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Structure of the Pyoverdin PVD 2908 – a new Pyoverdin from *Pseudomonas sp.* 2908

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Abstract

An unknown siderophore (pyoverdin) was isolated from the strain *Pseudomonas sp.* 2908. The structure of the pyoverdin – called PVD 2908 – was elucidated by spectroscopic methods and degradation studies. Some other siderophores were identified by LC/ESI-MS-screening based on the knowledge of PVD 2908.

Abbreviations: Common amino acids, 3-letter code; OHAsp – *threo*-β-hydroxy Asp; cOHOrn – cyclo-N^5-hydroxy Orn (3-amino-1-hydroxy-piperidone-2); Chr – pyoverdin chromophore; Kgl – 2-ketoglutaric acid residue; TAP – derivatives N/O-trifluoracetyl-amino acid-isopropyl esters; MS – mass spectrometry; CA – collision activation; ESI – electrospray ionization; FAB – fast atom bombardment; COSY – correlation spectroscopy; HMQC – heteronuclear multiple quantum coherence; HMBC – heteronuclear multiple bond coherence; ROESY – rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY – total correlated spectroscopy; DSS – 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA – ethylene diamine tetraacetic acid.

Introduction

Iron possesses two stable redox states and the redox potential between them can be influenced by complexing ligands. It plays, therefore, an important role for many redox processes in biological systems. Due to its low solubility under the living conditions of microorganisms, many bacteria excrete Fe^{3+}-chelators, the so-called siderophores. The typical siderophores of the fluorescent group of the genus *Pseudomonas* are named pseudobactins or more commonly pyoverdins.

The common structural feature of the pyoverdins is a dihydroxyquinoline chromophore responsible for the yellowish-green fluorescence. It is one of the binding sites for Fe^{3+}; the other two are part of a peptidic chain attached to the chromophore, comprising 6 to 12 amino acids (both D and L). In most cases the peptide chain is bound via its N-terminus to the carbonyl function of the chromophore. Only one exception – a connection through an ester bond between chromophore and peptide chain – is reported in literature (Yang and Leong, 1984), but this structure probably has to be revealed (Voßen *et al.* 2000). The third part is a small dicarboxylic acid, bound amidically to the amino function of the chromophore. Co-occuring with the pyoverdins other siderophores were found in many cases, differing in the chromophore part. So far, ferribactins, 5,6-dihydropyoverdins and 5,6-dihydropyoverdin-7-sulfonic acids were found; all of them are regarded to be precursors in the biogenesis of pyoverdins.

The various fluorescent *Pseudomonas* strains produce pyoverdins differing in their peptide chains responsible for the recognition at the cell surfaces (Hohnadel and Meyer 1988). Usually several pyoverdins produced by a strain differ only in the dicarboxylic side chain; only in three cases pyoverdins from one strain with slightly different peptide chains – a linear and a cyclic one – are reported (Khalil-Rizvi *et al.* 1997; Voßen *et al.* 2000). In some cases, different strains produce identical pyoverdins (e.g. Hohlneicher *et al.* 1995; Budzikiewicz *et al.* 1997).

In the following we wish to report the structure elucidation of PVD 2908, a pyoverdin produced by *Pseudomonas sp.* 2908, and the identification of several further siderophores by LC/ESI-MS-screening.

Materials and methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT HSQ-30 (FAB, matrix thioglycerol/dithiodiethanol) with FAB gun 11 NF, 8 kV, FAB gas Xe (Ion Tech Ltd., Teddington, GB), Finnigan 900 ST (ESI; 50 μM solutions in CH_3OH/H_2O 1:1, v/v); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale, CA, USA) GC 3400. For conditions of LC/ESI-MS-Screening see Kilz *et al.* (1999).

NMR: DRX 300 and DRX 500 (all Bruker, Karlsruhe). Chemical shifts are given relative to TMS with the internal standard DSS using the correlation δ(TMS) = δ(DSS) for ^{1}H and δ(TMS) = δ(DSS) - 1.61 for ^{13}C.

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

CD: Jasco J-715 (Jasco, Tokyo, J).

IEF: Pharmacia PhastSystem and PhastGel IEF (Pharmacia, Uppsala, S).

Chromatography: Servachrom XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), QAE Sephadex A-25 (Pharmacia, Uppsala, S); GC: column Chirasil-L-Val (Macherey-Nagel, Düren); Sep-Pak RP_{18} cartouche (Waters, Milford MA, USA); Thin-layer-chromatography: Polygram Polyamid-6 UV_{254} (Macherey-Nagel, Düren).

