The Pyoverdin of *Pseudomonas fluorescens* G173, a Novel Structural Type Accompanied by Unexpected Natural Derivatives of the Corresponding Ferribactin

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The siderophores produced by *Pseudomonas fluorescens* G173 are unusual in several respects. So far all pyoverdins with a C-terminal cyclopeptidic substructure have in common that the ε-amino group of an in-chain Lys is bound amidically to the carboxyl group of a C-terminal Ser or Thr and that N^5-formyl-N^5-hydroxy Orn (FoOHOrn) is the next amino acid after Lys. FoOHOrn may (cyclotetrapeptidic structures) be or may not (cyclotripeptidic structures) be followed by a further amino acid. In the pyoverdin described here Orn instead of Lys is the amino acid forming the cycle, FoOHOrn is replaced by AcOHOrn which does not follow the branching Orn but is the penultimate amino acid and finally the last amino acid is Asp. The producing strain which had been classified as *Pseudomonas fluorescens* may well be a new species.

Pyoverdins are frequently accompanied by ferribactins which are considered to be their biogenetic precursors. They always have the same amino acid chain as the co-occurring pyoverdins but the pyoverdin chromophore is replaced by a condensation product of L-Dab and D-Tyr with the amino group of Tyr bound to the γ-carboxyl group of Glu. A ferribactin having these structural characteristics is produced by the investigated strain, but it is accompanied by derivatives where the α-amino group of Glu is partially or completely transformed into a hydroxamic acid by substitution with a hydroxyl and/or acetyl group.

Key words: Pseudomonas fluorescens, Pyoverdin, Ferribactin

Introduction

Pyoverdins are the typical siderophores of the fluorescent members of the bacterial genus *Pseudomonas sensu stricto* (rRNA homology group I according to the original classification; Palleroni, 1984, 1992). They consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6 to 12 amino acids bound to its carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to the NH_2-group (cf. **1a**). So far the structures of over fifty pyoverdins differing in their peptide chains have been elucidated and recurring patterns could be recognized. Thus the second largest subgroup (Fuchs and Budzikiewicz, 2001a) is characterized by a cylopeptidic C-terminus comprising three or four amino acids. The cycle is formed by an amide bond between the ε-amino group of Lys and the carboxyl group of a C-terminal Ser or Thr, and the amino acid following Lys is always FoOHOrn. The C-terminal cycle of the pyoverdin from strain G173 differs from this pattern in two ways: Lys is replaced by Orn and FoOHOrn by AcOHOrn which in addition does not follow immediately upon Orn. *P. fluorescens* is an ill-defined conglomerate of saprophytic strains which currently is

Abbreviations: Common amino acids, 3-letter code; Dab, 2,4-diaminobutyric acid; Ac(Fo)OHOrn, N^5-acetyl(formyl)-N^5-hydroxy Orn; Chr, pyoverdin chromophore; Mala, malamide residue; Suca, succinamide 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; CA, collision activation; 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 CXII of the series "Bacterial Constituents". For Part CXI see Budzikiewicz *et al.* (2002).

being broken up into a number of newly defined species. Characterization by the pyoverdins produced plays an increasingly important role (Meyer *et al.*, 2002); G173 might well be a candidate for reclassification.

Pyoverdins are frequently accompanied by ferribactins which are considered to be their biogenetic precursors (Hohlneicher *et al.*, 2001). Ferribactins have the same peptide chain as the co-occurring pyoverdins, but the pyoverdin chromophore is replaced by a condensation product of D-Tyr and L-Dab with Glu bound by its γ-carboxyl group to the amino group of Tyr. Ferribactins lack the catecholate system of the pyoverdin chromophore. They have only two bidentate ligands available for the complexation of Fe^{3+} (Taraz *et al.*, 2000). For the ferribactin produced by the strain G173 accompanying compounds were found where the α-amino group of Glu is transformed into a hydroxamic acid, otherwise a typical ligand of the peptide chains.

Materials and Methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT 900 ST (ESI); GC/MS Incos 500 (both Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400. CA experiments were either performed in the ion trap or in the octapole region in front of the ion trap. The results differ due to the different residence time of the ions and due to the different amounts of energy transferred (Fuchs and Budzikiewicz, 2001a).

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.

