

In Vitro Mutasynthesis of Lantibiotic Analogues Containing Nonproteinogenic Amino Acids

Matthew R. Levensgood, Patrick J. Knerr, Trent J. Oman, and Wilfred A. van der Donk*

Howard Hughes Medical Institute and Roger Adams Laboratory, Department of Chemistry, University of Illinois at Urbana–Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

Received April 22, 2009; E-mail: vddonk@illinois.edu

The lantibiotics are a class of polycyclic peptide natural products that exhibit antimicrobial activity against a variety of clinically relevant bacterial pathogens.^{1,2} These compounds have a long history of use in food preservation and possess considerable promise as human therapeutics because of their potency and unique modes of action.³ Lantibiotics are ribosomally synthesized as linear prepeptides and are extensively post-translationally modified to their active forms. These modifications introduce thioether-containing cross-linked amino acids called lanthionine (Lan) and methyl-lanthionine (MeLan) as well as dehydroalanine (Dha) and dehydrobutyryne (Dhb) residues. The latter motifs result from enzymatic dehydration of Ser or Thr residues, respectively. (Me)Lan cross-links are then installed through enzymatic intramolecular Michael-type addition of Cys thiols onto the unsaturated amino acids. Despite their potential as antimicrobial therapeutics, significant hurdles must be overcome for lantibiotics to reach the clinic, including improving the solubility and stability of the compounds under physiological conditions.

The generation of lantibiotic analogues for structure–activity studies has traditionally been conducted through in vivo mutagenesis of lantibiotic prepeptides. Such studies have provided a wealth of knowledge regarding lantibiotic structure–activity relationships but have yielded only a few lantibiotics with enhanced bioactivity⁴ or improved therapeutic properties.⁵ The chemical synthesis of lantibiotics has been achieved for two family members.^{6,7} In principle, synthesis provides access to lantibiotic analogues with expanded chemical diversity by introduction of nonproteinogenic amino acids. At present, however, no such analogues have been reported. An alternative approach is to use biosynthetic enzymes with synthetic substrate analogues, a strategy we term in vitro mutasynthesis (IVM). We report herein the first preparation of lantibiotic analogues containing nonproteinogenic amino acids using IVM. Several of these analogues possess improved antimicrobial activity.

Lactacin 481 is a lantibiotic produced by *Lactococcus lactis* CNRZ 481 that contains three Me(Lan) rings and one Dhb residue.⁸ Lactacin 481 synthetase (LctM) introduces these post-translational modifications by catalyzing both dehydration and cyclization of its substrate prepeptide, LctA.⁹ LctA is a 51 amino acid peptide consisting of a C-terminal structural peptide that undergoes post-translational modification as well as an N-terminal leader peptide that is required for efficient processing by LctM.^{10,11} Several recent reports have characterized the promiscuous substrate specificity of LctM toward truncated LctA mutant substrates.^{12–14} A significant challenge for the introduction of nonproteinogenic amino acids into lactacin 481 is the preparation of full-length LctA prepeptides. For this purpose, we developed the synthesis of a triazole-linked LctA peptide analogue (**3**) via Cu(I)-catalyzed 1,3-dipolar cycloaddition of an alkyne-functionalized LctA leader peptide (**1**) and an azide-modified LctA structural region (**2**).¹⁴ This strategy was utilized here to prepare triazole-linked LctA substrates containing several

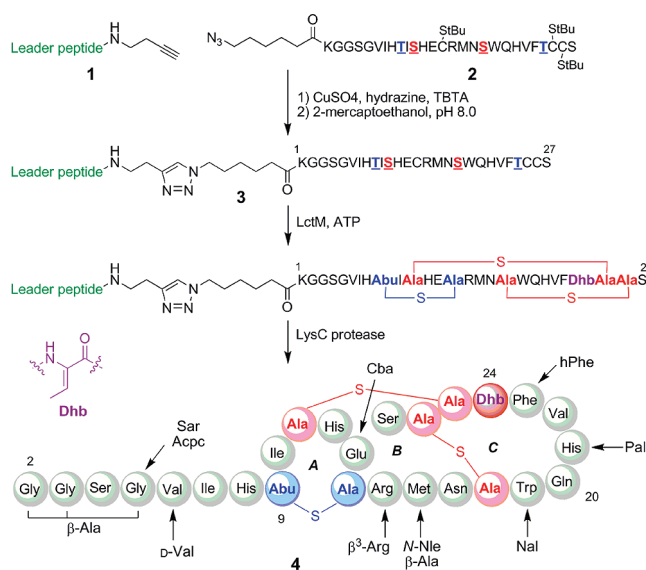


Figure 1. In vitro mutasynthesis of lactacin 481 analogues. Synthetic substrate analogues were prepared using copper-catalyzed [2 + 3] cycloaddition of two fragment peptides. The resulting LctA analogues were treated with LctM, which dehydrated the underlined Ser and Thr residues and incorporated the thioether rings shown. Mutant analogues generated are indicated with arrows. Lan residues are shown in red, and the Me(Lan) cross-link is shown in blue.

