

A New Pyoverdin from *Pseudomonas aureofaciens**

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Pseudomonas aureofaciens, Pyoverdin, Siderophore

From *Pseudomonas aureofaciens* a new pyoverdin was isolated and its structure was determined by various spectroscopic methods and by partial degradation.

Introduction

Iron, after oxygen, silicon and aluminum, is the element fourth in abundance by weight in the earth crust. For most living organisms, however, it is the critical determinant of primary production because of its low availability. This is due to the fact that Fe^{3+} is precipitated at physiological pH-values as insoluble ferric hydroxide ($K_s = 10^{-39}$). To overcome this iron stress, many bacteria (and eucariotic organisms as well) excrete siderophores, i. e. compounds, that form highly stable water soluble complexes with Fe^{3+} . The ferri-siderophore is recognized and taken up by proteins in the outer membrane of the bacterial cell. Bacteria of the genus *Pseudomonas* from the so-called fluorescent group produce characteristic siderophores, the pyoverdins (Budzikiewicz, 1993 and 1997). They have three structural elements: a peptide chain of 6–12 amino acids, the dihydroxyquinoline chro-

mophore and a side chain which is an acid derived from the citrate cycle (Teintze *et al.*, 1981, Michels *et al.*, 1991). The two hydroxy groups of the chromophore and two amino acids from the peptide chain form an octahedral complex with Fe^{3+} as central ion. The coordinating sites in the peptide chain can be hydroxamates derived from Orn or α -hydroxy-carboxylate amino acids such as β -hydroxy-Asp or β -hydroxy-His. Another important purpose of the peptide chain is the recognition of the iron complex on the cell surface. Most *Pseudomonas* strains produce their own pyoverdins distinguishable by the composition of their peptide chain. In this communication we describe the isolation and structure determination of the first pyoverdin from *Pseudomonas aureofaciens*.

Materials and Methods

Instruments

UV/VIS spectroscopy: HITACHI 200, LAMBDA 7 (both Perkin-Elmer, Überlingen), 1 mg of **1a** in 20 ml water, 20 ml 0.1 M acetic acid pH 2.7 and 20 ml 0.066 M phosphate buffer pH 7.

Mass spectrometry: FAB-MS: HSQ-30 (Finnigan-MAT, Bremen) with FAB-gun from IonTech Ltd. (Teddington, GB), FAB-gas: Xe, and VARIAN MAT 731 (Finnigan-MAT, Bremen) with the same FAB-gun, FAB-gas: Xe; sample preparation by adsorption on Sep-Pak RP-18, washing with H_2O , desorption with $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 1:1 and drying i.v.

NMR: AM 300 (Bruker, Rheinstetten), (^1H : 300 MHz, ^{13}C 75.5 MHz); 15 mg of **1a** were dissolved in 0.5 ml D_2O , purified on Biogel P2 with 0.1 M acetic acid as solvent; the acetate anions were replaced on a DEAE-column by Cl^- , H_2O as

Abbreviations: Common amino acids, 3-letter-code; AcOHOrn, N^5 -acetyl- N^5 -hydroxy-Orn; aThr, *allo*-Thr; FoOHOrn, N^5 -formyl- N^5 -hydroxy-Orn; c-OH-Orn, cyclo- N^5 -hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); TAP, trifluoroacetyl (amino acid) O-isopropylester; GC/MS, gas chromatograph coupled with a mass spectrometer; FAB-MS, fast atom bombardement mass spectrometer; HVPE, high voltage paper electrophoresis; RP-HPLC, reversed phase high performance liquid chromatography; TLC, thin layer chromatography; NMR-techniques: COSY, correlation spectroscopy; TOCSY, total correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; ROESY, rotating frame Overhauser and exchange spectroscopy.

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For part LXXVII see Risse *et al.* (1998).

