Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Introduction of Noncanonical Amino Acids into the Lasso Peptide Microcin J25

Frank J. Piscotta[1], Jeffery M. Tharp[3], Wenshe R. Liu[3]*, and A. James Link[1,2]*

Departments of [1]Chemical and Biological Engineering and [2]Molecular Biology, Princeton University, Princeton, NJ 08544 and [3]Department of Chemistry, Texas A&M University, College Station, TX, 77843

*to whom correspondence should be addressed: ajlink@princeton.edu or wliu@chem.tamu.edu

Methods

General. Oligonucleotides for this study were purchased from IDT. Restriction enzymes and T4 DNA ligase were from New England Biolabs. PicoMaxx DNA polymerase (Agilent) was used for all PCRs. ncAAs were purchased from Chem Impex and used as received.

Plasmids and primers. pEVOL-pylT-N346A/C348A, the plasmid containing the mutated pyrrolysine synthetase and the corresponding pyrrolysine tRNA has been previously described.[1] The pEVOL plasmid contains a single constitutively-expressed copy of the *pylT* tRNA gene as well as two copies of the mutant *pylS* synthetase gene. One of these copies is constitutively expressed while the other is under the control of an arabinose promoter. The plasmids pFP5, pFP7, pFP9, and pFP11, containing the gene cluster responsible for the synthesis, maturation, and export of the noncanonical amino acid (ncAA) -substituted MccJ25 variants, were based on the pJP73 plasmid. pJP73 is identical to the previously described pJP3[2] except that it also contains a copy of the *lacI*^{q} repressor in *cis* for tight expression of the *mcjA* gene. In this construct, the *mcjA* gene, encoding the MccJ25 precursor, is under control of an IPTG-inducible T5 promoter while the *mcjBCD* operon is constitutively expressed.[2] Within the *mcjA* gene, each plasmid has a single amber codon (TAG) mutation introduced using site-directed mutagenesis. The mutant *mcjA* genes were constructed by overlap PCR and cloned into pJP73 using the unique *Nco*I and *Hin*dIII restriction sites in the plasmid. A list of plasmids and primers used in this study can be found in Table S1.

MccJ25 Expression. For small-scale test expressions, BL21(DE3) harboring both the pEVOL plasmid and a MccJ25 plasmid were grown overnight in 5 mL LB (10 g tryptone, 5 g of yeast extract, 10 g NaCl per liter) containing 100 mg/L ampicillin and 25 mg/L chloramphenicol at 37 °C in an incubating shaker (250 rpm). An overnight culture (500 µL) was used to inoculate 50 mL of LB containing ampicillin and chloramphenicol, and this was grown at 37 °C with shaking to an OD_{600} of 0.5. At this point, the ncAA was added to a final concentration of 2 mM, followed by the addition of isopropyl-β-D-galactoside (IPTG) and arabinose to final concentrations of 1 mM and 0.2%, respectively. The culture was then grown for 20 hours at 37 °C with shaking. For large-scale expression of the F10 *m*-bromoPhe variant and F19 *m*-chloroPhe variant, 5 mL of the overnight culture in LB was added to 500 mL of M9 minimal media (3 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 0.25 g/L NaCl, 1.5 mg/L $CaCl_2$, 1 mM $MgSO_4$, 0.2% glucose, 0.00005% thiamine, 0.04 g/L of each aa). As for the smaller cultures, the M9 culture was induced at OD_{600} of 0.5 and allowed to grow for 20 h. M9 media was used for the larger cultures in order to simplify the purification of these peptides.

Solid Phase Extraction of Supernatant. Following expression, the cells were pelleted by centrifugation (10 min, 8000 x *g*, 4 °C). Though there are no reports of active MccJ25 variants being retained in the cell, we checked the cell pellets for the four I13-substituted variants for the presence of lasso peptides by mass spectrometry. There was no evidence of lasso peptide retention in the cell. The supernatant was extracted using a Strata C8 solid phase extraction column (500 mg packing/3 mL for small scale, 1 g packing/6 mL for large scale, Phenomenex). The column was primed with two column volumes of methanol and washed with two column volumes of ultrapure water. The supernatant was then added to the column using a peristaltic pump. The column was washed once more with two column volumes of ultrapure water, followed by elution with methanol (4 x 0.5 mL for small-scale experiments and 6 x 1 mL for large-scale).

HPLC and MALDI-TOF mass spectrometry. To prepare the SPE eluates for HPLC analysis, the methanol was evaporated to dryness under vacuum in a speedvac (Labnet DyNAvap centrifugal evaporator). The residue was resuspended in a 50% water, 50% acetonitrile (ACN) solution containing 0.1% trifluoroacetic acid (TFA) such that the samples were 50x concentrated relative to the original culture supernatant. The samples (5 µL) were analyzed via HPLC (Agilent) using a previously described gradient.[3] HPLC peaks unique to the ncAA-substituted MccJ25 variants were collected and subjected to subsequent MALDI-TOF mass spectrometry. To prepare samples (either extracted culture supernatants or HPLC-purified peaks) for MALDI, the samples were mixed with an equal volume (1 µL) of alpha-cyano-4-hydroxycinnamic acid matrix (2.5 mg/mL in 50/50 water/ACN with 0.1 % TFA) and analyzed on a MALDI-TOF mass spectrometer (ABSciex 4800 Plus MALDI TOF/TOF Analyzer).

Determination of relative production. Production of 15 ncAA-substituted MccJ25 variants and wild-type MccJ25 was characterized by peak area in the HPLC trace. Before determining variant production relative to wild-type, areas were normalized and background-subtracted against a negative control using peaks common to all traces at 16.1 min and 16.7 min (see Figure S1). For MccJ25 variant peaks that show no overlap with the rest of the HPLC trace, a ratio R = (16.1 min peak area of sample)/(16.1 min peak area of negative control) was determined. The peak area corresponding to the MccJ25 variant was divided by this value R to give a normalized area. In the cases where the MccJ25 peak overlapped with either the 16.1 min or 16.7 min peak (including wild-type MccJ25, which overlaps with the 16.1 min peak), the areas of the non-overlapping peaks were used to calculate the ratio R. This value of R was used to calculate a normalized peak area. The area of the overlapping peak present in the negative control was subtracted to find the area related to the lasso peptide. Finally, relative production was calculated as the ratio of the normalized variant area to normalized wild-type area.

Antimicrobial assays. To test the antimicrobial activity of the MccJ25 variants, a spot-on-lawn assay was used as previously described.[2] Briefly, 5 mL of M63 soft agar inoculated with approximately 10^7 cells/mL of exponential phase *Salmonella newport* was overlaid on an M63 agar plates. Drops of C8-extracted culture supernatant resuspended in 50% acetonitrile (5 µL, 20x concentrated relative to original supernatant) as well as positive and negative controls (wild type MccJ25 and 50% acetonitrile solution, respectively) were spotted on the *Salmonella newport* layer, and the plates were incubated overnight at 37 °C.

Spot on lawn assays were also performed to determine the last active dilution of the MccJ25 variants. M63 agar plates overlaid with M63 soft agar containing *Salmonella newport* were prepared, and C8 extracted culture supernatant resuspended in 50% acetonitrile was serially diluted two-fold in in LB, starting with a sample that was 1 x concentrated relative to supernatant. These serial dilutions were spotted (5 µL) on the M63 agar plates and incubated overnight at 37 °C. The last dilution producing a zone of inhibition was taken as relative measure of antimicrobial activity.

To determine the minimum inhibitory concentration (MIC) of the F10 *m*-BrPhe and F19 *m*-ClPhe MccJ25 variants, the peptides were purified via HPLC, and the collected fractions were pooled together and lyophilized. The resulting powder was resuspended in ultrapure water, and the concentrations were determined by measuring the absorbance at 280 nm via NanoDrop (Thermo Fisher Scientific) and by the BCA assay (Pierce). These two methods gave good agreement, and are consistent with the estimate of concentration from the HPLC peak area. The same procedure was followed to generate pure wild-type MccJ25. Wild-type MccJ25 contains two tyrosine residues, so an extinction coefficient of 2560 $M^{-1}cm^{-1}$ was used. Since *m*-BrPhe also has absorbance at 280 nm (Figure S6), an extinction coefficient of 2920 $M^{-1}cm^{-1}$ was used for the *m*-BrPhe substituted peptide. The concentration of the *m*-ClPhe substituted variant was measured only by BCA assay because, as mentioned above, the BCA assay and A_{280} methods give comparable results. Resuspended peptides were diluted to known concentrations in LB, and 5 µL of these samples were spotted on M63 plates overlaid with M63 soft agar containing *Salmonella newport* as described above. The lowest concentration still giving a visible zone of growth inhibition was taken as the minimal inhibitory concentration. The spot assays were repeated at least four times, and representative results are given in Figure 2.

