Supplementary Information

Ciprofloxacin as a tryptophan mimic within an antimicrobial peptide

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General information

15 M'Ω water supplied by ELGA Purelab water purifier. Piperidine was supplied by Thermo Fischer scientific (Ireland). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-Oxide Hexafluoro phosphate (HATU), Di-tert butyl dicarbonate, Diisopropyl ethyl amine, trifluoroacetic acid, N,N'-Diisopropylcarbodiimide (DIC), fmoc-Lysine(Mtt)-OH, ciprofloxacin and 1,2-Ethanedithiol (EDT) were supplied by Tokyo Chemical Industry (TCI, Japan). Oxyma pure, N,N-Dimethylformamide (DMF), acetonitrile, triisopropyl silane (TIPS), thioanisole, diethyl ether, Rink Amide PEGA resin, Muller-Hinton broth (MH), bovine serum albumin (BSA), acetic acid, alumina, dichloromethane (DCM) were supplied by Merck (Germany). Boc-Arginine(Pbf)-OH supplied by Fluorochem (Ireland). a-Cyano-4-hydroxycinnamic acid (HCCA) Maldi matrix supplied by Bruker (ireland). All other chemicals were supplied by CEM (UK).

Synthesis

SPPS

Amino acids were weighed and dissolved in appropriate volume of DMF to produce a 2M concentration. An appropriate amount of oxyma, DIC, 20% piperidine in DMF and resin were added to the CEM liberty blue automated peptide synthesiser and method ran to produce the peptide chain attached to the resin (PEGA rink amide). The resin was washed with DCM and filtered ready for further synthesis.

Mtt removal

The resin was swelled in 3 mL of DCM for 15 min. A solution of 1% TFA in DCM was made. The resin was drained, 5 ml of deprotection solution was then added and resin shaken for 30 mins. The resin was drained and the deprotection repeated until a colourless solution was present after the 30 mins treatment. The resin was drained and washed with 2xDCM, 2xMeOH, 2x 1% DIEA in DMF, 2xDMF and finally rinsed with DCM ready for further synthesis

Boc-Cip coupling

The resin was swelled in 3 mL of DMF for 45 minutes. A 5ml coupling solution of BOC-Cip and HATU in 1.75:1 DCM:DMF was prepared and stirred for 10 min after which time DIEA was added dropwise and stirring maintained for a further 10 min. The DMF was drained from the resin and the coupling solution added and the mixture shaken for 1 hour. After which time the solution was drained and the coupling

repeated a further two times. The solution was then drained and washed with 2xDCM, 2xMeOH, 2x DMF and rinsed with DCM ready for further synthesis.

Cleavage

All peptides underwent cleavage from the resin and final deprotection of any side-chain protecting groups as follows: a cleavage cocktail of 81.5% trifluoroacetic acid (TFA), 5% water, 1% triisopropylsilane (TIPS), 10% thianisole and 2.5% 1,2-ethanedithiol was produced and added to the amide resin. The reaction mixture was stirred for 2.5 hours. Upon completion the resin was filtered and washed with a small amount of TFA. Diethyl ether was added dropwise in aliquots to the solution, with vigorous mixing between additions, until precipitation of the peptide did not resolubilise upon mixing. The solid peptide was centrifuged and washed with diethyl ether twice, allowed to dry, then solubilised in water and lyophilised ready for analysis. Peptides percentage yields were as follows: Bac8c 45%, Bac8c(Cip^{3}) 35%, Bac8c(Cip^{6}) 45% and Bac8c(Cip^{3,6}) 45%

HPLC

HPLC was performed on a Shimadzu Prominence using mobile phase A and B consisting of dH_2O + 0.1% TFA and acetonitrile + 0.1% TFA respectively. Peptides were dissolved in H_2O up to 0.1 mg/ mL and 100 uL injected onto Gemini column (Phenomenex, 110Å, 5µ, C18, 4.6mmd/250 mL) with 1 mL/ min flow rate and a gradient A:B ran as the following 0-5 mins at 95:5, 5-30 min from 5:95 up to 35:65, 30-35min down to 5:95 then 35-42 min at 95:5. Peaks were detected using a Shimadzu SPD-M20A Photodiode Array detector and processed using Shimadzu LabSolutions.

