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Bacterial siderophores: structure and NMR assignment of pyoverdins Pa, siderophores of *Pseudomonas aeruginosa* ATCC 15692

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Summary. In iron-deficient conditions, *Pseudomonas aeruginosa* ATCC 15692 synthesizes two major siderophores, pyoverdins Pa and pyoverdin Pa B. Two other compounds, pyoverdin Pa A (occurring from hydrolysis of pyoverdin Pa during the culture) and pyoverdin Pa C (occurring artifactually during the purification procedure) were also isolated. All these compounds possess the same partly cyclic peptide chain

D-Ser-L-Arg-D-Ser-L-Orn(δOH·HCO)-L-Lys-L-Thr
                                  | |
                L-Orn(δOH·HCO)-L-Thr

where L-Orn(δOH·HCO) is N^{δ}-formyl, N^{δ}-hydroxy-L-ornithine. The chain is bound to a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline and having the (*S*) configuration. The four pyoverdins differ only in the acyl substituent bound to the nitrogen atom bound to carbon C3 of the chromophore. This is succinamide (pyoverdin Pa), succinic acid (pyoverdin Pa A), methyl succinate (pyoverdin Pa C) and 2-oxoglutaric acid (pyoverdin Pa B). The complete ^{1}H- and ^{13}C-NMR assignments, using two-dimensional total correlation NMR spectroscopy (TOCSY) and rotating-frame Overhauser enhancement spectroscopy (ROESY) procedures, as well as ^{1}H–^{13}C correlations, are reported. The complete sequence of the peptide using CHα–NH correlations was achieved by NMR and confirmed the partly cyclic structure earlier reported using fast-atom-bombardment mass spectrometry (FAB-MS) on the siderophores and their dansylated fragments [Briskot G, Taraz K, Budzikiewicz H (1989) *Liebigs Ann Chem:* 375–384]. The use of these NMR procedures appears to be a tool of choice and a complementary approach to FAB-MS in the structure determination of some complex pyoverdins.

Key words: Siderophores – Pyoverdins – *Pseudomonas aeruginosa* – FAB-MS – NMR

Introduction

In iron-deficient conditions, most microorganisms synthesize and secrete small molecules called siderophores. These compounds bind iron very firmly and transport it into cells by high-affinity transport systems (Neilands 1974, 1981, 1984). The first step in this transport is the chelation of the iron present in the external medium by the siderophores. This chelation generally occurs via bidentate chelating groups which are very often catecholates, hydroxamates or hydroxy acids (Hider 1984). In spite of the large variety of structures presented by these siderophores, they all have iron coordinated as octahedral complexes (Raymond and Carrano 1979).

Pseudomonas aeruginosa is an opportunistic pathogenic bacterium which is the cause of 10%–20% of the infections in hospitals, especially those involved with cystic fibrosis, acute leukemia or with patients having undergone organ transplantation (Bodey et al. 1983). The virulence of *P. aeruginosa* is closely related to its iron metabolism (Weinberg 1978, 1984). The iron proteins such as transferrin or lactoferrin occurring in the blood decrease the concentration of free iron, thereby playing a bacteriostatic role (Bullen 1981). In these iron-deficient conditions, the bacteria synthesize siderophores which facilitate their growth (Cox and Graham 1979; Wendenbaum et al. 1983). It was recently shown that siderophore-mediated iron acquisition from transferrin by *P. aeruginosa* can be enhanced by the concerted action of the elastase excreted by this bacterium, suggesting that both siderophore and elastase can be factors of virulence for *P. aeruginosa* (Döring et al. 1988).

Pseudomonas aeruginosa ATCC 15692 synthesizes two types of siderophores: pyochelin and pyoverdins. The structure of pyochelin **1** was determined by ^{1}H and

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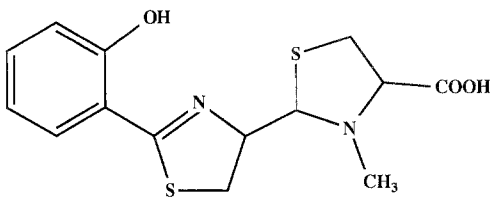
Abbreviations. Ser, serine; Arg, arginine; Thr, threonine; Lys, lysine; OHOrn, N^{δ}-hydroxyornithine; Chr, chromophore

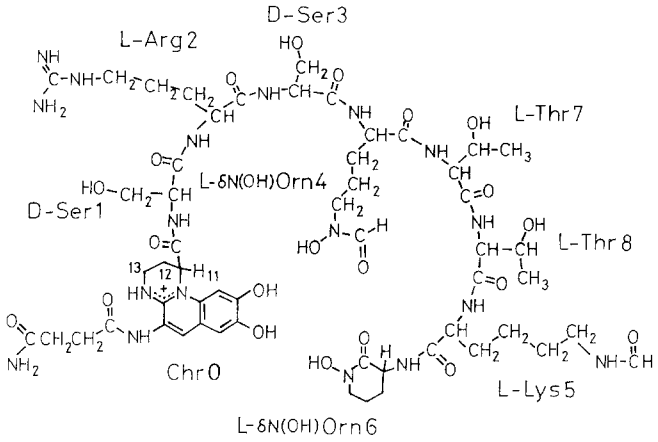


1

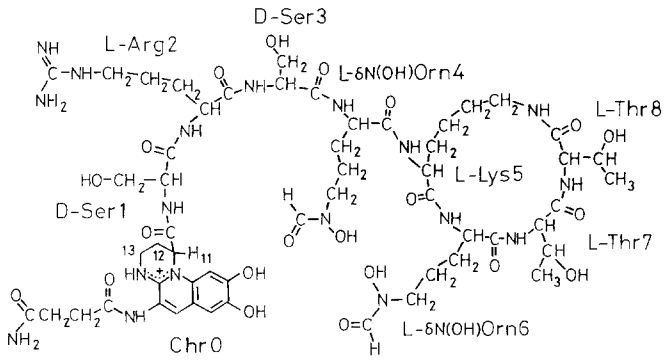
^{13}C NMR (Cox and Graham 1979; Cox et al. 1981). It is a phenolate bound to a thiazolinylthiazolidine moiety. Physicochemical studies of the pyochelin-Fe(III) complexes have shown that their stoichiometry is 2:1 and that their association constant is very low ($2.4 \times 10^5\ M^{-1}$; Cox and Graham 1979). This siderophore can be isolated from the culture supernatants only in small amounts (≈ 6 mg/l). Pyochelin is only responsible for 5–10% of the iron transport in *P. aeruginosa* (Ankenbauer et al. 1985).

The main siderophore of *P. aeruginosa* is in fact a mixture of compounds which we call the pyoverdins Pa. These compounds are synthesized in iron-deficient conditions up to 200 mg/l and their association constants are extremely high ($\approx 10^{32}\ M^{-1}$; Abdallah et al., unpublished results). The structure of the main pyoverdin of *P. aeruginosa*, pyoverdin Pa, has been described earlier (Wendenbaum et al. 1983; Demange et al. 1987). It was reported to be an octapeptide constituted with D and L amino acids bound to a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline (**2a**). Recently another structure (**2b**), based mainly on degradation and chemical transformation studies, has been proposed (Briskot et al. 1989).

In order to clarify this situation, we have performed additional two-dimensional (2D) NMR experiments using total correlation NMR spectroscopy (TOCSY) with the help of rotating-frame Overhauser spetroscopy (ROESY) and homonuclear Hartmann-Hahn spectroscopy (HOHAHA) and have unequivocally assigned for the first time all the resonances of pyoverdins Pa. This is the first application of TOCSY and ROESY NMR to the characterization of pyoverdins and clearly illustrates the power of NMR strategy for sequencing complex peptides when FAB-MS cannot give a complete structure determination. Further, we have investigated the biosynthesis of the pyoverdins and we also explain the possible contradictions related to the attachment of 2-oxoglutaric acid to the chromophore in pyoverdin Pa B.



2a



2b

Materials and methods

Strain and culture medium. Pseudomonas aeruginosa strain ATCC 15692 (PA01) was grown in aerobic conditions. The culture medium 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. It was adjusted to pH 7.0 before sterilization.

Isolation and purification of the pyoverdins

The bacteria were grown aerobically at 25° C in conical flasks each containing 0.5 l culture medium and subject to mechanical agitation. The pH of the culture was periodically adjusted to 7.0 by careful addition of hydrochloric acid (6 M). After 48 h, the culture medium (4.5 l overall) was centrifuged and the pyoverdins extracted from the supernatant. Two methods were used, as follows.

a) Complexation-decomplexation method. This is based on the solubility of the pyoverdin-iron(III) or pyoverdin-aluminum(III) complexes in phenol/methylene chloride mixtures. It is a modification of the procedure used by Meyer and Abdallah (1978) for the purification of pyoverdin Pf. At the end of the exponential phase (48-h culture), the cultures were centrifuged at 20000 *g* for 30 min at 4° C. The pH of the supernatant was adjusted to 5.0 by careful addition of formic acid. The supernatant was then extracted with ethyl acetate in order to remove secondary compounds synthesized by the bacteria (Itoh et al. 1971). The aqueous phase was treated with 5 ml 2 M iron chloride or aluminum chloride solution. The precipitate of inorganic phosphates was then removed by centrifugation and the supernatant was concentrated under reduced pressure to 0.5 l saturated by addition of sodium chloride and extracted three times with 100 ml of a mixture of methylene chloride/phenol (1:1, vol./mass). The organic phase containing the metal complexes was separated from the aqueous phase after centrifugation (4000 rpm, 10 min, room temperature).

