

stereochemical analogues were found to be inactive, highlighting the importance of the natural, enzymatically installed Lan/MeLan stereochemical configuration for biological activity.

Lactacin 481 is a tricyclic lantibiotic produced by *Lactococcus lactis* subsp. *lactis*. This natural product exerts its antibacterial activity via inhibition of transglycosylation involved in the biosynthesis of peptidoglycan, likely via binding to the peptidoglycan precursor lipid II.²⁸ The in vitro reconstitution of its biosynthesis in 2004²⁹ has led to the development of a chemoenzymatic platform to produce analogues containing nonproteinogenic amino acids,^{30,31} several of which display improved antimicrobial activity compared with the parent compound. However, as this approach relies on the biosynthetic machinery to install the desired post-translational modifications, alteration of the Lan/MeLan stereochemistry cannot be achieved. Indeed, attempts to produce MeLan stereoisomers biosynthetically from peptides containing *allo*-threonine were unsuccessful, as *allo*-threonine was not accepted as a substrate.³² Therefore, we drew upon previous total syntheses of lantibiotics via 9-fluorenylmethoxycarbonyl-based solid-phase peptide synthesis (Fmoc-SPPS)^{14,22} to construct **1** and the desired analogues bearing cross-link stereoisomers. Our approach involved the solid-supported construction of the peptide backbone incorporating orthogonally protected Lan/MeLan building blocks, each of which could be selectively deprotected and cyclized with the N-terminus of the growing peptide to yield the desired cross-links. For **1**, three such building blocks are necessary: an orthogonal pair of Lan building blocks (**2** and **3**) for the overlapping B and C rings, and one MeLan building block (**4**) for the A ring (Figure 2).

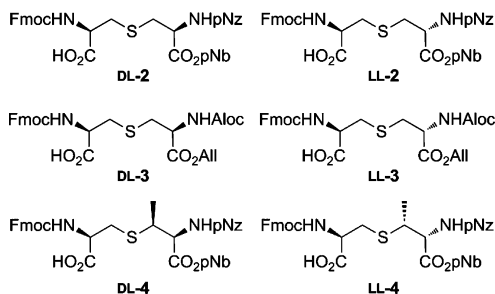
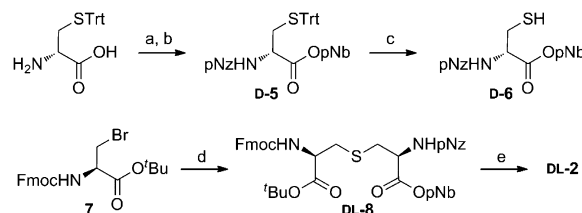


Figure 2. Structures of Lan/MeLan building blocks 2–4.

The syntheses of DL-3³³ and DL-4²² have been reported previously. The construction of the *p*-nitrobenzyl (pNb)-protected Lan DL-2 proceeded with full preservation of stereochemical integrity via a phase-transfer condensation of protected D-cysteine D-6 and bromoalanine **7** (Scheme 1). Importantly, as the cross-link stereochemistry was preinstalled into each building block, a simple exchange of the D-amino acid starting material with the L isomer afforded the LL diastereomers of all three building blocks (LL-2, LL-3, and LL-4) in similar overall yields [see the Supporting Information (SI)]. In the case of LL-4, the use of L-threonine as starting material generated a change in two stereocenters, yielding an overall configuration of (2*R*,3*R*,6*R*). Because of the anti addition observed during the biosynthesis of all naturally occurring MeLan cross-links to date, we chose to explore this stereoisomer rather than those that would result from net syn addition [i.e., (2*R*,3*S*,6*R*) and (2*S*,3*R*,6*R*)], which have not been found in natural lanthipeptides.^{13,16,32}

