The Pyoverdins of *Pseudomonas* sp. 96–312 and 96–318[§]

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The structures of the pyoverdins isolated from the *Pseudomonas* spp. 96–312 and 96–318 were elucidated by spectroscopic and degradation techniques. As observed before for *Pseudomonas* spp. producing pyoverdins with a C-terminal cyclopeptidic substructure, the two strains can recognize to some extent structurally different pyoverdins as long as they have also a similar cyclopeptidic C-terminus.

Introduction

From the bacterial family Pseudomonadaceae *sensu lato* several hundred species have been described over the years. This rather heterogenous conglomerate had been divided into five rRNA homology groups (Palleroni 1984, 1992). Recent systematic studies retained only homology group I as Pseudomonadaceae *sensu stricto* breaking up the other four groups into several new families. Group I can readily be subdivided into non-fluorescents and fluorescents, the latter ones being characterized by the production of pyoverdins as siderophores (Budzikiewicz, 1997a, b). By now over 50 pyoverdins are known differing in the structure of their peptide chains (Fuchs and Budzikiewicz, 2001). They are used increasingly for the classification of strains (Meyer, 2000) and for the identification of isolates (Fuchs *et al.*, 2001). For the analysis of a large number of isolates a method had to be developed which combined a minimum in purification techniques with a maximum of structural information. This can be achieved by electrospray ionization mass spectrometric analysis (ESI-MS) of a desalted crude extract (Kilz *et al.*, 1999; Fuchs and Budzikiewicz, 2001). Problems in the interpretation of the mass spectra arise when part of the peptide chain forms a cyclopeptidic substructure. Based on the rules developed for this type of pyoverdins (Fuchs and Budzikiewicz, 2000), structures had been proposed for several new representatives (Fuchs, 2000). For two of these the confirmation by "classical" means (NMR and degradation) will be presented here.

[§] Part CVII of the Series "Bacterial Constituents". For part CVI see Ongena *et al.* (2001).

Abbreviations: Common amino acids, 3-letter code; FoOHOrn, δ-N-formyl-δ-N-hydroxy-Orn; Suc (Suca), Mala, Kgl, succinic acid (amide), malamide and ketoglutaric acid residues; TAP, N/O-trifluoroacetyl (amino acid) isopropyl ester; ESI, electrospray ionization; FAB, fast atom bombardment; MS, mass spectrometry; CA, collision activation; COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlation spectroscopy; CFML, Collection de la Faculté de Médecine de Lille.

Materials and Methods

The *Pseudomonas* strains CFML 96–312 and 96–318 were provided by the Laboratoire de Microbiologie de la Faculté de Médecine de Lille, France, from a collection of mineral water isolates investigated for taxonomical purposes. As determined by numerical taxonomy studies, the two strains belong to phenotypic clusters together with *P. fluorescens* and *P. tolaasii* strains and, therefore, could be related to one of these species (Baïda, 2001). The bacteria were grown in a gluconate minimal medium (Beiderbeck *et al.*, 1999). For the

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work-up of the cultures and isolation of the ferri-complexes by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* (1999). The Biogel main fractions were subjected to ion-exchange chromatography on CM-Sephadex A-25 with a 0.02 M pyridinium acetate buffer (pH 5.0). Decomplexation was achieved with oxalate (Voss *et al.*, 1999).

The CM-Sephadex separation of the ferri-pyoverdins of *P.* sp. 96–312 yielded one main and two minor fractions which were purified by re-chromatography under the same conditions. From *P.* sp. 96–318 two fractions were obtained. The main fraction was rechromatographed on CM-Sephadex, the minor one could be separated into two fractions on DEAE-Sephadex using the same buffer as above.

For the qualitative and quantitative analysis of the amino acids, the determination of their configuration by GC/MS of their TAP-derivatives on a chiral column and the dansyl derivatization see Briskot *et al.* (1986) and Mohn *et al.* (1990). For instrumental details see Sultana *et al.* (2000).

