Bioorganic & Medicinal Chemistry Letters 19 (2009) 6189–6191

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Functional and biochemical analysis of a key series of ramoplanin analogues

Xiao Fang [a,b], Joonwoo Nam [c], Dongwoo Shin [c], Yosup Rew [c], Dale L. Boger [c,*], Suzanne Walker [b,*]

[a] Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA
[b] Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA
[c] Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA

ARTICLE INFO

Article history:
Received 1 August 2009
Revised 1 September 2009
Accepted 1 September 2009
Available online 6 September 2009

Keywords:
Ramoplanin
Antibiotic

ABSTRACT

Ramoplanin is a potent lipoglycodepsipeptide antibiotic that is active against a wide range of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococcus* (VRE). It acts as an inhibitor of peptidoglycan (PG) biosynthesis that disrupts glycan chain polymerization by binding and sequestering Lipid II, a PG precursor. Herein, we report the functional antimicrobial activity (MIC, *S. aureus*) and fundamental biochemical assessments against a peptidoglycan glycosyltransferase (*Escherichia coli* PBP1b) of a set of key alanine scan analogues of ramoplanin that provide insight into the importance and role of each of its individual amino acid residues.

Ramoplanin (**1**) is a 17-residue lipoglycodepsipeptide antibiotic that exhibits potent antimicrobial activity against a wide range of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococcus* (VRE), Figure 1.[1] Ramoplanin inhibits peptidoglycan biosynthesis, and we have established that the mechanism of inhibition involves binding to Lipid II, the substrate of the bacterial transglycosylases (TGases; also known as peptidoglycan glycosyltransferases, or PGTs) that form the glycan chains of peptidoglycan.[2,3] Ramoplanin is in clinical trials for the treatment of *Clostridium difficile* associated disease in the gastrointestinal (GI) tract, but its systemic use is currently limited by its hydrolytic instability and its propensity to aggregate.[1,4] Recently, we reported a total synthesis of the natural product aglycon[5,6] and detailed its extension to the preparation of [Dap^2]ramoplanin aglycon (**2**) and a series of related analogues.[7–9] In these studies, we were able to show that the lactam analogue **2**, Figure 2, maintains the full biological activity of the natural product and is chemically stable, addressing the problem of hydrolytic instability caused by the natural lactone linkage.[9] Additionally and in these studies, we were able to utilize a biochemical assay measuring transglycosylase inhibition alongside antimicrobial assays to not just assess the impact of such structural changes on functional activity, but to also probe the role of individual structural features found in the natural products. For example, we were able to demonstrate that **2** not only maintains the antimicrobial activity of **1**, but that it also binds Lipid II and inhibits transglycosylase as effectively as **1**.[9] Just as significantly, we were able to demonstrate that ramoplanin analogues lacking the Asn[1] lipid side chain are much less active in antimicrobial assays (>100-fold),[7,9] and that this is not the result of a loss in Lipid II binding affinity or capabilities for inhibition of transglycosylase.[9] Rather, we could attribute this loss in antimicrobial activity to the bacterial membrane delivery and anchoring effects of the hydrophobic side chain.[9] We have subsequently carried out an alanine scan of compound **2** in efforts to identify and define the role of the residues important for biological activity.[8] Herein, we report biochemical studies of a key set of these alanine analogues, which provide additional insight into the role of each amino acid residue in the biological activity of ramoplanin.

The minimum inhibitory concentrations (MICs) of the analogues of **2** containing alanine replacements at residues 3 through 12 against a representative *S. aureus* strain are shown in Table 1. The results indicate that residues 5, 6, and 9 play modest roles since replacement of each of these residues with alanine leads to only small increases in the MIC (<6-fold). For all other residues, the MICs increase >15-fold upon alanine replacement, with particularly dramatic increases observed for replacements at positions 4, 8, 10, and 12. Analogous observations were reported earlier[8] in antimicrobial assays conducted against a different strain of *S. aureus* and differ only in the relative importance observed for residue 12 (10-fold vs 80-fold herein).

