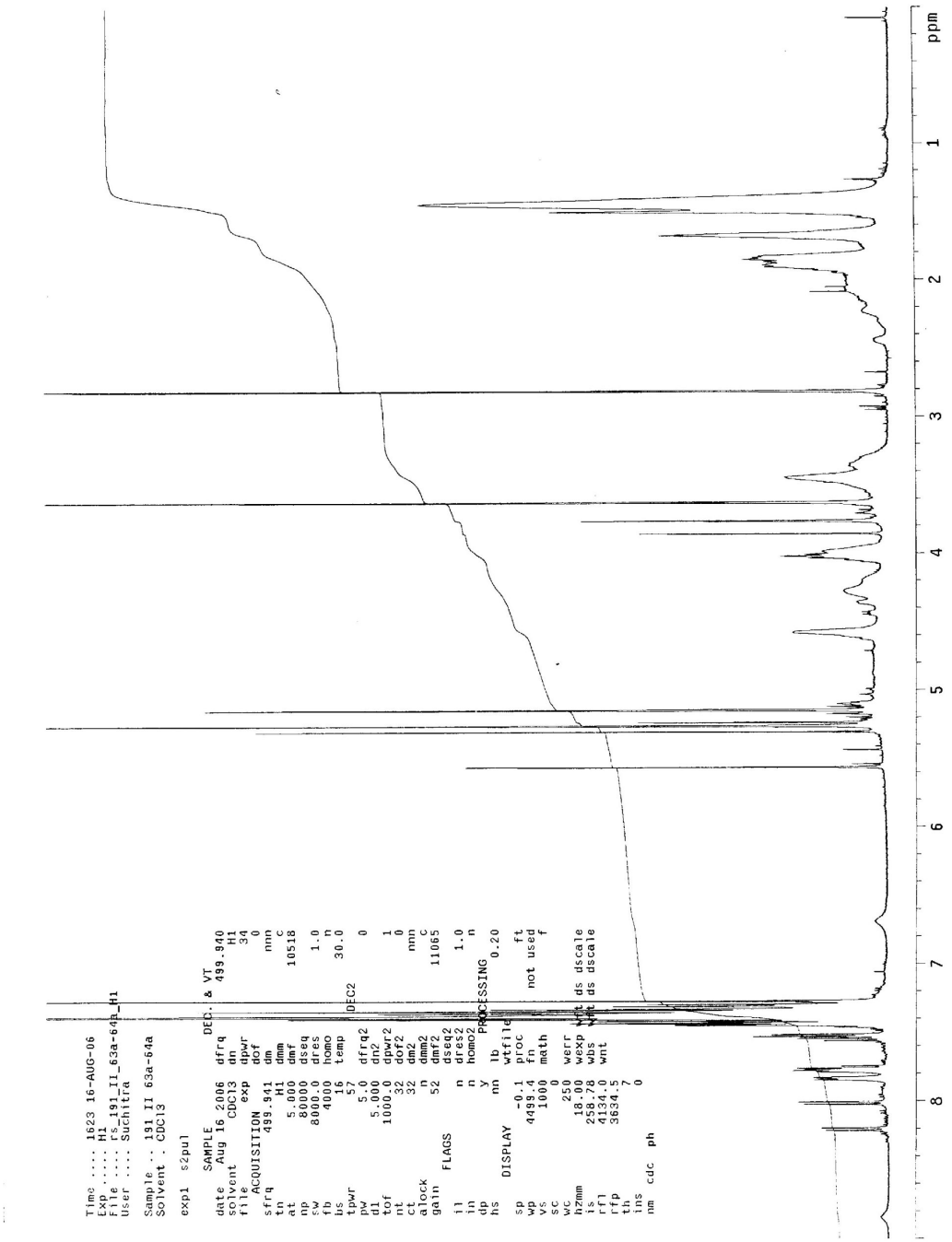


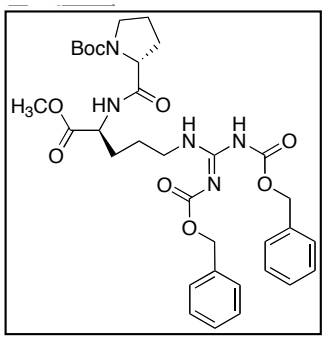
Compound 1 – NMR cyclized tetrapeptide 1a-2a-3a-4a



