# Natural product antibiotics: synthesis and next generation analogues

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# 2B

# Chapter 2B

## Total Synthesis, Structure Elucidation, and Bioactivity Evaluation of the Cyclic Lipopeptide Natural Product Paenilipoheptin A


### Abstract

In this study, we further investigated the structure of the recently reported cyclic lipopeptide natural product paenilipoheptin A. Here, we disclose the first total synthesis of the compound, allowing for its complete structural assignment. The route developed employs automated SPPS, providing access to the compound in quantities suitable for antibacterial and antifungal testing. These studies unequivocally establish the stereochemical framework of paenilipoheptin A and further reveal that the compound possesses moderate activity against Gram-positive bacteria.



Parts of this chapter have been published in:

Lysenko, V.*; Machushynets, N. V.*; van Dam, J. L.; Sterk, F. A. C.; Speer, A.; Ram, A. F. J.; Slingerland, C. J.; Van Wezel, G. P.; Martin, N. I. Total Synthesis, Structure Elucidation, and Bioactivity Evaluation of the Cyclic Lipopeptide Natural Product Paenilipoheptin A. *Org. Lett.* **2025**, *27* (12), 2826–2831.

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## Introduction

The growing threat posed by antibiotic resistance has sparked greater efforts toward the discovery and development of novel antibacterial compounds.[1,2] Many known antibiotics are derived from natural sources,[3,4] and there is reason to believe that more are still waiting to be discovered.[5–7] Soil-dwelling bacteria represent an important source of antibiotics, among which members of the *Paenibacillus* genus have yielded a variety of lipopeptides with a range of activities, including polymyxins, tridecaptins, paenibacterins, and fusaricidins.[8–14] Recently, a new class of cyclic lipopeptides termed the paenilipoheptins was detected in fermentations of *Paenibacillus*.[15,16] However, given their low production levels, the biological activities of the paenilipoheptins could not be fully established, and only partial structural assignments were possible based on genome-based predictions, Marfey's analysis, NMR spectroscopy, and mass spectrometry-based methods.[15,16] Given our prior experience in the synthesis and structural characterization of various cyclic- and lipopeptide antibiotics,[17–22] the paenilipoheptins presented an interesting new family to study, with our attention particularly drawn to paenilipoheptin A (**Figure 1**). While a structure of paenilipoheptin A in which the connectivity of the constituent amino acids was recently proposed,[16] a number of stereochemical ambiguities remained. This prompted us to pursue the total synthesis of paenilipoheptin A to both fully establish its structure and provide quantities of material suitable for evaluating the compound's biological activity.

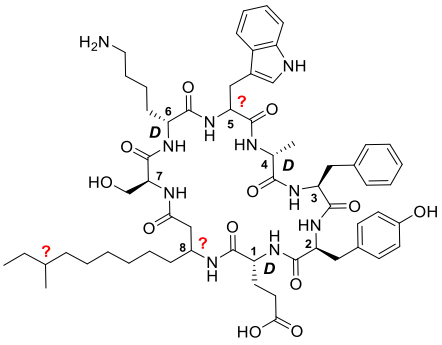



**Figure 1**. Recently proposed structure of paenilipoheptin A with stereochemical ambiguities indicated.[16]

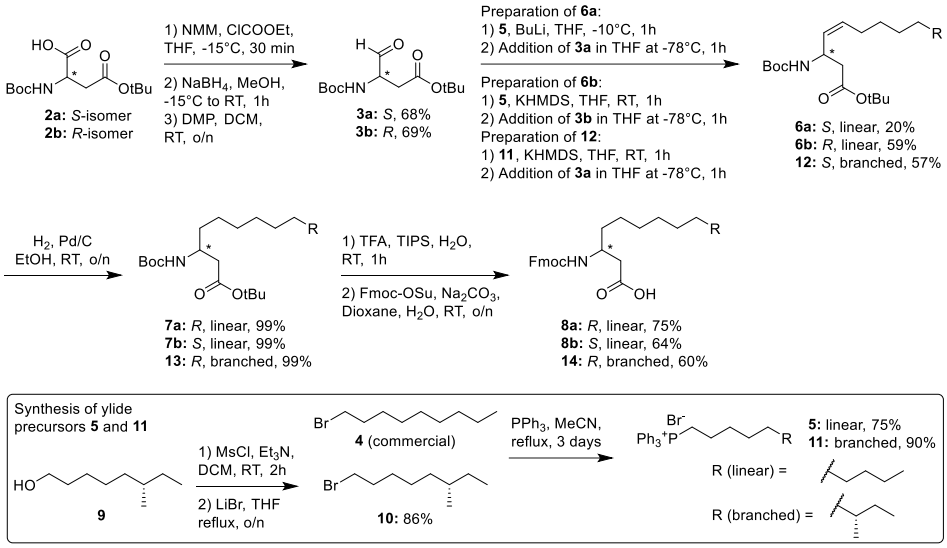
While structurally distinct, the macrocyclic lipopeptide connectivity of the paenilipoheptins is reminiscent of the iturin class of lipopeptides produced by *B. subtilis*.[23,24] As illustrated in **Figure 1**, paenilipoheptin A features a macrocyclic core composed of seven alpha amino acids, of which the residues at positions 2, 3, and 7 were previously shown to have L-stereochemistry while those at positions 1, 4, and 6 have D-stereochemistry (based on Marfey's analysis).[15,16] The macrocycle is closed by amide bond formation between the C-terminal D-Glu[1] residue and the amino group of an unusual beta-amino acid bearing a lipophilic side chain containing anteiso-type branching. Notably, the experimental approaches underscoring the previously proposed structure of

paenilipoheptin A were not sufficient to address the stereochemistry of the beta-amino acid at position 8 or to confirm the D-stereochemistry proposed for $Trp^5$ (based on analysis of the biosynthetic gene cluster). To address these remaining stereochemical details, we therefore turned to a total synthesis approach.

## Results and discussion



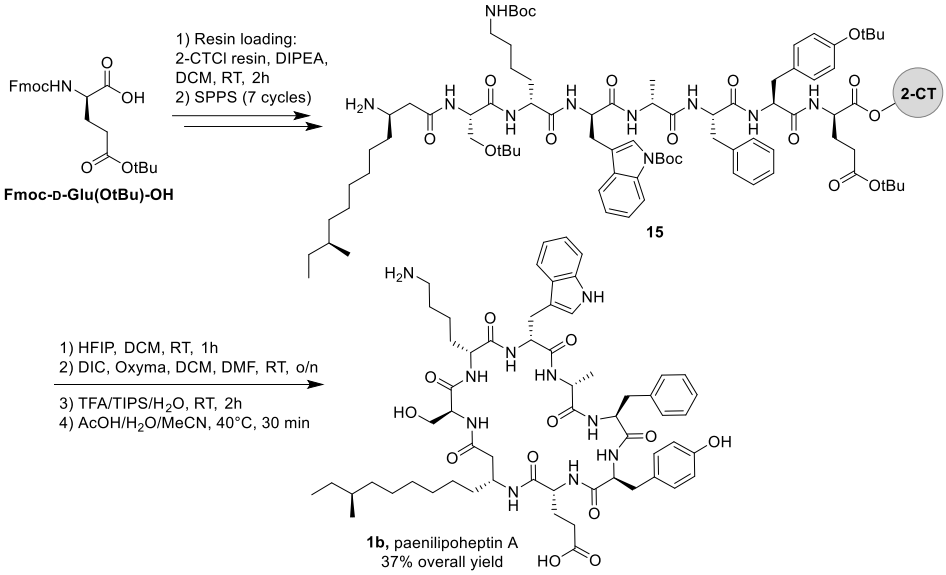
To begin, we focused our attention on the unusual 13-carbon branched tail beta-amino acid at position 8. Given that this amino acid is not commercially available in any of the four possible stereochemical configurations, we opted to first synthesize a simpler, structurally similar, linear analogue with the aim of gaining insight into the stereochemistry of the β-position. While methods have been reported for the synthesis of some beta-amino acids,[25,26] these approaches were not deemed to be optimal for our purposes. Instead, we elected to employ an approach starting from the available and suitably protected L- and D-Asp building blocks **2a** and **2b**, using Wittig chemistry to install the lipid side chain (**Scheme 1**).[27] Conversion of **2a** and **2b** to the corresponding aldehydes **3a** and **3b** was achieved as previously described.[28] Wittig reagent **5** was prepared from the corresponding halide **4** via an established protocol.[29] In our initial attempt at condensing **3a** with **5**, we employed a 2-fold excess of **5** treated with BuLi at −10 °C to generate the active ylide, followed by the addition of the aldehyde at −10 °C. While this approach failed to yield the desired product, we found that lowering the temperature to −78 °C during the aldehyde addition step led to the formation of product **6a** in 20% yield after column chromatography. Given this low yield, when preparing the enantiomeric **6b**, we explored the use of potassium bis(trimethylsilyl)amide (KHMDS) at room temperature to activate the ylide, followed by the addition of aldehyde **3b** at −78 °C. Gratifyingly, this approach was found to result in an improved 59% yield of the expected alkene product. With key intermediates **6a** and **6b** in hand, we proceeded with hydrogenation, which yielded the saturated **7a** and **7b** in quantitative yield. The Boc and tBu groups were then removed using TFA, followed by direct treatment with Fmoc-succinimide (FmocOSu) to yield Fmoc-protected **8a** and **8b** in 75% and 64% yields, respectively.