MALDI-TOF Mass Spectrometer

Mass Spectra were acquired in high-resolution reflectron mode on a Bruker Autoflex Max MALDI system. Samples were dissolved in H_2O + 1% TFA up to 0.1mg/ mL. 2 µL were taken and added with 2 µL of matrix solution (HCCA in H_2O + 1% TFA) and the solution mixed using a pipette. 0.5 µL were then spotted onto MALDI plate. Spectra were processed using mmass[1]

Boc-Cip synthesis

Ciprofloxacin (5g, 15.09 mmol) was dissolved in 75ml THF and cooled to 0°C in an ice bath. Di-tert-butyl dicarbonate (3.95g, 18.11 mmol) was added slowly while stirring the solution and then triethyl amine (2.52 mL, 18.11 mmol) was added dropwise. The solution was stirred for 10 mins, then allowed to warm to RT and stirred overnight. The mixture was concentrated *in vacuo* and 100ml of petroleum ether was added. The resulting precipitate was filtered and washed with cold Petroleum ether, H_2O, acetone and dried. The product was then purified via flash column chromatography using 10:90 MeOH:DCM on alumina to afford an off white product with 96.7% yield. Analytical data agrees with literature.[2]

^{1}H NMR (400 MHz, $CDCl_3$) δ 14.94 (s, 1H, -COO**H**), 8.71 (s, 1H, N-C**H**-COOH), 7.97 (d, $^{3}J_{H\text{-}F}$ = 12 Hz, 1H,F-C-C**H**-C), 7.35 (d, $^{4}J_{H\text{-}F}$ = 8 Hz, 1H, F-C-C-C**H**), 3.67-3.65 (m, 4H, **CH_2-**N-C**H_2**), 3.57 – 3.52 (m, 1H, N-C**H**-CH_2), 3.30 – 3.28 (m, 4H, **CH_2-**N-C**H_2**), 1.49 (s, 9H, C(C**H_3**)$_3$), 1.39 (m, 2H, N-CH-C**H_2**-CH_2), 1.23 (m, 2H, N-CH-CH_2-C**H_2**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.02 (d, ^{4}J = 2 Hz), 166.92, 154.89 (d, ^{1}J = 252.5 Hz), 154.57, 147.49, 145.82 (d, ^{2}J = 10.1Hz), 139.02, 120.08 (d, ^{4}J = 7 Hz), 112.57 (d, ^{2}J = 23.2 Hz), 108.09, 105.08 (d, ^{4}J= 3 Hz), 80.37, 49.74, 35.33, 28.41, 8.27. ^{19}F NMR (376 MHz, $CDCl_3$) δ 120.90 (m) Mass spectrometry (ESI, Advion) *M/z*: 432.2 $[M+H]^+$ Calc: 432.19

Antibacterial activity

MICs were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) as described previously.[3,4] Bacterial strains used include Gram-negative (*Acinetobacter baumannii* (DSM 30007), *Enterobacter cloacae* (DSM 30054), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (DSM 26371), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (CIP 80.39), and *Shigella sonnei* (ATCC 29930)) and Gram-positive bacteria (*Bacillus cereus* (DSM 31), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (DSM 2570), *Enterococcus faecium* (DSM 20477), Listeria monocytogenes (DSM 20600), *Staphylococcus epidermidis* (DSM 20044) and *Staphylococcus aureus* (ATCC 25923)). Overnight bacterial suspensions were prepared from single colonies grown on agar plates and were grown at 37°C under agitation at 200 rpm. The next day, exponentially growing bacterial suspensions were obtained by diluting 1:100 the overnight cultures in Mueller-Hinton (MH) media and growing them at 37°C and 200 rpm. After 2-3 h, optical densities were recorded at 600nm and bacteria suspensions were diluted to reach approximatively a bacterial density of 10^{e5} bacteria/mL. 100 µL of these suspensions were inoculated into polypropylene 96 well plates and exposed to increasing concentrations of molecules. After 24h, bacterial growth in wells was compared to positive (no molecules) and negative controls (no bacteria) and the MIC was determined as the lowest concentration at which no growth was observed. MBC were determined by platting on agar plates of 10 µl of the wells of the MIC plates and counting bacteria colonies after 18-24 h incubation at 37°C. The MBC were also determined by adding resazurin (Tox-8 kit from Sigma Aldrich) directly into 96 wells plates used for MIC (final dilution 1:10). After 4 h incubation at 37°C, the fluorescence signal of the wells was measured (Ex 530 nm/Em 590 nm) and the MBC were determined as the concentration of molecules leading to more than 99.9% reduction of the metabolism of resazurin by bacteria. MIC and MBC were determined at least two independent times. All bacteria were grown and tested in aerobic conditions, except *Listeria monocytogenes, Enterobacter cloacae, Enterococcus faecalis* and *E. faecium* that were grown and tested in microaerobic condition generated using GasPak units.

