The Siderophores of *Pseudomonas fluorescens* 18.1 and the Importance of Cyclopeptidic Substructures for the Recognition at the Cell Surface[#]

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The structure of the pyoverdin siderophore of *Pseudomonas fluorescens* 18.1 was elucidated by spectroscopic methods and chemical degradation. By cross feeding studies structurally closely related pyoverdins containing a C-terminal cyclopeptidic substructure were tested regarding the mutual recognition by the producing strains. Partial recognition of foreign pyoverdins was observed.

Introduction

Fluorescent members of the rRNA homology group I of the genus *Pseudomonas* are characterized by the production of so-called pyoverdins, i.e., siderophores consisting of a dihydroxyquinoline chromophore bound amidically 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). By now about 50 pyoverdins are known, for more than 30 complete or fairly complete structures have been established (Kilz *et al.,* 1999). The peptide chain has a two-fold function. It provides two of the ligand sites for Fe^{3+}, and it is responsible for the recognition of the iron complex at the surface of the producing cell. The variability of the peptide chain is closely connected with the second function: It safegards that a given ferri-pyoverdin is available only to the producing strain because of a highly specific interaction between the ferri-pyoverdin and a receptor outer membrane protein. There are, however, examples known where *Pseudomonas* strains can accept ferri-pyoverdins differing in the peptide chain from the proper one (for a summary as well preceding studies regarding common amino acid patterns see Georgias *et al.*, 1999).

Pyoverdins can roughly be divided into four sub-groups, viz.

– the most common variety having cOHOrn as C-terminal amino acid (cf. Georgias *et al.,* 1999);

– next in number are those characterized by a C-terminal cyclic part consisting of 3 or 4 amino acids, formed by an amide bond between the carboxyl group of the C-terminal amino acid and the α-amino group of an in-chain Lys; the amino acid following Lys for all structures established sofar is FoOHOrn (see below);

– pyoverdins with a C-terminal *cyclo*depsipeptidic substructure formed by an ester bond between the carboxyl group of the C-terminal amino acid and an in-chain Ser or Thr (cf. Voßen *et al.*, 2000);

– pyoverdins with a C-terminal free carboxyl group; some or all of them may be hydrolysis products of the rather labile cyclic esters which are

Abbreviations: Common amino acids, 3-letter code; OHAsp, *threo*-β-hydroxy Asp; Dab, 2,4-diaminobutyric acid; FoOHOrn, N^5-formyl-N^5-hydroxy-Orn; cOHOrn, *cyclo*-N^5-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); Chr; pyoverdin chromophore; Suc, succinic acid residue; TAP-derivatives, 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; HMBC, heteronuclear multibond 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.

[#] Part XCII of the series "Bacterial Constituents". For Part XCI see Beiderbeck *et al.* (2000).

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readily hydrolyzed at pH values > 9 (cf. Voßen *et al.*, 1999).

To the second group belong (D-amino acids are printed in bold)

Chr-**Ser**-Arg-**Ser**-FoOHOrn-(Lys-FoOHOrn-Thr-Thr) (*P. aeruginosa* ATCC 15692)

Chr-**Ser**-Lys-Gly-FoOHOrn-(Lys-FoOH**Orn**-Ser) (*P. fluorescens* ATCC 13525)

Chr-**Ser**-Lys-Gly-FoOHOrn-**Ser**-Ser-Gly-(Lys-FoOH**Orn**-Glu-Ser) (*P. fluorescens* 12)

(*P. a.* 15692: Briskot *et al.*, 1989; *P. f.* 13525: Hohlneicher *et al.*, 1995; *P. f.* 12: Geisen *et al.*, 1992).

The common structural parts Ser – basic amino acid – neutral amino acid – FoOHOrn next to the chromophore and the cyclic substructure are obvious. Actually, *P. a.* 15692 recognizes the ferripyoverdin of *P. f.* 13525 (Kinzel *et al.*, 1998). From *P. fluorescens* 18.1 a pyoverdin could be isolated which is accepted by all three strains mentioned and which in turn accepts two of the pyoverdins from these three strains. The elucidation of its structure (**1a**) and of those of co-occuring siderophores, viz.

