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Studies on Tridecaptin B_1, a Lipopeptide with Activity Against Multidrug Resistant Gram-Negative Bacteria

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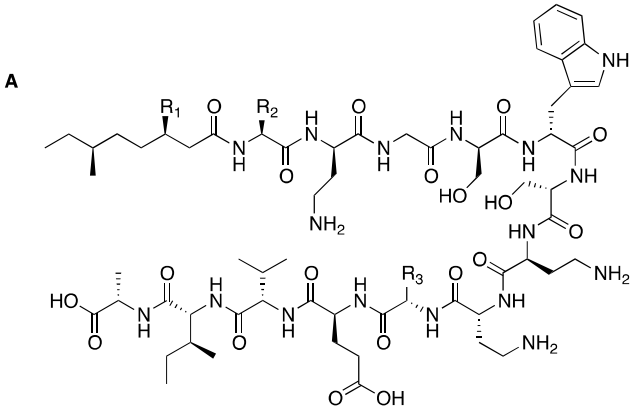
Previously other groups had reported that *Paenibacillus polymyxa* NRRL B-30507 produces SRCAM 37, a type IIA bacteriocin with antimicrobial activity against *Campylobacter jejuni*. Genome sequencing and isolation of antimicrobial compounds from this *P. polymyxa* strain show that the antimicrobial activity is due to polymyxins and tridecaptin B_1. The complete structural assignment, synthesis, and antimicrobial profile of tridecaptin B_1 is reported, as well as the putative gene cluster responsible for its biosynthesis. This peptide displays strong activity against multidrug resistant Gram-negative bacteria, a finding that is timely to the current problem of antibiotic resistance.

Introduction

Recently attention has focused on the problem of antibiotic resistance: about half of hospital-acquired infections are resistant to conventional therapy with the USA reporting 23,000 deaths per year at a direct cost of $20 billion.[1] If left unchecked, a post-antibiotic age may occur in which routine operations could become life-threatening procedures.[2] Compounding this problem is the dearth in new structural classes of antibiotics, with only four (linezolid, daptomycin, fidaxomicin and bedaquiline) entering the market in the past 40 years.[3,4] Of particular concern is that none of these classes are active against Gram-negative bacteria. Even the recently discovered "resistance-proof" antibiotic teixobactin is only active against Gram-positive organisms.[5] The 2014 antimicrobial resistance global report by the World Health Organisation indicates that multidrug resistant (MDR) strains of *Escherichia coli* and *Klebsiella pneumoniae* are emerging, against which many conventional antibiotics are ineffective.[6] In particular, carbapenem resistant *K. pneumoniae* infections have a mortality rate of >50% and are resistant to fluoroquinolones, aminoglycosides and all available β-lactams.[7]

Non-ribosomally synthesised lipopeptides are an attractive class of antibiotic candidates for targeting multidrug resistant organisms.[8] These compounds typically kill bacteria by membrane disruption, against which the development of resistance is limited.[9] The tridecaptins are a class of linear cationic lipopeptides, containing 13 amino acids and N-terminally acylated with a fatty acid chain.[10-13] These peptides are composed of a combination of L- and D-amino acids and display good stability to human plasma.[14] Furthermore, they are active against several Gram-negative strains, while showing low cytotoxicity and haemolytic activity.[15]

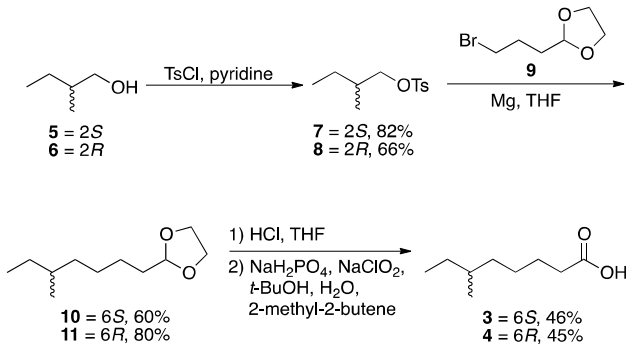
Our group recently isolated tridecaptins, non-ribosomal peptide synthetase (NRPS) products while searching for anti-



TriA$_1$: R_1 = OH, R_2 = $CH(CH_3)_2$, R_3 = Bn
TriB$_1$: R_1 = H, R_2 = H, R_3 = $CH((S)\text{-}CH_3)C_2H_5$

Figure 1. (A) Structure of tridecaptin B_1 and tridecaptin A_1; (B) proposed amino acid sequence of SRCAM 37.

Campylobacter bacteriocins (ribosomally synthesised peptides) reported to be produced by *Paenibacillus terrae* NRRL B-30644 and *Paenibacillus polymyxa* NRRL B-30509.[16,17] The active compounds were determined by our group to be tridecaptin A_1 (Figure 1), and tridecaptins A_3 and A_4, respectively.[12,18] We subsequently turned our attention to another reported anti-*Campylobacter* bacteriocin, SRCAM 37, produced by *Paenibacillus polymyxa* NRRL B-30507.[16] The proposed sequence of SRCAM 37 (Figure 1B) contains the characteristic YGNGV sequence associated with class IIa bacteriocins, but lacks the cysteines typically involved in a disulphide bond in this class of peptides. Our genome sequencing of this organism revealed no genetic determinants for this proposed sequence.



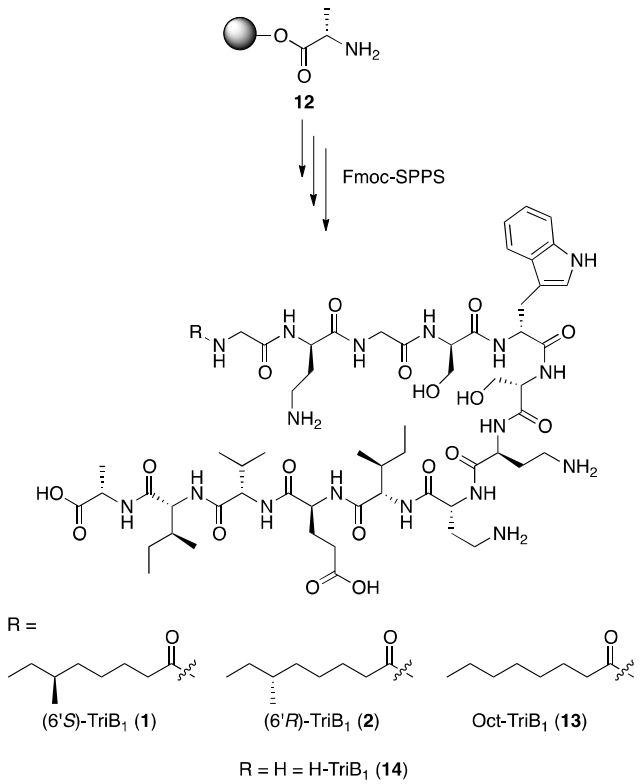
Scheme 1. Synthesis of (6*S*)- and (6*R*)-methyloctanoic acid.

Furthermore, there is a 252 Da discrepancy between the reported mass of SRCAM 37 and the mass of the proposed sequence. Herein we report the discovery of a new tridecaptin variant, tridecaptin B_1 ($TriB_1$) (Figure 1A), which displays antimicrobial activity against Gram-negative bacteria, including MDR strains of *K. pneumoniae* and *Acinetobacter baumannii*. Complete structural elucidation of this peptide was performed by a combination of chemical synthesis and degradative analysis. Genome sequencing and genetic analysis were also used to identify the gene cluster responsible for the biosynthesis of tridecaptin B_1.

