Supporting information for:

A convenient chemoenzymatic preparation of chimeric macrocyclic peptide antibiotics with potent activity against Gram-negative pathogens

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Reagents and General Procedures

All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. Fmoc-Dab(Boc)-OH, Fmoc-D-Dab(Boc)-OH, Fmoc-Hse(Trt)-OH, Fmoc-Tle-OH and Fmoc-Glu-OAllyl were obtained from Combi-Blocks. For compound characterization HRMS analysis was performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionisation) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000.

Purity of the peptides was confirmed to be ≥ 95% by analytical RP-HPLC using a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 5 µm) at 30 °C and equipped with a UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile, 95/5; solvent B, 0.1 % TFA in water/acetonitrile, 5/95. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 13 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min.

The compounds were purified via preparative HPLC using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 µm) and equipped with a ECOM Flash UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 45 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min.

Synthesis

*Tetra-Boc-protected PMEN/PMBN (**4a,b**)*

PMEN-Boc_4 (**4a**) and PMBN-Boc_4 (**4b**) were prepared as previously described for PMBN-Boc_4.[1]

*PMEN azide derivatives (**8a-c**)*

*PMEN-C_2-N_3 (**8a**)*

PMEN-Boc_4 (0.45 g, 0.34 mmol) was dissolved in DCM and DMF (8:2 v:v, 10 mL). In a separate flask, 2-azidoacetic acid (68 mg, 0.68 mmol) and BOP (0.30 g, 0.68 mmol) were dissolved in DCM (8 mL). The mixture of 2-azidoacetic acid and BOP was then added to the PMEN-Boc_4, followed by addition of DIPEA (0.24 mL, 1.4 mmol). The reaction was left to stir overnight at RT under N_2 atmosphere. After completion, the solvent was evaporated and the residue treated with TFA/TIPS/H_2O (95:2.5:2.5, 8 mL) for 1.5 hours. The reaction mixture was added to ice-cold MTBE/PE (2/1, 120 mL). The resulting precipitate was washed with MTBE/PE (2/1). Crude peptide was lyophilized from t-BuOH/H_2O and HPLC purified. Yield: 130 mg, 0.13 mmol, 39%.

*PMEN-C_5-N_3 (**8b**)*

Compound was prepared as PMEN-C_2-N_3, starting from PMEN(Boc)4 and 5-azidopentanoic. Yield: 85 mg, 0.08 mmol, 36%.

*PMEN-(PEG)3-N3 (**8c**)*

Compound was prepared as PMEN-C_2-N_3, starting from PMEN(Boc)4 and 3-(2-(2-(2-azidoethoxy)-ethoxy)ethoxy)propanoic acid. Yield: 120 mg, 0.11 mmol, 43%.

*Tri-Boc-protected PMEH (**10**)*

PMEH-Boc_3 (**10**) was prepared and purified as previously described before for PMBH-Boc_3.[2]

*PMEH-C_2-N_3 (**11**)*

PMEH-Boc_3 (0.10 g, 0.10 mmol) was dissolved in DCM (1 mL). In a separate flask, 2-azidoacetic acid (20 mg, 0.19 mmol) was dissolved in DCM (1 mL) and 2,4,6-trimethylpyridine (50 uL, 0.38 mmol) was added. HCTU (79 mg, 0.19 mmol) HOBt (26 mg, 0.19 mmol) were dissolved in DMF (1 mL) and added to the 2-azidoacetic acid. Pre-activation was run for 5 min. at RT, after which the mixture of 2-azidoacetic acid and coupling agent was added to the flask containing PMEH-Boc_3. The coupling reaction was run for 2-3 hours at RT under N_2 atmosphere. When needed, additional 2-azidoacetic acid and coupling agents were added and reaction time was extended. Once complete, the solvent was evaporated and the residue treated with TFA/TIPS/H_2O (95:2.5:2.5, 4 mL) for 1.5 hours. The reaction mixture was added to ice-cold MTBE (40 mL). The resulting precipitate was washed with MTBE. Crude peptide was lyophilized from t-BuOH/H_2O and HPLC purified. Yield: 41 mg, 0.05 mmol, 51%.