Chr-**Ser**-Lys-Gly-FoOHOrn-Ser-**Ser**-Gly-(Lys-FoOH**Orn**-Ser)

will be reported here.

Materials and Methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT HSQ-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: AM 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.

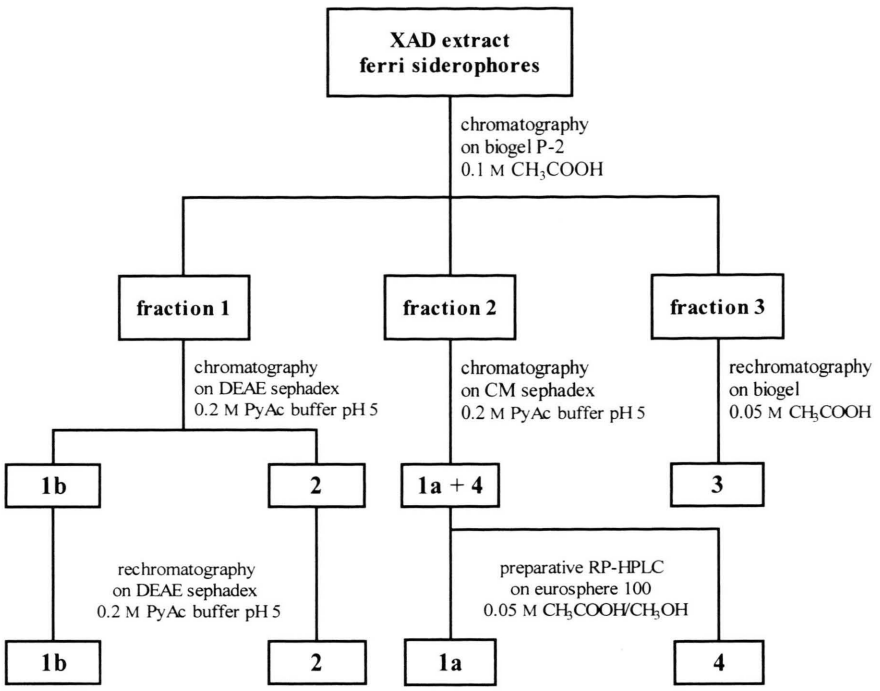
UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen).

Chromatography: RP-HPLC columns Nucleosil 100-C_{18} (5 µm) and 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 DEAE-Sephadex A-25 (Pharmacia, Uppsala, S), SepPak RP-18 cartouches (Waters, Milford MA, USA); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt).

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 p. a. quality.

Production, isolation and derivatisation of the siderophores

Pseudomonas fluorescens 18.1 (isolated from fish, Champomier-Verges and Richard, 1994) was grown for 72–96 hrs. in a gluconate medium (Beiderbeck *et al.*, 1999). For the work-up of the culture medium by chromatography on a XAD-4 column see Georgias *et al.* (1999). The separation of the various siderophores is summarized in Scheme 1. For decomplexation the ferribactin was dissolved in 1% aqueous citric acid and extracted six times with a 5% solution of 8-hydroxyquinoline in $CHCl_3$ and than with pure $CHCl_3$. The aqueous phase was chromatographed on Biogel P-2, solvent 0.1 M acetic acid. The other siderophores were dissolved in H_2O and adsorbed on an activated SepPak RP-18 cartouche, decomplexed with 6.5% aqueous oxalate, washed with H_2O and desorbed with CH_3OH/H_2O 3:1 (v/v). For qualitative and quantitative 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.* (1986) and Mohn *et al.* (1990). For partial hydrolysis 50 mg of **1a** were dissolved in 50 ml 6 M HCl and heated to 60 °C for 30 min., cooled and brought to dryness i.v., 3 times redissolved in H_2O, brought to dryness again and chromatographed on Biogel P-2 (solvent 0.1 M acetic acid). The fractions were analyzed with FAB-MS. The configuration of the amino acids was determined after total hydrolysis as above. The Ga^{3+} complex of **1a** was prepared by adding an 1.5fold excess of $Ga(NO_3)_3 \cdot 2H_2O$ to a solution of **1a** in 0.2 M pyridinium acetate buffer (pH 5.0). After 24 hrs. the solution was brought to dryness i.v., redissolved in the same buffer and chromatographed on Biogel P-2 with the same buffer. The fraction showing a blue fluorescence under 364 nm irradiation was collected.



