Total Synthesis and Stereochemical Assignment of the Antimicrobial Lipopeptide Cerexin A 1

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Total Synthesis and Stereochemical Assignment of the Antimicrobial Lipopeptide Cerexin A_1

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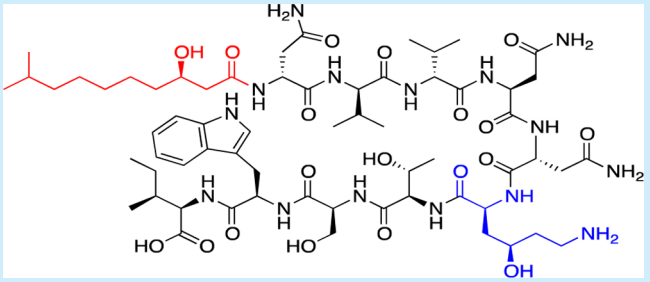
Supporting Information

ABSTRACT: The isolation and total synthesis of the antimicrobial lipopeptide cerexin A_1 is reported. This synthesis includes the preparation of orthogonally protected γ-hydroxylysine, utilizing a nitrile Reformatsky-type reaction as a key step to yield both diastereomers more efficiently than previously reported methods. The configuration of the β-hydroxyl in the lipid tail was determined by the use of a modified Ohrui−Akasaka approach. Furthermore, new cerexin analogues from *Bacillus mycoides* ATCC 21929 were isolated and characterized, revealing an ϵ-amino succinylation of a hydroxylysine residue that is unusual in a nonribosomal peptide synthetase product.



The continued emergence of multi-drug-resistant (MDR) bacteria is a major concern worldwide. A recent report by the Centers for Disease Control and Prevention estimates that MDR infections in the United States are resulting in 23,000 deaths per year, costing the economy up to $20 billion.[1] In the last 50 years, four new structurally and mechanistically distinct classes of antibiotics have been commercialized; linezolid, fidaxomicin, bedaquiline, and the lipopeptide daptomycin.[2,3] Lipopeptides are particularly attractive candidates for antibiotics, as it is difficult for bacteria to develop resistance mechanisms against them. This is because most lipopeptides target the cell membrane, which is difficult for bacteria to reorganize.[4] Our group recently isolated the tridecaptins, a class of linear cationic lipopeptides, from several *Paenibacillus* species.[5−7] Although these compounds were discovered decades earlier, no further investigations had been performed, and their strong activity against MDR Gram-negative bacteria remained unknown until recently.[8−10] We therefore sought to identify other understudied lipopeptides that may have interesting antimicrobial activities. Cerexins are a class of nonribosomally produced decapeptides reported to show moderate activity against Gram-positive organisms.[11,12] Cerexin A_1 ($CxnA_1$) (Figure 1) is the best characterized example, which contains seven D-amino acids and a β-hydroxylated N-terminal lipid tail; however, the original authors did not report the stereochemistry of the lipid tail.[12f] This lipopeptide also contains the novel L-threo-γ-hydroxylysine (γ-Hyl) residue, which is also found in collagen,[13] the glidobactins,[14] and cepafungins.[15]

We obtained the $CxnA_1$ producer strain, *Bacillus mycoides* ATCC 21929, to isolate this lipopeptide. A deferred inhibition assay revealed activity against *Staphylococcus aureus* ATCC 6538, and this organism was therefore used as an indicator strain for the activity-guided purification of cerexins. The supernatant from a 4 L culture was fractionated on Amberlite XAD 16 resin, followed by further purification by C18 solid-phase extraction. Final purification was achieved by HPLC, with four products identified with Gram-positive activity. Analysis by high-resolution MS and tandem mass spectrometry identified the first active HPLC fraction to elute as $CxnA_1$. Although the structure of $CxnA_1$ has been previously reported,[11] the configuration of the β-hydroxy group on its lipid tail remained unknown. We have previously had success in identifying the absolute configuration of tridecaptin A_1 by synthesis of the possible peptide diastereomers and comparison of these to the natural peptide by HPLC and NMR.[6] We therefore embarked on the synthesis of the possible $CxnA_1$ lipid tail isomers by Fmoc solid-phase peptide synthesis (SPPS). This first required the synthesis of orthogonally protected γ-Hyl, which was prepared according to a literature procedure.[16a] However, in our hands, some steps were much lower yielding than reported (see Supporting Information,