The siderotyping procedures involving pyoverdin-isoelectrofocusing (IEF) and pyoverdin-mediated ^{59}Fe-iron uptakes were performed as described previously (Meyer *et al.*, 1998; Munsch *et al.*, 2000). The pyoverdins of foreign origin used for heterologous uptakes are indicated in the legend of Fig. 1. They were purified by chromatography on XAD-4. The ^{59}Fe incorporation experimental values were corrected for blank values obtained in assays without bacteria. 100% incorporation corresponded to 5,000 cpm for *Pseudomonas* sp. 96–312 and 15,000 cpm for *Pseudomonas* sp. 96–318 of the 25,000 cpm available per assay.

Results

Siderotyping behavior of Pseudomonas sp. CFML 96–312 *and* CFML 96–318.

The IEF-pattern of the pyoverdin 96–312 accumulated in the culture supernatant displayed three isoform bands at pHi 8.60, 8.50 and 7.45, respectively, while two bands at pHi 8.55 and 7.30 characterized the IEF-pattern of the pyoverdin 96–318. For both pyoverdins the lowest band appeared as the major isoform present in the culture supernatants.

As shown in Fig. 1, the ferri-pyoverdins of the two strains were also recognized by several other strains, though with lower efficiency (12 to 76%) than the own (homologous) ferri-pyoverdin. Also cross-incorporation occurred between the two strains and their respective pyoverdins.

Pyoverdin 96–312

All three fractions show the typical UV/Vis absorptions (Budzikiewicz, 1997b) of the free pyoverdins as well as of their ferri complexes. The molecular masses of the free pyoverdins as determined by FAB-MS are 1189, 1190 (main fraction) and 1218 u. The mass differences correspond to the side chains Suca, Suc and Kgl. This is confirmed by the observation of *retro*-Diels-Alder fragments resulting in the loss of the quinoline part of the molecules together with the side chains at *m/z* 888 (Michels *et al.*, 1991), the mass differences between the molecular ions and *m/z* 888 corresponding to the three acid side chains. Amino acid analysis showed the presence of 2 L-Lys, 2 L-

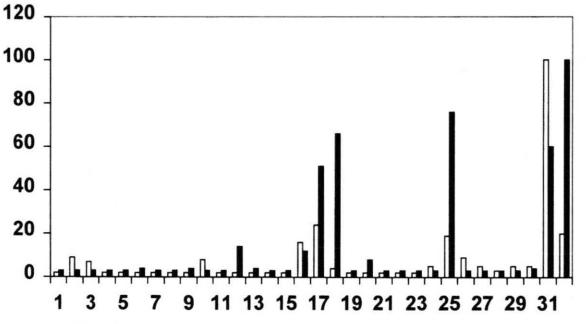


Fig. 1. $^{59}Fe^{3+}$-incorporation in *Pseudomonas* sp. 96–312 (white bars) and sp. CFML 96–318 (black bars) mediated by heterologous pyoverdins, expressed as percentage of incorporation compared with the respective homologous pyoverdin. The pyoverdins tested came from the following *Pseudomonas* strains: 1. *P.* sp. E8; 2. *P. syringae* ATCC 19310; 3. *P. fluorescens* 9AW; 4. *P. putida* ATCC 12633; 5. *P. fluorescens* 51W; 6. *P. aeruginosa* Pa6; 7. *P. fluorescens* CCM 2798; 8. *P. fluorescens* CHA0; 9. *P. tolaasii* LMG 2342; 10. *P. aeruginosa* ATCC 27853; 11. *P. fluorescens* ii; 12. *P. fluorescens* SB8.3; 13. *P. fluorescens* ATCC 17400; 14. *P. fluorescens* 1.3; 15. *P.* sp. 267; 16. *P. chlororaphis* ATCC 9446; 17. *P. aeruginosa* ATCC 15692; 18. *P. fluorescens* 18.1; 19. *P. fluorescens* 12; 20. *P. fluorescens* CFBP 2392; 21. *P. putida* CFBP 2461; 22. *P.* sp. ATCC 15915; 23. *P. putida* WCS358; 24. *P. 'mosselii'* CFML 90–77; 25. *P. rhodesiae* CFML 92–104; 26. *P. putida* CFML 90–33; 27. *P.* sp. CFML 90–51; 28. *P.* sp. CFML 90–52; 29, *P.* sp. strain 7SR1; 30. *P.* sp. A214; 31. *P.* sp. CFML 96–312; 32. *P.* sp. CFML 96–318.

