Supporting Information

Solid Phase Synthesis of Lysobactin (Katanosin B): Insights into Structure and Function

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General Experimental

Amino acids, coupling reagents, and general chemicals were purchased from Sigma Aldrich, Alfa Aesar, Nova Biochem, Chem Impex, and Santa Cruz Biotech. Tetrahydrofuran (THF) was distilled over sodium and benzophenone. All dimethylformamide (DMF) was HPLC grade and obtained from Omnisolv. All commercially available reagents were used as received.

Optical rotations were measured on a Perkin-Elmer polarimeter (Model 241) at 589 (sodium D line) using a 1 mL capacity quartz cell with a 10 cm path length. Concentrations (c) are given in g/100 mL. ^{1}H-NMR spectra were measured on a Varian INOVA-500 (500 MHz). ^{13}C-NMR spectra were measured on INOVA-500 (125 MHz). Reported NMR spectra were referenced to the acetonitrile peak at 1.96 ppm for ^{1}H NMR and 118.26 ppm for ^{13}C NMR. Mass spectral data were recorded on a Waters LCT Classic Electrospray time of flight analyzer with Agilet 1100 capillary HPLC inlet or Sciex API III electrospray quadropole with direct infusion inlet. Circular dichroism spectra were recorded on a Jasco spectrometer (Model J-715) ranging from 190 to 270 nm wavelength with a sample concentration of 133µg/mL.

Analytical thin layer chromatography (TLC) was performed using Whatman glass plates coated with a 0.25 mm thickness of silica gel containing PF 254 indicator, and compounds were visualized with UV light, cerium molybdate stain, or ninhydrin stain.

Analytical high performance liquid chromatography (HPLC) was performed on an Agilent 1100 instrument with PDA and ELSD detection. Analysis was carried out using Phenomenex Luna C18(2) reverse-phase column (5µ particle size, 100 Å pore size, 150 mm length x 1.0 mm diameter) with mobile phases consisting of water and acetonitrile with 0.1 % TFA. Preparatory HPLC purifications were performed with an Agilent 1100 Series HPLC purification system using a Phenomenex Luna C18(2) reverse-phase column (5µ particle size, 100 Å pore size, 250 mm length x 22 mm diameter).

Flash column chromatography was performed using Silicycle 60 Å, 35-75 µm silica gel. All compounds purified by chromatography were sufficiently pure for use in further experiments, unless otherwise noted.

All synthetic amino acids matched all spectral data as according to their literature precedents (threo-β-hydroxyasparagine[1], threo-β-hydroxyleucine[2], threo-phenylserine[3], allo-threonine[4], D-Arginine[5])

General Procedure for Solid Phase Couplings of Commercially Available Amino Acids (0.1 mmol scale)

2-Chlorotrityl resin was swollen with DMF for 30 min. A solution of the appropriate Fmoc-protected amino acid (0.5 mmol), DEPBT (150 mg, 0.5 mmol), and DIEA (155 µL, 1 mmol) in DMF (0.5 M) was added to the resin and the suspension was agitated for the specified amount of time (Table 1**)**. The resin was filtered and washed with DMF (5 x 4 mL).

Coupling of Unnatural β-Hydroxy Amino Acids and Fmoc-D-Arg(Boc)$_2$-OH (0.1 mmol scale)

2-Chlorotrityl resin was swollen with DMF for 30 min. A solution of the appropriate Fmoc-protected amino acid (0.15 mmol), DEPBT (45 mg, 0.15 mmol), and DIEA (46 µL, 0.3 mmol) in DMF (0.5 M) was added to the resin and the suspension was agitated for the specified amount of time (Table 1). The resin was filtered and washed with DMF (5 x 4 mL).

Table 1: Reaction Times for Amino Acid Couplings

Amino Acid	**Equivalency**	**Reaction Time**
Fmoc-aThr(OTBS)-OH	2	19h
Fmoc-Ile-OH	5	2.5h
Fmoc-D-Arg(Boc)$_2$	1.5	20h
Fmoc-Leu-OH	5	3h
Fmoc-HyLeu(OTBS)-OH	1.5	19h
Fmoc-HyPhe-OH	1.5	20h
Fmoc-Leu-OH	5	8h
Boc-D-Leu-OH	5	8h
Alloc-Ser(OtBu)-OH	10	17h
Fmoc-HyAsn(CONHTrt)-OH	1.5	24h

General Fmoc Deprotection Conditions*

The resin was treated with 20% piperidine/DMF (3 x 3 mL x 3 min). The resin was filtered and washed with DMF (5 x 4 mL).

* = These conditions were used for all Fmoc deprotections except for the final cleavage

Resin Cleavage Protocol

2-Chlorotrityl resin was treated with a solution of AcOH/trifluoroethanol/CH_2Cl_2 (1:1:3) and was agitated for 2 h. The resin was filtered and resubmitted to the same reaction condition for another 2 h. The resin was again filtered. Both filtrates were combined and concentrated *in vacuo*.

MIC Determination Protocol

Minimal inhibitory concentrations (MIC) of lysobactin and its analog **11** for *B.subtilis PY79* strain were calculated following the Broth microdilution method.[6] *B.subtilis PY79*, exponentially growing in cation-adjusted Mueller-Hinton Broth 2 (CAMHB2, Fluka), was diluted to final $5x10^5$ Colony forming units (CFU)/mL into wells of a 96-well polystyrene plate containing CAMHB2 (either supplemented with 0.0005 % Bovine Serum Albumin (BSA) + 0.01% Acetic Acid or not[6]) with 2-fold dilutions of both lysobactin and **11** spanning the final concentration range of 32 ug/mL – 0.0625 ug/mL. The plate was incubated 16-18 h at 37^{o}C and the MIC was determined as the lowest concentration of drug dilutions that inhibited growth completely. Presence of BSA + Acetic Acid did not affect the MIC significantly. The reported MIC is the average of two experiments conducted on two different days.

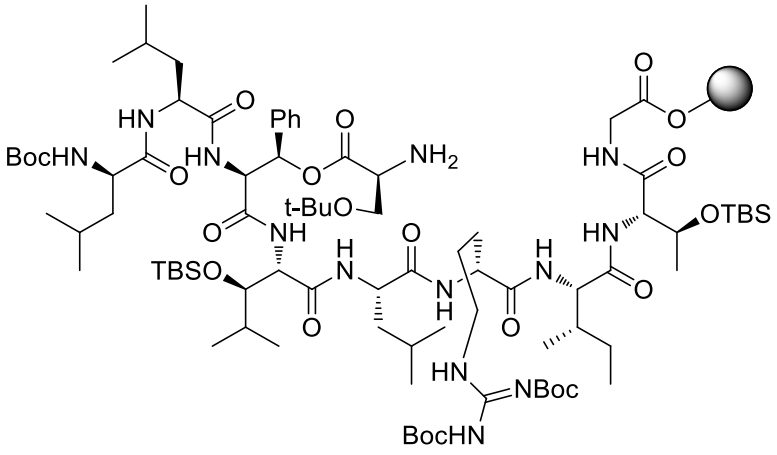
Membrane Permeablization Assay Protocol

To assess the effects of lysobactin and **11** on membrane permeablization, a well-established Propidium Iodide (PI) assay with the following modifications was used.[7,8] *B.subtilis PY79* or *E.coli MG1655,* exponentially growing in CAMHB2, was washed once with 0.85% saline and diluted to final $1x10^8$ CFU/mL in 0.85% saline containing 12.5 μg/mL PI. As soon as the cells were added to a black 96-well polystyrene plate (Corning)

containing 2-fold dilutions of drugs with the range of 64 μg/mL – 0.5 μg/mL together with wells of appropriate controls (e.g. no drug, PI photobleaching and buffer only controls), change in relative fluorescence units (RFUs) was measured with a top-read function of a SpectraMax M2 plate reader. Each well was excited at 535 nm and fluorescence was detected at 617 nm. The dead time until the first reading was approximately 45 seconds. The change in RFU, which is a result of normally membrane impermeable and vaguely fluorescent PI influx into the cells, was plotted against the duration of incubation after the data is normalized. The reported RFUs are replicate of two experiments.

Killing Kinetics Protocol

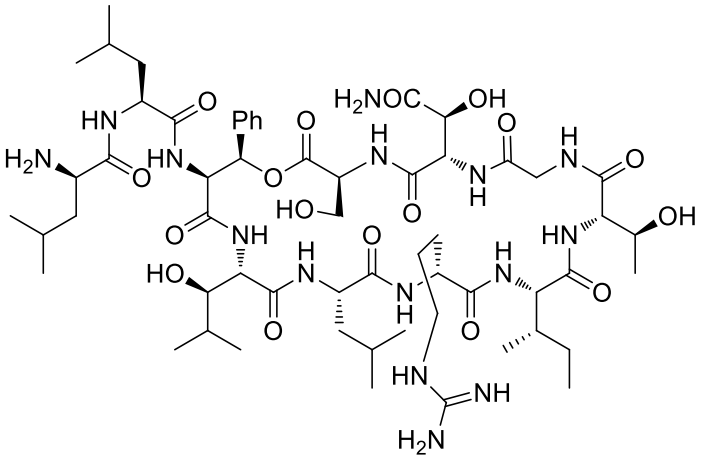
Exponentially growing *B.subtilis PY79* was washed once with 0.85 % saline and diluted to $5x10^5$ (CFU)/mL in 0.85 % saline with test drugs (lysobactin, **11**, and vancomycin) together with no drug control. At different time points, aliquots were taken, cells immediately diluted 10^3 and 10^4 times, plated on cation-adjusted Mueller-Hinton Agar 2 (CAMHA2, Fluka) plates, and incubated 16-18 h at 37°C as replicates of 3 plates. Colonies were counted in each plate and percent survival is calculated by comparison to no drug control.



Boc-D-Leu-L-Leu-L-*threo*-*O*-[H-L-Ser(OtBu)]-HyPhe-L-Leu(OTBS)-L-Leu-D-Arg(Boc)$_2$-L-Ile-L-*a*Thr(OTBS)-Gly-O(2-Cl-Trt) (7) (0.1 mmol) 2-Chlorotrityl resin was swollen in dry THF for 1 h in a reaction vessel purged with argon. A solution of Alloc-Ser(OtBu)-OH (246 mg, 1 mmol), DIC (139 mg, 1.1 mmol), and

DMAP (12.2 mg, 0.1 mmol) in dry THF (2.2 mL, 0.45 M) was added to the resin in a sealed vial under inert atmosphere. The suspension was agitated (no agitation gave very low yields) with a magnetic stirrer at 37˚C in an oil bath for 24 h. The resin was filtered and washed with THF (5 x 4 mL) then DMF (4 x 5 mL).

