Supporting Information for

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

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General information.

Materials, reactions and purification

Authentic lacticin 481 (1) was provided by Dr. Juan Velásquez (University of Illinois at Urbana-Champaign) after isolation from the producing organism *Lactococcus lactis* subsp. *lactis* CNRZ 481 as previously described. Protected and unprotected amino acids, resins for solid-phase peptide synthesis (SPPS), and peptide coupling reagents N,N'-diisopropylcarbodiimide (DIC), 1-hydroxy-7-azabenzotriazole (HOAt) and 1-hydroxybenzotriazole monohydrate (HOBt) were purchased from Chem-Impex International; 7-azabenzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from AAPPTec. Dimethylformamide (DMF), dichloromethane and tetrahydrofuran (THF) were purchased at reaction grade from Fisher Scientific and dried via a solvent dispensing system prior to use. Other chemical reagents and solvents were purchased from Sigma Aldrich or Alfa Aesar and used without further purification. All reactions were run under an atmosphere of N₂ unless otherwise stated. Reaction progress and chromatography fractions were monitored by thin layer chromatography (TLC) on silica-gel-coated glass plates with a F254 fluorescent indicator. Visualization was achieved by UV absorption by fluorescence quenching or permanganate stain (1.5 g KMnO₄, 10 g K₂CO₃, 1.25 mL 10% NaOH in 200 mL of H₂O). Flash chromatography was performed using Silicycle SiliaFlash P60, 230-400 mesh silica gel. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1260 Infinity system equipped with a Phenomenex Jupiter C12 (90 Å pore size, 250 mm × 4.6 mm × 10 μm) analytical column at a flow rate of 1 mL/min and a solvent gradient of 10-100% solvent B over 45 min, with detection at 220 nm. Preparatory RP-HPLC was performed on a Waters 600 system equipped with a Phenomenex Jupiter Proteo C12 (90 Å pore size, 250 mm × 15.0 mm × 10 μm) preparative column at a flow rate of 10 mL/min and solvent gradients as described for each peptide. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use. HPLC solvent compositions: solvent A, 0.1% trifluoroacetic acid (TFA) in H₂O; solvent B, 80:20 MeCN/H₂O with 0.087% TFA.

Characterization

NMR spectra were recorded on a Varian Unity 400 or Unity Inova 500 spectrometer. Small molecules (MW < 1000 Da) were analyzed by electrospray ionization/time-of-flight (ESI-TOF) mass spectrometry on a Waters Quattro II quadrupole spectrometer. Peptides (MW > 800 Da) were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry on a Bruker Daltonics UltraflleXtreme TOF/TOF spectrometer using a matrix solution consisting of saturated α-cyano-4-hydroxycinnamic acid in 1:1:0.1 H₂O/MeCN/TFA.
Synthetic procedures for Lan/MeLan building blocks.

Nitrobenzyl-protected lanthionine building blocks

L-Bromoalanine 7 was prepared as previously described.²

\[ \text{Nitrobenzyl-protected lanthionine building blocks} \]

\[ \begin{align*}
\text{H}_2\text{N} & \quad \text{OHN} \\
\text{O} & \quad \text{OH}
\end{align*} \]

\[ \begin{align*}
\text{pNzHN} & \quad \text{OpNb} \\
\text{OpNb} & \quad \text{OpNb}
\end{align*} \]

Compound \textbf{d-5}. \text{S-tryt}-\text{L-cysteine} (1.82 g, 5.00 mmol) and sodium carbonate (0.53 g, 5.00 mmol) were dissolved in water (10 mL) and MeCN (5 mL) and chilled in an ice bath. A solution of 4-nitrobenzyl chloroformate (1.08 g, 5.00 mmol) in MeCN (5 mL) was added dropwise over 10 min. The reaction was stirred for 15 h, gradually warming to room temperature, then concentrated under reduced pressure. The residue was taken up in DMF (10 mL), and sodium bicarbonate (0.50 g, 6.00 mmol) was added, followed by 4-nitrobenzyl bromide (1.30 g, 6.00 mmol). The reaction was stirred as a heterogeneous mixture for 21 h, then concentrated under reduced pressure and the residue taken up in EtOAc. The organic layer was washed with saturated NaHCO₃, 0.1 M KHSO₄ and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 2:1 hexane/EtOAc) to yield \textbf{d-5} (3.20 g, 4.72 mmol, 94%) as a yellow foam. \( R_f \) 0.28 (2:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 8.20 (m, 4H), 7.50 (d, \( J = 8.5 \) Hz, 2H), 7.46 (d, \( J = 8.5 \) Hz, 2H), 7.37 (m, 6H), 7.33-7.20 (m, 9H), 5.35 (d, \( J = 8.5 \) Hz, 1H), 5.28-5.16 (m, 4H), 4.38 (q, \( J = 6.5 \) Hz, 1H), 2.76 (dd, \( J = 12.5 \) Hz, 6.5 Hz, 1H), 2.65 (dd, \( J = 12.5 \) Hz, 4.5 Hz, 1H). \(^1\)C NMR (125 MHz, CDCl₃) \( \delta \) 170.2, 155.3, 148.0, 147.8, 144.2, 143.6, 142.3, 129.5, 128.6, 128.2, 128.1, 127.2, 123.9 (two overlapping signals that are resolved in compound 6), 67.3, 66.0, 65.7, 53.2, 34.0. HRMS (ESI) calc. [M+Na]⁺ for C₃₇H₃₁N₃O₈SNa 700.1730, found 700.1733. The enantiomer \textbf{l}-\text{5} was also prepared in this way from \text{S-tryt}-\text{L-cysteine} in 82% yield.