Orn (as shown by NMR, derivatized Orn gives too low values in the GC analysis) and 3 Ser with the ratio 2 L and 1 D. Isolation of ε-dansyl Lys demonstrated that the ε-NH_2 group of at least one Lys, but no α-amino group is free. The configuration of the C-1 of the chromophore was found to be *S* from the CD spectrum of the isolated hydroxychromophore (transformation of the 4-NH_2 into an OH-group during hydrolysis) (Michels *et al.*, 1991).

For the NMR-techniques applied see Sultana *et al.* (2000). The ^{1}H-data of **1b** are assembled in Table I. They correspond to those observed with other pyoverdins (Budzikiewicz, 1997a, b). The following ones deserve a comment: The NH-signal of the Ser bound directly to the carboxyl group of the chromophore is typically shifted downfield. The shift values of the CH_2-groups of three Ser (3.8–4.0 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected) (Budzikiewicz, 1997b). The downfield shift of the ε-CH_2 of Lys^1 as compared with that of Lys^2 suggest that Lys^1 is connected ε-amidically to another amino acid (cf. Hohlneicher *et al.*, 1995; Amann *et al.*, 2000). This is confirmed by ROESY and HMBC cross peaks of the Lys^1-ε-NH-signal (identified by a COSY cross peak with the ε-CH_2 signal of Lys^1) with the α-and β-protons of Ser^3 in accordance with the ring structure. The two FoOHOrn units can be recognized by the split formyl signals both in the ^{1}H- and the ^{13}C-spectra due to *E/Z*-isomerization with a prevalence of the *Z*-form (Budzikiewicz, 1997b). The ^{13}C-data agree with those reported for other pyoverdins (see e.g. Sultana *et al.* 2000) showing no pecularities. They are therefore not reported. The peptide sequence was derived from ROESY (correlation of amide NH protons with spatially close α- and β-H's of the preceding amino acid, C**H**-C**H**-CO-N**H**) and HMBC (correlating amide CO with the α-H of the following amino acid, **C**O-NH-C**H**) (cf. Sultana *et al.*, 2000). It confirms the sequence proposed from the ESI-CA-MS spectra (Fuchs and Budzikiewicz, 2000).

The location of D- and L-Ser could be established by analysing the cleavage products after partial hydrolysis (6 M HCl, 90 °C, 60 min). Small peptides are washed out with H_2O while species still containing the chromophore are retained on a Sep-Pak RP-18 cartridge and can be eluted with CH_3CN/CH_3COOH 100:1. Apparently hydrolysis had occurred overwhelmingly after the first Ser as further hydrolysis gave only D-Ser. Hence the Ser bound to the chromophore is D- and consequently the other two Ser are L-configurated. The structures of the pyoverdins from *P.* sp. 96–312 are given in Fig. 2.