Scheme 1. Synthesis of DL-2^a

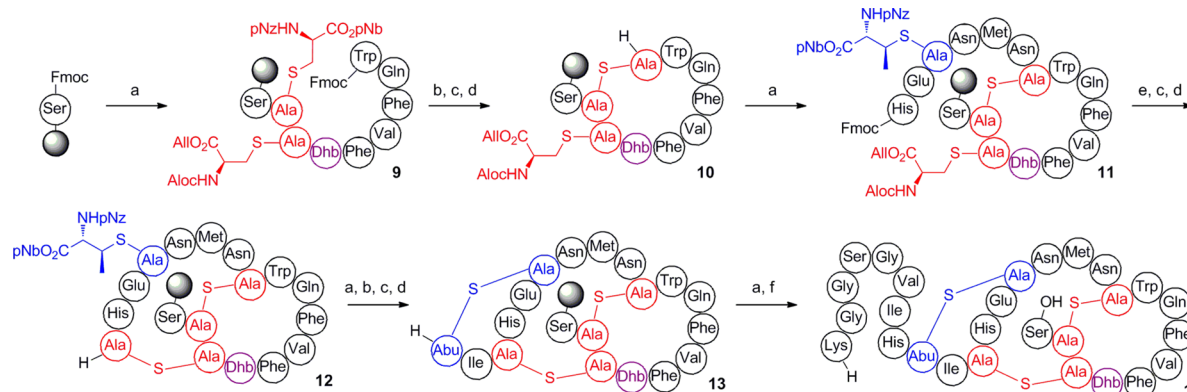


^aReagents and conditions: (a) pNzCl, Na₂CO₃, H₂O, 1,4-dioxane; (b) pNbBr, NaHCO₃, DMF, 94% (two steps); (c) CF₃CO₂H, ^tPr₃SiH, CH₂Cl₂, 88%; (d) D-6, NaHCO₃, Bu₄NBr, EtOAc, H₂O, Bu₃P, 78%; (e) CF₃CO₂H, PhSiH₃, CH₂Cl₂, 95%. Abbreviations: pNz, *p*-nitrobenzyloxycarbonyl; pNb, *p*-nitrobenzyl; Trt, trityl.

Following the successful syntheses of these three diastereomeric pairs of building blocks, we approached the construction of **1** containing only the natural DL configuration of the cross-links via Fmoc-SPPS (Scheme 2). Preloaded Wang resin with a low-density substitution of 0.1 mmol/g was utilized, effectively preventing intermolecular side reactions during the solid-supported cyclization reactions. Fmoc deprotection was performed using piperidine, and amino acids were activated for coupling using *N,N'*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt). To install the Dhb residue at position 24, the dipeptide Fmoc-Phe-(Z)-Dhb-OH²² was synthesized in solution and coupled under these standard conditions. After completion of intermediate **9**, the nitrobenzyl-based protecting groups of the Lan building block were removed by treatment with 6 M SnCl₂ and 5 mM HCl in *N,N'*-dimethylformamide (DMF), leaving the allyl-based groups unaffected. After removal of the Fmoc group from the N-terminus, cyclization was promoted using two 3 h treatments with (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP), HOAt, and 2,4,6-collidine to give **10** bearing the C ring of **1**. Further SPPS gave **11**, and subsequent removal of the allyl-based protecting groups with Pd(PPh₃)₄ and phenylsilane, Fmoc removal, and cyclization as described above yielded bicyclic intermediate **12**. Isoleucine coupling and installation of the A ring via the same chemical sequence used for **10** gave tricyclic intermediate **13**. After further SPPS to complete the amino acid sequence, cleavage from the resin and global deprotection were achieved using trifluoroacetic acid, water, and triisopropylsilane to give synthetic **1**.

To probe the importance of the cross-link stereochemistry for the biological activity, each ring of **1** was systematically changed from the natural DL configuration to the diastereomeric LL configuration. This goal was accomplished simply by replacing the DL-Lan/MeLan building block used in the synthesis of **1** with its LL counterpart. In this way, three additional peptides containing Lan/MeLan stereoisomers for the A ring (LL-A), the B ring (LL-B), and the C ring (LL-C) of lactacin 481 were constructed.

Reversed-phase high-performance liquid chromatography (RP-HPLC) purification gave milligram quantities of the desired peptides in average overall yields of 1.3%, corresponding to an average yield per step of 92%. Analysis via analytical RP-HPLC revealed that **1** coeluted with authentic lactacin 481 (Figure S1 in the SI). Interestingly, the analogues LL-A, LL-B, and LL-C all exhibited substantial deviations in retention time compared with **1**, which may indicate changes in the overall three-dimensional structure of the peptide (Figures S2–S4). The desired ring

Scheme 2. Solid-Supported Synthesis of **1**^{a,b}

^aReagents and conditions: (a) SPPS; (b) SnCl_2 , HCl, DMF; (c) piperidine, DMF; (d) PyAOP, HOAt, 2,4,6-collidine, DMF; (e) $\text{Pd}(\text{PPh}_3)_4$, PhSiH_3 , DMF, CH_2Cl_2 ; (f) $\text{CF}_3\text{CO}_2\text{H}$, H_2O , Pr_3SiH . ^bPrior to cleavage from the resin, all residues contained appropriate side-chain protecting groups for Fmoc-SPPS: *tert*-butoxycarbonyl (Boc) for Lys and Trp, *tert*-butyl (tBu) for Ser, *tert*-butyl ester (O^tBu) for Glu, and trityl (Trt) for Asn, Gln, and His. For experimental details, see the SI.

topology of **1** was confirmed using tandem mass spectrometry by comparison to authentic lactacin 481 (Figure S5). The entire SPPS, cleavage, and purification sequence could be completed in 10–12 days.

The desired absolute stereochemical configurations of the Lan/MeLan residues in each peptide were confirmed via chiral gas chromatography–mass spectrometry (GC–MS) analysis of the hydrolyzed and derivatized peptide using synthetic standards (Figure S6).^{15,21} During the course of each synthesis, an appreciable amount of epimerization in the Lan building blocks was observed, possibly from repeated exposure of the ester-protected building blocks to piperidine. Fortunately, these epimerization byproducts could be separated from the desired full-length peptides during HPLC purification and were isolated in submilligram quantities. Only the desired product of each synthesis, as identified by chiral GC–MS analysis, was used for subsequent biological evaluation.

