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The contribution of achiral residues in the laspartomycin family of calcium-dependent lipopeptide antibiotics†

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The growing threat of antibacterial resistance is a global concern. The so-called calcium-dependent lipopeptide antibiotics (CDAs) have emerged as a promising source of new antibiotic agents that are rich in structural and mechanistic diversity. Over forty unique CDAs have been identified to date and share a number of common features. Recent efforts in our group have provided new mechanistic and structural insights into the laspartomycin family of CDAs. We here describe investigations aimed at probing the role of the three glycine residues found in the laspartomycin peptide macrocycle. In doing so laspartomycin analogues containing the achiral 2-aminoisobutyric acid (AIB) as well as L- or D-alanine in place of glycine were prepared and their antibacterial activities evaluated.

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Introduction

The accelerated emergence of multi drug-resistant (MDR) bacteria is now considered a top priority and one of the most urgent global threats to human health.¹ According to a 2017 report from the Center for Disease Control and prevention (CDC) more than 2 million Americans acquire antibiotic-resistant infections each year leading to at least 23 000 deaths in the US alone.² Since the so-called “golden age” of antibiotic discovery spanning the 1940s–1960s, very few new antibiotics operating with novel modes of action have been introduced.³ Notable in this regard is daptomycin, the preeminent calcium-dependent antibiotic (CDA) and the most recent first-in-class antibiotic to have entered the clinic.^{4,5} Daptomycin's precise mechanism of action is a topic of some debate.^{6,7} By comparison, the mode of action of the structurally similar CDA laspartomycin C (Fig. 1) is more clearly understood.^{8,9} Unlike daptomycin, laspartomycin C specifically targets the essential bacterial phospholipid undecaprenyl phosphate (C₅₅-P). C₅₅-P represents a novel target and to date there are no clinically approved antibiotics that operate by binding C₅₅-P. Our group recently reported the first total synthesis of laspartomycin C¹⁰ as well as the co-crystal structure of laspartomycin C bound to both calcium and a C₁₀ truncated analogue of C₅₅-P.¹¹

While the CDAs comprise a diverse family of lipopeptide antibiotics with varying mechanisms of action, they share a number of key structural similarities. These include the positioning of key achiral or D-amino acids as well as the highly conserved Asp-X-Asp-Gly motif.¹² This Asp-X-Asp-Gly sequence is essential for the calcium binding that all CDAs utilize in achieving their full antibacterial activity. In the absence of calcium, the activity of the CDAs is significantly reduced or completely lost.¹³ Fig. 1 presents the structure of laspartomycin C and highlights the structural features common to many CDAs.

At present there are more than 40 unique CDAs known and their structural diversity continues to provide great opportunity for discovery.¹⁴ All CDAs, except for the newly reported malacindins, contain 10 amino acids in their macrocycle. Using the macrocycle of laspartomycin C as a reference point it can be

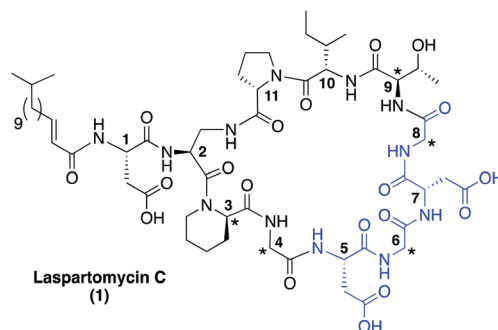


Fig. 1 Laspartomycin C. Indicated with a star are the achiral or D-amino acids and highlighted in blue is the conserved Asp-X-Asp-Gly calcium binding motif common to the CDAs.

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noted that: (1) The majority of CDAs bear an L-amino acid at position 4 (usually an ornithine, alanine, aspartic acid, or β -methyl-aspartic acid) with the exception being the laspartomycins which include a glycine at position 4; (2) position 6 is nearly always a D-amino acid, again with the exception of the laspartomycins (as well as the related friulimycins and amphomycins) which contain glycine at position 6; and (3) 100% of CDAs contain a glycine at position 8.