Cytotoxicity determination

The toxicity/innocuity of the peptides was evaluated using HepG2 cells as a model of human liver cells as previously described.[4] HepG2 were routinely grown on 75 cm^2 flasks in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (all from Thermo Fisher Scientific, Illkirch-Graffenstaden, France) in a 5% CO2 incubator at 37 °C. For the cytotoxicity assay, cells were detached using trypsin–EDTA solution, counted using Malassez counting chamber and seeded into 96-well cell culture plates (Greiner bio-One, Dominique Dutscher, Brumath, France) at approximately 10,000 cells per well. After 48–72 h incubation at 37 °C to allow cells to attach and to reach 80-90% confluence, wells were emptied and cells were exposed to increasing concentrations of compounds (from 0 to 300 µM) diluted in 100 µL of culture medium. After 48 h incubation at 37 °C in a 5% CO_2 incubator, wells were emptied and cell viability was measured using resazurin (in vitro toxicity assay (TOX8) kit from Sigma- Aldrich, Lyon, France) as previously described. The fluorescence values (Ex at 530 nm / Em at 590 nm) of the wells were normalized by the negative controls and expressed as percentage of viability. The CC_{50} values of the compounds (i.e. the concentration of compounds causing a reduction of 50% of the cell viability as compared to the control) were finally calculated using GraphPad® Prism 7 software (San Diego, CA, USA). Experiments were conducted in triplicate (n=3).

