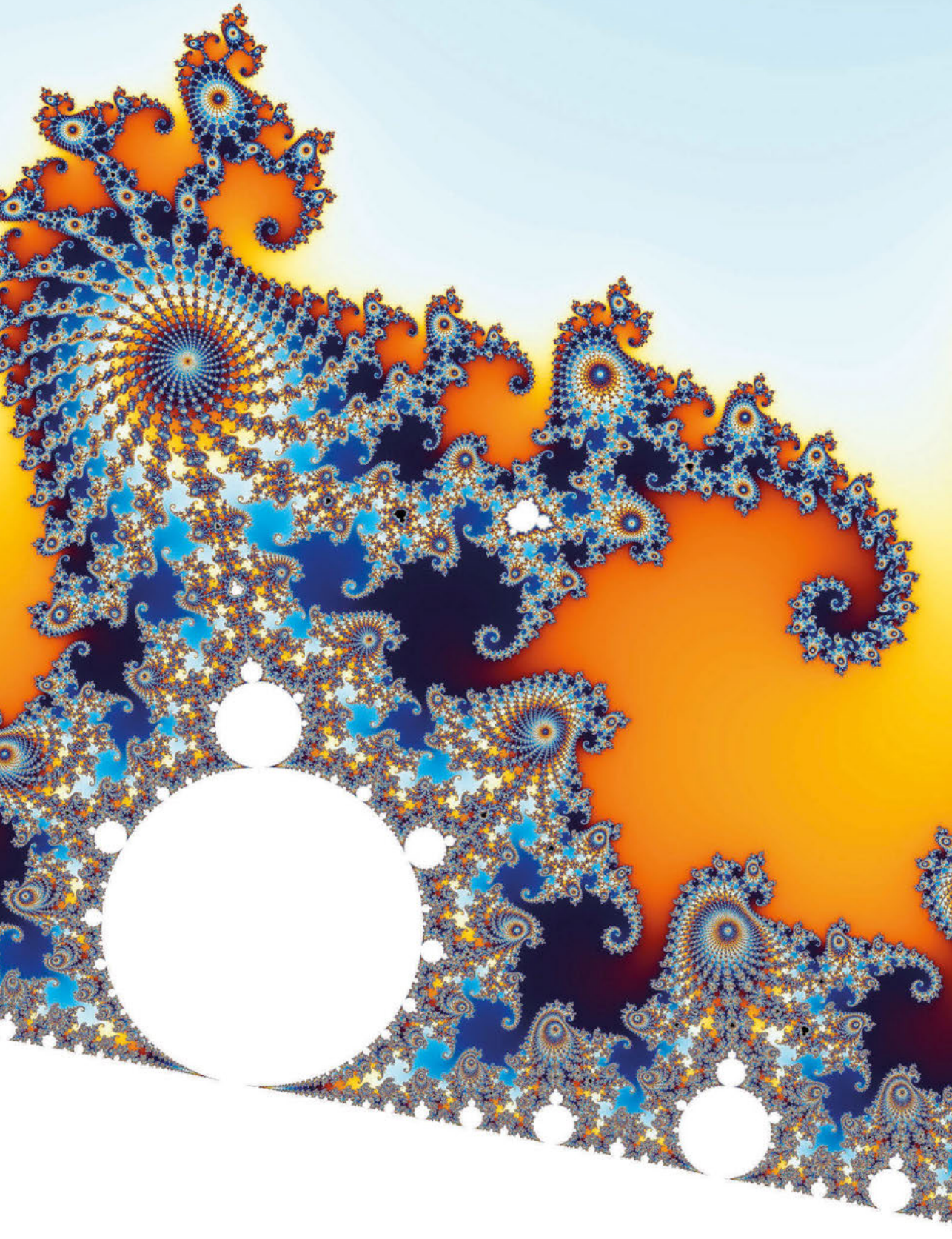
Next generation lipopeptide antibiotics

Al Ayed, U.K.



Chapter 2

Synthetic Studies with the Brevicidine and Laterocidine Lipopeptide Antibiotics Including Analogues with Enhanced Properties and *In Vivo* Efficacy

Abstract

Brevicidine and laterocidine are two recently discovered lipopeptide antibiotics with promising antibacterial activity. Possessing a macrocyclic core, multiple positive charges, and a lipidated N-terminus, these lipopeptides exhibit potent and selective activity against Gram-negative pathogens, including polymyxin-resistant isolates. Given the low amounts of brevicidine and laterocidine accessible by fermentation of the producing microorganisms, synthetic routes to these lipopeptides present an attractive alternative. We here report the convenient solid-phase syntheses of both brevicidine and laterocidine and confirm their potent anti-Gram-negative activities. The synthetic routes developed also provide convenient access to novel structural analogues of both brevicidine and laterocidine that display improved hydrolytic stability while maintaining potent antibacterial activity in both *in vitro* assays and *in vivo* infection models.

Parts of this chapter have been published in Chemical Science:

Al Ayed, K.*; Ballantine, R. D.*; Hoekstra, M.; Bann, S. J.; Wesseling, C. M. J.; Bakker, A. T.; Zhong, Z.; Li, Y.-X.; Brüchle, N. C.; Van Der Stelt, M.; Cochrane, S. A.; Martin, N. I. Synthetic Studies with the Brevicidine and Laterocidine Lipopeptide Antibiotics Including Analogues with Enhanced Properties and in Vivo Efficacy. Chem. Sci. **2022**, 13 (12), 3563–3570. https://doi.org/10.1039/D2SC00143H

Parts of the data in this chapter are part of a patent:

"Antibiotic natural product analogues"; Cochrane, S.A.; Ballantine, R. D.; Martin, N. I.; Al Ayed, K.; Hoekstra, M.; Zamarbide Losada, S.D.; Priority Date: 28 January 2021; Published: 4 August 2022; WO2022162332A1

*Authors contributed equally

Introduction

The accelerated appearance of multi-drug resistant bacterial pathogens has led to the worrying speculation that society may soon face a "post-antibiotic" era.[1-3] The gravity of the antimicrobial resistance (AMR) crisis is most clearly reflected by the spread of the "ESKAPE" pathogens (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species), a group of organisms that are increasingly difficult or impossible to treat with conventional antibiotics. Globally, deaths due to infections with drug-resistant bacteria are nearing one million per year.[4] Even more worrying are recent projections suggesting that by the year 2050 the number of AMR associated deaths will grow to a staggering ten million per year.[4]

To prioritize the greatest threats currently associated with AMR, the World Health Organization (WHO) recently published its list of priority pathogens.[5] Among these pathogens, it is exclusively the Gram-negative members of the ESKAPE family that are labeled as "critical", the highest threat level on the WHO list. This is due to the rapidly accelerating rise in antibiotic resistance among *Acinetobacter*, *Pseudomonas*, and various *Enterobacteriaceae* (including *Klebsiella* and *Escherichia coli*) causing severe and often deadly bloodstream and pulmonary infections. The AMR threat underscores the importance of pursuing new strategies in discovering and developing the antibiotics of the future.

Using a biosynthetic gene cluster mining strategy, Li and co-workers recently reported the discovery of a promising new class of macrocyclic lipopeptides termed the brevicidines and laterocidines (Figure 1).[6] Produced by strains of *Brevibacillus laterosporus*, brevicidine and laterocidine specifically kill all Gram-negative members of the ESKAPE family, including drug-resistant strains. The antibacterial activity of these lipopeptides is promising, with MIC values comparable to the polymyxins, the only class of lipopeptides presently used in the clinical treatment of serious Gram-negative infections. Of particular note is the finding that brevicidine and laterocidine effectively kill pathogenic strains featuring the recently reported MCR-type polymyxin resistance mechanism.[6] Furthermore, the initial report describing the discovery of brevicidine and laterocidine also indicates that these lipopeptide antibiotics show little propensity to induce resistance and have low toxicity towards mammalian cells.[6]

While brevicidine and laterocidine are promising new anti-Gram-negative antibiotics, both compounds are difficult to isolate in significant quantities from natural sources, presenting a major obstacle to investigate their full potential. Brevicidine and laterocidine can be obtained by fermentation of the producing microorganisms, however this labour-intensive process provides limited amounts of material (sub-milligram-per-litre yields in the case of laterocidine).[6] For this reason, reliable synthetic routes to these lipopeptides present an attractive alternative as a means of providing larger amounts of material for both clinical evaluation and mechanistic studies.[7,8] Herein we report the total syntheses of brevicidine and laterocidine by solid-phase peptide synthesis (SPPS).[9] The synthetic compounds and natural products have identical ^{1}H-NMR spectra, RP-HPLC retention times, and antibacterial activities. Using the same synthetic approach, the enantiomers of brevicidine and laterocidine were also prepared to probe the role of stereochemistry in the antibacterial mechanism of these unique lipopeptides. Furthermore, novel analogues wherein the ester moiety of the peptide macrocycle was replaced by an amide linkage, were prepared and their stability and antibacterial activities assessed both *in vitro* and *in vivo*.

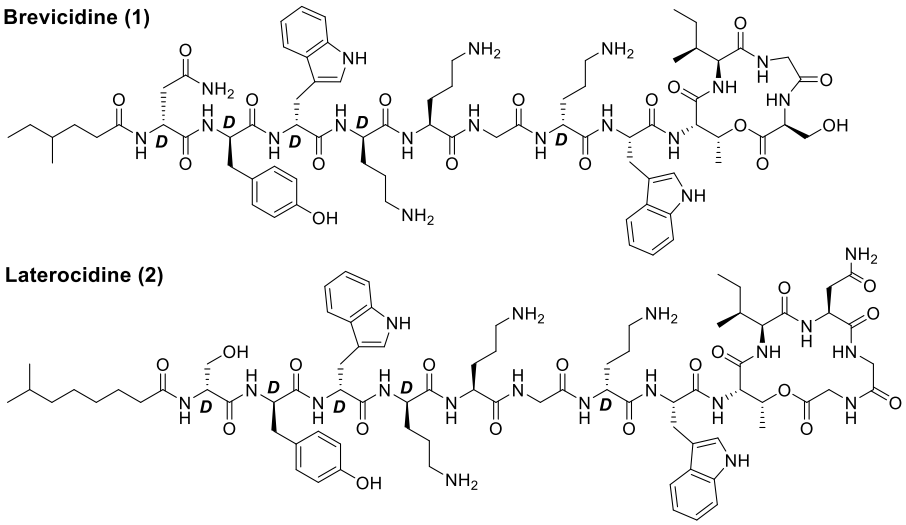


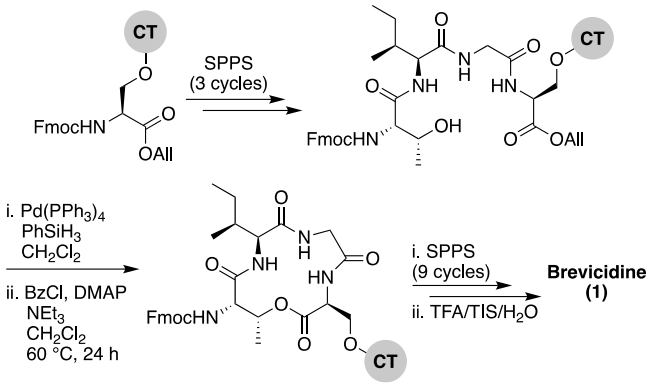
Figure 1. Structures of brevicidine (**1**) and laterocidine (**2**)

Results and discussion

Brevicidine and laterocidine share several structural features, including a C-terminal ester-linked macrocycle of 4- or 5- amino acids, a number of conserved residues including three positively charged ornithines, and a lipidated N-terminus. It was previously demonstrated that the macrocycle is necessary for antibacterial activity of these peptides.[6] Key to the syntheses of both brevicidine and laterocidine was therefore development of a reliable approach for the introduction of the macrocycle formed via an ester linkage between the C-terminal carboxylate and the corresponding threonine side chain hydroxyl group. For both brevicidine and laterocidine, convenient solid phase approaches were developed that allowed on-resin formation of the key macrocycle and installation of all other amino acids.

In approaching the synthesis of brevicidine, we initially investigated a strategy starting from Gly11 loaded on 2-chlorotrityl resin (CT) to generate a linear peptide that would subsequently be cyclized in solution. We envisaged installation of the required Thr9-Ser12 linkage as a preformed, ester-linked dipeptide. However, while incorporation of the Thr9-Ser12 unit was achieved, further elongation of the peptide failed due to an O→N acyl shift that occurred upon removal of the Thr9 Fmoc group (see supporting Scheme S1). As an alternative, we next examined formation of the macrocycle at an earlier stage to assess whether the ester linkage might be more stable when contained in the more conformationally restricted ring system. To implement this approach, Fmoc-Ser-OAll was loaded on to CT resin via its free side-chain hydroxyl group. Notably, the initial conditions used (1 h, RT) resulted in a lower loading than required (0.07 mmol/g), so the reaction time was extended to 24 h along with heating at 45 °C, resulting in an improved loading of 0.13 mmol/g. Resin-bound Fmoc-Ser-OAll was then extended to the tetrapeptide using standard Fmoc-SPPS (Scheme 1). At this stage, the C-terminal allyl ester was cleanly removed on resin using $Pd(PPh_3)_4/PhSiH_3$ in CH_2Cl_2. Subsequent closure of the macrolactone was then investigated using different coupling

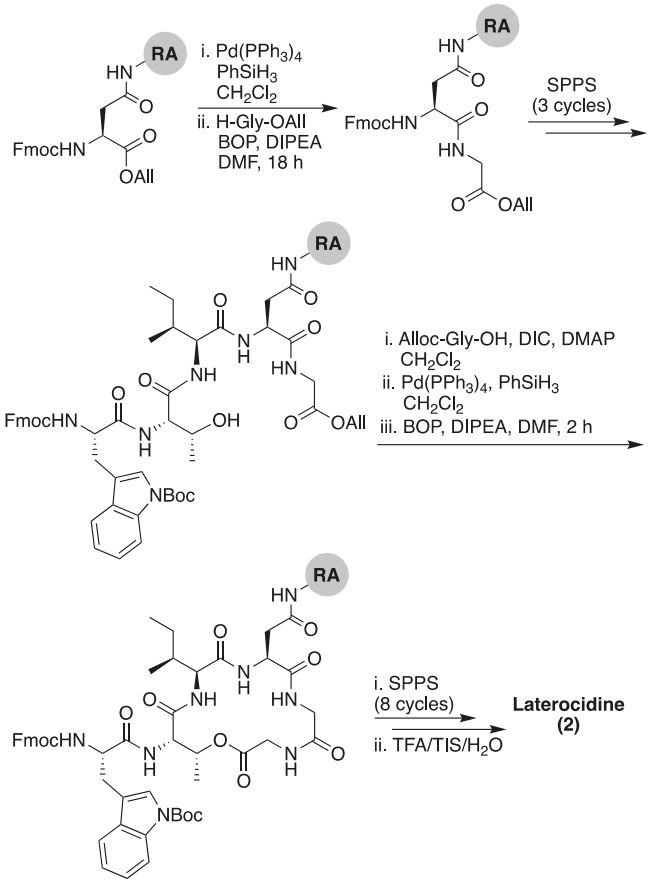
conditions. Cyclization of the on-resin tetrapeptide proved to be refractory to both the use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and diisopropylcarbodiimide (DIC), with only starting material being obtained after 48 h. To circumvent this lack of reactivity, we next attempted a modified Yamaguchi esterification, utilizing benzoyl chloride as the coupling reagent, along with a 24 h reaction time run at RT. Although only a small amount of the desired cyclic product was obtained using these conditions, increasing the reaction temperature to 60 °C resulted in near complete conversion. Building from the successful formation of the resin-bound tetrapeptide macrolactone, all that remained was to extend the peptide from the N-terminal threonine residue with SPPS. It was thought best to proceed with caution at the initial Fmoc deprotection to avoid unwanted *O*→*N* acyl migration. Therefore, less aggressive Fmoc deprotection conditions (10% piperidine in DMF) were used in the first deprotection. Gratifyingly, we did not detect any *O*→*N* acyl migration, even using standard Fmoc deprotection conditions, validating our hypothesis that preforming the C-terminal macrocycle would overcome this issue. The remainder of the peptide was constructed without incident along with coupling of the N-terminal 4-methylhexanoic acid. Following global deprotection and resin cleavage and purification by RP-HPLC, synthetic brevicidine was obtained in an overall yield of 9% over 28 steps.



Scheme 1. Total SPPS of brevicidine (**1**). CT = 2-chlorotrityl resin.

The synthetic strategy initially pursued for the preparation of laterocidine was inspired by the successful route developed for brevicidine (see supporting Scheme S2). Unfortunately, formation of the macrolactone proved refractory towards a variety of conditions, including the modified Yamaguchi esterification, with all failing to provide the desired product in appreciable yield. We ascribe this difficulty in ester formation to the larger five-amino-acid macrocycle found in laterocidine versus the four-amino-acid ring found in brevicidine. As an alternative, we next investigated the possibility of closing the macrocycle via amide bond formation between Gly12 and Gly13 (Scheme 2). Following allyl ester cleavage of Fmoc-Asp-OAll loaded Rink amide (RA) resin, an allyl ester protected Gly was coupled after which the peptide was built out to Trp8. The ester linkage between the free Thr9 side chain hydroxyl and the Gly13 C-terminal carboxylate was successfully introduced by coupling Alloc-Gly-OH using an on-resin Steglich esterification approach inspired by the Albericio group's synthesis

of pipecolidepsin A.[10] Following removal of the allyl and Alloc protecting groups, a BOP/DIPEA mediated macrolactamization resulted in the clean formation of the macrocyclic product. From there the peptide was completed using standard Fmoc-SPPS conditions, including N-terminal lipidation with isopelargonic acid. Following cleavage from resin and global deprotection, the crude lipopeptide was subsequently purified using RP-HPLC, yielding laterocidine in 2% purified yield over 29 steps.



Scheme 2. Total SPPS of laterocidine (**2**). RA = Rink amide resin.

Our route to brevicidine and laterocidine was originally disclosed in preprint form (uploaded to ChemRxiv on 10 Jan 2021).[9] Subsequently, Hermant and co-workers reported a different synthetic strategy also providing access brevicidine and laterocidine.[11] As described above, our syntheses are performed entirely on solid support, wherein a side-chain residue is immobilized (Ser 12 for brevicidine and Asn11 for laterocidine) allowing for on resin formation of the peptide macrocycle. Hermant and co-workers opted for an alternate approach combining solid-phase and solution phase synthesis, wherein linear peptide precursors were assembled on resin followed by macrolactamization in solution.

