The Structure of the Pyoverdin from *Pseudomonas fluorescens* 1.3. Structural and Biological Relationships of Pyoverdins from Different Strains*

Halka Georgias, Kambiz Taraz, Herbert Budzikiewicz, Valerie Geoffroy and Jean-Marie Meyer

a Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, D-50939 Köln, Germany
b Laboratoire de Microbiologie et Génétique, Université Louis Pasteur, UPRES-A n° 7010, 28 rue Goethe, F-67000 Strasbourg, France

Z. Naturforsch. 54c, 301–308 (1999); received February 26/March 12, 1999

**Pseudomonas fluorescens**, Pyoverdin, Iron Transport

The structure of the pyoverdin siderophore of *Pseudomonas fluorescens* 1.3 was elucidated by spectroscopic methods and chemical degradation. It shows structural similarities with the pyoverdins of several other strains. Whether mutual recognition occurs was investigated by growth tests.

**Introduction**

The detailed structural investigation of pyoverdins started almost 100 years after Gessard (1892) had first described their formation by fluorescent pseudomonads, namely when Teintze *et al.* (1981) published the structure of pseudobactin. In the meantime over 30 complete or fairly complete structures were elucidated and from preliminary studies it appears that many more are to be expected (Kilz *et al.*, 1999). Pyoverdins consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6 to 12 amino acids bound to its carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amicidally to the NH₂-group (cf. 1). Usually several pyoverdins co-occur having the same peptide chain, but differing in the nature of the dicarboxylic acid. The peptide chain has a twofold function. It provides two of the ligand sites for Fe³⁺, and it is responsible for the recognition of the iron complex at the surface of the producing cell (Hohnadel and Meyer, 1988; Budzikiewicz, 1997a). The variability of the peptide chain is closely connected with the second function: It safeguards that a given ferri-pyoverdin is available only to the producing strain because of a highly specific interaction between the pyoverdin and a receptor outer membrane protein (Meyer *et al.*, 1979). There are, however, examples known where *Pseudomonas* strains can accept pyoverdins differing in the peptide chain from the one produced by themselves, as certain *P. fluorescens* and *P. putida* strains (Jacques *et al.*, 1995) or *P. aeruginosa* ATCC 15692 that recognizes the pyoverdin of *P. fluorescens* ATCC 13525 (Hohnadel and Meyer, 1988; Kinzel *et al.*, 1998). It is still an open question whether the foreign ferri-pyoverdin is accepted by the receptor of the own pyoverdin due to structural similarities or whether a new receptor is developed (Koster *et al.*, 1993). The first hypothesis is favored by a recent report (Meyer *et al.*, 1999) demonstrating that FpvA, the ferripyoverdin receptor of *P. aeruginosa* ATCC 15692 efficiently recognizes the pyoverdin of *P. fluorescens* ATCC 13525. The...

---

**Abbreviations:** Common amino acids, 3-letter code; OHAsp, *threo*-β-hydroxy Asp; Dab, 2,4-diaminobutyric acid; FoO H O rrn, N⁵-formyl-N⁵-hydroxy-Orn; cOHOrn, OHAsp, /ira?-ß-hydroxy Asp; Dab, 2,4-diaminobutyric acid; TMS, tetramethylsilane.

* Part LXXII of the series “Bacterial Constituents”. For Part LXXXI see Münzinger *et al.* (1999), for Part LXX see Voss *et al.* (1999).

Reprint requests to Prof. Dr. H. Budzikiewicz.

Fax: +49–221–470–5057
E-mail: h.budzikiewicz@uni-koeln.de

0939–5075/99/0500–0301 $ 06.00 © 1999 Verlag der Zeitschrift für Naturforschung, Tübingen. www.znaturforsch.com
structure elucidation of the *P. fluorescens* pyoverdin Pf 1.3 (1), that shows structural similarities with the pyoverdins of *P. fluorescens* ATCC 17400, *P. fluorescens* 51W, *P. fluorescens* ii and one from a *P. putida* isolate (for the structures see Discussion) allows to study the cross uptake between these strains.

**Material and Methods**

**Instruments**

Mass spectrometry: Finnigan-MAT HSQ-30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI; 50 µm solutions in CH₃OH/H₂O 1:1 v/v); GC/MS Incos 500 (all Finnigan-MAT; Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: AM 300 (*H 300, ¹³C 75.5 MHz) and DRX 500 (*H 500, ¹³C 125 MHz) (both Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS: δ(TMS) = δ(DSS) for *H, δ(TMS) = δ(DSS) – 1.61 ppm for ¹³C.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen).

