SUPPORTING INFORMATION

N-Methyl and Peptoid Scans of an Autoinducing Peptide Reveal New Structural Features Required for Activation and Inhibition of AgrC Quorum Sensing Receptors in *Staphylococcus aureus*

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Contents.

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Experimental procedures – Chemistry.

Chemical reagents and instrumentation

All chemical reagents were purchased from commercial sources (Alfa-Aesar, Sigma-Aldrich, and Acros) and used without further purification. Solvents were purchased from commercial sources (Sigma-Aldrich and J.T. Baker) and used as obtained, with the exception of anhydrous dichloromethane (CH_2Cl_2), which was stored over molecular sieves. Water (18 MΩ) was purified using a Millipore Analyzer Feed System. Solid-phase resin was purchased from Chem-Impex International. Fmoc-protected *N*-methyl amino acids were purchased from Neuland Laboratories.

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. Peptide thioesters were purified using a linear gradient (75% → 45% A over 30 min). Cyclic peptides were purified using a linear gradient (70% → 55% A over 27 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 spectrometer. The samples were sprayed with a sample cone voltage of 20 V. MS/MS measurements were made with a Bruker ULTRAFLEX III (MALDI-TOF/TOF) mass spectrometer equipped with a SmartBeam laser and a LIFT cell.

Structures of the AIP-III analogues in this study

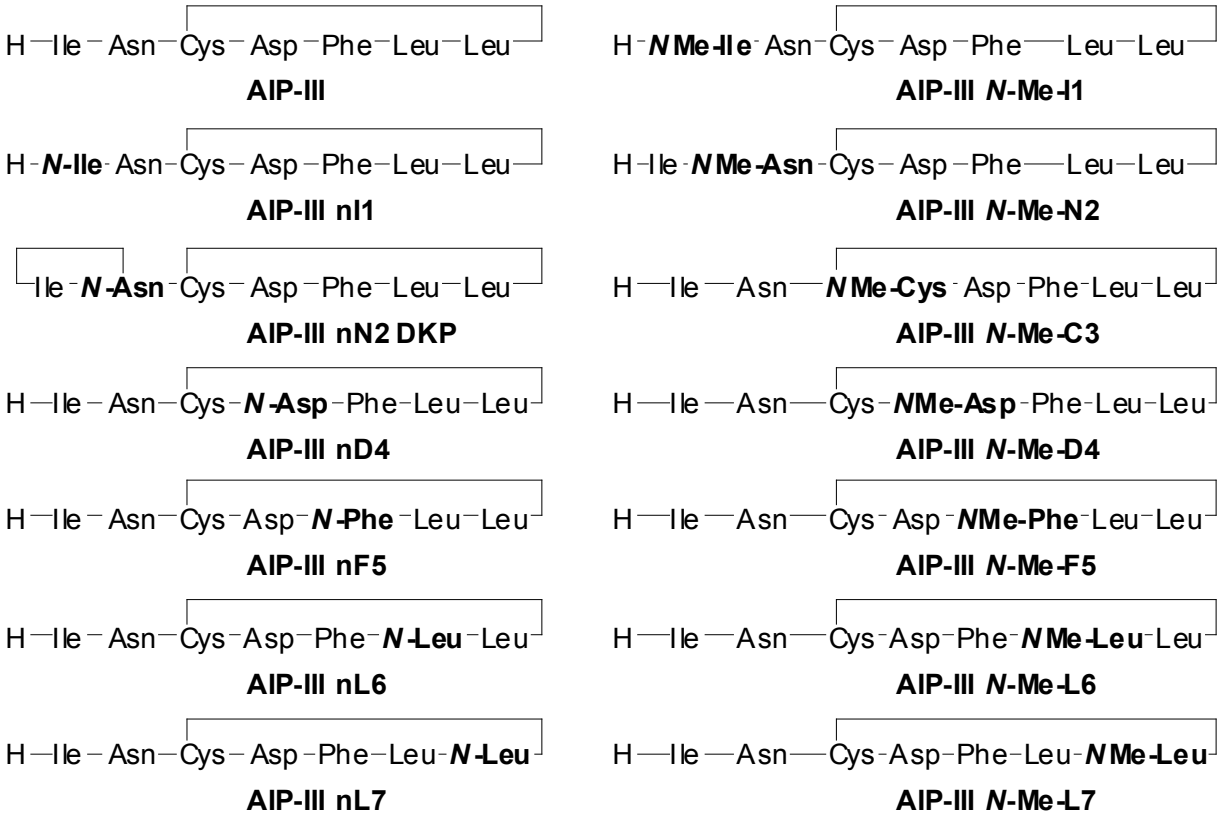


Figure S-1. Structures of AIP-III and the AIP-III analogues synthesized and evaluated in this study.

Overview of peptide synthesis approach

We recently reported a method for the expedient synthesis of AIPs and analogues thereof.[1] This route involves the solid-phase synthesis of linear peptide thioesters using Fmoc peptide assembly on 4-hydroxymethyl phenylacetamidomethyl (PAM) resin, followed by chemoselective macrocyclization. However, this protocol was not suitable for the synthesis of peptides containing *N*-alkylated amino acids (*e.g.*, peptoid residues) at the C-terminus due to a well-known side reaction, diketopiperazine (DKP) formation, observed for C-terminal proline-containing peptides and *N*-methylated peptides.[2-4] To circumvent this side reaction and minimize DKP formation, we took advantage of the acid-stability of PAM resin, a resin initially designed for Boc chemistry, and synthesized the linear peptide thioesters using Boc/Fmoc hybrid peptide assembly. The three C-terminal residues (Phe-Leu-Leu) were introduced using standard Boc chemistry, whereas the four N-terminal residues (Ile-Asn-Cys-Asp) were introduced using standard Fmoc chemistry. Peptoid residues were installed using the well-developed sub-monomer protocol,[5] while *N*-methylated amino acids were purchased and introduced through standard peptide coupling protocols. Using this synthetic route, we were able to synthesize all the

AIP-III analogues for this study in acceptable yields (5–20% isolated yields) with the exception of AIP-III nN2, which we found to serendipitously react in aqueous solutions to yield an alternate product (see below). All peptide products were purified to homogeneity by RP-HPLC (see Table S-1) and obtained on milligram scale. Full details of our synthesis and purification methods are provided below.

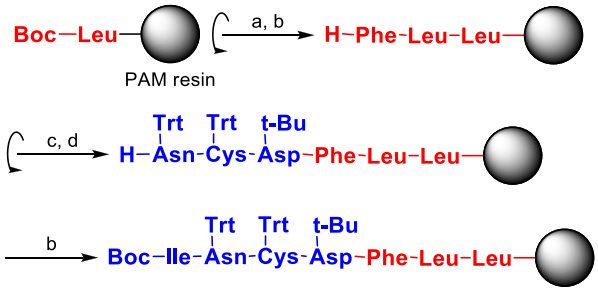
Linear peptide synthesis protocols

Typical solid-phase peptide synthesis (SPPS) was performed using Boc-protected, L-leucine pre-loaded PAM resin (0.8 mmol/g). However, *N*-Me-Leu7 and nLeu7 were manually loaded onto PAM resin to generate the two carboxy terminal modified AIP-III analogues (AIP-III *N*-Me-L7 and AIP-III nL7), as follows.

To generate the *N*-Me-Leu7 analogue, aminomethyl polystyrene (AM) resin (100 mg, 1.17 mmol/g) was swelled in diisopropylethylamine (DIPEA; 10% in CH_2Cl_2; 2 mL) for 10 min. The resin was drained, and washed with DIPEA (10% in CH_2Cl_2; 3 x 1 mL) and dimethylformamide (DMF; 3 x 2 mL). Next, 4-(bromomethyl) phenylacetic acid (1.4 equiv.) and *N,N'*-diisopropylcarbodiimide (DIC; 1.7 equiv.) were dissolved in CH_2Cl_2. This solution was added to the resin, and the suspension was agitated overnight at rt. The resulting PAM resin was drained, washed with DMF (3 x 2 mL) and CH_2Cl_2 (3 x 2 mL), and then suspended in DMF. Fmoc-*N*-Me-Leu-OH (2 equiv.) and DIPEA (4 equiv.) were dissolved in DMF and added to the resin, and the suspension was agitated overnight at rt. The resin was drained, washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL), and treated with a solution of acetic anhydride (10 equiv.) and DIPEA (7 equiv.) in DMF for 15 min. The resin was again drained, washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL), and subjected to a chloranil test to confirm the absence of free amines (see procedure below).

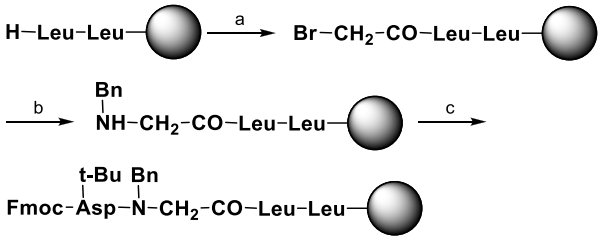
To generate the nLeu7 analogue, PAM resin (100 mg, 0.85 mmol/g) was swelled in a minimal amount of DMF (1 mL) for 30 min. Bromoacetic acid (10 equiv.) was dissolved in dry CH_2Cl_2 at 0 °C. DIC (5 equiv.) was added to this solution, the solution was stirred at 0 °C for 20 min, and the solvent was removed *in vacuo*. The resulting solid was dissolved in a minimal amount of DMF (1 mL), and 4-dimethylaminopyridine (DMAP; 0.1 equiv.) was added. The solution was added to the resin and agitated for 1 h at rt. The resin was drained and washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL). Isobutyl amine (10 equiv.) was dissolved in DMF (2 mL). DIPEA (10 equiv.) was added, and the solution was allowed to pre-activate for 1 min at rt. The solution was added to the resin, and the suspension was agitated overnight at 50 ºC. The resin was drained, washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL), and subjected to a chloranil test to assess to assess reaction completion.

