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Synthesis and Bioactivity of Diastereomers of the Virulence Lanthipeptide Cytolysin

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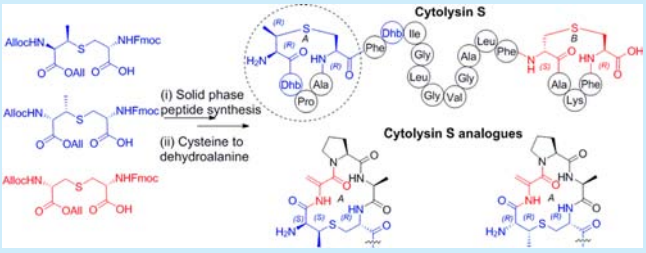
Supporting Information

ABSTRACT: Cytolysin, a two-component lanthipeptide comprising cytolysin S ($CylL_S''$) and cytolysin L ($CylL_L''$), is the only family member to exhibit lytic activity against mammalian cells in addition to synergistic antimicrobial activity. A subset of the thioether cross-links of $CylL_S''$ and $CylL_L''$ have LL stereochemistry instead of the canonical DL stereochemistry in all previously characterized lanthipeptides. The synthesis of a $CylL_S''$ variant with DL stereochemistry is reported. Its antimicrobial activity was found to be decreased, but not its lytic activity against red blood cells. Hence, the unusual LL stereochemistry is not responsible for the lytic activity.



Lanthipeptides belong to the class of ribosomally synthesized and post-translationally modified peptides (RiPPs) and bear lanthionine (Lan) and methyllanthionine (MeLan) structures as well as dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues (Figure 1).[1] Two-component lanthipeptides

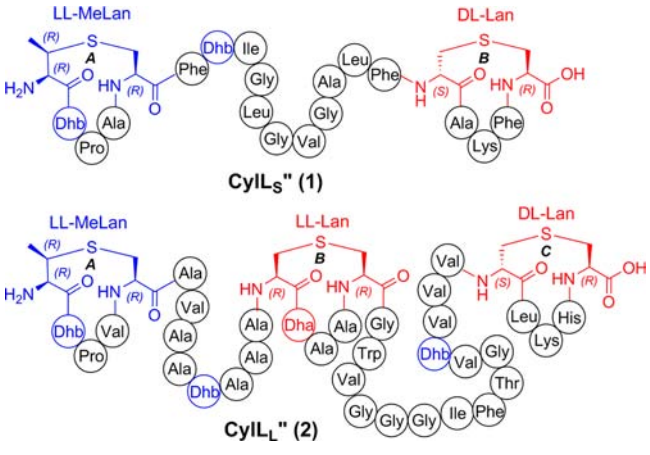


Figure 1. Structures of cytolysin S and L. Lan and Dha (both derived from Ser) are shown in red, and MeLan and Dhb (both derived from Thr) are shown in blue.

are an interesting subclass of lanthipeptides in which two peptides synergistically act to provide antibacterial activity.[2] Cytolysin is a two-component lanthipeptide and comprises cytolysin S ($CylL_S''$) and cytolysin L ($CylL_L''$).[3] In addition to exhibiting synergistic antimicrobial activity, cytolysin is the first and thus far only lanthipeptide shown to potently lyse mammalian cells.[4] Cytolysin is responsible for the enhancement of enterococcal virulence and is produced by many clinical isolates of *Enterococcus faecalis*.[5] Compared with most lanthipeptides, cytolysin exhibits unusual stereochemistry. This class of molecules generally contain Lan and MeLan with DL stereochemistry, i.e., (2*S*,6*R*)-lanthionine and (2*S*,3*S*,6*R*)-methyllanthionine.[6] In the case of cytolysin, the B ring of $CylL_S''$ and the C ring of $CylL_L''$ contain Lan with the canonical stereochemistry, but LL stereochemistry (i.e., (2*R*,6*R*)-Lan and (2*R*,3*R*,6*R*)-MeLan) was observed for the A ring of $CylL_S''$ and the A and B rings of $CylL_L''$ (Figure 1).[7] These observations suggested a possible correlation between the unique stereochemistry and the unusual lytic activity against mammalian cells. To test this hypothesis, we report the total synthesis of a diastereomer of cytolysin to investigate the effect of the stereochemistry of the thioether cross-links on the biological activity.