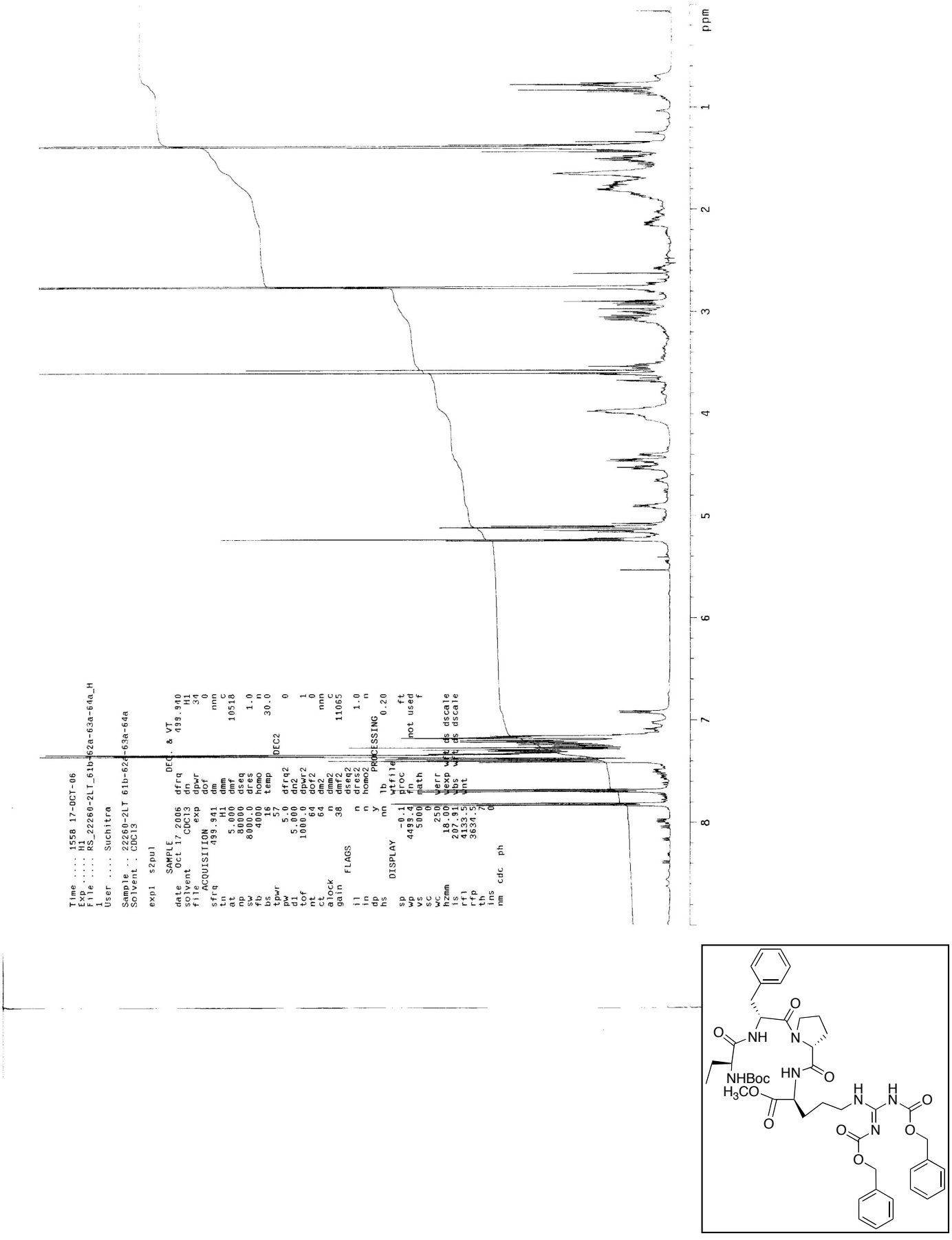


Compound 2 – NMR Dipeptide 1b-2a

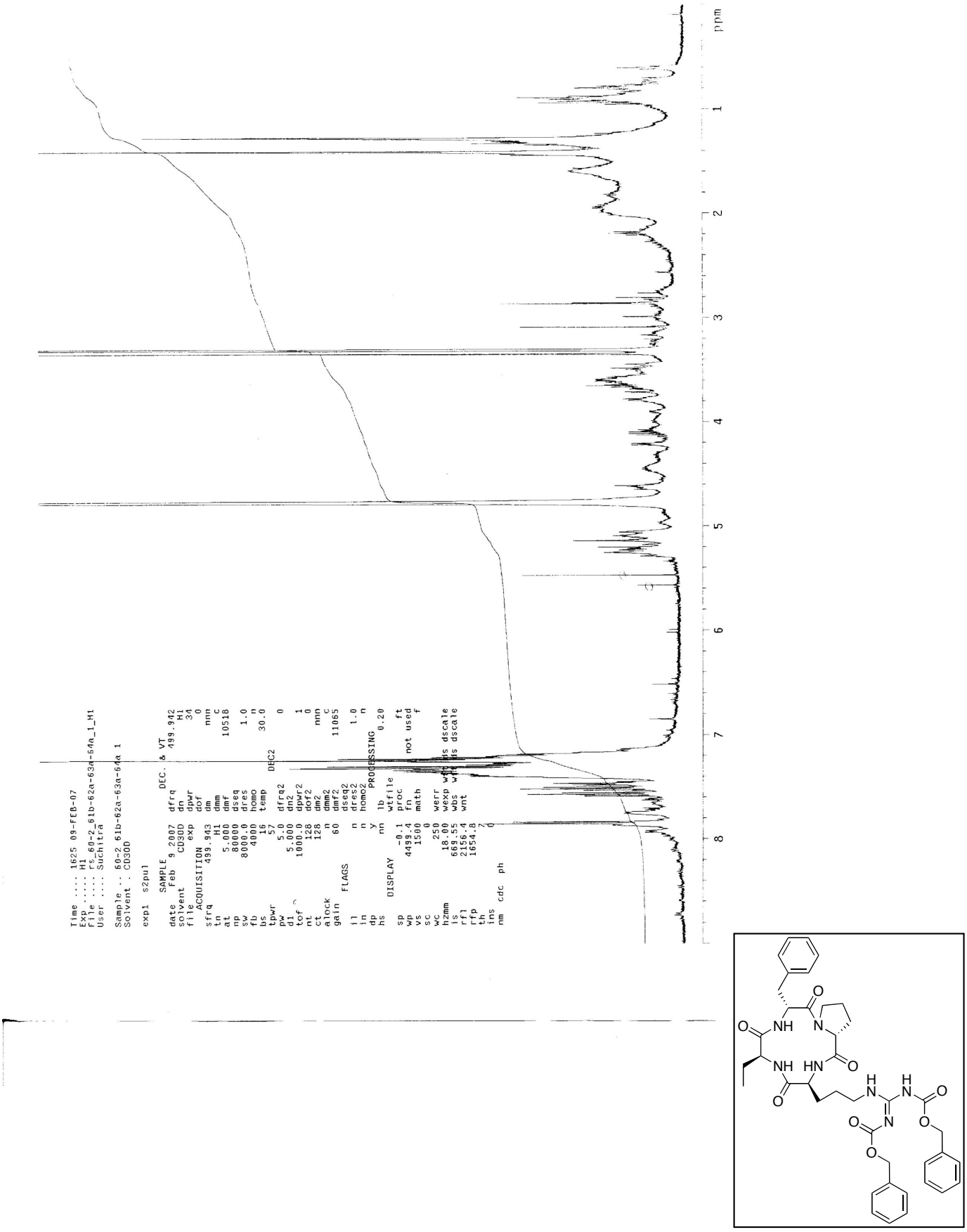




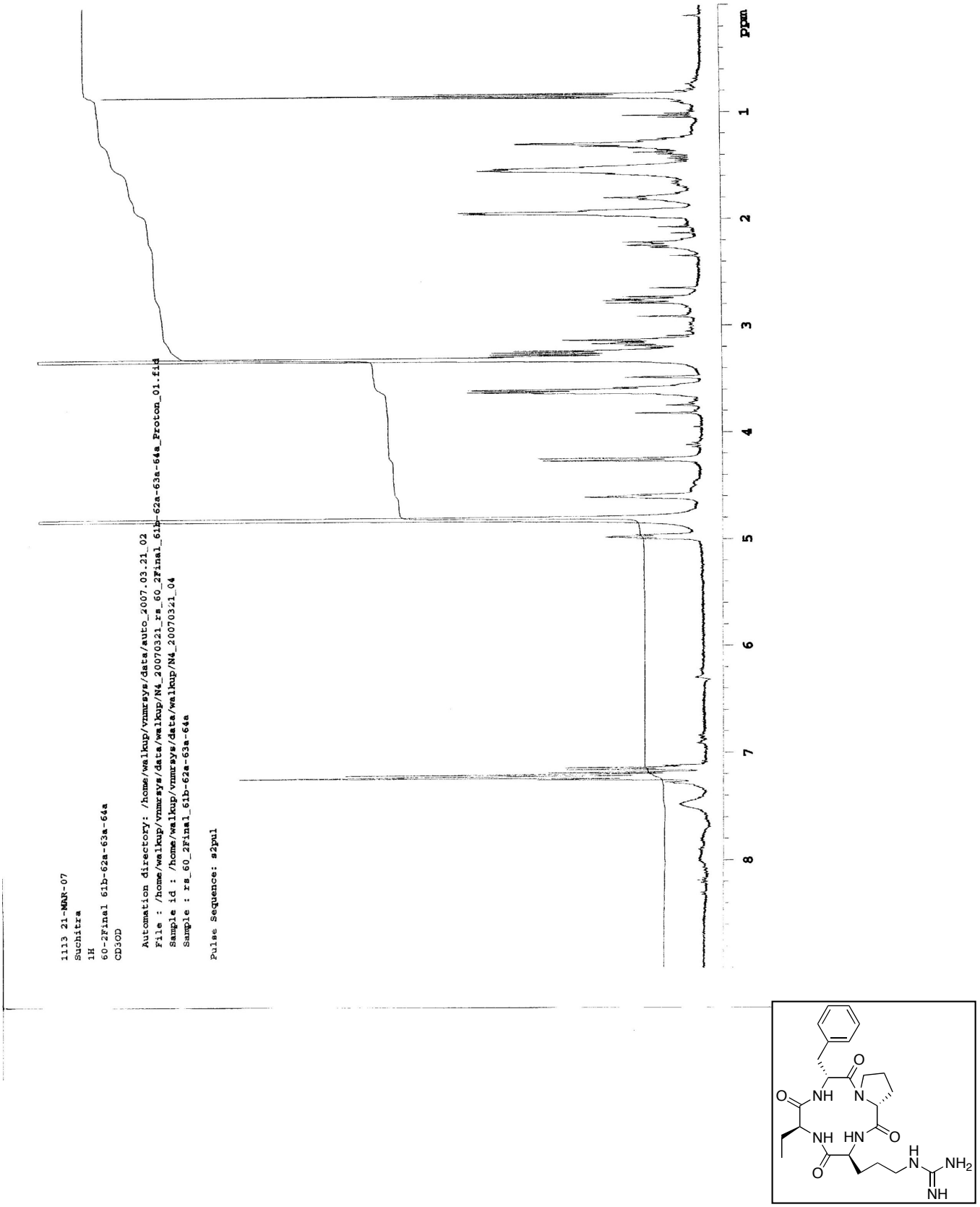
Compound 2 – NMR Dipeptide 3a-4a



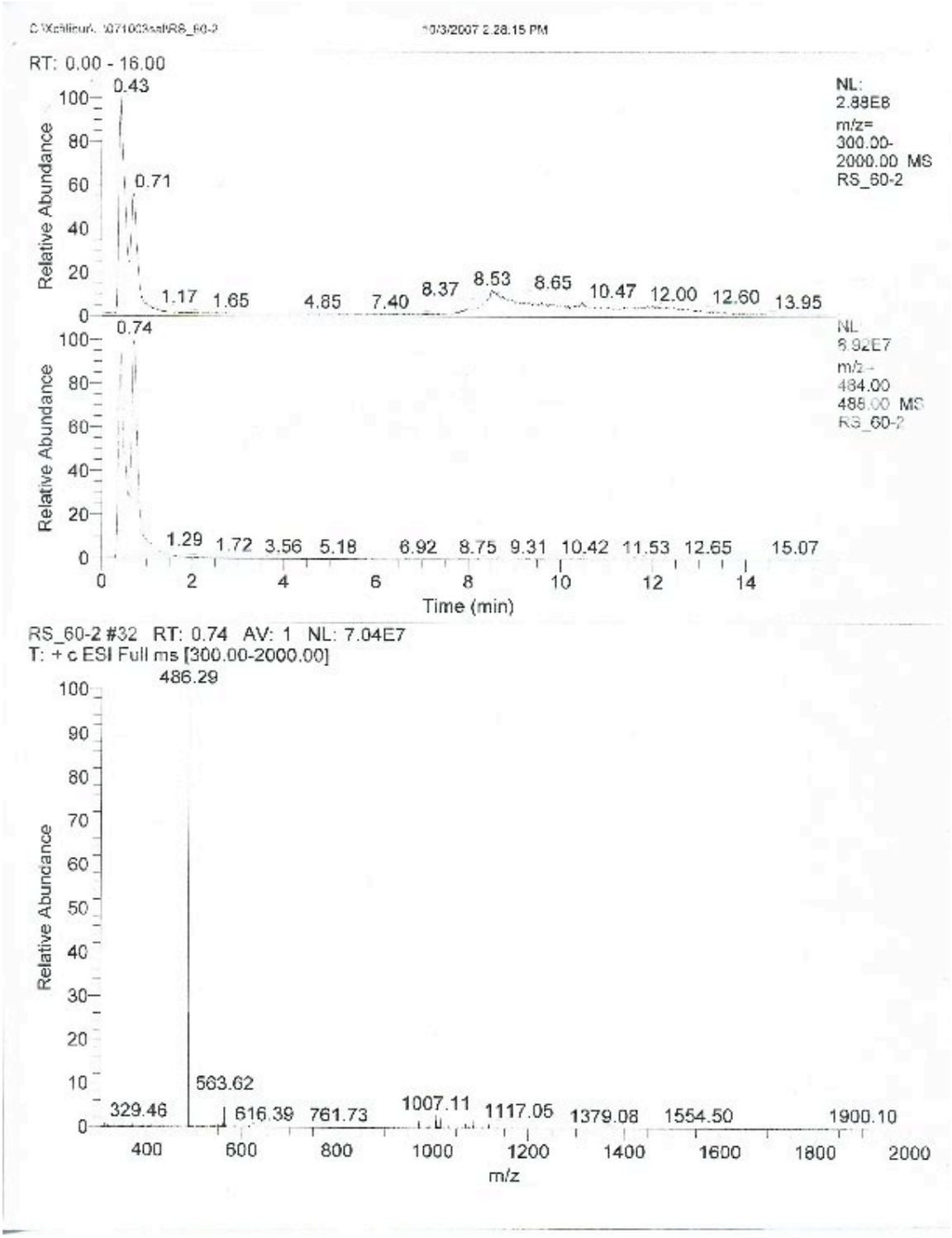
Compound 2 – NMR linear tetrapeptide 1b-2a-3a-4a

