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Structure of Azotobactin D, a Siderophore of *Azotobacter vinelandii* Strain D (CCM 289)†

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ABSTRACT: The structure elucidation of azotobactin D, the fluorescent siderophore excreted by *Azotobacter vinelandii* strain D, has been accomplished by using essentially NMR techniques and FAB mass spectrometry. It is a chromopeptide possessing a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline bound to a peptide chain of 10 amino acids constituted with D- and L-serine (2), L-homoserine (3), D-citrulline (1), D-N^{δ}-acetyl-N^{δ}-hydroxyornithine (1), L-aspartic acid (1), D-*threo*-β-hydroxyaspartic acid (1), and glycine (1). The chromophore is located at the N-terminus of the peptide, and one of the homoserines is at its C-terminus. The latter lactonizes readily, yielding a high amount of azotobactin δ, which is in fact the major compound isolated after the purification steps. The chromophore has an *S* chiral center. The structure of this siderophore differs significantly from the structure proposed by Fukasawa et al. [Fukasawa, K., Goto, M., Sasaki, K., Hirata, Y., & Sato, S. (1972) *Tetrahedron 28*, 5359–5365] for the fluorescent peptide excreted similarly by *A. vinelandii* strain O.

Iron is an essential element for all living cells.

In aerobic media, at physiological pH, the availability of iron for the cells is limited by the solubility of ferric hydroxide.

In iron-deficient conditions, most microorganisms synthesize very powerful iron-sequestering molecules of low molecular weight, called siderophores, which solubilize iron and transport it into the cells by a high-affinity iron transport system (Neilands, 1973).

Azotobacter vinelandii is a bacterium that transforms nitrogen into ammonia. It produces nitrogenase and other proteins involved in nitrogen fixation that require high amounts of iron as well as molybdenum.

In iron-deficient conditions, *A. vinelandii* excretes large amounts of a yellow-green water-soluble fluorescent compound, azotobactin, which is a siderophore of this bacterium (Fekete et al., 1983; Page & Huyer, 1984; Knosp et al., 1984).

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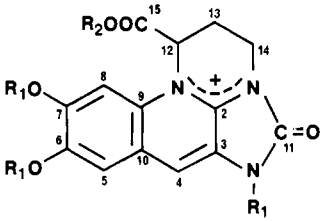
The structure of the pigment excreted by *A. vinelandii* strain O has been reported by Fukasawa and co-workers (Fukasawa et al., 1972). It is a chromopeptide possessing a peptide chain bound to a fluorescent chromophore, **1a**, derived from 2,3-diamino-6,7-dihydroxyquinoline.



1a $R_1 = H$ $R_2 = H$

1b $R_1 = CH_3$ $R_2 = H$

1c $R_1 = H$ $R_2 = CH_3$

The structure of compound **1a** was deduced from X-ray structure determination of its permethylated derivative **1b** (Corbin et al., 1970; Karle & Karle, 1971). It is very similar to the chromophore **2a** common to pyoverdin Pa (Wendenbaum et al., 1983) and pseudobactin (Teintze et al., 1981).

The stereochemistry of **2a** at its asymmetric center is *S* (Teintze et al., 1981; Wendenbaum et al., 1983). No data on the stereochemistry of chromophore **1a** are available since

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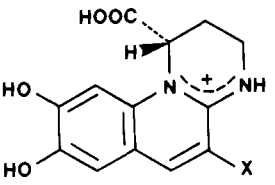


2a X = NH_2

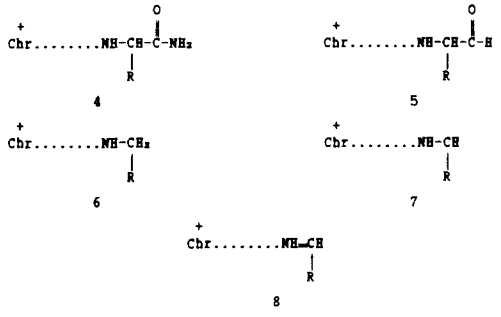
2b X = OH

compound **1b** was in fact synthesized from chromophore **1a** as a racemic mixture (Karle & Karle, 1971).

The peptide chain of chromopeptide **3** excreted by *A. vinelandii* strain O was found to be constituted with some unusual amino acids such as homoserine and citrulline, together with amino acids that occur more frequently in siderophores, namely, serine, aspartic acid, and β-hydroxyaspartic acid.

Chromophore-Aspartic acid-Homoserine-Serine-Homoserine-Citrulline-Serine-Glycine-β-*threo*-hydroxyaspartic acid

3



4 5 6 7 8

Chromophore-(Asp/Ser)-Hse-Gly-OHAsp-Ser-Cit-Hse-$N^δ$-Ac,$N^δ$-OHOrn-Hse

9

Chromophore-(Asp/Ser)-Hse-Gly-OHAsp-Ser-Cit-Hse (lactone)

10

Chromophore-(Asp/Ser)-Hse (lactone)

11

Gly-OHAsp-Ser-Cit-Hse-$N^δ$-Ac,$N^δ$-OHOrn-Hse (lactone)

12

Chromophore-Asp

13

Chromophore-Asp-Ser-Hse-Gly-OHAsp-Ser-Cit-Hse-$N^δ$-Ac,$N^δ$-OHOrn-Hse

14

The sequence of this peptide was deduced from Edman degradation carried out on hydrolytic fragments since blocked peptides cannot be sequenced by the Edman degradation.

