A Pyoverdin from *Pseudomonas* sp. CFML 95–275[§]

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From *Pseudomonas* sp. CFML 95–275 a pyoverdin was isolated with a cyclopeptidic substructure. It could be shown that this pyoverdin is identical with one obtained from *Pseudomonas fluorescens* BTP 7 for which a lactone structure had been deduced from the interpretation of a FAB spectrum. The elucidation of the correct structure of the pyoverdin is described.

Introduction

Pseudomonas species belonging to the so-called fluorescent group of its genus produce siderophores named pyoverdins. These are chromopeptides consisting of a dihydroxyquinoline chromophore bound amidically to the N-terminus of a peptide chain by its carboxyl group at C-1, and to a small dicarboxylic acid or its amide by the amino group at C-5 (cf. **1**) (Budzikiewicz, 1997a and 1997b). The peptide chain frequently comprises cyclic substructures. Larger cycles are built either by formation of an amide bond between the carboxyl group of the C-terminal amino acid and the ε-amino group of an in-chain Lys or by formation of an ester bond between the carboxyl group of the C-terminal amino acid and the OH-group of an in-chain Ser or Thr.

The structure of a pyoverdin is highly characteristic for the producing *Pseudomonas* strain and can, therefore, be used for classification purposes (Meyer, 2000). *Pseudomonas* CFML 95–275 belongs to a collection of isolates from natural mineral waters which has been analyzed extensively for taxonomic purposes (Verhille *et al.*, 1997) leading to the proposal of several new species. A screening by siderotyping methods (see below) of the pyoverdins produced by these strains showed that the patttern of CFML 95–275 differed from those of the other strains as well as from those of 33 reference strains with known pyoverdin structures.

The structure elucidation of the new pyoverdin will be described. Its characteristic feature is a lactamic C-terminal cycle comprising four amino acids which is formed by an ε-amino Lys link with the C-terminal Ser (**1**). The pyoverdin CFML 95–275 could be shown to be identical with the one obtained from *Pseudomonas fluorescens* BTP7 for which a lactone structure (**3**) had been proposed (Ongena *et al.*, 1998) and whose structure has to be corrected.

[§] Part XCIV of the series "Bacterial constituents". For part XCIII see Taraz *et al.* (2000).

Abbreviations: Common amino acids, 3-letter code; FoOHOrn (Fho in Fig. 2), 5-N-formyl-5-N-hydroxy Orn; Kgl, α-ketoglutaric acid residue; Chr, pyoverdin chromophore (see Fig. 1); TAP, N/O-trifluoroacetyl-(amino acid)-isopropyl ester; RP-HPLC, reversed phase high performance liquid chromatography; 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; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; CFML, Collection de la Faculté de Médicine de Lille.

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Materials and Methods

Instruments and Chemicals

Mass spectrometry: Finnigan-MAT H-SQ 30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: DPX 300 (^{1}H 300, ^{13}C 75.5 MHz) and DRX 500 (^{1}H 500, ^{13}C 125 MHz) (both 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. Suppresion of the H_2O signal by the WATERGATE puls sequence.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen), CD: Jasco 715 (Jasco, Tokyo, Japan).

Isoelectrofocussing (IEF): For the analysis of pyoverdin forms ("isoforms") see Meyer *et al.* (1998). The bacteria were grown under iron starvation in a CAA medium (Budzikiewicz *et al.*, 1997b).

^{59}Fe-uptake studies: For the technique see Munsch *et al.* (2000). The values given in Fig. 5 were measured after 20 min of incubation and corrected for blank values obtained in assays without bacteria.

Chromatography: RP-HPLC column Eurospher 100-C_{18} (7 μm) (Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex C-25 and SP-Sephadex C25 (Pharmacia, Uppsala, S; GC/MS: Chirasil-L-Val (Chrompack, Frankfurt); plate chromatography: Silicagel-60 No. 5745 without fluorescence indicator (Merck, Darmstadt), plates 20×20 cm, 2 mm layer.

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 were of *p. a.* quality.

