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Tuning the Activity of a Short Arg-Trp Antimicrobial Peptide by Lipidation of a C- or N-terminal Lysine Side-Chain

by

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Synthesis of the lipidated KR_3W_3 peptides.

Two batches of 0.25 mmol resin bound peptide were prepared: one with an *N*-terminal Lys(Mtt) and one with a *C*-terminal Lys(Mtt) residue. The respective sequences were as follows:

- Fmoc-Arg(Pbf)-Trp(Boc)-Arg(Pbf)-Trp(Boc)-Arg(Pbf)-Trp(Boc)-Lys(Mtt)-Rink-PS for the *C*-terminal lipidation, and
- Fmoc-Lys(Mtt)-Arg(Pbf)-Trp(Boc)-Arg(Pbf)-Trp(Boc)-Arg(Pbf)-Trp(Boc)-Rink-PS for the *N*-terminal lipidation.

Synthesis of these sequences was performed on a microwave assisted automated peptide synthesizer (CEM, Liberty). After the above-mentioned sequences were prepared, the Mtt-group was removed from the lysine residues using repeated treatments of the resin with 1% TFA in DCM. After this, each batch was dried using washing with DCM (3 times 2 min, 5 mL each) and Et_2O (3 times 2 min, 5 mL each) and subsequent storing under vacuum. Each of the dry batches was then divided into seven equal portions to which the fatty acids were coupled. For this, the respective fatty acid (4 equiv) was mixed with TBTU (3.8 equiv, 43.6 mg) and HOBt (4 equiv; 19.3 mg) and dissolved in DCE:NMP – 1:1 (v/v) (for C_5 - C_{13}). After this, D*i*PEA (8 equiv, 62.2 mL) was added to the solution, which was then added to the resin. The reaction was allowed to take place overnight, after which the resin was washed with NMP (3 times 2 min, 5 mL each) and DCM (3 times 2 min, 5 mL each). The resin-bound lipidated peptides were deprotected and cleaved from the resin using TFA:TIS:water – 92.5:5:2.5 (v/v/v). The cleaved products were then precipitated in cold (-20 ^{o}C) Et_2O:hexanes – 1:1 (v/v) followed by centrifugation. The supernatant was decantated and the pellet was again agitated in the same solution. After centrifugation, the final compound was obtained, for each peptide >90% purity was established.

ESI-MS analysis of the lipidated peptides

High definition mass spectrometry data were acquired using a Synapt G2-S HDMS instrument (Waters, UK) equipped with a lock spray source for electrospray ionization (ESI) and a time of flight (ToF) detector. Purified samples were diluted 1:1000 in 50 % (v/v) acetonitrile, 0.1 % (v/v) formic acid (Biosolve, NL) and a volume of 5 µl was subjected to mass spectrometry (10 min scan time, 2 µL/min solvent flow (50 % (v/v) acetonitrile and 0.1 % (v/v) formic acid). Spectra were recorded in positive ionization mode and high-resolution mode over a mass range of 50 to 2000 m/z with 1 s/scan. The following parameters were used for the NanoLockSpray source: capillary voltage, 3 kV; sampling cone voltage, 50 V; source temperature, 95 °C; desolvation temperature, 350 °C; cone gas flow; 50 L/h; desolvation gas flow, 650 L/h. [Glu1] - Fibrinopeptide B (Waters, UK) serving as lock mass analyte was fed through the lock spray channel (lock mass capillary voltage, 3 kV). Analysis of the spectra was performed with the MassLynx software (version 4.1 SCN851, Waters, UK).

Table S1. HR-ESI-MS data of the Lys-lipidated $(RW)_3$-peptides

	calculated		measured			measured		
	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+2H]^{2+}$	Δ	ppm	$[M+3H]^{3+}$	Δ	ppm
$(RW)_3K$	586.8387	391.5616	586.8417	0.0030	5.20	391.5638	0.0022	5.70
C-C_2	607.8440	405.5652	607.8467	0.0028	4.52	405.5675	0.0023	5.75
C-C_4	621.8596	414.9088	621.8590	-0.0006	-0.96	414.9109	0.0021	4.98
C-C_6	635.8753	424.2526	635.8767	0.0014	2.28	424.2526	0.0000	0.00
C-C_8	649.8909	433.5964	649.8947	0.0038	5.85	433.5982	0.0018	4.23
C-C_{10}	663.9066	442.9401	663.9066	0.0000	0.08	442.9398	-0.0003	-0.75
C-C_{12}	677.9222	452.2839	677.9224	0.0002	0.30	452.2834	-0.0005	-1.11
C-C_{14}	691.9379	461.6277	691.9397	0.0018	2.67	461.6306	0.0029	6.35
C-Fc	692.8349	462.2257	692.8367	0.0018	2.60	462.2271	0.0014	3.03
$K(RW)_3$	586.8387	391.5616	586.8417	0.0030	5.20	391.5638	0.0022	5.70
N-C_2	607.8440	405.5652	607.8467	0.0028	4.52	405.5675	0.0023	5.75
N-C_4	621.8596	414.9088	621.8590	-0.0006	-0.96	414.9109	0.0021	4.98
N-C_6	635.8753	424.2526	635.8767	0.0014	2.28	424.2526	0.0000	0.00
N-C_8	649.8909	433.5964	649.8947	0.0038	5.85	433.5982	0.0018	4.23
N-C_{10}	663.9066	442.9401	663.9078	0.0012	1.88	442.9398	-0.0003	-0.75
N-C_{12}	677.9222	452.2839	677.9224	0.0002	0.30	452.2834	-0.0005	-1.11
N-C_{14}	691.9379	461.6277	691.9397	0.0018	2.67	461.6306	0.0029	6.35
N-Fc	692.8349	462.2257	692.8367	0.0018	2.60	462.2271	0.0014	3.03

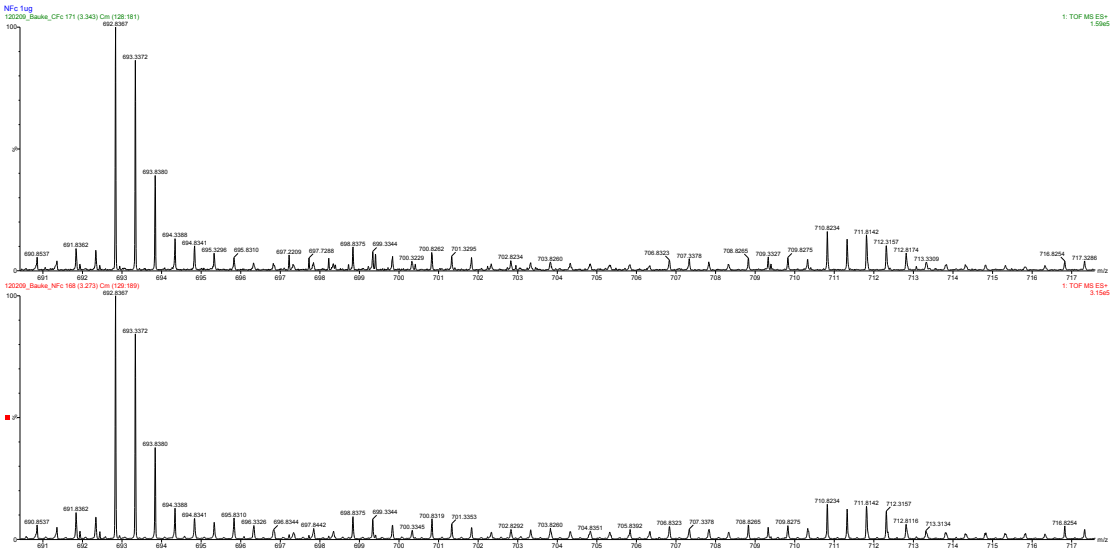
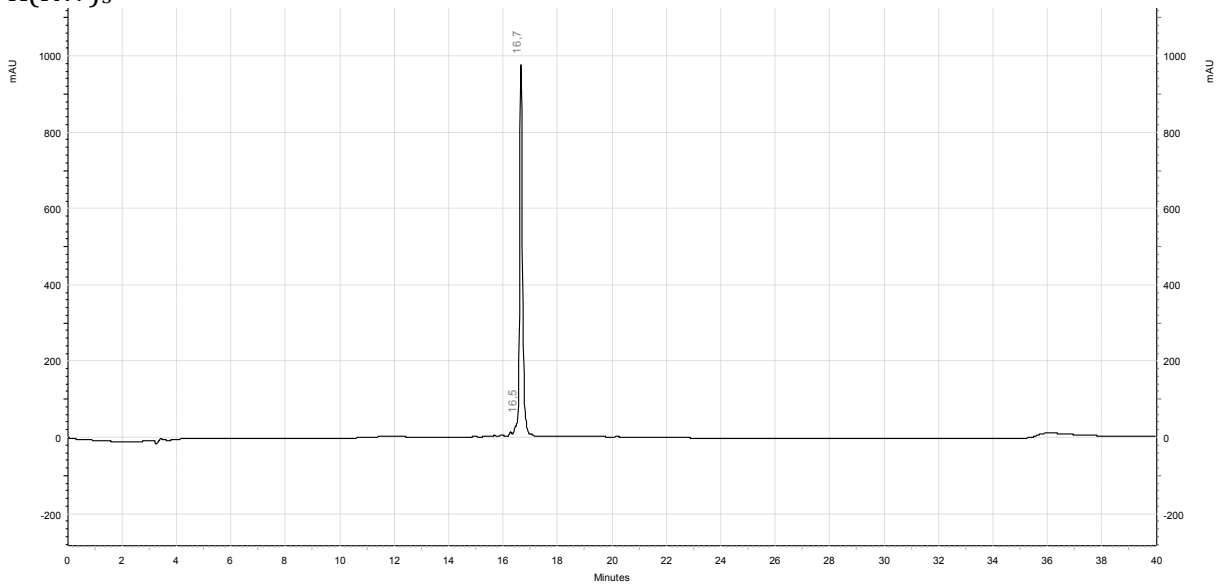