References

1 Y. S. Wang, X. Q. Fang, A. L. Wallace, B. Wu and W. S. R. Liu, *J. Am. Chem. Soc.*, 2012, **134**, 2950.

2 S. J. Pan, W. L. Cheung and A. J. Link, *Protein Expression and Purification*, 2010, **71**, 200.

3 W. L. Cheung, S. J. Pan and A. J. Link, *J. Am. Chem. Soc.*, 2010, **132**, 2514.

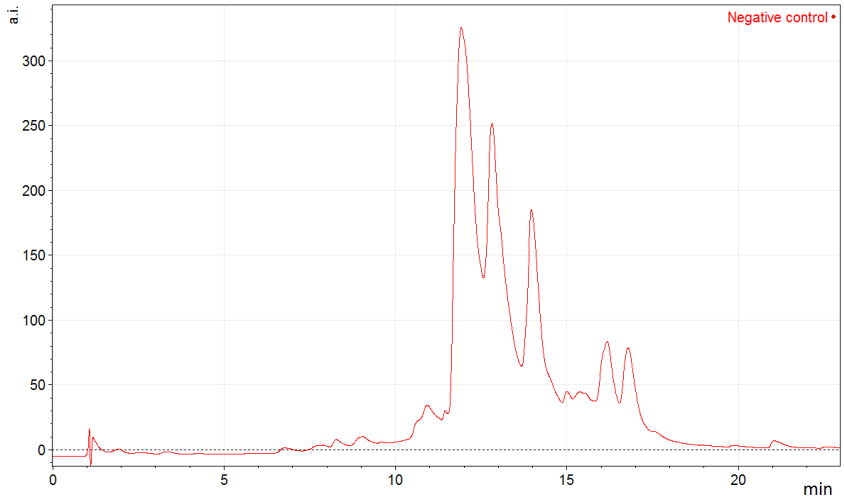
Supplementary Table

Table S1: Plasmids and primers used in this study

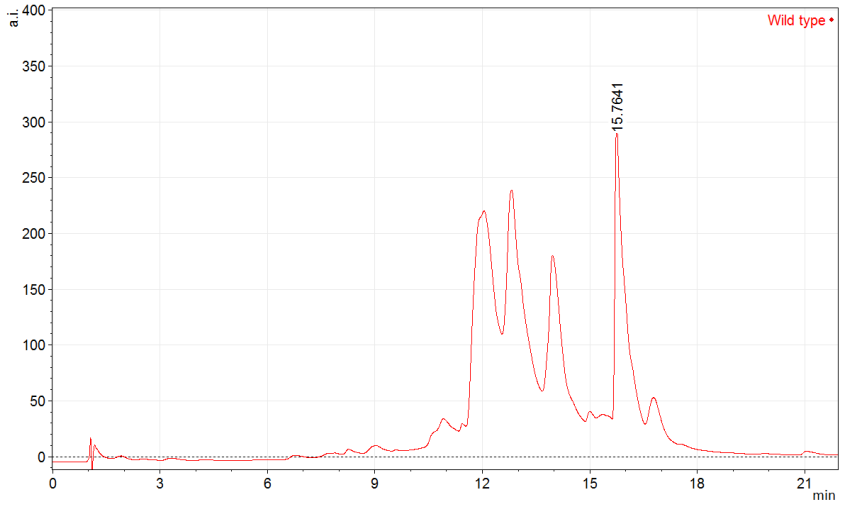
Plasmid or Primer	Description
Plasmid	
pJP3	MccJ25 gene cluster in pQE60
pJP73	MccJ25 gene cluster in pQE80
pFP5	MccJ25 gene cluster in pQE80 with V6TAG mutation
pFP7	MccJ25 gene cluster in pQE80 with F10TAG mutation
pFP9	MccJ25 gene cluster in pQE80 with I13TAG mutation
pFP11	MccJ25 gene cluster in pQE80 with F19TAG mutation
pEVOL-pylT-N346A/C348A	Pyrrolysine tRNA and mutated pylRS for UAA incorporation
Primer	
XhoI_F	GGCGTATCACGAGGCCCTTTCGTCTTCACC
F10TAG_R	CACCTAATACTCAGGCACATGTCCTGCACCACCT
F10TAG_F	ACATGTGCCTGAGTAT**TAG**GTGGGGATTGGTAC
V6TAG_R	GGCTAATGTCCCGCACCACCTTTTGTGAGTTGCG
V6TAG_F	GGTGCGGGACAT**TAG**CCTGAGTATTTTGTGGG
I13TAG_R	TCGGTGTGCCCTACCCAACAAAATACTCAGGC
I13TAG_F	GTTGGG**TAG**GGCACACCGATATCTTTCTATGGC
F19TAG_R	CATACTAAGATATAGGTGTGCCAATCCCC
F19TAG_F	GGCACACCTATATCT**TAG**TATGGCTGAAGC
F19TAG_R2	GCAGTAACGGATCATTTTGCACACTCCC
Mccj25_R	GGAGTTCTGAGGTCATTACTGGATCTATCAACAGG

*TAG in bold represents amber codons inserted into *mcjA*

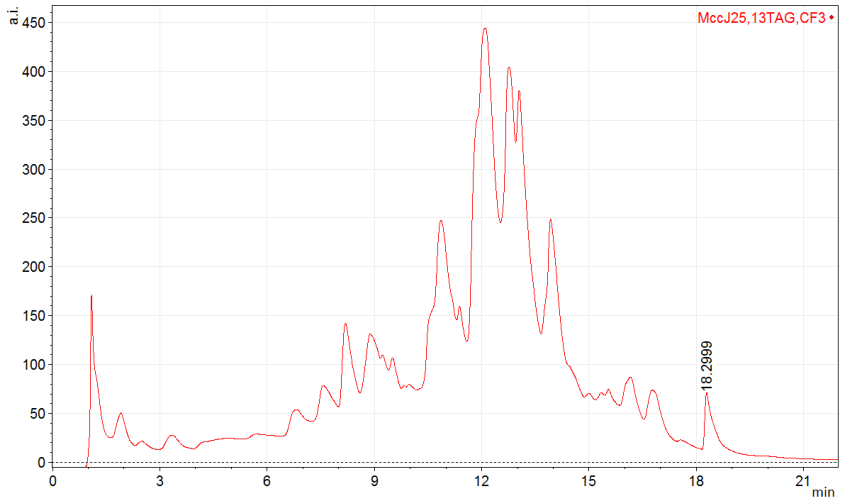
Supplementary Figures



Negative control (no *mcjA*)

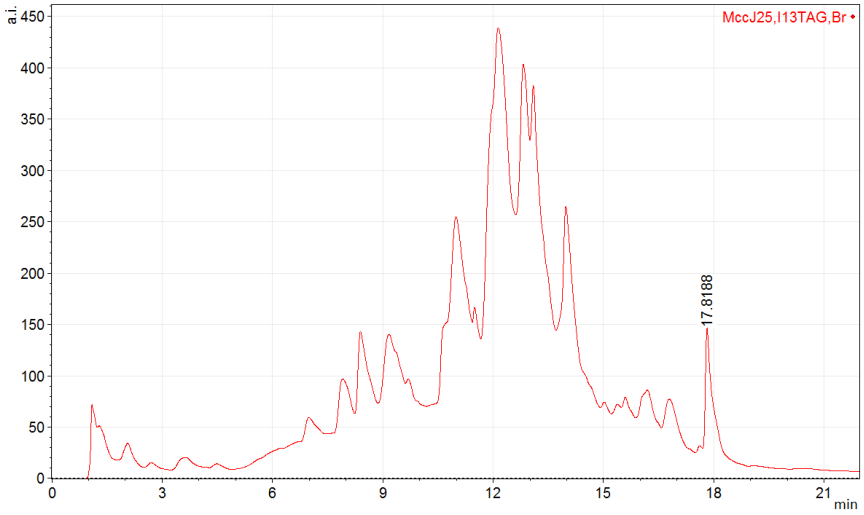


Wild-type MccJ25

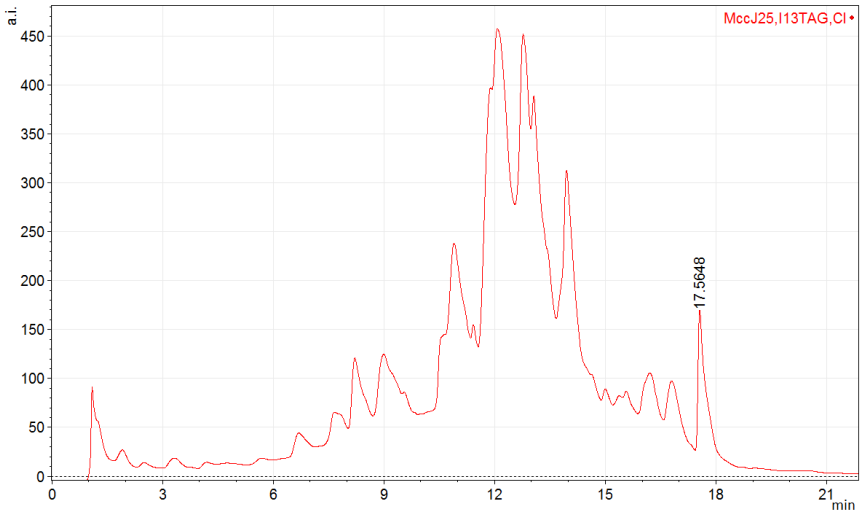


I13 *m*-CF_3Phe MccJ25

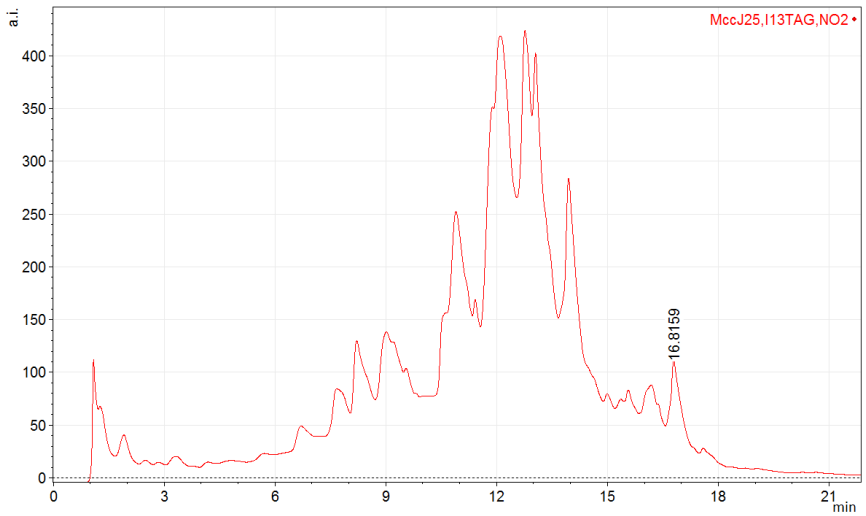
Figure S1: continued on next page.



I13 *m*-BrPhe MccJ25

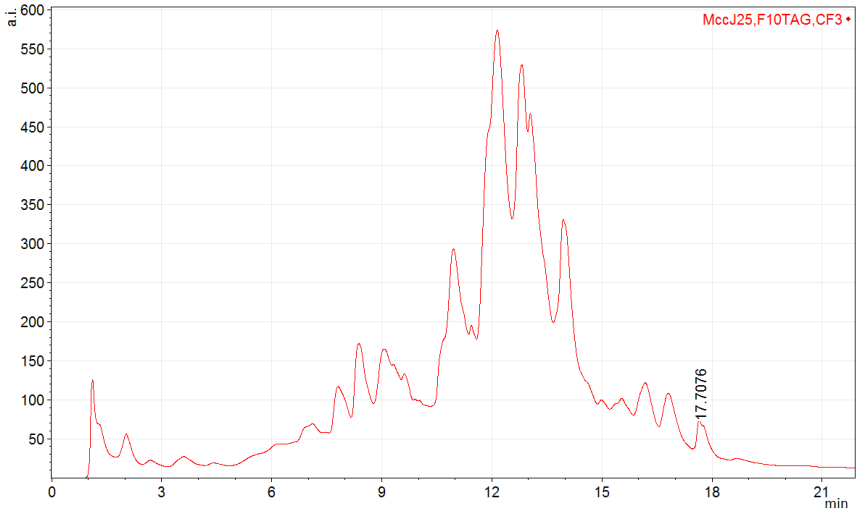


I13 *m*-ClPhe MccJ25

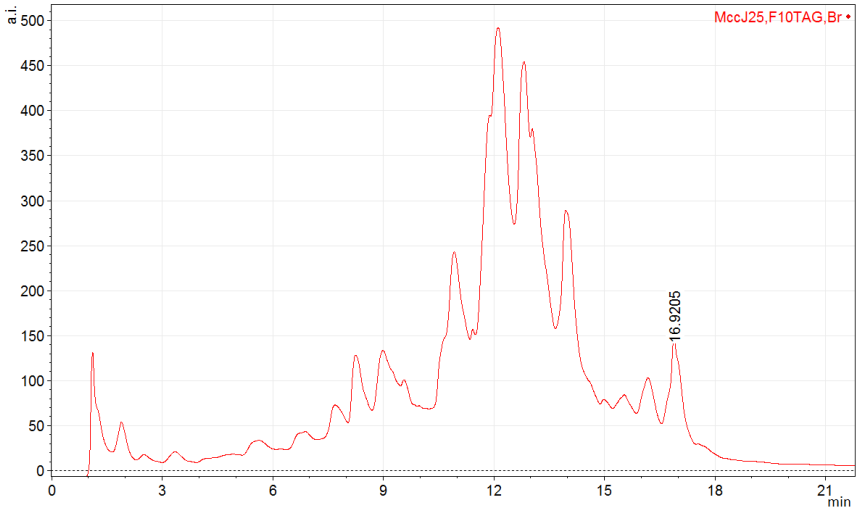


I13 *m*-NO_2Phe MccJ25

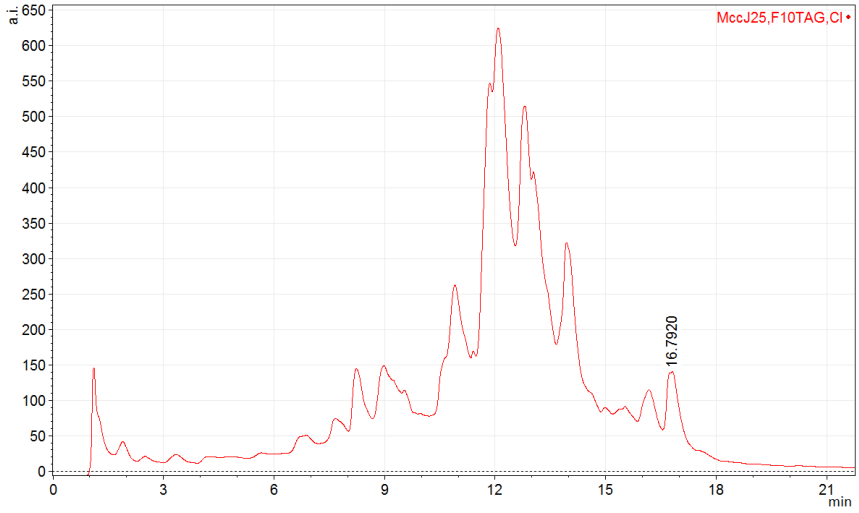
Figure S1: continued on next page.



