Supporting Information

Modulation of backbone flexibility for effective dissociation of antibacterial and hemolytic activity in cyclic peptides

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Content:

List of abbreviations

Boc, *tert*-butyloxycarbonyl group;

CFU, colony-forming units;

COMU, (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate;

DCM, dichloromethane;

DIEA, diisopropylethylamine;

Dmb, dimethoxybenzyl gorup

DMF, dimethylformamide;

DMTMM · BF_4, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate

Et_2O, diethyl ether;

EtOH, ethanol;

Fmoc, 9-Fluorenylmethoxycarbonyl group;

MeCN, acetonitrile;

MHB, Müller-Hinton broth;

MIC, minimum inhibitory concentration;

Oxyma, ethyl (hydroxyimino)cyanoacetate;

PTFE, polytetrafluoroethylene;

TFA, trifluoroacetic acid;

TIS, triisopropylsilane;

List of materials

Disposable 5-ml polypropylene reactors fitted with a PTFE filter were acquired from Thermo Scientific. Tentagel S RAM resin, COMU, Oxyma, TFA, piperidine and most Fmoc-protected amino acids were purchased from Iris-Biotech GmbH. Fmoc-Asp-ODmb was purchased from Bachem GmbH. DIEA, DMTMM · BF_4 and TIS were from Sigma-Aldrich. Lipids were from Avanti Ltd. All solvents were from VWR: DMF (synthesis grade), DCM (optical grade), MeCN (optical grade).

All reagents and solvents were used without further purification.

Methods

General procedure for solid-phase peptide synthesis. Peptides were synthesized manually in 5-ml polypropylene reactors fitted with a PTFE filter. The synthesis scale was 0.04 mmol. The resin

(Tentagel S RAM 0.2 mmol/g) was allowed to swell overnight in DMF then washed with DMF (5×). Chain elongation was achieved with single couplings using 5 equivalents of amino acid, Oxyma and COMU each, and 10 equivalents of DIEA (based on declared resin loading). Fmoc-protected amino acids were dissolved in DMF at a concentration of 0.6 M; they were then activated by the sequential addition of Oxyma/COMU (0.6 M in DMF) and of DIEA, and the mixture was immediately transferred into the reactor. Oxyma and COMU were kept dry until immediately before coupling. The reactor was placed on a shaker and couplings were allowed to proceed for 1:15h. The Fmoc-group was removed by treatment with a 20% (v/v) piperidine solution in DMF (3 × 4 min). Before and after the piperidine deprotection procedure, the resin was washed with DMF (3×), DCM (3×) and DMF again (5×).

Peptide Macrocyclization. After removal of the terminal Fmoc-group, the resin was washed with DCM (5×) and then treated with a 1.5% (v/v) TFA/DCM solution (6 × 5 min) to remove the Dmb-group. The resin was then washed again with DCM (5×) and dried *in vacuo*. The dry resin was allowed to re-swell in DMF for 2 h. A solution of DMTMM · BF_4 (3 equiv.) in sufficient DMF to cover the resin was added to the reaction vessel, followed by DIEA (7 equiv.). The reaction was allowed to proceed overnight and completion was verified with the TNBS test (Hancock et al., *Anal. Biochem.* **1976,** *71* (1), 260-264).

Peptide release from the solid support. After cyclization, the resin was washed with DMF (5×), DCM (3×), EtOH (5×) and dried *in vacuo*. The release of cyclic peptides from the solid support was performed with a 95% TFA/H_2O/TIS solution for 2h (approx. 3 ml per 100 mg of resin). The cleavage solution was collected and concentrated down to ~300 µl with a gentle stream of N_2, then the peptides were precipitated and washed with Et_2O (3×). After spontaneous evaporation of the residual Et_2O, peptides were dissolved in H_2O and minimal MeCN and freeze-dried before analysis.

Peptide characterization. Identity verification was performed on a Bruker Microflex MALDI-ToF-MS using an α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in H_2O:MeCN:TFA, 50:47.5:2.5). Observed *m/z* values were within a ±0.02% range from theoretical values. Peptide purity was assessed by analytical RP-HPLC on a system consisting of a Waters 600 pump connected to a Waters Symmetry C18 column and a Waters 2996 PDA detector. Crude peptides were subjected to preparative RP-HPLC and isolated in ≥95% purity.

Compound 1: t_R = 17.2 min (RP-HPLC); purity = 97.6%; *m/z* calculated for $C_{71}H_{93}N_{14}O_{10}$ $[MH^+]$ = 1301.71, found = 1301.68. Yield after purification: 22%.

Compound 2: t_R = 17.0 min (RP-HPLC); purity = 96.3%; *m/z* calculated for $C_{73}H_{97}N_{14}O_{10}$ $[MH^+]$ = 1329.74, found = 1329.69. Yield after purification: 18%.

Compound 3: t_R = 17.6 min (RP-HPLC); purity = 98.5%; *m/z* calculated for $C_{75}H_{101}N_{14}O_{10}$ $[MH^+]$ = 1357.77, found = 1357.61. Yield after purification: 16%.

Compound 4: t_R = 18.0 min (RP-HPLC); purity = 99.6%; *m/z* calculated for $C_{77}H_{105}N_{14}O_{10}$ $[MH^+]$ = 1385.81, found = 1385.77. Yield after purification: 16%.

Compound 5: t_R = 18.8 min (RP-HPLC); purity = 100%; *m/z* calculated for $C_{79}H_{109}N_{14}O_{10}$ $[MH^+]$ = 1413.84, found = 1413.73. Yield after purification: 14%.

Compound 6: t_R = 15.9 min (RP-HPLC); purity = 100%; *m/z* calculated for $C_{73}H_{98}N_{15}O_{10}$ $[MH^+]$ = 1344.75, found = 1344.73. Yield after purification: 17%.

Compound 7: t_R = 15.9 min (RP-HPLC); purity = 100%; *m/z* calculated for $C_{75}H_{102}N_{15}O_{10}$ $[MH^+]$ = 1372.79, found = 1372.76. Yield after purification: 21%.

Compound 8: t_R = 16.9 min (RP-HPLC); purity = 99.6%; *m/z* calculated for $C_{73}H_{97}N_{14}O_{11}$ $[MH^+]$ = 1345.74, found = 1345.69. Yield after purification: 23%.

Compound 9: t_R = 17.2 min (RP-HPLC); purity = 100%; *m/z* calculated for $C_{75}H_{101}N_{14}O_{12}$ $[MH^+]$ = 1389.76, found = 1389.47. Yield after purification: 16%.

Peptide quantification. Triplicate samples of **1** were subjected to thermogravimetric analysis (TGA) using a Perkin Elmer Pyris 1 with the following heating procedure: from room temperature (26 °C) to 120 °C at 10 °C/min; hold for 20 min at 120 °C; from 120 °C to 200 °C at 10 °C/min. In parallel, the UV absorbance of aqueous solutions of all peptides was measured in a 1 cm quartz cuvette using a Cary50 spctrophotometer. The molar absorptivity (ε) of **1** was calculated using the formula $\varepsilon = A / (l \times c)$, where A is the measured maximum UV absorbance (λ_{MAX} = 259 nm), l is the light path of the cuvette (1 cm) and c is the corrected concentration (M) based on the results from TGA. The ε value of **1** was employed for **2-9** as well, and each solution was quantified based on the respective UV absorbance.

Antimicrobial activity. Minimum inhibitory concentration (MIC) for all peptides was determined with the tube microdilution method according to CLSI standards. Bacteria were grown in Müller-Hinton broth enriched in divalent cations (MHB-II). Aliquots of 50 µl of bacterial suspension (≈1 × 10^6 CFU/ml) in exponential growth phase were mixed with 50 µl of peptide solutions in MHB-II arranged in a 2-fold dilution series. Plates were incubated at 37 °C for 18 h and MIC values were assigned upon visual assessment. Tests were performed in triplicate. In case of discording values, the value recurring two times out of three is reported (e.g. 4, 4, 8; MIC = 4), or the intermediate value for a trio differing by one dilution (e.g. 2, 4, 8; MIC = 4). A negative control (no antibiotic), a sterility control (no inoculum) and a positive control (gentamicin) were included. MIC values for gentamicin (µg/mL): *S. aureus*, 1; *E. coli*, 0.25; *E. faecium*, 128; *K. pneumoniae*, 8; *P. aeruginosa*, 1; *A. baumannii*, 128.

Time-kill curves were measured by growing single colonies of ATCC 29213 in MHB-II. An overnight cultures were diluted 1:100 in 50 ml of preheated (37 °C) MHB-II and transferred into Erlenmeyer flasks placed in a water bath under shaking. When the cultures reached OD_{600} = 0.1 they were diluted to OD_{600} = 0.002, divided into fresh flasks and treated with the test compound. Spot-plating was performed in triplicate at selected time points by transferring 10 µl of a 10-fold diluted suspension on an agar plate.

Hemolytic activity. Peptide-induced haemolysis was evaluated as previously described (Oddo et al., *Eur. J. Med. Chem.* **2015,** *102*, 574-581). Briefly, 75 µl of peptide solution in phosphate-buffered saline (PBS) were mixed in triplicate with 75 µl of a 0.5% (v/v) RBC suspension in PBS prepared from a fresh 0^+ blood sample in EDTA. After incubation at 37 °C for 1h, the plates were centrifuged. Hemoglobin content in the supernatant (60 µl) was measured with a Molecular Devices VersaMax Microplate Reader (λ = 414 nm) after transfer to a clear polystyrene plate. Results were normalized with respect of a positive (melittin) and negative (PBS) control.

Standard deviations (expressed in % hemolysis)

Peptide no.	Data point (sample concentration, µM)							
	300	**150**	**75**	**37.5**	**18.75**	**9.375**	**4.7**	**2.35**
1	1.56	1.70	2.45	0.82	0.47	0.47	0	0.47
2	1.25	0.81	0.47	0.47	0.47	0.81	1.25	1.26
3	1.81	1.70	6.60	2.87	1.70	1.25	2.36	3.56
4	3.12	3.56	3.09	5.25	2.16	0.94	1.25	0.00
5	3.73	3.68	2.45	3.68	2.45	1.88	3.30	2.05
6	1.7	0.47	0.94	0.47	0.47	0.47	0.81	0.81
7	2.16	1.25	1.25	0.47	0.47	0.94	0.82	0.82
8	7.3	1.70	0.47	0.47	0.00	1.25	0.00	0.00
9	0.47	0.95	0.47	0.47	0.82	0.94	0.94	0.47

General procedure for liposome preparation. $CHCl_3$ solutions of the selected phospholipids were mixed to achieve a 1:3 molar ratio. $CHCl_3$ was removed and the residue was dried *in vacuo* overnight, then resuspended in a 0.1 M inorganic buffer (90% NaCl + 10% $NaHCO_3$, 1 ml for each 1 mg of combined lipids). The mixture was freeze-thawed (5×) and extruded 10 times through a membrane (pore size: 100 nm) under N_2 pressure. The extruder was maintained at a temperature of 50 °C.

Circular dichroism. Spectra were acquired on a Jasco J-810. Peptides stock solution in water were diluted to 160 µM concentrations, then mixed with equal volumes of either inorganic buffer (0.1 M, 90% NaCl + 10% $NaHCO_3$) or liposome suspension (800 µM lipid concentration) in the same inorganic buffer. Spectra were acquired at 37 °C from a 2 mm quartz cuvette and processed as follows: the baseline (blank) was subtracted, then spectra were normalized and smoothed using a 5-point Savitzky-Golay algorithm in Jasco Spectra Analysis.

Marker release assays. $CHCl_3$ solutions of 1,2-dioleoyl-glycero-3-phosphocholine (DOPC) and 2-oleoyl-1-palmitoyl-glycero-3-phosphoglycerole (POPG) were mixed to achieve a 1:3 molar ratio. $CHCl_3$ was removed and the residue was dried *in vacuo* overnight, then resuspended in a 50 mM sulforhodamine B solution (1 ml for each 1 mg of combined lipids) in buffer (0.05 M, 90% NaCl + 10% $NaHCO_3$). The mixture was freeze-thawed (5×) and filtered through a PD-10 column, which had been conditioned with the same buffer. The darker fractions, not equilibrating with the gel

material, were collected and extruded as described in the general procedure. The liposome suspension was diluted with the same buffer to a concentration of approx. 12 μM with respect of total lipids. Aliquots of 100 μl were mixed with 1-5 μl of peptide solutions in water and rapidly introduced into a Biotek Synergy H-4 plate reader. Fluorescence emission intensity (Exc: 566 nm; Em: 586 nm) was measured in 1 min intervals for 20 min. Results have been normalized with respect of a negative (no peptide) and positive (10 μl of 20% v/v Triton-X) control and smoothed with a 3-point moving average in Excel. Experiments were performed in duplicate.

Reverse-phase HPLC Setup

For all experiments:

- **Buffer A:** 99.9% Water, 0.1% TFA
- **Buffer B:** 89.95% MeCN, 9.95% Water, 0.1% TFA

Analytical HPLC

- **Pump:** Waters™ 600 Pump
- **Detector:** Waters™ 2996 Photodiode Array Detector
- **Column:** Waters™ Symmetry™ C18, 4.6 x 250 mm, 5 μm

Gradient:

Time (min)	Flow (ml/min)	Buffer A	Buffer B
0.0	1.50	100%	0%
20.0	1.50	30%	70%
22.0	1.50	0%	100%
27.0	1.50	0%	100%
30.0	1.50	100%	0%

Equilibration delay between injections (both methods): 9 min with 100% Buffer A, 1.50 ml/min.

The ApexTrack™ algorithm has been used for peak integration. Peaks having an area <0.01% were not included in the purity calculations.

Spectra were processed as follows: the raw data were exported using the Waters Empower 3 software and imported in Microsoft Excel. The baseline (blank) was subtracted and negative absorbance values were corrected to 0 (zero).

