Identical Pyoverdines from *Pseudomonas fluorescens* 9AW and from *Pseudomonas putida* 9BW*

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*Pyoverdine, Siderophore, Pyoverdine, Siderophore, Bacterial Classification*

From *Pseudomonas fluorescens* 9AW and from *Pseudomonas putida* 9BW identical pyoverdine-type siderophores were isolated and their structures were elucidated by spectroscopic methods and degradation studies. These novel compounds are of interest as they contain L-threo-β-hydroxy histidine in their peptide chains, an amino acid sofar encountered in nature only rarely. The co-occurrence of the same pyoverdine in different *Pseudomonas* species and its significance for the classification is discussed.

**Introduction**

Iron possesses two stable oxidation states (Fe²⁺ and Fe³⁺) and the redox potential between them can be influenced strongly by complexing ligands. It plays, therefore, an important role for many redox processes in biological systems. Due to the low solubility of its various oxide hydrates the concentration of free Fe³⁺ in the soil is at best about 10⁻¹⁷ mol/l at pH values around 7. To maintain a sufficient supply of iron, soil bacteria excrete water soluble low molecular weight compounds with high complexing constants for Fe³⁺. A structurally interesting type of these so-called siderophores is produced by the fluorescent group of the genus *Pseudomonas* referred to as pseudobactins or more commonly as pyoverdines (Budzikiewicz, 1993). Their common structural feature is a dihydroxyquinoline nucleus responsible for the yellowish-green fluorescence which gave the name to the "fluorescent" pseudomonads. It is one of the binding sites for Fe³⁺; the other two necessary to form an octahedral complex are contained in a peptide chain attached to the quinoline chromophore. It contains 6 to 12 amino acid (both d and l). These binding sites are either two hydroxamate units derived from Orn or one hydroxamate and one α-hydroxy carboxylate. The various fluorescent *Pseudomonas* spp. or even strains produce pyoverdines differing in their peptide chains responsible for the recognition at the cell surface (Hohnadel and Meyer, 1988).

We now wish to report the isolation and structure elucidation of a pyoverdine which is remarkable in a twofold way: The α-hydroxy acid commonly encountered in pyoverdines is threo-β-hydroxy Asp while here threo-β-hydroxy His was found, an amino acid which has been sofar encountered in nature only in exochelin MN from *Mycobacterium neoaurum* (Sharman et al., 1995) and in a pyoverdine from *Pseudomonas fluorescens* 244 (Hancock et al., 1993), while the erythro-isomer was found in the bleomycins (Koyama...
et al., 1973). Secondly, pyoverdines are highly specific regarding the recognition by the excreting *Pseudomonas* strain. The fact that the same pyoverdine could be isolated both from a *Pseudomonas fluorescens* and a *P. putida* strain raises classification problems which will be discussed below.

**Material and Methods**

**Instruments**

Mass spectrometer: Finnigan-MAT HSQ-30 (FAB; matrix thioglycerol), Finnigan-MAT 900ST (ESI; aqueous solution 5 pmol/μl); Kratos MS 25 RFA (GC/MS). Sample preparation by adsorption on Sep-Pak RP-18, removing of inorganic material with H₂O, desorption with CH₃OH/H₂O 1:1 and drying i.v.

NMR: Bruker AM 300 (¹H 300, ¹³C 75.5 MHz), Bruker DRX 500 (¹H 500, ¹³C 125 MHz). Chemical shifts are given relative to TMS with the internal standard DSS silapentane-5- using the relations δ(TMS) = δ(DSS) for ¹H and δ(TMS) = δ(DSS) – 1.61 ppm for ¹³C. Samples: 12 mg desferri-1a were twice dissolved in 0.5 ml D₂O and brought to dryness i.v. at 30 °C. The dry substances were redissolved in 0.6 ml phosphate buffer (D₂O/H₂O 1:9 v/v; pH 4.3).

UV/Vis: Perkin-Elmer Lambda 7 and Hitachi 200; 1 mg substance in 20 ml 0.1 m phosphate buffer.