Structure **3** was proposed with no mention of the stereochemistry of the amino acids.

In view of what is known about siderophores, this structure is almost certainly wrong since it possesses only *two* chelating groups, the catechol of the chromophore and the hydroxy acid of β-hydroxyaspartic acid, instead of the *three* normally expected for such molecules (Teintze et al., 1981; Teintze & Leong, 1981; Philson & Llinas, 1982; Wendenbaum et al., 1983; Yang & Leong, 1984; Buyer et al., 1986).

In this paper we report the complete structure of azotobactin D, the yellow-green fluorescent siderophore produced by *A. vinelandii* strain D, and show that this compound differs significantly in its peptide sequence from that proposed for the related pigment produced by strain O. We describe how the techniques of FAB mass spectrometry and NMR spectroscopy together comprise a powerful combination for the rapid structural assignment of this type of peptide-based siderophore.

Materials and Methods

Strain and Culture Medium. Azotobacter vinelandii strain D (CCM 289) (van Lin & Bothe, 1972; Bothe & Falkenberg, 1972) was grown in aerobic conditions.

The culture medium had the following composition per liter: K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCO_3$, 1 g; NaCl, 0.2 g; mannitol, 10 g. It was adjusted to pH 7.0 before sterilization.

Isolation of Azotobactin D. The bacteria were grown aerobically at 25 °C in 10 1-L conical flasks each containing 0.5 L of culture medium and subject to mechanical agitation. After 6 days, the culture medium (5 L overall) was treated with a molar solution of barium chloride (35 mL) and centrifuged.

The supernatant was divided into two fractions of 2.5 L that were each applied on a Sephadex column [acetate form, outside diameter (ϕ) = 3 cm, length (l) = 40 cm].

The column was first eluted with water (500 mL) and then with a linear gradient of sodium chloride (0–0.5 M, 2 L overall).

Two fluorescent compounds were isolated: azotobactin D and azotobactin δ in the ratio of 60% to 40%.

Both compounds were separately chromatographed on a Bio-Gel P2 column (ϕ = 3 cm, l = 80 cm) eluted with water and then, after evaporation, on a DEAE-Sephadex[1] column made up in a 0.1 M pyridine–acetic acid, pH 5.0, buffer (ϕ = 3 cm, l = 40 cm) eluted with a linear gradient of the same buffer (0.1–0.5 M, 1 L overall).

The total yield after lyophilization was 100 mg of azotobactin δ and 160 mg of azotobactin D.

Alternatively, in another experiment, after centrifugation (15 000 rpm, 30 min), the bacterial supernatant was filtered through a 0.2-μm membrane (Millipore), adjusted to pH 4, and applied on a column of octadecylsilane (Lichroprep RP 18, 40–63 μm, Merck, Darmstadt) made up in 0.05 M pyridine–acetic acid buffer, pH 5.0. The column was eluted with the same buffer first (0.5 L), then with a mixture of buffer–acetonitrile (1:1), which eluted azotobactin D and azotobactin δ, and finally with a 1:4 mixture of buffer–acetonitrile, which eluted (dihydroxybenzoyl)lysine.

The fractions containing crude azotobactin were pooled, dried, dissolved in 0.05 M pyridine–acetic acid buffer, pH 5.0, and chromatographed on a DEAE-Sephadex A-25 column (ϕ = 4 cm, l = 40 cm) made up in the same buffer, and eluted first with 0.05 M of the same buffer (0.6 L) and then with a linear gradient of 0.05–1 M pyridine–acetic acid buffer, pH 5.0 (2 L overall).

In this case, the ratio between azotobactin D and azotobactin δ was found to be 1:4, namely, 80 mg of azotobactin D and 320 mg of azotobactin δ were isolated.

Electrophoresis. Electrophoretic analyses were performed on cellulose acetate sheets in 0.1 M pyridine–acetic acid, pH 5.0, buffer. The fluorescent spots were visualized with UV light at 350 nm.

NMR Spectroscopy. ^1H and ^{13}C NMR spectra were measured on either a Bruker 200- or 400-MHz spectrometer (Bruker Spectrospin, Wissembourg, France), using either deuteriated trifluoroacetic acid or ^2H_2O as solvent and sodium [$^2H_6$](trimethylsilyl)propanesulfonate as an internal standard.

^1H NMR spectra were determined at 200 or 400 MHz; ^{13}C NMR spectra were measured at 50 or 100 MHz. ^1H con-

[1] Abbreviations: Hse, homoserine; Cit, citrulline; OHAsp, β-*threo*-hydroxyaspartic acid; OHOrn, $N^δ$-hydroxyornithine; Chr, chromophore; DEAE, diethylaminoethyl.

ventional spectra were recorded at 20 °C.

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.

