Supporting Information for:

Inverted Polyamidoamine (i-PAMAM) Dendrimer Antimicrobials

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Contents

1. Antimicrobial Activities

i-PAMAM dendrimers cytotoxicity was assayed against *P. aeruginosa* PAO1, *P. aeruginosa* PA14, *P. aeruginosa* PA14 4.13, *P. aeruginosa* PA14 4.18, *P. aeruginosa* PA14 2P4, *K. pneumoniae* NCTC418, *E. coli* W3110, *A. baumannii* ATCC 19606, *S. aureus* COL (clinical isolate of MRSA) and *S. aureus* Newman.

To determine the Minimal Inhibitory Concentration (MIC), Broth Microdilution method was used.[1] A colony of bacteria from glycerol stock was grown in LB medium overnight at 37 °C and agitated at 180 rpm. The compounds were prepared as stock solutions of 2 mg/mL in sterilized MilliQ deionized water, added to the first well of a 96-well sterile, round bottom microtiter plates in polypropylene (Costar®, untreated) and diluted serially by ½. The concentration of the bacteria was quantified by measuring absorbance at 600 nm and diluted to an OD_{600} of 0.022 in 12.5% MH medium (pH 7.4 or 8.5). The sample solutions (150 µL), prepared at the desired concentration in 12.5% MHB (pH 7.4 or 8.5), were mixed with 4 µL diluted bacterial suspension with a final inoculation of about $5x10^5$ CFU. For each test, two columns of the plate were kept for sterility control (12.5% MH medium only, pH 7.4 or 8.5), growth control (12.5% MH medium with bacterial inoculum, no compound, pH 7.4 or 8.5). The positive control, Polymyxin B (starting with a concentration of 16 µg/mL) in 12.5% MH medium (pH 7.4 or 8.5) with bacterial inoculums, was introduced in the two first lines of the plate. The plates were incubated at 37 °C for ca. 18 hours under static conditions. 15 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[2] (1 mg/mL in sterilized MilliQ deionized water) were added to each well and the plates were incubated for 30 minutes at room temperature. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound that inhibits the visible growth of the tested bacteria (yellow) with the unaided eye.

2. Serum Stability Assay

Human serum was diluted in 0.1 M filtered TRIS buffer pH 7.4 (25%, 1:3, v/v). Selected peptide dendrimers were diluted in 0.1 M filtered TRIS buffer pH 7.4 to a concentration of 400 µM and 0.1 mg/mL of 4-hydroxybenzoic acid was added as internal standard. Aliquots of peptide dendrimer solution (50 µL) were added to aliquots of serum (50 uL) in sterile Eppendorf tubes, to reach a dendrimer concentration of 200 µM during the assay. Samples were incubated at 37 °C under gentle stirring (350 rpm). Different samples (triplicates) were quenched at different time points (0 / 1 / 6 / 10 / 24 h) by precipitating serum proteins through the addition of (0.1 M) $ZnSO_4 \cdot 7\ H_2O$/ACN (1:1) (0.1 M, 100 µL) and cooling in ice bath for 10 minutes. Protein precipitates were pelleted under centrifugation (5 min at 4'000 rpm) and the supernatants were analysed by LC-MS. Experiment controls included a precipitation control for each peptide dendrimer to test their resistance to the protein precipitation conditions and serum blanks to check reproducibility over different serum batches. Peaks corresponding to the internal standard and the undegraded dendrimers were integrated, with the ratio dendrimer/standard at t = 0 h as 100%.

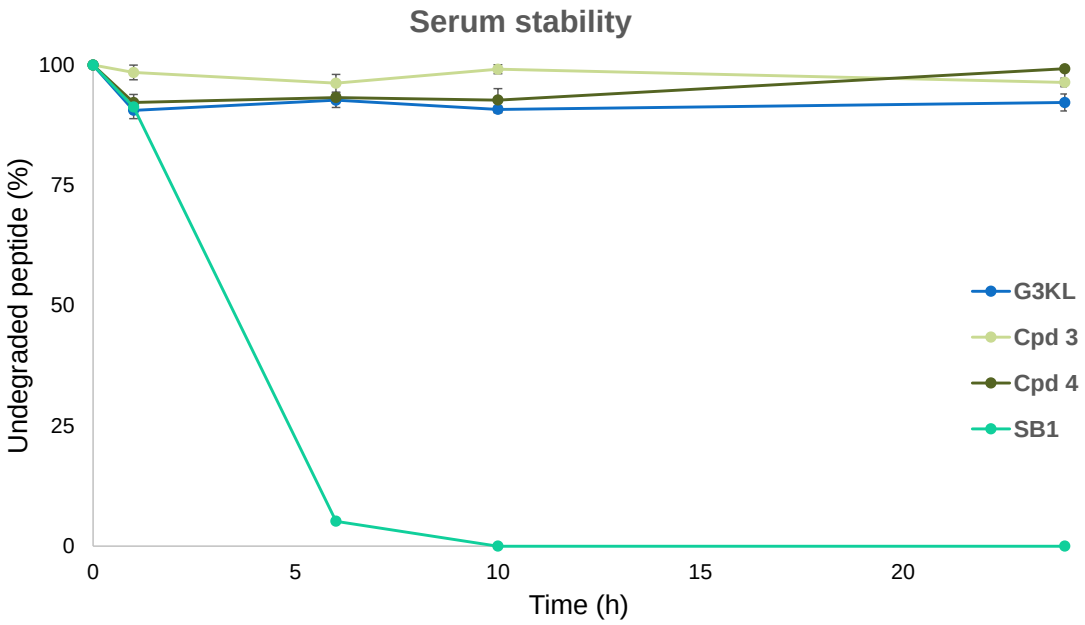


Figure S1. Serum stability assay of the respective compound (200 µM), incubated with human serum (12.5% in TRIS buffer, 0.1 M, pH 7.4) for different times, at 37 °C. Normalized undegraded dendrimer values determined by RP-HPLC analysis using 4-hydroxybenzoic acid as internal standard.

3. Acid-base titration

Powder dendrimer samples (ca. 2.6 mg) were diluted in Milli-Q water 4 mL (final concentration of dendrimers is 100 µM) and acidified to pH ~3 with 1 M HCl. Then, 0.1 M NaOH was added in step of 2 µL with a Dosimat plus (Metrohm, Zofingen, Switzerland) and pH was measured on a 692 pH/ion meter (Metrohm).

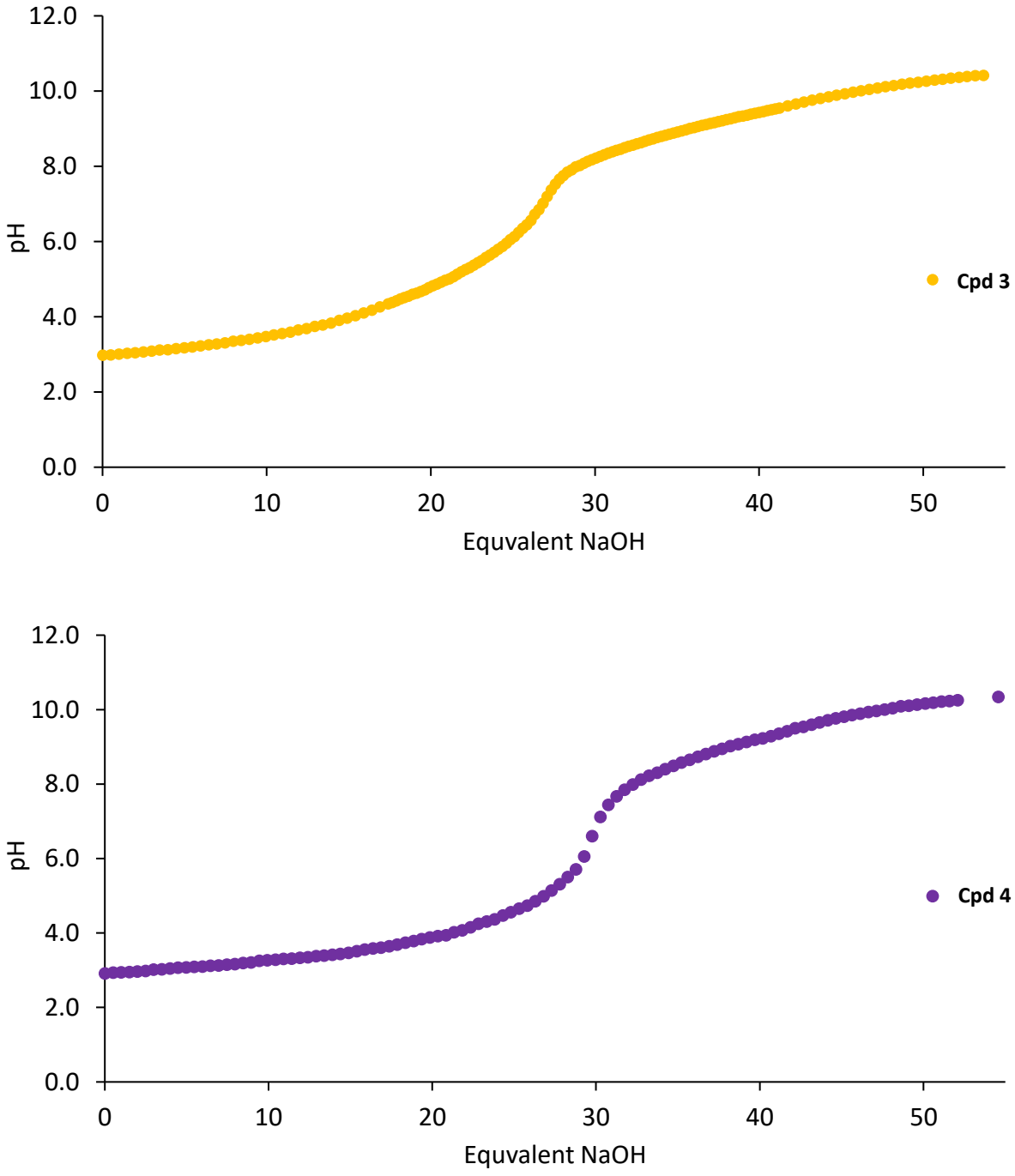


Figure S2. Titration curves for i-PAMAM **3** and **4** (100 µM) by addition of 2 µL NaOH (0.1 M).

4. pKa predictions – with Marvin

Marvin was used to predict the pKa of i-PAMAM **3** and **4**: Marvin 17.21.0, Chemaxon (https://www.chemaxon.com).

