SUPPORTING INFORMATION

CONTENTS.

GENERAL CHEMICAL METHODS AND ADDITIONAL DATA.

Chemical reagents and instrumentation.

All standard chemical reagents were purchased from commercial sources (Alfa-Aesar, Sigma-Aldrich, or Acros) and used without further purification. Solvents were purchased from commercial sources (Sigma-Aldrich or J.T. Baker) and used as obtained. Water (18 MΩ) was purified using a Millipore Analyzer Feed System. Solid-phase resin and protected L-diamino propionic acid (Dap) [Fmoc-L-Dap(Boc)-OH] were purchased from Chem-Impex.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Shimadzu system equipped with an SCL-10Avp controller, an LC-10AT pump, an FCV-10ALvp solvent mixer, and an SPD-10MAvp UV/vis diode array detector. An analytical Phenomenex Gemini C18 column (5 μm, 4.6 mm × 250 mm, 110 Å) was used for analytical RP-HPLC work. A semi-preparative Phenomenex Gemini C18 column (5 μm, 10 mm × 250 mm, 110 Å) was used for preparative RP-HPLC work. Standard RP-HPLC conditions were as follows: flow rates = 1 mL min^{-1} for analytical separations and 5 mL min^{-1} for semi-preparative separations; mobile phase A = 18 MΩ water + 0.1% trifluoroacetic acid (TFA); mobile phase B = acetonitrile (ACN) + 0.1% TFA. Purities were determined by integration of peaks with UV detection at 220 nm. Cyclic peptides were purified using a linear gradient (75% → 55% A over 30 min). Overall sample purity was determined using a linear gradient (90% → 5% A over 27 min).

MALDI-TOF mass spectrometry (MS) data were obtained on a Bruker RELEX II spectrometer equipped with a 337 nm laser and a reflectron. In positive ion mode, the acceleration voltage was 25 kV. Exact mass (EM) data were obtained on a Waters (Micromass) LCT ESI-TOF mass spectrometer, and the samples were sprayed with a sample cone voltage of 20 V to minimize in-source fragmentation. To assess peptide stability, EM data were obtained on a Thermo Q Exactive Plus™ ESI-Q-IT (orbitrap) mass spectrometer. Tandem MS (MS/MS) measurements were made with a Bruker ULTRAFLEX III (MALDI-TOF/TOF) mass spectrometer equipped with a SmartBeam laser and a LIFT cell.

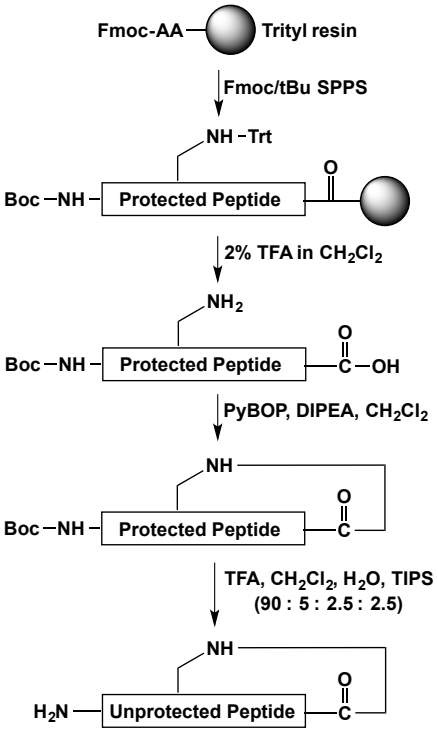
Peptide synthesis and purification.

The amide-bridged AIP-III analogues were synthesized on L-Leu preloaded 2-chlorotrityl resin (0.9 mmol/g) using standard Fmoc/tBu SPPS procedures (see Scheme S-1 below). Cys residues were replaced with Dap. For the full-length AIP analogues, the final residue added was Boc-Ile-OH. The truncated AIP analogue was capped with an acetyl group prior to cleavage. The peptidyl-resin was treated with 2 mL of 2% TFA in CH_2Cl_2 (5 x 2 min) to effect cleavage, followed by extensive washes with CH_2Cl_2 (3 x 5 mL). The washes were combined, and the solvent was removed under reduced pressure to afford a yellow oil. The oil was dissolved in H_2O:ACN (10 mL; 1:1 solution) and lyophilized.

To effect macrolactamization, the crude linear peptide (10 mg) was dissolved in CH_2Cl_2 (5 mL), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 2 equiv.) and *N*,*N*-Diisopropylethylamine (DIPEA, 4 equiv.) were added, and the solution was stirred overnight. The solvent was removed under reduced pressure. To effect deprotection, a 5 mL

solution of TFA:CH_2Cl_2:H_2O:triisopropylsilane (TIPS) (90:5:2.5:2.5) was added, and the solution was allowed to stir for 2 h. A cooled solution of diethyl ether:hexane (1:1 40 mL, 0 °C) was added, and the peptide was allowed to precipitate in a freezer at -20 °C overnight. The precipitated peptide solution was centrifuged, and the supernatant was removed to yield a white solid. The solid was dissolved in H_2O:ACN (1:1 solution), lyophilized, dissolved in H_2O:ACN (7:3 solution), and purified by semi-preparative RP-HPLC (25–30% isolated yields).

The purified peptides were characterized by analytical RP-HPLC, HRMS, and MS/MS (for full-length peptides to confirm correct lactam formation). See below for full characterization data.



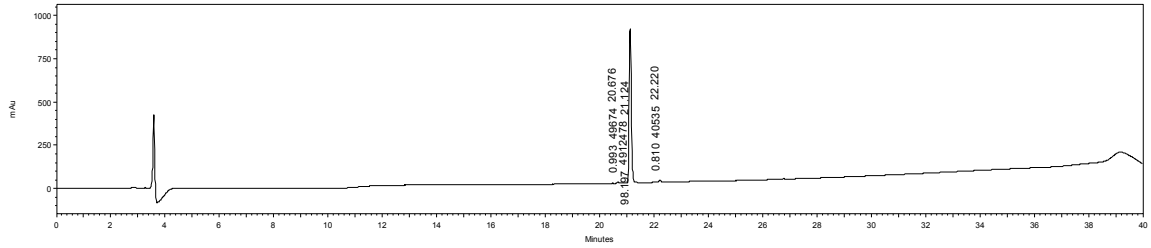
Scheme S-1. Solid-phase synthetic route to amide-bridged AIP-III analogues.

Table S-1. MS and HPLC data for AIP-III analogues in this study. EM = Exact Mass.