Two-dimensional correlated spectroscopy (COSYHG.AUR) was performed by using the two-pulse sequence $(90\text{–}t_1\text{–}45\text{–}t_2)_n$ (Aue et al., 1976), with presaturation of the signal of water for 2 s. Sixteen pulses were used for each of values with 2048 points in t_2. Zero filling in t_1 gave a 1024 × 2048 point frequency domain matrix. Both t_1 and t_2 were multiplied with a sine-bell function.

Heteronuclear shift correlated 2D ^1H–^{13}C NMR spectroscopy was performed by using a Bruker program (HCORR.AUR) based on polarization transfer from ^1H to ^{13}C via J ^{13}C–^1H coupling (Bax & Morris, 1981).

For each of 256 t_1 values 112 pulses were used with 2048 points in t_2. Zero filling in t_1 and t_2 gave a 1024 × 4096 point frequency domain matrix. Both t_1 and t_2 were multiplied with a sine-bell function.

UV–Visible Spectrophotometry. Absorption spectra were obtained by use of a Hewlett-Packard 8451A UV–vis spectrophotometer or a Varian Cary 219 instrument.

The extinction coefficient of ferric azotobactin D (and δ) at its absorption maximum was calculated from the absorbance of an aqueous solution whose iron concentration was measured by atomic absorption spectroscopy using either a Techtron 1200 or a Jobin-Yvon ICP 2500 instrument.

The extinction coefficient of azotobactin D (and δ) was deduced after titration with a 10^{-2} M ferric chloride solution.

Circular Dichroism. Circular dichroism (CD) spectra were determined on a Jobin-Yvon DC III instrument. The solvents were either 0.1 M pyridine–acetic acid buffer at pH 5.0 or 0.1 M phosphate buffer at pH 7.2.

Mass Spectrometry. Electron impact mass spectrometry was performed on a Thomson THN 208 high-voltage (8 kV) spectrometer (Thomson, 78-Chatou, France) using a direct inlet.

FAB mass spectrometry was determined on a VG Analytical (Manchester, U.K.) ZAB-HF mass spectrometer fitted with a FAB gun manufactured by M-Scan (Ascot, U.K.). Xenon was used as a gas.

Azotobactin D (and δ) was dissolved in 5% acetic acid (5–10 μg, 1:1) on the stainless steel target of the FAB probe. Aliquots of hydrolysis and esterification reactions were loaded directly into the matrix without drying down. The mass spectrometer was operated at 8-kV accelarating voltage, and the FAB gun was operated at 10 kV and 10 μA. Spectra were recorded on oscillograph paper and were manually counted.

High-Pressure Column Chromatography. Analytical HPLC was performed on a Chromatem-800 instrument (Touzart et Matignon, Vitry sur Seine, France) using reverse-phase octadecylsilane columns eluted with gradients of 0.017 M pyridine–acetic acid buffer, pH 5.3, in acetonitrile (up to 50% acetonitrile).

Preparative HPLC was performed on a Du Pont instrument using a Zorbax ODS column (ϕ = 2.1 cm, l = 25 cm) and eluting with the same buffer as above.

Amino acid analyses were performed either on a Durrum D 500 or on a Beckman Unichrom instrument.

Isolation and Purification of the Chromophore of Azotobactin (Compound **1a**). Azotobactin D (43 mg) was hydrolyzed with 6 M HCl (8 mL) at 110 °C in a sealed tube. After 40 h, the solvent was evaporated and the acid completely removed by sequential addition of water and evaporation. The residue was suspended in water, a few drops of trifluoroacetic acid were added in order to dissolve it, and the solution was applied on a Sephadex G-10 column (ϕ = 3 cm, l = 80 cm) eluted with water (200 mL) and then with 1% aqueous acetic acid to yield 7 mg of pure chromophore.

Preparation of the Methyl Ester of the Chromophore of Azotobactin (**1c**). Azotobactin D (50 mg) was treated with 6 M HCl (2.5 mL) in a sealed tube and heated at 110 °C. After 24 h, the reaction mixture was evaporated under reduced pressure and dried in a desiccator in the presence of phosphorus pentoxide and potassium hydroxide pellets. The crude product was treated with 6 M anhydrous HCl dissolved in methanol (3 mL) and heated at 110 °C for 3 h in a Teflon stoppered tube and then evaporated under a stream of argon.

The chromophore was purified by HPLC using a C-18 octadecylsilane column (Chromatem, Lichrosorb 5 μm) eluted with a gradient of trifluoroacetic acid at pH 2.2 (0.05%) and acetonitrile. The yield of pure compound was 85%.

Prolonged Acid Hydrolysis of the Chromophore of Azotobactin. Chromophore **1a** (0.5 mg) was dissolved in 2 M aqueous HCl (0.5 mL) in a sealed tube heated 72 h at 150 °C. The mixture was dried in a desiccator in the presence of phosphorus pentoxide and potassium hydroxide pellets and purified by HPLC as above. It yielded quantitatively a new chromophore, **2b**, which was separately obtained from the acid hydrolysis of pyoverdins (Demange et al., 1987).

The reaction was monitored as a function of time by HPLC (several samples were used), and the new chromophore was characterized by coelution with an authentic sample and by its ^1H NMR spectrum.

