Bacterial Siderophores: Structures of Pyoverdins Pt, Siderophores of *Pseudomonas tolaasii* NCPPB 2192, and Pyoverdins Pf, Siderophores of *Pseudomonas fluorescens* CCM 2798. Identification of an Unusual Natural Amino Acid†

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ABSTRACT: Pyoverdins were isolated and characterized respectively from the cultures of *Pseudomonas tolaasii* NCPPB 2192 (pyoverdins Pt, Pt A, and Pt B) and *Pseudomonas fluorescens* CCM 2798 (Pyoverdins Pf/1, Pf/2, Pf, Pf/3/1, and Pf/3/2) each grown in iron-deficient conditions. Their structures were established by using FAB-MS, NMR, and CD techniques. These siderophores are chromopeptides, and all but one (pyoverdin Pf/3/3) possess at the N-terminal end of their peptide chain the same chromophore that has been reported in pyoverdin Pa from *Pseudomonas aeruginosa* ATCC 15692 [Wendenbaum, S., Demange, P., Dell, A., Meyer, J. M., & Abdallah, M. A. (1983) *Tetrahedron Lett. 24*, 4877–4880] and pseudobactin B 10 from *Pseudomonas* B10 [Teintze, M., Hossain, M. B., Barnes, C. L., Leong, J., & Van der Helm, D. (1981) *Biochemistry 20*, 6446–6457] which is derived from 2,3-diamino-6,7-dihydroxyquinoline. In pyoverdins Pt this chromophore is bound to a linear peptide chain D-Ser-L-Lys-L-Ser-D-Ser-L-Thr-D-Ser-L-OHOrn-L-Thr-D-Ser-D-OHOrn(cyclic) which has its C-terminal end blocked by cyclic D-N^{δ}-hydroxyornithine. In pyoverdins Pf, the peptide chain is also linear, SerCTHPMD-Gly-L-Ser-D-*threo*-OHAsp-L-Ala-Gly-D-Ala-Gly-L-OHOrn(cyclic), and contains an unusual natural amino acid which is the result of the condensation of 1 mol of serine and 1 mol of 2,4-diaminobutyric acid, forming a cyclic amidine. The pyoverdins Pt differ only in substituent bound to the nitrogen on C-3 of the chromophore, which is succinic acid in pyoverdin Pt A, succinamide in pyoverdin Pt, and α-ketoglutaric acid bound to the chromophore by its C-5 carbon atom in pyoverdin Pt B. Similarly, pyoverdin Pf/1, pyoverdin Pf/2, pyoverdin Pf (the major compound), and pyoverdin Pf/3/2 are substituted respectively by L-malic acid, succinic acid, L-malic amide, and succinamide. Pyoverdin Pf/3/3 has the same chromophore as azotobactin, the peptidic siderophore of *Azotobacter vinelandii*. These pyoverdins are very similar to pseudobactin B 10, the siderophore of *Pseudomonas* B10: they are linear peptides containing three bidentate groups strongly chelating Fe(III) and blocked at their N-terminal end by the catecholic chromophore and at their C-terminal end by cyclic N^{δ}-hydroxyornithine. They differ therefore from other pyoverdins such as those from *P. aeruginosa* ATCC 15692 which contain a partly cyclic peptide [Briskot, G., Taraz, K., & Budzikiewicz, H. (1989) *Liebigs Ann. Chem.*, 375–384].

When microorganisms are grown in iron-deficient conditions, they produce small molecules called siderophores which transport this element into their cells by a high-affinity transport system (Neilands, 1974, 1981, 1984). These iron carriers give very stable octahedral complexes with Fe(III) (Raymond & Carrano, 1979). Some bacteria belonging to the *Pseudomonas fluorescens* or *Pseudomonas putida* strains are well-known to have an effect on the growth of plants. These bacteria, which are located in the rhizosphere of the plants, are also called plant growth promoting rhizobacteria (PGPR). The explanation of this phenomenon is now well-known, and many reports describe and discuss it (De Weger et al., 1987; Schroth & Hancock, 1982; Kloepper et al., 1980a,b). It is a process based on the ability of the strains to produce pyoverdins. The very strong chelating properties of these siderophores exert an antagonist action against plant parasites which are no longer able to acquire essential supplies of iron.

Lemanceau and Samson (1983) have studied the interaction which can exist between green beans (*Phaseolus vulgaris*), phytopathogenic fungi (*Pythium ultimum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, and *Fusarium solani*) and some fluorescent pseudomonads. In particular, they have shown that a strain of *Pseudomonas*, *Pseudomonas tolaasii* NCPPB 2192, very efficiently inhibits the growth of *P. ultimum*. In vitro experiments have suggested that the factor which is responsible for the growth inhibition of this fungus is pyoverdin Pt A, one of the three major siderophores of *P. tolaasii*.

P. fluorescens CCM 2798 is a Gram-negative bacterium which belongs to the fluorescent pseudomonads biotype B (Stanier et al., 1966). In iron-deficient conditions, this bacterium synthesizes green-yellow water-soluble compounds,

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pyoverdins Pf, which are the siderophores of this microorganism (Meyer & Hornsperger, 1978). The major siderophore, pyoverdin Pf, was shown to be an antagonist of the growth of *Pseudomonas aeruginosa* ATCC 15692 (Hohnadel & Meyer, 1988). The physicochemical properties of this compound, described by Meyer and Abdallah (1978), indicated that it was a chromopeptide containing a chromophore which could be derived from 2,3-diamino-6,7-dihydroquinoline. However, the complete structures of pyoverdins Pf have not previously been established.

In this paper, we report the structure elucidation using FAB mass spectrometry, NMR, and CD techniques of the three pyoverdins excreted by *P. tolaasii* NCPPB 2192 and the five pyoverdins occurring in the cultures of *P. fluorescens* CCM 2798. We compare these structures to those of already reported pyoverdins (Philson & Llinas, 1982; Buyer et al., 1986; Demange et al., 1987, 1988a; Teintze, 1981; Van der Helm, 1987; Yang & Leong, 1984; Briskot et al., 1986, 1989, Poppe et al., 1987). We also show that the peptide chain of pyoverdins Pf contains an unusual amino acid possessing a cyclic amidine skeleton.

Materials and Methods

Strains and Culture Media. *P. tolaasii* strain NCPPB 2192, *P. fluorescens* CCM 2798, and *P. fluorescens* CCM 2799 were grown in aerobic conditions, similar to those earlier described by Meyer and Abdallah (1978). The culture media had the following composition per liter: K_2HPO_4, 6 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; succinic acid, 4 g. They were adjusted to pH 7.0 before sterilization.

Isolation and Purification of the Pyoverdins. The bacteria were grown at 25 °C in conical flasks, each containing 0.5 L of culture medium and subjected to mechanical agitation. After 48 h, the culture medium (4.5 L overall) was centrifuged and the pyoverdins were extracted from the supernatant, by using the same hydrophobic chromatography as for azotobactin (Demange et al., 1988a) and pyoverdins Pa (Demange et al., 1990). Three major fractions were separated from the cultures of *P. tolaasii* NCPPB 2192: pyoverdin Pt B (150 mg), pyoverdin Pt A (350 mg), and pyoverdin Pt (200 mg). They were further purified by HPLC according to the procedure described previously for azotobactin (Demange et al., 1988a).

When needed, the corresponding Fe(III) complexes were prepared by addition of 5 equiv of a solution of ferric chloride per equivalent of pyoverdin dissolved in water and purified as described below for the pyoverdins Pf complexes. Each of the pyoverdin Pt, Pt A, and Pt B Fe(III) complexes obtained after CM-Sephadex column chromatography was found to be a single major compound after HPLC (see below).