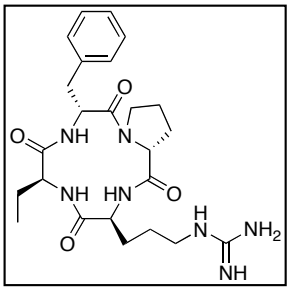


Compound 2 – NMR cyclized protected tetrapeptide 1b-2a-3a-4a

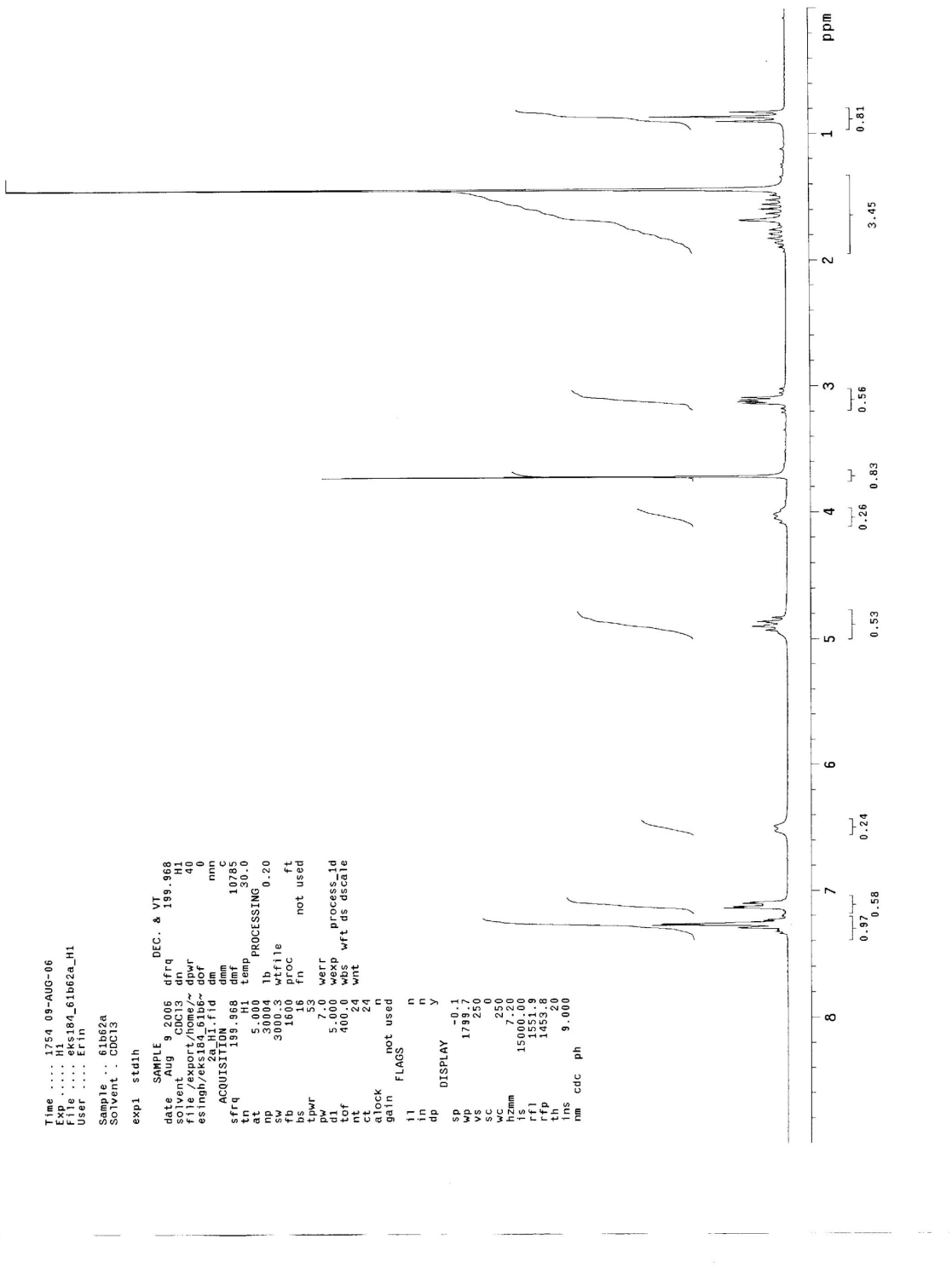


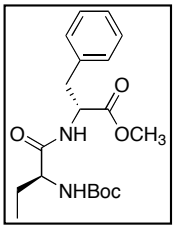
Compound 2 – NMR cyclized 1b-2a-3a-4a



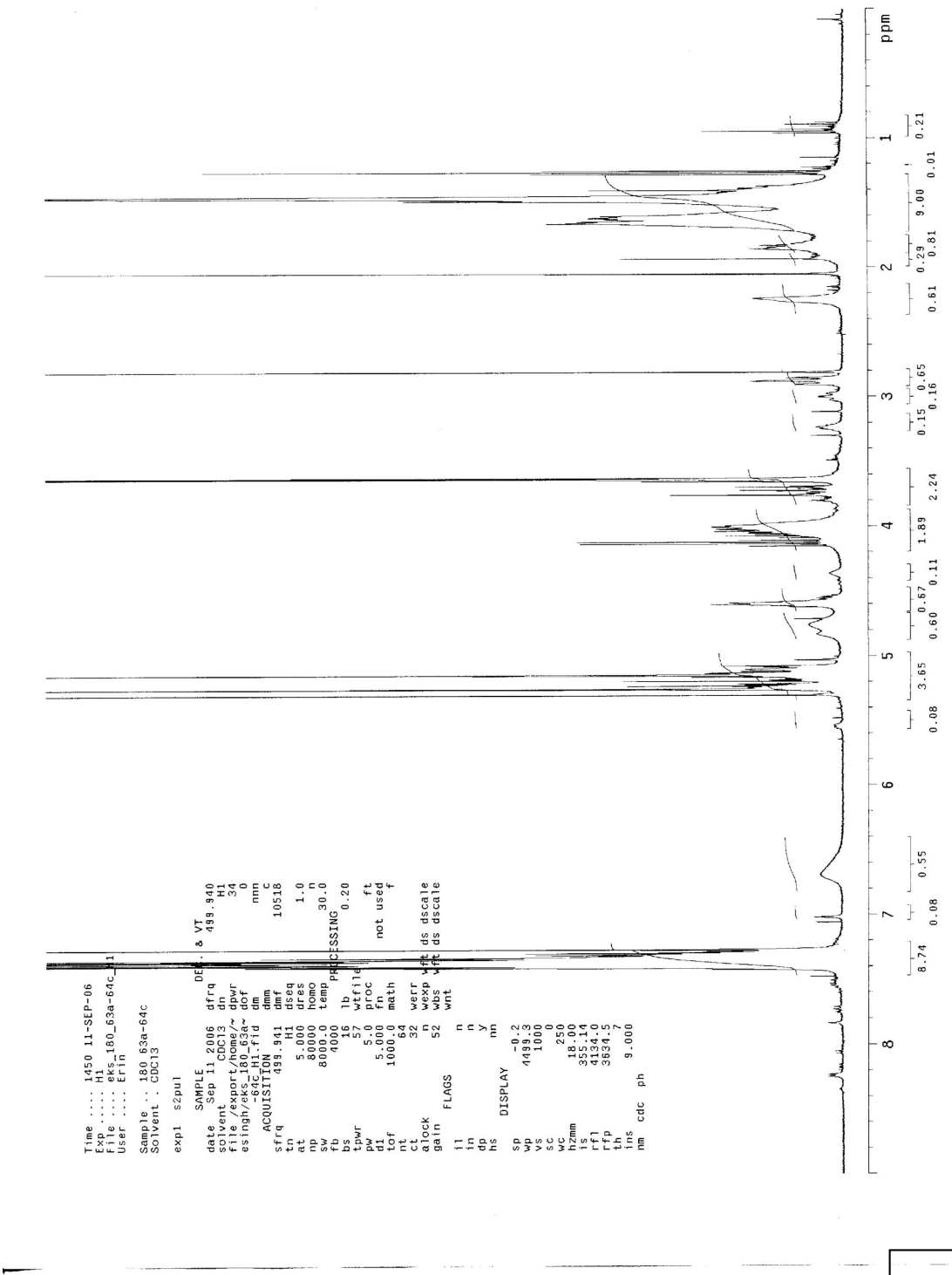


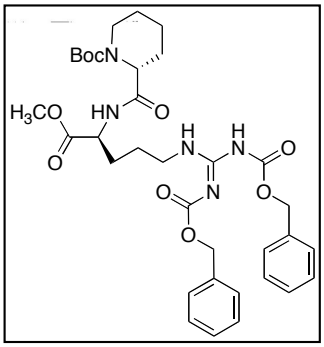
Compound 2 – LCMS cyclized tetrapeptide





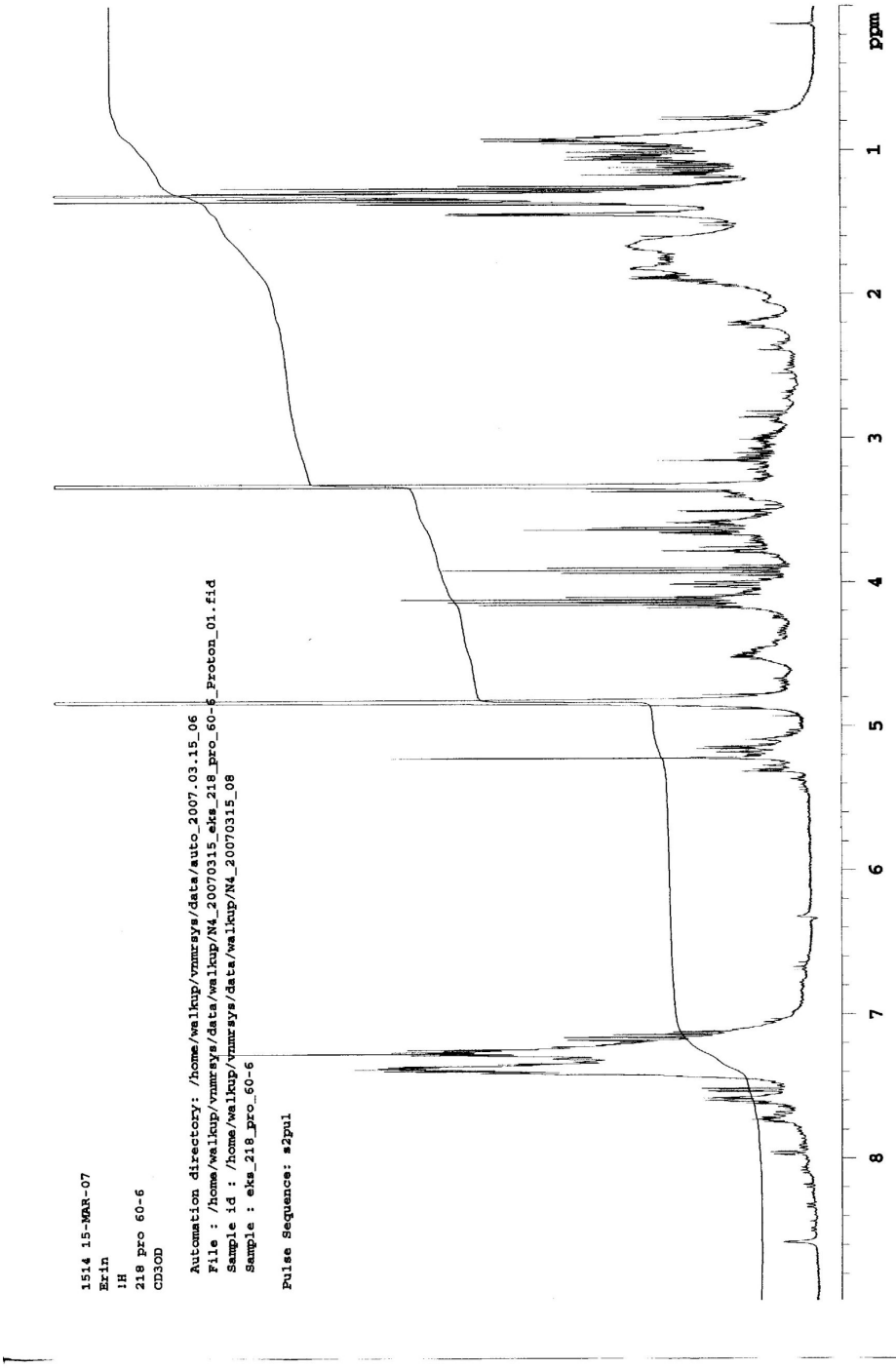
Compound 3 – NMR dipeptide 1b-2a

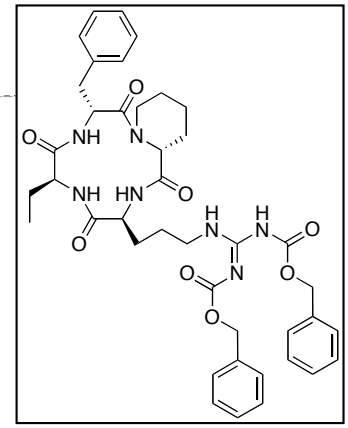




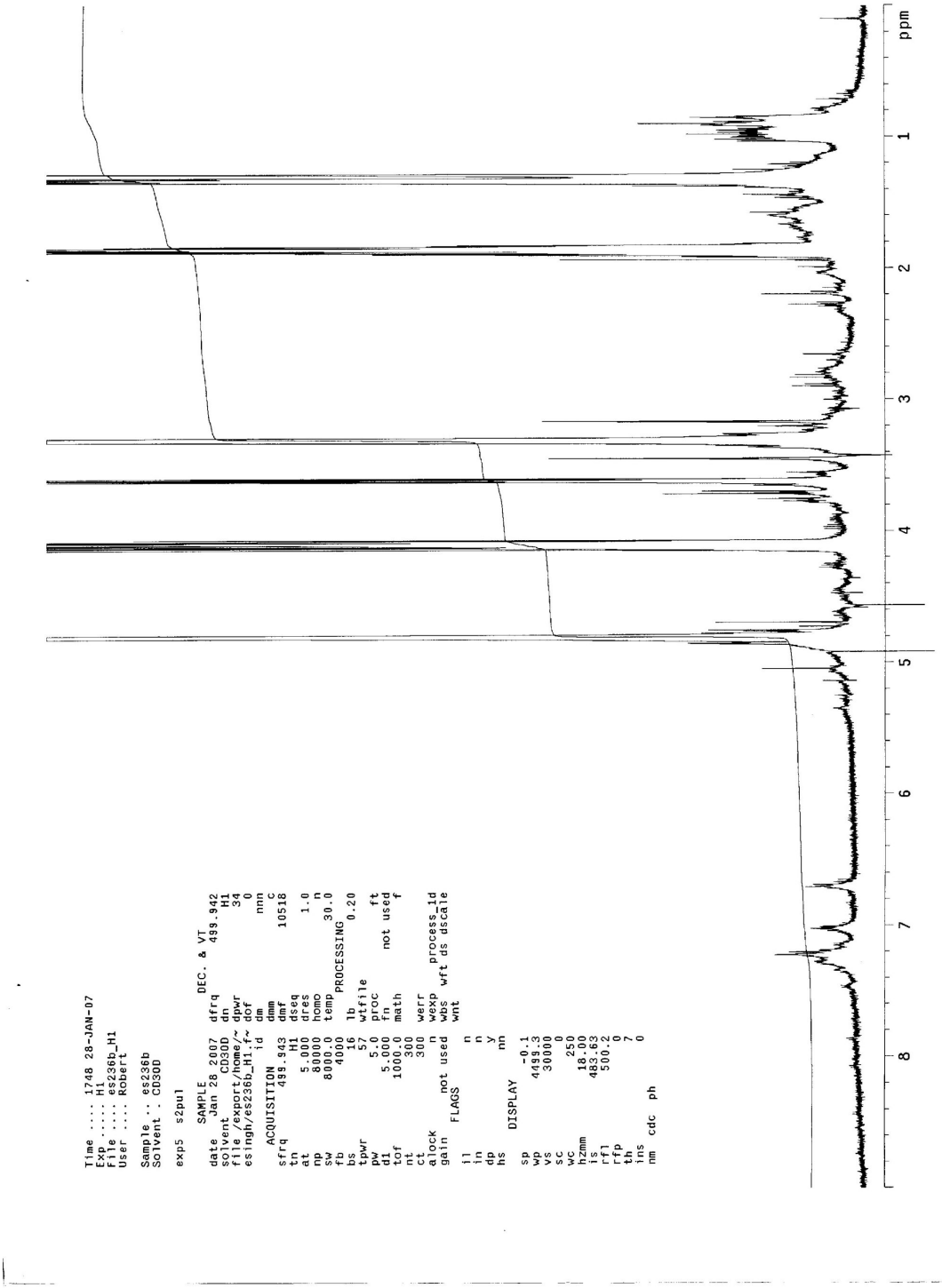
Compound 3 – NMR dipeptide 3a-4a

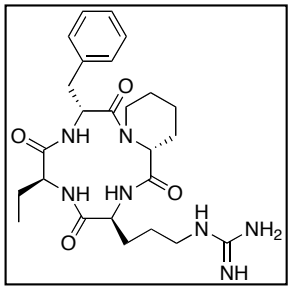
Compound 3 – NMR linear tetrapeptide 1b-2a-3a-4c



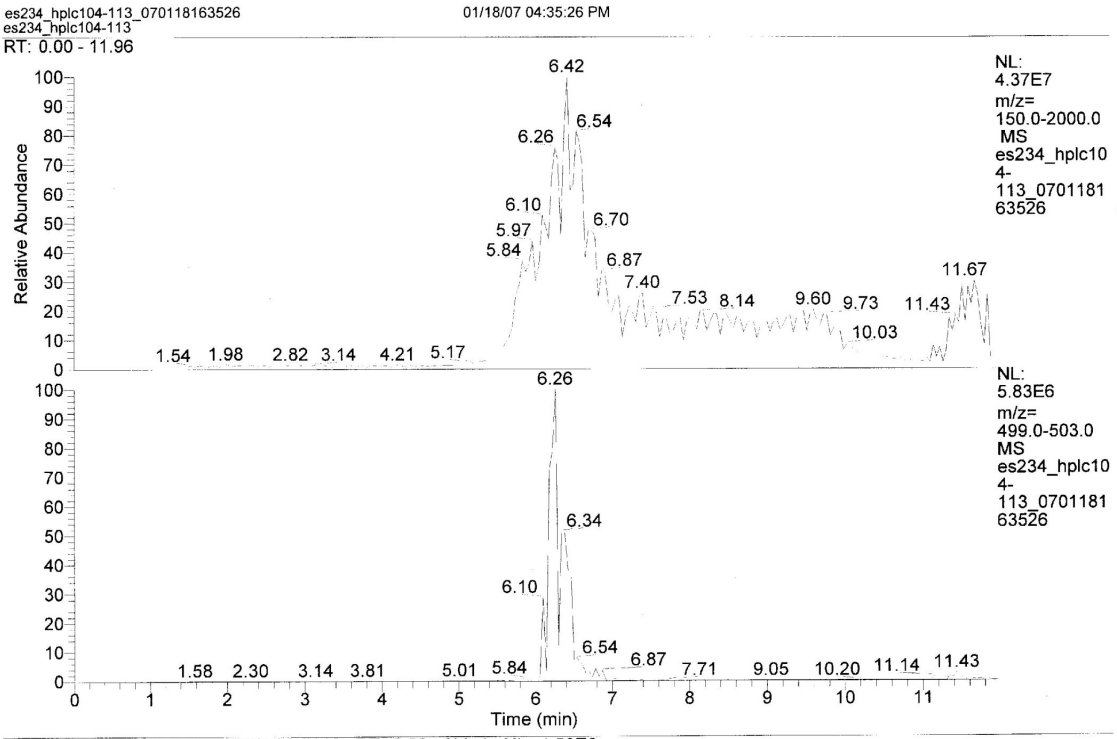


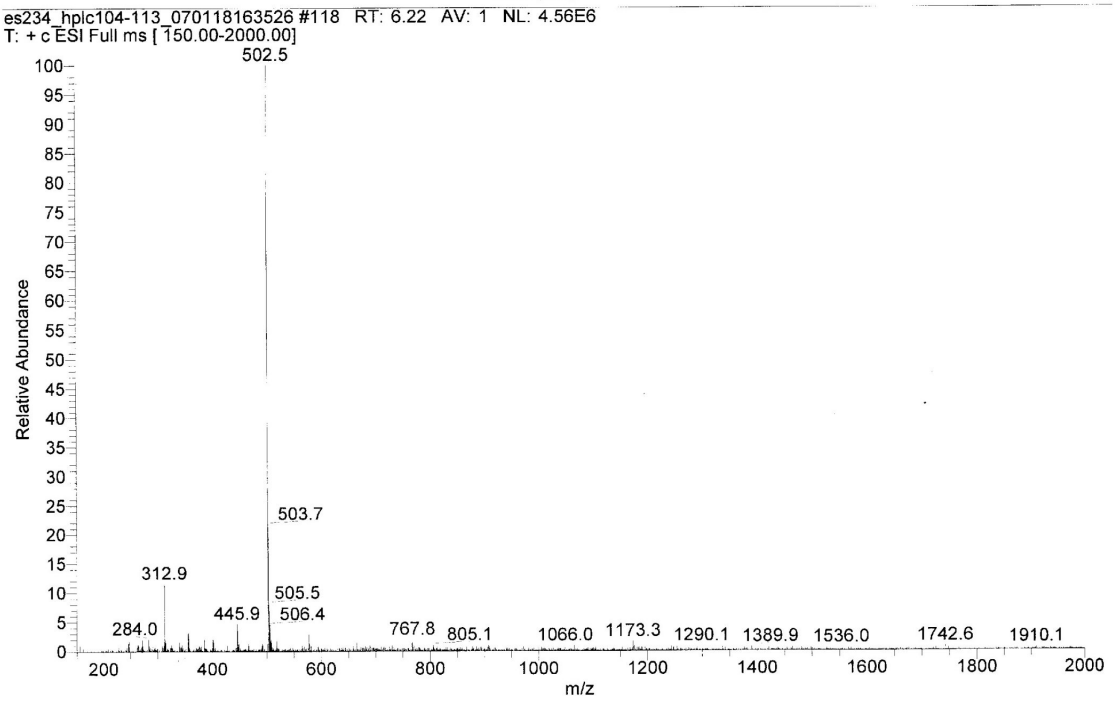
Compound 3 – NMR cyclized protected tetrapeptide 1b-2a-3a-4c