Preparative HPLC

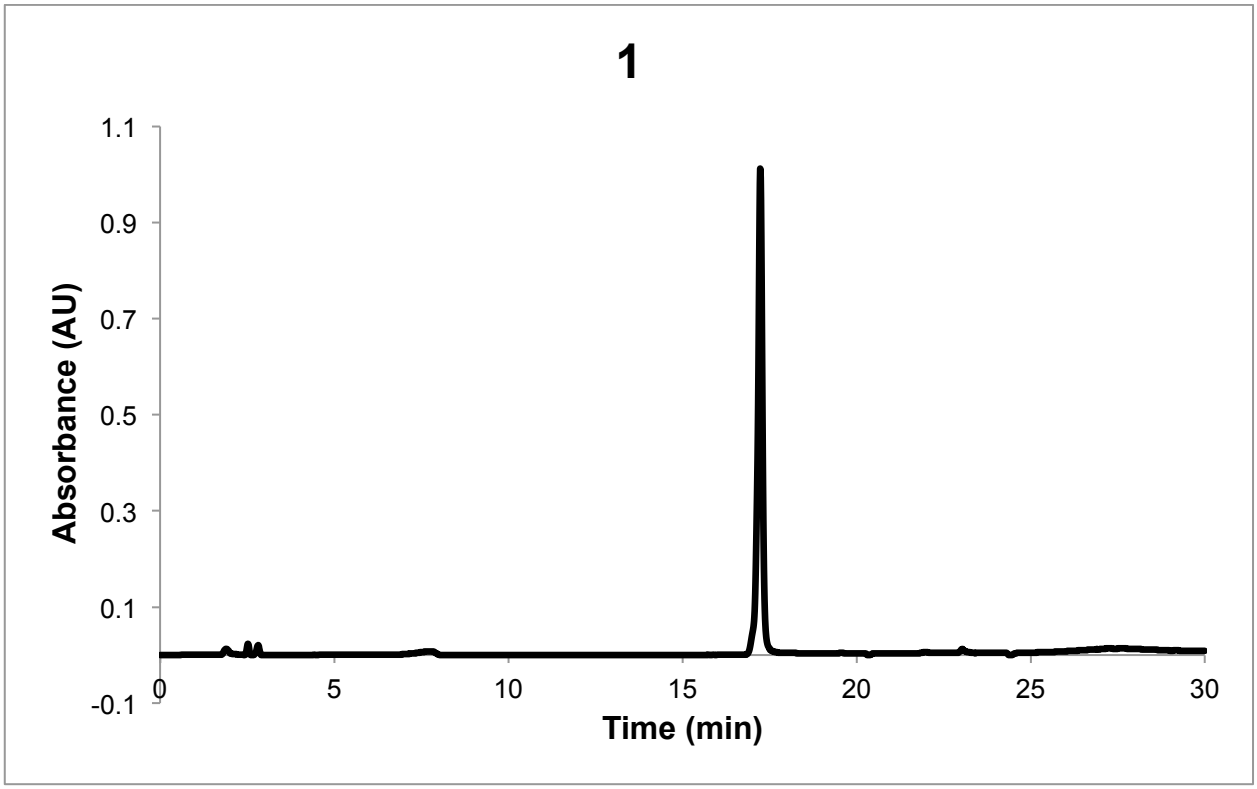
- Pump: Waters™ 600 Pump
- Detector: Waters™ 996 Photodiode Array Detector
- Column: Waters™ XBridge™ BEH130 C18, 10 x 250 mm, 5 µm

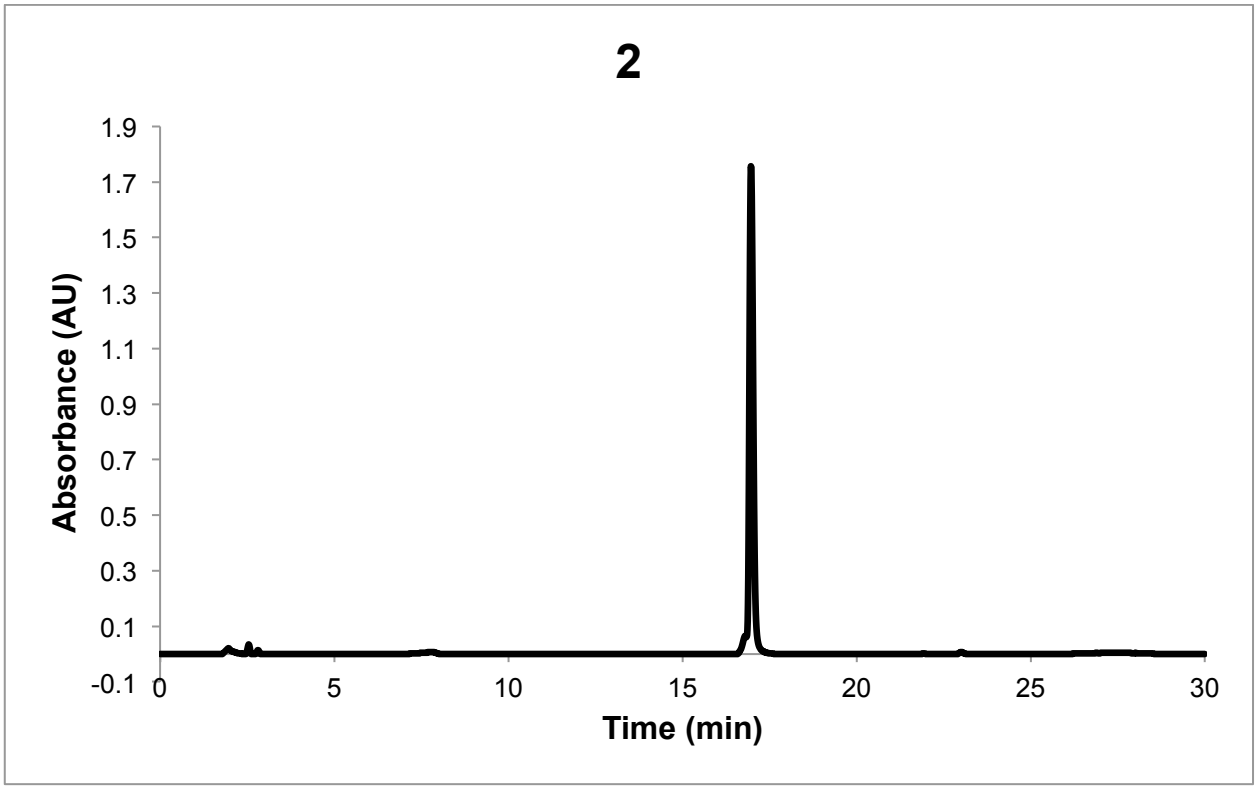
Gradient:

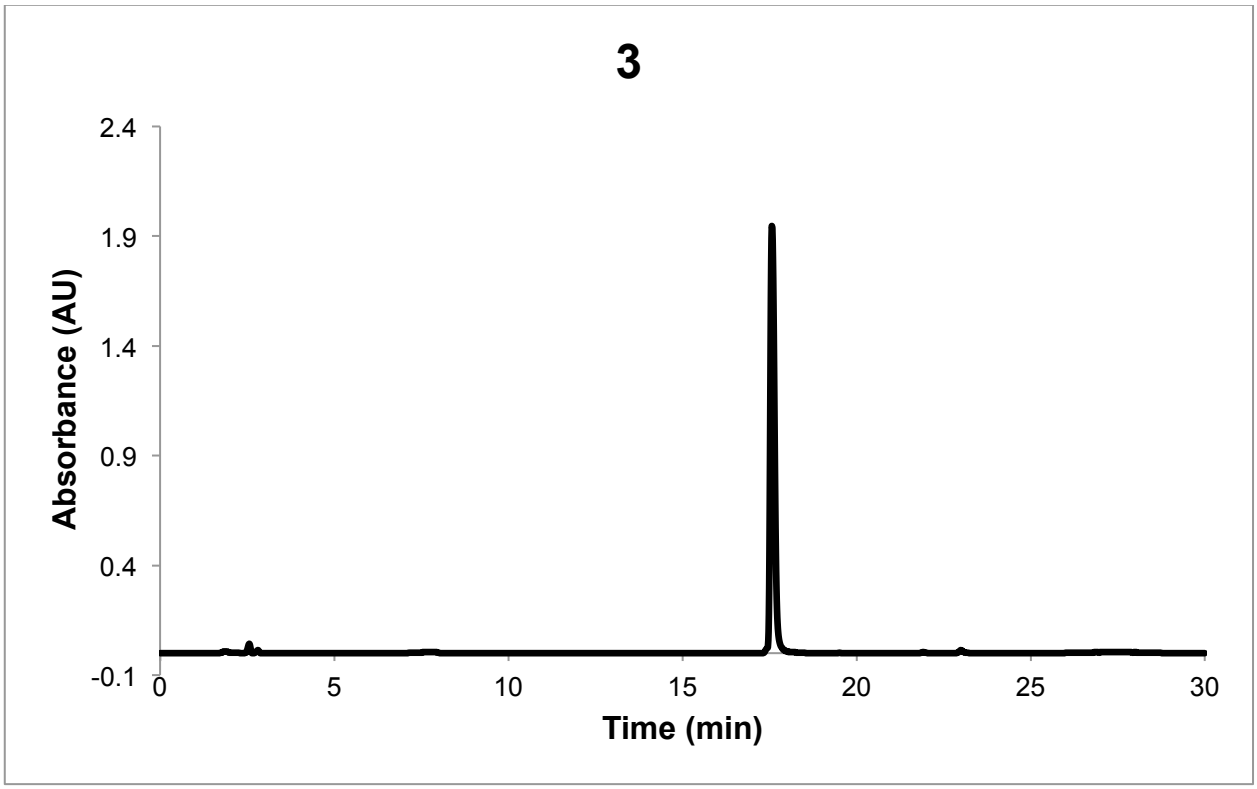
Time (min)	Flow (ml/min)	Buffer A	Buffer B
0.0	4.00	100%	0%
1.0	6.00	100%	0%
3.0	8.50	100%	0%
5.0	8.50	90%	10%
20.0	8.50	45%	55%
26.0	9.00	0%	100%
27.0	9.00	0%	100%
30.0	9.00	100%	0%
39.0	9.00	100%	0%

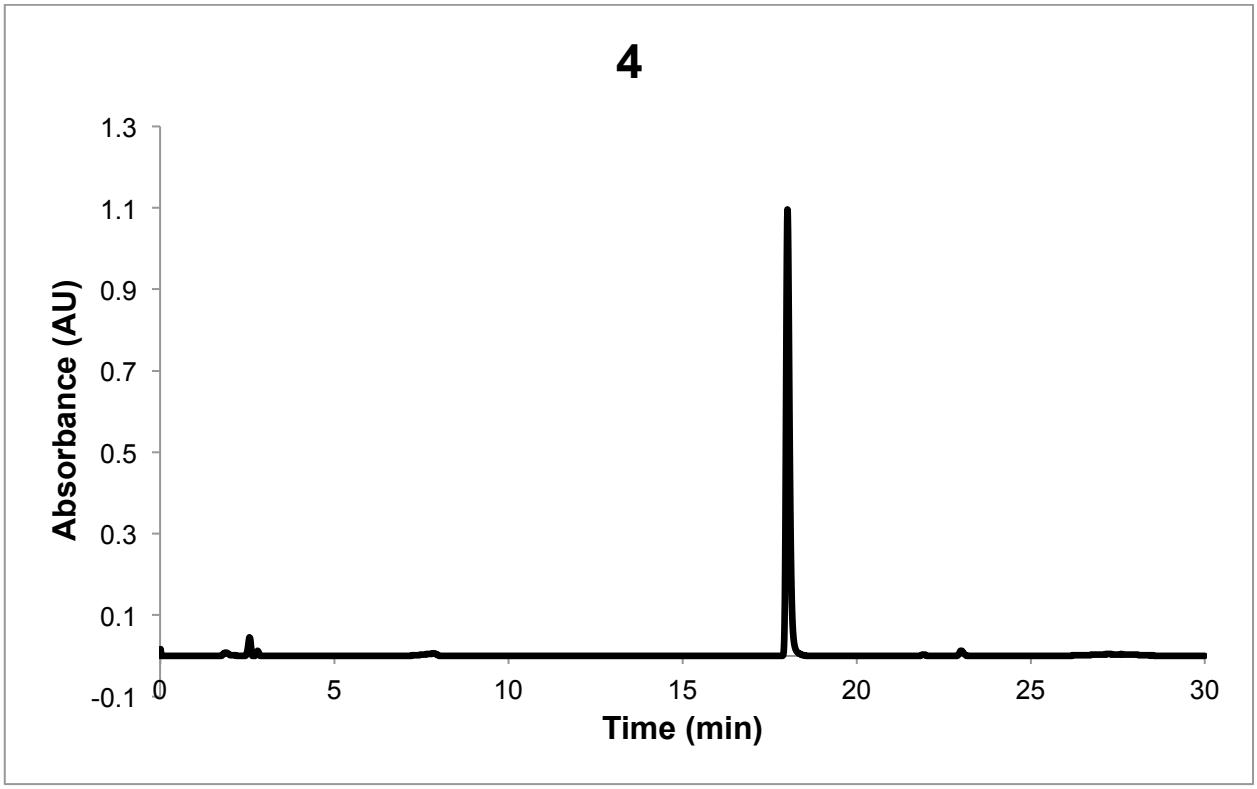
Purified fractions were collected manually.