nonproteinogenic amino acids in the structural peptide, including β -amino acids, D-amino acids, and *N*-alkylglycine (peptoid) residues. An additional two mutations, Asn15Arg and Phe21His, were included in substrate **3** to improve solubility.^{15,16} On the basis of our previous survey of the types of amino acids that are tolerated by LctM,¹⁴ substrates including the following nonproteinogenic amino acid mutations were prepared (Figure 1): sarcosine (Sar) and aminocyclopropanoic acid (Acpc) in place of Gly5, D-valine at position 6, 4-cyanoaminobutyric acid (Cba) in place of Glu13, β^3 -homoarginine (β^3 -Arg) at position 15, *N*-butylglycine (*N*-Nle) and β -Ala replacing Met16, naphthylalanine (Nal) at Trp19, 4-pyridinylalanine (Pal) at position 21, and homophenylalanine (hPhe) in place of Phe23. A final analogue contained three concomitant replacements of Gly2, Gly3, and Gly5 with β -Ala. The triazole-linked LctA substrate analogues (0.5–1.5 mg) were incubated with 0.5 μ M LctM in the presence of 10 mM Mg^{2+} and 1 mM ATP. Assay progress was monitored by MALDI-TOF MS. Each of the unnatural LctA substrates was dehydrated four times by LctM, as shown for representative examples in Figure 2 (all of the MS data is provided in the Supporting Information). Typically, a small amount of threefold-dehydrated product was also present and readily removed via reversed-phase HPLC. To produce bioactive lactacin 481 analogues, the leader peptide and triazole linker were removed

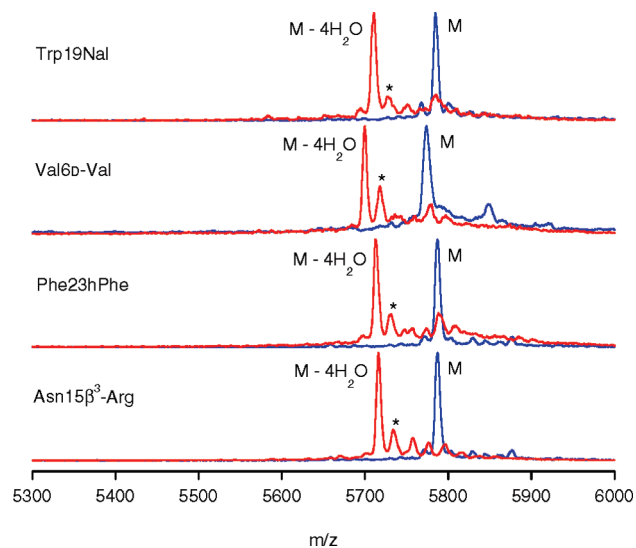


Figure 2. Representative MALDI-MS spectra after incubation of triazole-linked LctA substrate analogues (**3**, blue) with LctM in the presence of ATP and Mg^{2+} (red). Asterisks represent threefold-dehydrated products.

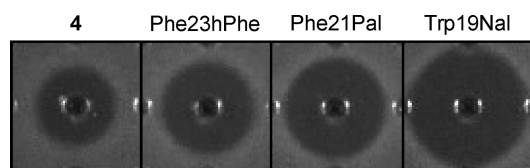


Figure 3. Antimicrobial assays for biological activity of selected lactacin 481 analogues against *L. lactis* HP.

by proteolysis using commercially available endoproteinase LysC, which efficiently cleaved the modified substrates C-terminal to Lys1.¹⁶ Thus, lactacin 481 analogues were produced in high purity (Figure S4 in the Supporting Information). To ensure that complete cyclization had occurred, the analogues were incubated with a thiol-modifying reagent, demonstrating that no free thiols remained in the LctM-treated peptides.