Results and Discussion

Physicochemical Properties of Azotobactin D and Azotobactin δ. The two major fluorescent compounds isolated from the culture media, azotobactin D and azotobactin δ, were purified either by a classical sequence of ion-exchange chromatography followed by gel filtration desalting or by a new and rapid procedure using octadecylsilane as a stationary phase for the direct filtration of the bacterial supernatant. This procedure was much more rapid than that we have previously reported for the purification of pyoverdins (Meyer & Abdallah, 1978), involving complexation with iron, purification of the iron complex, removal of the iron by treatment with 8-hydroxyquinoline, and final purification of the free ligands. Moreover, the older procedure could not be applied when the iron complexes were not extractible in benzyl alcohol or 1:1 mixtures of phenol and chloroform. The new purification procedure appeared to be superior to the method recently described (Briskot et al., 1986) using the XAD type of hydrophobic stationary phases that give weaker interactions with this type of siderophore. Using octadecylsilane, we could also, by increasing the relative concentration of acetonirile in buffer, isolate (dihydroxybenzoyl)lysine from the culture media of *Azotobacter vinelandii* strain D.

Film electrophoresis and ion-exchange chromatography showed that azotobactin D has one more negative charge than azotobactin δ.

The absorption spectra of both compounds were found to be identical and very close to those reported for azotobactin O (Fukasawa et al., 1972).

The spectra of the free ligands are pH dependent between pH 3 and pH 9, whereas the spectra of the corresponding iron(III) complexes are unaffected by a change of pH in the same range.

At pH 5.0, azotobactin D shows an absorption maximum at 380 nm (ϵ = 23 500 M^{-1} cm^{-1}) and a shoulder at 336 nm

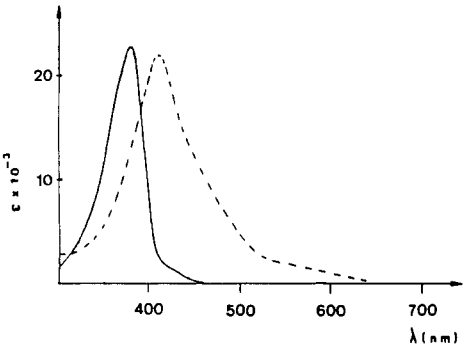


FIGURE 1: Absorption spectra of azotobactin D (—) and its iron(III) complex (---) at pH 5.0.

(ϵ = 19 600 M^{-1} cm^{-1}). Its iron complex has a maximum at 412 nm (ϵ = 23 000 M^{-1} cm^{-1}) and two shoulders at 450 nm (ϵ = 10 000 M^{-1} cm^{-1}) and at 550 nm (ϵ = 2000 M^{-1} cm^{-1}) (see Figure 1).

The circular dichroism spectrum of azotobactin D showed a negative Cotton effect in the 350–450-nm range, with two minima at 330 ($\Delta\epsilon$ = −1.9) and 412 nm ($\Delta\epsilon$ = −1.2). From the comparison of this spectrum with the circular dichroism spectrum of pyoverdin Pa (Demange et al., 1987), it was not possible to draw any conclusion on the stereochemistry of the asymmetric center of azotobactin D (see below).

Fast atom bombardment gave a molecular ion at 1411 u for azotobactin D and at 1393 u for azotobactin δ (18 u below). The 1:1 stoichiometry of the corresponding iron complexes was also established by FAB, which gave the corresponding molecular peaks, respectively, at 1464 and 1446 u.

Structure Elucidation of Azotobactin D. Total acid hydrolysis (48 h, 6 M HCl, 110 °C or 48 h, 6 M HI, 110 °C) of azotobactin D as well as azotobactin δ indicated that both siderophores were constituted with the same chromophore (unaltered during the hydrolysis step) and a peptide chain of 10 amino acids possessing serine (2), homoserine (3), citrulline (1), glycine (1), aspartic acid (1), N^{δ}-hydroxyornithine (1), and an unknown amino acid that eluted before aspartic acid and was further characterized as *threo*-β-hydroxyaspartic acid (see below for stereochemistry of the chromophore and the amino acids).

Identification of the Chromophore. Preliminary experiments suggested that the chromophore obtained after acid hydrolysis of azotobactin D and azotobactin δ was identical with chromophore **1a** derived from the pigment excreted by *A. vinelandii* strain O after the same treatment (Fukasawa et al., 1972; Corbin et al., 1970; Karle & Karle, 1971).

This was established after comparison of the spectrophotometric data and the electron impact mass spectrum as well as the ^1H NMR spectrum reported for compound **1a** with those we obtained for the chromophore derived from azotobactin from strain D and its methyl ester **1c** (the latter appeared to be more soluble in most solvents than compound **1a**).

However, the assignment of the chromophoric protons had never been truly established, nor had the ^{13}C NMR spectrum of this molecule ever been reported.

If proton H-4 can be easily recognized since it shifts readily when the substituent on C-3 varies (Demange et al., 1987), protons H-5 and H-8 are more difficult to distinguish. These protons were distinguished by measuring the nuclear Overhauser effect on proton H-8 (at 6.90 ppm), whose signal is enhanced on irradiation of proton H-12 (at 5.80 ppm).