To confirm the equivalency of the synthetic and natural lipopeptides, their ^{1}H-NMR spectra were compared with published data for the natural products, revealing them to be indistinguishable (Figure 2, also see supporting Figures S1-2). In addition, LC-MS/MS analysis of the synthetic lipopeptides and comparison to the natural products further verified their identity (see experimental methods Figure 5). Antibacterial assays were also performed against a range of Gram-negative bacteria, which confirmed that synthetic brevicidine and laterocidine possess the same activity profile as the natural products (Table 1). To confirm that the antibacterial activity of brevicidine and laterocidine is intrinsically dependent on the chirality of the molecules, we synthesized the enantiomeric forms of both lipopeptides. The syntheses of *ent*-brevicidine (***ent*-1**) and *ent*-laterocidine (***ent*-2**) were achieved by following the same routes developed for the natural lipopeptides, but employing the corresponding mirror image amino acid building blocks (see supporting Schemes S3 and S4). Of note, similar mirror-image strategies have been used to characterize the stereochemical aspects in the antibacterial mechanisms of other peptide antibiotics.[12-16] In cases where an achiral target is implicated, the enantiomeric peptide antibiotic typically shows activity on par with the natural product, as in the case of bacitracin, which targets undecaprenyl pyrophosphate (C55-PP),[12] and laspartomycin, which targets undecaprenyl phosphate (C55-P).[13] Conversely, in the case of tridecaptin A1, which targets the chiral cell wall precursor lipid II,[14] thanatin, which targets the LptA and LptD proteins involved in LPS biosynthesis,[15] and daptomycin, which targets phosphatidylglycerol,[16] the activities of the corresponding enantiomers is significantly reduced. In their initial investigations with brevicidine and laterocidine, Li and co-workers found that addition of exogenous lipopolysaccharides (LPS) strongly antagonized the activity of both peptides, suggesting an interaction with LPS as part of the mechanism of action.[6] Given the inherent chirality of LPS, we hypothesized that *ent*-brevicidine and *ent*-laterocidine might exhibit decreased antibacterial activities relative to the natural products in the event that stereochemically defined binding interactions with LPS, rather than nonspecific electrostatic interactions, are central to the working mechanism of the lipopeptides. As confirmation of this hypothesis, the antibacterial activities of *ent*-brevicidine and *ent*-laterocidine were found

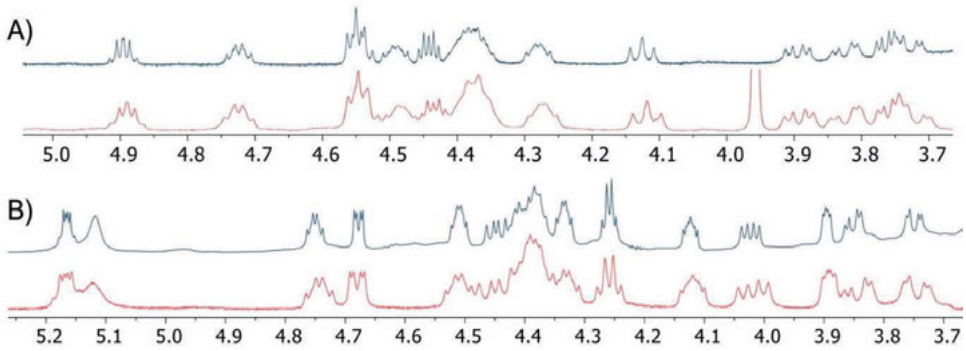
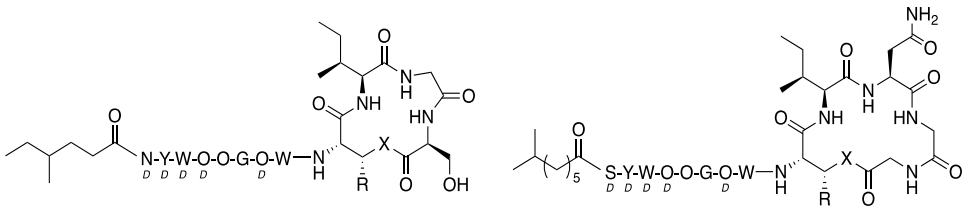


Figure 2. Overlaid portions of 1H-NMR spectra obtained for synthetic lipopeptides (blue traces) andpreviously published spectra (red traces) corresponding to: A) brevicidine (**1**) and B) laterocidine (**2**). The peak at ca. 3.96 ppm in the published spectrum of brevicidine is attributed to an impurity not present in the synthetic material. Spectra recorded in DMSO-d6 at RT.

to be consistently lower than the natural products, with the MIC values ranging from 8- to 32-fold higher (Table 1). Interestingly, the antibacterial activities of both laterocidine and *ent*-laterocidine were found to be antagonized upon addition of LPS (see experimental methods Figure 6). In the presence of LPS, the activities of laterocidine and *ent*-laterocidine decrease by 8-fold or more, indicating that the interaction with LPS may be driven by nonspecific electrostatic interactions. This finding suggest another chiral biomolecular target might be involved in the mechanism of action associated with these lipopeptides. Studies are underway to identify this target.

To probe the role of the Thr9 side chain methyl group on the conformational requirements of the brevicidine and laterocidine macrocycles, analogues were synthesized in which the Thr9 residue was substituted for Ser (Figure 3). The synthetic route used in preparing Ser9 analogues 3 and 4 was essentially the same as for the natural products (see supporting Schemes S5 and S6), although it was found that more strictly anhydrous conditions were necessary to prevent product degradation during the cyclization step. The antibacterial activities of the Ser9 analogues were similar to the natural products, suggesting the β-methyl group in Thr9 does not play a crucial role in dictating the biologically active conformation of either peptide.



Brev (**1**)**:** R = Me, X = O
Ser9-Brev (**3**)**:** R = H, X = O;
Dap9-Brev (**5**): R = H, X = NH
MeDap9-Brev (**7**): R = Me, X = NH

Lat (**2**)**:** R = Me, X = O
Ser9-Lat (**4**)**:** R = H, X = O;
Dap9-Lat (**6**): R = H, X = NH
MeDap9-Lat (**8**): R = Me, X = NH

Figure 3. Brevicidine and laterocidine analogues containing modified macrocycles. Non-ring amino acids shown as one-letter codes. D-amino acids labelled D.

We next explored the effect of replacing the ester linkage between the Thr9 side chain and the C-terminus with the corresponding amide. For several macrocyclic depsipeptide antibiotics, it has been shown that such amide for ester substitutions can have a positive effect on hydrolytic stability, as in the case of analogues of ramoplanin,[17] fusaricidin,[18] daptomycin,[19] and fengycin,[20] Additionally, from a synthetic perspective, macrocyclic ring closure via formation of an amide linkage can be more facile than formation of the corresponding macrolactone due to the enhanced reactivity of amines over alcohols. To this end, amide analogues of brevicidine and laterocidine were prepared, and their hydrolytic stability and antibacterial activities assessed. For both lipopeptides, we examined the effect of replacing Thr9 with either (*S*)-2,3-diaminopropanoic acid (Dap) or (2*S*, 3*R*)-2,3-diaminobutanoic acid (MeDap) to yield analogues **5**-**8** (Figure 3). The synthesis of Dap9-Brev (**5**) started from CT resin loaded with Fmoc-Ser-OAll via its side-chain hydroxyl group (Scheme 3). Iterative Fmoc-SPPS was then used to construct the linear protected on-resin tetrapeptide incorporating Fmoc-

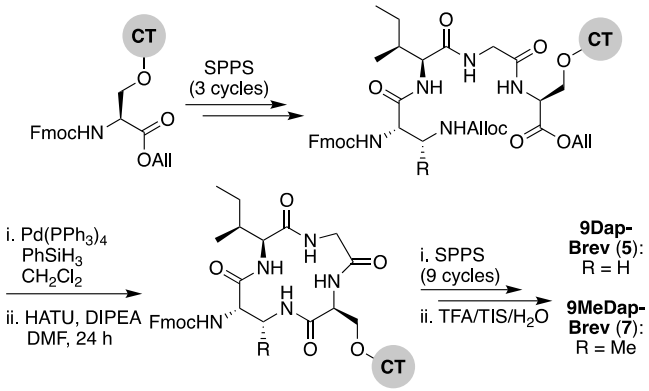
Table 1. *In vitro* minimum inhibitory concentrations (MICs) of peptide analogues determined using microbroth-dilution assays

	Brev (1)	*ent*-Brev (*ent*-1)	Ser9-Brev (3)	Dap9-Brev (5)	MeDap9-Brev (7)	Lat (2)	*ent*-Lat (*ent*-2)	Ser9-Lat (4)	Dap9-Lat (6)	MeDap9-Lat (8)	Col	PolyB
Ec ATCC 25922	1-2	16	4	8-16	16	1	8	0.5	1	1	0.25-0.5	0.5
Ec ATCC 25922 MCR-1	2	16	4	16	8	2	8-16	0.5	1-2	1	2-4	2-4
Ec MCR-1	1-2	16	2	8-16	8	0.5	8-16	0.5	1	0.5	2-4	2-4
Ec EQAS MCR-2	2	16-32	4-8	8-16	8-16	1	8-16	0.5-1	1	0.5	4	4
Kp ATCC 11228	1-2	32	2	16-32	8	2	16	1-2	2	1-2	<0.25	<0.25
Kp ATCC 13883	1	32	2	32	8	1	16	1-2	2	1-2	0.25	0.25
Kp 2048	1-2	32	4	16-32	8-16	2	16	2	2	2	0.5-1	0.5-1
Kp JS-123	1	8	2	8	4-8	0.5	4	0.5	0.5	0.5	<0.25	<0.25
Ab ATCC 17961	2-4	32	0.5-1	4	8-16	0.5	4	4	4	2	<0.25	<0.25
Ab ATCC 17978	4	16-32	2	16	16	0.5	4	2	4	2	<0.25	<0.25
Ab 2018-006	4	8-16	2	8	16	2	4	4	4	4	<0.25	<0.25
Ab MDR	4	16	1-2	8	16	1	4-8	2	4	4	<0.25	<0.25
Pa ATCC 27853	≤0.5	16	1	8	2-4	≤0.5	8	0.5-1	0.5-1	0.5	<0.25	<0.25
Pa PAO1	2	32	2-4	16-32	4	1	16	2	1-2	2	0.25-0.5	0.25-0.5
Pa NRZ-03961	2	32	4	16-32	8	1-2	16	1-2	2	2	0.25-0.5	0.25-0.5
Pa M-120	2-4	16	2	16	4	1	16	1	1-2	1	<0.25	<0.25
Sa USA300	64	>64	>64	>64	>64	64	64	>64	>64	32	>64	64

Ec = E. coli, Kp = K. pneumoniae, Ab = A. baumannii, Sa = S. aureus, Col = colistin, PolyB = polymyxin B. MICs reported in μg/mL.

Dap(Alloc)-OH. Both the allyl and Alloc groups were removed in a single deprotection with $Pd(PPh_3)_4/PhSiH_3$ in CH_2Cl_2. With the amine of Dap9 and the C-terminal carboxylic acid of the peptide liberated, an overnight macrolactamization was effected using HATU/DIPEA. Fmoc-SPPS was continued to complete the linear peptide backbone, followed by capping with 4-methylhexanoic acid. Finally, a global cleavage of the peptide from resin and subsequent purification by RP-HPLC yielded Dap9-Brev (**5**) in a 13% yield over 28 steps. The synthesis of the MeDap9-Brev (**7**) followed a similar strategy, with the exception that the corresponding Fmoc-MeDap(Alloc)-OH building block was incorporated at position 9. The antibacterial activities of brevicidine amide analogues **5** and **7** were evaluated, which in both cases revealed a significant loss of activity relative to brevicidine itself (Table 1). This reduced activity may be due to a loss of flexibility in the brevicidine macrocycle, caused by the ester to amide substitution preventing it from accessing its fully active conformation. In addition, the serum stability of Dap9-Brev (**5**) was assessed, revealing it to be much more stable than brevicidine

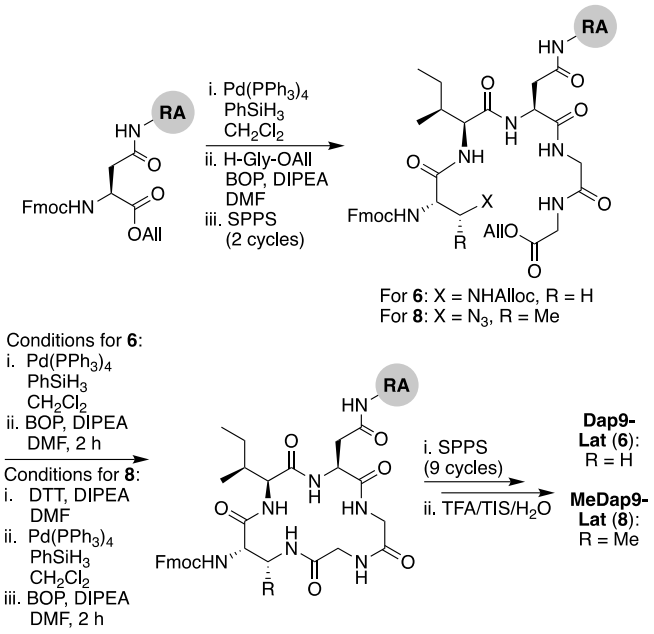
(see experimantal methods Figure 7). Dap9-Lat (**6**) was prepared from Fmoc-Asp-OAll loaded Rink amide resin (Scheme 4). Treatment with $Pd(PPh_3)_4/PhSiH_3$ in CH_2Cl_2, followed by activation and coupling to H_2N-Gly-Gly-OAll, yielded an on-resin tripeptide, which was then elongated to the macrocycle precursor. After removal of the Alloc and allyl groups, on-resin macrocyclization was effected using BOP/DIPEA. The peptide was then completed using standard Fmoc-SPPS conditions, including N-terminal lipidation with isopelargonic acid. After cleavage from the resin, and global deprotection, the crude lipopeptide was purified using RP-HPLC, yielding Dap9-Lat (**6**) in 3% overall yield over 27 steps. MeDap9-Lat (**8**) was synthesized following essentially the same on-resin protocol used for **6**, with the exception that the MeDap residue was installed as the corresponding azido species and reduced on-resin before the macrolactamization step (this strategy was also attempted for MeDap9-Brev (**7**) but the on-resin azide reduction step caused peptide decomposition).



Scheme 3. Total SPPS of Dap9-Brev (**5**) and MeDap9-Brev (**7**).

In addition to the total SPPS of Dap9-Lat (**6**), we also investigated an operationally more straightforward approach, wherein the amide linked macrocycle was formed in solution at the end of the synthesis (see supporting Scheme S7). This approach yielded Dap9-Lat (**6**) in an excellent overall yield of 29% (over 30 steps). Notably, this solution phase cyclization strategy was found to scale well, providing convenient access to **6** in multi-gram quantities. Assessment of the antibacterial activities of laterocidine amide analogues **6** and **8** revealed them to largely mirror the activity of laterocidine itself, with the exception of the *A. baumannii* strains tested (Table 1). This is in marked contrast to the significant loss of activity observed for the corresponding brevicidine amide analogues **5** and **7**, suggesting that the larger macrocycle present in laterocidine is more amenable to the ester-to-amide substitution. The serum stability of Dap9-Lat (**6**) was also found to be enhanced relative to laterocidine, with nearly 60% of the amide analogue still intact after 24 h incubation with serum, versus 45% for laterocidine itself (see experimantal methods Figure 7).

Building on these findings, brevicidine (**1**), laterocidine (**2**), and the synthetically more tractable amide analogues **5** and **6** were taken forward for further characterisation in cell toxicity assays. All compounds were found to be non-hemolytic up to the highest concentration tested (128 μg/mL) (see experimantal methods Figure 8). Brevicidine, laterocidine, and Dap9-Lat (**6**) were also found to be non-toxic to HepG2 cells at 128 μg/mL, while Dap9-Brev (**5**) showed



Scheme 4. Total SPPS of Dap9-Lat (**6**) and MeDap9-Lat (**8**).

a slight indication of toxicity at the same concentration (see experimantal methods Figure 9). Based on the favourable balance of antibacterial activity, stability, low cell toxicity, and synthetic accessibility, Dap9-Lat (**6**) was selected for further in vivo evaluation. To begin, the tolerability of the compound was assessed in naïve ICR mice, showing it to be well tolerated when dosed subcutaneously at 40 mg/kg every q8h over a 24 h period (total daily dose 120 mg/kg). Building from this, an efficacy study was performed wherein Dap9-Lat (**6**) was further assessed for its capacity to reduce thigh infection in neutropenic mice infected with *E. coli* ATCC 25922. To gain an indication of dose-response, Dap9-Lat (**6**) was administered subcutaneously q8h at 10, 20, and 40 mg/kg and compared with groups treated with vehicle or polymyxin B as a clinical reference antibiotic administered subcutaneously q8h at 20 mg/kg (Figure 4). A clear dose response was observed in the mice treated with Dap9-Lat (**6**), with the highest 40 mg/kg dose tested resulting in an approximate 5-log reduction in bacterial load relative to the untreated group, an antibacterial effect comparable to that observed for polymyxin B administered at 20 mg/kg. Notably, these *in vivo* activities reflect the results of the *in vitro* activity assays, wherein Dap9-Lat (**6**) and polymyxin B were found to have MIC values against *E. coli* ATCC 25922 of 1.0 and 0.5 μg/mL respectively (Table 1). It is worthy of mention that in the original report describing the discovery and characterization of brevicidine and laterocidine the quantities of material obtained from fermentation were not sufficient for comprehensive *in vivo* efficacy studies. In contrast, the convenient and scalable synthesis of laterocidine amide analogue **6** provides the opportunity for further optimization and evaluation of these promising lipopeptide antibiotics.

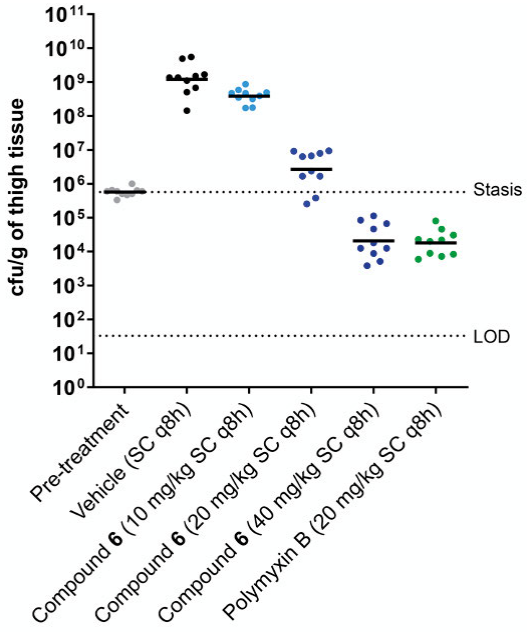


Figure 4. *In vivo* efficacy study. Scattergram of mouse thigh burdens (cfu/g) following infection with *E. coli* ATCC 25922 and treatment with test articles, as indicated on the x-axis. The geometric mean burden of each treatment is indicated by the horizontal bar. LOD = Limit of detection

Conclusions

In conclusion, we here report the total syntheses of the recently discovered lipodepsipeptide antibiotics brevicidine and laterocidine. In addition, a number of analogues of each were prepared including a particularly interesting variant of laterocidine wherein the ester linkage in the peptide macrocycle was substituted as an amide. This laterocidine analogue (**6**) maintains the potent anti-Gram-negative activity of the natural product *in vitro* and was also found to be efficacious *in vivo*. The *in vivo* efficacy of **6** is noteworthy given that in the original report describing the discovery of laterocidine, only modest *in vivo* activity was observed for the natural product,[6] likely attributable to the low quantities of material available for dosing, coupled with its hydrolytic instability. In contrast, laterocidine amide analogue **6** exhibits enhanced stability, is readily synthesized on gram-scale, and exhibits a clear dose-dependent effect *in vivo*.

The routes here reported also provide reliable access to the natural products themselves. Brevicidine was obtained in 28 steps from 2-chlorotrityl resin in an overall yield of 9%, and laterocidine was obtained in 29 steps from Rink-amide resin in an overall yield of 2%. In both syntheses, formation of the macrocycle on-resin at an early stage was found to be most effective, serving to limit deleterious O→N acyl shifts at the ester linkage on Thr9, and provided the desired peptides as the major products after cleavage from resin, allowing

for facile HPLC purification. Overall, both synthetic strategies are highly robust, yielding brevicidine and laterocidine in quantities that compare well with those obtained by isolation of the natural products from fermentation of the producing organisms. Synthetic brevicidine and laterocidine were shown to have identical 1H-NMR spectra and RP-HPLC elution profiles compared to their natural counterparts, confirming the previously reported structures. The antibacterial activities of synthetic brevicidine and laterocidine were also assessed against a panel of Gram-negative pathogens, demonstrating their potent antibacterial effect.

The methodology reported offers an efficient alternative to isolating these promising natural products from bacterial fermentation, and in doing so provides access to quantities of material suitable for further evaluation and mechanistic studies. Furthermore, the synthetic approaches we describe also provide access to novel analogues of both brevicidine and laterocidine. Of particular note is the finding that the enantiomeric forms of both brevicidine and laterocidine exhibit severely reduced antibacterial activities, a finding that supports a mechanism of action involving a stereospecific interaction with the bacterial target. Also of note is the finding that the macrocycles in brevicidine and laterocidine have varying tolerances for modification. Specifically, the ester-to-amide substitution, investigated as a means of both enhancing hydrolytic stability and increasing synthetic accessibility, was found to be detrimental to the activity of the brevicidines, while the laterocidine amide analogues largely maintain the activity of the natural product. Evaluation of the serum stability, haemolytic activity, and eukaryotic cell toxicity of the brevicidines and laterocidines here investigated in turn led the selection of laterocidine amide analogue **6** for further *in vivo* assessment. As noted above, these studies showed compound **6** to be well tolerated and capable of effectively reducing bacterial infection in a murine thigh-infection model. Also, while the clinically used polymyxins were found to be consistently more active (generally 2-to-4-fold lower MICs) than the brevicidines and laterocidines here studied, it is notable that in the case of *mcr*-positive polymyxin resistant strains, the brevicidines and laterocidines maintained their antibacterial activity. Given the "last-resort" status of the polymyxins, it is imperative that new antibacterial agents capable of overcoming polymyxin resistance be pursued.

In light of the increasing occurrence of Gram-negative pathogens with resistance to conventional antibacterial therapies, the brevicidine and laterocidine family of lipopeptide antibiotics represent promising leads for further development. In this regard, the recent discovery of the relacidines,[21] which show high structural similarity to laterocidine, indicates that these lipopeptide antibiotics may be widespread in nature. The encouraging results we here report pave the way for future investigations aimed at further developing these promising lipopeptides with an eye to more fully characterizing their therapeutic potential. To this end, the synthetic approaches here described provide a convenient means to access structurally diverse analogues. In addition, mechanistic studies into how the brevicidines and laterocidines prevent the growth of polymyxin resistant strains are ongoing and will be reported in due course.