It was then treated with equal volumes of diethyl ether and water (0.5 l). The aqueous phase was washed three times with 200 ml ether, evaporated under reduced pressure and lyophylized. The crude pyoverdin-iron(III) or pyoverdin-aluminum(III) complexes were dissolved in 5 ml 1 M pyridine/acetate pH 5.0 and chromatographed on a column of CM-Sephadex C-25 ($l = 40$ cm, $\Phi = 3$ cm) made up in the same buffer at a flow rate of 1 ml/min. The fractions (5 ml) were monitored by spectrophotometry at 403 nm. Three major pyoverdin-metal(III) complexes were isolated: pyoverdin-Pa-B-metal(III), pyoverdin-Pa-A-metal(III) and pyoverdin-Pa-metal(III). Their proportion varied according to the pH and the age of the culture. The fractions were pooled and lyophilized. The pyoverdin-Pa-iron(III) was decomplexed according to Meyer and Abdallah (1978). The pH of each fraction was adjusted to 3.0 by addition of a 10% solution of acetic acid; 2 vol. 5% 8-hydroxyquinoline in methylene chloride was added and the biphasic mixture was transferred into a stoppered flask which was vigorously shaken for 30 min on a shaker. The aqueous phase was then separated from the organic phase in a separatory funnel; its pH was readjusted to 3.0 before a new treatment with 8-hydroxyquinoline. Four treatments were necessary to remove iron(III) from the pyoverdins. The aqueous phase was finally washed three times with methylene chloride in order to remove the excess of 8-hydroxyquinoline. After evaporation under reduced pressure of the aqueous phase, each pyoverdin thus obtained was chromatographed on a CM-Sephadex C-25 column ($l = 15$ cm, $\Phi = 1.6$ cm) made up in 0.1 M pyridine/acetate pH 5.0. The column was first eluted isocratically with the same buffer (60 ml) then with a linear 0.1–2 M gradient of pyridine/acetate pH 5.0 (2×125 ml). The yields were 39 mg pyoverdin Pa B, 68 mg pyoverdin Pa A and 270 mg pyoverdin Pa/l culture.

b) Hydrophobic chromatography method. After centrifugation, the bacterial supernatant (4.5 l) was filtered through a 0.45-μm membrane (Millipore) adjusted to pH 4.0 and applied to a column of octadecylsilane (Lichroprep RP 18, 40–63 μm, Merck, Darmstadt) in the same conditions as described for the purification of azotobactin (Demange et al. 1988). The pyoverdins were eluted with a 1:1 mixture of acetonitrile and 0.05 M pyridine/acetate pH 5.0. The corresponding pyoverdin fractions were pooled and chromatographed on a CM-Sephadex C-25 column ($l = 30$ cm, $\Phi = 34$ cm) made up in 0.05 M pyridine/acetate pH 5.0. The column was first eluted isocratically with the same buffer (0.6 l), then with a linear gradient of 0.05–2 M pyridine/acetate pH 5.0 (2×1 l). The fractions (5 ml) were monitored by spectrophotometry at 380 nm. Three major fractions were separated: pyoverdin Pa B (150 mg), pyoverdin Pa A (350 mg) and pyoverdin Pa (200 mg).

Electrophoresis, absorption spectroscopy, circular dichroism, mass spectrometry, high-pressure column chromatography, amino acid analyses, ^1H-^1H and ^1H-^{13}C correlated NMR spectroscopy (COSY). These were all performed as described previously (Demange et al. 1988). For TOCSY, HOHAHA and ROESY NMR measurements, the samples were up at pH 4.0 to a concentration of 10 μM in 0.5 ml of a 90% H_2O/10% D_2O solution bubbled with argon in order to remove any dissolved oxygen. The spectra were recorded at 25° C on a Bruker AM-400 spectrometer in the phase-sensitive absorption mode with quadrature detection in both dimensions, using the time-proportional phase-incrementation method described by Marion and Wüthrich (1983). The through-bond connectivity was obtained from a TOCSY (Braunschweiler and Ernst 1983) or HOHAHA (Bax and Davis 1985b) spectrum recorded with the MLEV-17 pulse sequence (Bax and Davis 1985b) using a 30-μs 90° pulse. A mixing time of 70 ms was used with a spin-locking radiofrequency field strength of 8.3 kHz. In the ROESY experiment (Bothner-By et al. 1984; Bax & Davis 1985a), the spin-lock was achieved by a decoupler pulse of 350-ms duration, using a 2.5-kHz radiofrequency field. The carrier frequencies of both transmitter and decoupling channels were synchronized. In all experiments using spin-lock, the carrier frequency was centered on the water resonance and a relaxation delay of 1.2 s was used. The water signal was suppressed by a continuous low-power irradiation during the relaxation delay. The spectral width in F_1 and F_2 was 5000 Hz; 256 experiments with 128 scans of 1024 complex points in t_2 were collected. The data points in t_1 and t_2 were zero-filled and the squared sine-bell shifted ($\pi/4$) apodization was performed in both dimensions.

The hydrolysis kinetics of the formyl groups were followed overnight by automatic recording of NMR spectra at pH 1.0 and 40° C. The assignment of different spin system was made before (at pH = 4.0) and after hydrolysis using the TOCSY experiment.

Results and discussion

Purification and general properties of the pyoverdins Pa

These siderophores were purified according to two methods. The first and the more classical one is the method of complexation/decomplexation. This was first used by Zähner et al. (1963) for the purification of sideramines from Aspergillaceae and applied to *Pseudomonas fluorescens* CCM 2798 (Meyer and Abdallah 1978) and, with slight modifications, to *Pseudomonas aeruginosa* ATCC 15692 (Wendenbaum et al. 1983). This method is based on the ability of the siderophore-iron(III) complexes to be extracted into organic solvents. We introduced the second method in our work on azotobactin and showed that, using octadecylsilane, we could easily remove all the salts and small molecules and isolate the siderophores in excellent yield (Demange et al. 1988). This procedure was much faster than the complexation/decomplexation and gave a better yield of the pyoverdins Pa. Both methods yielded three major siderophores with very similar properties: pyoverdin Pa, pyoverdin Pa A and pyoverdin Pa B.

The criteria used to check the purity of these compounds and of their complexes were film electrophoresis and HPLC. In film electrophoresis, at pH 5.0, each pyoverdin (or its iron complex) is positively charged. HPLC analyses of the free ligands require the pretreatment of the columns by EDTA solutions to remove any trace of metallic cations which are the main source of artifacts for these types of compounds (Cramer er al. 1984). The eluent system, which contains EDTA and octylsulfonic acid, permitted a very good and reproducible separation of pyoverdin Pa, pyoverdin Pa A and pyoverdin Pa B, by ion-pair liquid chromatography in the presence of octylsulfonic acid (Knox 1978). The retention times were very different, being 3.7 min (pyoverdin Pa B), 8.4 min (pyoverdin Pa) and 9.6 min (pyoverdin Pa A); in addition, an extra peak eluted at 13.7 min. The latter was an unstable compound, pyoverdin Pa C, which upon further preparative purification yielded pyoverdin Pa A. The nature of this compound was etablished by FAB-MS (see below).

Biosynthesis of the pyoverdins as a function of time

HPLC analysis of bacterial supernatants as a function of culturing time showed that pyoverdin Pa is the major siderophore excreted by *P. aeruginosa*. After a 40-h cul-

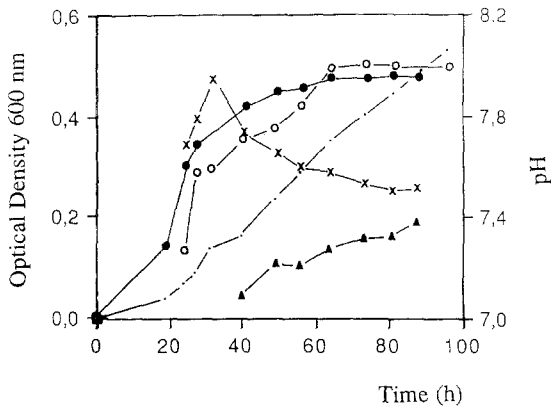


Fig. 1. Growth curve of *Pseudomonas aeruginosa* (●), variation of pH of the culture (- · - · -) and formation of pyoverdin Pa (×), pyoverdin Pa A (▲) and pyoverdin Pa B (○) as a function of the time of culture

ture, measurable amounts of pyoverdin Pa A were observed. The appearance of this compound coincided with the decrease of pyoverdin Pa (Fig. 1). This is a consequence of the increase of the pH of the culture medium and is due to the hydrolysis of pyoverdin Pa into pyoverdin Pa A (see below). This hydrolysis is faster as the pH becomes more alkaline. Pyoverdin Pa B is biosynthesized, like pyoverdin Pa, from the beginning of the culture but in lesser amounts. No pyoverdin Pa C was detected in this experiment suggesting that this compound was an artifact occurring during the purification process.

Physicochemical properties

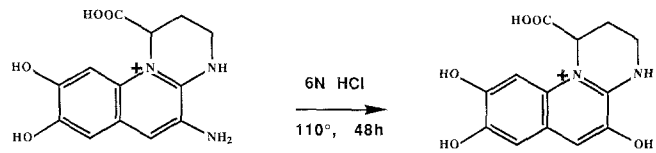
The absorption spectra of the pyoverdin-Fe(III) complexes are pH-insensitive. Pyoverdin-Pa-Fe(III) and pyoverdin-Pa-A-Fe(III) exhibit a maximum at 403 nm ($\varepsilon = 1.9 \times 10^4\ M^{-1} \times cm^{-1}$) and two shoulders at 460 nm ($\varepsilon = 4.0 \times 10^3\ M^{-1} \times cm^{-1}$) and 540 nm ($\varepsilon = 3.0 \times 10^3\ M^{-1} \times cm^{-1}$). The spectrum of pyoverdin-Pa-B-Fe(III) differs slightly from the former by showing a bathochromic effect of 2 nm with a maximum at 405 nm ($\varepsilon = 1.9 \times 10^4\ M^{-1} \times cm^{-1}$). At pH 4.2, pyoverdin Pa and pyoverdin Pa A gave two maxima at 365 nm ($\varepsilon = 1.4 \times 10^4\ M^{-1} \times cm^{-1}$) and 380 nm ($\varepsilon = 1.4 \times 10^4\ M^{-1} \times cm^{-1}$). The same bathochromic effect of 2 nm was observed for pyoverdin Pa B the maxima of which occur at 367 nm ($\varepsilon = 1.4 \times 10^4\ M^{-1} \times cm^{-1}$) and 382 nm ($\varepsilon = 1.4 \times 10^4\ M^{-1} \times cm^{-1}$). The spectra of the free ligands are very pH-sensitive and show isosbestic points at 373 nm for pyoverdin Pa and pyoverdin Pa A and at 375 nm for pyoverdin Pa B.

The circular dichroic spectra of the iron complexes are pH-independent and showed a positive Cotton effect at 400 nm ($\Delta\varepsilon = +1.9$) and two negative Cotton effects at 460 nm ($\Delta\varepsilon = -1.13$) and 540 nm ($\Delta\varepsilon = -0.3$). The circular dichroic spectra of the free pyoverdins are very pH-sensitive. At pH 4.0, they present a positive Cotton effect at 365 nm ($\Delta\varepsilon = +3.33$) and at 380 nm ($\Delta\varepsilon = +2.67$). These spectra are comparable to those reported by Teintze et al. (1981) for pseudobactin B10.

