

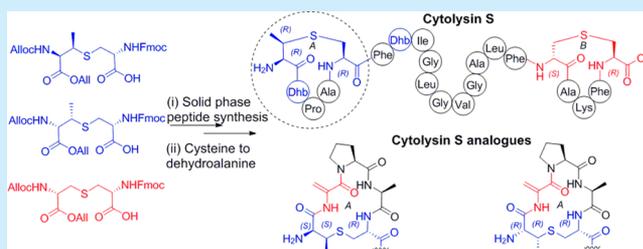
Synthesis and Bioactivity of Diastereomers of the Virulence Lanthipeptide Cytolysin

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S 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

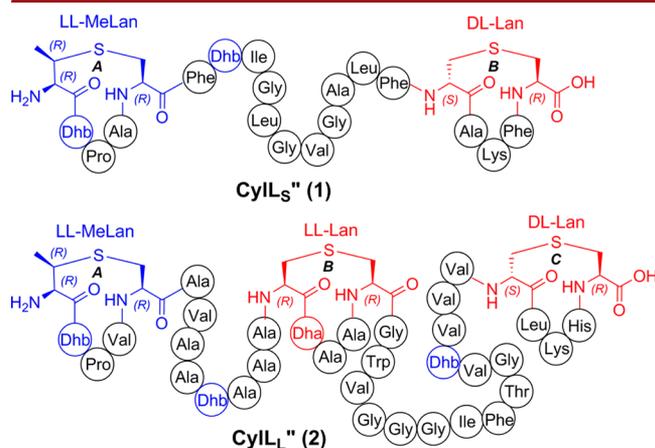


Figure 1. Structures of cytolysin S and L. Lan and Dha (both derived from Ser) are shown in red, and MeLan and Dhb (both derived from Thr) are shown in blue.

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., (2S,6R)-lanthionine and (2S,3S,6R)-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., (2R,6R)-Lan and (2R,3R,6R)-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 lactacin 3147, epilancin 15x, and lactacin 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

Received: October 29, 2016

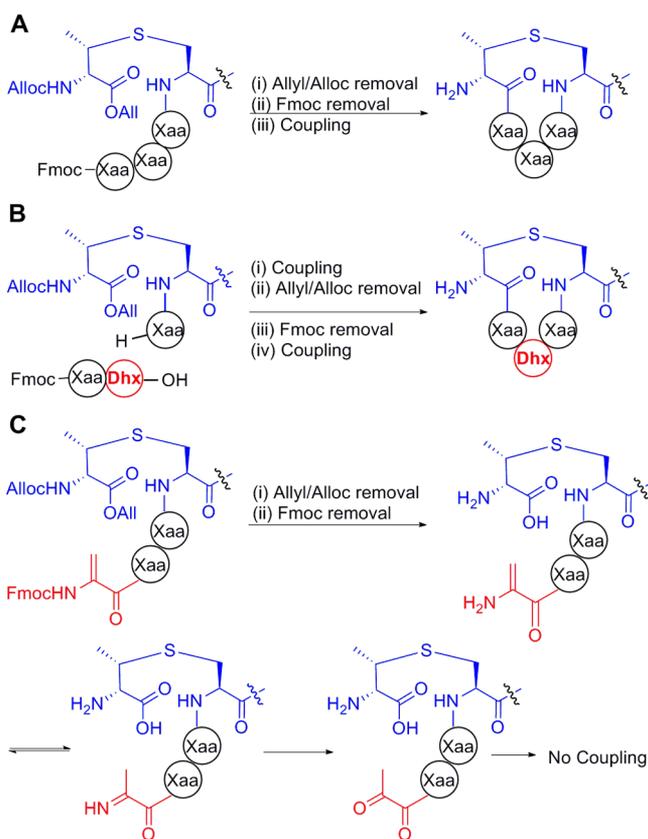


Figure 2. (A) Orthogonal protecting groups on a (methyl)lanthionine building block (DL-MeLan here) allow elongation and subsequent cyclization of a peptide. For alternative protecting group schemes, see ref 10. (B) Introduction of short oligopeptides containing dehydro amino acids (Dhx). (C) If the amine 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 present in building blocks 3, 4, and 5 (Figure 3).^{8b,e,11}

