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Analysis and configuration assignments of the amino acids in a pyoverdine-type siderophore by reversed-phase high-performance liquid chromatography

Diane K. Hancock* and Dennis J. Reeder

National Institute of Standards and Technology (NIST), Biotechnology Division, Gaithersburg, MD 20899 (USA)

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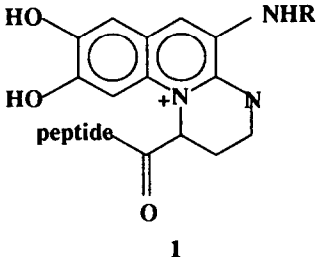
ABSTRACT

The amino acid composition of the siderophore pyoverdine Pf244 was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) of phenylthiocarbamyl derivatives of the acid-hydrolyzed pyoverdine. The siderophore contains an acid-labile amino acid, N^{δ}-hydroxyornithine, and an amino acid previously unknown in naturally occurring systems, L-*threo*-β-hydroxyhistidine. Amino acid chirality assignments were determined by RP-HPLC of the β-D-glucopyranosyl isothiocyanate tetraacetate derivatives. Reactions with amino acid oxidases established the L-configuration of *threo*-β-hydroxyhistidine.

INTRODUCTION

Siderophores are low-molecular-mass peptidic ferric-ion chelators synthesized and secreted by many bacteria and fungi in response to iron deprivation. *Pseudomonas* bacteria produce pyoverdine (also termed pseudobactin) siderophores. Structures of many pyoverdines have been determined using a combination of nuclear magnetic resonance spectroscopy, fast atom bombardment mass spectrometry, and amino acid analysis [1–6]. They all comprise a yellow–green fluorescent chromophore (**1**) covalently bound to the N-terminus of a hydrophilic oligopeptide of six to twelve amino acids.



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The peptide portion contains two amino acids that function as ferric-specific bidentate ligands, generally either two hydroxamates (as N^{δ}-hydroxyornithine, present in cyclic form, or as a N^{δ}-formyl or -acetyl derivative) or one hydroxamate and one β-hydroxyaspartic acid. These bidentate groups and the chromophore catechol unit octahedrally bind the ferric ion through six oxygen atoms. Pyoverdine-type siderophores are strain specific, differing in amino acid configuration, composition and sequence, with amino acid stereochemistry determining the possible coordination isomers. Problems encountered in stereochemical analysis of siderophore amino acids by gas chromatography have been previously addressed [7].

This paper describes (1) Pico-Tag HPLC identification of unusual amino acids (*i.e.*, *allo*-threonine, N^{δ}-hydroxyornithine and *threo*-β-hydroxyhistidine) present in pyoverdine Pf244, the siderophore expressed by *Pseudomonas fluorescens* 244, and (2) stereochemical assignments of the constituent amino acids by reversed-phase

* Corresponding author.

high-performance liquid chromatography (RP-HPLC) analysis of the amino acids derivatized with the chiral reagent, β-D-glucopyranosyl isothiocyanate tetraacetate (GITC).

EXPERIMENTAL

Chemicals[a]

Amino acid standard H, phenylisothiocyanate (PITC) and ninhydrin were obtained from Pierce, GITC from Polysciences, triethylamine (TEA, Gold Label) and (*SS*)-*allo*-threonine (L-*allo*-) from Aldrich, (*RR,SS*)-β-hydroxyaspartic acid (D,L-*threo*-), rhodotorulic acid, (*RR*)-*allo*-threonine (D-*allo*-) and other amino acids from Sigma. Ultrapure hydrochloric acid was prepared at NIST [8]. (*RS,SR*)-β-Hydroxyhistidine (D,L-*erythro*-) and the mother liquors from which (*RR,SS*)-β-hydroxyhistidine (D,L-*threo*-) was recovered were provided by Professor S. Hecht. $CH_3SiC^2H_2C^2H_2CO_2Na$ (TSP) was obtained from MSD Isotopes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) from Fluka. Pico-Tag eluents A and B and sample diluent were obtained from Waters or made according to Waters' instructions [9]. Acetonitrile and methanol were HPLC-grade solvents from EM Science. Water was purified with an Organex Milli-Q system (Millipore). The column selectivity test mixture, SRM 869, was obtained at NIST [10]. L-Amino acid oxidase (LAO), supplied in aqueous solution, and D-amino acid oxidase (DAO), supplied dry, were purchased from Worthington Biochemical.