Fast-atom-bombardment mass spectrometry (FAB-MS) gave molecular ions (M^+) at *m/z* 1333 (pyoverdin Pa), at *m/z* 1334 (pyoverdin Pa A), at *m/z* 1362 (pyoverdin Pa B) and at *m/z* 1348 (pyoverdin Pa C). The 1:1 stoichiometry of the corresponding iron complexes was also established by FAB-MS, which gave the corresponding molecular peaks at *m/z* 1386, *m/z* 1387, *m/z* 1415 and *m/z* 1401, respectively.

Structure elucidation of the pyoverdins

Total acid hydrolysis (48 h, 6 M HCl, 110° C or 48 h, 7.5 M HI, 110° C) of the three pyoverdins indicated that they are constituted with a chromophore bound to the same peptide moiety of 8 amino acids possessing serine (2), arginine (1), lysine (1), threonine (2) and N^{δ}-hydroxyornithine (2). After this hydrolysis step it was observed that the chromophore isolated from the hydrolyzates of pyoverdin Pa and pyoverdin Pa A differed in λ_{max} from the starting chromophore ($\lambda_{max} = 352$, 364 nm for the hydrolysis product compared to λ_{max} 364, 380 nm for the native pyoverdins). Hydrolysis of pyoverdin Pa B resulted in a change in colour of the solution which corresponded to a shift of the absorption maximum to 410 nm (see below).



3a **3b**

These observations, together with the NMR data (see below), indicated that the chromophores of pyoverdin Pa and pyoverdin Pa A are the same as those of pseudobactin (Teintze et al. 1981) and possess the structure **3a** giving upon acid hydrolysis compound **3b** (probably by addition of one molecule of water at C2, hydrolysis of the enamine thus obtained, and rearomatization). The structure of the chromophore of pyoverdin Pa B will be discussed below.

Sequence of the peptide chain

The peptide sequence of pyoverdin Pa A (and pyoverdin Pa) was determined from the data afforded by FAB-MS analysis of the intact siderophore and of products of partial hydrolysis.

The positive FAB spectrum of pyoverdin Pa A is reproduced in Fig. 2. The molecular ion M^+ at *m/z* 1334 dominates the spectrum. The major fragment at *m/z* 1031 corresponds to the loss of a chromophoric frag-

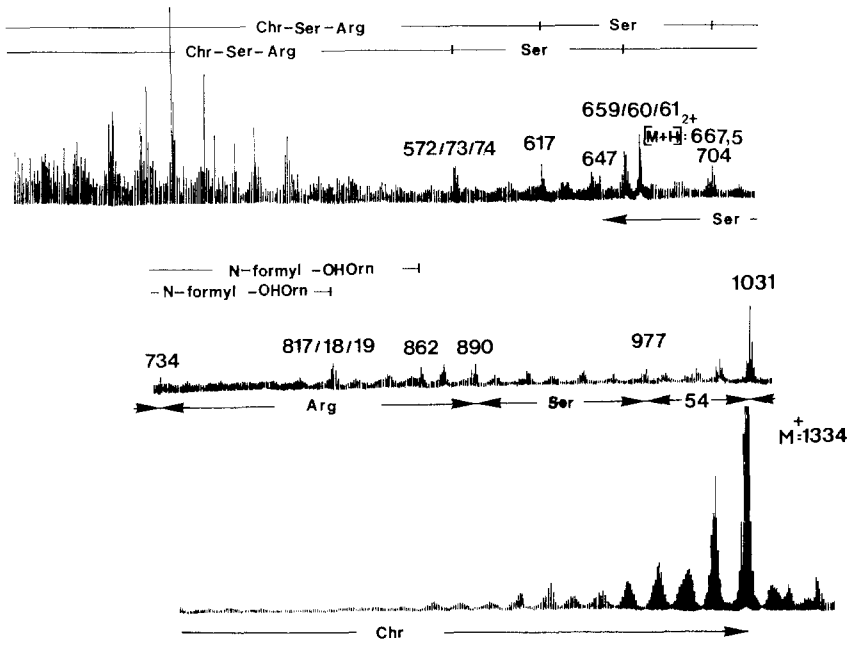
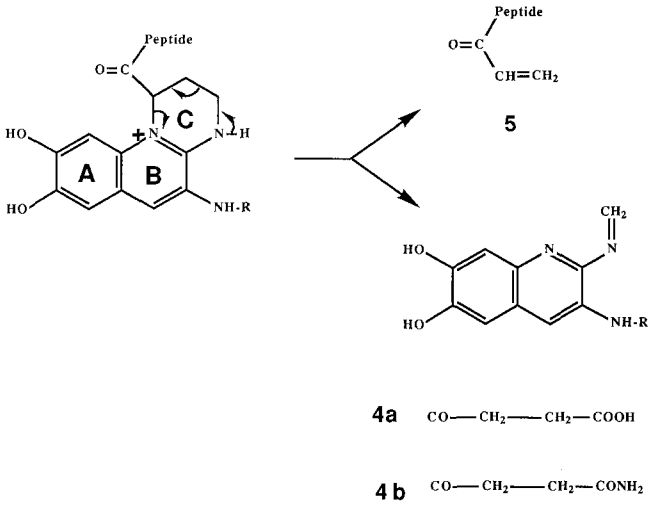


Fig. 2. FAB-MS spectrum of pyoverdin Pa A



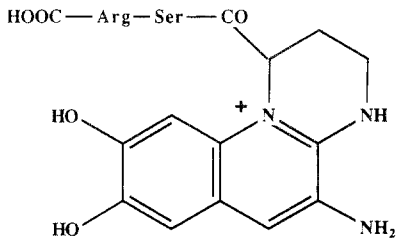
ment of 303 Da from the molecular ion. The loss of **4a** from pyoverdin Pa and pyoverdin Pa A is due to a characteristic cleavage of the pyoverdin through the saturated cycle C of the chromophore, resulting in a major fragment ion constituted of the complete peptide chain together with two carbons from the C ring of the chromophore (structure **5**). Some fragmentation within the peptide chain occurs to give minor N- and C-terminal sequence ions from which a partial sequence can be deduced. N-terminal amide fragmentations occur at *m/z* 617 [(Chr-Ser-Arg), *m/z* 704 (Chr-(Ser,Arg)-Ser] and *m/z* 862 [Chr-(Ser,Arg)-Ser-Orn(OH·CHO)]. Each is accompanied by a cluster of signals 43, 44 and 45 Da lower, resulting from cleavage of the Cα-carbonyl bond (Demange et al. 1988). C-terminal fragment ions (assigned in Table 1) provide further evidence for the N-terminal sequence of pyoverdin Pa A and resolved the Ser/Arg ambiguity. Mild acid hydrolysis of pyoverdin Pa A (6 M HCl, 30 min, 100° C) afforded chromopeptide as well as peptide fragments. Four fluorescent molecules were isolated in various yields from this hydrolysis, one containing only serine, two containing serine and arginine [**6** (major product) and **7**] and one containing both amino acids in the ratio of 2:1. The structures of **6** and **7** were determined by FAB-MS of native (molecular ions at *m/z* 519 and 619, respectively) and methyl-esterified material (molecular ions at *m/z* 533 and 647, respectively) and by ^{1}H NMR (Tables 2 and 3).

NMR spectra of pyoverdin Pa and pyoverdin Pa A

Both pyoverdin Pa and pyoverdin Pa A show similar NMR spectra, although slight differences were observed and interpreted. The spectra were very sensitive

Table 1. Interpretation of the C-terminal fragments in the FAB-MS spectrum of pyoverdin Pa A

Mass (Da)	C-terminal fragments $(M+H)^+$
647	H_2N-Orn(δOH·CHO)—[Thr-Thr,Lys-Orn(δOH)+CHO—H_2O]
734	H_2N-Ser-Orn(δOH·CHO)—[Thr-Thr,Lys-Orn(δOH)+CHO—H_2O]
890	H_2N-Arg-Ser-Orn(δOH·CHO)—[Thr-Thr,Lys-Orn(δOH)+CHO—H_2O]
977	H_2N-Ser-Arg-Ser-Orn(δOH·CHO)—[Thr-Thr,Lys-Orn(δOH)+CHO—H_2O]
1031	H_2C=CH—CO—NH-Ser-Arg-Ser-Orn(δOH·CHO)—[Thr-Thr,Lys-Orn(δOH)+CHO—H_2O]



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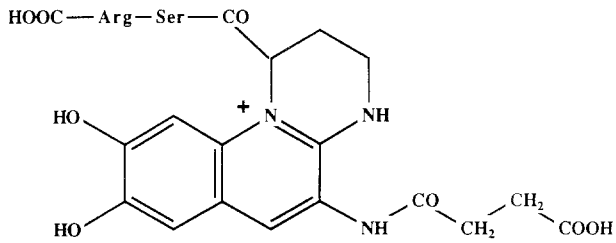

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Table 2. Assignment of the proton of the hydrolytic fragment **6**

Chemical shifts (ppm)	Multiplicity	Assignment
7.39	(s)	H4* Chr
7.09	(s)	H5* Chr
7.05	(s)	H8* Chr
5.84	(s)	H11 Chr
4.35	(t)	Hα Ser
4.23	(dd)	Hα Arg
3.99	(t)	Hβ Ser
3.75	(m)	H13, 13′ Chr
3.42		
2.70	(m)	Hδ Arg
2.57		
2.70–2.57		H12, 12′ Chr
1.77	(m)	Hβ Arg
1.52		
1.03	(m)	Hδ Arg

The chemical shifts were determined in ^2H_2O using sodium trimethylsilyl($^2H_6$)propanesulfonate as an internal standard. s indicates singlet, d doublet, t triplet and m multiplet

to pH variations. The detailed NMR assignments were made on pyoverdin Pa A which is produced in highest yields after the purification steps.

a) ^{1}H-NMR spectrum of pyoverdin Pa A

The ^{1}H-NMR spectrum of pyoverdin Pa A shows three singlets at 7.98 ppm, 7.24 ppm and 7.10 ppm and at higher field a singlet at 5.76 ppm. The values of these

