

COVER SHEET

TITLE: Synthetic Pan-Group Agonists of the Quorum Sensing Receptor AgrC in
Staphylococcus epidermidis

AUTHOR'S NAME: Wenqi Shen

MAJOR: Biochemistry

DEPARTMENT: Biochemistry

MENTOR: Helen E. Blackwell

DEPARTMENT: Chemistry

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ABSTRACT

Synthetic pan-group agonists of the quorum sensing receptor AgrC in *Staphylococcus epidermidis*

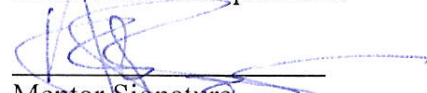
Staphylococcus epidermidis is a leading cause of hospital-acquired infections of indwelling medical devices, with robust biofilms and antibiotic resistances reducing the effectiveness of traditional antibiotic therapy. Its pathogenesis is controlled by the accessory gene regulator (*agr*) quorum sensing (QS) system. The activation of *agr* is achieved through binding of an autoinducing peptide (AIP) to its cognate receptor, AgrC. Divergent evolution has given rise to three *agr* specificity groups defined by the unique AIP sequence (AIPs-I–III), with observed cross-group inhibitory activities. As *agr* agonism has been shown to reduce biofilm accumulation, the development of pan-group activators of the *agr* system is of significant interest. To date, no such activators have been identified. Here, we report the first systematic SAR characterization of *S. epidermidis* AIP-II and AIP-III and the design of pan-group *agr* agonists in *S. epidermidis*. These new pan-group agonists represent new tools for studying QS modulation in *S. epidermidis*.

Wenqi Shen / Biochemistry
Author Name/Major



Author Signature

Helen E. Blackwell / Chemistry
Mentor Name/Department



Mentor Signature

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Date

Introduction

Many bacteria detect their local population density, alter their gene expression, and coordinate their collective behaviors using a chemical-based intercellular communication process called quorum sensing (QS).^{1, 2} QS controls various group behaviors and, for the ubiquitous skin-colonizing bacterium *Staphylococcus epidermidis*, the formation of surface-attached colonies called biofilms.³ QS modulation is therefore an attractive target for the development of non-antibiotic, or anti-virulence, approaches to limit pathogenicity of this opportunistic pathogen. which is capable of establishing chronic infections after surgery or other hospital-related procedures which allow an entry point of a common bacterium into the body.³⁻⁵ Recently, *S.epidermidis* has been identified as a leading cause of infections associated with medical-indwelling devices due to an uncanny ability to form robust biofilm on abiotic surfaces.⁶⁻⁸ As these robust biofilms are often recalcitrant to traditional antibiotic therapies,^{9, 10} *S. epidermidis* has gained notoriety as a public health burden.⁵ It is therefore critical to pursue anti-virulence strategies to control these infections and prevent biofilm development without increasing the spread of antibiotic resistances in organisms that are notably ubiquitous.

The QS circuit in *S. epidermidis* is the accessory gene regulator (*agr*) system, illustrated in Figure 1.¹¹ The *agr* system is operated by four proteins and a QS signal. AgrB is an integral membrane endopeptidase that processes the propeptide AgrD and secretes an autoinducing peptide (AIP), the QS signal of the *agr* system.^{12,13} Upon reaching a threshold concentration, the AIP activates its cognate receptor AgrC, which is a transmembrane histidine kinase.¹¹ AgrC then activates the response regulator AgrA through phosphorelay.¹¹ Thereafter, phosphorylated AgrA upregulates gene expression from the P2 and P3 promoters.¹⁴ The P2 promoter amplifies

expression of the entire *agr* operon, thus forming an auto-induction circuit typical of QS systems, whereas the P3 promoter drives the expression of RNAIII, the main effector molecule of the *agr* system that modulates downstream gene expression.^{15, 16} Phosphorylated AgrA also directly upregulates the expression of phenol-soluble modulins (PSMs), a group of small amphipathic peptides which along with RNAIII, play important roles in the biofilm life cycle and virulence production of *S. epidermidis*.^{17, 18} A number of studies have shown that *agr* is involved in *S. epidermidis* pathogenesis. Activation of the *agr* system is important for optimal skin colonization, protection against the defense mechanisms of the immune system, bacterial dissemination, and tissue infiltration.^{13, 19-21} Therefore, modulation of *agr* can attenuate virulence and limit infection.²²

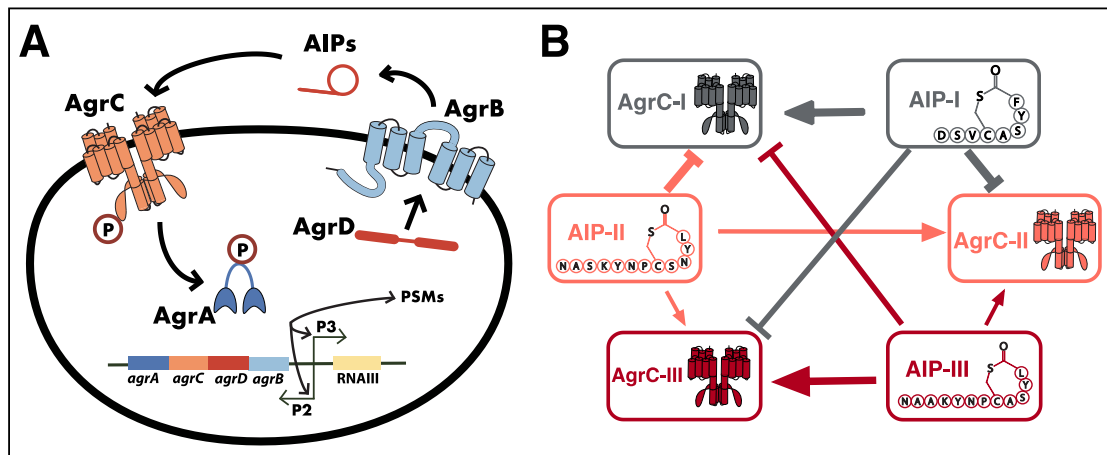


Figure 1. The *agr* system and the associated AIP signals of *S. epidermidis*. (A) Simplified diagram of the *agr* system. AgrD, which contains the sequence for the mature signal, is processed and secreted by AgrB to yield the autoinducing peptide (AIP) signal. Upon reaching a threshold extracellular concentration, AIP activates homodimeric AgrC. Activated AgrC signals AgrA through phosphorelay, and phosphorylated AgrA drives gene transcription from the P2, P3, and *psm* promoters. P2 amplifies *agr* expression, while P3 regulates RNAIII, the main effector molecule of the *agr* system. (B) Single-letter amino acid abbreviations of the known AIPs (AIP-I–III) for the three *agr* specificity groups (Groups I–III) of *S. epidermidis*.

The established role of the *agr* system in *S. epidermidis* biofilm formation is the primary mechanism by which *S. epidermidis* causes device-associated infection, and studies also suggest that intentional activation of the *agr* system may be beneficial.^{10, 23} Specifically, in strains that

form polysaccharide intercellular adhesion dependent biofilm, the *agr* system suppresses the expression of surface-attached AtIE protein, an important adhesion factor for initial attachment.²⁴ Also, PSMs, upregulated by the *agr* system, are found to facilitate bacterial detachment from biofilm.²² Therefore, artificial activation of the *agr* system could limit *S. epidermidis* infection by reducing biofilm growth on surfaces and rendering the dispersed bacteria more susceptible to antibiotic treatment.²⁵ Indeed, previous studies with *S. epidermidis* and the closely related human pathogen, *Staphylococcus aureus*, have demonstrated the reduction of biofilm accumulation via the activation of the *agr* system by both native and non-native agonists.^{26, 27}

While the artificial activation of the *agr* system could be readily achieved by the administration of the native peptide signal, the broad application of this approach to reduce biofilm accumulation of *S. epidermidis* is complicated due to the presence of three *agr* specificity groups of *S. epidermidis* (Group I–III), where each group is defined by the unique sequence of its AIP.^{19, 28} Driving the complexity is that the different AIPs (AIP-I–III) exhibit cross-group inhibitory behaviors, such that AIP-I significantly inhibits the *agr* systems in Group II and Group III, while AIP-II and AIP-III are strong inhibitors of the *agr* system in Group I.¹⁹ As such, applying the native agonist to reduce biofilm accumulation requires the identification of the *agr* specificity group of the target *S. epidermidis* strain, and this approach is only effective against one specificity group at a time.²⁹ Thus, biofilm formed by an unidentified strain or mixed groups would render the strategy ineffective.³⁰ This challenge could be overcome with a global agonist, or a cocktail of agonists, capable of targeting all three groups of *S. epidermidis* without any inhibitory activities between each group.²⁹ However, to date, no such pan-group agonist has

been identified. We recently reported the first systematic structure-activity relationship (SAR) study of the *S. epidermidis* AIP-I, and although we identified non-native agonists with enhanced potency against AgrC-I as well as potent pan-group antagonists targeting all three AgrC receptors, we did not uncover any AIP analogs with agonistic activities in all three groups.²⁶ Previous SAR studies in *S. aureus* have also yield a non-native pan-group antagonists against all *S. aureus agr* specificity groups but no pan-group agonists were revealed.³¹⁻³⁷

However, one analog of a *S. aureus* native AIP has been reported to activate the AgrC receptor from a different *S. aureus agr* group, despite the parent AIP being an inhibitor of that receptor, suggesting that a cross-group inhibitor can be converted into an activator.³¹ In addition, our NMR structural study of *S. epidermidis* AIP-I and its analogs (*in preparation*) suggests that the structural motif for AgrC-I activation is highly modular and could be introduced into a different AIP scaffold to turn that AIP scaffold into an agonist against AgrC-I. Lastly, *S. epidermidis* AIP-II and AIP-III share great similarity in sequence, and they do not exhibit cross-group inhibition against each other.¹⁹

Taken together, we hypothesize that a pan-group agonist, or a cocktail of agonists without cross-group inhibitory activities, could be designed for the *agr* systems in *S. epidermidis* by combining features in native AIPs that are important for receptor activation in each *agr* group. Currently, such features have only been identified for AIP-I, and little is known about the SARs of signal-receptor interactions for AIP-II and AIP-III.²⁶

Herein, we report the first systematic SAR characterization of *S. epidermidis* AIP-II and AIP-III, and the application of these new SAR data and the previously reported AIP-I SAR data for the identification of a first set of pan-group agonists for the *agr* systems in *S. epidermidis*.

These pan-group agonists represent powerful chemical tools to assess the effectiveness of targeting the *agr* system for biofilm dispersion. They serve as exciting new scaffolds for additional structural studies and probe design to elucidate the molecular basis of biofilm cycle and to improve our understanding of receptor activation of the *agr* system in *S. epidermidis*.

Methods

Biological Reagents, Strains, and General Methods.

Reagents were purchased from commercial sources and used according to company's instructions. The *S. epidermidis* fluorescence reporter strains AH3408 (Group I), AH3567 (Group II), and AH3409 (Group III) were grown in Tryptic Soy Broth (TSB, from Sigma) supplemented with 10 µg/mL of erythromycin and all cultures were incubated at 37 °C with shaking at 200 rpm unless noted otherwise.¹⁹ A Biotek Synergy 2 microplate reader was used to record absorbance and fluorescence measurements. IC₅₀/EC₅₀ values and the corresponding 95% confidence intervals were determined from sigmoidal curve fits (log [compound] vs response, 4-parameters) using GraphPad Prism software (v. 8.0.1).

Peptide Synthesis

AIP analogs and chimera compounds were synthesized according to the established Fmoc solid phase peptide synthesis (Fmoc-SPPS) protocols, purified by preparative reverse phase high performance liquid chromatography (RP-HPLC), analyzed for purity, and characterized by exact mass measurement to confirm identity.^{26, 38} Detail procedures about the synthesis, the related chemical reagents, and instrumentation information were provided in the Supporting Information (SI).

Fluorescence reporter assay

Agonism and antagonism assays for all three AgrC receptors were performed using a previously established protocol with two modifications.²⁶ First, to block AgrC activation by

endogenously produced AIP, AIP-I D1AS6AV3A, a pan-group antagonist uncovered in our prior study, at a final concentration of 25 nM was used for the agonism assay.²⁶ For the assessment of SARs of AIP-II and potential pan-group agonists, Group II cultures were grown for 8 hours (1 hour before the transition time from log phase to stationary phase on the growth curve) and augmented with 500 nM of AIP-II 9aa, an agonist against AgrC-II, to ensure consistent activation of the *agr* system (AIP-II 9aa was used over AIP-II due to compound availability).

Results and Discussion

1. SARs of AIP-II and AIP-III

To identify the chemical features important for activation of AgrC-II and AgrC-III of *S. epidermidis*, we first characterized the SARs for AIP-II and AIP-III by systematically replacing each amino acid with either alanine (Ala) or the corresponding D-amino acid to explore the roles of the side chains and the stereochemistry during the signal:receptor interaction. Cystine (Cys) 8 was not mutated to Ala due to its essentiality for forming the macrocycle, and the Ala residues that are part of the native sequence did not go through alanine substitutions. We also synthesized a series of analogs by removing residues from the exocyclic tail of the native AIPs to explore the impact of exocyclic tail length on the signal:receptor interaction. The results of the compound potency, as measured by the IC₅₀ and EC₅₀ values derived from dose-response analyses, are summarized in Figure 2.

Though the activity of the native AIP-I against AgrC-II and AgrC-III was in agreement with a previous report, the activity of AIP-II towards Group III and the activity of AIP-III towards Group II was different from the previous study.¹⁹ Specifically, AIP-II and AIP-III show no cross-group activities, and we observed cross-group activation between the two compounds, albeit only weakly so for AIP-II. This difference may be due to the variances in experimental procedures and the use of AgrC antagonists in our protocol to block the activation of the *agr* system by endogenous AIPs.

Given their structural similarities, we also observed comparable trends in SARs for AIP-II and AIP-III. Both AIPs require the bulky, hydrophobic residues Tyrosine 11 (Tyr11) and Leucine 12 (Leu12) within the macrocycle to be present for receptor binding and activation, as

replacement with Ala resulted in complete loss of activity. Residue Tyr11 in both AIPs is also intolerant to change in stereochemistry, as the substitution with the corresponding D-amino acid caused a complete loss of activity as well.

On the other hand, substitution of Leu12 with the corresponding D-amino acid only reduced the potency of, but did not abolish, the agonistic activity. We note that the impact of stereochemistry at these two residues in AIP-II and AIP-III on the AIP:AgrC interaction is the same as that of the equivalent residues in AIP-I, implying that the hydrophobic residues in all three AIPs likely adopt similar orientations.²⁶

We also identified three residues Tyr5, Asparagine 6 (Asn6), and Proline 7 (Pro7) in the exocyclic tail portion of both AIP-II and AIP-III to be important for receptor activation, as mutations to either Ala or the corresponding D-amino acid converted the resulting peptide from an agonist to an antagonist. The efficacies of the AIP-II analogs and AIP-III analogs containing D-amino acid substitutions at these three positions shared similar trends, further explaining the reason of no cross-group activities between Group II and Group III.

Overall, the above SAR trends for AIP-II and AIP-III are in agreement with the current model for activation of AgrC by its cognate AIP in *S. epidermidis* and *S. aureus*, where the hydrophobic residues of the AIP macrocycle interact with a proposed hydrophobic patch on the AgrC receptor for signal binding while the residues on the exocyclic tail of the AIP form contacts with the receptor to induce activation.^{26, 31, 39, 40}

Surprisingly, the first three residues from the N-terminus, Asn1, Ala2, and Serine 3 (Ser3)/Ala3, in both AIP-II and AIP-III were found to have little to no impact on AIP:AgrC binding or activation. Most of the Ala and D-amino acid replacements at these positions result in having

similar activities as that of native AIPs. AIP-III D-Ala3 is the only analog to show a reduction in potency, but it remained as an effective agonist towards the cognate receptor AgrC-III.

Moreover, AIP analogs without these three residues from the N-terminus (AIP-II/III 11aa, AIP-II/III 10aa, AIP-II/III 9aa) remained as agonists with similar or even better potency against the cognate AgrC receptors. These data suggest that these residues do not engage in meaningful contacts with the receptor and are likely solvent exposed.

However, removal of the fourth residue, Lysine 4 (Lys4), from the N-terminus of both AIPs resulted in an analog (AIP-II/III 8aa) with inhibitory activity towards the cognate receptors, indicating that this residue is required for receptor activation. Also, the side chain of Lys4 appears to be non-essential for receptor activation, as the Ala substitution in both AIPs maintained the agonistic activity towards the cognate receptors, though with a significant reduced potency compared to the native AIPs. For AIP-II, a similar effect was also observed for the D-amino acid substitution at Lys4. However, for AIP-III, D-amino acid substitution at Lys4 resulted in an antagonist, implying that Lys4, specifically its stereochemistry, contribute differently for AIP:AgrC-II and AIP:AgrC-III interactions.

Lastly, we turn to Cys8 and the two endocyclic residues that follow. Prior SAR studies in *S. aureus* and *S. epidermidis* have established the thioester linkage to be crucial for receptor binding, and ring-opened versions of the native AIPs were inactive towards their cognate receptors.^{26, 41}

Based on the previous studies, we assumed that the importance of thioester linkage is preserved for AIP-II and AIP-III. As such, we did not make the Cys to Ala substitutions for this SAR study. However, we did investigate the importance of the stereochemistry of the Cys. For

both the AIP-II and AIP-III, the D-amino acid replacements of Cys8 caused significant loss of activity, which is in agreement with our prior SAR characterization of *S. epidermidis* AIP-I.²⁶ This loss of activity could be attributed to either the stringent requirement for the local stereochemistry at the Cys residue or a global change in conformation of the macrocycle that prevented proper binding to the AgrC receptor.

The first two endocyclic residues that follow Cys8 are Ser9/Asn10 for AIP-II and Ala9/Ser10 for AIP-III. They are two of the three residues that differ between AIP-II and AIP-III, with the third residue being Ser3 for AIP-II and Ala3 for AIP-III. As mentioned earlier, residue Ser3 and Ala3 appear to play little to no role in receptor binding and activation for AIP-II and AIP-III. Therefore, the differences at the first two endocyclic residues between AIP-II and AIP-III are most likely to dictate the receptor specificity of the native AIPs.

For AIP-III, the role of the Ala9 side chain was not explored because the residue is already an Ala, and AIP-II Ser9 was able to tolerate the Ala substitution though the compound only retained a very weak agonistic activity. However, for AIP-II and AIP-III, the results D-amino acid mutations at residue 9 are similar. Specifically, replacement of Ala9 with D-Ala in AIP-III and Ser9 with D-Ser in AIP-II both resulted in complete loss of activities.

The residue 10 also play different roles between AIP-II and AIP-III. For AIP-II, both the Ala and D-amino acid mutations at Asn10 caused a significant loss of potency but did not change the agonistic profile of the peptides. However, in AIP-III, the Ala substitution at Ser 10 results in complete loss of activities. Unexpectedly, the D-Ser substitution at AIP-III Ser10 position resulted in an agonistic activity profile with a similar potency as the native AIP. This result indicated that the flipped stereochemistry may contribute to additional hydrogen

bonding within the binding pocket. As such, additional NMR study may help to reveal additional information about this residue.

Taken together, the above results support that residue 9 and 10 of AIP-II and AIP-III clearly make significant contributions to signal:receptor interactions that are likely responsible for the receptor specificity of the two AIPs.

