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Antimicrobial Leucocin Analogues with a Disulfide Bridge Replaced by a Carbocycle or by Noncovalent Interactions of Allyl Glycine Residues

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Type IIa bacteriocins are antimicrobial peptides from lactic acid bacteria that are useful for food preservation because of their nanomolar activity against pathogens such as *Listeria monocytogenes*.[1,2] They are ribosomally synthesized and have a characteristic YGNGV sequence near the N-terminus as well as a conserved disulfide bond. Leucocin A (LeuA, 37 residues) (**1**) from *Leuconostoc gelidum* was the first type IIa bacteriocin whose sequence was reported (Figure 1),[3] but more than 25 of such "pediocin class" of peptides are now known.[2,4] In all cases, the N-terminal portion (to residue 18) is highly conserved, whereas the C-terminal region has variable sequences. However, this variable section maintains an amphiphilic α-helical structure[5−9] that confers target specificity[10] and is probably involved in binding to a membrane-bound receptor.[4,11−13] Extensive studies indicate that the conserved disulfide bond between residues 9 and 14 is required for biological activity. Substitution of both cysteines in mesentericin Y105 (**2**) with serine[14] leads to nearly complete loss of antimicrobial effects. Similarly, replacement of a single cysteine in pediocin PA1 (**3**) with 11 different amino acids, including serine, glycine, valine, leucine, or phenylalanine abolishes detectable antibiotic activity.[15] Interest in olefin metathesis reactions in peptides[16,17] and in vivo stabilization of peptides by replacement of disulfide moieties with carbocyclic rings[16a] led us to examine formation of an analogue of leucocin A (carba-LeuA) wherein the sulfurs are replaced by an olefin. We also wanted to determine whether the disulfide is directly recognized by the receptor surface. We now report the synthesis of carba-LeuA **4** and the surprising observation that replacement of the disulfide bridge in Leu A with allylglycine residues *without ring formation* generates a derivative (diallyl-LeuA) **5** that has biological activity comparable to the natural parent compound **1**.

Solid supported synthesis of diallyl-LeuA **5** proceeds using standard Fmoc methodology with acid labile side chain protection (*t*-Bu, Boc, Trt) on Novasyn TGA resin (2.5% overall yield; avg 90.5% per residue) after cleavage/deprotection and HPLC purification) (Figure 2). Use of pseudoproline at residues 22−23 helps disrupt self-aggregation that otherwise hinders coupling of residues toward the N-terminus.[18,19] Our previous experience with on-resin ring closing metathesis (RCM) reactions to produce 20-membered ring analogues of oxytocin having an olefin in place of disulfide[16] suggested that the corresponding cyclization of the leucocin A analogue having allylglycines at positions 9 and 14 should proceed readily. However, this is not the case. Complexation of either first or second generation Grubbs catalysts[20,21] by interaction with amide bonds and/or hydrophobic residues in the protected resin-bound peptide completely blocks RCM processes. This leads to recovery of uncyclized material after cleavage from the resin. Extensive trials showed that successful RCM could be achieved by preliminary treatment of the uncyclized resin-bond precursor with 0.8 M LiCl[22] followed by addition of second generation Grubbs catalyst and reflux in 1,2-dichloroethane (83 °C) for 36 h with argon bubbling through the solution.[23] Removal of the catalyst with DMSO[16b]

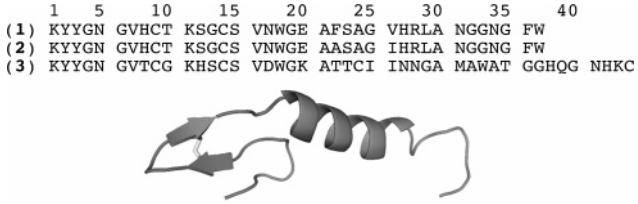

Figure 1. (Top) Type IIa bacteriocins: leucocin A (**1**), mesentericin Y105 (**2**), pediocin PA1 (**3**); all have a disulfide between Cys9 and Cys 14; **3** has an additional disulfide between Cys 24 and Cys 44. (Bottom) Solution structure of leucocin A displaying disulfide bridge and alpha helical portion of the bacteriocin.[5]

followed by acidic cleavage from the resin with concomitant deprotection afforded carba-LeuA **4** in 1.1% overall yield after HPLC purification. Two inseparable double bond isomers could be detected. Their ratio is 2:1 cis/trans based on ^{1}H NMR comparison to analogous carba-oxytocin analogues wherein a 20-membered ring is formed by RCM in a 4:1 cis/trans proportion.[16b] As a control for microbiological studies, the Cys9Ser and Cys14Ser double mutant **6** of LeuA was also synthesized in a fashion analogous to formation of **5**, except that Fmoc-Ser(*t*-Bu)-OH was used in place of Fmoc-L-allylglycine.