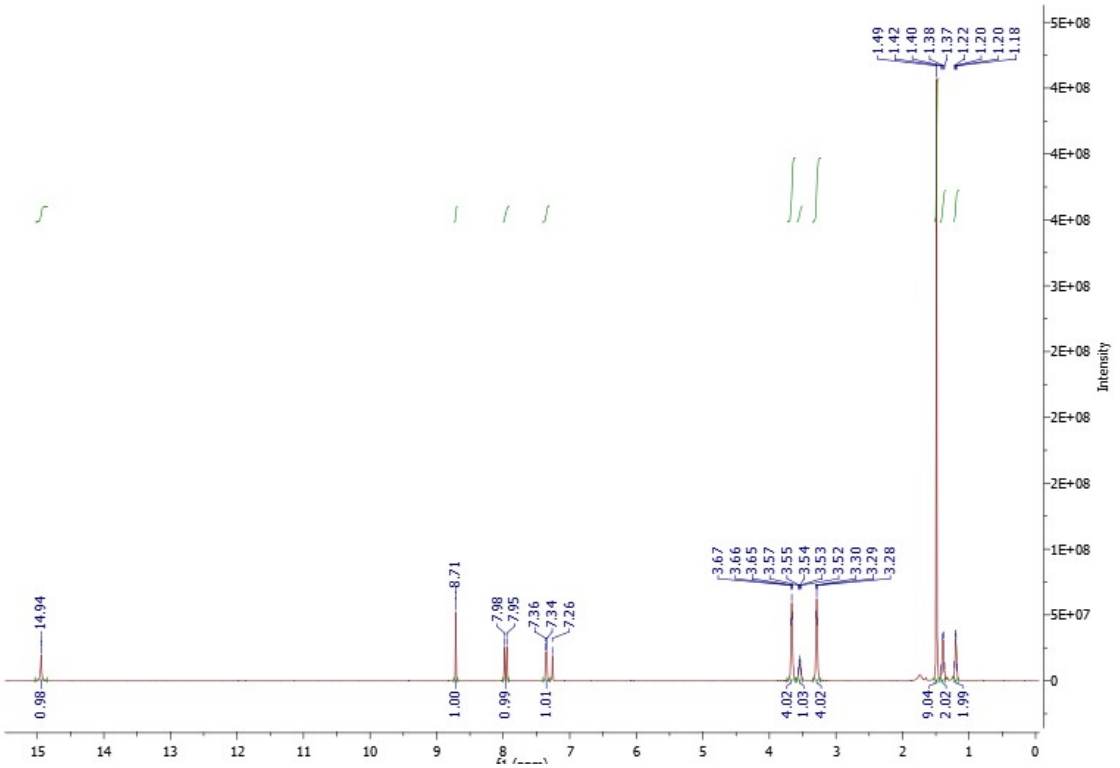


Figure S1: ^{1}H NMR for Boc-Cip analogue

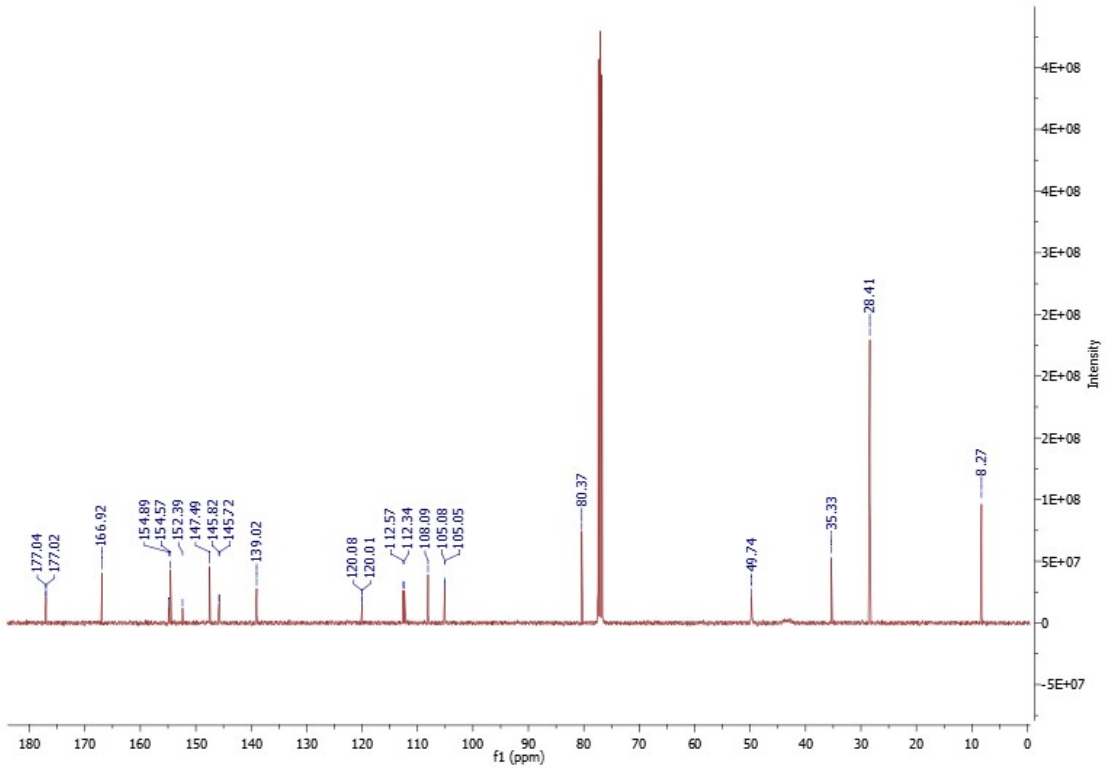


Figure S2: ^{13}C NMR for Boc-Cip analogue

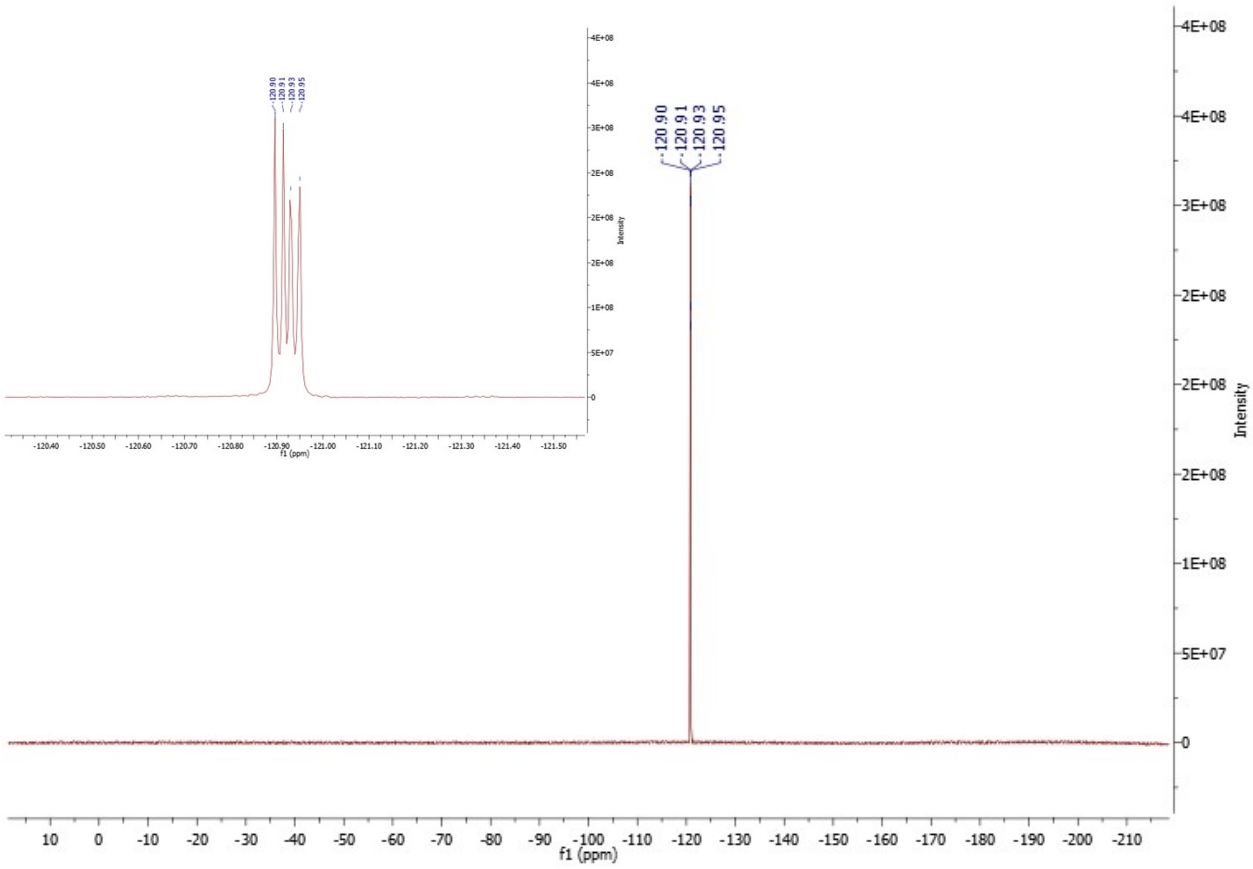


Figure S3: ^{19}F NMR for Boc-Cip analogue

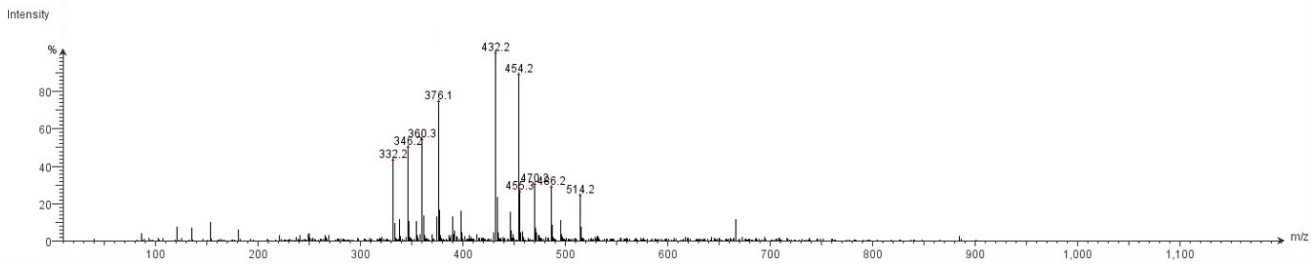
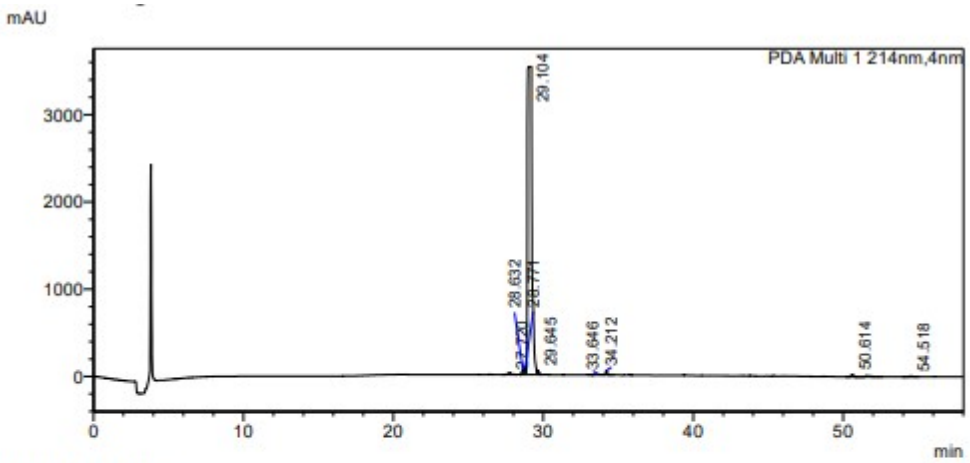


Figure S4: ESI Mass spectrum of Boc-Cip