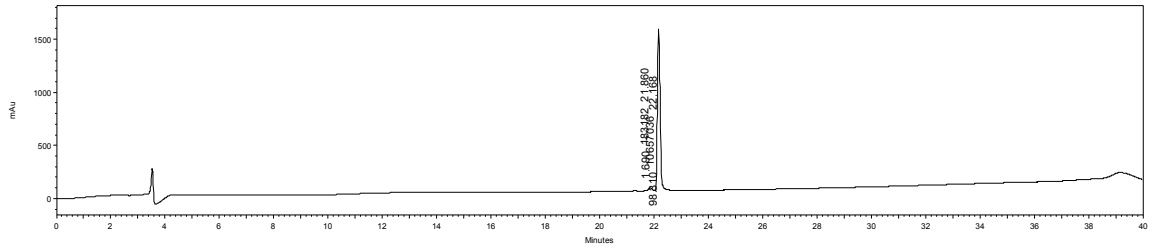
Compound name	Structure	Calc. EM	Obs. EM	Purity (%)
AIP-IIII Amide	Ile-Asn-(Dap-Asp-Phe-Leu-Leu)	802.4458	802.4447	>98
AIP-III D4A Amide	Ile-Asn-(Dap-Ala-Phe-Leu-Leu)	758.4560	758.4554	>98
tAIP-III D2A Amide	Ac-(Dap-Ala-Phe-Leu-Leu)	573.3396	573.3395	>99

HPLC traces for AIP-III analogues.

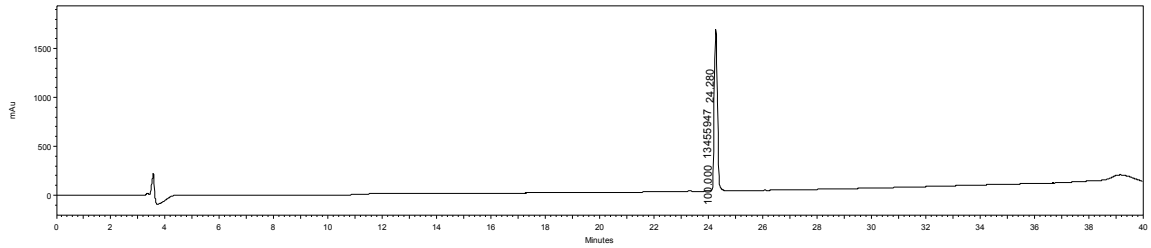
AIP-III Amide



AIP-III D4A Amide



tAIP-III D2A Amide



MS/MS analysis of select AIP-III analogues.

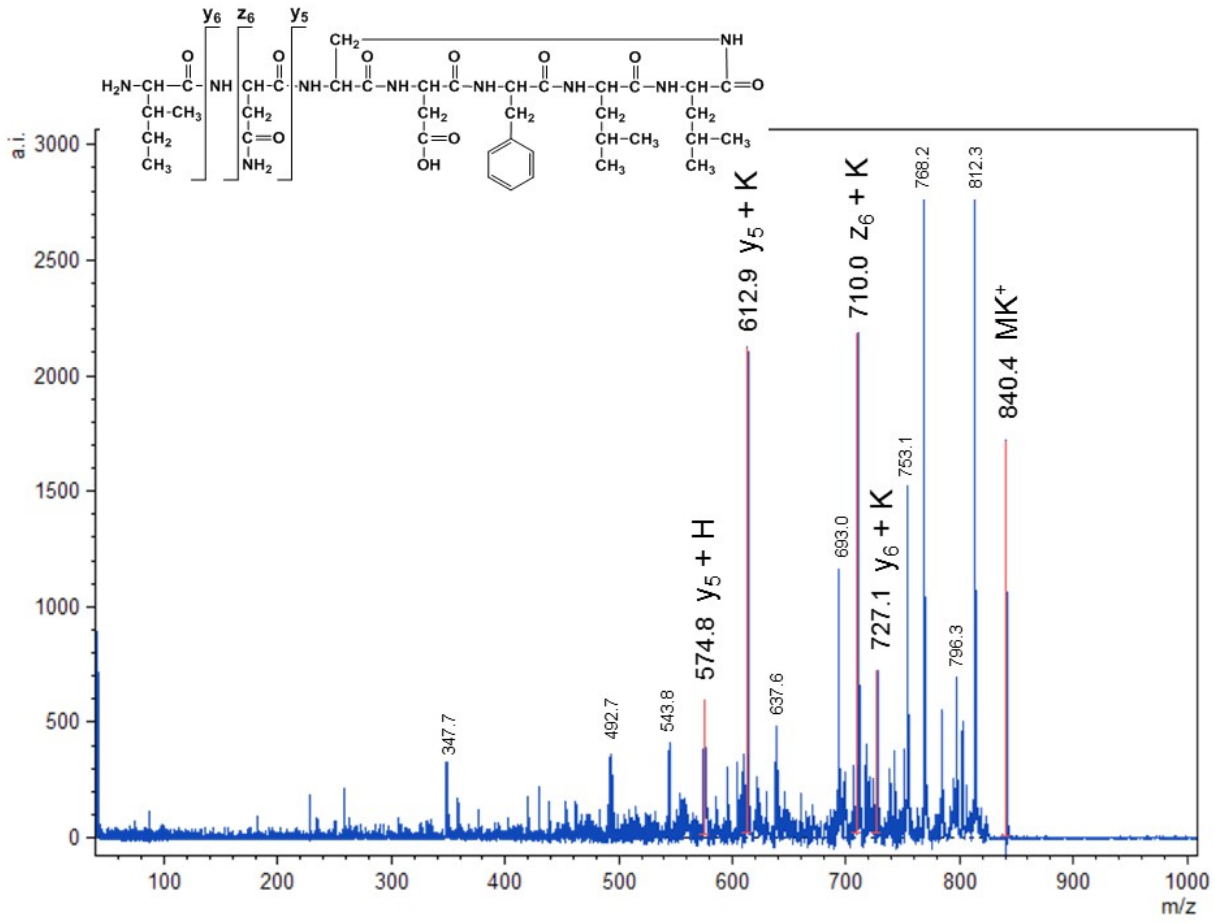


Figure S-1. Positive ion MS/MS analysis of AIP-III Amide. The absence of fragments smaller than y_5 supports macrocyclization between the carboxy terminus and the side chain of the Dap residue.