In the ^{13}C NMR spectrum of ester **1c**, the chemical shifts of the carbon atoms had nearly the same values as in azotobactin δ. This indicated that there was no alteration of the chromophore during the hydrolysis and esterification steps (see below and Table I).

Table I: ^1H and ^{13}C NMR Data of Chromophore **1c**

resonance	δ	coupling constants (Hz)
H-4	7.92	
H-5	7.28	
H-8	7.23	
H-12	6.06	J_3 H-12–H-13 = 5.35
		J_3 H-12–H-13′ = 2.05
H-13	2.43	J_2 H-13–H-13′ = −14.4
		J_3 H-13–H-14 = 12.4
H-13′	3.00	J_3 H-13′–H-14 = 4.3
		J_3 H-13–H-14′ = 1.5
H-14	3.56	J_2 H-14–H-14′ = −13.2
		J_4 H-14–H-12 = 0.8
H-14′	4.19	J_3 H-14′–H-13 = 4.84
		J_4 H-14′–H-12 = 1.0

constituent carbons	chemical shift	constituent carbons	chemical shift
C-15	171.6	C-10	122.7
C-11	156.0	C-5	116.0
C-7	153.9	C-8	103.2
C-2	148.4	C-12	58.8
C-6	142.4	OCH_3	57.3
C-9	131.7	C-14	37.7
C-3	124.4	C-13	25.1
C-4	123.8		

[a] The chemical shifts (δ) are in ppm with sodium [$^2H_6$](trimethylsilyl)propanesulfonate as internal standard. The coupling constants (J) are in hertz. The spectra were determined in ^2H_2O.

NMR Spectra of Azotobactin D and Azotobactin δ. Both azotobactin D and azotobactin δ show very similar NMR spectra, although slight differences were observed and interpreted. The spectra were very sensitive to pH variations.

It appeared to us to be more convenient to do most of the NMR measurements (2D ^{13}C–^1H correlation spectra and 2D ^1H–^1H COSY spectra) on azotobactin δ, since this compound was finally the most abundant after all the purification steps, which were performed at rather acidic pHs (4.0–5.0).

Therefore, all the assignments were made on this compound. The few differences that exist between the NMR spectra of both azotobactin D and azotobactin δ are interpreted below.

(*a*) *Description of the Spectra.* The ^1H NMR spectrum of azotobactin δ shows three rather sharp singlets at 6.91 (H-8), 7.02 (H-5), and 7.82 ppm (H-4) (at pH 3.4), corresponding to the three protons of the quinoline ring. The signal at 5.81 ppm represents proton H-12 on the asymmetric carbon atom of the ring C bound to the quinoline moiety. It was observed that the chemical shifts of protons H-4 and H-5 was markedly influenced by the concentration of the solution used for ^1H NMR measurements. In the concentration range usually used for ^{13}C spectra determination, H-5 can resonate at higher field than H-8 (see below for the ^{13}C resonance assignment). Under the conditions in which the 2D ^1H–^{13}C NMR spectrum was determined (i.e., ca. 120 mg/mL), the ^1H NMR chemical shifts were 7.32 (H-4), 6.95 (H-8), 6.50 (H-5), and 5.70 ppm (H-12). Upon dilution of the solution, the signals corresponding to H-8 and H-5 coalesced and became inverted, H-8 beginning resonating at higher field than H-5 (data not shown). The rest of the resonances at high field correspond to the signals due to the amino acids of azotobactin δ (and azotobactin D), an acetyl group at 1.95 ppm, as well as the protons of ring C. These values are presented in Table II, and the ^1H NMR spectrum of azotobactin δ in ^2H_2O is shown in Figure 2.

The assignments were determined by using a COSY 90 homonuclear correlation. All the corresponding values reported in Table II were found to be in agreement with those

Table II: Assignment of the Protons of Azotobactin δ[a]

proton	chemical shift (ppm)	multiplicity	proton	chemical shift (ppm)	multiplicity
a	1.28	m	m–n′	3.72	m
b	1.51–1.64	m	n	3.80	m
c	1.57	m	o	4.11–4.34	m
d–d′	1.59–1.82	m	p	4.12	m
e	1.76–1.84	m	q	4.23	m
z	1.96	s	r	4.24	m
f	2.11–2.41	m	s–s′	4.31	m
g	2.50–2.84	m	r′	4.32	m
h	2.68–2.80	m	t	4.38	d
i	2.88	m	u	4.46	t
j	3.40	m	v	5.81	s
k	3.44	m	w	6.91	s
j′	3.45	m	x	7.02	s
l	3.52–4.19	m	y	7.82	s

[a] For the numbering see Figure 7. The usual abbreviations are used for the multiplicities of the protons: m (multiplet), t (triplet), d (doublet), and s (singlet). The spectrum was determined in ^2H_2O with sodium [$^2H_6$](trimethylsilyl)propanesulfonate as an internal standard.