A				B			
Peptide Name	Sequence	IC ₅₀ [nM]	EC ₅₀ ^b [nM]	Peptide Name	Sequence	IC ₅₀ [nM]	EC ₅₀ ^b [nM]
AIP-II N1A	A-A-S-K-Y-N-P-(C-S-N-Y-L)	-	597.3	AIP-III N1A	A-A-A-K-Y-N-P-(C-A-S-Y-L)	-	81.54
AIP-II S3A	N-A-A-K-Y-N-P-(C-S-N-Y-L)	-	523.0	AIP-III K4A	N-A-A-A-Y-N-P-(C-A-S-Y-L)	-	>2000
AIP-II K4A	N-A-S-A-Y-N-P-(C-S-N-Y-L)	-	>2000	AIP-III Y5A	N-A-A-K-A-N-P-(C-A-S-Y-L)	123.0	-
AIP-II Y5A	N-A-S-K-A-N-P-(C-S-N-Y-L)	1298	-	AIP-III N6A	N-A-A-K-Y-A-P-(C-A-S-Y-L)	140.7	-
AIP-II N6A	N-A-S-K-Y-A-P-(C-S-N-Y-L)	557.2	-	AIP-III P7A	N-A-A-K-Y-N-A-(C-A-S-Y-L)	101.9	-
AIP-II P7A	N-A-S-K-Y-N-A-(C-S-N-Y-L)	547.4	-	AIP-III S10A	N-A-A-K-Y-N-P-(C-A-A-Y-L)	-	Inactive ^c
AIP-II S9A	N-A-S-K-Y-N-P-(C-A-N-Y-L)	-	>2000	AIP-III Y11A	N-A-A-K-Y-N-P-(C-A-S-A-L)	-	Inactive ^d
AIP-II N10A	N-A-S-K-Y-N-P-(C-S-A-Y-L)	-	>2000	AIP-III L12A	N-A-A-K-Y-N-P-(C-A-S-Y-A)	-	Inactive ^d
AIP-II Y11A	N-A-S-K-Y-N-P-(C-S-N-A-L)	Inactive ^e	-	AIP-III D-N1	^o N-A-A-K-Y-N-P-(C-A-S-Y-L)	-	77.13
AIP-II L12A	N-A-S-K-Y-N-P-(C-S-N-Y-A)	Inactive ^e	-	AIP-III D-A2	N- ^p A-A-K-Y-N-P-(C-A-S-Y-L)	-	104.1
AIP-II D-N1	^o N-A-S-K-Y-N-P-(C-S-N-Y-L)	-	421.8	AIP-III D-A3	N-A- ^o A-K-Y-N-P-(C-A-S-Y-L)	-	510.3
AIP-II D-A2	N- ^p A-S-K-Y-N-P-(C-S-N-Y-L)	-	553.8	AIP-III D-K4	N-A-A- ^p K-Y-N-P-(C-A-S-Y-L)	26.13	-
AIP-II D-S3	N-A- ^p S-K-Y-N-P-(C-S-N-Y-L)	-	432.6	AIP-III D-Y5	N-A-A-K- ^o Y-N-P-(C-A-S-Y-L)	48.59	-
AIP-II D-K4	N-A-S- ^p K-Y-N-P-(C-S-N-Y-L)	-	>2000	AIP-III D-N6	N-A-A-K-Y- ^o N-P-(C-A-S-Y-L)	1331	-
AIP-II D-Y5	N-A-S-K- ^o Y-N-P-(C-S-N-Y-L)	379.2	-	AIP-III D-P7	N-A-A-K-Y-N- ^p P-(C-A-S-Y-L)	642.5	-
AIP-II D-N6	N-A-S-K-Y- ^o N-P-(C-S-N-Y-L)	>2000	-	AIP-III D-C8	N-A-A-K-Y-N-P-(^o C-A-S-Y-L)	-	Inactive ^d
AIP-II D-P7	N-A-S-K-Y-N- ^p P-(C-S-N-Y-L)	822.0	-	AIP-III D-A9	N-A-A-K-Y-N-P-(C- ^o A-S-Y-L)	-	Inactive ^d
AIP-II D-C8	N-A-S-K-Y-N-P-(^o C-S-N-Y-L)	-	Inactive ^e	AIP-III D-S10	N-A-A-K-Y-N-P-(C-A- ^o S-Y-L)	-	145.3
AIP-II D-S9	N-A-S-K-Y-N-P-(C- ^o S-N-Y-L)	-	Inactive ^e	AIP-III D-Y11	N-A-A-K-Y-N-P-(C-A-S- ^o Y-L)	-	Inactive ^d
AIP-II D-N10	N-A-S-K-Y-N-P-(C-S- ^o N-Y-L)	-	>2000	AIP-III D-L12	N-A-A-K-Y-N-P-(C-A-S-Y- ^o L)	-	>2000
AIP-II D-Y11	N-A-S-K-Y-N-P-(C-S-N- ^o Y-L)	-	>2000	AIP-III 13aa	G-N-A-A-K-Y-N-P-(C-A-S-Y-L)	-	58.36
AIP-II D-L12	N-A-S-K-Y-N-P-(C-S-N-Y- ^o L)	-	>2000	AIP-III 11aa	A-A-K-Y-N-P-(C-A-S-Y-L)	-	48.97
AIP-II 13aa	G-N-A-S-K-Y-N-P-(C-S-N-Y-L)	-	450.6	AIP-III 10aa	A-K-Y-N-P-(C-A-S-Y-L)	-	29.89
AIP-II 11aa	A-S-K-Y-N-P-(C-S-N-Y-L)	-	292.3	AIP-III 9aa	K-Y-N-P-(C-A-S-Y-L)	-	28.86
AIP-II 10aa	S-K-Y-N-P-(C-S-N-Y-L)	-	183.3	AIP-III 8aa	Y-N-P-(C-A-S-Y-L)	20.17	-
AIP-II 9aa	K-Y-N-P-(C-S-N-Y-L)	-	83.50	AIP-III 7aa	N-P-(C-A-S-Y-L)	>2000	-
AIP-II 8aa	Y-N-P-(C-S-N-Y-L)	134.8	-	AIP-III 6aa	P-(C-A-S-Y-L)	>2000	-
AIP-II 7aa	N-P-(C-S-N-Y-L)	52.37	-	tAIP-II	Ac-(C-A-S-Y-L)	>2000	-
AIP-II 6aa	P-(C-S-N-Y-L)	663.5	-	AIP-I	D-S-V-(C-A-S-Y-F)	2.128	-
tAIP-II	Ac-(C-S-N-Y-L)	132.6	-	AIP-II	N-A-S-K-Y-N-P-(C-S-N-Y-L)	-	>2000
AIP-I	D-S-V-(C-A-S-Y-F)	13.90	-	AIP-III	N-A-A-K-Y-N-P-(C-A-S-Y-L)	-	71.77
AIP-II	N-A-S-K-Y-N-P-(C-S-N-Y-L)	-	226.1				
AIP-III	N-A-A-K-Y-N-P-(C-A-S-Y-L)	-	648.2				

Figure 2. IC₅₀ and EC₅₀ Values for Alanine and D-Amino Acid Scan Analogs of *S. epidermidis* (A) AIP-II in AgrC-II and (B) AIP-III in AgrC-III. ^a See Methods for details regarding the reporter strain and assay procedures. ^b Agonism assay performed in the presence of an inhibitor against AgrC-III (25nM AIP-I D1AS6AV3A) to block receptor activation by endogenously produced AIP-III. ^c Dose-response curves revealed neither agonism nor antagonism activities over the concentration range tested. ^d Dose-response curves revealed neither agonism nor antagonism activities over the concentration range tested.

2. Design and synthesis of pan-group agonists

Our SAR characterization of AIP-II and AIP-III has revealed key residues and features important for AgrC-II and AgrC-III activation (Figure 4). By integrating the SAR data from all three groups, we proposed that: 1) the macrocyclic hydrophobic residues of all three native *S. epidermidis* AIPs likely adopt similar orientations that are important for receptor binding; 2)

AIP-II and AIP-III can be shortened from 12-mers to 9-mers by removing the first three N-terminal residues and still maintain the potency of the native signal; 3) the remaining exocyclic tail residues of AIP-II and AIP-III are important for receptor activation; and 4) the first two endocyclic residues of AIP-II and AIP-III are likely responsible for the receptor specificity and can be altered without causing antagonism. Additionally, our prior study of AIP-I has identified the branched hydrophobic side chain of a valine residue (Val3) to be important for AgrC-I activation, and this structural motif must be properly oriented relative to the macrocycle.²⁶

Based on the existing SAR for all three native AIPs of *S. epidermidis*, we first tried to design a set of pan-group agonists by introducing the branched hydrophobic motif important for AgrC-I activation to the AIP-II 9aa scaffold with the K-Y-N-P-(C-S-N-Y-L).

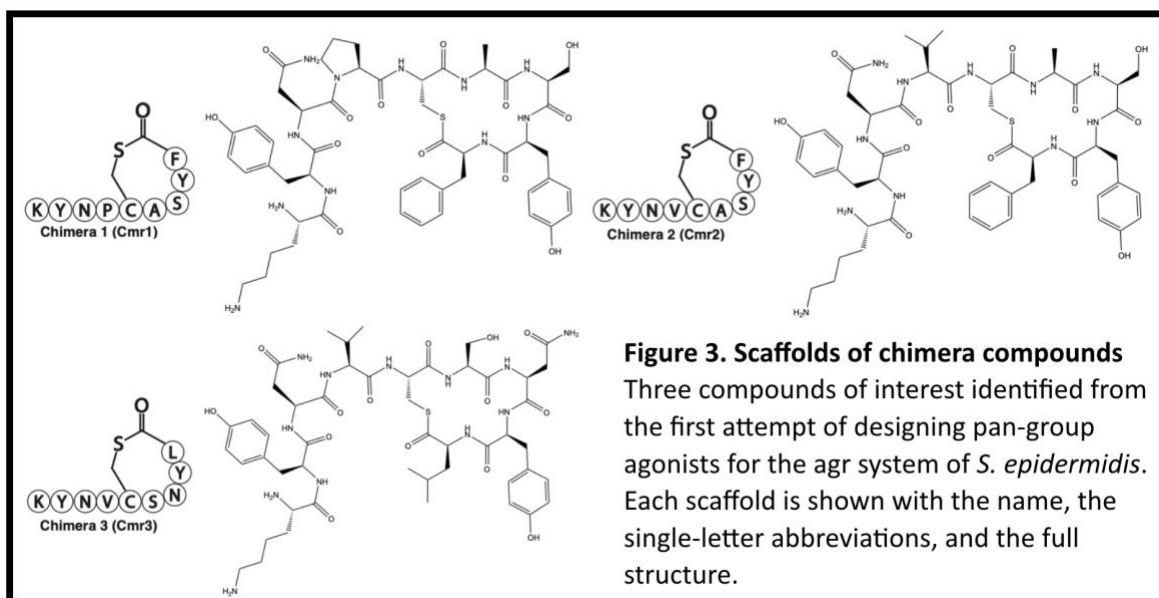
Although none of the resulting analogs showed agonistic activity towards more than one AgrC receptor, we were intrigued by the activity of the analog **Cmr3**, which is an antagonist against AgrC-I, but it remains as an agonist against AgrC-II. By replacing the macrocycle of **Cmr3** with that of AIP-I, we also discovered **Cmr2**, an agonist against AgrC-I with potency and efficacy similar to that of native AIP-I despite the significant differences in the exocyclic tail. However, it behaves as an antagonist against AgrC-II and an agonistic against AgrC-III, albeit with lower efficacy. Nevertheless, **Cmr2** represents the second case where a cross-group inhibitor of the *agr* system in *Staphylococci* is converted to an agonist.³¹

Since the difference in macrocycle sequences is responsible for the receptor agonism specificity of **Cmr2** and **Cmr3**, we further hypothesized that alterations in the macrocycle could lead to a peptide with agonistic activity toward all three receptors. The combination of the exocyclic tail of AIP-II/AIP-III 9aa with the macrocycle of AIP-I resulted in an AIP analog with

agonistic activity toward all three AgrC receptors. We named this compound **Cmr1**. Despite the relatively poor activity in Group I and Group II, **Cmr1** represents the first known pan-group agonist for the *agr* systems in *S. epidermidis*.

3. Optimizing the exist scaffolds towards additional pan-group agonists

The first attempt to design *S. epidermidis* pan-group agonist has yield one pan-group agonist (**Cmr1**) and two interesting scaffolds, **Cmr2** and **Cmr3**. We further investigate the three scaffolds as shown in Figure 3, aiming to incorporate the SAR results and to discover additional pan-group agonists.



We first focused on **Cmr2**, which is an agonist in both AgrC-I and AgrC-III. To broaden the receptor agonism specificity to include AgrC-II, we designed five analogs where the macrocycle structure was changed to be more like the native AIP-II. Despite the changes made to the residues in the macrocycle, none of the analogs was able to activate AgrC-II to any extent.

With a similar rationale as above, we next designed and synthesized five analogs based on the Cmr3 scaffold, where changes were also made within the macrocycle such that the

macrocycle is more AIP-I/AIP-III like. Within this set analog, another pan-group agonist, Cmr3_S6AN7A was discovered. Interestingly, one of the most potent pan-group antagonists against the AgrC receptors in *S. epidermidis* reported in our recent study also has two Ala residues at the first two endocyclic positions, suggesting that these residues may facilitate AIP recognition by all three receptors.²⁶

As mentioned at the beginning, our first pan-group agonist, **Cmr1**, has relatively poor activity in Group I and Group II. As such, we aim to improve the activity of this compound towards AgrC-I and AgrC-II. We designed and synthesized five more analogs with substitutions made in the macrocycle. Although this series of analogs did lead to peptides with improved activity towards AgrC-II when compared to the parent compound, we were not able to improve the activity in AgrC-I. In fact, Cmr1_S7A is the only one analog retained the pan-group agonist behavior within this set. Though this analog had a better efficacy in AgrC-II than the parent compound, the activity in AgrC-I actually worsened. Given that this set of peptides, including the parent compound, all lack a valine residue near the macrocycle, which is present in all of

Table 1. EC₅₀ values of native AIPs and pan-group analogs against AgrC I–III determined using *S. epidermidis* fluorescence reporter strains ^a

Peptide Name	Sequence	AgrC-I EC ₅₀ [nM] ^b	AgrC-II EC ₅₀ [nM] ^b	AgrC-III EC ₅₀ [nM] ^b
AIP-I	D-S-V-(C-A-S-Y-F)	170	— ^c	— ^c
AIP-II	N-A-S-K-Y-N-P-(C-S-N-Y-L)	— ^c	226	>2000
AIP-III	N-A-A-K-Y-N-P-(C-A-S-Y-L)	— ^c	>2000	138
Cmr1	K-Y-N-P-(C-A-S-Y-F)	>2000	61.3	0.93
Cmr2	K-Y-N-V-(C-A-S-Y-F)	71.8	— ^c	40.6
Cmr3	K-Y-N-V-(C-S-N-Y-L)	— ^c	>2000	— ^c
Cmr1_S7A	K-Y-N-P-(C-A-A-Y-F)	884	63.7	1.47
Cmr3_S6AN7A	K-Y-N-V-(C-A-A-Y-L)	134	192	1212

^aSee Method for details of reporter strains and experimental procedures. All assays performed in triplicate. ^bEC₅₀ values determined by testing peptides over a range of concentrations (0.5 nM – 1 mM). ^cDose response curve revealed antagonism and no agonism.

the potent agonists targeting AgrC-I, we suggested that future research can focus on developing analogs containing different substitutions at the proline position. The data of the compounds mentioned above was listed in Table 1, and the dose response curves were shown in the SI.

Conclusions

Herein, we expanded the SAR study to examine AIP-II and AIP-III, and our results have revealed residues important for AgrC-II and AgrC-III activation (Figure.4). With the SAR results, we designed a series of AIP analogs containing the known features important for AgrC activation in all three groups of *S. epidermidis*, and we were able to uncover three AIP analogs with agonistic activities against all three AgrC receptors, without confounding antagonistic activities against any of the receptors. Though the efficacy of these three peptides needs further optimization, they represent to our knowledge the first reported pan-group agonists of the *agr* systems in a Staphylococcal species. Characterization of the 3-dimensional structures of these agonists will provide us further insights into the structural motifs that enabled agonism towards all three AgrC receptors. Efforts towards this goal are underway.

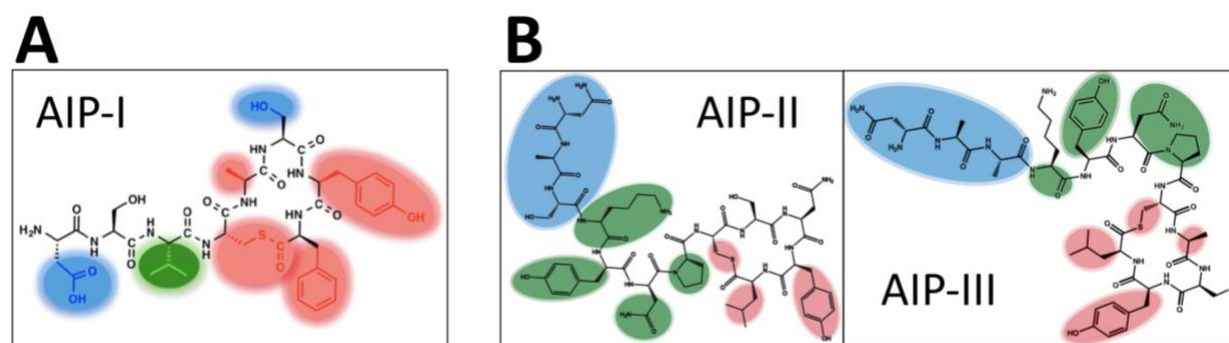


Figure 4. Summary of the key SAR trends for activation of *S. epidermidis* AgrC by AIPs. (A) SAR for AIP-I from previous study.²⁶ (B) SAR for AIP-II and AIP-III as revealed by the reporter assays in this study. Red, essential for AgrC receptor recognition. Green, essential for AgrC receptor activation. Blue, detrimental to AgrC receptor activation. Remaining residues contribute but are not critical to receptor recognition.

In this study, we also provided a deeper understanding of signal:receptor interactions for the *S. epidermidis* *agr* systems. Beyond the SARs gained from the systematic alanine and D-amino acid scans of AIP-II and AIP-III, we also observed that having a phenylalanine (Phe) at the C-terminal position improved the potency of the AIP analogs in all three AgrC receptors. In fact, the introduction of Phe residues resulted in a number of AIP analogs that are much more

potent agonists against AgrC-II and AgrC-III when compared to the native AIPs (between 5 to 50-fold). We also discovered that the two endocyclic positions next to Cys may contribute to group specificity and facilitate AIP recognition. More importantly, we identified a position in the AIP analogs that deserves more in-depth characterization in order to improve agonist efficacy towards all three AgrC receptors. Specifically, the structure of the exocyclic residue nearest to the macrocycle appears to dictate which receptors are better activated by the AIP analogs. The present study only examined two amino acids (Val and Pro) at that position extensively, and the effect of introducing other natural and unnatural amino acids at the position needs to be explored. Building on the pan-group agonist scaffolds uncovered in this study, even more effective pan-group agonists could be developed. Such compounds, along with the new agonists reported here and our previously reported pan-group antagonists, serve as new tools for better assessing the effects of targeting QS as an approach towards limiting the infectivity of *S. epidermidis*.

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This senior honor thesis is being prepared as a manuscript intended for publication, and the project was completed by these authors: Wenqi Shen^{‡,a,b}, Tian Yang^{‡,a,1}, Korbin H. J. West^a, Jeffery S. Kavanaugh^c, Sally R. Ruderman^{a,2}, Joseph K. Vasquez^a, Alexander R. Horswill^c, Helen E. Blackwell^a

^a Department of Chemistry, University of Wisconsin-Madison, 1101 University Ave., Madison, WI 53706, United States

^b Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock St, Madison, WI 53706, United States

^c Department of Immunology and Microbiology, Anschutz Medical Campus, University of Colorado, Aurora, CO 80045, United States

W. S[‡] and T. Y[‡] designed the experiments, screen compound. W. S also carried out the peptide synthesis and wrote this manuscript. K. H. J. W assisted with peptide synthesis and compounds screening. J. S. K and J. K. V helped bacterial reporter troubleshooting. S. R. R assisted with peptide synthesis. A. R. H provided the providing the *S. epidermidis* reporter strains. H. E. B guided the project.

‡These authors contributed equally.

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¹Current address: Promega Cooperation, 5399, 2800 Woods Hollow Rd, Fitchburg, WI 53711, United States

² Current address: Science Department, Canterbury School, 101 Aspetuck Avenue New Milford, CT 06776, United States

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Supporting Information

1. Chemical reagents and instrumentation

Reagents and solvents were purchased from commercial sources (Sigma-Aldrich or Chem-Impex International) and used as received, except for CH_2Cl_2 (DCM), which was distilled and dried over activated molecular sieves. Resins for solid-phase peptide synthesis were purchased from Chem-Impex and NovaBiochem. Water (18 M Ω) was purified using an arium[®] pro ultrapure water system (Sartorius). Reverse-phase high performance liquid chromatography (RP-HPLC) was carried out on a Shimadzu system equipped with a SCL-10Avp controller, a LC-20AT pump, an FCV-ALvp solvent mixer, and a SPD-M20A UV/Vis diode array detector. Peptide purification was performed on a semi-preparative Kromasil Eternity C18 column (10 mm x 250 mm, 5 μm particle size with 100 Å pore size) with a 5 mL/min flow rate, and analytical samples were run on an analytical Kromasil Eternity C18 column (4.6 mm x 250 mm, 5 μm particle size with 100 Å pore size) with a 1 mL/min flow rate.