Chromatograms (PDA @220 nm)






3
Absorbance (AU)
2.4
1.9
1.4
0.9
0.4
-0.1
0
5
10
15
20
25
30
Time (min)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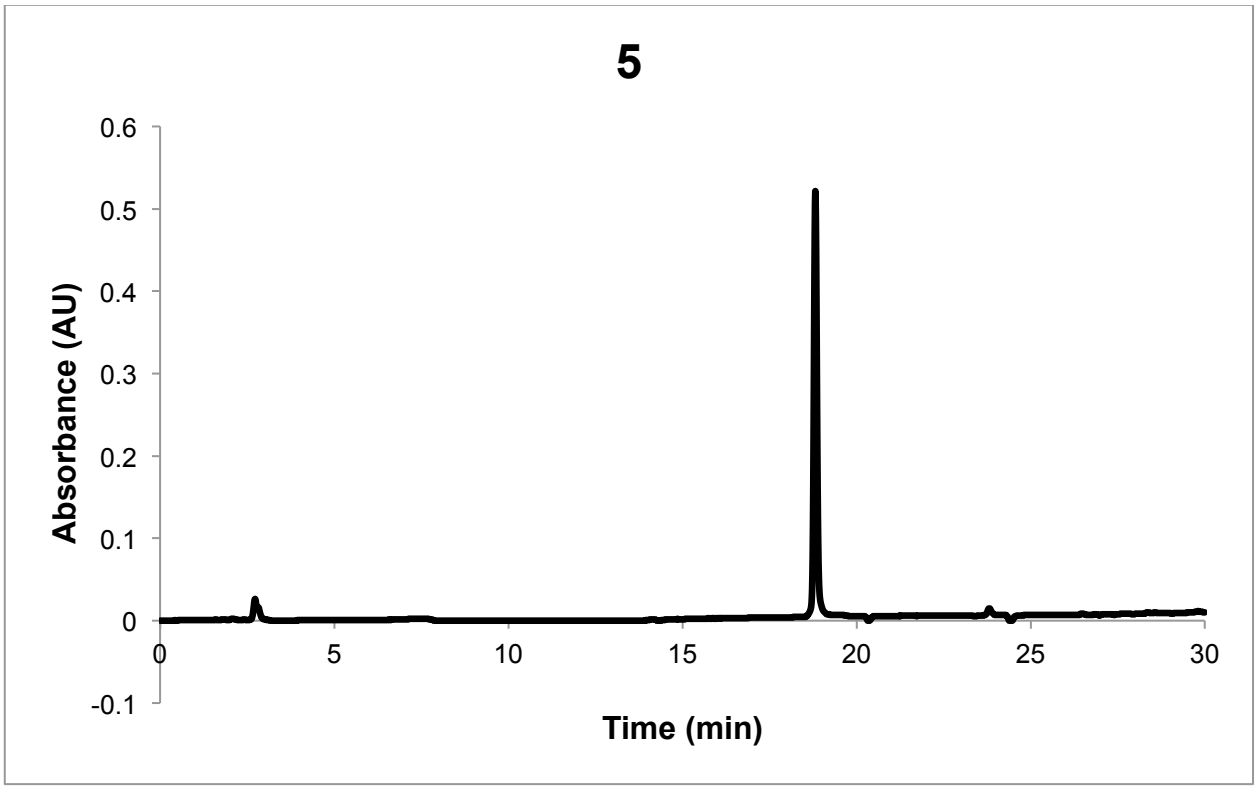
Absorbance (AU)
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
5
10
15
20
25
30
Time (min)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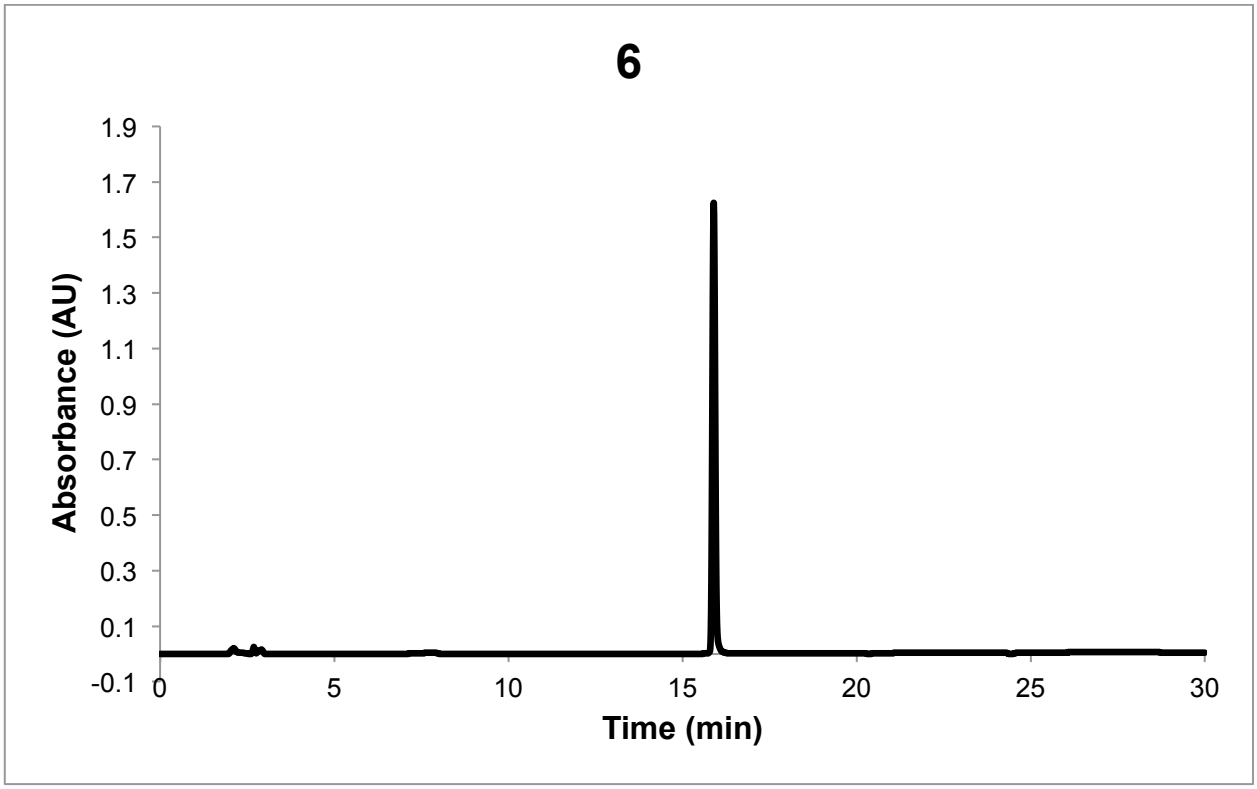
0
5
10
15
20
25
30
Time (min)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Absorbance (AU)
1.9
1.7
1.5
1.3
1.1
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0.5
0.3
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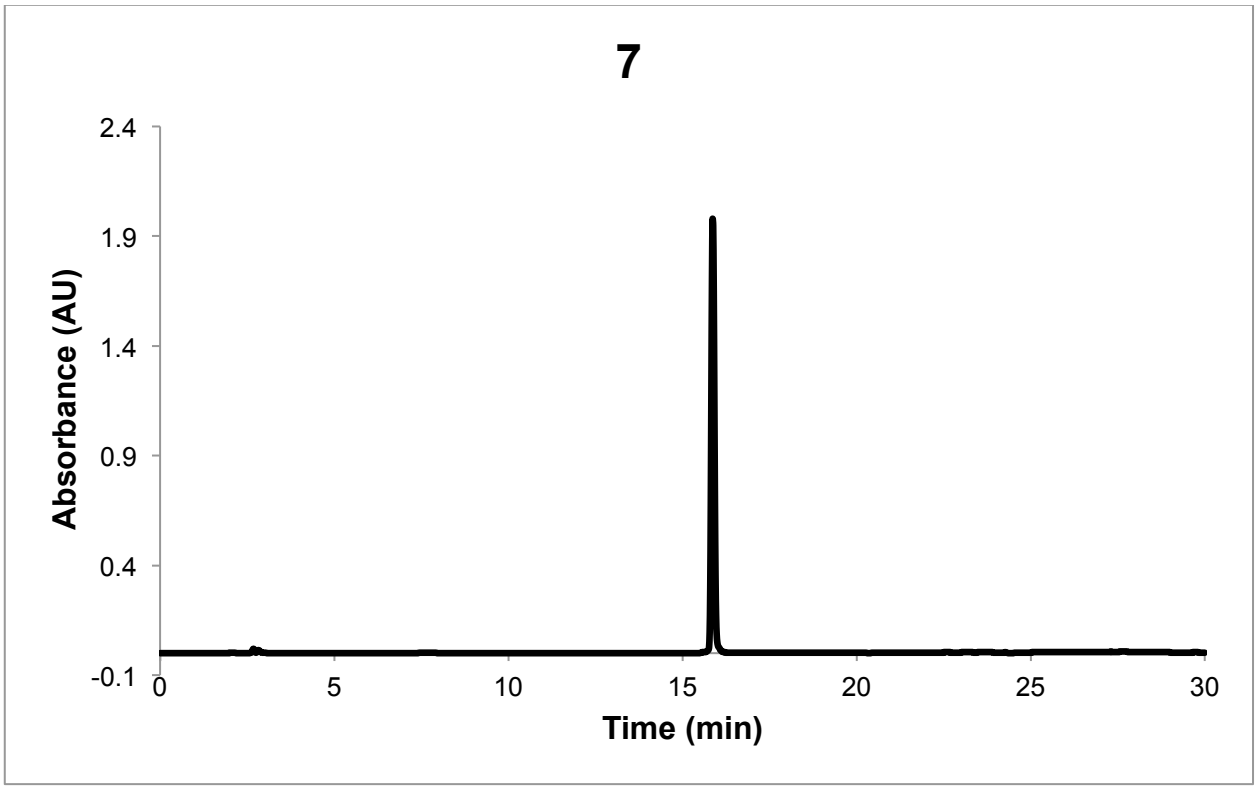
0
5
10
15
20
25
30
Time (min)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Absorbance (AU)
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1.4
0.9
0.4
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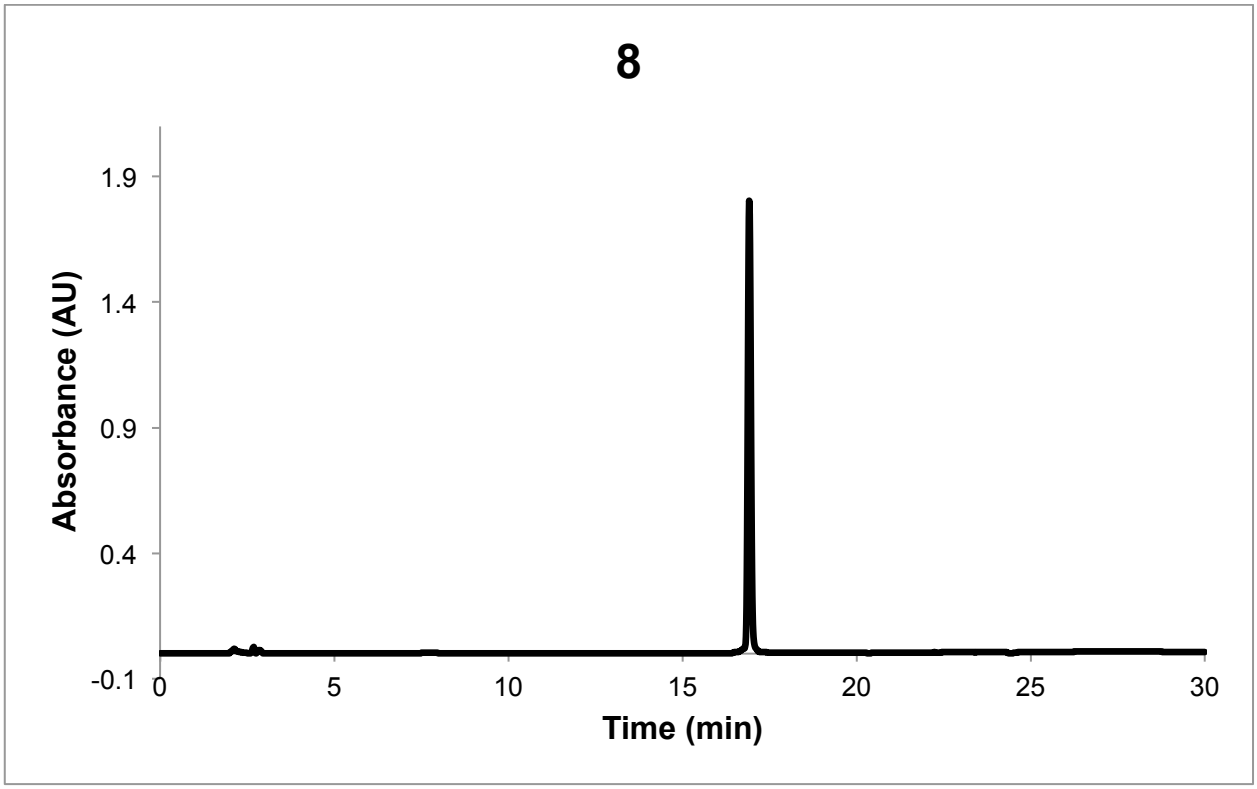
0
5
10
15
20
25
30
Time (min)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Absorbance (AU)
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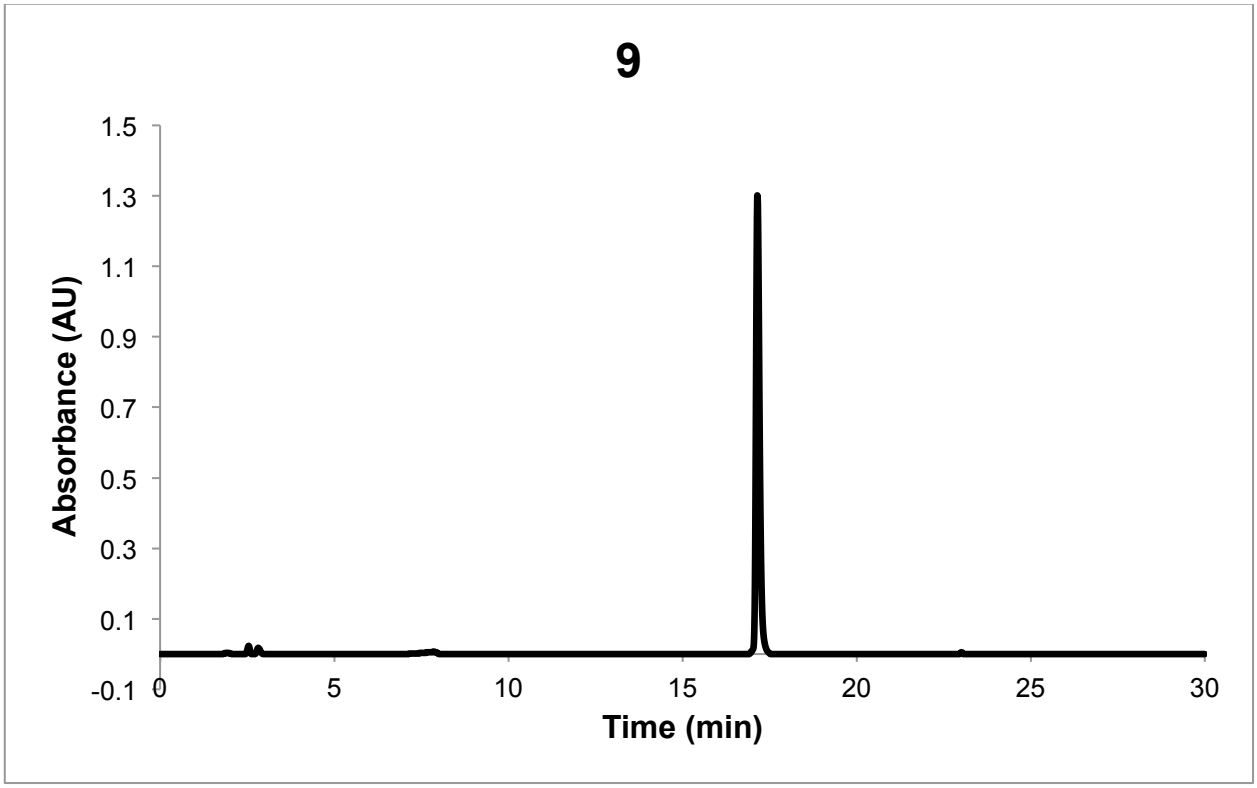
1.4
0.9
0.4
-0.1
0
5
10
15
20
25
30
Time (min)


