Analogues of Bacteriocins: Antimicrobial Specificity and Interactions of Leucocin A with Its Enantiomer, Carnobacteriocin B2, and Truncated Derivatives

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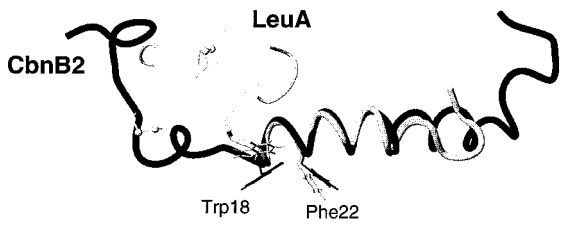
Introduction. Resistance of pathogenic bacteria to conventional antibiotics has encouraged the search for new antimicrobial agents that have alternative targets.[1] Cationic peptides are produced by a large variety of organisms including mammals (e.g. human defensins), amphibians (e.g. magainins), insects (e.g. cecropins, mellitin), plants (e.g. thionins), and bacteria (bacteriocins) and may constitute a key primary defense system against bacterial infection.[2] It has been generally accepted that most such peptides function by insertion into cell membranes of target bacteria with consequent formation of pores or channels that lead to cell leakage and death.[3] On this basis, enantiomeric all-D-amino acid peptides (e.g. cecropin A, magainin, mellitin) with full antimicrobial activity have been synthesized.[4] However, recent work demonstrates that certain naturally occurring peptides require relatively high concentrations to cause leakage in membranes, thereby indicating that at lower, physiologically relevant, concentrations interaction with other cellular targets may be the dominant mechanism of action.[5] In this regard, two lantibiotic bacteriocins,[6] mersacidin and nisin A, have been shown to bind strongly to lipid II, the precursor for peptidoglycan synthesis during bacterial cell wall formation.[7,8] It is likely that in certain cases binding of a chiral cell wall precursor or protein receptor by an antimicrobial peptide may initiate membrane disruption as well as directly inactivate the function of the target.

Type IIa bacteriocins from lactic acid bacteria (LAB), such as leucocin A (LeuA) and carnobacteriocin B2 (CbnB2) (Figure 1), possess considerable potential as nontoxic agents for food preservation. LAB may also act as probiotics for treatment of gastrointestinal infections in humans and animals.[9,10,] Although such bacteriocins disrupt membrane function in model systems and are claimed to form pores by self-association,[9b] substantial differences in their spectra of antimicrobial activity (i.e. susceptible organisms) hint at recognition of a chiral target as a key element of their mechanism of action. Our NMR studies on the solution structures of LeuA and CbnB2 in trifluoroethanol indicate that high sequence homology (67%) in the N-terminal half does not lead to substantial similarity in three-dimensional structures in this section.[11] However, the C-terminal portions, which have much greater variation in amino acid sequence, show highly conserved amphipathic α-helix structures that superimpose readily. Work by others on chimeric type IIa bacteriocins, in which N-terminal and C-terminal portions are interchanged, suggests that the C-terminal section determines antimicrobial specificity.[12] In this study we examine antimicrobial activity and antagonistic interactions of LeuA with peptide analogues, including CbnB2 and *ent*-LeuA (the enantiomer of LeuA), to determine whether chiral recognition of type IIa bacteriocins is a required feature of their mechanism of action.



CbnB2 VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP

LeuA KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW

Ped PA1 KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC

Ped PA1 20-34 Ac-KATTCIINNGAMAWA-NH_2

LeuA 18-37 WGEAFSAGVHRLANGGNGFW

N-Ac-LeuA 18-37 Ac-WGEAFSAGVHRLANGGNGFW

LeuA 18-32 WGEAFSAGVHRLANG

CbnB2 1-22 VNYGNGVSCSKTKCSVNWGQAF

Figure 1. Superimposition of three-dimensional solution structures of LeuA and CbnB2 based on the alignment of backbone atoms from Trp18 to Phe22 (see ref 11) with single-letter amino acid sequences and homology (gray shading) shown below. Sequences of pediocin PA1 (Ped PA1), its inhibitory fragment Ped PA1(20−34),[19] and peptide fragments synthesized in the present study (except for *ent*-LeuA) are also provided.

