An Exceptionally Large Pyoverdin from a *Pseudomonas* Strain Collected in Thailand[+]

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From a *Pseudomonas* strain obtained from a soil sample collected in Thailand a pyoverdin was obtained containing twelve amino acids in its peptide chain. The structure elucidation is described.

Introduction

Pyoverdins are the typical siderophores of the fluorescent species of the rRNA homology group I of the bacterial genus *Pseudomonas.* They have in common a dihydroxyquinoline chromophore connected to the N-terminus of a peptide chain consisting of 6 to 12 amino acids, L as well as D and partially modified (Budzikiewicz, 1997). In this contribution the structure elucidation of pyoverdin Pf 1547 will be described which had been isolated from a *Pseudomonas* sp. found in a soil sample from Thailand and subsequently from strains isolated from rice and corn. It is the second example of a pyoverdin comprising 12 amino acids with so far the highest molecular mass. The large distance between the two hydroxamic acid units (binding sites for Fe^{3+}) is unparalleled.

Materials and Methods

Instruments and Chemicals

Mass spectrometer: H-SQ 30 (FAB; matrix thioglycerol), 900 ST (ESI; aqueous solution 50 pmol/μl); Incos 500 (GC/MS) (all Finnigan-MAT, Bremen). Sample preparation by adsorption on Sep-Pak RP-18, removing of inorganic material with H_2O, desorption with CH_3OH/H_2O (1:1) and drying i.v.

NMR: Bruker AM 300 (^1H 300 MHz, ^{13}C 75.5 MHz), Bruker DRX 500 (^1H 500 MHz, ^{13}C 125 MHz) (Bruker, Karlsruhe). Chemical shifts are given relative to TMS with the internal standard DSS using the relations $\delta(DSS) = 0$ for ^1H and $\delta(DSS) = -1.61$ ppm for ^{13}C. Suppression of the H_2O signal by the WATERGATE puls sequence. Samples: 20 mM desferri-**1a** were twice dissolved in 0.5 ml D_2O and brought to dryness i.v. at 30 °C and redissolved in 0.6 ml phosphate buffer (D_2O/H_2O, 1:9, v/v; pH 4.3).

UV/VIS: Lambda 7 (Perkin-Elmer, Überlingen); 1 mg substance in 20 ml 0.1 M phosphate buffer.

Chromatography: RP-HPLC column Nucleosil 100-C_{18}, 5 μm (Knauer, Berlin); GC/MS, Chirasil-L-Val (Chrompack, Middelburg, NL); column

[+] Part LXXXVIII of the series "Bacterial Constituents". For Part LXXXVII see Voßen *et al.*, 2000.

Abbreviations: Common amino acids, 3-letter code; AcOHOrn, N^5-acetyl-N^5-hydroxy-Orn; cOHOrn, *cyclo*-N-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); Chr, pyoverdin chromophore; Suc, succinic acid side chain; TAP-derivates, N/O-trifluoroacetyl-(amino acid)-isopropyl esters; RP-HPLC, reversed phase high performance liquid chromatography; GC/MS, gas chromatograph coupled with a mass spectrometer; ESI, electrospray ionization; FAB, fast atom bombardment; CA, collision activation; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlation spectroscopy; DSS, $[d_6]$-2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; CAA, casamino acids; CFBP, Collection française de bactéries phytopathogènes de l'INRA (Angers).

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chromatography CM-Sephadex C-25 (Pharmacia, Uppsala, S), BioGel P2 (Bio-Rad, Richmond, USA), Sep-Pak RP-18 (Waters, Milford, GB).

Chemicals: Water was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column. Reagents were p. a. quality.