Figure S1. High-Resolution ESI-MS spectra of *C*-Fc (*top*) and N-Fc (*lower*) for $[M+2H]^{2+}$

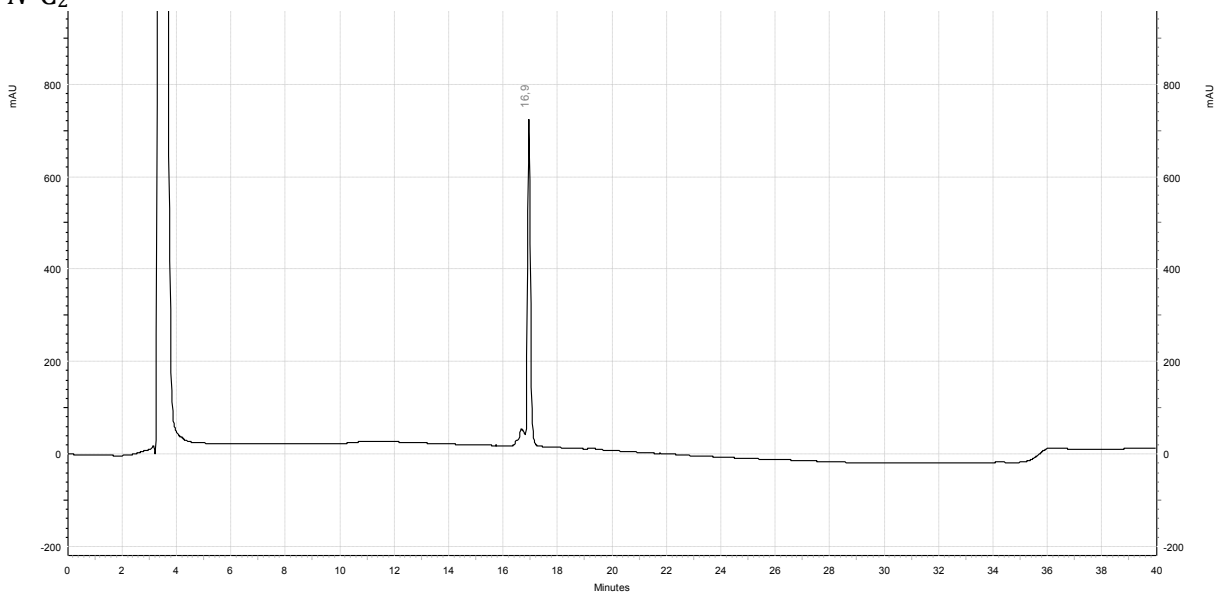
HPLC-analysis of the peptides

Some HPLCs were made from the DMSO-based stock-solutions that were used in the MIC-value determination. These can be recognized by the large DMSO-peak in the chromatogram eluting between 3 and 4 minutes.