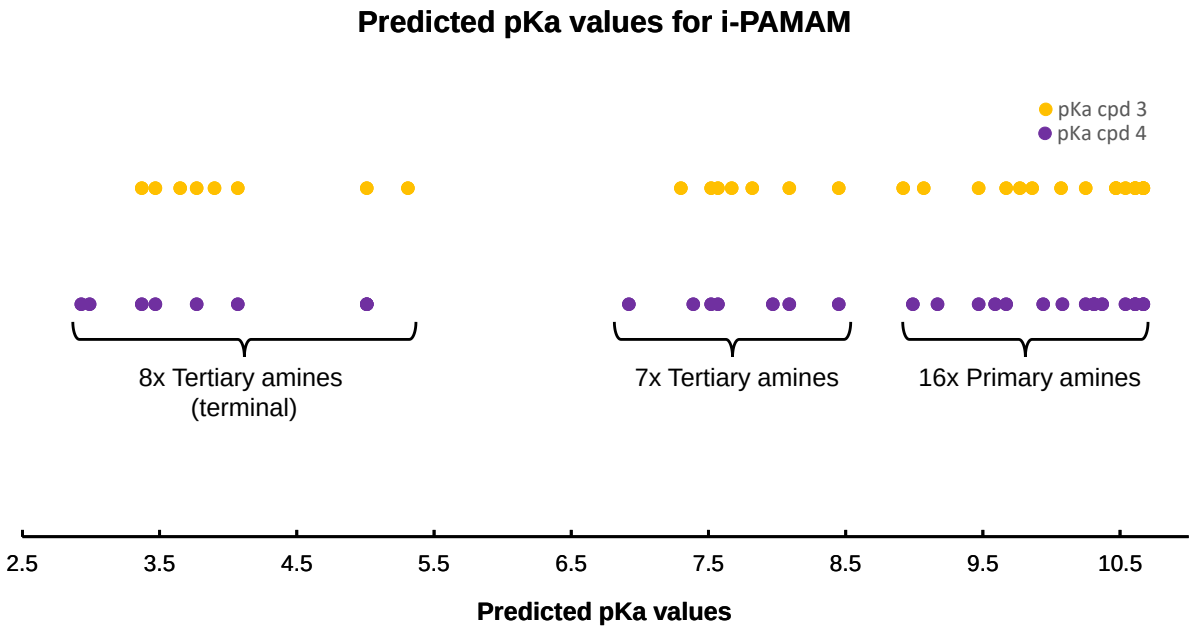


Figure S3. Predicted pKa for compound **3** and **4**, from the software Marvin from Chemaxon.

*Compound **3***

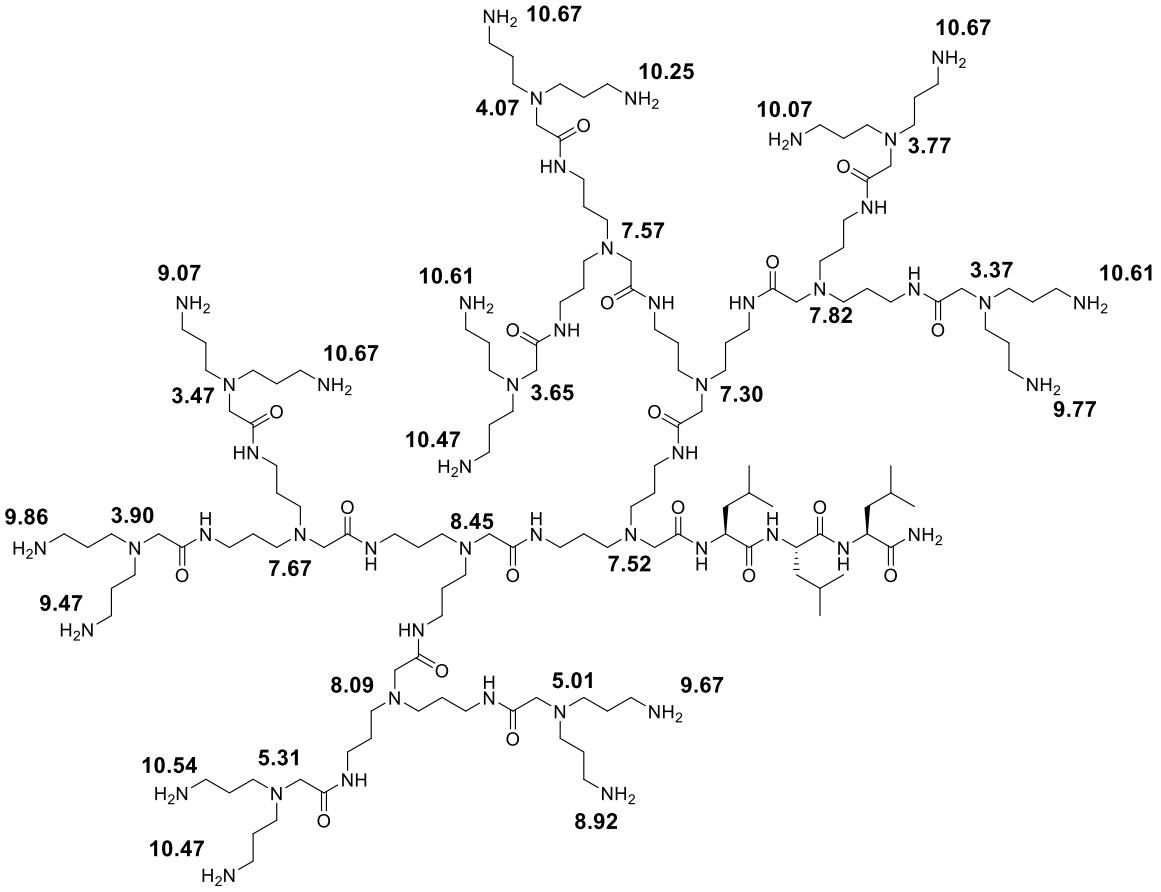


Figure S4. Chemdraw structure with the pKa values predicted with Marvin, for compound **3**.

*Compound **4***

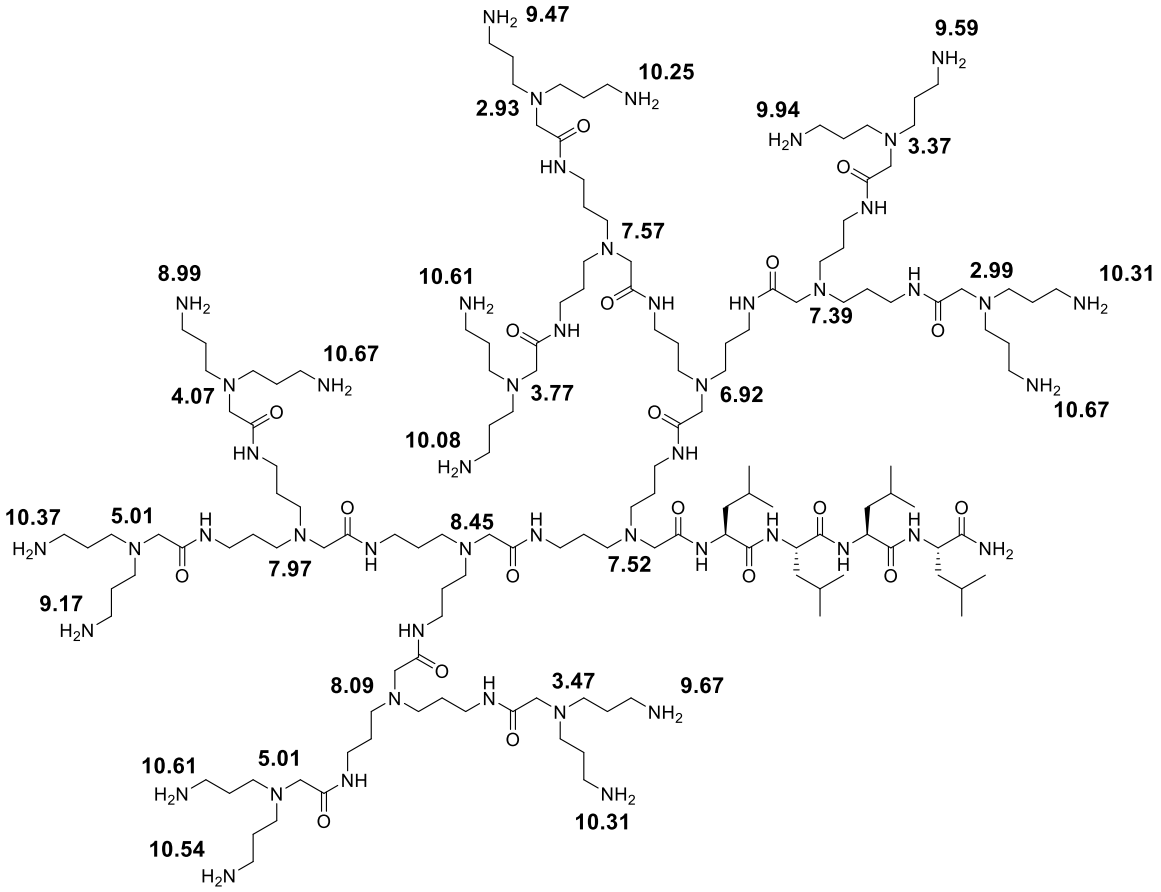


Figure S5. Chemdraw structure with the pKa values predicted with Marvin, for compound **4**.

5. DOSY NMR

Standard diffusion NMR experiments were performed using a Brucker Avance 400 MHz with diluted solutions of dendrimers in D_2O (ca. 7-10 mg mL^{-1}, pH~3, at 298 K). The gradient with a maximum strength of $50 \cdot 10^{-4}$ $T \cdot cm^{-1}$ was calibrated using the HOD proton signal in D_2O (99.997%). The diffusion time Δ was set at 125 ms and the gradient duration δ at 7 ms. Data analysis was performed by using MestReNova V14.2.0 (software from Mestrelab Research, https://mestrelab.com/) and the diffusion coefficient D [$m^2 \cdot s^{-1}$] was derived from peak integrals or intensities. The hydrodynamic radii were calculated from the diffusion coefficient D using the Stokes-Einstein equation $R_h = k_B \cdot T/(6 \cdot \pi \cdot \eta \cdot D)$ with Boltzmann constant $k_B = 1.38065 \cdot 10^{-23}$ $J \cdot K^{-1}$, temperature T in K and viscosity η = 1.089 mPa·s for D_2O.