Syntheses of just five lanthipeptides or their variants have been reported to date: nisin, lactosin S, both components of lacticin 3147, epilancin 15x, and lacticin 481.[8] Four of these have been completed on solid phase utilizing an orthogonal protection scheme that allows on-demand cyclization (Figure 2A). Cytolysin synthesis poses several challenges not present in these previously synthesized compounds. First, the sequences of $CylL_S''$ and $CylL_L''$ are extremely hydrophobic, with only a single charged residue in each peptide (Figure 1). Hydrophobic peptides are prone to incomplete coupling during solid-phase peptide synthesis (SPPS) because of inaccessibility of the reagents to the N-terminus of the elongating peptide chain.[9] Second, the structures of cytolysins contain a dehydro amino acid in the second position within the thioether rings. Dehydro amino acids cannot be incorporated via the usual elongation

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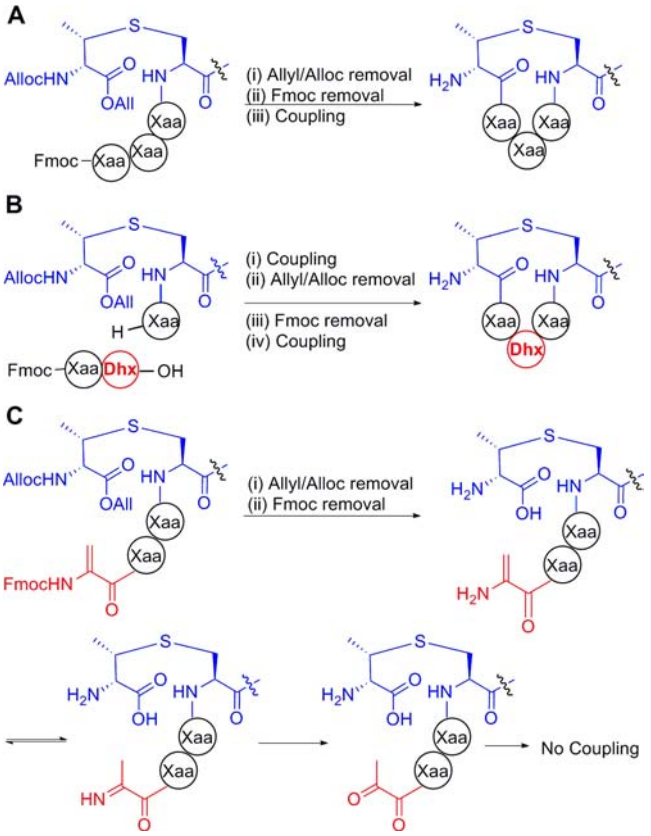

Figure 2. (A) Orthogonal protecting groups on a (methyl)lanthionine building block (DL-MeLan here) allow elongation and subsequent cyclization of a peptide. For alternative protecting group schemes, see ref 10. (B) Introduction of short oligopeptides containing dehydro amino acids (Dhx). (C) If the amine coupling partner for cyclization is a dehydro amino acid (Dha here), the low reactivity of the enamine promotes hydrolysis to the ketone, preventing cyclization.

methods of Fmoc SPPS because the enamine liberated upon Fmoc deprotection is very unreactive and would tautomerize to the imine followed by hydrolysis to the ketone, preventing further peptide coupling. Therefore, the traditional SPPS routes to lanthipeptides rely on the preparation of short oligopeptides containing preinstalled dehydro amino acids (e.g., Figure 2B).[8b−e] Unfortunately, this strategy does not work with a dehydro amino acid incorporated at the second position of a Lan/MeLan-containing ring because the dehydro amino acid is the point of cyclization (Figure 2C). The synthesis of such a structure has not been accomplished to date. Here we describe the synthesis of cytolysin S as well as a diastereomer by introduction of the dehydro amino acid after cyclization.