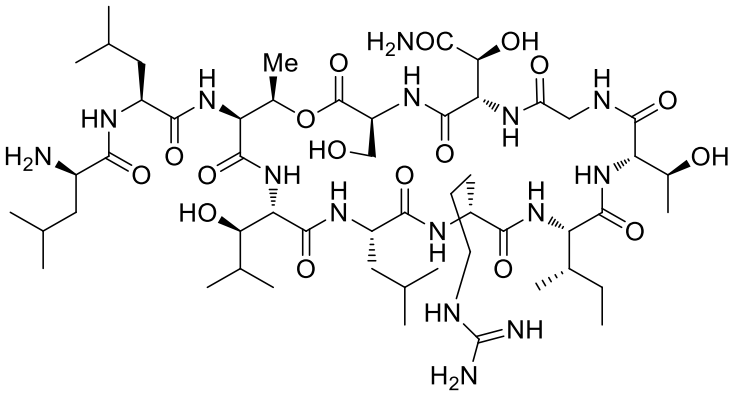
For Alloc deprotection, the resin was swollen in dry CH_2Cl_2 for 1 h in a reaction vessel purged with argon. A solution of $Pd(PPh_3)_4$ (11.6 mg, 0.01 mmol) and $PhSiH_3$ (296 μL, 2.4 mmol) in dry CH_2Cl_2 (3 mL, 0.033 M) was added to resin in inert atmosphere. The resin was agitated for 10 min (significant loss of yield was shown with extended reaction times). The resin was filtered and washed with CH_2Cl_2 (5 x 4 mL) then DMF (5 x 4 mL).



Lysobactin Bis-trifluoroacetate Salt (10). After coupling of Fmoc-β-HyAsn(CONHTrt)-OH to peptide **7** by the described standard protocol, the resin bound peptide was treated with 5% piperidine/DMF for 10 minutes. The resin was filtered and washed with DMF (5 x 4 mL).

After cleavage from the resin by the described standard protocol (155.7 mg, 0.073 mmol, 73%), the residue was treated with DEPBT (131 mg, 0.438 mmol), and DIEA (45 μL, 0.292 mmol) were dissolved in DMF (73 mL) at 0˚C. The reaction was stirred for 3 days at room temperature. The solvent was removed and the product was diluted with ethyl acetate (5 mL) and washed with 1 M aq. HCl (10 mL x 2), saturated aq. $NaHCO_3$ (10 mL x 2), and brine (10 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was sufficiently pure to carry on to the next step.

The yellow product residue was dissolved in a mixture of TFA/H_2O (95:5, 40 mL) and stirred at room temperature for 4 h. The reaction was concentrated *in vacuo*. The residue was purified by reverse-phase HPLC with 10-90% acetonitrile in H_2O. Lyophilization of the pure fractions provided lysobactin bis-trifluoroacetate salt (10.7 mg, 8.4% yield over entire synthesis) as a white solid. $[\alpha]^{20}_{D}$ -68.9˚ (c 0.07, MeOH); HRMS-ESI-TOF m/z calcd for $C_{58}H_{97}O_{17}N_{15}$ ($[M+H]^+$): 1276.7259, Found 1276.7297; HPLC: t_R = 22.6 min (10-90% MeCN/$H_2$0 over 1 h). ^{1}H NMR (50:50 D_2O/CD_3CN, 500 MHz) δ 7.74 (d, *J* = 8.5 Hz, 1 H), 7.40 (s, 2 H), 7.30 (m, 5 H), 6.99 (d, *J* = 5.8 Hz, 1 H), 6.94 (d, *J* = 9.6 Hz, 1 H), 6.13 (d, *J* = 10.4 Hz, 1 H), 5.69 (d, *J* = 10.4 Hz, 1 H), 4.84 (d, *J* = 2.0 Hz, 1 H), 4.60 (t, *J* = 5.9 Hz, 1 H), 4.49 (d, *J* = 2.0 Hz, 1 H), 4.33 (t, *J* = 9.8 Hz, 2 H, some overlap with H_2O peak), 4.24-4.18 (m, 1 H), 4.18-4.13 (m, 1 H), 4.11 (s, 1 H), 3.97-3.76 (m, 6 H), 3.75-3.67 (m, 2 H), 3.55-3.45 (m, 2 H), 2.94-2.86 (m, 1 H), 2.74-2.66 (m, 1 H), 1.90-1.73 (m, 5 H), 1.73-1.26 (m, 15 H), 1.23 (d, *J* = 6.3 Hz, 5 H), 1.20-1.10 (m, 1 H), 1.09-0.99 (m, 1 H), 0.98-0.72 (m, 39 H); ^{13}C NMR (50:50 D_2O/CD_3CN, 125 MHz) δ 176.4, 175.4, 174.2, 173.9, 173.8, 172.9, 172.4, 172.0, 171.7, 170.5, 169.9, 168.8, 162.0 (TFA salt), 156.4, 134.6, 129.9, 129.0 (2 C), 127.6 (2 C), 75.4, 74.8, 70.6, 70.0, 61.4, 60.7, 59.9, 59.3, 57.6, 56.0, 55.7, 55.4, 54.9, 52.4, 51.9, 43.1, 41.0, 40.5, 40.3, 39.0, 35.9, 30.5, 27.9, 25.9, 25.6, 24.4, 24.3, 24.1, 23.1, 23.0, 21.6, 21.3, 20.0, 19.7, 19.6, 18.7, 18.5, 15.1, 10.2



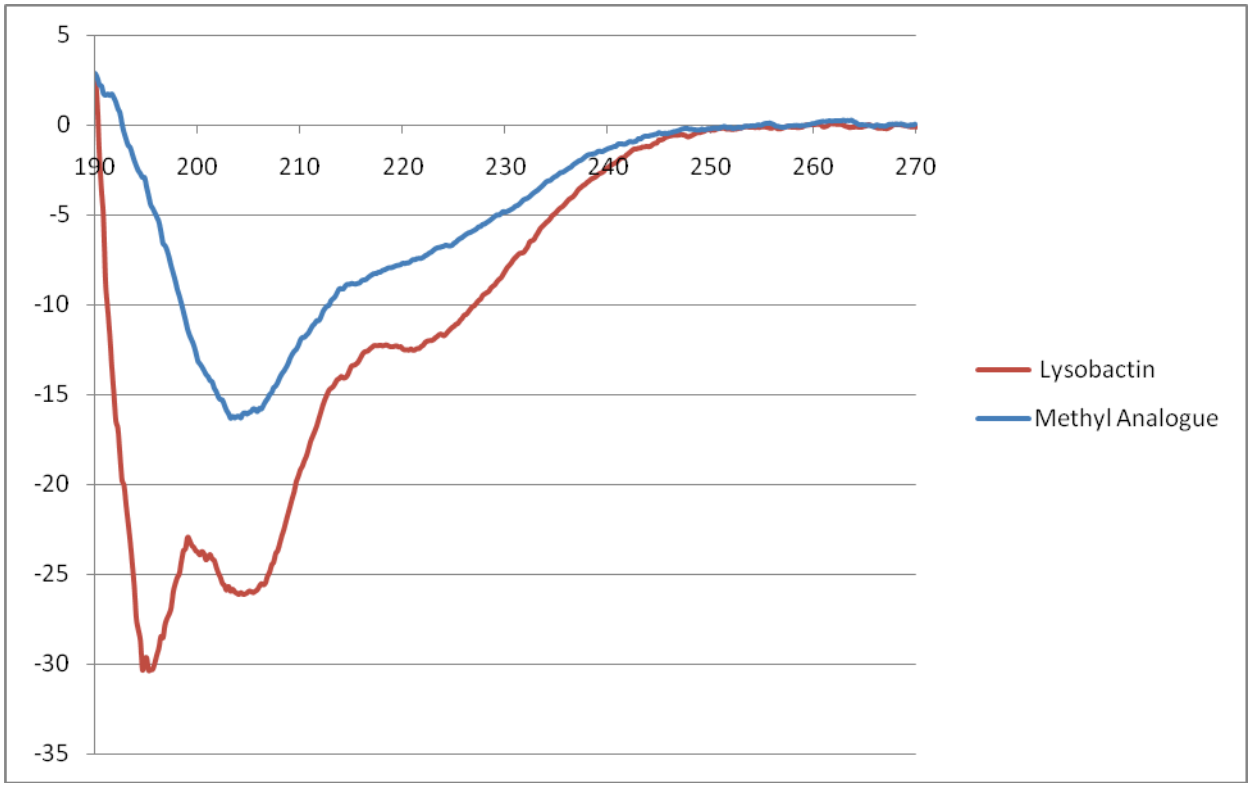
Δ3-Thr-lysobactin Bis-trifluroacetate salt (11). The solid phase synthesis for Δ3-Thr-lysobactin was carried out in an identical manner to that of the natural product with the exception of using Fmoc-Thr-OH (5 equiv) in place of L-threo-phenylserine and reacting with DEPBT (5 equiv) and DIPEA (10 equiv) in DMF (0.5 M) for 2 h.

The peptide residue (260 mg, 0.119 mmol), DEPBT (214 mg, 0.714 mmol), and DIEA (74 μL, 0.476 mmol) were dissolved in DMF (119 mL) at 0˚C. The reaction was stirred for 3 days at room temperature. The solvent was

removed and the product was diluted with ethyl acetate (5 mL) and washed with 1 M aq. HCl (10 mL x 2), saturated aq. $NaHCO_3$ (10 mL x 2), and brine (10 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was sufficiently pure to carry on to the next step.

