Supplemental Information for:

Synergy between the Clavanins as a weapon against multidrug-resistant *Enterobacter cloacae*

Marvin D. Naing[1], Samuel A. Juliano[1], Alfredo M. Angeles-Boza[1,2]*

1. Department of Chemistry, University of Connecticut, 55 N. Eagleville Road, Storrs, CT 06269, United States

2. Institute of Materials Science, University of Connecticut, 25 King Hill Road, Storrs, CT 06269, United States

*E-mail: alfredo.angeles-boza@uconn.edu

Table of Contents	Page #
Solid-Phase Peptide Synthesis	3
Purification using UPLC and Characterization using MS	3
Figure S1. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin A	4
Figure S2. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin B	5
Figure S3. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin C	6
Figure S4. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin D	7
Figure S5. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin E	8
Figure S6. HPLC (top) and ESI-MS (bottom) profile of the purified Clavaspirin	9
Scheme S1. Synthetic scheme for the synthesis of ClavD-FAM and ClavS-TAMRA	10
Figure S7. HPLC and ESI-MS profile of ClavD-FAM	11
Figure S8. HPLC and ESI-MS profile of ClavS-TAMRA	12
Table S1. Summary of calculated peptide masses compared with actual mass obtained from MS	13
CD Spectroscopy	14
Bacterial Culture Conditions	14
Broth Microdilution Assay and MIC Determination	15
Checkerboard Assay and FIC Calculation	15
Calculation of the Loewe Additivity Response	16
Table S2. FIC data obtained from the checkerboard assay and 1:1 combination broth microdilution assay.	16
Time-kill Kinetics Assay	17
Calculation of Bliss Independence Response	17
Confocal Microscopy	18
Transmission Electron Microscopy	18

Experimental Guidelines

Solid Phase Peptide Synthesis. All amino acids and reagents were purchased from Matrix Innovation (Quebec City, QC) unless otherwise specified. All peptides were synthesized using the Liberty Blue™ automated microwave peptide synthesizer (CEM Corporation, Matthews, NC) utilizing the standard fluorenlymethyloxycarbonyl (Fmoc) chemistry on Rink amide resin and using N,N'-diisopropylcarbodiimide (DIC) and ethyl 2-cyano-2-(hydroxyamino) acetate (Oxyma) as the activator and activator base, respectively. The peptides were deprotected and cleaved from the resin using a cleavage cocktail consisting of 5% nanopure water (Milli-Q), 5% phenol, 2% triisopropyl silane (TIS), and 88% trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO). Peptides were purified using Dionex UltiMate™ 3000 UPLC system utilizing a Jupiter® C18 semipreparative column (Phenomenex, Torrance, CA) and a water-acetonitrile gradient. Peptide identity was verified via electrospray ionization mass spectrometry in positive ion mode using a 4000 QTRAP (AB Sciex) mass spectrophotometer. Fractions were collected and lyophilized at least overnight or until a powder is obtained. For quantification, UV-vis spectrophotometry was employed using the molar extinction coefficients at 214 nm published in the literature[1]. Peptides that contain a fluorescent tag were synthesized the same way but with a Phe to Lys mutation close to the N-terminal as shown in scheme S1. Either 5(6)-carboxyfluorescein (FAM) (Acros Organics, NJ) or 5(6)-carboxytetramethylrhodamine (TAMRA) (Novabiochem, Burlington, MA) was used as the fluorescent label. They were also purified and quantified the same way as described above. All peptides are stored in nano pure water and kept at 4° C until further use.

Purification using UPLC and Characterization using Mass Spectrometry. The lyophilized powder obtained from the peptide synthesis were dissolved in 50:50 methanol and water mixture. If any solids remain undissolved, the solutions are filtered in a 0.45 μm syringe filter. The peptides were then purified using a Dionex UltiMate™ 3000 UPLC system utilizing a Jupiter® C18

semipreparative column using a water-acetonitrile gradient. Peaks were collected and pooled according to their retention times. All fractions were then lyophilized. The lyophilized fractions are then dissolved in 1 mL of nanopure water (milli Q). Fifty (50) microliters of the solution were then mixed with 50 μL of methanol containing 0.1% formic acid. Peptide identity was verified by manually injecting 20 μL of the sample into an electrospray ionization (ESI) mass spectrometer in positive ion mode using a 4000 QTRAP (AB Sciex) mass spectrophotometer. The MS is then reported below without any processing.