F10 *m*-CF_3Phe MccJ25

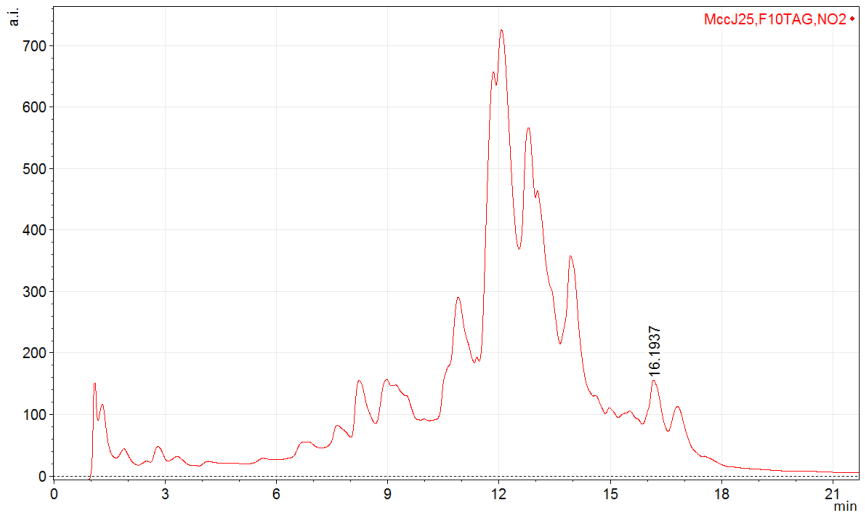


F10 *m*-BrPhe MccJ25

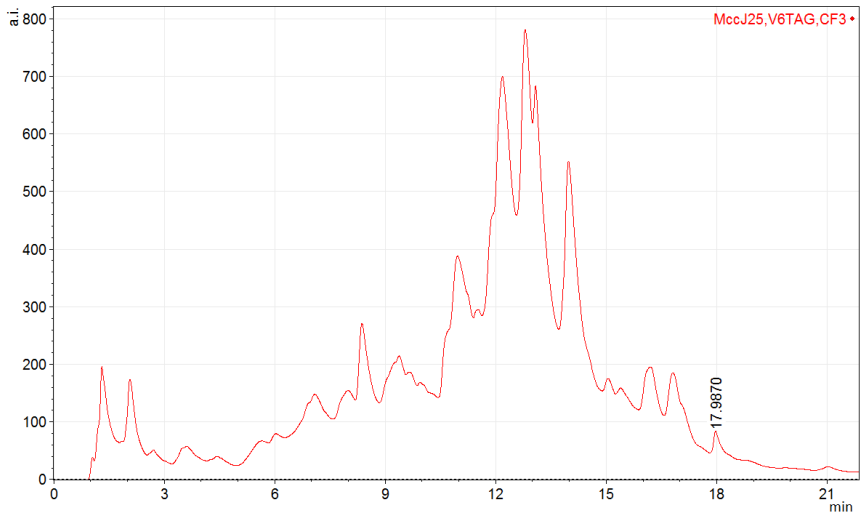


F10 *m*-ClPhe MccJ25

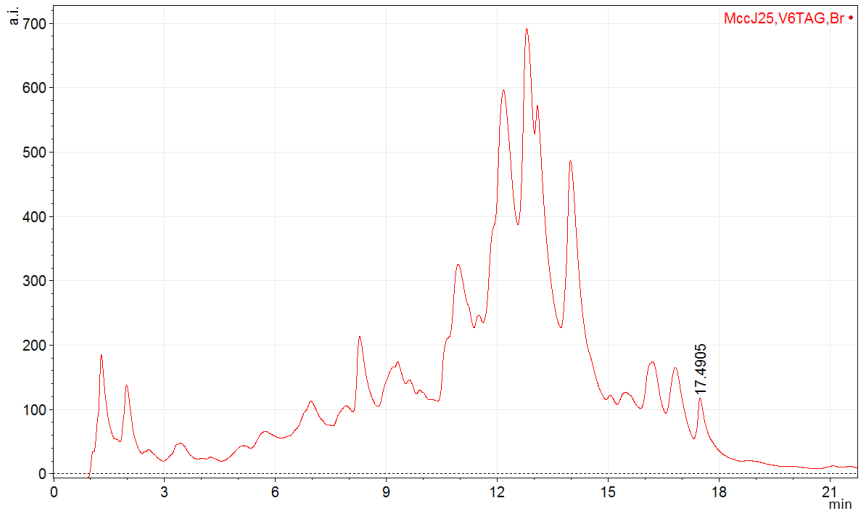
Figure S1: continued on next page.



F10 *m*-NO_2Phe MccJ25

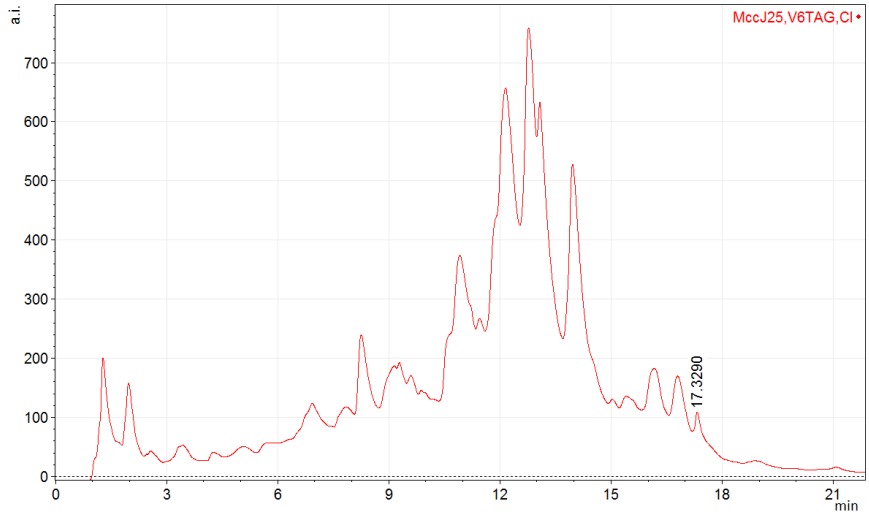


V6 *m*-CF_3Phe MccJ25

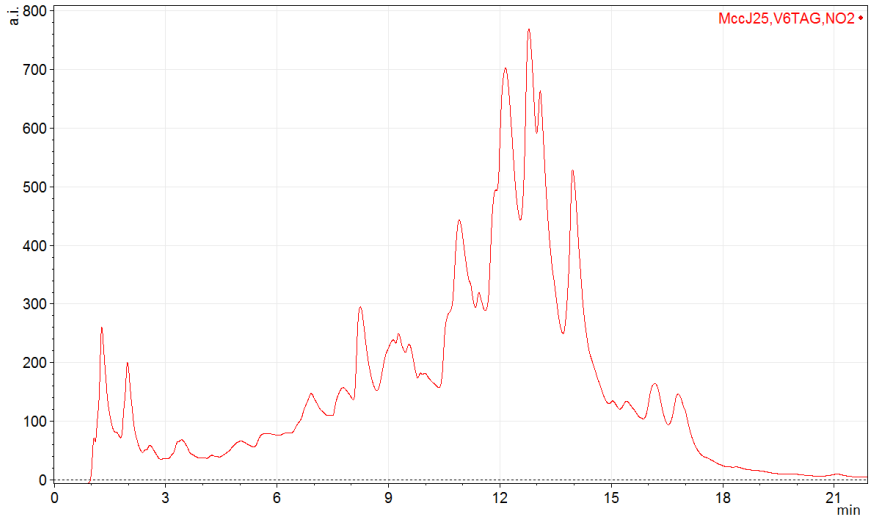


V6 *m*-BrPhe MccJ25

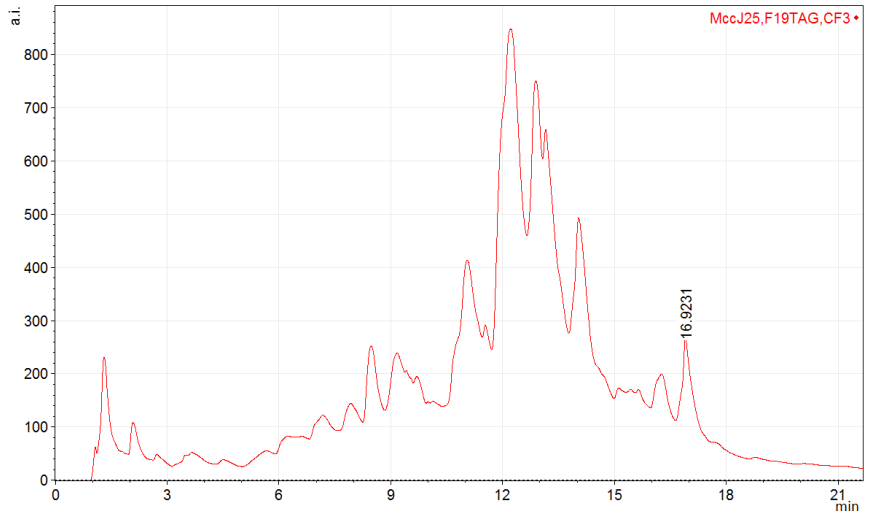
Figure S1: continued on next page.