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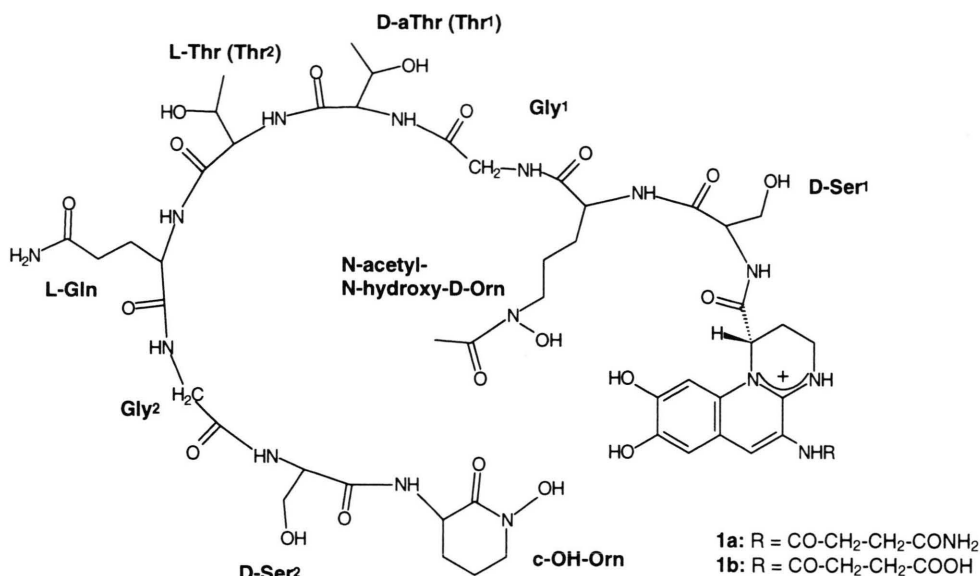


Fig. 1. Structure of the pyoverdinin from *P. aureofaciens*.

solvent. Chemical shifts are given relative to HDO as internal standard ($\delta = 4.8$ ppm).

Chromatography: GC-column Chirasil-L-Val (22 m \times 0.22 mm) (Chrompack, Frankfurt/M.); column chromatography: XAD Type 4 (Serva, Heidelberg), Biogel P2 (BioRad, Hercules, CA, USA), Sephadex G-25, CM-Sephadex C-25 and DEAE-Sephadex C-25 (Pharmacia, Uppsala, S), SepPak RP-18 (Waters, Milford, GB), TLC-plates: Silicagel 60 F₂₅₄ and HPTLC-Fertigplatten CHIR (Merck, Darmstadt).

HVPE: Camag HVE System 60600, paper MN261 (Camag, Muttens, CH); samples were dissolved in 0.1 M acetic acid (pH 2.7) and 0.1 M CH₃COONH₄ (pH 6.5), with glucose and desferal as standards (Poppe *et al.*, 1987).

Growth conditions, isolation and characterisation of the pyoverdinin

The strain of *P. aureofaciens* was obtained from the Institute for Microbiology, University Bonn. It was grown at 23 °C in a medium with gluconate as C-source (per l: 13 g Na-D-gluconate, 4 g KH₂PO₄, 5 g (NH₄)₂SO₄, 0.5 g MgSO₄·7H₂O) for 92 h. Then 20 ml of 5% iron(III) citrate solution was added per l of the culture and the pH-value was adjusted to 5.5 with 6 M hydrochloric acid. Cells were removed by tangential filtration (THLF-Sys-

tem, Millipore, Bedford, MA, USA). The brown filtrate was passed through a XAD-column. After washing with deionized water the pyoverdinin fraction was eluted with methanol/water 1:1 (v/v) and dried i.v. The residue was chromatographed on Biogel P2 with 0.2 M pyridinium acetate as solvent, the fraction absorbing at 405 nm was collected and dried i.v. The residue was applied to a CM-Sephadex column and desorbed with 0.2 M pyridinium acetate. Two fractions (**1a** and **1b**) were obtained, the latter being probably an artefact derived from **1a** by hydrolysis (Schäfer *et al.*, 1991). The fractions were rechromatographed with 0.02 M pyridinium acetate. The substances were dried i.v. and their purity was checked by TLC with methanol/H₂O/acetic acid 70:30:0.005 (v:v:v) as solvent. For mass spectrometry and NMR-spectroscopy the ferri-pyoverdins were decomplexed with 8-hydroxyquinoline/citrate and rechromatographed as described before (Briskot *et al.*, 1986).