*Compound **3***

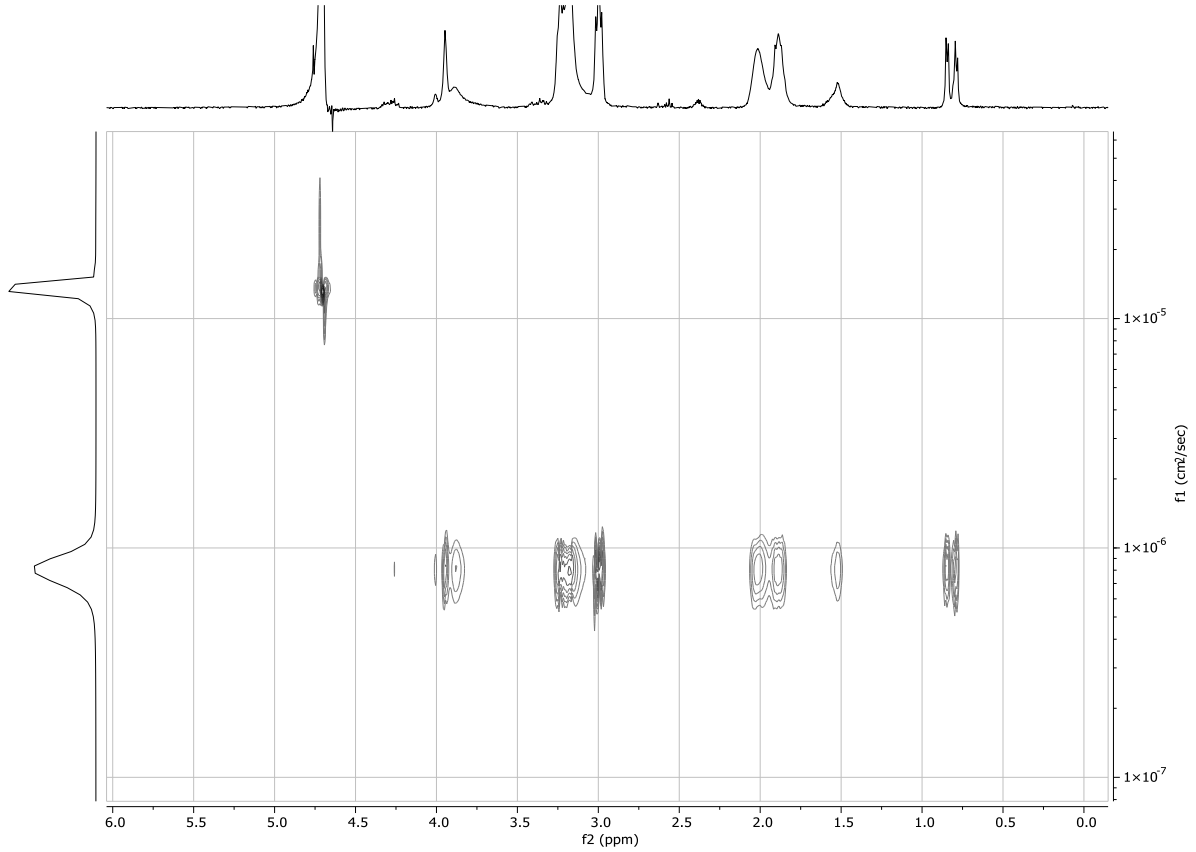


Figure S6. DOSY NMR for compound **3** measured in D_2O.

*Compound **4***

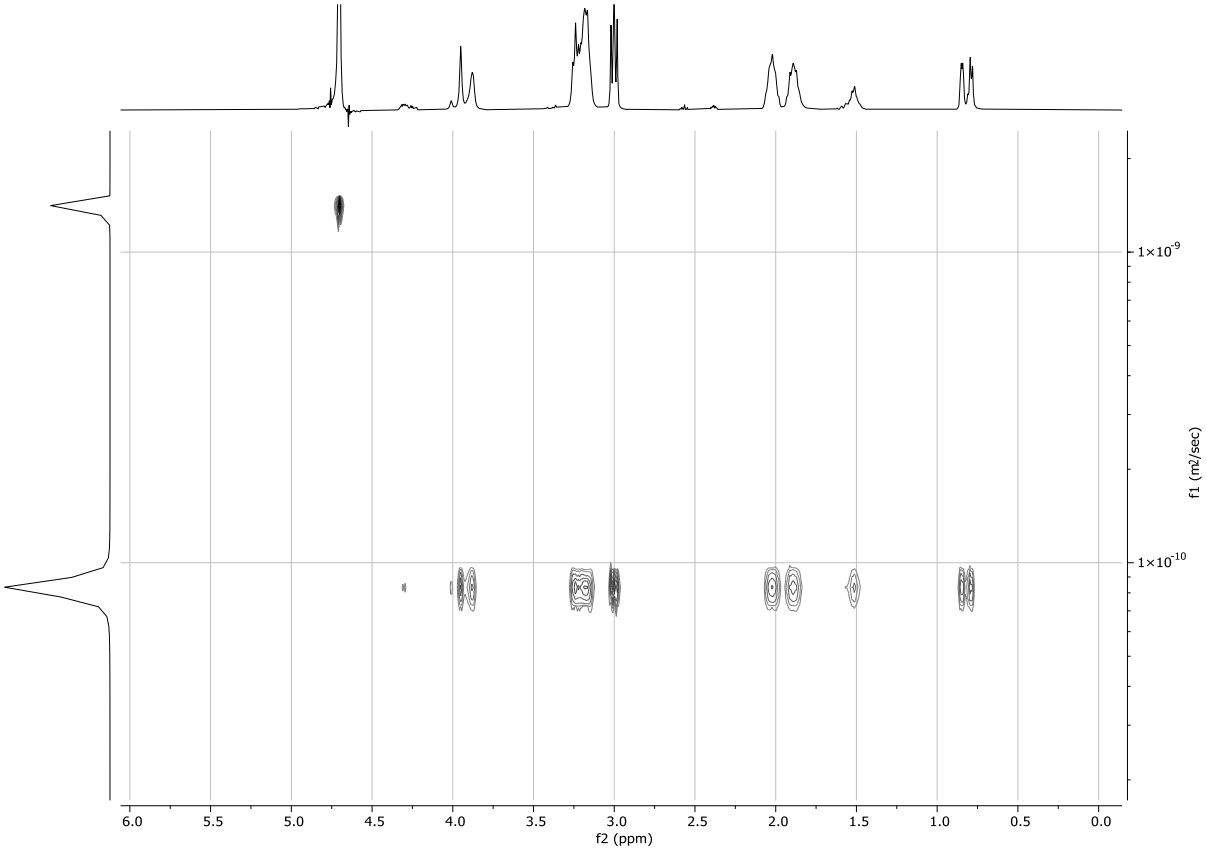
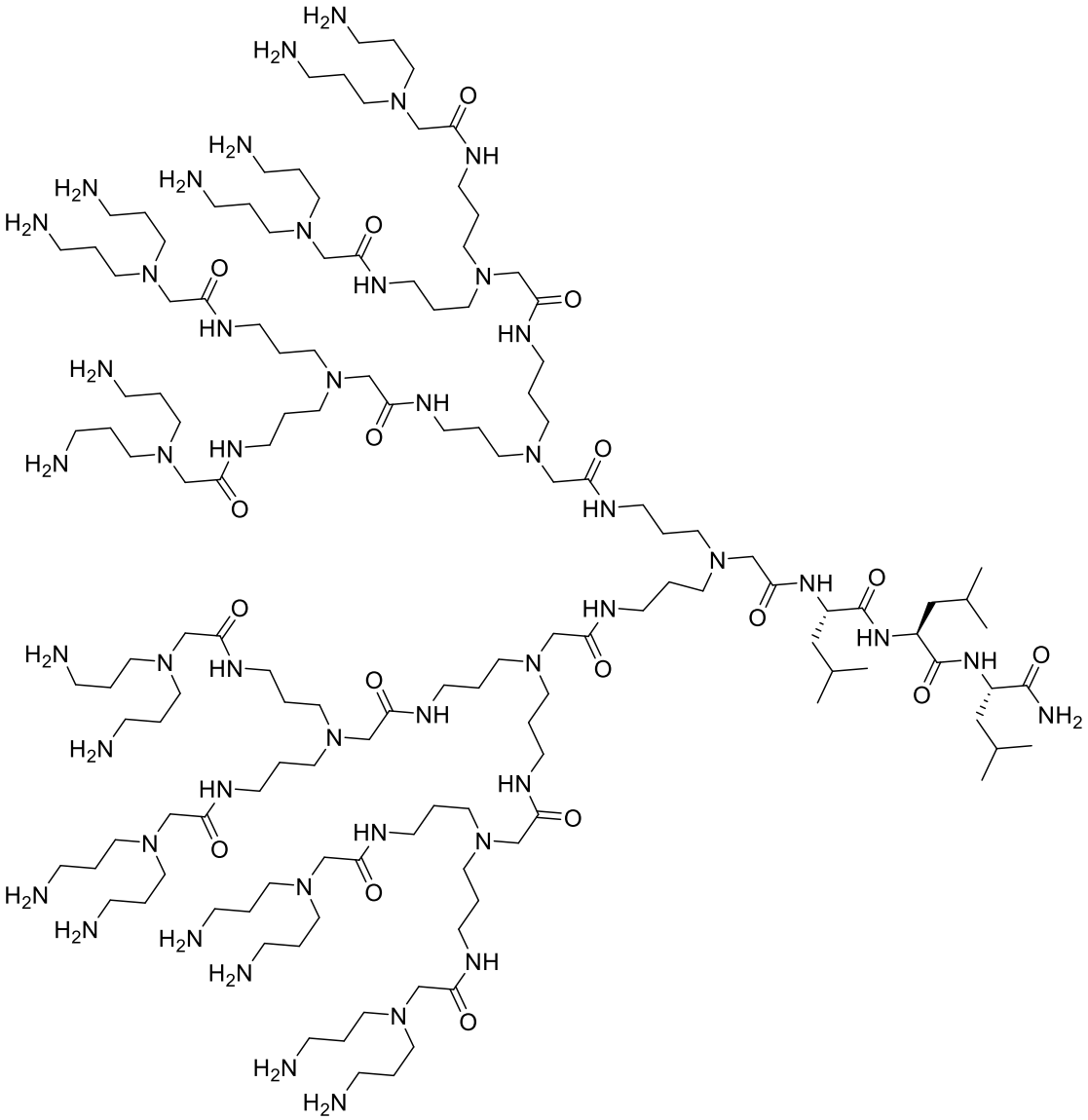


Figure S7. DOSY NMR for compound **4** measured in D_2O.