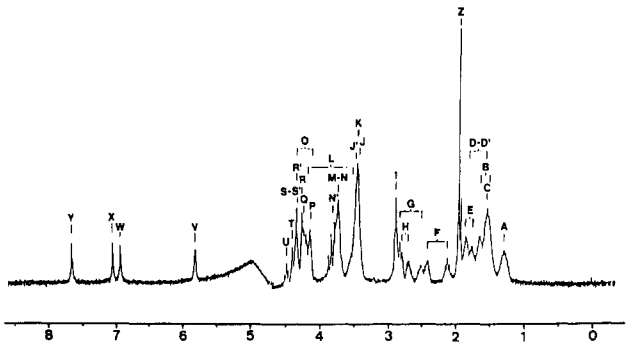


FIGURE 2: ^1H NMR spectrum of azotobactin δ determined in ^2H_2O with sodium [$^2H_6$](trimethylsilyl)propanesulfonate as an internal standard (the ^2HOH resonance is irradiated).

reported in literature for amino acids and peptides (Wüthrich, 1976a).

(*b*) *^{13}C NMR Spectra of Azotobactin D and Azotobactin δ.* The carbonyl region of the ^{13}C NMR spectrum of azotobactin δ, between 180.9 and 164.3 ppm, gives 15 peaks as expected. In the next region, there are 10 peaks; 9 of them belong to the quinoline ring, with three tertiary carbon atoms having their resonances at 101.8, 114.7, and 122.3 ppm and six quaternary carbon atoms having their resonances at 153.9, 148.3, 141.9, 130.4, 123.5, and 121.7 ppm. The 10th signal present in this region corresponds to the carbonyl group at C-11 of the chromophore and occurs at 155.5 ppm. All these signals correspond to the resonance previously observed for the chromophore **1c**. The rest of the signals from 74.5 to 22.2 ppm correspond to the amino acid resonances in addition to those of the three carbon atoms of ring C of the chromophore as well as the resonance of the methyl group of the acetyl bound to the N^{δ}-hydroxyornithine of the peptide chain (see below for the sequence of the peptide chain).

The chemical shifts of the resonances of all the carbon atoms of azotobactin δ are listed in Table III. The ^{13}C spectrum of the siderophore is represented in Figure 3.

(*c*) *Assignment of the Resonances of the Aliphatic Carbon Atoms.* The multiplicity of all the signals in the 74.5–22.2 ppm region was determined by DEPT sequence and by undecoupled ^{13}C NMR spectra. All the assignments were performed using the 2D ^1H–^{13}C correlation (see Figure 4). The results are reported in Table III.

All the correlations are present in Figure 4, except the correlation between C-13 of ring C of the chromophore and

Table III: Assignment of the Carbons of Azotobactin δ[a]

constituent carbons	chemical shift	constituent carbons	chemical shift
Hse CO (lactone) γ	180.9	Hse Cγ (lactone)	70.3
OHAsp CO γ	178.8	Ser Cβ	64.1 (2)
Asp CO γ	178.2	Hse Cγ	60.7 (2)
	177.1	OHAsp Cα	59.4
	176.8 (2)	Ser Cα	59.1
	176.7	Ser Cα–Chr C-12	58.7 (2)
	176.4	OHOrn Cα	56.7
	175.0	Cit Cα	56.3
	174.9	Hse Cα	54.3
	174.7	Hse Cα–Asp Cα	54.0 (2)
	174.6	Hse Cα (lactone)	52.2
	174.3	OHOrn Cδ	50.0
acetyl CO	170.5	Gly	45.6
Cit (–$NHCONH_2$)	164.3	Cit Cδ	42.1
Chr C-11	155.4	Asp Cβ	40.2
Chr C-7	153.9	Chr C-14	37.4
Chr C-2	148.3	Hse Cβ	36.1 (2)
Chr C-6	141.9	OHOrn Cβ	31.1
Chr C-9	130.4	Cit Cβ	30.7
Chr C-3	123.5	Hse Cβ (lactone)	30.6
Chr C-4	122.2	Cit Cγ	28.5
Chr C-10	121.7	Chr C-13	25.9
Chr C-5	114.7	OHOrn Cγ	25.3
Chr C-8	101.8	acetyl CH_3	22.3
OHAsp Cβ	74.5		

[a] See Figure 7. The spectrum was determined in ^2H_2O with sodium [$^2H_6$](trimethylsilyl)propanesulfonate as internal standard. Chr refers to the chromophore. In parentheses is the number of carbon atoms giving the same resonance.

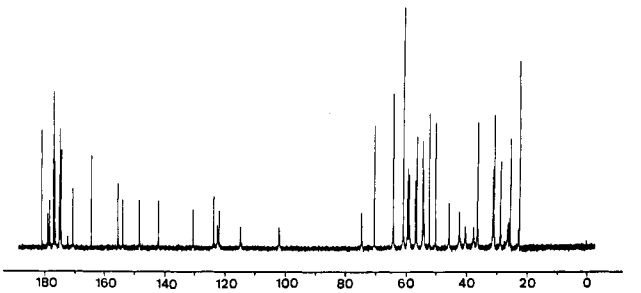


FIGURE 3: ^{13}C NMR spectrum of azotobactin δ in ^2H_2O with sodium [$^2H_6$](trimethylsilyl)propanesulfonate as an internal standard.