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

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 C-25 and DEAE-Sephadex A-25 (Pharmacia, Uppsala, S), SepPak RP-18 cartridges (Waters, Milford MA, USA); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt).

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

Bacterial strain: The strain G173, phenotypically identified as a *Pseudomonas fluorescens* biovar 3, belongs to a collection of 32 rhizobacteria isolated by the Laboratoire de Pathologie Végétale, INRA-Beaucouzé, France, and studied for their pyoverdin-siderotyping behavior (Fuchs *et al.*, 2002). Compared with the other strains of the group mentioned above and with 36 other *Pseudomonas* strains of the Strasbourg collection producing different well defined pyoverdins only G173 and G174 showed the same unique siderotyping features. A structural study seemed therefore warranted.

Results

Siderotyping

The pyoverdin-isoelectrophoretic pattern of strain G173 is characterized by three isoform-bands at pH_i 5.3, 4.7 and 4.1, respectively. The pyoverdin-mediated iron uptake capacity is not strictly specific to its own pyoverdin, but limited to a few pyoverdins each with a cyclic substructure in the peptidic part of the molecule. However, a much lower efficiency for pyoverdins of foreign origin was observed with a maximal value of 52% efficiency for the pyoverdin of *P. fluorescens* ATCC 13525 (Hohlneicher *et al.*, 1995). None of 28 pyoverdins with linear peptide chains was accepted by strain G173. This behavior is in agreement with the structural features described below for the G173 pyoverdin, i. e., the presence of Asp in the peptide chain responsible for the acidic pH_i values, and the presence of a cyclic substructure which is in agreement with a partial uptake specificity, as already described for other cyclic pyoverdins (Meyer *et al.*, 1998; Amman *et al.*, 2000; Weber *et al.*, 2000).

Production, isolation and derivatisation of the siderophores

For the growth of *Pseudomonas fluorescens* G173 on a succinate minimal medium, work-up of the culture medium, adsorption of the Fe^{3+} complexes after addition of Fe^{3+} citrate by chromatography on XAD and Biogel columns see Georgias *et al.* (1999). The only difference in work-up was that for the desorption of the ferri-siderophores from the XAD column a mixture of CH_3OH, H_2O and CH_3COOH 70:29:1 (v/v) was used. Desorp-

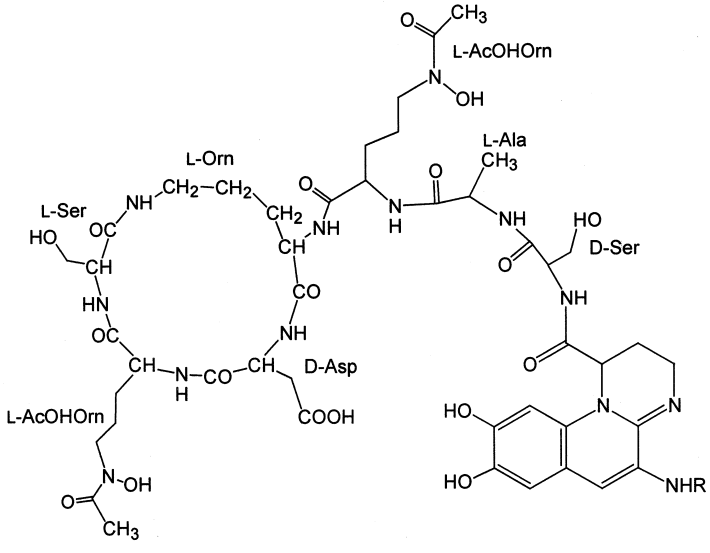
tion from the Biogel column with 0.2 M pyridinium acetate buffer (pH 5.0) gave a ferri-pyoverdin and a ferri-ferribactin fraction. The ferri-pyoverdin fraction was brought onto a Sephadex column and rechromatographed with the same buffer. In this way two compounds differing in the side chain (ferri-**1a** and -**1b**) could be obtained which were decomplexed after adsorption on a SepPak cartridge with a 6.5% aqueous oxalate solution, and subsequent rinsing with H_2O and desorption with CH_3OH/H_2O 3:1 (v/v). The purity was checked by HPLC. The ferri-ferribactin fraction was decomplexed with 8-hydroxyquinoline (Briskot *et al.*, 1986) and rechromatographed on Biogel. Cultures producing mainly ferribactins were worked up in the same way. For 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). Partial hydrolysis was performed with 6N HCl at 90 °C for 2 h. For experimental details see Schlegel *et al.* (2001).