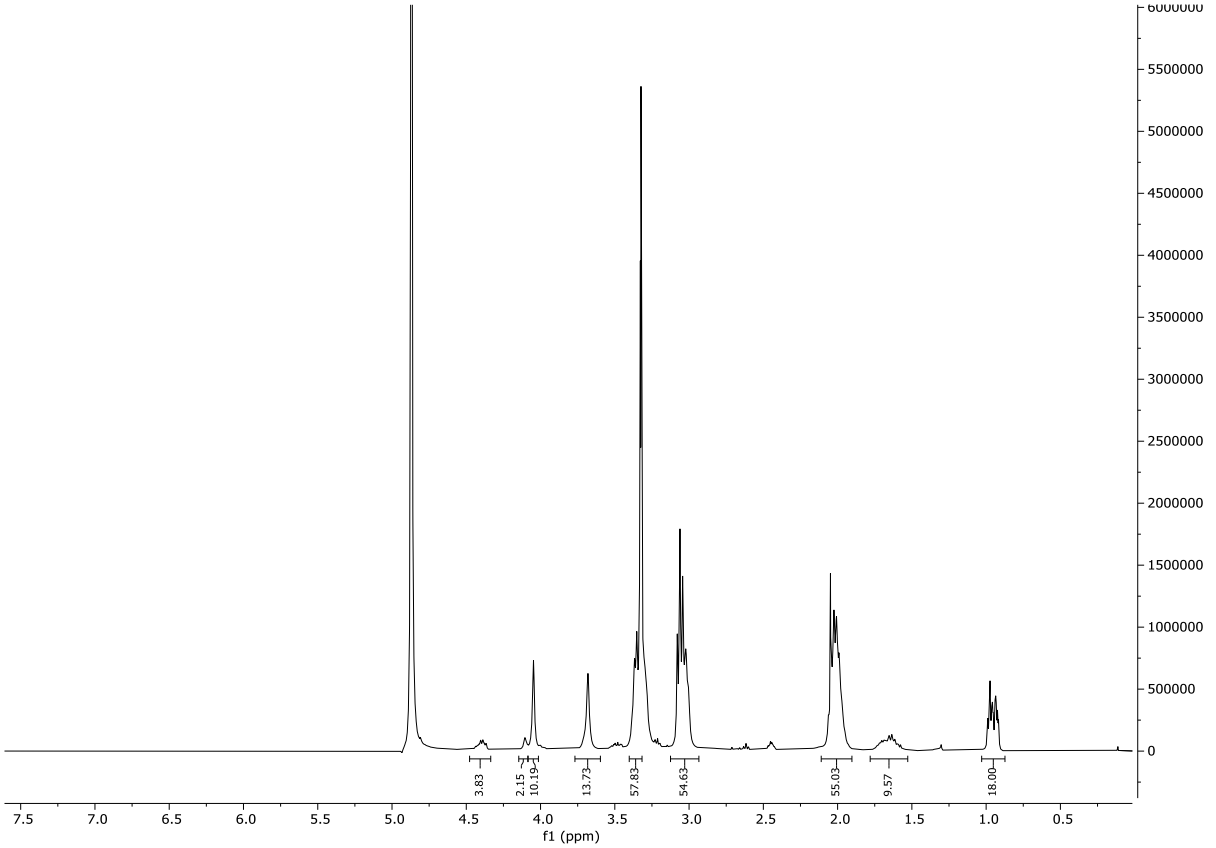
6. HPLC, MS and NMR data

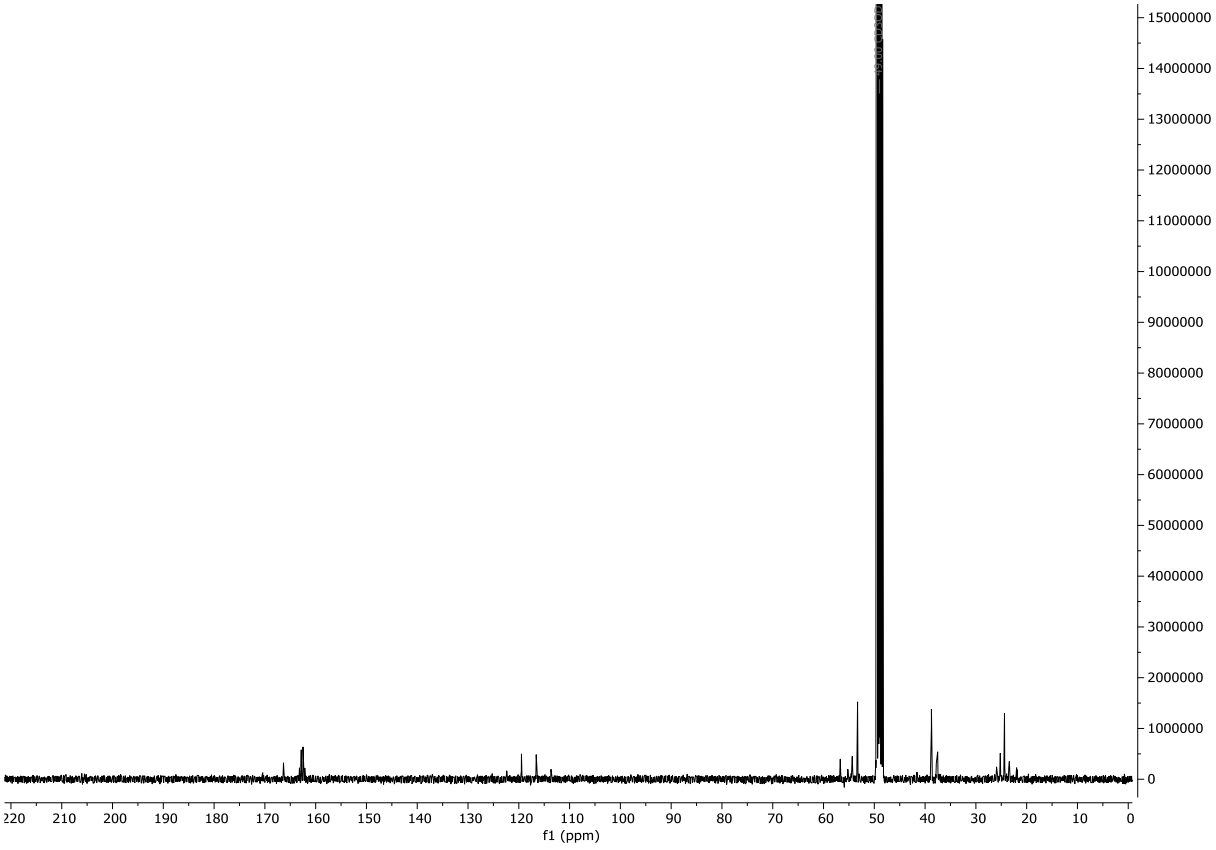
$(X)_8(X)_4(X)_2XLLL$ (3) was obtained as a foamy white solid after preparative RP-HPLC (43.1 mg, 10%). **Analytical RP-HPLC**: t_R = 1.80 min (A/D 100:0 to 0:100 in 7.50 min, λ = 214 nm). **MS** (ESI^+): $C_{138}H_{291}N_{49}O_{18}$ calc./obs. 2923.34/2923.3228 Da [M].

