Synthesis and Preliminary Biological Characterization of New Semisynthetic Derivatives of Ramoplanin

ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · JULY 2007

Impact Factor: 5.45 · DOI: 10.1021/jm070042z · Source: PubMed

Synthesis and Preliminary Biological Characterization of New Semisynthetic Derivatives of Ramoplanin

Romeo Ciabatti,* Sonia I. Maffioli, Gianbattista Panzone, Augusto Canavesi, Elena Michelucci, Paolo S. Tiseni, Ettore Marzorati, Anna Checchia, Matteo Giannone, Daniela Jabes, Gabriella Romanò, Cristina Brunati, Gianpaolo Candiani, and Franca Castiglione

Vicuron Pharmaceuticals, Gerenzano, Varese, Italy

Received January 12, 2007

Ramoplanin is a glycolipodepsipeptide antibiotic active against Gram-positive bacteria including vancomycin-resistant enterococci. Ramoplanin inhibits bacterial cell wall biosynthesis by a mechanism different from that of glycopeptides and hence does not show cross-resistance with these antibiotics. The systemic use of ramoplanin has been so far prevented because of its low local tolerability when injected intravenously. To overcome this problem, the fatty acid side chain of ramoplanin was selectively removed and replaced with a variety of different carboxylic acids. Many of the new ramoplanin derivatives showed antimicrobial activity similar to that of the natural precursor coupled with a significantly improved local tolerability. Among them the derivative in which the 2-methylphenylacetic acid has replaced the di-unsaturated fatty acid side chain (**48**) was selected as the most interesting compound and submitted to further in vitro and in vivo characterization studies.

Introduction

Ramoplanin (see Figure 1) is a glycolipodepsipeptide antibiotic obtained from fermentation of *Actinoplanes* ATCC33076. It is active against Gram-positive aerobic and anaerobic bacteria, including VRE.[a,1] Ramoplanin inhibits bacterial cell wall biosynthesis by a mechanism different from those of other cell wall synthesis inhibitors (*β*-lactams, glycopeptides such as vancomycin and teicoplanin, and lipopeptides such as daptomycin) and therefore does not show cross-resistance with them. Its unique mechanism of action has been the subject of a number of recent studies.[2] Because of its potent antimicrobial activity, ramoplanin could be an effective antibiotic for treating serious Gram-positive infections. However, while demonstrating excellent antimicrobial activity in the mouse septicemia infection model,[3] ramoplanin had poor local tolerability upon intravenous injection and also caused hematuria, which would be unacceptable in clinical practice.

A ramoplanin derivative with the antimicrobial activity of the natural product but without its tolerability issues could be a potential agent for the parenteral treatment of serious infections caused by multidrug-resistant Gram-positive bacteria. Initial derivatization utilized the simplest reactions feasible on such a complex molecule: (1) hydrogenation of the two double bonds of the fatty acid side chain to produce tetrahydroramoplanin,[4] (2) removal of the dimannosyl residue linked to the amino acid at position 11 to produce the ramoplanin aglycon,[5] and (3) guanylation of the amino groups of the two ornithines to produce the diarginine analogue.[6] While these analogues had antimicrobial activity comparable to that of ramoplanin, tolerability was not substantially improved. We then turned to modification of the lipidic part of the molecule, attaching various carboxylic acids to the N-terminal amino acid. We report on the chemical strategy we followed to selectively remove the fatty acid side chain of ramoplanin, the synthesis of new derivatives, and the biological activity of these compounds.

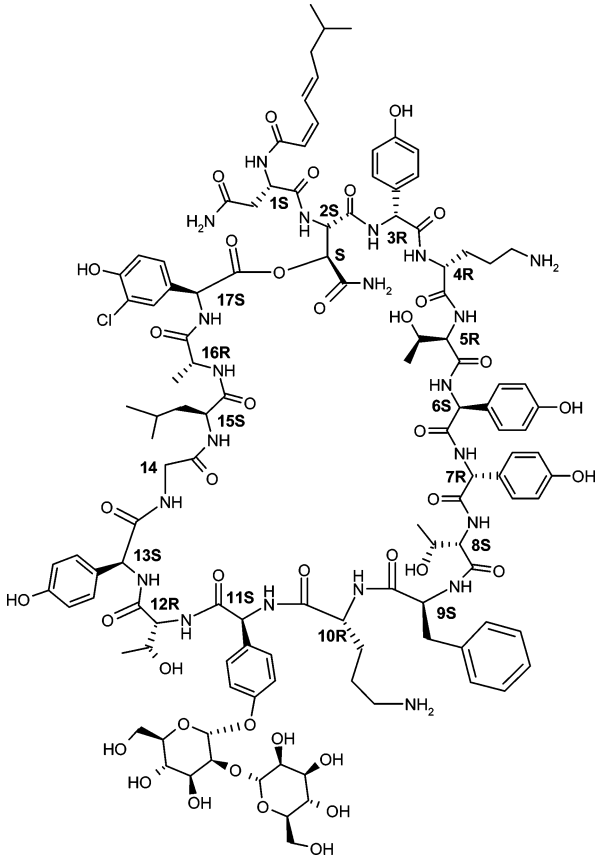


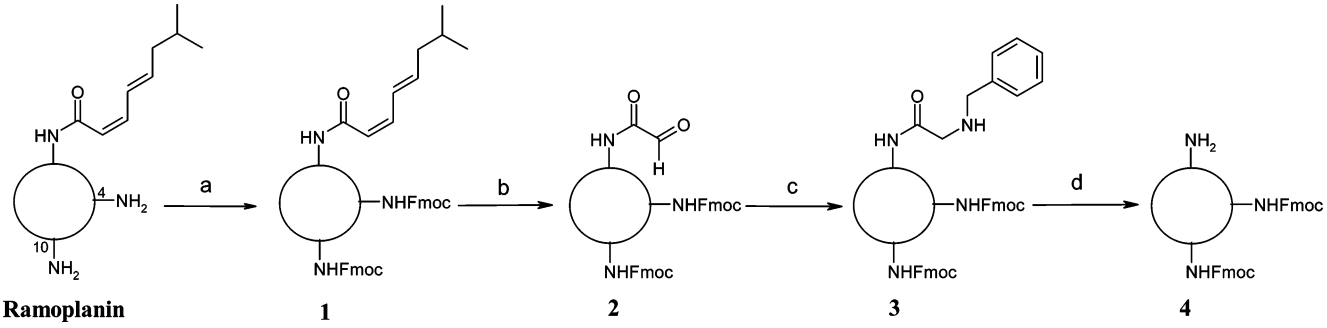
Figure 1. Structure of ramoplanin main component A2.

* To whom correspondence should be addressed. Phone: +39 023546509. Fax: +39 029 647 4358. E-mail: Romeo.Ciabatti@hotmail.it.

[a] Abbreviations: VRE, vancomycin-resistant enterococci; FmocOSu, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide; TEA, triethylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid; DCM, dichloromethane; PhNCS, phenyl isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyloxy; Cbz, carbobenzyloxy; PTSA, *p*-toluenesulfonic acid; TBAF, tetrabutylammonium fluoride; PyBOP, benzotriazol-1-yloxy-2-tris(pyrrolidin-1-yl)phosphonium hexafluorophosphate; DMSO, dimethylsulfoxide; MIC, minimal inhibitory concentration; PBS, phosphate buffer solution; OD, optical density.

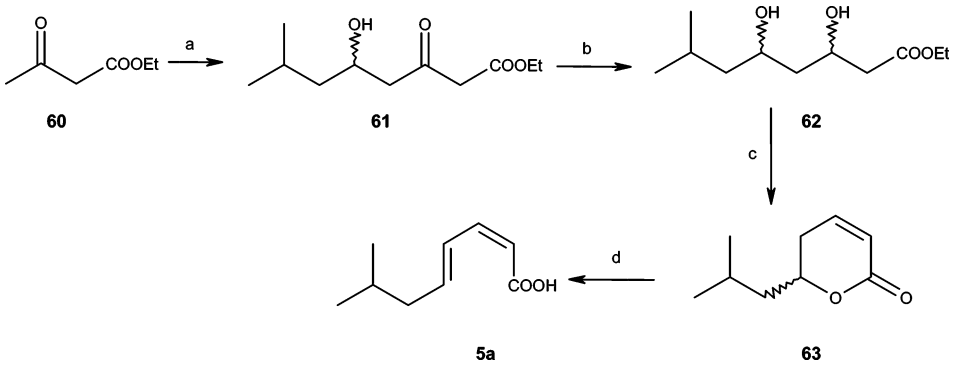
10.1021/jm070042z CCC: $37.00
Published on Web 06/02/2007

Scheme 1. Chemical Deacylation of Ramoplanin[a]



[a] Reagents and conditions: (a) FmocNOSu, TEA, DMF; (b) ozone, −78 °C, 3:1 methanol/DMF; (c) benzylamine, $NaCNBH_3$, DMF; (d) PhNCS, 1:1 H_2O/pyridine, then 1:1 TFA/DCM. Ramoplanin structure is schematized, with only the functional groups involved in the reactions illustrated. The ring represents the peptidic backbone (sugars not shown). The primary amines belong to the ornithines in positions 4 and 10.