9
Absorbance (AU)
1.5
1.3
1.1
0.9
0.7
0.5
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0.1
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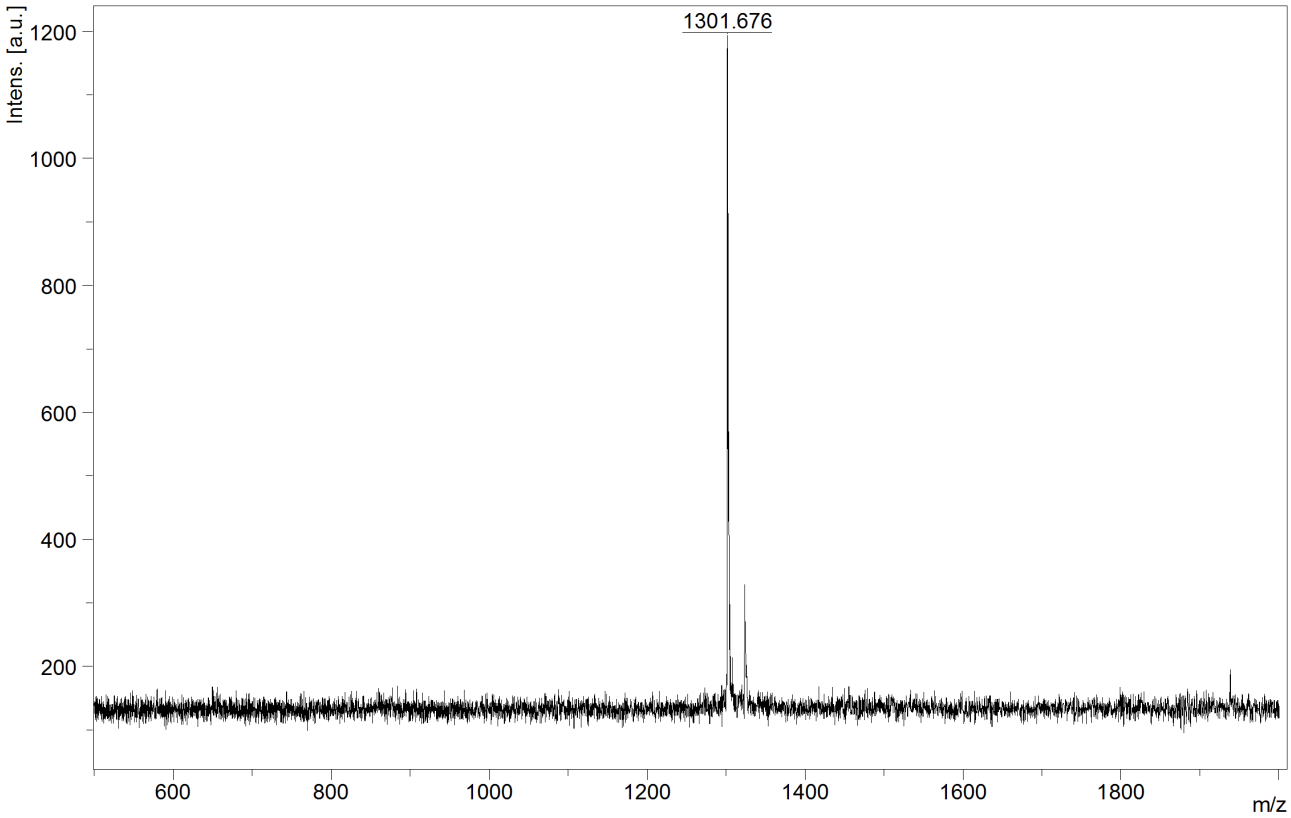
0
5
10
15
20
25
30
Time (min)

MALDI Spectra

Compound 1



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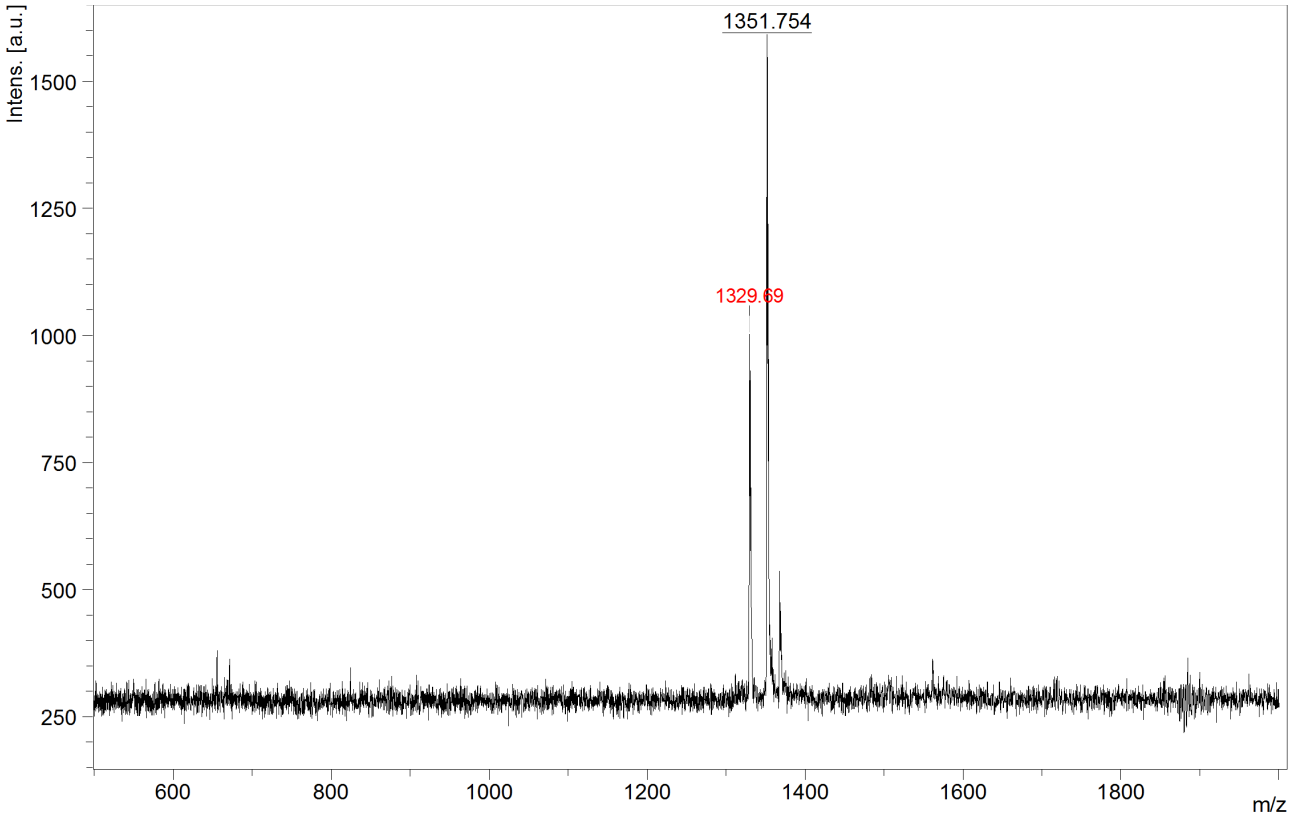

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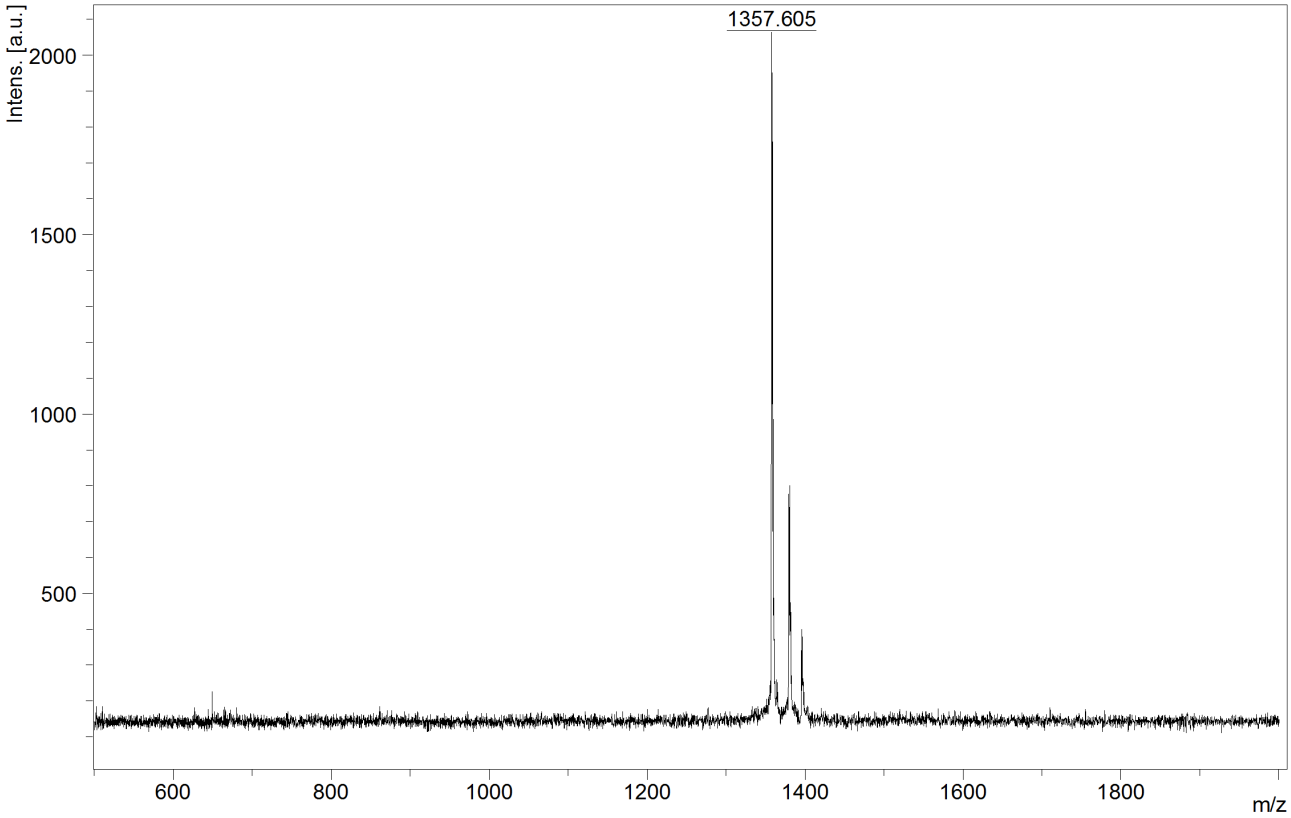

