A New Mechanism of Action Proposed for Ramoplanin

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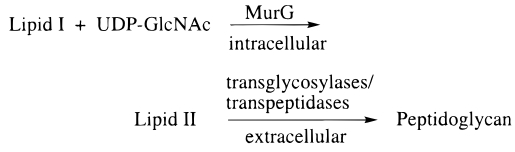
Ramoplanin (Figure 1) is a cyclic glycolipodepsipeptide antibiotic that kills gram positive bacteria by inhibiting cell wall biosynthesis. Ramoplanin was shown to block the conversion of Lipid I to Lipid II,[1] a reaction that is catalyzed by the intracellular GlcNAc transferase, MurG (Scheme 1). It was proposed that ramoplanin inhibits MurG by complexing Lipid I, which prevents it from being utilized as a substrate. Below we show that ramoplanin also inhibits the polymerization of Lipid II; therefore, we propose that another mechanism by which ramoplanin can kill bacterial cells is through inhibition of the transglycosylation step of peptidoglycan synthesis. Using a synthetic analogue of Lipid II, we present evidence that enzyme inhibition by ramoplanin involves substrate binding. Ramoplanin undergoes a conformational change upon substrate binding, and the resulting complexes self-associate to form fibrils. The significance of fibril formation is discussed.

Scheme 1[a]



[a] Lipids I and II are identical with **1** and **2** (Scheme 2) except that they contain an undecaprenyl (C55) instead of a citronellol (C10) chain.

The mechanism of action of ramoplanin has been investigated in permeabilized bacterial cells and membrane preparations by following the incorporation of radiolabel from a precursor into various intermediates along the pathway to peptidoglycan.[1−3] A limitation of these assays is that if one enzymatic step is blocked, then no information can be obtained about subsequent steps. Thus, because ramoplanin prevents the formation of Lipid II, it is not possible to determine whether it also inhibits the polymerization of Lipid II. We reinvestigated the ability of ramoplanin to block Lipid II polymerization using a modified membrane assay[4] in which the transglycosylases are selectively inhibited to permit the buildup of radiolabeled Lipid II. Following removal of the inhibitor, peptidoglycan synthesis commences. The effect of ramoplanin on Lipid II polymerization was evaluated by monitoring the amount of radioactive peptidoglycan formed in the presence of increasing concentrations of ramoplanin. Ramoplanin blocks the polymerization of Lipid II and thus is an inhibitor of the transglycosylation step of peptidoglycan synthesis (Figure 2).

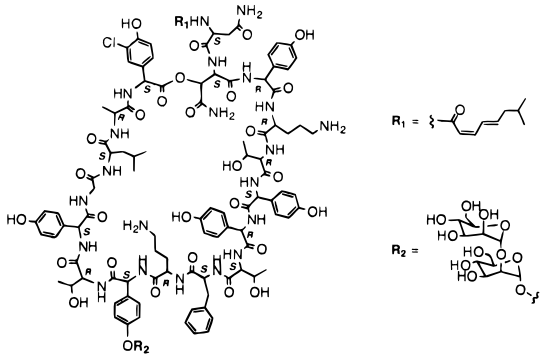


Figure 1. Ramoplanin (factor A2).

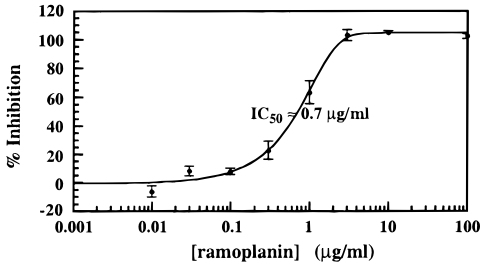
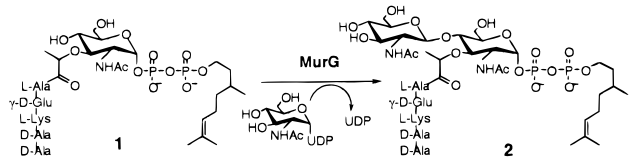


Figure 2. Inhibition of radiolabeled Lipid II polymerization by ramoplanin.[4] IC_{50} is approximately one-half the estimated Lipid II concentration.

