A New Pyoverdin from *Pseudomonas aeruginosa* R′

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From a *Pseudomonas aeruginosa* hospital isolate a new pyoverdin was isolated. It is identical with that of *Pseudomonas aeruginosa* strain R except that in the peptide chain L-Gln is missing.

Introduction

Pseudomonas aeruginosa is a dangerous opportunistic human-pathogenic bacterium responsible for frequently lethal hospital (nosocomial) infections (Botzenhart and Rüden, 1987). As a soil bacterium it is omnipresent especially due to the effect of the modern air conditioning systems. It is resistant against many disinfectant agents and – more important – against many of the common antibiotics (Pulverer, 1972; Neu, 1992; Kallová *et al.*, 1996; Niccolai *et al.*, 1997). The alginate film frequently surrounding the bacteria, the low permeability of their outer membrane and an active export mechanism for low molecular mass substances are the main reasons for the resistance. β-Lactamase activity inactivates in addition β-lactam antibiotics as penicillin or ampicillin. *Pseudomonas aeruginosa* affects especially severely injured patients (large wounds or burns) and those whose immune system is weakened. An extremely critical situation exists for persons suffering from mucoviscidosis (cystic fibrosis) when *Pseudomonas aeruginosa* infects the bronchial tubes.

Recent studies have shown (Meyer *et al.*, 1997) that three siderovars (sv.) could be defined in a collection of 88 strains of *Pseudomonas aeruginosa.* 42% of the strains were characterized by a pyoverdin identical to the one described for *P. aeruginosa* PAO1 (= ATCC 15692, sv. I, – Briskot *et al.,* 1989). Another 42% produced the same pyoverdin as *P. aeruginosa* ATCC 27853 (sv. II., Tappe *et al.*, 1993), while the remaining 16% correspond to the clinical isolate *P. aeruginosa* Pa6 (identical with the natural isolate R) (sv. III, Gipp *et al.*, 1991; Meyer *et al.*, 1997). Preliminary results on two more structures of pyoverdins allegedly isolated from *P. aeruginosa* strains can be found in a symposium report (Eng-Wilmot *et al.,* 1990). One of them stemming from *P. aeruginosa* 15152 upon re-examination was found to be identical with that of *P. aeruginosa* PAO1 (Beiderbeck, 1997). No further reports on the second one can be found in the literature. Now from a hospital isolate from Thailand a further sv. of *Pseudomonas aeruginosa* was obtained whose pyoverdin differs from that of the sv. III in the way that L-Gln is missing in the peptide chain. The structure elucidation and some of the implications of this discovery will be discussed.

§ Part CVIII of the series "Bacterial constituents". For part CVII see Schlegel *et al.* (2001).

Abbreviations: Common amino acids, 3-letter code; FoOHOrn, 5-N-formyl-5-N-hydroxy Orn; Chr, pyoverdin chromophore; Suca, succinamide side chain; TAP, N/O-trifluoroacetyl-(amino acid)-isopropyl ester; RP-HPLC, reversed phase high performance liquid chromatography; IEF, isoelectrofocussing; ESI, electrospray ionization; CA, collision activation; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple bond correlation NOESY, nuclear Overhauser and exchange spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane.

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Materials and Methods

Instruments and chemicals

Mass spectrometry: ESI: Finnigan-MAT 900 ST; GC/MS: Incos 500 (both Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: DRX 500 (^{1}H 500, ^{13}C 125 MHz) (Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ (TMS) = δ (DSS) for ^{1}H, δ (DSS) = −1.61 ppm for ^{13}C. Suppression of the H_2O signal by presaturation.

Chromatography: RP-HPLC column Nucleosil 100-C_{18} (5 μm) (Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex A-25 (Pharmacia, Uppsala, S); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt).

The siderotyping procedures involving isoelectrofocussing (IEF) and pyoverdin-mediated ^{59}Fe uptake were performed as described elsewhere (Fuchs *et al.*, 2001).