Results and Discussion

Isolation and characterisation of tridecaptin B_1

The compounds produced by *P. polymyxa* NRRL B-30507 inhibitory to Gram-negative bacteria were identified using an activity guided purification approach. The supernatant from a 1L overnight culture was fractionated on Amberlite XAD 16 resin, resulting in a single fraction with activity against the Gram-negative indicator strain *Escherichia coli*. Further purification by C_{18} solid-phase extraction cartridge and C_{18}-HPLC yielded three fractions active against *E. coli* (see supporting information S2). The first two fractions to elute on HPLC were identified as polymyxin B_1 and B_2 by tandem mass spectrometry (MS/MS) (see supporting information S3/S4). These polymyxins are known to be effective against most Gram-negative organisms, but show limited antimicrobial activity against *Campylobacter jejuni*.[19] The third fraction, which was also the most abundant, showed an $[M+H]^+$ signal of 1458.6 by MALDI-MS. MS/MS analysis of this peak suggested that it had a sequence similar to tridecaptin A_1 ($TriA_1$). From this information it was not possible to determine whether residue 9 was leucine or isoleucine. The identity of the N-terminal residue and structure of the lipid tail also remained elusive, therefore complete sequence assignment was achieved using TOCSY and NOESY experiments. After ordering and identification of the spin systems (see supporting information S5), residue 9 was identified as isoleucine, whereas the N-terminal residue was found to be glycine. This amino acid sequence bears similarities to previously reported tridecaptin B_α, however this variant has a C-terminal serine.[11] We therefore designated this antimicrobial peptide as tridecaptin B_1. In combination with ESI-HRMS and MS/MS, the NMR data suggested that the lipid tail was 6-methyloctanoic acid. Anteiso fatty acids are typically derived from L-isoleucine, resulting in an

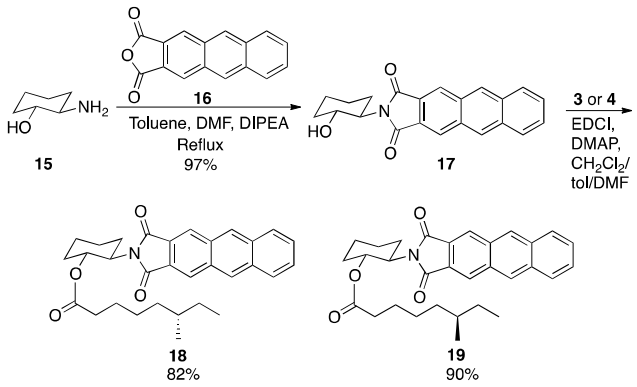


Scheme 2. Fmoc-SPPS of tridecaptin B_1 analogues.

S-configuration at the chiral methine, however this needed to be confirmed.[20] We have previously had success in the identification of the stereochemical components of N-terminal lipid tails by synthesising peptide diastereomers and comparing these to the natural peptide by HPLC and NMR. We therefore sought to synthesise both (6'*S*)-methyloctanoyl tridecaptin B_1 ((6'*S*)-$TriB_1$) (**1**) and (6'*R*)-methyloctanoyl tridecaptin B_1 ((6'*R*)-$TriB_1$) (**2**) for this purpose.

Lipid tail and peptide synthesis

The synthesis of (6'*R*)-$TriB_1$ and (6'*S*)-$TriB_1$ first required the preparation of enantiomerically pure (6*S*)-methyloctanoic acid (**3**) and (6*R*)-methyloctanoic acid (**4**). There have been two previously reported syntheses of these compounds, with the (6*S*)-acid synthesised in 7 steps from (*R*)-(+)-β-citronellol and the (6*R*)-acid in 9 steps from an Evans Auxilary.[21] We devised a more efficient synthesis to yield the desired acids in 4 steps from commercially available (2*S*)-methylbutanol (**5**) and synthetic (2*R*)-methylbutanol (**6**) (Scheme 1). (2*R*)-Methylbutanol (**6**) was synthesized in 3 steps using a previously reported literature procedure (see supporting information S6).[21] Tosylation of alcohols **5** and **6**, followed by a Grignard reaction with the commercially available bromo dioxolane **9** gave dioxolanes **10** and **11** in good yields. Subsequent acetal deprotection, followed by Pinnick oxidation of the resultant aldehydes, gave (6*S*)-methyloctanoic acid (**3**) and (6*R*)-methyloctanoic acid (**4**) in moderate yields. With the enantiomerically pure acids available, (6'*S*)-$TriB_1$ (**1**) and (6'*R*)-$TriB_1$ (**2**) were synthesised from H-Ala-2-chloro-2-trityl resin (**12**) using standard Fmoc solid-phase peptide synthesis (SPPS) (Scheme 2).[22] Oct-$TriB_1$ (**13**) and H-



Scheme 3. Synthesis of (6*S*)- and (6*R*)-anthracenyl derivatives.

$TriB_1$ (**14**) were also synthesised for comparison of antimicrobial activities.

Elucidation of lipid tail stereochemistry

Natural $TriB_1$ was co-injected with each of the synthetic standards on to an analytical C_{18} HPLC column. Both natural $TriB_1$ + (6'*S*)-$TriB_1$ and natural $TriB_1$ + (6'*R*)-$TriB_1$ eluted as a single peak (see supporting information S8) therefore we turned to NMR analysis to elucidate the stereochemistry of the anteiso methyl group. Unfortunately, the ^{1}H-NMR and TOCSY spectra of both synthetic isomers were identical in D_2O and d_6-DMSO. Although this method was successfully employed for the structural elucidation of $TriA_1$,[12] that peptide contains both an N-terminal valine and a β-hydroxy group. It is likely that this additional chiral character closer to the anteiso methyl group was responsible for the difference in NMR chemical shifts. We therefore sought an alternative method for identification of the stereochemistry of the lipid tail.

Previous efforts by our group to separate similar compounds by chiral GCMS have been unsuccessful, a difficulty that has also been observed by others.[23] Acid hydrolysis of the lipid tail from $TriB_1$, followed by derivatisation with a chiral reagent could allow determination of the lipid tail stereochemistry. However, due to the low biological production levels of $TriB_1$ (~1mg/L) detection may be an issue as the lipid tail accounts for only 10% of the total peptide mass. Also, derivatisation at the carboxylate would place the anteiso methyl group at least 7 atoms away from any chiral centre. A literature search identified the Ohrui-Akasaka method, wherein long chain chiral fatty acids are derivatised with anthracenyl derivative **17**.[24] The 100% chiral *gauche* conformation of the resultant esters places methyl groups at stereogenic centres in close proximity to the anthracene ring, thereby allowing separation of diastereomers by low temperature HPLC (-55 °C). The fluorescent anthracenyl group also allows for easy detection of small amounts of material. Cyclohexanol derivative **17** was synthesized in 97% yield from 2,3-anthracenedicarboxylic acid anhydride (**16**) and (1*R*,2*R*)-2-aminocyclohexanol (**15**) (Scheme 3). (6*S*)-methyloctanoic acid (**3**) and (6*R*)-methyloctanoic acid (**4**) were then derivatised with **17** to yield anthracenyl derivatives **18** and **19** in good yields. Gratifyingly, the (6*S*) and (6*R*) esters were easily distinguishable by comparison of the methyl shifts in their respective ^{1}H-NMR spectra (Figure 2). Hence, the natural peptide $TriB_1$ was

