An Isopyoverdin from *Pseudomonas putida* CFML 90–44

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From *Pseudomonas putida* CFML 90–44 an isopyoverdin was isolated. Its structure could be elucidated by chemical degradation and spectroscopic data.

**Introduction**

*Pseudomonas putida* is a bacterium commonly found in soil and water. It is able to use almost any carbon source and to degrade even polycyclic and chlorinated aromatic compounds (Silver et al., 1990). It is only potentially human pathogenic, i.e., it may infect only persons whose immune system is severely impaired (Graevenitz and Weinstein, 1971). *Pseudomonas putida* belongs to the so-called fluorescent pseudomonads which commonly produce siderophores named pyoverdins, chromopeptides consisting of a dihydroxyquinoline chromophore bound amidically to the N-terminus of a peptide chain by its carboxyl group at C-1, and to a small dicarboxylic acid or its amide by the amino group at C-5 (Budzikiewicz, 1997a and 1997b). In two instances a siderophore was isolated from a *P. putida* strain where the carboxyl group carrying the peptide chain is located at C-3 of the chromophore (Jacques et al., 1995; Sultana et al., 2001) thus differing from the rest of the about 50 pyoverdins described so far (Kilz et al., 1999). The only other example of this “iso”-chromophore has been reported for the siderophore of *Azomona macrocytogenes* ATCC 12334 (Michalke et al., 1996). We wish now to report the structure elucidation of a further example from *P. putida* CFML 90–44.

**Materials and Methods**

*Pseudomonas putida* CFML 90–44 is a hospital isolate from sputum. It was classified according to its phenotype as belonging to cluster II, subcluster Ib and according to its ribotype to cluster E (Elomari et al., 1994 and 1997). The strain was grown in a succinate minimal medium (Budzikiewicz et al., 1997). For the work-up of the culture and isolation of the ferri-complex by chromatography on XAD-4 and Biogel P-2 see Georgias et al. (1999). The Biogel fraction was subjected to ion-exchange chromatography on CM-Sephadex A-25 with a pyridinium acetate buffer (pH 5.0, 0.02 m). The second (major) fraction was pure as checked by analytical RP-HPLC with CH$_3$OH/CH$_3$COONa$_2$ buffer (pH 6.2) and was decomplexed with 8-hydroxyquinoline (Briskot et al., 1986); 1 was checked for purity by analytical RP-HPLC with a CH$_3$OH/0.1 m CH$_3$COONa$_2$/1 mm Na$_2$EDTA solution.

For the qualitative and quantitative analysis of the amino acids, the determination of their configuration by GC/MS of their TAP-derivatives on a chiral column and the dansyl derivatization see Briskot et al. (1986) and Mohr et al. (1990). For instrumental details see Sultana et al. (2000).

Siderotyping analysis of the strain was performed through pyoverdin isoelectrofocusing and $^{59}$Fe$_3$ incorporation as described previously (Meyer et al., 1998; Munsch et al., 2000). The following strains were tested for comparison (isopyoverdins in **bold**): *Pseudomonas* sp. E8; *P. syrin-
Results

Siderotyping behavior of P. putida CFML 90–44

P. putida CFML 90–44 is characterized by a pyoverdin-mediated iron uptake system specifically restricted to its own pyoverdin. None of 34 tested pyoverdins (see above) of foreign origin was able to facilitate iron incorporation into the strain. The isoelectrophoretic pattern developed by the pyoverdins (i.e. differing in the chromophore side chains) present in the growth culture supernatant (CAA growth medium, see Munsch et al., 2000) revealed a major band at pI 4.85 and three minor bands at pI 7.10, 4.25 and 3.90. This pattern was unique compared with those developed by the 34 pyoverdins mentioned above. Thus, both siderotyping methods suggested a novel structure for the pyoverdin of P. putida CFML 90–44.

Characterization of 1

The UV/Vis spectrum of 1 is characteristic for the isopyoverdin chromophore (Michalke et al., 1996): 402 nm at pH 7.0, split band at 366 and 376 nm at pH 3.0; ferri-1 400 nm and broad charge-transfer bands at ~475 and 550 nm. The molecular mass of 1 was determined by FAB-MS as 1437 u.