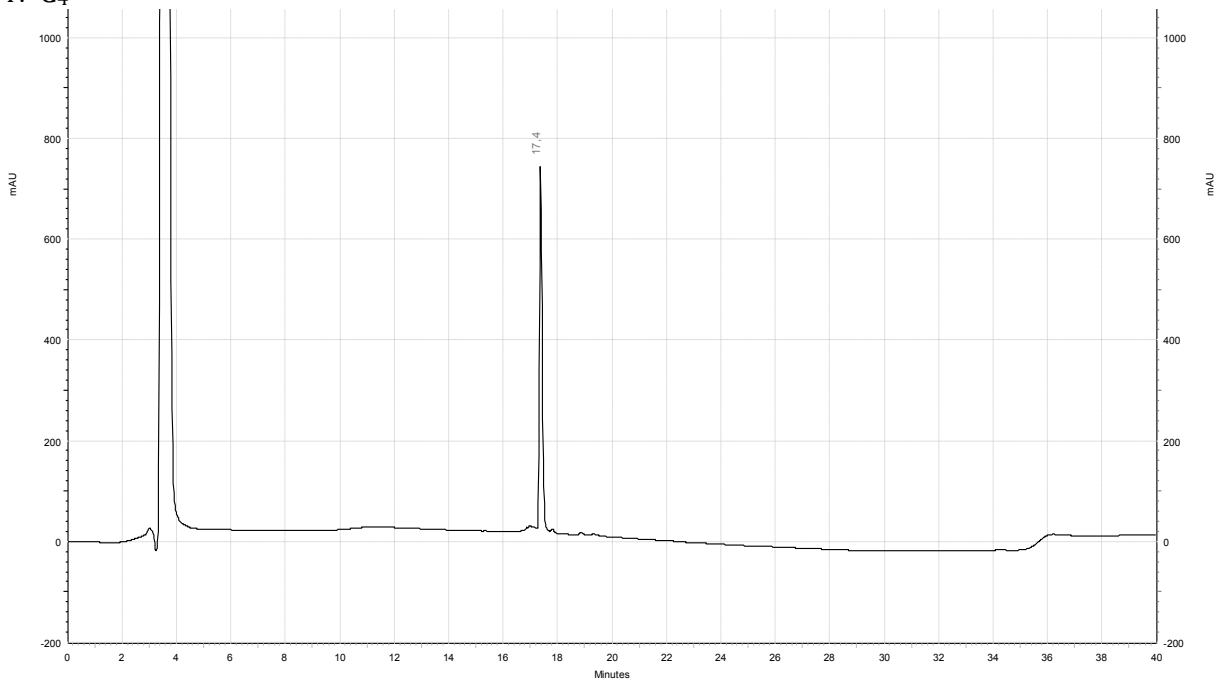
$K(RW)_3$



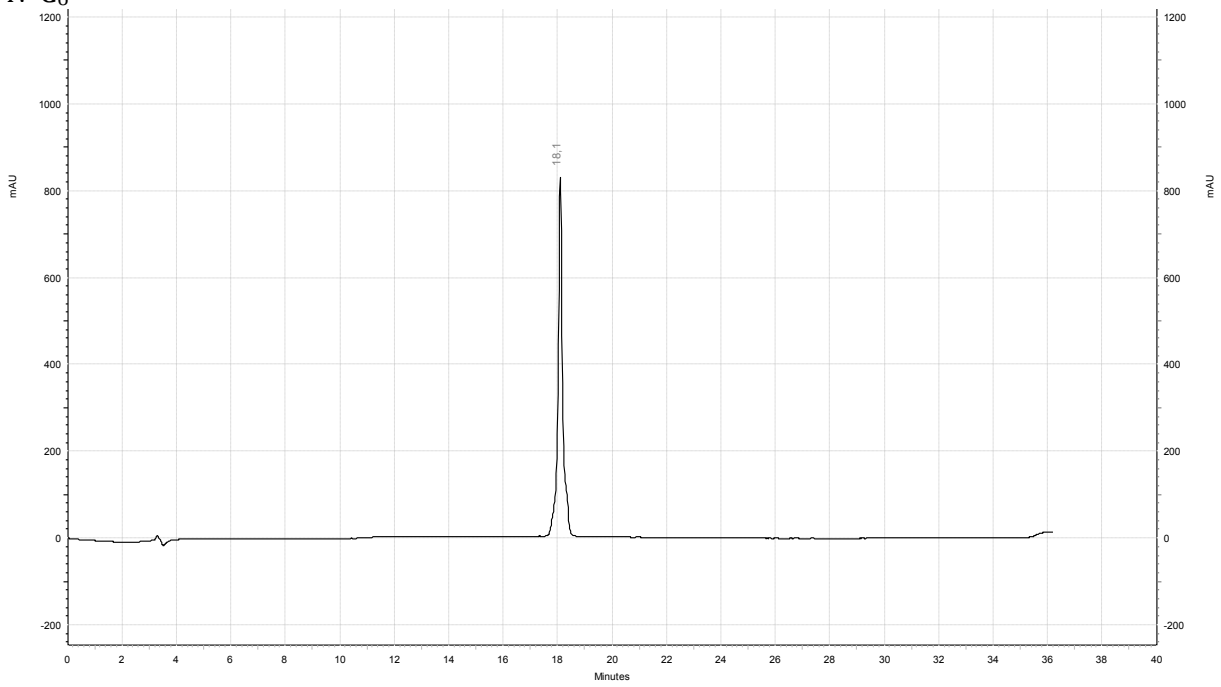
N-C_2



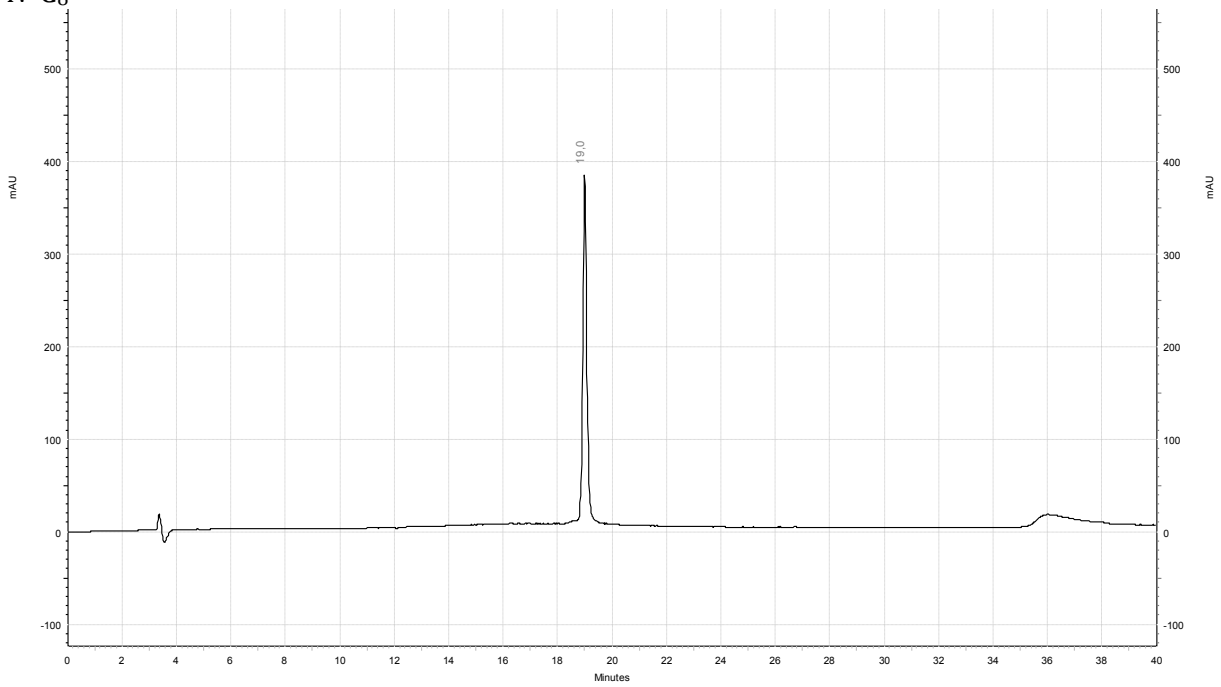
N-C_4