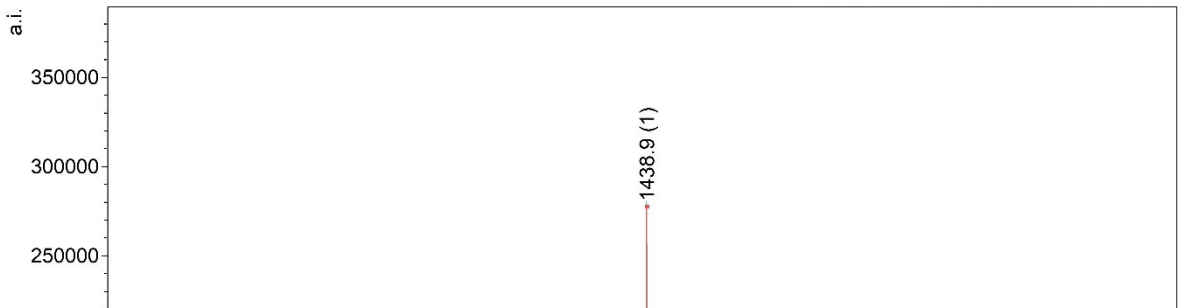
<Peak Table>

PDA Ch1 214nm

Peak#	Ret. Time	Area	Height	Area%
1	27.720	314404	17082	0.413
2	28.632	481657	61741	0.633
3	28.771	579296	73620	0.761
4	29.104	73115409	3528698	96.102
5	29.645	404426	51431	0.532
6	33.646	211505	7844	0.278
7	34.212	390059	50684	0.513
8	50.614	487342	28803	0.641
9	54.518	96729	8303	0.127
Total		76080827	3828207	100.000

