Chemistry and Biology of Ramoplanin: A Lipoglycodepsipeptide with Potent Antibiotic Activity

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1. Introduction

Before the introduction of antibiotics in the 1940s and 1950s, patients with bacteremia—bacteria in the blood stream—had almost no chance of survival.[1,2] Antibiotics (Figure 1) were hailed as miracle drugs because they rapidly cured infections that would otherwise have proven fatal. In the belief that antibiotics, vaccination, and modern sanitation methods had defeated infectious disease, the Surgeon General declared in 1970 that the United States was "ready to close the book on infectious disease as a major health threat." Twenty-five years later, hospital-acquired (nosocomial) infections cost several billion dollars and contribute to 100 000 deaths annually.[3] According to one estimate, 20% of patients admitted to hospitals have or will develop an infection and 70% of the bacteria that give rise to these infections are resistant to at least one of the main antimicrobial agents used to fight infection.[4]

More than one-third of nosocomial infections are caused by three Gram-positive pathogens—*Staphylococcus aureus*, coagulase-negative *staphylococci*, and *enterococci*. Acquired antibiotic resistance in these organisms is a major concern. Clinical isolates of

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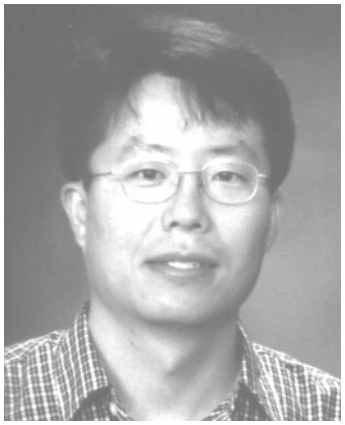
Suzanne Walker (above right) received her Ph.D. degree from Princeton University in 1992. She joined the Department of Chemistry at Princeton as a faculty member in 1995, where she initiated a program to study peptidoglycan biosynthesis and its inhibition. In 2004 she moved to the Department of Microbiology & Molecular Genetics at Harvard Medical School. She is interested in bacterial cell growth and division as well as the mechanism of action of unusual antibiotics.

Lan Chen (above left), born in Wuhan, China, obtained her M.S. degree in Chemistry from Wuhan University in 1993. She received her Ph.D. degree in 2000 from Rutgers University, where she studied mechanistic enzymology under the supervision of Professor W. Phillip Huskey. In 2000 she joined the laboratory of Professor Suzanne Walker as a postdoctoral associate, where she studied the kinetic mechanism of *E. coli* MurG, developed methods to study bacterial transglycosylases, and helped characterize the mechanism of action of ramoplanin. She joined Cubist (Lexington, MA) as a Research Scientist in 2004.

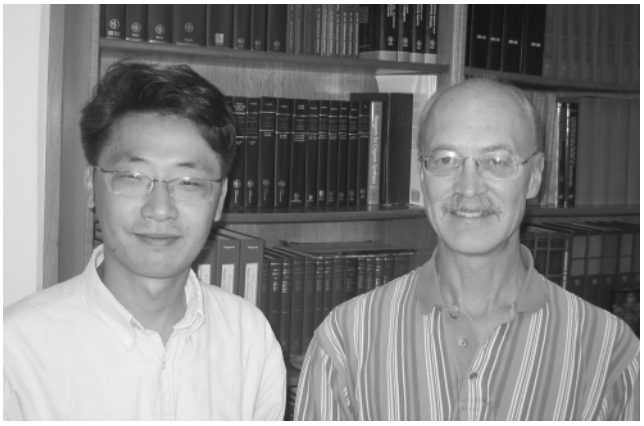
Yanan Hu was born in Dalian, China, and obtained her M.S. degree in Chemistry from Tsinghua University in Beijing in 2000. She joined the laboratory of Professor Suzanne Walker as a graduate research associate at Princeton University in 2000. She solved the X-ray structure of a co-complex of *E. coli* MurG with UDP-GlcNAc bound, developed a high-throughput screen for MurG inhibitors, and helped characterize the mechanism of action of ramoplanin. After receiving her Ph.D. degree in 2004, she joined Colgate-Palmolive (Piscataway, NJ) as a Research Scientist.

S. aureus, which infects burns, skin, and surgical wounds, are typically resistant to a range of antibiotics, including methicillin.[5] The glycopeptide antibiotic vancomycin is used to treat infections caused by drug-resistant *S. aureus* strains and other Gram-positive pathogens.[6] Unfortunately, vancomycin resistance has become increasingly common in enterococci and is now beginning to spread to other organisms.[7,8] For example, a methicillin-resistant *S. aureus* strain displaying high-level resistance to vancomycin was isolated recently from a dialysis patient in Michigan.[9,10] This isolate had apparently acquired the genes that confer vancomycin resistance from a coexisting *E. faecalis* strain and was now harboring these genes on a large multiresistance conjugative plasmid. Additional genes on the plasmid encoded resistance to trimethoprim, aminoglycosides, β-lactams, and disinfectants.[10]

As the foregoing discussion emphasizes, there is a clear need for new antibiotics to treat drug-resistant bacterial infections. Antibiotics that operate by new mechanisms or belong to new structural classes are particularly attractive because they are less likely to show cross-resistance with existing antibiotics. Un-



Yosup Rew, born in 1968 in Andong, South Korea, received his B.Sc. and M.Sc. degrees in Chemistry from the Seoul National University, South Korea, in 1990 and 1992, respectively. After working as a research scientist of the new fungicide discovery team in the agrochemical division of LG Chemical Ltd., Daejeon, South Korea. for 5 years, he continued his studies in chemistry at the University of California, San Diego. where he received his Ph.D. degree in 2002 under the supervision of the late Professor Murray Goodman in the field of peptide chemistry. Following this he was a postdoctoral fellow at The Scripps Research Institute working with Professor Boger and involved in the total synthesis of key analogues of ramoplanin. He joined Amgen (San Francisco) in 2004.



Dongwoo Shin (above left), born in 1971 in Seoul, South Korea, obtained his B.Sc. degree in Chemistry from Hanyang University, Seoul, South Korea in 1996. He received his Ph.D. degree (2002) at The University of Illinois at Chicago, where he worked on the design and synthesis of protease inhibitors under supervision of Professor Arun K. Ghosh. In 2002 he joined the laboratory of Professor Dale L. Boger as a postdoctoral associate. His current interests cover the total synthesis and structure–activity studies of bioactive molecules.

Dale L. Boger (above right) received his Ph.D. degree from Harvard University in 1980. He joined the Department of Medicinal Chemistry of the University of Kansas, moved to Purdue University in 1986, and joined the newly founded Department of Chemistry at The Scripps Research Institute in 1990. His research interests include the total synthesis and structural exploration of biologically active natural products.

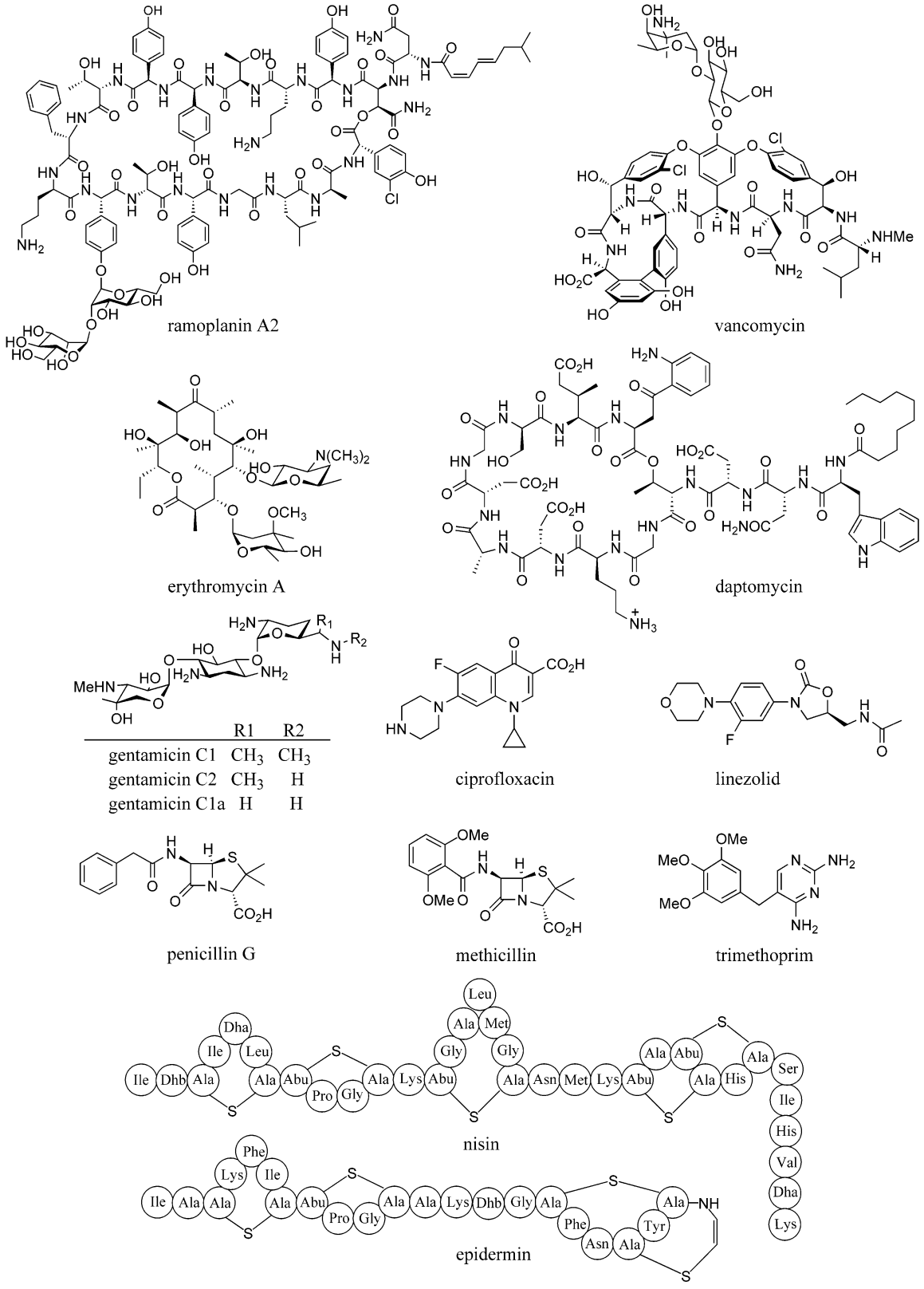


Figure 1. Structures of selected natural products.

fortunately, between 1962 and 2000 only one such antibiotic, linezolid, was introduced clinically, although others have since been introduced. All of the other agents that entered the market between 1962 and 2000 were analogues of existing drugs.[4,11] Collaborations between industrial and academic scientists may be required to meet the challenges involved in discovering, understanding, and developing new antimicrobial agents. Here we review an antibiotic that belongs to a new structural class of compounds and has not shown any cross-resistance to other antibacterial agents. This antibiotic, ramoplanin (Figure 2), is currently in Phase III clinical trials for the prevention of vancomycin-resistant enterococcal infections in hospitalized patients, and it possesses some potentially important advantages over many

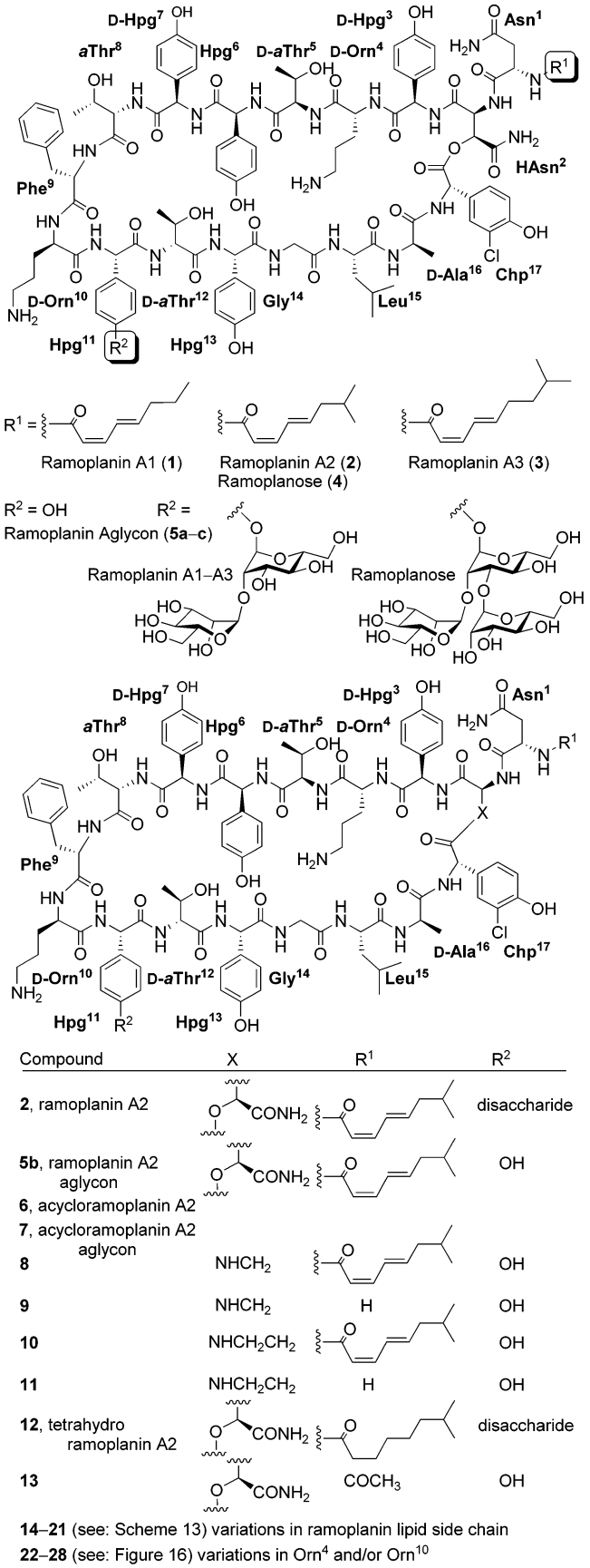


Figure 2. (a) Structures of the ramoplanins. (b) Structures of ramoplanin A2 and synthetic analogues described in the text.

other antibiotics.[12,13] Chief among them is that resistance to ramoplanin does not develop readily either in vitro or in vivo. However, ramoplanin has not yet been developed for systemic use, and its potential applications, albeit important, are currently limited.[14] A better understanding of the mechanism of action of ramoplanin, combined with the ability to prepare analogues, could lead to new derivatives with broader utility.

This review will present our understanding of the mechanism of action of ramoplanin. An introduction to the primary structure of ramoplanin, its spectrum of activity, and current clinical indications and progress will be followed by an overview of the early mechanistic investigations of this antibiotic. Technical limitations restricted the studies that were initially conducted on ramoplanin, and its mechanism of action continues to be refined and revised since the early investigations. Recent chemical and biochemical advances that have made it possible to probe ramoplanin's mechanism of action in greater detail will be described, and the current mechanistic understanding will be presented. This will be followed by a description of the synthetic studies that have been conducted on ramoplanin. The ability to synthesize ramoplanin and analogues is critical to addressing the relationship between structure and activity, which in turn is central to developing better antibiotics. The review will conclude with an assessment of where we are with respect to understanding ramoplanin and where we need to go.

2. Ramoplanin Basics

2.1. Structural Overview

The discovery, structure elucidation, and biosynthesis of ramoplanin have been reviewed recently by McCafferty et al., and only a few salient features will be repeated here.[15] Ramoplanin factors A1 (**1**), A2 (**2**), and A3 (**3**) were discovered in 1984 from a fermentation broth of Actinoplanes and identified as cell wall active antibiotics by Biosearch Italia (then Gruppo LePetit).[16,17] Ramoplanins A1−A3 consist of a 49-membered macrocyclic depsipeptide containing 17 amino acids joined by a lactone linkage between a beta hydroxy group on amino acid 2 ($HAsn^2$) and the carboxyl terminus of amino acid 17 (Chp^{17}). Ramoplanin is produced by nonribosomal peptide synthesis and, like many such natural products, contains a mixture of L and D amino acids (nine L, seven D) as well as several nonproteinogenic side chains, including ornithine (Orn), hydroxyphenylglycine (Hpg), and chlorohydroxyphenylglycine (Chp) in addition to β-hydroxyasparagine (β-OH-Asn, HAsn). Ten of the 16 residues in the ramoplanin macrocycle are β-branched: L-β-$HAsn^2$, D-Hpg^3, D-*allo*-Thr^5, L-Hpg^6, D-Hpg^7, L-*allo*-Thr^8, L-Hpg^{11}, D-*allo*-Thr^{12}, L-Hpg^{13}, and L-Chp^{17}. The first amino acid in the depsipeptide is acylated at the amino terminus with a lipid substituent. Ramoplanins A1−A3 differ in minor ways in the length and structure of this appended lipid substituent; however, all three have $\alpha,\beta,\gamma,\delta$-unsaturated chains. The lipid chains of ramoplanins A1−A3 were originally assigned the (2*Z*,4*Z*) stereochemistry around these double bonds,[18] but all three have been reassigned as (2*Z*,4*E*) based on work by

Kurz and Guba (A2)[19] and Boger and co-workers[20] (see section 6.3). Ramoplanin A2 is the compound now in clinical trials because it can be produced in much larger quantities than factors A1 and A3; however, the antimicrobial activities of the lipid variants are virtually indistinguishable. The ramoplanins also contain a saccharide moiety attached by an α-glycosidic linkage to the phenol of amino acid 11. In ramoplanins A1−A3 the saccharide moiety is an α-1,2-dimannosyl group; however, ramoplanin variants in which the terminal mannose has been removed or the saccharide consists of a branched trimannosyl group (ramoplanose, **4**) have been reported.[21] The activities of the saccharide variants appear to be similar to those of ramoplanins A1−A3.