V6 *m*-ClPhe MccJ25

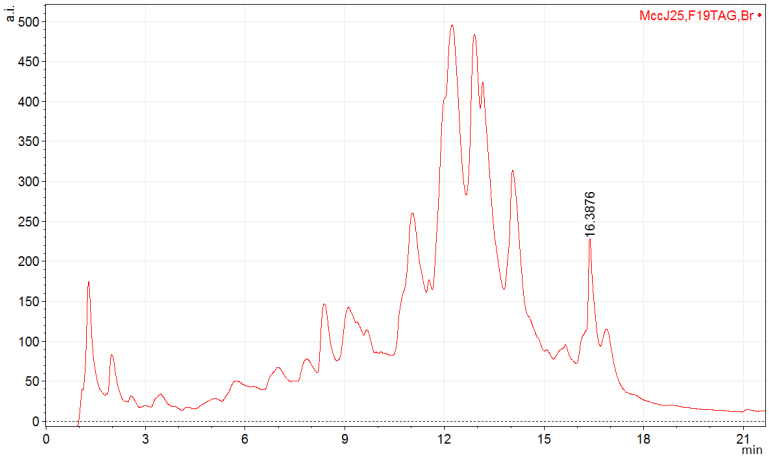


V6 *m*-NO_2Phe MccJ25

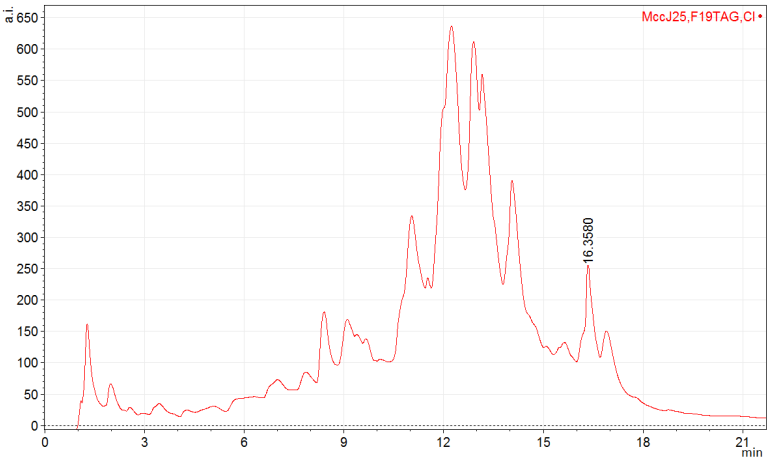


F19 *m*-CF_3Phe MccJ25

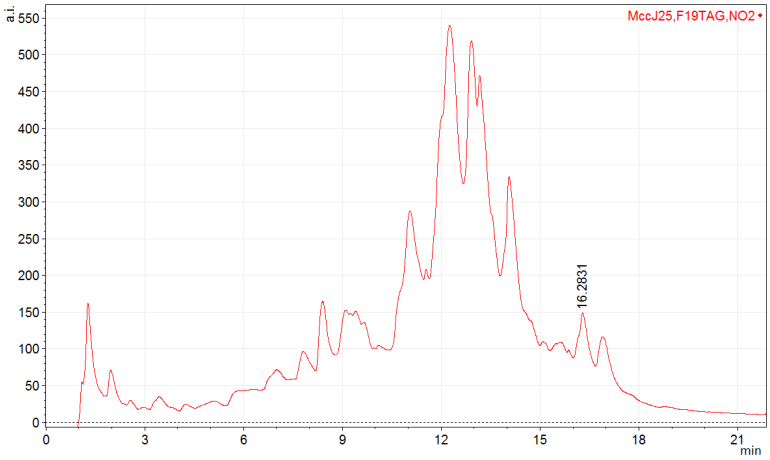
Figure S1: continued on next page.



F19 *m*-BrPhe MccJ25

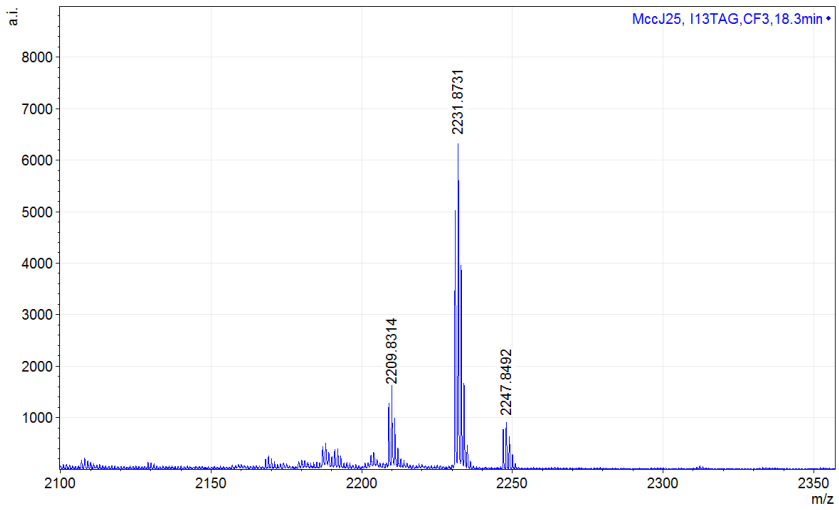


F19 *m*-ClPhe MccJ25

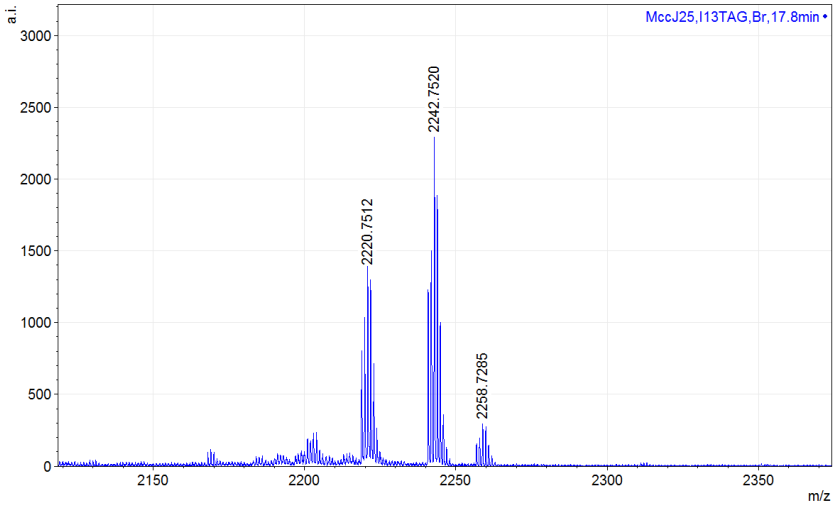


F19 *m*-NO_2Phe MccJ25

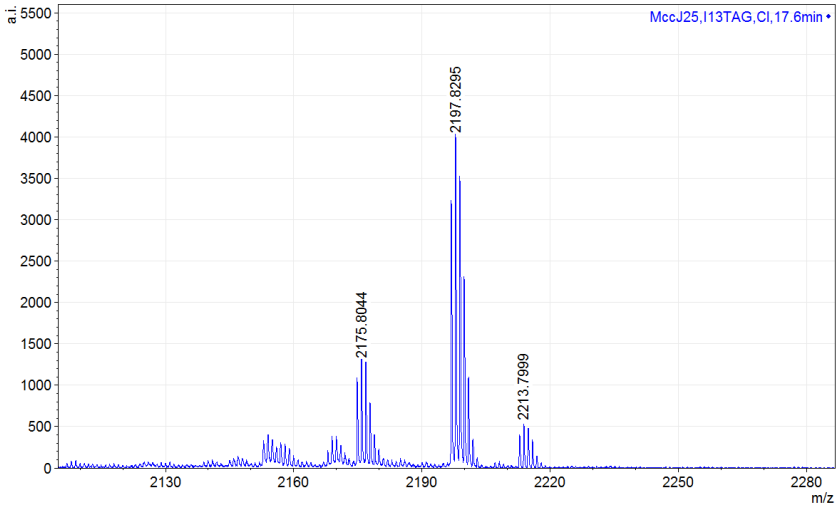
Figure S1. HPLC traces of wild-type MccJ25, a negative control lacking *mcjA*, and all 16 ncAA-substituted MccJ25 variants. All HPLC injections were 5 µL of a sample concentrated 50-fold relative to supernatant. Labeled peaks indicate the lasso peptide as determined by mass spectrometry. All variants were identified except for the V6 *m*-NO_2Phe peptide.



I13 *m*-CF_3Phe MccJ25

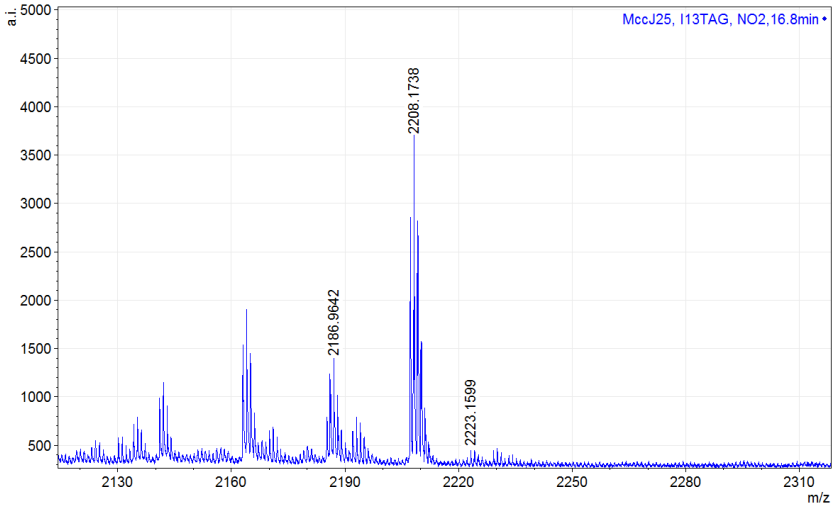


I13 *m*-BrPhe MccJ25

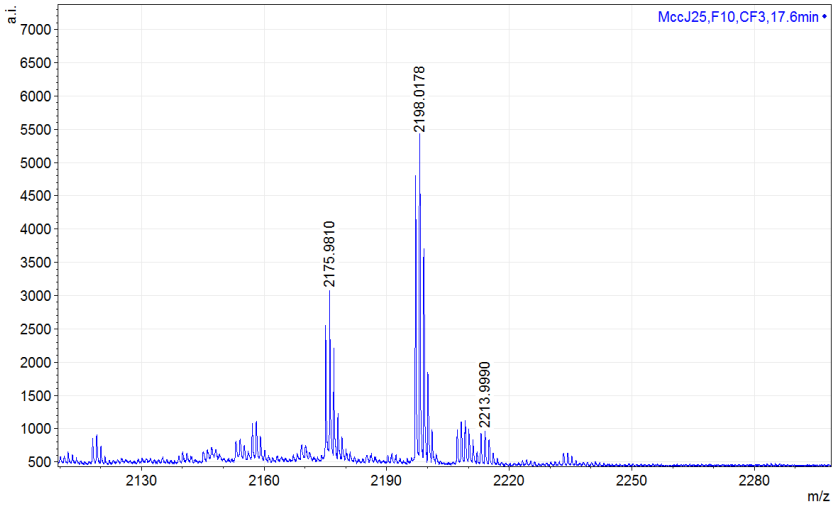


I13 *m*-ClPhe MccJ25

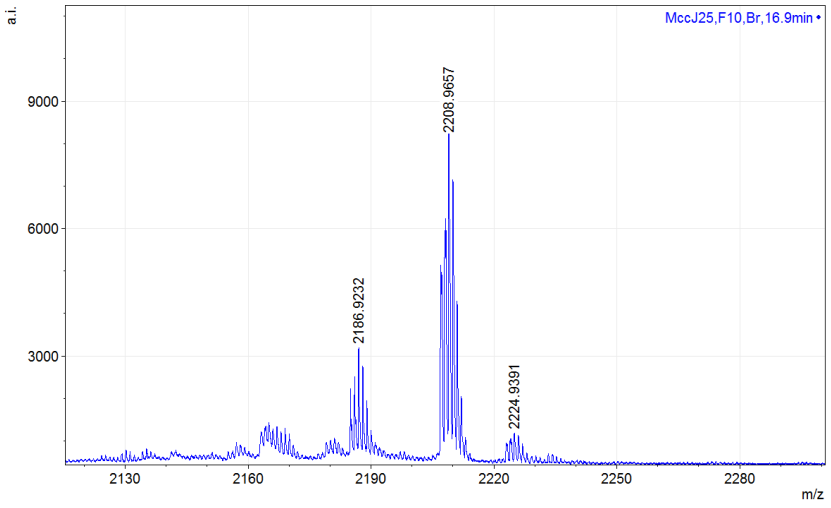
Figure S2: continued on next page.