Linear peptide elongation was performed using standard Boc *in situ* neutralization/activation protocols[6] for residues Phe5–Leu7 and our previously reported SPPS protocols[1] for residues Ile1–Asp4 (Scheme S-1).



Scheme S-1. Boc/Fmoc hybrid peptide assembly for the preparation of linear AIP-III analogues. Reagents: a. 50% TFA in CH_2Cl_2; b. Boc-AA-OH, HBTU, DIPEA (4:4:6 equiv.); c. Fmoc-AA-OH, HBTU, DIPEA (2:2:2 equiv.); d. 20% Piperidine in DMF.

Peptoid residues were installed using the two-step sub-monomer method (Scheme S-2).[5] First, bromoacetic acid (10 equiv.) was dissolved in DMF (2 mL). DIC (10 equiv.) was added, and the solution was pre-activated for 20 min at rt. The solution was added to the resin, and the suspension was agitated for 30 min at rt. The resin was drained, and the procedure was repeated. The resin was washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL), followed by a chloranil test to assess reaction completion. Next, the desired primary amine (10 equiv.) was dissolved in DMF (2 mL). DIPEA (10 equiv.) was added, and the solution was allowed to pre-activate for 1 min at rt. The solution was added to the resin, and the suspension was agitated overnight at 50 ºC. The resin was drained, washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL), and subjected to a chloranil test to assess reaction completion.



Scheme S-2. Introduction of peptoid residues. Reagents: a. $BrCH_2COOH$, DIC (10:10 equiv.); b. Benzylamine, DIPEA (10:10 equiv.); c. Fmoc-Asp(t-Bu)-OH, HATU, DIPEA (4:4:4 equiv.).

Fmoc-protected *N*-methylated amino acids were introduced using standard Fmoc-SPPS protocols, as we previously described.[1]

Couplings to secondary amines, either following an *N*-methylated amino acid or a peptoid residue, were performed as follows: a Boc or Fmoc-protected amino acid, depending on the position in the sequence (see above, 4 equiv.), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 4 equiv.), and DIPEA (4 equiv.) were dissolved in DMF (2 mL). The solution was allowed to pre-activate for 1 min, after which it was added to the resin and the resulting suspension was agitated for 2 h at rt. The resin was drained, washed with DMF (2 x 2 mL) and CH_2Cl_2 (2 x 2 mL), and subjected to a chloranil test to assess

reaction completion. If the test indicated the presence of free amines, the procedure was repeated and the reaction was left overnight at 50 ºC. After the second coupling cycle, peptide elongation was continued even if the chloranil test indicated that free amines were still present.

Choranil test protocol: A sample containing 1–3 mg of the resin was withdrawn and placed into a test tube. Acetaldehyde (2% in DMF; 2 drops) and chloranil (2% in toluene; 2 drops) were added to the sample. The solution was agitated at rt for 5 min. The beads turning a green or blue color was a positive indication of the presence of free amines, signifying either deprotection of the amine protecting group or an incomplete amide coupling reaction.

PAM resin cleavage protocol

Upon synthesis of a complete linear peptide sequence, the resin was washed with diethyl ether (1 x 2 mL) and dried under vacuum for 48 h. The linear peptidyl-resin (90 mg) was placed in a dry, three-neck round-bottom flask and suspended in anhydrous CH_2Cl_2 under argon at rt for 15 min. Me_2AlCl (20 equiv., 1 mL of 1 M hexane solution) and anhydrous CH_2Cl_2 (3 mL) were stirred in a separate, dry round-bottom flask under argon at 0 °C for 5 min. Ethanethiol (EtSH) (60 equiv.) was added drop-wise at 0 °C to this flask, and the solution was stirred for 15 min at 0 °C. This solution was then added to the suspended resin and stirred under argon for 5 h at rt to effect cleavage of the linear peptide as a thioester. The cleavage product solution (including the suspended resin) was transferred to a new round-bottom flask containing a TFA solution (95% (aq.), 3 mL), the solution was swirled around for 1 min, and the solvents were removed *in vacuo*. The resulting yellow or orange oil was subjected again to TFA solution (3 mL), allowed to stir at rt for 30 min, and the resin was removed by filtration. The resin was then washed with TFA solution (1 x 2 mL) to collect any additional peptide. The filtrates were combined, and a cooled solution of diethyl ether:hexane (1:1, 40 mL, 0 °C) was added to effect peptide precipitation. The peptide was allowed to further precipitate overnight in a freezer at -20 °C. The precipitated peptide solution was centrifuged, and the supernatant was removed to yield a white solid. This solid was dissolved in ACN (50% (aq.)), lyophilized, redissolved in ACN (50% (aq.)) and purified by semi-preparative RP-HPLC. Collected HPLC fractions were lyophilized to yield the peptide thioester as a white powder (5–20% isolated yields), and its identify was confirmed by MS (see Table S-1).

Peptide macrocyclization protocol

Purified peptide thioester was dissolved in a 60% guanidinium chloride (6 M solution in 0.1 M phosphate buffer):40% ACN solution to a final concentration ranging from 100 μM to 2 mM. The pH of the solution was adjusted to 6.8, and the solution was agitated at 50 ºC for 2 h. The macrocyclic peptide product was purified by semi-preparative RP-HPLC. Collected HPLC fractions were lyophilized to give a white powder (80–90% isolated yields) and analyzed by MS (see Table S-1). The cyclic product was then dissolved in 1 M hydrochloric acid (400 μL) and lyophilized prior to bioanalysis. The structures of the cyclic products are shown in Figure S-1.

Diketopiperazine (DKP) synthesis and quantification protocol in model peptides

Upon synthesis of a complete linear peptide sequence, the resin was washed with diethyl ether (1 x 2 mL) and air dried for 30 min. The resin (20–30 mg) was treated with TFA solution (95% (aq.), 2 mL) for 30 min, and the solution was filtered from the resin. The resin was washed with TFA (1 x 1 mL) to collect any additional peptide. The filtrates were combined, a cooled solution of diethyl ether:hexane (1:1, 10 mL, 0 °C) was added, and the peptide was allowed to precipitate for 2 h in a freezer at -20 °C. The precipitated peptide solution was centrifuged, and the supernatant removed to yield a white solid. This solid was dissolved in ACN (50% (aq.)), lyophilized, redissolved in ACN (10% (aq.)) and analyzed by RP-HPLC and MALDI-TOF MS to determine the percentage of DKP formation. See DKP section below for further details.

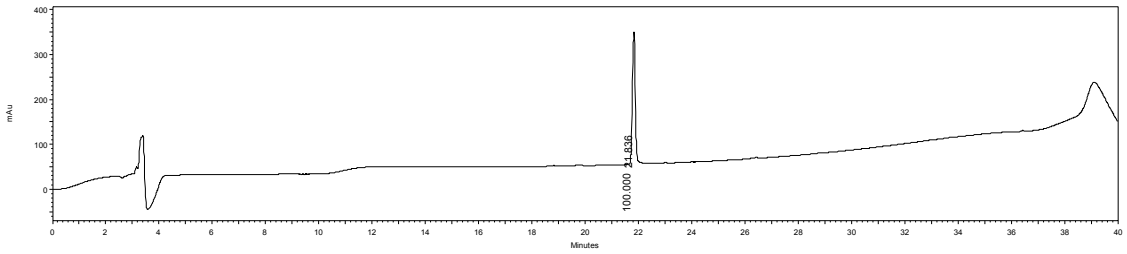
MS and HPLC data for AIP-III analogues.

Table S-1. MS and HPLC data for AIP analogues. Rt = retention time (minutes). EM = Exact Mass. See above for methods.

Compound Name	Structure	Thio-ester (-S-Et)							Cyclic peptide			
		Calc. MH^+	*Meas. MH^+*	*Calc. MNa^+*	*Obs. MNa^+*	*Calc. MK^+*	*Obs. MK^+*	*Rt HPLC*	*Calc. EM*	*Obs. EM*	*Rt HPLC*	*% Purity*
AIP-III *N*-Me-I1	***N*Me**I-N-(C-D-F-L-L)	895.4	895.3	917.4	917.3	933.4	933.3	22.7	833.4226	833.4214	21.8	>99
AIP-III *N*-Me-N2	I-***N*Me**N-(C-D-F-L-L)	895.4	895.3	917.4	917.4	933.4	933.4	22.9	833.4226	833.4236	21.9	>99
AIP-III *N*-Me-C3	I-N-(***N*Me**C-D-F-L-L)	895.4	895.3	917.4	917.3	933.4	933.3	22.9	833.4226	833.4214	21.7	>99
AIP-III *N*-Me-D4	I-N-(C-***N*Me**D-F-L-L)	895.4	895.1	917.4	917.1	933.4	933.1	22.9	833.4226	833.4243	22.1	>99
AIP-III *N*-Me-F5	I-N-(C-D-***N*Me**F-L-L)	895.4	-	917.4	917.3	933.4	933.3	23.1	833.4226	833.4235	21.8	>99
AIP-III *N*-Me-L6	I-N-(C-D-F-***N*Me**L-L)	895.4	-	917.4	917.3	933.4	933.3	23.4	833.4226	833.4246	22.6	>99
AIP-III *N*-Me-L7	I-N-(C-D-F-L-***N*Me**L)	895.4	895.2	917.4	917.4	933.4	933.3	23.4	833.4226	833.4229	22.4	>99
AIP-III nI1	**nI**-N-(C-D-F-L-L)	881.4	881.2	903.4	903.2	919.4	919.2	22.6	841.3889 (MNa^+)	841.3885	21.7	>99
AIP-III nN2	I-**nN**-(C-D-F-L-L)	881.4	881.2	903.4	903.2	919.4	919.2	22.4	Degrades to AIP-III nN2 DKP			
AIP-III nN2 DKP	(I-**nN**)-(C-D-F-L-L)	864.4	-	886.4	886.1	902.4	902.0	26.1	824.3624 (MNa^+)	824.3624	24.4	>99
AIP-III nD4	I-N-(C-**nD**-F-L-L)	881.4	881.3	903.4	903.3	919.4	919.3	22.5	819.4069	819.4053	21.6	>99
AIP-III nF5	I-N-(C-D-**nF**-L-L)	881.4	881.4	903.4	903.4	919.4	919.4	22.6	819.4069	819.4061	21.0	>99
AIP-III nL6	I-N-(C-D-F-**nL**-L)	881.4	881.2	903.4	903.2	919.4	919.3	22.8	819.4069	819.4088	21.4	>99
AIP-III nL7	I-N-(C-D-F-L-**nL**)	881.4	-	903.4	903.4	919.4	919.4	22.7	819.4069	819.4030	21.9	>99

HPLC traces for AIP-III analogues.