\[ \begin{align*}
\text{pNzHN} & \quad \text{OpNb} \\
\text{OpNb} & \quad \text{OpNb}
\end{align*} \]

Compound \textbf{d-6}. To a solution of \textbf{d-5} (2.03 g, 3.00 mmol) in CH₂Cl₂ (30 mL) under N₂ was added triisopropylsilane (0.92 mL, 4.50 mmol), followed by TFA (3.5 mL, 45.0 mmol). A vivid yellow color appeared immediately, then faded over the reaction time. After 1.5 h, the reaction was diluted with CH₂Cl₂ and water, then solid sodium bicarbonate (3.78 g, 45.0 mmol) was added to neutralize the residual acid. The organic layer was separated, washed with brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (SiO₂, 2:1 hexane/EtOAc) to yield \textbf{d-6} (1.15 g, 2.64 mmol, 88%) as an amber solid. \( R_f \) 0.53 (1:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 8.20 (m, 4H), 7.51 (app t, \( J = 8.5 \) Hz, 4H), 5.80 (d, \( J = 7.5 \) Hz, 1H), 5.35-5.26 (m, 2H), 5.22 (m, 2H), 4.73 (m, 1H), 3.10-2.98 (m, 2H), 1.38 (t, \( J = 9.0 \) Hz, 1H). \(^1\)C NMR (125 MHz, CDCl₃) \( \delta \) 169.7, 155.3, 148.0, 147.8, 143.5, 142.1, 128.8, 128.3, 124.0, 123.9, 66.2, 65.8, 55.4,
27.1. HRMS (ESI) calc. [M+H]⁺ for C₁₈H₁₈N₃O₈S 436.0815, found 436.0819. The enantiomer L-6 was also prepared in this way from L-5 in 77% yield.

Compound DL-8. Compounds 7 (0.58 g, 1.29 mmol) and D-6 (0.45 g, 1.03 mmol) were dissolved in N₂-sparged EtOAc (5 mL). Tetrabutylammonium bromide (1.33 g, 4.12 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 5 mL), then added to the organic solution. The biphasic mixture was stirred under N₂ for 20 h, with tributylphosphine (130 μL, 0.52 mmol) added to the reaction after 5 h. The organic layer was isolated, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 30% EtOAc/hexane) to yield DL-8 (0.64 g, 0.80 mmol, 78%) as a colorless foam. Rᶠ 0.39 (2:1 hexane/EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 8.15 (m, 4H), 7.74 (d, J = 7.5 Hz, 2H), 7.56 (m, 2H), 7.44 (app d, J = 8.5 Hz, 4H), 7.29 (t, J = 7.5 Hz, 2H), 5.96 (d, J = 7.5 Hz, 1H), 5.74 (d, J = 7.5 Hz, 1H), 5.17 (m, 2H), 4.67 (m, 1H), 4.48 (m, 1H), 4.34 (m, 2H), 4.20 (t, J = 7.0 Hz, 1H), 3.12 (dd, J = 14.0 Hz, 5.0 Hz, 1H), 3.08 (dd, J = 14.0 Hz, 5.0 Hz, 1H), 1.47 (s, 9H). ¹³C NMR (125 MHz, CDCl₃) δ 170.1, 169.2, 156.0, 155.5, 147.8, 147.6, 143.8, 143.67 & 143.53 (two signals for formally equivalent Fmoc carbons. Similar results have been reported previously for these types of compounds and are likely because of inequivalency due to rotamers. Additional examples in this work are hereafter simply referred to as Fmoc rotamers), 142.1, 141.3, 128.5, 128.0, 127.9, 127.2, 125.14 & 125.11 (Fmoc rotamers), 123.9, 123.8, 120.1, 83.4, 67.4, 66.1, 65.7, 54.5, 54.0, 47.1, 36.1, 35.4, 28.0. HRMS (ESI) calc. [M+H]⁺ for C₄₀H₄₁N₄O₁₂S 801.2442, found 801.2447.

Compound DL-2. To a solution of DL-8 (0.50 g, 0.62 mmol) in CH₂Cl₂ (3 mL) was added phenylsilane (80 µL, 0.66 mmol), followed by TFA (3 mL). The reaction was stirred under N₂ for 2.5 h, then concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 2% MeOH/CH₂Cl₂) to yield DL-2 (0.44 g, 0.59 mmol, 95%) as a colorless foam. ¹H NMR (500 MHz, CD₃OD) δ 8.10 (app t, J = 8.5 Hz, 4H), 7.75 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 7.5 Hz, 2H), 7.51 (app t, J = 7.5 Hz, 4H), 7.35 (dt, J = 7.5 Hz, 3.5 Hz, 2H), 7.25 (dt, J = 7.5 Hz, 3.5 Hz, 2H), 5.25-5.15 (m, 4H), 4.56 (m, 1H), 4.51 (m, 1H), 4.44 (m, 1H), 4.33 (m, 1H), 4.23 (m, 1H), 4.18 (t, J = 7.0 Hz, 1H), 3.10 (dd, J = 14.0 Hz, 5.0 Hz, 2H), 2.94 (dd, J = 14.0 Hz, 5.0 Hz, 1H), 1.47 (s, 9H). ¹³C NMR (125 MHz, CD₃OD) δ 173.7, 171.8, 158.4, 157.9, 148.8, 148.7, 145.7, 145.14 & 145.11 (Fmoc rotamers), 144.3, 142.4, 129.3, 128.9, 128.8, 128.2, 126.32, 126.28, 124.51, 124.47, 120.9, 68.2, 66.7, 66.3, 55.8, 55.4, 48.2, 35.7, 35.2. HRMS (ESI) calc. [M+H]⁺ for C₃₆H₃₃N₄O₁₂S 745.1816, found 745.1823.