*General procedure for formation of triazoles (**9a-9c, 12**)*

The alkyne modified peptidomimetic β-hairpin **7** was prepared using the same on-resin method employed in the synthesis of macrocycle **6** (see experimental section of main manuscript) but starting from Rink amide resin loaded with Fmoc-propargyl-glycine. The formation of the triazole linked bicyclic conjugates followed a previously described[3] protocol: To a solution of the alkyne modified peptidomimetic β-hairpin **7** (20.0 mg, 12.1 mmol, 1.0 eq) in ^{t}BuOH:H_20 (1:1, 1 mL) was added the polymyxin azide (**8a-8c, 11**) in 1.1 eq (19-22mg). Sodium ascorbate (0.25 mg, 1.2 mmol, 0.1 eq) was then added followed by $CuSO_4 \cdot 5H_2O$ (0.15 mg, 6 μmol, 0.05 eq). The mixture was stirred at room temperature for 1 hour at which time complete disappearance of starting materials was generally observed. The solution was diluted in 4 mL H_2O and directly subjected to RP-HPLC purification. Following lyophilization, the trialzole-linked conjugates were obtained as white powders in the following yields: **9a** (18mg 56%), **9b** (18mg 55%), **9c** (19 mg 60%), and **12** (21 mg 71%).

Antibacterial Assays

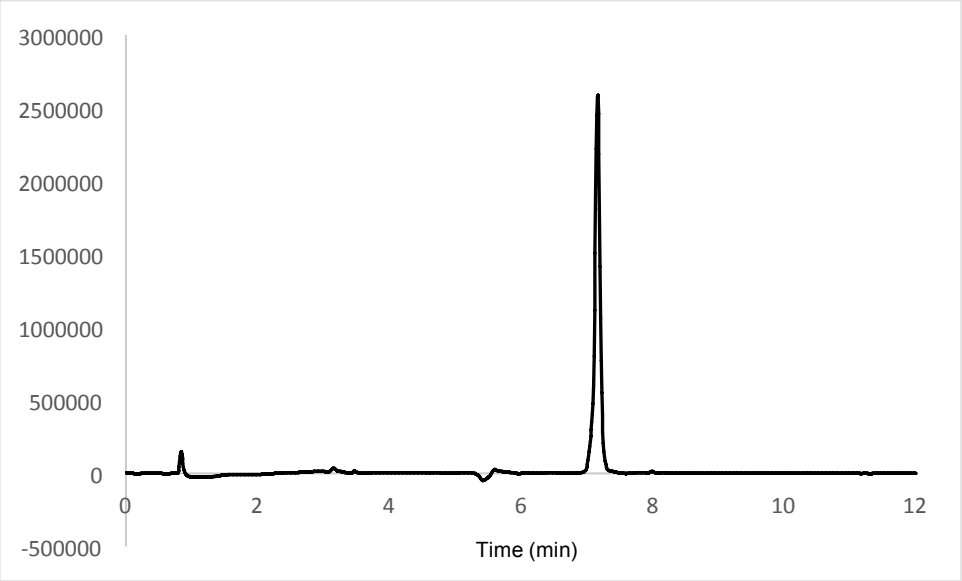
Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to CLSI guidelines.[4] Blood agar plates were inoculated with glycerol stocks of the chosen bacteria strains, followed by incubation for 16 hours at 37°C. Cation adjusted Mueller-Hinton broth (CAMHB) containing 10 $mg.L^{-1}$ Mg^{2+} and 25 $mg.L^{-1}$ Ca^{2+} was inoculated with individual colonies of the chosen bacteria, and incubated for 16 hours at 220 RPM. The peptides were dissolved in CAMHB, supplemented with polysorbate-80 (P-80 or Tween-80, sterile-filtered) at 0.002% v/v final concentration and serially diluted on polypropylene microtiter plates with a volume of 50 µL per well. Inoculated CAMHB ($2x10^5$ $CFU.mL^{-1}$) was added to reach a total volume of 100 µL per well. The microtiter plates were sealed with an adhesive membrane and after 16 hours of incubation at 37°C and 220 RPM the wells were visually inspected for bacterial growth. All reported MIC values result from three or more measurements performed on multiple days.