Production, isolation and chemical degradation of the pyoverdins

Pseudomonas sp. CFML 95–275 was grown in a succinate minimal medium (Budzikiewicz *et al.*, 1997a). 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 C-25 with a pyridinium acetate buffer (pH 5.0, gradient 0.02 to 0.2 M). The second (major) fraction was rechromatographed on SP-Sephadex C-25 with 0.02 M pyridinium acetate buffer (pH 5.0). Again, several fractions were obtained, the first of which (the main fraction, pure as checked by analytical RP-HPLC with CH_3OH/CH_3COONH_4 buffer pH 6.2) was decomplexed with 8-hydroxyquinoline (Briskot *et al.*,1986); **1** was finally purified by chromatography on Biogel P2 with 0.1 M acetic acid and checked for purity by analytical RP-HPLC as above.

For the qualitative and quantitative analysis of the amino acids, the determination of their configuration by GC/MS on a chiral column and the dansyl derivatization see Briskot *et al.* (1986) and Mohn *et al.* (1990). Partial hydrolysis for amino acid sequence determination (see Table III) was achieved with 6 N HCl at 50 °C for 30 min. The stereochemistry of Ser^1 was determined in the following way: **1** was hydrolyzed with 6 M HCl at 90 °C for 10 min, the hydrolysate was adsorbed on a Sep-Pak cartridge which retains only cleavage products still containing the chromophore when rinsed with 0.1 M CH_3COOH. The retained material was eluted with CH_3OH/H_2O (7:3, v/v), hydrolyzed again for 60 min, adsorbed on a Sep-Pak cartridge and treated as before. In this way a cleavage product was obtained containing only Ser^1 bound to the chromophore. It was hydrolyzed with 6 N HCl at 110 °C for 21 hrs and the obtained Ser was TAP derivatized and determined by GC on a chiral column as D-configurated. The material not retained on the cartridge after the first hydrolysis was separated by plate chromatography with isopropanol/pyridine/glacial acetic acid/H_2O 4:8:3:2 (v/v). 8 Fractions were obtained. They were eluted with 0.2 M CH_3COOH/CH_3OH 1:1 (v/v), subjected to total hydrolysis and analysed in form of their TAP derivatives as above. Fractions containing in adddition to Ser also Lys and Orn gave D/L-ratios of 1:2. Fraction No 8 which contained only 2 Ser (Ser^3 and Ser^4) gave a D/L-ratio of 1:1. Hydrolysis of **1** with 6 N HCl at 50 °C for 30 min gave a mixture from which by chromatography on Biogel with 0.1 M CH_3COOH a fraction could be separated which contained Ser^1, Ser^2 and Orn.

Subsequent hydrolysis as above and analysis of the TAP derivates gave D- and L-Ser in a ratio 1:1. Combination of these results shows that Ser^1 is D-, Ser^2 and Ser^5 are L-configurated and one Ser of Ser^3 and Ser^4 is D-, the other one L-configurated.

Results

Characterization of **1**

The UV/Vis spectrum of **1** is characteristic for a pyoverdin (Budzikiewicz, 1997a and 1997b): 402 nm at pH 7.0, split band at 366 and 376 nm at pH 3.0; ferri-**1** 398 nm and broad charge-transfer bands at 470 and 540 nm. The molecular mass of **1** was determined by FAB-MS as 1392 u. From the *retro*-Diels-Alder fragment at *m/z* 1062 it can be concluded that the acyl side chain of the chromophore is α-ketoglutaric acid (Michels *et al.*, 1991). Gas chromatographic analysis on a chiral column of the TAP derivatives after total hydrolysis showed the presence of only three amino acids, viz. L-Lys, L-Orn, D- and L-Ser 2:3. By total hydrolysis after dansylation ε-dansyl Lys was obtained as could be shown by chromatograhic comparison with samples of authentic α- and ε-dansyl Lys. Hence in **1** for one or both Lys the ε-amino group is free.

