Electronic Supplementary Material (ESI) for Chemical Communications.

A route to diastereomerically pure phenylglycine thioester peptides: crucial intermediates for investigating glycopeptide antibiotic biosynthesis

Julien Tailhades,[a,b] Melanie Schoppet,[a,b,c] Anja Greule,[a,b] Madeleine Peschke,[c] Clara Brieke,[c] and Max J. Cryle[† a,b,c]

[a] *EMBL Australia, Monash University, Clayton, Victoria 3800, Australia.*

[b] *The Monash Biomedicine Discovery Institute, Department of Biochemistry and Molecular Biology and ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Victoria 3800, Australia.*

[c] *Department of Biomolecular Mechanisms, Max Planck Institute for Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany.*

SUPPLEMENTARY INFORMATION

Abbreviations

ACN: acetonitrile

CoA: Coenzyme A

COMU: (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate

DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene

DMF: dimethylformamide

DCM: dichloromethane

ESI: Electrospray ionization

Et_2O: diethyl ether

HCl: hydrochloric acid

LC/MS: liquid chromatography–mass spectrometry

MeOH: methanol

NADH: Nicotinamide adenine dinucleotide

$NaNO_2$: sodium nitrite

NMR: nuclear magnetic resonance spectroscopy

PCP-X_{tei}: peptidyl carrier protein with the X domain issued from teicoplanin

RP-HPLC: reversed-phase high performance liquid chromatography

Sfp R4-4: synthase 4´-phosphopantetheinyl transferase

TFA: trifluoroacetic acid

TIS: triisopropylsilane

Trt: Trityl

SI1. Material and methods

SI1. 1. Materials

Solid phase peptide synthesis: Tribute synthesizer (Protein Technologies), 2-Chlorotrityl chloride resin (1 mmol/g, Merck), DCM (Chem-supply), hydrazine monohydrate 64-65% (Sigma-Aldrich), MeOH (Scharlau), DIEA (Sigma-Aldrich), Fmoc-L/D-3-chlorotyrosine (Merck), COMU (Merck), TEA (Merck), Ac_2O (Sigma-Aldrich), 2,6-lutidine (Sigma-Aldrich), DBU (Sigma-Aldrich), DMF (Ajax Finechem), TFA (Sigma-Aldrich), TIS (Sigma-Aldrich).

Peptide-CoA synthesis from peptide hydrazides: Urea (Sigma-Aldrich), NaH_2PO_4 (Sigma-Aldrich), $NaNO_2$ (Sigma-Aldrich), CoA (Affymetrix), TCEP (Sigma-Aldrich).

Peptide-PCP turnovers:

- Commercial: HEPES (Sigma-Aldrich), NaCl (Sigma-Aldrich), $MgCl_2$ (Sigma-Aldrich), glucose (Sigma-Aldrich), glucose deshydrogenase (Sorachim), NADH (Sigma-Aldrich).
- "In-house" expressed proteins: PCP-X tei, Sfp R4-4, $OxyB_{van}$, $OxyA_{tei}$, PuR, PuxBA105V.

SI1. 2. Methods

Solid Phase Peptide Synthesis (Fmoc/tBu): Peptide chain assembly was carried out on a Protein Technologies Tribute peptide synthesizer. Fmoc removal was performed using a 1% DBU solution in DMF (v/v) with UV feedback monitoring. Amino acid coupling was performed via activation of Fmoc-amino acids (3 eq.) in the presence of COMU (3 eq.) and 2,6-lutidine (3 eq.). Protecting groups and solid phase were cleaved using a solution of TFA/ TIS/ H_2O (95/ 2.5/ 2.5, v/v/v) for 1 h at room temperature. After cleavage, the resin was removed by filtration and washed twice with TFA. The filtrate was then concentrated under a stream of nitrogen to ~ 2mL volume. The peptide products were precipitated by the addition of ice cold Et_2O and washed by centrifugation three times.

Peptide-CoA synthesis from peptide hydrazide: the peptide hydrazide was dissolved in the buffer A to reach a final concentration of 4-5mM. The temperature of the reaction was maintained between -10 ^{o}C and -15 ^{o}C using a mixture of ice and sodium chloride. The addition of a 0.5 M $NaNO_2$ in water (0.95 eq.) led to the formation of the acyl azide in 10 minutes. Subsequently, CoA (1.2 eq.) in buffer A was added dropwise followed by the

solution B until the pH of the solution was 6-5-6.8. Reaction monitoring was performed using LC/MS after 1 hour. When the reaction had reached completion, purification was achieved using by preparative RP-HPLC.

Buffer A: 6 M urea buffer containing 0.2 M NaH_2PO_4; a solution of 1 M HCl was added until pH 3 was reached.
Buffer B: 1 M potassium phosphate buffer at pH 8.0; obtained by mixing 1M solutions of the monobasic and dibasic buffer in the appropriate ratio.

Preparation of peptidyl-PCP-X constructs: the loading of peptide CoA thioesters onto the PCP-X was achieved using 5 independent reactions, namely loading control (1), monocyclisation control (1) and bicyclisation reactions (3). To this end, the peptide CoA thioesters (18.5 μmol) were dissolved in buffer containing 67.4 μL of HEPES (0.5 M, pH 7.0 adjusted with 1M NaOH solution), 4.8 μL of aqueous $MgCl_2$ (1 M) and 9.6μL of NaCl (2.5 M). This solution was then diluted with 328.8 μL of water and 66.8 μL of PCP-X_{tei} (216 μM) was added. PCP loading was achieved by the addition of 3.8 μL of Sfp R4-4 mutant (384 μM) followed by incubation for 30 minutes at 30°C. Following the loading reaction, remaining peptidyl-CoA was removed by sequential concentration/ dilution (0.5 mL Ultracentrifugal filters, 10,000 MWCO Merck Millipore) using low salt buffer (50 mM Hepes pH 7.0, 50 mM NaCl). This procedure was repeated 4 times and the concentration of peptidyl-PCP-X constructs was adjusted to 30 μM by addition of low salt buffer (final volume of 481.3 μL).

Protocol for turnovers with $OxyB_{van}$ and $OxyA_{tei}$: the following solutions (Table 1) were mixed at 4 °C (same order than the table) in a 1.5ml Eppendorf tube to achieve a final volume of 105 μL.

SI Table 1. Volumes used to perform the loading control (1), monocyclisation control (1) and bicyclisation reactions (3).

<table>
<tr><th></th><th>Peptide-PCP-X</th><th>Low salt Buffer</th><th>20% Glucose</th><th>$OxyB_{van}$ (56 μM)</th><th>$OxyA_{tei}$ (100μM)</th><th>PuR (81μM)</th><th>PuxBA105V (137μM)</th><th>GluDes (2mg/ml)</th><th>NADH (75mM)</th></tr>
<tr><td>Loading control</td><td>87.5 μL</td><td>17.5 μL</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>Monocyclisation control</td><td>87.5 μL</td><td>5.2 μL</td><td rowspan="2">1.7 μL</td><td rowspan="2">0.9 μL</td><td>-</td><td rowspan="2">1.3 μL</td><td rowspan="2">3.8 μL</td><td rowspan="2">1.7 μL</td><td rowspan="2">2.8 μL</td></tr>
<tr><td>Bicyclisation reactions</td><td>87.5 μL</td><td>3.1 μL</td><td>2.1 μL</td></tr>
</table>

After gentle shaking for an hour at 30°C, the peptide was cleaved from the PCP-X construct by the addition of 15 μL of aqueous methylamine (~60%, v/v). After incubation for 15 minutes, the solution was neutralised through the addition of formic acid (diluted in water) and the peptides were purified by solid phase extraction using Strata-X-33 polymeric reversed phase columns (30 mg/mL, Phenomenex). The crosslinking state of the peptide was analysed via LC/MS.

Calculation of the turnover rate: the presence of 1 or 2 chlorine atoms requires deconvolution when MS signals are overlapping. The following calculation was performed to obtain the data presented in the tables in SI4 (highlighted in grey).

Sequence **6** and **8** (1 Cl)
Area (linear) = Area (linear) - 0.25*Area (monocyclic)
Area (monocyclic) = Area (monocyclic) - 0.25*Area (bicyclic)

Sequence **5** and **7** (2 Cl)
Area (linear) = Area (linear) - 0.375*Area (monocyclic) - 0.0675* Area (bicyclic)
Area (monocyclic) = Area (monocyclic) - 0.375*Area (bicyclic)

LC/MS: analyses were carried out on a Shimadzu High Performance Liquid Chromatograph coupled to Mass Spectrometer LCMS-2020 (ESI, operating both in positive and negative mode) equipped with a SPD-20A Prominence Photo Diode Array Detector and a LC-20AD solvent delivery module. Analytical separations were performed on a Waters XBridge BEH300 Prep C18 column (10 μm, 4.6 x 250 mm). The solvents used were water + 0.1% (v/v) formic acid (solvent A) and HPLC-grade ACN + 0.1% (v/v) formic acid (solvent B).

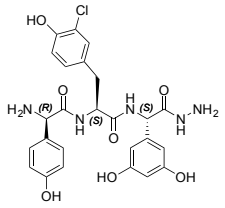
Preparative RP-HPLC: purifications were carried out on a Shimadzu High Performance Liquid Chromatograph equipped with a SPD-M20A Prominence Photo Diode Array Detector and two LC-20AP pumps. Preparative separations were performed on a Waters XBridge BEH300 Prep C18 column (5 μm, 19 x 150 mm) at a flow rate of 10 mL/min. The solvents used were water + 0.1% TFA (solvent A) and ACN + 0.1% TFA (solvent B).

NMR: Nuclear magnetic resonance spectra were recorded on a Bruker Avance III 600 in CD_3CN/ D_2O (v/v; 20 / 80).

SI2. Epimerisation control by NMR-^{1}H

SI2. 1. Tripeptide pilot study

1a



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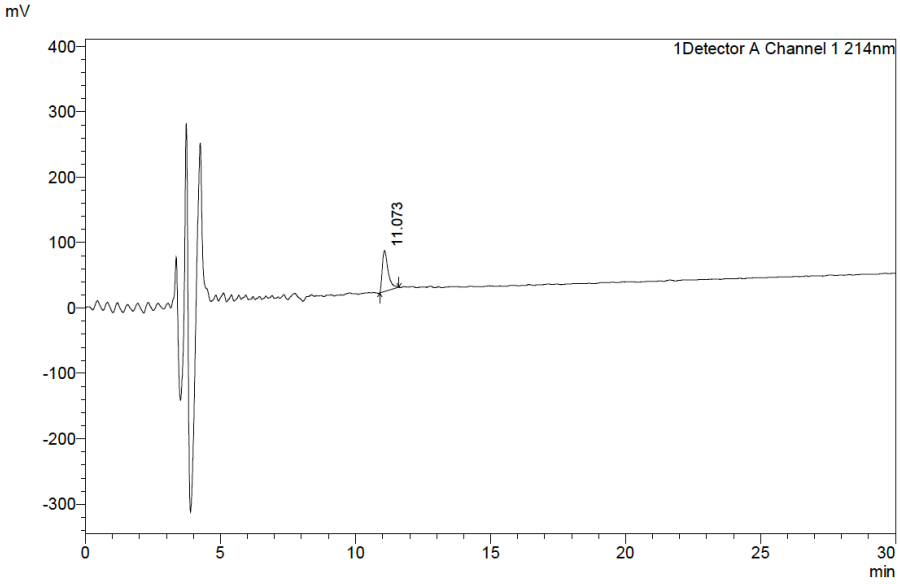
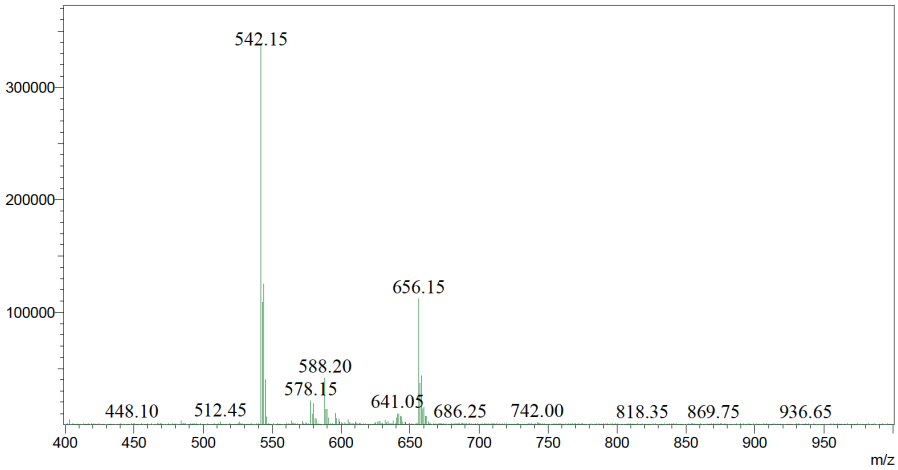
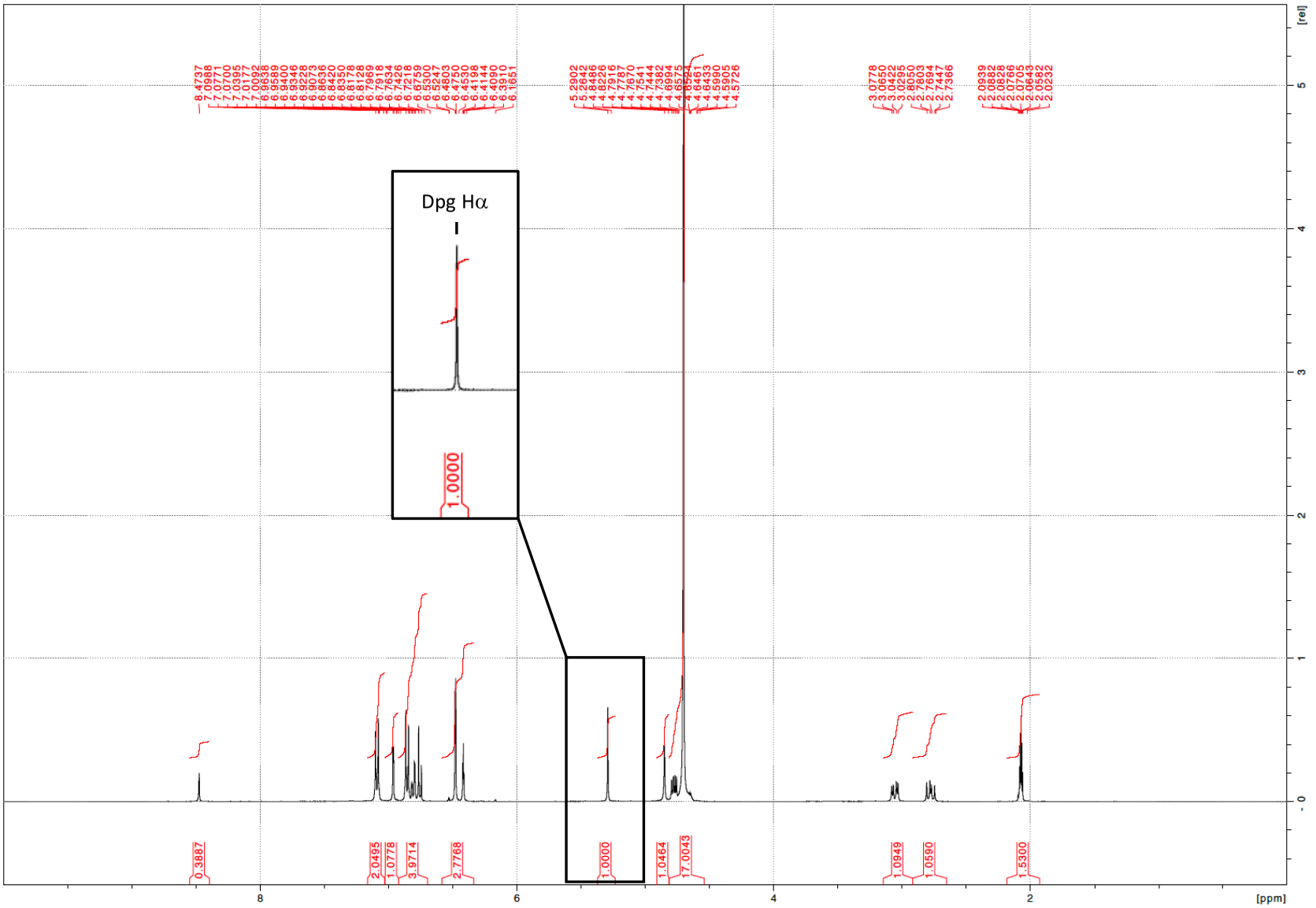