Experimental methods

Reagents and general methods

All reagents employed were of American Chemical Society (ACS) grade or higher and were used without further purification unless otherwise stated. Fmoc-Ser-OAll,[22] Fmoc-Asp-OAll,[23] TFA·NH_2-Gly-OAll,[24] TFA·NH_2-Gly-Gly-OAll[25] and (2S,3R)-2-((((9H-fluoren-9-yl) methoxy)carbonyl)amino)-3-azidobutanoic acid[26] were synthesized according to referenced literature procedures. The NMR characterization of brevicidine in DMSO-d_6 was obtained using a Bruker Ascend 600 (600 MHz). The NMR characterization of laterocidine in DMSO-d_6 was obtained on a Bruker AV850 spectrometer (850 MHz). LC-MS analyses were performed on a Shimadzu LC-20AD system with a Shimadzu Shim-Pack GISS-HP C18 column (3.0 x 150 mm, 3 μm) at 30°C and equipped with a UV 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, acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 13 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min. This system was connected to a Shimadzu 8040 triple quadrupole mass spectrometer (ESI ionisation). The NMR characterisation of brevicidine and derivatives in DMSO-d_6 was obtained using a Bruker Ascend 600 (600 MHz). LC-MS analyses were conducted on an Agilent 1260 HPLC (equipped with an Infinity II quaternary pump, vial sampler, integrated column compartment and a variable wavelength detector) and MSD single quadropole mass spectrometer. Samples were analysed using an Agilent Infinitylab poroshell 120 column (2.1 x 150 mm, 2.7 μm) under acetonitrile/water gradient with 0.1% formic acid as an additive. The following solvent system, at a flow rate of 0.3 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 to 0:100 (A/B) over 5 min, 0:100 (A/B) for 1 min, then reversion back to 95:5 (A/B) over 0.1 min.

Synthesis of brevicidine (1)

To a flame dried 25 mL round bottom flask was added Fmoc-Ser-OAll (110 mg, 0.300 mmol) and dry dichloromethane (DCM) (10.0 mL). 2-Chlorotrityl chloride resin (CT) (1.00 g, 0.81 mmol g^{-1}) and DIPEA (210 μL, 1.20 mmol) were added. The suspension was stirred under argon for 48 h, after which the resin was filtered through a manual SPPS vessel and washed with DCM (4 x 5 mL). The resin was then capped by adding a solution of methanol, DIPEA and DCM (3 mL, 10 : 5 : 85) and bubbled with argon for 1 h. The solution was discharged and the resin was washed with DCM (3 x 5 mL) before being dried under a stream of argon. A small portion of resin was then used to ascertain the loading. Estimation of loading level of first residue onto resin (0.15 mmol g^{-1}) was calculated via an Fmoc loading test, as described by Gude *et al.*[27]

Standard Fmoc SPPS protocol was used to extend the peptide to the linear Fmoc-Thr-Ile-Gly-Ser stage. Specifically, resin (670 mg, 0.1 mmol) was added to a manual SPPS vessel and bubbled in DMF (3 mL) to swell. The solvent was discharged and the resin was bubbled in an Fmoc deprotection solution of 20% piperidine in DMF (3 x 3 mL, 2 x 1 min then 1 x 5 min) with argon. The resin was washed with DMF (3 x 3 mL) and a coupling solution of amino acid (6 equiv), HATU (6 equiv) and DIPEA (12 equiv) in DMF (3 mL) was added. The solution was then bubbled with argon for 1 h, before the solution was discharged and the resin washed with DMF (3 x 3 mL). This process was repeated to obtain on-resin linear Fmoc-

tetrapeptide. At this stage the resin was split and a portion of this on-resin allyl-protected tetrapeptide (78.0 mg. 0.01 mmol) was added to a manual SPPS vessel and bubbled in DCM (3 mL) with argon for 15 min. The solvent was discharged and an allyl deprotection solution of tetrakis(triphenylphosphine) palladium (231 mg, 0.200 mmol) and phenylsilane (123 µL, 0.998 mmol) in DCM and DMF (1:1, 2 mL) was added. The solution was bubbled with argon for 2 h in darkness, after which the deprotection solution was discharged and the resin was washed with DMF (3 x 3 mL), 0.5 % sodium diethyldithiocarbamate solution in DMF (4 x 5 mL), DMF (4 x 5 mL) and DCM (4 x 5 mL). The resin was dried under argon, then added to a 5 mL flame dried round bottom flask under argon. Dry dichloromethane (3 mL) was added and the suspension stirred for 15 min. Benzoyl chloride (13.0 µL, 0.112 mmol), triethylamine (3.00 µL, 22.0 µmol) and catalytic DMAP (1 crystal) were added and the reaction mixture was stirred overnight at 60 °C. The resin was then filtered through a manual SPPS vessel and washed with DMF (3 x 5 mL) and DCM (3 x 5 mL) before being dried under argon. To ascertain reaction progress, a small sample was cleaved using a 2 % TFA solution in DCM (1 mL). The cleavage cocktail was gently agitated for 1 h and filtered through a glass wool plug. The filtrate was concentrated with a gentle stream of argon before being analysed by LC-MS. Desired cyclic product was identified ($[M+H]^+$ calculated for $C_{30}H_{36}N_4O_8$ 581.2, found (LC-MS) 581.5) Following this modified Yamaguchi esterification, the synthesis of brevicidine was completed using standard Fmoc SPPS protocols as described above, after which 4-methylhexanoic acid was coupled to the N-terminus. The dried resin was then added to a cleavage cocktail of TFA, TIPS and distilled water (10 mL, 95 : 2.5 : 2.5) and heated to 37°C for 1 h. The suspension was filtered through a glass wool plug and the filtrate concentrated under vacuum. Diethyl ether was used to precipitate the crude peptide, which was then centrifuged and washed with additional diethyl ether. The suspension was centrifuged and the pellet dissolved in a minimal amount of 1:1 acetonitrile and water solution with 0.1% TFA. The crude mixture was subsequently purified by RP-HPLC (See HPLC purification of synthetic peptides).Fractions were assessed by LC-MS and product-containing fractions were pooled, frozen and lyophilized to yield brevicidine as a white powder. Yield: 13 mg, 9% over 28 steps. HPLC retention time 23.3 min (Method A); $[M+3H]^{3+}$ calculated for $C_{74}H_{106}N_{18}O_{17}$ 507.2734, found (HR-MS) 507.2688.

Synthesis of *ent*-brevicidine (*ent*-1)

To a flame dried 25 mL round bottom flask was added Fmoc-D-Ser-OAll (112 mg, 0.305 mmol) and dry dichloromethane (10.0 mL). 2-Chlorotrityl chloride resin (CT) (1.00 g, 0.81 mmol g^{-1}) and DIPEA (210 µL, 1.20 mmol) were added. The suspension was stirred under argon for 48 h at 45 °C, after which the resin was filtered through a manual SPPS vessel and washed with DCM (4 x 5 mL). The resin was then capped and loading level of the first residue onto resin (0.25 mmol g^{-1}) was calculated as per the synthesis of brevicidine.

Standard Fmoc SPPS protocol was used to extend the peptide to the linear Fmoc-D-Thr-D-Ile-Gly-D-Ser peptide on a 0.1 mmol scale (400 mg) similar to the synthesis of brevicidine. Following SPPS of the tetrapeptide, an allyl deprotection solution of tetrakis (triphenylphosphine) palladium (231 mg, 0.200 mmol) and phenylsilane (123 µL, 0.998 mmol) in DCM and DMF (1:1, 2 mL) was added. The solution was bubbled with argon for 2 h in darkness, after which the deprotection solution was discharged and the resin was washed with DMF (3 x 3 mL), 0.5 % sodium diethyldithiocarbamate solution in DMF (4 x 5 mL),

DMF (4 x 5 mL) and DCM (4 x 5 mL). The resin was dried under argon, then added to a 5 mL flame dried round bottom flask under argon. Dry dichloromethane (3 mL) was added and the suspension stirred for 15 min. Benzoyl chloride (13.0 µL, 0.112 mmol), triethylamine (3.00 µL, 22.0 µmol) and catalytic DMAP (1 crystal) were added and the reaction mixture was stirred overnight at 60°C. The resin was then filtered through a manual SPPS vessel and washed with DMF (3 x 5 mL) and DCM (3 x 5 mL) before being dried under argon. A small sample was cleaved using a 2 % TFA solution in DCM (1 mL). The cleavage cocktail was gently agitated for 1 h and filtered through a glass wool plug. The filtrate was concentrated with a gentle stream of argon before being analysed by LC-MS ($[M+Na]^+$ calculated for $C_{30}H_{36}N_4O_8$ 603.2, found (LC-MS) 603.5). Following this modified Yamaguchi esterification, the synthesis of *ent*-brevicidine was completed using standard Fmoc SPPS protocols as described above after which 4-methylhexanoic acid was coupled to the N-terminus. The peptide was cleaved from resin, precipitated and purified by RP-HPLC (See HPLC purification of synthetic peptides) following the procedure for synthesising brevicidine. Yield: 3 mg, 2% over 28 steps. HPLC retention time 22.9 min; $[M-H]^-$ calculated for $C_{74}H_{106}N_{18}O_{17}$ 1517.7910, found (HR-MS) 1517.7943.

Synthesis of Ser9-brevicidine (3)

The desired Fmoc-tetrapeptide was synthesized from Fmoc-Ser-OAll as described above, with Fmoc-Ser used in place of Fmoc-Thr. This resin-bound tetrapeptide (0.065 mmol, 0.14 mmol/g) was added to a manual SPPS vessel and bubbled with DMF (5 mL) for 15 min then the solvent was discharged. An allyl deprotection solution of tetrakis (triphenylphosphine) palladium (150 mg, 0.130 mmol) and phenylsilane (80.0 µL, 0.649 mmol) in DCM and DMF (1:1, 2 mL) was added. The solution was bubbled with argon for 2 h in darkness, after which the deprotection solution was discharged and the resin was washed with DMF (3 x 3 mL), 0.5 % sodium diethyldithiocarbamate solution in DMF (4 x 5 mL), DMF (4 x 5 mL) and DCM (4 x 5 mL). The resin was dried under argon, then added to a 5 mL flame dried round bottom flask under argon. Dry dichloromethane (3 mL) was added and the suspension stirred for 15 min. Benzoyl chloride (8.00 µL, 68.9 µmol), triethylamine (20.0 µL, 0.143 mmol) and catalytic DMAP (1 crystal) were added and the reaction mixture was stirred overnight at 60°C. The resin was then filtered through a manual SPPS vessel and washed with DMF (3 x 5 mL) and DCM (3 x 5 mL) before being dried under argon. A small sample was cleaved using a 2 % TFA solution in DCM (1 mL). The cleavage cocktail was gently agitated for 1 h and filtered through a glass wool plug. The filtrate was concentrated with a gentle stream of argon before being analysed by LC-MS ($[M+H]^+$ calculated for $C_{30}H_{36}N_4O_8$ 567.2, found (LC-MS) 567.5). Following this modified Yamaguchi esterification, the synthesis of Ser9-brevicidine was completed using standard Fmoc SPPS protocols as described above. The peptide was cleaved from resin, precipitated and purified by RP-HPLC (See HPLC purification of synthetic peptides) following the procedure for synthesising brevicidine. Yield: 13 mg, 13% over 28 steps. HPLC retention time 22.1 min; $[M+2H]^{2+}$ calculated for $C_{73}H_{104}N_{18}O_{17}$ 753.3986, found (HR-MS) 753.3980

Synthesis of Dap9-brevicidine (5)

The desired Fmoc-tetrapeptide was synthesized from Fmoc-Ser-OAll as described above, with Fmoc-Dap(Alloc) used in place of Fmoc-Thr. This resin-bound tetrapeptide (0.1 mmol, 0.13 mmol/g) was added to a manual SPPS vessel and bubbled with DMF (5 mL) for 15 min then

the solvent was discharged. An allyl deprotection solution of tetrakis (triphenylphosphine) palladium (462 mg, 0.400 mmol) and phenylsilane (246 μL, 2.00 mmol) in DCM and DMF (1:1, 8 mL) was added. The solution was bubbled with argon for 2 h in darkness, after which the deprotection solution was discharged and the resin was washed with DMF (3 x 3 mL), 0.5 % sodium diethyldithiocarbamate solution in DMF (6 x 10 mL), DMF (4 x 5 mL) and DCM (4 x 5 mL). A solution of HATU (76.0 mg, 0.200 mmol) and DIPEA (70.0 μL, 0.402 mmol) in DMF (3 mL) and bubbled with argon overnight. The coupling solution was discharged, and the resin was washed with DMF (3 x 5 mL) then DCM (3 x 5 mL) and dried under a stream of argon. The synthesis of Dap9-brevicidine was completed using standard Fmoc SPPS protocols as described above. The peptide was cleaved from resin, precipitated and purified by RP-HPLC (See HPLC purification of synthetic peptides) following the procedure for synthesising brevicidine. Yield: 20 mg, 13% over 28 steps. HPLC retention time 21.6 min; $[M+2H]^{2+}$ calculated for $C_{73}H_{105}N_{19}O_{16}$ 752.9066, found (HR-MS) 752.9368.

Synthesis of MeDap9-brevicidine (7)

The desired Fmoc-tetrapeptide was synthesized from Fmoc-Ser-OAll as described above, with Fmoc-MeDap(Alloc)-OH (IUPAC name: (2S,3R)-2-[[(9H-fluoren-9-ylmethoxy)carbonyl] amino]-3-[[(2-propen-1-yloxy)carbonyl]amino]-butanoic acid) used in place of Fmoc-Thr. The synthesis of Fmoc-MeDap(Alloc)-OH was adapted from a previously reported literature precedent (R. Moran Ramallal, R. Liz & V. Gotor, *J. Org. Chem.*, 2010, 75, 19, 6614-6624) - To a flame dried 25 mL round bottom flask was added Fmoc-L-Abu(3*R*-N_3)-OH (146 mg, 0.398 mmol) and 10% Pd/C (63 mg, 59.2 μmol). The flask was evacuated and a balloon of hydrogen was attached. Methanol (10 mL) was added and the suspension was stirred for 30 min before being filtered through a celite plug. The filtrate was concentrated under vacuum and re-dissolved in dichloromethane (10 mL). The solution was stirred at 0 °C then allyl chloroformate (51 μL, 0.478 mmol) and DIPEA (83 μL, 0.478 mmol) were added. The reaction was stirred at 0 °C for 3 h and concentrated under vacuum. The crude solid was subsequently purified by flash chromatography (1 % MeOH in DCM with 1% acetic acid). The fractions containing product were pooled, concentrated and co-evaporated with toluene (3 x 10 mL) then chloroform (3 x 10 mL) to yield a white solid (70 mg, 41%); TLC: R_f 0.44 (10 % MeOH in DCM); α_D^{20}: +31.9 (0.6, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (2H, d, *J* = 7.5 Hz, Fmoc), 7.58 (2H, t, *J* = 6.7 Hz, Fmoc), 7.38 (2H, t, *J* = 7.3 Hz, Fmoc), 7.28 (2H, t, *J* = 7.3 Hz, Fmoc), 6.09 (1H, d, *J* = 7.7 Hz, -N**H**), 5.84 (1H, br, -OCH_2C**H**=CH_2), 5.32 (1H, d, *J* = 6.7 Hz, -$CHCH(CH_3)$N**H**-) 5.23 (1H, d, *J* = 17.1 Hz, -OCH_2CH=C**H**$_2$), 5.15 (1H, d, *J* = 9.9 Hz, -OCH_2CH=C**H**$_2$), 4.52 – 4.35 (6H, m, -C**H**$CH(CH_3)$NH-, -CHC**H**(CH_3)NH-, Fmoc-CHC**H**$_2$), 4.19 (1H, t, *J* = 6.9 Hz, Fmoc-C**H**CH_2), 1.23 (3H, br, -$CHCH(C$**H**$_3)$NH-); $[M+H]^+$ calculated for, $C_{23}H_{24}N_2O_6$ 425.1707, found (LC-MS) 425.4.

This resin-bound tetrapeptide (0.05 mmol, 0.14 mmol/g) was added to a manual SPPS vessel and bubbled with DMF (5 mL) for 15 min then the solvent was discharged. An allyl deprotection solution of tetrakis (triphenylphosphine) palladium (231 mg, 0.200 mmol) and phenylsilane (123 μL, 0.998 mmol) in DCM and DMF (1:1, 6 mL) was added. The solution was bubbled with argon for 2 h in darkness, after which the deprotection solution was discharged and the resin was washed with DMF (3 x 3 mL), 0.5 % sodium diethyldithiocarbamate solution in DMF (6 x 10 mL), DMF (4 x 5 mL) and DCM (4 x 5 mL). A solution of HATU (95.0 mg, 0.250 mmol) and DIPEA (87.0 μL, 0.499 mmol) in DMF (3 mL) and bubbled with argon overnight

at 50 °C. The coupling solution was discharged, and the resin was washed with DMF (3 x 5 mL) then DCM (3 x 5 mL) and dried under a stream of argon. A small sample was cleaved using a 2 % TFA solution in DCM (1 mL). The cleavage cocktail was gently agitated for 1 h and filtered through a glass wool plug. The filtrate was concentrated with a gentle stream of argon before being analysed by LC-MS ($[M+H]^+$ calculated for $C_{30}H_{36}N_4O_8$ 580.3, found (LC-MS) 580.6). The synthesis of MeDap9-brevicidine was completed using standard Fmoc SPPS protocols as described above. The peptide was cleaved from resin, precipitated and purified by RP-HPLC (See HPLC purification of synthetic peptides) following the procedure for synthesising brevicidine. Yield: 3 mg, 2% over 28 steps. HPLC retention time 21.2 min; $[M+H]^+$ calculated for $C_{74}H_{107}N_{19}O_{16}$ 1518.8216, found (HR-MS) 1518.4032.

Synthesis of laterocidine (2)

Rink Amide MBHA resin (5.0 g, 0.67 mmol g^{-1}) was loaded by overnight coupling via the free sidechain carboxylate of Fmoc-Asp-OAll (2.65 g, 6.70 mmol, 2 eq.) with BOP (2.96 g, 6.70 mmol, 2 eq.) and DiPEA (2.33 mL, 13.4 mmol, 4 eq.) in 150 mL of DMF. After capping with AcO_2 : pyridine (3 : 2, v/v) for 30 min the resin loading was determined to be 0.37 mmol g^{-1} . The loaded resin (680 mg, 0.25 mmol) was treated with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol, 0.3 eq.) and $PhSiH_3$ (0.75 mL, 7.5 mmol, 30 eq.) in DCM (ca. 15 mL) under nitrogen for 1 h. The resin was subsequently washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). TFA·H_2N-Gly-OAll (115 mg, 0.5 mmol, 2 eq.) was then coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 µL, 1.0 mmol, 4 eq.) under nitrogen flow for 1 h. The next 3 amino acids (Ile10, Thr9, Trp8) were coupled manually (1 h) under nitrogen flow via standard Fmoc solid-phase peptide synthesis (SPPS) (resin : Fmoc-AA : BOP : DiPEA, 1 : 4 : 4 : 8 molar eq.). DMF (5 mL) was used as solvent and Fmoc deprotections (2 min then 10 min) were carried out with 5 mL piperidine : DMF (1 : 4, v/v). The following Fmoc amino acids were used: Fmoc-Ile-OH, Fmoc-Thr-OH (used without side chain protection), and Fmoc-Trp(Boc)-OH. After coupling of Fmoc-Trp(Boc)-OH esterification of the Thr side chain was achieved by treating the resin-bound peptide with Alloc-Gly-OH (596 mg, 3.75 mmol, 15 eq.), DIC (0.59 mL, 3.75 mmol, 15 eq.) and DMAP (15 mg, 0.13 mmol, 0.5 eq.) in 8 mL DCM : DMF (3 : 1, v/v) for 18 h under nitrogen. The resin was treated with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol) and $PhSiH_3$ (0.75 mL, 7.5 mmol) in DCM (ca. 15 mL) under nitrogen for 2 h before being washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). The peptide was then cyclized using BOP (442 mg, 1.0 mmol, 4 eq.) and DiPEA (0.35 mL, 2.0 mmol, 8 eq.) for 2 h in 5 mL DMF under nitrogen flow. The remaining linear N-terminal section of the peptide was then synthesized using the standard SPPS protocol mentioned above. The following Fmoc amino acids were used: Fmoc-D-Ser(tBu)-OH, Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-Gly-OH. Following the final Fmoc removal step, isopelargonic acid (79 mg, 0.5 mmol, 2 eq.) was coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 µL, 1.0 mmol, 4 eq.) in 5 mL of DMF overnight, under nitrogen flow. Final deprotection was carried out by treating the resin with TFA : TIS : H_2O (95 : 2.5 : 2.5, 10 mL) for 90 min. The reaction mixture was filtered through cotton, the filtrate precipitated in MTBE : petroleum ether (1 : 1), and the resulting precipitate washed once more with MTBE : petroleum ether (1 : 1). The crude cyclic peptide was lyophilized from tBuOH : H_2O (1 : 1) and purified with reverse phase HPLC

(See HPLC purification of synthetic peptides). Pure fractions were pooled and lyophilized to yield laterocidine in >95% purity as a white powder. Yield: 8 mg, 2% over 29 steps. $[M+2H]^{2+}$ calculated for, $C_{78}H_{113}N_{19}O_{18}$ 802,9329, found (HR-MS) 802,9326.