Results and Discussion. *ent*-LeuA was synthesized by sequential coupling (DCC, HBTU) of Fmoc-protected D-amino acids using Wang resin as a solid support.[13,14] The following side chain protection was used: Arg (Pmc); Asn (Trt); Cys (Trt); Glu (tBu); His (Trt); Lys (Boc); Ser (tBu); Thr (tBu); Trp (Boc); Tyr (tBu). Cleavage with trifluoroacetic acid from the resin followed by HPLC purification gave the reduced peptide (cysteines as thiols, *m/z* 3932.11 ± 0.35, calcd 3932.33), which was quantitatively oxidized with oxygen to the corresponding disulfide, *ent*-LeuA, with an overall synthesis yield of 6% and ≥95% purity. HPLC comparison, electrospray mass spectrometry (*m/z* 3929.95 ± 0.59), N-terminal Edman amino acid sequencing, and CD spectra (mirror image in trifluoroethanol/water (90%) and 4 mM dodecylphosphocholine (DPC) micelles) confirmed the purity and structural identity of the enantiomeric peptide. This represents the first synthesis of an enantiomeric type IIa bacteriocin.

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ent-LeuA was tested for antimicrobial activity as well as possible agonist/antagonist effects with LeuA utilizing 10 strains of bacteria susceptible to the natural peptide.[14,15] Using comparable concentrations, pure *ent*-LeuA showed no antibacterial activity against any strain tested, thereby demonstrating that *interaction with a chiral receptor molecule is a critical feature of the mechanism of action of type IIa bacteriocins*. At very high concentrations (10^5 above minimum inhibitory concentration (MIC) of LeuA),[16] *ent*-LeuA displayed weak activity toward *Carnobacterium divergens* LV13, suggesting that nonspecific membrane disruption of the target organism could occur under these conditions. *ent*-LeuA was not able to antagonize the antimicrobial effect of natural LeuA at any concentration tested. A 10-fold excess with respect to the natural peptide did cause small decreases in MIC values of natural LeuA with about one-half of the strains examined. This may also be due to nonspecific effects on the membrane. These results contrast a report on the action of another class of less-active LAB bacteriocin, plantaricin A (26 amino acids, from *Lactobacillus plantarum*), which also assumes an amphiphilic α-helical conformation in TFE or DPC micelles.[17] Synthetic L- and D-enantiomers (PlnA-22L and PlnA-22D) missing the four N-terminal residues of plantaricin A both display full antimicrobial activity. Interestingly, PlnA-22D does not show the pheromone activity (induction of bacteriocin production) of plantaricin A or PlnA-22L, but it is able to inhibit the pheromone induction caused by the L-enantiomers at high concentration, thereby demonstrating that this is a receptor-mediated function. However, it should be noted that some all-D-amino acid peptides are biologically active and bind to receptor proteins normally targeted by natural L-peptides.[18]

It was recently reported that a 15-mer analogue (residues 20−34 with N-terminal acetyl and C-terminal primary amide) of pediocin PA1, a bacteriocin with considerable sequence homology to LeuA and CbnB2, was not antimicrobially active but significantly inhibited the activity of pediocin PA1 (ca. 20-fold increase in MIC).[19] This result also suggests that the 15-mer binds to a chiral receptor molecule which recognizes the corresponding portion of the parent bacteriocin. Hence, we employed L-amino acids to synthesize the corresponding C-terminal fragments of LeuA, namely LeuA(18−32), LeuA(18−37), and N-Ac-LeuA(18−37), by methods similar to those for *ent*-LeuA. An N-terminal fragment of CbnB2, CbnB2(1−22), was also prepared. None of these fragments displayed antimicrobial activity by themselves. When mixed in equal amounts with natural LeuA, the LeuA fragments weakly inhibited the activity of LeuA (2-fold increase in MIC), but less than was seen in the pediocin PA1 work.[19] The N-terminal fragment CbnB2(1−22) did not inhibit the activity of the parent CbnB2 even when present in 1000-fold excess. Since LeuA and CbnB2 have different but overlapping antimicrobial spectra,[14] interactions between the parent bacteriocins were examined. Only weak effects were seen in certain cases. Addition of a 10-fold molar excess of CbnB2 to LeuA increased the specific activity of the latter 2−4-fold against *Leuconostoc mesenteroides* 23386 and *Lactobacillus sakei* 20017, which are insensitive to CbnB2 alone. In contrast, with *Carnobacterium piscicola* UAL26/8B addition of a 1000-fold molar excess of CbnB2 reduced the specific activity of LeuA 16-fold. The results show that there is a requirement for a chiral receptor molecule for type IIa bacteriocins that probably recognizes the α-helical C-terminal half of the peptide and that this specific interaction accounts for the very potent but relatively narrow antimicrobial spectrum.