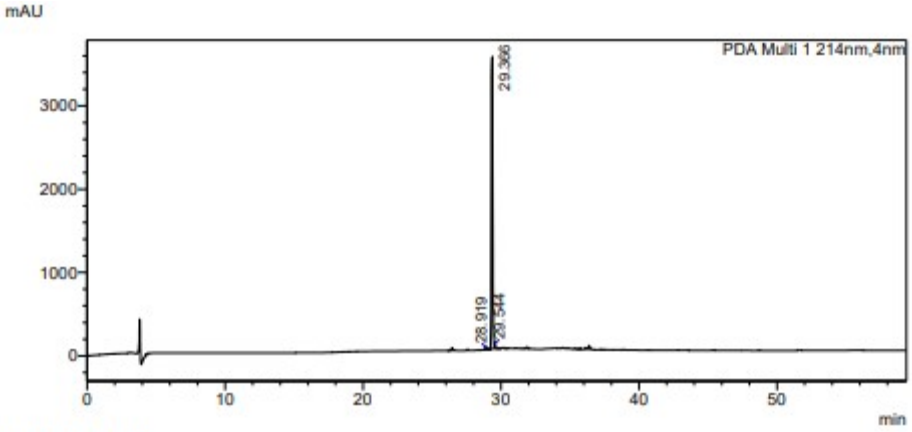
Figure S5: HPLC trace for Bac8c(Cip^3) peptide with peak table

Figure S6: MALDI-TOF mass spectrum of Bac8c(Cip^3)



bserved *M/z*: 1438.9

Calc *M/z*: 1438.9



<Peak Table>

PDA Ch1 214nm

Peak#	Ret. Time	Area	Height	Area%
1	28.919	204449	33660	0.868
2	29.366	23017258	3500628	97.721
3	29.544	332407	66661	1.411
Total		23554114	3600949	100.000

Figure S7: HPLC trace of Bac8c(Cip^6) with peak table

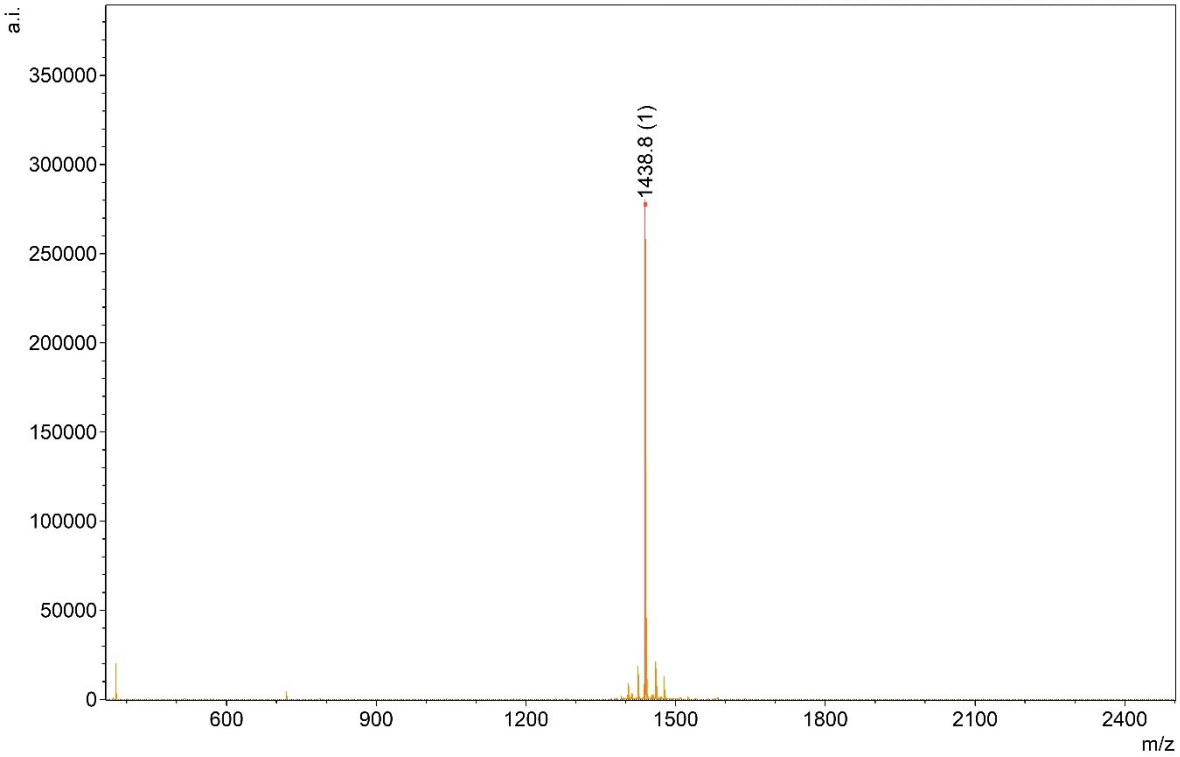
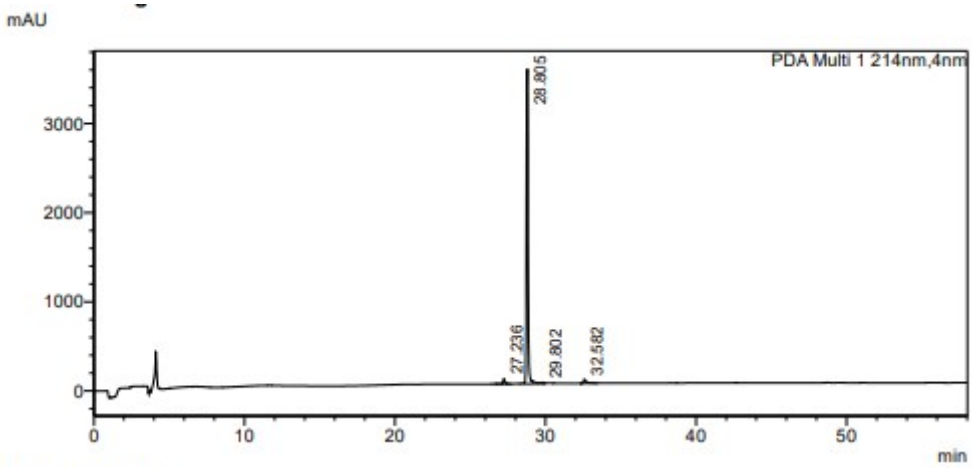