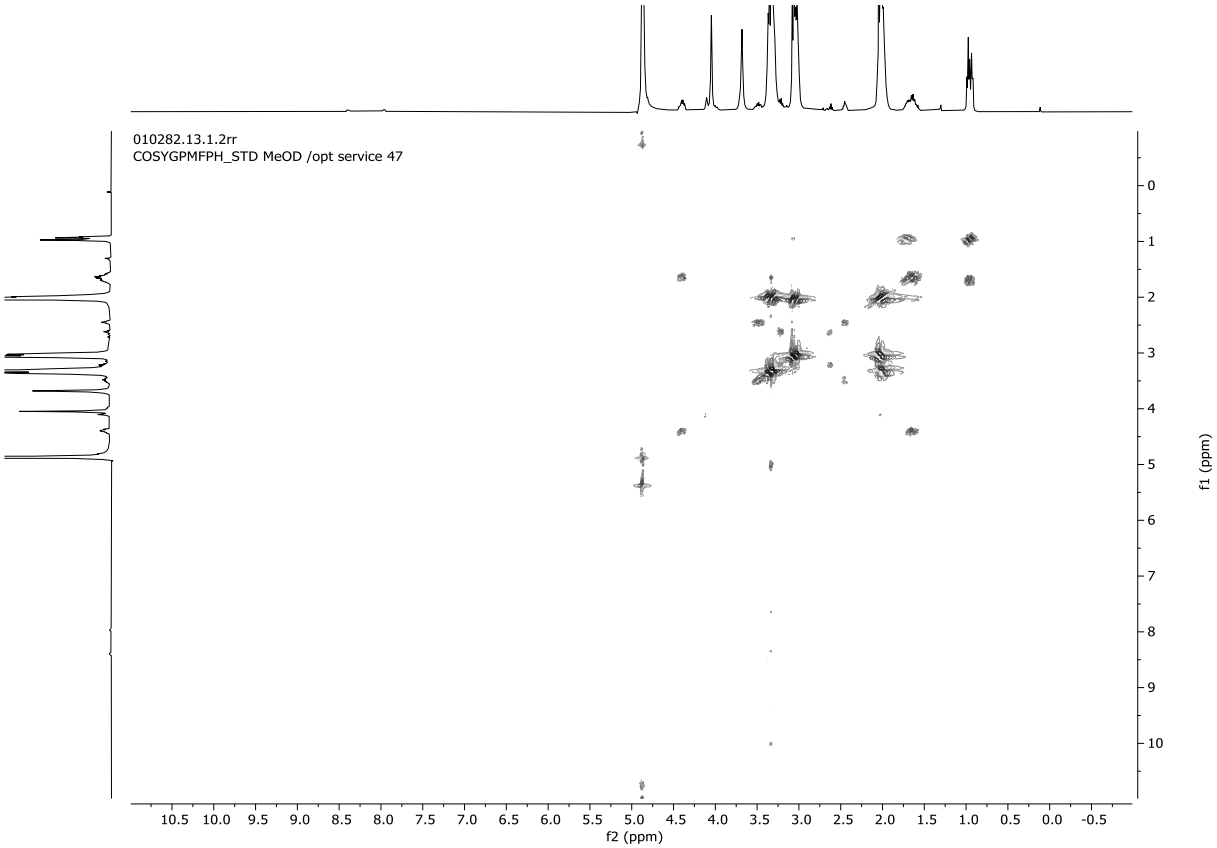


Chemical Formula: $C_{138}H_{291}N_{49}O_{18}$
Exact Mass: 2923.34

NMR



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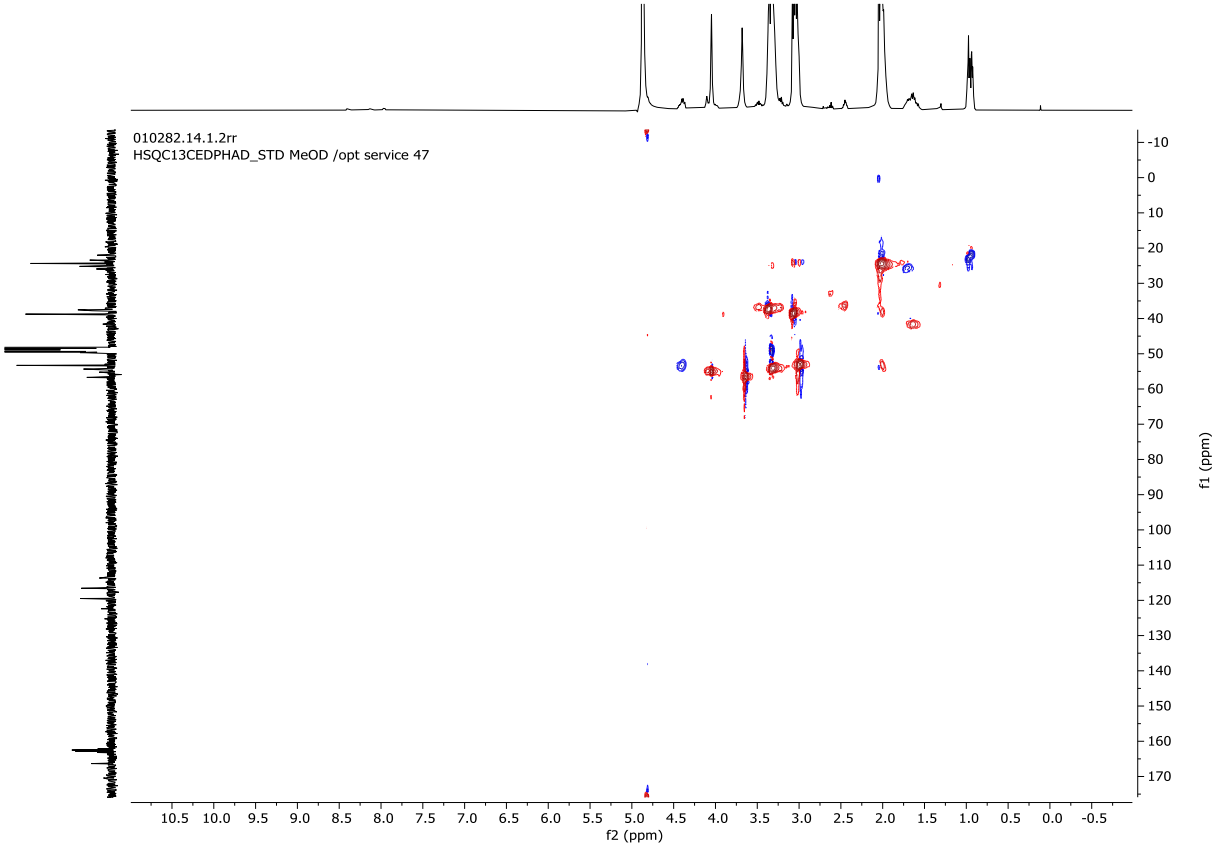
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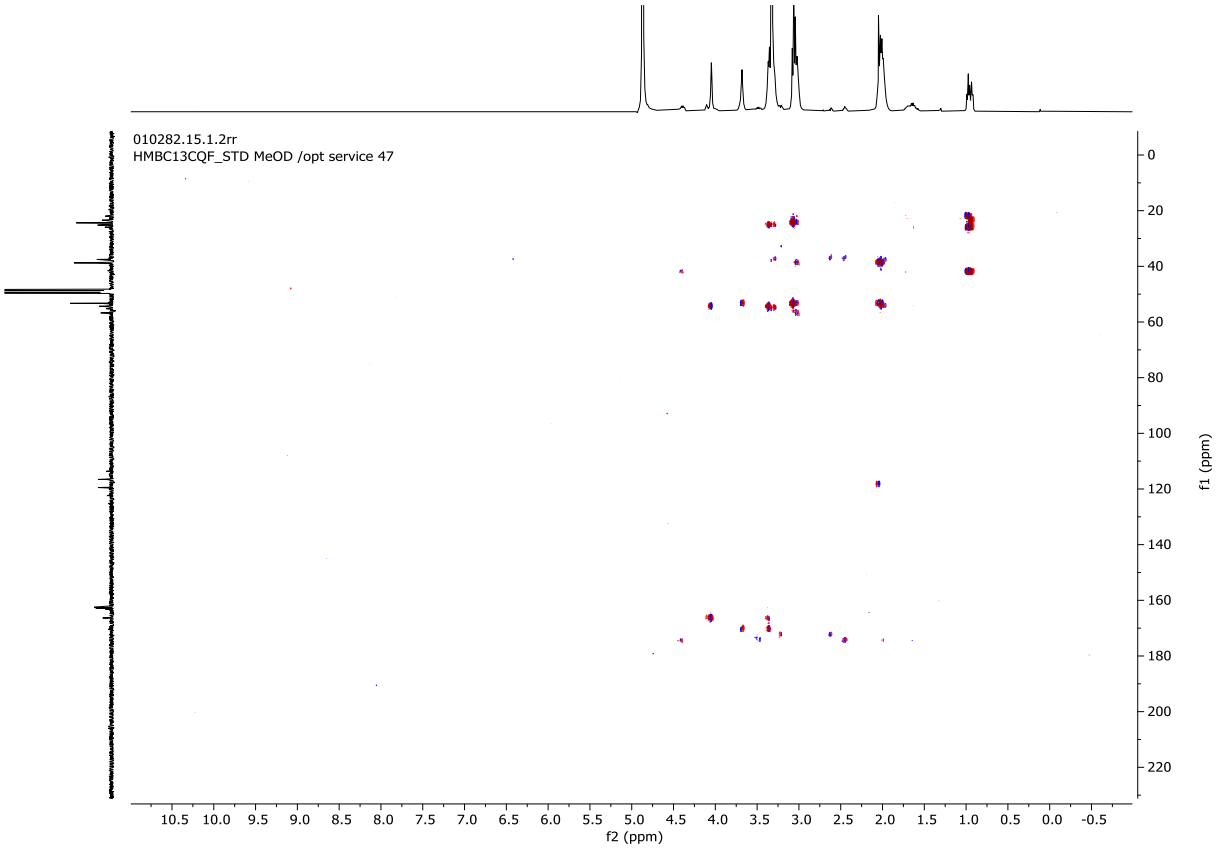
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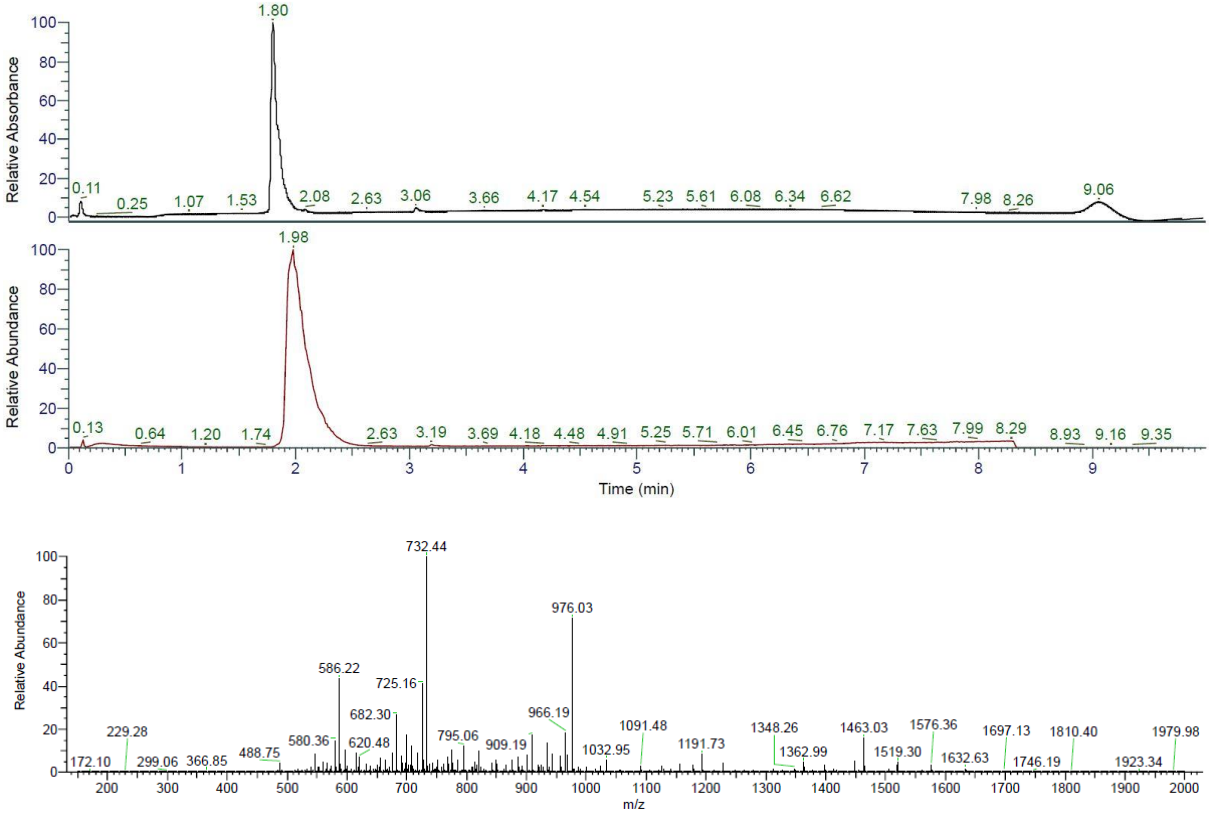
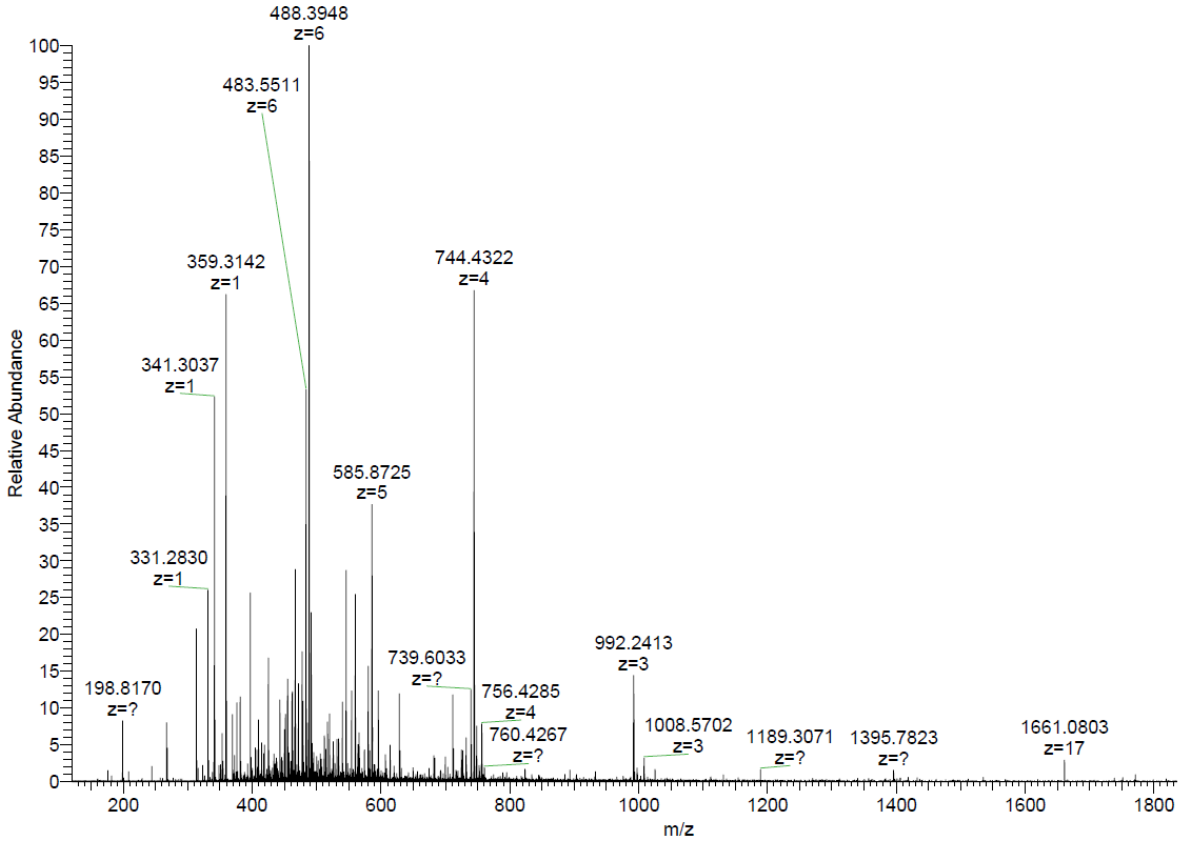
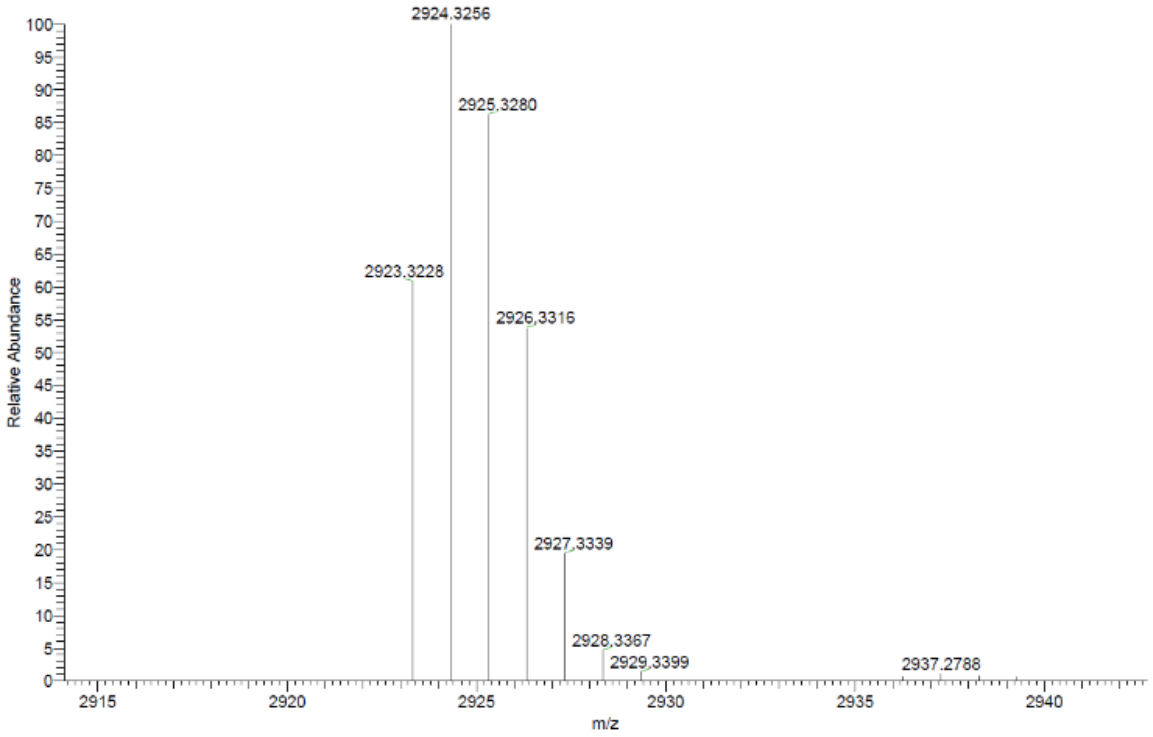
Analytical RP-HPLC