Scheme 1. Work-up of the XAD extract of the culture medium of *Pseudomonas fluorescens* 18.1 (PyAc ... pyridinium acetate).

Growth stimulation tests and pyoverdin isoelectrofocussing analysis

The methods previously described for growth stimulation (Georgias *et al.*, 1999) and for isoelectrofocussing (Meyer *et al.*, 1998) were used. For growth stimulation paper discs (6 mm ∅) impregnated with a solution of the respective pyoverdin were placed on an iron-free agar plate seeded with the *Pseudomonas* strain to be tested and the diameter of the colony developed under the disc was determined after 24 hrs. Isoelectrofocussing is based on the migratory aptitude of pyoverdins in an electric field in a pH gradient. The isoelectric pH values (pI) depend on the presence of basic (Arg or Lys) and acidic (Asp, side chain) groups present in the pyoverdin. Due to the co-occurance of several pyoverdins with different side chains (succinic, ketoglutaric acic, Glu etc.) usually several bands are observed.

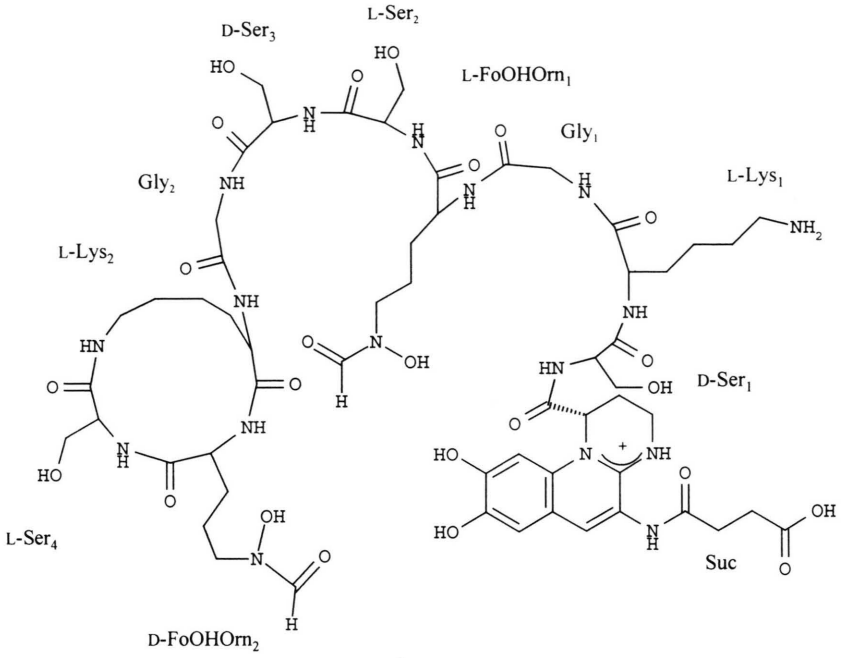
Results

Characterization of **1a**

1a and its iron complex gave the characteristic UV/Vis spectra of pyoverdins (Budzikiewicz, 1997). The molecular mass of **1a** as determined by FAB-MS amounts to 1391 u, a *retro*-Diels-Alder-fragment (loss of the dihydroxyquinoline unit together with the side chain; Michels and Taraz, 1991) at *m/z* 1089 (loss of 303 u) confirms the presence of a succinic acid side chain. Amino acid analysis after total hydrolysis showed the presence of Gly, L-Lys, D- and L-Orn, D- and L-Ser and succinic acid. The comparatively low intensity of the Orn signals suggests that Orn is present in a derivatized form.