The inclusion of glycine at residues 4, 6, and 8 in the laspartomycin C macrocycle is unique among the CDAs. This observation prompted us to investigate the contribution that the absence of chirality at these positions has on antibacterial activity. To this end we investigated the introduction of the achiral 2-aminoisobutyric acid (Aib) at these positions as well as the introduction of either L- or D-alanine residues. We here describe the preparation and evaluation of a series of novel laspartomycin analogues that provide new stereochemical structure–activity insights at positions 4, 6, and 8.

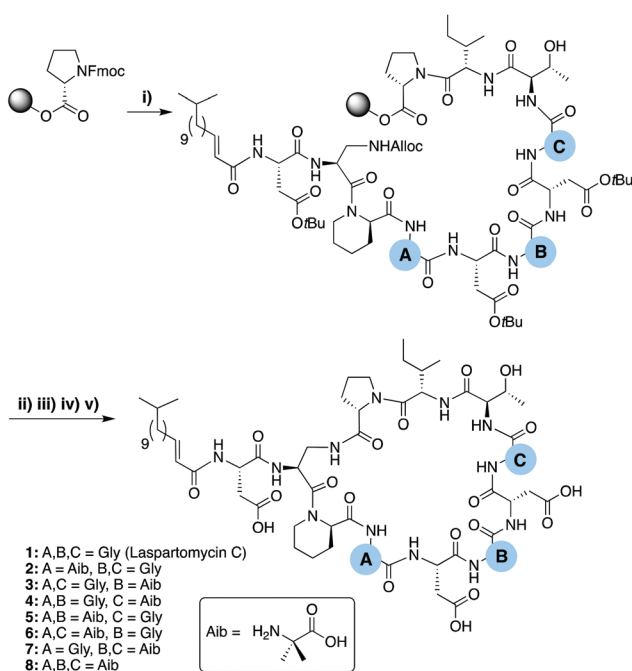
Results and discussion

To evaluate the role of the three achiral centers in the laspartomycin C macrocycle we began by preparing a series of analogues containing the achiral amino acid 2-aminoisobutyric acid (Aib) at each position. These laspartomycin C variants were prepared using a robust combined solid- and solution-phase approach that our group as well as Payne and coworkers previously described (Scheme 1).^{10,15} The linear lipopeptide

precursors were first assembled on solid support using the acid sensitive 2-chlorotrityl resin. Starting from C-terminal Pro₁₁ each peptide was assembled using standard Fmoc-SPPS strategies with the notable introduction of an Alloc side chain protected L-diaminopropionic acid (L-Dap) residue at position 2. Also, for analogues containing Gly at positions 6 and/or 8 (compounds 1–6) DMB-Gly was employed to avoid aspartamide formation. Following N-terminal lipidation, the Alloc side chain protecting of L-Dap₂ was removed and the partially protected peptide released from the resin using mildly acidic conditions. The partially protected peptide was then cyclized at high dilution by treatment with BOP/DIPEA resulting in the clean formation of the macrocyclic product. Global deprotection followed by RP-HPLC purification provided the various laspartomycin analogues in yields ranging from 5–13%.

To assess the contribution of the achiral positions 4, 6, and 8, analogues 2–8 were prepared wherein one or more of the glycine residues was replaced by the achiral Aib. The rationale for this replacement related to the previously reported ability of the Aib residue to predispose peptides towards their active conformation(s).¹⁶ The antimicrobial activity of analogues 2–8 was compared with that of authentic laspartomycin C (Table 1) using a serial dilution assay employing a clinically relevant strain of Methicillin-resistant *Staphylococcus aureus* (MRSA). Analogues 2 and 3 were found to exhibit calcium-dependent activity, albeit significantly less than laspartomycin C, while analogues 4–8, were essentially devoid of activity. Interesting, analogues 2 and 3, both containing a single Aib at position 4 and 6 respectively, maintain some activity. Conversely, Aib substitution at position 8 as in analog 4 led to complete loss of activity. Notably, analogue 8 containing Aib at all three positions, exhibits modest activity while showing no Ca²⁺ dependency. These results indicate that modification at positions 4 and 6 can compensate for an otherwise detrimental modification at position 8.