Isolation and purification of pyoverdine

Pyoverdine and ^{15}N-labelled pyoverdine were isolated from culture media and purified as their ferric chelates by the procedure of Hancock *et al.* [11]. Iron was removed from the ferric pyoverdines by extracting with a 5% (w/v) 8-hydroxyquinoline–chloroform solution.

[a] Commercial instruments or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the equipment or materials are the best available for the purpose.

High-performance liquid chromatography

Waters HPLC hardware was used: two Model 501 pumps, U6K injector, 440 detector (254 nm), 660 solvent programmer, TCM temperature control unit and 740 data module. A 150 × 3.9 mm Pico-Tag column was used for amino acid analysis and a 300 × 3.9 mm Vydac C_{18} (218TP5) column (Separations Group) for chiral analysis.

Amino acid analysis by the Pico-Tag method

Pyoverdine samples and amino acid standards were hydrolyzed and derivatized on a Pico-Tag work station equipped with a Varian SD200 vacuum pump. Batch hydrolyses of *ca.* 10-nmol pyoverdine samples were carried out in pyrolyzed (520°C, 24 h) 50 × 6 mm borosilicate glass tubes. Sample tubes were placed in a reaction vial with a Mininert-type valve (Waters) and vacuum dried. A 200-μl volume of 6 *M* ultrapure HCl and a small phenol crystal were added to the reaction vial, which was then alternately evacuated and purged with nitrogen three times, and again evacuated, sealed, and transferred to a 114°C oven for 21 h. Cooled sample tubes were transferred to a clean reaction vial and residual HCl was evaporated under vacuum. To assure basic derivatizing conditions 20 μl of ethanol–water–TEA (2:2:1) were added to each tube, vortex mixed and evaporated to constant 7 Pa (55 mTorr) pressure. Samples were derivatized with 20 μl of freshly prepared reagent (ethanol–water–TEA–PITC, 7:2:1:1) for 15 min at 20–25°C, then evaporated to constant pressure of 7 Pa to remove excess derivatizing reagent.

Phenylthiocarbamyl (PTC) derivatives of the amino acids were reconstituted in 200 μl of sample diluent (Waters) and separated on the Pico-Tag column. A concave gradient, 0-46% B over 8 min at 1 ml/min, was followed by holding the gradient 6 min at 46% B, then increasing to 100% B over 1 min, holding 2 min, reversing to 0% B over 1 min, and reequilibrating for 20 min. Eluent A was (0.14 *M* $NaC_2H_3O_2$ + 0.05% TEA, adjusted to pH 6.4 with acetic acid)–CH_3CN (94:6, v/v) and eluent B was CH_3CN–water (60:40, v/v). A 200-μl volume of 1000 ppm (w/w) aqueous Na_2EDTA was added to each liter of eluents A and B. Column tempera-

ture was maintained at 38°C. Additional analyses were carried out at 45°C with eluent A at pH 5.8. Amino acids in the pyoverdine sample were identified by comparison with PITC-derivatized standards and verified by coinjection of sample and standard under both conditions.

Determination of amino acid configurations

Enantiomeric composition of hydrolyzed pyoverdine samples was determined by RP-HPLC of the GITC-derivatized diastereomers, modifying Nimura *et al.*'s [12] procedure. Amino acids (5 nmol/μl) were mixed 1:1:1 (v/v/v) with 50 μl TEA/ml CH_3CN and 2.2 mg GITC/ml CH_3CN, and kept 30 min at 22°C, then analyzed directly on the same HPLC system used for the PTC-derivatives, except that a polymeric [13] Vydac C_{18} (218TP5) column was used at 24°C. A linear gradient of 10–75% B over 30 min was run at 1 ml/min, then rapidly increased to 100% B and held 14 min prior to reversing to 10% B with a 3-min linear gradient and reequilibrating 15 min. Eluent A was CH_3OH–10 m*M* phosphate buffer, pH 2.8 (1:3, v/v) and eluent B was CH_3CN–10 m*M* phosphate buffer, pH 2.8 (1:1, v/v). A 400-μl volume of 1000 ppm (w/w) aqueous Na_2EDTA was added to each liter of eluent.