Chemicals: Water was desalted and distilled twice in a quartz apparatus; for HPLC it was further purified on XAD-4 resin and filtered through a sterile filter. Organic solvents were distilled over a column. Reagents (Aldrich-Sigma, Deideshofen; Fluka, Neu-Ulm; Merck, Darmstadt; Riedel de Haen, Seelze) were p. a. quality.

Production, isolation and derivatisation of **1**

The strain *Pseudomonas sp.* 2908 came from the collection of Dr. J.-M. Meyer, Strasbourg. The culture in a succinate minimal medium, the isolation of a XAD-4 extract of the culture medium containing ferric siderophores and the first purification step by chromatography on Biogel P-2 were described elsewhere (Georgias *et al.* 1999). The main Biogel fraction was brought to dryness i.v., redissolved in 0.02 M pyridinium acetate buffer (pH 5.0) and chromatographed on QAE-Sephadex A-25 with a buffer gradient (detection 405 nm). The fraction containing **1** which eluted with 0.02 M buffer was brought to dryness i. v. and rechromatographed twice on QAE-Sephadex A-25 with a 0.1 M NaCl solution. Decomplexation was achieved with 8-hydroxyquinoline (Briskot *et al.* 1986).

For qualitative and quantitative analysis of the amino acids, determination of their configuration and dansylation of free amino groups see Briskot *et al.* (1986) and Mohn *et al.* (1990). For elucidation of complex constants see Mohn *et al.* (1990). The isolation of the chromophore to determine its configuration was described by Michels *et al.* (1991).

Results and discussion

Characterization of pyoverdin PVD 2908 *(***1***)*

The UV/Vis spectra of ferri-**1** (π-π^*: 400 nm; broad ct-bands: 470 and 560 nm) and **1** (pH 6.8: π-π^* 402 nm, pH 3.0: splitting of the band to 366 and 380 nm) correspond to those typically observed for pyoverdins (Meyer & Abdallah 1978). The isoelectric point of **1** is 4.92 $\pm$ 0.09. The molecular mass as determined by FAB- and ESI-MS is 1108 u. Retro-Diels-Alder fragmentation of the chromophore leads to the conclusion that the dicarboxylic side chain is ketoglutaric acid. By quantitative amino acid analysis after total hydrolysis and GC analysis of the TAP derivatives the composition of the peptide chain could be confirmed to be 2 D-Ser, 2 L-Ser, 1 L-*threo*-3-OH-Asp, 1 L-Orn and 1 L-cOHOrn. To determine which amino function (α or δ) is free in **1** ferri-**1** was dansylated and hydrolyzed. δ-Dansylamino-Orn could be identified by thin-layer-chromatography using authentic comparison material. The complex constants have been determined by following the change of the extinction at 450 nm after addition of EDTA. They are 9.8×10^{18} (pH 5.0) and 1.1×10^{25} (pH 7.0) and sofar comparable to those of other known pyoverdins.

Sequence determination by NMR

For a detailed discussion of the used NMR techniques see Evans (1995). H,H-COSY shows ^{3}J-coupling of H-C-C-H, while TOCSY allows to detect ^{4}J- and ^{5}J-coupling within one amino acid residue. Direct (^{1}J) C,H-correlations can be determined by HMQC, ^{2}J- and ^{3}J-C,H-coupling by HMBC. All amino acids, the chromophore and the 2-ketoglutaric acid can be identified by these techniques corroborated by shift values in comparison with literature data. ROESY establishes correlations between an amide proton and spatially close α- or β-protons of the preceeding amino acid.

Peptide sequencing can also be performed by interpretation of ^{2}J-coupling between a carbonyl carbon atom and the amide proton of the following amino acid in HMBC. Except of H,H-COSY and HMQC (11 mM) all experiments were performed in 22 mM solution in H_2O/D_2O 9:1 (v:v) at pH 4.3 (phosphate buffer) to minimize the exchange rate of the amide protons. Suppression of the H_2O signal was effected by presaturation (exception: TOCSY-experiment, suppression by Watergate). All experiments were performed at 25 °C, as the best separation of amide protons was achieved at that temperature.

The ^{1}H- and ^{13}C-NMR data are assembled in Tables 1 and 2. Those of the chromophore and of the 2-ketoglutaric acid side chain correspond to the ones observed for other pyoverdins with the same side chain. They deserve a comment. In aqueous solutions at pH larger than 2.0 2-ketoglutaric acid forms an equilibrium between at least two forms, the diastereomeric lactams II and III in scheme I (Cooper *et al.* 1974; Briskot *et al.* 1986; Drechsel *et al.* 1993). Therefore some resonances of the side chain and the chromophore are splitted. The shifts of the 4′CO atom (94.5 and 95.6 ppm) indicate the lactam forms, the 4′CO of the open chain form I in scheme I should resonance at approximately 200 ppm.