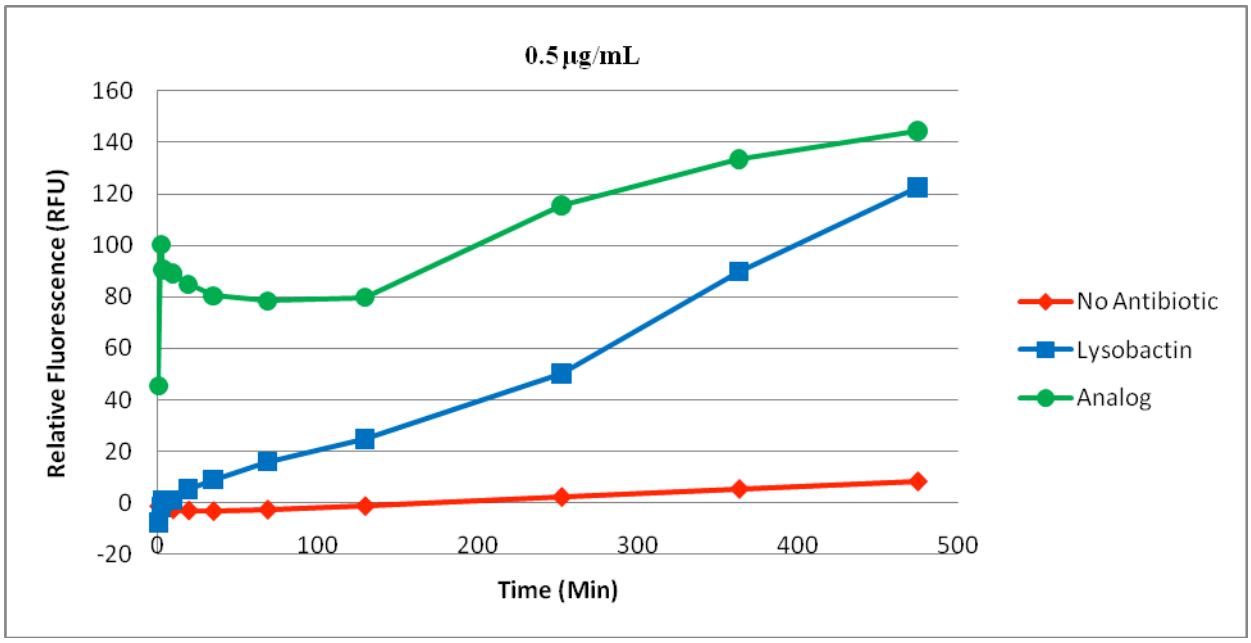
The yellow product residue was dissolved in a mixture of TFA/H_2O (95:5, 40 mL) and stirred at room temperature for 4 h. The reaction was concentrated *in vacuo*. The residue was purified by reverse-phase HPLC with 10-90% acetonitrile in H_2O over 1 h. Lyophilization of the pure fractions provided Δ3-Thr-lysobactin Bis-trifluroacetate salt (22.6 mg, 5.2% yield over entire synthesis) as a white solid. $[\alpha]^{20}_D$ -42.5° (c 0.32, 50:50 ACN/$H_2$0); HRMS-ESI-TOF m/z calcd for $C_{53}H_{95}O_{17}N_{15}$ ($[M+H]^+$): 1214.7108, Found 1214.7069; HPLC: t_R = 6.94 min (30-90% MeCN/$H_2$0 over 30 min). ^{1}H NMR (50:50 D_2O/CD_3CN, 500 MHz) δ 5.36-5.24 (m, 1 H), 5.18 (d, *J* = 9.27 Hz, 1 H), 4.83 (s, 1 H), 4.64 (t, *J* = 4.89 Hz, 1 H), 4.53 (s, 1 H), 4.35 (m, 1 H), 4.26-4.20 (m, 4 H, some overlap with H_2O), 4.10-4.04 (m, 2 H), 3.99 (s, 1 H), 3.95 (s, 1 H), 3.92 (m, 1 H), 3.89 (m, 1 H), 3.87 (m, 1 H), 3.85 (m, 1 H), 3.78 (d, *J* = 4.81 Hz, 1 H), 3.76 (d, 4.88 Hz, 1 H), 3.70 (d, *J* = 4.99, 1 H), 3.67 (s, 1 H), 3.64 (s, 1 H), 3.55 (dd, *J* = 3.10, 8.50 Hz, 1 H), 3.51-3.42 (m, 1 H), 3.12 (t, *J* = 7.13 Hz, 2 H), 3.07 (t, *J* = 6.93 Hz, 1 H), 1.95-1.92 (m, 1 H), 1.84-1.43 (m, 23 H), 1.42-1.29 (m, 2 H), 1.29-1.17 (m, 3 H), 1.14 (d, *J* = 6.19 Hz, 6 H), 1.12-1.08 (m, 1 H), 1.08-0.96 (m, 2 H), 0.96-0.83 (m, 23 H), 0.83-0.79 (m, 7 H), 0.79-0.70 (m, 16 H); ^{13}C NMR (50:50 D_2O/CD_3CN, 125 MHz) δ 175.28, 175.00, 173.61, 173.32, 173.11, 172.26, 172.00, 171.53, 171.21, 170.61, 169.79, 169.55, 156.41, 135.64, 124.72, 124.02, 115.21, 111.77, 74.54, 70.09, 69.71, 69.12, 61.07, 59.37, 58.24, 57.29, 55.53, 55.03, 53.98, 52.10, 51.54, 42.50, 40.44, 40.34, 40.20, 40.03, 39.49, 38.75, 35.57, 30.18, 27.56, 25.19, 25.03, 24.21, 24.06, 23.81, 22.55, 22.43, 22.05, 21.28, 20.79, 20.18, 19.56, 19.42, 18.04, 17.61, 16.06, 14.58, 9.83

CD Spectra (Figure 1)

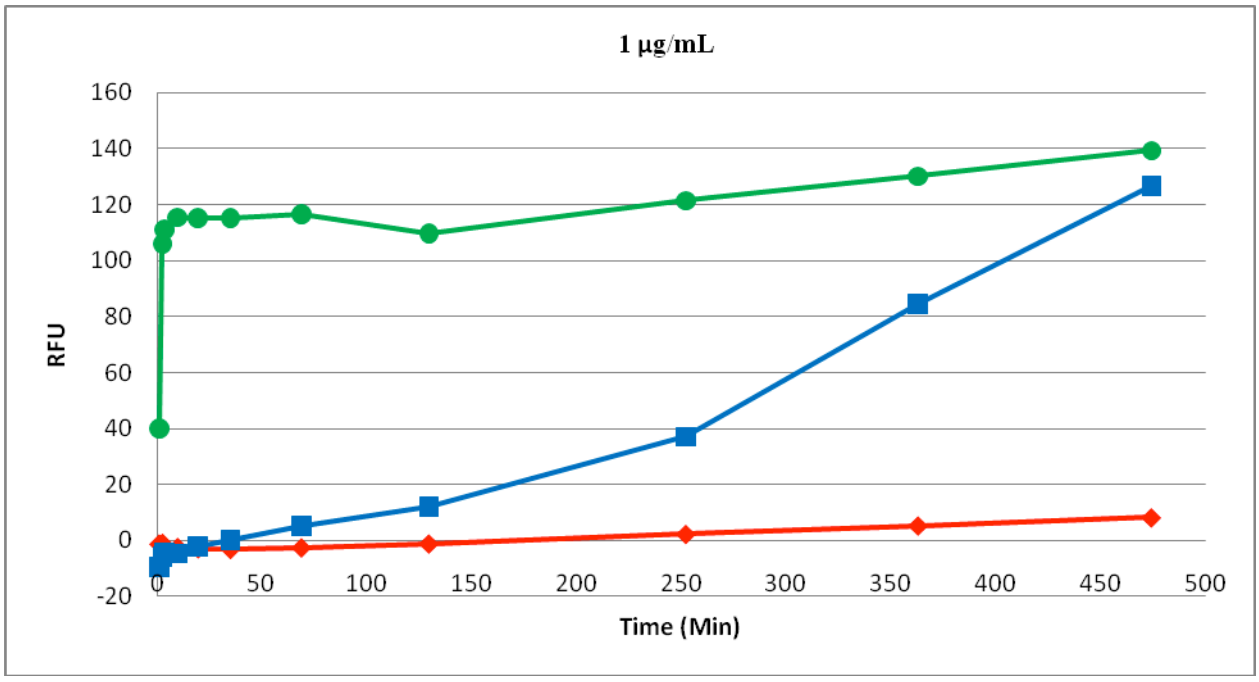


***B. subtilis* membrane disruption data at concentrations denoted in the graphs (Figure 2)**

2a



2b



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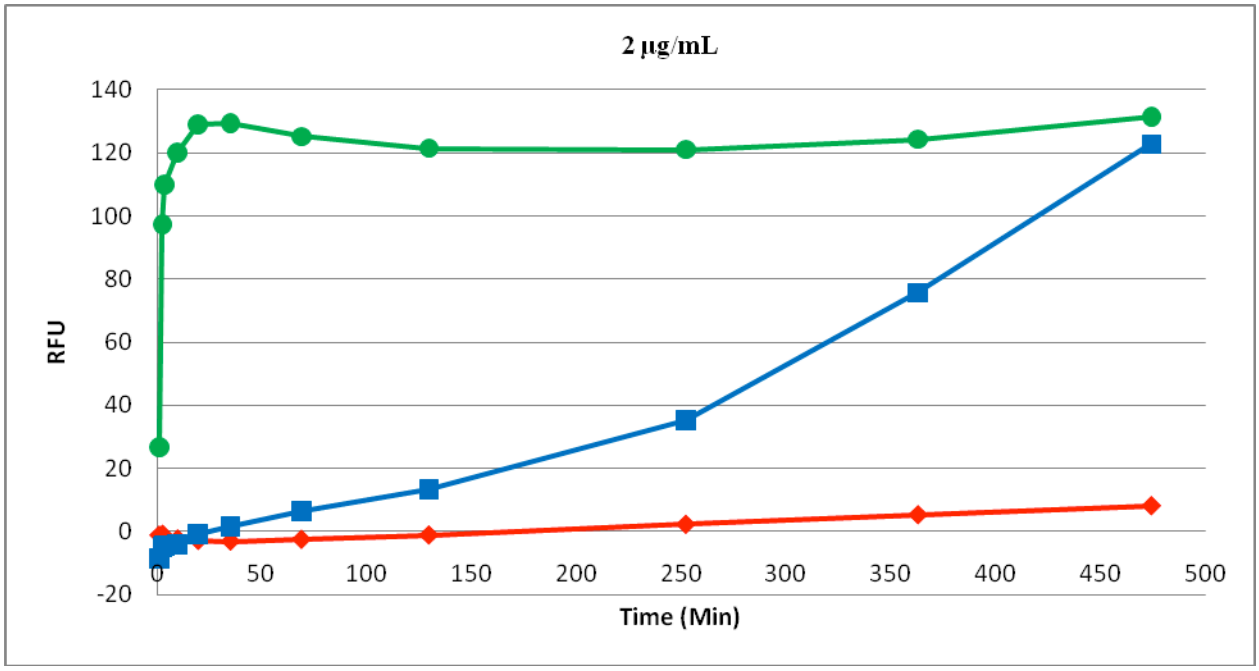
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RFU
Time (Min)
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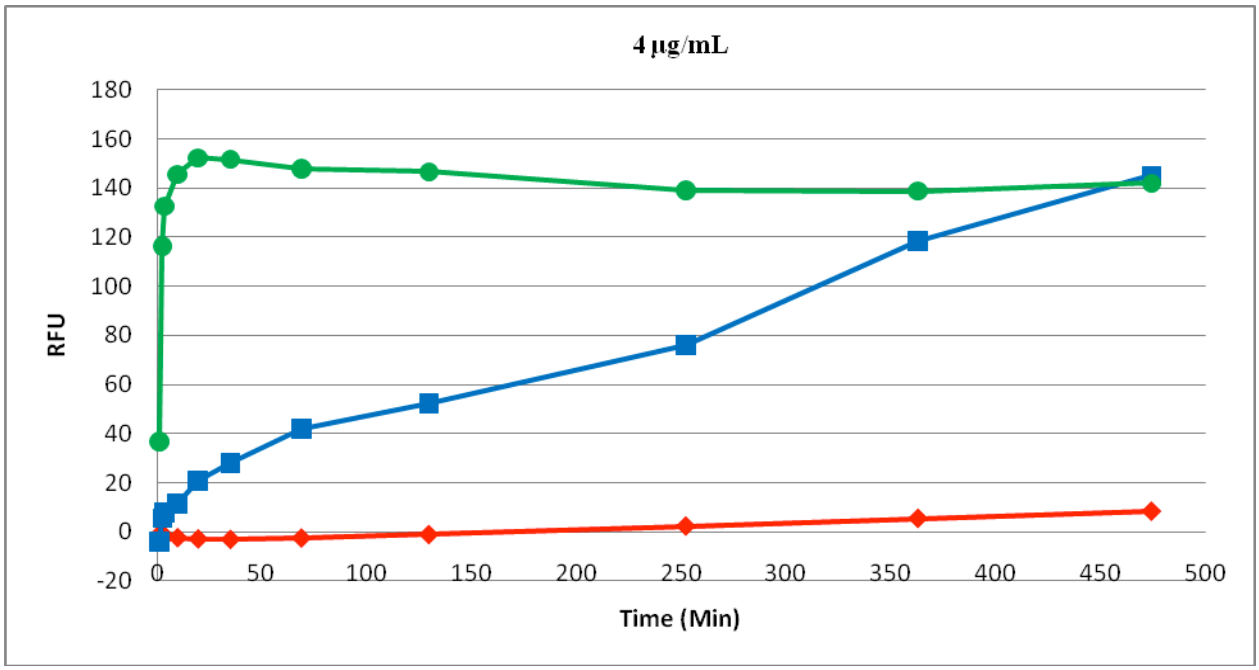
120
100
80
60
40
20
0
-20
0
50
100
150
200
250
300
350
400
450
500