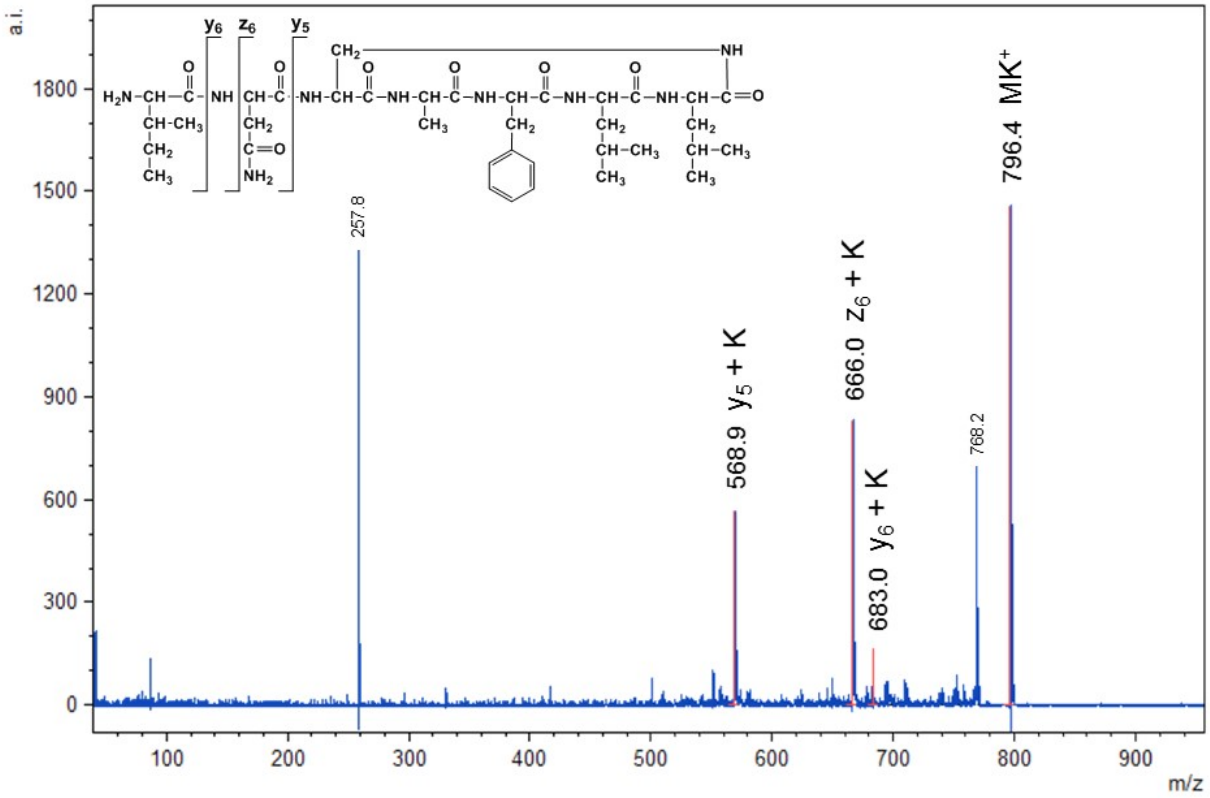


Figure S-2. Positive ion MS/MS analysis of AIP-III D4A Amide. The absence of fragments smaller than y_5 supports macrocyclization between the carboxy terminus and the side chain of the Dap residue.

Calculated logP values for representative peptides.

LogP values (for octanol/water) for AIP-III D4A and AIP-III D4A Amide were calculated using four common online programs. These values and associated websites are provided below. Lower values indicate a compound is more hydrophilic/polar.

http://www.molinspiration.com/

AIP-III D4A: 1.30
AIP-III D4A Amide: 0.01

http://www.organic-chemistry.org/prog/peo/

AIP-III D4A: -1.18
AIP-III D4A Amide: -1.62

http://vcclab.org/web/alogps/

AIP-III D4A: 1.06
AIP-III D4A Amide: 0.67

https://ilab.acdlabs.com/iLab2/index.php

AIP-III D4A: -0.13
AIP-III D4A Amide: -0.85

GENERAL BIOLOGICAL METHODS AND ADDITIONAL DATA.

Biological reagents and strain information.

All standard biological reagents were purchased from Sigma-Aldrich and used according to enclosed instructions. Brain heart infusion (BHI) was prepared as instructed with pH = 7.35.

The bacterial strains used in this study are listed in Table S-2. Bacterial cultures were grown in a standard laboratory incubator at 37 °C with shaking (200 rpm) unless noted otherwise. The bacterial dilutions and incubation periods were chosen in each assay to provide the greatest dynamic range between positive and negative controls for each bacterial strain. Absorbance and fluorescence measurements were obtained using a Biotek Synergy 2 microplate reader using Gen5 data analysis software. All biological assays were performed in triplicate. IC_{50} values were calculated using GraphPad Prism software (v. 4.0) using a sigmoidal curve fit.

Table S-2. *S. aureus* strains used in this study listed according to group.

Assay-type	Strain	Reference
Fluorescence		
Group-I	AH1677	[1]
Group-II	AH430	[1]
Group-III	AH1747	[1]
Group-IV	AH1872	[1]
β-Lactamase		
Group-I	RN9222	[2]
Group-II	RN9372	[2]
Group-III	RN9532	[2]
Group-IV	RN9371	[2]

Compound handling protocol.

Stock solutions of AIPs and AIP analogues (1 mM) were prepared in DMSO and stored at 4 °C in sealed vials. The amount of DMSO used in bacteriological assays did not exceed 4% (v/v). Black or clear polystyrene 96-well microtiter plates (Costar) were used for bacteriological assays.

Fluorescence assay protocol.

Peptide stock solutions were diluted with DMSO in serial dilutions (either 1:3 or 1:5), and 2 μL of the diluted solution was added to each of the wells in a black 96-well microtiter plate. An overnight culture of *S. aureus* GFP strain (Table S-2) was diluted 1:50 with fresh BHI (pH 7.35). A 198-μL portion of diluted culture was added to each well of the microtiter plate containing peptide. Plates were incubated at 37 °C for 24 h. Fluorescence (EX 500 nm / EM 540 nm) and OD_{600} of each well were then recorded using a plate reader, and IC_{50} values were calculated.

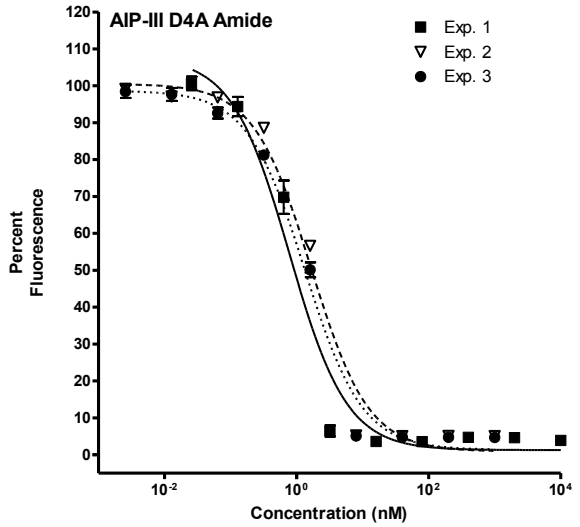
β-lactamase assay protocol.