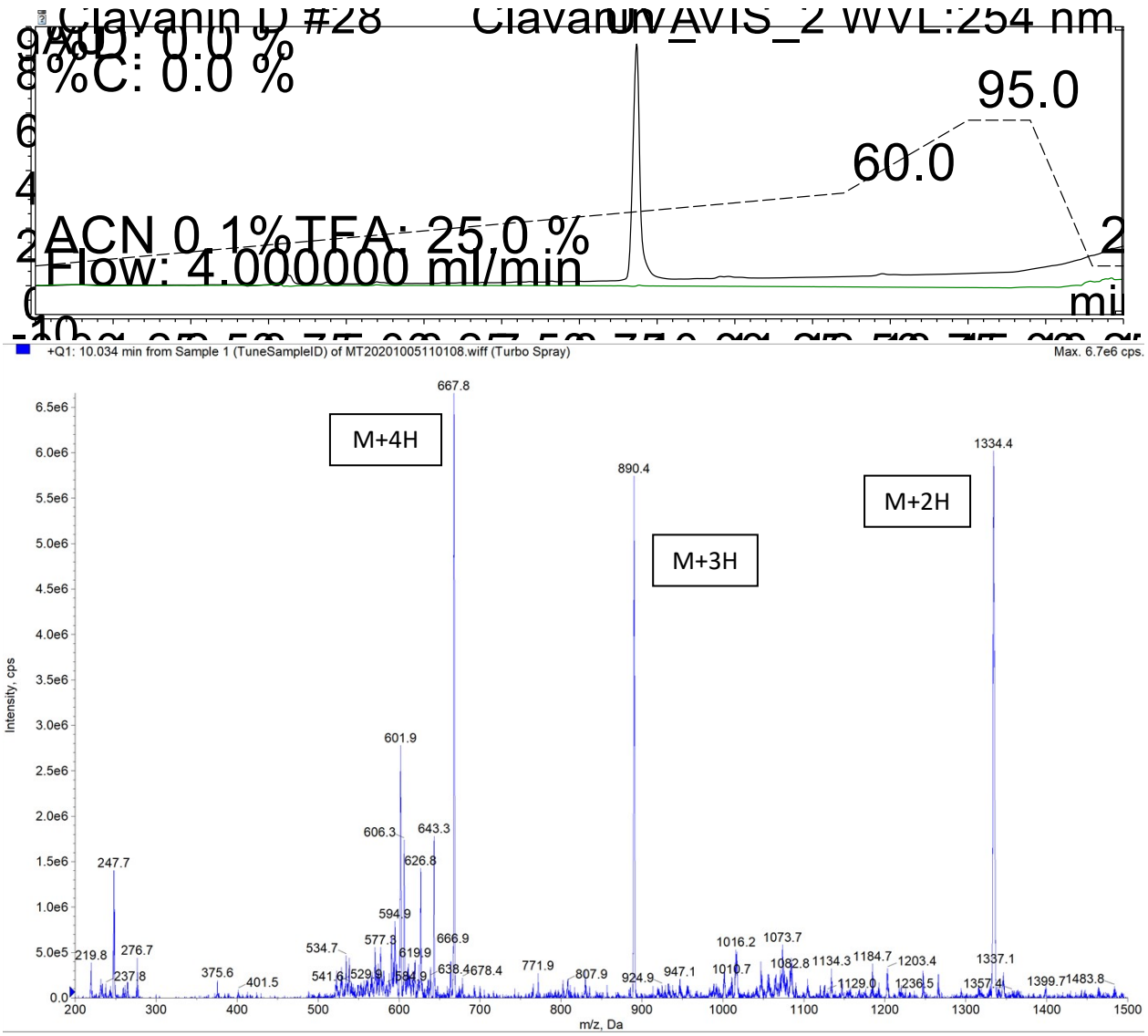


Figure S1. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin A (97.7% pure)

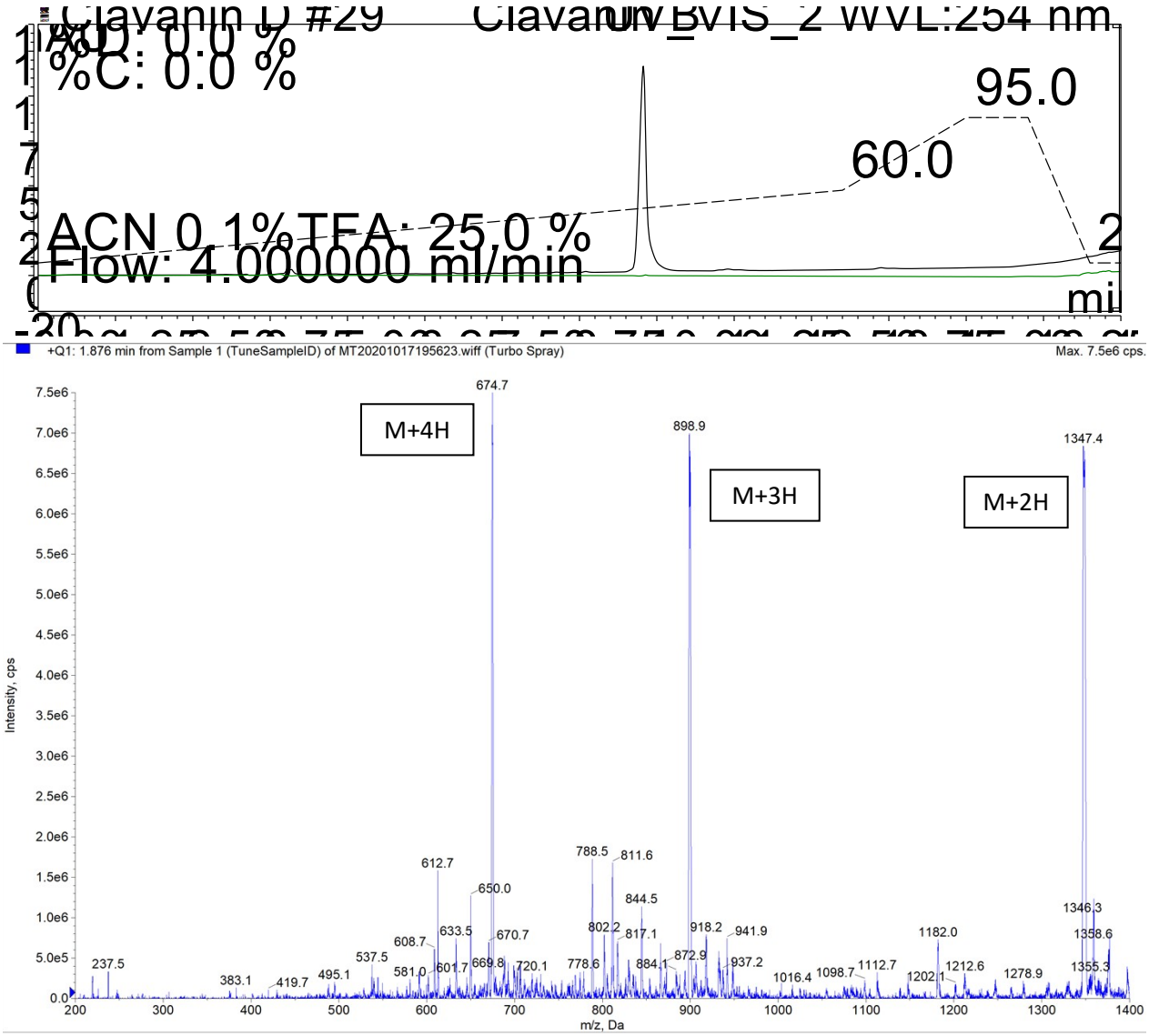


Figure S2. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin B (98.0% pure)