2.2. Antimicrobial Activity

Ramoplanin is active against a wide range of Gram-positive organisms, including many different species of *Staphylococcus, Enterococcus*, and *Bacillus* (Table 1). Like vancomycin and many other antibiotics, ramoplanin displays no activity against Gram-negative bacteria,[22,23] presumably because it cannot penetrate the outer membrane. In general, ramoplanin has MICs (MIC = minimum inhibitory concentration, defined as the lowest concentration at which bacterial growth ceases) below 1 μg/mL against most Gram-positive strains, although the measured MICs can vary widely based upon the method used to test growth inhibition.[24−28] It has been reported in two separate studies that the addition of BSA to the antibiotic stock solution or to plate wells containing bacteria that are used to carry out the measurements results in values up to 30-fold lower (more active) than without BSA.[29] This is consistent with the observation that ramoplanin tends to stick to nonpolar surfaces such as the plastic plates that are usually used for MIC measurements. BSA reduces this nonspecific binding, thereby increasing the amount of ramoplanin in solution.

Ramoplanin has been compared to vancomycin in a number of different in vitro studies and consistently has been found to be a more potent antibiotic on a molar basis regardless of the method of testing used.[15,30] Ramoplanin is also bactericidal at concentrations close to its MIC, which may provide it some advantages over vancomycin, which is bacteriostatic at concentrations near its MIC. Finally, ramoplanin retains full activity against vancomycin-resistant enterococcal strains and methicillin-resistant staphylococcal strains,[27,28] and significant ramoplanin resistance has not been observed to date, despite efforts to elicit it in the laboratory. The mechanism of action of ramoplanin makes the spontaneous development of high-level resistance improbable. However, as with other natural product antibiotics, resistance in clinically relevant pathogens could develop to ramoplanin by the horizontal transfer of genetic information from the producing organism to other microorganisms. The likelihood of this occurring rapidly depends on usage patterns of ramoplanin and related antibiotics, the biology of the producing microorganisms, and the mechanism of resistance in the producer organisms. It should be noted that the mechanism by which the ramoplanin producer resists the effects of ramoplanin has not yet been elucidated. It should also be noted that only one confirmed structurally related compound has been reported, enduracidin (Figure 3), and this compound is not used clinically or commercially.[31] Another potentially related antibiotic, janiemycin, has also been reported but not yet structurally characterized.[32]

Table 1. Minimum Inhibitory Concentrations (MICs) of Ramoplanin and Its Analogues Against Different Bacterial Strains (μg/mL; compound structures are shown in Figure 2b)

compound	*S. aureus*[a]	*S. aureus*[b]	*S. aureus*[c]	*E. faecium*[e]	*E. faecalis*[f]	*B. subtilis*[d]
2	0.5−1.56	0.125		0.1	0.1	0.03
5b	0.25−0.78		1			0.03
6	>128					64
7	>128					
8	0.39			0.3	0.1	
9	6.25−15			30	15	
10	>35−50			25	15	
11	>100					
12			2			
13	12.5−80			15	20	
14		8				
15		4				
16		0.125				
17		0.25				
18		0.25				
19		1				
20		0.06				
21		0.06				
22	>128					
23	4					
24				0.8	0.8	
25				50	50	
26						0.25
27						4
28						16

[a] *S. aureus* ATCC25923, refs 109 and 110. [b] *S. aureus* Smith SA819, ref 111. [c] *S. aureus* Tour, ref 112. [d] *B. subtilis* ATCC 8037, ref 30. [e] *E. faecium* L19624, refs 93 and 113. [f] *E. faecalis* Z9212, refs 36 and 113.

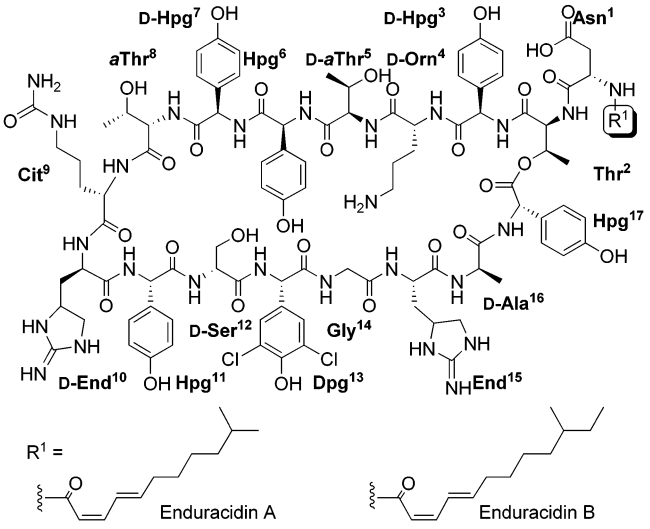


Figure 3. Structures of the enduracidins.

2.3. Clinical Status

Ramoplanin has not yet been developed for parenteral use due, inter alia, to problems associated with its stability in the blood stream.[33] However, it is currently in phase III clinical trials for the prevention of systemic nosocomial infections caused by vancomycin-resistant enterococci (VRE). Certain groups of patients, including those undergoing abdominal surgery and those receiving chemotherapy, are at high risk of developing systemic VRE infections in the hospital. It is believed that the GI tracts of many of these patients are colonized with VRE (possibly acquired in the hospital from other patients or contaminated instruments, etc.) and that damage to the intestinal mucosa caused by surgery or certain drugs can lead to systemic VRE infections. Therefore, eradicating VRE from the GI tracts of these patients prior to treatment may reduce the incidence of systemic VRE infections. Early clinical results are promising, and because the incidence of nosocomial VRE infections is increasing, the FDA has assigned ramoplanin Fast Track status for this indication.[14]

In addition to its use for the prevention of blood stream VRE infections, ramoplanin is also in clinical trials for the treatment of *Clostridium difficile*-associated diarrhea (CDAD).[3] *C. difficile*-associated diarrhea affects over 400 000 patients a year, and strains resistant to many commonly used therapeutic agents have appeared. As a result, the FDA recently designated this application of ramoplanin for Fast Track status as well.

3. Mechanism of Action of Ramoplanin—Early Work

3.1. Cellular Targets of Antibiotics

The majority of antibiotics act by inhibiting DNA synthesis, RNA synthesis, protein synthesis, or peptidoglycan synthesis.[34] DNA, RNA, and proteins are found in eukaryotic cells as well as prokaryotes, but peptidoglycan is unique to bacteria.[35] Peptidoglycan is a polymeric mesh that forms layers around bacterial cell membranes. One of its primary functions is to stabilize bacterial membranes so that they do not rupture as the osmotic pressure within the cells fluctuates.[36] Both the chemical structure of peptidoglycan and the process by which it is made are highly conserved in bacteria, even across genera that occupy very different evolutionary niches.[35] Microorganisms produce a range of natural products that kill other microorganisms by blocking peptidoglycan biosynthesis.[34] Because there are no analogous metabolic pathways in mammalian cells, many of these natural products have proven to be safe as well as efficacious antibiotics. Because peptidoglycan biosynthesis is regarded as an attractive pathway with which to interfere, many research groups in academia and industry have established programs to identify inhibitors of this pathway.[37,38] Ramoplanin was discovered in one such program. An overview of peptidoglycan structure and biosynthesis is provided in the following section to provide a context for discussing the current understanding of the mechanism of action of ramoplanin.

3.2. Peptidoglycan Structure and Biosynthesis

Peptidoglycan is comprised of linear chains of a repeating disaccharide held together by cross-links between peptides appended to one of the sugars of the disaccharide.[39−42] The repeating disaccharide unit consists of *N*-acetyl glucosamine attached via a β-glycosidic linkage to the C4 alcohol of an *N*-acetyl muramic acid derivative. Muramic acid, a sugar found only in bacteria, is identical to glucose except that the C3 hydroxyl is replaced by a lactic acid unit. The peptide chain that is involved in cross-linking the glycan strands is attached via its N-terminus to the lactate moiety. Prior to cross-linking, the peptide typically consists of a pentapeptide terminating in D-Ala-D-Ala. There are strain-dependent variations in the composition of the peptides, but all contain a nucleophilic group that attacks the D-Ala-D-Ala peptide bond to form cross-links.[39−42]

Peptidoglycan is synthesized in three stages (Figure 4), the first of which takes place in the cytoplasm and involves the conversion of UDP-*N*-acetyl glucosamine to UDP-*N*-acetyl muramic acid pentapeptide by a series of seven enzymes.[43−54] The first two, MurA and MurB, convert UDP-GlcNAc to UDP-MurNAc; the rest are ligases involved in the assembly of the peptide chain. The second stage of peptidoglycan biosynthesis, which takes place on the cytoplasmic surface of the bacterial membrane, commences when MraY, an enzyme containing several membrane-spanning regions, catalyzes a pyrophosphate exchange reaction in which membrane-bound undecaprenyl phosphate attacks the α-phosphate of UDP-MurNAc pentapeptide to form a lipid-anchored MurNAc pentapeptide known as Lipid I with release of UMP.[43,55,56] A membrane-associated glycosyltransferase, MurG, then catalyzes the transfer GlcNAc from UDP to the C4 hydroxyl of the MurNAc sugar of Lipid I to form Lipid II.[40,57−59] Once Lipid II is made, the disaccharide is somehow transported from the cytoplasmic surface of the bacterial membrane to the external surface where it is polymerized and cross-linked in the final stage of peptidoglycan bio-

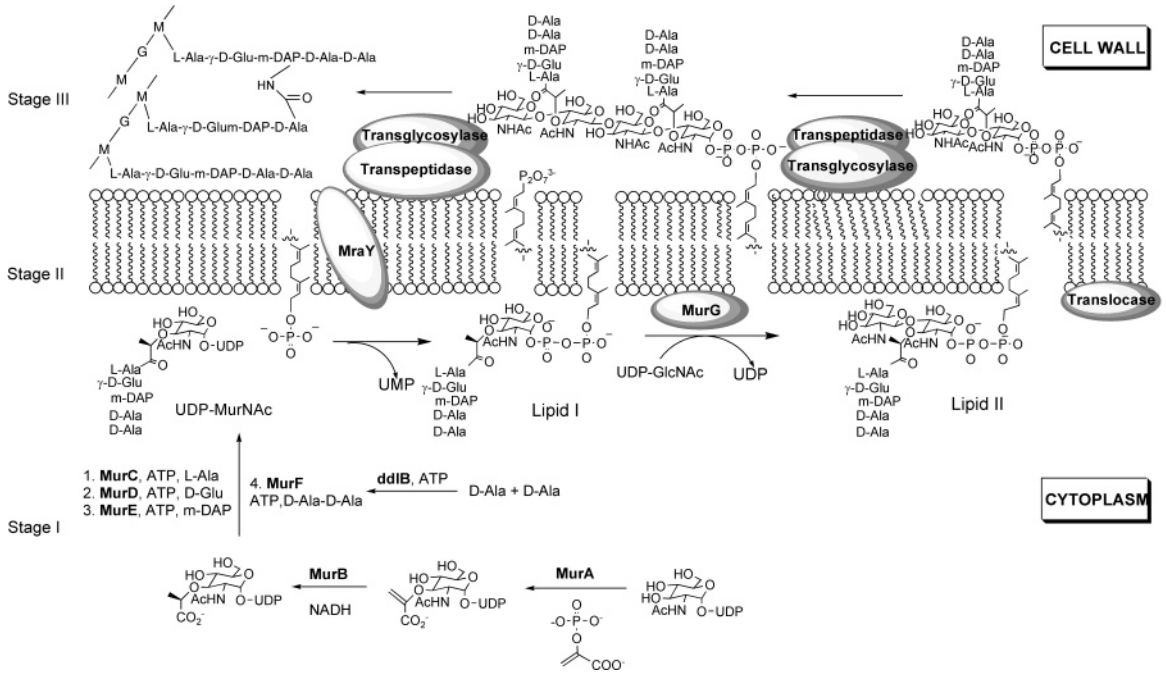


Figure 4. Three stages of peptidoglycan biosynthesis.

synthesis.[39,60] The enzymes responsible for polymerizing the GlcNAc-MurNAc disaccharide to form the glycan chains of peptidoglycan are known as transglycosylases; the enzymes involved in cross-linking the glycan chains are known as transpeptidases.[39] Bacteria contain several transglycosylases and transpeptidases that play different roles in cell growth and division.[61] Most, but not all, of the transglycosylases are found as domains in bifunctional enzymes that also contain transpeptidase domains. It is believed that the bulk of peptidoglycan biosynthesis is carried out by a subset of the transglycosylases.[62,63] In *E. coli*, for example, the major transglycosylases are contained in the bifunctional enzymes PBP1a and PBP1b, with PBP1b believed to be responsible for 85% of glycan strand synthesis.[64,65] Homologous enzymes in other bacterial strains can be readily identified based on sequence similarities and are assumed to play comparable roles.[62]

Several antibiotics in clinical use block key steps in peptidoglycan biosynthesis.[34] For example, fosfomycin, used to treat urinary track infections, inhibits MurA, which catalyzes the first committed step in the biosynthetic pathway.[66] Vancomycin and other glycopeptide antibiotics block glycan polymerization and cross-linking by binding to the D-Ala-D-Ala dipeptide terminus of Lipid II and nascent peptidoglycan.[6] Penicillin and other β-lactams covalently modify the active site of the enzymes involved in transpeptidation.[67,68]

3.3. MurG Is Proposed as the Target of Ramoplanin

Ramoplanin was discovered by scientists at Biosearch Italia in the course of screening fermentation products for compounds that killed a methicillin-resistant *S. aureus* strain without affecting a variant lacking a cell wall and grown under hypotonic conditions.[16,17] This screen was designed to identify compounds that block peptidoglycan biosynthesis. Experiments probing inhibition of macromolecular synthesis showed that ramoplanin blocks the incorporation of radiolabeled precursors into peptidoglycan without affecting the incorporation of radiolabeled building blocks into DNA, RNA, or protein, thereby confirming that ramoplanin inhibits cell wall synthesis. The challenge then became identifying the step that ramoplanin affects. At that time, identifying the site of inhibition of a compound that blocks peptidoglycan synthesis was a daunting challenge. While the structures of all the intermediates in the pathway were known, most of the enzymes involved in the individual chemical transformations had not been isolated or characterized, and, with few exceptions, direct assays for the enzymes did not exist.

In 1990, Somner and Reynolds reported detailed investigations of the mechanism of action of ramoplanin.[69,70] They first showed that bacteria treated with ramoplanin accumulate UDP-MurNAc-pentapeptide, implying that ramoplanin blocks one of the membrane-associated steps that takes place after the formation of the final cytoplasmic intermediate. They then tried to determine which membrane-associated step ramoplanin affects. In 1990, two related assays existed to probe the membrane-associated steps of peptidoglycan synthesis. One assay involved monitoring the conversion of UDP-MurNAc pentapeptide to nascent peptidoglycan using washed, particulate bacterial membranes as a source of the required membrane-bound enzymes, including MraY, MurG, and the transglycosylases/transpeptidases.[71,72] The second assay involved monitoring the conversion of UDP-MurNAc pentapeptide to mature peptidoglycan in bacterial cells rendered membrane permeable with an organic solvent such as toluene.[73] Methods to isolate UDP-MurNAc pentapeptide from bacterial cells, combined with techniques to separate Lipid I/II from peptidoglycan and to distinguish cross-linked

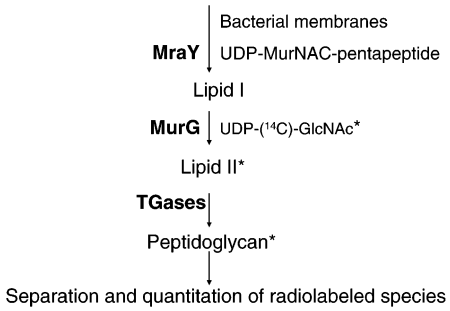


Figure 5. Schematic of the membrane assay used to determine the site of inhibition of ramoplanin.

from un-cross-linked peptidoglycan, had enabled the development of these assays.[71,72] By using isotopically labeled UDP-MurNAc pentapeptide and/or UDP-GlcNAc, Somner and Reynolds were able to follow the formation of various intermediates along the pathway and monitor changes in product formation upon addition of ramoplanin and other antibiotics. Somner and Reynolds noted that addition of ramoplanin to permeabilized bacterial cells supplemented with radiolabeled UDP-MurNAc-pentapeptide prevented incorporation of radiolabel into both un-cross-linked and cross-linked peptidoglycan. Un-cross-linked peptidoglycan was formed in the presence of an antibiotic known to block only the cross-linking step of peptidoglycan biosynthesis. Therefore, Somner and Reynolds concluded that ramoplanin acts before the transpeptidation step. They then showed that ramoplanin blocks the formation of Lipid II but does not prevent the formation of Lipid I. Thus, Somner and Reynolds proposed that ramoplanin acts by blocking peptidoglycan biosynthesis at the step catalyzed by the glycosyltransferase, MurG (Figure 5). On the basis of preliminary experiments that were mentioned but not described in the paper, Somner and Reynolds also proposed that ramoplanin inhibits its target by binding to and sequestering Lipid I.