From the cultures of *P. fluorescens* CCM 2798, three major fractions were also separated: pyoverdin Pf/1 (160 mg), pyoverdin Pf/2 (110 mg), and pyoverdin Pf/3 (485 mg). However, the last fraction corresponding to that described for young cultures (Meyer & Abdallah, 1978) was found to contain three compounds by HPLC analysis. It was therefore dissolved in water (5 mL) and treated with a solution of ferric chloride (1 mL, 2 M). The complex was purified by column chromatography on CM-Sephadex C-25 (2 cm × 40 cm), and the column was eluted with a gradient of pyridine–acetic acid buffer, pH 5.0 (50 mM–2 M, 2 × 0.5 L). The major fraction was purified by preparative HPLC on an octadecylsilane column eluted isocratically with acetonitrile/25 mM pyridine–acetic acid buffer, pH 5.0, to yield three pure iron complexes: pyoverdin Pf–Fe(III) (280 mg), pyoverdin Pf/3/2–Fe(III) (40 mg), and pyoverdin Pf/3/3–Fe(III) (120 mg). Treatment of pyoverdin Pf–Fe(III) with 8-hydroxyquinoline

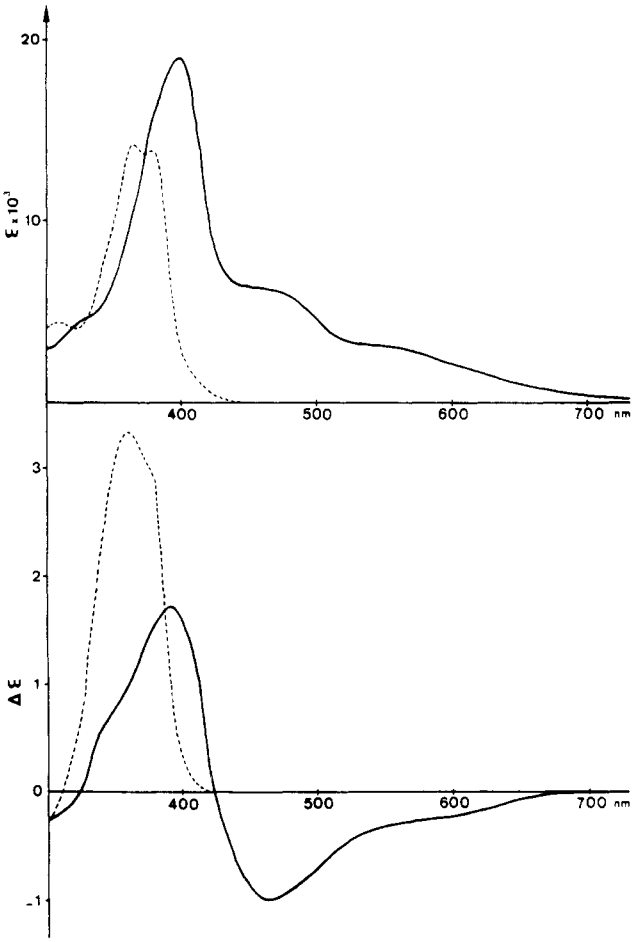


FIGURE 1: Absorption spectra (a) and circular dichroism spectra (b) of pyoverdin Pt A (···) and its Fe(III) complex (—) at pH 5.0.

(Meyer & Abdallah, 1978) and rechromatography on the CM-Sephadex C-25 column yielded pure pyoverdin Pf (150 mg).

Electrophoresis, NMR spectroscopy, UV–visible spectrophotometry, circular dichroism, mass spectrometry, high-pressure column chromatography, and amino acid analyses were performed as described previously (Demange et al., 1988a).

Results and Discussion

(A) The Pyoverdins of P. tolaasii NCPPB 2192. The spectral characteristics (UV–visible, circular dichroism) of pyoverdins Pt and their iron complexes are presented in Figure 1. They were found to be identical with those of pyoverdins Pa (Demange et al., 1987), and also very close to those of pseudobactin B 10 (Teintze et al., 1981). These similarities strongly suggest that the pyoverdins Pt chromophore is identical with that in pyoverdins Pa. The molecular weigths of pyoverdins Pt and their iron complexes were determined by FAB mass spectrometry. Molecular ions (M^+) were observed at m/z 1425, 1453, and 1424 for pyoverdin Pt A, pyoverdin Pt B, and pyoverdin Pt, respectively, and at m/z 1478, 1506, and 1477 for their corresponding iron complexes. The difference of 53 between the free ligands and their respective iron complexes shows that the stoichiometry of these complexes is 1:1. As in the case of the pyoverdins Pa (Demange et al., 1987), pyoverdin Pt A differs from pyoverdin Pt by 28 mass units and from pyoverdin Pt by 1 mass unit.

Total hydrolysis of the three pyoverdins (6 M HCl, 110 °C, 48 h, or 7.5 M HI, 110 °C, 48 h) gave the same amino acid composition for the three compounds: threonine (2 mol), serine

Table I: Assignment of the Protons of Pyoverdin Pt A[a]

resonance	δ (ppm)	multiplicity	assignment	resonance	δ (ppm)	multiplicity	assignment
a	1.04	m	Lys Hγ	n	3.67	m	N^{δ}-OHOrn(c)Hδ
b_1	1.20	d	Thr_1 Hγ	o	3.68	m	N^{δ}-OHOrn(l)Hδ
b_2	1.22	d	Thr_2 Hγ	p_1	3.75	d (6 Hz)	Ser_1 Hβ
c	1.32	m	Lys Hδ	p_2	3.87	d (6 Hz)	Ser_2 Hβ
	1.42	m		p_3	3.88	d (6 Hz)	Ser_3 Hβ
d	1.59	m	Lys Hβ	p_4	3.94	d (6 Hz)	Ser_4 Hβ
	1.79	m		q_1	4.23	m	Thr_1 Hβ
e	1.72	m	N^{δ}-OHOrn(l)Hβ	q_2	4.32	m	Thr_2 Hβ
	1.84	m		p_5	4.36	d (5 Hz)	Ser_5 Hβ
f	1.92	m	N^{δ}-OHOrn(l)Hγ	r	4.37	m	Lys Hα
g	1.83	m	N^{δ}-OHOrn(c)Hβ	s_1	4.38	m	Thr_1 Hα
	2.02	m		s_2	4.42	m	Thr_2 Hα
h	1.91	m	N^{δ}-OHOrn(c)Hγ	t_2	4.36	m	Ser_2 Hα
	2.03	m		t_1	4.45	m	Ser_1 Hα
i	2.13	s	acetyl	u	4.48	m	N^{δ}-OHOrn(l)Hα
j	2.45	m	Chr H-12′	v	4.49	m	N^{δ}-OHOrn(c)Hα
	2.60	m	Chr H-12	t_3	4.49	m	Ser_3 Hα
k	2.60	m	Lys Hε	t_4	4.50	m	Ser_4 Hα
	2.67	m		t_5	4.97	m	Ser_5 Hα
l	2.61	t (8 Hz)	Succ	w	5.70	bs	Chr H-11
	2.68	t (8 Hz)		x_1	6.87	bs	Chr H-8
m	3.37	t	Chr H-13′	x_2	7.08	s	Chr H-5
	3.70	m	Chr H-13	y	7.86	s	Chr H-4

[a] The spectra were determined in ^2H_2O at pH 5.0 by using sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. Multiplicity: (s) singlet, (bs) broad singlet, (d) doublet, (t) triplet, and (m) multiplet. The resonances were assigned by homonuclear correlation of the proton (COSY 90).

(5 mol), lysine (1 mol), and N^{δ}-hydroxyornithine (2 mol), which was totally reduced to ornithine upon HI hydrolysis.

(I) Structure of Pyoverdin Pt A. Pyoverdin Pt A is the major siderophore isolated from the culture supernatant of *P. tolaasii*. It is a stable compound formed from the hydrolysis of pyoverdin Pt in aqueous solutions.

(a) NMR of Pyoverdin Pt A. The ^1H NMR spectrum of pyoverdin Pt A is assigned in Table I. The chemical shifts of the chromophore are very similar to those previously reported for pyoverdins Pa (Demange et al., 1990), showing three aromatic protons as singlets at 7.90, 7.10, and 6.90 ppm. The signal at 2.13 ppm remains even after chromotography of pyoverdin Pt A on a DEAE-Sephadex A-25 column, chloride form, and corresponds to an acetyl group covalently bound to the molecule. The assignments of the amino acids protons (Table I) are in agreement with those reported in the literature (Wüthrich, 1976a).

The ^{13}C NMR spectrum of pyoverdin Pt A showed three distinct regions: the carbonyl region, the aromatic region, and the aliphatic region. The carbonyl signals resonate between 169.2 and 183.9 ppm (Table II). The 11 signals occurring between 173.6 and 176.9 ppm were assigned to the 10 carbonyls of the amino acids and to the carbonyl of the chromophore. This assignment was done by comparison of their chemical shifts with those reported in the literature (Wüthrich, 1976b; Kalinowski et al., 1984) (see Table II). The signals resonating at 180.4 and 183.9 ppm correspond respectively to the carboxamide group and the carboxylic acid group of the succinyl moiety bound to the chromophore. The signals at 169.2 and 172.2 ppm were assigned to the carbonyl of the acetyl group covalently bound to the molecule, and forming with the hydroxylamine of N^{δ}-hydroxyornithine a hydroxamate group. Both these signals were assigned, by comparison with several standard acetamides, to the cis (169.2 ppm) and trans (172.2 ppm) isomers of the hydroxamate (Stewart & Siddall, 1970). The signals of the nine aromatic carbon atoms resonate between 102.9 and 159.8 ppm. Three are tertiary carbons at 102.8 (C-8), 115.5 (C-5), and 141.9 ppm (C-4), and six are quaternary carbons at 116.0 (C-10), 118.4 (C-3), 135.8 (C-9), 148.2 (C-6), 151.9 (C-2) and 159.8 ppm (C-7). These

Table II: Assignment of the Carbonyls of Pyoverdin Pt A[a]

carbon atom	chemical shift (ppm)	carbon atom	chemical shift (ppm)
COOH Succ	183.9	CO Ser	174.8
CONH Succ	180.4	CO Ser	174.7
CO OHOrn	176.9	CO Ser	174.6
CO OHOrn	176.7	CO Ser	174.0
CO Lys	176.6	CO Ser	173.6
CO Thr	175.2	$COCH_3$ (trans)	172.2
CO Thr	175.1	$COCH_3$ (cis)	169.2
CO Chr	174.9		

[a] The spectrum was determined in ^2H_2O at pH 5.0 using sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard.

chemical shifts are very similar to those of the chromophore of pyoverdin Pa A (Demange et al., 1987, 1990), which indicates both chromophores possess the same structure. The chemical shifts of the aliphatic carbon atoms between 21.6 and 70.0 ppm are presented in Table III. Their assignment was performed after determination of their multiplicity, by comparison with the values obtained for pyoverdin Pa A (Demange et al., 1987, 1990) and those reported in the literature (Wüthrich, 1976b; Kalinowski et al., 1984).

