Supporting information

Understanding interactions of Citropin 1.1 analogs with model membranes and their influence on biological activity

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Experimental Section: The antimicrobial activity for additional strains and cytotoxicity studies performed by The Community for Antimicrobial Drug Discovery (CO-ADD), Australia, and Flow cytometry protocol.

1. Antimicrobial activity for additional strains and cytotoxicity: ***The protocols were adapted and slightly modified from the documentation provided by CO-ADD and also from the paper reported by Almeida et al. [1].*** The antimicrobial assays against 5 bacteria strains: *Staphylococcus aureus* ATCC 43300 MRSA; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853; *Klebsiella pneumoniae* ATCC 700603; *Acinetobacter baumannii* ATCC 19606, and 2 fungi: *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* var. grubii H99 ATCC 20882 were performed by CO-ADD (The Community for Antimicrobial Drug Discovery), funded by the Wellcome Trust (UK) and The University of Queensland (Australia). Antimicrobial screening was performed at a single concentration (32 μg/mL) and MIC was determined against those microorganisms that showed susceptibility to the compounds tested in the primary screen. In addition, the active compounds were screened for cytotoxicity against a human embryonic kidney cell line (HEK293) and human red blood cells. All the sample preparation was done using liquid handling robots.

Antibacterial assay: All bacteria were cultured in Cation-adjusted Mueller Hinton broth (CAMHB) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37 °C for 1.5-3 h. The resultant mid-log phase cultures were diluted then added to each well of the compound containing plates, giving a cell density of 5×10^5 CFU/mL. Plates were covered and incubated at 37 °C for 18 h without shaking. Inhibition of bacterial growth was determined measuring absorbance at 600 nm (OD600), using a Tecan M1000 Pro monochromator plate reader. The percentage of growth inhibition was calculated for each well, using the negative

control (media only) and positive control (bacteria without inhibitors) on the same plate as references. For the screening, the significance of the inhibition values was determined by modified Z-scores, calculated using the median and mean absolute deviation (MAD) of the samples (no controls) on the same plate. Samples with inhibition value above 80% and Z-Score above 2.5 for either replicate (n=2 on different plates) were classed as actives. The MIC was determined as the lowest concentration at which the growth was fully inhibited, defined by an inhibition ≥ 80%. In addition, the maximal percentage of growth inhibition is reported as DMax, indicating any compounds with partial activity. Hits were classified by MIC ≤ 16 μg/mL in either replicate (n=2 on different plates).

Antifungal assay: Fungi strains were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30 °C. A yeast suspension of 1 x 10^6 to 5 x 10^6 CFU/mL (as determined by OD530) was prepared from five colonies. The suspension was subsequently diluted and added to each well of the compound-containing plates giving a final cell density of fungi suspension of 2.5 ×10^3 CFU/mL and a total volume of 50 μL. All plates were covered and incubated at 35 °C for 24 h without shaking. Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD530), while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm (OD600-570), after the addition of resazurin (0.001% final concentration) and incubation at 35 °C for additional 2 h. The absorbance was measured using a Biotek Synergy HTX plate reader. The percentage of growth inhibition was calculated for each well, and the analysis was performed as described for antibacterial assay. Due to a higher variance in growth and inhibition, a lower threshold (≥ 70%) was applied to the data for *C. neoformans*.

Cytotoxicity Assay: HEK293 cells were counted manually in a Neubauer hemocytometer and then plated in the 384-well plates containing the compounds to give a density of 5000 cells/well in a final volume of 50 µL. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) was used as growth media and the cells were incubated together with the compounds for 20 h at 37 °C in 5% CO_2. 5 µL of 25 µg/mL resazurin (2.3 µg/mL final concentration) was added and incubated for further 3 h at 37 °C in 5% CO_2. The fluorescence intensity was measured using a Tecan M1000 Pro monochromator plate reader, using automatic gain calculation. CC_{50} (concentration at 50% cytotoxicity) were calculated by curve fitting the inhibition values vs. log(concentration) using a sigmoidal dose-response function, with variable fitting values for bottom, top and slope. In addition, the maximal percentage of cytotoxicity is reported as D_{Max}, indicating any compounds with partial cytotoxicity. Cytotoxic samples were classified by $CC_{50} \leq 32$ µg/mL or $CC_{50} \leq 10$ µM in either replicate (n=2 on different plates). In addition, samples were flagged as partial cytotoxic if $D_{Max} \geq 50\%$, even with CC_{50} > the maximum tested concentration.

Haemolysis Assay: Human whole blood was washed three times with 3 volumes of 0.9% NaCl and then resuspended in same to a concentration of 0.5×10^8 cells/mL, as determined by manual cell count in a Neubauer hemocytometer. The washed cells were then added to the 384-well compound-containing plates for a final volume of 50 µL. After a 10 min shake on a plate shaker the plates were then incubated for 1 h at 37 °C. After incubation, the plates were centrifuged at 1000 g for 10 min to pellet cells and debris, 25 µL of the supernatant was then transferred to a polystyrene 384-well assay plate. Hemolysis was determined by measuring the supernatant absorbance at 405 mm (OD405). The absorbance was measured using a Tecan M1000 Pro monochromator plate reader. HC_{10} and HC_{50} (concentration at 10% and 50% hemolysis,

respectively) were calculated by curve fitting the inhibition values vs. log(concentration) using a sigmoidal dose-response function with variable fitting values for top, bottom and slope. In addition, the maximal percentage of hemolysis is reported as D_{Max}, indicating any compounds with partial hemolysis. Hemolysis samples were classified by $HC^{10} \leq 32$ μg/mL in either replicate (n=2 on different plates). In addition, samples were flagged as partial hemolytic if $D_{Max} \geq 50\%$, even with HC10 > the maximum tested concentration.

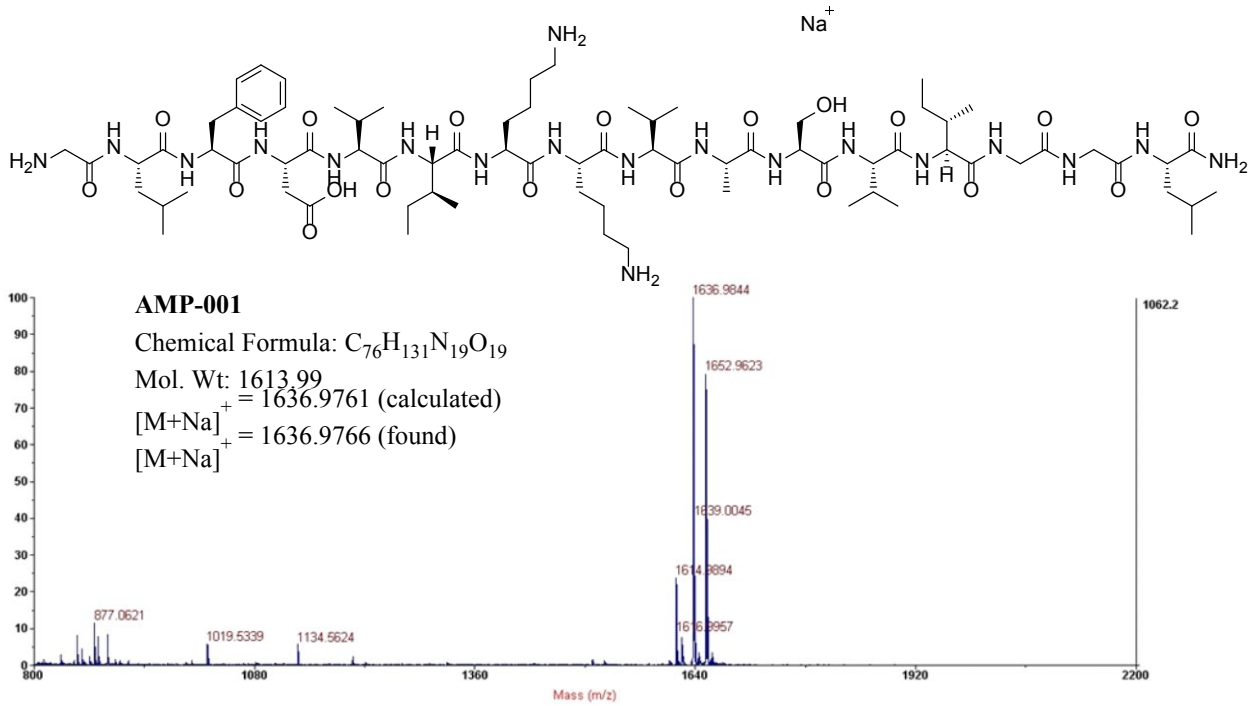
2. Flow Cytometry: *S. aureus* JE2 USA 300 and *E. coli* K12 were grown to the mid log phase in Muller Hinton Broth at 37ºC under constant shaking at 150 rpm, washed twice and diluted to 106 CFU/mL in saline 0.85%. AMPs at twice MIC values were incubated with the bacterial suspension for 30 minutes at 37ºC and stained with Propidium Iodide (PI) at concentration of 10 μg/mL. The cells were immediately washed and resuspended in saline 0.85%. As control we used cultures with PI, no PI and treated with EtOH 70%. Readings were obtained with a flow cytometry at a laser excitation wavelength of 488 nm.