At the time of their experiments Somner and Reynolds were missing an important piece of information about MurG, namely, that it is located on the intracellular surface of the cytoplasmic membrane.[74] Therefore, to reach MurG and/or its substrate, ramoplanin would have to pass through the cell membrane. Somner and Reynolds observed in 1990 that ramoplanin is unlikely to penetrate the cytoplasmic membrane because of its size and polar nature, but they did not consider alternative mechanisms of action because the cellular location of MurG had not been established. Moreover, the biochemical results were clear.

The hypothesis that ramoplanin inhibits peptidoglycan biosynthesis at the MurG step became widely accepted over the next decade. Although Bupp et al. reported in 1993 that MurG is located on the intracellular surface of the cytoplasmic membrane,[74] this finding did not initially prompt a reexamination of ramoplanin's mechanism. In fact, tools to test the hypothesis that ramoplanin inhibits MurG by binding to Lipid I were still not available. Lipid I, the MurG substrate that ramoplanin was proposed to bind, is present in miniscule quantities in bacterial cells, making isolation from natural sources all but impossible.[75,76] Because Lipid I could not be obtained, there was no direct assay to monitor MurG activity and no straightforward way to assess whether ramoplanin binds to Lipid I. Although Lipid II is found in somewhat larger amounts than Lipid I, it also presented major challenges for isolation. Furthermore, natural Lipid I and Lipid II both contain a 55-carbon undecaprenyl chain that renders them insoluble in water and thus difficult to manipulate. Until these issues of substrate availability and physical properties were addressed, it was not feasible to carry out detailed investigations of how ramoplanin inhibits individual membrane-associated enzymes of peptidoglycan synthesis.

3.4. Ramoplanin Is Shown To Bind to an Intermediate in Peptidoglycan Biosynthesis

The first direct evidence that ramoplanin is a substrate binder was reported by Brötz et al. in 1998.[77,78] Brötz, Sahl, and co-workers were studying several lantibiotics, including nisin and epidermin (Figure 1). Lantibiotics are ribosomally synthesized peptides that are posttranslationally dehydrated at serine and threonine side chains.[79,80] They then undergo a series of enzyme-catalyzed cyclizations in which cysteine residues attack the dehydroamino acids to form thioether-linked macrocycles. Lantibiotics display a range of mechanisms of action, but many appear to target Lipid II and other molecules presented on bacterial cell surfaces (the lantibiotics are reviewed by van der Donk in this issue). Brötz et al. showed that ramoplanin as well as the lantibiotics nisin and epidermin comigrate with Lipid II upon thin-layer chromatography, indicating that all three molecules interact with this peptidoglycan intermediate.[78] They also reported that nisin and epidermin block the formation of Lipid II in a particulate membrane assay similar to that employed by Somner and Reynolds in their study of ramoplanin. Thus, like ramoplanin, both nisin and epidermin can inhibit the MurG step of peptidoglycan biosynthesis. Brötz et al. did not conclude, however, that nisin and epidermin function in cells by inhibiting MurG because other information pointed to a different mechanism of action for these lantibiotics. By showing that nisin and epidermin behave similarly to ramoplanin with respect to MurG inhibition in a particulate membrane assay and yet are presumed not to inhibit MurG in cells, Brötz et al. thus provided the basis for questioning the interpretation that ramoplanin inhibits the MurG in a cellular context.

3.5. Ramoplanin Is Proposed To Block the Transglycosylation Step of Peptidoglycan Biosynthesis

The possibility that ramoplanin functions by inhibiting a different cellular target than MurG was addressed in 2000 by Lo et al., who noted that the original mechanistic investigations on the antibiotic were not conclusive.[81] Assays involving the use of a precursor substrate to monitor flux through several steps in a pathway can provide inaccurate information about cellular targets if more than one step in

the pathway is inhibited by a particular compound. Under these circumstances, such vectorial assays report only the first step to be inhibited, which may not be the relevant step in a cellular context. In the case of a proposed substrate binder, there is special cause for concern because the substrates along a particular pathway often bear a close resemblance to one another. Furthermore, in the case of peptidoglycan biosynthesis, it is necessary to carry out these vectorial assays under conditions in which the polar precursor substrates can access enzymes that are normally found on the internal surface of the bacterial membrane. Thus, the integrity of the membrane must be disrupted by organic solvents, detergents, or mechanical means for the assays to proceed, and so a major constraint on the mechanism of action—the membrane barrier—is removed. Lo et al. speculated that in the presence of an intact membrane barrier, ramoplanin might block the polymerization of Lipid II, which is catalyzed by bacterial transglycosylases located on the external surface of the bacterial membrane, instead of the synthesis of Lipid II, which is catalyzed by an enzyme located on the internal surface of the bacterial membrane. Inhibition of the transglycosylation step would not have been observed under assay conditions in which the formation of Lipid II is blocked.

The hypothesis that ramoplanin can block transglycosylation was tested by Lo et al. using a modified version of a particulate membrane assay in which UDP-MurNAc pentapeptide and radiolabeled UDP-GlcNAc were added as precursor substrates.[81,82] In this modified assay, Triton X-100 was added to the bacterial membranes, causing inhibition of the bacterial transglycosylases and allowing radiolabeled Lipid II to accumulate. The addition of a Triton X-100 scavenger led to reactivation of the transglycosylases and concomitant formation of peptidoglycan. This modified assay permits one to add inhibitors after Lipid II is formed and thus evaluate steps after the formation of this intermediate.[82] Ramoplanin was found to block transglycosylation in this assay, implicating a second target for this antibiotic. Thus, depending on the assay conditions, ramoplanin is capable of inhibiting either MurG or the bacterial transglycosylases. Lo et al. suggested that the bacterial transglycosylases were likely to be the primary targets of ramoplanin because they are extracellular and would be encountered first by ramoplanin. The validity of this argument depends on issues such as whether transglycosylase inhibition is comparable to or better than MurG inhibition and whether ramoplanin is able to penetrate cell membranes. The former issue is addressed in the sections below.

4. Mechanism of Action of Ramoplanin—Recent Work

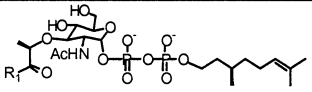
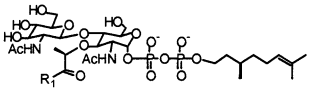
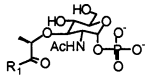
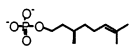
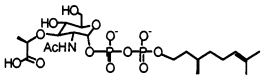
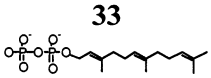
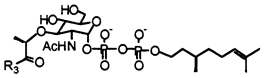
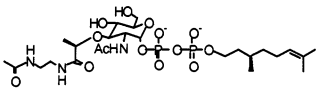
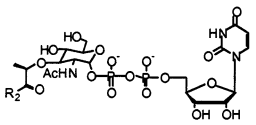
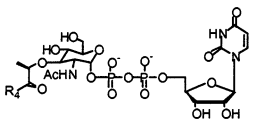
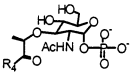
4.1. Technical Advances in the Study of Peptidoglycan-Synthesizing Enzymes

The development of better methods to obtain natural Lipid I and II substrates and analogues has made it possible to assay MurG and the bacterial transglycosylases directly, enabling in turn detailed studies of ramoplanin inhibition. For example, in 1997 Auger et al. reported a method to convert UDP-MurNAc pentapeptide, which can be isolated in large quantities from natural sources, to a heptaprenyl Lipid I analogue, potentially enabling the study of MurG.[83] Shortly thereafter Men et al. reported the chemical synthesis of a Lipid I analogue (**29**, Table 2) and its use in a biotin capture assay to monitor MurG activity.[84] Two total syntheses of natural Lipid I containing the 55-carbon undecaprenyl chain were reported in 2001.[85,86] *E. coli* MurG was purified and characterized using synthetic Lipid I analogues,[87,88] and several different fluorescence-based and radiometric assays to monitor enzymatic activity using synthetic substrate analogues have been reported.[84,87−94] Moreover, access to purified MurG has made possible the enzymatic conversion of synthetic Lipid I to Lipid II. The first synthesis of a Lipid II analogue containing a 10-carbon lipid chain (**30**, Table 2) was reported in 2000.[81] This accomplishment was followed in 2001 by the conversion of synthetic Lipid I containing the intact 55-carbon chain to Lipid II using MurG to form the glycosidic linkage.[85] This synthesis of Lipid II was followed by two alternative routes to the compound in which the glycosidic bond was formed by chemical methods.[95,96] Finally, in 2003 an efficient enzymatic strategy to produce quantities of Lipid II and analogues from UDP-MurNAc pentapeptide using bacterial membranes was reported.[97] Methods to study purified transglycosylases have followed developments in Lipid II synthesis, enabling the kinetic analysis of a variety of antibiotics proposed to block transglycosylation.[95,98,99]

4.2. Expected Inhibition Kinetics for Substrate Binders

The availability of substrates and assays to monitor MurG and the bacterial transglycosylases has made it possible to examine how ramoplanin inhibits these enzymes. Before detailing the results of recent studies on ramoplanin, it is worth describing the inhibition curves that would be expected for a substrate binder.[100] Figure 6a shows a calculated curve for inhibition of enzymatic activity by a substrate binder that forms a 1:1 complex and has a dissociation constant that is low relative to the K_m of the substrate. The reaction rate is negligible at low substrate concentrations because the substrate is fully bound and unable to react. When the substrate concentration increases to the level where free substrate becomes available, the reaction rate jumps rapidly. At high substrate concentrations inhibition is overcome. Thus, the plot of reaction rate as a function of substrate concentration has a pronounced sigmoidal shape. Compounds that bind the substrate less tightly still yield sigmoidal inhibition curves, but the behavior is not as pronounced. Figure 6b shows inhibition curves for three hypothetical substrate binders having K_ds ranging from 0.01 to 1 μM. Hence, the shape of the inhibition curves can provide information both on the mechanism of inhibition and on the affinity of substrate binding.

Table 2. Substrate Recognition of Ramoplanin[a]

Compound	Binds to ramoplanin (1)	Ref.
 **29**	Yes	30,93,103,104
 **30**	Yes	81,93,103
 **31**	No	103
 **32**	No	103
 **33**	No	30,104
 **34**	Yes	103
 **35**	Yes	103
 **36**	Yes	103
 **37**	Yes	30
 **38**	Yes	30
 **39**	No	104

[a] R_1 = L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala. R_2 = L-Ala-γ-D-Glu-L-Dap-D-Ala-D-Ala. R_3 = L-Ala-γ-D-Glu-$CONHCH_3$. R_4 = L-Ala-γ-D-Gln.

4.3. Inhibition Kinetics of Ramoplanin

4.3.1. Transglycosylase Inhibition

Plots of reaction rate versus substrate concentration for inhibition of *E. coli* PBP1b, a prototypical bacterial transglycosylase, as well as *E. coli* MurG are shown in Figure 7a and b.[93,101] The plots for inhibition of the transglycosylase are sigmoidal, and inhibition is overcome at high substrate concentrations, consistent with a substrate-binding mechanism for inhibition of this enzyme. The absence of enzymatic activity in the first part of the curve implies that ramoplanin binds to Lipid II with a dissociation constant that is low relative to the substrate concentrations used. In addition, the substrate concentration at which the reaction rate increases suggests that ramoplanin binds to Lipid II with a stoichiometry of 2:1. For example, when the ramoplanin concentration is 6 μM, the reaction rate jumps when the substrate concentration exceeds 3 μM; when the ramoplanin concentration is 8 μM, the reaction rate jumps when the substrate concentration exceeds 4 μM. Thus, the inhibition kinetics reveal that ramoplanin binds with submicromolar affinity and a 2:1 ramoplanin:Lipid II stoichiometry. Assuming that ramoplanin acts by a pure equilibrium binding mechanism, a dissociation constant of 50 nM can be calculated from the kinetic data.[101]

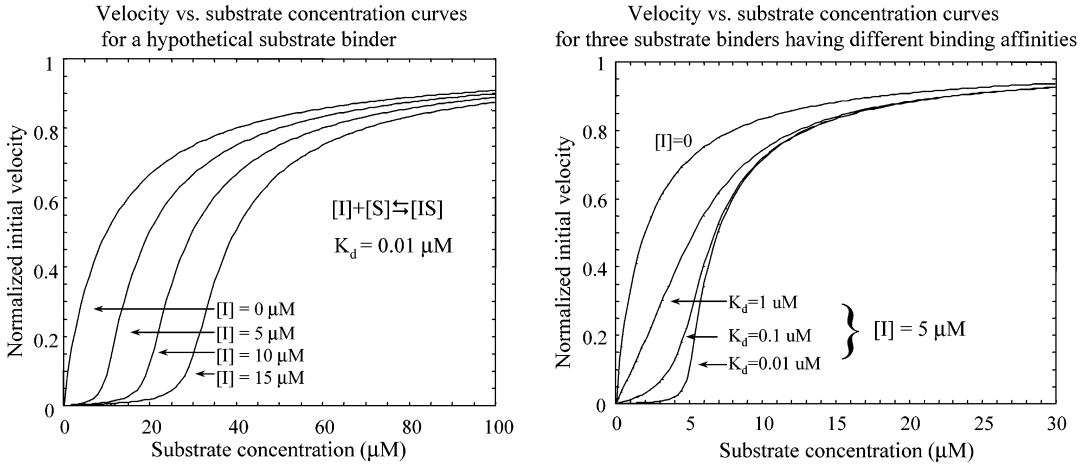


Figure 6. Calculated inhibition curves for hypothetical substrate binders. (a) Curves generated at four different inhibitor concentrations (0, 5, 10, and 15 mM) for a substrate binder that forms a 1:1 complex and has a K_d of 10 nM. (b) Curves generated at a single inhibitor concentration for three substrate binders that form 1:1 complexes and have K_ds of 1, 0.1, and 0.01 mM.

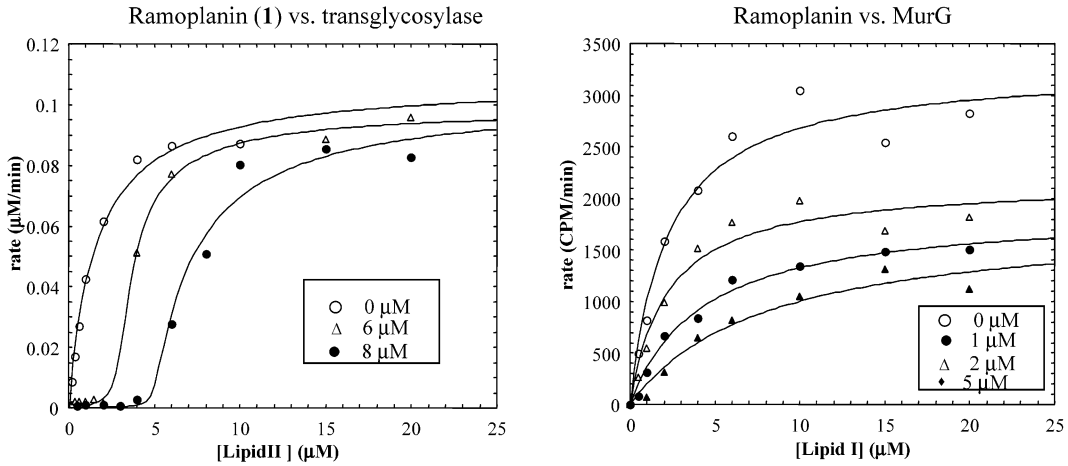


Figure 7. Inhibition kinetics of ramoplanin against transglycosylase PBP1b and MurG.

4.3.2. MurG Inhibition

The curves for MurG inhibition are different from those for transglycosylase inhibition in two respects (Figure 7).[93,101] First, inhibition cannot be overcome by increasing the substrate concentration. Second, the shapes of the curves are different. Whereas the curves for transglycosylase inhibition are strongly sigmoidal, those for MurG inhibition are not. In fact, only at the highest ramoplanin concentration is there any kinetic evidence that substrate binding may occur. This observation, combined with the fact that inhibition cannot be overcome, reveals that ramoplanin does not inhibit MurG simply by sequestering Lipid I. The kinetic data do not, however, rule out the possibility that ramoplanin forms a complex with Lipid I and that this complex inhibits MurG in a noncompetitive manner.