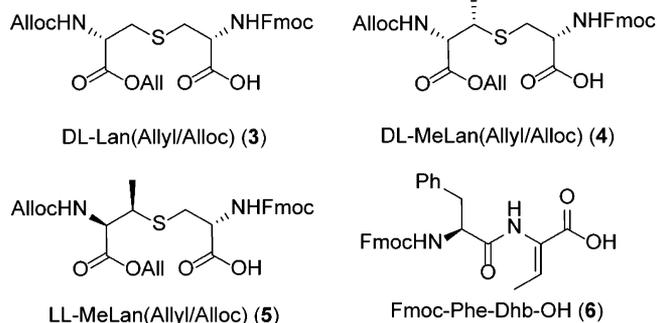
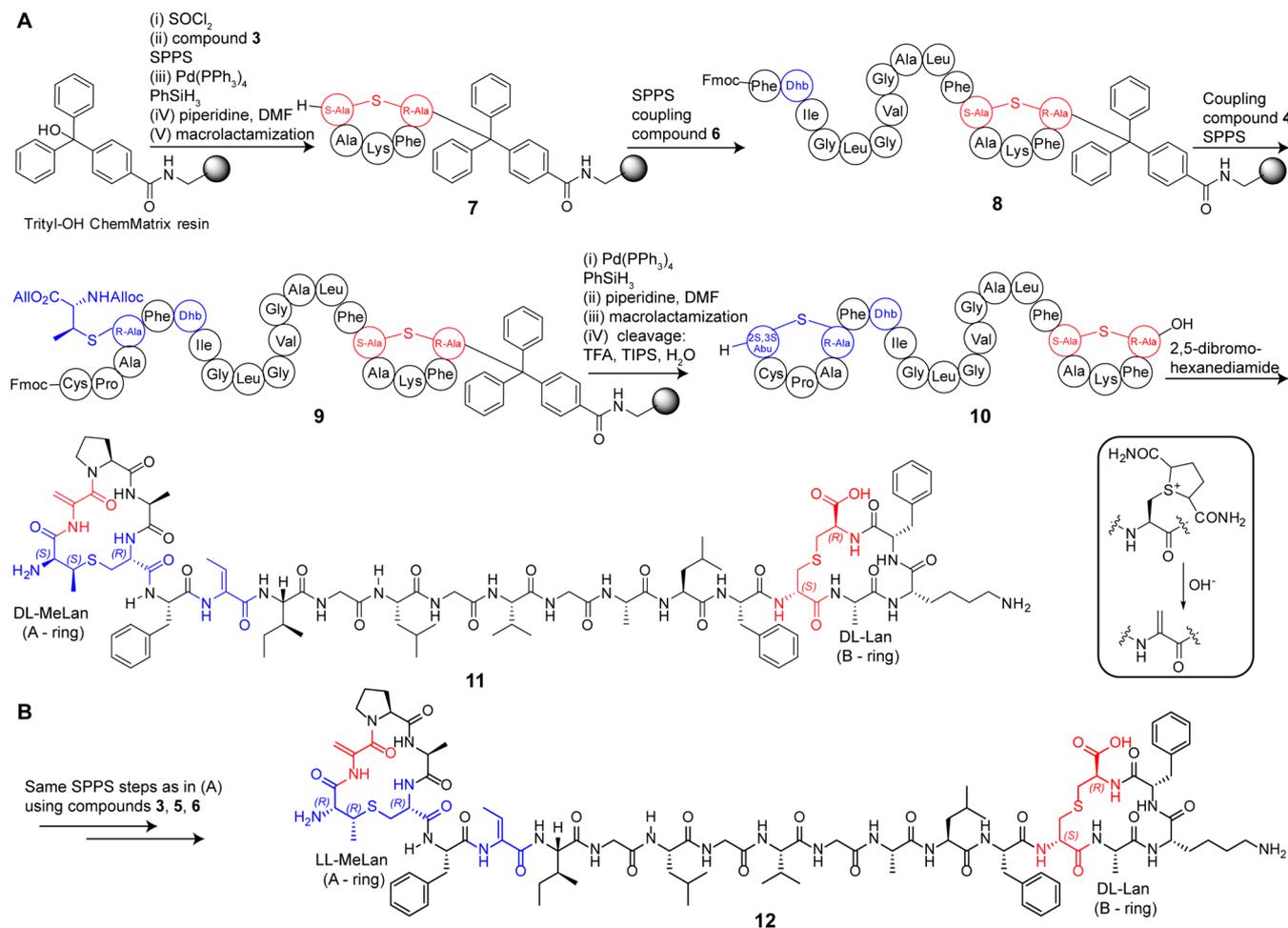


Figure 3. Building blocks used in SPPS of cytolysin analogues.

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.

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 |

^aNot 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

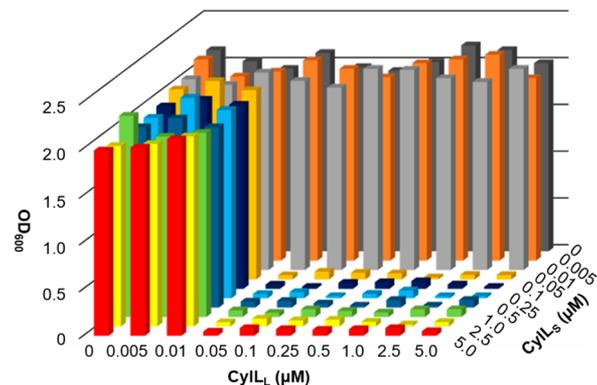


Figure 4. Antimicrobial activity assays of WT cytolysin against *Lactococcus lactis* 481. The isobologram demonstrates that the MIC of the combination of CylL_L^{''} and CylL_S^{''} is reached with each component at 0.05 μM, suggesting a 1:1 stoichiometry in the active species.

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

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.

■ ACKNOWLEDGMENTS

We thank Dr. Patrick Knerr (UIUC) for providing Boc-Phe-Thr-OtBu and DL-Lan building block for initial studies. We thank Dr. Weixin Tang (UIUC) for providing Lan/MeLan synthetic standards for GC/MS analysis. We thank Dr. Alexander V. Ulanov of the Metabolomics Center at UIUC for assistance with GC/MS analysis. This study was supported by the Howard Hughes Medical Institute. A Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer was purchased in part with a grant from the National Institutes of Health (S10 RR027109).

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