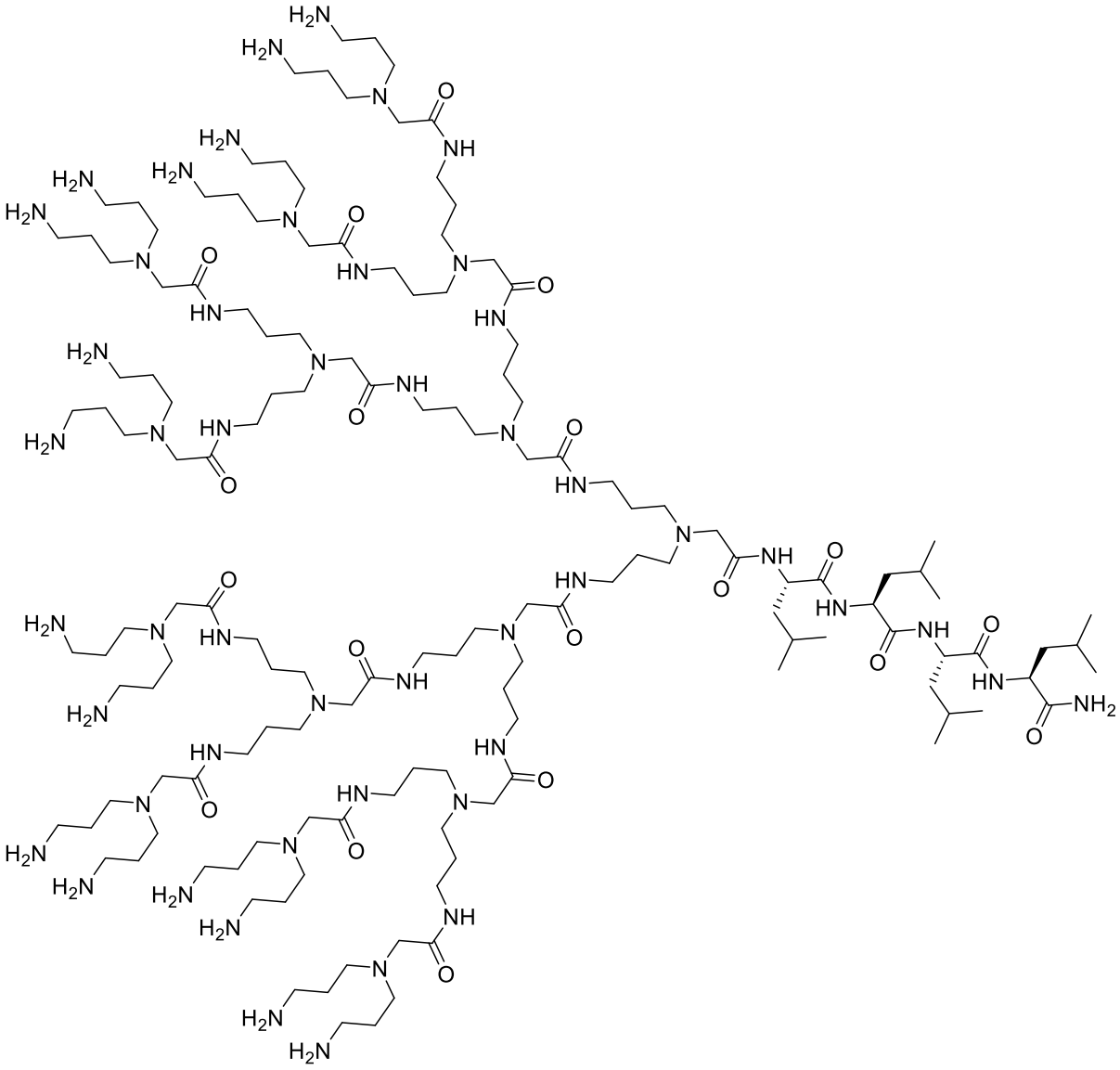


HRMS spectra



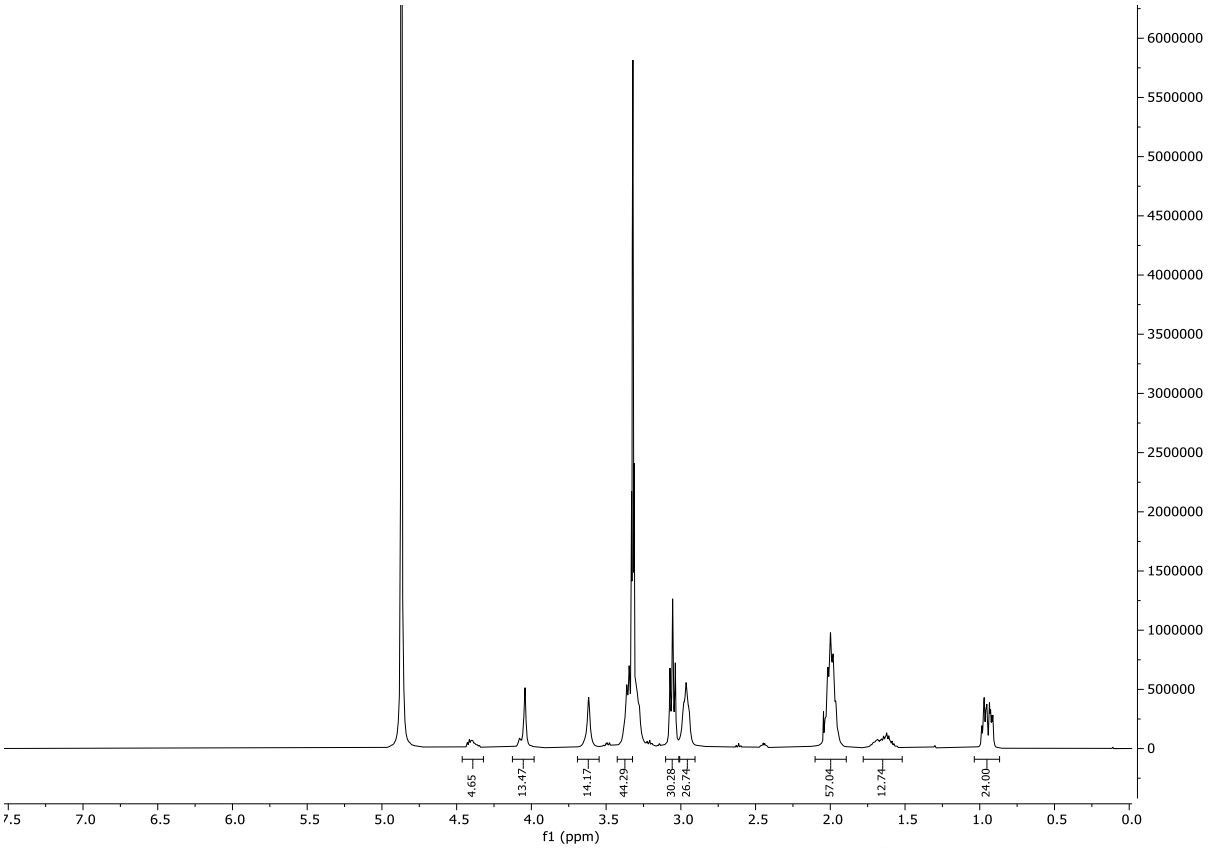


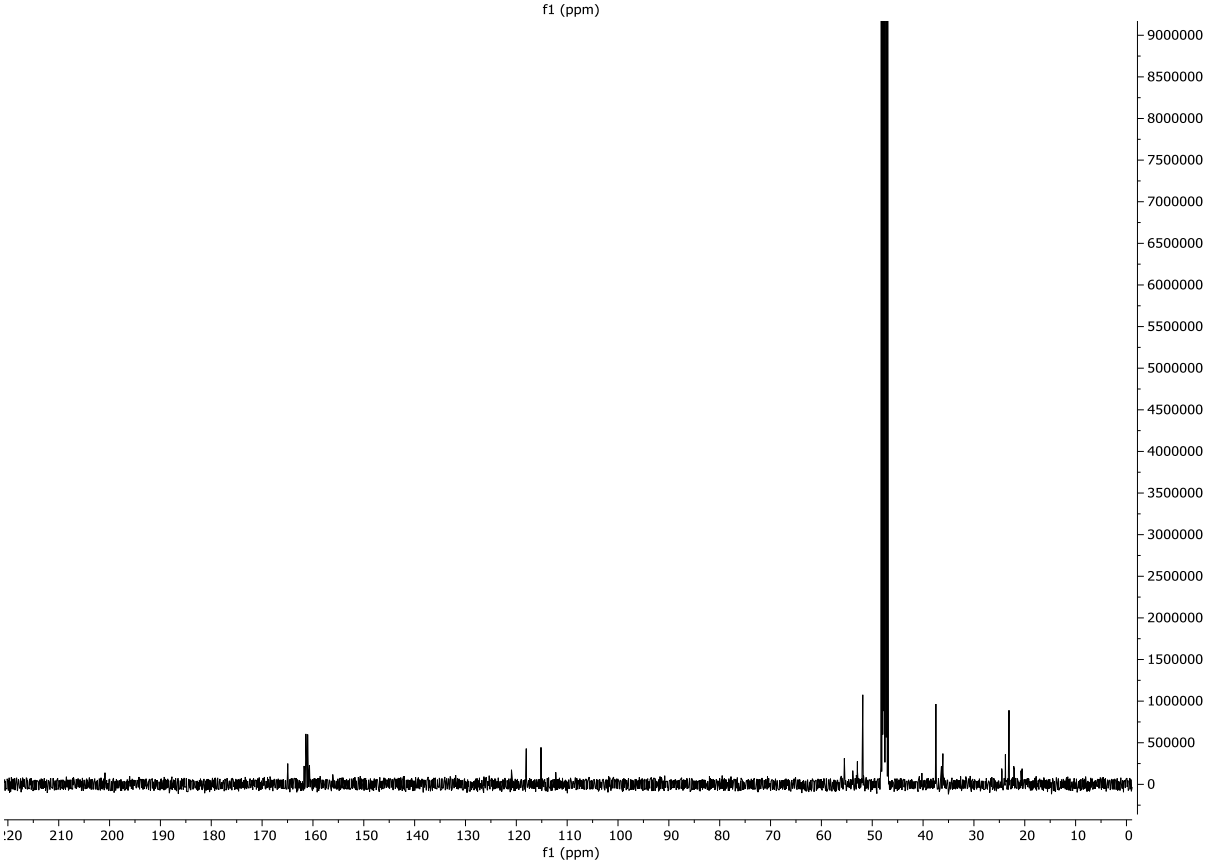
$(X)_8(X)_4(X)_2XLLLL$ (4) was obtained as a foamy white solid after preparative RP-HPLC (34.7 mg, 8%). **Analytical RP-HPLC**: t_R = 1.99 min (A/D 100:0 to 0:100 in 7.50 min, λ = 214 nm). **MS** (ESI^+): $C_{140}H_{302}N_{50}O_{19}$ calc./obs. 3036.42/3036.4061 Da [M].

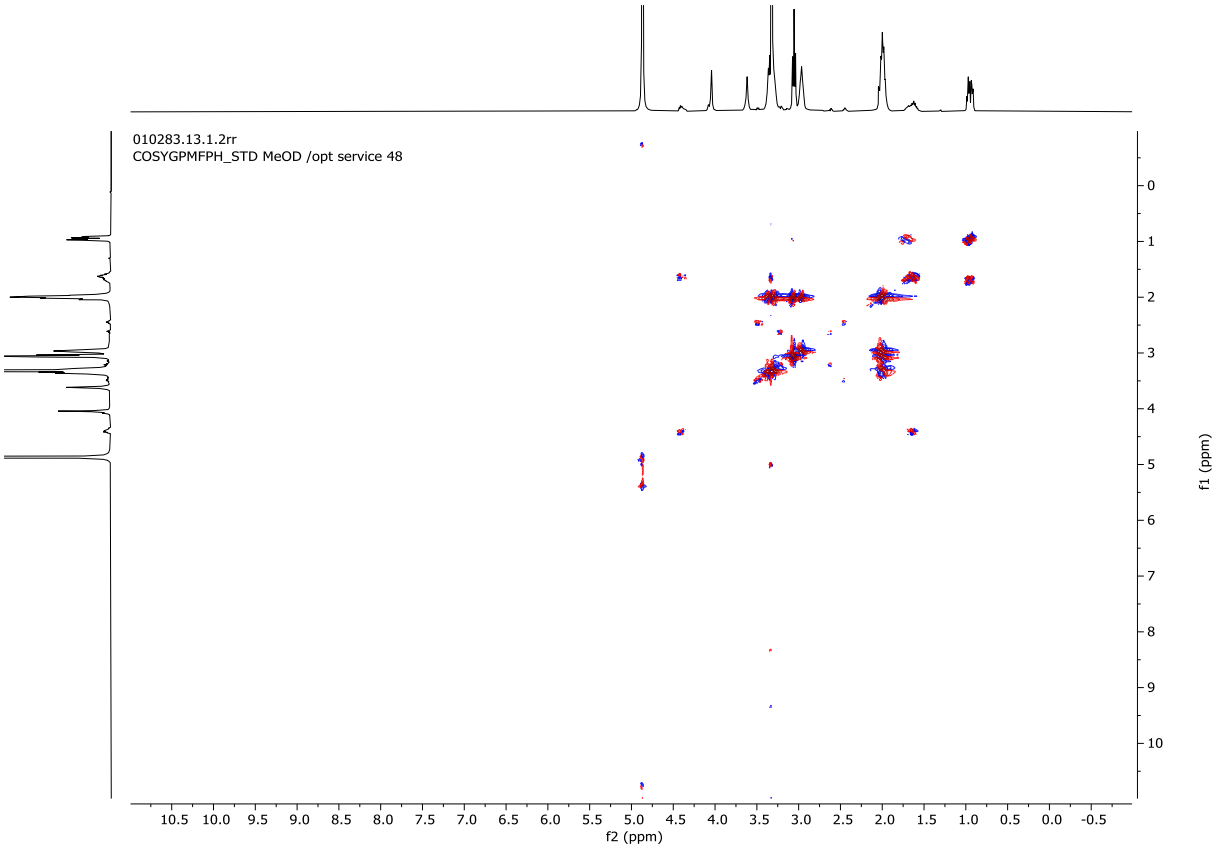


