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Bacterial siderophores: structure elucidation, and ^{1}H, ^{13}C and ^{15}N two-dimensional NMR assignments of azoverdin and related siderophores synthesized by *Azomonas macrocytogenes* ATCC 12334

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Two major azoverdins were isolated from the cultures of *Azomonas macrocytogenes* ATCC 12334 grown in iron-deficient medium. Their structures have been established using fast atom bombardment-mass spectroscopy, homonuclear and heteronuclear two-dimensional ^{15}N, ^{13}C and ^{1}H NMR, and circular dichroism techniques. These siderophores are chromopeptides possessing at the N-terminal end of their peptide chain the chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline common to pyoverdins. The linear peptide chain (L)-Hse–(D)-AcOHOrn–(D)-Ser–(L)-AcOHOrn–(D)-Hse–(L)-CTHPMD has at its C-terminal end a new natural amino acid which is the result of the condensation of 1 mol of homoserine and 1 mol of 2,4-diaminobutyric acid forming a cyclic amidine belonging to the tetrahydropyrimidine family: 2-homoseryl-4-carboxyl-3,4,5,6-tetrahydropyrimidine. The azoverdins differ only by a substitutent bound to the nitrogen on C-3 of the chromophore: azoverdin, the most abundant one, possesses a succinamide moiety, whereas azoverdin A bears a succinic acid moiety. ^{15}N-labelled azoverdin afforded readily, after the complete assignment of the ^{15}N spectrum of the siderophore, a sequence determination of the peptidic part of the molecule and gave evidence for the presence of two tetrahydropyrimidine groups on the molecule: one on the chromophore and the second at the C-terminal end of the siderophore.

Keywords: azoverdin, *Azomonas macrocytogenes*, ^{15}N NMR, siderophore, iron transport

Introduction

Most microorganisms respond to iron-limited growth conditions by producing low molecular mass iron-binding compounds, called siderophores, which are able to chelate iron from the environment, thereby making iron available to the cell for assimilation (Neilands 1981, 1984). While certain functional aspects of siderophore structure are constant, the chemical structure of these molecules varies widely depending on the producing organism (Raymond *et al.* 1984, Matzanke 1991, Winkelmann 1991). Pyoverdins, pseudobactins and azotobactin comprise a group of structurally similar siderophores which are produced by group 1 fluorescent *Pseudomonas* spp. or *Azotobacter vinelandii* growing under iron limitation (Abdallah 1991). Pyoverdins have a molecular mass of 1000–1500 and are composed of a chromophore, structurally based on 2,3-diamino-6,7-dihydroxyquinoline, to which a peptide of six to 10 amino acids is attached via the N terminus (Teintze *et al.* 1981, Philson & Lliñas 1982, Wendenbaum *et al.* 1983, Yang & Leong 1984, Buyer *et al.* 1986, Demange *et al.* 1987, 1988a, 1990a, Poppe *et al.* 1987, Abdallah 1991). In addition, the 3-amino moiety of the chromophore is substituted with various acyl groups derived from succinate, malate or α-ketoglutarate (Demange *et al.* 1987, Poppe *et al.* 1987, Briskot *et al.* 1989, Abdallah 1991) and many other diacids from the Krebs cycle (Linget *et al.* 1992a). In azotobactin, a 'pyoverdin-like' siderophore produced by *A. vinelandii*, the

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chromophore has a somewhat different structure, and the acyl group is replaced by a cabonyl group forming an extra imidazolone ring (Corbin *et al.* 1970, Fukawawa *et al.* 1972, Demange *et al.* 1988a).

Pyoverdins have a strong affinity for ferric iron with a stability constant $\log K1 = 30.8 \pm 0.9$ (Demange *et al.* 1989, Albrecht-Gary *et al.* 1994) relative to the equilibrium

$$L^{4-} + Fe^{3+} \rightleftarrows LFe^{-}$$

The three functional groups responsible for the octahedral coordination of one atom of Fe(III) are provided by the catechol function of the chromophore and either two hydroxamate functions or one hydroxy acid and one hydroxamate, present in the peptide moiety. Generally, N^{δ}-hydroxyornithine (OHOrn; formylated or acetylated) or *β-threo*-hydroxyaspartic acid, located in the middle of the peptide chain, supplies one hydroxamate group, whereas the second is provided either by a C-terminal cyclized OHOrn or by an N^{δ}-acyl-N^{δ}-hydroxyornithine (AcOHOrn) belonging to a larger ring (Teintze *et al.* 1981, Teintze & Leong 1981, Yang & Leong 1984, Demange *et al.* 1987, 1990a, Briskot *et al.* 1989, Abdallah 1991). Azotobactin differs from pyoverdins and pseudobactins in that the C-terminal OHOrn is not cyclized but is acetylated and followed by L-homoserine (Hse) which may or may not be lactonized (Demange *et al.* 1987, 1988a).

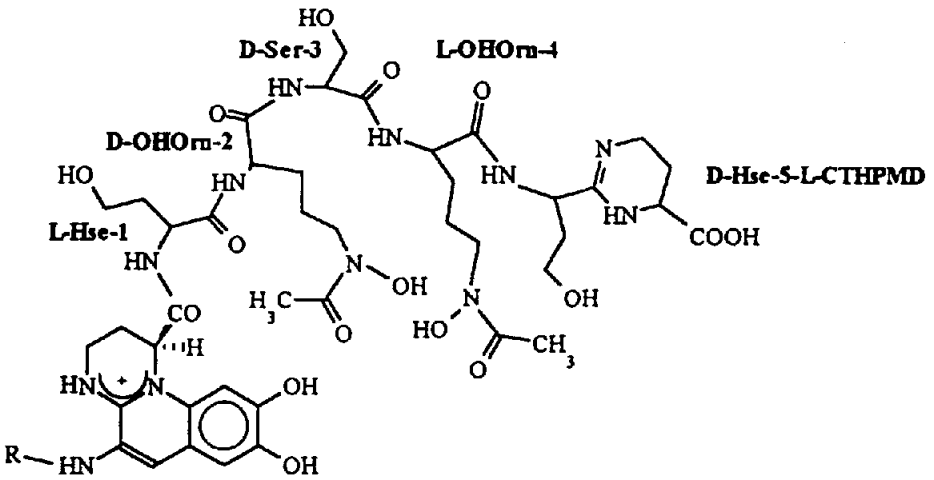
Structural elucidation of pyoverdins and their precursors has shown in some instances the presence in the peptide chain of new amino acids derived from carboxytetrahydropyrimidine. These compounds result from the condensation of one molecule of 2,4-diaminobutyric acid (Dab) with an other amino acid such as serine (Demange *et al.* 1990a), glutamine (Demange *et al.* 1990b) or tyrosine (Budzikiewicz *et al.* 1992, Linget *et al.* 1992b).

Recently, cell-free culture supernatants of *Azomonas macrocytogenes* ATCC 12334 containing a yellow–green water soluble fluorescent compound were found to exhibit a pH-dependent absorption spectrum analogous to pyoverdins and promote $^{55}Fe^{3+}$ assimilation by *A. macrocytogenes* (Collinson & Page 1989). In view of the apparent chemical and functional similarities of the fluorescent compound elaborated by *A. macrocytogenes* with that of pyoverdins, the name azoverdin has been proposed (Collinson *et al.* 1990).

Preliminary studies on azoverdin showed that azoverdin is a chromopeptide possessing at its N-terminal end a fluorescent chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline bound to a hexapeptide containing (D)- and (L)-Hse (2), (D)- and (L)-AcOHOrn (2), (D)-Ser (1) and (L)-Dab. The structure of azoverdin was tentatively reported by us to be (succinamide)–chromophore–(L)-Hse (D)-AcOHOrn (D)-Ser–(L)-AcOHOrn–(D)-Hse–(L)-cDab (Linget *et al.* 1992c).

In this paper, we report the complete structure elucidation of azoverdin (Figure 1). This structure has mainly been established by NMR assignments on fully labelled [^{15}N]azoverdin, fast atom bombardment-mass spectrometry (FAB-MS), circular dichroism (CD) and comparison with the results already reported for pyoverdins (Teintze & Leong 1981, Philson & Liñas 1982, Yang & Leong 1984, Buyer *et al.* 1986, Demange *et al.* 1987, Poppe *et al.* 1987, Briskot *et al.* 1989).