Synthesis of *ent*-laterocidine (ent-2)

Rink Amide MBHA resin (5.0 g, 0.67 mmol g^{-1}) was loaded by overnight coupling via the free sidechain carboxylate of Fmoc-D-Asp-OAll (2.65 g, 6.70 mmol, 2 eq.) with BOP (2.96 g, 6.70 mmol, 2 eq.) and DiPEA (2.33 mL, 13.4 mmol, 4 eq.) in 150 mL of DMF. After capping with AcO_2 : pyridine (3 : 2, v/v) for 30 min the resin loading was determined to be 0.37 mmol g^{-1} . The loaded resin (675 mg, 0.25 mmol) was treated with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol, 0.3 eq.) and $PhSiH_3$ (0.75 mL, 7.5 mmol, 30 eq.) in DCM (ca. 15 mL) under nitrogen for 1 h. The resin was subsequently washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). TFA·H_2N-Gly-OAll (115 mg, 0.5 mmol, 2 eq.) was then coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 µL, 1.0 mmol, 4 eq.) under nitrogen flow for 1 h. The next 3 amino acids (D-Ile10, D-Thr9, D-Trp8) were coupled manually (1 h) under nitrogen flow via standard Fmoc solid-phase peptide synthesis (SPPS) (resin : Fmoc-AA : BOP : DiPEA, 1 : 4 : 4 : 8 molar eq.). DMF (5 mL) was used as solvent and Fmoc deprotections (2 min then 10 min) were carried out with 5 mL piperidine : DMF (1 : 4, v/v). The following Fmoc amino acids were used: Fmoc-D-Ile-OH, Fmoc-D-Thr-OH (used without side chain protection), and Fmoc-D-Trp(Boc)-OH. After coupling of Fmoc-D-Trp(Boc)-OH esterification of the D-Thr side chain was achieved by treating the resin-bound peptide with Alloc-Gly-OH (596 mg, 3.75 mmol, 15 eq.), DIC (0.59 mL, 3.75 mmol, 15 eq.) and DMAP (15 mg, 0.13 mmol, 0.5 eq.) in 8 mL DCM : DMF (3 : 1, v/v) for 18 h under nitrogen. The resin was treated with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol) and $PhSiH_3$ (0.75 mL, 7.5 mmol) in DCM (ca. 15 mL) under nitrogen for 2 h before being washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). The peptide was then cyclized using BOP (442 mg, 1.0 mmol, 4 eq.) and DiPEA (0.35 mL, 2.0 mmol, 8 eq.) for 2 h in 5 mL DMF under nitrogen flow. The remaining linear N-terminal section of the peptide was then synthesized using the standard SPPS protocol mentioned above. The following Fmoc amino acids were used: Fmoc-L-Ser(tBu)-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-L-Trp(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-Gly-OH. Following the final Fmoc removal step, isopelargonic acid (79 mg, 0.5 mmol, 2 eq.) was coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 µL, 1.0 mmol, 4 eq.) in 5 mL of DMF overnight, under nitrogen flow. Final deprotection was carried out by treating the resin with TFA : TIS : H_2O (95 : 2.5 : 2.5, 10 mL) for 90 min. The reaction mixture was filtered through cotton, the filtrate precipitated in MTBE : petroleum ether (1 : 1), and the resulting precipitate washed once more with MTBE : petroleum ether (1 : 1). The crude cyclic peptide was lyophilized from tBuOH : H_2O (1 : 1) and purified with reverse phase HPLC (See HPLC purification of synthetic peptides). Pure fractions were pooled and lyophilized to yield *ent*-laterocidine in >95% purity as a white powder. Yield: 30 mg, 7.4% over 29 steps. $[M+2H]^{2+}$ calculated for, $C_{78}H_{113}N_{19}O_{18}$ 802,9329, found (HR-MS) 802,9327.

Synthesis of Ser9-laterocidine (4)

The loaded resin (274 mg, 0.1 mmol) was treated with $Pd(PPh_3)_4$ (30 mg, 0.03 mmol, 0.3 eq.) and $PhSiH_3$ (0.30 mL, 3.0 mmol, 30 eq.) in DCM (ca. 7 mL) under nitrogen for 1 h. The resin was

subsequently washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). TFA·H_2N-Gly-OAll (115 mg, 0.5 mmol, 2 eq.) was then coupled using BOP (88 mg, 0.2 mmol, 2 eq.) and DiPEA (87 µL, 0.4 mmol, 4 eq.) under nitrogen flow for 2 h. The next 3 amino acids (Ile10, Ser9, Trp8) were coupled manually (1 h) under nitrogen flow via standard Fmoc solid-phase peptide synthesis (SPPS) (resin : Fmoc-AA : BOP : DiPEA, 1 : 4 : 4 : 8 molar eq.). Dry DMF (3 mL) was used as solvent and Fmoc deprotections (2 min then 10 min) were carried out with 3 mL piperidine : DMF (1 : 4, v/v). The following Fmoc amino acids were used: Fmoc-Ile-OH, Fmoc-Ser-OH (used without side chain protection), and Fmoc-Trp(Boc)-OH. After coupling of Fmoc-Trp(Boc)-OH esterification of the Ser side chain was achieved by treating the resin-bound peptide with Alloc-Gly-OH (238 mg, 1.5 mmol, 15 eq.), DIC (0.24 mL, 1.5 mmol, 15 eq.) and DMAP (6 mg, 0.05 mmol, 0.5 eq.) in 3 mL DCM : DMF (3 : 1, v/v) for 18 h under nitrogen. The resin was treated with $PhSiH_3$ (0.30 mL, 3.0 mmol, 30 eq.) in DCM (ca. 7 mL) under nitrogen for 2 h before being washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). The peptide was then cyclized using BOP (177 mg, 0.4 mmol, 4 eq.) and DiPEA (0.14 mL, 0.8 mmol, 8 eq.) for 2 h in 3 mL DMF under nitrogen flow. The remaining linear N-terminal section of the peptide was then synthesized using the standard SPPS protocol mentioned above. The following Fmoc amino acids were used: Fmoc-D-Ser(tBu)-OH, Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-Gly-OH. Following the final Fmoc removal step, isopelargonic acid (79 mg, 0.5 mmol, 2 eq.) was coupled using BOP (88 mg, 0.2 mmol, 2 eq.) and DiPEA (87 µL, 0.4 mmol, 4 eq.) in 3 mL of DMF overnight, under nitrogen flow. Final deprotection was carried out by treating the resin with TFA : TIS : H_2O (95 : 2.5 : 2.5, 5 mL) for 90 min. The reaction mixture was filtered through cotton, the filtrate precipitated in MTBE : petroleum ether (1 : 1), and the resulting precipitate washed once more with MTBE : petroleum ether (1 : 1). The crude cyclic peptide was lyophilized from tBuOH : H_2O (1 : 1) and purified with reverse phase HPLC (See HPLC purification of synthetic peptides). Pure fractions were pooled and lyophilized to yield laterocidine in >95% purity as a white powder. Yield: 4 mg, 2% over 29 steps $[M+2H]^{2+}$ calculated for, $C_{78}H_{113}N_{19}O_{18}$ 795.9250, found (HR-MS) 795,9249.

Synthesis of Dap9-laterocidine (6)

The loaded resin (680 mg, 0.25 mmol) was treated with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol, 0.3 eq.) and $PhSiH_3$ (0.75 mL, 7.5 mmol, 30 eq.) in DCM (ca. 15 mL) under nitrogen for 1 h. The resin was subsequently washed with CH_2Cl_2 (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). TFA-H_2N-Gly-Gly-OAll (143 mg, 0.5 mmol, 2 eq.) was then coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 µL, 1.0 mmol, 4 eq.) under nitrogen flow for 1 h. The next two amino acids (Ile and Dap) were coupled manually (1 h) under nitrogen flow via standard Fmoc solid-phase peptide synthesis (SPPS) (resin : Fmoc-AA : BOP : DiPEA, 1 : 4 : 4 : 8 molar eq.). DMF (5 mL) was used as solvent and Fmoc deprotections (2 min then 10 min) were carried out with 5 mL piperidine : DMF (1 : 4, v/v). The following Fmoc amino acids were used: Fmoc-Ile-OH and Fmoc-Dap(Alloc)-OH. The resin was then treated two times with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol) and $PhSiH_3$ (0.75 mL, 7.5 mmol) in DCM (ca. 15 mL) under nitrogen for 3 h with washing in between with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL),

and DMF (5 × 10 mL). The macrocycle was then closed by treatment with BOP (442 mg, 1.0 mmol, 4 eq.) and DiPEA (0.35 mL, 2.0 mmol, 8 eq.) for 2 h in 5 mL DMF under nitrogen flow. Following cyclization the remaining linear N-terminal section of the peptide was added using a CEM Liberty Blue automated peptide synthesizer with microwave irradiation, on standard settings (resin : Fmoc-AA : DIC : Oxyma, 1 : 5 : 5 : 5 molar eq.). DMF was used as solvent and Fmoc deprotections were carried out with piperidine : DMF (1 : 4, v/v). The following Fmoc amino acids were used: Fmoc-D-Ser(tBu)-OH, Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-Gly-OH and Fmoc-Trp(Boc)-OH. Following the final Fmoc removal step, the resin was removed from the CEM Liberty Blue and washed with DCM and DMF before isopelargonic acid (79 mg, 0.5 mmol, 2 eq.) was coupled manually using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 µL, 1.0 mmol, 4 eq.) in 5 mL of DMF overnight, under nitrogen flow. Final deprotection was carried out by treating the resin with TFA : TIS : H_2O (95 : 2.5 : 2.5, 10 mL) for 90 min. The reaction mixture was filtered through cotton, the filtrate was precipitated in MTBE : petroleum ether (1 : 1) and the resulting precipitate washed once more with MTBE : petroleum ether (1 : 1). The crude cyclic peptide was lyophilized from tBuOH : H_2O (1 : 1) and purified with reverse phase HPLC (See HPLC purification of synthetic peptides). Pure fractions were pooled and lyophilized to yield Dap9-laterocidamide in >95% purity as a white powder. Yield 10 mg, 3 % yield over 27 steps. $[M+2H]^{2+}$ calculated for, $C_{77}H_{112}N_{20}O_{17}$ 795,4330 found (HR-MS) 795,43

Synthesis of Dap9-laterocidine (6) via Solution-Phase Cyclization.

2-Chlorotrityl resin (5.0 g, 1.60 mmol g−1) was loaded with Fmoc-Gly-OH. Resin loading was determined to be 0.67 mmol g^{-1}. The linear peptide was assembled manually on a 3 mmol scale under nitrogen flow via standard Fmoc solid-phase peptide synthesis (SPPS) (1 h couplings, resin : Fmoc-AA : BOP : DiPEA, 1 : 4 : 4 : 8 molar eq.). DMF (60 mL) was used as solvent and Fmoc deprotections (2 min then 10 min) were carried out with 60 mL piperidine : DMF (1 : 4, v/v). The following Fmoc amino acids were used: Fmoc-D-Ser(tBu)-OH, Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-Gly-OH and Fmoc-Trp(Boc)-OH, Fmoc-Dap(Alloc)-OH and Fmoc-Ile-OH. Following the final Fmoc removal step, isopelargonic acid (0.95 g, 6 mmol, 2 eq.) was coupled using BOP (2.65 g, 6 mmol, 2 eq.) and DiPEA (2.1 mL, 12 mmol, 4 eq.) in 60 mL of DMF overnight, under nitrogen flow. The resin was then treated three times with $Pd(PPh_3)_4$ (0.9 g, 0.075 mmol) and $PhSiH_3$ (9.0 mL, 7.5 mmol) in CH_2Cl_2 (ca. 180 mL) under nitrogen for 2 h with washing in between with CH_2Cl_2 (5 × 120 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 120 mL), and DMF (5 × 120 mL). The peptide was cleaved off the resin by treating it with HFIP : DCM (1 : 4, v/v, 240 mL) for 1 h and rinsed with additional HFIP : DCM and DCM. The combined washings were then evaporated to yield the linear protected peptide with a free C-terminus and Dap amino sidechain. The partially protected peptide was dissolved in DCM (1.8 L), treated with BOP (2.65 g, 6 mmol, 2 eq.) and DiPEA (2.1 mL, 12 mmol, 4 eq.) and the solution was stirred overnight under nitrogen atmosphere. The reaction mixture was concentrated and directly treated with TFA : TIS : H_2O (95 : 2.5 : 2.5, 120 mL) for 90 min. The reaction mixture was subsequently filtered through cotton, the filtrate was precipitated in MTBE : petroleum ether (1 : 1) and the resulting precipitate washed once more with MTBE : petroleum ether (1 : 1). The crude cyclic peptide was lyophilized from tBuOH : H_2O (1 : 1) and purified using a Buchi Pure C-815 Flash system with a Buchi FlashPure ID C18-WP

20 μm irregular 80 g reverse-phase column. The purification was performed while doing 1 g injections of crude peptide. The following solvent system, at a flow rate of 40 mL/min, was used: solvent A, 0.1 % TFA in water; solvent B, 0.1 % TFA in acetonitrile. Gradient elution was as follows: 100:0 to 50:50 (A/B) over 60 min. Pure fractions were pooled and lyophilized to yield Dap9-laterocidamide in >95% purity as a white powder. Yield 1.4 g, 29 % yield over 30 steps. $[M+2H]^{2+}$ calculated for, $C_{77}H_{112}N_{20}O_{17}$ 795,4330 found (HR-MS) 795,4327.

Synthesis of MeDap9-laterocidine (8)

Rink amide MBHA resin loaded with Fmoc-Asp-OAll (680 mg, 0.25 mmol) was was treated with $Pd(PPh_3)_4$ (75 mg, 0.075 mmol) and $PhSiH_3$ (0.75 mL, 7.5 mmol) in DCM (ca. 15 mL) under nitrogen for 2 h before being washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). TFA·H_2N-Gly-OAll (115 mg, 0.5 mmol, 2 eq.) was then coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 μL, 1.0 mmol, 4 eq.) under nitrogen flow for 1 h. The next 2 amino acids Fmoc-Ile-OH and (2S,3R)-Fmoc-azido-aminobutyric acid were coupled manually (1 h) under nitrogen flow via standard Fmoc solid-phase peptide synthesis (SPPS) (resin : Fmoc-AA : BOP : DiPEA, 1 : 4 : 4 : 8 molar eq.). DMF (5 mL) was used as solvent and Fmoc deprotections (2 min then 10 min) were carried out with 5 mL piperidine : DMF (1 : 4, v/v). The azide was then reduced using a DTT (2M) and DiPEA (1M) in DMF (ca. 15 mL) for 2H under N_2 followed by washings with DMF (5×10 mL). The Allyl protecting group on the C-terminus was then removed using $Pd(PPh_3)_4$ (75 mg, 0.075 mmol) and $PhSiH_3$ (0.75 mL, 7.5 mmol) in DCM (ca. 15 mL) under nitrogen for 2 h before being washed with DCM (5 × 10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and DMF (5 × 10 mL). The peptide was then cyclized using BOP (442 mg, 1.0 mmol, 4 eq.) and DiPEA (0.35 mL, 2.0 mmol, 8 eq.) for 2 h in 5 mL DMF under nitrogen flow. The remaining linear N-terminal section of the peptide was then synthesized using the standard SPPS protocol mentioned above. The following Fmoc amino acids were used: Fmoc-D-Ser(tBu)-OH, Fmoc-D-Tyr(tBu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Orn(Boc)-OH, Fmoc-Gly-OH. Fmoc-L-Trp(Boc)-OH. Following the final Fmoc removal step, isopelargonic acid (79 mg, 0.5 mmol, 2 eq.) was coupled using BOP (221 mg, 0.5 mmol, 2 eq.) and DiPEA (174 μL, 1.0 mmol, 4 eq.) in 5 mL of DMF overnight, under nitrogen flow. Final deprotection was carried out by treating the resin with TFA : TIS : H_2O (95 : 2.5 : 2.5, 10 mL) for 90 min. The reaction mixture was filtered through cotton, the filtrate precipitated in MTBE : petroleum ether (1 : 1), and the resulting precipitate washed once more with MTBE : petroleum ether (1 : 1). The crude cyclic peptide was lyophilized from tBuOH : H_2O (1 : 1) and purified with reverse phase HPLC (See HPLC purification of synthetic peptides). Pure fractions were pooled and lyophilized to yield MeDap9-laterocidine in (9.7 mg, 2.4% over 29 steps, purity >95%) $[M+2H]^{2+}$ calculated for, $C_{78}H_{114}N_{20}O_{17}$ 802.4408, found (HRMS) 802.4412.

HPLC purification of synthetic peptides

Brevicidine and analogues were purified using a Perkin Elmer HPLC system composed of a 200 series binary pump, UV/Vis detector monitoring at 220 nm, vacuum degasser and Rheodyne 7725i injector. **Method A (Preparative):** Phenomenex Luna C18 column (21.2 x 250 mm, 5 μm) with a 2 mL injection loop. The following solvent system, at a flow rate of 10 mL/min, was used: solvent A, 0.1 % TFA in water; solvent B, acetonitrile. Gradient elution

was as follows: 80:20 (A/B) for 5 min, 80:20 to 45:55 (A/B) over 30 min, 45:55 to 0:95 (A/B) over 3 min, 0:95 (A/B) for 3 min then reversion back to 80:20 (A/B) over 2 min, 80:20 (A/B) for 5 min. **Method B (Analytical):** Phenomenex C18 Luna column (4.6 x 150 mm, 5 μm) with a 200 μL injection loop. The following solvent system, at a flow rate of 2 mL/min, was used: solvent A, 0.1 % TFA in water; solvent B, acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 5:95 (A/B) over 18 min then reversion back to 95:5 (A/B) over 0.1 min, 95:5 (A/B) for 3.9 min. Laterocidine and analogues were purified using the following methods. **Method C (Preparative):** BESTA-Technik system equipped with a ECOM Flash UV detector monitoring at 214 nm and 254 nm with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 μm). 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: 100:0 (A/B) for 5 min, 100:0 to 50:50 (A/B) over 50 min, 50:50 to 0:100 (A/B) for 3min, then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 5 min. **Method D (Analytical):** 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 214 nm 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 2 min, 100:0 to 50:50 (A/B) over 45 min, 50:50 (A/B) to 0:100 (A/B) over 1 min, 0:100 (A/B) for 6 min then reversion back to 100:0 (A/B) over 1min, 100:0 (A/B) for 5min. HPLC traces of all peptides can be found in the online supplementary material at DOI https://doi.org/10.1039/D2SC00143H

MIC determinations

Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to CLSI guidelines in triplicate. Blood agar plates were inoculated with glycerol stocks of *E. coli* ATCC 25922, *E. coli* MCR-1 (clinical isolate from Utrecht Medical Centre, NL), *E. coli* EQAS MCR-2 (clinical isolate from Wageningen University and Research, NL), *K. pneumoniae* ATCC 11228, *K. pneumoniae* ATCC 13883, *K. pneumonia* 2048 (clnical isolate from Vrije Universiteit Amsterdam Medical Centre, NL), *K. pneumonia* JS-123 (clinical isolate from from Utrecht Medical Centre, NL), *A. baumannii* ATCC 17961, *A. baumannii* ATCC 17978, *A. baumannii* 2018-006 (clinical isolate from Rijksinstituut voor Volksgezondheid en Milieu, NL), *A. baumannii* MDR (clinical isolate from Vrije Universiteit Amsterdam Medical Centre, NL), *P. aeruginosa* ATCC 27853, *P. aeruginosa* PAO1, *P. aeruginosa* NRZ-03961 (Reference strain from Das Nationale Referenzzentrum für gramnegative Krankenhauserreger, DE), *P. aeruginosa* M-120 (clinical isolate from Leiden University Medical Centre, NL) and *S. aureus* USA300 (clinical isolate from Texas Children's Hospital, USA). *E. coli* 25922 MCR1 was grown on LB agar supplemented with kanamycin. The inoculated agar plates were then incubated for 16 h at 37°C. Individually grown colonies were subsequently used to inoculate 3 mL aliquots of TSB that were then incubated at 37°C with shaking at 220 rpm. In parallel, the lipopeptide antibiotics to be assessed were serially diluted with Mueller-Hinton broth (MHB) in polypropylene 96-well plates (50 μL in each well). Once the OD_{600} of the bacterial suspensions reached 0.5, the bacteria were diluted with MHB (final concentration 2×10^5 CFU mL^{-1}) and added to the microplates containing the test compounds (50 μL to each well, final volume: 100 μL). The well-plates were sealed with an adhesive membrane and after 16 h of incubation at 37°C with shaking at 220 rpm the wells were visually inspected for bacterial

growth. MIC values reported are based on three technical replicates and defined as the lowest concentration of the compound that prevented visible growth of bacteria.

