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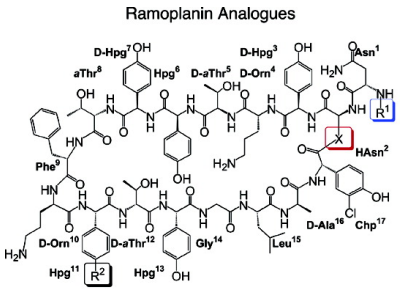

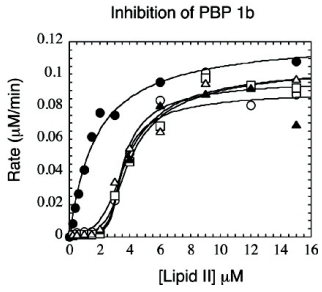

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Dissecting Ramoplanin: Mechanistic Analysis of Synthetic Ramoplanin Analogues as a Guide to the Design of Improved Antibiotics

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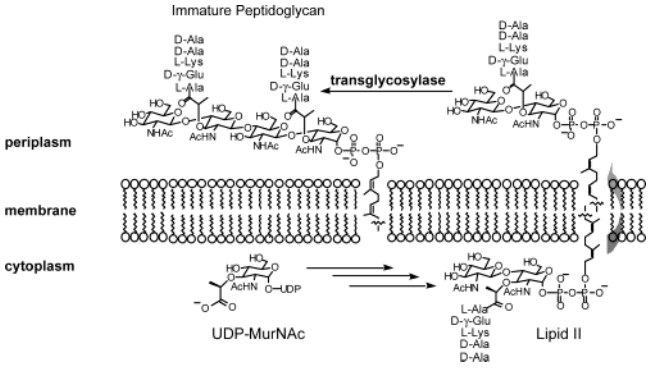
Ramoplanin is a cyclic lipoglycodepsipeptide antibiotic that disrupts bacterial cell wall synthesis by binding the peptidoglycan intermediate Lipid II.[1] Lipid II is the substrate for the bacterial transglycosylases (TGase), and ramoplanin binding blocks its polymerization to form the peptidoglycan carbohydrate chains (Scheme 1). Ramoplanin is currently in phase III clinical trials for the eradication of vancomycin-resistant enterococci (VRE) from the gastrointestinal tracts of hospitalized patients at risk for acquiring systemic VRE infections; it is also in phase II trials for the treatment of nasal methicillin resistant *Staphylococcus aureus* (MRSA) infections. Although the preliminary clinical results are promising, additional uses for ramoplanin are currently limited because the compound is not orally absorbed and the lactone bond hydrolyzes rapidly upon IV administration. However, it may be possible to overcome these limitations with appropriate analogues. The total synthesis of the ramoplanin aglycon has been reported and has been adapted to prepare analogues **2**−**5** in which key structural features have been altered.[2] Herein, we directly compare the ability of these analogues to inhibit transglycosylation, enlisting our recently introduced assay to quantitate the conversion of Lipid II to peptidoglycan. Combined with the antimicrobial activity of the analogues, the results described here provide insight into the roles of the key structural features required for Lipid II binding, inhibition of the transglycosylation reaction, and biological activity.

Scheme 1. Final Stage of Peptidoglycan Biosynthesis



Compounds **2**−**5** differ from ramoplanin in several ways. All four of the compounds are missing the carbohydrate moiety that is attached to the phenol of hydroxyphenylglycine 11 (Hpg11). Previous studies have established that removing the carbohydrate does not alter the biological activity; the synthesis of ramoplanin analogues is simplified considerably if the sugars are omitted.[3] Compounds **2** and **3** contain an amide linkage in place of the ester