Chemical Formula: $C_{144}H_{302}N_{50}O_{19}$
Exact Mass: 3036.42

NMR



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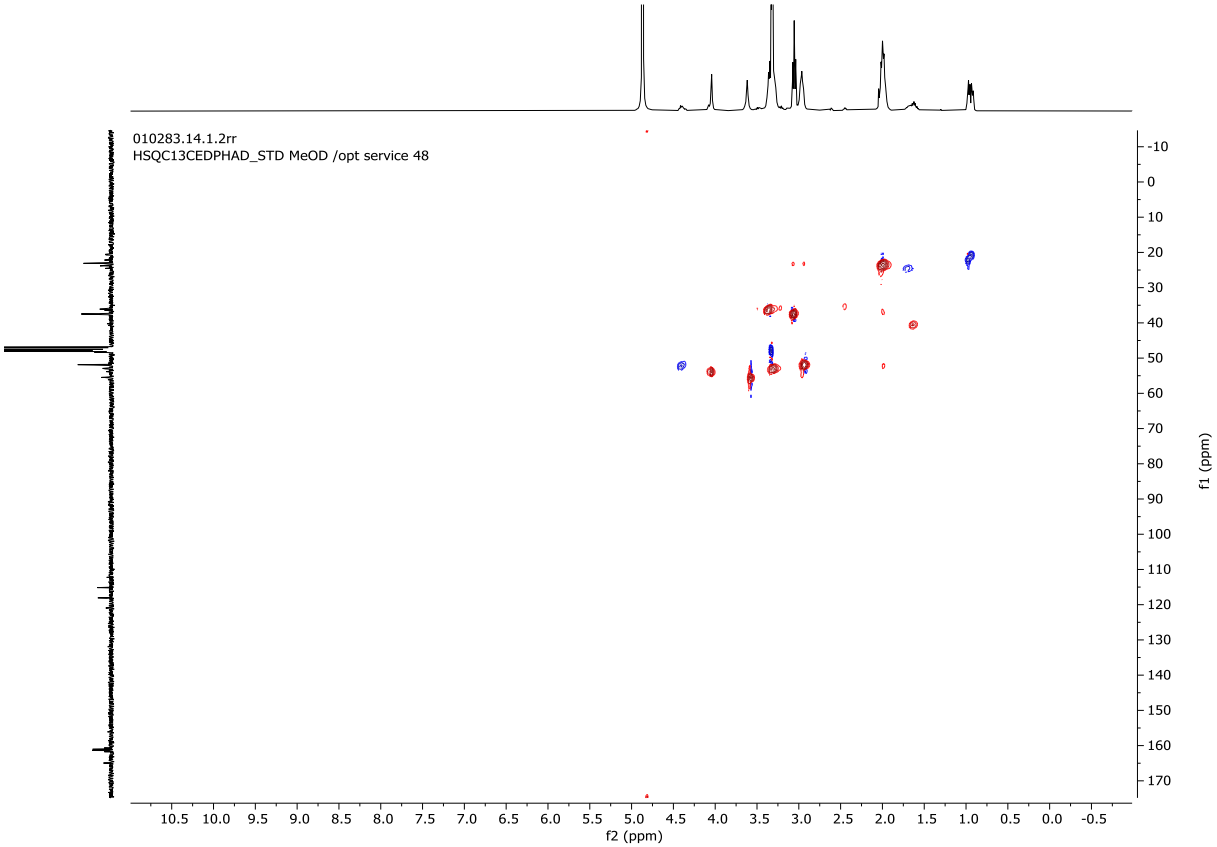
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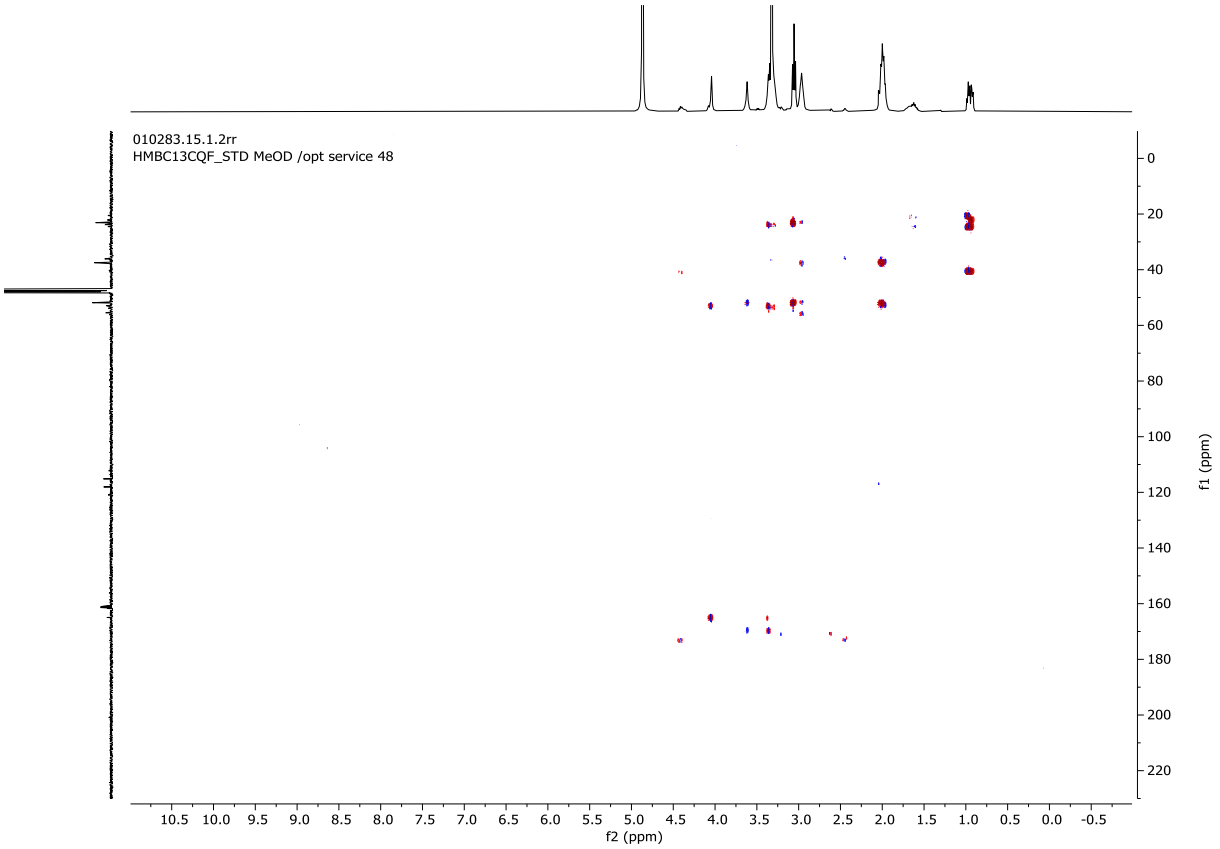
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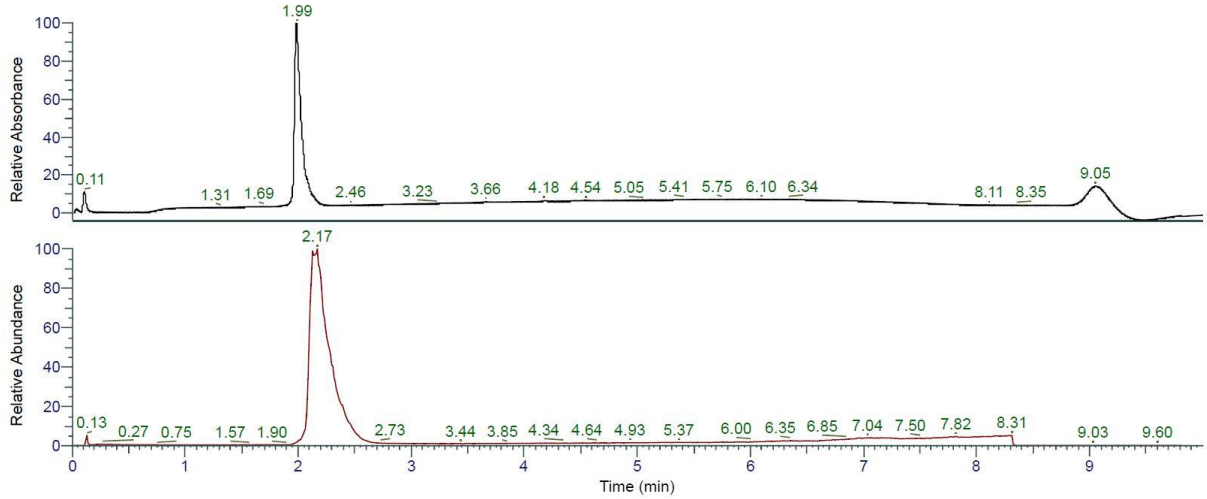
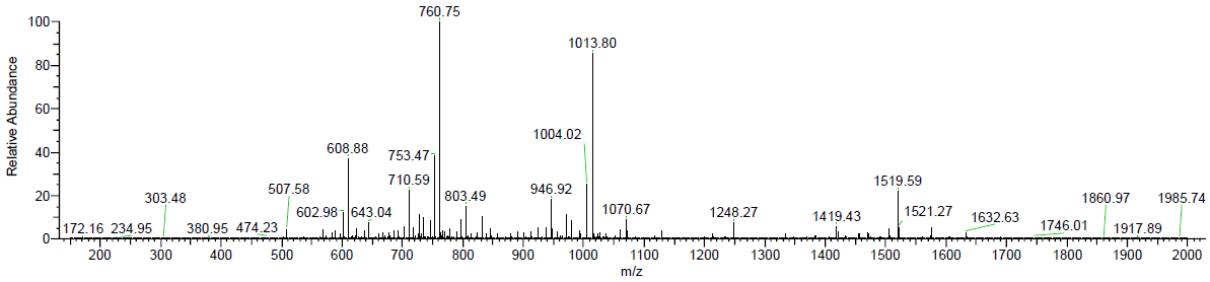
Analytical RP-HPLC