Enantiomer configurations in the hydrolyzed and GITC-derivatized pyoverdines were identified by comparison with GITC-derivatized optically active amino acid standards (commercially available for all except for *threo*-β-hydroxyhistidine) and verified by coinjection. The chirality of *threo*-β-hydroxyhistidine was determined by reacting *threo*- and *erythro*-β-hydroxyhistidine (see below) with LAO in 0.5-ml reaction vials with Mininert caps (Pierce). A 100-nmol amount of hydrolyzed pyoverdine in 75 μl 0.1 *M* HEPES buffer, pH 7.2, was treated with 2 μl of LAO, the vial flushed with oxygen, and incubated at 27°C. The reaction was monitored by HPLC of GITC-derivatized aliquots of the reaction mixture over four days, with additional LAO (10 μl) added after one day. Another 100-nmol portion of hydrolyzed pyoverdine was treated with 2 μg of DAO in 100 μl of 0.05 *M* sodium pyrophosphate, pH 8.3, and incubated at 37°C.

Isolation and purification of threo-β-hydroxyhistidine

threo-β-Hydroxyhistidine was recovered from mother liquors from synthesis of *erythro*-β-hydroxyhistidine [14], known to yield about 10% of the contaminating *threo*-isomer. A 13-g amount of mother liquors, containing mainly glycine and pyruvic acid and less than 0.1% of residual β-hydroxyhistidines, were dissolved in 0.1 *M* $NaC_2H_3O_2$, adjusted to pH 3.3, and chromatographed on a 21 × 2.5 cm SP-Sephadex C-25 cation-exchange column equilibrated with 0.1 *M* $NaC_2H_3O_2$, pH 3.3. The column was eluted with the acetate buffer at 1 ml/min, collecting 50-drop fractions. Portions of 1 μl of each fraction applied to filter paper and dried were spot-tested with 1 μl of ninhydrin reagent [15] (150 ml of methyl Cellosolve and 50 ml of 4 *M* $NaC_2H_3O_2$, pH 5.5, purged with argon before addition of 4 g of ninhydrin and 80 mg of $SnCl_2 \cdot 2H_2O$). At ambient temperature, color developed within 30 min. After glycine eluted the eluent was changed to 0.1 *M* $NaC_2H_3O_2$, pH 5.4. The β-hydroxyhistidine eluted over many fractions. Representative fractions were adjusted to pH 2, rotoevaporated at 31°C to remove acetate, and then reconstituted in 400 μl of ^2H_2O; 10-μl aliquots of each were analyzed by HPLC after GITC derivatization. The remaining reconstituted fractions were spiked with an additional 100 μl of stock ^2H_2O–TSP solution and analyzed by NMR, using TSP as an internal standard for relative quantitation of β-hydroxyhistidine.

Fractions were pooled, concentrated, adjusted to pH 4, and applied to 7 × 1.5 cm SP-Sephadex C-25 columns for desalting and further purification. Columns were eluted with 0.01 *M* $NH_4C_2H_3O_2$, pH 4, to remove residual glycine and salts, and then with 1 *M* NH_4OH. Fractions were monitored with ninhydrin spot tests, pH, and conductivity measurements, and checked for purity by NMR spectroscopy. Finally, pooled fractions were treated with Norit to remove small amounts of colored aromatic contaminants.

Nuclear magnetic resonance

All NMR spectra were measured on a Bruker WM (400 MHz) spectrometer using a 5-mm selective ^1H probe. Intact pyoverdine ^1H–^1H

two-dimensional correlated spectroscopy (COSY) spectra (^2H_2O, pH 5.3, TSP) were recorded using presaturation, with and without a fixed delay to enhance long-range coupling [16].

Fast atom bombardment mass spectrometry (FAB-MS)

FAB-MS was performed on a Varian MAT 731 double focusing mass spectrometer of Mattauch–Herzog geometry fitted with a Cs^+ gun. A glycerol–thioglycerol (1:1) matrix was used.