Scheme 2. Synthesis of 2Z,4E-7-Methyl-octa-2,4-dienoic Acid (Fatty Acid **5a** of Ramoplanin A2)[a]



[a] Reagents and conditions: (a) NaH, THF at 0 °C; then BuLi, THF at −78 °C, *i*-$PrCH_2CHO$; (b) $NaBH_4$, MeOH, −30 °C; (c) toluene, PTSA, reflux; (d) TBAF, THF, room temp.

Chemistry

Because of the chemical complexity of ramoplanin and the presence of many amidic bonds, selective hydrolytic removal of the fatty acid side chain appeared challenging. To overcome this problem, we took advantage of the presence of the double bonds on the fatty acid side chain. In particular, the transformation of the α,β-double bond in an aldehyde group and a subsequent conversion of it into an amine moiety would have given us access to the well-known Edman degradation, thus allowing a selective and smooth removal of the original fatty acid side chain. Following the above strategy, deacylation of ramoplanin was thus accomplished by chemical methods (see Scheme 1).

Additionally, in order to selectively derivatize the N-terminal amine, the amino groups of ornithines 4 and 10 had to be protected. Among the protective groups compatible with the degradation scheme, Fmoc was found to be the most suitable. Although Cbz could be used for deacylation, it was not suitable for the subsequent synthesis of unsaturated derivatives because of its hydrogenolytic cleavage. Fmoc groups were introduced by reaction of ramoplanin with Fmoc succinimidyl activated reagent in the presence of TEA. DiFmoc-ramoplanin **1** was treated with ozone in a mixture of 1:3 DMF/methanol at −78 °C, followed by addition of triphenylphosphine. The resulting aldehyde **2** was reductively aminated with benzylamine in the presence of $NaCNBH_3$ to give compound **3**, which has a new N-terminal amino acid (*N*-Bn glycine). This amino acid was easily removed by Edman degradation upon treatment with phenylisothiocyanate in 1:1 pyridine/water followed by acidification with anhydrous trifluoroacetic acid. The overall yield of the final key synthon, diFmoc-deacyl-ramoplanin **4**, was 40%.

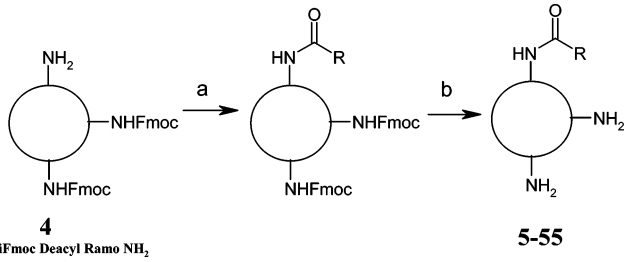
In order to verify that the degradation scheme did not affect the chirality of the natural scaffold, ramoplanin A2 (**5**) was resynthesized starting from synthon **4** (Scheme 3). Its biological activity and physicochemical characteristics (MIC, hemolytic activity, HPLC retention time, ^1H NMR, ^{13}C NMR, IR, $[\alpha]_D$) were identical to those of ramoplanin.

The fatty acid side chain of ramoplanin A2,**5a**, was synthesized following the procedure reported in Scheme 2. The α,β-unsaturated lactone **63** was the key intermediate in the synthesis of **5a** because it yielded the desired cis configuration at the α,β double bond.[7] It was prepared by the condensation of **60** with isovaleraldehyde, followed by the reduction of **61** to produce the racemic dialcohol **62**, which was then cyclized by refluxing with acid.

Several di-unsaturated carboxylic acids (**40a**−**45a**), analogues of the natural fatty acid chain of ramoplanin, were prepared and condensed with **4**. The di-unsaturated carboxylic acids were synthesized starting from the corresponding aldehyde. The synthesis was the same as that used to regenerate ramoplanin A2 (Scheme 2). In order to obtain the isomers with 2*E*,4*E* double bond geometry, the lactone was opened by basic treatment with 1 M NaOH for 2 h at room temperature, obtaining the more stable trans,trans isomers. The structures of the di-unsaturated acids are in Table 2.

Starting from the key intermediate diFmoc-deacyl-ramoplanin **4**, more than 100 semisynthetic analogues were produced.[8] To facilitate screening a large number of carboxylic acids, up to 12 reactions, each one carried out with 10−20 mg of diFmoc-deacyl-ramoplanin **4**, were run simultaneously; the progress of the reactions was followed by HPLC. DiFmoc-deacyl-ramoplanin **4** was condensed with carboxylic acids **5a**−**39a** in dry DMF, using PyBOP as the condensing agent (see Scheme 3). When the amidation step was complete (1−12 h), the Fmoc protection was removed in situ by adding 5% piperidine and allowing the reaction to proceed for 30 min at room temperature. The pH

Scheme 3. Synthesis of Ramoplanin Analogues[a]



[a] Reagents and conditions: (a) RCOOH (**5a**−**55a**) PyBOP in dry DMF; (b) 5% piperidine in DMF 30 min at room temp.

was adjusted to 6−7 with 1 N HCl. The reaction mixtures were analyzed by LC−MS to verify the identity of the derivatives. In each run, a previously characterized derivative was resynthesized as an internal reference. The solutions were neutralized and evaluated for antibacterial and hemolytic activity without purification. Reduced hemolysis in vitro was the parameter chosen to predict in vivo tolerability. Ramoplanin at 100 mg/L produces 90−100% hemolysis. The new analogues were tested at 90 and 180 mg/L. The uncondensed carboxylic acids were also tested as controls. Biological activity data for a selection of the most interesting derivatives (**6**^{s}−**39**^{s})[9] are in Table 1.

Results and Discussion

Among the aliphatic derivatives (**6**^{s}−**13**^{s}), compounds with substituents containing eight or nine carbon atoms (**10**^{s} and **11**^{s}) had better antimicrobial activity than compounds with shorter (**6**^{s}−**9**^{s}) or longer (**12**^{s} and **13**^{s}) chains. All of the aliphatic derivatives were less hemolytic than ramoplanin, although some hemolytic activity was seen at 180 mg/L with the two most hydrophobic derivatives (**12**^{s} and **13**^{s}).

Among the benzoic acid derivatives (**14**^{s} −**27**^{s}) benzoic acid **14**^{s} had little hemolytic activity, but its antibacterial activity was also greatly reduced. When the substituent was an aliphatic chain (**15**^{s}−**18**^{s}), antimicrobial activity increased with the length from C_1 to C_4 (**15**^{s}−**17**^{s}) but was greatly decreased with a C8 substituent (**18**^{s}). The hemolytic activity of **16**^{s}−**18**^{s} was higher than that of **14**^{s} and **15**^{s}. When the substituent was an ether moiety, there was a moderate effect on antibacterial activity, but there was a great reduction in hemolytic activity (**22**^{s} and **23**^{s} vs **16**^{s} and **17**^{s}). A double substitution (**26**^{s}) did not improve the antibacterial activity. The introduction of a chlorine group (**27**^{s}) reduced the antibacterial activity and increased hemolysis relative to compound **23**^{s}. Compounds with polar substituents (**20**^{s} and **21**^{s}) had greatly decreased antimicrobial activity. Most of the naphthyl derivatives (**28**^{s}−**31**^{s}) had good antimicrobial activity and were somewhat less hemolytic than ramoplanin. In the benzylic class (**32**^{s}−**39**^{s}), the benzylic derivative itself (**32**^{s}) had slightly better activity than the benzoic analogue **14**^{s} against staphylococci. The effects of substituents on the antimicrobial activity of the benzylic compounds were similar to that of the benzoic derivatives. The hemolytic activity of the benzylic derivatives was lower than that of all the other series. We therefore prepared a greater number of compounds of this class (Table 2 compounds **32**, **33**, **47**−**55**). New derivatives **40**−**55** and selected members of other classes (**10**, **31**) were prepared as powders to better evaluate their antibacterial and hemolytic activities

Among the di-unsaturated analogues (**40**−**45**), as for the saturated derivatives, the hemolytic activity decreased somewhat with the number of carbon atoms in the chain (**5**, **41**, **43**, **45**). The comparison of pairs of analogues differing only in double bond geometry showed the trans,trans conformation to be somewhat less hemolytic than the 2-cis,4-trans isomer (compare ramoplanin A2 vs **40**, **41** vs **42**, and **43** vs **44**). All of the di-unsaturated analogues had antibacterial activity comparable to that of ramoplanin. Comparison of aliphatic derivative **10** with its isomer **46**, both containing eight carbon atoms, showed an effect of the geometry of the fatty acid moiety; while the two compounds had similar antimicrobial activity, the hemolytic activity of **46** was somewhat lower than that of its linear isomer **10.**

