L-*threo*-β-Hydroxyhistidine, an Unprecedented Iron(III) Ion-binding Amino Acid in a Pyoverdine-type Siderophore from *Pseudomonas fluorescens 244*

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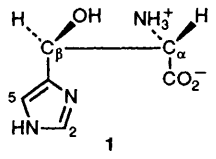
Nuclear magnetic resonance spectroscopy and mass spectrometric analysis of a unique pyoverdine-type siderophore isolated from the culture filtrate of *Pseudomonas fluorescens 244* reveals a new natural amino acid, L-*threo*-β-hydroxyhistidine, that functions as an iron(III) bidentate ligand.

Siderophores (Gk. iron bearer) are low-molecular-mass iron(III) ion chelators synthesized and secreted by most bacteria and fungi in response to iron starvation. The iron(III)-specific ligands in these molecules, though very limited in number, have served as models in the design and synthesis of new chelating agents for iron(III) and other ions with similar charge-to-ionic radius ratios, *e.g.* Al^{III}, Ga^{III}, Cr^{III} and the actinides(IV). Such compounds are of pharmacological interest as chelation therapy agents for treatment of iron and aluminium overload and of environmental interest for cleanup of radioactive actinides such as ^{238}Pu.[1]

Structure elucidation of pyoverdine Pf244, a siderophore



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expressed by *Pseudomonas fluorescens 244*, a bacterium isolated from Chesapeake Bay sediment, Baltimore Harbor,[2] has revealed the presence of L-*threo*-β-hydroxyhistidine **1** as one of the six amino acids in its peptide chain. Not only is **1** a highly unusual siderophore ligand, but it is also unique in its own right, the *threo*-isomer not having been previously reported as a naturally occurring amino acid.

Pseudomonas and *Azotobacter* species often produce pyoverdine (also termed pseudobactin or azotobactin)-type siderophores, which consist of essentially the same yellow–green fluorescent chromophore, identified as 2,3-diamino-6,7-dihydroxyquinoline, linked to the *N*-terminus of an oligopeptide of six to twelve amino acids. Structures of many pyoverdines have been reported.[3] Although these siderophores are strain-specific, with differing amino acid composition and sequence, each, with the exception of the present case, contains the same limited range of three iron(III)-specific bidentate ligands: (*i*) the catechol group of the chromophore, (*ii*) a hydroxamic acid, N^{δ}-hydroxyornithine, present in cyclized form or as a formyl or acetyl derivative, and (*iii*) an additional hydroxamic acid or a β-hydroxyaspartic acid. These bidentate groups bind the iron(III) ion octahedrally through six oxygen atoms. However, when *Pseudomonas fluorescens 244* is grown in a chemically defined iron-poor medium† it expresses a highly unusual pyoverdine. Mass spectrometry, NMR, and amino acid analysis were used to determine the structure of this novel siderophore, pyoverdine Pf244.

The presence of β-hydroxyhistidine was originally suggested by fast atom bombardment mass spectrometry (FABMS) of the intact pyoverdine and of a fully ^{15}N-labelled preparation of the pyoverdine, and by NMR of the unlabelled material. Analysis of the observed sequence ions in the FAB mass spectrum showed the presence of a residue with a mass of *m/z* 153 for the nonlabelled material and *m/z* 156 for the ^{15}N-labelled material, corresponding to the β-hydroxyhistidine and indicating the presence of three nitrogens in the residue. In addition to the peptide backbone fragmentation, fragment ions resulting from homolytic cleavage of the β-OH from the β-hydroxyhistidine residue were also observed. FABMS of pyoverdine and [^{15}N]pyoverdine subjected to partial acid hydrolysis (30 and 120 min at 105 °C, 6 mol l^{-1} HCl) further substantiated the presence of the β-hydroxyhistidine residue and the peptide sequence: seryl-lysyl-β-hydroxyhistidyl-threonyl-seryl-cyclo-N^{δ}-hydroxyornithine.

2D COSY NMR experiments on the intact pyoverdine revealed a spin system that also suggested the presence of the β-hydroxyhistidine residue.‡ ^{13}C NMR of pyoverdine Pf244 further supported the identity of this amino acid residue.§

Amino acid analysis of phenyl isothiocyanate (PITC)-derivatized pyoverdine acid hydrolysate and an authentic sample of PITC-derivatized DL-*erythro*-β-hydroxyhistidine (sample of the *erythro*-isomer provided by Sidney Hecht) proved that the unknown amino acid was not the *erythro*-isomer.¶ A pure standard sample of the *threo*-isomer was not available, but 1 mg of the *threo*-isomer was recovered as a 2 : 1 mixture with the *erythro*-isomer from the spent mother liquors of the *erythro* synthesis.[4] NMR analysis of the mixture verified its isomeric ratio and purity.‖ PITC derivatization of this preparation and HPLC analysis confirmed the identity of the *threo*-isomer in pyoverdine Pf244.