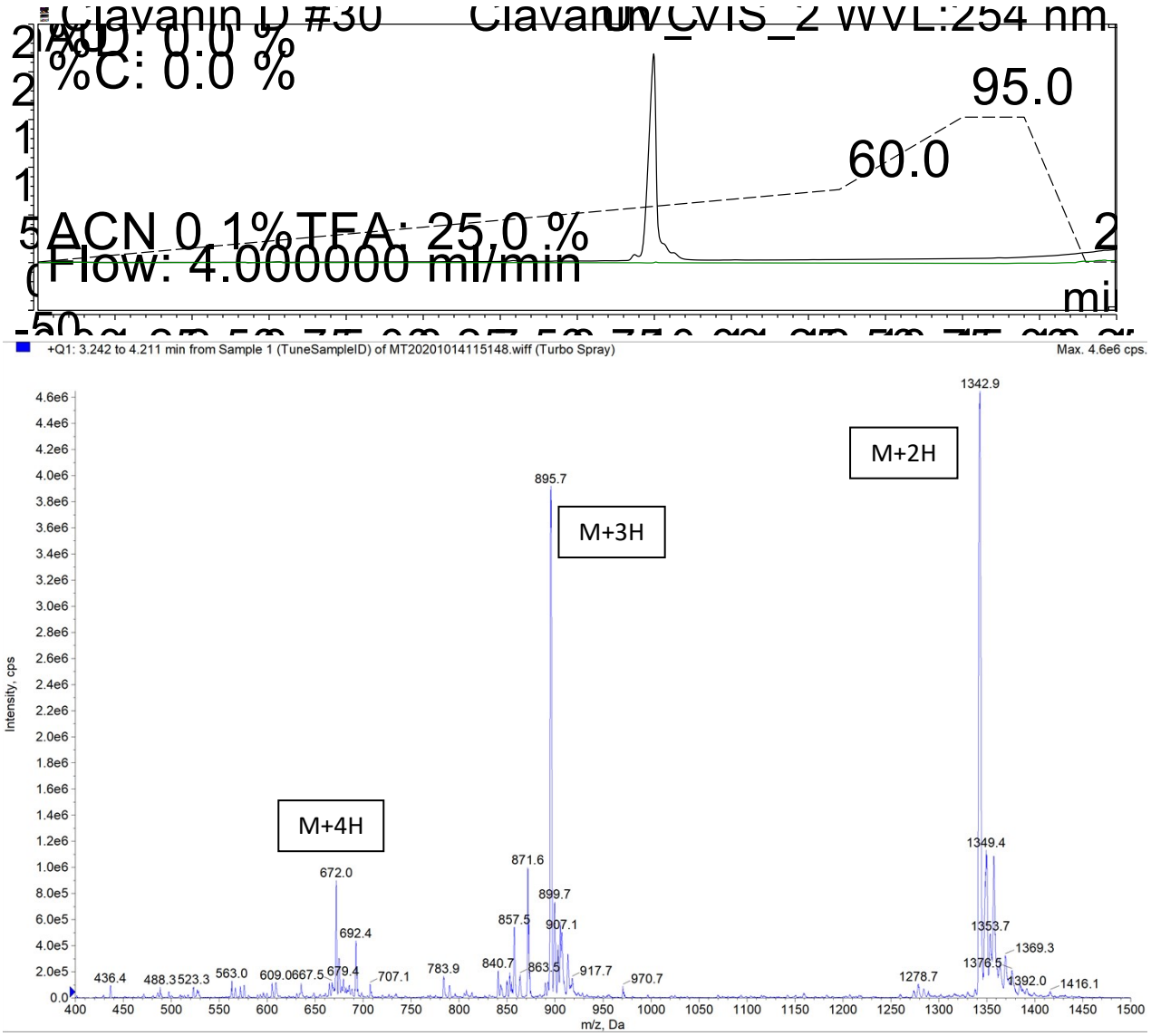


Figure S3. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin C (90.3% pure)

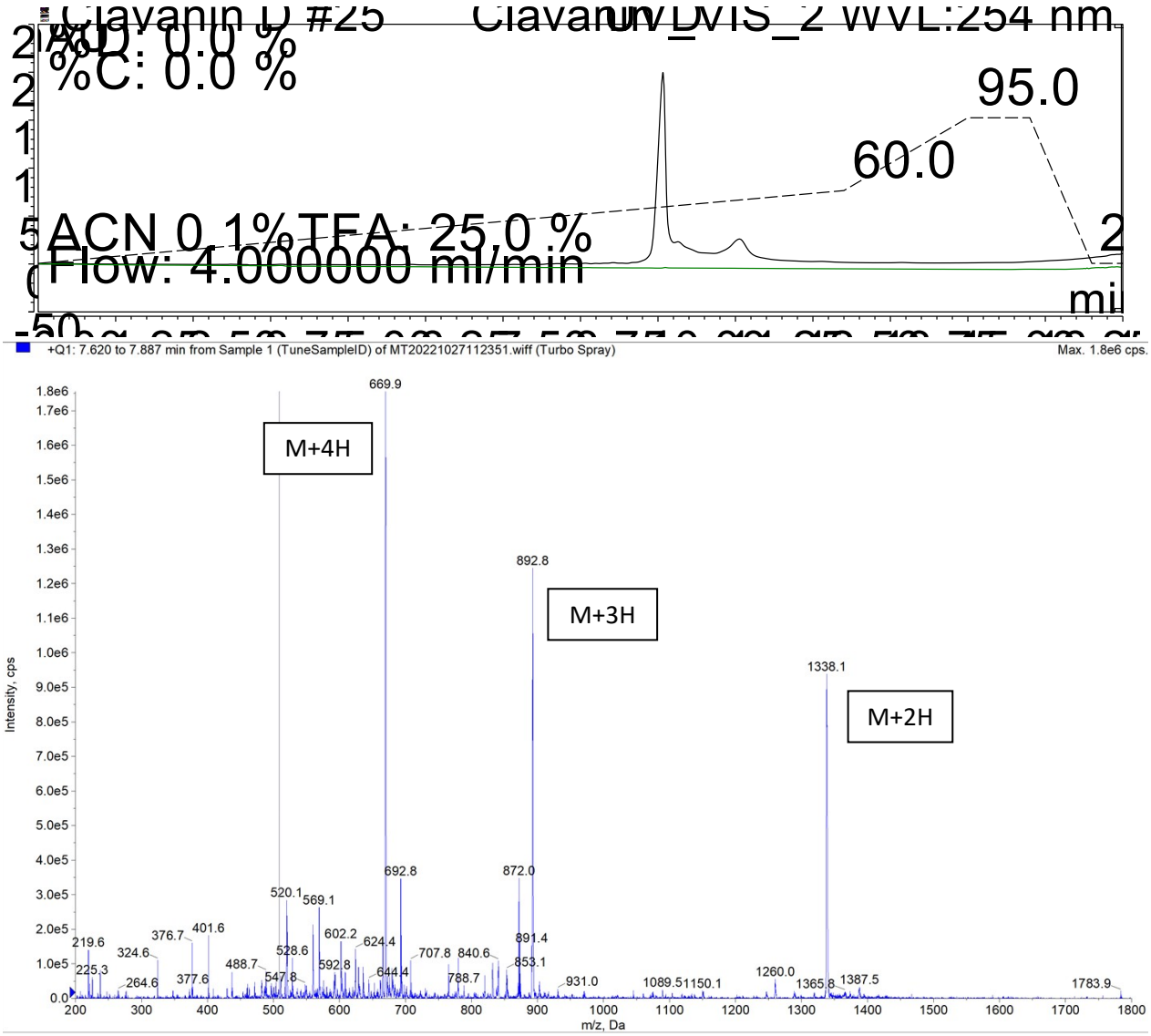


Figure S4. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin D (82.6%)