Characterization of **1a**

1a (Fig. 1) and its Fe^{3+}-complex gave the characteristic UV/Vis spectra of pyoverdins (Budzikiewicz, 1997). The molecular mass of **1a** as determined by ESI-MS amounts to 1174u, a *retro*-Diels-Alder-fragment of the chromophore in the octapole-CA spectrum at *m/z* 204 resulting in the loss of C-1 + C-2 together with the peptide chain (Fuchs and Budzikiewicz, 2001a) confirms the presence of pyoverdin chromophore. Amino acid analysis after total hydrolysis showed the presence of D-Ala, D-Asp, L-Orn, D- and L-Ser and succinic acid. After partial hydrolysis (for details see Schlegel *et al.*, 2001) fractions could be isolated which contained the chromophore and only one Ser. After total hydrolysis of these fractions the presence of D-Ser could be shown by GC/MS analysis on a chiral column.

Sequence determination by NMR and MS

Basis for the sequence determination is the unambiguous identification of all ^1H- 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 (^1J) C,H connections can be determined by a HMQC experiment, quaternary C-atoms can be identified with HMBC optimized for ^2J- and ^3J-coupling, CH-, CH_2- and CH_3-groups by DEPT. Sequence information is obtained by NOESY/ROESY which allows a correlation of an NH-proton with spatially close α- and



1a: R = $COCH_2CH_2CONH_2$
2a: R = $COCH_2CHOHCONH_2$

Fig 1. Pyoverdins G 173.

Table I. ^{1}H-NMR data of **1a** (H_2O/D_2O 9:1, pH 4.3, 25 °C), correlations by TOCSY and NOESY.

Suca	CH_2	CH_2								
	2.79	2.73								
Chr	1	2a	2b	3a	3b	$4NH^+$	6	7	10	5-NH
	5.71	2.47	2.67	3.41	3.74	*	7.94	7.16	7.10	8.69
Amino acid	α-NH	α	ß	γ	δ	δ-NH	CH_3CO			
Ser[1]	9.38	4.45	3.97							
Ala	8.71	4.37	1.37							
AcOHOrn[1]	8.05	4.03	1.95	1.22	3.43		2.01			
Orn	7.83	3.95	2.12	1.69	3.22	7.43				
Ser[2]	8.42	4.32	3.89							
AcOHOrn[2]	8.46	4.29	1.71	1.41	3.64		2.08			
Asp	8.03	4.52	2.68							

* Not observed.

Table II. ^{13}C-NMR data (δ [ppm]) of **1a** (H_2O/D_2O 9:1 v/v, pH 4.3; 25 °C); based on HMBC and HSQC spectra.

Suca	CO	CH_2	CH_2	$CONH_2$			
	176.4	31.9	31.1	177.3			
Chr	1-CO	1	2	3	4a	5	6
		57.9	23.1	36.2	150.6	118.4	140.2
	6a	7	8	9	10	10a	
	116.1	115.3	145.1	153.0	101.4	113.0	
Amino acid	CO	α	β	γ	δ	CH_3CO	
Ser[1]	172.7	57.6	62.1				
Ala	176.2	50.8	17.2				
AcOHOrn[1]	173.8	54.9	28.1	23.1	28.0	170.2	
Orn	174.5	55.8	29.1	23.5	39.7		
Ser[2]	172.8	57.8	61.5				
AcOHOrn[2]	174.4	54.6	28.5	23.4	48.5	170.2	
Asp	175.3	53.5	38.6	177.8			

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. 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, 1997). The following ones deserve a comment: The NH-signal of Ser[1] bound directly to the carboxyl group of the chromophore (9.38 ppm) is typically shifted downfield. The shift values of the CH_2-groups of the two Ser (3.96 and 3.89 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected). The signals for the *Z/E*-isomers of the N-acetyl groups of the two AcOHOrn units coincide; this had been observed before (Jacques *et al.*, 1995). The signals of the Orn residue incorporated into the cyclic substructure correspond to those of Lys in an analogous position. The α-NH couples with AcOHOrn[1] and the signal of its δ-CH_2 at 3.22 ppm suggests an amide bond in agreement with the observation of cross signals with α-CH and β-CH_2 of Ser[2]. The formation of cyclic substructure is reflected in the observed molecular mass (1174 u) calculated for a chromophore with a succinamide side chain and a peptide chain consisting of Ala, Asp, Orn, 2 AcOHOrn, and 2 Ser minus 1 H_2O.