Testing of the benzylic derivatives in powder form confirmed their relatively low hemolytic activity. The reduced antimicrobial activity of compound **32** was improved by addition of a methyl group, and the hemolytic activity was lowered. Among the three possible isomers (**48**, **49**, and **33**), the one with the methyl group in position 2 (**48**) was slightly less hemolytic than the other two. The same effect of the position of methyl groups was observed in comparing two dimethyl compounds, **53** and **54**. Compound **54**, with both methyl groups in the ortho position, was less hemolytic than **53**, while having comparable antibacterial activity. Additionally, comparison of the benzoic compound **47**, with its benzylic analogue **48**, again demonstrated that the benzylic compound had both more potent antimicrobial activity and lower hemolytic activity than the benzoic analogue. The lower hemolytic activity of the benzylic derivatives might be due to their having different amphipathic properties, as described for other molecules.[10] However, the assessment of the above hypothesis would deserve further investigation to completely elucidate the role of the lipid side chain. Recent studies demonstrated in fact that the lipid side chain of ramoplanin is not essential for the binding of ramoplanin with the biological target; nevertheless, it plays a key role in the biological activity probably as it helps target ramoplanin to bacterial membranes, thus positioning the antibiotic near its target, lipid II, which is located on the outer surface of the bacterial membrane.[11]

In the benzylic series, increasing the number of carbon atoms (**50** and **55**) did not affect antibacterial activity but led to an increase in hemolytic activity. A trifluoromethyl substitution (**52**) did not greatly affect antibacterial activity but resulted in greater hemolysis, while the introduction of a nitro moiety (**51**) decreased the antibacterial activity somewhat.

Among the new analogues, **45**, **46**, and **48** were selected for in vivo tolerability testing (Table 3). Ramoplanin, administered to rats at a dose of 10 mg/kg and at a drug concentration of 1 mg/mL, invariably caused red or dark urine (hematuria) within 24 h, while tails (injection sites) became dark or discolored 1−2 days postdose (data not shown). Lower regimens at 5 or 10 mg/kg both at a drug concentration of 0.5 mg/mL produced variable results.

Analogues were solubilized in 5% glucose and administered to rats by intravenous injection into tail vein. The primary goal of these experiments was to evaluate the tolerability profile of these compounds by visually examining the urine emitted by rats within the first several hours after treatment.

Compound **48** was the analogue with the highest flebotolerability profile in these experimental conditions, with no hematuria at a dose of 20 mg/kg and at a drug concentration of 8 mg/mL (heading "20, 8" in Table 3). After one or two treatments with compound **48**, urine samples were similar in color to those of rats given 5% glucose and no macroscopic lesions, such as dark tail, at injection sites or signs of suffering were observed. The other two analogues tested in this experiment, i.e., compounds **45** and **46**, had a tolerability profile lower than that of compound **48**. In fact, compound **45** caused hematuria at 10 mg/kg and 8 mg/mL, but hematuria was still present at a lesser

Table 1. Biological Activities of Derivatives Tested from Using Parallel Synthesis Solutions

Compound no.	A2 (5^s) ramo	6^s	7^s	8^s	9^s	10^s	11^s	12^s	13^s	14^s	15^s	16^s	17^s	18^s	19^s	20^s	21^s	22^s
Microorganism (code)	Antimicrobial activity (MIC in mg/L)																	
Staphylococcus aureus Smith (819)	<0.03	>32	8	4	1	0.25	0.25	2	16	8	4	0.5	0.25	8	0.5	32	8	1
S. aureus clin. isolate Met-R (613)	0.06	32	8	4	1	0.5	1	2	16	8	4	1	0.5	8	1	32	4	2
S. aureus clin. isolate VISA Met-R (3797)	1	>32	>32	32	8	4	8	8	32	>32	4	4	4	16	4	>32	32	32
S. aureus clin. isolate VISA (3798)	0.25	>32	32	16	4	2	4	4	32	32	8	4	2	16	2	>32	32	8
Streptococcus pyogenes C203 (49)	<0.03	0.5	0.125	0.125	<0.03	<0.03	0.125	0.25	4	0.125	<0.03	<0.03	<0.03	2	<0.03	0.5	0.06	<0.03
Enterococcus faecalis (559 isogenic of 560)	<0.03	16	8	2	0.5	0.125	0.06	0.125	4	4	2	1	0.25	4	0.5	16	4	2
E. faecalis VanA (560)	<0.03	16	8	2	0.5	0.06	0.06	0.06	4	4	2	1	0.25	4	0.5	32	8	2
E. faecium (568 isogenic of 569)	<0.03	32	8	4	1	0.25	0.125	0.25	8	8	4	1	0.25	4	0.5	32	8	2
E. faecium clin. isolate Van-A (569)	<0.03	16	8	4	1	0.125	<0.03	0.06	4	4	2	1	0.25	4	0.5	32	4	2
Escherichia coli SKF12140 (47)	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Candida albicans SKF2270 (145)	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Hemolysis																		
compound concentratiom (mg/L)	% hemolysis																	
180	79	2	0	8	1	16	5	41	59	4	3	62	91	72	13	4	3	1
90	81	0	0	0	2	0	0	0	1	0	5	36	47	95	5	0	2	1
condensed acid	**A2 (5a)**	**6a**	**7a**	**8a**	**9a**	**10a**	**11a**	**12a**	**13a**	**14a**	**15a**	**16a**	**17a**	**18a**	**19a**	**20a**	**21a**	**22a**
structure of the condensed acid	(2Z,4E)-7-Me-2,4-octadienoic acid	*n*-C_3H_7COOH	*n*-C_4H_9COOH	*n*-C_5H_{11}COOH	*n*-C_6H_{13}COOH	*n*-C_7H_{15}COOH	*n*-C_8H_{17}COOH	*n*-C_9H_{18}COOH	*n*-$C_{13}H_{27}$COOH	PhCOOH	4-Me-PhCOOH	4-C_2H_5-PhCOOH	4-*n*C_4H_9-PhCOOH	4-*n*C_8H_{17}-PhCOOH	4-Cl-PhCOOH	4-OH-PhCOOH	4-CN-PhCOOH	4-EtO-PhCOOH

Compound no.	A2 (5^s) ramo	23^s	24^s	25^s	26^s	27^s	28^s	29^s	30^s	31^s	32^s	33^s	34^s	35^s	36^s	37^s	38^s	39^s
Microorganism (code)	Antimicrobial activity (MIC in mg/L)																	
Staphylococcus aureus Smith (819)	<0.03	0.25	8	8	2	1	0.125	1	0.25	0.25	2	1	0.5	>32	8	16	4	1
S. aureus clin. isolate Met-R (613)	0.06	0.5	2	4	8	2	0.25	2	0.25	0.125	2	2	0.5	>32	8	2	8	2
S. aureus clin. isolate VISA Met-R (3797)	1	4	8	16	16	8	2	8	1	1	16	8	4	>32	32	8	32	8
S. aureus clin. isolate VISA (3798)	0.25	4	4	8	16	8	1	8	1	0.5	8	8	2	>32	32	8	16	8
Streptococcus pyogenes C203 (49)	<0.03	0.125	0.25	2	1	0.13	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	0.06	0.06	≤ 0.03	1	0.125	≤ 0.03	0.06	≤ 0.03
Enterococcus faecalis (559 isogenic of	<0.03	0.25	0.25	2	1	0.5	0.125	0.5	0.125	0.125	4	2	0.5	>32	8	2	8	1
E. faecalis VanA (560)	<0.03	0.25	0.25	2	1	0.13	0.25	0.5	0.125	0.125	4	2	0.5	>32	8	2	4	1
E. faecium (568 isogenic of 569)	<0.03	0.25	0.25	2	1	0.5	0.25	1	0.25	0.25	8	4	1	>32	16	1	4	2
E. faecium clin. isolate Van-A (569)	<0.03	0.25	0.25	1	1	0.25	0.25	1	0.25	0.25	4	2	1	>32	8	2	4	2
Escherichia coli SKF12140 (47)	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Candida albicans SKF2270 (145)	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Hemolysis																		
compound concentratiom (mg/L)	% of hemolysis																	
180	79	5	10	21	47	100	40	25	59	28	0	0	0	0	0	0	0	0
90	81	0	8	0	0	26	8	14	24	15	0	0	0	0	0	0	0	0
condensed acid	**A2 (5a)**	**23a**	**24a**	**25a**	**26a**	**27a**	**28a**	**29a**	**30a**	**31a**	**32a**	**33a**	**34**	**35a**	**36a**	**37a**	**38a**	**39a**
structure of the condensed acid	(2Z,4E)-7-Me-2,4-octadienoic acid	4-*n*C_4H_9O-PhCOOH	4-*n*C_5H_{11}O-PhCOOH	4-*n*C_7H_{15}O-PhCOOH	3,4-di-*n*C_4H_9O-PhCOOH	4-[3-Cl-PhCH$_2$O]PhCOOH	2-naphthylCOOH	1-naphthylCOOH	2-naphthylCH$_2$COOH	1-naphthylCH$_2$COOH	PhCH$_2$COOH	4-Me-PhCH$_2$COOH	4-Cl-PhCH$_2$COOH	4-OH-PhCH$_2$COOH	4-MeO-PhCH$_2$COOH	PhCH$_2$CH$_2$COOH	PhOCH$_2$COOH	PhCH$_2$CH$_2$CH$_2$COOH

extent at the doses of 10 mg/kg and 4 mg/mL or 20 mg/kg and 4 mg/mL. Compound **46** also developed hematuria at 10 mg/kg and 8 mg/mL but not at 10 mg/kg and 4 mg/mL or at 20 mg/kg and 4 mg/mL, indicating a decreased tolerability profile compared with that of compound **48**. The lower tolerability of compounds **45** and **46** was also accompanied by an equally lower local tolerability, since injection sites of tails after treatments were macroscopically in poor condition.