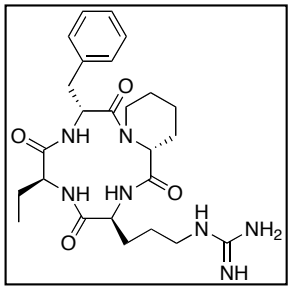




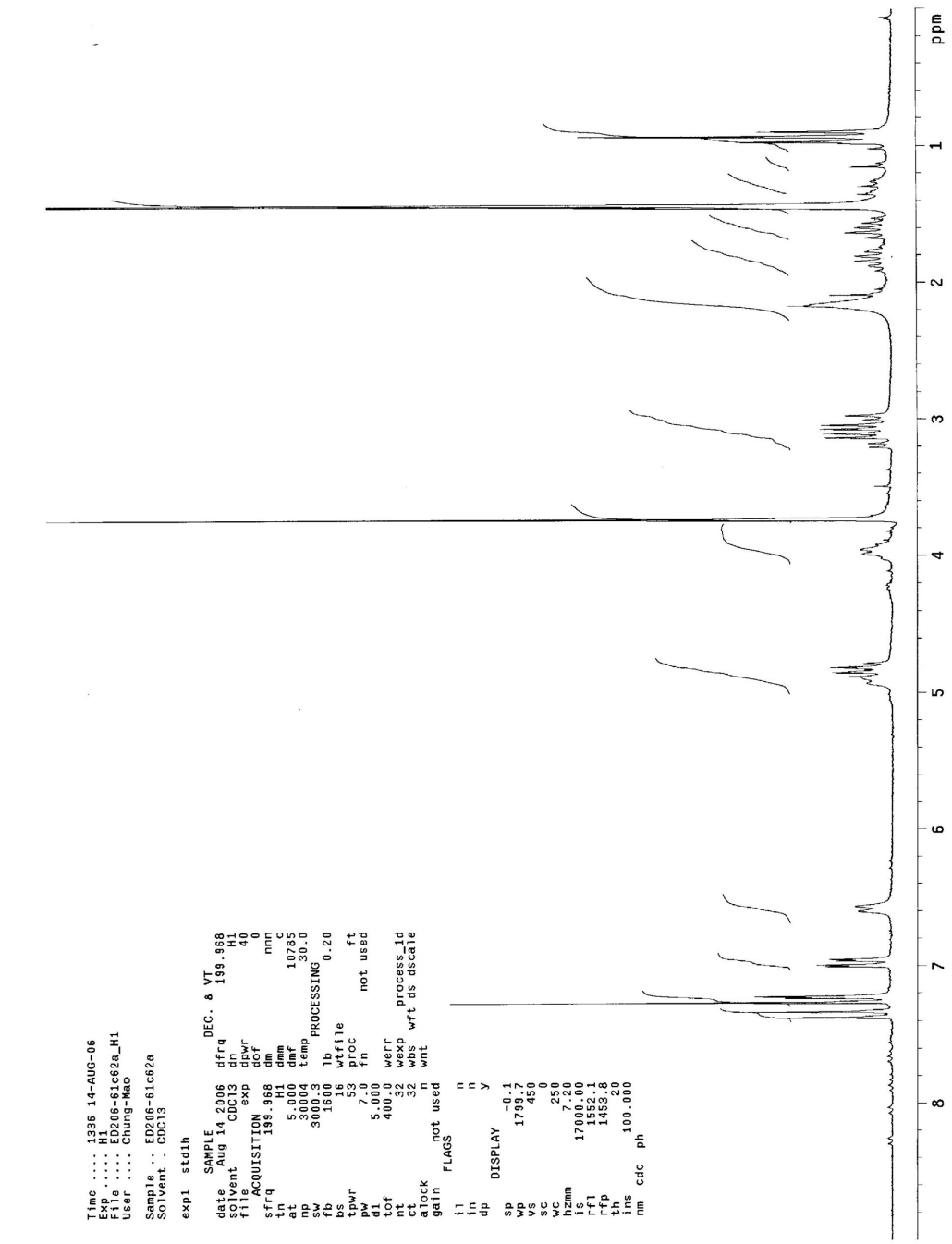
Compound 3 – NMR cyclized tetrapeptide 1b-2a-3a-4c