Table S1. HRMS for all newly reported compounds

Compound	Chemical formula	Calculated [M+H]	Calculated [M+2H]/2	Measured
3a	$C_{114}H_{187}N_{33}0_{32}$	2531.4098	1266.2088	1266.2085
3b	$C_{117}H_{185}N_{33}0_{32}$	2565.3942	1283.2010	1283.2017
6	$C_{70}H_{106}N_{18}0_{20}$	1519.7909	760.3994	760.3994
7	$C_{75}H_{111}N_{19}0_{21}$	1614.8280	807.9179	807.9180
8a	$C_{42}H_{77}N_{17}0_{12}$	1012.6016	506.8047	506.8043
8b	$C_{45}H_{83}N_{17}0_{12}$	1054.6485	527.8282	527.8278
8c	$C_{49}H_{91}N_{17}0_{15}$	1158.6959	579.8519	579.8515
9a	$C_{117}H_{188}N_{36}0_{33}$	2626.4218	1313.7148	1313.7106
9b	$C_{120}H_{194}N_{36}0_{33}$	2668.4687	1334.7383	1334.7359
9c	$C_{124}H_{202}N_{36}0_{36}$	2772.5161	1386.7619	1386.7555
11	$C_{34}H_{62}N_{14}0_9$	811.4902	406.2491	811.4903
12	$C_{109}H_{173}N_{33}0_{32}$	2425.3104	1213.1591	1213.1577

Analytical HPLC traces

3a



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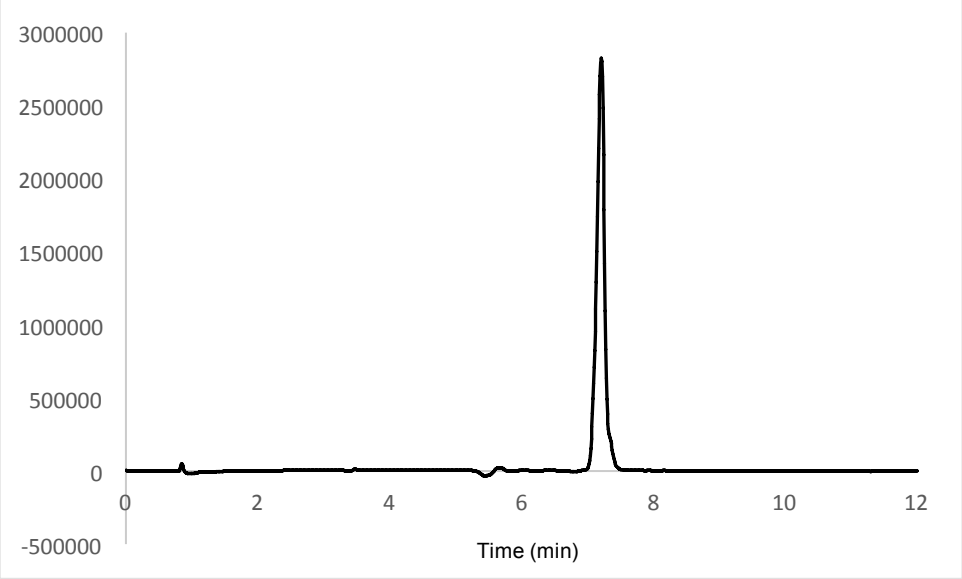