I13 *m*-NO_2Phe MccJ25

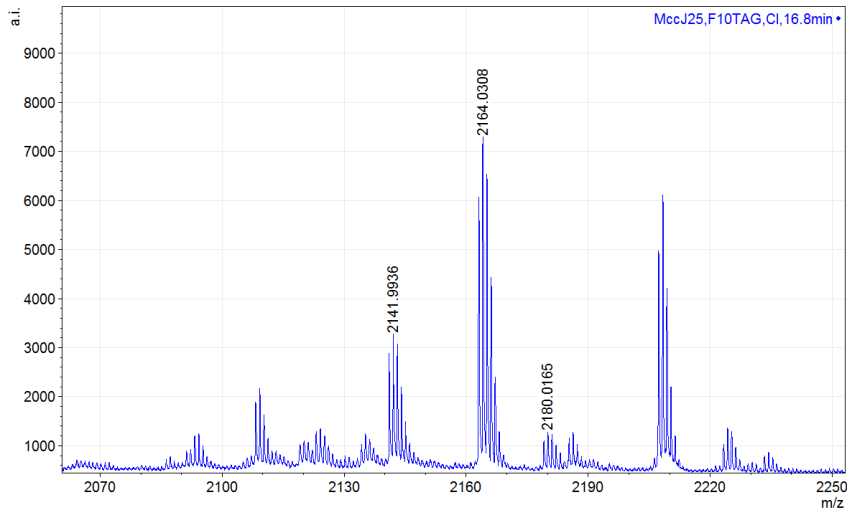


F10 *m*-CF_3Phe MccJ25

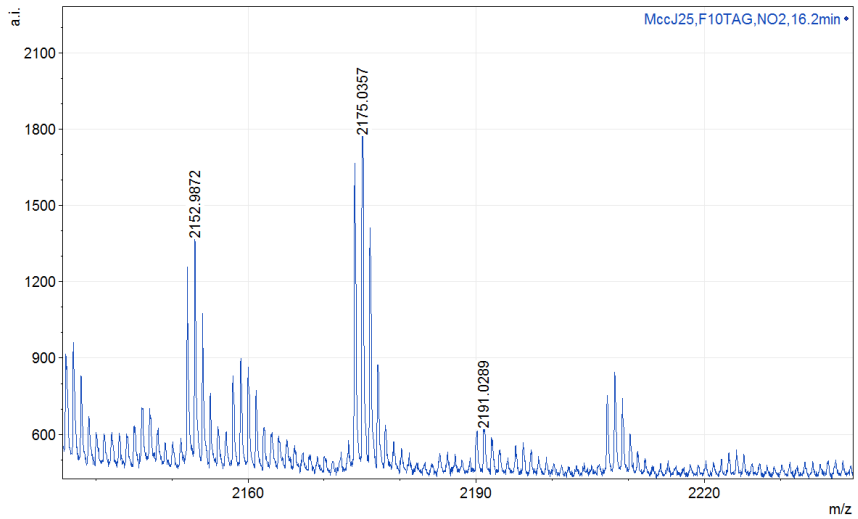


F10 *m*-BrPhe MccJ25

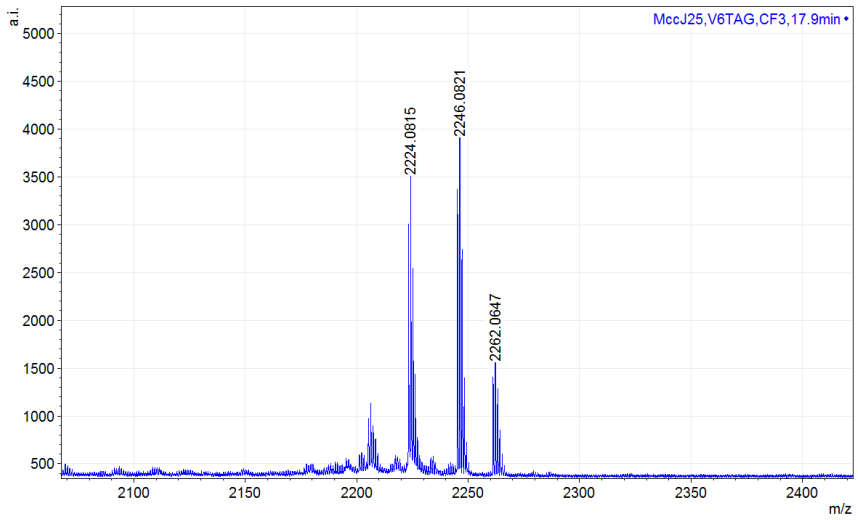
Figure S2: continued on next page.



F10 *m*-ClPhe MccJ25

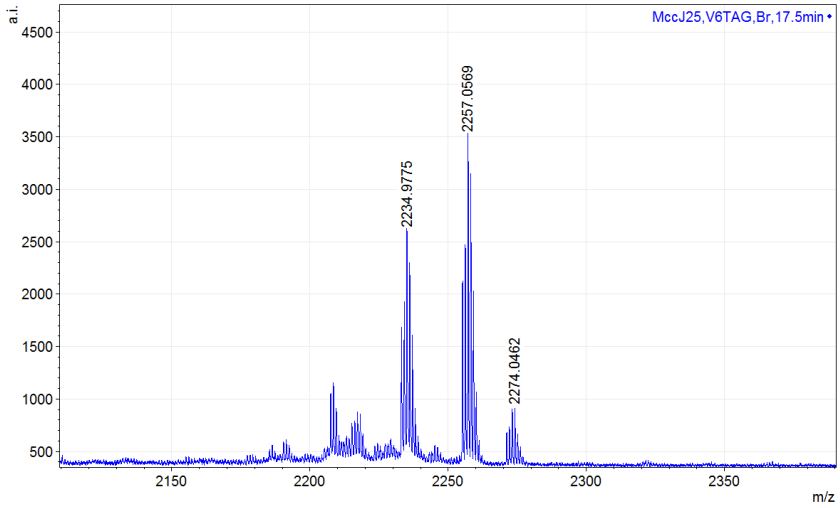


F10 *m*-NO_2Phe MccJ25

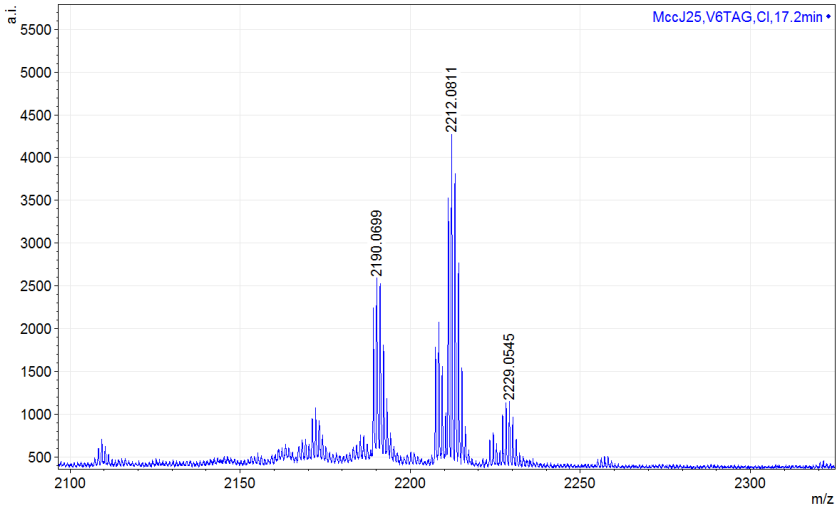


V6 *m*-CF_3Phe MccJ25

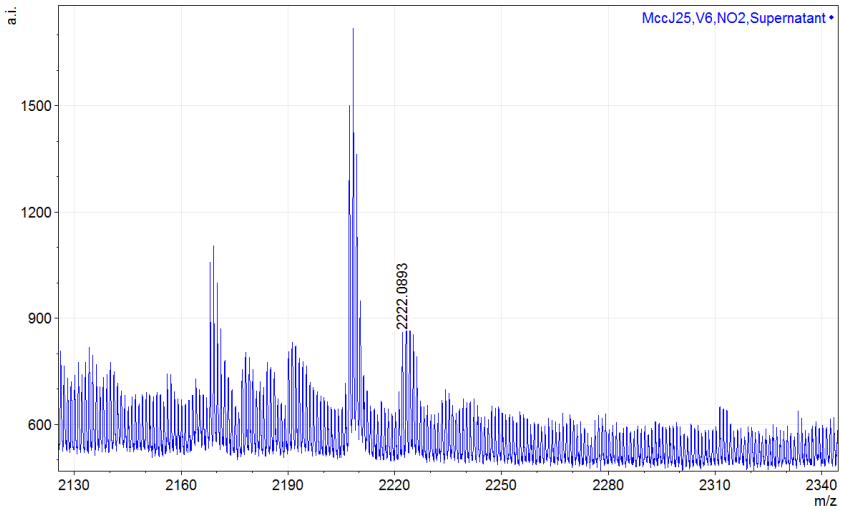
Figure S2: continued on next page.