In conclusion, these studies indicated that chemical modifications of the natural molecule of ramoplanin, in order to obtain analogues with a better flebotolerability profile, were possible. In particular, they indicated that the modifications play a crucial role in allowing us to administer to rats a higher injectable dose than the natural molecule of ramoplanin permitted and, at the same time, maintaining the same in vitro antimicrobial profile. Because of its improved tolerability profile,

Table 2. Biological Activity of Compounds Synthesized as Powder

	Ramo A2 (5)	40	41	42	43	44	45	10	46	31	32	47	48	49	33	50	51	52	53	54	55
Microorganisms (code)	**Antimicrobial activity (MIC in mg/L)**																				
Staphylococcus aureus Smith (819)	0.06	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25	0.125	0.25	≤0.06	1	1	≤0,125	≤0,125	≤0.125	0.125	0.5	≤0.125	≤0.125	≤0.125	0.25
+ 30% bovine serum	**0.5**	**1**	**0,5**	**1**	**0.5**	**0.5**	**0.5**	**1**	**0.5**	**0.25**	**1**	**1**	**0,25**	**1**	**0.5**	**1**	**1**	**1**	**1**	**0.5**	**1**
S. aureus clin. isolate Met-R (613)	0.03	≤0.125	≤0.125	0.25	≤0.125	≤0.125	0.25	0.25	0.25	≤0.06	1	1	≤0,125	0,25	≤0.125	0.5	1	≤0.125	≤0.125	≤0.125	0.5
S. aureus clin. isolate VISA Met-R (3797)	1	1	1	2	1	1	2	2	2	2	4	8	2	2	4	2	4	2	1	1	4
S. aureus clin. isolate VISA (3798)	0.25	0.5	0.5	1	0.5	0.5	1	1	1	0.25	2	8	1	1	2	1	2	1	0.5	0.25	2
+ 30% bovine serum	**2**	**4**	**2**	**4**	**2**	**2**	**2**	**2**	**2**	**1**	**2**	**4**	**1**	**4**	**4**	**4**	**4**	**4**	**4**	**2**	**4**
Streptococcus pyogenes C203 (49)	0.015	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	<0.06	≤0.125	≤0.06		≤0.125	≤0,125	≤0,125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
Enterococcus faecalis (559 isogenic of 560)	0.03	≤0.125	≤0.125	0.25	≤0.125	≤0.125	0.5	0.125	0.5	≤0.06	2	2	0,5	0,25	0.25	0.5	2	≤0.125	≤0.125	≤0.125	0.5
E. faecalis VanA (560)	0.03	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25	0.125	0.25	≤0.06	2	2	0,25	≤0,125	≤0.125	0.5	1	≤0.125	≤0.125	≤0.125	1
+ 30% bovine serum	**0.5**	**0.5**	**0.5**	**1**	**0.5**	**0.5**	**0.5**	**1**	**1**	**0.25**	**2**	**2**	**0,5**	**1**	**1**	**2**	**2**	**0.5**	**1**	**1**	**2**
E. faecium (568 isogenic of 569)	≤0.125	≤0.125	0.25	0.25	≤0.125	≤0.125	0.5	0.125	0.5	0.125	2	2	0,5	0,5	0.5	≤0.06	4	0.5	0.25	0.5	na
E. faecium clin. isolate Van-A (569)	0.015	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25	<0.06	0.5	≤0.06	1	4	0,5	0,5	0.5	0.5	2	0.25	≤0.125	0.25	1
+ 30% bovine serum	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0.5**	**1**	≤0.125	**1**	**4**	**2**	**4**	**8**	**2**	**2**	**4**	**8**
Escherichia coli SKF12140 (47)	>32	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Candida albicans SKF2270 (145)	>32	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Compound concentration (mg/L)	**% hemolysis**																				
1600									83			93	5								
1200														5	9	76	0	51	87	43	45
800							96		73	98		94	2								
600														0	11	1	0	5	18	13	4
400		78	97		94	78	51	59	8	60	2	83	1								
300														0	2	1	1	3	0	0	2
200		87	81	29	83	37	45	5	4	30	0	17	1								
100	87-100	85	64	30	65	23	38	0	0		3	2	1								
condensed acid	**A2 (5a)**	**40a**	**41a**	**42a**	**43a**	**44a**	**45a**	**10a**	**46a**	**31a**	**32a**	**47a**	**48a**	**49a**	**33a**	**50a**	**51a**	**52a**	**53a**	**54a**	**55a**
Structure of the condensed acid	(2Z,4E)-7-Me-2,4-octadienoic acid	(2E,4E)-7-Me-2,4-octadienoic acid	(2Z,4E)-6-Me-2,4-heptadienoic acid	(2E,4E)-6-Me-2,4-heptadienoic acid	(2Z,4E)-2,4-octadienoic acid	(2E,4E)-2,4-octadienoic acid	(2Z,4E)-2,4-heptadienoic acid	*n*-$C_9H_{18}COOH$	*n*-$C_4H_9CH(Et)COOH$	1-NaphthylCH$_2$COOH	PhCH$_2$COOH	2-MePhCOOH	2-MePhCH$_2$COOH	3-MePhCH$_2$COOH	4-MePhCH$_2$COOH	2-EtPhCH$_2$COOH	2-NO$_2$PhCH$_2$COOH	2-CF$_3$PhCH$_2$COOH	3,5-di-MePhCH$_2$COOH	2,6-di-MePhCH$_2$COOH	2-MePhCH(Me)COOH

Table 3. In Vivo Tolerability of Selected Ramoplanin Derivatives[a]

		treatment [dosage (mg/kg), drug concentration (mg/mL)]			
compd	vehicle	10, 8	10, 4	20, 4	20, 8
45	3/3 rats A	5/6 rats P	2/3 rats A	2/3 rats A	not tested
46	3/3 rats A	3/3 rats P	3/3 rats A	3/3 rats A	not tested
48	3/3 rats A	3/3 rats A	not tested	not tested	3/3 rats A

[a] P = presence of hematuria, pink or red urine. A = absence of hematuria, yellow or colorless urine.

compound **48** was chosen for further in vitro and in vivo characterization.

Conclusion

These studies demonstrated that the tolerability profile of ramoplanin can be improved by chemical modification while maintaining antibacterial activity. Replacement of the original fatty acid chain with different chemical residues has shown that the antimicrobial activity and hemolytic effect can be modulated, depending on the nature and the structure of the residue. In addition, we have synthesized a number of new derivatives in which the two activities were separated. Among them **48**, in which the 2-methylphenylacetic acid replaced the natural chain, was selected as the most interesting compound and was submitted to further in vitro and in vivo characterization studies.

Experimental Section

General Part. HPLC analysis details are as follows: (a) instrument, Shimadzu SCL-6B; column, Merck Lichrocart 125-4 Lichrosphere 100 RP-18 (5 μm); flow, 1 mL/min; detector UV λ = 270 nm; injection volume, 10 μL; phase A, 0.05 M $HCOONH_4$; phase B, MeCN; linear gradient from 35% B to 40% B over 15 min and then from 40% B to 70% B over 20 min. Details for (b) are the same as for (a) except for the following: instrument, Varian 9010; column, Merck Lichrocart 125-4 Lichrosphere 100 RP-8 (5 μm); linear gradient from 30% B to 40% B over 30 min and then from 40% B to 80% B over 5 min. Details for (c) are the same as for (a) except for the following: linear gradient from 15% B to 37% B over 20 min, from 37% B to 43% B over 5 min, and then from 43% B to 58% B over 5 min. Details for (d) are the same as for (a) except for the following: instrument, HP 1090. Details for (e) are the same as for (a) except for the following: instrument, Varian 9010; column, Merck Lichrocart 125-4 Lichrosphere 100 RP-8 (5 μm); linear gradient from 35% B to 70% B over 35 min. Details for (f) are the same as for (e) except for the following: linear gradient from 20% B to 40% B over 30 min and then from 40% B to 80% B over 5 min.