To determine whether the increases in the MICs correlate with decreased affinity for Lipid II, we examined the ability of the analogues to inhibit its incorporation into peptidoglycan by *Escherichia coli* PBP1b, a representative PGT, and we also assessed their ability to complex the fluorescent Lipid II analogue **3**, Figure 2. The results, summarized in Table 1, show that the antimicrobial potencies

* Corresponding authors. Tel.: +1 858 784 7522; fax: +1 858 784 7550 (D.L.B.); tel.: +1 617 432 5488; fax: +1 617 496 0215 (S.W.).
E-mail addresses: boger@scripps.edu (D.L. Boger), suzanne_walker@hms.harvard.edu (S. Walker).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.09.001

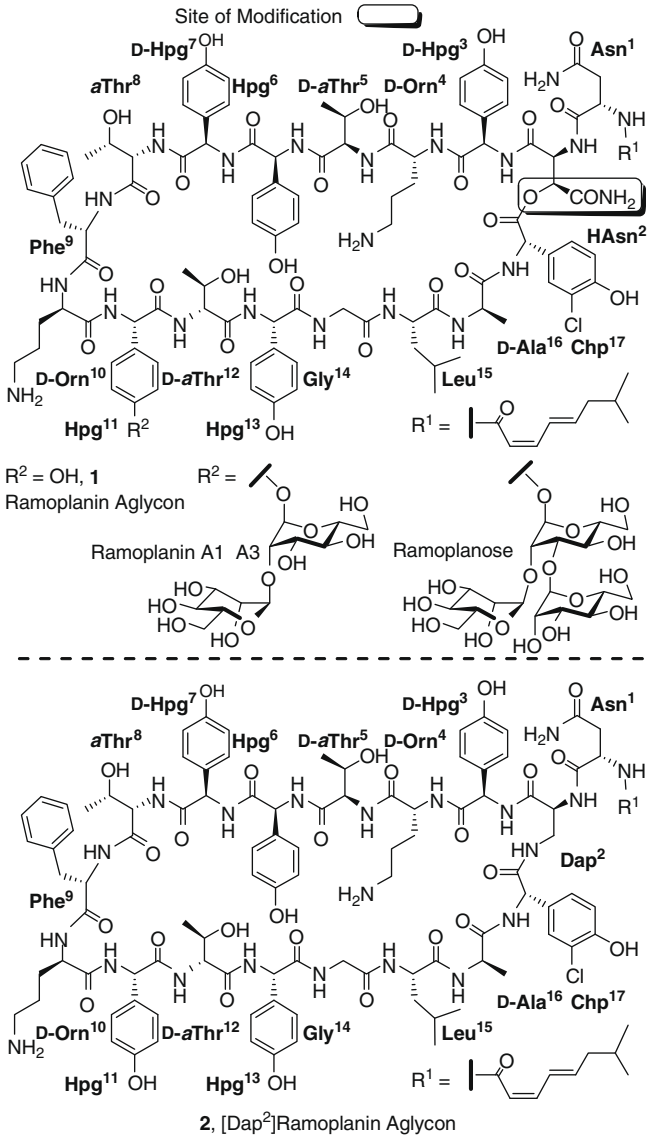

Figure 1. Structure of ramoplanin and [Dap^2]ramoplanin aglycon.

generally parallel an analogue's ability to bind to Lipid II. For example, the analogues containing alanine replacements at residues 5, 6, and 9 show characteristic sigmoidal velocity versus substrate concentration curves that are similar to those for ramoplanin (**1**) itself and the fully active analogue **2**. These sigmoidal curves arise because the substrate is completely sequestered by complexation with the ramoplanin and is therefore not available to react until it exceeds a critical concentration.[10] That concentration (3 μM) is half the inhibitor concentration (6 μM) because, as we have shown, ramoplanin binds Lipid II as a dimer (2:1 ramoplanin/Lipid II).[3] In contrast, the kinetic curves for the analogues containing alanine replacements at other positions show only slight inflections compared with the control curve (no inhibitor), implying that they still interact with Lipid II when assayed at 6 μM, but more weakly compared with the parent compound **2**, Figure 3.

Table 1
Summary of results of assays for ramoplanin analogues

Analogue	Antimicrobial potency, MIC[a] (μg/mL)	Inhibition vs PBP1b[b]	K_d with **3** (nM)
1	0.4	S	40 ± 3
2	0.4	S	33 ± 16
Ala^3	13	S	ND[c]
Ala^4	31	N	4300 ± 800
Ala^5	2.4	S	447 ± 41
Ala^6	1.6	S	378 ± 104
Ala^7	13	N	1200 ± 400
Ala^8	38	N	8700 ± 1300
Ala^9	1.6	S	383 ± 58
Ala^{10}	>50	N	6500 ± 2800
Ala^{11}	6.3	N	6300 ± 300
Ala^{12}	33	N	5400 ± 1200

[a] MIC values were measured using a standard microdilution assay against *S. aureus* ATCC 29213. Experimental MIC is defined as the lowest antibiotic concentration that results in no visible growth after incubation at 37 °C for 24 h.
[b] Inhibition assays were carried out as described in Ref. 10. S indicates the compound exhibited a sigmoidal curve in the assay while N stands for a non-sigmoidal shaped curve. Representative curves are shown in Figure 3.
[c] Not determined due to lack of sufficient material.