We also report for the first time the complete assignment of the ^{15}N NMR signals of a pyoverdin-like siderophore and show by two-dimensional (2D) heteronuclear NMR spectroscopy that the peptide chain of azoverdin contains the same new type of tetrahydropyrimidine-based amino acids previously reported in pyoverdins (Demange *et al.*



Azoverdin, **Azoverdin A,**

Figure 1. The structure of azoverdin and azoverdin A.

1990a,b) and we compare it to other amidines found in other pyoverdins or pyoverdin-like peptides (Demange *et al.* 1990a,b, Buzikiewicz *et al.* 1992, Linget *et al.* 1992b).

Materials and methods

Organisms and growth conditions

A. macrocytogenes strain ATCC 12334 was grown in liquid, iron-free Burk medium (Page & Sadoff 1976) containing 14 mM ammonium acetate and 1% mannitol (w/v) to replace glucose. To reduce iron contamination, deionized, distilled water was used and glassware was acid washed. Liquid medium, 400 ml per 1 l flask, was inoculated to an initial optical density at 620 nm of approximately 0.1 with *A. macrocytogenes* as previously described (Collinson *et al.* 1987). Cultures were incubated on a rotary shaker at 225 r.p.m. at 28°C for 48 h.

Quantification of azoverdin

The concentration of azoverdin-free ligand and azoverdin–Fe(III) complex present in various solutions was calculated using the spectrophotometric data previously determined for pyoverdin Pa and its iron complex at pH 5.0 ($\lambda_{max} = 380$ nm, $\varepsilon_{380} = 16500\ M^{-1} \times l$ for the ligand, and $\lambda_{max} = 400$ nm, $\varepsilon_{400} = 19000\ M^{-1} \times l$ for the FeIII) complex), considering that both molecules have the same chromophore (Demange *et al.* 1987, 1990a).

Isolation and purification of azoverdin Fe(III) complex

Bacterial cells were removed from the culture supernatant fluids of *A. macrocytogenes* grown for 48 h on iron-limited medium by centifugation (1000 *g*, 15 min, 4°C) and filtration (0.22 μm pore size, Minitan® filtration unit; Millipore, Bedford, MA). The resulting filtrate was acidified to pH 3.8 with formic acid and pumped (4.5 ml min^{-1}) through a column (3.0 × 20 cm) of octadecylsilane (ODS) (LiChroprep® RP-18; Merck, Darmstadt, Germany) (Demange *et al.* 1988a, 1990). The fluorescent material retained by the ODS was rinsed with at least five bed volumes of acidified distilled water (adjusted to pH 3.8 with acetic acid) then eluted from the column with 400 ml of 50% (v/v) acetonitrile in 0.05 M pyridine/acetic acid, pH 5.0. The fluorescent eluate was concentrated, lyophilized (258 mg), resuspended in 0.05 M pyridine/acetic acid, applied to a CM-Sephadex C25 cation exchange column (1.5 × 30 cm) and eluted from this column (1 ml min^{-1}) using a 1:1 linear gradient of 0.05–0.5 M pyridine/acetic acid, pH 5.0 (2 × 0.75 l, 6 ml fractions). Three fractions were separated: the first (18 mg), heterogeneous containing at least four compounds, was discarded; the second (20 mg) was called azoverdin A. Azoverdin, the third and major fraction (140 mg) recovered from this column, was treated with 20 molar excess Fe^{3+} (added as a solution of 1.7 M ferric chloride), the pH of the solution was adjusted to 3.8 before it was chromatographed on an ODS column (3.0 × 20 cm). The column was washed first with a solution of acetic acid, pH 3.8, in order to remove excess iron chloride, then with a 1:1 mixture of acetonitrile and 0.5 M pyridine/acetic acid buffer, pH 5.0, which eluted the azoverdin–Fe(III) complex (145 mg).

The bulk of complex was applied to a CM-Sephadex C-25 column (1.5 × 30 cm) eluted firstly isocratically with 200 ml 0.05 M pyridine/acetic acid buffer pH 5.0, then with a linear gradient of pyridine/acetic acid buffer, pH 5.0 (0.05–2 M, 2 × 100 ml), to separate the azoverdin–Fe(III) complex (96 mg) from a small amount of unchelated azoverdin (9 mg).

HPLC

Azoverdin–Fe(III) complex was purified by preparative reverse-phase HPLC using a column (2.25 × 25 cm) containing ODS (10 μm particle size; Nucleosil, SFCC, Neilly sur Marne, France) as the bonded phase. Samples of 2 5 mg were eluted with 0.025 M pyridine/acetic acid containing 5% (v/v) acetonitrile at a flow rate of 6 ml min^{-1} at 27.0°C. Elution of the azoverdin Fe(III) complex, the major compound (57 mg), was monitored spectrophotometrically at 400 nm.

Progression of the purification of the complex was monitored by analytical HPLC using a column (0.5 × 26 cm) containing ODS (5 μm particle size; Nucleosil). Samples of approximately 0.025 mg were eluted using the above conditions at a flow rate of 1.0 ml min^{-1}. The retention time of the azoverdin Fe(III) complex was 37 min.

Iron-free azoverdin was analysed by analytical HPLC using a column (0.5 × 26 cm) containing ODS (5 μm particle size; Supelco®). Samples were eluted with a buffer containing 0.2 M citric acid, 1.0 mM EDTA, 1 mM 1-octane sulphonic acid, 0.2 M Na_2HPO_4 and 10% (v/v) acetonitrile at pH 3.0. Elution of iron-free azoverdin was monitored with a spectrophotometer at a wavelength of 380 nm.

Preparation of the free ligand

Initially iron was removed from the azoverdin–Fe(III) complex using 8-hydroxyquinoline as described by Meyer & Abdallah (1978). Ferrated azoverdin (100 mg) was dissolved in 10 ml distilled H_2O, the solution was adjusted to pH 4.0 with 10% (v/v) acetic acid and then stirred vigorously for about 1 h with 30 ml 3% (v/v) 8-hydroxyquinoline in chloroform. The extraction procedure was repeated with fresh layers of 8-hydroxyquinoline/chloroform until virtually all of the iron was removed from the aqueous layers. Additional extractions of the aqueous layer with chloroform removed most of the residual 8-hydroxyquinoline.

In order to overcome the tedious aspects of this procedure, another procedure was designed using EDTA as the competitor chelate for Fe(III): 50 mg of azoverdin–Fe(III) complex was dissolved in 3 ml of water at pH 3.8 and 10 ml of a 0.2 M solution of EDTA sodium salt added. The solution was stirred for 1 h, diluted twice with water at pH 3.8 and chromatographed on an ODS reverse-phase column (30 × 20 cm), made up in water at pH 3.8. The column was

first washed with 50 ml of a 0.1 M solution of EDTA, then with 400 ml water at pH 3.8. Azoverdin was eluted as a free ligand with a 1:1 mixture (v/v) of acetonitrile–0.05 M pyridine/acetic acid buffer, pH 5.0.

Azoverdin-free ligand was lyophilized, resuspended in 0.05 M pyridine/acetic acid, and applied to and eluted from a CM-Sephadex C25 ion exchange column with a linear gradient of 0.05 M pyridine/acetic acid, pH 5.0 (0.05 0.5 M, 2×0.5 l), to remove any traces of azoverdin–Fe(III) complex and EDTA from the free ligand. A minor fraction (4 mg) corresponding to azoverdin A was separated from the bulk of azoverdin (44 mg). Purified azoverdin was lyophilized and stored desiccated in the dark at −20°C.

Preparation of ^{15}N-labelled azoverdin

For the preparation of fully ^{15}N-labelled azoverdin, ammonium acetate ($1.1\ g\ l^{-1}$) was replaced in the culture medium by 98.9% $(^{15}NH_4)_2SO_4$ ($0.2\ g\ l^{-1}$) (Dobišová *et al.* 1994). ^{15}N-labelled azoverdin was prepared and purified as described above.

Electrophoresis

Samples of approximately 0.05 mg were applied to the midpoint of cellulose acetate membranes (5.6×14 cm) and then subjected to electrophoresis (300 V, 30 min, 4°C) using 0.1 M pyridine/acetic acid, pH 5.0, as the buffer. Following electrophoresis, the cellulose acetate membranes were illuminated with UV light (366 nm) to detect fluorescent compounds and then sprayed with a solution of 1% (w/v) $FeCl_3$ in distilled H_2O to detect iron-binding compounds.