For NMR analysis, ^{1}H chemical shifts are in ppm. NMR measurements were carried out on a Bruker DRX 500 spectrometer operating at 500.13 MHz. Samples containing 5 mM of ramoplanin derivative in D_2O−DMSO-d_6 (4:1) were utilized. For identification and assignment of the spin systems, two-dimensional ^{1}H COSY and TOCSY spectra were recorded at 313 K. Amino acid residues are numbered according to the literature,[12] and only the peak resonances differing from natural ramoplanin are reported in this table.

For MS analysis, the mass spectra were recorded on an LCQ Advantage ThermoFinnigan instrument.

Chemistry. 4,10-diFmoc-Protected Ramoplanin (1). A solution of ramoplanin dihydrochloride (110.6 g, 40 mmol) in DMF (500 mL) was maintained at 0 °C with stirring under nitrogen atmosphere. To this solution Fmoc-ONSu (6.8 g, 20 mmol) and TEA (5.8 mL, 41.2 mmol) were added, maintaining the mixture at 0−5 °C. After 5 min additional Fmoc-ONSu (6.8 g, 20 mmol) and TEA (5.8 mL, 41.2 mmol) were added. After another 5 min, Fmoc-ONSu (13.6 g, 40 mmol) was added. The mixture temperature was allowed to rise to room temperature. The reaction was monitored by HPLC analysis (a) (retention time of 25.6 min). After HPLC control an additional 10.8 g of Fmoc-ONSu was necessary to complete the reaction. After 30 min, acetic acid (20 mL) was added

and the reaction mixture was poured into ethyl acetate (1L) filtered and dried. An amount of 133 g of a solid product was obtained. The solid was washed with stirring in methanol/water (1:9), and the mixture was adjusted to pH 4.5−5 with acetic acid. The solid was filtered and dried at 35 °C under reduced pressure, obtaining 126.8 g of white solid. Yield 100%.

4,10-diFmoc-Protected Ramo-NHCOCHO (2). Into a solution of 4,10-diFmoc protected ramoplanin (**1**) obtained in the previous step (126 g) in methanol/DMF (3:1, 3 L) and cooled to −78 °C, ozone was bubbled (170 mmol, at a flow rate of 100 L/h of oxygen containing 5% of ozone) with stirring. The mixture was maintained at −78 °C for 30 min. The reaction was monitored by HPLC analysis (a) (retention time of 7.5 min). The excess of ozone was eliminated by bubbling nitrogen into the solution. Triphenylphosphine was added (25 g), and the mixture was allowed to reach room temperature. Methanol was evaporated under reduced pressure, and the residual DMF solution was poured into ethyl acetate (8 L) with stirring. The precipitate was filtered, washed with ethyl acetate (3 × 150 mL), and dried at room temperature, obtaining 132 g of a solid that was used for the following step.

4,10-diFmoc Protected Ramoplanin-NHCOCH$_2$NHCH$_2$Ph (3). To a solution of 4,10-diFmoc-protected ramoplanin-CHO (**2**) (130 g, 45 mmol) and benzylamine hydrobromide (43 g, 224 mmol) in anhydrous DMF (1 L), $NaCNBH_3$ (4.23 g, 67 mmol) was added with stirring at room temperature. The mixture was stirred for 2 h. The reaction was monitored by HPLC analysis (a) (retention time 19.6 min). The solution was poured into water (10 L). The precipitate was filtered and dried at 35 °C under reduced pressure, obtaining 130 g of crude product. The crude product (107 g) was dissolved at 35−40 °C in 1.5 L of a 1:1 acetonitrile/water mixture at pH 2.5 (1 N HCl). To this solution, with stirring, silanized silica gel was added (300 g). After 30 min, acetonitrile was evaporated under reduced pressure and the water suspension was charged at the top of a silanized silica gel column (diameter 7.5 cm, height 100 cm) previously equilibrated with water. Elution was with a water/acetonitrile gradient from 85:15 to 1:1. Fractions containing the product were collected, and the acetonitrile was evaporated under reduced pressure. The precipitate was filtered, washed with water (100 mL), and dried at 35 °C under reduced pressure, obtaining 54.6 g of white solid. Yield was 42%, starting from ramoplanin.

4,10-diFmoc Protected Ramoplanin-NH$_2$ (4). To a solution of 4,10-diFmoc protected ramoplaninNHCOCH$_2$NHCH$_2$Ph (**3**) (17 g, 5.65 mmol) in 1:1 pyridine/water (340 mL), phenyl isothiocyanate (0.74 mL, 6.35 mmol) was added while stirring at room temperature. The reaction was monitored by HPLC analysis (a) (retention time of 24.7 min). After 1 h the solvent was evaporated and the residue was suspended in toluene (50 mL) and evaporated. This operation was repeated twice. The solid was then suspended in dichloromethane (100 mL) and added with TFA (100 mL). After 15 min at 40 °C and HPLC control (a) (retention time of 9.5 min), the mixture was evaporated under reduced pressure and the oil obtained was triturated with diethyl ether (100 mL) and dried at 35−40 °C under reduced pressure, obtaining 17 g of solid. The solid was suspended in water, and the suspension was stirred at room temperature for 2 h and filtered. The solid was dried at 35−40 °C under reduced pressure, obtaining 15 g of white solid. Yield 91%.

Preparation of Carboxylic Acids (5a−55a) To Be Condensed on 4,10-diFmoc-Protected Ramoplanin-NH$_2$ (4). Many of the carboxylic acids to be condensed with 4,10-diFmoc protected ramoplanin-NH_2 (**4**) are commercially available. Those carboxylic acids not commercially available were prepared according to the following procedure.

5-Hydroxy-7-methyl-3-oxooctanoic Acid Ethyl Ester (61). Sodium hydride, as a 60% mineral oil dispersion (6.8 g, 169 mmol), was weighed into a dry round bottomed flask, and dry tetrahydrofuran (220 mL) was added. The flask was cooled in ice and flushed with nitrogen. Ethyl acetoacetate (20 g, 154 mmol) was added dropwise to the cooled and stirred slurry, and the mixture was stirred for 10 min after the addition was complete. The solution was cooled at −78 °C. A solution of *n*-butyllithium (85 mL of 2 M solution in cyclohexane) was added dropwise to the reaction mixture, and stirring was continued for a further 10 min. Isovaleraldehyde (16.5 mL, 154 mmol) was then added in one portion. After a further 10 min the mixture was poured into a HCl solution (50 mL of 37% HCl in 400 mL of water). Ether was added, and the aqueous layer was removed and extracted again with 2 × 40 mL of ether. The ether extracts were combined, washed with a saturated brine solution, dried over sodium sulfate, and filtered, and the solvent was removed under reduced pressure. The oily residue was purified by flash chromatography using 8:2 hexane/ethyl acetate as eluent, obtaining 16.6 g of product (50%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.95 (d, 6H), 1.19 (m, 1H), 1.28 (t, 3H), 1.51 (m, 1H), 1.80 (m, 1H), 2.65 (dd, 1H), 2.68 (s broad, 1H), 2.73 (dd, 1H), 3.47 (s, 2H), 4.21 (q, 2H).

3,5-Dihydroxy-7-methyloctanoic Acid Ethyl Ester (62). Sodium borohydride (1.58 g, 41.6 mmol) was added to a stirred solution of **61** (9 g, 41.6 mmol) in MeOH (100 mL) at −30 °C. Stirring was continued at the same temperature for 2 h. Then a saturated ammonium chloride solution was added and methanol