Chromatography: HPLC Knauer, column Nucleosil 100-C₁₈, 5 μm, Kromasil 100-C₄ (5μm) and Nucleodex-β-OH (Macherey & Nagel, Düren); GC Carlo Erba HRGC 4160, FID detector, column Chirasil-L-Val (Chrompack, Middelburg, NL); column chromatography CM-Sephadex C-25 (Pharmacia, Uppsala, S), XAD-4 (Serva, Heidelberg), BioGel P2 (Bio-Rad, Richmond, USA), Sep-Pak RP-18 (Water, Milford, GB).

HVEP: Camag HVE system 60600, paper MN 261 (Camag, Muttenz, CH). The samples were dissolved in citrate/HCl buffer (Merck, Darmstadt) (pH 4.0) or in 0.5 m phosphate buffer (pH 6.9), standard desferal and glucose.

Isoelectrofocussing electrophoresis (IEF): Bio-Rad model 111 (Ivry sur Seine, F) using a Mini IEF cell with 125x65x0.4 mm polyacrylamide (5%) gel containing ampholine Byleyte 3/10 following the procedure recommended by the manufacturer.

**Production, Isolation and Derivatisation of the Pyoverdines**

*Pseudomonas fluorescens* 9AW or *Pseudomonas putida* 9BW, both isolated at the Schirmacher Oasis Antarctica (Shivaji et al., 1989) were grown for 72 hrs. in a medium consisting of 4 g succinic acid 6 g K₂HPO₄, 3 g KH₂PO₄, 1 g (NH₄)₂SO₄ and 0.2 g MgSO₄·7H₂O in 11 H₂O (pH adjusted to 6.5 with 10% KOH). After removal of the cell material by centrifugation the culture medium was adjusted to
pH 6.0 and passed through a XAD-4 resin column. The pyoverdine-containing fraction was subsequently eluted with CH$_3$OH/H$_2$O 1:1 (v/v) and lyophilized. To 180 mg of the lyophilized material dissolved in 25 ml H$_2$O (destilled) 3 ml of a 3% solution of Fe(III) citrate in H$_2$O were added and H$_2$O was removed i.v. at 30 °C. The dry residue was redissolved in 2 ml 0.1 M CH$_3$COOH (pH 5.0) and chromatographed on BioGel P-2 with 0.1 M CH$_3$COOH. From the fractions showing an absorption at 405 nm CH$_3$COOH was removed i.v. at 30 °C by adding several times H$_2$O, evaporation to dryness and subsequent drying at 0.1 torr. The fractions containing the ferri-pyoverdines were dissolved in 1 ml 0.2 M pyridinium acetate buffer (pH 5.0) and chromatographed on CM-Sephadex C-25 with 0.2 M pyridinium acetate buffer. Chromatography was repeated and from the brown fractions the buffer was removed i.v. at 30 °C by adding several times H$_2$O and bringing to dryness. For the structure elucidation described below the pyoverdines from *Pseudomonas fluorescens* 9AW were investigated in detail. Depending on the time needed for the work-up varying amounts of ferri-la and -lb could be obtained (la is a hydrolysis product of lb; cf. Schäfer et al., 1991), yield together ca. 17 mg. Ferri-lb was transformed into ferri-la by letting stand an aqueous solution (pH 9.0) for 10 days at room temperature (Geisen et al., 1992). Decomplexation was achieved by dissolving the ferri-la in 1% aqueous citric acid and shaking several times with a 5% solution of 8-hydroxyquinoline in CHCl$_3$ (Briskot et al., 1986). The free pyoverdines were purified by chromatography on Bio-Gel P2 with 0.1 M acetic acid. For qualitative and quantitative analysis of the amino acids, determination of their configuration and dansyl derivatisation of free amino groups see Briskot et al. (1986) and Mohn et al. (1990).

For the identification of OHHis 5 mg la were hydrolyzed with 6 M HCl at 110 °C for 21 hrs, the hydrolysate was adsorbed on a Sep-Pak RP-18 cartridge and the amino acids were eluted with H$_2$O. The eluate was brought to dryness i.v., three times redissolved in H$_2$O and finally dried for 30 min at 0.1 torr. After dansylation (see above) the purified dansylated amino acids were chromatographed on Kromasil using a gradient CH$_3$OH/acetate (pH 6.1) going from 10 to 100% CH$_3$OH v/v (detection at 254 nm) and comparison with authentic material including co-injection established the l-configuration.