Scheme 2. Enzymatic Synthesis of **2** from **1**[8]



Ramoplanin was proposed to act by complexing substrates required for peptidoglycan synthesis.[1] Unfortunately, difficulties in isolating Lipid intermediates from bacterial cells have hindered studies of their interactions with ramoplanin.[5,6] Moreover, the natural Lipid intermediates contain a 55 carbon polyprenol chain that renders them insoluble in water, and thus difficult to use in biophysical studies of complex formation. We recently developed a synthetic route to a soluble Lipid I analogue (**1**) to use in studying MurG,[7] the GlcNAc transferase that converts Lipid I to Lipid II. Using purified MurG, we have now made the corresponding Lipid II analogue **2** from **1**, as shown (Scheme 2).[8,9] Compound **2** is identical to natural Lipid II except that the 55 carbon chain has been replaced with a 10 carbon unit so that the compound is freely water soluble.[10]

The ability of ramoplanin to interact with **2** was investigated by NMR (Figure 3). Titration of ramoplanin with **2** caused the ramoplanin signals to disappear. Resonances for free **2** began to appear at a 1:1 ramoplanin:**2** ratio. The disappearance of the ramoplanin resonances connotes a change in relaxation properties

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(1) Somner, E. A.; Reynolds, P. E. *Antimicrob. Agents Chemother.* **1990**, *34*, 413−419.
(2) Reynolds, P. E.; Somner, E. A. *Drug. Exptl. Clin. Res.* **1990**, *16*, 385−389.
(3) Brötz, H.; Bierbaum, G.; Reynolds, P. E.; Sahl, H.-G. *Eur. J. Biochem.* **1997**, *246*, 193−199.
(4) The assay is described in: Goldman, R. C.; Baizman, E. R.; Longley, C. B.; Branstrom, A. A. *FEMS Lett.* **2000**, *183*, 209−214. Briefly, 20 μM UDP-MurNAc-pentapeptide and limiting quantities of [^{14}C]-UDP-GlcNAc (0.83 μM, 300 mCi/mmol) were added to *E. coli* membranes in the presence of Triton-X. Following depletion of UDP-GlcNAc, the Triton-X was removed, and the polymerization of radiolabled Lipid II was monitored as described in: Park, W.; Matsuhashi, M. *J. Bacteriol.* **1984**, *157*, 538−544.
(5) Ramoplanin alters the chromatographic mobility of Lipid II, but nothing else is known regarding the structure of the complex. See: Brötz, H.; Josten, M.; Wiedemann, I.; Schneider, U.; Götz, F.; Bierbaum, G.; Sahl, H.-G. *Mol. Microbiol.* **1998**, *30*, 317−327.
(6) NMR structures of ramoplanin and analogues have been reported previously. See: (a) Skelton, N. J.; Harding, M. M.; Mortishire-Smith, R. J.; Rahman, S. K.; Williams, D. H.; Rance, M. J.; Ruddock, J. C. *J. Am. Chem. Soc.* **1991**, *113*, 7522−7530. (b) Maplestone, R. A.; Cox, J. P. L.; Williams, D. H. *FEBS Lett.* **1993**, *326*, 95−100. (c) Kurz, M.; Guba, W. *Biochemistry* **1996**, *35*, 12570−12575.
(7) (a) Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1998**, *120*, 2484−2485. (b) Ha, S.; Chang, E.; Lo, M.-C.; Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1999**, *121*, 8415−8426.