Table 4. HPLC Retention Times and MS Data for Compounds **5**−**55**[a]

compd	t_R (min) of diFmoc derivatives	t_R (min) of final compd	lower isotope molecular weight
5	34.2 (b), 25.6 (d)	10.9 (b)	2552
6	15.5 (d)	2.8 (d)	2483
7	18.6 (d)	4 (d)	2500
8	21.3 (d)	4.51 (d)	2514
9	33.7 (b)	8.9 (b)	2528
10	24.7 (d)	5.5 (d)	2542
11	26.3 (d)	8.4 (d)	2556
12	27.5 (d)	12.7 (d)	2570
13	34.9 (d)	19.1 (d)	2625
14	17.1 (d)	8.4 (d)	2520
15	20.9 (d)	4.2 (d)	2534
16	21.7 (d)	5.1 (d)	2548
17	25.3 (d)	6.5 (d)	2576
18	31.6 (d)	14.2 (d)	2632
19	21.8 (d)	6.3 (d)	2554
20	10.2 (d)	2.1 (d)	2536
21	17.3 (d)	3.85 (d)	2562
22	32.4 (b)	5.6 (b)	2564
23	25.2 (d)	5.6 (d)	2592
24	26.8 (d)	5.8 (d)	2606
25	29.6 (d)	6.2 (d)	2634
26	27.6 (d)	5.9 (d)	2664
27	26.8 (d)	5.3 (d)	2660
28	22.1 (d)	4.9 (d)	2570
29	20.9 (d)	20.9 (d)	2570
30	22.6 (d)	5 (d)	2584
31	33.7 (b)	7.7 (b)	2584
32	30.2 (b)	4.2 (b)	2534
33	21.6 (d)	4.5 (d)	2548
34	22.0 (d)	4.9 (d)	2568
35	11.8 (d)	2.3 (d)	2550
36	9.2 (e)	14.4 (f)	2564
37	21.4 (d)	4.5 (d)	2548
38	20.7 (d)	4.15 (d)	2550
39	22.5 (d)	5 (d)	2562
40	34.2 (b)	13.7 (b)	2552
41	33.8 (b)	8.2 (b)	2538
42	33.7 (b)	9.5 (b)	2538
43	33.6 (b)	8 (b)	2538
44	33.9 (b)	9.6 (b)	2524
45	33.2 (b)	5.5 (b)	2524
46	25.0(a)	21.73 (c)	2542
47	18.9(a)	16.27 (c)	2534
48	21.7(a)	18.83 (c)	2548
49	10.4 (e)	17.9 (f)	2548
50	10.9 (e)	19.0 (f)	2562
51	9.3 (e)	13.9 (f)	2579
52	11.1 (e)	19.7 (f)	2602
53	12.0 (e)	21.1 (f)	2562
54	10.9 (e)	19.0 (f)	2562
55	11.3−12.0 (e)	17.3−18.9 (f)	2562

[a] Letters in parentheses refer to the HPLC conditions described in the Experimental Section.

Table 5. ^{1}H Chemical Shifts (in ppm) of Selected Compounds[a]

residue		22	31	32	45	48	54
side chain		1.37 (3H)	3.99 (2H)	3.55 (2H)	0.98 (3H)	3.52 (2H)	3.55 (2H)
		4.13 (2H)	7.95 (1H)	7.3 (2H)	5.56 (1H)	2.17 (3H)	2.19 (6H)
		7.57 (2H)	7.91 (1H)	7.35 (3H)	6.49 (1H)	7.1 (1H)	7.03 (2H)
		6.98 (2H)	7.87 (1H)		7.07 (1H)	7.7 (1H)	7.13 (2H)
			7.57 (1H)		6.17 (1H)	7.18 (1H)	
			7.56 (1H)		2.14 (2H)	7.21 (1H)	
			7.47 (1H)				
			7.39 (1H)				
Asn (1)	CHα	4.71	4.55	4.7	4.7	4.55	4.75
	CH$_2\beta$	1.88−1.22	1.83−2.24	1.79−2.21	1.66−2.18	1.68−2.18	1.6−2.2
Leu (15)	CHα	4.18	3.95	4.06	4.285	4.07	4.13
	CH$_2\beta$	1.4−1.44	1.16−1.22	1.42−1.46	1.503	1.39	1.46
	CHγ	1.4	1.33	1.44	1.503	1.45	1.46
	Me	0.68	0.43−0.56	0.62−0.74	0.765−0.8	0.67−0.76	0.72−0.79
Ala (16)	CHα	4.34	4.097	4.25	4.36	4.195	4.18
	Me	1.46	0.93	1.244	1.428	1.178	1.14

[a] NMR conditions are detailed in the Experimental Section.

was evaporated under reduced pressure. The mixture was extracted with ethyl acetate, and the organic layer was dried over sodium sulfate and concentrated to give the alcohol **62**, which was used without any further purification for the following step.

6-Isobutyl-5,6-dihydro-2-pyrone (63). Toluene (100 mL) and PTSA (800 mg) were added to (**62**), and the mixture was refluxed for 2 h. The reaction was quenched with water, and the mixture was extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated to give 6 g of lactone (**63**), which was used as such for the following step. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.97 (d, 6H), 1.41 (m, 1H), 1.80 (m, 1H), 1.92 (m, 1H), 2.31 (m, 2H), 4.52 (m, 1H), 6.04 (m, 1H), 6.90 (m, 1H).

2*Z*,4*E*-7-Methylocta-2,4-dienoic Acid (5a). A mixture of the lactone **63** (6 g) and *n*-$Bu_4NF\cdot 3H_2O$ (10 g) in THF (100 mL) was stirred at room temperature for 3 h under nitrogen. Water was added, and the mixture was extracted with ethyl acetate. The organic layer was washed with saturated brine solution, dried over sodium sulfate, and evaporated under reduced pressure to give 3.8 g of dienoic acid **5a**. Yield 60% from **61**. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.93 (d, 6H), 1.73 (m, 1H), 2.11 (m, 1H, J = 7.4 Hz), 5.6 (d, 1H, J = 11.4), 6.12 (dt, 1H, J = 15.2 and 7.4), 6.66 (dd, 1H, $J_1 = J_2$ = 11.3), 7.33 (m, 1H, J = 15.2 Hz). ^{13}C NMR ($CDCl_3$): δ 170.6, 147.0, 145.3, 127.4, 113.9, 41.7, 29.1, 22.2.

Following the same procedures but with different starting aldehydes, the following carboxylic acids were synthesized.

2*Z*,4*E*-6-Methyl-2,4-heptandienoic Acid (41a). Starting aldehyde was isobutyraldehyde, giving **41a**. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.05 (d, 6H), 2.48 (m, 1H), 5.6 (d, 1H, J = 11.31 Hz), 6.08 (m, 1H), 6.65 (dd, 1H), 7.3 (m, 1H).

2*Z*,4*E*-2,4-Octadienoic Acid (43a). Starting aldehyde was butyraldehyde, giving **43a**. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.98 (t, 3H), 1.45 (m, 2H), 2.17 (m, 2H), 5.58 (d, 1H, J = 11.33 Hz), 6.12 (m, 1H, J = 15.2 Hz), 6.65 (dd, 1H), 7.34 (m, 1H).

2*Z*,4*E*-2,4-Heptadienoic Acid (45a). Starting aldehyde was propionaldehyde, giving **45a**. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.07 (t, 3H), 2.25 (m, 2H), 5.59 (d, 1H, J = 11.35 Hz), 6.17 (m, 1H), 6.66 (dd, 1H), 7.35 (m, 1H).

2*E*,4*E*-7-Methyl-2,4-octadienoic Acid (5a). The trans,trans isomer of **40a** was synthesized following the same synthetic strategy of compound **5a** but changing the final step. A mixture of the lactone **63** (2.143 mmol) and 30% NaOH (11 mL) was stirred at reflux for 1 h. The mixture was acidified with 5 N HCl to pH 3 and then extracted with ethyl acetate. The organic layer was washed with saturated brine, dried over sodium sulfate, and evaporated under reduced pressure to give a crude product, which was purified by flash chromatography (9:1 DCM/MeOH). An amount of 0.37 g of the desired compound were obtained. Yield 60%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.9 (d, 6H, Me_2-CH), 1.76 (m, 1H, $CHMe_2$), 2.1 (t, 2H, $CH_2CH{=}CH$), 5.82 (d, 1H, J = 15.36 Hz, CHCOOH), 6.12−6.3 (m, 2H), 7.29 (m, 1H).

Following the same procedure but with different starting aldehydes, the following carboxylic acids were synthesized.

2*E*,4*E*-6-Methyl-2,4-heptadienoic acid (42a). Starting aldehyde was isobutyraldehyde, giving **42a**. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.07 (d, 6H), 2.43 (m, 1H), 5.8 (d, 1H, J = 15.2 Hz), 6.15 (m, 1H), 6.24 (m, 1H), 7.26 (m, 1H).

2*E*,4*E*-2,4-Octadienoic Acid (44a). Starting aldehyde was butyraldehyde, giving **44a**. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.93 (t, 3H), 1.45 (m, 2H), 2.17 (m, 2H), 5.77 (d, 1H, J = 15.3 Hz), 6.15−6.3 (m, 2H), 7.25 (m, 1H).

4-Butoxybenzoic Acid (23a). Step 1. A mixture of 4-hydroxybenzaldehyde (8.2 mmol), butyl bromide (8.2 mmol), K_2CO_3 (8.2 mmol), and KI (8.2 mmol) in acetone (15 mL) was stirred at reflux for 6 h. Acetone was evaporated, and the semisolid residue was dissolved in water and extracted with ethyl acetate. The organic layer was washed with 0.1 N NaOH and then with saturated brine, dried over sodium sulfate, and evaporated under reduced pressure to give a crude product, which was used as such for the following step. Yield 100%.

