JOURNAL OF
MEDICINAL
CHEMISTRY

© Copyright 1996 by the American Chemical Society

Volume 39, Number 19 September 13, 1996

Communications to the Editor

Antimicrobial α,α-Dialkylated Amino Acid Rich Peptides with *in-Vivo* Activity against an Intracellular Pathogen

T. Scott Yokum,† Philip H. Elzer,*,‡ and Mark L. McLaughlin*,†

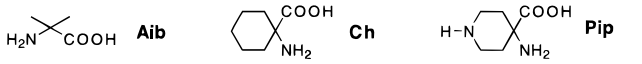
Departments of Chemistry and Veterinary Science, Louisiana State University, Baton Rouge, Louisiana 70803-1804

Received June 24, 1996

The development of antibiotic resistance is threatening a return to the preantibiotic era.[1] This threat is hastened by the reliance on only a handful of distinct mechanisms of action among the vast majority of the commercially available drugs.[2] This problem alone is sufficient to warrant development of novel antimicrobial agents with unique modes of action.[3] In addition, new methods of controlling intracellular pathogens are needed because conventional antibiotic therapy and the host's immune response are not very effective against sequestered microorganisms.[4] *Brucella abortus* is an intracellular pathogen which lives and replicates in macrophages and quickly establishes chronic experimental infections in mice.[5] Herein, we report the *in-vitro* antibacterial and *in-vivo* anti-brucellae activity of a series of peptides (**1**−**10**).

Peptides **2**−**10** are composed of 50−80% α,α-dialkylated amino acids. The design of these peptides is loosely based on the natural antimicrobial peptides peptaibols.[6−8] Peptaibols have several α-aminoisobutyric acid (Aib) residues, are acetylated on the N-terminus, and have an amino alcohol at the C-terminus.[7] *De-novo* peptides that include only a putative amphipathic helix have activity comparable to or greater than that of native antimicrobial peptides.[10] Amphipathic peptides with ≥18 residues can have very high cytotoxicity,[11] but simply shortening the peptides to 14 residues reduces cytotoxicity and retains much of the antimicrobial activity.[12] Peptides **2**−**10** were synthesized to test the hypothesis that the Aib or Aib-like residues would stabilize helical conformations and retain biological activity as the peptides were further shortened.[13] In addition, peptides with several α,α-dialkylated amino acids throughout the sequence were expected to be resistant to enzymatic hydrolysis which is likely to enhance *in-vivo* activity.[14] Combinations of lysine with Aib or 1-amino-1-cyclohexanecarboxylic acid (Ch) and combinations of lysine with Aib and the novel amino acid 4-aminopiperidine-4-carboxylic acid[15] (Pip) were incorporated into peptides **2**−**10**.

1	Al-14	LysAlaAlaLysLysAlaAlaLysAlaAlaLysLysAlaAla-NH_2
2	Ai-14	Lys**AibAib**LysLys**AibAib**Lys**AibAib**LysLys**AibAib**-NH_2
3	Ai-11	LysLys**AibAib**Lys**AibAib**LysLys**AibAib**-NH_2
4	Ai-11-ac	Ac-LysLys**AibAib**Lys**AibAib**LysLys**AibAib**-NH_2
5	Ch-14	Lys**ChCh**LysLys**ChCh**Lys**ChCh**LysLys**ChCh**-NH_2
6	Ch-11	LysLys**ChCh**Lys**ChCh**LysLys**ChCh**-NH_2
7	Ch-13	Lys**ChCh**LysLys**ChCh**Lys**ChCh**LysLys**Ch**-----NH_2
8	Ch-10	LysLys**ChCh**Lys**ChCh**LysLys**Ch**-----NH_2
9	Pi-10	**AibAibPip**Lys**AibAibPip**Lys**AibAib**-NH_2
10	Pi-10-ac	Ac-**AibAibPip**Lys**AibAibPip**Lys**AibAib**-NH_2



Until recently, the use of Aib and Aib-like residues was limited due to the difficulty of coupling or harsh conditions used for their incorporation. Carpino's development of new coupling methods have allowed simple solid-phase synthesis of Aib or Aib-like rich peptides.[16] Peptide **1** was synthesized using standard Fmoc solid-phase synthesis methods.[12] Peptides **2**−**10** were synthesized via Fmoc chemistry using preformed acid fluorides on a PAL resin to yield the C-terminus amides. The only synthetic difficulty encountered came in the coupling of the second residue in the Ch-11,14 peptides. Fortunately, the major peptides obtained were also amphipathic and showed good *in-vitro* antibacterial activity. To maximize the number of α,α-dialkylated amino acids and retain a high level of positive charge

* Corresponding authors.
† Department of Chemistry.
‡ Department of Veterinary Science.