All known CDAs contain a glycine at position 8 which is part of the Asp-X₆-Asp-Gly₈ calcium binding region. It therefore stands to reason that changes at position 8 significantly impact in the peptide's ability to chelate calcium and bind to its bacterial target. In this regard it is notable that position 6,



Scheme 1 (i) Fmoc SPPS; (ii) Pd[(C₆H₅)₃P]₄, C₆H₅SiH₃, CH₂Cl₂, 1 h; (iii) HFIP, CH₂Cl₂, 1 h; (iv) BOP, DIPEA, CH₂Cl₂, 16 h; (v) TFA, TIS, H₂O, 1 h (Fmoc-D-Thr was employed without side chain protection and incorporated without incident).

Table 1 MIC values for laspartomycin C and analogues 2–8^a

Compound	[Ca ²⁺]			
	0	2.5	5.0	10
1 (Laspc)	≥128	4	4	2
2	≥128	16	16	16
3	≥128	64	32	32
4	≥128	≥128	≥128	≥128
5	64	64	64	64
6	≥128	≥128	≥128	≥128
7	≥128	≥128	≥128	≥128
8	64	64	64	64

^a Minimum inhibitory concentrations reported in $\mu\text{g mL}^{-1}$ against MRSA USA300 at calcium concentration indicated.



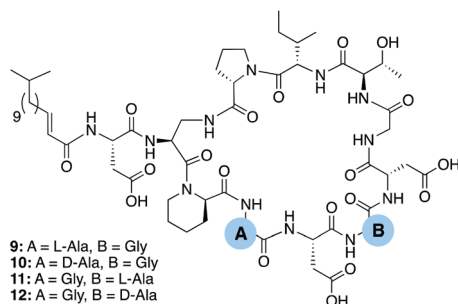


Fig. 2 Laspartomycin C analogues prepared to investigate the effect of introducing L- or D-alanine at positions 4 and 6.

Table 2 MIC values for laspartomycin C and analogues 9–12^a

Compound	[Ca ²⁺]			
	0	2.5	5.0	10
1 (LaspC)	≥128	8	4	2
9	≥128	16	8	4
10	≥128	16	8	2
11	≥128	64	32	16
12	64	16	8	1

^a Minimum inhibitory concentrations reported in $\mu\text{g mL}^{-1}$ against MRSA USA300 at calcium concentration indicated.

also part of the CDA calcium binding region, is more amenable to variation. In the case of the laspatomycins, friulimicins, and amphotomycins the inclusion of Gly at position 6 sets them apart from the majority of other CDAs which generally contain a D-amino acid at this site. In the case of daptomycin D-alanine is found at position 6 while in the A54145 class it is D-lysine and in the confusingly named “CDA-class” it is D-phenylglycine.¹⁴ With this in mind we prepared four additional analogues examining the effect in introducing L- or D-alanine at positions 4 and 6 (Fig. 2).

The antimicrobial activity of analogues 9–12 was compared with that of authentic laspartomycin C (Table 2). Using the same activity assay employing MRSA USA300 as an indicator strain, all four analogues were found to be more active than their Aib containing counterparts with analogues 10 and 12 showing activity on par with that of laspartomycin itself. Interesting, the introduction of L- or D-alanine at position 4 yields compounds of similar activity while at position 6 the D-Ala variant was 16-fold more active than the L-analogue. These findings further demonstrate the importance of a D-stereocenter at position 6 of the CDA calcium-binding motif.

Conclusion

In summary, we have generated a focused library of laspartomycin C analogues designed to explore the contribution of the achiral 4, 6, and 8 positions on antibacterial activity. Using a combined solid- and solution-phase approach, analogues were

first prepared wherein positions 4, 6 and 8 were modified to contain the achiral amino acid Aib. These analogues showed a significant reduction in activity compared to the parent compound. Incorporation of Aib at position 8 led to complete loss of activity while the same modification at positions 4 or 6 led to analogues that retained some activity. A second series of analogues incorporating either D or L-alanine at positions 4 and 6 were also prepared. The introduction of both D- and L-Ala at position 4 led to compounds that showed activity similar to laspartomycin C. However, at position 6 the D-Ala variant is 16-times more active than the L-Ala and even showed slightly better activity than laspartomycin C.