To establish the position of d- and l-Ser in the molecule la was hydrolyzed for 30 min. The hydrolysate was adsorbed on a Sep-Pak RP-18 cartridge which retains the fractions containing parts of the peptide chain bound to the chromophore while peptides and amino acids not bound to the chromophore can be eluted with 0.1 M CH$_3$COOH. The retained material was desorbed with CH$_3$OH/H$_2$O 8:2 v/v. The chromophore-containing peptides were separated by chromatography on Bio-Gel P2 with 0.1 M CH$_3$COOH (detection at 254 nm). The molecular masses of the various fractions were determined by ESI-MS; the one whose mass corresponded to chromophore plus Ser was hydrolyzed for 21 hrs. In this way d-Ser could be identified after TAP-derivatization and chromatography on a chiral column (see above).

Edman-degradation of la (cf. Tarr, 1977). To 1 mg la dissolved in 20 ml H$_2$O/pyridine 1:1 (v/v) 10 ml of a 10% solution of phenylisothiocyanate in pyridine was added. The solution was degassed with N$_2$ for 10 sec and heated to 37 °C for 1 hr. The reaction mixture was extracted twice with 30 ml hexane/ethyl acetate 10:1 (v/v) each and 4 times with hexane/ethyl acetate 1:1 (v/v). After removal of the solvents from the aqueous phase i.v. the residue was dried at 0.1 torr over P$_2$O$_5$ for 1 hr. The residue dissolved in 10 ml waterfree CF$_3$COOH was degassed with N$_2$ for 10 sec and maintained at 37 °C for 30 min. The solvent was removed i.v., the residue was kept over solid NaOH i.v. for 30 min., then dissolved in 20 ml pyridine/H$_2$O 1:2 (v/v) and extracted 3 times with 30 ml hexane/ethyl acetate 1:1 (v/v). The aqueous phase was brought to dryness i.v. The residue was than dansylated and subsequently hydrolyzed (cf. above).

**Results**

**Characterization of la from Pseudomonas fluorescens 9AW**

The UV/Vis-spectra of la (λ nm, E cm$^{-1}$ mmol$^{-1}$; pH 7.0: 399, 15511; 227, 30771; pH 3.0: 374, 6244;
362, 6516; 245, 8826; 218, 19904) and of ferri-\textbf{1a} (pH 7.0: 401, 15386; 262, 12821; 231 32701; plus broad charge-transfer bands at about 470 (465, 3618) and 560 nm (546, 1950)) are typical for pyoverdines (Budzikiewicz, 1993). The molecular mass as determined by FAB- and ESI-MS was 1043 u. After total hydrolysis the following amino acids could be identified: L-threo-OHHis (by HPLC), L-Lys, L-OH-Orn, d-Ser, L-Ser, d-alloThr (by GC as TAP derivatives). The electrophoretic mobility of \textbf{1a} (cf. Poppe et al., 1987) showed +1 charge at pH 6.9 and +2 charges at pH 4.0. The chromophore and Lys are protonated at pH 6.9, the succinate side chain provides -1 charge (\(\sum +1\)). The additional positive charge at pH 4.0 comes from the imidazole ring of OHHis (\(pK_{S1} 3.68, pK_{S2} 5.29\); Mooberry et al., 1980).

To determine which NH\(_2\)-group (\(\alpha\) or \(\varepsilon\)) is free in \textbf{1a} ferri-\textbf{1a} was dansylated and hydrolyzed. \(\varepsilon\)-Dansylamino-Lys could be identified by HPLC using authentic comparison material. Other dansylated amino acids (esp. \(\alpha\)-dansylamino-Lys) were not detected. This result was confirmed by the failure of an Edman degradation. Especially after dansylation and hydrolysis no dansyl-OHHis (or any other dansylated amino acid) could be detected. It follows that no cleavage of the molecule had occurred and hence no free \(\alpha\)-amino group was present. Note that no \(\varepsilon\)-dansylamino-Lys could be found either; apparently the \(\varepsilon\)-amino group had reacted with phenylisothiocyanate.