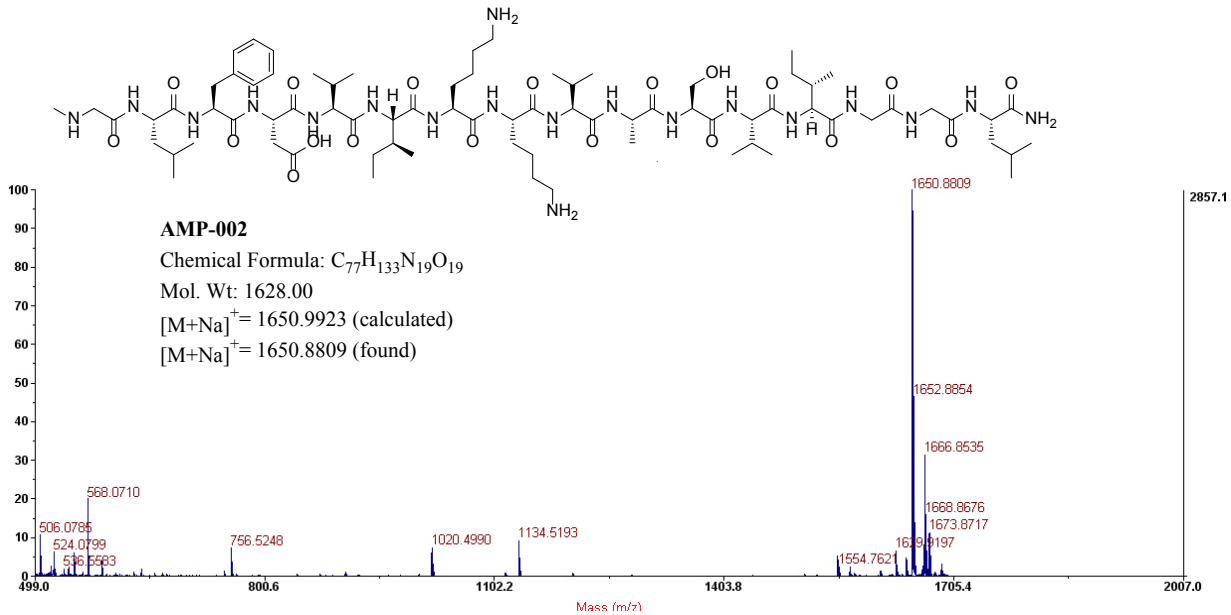
Table S1. Cit 1.1 and its analogues

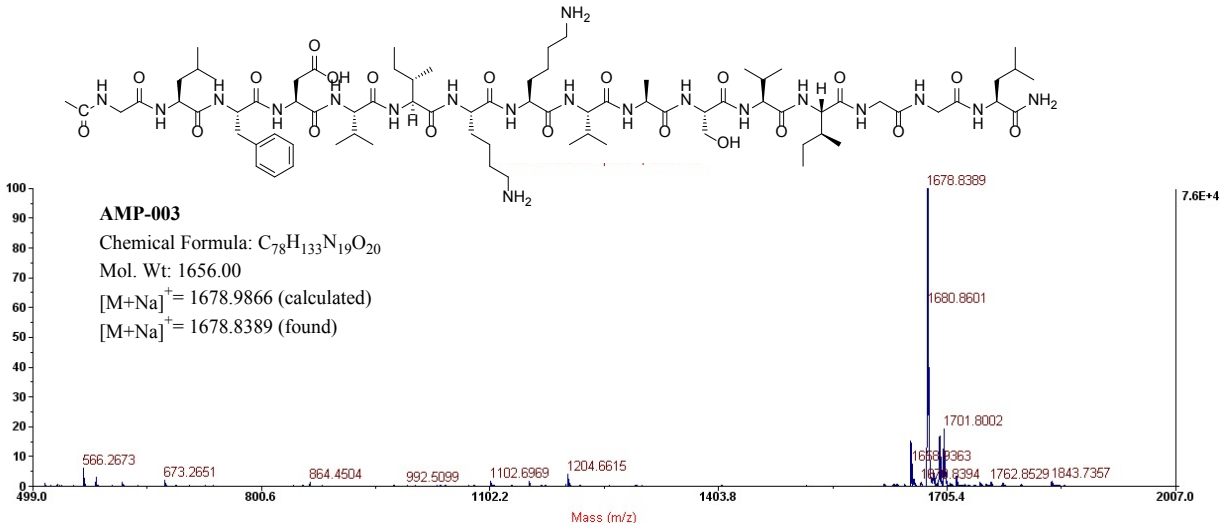
Peptide sequence	Peptide code	MW	Purity
GLFDVIKKVASVIGGL	AMP-001 (Cit 1.1)	1615.00	97.57%
SarLFDVIKKVASVIGGL	AMP-002	1628.00	98.47%
AcGLFDVIKKVASVIGGL	AMP-003	1656.00	99.80%
$(CH_3)_3$GLFDVIKKVASVIGGL	AMP-004	1658.03	98.25%
Hydrazine-LFDVIKKVASVIGGL	AMP-005	1615.97	100 %
GLFDVI(Orn)(Orn)VASVIGGL	AMP-006	1585.96	95.98%
GLFDVI(Dab)(Dab)VASVIGGL	AMP-007	1558.89	97.26%
GLFDVI(Dpr)(Dpr)VASVIGGL	AMP-008	1529.89	97.25%
GLFDVI(Cit)KVASVIGGL	AMP-009	1642.98	97.26%
$(CH_3)_3$GLFDVIK$(CH_3)_3$K$(CH_3)_3$VASVIGGL	AMP-010	1743.15	99.44%
GLFDVIK$(CH_3)_3$K$(CH_3)_3$VASVIGGL	AMP-011	1702.18	96.40%
GLFDVIRRVASVIGGL	AMP-012	1670.00	96.37%
GLFDVIRKVASVIGGL	AMP-013	1641.99	97.57%
GLFDVIKKGVASVIGGL	AMP-014	1671.01	94.65%
GLFEVIKKVASVIGGL	AMP-015	1628.00	99.28%
GLWDVIKKVASVIGGL	AMP-016	1653.00	96.71%
GLWRVIRKVASVIGGL	AMP-017	1724.02	94.10%
PhenylGlyLWDVIRKVASVIGGL	AMP-018	1757.04	100%
GL(Biphenylalanine)DVIKKVASVIGGL	AMP-019	1691.10	98.55%