**Scheme 1.** Syntheses of beta-amino acids **8a**, **8b**, and **14**.

With both enantiomers of the linear analogue of the beta-amino acid in hand, we proceeded to compare the retention times of their corresponding Marfey's derivatives with that of the Marfey's derivative obtained from hydrolysis of natural paenilipoheptin A (**Table S1**).[30,31] To do so, we retained a portion of intermediates **7a** and **7b**, which, after removal of the Boc and tBu groups, were directly treated with Marfey's reagent (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide). Our reason for doing so was based on the hypothesis that the branched beta-amino acid derived from the natural product would have a similar retention time to the linear analogue bearing the same stereochemistry at the β-position. When comparing the retention times of the corresponding adducts, we found that Marfey's derivative of amino acid **7a** elutes at nearly the same time as that derived from the natural product. On the other hand, the Marfey's adduct derived from the enantiomeric **7b** had a significantly different retention time. These findings provided a hint that the β-position of the N-terminal beta-amino acid in paenilipoheptin A is of *R* stereochemistry. With regard to the unknown stereocenter in the anteiso-branched lipid side chain, we made the assumption that it is of the *S* configuration, as commonly seen in other compounds also bearing anteiso-branched lipids produced by Paenibacillus spp.[12,13,32] Conveniently, (*S*)-6-methyloctan-1-ol (**9**) is commercially available, providing a short route to the corresponding Wittig reagent (**Scheme 1**). In doing so, alcohol **9** was first converted to the mesylate, followed by treatment with LiBr in THF to yield bromide **10**. To generate the phosphonium bromide **11**, we followed a similar method as used for the preparation of **5**, with purification achieved by precipitation from MTBE rather than column chromatography. With a limited supply of Wittig reagent **11**, we opted to use only 1.1 equiv of this compound in the subsequent Wittig reaction, otherwise following the same procedure used for the preparation of **6b**. The desired product **12** was thus formed in an acceptable 57% yield, after which hydrogenation yielded **13**, which, in turn, was

subjected to the same deprotection/ reprotection steps used for preparing **8a** and **8b**, to arrive at building block **14**.




**Scheme 2**. Combined solid- and solution-phase route developed for the synthesis of the compound **1b** (paenilipoheptin A).

With building block **14** in hand, we proceeded to synthesize paenilipoheptin A using a combined solid- and solution-phase strategy (**Scheme 2**). In doing so, we used microwave-assisted solid-phase peptide synthesis (SPPS) to prepare the linear protected precursor that was subsequently cleaved from the resin and cyclized in solution. Also of note, we specifically elected to install D-Trp at position 5 (as predicted based on analysis of the biosynthetic gene cluster; see **Figure S1**).[16] As illustrated in **Scheme 2**, protected linear peptide **15** was synthesized starting from the D-Glu[1] loaded via its $\alpha$-carboxyl group onto 2-chlorotrityl (2-CT) resin. In preparing the linear precursor peptide 10% piperazine in EtOH/NMP was used for Fmoc deprotections and HBTU and DIPEA for amino acid couplings. Given its limited supply, the final coupling of compound **14** was performed manually using a reduced number of equivalents in comparison to the standard protocol used for the other couplings. After the final Fmoc deprotection, the protected peptide **15** was then cleaved from the resin using hexafluoroisopropanol (HFIP) to maintain all side chain protecting groups. After evaporation, the crude was then directly treated with DIC/Oxyma (6:6) in DCM/DMF (5:1), which resulted in the clean and complete formation of the desired macrocycle via amide bond formation between the amino group of the N-terminal beta-amino acid and the α-carboxyl group of D-Glu[1]. Following this, global deprotection was performed by treatment with TFA/TIPS/$H_2O$, resulting in the desired **1b** as the main product, as judged by LC-MS. Interestingly, under these conditions, we did not achieve complete deprotection, with approximately 10% of the

product observed as a + 44 Da species that we ascribed to the persistence of a $CO_2$ adduct with the indole of D-Trp[5]. To resolve this issue, we further treated the crude product mixture with a 2% solution of AcOH in $H_2O$/MeCN at 40 °C for 30 min, which led to complete deprotection. Subsequent purification using reverse-phase high-performance liquid chromatography (RP-HPLC) resulted in a yield of 23 mg of compound **1b** (37% overall yield, corresponding to an average yield of 94.9% per step).

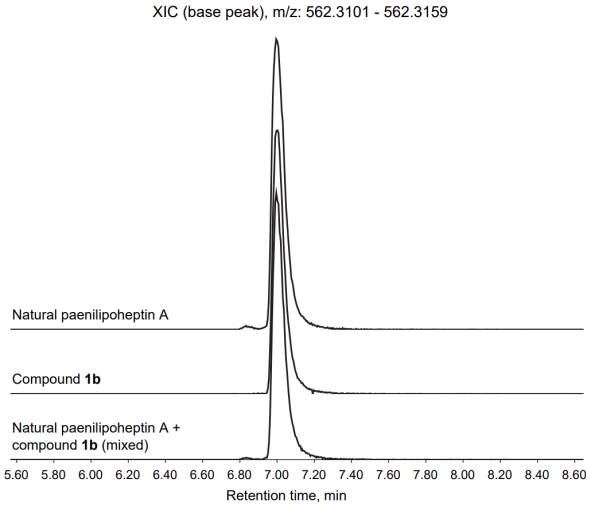



**Figure 2**. Extracted ion chromatograms of authentic paenilipoheptin A (*m/z* = 562.3130, calcd for $[M+2H]^{2+}$) isolated from fermentation of *Paenibacillus sp.* JJ-21 overlaid with trace obtained for synthetic compound **1b**.

We next proceeded to compare compound **1b** with natural paenilipoheptin A isolated from its producing Paenibacillus strain. We were delighted to find that both compounds have identical NMR spectra (**Figures S2** and **S3**) and show the same HPLC retention time, also eluting as a single peak when co-injected (**Figure 2**). These findings confirm that the structure of paenilipoheptin A is indeed that of compound **1b**.

This further supports the stereochemistry of D-Trp[5] and the 3*R* stereochemistry of the naturally occurring beta-amino acid. While these data also point to *S* stereochemistry for the anteiso-branched side chain, we cannot completely exclude the possibility of the natural product having the *R* configuration at this position.

In our previous work describing the isolation and partial characterization of paenilipoheptin A,[16] it was not possible to perform a broad assessment of the compound's bioactivity due to the limited amount of material isolated from the fermentation of the producing organism. Now, with access to larger quantities of material obtained via synthesis, we were able to perform a much wider screen against a range of bacteria and fungi (**Table 1** and **Table S2**). Due to its limited aqueous solubility, we selected 64 μg/mL as the maximum concentration of compound **1b** used in the activity assays.

**Table 1.** Minimal inhibitory concentrations (MICs) determined for naturally produced paenilipoheptin A and compound **1b.**

| Gram-positive bacterial strain tested | MIC (µg/mL) | | |
|---|---|---|---|
| | Paenilipoheptin A | **1b** | Vancomycin |
| *B. subtilis* 168 | 16 | 8 | 0.25 |
| *E. faecium* E980 | 16 | 8 | 0.5 |
| *E. faecium* VRE E155 | ND | 8 | >128 |
| *E. faecium* VRE E7314 | ND | 8 | >128 |
| *S. aureus* ATCC 29213 | 16 | 16 | 0.5 |
| *S. aureus* USA 300 | 16 | 16 | 0.5 |
| *S. aureus* VRS3b | ND | 16 | >128 |
| *S. aureus* LIM-2 | ND | 32 | 4 |
| *Paenibacillus sp.* JJ-21 | ND | 64 | 0.25 |

ND – not determined;

Interestingly, compound **1b** did not show any activity against the fungal strains tested, despite sharing some structural similarities with iturin A, a natural product reported to have antifungal activity.[23,33] The extent to which the antifungal activity observed for iturin A is driven by a targeted mechanism or nonspecific membrane lysis can be debated, considering its propensity to lyse red blood cells.[34] In contrast, when tested at the same concentration, **1b** exhibited no hemolytic activity (**Figure S4**).