Compound LL-8. Compounds 7 (0.56 g, 1.26 mmol) and L-6 (0.50 g, 1.15 mmol) were dissolved in N₂-sparged EtOAc (8 mL). Tetrabutylammonium bromide (1.48 g, 4.60 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 8 mL), then added to the organic solution. The biphasic mixture was stirred under N₂ for 24 h, with tributylphosphine (140 μL, 0.58 mmol) added to the reaction after 4 h. The organic layer was isolated, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 30%
EtOAc/hexane) to yield LL-8 (0.62 g, 0.78 mmol, 68%) as a colorless foam. RF 0.38 (2:1 hexane/EtOAc). 1H NMR (500 MHz, CDCl 3) δ 8.16 (m, 4H), 7.74 (d, J = 7.5 Hz, 2H), 7.56 (m, 2H), 7.46-7.36 (m, 6H), 7.29 (t, J = 7.5 Hz, 2H), 5.91 (d, J = 7.5 Hz, 1H), 5.65 (d, J = 7.0 Hz, 1H), 5.23-5.11 (m, 4H), 4.62 (m, 1H), 4.42-4.32 (m, 2H), 4.20 (t, J = 7.0 Hz, 1H), 3.12-3.03 (m, 3H), 2.91 (dd, J = 14.0 Hz, 5.5 Hz, 1H), 1.47 (s, 9H). 13C NMR (125 MHz, CDCl 3) δ 170.2, 169.3, 156.0, 155.5, 147.8, 147.5, 143.7, 143.6 & 143.5 (Fmoc rotamers), 142.1, 141.3, 128.5, 127.9, 127.8, 127.1, 125.1, 123.8, 123.7, 120.1, 83.4, 67.2, 66.0, 65.6, 54.3, 53.9, 47.1, 35.6, 34.8, 28.0. HRMS (ESI) calc. [M+H]+ for C40H41N4O12S 801.2442, found 801.2443.

Compound LL-2. To a solution of LL-8 (0.55 g, 0.69 mmol) in CH2Cl2 (5 mL) and phenylsilane (80 µL, 0.72 mmol) was added TFA (5 mL). The reaction was stirred for 2 h, concentrated under reduced pressure and repeatedly redissolved in CH2Cl2 and concentrated to remove residual acid. The crude material was purified by flash chromatography (SiO2, 2%-5% MeOH/CH2Cl2) to yield LL-2 (0.45 g, 0.61 mmol, 89%) as a white solid after lyophilization from 1:1 benzene/MeCN. RF 0.05 (EtOAc). 1H NMR (500 MHz, CD3OD) δ 8.10 (app t, J = 8.5 Hz, 4H), 7.74 (d, J = 7.5 Hz, 2H), 7.62 (d, J = 7.5 Hz, 2H), 7.49 (app t, J = 7.5 Hz, 4H), 7.34 (t, J = 7.5 Hz, 2H), 7.26 (t, J = 7.5 Hz, 2H), 5.26-5.11 (m, 4H), 4.55 (m, 1H), 4.43 (m, 1H), 4.31 (m, 1H), 4.24 (m, 1H), 4.18 (t, J = 7.0 Hz, 1H), 3.15 (dd, J = 14.0 Hz, 4.5 Hz, 2H), 2.99 (dd, J = 14.0 Hz, 3.5 Hz, 1H), 2.90 (dd, J = 14.0 Hz, 4.0 Hz, 1H). 13C NMR (125 MHz, CD3OD) δ 173.7, 171.9, 158.4, 157.9, 148.9, 148.7, 145.7, 145.13 & 145.11 (Fmoc rotamers), 144.3, 142.4, 129.3, 128.9, 128.8, 128.1, 126.3, 124.52, 124.47, 120.9, 68.2, 66.7, 66.3, 55.6, 55.2, 48.3, 35.4, 34.9. HRMS (ESI) calc. [M+H]+ for C36H33N4O12S 745.1816, found 745.1817.

Allyl-protected lanthionine building blocks

L-Cystine 15 and Lan building block DL-3 were prepared as previously described.2 L-Bromo-alanine L-14 was prepared from L-serine as reported for its enantiomer D-14.2