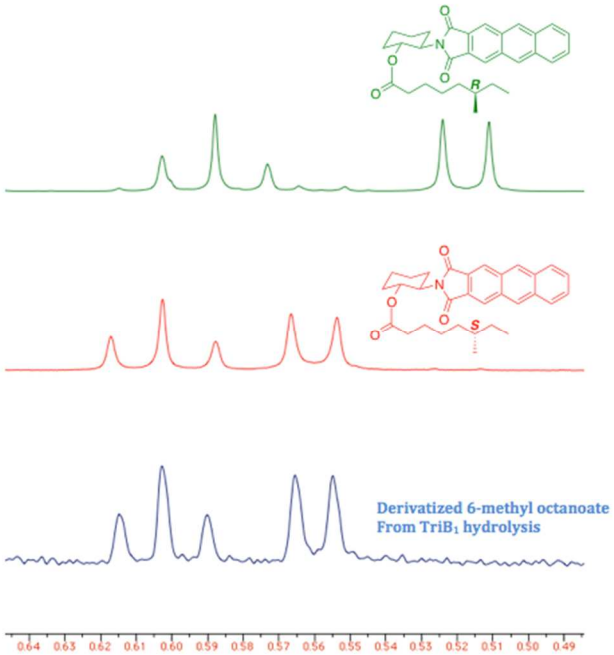


Figure 2. ^{1}H-NMR chemical shift (ppm) comparison of anthracene derivatives **18** and **19** and the corresponding derivative of 6-methyloctanoate obtained by hydrolysis of tridecaptin B_1.

hydrolyzed at 90 °C in 6M HCl for 2 hours, and its lipid tail as a carboxylic acid was extracted into diethyl ether. The crude acid was then coupled with alcohol **17** and the resulting ester compared with the (6*S*)-ester **18** and (6*R*)-ester **19** by ^{1}H-NMR (Figure 2) and low-temperature HPLC (see supporting information S9). This result clearly shows that the N-terminal lipid of tridecaptin B_1 is (6*S*)-methyloctanoic acid.

Antimicrobial testing of tridecaptin B_1 analogues

Having synthesized both lipid tail diastereomers of $TriB_1$, we decided to investigate if the lipid tail stereochemistry had any effect on antimicrobial activity. (6'*S*)-$TriB_1$ (**1**) and (6'*R*)-$TriB_1$ (**2**) were tested against a panel of Gram-positive and Gram-negative bacteria (Table 1). Oct-$TriB_1$ (**13**), wherein the chiral lipid tail has been substituted with octanoic acid, was also synthesized and tested. Replacement of the chiral lipid tail with a cheap, commercially available alternative would simplify the synthesis of $TriB_1$ analogues. Our group previously reported that a non-antimicrobial derivative of $TriA_1$ missing the lipid tail, H-$TriA_1$, acts as a sensitizer of the outer-membrane of Gram-negative bacteria, increasing the potency of some antibiotics.[14] H-$TriB_1$ (**14**) was therefore synthesized and tested both for individual antimicrobial activity and synergistic activity with the antibiotic rifampicin. Consistent with previous tridecaptin analogues, $TriB_1$ displays weak activity against Gram-positive bacteria, with no inhibition observed at the highest concentrations tested (100 μg/mL). The other analogues also showed no activity against these strains. $TriB_1$ displays strong activity (3.13-6.25 μg/mL) against *E. coli*, *Salmonella enterica*, and *K. pneumoniae*, including an MDR strain of *K. pneumoniae* (ATCC 700603). Weaker activity (12.5-25 μg/mL) was found against *Pseudomonas aeruginosa*, *A. baumannii* and MDR *A. baumannii* (ATCC BAA 1605). The incorrect 6-methyl stereochemistry in

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Table 1 Antimicrobial testing of tridecaptin B_1 analogues by microbroth dilution assay

	Minimum Inhibitory Concentration			
Organism	**(6'*S*)-TriB$_1$**	**(6'*R*)-TriB$_1$**	**Oct-TriB$_1$**	**H-TriB$_1$**
Escherichia coli ATCC 25922	6.25	12.5	12.5	>100
Salmonella enterica ATCC 13311	3.13	6.25	12.5	>100
Pseudomonas aeruginosa ATCC 27853	12.5	25	25	>100
Klebsiella pneumoniae ATCC 13883	6.25	6.25	12.5	>100
Klebsiella pneumoniae ATCC 700603	3.13	6.25	6.25	>100
Acinetobacter baumannii ATCC 19606	25	50	50	>100
Acinetobacter baumannii ATCC BAA 1605	25	25	50	>100
Enterococcus faecalis ATCC 29212	>100	>100	>100	>100
Staphylococcus aureus ATCC 29213	>100	>100	>100	>100
Bacillus cereus ATCC 21928	>100	>100	>100	>100
Bacillus mycoides ATCC 21929	>100	>100	>100	>100

[a] MIC values are reported in μg/mL.

(6'*R*)-TriB$_1$ (**2**) appears to decrease the activity of the peptide 2-fold. Oct-TriB$_1$ (**13**) is 2-4 fold less active than the natural peptide against the Gram-negative bacteria tested. Likely the decrease in hydrophobicity from 6-methyloctanoyl to octanoyl accounts for the small decrease in activity, as lipopeptides usually operate through membrane disruption. As expected, H-TriB$_1$ displayed no antimicrobial activity. However, the activity of rifampicin against *E. coli* was increased 64x in the presence of sub-MIC concentrations (12.5 μg/mL) of TriB$_1$ (see supporting information S9). The good activity of TriB$_1$ against the MDR strain of *K. pneumoniae* suggests that this class of lipopeptides should be considered for further development as antibiotic candidates.

Identification of the tridecaptin B_1 biosynthetic machinery

The genomic DNA of *P. polymyxa* NRRL B-30507 was isolated and sequenced. A candidate gene cluster responsible for the tridecaptin B_1 biosynthesis was identified by searching for the presence of NRPSs that are homologous to the previously reported NRPS gene products TriD and TriE postulated in tridecaptin A_1 biosynthesis.[12] Unfortunately this gene cluster was spread over multiple contigs. Extensive PCR was required to close the gaps between the contigs and assemble the complete gene cluster. These efforts revealed a gene cluster very similar to that previously reported for tridecaptin A_1, encoding five proteins (TrbA, TrbB, TrbC, TrbD and TrbE) (Figure 3). TrbA is a putative thioesterase which may be involved in correcting misacylation of thiolation domains in the NRPSs.[25] TrbB and TrbC are ABC transporter proteins, whilst TrbD and TrbE are NRPSs. TrbD and TrbE contain ten and three adenylation domains, respectively, and epimerase domains are present in each instance where a D-amino is found. When sequence prediction software was used to analyse each individual adenylation domain, the predicted amino acid specificities closely matched the sequence of tridecaptin B_1 (see supporting information S10). The greatest discrepancies lie in adenylation domains 1 and 9, which are predicted to select valine and phenylalanine respectively, rather than glycine and isoleucine, which are found in TriB$_1$. The predicted amino acids for those two positions are in fact those found in TriA$_1$. Further inspection of these adenylation domains revealed small differences in the active site residues between TrbD and TriD, as well as the TriD homologue in *P. polymyxa* NRRL B-30509 (see supporting information S11).[26] Whether the differences are responsible for the different amino acids at positions 1 and 9 in the tridecaptin A variants and tridecaptin B_1 remains to be investigated. The three domains selecting for diaminobutyrate residues resemble adenylation domains specific for ornithine. In addition, adenylations domains 5 and 13 in TrbD and TrbE predict different amino acids compared to the actual amino acids that are incorporated in TriB$_1$ (see supporting information S10). The same amino acid differences between what the adenylation domains predict and what is actually found at those two positions were also observed for tridecaptin A_1.