Sequence determination by NMR and MS

Basis for the sequence determination is the unambiguous identification of all ^{1}H- and ^{13}C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: TOCSY allows to detect the H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). Direct (^{1}J) C,H connections can be determined by a HMQC experiment, quaternary C-atoms can be identified with HMBC optimized for ^{2}J- and ^{3}J-coupling, CH-, CH_2- and CH_3-groups by DEPT. Sequence information is ob-



1a

tained 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

Table I. ^{1}H NMR data[b] of **1a** (H_2O/D_2O 9:1, pH 4.3), correlations by TOCSY and NOESY.

Suc	2′	3′								
	2.74	2.65								
Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.72	2.55	2.72	3.39	3.73	8.97	7.88	7.02	6.94	9.94
Amino acid	α-NH	α	β	γ	δ	ε	ε-NH$_{(2)}$	CHO$_Z$	CHO$_E$	
Lys^1	8.64	4.36	1.61 1.84	1.14	1.41 1.53	2.66 2.76	7.53			
Lys^2	8.05	4.21	1.62 1.93	0.95 1.35	1.53	3.15 3.31	7.45			
Ser^1	9.83	4.41	4.01							
Ser^2	8.74	4.52	3.93							
Ser^3	8.63	4.44	3.87							
Ser^4	9.06	4.36	3.89							
Gly^1	8.39	3.61								
Gly^2	8.59	3.96								
FoOHOrn^1	8.35	4.29	1.69[a] 1.78[a]	1.70[a]	3.57$_Z$[a] 3.60$_E$[a]			7.96	8.29	
FoOHOrn^2	8.27	4.45	1.76	1.63[a]	3.57$_Z$[a] 3.60$_E$[a]			7.96	8.29	

[a] Assignment by ^{1}H, ^{13}C-HSQC in D_2O at 25 °C, [b] *Z* and *E* at FoOHOrn signals refers to the configuration of the N-hydroxyformamide groups.

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

Suc	1′CO	2′CH_2	3′CH_2	COOH				
	178.7	33.1	33.5	182.4				
Chr	CO	1	2	3	4a	5	6	
	171.7	57.9	23.0	36.1	150.5	118.7	139.9	
	6a	7	8	9	10	10a		
	114.9	114.5	144.8	153.1	101.0	132.8		
Amino acid	CO	α	β	γ	δ	ε	CHO_Z	CHO_E
Lys^1	175.3	54.5	31.2	23.2	27.0	40.3		
Lys^2	176.3	56.2	29.7	19.8	27.0	40.3		
Ser^1	173.3	58.2	61.8					
Ser^2	173.7	56.8	62.2					
Ser^3	173.8	57.1	62.2					
Ser^4	173.2	57.8	60.8					
Gly^1	172.2	43.3						
Gly^2	172.8	43.8						
$FoOHOrn^1$	175.4	54.6	29.0	23.7	47.1_Z 51.1_E		160.9	165.3
$FoHOOrn^2$	174.8	54.5	26.5	23.7	47.1_Z 51.1_E		160.9	165.3

[a] Based on HMBC and HSQC spectra.