Table 3. Assignment of the protons of the hydrolytic fragment 7

Chemical shifts (ppm)	Multiplicity	Assignment
7.99	(s)	H4 Chr
7.25	(s)	H5 Chr
7.16	(s)	H8 Chr
5.87	(m)	H11 Chr
4.37	(t)	Hα Ser
4.27	(dd)	Hα Arg
4.02	(t)	Hβ Ser
3.75	(m)	H13, 13′ Chr
3.40		
2.75	(m)	H-Succ
2.67		
2.75–2.67	(m)	Hδ Arg
2.75–2.67	(m)	H12, 12′ Chr
1.85	(m)	Hβ Arg
1.61		
1.03	(m)	Hδ Arg

The chemical shifts were determined in ^2H_2O using sodium trimethylsilyl($^2H_6$)propanesulfonate as an internal standard. s indicates singlet, dd double doublet, t, triplet and m multiplet

chemical shifts are very close to those reported for pseudobactin B10. They correspond, respectively, to the proton H-11 and to the three aromatic protons H-4, H-5 and H-8 of the chromophore **3a** derived from 2,3-diamino-6,7-dihydroxyquinoline. In addition, at lower field there are two sets of signals, one at 7.94 and 7.98 ppm corresponding to 1.6 proton and the second at 8.30 ppm corresponding to 0.4 proton. During mild acid hydrolysis monitored by ^{1}H NMR, these two pairs of signals disappear progressively giving rise to a singlet at 8.22 ppm corresponding to the non-exchangeable proton of formic acid. Therefore the two protons correspond to formyl groups which are in a very similar chemical environment. The same observation was made by Philson and Llinas (1982) in NMR studies of the pyoverdin of *Ps. fluorescens* ATCC 13525.

The two signals exhibited by each of these protons were assigned to the presence of *cis* isomers at 7.94 and 7.98 ppm and to *trans* isomers at 8.30 ppm characteristics of *N*-formyl, *N*-alkylhydroxylamines or *N*-formyl, *N*-alkylamines (Kolasa 1983). This assignment is in agreement with the ^{1}H NMR of pyoverdin Pa A enriched with 98.9% ^{15}N. Both signals at 7.94 ppm and 7.98 ppm present a $^2J_{^1H\text{-}^{15}N}$ of 20 Hz whereas the signal at 8.30 ppm shows a couling constant $^2J_{^1H\text{-}^{15}N}$ of 12 Hz. FAB-MS experiments showed (see below) that the formyl groups are located on the peptide chain and must therefore be *N*-formylating hydroxyornithine and/or lysine.

In the higher-field region between 1–4.5 ppm, only a few resonances could be assigned without ambiguity. The two doublets centered at 1.22 ppm and 1.25 ppm correspond to the methyl group of the threonines. The singlet at 1.94 ppm was assigned to the methyl group of an acetate anion. It disappears after ion-exchange chromatography of pyoverdin Pa A on DEAE-Sephadex A-25 (chloride form).

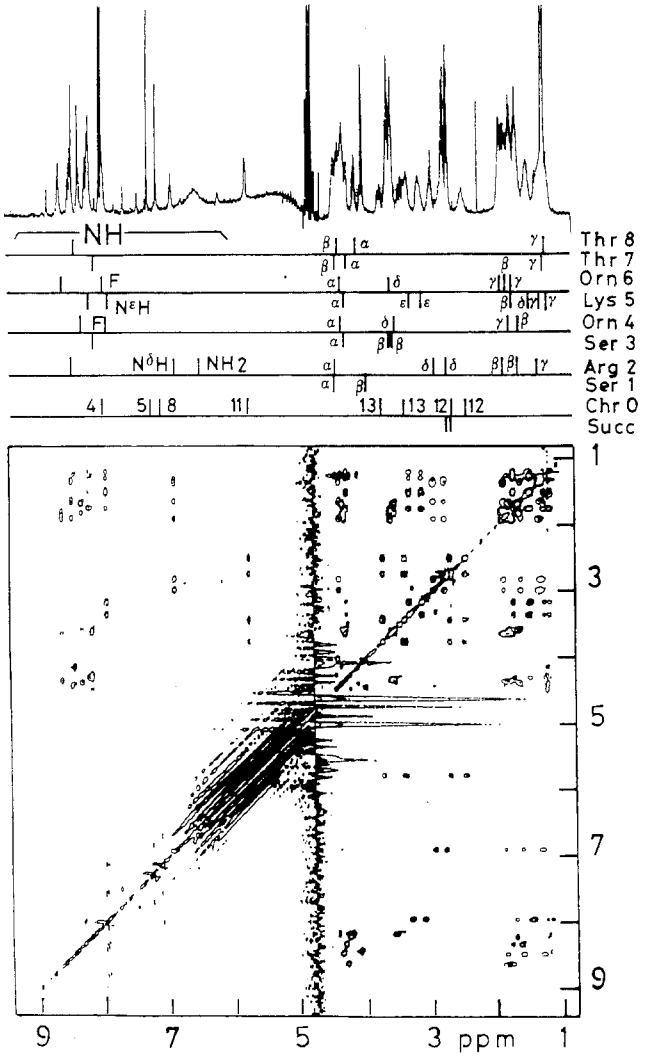


Fig. 3. Contour plot of the ^{1}H-TOCSY spectrum of pyoverdin Pa A in H_2O. The complete assignment is indicated in the upper part of the diagram. F refers to the formyl protons

The other signals were assigned by 2D-TOCSY NMR correlation. The correlation of the amino acids and the chromophore are presented in Fig. 3. Their chemical shifts are reported in Table 4. These values are in agreement with those reported in literature for amino acids (Wüthrich 1976a). The 2D-TOCSY spectrum of pyoverdin Pa A in H_2O is shown in Fig. 3. The assignment of the chromophore and the serine and threonine spin systems is straightforward. The ambiguity lies in the distinction of the four remaining connectivities of arginine, lysine and both hydroxyornithine residues. Undeuterated water preserves the NH signals and allows discrimination between the spin systems of arginine and lysine residues which each contain two NH protons, and those of the two hydroxyornithine residues which have only one NH proton each. The two NH resonances at 8.45 and 6.89 ppm and the two resonances at 2.97 and 2.80 ppm which exhibit the same number of cross-peaks with aliphatic protons correspond to the same residue. This is also the case for the two NH protons at 8.22 and 7.94 ppm which are connected to the signals at 3.34 and 3.14 ppm. The two triplet signals at 6.89 and 7.94 ppm are attributed to the NH protons bonded respectively to the $CH_2\delta$ and $CH_2\varepsilon$ side-chain groups of arginine and lysine. The complete assignment of the ^{1}H spectrum is given in Table 4. The aqueous solution of pyoverdin Pa A does not exhibit any NOE connectivities in 2D NOESY spectra. This result is generally observed for short flexible peptides due to the conformational averaging or to the unfavorable molecular correlation time ($\omega_0 \times \tau_c \leq 1$). Thus the ROESY experiment is essential for NOE analysis.

Table 4. ^{1}H-NMR assignments of pyoverdin Pa A from the 2D-TOCSY experiments

Residue δ ($\pm$0.01 ppm)						
	NH	CHα	CHβ	CHτ	CHδ	CHε
Ser 1	10.00	4.41	4.00			
Arg 2	8.45	4.40	1.64	1.34	2.80	6.80
			1.89		2.97	
Ser 3	8.14	4.27	3.56			
			3.61			
Orn(OH) 4	8.30	4.34	1.66	1.83	3.55	
Lys 5	8.22	4.30	1.75	1.19	1.50	3.14
				1.28		3.34
Orn(OH) 6	8.59	4.30	1.83	1.76	3.61	
				1.90		
Thr 7	8.14	4.23	4.46	1.25		
Thr 8	8.42	4.11	4.37	1.22		

Others	
Succinate	$COCH_2CH_2CO$ 2.69; 2.76
Chromophore	H-4 (7.98); H-5 (7.24); H-8 (7.10); H-11 (5.76); H-12 (2.48; 2.72); H-13 (3.40; 3.73)
Arg 2	NHε (6.89); $H_2NC=NH$ (6.53)
Orn(OH) 4	CHO *cis* (7.94); CHO *trans* (8.30)
Lys 5	NHζ (7.94)
Orn(OH) 6	CHO *cis* (7.98); CHO *trans* (8.30)

None of the NH(i)-NH($i+1$) cross-peaks is observed on the NOESY map for pyoverdin Pa A. However, the presence of CαH(i)-NH(i) and CαH(i)-NH($i+1$) NOE connectivities between neighboring residues can be used for the sequential assignment of the molecule. In Fig. 4, no cross-peak is observed between the NH(Arg2) at 8.45 ppm and the CαH(Ser) at 4.27 ppm. Consequently this resonance is attributed to the Ser3 residue and the resonance at 4.41 ppm to the Ser1 residue. Starting from arrow 1 for the CαH(Ser1)-NH(Arg2) cross-peak, the following CαH(Arg2)-NH/Ser3) (arrow 2) and CαH(Ser3)-NH(OHOrn4) (arrow 4) cross-peaks confirm the assignment of the Ser3 resonances. It also allows the assignment of the Ser1/Ser3 spin systems. In the same way, the three arrows 4, 5 and 6 allow discrimination between the Orn(δOH)4/Orn(δOH6) spin systems and location of the Lys5 residue between the two Orn(δOH) residues. All these observations are in favor of the structure proposed by Briskot et al. (1989).