RESULTS AND DISCUSSION

Amino acid composition of pyoverdine Pf244

Amino acids derivatized with PITC to give PTC-amino acids were analyzed by RP-HPLC using a modified Pico-Tag method. The RP-HPLC solvent program was empirically determined to resolve all 17 PITC-derivatized amino acids present in the Pierce amino acid standard H preparation (Fig. 1a). Identities of amino acids were confirmed by individual coinjections of PTC derivatives. A chromatogram of the PITC-derivatized pyoverdine hydrolysate (Fig. 1b) indicated that only two of the standard amino acids are present in this pyoverdine —serine and lysine. Three peaks were unidentified.

A COSY NMR spectrum of the intact pyoverdine Pf244 revealed spin systems [(αCH 4.30, βCH 4.05, CH_3 1.14 ppm), (αCH 4.46, βCH_2 1.80, 1.99; γCH_2 1.94, 1.99; δCH_2 3.63 ppm), (αCH 4.77, βCH 5.18 ppm)] suggesting the presence of threonine, ornithine and a β-hydroxyamino acid, respectively. Two additional peaks in the pyoverdine hydrolysate were identified as ornithine and *allo*-threonine by coelution with additional standards.

Analysis of ornithine

Ornithine is found commonly in pyoverdines, often at peptide's C terminus as an internally cyclized N^{δ}-hydroxyornithine (**2**), or as a N^{δ}-acyl-N^{δ}-hydroxyornithine (**3**) where acyl is either formyl or acetyl. Despite common occurrence, verification of the specific form of the ornithine

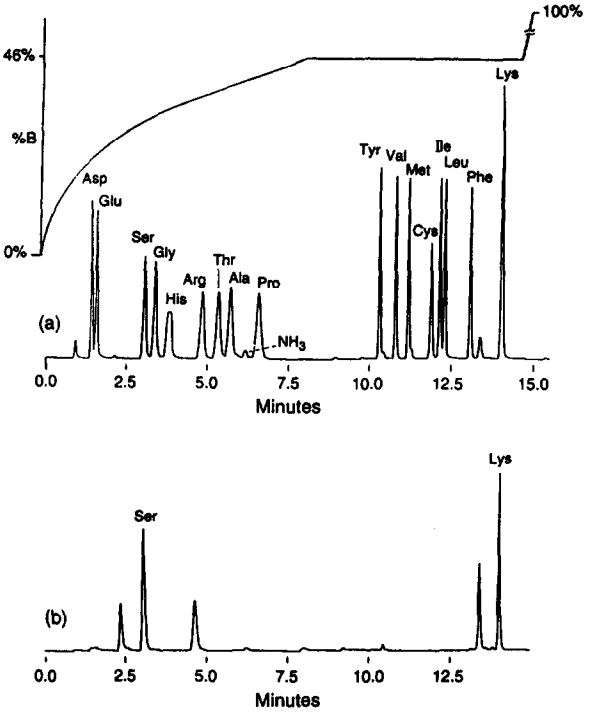
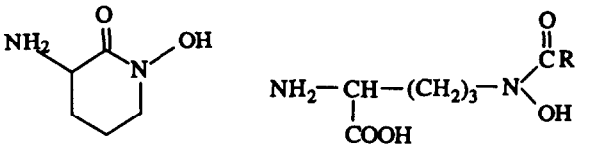


Fig. 1. Amino acid analysis of pyoverdine Pf244. (a) RP-HPLC chromatogram of PITC-derivatized amino acid standards, illustrating mobile phase gradient program: flow-rate 1 ml/min, 38°C, UV–Vis detection, 254 nm; solvent A: sodium acetate–TEA buffer, pH 6.4, and 6% CH_3CN; solvent B: CH_3CN–water (60:40). (b) RP-HPLC chromatogram of a PITC-derivatized ferric pyoverdine Pf244 hydrolyzate, same chromatographic conditions.

is not straightforward due to instability of hydroxamic acids under HCl hydrolysis [7,17–19]. Depending on conditions, hydrolysis of siderophores containing **2** or **3** may give either N^{δ}-hydroxyornithine or decomposition of the hydroxyamino function and only fractional recovery of ornithine. Iron(III) apparently catalyzes decomposition [18]. The absence of formyl or acetyl peaks in the pyoverdine ^1H NMR spectrum and the presence of a spin system with chemical shifts nearly identical to those reported for pseudobactin [1] suggested that ornithine is present in Pf244 as **2**. Under acid conditions **2** readily undergoes hydrolysis with ring opening to give a N^{δ}-hydroxyornithine. MS of partially hydrolyzed pyoverdine (6 *M* HCl, 105°C, 10–120 min) gave $(M + H + H_2O)^+$

and fragmentation peaks corresponding to a C-terminal N^{δ}-hydroxyornithine residue.



