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Alanine Scan of [L-Dap^2]Ramoplanin A2 Aglycon: Assessment of the Importance of Each Residue

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Abstract: In efforts that define the importance of each residue and that identify key regions of the molecule, an alanine scan of the ramoplanin A2 aglycon, a potent antibiotic that inhibits bacterial cell wall biosynthesis, is detailed. As a consequence of both its increased stability (lactam vs lactone) and its "relative" ease of synthesis, the alanine scan was conducted on [Dap^2]ramoplanin A2 aglycon, which possesses antimicrobial activity equal to or slightly more potent than that of ramoplanin A2 or its aglycon. Thus, 14 key analogues of the ramoplanin A2 aglycon, representing a scan of residues 3−13, 15, and 17, were prepared enlisting a convergent solution-phase total synthesis that consolidated the effort to a manageable level. The antimicrobial activity of the resulting library of analogues provides insight into the importance and potential role of each residue of this complex glycopeptide antibiotic.

Introduction

Because of the overuse of broad spectrum antibiotics in medicine and agriculture, the increasing frequency of bacterial resistance to existing drugs presents a serious threat to public health.[1] Such growing concerns have increased with the recent reports of the emergence of multi-drug-resistant organisms including methicillin-resistant *Staphylococcus aureus* (MRSA) or methicillin-resistant enterococci that exhibit a reduced vancomycin susceptibility.[2] As a result, it has become increasingly important to identify new antibiotics that can replace vancomycin as the antibiotic of last resort for such resistant bacterial infections.

Ramoplanin is a lipoglycodepsipeptide first isolated from the fermentation broths of *Actinoplanes* sp. ATCC 33076 in 1984 (Figure 1).[3] The ramoplanin complex, which consists of three closely related compounds **1**−**3**,[4] was found to be 2−10 times more active than vancomycin against Gram-positive bacteria (500 strains) and maintains full activity against vancomycin-resistant enterococci (VRE) (minimum inhibitory concentrations, MIC = 0.5 μg/mL) and all known strains of MRSA.[5] Although the mechanism of action of ramoplanin is not yet fully defined, it is a promising drug candidate for vancomycin-resistant bacterial infections since it is known to function in a unique manner.[6] Both antibiotics inhibit bacterial cell wall biosynthesis, but vancomycin prevents cell wall cross-linking by primarily inhibiting the late stage transpeptidase-catalyzed step by binding to its substrate D-Ala-D-Ala at the terminus of peptidoglycan precursors,[7] whereas ramoplanin inhibits the earlier stage

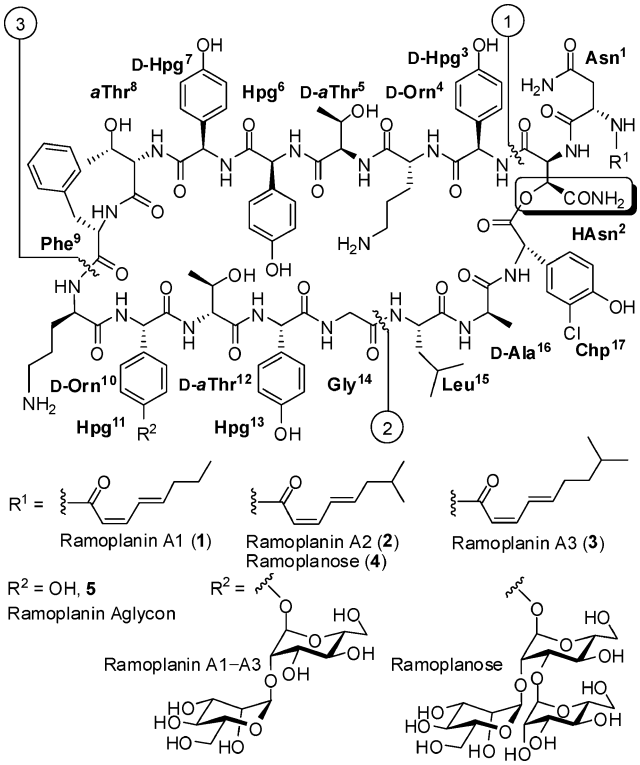

Figure 1. Structure of the ramoplanins.

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intracellular glycosyltransferase (MurG) and the more accessible extracellular transglycosylase (PBP1b)-catalyzed steps by binding their substrates Lipids I and II, thereby precluding maturation of the bacterial cell wall.[6,8] Consequently, no cross-resistance of ramoplanin with existing antibiotics, including vancomycin, has yet been observed. Currently, ramoplanin is in phase III clinical trials for topical, nasal, and GI infections.[5,9] However, the therapeutic applications of ramoplanin are limited because of its poor pharmacokinetics: it is not orally absorbed and it is not stable in plasma because of rapid hydrolysis of the labile lactone.

Ramoplanin A1−A3 consists of a 49-membered ring composed of 17 amino acids including 12 unnatural amino acids and seven possessing the D-configuration. The initial structure of ramoplanin was disclosed in 1989, and the three compounds that make up the ramoplanin complex differ only in the lipid side chains attached to the Asn1 N-terminus.[4] Originally, the two double bonds in the three different acyl groups were assigned the cis−cis stereochemistry,[4a] and this has since been corrected to be cis−trans.[10] The C-terminal 3-chloro-4-hydroxyphenylglycine (Chp^{17}) forms a lactone bond with the hydroxy group of β-hydroxyasparagine (HAsn^2). In 1991, the structure of ramoplanose (**4**) was disclosed by Williams and co-workers, and its composition was identical to ramoplanin A2 except for the branched mannose trisaccharide (vs mannose disaccharide) at Hpg^{11} and the stereochemistry of the acyl side chain (*cis,-trans*- vs *cis,cis*-7-methyloctadi-2,4-enoic acid).[11] Kurz and Guba subsequently corrected the olefin stereochemistry of ramoplanin A2 as cis−trans by 2D NMR in 1996.[10] They also established the Hpg^6 and Hpg^7 absolute stereochemistry and found that the solution conformation consists of two antiparallel β-strands (HAsn^2-D-Hpg^7 and D-Orn^{10}-Gly^{14}) stabilized by six transannular H-bonds and a cluster of hydrophobic aromatic side chains (D-Hpg^3, Phe^9, and Chp^{17}) providing a U-shape topology to the β-sheet with a reverse β-turn (*a*Thr^8-Phe^9) at one end and a more flexible connecting loop (Leu^{15}-Chp^{17}) at the other end in the solution structure.

Enduracidin A and B are additional members of the ramoplanin family that have been employed as feed additives and are known to inhibit Gram-positive bacterial cell wall biosynthesis[12] by the same mechanism as the ramoplanins[12f] (Figure 2). The enduracidins and ramoplanins share a high degree of structural similarity, including two antiparallel β-strands and a conserved D-Hpg^3-*a*Thr^8 region thought to be important for Lipid I and II recognition and binding. Moreover, many of the remaining residues in the enduracidins and ramoplanins are identical (Hpg^{11}, Gly^{14}, D-Ala^{16}) or represent conservative structural departures (D-Ser^{12} vs D-*a*Thr^{12}, Dpg^{13} vs Hpg^{13}, Hpg^{17} vs Chp^{17}, and Thr^2 vs HAsn^2). Even some of the significant departures (Cit^9 vs Phe^9 and D-End^{10} vs D-Orn^{10}) represent changes that maintain the stereochemical and potential functional features (D-End^{10} vs D-Orn^{10}) of the ramoplanins. Recently, Marazzi and co-workers disclosed a solution-phase conformation of the enduracidins determined by NMR exhibiting only subtle structural differences between the enduracidins and ramoplanins.[13] The enduracidins do not contain a di- or trisaccharide at Hpg^{11}, and the lipid side chains are longer than those found in the ramoplanins. Enduracidin includes an additional basic residue at End^{15} (vs Leu^{15}) and an acidic residue at Asp^1 (vs Asn^1) that are proximal and engaged in a transannular salt bridge, as well as a flexible side chain at the Cit^9, which is exposed to the solvent (H_2O/DMSO-d_6, 4:1), whereas ramoplanin incorporates a hydrophobic side chain at Phe^9 forming a well-packed hydrophobic core along with other aromatic side chains (D-Hpg^3, Chp^{17}) and the lipid side chain.[10] The net result is that the characteristic ramoplanin hydrophobic core is disrupted within the enduracidins. The significance of this difference is yet to be defined, and it is not reflected in different transglycosylase inhibition kinetics.[12f] Although less well characterized, janiemycin represents an additional member of the ramoplanin family.[14]