Compound LL-16. Tributylphosphine (310 µL, 1.23 mmol) was added to a solution of 15 (0.82 g, 1.03 mmol) in tetrahydrofuran (10 mL) and stirred under N2 for 15 min. Water (1.0 mL) was added, and the reaction was stirred an additional 3 h then concentrated under reduced pressure. To the resulting oil was added L-14 (0.60 g, 2.05 mmol) in N2-sparged EtOAc (10 mL). Tetrabutylammonium bromide (2.64 g, 8.20 mmol) was dissolved in N2-sparged 0.5 M aqueous NaHCO3 (pH adjusted to 8.5, 10 mL), then
added to the organic solution. The biphasic mixture was stirred under N₂ for 16 h, then washed with water and brine, dried over Na₂SO₄ and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 4:1 hexane/EtOAc) to yield LL-16 (0.87 g, 1.42 mmol, 70% over two steps) as a colorless oil. \( R_f \) 0.25 (3:1 hexane/EtOAc). \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 7.77 (d, \( J = 7.5 \) Hz, 2H), 7.62 (d, \( J = 7.5 \) Hz, 2H), 7.40 (t, \( J = 7.5 \) Hz, 2H), 7.32 (t, \( J = 7.5 \) Hz, 2H), 5.93-5.84 (m, 2H), 5.69 (app t, \( J = 7.0 \) Hz, 2H), 5.40-5.20 (m, 4H), 4.65-4.55 (m, 5H), 4.50 (m, 1H), 4.41 (m, 2H), 4.24 (t, \( J = 7.0 \) Hz, 1H), 3.10-2.95 (m, 4H), 1.49 (s, 9H). \(^{13}\)C NMR (125 MHz, CDCl₃) \( \delta \) 170.3, 169.5, 155.9, 155.7, 143.94 & 143.86 (Fmoc rotamers), 141.4, 132.6, 131.3, 127.8, 127.2, 125.26 & 125.22 (Fmoc rotamers), 120.1, 119.4, 118.1, 83.2, 67.3, 66.6, 66.1, 54.4, 53.9, 47.2, 35.6, 35.5, 28.1. HRMS (ESI) calc. [M+H]⁺ for C₃₂H₃₉N₂O₈S 611.2427, found 611.2431.

Compound LL-3. To a solution of LL-16 (0.80 g, 1.31 mmol) in CH₂Cl₂ (3 mL) was added phenylsilane (180 µL, 1.44 mmol), followed by TFA (3 mL). The reaction was stirred under N₂ for 2 h, then concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 1% MeOH/CH₂Cl₂) to yield LL-3 (0.66 g, 1.19 mmol, 91%) as a white solid after lyophilization from 1:1 benzene/MeCN. \( R_f \) 0.03 (1:1 EtOAc/hexane). \(^1\)H NMR (500 MHz, CD₃OD) \( \delta \) 7.73 (d, \( J = 7.5 \) Hz, 2H), 7.63 (m, 2H), 7.34 (t, \( J = 7.5 \) Hz, 2H), 7.27 (t, \( J = 7.5 \) Hz, 2H), 5.92-5.83 (m, 2H), 5.30-5.24 (m, 2H), 5.20-5.15 (m, 2H), 4.58 (d, \( J = 5.0 \) Hz, 2H), 4.51 (m, 2H), 4.44 (m, 2H), 4.30 (m, 2H), 4.18 (t, \( J = 7.0 \) Hz, 1H), 3.14-3.03 (m, 2H), 2.98-2.88 (m, 2H). \(^{13}\)C NMR (125 MHz, CD₃OD) \( \delta \) 173.7, 171.9, 158.4, 158.1, 145.12 & 145.04 (Fmoc rotamers), 142.4, 134.0, 133.0, 128.7, 128.1, 126.3, 120.9, 118.8, 117.7, 68.1, 67.0, 66.7, 55.3, 55.1, 48.2, 35.2, 34.9. HRMS (ESI) calc. [M+H]⁺ for C₂₈H₃₁N₂O₈S 555.1801, found 555.1801.

Nitrobenzyl-protected methyllanthionine building blocks

\[ \text{L-Bromoalanine } \text{7} \text{ and MeLan building block } \text{DL-4} \text{ were prepared as previously described.} \]

\[ \text{L-β-Methylcysteine } \text{L-17} \text{ (shown) was prepared from L-threonine as reported for its enantiomer } \text{D-17}. \]
Compound LL-18. Compounds 7 (0.66 g, 1.46 mmol) and L-17 (0.53 g, 1.17 mmol) were dissolved in N₂-sparged EtOAc (10 mL). Tetrabutylammonium bromide (1.51 g, 4.68 mmol) was dissolved in N₂-sparged 0.5 M aqueous NaHCO₃ (pH adjusted to 8.5, 10 mL), then added to the organic solution. The biphasic mixture was stirred under N₂ for 24 h, with tributylphosphine (150 μL, 0.59 mmol) added to the reaction after 7 h. The organic layer was isolated, washed with water and brine, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude material was purified by flash chromatography (SiO₂, 30% EtOAc/hexane) to yield LL-18 (0.74 g, 0.91 mmol, 78%) as a colorless foam. Rf 0.33 (2:1 hexane/EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 8.17 (d, J = 8.5 Hz, 2H), 8.12 (d, J = 8.5 Hz, 2H), 7.74 (d, J = 7.5 Hz, 2H), 7.56 (m, 2H), 7.49 (d, J = 8.5 Hz, 2H), 7.40 (m, 4H), 7.29 (t, J = 7.5 Hz, 2H), 5.89 (d, J = 8.5 Hz, 1H), 5.62 (d, J = 7.5 Hz, 1H), 5.29-5.10 (m, 4H), 4.56 (m, 1H), 4.45 (m, 1H), 4.36 (d, J = 7.0 Hz, 2H), 4.19 (t, J = 7.0 Hz, 1H), 3.50 (m, 1H), 3.02 (d, J = 14.0 Hz, 4.5 Hz, 1H), 2.89 (dd, J = 14.0 Hz, 4.5 Hz, 1H), 1.48 (s, 9H), 1.35 (d, J = 7.0 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 170.2, 169.3, 156.0, 147.9, 147.6, 143.8, 143.65 & 143.51 (Fmoc rotamers), 142.1, 141.3, 128.7, 128.0, 127.9, 127.2, 125.12 & 125.07 (Fmoc rotamers), 123.9, 123.8, 120.1, 83.4, 67.4, 66.1, 65.7, 58.9, 54.4, 47.1, 42.9, 34.0, 28.1, 19.4. HRMS (ESI) calc. [M+H]+ for C₄₁H₄₃N₄O₁₂S 815.2598, found 815.2598.