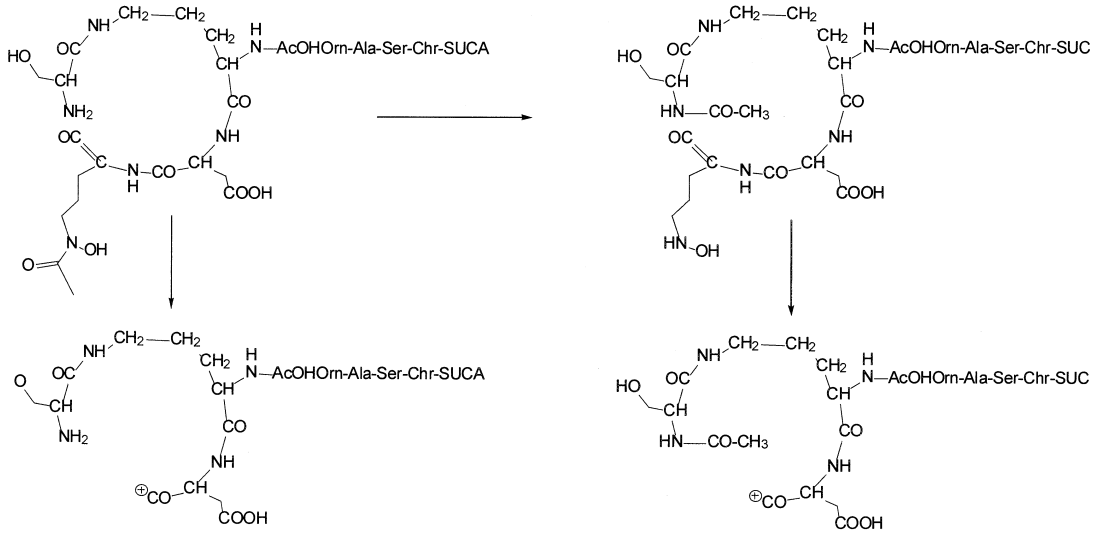
Owing to the new structural type of **1a** the mass spectral analysis deserves a more detailled discussion. The most important fragment ions of a peptide upon collision activation (CA) of $[M+2H]^{2+}$ are the N-terminal A- (~ NH-CHR$^+$) and B- (~ NH-CHR-CO$^+$) and the C-terminal Y″-ions (~ CO-CHR′-NH$_3^+$) (Roepstorff and Fohlman, 1984) (CA of the doubly protonated molecular ions gives a larger number of sequence-characteristic fragments, especially from pyoverdins with a cyclic substructure (Fuchs and Budzikiewicz, 2001a). In the octapole-CA spectrum pyoverdins show an abundant A_1^+ ion the mass of which for **1a** (*m/z* 416) is in agreement with Ser as the first amino acid and Suca as side chain; it shows consecutive loss of the Suca (*m/z* 317) and of Ser residues (*m/z* 204) (Fuchs and Budzikiewicz, 2001a). As had been observed for other pyoverdins with a C-terminal cyclic substructure, **1a** upon trap-CA of $[M+2H]^{2+}$ gives B_2^+ to B_4^+ (branching amino acid) and ions where parts of the cycle are attached to B_4 (Fuchs and Budzikiewicz, 2000), as well as the complete cycle (Y_4'', *m/z* 489). B_4 upon further CA loses consecutively Orn, AcOHOrn and Ala thus giving the ions B_3 to B_1. In the same way Y_4'' can be activated to lose parts of the ring (see Table III). All fragment ions show in addition loss of H_2O (not listed in Table III). Ions containing an AcOHOrn unit can lose CH_2CO (−42u) and/or $C_2H_7NO_2$ (− 77 u). More interesting is the migration of a CH_3CO group with back transfer of H which leads to the ions $[B_4 + Ser + CH_2CO]^+$ (*m/z* 930) and $[B_4 + Ser + Asp + CH_2CO]^+$ (*m/z* 1045) in the trap-CA spectrum of $[M+2H]^{2+}$. Such acyl migrations (CHO from FoOHOrn and CH_3CO from AcOHOrn) have been observed when a free ε-amino group of Lys can act as an acceptor (Fuchs and Budzikiewicz, 2001b). However, an amino acid with a free amino group is not present in **1a**. Yet, formation of $[B_4 + Ser]^+$ (*m/z* 888) and of $[B_4 + Ser + Asp]^+$ (*m/z* 1003) requires as a first step cleavage of the Ser-AcOHOrn peptide bond accompanied by an H-transfer to form a Ser-NH$_2$ group, followed by cleavage of the AcOHOrn-Asp and the Asp-Orn peptide bonds, respectively (formation of B-ions) (Scheme 1). The newly formed Ser-NH$_2$ group may then be the acceptor for the migrating acetyl group. **1a** is the first example with AcOHOrn in a C-terminal cyclic substructure, but it exists a number of pyoverdins with FoOHOrn in the cycle. For these compounds corresponding fragments are observed which could be formed by an analogous formyl transfer (+ CO) (Fuchs, 2000), but an alternative genesis could not be excluded, initiated by a cleavage of the CO-CHR bond instead of the NH-CO bond between Ser and FoOHOrn. The results obtained with **1a** favor the transfer mechanism also for FoOHOrn containing pyoverdins.