From partial hydrolysis a fragment containing the chromophore and Ser could be isolated; subsequent hydrolysis gave d-Ser as identified after TAP-derivatization by GC on a chiral column. Thus the N-terminal amino acid of the of the peptide chain bound to the chromophore is d-Ser.

**Sequence determination by NMR**

For a detailed discussion of the NMR-techniques see Evans (1995). H.H-COSY shows \(^3\)J-coupling of H-C-C-H while \(^4\)J- and \(^5\)J-coupling within one amino acid residue can be detected by TOCSY. The single amino acids can be identified by these techniques corroborated by shift values in comparison with literature data. Direct \(^1\)J C,H-connections can be determined by HMQC, \(^2\)J- and \(^3\)J-C,H-coupling by HMBC. Peptide sequencing is possible by ROESY which by resorting to Nuclear Overhauser Effects allows a correlation between an NH-proton and spatially close \(\alpha\)- and \(\beta\)-H of the preceding amino acid (-CH-CH-CO-NH-). \(^1\)H- and \(^13\)C-measurements and peptide sequence

The \(^1\)H- and \(^13\)C-data are assembled in Tables I and II. Those of the chromophore and of the succinic acid side chain correspond to the ones observed for other pyoverdines (Budzikiewicz, 1993). From the TOCSY spectrum the signals of two Ser can be identified. The shifts of the \(\beta\)-CH\(_2\)-groups (~4 ppm) indicate that the OH-groups are not esterified (esterifications results in downfield shift of ~0.5 ppm). The low-field resonance of one of the NH-protons (9.7 ppm) is in agreement with the direct connection with the chromophore-

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**Table I. \(^1\)H-NMR data of \textbf{1a} (pH 4.3).**

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<tr>
<td>c(OH)Orn</td>
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Table II. $^{13}$C-NMR data of 1a (pH 4.3).

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<td>139.5</td>
<td>115.4</td>
<td>114.6</td>
<td>144.2</td>
<td>152.0</td>
<td>100.9</td>
</tr>
</tbody>
</table>

| Ser  | 172.2 | 57.6 | 61.6 |
| Lys  | 174.4 | 54.0 | 30.7 | 22.6 | 26.5 | 39.7 |
| (OH)His | 170.5 | 58.2 | 65.6 | 134.6 | 117.0 |
| aThr | 171.8 | 59.9 | 67.4 | 19.1 |
| Ser' | 172.5 | 56.4 | 61.8 |
| (OH)Orn | 167.1 | 51.1 | 27.3 | 20.6 | 52.3 |

COOH (see above). From the shift of the $\beta$-CH of aThr (4.14 ppm) in the same way as for Ser esterification of the OH-group can be excluded. Also the values for the $\varepsilon$-CH$_2$-group of Lys agree with a free NH$_2$-group and exclude an amide bond in accordance with the degradation data. For the imidazol ring of OHHis two aromatic singlets are observed. The C-terminal N-hydroxy-cyclo-Orn shows the typical signals for this system (Mohn et al., 1990). Since all amide NH could be identified within the amino acid residues constituting the peptide chain (see Table I) sequence information could be obtained from the ROESY spectra as depicted in Fig. 1. In this way the partial structures L-Ser-D-aThr-D-OHHis-L-Lys and D-Ser-chromophore-succinic acid can be derived. L-cOHOrn can only be the C-terminus of the peptide chain. Hence the complete sequence of 1a amounts to L-cOHOrn-L-Ser-D-aThr-D-OHHis-L-Lys-D-Ser-chromophore-succinic acid.

**Mass spectrometric evidence**

In the ESI-MS spectrum of 1a after collision induced fragmentation either in the skimmer region or in the ion trap several sequence-characteristic ions could be observed arising from cleavages at the peptide bonds. They are summarized in Fig. 2 and confirm the conclusions derived from the NMR data.