2d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RFU
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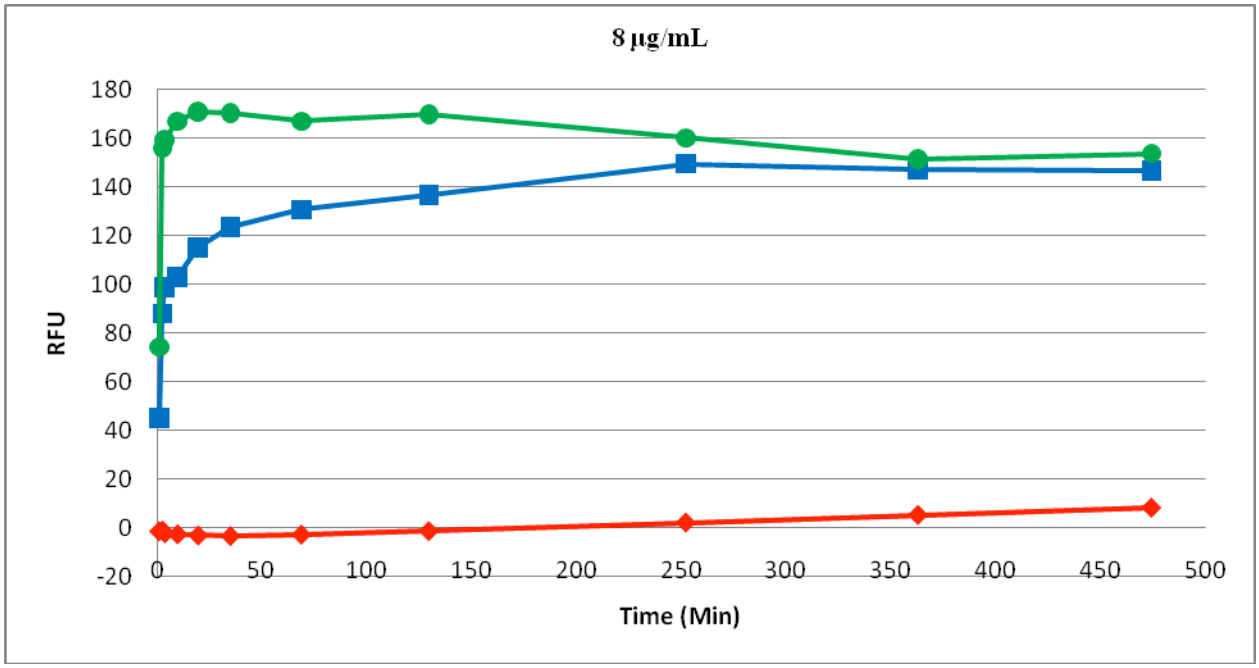
150
200
250
300
350
400
450
500

2e


8 µg/mL
RFU
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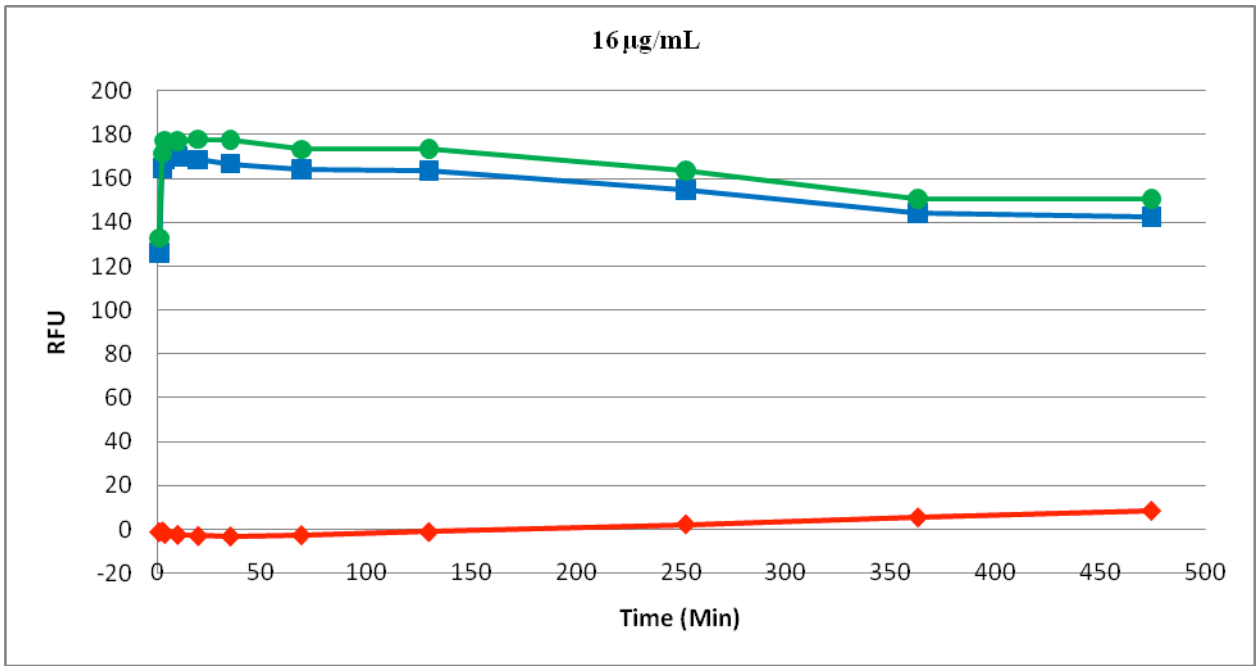
160
140
120
100
80
60
40
20
0
-20
0
50
100
150
200
250
300
350
400
450
500
Time (Min)

2f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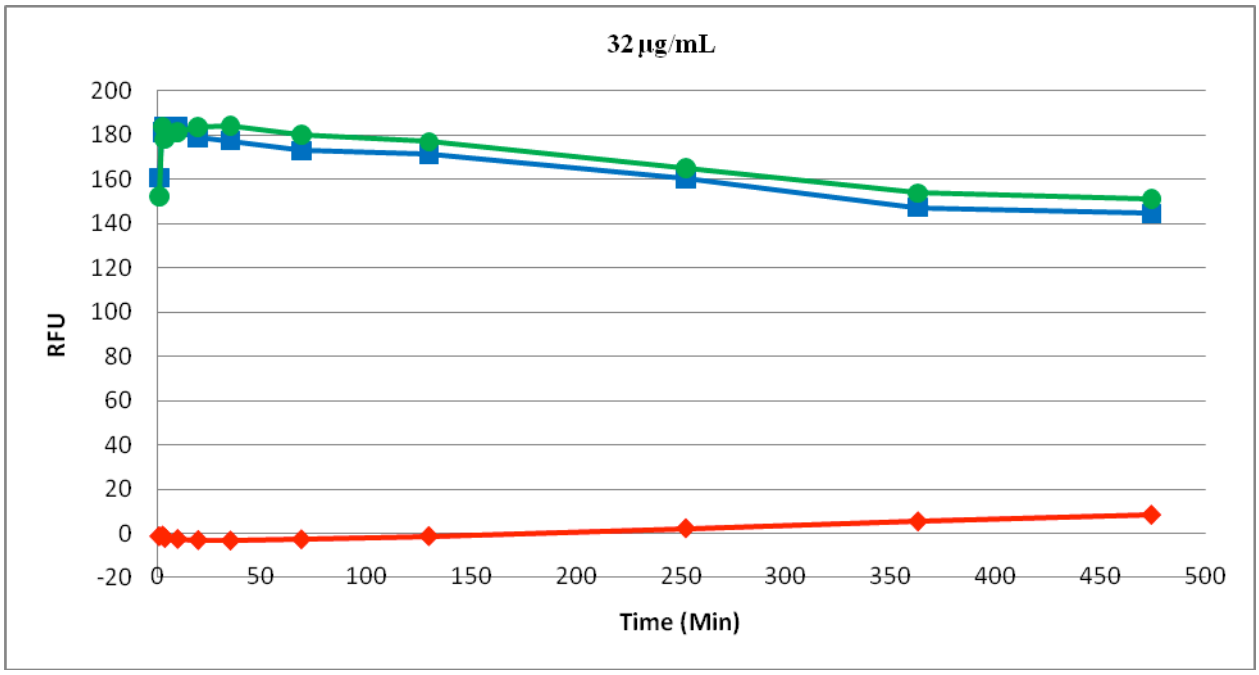
150
200
250
300
350
400
450
500
Time (Min)