Determination of the amino acid sequence by NMR

Basis for the sequence determination by NMR is the unambiguous identification of all ^{1}H- and ^{13}C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: COSY and TOCSY allows to detect the H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). Quaternery C-atoms can be identified with HMBC optimized for ^2J- and ^3J-coupling. C,H-^1J-coupling can be detected by HMQC. Sequence information is obtained by NOESY/ROESY which allows a correlation of an NH-proton with spatially close α- and β-H's of the preceding amino acid (C**H**-C**H**-CO-N**H**) and by HMBC correlating amide-CO with the α-H of the following amino acid (see Fig. 1). The ^{1}H- and ^{13}C-data of **1** are compiled in Tables I and II. They correspond to those observed with other pyoverdins (Budzikiewicz, 1997a and 1997b). 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 five Ser (3.83–3.95 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected) (Budzikiewicz, 1997b).

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

Kgl	2′	3′	3′							
	2.81	2.34	2.68							
Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.69	2.45	2.69	3.35	3.67	N. O.[b]	7.85	7.12	6.98	9.92[c]
Amino acid	α-NH	α	β	γ		δ	ε	ε-$NH_{(2)}$	CHO_Z	CHO_E
Lys^1	8.12	4.25	1.69	1.11 1.21		1.42	3.18	7.76		
Lys^2	8.54	4.06	1.89	1.45		1.67	2.99	7.65[c]		
Ser^1	9.31	4.52	3.93							
Ser^2	8.64	4.45	3.84							
Ser^3	8.35	4.45	3.83							
Ser^4	8.21	4.28	4.13	1.23						
Ser^5	8.25	4.21	3.95							
$FoOHOrn^1$	8.26	4.23	1.50 1.62	1.48		3.35 3.44			7.96	8.23
$FoOHOrn^2$	8.58	4.26	1.77	1.75		3.58 3.61			7.96	8.30

[a] Based on COSY and TOCSY correlations. [b] Not observed. [c] 5 °C.

Table II. ^{13}C-NMR data (δ [ppm]) of **1b** (pH 4.3; 25 °C; D_2O)[a,d].

Kgl	1′CO	2′CH_2	3′CH_2	4′C[b]	COOH			
	176.6	30.1 31.5	33.0 33.8	ca 95	180.5			
Chr	CO	1	2	3	4a	5	6	
	171.3	58.2	23.5	36.3	149.3	117.2	142.6	
	6a	7	8	9	10	10a		
	115.4	115.0	145.2	154.9	101.4	134.0		
Amino acid	CO	α	β	γ	δ	ε	CHO_Z	CHO_E
Lys[1]	174.6	54.6	31.7	22.5	28.6	39.9		
Lys[2]	174.6	55.7	29.9	23.5	27.4	40.2		
Ser[1]	173.0	57.3	62.2[c]					
Ser[2]	173.0	56.7	62.0[c]					
Ser[3]	172.2	56.6	61.9[c]					
Ser[4]	172.2	56.4	61.8[c]					
Ser[5]	172.2	57.2	61.2					
FoOHOrn[1]	175.1	54.7	28.1	23.0	47.0_E 50.7_Z		160.6	164.9
FoHOOrn[2]	173.3	54.8	28.6	24.1	47.0_E 50.8_Z		160.7	164.9

[a] Based on HMBC and HMQC spectra. [b] See text. [c] May be interchanged. [d] In connection with FoOHOrn Z and E refers to the *cis/trans* configuration of the formyl group.

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 NOESY, ROESY and HMBC cross peaks of the Lys[1]-ε-NH-signal (identified by TOCSY and COSY cross peaks with the ε-CH_2 signal of Lys[1]) with the α-, β- and NH-protons of Ser[5]. Due to the higher exchange rate of the hydrogens of an NH_2- as compared with a CONH-group those of the Lys[2]-ε-NH_2-group can only be seen at 5 °C.

The two FoOHOrn units can be recognized by the split formyl signals both in the ^{1}H- and the ^{13}C-spectra due to *cis/trans*-isomerization with a prevalence of the *Z*-form (Budzikiewicz, 1997b).

The NMR-data of an α-ketoglutaric acid side chain reflect the isomeric structures wich prevail over the one depicted in Fig. 1, viz. the stereoisomeric cyclization products (Fig. 3, **2a** and **2b**). (Budzikiewicz, 1997b). The CO-signal of the α-CO-group is hardly visible, instead broadened signals at about 95 ppm occur. Broadening of the chromphore H-signals is a consequence of these isomeric forms.