Compound LL-4. To a solution of LL-18 (0.50 g, 0.62 mmol) in CH₂Cl₂ (5 mL) and phenylsilane (70 µL, 0.66 mmol) was added TFA (5 mL). The reaction was stirred for 2 h, concentrated under reduced pressure and repeatedly redissolved in CH₂Cl₂ and concentrated to remove residual acid. The crude material was purified by flash chromatography (SiO₂, 0%-5% MeOH/CH₂Cl₂) to yield LL-4 (0.42 g, 0.56 mmol, 90%) as a white solid after lyophilization from 1:1 benzene/MeCN. Rf 0.05 (EtOAc). ¹H NMR (500 MHz, CD₃OD) δ 8.07 (d, J = 8.5 Hz, 2H), 8.03 (d, J = 8.5 Hz, 2H), 7.70 (d, J = 7.5 Hz, 2H), 7.59 (m, 2H), 7.51 (d, J = 8.5 Hz, 2H), 7.43 (d, J = 8.5 Hz, 2H), 7.32 (t, J = 7.5 Hz, 2H), 7.24 (t, J = 7.5 Hz, 2H), 5.26-5.04 (m, 4H), 4.52 (d, J = 4.5 Hz, 1H), 4.38 (m, 1H), 4.28-4.22 (m, 2H), 4.15 (t, J = 7.0 Hz, 1H), 3.46 (m, 1H), 3.08 (dd, J = 14.0 Hz, 4.5 Hz, 1H), 2.91 (dd, J = 13.5 Hz, 3.5 Hz, 1H), 1.34 (d, J = 6.5 Hz, 3H). ¹³C NMR (125 MHz, CD₃OD) δ 174.0, 171.6, 158.5, 158.2, 149.0, 148.7, 145.6, 145.20 & 145.10 (Fmoc rotamers), 144.3, 142.5, 129.7, 128.9, 128.8, 128.1, 126.25 & 126.23 (Fmoc rotamers), 124.53, 124.45, 120.9, 68.2, 66.8, 66.4, 60.5, 55.7, 48.3, 43.9, 34.7, 19.6. HRMS (ESI) calc. [M+H]+ for C₃₇H₃₅N₄O₁₂S 759.1972, found 759.1962.
Synthetic procedures for lacticin 481 and ring diastereomers.

Standard SPPS protocols. Unless noted otherwise, standard cycles for SPPS were performed as follows, using a fritted glass reaction vessel equipped with a N\textsubscript{2} inlet for resin/reagent agitation and a suction outlet for draining. Fmoc deprotection was achieved by agitating resin with 20% piperidine in DMF for 10-15 min. After draining the reaction vessel, the resin was washed with DMF (3 x 30 s) and CH\textsubscript{2}Cl\textsubscript{2} (2 x 30 s). The appropriately side-chain protected Fmoc-amino acid (5 equiv.) in DMF (5 mL) was pre-activated with DIC and HOBt (5 equiv. each) for 5 min, then added to resin and agitated for 60 min. After draining the reaction vessel, the resin was washed as before. The completion of all couplings was assessed by a Kaiser test; double couplings were performed for the residues following each cyclization and otherwise as needed. Test cleavages were performed after each cyclization step by removing a small portion of dry resin from the reaction vessel and treating with 90:5:5 TFA/H\textsubscript{2}O/triisopropylsilane for 1 h under N\textsubscript{2}. After removing the cleaved resin by filtration, the filtrate was concentrated under a stream of N\textsubscript{2}. The peptide was precipitated with cold Et\textsubscript{2}O, isolated by centrifugation at 12,000 \times g, dissolved in 1:1 H\textsubscript{2}O/MeCN, and spotted onto a MALDI-TOF MS target for analysis.

Intermediate 9: The substitution of the Fmoc-Ser(\textsuperscript{t}Bu)-Wang resin (initial substitution 0.55 mmol/g) was first reduced such that 1 equiv. corresponded to 0.10 mmol/g. The resin was swelled in DMF for 20 min, and the reaction vessel was drained. To ensure local as well as global substitution reduction, Lan building block \textbf{DL-2} (for 1, LL-A, and LL-B) or \textbf{LL-2} (for LL-C) (1 equiv.) and Boc-Ala-OH (3 equiv.) were pre-activated with DIC/HOAt (4 equiv.) for 5 min, then added to the resin. The reaction was performed for 3 h. Any remaining free resin sites were capped with 1:2:7 Ac\textsubscript{2}O/pyridine/DMF for 30 min. Adjusted resin substitution was calculated as follows: Fmoc-protected resin (20 mg) was agitated with 20% piperidine/DMF (1.0 mL) for 15 min. A 20 \textmu L aliquot of this solution was diluted 100:1 with DMF. The absorbance of this solution at 301 nm (\textit{A}\textsubscript{301}) was recorded after blanking with pure DMF, and the resin substitution was calculated using the equation: Substitution = 101(\textit{A}\textsubscript{301})/7.8(mg resin).\textsuperscript{6} After standard Fmoc deprotection, Lan building block \textbf{DL-3} (for 1, LL-A, and LL-C) or \textbf{LL-3} (for LL-B) (2 equiv.) was pre-activated with DIC/HOAt (2 equiv.) for 5 min, then added to resin and reacted for 2 h. After standard Fmoc deprotection, Fmoc-Phe-(\textsuperscript{Z})-Dhb-OH\textsuperscript{2} (3 equiv.) was pre-activated with DIC/HOAt (3 equiv.) in DMF for 10 min and reacted with the resin-bound peptide for 2 h; this coupling was repeated for an additional 2 h, then the peptide was Fmoc-deprotected by the standard protocol. Val22, Phe21 and Gln20 were coupled/Fmoc-deprotected by the standard protocol, then Trp19 was coupled but not deprotected to yield the resin-bound intermediate 9.