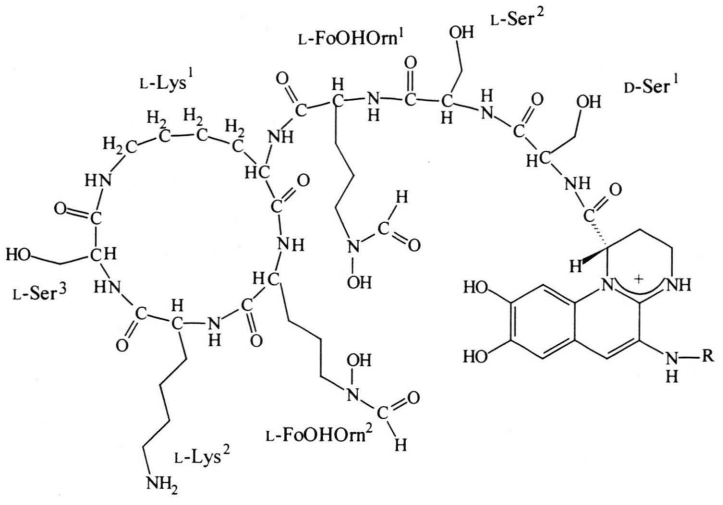
Pyoverdin 96–318

Again, all three fractions show the typical UV/Vis spectra of the free pyoverdins as well as of

Table I. ^{1}H-NMR data (δ [ppm]) of **1b** (pH 4.3; 5 °C; H_2O/D_2O 9:1)[a].

Suc	2′	3′								
	2.73	2.68								
Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.65	2.44	2.70	3.45	3.73	8.88	7.66	6.76	7.00	9.79
Amino acid	α-NH	α	ß	γ	δ		ε	ε-$NH_{(2)}$	CHO_Z	CHO_E
Lys^1	8.03	4.10	1.62	1.12	1.43		3.15 3.31	7.85		
Lys^2	8.72	4.06	1.91	1.42	1.70		3.00	7.64		
Ser^1	9.56	4.54	4.01							
Ser^2	8.83	4.52	3.83							
Ser^3	8.34	4.24	3.96							
FoOHOrn^1	8.29	3.89	1.48	1.32	3.32_Z 3.34_E				7.86	8.24
FoOHOrn^2	8.66	4.27	1.77	1.76	3.61_Z 3.63_E				7.98	8.32

[a] Based on H,H-COSY, NOESY and TOCSY correlations.



1a: R= CO-CH_2-CH_2-$CONH_2$
1b: R= CO-CH_2-CH_2-CO_2H
1c: R= CO-CH_2-CH_2-CO-CO_2H

Fig. 2. Structures of the pyoverdins from *Pseudomonas* sp. 96-312.

their ferri complexes. The molecular masses of the free pyoverdins as determined by FAB-MS are 1263, 1278 and 1291 u (main fraction). The mass differences correspond to the side chains Suc, Mala and Kgl. The *retro*-Diels-Alder fragments accordingly are observed at *m/z* 961 (Michels *et al.*, 1991). Amino acid analysis showed the presence of 1 L-Lys, 3 L-Orn (as shown by NMR, see above) and 4 Ser with the ratio 2 L and 2 D. Analysis of the hydrolysis products after dansylation gave δ-dansyl Orn and no dansyl Lys. Hence δ-NH_2 group of Orn is free and both NH_2 groups of Lys are substituted. The configuration of the C-1 of the chromophore is *S*.

The ^{1}H-data of **2c** are assembled in Table II. The NH-signal of the Ser bound directly to the car-

Table II. ^{1}H-NMR data (δ [ppm]) of **2c** (pH 4.3; 5 °C; H_2O/D_2O 9:1)[a].

Kgl[b]	2′			3′						
	2.80, 2.85 / 2.87			2.37, 2.66 / 2.36, 2.78						
Chr[b]	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.60	2.55	2.69	3.37	3.70	8.75	7.82, 7.84	7.01	7.11	not ob-
	5.69					8.90	7.90, 7.96		7.04	served
Amino acid	α-NH	α	ß	γ	δ	ε	ε-NH		CHO_Z	CHO_E
Lys	8.32	4.45	1.56	1.10	1.43	3.04	7.74			
			1.69			3.40				
Orn	8.68	4.38	1.54	1.43	2.61		δ-NH_2			
			1.84		2.79		7.55			
Ser[1]	9.86	4.40	4.00							
Ser[2]	8.67	4.44	3.84							
Ser[3]	8.73	4.45	3.93							
Ser[4]	8.46	4.32	3.80							
			3.94							
FoOHOrn[1]	8.50	4.22	1.75	1.59	3.47_Z				7.96	8.29
					3.52/					
					3.40_E					
FoOHOrn[2]	8.80	4.40	1.80	1.68	3.59/				7.96	8.27
					3.38_Z					
					3.61_E					

[a] Based on H,H-COSY , NOESY and TOCSY correlations.
[b] Different resonance frequences due to the equilibrium structures as determined in the HMQC spectrum.