The peptide sequence as derived from ROESY/NOESY and HMBC correlations is given in Fig. 1.

Determination of the amino acid sequence by ESI mass spectrometry

The amino acid sequence deduced from NMR data is confirmed by the fragment ions obtained after ESI by ion trap CA. As the analyzed pyoverdin contains the side chain ketoglutaric acid CA of $[M + 2H]^{2+}$ in the ion trap results in the loss of H_2O and $H_2O + CO_2$. Sequence relevant fragment ions can be observed by isolation and fragmentation of the ion $[M - H_2O - CO_2 + 2H]^{2+}$ leading to N-terminal ions. The B-Ions (Roepstorff and Fohlman, 1984) X-NH-CHR-CO^+ are present up to B_5 (cleavage after Ser[4]). B_5 is followed by a fragment *m/z* 958 corresponding to B_5 + Lys[1]. The branching after Lys[1] is reflected in the observation of two ions, viz. *m/z* 1045 (B_5 + Lys[1] + Ser[5]) and *m/z* 1116 (B_5 + Lys[1] + FoOHOrn[2]). In addition, losses of the three amino acids (elimination of NH-CHR-CO) completing the cyclic substructure after Lys[1] can be observed: *m/z* 1203 (- 128u, Lys[2]), *m/z* 587 (doubly charged corresponding to

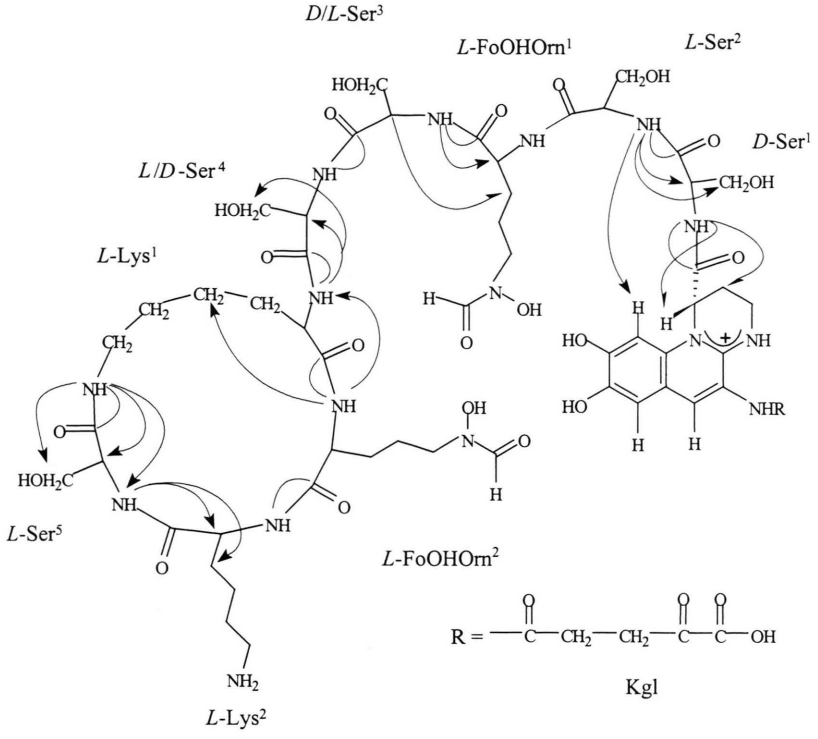

Fig. 1. Sequential information from NMR data of **1**.

m/z 1173 singly charged, doubly charged ions carry one H^+ more than singly charged ones; – 158, FoOHOrn^2) and *m/z* 622.5 (doubly charged corresponding to *m/z* 1244 singly charged; – 87, Ser^5). The series of B-ions is corroborated by several C-terminal Y"-ions (H_3N^+-CHR-COX') (Fig. 2) (Fuchs and Budzikiewicz, 2000).