When tested against a panel of bacteria, compound **1b** was found to display no activity against Gram-negative species, including a hypersensitive strain of *E. coli* with deletions of the bamB gene, along with knockout of the tolC porin gene.[35] However, when tested against Gram-positive species, **1b** was found to inhibit the growth of *B. subtilis* and *E. faecium* strains at a concentration of 8 µg/mL and also fully inhibited the growth of *S. aureus* at a concentration of 16 µg/mL, which is similar to the values obtained for paenilipoheptin A isolated from biological source. Interestingly, the same antibacterial activity was maintained against vancomycin-resistant strains of *S. aureus* and *E. faecium* with MICs ranging from 8 to 16 µg/mL, while vancomycin showed no activity at the highest concentration tested of 128 µg/mL. We also tested the effect of compound **1b** on the growth of the Gram-positive paenilipoheptin A producing organism, *Paenibacillus sp.* JJ-21. In this case, a much higher MIC value (64 µg/mL) was measured, relative to the other Gram-positives tested, which may be indicative of a self-protective resistance mechanism used by the organism to shield itself from the effects of its own antibacterial compound. Finally, compound 1b was also tested against *M. tuberculosis*, *M. smegmatis*, and *M. abscessus*, which, in all cases, indicated no impact on bacterial cell growth.

## Conclusions

In conclusion, we report here the total synthesis and full structure elucidation of the natural product paenilipoheptin A. This represents the first total synthesis of a member of the paenilipoheptin family of lipopeptides, of which a number of members have recently been identified via bacterial genome mining. The synthesis of paenilipoheptin A depended on the preparation of a unique beta-amino acid containing (3*R*, 10*S*) stereochemistry. Synthetic paenilipoheptin A and the natural product isolated from the producing microorganism were shown to have identical analytical data, supporting the fully assigned structure. Access to synthetic paenilipoheptin A enabled further assessment of its activity against a broad selection of bacterial and fungal strains, revealing the compound to have specific anti-Gram-positive activity.

## Acknowledgement

We thank A. M. C. H. van den Nieuwendijk and Dr. R. J. B. H. N. van den Berg for the assistance with optical rotation measurements. The work was financially supported by the Netherlands Organization for Scientific Research (NWO) through OTP grant no. 19384 to N.I.M. and NACTAR grant no. 16440 to G.P.vW., and N.I.M.

## Materials and methods

### General information

Extended supporting information, which includes NMR and HPLC figures, is available free of charge at https://doi.org/10.1021/acs.orglett.5c00232.

### Reagents

All reagents employed were of American Chemical Society (ACS) grade or higher and were used without further purification unless otherwise stated.

### HRMS

High-resolution mass spectra (HRMS) analyses were performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 μm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 10 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 4 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionization) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000. MZMine 3.2.8 was used to analyze the obtained data.[36]

**Analytical HPLC**

HPLC analyses were performed on a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 5 µm) at 30 °C and equipped with a UV detector monitoring at 214 and 254 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 100:0 (A/B) for 3 min, 100:0 to 0:100 (A/B) over 47 min, 0:100 (A/B) for 4 min, then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 5 min.

**NMR**

$^{1}H$ and $^{13}C$ NMR spectra were recorded on Bruker AV 400 MHz (at 400 ($^{1}H$), 162 ($^{31}P$), and 101 ($^{13}C$) MHz), AV 850 MHz (at 850 ($^{1}H$), and 214 ($^{13}C$) MHz). The temperature of the NMR experiments was 298K unless stated otherwise. Chemical shifts are reported in ppm (δ) and were calibrated using residual deuterated solvent as an internal reference. (δ $^{1}H$ NMR: $CDCl_3$ 7.26; DMSO 2.50; δ $^{13}C$ NMR: $CDCl_3$ 77.16; DMSO 39.52). The NMR data are processed as follows: chemical shift, multiplicity (br s = broad singlet, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, tt = triplet of triplets, m = multiplet), integration, coupling constants (*J*, reported in Hz) and a number of nuclei. NMR spectra were analyzed and processed using MestreNova version 14.2.0.

**Marfey's analysis**

The stereochemistries of chiral centers present at α carbons were assigned by applying derivatization methods coupled with chromatographic analysis. The advanced Marfey's method using L-FDAA (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide) established the absolute configurations of amino acids.[31]

The general method for Marfey's analysis (as described):[30]

A sample of authentic paenilipoheptin A (30 µg) in 6 M HCl (100 µL) was heated to 100 °C in a sealed vial for 15 h, after which the hydrolysate was concentrated to dryness at 40 °C under a stream of dry $N_2$. The hydrolysate was then treated with 1 M $NaHCO_3$ (20 µL) and L-FDAA (1% solution in acetone, 40 µL) at 40 °C for 1 h, after which the reaction was neutralized with 1 M HCl (20 µL). An aliquot of the analyte was diluted 50 times with $H_2O$/MeCN (1:1) and injected (2 µL) into an HRMS instrument following the standard protocol of the analysis (see general methods). The analyte amino acid content was assessed by comparison to authentic standards, which were prepared from **7a**, **7b**, and **13** by treating the compounds with pure TFA to perform Boc and tBu deprotection. After that, all volatile components were evaporated in vacuo, and the residue was treated with solutions of $NaHCO_3$ and L-FDAA, as described above.

**Optical rotations**

Optical rotations were measured on an automatic Anton Paar MCP100 polarimeter of sodium D-line, at $\lambda = 589$ nm.

**Antibacterial assay against Gram-negative and Gram-positive bacteria**

From glycerol stocks, bacterial strains were cultured on blood agar plates and incubated overnight at 37 °C. Following incubation, 3 mL of tryptic soy broth (TSB) was inoculated with an individual colony. The cultures were grown to exponential phase ($OD_{600nm} = 0.5$) at 37 °C. The bacterial suspensions were then diluted 100-fold in TSB (for *E. faecium* strains) or cation-adjusted Mueller-Hinton Broth (CAMHB) to reach a bacterial cell density of $10^6$ CFU $mL^{-1}$. In polypropylene 96-well microtiter plates, test compounds in assay media (e.g., CAMHB or TSB) were added in triplicate and two-fold serially diluted to achieve a final volume of 50 µL per well. An equal volume of bacterial suspension (50 µL, $10^6$ CFU $mL^{-1}$) was added to the wells. The plates were sealed with breathable membranes and incubated at 37 °C for 18-22 h with constant shaking (600 rpm). The MICs were determined by visual inspection as the median of a minimum of triplicates.

**Antibacterial assay against filamentous fungi and yeasts**

The filamentous fungi strains were cultivated on malt extract agar (MEA) plates for 7 days, *Penicillium expansum* ATCC 24692 at 25 ˚C and *Aspergillus fumigatus* Af293, *Aspergillus niger* N400, *Aspergillus oryzae* RIB40 and *Fusarium oxysporum* CBS 101587 at 30 ˚C. Spores were harvested in sterile saline solution (0.9% NaCl in demi water) and filtered. Spore concentrations were determined using a TC20 automated cell counter, and spore stocks were diluted to $10^6$ spores/mL.

The yeast strains *Saccharomyces cerevisiae* NSY220.1 and *Candida albicans* ATCC 10231 were cultivated on yeast extract peptone dextrose agar (YPDA, 1% yeast extract, 2% bacteriological peptone, 2% glucose, 1.5% agar) for 5 days at 30 ˚C, followed by overnight liquid cultivations in 100 mL YPDB (1% yeast extract, 2% bacteriological peptone, 2% glucose) at 30 ˚C, 200 rpm. Fresh cells were obtained by cultivation of 500 µL preculture in 100 mL fresh YPDB at 30 ˚C, 200 rpm, until $OD_{600nm}$ reached 0.4.

The MIC of the compounds on filamentous fungi and yeast strains was determined using a liquid assay utilizing 96-well micro test plates. Each well contains a total volume of 100 µL consisting of the tested compound in the growth medium, malt extract broth (MEB) for the filamentous fungi strains, and YPDB for the yeast strains. For each compound, a concentration range was tested using a 2-fold dilution series. Compound **1b** and Iturin were tested in triplicate, Nystatin was tested in duplicate. Each well was inoculated with 10 µL of spore stock ($10^4$ spores) or cell suspension. Growth was scored after 7 days of incubation at the corresponding growth temperatures for each organism mentioned above. To limit evaporation and dehydration, the plates were kept closed and incubated in closed boxes. The MIC values were defined as the highest dilution, showing no growth.

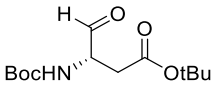
**Antibacterial assay against *M. tuberculosis* H37Rv, *M. smegmatis* mc²155, *M. abscessus* ATCC 19977**

The resazurin microtiter plate assay (REMA) was employed to assess the MIC of compounds against mycobacterial species. For this assay, the compound was prepared in a series of twofold dilutions within a 96-well plate using bacterial growth medium (7H9 Difco, 10% ADS, 0.2% glycerol, and 0.02% tyloxapol). Bacteria were grown until the mid-logarithmic phase, then collected by centrifugation, washed in phosphate-buffered saline (PBS) with 0.02% tyloxapol, resuspended in the growth medium, and introduced into the wells containing compound dilutions, aiming for a final $OD_{600}$ of 0.001 per well. The plates were sealed with parafilm and incubated at 37°C for 1 day for *M. smegmatis*, 2 days for *Mycobacterium abscessus*, and 6 days for *M. tuberculosis*. After incubation, resazurin solution (0.025% [wt/vol] resazurin sodium salt in Milli-Q water with 20% Tween-80 in a 3:1 ratio) was added to each well, and plates were further incubated. Color change was observed to indicate the extent of bacterial growth, and fluorescence was measured using a BioTek plate reader (Synergy H1) with bottom reading mode, excitation at 560 nm, and emission at 590 nm. Data from each 96-well plate were normalized to wells treated with DMSO (considered 100% viability) following background subtraction (medium only). The MIC values were defined as the highest dilution, showing no growth.