To minimize the problem of the high hydrophobicity of the cytolysins, we chose $CylL_S''$ rather than $CylL_L''$ as our synthetic target. In addition, the dehydrobutyrine in ring A of $CylL_S''$ was substituted with a dehydroalanine ($CylL_S''$-Dhb2Dha), which we envisioned could be accessed from a Cys. We first verified that this change would not alter the bioactivity of the peptide by preparing $CylL_S''$-Dhb2Dha biosynthetically via coexpression of the precursor peptide $CylL_S$-T2S with the lanthipeptide synthetase CylM in *Escherichia coli* using previously described methodology.[7] Characterization of the product by tandem mass spectrometry and GC/MS analysis of derivatized amino acids after acid hydrolysis of $CylL_S''$-Dhb2Dha demonstrated an LL-MeLan A ring and a DL-Lan B ring, identical to native $CylL_S''$ (Figure S1).[7] Purified $CylL_S''$-Dhb2Dha was found to have very similar antimicrobial activity as native $CylL_S''$ (Figure S2), thus making it a good target for synthesis. Hence, we set out to make both native $CylL_S''$-Dhb2Dha and its diastereomer with a DL-MeLan A ring instead of an LL-MeLan. The desired stereochemistry of the thioether cross-links was preset in building blocks **3**, **4**, and **5** (Figure 3).[8b,e,11]

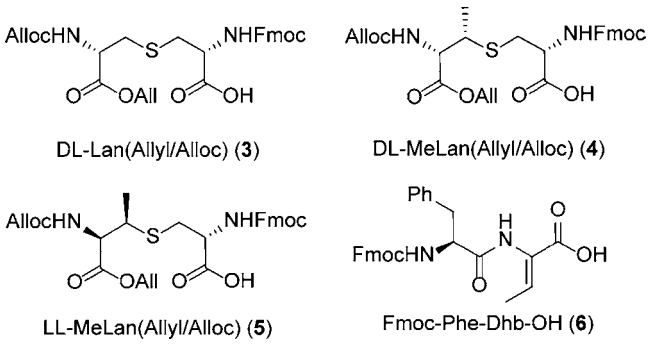

Figure 3. Building blocks used in SPPS of cytolysin analogues.

To aid in the synthesis of the hydrophobic peptide, poly(ethylene glycol) (PEG)-based ChemMatrix resin was employed instead of traditionally used polystyrene (PS) resin. ChemMatrix resin offers improved chemical stability, and because of its polar nature, this resin does not interact as much with the side-chain-protected peptides,[12] which we felt was important given the hydrophobic nature of $CylL_S''$. Additionally, ChemMatrix resin has enhanced swelling properties in a wide range of solvents, including solvents that minimize peptide self-association on the resin.[12a] A trityl-group-containing linker to the resin was employed to prevent racemization of the C-terminal Lan residue. The bulky linker was also envisioned to improve the stability of the C-terminal Lan building block, as C-terminal-protected Cys residues often suffer from base-catalyzed elimination and subsequent β-piperidylalanine formation.[13] The entire peptide was successfully synthesized on-resin (Scheme 1). In place of the dehydroalanine residue in the second position within the MeLan A ring of $CylL_S''$-Dhb2Dha, a cysteine was incorporated as a convenient precursor to Dha.[14] After cleavage of the peptide from the resin and purification, the peptide was reacted with 2,5-dibromohexanediamide, resulting in the formation of a cyclic sulfonium intermediate at Cys2. As reported by Davis and co-workers,[14a] under basic conditions, elimination generated the desired dehydroalanine (Scheme 1, inset). The purity of the final compound **11** was confirmed by analytical high-performance liquid chromatography and mass spectrometry (MS) (Figure S3). The desired stereochemistry of the thioether cross-links in compound **11** was confirmed by gas chromatography coupled to MS analysis employing a chiral stationary phase (Figure S4). For direct comparison in bioactivity assays, $CylL_S''$-Dhb2Dha with the natural LL-MeLan A ring and DL-Lan B ring (compound **12**) was also synthesized (Scheme 1). This synthetic compound was expected to be identical to the biosynthetically accessed $CylL_S''$-Dhb2Dha and thus was envisioned as a good control compound to assess the success of the synthetic procedure. For the synthesis of compound **12**, similar synthetic steps were employed using synthetic building blocks **3**, **5**, and **6**.