Figure S8: MALDI-TOF mass spectrum of Bac8c(Cip^6)

Observed *M/z*: 1438.8

Calc *M/z*: 1438.9



<Peak Table>

PDA Ch1 214nm

Peak#	Ret. Time	Area	Height	Area%
1	27.236	483089	57442	1.598
2	28.805	29042297	3527423	96.059
3	29.802	165108	12425	0.546
4	32.582	543262	43140	1.797
Total		30233756	3640430	100.000

Figure S9: HPLC trace of Bac8c(Cip^{1,6}) with peak table

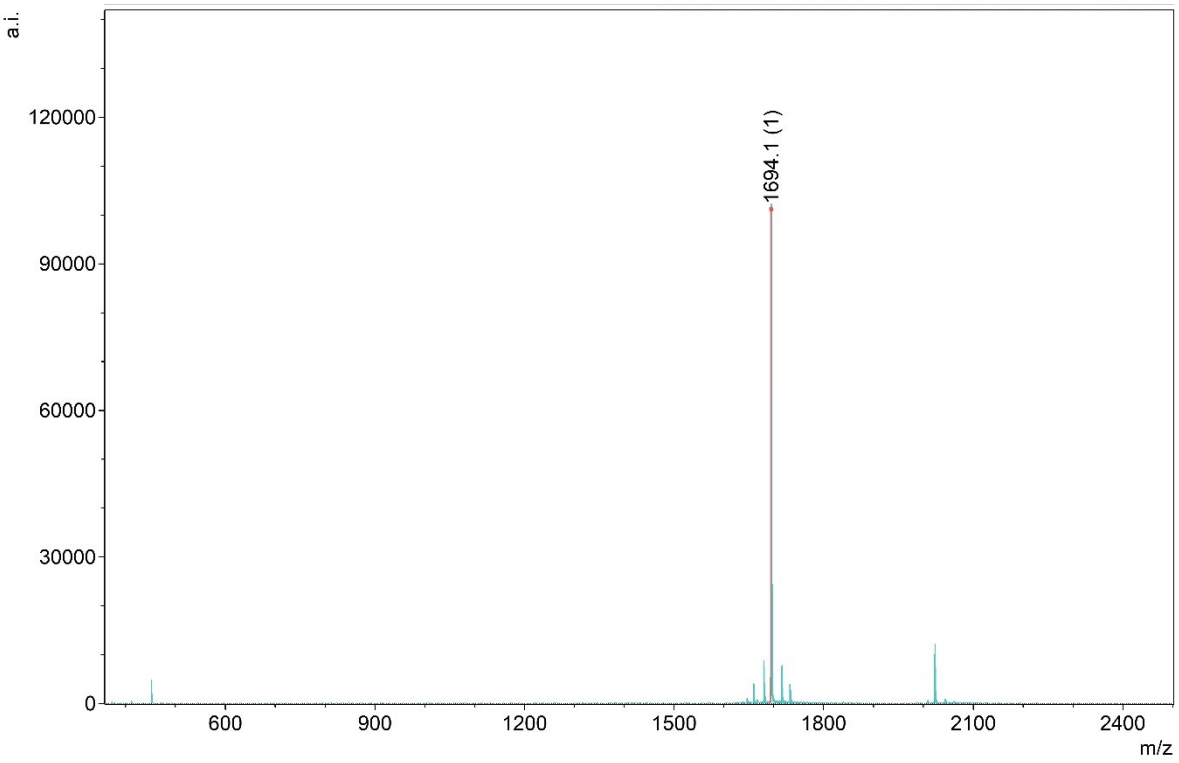
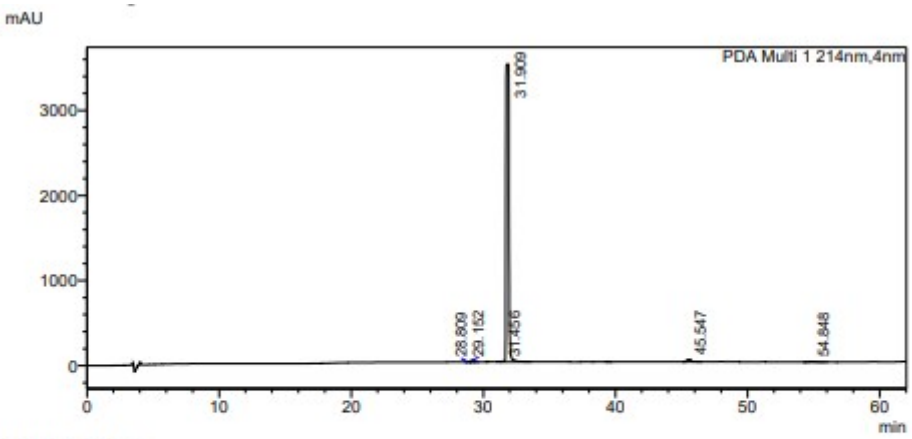