In 2002, we reported the first total synthesis of the ramoplanin A2 and ramoplanose aglycon (**5**) confirming the assigned

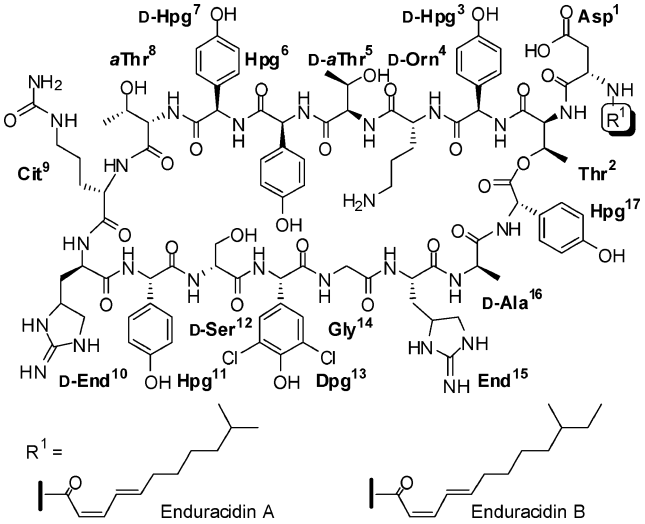

Figure 2. Structure of the enduracidins.

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structure.[15] Three key subunits composed of D-Hpg[3]-Phe[9] (subunit A), Leu[15]-Asn[1] (subunit B), and D-Orn[10]-Gly[14] (subunit C) were synthesized, sequentially coupled, and cyclized in a solution-phase approach to the 49-membered macrocyclic core of ramoplanin. Two macrocyclization sites, Phe[9]-D-Orn[10] and Gly[14]-Leu[15], were examined that maximize the convergency of the synthesis, minimize the use of protecting groups, prevent late stage opportunities for racemization of carboxylate-activated phenylglycine-derived residues, and benefit from a β-sheet preorganization of an acyclic substrate for ring closure.[16] Macrocyclization at the Phe[9]-D-Orn[10] site additionally benefits from closure at the corner of a β-turn incorporating a D-amine,[17] while the alternative closure at the Gly[14]-Leu[15] site represents a nonhindered glycine site incapable of racemization. Additional keys to the success of the approach were the choice of a SES protecting group for Orn[4] and Orn[10] and Fmoc protection for Asn[1] furnishing orthogonal protecting groups to Boc, Cbz, and benzyl ester deprotections yet capable of sequential and selective removal in the presence of the unstable depsipeptide ester bond. Deliberate final stage incorporation of the Asn[1] lipid side chain provided late stage access to all three ramoplanins as well as side chain analogues of the aglycons. As such, the total synthesis of the two minor components of the ramoplanin complex (A1 and A3) was achieved, confirming a reassigned cis−trans stereochemistry for the lipid side chains.[18]

This approach has since been extended to the synthesis of two key analogues of ramoplanin containing an amide linkage in place of the labile ester between HAsn[2] and Chp[17] in which HAsn[2] was replaced with L-2,3-diaminopropionic acid (Dap) or L-2,4-diaminobutyric acid (Dab).[19] The two derivatives are both much more stable and synthetically more accessible than the natural ramoplanin aglycon. In a subsequent mechanistic analysis of these analogues, both amide linkage substitutions as well as removal of the lipid chain did not affect Lipid II binding, indicating that the residue 2 modifications and the acyl side chain do not play an important role in substrate recognition and binding.[20] However, the antimicrobial activities of the Dab[2] analogue and compounds containing truncated side chains were not comparable to those of ramoplanin or the Dap[2] analogue. The ring-expanded 50-membered Dab[2] analogue, but not the 49-membered Dap[2] analogue, was found to aggregate extensively in aqueous buffer where its increased conformational flexibility perhaps permits the β-strands in the molecule to associate in an intermolecular manner (aggregation),[20] whereas the lipid side chain presumably helps ramoplanin localize to the bacterial cell membrane.[20]

In 1998, Brötz et al. reported the first direct evidence that ramoplanin binds to a substrate involved in peptidoglycan biosynthesis.[21] In 2000, Walker and co-workers showed that ramoplanin inhibits the transglycosylation step of peptidoglycan biosynthesis by binding to Lipid II.[6d] Shortly following that, insights into the interaction between ramoplanin A2 and a peptidoglycan precursor (Park's nucleotide) were disclosed by McCafferty et al. using NMR studies suggesting that the octapeptide sequence (D-Hpg[3]-D-Orn[10]),[8a] which is highly conserved among ramoplanins and enduracidins, constitutes the substrate recognition domain. Contemporary with these studies, inhibition kinetic studies[6g] and NMR titration experiments[6f] performed by Walker and co-workers suggest that the inhibitory species binds with a stoichiometry of 2:1 ramoplanin/Lipid II, and in a second NMR study, Lo et al. observed that ramoplanin A2 exists as a mixture of monomer and dimer in methanol.[6e] This latter series of studies led Walker to propose that Lipid II may bind in a cleft formed by the dimerization of two ramoplanin molecules and defined the dimer interface region as D-Orn[10]-Hpg[13].

Herein, we report an alanine scan of ramoplanin A2 aglycon and the resulting antimicrobial properties of the derivatives in efforts to define the importance of the individual residues and to identify key regions of the molecule.[22] As a consequence of its "relative" ease of synthesis and its resulting stability (lactam vs lactone), the alanine scan was conducted on the [Dap[2]]-ramoplanin A2 aglycon (**6**) which possesses antimicrobial activity equal to or slightly more potent than the authentic ramoplanin A2 aglycon itself (Figure 3).[19] Notably, the efforts constitute a scan of residues 3−13, 15, and 17 conducted implementing a convergent solution-phase total synthesis of the 14 key analogues.

Chemistry

Following the completion of the total synthesis of the natural product aglycons[15,18] and the identification of an active and stable amide (vs depsipeptide ester) template, [Dap[2]]ramoplanin A2 aglycon,[19] we initiated and herein report an alanine scan of the ramoplanin A2 aglycon enlisting [Dap[2]]ramoplanin A2 as the template. These efforts include a full alanine scan of the subunit A (D-Hpg[3]-Phe[9]) and C (D-Orn[10]-Gly[14]), which contain the putative substrate recognition domain (D-Hpg[3]-D-Orn[10]) proposed by McCafferty et al.,[8a] the dimer interface region (D-Orn[10]-Hpg[13]) defined by Walker et al.,[6e] and D-Orn[4] and D-Orn[10], which have been established to be important to the biological properties of ramoplanin.[6f,8a,15b,19] Selective semisynthetic modifications of D-Orn[4] or D-Orn[10] are notorious for their low yields or poor selectivity. Consequently, the identification of the modified site is difficult and the results derived from their examination have not been entirely conclusive.[4c−e] Therefore, the site-specific incorporation of D-Ala[4] and D-Ala[10] by the synthetic methods detailed herein unambiguously addresses these issues. In addition and to more clearly elucidate the role of the chlorine substitution at Chp[17], a Hpg[17] as well as an Ala[17] analogue were prepared.