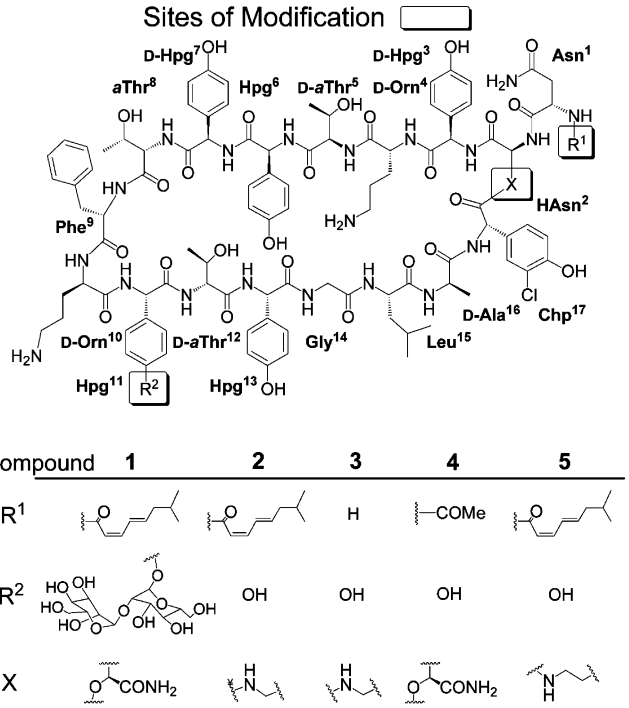


Figure 1. Structures of ramoplanin (**1**) and analogues **2**−**5**.

linkage found between the β-hydroxy group of L-threo-β-hydroxyasparagine 2 (HAsn2) and the carboxyl terminus of Chp17 in ramoplanin. The ester linkage, which is responsible for the instability of the natural product,[4] is difficult to install,[5,6] and compounds **2** and **3** are both more stable and more synthetically accessible than the ramoplanin aglycon. These two amide analogues differ from one another in that the amino terminus of **2** is acylated with the 10 carbon lipid chain found in the natural product, whereas **3** contains the unacylated free amine. Compound **4** is identical to the ramoplanin aglycone except that the amino terminus contains a simple acetyl group rather than the longer lipid chain of the natural product. Finally, compound **5** contains an amide linkage as in **2** but has an extra methylene unit in the macrocycle.

Compounds **2**−**5** were evaluated along with ramoplanin itself in a kinetic assay to monitor the activity of *E. coli* PBP1b. *E. coli* PBP1b is the major synthetic TGase in *E. coli* and shares significant sequence homology with TGases from other organisms.[7] We have recently reported that ramoplanin itself inhibits PBP1b by binding to the Lipid II substrate.[1] Furthermore, we showed that the kinetics of inhibition provided insight into both the affinity and the stoichiometry of Lipid II binding; hence, the inhibition assay is a sensitive measure of substrate binding. Kinetic data for the rate of reaction versus the concentration of Lipid II at identical inhibitor concentra-

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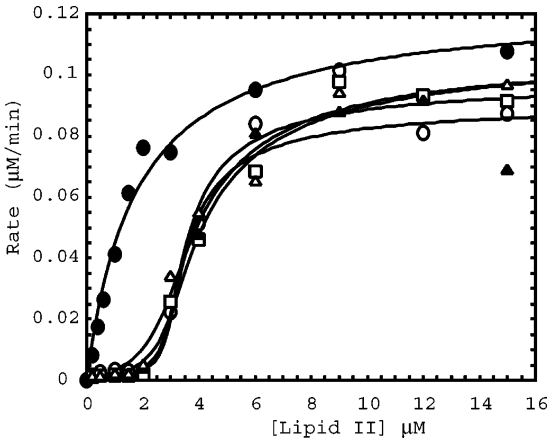

Figure 2. Inhibition of *E. coli* PBP1b by ramoplanin **1** (○), **2** (□), **4** (▲), **5** (△) at 6 μM concentration, and no inhibitor (●). The curve for **3** was omitted for clarity but is similar to curves for **1**, **2**, **4**, and **5**. Assay conditions are described in ref 11. Experiments were done in duplicate, and each point represents the average of two measurements.