Peptide was purified on a semi-preparative Kromasil Eternity C18 column (10 mm x 250 mm, 5 μm particlesize with 100 Å pore size) with 5 mL/min flow rate, and purity of each peptide was checked using analytical Kromasil Eternity C18 column (4.6 mm x 250 mm, 5 μm particle size with 100 Å pore size) with 1 mL/min flow rate. Solvent A = 18.2 M Ω water + 0.1% trifluoroacetic acid (TFA); solvent B = acetonitrile (ACN) + 0.1% TFA. Linear peptides were purified over a linear gradient of 20%→ 50% solvent B over 30 min, and cyclic peptides were purified over a linear gradient of 23%→38% solvent B over 36 min. Peptide purity was determined using a linear gradient of 10%→ 95% solvent B over 27 min, with integration of peaks detected at 220 nm.

MALDI-TOF mass spectrometry (MS) data were obtained using a Bruker microflex LRF spectrometer equipped with a 337 nm laser and a reflectron. Exact mass (EM) data were obtained using a Thermo Q Exactive Plus ESI-Q-IT (orbitrap) mass spectrometer.

2. Mass spectrometer (MS) and HPLC data

Table SI.1: MS and HPLC data for the *S. epidermidis* AIP-II analogs synthesized in this study. EM = exact mass. Rt = retention time.

Peptide Name	Sequence	Calc. EM	Obs. EM	Rt (min)	Purity (%)
AIP-II N1A	A-A-S-K-Y-N-P-(C-S-N-Y-L)	1312.5991	1312.6010	18.5	>99
AIP-II S3A	N-A-A-K-Y-N-P-(C-S-N-Y-L)	1339.6100	1339.6121	16.0	>99
AIP-II K4A	N-A-S-A-Y-N-P-(C-S-N-Y-L)	1298.5471	1298.5459	16.7	>99
AIP-II Y5A	N-A-S-K-A-N-P-(C-S-N-Y-L)	1263.5787	1263.5764	17.1	>97
AIP-II N6A	N-A-S-K-Y-A-P-(C-S-N-Y-L)	1312.5991	1312.6021	17.6	>99
AIP-II P7A	N-A-S-K-Y-N-A-(C-S-N-Y-L)	1329.5893	1329.5895	17.3	>99
AIP-II S9A	N-A-S-K-Y-N-P-(C-A-N-Y-L)	1339.1600	1339.1606	17.3	>99
AIP-II N10A	N-A-S-K-Y-N-P-(C-S-A-Y-L)	1312.5991	1312.6014	17.7	>99
AIP-II Y11A	N-A-S-K-Y-N-P-(C-S-N-A-L)	1263.5787	1263.5811	16.3	>99
AIP-II L12A	N-A-S-K-Y-N-P-(C-S-N-Y-A)	1313.5580	1313.5575	15.3	>99
AIP-II D-N1	DN-A-S-K-Y-N-P-(C-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3054 [M+2H] ²⁺	16.0	>97
AIP-II D-S3	N-A-DS-K-Y-N-P-(C-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3051 [M+2H] ²⁺	15.9	>97
AIP-II D-K4	N-A-S-DK-Y-N-P-(C-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3063 [M+2H] ²⁺	16.0	>96
AIP-II D-Y5	N-A-S-K-DY-N-P-(C-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3061 [M+2H] ²⁺	16.1	>95
AIP-II D-N6	N-A-S-K-Y-DN-P-(C-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3049 [M+2H] ²⁺	15.9	>94
AIP-II D-P7	N-A-S-K-Y-N-DP-(C-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3065 [M+2H] ²⁺	16.0	>97
AIP-II D-C8	N-A-S-K-Y-N-P-(DC-S-N-Y-L)	678.3056 [M+2H] ²⁺	678.3066 [M+2H] ²⁺	16.8	>97
AIP-II D-S9	N-A-S-K-Y-N-P-(C-DS-N-Y-L)	678.3056 [M+2H] ²⁺	678.3041 [M+2H] ²⁺	16.2	>97
AIP-II D-N10	N-A-S-K-Y-N-P-(C-S-DN-Y-L)	678.3056 [M+2H] ²⁺	678.3040 [M+2H] ²⁺	15.8	>98
AIP-II D-Y11	N-A-S-K-Y-N-P-(C-S-N-DY-L)	678.3056 [M+2H] ²⁺	678.3044 [M+2H] ²⁺	15.5	>95
AIP-II D-L12	N-A-S-K-Y-N-P-(C-S-N-Y-DL)	678.3056 [M+2H] ²⁺	678.3047 [M+2H] ²⁺	15.6	>99

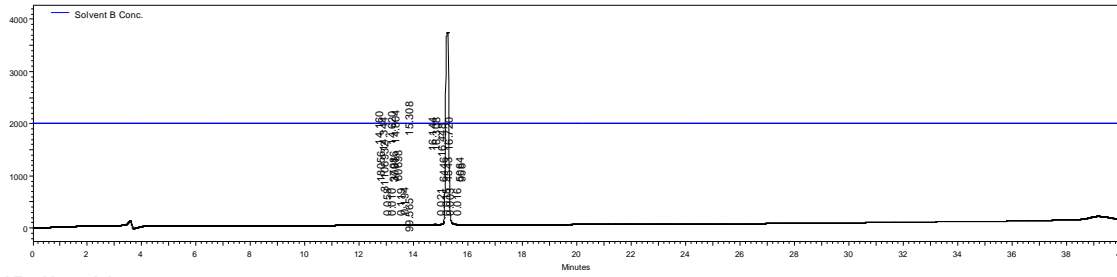
Table SI.2: MS and HPLC data for the *S. epidermidis* AIP-III analogs synthesized in this study. EM = exact mass. Rt = retention time.

Peptide Name	Sequence	Calc. EM	Obs. EM	Rt (min)	Purity (%)
AIP-III N1A	A-A-A-K-Y-N-P-(C-A-S-Y-L)	627.3028 [M+2H] ²⁺	627.3029 [M+2H] ²⁺	18.4	>96
AIP-III K4A	N-A-A-A-Y-N-P-(C-A-S-Y-L)	620.2768 [M+2H] ²⁺	620.2776 [M+2H] ²⁺	18.8	>99
AIP-III Y5A	N-A-A-K-A-N-P-(C-A-S-Y-L)	602.7926 [M+2H] ²⁺	602.7935 [M+2H] ²⁺	17.8	>97
AIP-III N6A	N-A-A-K-Y-A-P-(C-A-S-Y-L)	627.3028 [M+2H] ²⁺	627.3038 [M+2H] ²⁺	18.4	>99
AIP-III P7A	N-A-A-K-Y-N-A-(C-A-S-Y-L)	635.7979 [M+2H] ²⁺	635.7994 [M+2H] ²⁺	18.1	>99
AIP-III S10A	N-A-A-K-Y-N-P-(C-A-A-Y-L)	640.8082 [M+2H] ²⁺	640.8109 [M+2H] ²⁺	19.4	>99
AIP-III Y11A	N-A-A-K-Y-N-P-(C-A-S-A-L)	602.7926 [M+2H] ²⁺	602.7922 [M+2H] ²⁺	17.7	>96
AIP-III L12A	N-A-A-K-Y-N-P-(C-A-S-Y-A)	627.7822 [M+2H] ²⁺	627.7824 [M+2H] ²⁺	16.8	>97
AIP-III D-N1	DN-A-A-K-Y-N-P-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8054 [M+2H] ²⁺	16.7	>99
AIP-III D-A2	N-DA-A-K-Y-N-P-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8050 [M+2H] ²⁺	16.8	>99
AIP-III D-A3	N-A-DA-K-Y-N-P-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8056 [M+2H] ²⁺	16.7	>99
AIP-III D-K4	N-A-A-DK-Y-N-P-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8052 [M+2H] ²⁺	16.7	>98
AIP-III D-Y5	N-A-A-K-DY-N-P-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8055 [M+2H] ²⁺	16.7	>99
AIP-III D-N6	N-A-A-K-Y-DN-P-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8052 [M+2H] ²⁺	16.5	>99
AIP-III D-P7	N-A-A-K-Y-N-DP-(C-A-S-Y-L)	648.8057 [M+2H] ²⁺	648.8061 [M+2H] ²⁺	16.6	>99
AIP-III D-A9	N-A-A-K-Y-N-P-(C-DA-S-Y-L)	648.8057 [M+2H] ²⁺	648.8063 [M+2H] ²⁺	16.9	>99
AIP-III D-S10	N-A-A-K-Y-N-P-(C-A-DS-Y-L)	648.8057 [M+2H] ²⁺	648.8063 [M+2H] ²⁺	16.5	>97
AIP-III D-Y11	N-A-A-K-Y-N-P-(C-A-S-DY-L)	648.8057 [M+2H] ²⁺	648.8062 [M+2H] ²⁺	15.9	>99
AIP-III D-L12	N-A-A-K-Y-N-P-(C-A-S-Y-DL)	648.8057 [M+2H] ²⁺	648.8060 [M+2H] ²⁺	16.2	>99
AIP-III 11aa	A-A-K-Y-N-P-(C-A-S-Y-L)	1182.5612	1182.5618	17.9	>98
AIP-III 10aa	A-K-Y-N-P-(C-A-S-Y-L)	1111.5241	1111.5226	18.0	>96
AIP-III 9aa	K-Y-N-P-(C-A-S-Y-L)	1040.4870	1040.4851	18.0	>97
AIP-III 8aa	Y-N-P-(C-A-S-Y-L)	912.3920	912.3917	19.4	>97

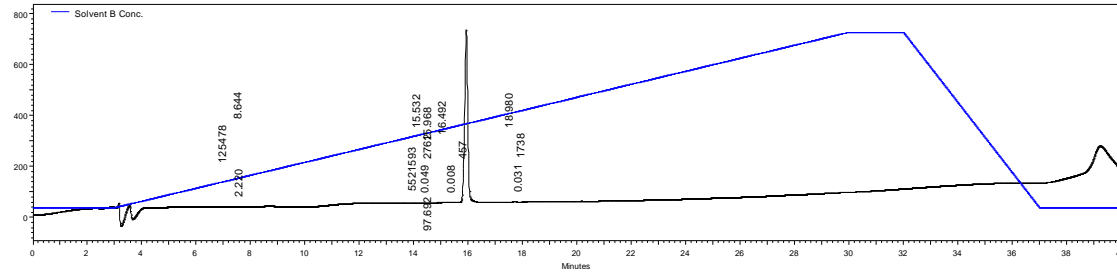
Table SI.3: MS and HPLC data for the *S. epidermidis* AIP analogs synthesized for pan-group agonist evaluation. EM = exact mass. Rt = retention time. α = L-2-Aminobutyric acid

Peptide Name	Sequence	Calc. EM	Obs. EM	Rt (min)	Purity (%)
AIP19aaN3L	K-Y-L-P-(C-S-N-Y-L)	541.7706 [M+2H] ²⁺	541.7706 [M+2H] ²⁺	17.7	>92
AIP19aaN3V	K-Y-V-P-(C-S-N-Y-L)	534.7628 [M+2H] ²⁺	534.7624 [M+2H] ²⁺	17.0	>97
Cmr3	K-Y-N-V-(C-S-N-Y-L)	543.2579 [M+2H] ²⁺	543.2575 [M+2H] ²⁺	17.0	>95
AIP19aaP4L	K-Y-N-L-(C-S-N-Y-L)	1099.5241	1099.5240	17.7	>97
Cmr2	K-Y-N-V-(C-A-S-Y-F)	538.7471 [M+2H] ²⁺	538.7466 [M+2H] ²⁺	17.8	>96
AIP1tail AIP1ring	D-S-V-(C-S-N-Y-L)	882.3662	882.3660	17.0	>94
AIP1tail AIP1ring	D-S-V-(C-A-S-Y-L)	839.3604	839.3613	18.8	>96
Cmr1	K-Y-N-P-(C-A-S-Y-F)	1074.4713	1074.4710	17.6	>95
Cmr2_A6S	K-Y-N-V-(C-S-S-Y-F)	1092.4819	1092.4828	18.1	>92
Cmr2_A6SS7A	K-Y-N-V-(C-S-A-Y-F)	1076.4870	1076.4878	19.0	>91
Cmr2_S7N	K-Y-N-V-(C-A-N-Y-F)	1103.4979	1103.4988	18.4	>98
Cmr2_S7A	K-Y-N-V-(C-A-A-Y-F)	1060.4921	1060.4928	19.4	>93
Cmr2_F9L	K-Y-N-V-(C-A-S-Y-L)	1042.5026	1042.5037	17.9	>97
Cmr3_S6A	K-Y-N-V-(C-A-N-Y-L)	535.2604 [M+2H] ²⁺	535.2603 [M+2H] ²⁺	17.6	>98
Cmr3_N7A	K-Y-N-V-(C-S-A-Y-L)	521.7550 [M+2H] ²⁺	521.7549 [M+2H] ²⁺	18.0	>97
Cmr3_N7S	K-Y-N-V-(C-S-S-Y-L)	1058.4975	1058.4988	17.7	>95
Cmr3_S6AN7A	K-Y-N-V-(C-A-A-Y-L)	513.7575 [M+2H] ²⁺	513.7578 [M+2H] ²⁺	18.6	>94
Cmr3_L9F	K-Y-N-V-(C-S-N-Y-F)	560.2500 [M+2H] ²⁺	560.2503 [M+2H] ²⁺	17.8	>99
Cmr3_N7 α	K-Y-N-V-(C-S- α -Y-L)	528.7623 [M+2H] ²⁺	528.7626 [M+2H] ²⁺	19.4	>97
Cmr3_S6 α	K-Y-N-V-(C- α -N-Y-L)	542.2682 [M+2H] ²⁺	542.2680 [M+2H] ²⁺	18.5	>96
Cmr3_S6G	K-Y-N-V-(C-G-N-Y-L)	1055.4979	1055.4988	17.9	>95
Cmr3_S6T	K-Y-N-V-(C-T-N-Y-L)	1099.5241	1099.5251	18.3	>93
Cmr3_N7L	K-Y-N-V-(C-S-L-Y-L)	1084.5496	1084.5497	19.9	>96
Cmr3_N7T	K-Y-N-V-(C-S-T-Y-L)	1072.5132	1072.5137	18.7	>95
Cmr3_N7G	K-Y-N-V-(C-S-G-Y-L)	1028.4870	1028.4875	18.0	>96
Cmr3_N7V	K-Y-N-V-(C-S-V-Y-L)	535.7706 [M+2H] ²⁺	535.7705 [M+2H] ²⁺	19.8	>93
Cmr3_N7Q	K-Y-N-V-(C-S-Q-Y-L)	550.2657 [M+2H] ²⁺	550.2654 [M+2H] ²⁺	18.5	>96