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The Pf244 amino acid identified as ornithine coeluted with both PITC-derivatized ornithine and hydrolyzed rhodotorulic acid. Acid hydrolysis of rhodotorulic acid (the diketopiperazine ring dimer of N^{δ}-acetyl-N^{δ}-hydroxyornithine) also gives ornithine rather than N^{δ}-hydroxyornithine. Baseline chromatographic separation of reagent and ornithine peaks, allowing quantitation of ornithine, was achieved on the Pico-Tag column at pH 5.8, 45°C, but not at higher pH and lower temperatures. Both iron-free and ferric pyoverdine, gave *ca.* one-half equivalent of ornithine per pyoverdine hydrolyzed. The PTC derivatives of hydrolyzed rhodotorulic acid and pyoverdine still coeluted with PTC-ornithine, further supporting that the product was PTC-ornithine. Whether the reduction of N^{δ}-hydroxyornithine to ornithine occurs during acid hydrolysis or during alkaline derivatization was not determined. Though the precise form of ornithine present in Pf244 cannot be established solely by Pico-Tag amino acid analysis, it was readily confirmed as the cyclic N^{δ}-hydroxy species with MS and NMR data.

Identification of a new amino acid

It was deduced that the unidentified amino acid in pyoverdine Pf244 was probably a bidentate ligand since otherwise iron coordination could be only four-fold —by the quinoline catechol group and the hydroxyornithine hydroxamic acid moiety. However, amino acid analysis had confirmed that *threo*-β-hydroxyaspartic acid, the only other chelating group known in previously studied pyoverdines, was not present. The sequence ions in MS of intact pyoverdine and of a fully ^{15}N-labelled preparation indicated that the unidentified residue had a mass of 153 u for the non-labelled and 156 u for labelled material, confirming three nitrogens in the amino acid residue and suggesting it was β-hydroxyhistidine. The β-hydroxy spin system in a two-dimensional COSY NMR spectrum supported this conclusion, revealing extended connectivity from the αCH and βCH to singlets at 7.21 and 8.14 ppm.

The siderophore literature has no record of β-hydroxyhistidine. β-Hydroxyhistidine has been found naturally only as the *erythro*-isomer in the bleomycin family of anti-tumor antibiotics [20]. Amino acid analyses of a sample of DL-*erythro*-β-hydroxyhistidine containing a trace of the *threo*-isomer [14, gift of S. Hecht] showed that the *erythro*-isomer was not the amino acid present in pyoverdine, but the small ($\leq 1\%$) contaminant did have the same retention time as the unidentified pyoverdine amino acid. Cation-exchange column chromatography of the concentrated mother liquors from Hecht *et al.*'s synthesis of the DL-*erythro*-β-hydroxyhistidine [14] yielded 0.01% (w/w) of the *threo*-isomer in isomeric mixtures. One cut of column fractions that by ^{1}H NMR contained a 1:1 mixture of *threo*- and *erythro*-isomers was PITC-derivatized and the derivatives could then be separated on the Pico-Tag column (Fig. 2b). Comparison with

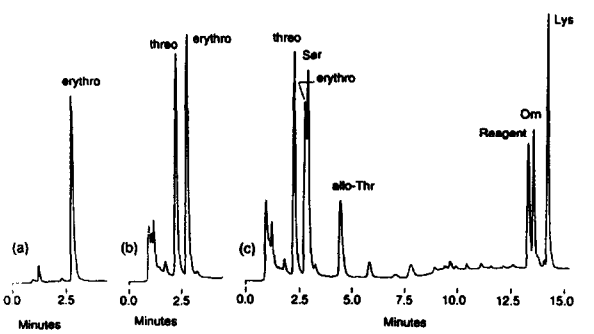
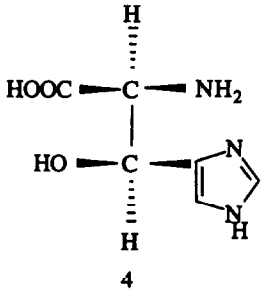