Two additional observations complete the MS structural studies. The high abundance of the B_3-ion is in agreement that Orn is bound with its α-NH$_2$ group in the peptide chain and incorporated with δ-NH$_2$ group into the cycle (binding with the δ-NH$_2$ group to AcOHOrn in the linear part of the molecule would make the formation of B_3 less likely: B-ion formation in normal peptides is fostered by nucleophilic attack of the CO group of the preceding amino acid yielding a five membered cycle (Schlosser and Lehmann, 2000). This would not be possible due to the larger distance if Orn were bound by its δ-amino group to AcOHOrn (cf. analogous observations with Lys bound with its ε-amino group, Fuchs and Budzikiewicz, 2000). The observation of an ion formed by the partial loss of AcOHOrn from the cycle, viz. $[H_2N$-Ser-(Orn ~ Chr-Suca)-Asp-CONH$_3]^{2+}$ which upon CA loses (HN-Asp-CONH$_3$) confirms the C-terminal sequence in the cycle Orn-Asp-AcOHOrn-Ser distinguishing it from the *retro*-sequence Orn-Ser-AcOHOrn-Asp where the corresponding ion $[H_2N$-Asp-(Orn ~ Chr-Suca)-Ser-CONH$_3]^{2+}$ would have lost HN-Ser-CONH$_3$). For a detailled discussion see Fuchs and Budzikiewicz, 2001a.

Table III. Structure relevant ions obtained from **1a** after CA of $[M+2H]^{2+}$ (nominal masses).

Ion	Mass	Ion	Mass
B_1[a]	444	Y_5''	661
B_2	515	Y_4''	489
B_3	687	Y_4-AcOHOrn[b]	317
B_4	801	Y_4-AcOHOrn-Ser[b]	230
B_4 + Ser	888	Y_4-Orn[b]	375
B_4 + Asp	916		
B_4 + Ser + Asp	1003		

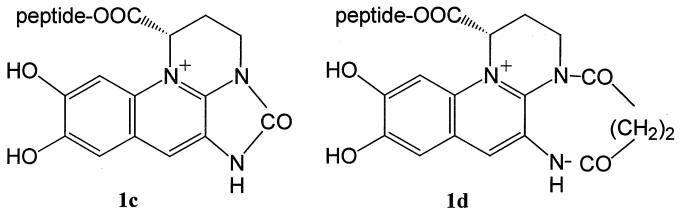
[a] Obtained after CA of B_4.
[b] Obtained after CA of Y_4''.