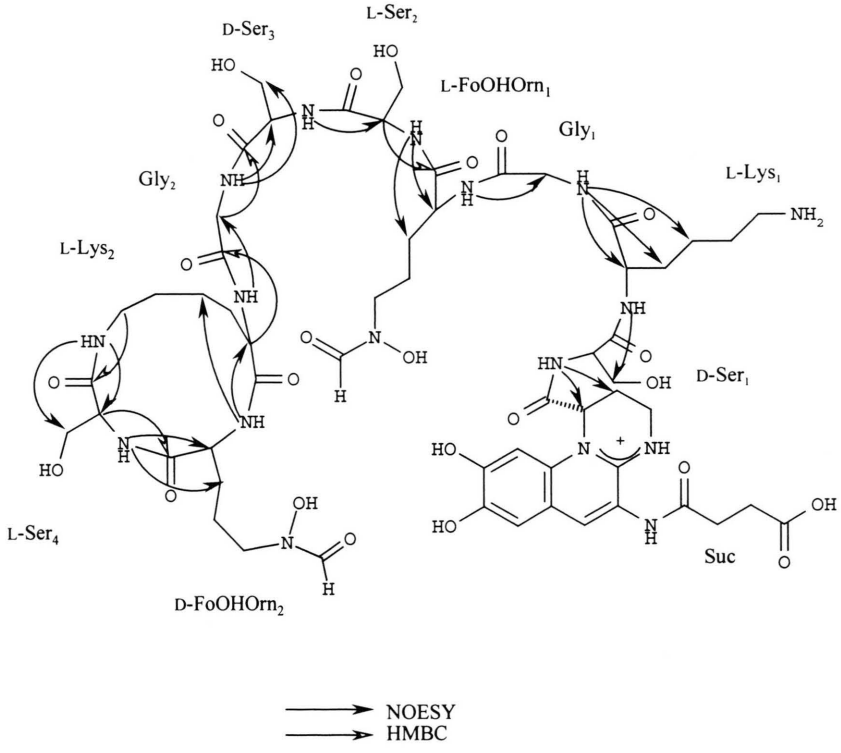


Fig. 1. Sequence specific NMR cross peaks of **1a**.

I and II. They correspond to those observed with other pyoverdins (Budzikiewicz, 1997). The following ones deserve a comment: The NH-signal of Ser^1 bound directly to the carboxyl group of the chromophore is typically shifted downfield. The shift values of the CH_2-groups of all four Ser (3.87–4.01 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected). The *Z/E*-ratio of the formyl protons is 3:1. The signals of the two Lys residues can readily be distinguished. The αNH of Lys^1 couples with β-CH_2 of Ser^1. The shift value of ε-CH_2 and the broad ε-NH_2 signal due to a faster exchange than observed for amide protons is in agreement with a free ε-amino group. The α-NH of Lys^2 couples with Gly^2; the signals of its ε-CH_2 are shifted downfield by about 0.5 ppm suggesting an amide bond in agreement with the observation of cross signals with α-CH and β-CH_2 of Ser^4. The formation of cyclic substructure is reflected in the observed molecular mass (1391 u) calculated for a chromophore with a succinic acid side chain and a peptide chain consisting of 2 Gly, 2 FoOHOrn, 2 Lys and 4 Ser minus 1 H_2O.

Ga(III) complexes can be used to simulate the Fe(III) complexes not amenable to NMR studies (Mohn *et al.*, 1994). Their UV/Vis spectra show the pH independant chromophore absorption maximum at 400 nm also observed for the Fe(III) complex, but no charge transfer band as Ga^{3+} does not have free d-orbitals. The ESI-MS shows $[M - 2H + Ga]^+$ ions at *m/z* 1458 and 1460 (due to ^{69}Ga and ^{71}Ga). The effect on the NMR spectra is a twofold one (the signals were assigned by two-dimensional techniques as discussed before): Differences in the shifts of the ^{13}C resonances as compared with those of the free pyoverdin reflect the influence of the metal ion on the binding sites. Thus, large differences can be noted for the *Z*-formyl groups (−6.1 ppm for $FoOHOrn^1$ and −6.8 ppm for $FoOHOrn^2$) and for the C-atoms carrying the catecholate hydroxyl groups of the chromophore (+7.2 ppm for C-8 and +9.7 ppm for C-9) in agreement with the Ga(III) complexes of other pyoverdins (e.g., Mohn *et al.*, 1994; Voss *et al.*, 1999). The ^1H spectra are influenced by the restricted dynamics of the molecule (only *Z*-formyl signals, non-equivalence of some CH_2-protons) and the fixation above or below the aromatic system of the chromophore (high field shifts) or in the ring plane (low field shifts) (see Table III). Especially remarkable are the protons of the CH_2 group of Gly^2 ; they are not any more equivalent and shifted upfield by 0.82 and 1.67 ppm, respectively. Obviously, the C-terminal cyclopeptide must also be in the vicinity of the chromophore:

Table III. ^1H-NMR data[c] of the peptide chain protons of Ga-**1a** (H_2O/D_2O 9:1, pH 4.3), correlations by TOCSY and NOESY.