LC-MS/MS

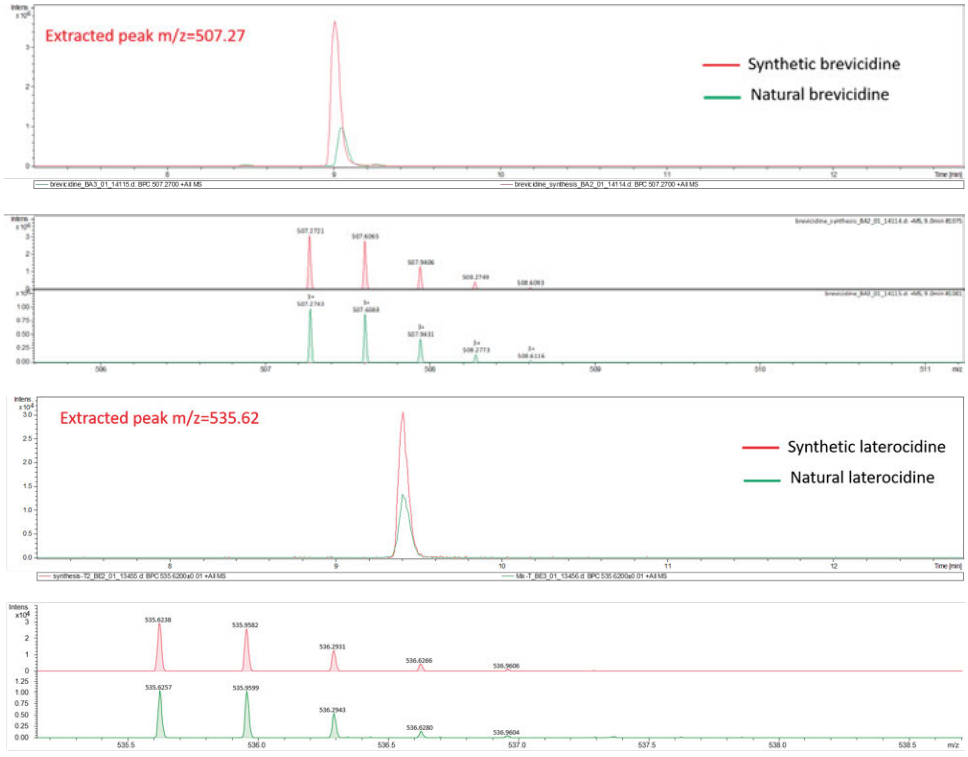


Figure 5. Comparison of synthetic lipopeptides with their natural counterparts by LC-MS/MS.

UltiMate 3000 UHPLC Systems coupled to Bruker impact™II Mass Spectrometer (TOF). **Column:** Waters Acquity UPLC BEH C18 column (1.7 µm, 130 Å, 2.1 × 150 mm). **LC method:** The column was maintained at 40˚C and run at a flow rate of 0.2 mL/min, using 0.1% formic acid in H2O as solvent A and 0.1% formic acid in acetonitrile as solvent B. A gradient was employed for chromatographic separation starting at 5% B for 2 min, then 5% to 95% B for 15 min, and finally held at 95% for 4 min, The column was re-equilibrated to 5% B for 1 min before the next run was started. **MS method:** The MS system was tuned using standard sodium formate solution. The same solution was used to calibrate the system before starting. All the samples were analyzed in positive polarity, using data-dependent acquisition mode. Detection range: 100-1500 m/z.

LPS antagonization assay

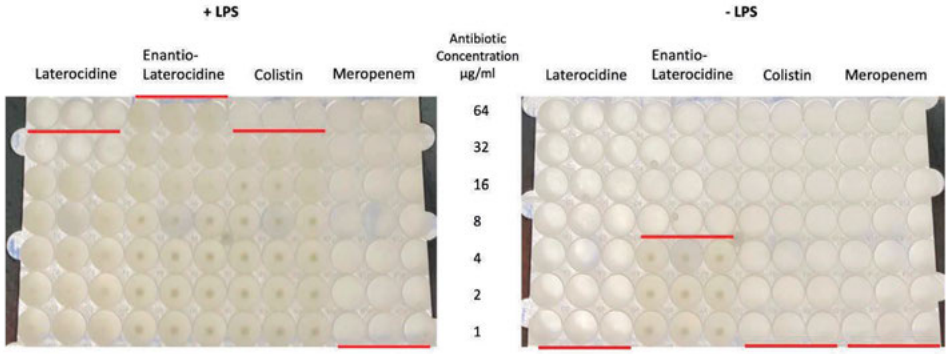


Figure 6. LPS antagonization assay.

A blood agar plate was inoculated with a glycerol stock of *E. coli* ATCC 25922. The inoculated agar plate was then incubated for 16 h at 37 °C. An individually grown colony was subsequently used to inoculate 3 mL of TSB that was then incubated at 37 °C with shaking at 220 rpm. In parallel, the compounds to be assessed were serially diluted with Mueller-Hinton broth (MHB) in polypropylene 96-well plates (50 µL in each well). Once the OD600 of the bacterial suspensions reached 0.5, the bacteria were diluted with MHB (final concentration 2×10^5 CFU mL^{-1}). The media were then either supplemented with 1 mg/mL of LPS (lipopolysaccharides from *E. coli* O55:B5, Sigma-Aldrich) or added directly to the microplates containing the test compounds (50 µL to each well, final volume: 100 µL). The well-plates were sealed with an adhesive membrane and after 16 h of incubation at 37°C with shaking at 220 rpm. The wells were visually inspected for bacterial growth.

Serum stability assay

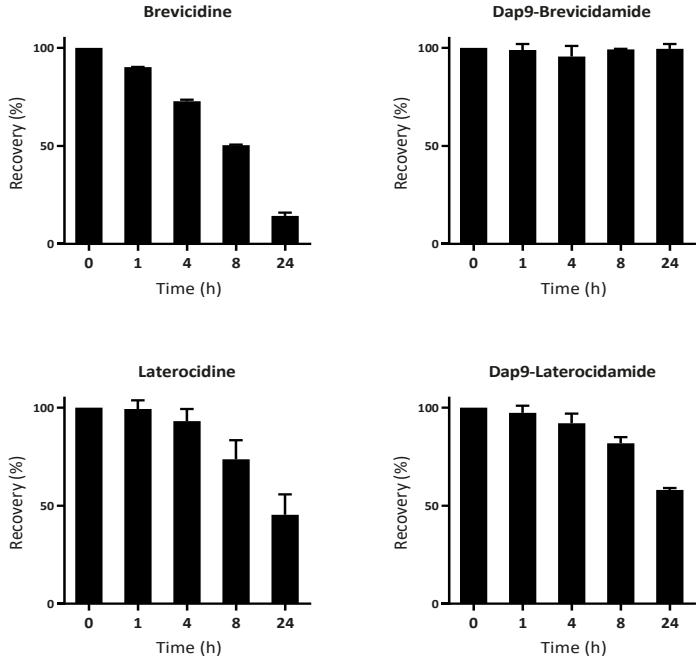


Figure 7. Serum stability assays comparing natural lipopeptides to synthetic amide analogues.

10 mg/mL peptide solutions were prepared in in Milli-Q water. Samples were prepared with 42 µL peptide solution and 518 µL human serum (obtained from Sigma Aldrich, product number: H4522) and incubated at 37 °C. Samples were taken at t = 0, 1, 4, 8 and 24 h. To 100 µL of serum, 100 µL of 6% TCA in ACN (containing 0.2 µg/mL D-Phenylalanine as internal standard) was added to precipitate the proteins. The samples were vortexed, left for 15 min at room temperature and stored at -20 °C. Before analysis the samples were centrifuged for 5 min at 13 000 rpm. The supernatant was analyzed by RP-HPLC using 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 220 nm 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 2 min, 100:0 to 50:50 (A/B) over 45 min, 50:50 (A/B) to 0:100 (A/B) over 1 min, 0:100 (A/B) for 6 min then reversion back to 100:0 (A/B) over 1 min, 100:0 (A/B) for 5 min. The peaks were integrated and normalized to the internal standard. Recovery of the peptides at t=0 was compared to control samples without serum and was within the 85%-115% range (data not shown). The t=0 value was then set at 100% for each analogue and all time-points were calculated as a percentage of t=0. Biological duplicates of the experiment were performed.

Hemolysis assay

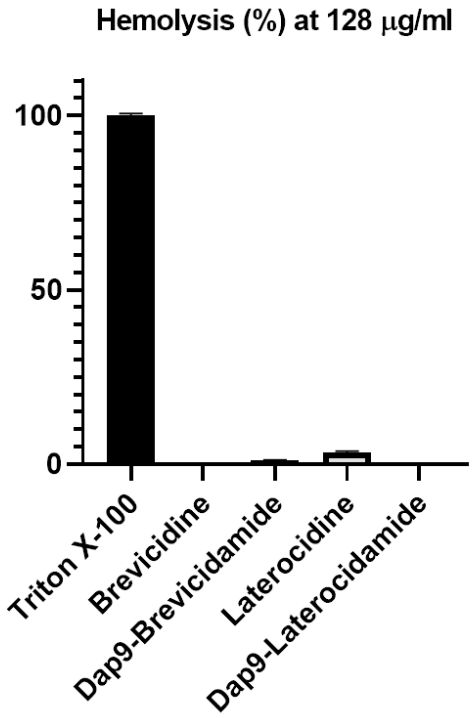


Figure 8. Hemolytic activity of selected analogues against sheep red blood cells.

Experiments were performed in triplicate and Triton X-100 used as a positive control. Red blood cells from defibrinated sheep blood obtained from Thermo Fisher were centrifuged (400 g for 15 min at 4°C) and washed with Phosphate-Buffered Saline (PBS) containing 0.002% Tween20 (buffer) for five times. Then, the red blood cells were normalized to obtain a positive control read-out between 2.5 and 3.0 at 415 nm to stay within the linear range with the maximum sensitivity. A serial dilution of the compounds (256 – 8 μg/mL, 75 μL) was prepared in a 96-well plate. The outer border of the plate was filled with 75 μL buffer. Each plate 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 plates were centrifuged (800 g for 5 min at room temperature) 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.

Cytotoxicity assay

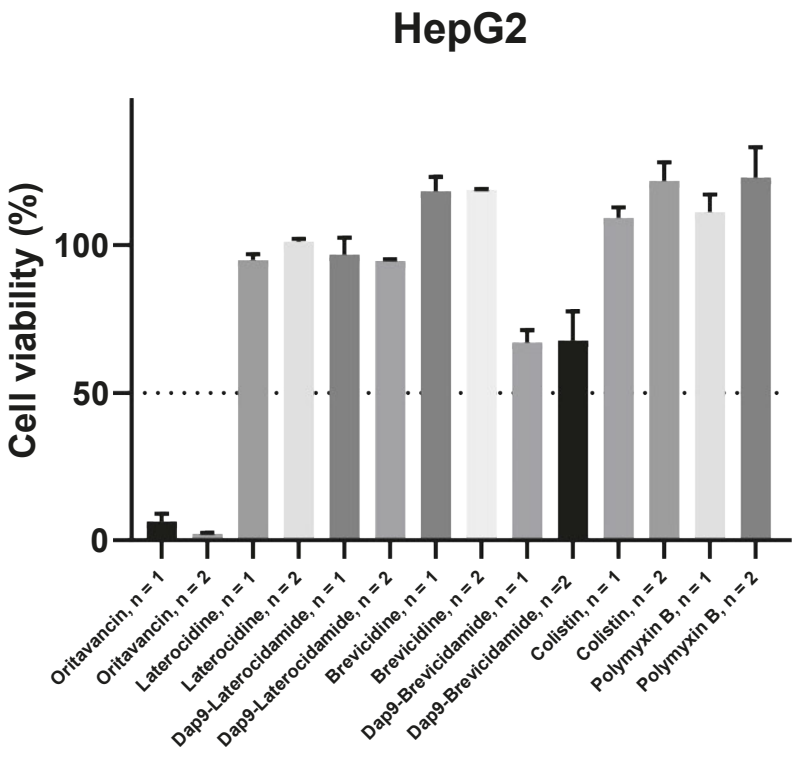


Figure 9. Cytotoxicity of selected brevicidine and laterocidine analogues against HepG2 cells compared to oritavancin, colistin and polymyxin B using a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

HepG2 cells were seeded at a density of 1.5×10^4 cells per well in a clear 96-well tissue culture treated plate in a final volume of 100 µL of Dulbecco's Modified Eagle Medium (DMEM), supplemented with Fetal Bovine Serum (1%), Glutamax and Pen/Strep. Cells were incubated for 24 h at 37°C, 7% CO_2 to allow cells to attach to the plates. In addition to a single vehicle control, compounds (diluted from DMSO stock) were added to each well to obtain in a final 128 µg/mL concentration (max. final DMSO concentration 0.5%) for all compounds except for oritavancin which was administered at 50 µM. Incubation was done for 24 h at 37 °C, 7% CO_2. After the incubation, MTT was added to each well at a final concentration of 0.40 mg/mL. The plates were then incubated for 2 h at 37 °C, 7% CO_2. Medium was carefully removed via suction, and purple formazan crystals were resuspended in 100 µL DMSO. Absorbance was read at 570 nm using a Clariostar plate reader. The data was then analysed with GraphPad Prism software. Technical triplicates for each condition were used, along with biological duplicates.

In vivo tolerability and efficacy studies

Ethical Issues. Animal experiments were performed under UK Home Office Licences P89653310 (tolerability and PK) and PA67E0BAA (thigh efficacy), with local ethical committee clearance.

Animal Strain. Mice used in these studies were supplied by Charles River (Margate UK) and were specific pathogen free. The strain of mice used was ICR (also known as CD1 Mice) which is a well characterized outbred murine strain. Mice (male) were 11-15 g on receipt and were allowed to acclimatise for at least 7 days.

Animal Housing. Mice were housed in sterilised individual ventilated cages exposing the mice at all times to HEPA filtered sterile air. Mice had free access to food and water and had aspen chip bedding (changed at least once weekly). The room temperature was 22°C +/- 1°C, with a relative humidity of 60% and maximum background noise of 56 dB. Mice were exposed to 12 h light/dark cycles.

Test compounds. Compound **6** was dissolved in water for injection in which it formed a clear colourless solution. Polymyxin B was dissolved in saline for injection to produce a clear colourless solution .

Tolerability study. The tolerability of compound **6** was assessed in the same mouse strain used for the efficacy studies. Compound 6 was administered via subcutaneous administration route at 3 8-h intervals indicating good tolerability up to 40 mg/kg. The mice used in the tolerability study were naïve and were not immunosuppressed or infected.

Efficacy study. The in *vivo* efficacy of compound **6** was assessed in a mouse thigh abscess model where both thighs of each mouse were infected with *E. coli* ATCC 25922

Immunosuppression. Mice were rendered neutropenic with subcutaneous injections of cyclophosphamide at 150 mg/kg 4 days before infection and 100 mg/kg 1 day before infection. The immunosuppression regime leads to neutropenia starting 24 h post administration of the first injection, which continues throughout the study.

Infection. The bacterial strain used was *E. coli* ATCC 25922. An aliquot of a previously prepared frozen stock of the strain was thawed and diluted in sterile PBS to the desired inoculum just prior to infection. Mice were infected with 0.05 mL of the bacterial strain suspensions by intramuscular (IM) injection under temporary inhaled anaesthesia (2.5% isofluorane for 3-4 min) into both thighs. The inoculum was 6×10^6 cfu/mL, 3×10^5 cfu/thigh.

Analgesia. At the time of thigh infection, buprenorphine analgesia was administered at 0.03mg/kg subcutaneously using a 0.015mg/mL solution delivered at 2 mL/kg. The same dose was administered again 9 and 17 h post-infection.

Treatment. Compound **6** was administered SC every 8 h starting 1 h post-infection at does of 10, 20, and 40 mg/kg. Additional control groups comprising an infected pre-treatment group, which was euthanised 1 h after infection, a vehicle (WFI) treated group and a group that

received comparator Polymyxin B SC every 8 h dosed at 20 mg/kg were included.

Endpoints. One h and 20.5 (planned 25) h post-infection, the clinical condition of all animals was assessed prior to humane euthanasia using pentobarbitone overdose, and the thighs were removed and weighed. Thigh samples were homogenized in 3mL ice cold sterile PBS; the homogenates were quantitatively cultured onto CLED agar and incubated at 37°C for 18 - 24 h before colonies were counted.

Data analysis. The data from the culture burdens were analysed using appropriate non-parametric statistical models (Kruskal-Wallis using Conover-Inman to make all pairwise comparisons between groups) with StatsDirect software v. 3.3.5, and compared to vehicle control. For all calculations the thighs from each animal were treated as two separate data points.