From the above experiments, it can be concluded that pyoverdin Pt A is constituted with a peptide chain of 10 amino acids (5 mol of serine, 2 mol of threonine, 1 mol of lysine, and 2 mol of N^{δ}-hydroxyornithine) bound to a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline. In addition, an acetyl group is linked to N^{δ}-hydroxyornithine and a succinyl group acylates an amine function, probably the amine at position C-3 of the chromophore.

(b) Sequence of the Peptide Chain. The sequence of the peptide chain was determined by FAB mass spectrometry. Both N- and C-terminal fragments, together with the major fragment ion (m/z 1122) formed by the cleavage across the chromophore, were observed (Demange et al., 1987, 1990). The N- and C-terminal ions are assigned in Tables IV and V. The nature of the acylating groups is confirmed by the presence of a peak at m/z 1383, corresponding to the loss of a ketene derived from an acetyl group (42 mass units), and

Table III: Assignment of the Aliphatic Carbons of Pyoverdin Pt A[a]

carbon atom	chemical shift (ppm)	carbon atom	chemical shift (ppm)
Thr Cβ	70.0 (d)	HOOrn Cα (c)	53.5 (d)
Thr Cβ	69.9 (d)	HOOrn Cδ (l)	50.0 (t)
Ser Cβ (3)	63.9 (t)	Lys Cε	41.9 (t)
Ser Cβ	63.8 (t)	Chr C_{13}	37.9 (t)
Ser Cβ	63.6 (t)	Succ C_1	35.6 (t)
Thr Cα	62.3 (d)	Succ C_2	35.0 (t)
Thr Cα	62.2 (d)	Lys Cδ	32.8 (t)
Ser Cα	60.1 (d)	HOOrn Cβ (l)	30.8 (t)
Chr C_{11}	59.4 (d)	HOOrn Cβ (c)	29.4 (t)
Ser Cα	58.8 (d)	Lys Cβ	28.7 (t)
Ser Cα	58.7 (d)	CH_3 acetyl	26.0 (q)
Ser Cα	58.5 (d)	HOOrn Cγ (l)	25.3 (t)
Ser Cα	58.4 (d)	Chr C_{12} + Lys Cγ	24.9 (t)
Lys Cα	56.3 (d)	HOOrn Cγ (c)	22.9 (t)
HOOrn Cα (l)	56.0 (d)	Thr Cγ	21.7 (q)
HOOrn Cδ (c)	54.5 (t)	Thr Cγ	21.6 (q)

[a] The spectra were determined at pH 5.0 in ^2H_2O using sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. In the case of N^δ-hydroxyornithine, (l) refers to the one which is in the middle of the peptide chain and (c) to the one which is cyclic at the C-terminal end of the peptidic siderophore. The values in parenthesis indicate the number of carbon atoms. Multiplicity: (d) means doublet, (t) triplet and (q), quartet.

Table IV: C-Terminal Sequence Fragment Ions of Pyoverdin Pt A Together with Their Assignments

mass units	C-terminal fragments $(M + H)^+$
491	H_2N-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn(cyclic)
679	(H_2N-Thr-Ser)-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn(cyclic)
766	H_2N-Ser-(Thr-Ser)-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn(cyclic)
853	H_2N-Ser-Ser-(Thr-Ser)-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn-(cyclic)
981	H_2N-Lys-Ser-Ser-(Thr-Ser)-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn(cyclic)
1068	H_2N-Ser-Lys-Ser-Ser-(Thr-Ser)-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn(cyclic)
1122	H_2C=CHCONH-Ser-Lys-Ser-Ser-(Thr-Ser)-N^δ-acetyl-OHOrn-Thr-Ser-OHOrn(cyclic)

Table V: N-Terminal Sequence Fragments of Pyoverdin Pt A[a]

Cα ions	amide	assignment
417, 418, 419	462	Chr-Ser
545, 546, 547	590	Chr-Ser-Lys
632, 633, 634	677	Chr-Ser-Lys-Ser
719, 720, 721	764	Chr-Ser-Lys-Ser-Ser
820, 821, 822	865	Chr-Ser-Lys-Ser-Ser-Thr
907, 908, 909	952	Chr-Ser-Lys-Ser-Ser-Thr-Ser
1180, 1181, 1182	1225	Chr-Ser-Lys-Ser-Ser-Thr-Ser-(273)
1267, 1268, 1269	1318	Chr-Ser-Lys-Ser-Ser-Thr-Ser-(273)-Ser
	1425 (M^+)	Chr-Ser-Lys-Ser-Ser-Thr-Ser-(273)-Ser-cOHOrn

[a] The amide ions are formed by cleavage of the N–Cα bonds; the Cα ions result from cleavage of Cα–CO bonds. The presence of the clusters of Cα ions is due to hydrogen-transfer reactions.

a peak at m/z 1325, corresponding to the loss of a succinyl group (100 mass units), from the molecular peak at m/z 1425. From the data in Tables IV and V, the following partial sequence was assigned: Chr-Ser-Lys-Ser-Ser-Thr-Ser-Z-Ser-OHOrn(cyclic).[1]

The clarification of Z (273 mass units) and confirmation of the remainder of the sequence were obtained by FAB-MS analyses of derivatives and hydrolysates. Acetylation for 5 min of pyoverdin Pt A with a 1:1 mixture of acetic anhydride and deuterated acetic anhydride in methanol yielded signals at m/z 1551, 1554, and 1557 which corresponded to the addition of three acetyl groups on the siderophore (M^+ at m/z 1425). However, the labeling pattern that would be expected for three 1:1 labeled groups, that is, 1:3:3:1, was not seen; rather, the pattern resembled a distorted 1:4:6:4:1 pattern. This is in agreement with the fact that the molecule already contains an acetyl function which can be partially exchanged for a labeled acetyl group.

Table VI: Major Molecular Ions Observed in the FAB Mass Spectrum of Pyoverdin Pt A Hydrolysates (6 M HCl, 90 °C, 20 min)[a]

mass (u)	molecular ion assignment
591	Chr-Ser-Lys-COOH
765	Chr-Ser-Lys-Ser-Ser-COOH
1083	Chr-Ser-Lys-Ser-Ser-Thr-Ser-OHOrn-COOH
1184	Chr-Ser-Lys-Ser-Ser-Thr-Ser-OHOrn-Thr-COOH
1271	Chr-Ser-Lys-Ser-Ser-Thr-Ser-OHOrn-Thr-Ser-COOH
1401	Chr-Ser-Lys-Ser-Ser-Thr-Ser-OHOrn-Thr-Ser-OHOrn-COOH

[a] Each of these ions was accompanied by a signal at 18 u lower which probably arises from dehydration of the succinic acid to succinimide.

Pyoverdin Pt A is rapidly esterified after 5-min reaction (2 M HCl in methanol/deuterated methanol, 1:1 v/v), yielding a monoester at m/z 1439 and 1442. After 30 min, a second doublet occurs at m/z 1457 and at m/z 1460 (18 mass units above the former) together with a triplet at m/z 1471, 1474, and 1477. This indicates that there is an addition of a water molecule on the monoester followed by esterification of the species thus formed. We interpreted this as the hydrolysis of the cyclic hydroxyornithine followed by the esterification of the acid function thus formed.