The antimicrobial activity of **1** and its diastereomers LL-A, LL-B, and LL-C were assessed and compared to authentic lactacin 481 using growth inhibition assays in liquid culture. Cultures of the indicator strain *L. lactis* subsp. *cremoris* HP were treated with a 2-fold serial dilution of each peptide in a 96-well plate format. Half-maximal inhibitory concentration (IC_{50}) and minimal inhibitory concentration (MIC) values were determined from plots of culture optical density at 600 nm (OD_{600}) versus peptide concentration (Figure 3). As expected, the activity of synthetic **1** ($\text{IC}_{50} = 300 \pm 70$ nM; MIC = 625 nM) was the same as that of the authentic natural product ($\text{IC}_{50} = 250 \pm 50$ nM; MIC = 625 nM) within experimental error. However, none of the three diastereomeric analogues possessed any activity up to the highest concentration tested (10 μM). These observations indicate that the natural DL configuration of each cross-link is essential for the biological activity of **1**.

The ability of each analogue to antagonize the antibacterial activity of authentic lactacin 481 was also tested. Several lantibiotics form multimeric complexes with lipid II;^{34,35} indeed, haloduracin α , which contains the mersacidin-like lipid II binding motif that is also present in lactacin 481, binds lipid II with 2:1 stoichiometry.³⁶ It was therefore possible that the inactive lactacin 481 analogues could still bind the same biological target as wild-type lactacin 481 but lack the ability to form the complexes necessary for full activity. If this were the case,

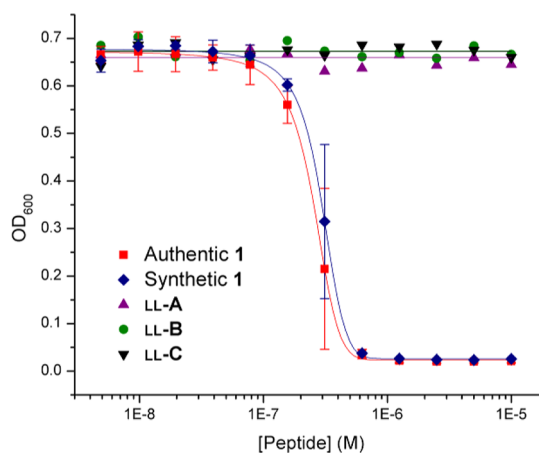


Figure 3. Evaluation of the antibacterial activities of authentic and synthetic **1** and the analogues LL-A, LL-B, and LL-C against *L. lactis* subsp. *cremoris* HP in liquid culture. Error bars represent standard deviations of three replicates.

antagonism could result when active and inactive species are supplied together. This possibility was investigated by applying authentic lactacin 481 and each of the three analogues on agar plates cultured with *L. lactis* HP. In each case, no antagonism was observed (Figures 4 and S8), likely indicating that the inactive analogues lack the ability to bind the biological target of lactacin 481.

In this work, both the first total synthesis of lactacin 481, a lantibiotic possessing a complex tricyclic topology, and the first investigation of the importance of the Lan/MeLan stereochemistry for the antibacterial activity of a lantibiotic have been reported. In the case of lactacin 481, substitution of any of the three DL-Lan/MeLan residues with the corresponding LL stereoisomers completely abolished the activity. As it has recently been shown that some of the homologous enzymes that determine the stereochemistry of the Lan/MeLan residues can make both DL and LL isomers,¹⁶ the results provided herein suggest that the stereochemistry of lactacin 481 evolved specifically to optimize tight binding of its biological target and not because its biosynthetic machinery is limited to generating only the DL configuration. The substantial difference between the RP-HPLC retention times of **1** and its stereoisomeric analogues

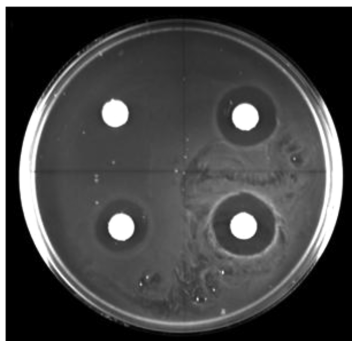


Figure 4. Evaluation of antagonism between **1** and the analogue LL-C on an agar plate cultured with *L. lactis* subsp. *cremoris* HP. Paper discs were treated with 10 μ L aliquots of (clockwise from top left) 10 μ M LL-C, 10 μ M authentic **1**, a mixture of **1** and LL-C (10 μ M each), and a mixture of **1** and LL-C (5 μ M each). Similar results were obtained for LL-A and LL-B (Figure S8).

(Figures S1–S4) indicates an alteration in the three-dimensional structure that likely prevents binding of the target and thus eliminates the activity, a conclusion reinforced by the lack of antagonism when active and inactive species were applied simultaneously. However, these findings leave unaddressed the potential importance of the newly discovered LL stereo-configuration of cross-links in several other natural lantibiotics, including both peptides of the enterococcal cytotoxin and the β -peptide of haloduracin.¹⁶ Further synthetic efforts using this solid-supported strategy may shed additional light on the stereochemistry–activity relationships of these compounds.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental procedures, characterization of novel molecules, and GC–MS results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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