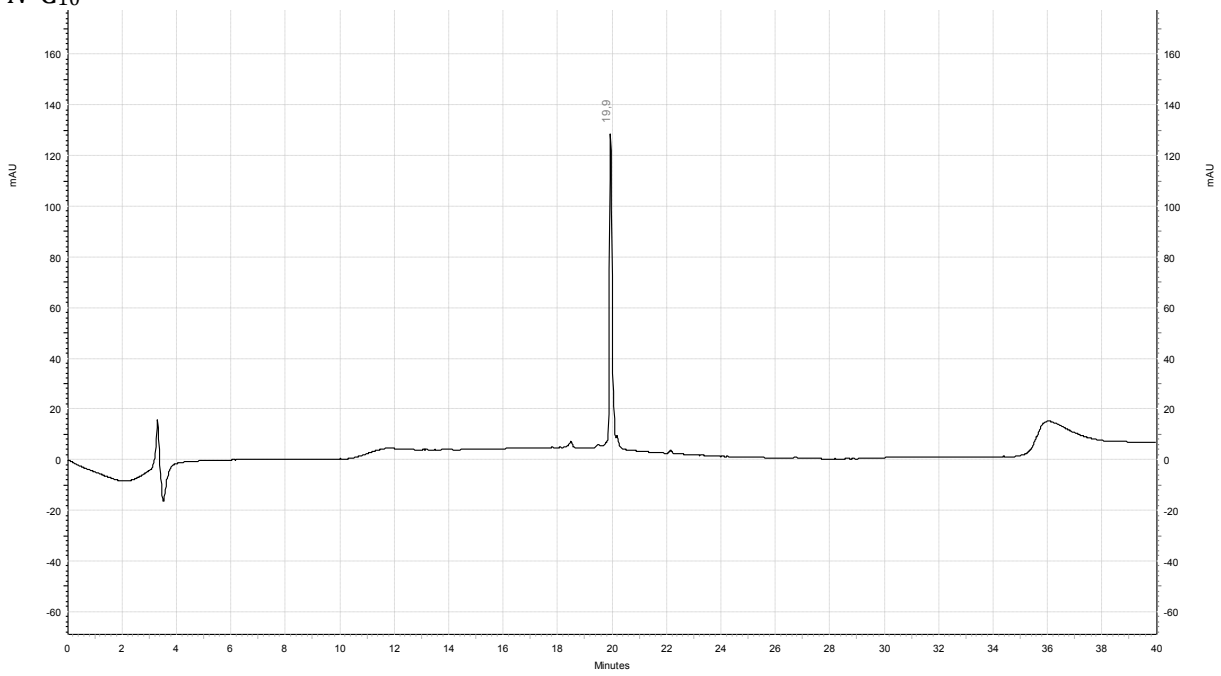
N-C_6



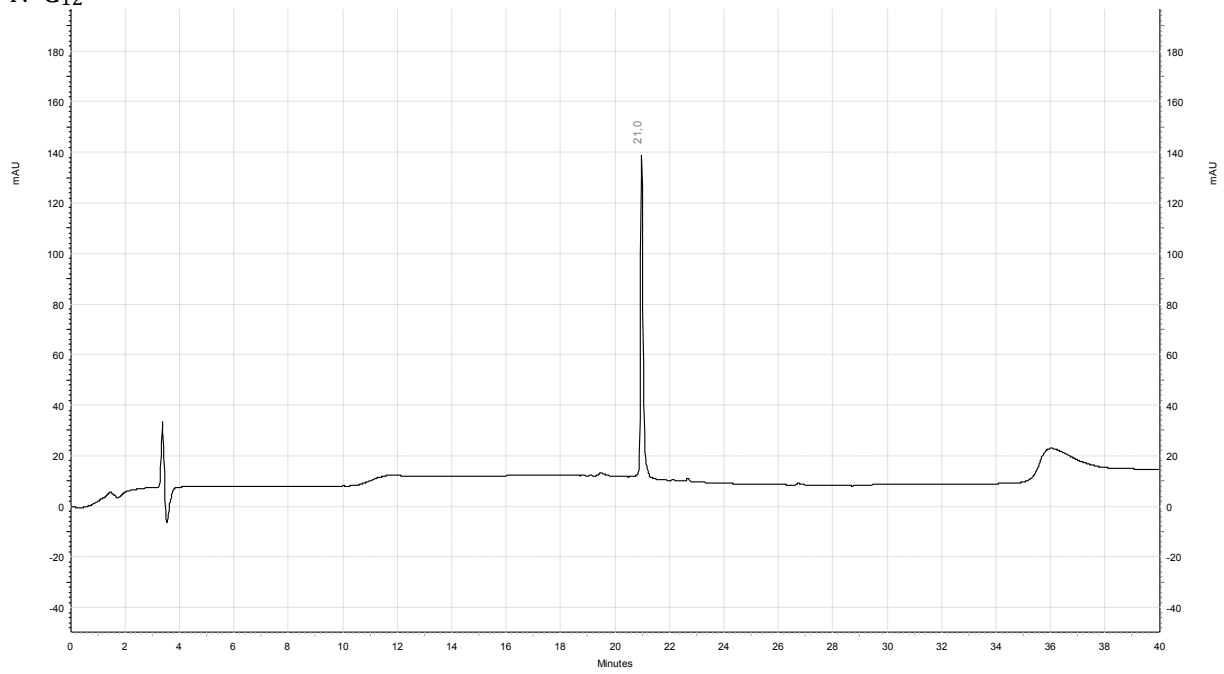
N-C_8



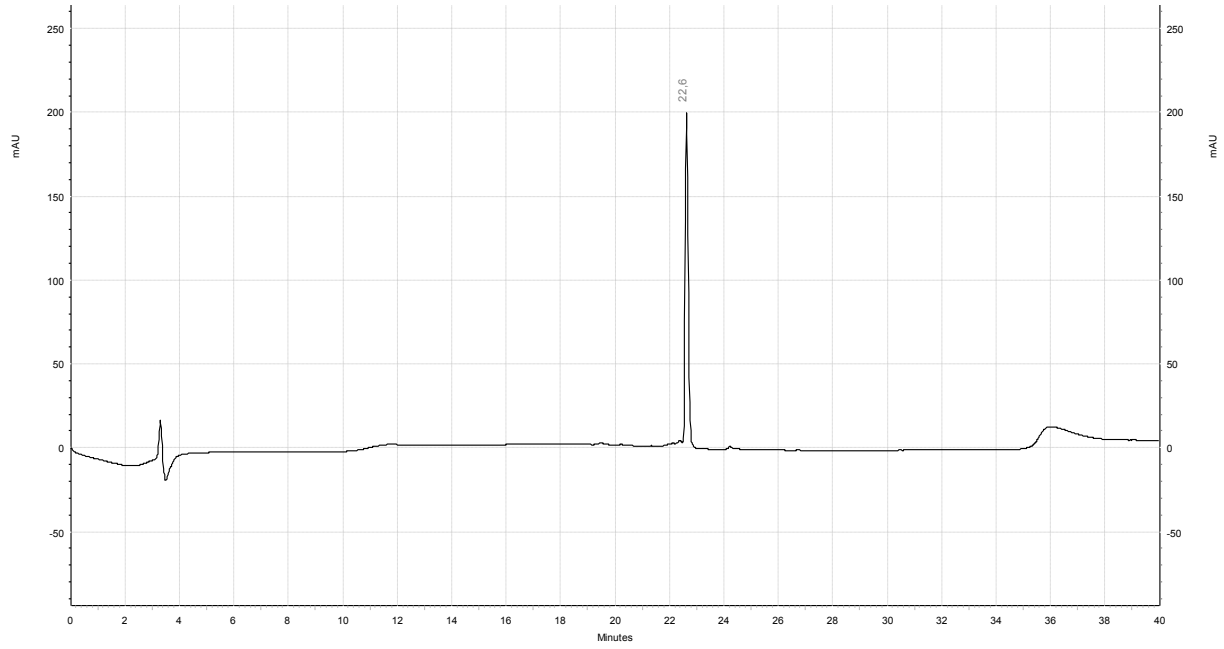
N-C_{10}



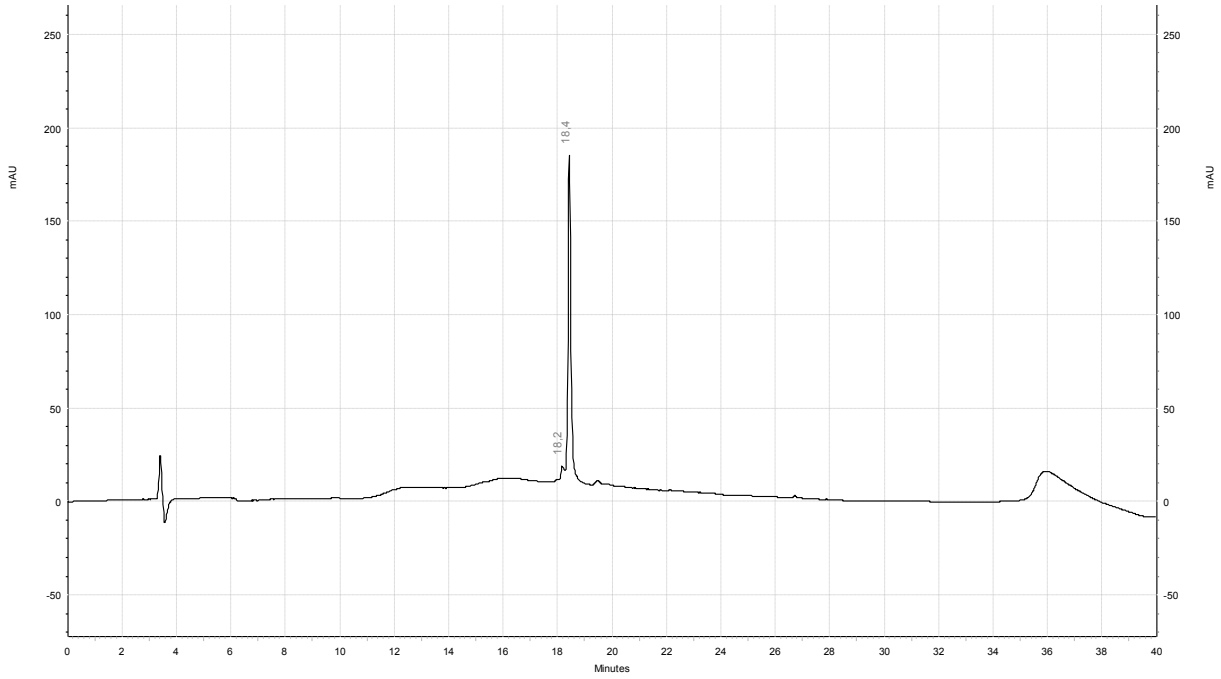