Acid hydrolysis of pyoverdin Pt A (6 M HCl, 90 °C) was monitored by FAB mass spectrometry at 5-min time intervals. The first step in this hydrolysis is the addition of a molecule of water to yield a signal at m/z 1443 corresponding to the hydrolysis of the terminal cyclic N^δ-hydroxypiperidinone. These results are consistent with the ^{13}C NMR spectrum of pyoverdin Pt A, where differences were observed for both carbons Cα and Cδ of the hydroxyornithines. Further hydrolysis gave peaks at m/z 1401 and 1383 corresponding to the loss of the acetyl group from the species that give rise to ions at m/z 1443 ($M^+ + H_2O$) and at m/z 1425 (M^+). After 20 min (Table VI), extensive hydrolysis of the peptide backbone had occurred, giving ions containing useful sequence information. An additional signal occurring at m/z 829 was assigned to the peptide H_2N-Ser-Ser-Thr-Ser-N^δ-OHOrn-COOH. This hydrolytic fragment arises from cleavage of the amide bond between lysine (at position 2) and serine (at position 3), which is the preferred cleavage in acid hydrolysis of pyoverdin Pt A. The data from the hydrolysis experiments define the structure of Z as OHOrn-Thr, and therefore, the complete sequence of pyoverdin Pt A is Chr-Ser-Lys-Ser-Ser-Thr-Ser-OHOrn-Thr-Ser-OHOrn(cyclic).

(*c*) *Configuration of the Amino Acids and of the Chromophore of Pyoverdin Pt A.* The stereochemistry of the chromophore was determined from its circular dichroism spectrum which was found to be identical with the spectra of pyoverdin Pa A (Demange et al., 1987) and pseudobactin B 10 (Teintze et al., 1981), the structure of the latter having been determined by X-ray diffraction. Thus the chiral center of the chromophore has the *S* configuration. The configuration of the amino acids was determined by chiral gas chromatography on a capillary Chirasil column (Frank et al., 1977;

[1] Abbreviations: OHAsp, D-β-*threo*-hydroxyaspartic acid; DABA, 2,4-diaminobutyric acid; OHOrn, N^δ-hydroxyornithine; cOHOrn, cyclic N^δ-hydroxyornithine; SerCTHPMD, 2-seryl-6-carboxy-3,4,5,6-tetrahydropyrimidine; Chr, chromophore.

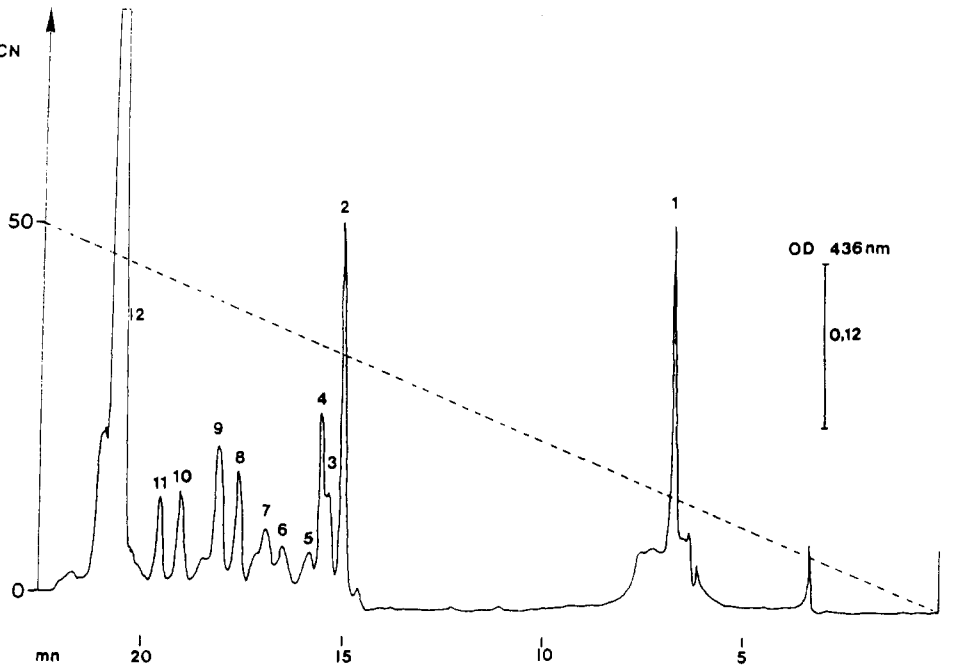


FIGURE 2: HPLC chromatogram of the mixture of the DABIT peptides obtained after partial acid hydrolysis of pyoverdin Pt A (20 min, 6 M HCl, 90 °C) and derivatization of the hydrolysate with DABITC. Peptide 12 coelutes with excess DABITC.

Demange et al., 1988b) after derivatization of acid hydrolysates (HCl and HI) of pyoverdin Pt A or its partial hydrolysis fragments. The derivatives used were N^{δ}-(heptafluoropropionyl)-*O*-propyl or *N*-(pentafluoropropionyl)-*O*-methyl esters. It was found that the configuration of lysine, one serine, and both threonines was L. The configuration of the four other serines was D, and the hydroxyornithines had opposite configurations.

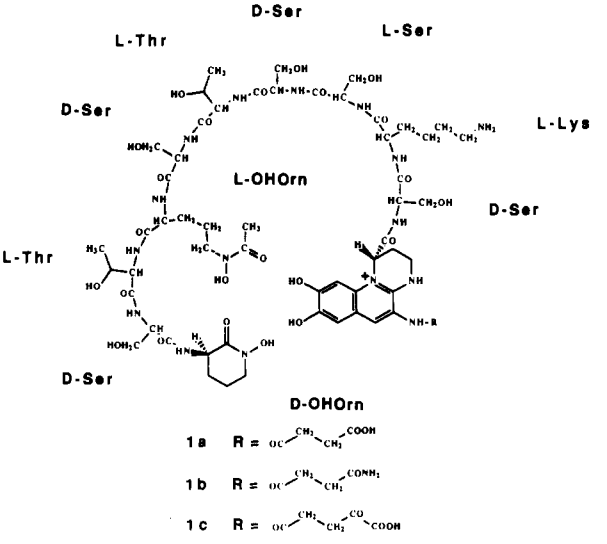
Partial hydrolytic fragments were isolated and purified in order to locate the L-serine and the L-N^{δ}-hydroxyornithine in the decapeptide bound to the chromophore. The hydrolysis conditions (20 min, 6 M HCl, 90 °C) afforded a large number of hydrolytic fragments of low molecular weight. The crude hydrolysis mixture was treated by 4-(dimethylamino)azobenzene 4′-isothiocyanate (DABITC) according to the method of Edman–Chang (Chang, 1981) and then purified by preparative HPLC on an octadecylsilane stationary phase (Figure 2). Two types of hydrolytic fragments were obtained. Those showing an absorption at 380 nm had not been derivatized by the DABITC reagent. They contained the chromophore of pyoverdin Pt A and did not have a free N-terminus. The major fraction 1 corresponds to Chr-Ser-Lys-OH, obtained after cleavage of the bond between lysine-2 and serine-3. The stereochemical analysis of this fragment showed that the configuration of lysine is L and that of serine is D. Peptide fragments which had been derivatized by DABITC gave a maximum of absorption at 436 nm. After acid hydrolysis (6 M HCl, 24 h, 110 °C), the amino acids of each hydrolytic fragment were analyzed on an amino acid analyzer and by gas chromatography on a L-chirasyl-Val capillary column.

DABITC-peptide 12 (Figure 2 and Table VII) corresponds to two dipeptides, D-Ser-L-OHOrn and D-Ser-D-OHOrn, indicating that both serines bound to the N^{δ}-hydroxyornithines possess the D configuration. The position of the L-N^{δ}-hydroxyornithine in pyoverdin Pt A was obtained after determination of the configuration of the amino acids of the tripeptide Ser-OHOrn-Thr corresponding to peak 10 of the HPLC chromatogram (Figure 2). This tripeptide arises from the cleavage of the amide bonds between threonine-5 and serine-6 and threonine-8 and serine-9. In this peptide, the configuration of the serine at the N-terminal end is D, whereas those of N^{δ}-hydroxyornithine and threonine are L. Therefore, the other N^{δ}-hydroxyornithine at the C-terminus of the pyoverdin has a D configuration. The location of the L-serine can thus be deduced by difference, taking into account that the tripeptide corresponding to peak 2 of the HPLC chromatogram is D-Ser-L-Thr-D-Ser. The only serine left has an L configuration and is located at position 3 of the peptide chain. The complete structure of pyoverdin Pt A is given by structure **1a**.

Table VII: Determination of the Amino Acid Composition and Configuration of the N-Terminal Peptidic Fragments 2, 10, and 12 of Pyoverdin Pt A[a]

DABITC-peptide	amino acid composition	configuration of the amino acids	N-terminal amino acids
2	Ser_2, Thr	D-Ser, L-Thr	Ser
10	Ser, Thr, OHOrn, Ser_2, Thr_2, $OHOrn_2$	D-Ser, L-Thr, L-OHOrn	Ser
12	Ser, OHOrn	D-Ser, (*R*,*S*)OHOrn	Ser

[a] See Figure 2 for the numbering of these peptides.