Chromatography: RP-HPLC Knauer (Berlin), column Nucleosil 100-C₁₈ (preparative 7, analytical 5 µm) (Knauer, Berlin); low pressure chromatography column Biogel P-2 (Bio-Rad, Richmond CA, USA), XAD-4 (Serva, Heidelberg); GC: columns SE 54 (CS, Langerwehe), Chirasil-L-Val (Macherey-Nagel, Düren).

**Production, isolation and derivatisation of 1**

*Pseudomonas fluorescens* 1.3 (a fish isolate, Champomier and Richard, 1994) was grown for 72 hrs. in 6 l of a succinate medium (Budzikiewicz et al., 1997). After addition of 10 ml 5% Fe(III)-citrate solution cell material was removed by tangential filtration. The solution was adjusted to pH 5.5-6.0 with 6 M HCl and passed through a XAD-4 resin column. Inorganic material was washed out with 15 l H₂O, then ferri-1 was eluted with 2.5 l CH₃OH/H₂O 8:2 (v/v), brought to dryness i.v. at 30 °C, redissolved in 0.2 M pyridinium acetate buffer (pH 5.0) and chromatographed on Biogel P-2 with the same buffer. The fraction containing ferri-1 (detection at 405 nm) was brought to dryness i.v. at 30 °C. For complete removal of the buffer substances were redissolved in H₂O, brought to dryness again and further purified by RP-HPLC on Nucleosil 100-C₁₈ (solvent 0.1 M CH₃COONH₄-buffer, pH 6.2). Purity was controlled by analytical RP-HPLC. Decomplexation was achieved with 8-hydroxyquinoline (Briskot et al., 1986), free 1 was purified by chromatography on Bio-Gel P-2 with 0.1 M CH₃COOH. For qualitative and quantitative analysis of the amino acids, determination of their configuration and dansyl derivatisation of the free ε-amino group of Lys see Briskot et al. (1986) and Mohn et al. (1990).

**Growth stimulation tests**

Pyoverdin-induced growth stimulation tests were performed by plating the bacteria (100 µl of a 1/10 diluted overnight culture in a rich medium containing of 10 g peptone, 5 g meat extract and 5 g NaCl) in KingB-agar (Difco, Detroit MI, USA) medium supplemented with 500 mg/l of ethylene-diamine dihydroxyphenyl acetic acid. UV-sterilized filter paper discs (6 mm diameter) were impregnated with 10 µl of 1 mM XAD-purified pyoverdin solutions and applied at the surface of the seeded agar. Bacterial growth was determined after 24 h incubation at 25 °C and scored as (++) for growth diameter ≥ 15 mm, (+) for growth diameter < 15 mm, or (−) for no stimulated growth around discs (Table I).

**Results**

**Characterization of 1**

The UV/Vis spectra of 1 (λ, 401 nm: pH 6.8; 379 and 368 nm: pH 3.0) and of ferri-1 (402, 470 and 550 nm: pH 6.8) are typical for pyoverdins (Budzikiewicz, 1997b). The molecular mass as determined by FAB- and ESI-MS is 1285 u. After total hydrolysis the following amino acids could be identified: 1 D-Ala, 1 L-OHAsp, 1 L-Dab, 3 Gly, 1 d-Glu, 1 L-Lys, 1 L-OHOrn, 1 L-Ser plus succinic acid. After dansylation and total hydrolysis ε-dan-
The \(^{1}H\) and \(^{13}C\)-data are assembled in Tables II and III. Those of the chromophore and of the succinic acid side chain correspond to the ones observed for other pyoverdins (Budzikiewicz, 1997b). The signals of the amino acids including the tetrahydropyrimidine condensation product between Dab and the preceding Gin (esp. the low-field shift of the \(\alpha\)-CH at 4.58 ppm; Gwose and Taraz, 1992) and cOHOrn (Mohn et al., 1990) could be identified by TOCSY experiments and comparison with literature data. The shift of the CH\(_{2}\)-group of Ser (3.89 ppm) indicates that the OH-group is not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected). The low-field resonance of the Ala-NH (9.60 ppm) is in agreement with the direct connection with the chromophore. The shift values of the e-CH\(_{2}\)-group of Lys agree with the free NH\(_{2}\)-group in accordance with the dansylation experiment (\textit{v. supra}). The presence of Gin rather than Glu is confirmed by the NOESY spectrum (NOE between the amide protons and those of the \(\gamma\)-CH\(_{2}\)-group). Since all amide NH of the peptide chain could be identified (see Table II) the sequence could be determined from the NOESY and ROE SY spectra as depicted in Fig. 1. The result is confirmed by the coupling between the carbonyl-C signals of the amide.

**Sequence determination by NMR**

For a detailed discussion of the NMR techniques see Evans (1995). TOCSY allows to detect \(^4\)J- and \(^3\)J-coupling within one amino acid residue, NOESY and ROE SY allows a correlation between an NH-proton and spatially close \(\alpha\)- and \(\beta\)-H of the preceding amino acid (CH-CH-CO-NH-). \(^2\)J- and \(^3\)J-CH-coupling can be established by HBMC.