Conclusions

Genome sequencing of *P. polymyxa* NRRL B-30507 revealed no genetic determinants for the previously reported antimicrobial peptide SRCAM 37.[16] An activity guided purification approach revealed that, in addition to polymyxins B_1 and B_2, the major component active against Gram-negative bacteria from this organism was a new tridecaptin variant, tridecaptin B_1. The sequence of tridecaptin B_1 was elucidated by a combination of tandem mass spectrometry and 2D-NMR experiments. A novel synthesis of (6*S*)- and (6*R*)-methyloctanoic acid was used in the synthesis of TriB$_1$ analogues, however identification of the natural lipid tail was not possible using our previously reported method.[12] Derivatisation of TriB$_1$ using the Ohrui-Akasaka method allowed ^{1}H-NMR identification of the lipid tail as (6*S*)-methyloctanoic acid.[26] A small structure activity relationship study revealed that the stereochemistry and structure of the lipid

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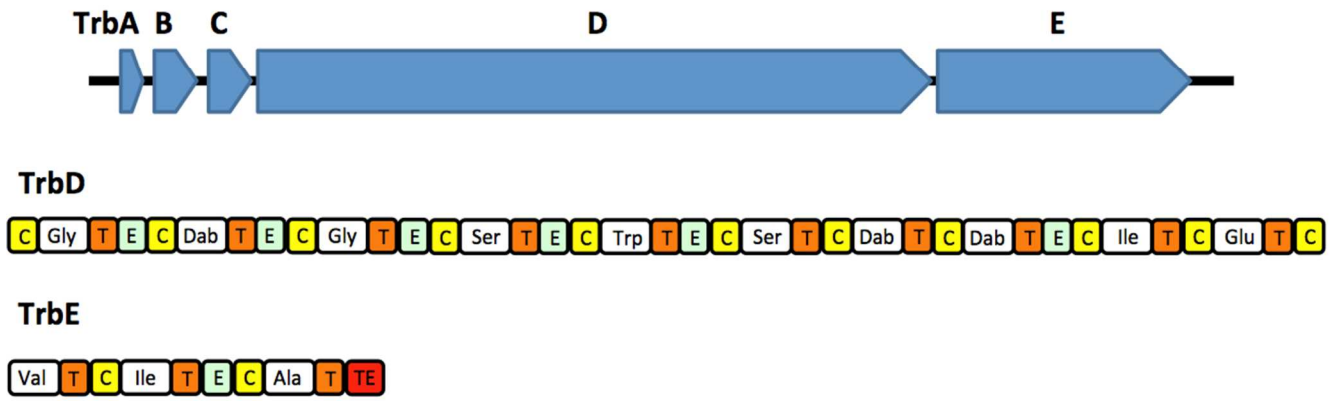

Figure 3. Tridecaptin B_1 biosynthetic gene cluster. TrbA is a putative thioesterase, TrbB and TrbC are ABC transporter proteins and TrbD and TrbE are NRPSs. The domains in TrbD and TrbE are denoted by the following colours: yellow = condensation domain, white = adenylation domain, orange = thiolation domain, mint = epimerase domain and red = thioesterase domain.

tail is important for antimicrobial activity. TriB_1 shows strong activity against multidrug resistant *Klebsiella pneumoniae*, as well as other Gram-negative organisms. A bioinformatic analysis of the producer strains genome and extensive PCR was used to identify the putative gene cluster responsible for the biosynthesis of TriB_1. With the current problem of antibiotic resistance, we believe these discoveries constitute an important addition to other known Gram-negative targeting antimicrobial agents.

Experimental Section

Isolation of tridecaptin B_1: *P. polymyxa* NRRL B-30507 was obtained from the United States Department of Agriculture-Agricultural Research Service. 1 L of Mueller Hinton (MH) broth was inoculated with a 10 mL overnight culture of *P. polymyxa* NRRL B-30507 in MH broth and shaken at 37 °C for 40 hours. The cells were removed by centrifugation (11 000 *g*, 4 °C, 10 min) and the supernatant loaded on to Amberlite XAD16 resin (20 – 60 mesh, Sigma, 40 g), which had been pre-washed with deionized H_2O (500 mL), at 10 mL/min. The flow through was collected and the resin washed sequentially with 250 mL of deionized H_2O, 20% isopropanol (IPA), 40% IPA and 80% IPA + 0.1% TFA. 200 µL of each wash fraction was concentrated to approximately 20 µL and tested for activity against *E. coli* ATCC 25922. The active fraction was concentrated by rotary evaporator to ~30 mL and loaded on to a C_{18}-SPE cartridge (Phenomenex) at 3 mL/min. The flow through was collected and the column was washed sequentially with 50 mL of 30% EtOH, 40% IPA and 80% IPA + 0.1% TFA. Activity of each fraction was tested and the active fractions concentrated and purified by HPLC.

General Information: Chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. All fluorenylmethyloxycarbonyl (Fmoc) amino acids and resins used in peptide synthesis were purchased from ChemImpex (Wood Dale, IL, USA). 2-(3-Bromopropyl)-1,3-dioxolane and 2,3-anthracenedicarboxylic anhydride were purchased from TCI Chemicals (Portland, OR, USA) and (1*R*, 2*R*)-2-aminocyclohexanol (>99%) was purchased from Oakwood Chemicals (West Columbia, SC, USA). Peptide grade *N*,*N*-dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) and HPLC grade acetonitrile (MeCN) were purchased from Caledon Labs (Georgetown, ON, Canada). Media and bacterial strains were purchased from ATCC (Manassas, VA, USA). Reactions were monitored by Thin Layer Chromatography using Silica Gel GF glass TLC plates (Analtech) with visualisation by an ultraviolet lamp (254 or 365 nm) or chemical staining (phosphomolybdic acid, potassium permanganate or bromocresol). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 500 or 600 MHz spectrometer. For ^{1}H NMR spectra, δ values were referenced to $CDCl_3$ (7.26 ppm), d_6-DMSO (2.50 ppm) or D_2O (4.79 ppm). For ^{13}C NMR spectra, δ values were referenced to $CDCl_3$ (77.16 ppm) or d_6-DMSO (39.5 ppm). High resolution mass spectrometry (HRMS) spectra were recorded on a Bruker 9.4T Apex-Qe Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Peptides were purified on a Gilson preparative high-performance liquid chromatography (HPLC) system equipped with a model 322 HPLC pump, GX-271 liquid handler, 156 UV/vis detector and a 10 mL sample loop. Analytical runs were performed on a Gilson analytical HPLC system equipped with a model 322 HPLC pump, 171 diode array detector, FC203B fraction collector, Rheodyne 7725i injector and 1 mL sample loop. A Phenomenex C_{18} column (21.2 x 250 mm, 100 Å, 5 micron) was used for preparative scale purification and a Vydac C_{18} column (5 µm, 4.6 x 250 mm) for analytical scale.

General procedure for HPLC purification of peptides: Natural extracts and synthetic peptides were purified using C_{18}-RP-HPLC: Phenomenex C_{18} column, flow rate 10 mL/min, detected at 220 nm. Gradient: starting from 20% MeCN (0.1% TFA) and 80% water (0.1% TFA) for 5 min, ramping up to 55% MeCN over 30 min, then ramping up to 95% MeCN over 3 min,

staying at 95% MeCN for 3 min, ramping down to 20% MeCN over 2 min, then staying at 20% MeCN for 5 min. Product containing fractions were pooled and lyophilized.

