Amonabactin: Characterization of a Series of Siderophores from *Aeromonas hydrophila*

Jason R. Telford,† Julie A. Leary,† Linda M. G. Tunstad,† B. Rowe Byers,‡ and Kenneth N. Raymond*,†

Department of Chemistry
University of California
Berkeley, California 94720
Department of Microbiology
University of Mississippi Medical Center
2500 North State Street
Jackson, Mississippi 39216

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Iron is increasingly implicated as a major determinant of bacterial infection.[1] In an aerobic environment, iron concentrations are limited to 10^{-18} M by the insolubility of $Fe(OH)_3$. However, in biological systems, available iron is moderated to even lower concentrations by protein-dependent sequestering agents, such as transferrin and lactoferrin.[2,3] The consequence of freely available iron *in vivo* is a loss of the bacteriostatic nature of serum.[4] Pretreatment of mice with the siderophore desferrioxamine plus iron reduced the LD_{50} dose of *Yersinia enterocolitica* from more than 10^8 to less than 10 organisms.[5] Iron has also been shown to be the regulatory stimulus for a number of virulence factors from several bacterial genera such as *Yersinia*,[6] *Corynebacteria*,[7] *Mycobacterium*,[8] *Vibrio*,[9] and others. Additionally, several plasmids which are associated with virulence carry genes encoding synthesis of siderophores.[10]

Siderophores are low-molecular-mass compounds secreted by microorganisms in response to low iron conditions.[11] These chelating agents strongly and specifically bind, solubilize, and deliver iron to the iron-depleted cells via specific cell surface receptors. The siderophores of pathogens, *in vivo*, must be capable of extracting iron at significant rates against a strong free energy bias.[12]

The aeromonads are mesophilic, Gram-negative, fresh water, opportunistic pathogens causing a wide range of diseases in poikilothermic and homeothermic animals. These range from red leg and furunculosis in frogs and fish to septicemia and soft tissue infections in mammals. They produce a hemolysin under iron stress,[13] which is important to virulence, and most produce one of two siderophores: either enterobactin or amonabactin.[14] The amonabactins were originally isolated, and given their trivial name, by Barghouthi et al.[15] Their early characterization of these siderophores indicated two compounds, containing glycine, lysine, and either tryptophan or phenylalanine, and catechol.

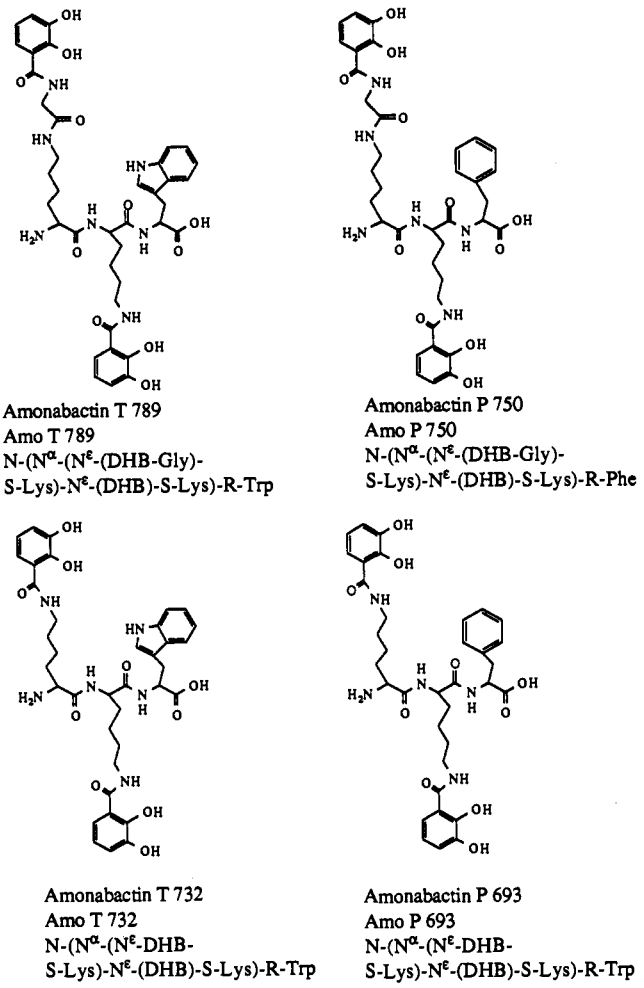

Figure 1.