2g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RFU
Time (Min)
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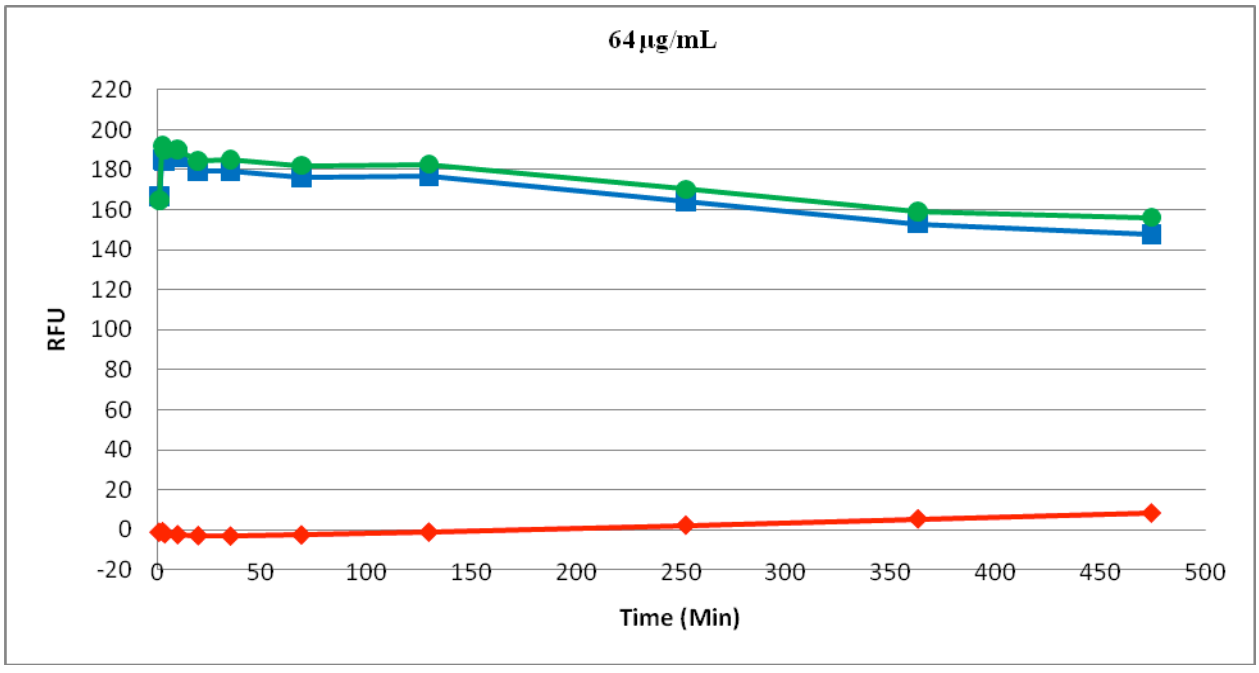
400
450
500

2h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RFU
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100
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60
40
20
0
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150
200
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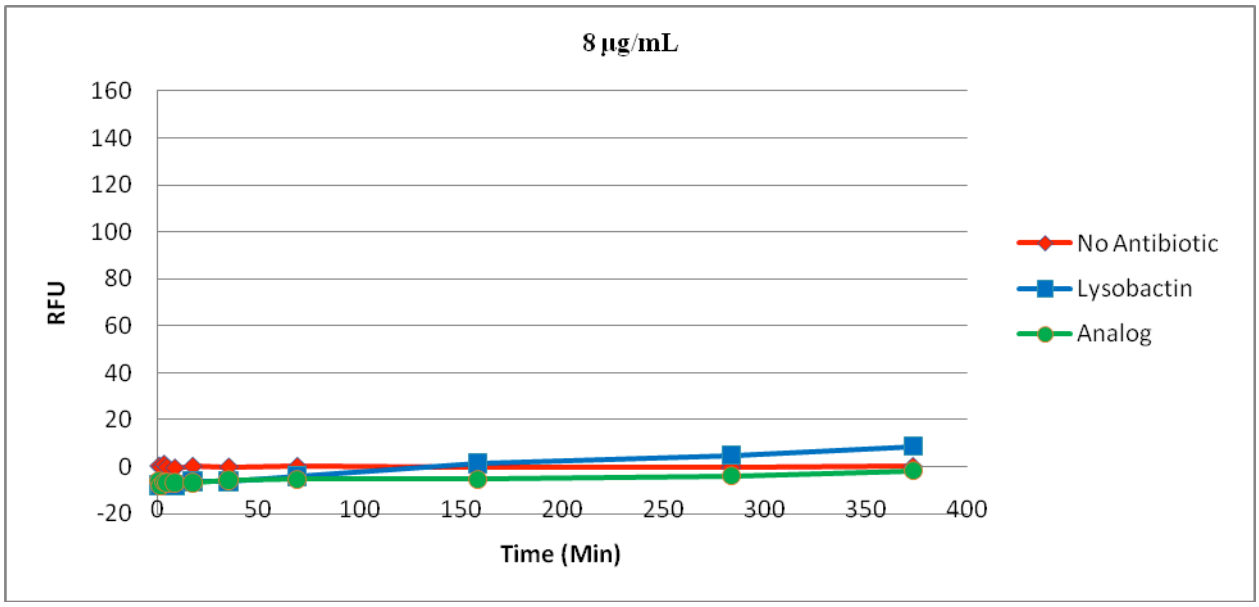
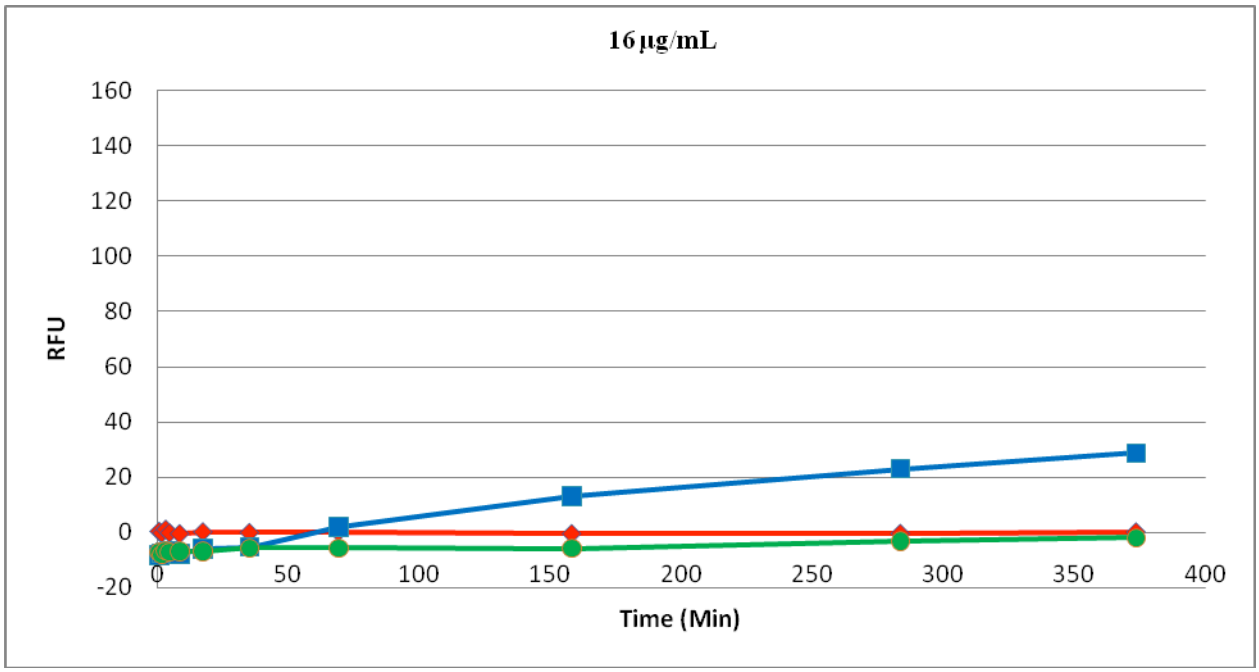
300
350
400
450
500

E. Coli Membrane Disruption at concentrations denoted in the graphs (Figure 3)

3a



3b



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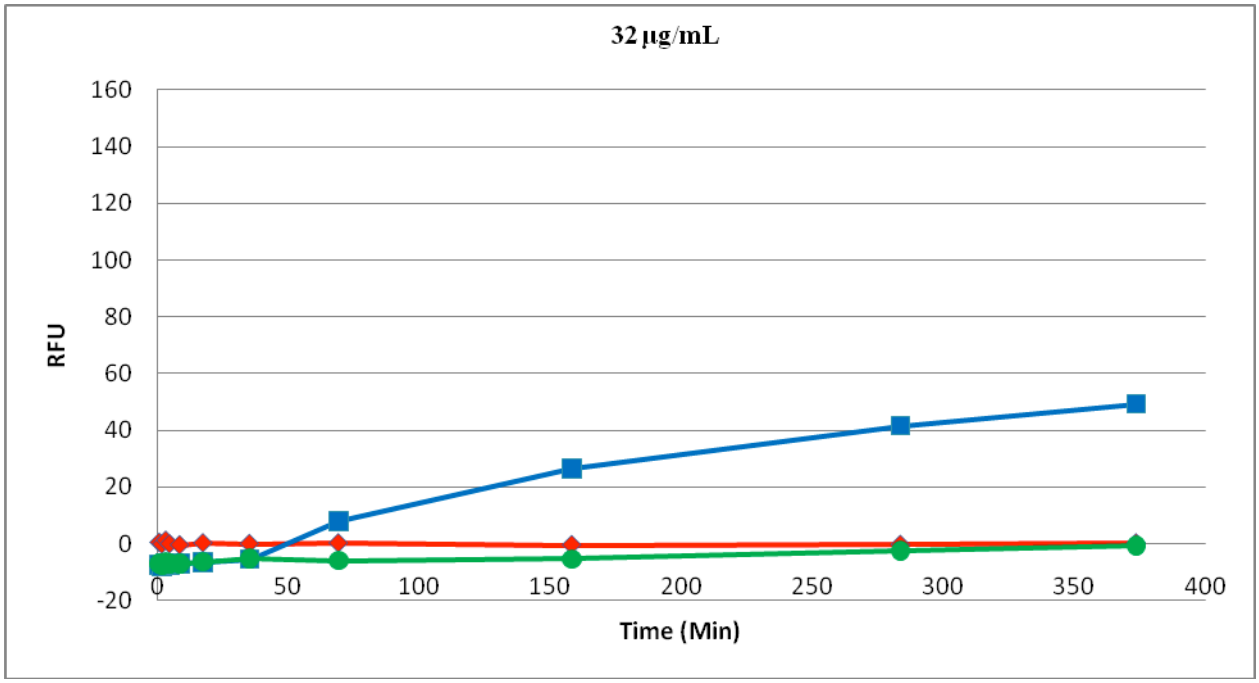
32 µg/mL
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140
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100
80
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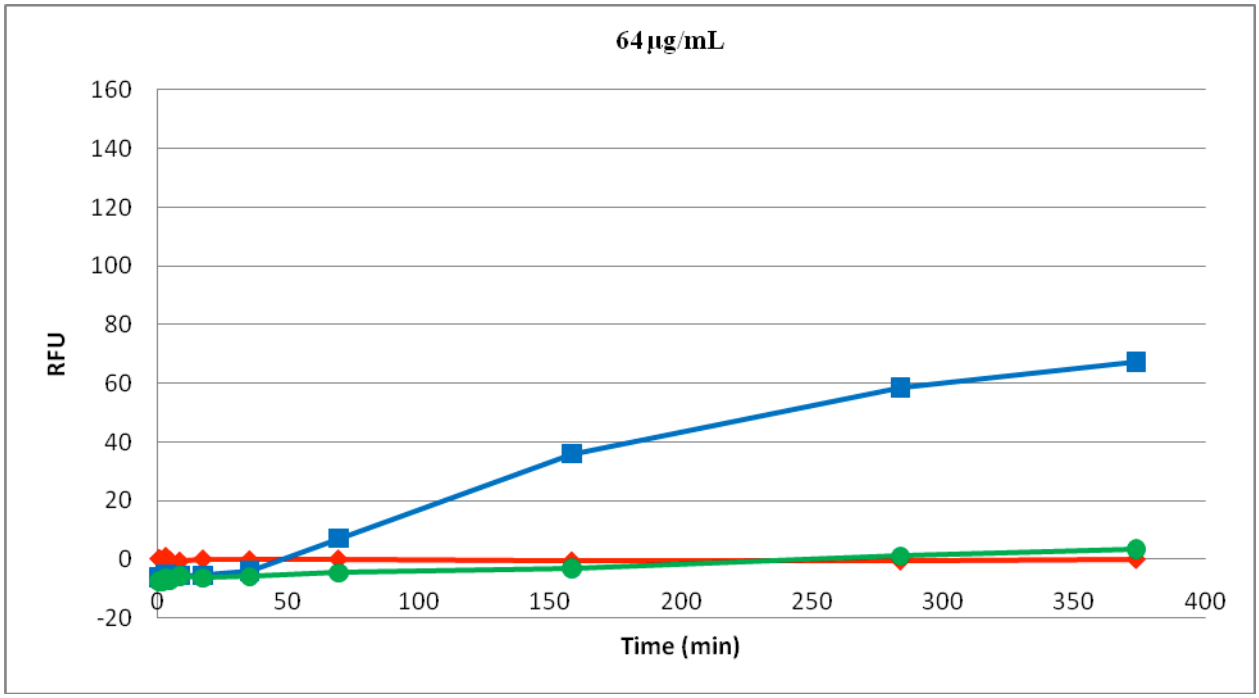
-20
RFU
0
50
100
150
200
250
300
350
400
Time (Min)