AIP-III *N*-Me-I1



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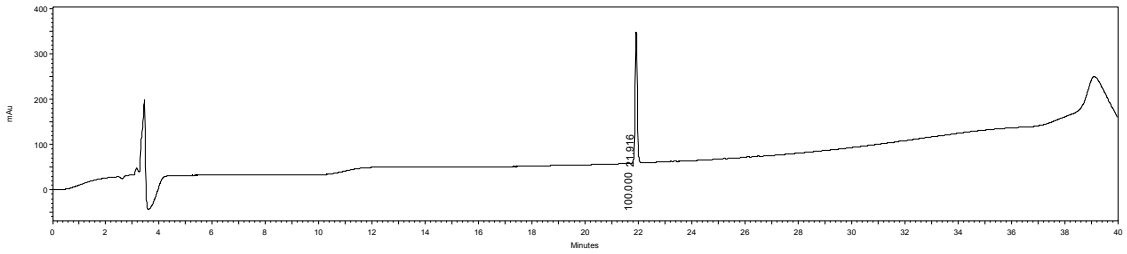

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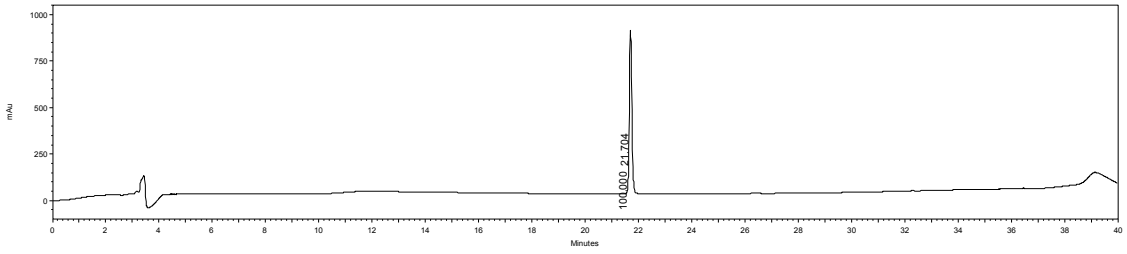
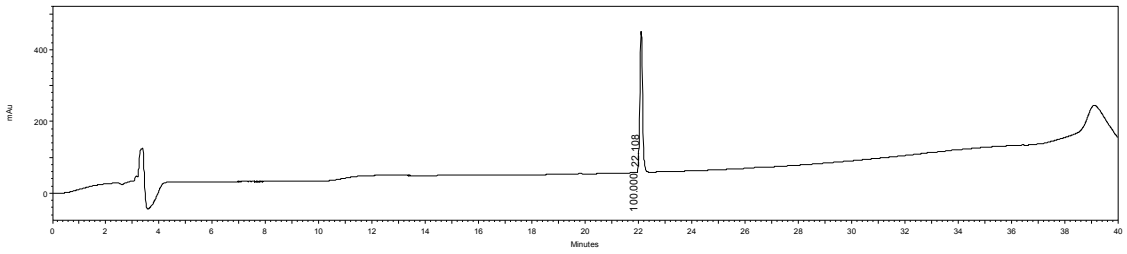
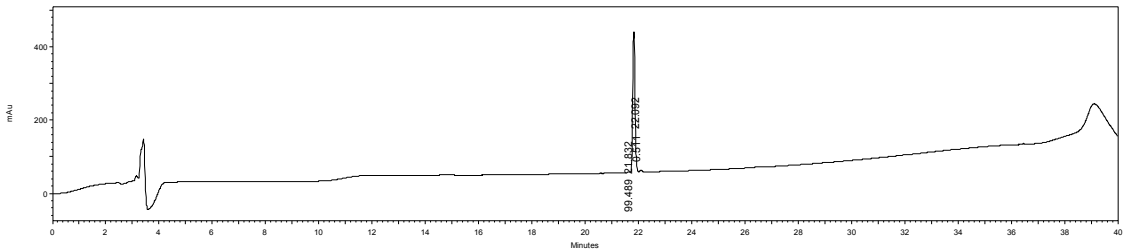


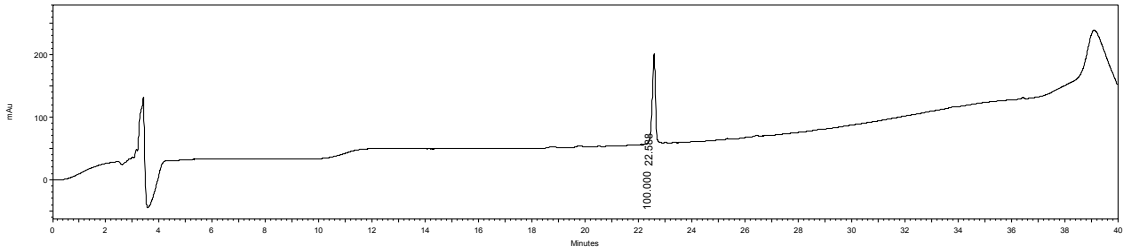
AIP-III *N*-Me-D4



AIP-III *N*-Me-F5



AIP-III *N*-Me-L6



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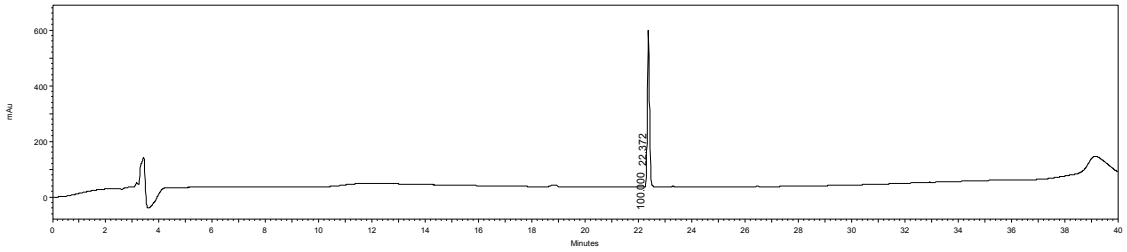
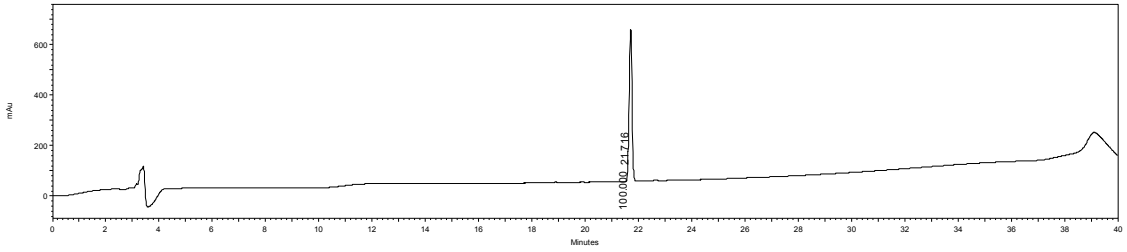
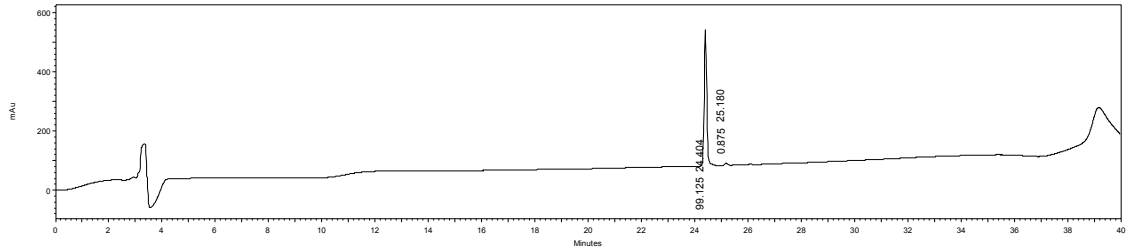