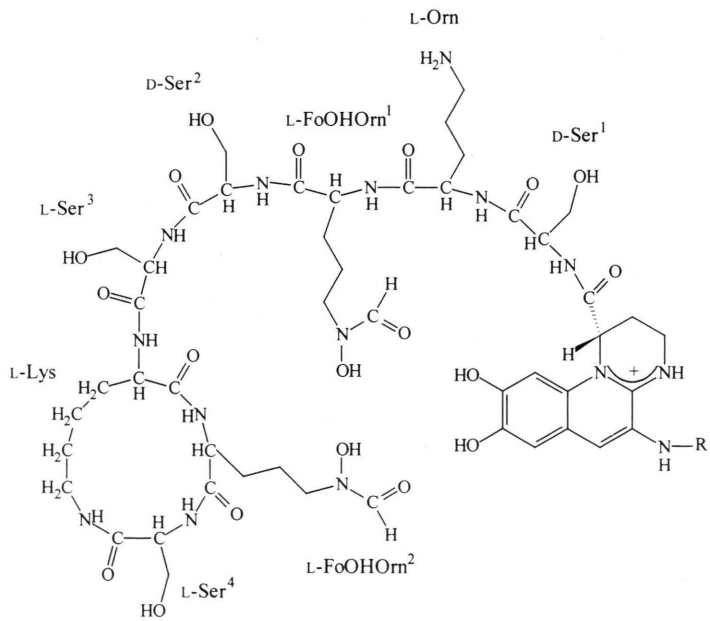
boxyl group of the chromophore is also here shifted downfield. The shift values of the CH_2-groups of four Ser (3.8–4.0 ppm) show that the OH-groups are not esterified. The shift values of the ε-CH_2 of Lys suggest as above that Lys is connected ε-amidically to another amino acid, as confirmed by ROESY and HMBC cross peaks of the Lys-ε-NH-signal (identified by a COSY cross peak with the ε-CH_2 signal of Lys) with the α-and β-protons of Ser^4 in accordance with the ring structure. The two FoOHOrn units show split formyl signals both in the ^{1}H- and the ^{13}C-spectra due to *E/Z*-isomerization with a prevalence of the *Z*-form (Budzikiewicz, 1997b).

The NMR-data of the α-ketoglutaric acid side chain reflect the isomeric structures which prevail over the one depicted in Fig. 3, viz. the stereoisomeric lactamic cyclization products (Sultana *et al.*, 2000). The CO-signal of the α-CO-group is hardly visible, instead broadened signals at about 95 ppm occur. Broadening or splitting of the chromophore H-signals is a consequence of these isomeric forms. Also several signals of the amino acid residues are split. This phenomenon caused by more than one conformation of the peptide chain has been observed occasionally. The ^{13}C-data agree with those reported for other pyoverdins and are therefore not reported. The peptide sequence was derived from ROESY and HMBC. As above, it confirms the sequence proposed from the ESI-CA-MS spectra (Fuchs and Budzikiewicz, 2000).