Monocyclic intermediate 10: The nitrobenzyl protecting groups of 9 were removed with two treatments of 6 M SnCl\textsubscript{2} and 5 mM HCl/dioxane in DMF (5 mL) for 1 h each. Following the second treatment, the reaction vessel was drained, and the resin was washed with DMF (3 x 30 s), 1:1 DMF/H\textsubscript{2}O (3 x 1 min), 1:1 THF/H\textsubscript{2}O (3 x 1 min), DMF (3 x 30 s) and CH\textsubscript{2}Cl\textsubscript{2} (2 x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 30 s), CH\textsubscript{2}Cl\textsubscript{2} (3 x 30 s) and DMF (2 x 30 s) to remove all traces of
piperidine. Cyclization was promoted by adding PyAOP and HOAt (5 equiv. each) in DMF to the resin and agitating for 5 min, then adding 2,4,6-collidine (10 equiv.) and agitating for 3 h. After draining, this treatment was repeated for an additional 3 h to yield the resin-bound intermediate \textbf{10}. Test cleavage analysis on a small sample was performed as described above under standard SPPS protocols to confirm completed cyclization. HRMS (MALDI-TOF) calc. [M+Na]^+ for C_{65}H_{83}N_{13}O_{17}S_{2}Na 1404.537, found 1404.530.

Bicyclic intermediate \textbf{12}: Asn17, Met16 and Asn15 were coupled/Fmoc-deprotected to \textbf{10} by the standard protocol. MeLan building block \textbf{DL-4} (for \textbf{1}, \textbf{LL-B} and \textbf{LL-C}) or \textbf{LL-4} (for \textbf{LL-A}) (2 equiv.) was pre-activated with DIC/HOAt (2 equiv) for 5 min, then added to resin and reacted for 2 h. After standard Fmoc-deprotection, Glu13 was coupled and Fmoc-deprotected by the standard protocol, then His12 was coupled but not deprotected. The allyl protecting groups were then removed by agitating resin with tetrakis(triphenylphosphine)palladium(0) (1 equiv.) and phenylsilane (10 equiv.) in 1:1 DMF/CH_2Cl_2 (10 mL) for 2 h, protected from light. After draining the reaction vessel, the resin was washed with CH_2Cl_2 (3 x 1 min), 0.5% diethylthiocarbamate in DMF (3 x 1 min), DMF (3 x 30 s) and CH_2Cl_2 (2 x 30 s). The Fmoc group was removed by the standard protocol, followed by washing with DMF (5 x 30 s), CH_2Cl_2 (3 x 30 s) and DMF (2 x 30 s) to remove all traces of piperidine. Cyclization was promoted as for \textbf{10} to yield the resin-bound intermediate \textbf{12}. Test cleavage analysis was performed to confirm completed cyclization. HRMS (MALDI-TOF) calc. [M+Na]^+ for C_{104}H_{130}N_{26}O_{32}S_{4}Na 2405.813, found 2405.835.

Tricyclic intermediate \textbf{13}: Ile10 was coupled to resin-supported \textbf{12} by the standard protocol but not Fmoc-deprotected. Removal of the nitrobenzyl and Fmoc groups, peptide cyclization and test cleavage analysis were performed as described for \textbf{10} to yield resin-bound intermediate \textbf{13}. HRMS (MALDI-TOF) calc. [M+Na]^+ for C_{95}H_{129}N_{25}O_{26}S_{4}Na 2186.832, found 2186.859.

Lacticin 481 (\textbf{1}) and analogues \textbf{LL-A}, \textbf{LL-B}, and \textbf{LL-C}: The remaining eight residues were coupled to resin-supported \textbf{13} and deprotected by the standard protocol. The peptide was cleaved from resin and globally deprotected with 92.5:5:2.5 TFA/H_2O/triisopropylsilane under N_2 for 2 h. The cleaved resin was removed by filtration, and the filtrate was concentrated under a stream of N_2. The peptide was precipitated with cold Et_2O, isolated by centrifugation at 12,000 \times g, dissolved in 1:1 H_2O/MeCN and lyophilized to dryness. Crude peptide was dissolved to 10 mg/mL in 10% MeCN/H_2O with 0.1% TFA and purified by preparatory RP-HPLC using a solvent gradient of 10% solvent B (see General Information) for 1 min, then 10-25% over 4 min, then 25-50% over 25 min, then 50-100% over 1 min. Partially-pure peptide eluted in fractions collected 22-28 min. These fractions were lyophilized and repurified under the same conditions, with pure product eluting as described for each peptide.
Lyophilization yielded the desired peptides 1, LL-A, LL-B, and LL-C (average yield 1.9 mg, 0.66 μmol, 1.3% from 50 μmol scale syntheses; 92% per step over 52 steps) as white powders. Byproducts resulting from Lan epimerization were also observed and were separated from the desired products (average yield 0.5 mg, 0.17 μmol, 0.3% from 50 μmol scale syntheses).