The two threonine spin systems are delineated by the CαH(Orn6)-NH(Thr7) (arrow 6) and CαH(Thr7)-

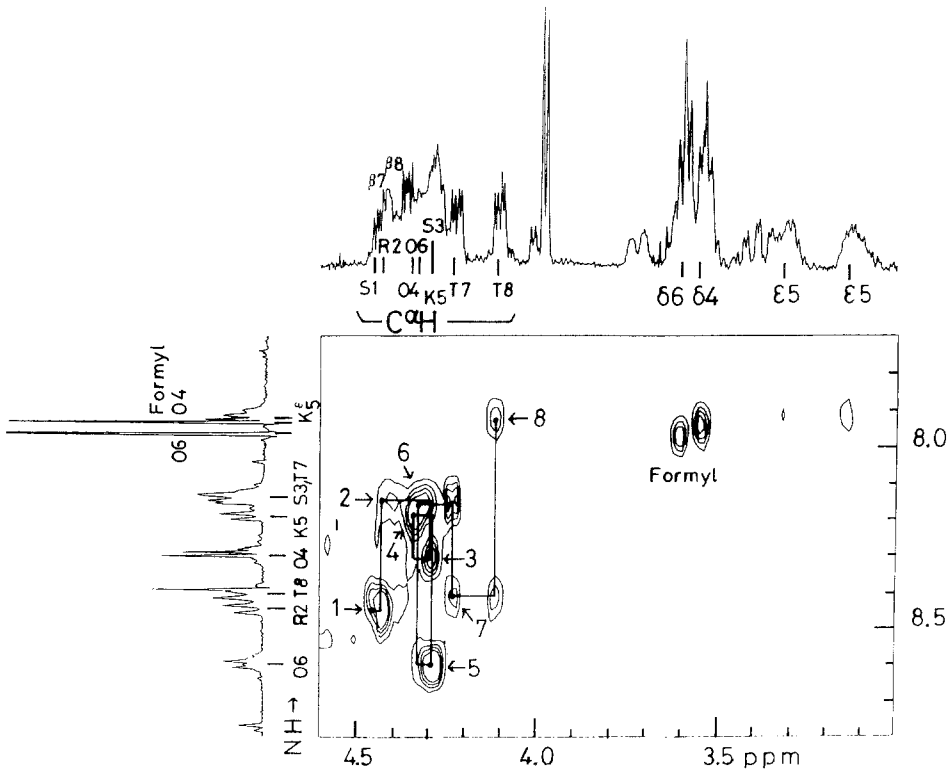


Fig. 4. ^{1}H-ROESY map showing the cross-peaks in X-CHα and Y-NH correlated region. The number assigned to each arrow indicates the position *i* of the CαH(*i*) in the amino acid sequence. The correlations for the following X-Y couples S_1-R_2 (arrow 1), R_2-S_3 (arrow 2), S_3-O_4 (arrow 3), O_4-K_5 (arrow 4), K_5-O_6 (arrow 5), O_6-T_7 (arrow 6), T_7-T_8 (arrow 7) and CHα(T_8)-NHε(K_5) (arrow 8) allow a distinction between Ser_1/Ser_3, Orn_4/Orn_6 and Thr_7/Thr_8 spin systems. Amino acid residues are represented in the one-letter code

NH(Thr8) (arrow 7) cross-peaks. Finally the connecting lines stop at the CαH(Thr8)-NζH(Lys5) (arrow 8) cross-peak. This connectivity proves that Lys5-NζH is bonded to the terminal carbonyl of the peptide backbone.

Assignment of the formyl groups

By analogy to *N*-methylformamide which gives two formyl signals at 8.0 ppm and 7.9 ppm corresponding to *trans* and *cis* forms respectively, we assigned the signal at 8.3 ppm to the *trans* and the signals at 7.94 ppm and 7.98 ppm to the *cis* form respectively.

In the ^{1}H-NMR spectrum of pyoverdin Pa A, the two formyl groups are split into two sets of signals with an intensity ratio of about 1:3 (*trans/cis*; Table 4). All the formyl signals appear as singlets. This indicate the absence of NH protons in the vicinity and invalidates our proposition that one formyl group is bound to Nε atom of lysine. The two formyl groups are thereby located on the Nδ atoms of hydroxyornithines as proposed by Briskot et al. (1989).

At different hydrolysis rates, both formyl proton resonances exhibit similar evolutions (Fig. 5). Simultaneously with the decrease in the peak intensity at 3.55 ppm and 3.61 ppm, an increase of the two triplet signals at 3.25 ppm and 3.35 ppm is observed. The TOCSY spectra performed before and after hydrolysis

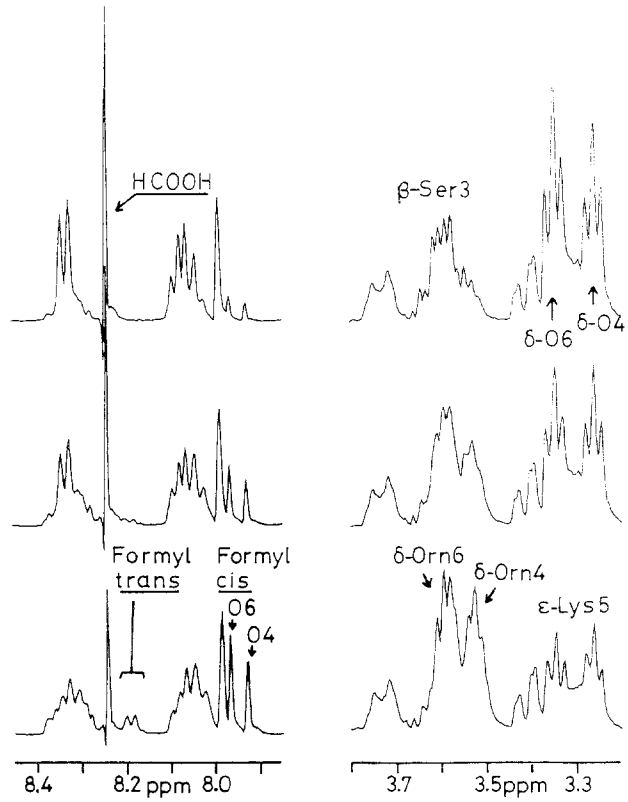


Fig. 5. Evolution of the formyl and $CH_2\delta$ proton resonances at different hydrolysis stages

indicate that all these signals arise from the $CH_2\delta$ of both Orn(δOH) residues. In addition, for both these residues, NOE cross-peaks between the formyl and the $CH_2\delta$ protons are observed on the ROESY spectrum (Fig. 4).

b) ^{13}C-NMR spectrum of pyoverdin Pa and pyoverdin Pa A

The ^{13}C spectrum of pyoverdin Pa A is presented in Fig. 6. It is characteristic of a chromopeptide. It can be divided into three regions: the carbonyl region (160–185 ppm), the aromatic region (100–160 ppm) and the aliphatic region (20–70 ppm). The assignment of each carbon atom was performed by measuring the $J_{^1H\text{-}^{13}C}$ coupling constants of the proton-undecoupled spectrum, the $J_{^{13}C\text{-}^{15}N}$ coupling constants of the 98.9% ^{15}N-enriched pyoverdin Pa A of the proton-decoupled ^{13}C-NMR spectrum as well as by ^{1}H-^{13}C heteronuclear correlation.

The carbonyl region between 183.8–159.8 ppm gives 16 peaks as expected. Only some of them were assigned without ambiguity. The carbonyl (180.2 ppm) and the carboxyl (183.8 ppm) of the succinyl group are readily distinguished from the other signals by the presence of two $^2J_{^1H\text{-}^{13}C}$ couplings. The carbonyl (180.2 ppm) bound to the nitrogen atom at the position C3 of the chromophore has in addition an extra $^1J_{^{13}C\text{-}^{15}N}$. The carbonyls of both formyl groups were assigned by heteronuclear 2D ^{1}H-^{13}C NMR. They are also characterized by the presence of a $^1J_{^{13}C\text{-}^{15}N}$ coupling (see Table 5). Each is present as two signals characteristic of a *cis-trans* isomerism.

The signals at 162.0 and 162.3 ppm correspond to the *cis* isomer of both N^δ-formyl-N^δ-hydroxyornithine and N^ε-formyllysine, whereas those at 166.50 ppm and 166.55 ppm correspond to the *trans* isomers. They were assigned using 2D ^{1}H-^{13}C correlation and by comparison of their chemical shifts to those of *N*-methylformamide (Levy and Nelson 1972). The carbon at 159.8 ppm corresponds to the guanidinium group of arginine

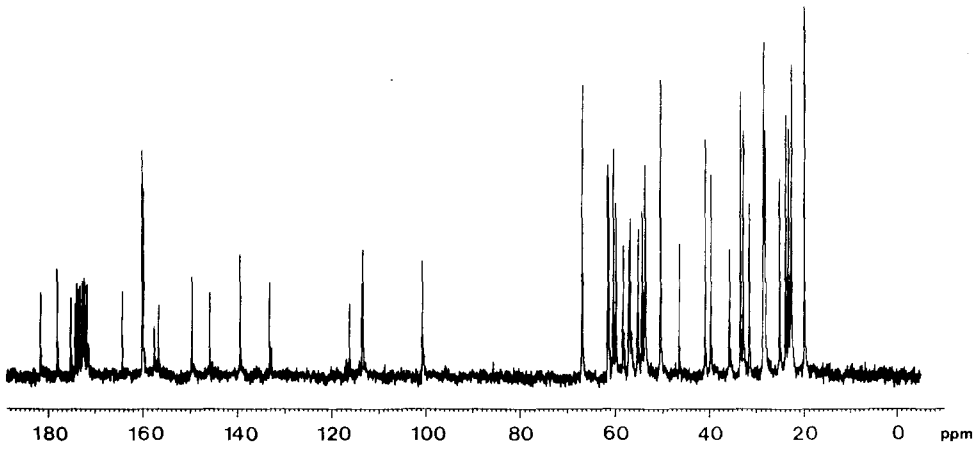


Fig. 6. ^{13}C-NMR spectrum of pyoverdin Pa A determined in ^2H_2O with sodium trimethylsilyl ($^2H_6$) propane sulfonate as an internal standard

Table 5. Assignment of the carbonyls of pyoverdin Pa A

Chemical shift (ppm)	$^1J_{^{13}C\text{-}^{15}N}$ (Hz)	$^1J_{^{13}C\text{-}^1H}$ (Hz)	$^3J_{^{13}C\text{-}^1H}$ (Hz)	Assignment
183.8	/	/	7.5 (t)	COOH Succ
180.2	13.0 (d)	/	7.5 (t)	CO-Succ
177.3	15.0 (d)	/	/	CO Orn(OH)
176.4	15.0 (d)	/	3.5 (d)	CO Arg
176.2	15.0 (d)	/	3.5 (d)	CO Lys
175.7	15.0 (d)	/	3.5 (d)	CO Orn(OH)
175.5	15.0 (d)	/	/	CO Thr
174.9	15.0 (d)	/	3.5 (d)	CO Thr
174.8	15.0 (d)	/	17.5; 3.5 (dd)	CO Chr
174.5	15.0 (d)	/	3.5 (d)	CO Ser
173.9	15.0 (d)	/	3.5 (d)	CO Ser
166.55	17.5 (d)	202.5 (d)	/	CHO(*trans*)
166.50	17.5 (d)	202.5 (d)	/	CHO(*trans*)
162.3	15.0 (d)	200.0 (d)	/	CHO(*cis*)
162.0	15.0 (d)	200.0 (d)	/	CHO(*cis*)
159.8	20.0 (d)	/	3.5 (m)	Cζ Arg

The chemical shifts were determined in ^2H_2O with sodium trimethylsilyl(^2H)$_6$propanesulfonate as an internal standard. d indicates doublet, t triplet and m multiplet

(Wüthrich 1976b; Kalinowski et al. 1984). The remaining resonances between 173.9–177.3 ppm correspond to the carbonyls of the amino acids. Their assignment was performed by comparison with values in the literature (Wüthrich 1976b; Kalinowski et al. 1984). The carbonyl of the chromophore (174.8 ppm) can be distinguished by the presence of two $^3J_{^{13}C-^1H}$ couplings of 3.5 Hz and 17.5 Hz.