To determine whether Lipid I binding is required for inhibition of MurG by ramoplanin, alanine was attached to the Orn^4 and Orn^{10} amines to produce derivatives **24** and **25** (Figure 16). The Orn^{10} derivative was found to be incapable of binding to Lipid I, while the Orn^4 derivative retained the ability to bind Lipid I.[93] The compounds were tested for MurG inhibition, and both had IC_{50}s comparable to the parent compound, indicating that Lipid I binding is not required for MurG inhibition. On the basis of these results, it was proposed that ramoplanin inhibits *E. coli* MurG not by binding to Lipid I, as originally suggested by Somner and Reynolds,[69] but by binding directly to the enzyme. This hypothesis is supported by studies showing that fluorescent ramoplanin derivatives bind directly to *E. coli* MurG at concentrations comparable to the concentrations required to inhibit enzymatic activity.[93]

4.4. Evaluating the Proposed Cellular Targets of Ramoplanin

The aforementioned studies have revealed that ramoplanin inhibits MurG and the bacterial transglycosylase, PBP1b, by different mechanisms. These differences in the mode of inhibition are unexpected and, therefore, interesting; however, the results do not by themselves provide insight into which target is preferred in a cellular context. To draw conclusions as to the cellular target from the biochemical experiments, it is necessary to consider other issues as well. One issue that has already been raised is that of accessibility. Lipid II and the bacterial transglycosylases are located on the external surface of the bacterial membrane, where they are accessible to ramoplanin. MurG, however, is located on the cyto-

plasmic surface of the bacterial membrane, and ramoplanin would have to penetrate the membrane to reach this enzyme. Ramoplanin has a molecular weight of more than 2500 and an estimated water solubility of greater than 100 mg/mL. In the absence of a transport system, a molecule having these properties is unlikely to penetrate bacterial membranes efficiently. No experiments addressing whether ramoplanin accumulates inside bacterial cells have yet been reported, and the existence of a transport mechanism cannot be discounted.

Another issue that bears on the mechanism of action is that of the correspondence between the concentrations of antibiotic needed for substrate binding or enzyme inhibition relative to those needed to inhibit bacterial growth. As noted earlier, ramoplanin is a potent antibiotic that inhibits a range of Gram-positive bacterial strains at submicromolar concentrations. Ramoplanin binds to and inhibits *E. coli* MurG at concentrations at least 10-fold higher than these MICs. Unless ramoplanin inhibits MurG homologues from clinically relevant Gram-positive microorganisms at significantly different concentrations (or somehow becomes concentrated at the site of action), these low MICs cannot be explained by inhibition of MurG. In contrast, the kinetics for transglycosylase inhibition indicate that ramoplanin binds to Lipid II very tightly, with an estimated dissociation constant below 10^{-7} M.[101] Moreover, a qualitative comparison of ramoplanin binding to Lipid I and Lipid II analogues shows that the molecule binds to Lipid II better than to Lipid I (see below). Thus, a mechanism of action involving inhibition of enzymes that utilize Lipid II would be consistent with the experimental evidence. On the basis of both target accessibility and the correspondence between the results of biochemical and cell-based assays, a strong argument can now be made that ramoplanin inhibits bacterial transglycosylases rather than MurG in bacterial cells.

Recently, another set of experiments addressing the cellular target of ramoplanin was reported. Scientists at Genome Therapeutics constructed a *S. aureus* strain in which the expression of MurG is increased. Compounds that inhibit MurG directly should show an increase in MICs against this strain relative to typical *S. aureus* strains. The MIC of ramoplanin against this strain was found to be comparable to its MICs against other strains, implying that the MurG enzyme itself is not the cellular target of the antibiotic.[102]

5. Molecular Recognition by Ramoplanin

Brötz et al. provided the first direct evidence for an interaction between ramoplanin and a substrate involved in peptidoglycan biosynthesis when they showed that ramoplanin comigrates with Lipid II.[77] Subsequent work from members of the Walker lab has shown that ramoplanin inhibits the transglycosylation step of peptidoglycan biosynthesis by binding to Lipid II.[81,101] Thus, understanding how ramoplanin recognizes Lipid II is essential for understanding its mechanism of action. As described below, the recognition of Lipid II by ramoplanin has proven extremely challenging to study because ramoplanin does not form a discrete complex with Lipid II in vitro. Rather, it associates to form ordered fibrils that do not yield readily to structural analysis.[81]

5.1. Problem of Fibril Formation

The first attempts to characterize the interaction between ramoplanin and Lipid II were reported by Lo et al., who prepared a water-soluble analogue of Lipid II containing a citronellyl chain in order to probe the interaction of this molecule with ramoplanin.[81] Natural Lipid II contains a 55-carbon lipid chain, which causes it to aggregate extensively in water, and it was expected that the water-soluble analogue would facilitate structural analysis of the complex. To address this possibility, Lo et al. carried out an NMR titration of ramoplanin with the Lipid II analogue (**30**) (Figure 8).[81] Unexpectedly, the NMR resonances of ramoplanin disappeared upon addition of approximately 0.5 equiv of Lipid II analogue. A CD titration of ramoplanin with the Lipid II analogue revealed significant changes in the peptide backbone region of the antibiotic along with the appearance of a new band in the far UV region of the spectrum, implying a nonrandom orientation of the aromatic side chains in the complex (Figure 9). The presence of isosbestic points in spectra acquired at different Lipid II concentrations indicated a two state transi-

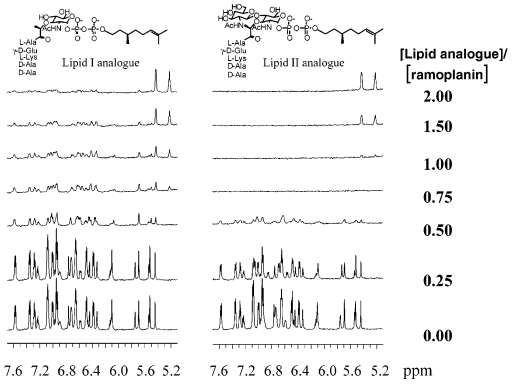


Figure 8. NMR titrations of ramoplanin with Lipid I and Lipid II analogues. Spectra show downfield region of ramoplanin in D_2O upon addition of increasing amounts of Lipid I/II analogues.

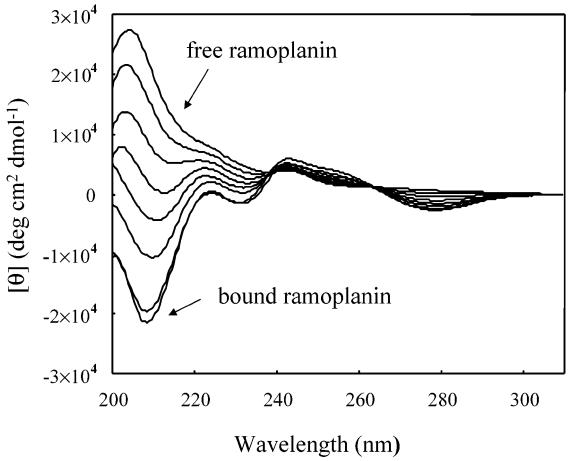


Figure 9. CD spectra of ramoplanin upon titration with Lipid II analogue.

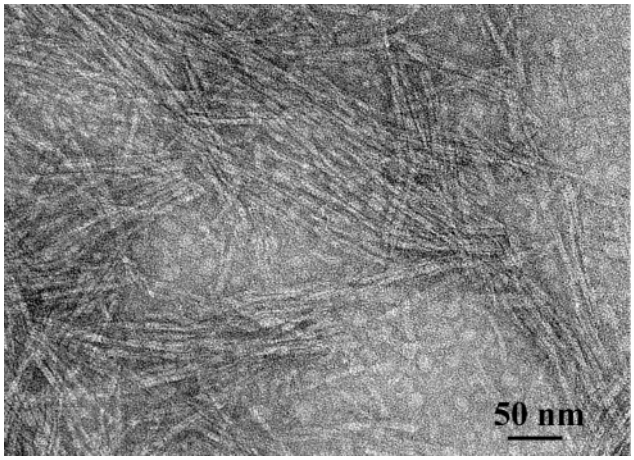


Figure 10. Transmission electron micrograph of ramoplanin with Lipid II analogue.

tion between free ramoplanin and the complexed species. These data, combined with the NMR results, suggested that ramoplanin forms an ordered assembly in the presence of the Lipid II analogue. Electron-microscopic analysis of the mixture of ramoplanin and the substrate analogue revealed, in fact, that the ramoplanin complexes assemble to form fibrils (Figure 10).[81,103] The formation of these fibrils explained the disappearing NMR resonances but raised questions about whether the ability of ramoplanin to self-assemble in the presence of substrate is in some way biologically relevant. This is a question that has not yet been answered.

The propensity of ramoplanin complexes to form fibrils in the presence of Lipid II greatly complicates structural analysis and makes it difficult to evaluate differences in the binding affinities of different substrates. Therefore, the relative contributions of the different parts of Lipid II to binding are not well understood. Lo and Cudic et al. evaluated the behavior of ramoplanin in the presence of different Lipid I analogues or fragments thereof using CD changes and fibril formation as a measure of binding. From these studies it is known that the diphosphate portion of the molecule is essential because the MurNAc monophosphate derivatives **31** and **39** do not interact with ramoplanin nor does the lipid monophosphate derivative **32** (Table 2).[30,81,103,104] However, lipid diphosphates such as farnesyl pyrophosphate **34** do interact, promoting the formation of ramoplanin fibrils.[103] It is also known that the GlcNAc sugar plays a role in binding of Lipid II because NMR titrations with Lipid I and Lipid II analogues show that ramoplanin binds Lipid II more tightly than it binds Lipid I (Figure 8). A comparison of the inhibition kinetics of transglycosylase and MurG also suggests that Lipid II is a better ligand for ramoplanin than Lipid I.[93,101] Finally, Cudic et al. also suggested that the peptide chain on the MurNAc sugar is essential for recognition based on studies showing that compound **33** (Table 2) does not interact with ramoplanin.[30] Lo reported, however, that compounds **34**, **35,** and **36** (Table 2) do interact with ramoplanin, with the binding of **35** and **36** qualitatively indistinguishable from **29**, which contains the intact peptide chain.[103] It is possible that the free lactate in **33** disfavors binding. In any event, the peptide chain does not appear to be essential for binding of lipid intermediates to ramoplanin. There is evidence, however, that the intact peptide chain plays a role in the affinity of UDP-MurNAc-pentapeptide for ramoplanin.[104]

5.2. Comparison of Substrate-Binding Affinities

A quantitative binding assay is required to understand the structural features that are important for substrate recognition by ramoplanin. In an effort to develop such an assay, Cudic et al. explored the behavior of a variety of ramoplanin complexes (i.e., ramoplanin with UDP-MurNAc pentapeptide and several Lipid I analogues) under different solution conditions and found that some complexes remain in solution even at relatively high concentrations (10^{-4} M) provided that 20% DMSO is added.[104] Therefore, it is possible to measure dissociation constants for the compounds that form these soluble complexes using NMR methods.[104] Cudic et al. measured dissociation constants for UDP-MurNAc-pentapeptide **37** and the corresponding dipeptide analogue **38** and found a 10-fold difference in affinity, indicating that the terminal L-Dap-D-Ala-D-Ala tripeptide plays a role in the binding of these substrate analogues to ramoplanin. Whether this is also true for Lipid II is not clear because there may be significant differences in how the UDP analogues and Lipid II bind to ramoplanin (see below).

To characterize the binding interface of the DMSO-solubilized ramoplanin:UDP-MurNAc pentapeptide complex, Cudic et al. analyzed NMR chemical shift changes and intermolecular NOEs. The data showed that ramoplanin binds to UDP-MurNAc-pentapeptide in a 1:1 ratio and that the majority of the contacts from ramoplanin to UDP-MurNAc-pentapeptide involve residues 2−8. Cudic et al. suggested that both ornithine 4 and ornithine 10 make stabilizing electrostatic contacts to the ligand, helping to orient it. They proposed that a contact from Orn^4 to the carboxylate of the terminal D-Ala-D-Ala dipeptide plays a role in discriminating the pentapeptide from the dipeptide. This NMR study was carried out based on the assumption that structural information on weakly binding peptidoglycan intermediates, which are not as susceptible to fibril formation as Lipid II, would be relevant to understanding how ramoplanin recognizes Lipid II, which is presumed to be the biologically relevant substrate. The validity of this assumption has been called into question by the kinetic results of Hu et al., showing that ramoplanin binds to Lipid II with a stoichiometry of 2:1.[101] A Job titration has confirmed ramoplanin binds to Lipid II in a 2:1 mode rather than the 1:1 mode reported for the UDP-MurNAc-pentapeptide complex (Figure 11). Therefore, UDP-MurNAc-pentapeptide may not be a good model system for Lipid II. There is clearly still a need for direct structural information on ramoplanin bound to Lipid II or a suitable analogue. It may be necessary to produce isotopically labeled ramoplanin and Lipid II analogues in order to obtain the required structural information.

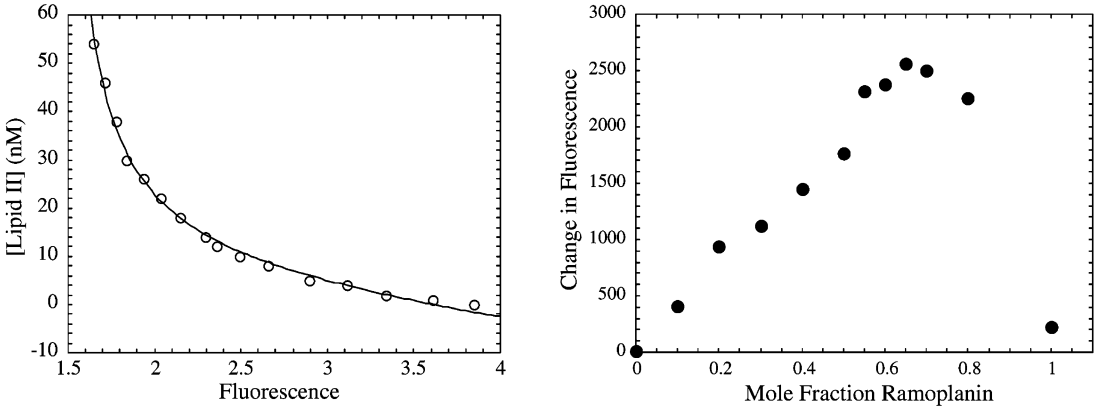


Figure 11. (a) Titration of a fluorescently labeled ramoplanin analogue (Orn4-fluorescein) with Lipid II. (b) Job titration of Orn^4-fluorescein ramoplanin with Lipid II.

5.3. Structural Studies on Ramoplanin and Ramoplanin Complexes

In the absence of direct structural information on the ramoplanin complex, models for how ramoplanin interacts with Lipid II must be pieced together based on the solution structure of free ramoplanin combined with available data on substrate recognition. The first solution structure of a ramoplanin factor was determined more than a decade ago by Williams and co-workers, who were studying an analogue of ramoplanin A2 called ramoplanose.[21] Ramoplanose is identical to ramoplanin A2 except that it contains a branched trisaccharide rather than a disaccharide moiety at position Hpg^{11}. In water, ramoplanose was found to have an antiparallel β-sheet structure with a reverse turn at L-Thr^8/L-Phe^9 and another turn in the vicinity of Gly^{14}-D-Ala^{16}. The chirality of the amino acids flanking L-Thr^8/L-Phe^9 alternates in a D,L,D pattern, with the result that the R groups on both sides of this turn are located on the same surface of the β-sheet. Williams and co-workers noted that the β-sheet curves upward slightly in order to alleviate the steric congestion between side chains on the same side of the molecule.

The first solution structure for ramoplanin A2 itself was reported by Kurz and Guba in 1996.[19] The structure of ramoplanin A2 was found to be similar in many respects to that reported for ramoplanose, as expected based on the structural similarities between the molecules. For example, like Williams and co-workers, Kurz and Guba observed that ramoplanin adopts an antiparallel β-stranded structure with turns in the vicinity of residues 8−9 and 14−16.[19,21] However, there were also significant differences between the two NMR structures, and the overall topologies were different. For example, whereas ramoplanose was reported to have a slight curvature, ramoplanin A2 was found be distinctly U-shaped, with amino acid side chains located at the two ends of the antiparallel sheet structure coming into contact. There were minor differences in the solution conditions under which the NMR data were acquired. Ramoplanose was studied in water, and ramoplanin was studied in a 90:10 water:DMSO mixture. In addition, the carbohydrate moieties on the two compounds were slightly different. However, the differences in the structures are probably related to the

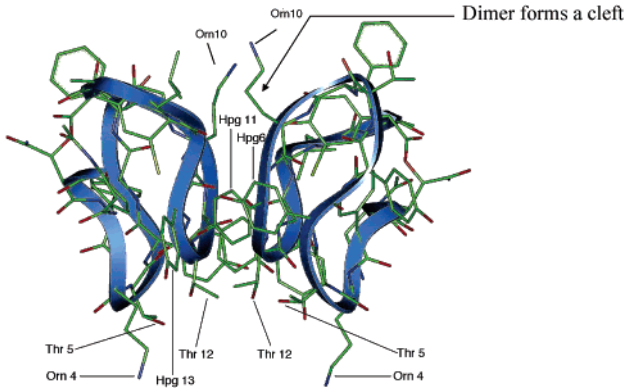


Figure 12. NMR structure of ramoplanin dimer in methanol.

fact that Kurz and Guba used many more NOE constraints in their calculations, including constraints between amino acids 9 and 17 at opposite ends of the molecule. Williams and co-workers noted that they were unable to achieve convergence due to limitations in the modeling packages unless the structural calculations were performed with a reduced set of distance constraints and atoms.[21] Subsequent NMR studies on ramoplanin A2 by Lo et al. and by Cudic et al. support the U-shaped topology observed by Kurz and Guba.[104,105]

The third NMR study of ramoplanin in the absence of substrate was reported by Lo et al., who observed that ramoplanin A2 exists as a mixture of monomer and dimer forms in slow exchange in methanol.[105] The structure of the dimer was solved in the hope that it might shed light on how ramoplanin binds to Lipid II or on how it self-associates or both. The ramoplanin dimer in methanol was found to consist of a C_2-symmetric antiparallel structure in which the two monomer units are held together by interactions between amino acids 10−14 (Figure 12). The structures of the monomer units themselves are very similar to the monomer structure reported by Kurz and Guba. The chirality of the residues in the β-strands spanning residues 10−14 alternates in a D,L,D,L,Gly pattern. Cyclic peptides consisting of alternating D,L residues are known to self-associate via a combination of hydrogen bonds and interactions between side chains aligned on the same side of the peptide backbone, and the interface between ramoplanin monomers is reminiscent of the interface

between these self-associating peptides.[106−108] The ramoplanin dimer contains a cleft that provides a possible binding site for Lipid II. An Orn[10] residue from each monomer flanks this cleft. The Orn[4] residues are presented on opposite side of the dimer. Derivatives of ramoplanin containing alanine at either Orn[4] or Orn[10] have been prepared and tested for biological activity and the ability to bind peptidoglycan intermediates.[93] The Orn[4]Ala derivative retained biological activity and the ability to bind Lipid I substrate analogues, but the Orn[10]Ala derivative was inactive and unable to bind Lipid I analogues. These results suggest that Orn[10] makes critical contacts to the substrate but Orn[4] does not. The results are consistent with a model in which Lipid II binds in a dimer cleft flanked by the two Orn[10] residues (Figure 12). However, confidence in the model will require additional experiments, particularly since results that might not be consistent with this model have also been reported (see section 8). Work on various synthetic and semi-synthetic derivatives of ramoplanin that shed more light on the Lipid II recognition event and on the structural requirements for biological activity is discussed in the following section.