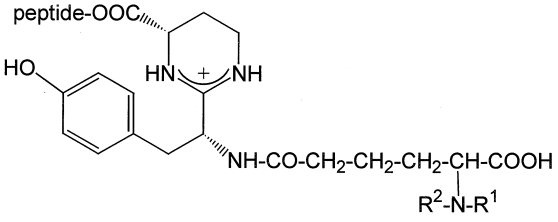


Scheme 1. Formation of the ions $[B_4+Ser]^+$ and $[B_4 + Ser + CH_2CO]^+$.

Related compounds

From an analysis of the ESI-CA (octapole and trap) spectra of the $[M+2H]^{2+}$ ions from the crude XAD extract at *m/z* 596.3, 579.7 and 551.7 the presence of the following compounds can be deduced: a pyoverdin with a Mala side chain (**1b**) and congeners with an azotobactin **1c** (cf. Hohlneicher *et al.*, 1995) and a succinopyoverdin **1d** (cf. Lenz *et al.*, 2000) chromophore (Fig. 2). All three show the same characteristic ions of the peptide part, N-terminal ions shifted in mass according to the respective chromophores.





2a: $R^1 = R^2 = H$
2b: $R^1 = OH$, $R^2 = H$
2c: $R^1 = H$, $R^2 = COCH_3$
2d: $R^1 = R^2 = COCH_3$

Fig. 2. Azotobactin (**1c**), succinopyoverdin (**1d**), and ferribactins (**2a–2d**) accompanying the pyoverdin G173.

Ferribactins

Ferribactins are the biosynthetic precursors of the pyoverdins (Böckmann *et al.*, 1997; Hohlneicher *et al.*, 2001). Depending on the bacterial strain under investigation and on the culture conditions they may or may not be found in the culture medium together with the pyoverdins and their congeners. Their chromophore is a condensation product of L-Dab with D-Tyr giving a tetrahydropyrimidine ring. The Tyr-NH_2 group is bound amidically to the γ-carboxyl group of L-Glu (see **2a**). The peptide part is identical with that of the corresponding pyoverdin. Accordingly, the molecular mass of **2a** was determined as 1192u. The amino acid analysis in agreement with that of **1a** gave D-Ala, D-Asp, L-Orn, D- and L-Ser and in addition L-Glu. The ^{1}H- and ^{13}C-shift values of the chromophore part correspond to those found in the literature (e.g., Amann *et al.*, 2000). The ^{1}H-shifts of the peptide part (Table IV) differ in some instances from those given in Table I for **1a**, probably due to differences in (de)shielding effects of the different chromophores. The influence on the ^{13}C-shifts is less pronounced; they will, therefore, not be reported. Sequence relevant connectivities were determined by two-dimensional NMR techniques as for **1a**.

So far when present in the culture medium the ferribactin had always the structural characteristics described above for **2a**. *Pseudomonas fluorescens* G173 shows a drastically different behavior. While the strain usually produced only the pyoverdins **1a** and **1b** etc., accumulation of **2a** and its novel derivatives together with hardly any pyoverdins occurred occasionally under not clearly understood circumstances. Defined variations of culture and growth parameters did not give an answer.

Fig. 3 shows the singly charged molecular ion region obtained by ESI of the culture extract, where in addition to **2a** ($[M+H]^+$ *m/z* 1193) a whole series of compounds can be seen. Octapole-CA of $[M+2H]^{2+}$ of **2a** (*m/z* 597) yields A_1 and ions due to the loss of H_2O and of Glu (see Table V). In Fig. 2 (trap-CA) in analogy to the fragments obtained from **1a** the series of sequence relevant ions is extant. Confirmation is obtained by CA of the $[M-Glu+2H]^{2+}$ fragment ion. CA of the other molecular species allows to recognize where structural modifications had occurred.

The first astonishing result is that the free NH_2 group of Glu is transformed into a hydroxamic acid as present in the two AcOHOrn residues. Both the -NHOH (+ 16, **2b**) and the -$NHCOCH_3$ derivative (+ 42, **2c**) are formed. The A- and B-ions containing the modified Glu are shifted accordingly, while the Y-ions and those where the modified Glu is lost have the same mass as in the spectrum of **2a** (see Table V). The complete hydroxamic acid **2d** is also present ($[M+H]^+$ *m/z* 1251 in Fig. 1), but its mass coincides with the $^{13}C_2$-satellite of *m/z* 1249 and therefore a mixture spectrum is obtained by CA. Several abundant ions are, however, clearly discernible in the CA spectrum of $[M+H]^+$ as (cf. Table V) $[M+H-Glu]^+$ (*m/z* 1064) showing that the modifications had occurred only in the Glu side chain, A_1 (*m/z* 492), B_3 (*m/z* 763) and B_4 (*m/z* 877) each with a mass increase of 58u, while A_1-Glu occurs at *m/z* 305 (loss of the mass increment).