In the next region, between 100–159 ppm there are nine signals, corresponding to three tertiary carbons at 102.9 ppm, 115.6 ppm and 141.5 ppm, and six quaternary carbon atoms at 115.8 ppm, 118.4 ppm, 135.4 ppm, 148.0 ppm, 151.8 pm and 158.9 ppm. These values obtained at pH 6.5 were compared to those reported for pseudobactin B10, the structure of which was determined by X-ray diffraction (Teintze et al. 1981). From the very close values of the chemical shifts, we concluded that pyoverdin Pa A (and pyoverdin Pa) possess the same chromophore **3a** as pseudobactin B10. The assignments of the three tertiary carbons C4 (141.5 ppm), C5 (115.6 ppm) and C8 (102.9 ppm) have been made by using the 2D ^1H-^{13}C correlation.

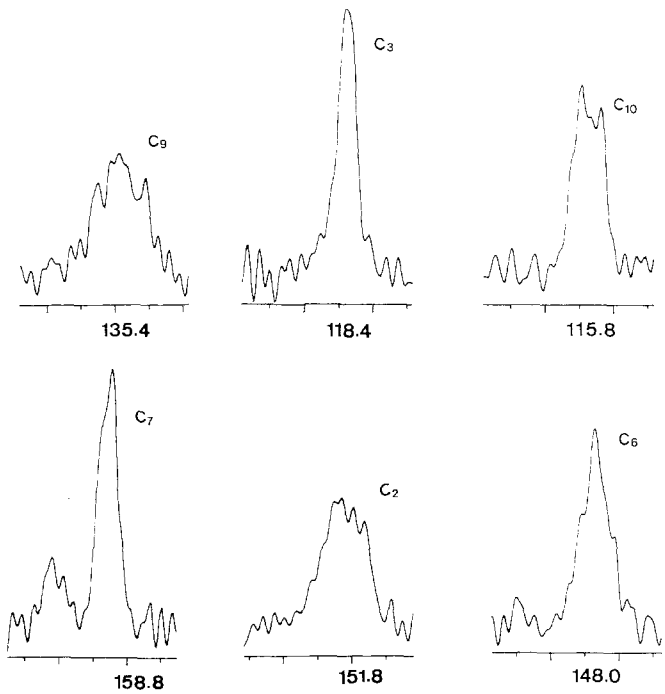


Fig. 7. Assignment of the quaternary carbon atoms of the chromophore of pyoverdin Pa A from their long-range coupling constants at 100 MHz

The quaternary carbon atoms were assigned by considering their J_2 and J_3 long-range couplings as well as the $^1J_{^{13}C-^{15}N}$ couplings. J_2 couplings are generally small. They cannot exceed 4 Hz and are generally in the range of 0.5–1 Hz for aromatic rings. On the other hand, J_3 couplings are much larger and are in the range of 10 Hz (Hansen 1979). C2 is substituted by two nitrogen atoms bearing a delocalized positive charge. C6 and C7 are each substituted with a hydroxyl group. These three carbon atoms resonate at lower field, precisely at 148.0 ppm, 151.8 ppm and 158.8 ppm. Carbon C2 (151.8 ppm) is distinguishable from C6 and C7 by the presence of two $^1J_{^{13}C-^{15}N}$ couplings of 20 Hz. For carbons C6 and C7 the latter is the more deshielded since it is situated in a *meta*-position to the carbon atom bearing the positively charged nitrogen N1. Therefore C7 resonates at 158.8 ppm ad C6 at 148.0 ppm.

The three remaining resonances at 115.8 ppm, 118.4 ppm and 135.4 ppm correspond to carbons C10, C3 and C9. C10 shows a $^3J_{^{13}C-^1H}$ with proton H8. It is represented as a doublet centered at 115.8 ppm (Fig. 7). C3 shows no ^3J long-range coupling and only one ^2J coupling, and is therefore assigned to the expected singlet occurring at 118.4 ppm. Moreover, the presence of a $^1J_{^{13}C-^{15}N}$ coupling confirms that this carbon is substituted by a nitrogen atom (Table 6). The signal at 135.4 ppm is a doublet of doublets (Fig. 7). It corresponds to carbon C9 which shows $^3J_{^{13}C-^1H}$ couplings with H4 and H5. In addition the assignment of this carbon is confirmed by the presence of a $^1J_{^{13}C-^{15}N}$ coupling.

The assignment of the aliphatic carbon atoms was performed by heteronuclear 2D ^{13}C-^1H correlation (Fig. 8). The multiplicity of each signal, in addition to the $^1J_{^{13}C-^{15}N}$ coupling constants obtained from the ^{13}C-NMR spectrum of the 98.9% ^{15}N-labelled pyoverdin Pa A, confirms these assignments. The chemical shifts of the aliphatic carbons and the $^1J_{^{13}C-^1H}$ and $^1J_{^{13}C-^{15}N}$ cou-

Table 6. Assignment of the aromatic carbon atoms of pyoverdin Pa A

Chemical shift (ppm)	$^1J_{^{13}C-^{15}N}$ (Hz)	$^1J_{^{13}C-^1H}$ (Hz)	$^3J_{^{13}C-^1H}$ (Hz)	Assignment
158.8	/	/	/	Chr C7
151.8	20.0 (t)	/	(m)	Chr C2
148.0	/	/	/	Chr C6
141.5	/	167.5 (dd)	/	Chr C4
135.4	15.0 (d)	/	7.5 (t)	Chr C9
118.4	15.0 (d)	/	/	Chr C3
115.8	/	/	6.0 (d)	Chr C10
115.6	/	162.5 (dd)	/	Chr C5
102.9	/	157.5 (d)	/	Chr C8

The chemical shifts were determined in ^2H_2O with sodium trimethylsilyl($^2H_6$)propanesulfonate as an internal standard. d indicates doublet, t triplet and dd double doublet

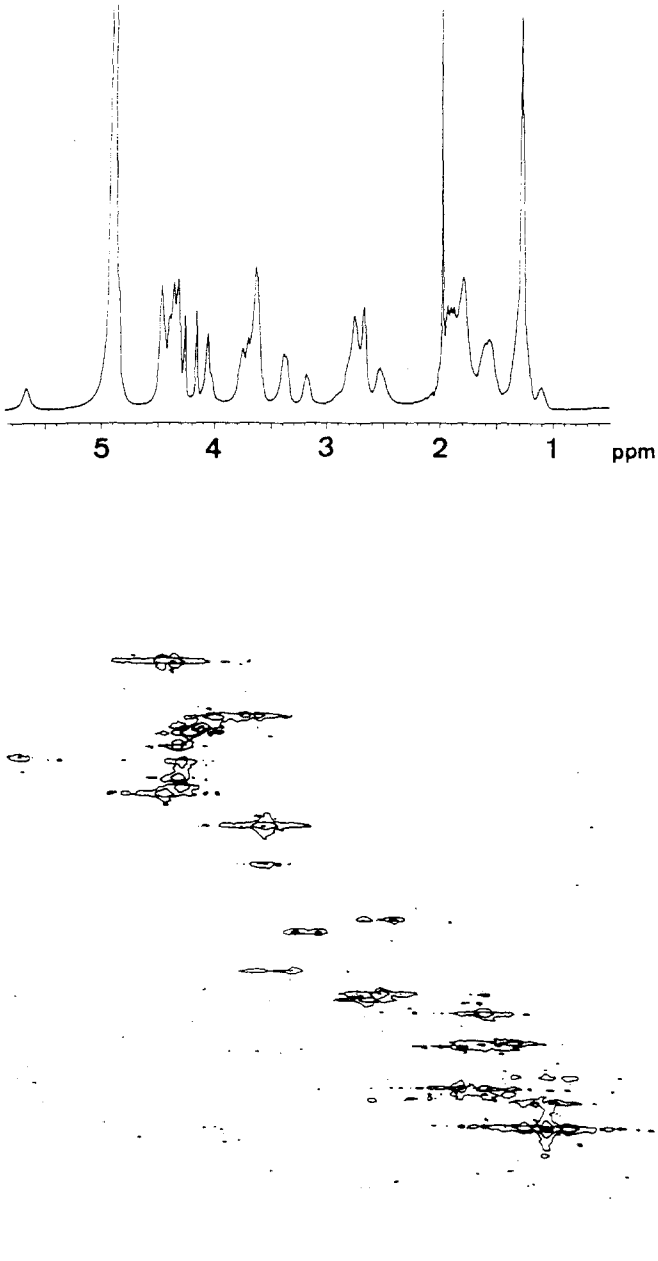


Fig. 8. ^{1}H-^{13}C heteronuclear correlation NMR spectrum of pyoverdin Pa A determined in $^{2}H_2O$ with sodium trimethylsilyl($^{2}H_6$)propane sulfonate as an internal standard

pling constants are presented in Table 7. These assignments are also in agreement with those reported in literature (Wüthrich 1976b; Kalinowski et al. 1984).

Structure determination of pyoverdin Pa

FAB-MS analysis of pyoverdin Pa showed that it differed by 1 Da in molecular mass from pyoverdin Pa A. (M^+ for pyoverdin Pa is at *m/z* 1333) and that the structural difference was located in the chromophore (all fragment ions including the major ion resulting from loss of the chromophore were identical in the spectra of pyoverdin Pa and pyoverdin Pa A). FAB-MS of ^{15}N-labelled pyoverdin Pa and pyoverdin Pa A gave identical molecular ions at *m/z* 1351 indicating that pyoverdin Pa A possesses one less nitrogen atom than pyoverdin Pa (the latter having 18 nitrogens). Esterification of pyoverdin Pa A with a 1:1 mixture of methanol/deuterated methanol gave signals at *m/z* 1346 and 1349 indicating that this compound has one free carboxylic acid group not present in pyoverdin Pa which does not show any mass shift after the same treatment.