3d


64 µg/mL
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140
120
100
80
60
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20
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RFU
0
50
100
150
200
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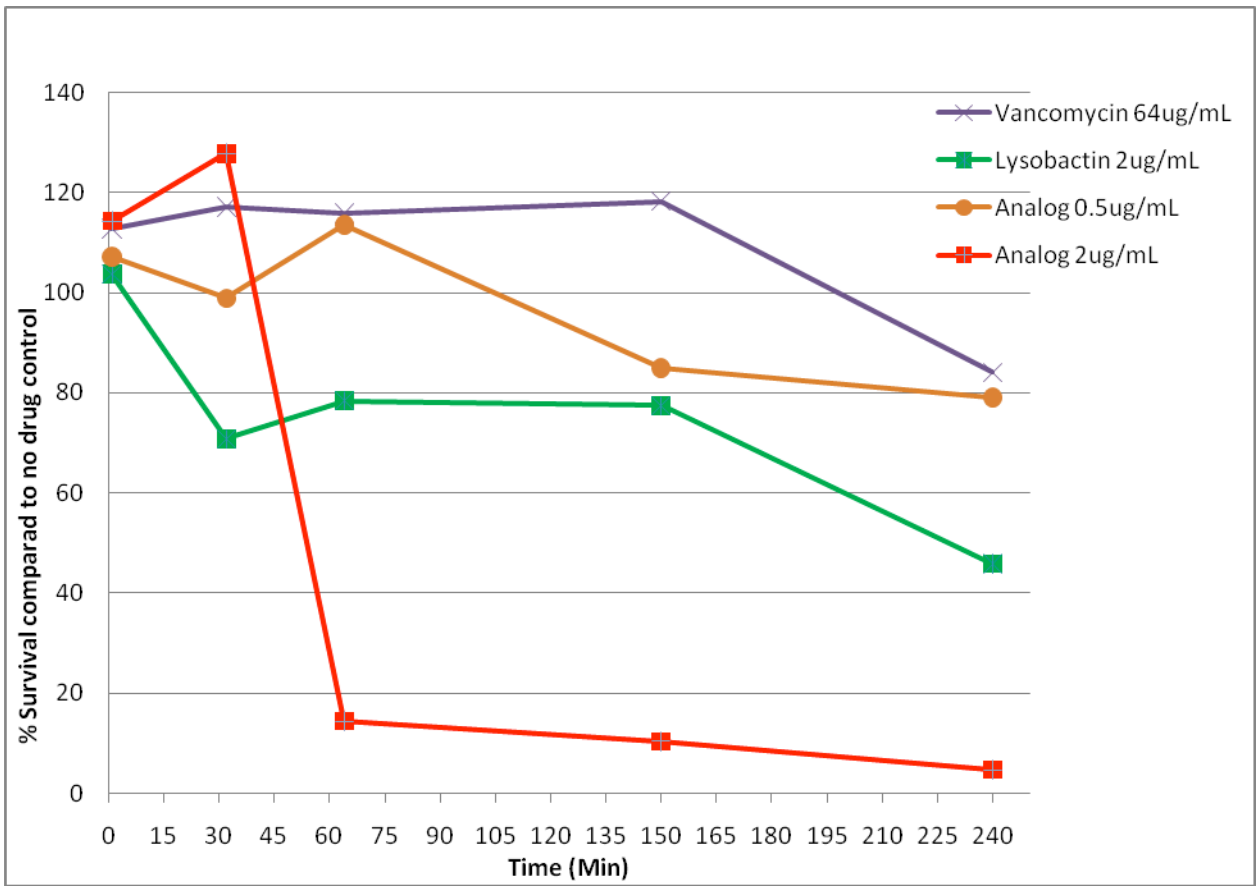
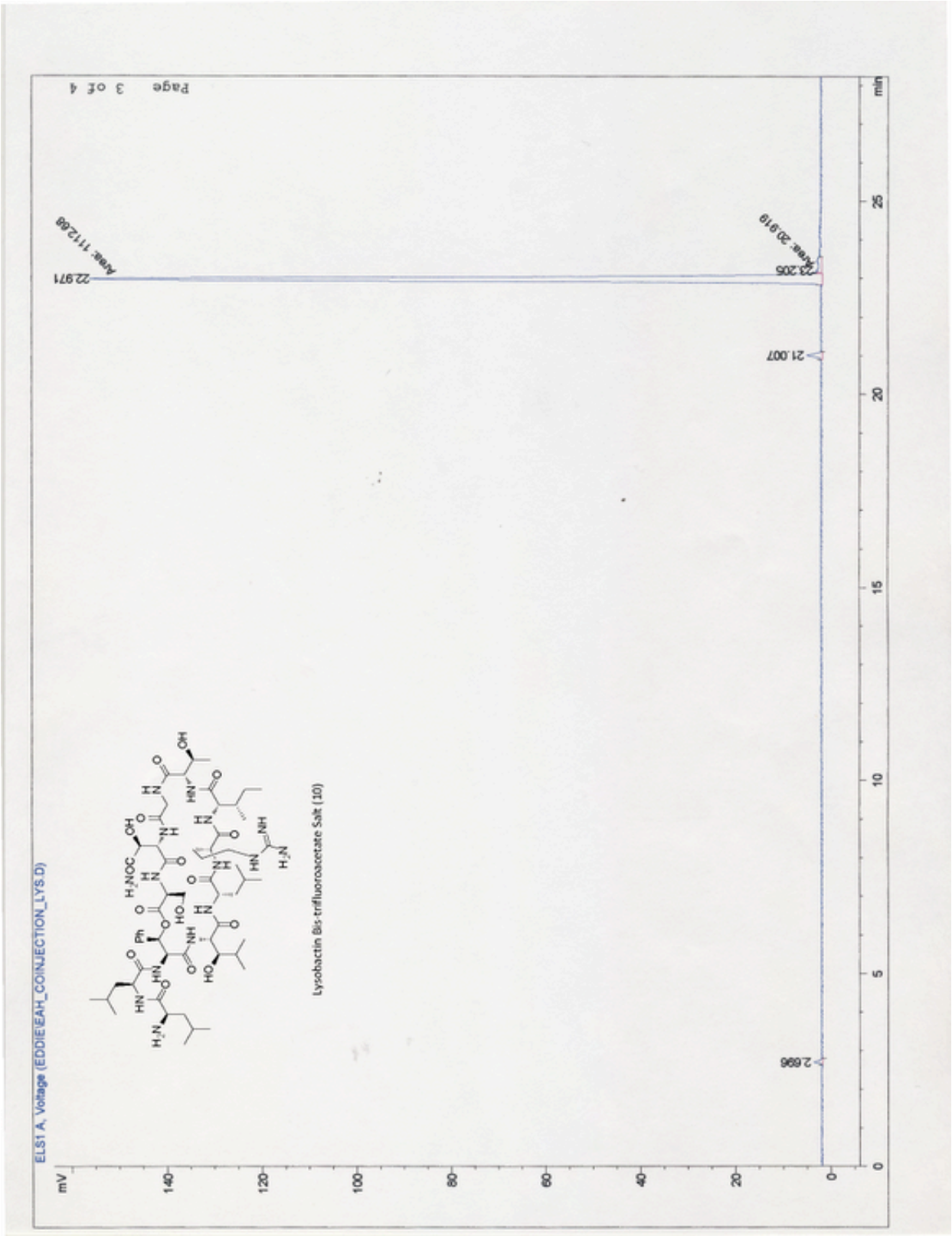
300
350
400
Time (min)