Synthesis of (2*S*)-methylbutyltosylate (7): (2*S*)-Methylbutanol (5.0 g, 57.0 mmol) was dissolved in pyridine (100 mL). Tosyl chloride (21.6 g, 113 mmol) was added and the resulting solution stirred at ambient temperature for 16 h. The reaction was diluted with H_2O (300 mL) and Et_2O (300 mL) and the organic phase separated. The aqueous phase was washed with Et_2O (2 x 150 mL) and the combined organic extracts washed with H_2O (100 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield tosylate **7** as a colourless oil (11.2 g, 82%). $[\alpha]_D^{25}$ = 4.78 (*c* = 1.1 g/100mL, $CHCl_3$); IR ($CHCl_3$ cast) 2966, 2934, 2879, 1361 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.79 (d, 2H, *J* = 8.5 Hz, ortho-ArH), 7.34 (d, 2H, *J* = 7.9 Hz, meta-ArH), 3.88 (dd, 1H, *J* = 9.4, 5.90, OCHH), 3.81 (dd, 1H, *J* = 9.4, 6.40, OCHH), 2.45 (s, 3H, $ArCH_3$), 1.75-1.66 (m, 1H, $CHCH_2O$), 1.44-1.34 (m, 1H, $CHHCH_3$), 1.20-1.09 (m, 1H, $CHHCH_3$), 0.88 (d, 3H, *J* = 6.8 Hz, $CHCH_3$), 0.83 (t, 3H, *J* = 7.5 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 144.7, 133.4, 129.9, 128.0, 75.0, 34.5, 25.6, 21.8, 16.1, 11.1; HRMS (ES) Calcd for $C_{12}H_{18}O_3SNa$ $[M+Na]^+$ 265.0869, found 265.0867.

(2*R*)-methylbutyltosylate (8): Product was isolated as a colourless oil (5.45 g, 66%). $[\alpha]_D^{25}$ = -4.3 (*c* = 1.1 g/100mL, $CHCl_3$); IR ($CHCl_3$ cast) 2966, 2934, 2879, 1360 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 7.79 (d, 2H, *J* = 8.3 Hz, ortho-ArH), 7.34 (d, 2H, *J* = 7.4 Hz, meta-ArH), 3.83 (dd, 1H, *J* = 9.38, 5.90, OCHH), 3.77 (dd, 1H, *J* = 9.38, 6.40, OCHH), 2.40 (s, 3H, $ArCH_3$), 1.66 (ddt, 1H, *J* = 13.1, 6.6 Hz, $CHCH_2O$), 1.38-1.30 (m, 1H, $CHHCH_3$), 1.14-1.05 (m, 1H, $CHHCH_3$), 0.83 (d, 3H, *J* = 6.8 Hz, $CHCH_3$), 0.78 (t, 3H, *J* = 7.5 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 144.7, 133.3, 129.9, 128.0, 74.9, 34.4, 25.5, 21.7, 16.0, 11.0; HRMS (ES) Calcd for $C_{12}H_{18}O_3SNa$ $[M+Na]^+$ 265.0869, found 265.0868.

Synthesis of 2-(1-(6*S*)-methyloctyl)-1,3-dioxolane (10): Magnesium turnings were removed from the oven and ground with a mortar and pestle. The magnesium (0.54 g, 22.0 mmol) was then added to a flame-dried flask under argon and suspended in dry THF (6 mL). A small crystal of I_2 was added and the mixture refluxed until the brown colour disappeared. The flask was then removed from the oil bath and the bromo dioxolane (1.52 mL, 11.0 mmol) added slowly. Upon initiation of the reaction, the addition was adjusted to maintain a smooth reflux. The mixture was then heated to reflux for 3 h and cooled to room temperature. The Grignard reagent was titrated using the I_2/LiCl method (Knochel, *Synthesis-Stuttgart*, **2006**, *5*, 890) and found to have a concentration of 1 M. In a separate flask, tosylate **7** (0.5 g, 2.1 mmol) and anhydrous $CuCl_2$ (24 mg, 0.11 mmol) were dissolved in dry THF (6 mL) under argon and cooled to – 10 °C. The Grignard solution (1 M in THF, 2.5 mL, 2.5 mmol) was added dropwise and the resulting brown solution stirred at ambient temperature for 2 h. The mixture was diluted with saturated NH_4Cl (5 mL) and washed with Et_2O (3 x 10 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, concentrated *in vacuo* and purified by flash column chromatography (SiO_2, 9:1 hexanes:EtOAc) to yield the product as a colourless oil (235 mg, 60%). $[\alpha]_D^{25}$ = 6.98 (*c* = 0.55 g/100mL, $CHCl_3$); IR ($CHCl_3$ cast) 2957, 2928, 2875 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 4.84 (t, 1H, *J* = 4.90, OCHO), 3.97-3.93 (m, 2H, OCHH), 3.87-3.83 (m, 2H, OCHH), 1.67-1.64 (m, 2H, $OCHCH_2$), 1.42-1.28 (m, 7H), 1.13-1.09 (m, 2H, CH_2CH_3), 0.86-0.83 (m, 6H, 2 x CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 104.9, 65.0, 36.6, 34.4, 34.1, 29.6, 27.2, 24.6, 19.4, 11.5.

2-(1-(6*R*)-methyloctyl)-1,3-dioxolane (11): Product isolated as a colourless oil (0.65 g, 85%). $[\alpha]_D^{25}$ = -7.02 (*c* = 0.55 g/100mL, $CHCl_3$); IR ($CHCl_3$ cast) 2957, 2928, 2875 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 4.84 (t, 1H, *J* = 4.90, OCHO), 3.97-3.95 (m, 2H, OCHH), 3.85-3.83 (m, 2H, OCHH), 1.67-1.63 (m, 2H, $OCHCH_2$), 1.46-1.25 (m, 7H), 1.16-1.07 (m, 2H, CH_2CH_3), 0.86-0.83 (m, 6H, 2 x CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 104.9, 65.0, 36.6, 34.4, 34.1, 29.6, 27.2, 24.6, 19.3, 11.5.

Synthesis of (6*S*)-methyloctanoic acid (3): Dioxolane **10** (186 mg, 1.0 mmol) was dissolved in THF (10 mL) and 1 M HCl (10 mL) and stirred at 50 °C for 5 h. The reaction mixture was then added to a stirring solution of sat. $NaHCO_3$ (30 mL) and the organic layer separated. The aqueous layer was washed with CH_2Cl_2 (3 x 15 mL) and the combined organic extracts washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude fragrant aldehyde (1.0 mmol) was then dissolved in *t*-BuOH (5 mL) and H_2O (1 mL) and stirred at ambient temperature. 1-Methyl-2-butene (3.18 mL, 30.0 mmol), NaH_2PO_4 (360 mg, 3.0 mmol) and $NaClO_2$ (181 mg, 2.0 mmol) were added sequentially and the resulting yellow solution stirred at ambient temperature for 1 h. Additional H_2O should be added if the reaction is not monophasic. A solution of sat. NH_4Cl (5 mL) and CH_2Cl_2 (30 mL) were added and the organic layer separated. The aqueous layer was washed with CH_2Cl_2 (2 x 10 mL) and the combined organic extracts dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude reaction was purified by flash column chromatography (SiO_2, 9:1 hexanes:EtOAc + 0.1 % AcOH) to yield (6*S*)-Methyloctanoic acid (**3**) as a colourless liquid (73 mg, 46 %). $[\alpha]_D^{25}$ = 7.38 (*c* = 1.0 g/100mL, $CHCl_3$), lit (ref 21) $[\alpha]_D^{25}$ = -7.9 (*c* = 0.9 g/100mL, $CHCl_3$); IR ($CHCl_3$ cast) 2961, 2931, 2874, 1711 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 2.39-2.37 (m, 2H, $C(O)CH_2$), 1.66-1.54 (m, 2H, $C(O)CH_2CH_2$), 1.36-1.08 (m, 9H), 0.87-0.84 (m, 6H, 2 x CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 180.4, 36.4, 34.4 (2 overlapping signals), 29.6, 26.8, 25.4, 19.3, 11.5; HRMS (ES) Calcd for $C_9H_{17}O_2$ $[M-H]^-$ 157.1234, found 157.1232.