GL(1-Nap)DVIKKVASVIGGL	AMP-020	1665.06	98.27%
SarLWDVIRKVASVIGGL	AMP-021	1695.02	95.66%

Figure S1: Full chemical structure of AMPs designed and MALDI-TOF-MS



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1678.8389
7.6E+4
1680.8601
1701.8002
566.2673
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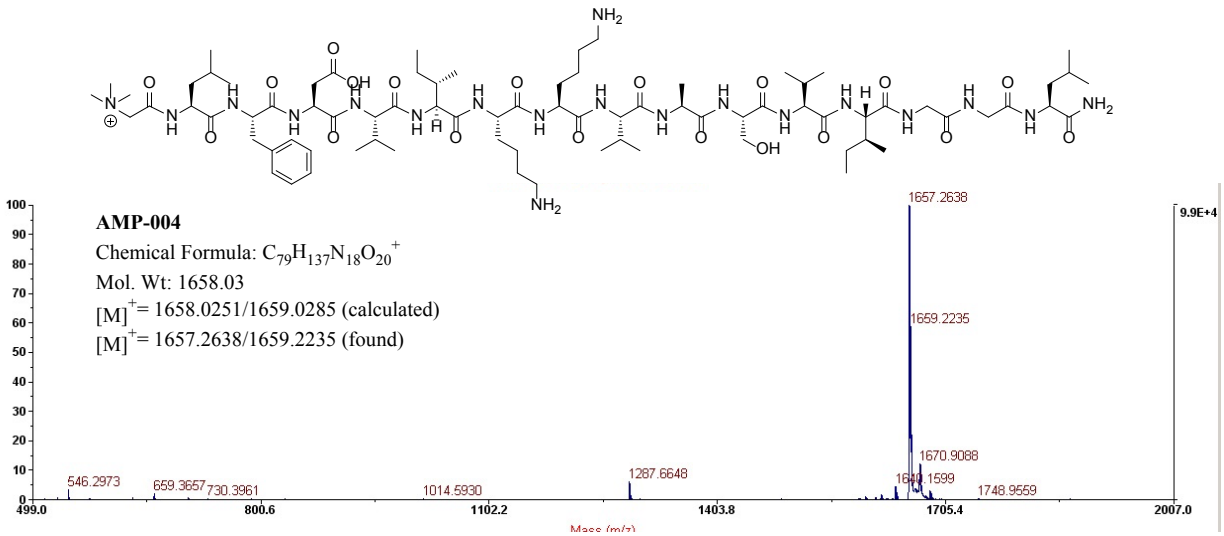
Mass (m/z)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$[M]^+$= 1657.2638/1659.2235 (found)
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9.9E+4
1659.2235
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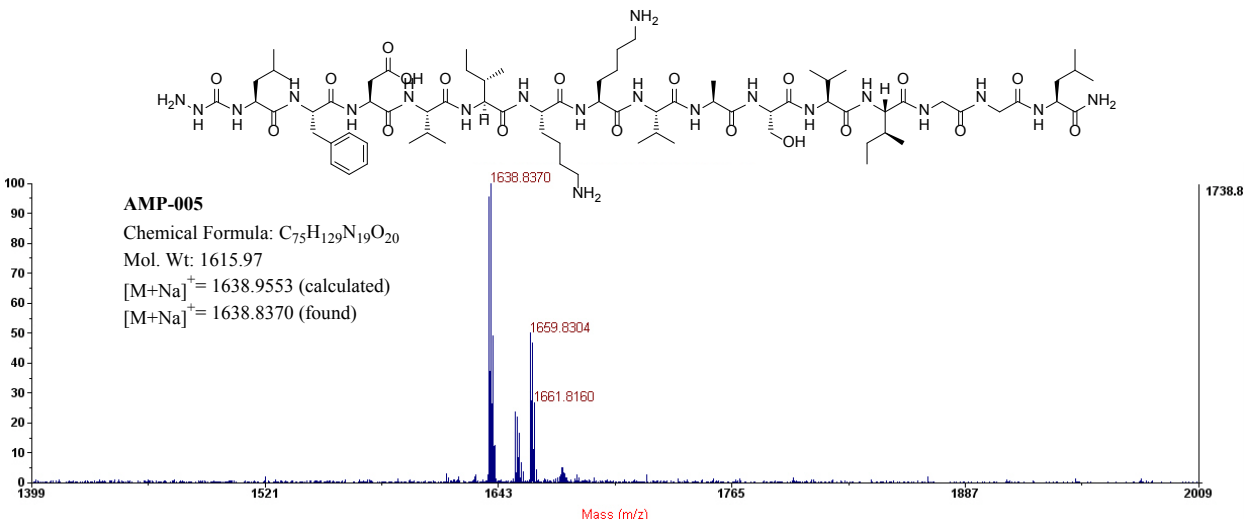
Mass (m/z)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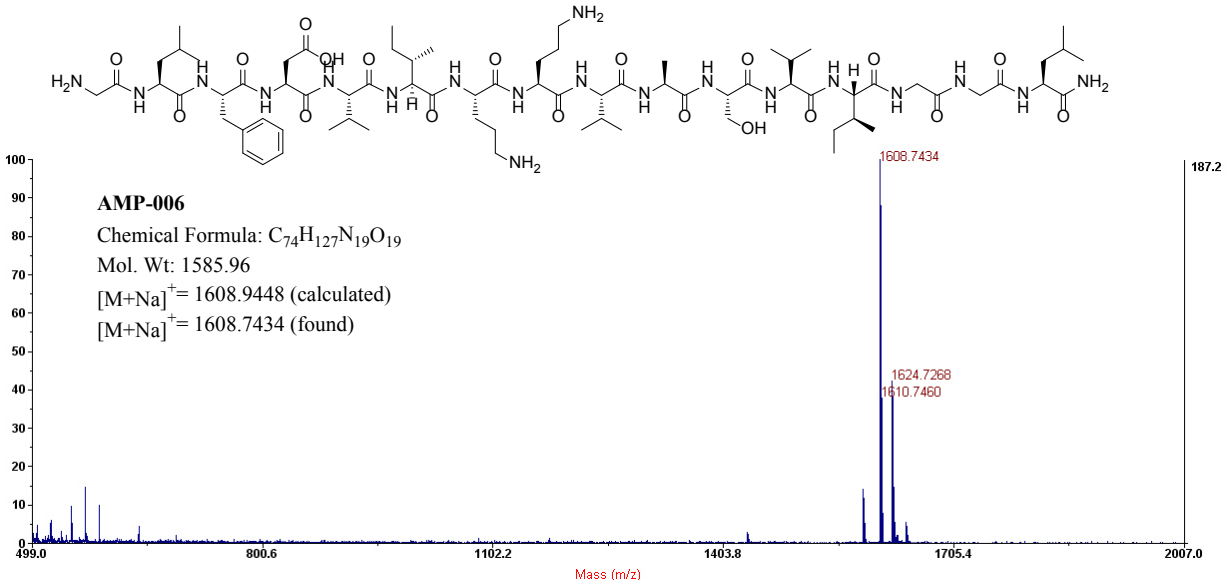
Mass (m/z)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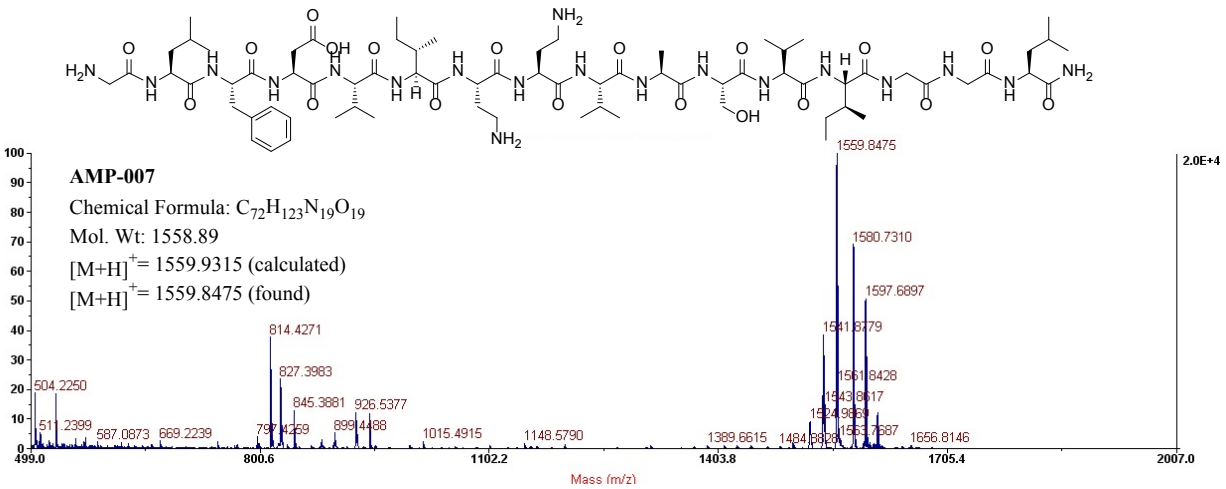
Mass (m/z)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1559.8475
1580.7310
1597.6897
814.4271
827.3983
504.2250
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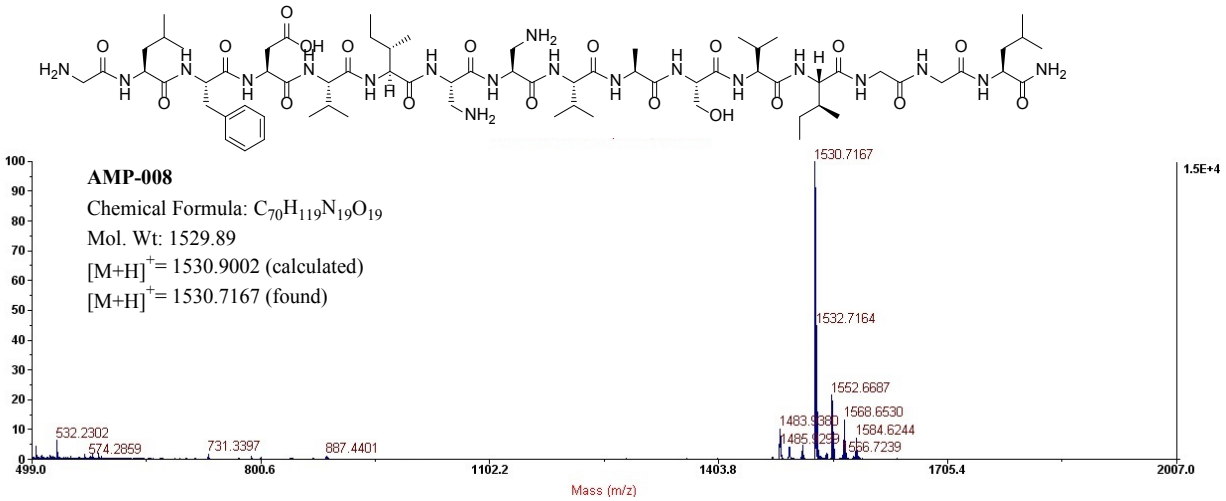
Mass (m/z)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$[M+H]^+$= 1530.7167 (found)
1530.7167
1532.7164
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532.2302
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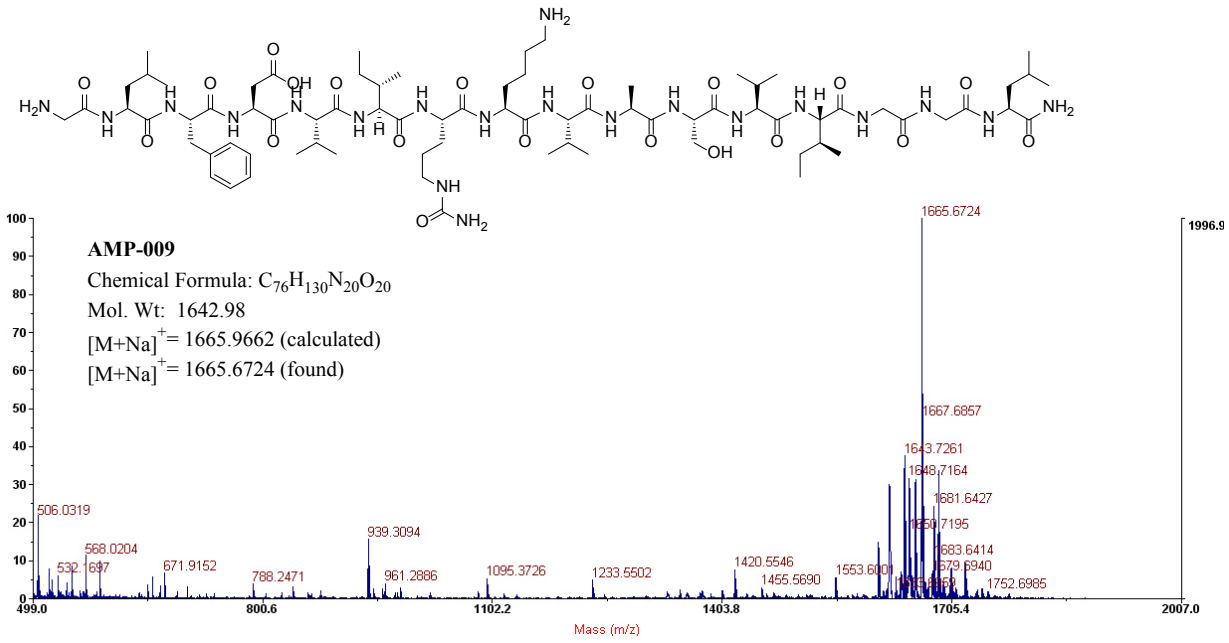
Mass (m/z)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1665.6724
1667.6857
1643.7261
1648.7164
1681.6427
1650.7195
1683.6414
1679.6940
1752.6985
506.0319
568.0204
532.1697
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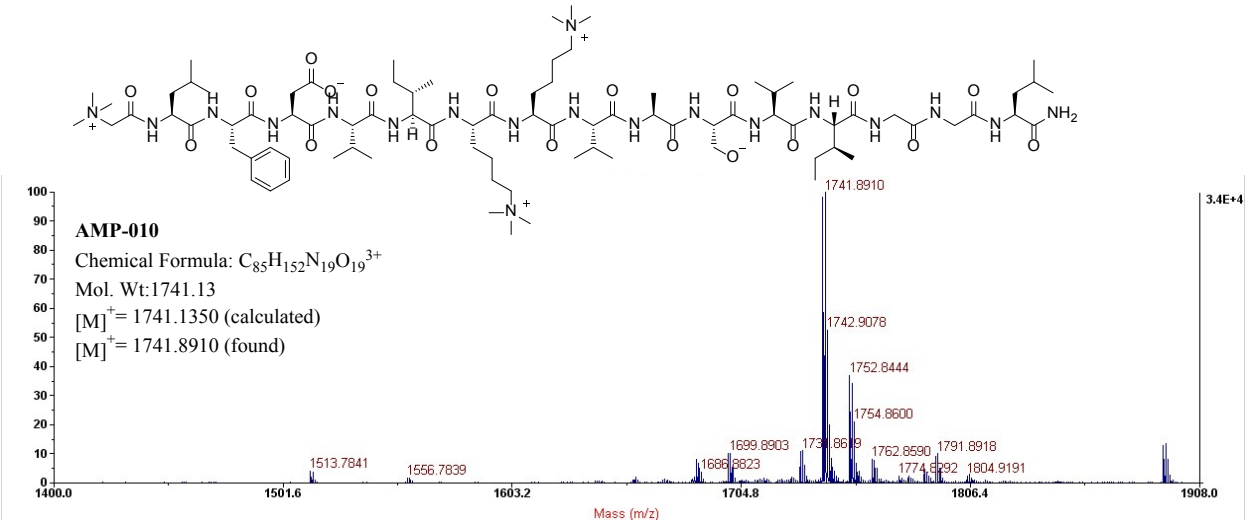
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$[M]^+$= 1741.8910 (found)
1741.8910
1742.9078
1752.8444
1754.8600
1699.8903
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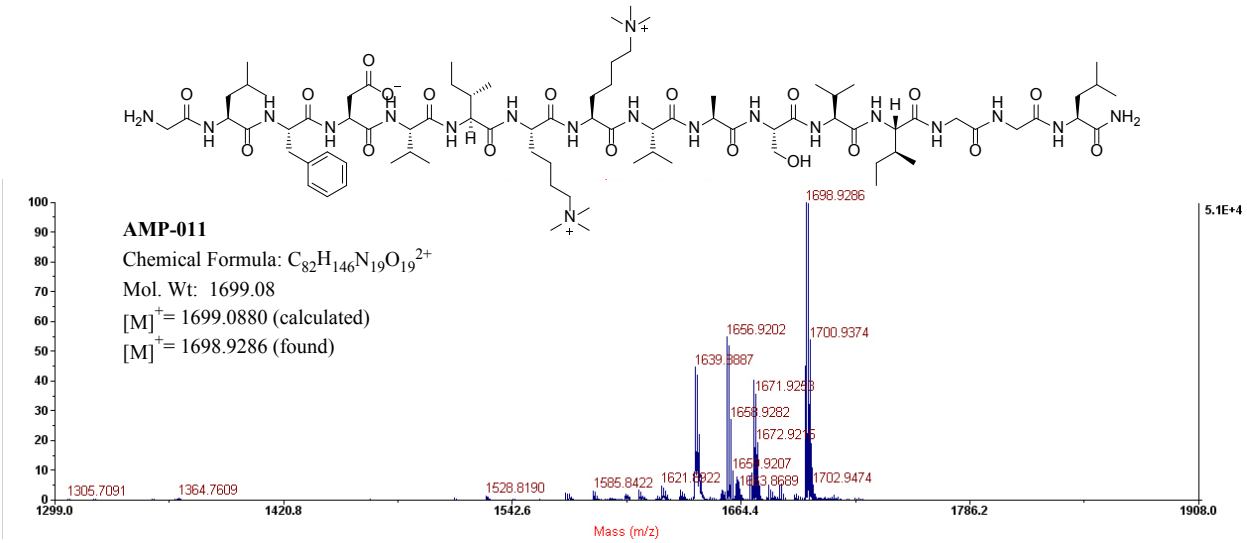
Mass (m/z)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1698.9286
1656.9202
1700.9374
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1671.9253
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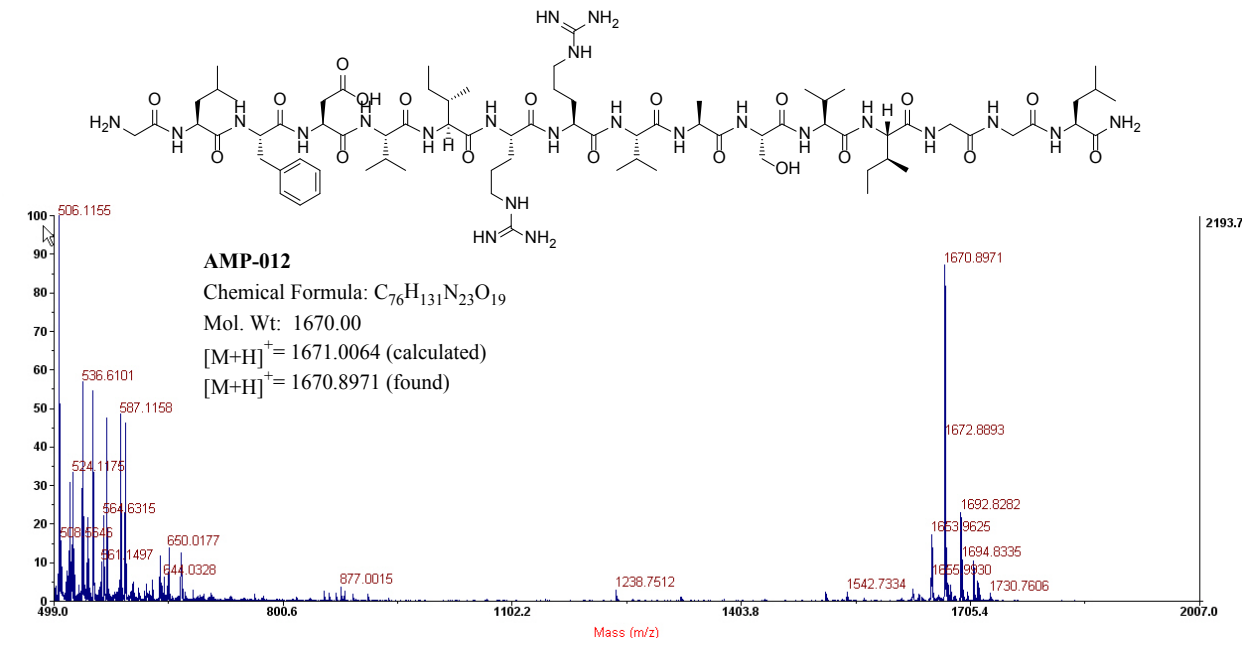
Mass (m/z)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1670.8971
536.6101
587.1158
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524.1175
564.6315
1692.8282
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650.0177
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1694.8335
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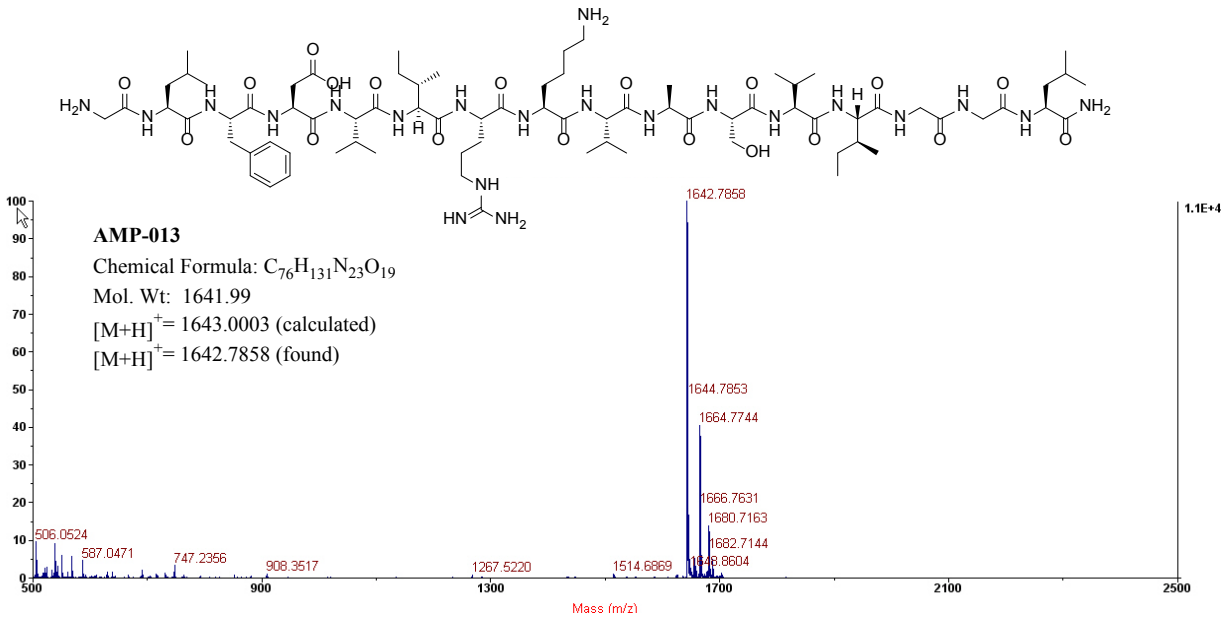
Mass (m/z)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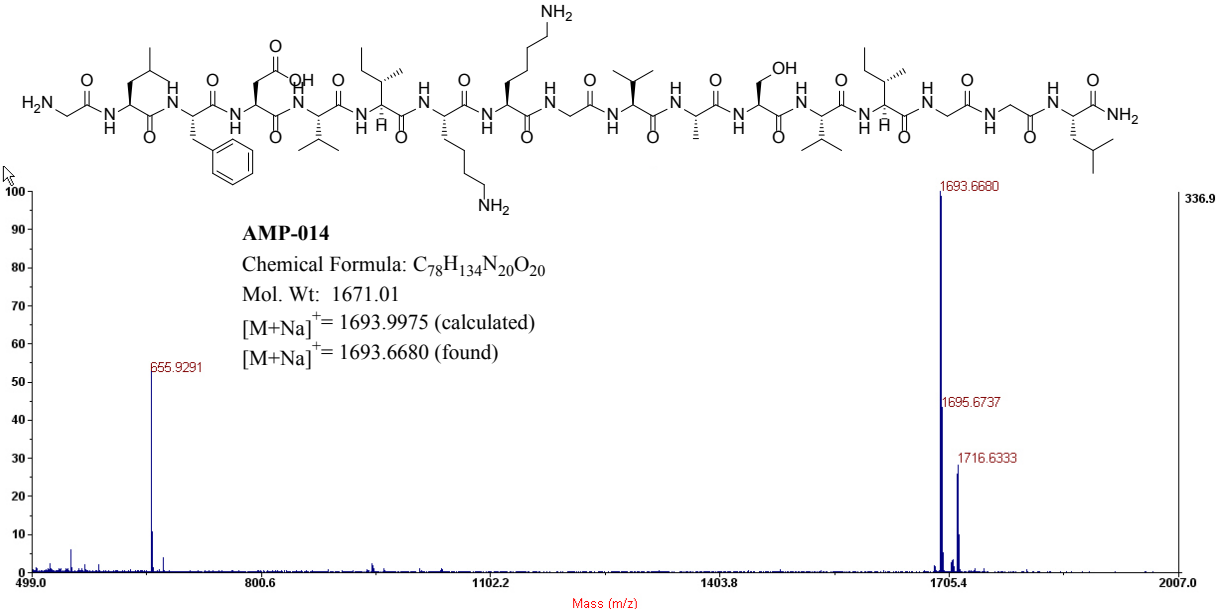
Mass (m/z)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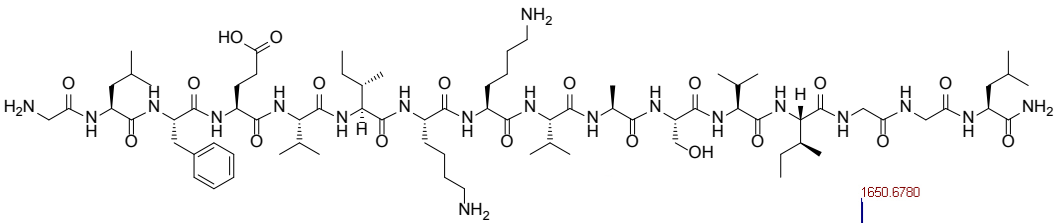
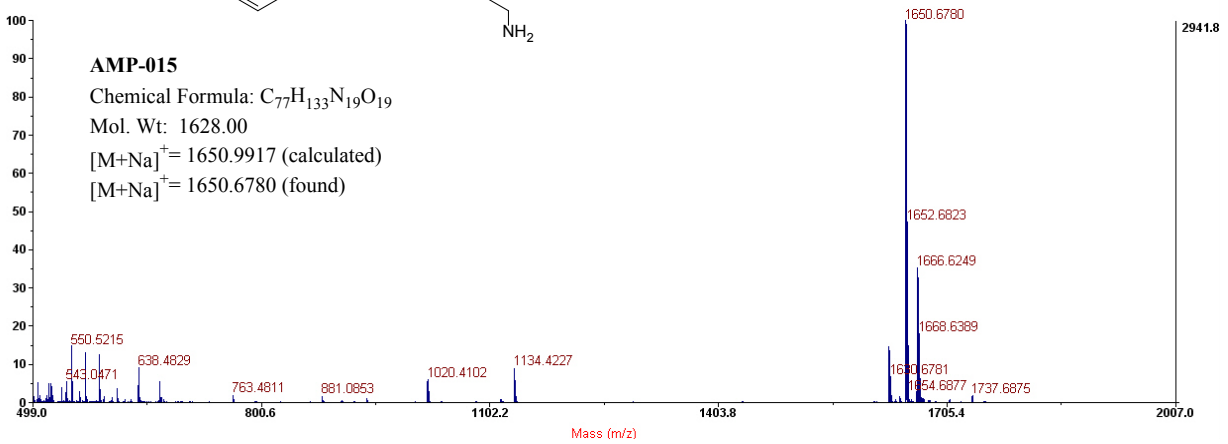
Mass (m/z)