Qualitative analysis of the amino acids was performed by TAP-derivatisation and GC-MS as described earlier (Jacques *et al.*, 1995). Partial hydrolysis: To determine the position of L-Thr and D-aThr in the peptide chain 1 mg of the pyoverdinin **1a** was hydrolyzed with 1 ml 3 M formic acid for 10 min at 90 °C. The solution was dried i.v., FAB-MS of the residue showed that the peptide chain

was hydrolyzed at the amide bond between Gly¹ and Thr¹ (see Fig. 1). The material was subsequently dansylated and hydrolyzed again with 1 ml of 6 M HCl at 90 °C for 8 h (Risse *et al.*, 1998). Dansyl-derivatives of the amino acids were adsorbed on SepPak cartridges and further investigated by TLC on chiral plates with methanol/H₂O/ acetonitrile 50:40:20, 0.05 M KH₂PO₄ as solvent. The aqueous eluate of the SepPak cartridges was dried i.v. and TAP-derivatized.

Results and Discussion

The UV/Vis spectra are typical for pyoverdins: ferri-**1a**: 399 nm at pH 5.3; desferri-**1a**: 363 nm at pH 3.2 und 399 at pH 7.1 (Briskot *et al.*, 1986). The molecular mass as determined by FAB-MS was 1277 u for **1a** and 1278 u for **1b**. After total hydrolysis and TAP-derivatisation L-Glu, Gly, D-Orn, D-Ser, L-Thr, D-aThr could be observed. The electrophoretic mobility of the free pyoverdinin **1a** is +0.6 (at pH 2.7) and +0.41 (at pH 6.5).

Partial hydrolysis resulted in a cleavage of the peptide chain between Gly¹ and Thr¹ (s. Fig. 1). The material was dansylated, completely hydrolyzed and dried i.v. The dansylated amino acids contained only dansyl-D-aThr. Therefore Thr¹ is D-aThr. After TAP-derivatisation the free amino acids showed a L-Thr/D-aThr ratio of 1.5. There-

fore the Thr closer to the C-terminus (Thr²) is L-Thr. The fact that D-aThr was observed in the TAP-derivatives is probably due to incomplete dansylation of the partial hydrolysate.

NMR-spectroscopy

The pyoverdinin from *P. aureofaciens* contains several bifunctional amino acids (Thr, Ser, Gln and Orn). It had to be shown in which way these amino acids were inserted in the peptide chain. Furthermore sequence-specific signals should be obtained in the ROESY-experiment. The data were assembled by a variety of two-dimensional experiments: H,H-COSY shows ³J-coupling of H-C-C-H, whereas ⁴J- and higher couplings could be observed by TOCSY. Carbon shifts were obtained by HMQC and HMBC for the carbonyl carbon atoms. For a more detailed description of the experiments see Evans (1995), Braun *et al.* (1996) and Schaffner *et al.* (1996).

The ¹H- and ¹³C-data of **1a** are assembled in Tables I and II. The signals of the chromophore and the succinic acid amide side chain correspond to those of other pyoverdins published before (Briskot *et al.*, 1989, Geisen *et al.*, 1992). The chemical shifts of the β-protons of the Ser and Thr indicate that the OH-groups are free; otherwise their chemical shifts should be shifted downfield