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(16) (a) Nonpolar solvents (CH_2Cl_2, $CHCl_3$, EtOAc) should be used to take advantage of the β-sheet preorganization. However, the linear substrates typically proved insoluble in such solvents. Consequently, variable amounts of DMF are added until the substrates were dissolved. (b) Maplestone, R. A.; Cox, J. P. L.; Williams, D. H. *FEBS Lett.* **1993**, *326*, 95.
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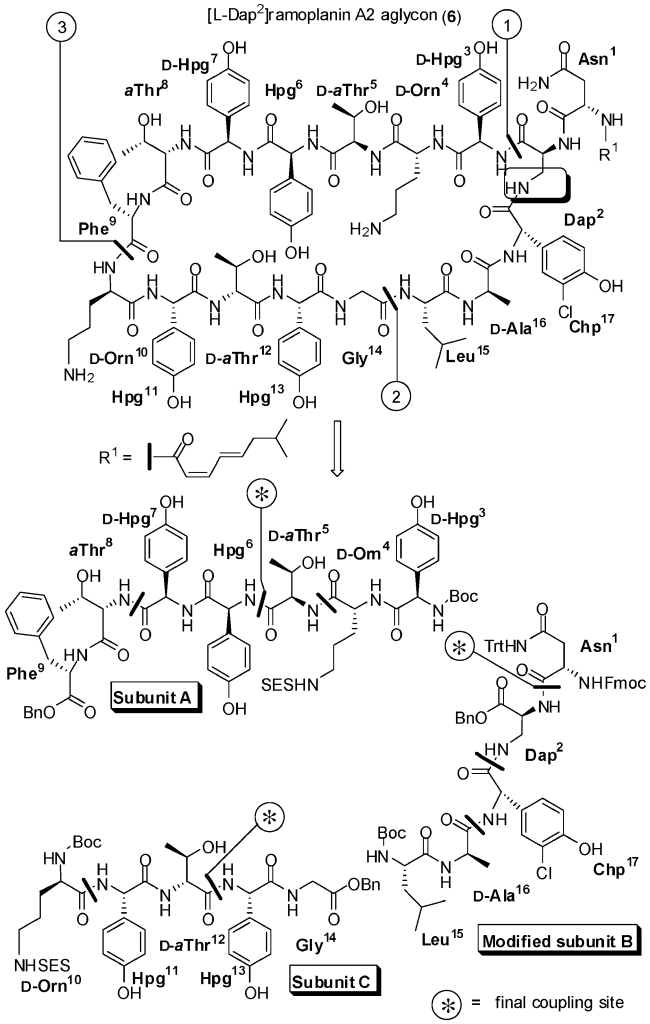


Figure 3. Structure of [Dap^2]ramoplanin A2 aglycon (**6**) and the three key subunits A−C.

The synthesis and assembly of three key subunits were facilitated by the convergent nature of the solution-phase approach (Figure 3). For example, each change within the A subunit utilized the common [Dap^2]B and C subunits for completion of the synthesis, and this expedited the parallel synthesis of the library. Similarly, each of the subunits A, B, and C was also assembled in a convergent fashion (e.g., Ala^3 through Ala^5 were prepared by coupling the modified tripeptide to a common Hpg^6-Phe^9 tetrapeptide), and this consolidated the work to a manageable level, reducing the complexity of the syntheses and number of new intermediates. Thus, each residue analogue required at most five coupling reactions to assemble the linear peptide, a macrocyclization, the side chain introduction (two steps), and a final global deprotection (nine operations total) enlisting unmodified subunits used in the synthesis of [Dap^2]-ramoplanin A2 aglycon.

Synthesis of the A Subunit Ala Derivatives: Heptapeptide D-Hpg^3-Phe^9. The seven synthetic targets of the alanine-substituted subunit A (**8**−**14**) and details of the preparation of the [Ala^5] analogue (**10**) are summarized in Scheme 1.[23] Following the approach defined for synthesis of authentic

Scheme 1.[23] Alanine-Substituted Subunit A Analogues and Synthesis of [Ala^5] Subunit A (**10**)

Alanine-substitued subunit A analogues:

7: Boc-D-Hpg^3-D-Orn^4(SES)-D-*a*Thr^5-Hpg^6-D-Hpg^7-*a*Thr^8-Phe^9-OBn

8: Boc-D-**Ala^3**-D-Orn^4(SES)-D-*a*Thr^5-Hpg^6-D-Hpg^7-*a*Thr^8-Phe^9-OBn (407 mg)

9: Boc-D-Hpg^3-D-**Ala^4**-D-*a*Thr^5-Hpg^6-D-Hpg^7-*a*Thr^8-Phe^9-OBn (197 mg)

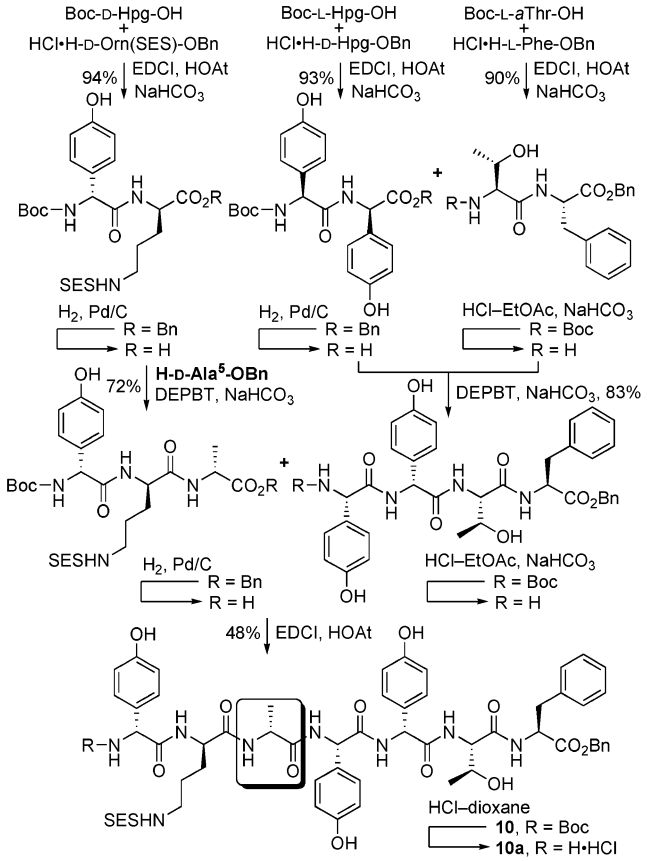
10: Boc-D-Hpg^3-D-Orn^4(SES)-D-**Ala^5**-Hpg^6-D-Hpg^7-*a*Thr^8-Phe^9-OBn (70 mg)

11: Boc-D-Hpg^3-D-Orn^4(SES)-D-*a*Thr^5-**Ala^6**-D-Hpg^7-*a*Thr^8-Phe^9-OBn (463 mg)

12: Boc-D-Hpg^3-D-Orn^4(SES)-D-*a*Thr^5-Hpg^6-D-**Ala^7**-*a*Thr^8-Phe^9-OBn (373 mg)

13: Boc-D-Hpg^3-D-Orn^4(SES)-D-*a*Thr^5-Hpg^6-D-Hpg^7-**Ala^8**-Phe^9-OBn (362 mg)

14: Boc-D-Hpg^3-D-Orn^4(SES)-D-*a*Thr^5-Hpg^6-D-Hpg^7-*a*Thr^8-**Ala^9**-OBn (175 mg)