Figure S10: MALDI-TOF mass spectrum of Bac8c(Cip^{3,6})

Observed *M/z*: 1694.1

Calc *M/z*: 1694.0



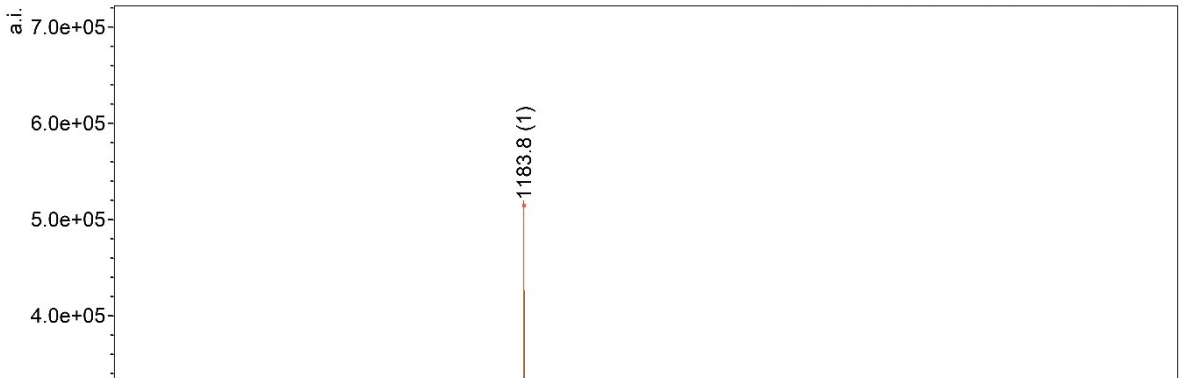
<Peak Table>

PDA Ch1 214nm

Peak#	Ret. Time	Area	Height	Area%
1	28.809	41943	5052	0.074
2	29.152	159096	19392	0.280
3	31.456	89077	8623	0.157
4	31.909	55793295	3497549	98.171
5	45.547	508161	26676	0.894
6	54.848	241323	6666	0.425
Total		56832895	3563958	100.000

Figure S11: HPLC trace of Bac8c with peak table

Figure S12: MALDI-TOF mass spectrum of Bac8c



Observed *M/z*: 1183.8

Calc *M/z*: 1183.7

1 T. H. J. Niedermeyer and M. Strohalm, *PLoS One*, 2012, **7**, e44913.
2 J. Azéma, B. Guidetti, J. Dewelle, B. Le Calve, T. Mijatovic, A. Korolyov, J. Vaysse, M. Malet-Martino, R. Martino and R. Kiss, *Bioorganic & Medicinal Chemistry*, 2009, **17**, 5396–5407.
3 E. Forde, H. Humphreys, C. M. Greene, D. Fitzgerald-Hughes and M. Devocelle, *Antimicrob Agents Chemother*, 2014, **58**, 978–985.
4 H. Olleik, T. Yacoub, L. Hoffer, S. M. Gnansounou, K. Benhaiem-Henry, C. Nicoletti, M. Mekhalfi, V. Pique, J. Perrier, A. Hijazi, E. Baydoun, J. Raymond, P. Piccerelle, M. Maresca and M. Robin, *Antibiotics*, 2020, **9**, 381.