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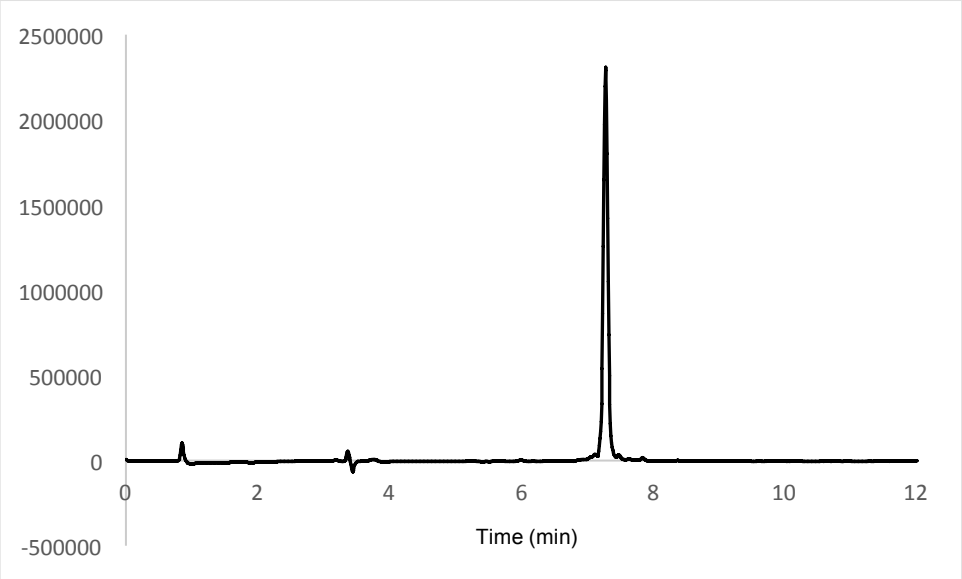
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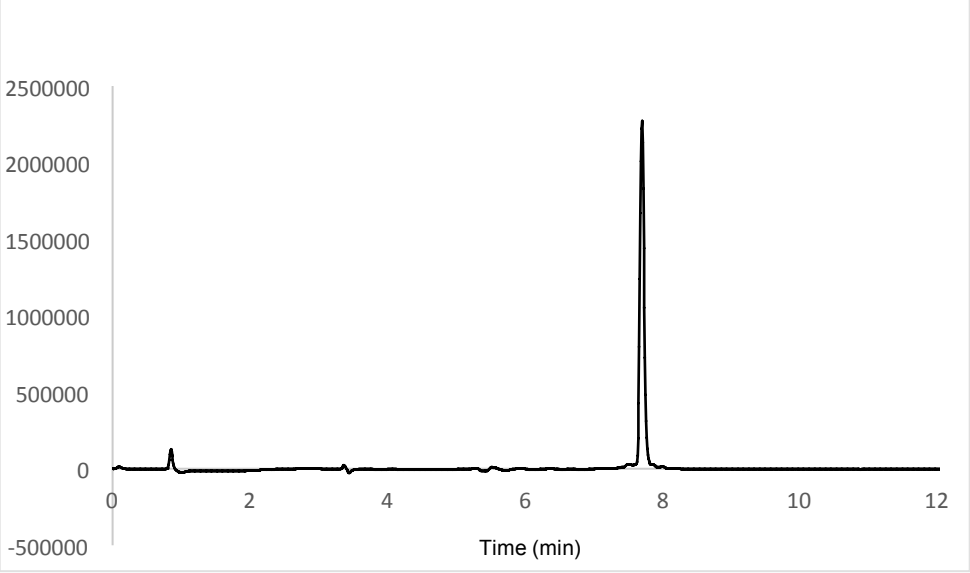
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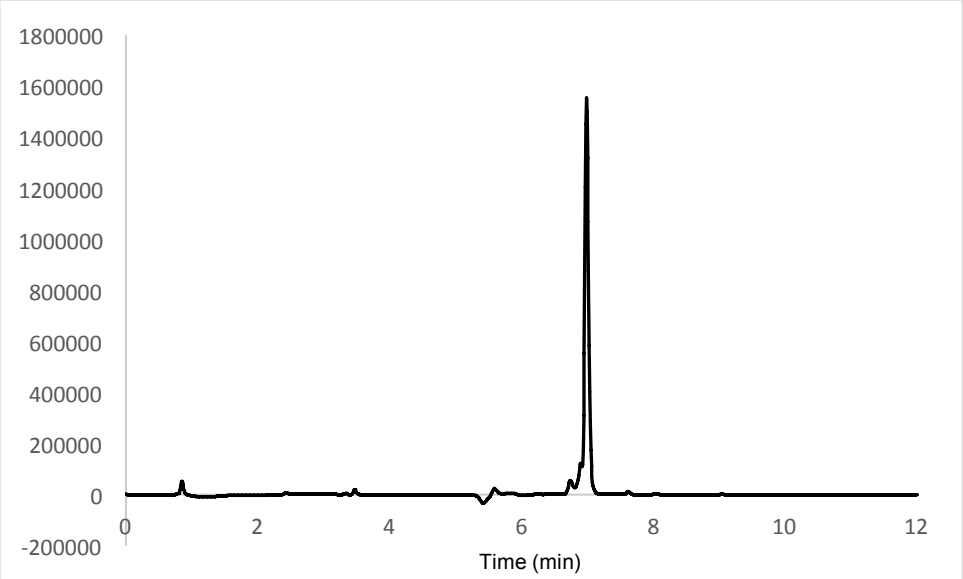