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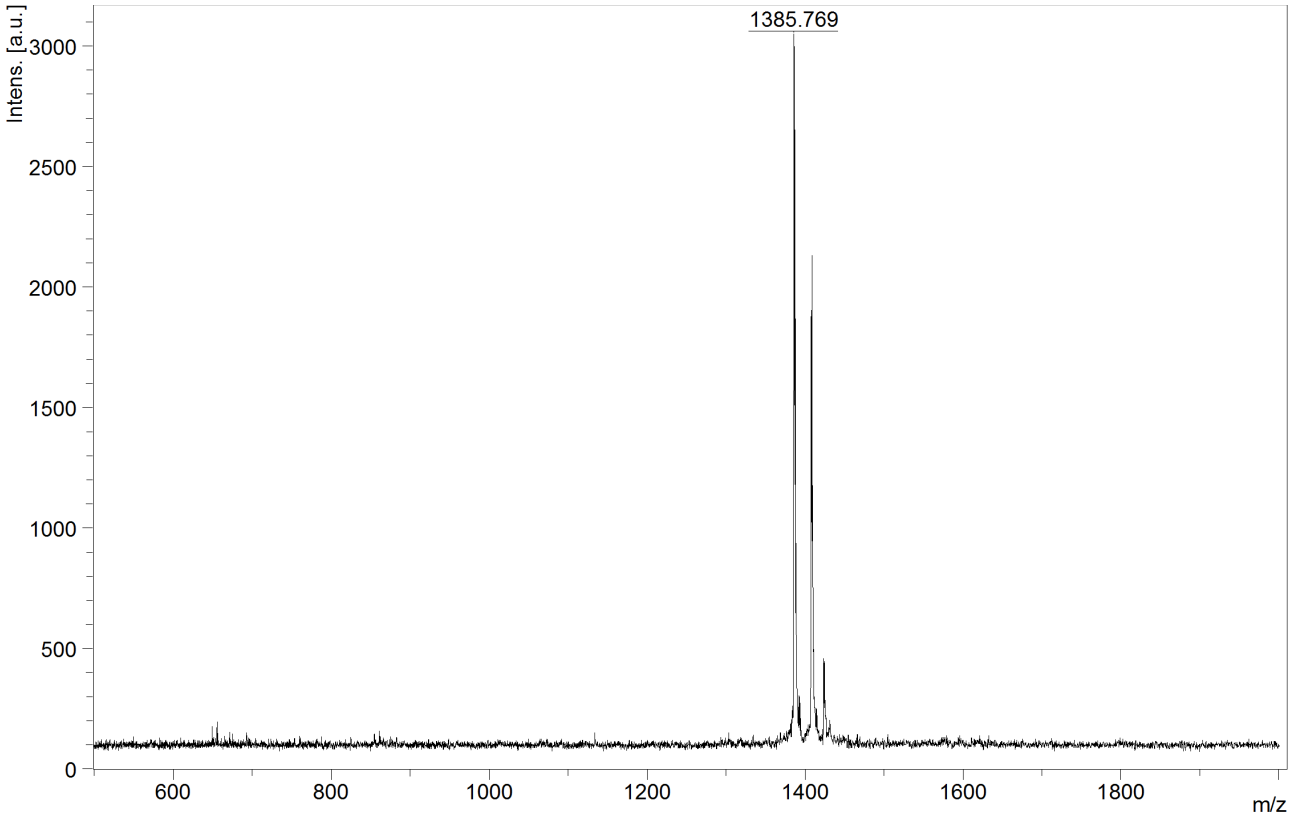

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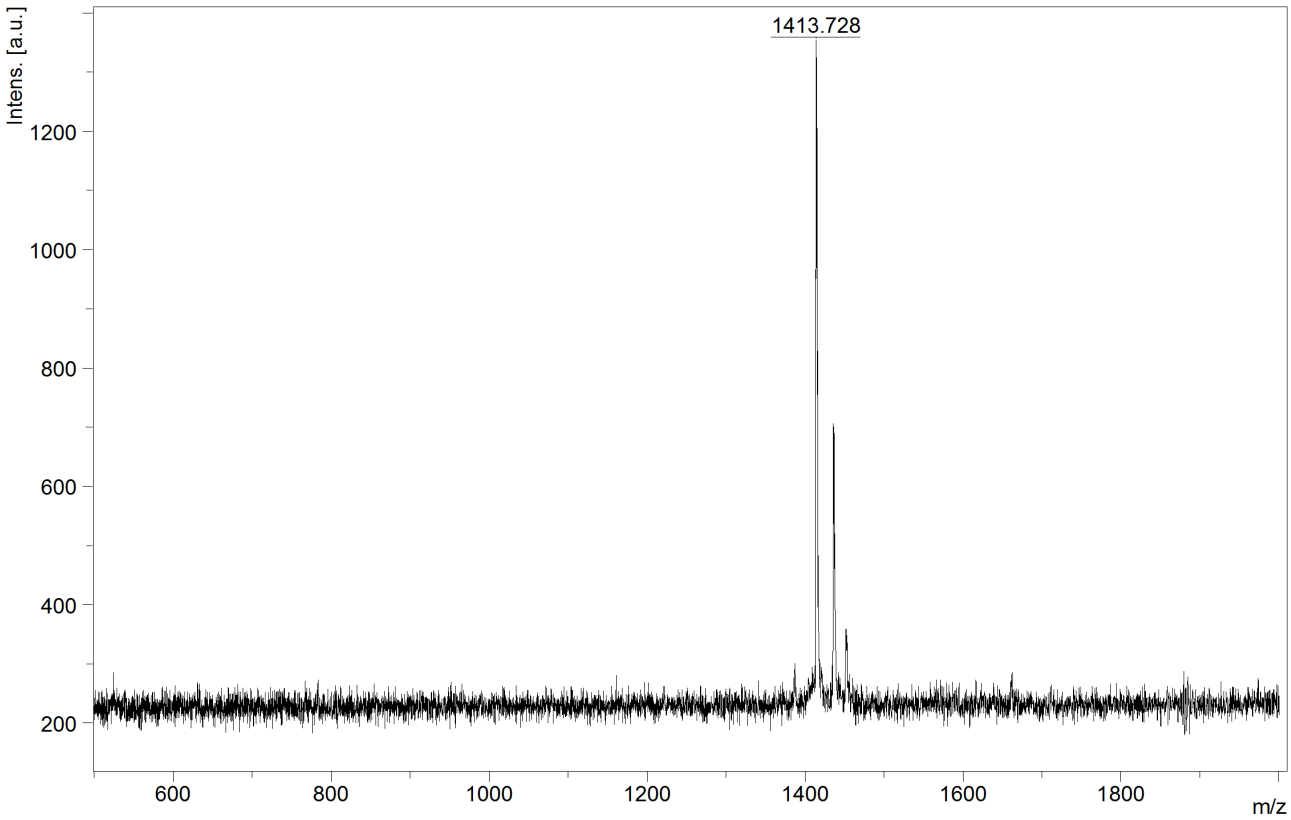

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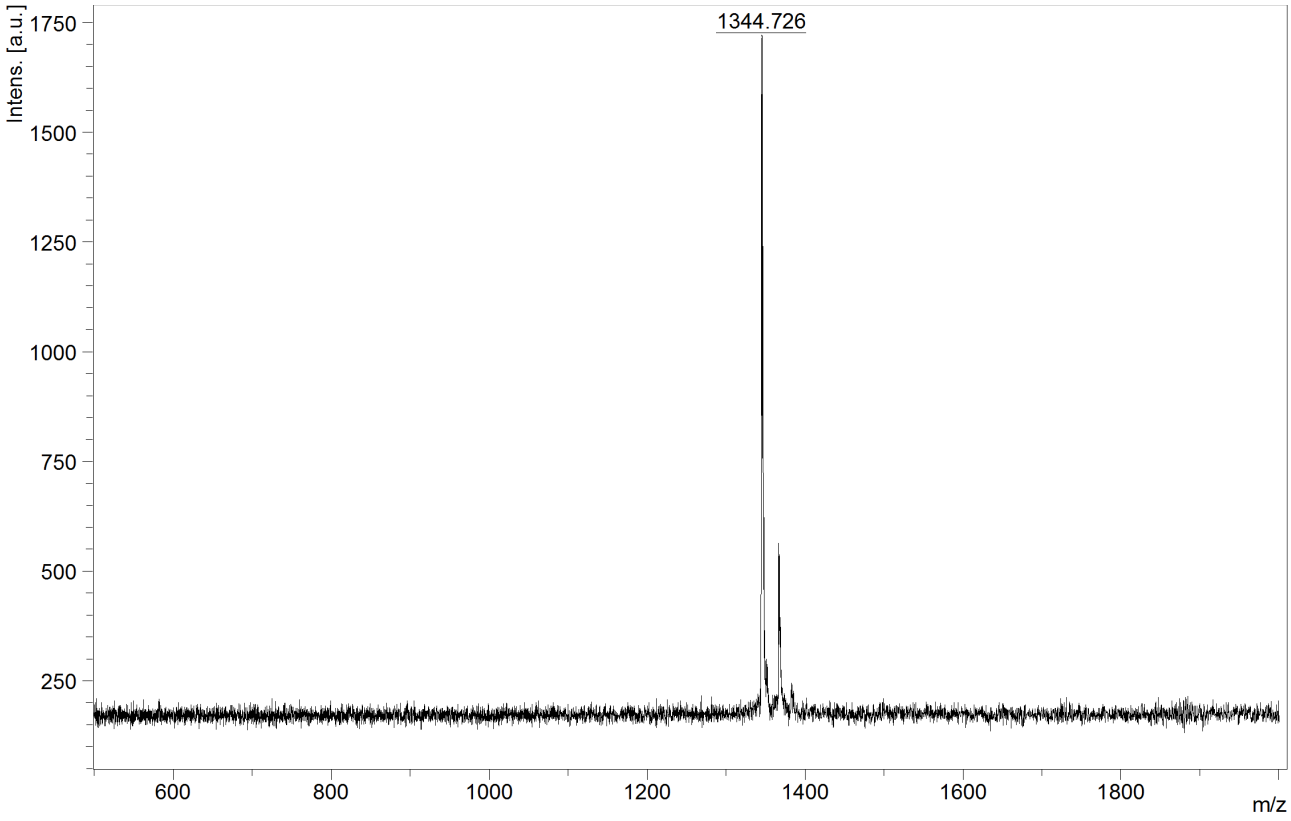

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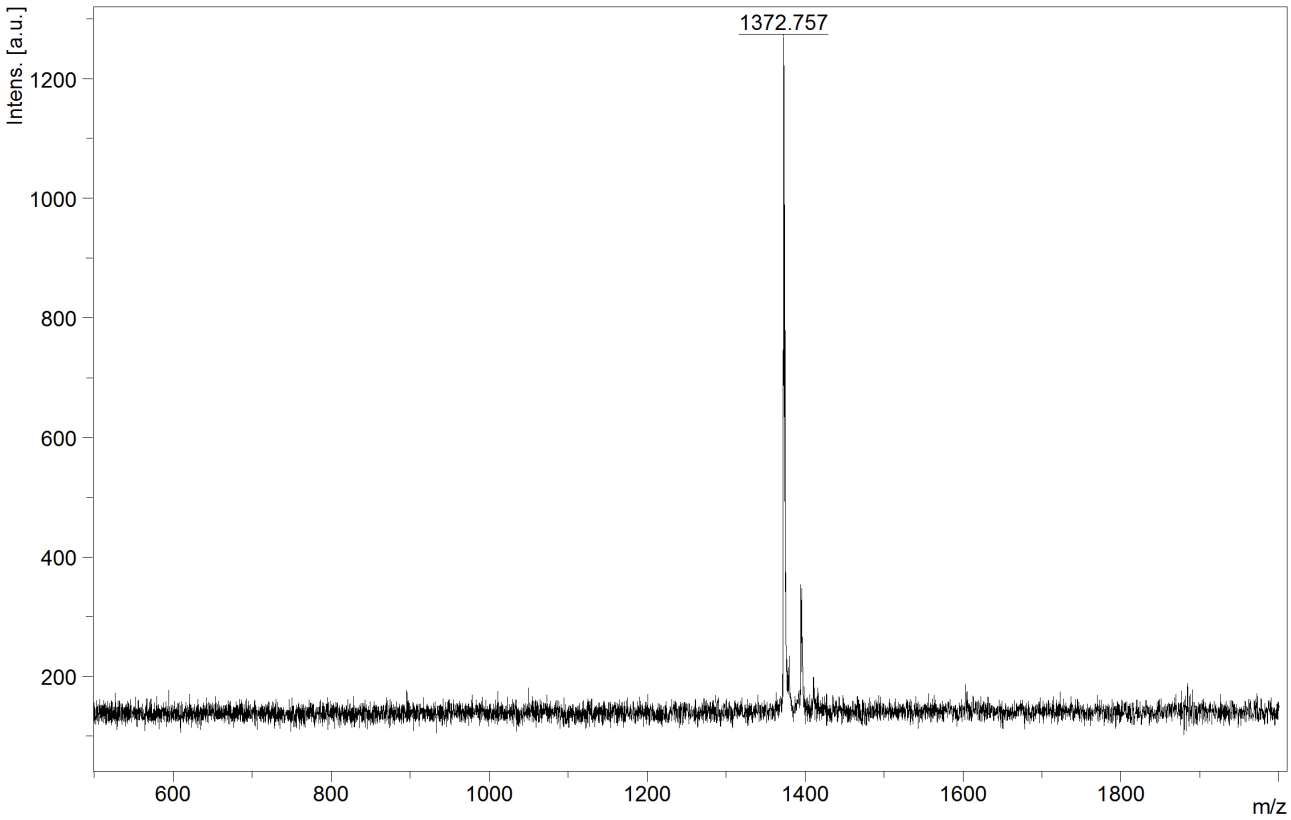