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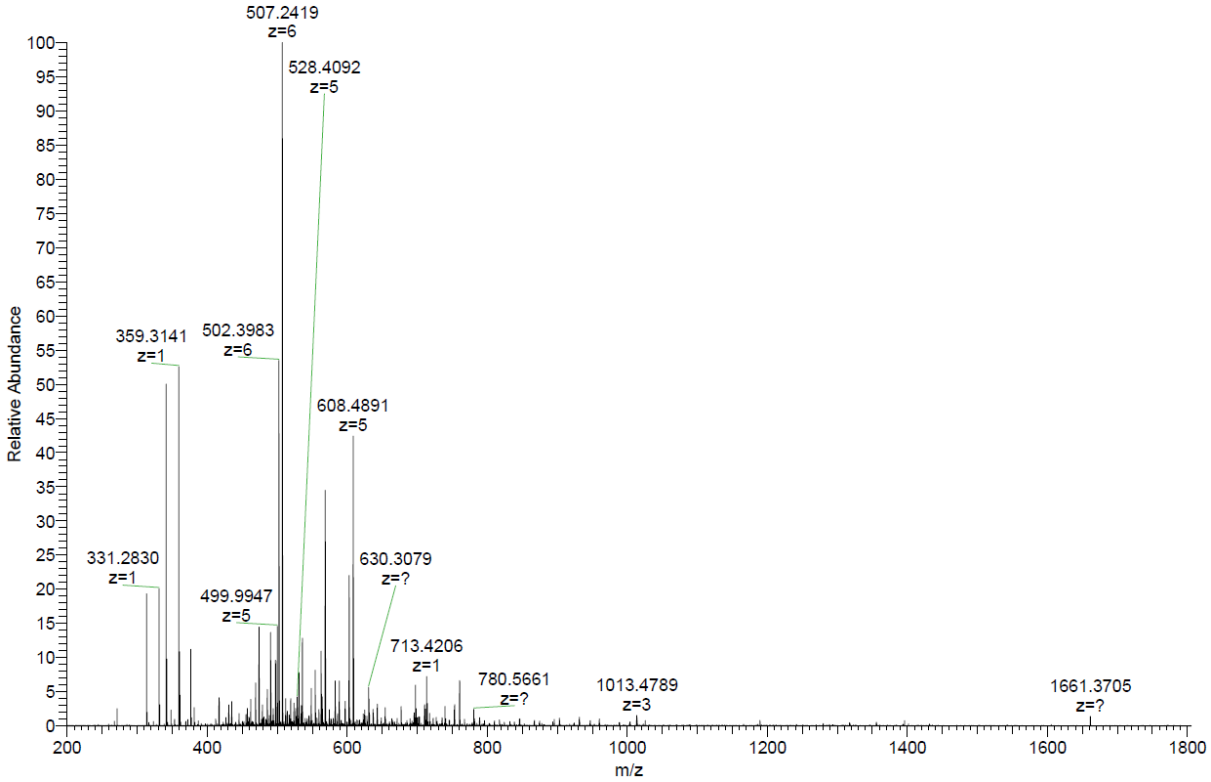
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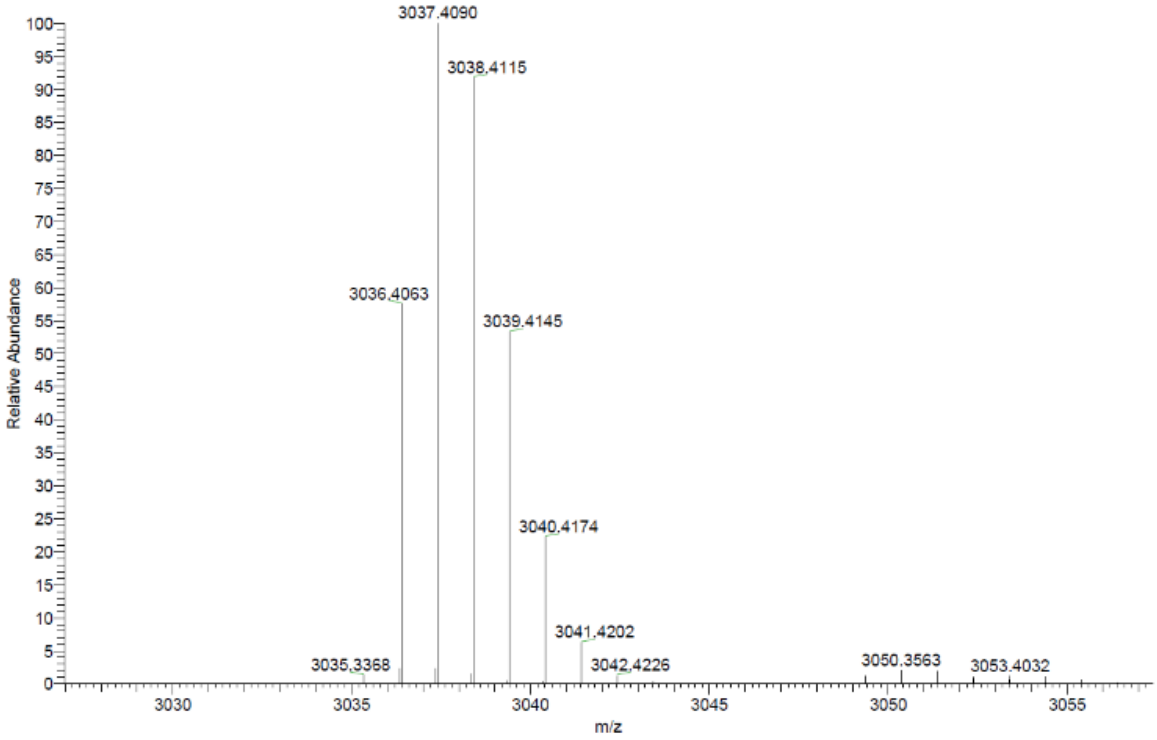
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Relative Abundance
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7. References

(1) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, *3* (2), 163–175. https://doi.org/10.1038/nprot.2007.521.

(2) Berridge, M. V.; Herst, P. M.; Tan, A. S. Tetrazolium Dyes as Tools in Cell Biology: New Insights into Their Cellular Reduction. In *Biotechnology Annual Review*; Elsevier, **2005**; Vol. 11, pp 127–152. https://doi.org/10.1016/S1387-2656(05)11004-7.