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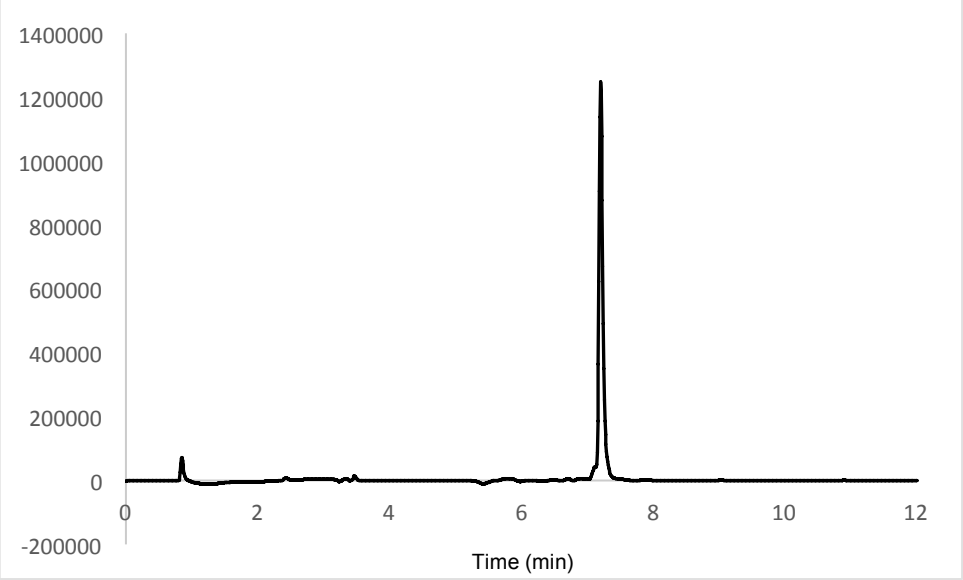
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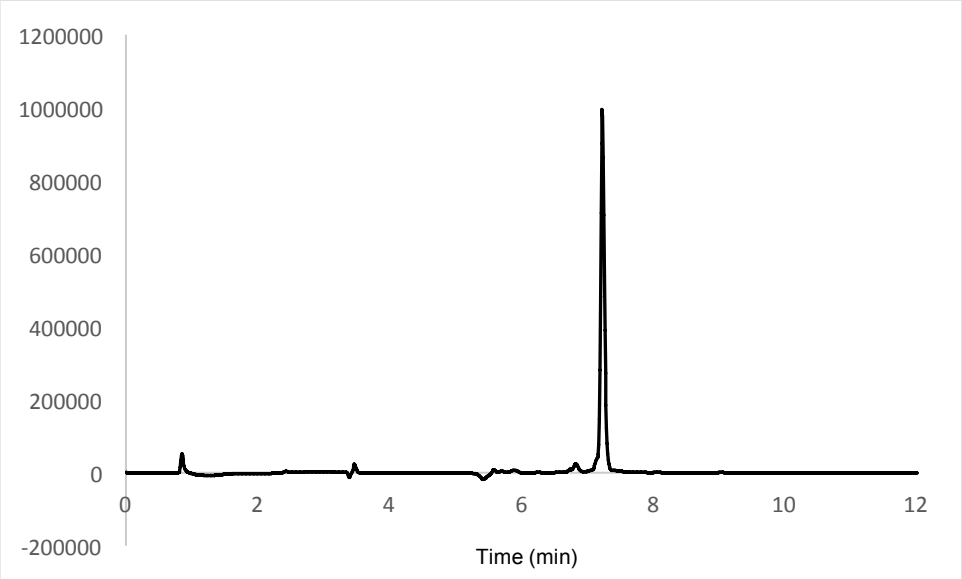