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

Production and isolation of the pyoverdin **1**

The *Pseudomonas aeruginosa* strain under investigation was isolated from a patient's urine at Srinakharin Hospital, Khon Kaen University, Khon Kaen, Thailand, and identified by the tests published by MacFaddin (1974) (Collection *Pseudomonas aeruginosa* Khon Kaen University No. 1). It was grown in a medium containing 20.0 g sucrose, 2.0 g L-Asp, 1.0 g K_2HPO_4 and 15.0 g agar per liter (Ruangviriyachai *et al.*, 1995). For the work-up of the culture and isolation of the ferri-pyoverdins by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* (1999). The Biogel fraction containing ferri-pyoverdins was subjected to ion-exchange chromatography on CM-Sephadex A-25 with a pyridinium acetate buffer (pH 5.0, gradient 0.02 to 0.2 M). The major fraction (ferri-**1a**) was rechromatographed with 0.02 M pyridinium acetate buffer (pH 5.0). The resulting main fraction (pure as checked by analytical RP-HPLC with CH_3OH/H_2O) was decomplexed with K-oxalate (Voss *et al.*, 1999). For the analysis of the amino acids and the determination of their configuration by GC/MS of their TAP derivatives on a chiral column see Briskot *et al.* (1989) and Mohn *et al.* (1990).

Results

Structure of **1**

The molecular mass of **1a** was determined by ESI-MS as 1045 u. In the crude extract in addition to **1a** (with a succinamide side chain) by ESI-MS also **1b** (with a succinic acid side chain) and **1c** (with an α-ketoglutaric acid side chain) could be identified. Gas chromatographic analysis of the TAP derivatives after total hydrolysis of **1a** showed the presence of L-Dab, D-Glu, Gly, L-Lys, D- and L-Orn and D-Ser. Thus the amino acid composition differs from that of pyoverdin R

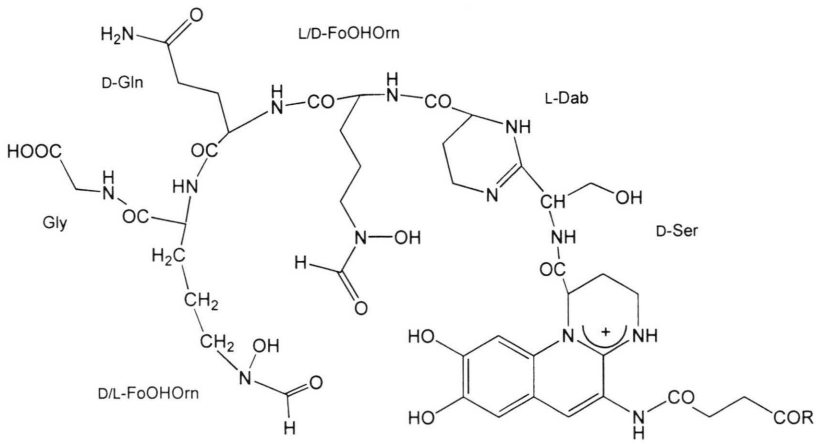

Fig. 1. Structure of pyoverdin **1** (**1a:** R = NH_2, **1b**: R = OH, **1c**: R = COOH)

(Gipp *et al.*, 1991) only by the absence of L-Glu. The ^{1}H- and ^{13}C-data of **1a** are compiled in Tables I and II. They were confirmed by COSY- and TOCSY-correlations within the chains of the single amino acids and by connection of the α-CH with its α-NH group by NOESY. The data correspond to those observed for pyoverdin R. Especially should be noted the downfield shift of the Ser-NH due to the connection with the carboxyl group of the chromophore, the shift value of the CH_2 group of Ser showing that the hydroxyl group is not esterified (Budzikiewicz, 1997), and the typical shift values for the tetrahydropyrimidine ring formed by condensation of the γ-NH_2 group of Dab with the amide carbonyl group of Ser (Gipp *et al.*, 1991; Risse *et al.*, 1998). The amino acid sequence follows from HMBC cross peaks connecting the CHα-signals with the CO-signals of the preceding amino acid.