**Hemolysis assay**

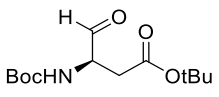
The hemolytic activity of compound **1b** was assessed in triplicate. Red blood cells from defibrillated sheep blood obtained from Thermo Fisher were centrifuged (400 g for 15 min at 4 °C) and washed with PBS containing 0.002% Tween20 (buffer) five times. Then, the red blood cells were normalized to obtain a positive control read-out of 2.5 at 415 nm to stay within the linear range with the maximum sensitivity. A serial dilution of the compounds (64 to 2 µg/mL, 75 µL) was prepared in a 96-well plate. The outer border of the plate was filled with 75 µL buffer, the plate also contained a positive control (0.1% Triton-X final concentration, 75 µL) and a negative control (buffer, 75 µL) in triplicate. The normalized blood cells (75 µL) were added, and the plates were incubated at 37 °C for 1 h while shaking at 500 rpm. A flat-bottom plate of polystyrene with 100 µL buffer in each well was prepared. After incubation, the plate was centrifuged (800 g for 5 min at RT), and 25 µL of the supernatant was transferred to their respective wells in the flat-bottom plate. The values obtained from a read-out at 415 nm were corrected for background (negative control) and transformed to a percentage relative to the positive control (0.1% Triton-X).

**<u>Synthesis methods and analytical data on small molecules</u>**

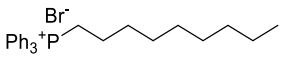



***tert*-butyl (*S*)-3-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (3a):** (*S*)-4-*tert*-Butoxy-2-(*tert*-butoxycarbonylamino)-4-oxobutanoic acid (**2a**) (4 g, 13.83 mmol) was dissolved in anhydrous THF (50 mL) and the solution was cooled to -20°C while stirring under Ar

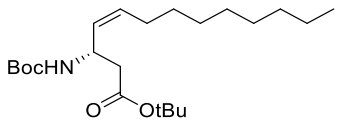
atmosphere before the addition of *N*-methylmorpholine (1.52 mL, 13.83 mmol). After the reaction had been stirred for 10 min, ethyl chloroformate (1.32 mL, 13.83 mmol) was added dropwise, and the reaction was stirred for 1h. The sodium borohydride (1.57 g, 41.48 mmol) was then added, followed by dropwise addition of MeOH (5 mL) over 10 min at -20°C. After 1 hour, the reaction mixture was allowed to warm to RT and was left stirring for another hour before quenching with 1 M HCl (50 mL). The organic solvents were separated and evaporated under reduced pressure, and the residue was combined with the water fraction and extracted with MTBE (2 × 100 mL). The combined organic layer was washed with 1 M HCl (70 mL), water (70 mL), sat. aqueous $NaHCO_3$ (70 mL) and brine (40 mL), then dried over $Na_2SO_4$ and evaporated under reduced pressure. The residue was dissolved in anhydrous DCM (80 mL), and Dess-Martin periodinane (6.45 g, 15.21 mmol) was added at RT to this solution. After 1h, the reaction was completed, as judged by TLC, the solvent was evaporated, and the residue was quenched with a 10% aqueous solution of $Na_2S_2O_3$ (70 mL) and extracted with MTBE (2 × 100 mL). The combined organic layer was washed with a saturated aqueous solution of $NaHCO_3$ (80 mL), water (80 mL), and brine (40 mL), dried over $Na_2SO_4$, filtered, and evaporated to give the crude product that was purified using column chromatography (MTBE/PE = 1:2, $R_f$ = 0.3) which yielded the pure aldehyde **3a** (2.52 g, 68% yield) as a yellow semi-solid. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 9.61 (s, 1H), 5.62 (d, *J* = 7.7 Hz, 1H), 4.34 – 4.25 (m, 1H), 2.87 (dd, *J* = 17.0, 4.8 Hz, 1H), 2.71 (dd, *J* = 17.0, 5.0 Hz, 1H), 1.43 (s, 9H), 1.41 (s, 9H). **$^{13}$C{$^1$H} NMR** (101 MHz, $CDCl_3$) δ 199.6, 170.4, 155.6, 82.1, 80.5, 56.3, 35.9, 28.4, 28.1. Spectral data is in agreement with those reported in the literature.[28]



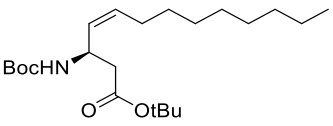

***tert*-butyl (*R*)-3-((*tert*-butoxycarbonyl)amino)-4-oxobutanoate (3b):** The compound was prepared with a similar procedure to that employed for the synthesis of compound **3a** starting from *tert*-butyl (*R*)-3-((*tert*-butoxycarbonyl)amino)-4-hydroxybutanoate (**2b**) (4 g, 13.83 mmol). Yield: 2.6 g, 69%, yellow semi-solid. Spectral data is consistent with the structure and identical to *S*-isomer.



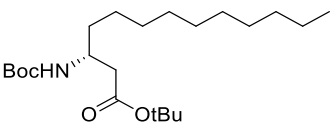

**Nonyltriphenylphosphonium Bromide (5):** To a stirred solution containing triphenylphosphine (4.11 g, 15.7 mmol) in 80 mL of anhydrous MeCN was added 1-bromononane (3 mL, 15.7 mmol) at RT. The reaction mixture was stirred at reflux for 3 days to achieve full conversion of starting materials as judged by $^1$H NMR. The cooled reaction mixture was concentrated and purified via column chromatography (DCM/MeOH = 10:1, $R_f$ = 0.4) to afford compound **5** (5.52 g, 75% yield) as a white solid. Prior to use in the next step, the Wittig reagent was co-evaporated with anhydrous THF. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 7.70 – 7.60 (m, 9H), 7.60 – 7.51 (m, 6H), 3.49 – 3.37 (m, 2H), 1.52 – 1.37 (m, 4H), 1.14 – 0.95 (m, 10H), 0.65 (t, *J* = 6.9 Hz, 3H). **$^{13}$C{$^1$H} NMR** (101 MHz, $CDCl_3$) δ 134.84, 134.81, 133.3, 133.2, 130.3, 130.2, 118.2, 117.4, 31.4, 30.12, 30.0, 28.8, 22.6, 22.23, 22.20, 22.1, 13.8. **$^{31}$P{$^1$H} NMR** (162 MHz, $CDCl_3$) δ 24.5. Spectral data is in agreement with those reported in the literature.[29]




***tert*-butyl (*S*,*Z*)-3-((*tert*-butoxycarbonyl)amino)-tridec-4-enoate (6a):** BuLi (4 mL, 2.5 M in Hexane, 10.00 mmol) was added dropwise to a stirred solution of Wittig reagent **5** (5.03 g, 10.71 mmol) in anhydrous THF (25 mL) at -15°C under Ar atmosphere. After 1 h of stirring at this temperature, the reaction was cooled to -78°C, and the aldehyde **3a** (1.3 g, 4.76 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was allowed to warm up to RT and stirred for an additional hour. The reaction mixture was quenched with saturated aqueous $NH_4Cl$ (25 mL) and extracted with $Et_2O$ (2 × 50 mL). The combined organic phases were washed with 1 M HCl (50 mL), sat. $NaHCO_3$ (50 mL) and brine (30 mL), then dried over $Na_2SO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (MTBE/PE = 1:8, $R_f$ = 0.42) afforded compound **6a** (0.36 g, 20% yield) as a mixture of diastereomers with the ratio 91:9 based on $^1$H-NMR integration. NMR is reported for major isomer. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 5.49 – 5.41 (m, 1H), 5.37 – 5.29 (m, 1H), 5.05 (s, 1H), 4.75 – 4.63 (m, 1H), 2.45 (d, *J* = 5.7 Hz, 2H), 2.22 – 2.02 (m, 2H), 1.42 (d, *J* = 4.1 Hz, 18H), 1.37 – 1.19 (m, 12H), 0.86 (t, 3H). **$^{13}$C{$^1$H} NMR** (101 MHz, $CDCl_3$) δ 170.7, 155.0, 133.1, 128.9, 81.0, 45.2, 41.6, 32.0, 29.7, 29.6, 29.42, 29.41, 28.5, 28.2, 27.8, 22.8, 14.2.