Determination of the amino acid sequence by partial hydrolysis

After partial hydrolysis the masses of the $[M + H]^+$ ions of the products assembled in Table III could be determined by FAB mass spectrometry. Note that due to the acid instability of hydroxamic acids the formyl group is lost (- CO) from all hy-

Table III. $[M+H]^+$ ions after partial hydrolysis of **1** as determined by FAB-MS.

m/z	Assignment
445	Kgl-Chr-Ser – H_2O – CO_2
473	Kgl-Chr-Ser – H_2O
491	Kgl-Chr-Ser
690	Kgl-Chr-Ser-Ser-FoOHOrn – CO – H_2O
708	Kgl-Chr-Ser-Ser-FoOHOrn – CO
777	Kgl-Chr-Ser-Ser-FoOHOrn-Ser – CO – H_2O
795	Kgl-Chr-Ser-Ser-FoOHOrn-Ser – CO
561	Ser-Lys-FoOHOrn-Lys-Ser – CO – H_2O
579	Ser-Lys-FoOHOrn-Lys-Ser – CO – H_2O
648	Ser-Ser-Lys-FoOHOrn-Lys-Ser – CO – H_2O
666	Ser-Ser-Lys-FoOHOrn-Lys-Ser – CO

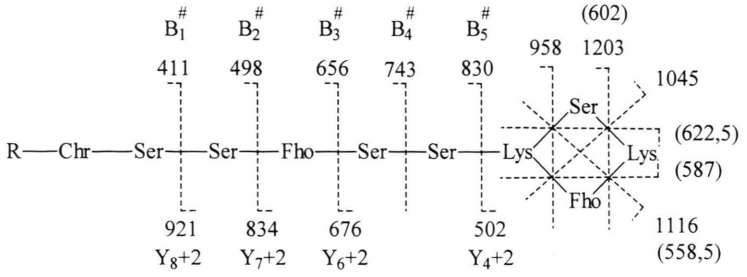

Fig. 2. Ions observed after CA of $[M - H_2O - CO_2 + 2H]^{2+}$.$B^{\#}$-ions after the loss of H_2O and CO_2 from the ketoglutaric acid side chain. Doubly charged ions in parentheses.

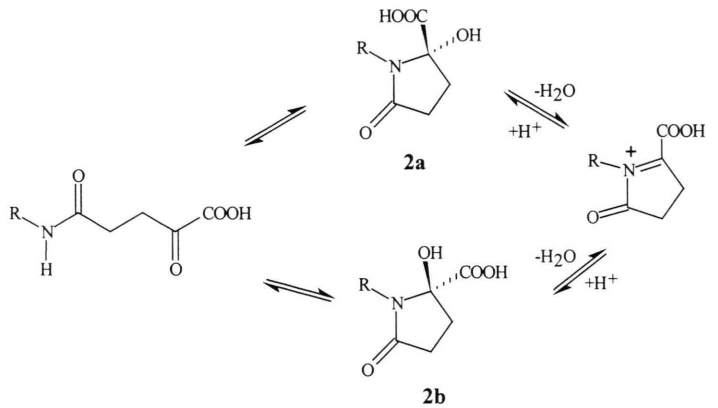

Fig. 3. Equilibrium structures of Kgl.