The amino acid sequence could be confirmed by collision activation (CA) of $[M + 2H]^{2+}$ of **1a** (Table III). Due to the good charge stabilization in

Table I. ^{1}H-NMR data (δ [ppm]) of **1** (pH 4.3; 25 °C; H_2O/D_2O 9:1).

Suca	2′	3′						
	2.83	2.75						
Chr	1a	2a	2b	3a	3b	6	7	10
	5.72	2.51	2.70	3.34	3.69	7.91	7.15	7.09
Amino acid	NH	α	ß	γ		δ	CHO_{cis}	CHO_{tra}
Ser	9.38	4.67	4.03					
Dab	8.84	4.44	2.08	3.24				
			2.14	3.46				
FoOHOrn[1]	8.39	4.13	1.92	1.64		3.49	7.97	8.31
			2.01	1.79				
Gln	8.50	4.31	1.95	2.32				
			2.12					
FoOHOrn[2]	8.19	4.29	1.93	1.59		3.45	7.88	8.25
			2.09	1.69				
Gly	8.04	3.78						

Table II. ^{13}C-NMR data[a] (δ [ppm]) of **1a** (pH 4.3; 25 °C; D_2O/H_2O 9:1).

Suca	CONH	2′	3′				
	176.9	31.9	31.0				
Chr	CO	1	2	3	4a	5	6
	172.0	57.9	22.8	36.1	153.7	118.6	140.0
	6a	7	8	9	10	10a	
	115.9	115.3	145.3	152.1	101.5	133.4	
Amino acid	CO	α	β	γ	δ	CHO	
Ser	163.6[b]	55.7	61.8				
Dab	174.6	52.1	20.0	37.4			
FoOHOrn[1]	175.2	54.4	23.7	28.5	51.0	160.9	
					47.2	164.7	
Gln	174.5	53.2	27.5	32.2	178.0		
FoOHOrn[2]	174.2	55.2	27.3	23.1	50.8	160.6	
					47.2	164.7	
Gly	170.8	44.1					

[a] Based on HMBC and HMQC spectra.
[b] -HN-CR=N-.

Table III. MS-CA spectrum of **1a**, A-, B-, C-ions.

Amino acid	n	N-terminal fragments					
		A_n	A_n-CH_2O	B_n	B_n-CH_2O	C_n''	C_n''-CH_2O
Ser/Dab	1/2	498	**468**	**526**	496	**543**	513
Fho	3	**656**	**626**	**684**	**654**	701	671
Gln	4	784 (**392,5**)	**754**	**812** (**406,5**)	**782**	**829** (**415**)	**799**
Fho	5	942 (**471,5**)	912	970 (**485,5**)	940	987 (**494**)	957
Gly	6	–		–		–	

Calculated fragment ions of the pyoverdin with succinamide side chain. Observed fragment ions (obtained by CA of $[M+2H]^{2+}$) are printed in bold. A: X-NH$^+$=CHR, B: X-NH-CHR-CO$^+$, C'': X-NH-CHR-CO-NH_3 (Roepstorff and Fohlman, 1984). Masses of doubly charged ions in parentheses (note that they carry one proton more than singly charged ions, hence $2\ m^{2+} = m^+ + 1$).

the chromophore and in the tetrahydropyrimidine ring (protonated amidine systems) mainly N-terminal fragments are observed. The mass difference between B_3 and B_4 (128 u) shows that Glu found in the amino acid analysis stems from Gln present in the peptide chain. $[M + 2H]^{2+}$ and a series of N-terminal ions is accompanied by species which have lost CH_2O. This fragmentation is a typical process when the partner for the condensation product with Dab is a β-hydroxy amino acid as Ser in the case of **1**. It can be formulated as a McLafferty rearrangement (Fig. 2) and it is especially evident after CA (Risse *et al.*, 1998). A mass spectrometric analysis of **1c** gave the analogous sequentional ions starting from $[M - CO_2 - H_2O + 2H]^{2+}$ (the loss of CO_2 and H_2O is typical for the ketoglutaric acid side chain; cf. Voßen and Taraz, 1999).