Gradient 5-35% in ACN in 30 minutes

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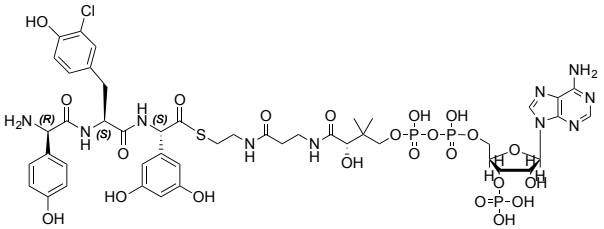
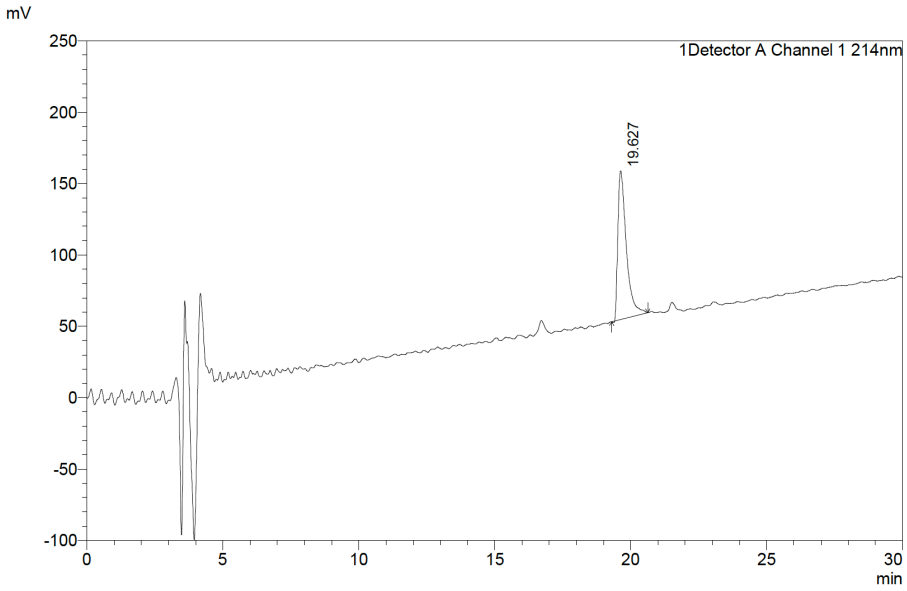
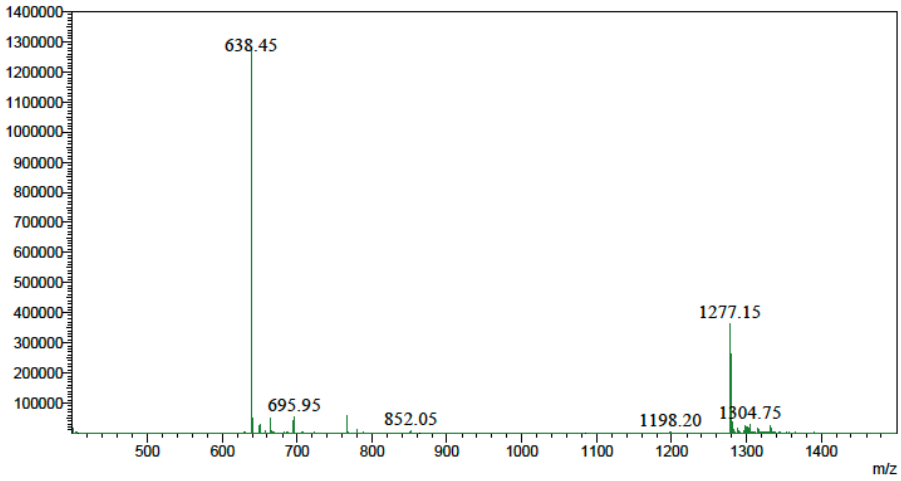
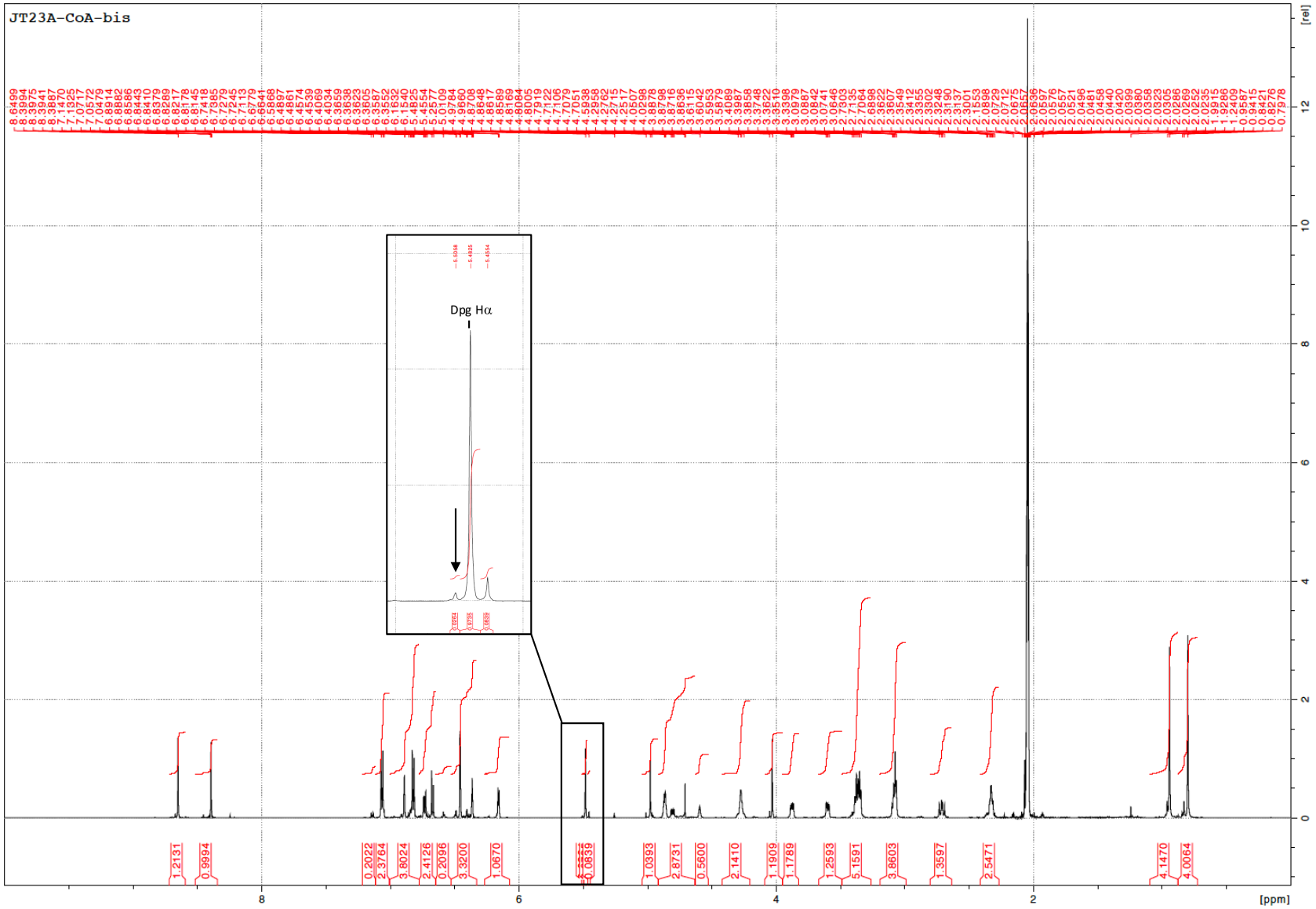


Chemical Formula: $C_{46}H_{58}ClN_{10}O_{23}P_3S$
Exact Mass: 1278.2 Da
Experimental mass: 1278.15 Da
(m/z: 638.45 Da; $[M-2H]^-/2$)



Gradient 5-35% in ACN in 30 minutes

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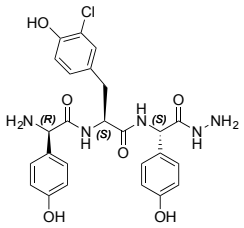
2a



Chemical Formula: $C_{25}H_{26}ClN_5O_6$
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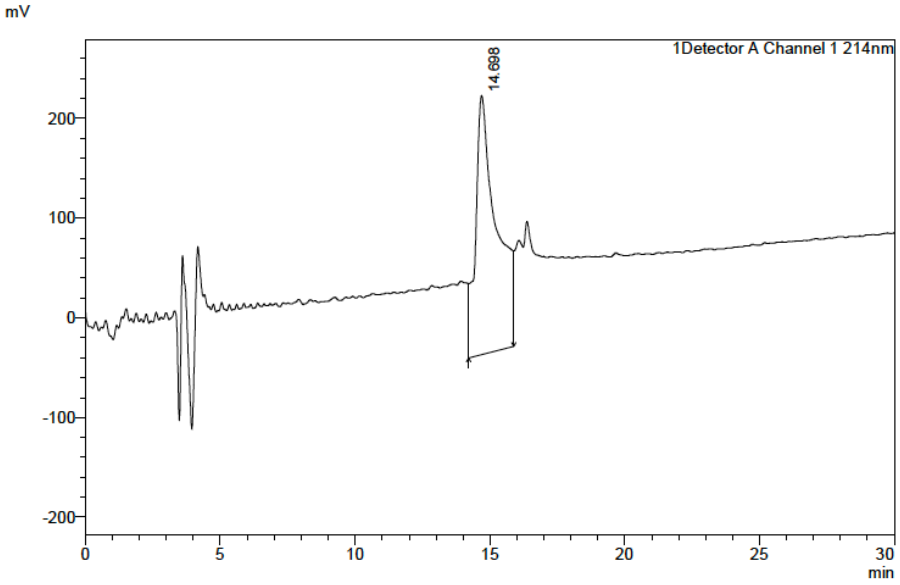
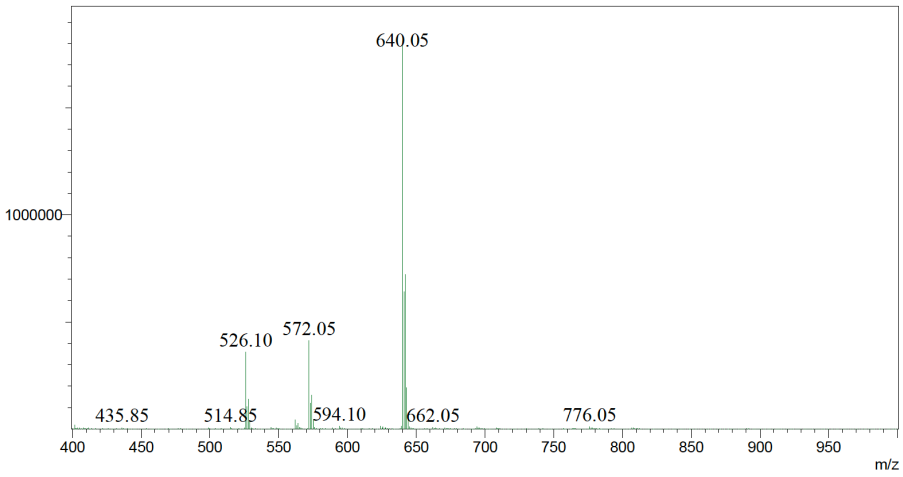
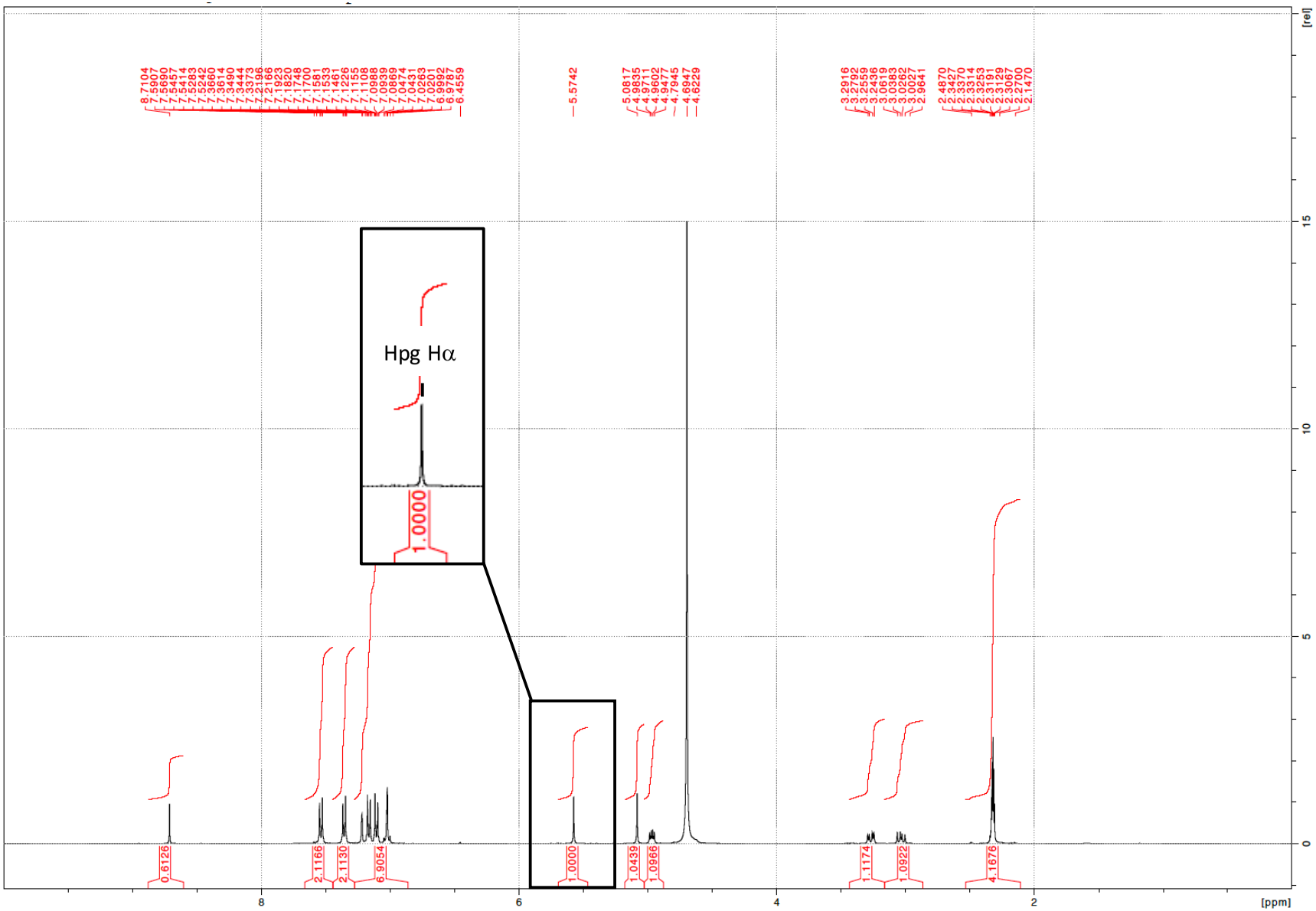


Gradient 5-35% in ACN in 30 minutes

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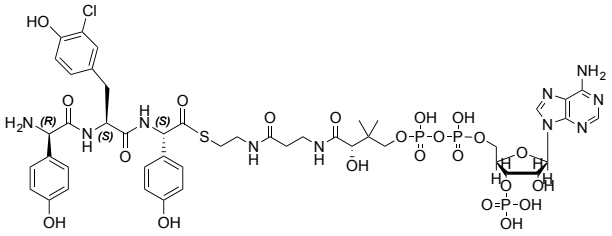