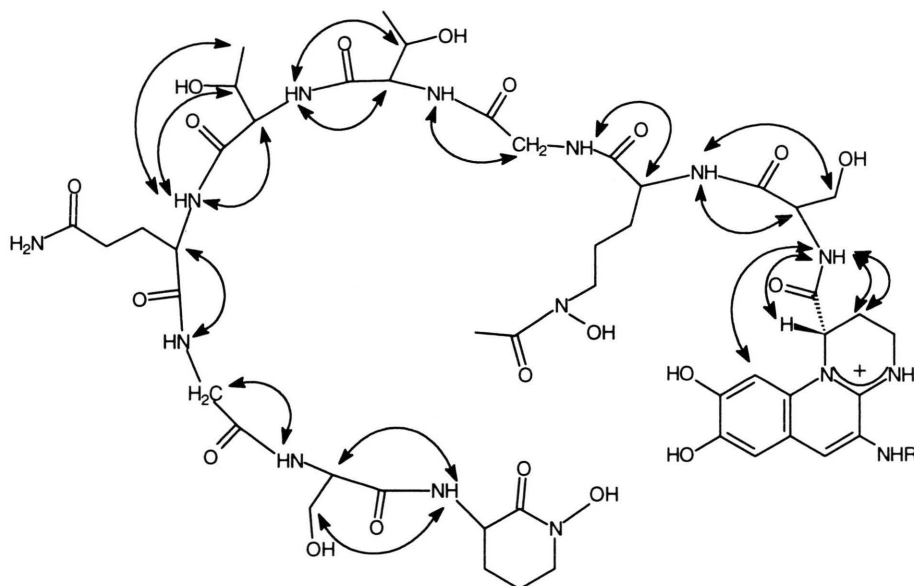


Fig. 2. Sequence-specific NOEs as observed in the ROESY-spectrum.

Table I. ¹H-NMR-Data of **1a**.

| Suca | 2' | 3' | NH ₂ | | | | | | | |
|--------|------|------|-----------------|------|------|------------------|-----|------|------|------|
| | 2.78 | 2.73 | 9.81 | | | | | | | |
| Chr | 1 | 2a | 2b | 3a | 3b | 4HN ⁺ | 5HN | 6 | 7 | 10 |
| | 5.68 | 2.49 | 2.69 | 3.39 | 3.69 | 8.81 | – | 7.93 | 7.18 | 6.97 |
| | HN | α | β | γ | δ | acetyl | | | | |
| Ser 1 | 9.60 | 4.52 | 3.93 | | | | | | | |
| Ser 2 | 8.47 | 4.46 | 3.88 | | | | | | | |
| AcOrn | 8.83 | 4.44 | 1.61/1.79 | 1.45 | 3.35 | 2.00/2.05 | | | | |
| Gly 2 | 8.62 | 3.98 | | | | | | | | |
| Gly 1 | 8.41 | 3.98 | | | | | | | | |
| Thr 1 | 8.30 | 4.32 | 4.14 | 1.23 | | | | | | |
| Thr 2 | 8.58 | 4.34 | 4.25 | 1.21 | | | | | | |
| Gln | 8.79 | 4.38 | 2.16 | 2.35 | | | | | | |
| cOHOrn | 8.66 | 4.45 | 1.86/2.04 | 2.00 | 3.63 | | | | | |

Table II. ¹³C-NMR-Data of **1a**.

| Suca | CO(1') 2' | 3' | CONH ₂ (4') | | | | | | | |
|--------|-----------|-------|------------------------|-------|-------|------------|-------|--|--|--|
| | 177.5 | 31.7 | 30.7 | 178.9 | | | | | | |
| Chr | CO | 1 | 2 | 3 | 4a | 5 | | | | |
| | 171.4 | 58.2 | 23.1 | 36.2 | 150.6 | 118.7 | | | | |
| | 6 | 6a | 7 | 8 | 9 | 10 | 10a | | | |
| | 140.3 | 116.1 | 115.3 | 144.8 | 152.5 | 101.4 | 133.0 | | | |
| | CO | α | β | γ | δ | acetyl | | | | |
| Ser 1 | 172.4 | 56.7 | 62.3 | | | | | | | |
| Ser 2 | 172.4 | 57.1 | 61.7 | | | | | | | |
| AcOrn | 174.9 | 54.4 | 29.2 | 23.4 | 48.0 | 175.1/20.3 | | | | |
| Gly 2 | 172.5 | 43.6 | | | | | | | | |
| Gly 1 | 172.7 | 43.7 | | | | | | | | |
| Thr 1 | 173.2 | 60.7 | 68.0 | 20.0 | | | | | | |
| Thr 2 | 173.4 | 60.2 | 68.4 | 20.0 | | | | | | |
| Gln | 174.9 | 54.4 | 27.7 | 32.2 | 178.9 | | | | | |
| cOHOrn | 167.6 | 51.6 | 27.7 | 21.2 | 52.7 | | | | | |

by approximately 0.5 ppm (Risse *et al.*, 1998). Glu found in the amino acid analysis is derived from Gln as shown by the chemical shift of the carbonyl carbon at 174.9 ppm. The carbonyl group of the free acid has a chemical shift of approximately 182.2 ppm (Geisen *et al.*, 1992). The data for the two Orn show that one of them is cOHOrn at the C-terminus and the other one bears a hydroxamate end group, the nitrogen being acetylated and hydroxylated (AcOHOrn).