UV-visible spectrophotometry, CD, MS, amino acid analyses and chiral phase gas chromatography

All these measurements were performed as described previously (Demange *et al.* 1988a, 1990a).

NMR Spectroscopy

^1H, ^{13}C and ^{15}N NMR spectra were determined either on a Bruker AM 400 spectrometer or on a Bruker ARX 500 spectrometer (Bruker Spectroscopin, Wissembourg, France), using as solvent water containing 1% of fully deuterated $[^2H_{10}]t$-butanol (CEA-Saclay, France) as an internal standard. $(^{15}NH_4)_2SO_4$ (Aldrich, Saint Quentin-Fallavier, France) was used as an external standard in the determination of the ^{15}N NMR spectra.

^1H, ^{13}C and ^{15}N NMR spectra were measured respectively at 400, 100 and 40 MHz on the Bruker AM 400 instrument or at 500, 125 and 50 MHz on the Bruker ARX 500 spectrometer, at two temperatures, 300 and 323 K.

^{15}N NMR spectra were determined at pH 2.6 and 4.0. The resolution enhancement was performed by exponential multiplication with a negative line broadening factor followed by Gaussian multiplication with a positive line broadening factor. Each factor was set up empirically.

^1H-coupled ^{15}N NMR spectra were measured with the EKLONG.AUR programme (Bruker Spectroscopin). ^1H-decoupled ^{15}N NMR spectra with no NOE effect were measured using the INVERSEGATE.AUR programme (Bruker Spectroscopin). ^1H-decoupled ^{15}N NMR spectra with the NOE effect were measured using the EKLONG.AUR programme (Bruker Spectroscopin).

The $^{15}NH_2$ and ^{15}NH resonances were assigned from distortionless enhancement polarization transfert (DEPT) experiments using the DEPTSAT.AUR (DEPT 90 and 135) programme (Bruker Spectroscopin), as well as from 2D heteronuclear ^1H–^{15}N correlations. The fully substituted ^{15}N signals were assigned from Quaternary Decoupled (QUAT-D) experiments (Bendall & Pegg 1983).

The 2D homonuclear Hartmann Hahn spectroscopy (HOHAHA) NMR spectra were recorded at 300 and 323 K. The 2D rotating frame Overhauser effect spectroscopy (ROESY) and the 2D heteronuclear multiple quantum coherence ^1H–^{13}C (HMQC) correlations and heteronuclear multiple bound coherence (HMBC) spectra were determined at 300 K. All spectra were measured in the phase sensitive absorption mode with quadrature detection in both dimensions, using the time proportional incrementation method described by Marion & Wuthrich (1983). The trough bound connectivities were obtained from a HOHAHA spectrum determined with the MLEV-17 sequence (Bax & Davis 1985a) using a 34 μs 90° pulse. A mixing time of 700 ms was used with a spin-locking radiofrequency field strength of 7.4 kHz. In the ROESY experiments (Bothner-By *et al.* 1984, Bax & Davis 1985b), the spin-lock was achieved by a decoupler pulse of 300 ms duration, using a 2.3 kHz radiofrequency field. The carrier frequencies of both transmitter and decoupled channels were synchronized. In all the experiments using spin-lock, the carrier frequency was centred on the water resonance. A relaxation delay of 1.5 s was used. The water signal was suppressed by a low continuous power irradiation during the relaxation delay. The spectral width in F_1 and F_2 was 4400 Hz; 512 experiments with 40 and 48 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 assignment of the carbonyl signals was performed using 2D heteronuclear ^1H ^{13}C correlation HMQC (Bax *et al.* 1983, Müller 1979) and 2D ^1H–^{13}C heteronuclear correlation HMBC. These spectra were determined on a Bruker ARX 500 spectrometer at 300 K with 512 t_1 points and 2048 t_2 points (512 experiments with 32 and 64 scans of 2048 complex points in t_2 were collected in both cases). The spectral width was set to 33334.10 Hz in the F_1 dimension and 6024.60 Hz in the F_2 dimension and the FID resolution was set to 130.21 Hz in the F_1 dimension and to 2.94 Hz in the F_2 dimension. As for the ROESY NMR experiment, the ^1H carrier was set on the water resonance and the suppression of the solvent resonance was achieved by presaturing during the 1.5 s relaxation delay.

Results and discussion

Isolation and purification of azoverdin, azoverdin A and ^{15}N-labelled azoverdin from cultures of A. macrocytogenes

All the ligands were purified extensively similarly to azoverdin as described in Material and methods. Azoverdin A, the second major fluorescent siderophore excreted by *A. macrocytogenes*, was isolated during the first CM-Sephadex C-25 ion exchange chromatography of the crude azoverdin.

From the cultures of *A. macrocytogenes* yielding ^{15}N-labelled azoverdin (Dobišová *et al.* 1994), it was possible to isolate and purify ^{15}N-labelled azoverdin A as well. The labelling experiment was carried out using $0.2\,g\,l^{-1}$ of ^{15}N-labelled ammonium sulphate as a source of nitrogen and a cultivation time of 48 h. Azoverdin produced under these conditions contained 95% of ^{15}N. This was further corroborated by the FAB-MS analysis of the ^{15}N-labelled sample of azoverdin A (succinic acid form of azoverdin) which showed the presence of a very small signal at 1091 mass units (unlabelled siderophore) with about 5% of the intensity of the major signal at 1103 mass units (azoverdin and 12 ^{15}N nitrogen atoms) originating from the residual unlabelled ammonium sulphate of the inoculum. We can conclude that the method described here represents a general technique. It is based on the optimized assimilation of an inorganic source of nitrogen and is useful for the ^{15}N-labelling of related siderophores produced by other nitrogen-fixing bacteria.

Spectrophotometric properties of purified azoverdin

The UV-visible spectra of the free ligand and of its iron complex are similar to those determined for pyoverdins, the peptides siderophores of the fluorescence pseudomonads, with two maxima at 368 and 380 nm for the pH-dependent spectrum of the free ligand ($\varepsilon = 16000\,M^{-1}\,l$ at pH 5.0 in a buffered aqueous solution). In the iron complex there is only one maximum at 400 nm ($\varepsilon = 19500\,M^{-1}\,l$), and two shoulders at 460 nm ($\varepsilon = 6000\,M^{-1}\,l$) and 540 nm ($\varepsilon = 3000\,M^{-1}\,l$), and these values are pH independent. Azoverdin and its iron complex therefore possess a chromophore with the same spectral characteristics as pyoverdins (Collinson *et al.* 1990), suggesting that azoverdin has a pyoverdin-like structure.

Amino acids analysis

Total acid hydrolysis of purified azoverdin (6 N HCl, 110°C, 48 h and 57% HI, 110°C, 48 h) indicated that this compound was constituted of a chromophore bound to a peptide moiety of six amino acids: Hse (2), Ser (1), OHOrn (2) and Dab (1).

FAB mass spectroscopy

Unlabelled azoverdin. Azoverdin and its Fe(III) complex gave molecular peaks at *m/z* 1090 u and at 1143 u, respectively, showing a 1:1 stoichiometry. Azoverdin–Fe(III), on treatment with methanolic HCl, produced signals at *m/z* 1104 (molecular iron minus iron, monoesterified), 1119 (molecular ion minus iron, diesterified), 1140 (molecular ion minus ammonia, monoesterified), 1157 (molecular iron, monoesterified) and 1172 (molecular ion of the acid form, diesterified).