10: Boc-D-Hpg^3-D-Orn^4(SES)-D-**Ala^5**-Hpg^6-D-Hpg^7-*a*Thr^8-Phe^9-OBn

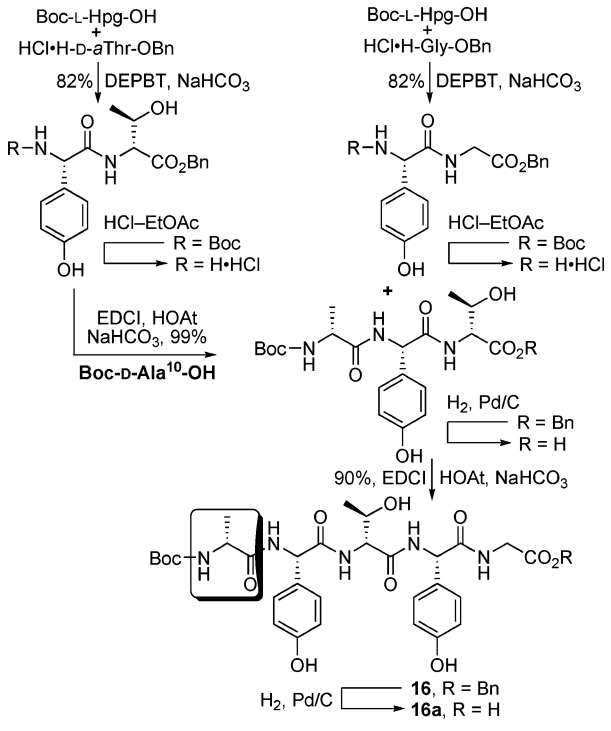
subunit A (**7**), we assembled heptapeptides **8**−**14**, which contain all but Orn^{10} of the putative Hpg^3-Orn^{10} recognition domain, from the D-Hpg^3-D-*a*Thr^5 tripeptide and Hpg^6-Phe^9 tetrapeptide.[15] In turn, the tripeptide for **10** was obtained by the coupling of Boc-D-Hpg^3-D-Orn^4(SES)-OH and H-D-Ala-OBn (72%). Benzyl ester hydrogenolysis followed by coupling with H-Hpg^6-D-Hpg^7-*a*Thr^8-Phe^9-OBn gave the heptapeptide **10** (48%). In similar fashion, the remainder of the alanine-substituted A subunits was prepared (Supporting Information).

Synthesis of the C Subunit Ala Derivatives: Pentapeptide D-Orn^{10}-Gly^{14}. The preparation of the pentapeptide **15**,[15] which contains the entire dimer interface domain,[6e] and the synthetic alanine-substituted C subunits (**16**−**19**) is summarized in Scheme 2 along with details of the key [Ala^{10}] derivative. Coupling of HCl•H-L-Hpg^{11}-D-*a*Thr^{12}-OBn with Boc-D-Ala-OH provided the tripeptide in 99% yield. Benzyl ester deprotection and coupling with H-L-Hpg^{13}-Gly^{14}-OBn gave pentapeptide **16** (90% yield). The synthesis of remaining alanine-substituted C subunits proceeded similarly (Supporting Information). Since residue 14 is glycine, its replacement with an alanine was not conducted.

(23) EDCI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOAt = 1-hydroxy-7-azabenzotriazole, DEPBT = 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one, SES = 2-trimethylsilylethanesulfonyl, BCB = *B*-bromocatecholborane.

Scheme 2. [23] Alanine-Substituted Subunit C Analogues and Synthesis of [Ala[10]] Subunit C (**16**)

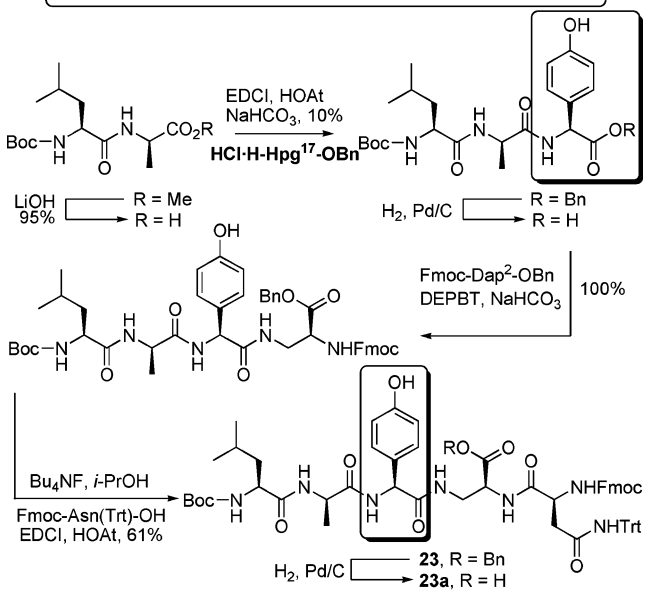
Alanine-substitued subunit C analogues:
15: Boc-D-Orn[10](SES)-Hpg[11]-D-*a*Thr[12]-Hpg[13]-Gly[14]-OBn
16: Boc-**D-Ala[10]**-Hpg[11]-D-*a*Thr[12]-Hpg[13]-Gly[14]-OBn (152 mg)
17: Boc-D-Orn[10](SES)-**Ala[11]**-D-*a*Thr[12]-Hpg[13]-Gly[14]-OBn (87 mg)
18: Boc-D-Orn[10](SES)-Hpg[11]-**D-Ala[12]**-Hpg[13]-Gly[14]-OBn (100 mg)
19: Boc-D-Orn[10](SES)-Hpg[11]-D-*a*Thr[12]-**Ala[13]**-Gly[14]-OBn (114 mg)



[Ala[10]] subunit C, **16**: Boc-**D-Ala[10]**-Hpg[11]-D-*a*Thr[12]-Hpg[13]-Gly[14]-OBn

Scheme 3. [23] Modified Subunit B and Its Analogues

Modified subunit B analogues:
20: Fmoc-Asn[1](Trt)-(Boc-Leu[15]-D-Ala[16]-Chp[17])Dap[2]-OBn
21: Fmoc-Asn[1](Trt)-(Boc-**Ala[15]**-D-Ala[16]-Chp[17])Dap[2]-OBn (85 mg)
22: Fmoc-Asn[1](Trt)-(Boc-Leu[15]-D-Ala[16]-**Ala[17]**)Dap[2]-OBn (130 mg)
23: Fmoc-Asn[1](Trt)-(Boc-Leu[15]-D-Ala[16]-**Hpg[17]**)Dap[2]-OBn (79 mg)