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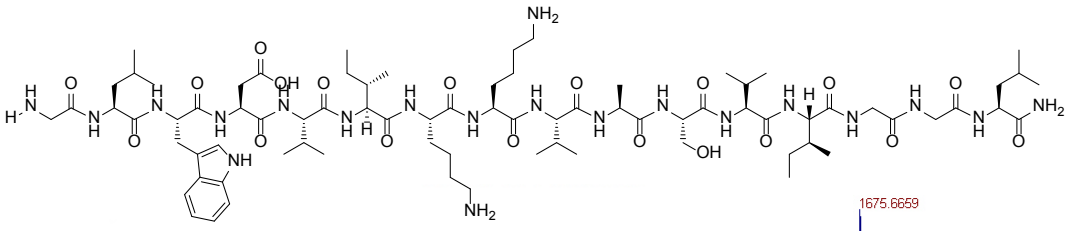
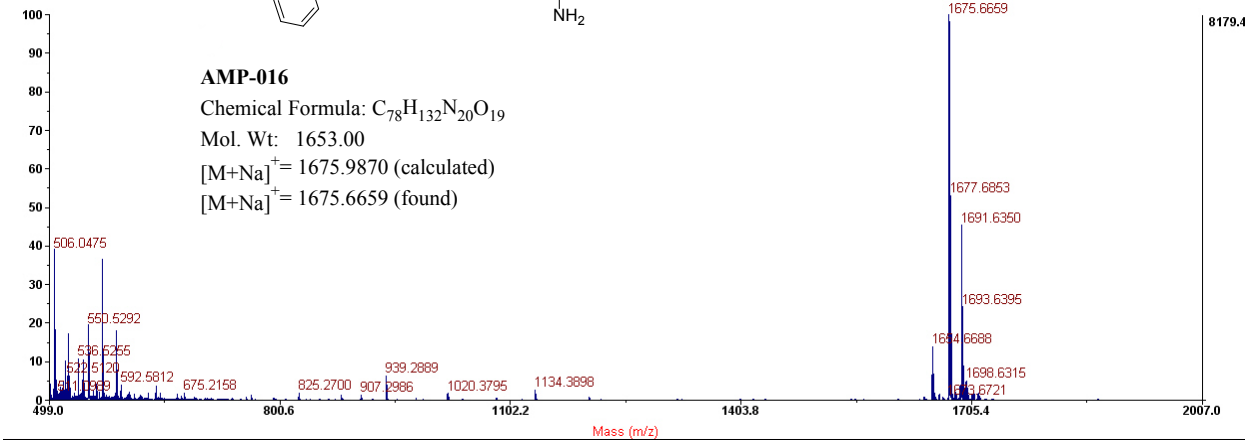
Mass (m/z)