Fig. 2. Amino acid analyses to verify that the β-hydroxyhistidine in pyoverdine Pf244 is the *threo* isomer. Mobile phase solvent program as in Fig. 1 except eluent A at pH 5.8 and temperature at 45°C to resolve ornithine from reagent peak. (a) Chromatogram of PITC-derivatized DL-*erythro*-β-hydroxyhistidine. (b) Chromatogram of *ca.* 1:1 mixture of PITC-derivatized *threo*- and *erythro*-β-hydroxyhistidine isolated from mother liquors. (c) Chromatogram of coinjected PITC-derivatized hydrolyzed pyoverdine Pf244 and the β-hydroxyhistidines from the mother liquors. The *threo*-β-hydroxyhistidine coelutes with the pyoverdine β-hydroxyhistidine.

the retention time in the chromatogram of an *erythro*-standard (Fig. 2a), allowed identification of the PTC-*threo*-isomer. Coinjection of PITC-derivatized pyoverdine Pf244 and the *threo*- and *erythro*-β-hydroxyhistidine mixture (Fig. 2c) confirmed the identity of the remaining unidentified amino acid as *threo*-β-hydroxyhistidine (**4**).



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Quantitation of the amino acids in pyoverdine

Absorbance measurements of the PITC derivatives were employed to quantify the amino acid composition of pyoverdine Pf244. PITC reacts quantitatively with both primary and secondary amino acids, giving derivatives with nearly identical molar extinction coefficients at 254 nm on a per amino group basis [21]. Table I summarizes the relative response factors for the PTC-amino acids obtained from analyses of standards, and the experimental results for the composition of pyoverdine Pf244: 1 mol equivalent each of β-hydroxyhistidine, *allo*-threonine and lysine; 2 mol of serine; and one-half equivalent of ornithine. Response factors for β-hydroxyhistidine and hydrolyzed rhodotorulic acid are aberrant. The extinction coefficient for PTC-β-hydroxyhistidine does, in fact, appear to be different. However, the PTC derivative of hydrolyzed rhodotorulic acid is PTC-ornithine which arises from reduction of the HCl hydrolysis intermediate, N^{δ}-hydroxyornithine. Acid-catalyzed degradation of this intermediate, also present in the pyoverdine hydrolysis, accounts for both the low response factor for rhodotorulic acid and the low ornithine value for pyoverdine. The amino acid composition determined by 254-nm absorbance of the component PITC derivatives is consistent with the MS observation of a molecular $(M+H)^+$ ion at m/z 1044 for non-chelated pyoverdine Pf244, where the R group on the chromophore (**1**) is a succinic acid moiety.

TABLE I

MOLAR RESPONSE FACTORS FOR CHROMATOGRAPHIC ANALYSIS OF PITC-DERIVATIZED AMINO ACIDS AND THEIR APPLICATION TO AMINO ACID ANALYSIS OF IRON-FREE PYOVERDINE

Amino acid	Response factor normalized to lysine[a]	Amino acid composition of pyoverdine (mol)
β-Hydroxyhistidine	0.58[b]	1.05
Serine	0.96	1.80
allo-Threonine	0.97	0.96
Ornithine	1.93	0.55
Lysine	2.00	1.00
Rhodotorulic acid[c]	0.43	

[a] Determined at pH 5.8, 45°C.
[b] Determined on *erythro*-isomer.
[c] Hydrolyzed in 6 *M* HCl, 114°C, 21 h; molarity based on molecular mass of monomer.

Configuration of amino acids in pyoverdine Pf244

Stereochemical analysis of the component amino acids employed RP-HPLC with GITC as the precolumn chiral derivatizing agent. Separation was accomplished on a polymeric Vydac column with 30-nm pore size, following Sander and Wise [13], who reported that polymeric C_{18} phases with large pore sizes showed enhanced shape selectivity. Parallel efforts with a monomeric (selectivity coefficient 1.7 [10,22]) Beckman Ultrasphere ODS column and a methanol–phosphate buffer mobile phase [23,24] were unsuccessful. Using a methanol–acetonitrile–phosphate buffer gradient system adapted from a system of Nimura *et al.* [12], separation on the Vydac column at 24°C provided nearly baseline resolution of both enantiomers of GITC-derivatized DL-serine, -*allo*-threonine, -ornithine, -lysine and hydrolyzed pyoverdine with L enantiomers eluting before D.