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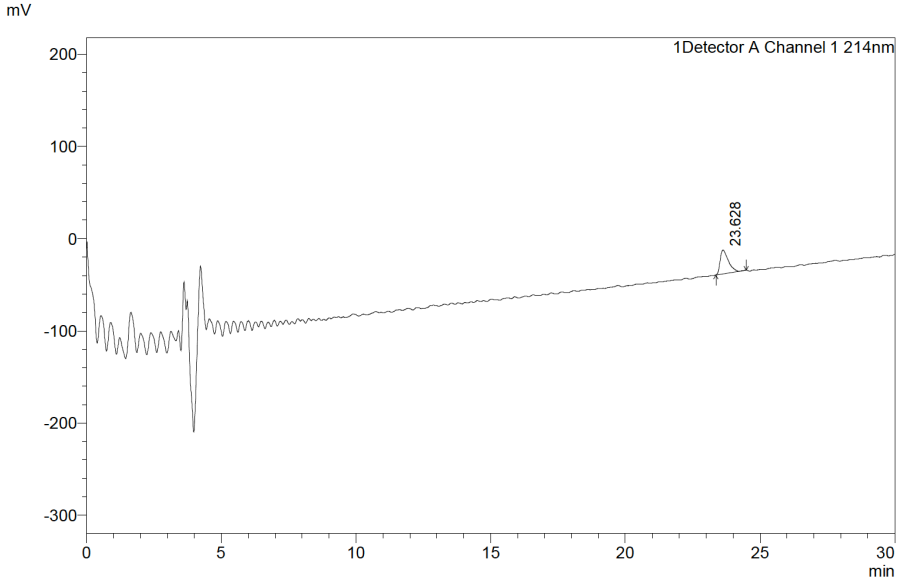
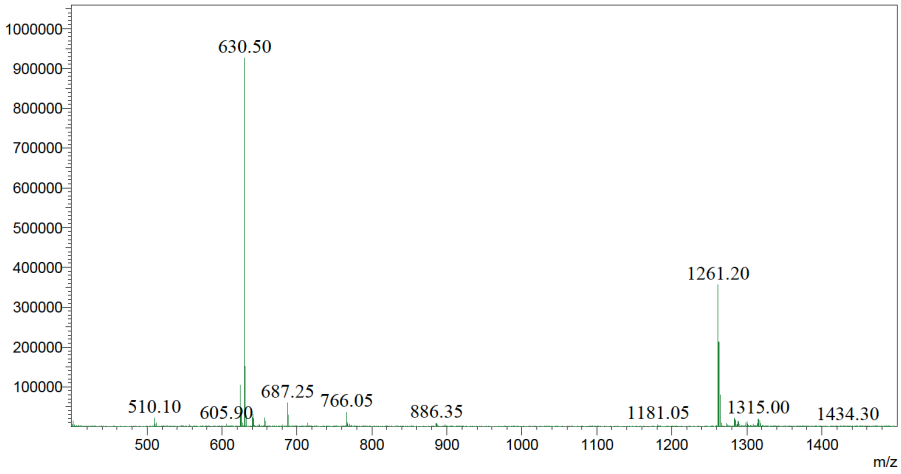
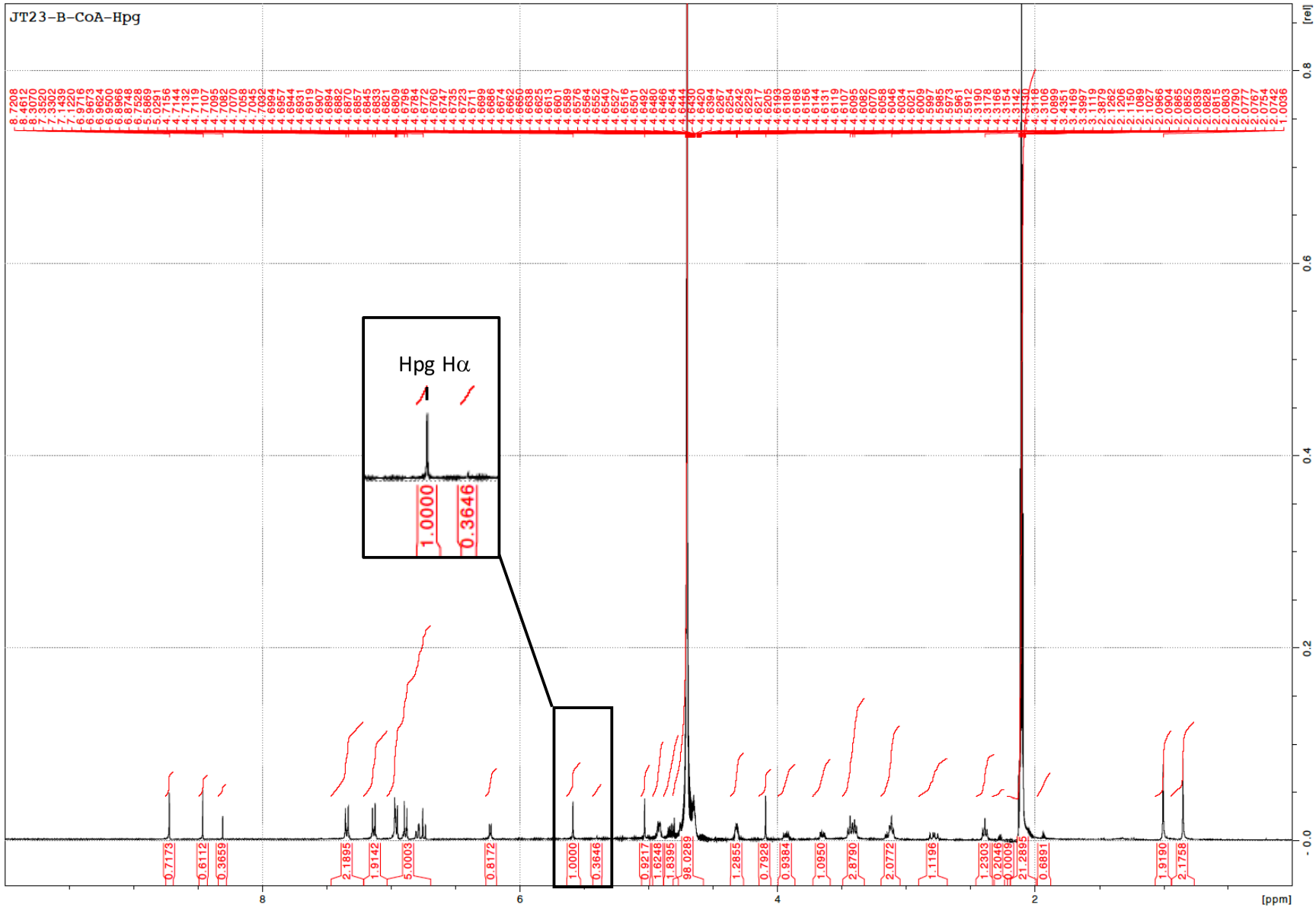


Gradient 5-35% in ACN in 30 minutes

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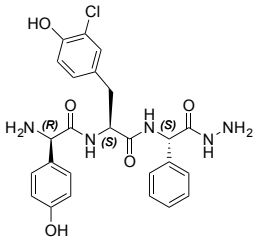

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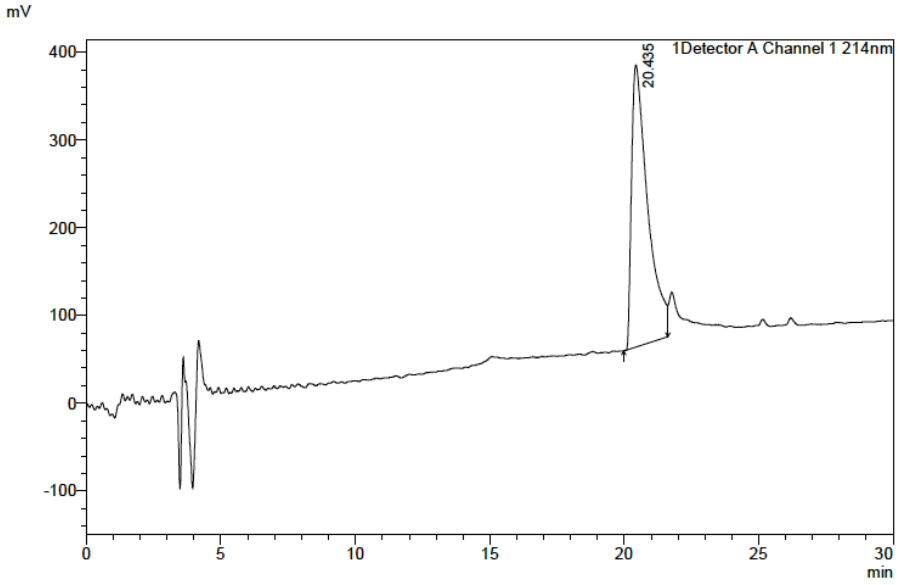
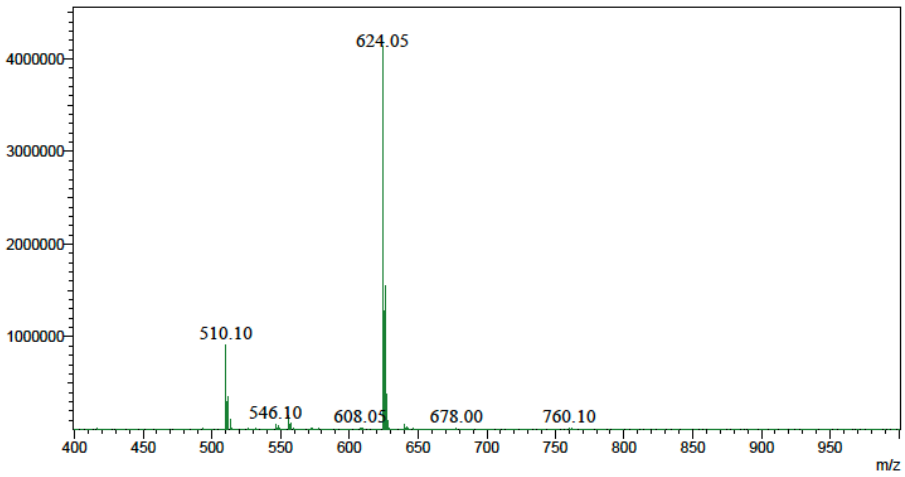
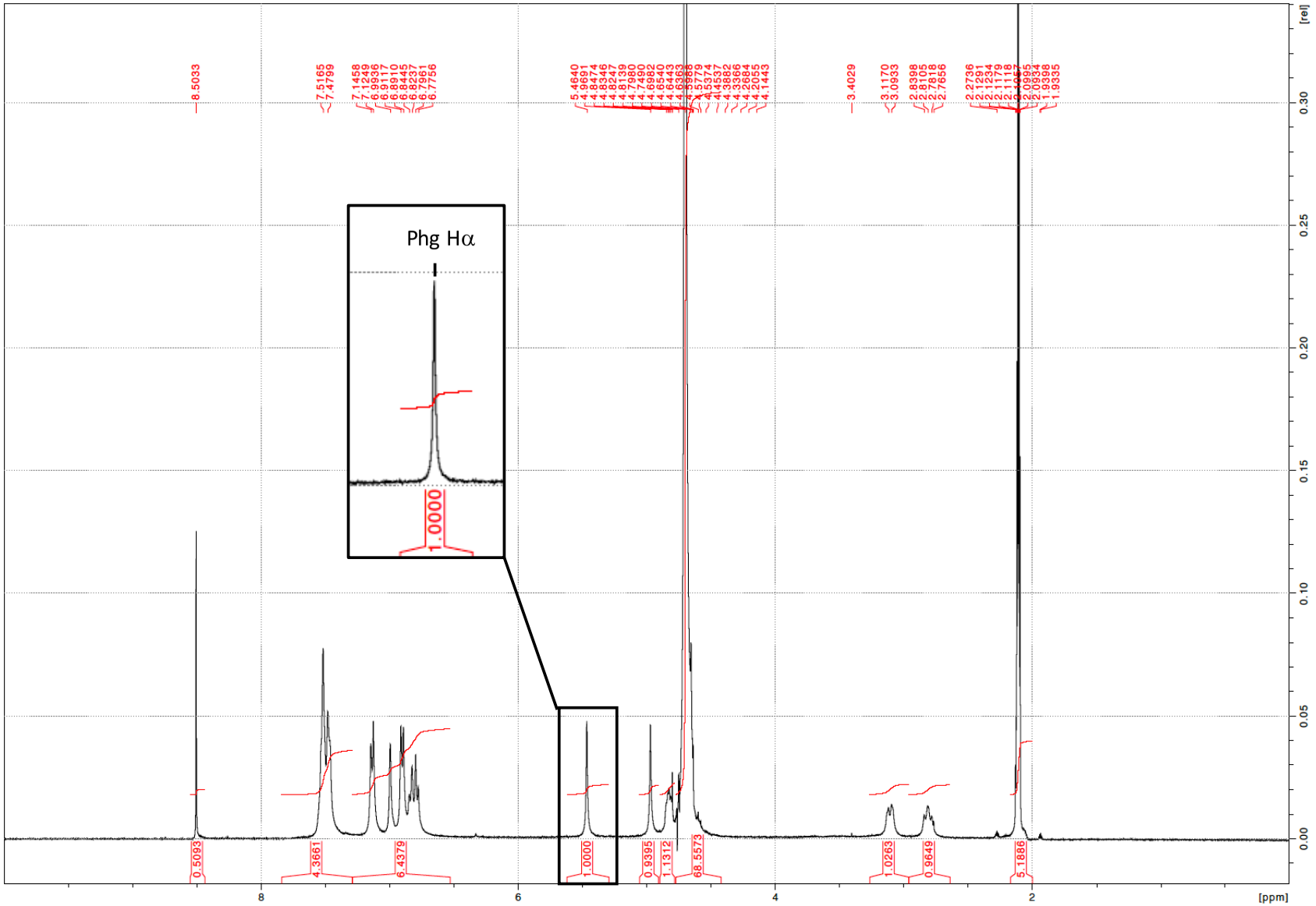


Gradient 5-35% in ACN in 30 minutes

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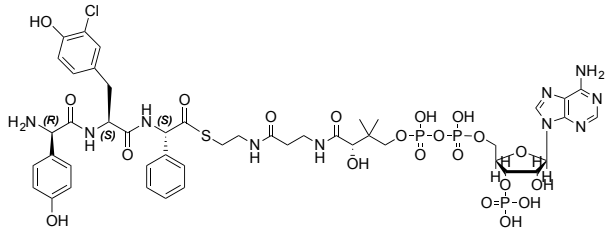