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8179.4
1677.6853
1691.6350
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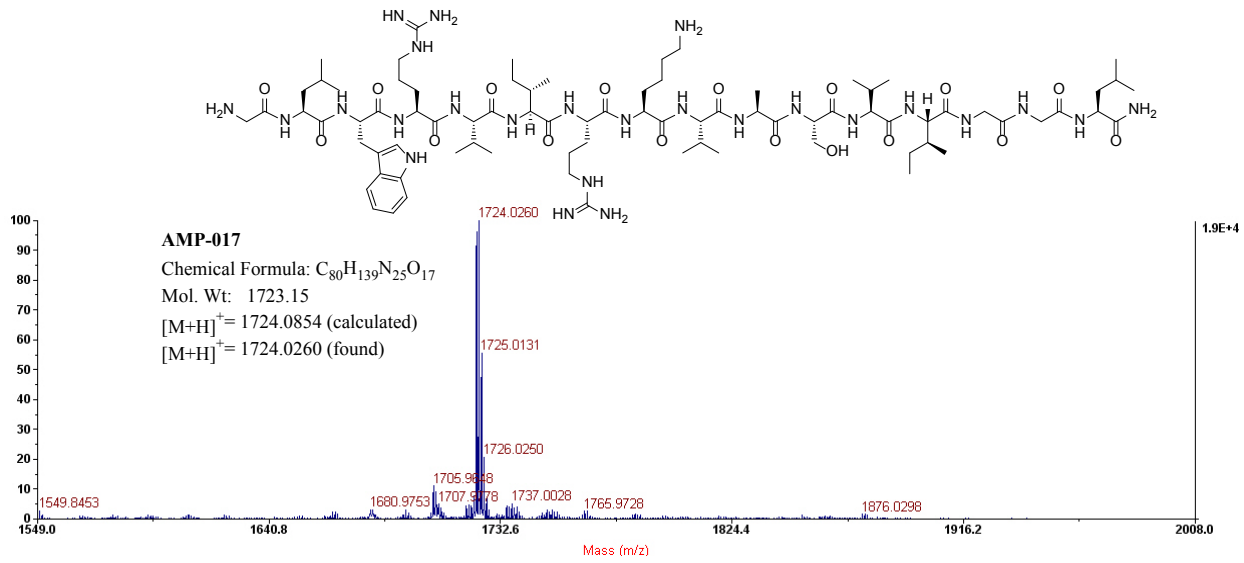
Mass (m/z)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$[M+H]^+$= 1724.0260 (found)
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1726.0250
1705.9228
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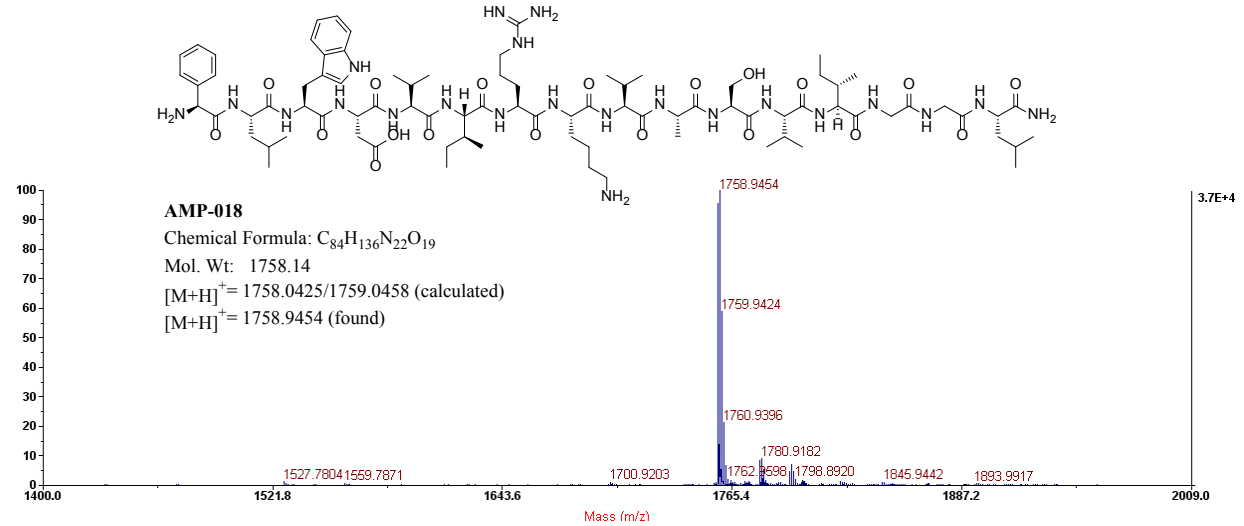
Mass (m/z)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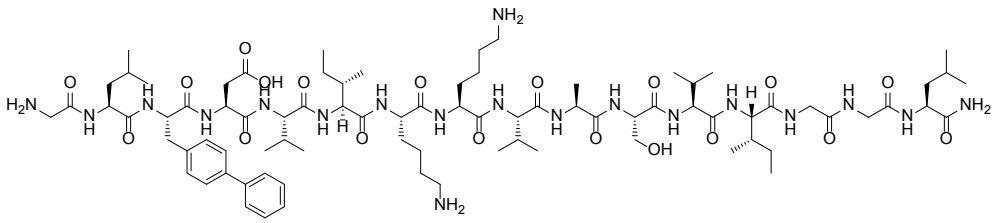
Mass (m/z)



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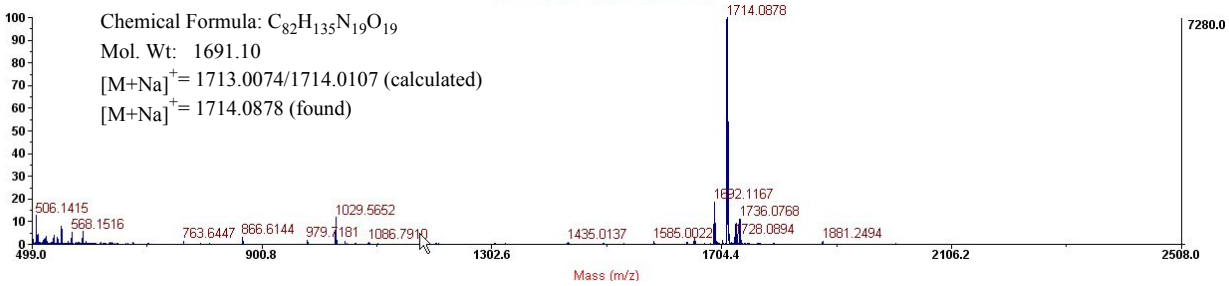
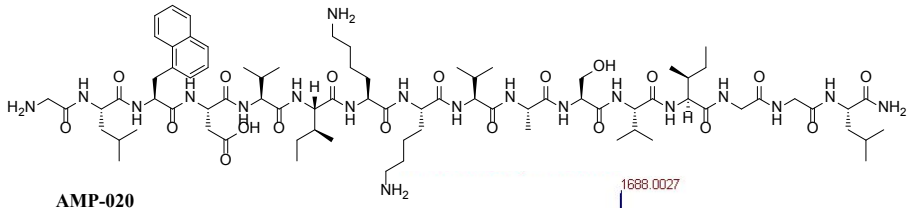
$[M+Na]^+$= 1714.0878 (found)





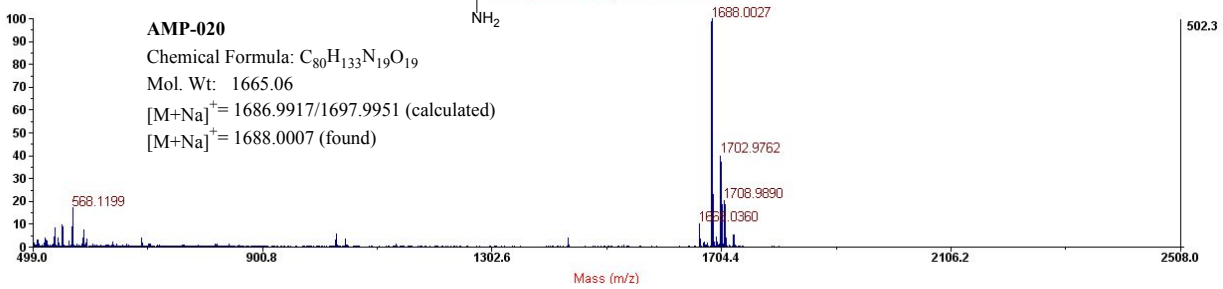
AMP-020

Chemical Formula: $C_{80}H_{133}N_{19}O_{19}$

Mol. Wt: 1665.06

$[M+Na]^+$= 1686.9917/1697.9951 (calculated)

$[M+Na]^+$= 1688.0007 (found)



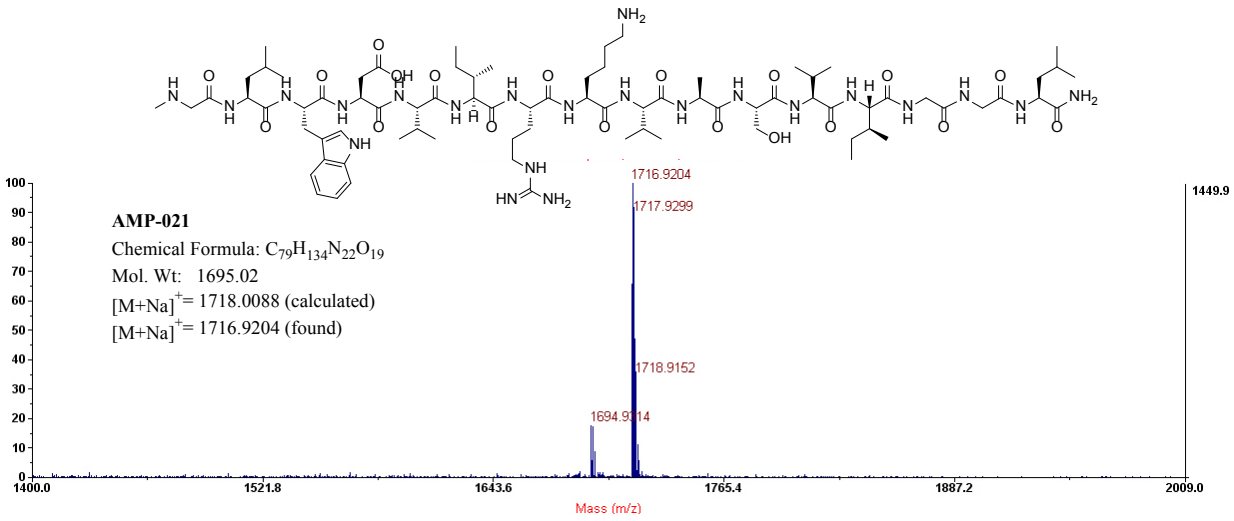
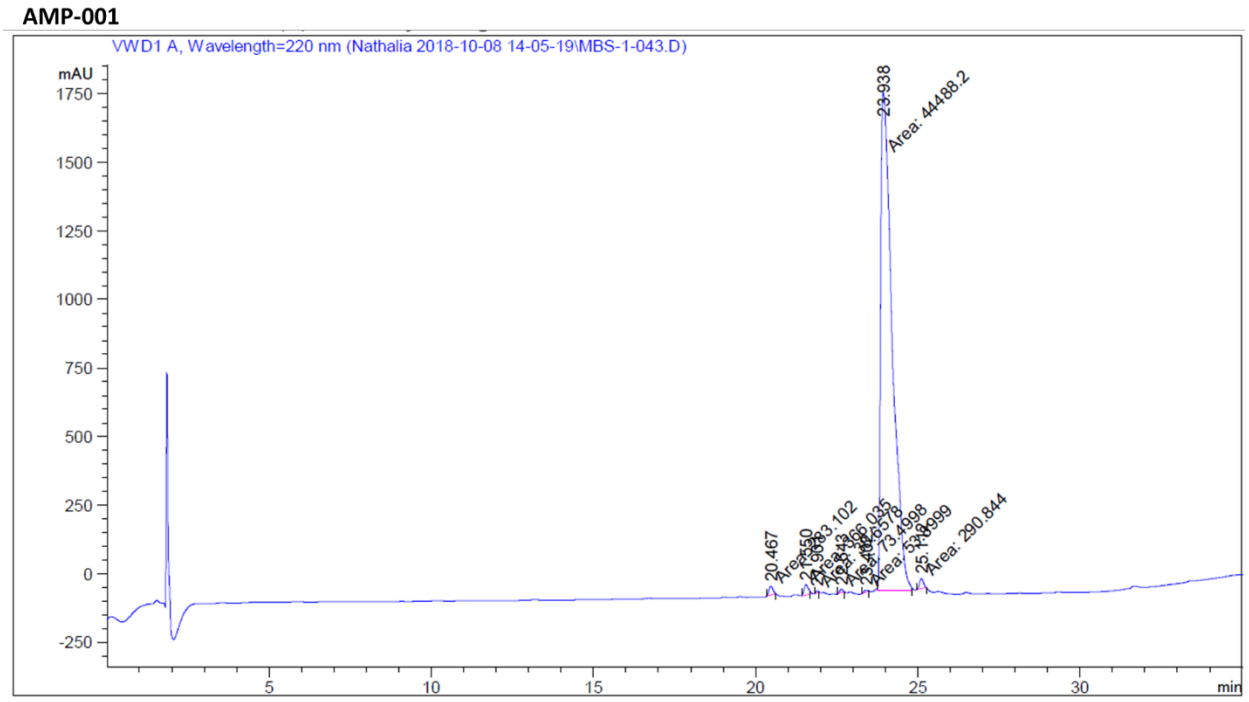
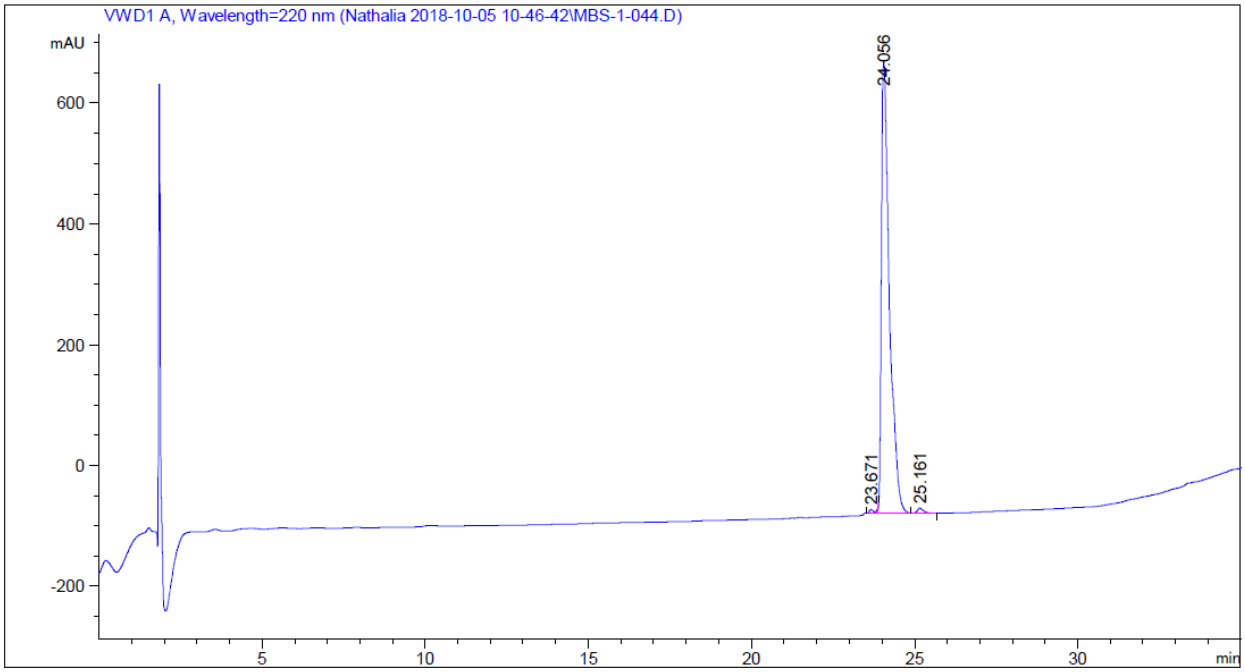