S0022-2623(96)00451-7 CCC: $12.00 © 1996 American Chemical Society

Table 1. Peptide Antibacterial Activity[a] and Percent Helicity

peptide	*E. coli*	*S. aureus*	% helicity[b]
Al-14	>178	>178	ND[c]
Ai-14	5.5	11	42
Ai-11	55	>220	35
Ai-11-ac	6.6	212	ND[d]
Ch-13	4.5	2.2	61
Ch-11	5.2	2.6	ND[d]
Ch-10	5.7	2.8	43
Pi-10	7.7	123	43

[a] These MIC (in μM) are corrected for the actual peptide concentration using quantitative amino acid analysis according to ref 12. [b] The percent α-helix = $-100([\theta]_{222} + 3000)/33000$; $[\theta]_{222} = [\theta]_{obs}$(MRW/10lc) according to ref 22 taken in 25 mM SDS micelles with 100−200 μM peptide. [c] This peptide precipitates when the 25 mM SDS is added, precluding CD spectroscopic measurement. [d] Insufficient quantities of these peptides were made for CD spectroscopic measurements.

Table 2. Peptide-Mediated *B. abortus* Reductions in Chronically Infected BALB/c Mice

treatment (dose)	% bacterial reduction	significance[a]
Ai-14 (500 μg)	73	<0.05
Ai-11 (500 μg)	63	<0.05
Ai-11-ac (500 μg)	30	<0.05
Pi-10 (500 μg)	90	<0.05
Al-14 (1 mg)	−6	NS

[a] NS means not significant.

on the polar face of these peptides, the lysine-like α,α-dialkylated amino acid Pip is introduced. This residue with the γ-amine protected as the Boc derivative allows the synthesis of an amphipathic peptide containing no natural amino acids. We have not yet attempted to synthesize a peptide devoid of natural amino acids because of the perceived problems in the synthesis and characterization. A peptide with only achiral α,α-dialkylated amino acids would show no CD spectra because there would be no preference for a left- or right-handed helix.

The replacement of Ala residues in Al-14 with Aib residues in Ai-14 has a substantial effect on the biological activity. MIC assays were done with *Escherichia coli* and *Staphylococcus aureus*.[12] Ai-14 is at least 8−16 times more active than Al-14. Al-14 precipitates out of 25 mM SDS micelles, so a comparison of secondary structure is precluded. Shortening Ai-14 to make Ai-11 showed diminished but substantial activity against *E. coli* and no activity against *S. aureus*. While Aib helps to promote helical conformations, it is not a very hydrophobic residue. The Ch residue was introduced to determine whether greater hydrophobic character would increase the activity of peptides with otherwise similar designs. Unfortunately, sufficient amounts of the analogous peptide sequence were not produced due to coupling problems with the second Ch residue from the C-terminus. Ch-13 had essentially the same activity as its closest Aib peptide counterpart, Ai-14, but Ch-10 with only 10 residues had increased activity over Ch-13 and much higher activity than its closest Aib peptide counterpart, Ai-11. Pi-10 with only 10 residues and only two natural amino acids also showed good antibacterial activity against *E. coli* and moderate activity against *S. aureus*.

In-vivo peptide activity was tested using BALB/c mice infected with a virulent strain of *B. abortus*. Both the humoral and cell-mediated immune responses developed 3−4 weeks after the infection of the mice.[5] Both of these responses are needed to control the infection, and seldom do they eliminate the infection because the organism is hidden within the macrophages.[17−19] The mice were treated intravenously with peptide, and 24 h later the spleens of the treated and control (saline) animals were cultured for viable organisms.[20] Percent bacterial reduction was determined by subtracting the total number of organisms in the treated spleens from the total number of organisms in the control spleens. This difference was divided by the total number of organisms in the control spleens and multiplied by 100. Infected mice treated with 100 μg of Ch-10, Ch-13, or Pi-10 showed no significant differences compared to the controls. However infected mice treated with 100 μg of the Ai series show some reductions compared to the controls, but the reductions were not statistically significant. Noninfected mice were unharmed, but infected mice treated with a single 500 μg dose of Ch-10 or Ch-13 died immediately after treatment. As shown in Table 2, this dose of Pi-10 and the Ai peptides resulted in significant reductions of bacterial load compared to the controls. *B. abortus* is one of the few bacteria that are resistant to the direct antimicrobial effect of these peptides.[12,21] We speculate that the peptides are selectively lysing the infected macrophages exposing a portion of the *B. abortus* load to the immune system, which is then destroying the released bacteria. *In-vitro* peptide testing with infected and noninfected macrophages shows a selective destruction of infected macrophages.[21]