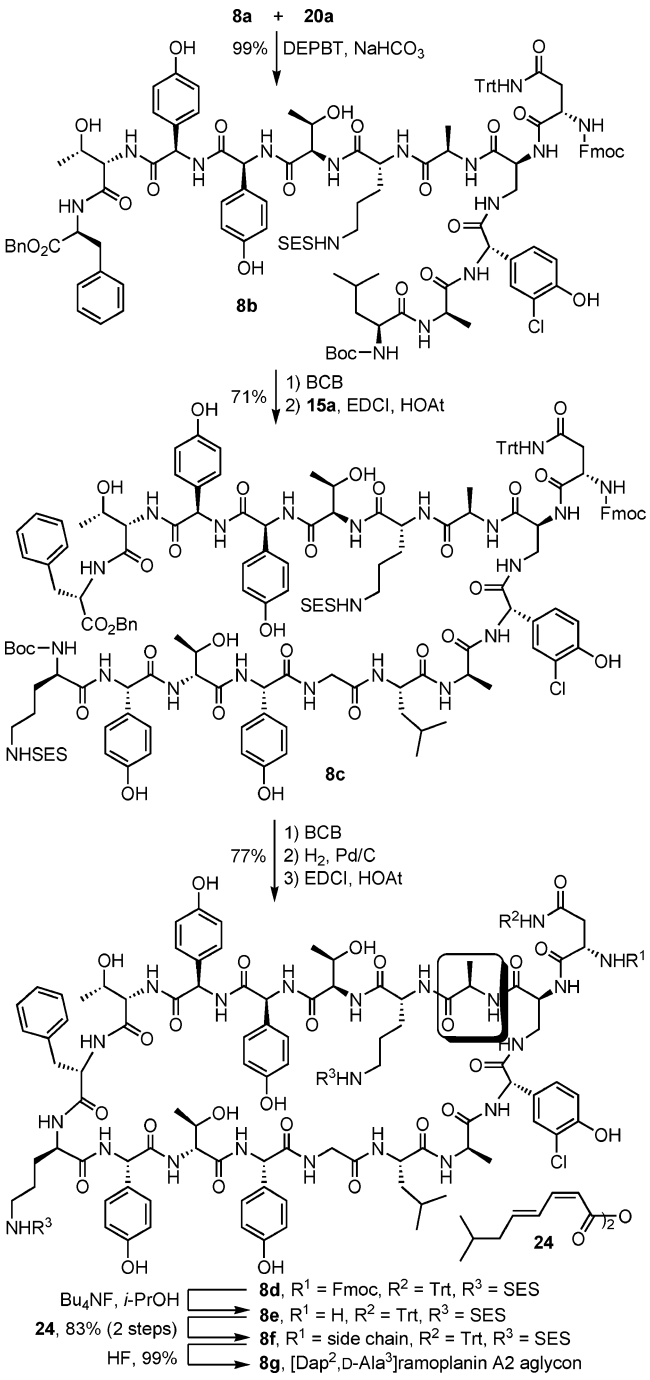


23: Fmoc-Asn[1](Trt)-(Boc-Leu[15]-D-Ala[16]-**Hpg[17]**)-Dap[2]-OBn

Synthesis of the B Subunit Derivatives: Pentapeptide [L-Dap[2]]B. Within the B subunit, residue 2 was fixed as the simplified and stable Dap[2] where the side chain linkage is attached to the coupled and similarly unmodified Asn[1].[19] Additionally, residue 16 already constitutes a D-Ala, and it was not further modified. Rather, our interest focused on Leu[15] as well as Chp[17], which was replaced with Hpg[17] to directly assess the role of the unusual chloride substituent as well as Ala[17] (Scheme 3). The modified pentapeptide subunit **23**, incorporating the Hpg[17] (or Ala[17] for **22**) residue and the Dap[2] amide, was obtained from an intermediate tripeptide, which in turn was prepared by coupling Boc-Leu[15]-D-Ala[16]-OH with HCl·H-Hpg[17]-OBn (EDCI, HOAt, $NaHCO_3$, 20% DMF−CH_2Cl_2, 0 °C, 20 h) followed by deprotection of the benzyl ester (H_2, 10% Pd/C, MeOH, 25 °C, 20 min, 100%). Fmoc-Dap[2](NH_2·HCl)-OBn obtained from Fmoc-Dap[2](Boc)-OH (HCl−EtOAc) was coupled with this tripeptide using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT)[23] ($NaHCO_3$, DMF, 0 °C, 1 h, then 25 °C, 52 h, 100%) to give a single diastereomer of the corresponding tetrapeptide with no detectable racemization of the sensitive Hpg[17] residue. Fmoc removal (Bu_4NF, *i*-PrOH, DMF, 25 °C, 1 h, sonication), coupling the free amine with Fmoc-Asn(Trt)-OH (EDCI, HOAt, DMF, 0 °C, 48 h, 61% for two steps), and benzyl ester hydrogenolysis (H_2, 10% Pd/C, EtOH, 25 °C, 45 min, 100%) provided **23a**.[19] The original pentapeptide subunit B and the simplified [Dap[2]]B subunit **20** were found to be sensitive to the benzyl ester deprotection step, potentially generating mixtures with a contaminate dechlorinated side product if not closely monitored. In an effort to take advantage of this observation and to reduce the number of synthetic steps for the preparation of **23a**, direct dechlorination of the [Dap[2]]B subunit **20** or [Dap[2]]ramoplanin A2 aglycon itself under various conditions was examined. However, the rate of dechlorination was too slow, and the approach proved to be impractical.

Synthesis of [Dap[2],Ala[m]]ramoplanin A2 Aglycon. The convergent strategy developed for the total synthesis of the ramoplanin and [Dap[2]]ramoplanin A2 aglycons was utilized for the preparation of the alanine-substituted [Dap[2]]ramoplanin A2 aglycon analogues.[19] For example, assemblage of the alanine-substituted subunit A analogues (**8**−**14**) required coupling with the common unmodified B and C subunits and maximized the convergent nature of the synthesis. Representative of the final stages of the analogue preparations, the synthesis of [Dap[2],D-Ala[3]]ramoplanin A2 aglycon begins with the treatment of **8** with 4 N HCl−dioxane followed by coupling of resulting amine with the B subunit carboxylic acid **20a** to provide **8b** without competitive β-elimination (Scheme 4). An excess of **20a** was employed to consume all **8a**, which proved difficult to remove from the product enlisting either an acid wash or chromatography. Boc removal of **8b** under mild conditions (*B*-bromocatecholborane ≡ BCB, CH_3CN, 0 °C, 3 h) that do not affect the Asn[1] trityl group gave the corresponding amine.[19] The resulting amine was then coupled with the unmodified subunit C (**15a**) to yield **8c**. Successive Boc removal, benzyl ester hydrogenolysis, and macrocyclization provided the cyclic peptide core **8d** in superb yield attributable to the β-sheet preorganization of the cyclization substrate as well as closure at a D-amine terminus.[17] Fmoc removal under specially developed conditions (8 equiv of Bu_4NF, 10 equiv of *i*-PrOH, DMF, 25 °C, 1 h, sonication), acyl side chain introduction, and final global

Scheme 4. [23] Synthesis of [Dap^2,Ala^3]ramoplanin A2 Aglycon (**8g**)



deprotection (HF, anisole, 0 °C, 90 min) yielded [Dap^2,D-Ala^3]-ramoplanin A2 aglycon (**8g**).[15] In a similar manner, each of the ramoplanin A2 aglycon analogues was synthesized, and details of their synthesis and their intermediates are provided in the Supporting Information. The final products were purified by reverse-phase HPLC to provide homogeneous materials that were used for characterization and the biological evaluation of the final products. However, it is notable that the final products were obtained in purities ranging from 46−91% (av = 73%, see Supporting Information) even before this final purification. The ^{1}H NMR spectroscopic properties of each of the analogues did not reveal a loss of conformational rigidity that might contribute to a change or loss of antimicrobial activity, and only Ala^8, Ala^{10}, Ala^{11}, and most notably Ala^{17} exhibited slightly less crisp spectra that might be attributable to a conformational heterogeneity (line broadening).