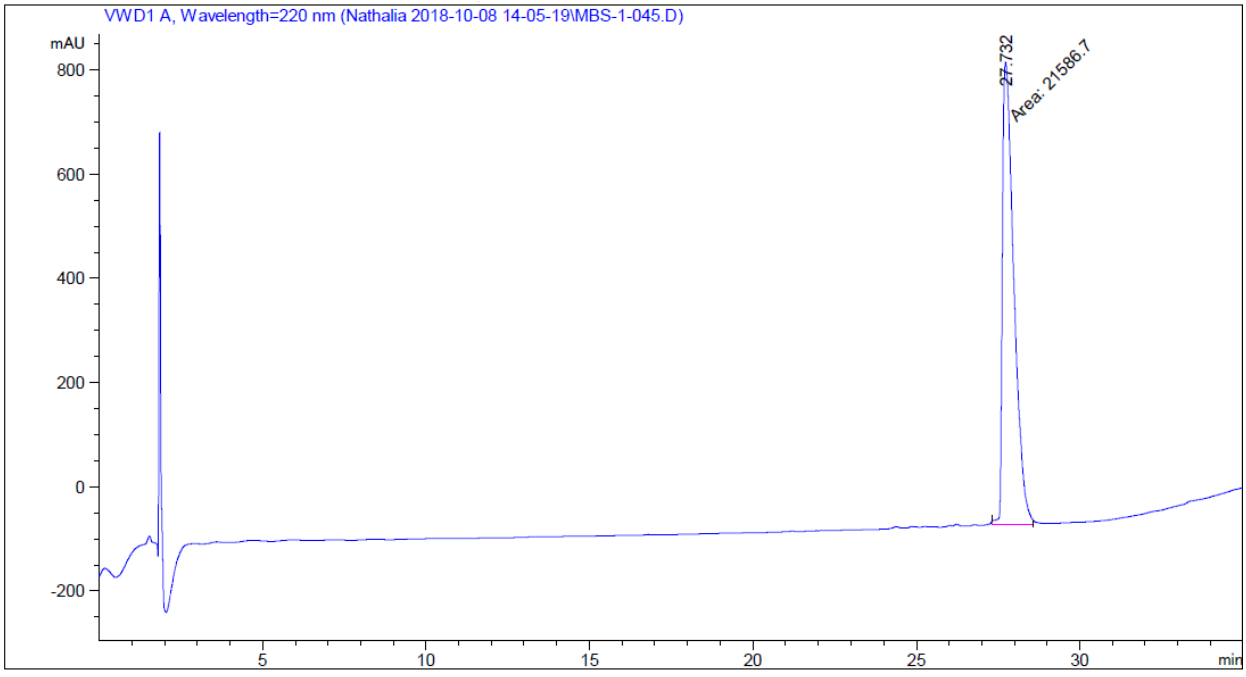
Figure S2: Selected HPLCs traces of AMPS



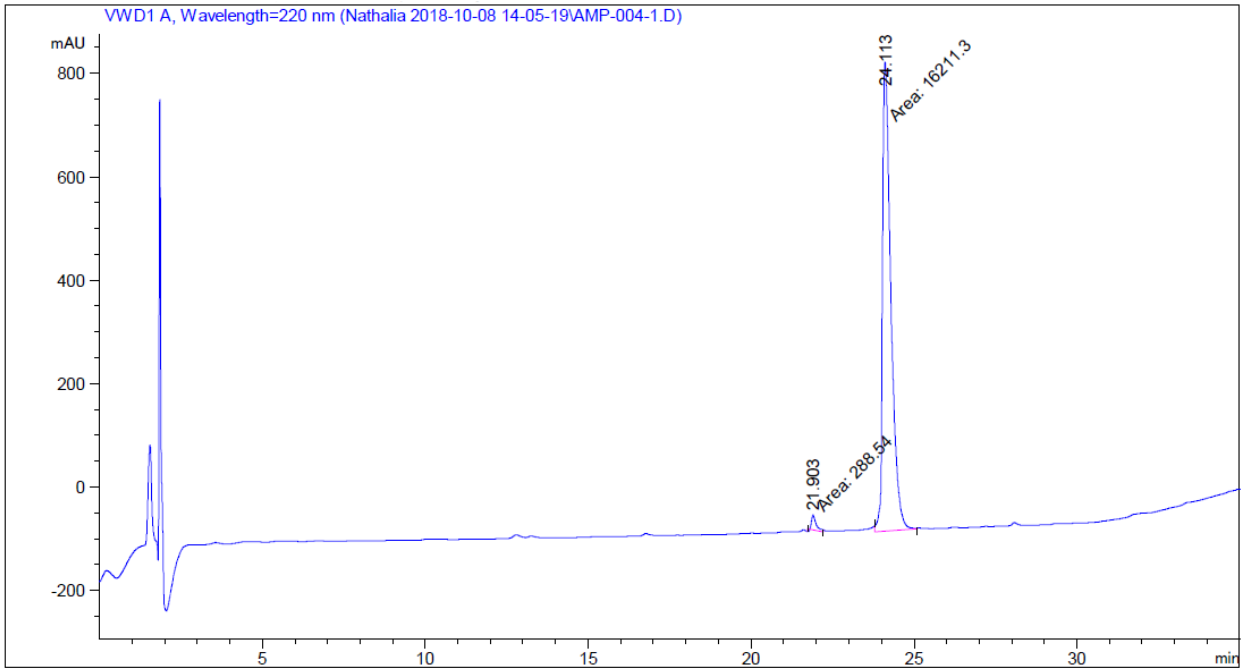
AMP-002



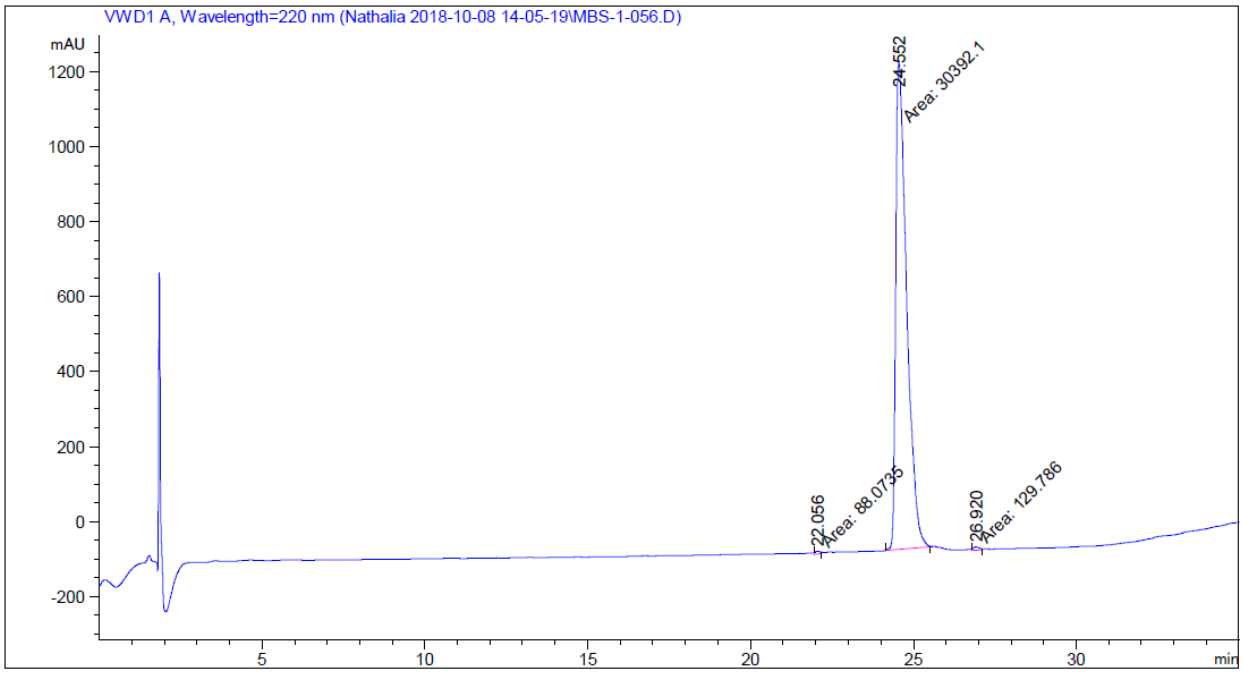
AMP-003



AMP-004



AMP-015



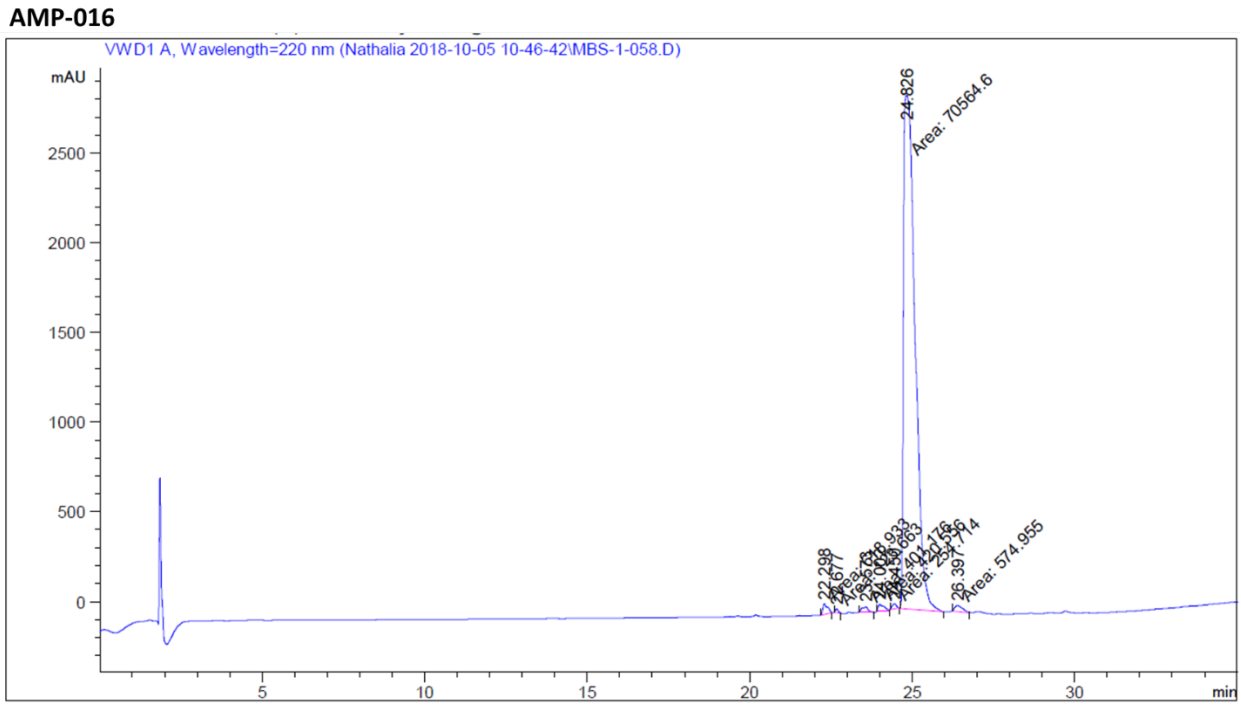


Figure S3: CD spectra of **AMP-001** Cit 1.1, **AMP-016**, **AMP-018**, **AMP-019**, **AMP-020** and **AMP-021** peptide solutions at 2 Mm in MilliQ water. Spectra was subtracted from MilliQ water (no AMP).