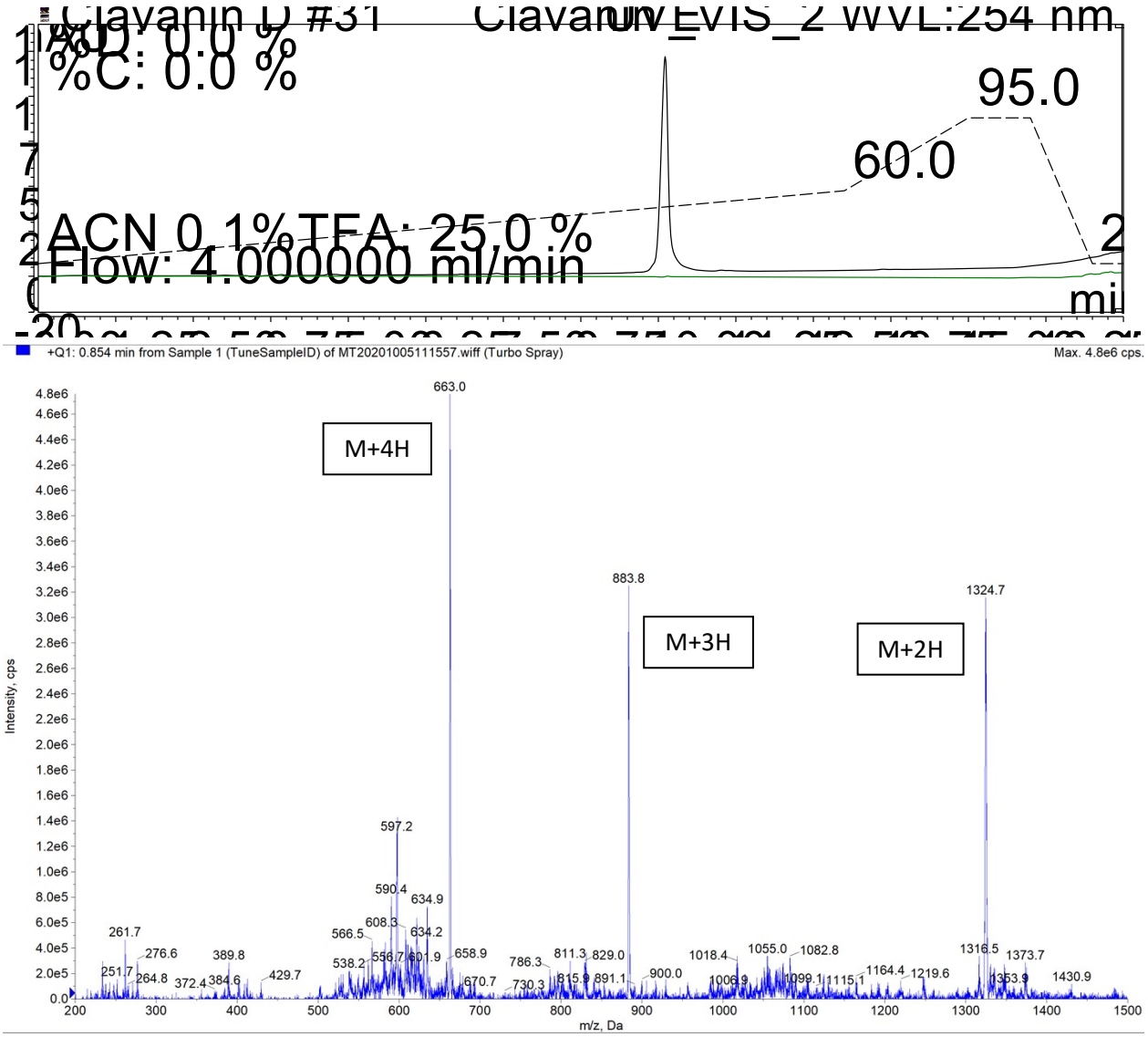


Figure S5. HPLC (top) and ESI-MS (bottom) profile of the purified Clavanin E (99.0%)