The sequence of the peptide (Table 1) was deduced from the data afforded by FAB-MS of the products of mild hydrolysis (6 M HCl, 60°C, 5–40 min). After 5 min of acid treatment, two types of fragments were obtained:

Table 1. Hydrolytic chromophoric and peptidic fragments of azoverdin (6 M HCl, 60°C, 5 min) and their interpretation [the Hse,Dab moeity was shown by NMR to be Hse–CTHPMD (*vide infra*)]

Mass	Assignment
202	(Hse,Dab)
332	N^{δ}-OHOrn–(Hse,Dab)
359	chromophore–Hse lactone
419	Ser–N^{δ}-OHOrn–(Hse,Dab)
441	(succinimide)–chromophore–Hse lactone
458	(succinamide)–chromophore–Hse lactone
459	(succinic acid)–chromophore–Hse lactone
549	N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–(Hse,Dab)
591	(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab + 1 acetyl)
633	(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab + 2 acetyls)
989	(succinimide)–chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab)
1006	(succinamide)–chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab)
1007	(succinic acid)–chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab)
1031	(succinimide)–chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab + 1 acetyl)
1048	(succinamide)–chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab + 1 acetyl)
1049	(succinic acid)–chromophore–Hse–(N^{δ}-OHOrn–Hse,Dab + 1 acetyl)
1073	(succinimide)–chromophore–Hse–(N^{δ}-OHOrn–Hse,Dab + 2 acetyls)
1090	(succinamide)–chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab + 2 acetyls)
1091	(succinic acid) chromophore–Hse–(N^{δ}-OHOrn–Ser–N^{δ}-OHOrn–Hse,Dab + 2 acetyls)

chromophoric hydrolytic fragments and peptidic hydrolytic fragments.

These results are consistent with the presence of two acetyl groups bound to the peptidic moiety, that the N-terminal amino acid is Hse and that succinamide is bound to the chromophore (succinamide is converted into succinic acid and succinimide during the course of hydrolysis). Similar results have been obtained for azotobactin (Demange *et al.* 1988a) where the preferential cleavage is between the carboxy groups of Hse and the amine function of the next amino acid bound to them, followed by lactonization of homoserines.

The smallest fragment with the composition Hse,Dab, could be either a lactam form of Dab at the C-terminal end of the peptide or a tetrahydropyrimidine ring similar to those present in other pyoverdins (Demange *et al.* 1990a,b). Treatment of azoverdin with concentrated ammonia (90°C, 30 min) afforded a mixture of products yielding a FAB spectrum containing two equally intense peaks at *m/z* 1091 and 1109 u. The first would correspond to the hydrolysis of the succinamide moiety into succinic acid, and the second to a further hydrolysis of the lactam or the tetrahydropyrimidine ring. On further treatment with PITC, only the ring-opened material reacted to a compound giving a peak at *m/z* 1244 u, corresponding to the addition of 1 mol of reagent to an amine group formed in the ammonia reaction. This could still occur on either a ring-opened lactam or tetrahydropyrimidine. Thus the experiment does not distinguish between these two structures though it unambiguously confirms that an amino group can be formed by base hydrolysis.

Methanolysis of azoverdin using a 1:1 mixture of methanol-*d*4-methanol/HCl showed that the molecule breaks between the first Hse and the first OHOrn to give three major components: a 1:2:1 triplet at 505/508/511 corresponding to the diester of the succinylated chromophore with Hse attached; a 1:1 doublet at 473/476 corresponding to the monoester of the succinylated chromophore with Hse lactone attached; and a 1:1 doublet at 647/650 which corresponds to the rest of the peptide as the monoester indicating the occurrence of a carboxyl group somewhere in the AcOHOrn–Ser–AcOHOrn–(Hse/Dab) sequence, presumably at the end of the peptide chain. From all these results, we concluded that the peptide moiety is linear and consistent with the sequence

Hse–AcOHOrn–Ser–AcOHOrn–(Hse,Dab-H_2O).

^{15}N-labelled azoverdins. ^{15}N-labelled azoverdin and ^{15}N-labelled azoverdin A show electrophoretic and HPLC characteristics identical to those of the unlabelled compounds.

[^{15}N]Azoverdin A (Figure 1, R = $COCH_2CH_2COOH$) gave a molecular ion at 1103, consistent with the incorporation of 12 ^{15}N atoms. A dimer is present at 2206 (formed in the MS experiment) and other minor signals occur at 1209 and 1317.

Methanol-*d*4-methanol (1:1)/HCl treatment at room temperature shows evidence of deuterium exchange (cluster centered at 1103 which has shifted to 1105). Heating the sample at 60°C showed that esterification as well as cleavage occur as the reaction proceeds. The cleavage appears as fast or faster than the esterification so the molecular ion region of the intact molecule is still showing incomplete esterification even after 4 min at 60°C. The cleaved fragments are fully consistent with the data obtained on unlabelled azoverdin: the 1:1 doublet at *m/z* 647/650 has shifted to 655/658 showing that eight ^{15}N nitrogen atoms are present in this peptide. whilst the 1:2:1 triplet at 505/508/511 has shifted to 509/512/515 consistent with only four ^{15}N nitrogen atoms in the chromophore + Hse dimethylester moiety.

[^{15}N]Azoverdin gave the same molecular ion at 1103 as [^{15}N]azoverdin A. This is consistent with an extra amide function on the chromophore. Under the same treatment as above it gave identical data, showing the very easy hydrolysis of the succinamide moiety to succinic acid.

NMR studies on unlabelled and ^{15}N-labelled azoverdin

Preliminary 2D NMR epxeriments seemed consistent with a lactam form for the Dab moiety (Linget *et al.* 1992c). However, ^{15}N NMR contradicted this result. Therefore in order to clarify the structure of the peptide at the C-terminal end of azoverdin, extensive NMR studies were undertaken.

^{1}H and ^{13}C NMR spectroscopy confirmed most of the sequence of the peptide chain (HOHAHA, ROESY and ^{13}C–^{1}H heteronuclear correlation, HMBC, HMQC). ^{15}N-labelled azoverdin showed ^{1}H and ^{13}C NMR chemical shifts identical to those of the unlabelled azoverdin.

^{1}H NMR studies. The ^{1}H NMR spectra of unlabelled azoverdin and ^{15}N-labelled azoverdin (water/[$^2H_{10}$]*t*-butanol 99:1, 323°K, pH 4.0) shows three singlets at 7.98, 7.24 and 7.12 p.p.m. The values of these chemical shifts are very close to those reported for pyoverdin Pa A (Demange *et al.* 1990c). They correspond to the three aromatic protons H-4, H-5 and H-8 of the chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline.

HOHAHA NMR spectroscopy (water/[$^2H_{10}$]*t*-butanol 99:1) (Bax & Davis 1985, Bax 1989, Demange *et al.* 1990c) performed at two temperatures, 323 and 300 K, allowed the assignment of the NH protons as well as the protons of the side chains of the amino acids. The chemical shifts vary with temperature and are in agreement with the values reported in the literature (Wüthrich 1976a, Sumikawa *et al.* 1988, Yu *et al.* 1988).

At 323 K, the proton H-11 of the tetrahydropyrimidine ring of the chromophore does not occur at 5.58 p.p.m. as it does usually with all the other pyoverdins so far investigated (Abdallah 1991), but instead at 4.62 p.p.m. In addition the protons H-13 and H-13′ of the same ring are deshielded to 4.10 p.p.m. and do not occur as an AA′BB′X system but as an ABB′X system, indicating that the coupling constant between H-13 and H-13′ is very small and therefore not measurable.

At 300 K azoverdin shows two distinct conformers, one of which shows the usual chemical shifts of pyoverdins (in

Table 2. Assignment of the protons of ^{15}N-labelled azoverdin at 323 K in H_2O/deuterated *t*-butanol 99:1, using deuterated *t*-butanol as an internal standard

	NH	α	β	γ	δ	Others
Hse-1	8.82	4.82	2.25	3.82–3.89		
AcOHOrn-2	8.71	4.43	1.87–1.93	1.75–1.79	3.72	2.12 (acetyl)
Ser-3	8.36	4.60	3.98			
AcOHOrn-4	7.79	4.35	1.88–1.92	1.73–1.78	3.71	2.12 (acetyl)
Hse-5	8.45	4.55	2.12	3.69		
Dab		4.45	2.09	3.39		
Chromophore	H4	H5	H8	H11	H12–12′	H13–13′
	7.98	7.24	7.12	4.70	2.60–2.82	4.10
Succinamide		2.84 2.89				

Table 3. Assignment of the protons of ^{15}N-labelled azoverdin at 300 K in H_2O/deuterated *t*-butanol 99:1, using deuterated *t*-butanol as an internal standard

	NH	α	β	γ	δ	Others
Hse-1	9.13	4.80	2.17	3.75–3.80		
AcOHOrn-2	8.74	4.32	1.80	1.73	3.64	2.10–2.15 (acetyl)
Ser-3	8.39	4.52	3.92			
AcOHOrn-4	7.85	4.30	1.71	1.90	3.66	2.10–2.15 (acetyl)
Hse-5	8.69	4.48	2.10	3.70		
Dab		4.46	2.05	3.30		
Chromophore	H4	H5	H8	H11	H12–12′	H13–13′
	7.91	7.20	7.18	4.62	2.50–2.72	4.01
				5.58	2.90–3.10	4.27
Succinamide		2.70–2.78				

Table 4. Connectivities between chromophoric protons and NH and CHα protons obtained from ROESY

	Chr H-8		Chr H-11		Hse 1		OHOrn2		Ser3		OHOrn 4		Hse 5
	7.18	1 ⇨	4.62										
				2 ⇩									
NH					9.13		8.74		8.39		7.85		8.69
					⇩	3 ⇗	⇩	4 ⇗	⇩	5 ⇗	⇩	6 ⇗	⇩
CHα					4.80		4.32		4.52		4.30		4.48

particular for H-11, H13 and H13′) (Demange *et al.* 1990c, Abdallah 1991), while the second one possesses the same chemical shifts as above at 323 K.