(6*R*)-methyloctanoic acid (4): $[\alpha]_D^{25}$ = -5.38 (*c* = 1.0 g/100mL, $CHCl_3$), lit (ref 21) $[\alpha]_D^{25}$ = -5.7 (*c* = 0.8 g/100mL, $CHCl_3$); IR ($CHCl_3$ cast) 2961, 2932, 2874, 1711 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 2.36 (t, 2H, *J* = 7.5, $C(O)CH_2$), 1.67-1.57 (m, 2H, $C(O)CH_2CH_2$), 1.39-1.28 (m, 5H), 1.15-1.09 (m, 2H, CH_2CH_3), 0.86-0.84 (m, 6H, 2 x CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 180.4, 36.3, 34.3 (2 overlapping signals), 29.6, 26.7, 25.2, 19.3, 11.5; HRMS (ES) Calcd for $C_9H_{17}O_2$ $[M-H]^-$ 157.1234, found 157.1234.

General procedure for peptide synthesis: Peptides were synthesized using Fmoc chemistry[22] on preloaded L-alanine-2-chlorotrityl resin (30 mg, 0.025 mmol, 0.824 mmol/g loading). Reactions were performed in a custom-built 20 mL glass fritted column fitted with a T-joint and three-way T-bore teflon stopcock. The resin was pre-swollen in DMF (5 mL, 10 min) by bubbling with argon. Between deprotections and couplings the vessel was drained under argon pressure and washed with DMF

(3 x 5 mL). The Fmoc group was removed by bubbling with 20% piperidine in DMF (3 x 5 mL x 5 min). The deprotection steps were monitored by UV absorbance. Fmoc-D-*allo*-isoleucine (5 equiv) was pre-activated by shaking with 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5 b]pyridinium 3-oxid hexafluorophosphate (HATU) (5 equiv) and *N*,*N*-diisopropylethylamine (DIPEA) (10 equiv) in DMF (5 mL) for 5 min. The resin was bubbled with Ar in the coupling solution for 1 h, drained and washed with DMF (3 x 5 mL). Appropriate deprotection and coupling steps were continued to complete the peptide synthesis. The resin-bound peptide was washed with CH_2Cl_2 (3 x 5 mL) and dried under argon for 20 min. The resin was transferred to a screw top vial containing trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O (95:2.5:2.5, 2 mL) and shaken for 2 h. The cleavage solution was filtered and concentrated *in vacuo* and the crude peptide was precipitated with cold diethyl ether.

(6'*S*)-TriB$_1$ (1): Product eluted at 31.1 min and was isolated as a white powder (7 mg, 19%). ^{1}H NMR (D_2O, 600 MHz): δ 7.57 (d, 1H, *J* = 8.1 Hz, D-Trp5-ArH), 7.46 (d, 1H, *J* = 8.1 Hz, D-Trp5-ArH), 7.21-7.19 (m, 2H, D-Trp5-ArH), 7.11 (t, 1H, *J* = 7.1 Hz, D-Trp5-ArH), 4.63-4.60 (1H, m, D-Trp5-Hα), 4.46-4.44 (m, 1H, D-Ser4-Hα), (m, 2H, D-Dab2-Hα + D-Dab8-Hα), 4.36-4.31 (m, 3H, Dab7-Hα + Glu10-Hα + D-aIle12-Hα), 4.22-4.18 (m, 1H, Ala13-Hα), 4.16-4.10 (m, 3H, Ser6-Hα + Ile9-Hα + Val11-Hα), 3.90 (s, 2H, Gly3-Hα), 3.88 (s, 2H, Gly1-Hα), 3.80-3.73 (m, 2H, D-Ser4-Hβ), 3.56-3.54 (m, 1H, Ser6-Hβ), 3.30-3.25 (m, 3H, D-Trp5-Hβ + Ser6-Hβ), 3.06-2.94 (m, 6H, D-Dab2-Hγ + Dab7-Hγ + D-Dab8-Hγ), 2.36-1.79 (m, 15H, Lipid-Hα + D-Dab2-Hβ + Dab7-Hβ + D-Dab8-Hβ + Ile9-Hβ + Glu10-Hβ + Glu10-Hγ + Val11-Hβ + D-aIle12-Hβ), 1.53-1.47 (m, 2H, Lipid-Hβ), 1.41-0.99 (m, 14H, Lipid-Hγ, Hδ, Hε, Hζ + Ile9-Hγ + D-aIle12-Hγ Ala13-Hβ), 0.92-0.75 (m, 24H, Lipid-Hζ, Hη + Ile9- Hγ, Hδ + Val11-Hγ + D-aIle12-Hγ, Hδ). HRMS (ES) Calcd for $C_{67}H_{112}N_{17}O_{19}$ $[M+H]^+$ 1458.8315, found 1458.8327.

(6'*R*)-TriB$_1$ (2): Product eluted at 31.1 min and was isolated as a white powder (8 mg, 22%). ^{1}H NMR (D_2O, 600 MHz): ^{1}H NMR (D_2O, 600 MHz): δ 7.57 (d, 1H, *J* = 8.1 Hz, D-Trp5-ArH), 7.47 (d, 1H, *J* = 8.1 Hz, D-Trp5-ArH), 7.22-7.19 (m, 2H, D-Trp5-ArH), 7.12 (t, 1H, *J* = 7.4 Hz, D-Trp5-ArH), 4.62 (1H, t, *J* = 7.3 Hz, D-Trp5-Hα), 4.45 (t, 1H, *J* = 5.1 Hz, D-Ser4-Hα), 4.43-4.39 (m, 2H, D-Dab2-Hα + D-Dab8-Hα), 4.37-4.32 (m, 3H, Dab7-Hα + Glu10-Hα + D-aIle12-Hα), 4.22-4.18 (m, 1H, Ala13-Hα), 4.15-4.11 (m, 3H, Ser6-Hα + Ile9-Hα + Val11-Hα), 3.90 (s, 2H, Gly3-Hα), 3.88 (s, 2H, Gly1-Hα), 3.80-3.73 (m, 2H, D-Ser4-Hβ), 3.56-3.54 (m, 1H, Ser6-Hβ), 3.30-3.25 (m, 3H, D-Trp5-Hβ + Ser6-Hβ), 3.06-2.94 (m, 6H, D-Dab2-Hγ + Dab7-Hγ + D-Dab8-Hγ), 2.36-1.79 (m, 15H, Lipid-Hα + D-Dab2-Hβ + Dab7-Hβ + D-Dab8-Hβ + Ile9-Hβ + Glu10-Hβ + Glu10-Hγ + Val11-Hβ + D-aIle12-Hβ), 1.53-1.47 (m, 2H, Lipid-Hβ), 1.41-0.99 (m, 14H, Lipid-Hγ, Hδ, Hε, Hζ + Ile9-Hγ + D-aIle12-Hγ Ala13-Hβ), 0.92-0.75 (m, 24H, Lipid-Hζ, Hη + Ile9- Hγ, Hδ + Val11-Hγ + D-aIle12-Hγ, Hδ). HRMS (ES) Calcd for $C_{67}H_{112}N_{17}O_{19}$ $[M+H]^+$ 1458.8315, found 1458.8322.