Identification of the iso-chromophore and the amino acids of 1

The NMR characteristics of the isopyoverdin (iPyo) as compared with those of the pyoverdin (Pyo) chromophore were discussed in detail before (Jacques et al., 1995; Michalke et al., 1996; Sultana et al., 2001). In the 1H spectrum the shifts of the protons of the 1-CH- and of the 3-CH2-group of Pyo and of the 3-CH- and of the 1-CH2-group of iPyo show significant differences due to their respective relative proximity to the aromatic part of the chromophore. Especially the downfield-shifted signal of the CH-1 of Pyo (ca. 5.7 ppm) is missing in the iPyo spectrum. The presence of a CH2-group at C-1 of 1 follows from two-dimensional correlations: the two protons of C-1 (3.88 and 4.50 ppm) exhibit NOE cross peaks to the H of C-10 at 7.10 ppm. Also the 13C shifts of C-1 and C-3 (43.8 and 51.9 ppm, identified in the HMQC spectrum which offers 1J-CH correlations) differ from those of a pyoverdin chromophore in correspondence with the literature data. Typically the NH resonance value of the amino acid (Asp) attached to the carboxyl group of the chromophore is not shifted downfield relative to the other NH signals as it is observed for pyoverdins: it is not in the influence sphere of the aromatic part of the chromophore.

After total hydrolysis and GC-MS analysis of the TAP-derivatives on a chiral column the following amino acids could be identified: l-Asp, l-Glu, Gly, l-Lys, d-Orn, d- and l-Ser (1:1) and l-Thr. Which of the Ser are d- and which are l-configurated remains open. Hydrolysis after dansylation yielded e-dansyl Lys as shown by chromatographic comparison with authentic α- and e-dansyl Lys. Hence, in 1 the ε-amino group of Lys is free.

Determination of the amino acid sequence

For a discussion of the various NMR techniques applied for the identification of the 1H- and 13C-signals see e.g. Sultana et al. (2000 and 2001). Those of the peptide chain and the Glu side chain correspond to those observed with other pyoverdins (Budzikiewicz, 1997a and 1997b). Only the following ones deserve a comment: the shift values of the β-CH-groups of Thr (4.29 ppm) and of the four Ser (3.90 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected) (Budzikiewicz, 1997b). The shift values of Asp correspond to literature data (e.g., Jacques et al., 1995) in ac-
c-time 305
DIL -Sen  
HOH2 C
DtL-Sev2
HOH2 C.
DIL- Ser3
HOH2 C ' o  
D/L-Seu
HO 'H  
HO ' ^ .^ " " N H R
R = — ... 305 3
Ser4 9 218 2
cOHOrn 131b 1
a  CA of \([M-H_2O + 2H]^2+\) in the ion trap, b  CA of  
\([M+2H]^2+\) in the octapole.

**Table I.** MS-CA spectrum of 1, \([B-H_2O]\) and \(Y^\prime\)-ions.

<table>
<thead>
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<th>Amino acid</th>
<th>n</th>
<th>(B_n-H_2O)</th>
<th>(Y_n^\prime)</th>
<th>n</th>
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<tr>
<td>Glu-Chr</td>
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<tr>
<td>Asp</td>
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<td>612</td>
<td>937</td>
<td>9</td>
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<td>Lys</td>
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<td>784</td>
<td>809</td>
<td>8</td>
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<tr>
<td>AcOHOrn</td>
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<td>885</td>
<td>7</td>
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<tr>
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<tr>
<td>cOHOrn</td>
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</table>

**Discussion**

According to the current biosynthesis scheme the chromophore of pyoverdins originates from a condensation product of d-Tyr and l-Dab found

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**Fig. 1.** Structure of the isopyoverdin 1 (2 d-Ser and 2 l-Ser).
in the ferribactins (Böckmann et al., 1997). Connection of the α-N of Dab with the 2-position of the phenyl ring of Tyr results in the isoquinoline ring of the pyoverdins. An analogous reaction sequence with the γ-N of Dab leads to the isopyoverdins. Pseudomonas isopyoverdins were found to be produced so far only by P. putida strains and they exhibit some structural analogues (L-Asp as the first amino acid, Glu as side chain). How and why isopyoverdins rather than pyoverdins are produced is still a moot point.

It is important to note that P. putida CFML 90–33 and P. putida BTP1 are unable to mediate iron uptake in P. putida 90–44, neither does P. putida CFML 90–33 accept the ferri-pyoverdin of P. putida BTP1 (Sultana et al., 2001). Obviously the iso-chromophore is not involved primarily in the recognition at the receptor site and differences in the peptide chains, viz. l-Asp-l-Lys-d-AcOHOrn-l-Thr-Ser-Ser-Gly-Ser-Ser-d-cOHOrn (90–44) l-Asp-l-Lys-l-Thr-d-OhAsp-l-Thr-d-aThr-l-cOHOrn (90–33) l-Asp-l-Ala-l-Asp-d-AcOHOrn-l-Ser-l-cOHOrn (BTP1) are responsible for the negative cross-uptake results.

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