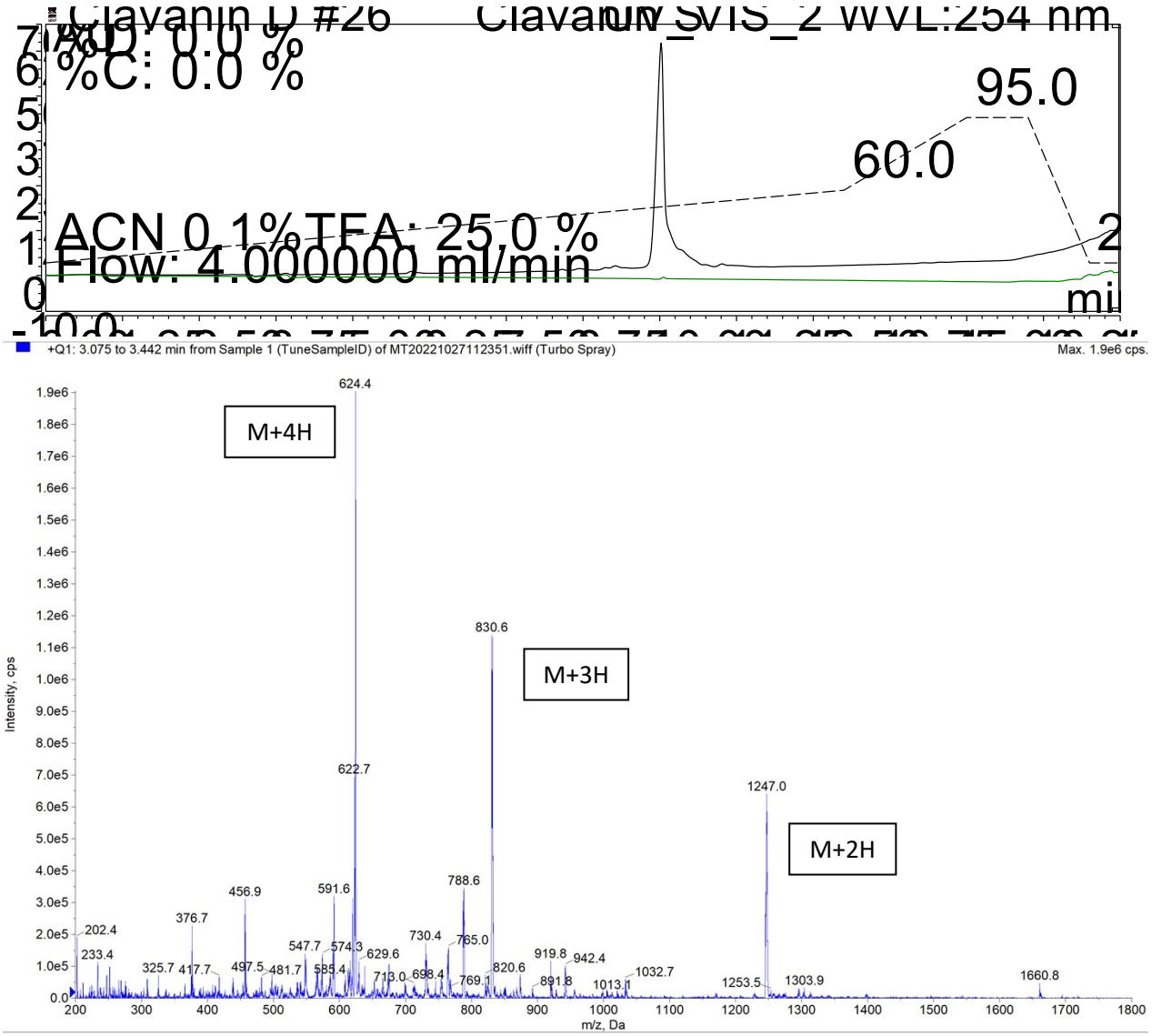
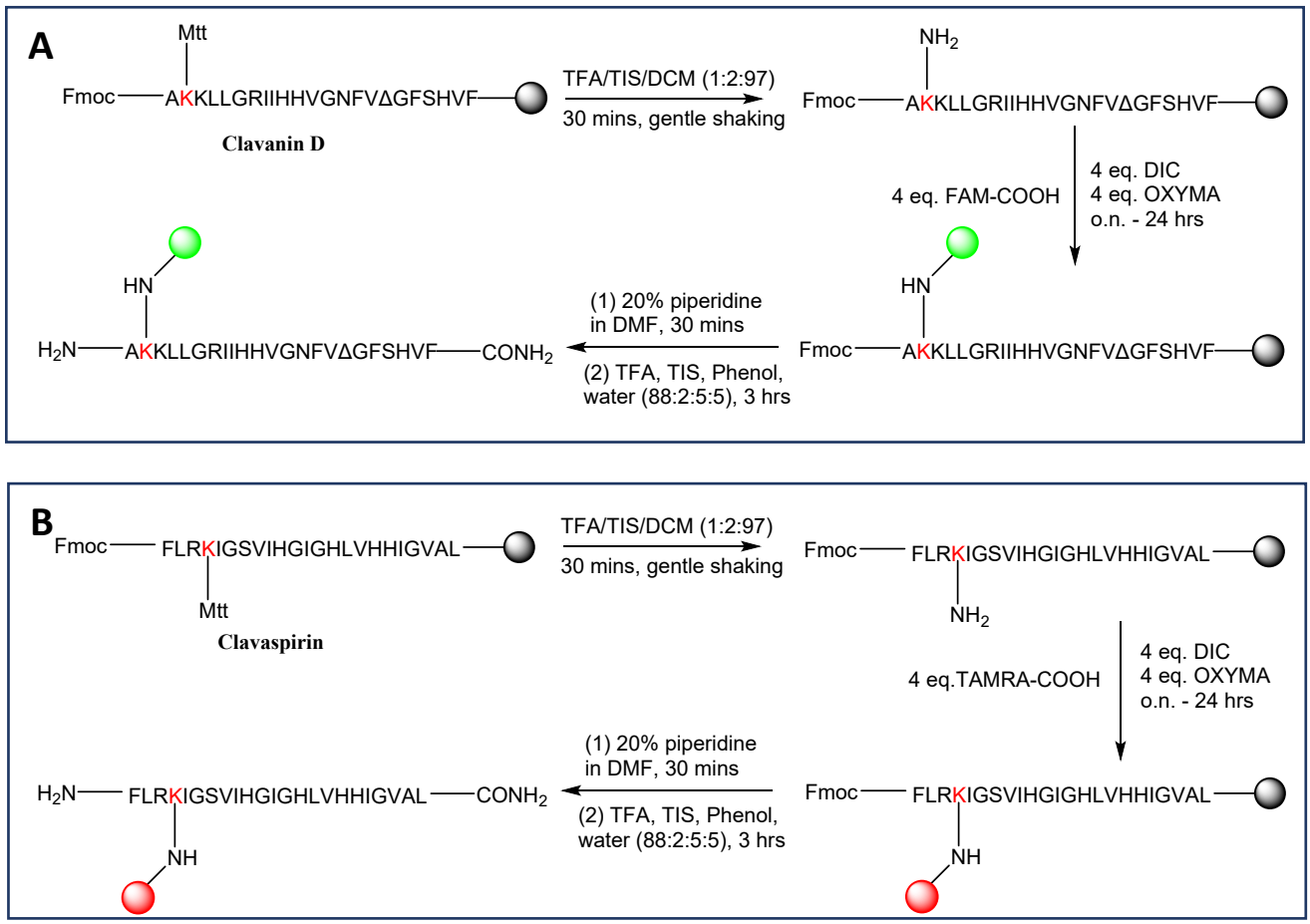


Figure S6. HPLC (top) and ESI-MS (bottom) profile of the purified Clavaspirin (Clavanin S) (89.9%)



Scheme S1. Synthetic scheme showing the synthesis of (A) ClavD-FAM and (B) ClavS-TAMRA. Each synthesis utilizes a Lys-Mtt in place of a Phe residue that can be selectively deprotected using 1% TFA.