The complete assignment of the ^{1}H spectrum at the two temperatures is given in Tables 2 and 3.

ROESY NMR experiments (Bothner-By *et al.* 1984) were performed in a similar fashion as for pyoverdin Pa A (Demange *et al.* 1990c) (Table 4). The presence of CαH(*i*)–NH(*i*) and CαH(*i*)–NH(*i* + 1) NOE connectivities between neihbouring residues was used for sequential assignment of azoverdin, independently from the FAB-MS determination. A cross-peak is observed between H-8 of the chromophore at 718 and H-11 of the chromophore at 4.62 p.p.m., confirming the assignment made above. A cross-peak is also observed (arrow 2) between H-11 and NH(Hse 1) at 9.13 p.p.m. Consequently this resonance is attributed to the Hse-1 residue. Starting from arrow 3 for the CαH(Hse 1)–NH(AcOHOrn 2) cross-peak, the following CαH(AcOHOrn 2)–NH(Ser 3) (arrow 4) and CαH(AcOHOrn 4)–NH(Hse 5) cross-peaks confirm the previous assignments. No cross-peak was observed between CαH(Hse 5) and NH(Dab), suggesting that this latter was readily exchanged.

This experiment confirmed the sequence of the peptide chain of azoverdin

chromophore–Hse–AcOHOrn–Ser–AcOHOrn–(Hse,Dab)

as determined by FAB-MS, but did not clear cut for the structure of the C-terminal end of the siderophore.

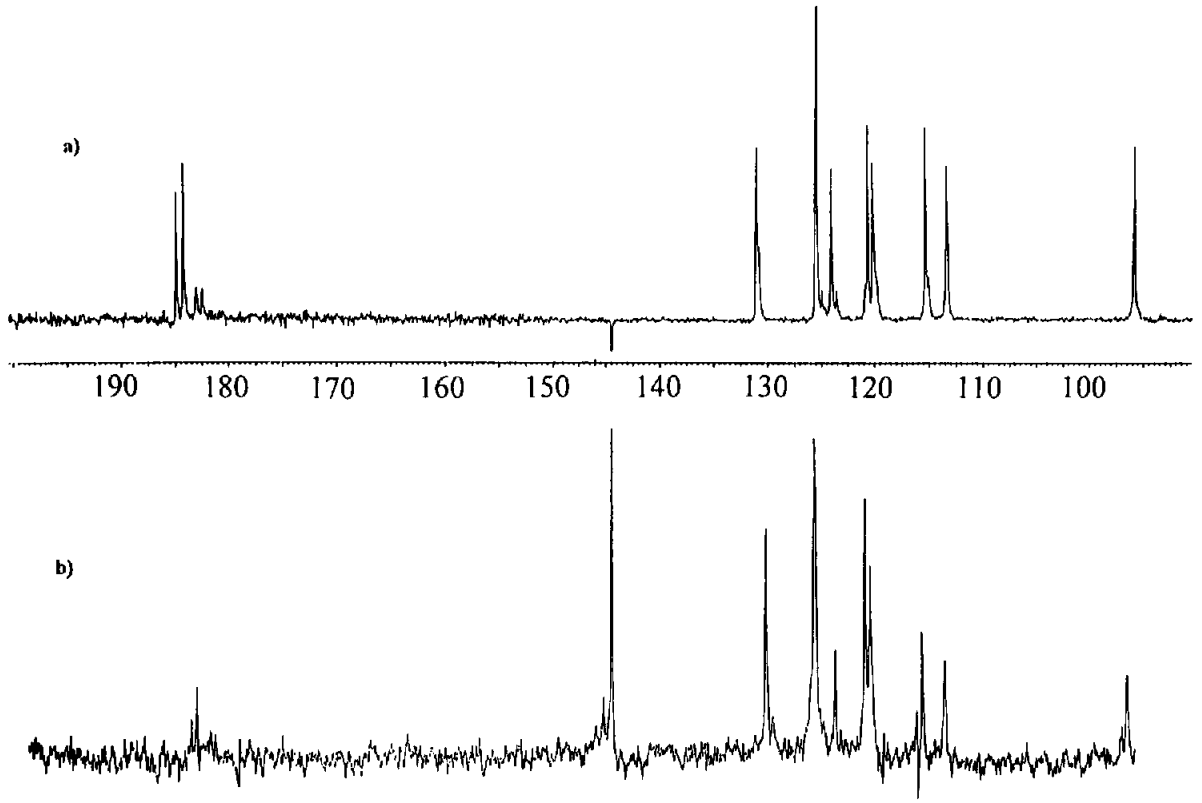


Figure 2. Proton decoupled ^{15}N NMR spectra of azoverdin at pH 4.0 (**a**) and after 24 h at pH 2.6 (**b**).

^{15}N NMR studies. At pH 4.0, the proton decoupled ^{15}N spectra of azoverdin presents 13 signals between 95.00 and 185.00 p.p.m. (Figure 2a). An additional signal at 25.00 p.p.m. corresponds to ammonia occurring from the partial hydrolysis of the succinamide group of azoverdin into succinic acid, as for pyoverdin Pa (Demange *et al.* 1990). From the comparison with the proton undecoupled ^{15}N spectrum and using distortionless enhancement polarization transfer (DEPT 90 and 135), it was possible to show the presence of (i) one signal corresponding to a $CONH_2$ group at 115.44 p.p.m., occurring as a triplet ($^1J_{^1H-^{15}N} = 89.1$ Hz); (ii) seven signals, between 95.92 and 131.05 p.p.m. with a $^1J_{^1H\ ^{15}N}$ of 95.0 Hz, corresponding to CONH groups; and five singlets corresponding to as many fully susbstituted nitrogen atoms.

The five amide groups of the peptidic chain, the secondary amide as well as the primary amide groups of the succinyl moiety bound to the chromophore were unambiguously assigned using the HOHAHA and 2D ^1H–^{15}N heteronuclear correlation spectra of azoverdin. The signal at 125.50 p.p.m. corresponded to both ^{15}NH of Hse-1 and AcOHOrn-2 (Table 5).

The four signals between 182.45 and 184.95 p.p.m. were identified as the two *cis* and *trans* isomeric forms of the hydroxamates borne by the two AcOHOrn residues. The two first signals occur probably from the *cis* form, whereas the two others occur from the *trans* form (Brown *et al.* 1988).

The ^{13}C NMR study of the ^{15}N-labelled azoverdin suggests that two tetrahydropyrimidine rings are present in azoverdin (Figure 3). The first belongs to the chromophoric moiety of the molecule and the second, occurring from the condensation of Dab with the caboxyl of Hse-5 residue, is located at the C-terminal end of the peptide.

The signal at 95.92 p.p.m. was assigned as the ^{15}NH of the chromophoric tetrahydropyrimidine moiety. Its tertiary ^{15}N partner is located at 144.12 p.p.m. This latter is the only ^{15}N in azoverdin to show a positive NOE effect.

At pH 4.0, it was not possible to distinguish between the two ^{15}NH of the second tetrahydropyrimidine moiety because these resonances appeared to be hidden by the signal of the primary amide of the succinamide moiety.