N-C_{12}



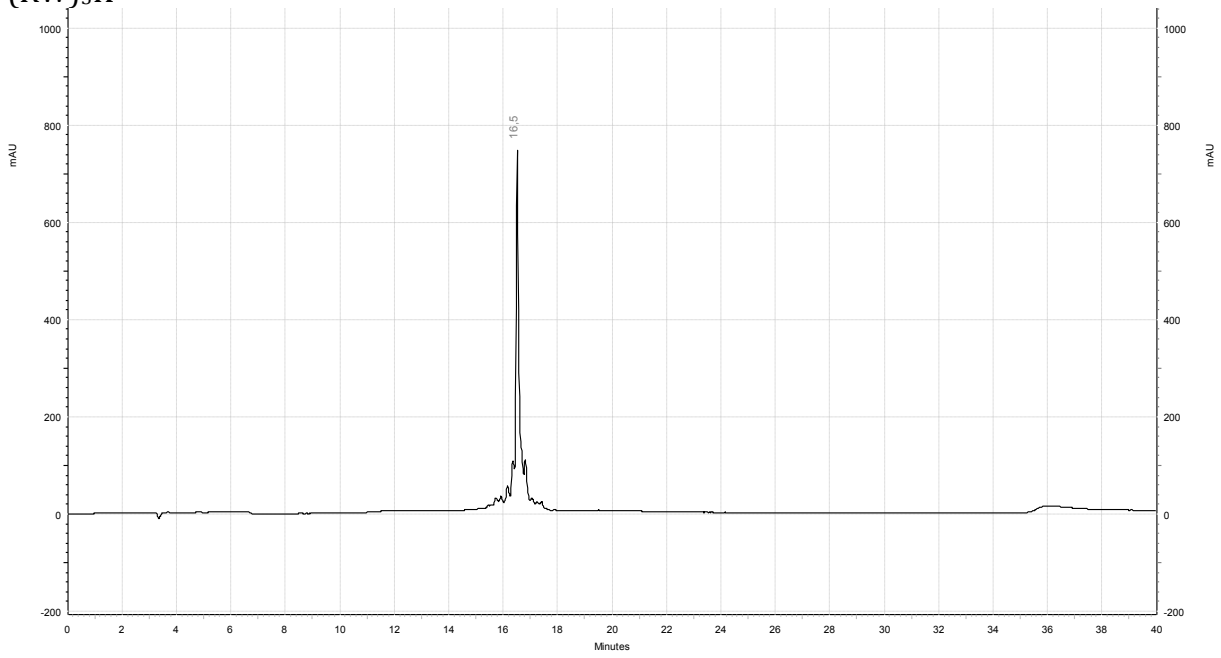
N-C_{14}



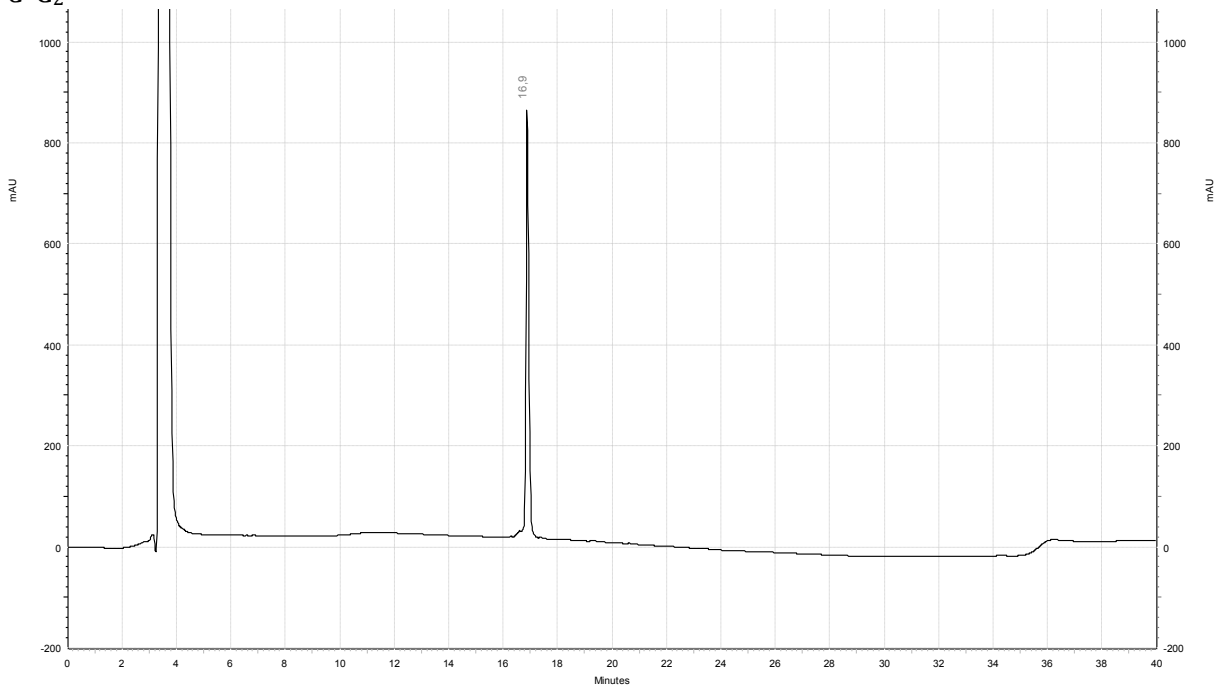
N-Fc



$(RW)_3K$



C-C_2