Antimicrobial peptides and the organisms that produce them are being investigated as possible therapeutic agents, especially for the treatment of gastrointestinal infections and organisms resistant to current antibiotics.[6−9] Even though the antimicrobial spectra of LAB bacteriocins is limited, they show considerable promise because they are nontoxic to mammals (many occur naturally on food) and are much more potent than conventional antibiotics. The present work confirms earlier studies that show that for type IIa bacteriocins, the entire peptide is necessary for significant activity. More importantly, the demonstration of a requirement for interaction with a chiral receptor molecule affords a novel possibility for future antibiotic development. Very recent genetic experiments on resistance to bacteriocins provide circumstantial evidence that the protein components of the sugar phosphotransferase systems (PTS) may be the target of these peptides.[20] Additional studies to confirm this hypothesis are currently underway.

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Supporting Information Available: Experimental procedures for peptide syntheses, microbiological studies, and characterization (HPLC, MS, CD) of *ent*-LeuA. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Levy, S. B. The challenge of antibiotic resistance. *Sci. Am.* **1998**, *278*, 46−53. (b) Domagala, J. M.; Sanchez, J. P. New approaches and agents to overcome bacterial resistance. *Annu. Rep. Med. Chem.* **1997**, *32*, 111−120. (c) Henry, C. M. Antibiotic resistance. *Chem. Eng. News* **2000**, *March 6*, 41−58.

(2) (a) *Antimicrobial Peptides*; Boman, H. G., Marsh, J., Goode, J. A., Eds.; Wiley: New York, 1994. (b) Nissen-Meyer, J.; Nes, I. F. Ribosomally synthesized antimicrobial peptides. Their function, structure, biogenesis, and mechanism of action. *Arch. Mirobiol.* **1997**, *167*, 67−77.

(3) (a) Epand, R. M.; Shai, Y.; Segrest, J. P.; Anantharamaiah, G. M. Mechanisms for the modulation of membrane bilayer properties by amphipathic helical peptides. *Biopolymers* **1995**, *37*, 319−338. (b) Subczynski, W. K.; Lewis, R. N. A. H.; McElhaney, R. N.; Hodges, R. S.; Hyde, J. S.; Kusumi, A. Molecular organization and dynamics of 1-palmitoyl-2-oleoylphosphatidylcholine bilayers containing a transmembrane α-helical peptide. *Biochemistry* **1998**, *37*, 3156−3164. (c) Matsuzaki, K.; Mitani, Y.; Akayada, K.; Murase, O.; Yoneyama, S.; Zasloff, M.; Miyajima, K. Mechanism of synergism between antimicrobial peptides magainin 2 and PGLa. *Biochemistry* **1998**, *37*, 15144−15153. (d) Moll, G. N.; Konings, W. N.; Driessen, A. J. M. Bacteriocins: mechanism of membrane insertion and pore formation. *Antonie van Leeuwenhoek* **1999**, *76*, 185−198.

(4) (a) Wade, D.; Bowman, A.; Wahlen, B.; Drain, C. M.; Andreu, D.; Bowman, H. G.; Merrifield, R. B. All-D-amino acid-containing channel-forming antibiotic peptides. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4761−4765. (b) Maloy, W. L.; Kari, U. P. Structure−activity studies on magainins and other host defense peptides. *Biopolymers* **1995**, *37*, 105−122 and references therein.

(5) Wu, M.; Maier, E.; Benz, R.; Hancock, R. E. W. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* **1999**, *38*, 7235−7242.

(6) For reviews of lantibiotics see: (a) van Kraaij, C.; de Vos, W. M.; Siezen, R.; Kuipers, O. Lantibiotics: biosynthesis, mode of action and applications. *Nat. Prod. Rep.* **1999**, *16*, 575−587. (b) Guder, A.; Wiedemann, I.; Sahl, H.-G. Posttranslationally modified bacteriocins − the lantibiotics. *Biopolymers* **2000**, *55*, 62−73.

(7) Brötz, H.; Bierbaum, G.; Leopold, K., Reynolds, P. E.; Sahl, H. G. The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrob. Agents Chemother.* **1998**, *42*, 154−160.

(8) Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O. P.; Sahl, H. G.; de Kruijff, B. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **1999**, *286*, 2361−2364.