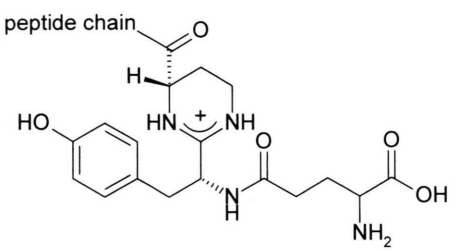
Amino acid	α-NH	α	β	γ	δ	ε	ε-$NH_{(2)}$	CHO_Z	CHO_E
Lys^1	8.44	4.63	1.23[a]	0.55	1.08	2.16	7.40		
			1.92	0.83	1.22	2.23			
Lys^2	7.80	3.81	1.35[a]	*1.07*	1.32	*3.13*	7.46		
			1.90		1.66	*3.23*			
Ser^1	9.68	4.28	4.14						
Ser^2	*8.18*	*5.02*[b]	3.97						
			4.04						
Ser^3	*9.59*	4.34	4.00						
			4.08						
Ser^4	*8.52*	4.40	3.84						
Gly^1	*8.80*	3.97							
		4.22							
Gly^2	8.55	*2.29*							
		3.14							
$FoOHOrn^1$	8.71	*4.75*	*2.29*	1.50	3.58			8.16	
			2.36	1.62	3.99				
$FoOHOrn^2$	7.98	4.27	1.68	1.44	3.46			7.98	
			1.84[a]	1.64[a]	3.72				

[a] Assignment by $^1H,^{13}C$-HSQC in D_2O at 25 °C, [b] Assignment by TOCSY at 25 °C, [c] Shifts of Ga-**1a** differing from those of **1a** by 0.5 ppm and more are printed in *italics*.

Table IV. MS-CA spectrum of **1a**.

Amino acid	n	B_n **1a**	$-H_2O$ **1a**	B_n **3**	$-H_2O$ **3**	Y" **3**	n
Ser	1	445 [a, b]	427				10
Lys	2	573[c]	555	461		948	9
Gly	3	630	612	518		820	8
FoOHOrn	4[a]	788[a]	770[a]	676[a]	658	763	7
Ser	5	875	857	763	745	605	6
Ser	6	962	944	850	832	518	5
Gly	7	1019 [b]	1001	907 [a]		431	4

[a] Also the corresponding A-ion is observed, [b] Also the corresponding C-ion is observed, [c] Also the corresponding Y"-ion is observed.



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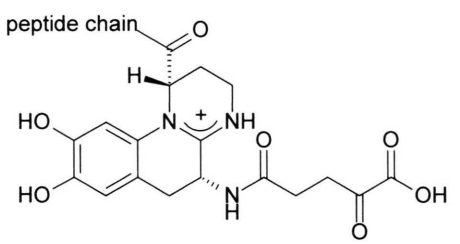

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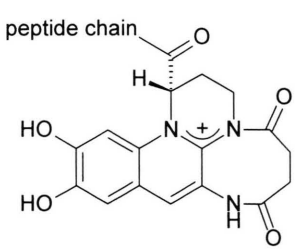

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note the downfield shift of 0.96 ppm of the NH of Ser^4.

The most important fragment ions of a peptide upon collision activation (CA) of $[M + H]^+$ are the so-called B-ions ($RNH\text{-}CHR\text{-}CO^+$ etc.) (Roepstorf and Fohlman, 1984). The whole series accompanied by $[B - H_2O]^+$-ions can be observed up to B_7 (after Gly^2) formed by the loss of the C-terminal cycle; they confirm the sequence determined by NMR. The A ($RNH\text{-}CHR^+$) and C"-ions ($RNHCONH_3^+$) can be seen in addition in some instances (see Table IV).