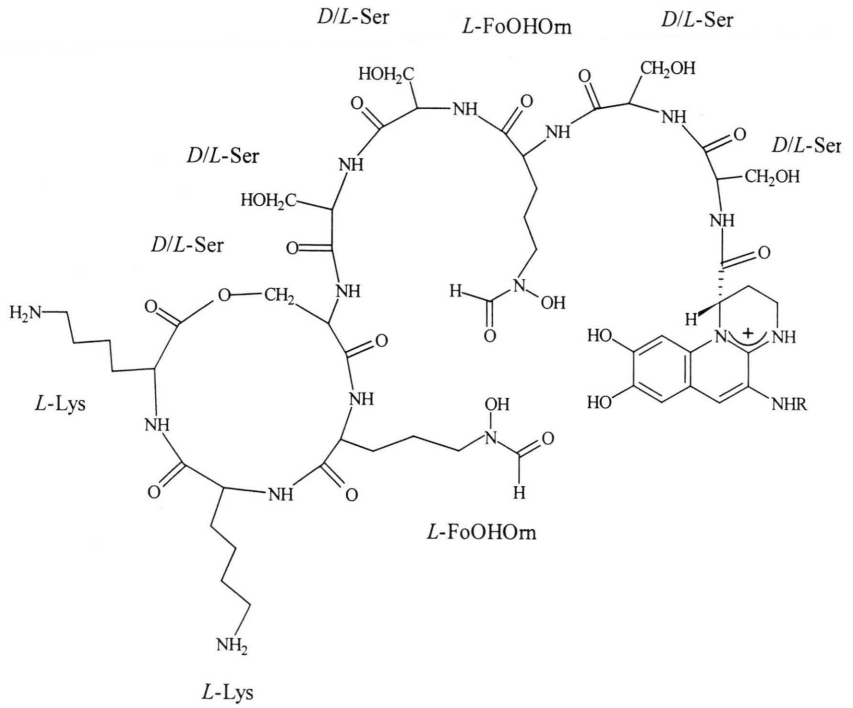

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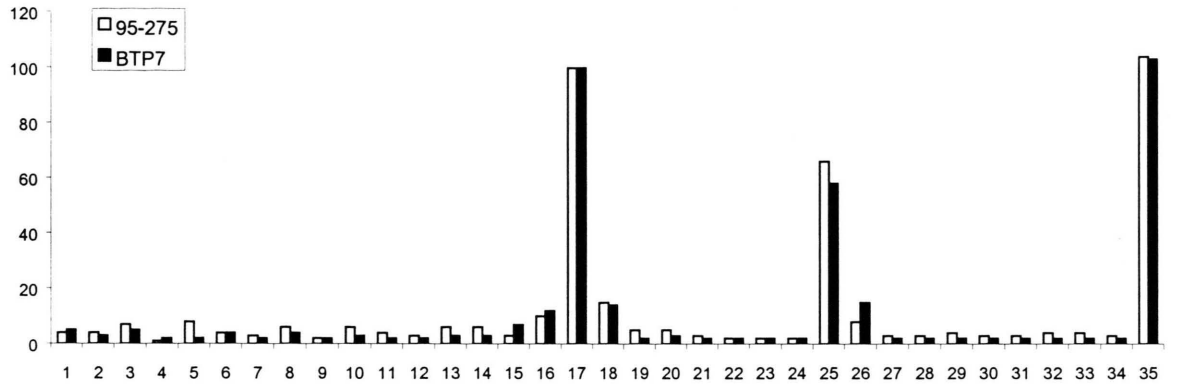
Fig. 4. Proposed structure for the pyoverdin BPT 7.



The pyoverdins tested originated from: 1. *P.* sp. A225; 2. *P. syringae* ATCC 19310; 3. *P. fluorescens* 9AW; 4. *P. putida* ATCC 12633; 5. *P. fluorescens* 51W; 6. *P. aeruginosa* Pa 6; 7. *P. fluorescens* CCM 2798; 8. *P. fluorescens* CHA0; 9. *P. tolaasii* NCPPB 2192; 10. *P. aeruginosa* ATCC 17853; 11. *P. fluorescens* ii; 12. *P. fluorescens* SB 8.3; 13. *P. fluorescens* ATCC 27400; 14. *P. fluorescens* 1.3; 15. *P.* sp. 267; 16. *P. fluorescens* ATCC 13525; 17. *P.* sp. CFML 95–275; 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. monteilii* CFML 90–54; 24. *P. "mosselii"* CFML 90–77; 25, *P. rhodesiae* CFML 92–104; 26, *P. veronii* CFML 92–124; 27, *P.* sp. CFML 90–40; 28, *P.* sp. CFML 90–42; 29, *P.* sp. CFML 90–51; 30. *P.* sp. CFBP 4396; 31, *P.*sp. 7SR1; 32, *P.* sp. 2908; 33, *P.*sp. A 214; 34, *P.* sp. Ps 3a; 35, *P. fluorescens* BTP7.