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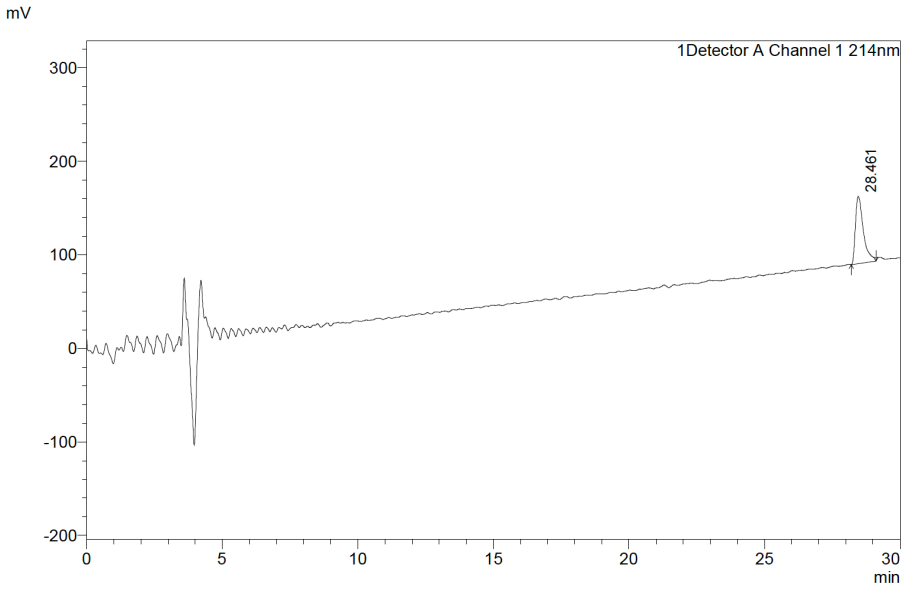
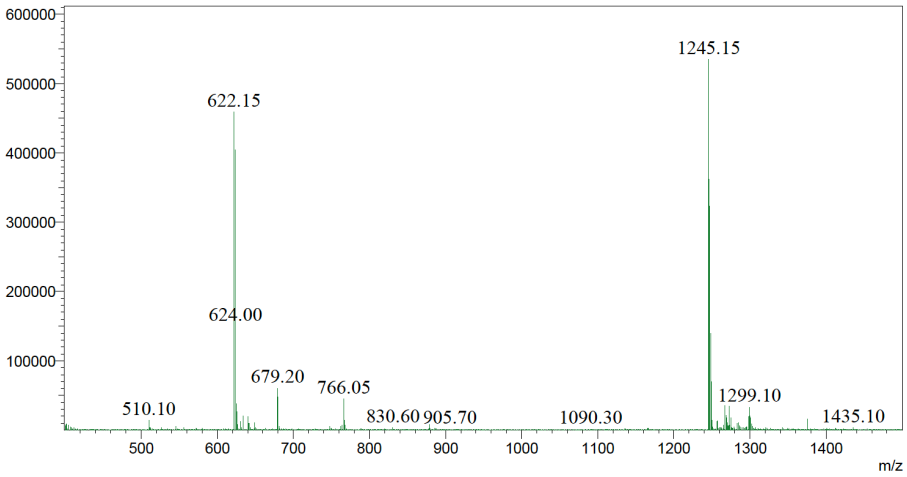
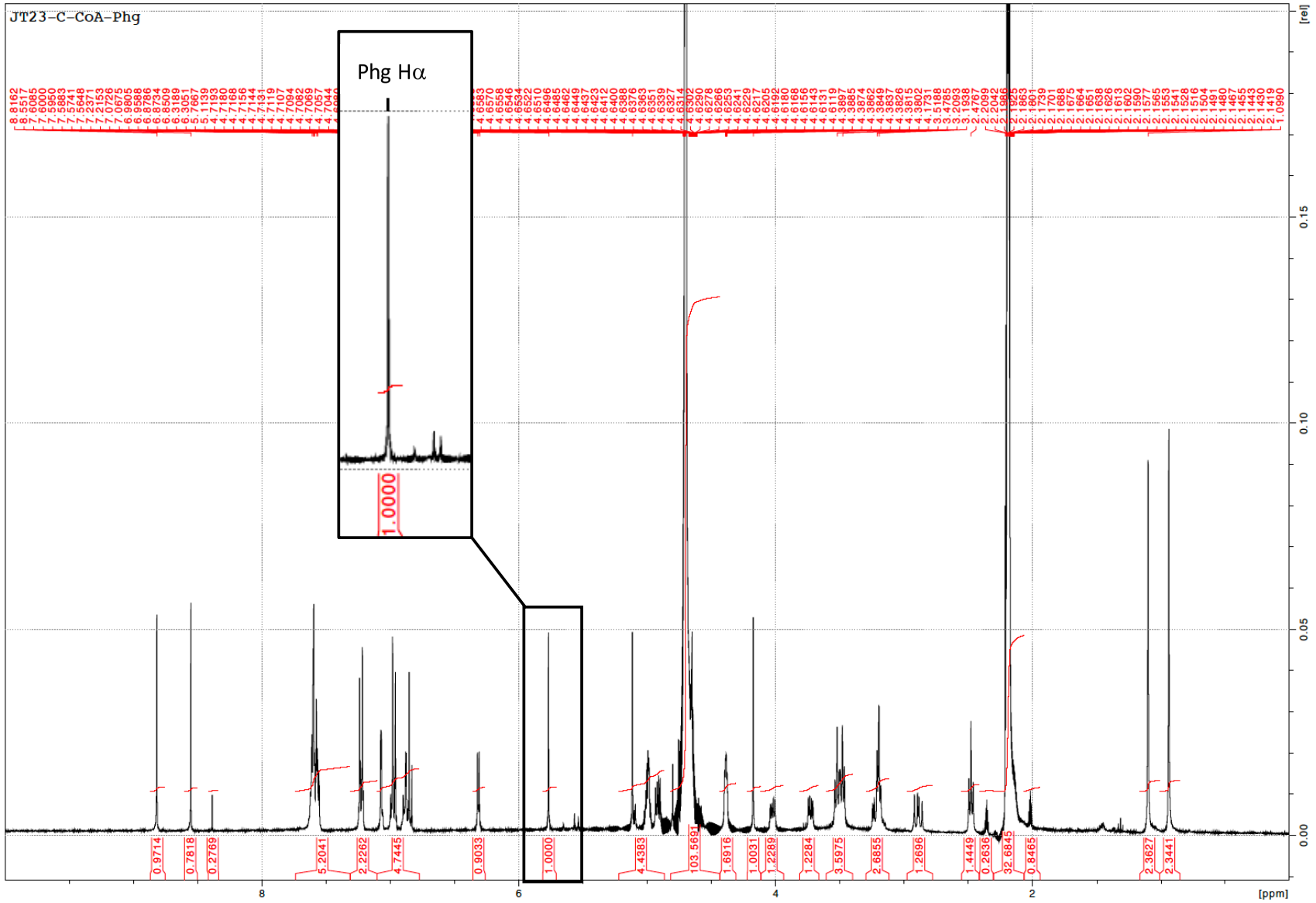


Gradient 5-35% in ACN in 30 minutes

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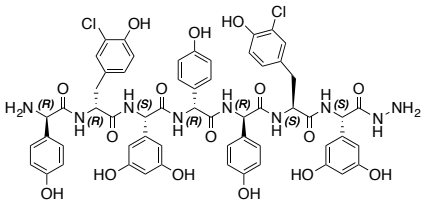
SI3. Characterisation of peptide sequences 5-8

5a



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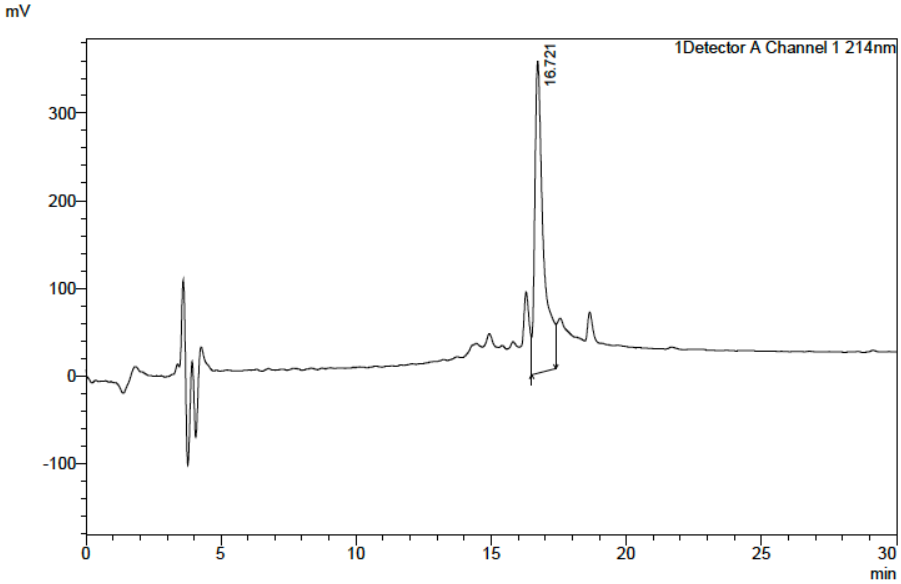
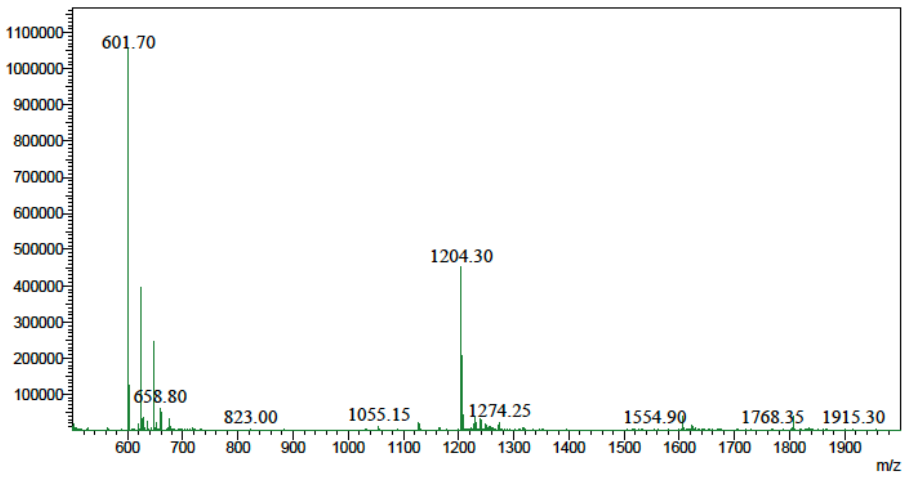
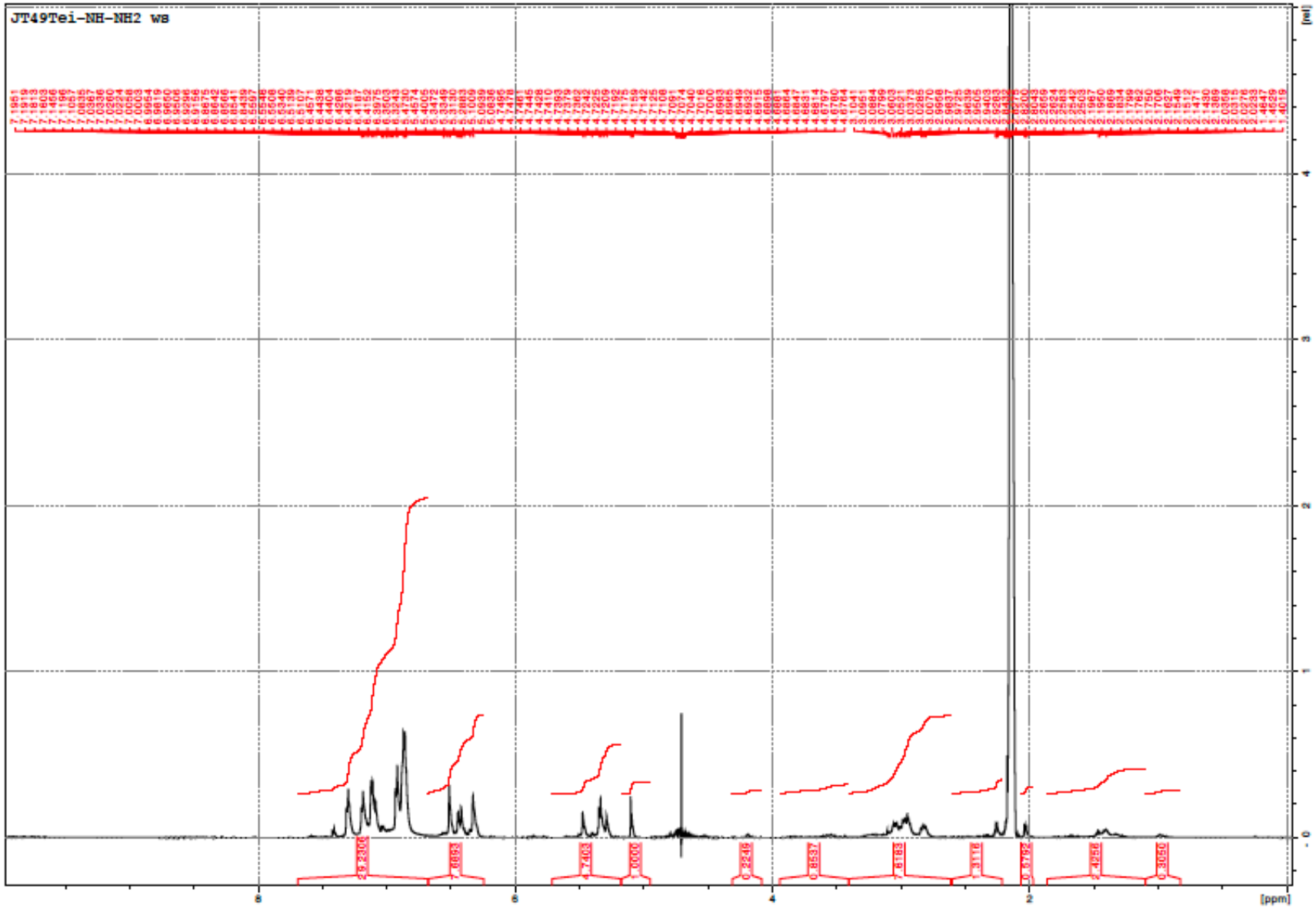
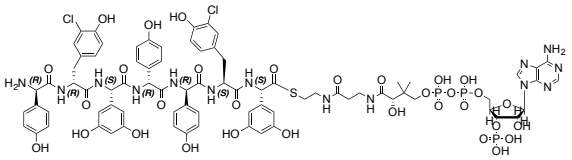


Gradient 10-40% in ACN in 30 minutes

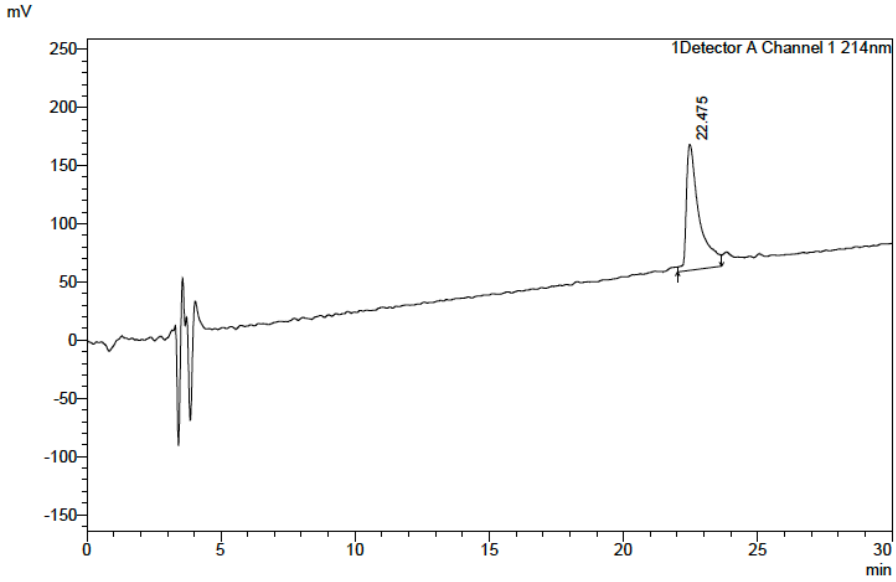



JT49Tei-NH-NH2 ws
[ppm]