Localization of the amino acids present both in the D- *and* L-*form*

FoOHOrn and Ser are present in both configurations. After partial hydrolysis the reaction mixture could be divided by chromatography on Biogel P-2 into two fractions. One contained fragments comprising the chromophore and N-terminal amino acids (Chr-Ser^1-Lys^1, Chr-Ser^1-Lys^1-Gly, Chr-Ser^1-Lys^1-Gly-$OHOrn^1$ and Chr-Ser^1-Lys^1-Gly-$OHOrn^1$-Ser^2) with decreasing abundance as determined by FAB-MS. After total hydrolysis of the mixture L-Lys, L-Orn, D-Ser and L-Ser in a ratio of about 10:1 could be detected by GC on a chiral column after TAP derivatization. Hence Ser^1 is D-configurated (sofar for all pyoverdins with Ser bound to the chromophore D-configuration was observed with only one not absolutely convincing exception; for a Table see Kilz *et al.*, 1999) Ser^2 and Orn^1 are L-configurated. The second fraction contained overwhelmingly the C-terminal cycle with admixtures of larger C-terminal peptides (5 to 10%) up to Gly^1. Analysis of the total hydrolysate gave L-Lys, mainly D-Orn (Orn^2) in agreement with the result mentioned above and D- to L-Ser in a ratio of about 1:10. Hence Ser^4 in the cycle is L-configurated. Since Ser^2 is L, Ser^3 must be D. It follows the complete structure **1a**.

Pyoverdin **1b**

Usually several pyoverdins co-occur differing only in the nature of the dicarboxylic acid bound to the chromophore (Budzikiewicz, 1997). As can be seen from Scheme 1, a second pyoverdin with an α-ketoglutaric acid side chain could also be isolated. Its molecular mass amounts to 1419 u. The amino acid analysis gave the same result as **1a** ex-

cept that succinic acid was absent (ketoglutaric acid decomposes under the hydrolysis conditions).

Dihydropyoverdin **2**, *ferribactin* **3** *and succinopyoverdin* **4**

According to the current biogenetic scheme 5,6-dihydropyoverdins and ferribactins are the precursors of pyoverdins (Böckmann *et al.*, 1997); usually they can be found together with the pyoverdins in the fermentation broth and have the same peptide chain as the pyoverdins. A dihydropyoverdin (**2**) with a ketoglutaric acid side chain could be identified by its UV/Vis spectrum (a not pH dependant maximum at 300 nm; the Fe(III) complex shows a maximum at 320 nm and a broad charge-transfer band between 500 and 600 nm) and its molecular mass as determind by FAB-MS of 1421 u (2 units higher than **1b**).

The molecular mass of the ferribactin **3** amounts to 1408 u. In the total hydrolysate of **3** in addition to the amino acids found for **1a** L-Glu (side chain) and L-Dab plus D-Tyr (after alkaline hydrolysis to cleave the tetrahydropyrimidine ring; Taraz *et al.*, 1991) were found. The ^{1}H and ^{13}C NMR spectra confirmed the presence of the same peptide chain in **3** as in **1a** (connectivities were established in the same way as described above for **1a**). As can be seen from Table V the now absent influence of the aromatic pyoverdin chromophore shows its effect on the first four amino acids. It is especially obvious for the NH of Ser which in **1a** is located in the deshielding region of the quinoline system (upfield shift of 1.27 ppm). The chromophore protons obviously are missing, instead those of the Dab/Tyr system and of the Glu side chain can be seen. They conform with literature values. The chemical shifts of the ^{13}C-signals differ from those observed for **1a** by ≤ 0.5 ppm with the exception of Ser^{1} (upfield shift by 1.3 ppm). Therefore, in Table VI only the values for the Dab/Tyr system and for Glu are given.

In the CA-spectrum of **1a** essentially only B- and [B – H_2O]-ions had been observed due to the charge stabilization in the aromatic chromophore. These series with the appropriate mass shifts (Δ 112 u) can also be observed for **3** if the [M – Glu + H]$^{+}$ ion (*m/z* 1280) is collision activated ([M + H]$^{+}$ upon CA yields only ions due to the loss of H_2O and of Glu). For **3** the positive charge can also be stabilized in the C-terminal fragments. Therefore, the series of Y″-fragments (Roepstorf and Fohlman, 1984) starting from the [cyclopeptide-NH-CO-CH_2-NH_3]$^{+}$ ion, (*m/z* 431) are present with comparable abundance. Both series (Table IV) confirm the peptide sequence.

The structure of the succinopyoverdin **4** with a new chromophore was described elsewhere (Lenz *et al.*, 2000).