The second set of derivatives comprises compounds formed by addition of a CH_2-group (+ 14 u). Such species were mentioned twice in the literature for pyoverdins, viz. the formation of the methyl ester of a succinic acid side chain as an artifact during work-up (Demange *et al.*, 1990) and the possible replacement of the formyl by an ace-

Table IV. ^{1}H-NMR data of **2a** (H_2O/D_2O 9:1 v/v, pH 4.3, 25 °C), correlations by TOCSY and NOESY.

Amino acid	α-NH	α	β	γ	δ	δ-NH	CH_3CO
Ser[1]	9.03	4.44	3.87				
Ala	8.67	4.34	1.41				
AcOHOrn[1]	8.47	4.52	2.04	1.68	3.12		2.08
Orn	8.39	3.89	2.19	1.66	3.25	7.53	
Ser[2]	8.75	4.32	3.90				
AcOHOrn[2]	8.51	4.34	1.81	1.67	3.31		2.08
Asp	8.23	4.63	2.91				

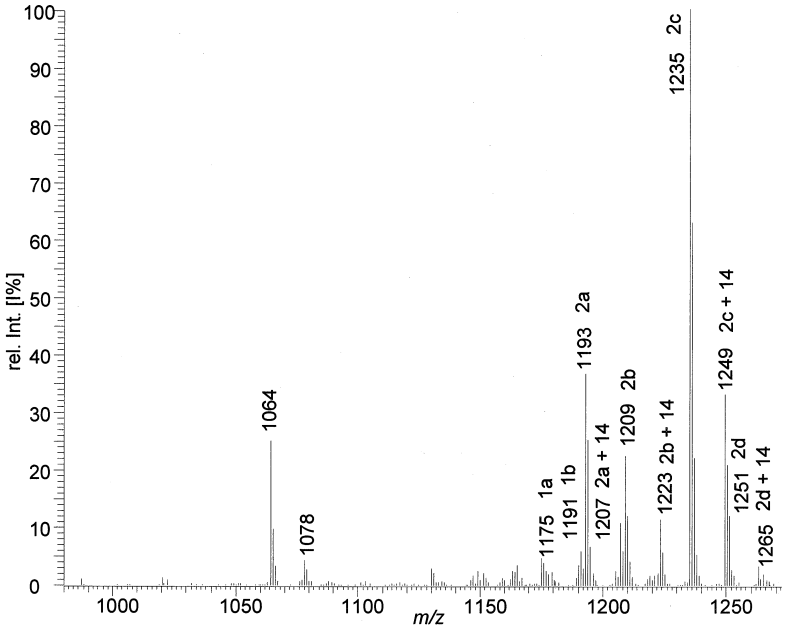


Fig. 3. Singly charged molecular ion region ($[M+H]^+$ ions) of *Pseudomonas fluorescens* G173. The numbers 1a, 1b, 2a-2d refer to the structural formulas of the pyoverdins and ferribactins. For *m/z* 1064 and 1078 see text (loss of Glu or modified Glu).

tyl group in FoOHOrn (Kilz *et al.*, 1999). Addition of the CH_2 unit can apparently occur at two different parts of the ferribactin molecule. Form the mass shifts of the main part of ″**2a** + 14″ follows that the additional CH_2 group must be located in the C-terminal cycle (no shift of A_1 and B_3, shift of Y_4″). From the four amino acids making up the cycle Orn and Ser can be excluded (no shift of B_4 and B_4 + Ser, shift of Y_4″-Orn and Y_4″-Orn-Ser). It must be present in Asp (shift of B_4 + Asp and of B_4 + Ser + Asp). Accordingly the [M-Glu]-ion is shifted to *m/z* 1078.