In summary, three different solution structures of ramoplanin factors have now been reported.[19,21,105] Two of the structures are of monomers that differ in overall topology,[19,21] and one is of a dimer comprised of two molecules that closely resemble one of the reported monomers.[105] The two monomer structures were determined in largely aqueous media, while the dimer structure was determined in methanol. Ramoplanin is believed to act at a membrane interface, and Lo et al. suggested that methanol may mimic the conditions under which ramoplanin acts better than water.[105] Whether this is so remains to be established. However, it is worth noting that the dimer structure is consistent with other evidence showing that ramoplanin complexes Lipid II with a stoichiometry of 2:1. Structural studies of ramoplanin in the presence of mono- or bilayers could provide more insight into the bioactive conformation of ramoplanin. At this point, however, it is evident that ramoplanin can exist as a monomer, dimer, and, in the presence of Lipid II, higher order assembly with a 2:1 stoichiometry.[101] The changeable nature of ramoplanin is remarkable.

6. Total Synthesis of Ramoplanin and Key Analogues

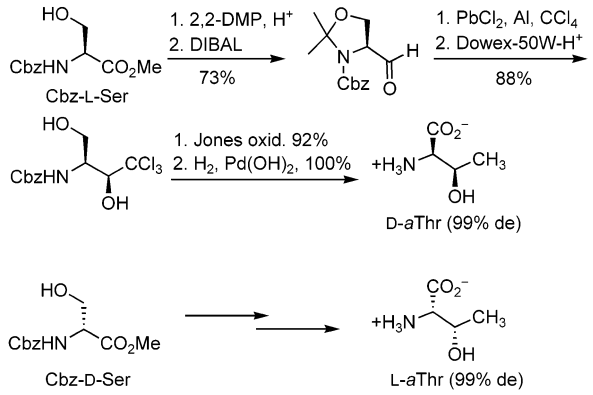
6.1. Preparation of Key Amino Acids

Ramoplanin consists of 17 amino acids of which seven possess the nonproteinogenic D-configuration and 12 possess nonstandard side chains. Of these, the β-hydroxy α-amino acids (D/L-*allo*-threonines and L-*threo*-β-hydroxyasparagine) are the most abundant components of the ramoplanins whose preparations constitute significant elements of any projected total synthesis. Since β-hydroxy α-amino acids are important components of many complex natural products, a number of approaches have been reported for their synthesis. These can be classified as (a) aldol-type additions of anions or enolates possessing chiral auxiliaries including bis-lactams,[114] oxazolidinones,[115,116] oxazinones,[117] and imidazolidinones,[118] (b) the use of glycine Schiff bases,[119] (c) asymmetric epoxidation,[120,121] dihydroxylation,[122,123] and aminohydroxylation of olefins,[124,125] (d) enantioselective hydrogenation,[126−128] (e) preparation from naturally occurring amino acids,[129−133] and (f) enzymatic synthesis.[134−137] Summarized below are the approaches implemented in efforts leading to the total synthesis of ramoplanin.

6.1.1. aThr (allo-Threonines)

Despite their biological significance, the availability of *allo*-threonines, nonproteinogenic amino acids, is limited. Consequently, various methods for their preparation have been developed. Of these, derivatization of a readily available natural amino acid is the simplest. For efforts directed at ramoplanin, the approach described by Beaulieu where D- or L-serine is converted to L- or D-γ,γ,γ-trichloro-*allo*-threonine, followed by catalytic hydrogenolysis to provide diasteromerically pure L- or D-*allo*-threonine, was employed (Scheme 1).[132]

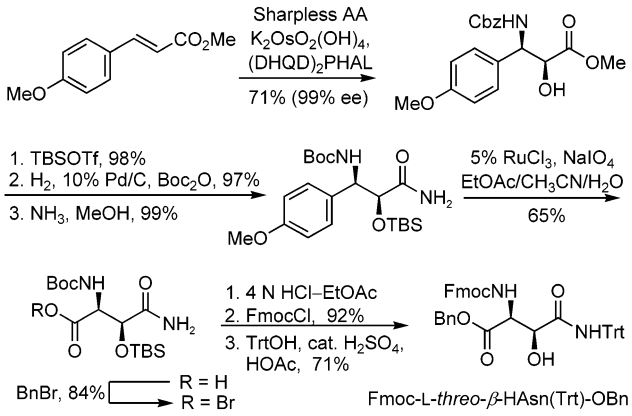
Scheme 1



6.1.2. HAsn (L-threo-β-Hydroxyasparagine)

In the ramoplanins the hydroxyl group of L-*threo*-β-HAsn[2] forms a labile ester bond with the C-terminus of 3-chloro-4-hydroxyphenylglycine (Chp[17]), and the preparation of this unnatural amino acid, L-*threo*-β-HAsn, constitutes one of the key elements in the total synthesis of the ramoplanin aglycons. The isomers of β-HAsn were originally isolated from human urine[138] and had been synthesized as a racemic mixture that was separated by resolution.[139,140] Although there are reports of the enantioselective synthesis of the isomers of L-β-hydroxyaspartic acid (L-β-HAsp)[141−144] and L-*erythro*-β-HAsn,[145] an asymmetric synthesis of L-*threo*-β-HAsn had not been disclosed. In efforts leading to the total synthesis of the ramoplanin aglycons, Boger et al. disclosed the preparation of L-*threo*-β-HAsn enlisting the Sharpless asymmetric aminohydroxylation (AA) of methyl 4-methoxycinnamate to provide the corresponding amino alcohol in 71% yield and >99% ee (Scheme 2).[109,146] Sequential protection of the alcohol (TBDMSOTf), one-pot *N*-Cbz/Boc exchange, and direct aminolysis of the methyl ester afforded the corresponding amide in 94% overall yield with no

Scheme 2



evidence of epimerization. The aryl ring was oxidatively cleaved with RuO_4 to release the carboxylic acid, which was subsequently protected to provide the benzyl ester in 65% and 84% yields, respectively. Treatment of Boc-L-*threo*-HAsn(OTBS)-OBn with 4 N HCl−EtOAc led to the single-step removal of the Boc and TBS protecting groups, and the resulting amine was Fmoc protected (92%, 2 steps). A final treatment with trityl alcohol and acetic anhydride under acidic conditions provided Fmoc-L-*threo*-β-HAsn(Trt)-OBn in 71% yield and suitably protected for direct incorporation into the following synthetic efforts.

6.2. Total Synthesis of the Ramoplanin A2 and Ramoplanose Aglycon

The first, and to date only, total synthesis of the ramoplanin A2 and ramoplanose aglycon was disclosed in 2001.[109,147,148] Three key subunits composed of residues 3−9 (heptapeptide **53**), the pentadepsipeptide **64** (residues 1, 2, and 15−17), and pentapeptide **72** (residues 10−14) were sequentially coupled and cyclized in a solution-phase, convergent approach to the 49-membered depsipeptide core of **1**−**3** (Figure 13).

The indicated coupling sites were chosen to maximize the convergency of the synthesis including that of the three subunits, to minimize the use of protecting groups, to prevent late-stage opportunities for racemization of carboxylate-activated phenylglycine-derived residues, and to enlist β-sheet preorganization of an acyclic macrocyclization substrate for ring closure. Macrocyclization at the Phe^9-D-Orn^{10} site proved unusually successful and represents a site found at the corner of a β-turn at the end of the H-bonded antiparallel β-strands of ramoplanin (Figure 14). Consequently, closure at this site may benefit from both β-sheet preorganization of the substrate[149] as well as closure at a D-amine terminus.[150,151] A second alternative cyclization site at Gly^{14}-Leu^{15} site was also successfully examined and lies within a small flexible loop at the other end of the H-bonded antiparallel β-strands. In addition to potentially benefiting from β-sheet preorganization of the macrocyclization substrate, it represents closure at a nonhindered site incapable of racemization. Also key to the implementation of the approach was the judicious choice of the Orn^4/Orn^{10} SES protection and the Asn^1

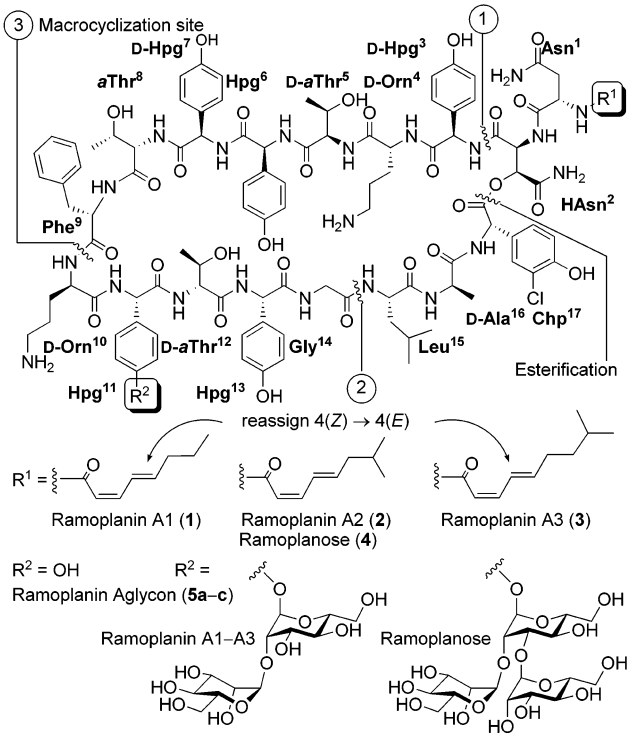
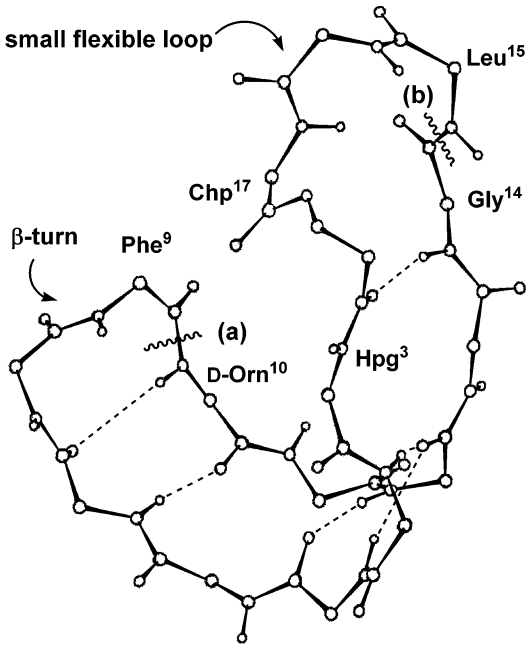


Figure 13. Key disconnections for synthesis of the ramoplanins.

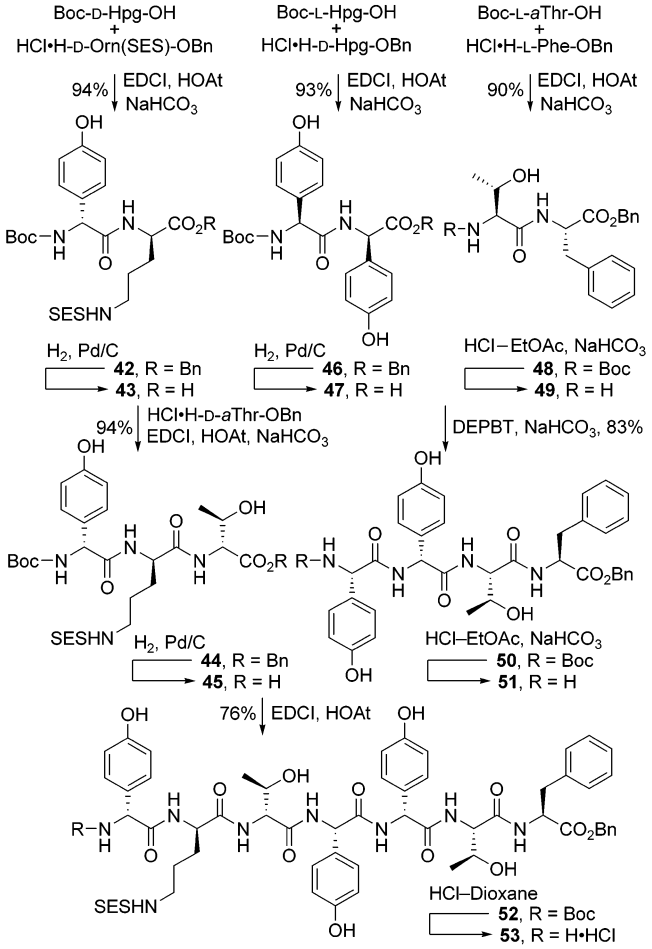


- Rigid β-Sheet conformation stabilized by intramolecular H-bonding and a cluster of aromatic side chains
- Macrolactamizations that may benefit from β-sheet preorganization of substrates
  - (a) Cyclization at L-Phe^9–D-Orn^{10}:
    - closure at the corner of β-turn with a D-amine
  - (b) Cyclization at Gly^{14}–Leu^{15}:
    - No racemization with glycine activation

Figure 14. Macrolactamization sites.

Fmoc protection providing orthogonal protecting groups stable to Boc, Cbz, and benzyl ester deprotections yet capable of sequential and selective removal in the presence of the labile depsipeptide ester.

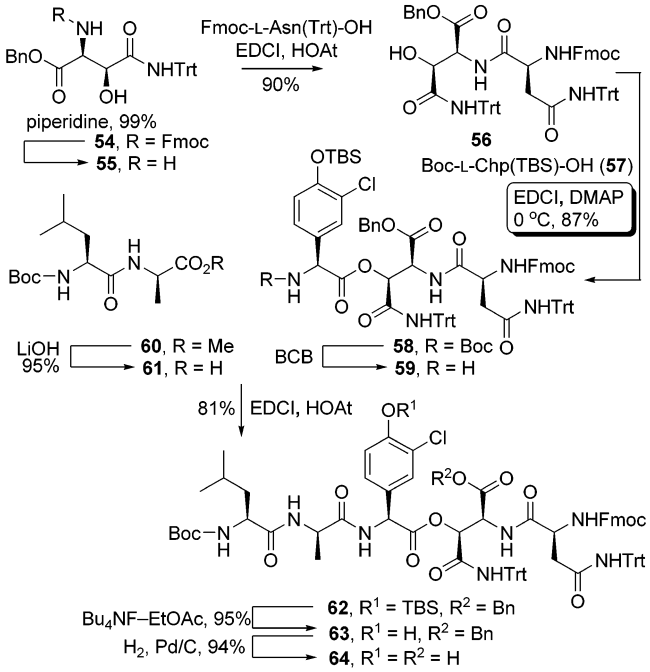
Scheme 3



Heptapeptide **53**, the first of the three key subunits which contains all but Orn[10] of the putative Hpg[3]-Orn[10] recognition sequence,[30] was assembled as shown in Scheme 3 from the tripeptide **45** and tetrapeptide **51** followed by Boc deprotection. This coupling cleanly provided **52** without deliberate protection of the free alcohols and with no evidence of competitive β-elimination or trace racemization. Tripeptide **45** was prepared by benzyl ester deprotection of **44** obtained by coupling D-*a*Thr-OBn[132,152] with the dipeptide **43**. In turn, **43** was obtained by benzyl ester deprotection of **42** derived from coupling Boc-D-Hpg-OH and D-Orn(SES)-OBn.[153,154] Tetrapeptide **51** was prepared from the dipeptides **46** and **48** secured by the coupling of Boc-L-Hpg-OH with D-Hpg-OBn and Boc-L-*a*Thr-OH[132,152] with L-Phe-OBn, respectively. Notably, benzyl ester hydrogenolysis of **46** followed by coupling with **49** activated by DEPBT[155,156] was accomplished with no detectable racemization of the sensitive D-Hpg residue (>99% de) or β-elimination of the hindered L-*a*Thr, whereas alternative coupling reagents gave lower conversions accompanied by substantial epimerization.