From the molecular mass and the NMR-data it follows that every amino acid besides Gln is present twice in the peptide chain. Figure 2 shows the sequence specific signals obtained in the ROESY-

experiment. It follows that the structure of the new pyoverdin is **1a**.

Conclusion

1a is the first pyoverdin obtained from a *Pseudomonas aureofaciens* culture. *P. aureofaciens* was originally classified as biovar E of *P. fluorescens*, but was separated as the most notable producer of phenazine pigments (Römer *et al.*, 1981), and later on combined with *P. chlororaphis*, the original biovar D (Palleroni, 1992), pigmented by phenazine-1-carbamide (chlororaphin). Several phenazine derivatives were also obtained from cultures of a *P. fluorescens* strain, especially when grown under iron deficiency in the presence of rather high concentrations of Be²⁺ (Taraz *et al.*, 1990). The only other species within the fluorescent *Pseudomonas* group that produces a phenazine derivative is *P. aeruginosa* characterized by the betaine pyocyanin (responsible for the color of the blue-green pus from which the bacterium had its original name "*Bacillus pyocyaneus*") derived from 1-hydroxy-phenazine-5-oxide. Pyocyanin has probably nothing to do with the iron transport. *P. fluorescens* Y₄, however, produces under iron deficiency no pyoverdins, but rather phenazine derivatives containing the structural element 2,3-dihydroxybenzoic acid, the constituent of catecholate siderophores (Taraz *et al.*, 1991). In the iron deficient culture of the *P. aureofaciens* strain under investigation no phenazines were observed. To our knowledge, other phenazine producers were not

investigated in view of their ability to use pyoverdins as siderophores.

Pyoverdins have been used before to establish relationships between *Pseudomonas* spp. (Budzikiewicz *et al.*, 1997). It seemed, therefore, of interest to compare the amino acid sequence of **1a** with those of other pyoverdins (Kilz *et al.*, 1998). The most frequently encountered pattern amongst *P. fluorescens* strains shows at the N-terminus a small neutral amino acid (Ala, Ser) followed by Lys. The N-terminus D-Ser-D-AcOHOrn was encountered so far only once in a pyoverdin (which shows similarities with **1a** insofar as it also has only neutral amino acids between AcOHOrn and the C-terminal cOHOrn) from a *P. fluorescens* (Barelmann,

unpublished); D-Ser-D-FoOHOrn is the N-terminus of the pyoverdin from one of the siderovars of *P. aeruginosa* (Tappe *et al.*, 1993). It is thus too early to speculate whether a second characteristic pattern may evolve.