We have reported the use of the fluorescent compound **3** in an assay to quantify the dissociation constants of the ramoplanin–Lipid II complexes,[10] and this assay was used to assess the K_d of each of the Ala^4 through Ala^{12} analogues. The results, shown in Table 1, parallel the MIC measurements. Ala^5, Ala^6, and Ala^9 have the lowest K_ds among the tested analogues; the K_ds for most of the other residues increase by factors of 100 or more compared with that for the parent compound **2**. Ala^8, Ala^{10}, and Ala^{12} are the poorest binders. Significantly, for this series of compounds, the impact of the structural changes on the functional antimicrobial activity (MIC) reflects the ability of the analogues to bind Lipid II and thereby inhibit enzyme-mediated glycan polymerization.

High resolution NMR structures of ramoplanin have defined conformations for the natural product in both monomeric and dimeric states,[11,12] and the results reported here indicate that many of the residues having the greatest impact on binding are located in the β-turn comprising residues 7–10[12] and along the

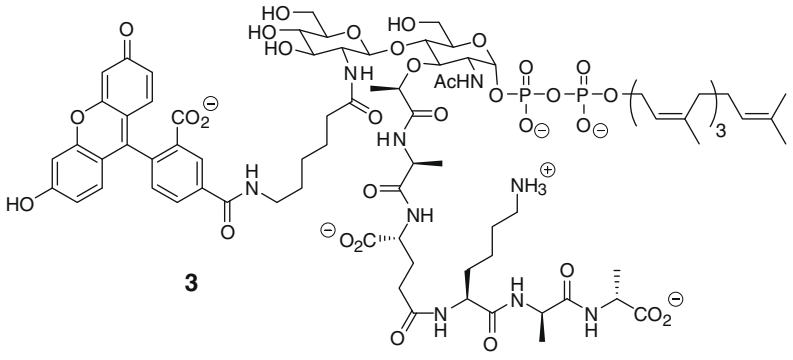

Figure 2. Structure of fluorescently labeled Lipid II analogue fl-LPII (**3**).

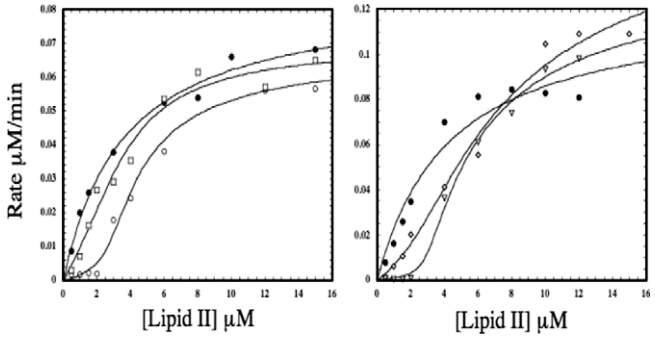

Figure 3. Representative curves of inhibition of *E. coli* transglycosylase PBP1b by ramoplanin analogues. Assay conditions are described in Ref. 10. Concentration of inhibitors is 6 μM for each reaction. (A) Inhibition of *E. coli* PBP1b by **2** (o), Ala^{10} (□) and no inhibitor (●); and (B) inhibition of *E. coli* PBP1b by Ala^{8} (o), Ala^{9} (▽) and no inhibitor (●).

dimer interface, which spans residues 10–14.[12] Based on a comparison of ramoplanin and the related compound enduracidin, McCafferty and coworkers suggested that ramoplanin residues 3–10 comprise the minimal pharmacophore for substrate complexation.[13] The results reported here indicate that residues 11 and 12, which lie outside this region, are critical for substrate complexation whereas residues 5, 6, and 9, which lie inside the putative binding domain, play only modest roles in complexation. This indicates that the minimal pharmacophore for substrate complexation extends beyond the region identified by McCafferty and coworkers. Since substrate complexation does not simply involve formation of a 1:1 complex, some of the residues identified as important for binding may be involved in ramoplanin:ramoplanin contacts.