We have elucidated the structures of this novel family of siderophores. To characterize the amonabactins, *Aeromonas hydrophila* was cultured in a low-iron medium supplemented with either 3 mM L-tryptophan or 30 mM L-phenylalanine.[16] Amonabactin was purified by polyamide chromatography, followed by HPLC.[17] It was found that, instead of two amonabactins as originally thought (a tryptophan- and a phenylalanine-containing form), there are four varieties, with either tryptophan or phenylalanine, and either containing glycine or being a glycine-deleted form. The full structures of the amonabactins have been deduced using amino acid analysis, tandem mass spectroscopy, chiral GC–MS, and 2-D NMR. As a final proof, each of the four amonabactins was synthesized and their spectral properties were compared with those of the natural product.[18]

* Author to whom correspondence should be addressed.
† University of California.
‡ University of Mississippi Medical Center.

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(16) This is a modification of the medium used in ref 15 and is composed of (per liter) glucose, 10 g; $(NH_4)_2HPO_4$, 1 g; K_2HPO, 4 g; KH_2PO_4, 2.7 g; $Mn(SO_4)$, 0.007 g; and $Mg(SO_4)$, 0.127 g.
(17) Rainin HPLC system with a C18 reverse phase column. The mobile phase consisted of 28% acetonitrile, 72% water, each with 0.1% trifluoroacetic acid.

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Tandem mass spectra of the purified amonabactins[19] (MH^+ = 790, Amo T 789; MH^+ = 733, Amo T 732; MH^+ = 751, Amo P 750; MH^+ = 694, Amo P 693) indicated a linear peptide of the sequence DHB-(Gly)-Lys-DHB-Lys-Trp(or Phe) (where DHB is 2,3-dihydroxybenzoic acid).

The phenylalanine-containing amonabactins were first synthesized with an assumption of standard peptide linkages and all L-amino acids. However, their spectral signatures were not identical to those of the natural products. The differences could arise from either the backbone linkage or the chirality of the amino acids. Supplementation studies with L and D amino acids were inconclusive. Hence, the chirality of the amino acids was determined by direct methods; the peptides were hydrolyzed in 6 N HCl, and the amino acids were converted to the *N*-trifluoroacetyl *O*-isopropyl derivatives.[20] These were then analyzed by chiral GC–MS.[21] In all cases, the lysines were of the L form and the aromatic amino acid was of the D form.

2D-NMR, utilizing an HMBC pulse sequence,[22] was then used to resolve whether the peptide backbone was linked through the α or ϵ amines of lysine. Cross peaks due to $^{2,3}J_{C,H}$ between chiral protons, glycine protons, of C^{ϵ} protons and either DHB or amino acid carbonyl carbons are sufficient to establish connectivities. For Amo T 789, the N terminus lysine chiral proton shows a $^{2}J_{C,H}$ cross peak to its own carbonyl carbon. The C terminus lysine shows a $^{2}J_{C,H}$ coupling to its own carbonyl carbon and a $^{3}J_{C,H}$ coupling to the N terminus lysine carbonyl carbon. The tryptophan chiral proton shows a $^{2}J_{C,H}$ coupling to its own carbonyl and a $^{3}J_{C,H}$ coupling to the C terminus lysine carbonyl carbon. Glycyl protons were coupled to a DHB carbonyl and the glycyl carbonyl carbons. The C^{ϵ} protons of the C terminus lysine were coupled to a DHB carbonyl carbon, and the C^{ϵ} protons of the N terminus lysine showed cross peaks due to $^{3}J_{C,H}$ coupling to the glycyl carbonyl carbon. This architecture is seen in the other amonabactins as well. This allowed the unambiguous assignment of the structures in Figure 1.

In the final proof, the amonabactins were synthesized and their spectroscopic properties compared to those of the natural products.[18] In all cases they were identical. The biological activity of the peptides has not yet been determined. While it is not yet known whether the production of amonabactin is significant in the virulence of the Aeromonads, there is evidence that the amonabactin-producing isolates of *Aeromonas* are able to remove iron from transferrin and show greater resistance to the complement activity of serum.[13] Whether amonabactin is a cause or consequence of this is a question that remains to be answered.[23]

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Supplementary Material Available: Experimental procedures, mass spectra, and tables of chemical shifts and relevant proton/carbon correlations (31 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(18) A full manuscript describing the synthesis and characterization of the amonabactins is in preparation.

(19) Mass spectra were acquired at the U.C. Berkeley College of Chemistry facility on a VG ZAB2-EQ mass spectrometer of BEqQ geometry.

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(21) Chiral GC–MS analyses were performed at the Facility for Advanced Instrumentation, University of California, Davis.

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