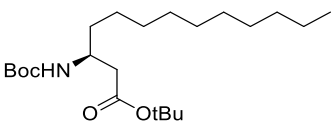



***tert*-butyl (*R*,*Z*)-3-((*tert*-butoxycarbonyl)amino)-tridec-4-enoate (6b):** KHMDS (20.3 mL, 0.5 M in Toluene, 10.14 mmol) was added dropwise to a stirred solution of Wittig reagent **5** (5.1 g, 10.87 mmol) in anhydrous THF (25 mL) at RT under Ar atmosphere. After 1 h, the reaction was cooled to -78°C, and the aldehyde **3b** (1.32 g, 4.83 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was allowed to warm up to RT and stirred for an additional hour. The reaction mixture was quenched with saturated aqueous $NH_4Cl$ (25 mL) and extracted with $Et_2O$ (2 × 50 mL). The combined organic phases were washed with 1 M HCl (50 mL), sat. $NaHCO_3$ (50 mL) and brine (30 mL), then dried over $Na_2SO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (MTBE/PE = 1:8, $R_f$ = 0.42) afforded compound **6b** (1.09 g, 59% yield) as a mixture of diastereomers with the ratio 91:9 based on $^1$H-NMR integration. Spectral data is consistent with the structure and identical to *S*-isomer.

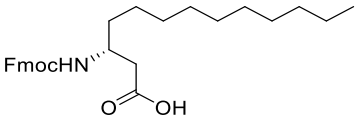



***tert*-butyl (*R*)-3-((*tert*-butoxycarbonyl)amino)-tridecanoate (7a):** A mixture of alkene **6a** (360 mg, 0.94 mmol) and Pd/C (50 mg, 10% Pd on charcoal) in EtOH (5 mL) was stirred under an atmosphere of $H_2$ baloon (1 atm) overnight. After the completion of the reaction, the catalyst was filtered off, the precipitate was washed with EtOH (3 × 10 mL), and the solution was concentrated in vacuo to give compound **7a** (360 mg, 99%) as a colorless oil. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 4.91 (d, *J* = 9.3 Hz, 1H), 3.91 – 3.78 (m, 1H), 2.46 – 2.31 (m, 2H), 1.47 – 1.39 (m, 20H), 1.34 – 1.20 (m, 16H), 0.87 (t, *J* = 6.7 Hz, 3H). **$^{13}$C{$^1$H} NMR** (101 MHz,

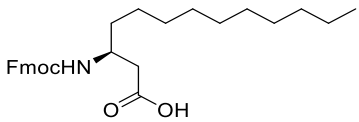
$CDCl_3$) δ 171.3, 155.5, 80.9, 48.0, 40.7, 35.0, 32.0, 29.73, 29.68, 29.54, 29.46, 28.5, 28.2, 26.2, 22.8, 14.2.



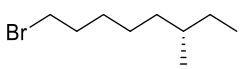

***tert*-butyl (*S*)-3-((*tert*-butoxycarbonyl)amino)-tridecanoate (7b):** The compound was prepared with a similar procedure to that employed for the synthesis of compound **7a** starting from alkene **6b** (1.05 g, 2.74 mmol). Yield: 1.05 g, 99%, colorless oil. Spectral data is consistent with the structure and identical to *R*-isomer.




**(*R*)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)-amino)tridecanoic acid (8a):** The compound **7a** (360 mg, 0.93 mmol) was dissolved in a mixture of TFA/TIPS/$H_2O$ (95:2.5:2.5, 3 mL) and was left stirring for 1h. Then, the reaction mixture was concentrated in vacuo, and the residue was dissolved in a mixture of water/Dioxane (1:1, 10 mL). $Na_2CO_3$ (297 mg, 2.80 mmol) was added to this suspension, and the resulting mixture was cooled to 0°C, followed by a dropwise addition of a solution of Fmoc-OSu (347 mg, 1.03 mmol) in dioxane (2 mL). The reaction mixture was stirred at RT overnight and the next day diluted with water (20 mL) and extracted with $Et_2O$ (25 mL). The aqueous layer was separated, acidified with 1 M HCl till pH 2-3, and extracted with EtOAc (2 × 20 mL). The combined organic layers were washed with water (20 mL), brine (15 mL), dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was triturated with PE (5 mL), and the precipitate was filtered, washed with additional PE (2 × 5 mL), and dried to obtain Fmoc amino acid **8a** (320 mg, 76%) as a white powder. **$[\alpha]_D^{25}$** = +13.1° (c = 1, $CHCl_3$). **$^1$H NMR** (400 MHz, DMSO-$d_6$) δ 12.15 (br s, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.68 (dd, *J* = 7.6, 3.8 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.31 (td, *J* = 7.4, 3.3 Hz, 2H), 7.21 (d, *J* = 8.7 Hz, 1H), 4.34 – 4.16 (m, 3H), 3.82 – 3.70 (m, 1H), 2.41 – 2.20 (m, 2H), 1.44 – 1.33 (m, 2H), 1.28 – 1.14 (m, 16H), 0.82 (t, *J* = 6.6 Hz, 3H). **$^{13}$C{$^1$H} NMR** (101 MHz, DMSO-$d_6$) δ 172.6, 155.6, 144.0, 143.9, 140.8, 127.6, 127.0, 125.2, 120.1, 65.1, 47.8, 46.8, 34.2, 31.3, 29.04, 28.99, 28.8, 28.7, 25.3, 22.1, 14.0. **HRMS** (ESI) *m/z*: $[M+H]^+$ calcd for $C_{28}H_{38}NO_4+H^+$: 452.2795; found: 452.2799.

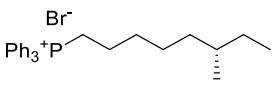



**(*S*)-3-((((9H-fluoren-9-yl)methoxy)carbonyl) amino)tridecanoic acid (8b):** The compound was prepared with a similar procedure to that employed for the synthesis of compound **8a** starting from compound **7b** (1.02 g, 2.645 mmol). Yield: 760 mg, 64% white powder. **$[\alpha]_D^{25}$** = -11.6° (c = 1, $CHCl_3$). **HRMS** (ESI) *m/z*: $[M+H]^+$ calcd for $C_{28}H_{38}NO_4+H^+$: 452.2795; found: 452.2820. Spectral data are consistent with the structure and identical to *R*-isomer.

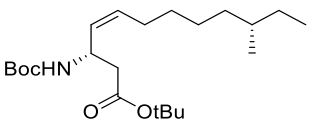



**(*S*)-1-bromo-6-methyloctane (10):** MsCl (350 mg, 3.05 mmol) was added dropwise to the solution of (*S*)-6-methyl octanol (400 mg, 2.77 mmol) and $Et_3N$ (970 mkl, 6.94 mmol) in anhydrous DCM (10 mL) at 5°C under

an Ar atmosphere. After 2 h, the solvent was evaporated, and the residue was diluted with water (10 mL) and extracted with $Et_2O$ (40 mL). The organic layer was separated and washed with 1 M HCl (10 mL), sat. aq. $NaHCO_3$ (10 mL), water (10 mL), brine (10 mL), then dried over $Na_2SO_4$, filtered, and concentrated in vacuo to afford the corresponding mesylate that was then dissolved in THF (10 mL). LiBr (962 mg, 11.08 mmol) was added to this solution, and the reaction was refluxed overnight. The next day, the solvent was evaporated, and the residue was diluted with water (10 mL) and extracted with $Et_2O$ (25 mL). The organic layer was washed with sat. aq. $NaHCO_3$ (10 mL), water (10 mL), brine (10 mL), then dried over $Na_2SO_4$, filtered, and concentrated in vacuo to afford bromide **10** (493 mg, 86% yield) as a colorless liquid. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 3.41 (t, $J$ = 6.9 Hz, 2H), 1.86 (p, $J$ = 7.0 Hz, 2H), 1.47 – 1.21 (m, 7H), 1.20 – 1.02 (m, 2H), 0.90 – 0.81 (m, 6H). **$^{13}$C{$^1$H} NMR** (101 MHz, $CDCl_3$) δ 36.5, 34.4, 34.2, 33.0, 29.6, 28.7, 26.4, 19.3, 11.5. Spectral data is in agreement with those reported in the literature.[37]

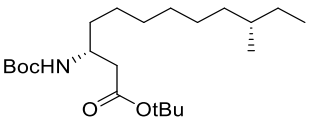



**(*S*)-6-methyloctyltriphenylphosphonium bromide (11):** To a stirred solution containing triphenylphosphine (625 mg, 2.38 mmol) in 20 mL of anhydrous MeCN was added bromide **10** (493 mg, 2.38 mmol) at RT. The reaction mixture was stirred at reflux for 3 days to achieve full conversion of starting materials as judged by NMR. The cooled reaction mixture was concentrated in vacuo, dissolved in a minimum amount of MeCN (2 mL), and crushed out from MTBE (20 mL). The liquid layer was decanted, and the precipitate was dried to afford compound **11** (1g, 90% yield) as a white solid. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 7.90 – 7.75 (m, 12H), 7.73 – 7.63 (m, 6H), 3.85 – 3.74 (m, 2H), 1.61 (d, $J$ = 5.6 Hz, 4H), 1.31 – 1.12 (m, 5H), 1.10 – 0.93 (m, 2H), 0.83 – 0.73 (m, 6H). **$^{13}$C{$^1$H} NMR** (101 MHz, $CDCl_3$) δ 135.09, 135.06, 133.9, 133.8, 130.7, 130.5, 119.0, 118.2, 36.2, 34.4, 30.9, 30.8, 29.5, 26.9, 23.2, 22.83, 22.79, 22.65, 19.3, 11.5. **$^{31}$P{$^1$H} NMR** (162 MHz, $CDCl_3$) δ 25.0.