Oct-TriB$_1$ (13): Product eluted at 36.5 min and was isolated as a white powder (10 mg, 28%). ^{1}H NMR (D_2O, 600 MHz): δ 7.57 (d, 1H, *J* = 7.9 Hz, D-Trp5-ArH), 7.47 (d, 1H, *J* = 8.1 Hz, D-Trp5-ArH), 7.22-7.18 (m, 2H, D-Trp5-ArH), 7.12 (t, 1H, *J* = 7.1 Hz, D-Trp5-ArH), 4.62 (1H, t, *J* = 7.4 Hz, D-Trp5-Hα), 4.46-4.44 (m, 1H, D-Ser4-Hα), 4.43-4.39 (m, 2H, D-Dab2-Hα + D-Dab8-Hα), 4.38-4.31 (m, 3H, Dab7-Hα + Glu10-Hα + D-aIle12-Hα), 4.22-4.11 (m, 4H, Ala13-Hα + Ser6-Hα + Ile9-Hα + Val11-Hα), 3.91 (s, 2H, Gly3-Hα), 3.88 (s, 2H, Gly1-Hα), 3.81-3.73 (m, 2H, D-Ser4-Hβ), 3.58-3.53 (m, 1H, Ser6-Hβ), 3.31-3.26 (m, 3H, D-Trp5-Hβ + Ser6-Hβ), 3.07-2.96 (m, 6H, D-Dab2-Hγ + Dab7-Hγ + D-Dab8-Hγ), 2.36-1.81 (m, 15H, Lipid-Hα + D-Dab2-Hβ + Dab7-Hβ + D-Dab8-Hβ + Ile9-Hβ + Glu10-Hβ + Glu10-Hγ + Val11-Hβ + D-aIle12-Hβ), 1.54-1.49 (m, 2H, Lipid-Hβ), 1.39-1.08 (m, 14H, Lipid-Hγ, Hδ, Hε, Hζ + Ile9-Hγ + D-aIle12-Hγ Ala13-Hβ), 0.92-0.79 (m, 21H, Lipid-Hζ, Hη + Ile9- Hγ, Hδ + Val11-Hγ + D-aIle12-Hγ, Hδ). HRMS (ES) Calcd for $C_{66}H_{110}N_{17}O_{19}$ $[M+H]^+$ 1444.8160, found 1444.8158.

H-TriB$_1$ (14): Product eluted at 21.1 min and was isolated as a white powder (5 mg, 15%). ^{1}H NMR (D_2O, 600 MHz): δ 7.58 (d, 1H, *J* = 8.2 Hz, D-Trp5-ArH), 7.48 (d, 1H, *J* = 8.2 Hz, D-Trp5-ArH), 7.23-7.20 (m, 2H, D-Trp5-ArH), 7.12 (t, 1H, *J* = 7.4 Hz, D-Trp5-ArH), 4.64 (1H, t, *J* = 7.3 Hz, D-Trp5-Hα), 4.50-4.46 (m, 2H, D-Ser4-Hα + D-Dab2-Hα), 4.42 (dd, 1H, *J* = 9.3, 5.6 Hz, D-Dab8-Hα), 4.37-4.31 (m, 3H, Dab7-Hα + Glu10-Hα + D-aIle12-Hα), 4.18-4.11 (m, 4H, Ala13-Hα + Ser6-Hα + Ile9-Hα + Val11-Hα), 3.96-3.89 (m, 2H, Gly1-Hα), 3.85 (s, 2H, Gly3-Hα), 3.79-3.73 (m, 2H, D-Ser4-Hβ), 3.52 (dd, 1H, *J* = 11.4, 4.8 Hz, Ser6-Hβ), 3.28-3.26 (m, 3H, D-Trp5-Hβ + Ser6-Hβ), 3.07-2.96 (m, 6H, D-Dab2-Hγ + Dab7-Hγ + D-Dab8-Hγ), 2.31-1.81 (m, 13H, D-Dab2-Hβ + Dab7-Hβ + D-Dab8-Hβ + Ile9-Hβ + Glu10-Hβ + Glu10-Hγ + Val11-Hβ + D-aIle12-Hβ), 1.39-1.09 (m, 7H, Ile9-Hγ + D-aIle12-Hγ Ala13-Hβ), 0.92-0.79 (m, 18H, Ile9-Hγ, Hδ + Val11-Hγ + D-aIle12-Hγ, Hδ). HRMS (ES) Calcd for $C_{58}H_{96}N_{17}O_{18}$ $[M+H]^+$ 1318.7104, found 1318.7114.

Synthesis of (1*R*, 2*R*)-2-(2,3-anthracenedicarboximido)cyclohexanol (17): This compound was synthesized according to a modified literature procedure.[16] 2,3-Anthracenedicarboxylic acid anhydride (**16**) (100 mg, 0.403 mmol) was added to dry toluene (35 mL) and heated to reflux (oil bath, 140 °C). A solution of (1*R*,2*R*)-2-aminocyclohexanol (**15**) (65 mg, 0.564 mmol) in dry DMF (10 mL) was added to the reaction mixture, at which point all solids dissolved. Dry DIPEA (3 mL) was then added and the resulting solution refluxed for 16 h. Most of the solvent (35 mL) was then removed using a Dean-Stark tube and the resulting solution cooled to ambient temperature. Ethyl acetate (70 mL) was added and the resulting solution washed with 0.2 M NaOH (50 mL), 0.2 M HCl (50 mL) and saturated $NaHCO_3$ (50 mL). The organic phase was then dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield alcohol **17** as a yellow powder (135 mg, 97%). $[\alpha]_D^{25}$ = -34.99 (*c* = 0.212 g/100mL, DMF); IR ($CHCl_3$ cast) 3525, 2926, 2856, 1764, 1699 cm^{-1}; ^{1}H NMR (d_6-DMSO, 500 MHz): δ 8.87 (s, 2H, H8), 8.59 (s, 2H, H7), 8.15-8.13 (m, 2H, H10), 7.64-7.63 (m, 2H, H9), 4.93 (d, 1H, *J* = 4.72 Hz, OH), 4.12-4.06 (m, 1H, H1), 3.84 (ddd, 1H, *J* = 12.8, 9.7, 3.4 Hz, H2), 2.14-2.06 (m, 1H, H3(eq)), 1.96-1.90 (m, 1H, H6(eq)), 1.73-1.64 (m, 3H, H3(ax) + H4(eq) + H4(ax)), 1.32-1.17 (m, 3H, H6(ax) + H5(eq) + H5(ax)); ^{13}C NMR (d_6-DMSO, 125 MHz): δ 167.5, 132.6, 131.6, 129.9, 128.3, 127.5, 126.6, 125.0, 67.6, 57.3, 34.7, 28.2, 25.0, 24.2; HRMS (ES) Calcd for $C_{22}H_{20}NO_3$ $[M+H]^+$ 346.1438, found 346.1432.

