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Chemical Synthesis of the Lantibiotic Lacticin 481 Reveals the Importance of Lanthionine Stereochemistry

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Supporting Information

ABSTRACT: Lantibiotics are a family of antibacterial peptide natural products characterized by the posttranslational installation of the thioether-containing amino acids lanthionine and methyllanthionine. Until recently, only a single naturally occurring stereochemical configuration for each of these cross-links was known. The discovery of lantibiotics with alternative lanthionine and methyllanthionine stereochemistry has prompted an investigation of its importance to biological activity. Here, solid-supported chemical synthesis enabled the total synthesis of the lantibiotic lacticin 481 and analogues containing cross-links with non-native stereochemical configurations. Biological evaluation revealed that these alterations abolished the antibacterial activity in all of the analogues, revealing the critical importance of the enzymatically installed stereochemistry for the biological activity of lacticin 481.

ibosomally synthesized and post-translationally modified Repetides (RiPPs) have become recognized as a major class of natural products. 1,2 The structural and functional diversity of RiPPs has expanded greatly in recent years because of the growing availability of sequenced genomes and the application of bioinformatic analyses to the discovery of new compounds.³⁻ One of the largest and best-studied classes of RiPPs are the lanthipeptides, which are polycyclic peptides with complex topologies enforced by the thioether-containing cross-links mesolanthionine (Lan) and (2S,3S,6R)-3-methyllanthionine (MeLan) (Figure 1).8 Many members of this family, termed lantibiotics, possess potent antimicrobial activity against a variety of human pathogens, and have therefore garnered substantial attention for clinical applications. 9-12 The biosynthesis of lanthipeptides involves the enzymatic dehydration of select serine and threonine residues in a linear, ribosomally synthesized precursor peptide to yield 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), followed by enzymatic cyclization via intramolecular Michael-type anti addition of cysteinyl thiols to afford the Lan/MeLan structures.8 Until recently, all characterized Lan and MeLan structures were reported to possess D stereochemistry at the newly formed α -stereocenter and thus an overall DL configuration (Figure 1); 13-15 this enforced the assumption that all lanthipeptide cross-links possess this configuration because they are made by homologous enzymes. The very recent discovery of lanthipeptides containing cross-links with an LL configuration 16 has called into question the

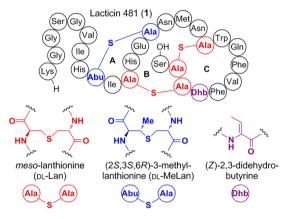


Figure 1. Sequence and ring topology of the lantibiotic lacticin 481 (1) and chemical structures of its post-translational modifications.

importance of the cross-link stereochemistry to the biological activity and mirrors a growing appreciation of stereoisomeric natural product biosynthesis.17

To engineer lantibiotics for therapeutic use, a variety of in vivo and in vitro platforms have been developed to produce analogues for the exploration of structure—activity relationships and mechanisms of action. ^{8,18,19} Of these approaches, total chemical synthesis presents the opportunity to remove any dependence on the biosynthetic machinery of the producing organism, thus opening up a wider chemical space for potential exploration.²⁰ The recent solid-supported total syntheses of lactocin S,²¹ both peptides of lacticin 3147, 14 and analogues of epilancin 15X²² have demonstrated that complex lantibiotics, including those containing overlapping topologies and MeLan cross-links, are feasible synthetic targets. This approach has also been used by Vederas and co-workers to produce lantibiotic analogues containing non-thioether-based cross-links,²³ an achievement inaccessible to the biosynthetic system. Other recent synthetic advances have focused on individual rings in various lanti-biotics.^{24–27} However, the role of Lan/MeLan stereochemistry in antibacterial activity has not been addressed to date. In this study, chemical synthesis was used to construct the lantibiotic lacticin 481 (1) (Figure 1). Systematic replacement of each DL-Lan/MeLan cross-link with its LL stereoisomer enabled the first assessment of the effect of the cross-link stereochemistry on the antibacterial activity. While synthetic 1 possessed biological activity comparable to the authentic natural product, all of the

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

Lacticin 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 (2R,3R,6R). 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., (2R,3S,6R) and (2S,3R,6R)], which have not been found in natural lanthipeptides.