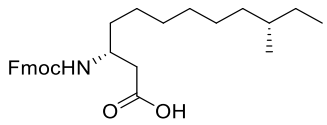



***tert*-butyl (3*S*,10*S*,*Z*)-3-((*tert*-butoxycarbonyl)amino)-10-methyldodec-4-enoate (12):** KHMDS (1.83 mL, 0.5 M in Toluene, 0.915 mmol) was added dropwise to a stirred solution of Wittig reagent **11** (473 mg, 1.01 mmol) in anhydrous THF (10 mL) at RT under Ar atmosphere. After 1 h, the reaction was cooled to -78°C, and the aldehyde **3a** (250 mg, 0.915 mmol) in anhydrous THF (2 mL) was added dropwise. The reaction was allowed to warm up to RT over 1 h and stirred for an additional hour. The reaction mixture was then quenched with saturated aqueous $NH_4Cl$ (15 mL) and extracted with $Et_2O$ (2 × 30 mL). The combined organic phases were washed with 1 M HCl (30 mL), sat. $NaHCO_3$ (30 mL), and brine (10 mL), then dried over $Na_2SO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (MTBE/PE = 1:8, Rf = 0.43) afforded compound **12** (200 mg, 57% yield) as a mixture of diastereomers with the ratio 92:8. NMR is reported for major isomer. **$^1$H NMR** (400 MHz, $CDCl_3$) δ 5.48 – 5.36 (m, 1H), 5.36 – 5.25 (m, 1H), 5.16 – 4.95 (m, 1H), 4.66 (p, $J$ = 6.6 Hz, 1H), 2.42 (d, $J$ = 5.7 Hz, 2H), 2.19 – 2.00 (m, 2H), 1.46 – 1.35 (m, 18H), 1.34 – 1.17

(m, 7H), 1.13 – 0.99 (m, 2H), 0.85 – 0.77 (m, 6H). **$^{13}C\{^1H\}$ NMR** (101 MHz, $CDCl_3$) δ 170.7, 155.0, 133.0, 132.0, 128.8, 80.9, 45.2, 41.5, 36.6, 34.4, 30.0, 29.5, 28.5, 28.1, 27.8, 26.8, 19.3, 11.5.




***tert*-butyl (3*R*,10*S*)-3-((*tert*-butoxycarbonyl)amino)-10-methyldodecanoate (13):** The compound was prepared with a similar procedure to that employed for the synthesis of compound **7a** starting from alkene **12** (200 mg, 0.52 mmol). Yield: 200 mg, 99%, colorless oil. **$^1H$ NMR** (400 MHz, $CDCl_3$) δ 4.92 (d, *J* = 9.4 Hz, 1H), 3.92 – 3.79 (m, 1H), 2.45 – 2.30 (m, 2H), 1.48 – 1.37 (m, 20H), 1.35 – 1.17 (m, 11H), 1.16 – 1.01 (m, 2H), 0.88 – 0.78 (m, 6H). **$^{13}C\{^1H\}$ NMR** (101 MHz, $CDCl_3$) δ 171.2, 155.5, 80.9, 79.1, 47.9, 40.7, 36.7, 35.0, 34.5, 30.0, 29.59, 29.56, 28.5, 28.2, 27.1, 26.2, 19.3, 11.5.




**(3*R*,10*S*)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)-amino)-10-methyldodecanoic acid (14):** The compound was prepared with a similar procedure to that employed for the synthesis of compound **8a** starting from compound **13** (200 mg, 0.52 mmol). Yield: 140 mg, 60%. **$[\alpha]_D^{25}$** = +12.7° (c = 1, $CHCl_3$). **$^1H$ NMR** (400 MHz, $CDCl_3$) δ 9.37 (br s, 1H), 7.75 (d, *J* = 7.6 Hz, 2H), 7.59 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.90 (d, *J* = 8.9 Hz, 0.25H, minor rotamer), 5.19 (d, *J* = 9.1 Hz, 0.75H, major rotamer), 4.65 – 4.34 (m, 2H), 4.22 (t, *J* = 6.9 Hz, 1H), 4.05 – 3.92 (m, 0.75H, major rotamer), 3.81 – 3.69 (m, 0.25H, minor rotamer), 2.69 – 2.51 (m, 1.5H, major rotamer), 2.45 – 2.28 (m, 0.5H, minor rotamer), 1.62 – 1.17 (m, 13H), 1.17 – 1.03 (m, 2H), 0.89 – 0.81 (m, 6H). **$^{13}C\{^1H\}$ NMR** (101 MHz, $CDCl_3$) δ 177.1, 156.1, 144.1, 144.0, 141.4, 127.8, 127.2, 125.21, 125.17, 120.1, 67.5, 66.8, 48.9, 48.2, 47.4, 39.0, 36.7, 34.5, 30.00, 29.6, 29.5, 27.1, 26.3, 19.3, 11.5. **HRMS** (ESI) *m/z*: $[M+H]^+$ calcd for $C_{28}H_{38}NO_4+H^+$: 452.2795; found: 452.2820.

**Detailed procedures for solid-phase peptide synthesis of the compound 1b**

**Resin loading**

2-Chlorotrityl chloride resin (2-CTCl) (4 g, 1.51 mmol/g) was loaded by coupling via the free carboxyl group of Fmoc-D-Glu(OtBu)-OH (5.14 g, 12.08 mmol, 2 eq.) with DIPEA (5.26 mL, 30.2 mmol, 5 eq.) in 70 mL of DCM. After 3 h at RT, an extra amount of DIPEA (5.26 mL, 30.2 mmol, 5 eq.) and 24 mol of MeOH were added, and the reaction was left shaking for another 15 minutes. The resin was then filtered, washed with DMF, EtOH, DCM, $Et_2O$, and dried overnight under a stream of $N_2$. The resin loading was then determined to be 0.555 mmol/g.

**Automated solid-phase peptide synthesis**

A CEM Liberty Blue automated peptide synthesizer with microwave irradiation was used to perform SPPS. Synthesis was performed on a 0.05 mmol scale using the following

system: 5 eq. HBTU (0.25 M in DMF), 10 eq. DIPEA (0.5 M in DMF), 5 eq. of amino acid (0.2 M in DMF). Fmoc group removal was performed using Piperazine/EtOH/NMP (1:1:9, m/v/v). A detailed overview of the automated protocols can be found below.

**Resin swelling**

The resin was swollen in 10 mL of DMF for 300 s prior to the first coupling.



***Protocol 1: Standard Coupling protocol***

| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 50 s at 25°C then 250 s at 50°C |
| **2** | Wash (DMF) | RT |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |
| **5** | Coupling amino acid | 150 s at 25°C then 750 s at 50°C |

***Protocol 2: Final deprotection N-terminus***

| Step | Function | Duration/Temperature |
|---|---|---|
| **1** | Deprotection N-terminus | 50 s at 25°C then 250 s at 50°C |
| **2** | Wash (DMF) | RT |
| **3** | Wash (DMF) | RT |
| **4** | Wash (DMF) | RT |

**Procedure for the synthesis of the compound 1b**

2-CT resin loaded with Fmoc-D-Glu(tBu)-OH was transferred into a CEM Liberty Blue µwave peptide synthesizer at a 0.05 mmol scale. The amino acids were coupled using standard coupling protocol 1 in the following order: 1) Fmoc-Tyr(tBu)-OH; 2) Fmoc-Phe-OH; 3) Fmoc-D-Ala-OH; 4) Fmoc-D-Trp(Boc)-OH; 5) Fmoc-D-Lys(Boc)-OH; 6) Fmoc-Ser(tBu)-OH followed by a final deprotection using protocol 2. The resin was then transferred to a manual reactor for the SPPS connected to nitrogen flow to perform the last coupling of the unnatural amino acid X. After the resin was swollen in DMF (7 mL) for 1 min, protected amino acid **12** (45.16 mg, 0.1 mmol), HBTU (37.92 mg, 0.1 mmol) and DIPEA (35.6 mkl, 0.2 mmol) were added. After bubbling with $N_2$ for 3h, the resin was filtered, washed with DMF (2 × 7 mL), and then treated with 7 mL of Piperazine/EtOH/NMP (1:1:9, m/v/v) for 20 min, followed by washing with DMF (2 × 7 mL), DCM (2 × 7 mL). The peptide was then detached from the resin using 7 mL of the 20% HFIP in DCM and filtered. Filtrate was collected, and the solvents were removed by rotary evaporation, yielding the protected linear peptide, which was used directly in the next step.