The pyoverdine isolated from *Pseudomonas putida* 9BW has the same molecular mass as the one obtained from *P. fluorescens* 9AW as determined by FAB-MS, the same aminoacid composi-
Fig. 2. Characteristic ions in the ESI mass spectrum of 1a (mass numbers without marks: sequence ions observed only by skimmer collision activated decomposition (skimmer CA); with *: observed by skimmer CA and in the ion trap; with **: observed only in the ion trap).

Discussion

Hancock and coworkers (Wang et al., 1990; Hancock et al., 1993; Hancock and Reeder, 1993; Hancock, 1994) reported a pyoverdine from Pseudomonas fluorescens 244 (pyoverdine Pf244) which is probably identical with 1a. The sequence and the stereochemistry of the amino acids of the peptide chain are the same (the position of d- and L-Ser had not been determined). The only difference is that in the preliminary report (Wang et al., 1990) a linkage between a Ser carboxyl group and the ε- rather than the α-amino group of Lys is proposed “as (NMR) studies on the Ga-chelated material suggested”, but no data were given and the claim was not repeated (and substantiated) in the subsequent publications.

The occurrence of L-threo-β-hydroxy His in 1a is of interest as this rare amino acid had not been encountered before in any one of the over 30 known pyoverdines. But of greater importance is the fact that 1a is produced both by a P. fluorescens and a P. putida strain.

Pseudomonas species of the fluorescent group can be subdivided into strains (“siderovars”) which produce pyoverdines differing in their peptide chains and which are recognized for the most part only by the producing strain. Thus, 3 siderovars of P. aeruginosa are well established (Meyer et al., 1997) and for P. fluorescens the complete structures of 10 pyoverdines and partial ones for 10 more are reported in the literature (cf. Budzikiewicz, 1993). Occasionally, enhanced bacteria growth has been observed upon addition of a “foreign” pyoverdine to a culture, as for various P. putida and P. fluorescens strains (Jacques et al., 1995). Both, induction of an appropriate receptor protein (Koster et al., 1993) and iron exchange between the Fe(III) complex of the added and the newly formed own pyoverdine (Gipp, 1987) has been invoked as an explanation. It is more remarkable when pseudomonads classified as different species produce the same pyoverdine. The only unequivocal example so far is P. fluorescens ATCC 1352 and P. chlororaphis ATCC 9446 (Hohlneiche et al., 1995). In this paper we report the production of 1a both by P. fluorescens 9AW and P. putida 9BW (Shivaji et al., 1989).

P. fluorescens (Palleroni, 1984; Palleroni, 1992; Elomari et al., 1996) is a collective species which originally had been subdivided into 7 biovar...
The differing nutritional patterns of the various biovars are less clear cut. *P. fluorescens* ATCC 13525 and *P. chlororaphis* ATCC 9446 may well be borderline species which would explain the formation of identical pyoverdines.

The main distinguishing feature for *P. putida* is the lack of gelatinase. The species is subdivided into the biovars A and B. Biovar B (to which *P. putida* 9BW apparently belongs, Shivaji et al., 1989) "is phenotypically closer to *P. fluorescens* than to typical *P. putida* (biovar A)" (Palleroni, 1992, p. 3077). The present results confirm this conclusion; should other strains of *P. putida* (biovar B) also produce pyoverdines typical for *P. fluorescens* strains a reclassification should be considered.

In any case "in [Palleroni's (1992, p. 3095)] opinion, differentiation of *P. aeruginosa* from all other fluorescent organisms is sharp, but distinction among the remaining fluorescent organisms ... is not as clear cut." The formation of fluorescent pigments particularly in iron-deficient media has been taken so far as a general characteristic for all species belonging to the fluorescent group, but an identification as pyoverdines and a consideration of their structural differences has not been taken into account. As more than 30 different pyoverdines are known today they should be considered as indicators for the classification of *Pseudomonas* strains of the fluorescent group which could be more reliable than nutritional patterns or the formation or absence of phenazines (from the published Tables, Palleroni 1984 and 1992, it can be seen that characteristic features as nutritional characteristics are rarely observed for 100% of the investigated strains).

**Acknowledgement.**

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