The importance of the two positively charged residues in ramoplanin, Orn^{4} and Orn^{10}, has been the subject of considerable investigation. The studies here show that Ala^{10} has the highest MIC among the tested analogues, indicating that Orn^{10} plays a critical role in ramoplanin's activity. The inhibition kinetics and binding experiments show that much of the loss in biological activity can be attributed to decreased Lipid II binding. Replacement of Orn^{4} with alanine also has a substantial deleterious effect on both biological activity and Lipid II complexation, which would seem to suggest that Orn^{4} also plays a critical role in substrate binding. We note, however, that when Orn^{4} and Orn^{10} are acylated with alanine, a conservative modification that moves the positive charge of a protonated amine further away from the natural product core, the Orn^{4} analogue retains almost full activity and the ability to bind substrate, whereas the Orn^{10} analogue is virtually inactive and is incapable of binding to substrates.[14] Similarly, analogues of ramoplanin containing fluorophores on Orn^{4} have been shown to be capable of binding to Lipid II analogues whereas the corresponding Orn^{10} derivatives are incapable of such binding.[15] These differences between the results obtained with different sets of Orn^{4} and Orn^{10} analogues suggests that Orn^{10} plays an essential and specific role in substrate binding, most likely by ion pairing with the diphosphate of Lipid II. Orn^{4} is also important since the Ala derivative is much less active and exhibits weak substrate binding. However, modifications of this ornithine amine that move or remove the positive charge are tolerated.

Because ramoplanin binds Lipid II substrates as a dimer and also forms higher order complexes in solution, the analysis of the data on analogues is complicated. It is likely that structural information on dimeric complexes with suitable substrate analogues will be required for a more complete understanding of the role of each of the side chains. Nevertheless, the studies reported here provide insight into which residues are tolerant of significant structural changes and which are not. This information is being used for the design of analogues that have improved physical properties and those that may be used to further probe ramoplanin's mechanism of action.[16]

Acknowledgement

We thank the National Institutes of Health (CA041101, D.L.B.; GM076710, S.W.) for financial support of the work.

References and notes

1. Walker, S.; Chen, L.; Hu, Y.; Rew, Y.; Shin, D.; Boger, D. L. *Chem. Rev.* **2005**, *105*, 449.
2. Lo, M. C.; Men, H.; Branstrom, A.; Helm, J.; Yao, N.; Goldman, R.; Walker, S. *J. Am. Chem. Soc.* **2000**, *122*, 3540.
3. Hu, Y.; Helm, J. S.; Chen, L.; Ye, X. Y.; Walker, S. *J. Am. Chem. Soc.* **2003**, *125*, 8736.
4. http://investors.oscient.com/phoenix.zhtml?c=97238&p=irol-newsArticle_Print&ID=440258&highlight=.
5. Jiang, W.; Wanner, J.; Lee, R. J.; Bounaud, P. Y.; Boger, D. L. *J. Am. Chem. Soc.* **2002**, *124*, 5288.
6. (a) Jiang, W.; Wanner, J.; Lee, R. J.; Bounaud, P. Y.; Boger, D. L. *J. Am. Chem. Soc.* **2003**, *125*, 1877; (b) Shin, D.; Rew, Y.; Boger, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11977.
7. Rew, Y.; Shin, D.; Hwang, I.; Boger, D. L. *J. Am. Chem. Soc.* **2004**, *126*, 1041.
8. Nam, J.; Shin, D.; Rew, Y.; Boger, D. L. *J. Am. Chem. Soc.* **2007**, *129*, 8747.
9. Chen, L.; Yuan, Y.; Helm, J. S.; Hu, Y.; Rew, Y.; Shin, D.; Boger, D. L.; Walker, S. *J. Am. Chem. Soc.* **2004**, *126*, 7462.
10. Fang, X.; Tiyanont, K.; Zhang, Y.; Wanner, J.; Boger, D.; Walker, S. *Mol. Biosystems* **2006**, *2*, 69.
11. Kurz, M.; Guba, W. *Biochemistry* **1996**, *35*, 12570.
12. Lo, M. C.; Helm, J. S.; Sarngadharan, G.; Pelczer, I.; Walker, S. *J. Am. Chem. Soc.* **2001**, *123*, 8640.
13. 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. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7384.
14. Helm, J. S.; Chen, L.; Walker, S. *J. Am. Chem. Soc.* **2002**, *124*, 13970.
15. Tiyanont, K.; Doan, T.; Lazarus, M. B.; Fang, X.; Rudner, D. Z.; Walker, S. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11033.
16. *Note added in proof*: While this Letter was under review, an X-ray crystal structure of a ramoplanin dimer was reported that further confirms the C2-symmetric, antiparallel ramoplanin dimer structure solved by NMR.[1,3,12] See: Hamburger J. B.; Hoertz, A. J.; Lee, A.; Senturla, R. J.; McCafferty, D. G.; Loll, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 13759.