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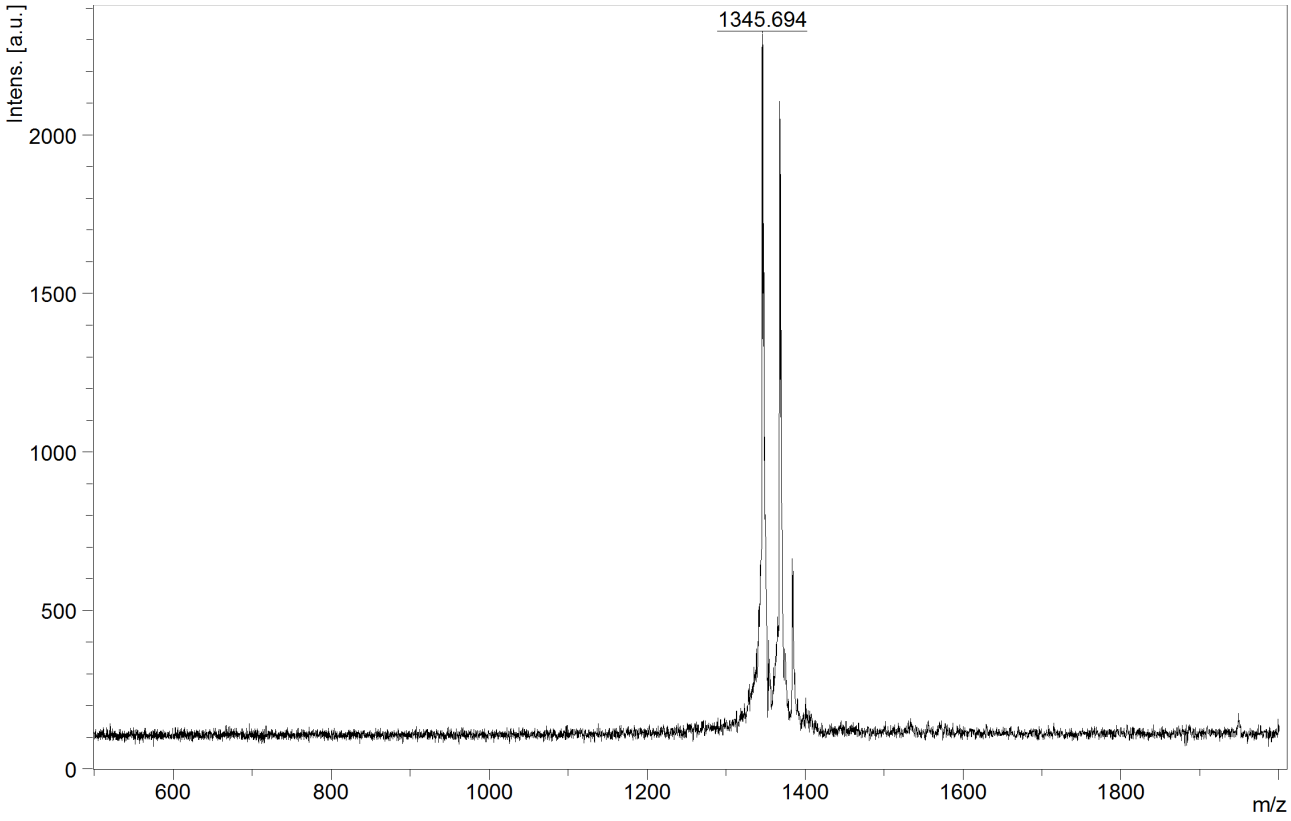

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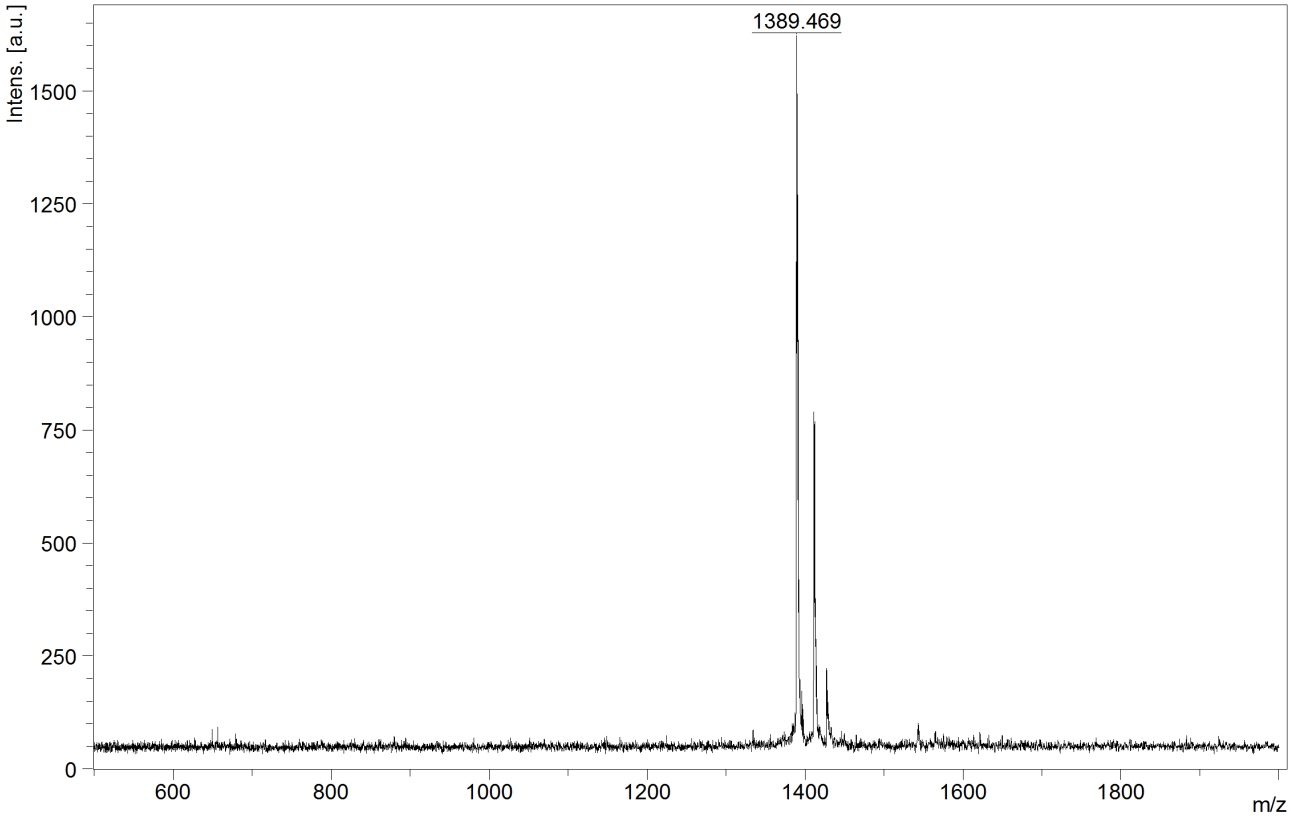
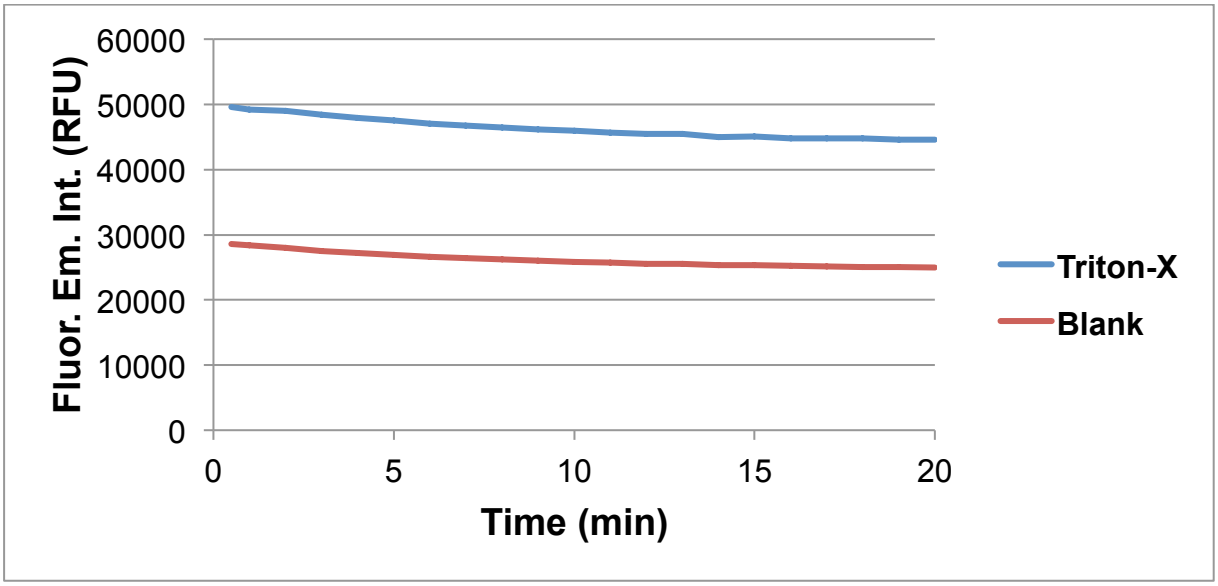
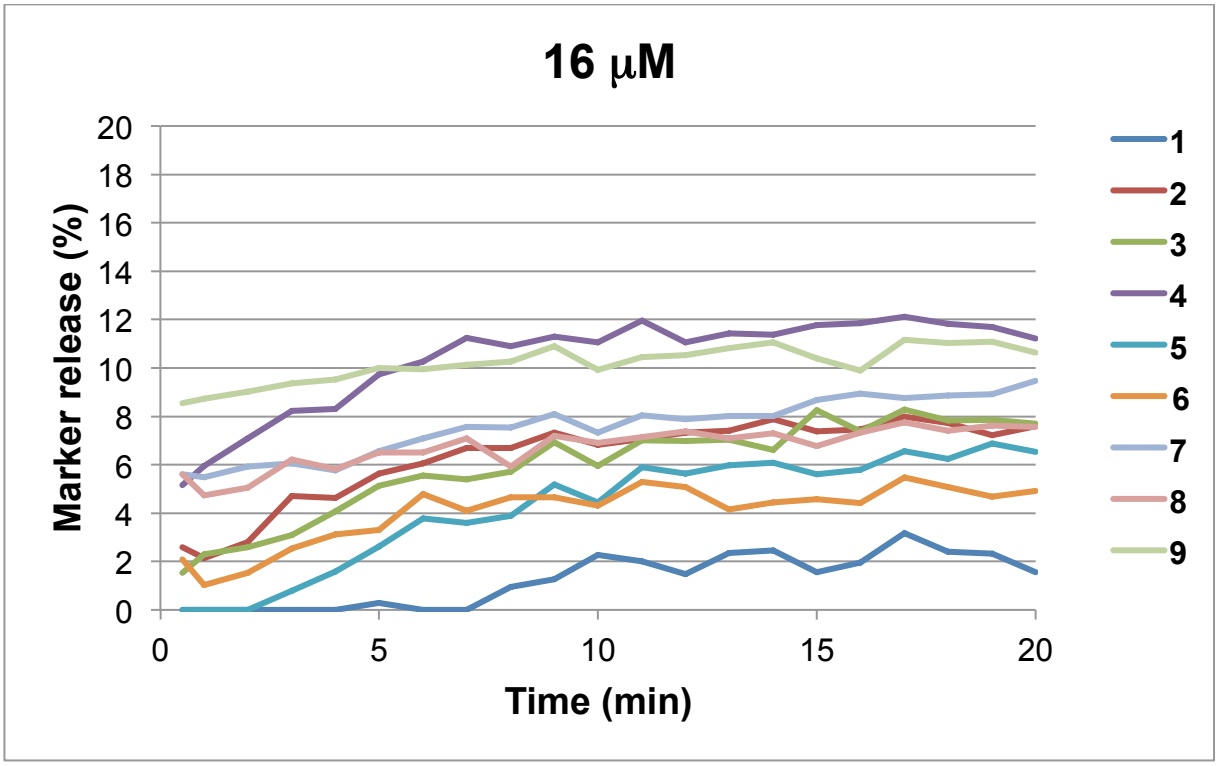


Marker Release Assays

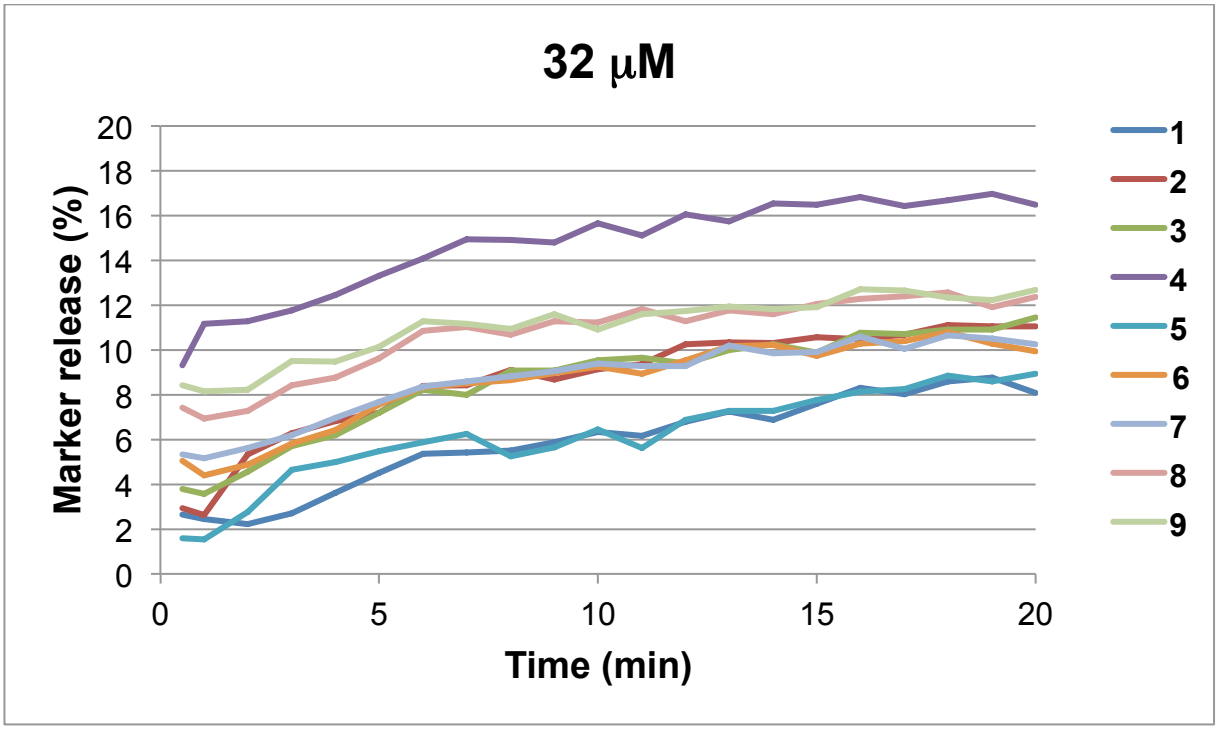
Normalized raw data (non-smoothed)