*Synthetic lacticin 481* (1). R_t = 26.3-27.0 min. Yield (50 μmol scale): 2.0 mg product; 0.6 mg epimerized byproducts. HRMS (MALDI-TOF) calc. [M+H]^+ for C_{127}H_{183}N_{36}O_{35}S_{4} 2900.252, found 2900.244. See Fig. S1 for HPLC and MS analysis, and Fig. S5 for MS/MS analysis.

*Lacticin 481 LL-A*. R_t = 22.6-23.3 min. Yield (50 μmol scale): 1.8 mg product; 0.4 mg epimerized byproducts. HRMS (MALDI-TOF) calc. [M+H]^+ for C_{127}H_{183}N_{36}O_{35}S_{4} 2900.252, found 2900.278. See Fig. S2 for HPLC and MS analysis.

*Lacticin 481 LL-B*. R_t = 22.7-23.4 min. HRMS (MALDI-TOF) calc. [M+H]^+ for C_{127}H_{183}N_{36}O_{35}S_{4} 2900.252, found 2900.279. See Fig. S3 for HPLC and MS analysis.

*Lacticin 481 LL-C*. R_t = 23.0-23.6 min. Yield (50 μmol scale): 1.8 mg product; 0.5 mg epimerized byproducts. HRMS (MALDI-TOF) calc. [M+H]^+ for C_{127}H_{183}N_{36}O_{35}S_{4} 2900.252, found 2900.256. See Fig. S4 for HPLC and MS analysis.

Note, since the epimerized products are seen by HPLC prior to acidic hydrolysis and chiral GC-MS analysis, the epimers must be formed during the peptide synthesis and not because of partial epimerization during the acid hydrolysis. In addition, because no diastereomers were observed in the protected Lan/MeLan building blocks to the limit of ¹H and ¹³C NMR detection, the epimerization must have taken place during the solid phase peptide synthesis. The ester protected Lan building blocks are introduced in positions 2 and 3 from the C-terminus and then are subjected to many rounds of Fmoc deprotection with piperidine prior to ring closure that removes the epimerization prone ester groups. We suspect that the repeated exposure to piperidine of the esters for a large number of deprotection/coupling sequences, results in small amounts of epimerization in each round.
Figure S1. Characterization of synthetic lacticin 481 (1). (a) Analytical RP-HPLC chromatogram, showing co-elution with authentic lacticin 481 (red dashed line). (b) MALDI-TOF mass spectrum.

Figure S2. Characterization of lacticin 481 ring diastereomer LL-A. (a) Analytical RP-HPLC chromatogram. (b) MALDI-TOF mass spectrum.
**Figure S3.** Characterization of lacticin 481 ring diastereomer LL-B. (a) Analytical RP-HPLC chromatogram. (b) MALDI-TOF mass spectrum.

**Figure S4.** Characterization of lacticin 481 ring diastereomer LL-C. (a) Analytical RP-HPLC chromatogram. (b) MALDI-TOF mass spectrum.
Figure S5. MALDI-MS/MS analysis of (a) authentic and (b) synthetic lacticin 418 (1). Identical fragmentation patterns are seen for both peptides, confirming identical ring topologies. For a discussion of tandem MS for the analysis of lanthipeptides, see Li et al.7
Chiral gas chromatography-mass spectrometry analysis.

The enantiomeric purity of Lan/MeLan amino acids produced by hydrolysis of synthetic 1 and stereoisomers LL-A, LL-B, and LL-C was determined by chiral GC/MS, using a procedure modified from previous reports.8-10 Lyophilized peptide (0.1-0.2 mg) was dissolved in 6 M HCl (3 mL) and heated at 100 °C in a sealed, high-pressure reaction vessel for 18 h. The reaction was cooled and concentrated with a stream of N₂ over several hours. Methanol (3 mL) was chilled in an ice bath, and acetyl chloride (1 mL) was added dropwise. This solution was added to the dry hydrolysate, and the mixture was sealed and heated at 100 °C for 1 h. The reaction was allowed to cool, then concentrated with a stream of N₂. The dry residue was suspended in CH₂Cl₂ (3.5 mL) and chilled in an ice bath. Pentafluoropropionic anhydride (0.5 mL) was added, and the mixture was sealed and heated at 100 °C for 20 min. The reaction was allowed to cool, then concentrated with a stream of N₂. The residue was dissolved in methanol and re-concentrated, then dissolved again in methanol (50 μL) for analysis. Lan/MeLan standards of differing stereochemical configurations (DD, DL and LL for Lan; DL and LL for MeLan), synthesized using published procedures and similarly derivatized as their pentafluoropropionamide methyl esters, were provided by Weixin Tang (University of Illinois at Urbana-Champaign) as solutions in methanol.11,12