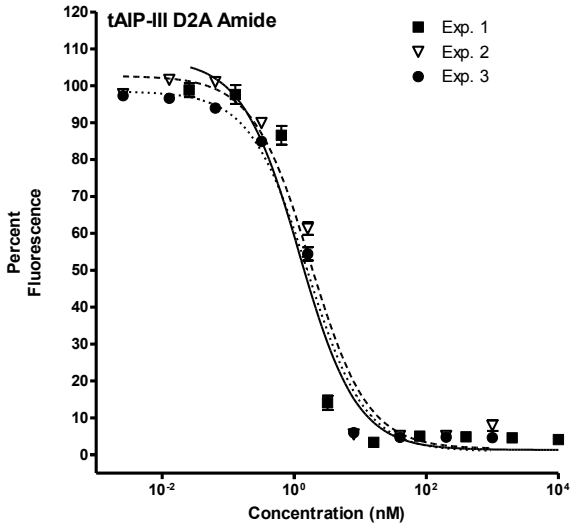
We utilized a β-lactamase/nitrocefin assay protocol analogous to that of Novick and co-workers,[3] except that β-lactamase activity was monitored at a single time point (i.e., 20 min) as opposed to determining a rate profile for enzyme activity (the single time point reading gave comparable data trends). In brief, peptide stock solutions were diluted with DMSO in serial dilutions, and 2 μL aliquots of the diluted solutions were added to each of the wells of a clear 96-well microtiter plate. An overnight culture of *S. aureus* β-lactamase strain (Table S-2) was diluted 1:50 with fresh BHI, and the bacteria were incubated until reaching early exponential phase (OD_{600} = 0.16). A 50-μL portion of culture was added to each well of the microtiter plate containing peptide. Plates were incubated for 1 h. The OD_{600} of each well was recorded using a plate reader, followed by the addition of sodium azide (5 μL of a 50 mM solution) and nitrocefin solution (the β-lactamase substrate; 50 μL of a 132 μg/mL solution in 0.1 M sodium phosphate buffer, pH 5.8). The plate was incubated in the dark for 20 min, and the absorbance (at 495 nm) of each well was recorded. No EC_{50} values were calculated in this study, as no compounds showed appreciable agonistic activity at concentrations up to 40 mM.

Fluorescence antagonism dose response curves.

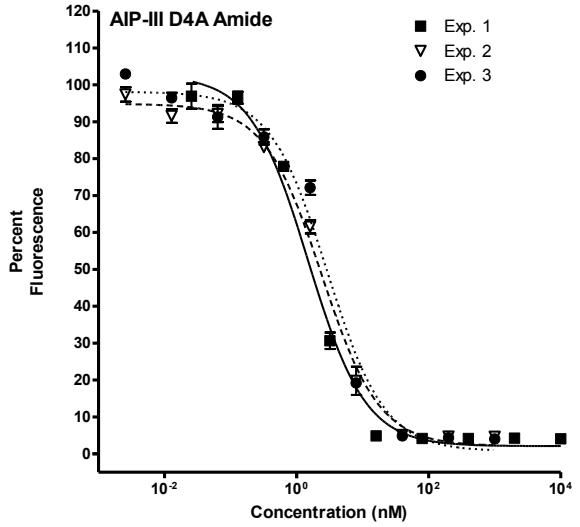
Peptides were screened over varying concentrations in the four *S. aureus* GFP reporter strains (Table S-2). The peptide for which data is shown in each plot below is indicted in bold at top left. Each dose response experiment was performed in triplicate on three separate occasions [i.e., experiments (Exp.) #1–3; shown for each peptide below]. Error bars indicate standard error of the mean of triplicate values. See section above for details of assay and strains.

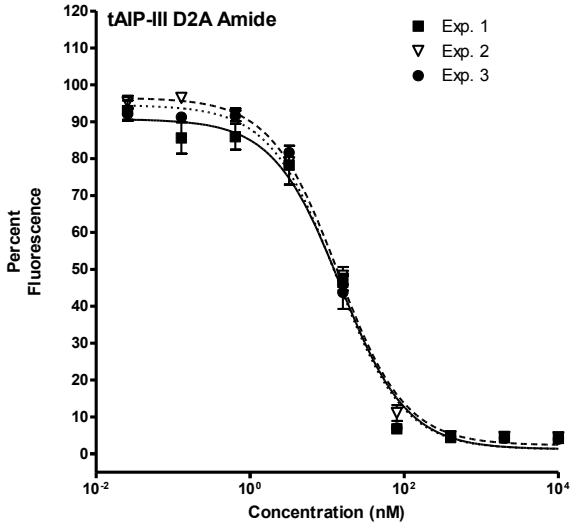
S. aureus AH1677 (Group-I)



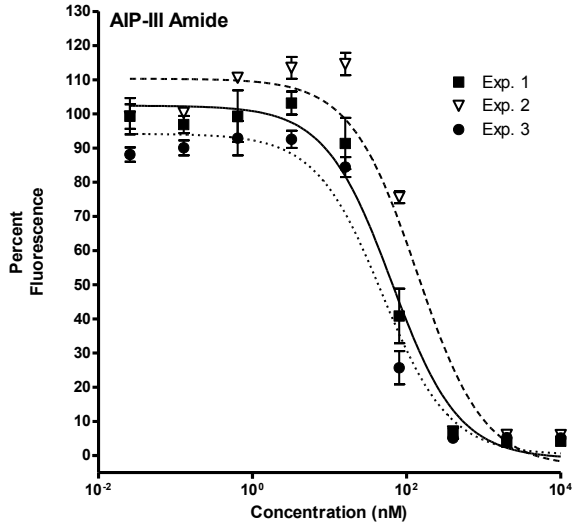


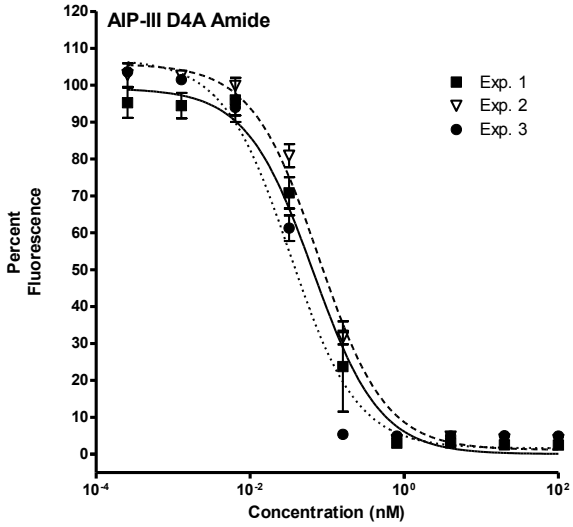
S. aureus AH430 (Group-II)