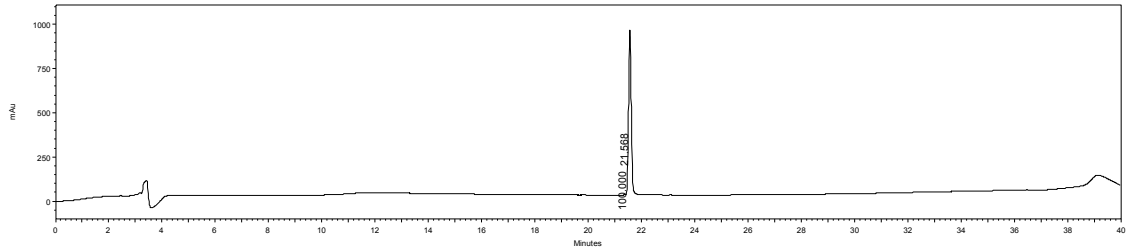
AIP-III nI1



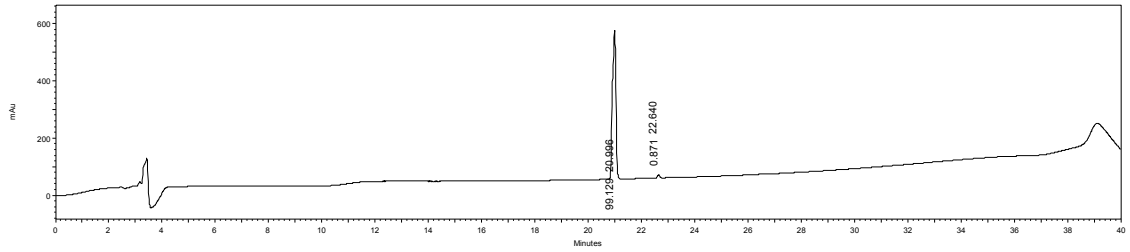
AIP-III nN2 DKP



AIP-III nD4



AIP-III nF5



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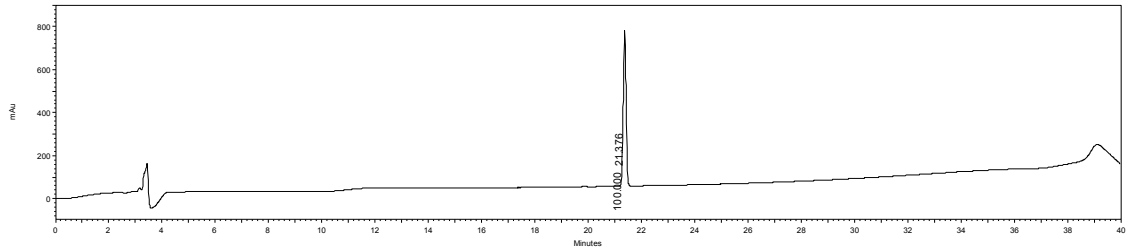
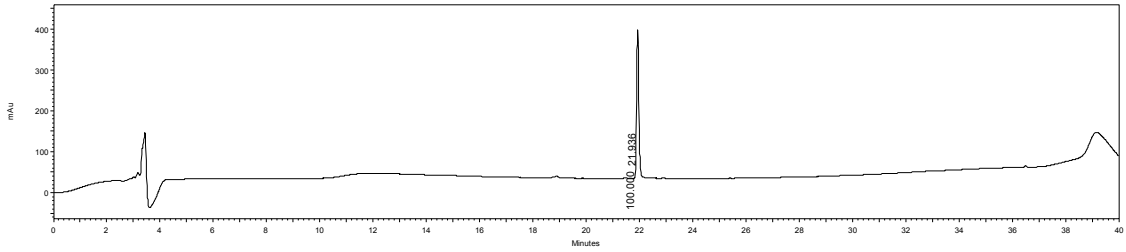


AIP-III nL7


mAu
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40
Minutes

MS/MS analysis of AIP-III nN2 thioester.

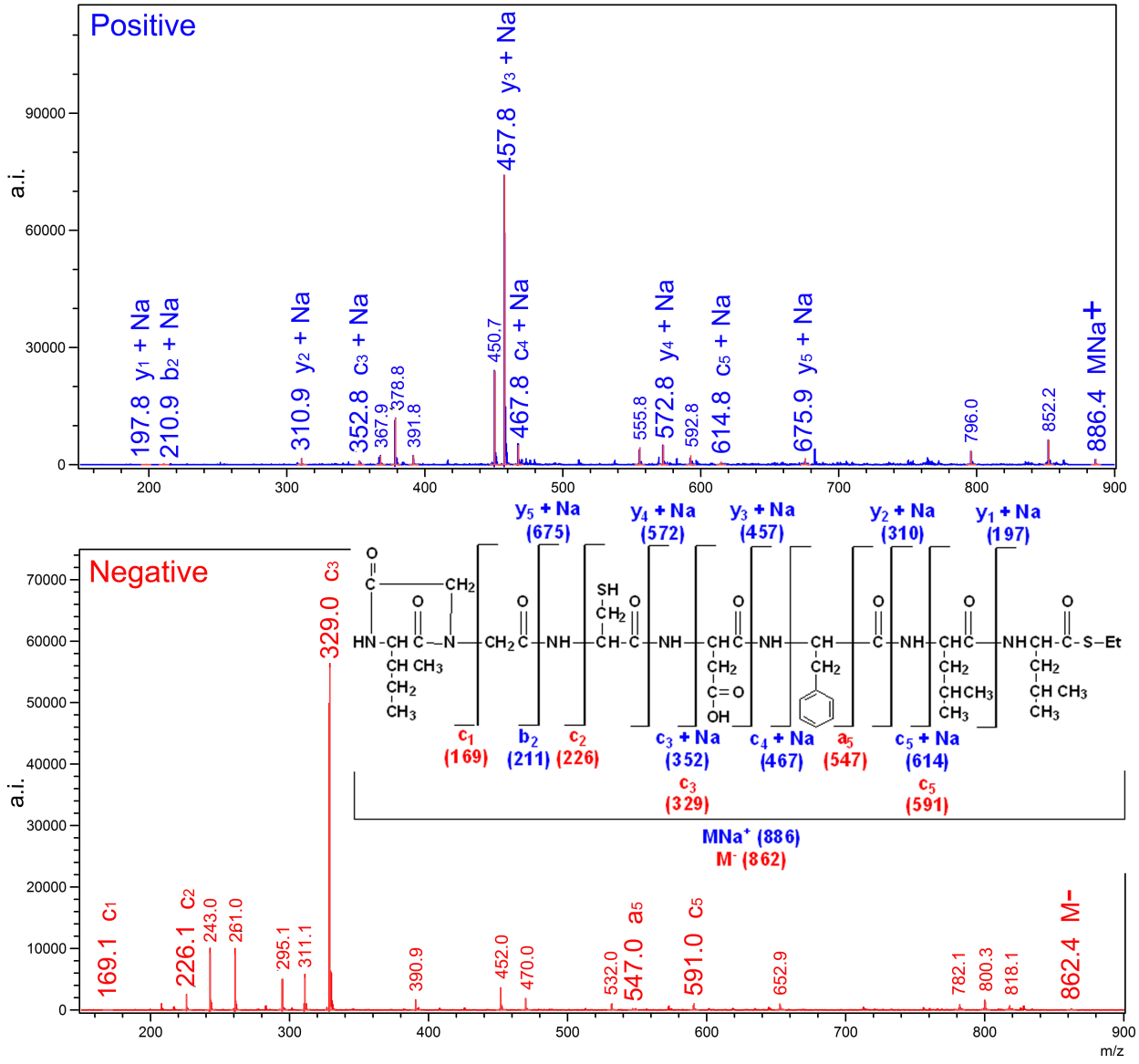


Figure S-2. Positive ion (blue spectrum) and negative ion (red spectrum) MS/MS analysis of the AIP-III nN2 thioester revealed the formation of a diketopiperazine (DKP) between the amino terminus and the side chain amide of the nAsn2 peptoid residue.

DKP formation study.

We observed clean formation of the bicyclic product AIP-III nN2 DKP upon subjection of the precursor AIP-III nN2 thioester to the macrocyclization conditions (Figures S-2 and S-3). We performed additional experiments to further explore this phenomenon, as described below.

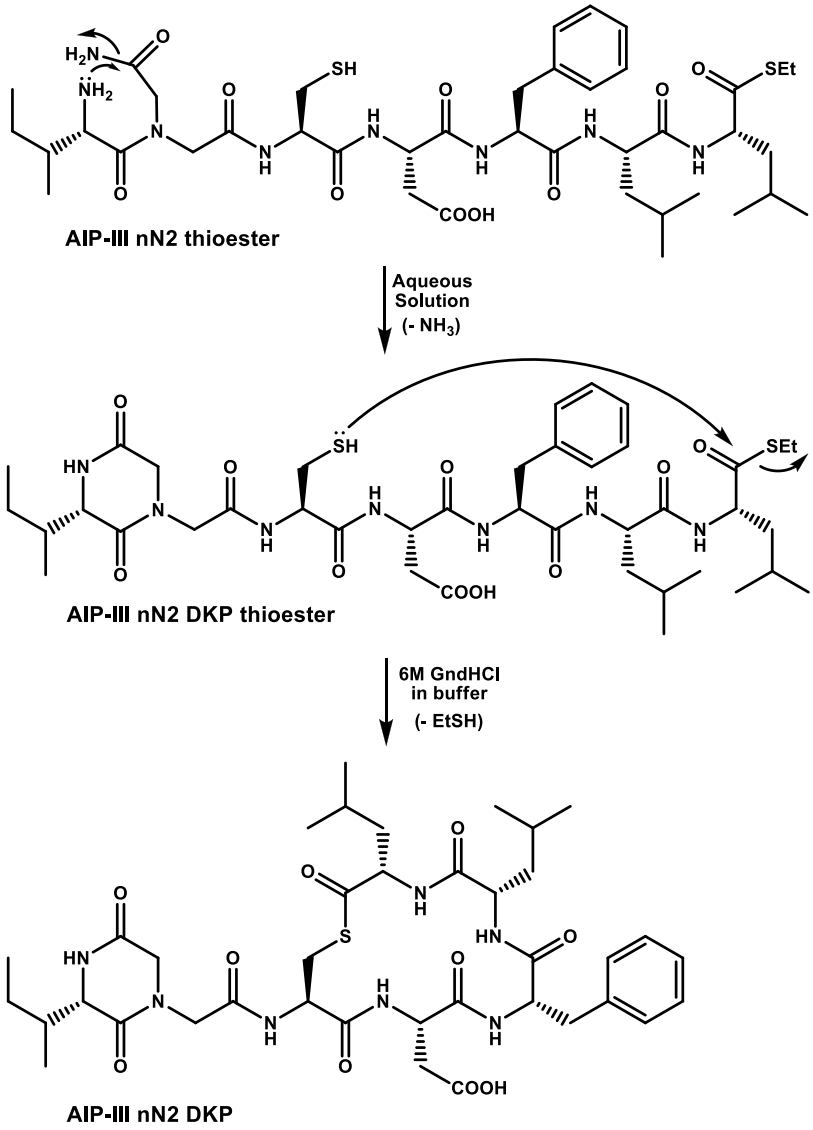


Figure S-3. Proposed pathway to AIP-III nN2 DKP. The linear AIP-III nN2 thioester undergoes a spontaneous DKP formation. The AIP-III nN2 DKP thioester then undergoes the second cyclization to form the bicyclic product AIP-III nN2 DKP.