Excepting *threo*-β-hydroxyhistidine, enantiomer configurations in the pyoverdine sample were easily identified as: one D-serine, one L-serine, one L-lysine, one L-ornithine and one D-*allo*-threonine. Assignment of configuration to

the remaining *threo*-β-hydroxyhistidine was not straightforward because no optically pure standards were available. Although elution order for the other amino acid enantiomers was L before D, Nimura *et al.* [12] had observed inversion of this order for histidine and arginine.

Determination of configuration of threo-β-hydroxyhistidine by amino acid oxidase reactivity

Absolute assignment of amino acid configuration was accomplished by reactions with LAO and DAO, which catalyze the oxidative deamination of many L- and D-amino acids:

$$RCHNH_2COOH + O_2 + H_2O \rightarrow RCOCOOH + NH_3 + H_2O_2$$

Since GITC couples with both primary and secondary amines, reaction of LAO or DAO with a given amino acid enantiomer provides configuration identification that is detected as a decrease in its peak size and an increase in the ammonia peak in chromatograms of the GITC-derivatized reaction products. However, these oxidase enzymes are unreactive to some amino acids and enzyme activity is often species-specific [25].

For the β-hydroxyhistidines, LAO/DAO analyses were complicated by overlapping chromatographic peaks, despite extensive variation of solvent gradients, eluents and temperatures. The chromatogram of GITC-derivatized DL-*erythro*-β-hydroxyhistidine showed well-resolved D- and L-peaks (Fig. 3a). However, a GITC-derivatized *threo–erythro* (2:1) mixture gave only three, not the expected four, peaks because of overlap of one *threo* and one *erythro* isomer (Fig. 3b). *threo*-β-Hydroxyhistidine in GITC-derivatized hydrolyzed pyoverdine coeluted with the first of the *erythro*-β-hydroxyhistidine peaks (Fig. 3c), verifying coelution of one *threo* enantiomer with one *erythro* enantiomer.

LAO and DAO enzyme reaction variables (pH, temperature, buffer system, oxygen level) [25–27], were selected to maximize reaction rates and yet permit direct HPLC analysis of the reaction mixture after GITC derivatization. RP-

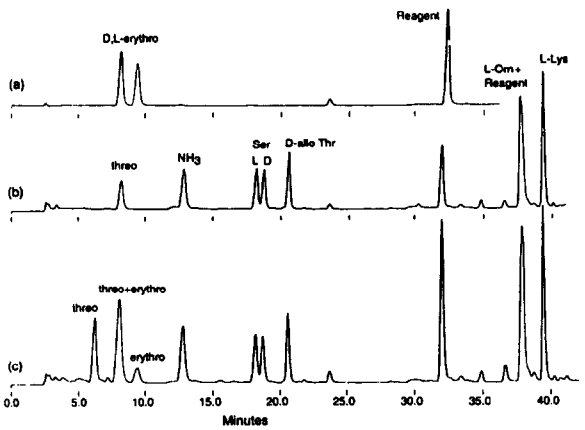

Fig. 3. Chiral amino acid analyses on a Vydac C_{18} column to confirm the identity of the β-hydroxyhistidine enantiomer in pyoverdine Pf244. (a) Chromatogram of GITC-derivatized DL-*erythro*-β-hydroxyhistidine. (b) Chromatogram of GITC-derivatized pyoverdine hydrolysate. (c) Chromatogram of GITC-derivatized mother liquor 2:1 *threo*- to *erythro*-β-hydroxyhistidine isolate coinjected with GITC-derivatized pyoverdine hydrolysate. See text for D,L-assignments.

HPLC chromatograms of GITC derivatives of LAO-treated and non-treated DL-*erythro*-β-hydroxyhistidine showed disappearance of the second *erythro* peak in the treated sample after 66 h and concomitant appearance of a peak corresponding to GITC–ammonia. This indicates that the second of the GITC *erythro* peaks is the L-enantiomer, in accord with the D-before-L inverted elution order for GITC–*erythro*-β-hydroxyhistidine previously observed for histidine [12]. Treatment of DL-*erythro*-β-hydroxyhistidine with DAO showed no chromatographic changes, signifying that DAO was inactive towards the D-enantiomer under the conditions used, although under the same conditions it fully reacted with D-alanine.