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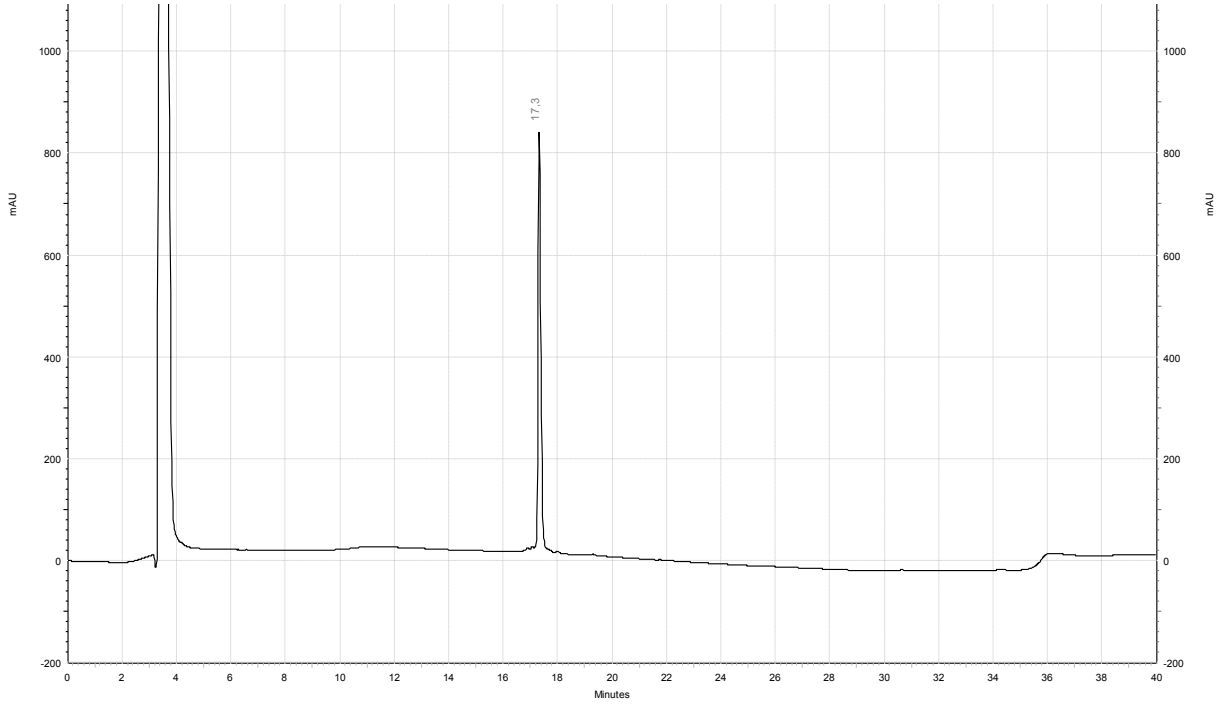
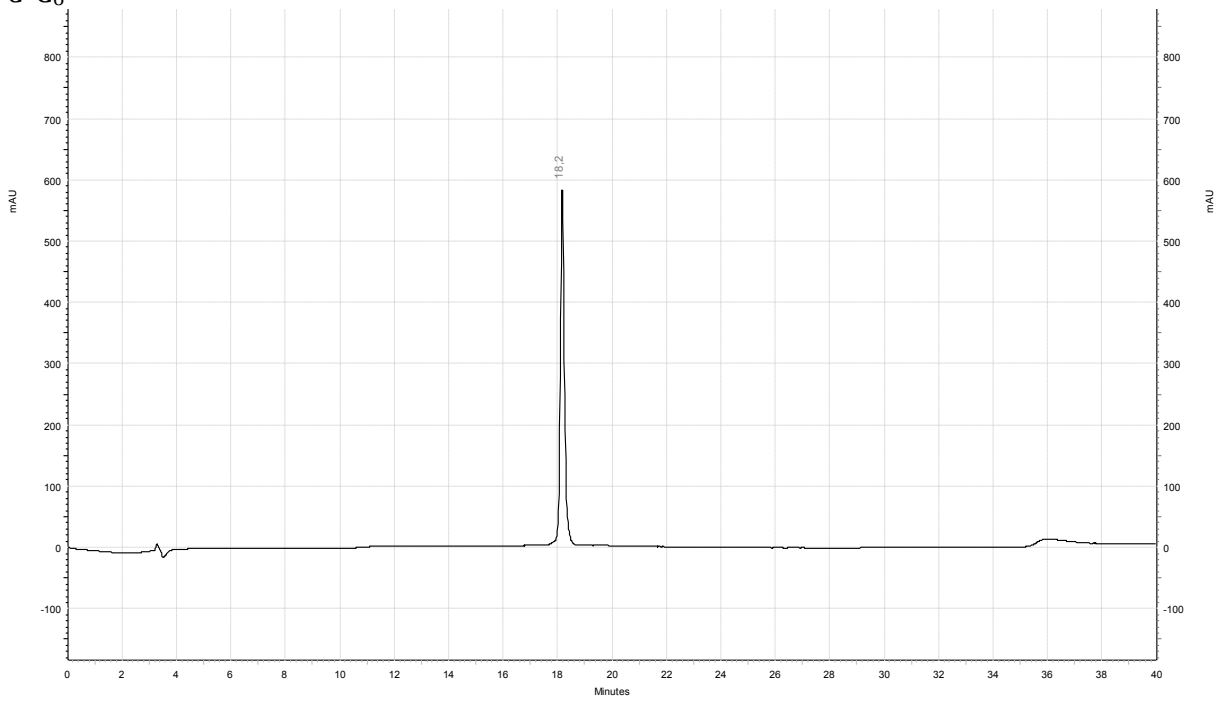
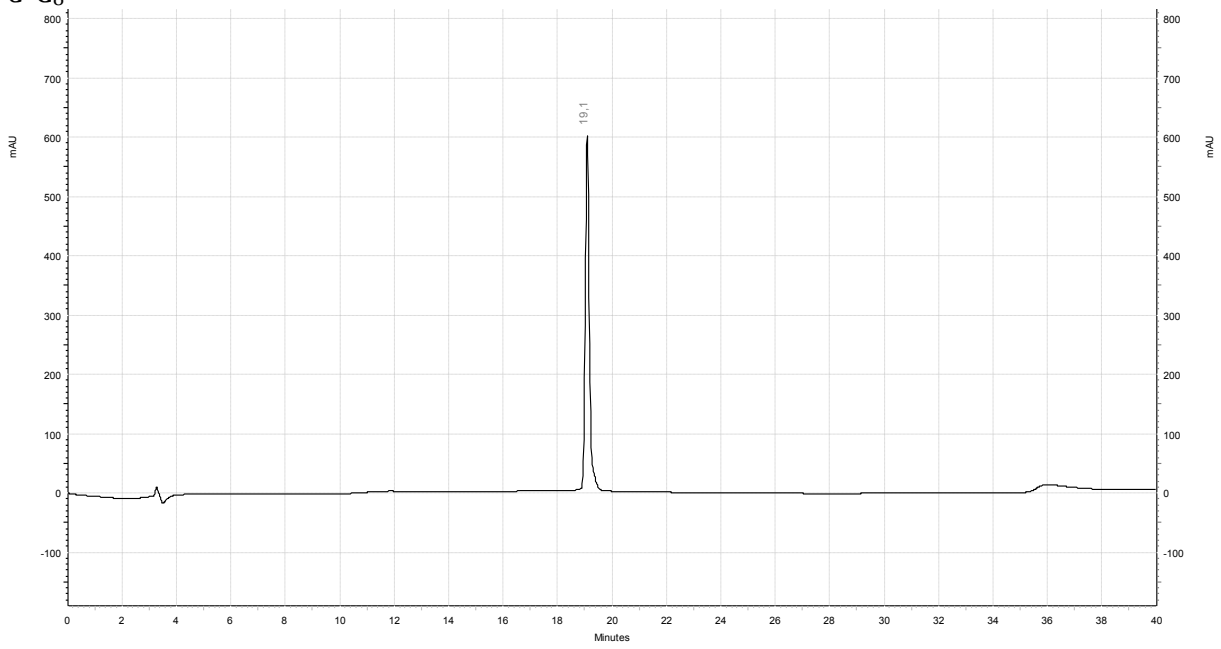