While the various members of the CDA family contain diverse amino acids at position 4 they are always either glycine or a L-amino acid. Our findings indicate that the incorporation of D-Ala at position 4 does not negatively affect activity. Position 6 plays a key role in the calcium-binding motif (Asp-X₆-Asp-Gly) and is always either a glycine or a D-amino acid. Our findings demonstrate that positions 4 and 6 of Laspartomycin C are amenable to substitution provided the correct stereochemical constraints are respected. To this end ongoing studies in our laboratory are aimed at establishing whether the antibacterial activity of the laspartomycins can be enhanced by structural variation at these positions.

Experimental

Solid phase peptide synthesis

Chlorotrityl resin (5.0 g, 1.60 mmol g⁻¹) was loaded with Fmoc-Pro-OH. Resin loading was determined to be 0.41–0.62 mmol g⁻¹. Linear peptide encompassing Pro11 to Asp1 were assembled manually *via* standard Fmoc solid-phase peptide synthesis (SPPS) (resin bound AA:Fmoc-AA:BOP:DiPEA, 1:4:4:8 molar eq.) on a 0.1 mmol scale. DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF (1:4 v:v). Amino acid side chains were protected as follows: ^tBu for Asp, Alloc for DAP, and DMB for Gly6 and Gly8. D-*allo*-Thr was introduced without side chain protection. Following coupling and Fmoc deprotection of Asp1, N-terminal acylation was achieved by coupling (E)-13-methyltetradec-2-enoic acid using the same coupling conditions used for SPPS. The resin-bound, Alloc protected intermediate was next washed with CH₂Cl₂ and treated with Pd(PPh₃)₄ (30 mg, 0.03 mmol) and PhSiH₃ (0.30 mL, 3.0 mmol) in CH₂Cl₂ (*ca.* 7 mL) under argon for 1 hour. The resin was subsequently washed with CH₂Cl₂ (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 resin was treated with (CF₃)₂CHOH:CH₂Cl₂ (1:4, 10 mL) for 1 hour and rinsed with additional (CF₃)₂CHOH:CH₂Cl₂ and CH₂Cl₂. The combined washings were then evaporated to yield the linear protected peptide with free C- and N-termini. The residue was dissolved in CH₂Cl₂ (150 mL) and treated with BOP (0.22 g, 0.5 mmol) and DiPEA (0.17 mL, 1.0 mmol) and the solution was stirred overnight after which TLC indicated



complete cyclization. The reaction mixture was concentrated and directly treated with TFA:TIS:H₂O (95:2.5:2.5, 10 mL) for 90 minutes. The reaction mixture was added to MTBE:hexanes (1:1) and the resulting precipitate washed once more with MTBE:hexanes (1:1). The crude cyclic peptide was lyophilized from ^tBuOH:H₂O (1:1) and purified with reverse phase HPLC. Pure fractions were pooled and lyophilized to yield the desired cyclic lipopeptide products in >95% purity as white powders, typically in 10–20 mg quantities (4.2–9.3% yield based on resin loading).

MIC determinations

Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to CLSI guidelines.¹⁷ Blood agar plates were inoculated with glycerol stocks of MRSA USA300 and *S. simulans* 22 (see ESI†) followed by incubation for 16 hours at 37 °C and 30 °C respectively. Cation adjusted Mueller-Hinton broth (MHB) containing 10 mg L⁻¹ Mg²⁺ was inoculated with individual colonies of *S. aureus* and Methicillin-resistant *S. aureus* (MRSA USA300), and incubated for 16 hours at 220 RPM. The peptides were dissolved in MHB (10 mg L⁻¹ Mg²⁺) and serially diluted on polypropylene microtiter plates with a volume of 50 µL per well. Inoculated MHB (2 × 10⁵ CFU mL⁻¹) containing 10 mg L⁻¹ Mg²⁺ and varying concentrations of Ca²⁺ (0–10 mM) was added to reach a total volume of 100 µL per well. The microtiter plates were sealed with an adhesive membrane and after 16 hours of incubation at 37 °C or 30 °C and 220 RPM the wells were visually inspected for bacterial growth. All reported MIC values result from two or more measurements.

Abbreviations

Alloc	Allyloxycarbonyl
BOP	(Benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMB	2,4-Dimethoxybenzyl
Fmoc-SPPS	Fluorenylmethyloxycarbonyl solid phase peptide synthesis
RP-HPLC	Reverse phase high performance liquid chromatography

Conflicts of interest

There are no conflicts to declare.

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