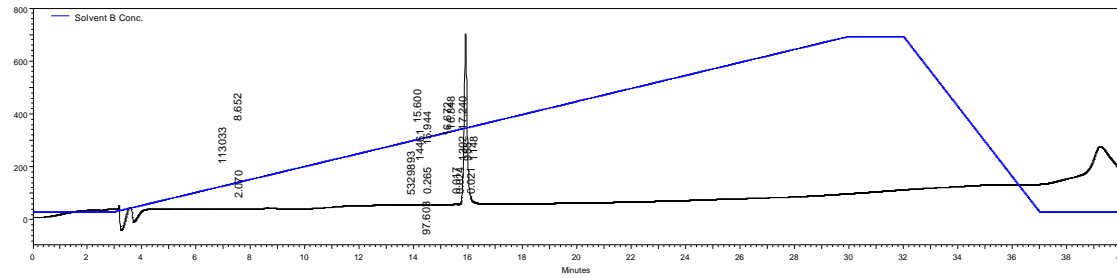
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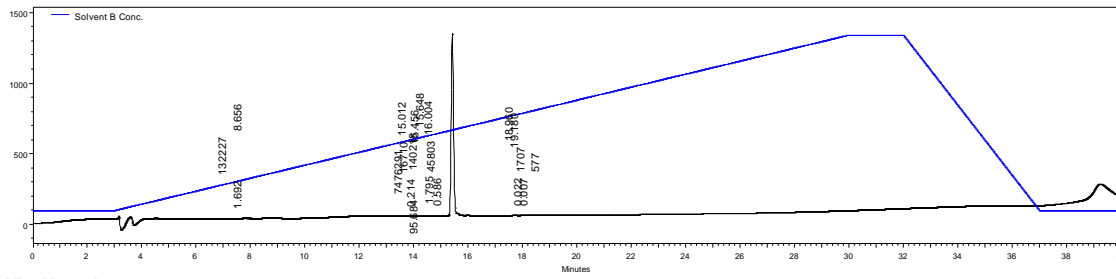
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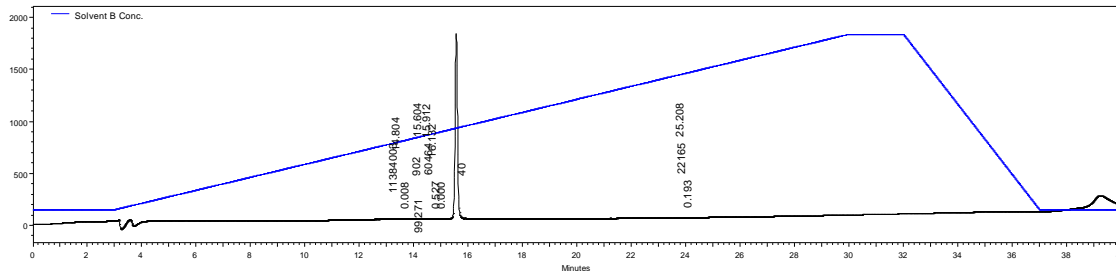
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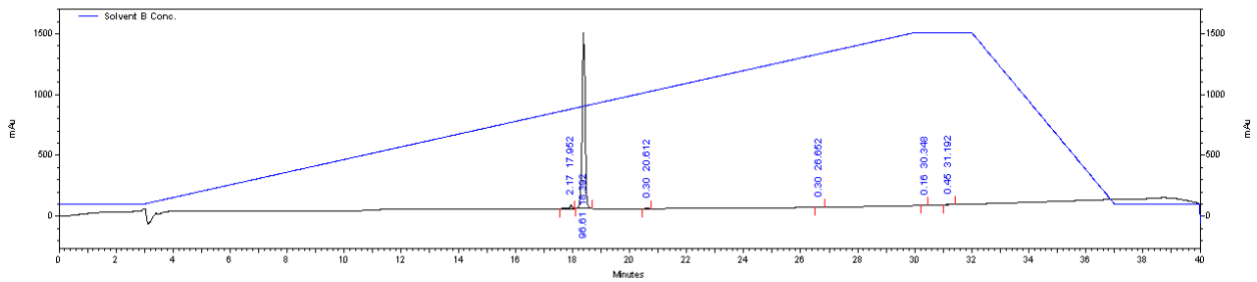
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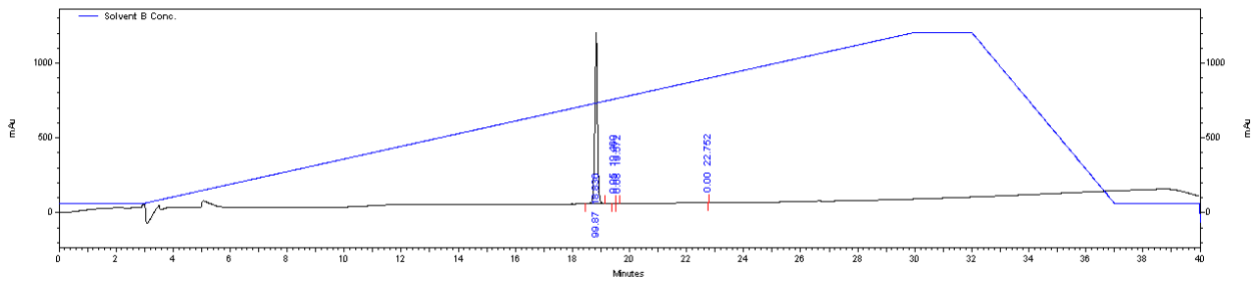
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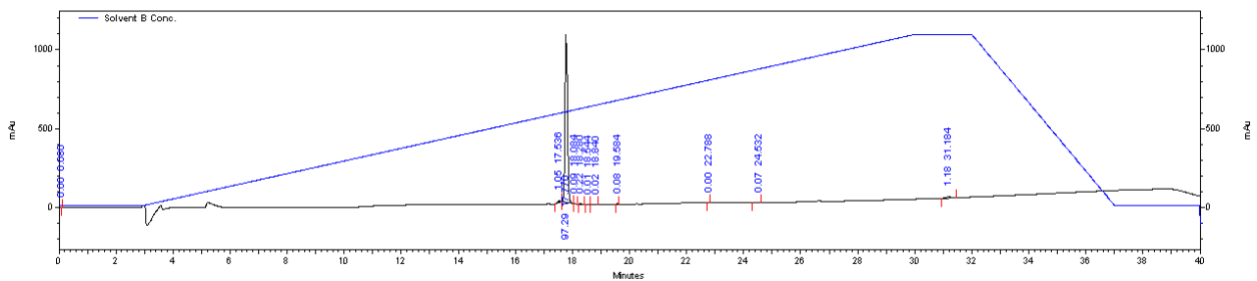
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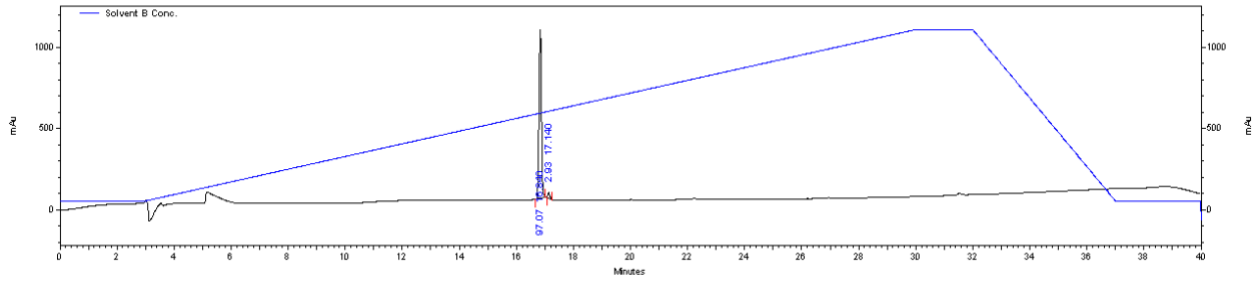
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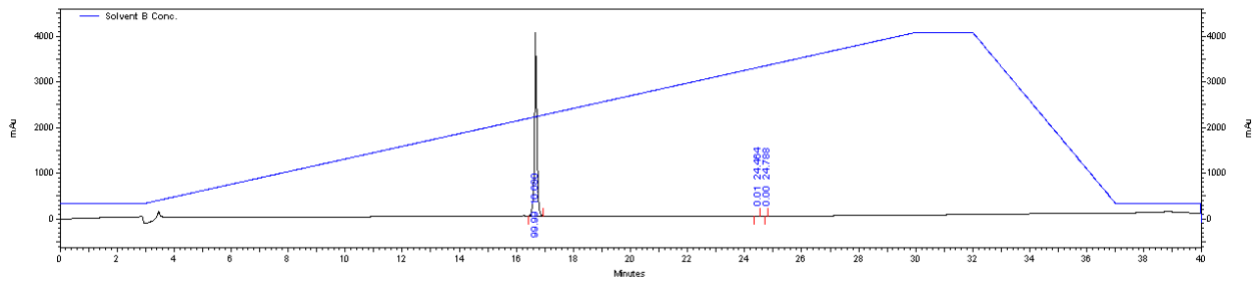
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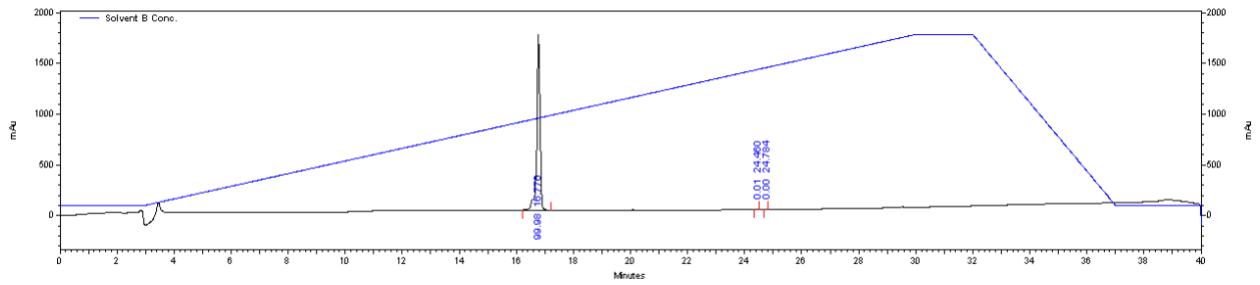
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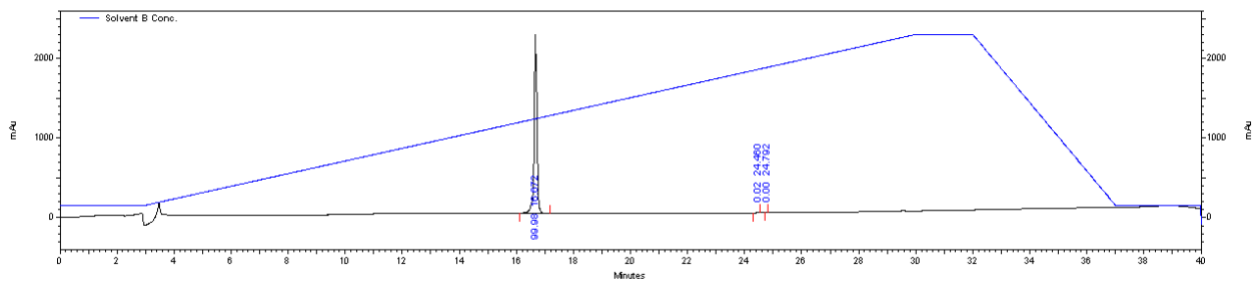
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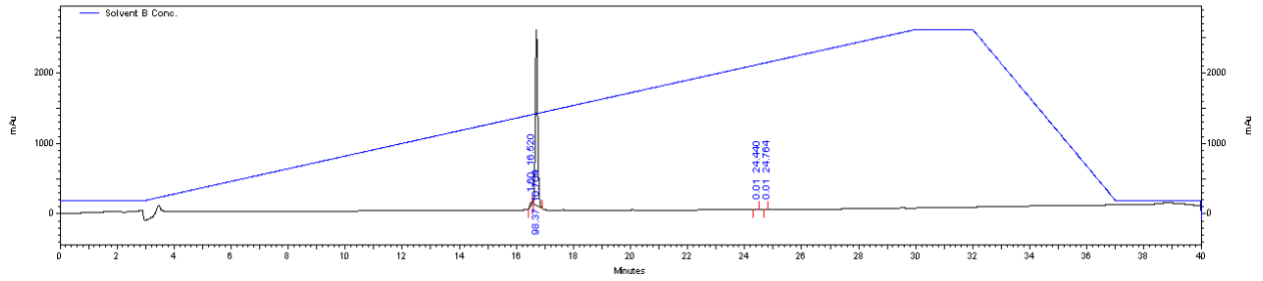
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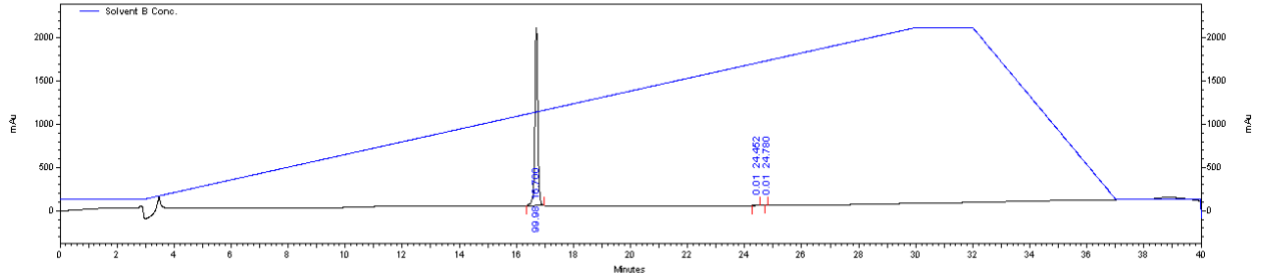
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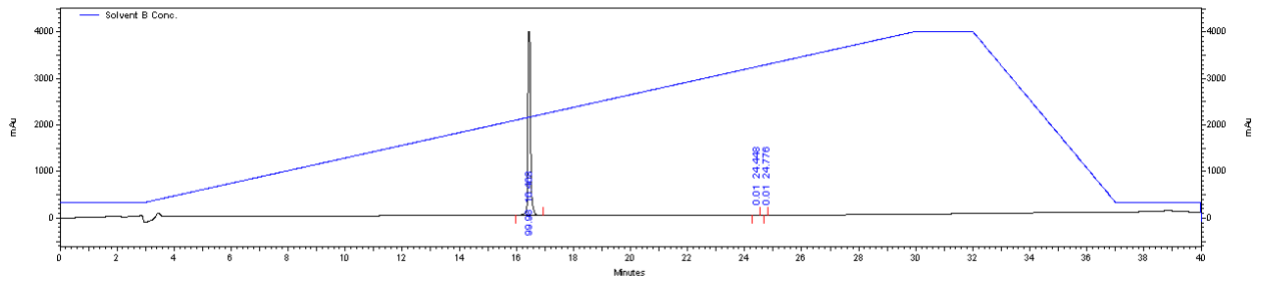
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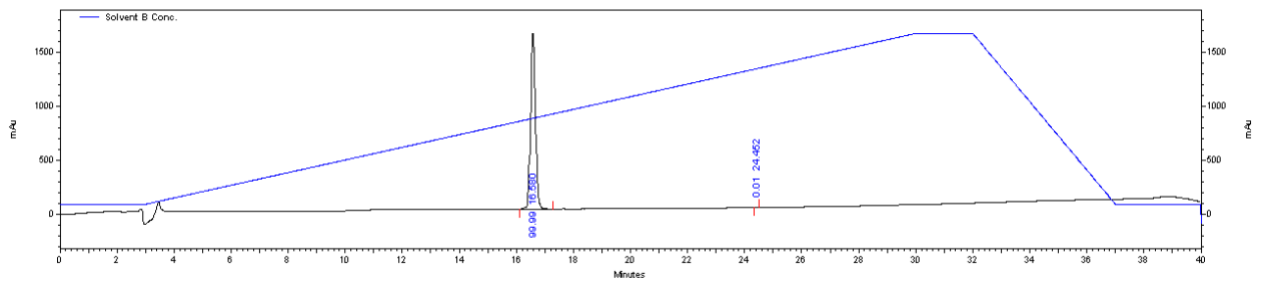
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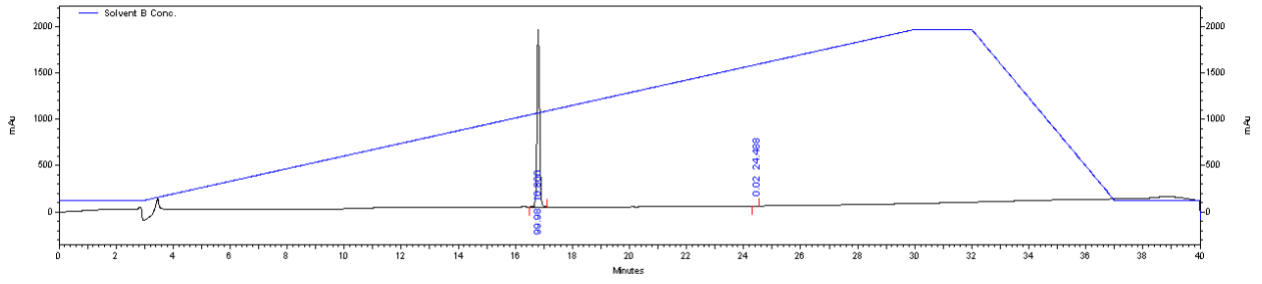
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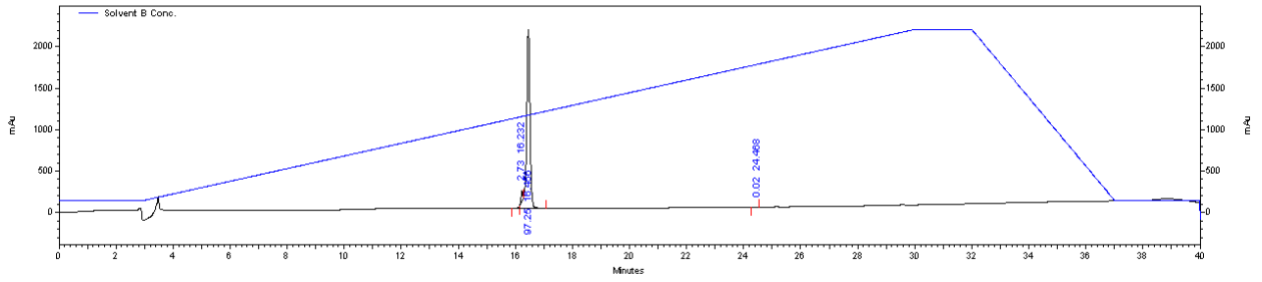
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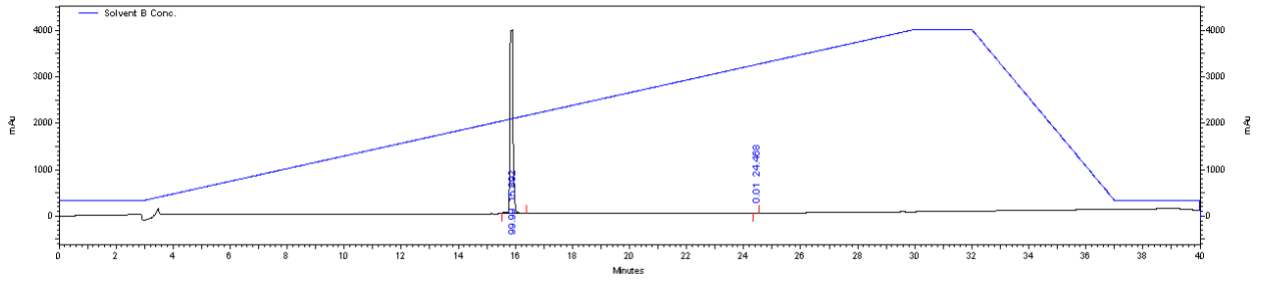
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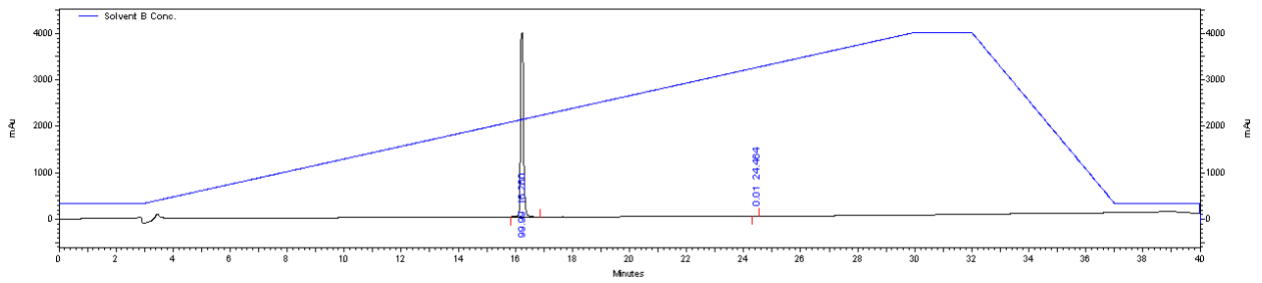
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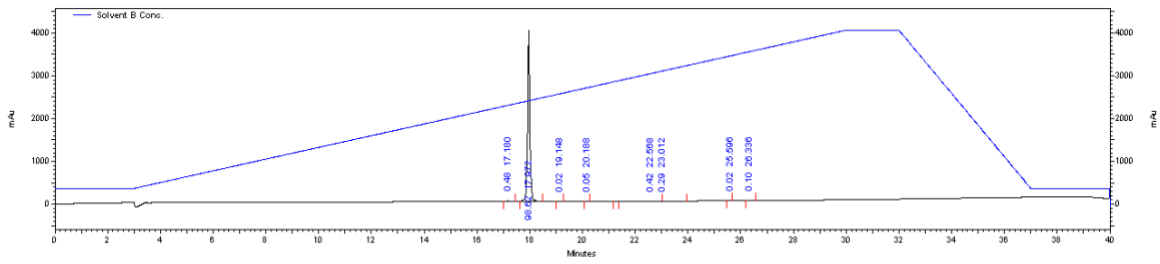
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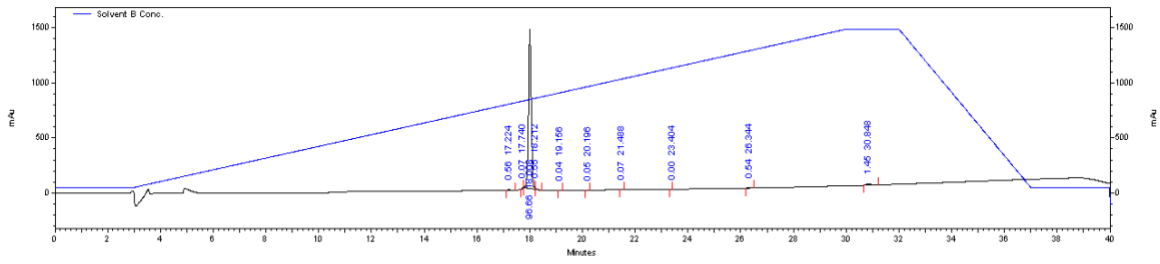
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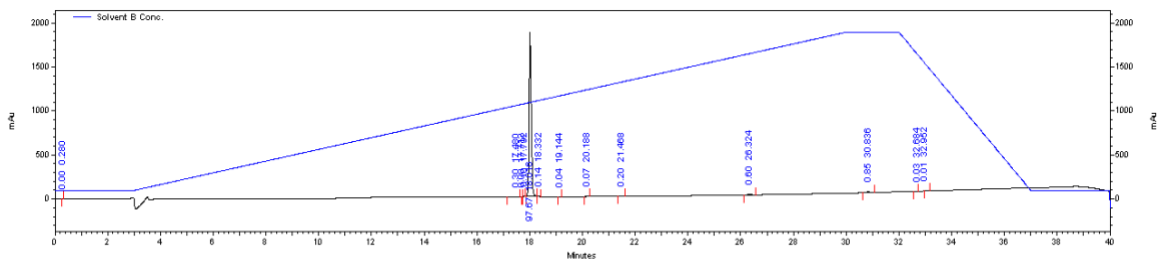
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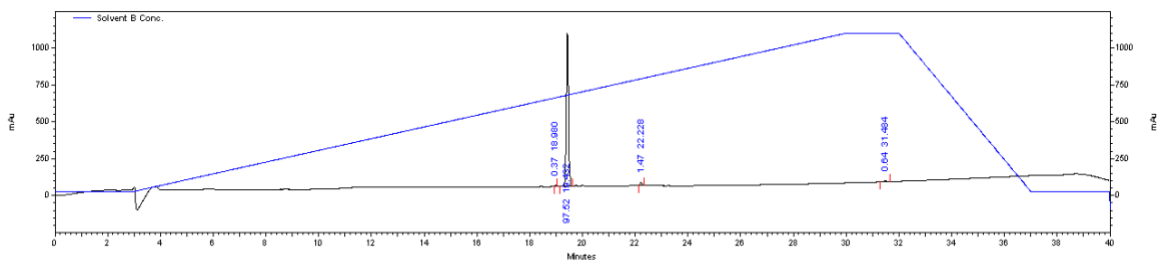
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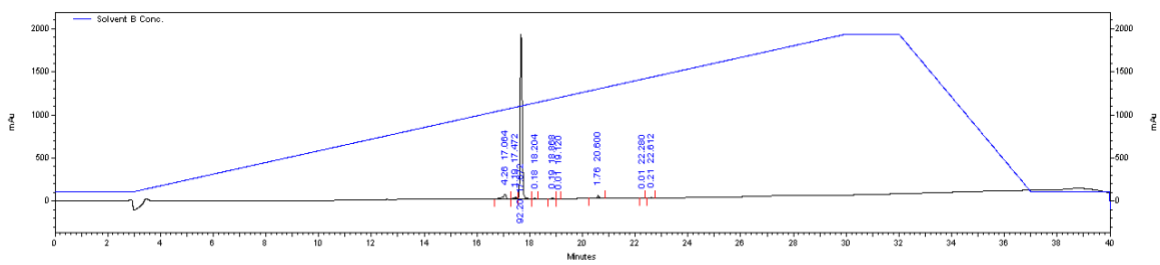
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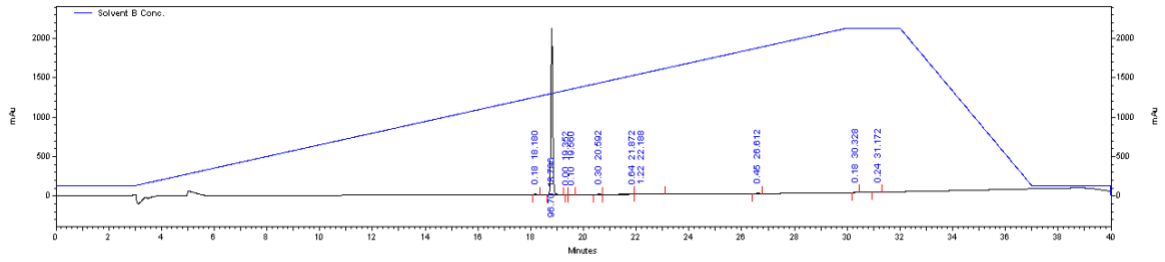
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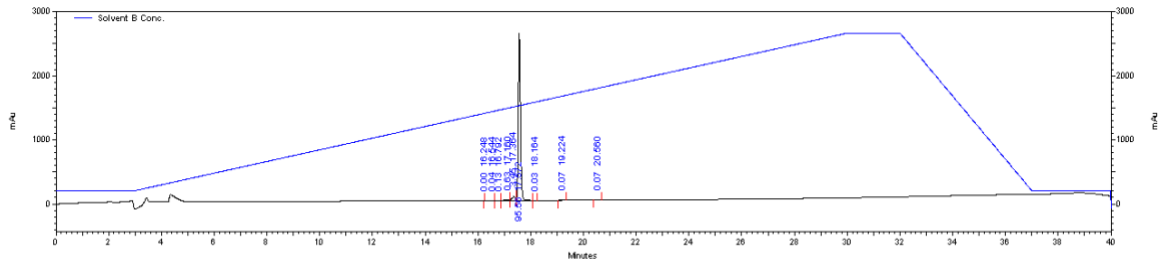
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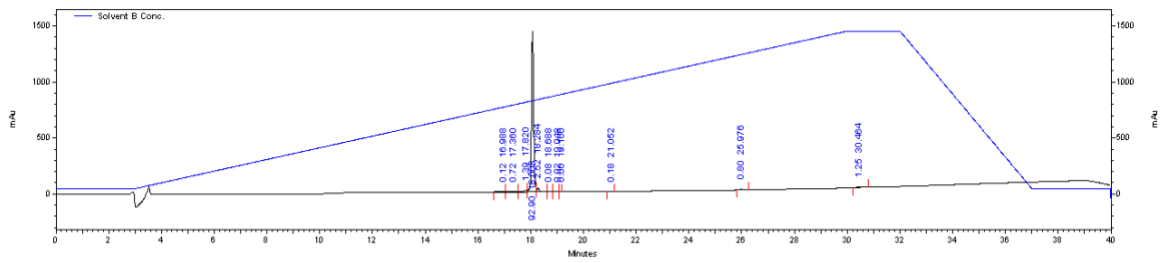
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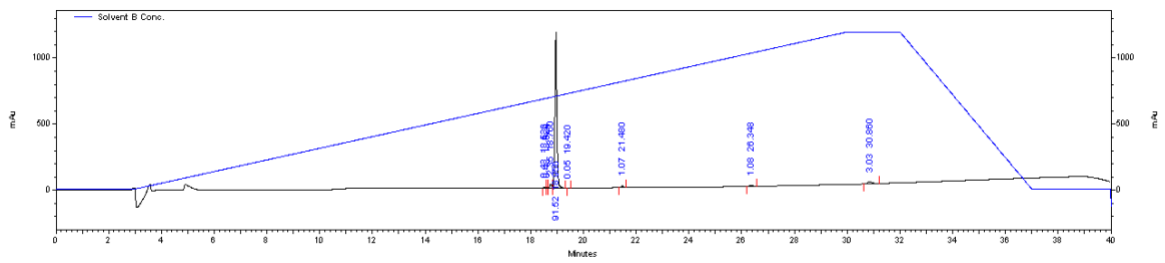
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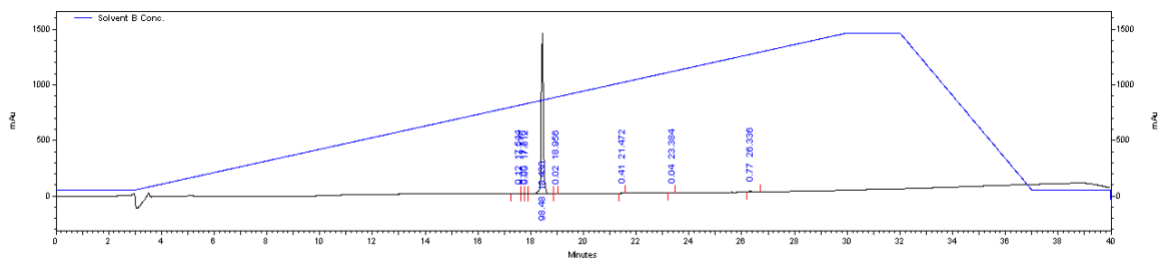
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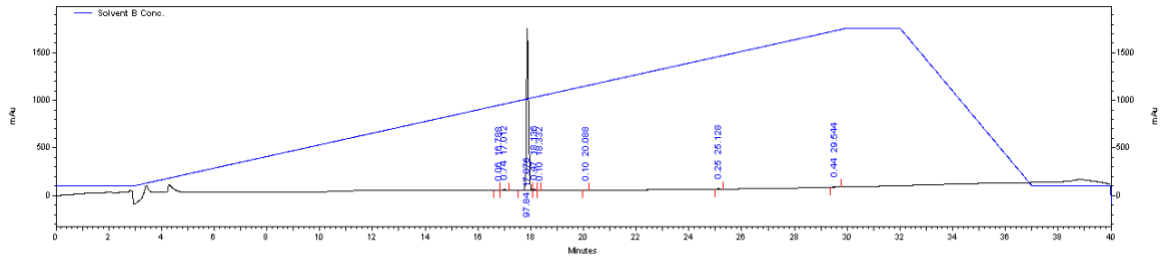
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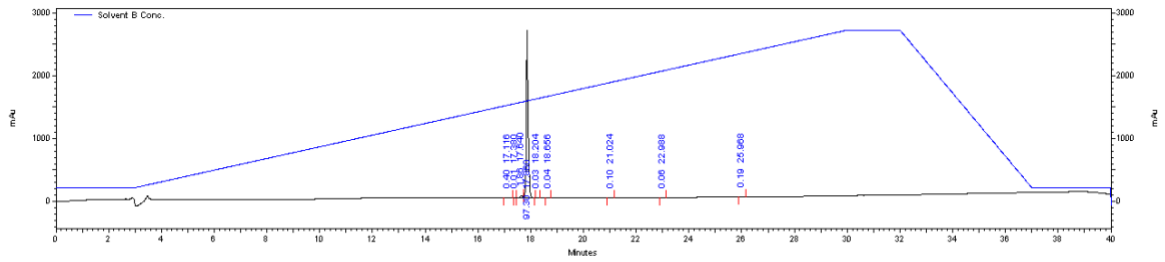
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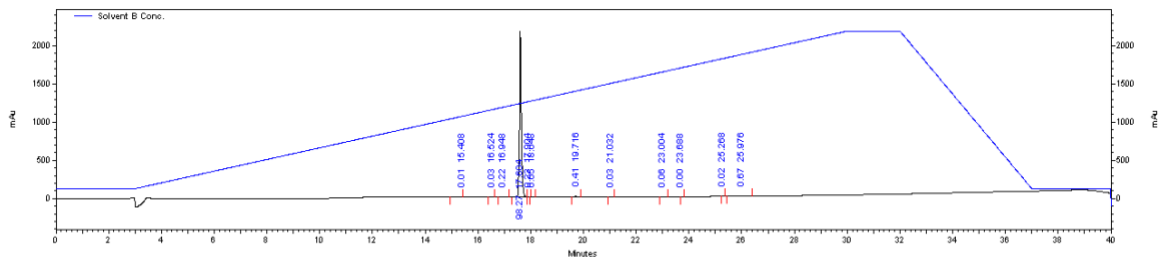
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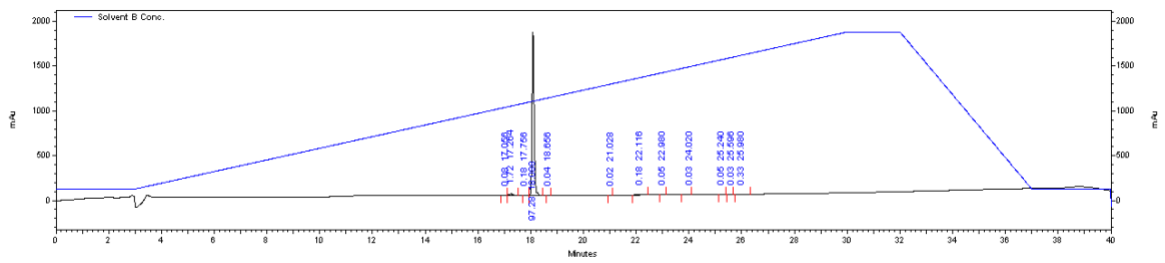
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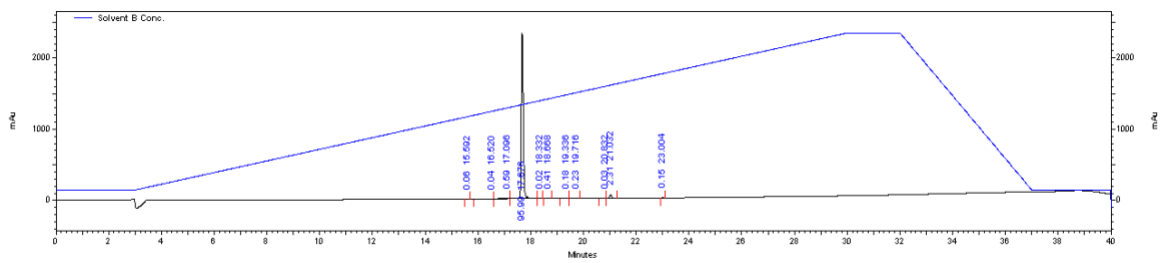
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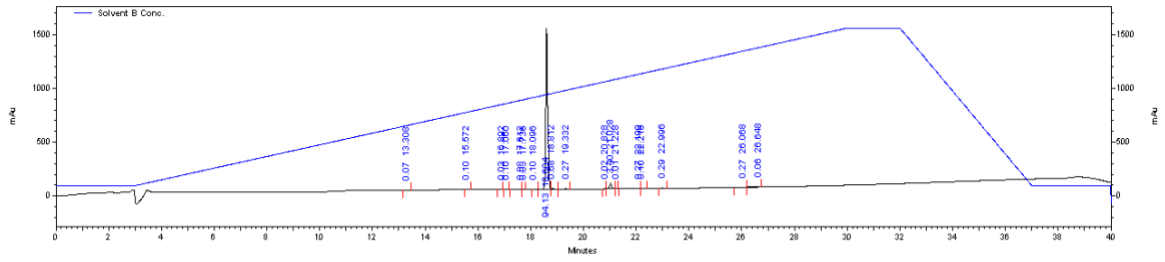
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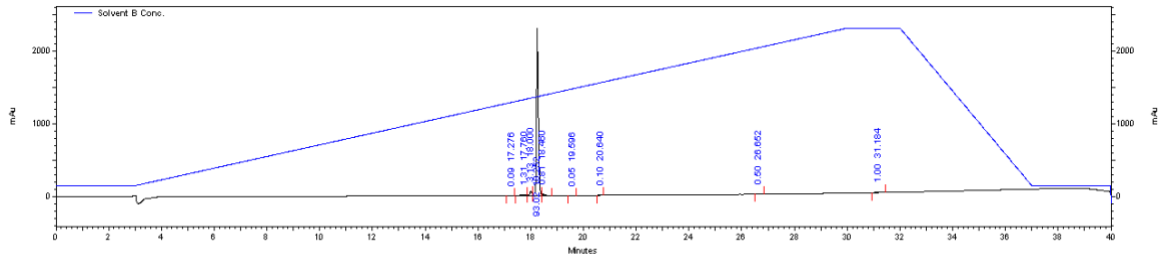
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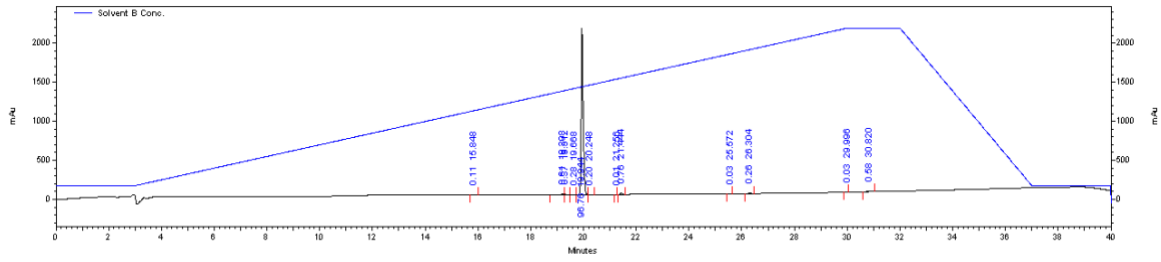
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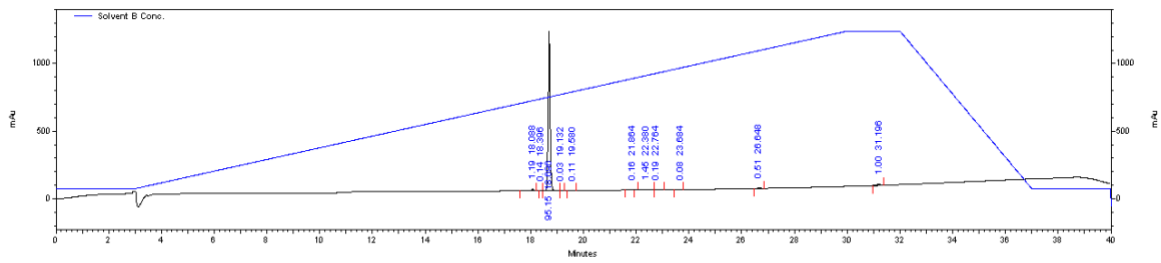
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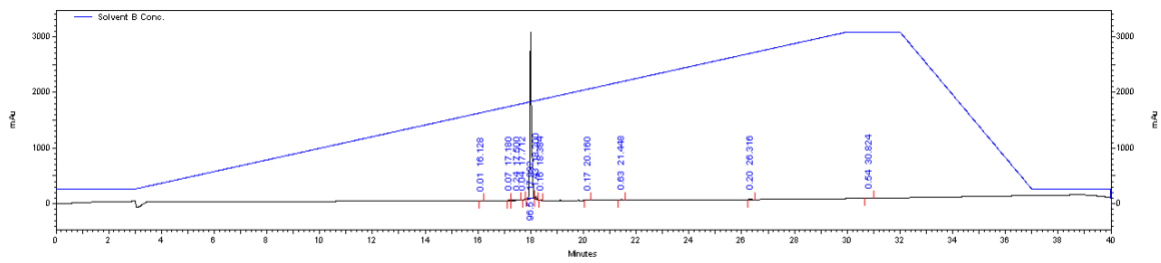
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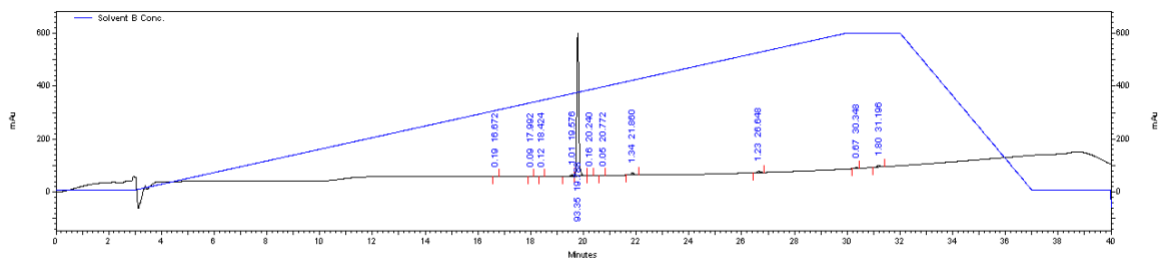
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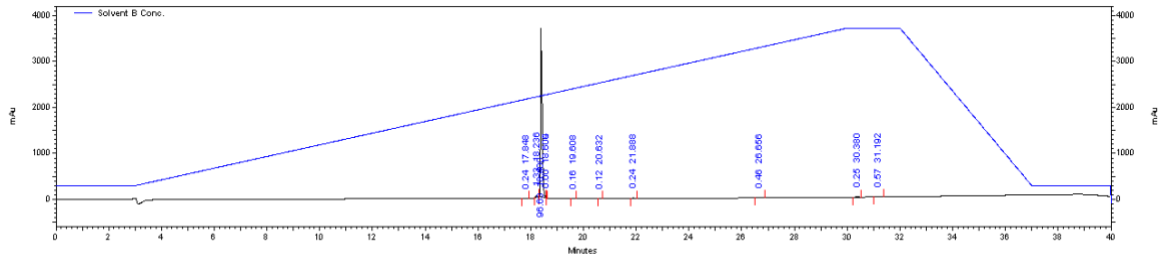
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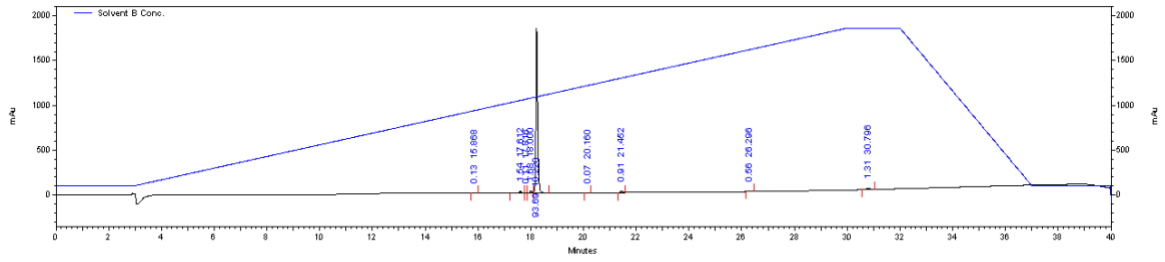
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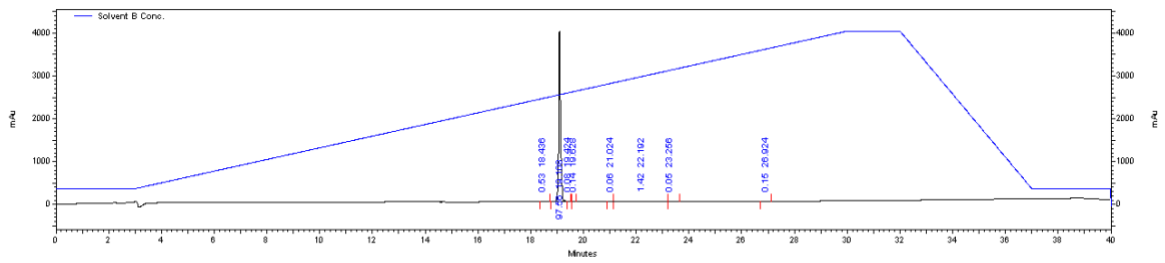
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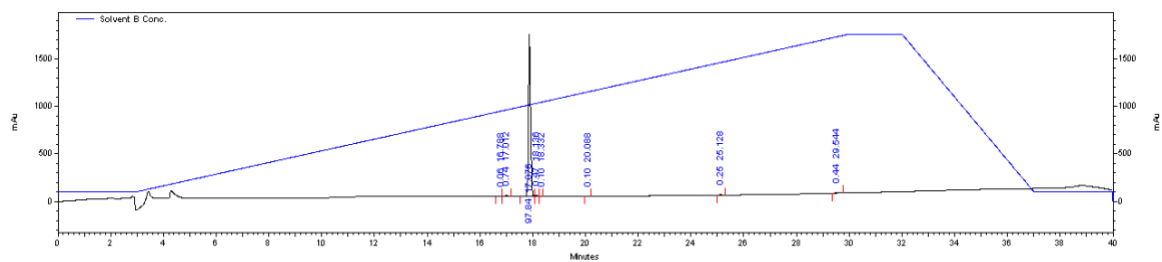
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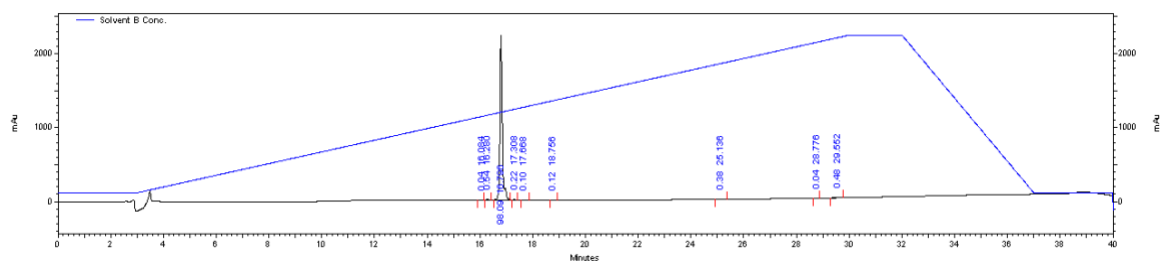
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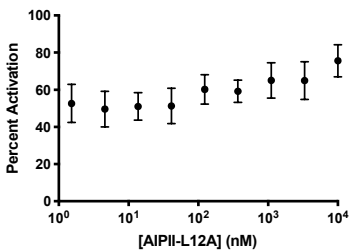
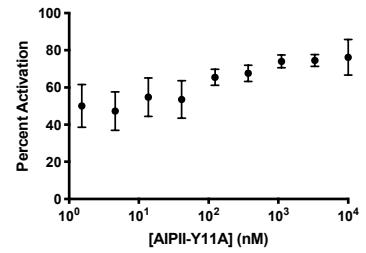
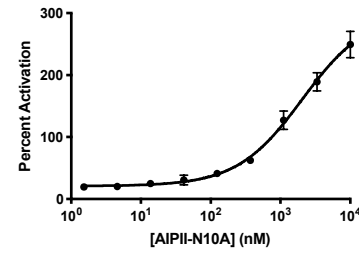
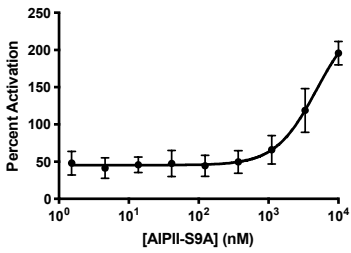
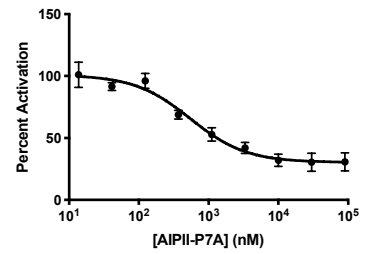
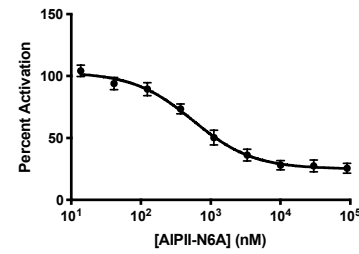
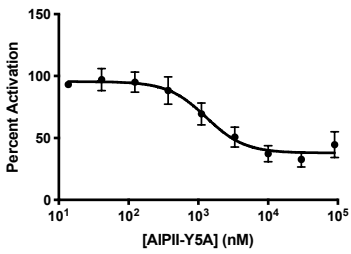
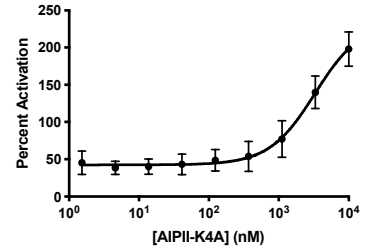
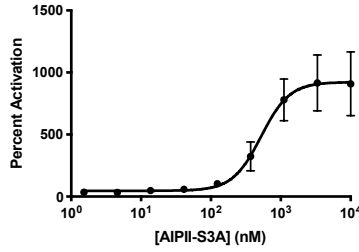
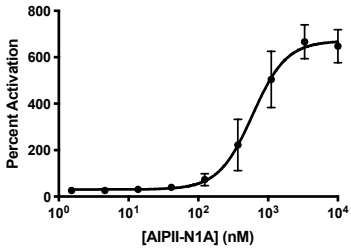