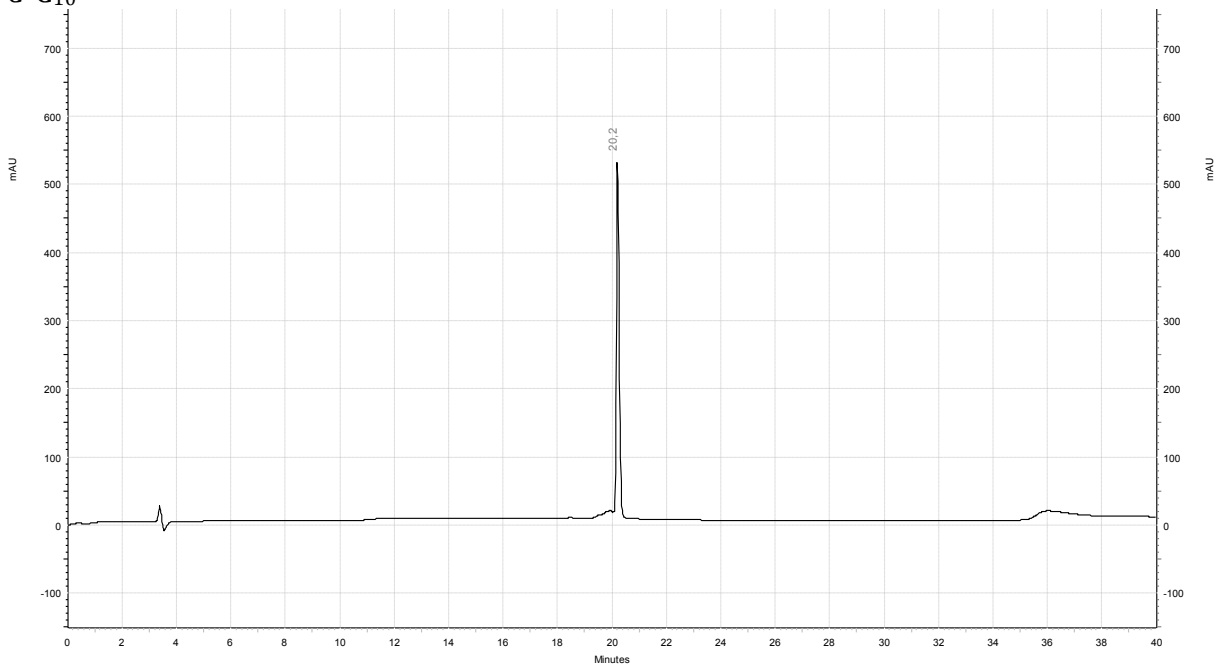
C-C_6



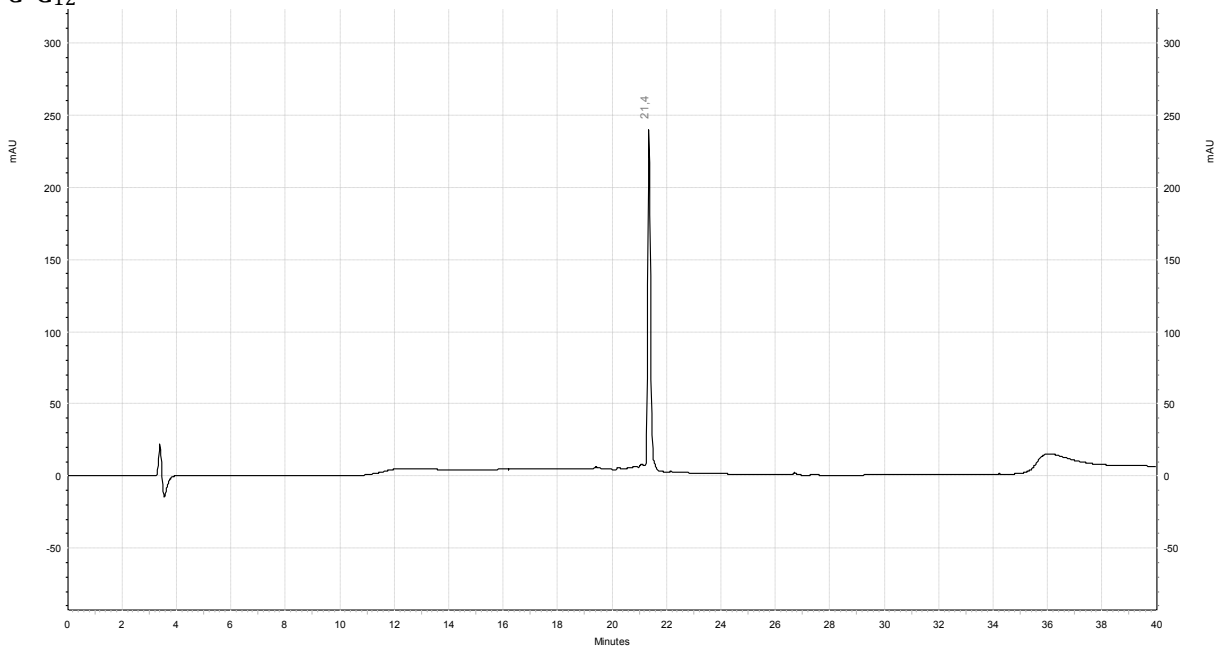
C-C_8



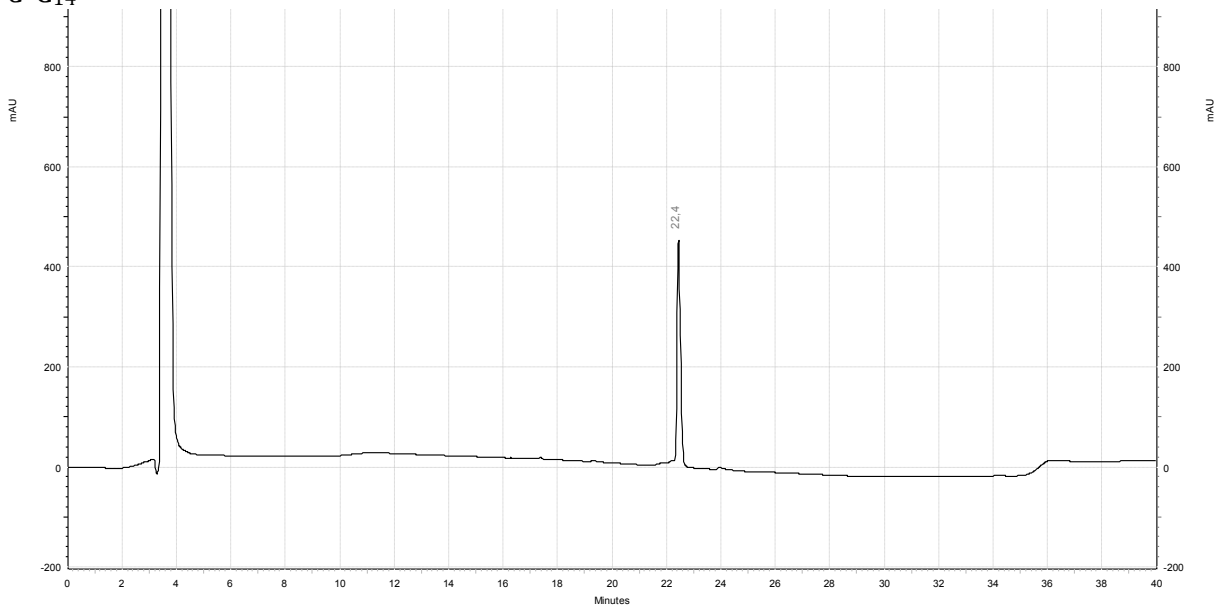
C-C_{10}



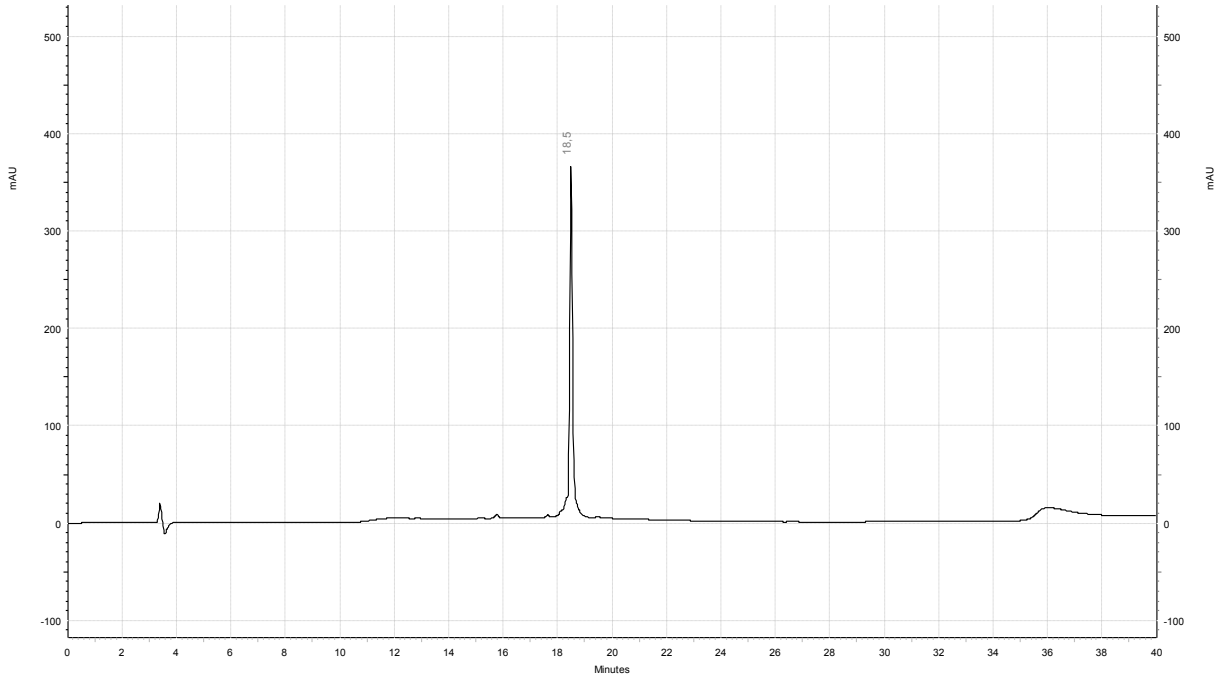
C-C_{12}



C-C_{14}


mAU
800
600
400
200
0
-200
22,4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Minutes

C-Fc


mAU
500
400
300
200
100
0
-100
18,5
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Minutes

Determination of MICs (table 1)

The minimal inhibitory concentrations (MIC) were tested against *Escherichia coli* DSM 30083, *Acenitobacter baumannii* DSM 30007, *Pseudomonas aeruginosa* DSM 50071, *Bacillus subtilis* DSM 402, *Staphylococcus aureus* DSM 20231 (type strain), and *Staphylococcus aereus* ATCC 43300 (MRSA) in a microtiter plate assay according to CSLI guidelines.[1] *E. coli, A. baumannii, S. aureus*, and *B. subtilis* were grown in Mueller Hinton broth, *P. aeruginosa* in cation adjusted Mueller Hinton II. Peptides were dissolved in DMSO to give 10 mg/mL stock solutions. Serial dilution in culture media was carried out automatically with the Tecan Freedom Evo 75 liquid handling workstation (Tecan, Männedorf, Switzerland) from 512 to 0.5 µg/mL. Peptide dilutions were inoculated with 10^5 bacteria/mL taken from late exponential cultures grown in the same media in a total volume of 200 µL per well. Cells were incubated for 16 hours at 37 °C. The lowest peptide concentration inhibiting visible bacterial growth was taken as MIC.

Bacterial survival rates

To determine survival of *Bacillus subtilis* 168 (*trpC2*)[2] after antibiotic treatment, 2 ml of Mueller Hinton broth (BD, Franklin Lakes, NJ, USA) were inoculated with $5x10^5$ cells per mL. Crude peptides that were >95% pure, were added at twice MIC and the culture incubated at 37 °C for 15 minutes under steady agitation. Colony forming units (CFU) were determined by plating serial dilutions of antibiotic-treated cultures on Mueller Hinton agar plates followed by incubation at 37 °C for 18 hours. Survival rates of peptide-treated bacteria are expressed relative to the untreated control as average over three independent biological replicates.