The NMR data of the amino acids correspond to the ones described in literature. The low-field shift of one Ser-NH (Ser_1, 9.53 ppm) is in agreement with the direct connection by an amide bond with the chromophore, as could be confirmed by ROESY. It results from the shielding of the aromatic system. The shift values of the CH_2-groups of the four Ser (3.87–3.96 ppm) show that the OH-groups are not esterified, as well as the shift value of the δ-CH_2-group of Orn (2.60/2.75 ppm) indicates a free amino function in agreement with the results of the dansylation experiment (otherwise a downfield shift would have been expected in both cases).

As all amide resonances could be identified the sequence of the petide chain could be determined by ROESY as depicted in Figure 1 (full arrows). Sequence information from HMBC (half arrows in Figure 1) confirmed the results.

Sequence determination by CA mass spectrometry

After collision of ions with molecules of an auxilliary gas, fragmentation is induced by transforming translational energy into vibrational energy (CA – 'collision activation'). Peptides fragment primarily by cleaving the peptide bond, so that a series of N- and/or C-terminal fragment ions are detected. The first activation (MS^2-experiment) of the double charged quasimolecular ion of **1** ($[M+2H]^{2+}$ *m/z* 555.1) effects only the loss of a molecule H_2O and/or CO_2 – probably from the 2-ketoglutaric acid. After further activation (MS^3-experiment) of the ion $[M\text{-}CO_2\text{-}H_2O+2H]^{2+}$ all N-terminal fragment ions of the B-type and additionally some C-terminal ions of the X- or Y-type are detected (Figure 2 and Table 3). Several ions are formed by an additional loss of H_2O (*m/z* 899.2, 881.2, 812.2 ...; *m/z* 515.2 and 506.2 – double-charged) or CO_2 (*m/z* 487.1). The B_6 ion (including those with additional loss of H_2O) also occurs as double-charged ion (*m/z* 459.1 ⇒ 450.0, 441.2 amd 432.1). As a result the sequence determined by NMR investigations is confirmed.

Investigations on configuration

In order to locate D- and L-Ser in the peptide chain, which cannot be distinguished by NMR or MS techniques, different partial hydrolysis of **1** were performed (2 M HCl, 57 °C, 30 min; 4 M HCl, 50 °C, 30 min; 6 M HCl, 60 °C, 30 min). The hydrolysate was separated by chromatography on Bio-Gel P-2 with 0.1 M acetic acid. The pure samples were identified by ESI-MS; the amino acid compositions of samples of interest for the location of Ser were then determined as described above (Table 4).

For determination of the configuration of chromophore-C1 an acid hydrolysis (3 M HCl, 110 °C, 7 d) was performed. The 5-OH-chromophore (Scheme II), which is more stable under conditions of hydrolysis (see Michels *et al.* 1991), was separated via a Sep-Pak RP_{18} cartouche. After further purification by RP-HPLC it was identified by PI-FAB-MS ($[M+H]^+$ *m/z* 277). The configuration of chromophore-C1 was then determined by CD spectroscopy. The CD-spectrum showed positive Cotton-effects at 250 nm (intensive) and 370–390 nm (weak), and a strong negative Cotton-effect at 300 nm. Therefore, it was concluded that the chromophore was *S*-configurated as in all pyoverdins, related to the proposed biogenesis starting from L-Dab and D-Tyr forming the ferribactin-chromophore (Böckmann *et al.* 1997).

Identification of co-occuring siderophores

Based on the knowledge of one siderophore from a culture broth of a *Pseudomonas* sp., via LC/ESI-MS-screening other siderophores (with different di-

Table 1. ^{1}H-NMR data (d[ppm]) of **1** (H_2O/D_2O, 25°C, pH 4.3)

Kgl	2′	3′						
	2.83	2.36						
		2.66						
Chr	1	2a	2b	3a	3b	$4NH^+$	6	7	10
	5.74	2.52	2.72	3.38	3.72	8.77	7.90	7.13	7.13
	NH	*α*	*β*	*γ*	*δ*	NH_2			
Ser_1	9.53	4.42	3.96						
Orn	8.21	4.47	1.50	1.37	2.60	7.44			
			1.86		2.75				
OHAsp	8.18	4.89	4.56						
Ser_2	8.39	4.51	3.87						
			3.92						
Ser_3	8.50	4.52	3.93						
Ser_4	8.29	4.46	3.88						
			3.93						
cOHOrn	8.36	4.52	1.77	2.00	3.63				
			2.04		3.69				