Table V. ^{1}H NMR data[b] of **3** (δ[ppm]; H_2O 5.1 ppm; H_2O/D_2O 9:1, pH 4.3), correlations by TOCSY and NOESY.

Amino acid	α-NH	α	β	γ	δ	ε	ε-$NH_{(2)}$	CHO_Z	CHO_E
Lys^{1}	8.87	4.44	1.77[a] 1.91	1.45	1.68[a]	3.01	n.o.[b]		
Lys^{2}	8.10	4.27	1.64 1.94	0.97 1.36	1.54	3.19 3.33	7.48		
Ser^{1}	8.56	4.55	3.93						
Ser^{2}	8.82	4.55	3.96						
Ser^{3}	8.67	4.49	3.93						
Ser^{4}	9.08	4.39	3.93						
Gly^{1} + Gly^{2}	8.63	3.96							
FoOHOrn^{1}	8.54	4.39	1.73[a] 1.71[a]	1.70[a]	3.57_Z 3.60_E			7.95	8.29
FoOHOrn^{2}	8.31	4.40	1.64[a] 1.71[a]	1.63[a]	3.57_Z[a] 3.60_E[a]			7.95	8.29
Glu	n.o.	3.71	2.48						
Dab	n.o.	4.47	2.11 2.15[a]	3.31 3.37					
Tyr	8.81	4.71	3.10 3.17	7.18[c]	6.87[d]				

[a] Assignement by ^{1}H, ^{13}C-HSQC in D_2O at 25 °C. [b] not observed. [c] H2/6. [d] H3/5.

Table VI. Partial ^{13}C NMR data (δ [ppm]; Tyr C3/5 116.8 ppm) of **3** (D_2O, pH 4.3; 25 °C)[a].

Tyr	α	β	C-1	C-2/6	C-3/5	C-4	C=N
	54.9	37.4	127.2	131.8	116.8	156.1	164.4
Glu, Dab	CO-α	α	β	γ	CO-γ		
Glu	175.2	55.0	26.9	31.9	175.2		
Dab	172.1	55.2	22.1	37.4			

Table VII. Growth stimulation tests.

Strain	Pyoverdin isoforms (pI)[1]	Growth promotion with the pyoverdin of the strain[2]			
		P. f. 18.1	P. a. 15692	P. f. 13525	P. f. 12
P. f. 18.1	8.5, 7.4, 7.1	++	+	±	++
P. a. 15692	8.8, 7.0	++	++	++	–
P. f. 13525	8.7, 7.3, 7.1	–	+	++	–
P. f. 12	7.1, 4.9, 4.8	+	–	±	++

[1] Isoelectric pH values, [2] Culture diameters: (++) = 15 mm, (+) < 15 mm, (±) < 15 mm with slight bacterial growth, (–) no growth stimulation.

Growth stimulation tests

P. fluorescens 18.1 was tested for growth promotion by addition of a number of structurally known pyoverdins. The pyoverdins from the three strains mentioned in the introduction were active, though with different degrees of effectiveness (see Table VII). All four pyoverdins show clearly different isoelectrofocussing patterns as it was to be expected for the different structures.

Discussion

The earlier observation that *P. aeruginosa* PAO (= ATCC 15692) accepts the ferri-pyoverdin from *P. fluorescens* ATCC 13525 (Kinzel *et al.*, 1998) had suggested that the C-terminal cyclic substructure might play the crucial role for the recognition at the cell surface. Cross feeding experiments (Table VII) show that at least partial recognition of the foreign pyoverdins among the four strains is observed. Two points become, however, evident: The compatibility is not strictly reciprocal (a similar observation had been made with pyoverdins not containing a cyclopeptidic substructure; Georgias *et al.*, 1999) and an identical C-terminal cyclopeptidic substructure is definitely not sufficient, as the pair *P. f.* 13525 and *P. f.* 18.1 show. The most important corollary of the results reported here and by Georgias *et al.* (1999) is that positive growth promotion tests are not sufficient to establish the identity of strains with respect of the production of identical pyoverdins.

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