Production, isolation and derivatization of the pyoverdin Pf 1547 (**1a**)

The pyoverdin Pf 1547 (**1**) was first obtained from a *Pseudomonas* sp. strain isolated from dried termite soil collected in the province Udornthani (Thailand) by the Dept. of Microbiology, Faculty of Science, Khon Kaen University. Subsequently it was also found to be produced by *Pseudomonas* sp. CFBP 4396 isolated from *Oryza sativa* (rice) and from *Pseudomonas fluorescens* bv V CFBP 4975 from *Zea mays* (corn). The bacteria were grown in a sucrose/asparagine medium at pH 6.5 (phosphate buffer) (Thai strain) or in a CAA medium (French strains). The isolation of **1a** was effected by a procedure reported earlier (Glennon *et al.*, 1994). After removal of the cell material by centrifugation a 5% solution of $FeCl_3$ in H_2O was added, the culture medium was adjusted to pH 6.0 and passed through a XAD-4 resin column. The pyoverdin-containing fraction was subsequently eluted with CH_3OH/H_2O 1:1 (v/v) and lyophilized. The lyophilized material was dissolved in 2 ml 0.02 M pyridinium acetate buffer (pH 5.0) and chromatographed on BioGel P-2 with 0.02 M pyridinium acetate buffer (pH 5.0). From the fractions showing an absorption at 405 nm the buffer was removed i.v. at 30 °C by adding several times H_2O. The fractions containing the ferri-pyoverdins were dissolved in 1 ml 0.02 M pyridinium acetate buffer (pH 5.0) and chromatographed on CM-Sephadex C-25 using a gradient from 0.02 M to 0.2 M pyridinium acetate buffer (pH 5.0) in 2 hours. Chromatography was repeated and from the brown fractions the buffer was removed i.v. at 30 °C by adding several times H_2O and bringing to dryness. Purity was proved by using RP-HPLC on Nucleosil C-18 with a gradient of CH_3OH/ 0.1 M CH_3COONH_4. Ferri-**1a** was adsorbed on a Sep-Pak RP-18 cartridge. Decomplexation was achieved by using oxalic acid (6.5 %, pH 4.3). After removal of the salt with water the free pyoverdin was eluted with CH_3OH/H_2O 1:1 (v/v). The solvent was removed i.v. and the residue was dried for 30 minutes at 10 Pa.

For the structure elucidation described below **1a** was investigated in detail. **1a** (with a succinic acid side chain, -CO-CH_2-CH_2-COOH, see Fig. 1) is actually the hydrolysis product of a pyoverdin with a succinamide (-CO-CH_2-CH_2-$CONH_2$) side chain (**1b**) (Schäfer *et al.*, 1991) which depending on the work-up can be found to accompany **1a**. Ferri-**1b** can be transformed into ferri-**1a** by letting stand an aqueous solution (pH 9.0) for 14 days at room temperature (Geisen *et al.*, 1992).

Results

Characterization **1a**

The UV/VIS-spectra of **1a** (pH 7.0: 399 nm; pH 3.0: 375 and 363 nm) and of ferri-**1a** (pH 7.0: 401 nm; plus broad charge-transfer bands at about 470 and 560 nm) are typical for pyoverdins (Budzikiewicz, 1997). The molecular mass of **1a** was determined by FAB- and ESI-MS as 1547 u. The typical fragment ion in the FAB MS spectra of pyoverdins formed by a *retro*-Diels-Alder mechanism (Michels *et al.*, 1991) is observed at *m/z* 1244. The mass difference of 303 u between the molecular mass and that of the fragment corresponds to a pyoverdin with succinic acid as the sidechain of the chromophore.

After total hydrolysis the following amino acids could be identified by GC/MS of their TAP derivates: D-Ala, D-Glu, Gly, L-Lys, L-Orn, D- and L-Ser, L-Thr. In addition succinic acid di-isopropyl ester was detected. The configuration of Ser^1 was established as D from a fragment obtained by partial hydrolysis containing the chromophore and one Ser. However, no suitable hydrolysis products could be obtained that contained either Ser^2 or Ser^3 alone; hence, which of the two Ser is L- and which is D-configurated must remain open.

Determination of the amino acid sequence of **1a** *by MS*

In the ESI-MS spectrum of **1a** after collision activation (CA) either in the octapole region of the ion trap or in the ion trap several sequence-characteristic ions could be observed arising from cleavage at the peptide bonds (B-ions: N-terminal

Table I. CA fragment ions observed for **1a**.