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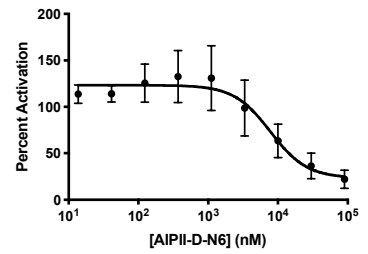
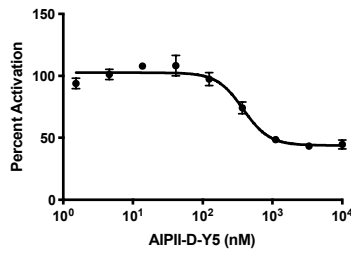
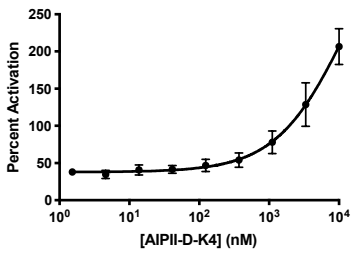
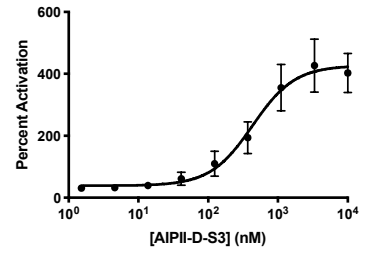
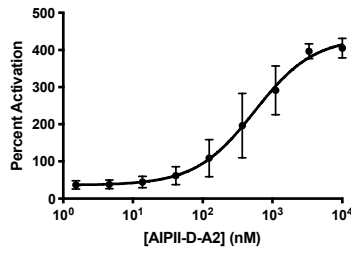
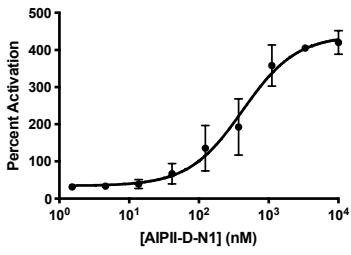