The selective destruction of a wide range of bacteria by amphipathic peptides relative to mammalian cells has been attributed to distinct differences in the cell membranes.[12] Bacteria have negatively charged phospholipids on the exterior membrane. Normal mammalian cells do not. Thus, one potential reason for the selective destruction of *B. abortus*-infected macrophages could be a greater negative charge on the exterior membrane of infected macrophages. Future studies are directed toward defining what membrane differences result in the selective destruction of infected macrophages.

Acknowledgment. We greatly appreciate the technical assistance of Ms. Martha M. Juban, Ms. Sue D. Hagius, and Ms. Paula A. Young. We thank Dr. Guillermo A. Morales, Mr. Chrisopher L. Wysong, Ms. Rebekah L. Gundry, and Prof. Robert P. Hammer for help with the synthesis of 1-Boc-4-Fmoc(amino)piperidine-4-carboxylic acid. We thank the National Science Foundation and the Louisiana Education Quality Support Fund for support of this research via the NSF/EPSCoR Grant [(RII/EPSCoR)LEQSF(1992−96)-ADP-01].

Supporting Information Available: *B. abortus* normal macrophage (Table A) and *B. abortus*-infected macrophage (Table B) susceptibility to peptide treatment, synthesis of 1-Boc-4-Fmoc(amino)piperidine-4-carboxylic acid, and a representative synthesis of an acid fluoride (5 pages). Ordering information is given on any current masthead page.

References

(1) Travis, J. Reviving the Antibiotic Miracle. *Science* **1994**, *264*, 360−362.
(2) Davies, J. Inactivation of Antibiotics and the Dissemination of Resistance Genes. *Science* **1994**, *264*, 375−382.
(3) Gabay, J. E. Ubiquitous Natural Antibiotics. *Science* **1994**, *264*, 373−374.