1a R = $-CO-CH_2-CH_2-COOH$

1b R = $-CO-CH_2-CH_2-CONH_2$

1c R = $-CO-CH_2-CH_2-CO-COOH$

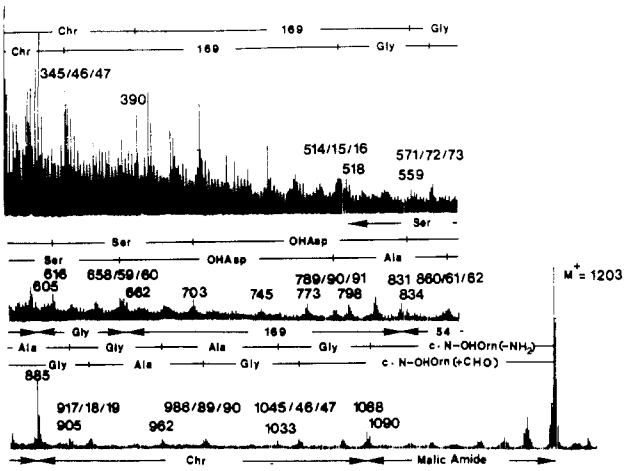


FIGURE 3: FAB-MS spectrum of pyoverdin Pf.

(II) Structure of Pyoverdin Pt and Pyoverdin Pt B. The structures of pyoverdins Pt B and Pt were deduced after comparison of their FAB mass spectra with that afforded by pyoverdin Pt A. The FAB spectra of these three pyoverdins possess a common major C-terminal ion at 1122 u which results from the characteristic pyoverdin cleavage across the saturated ring of the chromophore. This shows that the mass differences between the three pyoverdins occur in the chromophore moiety, most likely in the acyl group bound to the amine at position 3.

The mass of pyoverdin Pt (M^+ at m/z 1424) (**1b**), which is one unit less than that of pyoverdin Pt A, indicates that it possesses a succinamide group instead of the succinic acid group of pyoverdin Pt A. It is likely that pyoverdin Pt is initially the major siderophore synthesized by the bacterium, but since the pH of the culture increases rapidly, this compound is readily hydrolyzed into pyoverdin Pt A by an intramolecular reaction already reported for pyoverdin Pa (Demange et al., 1987, 1990).

The molecular ion M^+ of pyoverdin Pt B (**1c**) is 28 mass units higher in mass than that of pyoverdin Pt A, which corresponds to the substitution of succinic acid by α-ketoglutaric acid in the chromophore. Comparison of the ^{13}C NMR spectra of pyoverdin Pt A and pyoverdin Pt B reveals three signals at 31.5, 33.0, and 34.5 ppm in pyoverdin Pt B replacing the two methylenic signals at 35.0 and 35.6 ppm of succinic acid. In α-ketoglutaric acid bound to the chromophore, the carbon vicinal to the ketone group gives two signals at 33.0 and 34.5 ppm due to the simultaneous presence of a ketone and a *gem*-diol form in equilibrium in solution (Viswanathan et al., 1982). The orientation of α-ketoglutarate in pyoverdin Pt B is the same as in pyoverdin Pa B with a C-5 attachment to the chromophore. On acid hydrolysis of this compound (6 M HCl, 90 °C, 30 min), a fragment Chr-Ser-Lys is obtained showing an important modification in the chomophore. The absorption maximum of this molecule occurs at 410 nm ($\epsilon = 2.2 \times 10^4$ M^{-1} cm^{-1}) at acid pHs and shifts to 460 nm ($\epsilon = 2.95 \times 10^4$ M^{-1} cm^{-1}) at pH 6.7 with an isosbestic point at 426 nm. In this respect, pyoverdin Pt B and pyoverdin Pa B have a similar behavior and both give on acidic treatment the same chromophore with an extra pyrazinone ring (Demange et al., 1990); therefore, they both possess α-ketoglutaric acid bound to the nitrogen atom at C-3 of the chromophore with the same orientation.

(B) The Pyoverdins of P. fluorescens CCM 2798. We investigated smooth (CCM 2798) and rough (CCM 2799) forms of *P. fluorescens* CCM 2798 and found no observable differences between the mixture of siderophores excreted by each strain. The experiments reported below were carried out on the smooth form of the bacteria. Pyoverdin Pf, the major product occurring from the cultures of the bacteria, was used for the structure elucidation.

Table VIII: N-Terminal Sequence Fragments of Pyoverdin Pf[a]

Cα ions	amide	assignment
345, 346, 347	390	Chr
514, 515, 516	559	Chr-(169)
571, 572, 573	616	Chr-(169)-Gly
658, 659, 660	703	Chr-(169)-Gly-Ser
789, 790, 791	834	Chr-(169)-Gly-Ser-OHAsp
860, 861, 862	905	Chr-(169)-Gly-Ser-OHAsp-Ala
917, 918, 919	962	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly
988, 989, 990	1033	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala
1045, 1046, 1047	1090	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly
	1203	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)
	(M^+)	

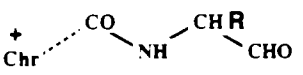
[a] The amide ions are formed by cleavage of the N–Cα bonds; the Cα ions result from cleavage of Cα–CO bonds. The presence of the clusters of Cα ions is due to hydrogen-transfer reactions.

Table IX: C-Terminal Sequence Fragments of Pyoverdin Pf

mass (m/z)	interpretation $(M + H)^+$
518	H_2N-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)
605	H_2N-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)
662	H_2N-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)
831	H_2N-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)
885	H_2C=CHCO-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)
1088	H_2N-Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn-(cyclic)
1203	(malic amide)-Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn(cyclic)

Acid hydrolysis (6 M HCl, 110 °C, 48 h) of the siderophore and amino acid analysis gave serine (1), glycine (3), alanine (1), N^{δ}-hydroxyornithine (1), and *β-threo*-hydroxyaspartic acid (1) (Hamilton, 1963). The presence of hydroxyaspartic acid was further confirmed by FAB-MS and during the stereochemical analysis of the amino acids on L-Chirasyl-Val stationary phase (see below).

(I) Structure of Pyoverdin Pf. (a) FAB Mass Spectrometry. FAB-MS of pyoverdin Pf (Figure 3) afforded an M^+ ion at m/z 1203, a major ion resulting from the characteristic chromophoric cleavage at 885 u, and sequence determining ions (Tables VIII and IX). The mass difference between the molecular ion and the chromophoric fragment ion suggests that malic amide substitutes the chromophore. The iron complex of pyoverdin Pf gave a signal at m/z 1256 corresponding to a 1:1 stoichiometry. The presence of β-hydroxyaspartic acid (increment of 131 mass units between the amide ions at m/z 834 and 703) is defined by fragment ions at m/z 773 and 745, which can be respectively assigned to the aldehyde ions **2a** and **2b**. Further evidence for β-hydroxyaspartic acid was afforded



2a R = H

2b R = CHO

by esterification experiments. After 5-min esterification with a 1:1 mixture of methanol/deuterated methanol acidified by HCl gas, the FAB-MS spectrum showed a doublet at m/z 1217 and 1220 indicating that one acid function has been

Table X: Hydrolytic Fragments of Pyoverdin Pf Obtained after Acid Hydrolysis as a Function of Time (6 N HCl, 90 °C)

time	major signals (m/z)
5 min	1221, 1203, 1188, 1170, 1106, 1091, 1088, 1034, 963
10 min	1221, 1203, 1106, 976, 919, 848, 791, 623, 618, 502
15 min	1106, 1091, 1034, 976, 919, 907, 848, 791, 720, 623, 618, 605, 602, 584, 502, 493, 436, 365
20 min	618, 502
2 h	502, 445

Table XI: Interpretation of Chromophoric Hydrolytic Fragments of Pyoverdin Pf Obtained after 15-min Acid Hydrolysis (6 N HCl, 90 °C)

mass	acid hydrolytic chromophoric fragments of pyoverdin Pf
963	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-COOH
1034	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-COOH
1091	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-COOH
1221	Chr-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-OHOrn-COOH

Table XII: Interpretation of Chromophoric Hydrolytic Fragments of Pyoverdin Pf Obtained after 15-min Acid Hydrolysis (6 N HCl, 90 °C)

mass	acid hydrolytic chromophoric fragments of pyoverdin Pf
502	Chr-(malic amide)-(169)-Gly-COOH
720	Chr-(malic amide)-(169)-Gly-Ser-OHAsp-COOH
791	Chr-(malic amide)-(169)-Gly-Ser-OHAsp-Ala-COOH
848	Chr-(malic amide)-(169)-Gly-Ser-OHAsp-Ala-Gly-COOH
919	Chr-(malic amide)-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-COOH
976	Chr-(malic amide)-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-COOH
1106	Chr-(malic amide)-(169)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrnCOOH

Table XIII: Interpretation of Peptidic Hydrolytic Fragments of Pyoverdin Pf Obtained after 15-min Acid Hydrolysis (6 N HCl, 90 °C)

mass	hydrolytic peptidic fragments of pyoverdin Pf
365	H_2N-Ser-OHAsp-Ala-Gly-COOH
436	H_2N-Ser-OHAsp-Ala-Gly-Ala-COOH
493	H_2N-Ser-OHAsp-Ala-Gly-Ala-Gly-COOH
623	H_2N-Ser-OHAsp-Ala-Gly-Ala-Gly-OHOrn-COOH

esterified (γ-carboxyl of *β-threo*-hydroxyaspartic acid). After 30-min reaction, the doublet of the monoester is shifted by 18 mass units higher to m/z 1235 and 1238. It is accompanied by a triplet at m/z 1249, 1252, and 1255. This is analogous behavior to that observed for pyoverdin Pt A (see above) and is rationalized as follows: the C-terminal end of the peptide chain which is blocked as a result of the cyclization of N^{δ}-hydroxyornithine into N^{δ}-hydroxypiperidinone is hydrolyzed in acid conditions, resulting in ring opening of the piperidinone and subsequent esterification of the new C-terminal group.