Amino acid	N-terminal		C-terminal	
	n	B_n	Y_n''	n
Ser[1]	1	445.1	–	12
Lys	2	573.2		11
Ala[1]	3	644.3		10
AcOHOrn	4	816.4		9
Thr	5	917.4	733.4	8
Ala[2]	6	988.4	632.3	7
Gly	7	1045.5	561.3	6
Gln	8	1173.6	504.3	5
Ala[3]	9	1244.6	376.2	4
Ser[2]	10	1331.6	305.1	3
Ser[3]	11	1418.7		2
cOHOrn	MH	1548.7	131.1	1

R-NH-CHR-CO^+, C-terminal Y"-ions $^+NH_3$-CHR-CO-R'; Roepstorff and Fohlman, 1984); they are summarized in Table I. The B-series is complete and is confirmed by several Y"-ions. From the mass of B_4 it can be seen that Orn is actually present as AcOHOrn, and from B_8, that the Glu observed in the total hydrolysate stems from Gln. As far as the mass spectral data are concerned, the positions in the peptide chain of Lys and Gln could be interchanged as they are isobaric.

Determination of the amino acid sequence **1a** *by* ^{1}H- *and* ^{13}C-*measurements*

H,H-COSY shows ^3J-coupling of **H**-C-C-**H**, while ^4J- and ^5J-coupling within one amino acid residue can be detected by TOCSY. The various amino acids can be identified by these techniques corroborated by shift values in comparison with literature data. Direct (^1J) **C-H**-connections can be determined by HMQC, ^2J- and ^3J-**C-H**-coupling by HMBC. Peptide sequencing is possible by ROESY and NOESY resorting to Nuclear Overhauser Effect correlations between a NH-proton and spacially close α- and β-H of the preceeding amino acid (-C**H**-C**H**-CO-N**H**-). The ^{1}H- and ^{13}C-data are summarized 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, 1997).

From the TOCSY spectrum the signals of three Ser residues can be identified. The shifts of the β-CH_2-groups (~4.4 ppm) indicate that the OH-groups are not esterified (esterification results in a downfield shift of ~0.5 ppm). In the same way from the shift value of the β-CH of Thr (4.17 ppm) esterification of the OH-group can be excluded. The low-field resonance of the Ser[1]-HN (9.73 ppm

Table II. ^{1}H NMR data of **1a** (pH 4.3, 25 °C).

Suc	2′	3′							
	2.79	2.73							
Chr[a]	5	1	2a	2b	3a	3b	6	7	10
	–	5.73	2.49	2.71	3.39	3.70	7.94	7.20	7.09
Amino acid	NH	α	β	γ	δ	ε	NH_2	CH_3[c]	
Ser[1]	9.59	4.39	3.97						
Lys	8.28	4.27	1.60	1.16	1.49	2.77	7.64[b]		
			1.83						
Ala[1]	7.91	4.07	0.96						
AcOHOrn	8.53	4.35	1.63	1.60	3.64			2.14_c	
			1.76					2.07_t	
Thr	8.17	4.30	4.17	1.17					
Ala[2]	8.46	4.36	1.41						
Gly	8.40	3.94							
Gln	8.27	4.33	1.96	2.34					
			2.10						
Ala[2]	8.50	4.35	1.41						
Ser[2]	8.50	4.47	3.94						
Ser[3]	8.34	4.47	3.89						
cOHOrn	8.42	4.50	1.79	2.04	3.65				
			2.04						

[a] The 4-NH^+-signal could not be observed. [b] Observed at 5 °C. [c] *cis*- and *trans*-acetyl group of AcOHOrn.

Table III. ^{13}C-NMR data of **1a** (pH 4.3, 25 °C).

Suc	CO (1′)	2′	3′	COOH			
	178.3	32.1	31.8	181.8			
Chr	CO	1	2	3	4a	5	
	171.6	57.6	22.9	36.4	150.5	118.8	
	6	6a	7	8	9	10	10a
	139.9	115.8	115.1	144.9	151.1	101.3	132.9
	CO	α	β	γ	δ	ε	CH_3a
Ser[1]	173.4	58.3	61.7				
Lys	174.4	54.4	30.8	23,6	27.1	40.5	
Ala[1]	176.0	51.0	17.3				
AcOHOrn	175.1	53.9	28.8	23.0	47.9		
Ac[a] *cis*	174.7						20.4
trans	170.1						20.7
Thr	172.7	60.4	68.4	19.9			
Ala[2]	176.5	51.2	17.8				
Gly	172.4	43.6					
Gln	174.2	54.0	27.9	32.1			
Ala[2]	176.5	51.2	17.2				
Ser[2]	173.2	56.9	62.3				
Ser[3]	172.2	56.9	62.3				
cOHOrn	167.4	51.4	27.7	20.8	52.6		

[a] Acetyl group of AcOHOrn.