V6 *m*-BrPhe MccJ25

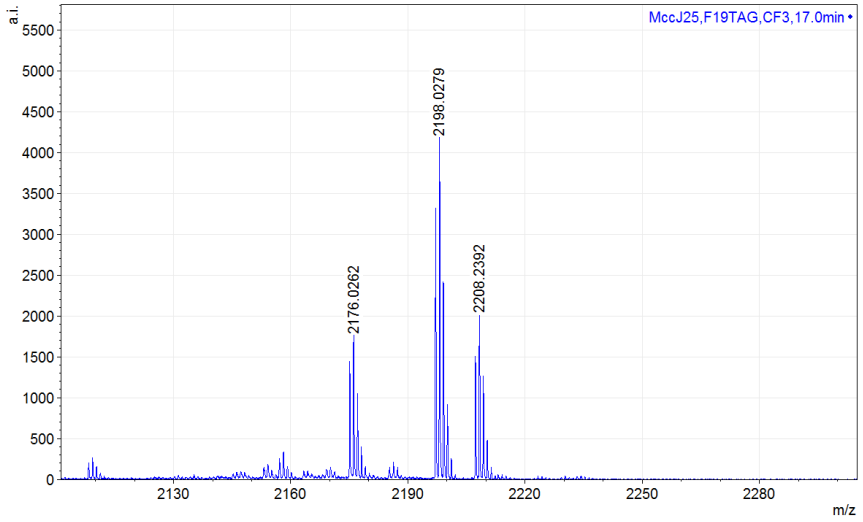


V6 *m*-ClPhe MccJ25

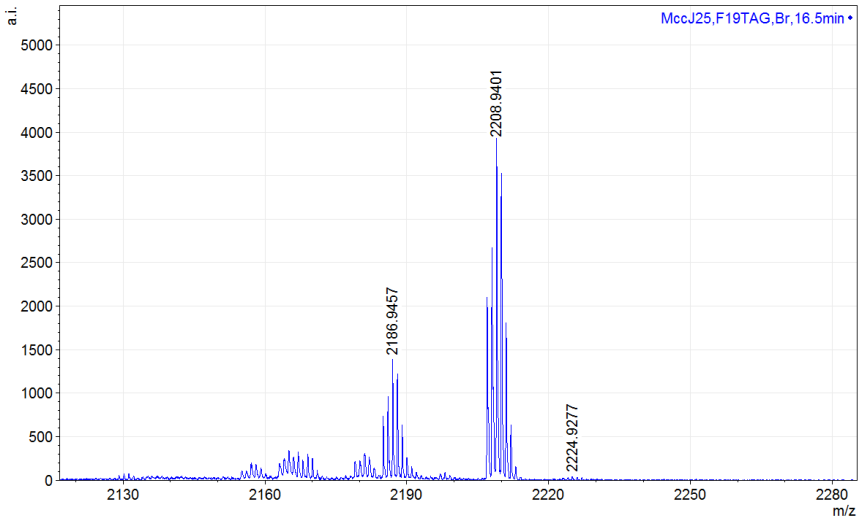


V6 *m*-NO_2Phe MccJ25

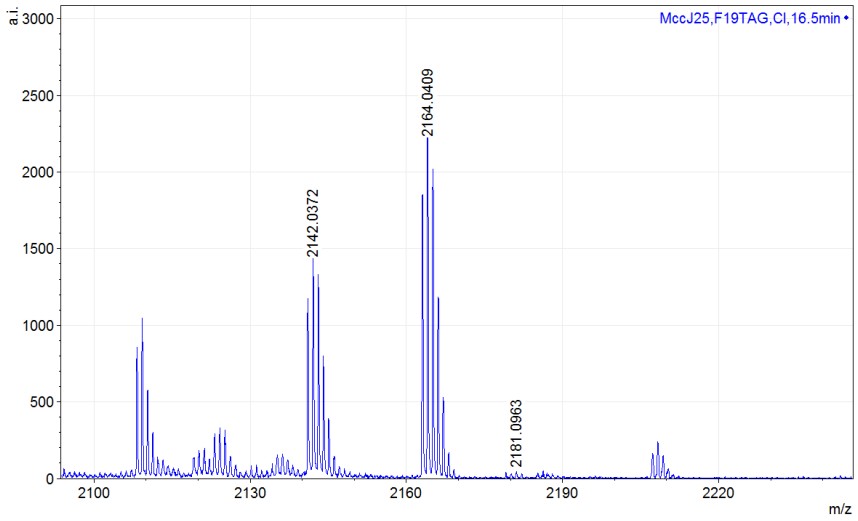
Figure S2: continued on next page.



F19 *m*-CF_3Phe MccJ25

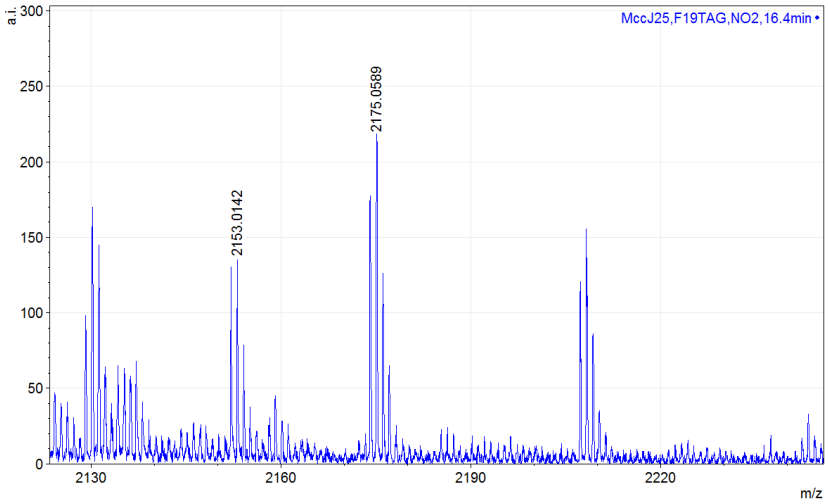


F19 *m*-BrPhe MccJ25



F19 *m*-ClPhe MccJ25

Figure S2: continued on next page.



F19 *m*-NO_2Phe MccJ25

Figure S2. MALDI-TOF mass spectra for ncAA-substituted MccJ25 variants fraction collected via HPLC. V6 *m*-NO_2Phe MccJ25 could not be identified on HPLC, so the spectrum presented here for that particular variant is from the SPE supernatant instead. All samples spotted were at the same concentration as the original supernatant and mixed with an equal volume of α-cyano-4-hydroxycinnamic acid matrix. Labeled peaks indicate H^+, Na^+, and K^+ adducts of the peptides.

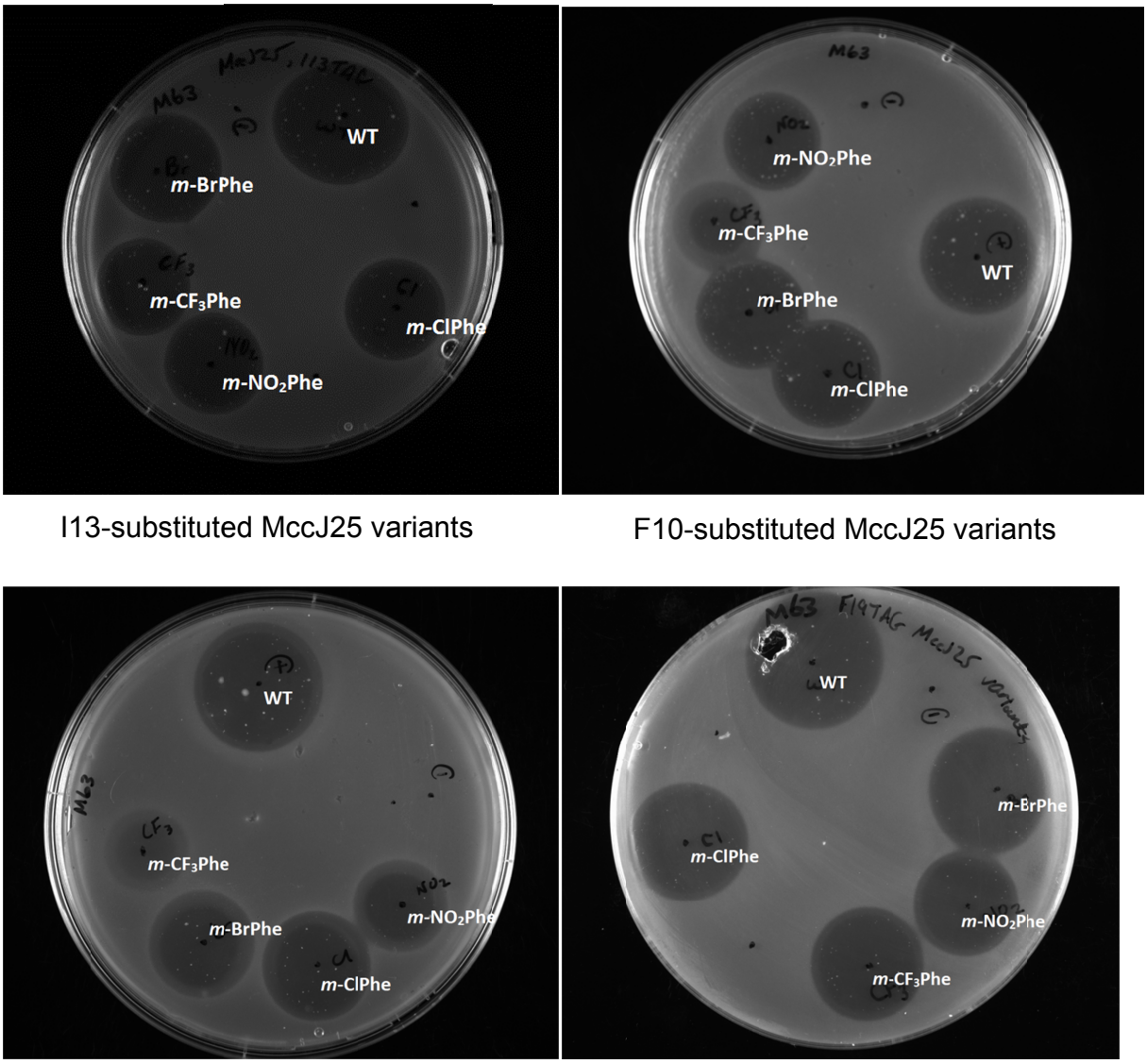
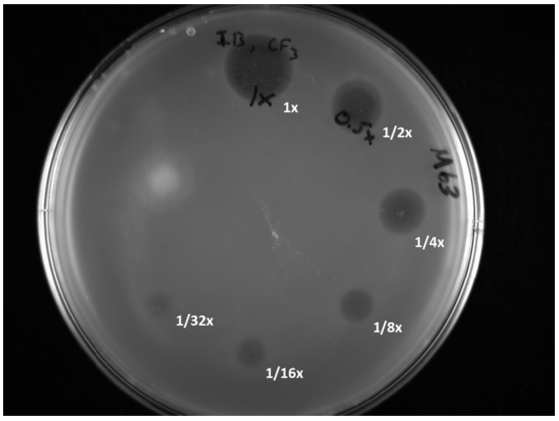
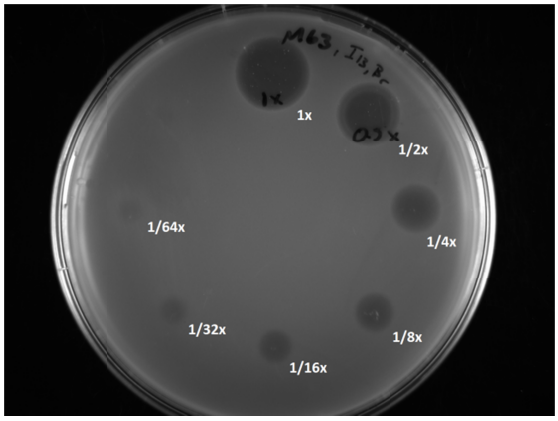


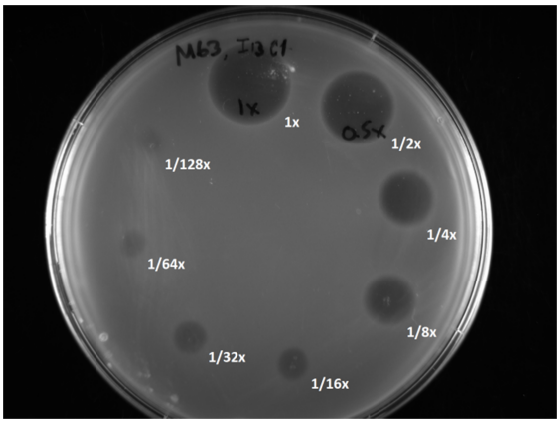
Figure S3. Spot-on-lawn assays against *Salmonella newport* to test for antimicrobial activity of the ncAA-substituted MccJ25 variants. Each spot is 5 µL of a 20-fold concentrated sample relative to the original supernatant. Wild-type MccJ25 and 50/50 ACN/water were used as positive and negative controls, respectively.