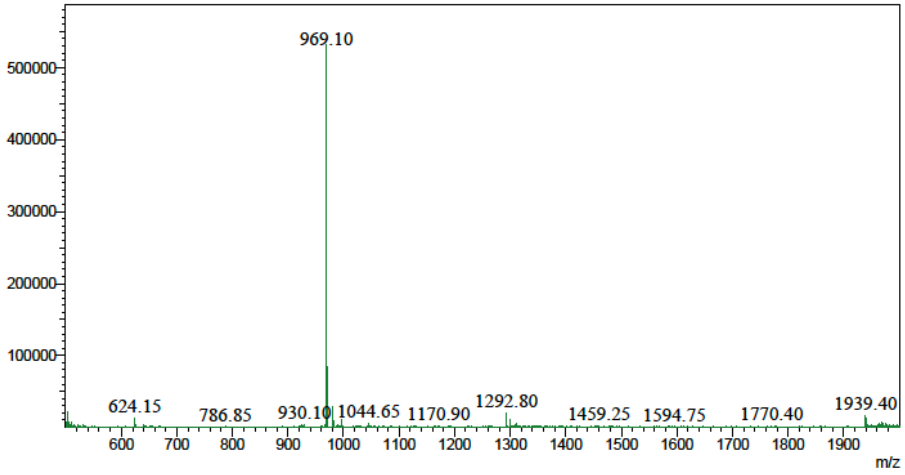
5

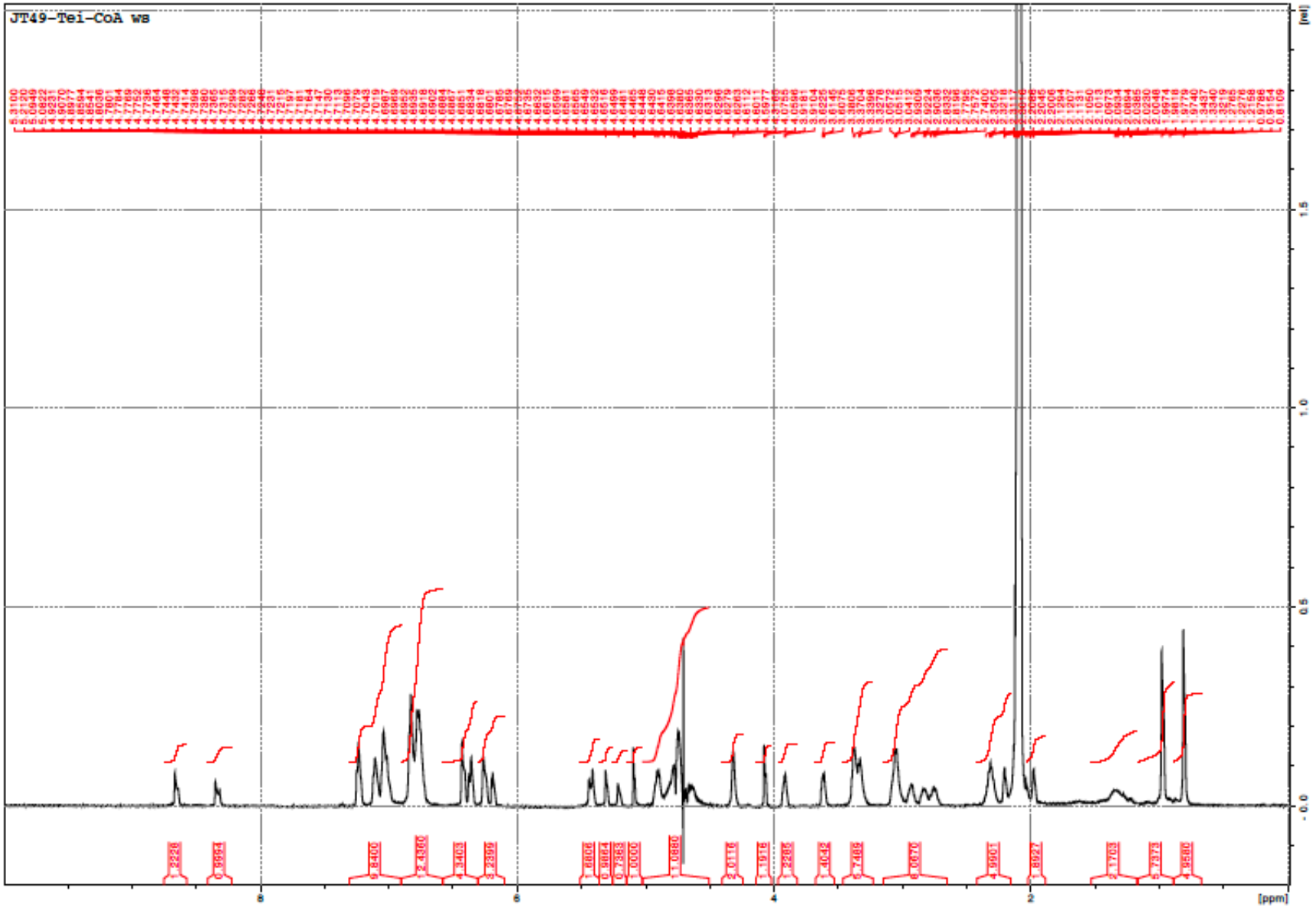


Chemical Formula: $C_{79}H_{87}Cl_2N_{14}O_{32}P_3S$
Exact Mass: 1938.39 Da
Experimental mass: 1940.2 Da
(m/z: 969.1 Da; $[M-2H]^-/2$)



Gradient 10-40% in ACN in 30 minutes




JT49-Tei-CoA ws
[rel]
1.5
1.0
0.5
- 0.0
8
6
4
2
[ppm]
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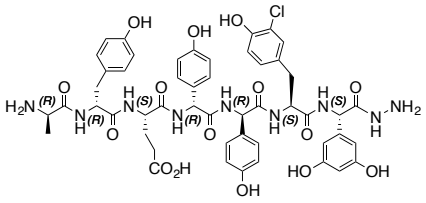
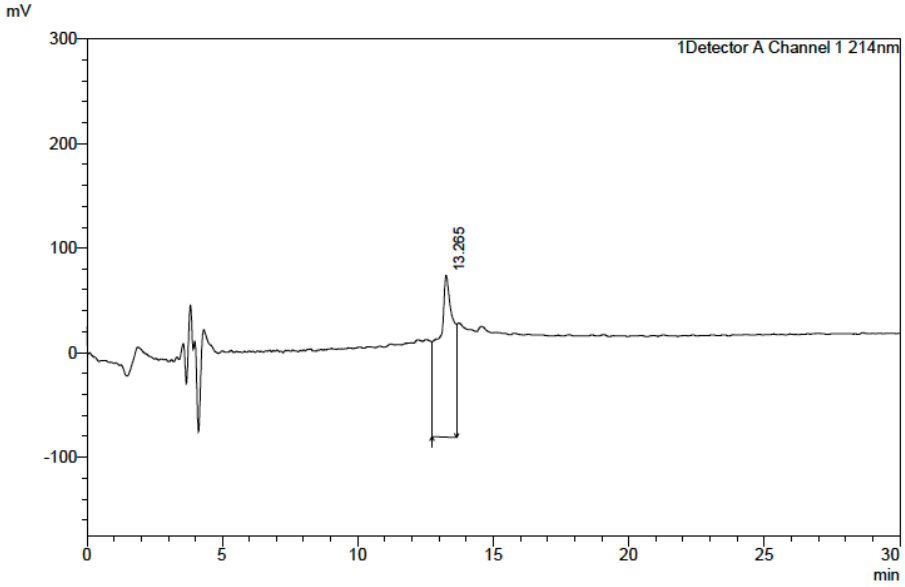
0.9994
9.8400
12.4300
4.3403
3.2399
0.6806
0.9884
0.7368
1.0000
11.0880
2.0116
1.1916
1.2288
4.4042
9.7469
6.0670
4.9961
1.8927
2.1703
5.7373
4.9540

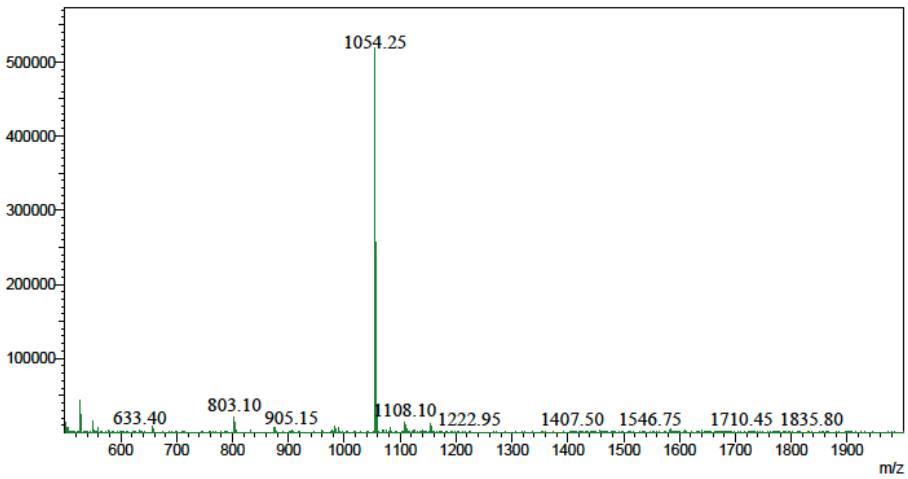
6a

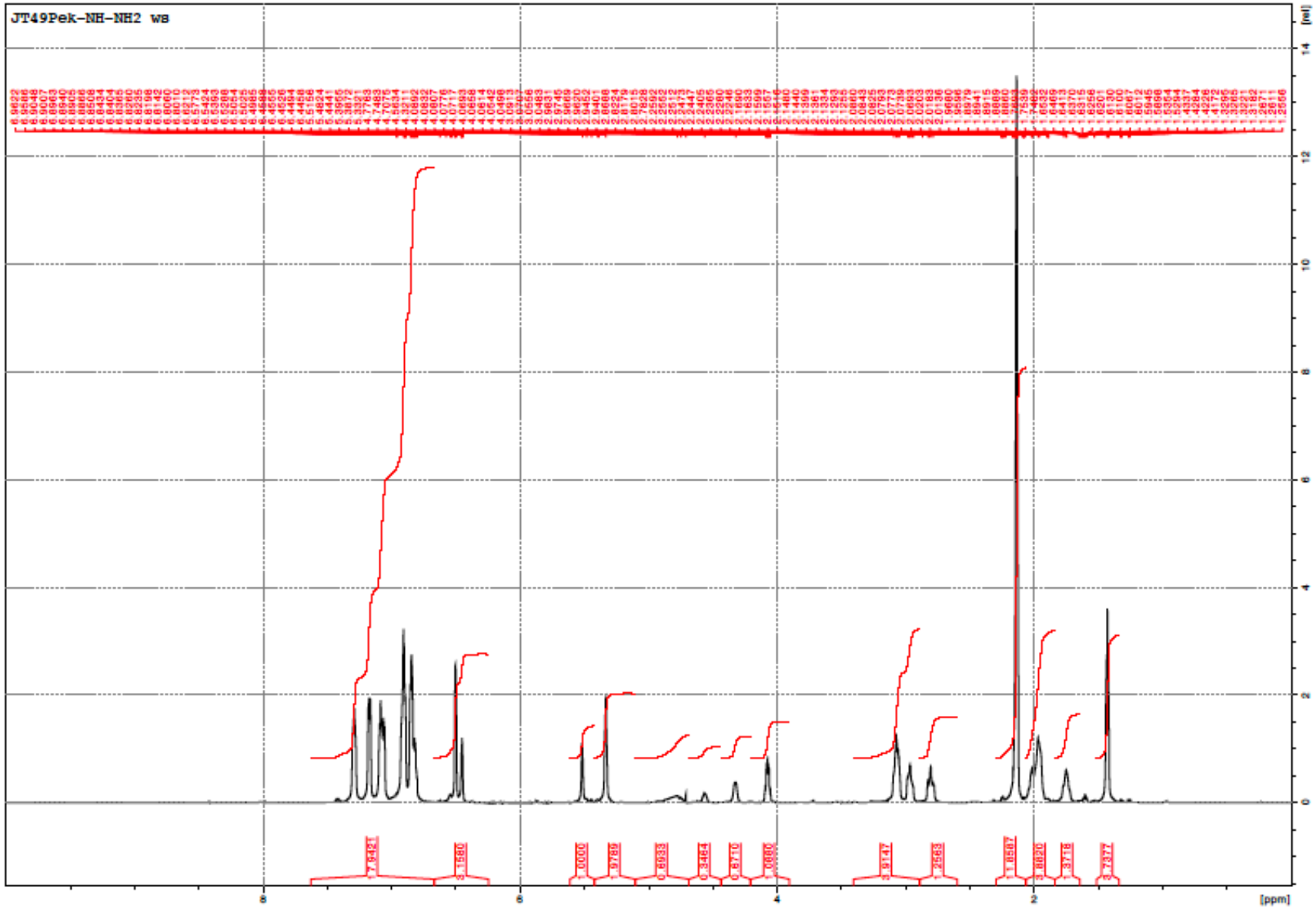


Chemical Formula: $C_{50}H_{54}ClN_9O_{15}$
Exact Mass: 1055.34 Da
Experimental mass: 1055.25 Da

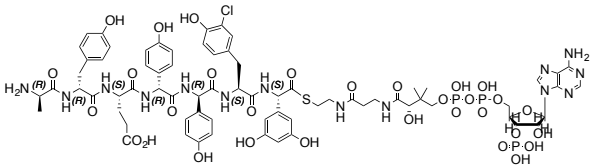


Gradient 10-40% in ACN in 30 minutes

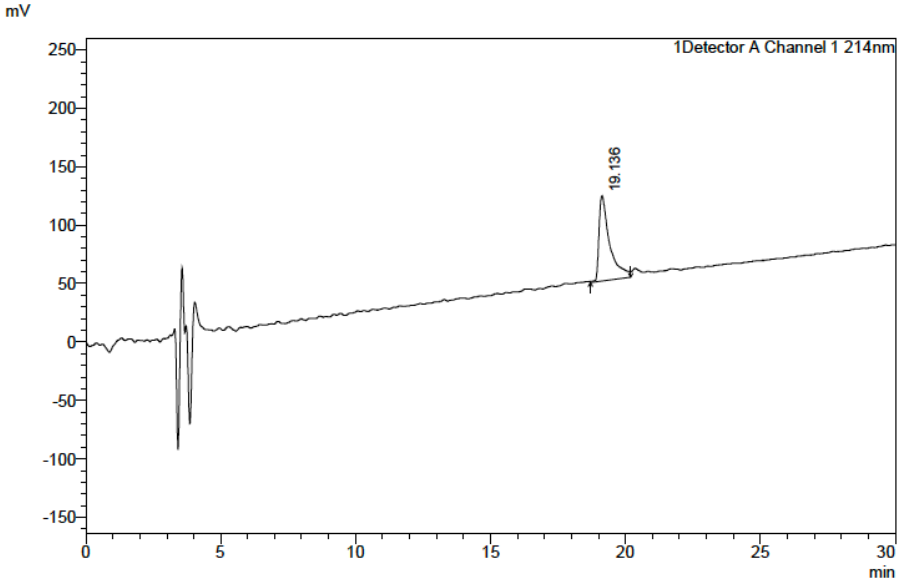



JT49Pek-NH-NH2 wb
[rel]
[ppm]