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- Braun S., Kalinowski H.-O. and Berger S. (1996), 100 and More Basic NMR Experiments. VCH, Weinheim.
- Briskot G., Taraz K. and Budzikiewicz H. (1986), Siderophore vom Pyoverdin-Typ aus *Pseudomonas aeruginosa*. Z. Naturforsch. **41c**, 497–506.
- Briskot G., Taraz K. and Budzikiewicz H. (1989), Pyoverdine-type siderophores from *Pseudomonas aeruginosa*. Liebigs Ann. chem. **375**, 375–384.
- Budzikiewicz H. (1993), Secondary metabolites from fluorescent pseudomonads. FEMS Microbiol. Rev. **104**, 209–228.
- Budzikiewicz H. (1997), Siderophores of fluorescent pseudomonads. Z. Naturforsch. **52c**, 413–420.
- Budzikiewicz H., Kilz S., Taraz K. and Meyer J.-M. (1997), Identical pyoverdines from *Pseudomonas fluorescens* 9AW and *Pseudomonas putida* 9BW. Z. Naturforsch. **52c**, 721–728.
- Evans, J. N. S. (1995), Biomolecular NMR Spectroscopy. Oxford Univ. Press, Oxford.
- Geisen K., Taraz K. and Budzikiewicz H. (1992), Neue Sideropore des Pyoverdin-Typs aus *Pseudomonas fluorescens*. Monatsh. Chem. **123**, 151–178.
- Jacques Ph., Ongena M., Gwose I., Seinsche D., Schröder H., Delfosse Ph., Thonart Ph., Taraz K. and Budzikiewicz H. (1995), Structure and characterisation of isopyoverdin from *Pseudomonas putida* BTP1 and its relation to the biogenetic pathway leading to pyoverdins. Z. Naturforsch. **50c**, 622–629.
- Kilz S., Lenz Ch., Fuchs R. and Budzikiewicz H. (1998), A fast screening method for the identification of siderophores from fluorescent *Pseudomonas* spp. by liquid chromatography/electrospray mass spectrometry. J. Mass. Spectrom., in press.
- Michels J., Benoni H., Briskot G., Lex J., Schmickler H., Taraz K., Budzikiewicz H., Korth H. and Pulverer G. (1991), Isolierung und spektroskopische Charakterisierung des Pyoverdin-Chromophors sowie seines 5-Hydroxy-Analogen. Z. Naturforsch. **46c**, 993–1000.
- Palleroni N. J. (1992), "Introduction to the family of Pseudomonadaceae" and "Human- and animal-pathogenic pseudomonads". The Prokaryotes (Balows A., Tröger H. G., Dworkin M., Harder W. and Holt J. G., eds.), Springer, New York; vol. **3**, chapters 160 and 161.
- Poppe K., Taraz K. and Budzikiewicz H. (1987), Pyoverdine type siderophores from *Pseudomonas fluorescens*. Tetrahedron **43**, 2261–2272.
- Risse D., Beiderbeck H., Taraz K., Budzikiewicz H. and Gustine D. (1998), Corrugatin, a lipopeptide siderophore from *Pseudomonas corrugata*. Z. Naturforsch. **53c**, 295–304.
- Römer A., Scholl H., Budzikiewicz H., Korth H. and Pulverer G. (1981), Phenazine aus Pseudomonaden. Z. Naturforsch. **36b**, 1037–1046.
- Schaffner E. M., Hartmann R., Taraz K. and Budzikiewicz H. (1996), Structure elucidation of Azotobactin 87, isolated from *Azotobacter vinelandii* ATCC 12837. Z. Naturforsch. **51c**, 139–150.
- Schäfer H., Taraz K. and Budzikiewicz H. (1991), Zur Genese der amidisch an den Chromophor von Pyoverdinen gebundenen Dicarbonsäuren. Z. Naturforsch. **46c**, 398–406.
- Tappe R., Taraz K., Budzikiewicz H., Meyer J.-M. and Lefèvre J. F. (1993), Structure elucidation of a pyoverdin produced by *Pseudomonas aeruginosa* ARCC 27853. J. prakt. Chemie **335**, 83–87.
- Taraz K., Schaffner E. M., Budzikiewicz H., Korth H. and Pulverer G. (1990), 2,3,9-Trihydroxyphenazin-1-carbonsäure – ein unter Berylliumeinwirkung gebildetes neues Phenazinderivat aus *Pseudomonas fluorescens*. Z. Naturforsch. **45b**, 552–556.
- Taraz K., Schaffner E. M., Budzikiewicz H., Korth H. and Pulverer G. (1991), Die Bildung hydroxylierter Phenazine durch *Pseudomonas fluorescens* Y₄ bei Be²⁺-Zusatz zum Kulturmedium – ein Abwehrmechanismus? Z. Naturforsch. **46c**, 194–196.
- Teintze M., Hossain M. B., Barnes C. L., Leong J. and van der Helm D. (1981), Structure of ferric pseudobactin, a siderophore from a plant growth promoting *Pseudomonas*. Biochemistry **20**, 6446–6457.