In order to overcome this problem, ^{15}N-labelled azoverdin was dissolved and kept for 24 h at pH 2.6 in a molar solution of acetic acid. This treatment resulted in the smooth hydrolysis of the primary amide of the succinyl moiety into a carboxylic acid function, as shown by the ^{15}N NMR proton decoupled spectrum at pH 2.6, where 13 signals were observed between 96.00 and 186.00 p.p.m. (Figure 2b). The signal at 115.44 p.p.m., occurring as a triplet in the corresponding proton-undecoupled spectrum and assigned to the $^{15}NH_2$ primary amide group of succinamide, had completely disappeared. Instead, two signals at 113.25 and 115.36 p.p.m. (doublets in the corresponding proton-undecoupled spectrum) were clearly observed. They were assigned to the two ^{15}N atoms of the C-terminal tetrahydropyrimidine and behaved like two ^{15}NH, in

Table 5. Assignment of the nitrogen atoms in azoverdin at 300 K using ^{1}H coupled, ^{1}H uncoupled, DEPT 90, DEPT 135 and QUAT D ^{15}N NMR spectra

	δ (p.p.m.) pH 4.0	δ (p.p.m.) pH 2.6	$^{1}J_{^{1}H\ ^{15}N}$ (Hz)	Multiplicity	Assignment
NH_4^+	25.00	25.00		m	ammonium
NH	95.92	96.19	92.0	d	chromophoric tetrahydropyrimidine
NH	113.47	113.25	95.0	d	C-terminal tetrahydropyrimidine
NH	NO	115.36	95.0	d	C-terminal tetrahydropyrimidine
NH_2	115.44	NO	89.1	t	succinamide
NH	120.26	120.21	95.0	d	Ser-3
NH	120.74	120.67	95.0	d	Hse-5
NH	124.13	124.52	95.0	d	NH succinamide
NH	125.50	125.32	95.0	d	Hse-1
NH	125.50	125.45	95.0	d	AcOHOrn-2
NH	131.07	129.98	95.0	d	AcOHOrn-4
N	144.12	144.40		s	chromophoric tetrahydropyrimidine
N	182.45	182.36		s	*cis*-hydroxamate
N	183.90	182.82		s	*cis*-hydroxamate
N	184.28	183.85		s	*trans*-hydroxamate
N	184.95	184.67		s	*trans*-hydroxamate

The chemical shifts were determined using $(^{15}NH_4)_2SO_4$ as an external reference. The chemical shifts were measured at pH 2.6 and 4.0. NO, not observed.

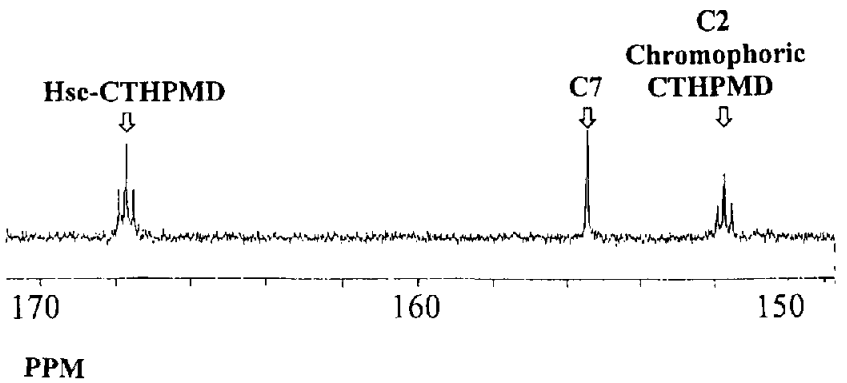


Figure 3. Proton decoupled ^{15}N-coupled ^{13}C spectrum of fully ^{15}N-labelled azoverdin, between 150 and 170 p.p.m.

agreement with the results of Inbar & Lepidot (1988). Moreover, at pH 2.6, the ^{15}NH signals of Hse-1 and AcOHOrn-2 were distinguishable, resonating respectively at 125.32 and 125.45 p.p.m.

These data show that the ^{15}N of the two tetrahydropyrimidine systems present a different behaviour: the chromophoric tetrahydropyrimidine shows no positive charge delocalization between the two nitrogen atoms. This results in the presence of an uncoupled ^{15}N signal and a proton coupled ^{15}NH. Similar results were observed with pyoverdin Pa A (data not shown).

On the contrary, the C-terminal tetrahydropyrimidine shows an important proton delocalization between the two nitrogen atoms, resulting in the observation of two ^{15}NH signals (Inbar & Lepidot 1988).

^{13}C NMR spectra. The ^{13}C NMR spectrum of azoverdin is characteristic of a chromopeptide and can be divided into three regions: the aliphatic region (from 20 to 65 p.p.m., 26 signals), the aromatic region (from 100 to 156 p.p.m., nine signals) and the carbonyl region (from 165 to 185 p.p.m., 11 signals). The assignment of each carbon atom was performed by measuring the $J_{^{1}H\ ^{13}C}$ coupling constants of the proton undecoupled spectrum, the $J_{^{15}N-^{13}C}$ coupling constants of the fully ^{15}N enriched azoverdin and finally by using the $^{1}H-^{13}C$ heteronuclear correlations.

The aliphatic region presents 26 signals. These were assigned by DEPT, by heteronuclear 2D $^{13}C-^{1}H$ correlation and by reverse heteronuclear correlation $^{1}H-^{13}C$ at 323 and 330 K. The multiplicity of each signal in addition to the $J_{^{15}N-^{13}C}$ coupling constants obtained from the ^{13}C NMR spectrum of the fully ^{15}N-labelled azoverdin confirmed these assignments which were also found to be in agreement with literature data (Wüthrich 1976b) (Table 6).

The assignments of the aromatic carbon atoms were readily performed by comparison with the corresponding

Table 6. Assignment of the carbon atoms of the aliphatic region of azoverdin at 300 K in H_2O/deuterated *t*-butanol using 2D ^1H ^{13}C heteronuclear correlation

	δ (p.p.m.)	Multiplicity	$^1J_{^{15}N\ ^{13}C}$ (Hz)
Cα Hse-1	53.27	d	10.5
Cβ Hse-1	37.23	s	
Cγ Hse-1	60.98	s	
Cα AcOHOrn-2	57.74	d	10.0
Cβ AcOHOrn-2	31.52	s	
Cγ AcOHOrn-2	24.70	s	
Cδ AcOHOrn-2	50.71	d	10.0
Cα Ser-3	59.29	d	10.00
Cβ Ser-3	64.75	s	
Cα AcOHOrn-4	58.23	d	10.2
Cβ AcOHOrn-4	32.58	s	
Cγ AcOHOrn-4	24.99	s	
Cδ AcOHOrn-4	50.99	d	9.9
Cα Hse-5	55.72	d	10.1
Cβ Hse-5	36.43	s	
Cγ Hse-5	61.37	s	
Cα Dab	54.97	d	10.0
Cβ Dab	26.54	s	
Cγ Dab	40.21	d	10.0
Cα succinate	35.86	s	
Cβ succinate	35.37	s	
Cα chromophore	53.97	d	10.0
Cβ chromophore	25.97	s	
Cγ chromophore	46.22	d	
Acetyl	23.15	s	
Acetyl	22.84	s	

Table 7. Assignment of the chromophoric carbon atoms of azoverdin in 1% deuterated *t*-butanol in H_2O

	δ (p.p.m.)	Multiplicity	$^1J_{^{15}N\ ^{13}C}$ (Hz)
C2	151.75	t	19.8
C3	121.19	d	17.0
C4	141.88	s	
C5	116.35	s	
C6	147.76	s	
C7	155.45	s	
C8	104.25	s	
C9	135.53	d	15.0
C10	118.80	s	

ones in pyoverdin Pa A and the chemical shifts were in complete agreement with those reported earlier by Demanege *et al.* (1990a). In particular the quaternay carbons C-3 and C-9, which are both in the vicinity of one ^{15}N atom, occur as doublets respectively at 121.19 p.p.m. ($^1J_{^{15}N-^{13}C}=17.0$ Hz) and 135.53 p.p.m. $^1J_{^{15}N-^{13}C}=15.0$ Hz), whereas carbon C-s at 151.75 occurs as a triplet ($^1J_{^{15}N\ ^{13}C}=19.8$ Hz) (see Figure 3 and Table 7).

The carbonyl region presents 11 signals, nine signals occurring as doublets, one at 183.27 p.p.m. occurring as a singlet and one at 167.74 p.p.m. as a triplet ($^1J_{^{15}N-^{13}C}=19.0$ Hz). This second triplet is in favour of the amide structure of Dab acid in azoverdin (Table 8 and Figure 3).