Table 2. ^{13}C-NMR data (d[ppm]) of **1** (H_2O/D_2O, 25°C, pH 4.3)

Kgl	CO(1)	2′	3′	CO(4′)	COOH(5′)							
	176.4	30.5	32.9	94.5	180.5							
		31.6		95.6								
Chr	CO	1	2	3	4a	5	6	6a	7	8	9	10	10 a
	171.6	57.9	22.7	36.3	151.1	117.8	142.6	115.5	115.7	144.8	153.2	101.0	133.4
	CO	*α*	*β*	*γ*	*δ*								
Ser_1	173.0	58.2	61.9										
Orn	174.1	54.0	28.7	24.1	39.7								
OHAsp	172.7	57.7	72.9	177.6									
Ser_2	173.1	57.1	62.1										
Ser_3	173.2	57.3	62.3										
Ser_4	172.3	57.2	62.2										
cOHOrn	167.4	51.5	27.6	20.9	52.7								

Table 3. MS-CA spectra of **1** – specific fragment ions

B_6	$[B_6+H]^{2+}$	B_5	B_4	B_3	B_2	B_1
917.2	459.1	830.0	743.1	656.1	525.3	411.0[a]
Y_2	Y_3	Y_4		$[X_5-H_2O-CO_2]^+$		
218.0	305.1	392.5		487.1		

[a] observed only with additional loss of H_2O (*m/z* 392.8).

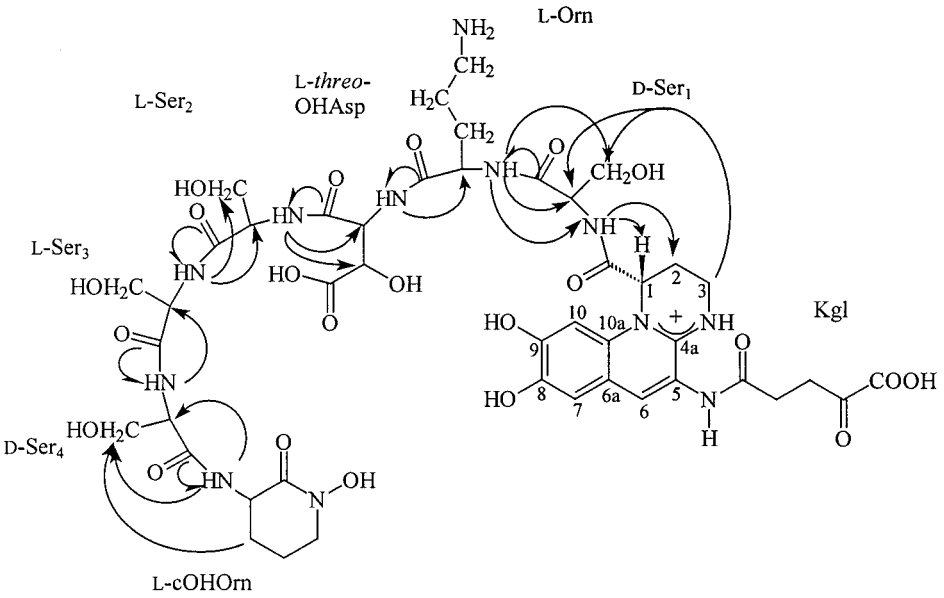


Fig. 1. NMR connectivities in the NMR spectra of **1** (ROESY full arrows, HMBC half arrows). Note that the 2-ketoglutaric acid forms several equilibrium forms (Scheme 1).

Table 4. Mass and composition of partial hydrolysis products of **1**

Sample	Mass	Composition
1	604 u	Kgl-Chr-D-Ser-L-Orn
2	822 u	Kgl-Chr-D-Ser-L-Orn-L-OHAsp-L-Ser
3	881 u	Kgl-Chr-D-Ser-L-Orn-L-OHAsp-L-Ser-D-Ser
4	304 u	L-cOHOrn-L-Ser-D-Ser