---

**Table II. \(^1\)H-NMR data (\(\delta[ppm]\)) of Pf 1.3 (pH 4.3, 5 °C, H\(_2\)O/D\(_2\)O).**

<table>
<thead>
<tr>
<th>Suc</th>
<th>2'</th>
<th>3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HN</td>
<td>2.70</td>
<td>2.61</td>
</tr>
<tr>
<td>1</td>
<td>2a</td>
<td>2b</td>
</tr>
<tr>
<td>2</td>
<td>3a</td>
<td>3b</td>
</tr>
<tr>
<td>4HN+</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>H(_2)N</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>2'</th>
<th>3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>9.60</td>
<td>4.37</td>
</tr>
<tr>
<td>Lys</td>
<td>8.67</td>
<td>4.24</td>
</tr>
<tr>
<td>Gly 1</td>
<td>8.47</td>
<td>3.88</td>
</tr>
<tr>
<td>Gly 2</td>
<td>8.40</td>
<td>3.95</td>
</tr>
<tr>
<td>(OH)Asp</td>
<td>8.38</td>
<td>4.82</td>
</tr>
<tr>
<td>Gln</td>
<td>8.94</td>
<td>4.58</td>
</tr>
<tr>
<td>Dab</td>
<td>9.76</td>
<td>4.49</td>
</tr>
<tr>
<td>Gly 3</td>
<td>8.54</td>
<td>4.05</td>
</tr>
<tr>
<td>Ser</td>
<td>8.58</td>
<td>4.48</td>
</tr>
<tr>
<td>c(OH)Orn</td>
<td>8.67</td>
<td>4.48</td>
</tr>
</tbody>
</table>
bonds with the following NH- and CH-signals of the following amino acids (Fig. 2).

**Mass spectral evidence**

In the ESI-MS spectrum of 1 after collision induced fragmentation either in the skimmer region or in the ion trap several sequence-characteristic ions could be observed arising from cleavages of the peptide bonds. They are summarized in Fig. 3 and confirm the conclusions derived from the NMR data. In addition the product of a partial hydrolysis (6 M HCl, 10 min, 90 °C) was investigated by ESI-MS. The hydrolysis products (Chr.. pyoverdin chromophor) Chr-Ala (m/z 347), Chr-Ala-Lys (m/z 475), [Glu-Dab – H2O] (m/z 230) and [Glu-Dab – 2 H2O] (m/z 212) (characteristic for the condensation product giving the tetrahydro-pyrimidine ring, Gwose and Taraz, 1992), OHAsp-[Glu-Dab – H2O] (m/z 361) and Gly-OHAsp-[Glu-Dab – H2O] (m/z 418) could be identified.
After prolonged hydrolysis (6 M HCl, 40 min, 110 °C) and TAP derivatization (Dallakian and Budzikiewicz, 1997) the derivatives of all amino acids of 1 except those of Dab and Glu could be detected by GC/MS. The dihydropyrimidine ring is generally more stable against acid hydrolysis than amide bonds. Instead, the derivative 2 typical for these condensation products (Filsak et al., 1994) could be observed again confirming the presence of this structural element.

Discussion

The standard opinion is that ferri-pyoverdins are recognized only by the receptor outer membrane protein of the producing strain. As stated in the Introduction exceptions to this rule can be found in the literature. The examples presented so far do not allow any conclusions regarding structural prerequisites for the recognition of a pyoverdin by a strain that produces a pyoverdin with another amino acid sequence: From the six siderophores tested by Jacques et al. (1995) one (BTP 1) is an isopyoverdin with a different chromophore, and BTP 7 and BTP 16 are structurally so different (Ongena et al., 1998) that a negative cross-recognition is not surprising. The structures of those pyoverdins where cross-recognition was detected (BTP 2, 7 and 9 as well as BTP 14 accepted by 16) are still unknown. The best-documented (Hohnadel and Meyer, 1988; Kinzel et al.,
Fig. 3. Characteristic ions in the ESI mass spectrum of Pf 1.3 observed in the ion trap (dotted lines); fragments observed in the MS3 spectrum of m/z 468 (solid lines): 1: m/z 338, 2: m/z 310, 3: m/z 251, 4: m/z 223, 5: m/z 194.