(9) For reviews see: (a) Stiles, M. A. Biopreservation by lactic acid bacteria. *Antonie van Leeuwenhoek* **1996**, *70*, 331−345. (b) Ennahar, S.; Sashihara, T.; Sonomoto, K.; Ishizaki, A. Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol. Rev.* **2000**, *24*, 85−106. (c) Nes, I. F.; Holo, H. Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* **2000**, *55*, 50−61.

(10) (a) *Probiotics*; Tannock, G. W., Ed.; Horizon Scientific Press: Norfolk, U.K., 1999. (b) *Lactic Acid Bacteria: Genetics, Metabolism and Applications*; Konings, W. N., Kuipers, O. P., Huis in t'Veld, J. H. J., Eds.; Kluwer Academic Publishers: Norwell, MA, 1999.

(11) (a) Wang, Y.; Henz, M. E.; Fregeau Gallagher, N. L.; Chai, S.; Gibbs, A. C.; Yan, L. Z.; Stiles, M. E.; Wishart, D. S.; Vederas, J. C. Solution Structure of Carnobacteriocin B2 and Implications for structure−activity relationships among type IIa bacteriocins from lactic acid bacteria. *Biochemistry* **1999**, *38*, 15438−15447. (b) Fregeau Gallagher, N. L.; Sailer, M.; Niemczura, W. P.; Nakashima, T. T.; Stiles, M. E.; Vederas, J. C. Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. *Biochemistry* **1997**, *36*, 15062−15072.

(12) Fimland, G.; Blingsmo, O.; Sletten, K.; Jung, G.; Nes, I. F.; Nissen-Meyer, J. New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region is important for determining dpecificity. *Appl. Environ. Microbiol.* **1996**, *62*, 3313−3318.

(13) Bodanszky, M. *Principles of Peptide Synthesis*; Springer-Verlag: Berlin, 1993.

(14) See Supporting Information for experimental details.

(15) The bacteria examined were *Carnobacterium divergens* LV13, *Carnobacterium piscicola* N5, *Carnobacterium piscicola* LV17A, *Carnobacterium piscicola* LV17B (Bac+ and Bac−), *Enterococcus faecium* BFE 900, *Leuconostoc gelidum* UAL187, *Leuconostoc gelidum* UAL187.13, *Leuconostoc mesenteroides* 23386, *Lactobacillus sakei* 20017, and *Listeria monocytogenes* LI0502. See Supporting Information.

(16) MIC of natural LeuA toward a typical target such as *C. divergens* LV13 is 11 ng/mL.

(17) Hauge, H. H.; Mantzilas, D.; Moll, G. N.; Konings, W. N.; Driessen, A. J. M.; Eijsink, V. G. H.; Nissen-Meyer, J. Plantaricin A is an amphiphilic α-helical bacteriocin-like pheromone which exerts antimicrobial and pheromone activities through different mechanisms. *Biochemistry* **1998**, *37*, 16026−16032.

(18) Li, C.; McCarthy, J. B.; Furcht, L. T.; Fields, G. B. An all-D amino acid peptide model of α1(IV)531−543 from type IV collagen binds the $\alpha_3\beta_1$ integrin and mediates tumor cell adhesion, spreading, and motility. *Biochemistry* **1997**, *36*, 15404−15410.

(19) Fimland, G.; Jack, R.; Jung, G.; Nes, I. F.; Nissen-Meyer, J. The bactericidal activity of pediocin PA-1 is specifically inhibited by a 15-mer fragment that spans the bacteriocin from the center toward the C terminus. *Appl. Environ. Microbiol.* **1998**, *64*, 5057−5060.

(20) (a) Gravesen, A.; Warthoe, P.; Knochel, S.; Thirstrup, K. Restriction fragment differential display of pediocin-resistant Listeria monocytogenes 412 mutants shows consistent overexpression of a putative beta-glucoside-specific PTS system. *Microbiology* **2000**, *146*, 1381−1389. (b) Dalet, K.; Héchard, Y. Resistance towards class IIa bacteriocins, a sigma 54 story. Abstract at Workshop on Bacteriocins of Lactic Acid Bacteria, Banff, Alberta, Canada, May 2000. (c) Ramnath, M.; Beukes, M.; Tamura, K.; Hastings, J. W. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis. *Appl. Environ. Microbiol.* **2000**, *66*, 3098−3101.

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