RP-HPLC of a GITC-derivatized β-hydroxyhistidine mother liquor isolate that had been treated with LAO for 48 h, showed a 68% reduction in the L-*erythro* peak, and no decrease in the other two peaks, indicating that the L-*erythro* isomer was more reactive than the L-*threo* isomer. Treatment of a mother liquor preparation with DAO showed no chromatographic changes.

Results of RP-HPLC analyses of hydrolyzed pyoverdine incubated with increased amounts of

TABLE II

REACTION OF L-AMINO ACID OXIDASE WITH HYDROLYZED PYOVERDINE

Amino acid	HPLC peak areas of GITC-derivatized reaction mixture[a]			
	0 h	15 h	60 h	80 h[c]
threo-β-OH-His	0.59	0.53	–	0.34 ± 0.01
L-Ser	0.84	0.69	0.16	0.13 ± 0.001
D-Ser	0.82	0.79	0.82	0.82 ± 0.002
D-*allo*-Thr	1.00	1.00	1.00	1.00
L-Orn[b]	5.47	3.96	3.00	3.02 ± 0.2
L-Lys	2.12	0.92	0.42	0.37 ± 0.03
NH_3	1.35	2.44	3.79	4.73 ± 0.4

[a] Peak areas normalized to *allo*-threonine.
[b] A reagent peak coelutes with ornithine.
[c] Values shown are the averages of two determinations and the standard deviation of the mean.

LAO in an oxygen atmosphere are summarized in Table II and illustrated in Fig. 4a and b. RP-HPLC of pyoverdine solutions showed that without oxygen blanketing both D- and L-serine enantiomers were unstable in 0.1 *M* HEPES buffer, pH 7.5, in the presence or absence of LAO. However after 80 h in an oxygenated system, analyses showed significant reduction in *threo*-β-hydroxyhistidine, L-serine, L-lysine and L-ornithine peaks and no change in the D-serine or D-*allo*-threonine peaks. Although reaction of LAO with the *threo*-β-hydroxyhistidine was slow, heavy oxidation confirmed that the *threo*-β-hydroxyhistidine amino acid had the L-configuration.

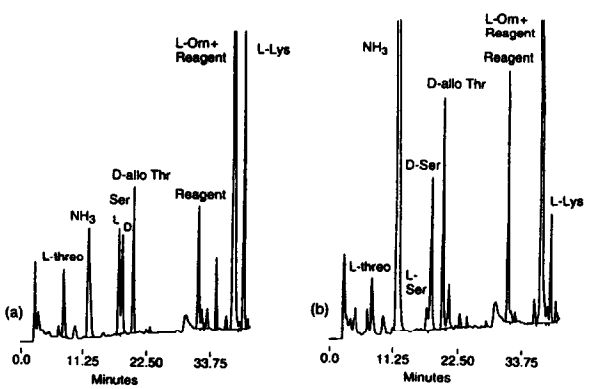

Fig. 4. Analyses of products from the reaction of L-amino acid oxidase on pyoverdine Pf244. (a) Chromatogram of GITC-derivatized reaction mixture at 0 h. (b) Chromatogram of reaction mixture after 80 h. Comparison of the 0 h and 80 h chromatograms confirms that the *threo*-β-hydroxyhistidine in pyoverdine Pf244 has the L-configuration.

CONCLUSIONS

Elucidation of the structure of a new pyoverdine containing a previously unseen amino acid demonstrates the potential of RP-HPLC for identification and stereochemical analysis of unusual amino acids found in small oligopeptide structures. Such approaches could be extended, for example, to other bacterial and fungal proteins. RP-HPLC of phenylthiocarbamyl derivatives of the amino acid constituents of pyoverdine hydrolysates may be used to identify and quantitate the peptide chain components, except where an N-hydroxylated form of ornithine requires confirmation by MS analysis of non-hydrolyzed material. Stereochemical configurations of the amino acids may be assigned with the chiral derivatizing agent, GITC, and RP-HPLC of the derivatized amino acids components on a Vydac 30-nm pore column. Reactivity with amino acid oxidases can be used to establish the configuration of unknown amino acids when preparations of pure enantiomers are lacking.

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