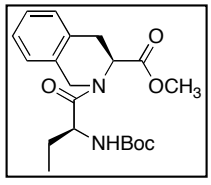




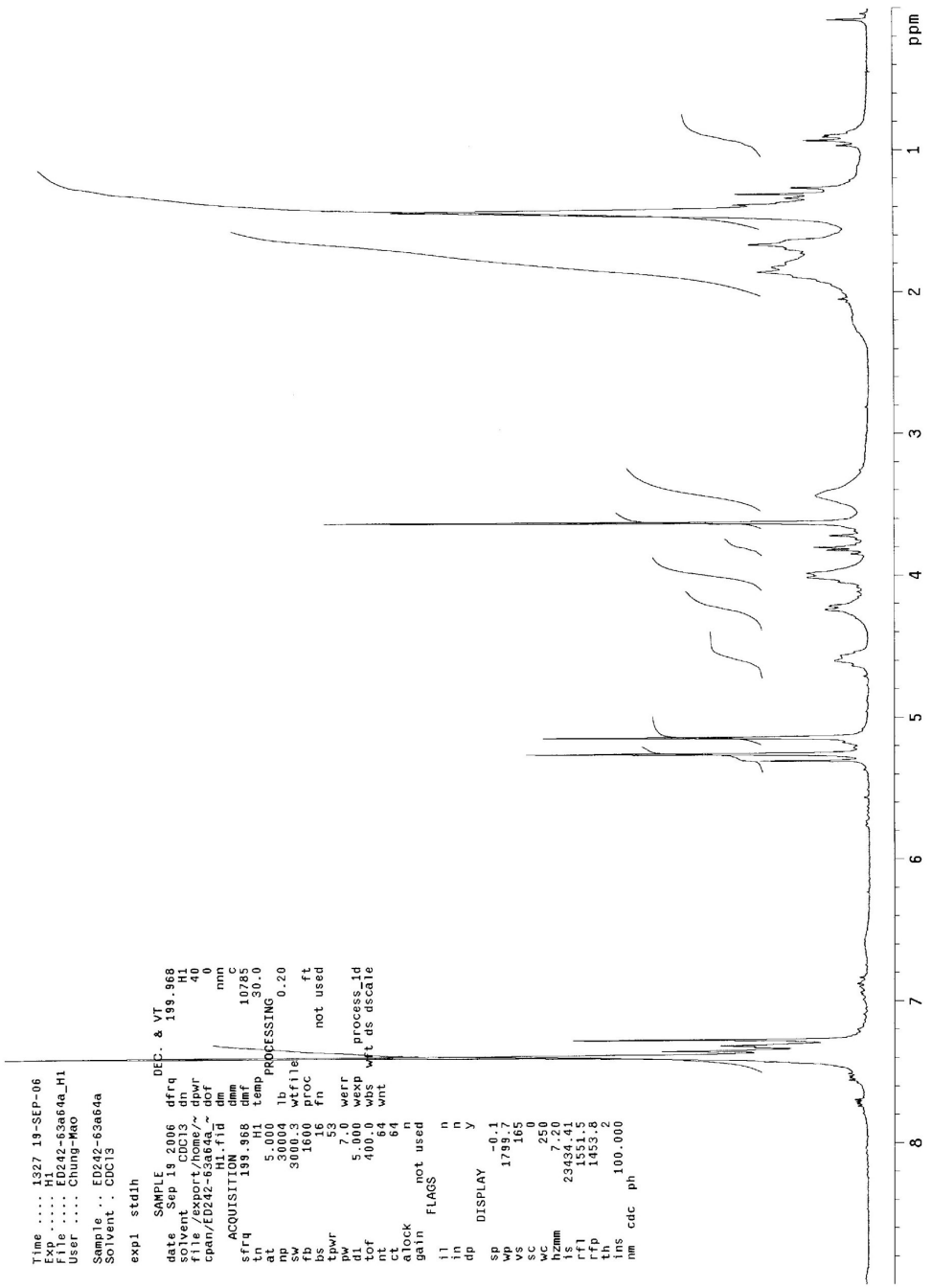


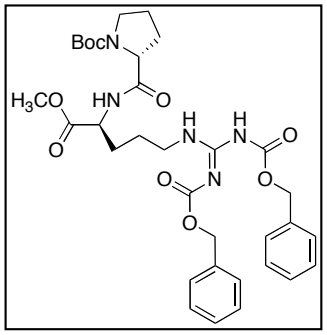
Compound 3 – LCMS cyclized tetrapeptide



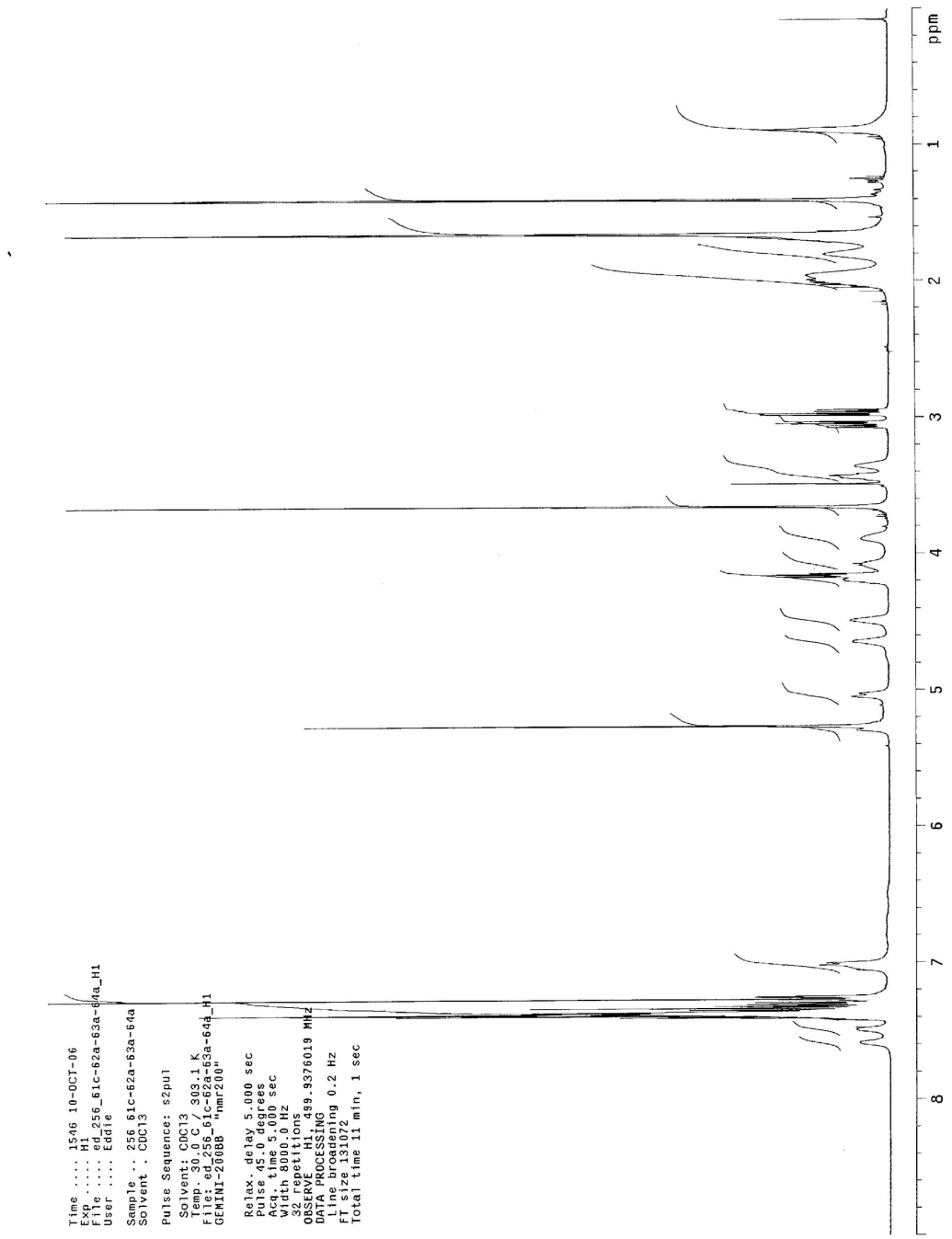


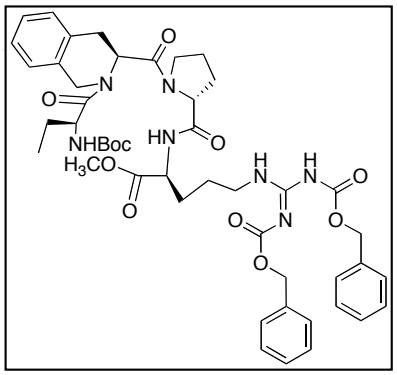
Compound 4 – NMR dipeptide 1c-2a



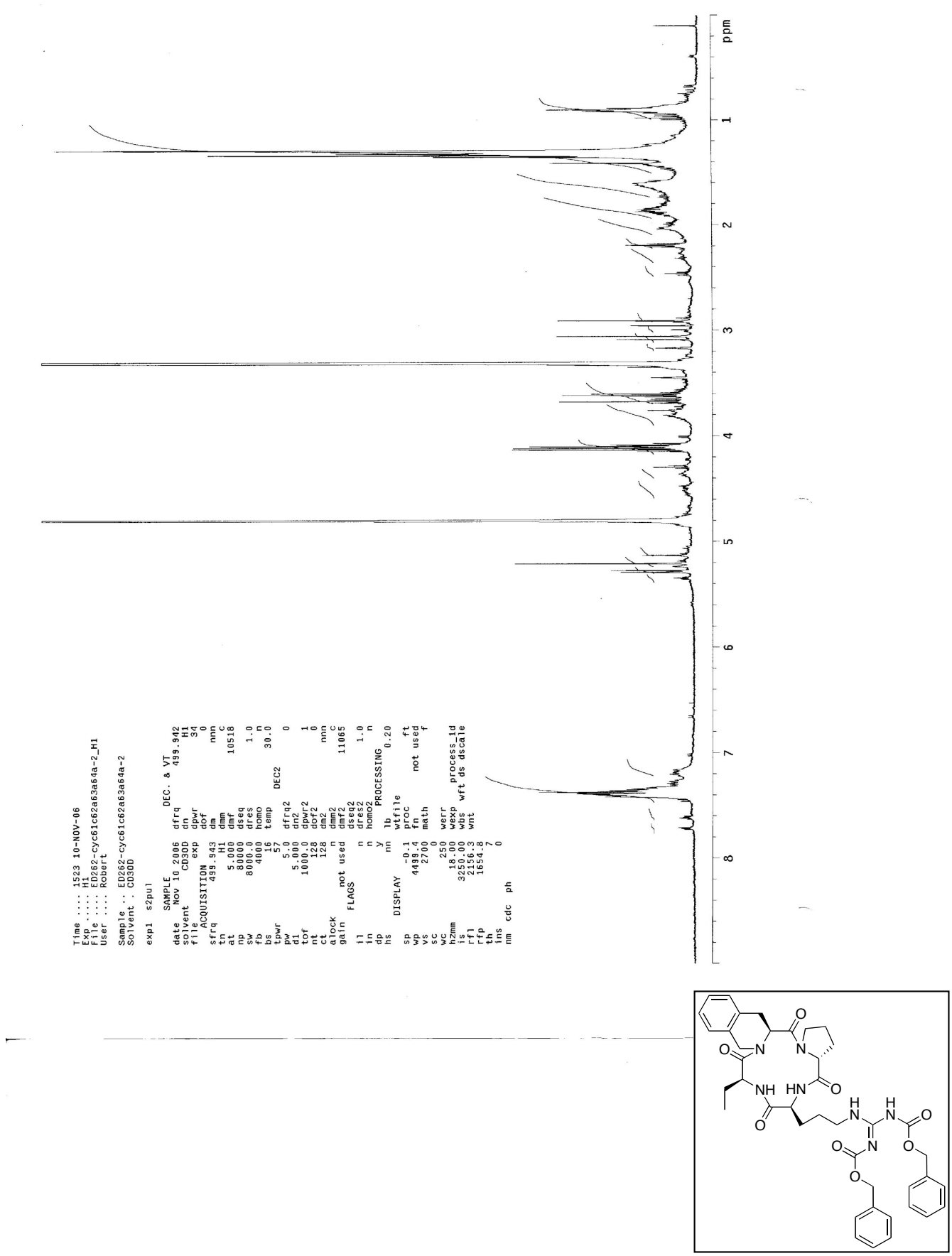


Compound 4 – NMR Dipeptide 3a-4a

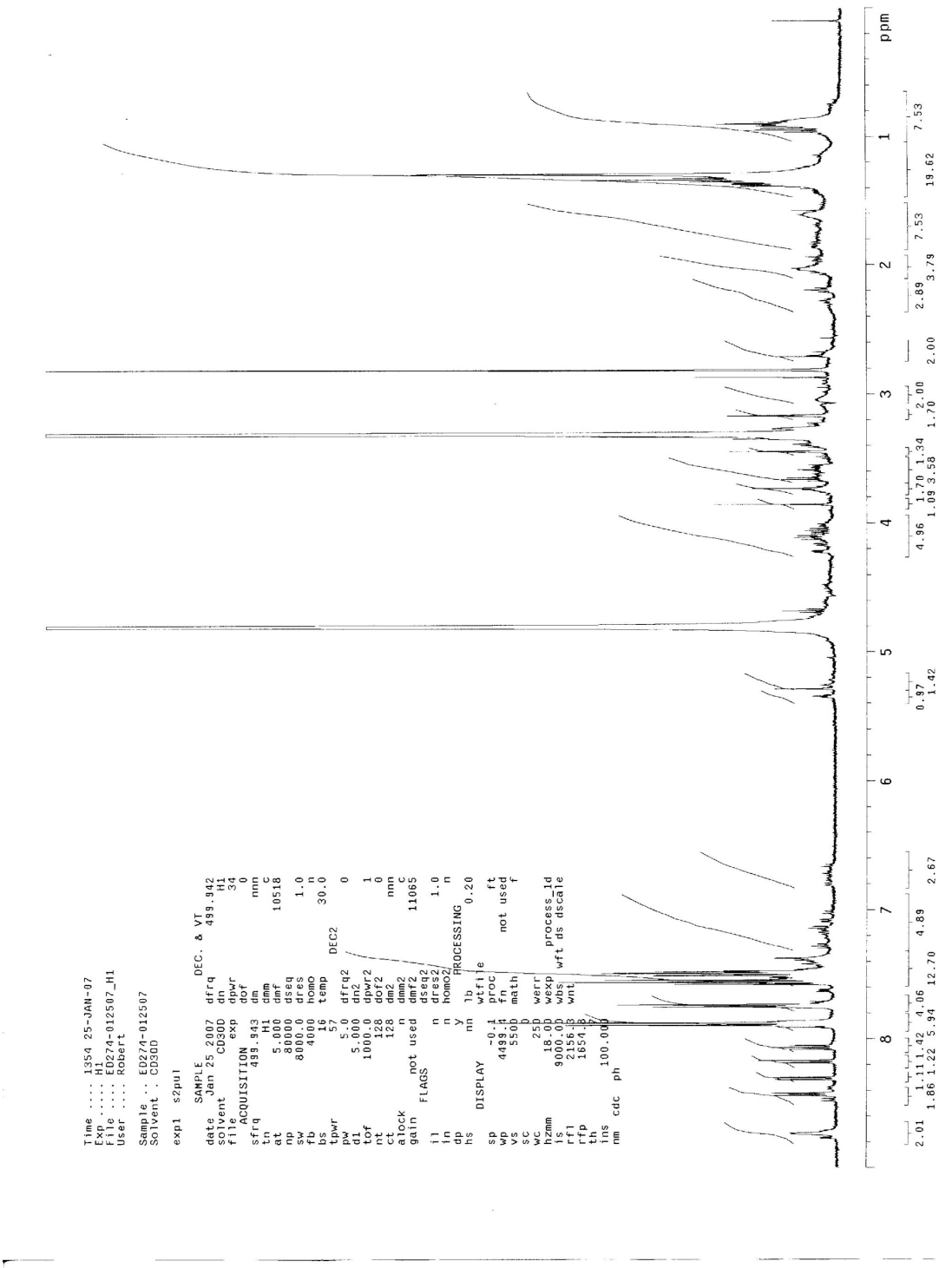


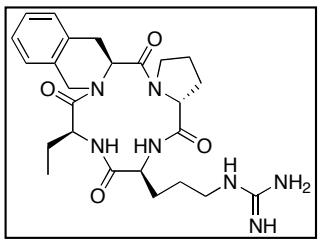


Compound 4 – NMR linear tetrapeptide 1c-2a-3a-4a

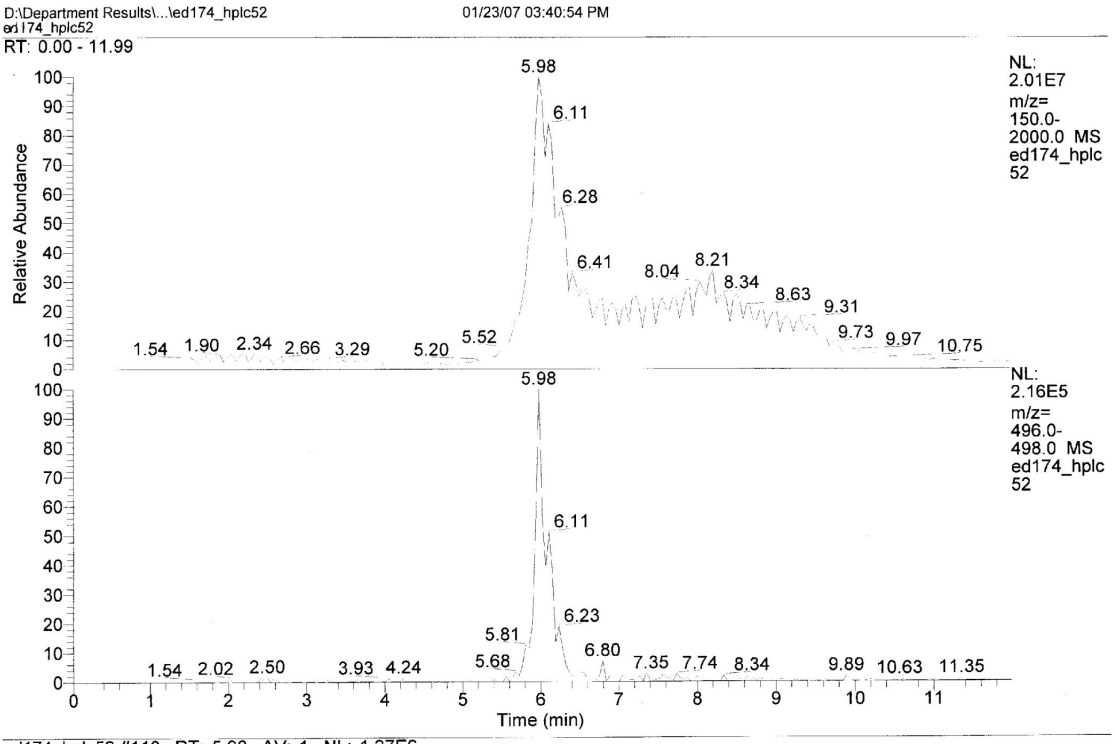


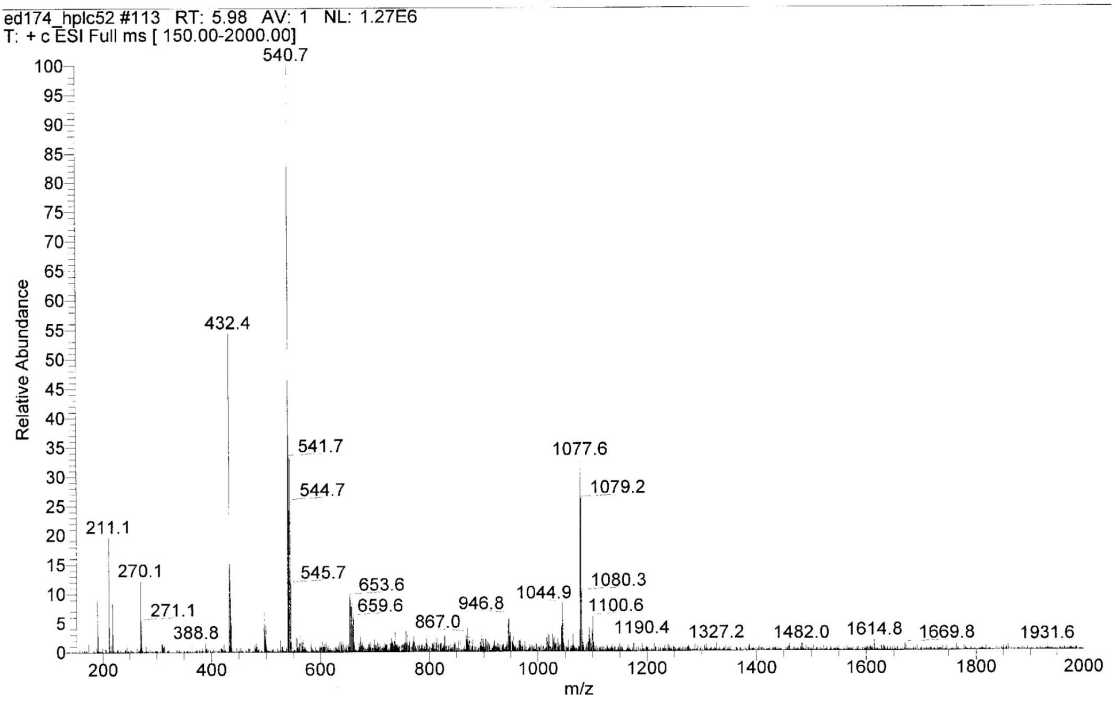
Compound 4 – NMR cyclized protected 1c-2a-3a-4a