To explore the parameters allowing for the DKP side reaction at Asn2, we synthesized a series of short model peptides (using methods described above) containing either nAsp or nAsn followed by either Boc-Gly or Fmoc-Gly at the N-terminus (see Table S-2 for structures). After Fmoc deprotection of Gly, the peptides were cleaved from the resin and DKP formation was monitored by analytical RP-HPLC and MS (using methods described above). We note that for the Fmoc-Gly analogues, DKP formation could have occurred on the resin, after Fmoc removal, or in the aqueous solutions prepared for HPLC and MS analysis. However, for the Boc-Gly analogues, DKP formation could only occur once dissolved in aqueous solution.

Table S-2. Structures of model peptides and conversion to DKP as observed by HPLC and MS.

Xaa-nAsx-Gly-Tyr-Lys-Gly-NH_2		
Xaa	nAsp	nAsn
Fmoc-Gly	DKP (> 90%)	No DKP (< 5%)[a]
Boc-Gly	No DKP (< 2%)	No DKP (< 4%)[a]
Fmoc-Ala	DKP (> 92%)	Low DKP (< 12%)[a]
Fmoc-Ile	-	Low DKP (< 11%)[a]
Fmoc-Cys(trt)	DKP (> 88%)	-
Xaa-nAsx-Phe-Leu-Leu-NH_2 (C-terminal AIP-III sequence)		
Fmoc-Cys(trt)	Partial DKP (35%)	-

[a] Only slow DKP formation was observed in aqueous solution. The rate was increased by the addition of acid. Rate of DKP formation: Ile > Ala > Gly.

For the model peptide with an nAsp residue, DKP formation did not occur at any point during or after Boc deprotection of Gly. However, as normally anticipated under basic conditions (but not observed during AIP-III nD4 synthesis), the nAsp residue was converted to the DKP immediately after Fmoc deprotection of Gly (Table S-2). As Fmoc deprotection was used during the synthesis of the AIP-III nD4 sequence, we wanted to further explore this phenomenon. We repeated the nAsp model peptide synthesis, but this time replaced the preceding Gly residue with either Fmoc-Ala, to introduce chirality and reduce flexibility, or Fmoc-Cys(trt) residue, as in the sequence of AIP-III. Again, we observed complete conversion to the DKP product immediately after Fmoc deprotection. We reasoned that DKP formation at nAsp4 in AIP-III nD4 might be hindered by the more sterically hindered, full AIP-III sequence extending to the C-terminus, so we attempted to synthesize the full length AIP-III nD4 sequence on the Rink Amide MBHA resin. As expected, only partial DKP formation was observed between the nAsp4 side chain and the Cys3 amine (35%, Table S-2), and the linear full length sequence was obtained (in 65%).

Unlike the nAsp model peptides, analogous nAsn model peptides did not undergo DKP formation immediately upon deprotection of either Fmoc-Gly or Boc-Gly. Evaluation of these linear peptides in aqueous solution over time revealed only slow conversions to DKP. This conversion was enhanced by the addition of acid, as in the case of HPLC solvents with 0.1%

TFA; however, DKP formation in the model peptides occurred at a much slower rate than observed with the AIP-III nN2 analogue (~24 h vs. ~2–3 h, respectively). We repeated the nAsn model peptide synthesis, but this time replaced the preceding Gly residue with either Fmoc-Ala or Fmoc-Ile, as in the normal AIP-III sequence. Again, the nAsn analogues did not yield much DKP product (Table S-2), but DKP conversion was enhanced when a larger alkyl side was present, presumably due to the increased nucleophilicity of the N-terminus. These observations suggest that the introduction of nAsn peptoid residues into peptide sequences may only be problematic when immediately adjacent to the N-terminal residue. We note that a recent study by Bräse and co-workers supports our finding, as they also reported the formation of DKPs when nAsn-type residues were introduced at positions adjacent to the N-terminal residue in peptides.[7]

Experimental procedures – Biology.

Biological reagents and instrumentation

All biological reagents and media were purchased from Sigma-Aldrich and used according to enclosed instructions. Tryptic soy broth (TSB) and brain heart infusion (BHI) were prepared as instructed with pH = 7.35.

The bacterial strains used in this study are listed in Table S-3. Bacterial cultures were grown in a standard laboratory incubator at 37 °C with shaking (at 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-3. *S. aureus* strains used in the two assays in this study listed according to group.

Assay-type	Strain	Reference
Fluorescence		
group-I	AH1677	8
group-II	AH430	8
group-III	AH1747	8
group-IV	AH1872	8
β-lactamase		
group-I	RN9222	9
group-II	RN9372	9
group-III	RN9532	9
group-IV	RN9371	9

Compound handling protocol

Stock solutions of synthetic peptides (1 mM) were prepared in DMSO and stored at 4 °C in sealed vials. The amount of DMSO used in biological screens did not exceed 4% (v/v). Polystyrene 96-well multititer plates (Costar) were used for screening.

Fluorescence assay protocol

Peptide stock solutions were diluted with DMSO in serial dilutions (either 1:3, 1:5, or 1:10), and 2 μL of the diluted solution was added to each of the wells in a black 96-well multititer plate. An overnight culture of *S. aureus* GFP strain was diluted 1:50 with fresh TSB. A 198-μL portion of diluted culture was added to each well of the multititer 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.

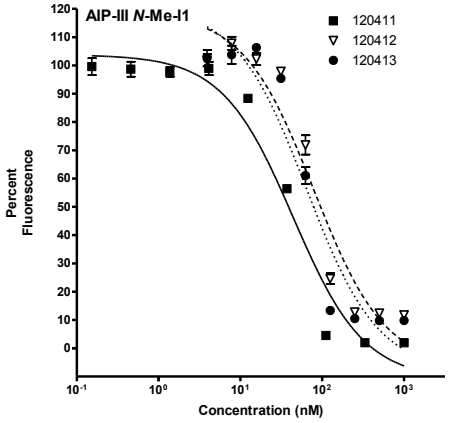
β-lactamase assay protocol

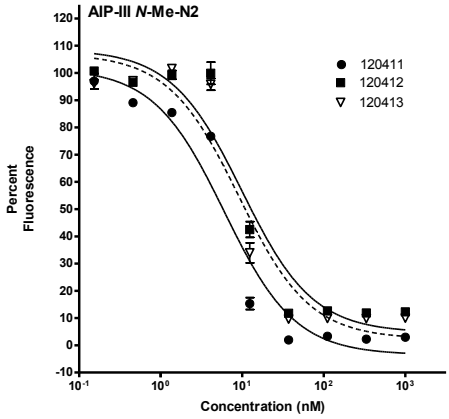
Our β-lactamase/nitrocefin assay protocol was analogous to that of Novick and co-workers,[10] except that we monitored β-lactamase activity at a single time point (at 20 min) as opposed to determining a rate profile for enzyme activity (the single time point reading gave analogous data trends). In brief, 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 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, the absorbance (at 495 nm) of each well was recorded, and EC_{50} values were calculated.

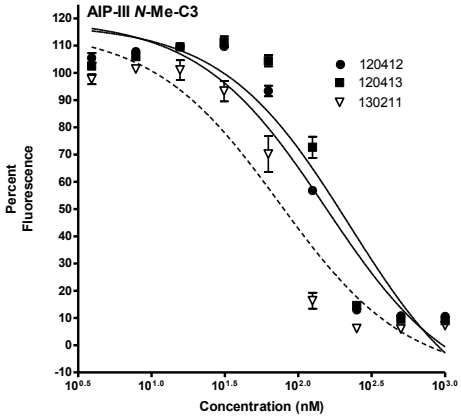
Fluorescence antagonism dose response curves.

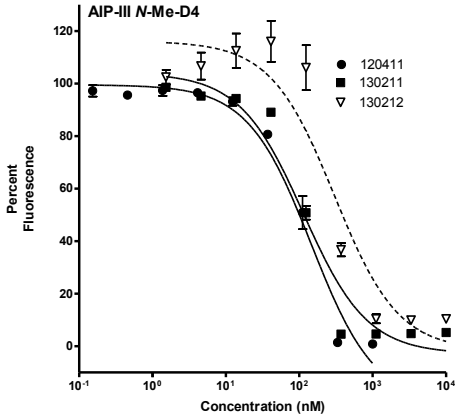
Peptides were screened over varying concentrations in the four indicated *S. aureus* reporter strains. The peptide tested in each plot below is indicted in bold at top left. Each dose response experiment was performed in triplicate on three separate occasions (*i.e.*, experiments #1–3; shown for each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

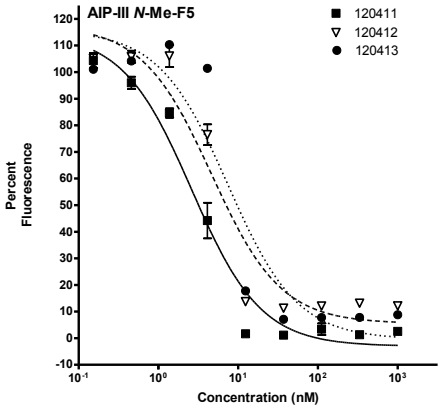
S. aureus AH1677 (group-I)