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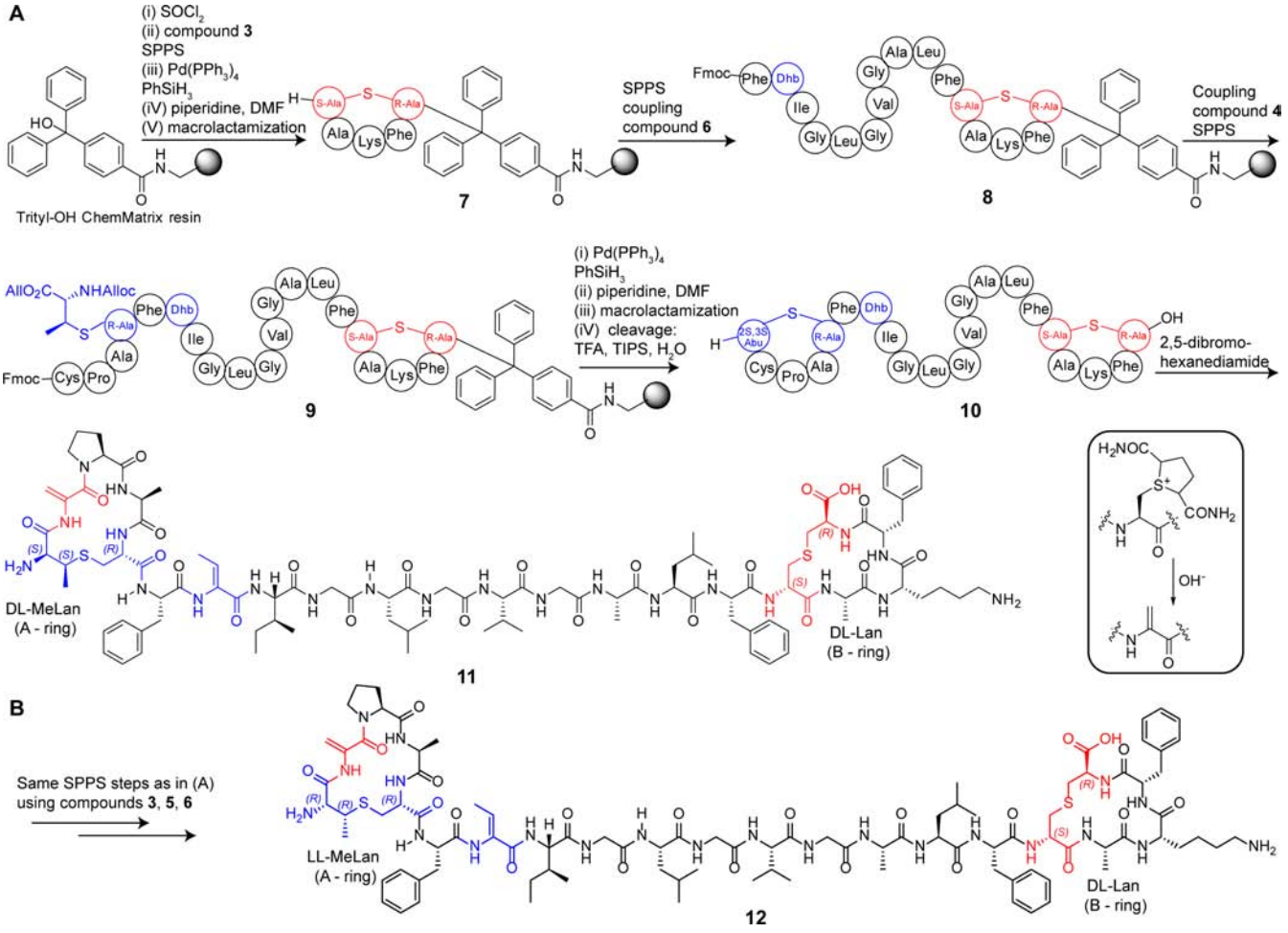
Scheme 1. Synthesis of (A) CylL$_S$″-Dhb2Dha (DL A ring, DL B ring) and (B) CylL$_S$″-Dhb2Dha (LL A ring, DL B ring)