Synthesis of (1*R*, 2*R*)-1-((6*S*)-methyloctyl)-2-(2,3-

anthracenedicarboximido) cyclohexanoate (18): (6*S*)-Methyloctanoic acid (**3**) (6.0 mg, 37.9 μmol) and EDCI.HCl (12.0 mg, 62.6 μmol) were dissolved in a 1:1:1 mixture of dry CH_2Cl_2, toluene and DMF (0.9 mL). Alcohol **17** (14.4 mg, 41.7 μmol) was added, followed by DMAP (1.5 mg, 12.2 μmol). The resulting cloudy yellow solution was stirred at ambient temperature for 16 h, clarifying as the reaction progressed. The reaction mixture was diluted with EtOAc (9 mL) and washed with sat. $NaHCO_3$ (5 mL), 10% citric acid (5 mL), water (5 mL) and brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was dissolved in CH_2Cl_2 (0.5 mL) and purified by preparatory TLC (Analtech Silica Gel GF, 20 x 20 cm, 500 microns), eluting with 2:1 hexanes:EtOAc. The product eluted as a thin yellow band (R_f ~ 0.6) and was isolated as a yellow solid (15 mg, 82%). $[\alpha]_D^{25}$ = -29.46 (*c* = 0.30 g/100mL, DMF); IR ($CHCl_3$ cast) 2928, 2859, 1772, 1731, 1707 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.63 (s, 2H, Anth9, Anth10), 8.49 (s, 2H, Anth5, Anth8), 8.10-8.07 (m, 2H, Anth2, Anth3), 7.63-7.60 (m, 2H, Anth1, Anth4), 5.57 (ddd, 1H, *J* = 10.7, 4.7, 4.7 Hz, NCH), 4.31 (ddd, 1H, *J* = 12.6, 10.5, 4.3 Hz, OCH), 2.48 (dddd, 1H, *J* = 13.0, 13.0, 13.0, 3.4 Hz, OCHCH(eq)), 2.26-2.21 (m, 1H, NCHCH(eq)), 2.09 (t, 2H, *J* = 7.5 Hz, lipid-Hα), 1.93-1.81 (m, 3H, OCHCH(ax) + $OCHCH_2CH$(eq) + $NCHCH_2CH$(eq)), 1.57-1.26 (m, 5H, NCHCH(ax) + $NCHCH_2CH$(ax) + $OCHCH_2CH$(ax) + lipid-Hβ), 1.05-0.66 (m, 7H, lipid-Hγ, Hδ, Hε, Hζ), 0.60 (t, 3H, *J* = 7.4 Hz, Lipid-Hη), 0.56 (d, 3H, *J* = 6.5 Hz, Lipid-Hζ); ^{13}C NMR ($CDCl_3$, 125 MHz): δ173.2, 167.8, 133.4, 132.2, 130.2, 128.6, 127.6, 126.5, 125.9, 71.7, 54.3, 36.2, 34.7, 34.1, 31.9, 29.4, 28.7, 26.5, 25.5, 25.3, 24.1, 19.0, 11.3; HRMS (ES) Calcd for $C_{31}H_{35}NO_4Na$ $[M+Na]^+$ 508.2458, found 508.2448.

(1*R*, 2*R*)-1-((6*R*)-methyloctyl)-2-(2,3-anthracene dicarboximido) cyclohexanoate (19): Product isolated as a yellow solid (16.5 mg, 90%). $[\alpha]_D^{25}$ = -37.39 (*c* = 0.30 g/100mL, DMF); IR ($CHCl_3$ cast) 2929, 2859, 1764, 1731, 1706 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ 8.61 (s, 2H, Anth9, Anth10), 8.48 (s, 2H, Anth5, Anth8), 8.08-8.06 (m, 2H, Anth2, Anth3), 7.62-7.60 (m, 2H, Anth1, Anth4), 5.57 (ddd, 1H, *J* = 10.7, 4.7, 4.7 Hz, NCH), 4.31 (ddd, 1H, *J* = 12.6, 10.5, 4.2 Hz, OCH), 2.48 (dddd, 1H, *J* = 13.0, 13.0, 13.0, 3.4 Hz, OCHCH(eq)), 2.25-2.21 (m, 1H, NCHCH(eq)), 2.14-2.04 (m, 2H, lipid-Hα), 1.91-1.82 (m, 3H, OCHCH(ax) + $OCHCH_2CH$(eq) + $NCHCH_2CH$(eq)), 1.59-1.20 (m, 5H, NCHCH(ax) + $NCHCH_2CH$(ax) + $OCHCH_2CH$(ax) + lipid-Hβ), 1.05-0.67 (m, 7H, lipid-Hγ, Hδ, Hε, Hζ), 0.59 (t, 3H, *J* = 7.4 Hz, Lipid-Hη), 0.52 (d, 3H, *J* = 6.5 Hz, Lipid-Hζ); ^{13}C NMR ($CDCl_3$, 125 MHz): δ173.1, 167.8, 133.4, 132.1, 130.1, 128.6, 127.6, 126.5, 125.9, 71.7, 54.3, 36.1, 34.7, 34.0, 31.9, 29.4, 28.6, 26.5, 25.5, 25.3, 24.1, 18.9, 11.3; HRMS (ES) Calcd for $C_{31}H_{36}NO_4$ $[M+H]^+$ 486.2639, found 486.2639.

Hydrolysis of tridecaptin B_1 and derivatisation of resulting methyloctanoate: Tridecaptin B_1 (3 mg, 2.06 μmol) was dissolved in 6M HCl (4 mL) and stirred at 110 °C for 3 h in a pressure tube. The reaction mixture was then cooled to ambient temperature and extracted with diethyl ether (3 x 5 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield crude 6-methyloctanoic acid (assumed 2.06 μmol). This residue was dissolved in a dry 1:1:1 mixture of CH_2Cl_2/toluene/DMF (150 μL) and to this solution was added EDCI.HCl (1.2 mg, 6.17 μmol), alcohol **17** (2.1 mg, 6.17 μmol) and DMAP (0.2 mg, 1.64 μmol). The resulting yellow solution was stirred at ambient temperature for 22 h and then loaded directly on to a preparatory TLC plate (Analtech Silica Gel GF, 20 x 20 cm, 500 microns) and eluted with 2:1 hexanes:EtOAc. The product eluted as a thin yellow band (R_f = 0.60) and was isolated as a yellow oil (300 μg, 30% over 2 steps). This product was dissolved in $CDCl_3$ (300 μL) and analysed by ^1H-NMR for comparison with **18** and **19** (see figure 2). HPLC analysis was then performed.

HPLC analysis of anthracene derivatives: Anthracenyl derivatives (50 μg) were dissolved in 100 μL of the HPLC eluent (methanol/acetonitrile/*n*-hexane = 300:200:5) and injected on to a Develosil ODS-3 column (Phenomenex, 3 μm, 4.6 mm x 150 mm), cooled to -55 °C and using a flow rate of 0.8 mL/min. An isocratic flow was maintained for 60 min. The column was cooled by immersion in a dry-ice/acetone bath regulated with a Thermo Neslab CC-100 Immersion Chiller and the absorbance of the anthracene ring was monitored at 370 nm. The (6*R*)-derivative **19** eluted at 42 min whilst the (6*S*)-derivative **18** eluted at 45 min (see supporting information S9).

Growth of bacterial strains: All cultures were grown from glycerol stocks. All organisms were grown in Mueller–Hinton (MH) broth at 37 °C with shaking at 225 rpm for 16 – 24 h.

Antimicrobial testing: All minimum inhibitory concentrations were determined using microbroth dilution assays, according to Clinical and Standards Laboratory Institute (CLSI) guidelines.[27] Briefly, peptides were dissolved in MH broth and serial dilutions made across a 96 well plate. Each well was inoculated with a suspension of the required bacterial strain to reach a final inoculum of 5 x 10^5 colony forming units per mL. The MIC was taken as the lowest concentration with no visible growth after 18 hours.

Genome sequencing: Genomic DNA from *P. polymyxa* NRRL B-30507 was isolated using a DNeasy Blood and Tissue Kit (Qiagen). The genome was sequenced by using an Illumina HiSeq 2500 system at the University of Illinois High-Throughput Sequencing and Genotyping Unit. These results were assembled into contigs by HPCBio (University of Illinois) using Velvet assembler and analysed using Artemis software.

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Notes and references

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