(4) Moulder, J. W. Comparative Biology of Intracellular Parasitism. *Microbiol. Rev*. **1985**, *49*, 298−337.
(5) Araya, L. N.; Elzer, P. H.; Rowe, G. E.; Enright, F. M.; Winter, A. J. Temporal Development or Protective Cell-Mediated and Humoral Immunity in BALB/c Mice Infected with *Brucella abortus*. *J. Immumol*. **1989**, *53*, 3330−3337.
(6) Benedetti, E.; Bavoso, A.; Di Blasio, B.; Pavone, V.; Pedone, C.; Toniolo, C.; Bonora, G. M. Peptaibol Antibiotics: A Study on the Helical Structure of the 2−9 Sequence of Emerimicins III and IV. *Proc. Natl. Acad. Sci. U.S.A*. **1982**, *79*, 7951−7954.
(7) Nagaraj, R.; Balaram, P. Alamethicin, a Transmembrane Channel. *Acc. Chem. Res*. **1981**, *14*, 356−362.
(8) Sansom, M. S. P. The Biophysics of Peptide Models of Ion Channels. *Prog. Biophys. Mol. Biol*. **1991**, *55*, 139−236.
(9) (a) Stewart, J. M. Amphipathic Helices in Designed Peptide Structures. In *The Amphipathic Helix*; Epand, R. M., Ed.; CRC Press: Boca Raton, FL, 1993; pp 21−37. (b) Saberwal, G.; Nagaraj, R. Cell-Lytic and Antibacterial Peptides that Act by Perturbing the Barrier Functions of Membranes: Facets of Their Conformational Features, Structure-Function Correlations and Membrane-Perturbing Abilities. *Biochim. Biophys. Acta* **1994**, *1197*, 109−131.
(10) (a) Lee, S.; Mihara, H.; Aoyagi, H.; Kato, T.; Izumuja, N.; Yamasaki, N. Relationship Between Antimicrobial Activity and Amphiphillic Property. *Biochim. Biophys. Acta* **1986**, *862*, 211−219. (b) Blondelle, S. E.; Houghten, R. A. Design of Model Amphipathic Peptides Having Potent Antimicrobial Activity. *Biochemistry* **1992**, *31*, 12688−12694.
(11) Cornut, I.; Buttner, K.; Dasseux, J.-L.; Dufourcq, J. The Amphipathic Helix Concept. Application of the *De Novo* Design of Ideally Amphipathic Leu, Lys Peptides with Hemolytic Activity Higher than that of Melittin. *FEBS Lett*. **1994**, *349*, 29−33.
(12) Javadpour, M. M.; Juban, M. M.; Lo, W.-C. J.; Bishop, S. M.; Alberty, J. B.; Cowell, S. M.; Becker, C. L.; McLaughlin, M. L. *De Novo* Antimicrobial Peptides with Low Mammalian Cell Toxicity. *J. Med. Chem*. **1996**, *39*, 3107−3113.
(13) (a) Degrado, W. F.; Raleigh, D. P.; Handel, T. *De novo* Protein Design: What Are We Learning? *Curr. Opin. Struct. Biol*. **1991**, *1*, 984−993. (b) Karle, I. L.; Balaram, P. Structural Characteristics of α-Helical Peptide Molecules Containing Aib Residues, *Biochemistry* **1990**, *29*, 6747−6756. (c) Balaram, P. Non-Standard Amino Acids in Peptide Design and Protein Engineering. *Curr. Opin. Struct. Biol*. **1992**, *2*, 845−851.
(14) Spatola, A. Peptide Backbone Modifications: A Structure-Activity Analysis of Peptides Containing Amide Bond Surrogates, Conformational Constraints, and Backbone Replacements. In *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*; Weinstein, B., Ed.; Dekker: New York, 1983; Vol. VII, pp 267−357.
(15) Please see Supporting Information and: Wysong, C. L.; Yokum, T. S.; Morales, G. A.; Gundry, R. L.; McLaughlin, M. L.; Hammer, R. P. Unpublished results.
(16) (a) Wenschuh, H.; Beyermann, M.; Krause, E.; Brudel, M.; Winter, R.; Schumann, M.; Carpino, L.; Bienert, M. Fmoc Amino Acid Fluorides: Convenient Reagents for the Solid-Phase Assembly of Peptides Incorporating Sterically Hindered Residues. *J. Org. Chem*. **1994**, *59*, 3275−3280. (b) Wenschuh, H.; Beyermann, M.; Haber, H.; Seydel, J. K.; Krause, E.; Bienert, M.; Carpino, L.; El-Faham, A.; Albericio, F. Stepwise Automated Solid Phase Synthesis of Naturally Occurring Peptaibols Using FMOC Amino Acid Fluorides. *J. Org. Chem*. **1995**, *60*, 405−410.
(17) Jones, S. M.; Winter, A. J. Survival of Virulent and Attenuated Strains of *Brucella abortus* in Normal and Gamma Interferon-Activated Murine Peritoneal Macrophages. *Infect. Immun*. **1992**, *60*, 3011−3014.
(18) Baldwin, C. L.; Winter, A. J. Macrophages and Brucella. In *Macrophage-Pathogen Interactions*; Zwilling, B. S., Eisenstein, T. K., Eds.; Marcel Dekker: New York, 1994; pp 363−380.
(19) Elzer, P. H.; Jacobson, R. H.; Jones, S. M.; Nielsen, K. H.; Douglas, J. T.; Winter, A. J. Antibody-Mediated Protection Against *Brucella abortus* in BALB/c Mice at Sucessive Periods After Infection: Variation Between Virulent Strain 2308 and Attenuated Vaccine Strain 19. *Immunology* **1994**, *82*, 551−558.
(20) Monteraz, J. A.; Winter, A. J. Comparison of Living and Nonliving Vaccines for *Brucella abortus* in BALB/c mice. *Infect. Immun*. **1986**, *53*, 245−251.
(21) The BALB/c mice weighed 20−22 g. *In-vitro* peptide treatment shows that *B. abortus*-infected macrophages are selectively killed. Please see Supporting Information for details.
(22) McLean, L. R.; Hagaman, K. A.; Owen, T. J.; Krstenansky, J. L. Minimal Peptide Length for Interaction of Amphipathic α-Helical Peptides with Phosphatidylcholine Liposomes. *Biochemistry* **1991**, *30*, 31−37.

JM960451N