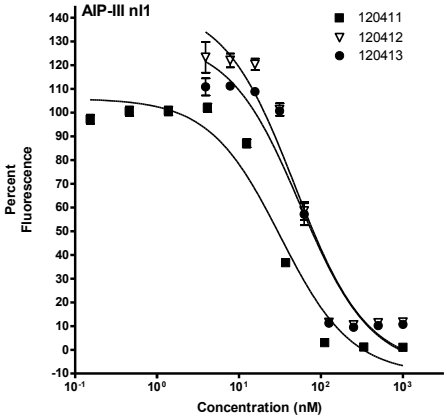


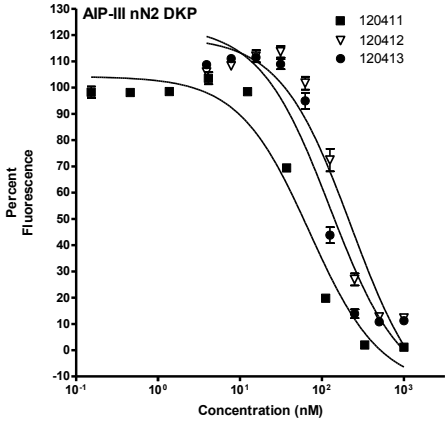


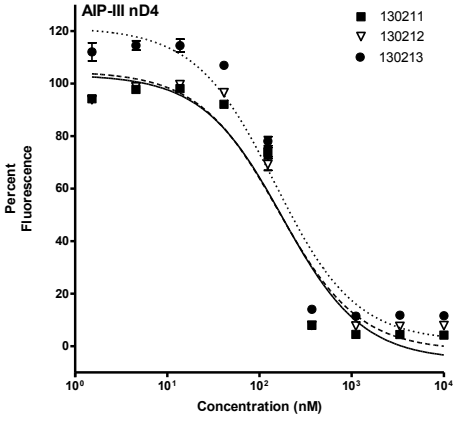


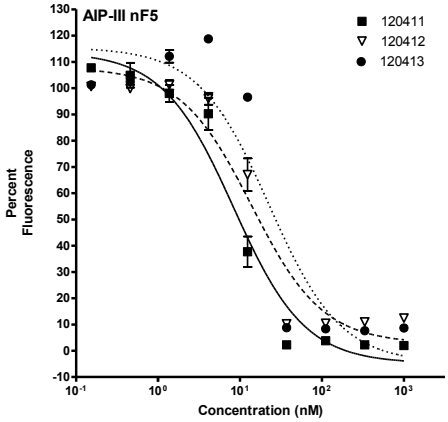



AIP-III *N*-Me-F5
120411
120412
120413
Percent Fluorescence
Concentration (nM)

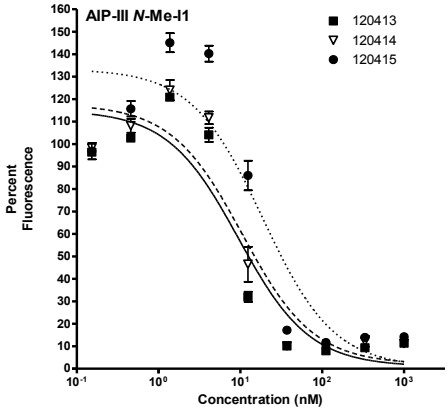

AIP-III nI1
120411
120412
120413
Percent Fluorescence
Concentration (nM)

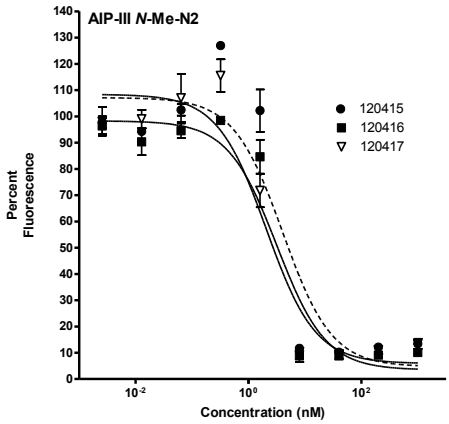

AIP-III nN2 DKP
120411
120412
120413
Percent Fluorescence
Concentration (nM)

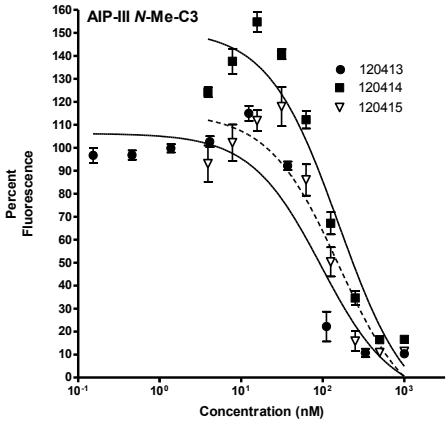

AIP-III nD4
130211
130212
130213
Percent Fluorescence
Concentration (nM)

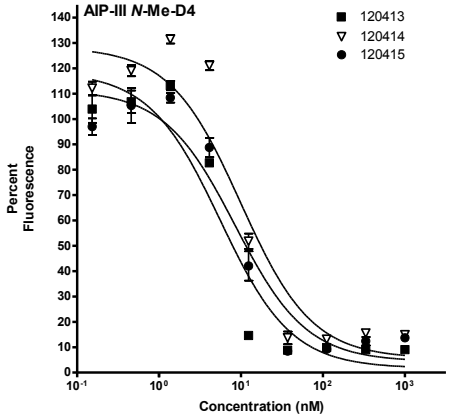

AIP-III nF5
120411
120412
120413
Percent Fluorescence
Concentration (nM)

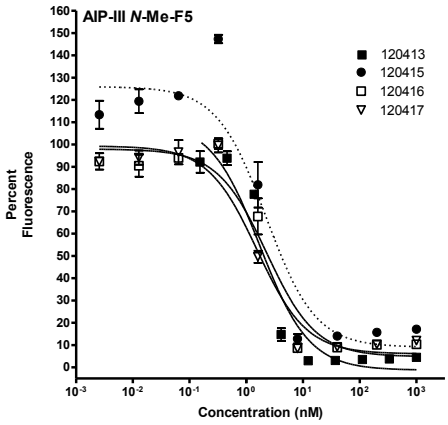
S. aureus AH430 (group-II)