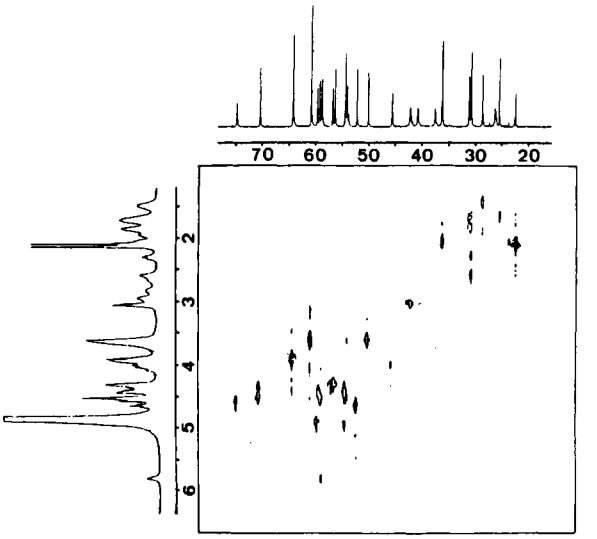


FIGURE 4: ^1H–^{13}C correlation for azotobactin δ in the aliphatic region of the spectrum.

the two protons on this carbon atom, H-13 and H-13′. However, it was proved that C-13 occurred at 26.0 ppm: the projection of the ^1H NMR spectrum corresponding specifically

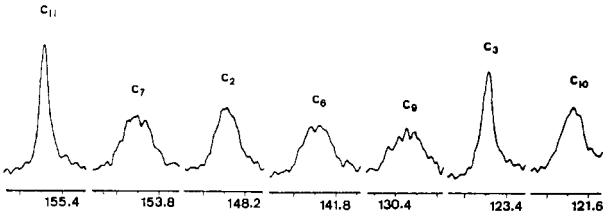


FIGURE 5: Assignment of the carbon atoms of the chromophoric part of azotobactin δ from their long-range coupling constants.

to this carbon atom (data not shown) showed a correlation with two protons at 2.40 and 2.79 ppm. This value of 26.0 ppm for the chemical shift of C-13 is also very close to the corresponding value of 25.1 ppm found in compound **1c**.

(*d*) *Assignment of the Aromatic Resonances.* The assignments of the aromatic ^{13}C resonances were performed after examination of the multiplicity of the signals in the ^{1}H undecoupled ^{13}C spectrum as well as the long-range J_2 and J_3 couplings for the quaternary carbon atoms.

The assignments of the three tertiary carbons C-4 (122.3 ppm), C-5 (115.6 ppm), and C-8 (102.8 ppm) have been made by using the 2D ^{1}H–^{13}C correlation. The ^{13}C chemical shifts were, in contrast to the ^{1}H shifts, not affected by changes in concentration of the solution used.

The quaternary carbon atoms were assigned by considering their J_2 and J_3 long-range 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 contrary, J_3 couplings are much larger and are in the range of 10 Hz (Hansen, 1979).

The six quaternary carbon atoms of the chromophore are C-2, C-3, C-6, C-7, C-9, and C-10. C-2 is substituted with two nitrogen atoms with a positive charge; C-6 and C-7 are each substituted with an OH group. These three carbons are therefore the most unshielded, and their resonances are expected at low field, namely, at 153.9, 148.3, and 141.9 ppm. The three remaining resonances at 121.7, 123.5, and 130.4 ppm correspond to C-10, C-3, and C-9, respectively. C-3 shows no J_3 long-range coupling and only one J_2 coupling. It is therefore expected to be nearly a singlet, as is the resonance at 123.5 ppm. C-9 is coupled with H-4 and H-5 (two J_3 couplings) and with H-8 (one J_2 coupling). It should show as a doublet of doublets or even as a multiplet, as is the signal at 130.4 ppm. Moreover, it occurs at lower field than C-10 because of the proximity of the positively charged nitrogen atom. Finally, C-10, which possesses one J_3 and two J_2 couplings, resonates at 121.7 ppm (see Figure 5).

The three resonances at low field correspond to C-2, C-6, and C-7. C-6 and C-7 both show one J_2 and one J_3 coupling and are expected to be very similar in shape and pH dependent, as are the signals at 141.9 and 153.9 ppm. The latter resonance is more likely due to C-7, which should be at lower field than C-6, as observed in similarly substituted compounds (Katritzky et al., 1981; Levy et al., 1980). The last quaternary carbon atom of the chromophore is C-2, and it resonates at 148.3 ppm (see Figure 5 and Table III). The resonance at 155.5 ppm corresponds to the carbonyl group of ring D of the chromophore and possesses a similar chemical shift as in compound **1c**.

(*e*) *Carbonyls of Azotobactin δ.* Azotobactin δ possesses 15 signals in the carbonyl region, from 180.9 to 164.4 ppm. Some of them could be assigned without ambiguity, like the resonance at 164.4 ppm that corresponds to the citrulline carbonyl $NHCONH_2$ and the resonance at 170.5 ppm that corresponds to the *N*-acetyl group bound to N^{δ}-hydroxyornithine, which is very close to the value it possesses in tetraglycylferrichrome (Deml et al., 1984).