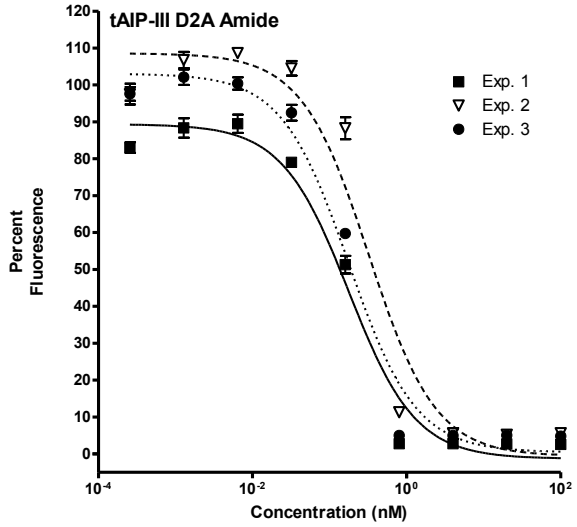




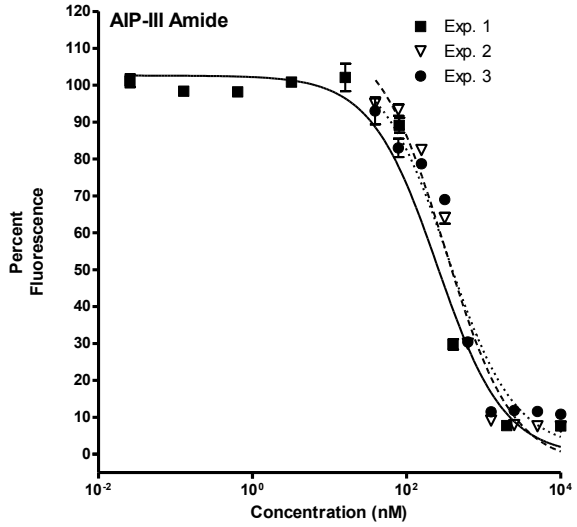
S. aureus AH1747 (Group-III)

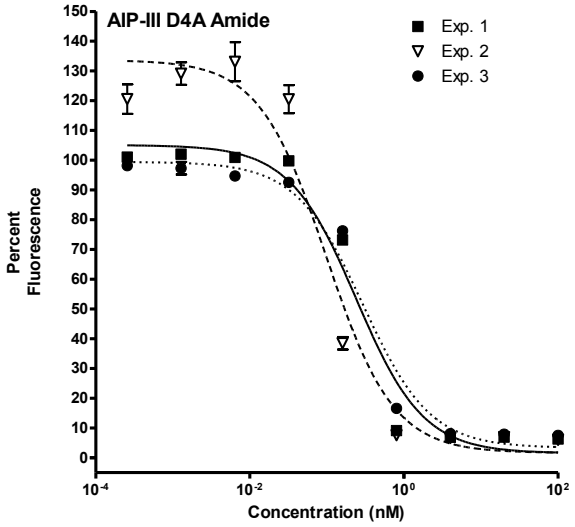






S. aureus AH1872 (Group-IV)





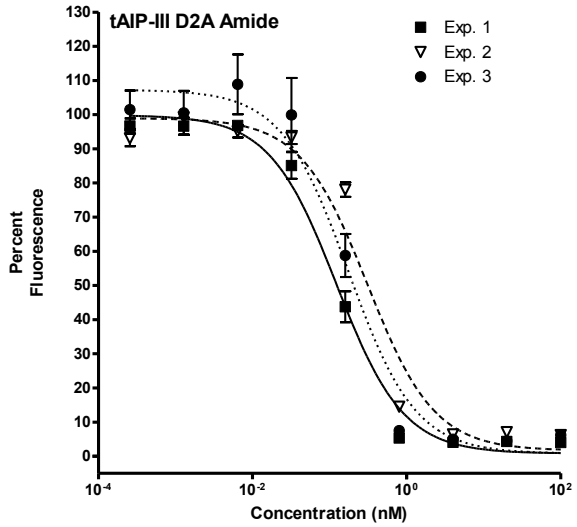


Table S-3. Complete set of IC_{50} values and 95% confidence ranges for the amide-bridged AIP analogues and control AIPs against AgrC I–IV as determined using the *S. aureus* fluorescence reporter strains.[a]

Peptide name	Sequence	Inhibition (IC_{50} nM)[b]			
		AgrC-I	AgrC-II	AgrC-III	AgrC-IV
AIP-III Amide	Ile-Asn-(Dap-Asp-Phe-Leu-Leu)	>1000	>1000	75.2 (26.3–215)	299 (220–408)
AIP-III D4A Amide	Ile-Asn-(Dap-Ala-Phe-Leu-Leu)	1.20 (0.558–2.59)	2.19 (1.12–4.31)	0.0551 (0.0227–0.134)	0.192 (0.0608–0.603)
tAIP-III D2A Amide	Ac-(Dap-Ala-Phe-Leu-Leu)	1.50 (1.02–2.20)	13.9 (12.6–15.3)	0.216 (0.110–0.426)	0.189 (0.0756–0.472)
AIP-III[c]	Ile-Asn-(Cys-Asp-Phe-Leu-Leu)	5.05 (2.46–10.4)	5.63 (1.89–16.7)	–[d]	8.53 (4.15–17.5)
AIP-III D4A[c]	Ile-Asn-(Cys-Ala-Phe-Leu-Leu)	0.485 (0.289–0.813)	0.429 (0.208–0.886)	0.0506 (0.0227–0.113)	0.0349 (0.0123–0.0990)
tAIP-III D2A[c]	Ac-(Cys-Ala-Phe-Leu-Leu)	0.257 (0.102–0.646)	0.900 (0.842–0.963)	0.329 (0.215–0.502)	0.0957 (0.0375–0.245)

[a] See above for details of reporter strains, experimental procedures, and plots of antagonism dose response curves. All assays performed in triplicate. [b] IC_{50} values determined by testing peptides over a range of concentrations (200 fM – 10 μM). Range indicates 95% confidence interval of triplicate experiments. [c] Control peptides, data reproduced from reference [4]. [d] Receptor agonist.