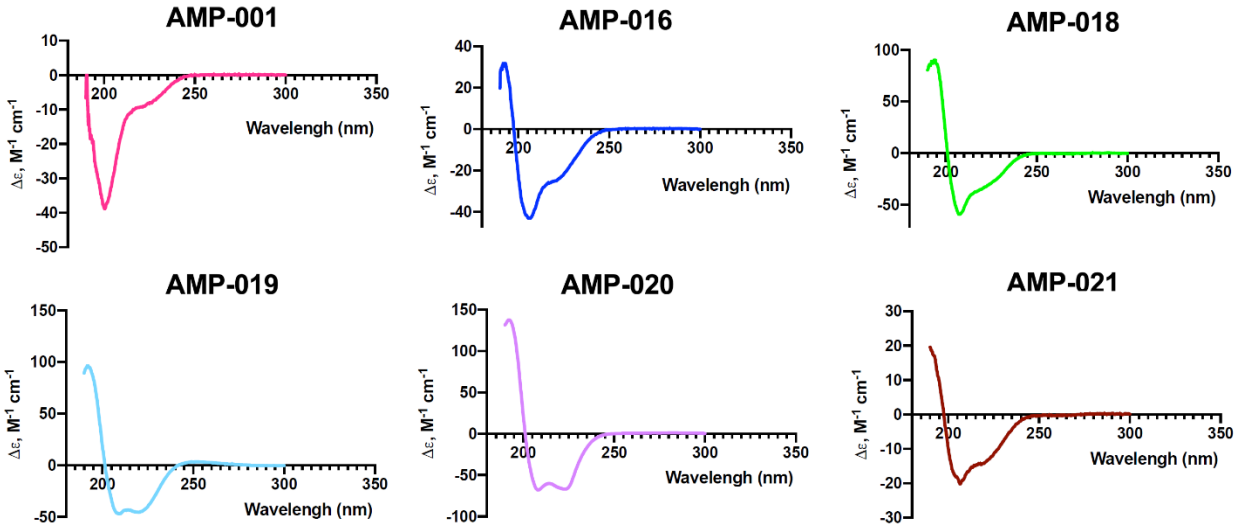


Figure S4: CD spectra of peptide + bacteria (A) **AMP-001** Cit 1.1 and **AMP-001** Cit 1.1 after incubation with live *E. coli* K12 cells and (b) **AMP-016** and **AMP-016** (blue) after incubation with live *E. coli* K12 cells. Cells were suspended in PBS (10^8 CFU/mL) with the addition of the AMPs at 2 mM. Spectra was subtracted from *E. coli* K12 (no AMP).

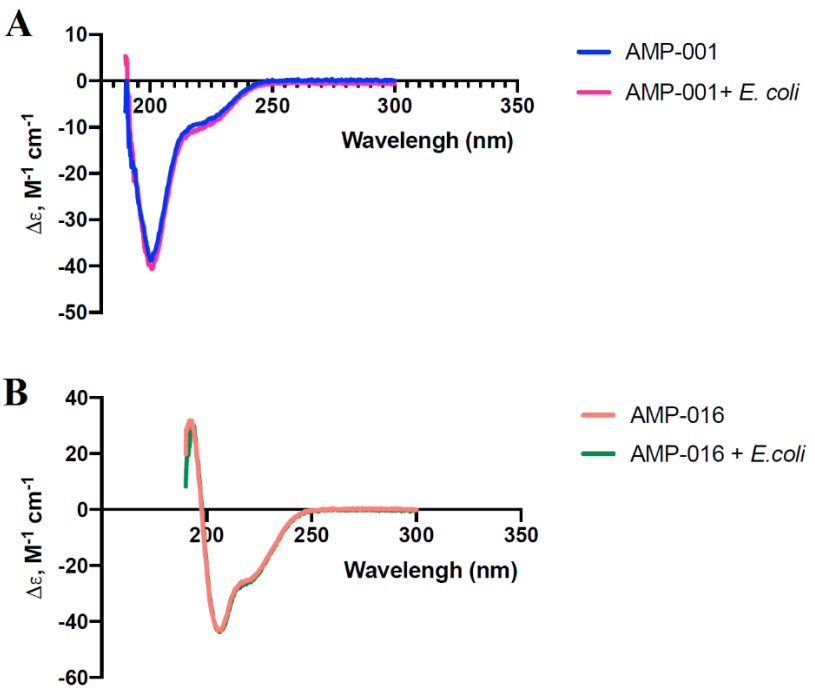


Table S2. Distance restraints and structure evaluation for **AMP-001** (**Cit 1.1**), **AMP-003**, and **AMP-016**.

Structure statistics for:	**AMP-001**	**AMP-003**	**AMP-016**
NOE Restraints	622	552	536
Short Range (|i-j|<=1)	414	390	362
Medium Range (1<|i-j|<5)	208	162	174
Long Range (peptide-peptide)	16	16	16
ϕ/ψ in most favored region	91.70%	94.20%	92.90%
ϕ/ψ in additionally allowed region	8.30%	5.80%	7.10%
ϕ/ψ in generously allowed	0%	0%	0
ϕ/ψ in disallowed region	0%	0%	0
Backbone atoms rmsd (Å)	0.3	0.7	0.4
Heavy atoms rmsd (Å)	0.5	0.7	0.6
NOE violations (>0.5Å)	0	0	0
PROCHECK (all) Z-score	-0.3	-0.95	-0.47

Figure S5. Temperature titration data for **Cit 1.1**. The change in the amide proton chemical shift (ΔH) was monitored as a function of temperature. Labels are found right of the graph and follow the peptide sequence in order. The largest changes in chemical shifts correspond to structurally stable amino acids becoming disordered at elevated temperature. Coloring corresponds to binned amide proton temperature titration data according to the ΔH ranges 0 to -0.05 (minor chemical shift, yellow), -0.05 to -0.25 (intermediate chemical shift, orange), and -0.25 to -0.45 (major chemical shift, red).

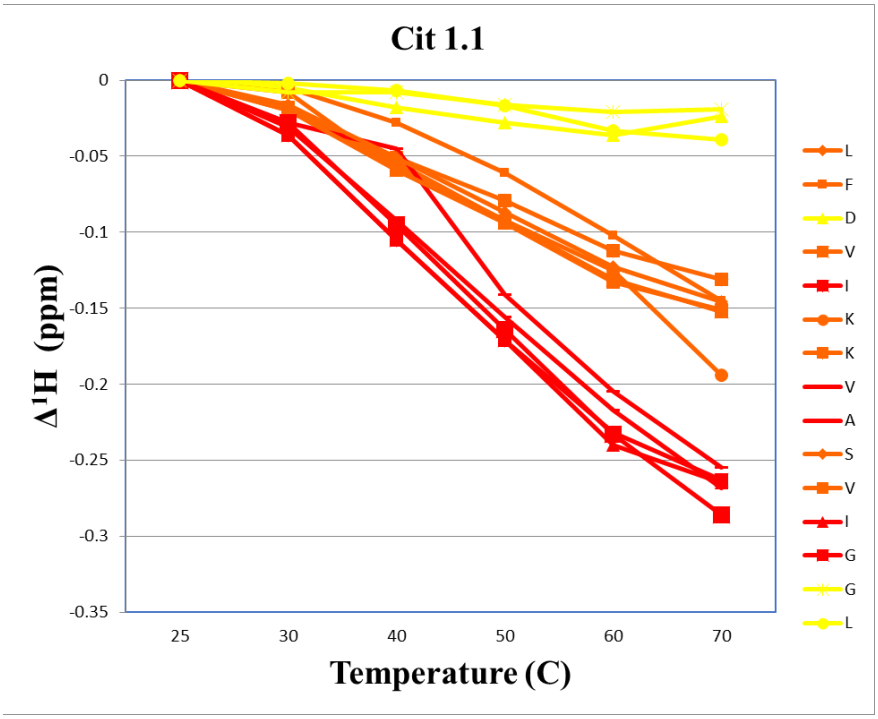


Figure S6. Temperature titration data for AMP-003. The change in the amide proton chemical shift (ΔH) was monitored as a function of temperature. Labels are found right of the graph and follow the peptide sequence in order. The largest changes in chemical shifts correspond to structurally stable amino acids becoming disordered at elevated temperature. Coloring corresponds to binned amide proton temperature titration data according to the ΔH ranges 0 to -0.05 (minor chemical shift, yellow), -0.05 to -0.25 (intermediate chemical shift, orange), and -0.25 to -0.45 (major chemical shift, red).

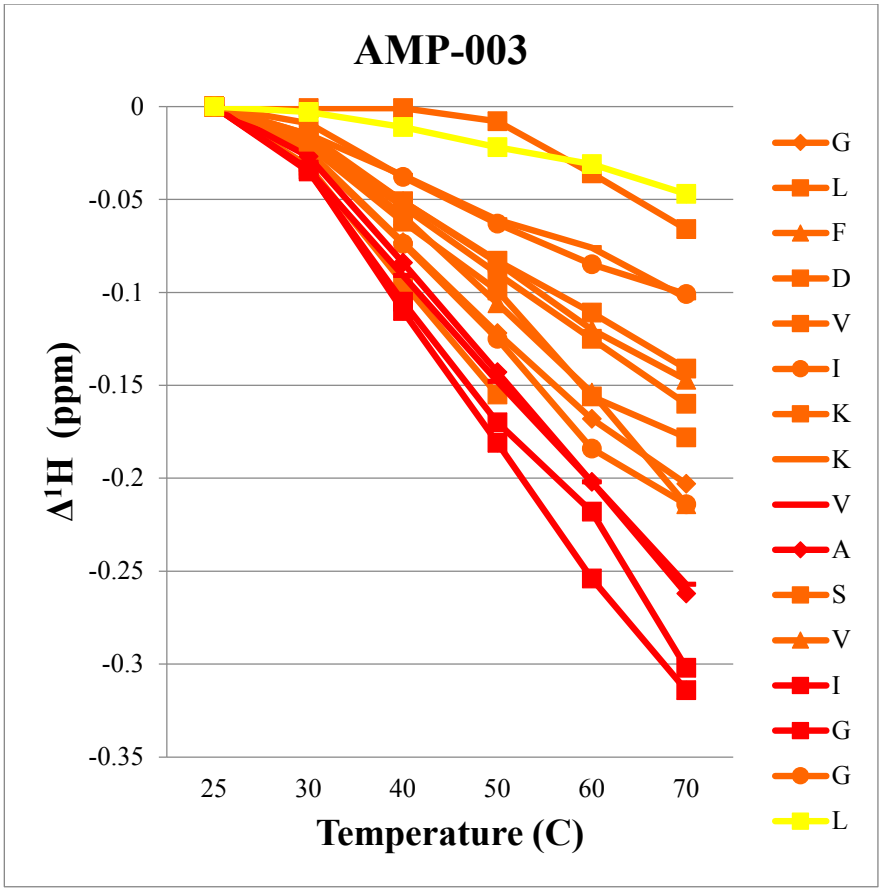


Figure S7: Temperature titration data for AMP-016. The change in the amide proton chemical shift (ΔH) was monitored as a function of temperature. Labels are found right of the graph and follow the peptide sequence in order. The largest changes in chemical shifts correspond to

structurally stable amino acids becoming disordered at elevated temperature. Coloring corresponds to binned amide proton temperature titration data according to the ΔH ranges 0 to -0.05 (minor chemical shift, yellow), -0.05 to -0.25 (intermediate chemical shift, orange), and -0.25 to -0.45 (major chemical shift, red).