Step 2. $AgNO_3$ solution (4.6 M in water, 0.56 mL) was added to the solution of the compound obtained according to step 1 (1.12 mmol) in ethanol (6.7 mL). KOH (5.6 mL of a 1 M solution in water) was added, and the reaction mixture was stirred at room temperature for 2 h. The solid was filtered, and the aqueous solution was acidified with concentrated HCl and extracted with diethyl ether. The organic phase was washed with water, dried over sodium sulfate, and evaporated under reduced pressure to give the desired 4-butoxybenzoic acid **23a**, which was used without further purification.

Following the same procedure but using the appropriate benzoic acid and alkyl bromide, the following carboxylic acids were synthesized.

4-Pentyloxybenzoic Acid (24a). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.93 (t, 3H), 1.41 (m, 4H), 1.74 (m, 2H), 4.05 (t, 2H), 7.00 (d, 2H), 7.88 (d, 2H).

4-Heptyloxybenzoic Acid (25a). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.88 (t, 3H), 1.3 (m, 6H), 1.74 (m, 2H), 4.05 (t, 2H), 7.00 (d, 2H), 7.88 (d, 2H).

3,4-Dibutoxy-benzoic acid (26a). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.94 (t, 6H), 1.44 (m, 4H), 1.71 (m, 4H), 3.98 (t, 2H), 4.03 (t, 2H), 7.05 (d, 1H), 7.45 (s, 1H), 7.52 (d, 1H).

Compounds 5−55. To a solution of the 4,10-diFmoc protected ramoplanin-NH_2 **4** (0.35 mmol), triethylamine (1.05 mmol), and the suitable carboxylic acid (**5a**−**55a**) (0.525 mmol) in DMF (12.5 mL) was added PyBOP (0.52 mmol) with stirring at room temperature. The reaction was monitored by HPLC analysis (see Table 4). The mixture was allowed to react at room temperature, and after 5 h, piperidine (0.6 mL) or alternatively 2,2,6,6-tetramethylpiperidine (1.8 mL) was added to remove the protecting group from the ornithine moieties. The reaction was continued at room temperature and monitored by HPLC (see Table 4). After 30

min, diluted HCl was added (6.5 mL of a 1 M solution). The resulting solutions can be tested for antimicrobial and hemolytic activities. Alternatively, the product can be purified by preparative HPLC and lyophilized. The derivatives were characterized by MS spectrometry (see Table 4) and some of them by NMR spectroscopy (see Table 5).

MICs were determined by broth microdilution method according to the NCCLS procedure.[13] Microorganisms were grown in cation-adjusted Muller−Hinton broth, in Todd−Hewitt broth (streptococci only), or in RPMI medium (*Candida albicans* only). Inocula were 5×10^5 CFU/mL. MICs were read after 24 h of incubation at 35 °C.

Hemolytic Activity. Hemolysis of erythrocytes is considered to be an indicator of the local tolerability of ramoplanin analogues. Hemolysis testing was performed according to the method suggested in the literature. Initial experiments were carried out directly on the solutions derived from the reaction of the amidation of 4,10-diFmoc protected ramoplanin-NH_2 **4** as previously described. The reaction solutions (resulting from the addition of 1 M hydrochloric acid) were diluted at 180 mg/L by adding 0.1% peptone and 0.8% NaCl (PBS). Additional experiments were carried out on the powdered compound: the ramoplanin analogues were dissolved at 40.000 μg/mL in DMSO and then diluted 1:5 in 0.1% peptone and 0.8% NaCl (PBS). Whole-blood sample was obtained from the abdominal aorta of rats and diluted 1:100 in PBS before the test.

PBS and 3% saponin in distilled water were the 0% and 100% hemolysis controls, respectively. The reaction solutions or solubilized powders to be tested were diluted 1:5 in triplicate into the diluted blood and incubated in a water bath at 37 °C for 45 min. The blood was then centrifuged at 2500−3000*g* for 10 min, and 0.1 mL of each supernatant was diluted in 0.9 mL of Drabkin's reagent. The OD of the samples was measured at 540 nm versus a blank consisting of Drabkin's reagent plus 0.1 mL of PBS. The percent hemolysis was calculated as $\Delta x/\Delta t \times 100$, where $\Delta x =$ mean OD_{540} (sample minus blank) and $\Delta t =$ mean OD_{540} (minus blank) of the positive control.

In Vivo Tolerability. Compounds were solubilized in 5% glucose. Three to six rats for dosing of test compound were treated by intravenous injection at 24 h intervals for 1−2 days. Urine samples were examined for the presence of blood (hematuria), and macroscopic observation of the injection sites and general behavior were also recorded. Rats were killed 24 h after the last treatment. Positive controls consisting of three rats given 5% glucose were used. Our previous studies performed in our labs have demonstrated that ramoplanin, administered to rats at a dose of 10 mg/kg and at a drug concentration of 1 mg/mL, invariably caused red or dark urine (hematuria) within 24 h while tails (injection sites) became dark or discolored 1−2 days postdose (data not shown). Lower regimens at 5 or 10 mg/kg, both at a drug concentration of 0.5 mg/mL, produced variable results. These in vivo ramoplanin studies also demonstrated that the drug concentration (mg/mL) more than the dosage (mg/kg) itself played a crucial role in causing hematuria. Therefore, in order to find a derivative with enhanced flebotolerability properties, it has been decided to start these in vivo studies with an initial dose of 10 mg/kg at a drug concentration of 8 mg/mL (10 mg/kg and 8 mg/mL). As second step, and in the case of a positive result, other rats were immediately dosed with the same test compound at 20 mg/kg at the same drug concentration (20 mg/kg and 8 mg/mL), which was the highest dose-concentration tested. In the case of a negative result at 20 mg/kg and 8 mg/mL, the same dose but at a lower drug concentration (20 mg/kg and 4 mg/mL) was assessed. Conversely, in the case of a negative result with an initial dose of 10 mg/kg and 8 mg/mL, the same dose administered at a lower concentration (10 mg/kg at a drug concentration of 4 mg/mL; 10 mg/kg and 4 mg/mL) was tested. In this case, whether a positive result was found, the corresponding higher dose as mg/kg at the same drug concentration was tested. With this experimental design, it was possible to verify different conditions and, at the same time, to determine the influence of the drug concentration versus the dose on the tolerability.

Acknowledgment. This work was partially supported by the Italian Government throught grants to the Progetto ex art.11 L451/94 Rif.3932 "Ricerca, Caratterizzazione e Sviluppo di Nuovi Antibiotici". Part of this work was presented at 43rd Annual ICAAC, Chicago, September 14−17, 2003.

References

(1) (a) Rolston, K. V. I.; Dholakia, N.; Ho, D. H.; LeBlanc, B.; Dvorak, T.; Streeter, H. In-vitro activity of ramoplanin (a novel lipoglycopeptide), vancomycin and teicoplanin against Gram-positive clinical isolates from cancer patients. *J. Antimicrob. Chemother.* **1996**, *38*, 265−269. (b) O'Hare, M. D.; Felmingham, D.; Gruneberg, R. N. The in vitro activity of ramoplanin (A-16686/MDL 62,128), vancomycin and teicoplanin against methicillin-susceptible and methicillin-resistant staphylococcus spp. *Drugs Exp. Clin. Res.* **1988**, *14*, 617−619. (c) Lawrence, T.; Rotstein, C.; Beam, T. R., Jr.; Gorzynski, E. A.; Amsterdam, A. In vitro activities of ramoplanin, selected glycopeptides, fluoroquinolones and other antibiotics against bloodstream isolates of Gram-positive cocci. *Antimicrob. Agents Chemother.* **1993**, *37*, 896−900. (d) Johnson, C. C.; Taylor, S.; Pitsakis, P.; May, P.; Levison, M. E. Bactericidal activity of ramoplanin against antibiotic-resistant enterococci. *Antimicrob. Agents Chemother.* **1992**, *36*, 2342−2345. (e) Walker, S.; Chen, L.; Hu, Y.; Rew, Y.; Shin, D.; Boger, D. L. Chemistry and biology of ramoplanin: a lipoglycodepsipetide with potent antibiotic activity. *Chem. Rev.* **2005**, *105*, 449−475.