Dry DMF (5 mL) was poured into the round-bottom flask containing a stirring bar, protected peptide (0.5 mmol), and Oxyma (43 mg, 0.3 mmol), followed by the addition of dry DCM (55 mL). The DIC (47 μL, 0.3 mmol) was added to the resulting mixture, and the reaction was left stirring overnight under an Ar atmosphere. The next day, the solvents were evaporated under reduced pressure, and the residue was diluted with water (20 mL) and extracted with EtOAc (2 × 30 mL). The combined organic layers were washed with 1 M $NaHCO_3$ (25 mL), water (25 mL), brine (20 mL), dried under $Na_2SO_4$, and concentrated using rotary evaporation. Final sidechain deprotection was carried out by treating the obtained powder with 3 mL of the TFA/TIPS/$H_2O$ (95:2.5:2.5) mixture for 2.5 h. The reaction mixture was precipitated in MTBE/PE (1:1) and centrifuged (4500 rpm, 5 min). The pellet was then resuspended in MTBE/PE (1:1) and centrifuged again (4500 rpm, 5 min). Finally, the pellet containing the crude peptide was dissolved in 10 mL of 2% AcOH in the $H_2O$/MeCN (70:30) and left at 40°C for 40 min, after which the solution was allowed to cool down to RT.

Peptide was purified via preparative HPLC using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 μm) and equipped with an ECOM Flash UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95:5; solvent B, 0.1 % TFA in water/acetonitrile 5:95. Gradient elution was as follows: 70:30 (A/B) for 3 min, 70:30 to 0:100 (A/B) over 49 min, 0:100 (A/B) for 4 min, then reversion back to 70:30 (A/B) over 1 min, 70:30 (A/B) for 3 min. The fractions containing the product were combined and lyophilized to obtain compound **1b** as a white fluffy powder (23 mg, 37% yield, calcd for 1×TFA salt) with >95% purity as determined by HPLC.

**HRMS** (ESI) *m/z*: $[M+2H]^{2+}/2$ calcd for $C_{59}H_{82}N_{10}O_{12}+2H^+$: 562.3130; found: 562.3134.

# Supplementary information

**Table S1.** Retention times ($t_R$, min) of the FDAA derivatives for the fatty β-amino acid (FAA) derived from authentic paenilipoheptin and compounds **7a**, **7b**, **13**. Marfey's adduct made from compound **13** showed the same retention time as the one derived from the natural product.

| Amino acid | $[M+H]^+$ | $t_R$, min | | | | |
|---|---|---|---|---|---|---|
| | | **7a** (L-FDAA) | **7b** (L-FDAA) | **13** (L-FDAA) | **13** (D-FDAA) | Paenilipoheptin A (L-FDAA) |
| **FAA** | 482.2609 | 10.35 | 9.70 | 10.17 | 9.53 | 10.17 |

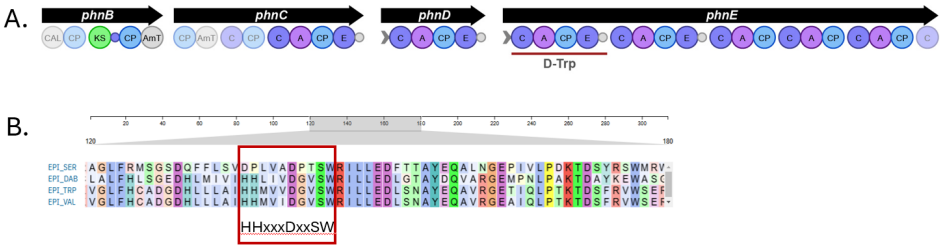



**Figure S1**. A. Module architecture of NRPS enzymes involved in the biosynthesis of paenilipoheptin A. Note that module with predicted substrate specificity of A domains for Trp contains epimerization (E) domain, indicating D-configuration. B. Clustal2 multiple sequence alignments of the epimerization domains of the paenilipoheptin A BGC from *Paenibacillus* sp. JJ-21. This revealed that the conserved active site motif HHxxxD of Trp is intact. Therefore, we believe that the epimerization (E) domain detected in this module is functional.

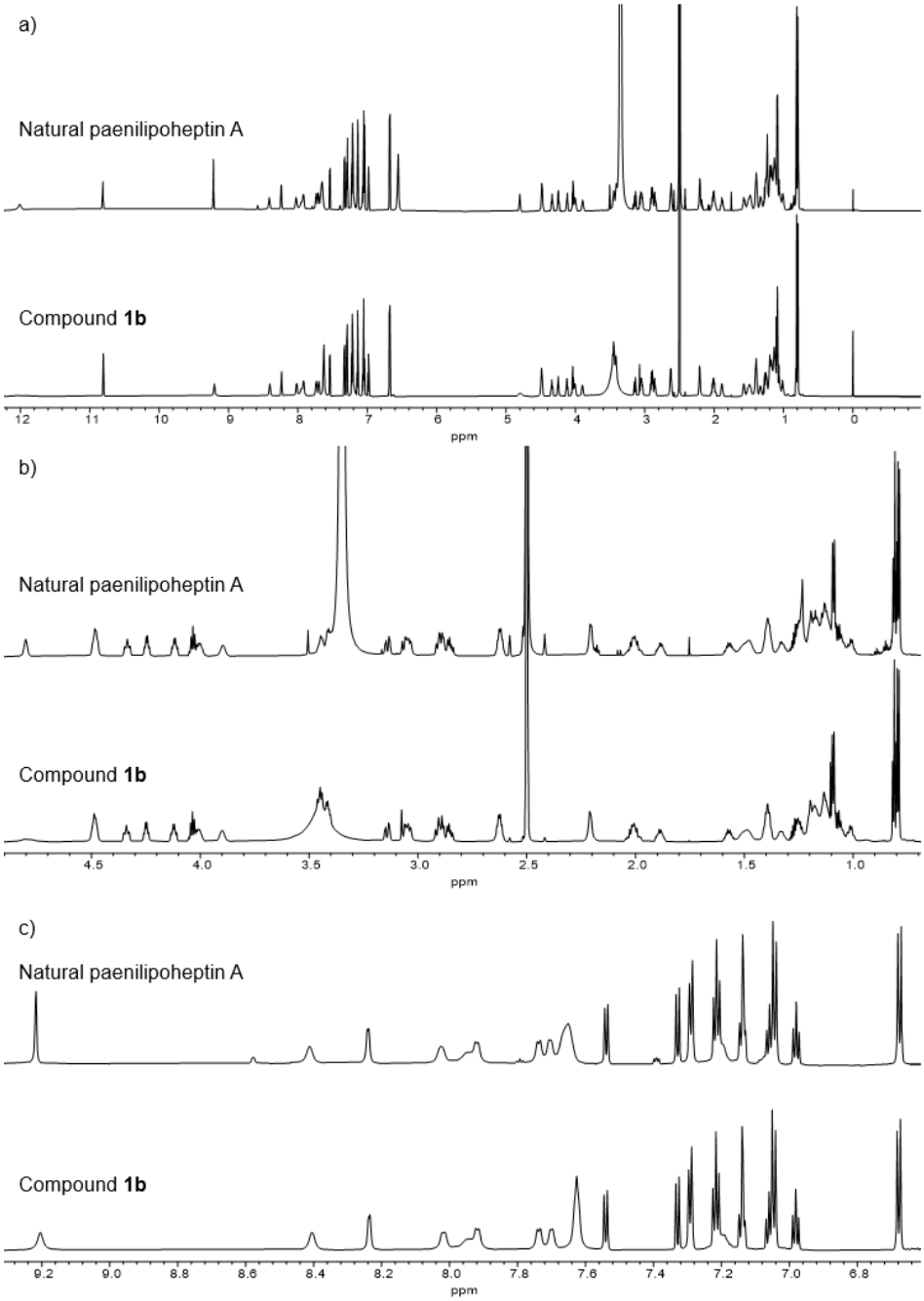



**Figure S2.** NMR comparison of the $^{1}$H-NMR (850 MHz, DMSO-$d_6$) spectrum of natural paenilipoheptin A isolated after fermentation of the producing organism overlaid with $^{1}$H-NMR (850 MHz, DMSO-$d_6$) spectra of synthetic compound **1b**: a) full spectrum, 12.5 – -1 ppm region; b) aliphatic protons region, 4.9 – 0.7 ppm; c) aromatic/amide protons region, 9.3 – 6.6 ppm.