Peptide stability studies.

To monitor hydrolytic stability, the peptides (~0.3 mg) were dissolved in 1 mL of either PBS (pH 7.4) or aqueous ammonium carbonate (200 mM, pH 8.0) to yield ~0.33 mM solutions. The peptide solutions were incubated with shaking at 37 °C. Samples (100 μL) were taken at 0, 0.5, 1, 2, 4, 8, 24, 48, and 72 h, and evaluated immediately for peptide degradation using analytical RP-HPLC (see above for details of instrumentation and method).

To monitor enzymatic stability, the peptides (0.3 mg) were dissolved in 1 mL of aqueous ammonium carbonate (200 mM, pH 8.0), and a 10-μL aliquot of 25 μg/mL Trypsin and Chymotrypsin solution (diluted from a 2.5 mg/mL Trypsin solution; Sigma) in aqueous ammonium carbonate (200 mM) was added prior to incubation with shaking at 37 °C. Sample stability over time was analysed using RP-HPLC (analogous to the hydrolysis studies above). The total amount of Trypsin and Chymotrypsin in these experiments (0.25 μg/mL) is comparable to the natural amount in serum.[5]

As highlighted in the main text, we observed ~10% and ~40% degradation of AIP-III D4A Amide in 200 mM ammonium carbonate at pH 8 over 24 h and 72 h, respectively. We were initially surprised by this result, but after further study, we hypothesized that deamidation of the Asparagine side chain (Asn2) was responsible for the degradation product in ammonium carbonate. Nonenzymatic deamidation of Asn has been shown to occur in proteins and peptides, and ammonia and carbonate are proposed to catalyze such reaction.[6] To test our hypothesis, we incubated AIP-III D4A Amide in 200 mM ammonium carbonate solution at pH 8 for 72 h and separated the starting material from the degradation product using semi-preparative RP-HPLC (see Methods section above; linear gradient 62% → 52% A over 27 min). The two compounds isolated were submitted for exact mass (EM) measurements. Based on the exact masses determined, we were able to confirm that the major compound in the solution after 72 h was the starting material AIP-III D4A Amide (Calculated EM $[M+H]^+$: 758.4559; Experimental EM $[M+H]^+$: 758.4559), and the exact mass of the minor degradation product only differed from the theoretical mass of the deamidation product by 4 ppm (Calculated EM $[M+H]^+$: 759.4400; Experimental EM 759.4397), strongly supporting our hypothesis. We suspect that this minor product may be an artifact of working in ammonium carbonate (chosen here to mimic the conditions for the enzymatic stability studies) and would not be observed in a significant amount in other common buffers. Nevertheless, as only ~10% of AIP-III D4A Amide was converted to this product over 24 h at pH 8, whilst 100% of AIP-III was degraded over 8 h, our data support the heightened hydrolytic stability of AIP-III D4A Amide.

We performed similar HPLC and MS experiments with AIP-III to demonstrate the susceptibility of the thioester linkage to hydrolysis under alkaline conditions. Unexpectedly, we did not observe a simple hydrolysis product in the fractions isolated using semi-preparative RP-HPLC (linear gradient 70% → 55% A over 27 min). Instead, the EM data showed one major product with a mass close to that of an oxidation product resulting from the addition of a single oxygen atom to AIP-III (Calculated EM $[M+H]^+$: 835.4024; Experimental EM $[M+H]^+$: 835.4258) and two minor products (Experimental EM $[M+H]^+$: 846.4163 and 854.3994). Since we could not match the mass of any proposed degradation product to the masses obtained experimentally, we suspect that the presence of ammonium carbonate and the reactive groups in AIP-III combined to

produce a more complex set of degradation products. In support of our hypothesis, when we incubated AIP-III in PBS buffer at pH 8 instead of ammonium carbonate for 72 h, our preliminary MS data suggest that the main degradation product is derived from thioester hydrolysis (Calc. EM $[M+Na]^+$: 859.4000; Experimental MALDI $[M+Na]^+$: 860.5). Additional experiments are in progress to confirm the identification of this product and to better delineate the reactions taking place with AIP-III and AIP-III D4A Amide in ammonium carbonate buffer.

NMR METHODS AND STRUCTURAL ANALYSES.

General NMR methods.

All NMR spectra were recorded on a Varian Inova 600 MHz spectrometer at 298 K, using 1.0–1.5 mM solutions of the peptides in 70% H_2O/30% CD_3CN, pH 3.65 (conditions analogous to our prior study).[7] Spectra were processed using the Vnmr software package (v. 6.1C; Varian) and NMRPipe software.[8] Chemical shifts were referenced to CD_3CN at 1.94 ppm.

Two-dimensional (2-D) homonuclear experiments gcosy, wgtocsy (tocsy[9] using DIPSI spinlock and the 3-9-19 water suppression sequence) and wgroesy (rotating frame NOE experiment with pulsed T-Roesy[10] spin lock and the 3-9-19 water suppression sequence) were acquired. The gcosy experiments were collected with 1754 and 512 real data points in the direct and indirect dimensions, respectively, with 16 scans per data point. For the wgtocsy experiments, 1536 and 256 real data points were collected in the direct and indirect dimensions, respectively, with 16 scans per data point. A relaxation delay of 2 sec was used for both the gcosy and wgtocsy experiments, with a mixing time of 80 ms for the wgtocsy. For the wgroesy experiments, 3080 real data points were used in the direct dimension, with 300 data points in the indirect dimension, and 64 scans per data point. A relaxation delay of 3 sec and a mixing time of 300 ms were used. Presaturation water suppression was used in the 32 scan ^{1}H 1-D experiments with 30272 real data points.

All spectra were analyzed using SPARKY software.[11] Assignment of resonances for each peptide (listed in Tables S-4–S-6) was achieved using standard sequential assignment methodology.[12] The number of ROEs observed for each peptide is listed in Table S-7. The volumes of the ROE peaks were calculated by SPARKY and converted into a continuous distribution of interproton distance restraints, with a uniform 20% distance error applied to take into account spin diffusion. Three-dimensional (3-D) structure calculations and refinements made use of the torsion angle molecular dynamics and the internal variable dynamics modules[13] of Xplor-NIH (v. 2.31),[14] with patches for the amide bridge and ring closure. The target function minimized was comprised of the experimental NMR restraints (ROE-derived interproton distances and torsion angles), a repulsive van der Waals potential for the non-bonded contacts,[15] a torsion angle database potential of mean force,[16] and a gyration volume potential.[17] The RMSD values for each peptide structure are listed in Table S-7. PyMOL[18] and Chimera[19] were used for visual analysis and presentation of the peptide structures.