Synthesis of the second key subunit, depsipentapeptide **64** which contains the labile backbone ester, is summarized in Scheme 4. Fmoc deprotection of Fmoc-L-*threo*-β-HAsn(Trt)-OBn (**54**, synthesis described in section 6.1.2) and coupling of **55** with Fmoc-L-Asn(Trt)-OH provided **56**. The key esterification of **56** with **57**[157] required activation with EDCI

Scheme 4

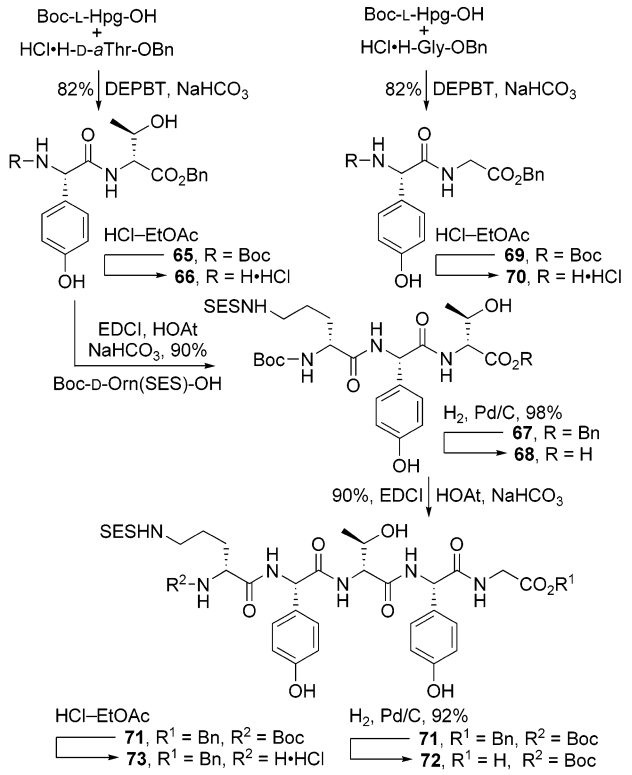


conducted in the presence of DMAP[158,159] catalyst. A wide range of alternative esterification protocols were examined, but these and EDCI- or DCC-promoted couplings in the absence of DMAP provided no or lower conversions and lower diastereomeric ratios or suffered competitive β-elimination. Even the EDCI–DMAP, or a less satisfactory DCC–DMAP,[158,159] reaction required carefully controlled reaction conditions with higher reaction temperatures (25 vs 0 °C), longer reaction times, or more DMAP (0.5–2 equiv vs 0.15–0.2 equiv) offering less satisfactory conversions. Boc removal,[160] coupling with **61**, buffered TBS deprotection,[161,162] and benzyl ester hydrogenolysis provided **64**. Efforts to shorten this sequence by first coupling **61** with L-Chp(OTBS)-OBn followed by benzyl ester deprotection and esterification with **57** were not successful, and an unbuffered Bu_4NF deprotection of **62** (no HOAc) led to competitive epimerization of the Chp α-aminoester center.

The preparation of the final subunit **72** is detailed in Scheme 5. D-*a*Thr-OBn[132,152] was coupled with Boc-L-Hpg-OH to give dipeptide **65** devoid of diastereomeric contaminants. Boc deprotection and coupling of **66** with Boc-D-Orn(SES)-OH provided tripeptide **67**. Benzyl ester deprotection and coupling of **68** with **70** gave pentapeptide **71**. In turn, the dipeptide **70** was obtained in two steps by coupling Boc-L-Hpg-OH and Gly-OBn hydrochloride to give dipeptide **69**, followed by Boc deprotection (HCl–EtOAc). Notably, C-terminus protection of D-*a*Thr as its benzyl ester in **67** and elsewhere throughout the synthesis permitted hydrogenolysis deprotection, avoiding base-catalyzed β-elimination. Similarly, coupling each sensitive Hpg-OH residue in **72**, like that throughout the synthesis, conducted upon activation with DEPBT[156] minimized epimerization especially when coupled with a hindered and sensitive *a*Thr amine terminus.

Assembly of three key fragments and macrocyclization at the Phe[9]-Orn[10] site are detailed in

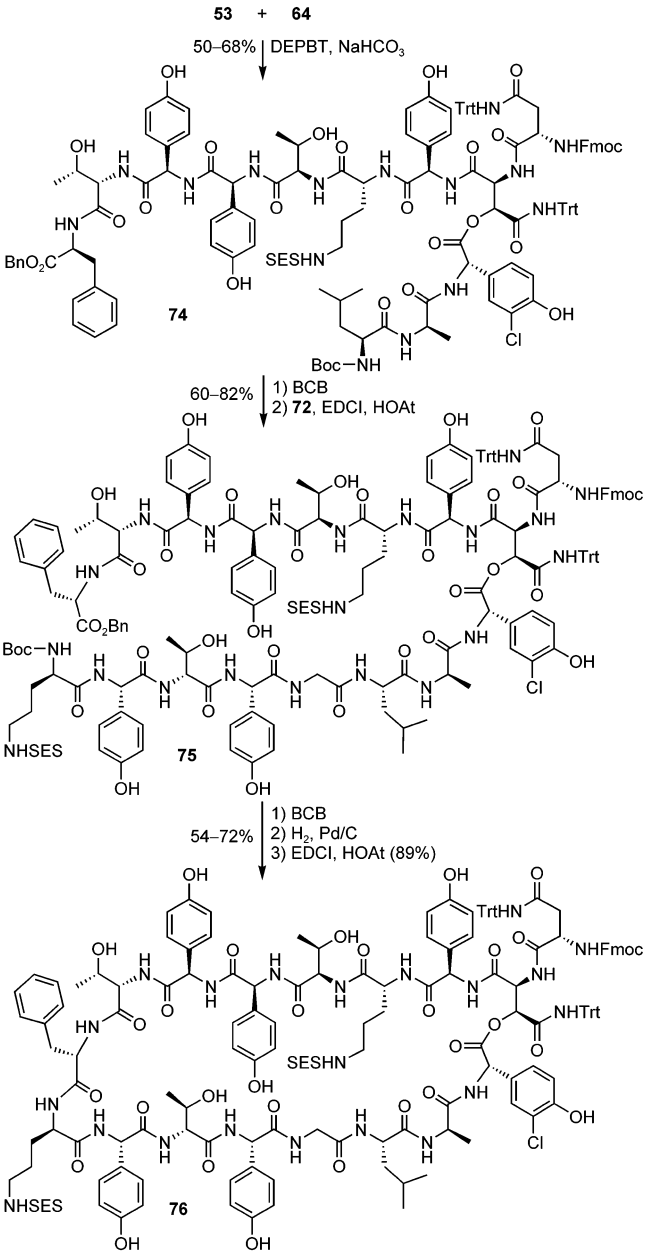
Scheme 5



Scheme 6. The coupling of subunits **53** and **64** proved to be the most challenging step of the synthesis. Carboxylate activation of **64** typically resulted in preferential β-elimination of the acyloxy substituent. This can be attributed to the combination of a superb leaving group, the hindered nature of the activated carboxylate resulting from its α,β,β-trisubstitution and the large protecting groups (trityl and Fmoc), and the enhanced α-carbon acidity of the activated carboxylate derived from **64** resulting from the use of a *N*-acyl versus carbamate derivative. Only DEPBT[156] promoted the coupling to provide **74** in superb yields with *no* competitive β-elimination, whereas all other alternative coupling reagents and conditions surveyed provided predominantly β-elimination products. Boc removal was accomplished under mild conditions[160] that preserved the trityl protecting groups. Coupling of the crude free amine with **72** provided the key acyclic depsipeptide **75**. Successive Boc removal, benzyl ester hydrogenolysis, and macrocyclization afforded the cyclic depsipeptide core **76**. Presumably, β-sheet preorganization of the cyclization substrate[149] and closure at a D-amine terminus[150,151] at the corner of a β-turn contributes to the superb conversions for closure of the 49-membered ring (89% when conducted with purified amino acid).

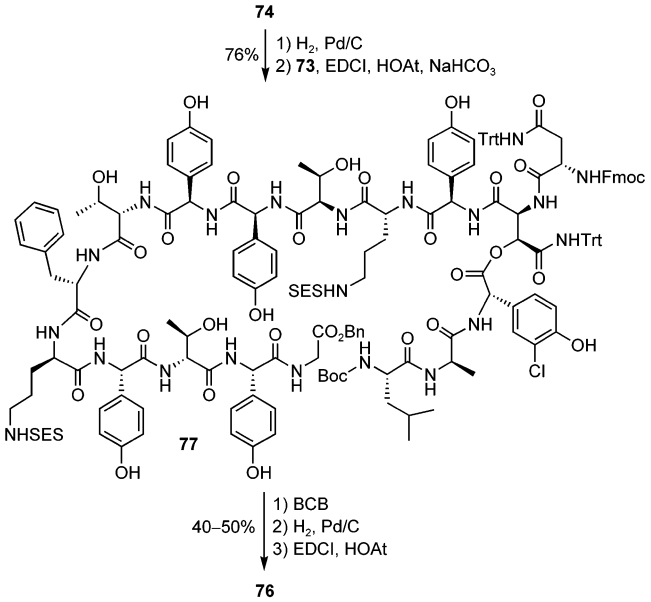
The alternative Gly^{14}-Leu^{15} site was also examined for the closure of the linear peptide (Scheme 7). This approach would be beneficial for the preparation of analogues of the sensitive depsipeptide region of the natural products enlisting an alternative coupling order of first assembling Hpg^{3}-Gly^{14} as a common precursor, followed by coupling with modified depsipentapeptide subunits, and a final key macrocyclization at the Gly^{14}-Leu^{15} site. It constitutes closure at

Scheme 6

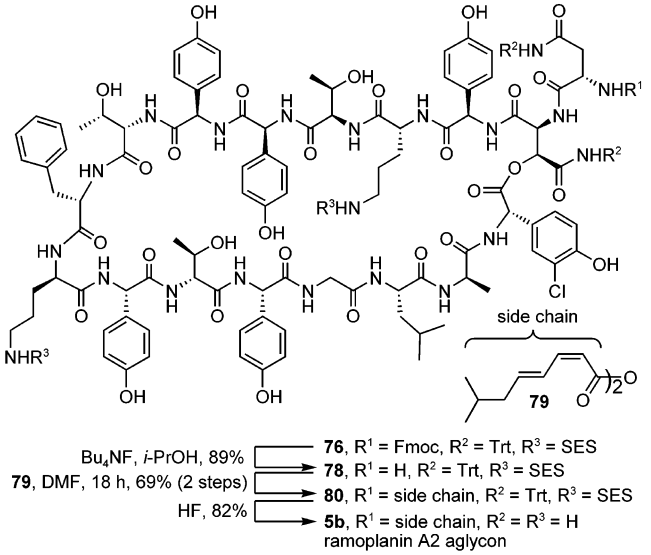


the other end of the H-bonded antiparallel β-strands at a site within a small flexible loop and should benefit from β-sheet preorganization of the cyclization substrate like closure at the Phe^{9}-Orn^{10} site. Unlike the Phe^{9}-Orn^{10} site it does not benefit from closure at a D-amine terminus, although the carboxylate terminus now lacks a L-amino acid side chain that may decelerate such ring closures. It also precludes competitive racemization of the activated carboxylate. As such, and based on these criteria alone, the two closures might be expected to perform comparably. The distinction between the two sites rests with Phe^{9}-Orn^{10} lying at the corner of a conformationally defined β-turn adjacent to the H-bonded antiparallel β-strands whereas the Gly^{14}-Leu^{15} site is embedded in what appears to be a conformationally more flexible region of the molecule. Benzyl ester deprotection of **74** followed by coupling with **73** provided **77**. Boc deprotection, benzyl ester deprotection, followed by macrocyclization provided **76** in good con-

Scheme 7



Scheme 8



versions (40−50%). This preliminary observation, which was not subjected to optimization efforts, suggests that closure at the Gly[14]-Leu[15] site, while perfectly acceptable, may not be as facile as closure at the Phe[9]-Orn[10] site.

Fmoc removal of **76** under specially developed conditions (8 equiv of Bu_4NF, 10 equiv of *i*-PrOH, DMF, 25 °C, 1 h) that do not promote competitive Asn[2] β-elimination followed by side-chain acylation of crude free amine **78** with anhydride **79** provided **80** in excellent overall conversions, Scheme 8. A single-step HF deprotection of the trityl and SES groups[163,164] or sequential trityl and SES deprotection provided the ramoplanin A2 and ramoplanose aglycon **5b**. Notably, the Fmoc deprotection required the development of a new method of Fmoc cleavage that preserves the sensitive depsipeptide ester and the Orn[4] and Orn[10] SES deprotections upon treatment with HF enlisted conditions first introduced and developed to avoid a competitive depsipeptide cleavage under typical strongly basic conditions.[163,164]

6.3. Total Synthesis and Structure of the Ramoplanin A1 and A3 Aglycons

The structure of ramoplanins (**1**−**3**) differs only in the acyl group attached to the Asn[1] N-terminus.[15,17,18,165] Initially the stereochemistry of the two double bonds in the three different acyl groups was assigned as *cis*−*cis*.[18] Three years after its structural elucidation, Williams disclosed the structure of ramoplanose (**4**) whose composition was identical to ramoplanin A2 with the exception of the branched mannose trisaccharide attached at Hpg[11] and the stereochemistry of the lipid side chain (*cis,trans*- vs *cis,cis*-7-methyloctadi-2,4-enoic acid).[21] Soon thereafter, the stereochemistry of the 7-methyloctadi-2,4-enoic acid side chain of ramoplanin A2 was also revised to *cis*−*trans* by Kurz et al. in studies that also served to provide a solution-phase conformation of the natural product.[19] The synthesis of the ramoplanin A2 aglycon detailed above confirmed this revised structure of ramoplanin A2.[109,147,148] Key to the strategic planning of the approach was the introduction of the lipid side chain onto the fully functionalized cyclic depsipeptide core, thereby potentially providing direct access to all natural aglycons from a common, late-stage intermediate. In recent studies this has been implemented for the total synthesis of the ramoplanin A1 and A3 aglycons, which confirmed an expected analogous structural revision of the lipid side-chain stereochemistry of ramoplanins A1 and A3.[20]

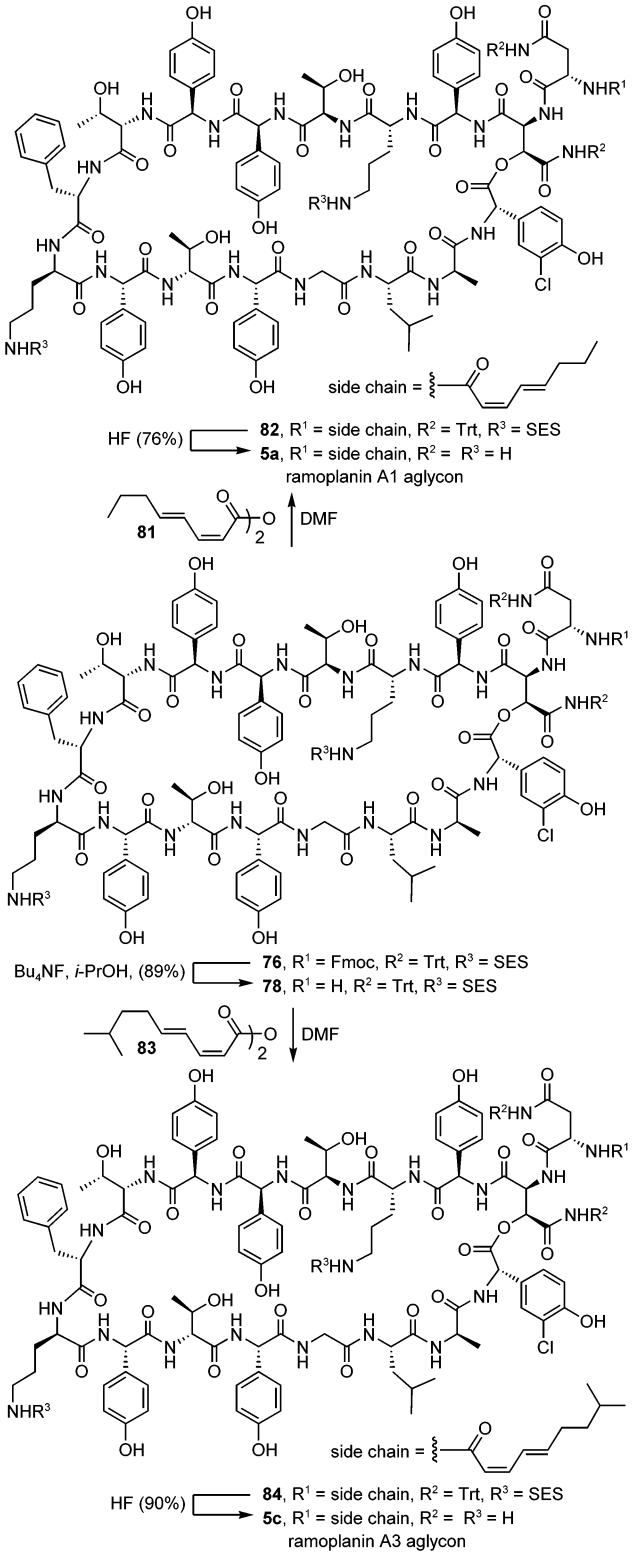
Removal of the Fmoc protecting group from **76** (Bu_4NF, *i*-PrOH) afforded the free amine, the advanced synthetic intermediate prepared en route to the ramoplanin A2 aglycon (Scheme 9).[20,109,148] Subsequent treatment of the resulting free amine with the anhydrides **81/83** provided the protected aglycons **82/84**. Global deprotection using HF furnished the ramoplanin A1 and A3 aglycons (**5a/5c**).