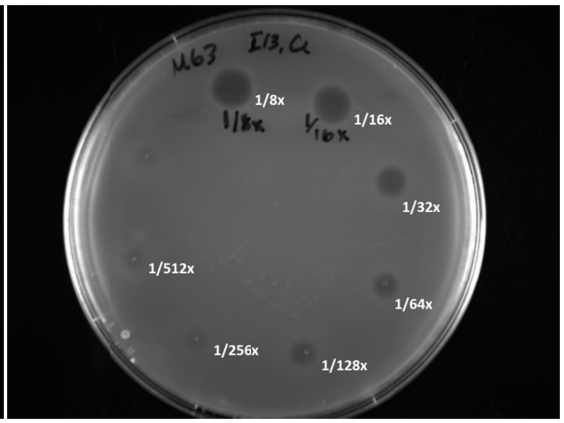
I13 *m*-CF_3Phe MccJ25



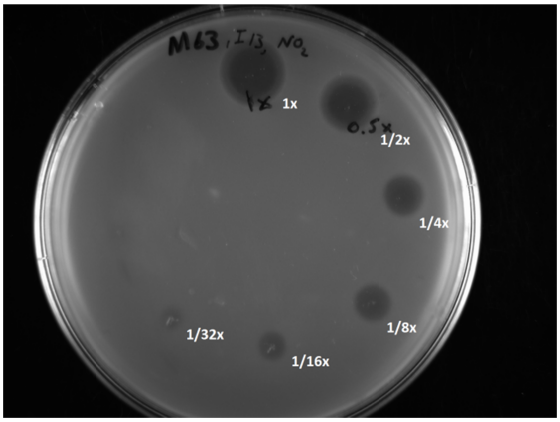
I13 *m*-BrPhe MccJ25



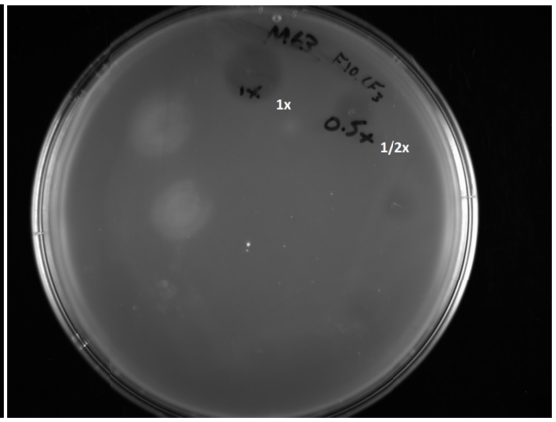
I13 *m*-ClPhe MccJ25 (#1)



I13 *m*-ClPhe MccJ25 (#2)

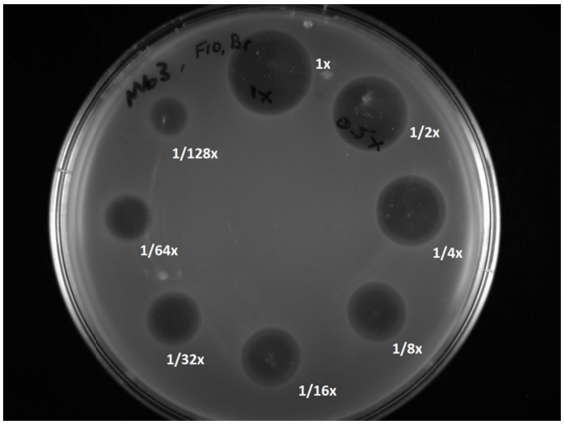


I13 *m*-NO_2Phe MccJ25

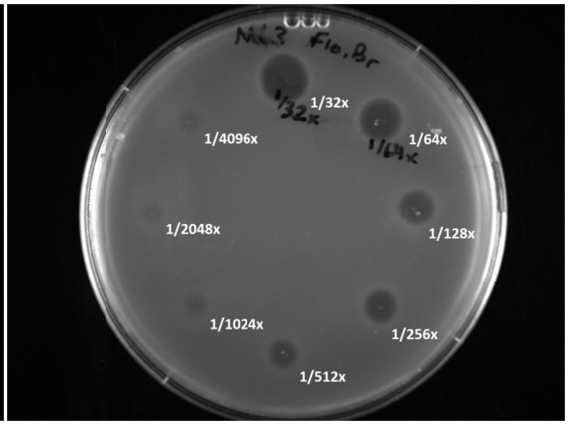


F10 *m*-CF_3Phe MccJ25

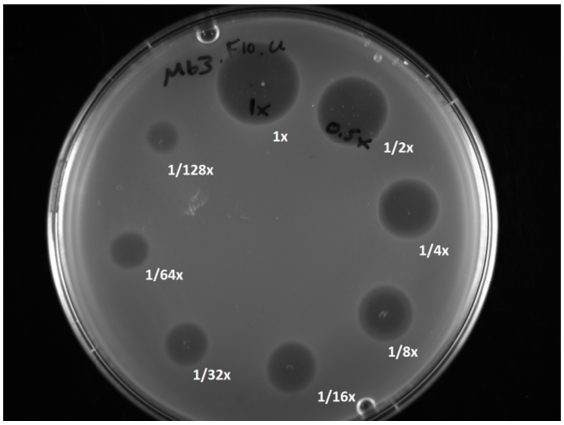
Figure S4: continued on next page.



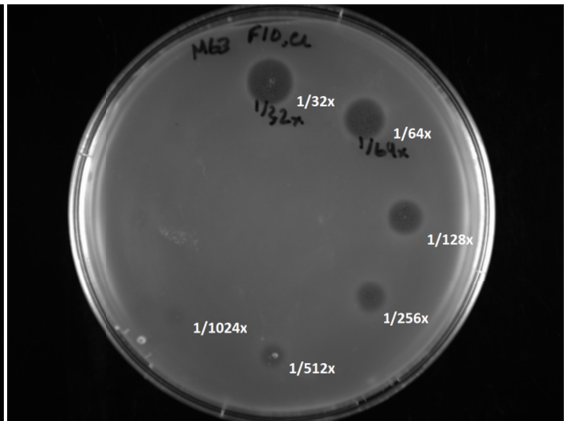
F10 *m*-BrPhe MccJ25 (#1)



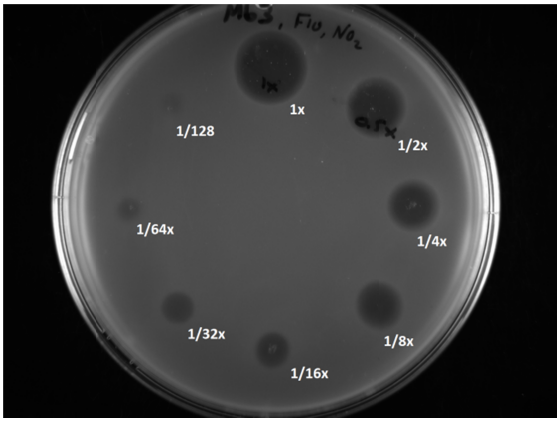
F10 *m*-BrPhe MccJ25 (#2)



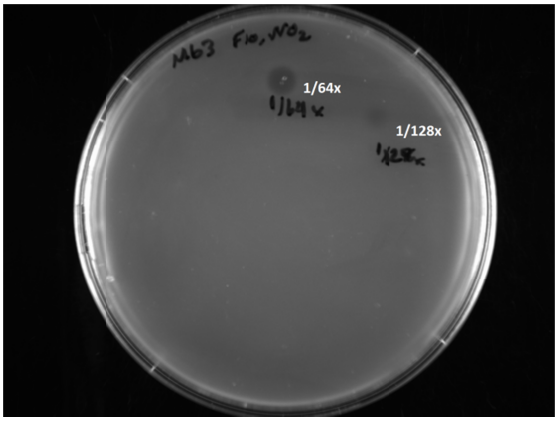
F10 *m*-ClPhe MccJ25 (#1)



F10 *m*-ClPhe MccJ25 (#2)

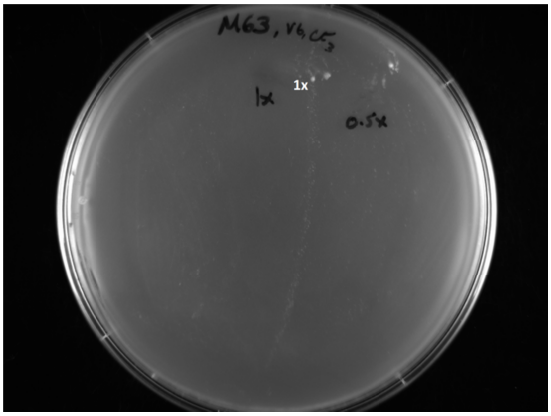


F10 *m*-NO_2Phe MccJ25 (#1)

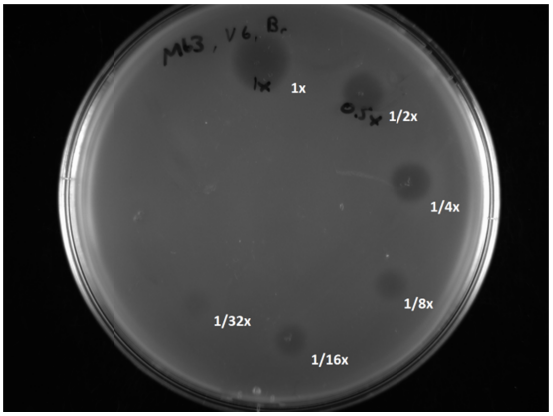


F10 *m*-NO_2Phe MccJ25 (#2)

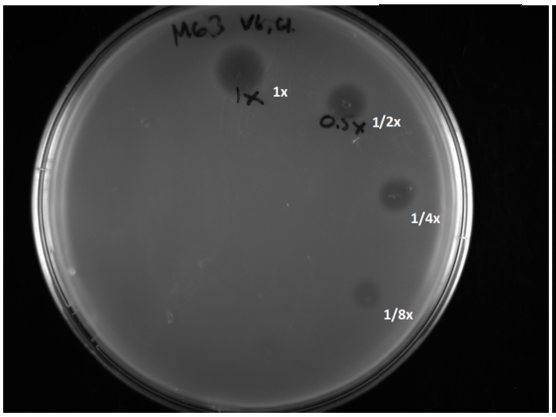
Figure S4: continued on next page.