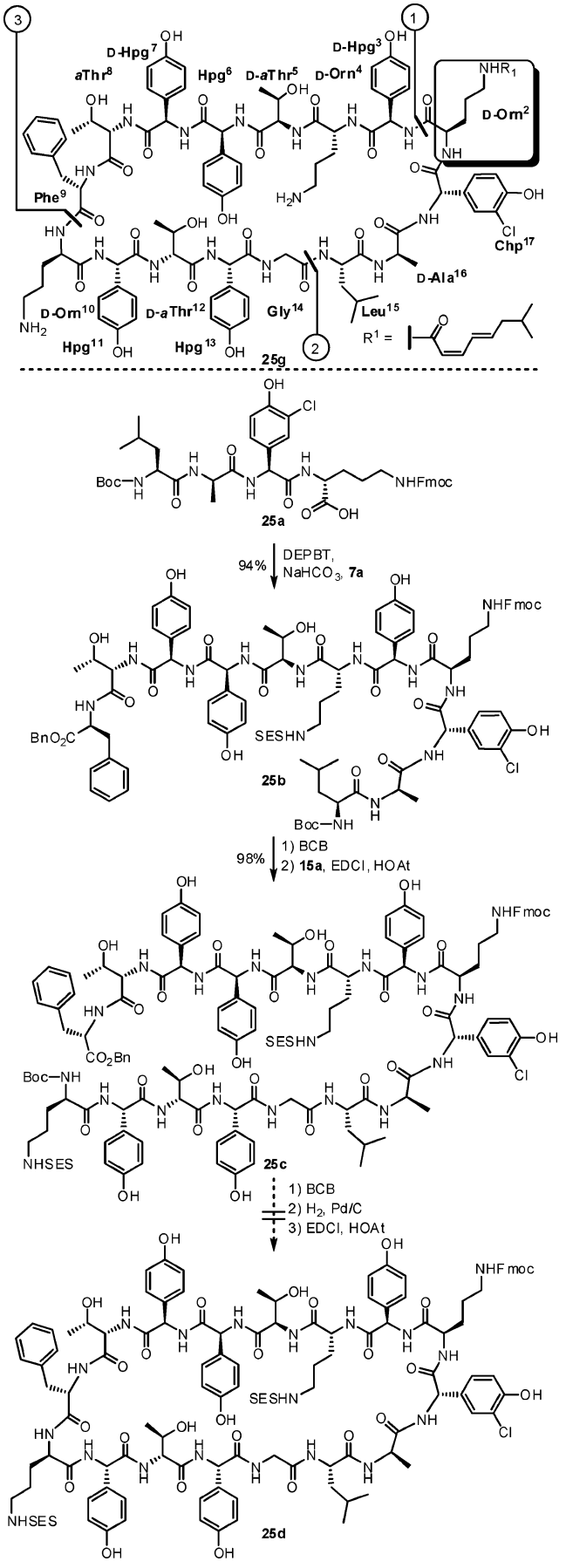
A 48-Membered Ramoplanin A2 Aglycon Analogue. One of the more successful structural modifications of the ramoplanin aglycon has been the replacement of L-HAsn^2 with L-Dap^2, simplifying the synthesis and removing the hydrolysis labile depsipeptide ester. Surprisingly, the analogous replacement with L-Dab^2, which results in a ring expansion to a 50-membered ring, resulted in a >100-fold loss in antimicrobial activity and provided an analogue with a propensity for aggregation in aqueous buffer. As a consequence, we now examined the ring-contracted 48-membered analogue **25g** incorporating a D-Orn^2 in place of L-HAsn^2 where the side chain also incorporates an amine for lipid side chain attachment, removing the need for Asn^1 (Scheme 5). The simplified B subunit **25a** was prepared and sequentially coupled with the authentic A (**7a**) and C (**15a**) subunits (Scheme 5). To our disappointment, all efforts to promote the macrocyclization of the linear peptide **25c** following C- and N-terminus deprotection failed to provide the macrocyclic peptide **25d**. Presumably, the decreased flexibility of **25c** resulting from deleting the one methylene is sufficient to preclude macrocyclization. These observations along with the behavior of the 50-membered [Dap^2]ramoplanin A2 aglycon underscore the special role residue 2 plays in conveying antimicrobial activity to the compounds and highlight how remarkable the stable L-Dap^2 for L-HAsn^2 substitution is.

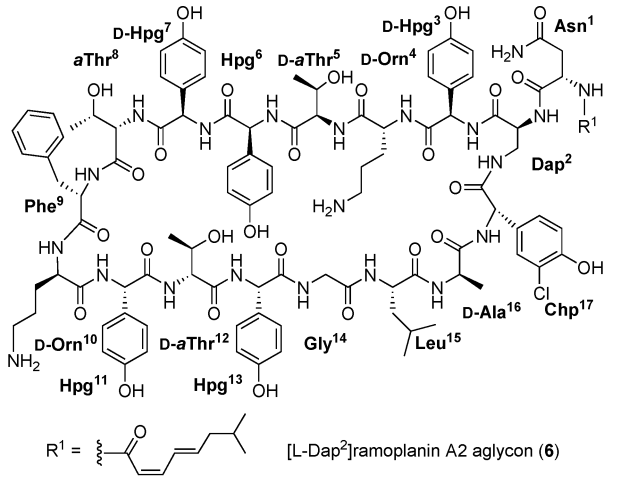
Antimicrobial Activity

The results of the antimicrobial assay of the analogues against *S. aureus* alongside the ramoplanin complex (MIC = 0.19 μg/mL) and [Dap^2]ramoplanin A2 aglycon (**6**, MIC = 0.07 μg/mL) are summarized in Figure 4. Analogous to observations first reported for [Dap^2]ramoplanin A2 aglycon (**6**) upon its initial disclosure,[19] it proved to be 2−3-fold more potent than the natural ramoplanin complex and slightly more active than the ramoplanin A2 aglycon.[19,20] Perhaps the most important of the comparisons to highlight first are the activities of the Ala^4 and Ala^{10} analogues probing the importance of Orn^4 and Orn.^{10} Semisynthetic modifications of these sites have indicated that both contribute significantly to ramoplanin's activity, but their relative importance and potential role remained unclear.[6f,8a,15b,19] Consistent with Walker's observations with such semisynthetic derivatives,[6f] the Ala^{10} analogue **16g** (MIC = 38 μg/mL) proved to be 540-fold less active than **6**, whereas the Ala^4 analogue **9g** (MIC = 3.1 μg/mL) was 45-fold less potent. This clearly indicates that Orn^{10} is essential to the activity of ramoplanin and plays a much more fundamental role than Orn^4, although both Ala^{10} and Ala^4 are among the least effective of the Ala analogues examined.

Within the remainder of the putative Lipid II binding domain proposed by McCafferty et al. (Hpg^4-Orn^{10}),[8a] the additional largest impacts on activity were observed with D-Ala^3 (vs D-Hpg^3, MIC = 5.2 μg/mL), D-Ala^7 (vs D-Hpg^7, MIC = 3.7 μg/mL), and Ala^8 (vs *a*Thr^8, MIC = 2.5 μg/mL), representing 75-fold, 50-fold, and 35-fold losses in antimicrobial activity, respectively. Thus, both D-Hpg^3 and D-Hpg^7 proved more significant than D-Orn^4, and *a*Thr^8 approaches this level of importance. Notably, the importance of *a*Thr^8 is unique among the three *a*Thr sites in ramoplanin, and each of the remaining D-Ala/D-*a*Thr substitutions (residues 5 and 12) resulted in ≤10-

Scheme 5.[23] Attempted Synthesis of the 48-Membered [D-Orn^2]ramoplanin A2 Aglycon (**25g**)