The pdb files for AIP-III Amide, AIP-III D4A Amide, and tAIP-III D2A Amide are included as additional Supporting Information.

Tables of NMR chemical shift assignments, numbers of assigned ROEs, and RMSD values.

Table S-4. Chemical shift assignments for AIP-III Amide.

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ile1		3.76		Hγ 1.40, 1.11, 0.89; Hδ 0.82
Asn2	8.58	4.51	2.72, 2.66	Hδ 7.40, 6.69
Dap3	8.26	4.34	3.70, 3.34	Hγ 7.46
Asp4	8.42	4.26	2.62	
Phe5	7.58	4.56	2.94, 2.88	Hδ 7.14; Hε 7.27; Hζ 7.23
Leu6	8.74	3.80	1.50	Hγ 1.13; Hδ 0.78, 0.71
Leu7	7.94	4.24	1.56	Hγ 1.52; Hδ 0.81, 0.74

Table S-5. Chemical shift assignments for AIP-III D4A Amide.

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ile1		3.74	1.84	Hγ 1.38, 1.09, 0.87; Hδ 0.82
Asn2	8.51	4.58	2.69, 2.59	Hδ 7.40, 6.70
Dap3	7.92	4.30	3.53, 3.27	Hγ 7.14
Ala4	8.20	4.08	1.14	
Phe5	8.33	4.06	3.12	Hδ 7.14; Hε 7.29
Leu6	7.89	3.95	1.82, 1.52	Hγ 1.39; Hδ 0.82, 0.77
Leu7	7.84	4.21	1.74	Hγ 1.61; Hδ 0.89, 0.80

Table S-6. Chemical shift assignments for tAIP-III D2A Amide.

Residue	HN (ppm)	Hα (ppm)	Hβ (ppm)	Others (ppm)
Ac0		1.87		
Dap1	7.85	4.29	3.54, 3.23	Hγ 7.11
Ala2	8.16	4.11	1.15	
Phe3	8.34	4.06	3.13	Hδ 7.14; Hε 7.29; Hζ 7.23
Leu4	7.86	3.97	1.81, 1.53	Hγ 1.39; Hδ 0.82, 0.77
Leu5	7.84	4.23	1.75	Hγ 1.63; Hδ 0.90, 0.80

Table S-7. Number of assigned ROEs and RMSD values for each peptide.

Peptide name	Total ROEs	Intra-residue ROEs	Inter-residue ROEs			Backbone RMSD (Å)[a]	Heavy atom RMSD (Å)[a]
			i - i+1	*i - i+2*	other		
AIP-III[b]						0.25	0.84
AIP-III D4A[b]						0.42	0.84
tAIP-III D2A[b]						0.22	0.79
AIP-III Amide	105	66	29	5	5	0.13	0.64
AIP-III D4A Amide	112	70	27	6	9	0.43	0.85
tAIP-III D2A Amide	85	53	21	3	8	0.01	0.32

[a] RMSD values calculated for the 10-structure ensemble of each peptide. [b] Control compound. Data reproduced from reference [7].

Additional images of calculated peptide structures.

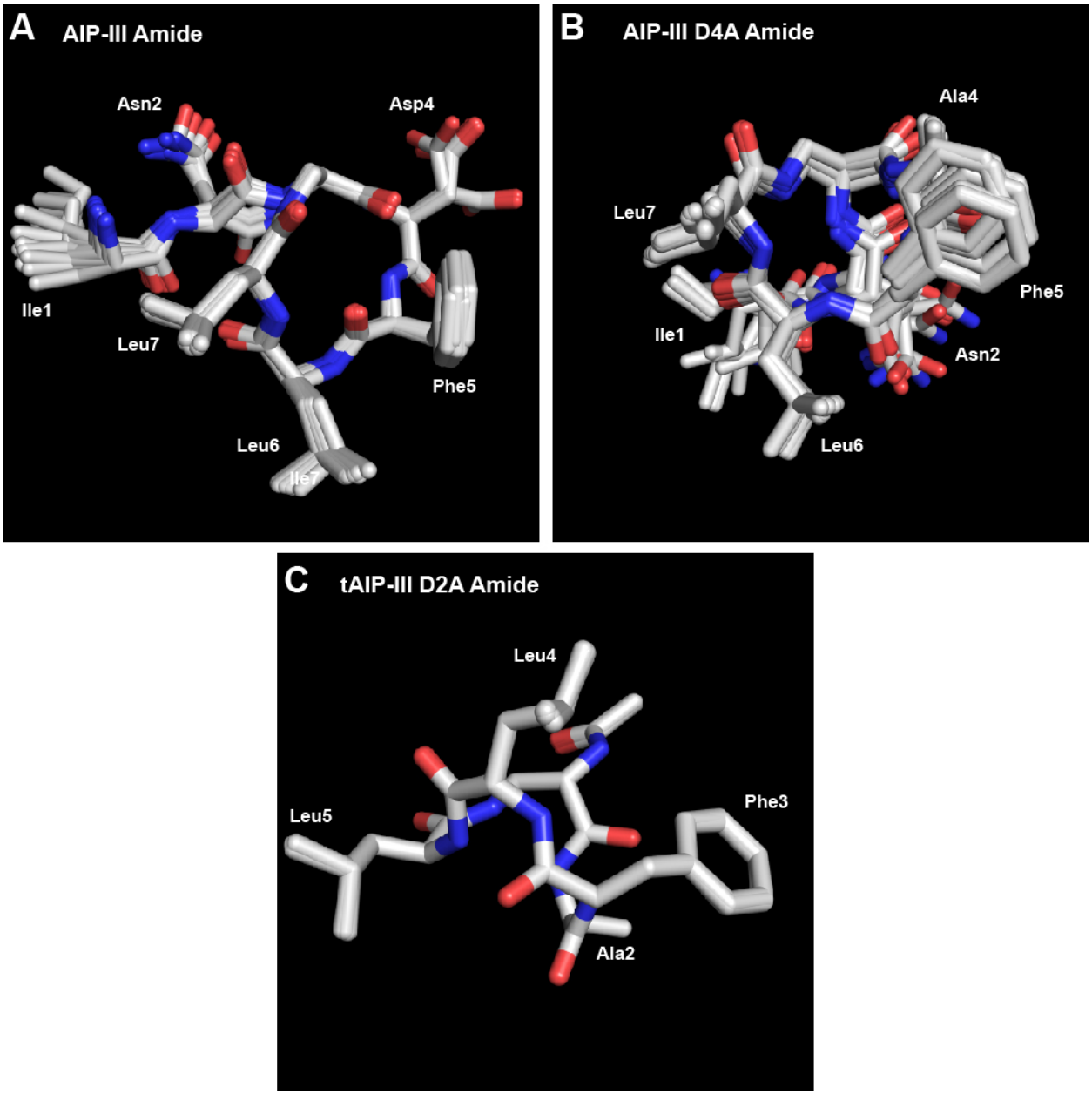


Figure S-3. Images of the heavy atom 10-structure ensembles for A) AIP-III Amide; B) AIP-III D4A Amide; and C) tAIP-III D2A Amide. For all structures, carbon is silver, oxygen is red, and nitrogen is blue.