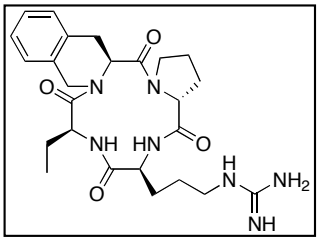




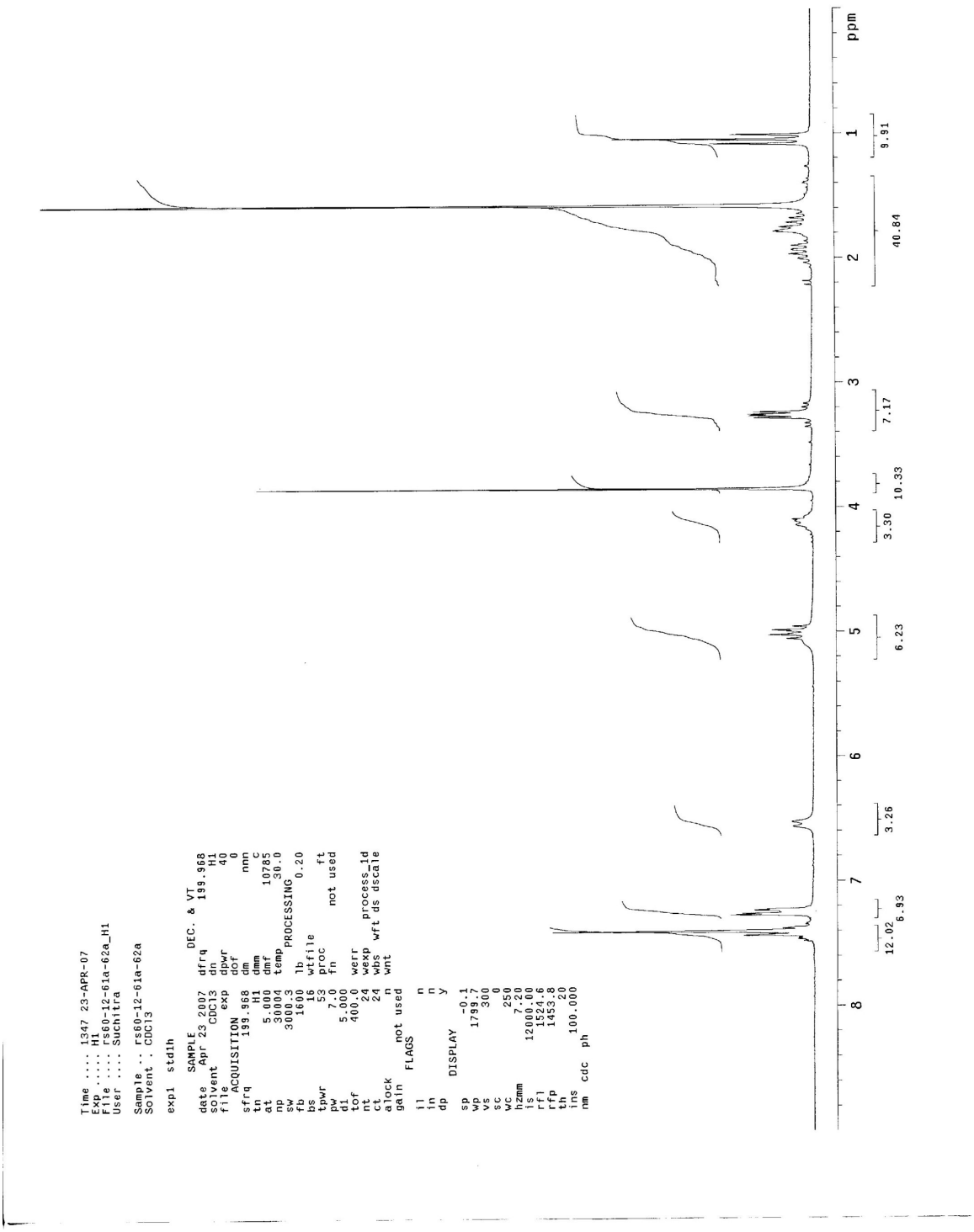
Compound 4 – NMR cyclized 1c-2a-3a-4a

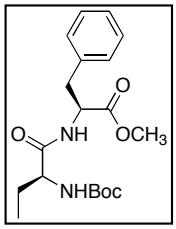




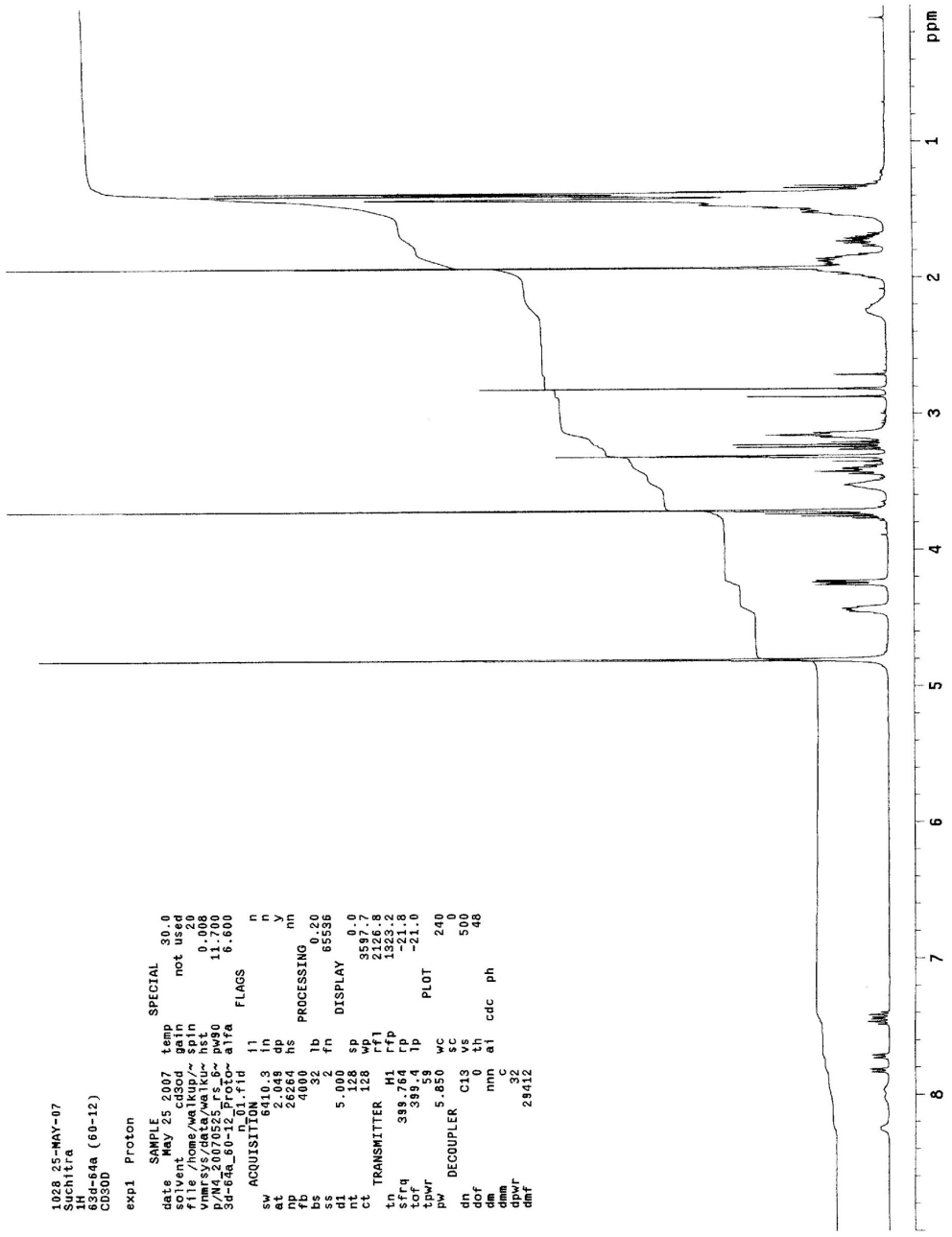


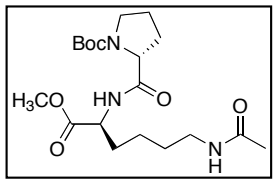
Compound 4 – LCMS cyclized 1c-2a-3a-4a



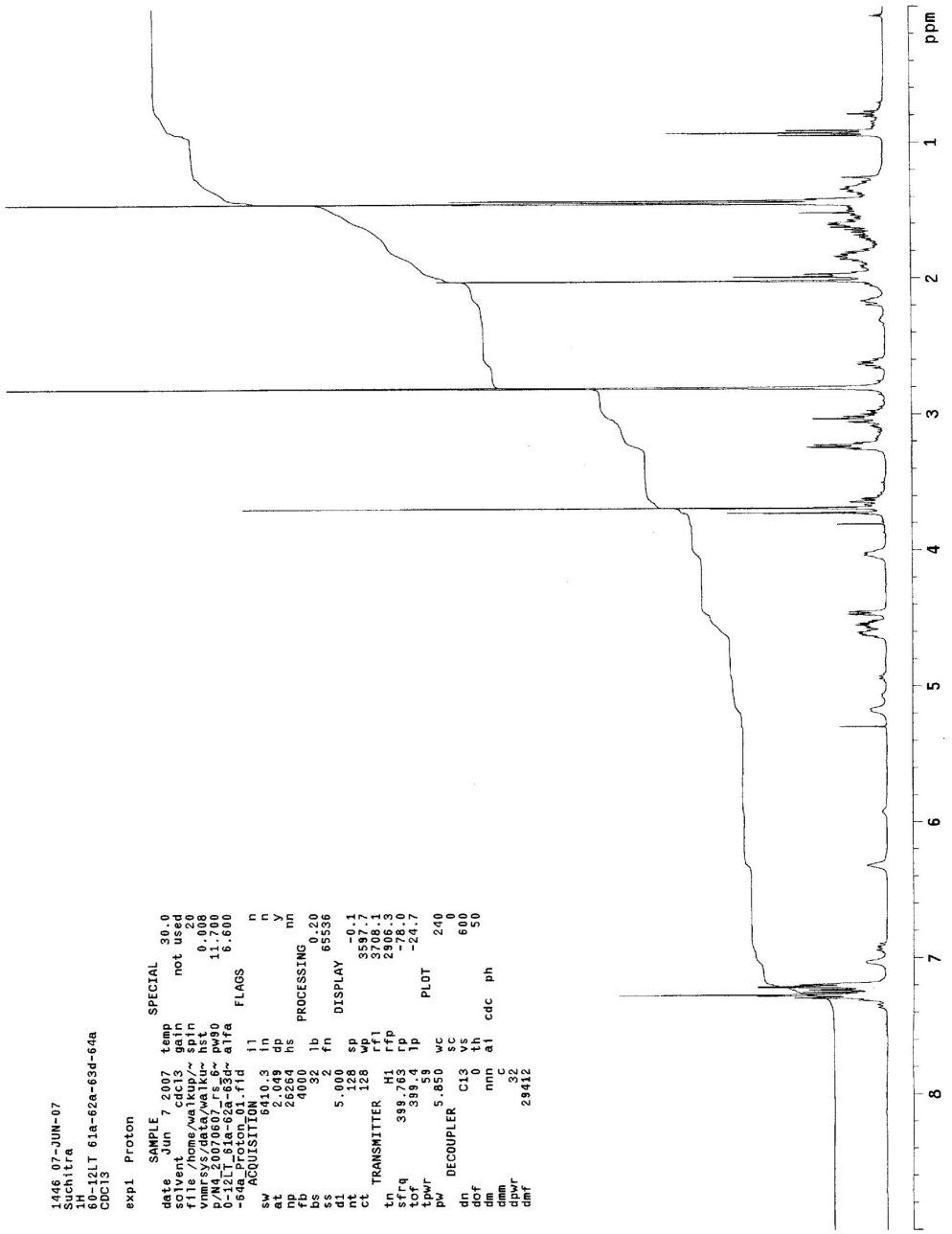


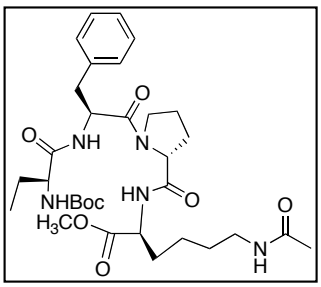
Compound 5 – NMR dipeptide 1a-2a



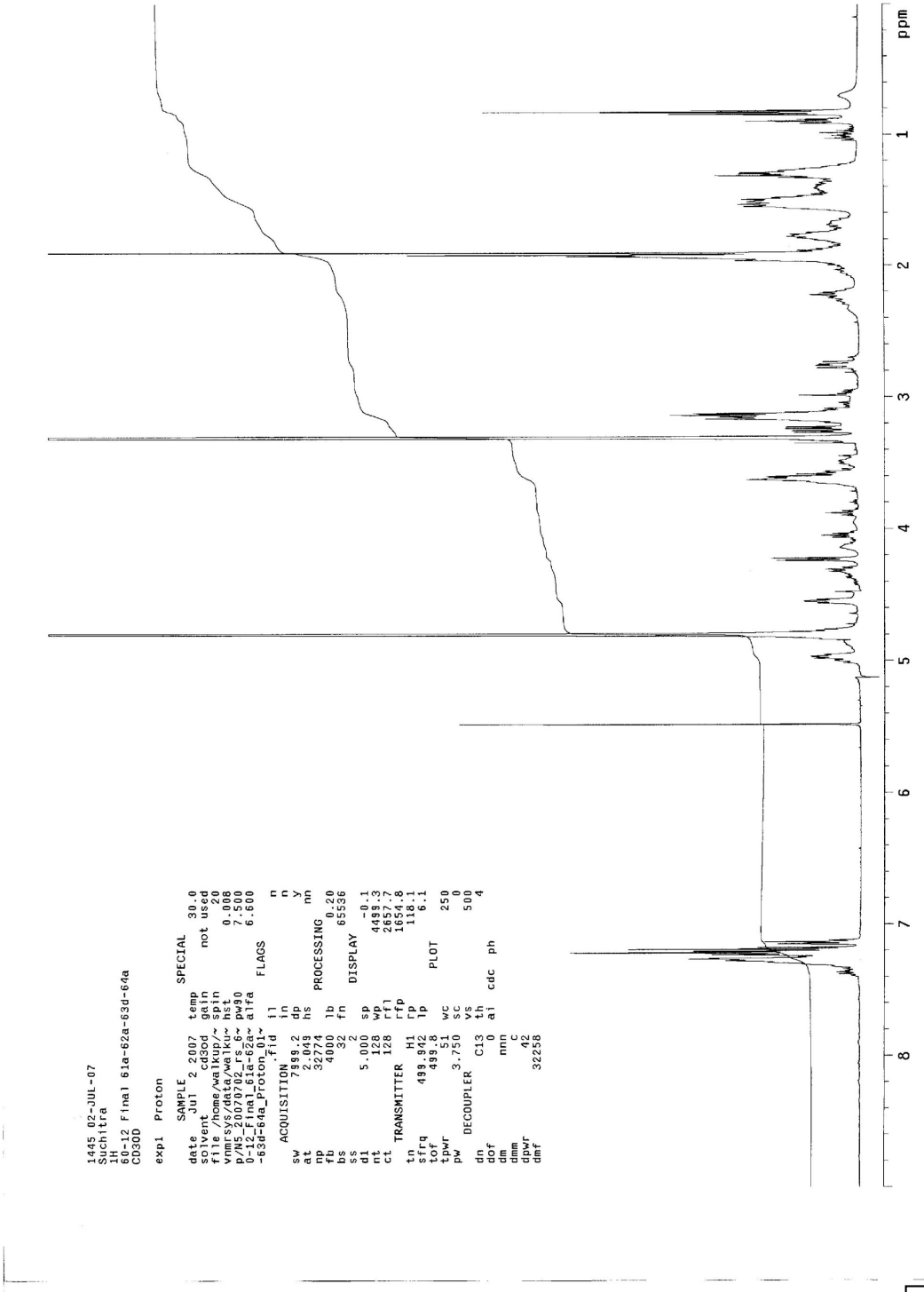