Compound	Authentic residue	MIC (μg/mL)[a,b]	Fold difference
ramoplanin complex **1**–**3**		0.19	2.7
5, ramoplanin A2 aglycon		0.11	1.6
6, [Dap^2]ramoplanin A2 aglycon		0.07	1
8g, [Dap^2,D-Ala^3]ramoplanin A2 aglycon	D-Hpg^3	5.2	74
9g, [Dap^2,D-Ala^4]ramoplanin A2 aglycon	D-Orn^4	3.1	44
10g, [Dap^2,D-Ala^5]ramoplanin A2 aglycon	D-*a*Thr^5	0.16	2.3
11g, [Dap^2,Ala^6]ramoplanin A2 aglycon	Hpg^6	0.9	13
12g, [Dap^2,D-Ala^7]ramoplanin A2 aglycon	D-Hpg^7	3.7	53
13g, [Dap^2,Ala^8]ramoplanin A2 aglycon	*a*Thr^8	2.5	36
14g, [Dap^2,Ala^9]ramoplanin A2 aglycon	Phe^9	0.6	8.6
16g, [Dap^2,D-Ala^{10}]ramoplanin A2 aglycon	D-Orn^{10}	38	540
17g, [Dap^2,Ala^{11}]ramoplanin A2 aglycon	Hpg^{11}	2.5	36
18g, [Dap^2,D-Ala^{12}]ramoplanin A2 aglycon	D-*a*Thr^{12}	0.7	10
19g, [Dap^2,Ala^{13}]ramoplanin A2 aglycon	Hpg^{13}	1.4	20
21g, [Dap^2,Ala^{15}]ramoplanin A2 aglycon	Leu^{15}	3.0	40
22g, [Dap^2,Ala^{17}]ramoplanin A2 aglycon	Chp^{17}	0.3	4
23g, [Dap^2,Hpg^{17}]ramoplanin A2 aglycon	Chp^{17}	0.09	1.3

[a] MIC values (μg/mL) were obtained using a standard microdilution assay. The MIC is defined as the lowest antibiotic concentration that resulted in no visible growth after incubation at 37 °C for 24 h.
[b] Bacterial strain *S. aureus* ATCC 25923.

Figure 4. Antimicrobial MIC values.

fold reductions in activity. Surprisingly, Ala^6 (vs Hpg^6, MIC = 0.9 μg/mL) as well as D-Ala^5 (vs D-*a*Thr^5, MIC = 0.16 μg/mL) central to this region had the least impact on ramoplanin's properties, resulting in only 10-fold and 2-fold reductions in antimicrobial activity. In fact, Hpg^6 proved to be the least important of the phenylglycines imbedded in the ramoplanin structure and each of the Ala/Hpg substitutions elsewhere resulted in >20-fold reductions in activity, and the Ala^5 analogue [Dap^2, D-Ala^5]ramoplanin A2 aglycon still proved to be slightly more active than the natural ramoplanin complex despite its 2-fold reduction in activity versus **6**.

The Ala^9 analogue (MIC = 0.6 μg/mL) with modification of Phe^9 resulted in a modest 8−9-fold reduction in antimicrobial activity despite its role in capping the hydrophobic core and important location at the corner of a β-turn in ramoplanins structure adjacent to D-Orn^{10}.[10] Given that the Orn^{10} importance almost certainly arises as a consequence of a key stabilizing binding interaction with the diphosphate central to the structure of Lipids I and II,[6,8] it is surprising that the adjacent residue Phe^9, unlike Hpg^{11}, would have such a modest effect. Nonetheless, it is consistent with the departure in structure observed with the enduracidins which possess a solvent accessible Cit^9 residue and suggests this may represent a useful modification site for further functionalization or conjugation efforts.

The Ala analogues spanning the dimerization domain defined by Walker et al. and observed in MeOH (Hpg[11]-Gly[14]),[6e] which resides adjacent to the key Orn[10] and transannular to the putative Lipid II recognition domain defined by McCafferty et al.,[8a] proved especially interesting. Each of the Ala substitutions resulted in a ≥10-fold reduction in antimicrobial activity, with that of Ala[11] (vs Hpg[11], MIC = 2.5 μg/mL) being the greatest (35-fold), followed by Ala[13] (vs Hpg[13], MIC = 1.4 μg/mL, 20-fold), and D-Ala[12] (vs D-*a*Thr[12], MIC = 0.7 μg/mL, 10-fold). In addition to illustrating a significant role that residues in this potential dimerization domain may play in ramoplanin's interaction with its biological target(s), it also defines D-*a*Thr[12] as the residue at which to probe such roles. In this regard, it is interesting to note that the enduracidins incorporate a D-Ser[12] in place of D-*a*Thr[12] and that the ramoplanin dimer structure established by NMR places its residue 12 hydroxyls within 3.87 Å of one another on the same face of the dimerization interface. Clearly, this represents a unique residue whose functionalization, deliberate dimerization, or conjugation may be exploited to probe mechanistic questions without perturbing the intrinsic structure of the ramoplanin monomer or dimer.

Finally, the examination of several additional analogues within the less well-defined Leu[15]-Asn[1] segment, which adopts a more flexible loop at one end of the ramoplanin structure and contains depsipeptide ester as well as the lipid side chain, has shed additional insights into its importance. Previously, we have shown that the lipid side chain is essential for antimicrobial activity (200−800-fold reductions),[19,20] but it does not impact Lipid II binding presumably serving to deliver or anchor ramoplanin to the bacterial cell wall.[20] Additionally, L-Dap[2] (49-membered ring), but not L-Dab[2] (50-membered ring), effectively replaces HAsn[2] (49-membered ring) providing a stable amide replacement for the labile depsipeptide ester.[19,20] Herein, the extensions of this work to an attempted replacement with D-Orn[2] resulting in a ring-contracted 48-membered macrocyclic amide revealed that the macrocyclization ring closure failed to provide the core structure. The ease of 49-membered ring macrocyclization, the failure of the corresponding (more rigid) 48-membered ring closure, and the aggregation of the 50-membered ring system[20] suggest a significant structural role for this corner of ramoplanin structure and highlight how special the properties of [Dap[2]]ramoplanin A2 aglycon are. Additionally, we probed the importance of the adjacent Chp[17] residue in this region. Its replacement with Hpg[17] representing the removal of the aromatic chlorine substituent had virtually no impact on the antimicrobial properties (MIC = 0.09 μg/mL).[24] Consistent with this lack of functional role for the chlorine substituent, the enduracidins incorporate Hpg[17] in their structure at this site. More significantly, the Ala[17] replacement for Chp[17] with **22g** (MIC = 0.3 μg/mL) had a similar lack of effect in the antimicrobial activity resulting in only a modest 4-fold reduction in antimicrobial potency. Despite the apparent significance of the structural change, this Ala modification at residue 17 was among those that had the least effect of any of the Ala substitutions although it was among the residues including Phe[9] that perturb the rigid solution conformation characteristic of ramoplanin. We have interpreted this behavior to suggest that it is only the stereochemistry at this center that might be important for its antimicrobial activity and that residues 16−1, constituting a more flexible loop including a D-Ala[16] at one end of the antiparallel β-sheet, do not directly interact with the biological target Lipid I or II and do not play a direct functional role. However, Phe[9] and Chp[17] do stabilize the intrinsic solution structure of ramoplanin by forming a hydrophobic core buried within the U-shaped conformation of the natural product and may indirectly contribute to its properties. Significantly, the results with Ala[17] indicate that this residue, and perhaps the adjoining residue Ala[16], represent prime sites for modification or conjugation in efforts to probe ramoplanin's mechanism of action. Finally, it is similarly interesting and surprising that the conservative Ala[15] replacement for Leu[15] at the end of this region of the molecule resulted in a 40-fold loss in antimicrobial activity.

(24) In contrast, removal of the aromatic chlorines from vancomycin diminished dimerization and altered its antimicrobial activity. See: (a) Harris, C. M.; Kannan, R.; Kopecka, H.; Harris, T. M. *J. Am. Chem. Soc.* **1985**, *107*, 6652. (b) Gerhard, U.; Mackay, J. P.; Maplestone, R. A.; Williams, D. H. *J. Am. Chem. Soc.* **1993**, *115*, 232.