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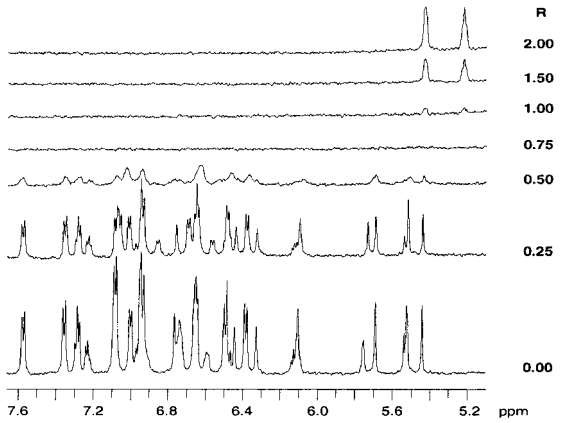

Figure 3. Downfield region of the 1D ^{1}H NMR spectra of ramoplanin in D_2O at pH 7.0 upon stepwise addition of **2**. The molar ratios (*R*) of **2**:ramoplanin are shown. Ramoplanin assignments have been reported previously.[6] The signals at 5.4 and 5.2 ppm are the anomeric proton of the MurNAc sugar and the vinylic proton of **2**.

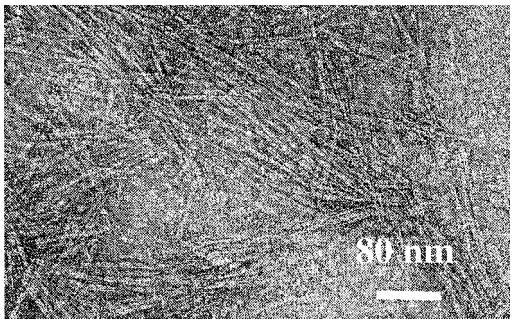

Figure 4. Transmission electron micrographs of ramoplanin + **2**. Neither ramoplanin nor **2** alone formed fibrils.

consistent with the formation of a high molecular weight species. A striking increase in the viscosity of the ramoplanin samples was evident upon the addition of **2**. In addition, electron micrographs of the ramoplanin complexes show long fibrils (Figure 4). Thus, ramoplanin undergoes a ligand-induced polymerization process in the presence of **2**.

Ligand-induced polymerization of ramoplanin complexes implies a conformational change in ramoplanin. Therefore, one would expect a significant change in the far-UV CD spectrum of ramoplanin upon adding **2**. In fact, the CD spectrum for ramoplanin changes dramatically upon titration with **2** (Figure 5). The appearance of a minimum at ~210 nm is consistent with a model in which assembly into fibrils is mediated by hydrogen bonds between amides in the ramoplanin complexes, and may be related to the unusual pattern of D and L amino acids in the molecule.[11]

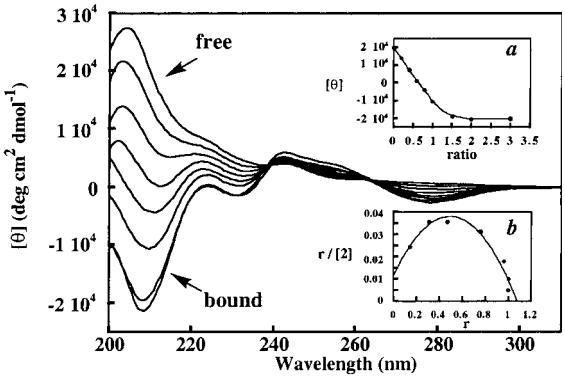

Figure 5. CD spectra of ramoplanin (100 uM) in pH 7.0 phosphate buffer upon titration with **2** (20−300 uM). Free ramoplanin has a maximum at ~204 nm and bound ramoplanin has a minimum at ~210 nm. Inset a is a plot of molar ellipticity ([θ]) at 210 nm vs **2**:ramoplanin ratio. Inset b is a Scatchard plot of the data. (r = fraction bound; [**2**] = concentration of free **2** (μM).)

The change in ellipticity of ramoplanin upon adding **2** levels off at a ratio of approximately 1:1 (Figure 5, inset a), the same ratio at which signals for free **2** begin to show up in the NMR titrations. Hence, the *apparent* stoichiometry for complex formation is 1:1; however, Scatchard analysis of the CD data produces a concave down curve (Figure 5, inset b), which is consistent with a ligand-dependent polymerization process.[12] The IC_{50} for inhibition of Lipid II polymerization suggests that the apparent stoichiometry for ramoplanin complex formation at membrane interfaces is also 1:1. Whether self-association of ramoplanin complexes occurs at membrane interfaces cannot be established from the inhibition data. We note, however, that cooperative interactions between ramoplanin complexes would enhance binding to membrane-anchored substrates, making ramoplanin a more effective inhibitor.[13] It is also possible that the ligand-induced conformational change in ramoplanin promotes binding to proteins involved in transglycosylation.