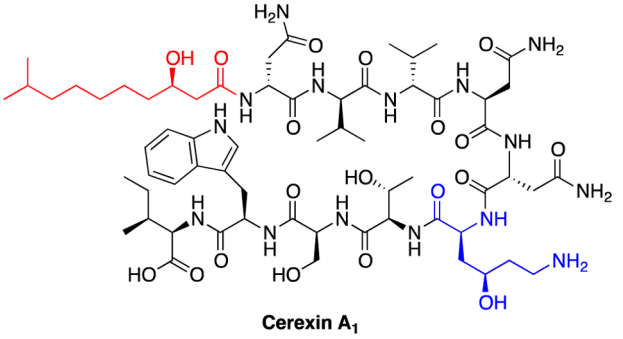


Figure 1. Structure of cerexin A_1.

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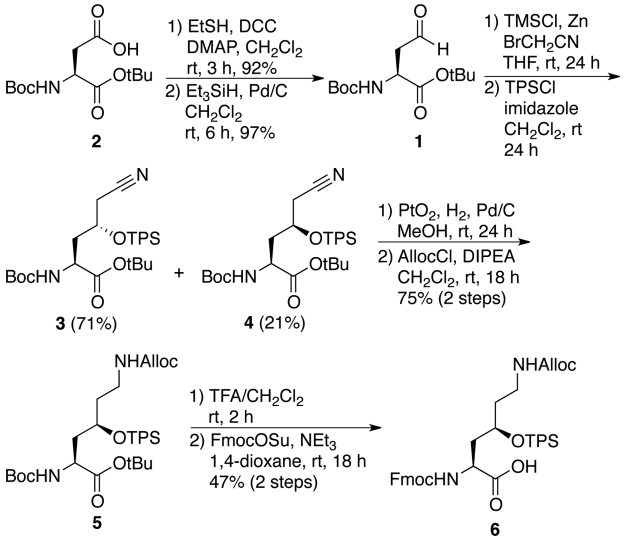
SI),[16a] so we devised an alternative synthesis with a nitrile Reformatsky reaction with aspartic acid semialdehyde (Asa) derivative **1** to give orthogonally protected γ-Hyl (Scheme 1).

Scheme 1. Synthesis of γ-Hyl Derivative 6

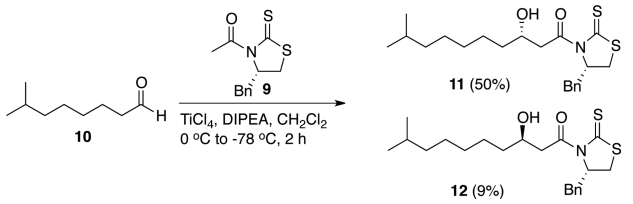


Boc-Asa-O*t*Bu (**1**) is prepared from Boc-Asp-O*t*Bu (**2**) via thioesterification and the subsequent reduction of the resulting thioester (Scheme 1).[16b] Treatment of aldehyde **1** with an in situ generated Reformatsky reagent yields a mixture of *threo*- and *erythro*-γ-hydroxy nitriles. Although these diastereomers are separable by column chromatography, purification is more facile after the alcohols have been protected as silyl ethers. Therefore, the crude diastereomeric mixture was directly treated with *tert*-butyldiphenylsilyl chloride and imidazole, yielding a 7:2 mixture of *erythro*- and *threo*-silyl ethers, **3** and **4**, respectively, in 92% overall yield. A crystal structure of the *erythro*-isomer **3** was obtained (CCDC 1430305), allowing assignment of stereochemistry (Figure S1). Reduction of nitrile **4**, followed by protection of the resulting amine, affords Alloc-carbamate **5** in good yield. Finally, removal of the Boc and *t*Bu groups with TFA, followed by protection as an Fmoc-carbamate, yields orthogonally protected γ-Hyl derivative **6** in 7% overall yield (8 steps). Using the previously reported procedure,[16a] we obtained analogous material in 3% overall yield (9 steps). Although the desired *threo*-isomer is the minor product, the facile synthesis and separation of both diastereomers should prove useful in future syntheses of enatiomerically diverse lipopeptide libraries. Furthermore, *erythro*-γ-Hyl is found in the potent antitumor agent glidobactin A.[17]