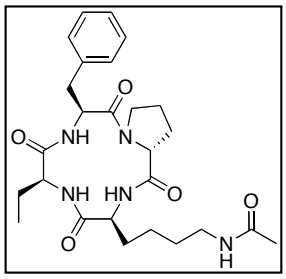
Compound 5 – NMR dipeptide 3b-4a



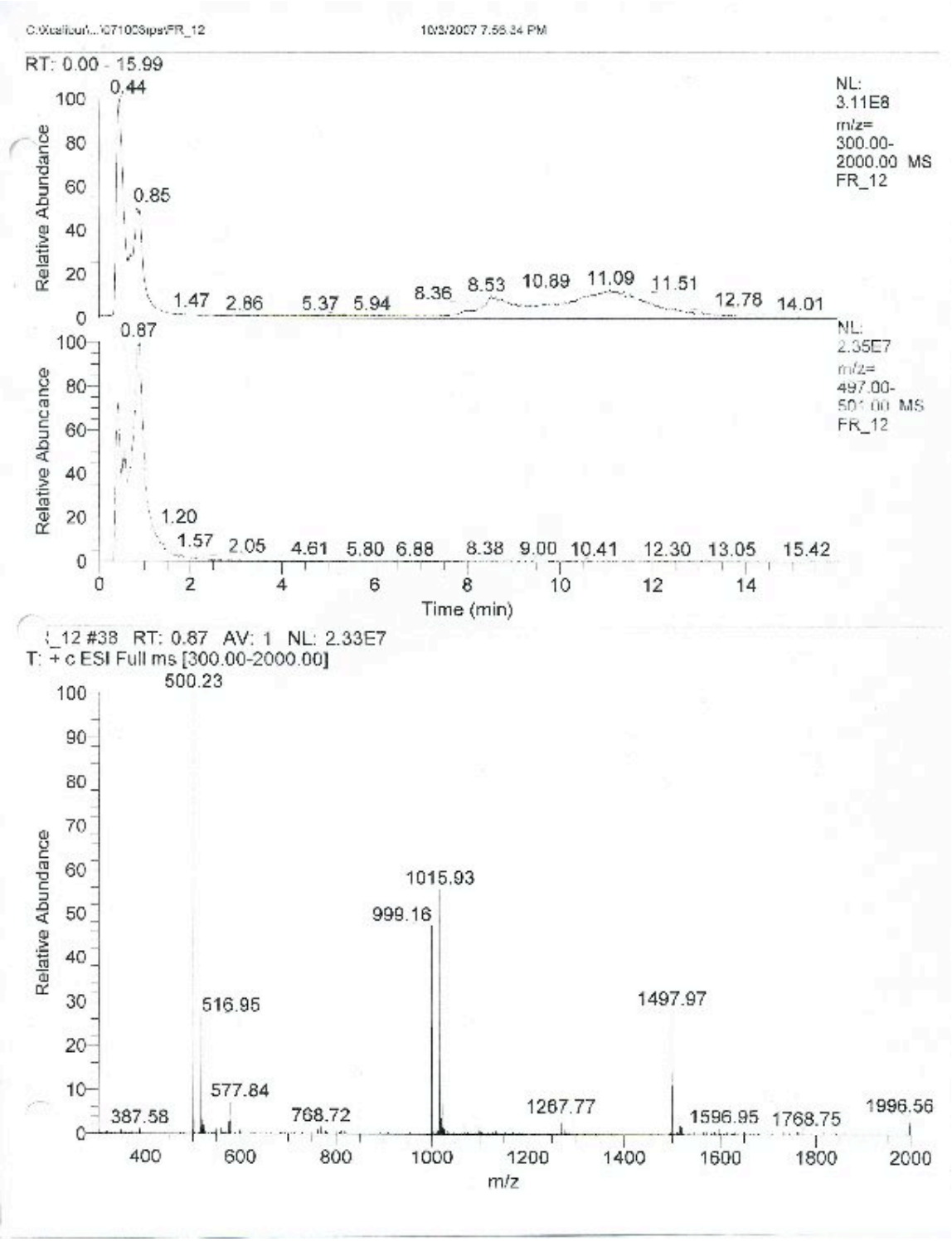


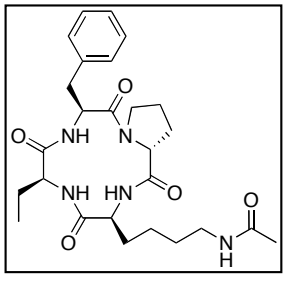
Compound 5 – NMR linear tetrapeptide



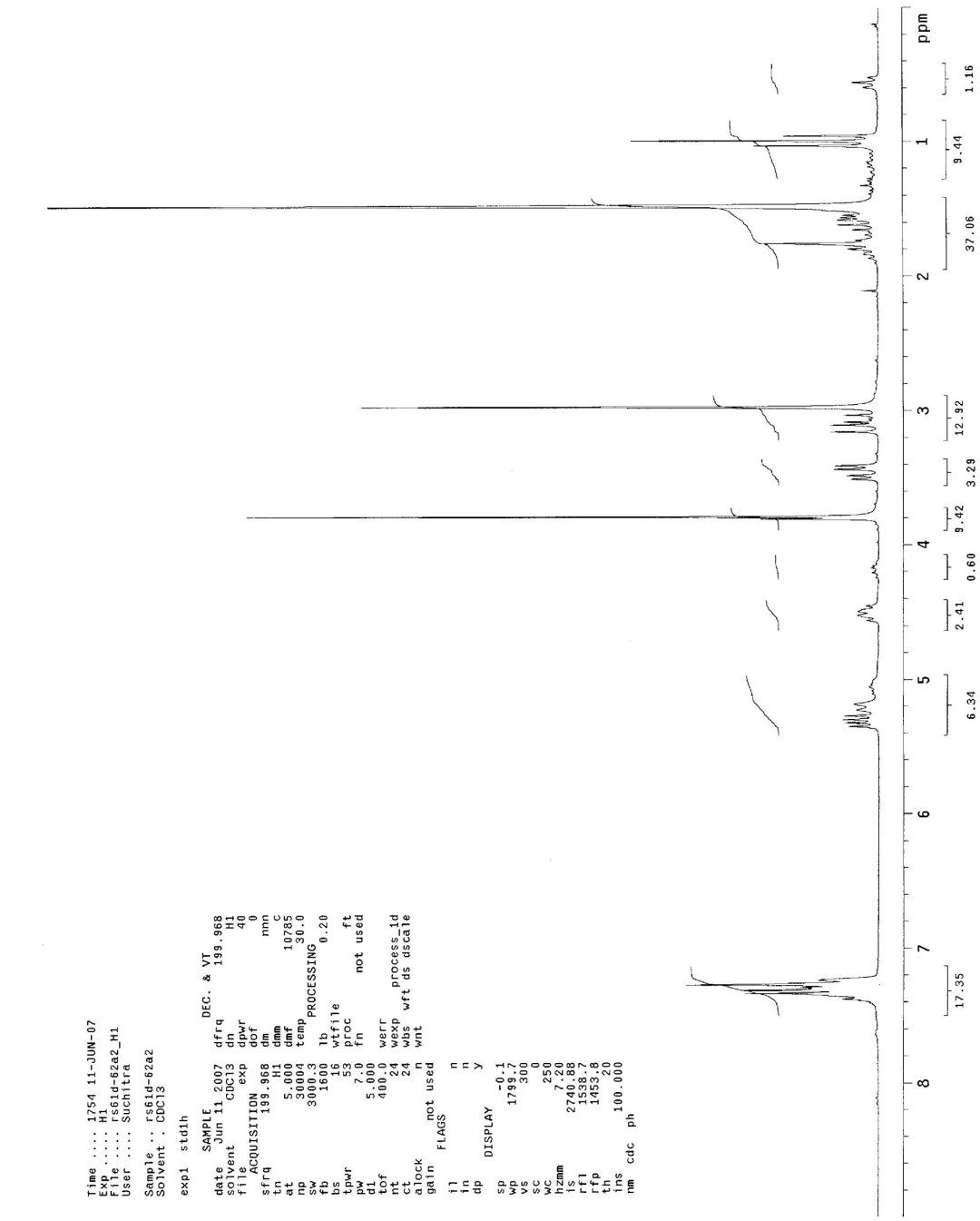


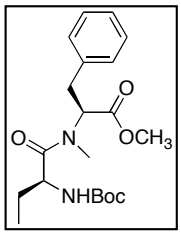
Compound 5 – NMR cyclized 1a-2a-3b-4a



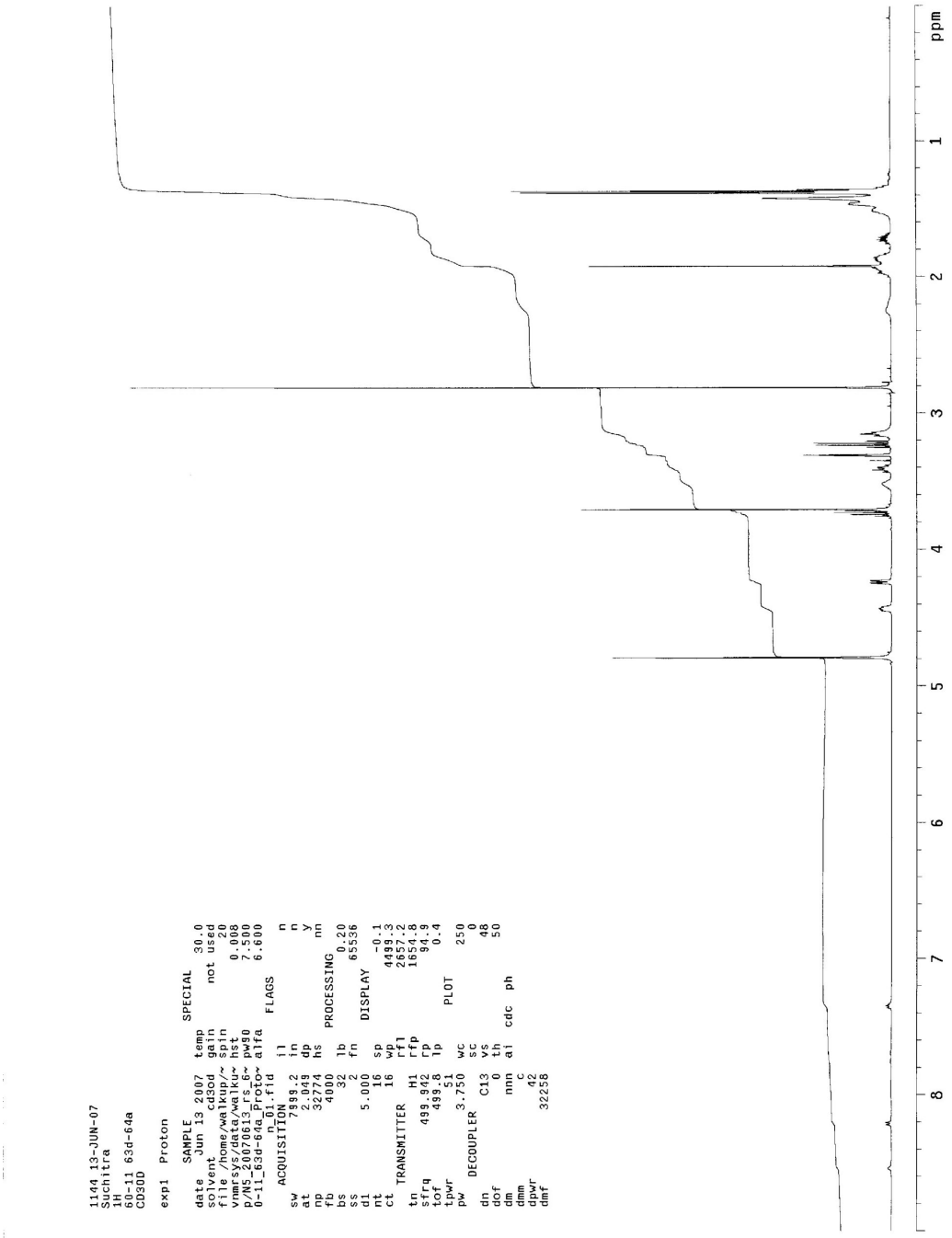


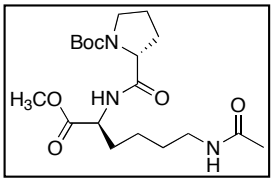
Compound 5 – LCMS cyclized tetrapeptide