For comparison, the authentic ramoplanin A1 and A3 aglycons were obtained from the natural ramoplanin complex by deglycosidation (HF) followed by HPLC purification.[166] The ^{1}H NMR spectroscopic data of the synthetic ramoplanin A1 and A3 aglycons (**5a/5c**) were in complete agreement with the authentic compounds. Although the H_β and H_γ proton signals of the lipid side chains are not clearly resolved in the ^{1}H NMR spectra due to partial coincidence with other resonances, $J_{\alpha\beta}$ and $J_{\gamma\delta}$ can be measured directly from the remaining two olefin proton signals, H_α and H_δ, in the spectra. Not only are their values very similar in ramoplanin A1−A3 (Figure 15), but the coupling constants of 11.3−11.8 Hz and 14.9−15.4 Hz for $J_{\alpha\beta}$ and $J_{\gamma\delta}$, respectively, define a cis stereochemistry for the C_α−C_β double bonds and a revised trans stereochemistry for C_γ−C_δ double bonds.

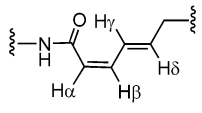
6.4. [*N*-Acetyl-Asn[1]]ramoplanin Aglycon

[*N*-Acetyl-Asn[1]]ramoplanin aglycon (**13**) was prepared and assessed in which the variable Asn[1] lipid *N*-acyl group was replaced with a minimal *N*-acetyl group. Semisynthetic modifications of the natural products and their aglycons have established that removal of the side-chain unsaturation (hydro-

Scheme 9



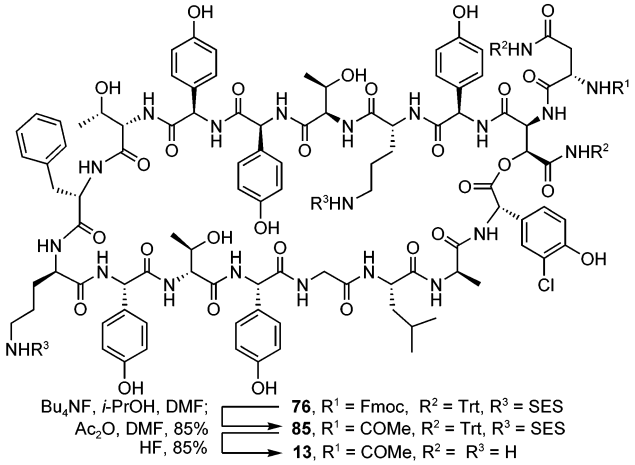
genation) appears to have a minimal effect,[167] but the impact of the lipid side chain presence and its potential role was not known. This was addressed with the total synthesis of **13** as shown in Scheme 10.[110] Removal of the Fmoc protecting group from **76**[109,110,148] (Bu_4NF, *i*-PrOH), the advanced synthetic intermediate we prepared en route to the ramoplanin aglycon, followed by treatment with Ac_2O provided the protected *N*-acetyl derivative **85**. Global deprotection using HF furnished **13**.



Compound	$J_{\alpha\beta}$	$J_{\gamma\delta}$
A1 Aglycon	11.8 Hz	14.9 Hz
A2 Aglycon	11.4 Hz	15.3 Hz
A3 Aglycon	11.3 Hz	15.4 Hz

Figure 15. Diagnostic coupling constants.

Scheme 10



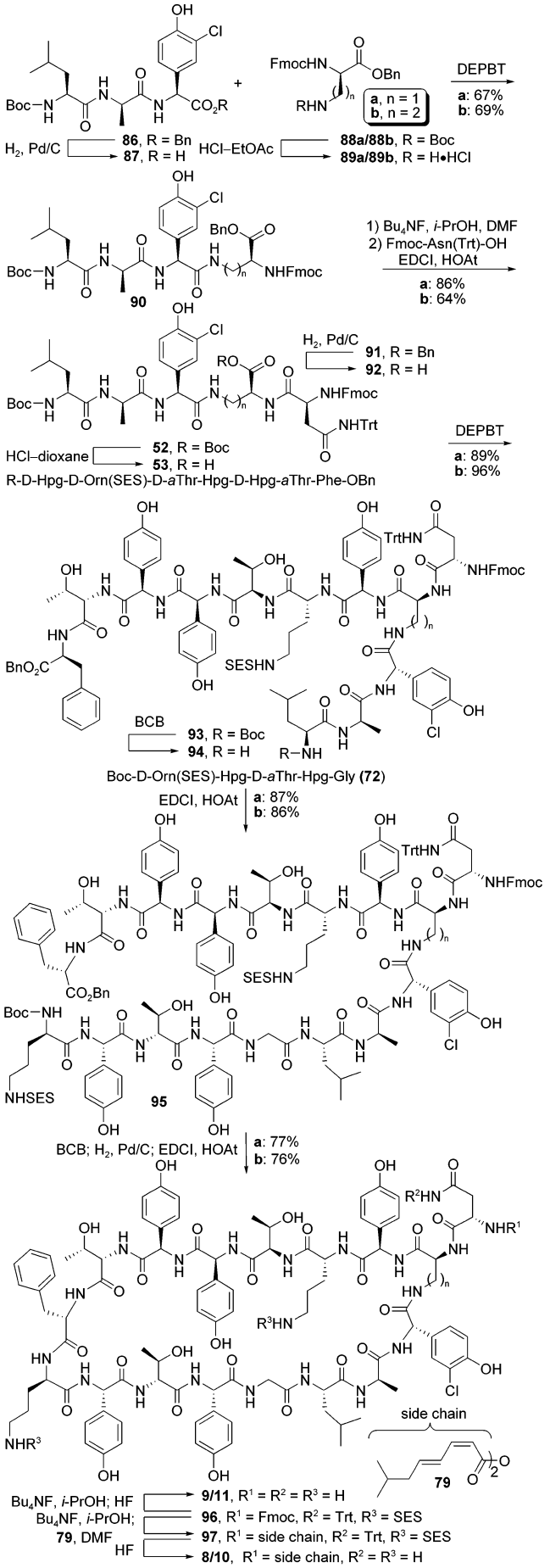
6.5. Total Synthesis of Ramoplanin Amide Analogues

Recently, Boger et al. described the total synthesis of two key analogues of ramoplanin which entailed the replacement of $HAsn^2$ with L-2,3-diaminopropionic acid (Dap) and L-2,4-diaminobutyric acid (Dab).[110] These deep-seated modifications provide a new generation of stable ramoplanin analogues incorporating a hydrolytically stable amide bond (vs ester) between residues 17 and 2.

Not only is the $HAsn^2$ residue the most lengthy to secure,[146] but its incorporation into and maintenance throughout the late stages of synthesis constitutes the most difficult challenge limiting the ease of a total synthesis of ramoplanin.[109,147,148] More significantly, the depsipeptide ester is the most fragile linkage in the molecule and is central to the instability of the natural products. Williams described the mild hydrolysis of ramoplanose providing the inactive linear peptide.[149] McCafferty and co-workers reported the time-dependent depsipeptide cleavage of ramoplanin A2 aglycon in acidic solutions,[104] and Boger and coworkers described depsipeptide cleavage of ramoplanin A2 or its aglycon that occurs rapidly even under mild basic conditions (1% Et_3N-H_2O, 25 °C, 40% hydrolysis in 2 min).[109,147,148] Since it lies at the end of the antiparallel β-sheet with the ester adopting a transoid carbonyl-eclipsed conformation analogous to that of an amide and forms the link to the short flexible loop (residue 15−17, see Figure 14),[19] ester replacement with an amide with incorporation of L-Dap or L-Dab to provide **8** and **10** was expected to have a minimal impact on the ramoplanin conformation but would preclude hydrolysis (**8** and **10**) and significantly reduce (**8**) or preclude (**10**) β-elimination-derived cleavage. Both would simplify the synthetic challenges associated with assembling a library of ramoplanin analogues required to probe each residue and would be expected to improve in vivo stability.

The modified pentapeptide subunits **92a** and **92b**, incorporating the Dap^2 and Dab^2 residues in place

Scheme 11



of HAsn[2], were prepared from tripeptide **86** (Scheme 11), which in turn was obtained by coupling Boc-Leu-D-Ala-OH[109,148] with Chp-OBn[109,148] followed by deprotection of the benzyl ester. Fmoc-Dap-OBn (**89a**) and Fmoc-Dab-OBn (**89b**), obtained from Fmoc-Dap(Boc)-OH/Fmoc-Dab(Boc)-OH, were coupled with **87** using DEPBT to give a single diastereomer of each tetrapeptide **90a** and **90b**. Fmoc removal upon treatment with the Bu_4NF and *i*-PrOH,[109,148] coupling with Fmoc-Asn(Trt)-OH, and benzyl ester hydrogenolysis provided **92a** and **92b**.

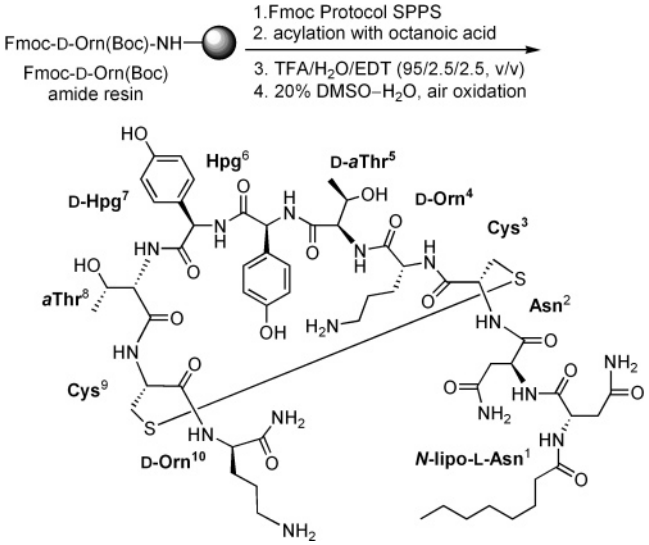
The convergent assembly of the modified pentapeptide subunits into the linear precursors for macrocyclization highlights a strategic advantage of the solution-phase modular synthesis developed for the ramoplanin synthesis. DEPBT coupling of **92a** and **92b** with heptapeptide **53**[109,148] provided **93a/93b**. Boc removal was accomplished under conditions that preserve the trityl protecting group, and the crude amines **94a** and **94b** were coupled with pentapeptide **72**[109,148] to yield the macrocyclization substrates **95a** and **95b**. Successive Boc removal, benzyl ester hydrogenolysis, and macrocyclization afforded the cyclic peptide cores **96a** and **96b**. Fmoc removal (Bu_4NF and *i*-PrOH),[109,148] Asn[1] acyl side-chain introduction, and global deprotection of the trityl and SES groups upon HF treatment provided the key amide analogues **8** and **10**.

The amide analogues lacking the Asn[1] side chain, **9** and **11**, were also prepared from **96a** and **96b** by successive Fmoc removal and HF deprotection of the trityl and SES groups.

6.6. Solid-Phase Synthesis of a Simplified Analogue

Although a total synthesis of ramoplanin using solid-phase chemistry has not yet been reported, a simplified analogue has been assembled. McCafferty and co-workers[30] synthesized a cyclic peptide, derived from the putative ramoplanin peptidoglycan recognition sequence, N^{α}-octanoyl-Asn[1]-Asn[2]-c[Cys[3]-D-Orn[4]-D-*a*Thr[5]-Hpg[6]-D-Hpg[7]-*a*Thr[8]-Cys[9]]-D-Orn[10]-NH_2, by solid-phase peptide synthesis of the linear peptide,

Scheme 12



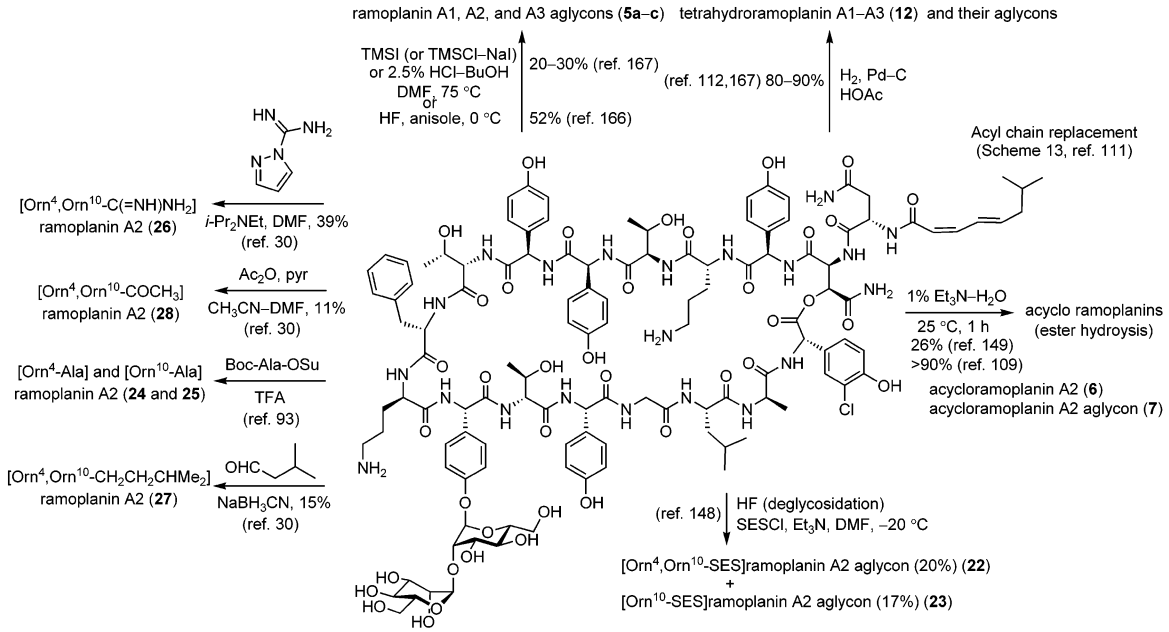


Figure 16. Degradation and derivatization of the ramoplanins.

Asp[1] N-acylation, resin cleavage, and oxidative intramolecular cyclization (Scheme 12).

7. Degradation and Semisynthetic Studies

In efforts to examine the role of structural features and functional groups in ramoplanin, accessible moieties have been chemically deleted, masked, or modified in the naturally occurring structures by several groups. Figure 16 summarizes the semisynthetic analogues examined to date and conditions used for their synthesis.

7.1. Ramoplanin Aglycons

In initial studies treatment of the ramoplanin complex with either (1) TMSI or TMSCl/NaI followed by hydrolysis or (2) HCl in anhydrous BuOH provided the deglycosylated derivatives of ramoplanin in 20−30% yield.[167] A recent improved procedure relied on reaction of the ramoplanin complex with anhydrous HF.[166] Reverse-phase HPLC purification of the crude mixture which serves to separate the A1−A3 aglycons gave pure A1 (3%, **5a**), A2 (46%, **5b**), and A3 (3%, **5c**).

7.2. Depsipeptide Hydrolysis

The most fragile linkage in the ramoplanins, the depsipeptide ester formed between HAsn[2] and Chp[17], can be hydrolyzed under mildly basic conditions to afford acyclic ramoplanin derivatives.[109,149] Williams first reported this hydrolysis with ramoplanose (1% Et_3N-H_2O, 25 °C, 1 h), and more recently the ease of hydrolysis was examined by Boger and co-workers in the course of synthetic studies. Treatment of either ramoplanin A2 or the ramoplanin A2 aglycon under these conditions resulted in rapid hydrolysis of the depsipeptide ester to cleanly provide the linear acyclic derivatives **6** and **7** (>90% isolated yield, 20 min; 80% conversion, 5 min; 30% conversion, 1 min) with little or no difference in the rates of reaction. Significantly, Williams and co-workers observed that the corresponding acyclic ramoplanose partially retained the secondary β-sheet structure of the parent molecule (^{1}H NMR NOEs). On the basis of this presumed preorganized β-sheet conformation of a linear peptide derivative, the Phe[9]-D-Orn[10] macrocyclization site found at the corner of the β-turn and the end of the H-bonded antiparallel β-sheet proved unusually successful in the total synthesis of the ramoplanin aglycons.

7.3. Lipid Side-Chain Reduction

Tetrahydroramoplanin (e.g., tetrahydroramoplanin A2, **12**) and ramoplanin aglycon derivatives[167,112] can be easily accessed by catalytic hydrogenation (5% Pd/C) of the natural products or their aglycons.