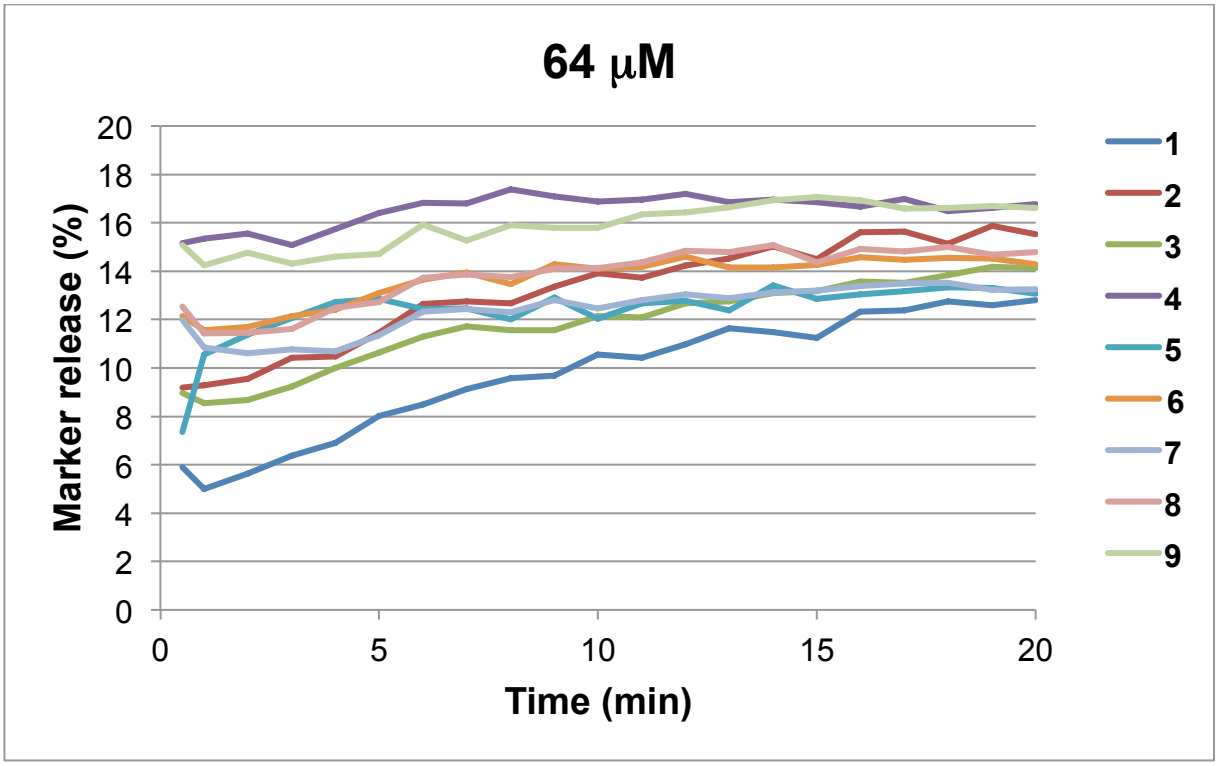
Standard deviation (expressed in % marker release)

t =	1	2	3	4	5	6	7	8	9
0.5	0.35	0.10	0.81	1.58	0.26	1.32	1.42	1.23	0.00
1	0.36	0.69	0.84	2.27	0.95	1.65	1.74	0.73	0.53
2	0.35	0.07	0.83	1.50	0.60	1.29	1.60	1.28	0.10
3	0.37	0.22	0.86	2.53	0.22	1.07	2.20	0.40	0.23
4	0.36	0.08	0.85	2.62	0.03	0.92	2.22	0.69	0.02
5	0.38	0.11	0.89	2.69	0.60	0.95	2.11	0.57	0.27
6	0.36	0.66	0.87	2.70	0.02	0.90	2.06	0.79	0.62
7	0.38	0.58	0.89	2.82	0.40	0.36	2.54	0.22	0.21
8	0.37	0.56	0.88	2.80	0.30	0.46	1.72	0.80	0.40
9	0.38	0.49	0.91	3.16	0.40	0.35	1.87	1.34	0.06
10	0.38	0.56	0.88	3.23	0.80	0.82	1.97	1.00	0.16
11	0.39	0.94	0.91	3.09	0.49	0.84	2.11	0.85	0.30
12	0.38	0.08	0.89	3.14	0.41	0.65	1.97	0.96	0.75
13	0.39	0.34	0.92	2.53	0.61	0.47	1.75	1.68	0.27
14	0.38	0.65	0.89	2.35	0.43	1.15	1.57	1.36	0.45
15	0.39	0.04	0.92	2.54	0.22	0.80	1.84	1.72	0.16
16	0.38	0.14	0.91	2.54	0.04	1.32	1.91	1.23	0.20
17	0.39	0.30	0.92	2.73	0.27	1.17	1.71	1.99	0.39
18	0.38	0.67	0.90	2.64	0.49	0.89	1.65	1.48	0.25
19	0.39	0.17	0.92	2.76	0.62	1.08	1.84	1.50	0.37
20	0.38	0.28	0.90	3.38	0.09	0.50	1.37	1.83	0.18



Standard deviation (expressed in % marker release)

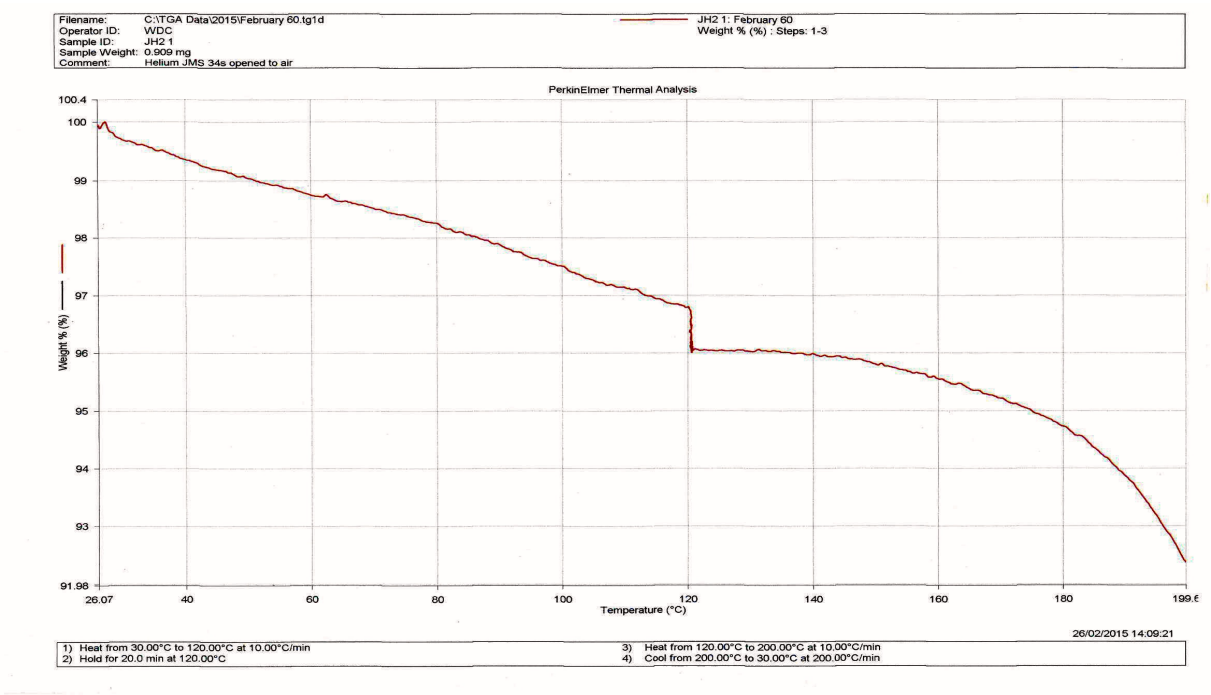
t =	1	2	3	4	5	6	7	8	9
0.5	2.06	0.37	0.36	0.83	0.48	1.04	0.78	0.81	0.69
1	1.84	0.28	0.15	0.16	0.26	0.95	0.44	0.94	0.69
2	1.72	1.70	0.53	0.04	1.45	0.74	0.96	1.17	1.10
3	1.74	1.53	0.30	0.00	2.49	0.67	0.74	0.78	0.97
4	1.56	1.96	0.45	0.19	2.91	0.65	0.76	0.88	0.77
5	1.76	1.05	0.18	0.62	2.50	1.09	0.92	1.27	1.11
6	1.65	1.64	0.12	0.00	2.70	1.22	1.02	1.26	1.00
7	1.56	1.26	0.29	0.40	1.98	0.52	0.90	1.30	0.82
8	1.77	1.74	0.46	0.51	1.65	0.87	1.17	1.26	0.98
9	2.03	1.48	0.30	0.39	2.00	0.32	1.26	1.49	1.22
10	1.81	1.61	0.24	0.09	2.08	0.42	0.90	0.98	1.15
11	2.38	1.61	0.47	1.10	2.20	0.91	0.99	1.04	0.96
12	2.01	1.78	0.09	0.40	2.29	0.80	1.00	1.23	1.09
13	2.23	2.00	0.51	0.26	1.62	0.83	0.82	0.98	1.11
14	1.87	1.48	0.10	0.30	1.92	0.49	0.96	1.05	0.76
15	1.86	1.58	0.25	0.34	2.14	0.81	1.14	1.23	0.83
16	2.21	1.04	0.36	0.44	1.89	1.23	0.98	1.27	0.97
17	1.83	1.23	0.10	0.26	2.04	0.95	0.97	0.76	1.15
18	1.71	1.51	0.29	0.50	1.75	0.61	0.65	0.88	0.93
19	1.68	1.43	0.33	0.61	2.09	0.77	0.89	1.31	1.12
20	1.85	1.39	0.49	0.08	1.91	0.69	1.07	1.22	0.73



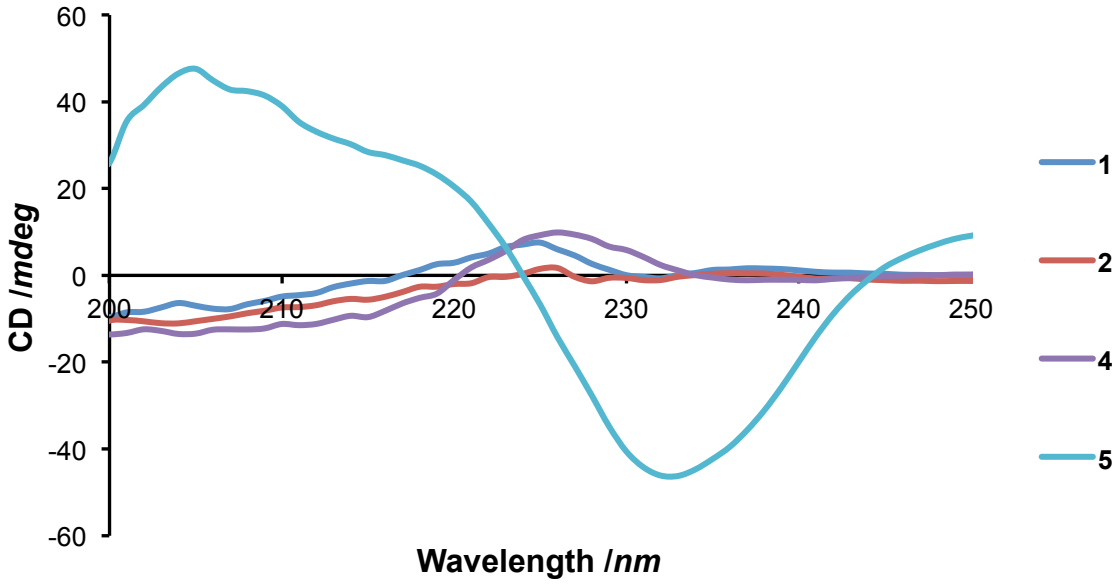
Standard deviation (expressed in % marker release)

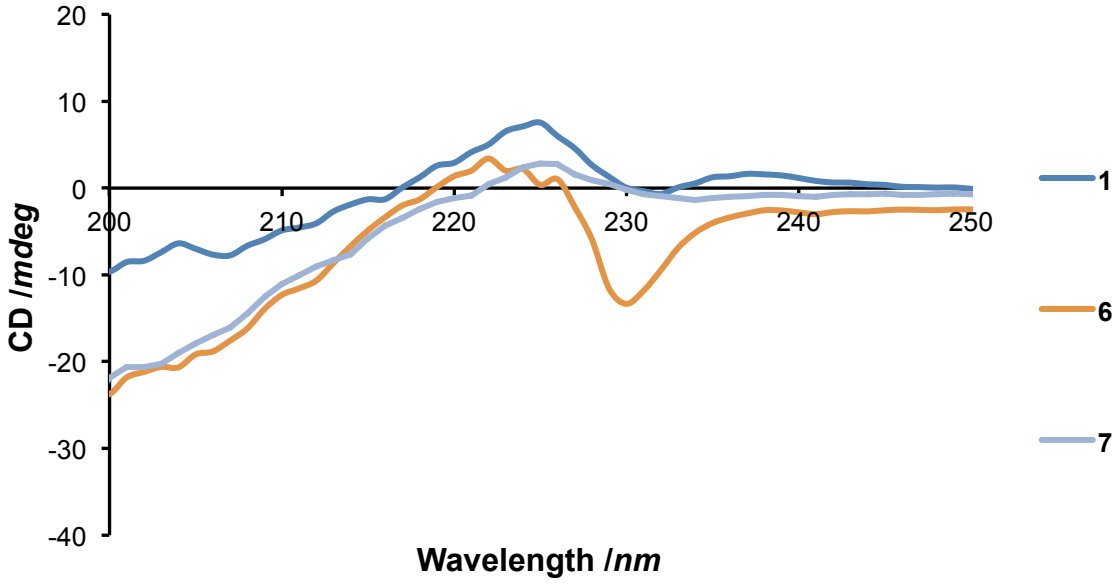
t =	1	2	3	4	5	6	7	8	9
0.5	1.20	1.15	1.53	2.27	1.79	0.45	0.56	2.19	0.90
1	1.39	1.10	2.26	0.99	0.10	0.48	0.16	1.15	1.72
2	0.76	1.00	1.80	0.23	0.57	1.01	0.13	1.41	0.70
3	1.05	0.60	1.83	0.13	0.45	1.20	0.40	1.52	1.44
4	1.56	1.24	1.92	0.25	0.92	0.87	0.32	0.95	1.67
5	1.30	0.91	1.94	0.34	0.37	1.34	0.25	1.30	1.68
6	1.30	0.95	2.01	0.08	0.91	1.37	0.42	1.19	1.18
7	1.46	0.98	1.75	0.08	0.97	0.94	0.40	1.14	1.66
8	1.54	0.66	1.99	0.28	1.81	1.08	0.07	1.32	1.82
9	1.13	0.24	2.31	0.11	1.67	1.20	0.68	1.21	1.62
10	1.42	0.42	2.41	0.14	1.09	1.00	0.08	1.08	1.13
11	1.42	0.69	2.36	0.04	0.28	0.95	0.05	1.10	1.19
12	1.55	0.56	2.12	0.35	0.76	1.26	0.28	1.57	1.25
13	1.40	0.82	2.27	0.58	0.11	1.04	0.42	1.82	1.66
14	1.54	0.77	2.23	0.60	1.10	0.54	0.21	1.66	0.96
15	1.49	0.90	2.30	0.98	0.87	0.86	0.50	1.40	1.05
16	1.34	0.90	2.22	0.15	1.15	0.89	0.18	1.26	1.35
17	1.23	0.51	2.43	0.37	1.69	1.13	0.22	1.50	1.37
18	1.39	0.62	2.08	0.57	0.51	0.94	0.18	1.38	1.29
19	1.15	0.80	2.24	0.05	1.12	0.68	0.08	1.57	0.61
20	1.47	0.83	2.23	0.35	1.61	0.73	0.14	1.56	1.45