Siderotyping of P. aeruginosa R'

As shown in Table IV, the *P. aeruginosa* strain R' presented an identical pyoverdin isoelectrofocussing pattern as strain Pa6, the reference strain of *P. aeruginosa* sv. III. Moreover, cross-incorporation studies involving the two strains and their respective pyoverdins demonstrated a similar efficiency in iron uptake, whereas both strains showed no cross-reactions with the strains *P. aeruginosa* PAO1 (reference strain of *P. aeruginosa* sv. I) and *P. aeruginosa* ATCC 27853 (reference strain of *P. aeruginosa* sv. II) and their respective pyoverdins. Accordingly, the strain R' should be classified within the same siderovar as strain Pa6 (sv. III). However, because of the difference in pyoverdin structures of the two strains, it is proposed to classify strain R' within a sub-group (sv. III-2) of *P. aeruginosa* sv. III, distinct from the Pa6 sub-group (sv. III-1). At least a MS analysis of the pyoverdins produced by the other strains belonging to sv. III (Meyer *et al.*, 1997) would now be required to precisely define their respective sub-grouping. A comparison of the peptide-synthetases involved in the biosynthesis of pyoverdin R' and pyoverdin Pa6 (R), might also allow a distinction and should be informative on the differences in biosynthetic pathways of the two closely related molecules.

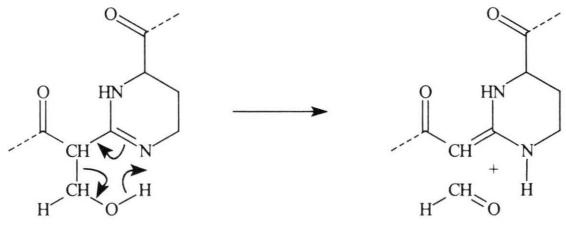

Fig. 2. McLafferty rearrangement of the tetrahydropyrimidine ring of **1**.

Discussion

The isolation of the new pyoverdin from *P. aeruginosa* R' (the designation R' was chosen to demonstrate the close structural relationship with the pyoverdin from *P. aeruginosa* R from which it differs only in the way that L-Gln is missing from the peptide chain) is remarkable in several ways. Among the ca. 50 pyoverdins whose structures have been established (Fuchs *et al.*, 2001) it is the first instance where two pyoverdins differing in their peptide chains show the same IEF pattern. The migration in the pH-gradient depends on the presence of basic and/or acidic groups in the peptide part and in the side chain of the chromophore. It must be concluded that here it makes no differ-

Table IV. Isoelectric pH (pI)values and $^{59}Fe^{3+}$-uptake data of the *P. aeruginosa* strains.

Strain	$^{59}Fe^{3+}$-uptake[a] as mediated by the pyoverdin of				pI
	R'	Pa6	PAO1	Pa27853	
R'	100	92	0	0	7.3; 5.2
Pa6	110	100	0	0	7.3; 5.2
PAO1	0	0	100	0	8.8; 7.0
Pa27853	0	0	0	100	8.9; 8.6; 7.5; 7.4

[a] Uptake of the strain specific pyoverdin 100%.

ence whether two neighboring or just one Gln are present in the molecule. The reciprocal uptake of the ferri-pyoverdins by the two strains poses a lesser problem. This has been observed occasionally when pyoverdins differ in their structures only in parts of the peptide chain not responsible for the recognition at the cell surface.

It has been almost a dogma in the literature after a screening of 88 strains mainly from hospital isolates (Meyer *et al.*, 1997) that *P. aeruginosa* is a well-defined species amongst the saprophytic so-called fluorescents (in contrast to the conglomerate *P. fluorescens/putida*) which comprises only three siderovars (regarding the two spurious pyoverdins mentioned in a conference report see the Introduction). The structural variant R' of sv. III may well be a local Asian mutant, nevertheless it would be interesting to test European isolates accordingly. However, since the relatively fast and simple IEF method does not work here, the costs for a series of MS investigations will probably be prohibitive. But even more interesting it would be to find out whether there are differences in pathogenicity. Even a thoroughly investigated species as *P. aeruginosa* occasionally offers unexpected new aspects!

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