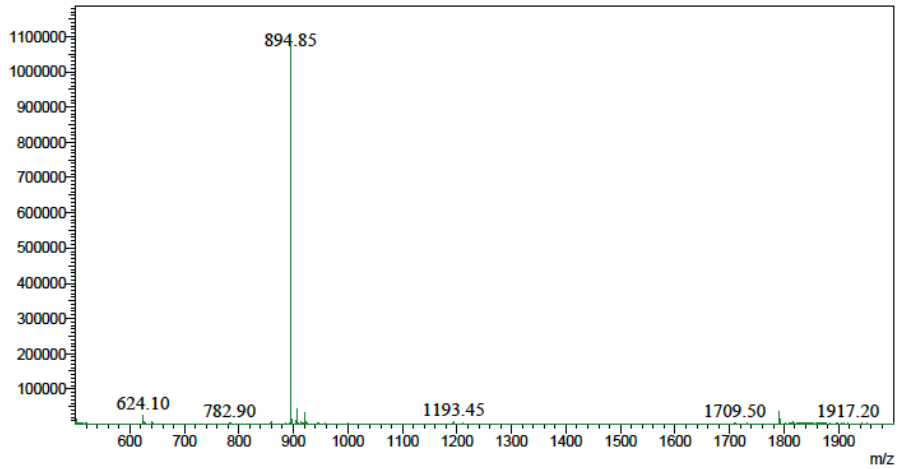
6

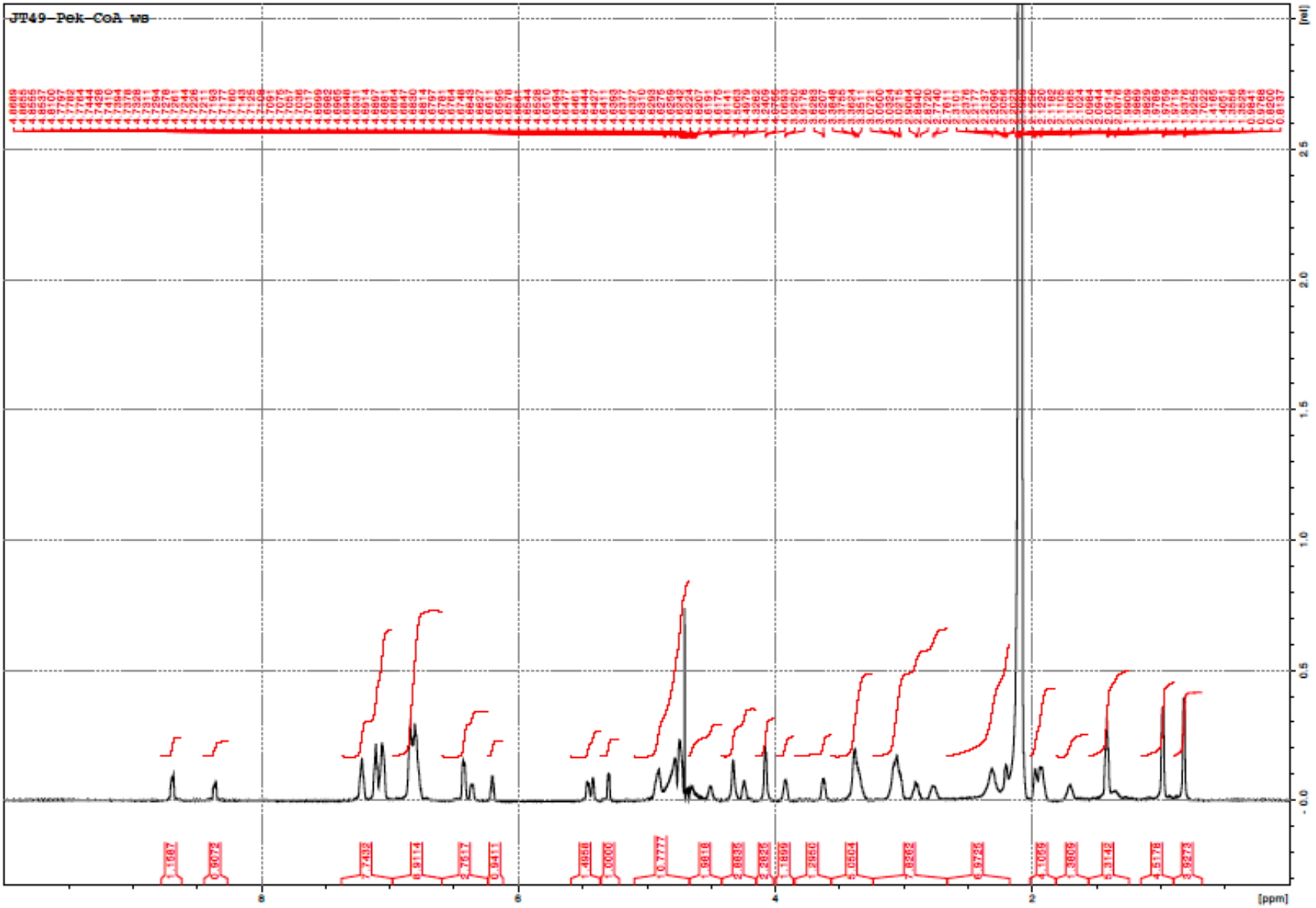


Chemical Formula: $C_{71}H_{86}ClN_{14}O_{31}P_3S$
Exact Mass: 1790.42 Da
Experimental mass: 1791.7 Da
(m/z: 894.85 Da; $[M-2H]^-/2$)



Gradient 10-40% in ACN in 30 minutes




JT49-Pek-CoA wb
[rel]
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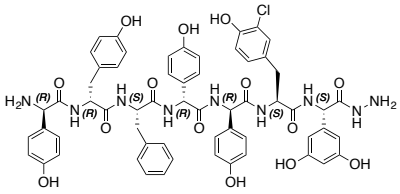
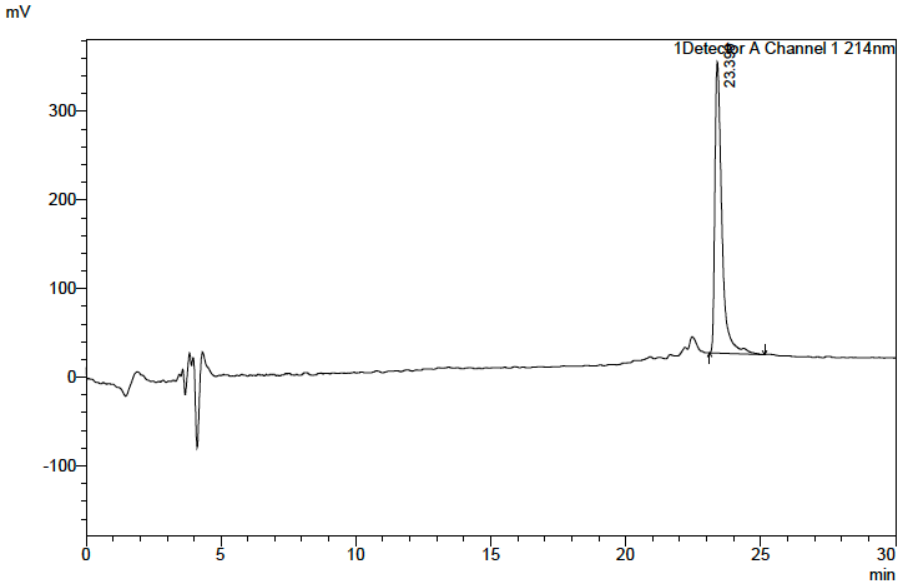
2.0
1.5
1.0
0.5
-0.0
8
6
4
2
[ppm]
1.1567
0.5072
7.7432
8.9114
2.7617
0.9411
1.4856
1.0000
0.7777
1.5818
2.0833
2.2825
1.1899
2.2950
5.0504
7.8262
6.9726
1.1066
3.8009
5.3142
4.5178
5.9273

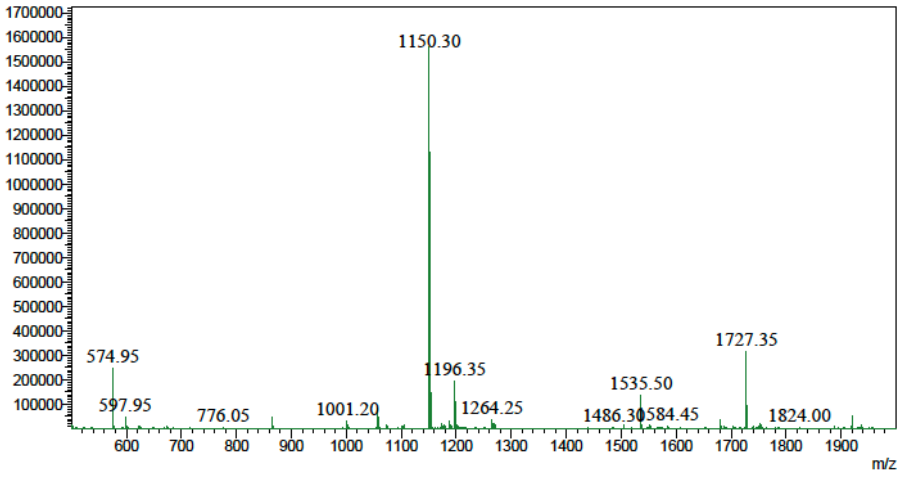
7a

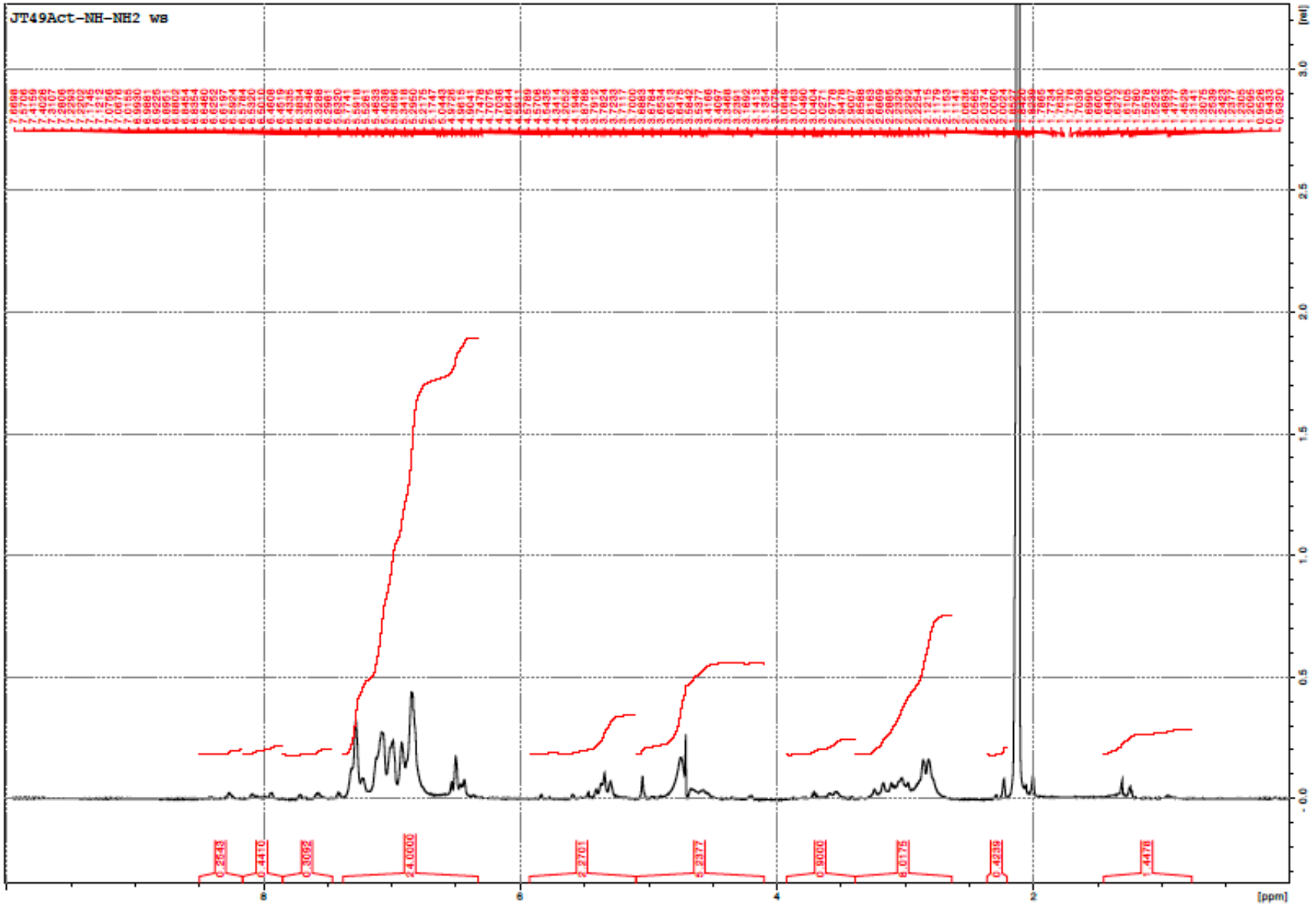


Chemical Formula: $C_{59}H_{58}ClN_9O_{14}$
Exact Mass: 1151.38 Da
Experimental mass: 1151.30 Da

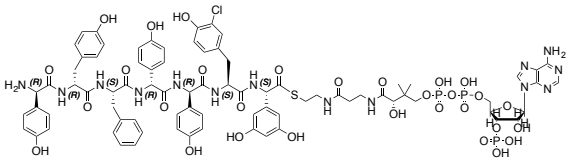


Gradient 10-40% in ACN in 30 minutes

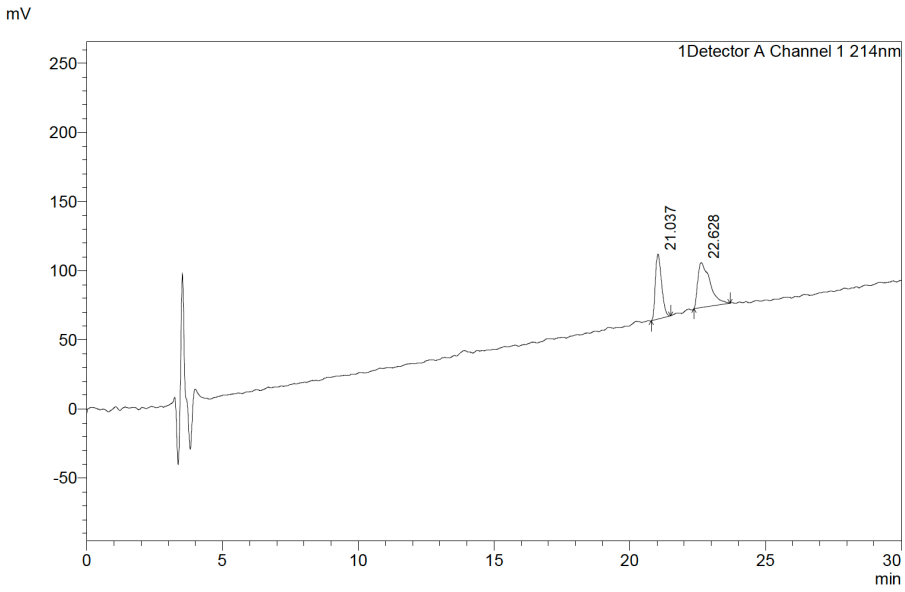



JT49Act-NH-NH2 ws
[rel]
3.0
2.5
2.0
1.5
1.0
0.5
-0.0
8
6
4
2
[ppm]
2.543
0.4470
0.3092
24.0000
2.2701
5.2377
0.9000
8.0175
0.4239
1.4476