Examination of the N-terminal sequence fragments shows the presence of a residue X of 169 mass units, which is bound to the chromophore and which does not correspond to any known amino acid or to a simple combination of amino acids. The N-terminal fragmentation pattern obtained for this residue with the signals at m/z 554, 516, 515, and 514 is characteristic of the fragmentation of the amino acid constituents of pyoverdins and indicates that this residue is very likely an amino acid.

The C-terminal fragments are interpreted in Table IX. The peak at m/z 1088 corresponds to loss of malic amide from the chromophore. The major fragment at m/z 885 results from the characteristic cleavage across the chromophore (see above). The following peptidic sequence is assigned from these data: Chr-(X = 169 mass units)-Gly-Ser-OHAsp-Ala-Gly-Ala-cO-HOrn. This sequence was corroborated by the results obtained by FAB-MS on mild acid hydrolysis of pyoverdin Pf which are presented in Table X. After a short time of hydrolysis (5 min), the main reaction is the hydrolysis of the C-terminal N^{δ}-hydroxypiperidinone ring into N^{δ}-hydroxyornithine. This is characterized by the presence of a peak at m/z 1221, 18 mass units above the molecular peak. The signals at m/z 1091 and 1106 correspond to the loss of the malic amide group bound to the chomophore.

The hydrolytic fragments at m/z 1091, 1034, 963, 976, 919, 848, 751, 720, 502, and 445 obtained after 15-min hydrolysis are hydrolytic *chromophoric* fragments, whereas the hydrolytic fragments at m/z 623, 493, 436, and 365 are *peptidic* fragments. They are all assigned in Tables XI–XIII. The hydrolysis products are exactly those expected for the proposed sequence, and these experiments indicate that the "169" moiety is stable under these hydrolysis conditions.

The product of mass 502 which contains the chromophore and the unassigned 169 moiety was purified and analyzed by NMR and FAB-MS (see below).

(*b*) *NMR of Pyoverdin Pf.* The ^1H NMR spectrum of pyoverdin Pf is assigned in Table XIV. The chemical shifts of the chromophore are similar to those in pyoverdin Pt A (see above). The other resonances corresponding to malic amide

Table XIV: Assignment of the Protons of Pyoverdin Pf[a]

resonance	δ (ppm)	multiplicity	assignment	resonance	δ (ppm)	multiplicity	assignment
a	1.39	d (7 Hz)	Ala_1 Hβ	l	3.83	d	Ser Hβ
b	1.42	d (7 Hz)	Ala_2 Hβ	m	3.94	s	Gly_2, Gly_3
c	1.81	m	OHOrn Hβ	n	4.06	s	"X"
	2.03	m		o	4.32	q (7 Hz)	Ala_2 Hα
d	1.94	m	OHOrn Hγ	p	4.37	q (7 Hz)	Ala_1 Hα
	2.03	m		q	4.44		"X"
e	2.08	m (14 Hz)	"X"	r	4.45		Ser Hα
	2.22	d, d (14 Hz; 3.5 Hz)		s	4.50		OHOrn Hα
g	2.54	m	Chr H-12	t	4.67	t	"X"
	2.73	m		u	4.72		malic amide
h	2.96	d, d (15.7 Hz; 4.8 Hz)	malic acid	v	5.71	m	Chr H-11
i	3.36	m (15.8 Hz)	"X"	w	6.92	s	Chr H-8
	3.56	d (15.8 Hz)		x	7.15	s	Chr H-5
j	3.58	d (16.9 Hz)	Gly_1 (AB)	y	7.92	s	Chr H-4
	3.85	d (16.9 Hz)					
k	3.64	m	OHOrn Hδ				
	3.70	m					

[a] The spectra were determined in ^2H_2O at pH 5.0 using sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. Multiplicity: (s) singlet, (d) doublet, (dd) double doublet, (t) triplet, (q) quartet, and (m) multiplet. The resonances were assigned by homonuclear correlation of the proton (COSY 90).

Table XV: Assignment of the Carbonyls and the Chromophoric Carbons of Pyoverdin Pf[a]

carbon atom	chemical shift (ppm)	carbon atom	chemical shift (ppm)
COOH OHAsp	183.7	CO Ser	169.5
$CONH_2$ malic acid	179.9	C "X"	165.3
CONH malic amide	179.3	Chr C-7	156.4
CO OHOrn	178.4	Chr C-2	152.0
CO OHAsp	177.9	Chr C-6	147.0
CO Chr	174.9	Chr C-4	142.2
CO Ala	174.6	Chr C-9	135.0
CO Ala	174.3	Chr C-3	119.2
CO Gly	173.9	Chr C-10	117.0
CO Gly	173.8	Chr C-5	116.3
CO Gly	173.4	Chr C-8	102.9

[a] The spectra were determined in ^2H_2O at pH 5.0 with sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard.

Table XVI: Assignment of the Aliphatic Carbon Atoms of Pyoverdin Pf[a]

carbon atom	chemical shift (ppm)	carbon atom	chemical shift (ppm)
OHAsp Cβ	74.7 (d)	Gly	45.4 (t)
malate C-1	71.1 (d)	Gly	45.3 (t)
Ser Cβ	64.3 (t)	Gly	44.9 (t)
(CH_2) "X"	63.1 (t)	malate C-2	42.1 (t)
OHAsp Cα	59.6 (d)	(CH_2) "X"	39.3 (t)
Chr C-11	59.5 (d)	Chr C-13	37.9 (t)
Ser Cα	58.5 (d)	OHOrn Cβ	29.5 (t)
(CH) "X"	57.5 (d)	Chr C-12	24.6 (t)
OHOrn Cδ	54.5 (t)	(CH_2) "X"	23.8 (t)
(CH) "X"	54.1 (d)	OHOrn Cγ	22.9 (t)
Ala Cα	53.1 (d) (2)	Ala Cβ	19.2 (q) (2)
OHOrn Cα	52.9 (d)		

[a] The spectra were determined in ^2H_2O at pH 5.0 using sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. In the case of N^δ-hydroxyornithine, l refers to the one located in the middle of the peptide chain and c to the cyclic one at the end of the peptide chain. The numbers in parentheses represent the number of carbon atoms. Multiplicity: (d) doublet, (t) triplet, and (q) quartet.

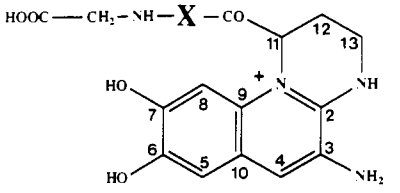
and the amino acids were assigned by using ^1H–^1H homonuclear correlation. These values are in agreement with those reported in the literature (Wüthrich, 1976a). A set of five resonances was assigned to amino acid X.

The ^{13}C NMR spectrum of pyoverdin Pf is assigned in Tables XV and XVI. It showed the same characteristics as all the pyoverdins so far investigated (Demange et al., 1987, 1990). The assignment of the signals of the carbon atoms was performed after determination of their multiplicity (by distortionless enhancement by polarization transfer) and by comparison of the chemical shift of each signal with those observed for other pyoverdins (see above) as well as with those reported in the literature (Wüthrich, 1976b; Kalinowski et al., 1984).

In the carbonyl region, 14 resonances were observed between 183.7 and 165.3 ppm. The signal at 183.7 ppm corresponds to the carboxyl group of β-hydroxyaspartic acid. The other resonances were assigned to the carbonyls of malic amide (179.9 and 179.2 ppm) and to the carbonyls of the amino acids including the amino acid X and the carbonyl of the chromophore. The signal at 165.3 ppm was assigned to a quaternary carbon atom belonging to the amino acid X.