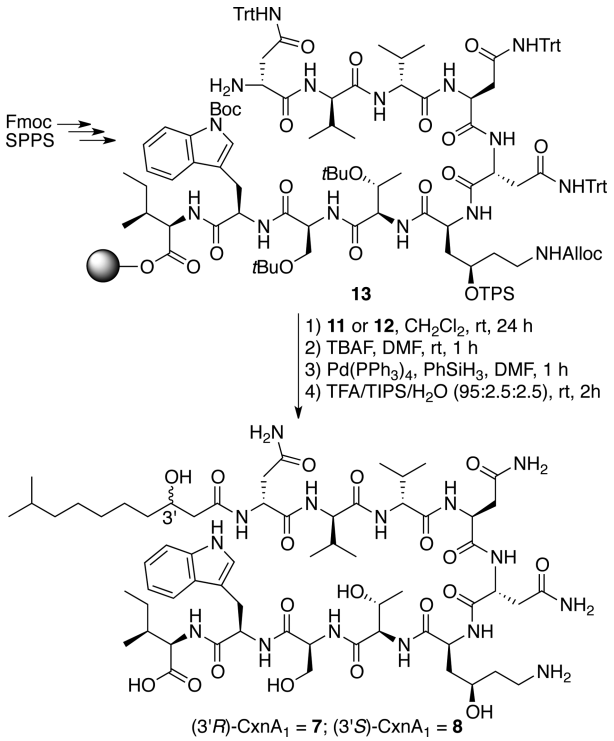
The synthesis of (3′*R*)-CxnA$_1$ (**7**) and (3′*S*)-CxnA$_1$ (**8**) also required the synthesis of the chiral lipid tails. An aldol reaction between Crimmins thiazolidinethione acetate **9** and 7-methyloctanal (**10**) yields both alcohol diastereomers, which are separable by column chromatography (Scheme 2). Analysis of the chemical shifts and coupling constants of the α-protons in **11** and **12** allowed their 3-OH configurations to be assigned.[18] The thiazolidinethione moiety is a good leaving group and can be used to acylate amines on-resin.[6]

The CxnA$_1$ peptide chain **13** was then synthesized using Fmoc-SPPS, and the N-terminus was acylated with thiazolidinethione **11** or **12** (Scheme 3). Treatment of these resin-bound peptides with TBAF in DMF removes the γ-Hyl TPS group. The Alloc protecting group was then removed using Pd(PPh$_3$)$_4$ and

Scheme 2. Synthesis of Activated Lipids 11 and 12



Scheme 3. Synthesis of CxnA$_1$ Lipid Tail Isomers 7 and 8



PhSiH$_3$, followed by global deprotection and cleavage of the peptides from resin using TFA to yield the synthetic standards **7** and **8**.