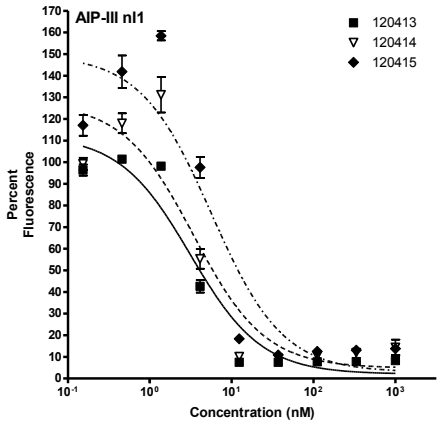


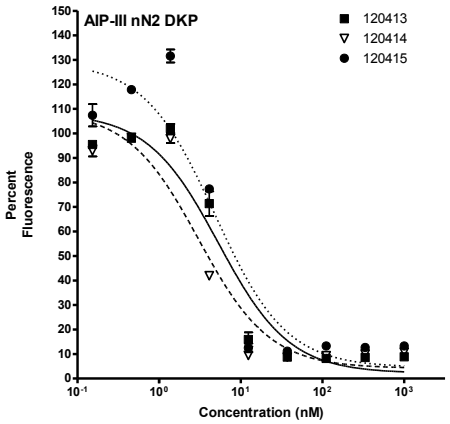


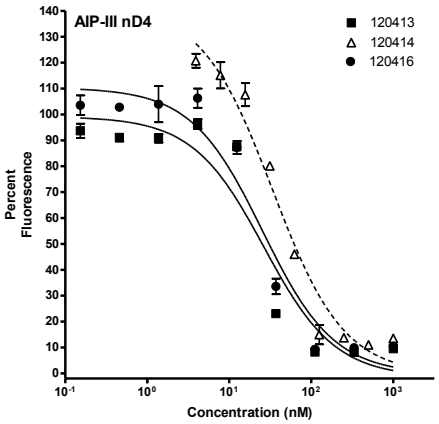


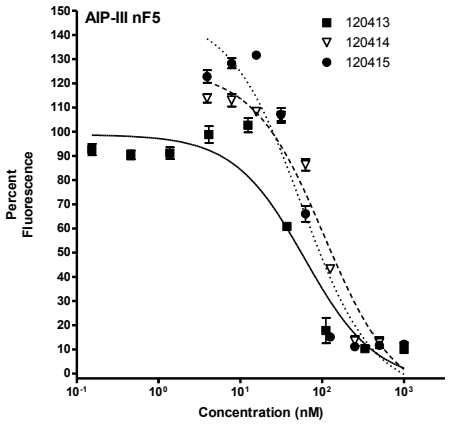




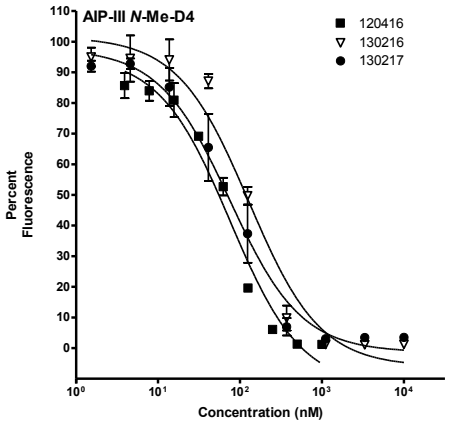


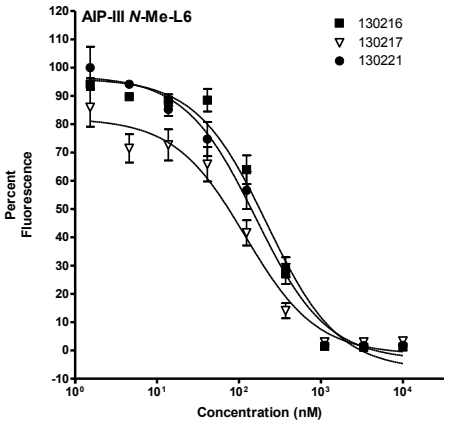


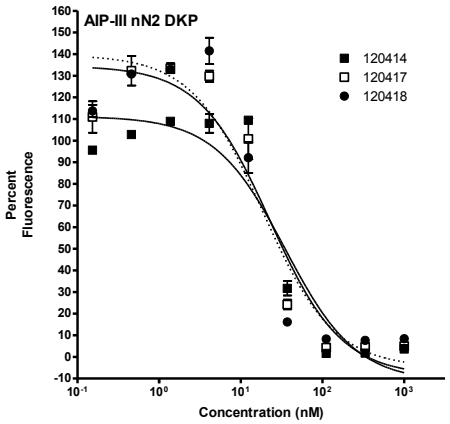


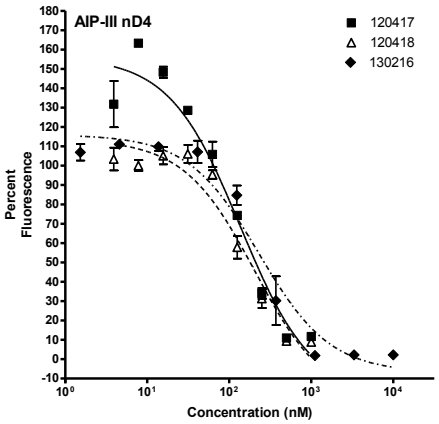


S. aureus AH1747 (group-III)

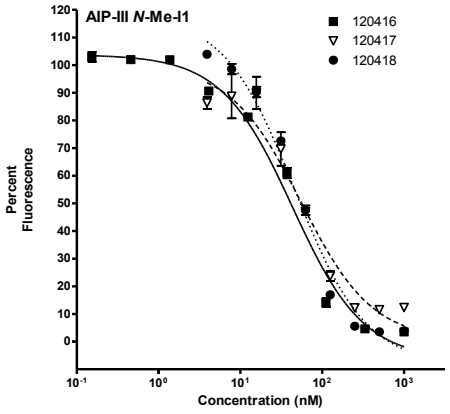


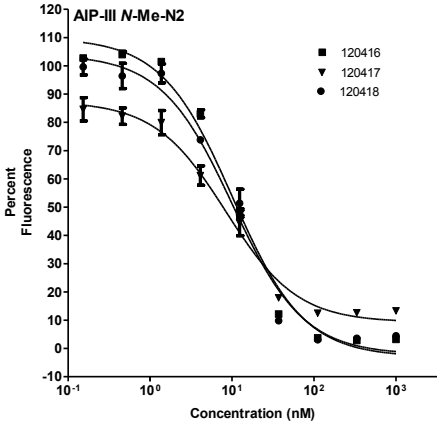


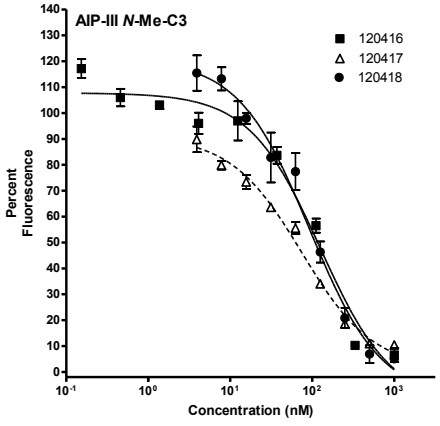


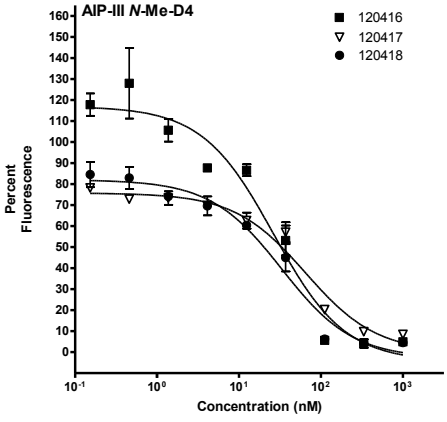


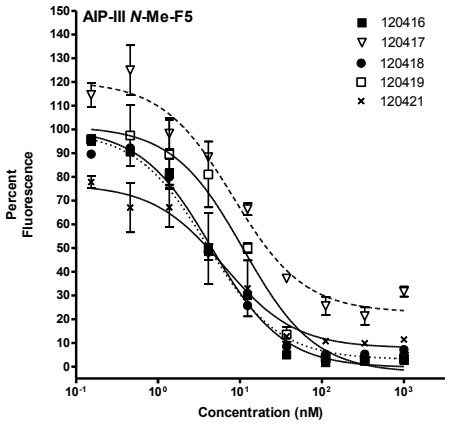
S. aureus AH1872 (group-IV)

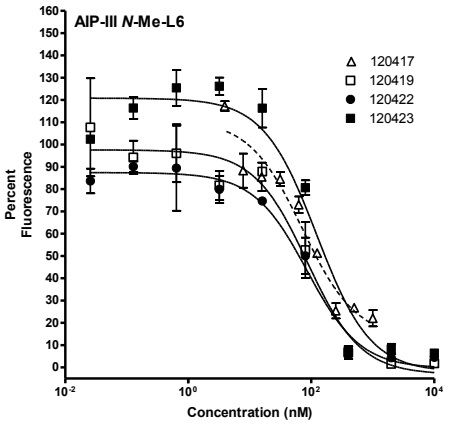


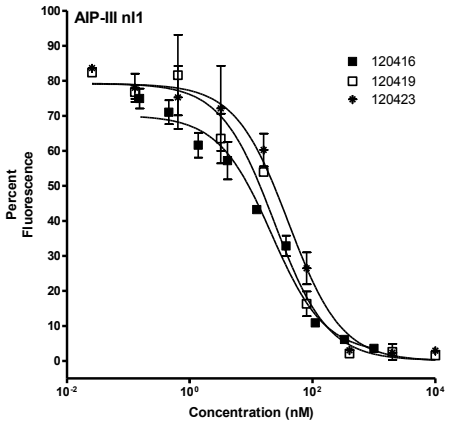


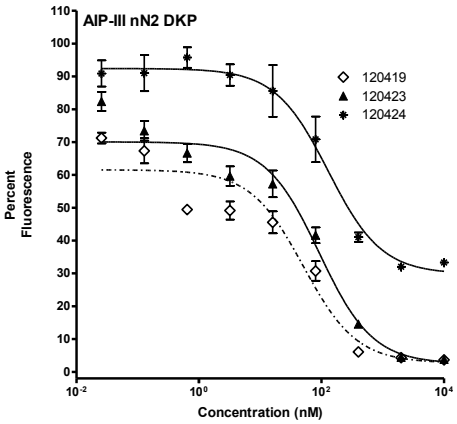


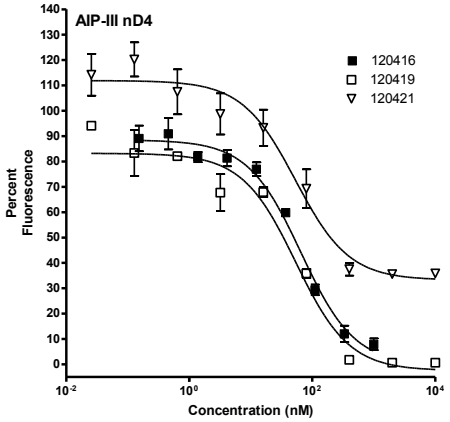


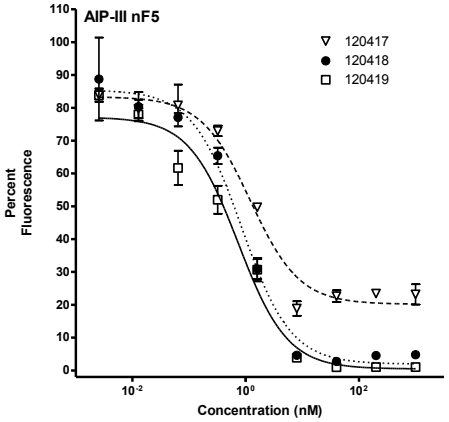

AIP-III *N*-Me-F5
120416
120417
120418
120419
120421
Percent Fluorescence
Concentration (nM)


AIP-III *N*-Me-L6
120417
120419
120422
120423
Percent Fluorescence
Concentration (nM)


AIP-III nI1
120416
120419
120423
Percent Fluorescence
Concentration (nM)


AIP-III nN2 DKP
120419
120423
120424
Percent Fluorescence
Concentration (nM)

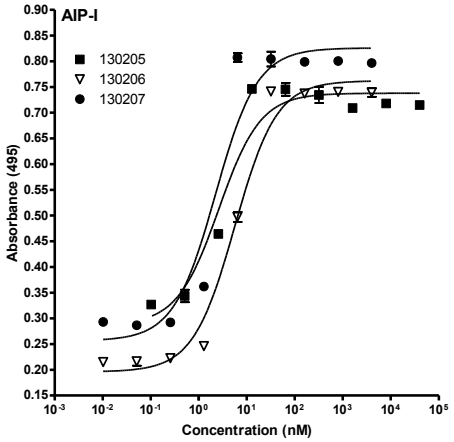

AIP-III nD4
120416
120419
120421
Percent Fluorescence
Concentration (nM)


AIP-III nF5
120417
120418
120419
Percent Fluorescence
Concentration (nM)

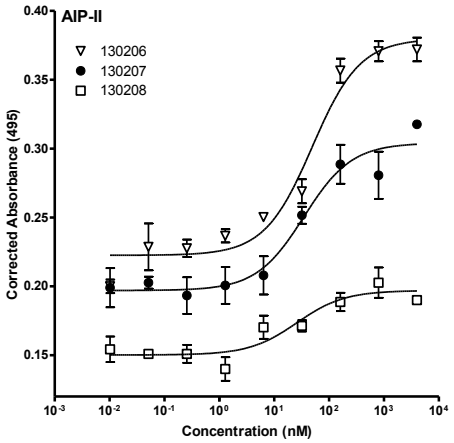
β-lactamase agonism dose response curves.