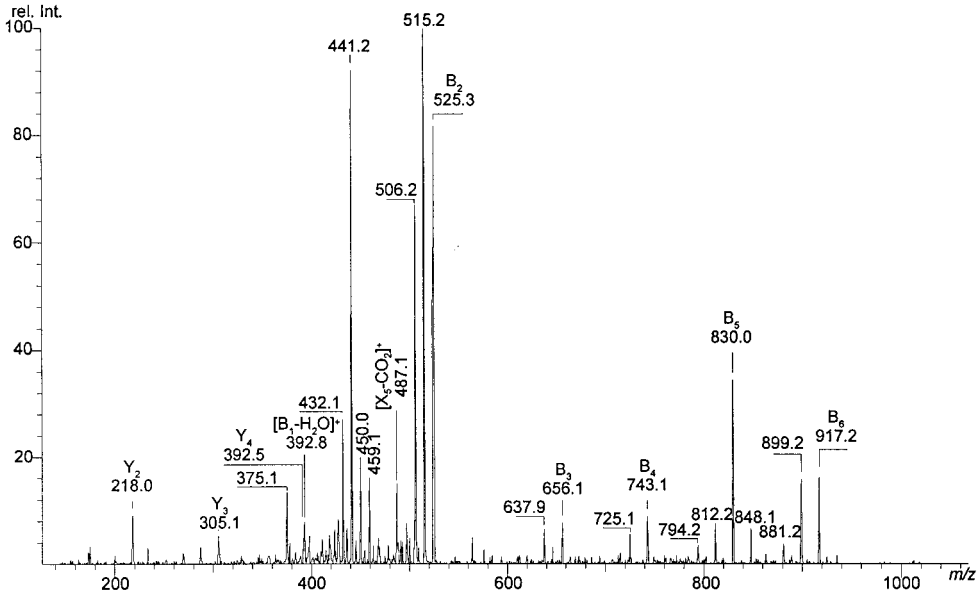
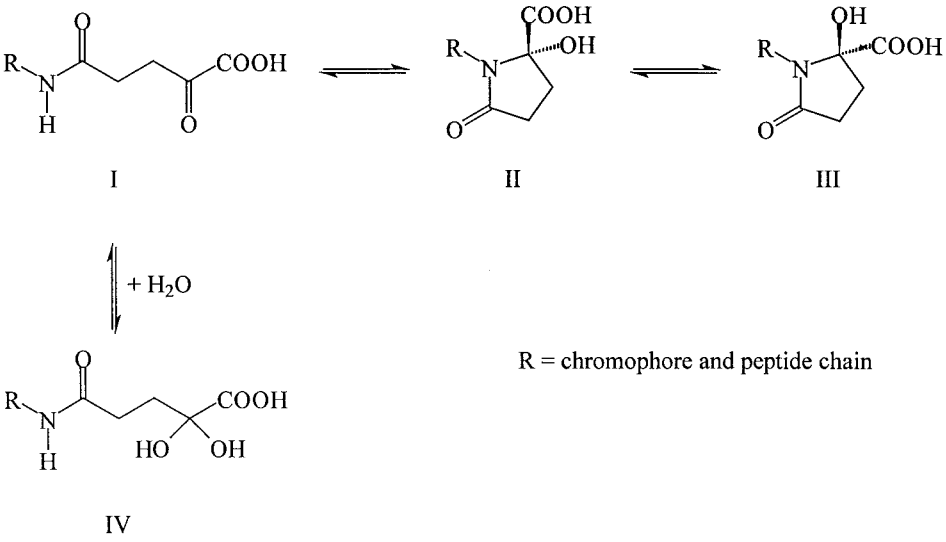
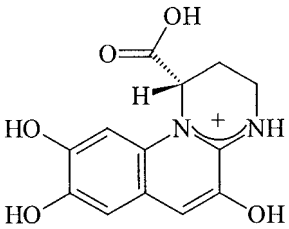


Fig. 2. Ion trap MS-CA spectra of the $[M\text{-}H_2O\text{-}CO_2+2\ H]^{2+}$ ion of **1** (for unassigned peaks see text).



Scheme 1. Equilibrium forms of 2-ketoglutaric acid as side chain.



Scheme 2. 5-OH-chromophore.

carboxylic acids or chromophores, see above) can be identified quickly, using a diode-array-detector for HPLC (Kilz *et al.* 1999). Screening the iron-containing biogel fraction of *Pseudomonas* sp. 2908, the congeners with succinic acid, succinic acid amide and glutamic acid as side chains could be detected. Additionally the 5,6-dihydropyoverdins with succinic acid amide and ketoglutaric acid and the 5,6-dihydropyoverdine-7-sulfonic acids with succinic acid amide, ketoglutaric acid and glutamic acid as side chains were identified.

Conclusion

A new pyoverdin form the culture broth of *Pseudonomas* sp. 2908 was isolated and its structure elucidated by spectroscopic investigations and chemical degradation experiments. The sequence was detemined to be

Kgl–1*S*-Chr–D-Ser–L-Orn–L-OHAsp-L-Ser–D-Ser–L-cOHOrn.

Co-occuring some more siderophores were identified, differing in the side chain and/or the chromophore (being precursors of the pyoverdin chromophore).

Acknowledgements

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