Scheme 1. Synthesis of DL-2^a

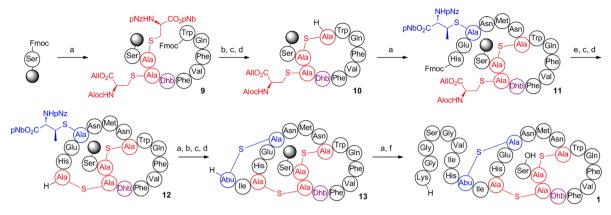
"Reagents and conditions: (a) pNzCl, Na₂CO₃, H₂O, 1,4-dioxane; (b) pNbBr, NaHCO₃, DMF, 94% (two steps); (c) CF₃CO₂H, [†]Pr₃SiH, CH₂Cl₂, 88%; (d) D-6, NaHCO₃, Bu₄NBr, EtOAc, H₂O, Bu₃P, 78%; (e) CF₃CO₂H, PhSiH₃, CH₂Cl₂, 95%. Abbreviations: pNz, *p*-nitrobenzyloxycarbonyl; pNb, *p*-nitrobenzyl; Trt, trityl.

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 lowdensity substitution of 0.1 mmol/g was utilized, effectively preventing intermolecular side reactions during the solidsupported cyclization reactions. Fmoc deprotection was performed using piperidine, and amino acids were activated for coupling using N_iN' -diisopropylcarbodiimide (DIC) and 1hydroxybenzotriazole (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 lacticin 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 lacticin 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}



"Reagents and conditions: (a) SPPS; (b) SnCl₂, HCl, DMF; (c) piperidine, DMF; (d) PyAOP, HOAt, 2,4,6-collidine, DMF; (e) Pd(PPh₃)₄, PhSiH₃, DMF, CH₂Cl₂; (f) CF₃CO₂H, H₂O, Pr₃SiH. Prior 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 (Bu) for Ser, tert-butyl ester (O'Bu) 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 lacticin 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). During the course of each synthesis, an appreciable amount of epimerization in the Lan building blocks was observed, possibly from repeated exposure of the esterprotected 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 lacticin 481 using growth inhibition assays in liquid culture. Cultures of the indicator strain L. lactis subsp. cremoris HP were treated with a 2fold serial dilution of each peptide in a 96-well plate format. Halfmaximal inhibitory concentration (IC₅₀) and minimal inhibitory concentration (MIC) values were determined from plots of culture optical density at 600 nm (OD₆₀₀) versus peptide concentration (Figure 3). As expected, the activity of synthetic 1 $(IC_{50} = 300 \pm 70 \text{ nM}; MIC = 625 \text{ nM})$ was the same as that of the authentic natural product (IC₅₀ = 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 μ M). 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 lacticin 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 lacticin 481, binds lipid II with 2:1 stoichiometry. It was therefore possible that the inactive lacticin 481 analogues could still bind the same biological target as wild-type lacticin 481 but lack the ability to form the complexes necessary for full activity. If this were the case,

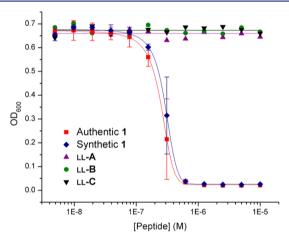


Figure 3. Evaluation of the antibacterial activities of authentic and synthetic 1 and the analogues LL-A, LL-B, and LL-C against *L. lactis* subsp. *cremoris* HP in liquid culture. Error bars represent standard deviations of three replicates.

antagonism could result when active and inactive species are supplied together. This possibility was investigated by applying authentic lacticin 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 lacticin 481.

In this work, both the first total synthesis of lacticin 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 lacticin 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 lacticin 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 stereoconfiguration of cross-links in several other natural lantibiotics, including both peptides of the enterococcal cytolysin and the β -peptide of haloduracin. Further synthetic efforts using this solid-supported strategy may shed additional light on the stereochemistry—activity relationships of these compounds.

ASSOCIATED CONTENT

S 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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