3. Dose-response curves of SARs in *S.epidermidis* Group-II and -III

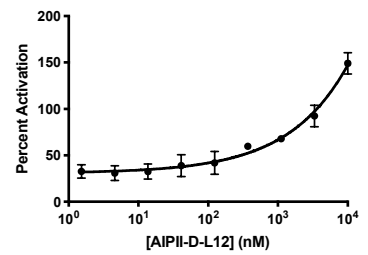
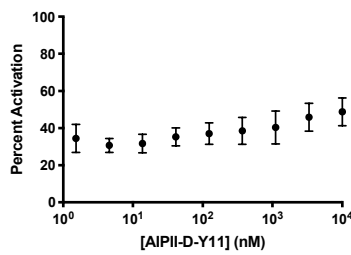
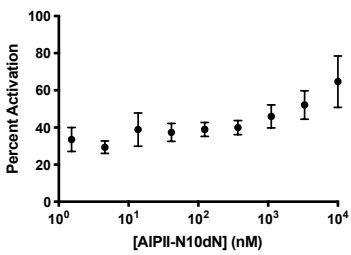
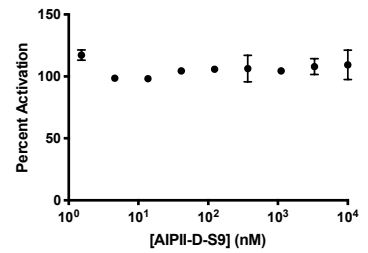
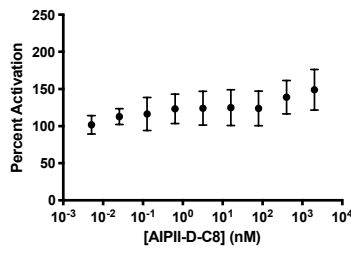
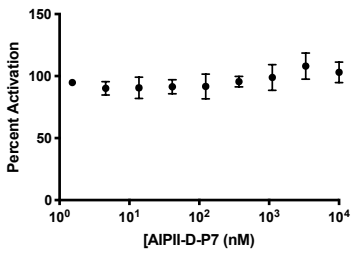
- Dose-response curves for Alanine scan in *S.epidermidis* Group II (AH3567)



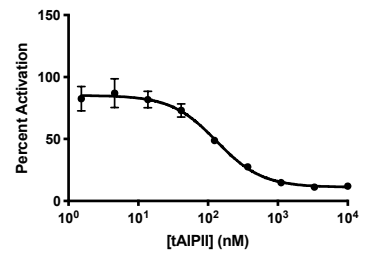
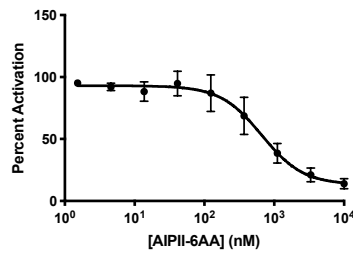
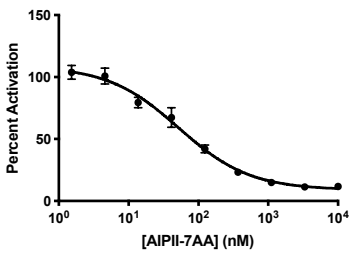
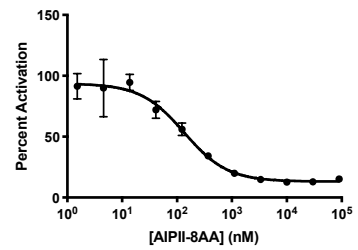
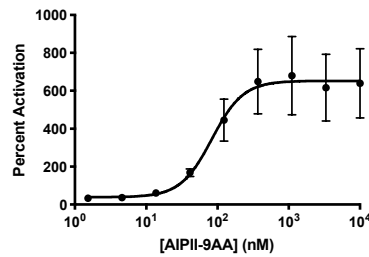
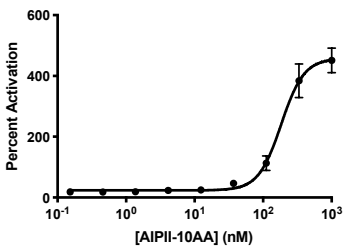
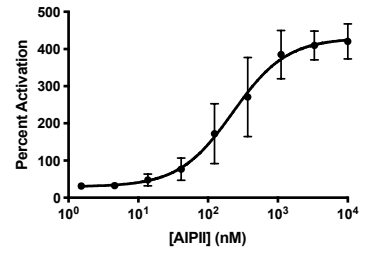
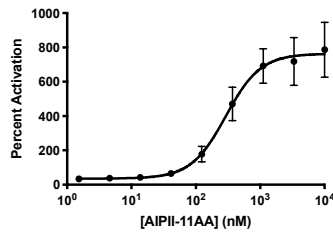
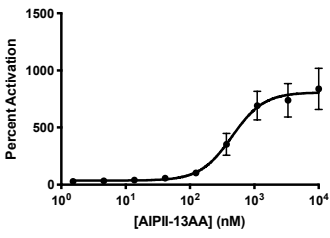
- Dose-response curves for D-amino acids scan in *S.epidermidis* Group II (AH3567)



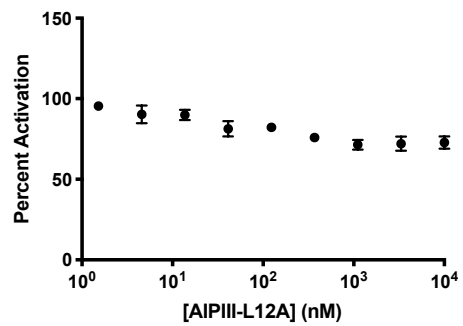
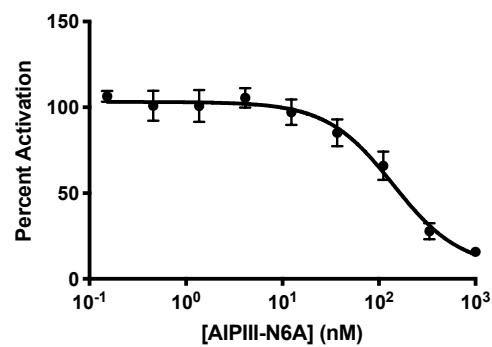
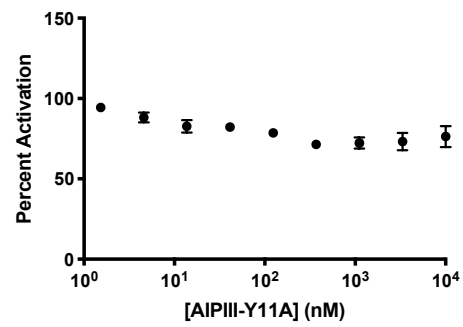
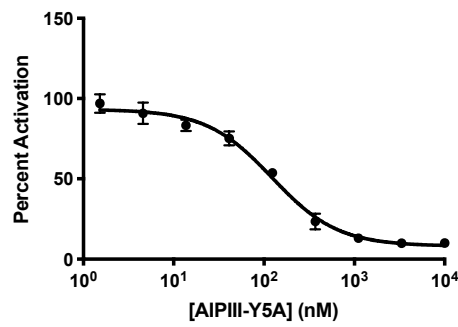
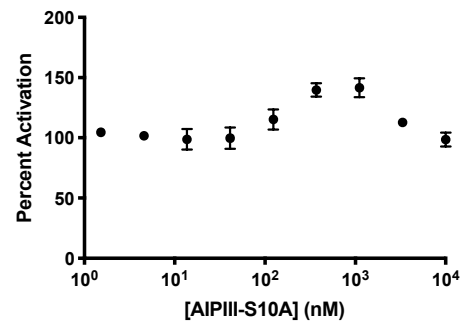
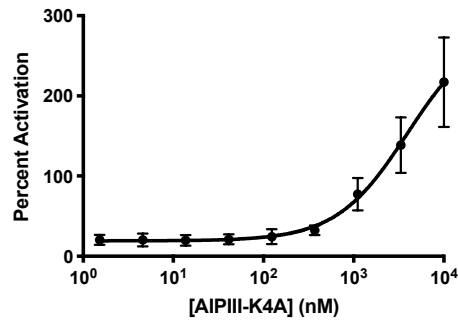
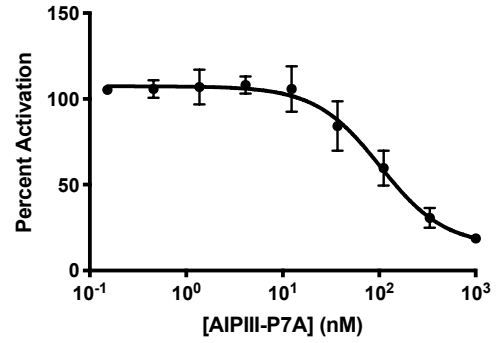
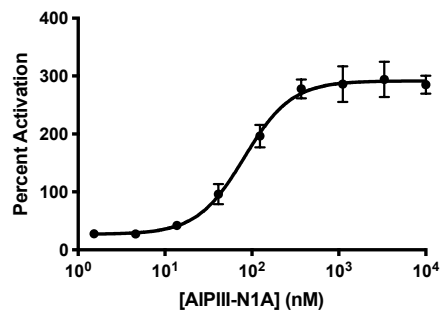
AIPII-P7dP



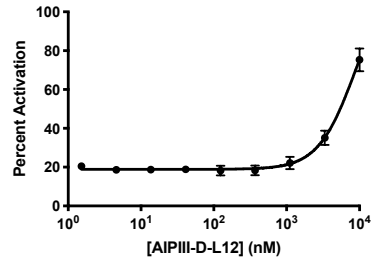
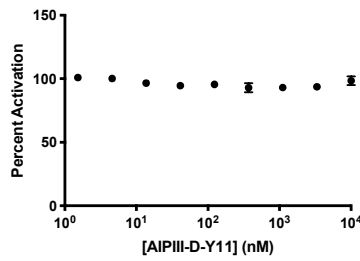
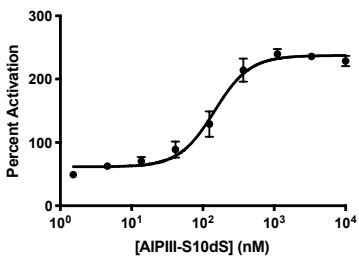
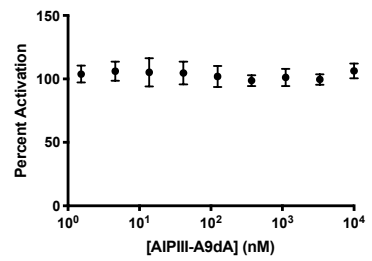
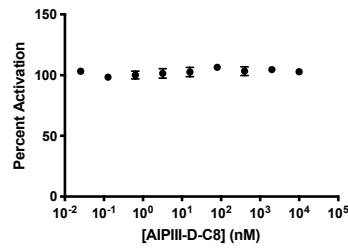
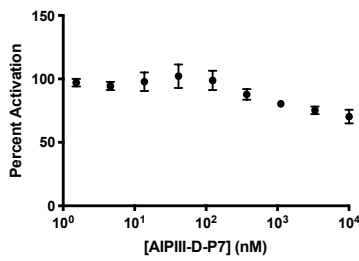
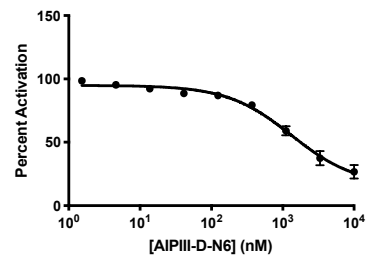
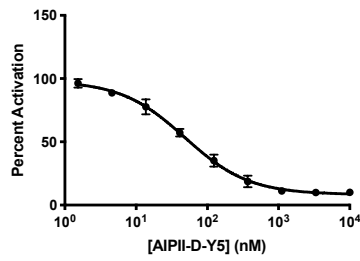
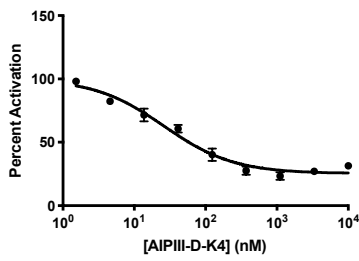
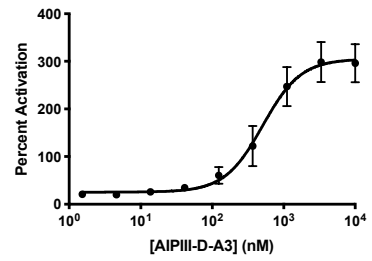
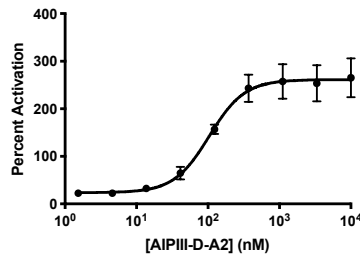
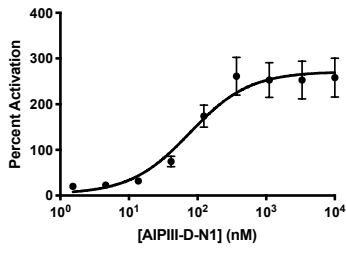
- Dose-response curves for tail truncation in *S.epidermidis* Group II (AH3567)



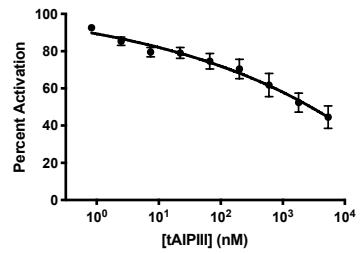
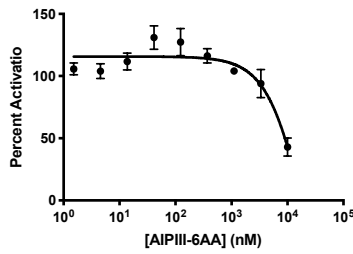
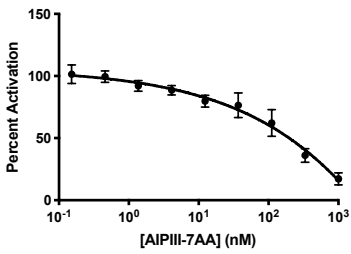
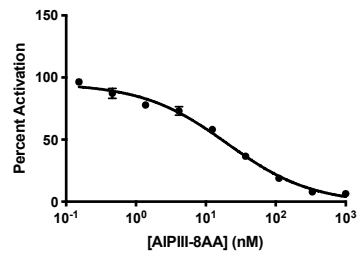
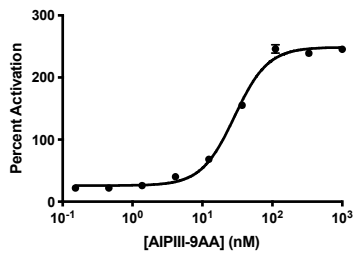
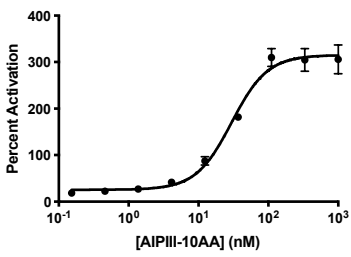
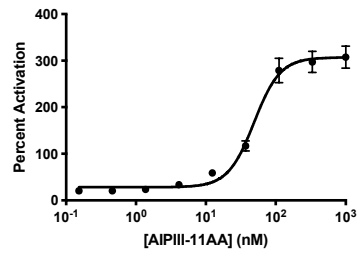
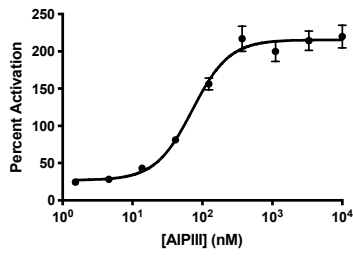
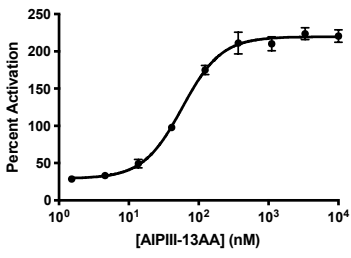
- Dose-response curves for Alanine scan in *S.epidermidis* Group III (AH3409)