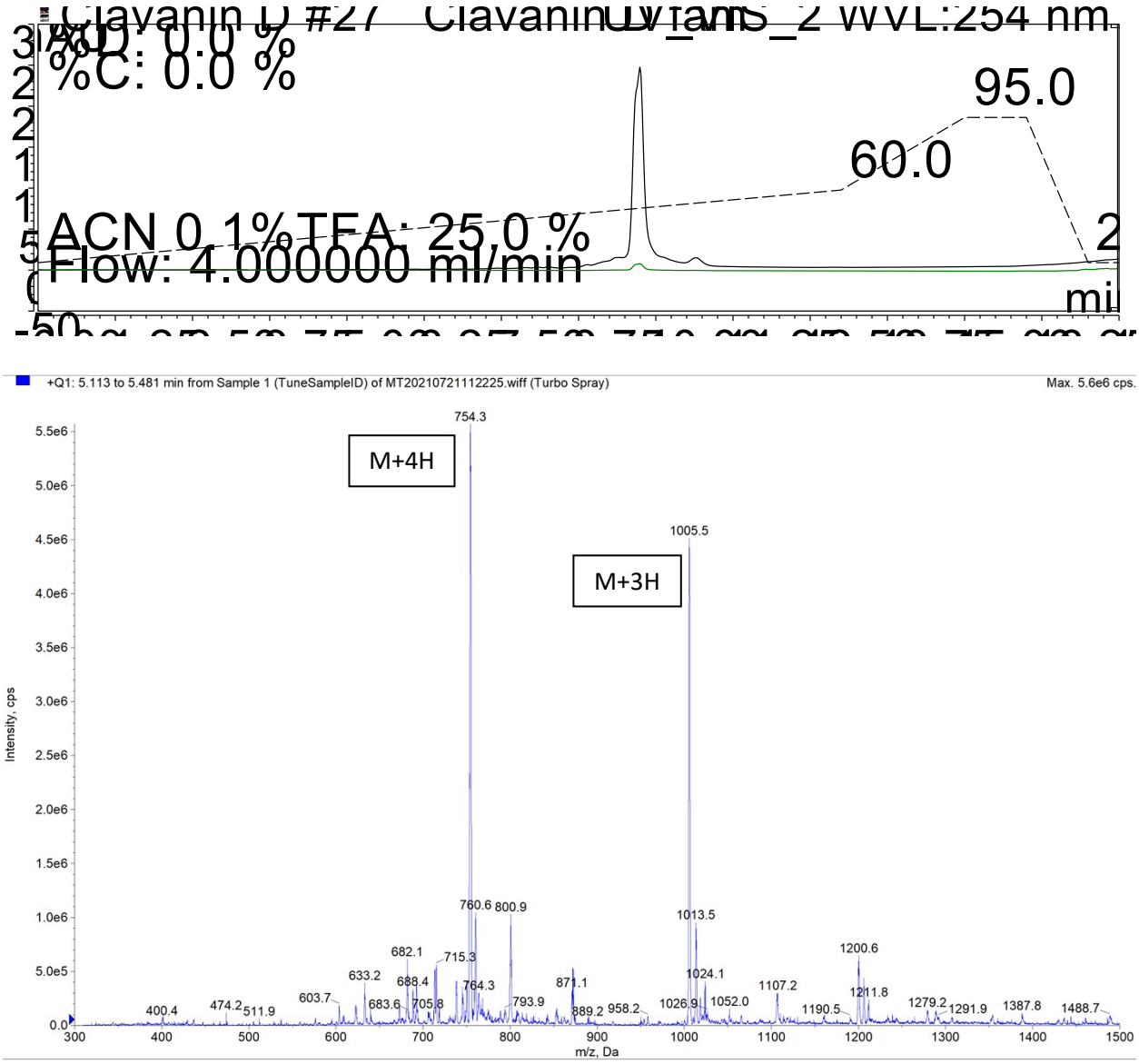


Figure S7. HPLC (top) and ESI-MS (bottom) profile of ClavD-FAM. The HPLC shows a broad peak on top due to the mixture of 5 and 6-carboxy isomers of fluorescein used in the synthesis

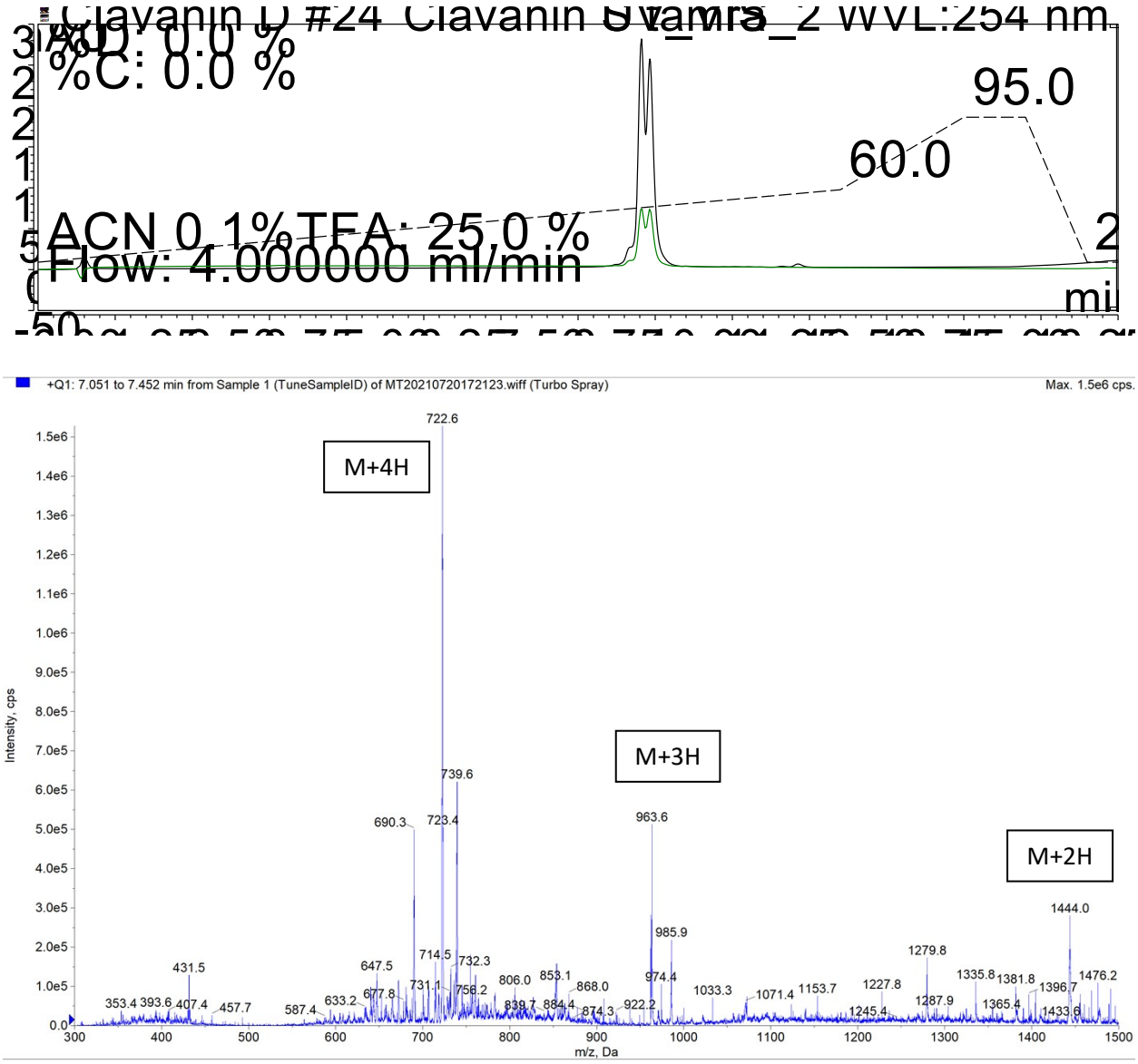


Figure S8. HPLC (top) and ESI-MS (bottom) profile of ClavS-TAMRA. The HPLC shows a splitting at the peak accounting for the 5 and 6-carboxy isomers of rhodamine used in the synthesis.