NMR characterization of compounds

Synthetic brevicidine (1)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Asn1	8.01 (1H, d, *J* = 7.8 Hz)	4.54 (1H, m)	2.53 (1H, under DMSO peak) & 2.35 (1H, dd, *J* = 15.7 & 6.8 Hz)			
D-Tyr2	7.98 (1H, d, *J* = 7.3 Hz)	4.27 (1H, m)	2.78 (1H, m) & 2.58 (1H, m)	Aromatic: 6.85 (2H, d, J = 8.6 Hz) & 6.55 (2H, d, *J* = 8.6 Hz)		
D-Trp3	8.11 (1H, m)	4.48 (1H, m)	3.17 (1H, dd, *J* = 14.6 & 4.3 Hz) & 3.02 (1H, dd, *J* = 14.8 & 9.9 Hz)	Indole: 10.89 (2H, br d, *J* = 6.5 Hz), 7.57 (2H, app dd, *J* = 8.2 & 2.2 Hz), 7.32 (2H, app dd, *J* = 14.2 & 8.2 Hz), 7.16 (1H, d, *J* = 2.2 Hz), 7.05 (2H, m) & 6.97 (3H, m)		
D-Orn4	8.07 (1H, d, *J* = 8.6 Hz)	4.37 (1H, m)	1.73 (1H, m) & 1.53 (1H, m)	1.53 (2H, m)	2.78 (2H, m)	7.76 (2H, br)
Orn5	7.92 (1H, d, *J* = 8.2 Hz)	4.37 (1H, m)	1.73 (1H, m) & 1.53 (1H, m)	1.53 (2H, m)	2.78 (2H, m)	7.76 (2H, br)
Gly6	8.23 (1H, t, *J* = 5.4 Hz)	3.82 (1H, dd, *J* = 16.6 & 6.1 Hz) & 3.73 (1H, dd, *J* = 18.4 & 7.8 Hz)				
D-Orn7	8.11 (1H, m)	4.37 (1H, m)	1.40 (1H, m) & 1.29 (1H, m)	1.29 (2H, m)	2.58 (2H, m)	7.68 (2H, br)
Trp8	8.19 (1H, m)	4.72 (1H, app q, *J* = 7.8 Hz)	3.08 (1H, dd, *J* = 14.6 & 5.6 Hz) & 2.96 (1H, dd, *J* = 14.4 & 8.6 Hz)	Indole: 10.89 (2H, app d, *J* = 6.5 Hz), 7.57 (2H, app dd, *J* = 8.2 & 2.2 Hz), 7.32 (2H, app dd, *J* = 14.2 & 8.2 Hz), 7.10 (1H, d, *J* = 2.6 Hz), 7.05 (2H, app q, *J* = 7.8 Hz) & 6.97 (3H, m)		
Thr9	8.19 (1H, m)	4.54 (1H, m)	4.88 (1H, m)	1.08 (3H, d, *J* = 6.5 Hz)		
Ile10	8.54 (1H, d, *J* = 9.6 Hz)	4.12 (1H, t, J = 10.8 Hz)	1.84 (1H, br m)	0.84 (3H, m), 1.51 (1H, m) & 1.06 (1H, m)	0.84 (3H, m)	
Gly11	8.44 (1H, t, *J* = 6.3 Hz)	3.89 (1H, dd, *J* = 15.5 & 6.5 Hz) & 3.55 (under H_2O)				
Ser12	7.62 (1H, d, *J* = 9.1 Hz)	4.43 (1H, dt, *J* = 9.0 & 4.7 Hz)	3.76 (1H, dd, *J* = 11.2 & 4.7 Hz) & 3.60 (1H, dd, *J* = 10.8 & 4.7 Hz)			
Lipid	2.06 (2H, m, O=CC**H**$_2$-), 1.53 (2H, O=CCH$_2$C**H**$_2$-), 1.29 (5H, -C**H**$_2$C**H(**CH$_3$)C**H**$_2$CH$_3$), 1.07 (2H, -C**H**$_2$CH$_3$) & 0.81 (6H, -CH(C**H**$_3$)CH$_2$C**H**$_3$)					

ent-brevicidine (*ent*-1)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
Asn1	8.02 (1H, d, J = 7.9 Hz)	4.54 (1H, m)	2.54 (1H, m) & 2.35 (1H, dd, J = 15.2 & 7.0 Hz)			
Tyr2	7.98 (1H, d, J = 7.5 Hz)	4.49 (1H, m)	2.79 (1H, m) & 2.62 (1H, m)	6.86 (2H, d, J = 8.5 Hz), 6.56 (2H, d, J = 8.5 Hz)		
Trp3	8.13 (1H, d, J = 8.2 Hz)	4.27 (1H, br)	3.17 (1H, dd, J = 14.6 & 3.4 Hz), 3.02 (1H, dd, J = 15.1 & 10.1 Hz)	Indole: 10.80 (2H, app d, J = 12.2 Hz), 7.57 (2H, app dd, J = 7.8 & 2.2 Hz), 7.32 (2H, app dd, J = 12.7 & 8.1 Hz), 7.17 (1H, d, J = 2.1 Hz), 7.05 (2H, m), 6.97 (2H, m)		
Orn4	8.11 (1H, m)	4.39 (1H, m)	1.72 (1H, m) & 1.56 (1H, m)	1.56 (2H, m)	2.78 (2H, m)	7.82 (2H, br m)
D-Orn5	7.92 (1H, d, J = 8.1 Hz)	4.38 (1H, m)	1.76 (1H, m) & 1.56 (1H, m)	1.56 (2H, m)	2.78 (2H, m)	7.82 (2H, br m)
Gly6	8.25 (1H, t, J = 5.4 Hz)	3.81 (1H, dd, J = 17.0 & 6.6 Hz), 3.73 (m)				
Orn7	8.09 (1H, m)	4.36 (1H, m)	1.41 (1H, br m) & 1.32 (1H, m)	1.32 (2H, m)	2.59 (2H, br m)	7.72 (2H, br)
D-Trp8	8.20 (1H, d, J = 8.6 Hz)	4,71 (1H, app q, J = 8.0 Hz)	3.09 (1H, dd, J = 13.8 & 4.9 Hz), 2.96 (1H, dd, J = 15.1 & 8.3 Hz)	Indole: 10.80 (2H, app d, J = 12.2 Hz), 7.57 (2H, app dd, J = 7.8 & 2.2 Hz), 7.32 (2H, app dd, J = 12.7 & 8.1 Hz), 7.11 (1H. d, J = 2.3 Hz), 7.05 (2H, m), 6.97 (2H, m)		
D-Thr9	8.19 (1H, d, J = 9.0 Hz)	4.55 (1H, m)	4.89 (1H, m)	1.09 (3H, d, J = 6.4 Hz)		
D-Ile10	8.58 (1H, d, J = 9.7 Hz)	4.12 (1H, app t, J = 10.5 Hz)	1.86 (1H, br m)	0.84 (3H, m), 1.51 (1H, m), 1.07 (1H, m)	0.84 (3H, m)	
Gly11	8.45 (1H, t, J = 6.3 Hz)	3.87 (1H, dd, J = 14.8 & 5.6 Hz), 3.54 (1H, dd, J = 15.2 & 6.1 Hz)				
D-Ser12	7.68 (1H, d, J = 8.8 Hz)	4.44 (1H, dt, J = 8.9 & 4.4 Hz)	3.78 (1H, m) & 3.62 (1H, br m)			
Lipid	2.06 (2H, m, O=CC**H**$_2$-), 1.50 (2H, O=CCH$_2$C**H**$_2$-), 1.27 (5H, -C**H**$_2$C**H**(CH)$_3$C**H**$_2$CH$_3$), 1.09 (2H, -C**H**$_2$CH$_3$), 0.82 (6H, -CH(C**H**$_3$)CH$_2$C**H**$_3$)					

Ser9-brevicidine (3)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Asn1	8.02 (1H, d, *J* = 7.6 Hz)	4.54 (1H, app q, *J* = 7.1 Hz)	2.53 (1H, under DMSO peak) & 2.35 (1H, dd, *J* = 15.3 & 6.7 Hz)			
D-Tyr2	7.98 (1H, d, *J* = 7.2 Hz)	4.27 (1H, m)	2.79 (1H, m) & 2.62 (1H, m)	Aromatic: 6.86 (2H, d, J = 8.2 Hz) & 6.55 (2, d, *J* = 8.2 Hz)		
D-Trp3	8.13 (1H, d, *J* = 7.2 Hz)	4.49 (1H, br m)	3.17 (1H, br m) & 3.02 (1H, dd, *J* = 14.4 & 9.8 Hz)	Indole: 10.80 (2H, s), 7.58 (2H, app dd, *J* = 18.9 & 7.8 Hz), 7.32 (2H, app dd, *J* = 14.9 & 8.1 Hz), 7.15 (1H, m), 7.05 (2H, m) & 6.97 (3H, m)		
D-Orn4	8.08 (1H, br d, *J* = 7.8 Hz)	4.38 (1H, m)	1.72 (1H, m) & 1.55 (1H, m)	1.55 (2H, m)	2.78 (6H, m)	7.78 (2H, br)
Orn5	7.93 (1H, d, *J* = 8.1 Hz)	4.37 (1H, m)	1.72 (1H, m) & 1.55 (1H, m)	1.55 (2H, m)	2.78 (6H, m)	7.78 (2H, br)
Gly6	8.22 (1H, m)	3.81 (1H, m) & 3.73 (1H, m)				
D-Orn7	8.05 (1H, d, *J* = 8.3 Hz)	4.34 (1H, m)	1.38 (1H, m) & 1.33 (1H, m)	1.33 (2H, m)	2.59 (2H, m)	7.72 (2H, br)
Trp8	8.19 (1H, d, *J* = 7.9 Hz)	4.64 (1H, m)	3.08 (1H, dd, *J* = 14.2 & & 2.91 (1H, dd, *J* = 14.2 & 8.8 Hz)	Indole: 10.80 (2H, s), 7.58 (2H, app dd, *J* = 18.9 & 7.8 Hz), 7.32 (2H, app dd, *J* = 14.9 & 8.1 Hz), 7.10 (1H, m), 7.05 (2H, m) & 6.97 (3H, m)		
Ser9	8.38 (1H, d, *J* = 7.2 Hz)	4.69 (1H, m)	4.20 (1H, dd, *J* = 9.8 & 5.1 Hz) & 3.84 (1H, m)			
Ile10	8.71 (1H, d, *J* = 9.7 Hz)	4.09 (1H, app t, J = 10.1 Hz)	1.83 (1H, br m)	0.84 (3H, m), 1.54 (1H, m) & 1.11 (1H, m)	0.84 (3H, m)	
Gly11	8.53 (1H, t, *J* = 6.3 Hz)	3.94 (1H, dd, *j* = 15.1 & 6.4 Hz) & 3.47 (1H, dd, *J* = 15.0 & 6.3 Hz)				
Ser12	7.46 (1H, br)	4.43 (1H, m)	3.71 (1H, m) & 3.57 (1H, m)			
Lipid	2.06 (2H, m, O=CC**H**$_2$-), 1.50 (2H, O=CCH$_2$C**H**$_2$-), 1.27 (5H, -C**H**$_2$C**H(**CH$_3$)C**H**$_2$CH$_3$), 1.10 (2H, -C**H**$_2$CH$_3$) & 0.81 (6H, -CH(C**H**$_3$)CH$_2$C**H**$_3$)					

Dap9-brevicidine (5)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Asn1	8.01 (1H, d, *J* = 7.8 Hz)	4.53 (1H, q, *J* = 7.2 Hz)	2.54 (under solvent) & 2.35 (1H, dd, *J* = 15.5 & 7.1 Hz)			
D-Tyr2	7.98 (1H, d, *J* = 7.3 Hz)	4.28 (1H, m)	2.77 (1H, m) & 2.61 (1H, m)	Aromatic: 6.85 (2H, d, *J* = 8.5 Hz), 6.55 (2H, d, *J* = 8.4 Hz)		
D-Trp3	8.11 (1H, d, *J* = 7.3 Hz)	4.46 (1H, m)	3.17 (1H, m) & 3.01 (1H, m)	Indole: 10.78 (2H, br m), 7.32 (2H, dd, *J* = 14.9 & 8.0 Hz),7.17 (1H, br d, *J* = 2.0 Hz), 7.06 (3H, m) & 6.98 (3H, m)		
D-Orn4	8.05 (1H, d, *J* = 8.3 Hz)	4.35 (1H, m)	1.70 (1H, m) & 1.52 (1H, m)	1.52 (2H, m)	2.77 (2H, m)	7.71 (2H, br)
Orn5	7.92 (1H, d, *J* = 7.6 Hz)	4.35 (1H, m)	1.74 (1H, m) & 1.54 (1H, m)	1.55 (2H, m)	2.77 (2H, m)	7.69 (2H, br)
Gly6	8.22 (1H, t, *J* = 5.3 Hz)	3.82 (1H, dd, *J* = 17.1 & 5.3 Hz) & 3.74 (1H, dd, *J* = 16.8 & 4.8 Hz)				
D-Orn7	8.05 (1H, d, *J* = 8.3 Hz)	4.35 (1H, m)	1.28 (2H, m)	1.28 (2H, m)	2.58 (2H, m)	7.61 (2H, m)
Trp8	8.27 (1H, d, *J* = 8.2 Hz)	4.60 (1H, m)	3.05 (1H, m) & 2.88 (1H, m)	Indole: 10.89 (2H, br m), 7.32 (2H, dd, *J* =14.9 & 8.0 Hz), 7.06 (3H, m) & 6.98 (3H, m)		
Dap9	8.30 (1H, m)	4.46 (1H, m)	3.44 (1H, dd, *J* = 12.7 & 6.0 Hz) & 3.03 (1H, m)			
Ile10	8.29 (1H, m)	4.05 (1H, app t. *J* = 10.0 Hz)	1.76 (1H, m)	1.48 (1H, m), 1.09 (1H, m) & 0.82 (3H, m)	0.82 (3H, m)	
Gly11	8.29 (1H, m)	3.86 (1H, dd, *J* = 14.7 & 6.1 Hz) & 3.53 (1H, dd, *J* = 14.7 & 6.2 Hz)				
Ser12	7.34 (1H, d, *J* = 8.4 Hz)	4.20 (1H, m)	3.57 (1H, m) & 3.34 (1H, m)			
Lipid	2.06 (2H, m, O=CC**H**$_2$-), 1.49 (2H, O=CCH$_2$C**H**$_2$-), 1.25 (5H, -C**H**$_2$C**H**(CH$_3$)C**H**$_2$CH$_3$), 1.08 (2H, m, -C**H**$_2$CH$_3$) 0.80 (6H, -CH(C**H**$_3$)CH$_2$C**H**$_3$).					

MeDap9-brevicidine (7)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Asn1	8.03 (1H, d, *J* = 7.8 Hz)	4.53 (1H, app q, *J* = 7.2 Hz)	2.52 (Under DMSO peak) & 2.35 (1H, dd, *J* = 15.2 & 7.0 Hz)			
D-Tyr2	7.98 (1H, d, *J* = 7.5 Hz)	4.27 (1H, m)	2.79 (1H, m) & 2.62 (1H, m)	Aromatic: 6.86 (2H, d, *J* = 8.4 Hz), 6.56 (2H, d, *J* = 8.5 Hz)		
D-Trp3	8.15 (1H, m)	4.42 (1H, m)	3.17 (1H, dd, *J* = 14.9 & 4.0 Hz) & 3.02 (1H, dd, *J* = 14.5 & 8.9 Hz)	Indole: 10.81 (2H, d, *J* = 15.8 Hz), 7.57 (2H, d, *J* = 7.8 Hz), 7.32 (2H, app dd, *J* = 11.1 & 8.1 Hz), 7.17 (1H, d, *J* = 2.1 Hz), 7.05 (2H, m) & 6.97 (2H, m).		
D-Orn4	8.14 (1H, m)	4.37 (1H, m)	1.73 (1H, m) & 1.55 (1H, m)	1.55 (2H, m)	2.78 (2H, m)	7.86 (2H, br m)
Orn5	7.92 (1H, d, *J* = 8.1 Hz)	4.38 (1H, m)	1.74 (1H, m) & 1.58 (1H, m)	1.58 (2H, m)	2.78 (2H, m)	7.86 (2H, br m)
Gly6	8.27 (1H, t, *J* = 5.6 Hz)	3.81(1H, dd, *J* = 16.7 & 5.6 Hz) & 3.73 (1H, dd, *J* = 17.1 & 5.2 Hz)				
D-Orn7	8.09 (1H, d, *J* = 8.4 Hz)	4.36 (1H, m)	1.34 (2H, m)	1.34 (2H, m)	2.59 (2H, m)	7.73 (2H, br)
Trp8	8.24 (1H, d, *J* = 8.2 Hz)	4.68 (1H, m)	3.11 (1H, dd, *J* = 15.6 & 4.5 Hz) & 2.95 (1H, dd, *J* = 14.4 & 8.7 Hz)	Indole: 10.81 (2H, d, *J* = 15.8 Hz), 7.57 (2H, d, *J* = 7.8 Hz), 7.32 (2H, app dd, *J* = 11.1 & 8.1 Hz), 7.12 (1H, d, *J* = 2.1 Hz), 7.05 (2H, m) & 6.97 (2H, m).		
MeDap9	8.14 (1H, m)	4.41 (1H, dd, *J* = 8.4 & 4.4 Hz)	4.18 (1H, m)	1.01 (3H, d, *J* = 6.8 Hz), NH: 6.86 (2H, d, *J* = 8.4 Hz)		
Ile10	8.59 (1H, d, *J* = 9.5 Hz)	4.07 (1H, app t, *J* = 10.2 Hz)	1.85 (1H, br)	0.86 (3H, m), 1.54 (1H, m) & 1.07 (1H, m)	0.86 (3H, m)	
Gly11	8.65 (1H, t, *J* = 5.7 Hz)	3.93 (1H, dd, *J* =14.7 & 6.8 Hz) & 3.42 (under H_2O)				
Ser12	7.54 (1H, d, *J* = 8.9 Hz)	4.20 (1H, m)	3.66 (1H, m) & 3.52 (1H, m)			
Lipid	2.06 (2H, m, O=CC**H**$_2$-), 1.50 (2H, O=CCH$_2$C**H**$_2$-), 1.26 (5H, -**H**$_2$C**H**(CH$_3$)C**H**$_2$CH$_3$), 1.06 (2H, -C**H**$_2$CH$_3$) & 0.81 (6H, -CH(C**H**$_3$)CH$_2$C**H**$_3$).					

Synthetic laterocidine (2)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Ser1	7.85 (1H, d, *J* = 7.4 Hz)	4.26 (1H, dd, *J* = 13.5 & 6.4 Hz)	3.50 (2H, m)	5.12 (1H, t, *J* = 5.4 Hz)		
D-Tyr2	8.00 (1H, m)	4.34 (1H, m)	2.83 (1H, dd, *J* = 14.3 Hz & 4.4 Hz) & 2.62 (1H, dd, *J* = 14.1 & 9.5 Hz)	Aromatic: 6.89 (2H, d, *J* = 8.5 Hz), 6.56 (2H, d, *J* = 8.5 Hz)		
D-Trp3	8.00 (1H, m)	4.52 (1H, m)	3.15 (1H, m) & 2.93 (1H, m)	Indole: 10.79 (1H, s), 7.57 (1H, d, *J* = 7.8 Hz), 7.33 (1H, d, *J* = 8.0 Hz), 7.14 (1H, br s), 7.06 (1H, t, *J* = 8.0 Hz) & 6.98 (1H, t, *J* = 7.4 Hz)		
D-Orn4	8.03 (1H, m)	4.39 (1H, m)	1.73 (1H, m) & 1.56 (1H, m)	1.56 (2H, m)	2.82 (2H, m)	7.71 (2H, m)
Orn5	8.09 (1H, d, *J* = 8.3 Hz)	4.41 (1H, m)	1.70 (1H, m) & 1.52 (1H, m)	1.52 (2H, m)	2.76 (2H, m)	7.71 (2H, m)
Gly6	8.24 (1H, t, *J* = 5.2 Hz)	3.85 (1H, dd, *J* = 17.2 & 5.7 Hz) & 3.75 (1H, dd, *J* = 16.8 & 5.0 Hz)				
D-Orn7	8.07 (1H, d, *J* = 8.5 Hz)	4.40 (1H, m)	1.43 (1H, m) & 1.29 (1H, m)	1.43 (2H, m)	2.59 (2H, m)	7.63 (2H, m)
Trp8	8.32 (1H, d, *J* = 8.1 Hz)	4.75 (1H, m)	3.14 (1H, m) & 2.93 (1H, m)	Indole: 10.76 (1H, s), 7.59 (1H, d, *J* = 8.0 Hz), 7.32 (1H, d, *J* = 8.1 Hz), 7.12 (1H, br s), 7.04 (1H, t, *J* = 7.6 Hz) & 6.96 (1H, t, *J* = 7.6 Hz)		
Thr9	7.92 (1H, d, *J* = 6.4 Hz)	4.68 (1H, dd, *J* = 8.8 & 3.1 Hz)	5.17 (1H, m)	1.09 (3H, d, *J* = 6.4 Hz)		
Ile10	8.30 (1H, d, *J* = 3.1 Hz)	3.90 (1H, m)	1.57 (1H, m)	1.57 (1H, m), 1.11 (1H, m) & 0.86 (3H, m)	0.86 (3H, m)	
Asn11	9.40 (1H, d, *J* = 6.7 Hz)	4.12 (1H, m)	2.89 (1H, m) & 2.65 (1H, m)			
Gly12	8.03 (1H, m)	4.02 (1H, dd, *J* = 16.9 & 8.3 Hz) & 3.58 (1H, dd, *J* = 17.1 & 4.6 Hz)				
Gly13	7.67 (1H, m)	4.45 (1H, dd, *J* = 16.6 & 10.0 Hz) & 3.37 (under H_2O)				
Lipid	2.10 (2H, m, O=CC**H**$_2$-), 1.45 (3H, O=CCH$_2$C**H**$_2$- & -CH$_2$C**H**(CH$_3$)$_2$), 1.20 (4H, -C**H**$_2$C**H**$_2$CH(CH$_3$)$_2$), 1.11 (2H, -C**H**$_2$CH(CH$_3$)$_2$) & 0.83 (6H,-CH$_2$CH(C**H**$_3$)$_2$)					