- Dose-response curves for D-amino acids scan in *S.epidermidis* Group II (AH3409)

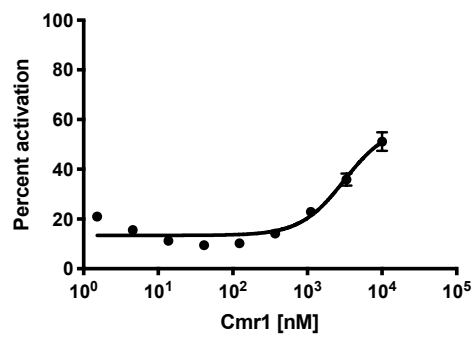
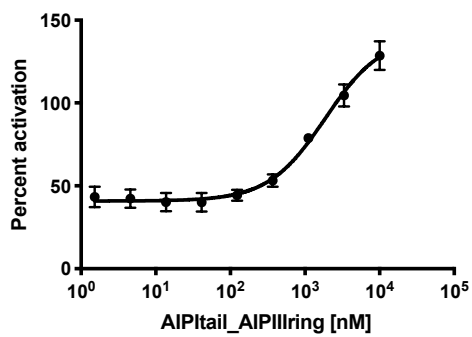
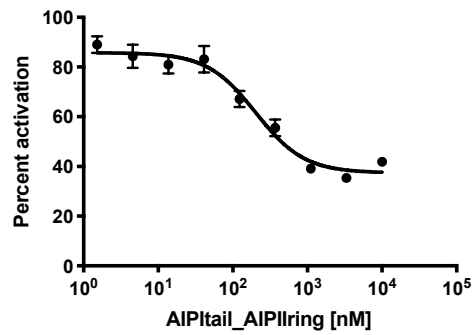
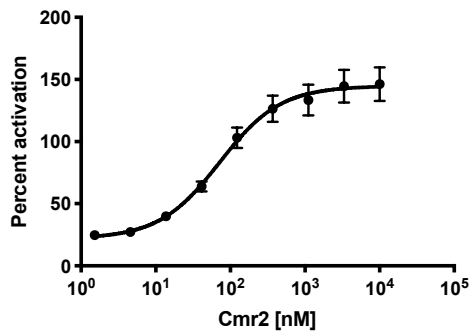
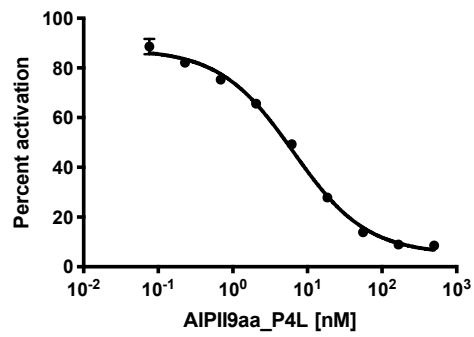
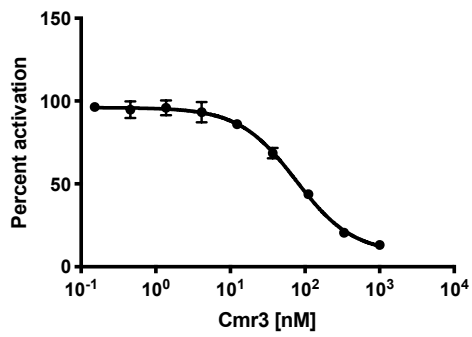
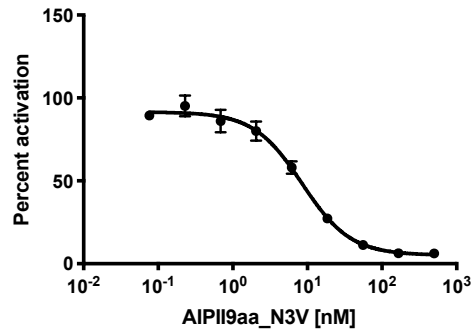
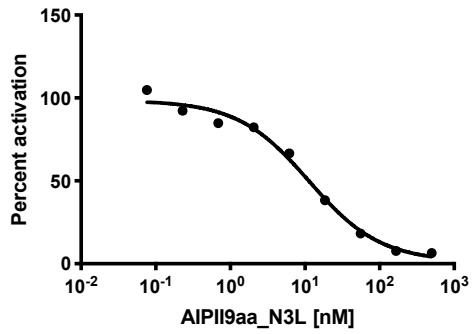


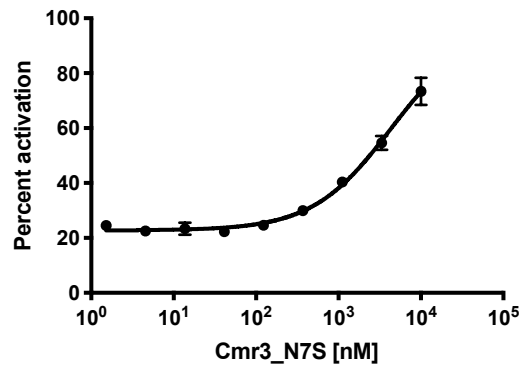
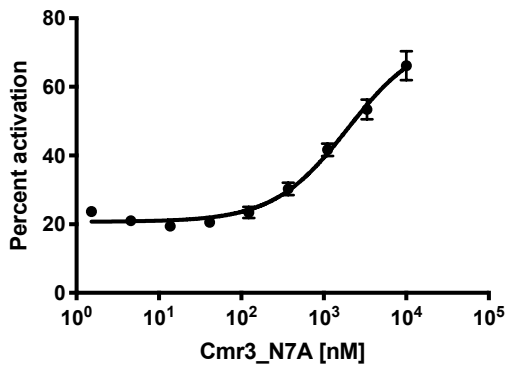
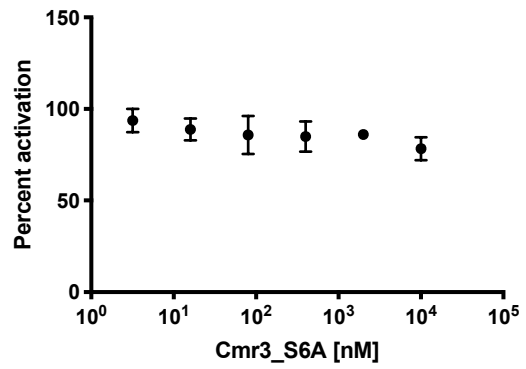
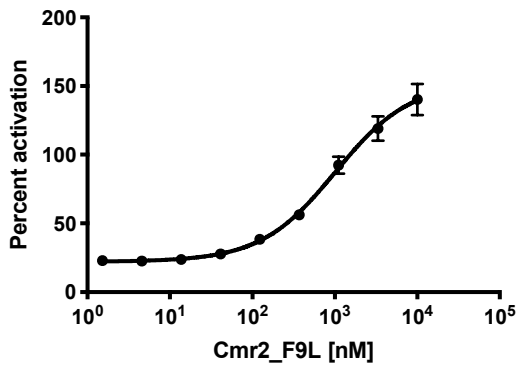
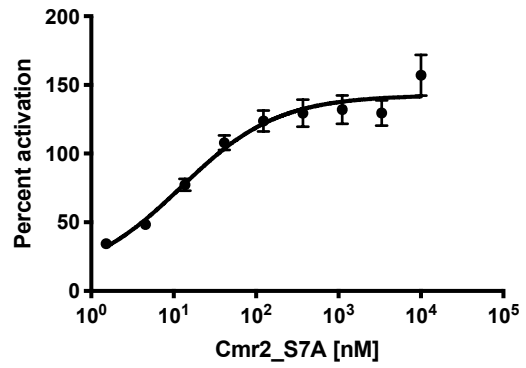
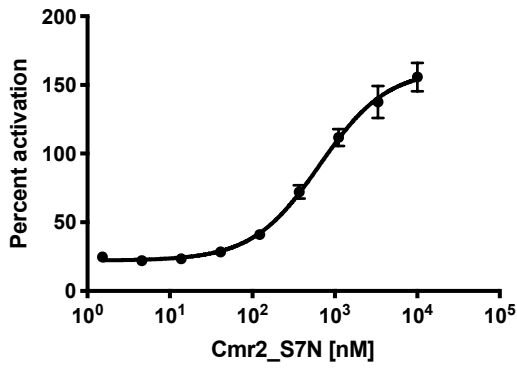
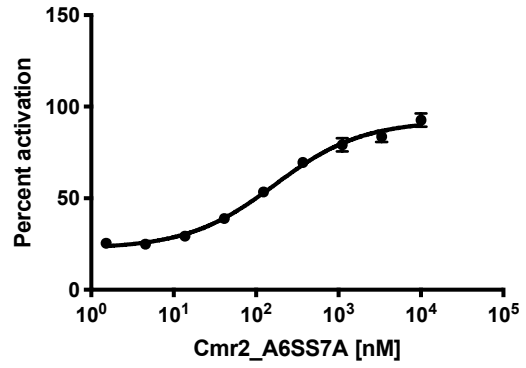
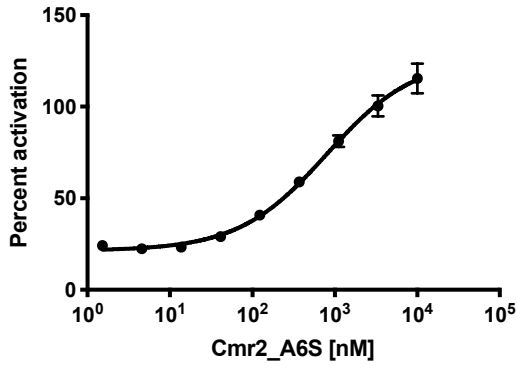
- Dose-response curves for tail truncation in *S.epidermidis* Group II (AH3409)

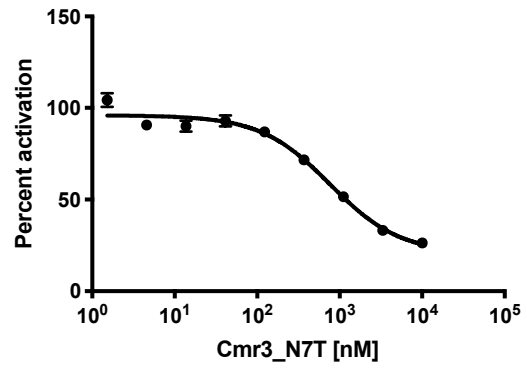
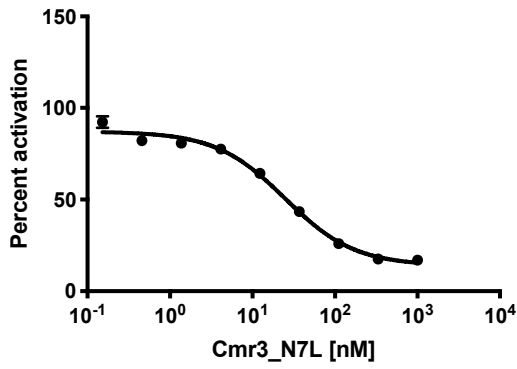
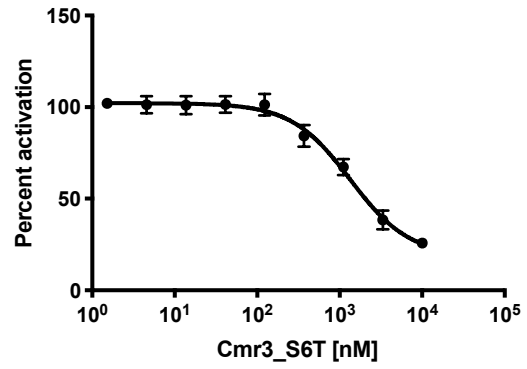
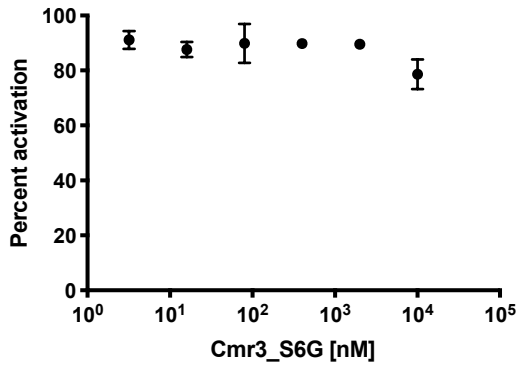
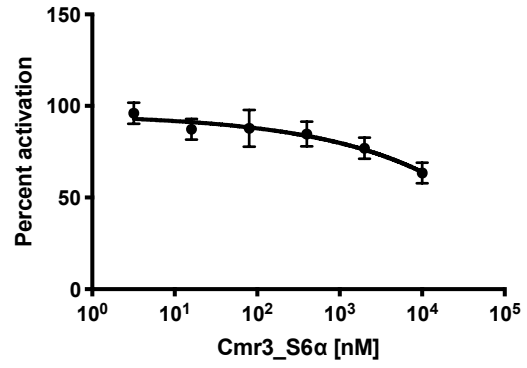
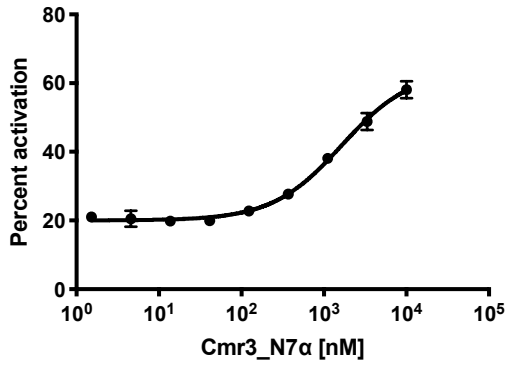
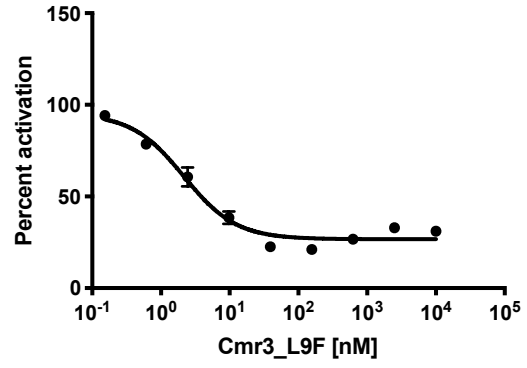
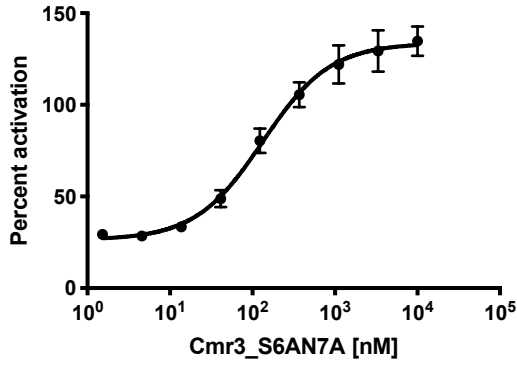


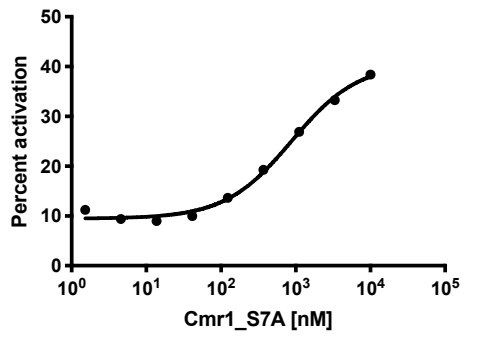
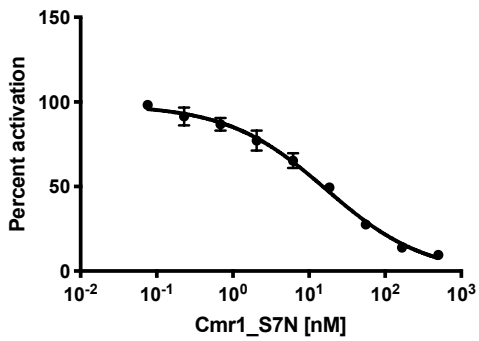
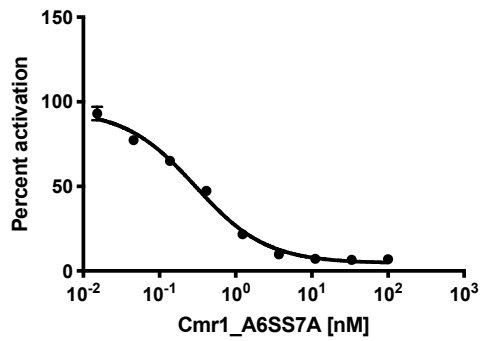
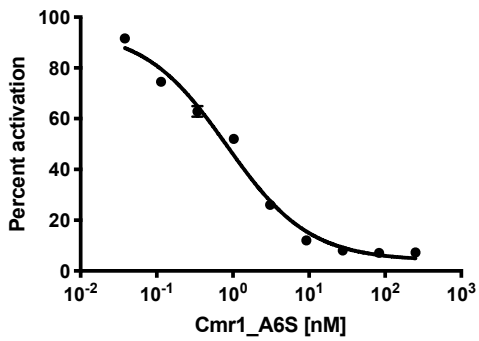
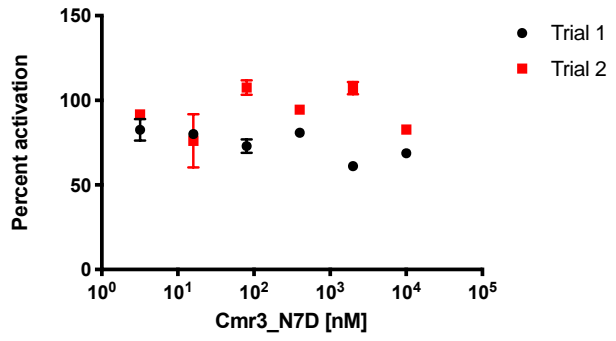
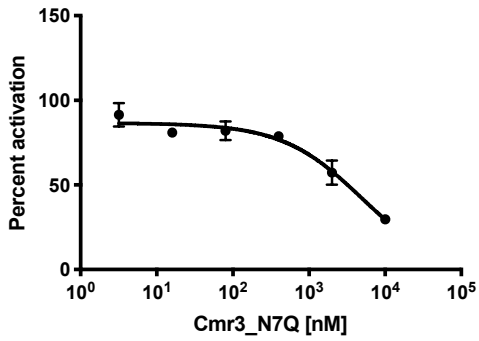
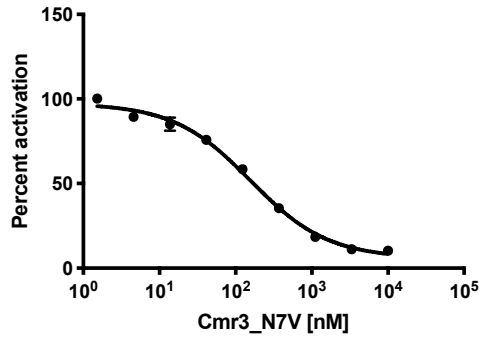
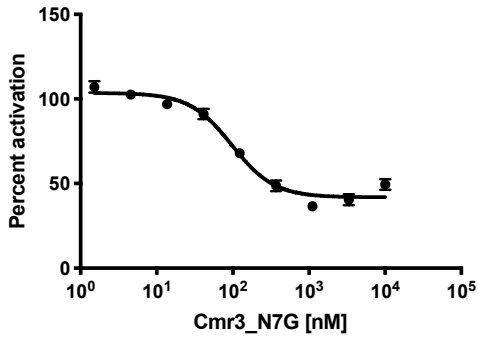
4. Dose-response curves of pan-group compounds in *S.epidermidis* Group I-III

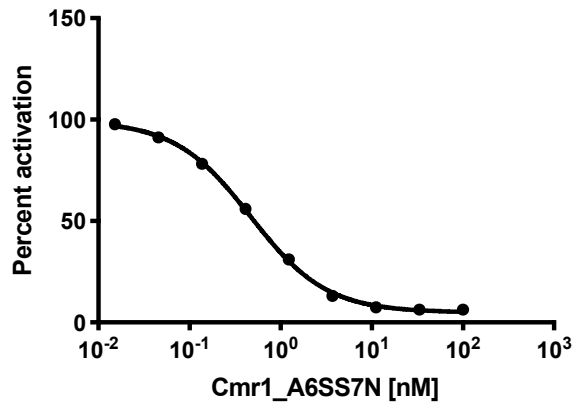
- Dose-response curve in *S.epidermidis* Group I (AH3408)