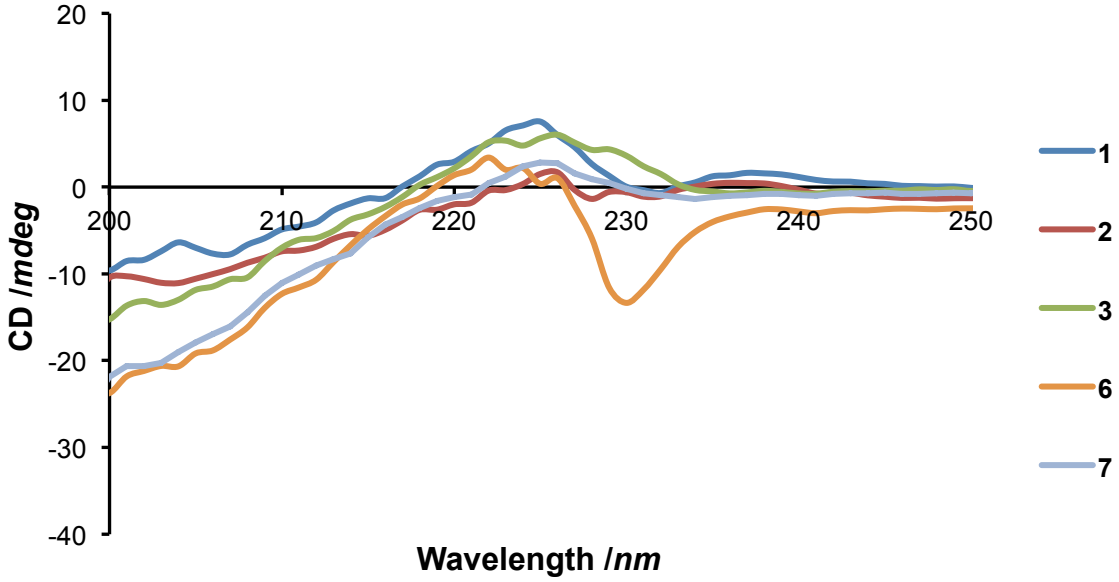
Thermogravimetric analysis of 1



Circular dichroism, selected comparisons (inorganic buffer)



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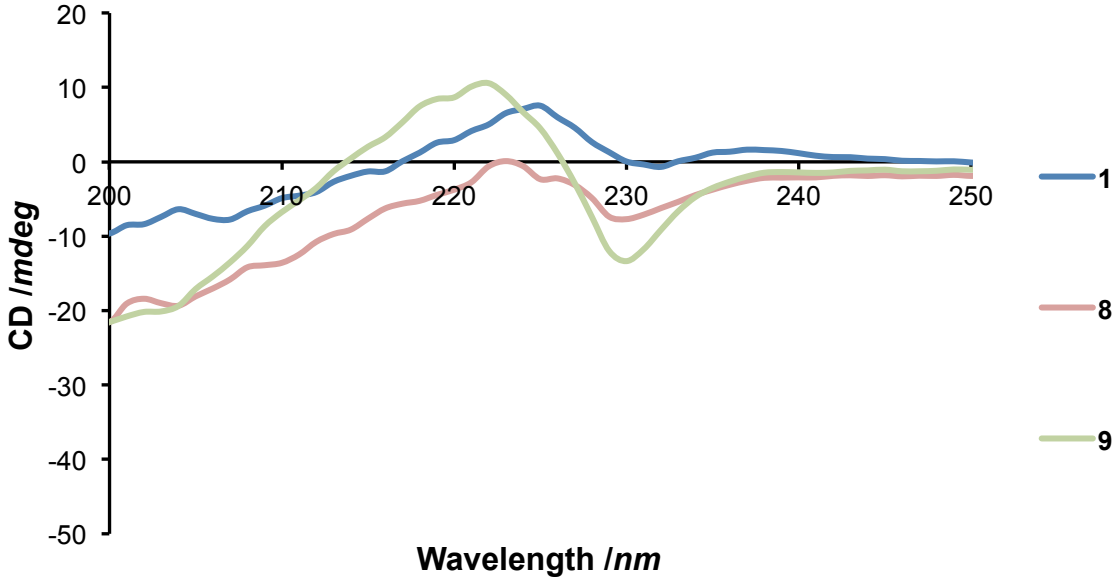
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CD /mdeg
Wavelength /nm
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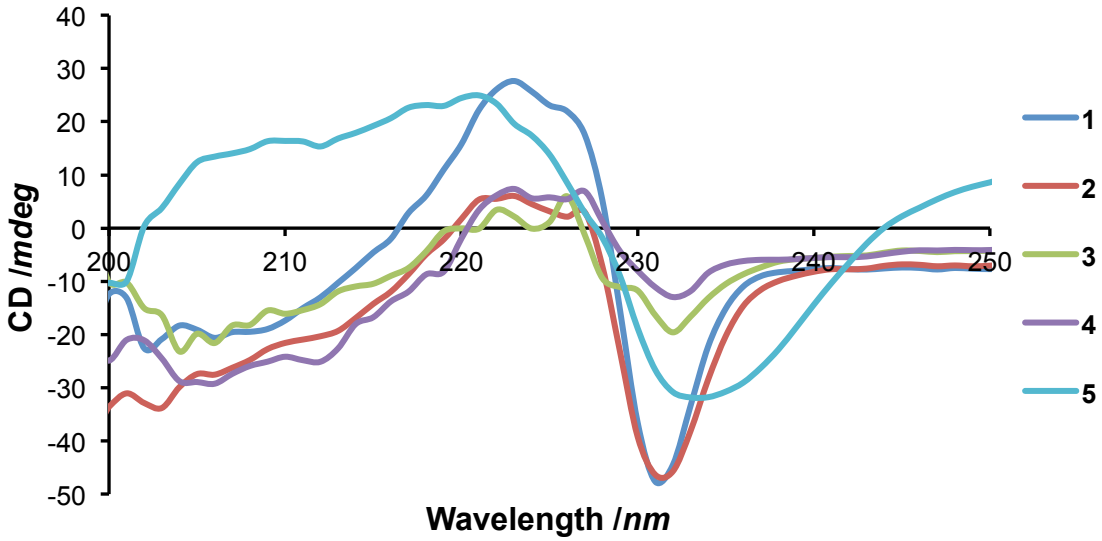
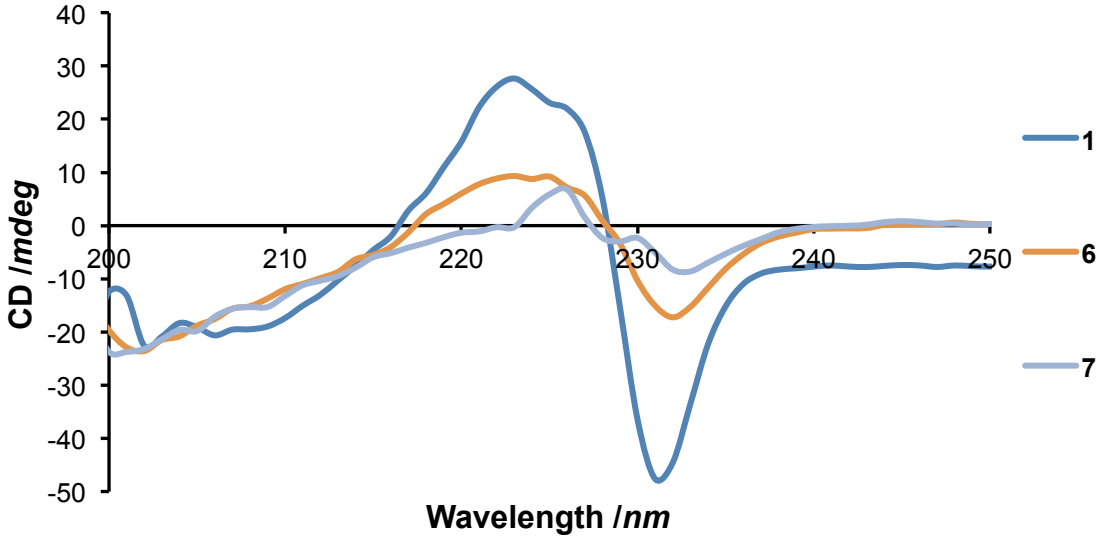
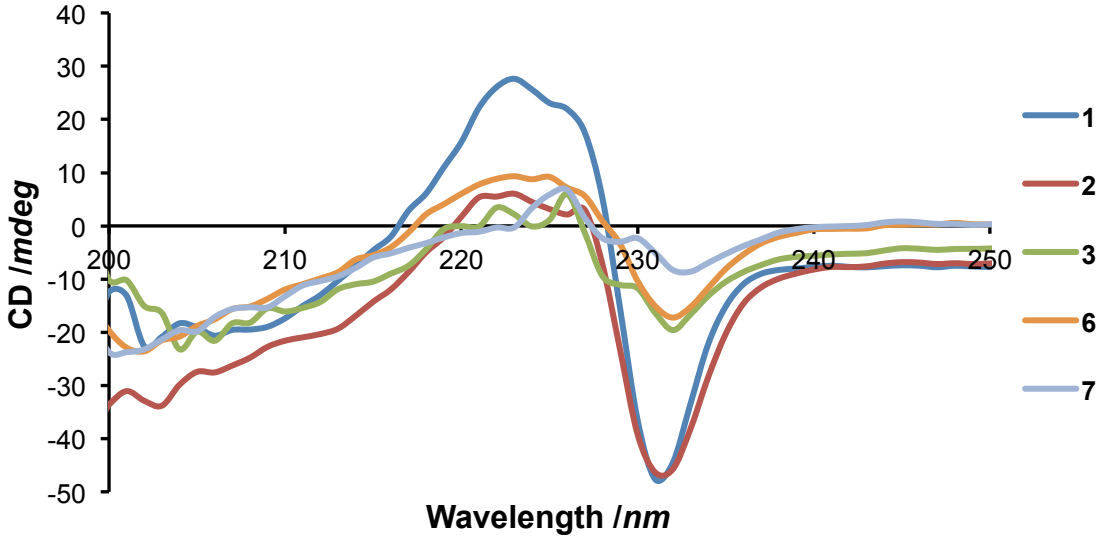
Circular dichroism, selected comparisons (membrane model)



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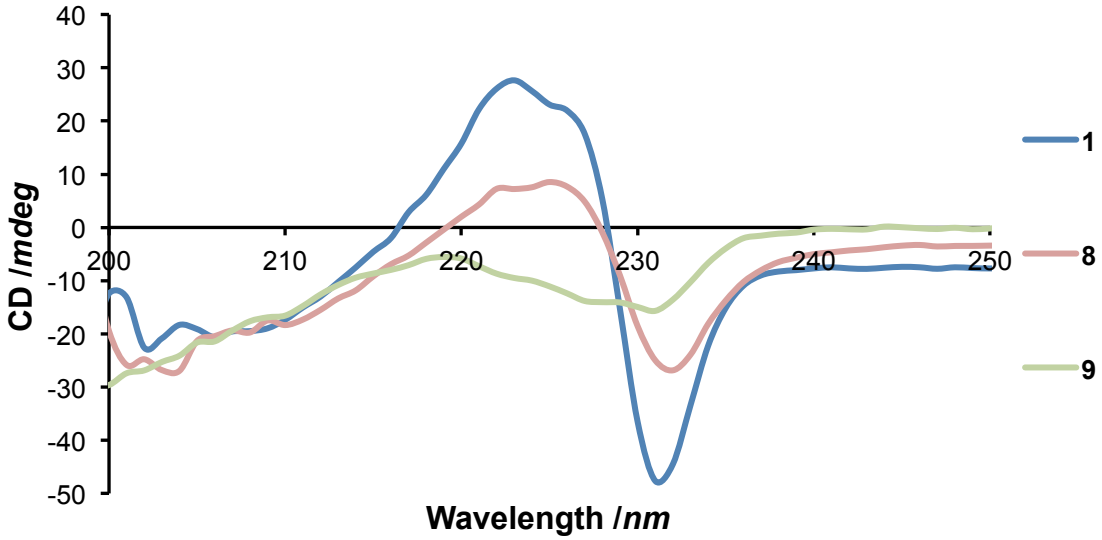
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Wavelength /nm
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