All analogues were tested against a variety of bacterial strains sensitive to LeuA (**1**), including *Carnobacterium maltaromaticum* UAL26, *C. divergens* LV13, and *L. monocytogenes* ATCC43256. The leucocin A producing organism, *Leuconostoc gelidum* UAL 187, was used as a control to ensure the peptides were inactive against the producing strain. As expected based on previous reports on mesentericin[14] and pediocin,[15] the C9S, C14S double mutant **6** was completely inactive. The carbocyclic analogue **4** (cis/trans mixture) is approximately an order of magnitude less active than LeuA (**1**) but is still a very potent antibiotic. Thus, the concentration necessary to achieve 50% inhibition of *C. maltaromaticum* in fermentation broth is 370 nM for **4** compared to 35 nM for **1**. The ability of methines to replace sulfur in 20-membered rings in peptides with retention of significant biological activity is in accord with previous studies on oxytocin analogues.[16] Surprisingly, in view of the fact that all other type IIa bacteriocin analogues examined to date which lack the disulfide bridge are inactive,[14,15] the acyclic diallyl-LeuA **5** is nearly as potent an antimicrobial agent (50 nM) as Leu A (**1**). In spot-on-lawn plate assays with a variety of bacterial strains, no difference in activity between **1** and **5** could be detected.

There is substantial evidence that this group of bacteriocins functions by binding to a membrane-bound receptor, namely proteins of the mannose phosphotransferase (*mpt*) system of the target bacteria.[4,11−13] This is probably followed by pore formation or disruption of the cell membrane with leakage of cellular contents. The conserved disulfide bond in type IIa bacteriocins appears to maintain the correct geometry of other residues along the backbone of the N-terminal region necessary for its function. Replacement of sulfurs by methines in **4** approximates the required structure quite

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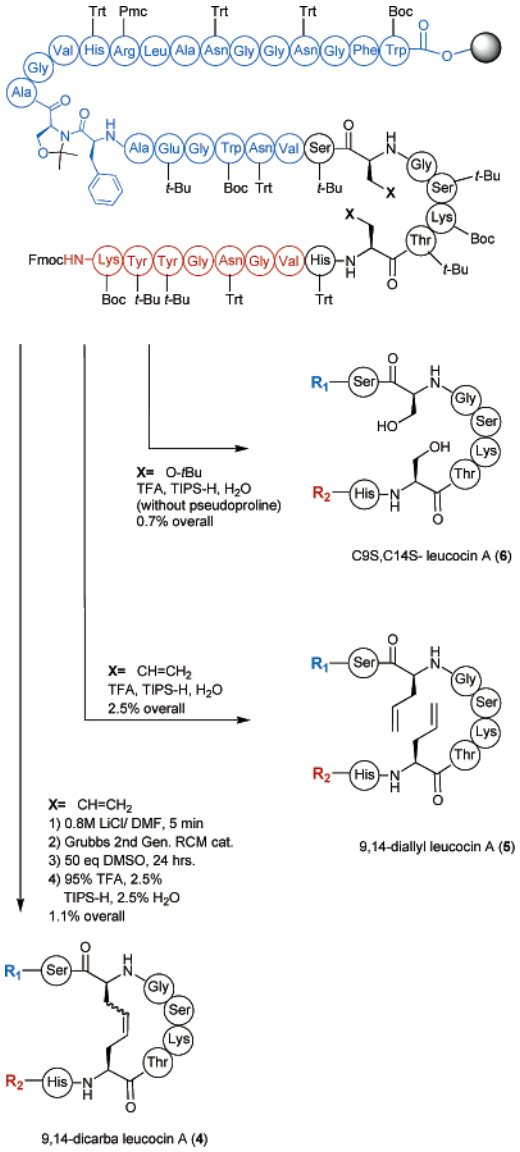

Figure 2. Syntheses of carba-leucocin A **4**, diallyl-leucocin A **5**, and [C9S,C14S]- leucocin A (**6**).

closely, resulting in only a modest reduction of activity. This suggests that the disulfide moiety holds the correct peptide conformation for receptor recognition but does not itself bind directly to the receptor surface. Apparently the hydrophobic interactions of the allyl side chains in **5** not only afford the correct conformation, but also provide sufficient flexibility (slippage) to attain the optimal geometry necessary for full activity comparable to natural leucocin A (**1**). We expect that acyclic diallyl moieties may successfully substitute for disulfide bridges in other peptide and protein systems. Single substitutions of cysteines in pedicocin PA1 (**3**) with hydrophobic residues such as leucine or phenylalanine abolish antimicrobial activity.[15] Similarly, attachment of acetamidomethyl (Acm) groups to both thiols of reduced mesentericin Y105 gives inactive peptide.[14] However, the Acm groups are relatively polar (as are serines) and single substitution may cause a mismatch of the lone hydrophobic residue with the remaining cysteine. It may prove possible to mimic the diallyl effect observed in the present work by dual substitution with other hydrophobic residues. We are currently exploring the scope of allowable replacements for the disulfide bridge in bacteriocins and other bioactive peptides.