With lipid tail isomers **7** and **8** available, we attempted to assign the stereochemistry of the natural peptide by comparison with these synthetic standards. HPLC coinjections of **7** + natural CxnA$_1$ and **8** + natural CxnA$_1$ both gave just one peak (Figure S2), and although this supported the previously reported peptide sequence of CxnA$_1$, it did not reveal the lipid tail stereochemistry. Unfortunately, the ^{1}H NMR spectra of these compounds are also identical (Figure S3). We therefore turned to the Ohrui–Akasaka method,[19] which was recently used by our group to deduce the stereochemistry of the lipid tail of tridecaptin B$_1$.[7] It seemed that derivatization of enantiomerically pure β-hydroxy isoundecanoic acids **14** and **15** with the anthracenyl acid **16** could allow differentiation between the *R* and *S* enantiomers by ^{1}H NMR spectroscopy. This would be due to placement of their isoundecanoyl chains in different proximities to the deshielding anthracene ring. Hydrolysis of thiazolidinethiones **11** and **12** gives enantiomerically pure acids **14** and **15** in good yields (Scheme 4). Treatment of these acids with TMS-diazomethane, followed by coupling to acid **16** using EDCI/DMAP, yields anthracenyl derivatives **17** and **18** in moderate yields. Gratifyingly, analysis by ^{1}H NMR revealed obvious differences between the ^{1}H NMR spectra of **17** and **18**. The most pronounced is the change in chemical shift and coupling pattern of the isopropyl

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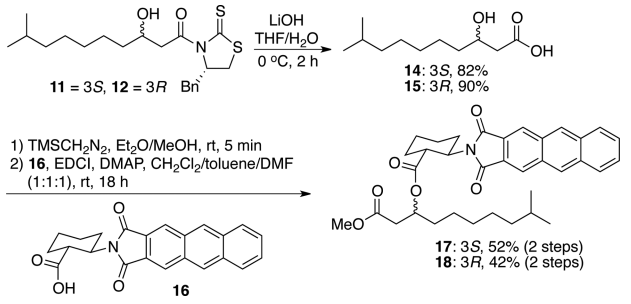
Scheme 4. Synthesis of Esters 17 and 18



signal (Figure 2). The lipid tail was then hydrolyzed from $CxnA_1$ by heating at 90 °C in 6 M HCl for 2 h, followed by derivatization

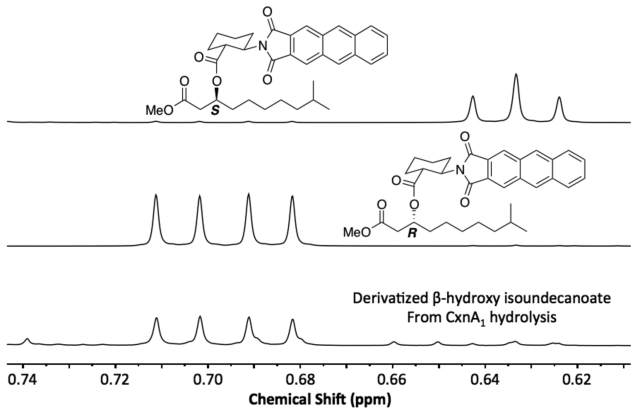


Figure 2. ^1H NMR analysis of the derivatized $CxnA_1$ lipid tail.

using the methodology reported in Scheme 4. ^1H NMR analysis clearly shows that the β-hydroxy group on $CxnA_1$ has the *R* configuration.

We next focused our attention on the identification of the other active compounds isolated from *Bacillus mycoides* ATCC 21929 (Figure 3). High-resolution MS revealed that the

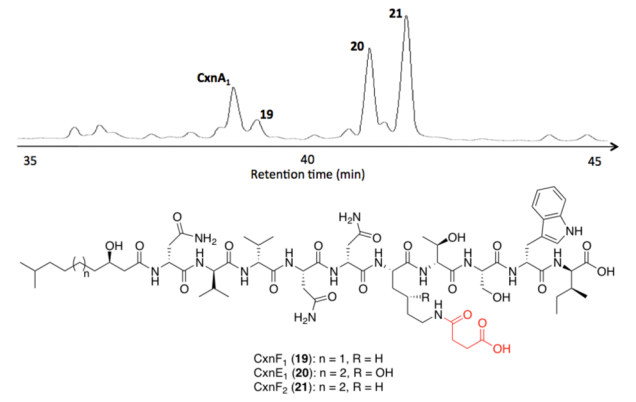


Figure 3. HPLC trace and structures of other active compounds isolate from *B. mycoides* ATCC 21929.