The nine resonances between 156.4 and 102.9 ppm correspond to the nine aromatic carbon atoms of the chromophore. The aliphatic carbon atoms of X were determined after assignment of all the other resonances. This amino acid contains three secondary carbon atoms at 23.8, 39.3, and 63.1 ppm and two tertiary carbon atoms at 54.1 and 57.5 ppm.

(*c*) *Identification of the Amino Acid X.* Mild acid hydrolysis of pyoverdin Pf (6 M HCl, 90 °C, 30 min) yielded essentially one major chromophoric compound (compound **3**)



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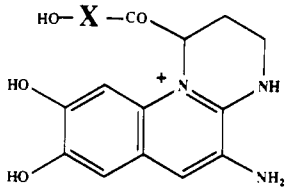
which gave an M^+ molecular ion in the FAB mass spectrum at m/z 502 which corresponds to Chr-X-Gly-OH. On treatment with a mixture of methanol/deuterated acetic anhydride (1:1) a cluster of signals at m/z 628, 631, 634, and 637 in 1:2:2:1 ratio is obtained, corresponding to the addition of three acetyl or deuterioacetyl groups. Since this reagent is specific for amino groups, it is likely that two of the introduced acetyl groups are located on the chromophore which possesses a primary amino group in position C-3 and a secondary amino group in position C-2. The third acetyl group is therefore probably located on amino acid X.

Chromopeptide **3** was purified by column chromatography on CM-Sephadex C-25 and showed a UV–vis spectrum characteristic of the pyoverdins with two maxima at 360 and 375 nm (at pH 5.0). The hypsochromic effect of 5 nm with respect to the spectrum of pyoverdin Pf is due to the loss of the malic amide substituent bound to the amine function at C-3 of the chromophore.

Amino acid analysis of a total hydrolysate of **3** (6 M HCl, 110 °C, 48 h) gave glycine as the only amino acid observed. The amino acid X was not detected under these conditions. Less vigorous acid hydrolysis of **3** (6 M HCl, 90 °C, 5 h) gave a compound **4** which had lost its glycine and contained only



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the amino acid X bound to the chromophore. The precise mass of compound **4** measured by FAB-MS with respect to poly(ethylene glycol) (MW = 415.2533) was found to be 445.1765 mass units. Nine possible combinations of C, H, N, and O fall within 10 mmu of this value. Of these, only one, $C_{20}H_{25}N_6O_6$, is compatible with the structure containing the chromophore in addition to the unknown moiety. In order to determine the complete structure of X, an NMR study of compound **3** was undertaken.

(*d*) *NMR Study of Compound* **3**. The ^1H NMR spectrum of **3** is assigned in Table XVII. It is characteristic of a chromopeptide related to the pyoverdins. It possesses three aromatic protons, H-4 (7.40 ppm), H-5 (7.03 ppm), and H-8 (6.90 ppm). The chemical shift of H-4 has a different value from that of H-4 in pyoverdin Pf. This is due to the loss of the malic amide substituent bound to the nitrogen atom at C-3 of the chromophore. The correlations between the aliphatic protons were established by 2D COSY NMR and are presented in Figure 4. They show that amino acid X possesses two independent spin systems: an A_2X system and a AA'BB'X system comparable to the system observed for the saturated ring of the chromophore. The chemical shifts of the protons belonging to the A_2X system are very close to those observed

Table XVII: Assignment of the Protons of the Hydrolytic Fragment "502", Chr-X-Gly-OH[a]

resonance	δ (ppm)	multiplicity	assignment
a	2.08	m (14.1 Hz; 4.8 Hz)	"X"
	2.22	d, d (14 Hz; 3.5 Hz)	
b	2.54	t, d (13.5 Hz; 5.4 Hz)	CHr H-12
	2.73	d (14.2 Hz)	
c	3.29	d (18.7 Hz)	Gly (AB)
	3.85	d (18.7 Hz)	
d	3.39	m	Chr H-13
	3.76	d, d (15.8 Hz; 3.7 Hz)	
e	3.40	t, d (13.2 Hz; 5.8 Hz)	"X"
	3.53	d, d (15.8 Hz; 3.5 Hz)	
f	4.04	d (5.4 Hz)	"X"
g	4.40	t (4.5 Hz)	"X"
h	4.63	t (5.4 Hz)	"X"
i	5.71	m	Chr H-11
j	6.90	s	Chr H-8
k	7.03	s	Chr H-5
l	7.40	s	Chr H-4

[a] The spectra were determined in ^2H_2O at pH 5.0 with sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. Multiplicity: (s) singlet, (d) doublet, (t) triplet, and (m) multiplet. The resonances were assigned by homonuclear correlation of the proton (COSY 90).

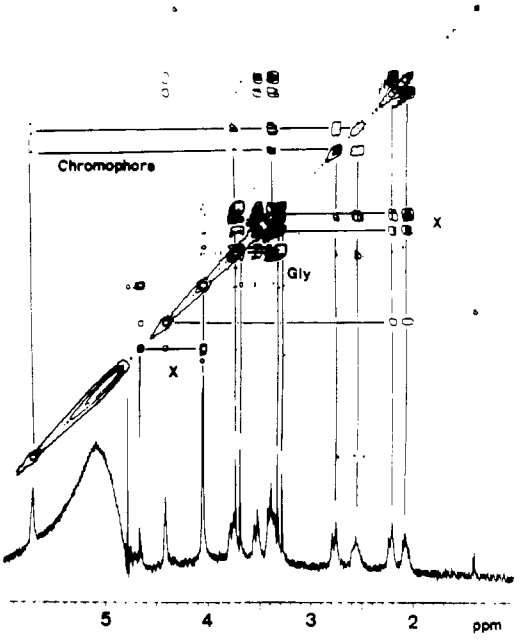


FIGURE 4: COSY spectrum of compound **3** in ^2H_2O. Chemical shifts are in ppm with sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. The lines trace out the connectivities for each group of the molecule, chromophore, glycine, and X (SerCTHPMD). The pH of the solution was 5.0.

for serine bound to a similar chromophore (see above). The protons of the AA'BB'X system have chemical shifts very close to those of 2,4-diaminobutyric acid, which is a constituent of polymixin B (Perkins et al., 1978).

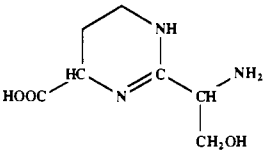
The ^{13}C NMR spectrum of the hydrolytic fragment **3** is assigned in Table XVIII. The multiplicity of each carbon atom was determined by distortionless enhancement by polarization transfer. This spectrum confirms the presence of serine. The chemical shifts measured for Cα (57.2 ppm) and Cβ (63.1 ppm) are in agreement with those published in the literature (Wüthrich, 1976b; Kalinowski et al., 1984). The signals of the other carbon atoms constituting the amino acid X are very close to those corresponding to the saturated ring of the chromophore. The signal at 165.3 ppm is also very close to the signal of a carbon bearing a guanidino group (δ = 157.8 ppm).

Table XVIII: Assignment of the Carbon Atoms of the Hydrolytic Fragment "502"[a]

chemical shift (ppm)	assignment	chemical shift (ppm)	assignment
178.7	COOH Gly	114.9 (d)	Chr C-5
174.1	CO Chr	102.8 (d)	Chr C-8
173.5	CO "X"	63.1 (t)	CH_2 "X"
165.1	C "X"	59.6 (d)	Chr C-11
151.4	Chr C-7	57.2 (d)	CH "X"
150.2	Chr C-2	54.4 (d)	CH "X"
146.4	Chr C-6	46.1 (t)	CH_2 Gly
130.4	Chr C-9	39.4 (t)	CH_2 "X"
128.3	Chr C-3	37.8 (t)	Chr C-13
125.2 (d)	Chr C-4	24.8 (t)	Chr C-12
119.0	Chr C-10	23.8 (t)	CH_2 "X"

[a] The chemical shifts (ppm) were determined with sodium ($^2H_6$)(trimethylsilyl)propanesulfonate as an internal standard. Multiplicity: (d) doublet and (t) triplet.

(*e*) *Structure of the Amino Acid X.* Taking into account all the results obtained by mass spectrometry and NMR on compound **3**, it was deduced that X has the composition $C_7H_{13}N_2O_2$ and is constituted with a serine to which is bound, via an amidine linkage, 2,4-diaminobutyric acid. The complete structure of this amino acid is represented by structure **5**.