Peptides were screened over varying concentrations in the four indicated *S. aureus* reporter strains. The peptide tested 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 #1–3; shown for each peptide below). Error bars indicate standard error of the mean of triplicate values. See above for details of methods and strains. [None of the analogs could activate the group-I, -II, and –IV strains. Only the native AIPs (AIP-I, -II, and -IV) were active in these strains (synthesized using our previously reported method),[1] and these dose response curves are shown below.]

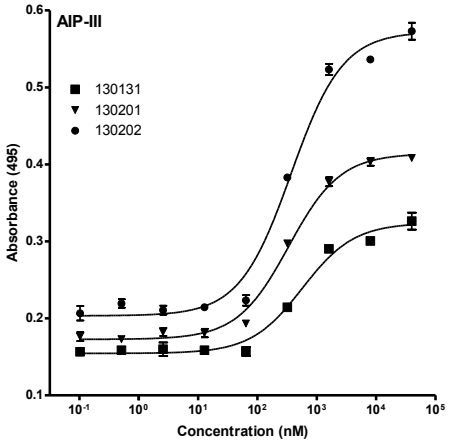
S. aureus RN9222 (group-I)

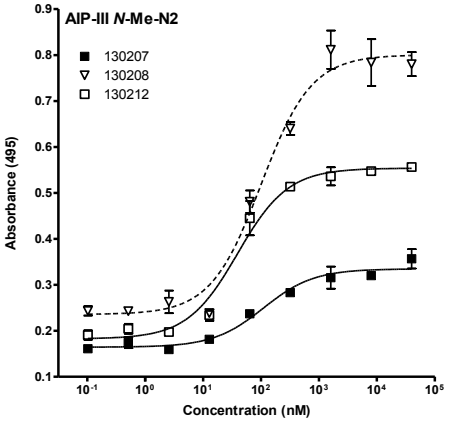


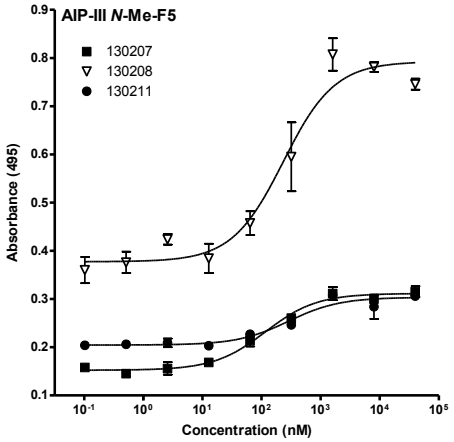
S. aureus RN9372 (group-II)

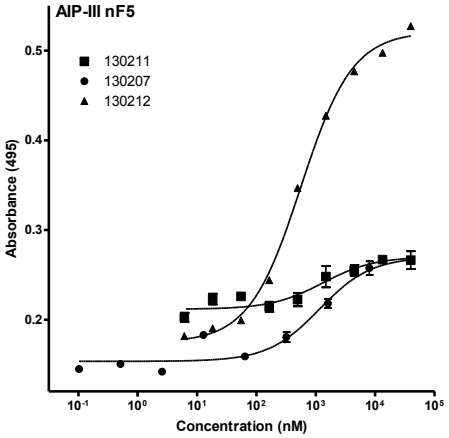


S. aureus RN9532 (group-III)

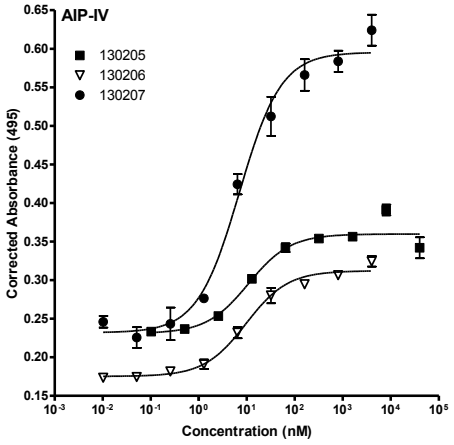








S. aureus RN9371 (group-IV)



Complete table of IC_{50} values for fluorescence assay.

Table S-4. IC_{50} values and 95% confidence ranges for the *N*-methyl and peptoid scan analogues of AIP-III against AgrC I–IV as determined using the *S. aureus* fluorescence reporter strains.[a]

Peptide name	Sequence	AgrC-I IC_{50} (nM)[b]	AgrC-II IC_{50} (nM)[b]	AgrC-III IC_{50} (nM)[b]	AgrC-IV IC_{50} (nM)[b]
AIP-III	I-N-(C-D-F-L-L)	5.05 (2.46-10.4)	5.63 (1.89-16.7)	N.T.[c]	8.53 (4.15-17.5)
AIP-III *N*-Me-I1	***N*Me**I-N-(C-D-F-L-L)	60.8 (34.0-109)	12.8 (6.52-25.1)	>1000[d]	49.2 (37.9-64.0)
AIP-III *N*-Me-N2	I-***N*Me**N-(C-D-F-L-L)	8.04 (4.91-13.2)	3.19 (2.12-4.79)	-[e]	9.54 (7.77-11.7)
AIP-III *N*-Me-C3	I-N-(***N*Me**C-D-F-L-L)	137 (45.8-410)	134 (76.4-234)	-	106 (57.8-193)
AIP-III *N*-Me-D4	I-N-(C-***N*Me**D-F-L-L)	172 (63.4-464)	7.98 (4.03-15.8)	90.2 (49.6-164)	36.9 (13.4-102)
AIP-III *N*-Me-F5	I-N-(C-D-***N*Me**F-L-L)	4.49 (1.61-12.5)	1.95 (1.30-2.93)	-	6.59 (2.68-16.2)
AIP-III *N*-Me-L6	I-N-(C-D-F-***N*Me**L-L)	>1000[c]	-	162 (89.3-293)	81.6 (46.8-142)
AIP-III *N*-Me-L7	I-N-(C-D-F-L-***N*Me**L)	-	-	>1000[d]	-
AIP-III nI1	**nI**-N-(C-D-F-L-L)	44.0 (25.1-77.2)	4.25 (2.48-7.28)	>1000[d]	22.2 (8.20-59.9)
AIP-III nN2 DKP	(I-**nN**)-(C-D-F-L-L)	126 (40.2-396)	4.28 (2.39-7.67)	25.1 (14.0-44.9)	84.8 (41.9-171)
AIP-III nD4	I-N-(C-**nD**-F-L-L)	162 (137-192)	28.3 (21.0-38.3)	206 (151-282)	53.2 (40.1-70.6)
AIP-III nF5	I-N-(C-D-**nF**-L-L)	13.8 (5.18-36.8)	75.6 (40.3-142)	>1000[d]	0.839 (0.532-1.32)
AIP-III nL6	I-N-(C-D-F-**nL**-L)	-	-	>1000[d]	>1000[c]
AIP-III nL7	I-N-(C-D-F-L-**nL**)	-	-	-	-

[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 (2.5 pM – 10 μM). Range indicates 95% confidence interval of triplicate experiments. [c] N.T. = Not tested. [d] 100% inhibition was not observed over the concentrations tested. [e] - = No inhibition was observed at the concentrations tested.

Complete table of EC_{50} values for β-lactamase assay.

Table S-5. EC_{50} values and 95% confidence ranges for the *N*-methyl and peptoid scan analogues of AIP-III in AgrC I–IV as determined using the *S. aureus* β-lactamase reporter strains.[a]

Peptide name	Sequence	AgrC-I EC_{50} (nM) [b]	AgrC-II EC_{50} (nM) [b]	AgrC-III EC_{50} (nM) [b]	AgrC-IV EC_{50} (nM) [b]
AIP-I	Y-S-T-(C-D-F-I-M)	3.21 (1.21-8.57)	N.T [c]	N.T	N.T
AIP-II	G-V-N-A-(C-S-S-L-F)	N.T	40.9 (30.3-55.3)	N.T	N.T
AIP-III	I-N-(C-D-F-L-L)	N.T	N.T	406 (281-586)	N.T
AIP-IV	Y-S-T-(C-Y-F-I-M)	N.T	N.T	N.T	7.90 (4.93-12.7)
AIP-III *N*-Me-I1	***N*Me**I-N-(C-D-F-L-L)	N.T	N.T	- [d]	N.T
AIP-III *N*-Me-N2	I-***N*Me**N-(C-D-F-L-L)	N.T	N.T	75.8 (24.8-232)	N.T
AIP-III *N*-Me-C3	I-N-(***N*Me**C-D-F-L-L)	N.T	N.T	>4000	N.T
AIP-III *N*-Me-F5	I-N-(C-D-***N*Me**F-L-L)	N.T	N.T	198 (78.2-503)	N.T
AIP-III *N*-Me-L6	I-N-(C-D-F-***N*Me**L-L)	-	-	N.T	N.T
AIP-III *N*-Me-L7	I-N-(C-D-F-L-***N*Me**L)	-	-	- [c]	-
AIP-III nI1	**nI**-N-(C-D-F-L-L)	N.T	N.T	-	N.T
AIP-III nF5	I-N-(C-D-**nF**-L-L)	N.T	N.T	947 (385-2330)	N.T
AIP-III nL6	I-N-(C-D-F-**nL**-L)	-	-	-	-
AIP-III nL7	I-N-(C-D-F-L-**nL**)	-	-	-	-

[a] See above for details of reporter strains, experimental procedures, and plots of agonism dose response curves. All assays performed in triplicate. [b] EC_{50} values determined by testing peptides over a range of concentrations (10 pM – 40 μM). Range indicates 95% confidence interval of triplicate experiments. [c] N.T. = Not tested. [d] - = No activation was observed at the concentrations tested.

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