molecular formulas of compounds **19**, **20**, and **21** differed from $CxnA_1$ by $+C_3H_2O_2$, $+C_4H_4O_3$, and $+C_4H_4O_2$, respectively. MS/MS analyses showed that **20** and **21** had similar sequences to $CxnA_1$, with the extra mass units present on residue 6 (see SI). Compound **19** has a lipid tail one methylene shorter than that found in $CxnA_1$; therefore, this analogue, like **21**, has $+C_4H_4O_2$ at residue 6. The molecular formulas of **19**, **20**, and **21** indicated that these compounds have two additional degrees of unsaturation relative to $CxnA_1$. In previously reported cerexin analogues, residue 6 is L-threo-γ-hydroxylysine in $CxnA_1$ and lysine in CxnC.[11] This suggested that the modification might be an acylation of the ε-amino group on γ-Hyl/Lys, with $+C_4H_4O_3$ corresponding to a succinyl or methyl malonyl group. Recently, succinylation has been identified as a post-translational modification found in many ribosomally synthesized proteins.[20] This modification was found in unnatural thiocillin variants produced by *B. cereus*,[21] as well as in subtilin, another ribosomal peptide produced by *Bacillus* species.[22] Therefore, we considered that compound **20** may be succinylated $CxnA_1$. Complete proton assignment of compound **20** by TOCSY and NOESY experiments (Figures S4 and S5 and Table S1) revealed that the ε-amino of γ-Hyl is indeed succinylated. Furthermore, treatment of $CxnA_1$ with 100 equiv of succinic anhydride yielded a new succinylated product with a retention time identical to that of **20** (Figure S6), thereby confirming that **20** is a succinylated analogue of $CxnA_1$, which we have designated as $CxnE_1$. This led us to conclude that compounds **19** and **21**, which contain one less oxygen at residue 6, are composed of succinylated lysine (Figure 3).

The antimicrobial activities of cerexin analogues **7**, **8**, **19**, **20**, and **21** were then determined against a panel of Gram-positive and Gram-negative bacteria. No activity was observed against the Gram-negative organisms tested (*Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*) at concentrations of 500 μg/mL. This poor activity also translated to most Gram-positive organisms, with the exception of *B. subtilis*. (3′*R*)-$CxnA_1$ and (3′*S*)-$CxnA_1$ have comparable activities (MIC = 62.5−31.3 μg/mL), whereas the succinylated analogues **19**, **20**, and **21** are over 30-fold less active (MIC > 500 μg/mL) against *B. subtilis*. This highlights the importance of the positive charge on the Hyl residue for biological activity. Others have suggested that lysine acylation of peptides may be a self-protection strategy for the producer organism.[21] However, as high concentrations of $CxnA_1$ (1 mg/mL) are not toxic to *B. mycoides* ATCC 21929, the extensive succinylation of the cerexins by this organism may serve another purpose.

In conclusion, we completed the first total synthesis of the antimicrobial lipopeptide cerexin A_1. The previously unknown lipid tail β-hydroxyl chirality was assigned through the Ohrui−Akasaka method, with the isopropyl groups acting as diagnostic markers in ^1H NMR. We also reported a new synthesis of orthogonally protected *threo-* and *erythro-*γ-hydroxylysine derivatives utilizing a nitrile Reformatsky-type reaction as the key step. Finally, we have identified new natural cerexin analogues in which the ε-amino group of lysine and hydroxylysine are succinylated. This modification is the first reported example of natural nonribosomal peptide synthetase products and was found to drastically decrease the antimicrobial activity of these compounds, highlighting the importance of the lysine amino group in the cerexin mechanism of action.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02779.

Detailed descriptions of synthetic and microbiology techniques and characterization of all compounds (PDF)

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Notes

The authors declare no competing financial interest.

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