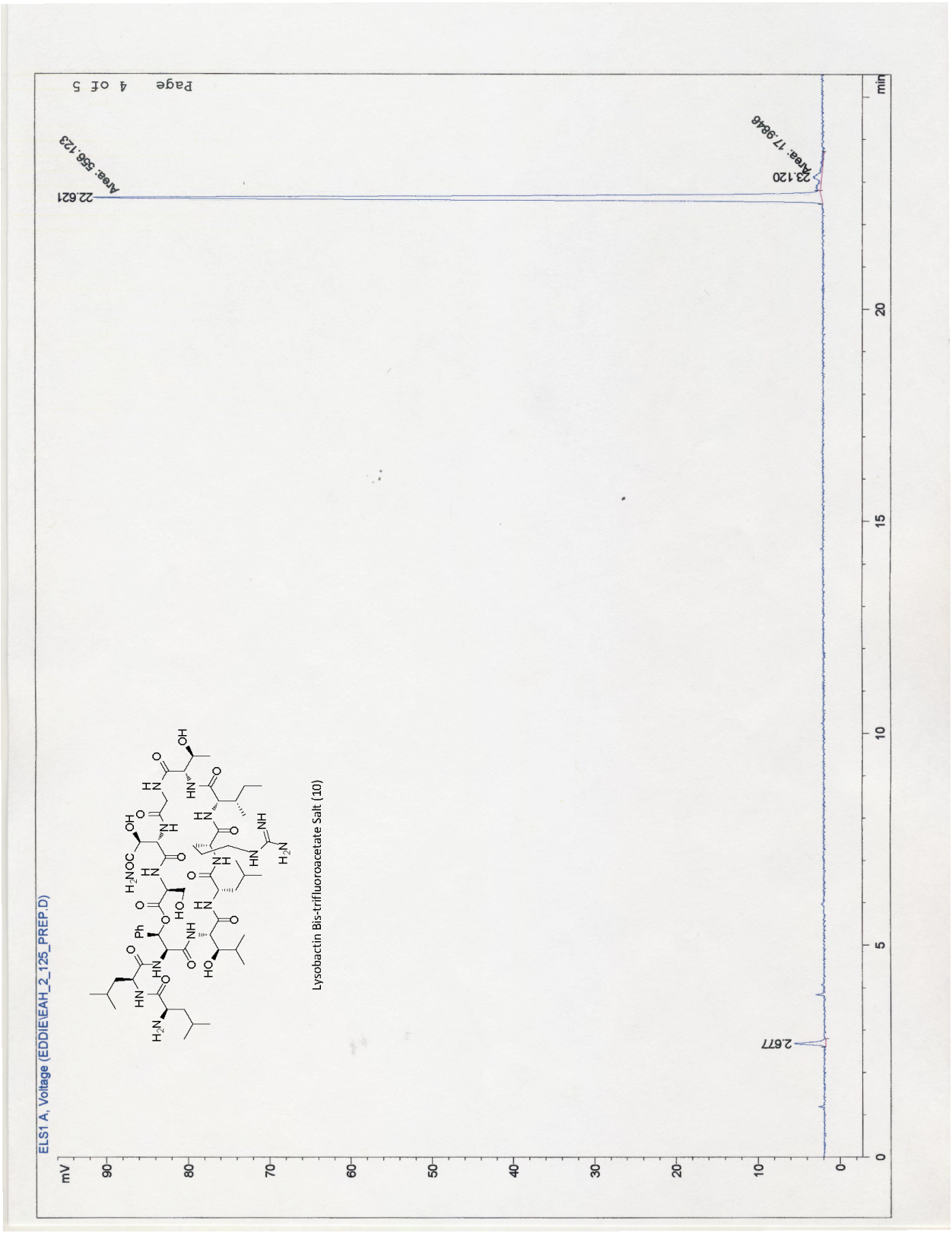
Killing Kinetics Assay (Figure 4)



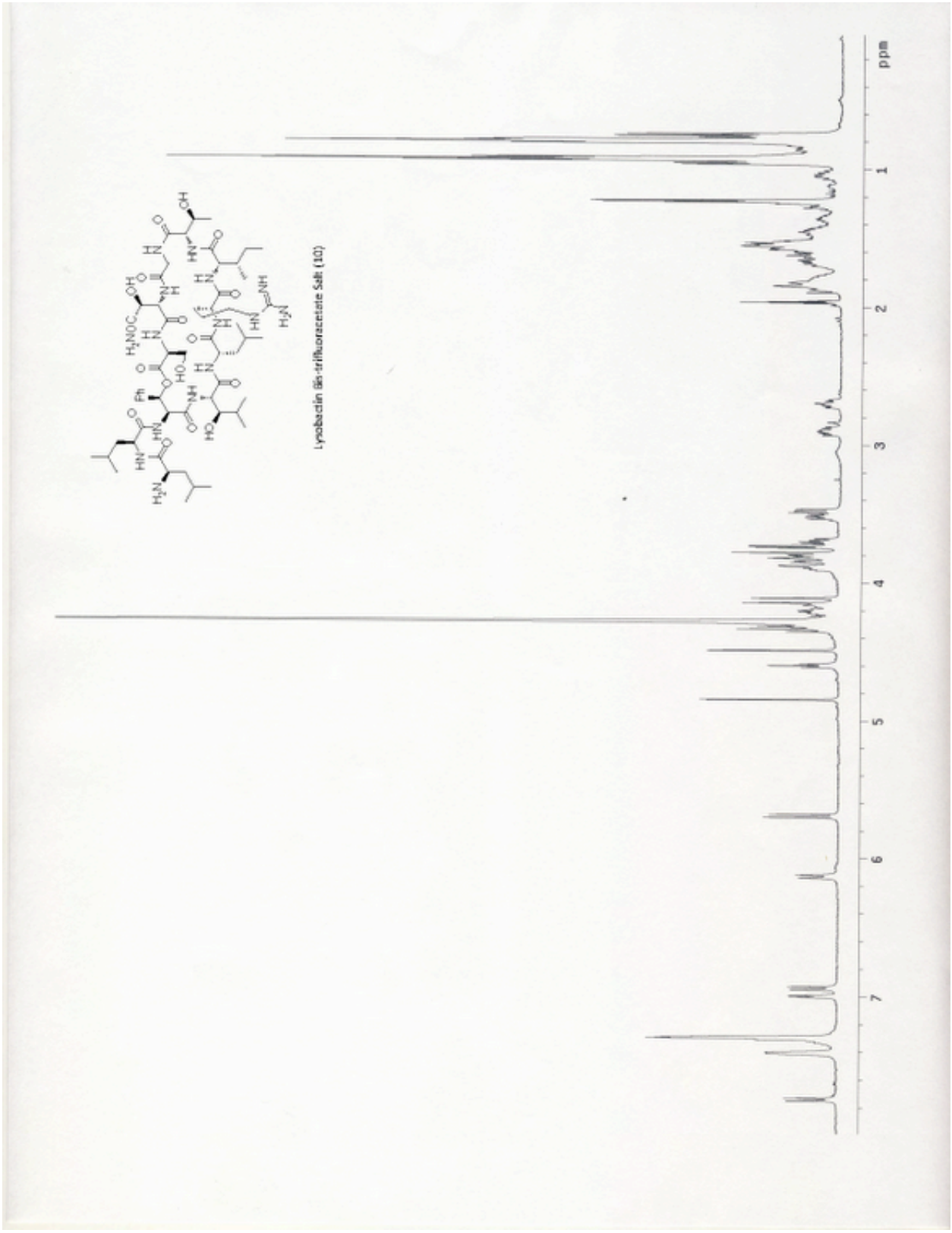
Synthetic and authentic lysobactin HPLC coinjection: 10-90% ACN/H_2O over 1 h



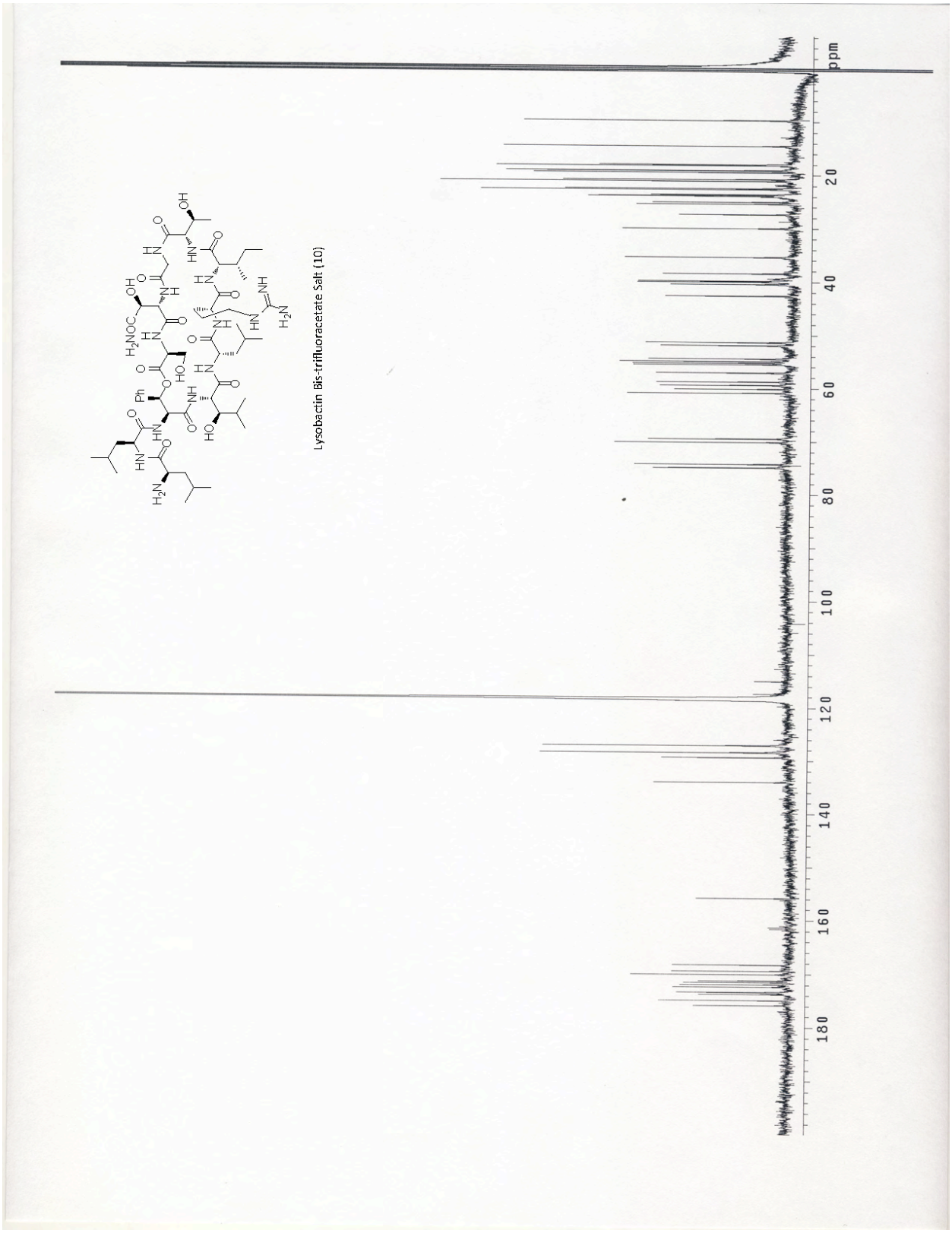
Synthetic lysobactin HPLC: 10-90% ACN/H_2O over 1 h