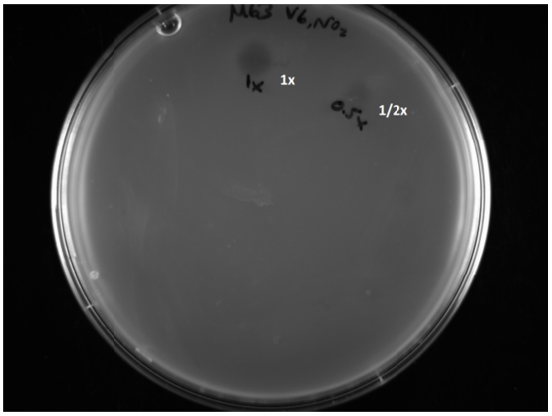
V6 *m*-CF_3Phe MccJ25



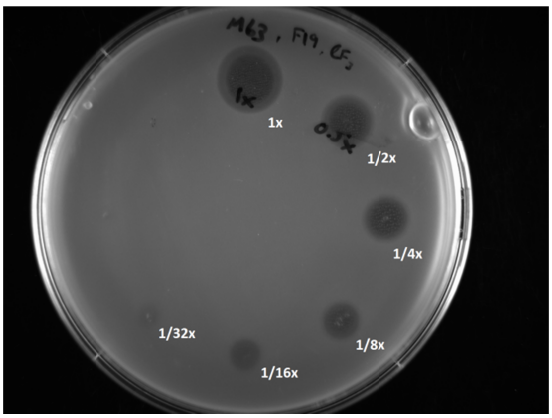
V6 *m*-BrPhe MccJ25



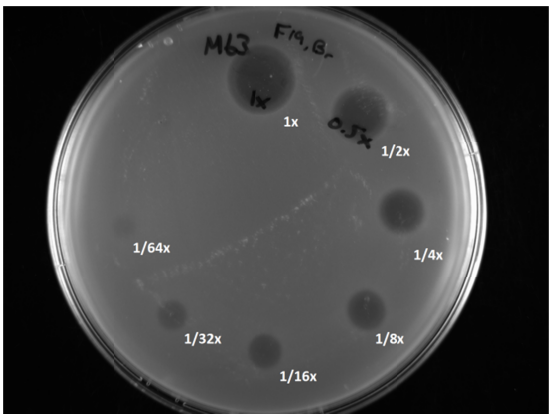
V6 *m*-ClPhe MccJ25



V6 *m*-NO_2Phe MccJ25

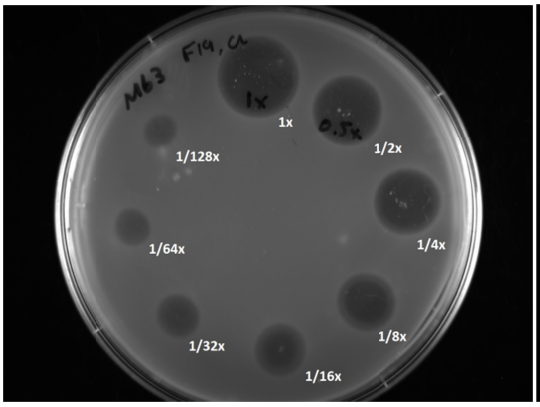


F19 *m*-CF_3Phe MccJ25

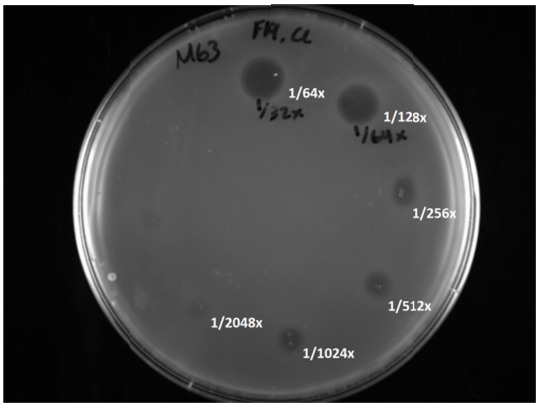


F19 *m*-BrPhe MccJ25

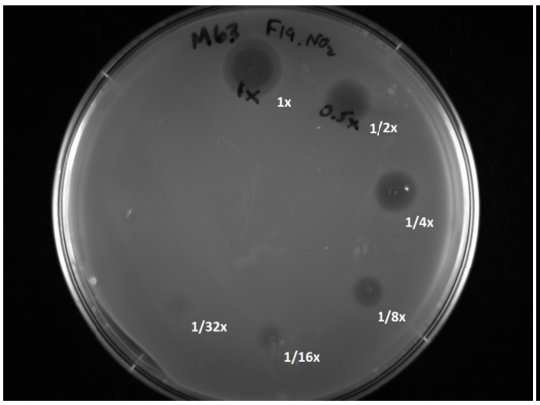
Figure S4: continued on next page.



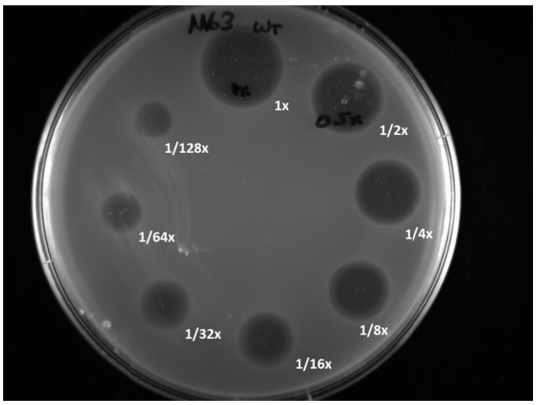
F19 *m*-ClPhe MccJ25 (#1)



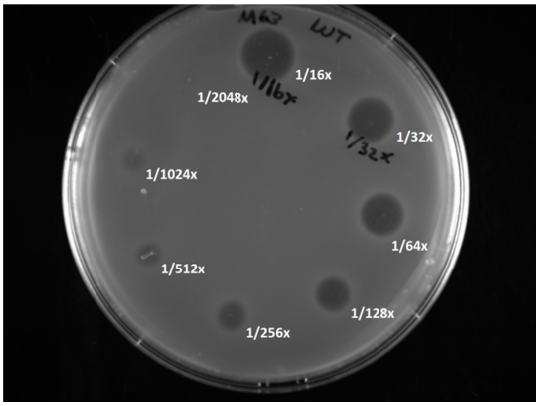
F19 *m*-ClPhe MccJ25 (#2)



F19 *m*-NO_2Phe MccJ25



Wild-type MccJ25 (#1)



Wild-type MccJ25 (#2)

Figure S4. Spot-on-lawn assays against *Salmonella newport* to determine the last active dilution for each MccJ25 variant and wild-type MccJ25. All spots are 5 µL with the 1x spot being at equivalent concentration to the culture supernatant. All subsequent spots are serial dilutions of this 1x sample.

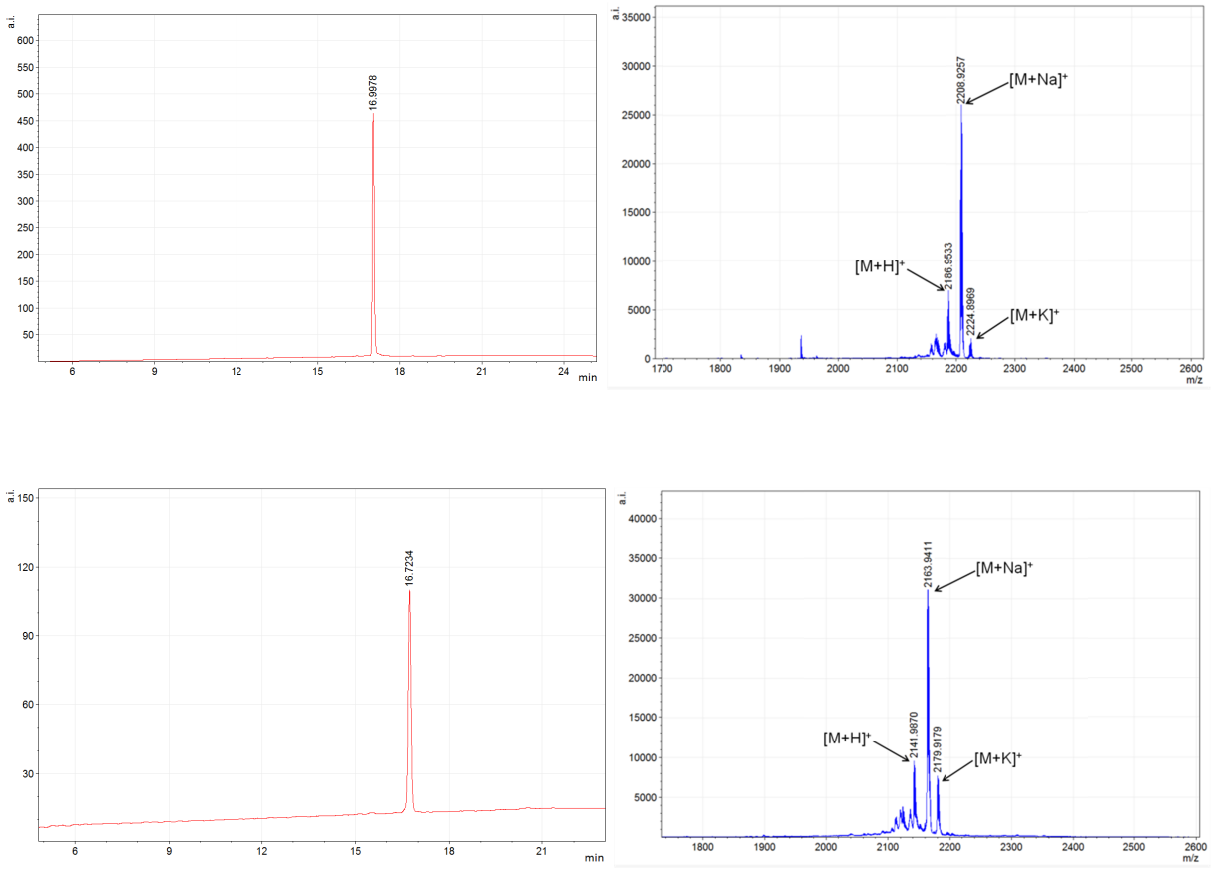


Figure S5. HPLC traces (left, 15 µL injections) and MALDI mass spectra (right) for purified MccJ25 variants. Top: F10 *m*-BrPhe MccJ25. Bottom: F19 *m*-ClPhe MccJ25.

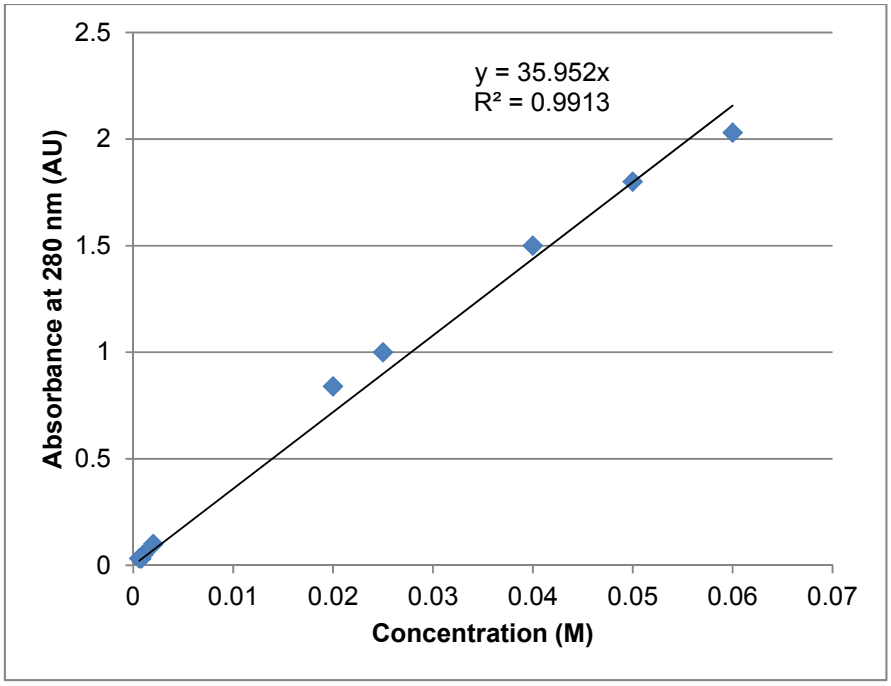


Figure S6. Determination of extinction coefficient of *m*-BrPhe. The path length of the nanodrop is 0.1 cm resulting in an extinction coefficient of 360 $M^{-1}cm^{-1}$.