Antimicrobial activity was tested in combination with wild-type (WT) CylL$_L$″ against *Lactococcus lactis* HP and *L. lactis* CNRZ 481. None of the peptides displayed antimicrobial activity without its partner, and all of the CylL$_S$″ peptides were active in combination with CylL$_L$″ (Table 1). Isobolograms

Table 1. Minimal Inhibitory Concentrations of Cytolysin and Derivatives against *L. lactis* 481

entry	individual MIC (μM)	combined with CylL$_L$″ MIC (μM)
CylL$_L$″	>50	n.a.[a]
CylL$_S$″	>50	0.05
11	>50	1.0
synthetic **12**	>50	0.1
biosynthetic **12**	>50	0.1

[a]Not applicable.

demonstrated that WT CylL$_L$″ and CylL$_S$″ act in 1:1 stoichiometry with a minimal inhibitory concentration (MIC) of 0.05 μM for each component (Figure 4 and Table 1). Expressed CylL$_S$″-Dhb2Dha also acted with 1:1 stoichiometry but with a 2-fold decrease in MIC (0.1 μM). Synthetic **12** exhibited antimicrobial activity identical to that of biosynthetic CylL$_S$″-Dhb2Dha, confirming the fidelity of the synthesis (Table 1 and Figure S5). Conversely, diastereomer **11** exhibited decreased antimicrobial activity (Figure S5) when combined with CylL$_L$″, as illustrated by a markedly smaller zone of growth

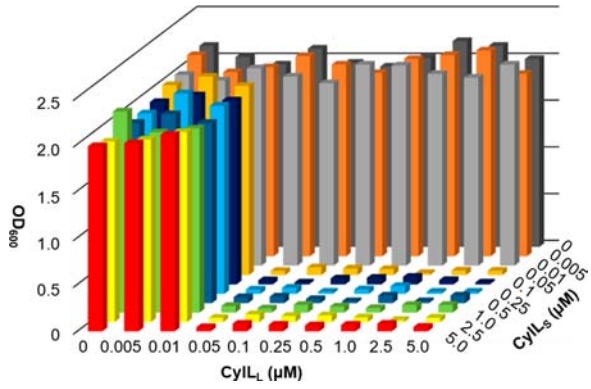


Figure 4. Antimicrobial activity assays of WT cytolysin against *Lactococcus lactis* 481. The isobologram demonstrates that the MIC of the combination of CylL$_L$″ and CylL$_S$″ is reached with each component at 0.05 μM, suggesting a 1:1 stoichiometry in the active species.

inhibition and a MIC in liquid culture that was increased 10-fold (Table 1).

The cytolysin S peptides were also tested in combination with CylL$_L$″ for synergistic hemolytic activity against rabbit red blood cells. WT CylL$_S$″ and expressed CylL$_S$″-Dhb2Dha exhibited very similar hemolytic activities. Surprisingly, **11** with a DL-MeLan A ring and DL-Lan B ring exhibited no decrease in hemolytic activity (Figure S6). This result shows

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that the influence of stereochemistry is different on the two activities of cytolysin. Similar findings were reported for mutants of cytolysin that affected antimicrobial and lytic activities differently.[15]

In summary, it appears that $CylL_S''$ with the LL stereochemistry of the A ring has evolved for optimal complementarity with native $CylL_L''$ with respect to antimicrobial activity. In $CylL_S''$ with DL stereochemistry of the A ring, the synergy with native $CylL_L''$ is clearly attenuated. Regarding the hemolytic activity, the stereochemistry of the A ring of $CylL_S''$ does not appear to be important. These findings further reinforce previous conclusions that these two activities have different structure−activity relationships.[15] They are also consistent with the proposal that cytolysin evolved predominantly for its antimicrobial activity since *E. faecalis* is mostly a commensal organism.[16]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03246.

Materials and experimental procedures, synthesis and characterization of small molecules and peptides, and biosynthesis of cytolysin analogues and bioactivity assays (PDF)

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Notes

The authors declare no competing financial interest.

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