Therefore we conclude that pyoverdin Pa has a succinamide group bound to the chromophore replacing the succinic acid present in pyoverdin Pa A.

Structure determination of pyoverdin Pa B

Pyoverdin Pa B (M^+ at 1362) is 28 Da heavier than pyoverdin Pa A and exhibits the same C-terminal fragment ions as pyoverdin Pa A and pyoverdin Pa. In par-

Table 7. Assignment of the aliphatic carbon atoms of pyoverdin Pa A

Chemical shift (ppm)	$^1J_{^{13}C-^{15}N}$ (Hz)	$^1J_{^{13}C-^1H}$ (Hz)	Assignment
69.1	/	147.5 (d)	Cβ Thr
69.0	/	147.5 (d)	Cβ Thr
63.7	/	147.5 (t)	Cβ Ser
63.5	/	147.5 (t)	Cβ Ser
62.5	7.5 (t)	140.0 (d)	Cα Thr
62.0	7.5 (t)	145.0 (d)	Cα Thr
60.5	7.5 (t)	145.0 (d)	Cα Ser
59.2	9.0 (t)	145.0 (d)	Cα Chr
59.0	7.5 (t)	145.0 (d)	Cα Ser
57.3	7.5 (t)	145.0 (d)	Cα Arg
56.5	7.5 (t)	145.0 (d)	Cα Lys
55.9	7.5 (t)	145.0 (d)	Cα Orn(OH)
55.8	7.5 (t)	145.0 (d)	Cα Orn(OH)
52.7	9.0 (d)	137.5 (t)	Cδ Orn(OH)
52.6	9.0 (d)	137.5 (t)	Cδ Orn(OH)
48.7	9.0 (d)	140.0 (t)	Cδ Orn(OH)
43.1	10.0 (d)	140.0 (t)	Cδ Arg
41.9	9.0 (d)	137.5 (t)	Cε Lys
37.8	5.0 (d)	145.0 (t)	Cβ Chr
35.8	/	127.5 (t)	C1 Succ
34.9	7.5 (d)	135.0 (t)	C2 Succ
33.5	/	130.0 (t)	Cδ Lys
30.6 (2)	/	125.0 (t)	Cβ Orn(OH)
30.5	/	125.0 (t)	Cβ Orn(OH)
30.3	/	125.0 (t)	Cβ Lys
30.2	/	125.0 (t)	Cβ Arg
27.2	/	130.0 (t)	Cγ Arg
26.0	/	127.5 (q)	CH_3 Acetyl
25.4	/	130.0 (t)	Cγ Orn(OH)
24.8 (2)	/	130.0 (t)	Cγ Lys + Cγ Orn(OH)
22.2	/	127.5 (q)	Cγ Thr
22.1	/	127.5 (q)	Cγ Thr

The chemical shifts were determined in ^2H_2O using sodium trimethyl($^2H_6$)propanesulfonate as an internal standard. The values in brackets indicate the number of carbon atoms. d indicates doublet, t triplet and q quartet

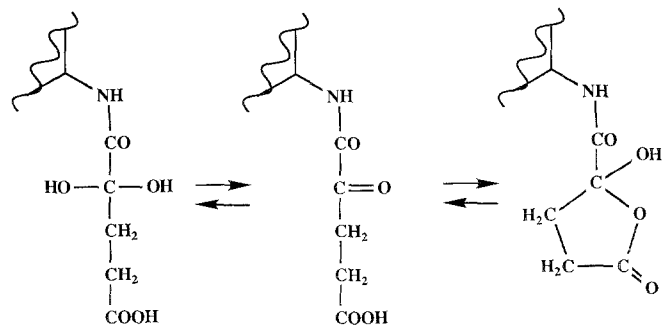
Table 8. Chemical shifts of the carbon atoms of pyoverdin Pa B compared to those of pyoverdin Pa A

Pyoverdin Pa A (ppm)	Pyoverdin Pa B (ppm)	Pyoverdin Pa A (ppm)	Pyoverdin Pa B (ppm)
	210.0	69.1	69.2
	182.4	69.0	69.1
183.7	**182.1**	63.7	63.8
180.2	**179.5**	63.5	63.5
177.3	177.5	62.5	62.6
176.4	176.5	62.0	62.1
176.2	176.2	60.5	60.4
175.7	175.8	59.2	59.7
175.5	175.4	59.0	58.8
174.9	175.0	57.3	57.6
174.8	174.7	56.5	56.6
174.5	174.6	55.9	56.0
173.9	173.8	55.8	55.9
166.55	166.70	52.7	52.8
166.50	166.75	52.6	52.7
162.3	162.5	48.7	48.8
162.0	162.4	43.1	43.2
159.8	158.9	41.9	41.9
		37.8	38.0
158.9	157.4	**35.5**	**35.6**
151.8	152.8	**34.9**	**34.5**
148.0	147.3	33.5	33.6
141.5	144.1		**32.0**
135.4	135.8	30.6 (2)	30.7 (2)
118.4	118.9	30.5; 30.3; 30.2	30.4 (3)
115.8	117.0	27.2	27.1
115.6	116.9	26.0	26.1
102.9	102.9	25.4	25.5
	99.2	24.8 (2)	25.1; 24.8
		22.2	22.3
		22.1	22.1

The values were determined in ^2H_2O with sodium trimethylsilyl(^2H)$_6$propanesulfonate as an internal standard. The values in brackets indicate the number of atoms. Bold-face indicates values which differ between the two compounds

ticular, the major fragment at *m/z* 1031 corresponding to loss of the chromophore indicates that the 28-Da increment is located on the chromophore. This increment corresponds to an extra ethyl group, to two methyl groups or to a carbonyl. The ^{1}H-NMR spectrum of pyoverdin Pa B is very similar to that of pyoverdin Pa A. However, the signal corresponding to proton H4 of the chromophore (7.94 ppm) is broadened, probably due to the interconversion of two species in solution.

By comparing the ^{13}C chemical shifts of both siderophores (see Table 8), it appears that the 28-Da difference between the two molecules is not due to a modification to the chromophore ring system or to the peptide chain since all these groups possess the same chemical shifts in both compounds. The only differences in the ^{13}C-NMR spectra are attributable to signals from the succinyl group of pyoverdin Pa A. The two signals at 35.5 ppm and 34.9 ppm in pyoverdin Pa A are replaced by three broader signals in pyoverdin Pa B at 35.6 ppm, 34.5 ppm and 32.0 ppm. The carbonyl signals of the succinyl group at 180.2 ppm and 183.7 ppm are replaced by signals at 210.0 ppm, 182.4 ppm, 182.1 ppm and 179.5 ppm. There is in addition an extra broad signal in pyoverdin Pa B at 99.2 ppm which is not observed in pyoverdin Pa A. All these signals can be assigned to 2-oxoglutaric acid (Viswanathan et al. 1982)



8 9 10

Table 9. Chemical shifts (in ppm) and assignment of the carbon atoms of 2-oxoglutaric, free and found in pyoverdin Pa B

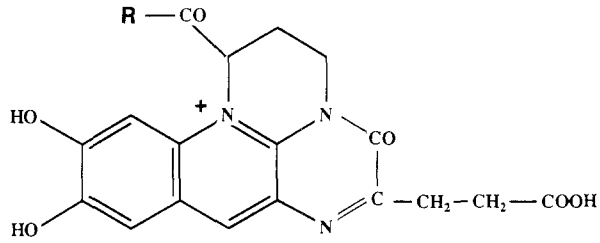
Carbons	2-Oxoglutaric acid		Pyoverdin Pa B	
	ketone oxo	gem-diol	oxo	gem-diol
C1	170.6	nd	179.5	nd
C2	206.7	94.8	210.0	99.2
C3	36.7	36.7	35.6	34.5
C4	32.7	32.7	32.0	32.0
C5	181.9	nd	182.4	182.1

nd, not determined

which differs from succinic acid by an extra carbonyl corresponding to an addition of 28 Da. 2-Oxoglutaric acid itself can exist in solution in three forms which are in equilibrium: a hydrated form **8** (gem-diol), an oxo form **9** and a cyclic form **10**. No signals attributable to the cyclic form were present in the ^{13}C-NMR spectrum of pyoverdin Pa B. This is probably due to a very rapid interconversion between the cyclic form and the corresponding oxo form. In contrast, a slower rate of hydration is suggested by the NMR data. The broadening of the 2-oxoglutaryl group signals is very likely due to the interconversion between the forms existing in solution. Broadening is also observed for some of the aromatic carbon atoms of the chromophore and also for the proton H4 which is very close to the 2-oxoglutaryl group. Table 9 presents the ^{13}C chemical shifts values of free 2-oxoglutaric acid and bound in pyoverdin Pa B.

The orientation of this 2-oxoglutaryl group was initially investigated using the oxidative decarboxylation reported by Briskot et al. (1986). The reaction of pyoverdin-Pa-A-Fe(III) and pyoverdin-Pa-B-Fe(III) with hydrogen peroxide in alkaline medium was monitored by film electrophoresis, absorption spectrophotometry, FAB-MS and NMR spectroscopy (after removal of iron from the complex). These experiments showed that the first stage of the reaction of hydrogen peroxide on pyoverdin Pa B was an oxidative decarboxylation of the 2-oxoglutarate moiety bound to the chromophore and the main product of the reaction, in less than 1 h, was pyoverdin Pa A.

These data suggest that the 2-oxoglutaric acid is bound to the chromophore at its C5 position. To confirm this assignment, we performed partial acid hydrolysis experiments and isolated chromopeptide fragments which were analyzed by FAB-MS and also by ^{1}H- and ^{13}C-NMR. The main product **11** of this reaction is constituted with a new chromophore bound to serine and the minor product **12** possesses the same new chromophore but bound to a peptide containing serine and arginine. During the course of the hydrolysis the initially pale yellow solution becomes deep orange-red. At pH 4.2 the absorption spectra of both compounds **11** and **12** gave a maximum at 410 nm ($\varepsilon = 2.2 \times 10^4\ M^{-1} \times cm^{-1}$) which shifts to 460 nm ($\varepsilon = 2.95 \times 10^4\ M^{-1} \times cm^{-1}$) at pH 6.7 with an isosbestic point at 426 nm. FAB-MS gave molecular ions at *m/z* 473 and 629 for fragments **11** and **12** respectively, the difference of 156 Da corresponding to arginine. Compound **12** is closely related to the hydrolytic fragment **7** obtained from partial hydrolysis of pyoverdin Pa A, differing from it by 10 Da. This difference is due to the addition of a carbonyl group (+28 Da: replacement of a succinyl group by 2-oxoglutaryl group) and to the loss of a molecule of water (−18 Da).