Table S1. Summary of calculated peptide mass for the Clavanins alongside the actual masses obtained from ESI-MS (in bold).

Peptide	Parent Mass	M+2H	M+3H	M+4H
Clavanin A	2666.2	1334.1	889.7	667.2
		1334.4	**890.4**	**667.8**
Clavanin B	2694.2	1347.6	899.1	674.5
		1347.4	**898.9**	**674.7**
Clavanin C	2683.2	1342.6	895.4	671.8
		1342.9	**895.7**	**672.0**
Clavanin D	2674.2	1338.1	892.4	669.6
		1338.1	**892.8**	**669.9**
Clavanin E	2646.2	1324.1	883.1	662.6
		1323.7	**883.8**	**663.0**
Clavaspirin	2492.0	1247.0	831.7	624.0
		1247	**830.6**	**624.4**
Clav D - FAM	3013.2	1507.6	1005.4	754.3
		-	**1005.5**	**754.3**
Clav S - TAMRA	2885.0	1443.5	962.7	722.3
		1444.0	**963.6**	**722.6**

CD Spectroscopy. We employed the use of CD spectroscopy in order to determine the secondary structure of the synthesized peptides. The purified peptides were prepared in 1:1 2,2,2-trifluoroethanol (TFE) and water at a concentration of 50 μM. The lipophilic solvent, TFE, is a known inducer of secondary structure in peptides and smaller proteins. Using this solvent, the hydrogen bonding between backbone amides and the solvent is reduced, which strengthens intramolecular hydrogen bonding that stabilizes secondary structures such as an α-helix or β-sheet.[2] Spectral scanning was done from 190-260 nm using a Jasco-J715 Spectropolarimeter and a 0.1 mm quartz cuvette. The resulting molar ellipticity vs wavelength graph is shown in Figure 1. The %helicity was calculated using the equation below.

$$\%\alpha - helix = -100\left(\frac{\Theta_{222} + 3000}{33000}\right) \quad (1)$$

Bacterial Culture Conditions. *E. cloacae* AR#0136 were grown in standard MHB media at either pH 5.5 or 7.4, following the tests done from previous works. The media was first prepared by dissolving the recommended amount according to manufacturer instructions. The pH was adjusted to 5.5 using 0.1 M HCl prior to autoclaving. No pH adjustments were made for the media at pH 7.4. Cells were incubated at 37° C with shaking at 234 rpm to mid-log phase (OD_{600} = 0.4-0.6) before each test.

Broth Microdilution Assay and MIC Determination. To get the minimum inhibitory concentration (MIC) of the AMPs, the broth microdilution assay was used.[3] Briefly, a bacterial culture was first grown according to the method mentioned above. The cells were then diluted to a final concentration of 1 x 10^6 CFU/mL prior to treating with the peptides. Prepare 2× the desired concentration of the peptides then dilute it serially across a 96-well plate. The last two wells do not contain any peptide and will serve as the growth control (GC) and the sterile control (SC), respectively. Finally, add the prepared bacterial culture into all the wells that contain the peptide and the GC. This will give the desired concentration of the peptide with the starting bacterial cell concentration of 5 x 10^5 CFU/mL. Incubate the whole plate at 37° C for 18-20 h. MIC was determined as the lowest concentration of peptide at which no bacterial growth could be visually observed.

Checkerboard Assay and FIC calculation. A standard checkerboard assay[4] was performed to assess synergistic activity between peptides. Briefly, the bacterial culture is prepared exactly as discussed above. The peptides were prepared at 4× the desired concentration and was serially diluted in separate 96-well plates with one peptide serially diluted on the columns of the first plate and the other peptide on the rows of the second plate. The dilutions on the second plate were then transferred to the first plate which makes the concentration of each well at 2× the desired

peptide concentration. The bacterial culture is added last to the 96-well plate prior to incubation at 37°C for 18-20 h. The plate is read visually and the wells that show no bacterial growth were recorded. The formula below was used to calculate the fractional inhibitory concentration index (FIC_{index}).[5]

$$FIC_{index} = FIC_A + FIC_B \quad (2)$$

Where, $FIC_x = \frac{MIC_{x\ in\ the\ combination}}{MIC_{x\ alone}}$, and the MIC's used in this equation are the MIC's determined in the same plate as the checkerboard to make sure all determinations undergo the same treatments. Synergy is defined as having an FIC_{index} of ≤ 0.5. An FIC_{index} between 0.5 and 1 signifies additivity, between 1-4 means indifference, and > 4 is antagonism.[5,6] FIC indices reported here are the mode of three trials that were done in duplicate.

Calculation of the Loewe Additivity Response. The Loewe additivity model is based on the assumption that when a drug or a compound interacts with itself, then there will be no effect on the response or simply a non-interaction response will be observed. This can be expressed using the following equation:

$$1 = \frac{x_1}{x_1^*} + \frac{x_2}{x_2^*} \quad (3)$$

Where, x_n is the dose in combination that gave the desired response, and x^*_n is the individual doses to which the same response is observed.[7] It follows then that if no interaction is observed between the two drugs, all possible dose combinations would produce the exact same response, which is one. This equation is largely similar to the FIC index used in the checkerboard assay.

Table S2. FIC data obtained from the checkerboard assay and 1:1 combination broth microdilution assay. Reported here are the lowest FIC values obtained in three trials.