The location of D- and L-Ser could be established by analysing the cleavage products after partial hydrolysis (6 M HCl, 90 °C, 60 min). The hydrolysate was chromatographed on Sep-Pak RP-18. The retained material still containing the chromophore (see above) gave after hyodrolysis only D-Ser, hence the first Ser is D-configurated. The material not retained consisting of small peptides from the C-terminal part which contains the other three Ser (1 D and 2 L) was separated on a preparative silicagel plate with isopropanol, pyridine, glacial acetic acid and H_2O 8:4:3:2 (v/v) into eight fractions. They were scratched from the plate, eluted with CH_3OH and 0.2 M CH_3COOH 1:1 (v/v), hydrolyzed etc. as above. The first fraction contained only L-Orn, L-Lys and L-Ser. This combination can only be the C-terminal cycle. Fraction 2 contains L-Orn, L-Lys and L-Ser, but with an L-Lys:L-Ser ratio twice as big as in fraction 1, fraction 3 shows the same L-Lys:L-Ser ratio as fraction 2, but it contains in addition D-Ser. It follows that Ser^4 and Ser^3 are L- and Ser^2 and Ser^1 are D-configurated.

Discussion

In an earlier publication (Fuchs and Budzikiewicz, 2000) the possibilities for structure proposals



2a: R= CO-CH_2-CH_2-CO_2H
2b: R= CO-CH_2-CHOH-$CONH_2$
2c: R= CO-CH_2-CH_2-CO-CO_2H

Fig. 3. Structures of the pyoverdins from *Pseudomonas* sp. 96-318.

of pyoverdins with a cyclopeptidic C-terminus by mass spectrometry were discussed. Fragmentation of the linear part of the peptide residue linked to the chromophore follows the rules established for peptides, when $[M+H]^+$ or $[M+2H]^{2+}$ obtained by ESI is subjected to collision activation (CA). Only starting from $[M+2H]^{2+}$ bond cleavages in the cyclic part can be observed. The fragments thus obtained allow to identify the amino acids present in the cycle of the molecule. A special problem for the mass spectrometric analysis of cyclopeptides is to distinguish between sequence and *retro*-sequence (here to differentiate between the peptidic sequence Lys-Fho-Lys-Ser with an amide bond between the C-terminal carboxyl group of Ser and the ε-amino group of Lys, and Lys-Ser-Lys-Fho with an amide bond between Fho and Lys). Without going into details, the presence of fragment ions is necessary that are not formed by the prevalent cleavage of the CO-NH bonds, and that can be induced by CA to further fragmentation. For the pyoverdins 96–312 and 96–318 ions of this type were detected and the sequence of the cycle was predicted correctly. So far pyoverdins with a cyclopeptidic C-terminus were only encountered with Lys as branching amino acid and in every case where the structure was based on NMR or degradation studies, Lys was incorporated by its α-amino group into the peptide chain and formed the cycle by an amide bond between its ε-amino group and the carboxyl group of the C-terminal amino acid (see **1** and **2**). A reverse incorporation (ε in the chain and α in the cycle) can not be excluded *a priori* (cf. Budzikiewicz *et al.*, 1999). A consistent fragmentation pattern has been observed for the pyoverdins with known structure as well as for the present two pyoverdins. This gives additional weight to the conclusions regarding the incorporation of Lys in new pyoverdins. It is comforting that the structures of **1** and **2** had been deduced correctly from the ESI-MS-CA spectra (certainly, MS can say nothing about D- and L-amino acids).

The ^{59}Fe uptake data are in accordance with earlier observations (Meyer *et al.*, 1999; Amann *et al.*, 2000; Weber *et al.* 2000) that cross-incorporation is frequently observed between strains producing pyoverdins with a cyclo-tri- or -tetrapeptidic C-terminal part of the peptide chain, while a strictly specific recognition seems to be characteristic for strains producing pyoverdins with linear peptide chains. It is worth noting, that in every case the cyclopeptidic part comprises Lys forming a peptide bond between its ε-amino group and the C-terminal carboxyl group of Ser (rarely Thr) and that the amino acid following Lys is always FoOH-Orn. Whether a further amino acid is present between FoOHOrn and the terminal amino acids (cyclotetrapeptide as in **1**) or not (cyclotripeptide as in **2**), and if so, whether this amino acid is a small neutral (Ser, Thr), basic (Lys as for **1**) or acidic (Glu, Weber *et al.*. 2000; see also the discussion there) seems not to play a major role. As nothing is known about the structure of the receptor site no further discussion is possible.

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