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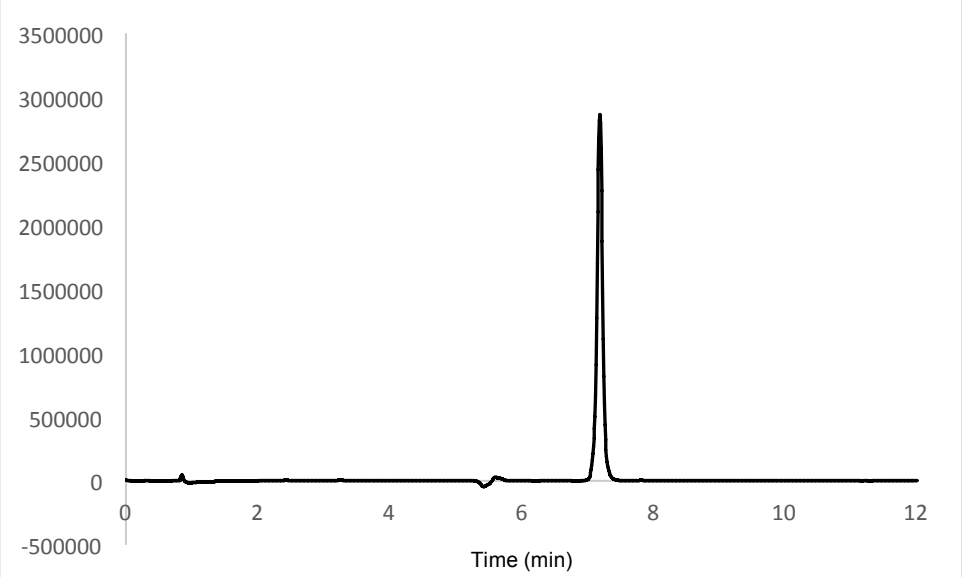
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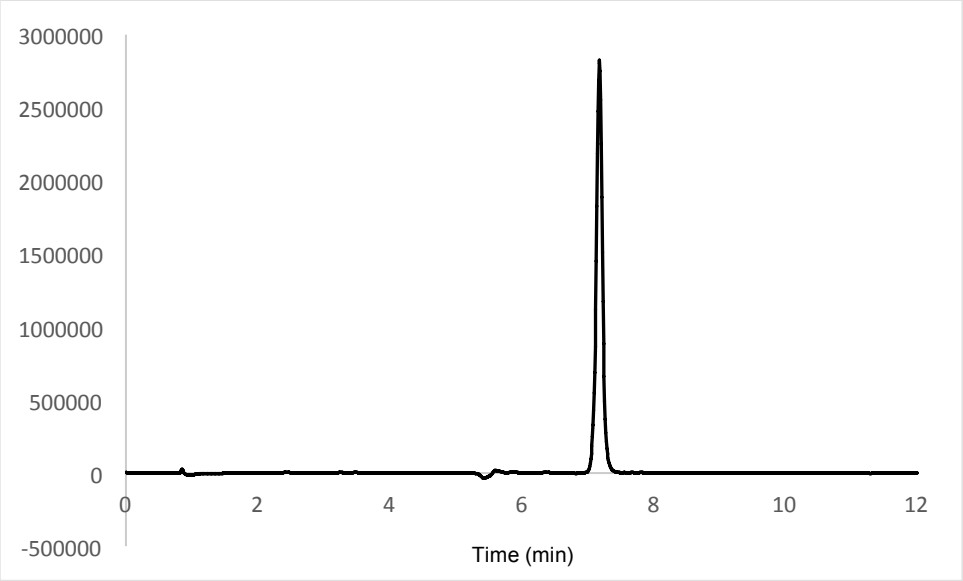
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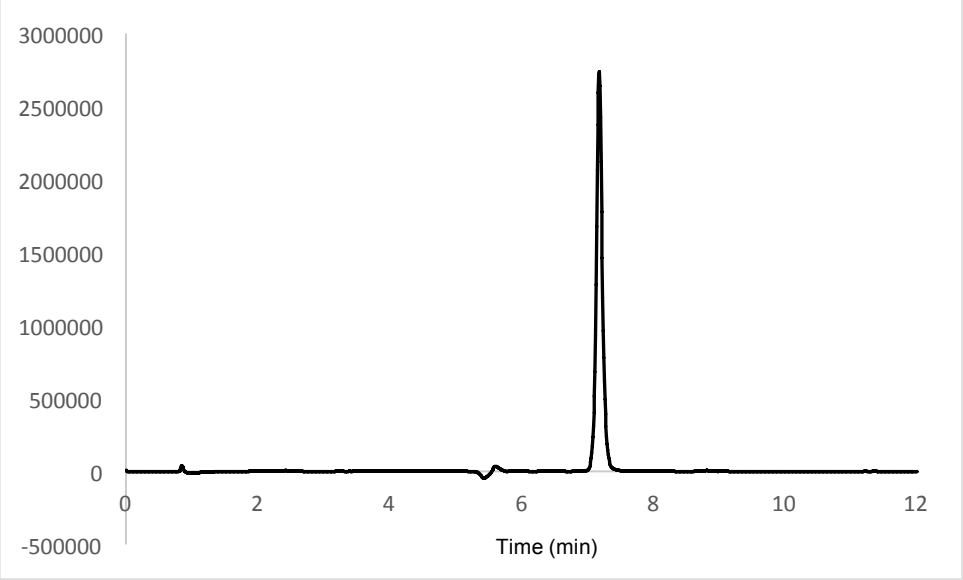