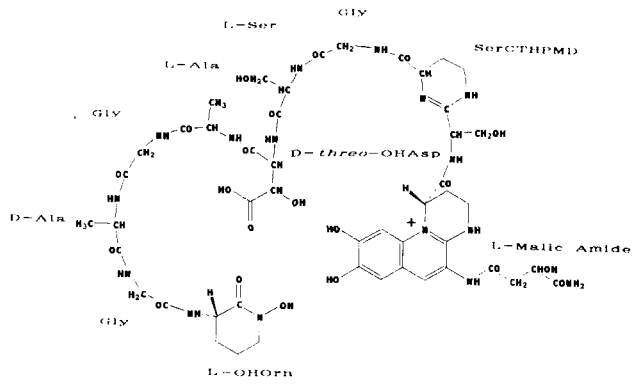
5

Under the hydrolytic conditions used (6 M HCl, 110 °C, 24 h), the amidine group is stable, but it was cleaved under more drastic acid conditions (12 M HCl, 180 °C, 12 h), as reported for cyclic amidines (Hawkins & Biggs, 1949). Serine as well as 2,4-diaminobutyric acid was characterized from such hydrolysates by GC–MS of their *O*-methyl, *N*-(pentafluoropropionyl) derivatives (see below).

(*f*) *Stereochemistry of the Different Constituents of the Siderophore.* The circular dichroism spectra of pyoverdin Pf and its iron complex were found to be very similar to those of pyoverdin Pt A (see Figure 1), showing that the chromophore has the same (*S*) configuration and that the coordination of the iron complex in solution is very similar. These data are very close to those reported for pseudobactin B 10 (Teintze et al., 1981) and pyoverdin Pa A (Demange et al., 1987, 1990). The configuration of the amino acids was determined by chiral gas chromatography in the same manner as for pyoverdin Pt A. The presence of hydroxyaspartic acid necessitates a predeactivation of the chiral column with diphenyltetramethylsilazane, since the *O*-methyl, *N*-pentafluoropropionyl ester of hydroxyaspartic acid tends to decompose on exposure to silicic acid, eliminating a pentafluoropropionyl group (Demange et al., 1988b). The data indicated L-serine, D-*threo*-hydroxyaspartic acid, L-N^δ-hydroxyornithine, D-alanine, and L-alanine. The location of L-alanine was determined after purification of a tripeptide Ser-OHAsp-Ala, isolated from a partial hydrolysate of pyoverdin Pf. The configuration of the tetrahydropyrimidine moiety could not be established since the amidine was only hydrolyzed under very drastic acidic conditions (180 °C, 48 h) or basic conditions (110 °C, 2 N NaOH). In both cases, serine and 2,4-diaminobutyric acid were characterized but as racemates. Malic amide was found to be L after total acid hydrolysis of pyoverdin Pf, esterification with methanol/HCl, and gas chromatography of the dimethyl ester of malic acid on capillary Chirasil-L-Val column. Au-

thentic samples of D,L- and L-malic acid were used as standards.

The complete structure of pyoverdin Pf is given by structure **6**.



6

(II) Structures of the Other Pyoverdins from P. fluorescens CCM 2798. The structures of the other siderophores isolated from the bacteria were deduced by using FAB mass spectrometry. They all possess the same peptide chain as pyoverdin Pf and differ in the chromophoric part of the molecule.

Pyoverdin Pf/1 with a mass of 1204 has a malic acid substituent, whereas pyoverdin Pf/2 with a mass of 1188 has a succinic acid substituent on the chromophore. Pyoverdin Pf/3/2–iron complex has a mass of 1240 and is decomplexed by acid, yielding the free ligand with a mass of 1187 whose substituent is therefore succinamide. The pyoverdin Pf/3/3 iron complex, with a mass of 1167, is different from the preceding pyoverdins since its chromophore is identical with the chromophore of azotobactin. This was confirmed after comparison of the spectrophotometric properties of both siderophores.

Conclusion

Most of the pyoverdins described in the present study are constituted with the same chromophore as pyoverdin Pa (Wendenbaum et al., 1983; Demange et al., 1987, 1990; Briskot et al., 1986, 1989) and all the similar siderophores reported in the literature (Philson & Llinas, 1982; Van der Helm et al., 1987; Teintze et al., 1981; Yang & Leong, 1984; Buyer et al., 1986; Poppe et al., 1987). One of the pyoverdins of *P. fluorescens* (pyoverdin Pf/3/3) possesses the same chromophore as azotobactin (Demange et al., 1988a). To the chromophore of each pyoverdin is bound a peptide chain differing for each strain. This chain is linear for both types of pyoverdins in contrast to the pyoverdins of *P. aeruginosa* ATCC 15692 for which it was proved to be partly cyclic (Briskot et al., 1989; Demange et al., 1990).

Pyoverdins Pf contain an unusual natural amino acid which is composed of 1 mol of serine condensed to 1 mol of 2,4-diaminobutyric acid, forming an amidine linkage. This type of naturally occurring amino acid has not previously been reported (Wagner & Musso, 1983). The new amino acid, which we call 2-seryl-6-carboxy-3,4,5,6-tetrahydropyrimidine, was relatively stable to acid hydrolysis and was not easily cleaved to its constituent amino acids under standard conditions. However, cleavage was readily achieved in basic conditions as expected for an amidine (Shriner & Neumann, 1944) and resulted in total racemization.

This amino acid can be formed from the attack of the γ-nitrogen atom of a diaminobutyryl residue on the seryl carboxyl group and elimination of a water molecule. Such a possibility appears feasible in view of the data from recent studies of specific cleavage of transpeptidation of synthetic peptides containing 2,4-diaminobutyric acid (Blodgett & Loudon, 1989). The location of a basic amino acid at position 2 seems to be a general feature in most pyoverdins (Demange et al., 1987, 1989). In pyoverdin Pf, this basic function is contained in the amidine part of the tetrahydropyrimidine ring of this unusual amino acid.

In pyoverdin Pf, the acyl group at position C-3 of the chromophore [which is common to all the pyoverdins so far investigated (Wendenbaum et al., 1983; Demange et al., 1987, 1989)], is malic amide with the *S* configuration. Malic acid was previously found in pseudobactin 7SR1 (Yang & Leong, 1984), but no stereochemistry was reported.

All these pyoverdins (Demange et al., 1987, 1989) possess three bidentate groups which strongly bind Fe(III), the catechol from the chromophore, the C-terminal N^{δ}-hydroxypiperidine, and either β-D-*threo*-hydroxyaspartic acid (pyoverdins Pf) or N^{δ}-acetyl-N^{δ}-hydroxyornithine (pyoverdins Pt) in the middle of the peptide chain, and give very stable octohedral metal(III) complexes of 1:1 stoichiometry with Fe(III), Al(III), and Ga(III) as shown by most siderophores (Raymond & Carrano, 1979). Pyoverdins Pf have the same bidentate groups as pseudobactin B 10 (Teintze et al., 1981) although the C-terminal cyclic L-N^{δ}-hydroxyornithine has the opposite configuration to that in pseudobactin B 10. In contrast, pyoverdins Pt have the same configuration as pseudobactin B 10 for this C-terminal residue. In spite of their structural differences, the circular dichroism spectra of the complexes of these pyoverdins Pf, pyoverdins Pa, pyoverdins Pt, and pseudobactin B 10 are very similar, which suggests that they have the same type of coordination around the metal in solution.

Acknowledgments

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Registry No. Pyoverdin Pt A, 130145-72-1; pyoverdin Pt, 130167-80-5; pyoverdin Pt B, 130167-81-6; pyoverdin Pf, 130145-73-2; pyoverdin Pf/1, 130145-74-3; pyoverdin Pf/2, 130145-75-4; pyoverdin Pf/3/2, 130145-76-5; pyoverdin Pf/3/3, 130167-82-7.

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Cooperative Binding of R17 Coat Protein to RNA†

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ABSTRACT: The binding of the R17 coat protein to synthetic RNAs containing one or two coat protein binding sites was characterized by using nitrocellulose filter and gel-retention assays. RNAs with two available sites bound coat protein in a cooperative manner, resulting in a higher affinity and reduced sensitivity to pH, ionic strength, and temperature when compared with RNAs containing only a single site. The cooperativity can contribute up to −5 kcal/mol to the overall binding affinity with the greatest cooperativity found at low pH, high ionic strength, and high temperatures. Similar solution properties for the encapsidation of the related fr and f2 phage suggest that the cooperativity is due to favorable interactions between the two coat proteins bound to the RNA. This system therefore resembles an intermediate state of phage assembly. No cooperative binding was observed for RNAs containing a single site and a 5′ or 3′ extension of nonspecific sequence, indicating that R17 coat protein has a very low nonspecific binding affinity. Unexpectedly weak binding was observed for several RNAs due to the presence of alternative conformational states of the RNA.

R17 coat protein binds tightly to a single hairpin in the 3600-nt R17 genomic RNA, resulting in repression of phage replicase synthesis. This system has been extensively studied as an example of a specific RNA–protein interaction (Romaniuk et al., 1987). The RNA–protein complex has also been shown to act as a precursor for assembly of the phage capsid, with the translational operator acting as a specific nucleation site (Hung et al., 1969; Ling et al., 1969). Qualitative studies have shown that capsid formation is very cooperative with no observed intermediates, presumably due to extensive protein–

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