Discussion and Conclusions

A full alanine scan of [Dap[2]]ramoplanin aglycon (**6**), a hydrolytically stable and slightly more potent analogue of the ramoplanin aglycon (**5**), was conducted, providing insights into the importance and potential role of each residue. By far the most important residue in ramoplanin is D-Orn.[10] Its replacement with Ala[10] resulted in a >500-fold reduction in antimicrobial activity consistent with its proposed integral role in Lipid II diphosphate binding. In contrast and more surprising to us, the conserved Orn[4] was found to be substantially less important than Orn[10], suggesting its role in binding Lipid I or II is not as critical. Both these residues lie in the putative recognition and binding domain proposed by McCafferty et al.[8a] Two of the remaining residues in this region (residues 3−10) exhibit a larger impact than Orn[4] (D-Hpg[3] and D-Hpg[7]), a third is comparable (*a*Thr[8]), and three appear much less important. Significantly, two of these (D-*a*Thr[5], Hpg[6]) lie central to this putative Lipid II recognition domain, and it is difficult to rationalize such a marginal impact central to a contiguous binding interface. An alternative explanation that might account for this behavior is that Orn[4] may not be involved in Lipid I or II substrate binding, but rather that it may interact with the membrane phosphates and collaborate with the lipid side chain to deliver and anchor ramoplanin to the bacterial cell wall. In the monomer solution structure of ramoplanin, Leu[15], Ala[16], the lipid side chain, and perhaps Hpg[13]/Gly[14] form a hydrophobic face on one side of the molecule adjacent to Orn[4] that could serve as the membrane binding domain (Figure 5). As such, the enduracidin's incorporation of End[15] for ramoplanin's Leu[15] would represent incorporation of an additional proximal membrane phosphate binding residue spatially bracketing this hydrophobic face of ramoplanin. The cluster of the remaining most prominent residues including Orn[10] (red, >100-fold reduction) and D-Hpg[3], D-Hpg[7], *a*Thr[8], and Hpg[11] (orange, 100−25-fold reduction) traverses the opposite end of ramoplanin at one corner of its reverse β-turn at *a*Thr[8]-Phe[9], potentially representing an alternative recognition domain for Lipid II. In the dimer structure, a potential and similar discontiguous recognition motif is defined by the red and orange residues, and Orn[4] is found proximal to the same hydrophobic face and lipid side chain. Although now speculative, such roles will become clearer upon examination of the derivatives herein for Lipid I or II binding in a transglycosylation inhibition assay that can dissect such roles.[20]

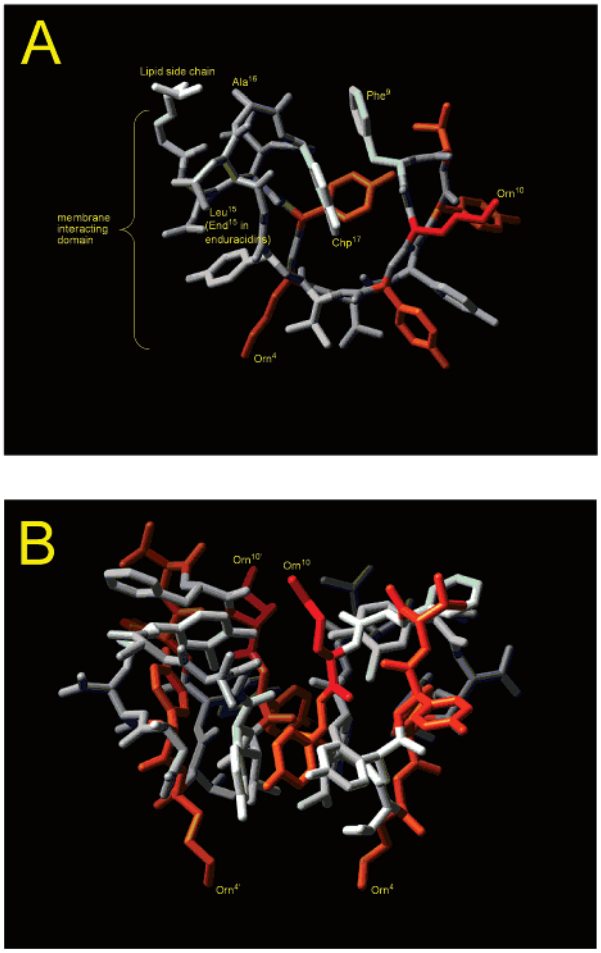


Figure 5. (A) Monomer and (B) dimer solution structures of ramoplanin (red: >100, orange: 100−25, white: <25-fold reductions in antimicrobial activity by alanine scan).

Within the residue 11−14 domain, all residues exhibit a significant effect even though they lie outside McCafferty's putative Lipid II recognition domain. The magnitude of their impact (10−40-fold) suggests a prominent role in establishing ramoplanin's activity. Although there may be many explanations for this behavior, this would be consistent with their stabilization of the ramoplanin dimerization interface observed by Walker et al.[6e] It is noteworthy that the most significant of these residues is Hpg[11], which also lies adjacent to the critical Orn[10] and is the site of glycosylation. Given the impact of Hpg[11], it is possible that it plays a larger role in binding Lipid II than present models suggest.

One of the most interesting regions of the molecule spans residues 15−2. Attached to residue 2 is Asn[1], which is external to the cyclic ring system and which is acylated with the unsaturated lipid side chain. We have suggested that this serves to anchor the antibiotic in the bacterial cell wall, ensuring its localization at its site of action.[20] Consistent with this, removal of the side chain or its replacement with a minimal acetyl group results in 200−800-fold reductions in antimicrobial activity but has no effect on Lipid II binding or transglycosylase inhibition.[20] This suggests that Leu[15]-HAsn[2] resides adjacent to a membrane anchoring center where this region constitutes a "relatively" flexible loop at one end of the otherwise rigid U-shaped antiparallel β-sheet. As mentioned above, it is also possible that the proximal Orn[4] collaborates with this lipid side chain by capping this membrane binding domain with a positively charged amine that binds membrane phosphates. We have also shown and continue to highlight herein that HAsn[2] may be effectively replaced with Dap[2], providing a hydrolytically stable 49-membered ring system in which an amide replaces the labile depsipeptide ester. The HAsn[2] β-carboxamide clearly does not contribute to ramoplanin's interaction with its biological target (but may sterically hinder ester hydrolysis), and the Dap[2] rigid secondary trans amide bond assuredly mimics the analogous trans ester conformation observed in the solution conformations of ramoplanin and enduracidin. Although flexible, efforts to expand[19,20] or contract the macrocycle by a single carbon atom at this site have failed or resulted in nonfunctional compounds, suggesting this region plays important conformationally related roles helping to confine ramoplanin to productive conformations. With the possible exception of Leu[15], the side chains of the remainder of this "flexible" loop, Leu[15]-D-Ala[16]-Chp[17], do not appear to directly contribute to ramoplanin's antimicrobial activity. The surprisingly small impact of replacing Chp[17] with Ala[17] (4-fold), the disparate Leu[15] versus solubilizing End[15] residues found in the ramoplanins versus enduracidins, and the conserved minimal side chain at D-Ala[16] suggest not only that this "connecting" region of the molecule may represent a membrane interacting region of the molecule, but also that it may be a good site for modification or conjugation studies.

Finally, the impact of nearly each residue is so significant that it is difficult to imagine deriving a simplified ramoplanin by excision of a substantial portion of its structure (i.e., cyclization or alternative presentations of only the putative recognition domain).[8b] The studies serve to define residues and regions amenable to further funtionalizations for detailed mechanistic studies in progress. Further insights into the individual roles of each residue may be forthcoming from their examination in assays establishing Lipid II binding or transglycosylation inhibition.[20]

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Supporting Information Available: Full experimental details and compound characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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