Table 1. Minimum Inhibitory Concentrations (MICs)[a]

ramoplanin analogues	*E. faecalis*[b]	*E. faecium*[c]	*S. aureus*[d]
1	0.1	0.1	0.1
2	0.1	0.3	0.3
3	15	30	15
4	20	15	80
5	15	25	35

[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 35 °C for 22 h. [b] Bacterial strain 29212. [c] Bacterial strain 49624. [d] Bacterial strain 29213.

tions (6 μM) for compounds **2**−**5** as well as ramoplanin were obtained (Figure 2). Compounds **2**−**5** behave similarly to ramoplanin in that at low Lipid II concentrations the reaction rates are negligible because no free substrate is available to react with the enzyme; however, when the Lipid II concentration approaches one-half the inhibitor concentration (~3 μM), the reaction rate begins to increase rapidly, and at high substrate concentrations inhibition is largely overcome. These data indicate that compounds **2**−**5** bind to Lipid II with comparable affinity and the same 2:1 (inhibitor: Lipid II) stoichiometry as ramoplanin itself. We have concluded that replacing the HAsn2 ester with an amide linkage does not affect Lipid II binding significantly, even when an additional methylene unit is inserted. Furthermore, truncating or removing the lipid chain does not affect the inhibition kinetics, showing that the lipid chain does not play a direct role in substrate binding or inhibition of transglycosylation. The biological activities of compounds **2**−**5** were evaluated, and the MIC of compound **2** proved to be comparable to that of ramoplanin itself. However, the activities of compounds **3** and **4**, both of which lack a lipid chain, are 2 orders of magnitude higher than ramoplanin (**1**) or the amide analogue containing the natural lipid chain (**2**). Thus, although the lipid chain does not play a role in substrate binding or in vitro inhibition of the transglycosylation reaction, it plays a key role in biological activity. Other antibiotics, including both natural and semisynthetic glycopeptide antibiotics, contain lipid moieties that contribute to biological activity, and it has been proposed that the lipids in these molecules function to localize the compounds to the cell membrane.[8,9] The lipid moiety in ramoplanin may play a similar role and thus help position the antibiotic near its target, Lipid II, which is located on the outer surface of the cytoplasmic membrane.

Compound **5**, which contains an extra methylene group in the macrocyclic ring, is also 100-fold less potent than **1** and **2** even though it contains a lipid chain and inhibits PBP1b in a manner comparable to ramoplanin itself. NMR analysis reveals that compound **5**, unlike ramoplanin and analogues **2**−**4**, aggregates extensively in aqueous buffer. Williams and co-workers have previously reported that the ring-opened form of ramoplanin maintains the same general conformation as the cyclized parent compound but shows a pronounced tendency to aggregate, presumably because the increased flexibility permits the β-strands in the molecule to associate in an intermolecular fashion.[10] The extra methylene in compound **5** evidently increases macrocycle flexibility, promoting self-association and perhaps enabling undesirable interactions with other molecules as well. Unfavorable partitioning of the more flexible ramoplanin analogue **5** in cell-based assays would explain its higher MICs.[11]

The results reported here provide insight into the function of key structural features in ramoplanin and may serve as a foundation for the design of improved analogues. We have shown that substituting an amide linkage lacking the HAsn2 carboxamide side chain for the more labile and more complex ester linkage does not affect substrate binding, in vitro activity, or in vivo activity *provided that the ring size is maintained and the lipid chain is not removed*. The amide-linked macrocycle is considerably more stable than the ester and may have significant advantages as a therapeutic agent. Increasing the ring size does not affect substrate binding or inhibition of the TGase reaction but greatly increases the tendency of the molecule to associate, which likely leads to the decrease in biological activity. Any modifications that increase the flexibility of the molecule may, therefore, have a generally deleterious effect on biological activity. Most importantly, we have found that the lipid chain plays a key role in biological activity without directly influencing binding to Lipid II or in vitro inhibition of transglycosylation.[12] We propose that the lipid helps target ramoplanin to bacterial membranes. If so, substitution of the lipid chain with other groups that also facilitate localization may lead to analogues with improved activity.[13] Analogues containing targeting elements that are selective for bacterial cell surfaces might have considerable advantages as therapeutic agents.

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