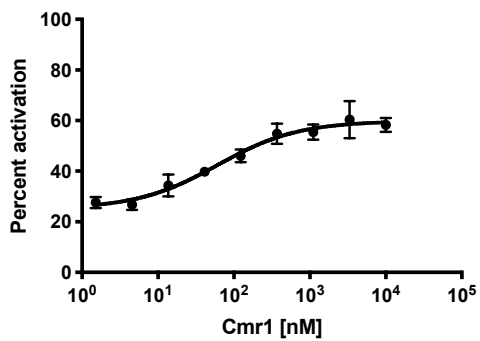
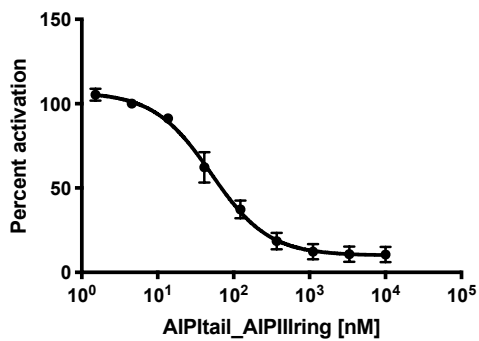
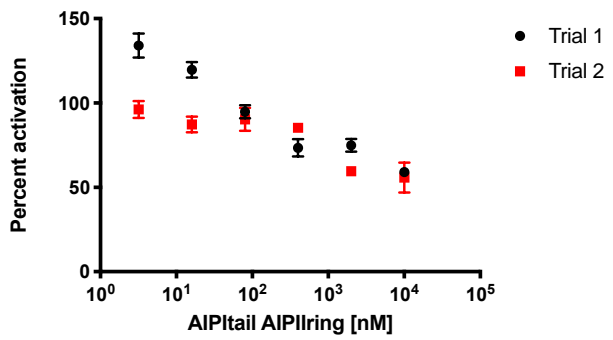
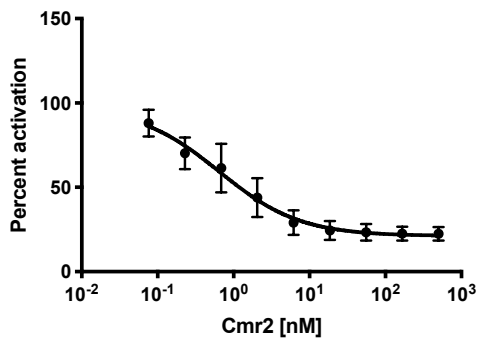
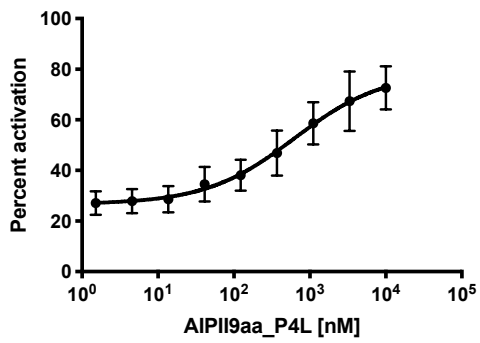
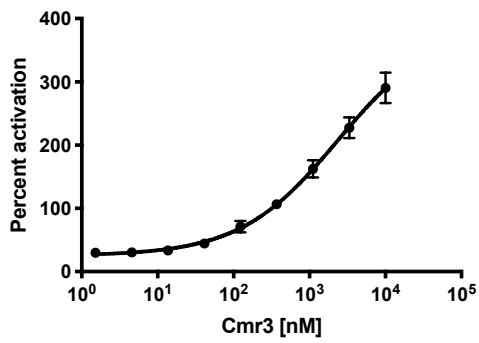
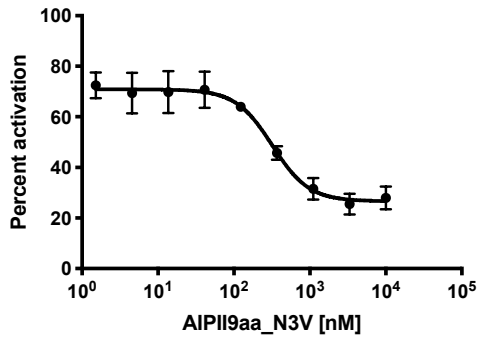
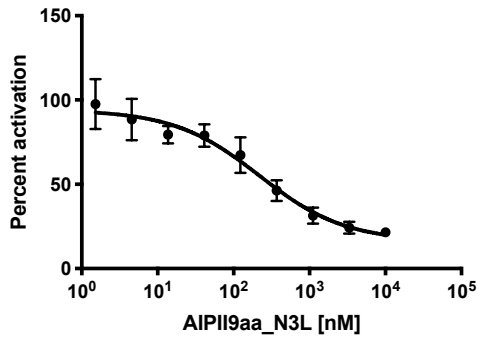


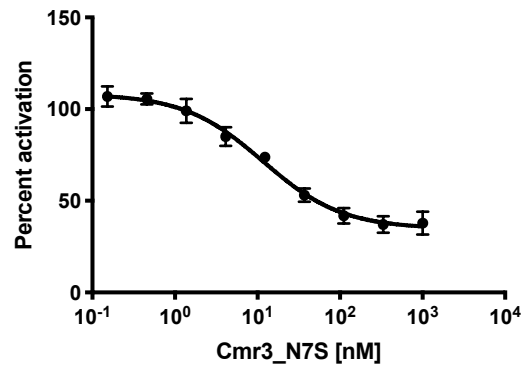
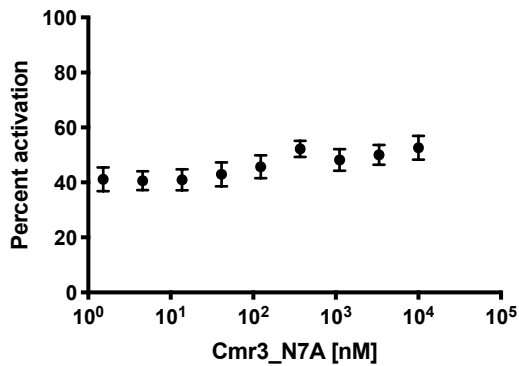
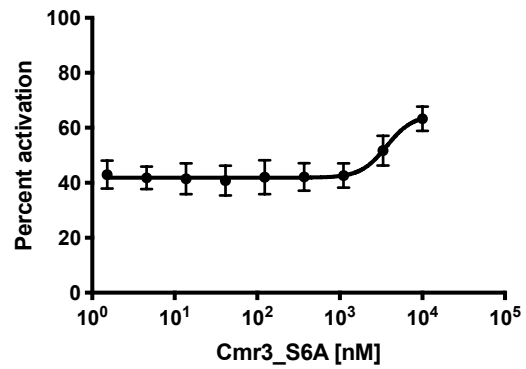
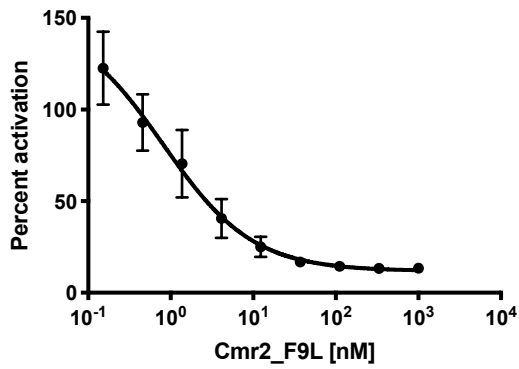
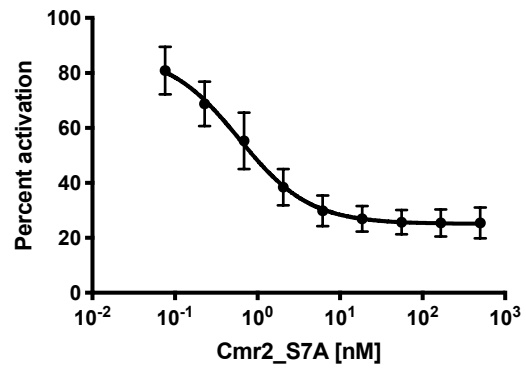
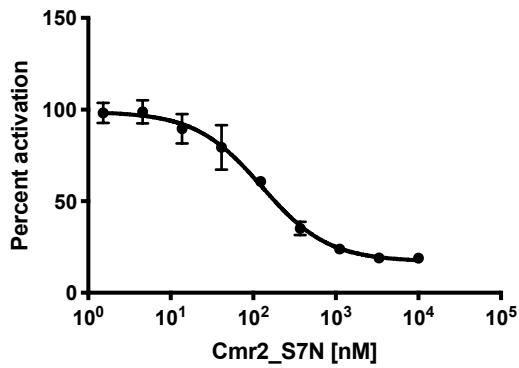
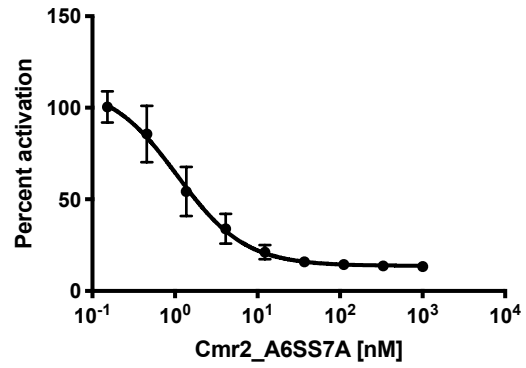
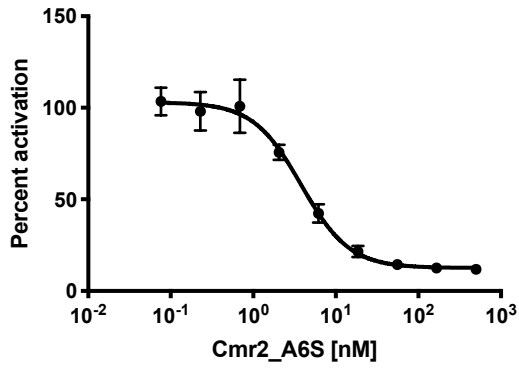


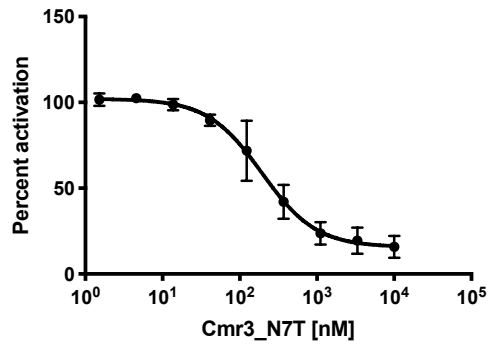
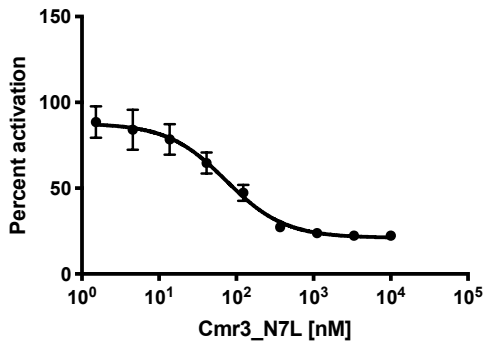
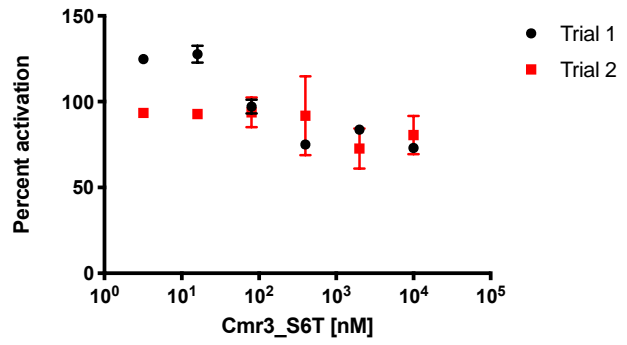
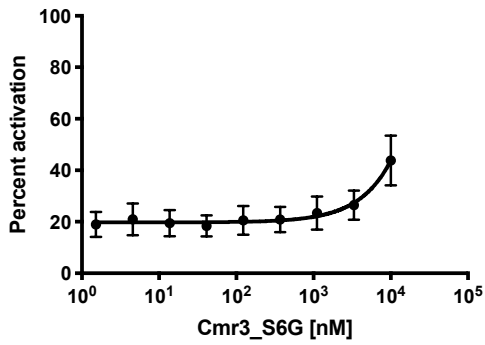
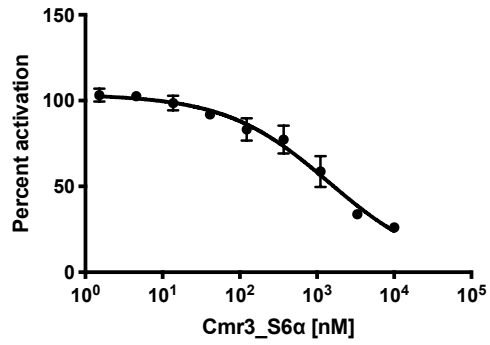
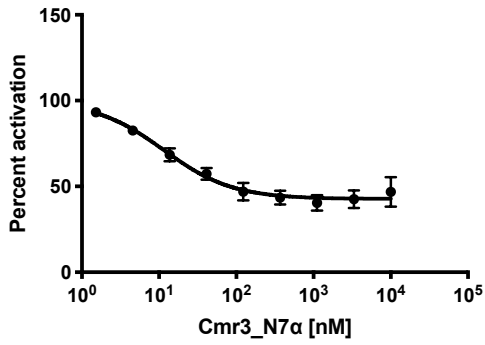
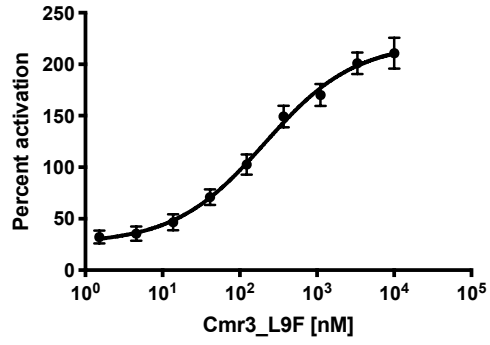
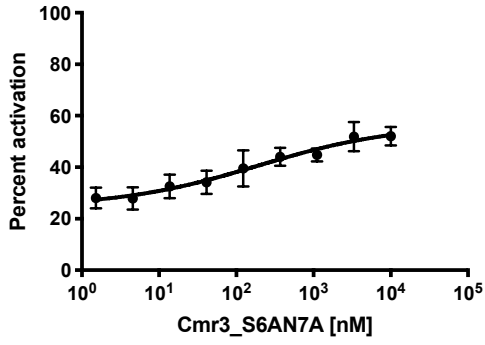


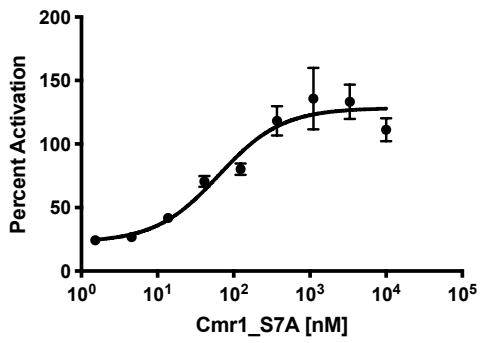
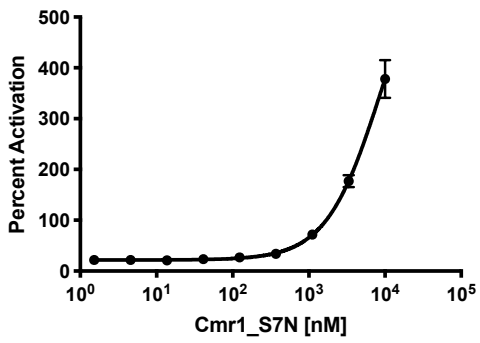
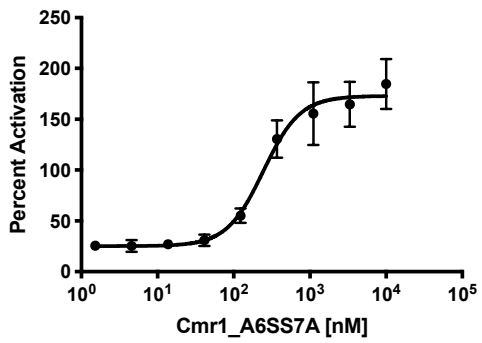
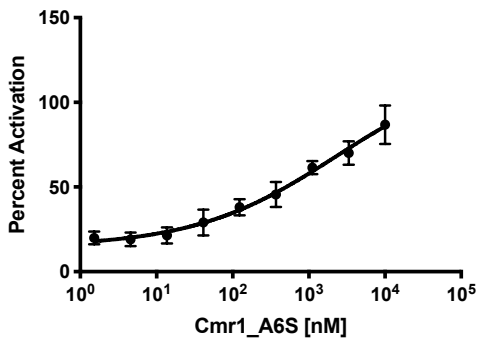
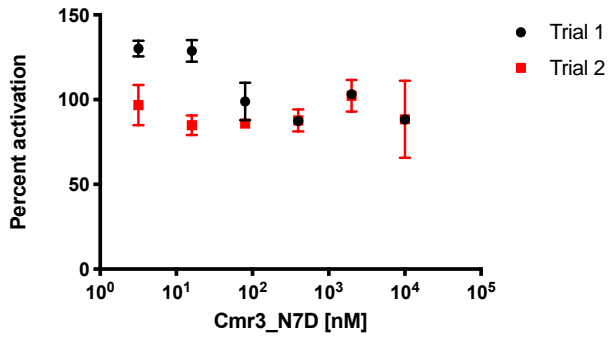
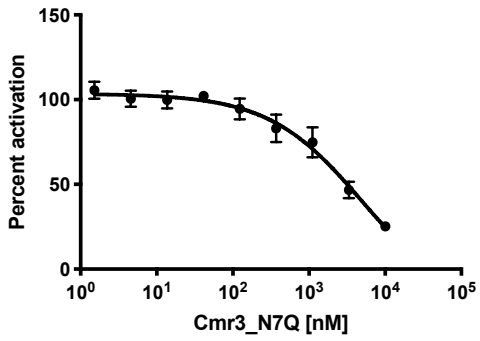
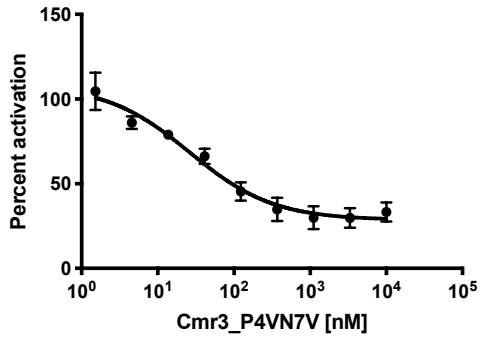
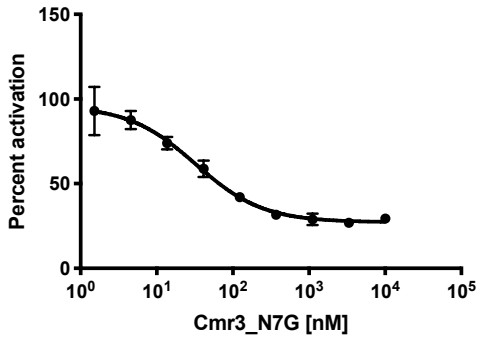


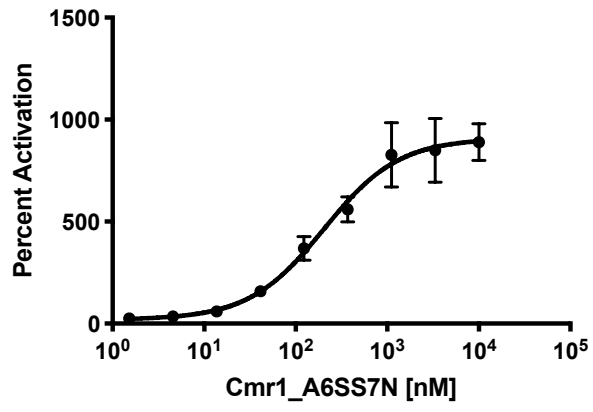
- Dose-response curve in *S. epidermidis* Group II (AH3567)











- Dose-response curve in *S.epidermidis* Group III (AH3409)