We propose that ramoplanin is an inhibitor of the transglycosylation step of peptidoglycan synthesis, joining a growing number of microbial and semisynthetic compounds that block this step.[14−16] The transglycosylases and Lipid II are found on the external surface of the bacterial membrane and would be the first targets encountered by ramoplanin. MurG, which is found inside the bacterial cell along with its Lipid I substrate, may be a secondary target. We have also shown that ramoplanin binds to a synthetic analogue of Lipid II, supporting the hypothesis that enzyme inhibition involves substrate binding.[1]

In closing, we note that it should be possible to use our soluble Lipid I/II analogues to characterize the mechanisms of action of other antibiotics proposed to interact with the Lipid I/II substrates involved in cell wall biosynthesis.[14]

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Supporting Information Available: Spectra for **1** and **2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) To 2.7 mg of **1** (2.43 umol) in 12.91 mL of buffer (50 mM HEPES pH 7.9, 5 mM $MgCl_2$) was added 0.32 mL of a 10 mg/mL aqueous stock of UDP-GlcNAc followed by 0.27 mL of a 10 mg/mL stock of MurG. The mixture was incubated for 40 min (room temperature) and another 0.32 mL aliquot of UDP-GlcNAc was added. After another 13 min, the solution was poured into 40 mL of MeOH, filtered, concentrated to 4 mL, and purified over a C18 column (8 mm × 80 mm, particle size 40 μm, pore size 60 Å; elution conditions: CH_3CN/0.1% NH_4HCO_3 from 0% to 30% over 60 mL). 3.2 mg of **2** was obtained (99%). ^{1}H NMR (D_2O, 600 MHz): see Supporting Information. ESI-MS calcd for $C_{49}H_{87}N_8O_{26}P_2$ [M + H$^+$], 1265.6; found, 1266.

(9) Imperiali and co-workers used a chemo-enzymatic route to make the substrate for oligosaccharyl transferase. See: Imperiali, B.; Zimmerman, J. W. *Tetrahedron Lett.* **1990**, *31*, 6485−6488.

(10) We have established that ramoplanin inhibits the MurG-catalyzed transfer of GlcNAc to **1** in the absence of membranes, showing that the molecule retains inhibitory potency in solution, and providing a justification for studying ramoplanin complexes in solution.

(11) Synthetic cylic peptides comprised of alternating D and L amino acids have been observed to form self-assembling peptide nanotubes. See: (a) Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. *Nature* **1993**, *366*, 324−327. (b) Hartgerink, J. D.; Granja, J. R.; Milligan, R. A.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1996**, *118*, 43−50.

(12) Cann, J. R. *Methods Enzymol.* **1978**, *48*, 299−307.

(13) Vancomycin and some other glycopeptide antibiotics are proposed to utilize cooperative interactions between complexes to enhance substrate binding. See inter alia: Williams, D. H. *Nat. Prod. Rep.* **1996**, *13*, 469−477.

(14) (a) Sahl, H.-G.; Bierbaum, G. *Annu. Rev. Microbiol.* **1998**, *52*, 41−79. (b) Brötz, H.; Bierbaum, G.; Leopold, K.; Reynolds, P. E.; Sahl, H.-G. *Antimicrob. Agents Chemother.* **1998**, *42*, 154−160. (c) Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O. P.; Sahl, H.-G.; de Kruijff, B. *Science* **1999**, *286*, 2361−2364.

(15) Malabarba, A.; Nicas, T. I.; Thompson, R. C. *Med. Res. Rev.* **1997**, *17*, 69−137.

(16) Ge, M.; Chen, Z.; Onishi, H. R.; Kohler, J.; Silver, L. L.; Kerns, R.; Fukuzawa, S.; Thompson, C.; Kahne, D. *Science* **1999**, *284*, 507−511.