1998; Kinzel et al., 1999) pair are the pyoverdins of Pseudomonas aeruginosa ATCC 15692 (3) (Briskot et al., 1989) and of P. fluorescens ATCC 13525 (4) (Hohlneicher et al., 1995). Their common feature is the C-terminal cyclic substructure formed by an amide bond between the carboxyl group of the C-terminal amino acid and the ε-amino group of Lys following FoOHOrn (note, that even the first three amino acids show a comparable pattern – D-Ser – basic – small neutral). The similarity of the two structures is obvious. A detailed investigation of the cross-recognition of pyoverdins with a C-terminal macrocycle is on the way. (In the following formulas D-amino acids are underlined, for modified amino acids see Abbreviations, Chr indicates the pyoverdin chromophore with the acid side chain. Gln/Dab indicates the tetrahydropyrimidine ring formed by condensation of Gln and Dab as in Fig 1).

Chr-Ser-Arg-Ser-FoOHOrn-(Lys-FoOHOrn-Thr-Thr) (3)
Chr-Ser-Lys-Gly-FoOHOrn-(Lys-FoOHOrn-Ser) (4)

With the structure elucidation of 1 the mutual recognition of four pyoverdins without a cyclic subunit from Pseudomonas fluorescens strains can now be compared which show remarkable structural similarities, viz. 5 from P. f. 17400 (Demange et al., 1990), 6 from P. f. 51W (Voss et al., 1999) and 7 from P. f. ii (Mohn et al., 1990). 8 from P. putida (Gwose et al., 1992) is added to the list because it also contains the tetrahydropyrimidine ring formed by condensation of Gln and Dab following OHAsp.

Chr-Ala-Lys-Gly-OHAsp-Gln/Dab-Gly-Ser-cOHOrn (1)
Chr-Ala-Lys-Gly-OHAsp-Gln/Dab-Ser-Ala-cOHOrn (5)
Chr-Ala-Lys-Gly-OHAsp-Gln-Ser-Ala-Gly-aThr-cOHOrn (6)
Chr-Ala-Lys-Gly-OHAsp-Gln-Ser-Ala-Ala-Ala-Ala-cOHOrn (7)
Chr-Ser-Thr-Ser-Orn-OHAsp-Gln/Dab-Ser-aThr-cOHOrn (8)

Recognition of foreign pyoverdins was tested by growth experiments involving the five strains mentioned and their corresponding pyoverdins. As shown in Table I, the best growth stimulation for a given strain was always observed with its own pyoverdin. Those of foreign origin gave variable responses from no effect to growth stimulation which, in some cases, reached an efficiency comparable to the one observed with the homologous pyoverdin.

The N-terminal part of the peptide chains of 1 and 5 is identical; it then follow two small neutral amino acids and the common C-terminal cOHOrn. The strain producing 1 shows the same acceptance for 5 as for 1, but the strain producing 5 accepts 1 only to a lesser degree than its own pyoverdin.
The second pair are 6 and 7 where again the first six amino acids are identical. The same divergence is observed as for the preceding pair: The strain producing 7 accepts 6 though to a lesser degree than 7, but the strain producing 6 does not accept 7.

8 has in common with 1 and 5 OHAsp-Gln/Dab and with 5 the following Ser. The producer of 8 recognizes 5 in the same way as 8, the producer of 5 accepts 8 and 1, though to a lesser degree than 5, and the producer of 1 accepts 8, but again not as well as 1.

As had been stated in the Introduction that the acceptance of a pyoverdin produced by a different strain and characterized by a peptide chain different from that of the own pyoverdin may be due (a) to identical or very similar structures in the part of the peptide chain responsible for the recognition by the own receptor outer membrane protein, (b) by the presence of multiple receptors each being specific to one type of pyoverdin, or (c) by the induced development of a new receptor. A less specific porin-mediated transport may also be taken into consideration (Meyer, 1992). The growth results reported above seem to show that identical or structurally very similar “key-sequences” can lead to a mutual acceptance, but that the requirements for the recognition are not of the same stringency for every strain, otherwise the same acceptance would be expected in both directions. The amino acids close to the chromophore apparently are not decisive for the recognition in agreement with the observation that a modification of the basic amino acid (Lys in 4 or Arg in 3) does not hinder the transport into the cell (Kinzel et al., 1998; Kinzel and Budzikiewicz, 1999). A decision, which of the possible explanations mentioned above for the acceptance of a foreign pyoverdin actually operates for a given strain, will only be possible when kinetic studies have been performed and the identification of the receptor proteins has been effected. It must also be said that too little is known about the three-dimensional structures of the iron complexes of the ferri-pyoverdins which may play an additional role (information is available only for the solution structures of three ferri-pyoverdins: Mohn et al., 1994; Tappe, 1995; Atkinson et al., 1998).

Acknowledgement

This research was supported by the European Commission DG XII under the project “Cell factories for the production of bioactive peptides from Bacillus subtilis and Pseudomonas” (Bio4-CT95–0176). The authors thank M. C. Champomier-Verges (INRA, Jovy en Josas, France) for the gift of the strain Pseudomonas fluorescens 1.3.