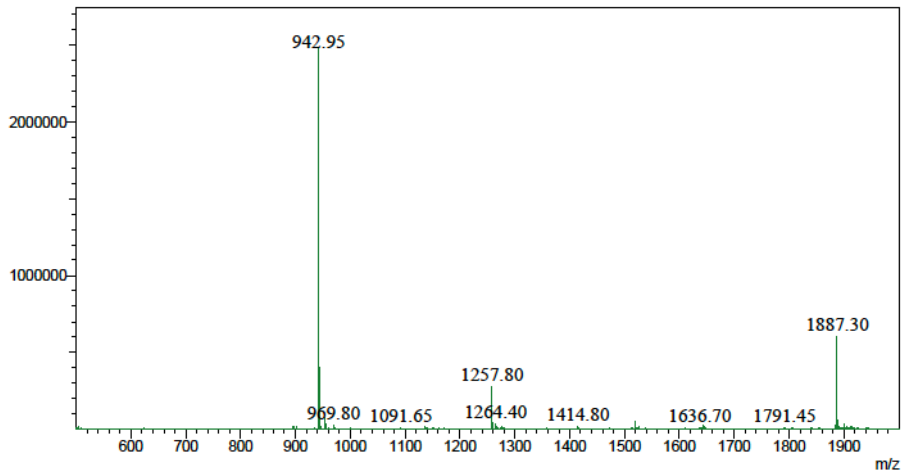
7



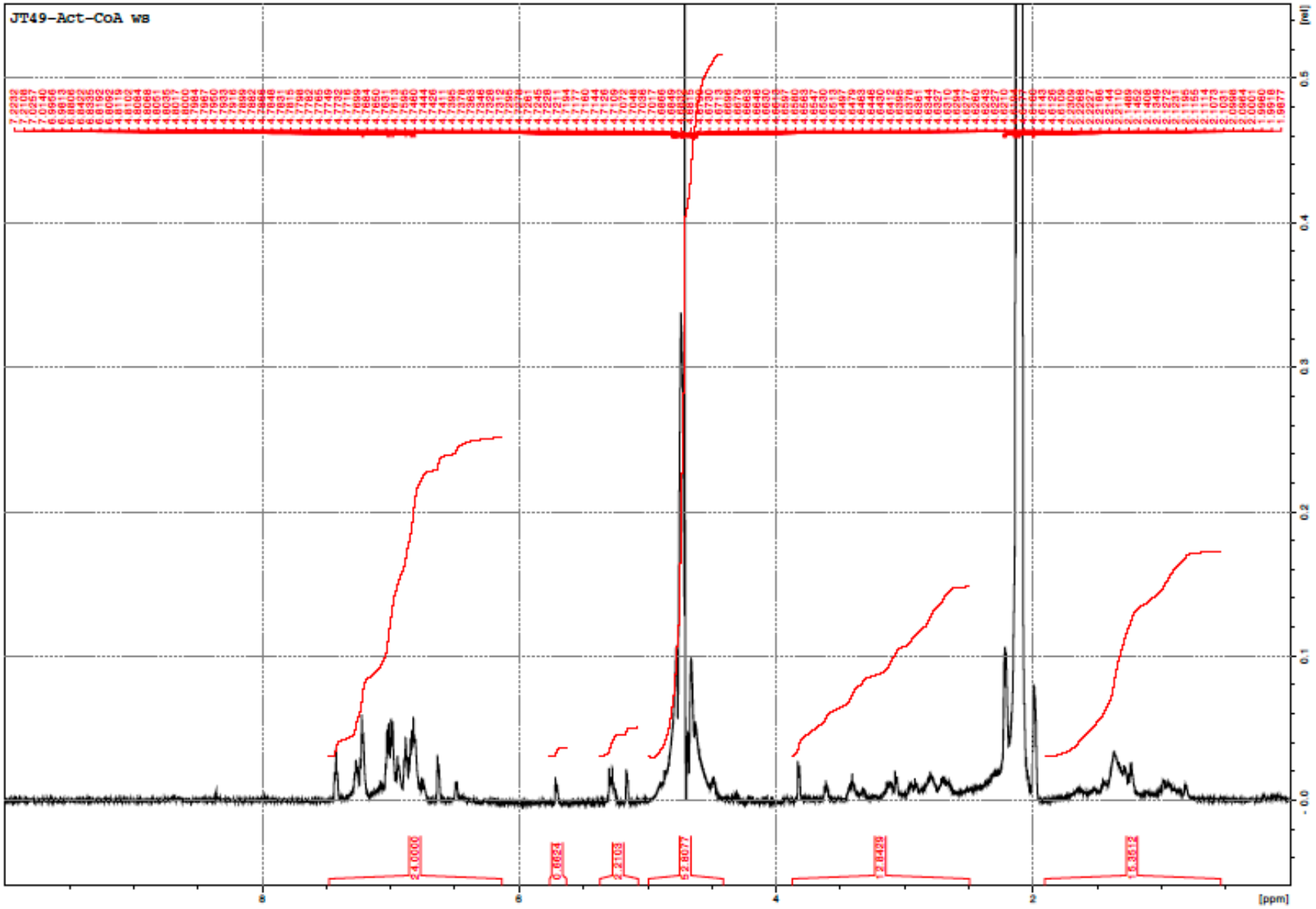
Chemical Formula: $C_{80}H_{90}ClN_{14}O_{30}P_3S$
Exact Mass: 1886.46 Da
Experimental mass: 1887.9 Da
(m/z: 942.95 Da; $[M-2H]^-/2$)



Gradient 15-45% in ACN in 30 minutes



Peak at 22.628 min
Peak at 21.037 min: 7 hydrolysed (Co-elution)


JT49-Act-CoA ws
24.0000
0.1824
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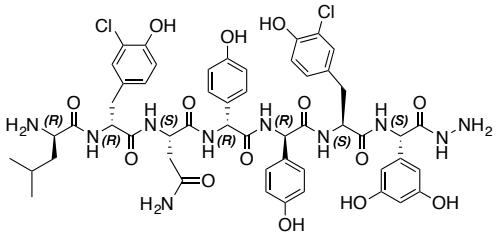
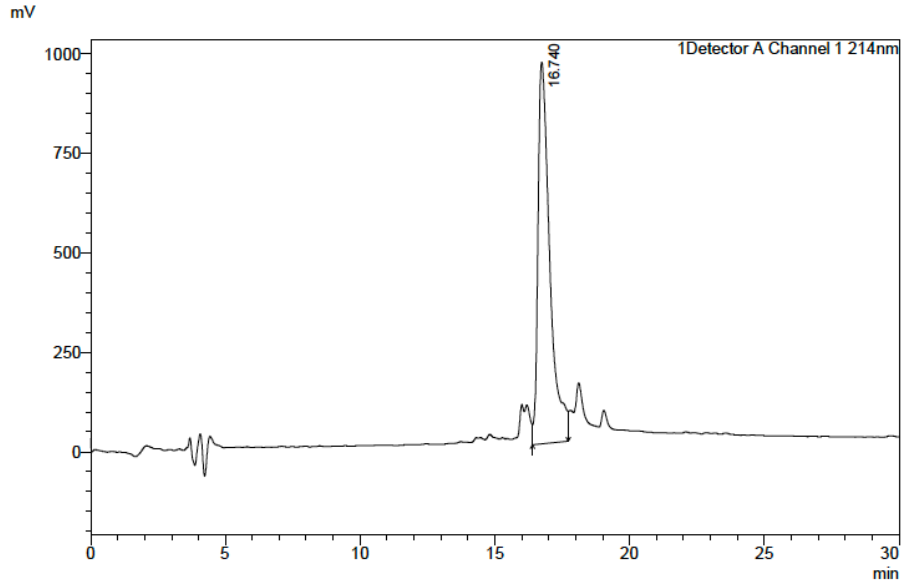
12.8077
12.8429
15.3513
8
6
4
2
[ppm]
[rel]
0.5
0.4
0.3
0.2
0.1
-0.0

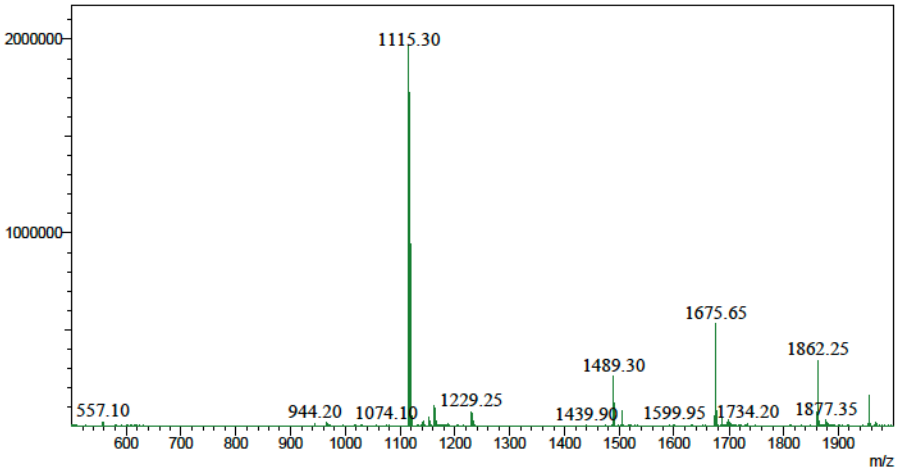
8a

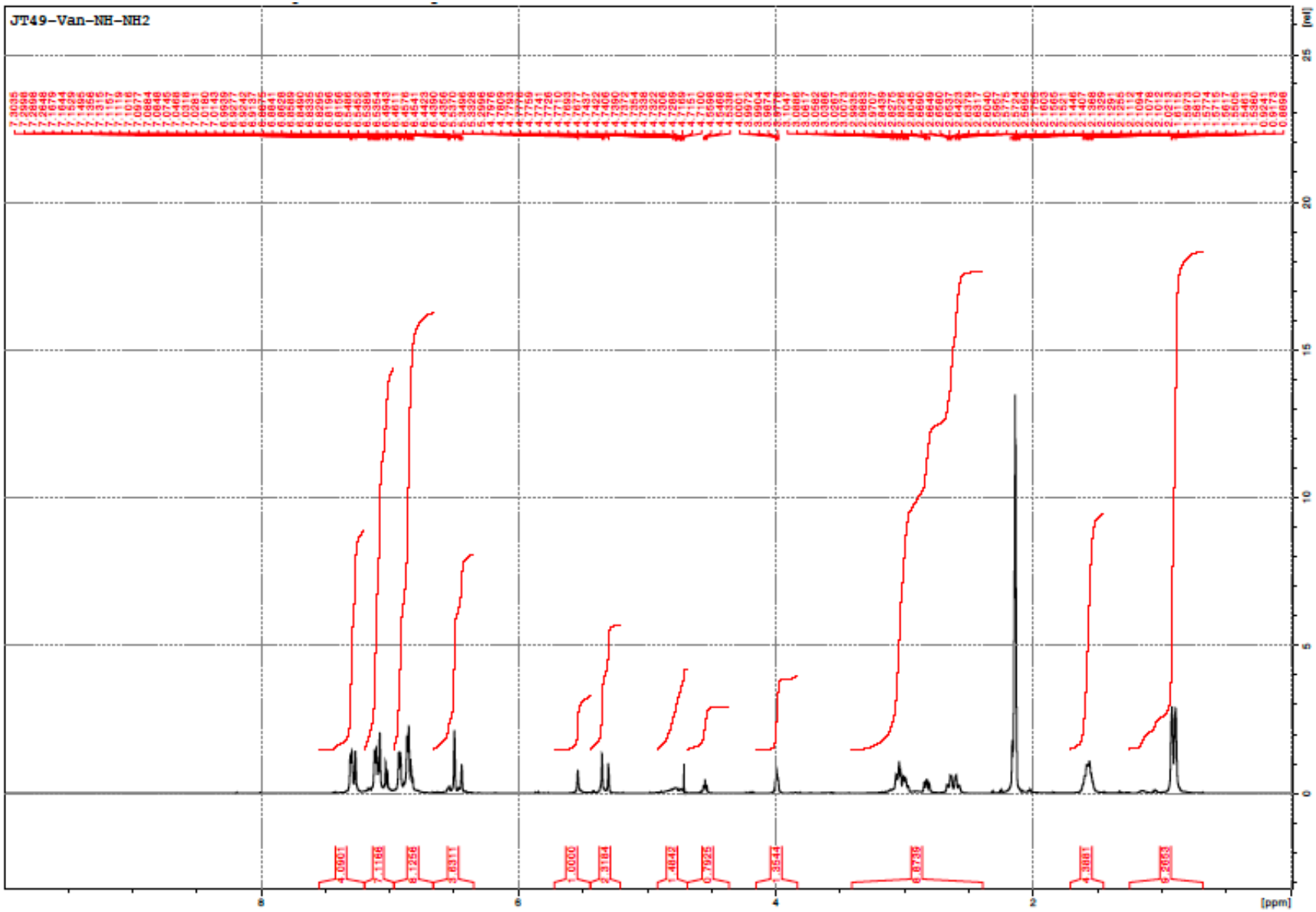


Chemical Formula: $C_{52}H_{58}Cl_2N_{10}O_{14}$
Exact Mass: 1116.35 Da
Experimental mass: 1116.30 Da

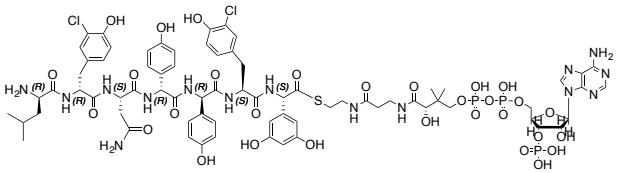


Gradient 10-40% in ACN in 30 minutes

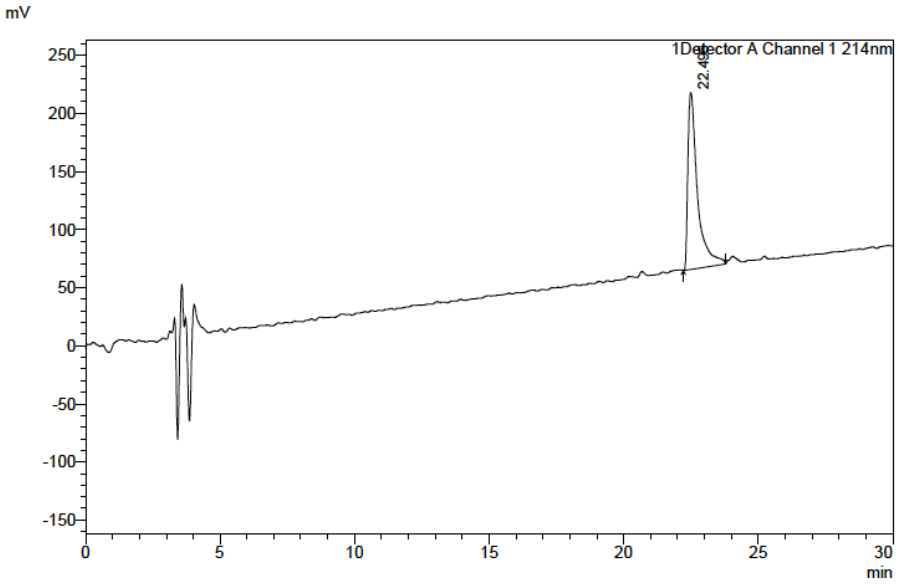



JT49-Van-NH-NH2
[ml]
25
20
15
10
5
0
8
6
4
2
[ppm]
4.0901
4.1166
6.1256
5.8311
1.0000
2.3184
1.4842
0.7925
1.3544
6.8739
4.3881
5.2863