Peptide	MIC_A (μM)	MIC_B (μM)	MIC_{combo} (μM)	FIC index	Interpretation
ClavA + ClavB	64	64	16/16	0.5	Synergy
ClavA + ClavC	64	4	8/4	1.125	Indifferent
ClavA + ClavD	64	4	8/4	1.125	Indifferent
ClavA + ClavE	64	4	4/4	1.0625	Indifferent
ClavA + ClavS	128	4	16/4	1.125	Indifferent
ClavB + ClavC	64	4	8/4	1.125	Indifferent
ClavB + ClavD	64	4	8/4	1.125	Indifferent
ClavB + ClavE	64	4	4/4	1.0625	Indifferent
ClavB + ClavS	128	4	16/4	1.125	Indifferent
ClavC + ClavD	4	4	4/4	2.0	Indifferent
ClavC + ClavE	4	4	4/4	2.0	Indifferent
ClavC + ClavS	16	4	4/4	1.25	Indifferent
ClavD + ClavE	4	4	4/4	2.0	Indifferent
ClavD + ClavS	8	4	2/1	0.5	Synergy
ClavS + ClavE	8	4	0.5/2	0.562	Additive
ClavD-FAM + ClavS-TAMRA	8	4	2/1	0.5	Synergy

Time-Kill Kinetics Assay.[4] *E. cloacae* AR#0136 bacterial culture was prepared using the same procedure as described above. A 350 μL of bacterial cells at 5 x 10^5 CFU/mL concentration were added to culture tubes containing 350 μL of 2× the desired concentration of each peptide. At 0, 1, 2, and 4 h time points, a 10 μL aliquot from each tube was taken and diluted to the desired concentration. Then, a 100 μL aliquot from each dilution were spread on LB Agar plates. Colonies were counted manually after overnight incubation of the plates at 37° C. The colony forming units (CFU) was then calculated from the number of colonies using the equation below:

$$\frac{CFU}{mL} = \frac{\#\ of\ colonies\ x\ 10}{10^{-x}} \quad (4)$$

Where x is the dilution of the solution that was plated (i.e., if the 10^{-2} dilution showed 100 colonies, the CFU/mL would be 10^4). The plotted curves show bacterial titer data obtained from three independent trials.

Calculation of Bliss Independence Response. The Bliss response or the non-interaction response was calculated using the formula below:

$$y_{bliss} = y_A + y_B - y_A y_B \quad (5)$$

Where y_{bliss} is the non-interaction response or the expected response if drug A and drug B does not interact with each other, y_A is the response of drug A alone, and y_B is the response of drug B. Any departure from the y_{bliss} response implies an interaction between the two drugs. There is synergy if the response is greater than y_{bliss} and antagonism if the response is lower than y_{bliss}.[7] Further, a deviation that is ≥ 2 log decrease in CFU in a time-kill assay is considered a strong synergy and less than that is considered weak synergy.

Confocal Microscopy. Bacteria was grown according to the same protocol outline above to an OD_{600} between 0.4 and 0.6 in MHB media at pH 5.5. Cells were washed and resuspended in the same media to remove cellular debris that might have accumulated. The fluorescently labeled peptides were prepared at 2× the desired concentration and was then mixed in a 1:1 ratio with the bacterial culture to get the desired concentration. The mixture was then allowed to incubate at 37° C with gentle mixing for 30 min. After incubation, the cells were washed to remove excess tagged peptide floating in the solution in order to reduce background noise. The cells were then resuspended in sterile nano pure water. After washing, 10 μL of the cell mixture was plated on microscope slides containing 1.5% agarose pads to immobilize the cells. Cells were then imaged immediately with a Nikon A1R spectral confocal microscope using a 60× oil immersion lens.

Transmission Electron Microscopy (TEM). TEM was accomplished with the help of the Bioscience Electron Microscopy Laboratory (BEML) at UConn, specifically Dr. Maritza Abril and Dr. Xuanhao Sun. Sample preparation was achieved via an agarose enrobing method. Overnight cultures of *E. cloacae* 0136 were diluted 1:100 and was allowed to grow up to an OD_{600} of 0.4 in

a 37°C incubator with shaking. For cells treated with the AMPs, sub-MIC concentrations were added after the 1:100 dilution. The cells were then centrifuged and washed with PBS. The bacterial samples were fixed with a fixative (2% glutaraldehyde and paraformaldehyde in 0.1 M sodium cacodylate buffer + 3 mM $MgCl_2$, pH 7.4) for 30 min. The cells were spun down at 10,000 rpm for 1 min and the fixative was removed. Cell pellets were resuspended in 500 µL of 0.1 M sodium cacodylate buffer. Melted 2% Type IX agarose (Sigma A5030) was then layered and the tubes were immediately centrifuged at 10,000 rpm for 1 min. Let solidify for 1 h in a bucket with ice in the refrigerator. The agarose plug was then carefully removed and placed in a petri dish with buffer. The excess agarose was removed and the enrobed cells were cut into 1 mm pieces using a microtome. Cell pellets were then rinsed with 0.1 M sodium cacodylate buffer + 3 mM $MgCl_2$ at pH 7.4 (3 × 15 min). The cells were post fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 60 min. The cells were then washed with sodium cacodylate buffer (3 × 15 min) and rinsed with maleate buffer pH 5.2 (1 × 15 min). The cells were further fixed by 1% uranyl acetate in maleate buffer for 60 min and washed with maleate buffer (3 × 5 min). The cells were dehydrated by the following steps: 25% ethanol (2 × 10 min); 50% ethanol (2 × 10 min); 70% ethanol (2 × 10 min); 95% ethanol (2 × 10 min); 100% ethanol (3 × 15 min); and 100% propylene oxide (3 × 15 min). The cells were then infiltrated by the following steps: 2:1 propylene oxide:spurr resin (2 × 30 min); 1:1 propylene oxide:spurr resin (2 × 30 min and overnight); 100% Glauert resin (6 × 60 min). The spurr was polymerized with embedded cells in 70°C oven for 1–2 days and then 90 nm sections were cut by Reichert-Jung Ultracut E. The samples were further stained with 2% uranyl acetate and 2.5% Sato's lead citrate and imaged under 80 KV using a FEI Tecnai Biotwin Transmission electron microscope.

References:

1. Kuipers, B. J. H. & Gruppen, H. Prediction of Molar Extinction Coefficients of Proteins and Peptides Using UV Absorption of the Constituent Amino Acids at 214 nm To Enable Quantitative Reverse Phase High-Performance Liquid Chromatography−Mass Spectrometry Analysis. *J. Agric. Food Chem.* **55**, 5445–5451 (2007).
2. Sonnichsen, F. D., Van Eyk, J. E., Hodges, R. S. & Sykes, B. D. Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. *Biochemistry* **31**, 8790–8798 (1992).
3. 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.* **3**, 163–175 (2008).
4. *Antimicrobial Susceptibility Testing Protocols*. (CRC Press, 2007). doi:10.1201/9781420014495.
5. Doern, C. D. When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing. *J. Clin. Microbiol.* **52**, 4124–4128 (2014).
6. Bonapace, C. R., Bosso, J. A., Friedrich, L. V. & White, R. L. Comparison of methods of interpretation of checkerboard synergy testing. *Diagn. Microbiol. Infect. Dis.* **44**, 363–366 (2002).
7. Tang, J., Wennerberg, K. & Aittokallio, T. What is synergy? The Saariselkä agreement revisited. *Front. Pharmacol.* **6**, (2015).