From the two alternatives, replacement of Asp by Glu and formation of a Asp methyl ester the second one is more likely since *Pseudomonas* spp. are very conservative regarding the incorporation of amino acids into the peptide part, though recently examples were found where in a minor

Table V. Structure relevant ions obtained from **2a** and from its derivatives after CA of the respective $[M + 2H]^{2+}$ ions (A-ions in the octapole, otherwise in the trap; all masses are nominal masses).

Ion/Comp.	**2a**	**2a**-Glu	**2b** (**2a** + 16)	**2c** (**2a** + 42)	**2a** + 14	**2b** + 14	**2c** + 14
$[M + H]^+$	1193	1064	1209	1235	1207	1223	1249
A_1	434		450	476	434	450	476/490
A_1-H_2O	416		432	458	416	432	458/472
A_1-Glu[a]	305		305	305	305	305	305
B_3	705	576	721	747	705[b]	721[b]	747/761
B_4	819	690	835	861	819[b]	835	861/875
B_4 + Ser	906	777	922	948	906[b]	922[b]	948/962
B_4 + Asp	934	805	950	976	948	964	990
B_4 + Ser + Asp	1021	892	1037	1063	1035	1051	1077
Y_5″	661	661	661	661	675	675	661/675
Y_4″	489	489	489	489	503[c]	503[c]	489/503
Y_4″-Orn	375	375	375	375	389	389	375/385
Y_4″-Orn-Ser	288	288	288	288	302	302	288/302
$[M + 2H\text{-Glu}]^{2+}$	532.5		532.5	532.5	539.5	539.5	532.5, 539.5

[a] Glu or modified Glu; [b] with a satellite + 14 u of low abundance, [c] with a satellite – 14 u of low abundance.

component of the mixture of siderophores the Ala of a pyoverdin is replaced by Gly (Barelmann *et al.*, 2002). The same conclusion can be reached for ″**2b** + 14″. ″**2c** + 14″ is an almost equal mixture of two components. One of them again carries the additional CH_2 group in the Asp, the other one in the N-acetyl Glu unit: ions containing acetyl-Glu, but not Asp (A_1, B_3, B_4, B_4 + Ser) occur with and without the 14u shift, A_1-acetyl-Glu is not shifted. The ions containing Asp, but not acetyl-Glu (the Y″-ions) occur with and without 14 u shift, while the ions containing both Asp and acetyl-Glu (B_4 + Asp and B_4 + Ser + Asp) are found only to be shifted by 14 u. Methylation of the Glu unit has occurred to a small extent also in the case of ″**2a** + 14″ and of ″**2b** + 14″, but the additional signals can only be seen for abundant ions (see Table V).

Discussion

From the about fifty pyoverdins for which complete or fairly complete structures have been established circa one fourth is characterized by a C-terminal cyclic substructure comprising three or four amino acids. In every case the cycle is formed by condensation of the ε-NH_2 group of Lys with the carboxyl group of a C-terminal Ser (only in one case Thr) and it contains FoOHOrn as one of the complexing sites for Fe^{3+}. In the pyoverdin of *Pseudomonas fluorescens* G173 Lys is replaced by Orn and FoOHOrn by AcOHOrn. While in the pyoverdins with a tetrapeptidic cycle FoOHOrn always follows in row the branching Lys, in the present case AcOHOrn is the penultimate amino acid. The pyoverdin G173 constitutes therefore a new structural variety. In this context it is worth noting that the fragmentation rules allowing structural assignments from the ESI-CA mass spectra which had been developed for cyclic substructures with Lys as the branching amino acid, were found to be valid also for **1a**.

More interesting is the behavior of the bacterial strain when producing mainly ferribactin. Ferribactins are much less efficient siderophores then pyoverdins possessing only two bidental ligands (Taraz *et al.*, 2000). The derivatisation of the free amino group of Glu which has not been observed before, could be interpreted as an attempt of the bacterium to create a siderophore with three ligand sites necessary for an efficient binding of Fe^{3+} (**2d**). The fact that the precursors **2b** and **2c** are also present in the culture extract suggests that **2a** is transformed. This would however be in contrast with earlier data which indicate that the FoOHOrn units are preformed and incorporated as such into the peptide chain during the pyoverdin biosynthesis (Visca *et al.*, 1992).

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