The assignments of most of the signals of the carbonyl region of the spectra were performed using 2D heteronuclear ^1H–^{13}C HMQC and HMBC correlations (Table 8). Cross peaks were observed between $^{15}NH(i)$ and $CO(i-1)$, between $CH\alpha(i)$ and $CO(i)$, and, finally, between the $CH\beta(i)$ and $CO(i)$. Other correlations between ^1H and ^{13}C of the side chain in each residue of azoverdin were also observed (Figure 4). From all these correlations it was possible to (i) to assign completely the carbonyl signals in azoverdin and (ii) confirm the sequence of the peptide moiety in azoverdin.

The singlet at 183.32 p.p.m. was unambiguously identified as the carboxylic acid function borne by the cyclized Dab.

The first carboxyl presents two cross peaks with the methylenic protons of the succinyl substituent of the molecule (2.70 and 2.78 p.p.m.), whereas the second presents only one cross peak with the $H\beta$ of the cyclized Dab (2.05 p.p.m.). The doublet at 180.70 p.p.m. was identified as the carbonyl of the secondary carboxamide group of the succinic moiety of the molecule. It presents two cross peaks with the methylenic protons of succinic acid.

The triplet at 167.74 p.p.m. corresponds to an amidine function formed by the cyclization of Dab. This is confirmed by the presence of two cross peaks with $H\alpha$ (4.48 p.p.m.) and $H\gamma$ (3.30 p.p.m.) of Dab, establishing clearly the sequence **Hse-5-CTHPMD**.

The two signals at 172.66 and 177.28 p.p.m. were identified as the two carbonyls of the acetyl functions located at the end of the side chain of the two OHOrn. Each signal gave cross peaks with the $H\delta$ of the side chain of the AcOHOrn (3.66 and 3.64 p.p.m.) and with the methyl protons of the acetyl groups (2.10 and 2.07 p.p.m.), suggesting the presence of the two isomeric forms *cis* and *trans* of the hydroxamates borne by the two OHOrn (Brown *et al.* 1988).

The signal at 174.04 p.p.m. which presented cross peaks with ^{15}NH of AcOHOrn-4 (7.85 p.p.m.) and with $CH\alpha$ (4.52 p.p.m.) and $CH\beta$ (3.92 p.p.m.) of Ser-3 was assigned to the carbonyl of Ser-3. It also presented a cross peak with $CH\alpha$ of AcOHOrn-2 (4.32 p.p.m.), confirming the sequence **AcOHOrn-2–Ser-3–AcOHOrn-4**.

The signal at 177.21 p.p.m. was assigned to the carbonyl of AcOHOrn-S: it presented cross peaks with $CH\alpha$ (4.32 p.p.m.) and $CH\beta$ (1.80 p.p.m.) of this residue as well as with ^{15}NH of Ser-3, in agreement with the same sequence as above.

The signal at 178.10 p.p.m. was assigned to the carbonyl of the chromophore. It showed cross peaks with the signals of ^{15}NH (9.13 p.p.m.) and H11 of the chromophore (4.58 p.p.m.). The signal at 174.99 p.p.m. was correlated with ^{15}NH of AcOHOrn-2 (8.74 p.p.m.) and was assigned to the carbonyl of Hse-1, whereas the signal at 181.60 p.p.m. corresponded to the carbonyl of AcOHOrn-4 and was correlated to $CH\alpha$ (4.30 p.p.m.), $CH\beta$ (1.71 p.p.m.) and ^{15}NH of AcOHOrn-4 (7.85 p.p.m.). The signal at 175.50 p.p.m. was assigned to the carbonyl of the succinamide moiety bound to the chromophore.

These results clearly confirm the sequence **succinamide-chromophore–Hse-1–AcOHOrn-2.**

In conclusion ROESY gave the sequence **chromophore–Hse-1–AcOHOrn-2–Ser-3–AcOHOrn-4–Hse-5** and HMQC

Table 8. Assignment of the carbonyls of ^{15}N-labelled azoverdin (1% deuterated *t*-butanol is used as an internal standard)

	δ (p.p.m.)	$^1J_{^{15}N,^{13}C}$ (Hz)	Multiplicity	Assignment
NH–C=N	167.74	19.0	t	Hse-5–CTHPMD
CO	172.66	15.0	d	acetyl *cis*-form
CO	174.04	15.0	d	Ser-3
CO	174.99	15.0	d	Hse-1
CO	175.50	15.0	d	succinamide
CO	177.21	15.0	d	AcOHOrn-2
CO	177.28	15.0	d	acetyls *trans*-form
CO	178.10	15.0	d	chromophore
CO	180.70	15.0	d	succinate
CO	181.60	15.0	d	AcOHOrn-4
COOH	183.32		s	Dab

The coupling constants are expressed in Hz.

gave the two sequences **chromophore–Hse-1–AcOHOrn-2–Ser-3–AcOHOrn-4** and **Hse-5–CTHPMD**. Therefore, from all these NMR studies, we can conclude that the structure of the peptidic moiety of azoverdin is:

Hse-1–AcOHOrn-2–Ser-3–AcOHOrn-4–Hse-5–CTHPMD.

Comparison of 2-homoseryl-4-carboxy-3,4,5,6-tetrahydropyrimidine (Hse CTHPMD) with other tetrahydropyrimides occurring in pyoverdin-like siderophores

Similar carboxytetrahydropyrimidine structures were already reported by us in pyoverdins from *Pseudomonas fluorescens* CCM 2798 and 2799 (Ser-CTHPMD) (Demange *et al.* 1990a), in pyoverdin from *P. fluorescens* ATCC 17400 (Gln-CTHPMD) (Demange *et al.* 1990b) and in desferri-ferribactin from *P. fluorescens* ATCC 13525 (Tyr–CTHPMD) (Linget *et al.* 1992b), a possible biogenic precursor of the corresponding pyoverdins.

The ^{13}C NMR shifts to the tetrahydropyrimidine moieties common to these compounds are reported in Table 9 together with the corresponding shifts occurring in azoverdin (for the numbering see Figure 5). The largest difference occurs from the free C-terminal caboxyl group in azoverdin with a difference of about 12 p.p.m. as compared with the corresponding carboxamide groups for the three other compounds. For the other carbon atoms, the differences are much smaller, illustrating the common presence of this tetrahydropyrimidine ring.

Configuration of the chromophore and of the amino acids

CD spectra of azoverdin show a positive Cotton effect at 380 nm ($\Delta\varepsilon = +23$) for the free ligand, whereas the iron complex shows a positive Cotton effect at 400 nm ($\Delta\varepsilon = +21$) and a negative one at 460 nm ($\Delta\varepsilon = -9$).

These results are very similar to those determined for pyoverdin Pa (Wendenbaum *et al.* 1983, Demange *et al.* 1990c) and pseudobactin (Teintze *et al.* 1981), and show that the configuration of the asymmetric carbon bound to the chromophore is *S* in azoverdin and that its iron complex has the same coordination as pyoverdin Pa and pseudobactin.

The configuration of the amino acids forming the peptide moiety was determined by gas chromatography on the Chirasil-L-val stationary phase of *O*-methyl, *N*-pentafluoro-propionyl derivatives of the amino acids after total hydrolysis of azoverdin (Demange *et al.* 1988b). It was found that serine has the D configuration, Dab has the L configuration, whereas Hse and OHOrn exist here under both configurations.

In order to locate the enantiomers of these two amino acids in the peptide, partial hydrolysis of azoverdin was undertaken (6 N HCl, 90°C, 15 min). Two types of fragments were obtained.

The chromophoric yellow ones, presenting either a yellow or a blue fluorescence, and detected by UV-vis spectrophotometry at 380 nm, and the peptidic ones detected after spraying with ninhydrin. Ten fractions were separated after CM-Sephadex chromatography of the hydrolyzate: six chromophoric (I–VI) and four peptide. Each fraction was purified by HPLC (trifluoroacetic acid 0.05%/acetonitrile 8% in water, spectrophotometric detection at 220 and 380 nm).

The chromophoric fragments gave each a major compound after HPLC purification (Table 10). The main compounds of the acid hydrolysis, i.e. fractions IV and VI (respectively R_t values of 20 min 9 s and 19 min 30 s) were hydrolysed, derivatized and analysed by gas chromatography on Chirasil-L-val capillary columns. Both contained L-Hse and D-OHOrn.