Fig. 5. Heterologous ^{59}Fe incorporation into *P.* sp CFML 95–275 and *P. fluorescens* BTP7.

drolysis products originally containing FoOHOrn. From these fragments two sequences can be deduced, viz. the N-terminal Kgl-Chr-Ser-Ser-FoOH-Orn-Ser and the C-terminal Ser-Ser-Lys FoOH-Orn-Lys-Ser. In the ROESY, NOESY and HMBC spectra cross peaks between Ser^2 and $FoOHOrn^1$ were not observed (see Fig. 1). The CA spectra as well as the partial hydrolysis data close this gap. The molecular mass of **1** (1392 u) corresponds to the structural details discussed above (amino acids, one cyclic substructure, α-ketoglutaric acid side chain). They allow to propose the structure **1** depicted in Fig. 1 for the pyoverdin CFML 95–275; only the location of the D- and L-Ser for Ser^3 and Ser^4 remains open.

Siderotyping studies

By IEF of the culture supernatant of the strain CFML 95–275 four pyoverdin isoforms could be characterized by their isoelectric pH values 5.3, 7.4, 8.3 and 8.4, a pattern that is unparallelled in the Strasbourg collection of pyoverdins. This novel character was confirmed by the pyoverdin-mediated iron uptake as illustrated in Fig. 5. Amongst the 33 strains tested only the pyoverdin of *Pseudomonas rhodesiae* CFML 104 showed an appreciable incorporation (60% of the homologous system). However, it gives different isoelectric pH-values (7.0, 7.1, 8.0 and 8.2) and, therefore, its structure should differ from that of the strain CFML 95–275. The problem of iron uptake mediated by non-identical heterologous pyoverdins is discussed by Georgias *et al.* (1999), Amann *et al.* (2000) and by Weber *et al.* (2001).

Discussion

The pyoverdin CFML 95–275 is remarkable as its amino acid chain comprises only three types of amino acids, viz. Lys, Orn and Ser – in contrast to the larger variety of amino acids contained in all other pyoverdins known sofar (Kilz *et al.*, 1999). A pyoverdin from *Pseudomonas fluorescens* (Pf BTP7) with the same amino acid composition had been described earlier (Ongena *et al.*, 1998). The structure proposed for it is identical with that of **1** up to Ser^4, but it continues Ser^5-FoOHOrn-Lys-Lys with a lactone linkage between Ser^5 and the C-terminal Lys (**3**, Fig. 4). By comparison of the ESI-CA and the NMR spectra of the two pyoverdins their identity could be established. Of importance is that the pyoverdins with an ester *cyclo*depsipeptidic substructure show characteristic pecularities in their NMR spactra: In cases where the hydroxyl part of an ester linkage is a Ser resi-

due, the ^{1}H-resonance of the CH_2 group is shifted from ~4.0 to ~4.5 ppm and the ^{13}C-resonance from ~62 to ~65 ppm (Budzikiewicz, 1997b). In a HMQC spectrum it could be shown that cross peaks exist only in the 3.8–4.0/61–62 ppm realm (cf. Tables I and II). Hence an ester bond can be excluded. Both, IEF and the iron uptake studies (Fig. 5) confirmed the identity of the two pyoverdins.

Since the structure of **1** follows beyond doubt especially from the two-dimensional NMR data, the FAB-data which had lead to structure **3** have to be scrutinized. The A- and C-ions observed in FAB spectrum up to Ser^4 (A_5 and C_5) and the data obtained from products of partial hydrolysis comprising the cycle (both the cycles of **1** and **3** have the same mass) are compatible with either structure. Recent results make it unlikely that products of partial hydrolysis containing a cyclic structure with a lactone linkage could have been isolated; the lactone is much more sensitive to hydrolysis than any amide bond (Voßen *et al.*, 2000). The positive ninhydrine test proves that at least one, but not necessarily that two NH_2-groups are free. The ions obtained after CA containing 2 Lys and 1 or 3 Ser residues can be explained from *either* structure by the cleavage of the CO-NH-bond after Ser^4 or before Ser^3 and elimination of FoOHOrn. There remain three ions the genesis of which we cannot explain starting from structure **1**: *m/z* 951 (B_6, i.e. B_5 + Ser) and 1063/1126 (+ FoOHOrn, $A_7 - H_2O$ and $C_7 + 2H$) all of them after opening of the lactone ring of **3**. Why just these three erratic fragments may have been formed is another question. In any case, structures based essentially on the interpretation of FAB spectra can only be considered as tentative.

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