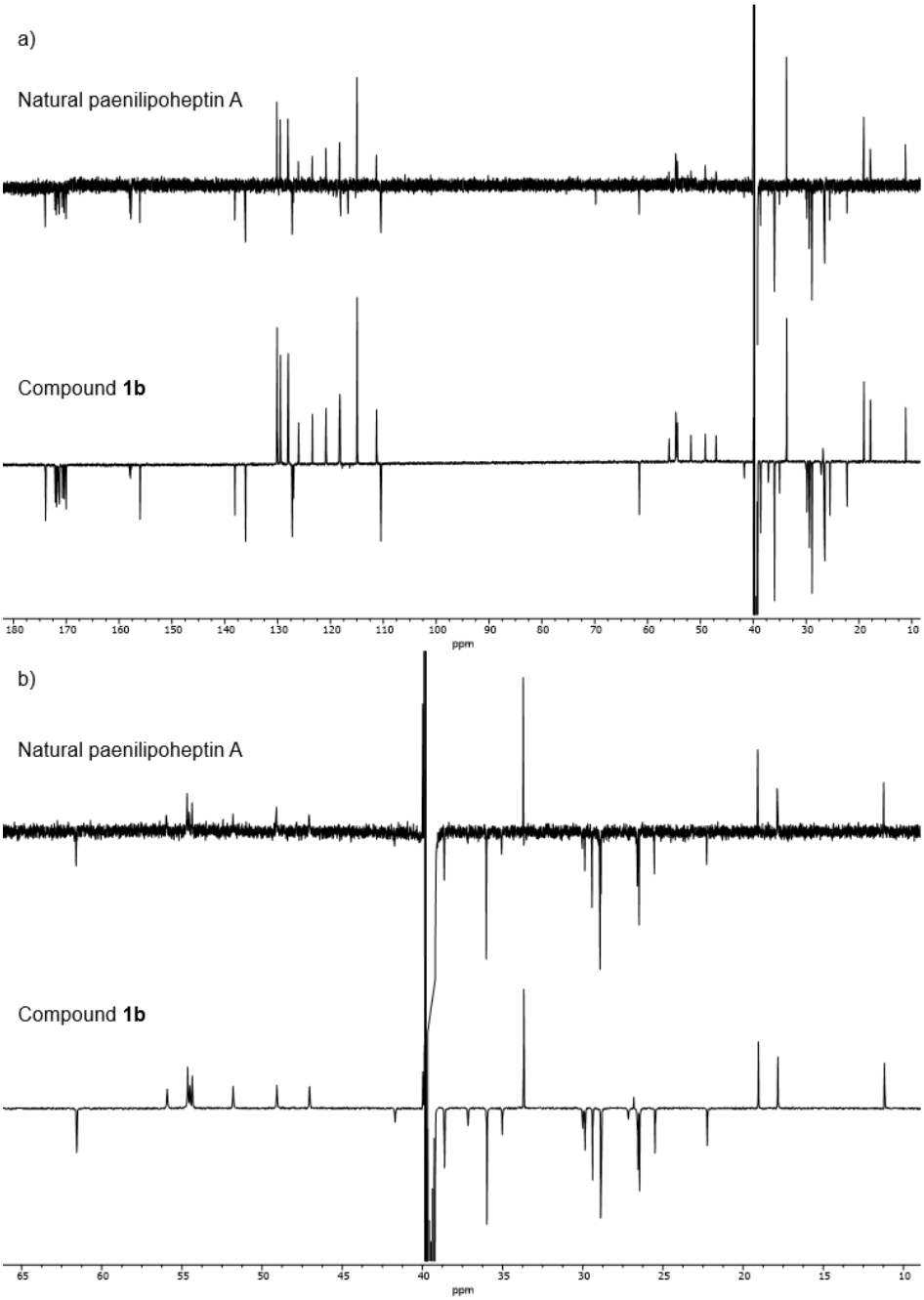



**Figure S3.** NMR comparison of the $^{13}$C-NMR (850 MHz, DMSO-$d_6$) spectrum of natural paenilipoheptin A isolated after fermentation of the producing organism overlaid with $^{13}$C-NMR (850 MHz, DMSO-$d_6$) spectra of synthetic compound **1b**: a) full spectrum, 180 – 10 ppm region; b) aliphatic carbons region, 65 – 10 ppm

**Table S2.** MICs determined for the natural paenilipoheptin A, compound **1b**, and control antibiotics.

| Microorganism/strain tested | MIC (µg/mL) | | | | |
|---|---|---|---|---|---|
| | Paenilipoheptin A | **1b** | Vancomycin/ Polymyxin B | Iturin A | Nystatin |
| **Gram-positive bacteria**[1] | | | | | |
| *B. subtilis* 168 | 16 | 8 | 0.25 | ND | ND |
| *E. faecium* E980 | 16 | 8 | 0.5 | ND | ND |
| *E. faecium* VRE E155 (VanA) | ND | 8 | >128 | ND | ND |
| *E. faecium* VRE E7314 (VanB) | ND | 8 | >128 | ND | ND |
| *S. aureus* ATCC 29213 | 16 | 16 | 0.5 | ND | ND |
| *S. aureus* USA 300 (MRSA) | 16 | 16 | 0.5 | ND | ND |
| *S. aureus* VRS3b | ND | 16 | >128 | ND | ND |
| *S. aureus* LIM-2 | ND | 32 | 4 | ND | ND |
| *Paenibacillus* sp. JJ-21 | ND | 64 | 0.25 | ND | ND |
| **Gram-negative bacteria**[2] | | | | | |
| *E. coli* ATCC 25922 | ND | >64 | 2 | ND | ND |
| *E. coli* BW 25113 *ΔbamB ΔtolC* | ND | >64 | 1 | ND | ND |
| *K. pneumoniae* ATCC 13883 | ND | >64 | 1 | ND | ND |
| *A. baumannii* ATCC 19606 | ND | >64 | 1 | ND | ND |
| *P. aeruginosa* ATCC 27853 | ND | >64 | 2 | ND | ND |
| **Mycobacteria** | | | | | |
| *M. tuberculosis* H37Rv | ND | >64 | ND | ND | ND |
| *M. smegmatis* mc2155 | ND | >64 | ND | ND | ND |
| *M. abscessus* ATCC 19977 | ND | >64 | ND | ND | ND |
| **Fungal species** | | | | | |
| *S. cerevisiae* NSY220.1 | ND | >64 | ND | 32 | 32 |
| *C. albicans* ATCC 10231 | ND | >64 | ND | 32 | 32 |
| *P. expansum* ATCC 24692 | ND | >64 | ND | 16 | 4 |
| *A. fumigatus* Af293 | ND | >64 | ND | 64 | 32 |
| *A. niger* N400 | ND | >64 | ND | 16 | 16 |
| *A. oryzae* RIB40 | ND | >64 | ND | 64 | 4 |
| *F. oxysporum* CBS 101587 | ND | >64 | ND | 64 | 32 |

ND – not determined;
[1] Vancomycin used as a control
[2] Polymyxin B used as a control

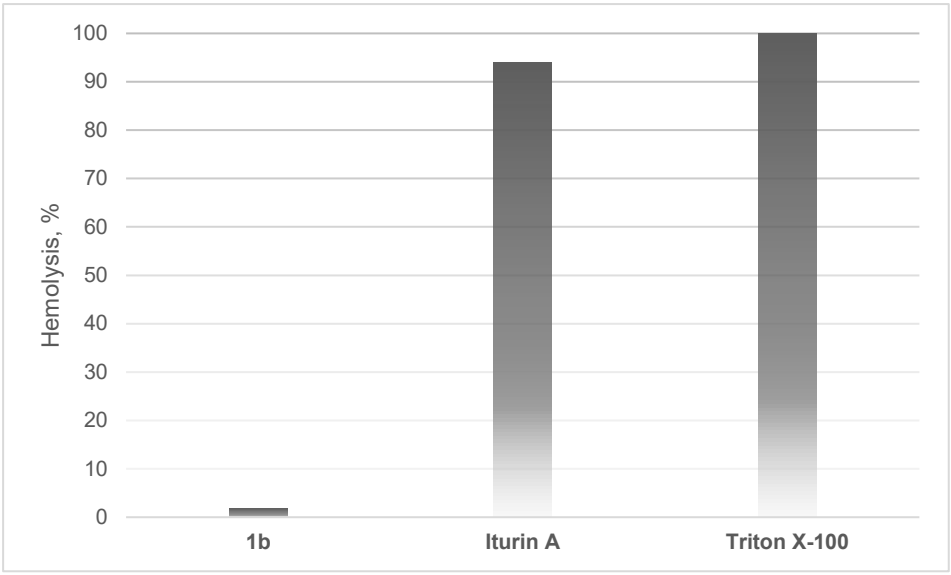



**Figure S4.** Hemolysis data for the compound 1b and Iturin A at a concentration of 64 µg/mL after 1 hour. Percentage of the hemolysis: 1b – 1.9%, Iturin A – 93.9% in comparison to Triton X-100 (100%). The obtained values were used as an average based on n = 3 technical replicates.

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