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Supporting Information Available: Experimental procedures for peptide synthesis and RCM reaction, spectral data, and protocols for antimicrobial testing. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Van Belkum, M. J.; Stiles, M. E. *Nat. Prod. Rep.* **2000**, *17*, 323−335.
(2) Fimland, G.; Johnsen, L.; Dalhus, B.; Nissen-Meyer, J. *J. Pept. Sci.* **2005**, *11*, 688−696.
(3) Hastings, J. W.; Sailer, M.; Johnson, K.; Roy, K. L.; Vederas, J. C.; Stiles, M. E. *J. Bacteriol.* **1991**, *173*, 7491−7500.
(4) Drider, D.; Fimland, G.; Hechard, Y.; McMullen, L. M.; Prevost, H. *Microbiol Mol. Biol. Rev.* **2006**, *70*, 564−582.
(5) Gallagher, N. L. F.; Sailer, M.; Niemczura, W. P.; Nakashima, T. T.; Stiles, M. E.; Vederas, J. C. *Biochemistry* **1997**, *36*, 15062−15072.
(6) Kaur, K.; Andrew, L. C.; Wishart, D. S.; Vederas, J. C. *Biochemistry* **2004**, *43*, 9009−9020.
(7) Wang, Y.; Henz, M. E.; Fregeau-Gallagher, N. L.; Chai, S.; Yan, L. Z.; Stiles, M. E.; Wishart, D. S.; Vederas, J. C. *Biochemistry* **1999**, *38*, 15438−15447.
(8) Uteng, M.; Hauge, H. H.; Markwick, P. R. L.; Fimland, G.; Mantzilas, D.; Nissen-Meyer, J.; Muhle-Goll, C. *Biochemistry* **2003**, *42*, 11417−11426.
(9) Sprules, T.; Kawulka, K. E.; Gibbs, A. C.; Wishart, D. S.; Vederas, J. C. *Eur. J. Biochem.* **2004**, *271*, 1748−1756.
(10) Jack, R. W.; Wan, J.; Gordon, J.; Harmark. K.; Davidson, B. E.; Hillier, A. J.; Fimland, G.; Blingsmo, O. R.; Sletten, K.; Jung, G.; Nes, I. F.; Nissen- Meyer, J. *Appl. Environ. Microbiol.* **1996**, *62*, 3313−3318.
(11) Yan, L. Z.; Gibbs, A. C.; Stiles, M. E.; Wishart, D. S.; Vederas, J. C. *J. Med. Chem.* **2000**, *43*, 4579−4581.
(12) Dalet, K.; Cenatiempo, Y.; Cossart, P.; Hechard, Y. *Microbiology* **2001**, *147*, 3263−3269.
(13) Ramnath, M.; Arous, S.; Gravesen, A.; Hastings, J. W.; Hechard, Y. *Microbiology* **2004**, *150*, 2663−2668.
(14) Fleury, Y.; Dayem, M. A.; Montagne, J. J.; Chaboisseau, E.; LeCaer, J. P.; Nicolas, P.; Delfour, A. *J. Biol. Chem.* **1996**, *271*, 14421−14429.
(15) Tominaga, T.; Hatakeyama, Y. *Appl. Environ. Microbiol.* **2006**, *72*, 1141−1147.
(16) (a) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas, J. C. *J. Org. Chem.* **2005**, *70*, 7799−7809 and references therein. (b) Stymiest, J. L.; Mitchell, B. F.; Wong, S.; Vederas, J. C. *Org. Lett.* **2003**, *5*, 47−49.
(17) Ghalit, N.; Rijkers, D. T. S.; Liskamp, R. M. J. *J. Mol. Catal. A: Chem.* **2006**, *254*, 68−77 and references therein.
(18) Woehr, T.; Wahl, F.; Nefzi, A.; Rohwedder, B.; Sato, T.; Sun, X.; Mutter, M. *J. Am. Chem. Soc.* **1996**, *118*, 9218−9227.
(19) Sampson, W. R.; Patsiouras, H.; Ede, N. J. *J. Pept. Sci.* **1999**, *5*, 403−409.
(20) Grubbs, R. H. *Tetrahedron* **2004**, *60*, 7117−7140.
(21) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 9606−9614.
(22) Synthesis Notes 3.5. *NovaBiochem Catalog*; EMD Biosciences: San Diego, CA, 2006−2007.
(23) Kazmaier, U.; Hebach, C.; Watzke, A.; Maier, S.; Mues, H.; Huch, V. *Org. Biomol. Chem.* **2005**, *3*, 136−145.

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