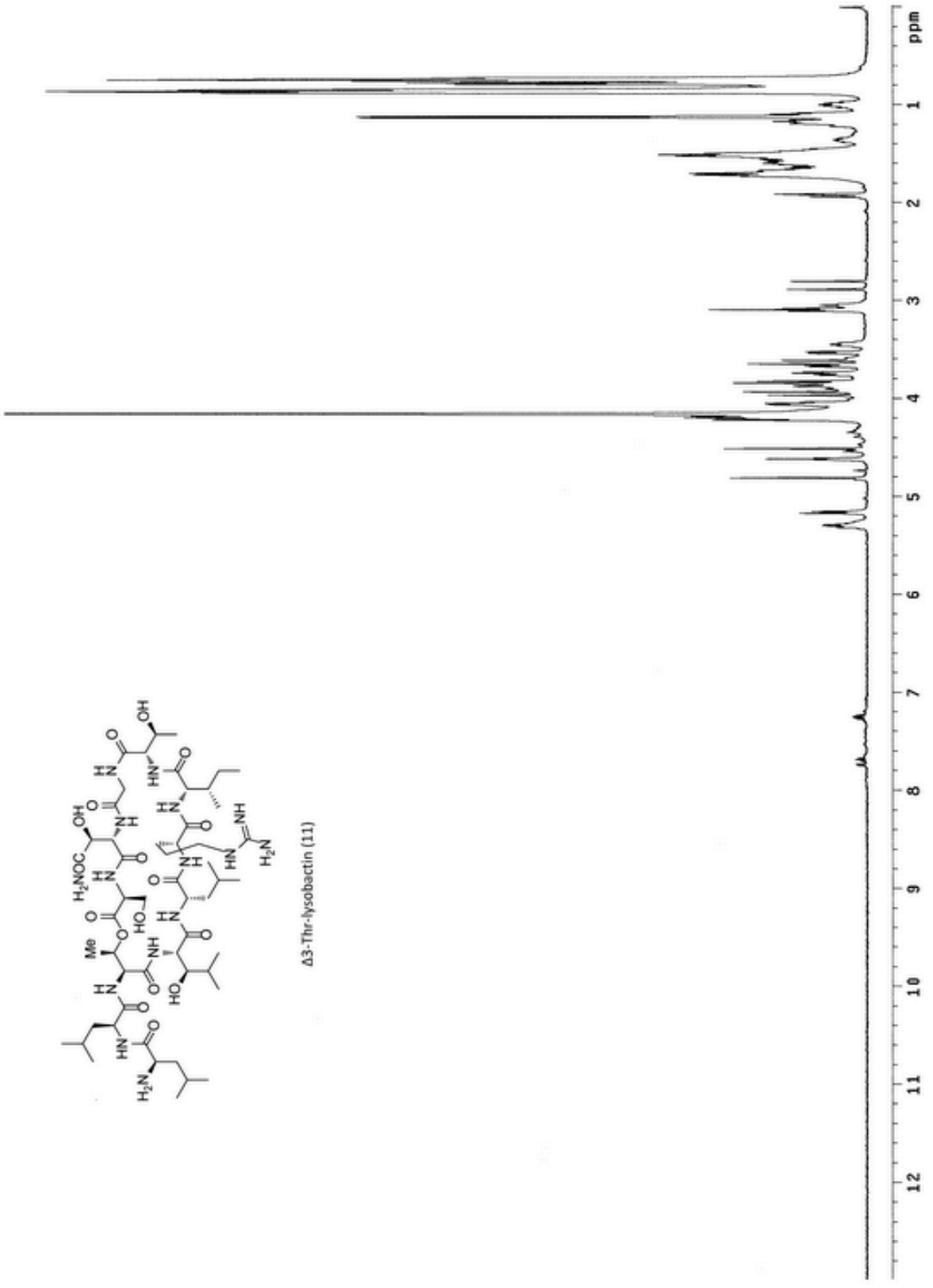
Lysobactin ^{1}H NMR: 50/50 CD_3CN/D_2O



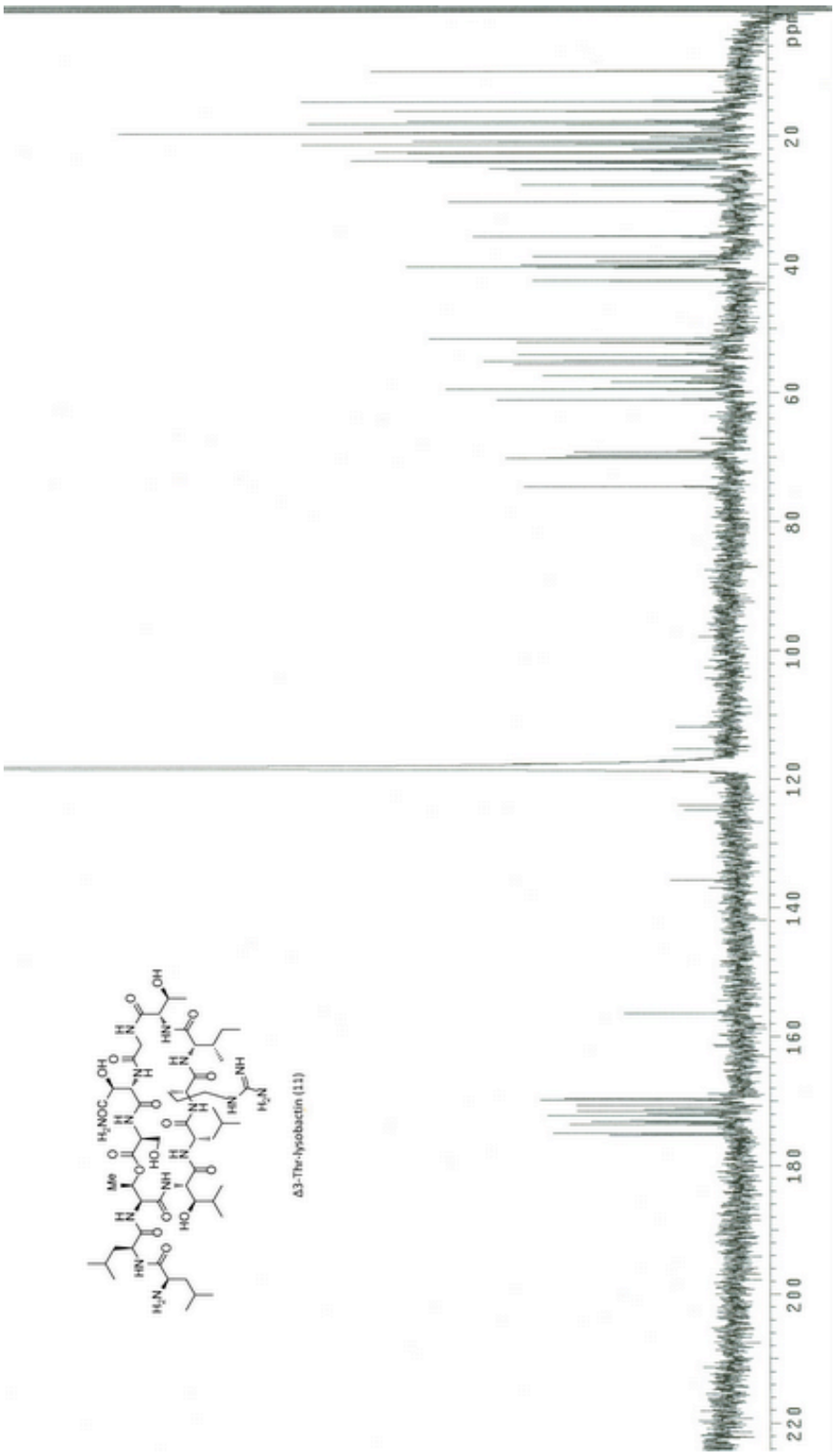
Lysobactin ^{13}C NMR: 50/50 CD_3CN/D_2O



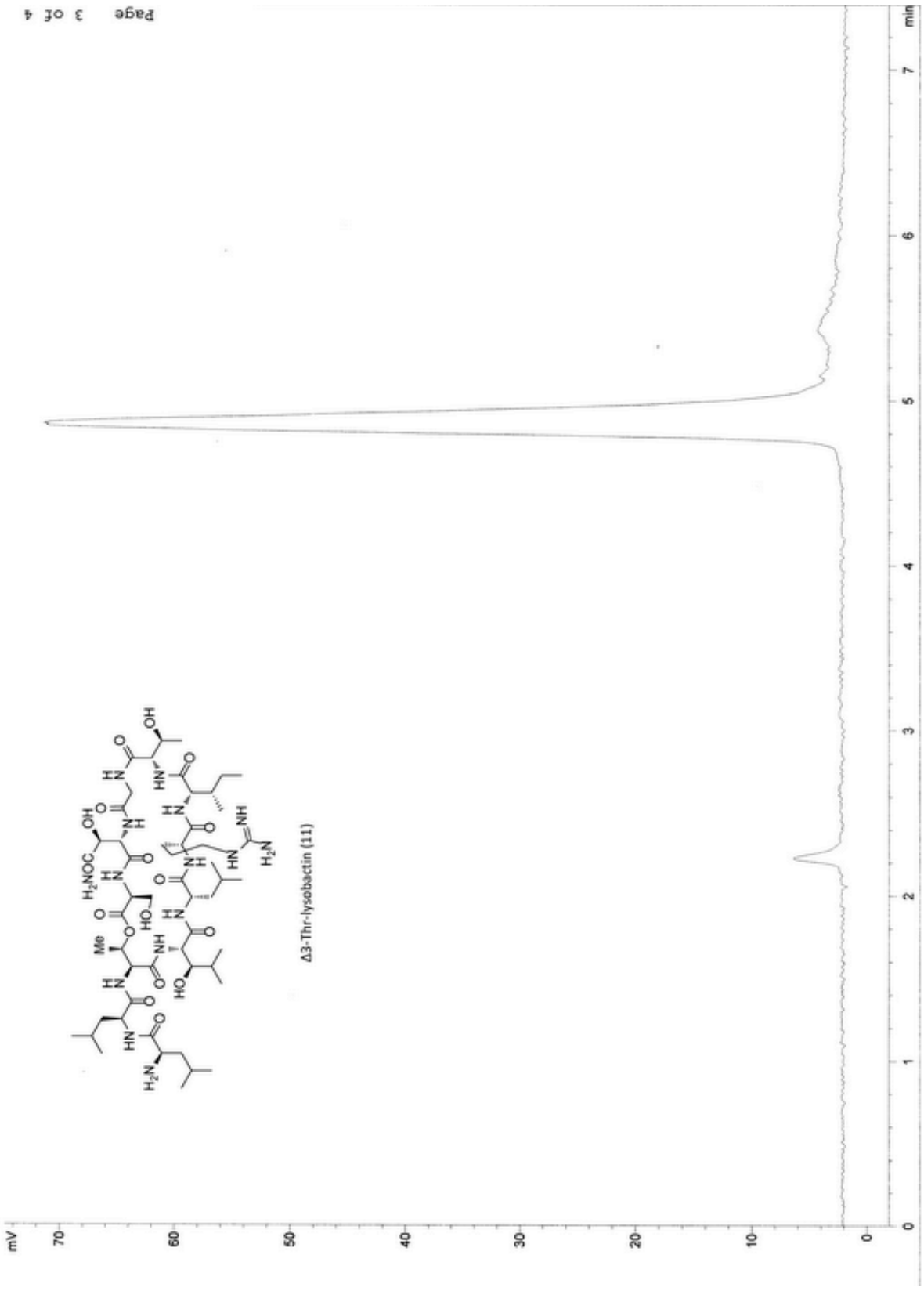
Δ3-Thr-lysobactin ^{1}H NMR: 50/50 CD_3CN/D_2O



Δ3-Thr-lysobactin ^{13}C NMR: 50/50 CD_3CN/D_2O



Δ3-Thr-lysobactin HPLC trace: 35% ACN/H_2O isocratic



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