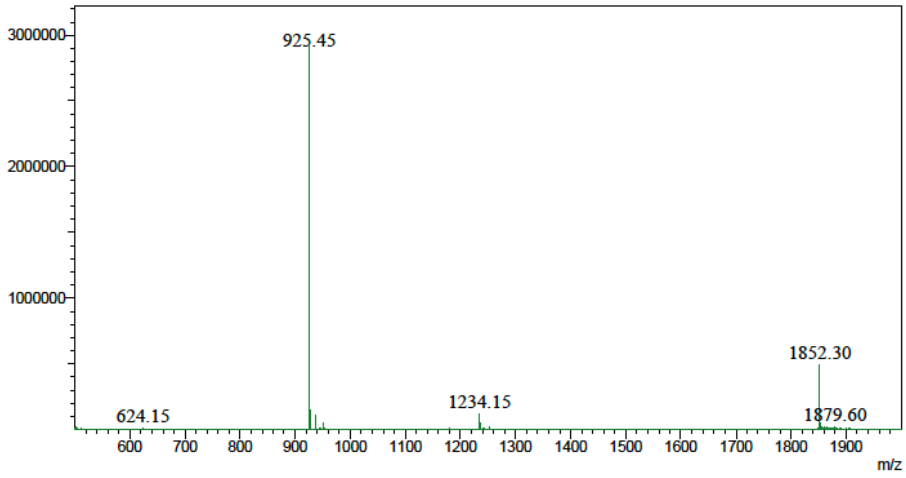
8

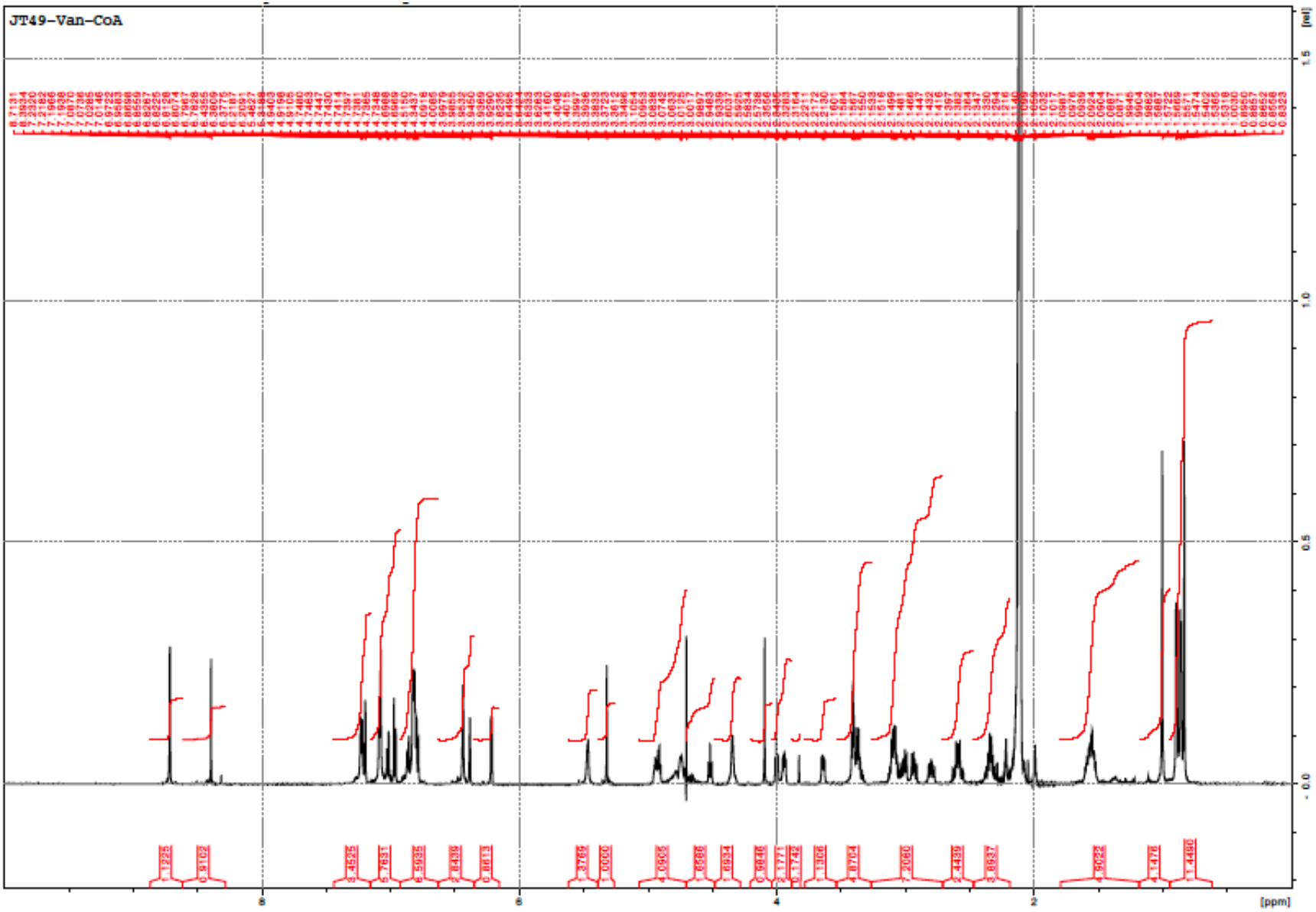


Chemical Formula: $C_{73}H_{90}Cl_2N_{15}O_{30}P_3S$
Exact Mass: 1851.43 Da
Experimental mass: 1852.9 Da
(m/z: 925.45 Da; $[M-2H]^-/2$)

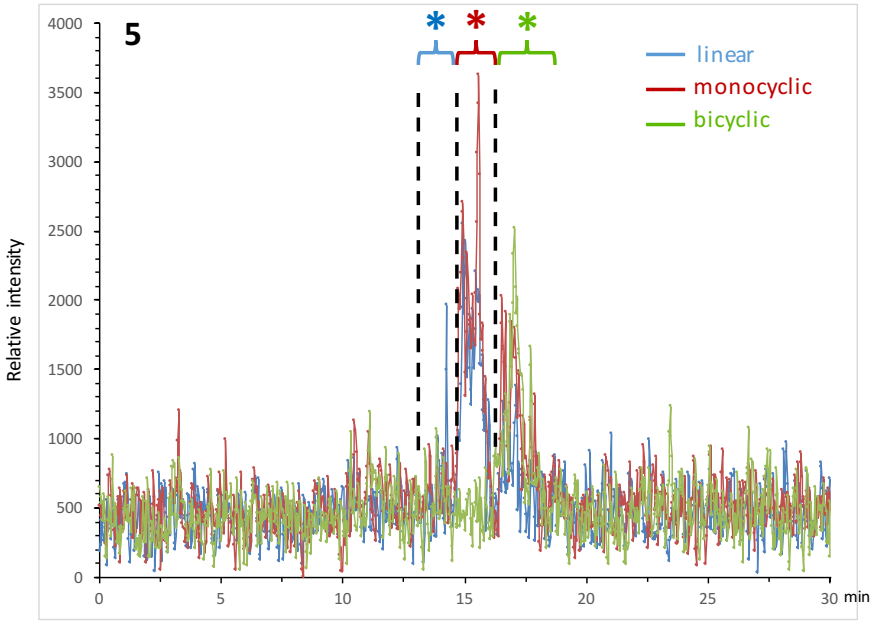


Gradient 10-40% in ACN in 30 minutes

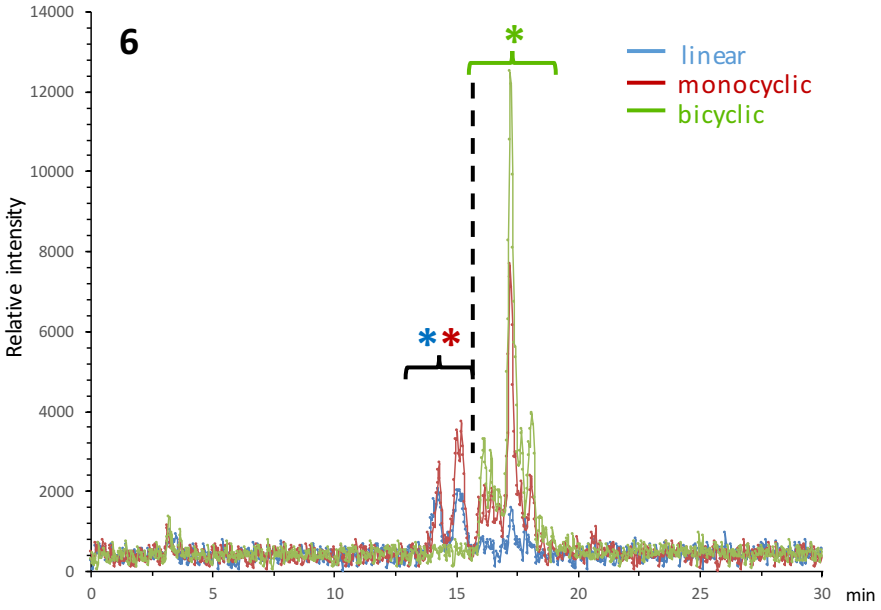



JT49-Van-CoA
[rel]
1.5
1.0
0.5
0.0
[ppm]
8
6
4
2
1.225
0.9102
3.4525
2.7631
2.5935
2.1439
0.8613
0.3765
1.0000
4.0606
2.1686
1.6834
0.5646
2.1771
0.7142
1.1306
4.8704
7.2060
2.4439
3.8837
4.9022
4.1476
1.4490

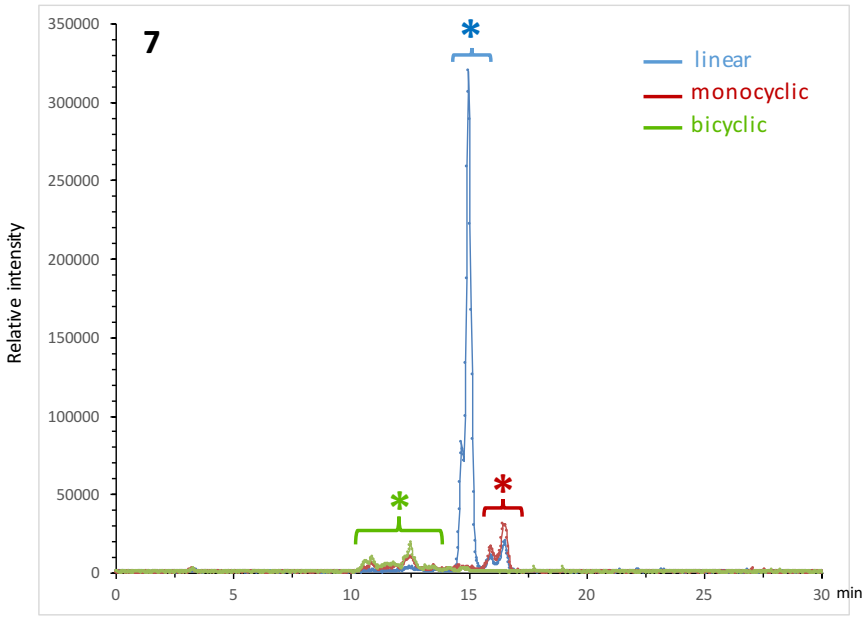
SI4. Turnover results using peptide sequences 5-8



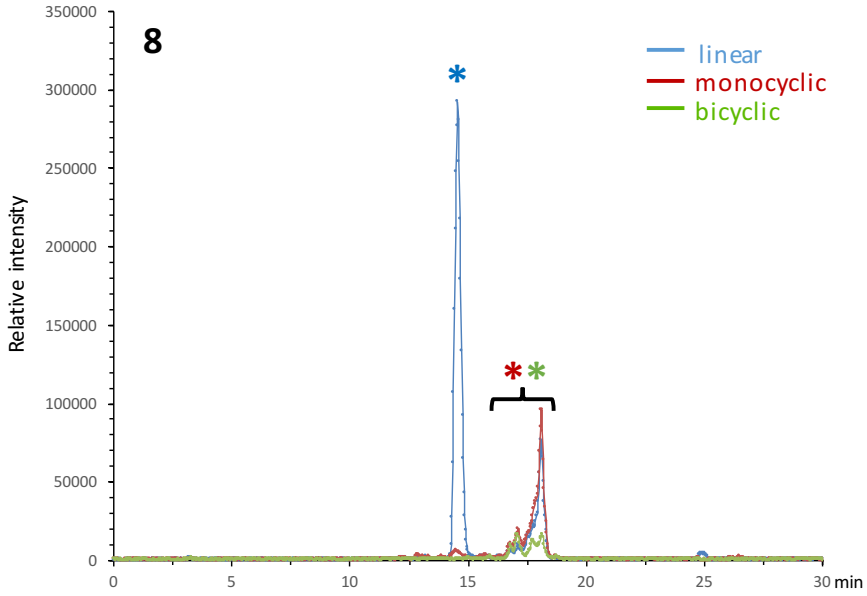
	MS Area		
	1	2	3
linear	173030.0	280823.0	162922.0
monocyclic	199583.0	348977.0	206134.0
bicyclic	169340.0	206147.0	187296.0
Total	541953.0	835947.0	556352.0
	MS Area adjustment (2 Cl)		
	1	2	3
linear	86755.9	136041.7	72979.3
monocyclic	136080.5	271671.9	135898.0
bicyclic	168678.0	207548.0	189304.0
Total	391514.4	615261.6	398181.3
	Ratio linear/monocyclic/bicyclic		
	1	2	3
linear	22.2	22.1	18.3
monocyclic	34.8	44.2	34.1
bicyclic	43.1	33.7	47.5
Total	100.0	100.0	100.0
	Turnover reates		
	1	2	3	Average	SD
monocyclic	77.8	77.9	81.7	79.1	2.2
bicyclic	43.1	33.7	47.5	41.5	7.1
OxyA activity	55.3	43.3	58.2	52.3	7.9



	MS Area				
	1	2	3		
linear	69564.0	43586.0	95374.0		
monocyclic	103953.0	44804.0	121591.0		
bicyclic	155664.0	108923.0	521930.0		
Total	329181.0	197313.0	738895.0		
	MS Area adjustment (1 Cl)				
	1	2	3		
linear	43575.8	32385.0	64976.3		
monocyclic	103953.0	44804.0	121591.0		
bicyclic	155664.0	108923.0	521930.0		
Total	303192.8	186112.0	708497.3		
	Ratio linear/monocyclic/bicyclic				
	1	2	3		
linear	14.4	17.4	9.2		
monocyclic	34.3	24.1	17.2		
bicyclic	51.3	58.5	73.7		
Total	100.0	100.0	100.0		
	Turnover reates				
	1	2	3	Average	SD
monocyclic	85.6	82.6	90.8	86.4	4.2
bicyclic	51.3	58.5	73.7	61.2	11.4
OxyA activity	60.0	70.9	81.1	70.6	10.6

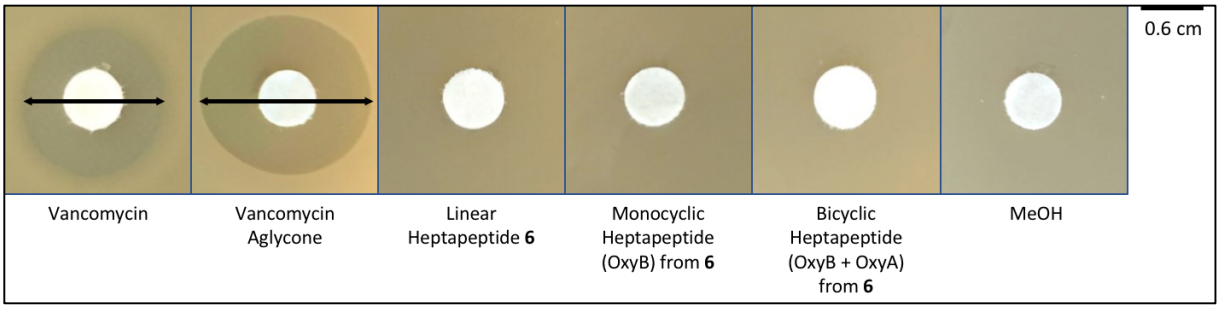


	MS Area				
	1	2	3		
linear	3387854.0	2531332.0	2765133.0		
monocyclic	370486.0	580644.0	619688.0		
bicyclic	441223.0	499486.0	891000.0		
Total	4199563.0	3611462.0	4275821.0		
	Ratio linear/monocyclic/bicyclic				
	1	2	3		
linear	80.7	70.1	64.7		
monocyclic	8.8	16.1	14.5		
bicyclic	10.5	13.8	20.8		
Total	100.0	100.0	100.0		
	Turnover reates				
	1	2	3	Average	SD
monocyclic	19.3	29.9	35.3	28.2	8.1
bicyclic	10.5	13.8	20.8	15.1	5.3
OxyA activity	54.4	46.2	59.0	53.2	6.4



	MS Area				
	1	2	3		
linear	3022883.0	2667505.0	2665704.0		
monocyclic	1136922.0	1592448.0	1869340.0		
bicyclic	475999.0	661774.0	460546.0		
Total	541953.0	835947.0	556352.0		
	MS Area adjustment (2 Cl)				
	1	2	3		
linear	3022883.0	2667505.0	2665704.0		
monocyclic	958422.4	1344282.8	1696635.3		
bicyclic	475999.0	661774.0	460546.0		
Total	4457304.4	4673561.8	4822885.3		
	Ratio linear/monocyclic/bicyclic				
	1	2	3		
linear	67.8	57.1	55.3		
monocyclic	21.5	28.8	35.2		
bicyclic	10.7	14.2	9.5		
Total	100.0	100.0	100.0		
	Turnover reates				
	1	2	3	Average	SD
monocyclic	32.2	42.9	44.7	39.9	6.8
bicyclic	10.7	14.2	9.5	11.5	2.4
OxyA activity	33.2	33.0	21.3	29.2	6.8

SI5. Agar diffusion assay of for linear, monocyclic and bicyclic forms of peptide 6 with *Bacillus subtilis* ATCC 23857



Sample [50 µg starting material]	**Inhibition zone [cm]**
Vancomycin (positive control)	1.4
Vancomycin Aglycone (positive control)	1.7
Linear heptapeptide **6**	-
Monocyclic heptapeptide (OxyB) from **6**	-
Bicyclic heptapeptide (OxyB+OxyA) from **6**	-
MeOH (negative control)	-

To test the antimicrobial activity of the turnover products, a standard agar diffusion assay was performed. To this end, 50 µg of vancomycin, vancomycin aglycone, the linear heptapeptide **6** and the monocyclic/bicyclic turnover products from **6** were dissolved in MeOH and pipetted onto sterile 0.6 cm filter papers. Methanol was used as a negative control. After 30 min, the dry filter papers were transferred onto LB plates spread with the test organism *Bacillus subtilis* ATCC 23857 and incubated at 37 °C overnight. The antimicrobial activity was analysed by measuring the inhibition zone around the disc.