The derivatized hydrolysates and standards were analyzed by GC-MS using an Agilent 7890A gas chromatograph equipped with a Waters Micromass GCT Premier detector and a Varian CP-Chirasil-L-Val fused silica column (25 m × 250 μm × 0.12 μm). Sample solutions in methanol were introduced to the instrument via split injection at an inlet temperature of 190 °C and flow rate of 1.7 mL/min helium gas. The temperature gradient used was held at 160 °C for 5 min, then ramped from 160 °C to 180 °C at 3 °C/min, then held at 180 °C for 1 min. The MS was operated in scan mode, and data was analyzed by selected ion monitoring (SIM) at unique fragment masses of 365 Da for Lan and 379 Da for MeLan. All standards eluted as distinct peaks (Fig. S6). For Lan, the DD-isomer eluted at 10.4 min, the DL-isomer at 10.6 min, and the LL-isomer at 10.8 min; for MeLan, the DL-isomer eluted at 8.1 min and the LL-isomer at 8.3 min. The derivatized hydrolysate of 1 confirmed the desired DL-configuration of both Lan and MeLan, while the analogues LL-A, LL-B, and LL-C showed the expected differences from 1 (Fig. S6). Amounts of non-DL-Lan configurations in the hydrolysate of 1 are believed to result from epimerization during hydrolysis, which has been reported previously.9,11,13
Figure S6. GC-MS analysis of the derivatized hydrolysate of (a) synthetic 1; (b) LL-A containing one LL-MeLan (A-ring), and two DL-Lan; (c) LL-B containing one DL-MeLan (A-ring), one LL-Lan (B-ring), and one DL-Lan (C-ring); (d) LL-C containing one DL-MeLan (A-ring), one DL-Lan (B-ring) and one LL-Lan (C-ring) compared to (e) synthetic standards. SIM was set to 365 Da for Lan (solid lines) and 379 Da for MeLan (dashed lines). Lan partially epimerized during synthesis (see main text) and during stereochemical analysis as previously reported.13
**Bacterial growth inhibition assays**

To generate stock concentrations of each peptide, lyophilized peptide was weighed using a Mettler-Toledo MT5 microbalance and dissolved in sterile deionized water (SDW) to give 200 μM stock solutions. Aliquots of these solutions were analyzed by analytical HPLC, detecting at 220 nm, and concentrations were adjusted as necessary based on the integration under the expected peptide peak.

Cultures of *Lactococcus lactis* subsp. *cremoris* HP (ATCC 11602) were grown overnight at 30 °C in GM17 medium (40 g/L M17, 0.5% glucose), then diluted with fresh GM17 to an optical density at 600 nm (OD$_{600}$) of 0.1 corresponding to ~1 × 10$^8$ colony forming units/mL.$^{14}$ Serial two-fold dilutions of stock solutions of authentic 1, synthetic 1, and ring diastereomers LL-A, LL-B, and LL-C were prepared in SDW to give 4x working concentrations. Corning Costar 96-well flat-bottom assay plates were used to determine the activity of each peptide against *L. lactis*, and experiments were performed in triplicate. Experimental wells contained 150 μL of diluted culture and 50 μL of 4x peptide solution. Control wells contained 150 μL GM17 and 50 μL SDW (negative control) or 150 μL diluted culture and 50 μL SDW (positive control). OD$_{600}$ was recorded at hourly intervals using a BioTek Synergy H4 plate reader, and plates were incubated at 30 °C between readings. After subtraction of blank measurements from experimental measurements, plots of OD$_{600}$ vs. peptide concentration were fitted to a dose-response curve with the equation: $y = A_1 + (A_2-A_1) / (1 + 10^{\log x_0 - x} p)$, where $p$ = variable Hill slope. Half maximal inhibitory concentration (IC$_{50}$) and minimal inhibitory concentration (MIC) values were calculated from this fit for each peptide after 6 h incubation, and triplicate calculations were averaged. While standard protocols from the Clinical and Laboratory Standards Institute recommend 20-24 h incubation,$^{15}$ we found that *L. lactis* cells in negative control wells began to die by 20 h, and that a 6 h incubation permitted the measurement of data values between the two plateau regions of the data fit (Fig. S7). Only marginal changes in the IC$_{50}$ values, and no change in MIC values, were observed between the data using 6 and 20 h.

![Figure S7](image-url)  
**Figure S7.** Analysis of the effect of incubation time on observed OD$_{600}$ values of *L. lactis* HP during growth inhibition assays with synthetic lacticin 481 (1).
**Test for antagonistic activities**

Cultures of *Lactococcus lactis* subsp. *cremoris* HP were grown at 30 °C in GM17 medium for 12-18 h, then diluted with fresh GM17 to an OD$_{600}$ of 1.0. A 250 μL aliquot of this culture and 500 μL of 20% aqueous glucose were added to 25 mL melted and cooled GM17 containing 1.5% agar, poured into a sterile petri dish, and allowed to solidify over 1 h. Sterilized filter paper discs were placed in four quadrants of each dish and impregnated with 10 μL of one of the following: 10 μM authentic lacticin 481, 10 μM stereoisomeric analogue, or a pre-mixed sample of authentic lacticin 481 and analogue at a concentration of 5 μM or 10 μM for each peptide. Plates were incubated at 30 °C for 24 h, then analyzed.

**Figure S8.** Evaluation of antagonism of the antimicrobial activity of 1 by the stereoisomeric analogues (a) LL-A and (b) LL-B on an agar plate cultured with *Lactococcus lactis* subsp. *cremoris* HP. Paper discs were impregnated with a 10 μL aliquot of (clockwise from top left) 10 μM analogue, 10 μM authentic 1, a mixture of 1 and analogue at 10 μM each, and a mixture of 1 and analogue at 5 μM each.
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NMR Spectra of novel small molecules