7.4. Orn[4] and Orn[10] Derivatization

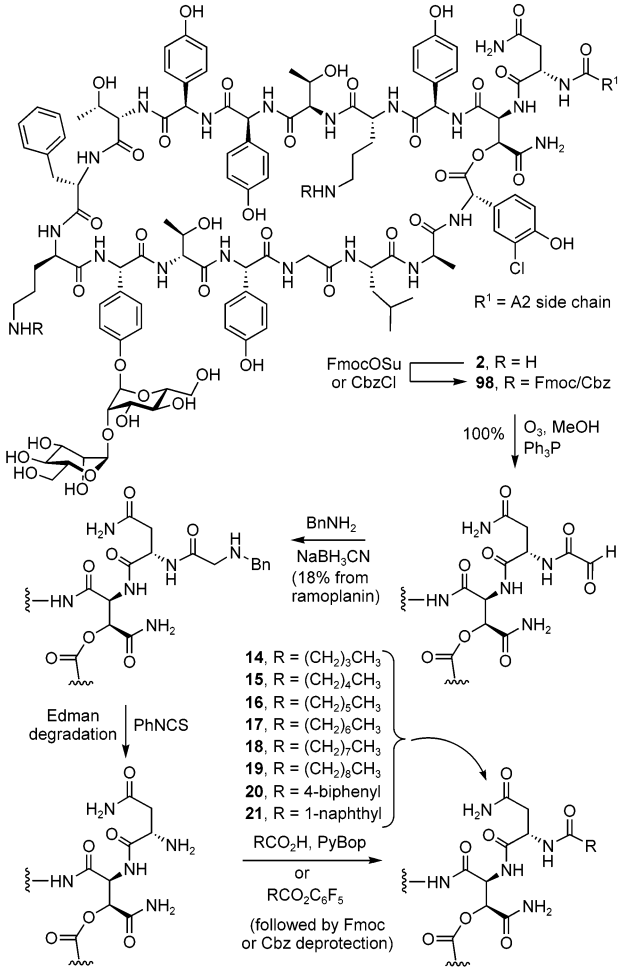
In efforts to probe the biological importance of the Orn[4] and Orn[10] δ-amino groups, several groups have described methods for their derivatization. McCafferty and co-workers converted the terminal amines of both Orn[4] and Orn[10] to the diguanidylated, diisovaleryl, and diacetyl derivatives.[30] Thus, treatment of ramoplanin A2 with either 1*H*-pyrazole-1-carboxamidine or acetic anhydride in the presence of pyridine provided the [Orn[4], Orn[10]]-diguanidylated (**26**) or -diacetylated (**28**) products. Reductive amination of ramoplanin A2 with isovaleraldehyde and $NaBH_3CN$ afforded [Orn[4], Orn[10]]-diisovaleryl ramoplanin A2 (**27**). In elegant studies designed to address the relative role of each Orn residue, Walker and coworkers disclosed the reaction of ramoplanin A2 with Boc-Ala-OSu followed by removal of Boc groups with TFA to provide the [Orn[4]-Ala] (**24**) and [Orn[10]-Ala] (**25**) derivatives.[93] Both retain the charged (protonated) amine of the parent molecule but displace its positioning by three atoms. Finally, treatment of the ramoplanin A2 aglycon with SESCl (Et_3N, DMF, −20 °C) afforded a mixture of the [Orn[4],Orn[10]]-diSES

derivative **22** and the [Orn^{10}]-monoSES derivative **23** which were separated by reverse-phase HPLC.[109]

7.5. Lipid Side-Chain Replacement

Complementary to the totally synthetic preparation of the [*N*-acetyl-Asn^1]ramoplanin aglycon, Ciabatti recently detailed the preparation of an extensive series of semisynthetic side-chain-modified ramoplanin analogues.[111] The Orn^4 and Orn^{10} δ-amino groups of ramoplanin A2 were first masked with Fmoc or Cbz protecting groups. Ozonolysis of the ramoplanin derivatives **98** and subsequent reductive amination of the resulting *N*-acyl glyoxal with benzylamine afforded a precursor suitable for Edman degradation with phenylisothiocyanate in pyridine$-H_2O$ to provide the ramoplanin-free amine. The resulting amine was then acylated with an extensive series of lipid side-chain replacements and the Orn protecting groups removed to give over 130 analogues of the natural products (Scheme 13).

Scheme 13



7.6. Summary

Notably, degradative and semisynthetic modifications of the ramoplanins have been limited to date and probed only small regions of the natural product. A renewed effort in such studies could shed insight into additional roles of undefined peripheral functional groups complementary to more deep-seated structural modifications and amino acid side-chain substitutions that are now accessible by total synthesis.

8. Structure–Activity Studies on Ramoplanin and Its Synthetic and Semisynthetic Analogues

8.1. Antimicrobial Activity of Key Derivatives and Analogues

Ramoplanin A2 is the most abundant component of the ramoplanin complex. Consequently, synthetic and semisynthetic analogues of ramoplanin A2 have been the subject of most investigations seeking to define the key structural requirements for activity. The antimicrobial activities of such key derivatives are summarized in Table 1.

The in vitro antimicrobial activity of ramoplanin A2 (**2**) and its aglycon (**5b**) are not distinguishable[109,110,167] with the latter typically displaying slightly greater in vitro potency.[109,110] Consequently, glycosidation does not contribute to the intrinsic in vitro antimicrobial activity and the role of the natural product di- or trisaccharides remains to be established. Acycloramoplanin A2 (**6**) and its aglycon (**7**), in which the macrocyclic lactone was hydrolyzed, have been reported to be inactive (MIC >128 μg/mL) and >250- to 500-fold less potent than the natural product[109] or roughly 2000-fold less active than ramoplanin A2 when tested against a more sensitive organism.[30] Although it is difficult to establish whether such residual activity ($\leq$0.05%) can be attributed to contaminant natural product in such samples, it is clear that the intact macrocyclic ring of the ramoplanin is key to their properties.

Two depsipeptide amide analogues were recently synthesized, one of which was found to be slightly more potent than the natural aglycon in antimicrobial assays providing a new lead structure with an improved profile and a more stable and accessible macrocyclic template on which to conduct structure–function studies.[110] The depsipeptide amine analogue **8** of the ramoplanin A2 aglycon, in which the backbone ester was replaced with an amide, retained or exhibited a slightly increased antimicrobial potency (MIC = 0.39 μg/mL) relative to the ramoplanin A2 aglycon (MIC = 0.78 μg/mL). Not only did this demonstrate that the depsipeptide ester may be replaced with a more stable amide without impacting the in vitro antimicrobial activity, but also that the HAsn^2 carboxamide found in ramoplanin (but is absent in **8**) does not appear to contribute directly to its properties. Notably, the ramoplanin ester adopts a typical trans (carbonyl eclipsed) conformation within the flexible loop capping one end of the antiparallel β-sheet, and it might not be surprising that its replacement with an amide was well tolerated. Just as importantly, **8** proved to be completely stable to mildly basic conditions (1% Et$_3$N$-$H$_2$O, 25 °C, 24 h, 0% disappearance)[110] that rapidly consume the ramoplanin aglycon (80% within 5 min at 25 °C).[109] In sharp contrast, the depsipeptide amide analogue **10** containing the single additional methylene in the macrocycle was inactive (MIC $\geq$ 35–50 μg/mL), exhibiting a >100-fold loss in activity relative

to the ramoplanin A2 aglycon and **8**.[110] Thus, even a simple methylene insertion with incorporation of the Dab[2] residue into the cyclic peptide abolished activity completely, suggesting not only that the cyclic structure is critical to ramoplanin's properties, but that subtle perturbations to even the flexible loop can have a pronounced impact. The impact of this modification was subsequently explored in greater detail as described in the next section.

Recent studies have also defined the impact of the lipid side chain. The tetrahydroramoplanin A1−A3, which are under investigation for use as topical antibiotics for treatment of wound infections, exhibited slightly reduced activities (e.g., **12**, Table 1).[112] The active ramoplanin amide analogue (**9**), lacking the Asn[1] lipid side chain, and [*N*-acetyl-Asn[1]]ramoplanin aglycon (**13**) both were approximately 16-[110] or 100-fold[113] less potent than the corresponding compounds containing the natural A2 side chain. These results indicate that the lipid side chain of ramoplanin A2 contributes significantly to its properties but is not essential.[110] These observations are in accord with those of Ciabatti and co-workers, who prepared an extensive series of over 130 semisynthetic ramoplanin analogues with modifications to the lipid side chain. When the length and composition of the aliphatic side chain is similar to that of the naturally occurring lipid side chains, the antimicrobial activities of the corresponding analogues are retained, whereas the activities are significantly reduced with longer and shorter lengths (e.g., **14**−**19** in Table 1 and Scheme 13). Interestingly, some analogues which possess aromatic side chains including naphthyl or biphenyl were found to possess the same or more potent antimicrobial activity than ramoplanin (e.g., **20** and **21**).[111] Further insight into the role of the lipid side chain was obtained from studies detailed in the following section.

The importance of the free amines of the two ornithine residues (Orn[4] and Orn[10]) has been studied extensively. McCafferty and co-workers found that [Orn[4],Orn[10]-diacetyl]ramoplanin A2 (**28**), in which both amines are acetylated, was 500-fold less potent than ramoplanin A2, highlighting the importance of these two basic amines which are conserved among all members of this class of natural products. They also found that preservation of cationic charge by guanidylation (**26**) or transformation to a secondary amine (**27**) by reductive amination resulted in only small to modest alterations in the antimicrobial activity.[30] Similarly, the [Orn[4],Orn[10]-diSES]ramoplanin A2 aglycon (**22**), in which both amines were converted to sulfonamide derivatives, was found to be inactive (MIC > 128 μg/mL, >500-fold loss in activity).[109] Much more interestingly, [Orn[10]-SES]-ramoplanin A2 aglycon (**23**), in which only the Orn[10] δ-amino group is protected, was 16-fold less potent than the free aglycon but >32-fold more potent than the diSES derivative.[109] Clearly, both the Orn[4] and Orn[10] amines contribute to the antimicrobial activity, and the latter result suggested that the Orn[4] free amine might be more important than the Orn[10] amine. However, more recent work of Walker's,[93] enlisting alanine derivatives of the Orn[4] and Orn[10] δ-amines, which maintain but move or extend the position of the free amines, found that [Orn[4]-Ala]-ramoplanin A2 (**24**) was an active antimicrobial agent (MIC = 0.8 μg/mL) whereas [Orn[10]-Ala]ramoplanin A2 (**25**) was nearly inactive (MIC = 50 μg/mL), implicating Orn[10] versus Orn[4] as the more important of the two residues. Notably, the positively charged amine of Orn[4] flanks the one side of the ramoplanin solution structure, whereas that of Orn[10] flanks the other, and they lie at opposite ends of the conserved putative peptidoglycan-binding domain Hpg[3]-Hpg.[10] Further studies on these and additional synthetic and semisynthetic derivatives should serve to clarify the role of Orn[4] and Orn[10] amines and the putative Hpg[3]-Orn[10] recognition domain and offer insight into the mechanism of action of ramoplanin.

8.2. Mechanistic Analysis of Ramoplanin Analogues—The Path Forward

Most recently, several of the key ramoplanin analogues have been examined for transglycosylation inhibition enlisting the assay introduced by Walker and co-workers[113] to quantitate the direct conversion of Lipid II to peptidoglycan. Combined with the antimicrobial activity of the analogues, the results provide insight into the roles of the key structural features required for Lipid II binding, inhibition of the transglycosylation reaction, and biological activity. Just as importantly, the work defines a protocol of assays from which key insights can be garnered about the functional role of ramoplanin structural features that can be used to design new, related, and improved analogues or derivatives. Thus, the active amide analogue **8** of the ramoplanin A2 aglycon, its free amine derivative **9** lacking the lipid side chain, [*N*-acetyl-Asn[1]]ramoplanin aglycon (**13**), and the inactive ring-expanded amide analogue **10** were examined in the kinetic assay for inhibition of the transglycosylase PBP1b. Notably, the kinetics of inhibition in this assay is a sensitive measure of substrate binding and provides insight into both the affinity and stoichiometry of Lipid II binding. As shown in Figure 17 each analogue bound to Lipid II with comparable affinity and the same 2:1 stoichiometry as ramoplanin itself. Thus, replacing the Asn[2] ester with an amide does not affect Lipid II binding, even when an additional methylene unit is inserted. Furthermore, truncating or removing the lipid side chain does not affect the inhibition kinetics, indicating that it does not play a direct role in substrate binding or transglycosylase inhibition.

Nonetheless, and with the exception of **8,** which was equipotent with **2**, each of these analogues was at least 2 orders of magnitude less potent than ramoplanin A2 in antimicrobial assays. Thus, although the lipid side chain does not play a role in substrate binding or in vitro inhibition of the transglycosylase reaction, it plays a key role in the biological activity. Most likely this arises by membrane targeting and localization by the lipid side chain, positioning the antibiotic near its target, Lipid II, which is located on the outer surface of the bacterial membrane.

Similarly, **10,** which contains an extra methylene in the macrocyclic ring, is also 100-fold less potent

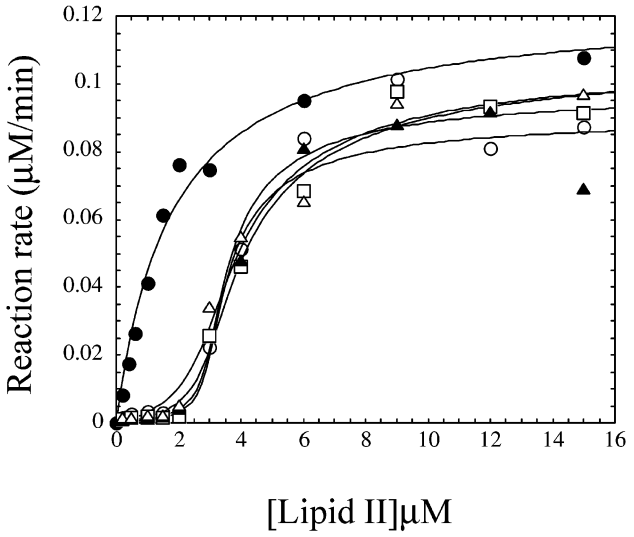


Figure 17. Inhibition of *E. coli* PBP1b by ramoplanin and its key analogues: **2** (○), **8** (□), **10** (△), and **13** (▲) at 6 μM concentration and no inhibitor (●).

than **2** or **8**. NMR analysis revealed that **10**, unlike ramoplanin and **8**, aggregates extensively in aqueous buffer. Williams and co-workers 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.[149] The extra methylene in **10** may increase macrocycle flexibility, promoting self-association and perhaps enabling undesirable interactions with other molecules as well. Unfavorable partitioning of the more flexible ramoplanin analogue **10** in cell-based assays would explain its higher MICs.

Thus, substituting an amide linkage lacking the HAsn[2] carboxamide side chain for the more labile and more complex ester linkage does not affect substrate binding, in vitro activity, or in vivo antimicrobial 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 appear to affect substrate binding or inhibition of the transglycosylation 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, the lipid chain plays a key role in biological activity without directly influencing binding to Lipid II or in vitro inhibition of transglycosylation. It is proposed 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.

9. Conclusion

Ramoplanin is an antibiotic with a novel structure and distinct mechanism of action. As such, it represents an excellent target for clinical development to treat bacteria resistant to the existing panel of clinically used antibiotics. Ramoplanin is currently in Phase III trials for the treatment of VRE; however, further development of this promising antibiotic requires a deeper understanding of its mechanism and the development of structural analogues that may address its present limitations (e.g., stability). Though it was originally believed to inhibit MurG, a series of experiments has shown that the transglycosylation step of peptidoglycan biosynthesis is the likely target for this molecule. The results of the mechanistic work also show that ramoplanain may bind to its target, Lipid II, with a 2:1 stoichiometry. The reevaluation of the enzymatic target and mechanism of action for ramoplanin has important implications for the design and synthesis of analogues with potentially improved potency, stability, and solubility.

Concurrent with the mechanistic work, a series of elegant synthetic studies resulted in the first total synthesis of the ramoplanin aglycon. The total synthesis enabled the construction of ramoplanin analogues that probed the contribution of specific aspects of the ramoplanin structure to biological activity. In particular, the role of the lipid tail, the size of the macrocycle, and the contribution of the ornithine residues to activity have been examined to date. Most significantly, a synthetic stable amide analogue of the ramoplanin aglycon has been identified (**8**, [L-Dap[2]]-ramoplanin A2 aglycon) that can now serve as an accessible macrocyclic template upon which systematic modifications to the structure can be conducted. Using this stable and accessible template, the key structural elements of the putative recognition domain and the dimerization domain can be probed with subsequent synthetic analogues to provide insight into the mechanism of action and potentially provide compounds that address issues limiting the clinical use of ramoplanin itself (e.g., stability). Thus, development of synthetic methodology and a synthesis of ramoplanin analogues have provided a means to further probe the mechanism of action.

The combination of synthetic and mechanistic work has furthered our understanding of ramoplanin. However, a number of questions remain about the drug, in particular, the role of ligand-induced polymerization in antibiotic activity and the structure of the ramoplanin:Lipid II complex. The ability to synthesize ramoplanin analogues now makes it possible to prepare specific derivatives for such structural and mechanistic analysis.

10. Abbreviations

BCB	B-bromocatecholborane
Chp	L-chlorohydroxyphenylglycine
Dab	L-2,3-diaminobutyric acid
Dap	L-2,3-diaminopropionic acid
DCC	1,3-dicyclohexylcarbodiimide
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one
DMAP	4-(dimethylamino)pyridine
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
HOAt	1-hydroxy-7-azabenzotriazole

HOBt	1-hydroxybenzotriazole
Hpg	L-hydroxyphenylglycine
SES	2-trimethylsilylethanesulfonyl

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