(2) (a) Lo, M.; Men, H.; Branstrom, A.; Helm, J.; Yao, N.; Goldman, R.; Walker, S. A new mechanism of action proposed for ramoplanin. *J. Am. Chem. Soc.* **2000**, *122*, 3540−3541. (b) Lo, M.; Helm, J.; Sarngadharan, G.; Pelczer, I.; Walker, S. A new structure for the substrate-binding antibiotic ramoplanin. *J. Am. Chem. Soc.* **2001**, *123*, 8640−8641. (c) Helm, J.; Chen, L.; Walker, S. Rethinking ramoplanin: the role of substrate binding in inhibition of peptidoglycan biosynthesis *J. Am. Chem. Soc.* **2002**, *124*, 13970−13971. (d) Cudic, P.; Kranz, J. K.; Behenna, D. C.; Kruger, R. G.; Tadesse, H.; Wand, A. J.; Veklich, Y. I.; Weisel, J. W.; McCafferty, D. G. Complexation of peptidoglycan intermediates by the lipoglycodepsipeptide antibiotic ramoplanin: minimal structural requirements for intermolecular complexation and fibril formation. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7384−7389. (e) Chen, L.; Yuan, Y.; Helm, J. S.; Hu, Y.; Rew, Y.; Shin, D.; Boger, D. L.; Walker, S. Dissecting ramoplanin: mechanistic analysis of synthetic ramoplanin analogues as a guide to the design of improved antibiotics. *J. Am. Chem. Soc.* **2004**, *126*, 7462−7463 (f) McCafferty, D. G.; Cudic, P.; Frankel, B. A.; Barkallah, S.; Kruger, R. G.; Li, W. Chemistry and biology of the ramoplanin family of peptide antibiotics. *Biopolymers* **2002**, *66*, 261−284. (g) Jiang, W.; Wanner, J.; Lee, R. J.; Bounaud, P. Y.; Boger, D. L. Total synthesis of the ramoplanin A2 and ramoplanose aglycon. *J. Am. Chem. Soc.* **2002**, *124*, 5288−5290. (h) Jiang, W.; Wanner, J.; Lee. R. J.; Bounaud, P. Y.; Boger, D. L. Total synthesis of the ramoplanin A2 and ramoplanose aglycon. *J. Am. Chem. Soc.* **2003**, *125*, 1877−1887. (i) Hu, Y.; Helm, J.; Chen, L.; Ye, X.; Walker, S. Ramoplanin inhibits bacterial transglycosilases by binding as a dimer to lipid II. *J. Am. Chem. Soc.* **2003**, *125*, 8736−8737. (j) Rew, Y.; Shin, D.; Hwang, I.; Boger, D. L. Total synthesis and examination of three key analogues of ramoplanin: a lipoglycodepsipeptide with potent antibiotic activity. *J. Am. Chem. Soc.* **2004**, *126*, 1041−1043.

(3) Pallanza, R.; Berti, M.; Scotti, R.; Randini, E.; Arioli, V. A-16686, a new antibiotic from Actinoplanes II. Biological properties. *J. Antibiot.* **1984**, *37*, 318−324.

(4) Ciabatti, R.; Cavalleri, B. Hydrogenated Derivatives of Antibiotic A/16686. U.S. Patent 5108988, 1988.

(5) (a) Ciabatti, R.; Cavalleri, B. Aglycons of A/16686 Antibiotics. European Patent 337203, 1989; *Chem. Abstr.* **1990**, *112*, 179893. (b) Jiang, W.; Wanner, J.; Lee, R. J.; Bounaud, P.-Y.; Boger, D. L. Total synthesis of the ramoplanin A2 and ramoplanose aglycon. *J. Am. Chem. Soc.* **2003**, *125*, 1877. (c) Rew, Y.; Shin, D.; Hwang, I.; Boger, D. L. Total synthesis and examination of three key analogs of ramoplanin: a lipoglycodepsipeptide with potent antibiotic activity. *J. Am. Chem. Soc.* **2004**, *126*, 1041. (d) Wanner, J.; Tang, D.; McComas, C. C.; Crowley, B. M.; Jiang, W.; Moss, J.; Boger, D. L. A new and improved method for deglycosidation of glycopeptide antibiotics exemplified with vancomycin, ristocetin and ramoplanin. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1169.

(6) Guanylated derivatives were synthesized in our laboratories, but the results have not been published. (a) For the present study, the derivatives were resynthesized as described in the following: Cudic, P.; Behenna, D. C.; Kranz, J. K.; Kruger, R. G.; Wand, A. J.; Veklich, Y. I.; Weisel, J. W.; McCafferty, D. G. Functional analysis of the lipoglycodepsipeptide antibiotic ramoplanin. *Chem. Biol.* **2002**, *9*, 1−20. For other ornithines derivatives, see the following: (b) Helm, J. S.; Chen, L.; Walker, S. Rethinking ramoplanin: the role of

substrate binding in inhibition of peptidoglycan biosynthesis. *J. Am. Chem. Soc.* **2002**, *124*, 13970. (c) Jiang, W.; Wanner, J.; Lee, R. J.; Bounaud, P.-Y.; Boger, D. L. Total synthesis and examination of three key analogs of ramoplanin: a lipoglycodepsipeptide with potent antibiotic activity. *J. Am. Chem. Soc.* **2003**, *125*, 1877.

(7) (a) Huckin, S. N.; Weiler, L. Aldol reactions of the dianion of β-ketoester. *Tetrahedron Lett.* **1971**, *50*, 4835−4838. (b) Masamune, S.; Imperiali, B.; Garvey, D. S. Synthesis of ansamycins: the ansa chain of rifamycin S. *J. Am. Chem. Soc.* **1982**, *104*, 5528−5531. (c) Roush, W. R.; Spada, A. Enantio- and stereoselective synthesis of the dihydroxyactadienoic acid fragments of the roridins and trichoverrins. *Tetrahedron Lett.* **1982**, *23*, 3773−3776. (d) Yoshida, T.; Saito, S. A new method for synthesis of α,β-unsaturated δ-lactones via Michael addition using methyl (phenylsulfinyl)acetate. *Chem. Lett.* **1982**, 1587−1590. (e) Nakata, T.; Hata, N.; Oishi, T. A stereoselective synthesis of (2*Z*,4*E*)-dienoic acid involving masked functional groups: nBu4NF induced ring-opening of α,β-unsaturated δ-lactone. *Heterocycles* **1990**, *30*, 333−334. (f) Corey, E. J.; Schmidt, G. Studies on the total synthesis of rifamycin. A method for the stereospecific synthesis of the carbonyl-conjugated 1,3 dienic unit [C(15) to C(20)]. *Tetrahedron Lett.* **1979**, *25*, 2317−2320.

(8) (a) Ciabatti, R.; Maffioli, S. I.; Checchia, A.; Romanò, G.; Candiani, G.; Panzone, G. A Process for the Production of Ramoplanin-like Amide Derivatives. WO 2003076460, 2003; *Chem.Abstr.* **2003**, *139*, 261566. (b) Raju, B.; Ciabatti, R.; Maffioli, S. I.; Singh, U.; Romanò, G.; Michelucci, E.; Tiseni, P. S.; Candiani, G.; Kim, B.; O'Dowd, H. Ramoplanin Derivatives Possessing Antibacterial Activity. WO 2007001335, 2007.

(9) The superscript(s) is used when the derivatives were tested for biological activity without isolation from the reaction solution.

(10) (a) Kondejewski, L. H.; Jelokhani-Niaraki, M.; Farmer, S. W.; Lix, B.; Kay, C. M.; Sykes, B. D.; Hancock, R. E. W.; Hodges, R. S. Dissociation of antimicrobial and hemolytic activities in cyclic peptides diastereomers by systematic alterations in amphipathicity. *J. Biol. Chem.* **1999**, *274*, 13181−13192. (b) Yamada, K.; Shinoda, S.-s.; Oku, H.; Komagoe, K.; Katsu, T.; Katakai, R. Synthesis of low-hemolytic antimicrobial dehydropeptides based on Gramicidin S. *J. Med. Chem.* **2006**, *49*, 7592−7595.

(11) (a) Walker, S.; Chen, L.; Hu, Y.; Rew, Y.; Shin, D.; Boger, D. L. Chemistry and biology of ramoplanin: a lipoglycodepsipetide with potent antibiotic activity. *Chem. Rev.* **2005**, *105*, 449−475. (b) Shin, D.; Rew, Y.; Boger, D. L. Natural product synthesis special feature: total synthesis and structure of the ramoplanin A1 and A3 aglycons: two minor components of the ramoplanin complex. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11977−11979.

(12) (a) Ciabatti, R.; Kettenring, J. K.; Winters, G.; Tuan, G.; Zerilli, L.; Cavalleri, B. Ramoplanin (A-16686), a new glycolipodepsipeptide antibiotic. III. Structure elucidation. *J. Antibiot.* **1989**, *42*, 254−267. (b) Kettenring, J. K.; Ciabatti, R.; Winters, G.; Tamborini, G.; Cavalleri, B. Ramoplanin (A-16686), a new glycolipodepsipeptide antibiotic. IV. Complete sequence determination by homonuclear 2D NMR spectroscopy. *J. Antibiot.* **1989**, *42*, 268−275. (c) Kurz, M.; Guba, W. 3D structure of ramoplanin: a potent inhibitor of bacterial cell wall synthesis. *Biochemistry* **1996**, *35*, 12570−12575.

(13) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*; Approved Guideline; The National Committee for Clinical Laboratory Standards (NCCLS), January 2003.

JM070042Z