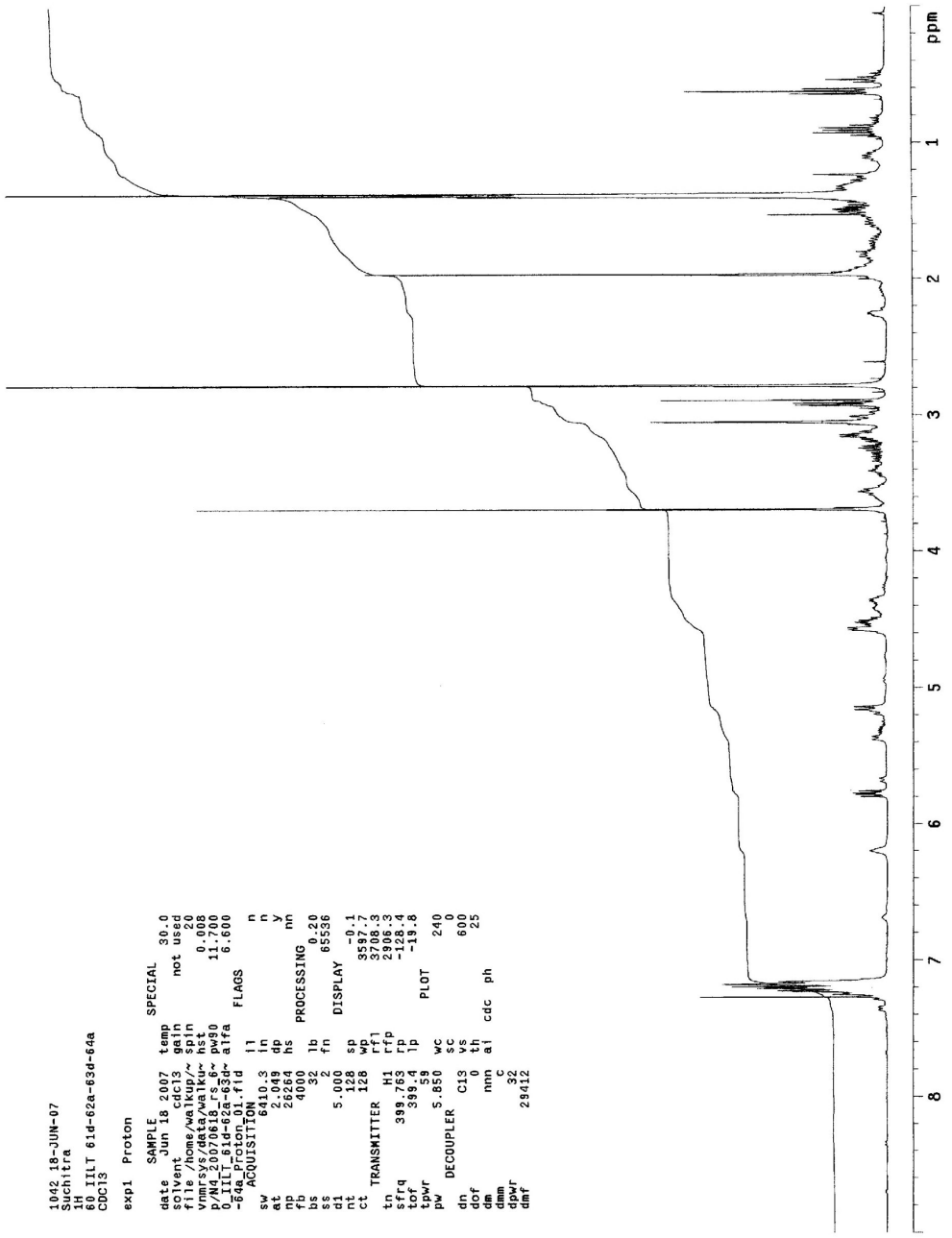


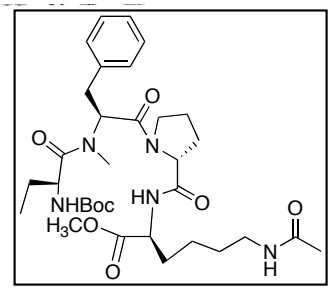
Compound 6 – NMR dipeptide 1d-2a



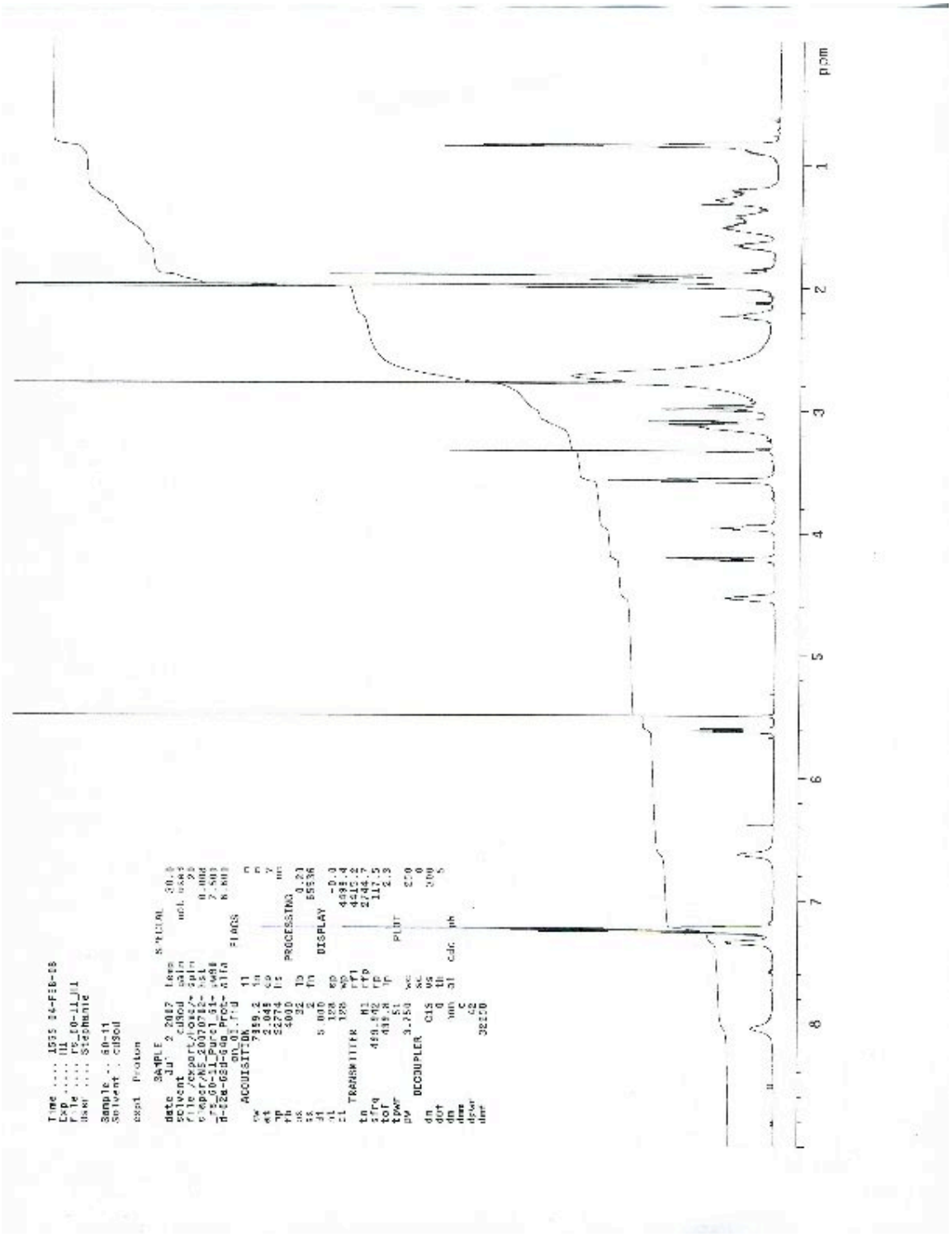


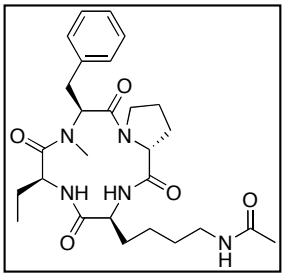
Compound 6 – NMR dipeptide 3-4a



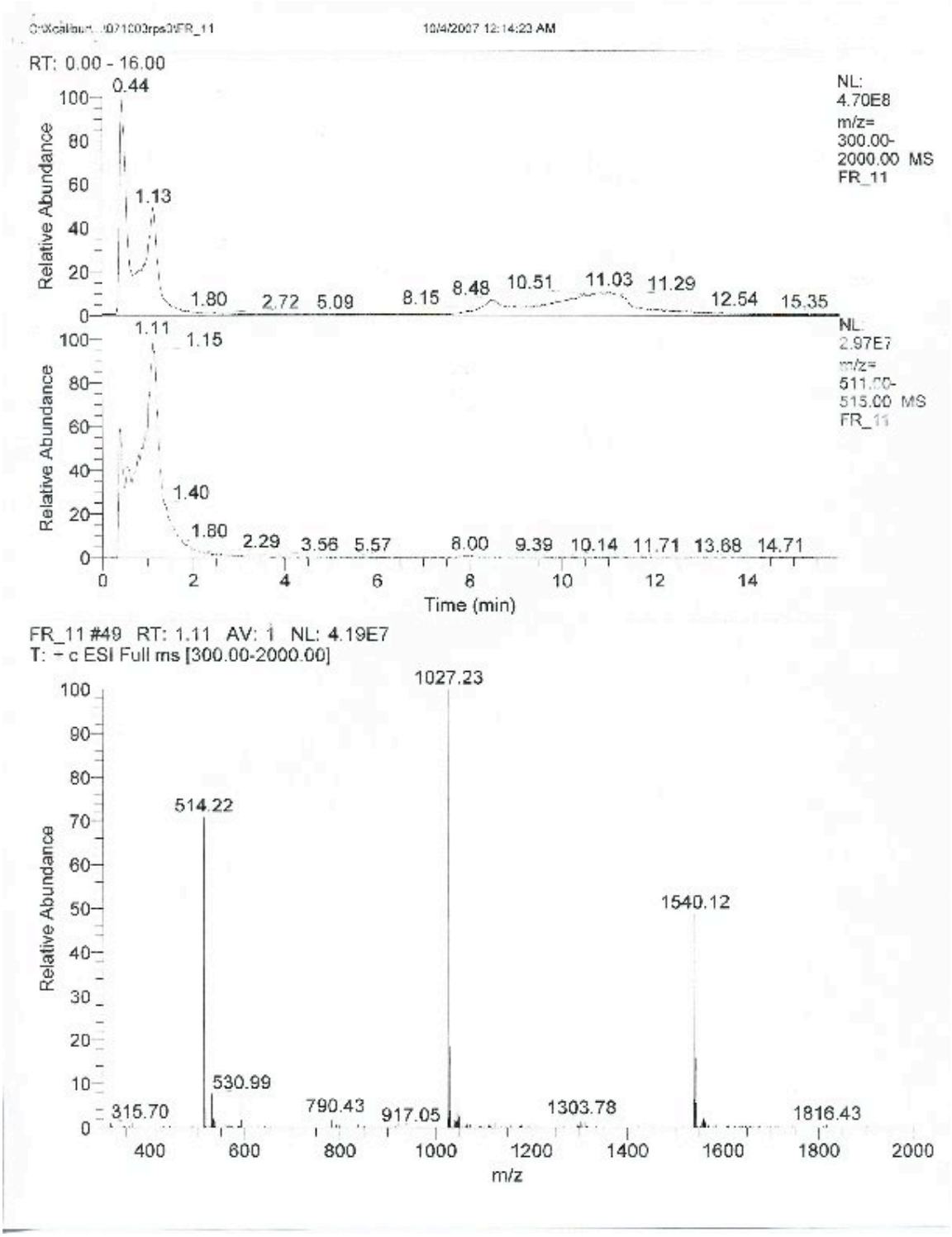


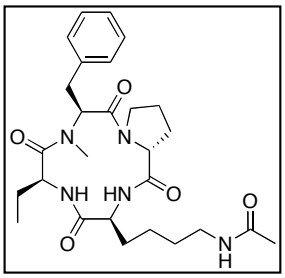
Compound 6 – NMR linear tetrapeptide 1d-2a-3b-4a





Compound 6 – NMR cyclized 1d-2a-3b-4a





Compound 6 – LCMS cyclized tetrapeptide