ent-laterocidine (*ent*-2)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
Ser1	7.85 (1H, d, *J* = 7.4 Hz)	4.27 (1H, q, *J* = 6.6 Hz)	3.50 (2H, m)	-OH: 5.13 (1H, t, *J* = 5.3 Hz)		
Tyr2	8.02 (1H, m)	4.34 (1H, td, *J* = 8.3, 4.7 Hz)	2.82 (1H, dd, *J* = 14.1, 3.9 Hz) & 2.62 (1H, dd, *J* = 14.2, 9.8 Hz)	Aromatic: 6.89 (2H, d, *J* = 8.4 Hz) & 6.56 (2H, d, *J* = 8.3 Hz) & 9.17 (1H, s, -OH)		
Trp3	8.00 (1H, m)	4.52 (1H, app dd, *J* = 13.3, 8.0 Hz)	3.15 (1H, dd, *J* = 15.3, 4.2 Hz) & 2.94 (m)	Indole: 10.79 (1H, s), 7.57 (1H, d, *J* = 7.9 Hz), 7.33 (1H, d, *J* = 8.1 Hz), 7.14 (1H, app s), 7.06 (1H, t, *J* = 7.6 Hz) & 6.98 (1H, t, *J* = 7.5 Hz)		
Orn4	8.02 (1H, m)	4.38 (1H, m)	1.72 (1H, m) & 1.55 (1H, m)	1.55 (2H, m)	2.78 (2H, m)	7.73 (2H, br)
D-Orn5	8.10 (1H, d, *J* = 8.1 Hz)	4.41 (1H, m)	1.70 (1H, m) & 1.52 (1H, m)	1.51 (2H, m)	2.76 (2H, m)	7.73 (2H, br)
Gly6	8.24 (1H, app t, *J* = 5.3 Hz)	3.85 (1H, dd, *J* = 16.7 & 5.7 Hz) & 3.75 (1H, dd, *J* = 16.7 & 4.9 Hz)				
Orn7	8.07 (1H, d, *J* = 8.2 Hz)	4.39 (1H, m)	1.43 (1H, m) & 1.29 (1H, m)	1.29 (1H, m)	2.58 (2H, br s)	7.65 (2H, m)
D-Trp8	8.32 (1H, d, *J* = 7.7 Hz)	4.75 (1H, app dd, *J* = 13.9, 8.2 Hz)	3.13 (1H, dd, *J* = 15.9, 5.5 Hz) & 2.93 (1H, m)	Indole: 10.76 (1H, s), 7.59 (1H, d, *J* = 7.9 Hz), 7.32 (1H, d, *J* = 8.0 Hz), 7.13 (1H, app s), 7.04 (1H, t, *J* = 7.4 Hz) & 6.96 (1H, t, *J* = 7.4 Hz)		
D-Thr9	7.93 (1H, d, *J* = 8.6 Hz)	4.69 (1H, dd, *J* = 8.6, 3.0 Hz)	5.17 (1H, m)	1.09 (2H, d, *J* = 6.2 Hz)		
D-Ile10	8.30 (1H, d, *J* = 2.6 Hz)	3.90 (1H, dd, *J* = 6.6, 3.8 Hz)	1.58 (1H, m)	1.58 (1H, m), 1.10 (1H, m) & 0.87 (2H, m)	0.87 (3H, m)	
D-Asn11	9.41 (1H, d, *J* = 6.7 Hz)	4.12 (1H, m)	2.89 (1H, dd, *J* = 16.1, 3.5 Hz) & 2.67 (1H, dd, *J* = 16.1, 9.5 Hz)	$-NH_2$: 7.37 (1H, br s)		
Gly12	8.03 (1H, m)	4.02 (1H, dd, *J* = 17.0, 8.2 Hz), 3.58 (1H, dd, *J* = 16.9, 4.3 Hz)				
Gly13	7.67 (1H, m)	4.46 (1H, dd, *J* = 16.6, 9.9 7 Hz), 3.38 (under H_2O) (1H, s)				
Lipid	2.11 (2H, m, O=CC**H**$_2$-), 1.48 (1H, -CH_2C**H**(CH_3)$_2$), 1.45 (2H, O=CCH_2C**H**$_2$-), 1.20 (2H, -C**H**$_2$$CH_2$CH($CH_3$)$_2$), 1.19 (2H, br m, O=C($CH_2$)$_2$C**H**$_2$-), 1.10 (2H, -C**H**$_2$CH($CH_3$)$_2$), 0.83 (6H,-$CH_2$CH(C**H**$_3$)$_2$)					

Ser9-laterocidine (4)

Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Ser	7.85 (1H, d, *J* = 7.2 Hz)	4.26 (1H, q, *J* = 4.8 Hz)	3.50 (m)			
D-Tyr2	8.00 (1H, m)	4.34 (1H, m)	2.83 (1H, dd, *J* = 14.3 & 3.8 Hz) & 2.62 (m)	6.89 (2H, d, *J* = 8.3 Hz) & 6.56 (2H, d, *J* = 8.3 Hz)		
D-Trp3	7.99 (1H, m)	4.52 (1H, m)	3.15 (1H, dd, *J* = 14.7 & 4.0 Hz) & 2.94 (m)	10.79 (1H, s), 7.57 (2H, app t, *J* = 8.2 Hz), 7.33 (1H, d, *J* = 8.2 Hz), 7.14 (1H, br s), 7.07 (1H, t, *J* = 7.3 Hz), 6.98 (1H, t, *J* = 7.5 Hz)		
D-Orn4	8.03 (1H, m)	4.38 (1H, m)	1.73 (1H, m), 1.56 (1H, m)	1.56 (2H, m)	2.78 (2H, m)	7.69 (2H, m)
Orn5z	8.08 (1H, d, *J* = 8.7 Hz)	4.41 (1H, m)	1.70 (1H, m), 1.52 (1H, m)	1.52 (2H, m)	2.77 (2H, m)	7.69 (2H, m)
Gly6	8.23 (1H, m)	3.82 (1H, dd, *J* = 17.0 & 5.9 Hz) & 3.76 (1H, dd, *J* = 17.4 & 4.1 Hz)				
D-Orn7	8.08 (1H, m)	4.40 (1H, m)	1.39 (1H, m), 1.29 (1H, m)	1.30 (2H, m)	2.58 (2H, m)	7.61 (2H, m)
Trp8	8.22 (1H, m)	4.69 (1H, m)	3.09 (2H, dd, *J* = 14.5 & 4.6 Hz) & 2.92 (m)	10.76 (1H, s), 7.57 (2H, app t, *J* = 8.2 Hz), 7.31 (1H, d, *J* = 8.1 Hz), 7.10 (1H, br s), 7.04 (1H, t, *J* = 7.5 Hz), 6.95 (1H, t, *J* = 7.4 Hz)		
Ser9	8.23 (1H, m)	4.62 (1H, m)	4.54 (1H, dd, *J* = 11.0 & 3.4 Hz) & 3.87 (1H, dd, *J* = 10.4 & 5.0 Hz)			
Ile10	8.31 (1H, d, *J* = 5.0 Hz)	4.06 (1H, m)	1.65 (1H, m)	1.52 (1H, m), 1.14 (1H, m) & 0.87 (3H, m)	0.86 (3H, m)	
Asn11	9.21 (1H, d, *J* = 6.2 Hz)	4.17 (1H, m)	2.87 (1H, dd, *J* = 16.0 & 3.8 Hz) & 2.62 (m)			
Gly12	7.98 (1H, m)	4.04 (1H, m) & 3.50 (1H, m)				
Gly13	7.60 (1H, m)	4.39 (1H, m) & 3.56 (1H, br m)				
Lipid	2.11 (2H, m, O=CC**H**$_2$), 1.46 (3H, O=CCH$_2$C**H**$_2$ & (CH$_3$)$_2$C**H**-), 1.20 (4H, (CH$_3$)$_2$CHCH$_2$C**H**$_2$C**H**$_2$-), 1.11 (2H, (CH$_3$)$_2$CHC**H**$_2$-), 0.83 (6H, (C**H**$_3$)$_2$CHCH$_2$-)					

Dap9-Laterocidine (6)

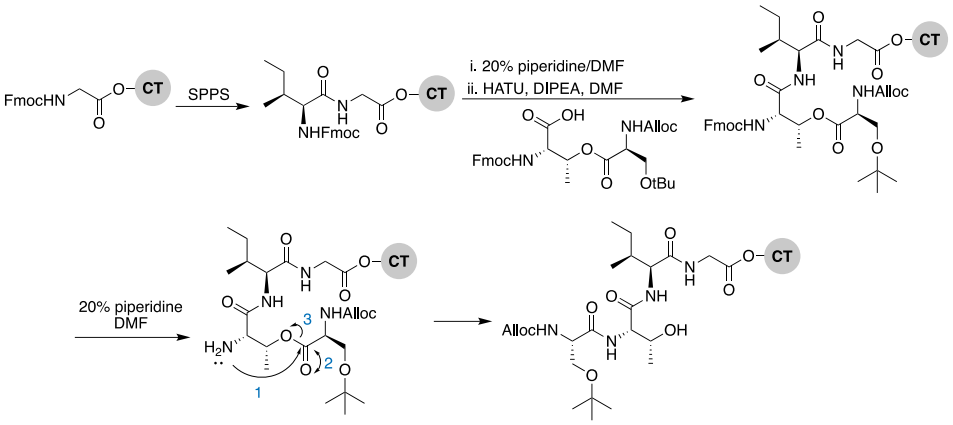
Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Ser1	7.86 (1H, d, *J* = 7.3 Hz)	4.26 (1H, q, *J* = 6.7 Hz)	3.50 (1H, m) -OH: 5.14 (1H, br s)			
D-Tyr2	8.03 (1H, m)	4.33 (1H, m)	2.83 (1H, dd, *J* = 14.3, 4.2 Hz) & 2.62 (1H, m)	Aromatic: 6.89 (2H, d, *J* = 8.4 Hz) & 6.56 (2H, d, *J* = 8.3 Hz) & 9.18 (1H, s, -OH)		
D-Trp3	8.02 (1H, m)	4.51 (1H, m)	3.15 (1H, dd, *J* = 14.5, 3.8 Hz) & 2.94 (1H, dd, *J* = 14.5, 9.3 Hz)	Indole: 10.80 (1H, s), 7.57 (1H, d, *J* = 7.9 Hz), 7.33 (1H, d, *J* = 8.1 Hz), 7.14 (1H, d, *J* = 1.7 Hz), 7.06 (1H, t, *J* = 7.5 Hz) & 6.98 (1H, t, *J* = 7.4 Hz)		
D-Orn4	8.03 (1H, m)	4.38 (1H, m)	1.74 (2H, m)	1.56 (2H, m)	2.78 (2H, m)	7.75 (2H, br)
Orn5	8.09 (1H, m)	4.40 (1H, m)	1.70 (2H, m)	1.53 (2H, m)	2.78 (2H, m)	7.76 (2H, br)
Gly6	8.26 (1H, t, *J* = 5.1 Hz)	3.79 (1H, dd, *J* = 16.8, 5.7 Hz) & 3.74 (1H, m)				
D-Orn7	8.07 (1H, m)	4.33 (1H, m)	1.36 (1H, m) & 1.29 (1H, m)	1.29 (2H, m)	2.57 (2H, m)	7.67 (2H, m)
Trp8	8.15 (1H, d, *J* = 8.3 Hz)	4.65 (1H, m)	3.09 (1H, dd, *J* = 14.1, 3.8 Hz) & 2.88 (1H, br m)	Indole: 10.78 (1H, s), 7.61 (1H, d, *J* = 7.9 Hz), 7.30 (1H, d, 8.1 Hz), 7.11 (1H, d, *J* = 1.8 Hz), 7.04 (1H, t, *J* = 7.4 Hz) & 6.96 (1H, t, *J* = 7.4 Hz)		
Dap9	8.20 (1H, *J* = 7.0 Hz)	4.29 (1H, br m)	3.74 (1H, m) & 2.67 (1H, m)	-NH: 7.67 (1H, m)		
Ile10	7.96 (1H, d, *J* = 9.1 Hz)	4.51 (1H, m)	1.62 (1H, m)	1.05 (1H, br m), 1.40 (1H, m) & 0.85 (3H, m)	0.81 (3H, m)	
Asn11	9.22 (1H, d, *J* = 6.2 Hz)	4.16 (1H, m)	2.92 (1H, m) & 2.56 (1H, m)	-NH_2: 7.39 (1H, br s)		
Gly12	8.23 (1H, dd, *J* = 9.1, 2.5Hz)	4.23 (1H, dd, *J* = 17.1, 9.6 Hz) & 3.28 (1H, br m)				
Gly13	7.72 (1H, dd, *J* = 9.0, 3.5 Hz)	4.15 (1H, m) & 3.39 (under H_2O)				
Lipid	2.10 (2H, m, O=CC**H_2**-), 1.46 (3H, -CH_2**CH**$(CH_3)_2$ & O=CCH_2C**H_2**-), 1.20 (4H, m, -C**H_2**$CH_2CH(CH_3)_2$ & O=$C(CH_2)_2$C**H_2**-), 1.11 (2H, -C**H_2**$CH(CH_3)_2$) & 0.83 (6H, -CH_2CH(C**H_3**$)_2$)					

MeDap9-laterocidine (8)

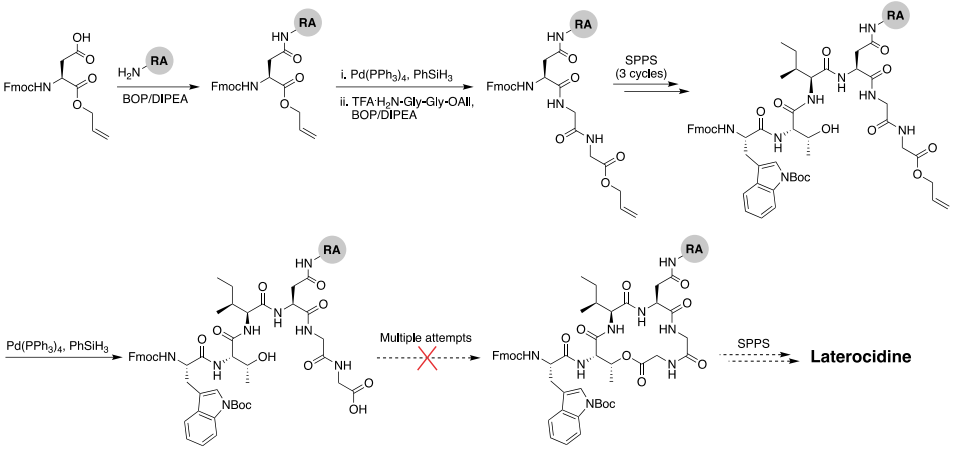
Residue	-NH	Hα	Hβ	Hγ	Hδ	Hε
D-Ser1	7.85 (1H, d, *J* = 7.4 Hz)	4.26 (1H, q, *J* = 6.7 Hz)	3.50 (2H, m) -OH: 5.12 (1H, *J* = 5.5 Hz)			
D-Tyr2	8.01 (1H, m)	4.33 (1H, m)	2.83 (1H, dd, *J* = 14.5, 4.2 Hz) & 2.62 (1H, m)	Aromatic: 6.89 (1H, d, *J* = 8.4 Hz) & 6.56 (1H, d, *J* = 8.3 Hz), 9.17 (1H, s, -OH)		
D-Trp3	8.00 (1H, m)	4.51 (1H, m)	3.15 (1H, dd, *J* = 14.3, 3.6 Hz) & 2.93 (1H, br m)	Indole: 10.79 (1H, s), 7.57 (1H, d, *J* = 7.8 Hz), 7.33 (1H, d, *J* = 8.1 Hz), 7.14 (1H, br s), 7.06 (1H, t, *J* = 7.5 Hz) & 6.98 (1H, t, *J* = 7.4 Hz)		
D-Orn4	8.03 (1H, d, *J* = 7.8 Hz)	4.38 (1H, m)	1.73 (1H, m) & 1.56 (1H, m)	1.53 (2H, m)	2.78 (2H, m)	7.71 (2H, m)
Orn5	8.08 (1H, m)	4.40 (1H, m)	1.70 (1H, m) & 1.53 (1H, m)	1.53 (2H, m)	2.77 (2H, m)	7.71 (2H, m)
Gly6	8.26 (1H, t, *J* = 5.0 Hz)	3.82 (1H, dd, *J* = 17.2, 5.8 Hz) & 3.73 (1H, dd, *J* = 16.9, 4.8 Hz)				
D-Orn7	8.13 (1H, d, *J* = 8.4 Hz)	4.38 (1H, m)	1.40 (1H, m) & 1.31 (1H, m)	1.31 (2H, m)	2.58 (2H, m)	7.63 (2H, br m)
Trp8	8.20 (1H, d, *J* = 7.8 Hz)	4.69 (1H, m)	3.08 (1H, dd, *J* = 14.6 & 4.1 Hz) & 2.92 (1H, m)	Indole: 10.78 (1H, s), 7.60 (1H, d, *J* = 7.7 Hz), 7.31 (1H, d, 8.1 Hz), 7.12 (1H, d, *J* = 1.7 Hz), 7.04 (1H, t, *J* = 7.5 Hz) & 6.96 (1H, t, *J* = 7.3 Hz)		
MeDap9	8.07 (1H, m)	4.52 (1H, m)	4.22 (1H, m)	1.05 (2H, d, *J* = 7.0 Hz), 6.88 (1H, m, NH)		
Ile10	7.91 (1H, d, *J* = 8.0 Hz)	4.35 (1H, m)	1.62 (1H, m) & 0.84 (1H, m)	1.43 (1H, m), 1.08 (1H, m) & 0.84 (3H, m)		
Asn11	9.25 (1H, d, *J* = 6.3 Hz)	4.14 (1H, m)	2.90 (1H, dd, *J* = 15.9 & 3.1 Hz) & 2.62 (1H, m)			
Gly12	8.35 (1H, br d, *J* = 7.6 Hz)	4.12 (1H, m) & 3.41 (1H, dd, *J* = 17.1 & 3.1 Hz)				
Gly13	7.73 (1H, m)	4.17 (1H, dd, *J* = 16.9 & 9.3 Hz) & 3.36 (1H, under H_2O)				
Lipid	2.11 (2H, m, O=CC**H**$_2$-), 1.46 (3H, -CH_2C**H**$(CH_3)_2$ & O=CCH_2C**H**$_2$-), 1.20 (4H, -C**H**$_2$$CH_2$$CH(CH_3)_2$) & O=C$(CH_2)_2$C**H**$_2$-), 1.11 (2H, -C**H**$_2$$CH(CH_3)_2$) & 0.83 (6H, -$CH_2$$CH(CH_3)_2$					

NMR traces of all peptides can be found in the online supplementary material at DOI https://doi.org/10.1039/D2SC00143H

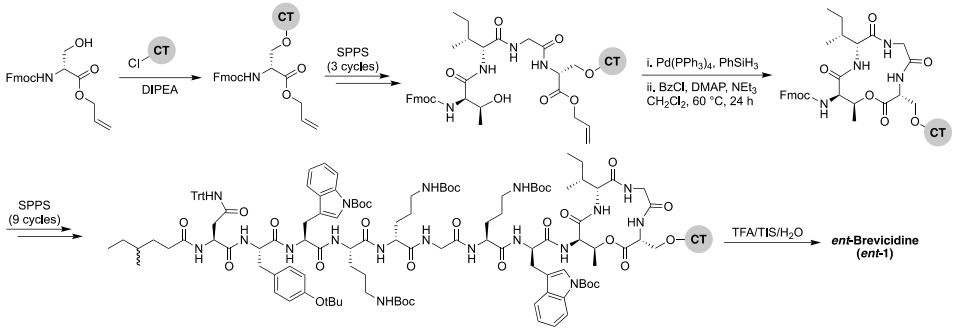
Supplemental Schemes



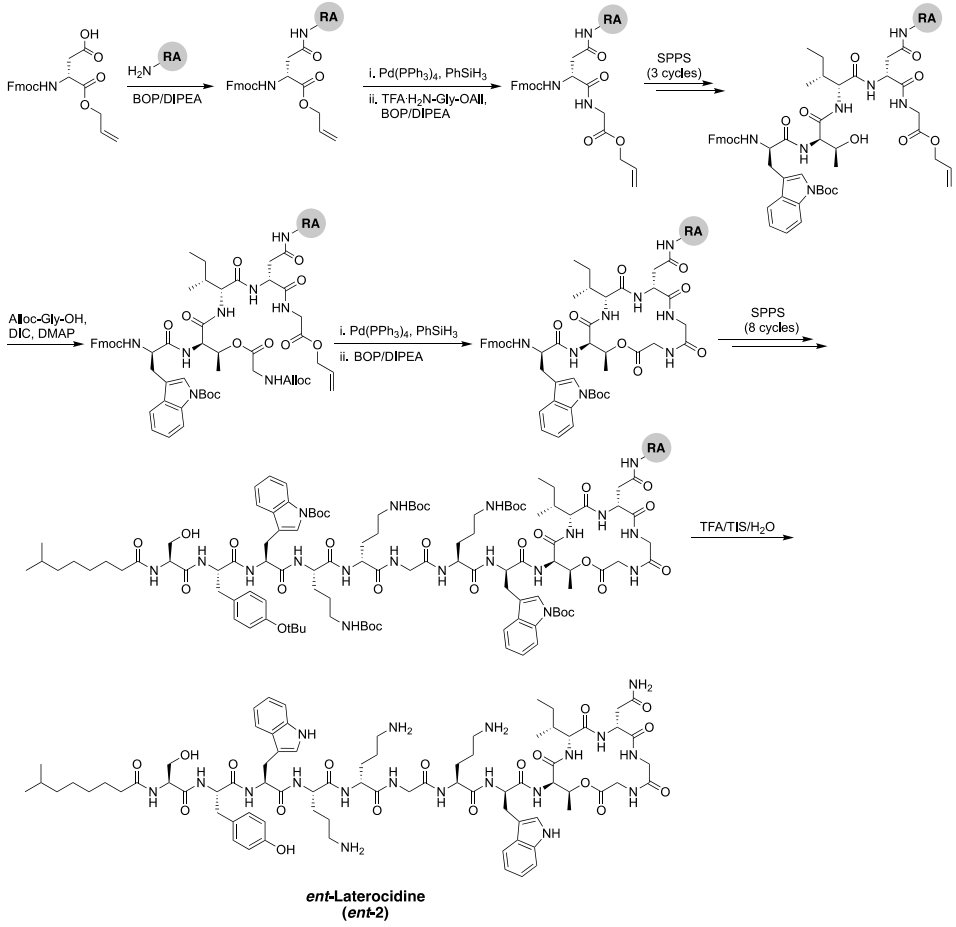
Scheme S1. Attempted synthesis of brevicidine failed due to deleterious *O*→*N* acyl shift. CT = 2-chlorotrityl resin.