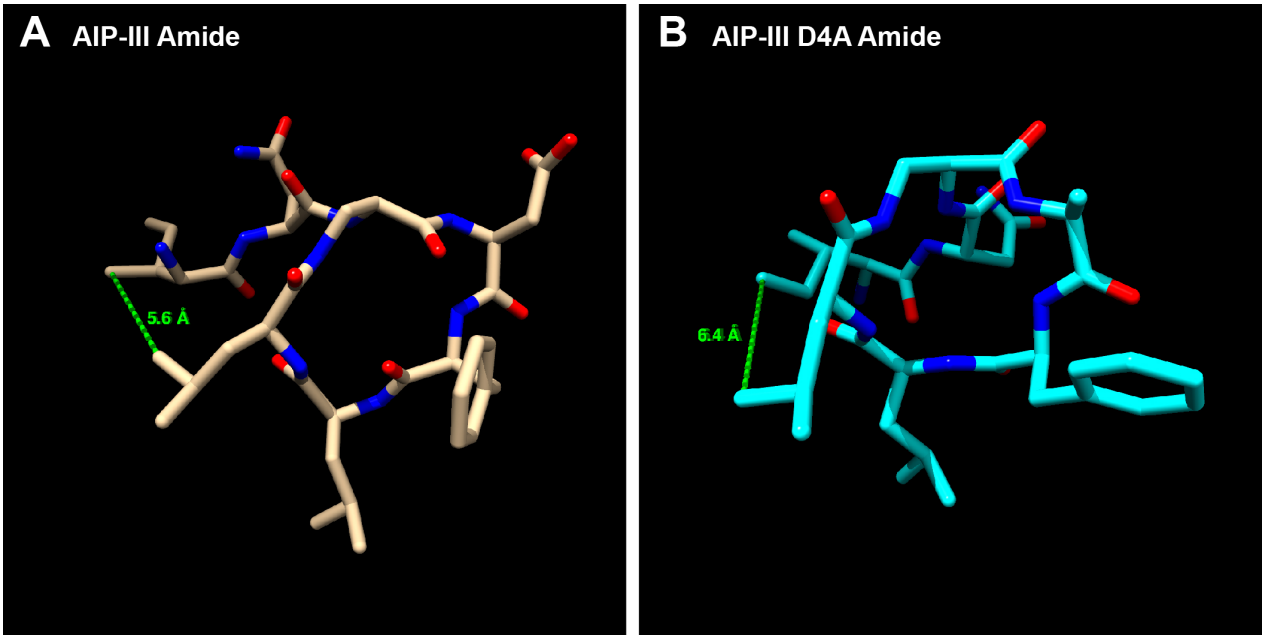


Figure S-4. Images of the heavy atom lowest energy structures for A) AIP-III Amide and B) AIP-III D4A Amide showing the distance between the Ile1 and Leu7 side chains. The Ile1 side chain in AIP-III Amide is almost 1 Å closer to the triangular knob than the same side chain in AIP-III D4A Amide. The difference in the activity profiles of these two analogues could result, in part, from the proximity differences between the exocyclic Ile1 side chain and the endocyclic knob motif. See main text.

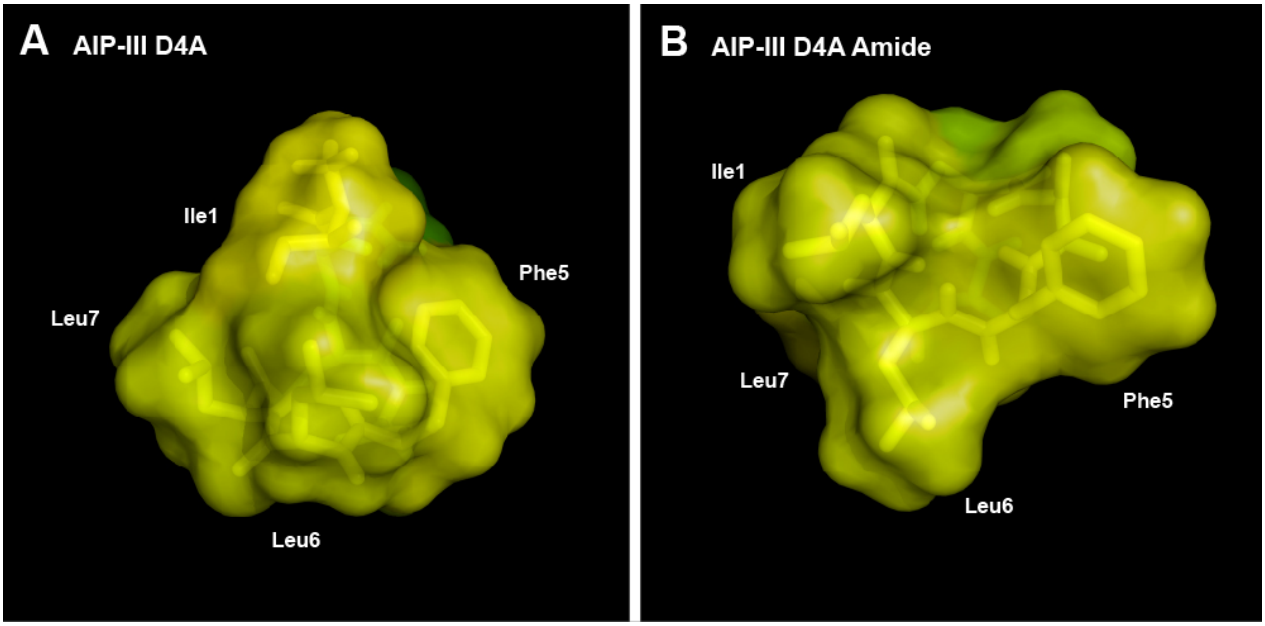


Figure S-5. Space-filling models of A) AIP-III D4A and B) AIP-III D4A Amide displaying hydrophobic (yellow) and hydrophilic (green) surfaces. These models are positioned to showcase the endocyclic, hydrophobic knob motif.

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