The complete structure of azoverdin is reported in Figure 1.

Conclusion

The purification and the complete structure elucidation of the two major azoverdins excreted by iron-limited *A. macrocytogenes* ATCC 12334 has been achieved and showed these siderophores to be extracellular pyoverdin-like compounds. *Azomonas* is only the third genus, together with *Pseudomonas* and *Azotobacter*, known to produce iron-binding, fluorescent compounds of this class. *A. macrocytogenes* NCIB 8700 (ATCC 12335), NCIB 10958 and *Azomonas agilis* similarly produce a yellow–green fluorescent

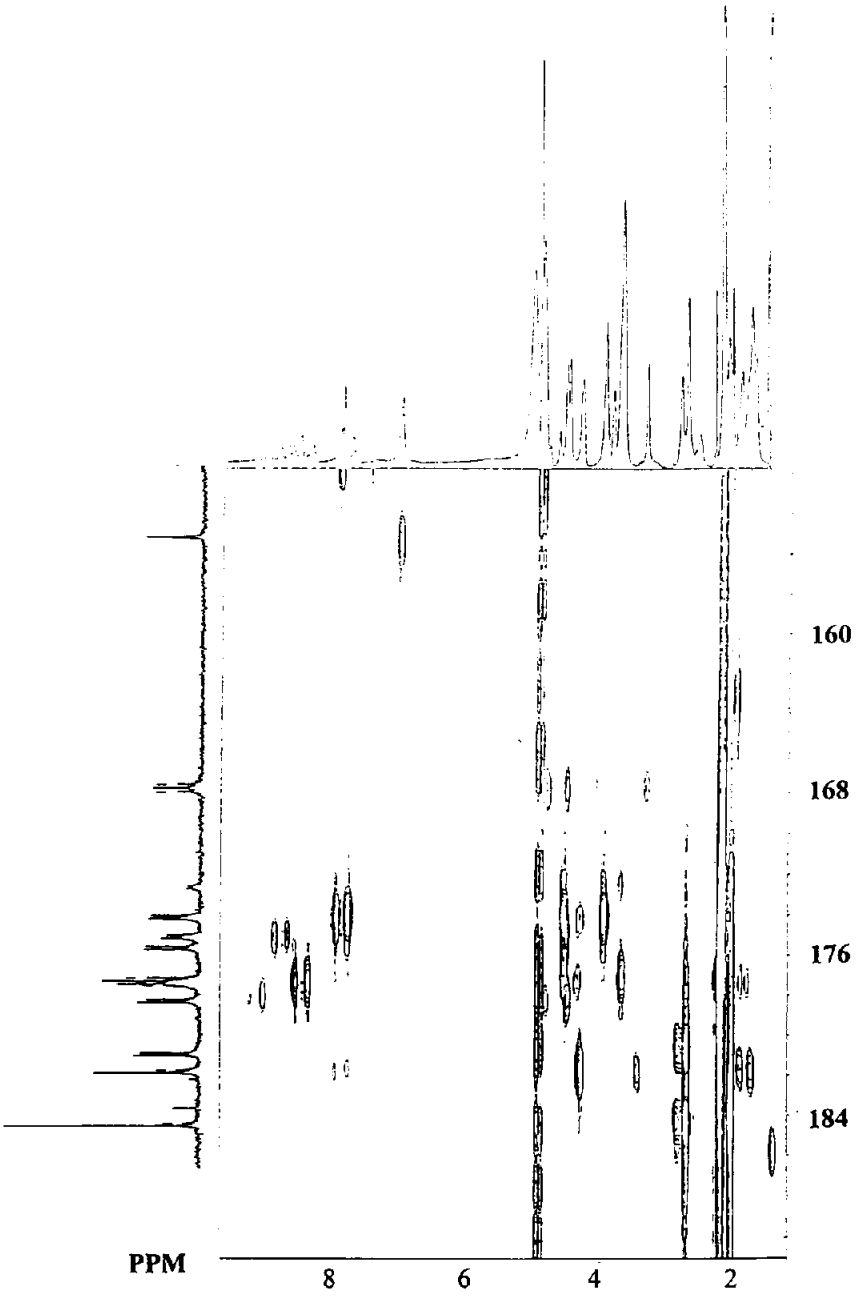


Figure 4. HMQC ^{1}H–^{13}C spectrum of azoverdin.

Table 9. ^{13}C NMR shifts of the tetrahydropyrimidine moieties in the natural pyoverdins and pyoverdin-like siderophores.

Compounds	C2	C4	C5	C6	C7	C8	Reference
Ser–CTHPMD (pyoverdin Pf 2798)	165.10	54.40	23.80	39.40	173.50	57.20	Demange *et al.* 1990a
Tyr–CTHPMD (desferriferribactin)	164.49	52.21	22.28	37.58	172.02	55.11	Linget *et al.* 1992b
Gln–CTHPMD (pyoverdin Pf 17400)	164.60	52.30	22.20	37.60	172.30	52.70	Demange *et al.* 1990b
Hse–CTHPMD (azoverdin)	167.74	54.97	26.54	40.21	184.10	55.77	this work

For the numbering see Figure 5.

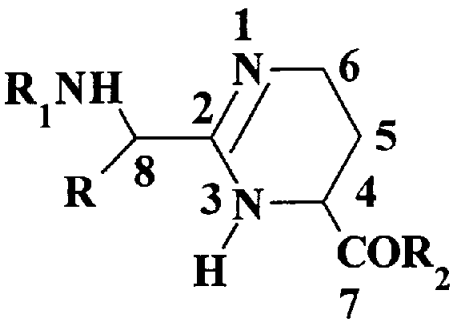


Figure 5. The 4-carboxy-3,4,5,6-tetrahydropyrimidine ring of pyoverdins and pyoverdin-like siderophores.

Table 10. Retention times (R_t) of the different chromophoric fractions of the mild acid hydrolysate of azoverdin after CM-Sephadex chromatography and HPLC purification on a ODS column

	R_t chromophoric fragments		R_t peptidic fragments	
I	major	9 min, 30 s 21 min, 33 s	minor	12 min
II	major	12 min, 45 s 12 min, 15 s	minor	9 min, 54 s
III	major	12 min		
IV	major	20 min, 9 s 4 min	minor	13 min, 15 s
V	major	21 min, 9 s 24 min, 36 s 7 min	minor	13 min, 15 s 5 min, 27 s
VI	major	19 min, 30 s	minor	11 min, 30 s

Buffer composition: 0.05% (v/v) and 8.0% (v/v) water solution of TFA and CH_3CN respectively.

compound when grown under iron limitation (Collinson & Page 1989). The techniques previously used to perform the structure elucidation of pyoverdins (Abdallah 1991) were successfully applied to azoverdin and improved, in particular, by introducing a new decomplexation procedure of ferriazoverdins using EDTA and the ODS column.

Most of the sequence of the peptide chain could be determined using FAB-MS and ROESY NMR. The configuration of the amino acids was established by gas chromatography on a capillary chiral column after derivatization of acid hydrolysates (HCl and HI) and its partial hydrolysis fragments.

The configuration of the chromophore was found to be (*S*), as in all the pyoverdins so far investigated. This was deduced from the CD spectra of azoverdin and by comparison with the spectra of other pyoverdins (Abdallah 1991) and pseudobactin B10, the structure of this latter having been determined by X-ray diffraction (Teintze *et al.* 1981).

With the introduction of the ^{15}N nucleus in azoverdin for its extensive NMR studies, it was possible to perform, for the first time in the pyoverdins family, a complete assignment of the nitrogen atoms of the peptidic siderophore.

From the HOHAHA and the 2D ^{15}N–^{1}H correlations (short and long distance), it was possible to assign all the peptidic nitrogen atoms, as well as the hydroxamic acid nitrogen atoms of the hydroxyornithines and the amidine nitrogen atoms of the two tetrahydropyrimidine moieties, in agreement with literature data (Brown *et al.* 1988, Inbar & Lepidot 1988).

From the $\underline{CH}\alpha$, $^{15}\underline{NH}$ and $^{13}\underline{CO}$ correlations, it was possible to assign all the carbonyl groups of azoverdin, and this gave some evidence of the presence of two 4-carboxyl-3,4,5,6-tetrahydropyrimidine rings in azoverdins.

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