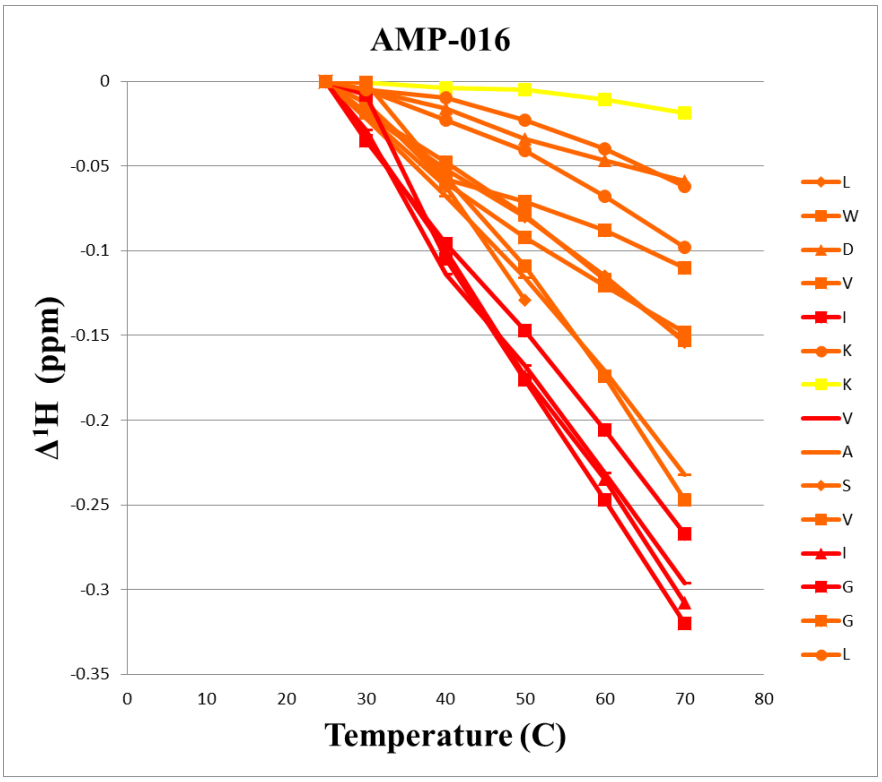


Figure S8: Gd-DTPA titration of Cit 1.1. The intensities of the amide proton peaks for each amino acid were monitored upon titration with Gd-DTPA at the listed concentrations. Intensities for each peak (I(x)) are normalized to the corresponding peak in the initial spectrum (I(0)).

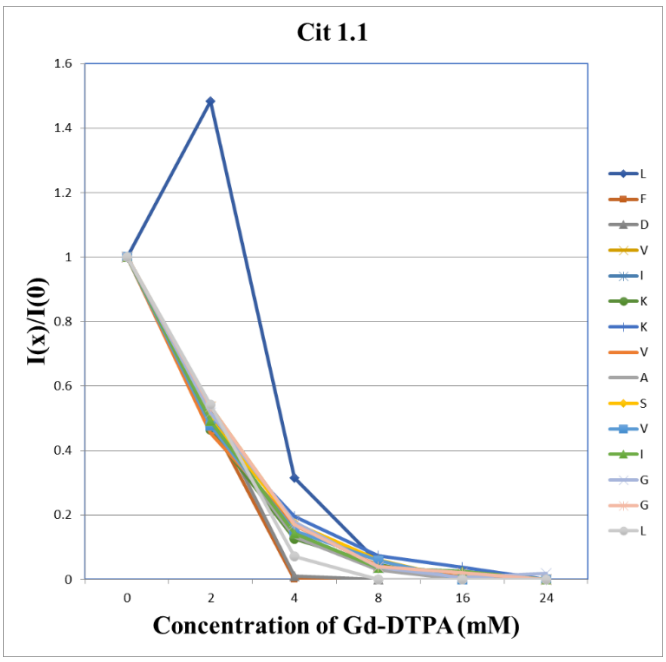


Figure S9: Gd-DTPA titration of AMP-003. The intensities of the amide proton peaks for each amino acid were monitored upon titration with Gd-DTPA at the listed concentrations. Intensities for each peak (I(x)) are normalized to the corresponding peak in the initial spectrum (I(0)).

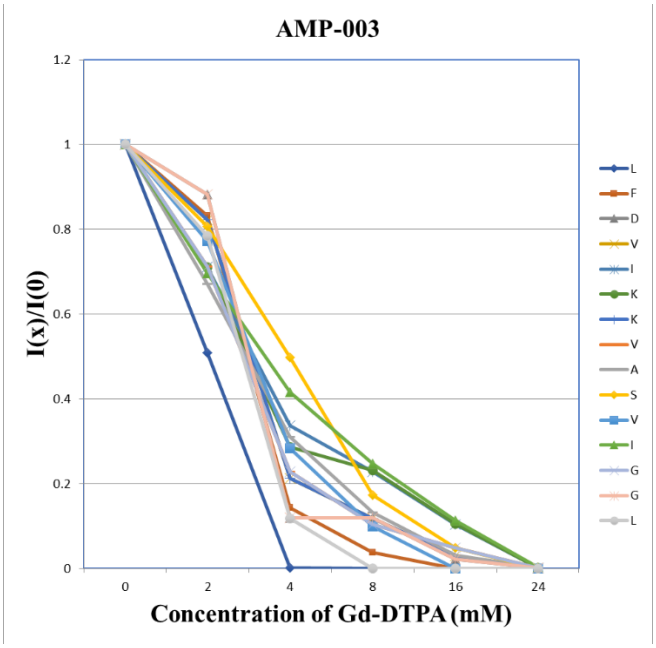


Figure S10: Gd-DTPA titration of AMP-016. The intensities of the amide proton peaks for each amino acid were monitored upon titration with Gd-DTPA at the listed concentrations. Intensities for each peak (I(x)) are normalized to the corresponding peak in the initial spectrum (I(0)).

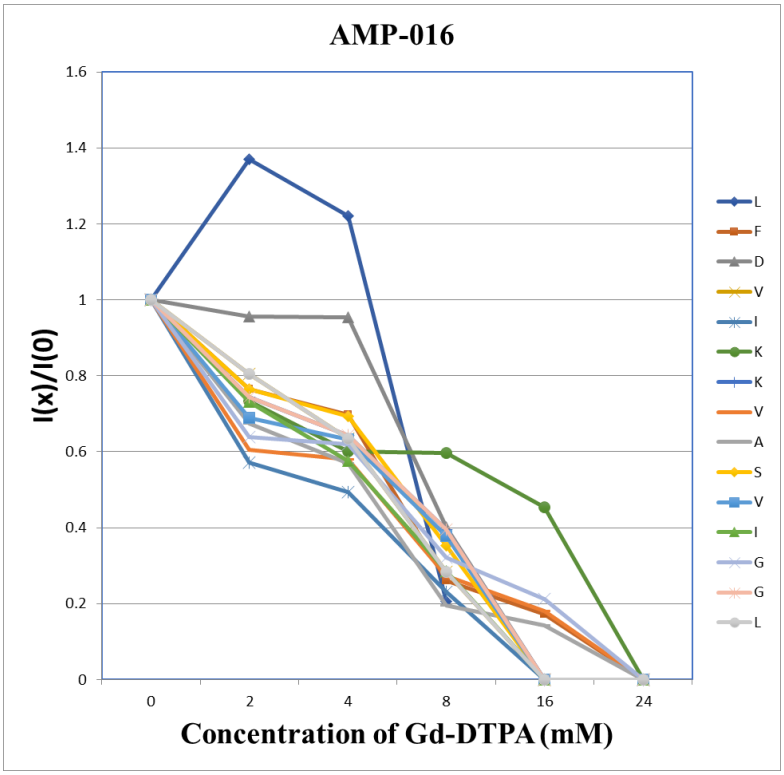
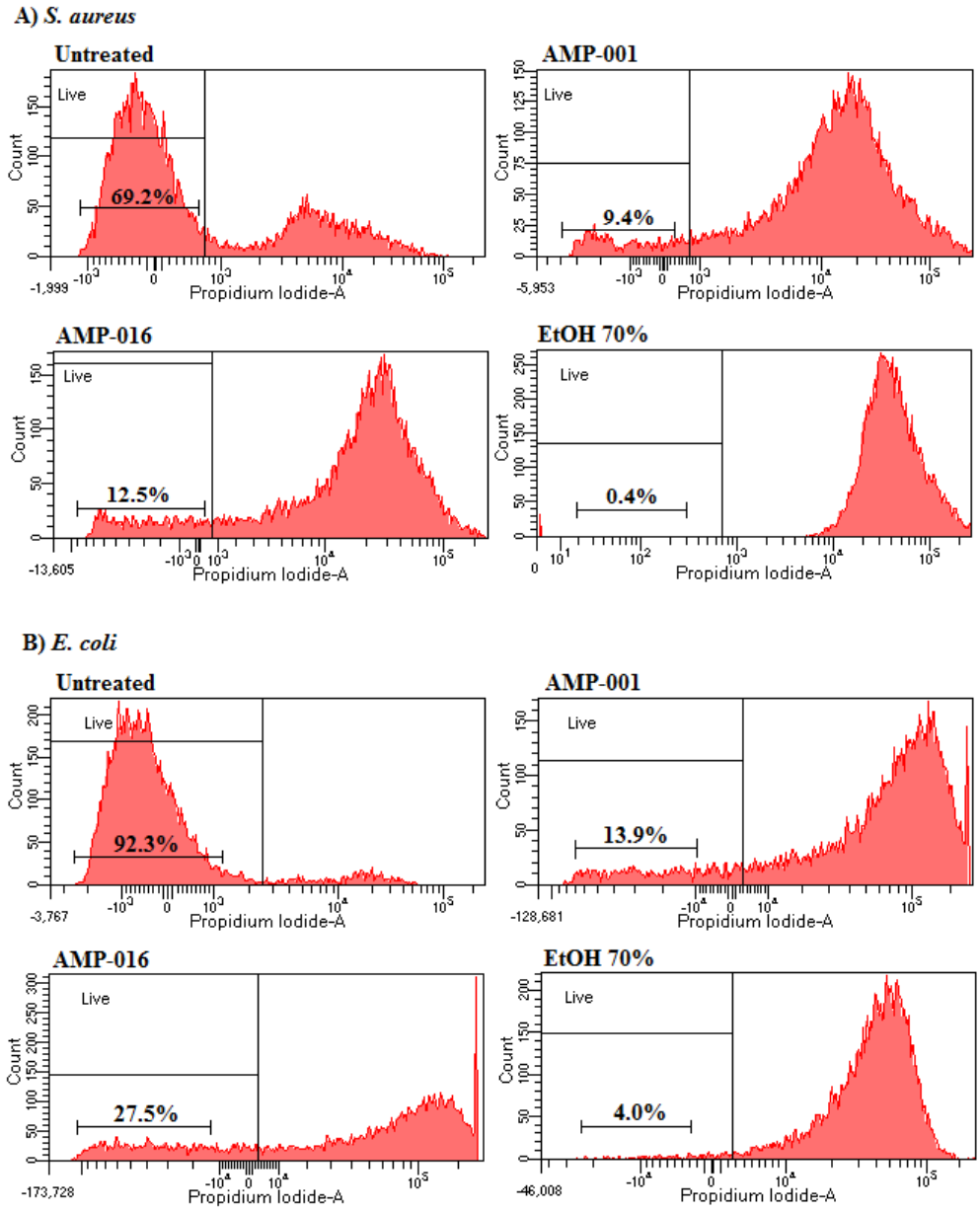


Figure S11: Flow cytometry analysis. Cell membrane disruption of *S. aureus* JE2 and *E. coli* K12 treated with **AMP-001 (Cit 1.1)** and **AMP-016** at MIC and 2 × MIC was determined by an increase in fluorescent intensity of PI. Untreated - No PA; Treatment with EtOH 70% was used as positive control.



References

[1] N.R. Almeida, Y. Han, J. Perez, S. Kirkpatrick, Y. Wang, M.C. Sheridan, Design, Synthesis, and Nanostructure-Dependent Antibacterial Activity of Cationic Peptide Amphiphiles, ACS Applied Materials & Interfaces, 11 (2019) 2790-2801 DOI: 10.1021/acsami.8b17808.