11 R = Ser— COOH

12 R = Ser— Arg— COOH

These results strongly suggest that compound **12** is derived from **7** by a nucleophilic attack on carbon C1 of 2-oxoglutaric acid, loss of water and aromatization. In addition the comparison of the ^{1}H chemical shifts of compounds **11** or **12** with those of pyoverdin Pa B or pyoverdin Pa A (see Table 4), show that the signals corresponding to H4, H5, H8 as well as H11, H13 and H13′ are shifted downfield as expected from the formation of an extra fused ring on the chromophore (see Tables 10 and 11 for the proton chemical shifts of compounds **11** and **12**).

The assignment of the carbon atoms was performed, after determination of their multiplicity by distorsionless enhancement polarization transfer (DEPT), by

Table 10. Assignment of the protons of the hydrolytic fragment **11**

δ (ppm)	Multiplicity	Assignment
8.96	s	Chr H4
7.59	s	Chr H5
7.46	s	Chr H8
6.15	s	Chr H11
4.59	t (3.1 Hz)	Ser-Hα
4.09	dd (3.7 Hz; 11.6 Hz)	Ser-Hβ
3.98	dd (3.7 Hz; 11.6 Hz)	Ser Hβ
3.72	t (14.9 Hz)	Chr H13
3.25	t (6.5 Hz)	CO·Glt H3
3.06	d (15.5 Hz)	Chr H12′
2.95	t (6.5 Hz)	CO·Glt H4
2.65	t (14.9 Hz)	Chr H12

The chemical shifts were determined in $^{2}H_2O$ using sodium trimethylsilyl($^{2}H_6$)propanesulfonate as an internal standard. s indicates singlet, d doublet, t triplet and m multiplet. Chr = chromophore and CO·Glt = 2-oxoglutaric acid

Table 11. Assignment of the protons of the hydrolytic fragment **12**

δ (ppm)	Multiplicity	Asignment
8.99	s	Chr H4
7.60	s	Chr H5
7.43	s	Chr H8
6.21	s	Chr H11
4.46	t (5.3 Hz)	Ser Hα
4.34	t (4.5 Hz)	Arg Hα
3.97	d (5.3 Hz)	Ser Hβ
3.73	t (14.9 Hz)	Chr H13
3.30	t (6.5 Hz)	CO·Glt H3
3.06	d (15.5 Hz)	Chr H12′
2.93	t (6.5 Hz)	CO·Glt H4
2.86	m	Arg Hδ
2.6	t (14.9 Hz)	Chr H12
1.84	m	Arg Hβ
1.64	m	Arg Hβ
1.24	m (8.1 Hz)	Arg Hγ

The chemical shifts were determined in ^2H_2O using sodium trimethylsilyl($^2H_6$)propanesulfonate as an internal standard. s indicates singlet, d doublet, t triplet and m multiplet. Chr = chromophore and CO·Glt = 2-oxoglutaric acid

Table 12. Assignment of the carbon atoms of the hydrolytic fragment **11**

δ (ppm)	Assignment
179.9	CO·Glt C5
175.5	Ser COOH
171.4	Chr CO
161.6	CO·Glt C1
159.9	CO·Glt C2
156.7	Chr C7
149.6	Chr C2
146.6 (d)	Chr C4
143.2	Chr C6
137.4	Chr C9
126.8	Chr C3
123.1	Chr C10
115.8 (d)	Chr C5
103.3 (d)	Chr C8
63.3 (t)	Ser Cβ
61.1 (d)	Chr C11
58.0 (d)	Ser Cα
39.1 (t)	Chr C13
31.8 (t)	CO·Glt C3
30.4 (t)	CO·Glt C4
24.3 (t)	Chr C12

The chemical shifts were determined in ^2H_2O using sodium trimethylsilyl($^2H_6$)propanesulfonate as an internal standard. d indicates doublet and t triplet

comparing their chemical shifts with those they possess in pyoverdin Pa B. The signals of the amino acids are in agreement with the values reported in literature (Wüthrich 1976b; Kalinowski et al. 1984). A significant feature of the ^{13}C-NMR spectra of fragments **11** and **12** is the presence of two signals at 162.0 ppm and 160.8 ppm which are assigned to carbons C1 and C2 of 2-oxoglutaric acid. The values observed for their chemical shifts cannot correspond to an oxo carbonyl in the vicinity of a carboxyamide or a carboxyl group (see Tables 12 and 13 for the carbon chemical shifts of compounds **11** and **12**). This seems to contradict the previously established orientation of the 2-oxoglutarate moiety (Briskot et al. 1986) and also does not appear to be consistent with the oxidative decarboxylation experiments. However we offer an alternative explanation for these changes on the chromophore assuming a C5 attachment.

Table 13. Assignment of the carbon atoms of the hydrolytic fragment **12**

δ (ppm)	Assignment
180.2	CO·Glt C5
177.9	Arg COOH
174.1	Ser CO
171.8	Chr CO
162.0	CO·Glt C1
160.8	CO·Glt C2
157.1	Chr C7
150.1	Chr C2
146.6 (d)	Chr C4
143.2	Chr C6
137.8	Chr C9
127.2	Chr C3
123.4	Chr C10
116.3 (d)	Chr C5
103.2 (d)	Chr C8
63.7 (t)	Ser Cβ
61.2 (d)	Chr C11
59.7 (d)	Ser Cα
55.4 (d)	Arg Cα
43.3 (t)	Arg Cδ
39.6 (t)	Chr C13
32.3 (t)	CO·Glt C2
30.8 (t)	CO·Glt C4
30.6 (t)	Arg Cβ
26.9 (t)	Arg Cγ
24.6 (t)	Chr C12

The chemical shifts were determined in ^2H_2O using sodium trimethylsilyl($^2H_6$)propanesulfonate as an internal standard. d means doublet and t triplet

The first step would be the removal of the oxoglutarate moiety after hydrolysis and formation of compound **6** bearing on *o*-diamino aromatic group (together with its analogue without arginine), followed by a recombination of the oxoglutarate moiety via its C1 and C2 and cyclization to **11** and **12**. This plausible mechanism can explain the formation of pyoverdin Pa A from pyoverdin Pa B on treatment with hydrogen peroxide by a C5-linked 2-oxoglutarate moiety as well as the large change in the spectral properties of the chromophore after partial hydrolysis. This is in agreement with the ^{1}H- and ^{13}C-NMR spectra of fragments **11** and **12** which no longer possess free oxo or oxoacid groups but instead an extra fused pyrazinone ring (Cheeseman and Westiuk 1978; Barlin and Pfleiderer 1969).

Conclusion

When *Pseudomonas aeruginosa* ATCC 15692 is cultivated in iron-deficient conditions, several pyoverdins

can be isolated from the culture media: pyoverdin Pa, pyoverdin Pa A, pyoverdin Pa B and pyoverdin Pa C. The bacteria in fact produce only two of them: pyoverdin Pa and pyoverdin Pa B. Pyoverdin Pa A occurs probably from ther hydrolysis of pyoverdin Pa, and pyoverdin Pa C, which was not found in the culture media, is probably due to an artifact of the purification procedure.

All these compounds are octapeptides with the same partly cyclic basic peptide

D-Ser-L-Arg-D-Ser-L-Orn(δOH)-L-Lys L-Thr
                                  | |
                  L-Orn(δOH)-L-Thr

bound to the same chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline which has the same configuration (*S*) as pseudobactin (Teintze et al. 1981).

The four pyoverdins differ only in the acyl substituent bound to the amino group on C3 of the chromophore which is succinamide (pyoverdin Pa), succinic acid (pyoverdin Pa A), succinate methyl ester (pyoverdin Pa C) and 2-oxoglutarate (pyoverdin Pa B). Since FAB-MS was not sufficient in the case of these pyoverdins to give a complete sequence of the molecules, novel NMR procedures such as 2D-TOCSY and ROESY were used in addition in order to assign unambiguously the amino acids, the formyl groups present on the molecules and to give the complete sequence of the peptide. The procedures appeared to be very useful in this particularly complex case of structure determination since any slight error in the NMR assignment can lead to an interpretation of the structure.

The diversity in the structure of the pyoverdins so far reported show that there are large structural differences between them: they can be linear, partly cyclic or completely cyclic. Many linear pyoverdins have been reported: the structure of pseudobactin B10 was elucidated by X-ray crystallography and considered as the structure of reference for pyoverdins (Teintze et al. 1981; van der Helm et al. 1987). Pseudobactin 214 is linear but has its C-terminal free, in contrast to pseudobactin B10. Its structure was established using classical nuclear Overhauser enhancement NMR (Buyer et al. 1986). Other linear pyoverdins were found in several strains of *Pseudomonas*. Their structures were elucidated using FAB-MS in combination with NMR (Demange et al. 1987).

Partly cyclic pyoverdins were reported (Poppe et al. 1987). They have their N-terminal blocked by the chromophore and their C-terminal belongs to a cyclic pseudodepsipeptide. The partly cyclic structures of pyoverdins C, D and E, which are identical to pyoverdins Pa, Pa A and Pa B, were also elucidated using essentially FAB-MS on the siderophores and on dansylated hydrolytic fragments, but no evidence for the NMR assignments were reported (Briskot et al. 1989). A totally cyclic structure was published for Pseudobactin 7SR1 where the chromophore is bound to a serine of the cyclic peptide via an ester linkage (Yang and Leong 1984).

The diversity of these structures illustrated the complexity of the problem of the structure elucidation of the pyoverdins. Unfortunately, the best way, which is structure determination by X-ray crystallography, is not always available since it appears to be very difficult to crystallize these siderophores, even when they have been very carefully purified.

In many instances FAB-MS can easily give the sequence of the peptide. However in some cases, such as for the pyoverdins Pa, an additional NMR study in H_2O using 2D-TOCSY (for the unambiguous assignment of the signals) completed with 2D-ROESY NMR in order to determine the sequential correlations between the CHα and the NH protons can be essential.

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