Table S2. Survival rates of *B. subtilis* treated with lipidated peptides.

peptide	MIC (µg/mL)	concentrations used (µg/mL)	%-survival rate	%-variance
$(RW)_3$	4	8	2.8	
$K(RW)_3$	8	16	6.950	1.661
N-C_6	2	4	0.211	0.136
N-C_8	2	4	0.006	0.011
N-C_{10}	4-8	12	0.016	0.016
N-C_{12}	8-16	24	0.011	0.018
N-C_{14}	16	32	0.033	0.004
C-C_6	2	4	0.687	0.550
C-C_8	2	4	0.261	0.235
C-C_{10}	2	4	0.053	0.036
C-C_{12}	8	16	0.004	0.006
C-C_{14}	16	32	0.000	0.000

[1] *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard*, Mo7-A8, vol 29, no. 2, **2009**, 8th edition.

[2] Agnostopoulos, C.; Spizizen. J. *J. Bacteriol.* **1961**, *81*, 741–746.

Hemolysis (after Oren 1997)
The peptides were tested for their hemolytic activities against human red blood cells (hRBCs).

Preparation of the hRBCs
After drawing whole blood into anticoagulant containing tubes (BD Vacutainer®, K2 EDTA 3.6 mg, Ref 368841, Lot 1248213), its fractionation was executed with one volume whole blood adding nine volumes sterile 0.9% NaCl and centrifuged (800g, 10 min, 4 °C). Subsequently, the lowest fraction containing all hRBCs was washed twice with nine volumes 1×PBS (PAA), triturating carefully. The concentrated hRBCs were re-suspended with 1×PBS to an erythrocyte concentration of 5% (v/v). Peptides, dissolved in 1×PBS and low DMSO concentration for dissolving reasons, were mixed each with 100 µL hRBCs 5% (v/v) and incubated under agitation on a flat shaker (170 rpm, 30 min, 37 °C). After sedimenting all probes (800g, 10 min, 4 °C), all supernatants were transferred into a fresh 96-well plate. Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 550 nm. Controls for 0% and 100% hemolysis consisted of hRBC 5% (v/v) suspended in PBS containing DMSO in according concentrations and 1% Triton X-100, respectively.

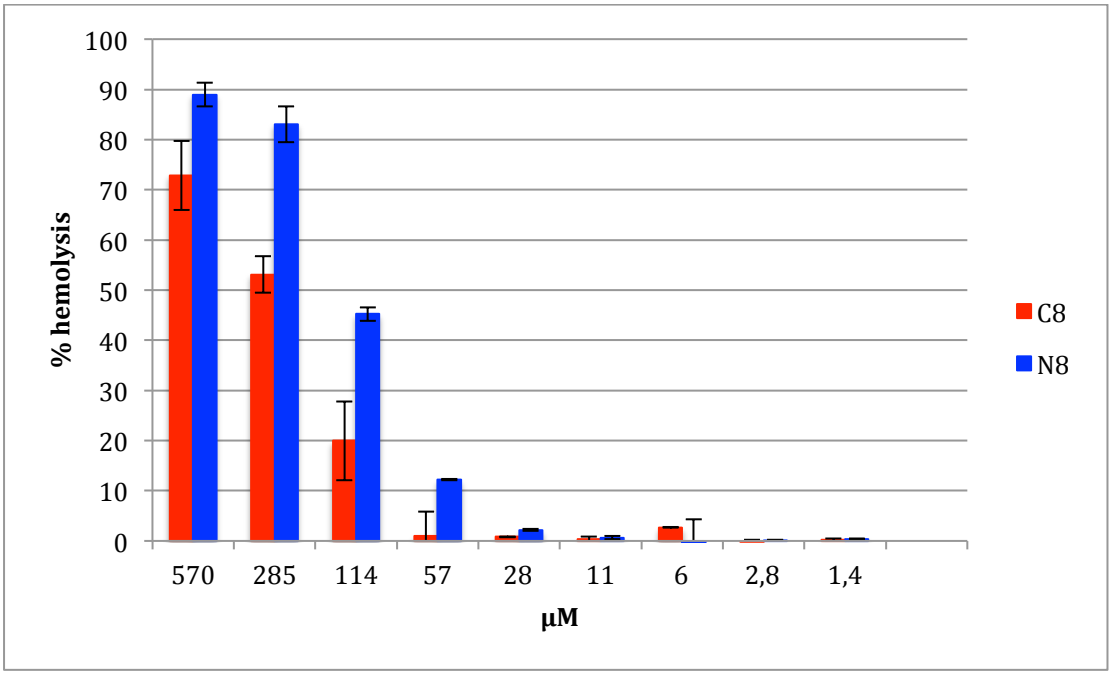


Figure S2. Determination of the 50% haemolytic concentration of *N*-C_8 (blue) and *C*-C_8 (red).

For the time-dependent (kinetics) comparison in hemolytic activity between *N*-C_8 and *C*-C_8, two tubes with hRBCs mixed with one of the two peptides were prepared (V_{tot} 630 µL) and 105 µL removed according to the time schedule (5, 10, 20, 30, and 60 minutes), immediately centrifuged and when all supernatants were present, measured at 550 nm.

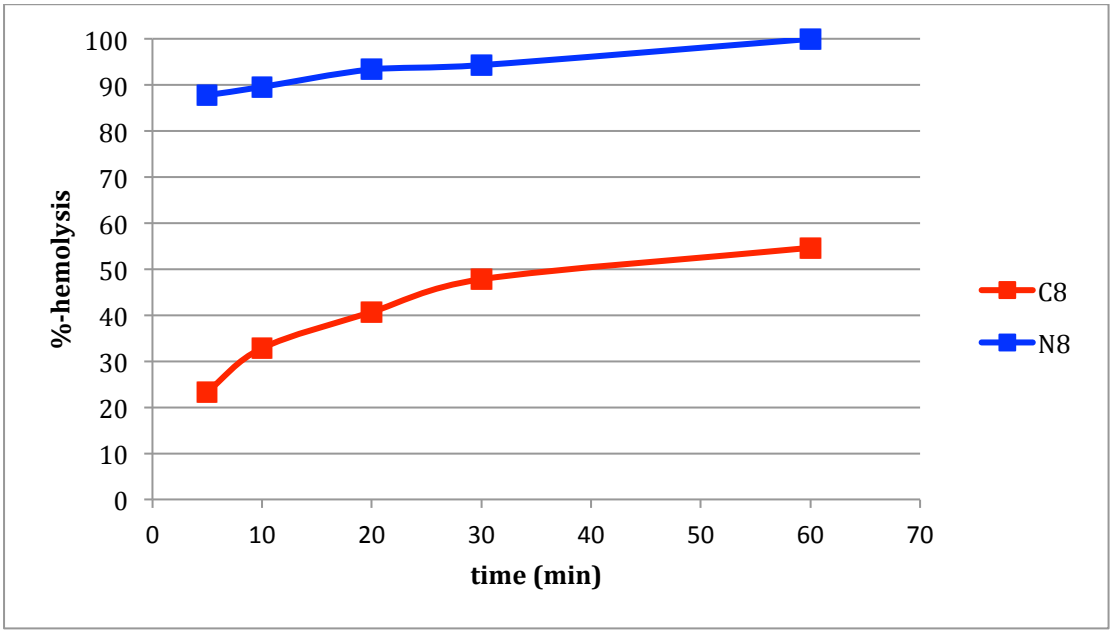


Figure S3. Time-dependent hemolysis of *N*-C_8 and *C*-C_8 with 500 µg/mL (285 µM).

IC_{50} values

Toxicity on human cancer cell lines was determined according to previously described procedures.[3]

[3] Bernhardt, G.; Reile, H.; Birnböck, H.; Spruß, T.; Schönenberger, H. *J. Cancer Res. Clin. Oncol.* **1992**, *118*, 35-43. Neukamm, M. J.; Pinto, A.; Metzler-Nolte, N, *Chem. Commun.* **2008**, 232-234.