The bioactivity of the analogues was evaluated by agar diffusion assays against the indicator strain *L. lactis* HP (Figure 3). The analogues and the parent compound **4** that did not contain any nonproteinogenic amino acids were spotted against *L. lactis* HP in a 96-well format. For initial characterization, each compound was spotted and the zone of inhibition measured following overnight incubation. Three of the lactacin 481 analogues, the ones containing Trp19Nal, Phe21Pal, and Phe23hPhe mutations, provided zones of inhibition larger than the parent compound **4**, as shown in Figure 3. The Gly5Sar, Gly5Acpc, Met16 β -Ala, and Asn15 β^3 -Arg mutant lantibiotics displayed a significant decrease in activity (~ 16 – 34 fold; see Figure S6), while the Glu13Cba, Met16N-Nle, and Val6D-Val mutants as well as the analogue with three concomitant β -Ala mutations displayed no bioactivity within the limits of the assay. The compounds containing Trp19Nal, Phe21Pal, and Phe23hPhe

mutations were also tested against *Bacillus subtilis* ATCC 6633 and provided trends in activity analogous to those observed with *L. lactis* HP (Figure S7). Minimum inhibitory concentration (MIC) values were determined against *L. lactis* HP for the three variants with improved activity in the agar diffusion assay. The obtained MIC values were $3.12 \mu M$ for Trp19Nal and Phe23hPhe, compared with $6.25 \mu M$ for the parent **4** and $12.5 \mu M$ for Phe21Pal; the improved agar diffusion activity for the latter compound may have been the result of differential diffusion rates.

The results presented here provide guidelines for further engineering. For example, the two analogues with improved bioactivity each contained nonproteinogenic amino acid substitutions in the C-ring of lactacin 481, suggesting this region as a site for additional mutation. Furthermore, each of these analogues contained non-natural side-chain substituents, whereas analogues with an altered amide backbone (β -amino acids or peptoids) had diminished activity. Finally, a dramatic decrease in activity was noted with all mutants containing substitutions in the N-terminal residues of lactacin 481.

In summary, we have presented the first preparation of lantibiotic analogues containing nonproteinogenic amino acids, suggesting that in vitro engineering of lantibiotics can be a viable method for identifying improved bioactive compounds.

Acknowledgment. This work was supported by the National Institutes of Health (GM58822 to W.A.v.d.D.) and a Ruth L. Kirschstein National Research Service Award (T32 to M.R.L.).

Supporting Information Available: Experimental procedures, mass spectral characterization of all compounds and enzymatic assays, and description and results of bioactivity assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Willey, J. M.; van der Donk, W. A. *Annu. Rev. Microbiol.* **2007**, *61*, 477.
- (2) Cotter, P. D.; Hill, C.; Ross, R. P. *Curr. Protein Pept. Sci.* **2005**, *6*, 61.
- (3) Breukink, E.; de Kruijff, B. *Nat. Rev. Drug Discovery* **2006**, *5*, 321.
- (4) Field, D.; Connor, P. M.; Cotter, P. D.; Hill, C.; Ross, R. P. *Mol. Microbiol.* **2008**, *69*, 218.
- (5) Rollema, H. S.; Kuipers, O. P.; Both, P.; de Vos, W. M.; Siezen, R. J. *Appl. Environ. Microbiol.* **1995**, *61*, 2873.
- (6) Fukase, K.; Kitazawa, M.; Sano, A.; Shimbo, K.; Horimoto, S.; Fujita, H.; Kubo, A.; Wakamiya, T.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2227.
- (7) Pattabiraman, V. R.; McKinnie, S. M.; Vederas, J. C. *Angew. Chem., Int. Ed.* **2008**, *47*, 9472.
- (8) van den Hooven, H. W.; Lagerwerf, F. M.; Heerma, W.; Haverkamp, J.; Piard, J. C.; Hilbers, C. W.; Siezen, R. J.; Kuipers, O. P.; Rollema, H. S. *FEBS Lett.* **1996**, *391*, 317.
- (9) Xie, L.; Miller, L. M.; Chatterjee, C.; Averin, O.; Kelleher, N. L.; van der Donk, W. A. *Science* **2004**, *303*, 679.
- (10) Levengood, M. R.; Patton, G. C.; van der Donk, W. A. *J. Am. Chem. Soc.* **2007**, *129*, 10314.
- (11) Patton, G. C.; Paul, M.; Cooper, L. E.; Chatterjee, C.; van der Donk, W. A. *Biochemistry* **2008**, *47*, 7342.
- (12) Zhang, X.; Ni, W.; van der Donk, W. A. *Org. Lett.* **2007**, *9*, 3343.
- (13) Zhang, X.; van der Donk, W. A. *J. Am. Chem. Soc.* **2007**, *129*, 2212.
- (14) Levengood, M. R.; Kerwood, C. C.; Chatterjee, C.; van der Donk, W. A. *ChemBioChem* **2009**, *10*, 911.
- (15) You, Y. O.; van der Donk, W. A. *Biochemistry* **2007**, *46*, 5991.
- (16) The parent analogue **4** differs from authentic lactacin 481 at three positions. In addition to the Asn15Arg and Phe21His mutations, treatment with LysC removes the N-terminal Lys. Lactacin 481 is a factor of 2.5–3 more active than **4**.

JA903239S