The *threo*-amino acid was determined to be the L-enantiomer from the reactions of D- and L-amino acid oxidases (DAO and LAO) with pyoverdine hydrolysates and subsequent amino acid analysis of the reaction products derivatized with the chiral reagent, β-D-glucopyransoyl isothiocyanate tetraacetate (GITC).**

Consideration of the pyoverdine Pf244 amino acid composition suggests that the β-hydroxyhistidine moiety serves as one of the bidentate chelating groups, the chromophore catechol and the cyclo-N^{δ}-hydroxyornithine hydroxamic acid groups serving as the other two. Initial speculation that the chelation occurs though the β-hydroxy oxygen atom and the N-3 nitrogen atom of the β-hydroxyhistidine residue appear to be substantiated by the large shifts observed in the ^{1}H and ^{15}N NMR spectra on chelation of the pyoverdine and the ^{15}N-labelled pyoverdine with Ga^{III}.[5]††

In conclusion, we have found a new amino acid, L-*threo*-β-hydroxyhistidine, in pyoverdine Pf244. Not only is this an amino acid hitherto unreported as occurring in nature, but it also appears to be one of the ligands that bind the siderophore to the iron(III) ion. The occurrence of this new iron(III) ligand in nature suggests that it may have significant potential in the design and synthesis of new chelating agents for removal of an excess of iron in thalassemia patients, aluminium in chronically dialysed patients, or $^{238}Pu^{IV}$ in contaminated patients and

† Succinate medium containing per litre: $K_2HPO_4 \cdot 3H_2O$, 7.9 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$ or $(^{15}NH_4)_2SO_4$, 1 g; succinic acid, 4 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 2 mg; NaOH to pH 7.

‡ All NMR experiments were performed with a 400 MHz spectrometer; ^{1}H–^{1}H 2D COSY NMR spectrum [H_2O–D_2O 85 : 15 v/v, pH 4.6, $Me_3SiCD_2CD_2CO_2Na$ (TSP)]: δ 8.35 (α-NH), 4.85 (α-CH), 5.29 (β-CH), 7.37 (H-5) and 8.59 (H-2).

§ ^{13}C NMR spectra (H_2O–D_2O 85 : 15 v/v, pH 2, ext. TSP, coupled and BIRD-decoupled, H-bearing C assigned by H–X 2D COSY) δ 60.6 (C-α, d, ^{1}J 144 Hz), 68.3 (C-β, d, ^{1}J 150 Hz), δ 119.8 (C-5, d, ^{1}J 205 Hz) and 137.1 (C-2, d, ^{1}J 222 Hz).

¶ Amino acid analysis was carried out by reversed-phase HPLC on a 3.9 × 150 mm Waters Pico-Tag column at 45 °C with a concave gradient, 0% B to 46% B over 8 min at 1 ml min^{-1}, followed by holding at 46% B for 6 min. Eluant A: 94 : 6 v/v (0.14 mol l^{-1} $NaC_2H_3O_2$ + 0.05% triethylamine buffer adjusted to pH 5.8 with acetic acid)–acetonitrile. Eluant B: 60% acetonitrile in water. This system provided baseline resolution of all PITC-derivatized pyoverdine amino acids and the β-hydroxyhistidine isomers.

‖ ^{1}H NMR spectrum (D_2O, pH 2.5, TSP) *threo*: δ 4.40 (α-CH, d, $^{3}J_{\alpha,\beta}$ 4.4 Hz), 5.60 (β-CH, dd, $^{3}J_{\alpha,\beta}$ 4.4, $^{4}J_{\beta,5}$ 1.1 Hz), 7.58 (H-5, t, width 2.4 Hz = $^{4}J_{2,5}$ + $^{4}J_{\beta,5}$ = 1.4 + 1.1 Hz) and 8.78 (H-2, d, $^{4}J_{2,5}$ 1.4 Hz); *erythro*: δ 4.47, (α-CH, d, $^{3}J_{\alpha,\beta}$ 3.4 Hz), 5.58 (β-CH, dd, $^{3}J_{\alpha,\beta}$ 3.4 Hz, $^{4}J_{\beta,5}$ 1.1 Hz), 7.50 (H-5, t, width 2.4 Hz = $^{4}J_{2,5}$ + $^{4}J_{\beta,5}$ = 1.4 + 1.1 Hz) and 8.74 (H-2, d, $^{4}J_{2,5}$ 1.4 Hz).

** Analysis of the GITC-derivatized diastereoisomers was carried out by reversed-phase HPLC at 24 °C on a 3.9 × 300 mm Vydac C18 (218TP5) column with a linear gradient of 10% B to 75% B over 30 min at 1 ml min^{-1}. Eluant A: 1 : 3 v/v methanol–10 mmol l^{-1} phosphate buffer, pH 2.8. Eluant B: 1 : 1 v/v acetonitrile–10 mmol l^{-1} phosphate buffer, pH 2.8. Hydrolysed pyoverdine incubated with LAO in 0.1 mol l^{-1} HEPES buffer, pH 7.2, at 27 °C, monitored by HPLC showed decreasing amounts of the *threo*-β-hydroxyhistidine over a 90 h reaction time.

†† Ga^{III} serves as a good analogue for paramagnetic Fe^{III} owing to its nearly identical ionic radius and kinetic lability. ^{1}H NMR spectra of pyoverdine (Py) and Ga^{III}–pyoverdine (Ga–Py) (H_2O–D_2O, 85 : 15 v/v, pH 5.0, 27 °C, TSP) β-hydroxyhistidine residue β-CH: δ 5.18, for Py, δ 5.48 for Ga–Py. ^{15}N NMR spectra [H_2O–D_2O, 85 : 15 v/v, pH 4.8, 31 °C, relative to external anhydrous liq. NH_3 *via* the shift (δ_N 20.68) of $^{15}NH_4^+$ in a saturated aqueous solution of $^{15}NH_4{}^{15}NO_3$], β-hydroxyhistidine ring N-3: δ_N 182 for Py, δ_N 215 for Ga–Py; ring N-1: δ_N 173 for Py, δ_N 174 for Ga–Py.

perhaps for treatment of rheumatoid arthritis and other autoimmune diseases, tissue injury, and protozoal infection.[1,6]

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