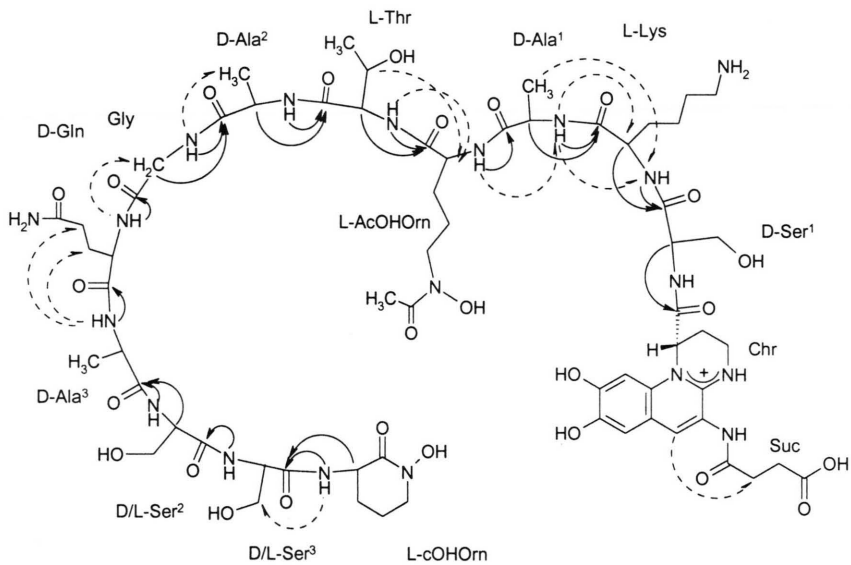

Fig. 1. Sequence specific cross-peaks for **1a** (HMBC →, ROESY ---›).

at 5 °C) is due to the influence of the aromatic system of the chromophore and shows that Ser[1] is connected directly with the chromophore-COOH. Starting from the doublets of their methyl groups (~1.0–1.2 ppm) the three spin systems of the Ala residues can be identified. Both the CH_3- and CO-group of the acetyl residue of AcOHOrn give two signals (2.07/2.14 ppm and 170.1/174.7 ppm, respectively) due to *cis/trans*-isomery (Budzikiewicz, 1997). The C-terminal N^5-hydroxy-*cyclo*-Orn

shows the typical signals for this system, especially the characteristic CO-resonance at 167.4 ppm (Mohn *et al.*, 1990).

Recently it was shown that Lys can be incorporated into the peptide chain both by its α- and by its ε-amino group (Budzikiewicz *et al.*, 1999; Sultana *et al.*, 2000). The shift values for the α-CH- and for the ε-CH_2-groups fall into the characteristic regions, while that of the α-NH lies between the typical values for α- and ε-NH (Budzikiewicz *et al.*, 2000). Connection via the α-NH Lys for **1a** is established by the observation of a HMBC cross peak between the Lys α-CH and the Ser^1 CO as well as a ROESY cross peaks with the Ala^1-NH, α-H and β-H of Ala^1 (see Fig. 1).

Since all amide NH resonances could be identified (see Table II) sequence information could be obtained from the ROESY spectra as depicted in Fig. 1. From ROESY data the partial structures cOHOrn-Ser^3 and Ser^2-Ala^3-Gln-Gly-Ala^2-Thr-AcOHOrn-Ala^1-Lys could be obtained. The sequence information obtained from the HMBC spectra (see also Fig. 1) comprises the complete structure cOHOrn-Ser^3-Ser^2-Ala^3-Gln-Gly-Ala^2-Thr-AcOHOrn-Ala^1-Lys-Ser^1-Chr for the **1a** in agreement with the results obtained from ESI-MS/MS (see above).

The unusual structure of the pyoverdin **1a** (cf. **Introduction)** explains why no heterologous incorporation with any of the pyoverdins of known structure could be observed (J.-M. Meyer, private communication).

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