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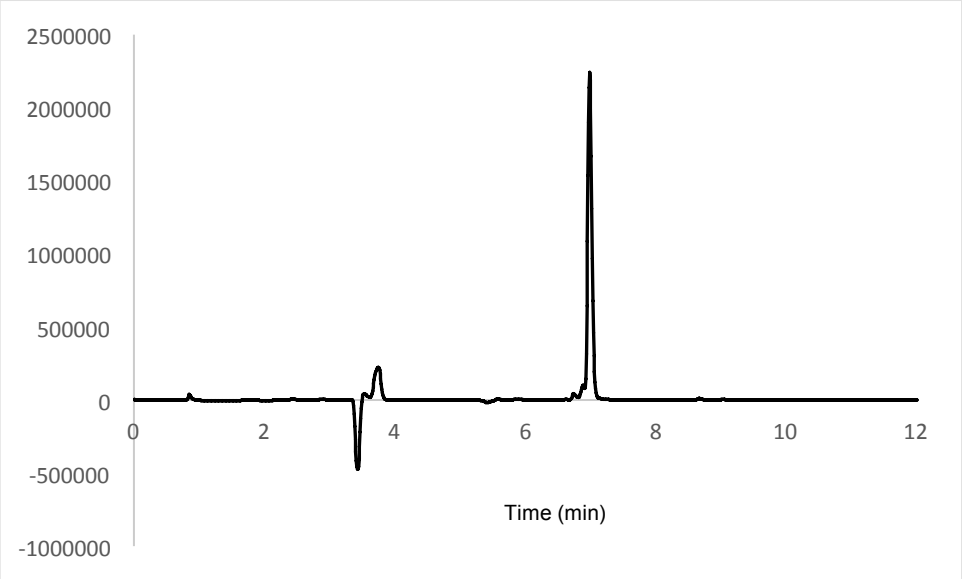
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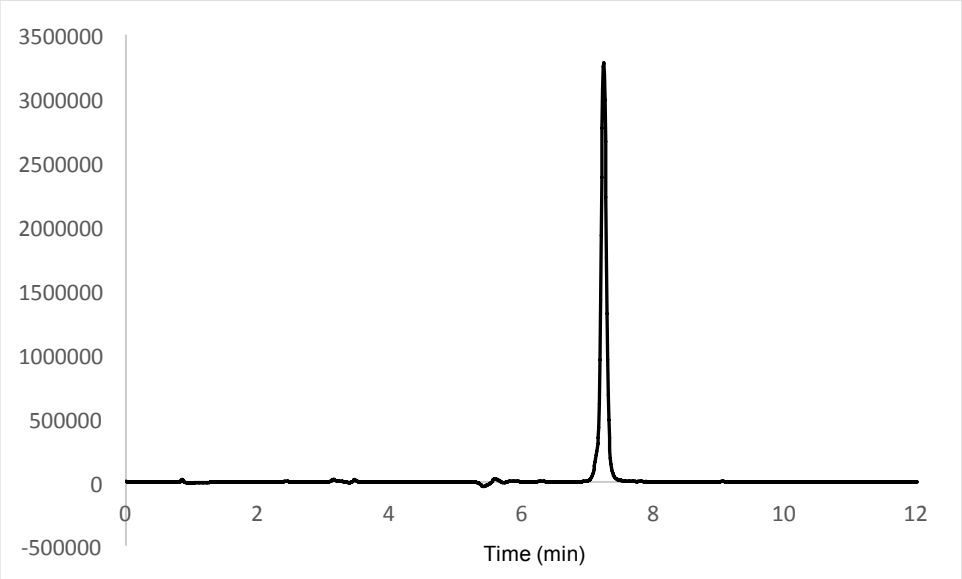
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