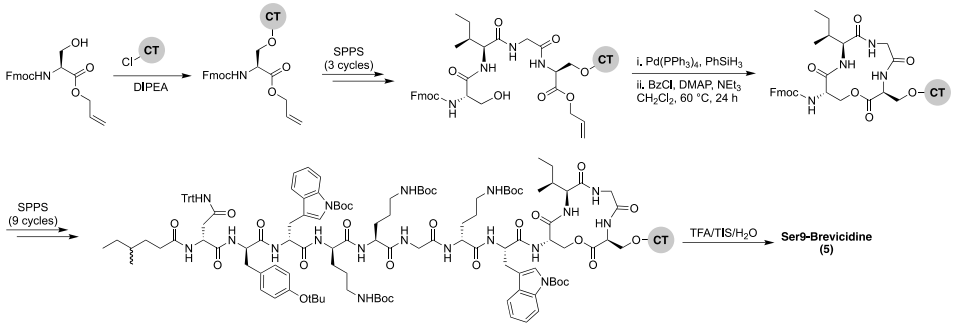
Scheme S2. Attempted synthesis of laterocidine failed at macrolactonization stage. RA = Rink amide resin.



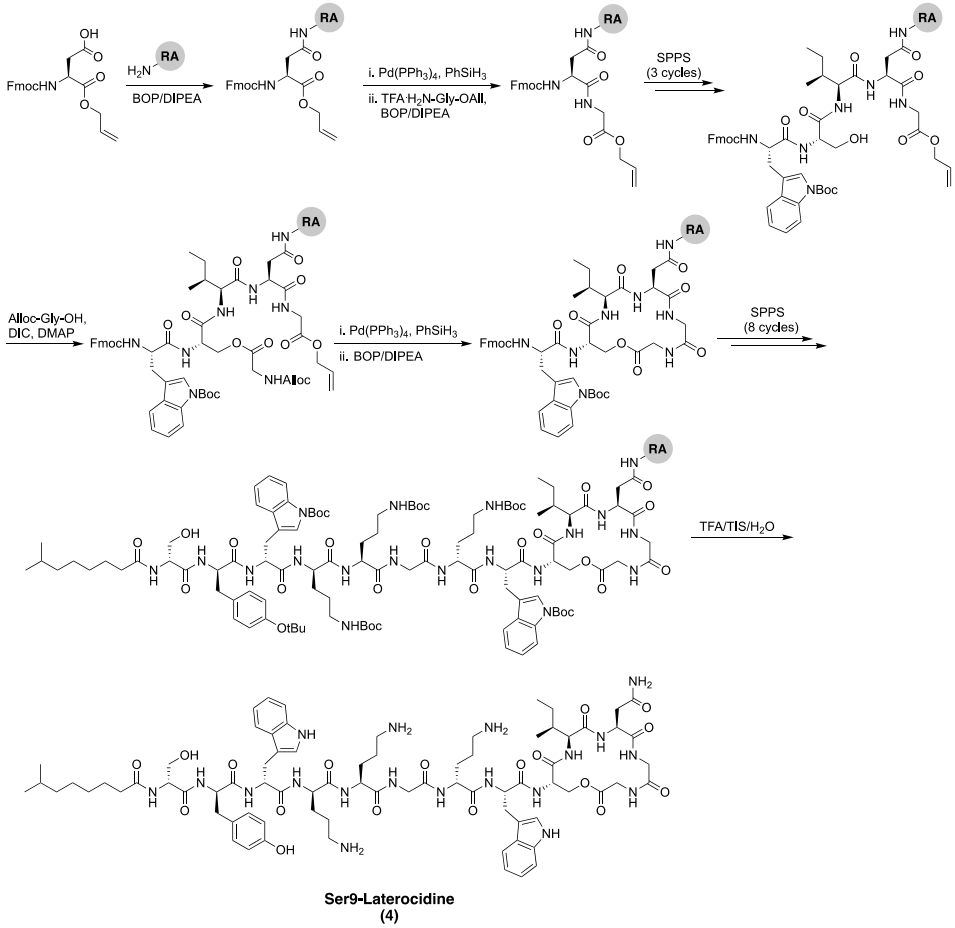
Scheme S3. Total SPPS of *ent*-brevicidine (***ent-1***). CT = 2-chlorotrityl resin.



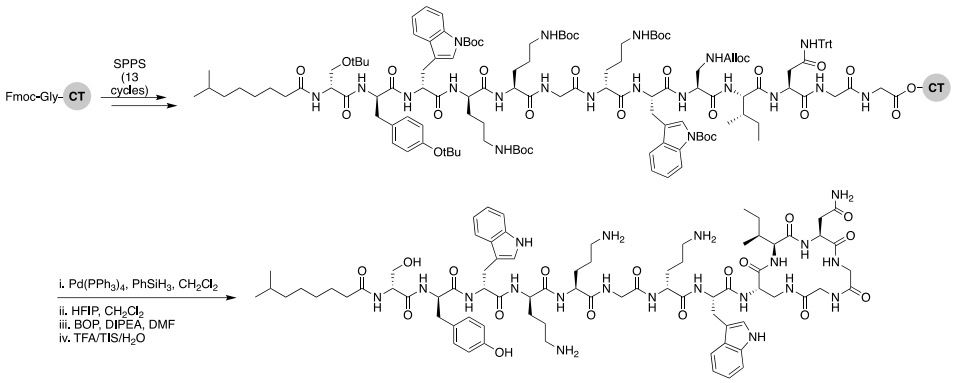
Scheme S4. Total SPPS of *ent*-laterocidine (***ent-2***). RA = Rink amide resin.



Scheme S5. Total SPPS of Ser9-brevicidine (**3**). CT = 2-chlorotrityl resin.



Scheme S6. Total SPPS of Ser9-laterocidine (**4**). RA = Rink amide resin.



Scheme S7. Combination synthesis of Dap9-laterocidine (**6**) where the entire protected linear peptide is assembled by SPPS, and cyclized and deprotected in solution. CT = 2-chlorotrityl resin.

Supplemental figures

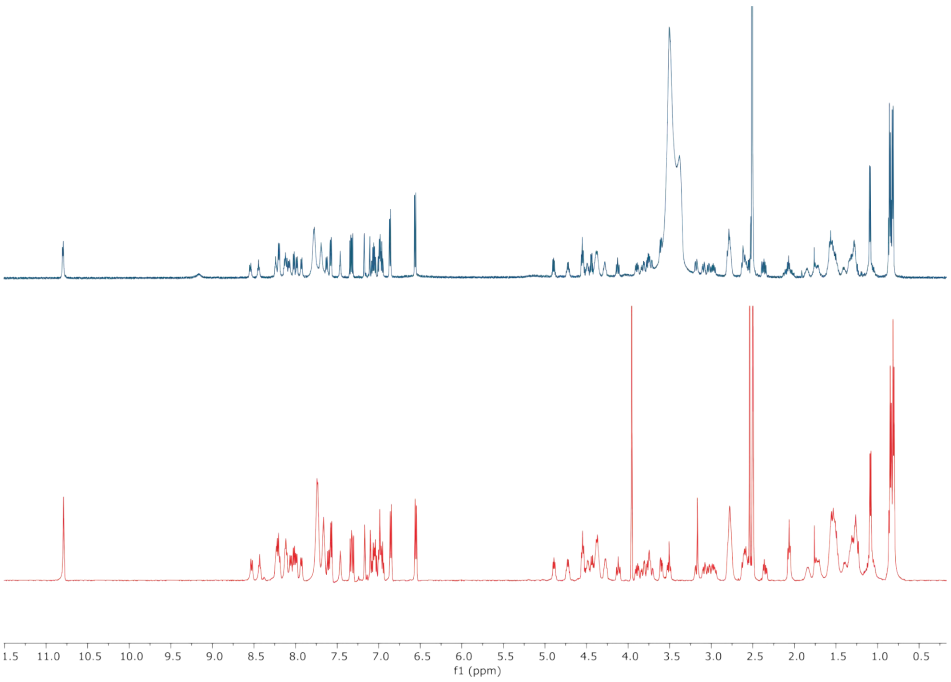


Figure S1. *^{1}H NMR (600 MHz, DMSO-d_6) of synthetic brevicidine (top) overlaid with the previously published ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of natural brevicidine (bottom) isolated from fermentation of the producing organism. Spectra were recorded at room temperature. The spectrum for the synthetic material contains broad signals between 3.30-3.65 ppm due to $H_2$0/HDO present in the NMR solvent. The peak at ca. 3.96 ppm in the published spectrum of brevicidine is attributed to an impurity not present in the synthetic material.*

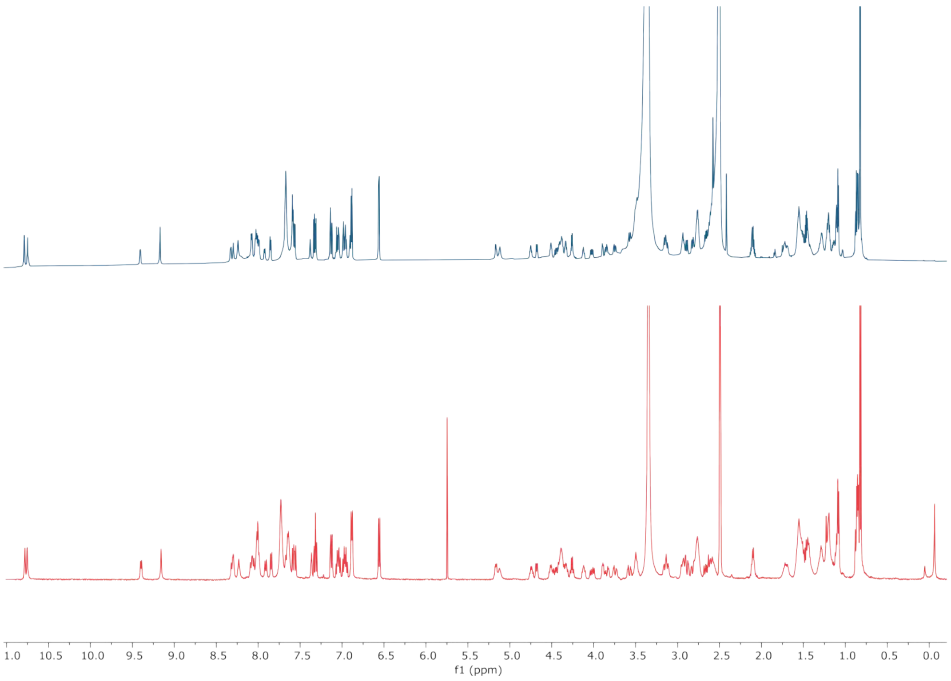


Figure S2. *^{1}H NMR (850 MHz, DMSO-d_6) of synthetic laterocidine (top) overlaid with the previously published ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of natural laterocidine (bottom) isolated from fermentation of the producing organism. Spectra were recorded at room temperature.*

References

(1) Bierbaum, G.; Sahl, H.-G. The Search for New Anti-Infective Drugs: Untapped Resources and Strategies. *International Journal of Medical Microbiology* **2014**, *304* (1), 1–2. https://doi.org/10.1016/j.ijmm.2013.10.003.

(2) Brown, E. D.; Wright, G. D. Antibacterial Drug Discovery in the Resistance Era. *Nature* **2016**, *529* (7586), 336–343. https://doi.org/10.1038/nature17042.

(3) Brown, E. D. Gram-Negative Resistance. *ACS Infect Dis* **2015**, *1* (11), 507–507. https://doi.org/10.1021/acsinfecdis.5b00123.

(4) O'Neill, J. *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations.*; 2014. https://amr-review.org/.

(5) WHO. *https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed*.

(6) Li, Y. X.; Zhong, Z.; Zhang, W. P.; Qian, P. Y. Discovery of Cationic Nonribosomal Peptides as Gram-Negative Antibiotics through Global Genome Mining. *Nat Commun* **2018**, *9* (1). https://doi.org/10.1038/s41467-018-05781-6.

(7) Morales, F. E.; Garay, H. E.; Muñoz, D. F.; Augusto, Y. E.; Otero-González, A. J.; Reyes Acosta, O.; Rivera, D. G. Aminocatalysis-Mediated on-Resin Ugi Reactions: Application in the Solid-Phase Synthesis of N -Substituted and Tetrazolo Lipopeptides and Peptidosteroids. *Org Lett* **2015**, *17* (11), 2728–2731. https://doi.org/10.1021/acs.orglett.5b01147.

(8) Diamandas, M.; Moreira, R.; Taylor, S. D. Solid-Phase Total Synthesis of Dehydrotryptophan-Bearing Cyclic Peptides Tunicyclin B, Sclerotide A, CDA3a, and CDA4a Using a Protected β-Hydroxytryptophan Building Block. *Org Lett* **2021**, *23* (8), 3048–3052. https://doi.org/10.1021/acs.orglett.1c00717.

(9) Al-Ayed, K.; Ballantine, R. D.; Zhong, Z.; Li, Y.; Cochrane, S.; Martin, N. Total Synthesis of the Brevicidine and Laterocidine Family of Lipopeptide Antibiotics. *ChemRxiv* **2021**, 1–8. https://doi.org/10.26434/CHEMRXIV.13660949.V1.

(10) Pelay-Gimeno, M.; García-Ramos, Y.; Jesús Martin, M.; Spengler, J.; Molina-Guijarro, J. M.; Munt, S.; Francesch, A. M.; Cuevas, C.; Tulla-Puche, J.; Albericio, F. The First Total Synthesis of the Cyclodepsipeptide Pipecolidepsin A. *Nat Commun* **2013**, *4* (1), 2352. https://doi.org/10.1038/ncomms3352.

(11) Hermant, Y.; Palpal-latoc, D.; Kovalenko, N.; Cameron, A. J.; Brimble, M. A.; Harris, P. W. R. The Total Chemical Synthesis and Biological Evaluation of the Cationic Antimicrobial Peptides, Laterocidine and Brevicidine. *J Nat Prod* **2021**, *84* (8), 2165–2174. https://doi.org/10.1021/acs.jnatprod.1c00222.

(12) McDougal, P. G.; Griffin, J. H. Enantiotracin. *Bioorg Med Chem Lett* **2003**, *13* (13), 2239–2240. https://doi.org/10.1016/S0960-894X(03)00252-X.

(13) Kleijn, L. H. J.; Vlieg, H. C.; Wood, T. M.; Sastre Toraño, J.; Janssen, B. J. C.; Martin, N. I. A High-Resolution Crystal Structure That Reveals Molecular Details of Target Recognition by the Calcium-Dependent Lipopeptide Antibiotic Laspartomycin C. *Angewandte Chemie International Edition* **2017**, *56* (52), 16546–16549. https://doi.org/10.1002/anie.201709240.

(14) Cochrane, S. A.; Findlay, B.; Vederas, J. C.; Ratemi, E. S. Key Residues in Octyl-Tridecaptin A 1 Analogues Linked to Stable Secondary Structures in the Membrane. *ChemBioChem* **2014**, *15* (9), 1295–1299. https://doi.org/10.1002/cbic.201402024.

(15) Vetterli, S. U.; Zerbe, K.; Müller, M.; Urfer, M.; Mondal, M.; Wang, S. Y.; Moehle, K.; Zerbe, O.; Vitale, A.; Pessi, G.; Eberl, L.; Wollscheid, B.; Robinson, J. A. Thanatin Targets the Intermembrane Protein Complex Required for Lipopolysaccharide Transport in Escherichia Coli. *Sci Adv* **2018**, *4* (11). https://doi.org/10.1126/SCIADV.AAU2634/SUPPL_FILE/AAU2634_SM.PDF.

(16) Moreira, R.; Taylor, S. D. The Chiral Target of Daptomycin Is the 2R, 2'S Stereoisomer of Phosphatidylglycerol. *Angewandte Chemie International Edition* **2022**, *61* (4), e202114858. https://doi.org/10.1002/anie.202114858.

(17) Nam, J.; Shin, D.; Rew, Y.; Boger, D. L. Alanine Scan of [L-Dap2]Ramoplanin A2 Aglycon: Assessment of the Importance of Each Residue. *J Am Chem Soc* **2007**, *129* (28), 8747–8755. https://doi.org/10.1021/JA068573K/SUPPL_FILE/JA068573KSI20070517_065843.PDF.

(18) Bionda, N.; Stawikowski, M.; Stawikowska, R.; Cudic, M.; López-Vallejo, F.; Treitl, D.; Medina-Franco, J.; Cudic, P. Effects of Cyclic Lipodepsipeptide Structural Modulation on Stability, Antibacterial Activity, and

Human Cell Toxicity. *ChemMedChem* **2012**, *7* (5), 871–882. https://doi.org/10.1002/cmdc.201200016.

(19) Hart, P. T.; Kleijn, L. H. J.; de Bruin, G.; Oppedijk, S. F.; Kemmink, J.; Martin, N. I. A Combined Solid- and Solution-Phase Approach Provides Convenient Access to Analogues of the Calcium-Dependent Lipopeptide Antibiotics. *Org. Biomol. Chem.* **2014**, *12* (6), 913–918. https://doi.org/10.1039/C3OB42238K.

(20) Gimenez, D.; Phelan, A.; Murphy, C. D.; Cobb, S. L. Fengycin A Analogues with Enhanced Chemical Stability and Antifungal Properties. *Org Lett* **2021**, *23* (12), 4672–4676. https://doi.org/10.1021/ACS.ORGLETT.1C01387/ASSET/IMAGES/LARGE/OL1C01387_0004.JPEG.

(21) Li, Z.; Chakraborty, P.; de Vries, R. H.; Song, C.; Zhao, X.; Roelfes, G.; Scheffers, D. J.; Kuipers, O. P. Characterization of Two Relacidines Belonging to a Novel Class of Circular Lipopeptides That Act against Gram-Negative Bacterial Pathogens. *Environ Microbiol* **2020**, *22* (12), 5125–5136. https://doi.org/10.1111/1462-2920.15145.

(22) Mukherjee, S.; van der Donk, W. A. Mechanistic Studies on the Substrate-Tolerant Lanthipeptide Synthetase ProcM. *J Am Chem Soc* **2014**, *136* (29), 10450–10459. https://doi.org/10.1021/ja504692v.

(23) Ge, J.; Li, L.; Yao, S. Q. A Self-Immobilizing and Fluorogenic Unnatural Amino Acid That Mimics Phosphotyrosine. *Chemical Communications* **2011**, *47* (39), 10939. https://doi.org/10.1039/c1cc14653j.

(24) Freire, F.; Fisk, J. D.; Peoples, A. J.; Ivancic, M.; Guzei, I. A.; Gellman, S. H. Diacid Linkers That Promote Parallel β-Sheet Secondary Structure in Water. *J Am Chem Soc* **2008**, *130* (25), 7839–7841. https://doi.org/10.1021/ja802042c.

(25) Chun, C.; Lee, S. M.; Kim, S. Y.; Yang, H. K.; Song, S.-C. Thermosensitive Poly(Organophosphazene)–Paclitaxel Conjugate Gels for Antitumor Applications. *Biomaterials* **2009**, *30* (12), 2349–2360. https://doi.org/10.1016/j.biomaterials.2008.12.083.

(26) Moreira, R.; Barnawi, G.; Beriashvili, D.; Palmer, M.; Taylor, S. D. The Effect of Replacing the Ester Bond with an Amide Bond and of Overall Stereochemistry on the Activity of Daptomycin. *Bioorg Med Chem* **2019**, *27* (1), 240–246. https://doi.org/10.1016/J.BMC.2018.12.004.

(27) Gude, M.; Ryf, J.; White, P. D. An Accurate Method for the Quantitation of Fmoc-Derivatized Solid Phase Supports. *Letters in Peptide Science 2002 9:4* **2002**, *9* (4), 203–206. https://doi.org/10.1023/A:1024148619149.