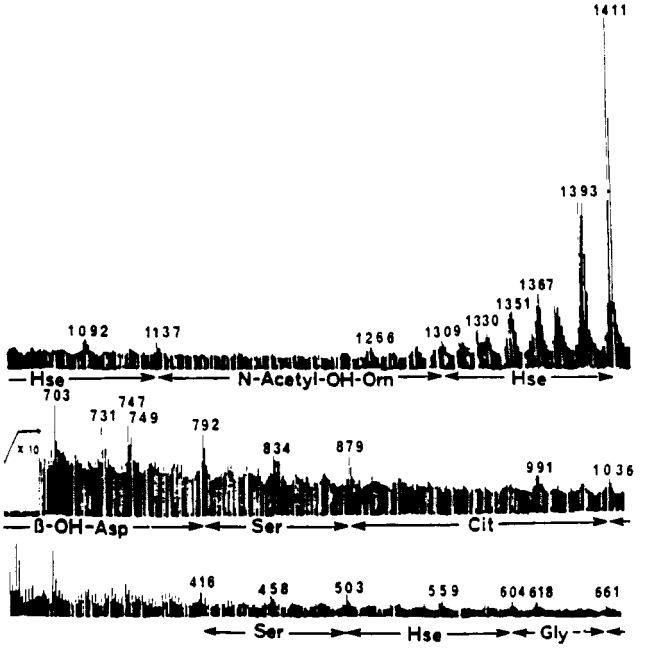


FIGURE 6: FAB spectrum of azotobactin D.

The carbonyl group of the terminal homoserine was found to resonate at 180.9 ppm since it shifts when azotobactin δ is changed to azotobactin D (see below the relationship between azotobactin D and azotobactin δ).

The rest of the resonances that occur between 174.3 and 178.8 ppm were tentatively assigned by comparison with values published in the literature for the amino acids (Wüthrich, 1976) (see Table III).

Sequence of the Peptide Chain. The peptide sequence of azotobactin D was determined from the data afforded by FAB mass spectrometric analysis of the intact siderophore and products of partial hydrolysis.

The positive FAB spectrum of azotobactin D is reproduced in Figure 6. The molecular ion M^+ at m/z 1411 dominates the spectrum. This ion is not protonated because of the presence of the permanent positive charge in the chromophore. Azotobactin δ gave a molecular ion at m/z 1393, 18 units below that of azotobactin, suggesting that the former was derived from the latter by loss of a water molecule. The molecules could be interconverted by changing the pH. The molecular ion at 1411 shifted to 1393 after azotobactin D had been allowed to stand for a short time in 0.1 M HCl (data not shown), while in basic medium azotobactin δ was converted to azotobactin D, either partially or fully, depending on the reaction conditions. Although the majority of the ion current was carried by the molecular ions, a few spectra were obtained of sufficient quality to allow fragment ions to be distinguished from the background signals. The spectrum in Figure 6 was the best spectrum acquired from azotobactin D. A series of N-terminal fragment ions was present from which an almost complete sequence could be deduced. Although azotobactin D contains normal peptide linkages, the presence of the fixed positive charge in the chromophore precludes the formation of ions normally observed in the FAB spectra of peptides (Morris et al., 1981; Williams et al., 1982) that carry a charge at the point of cleavage.

Instead, the following family of N-terminal structures give rise to characteristic sequence ion clusters with the amide ion **4** at highest mass, and **5**, **6**, **7**, and **8** separated from **4** by 15, 43, 44, and 45 mass units, respectively.

In the spectrum of azotobactin D (Figure 6) the first such cluster occurs at m/z 503 accompanied by 488 (weak), 460,

459, and 458. These can be assigned the composition chromophore plus serine plus aspartic acid, with the order of the two amino acids undefined. The weak signal at m/z 416 is indicative of chromophore-Asp, but the absence of corroborating ions 43, 44, and 45 below m/z 416 made this a very tentative assignment. In summary, the FAB fragmentation data suggested the sequence **9** for azotobactin D.

This sequence was corroborated and the Asp/Ser order firmly established by acid hydrolysis experiments in which the products were monitored by FAB-MS. Mild acid hydrolysis (0.1 M HCl, 90 °C, up to 30 min) rapidly converted azotobactin D to azotobactin δ (m/z 1411 → 1393), and this was followed by deacetylation to give a product at m/z 1351. No cleavage of the peptide chain occurred under these conditions. More vigorous conditions (6 M HCl, 50 °C) resulted in peptide bond cleavage, giving m/z 1120 (**10**) (corresponding to loss of the C-terminal homoserine and hydroxyornithine) and m/z 587 (**11**) (corresponding to the cleavage of the Hse–Gly bond). A minor signal at m/z 807 was assigned to the C-terminal peptide **12** arising from acid hydrolysis. A cyclic homoserine lactone residue was present at each of the new C-termini of the hydrolytic fragments.

At higher temperatures (6 M HCl, 90 °C, 8 min) fragment **11** was the major product. More prolonged hydrolysis led to the formation of a species of molecular mass 417, precisely that expected for chromophore-Asp **13**. This result provided firm evidence for the sequence chromophore-Asp-Ser... rather than chromophore-Ser-Asp... in azotobactin D. Compounds **11** and **13** were purified by HPLC, and upon amino acid analysis, **11** gave 1 mol each of aspartic acid, serine, and homoserine, while compound **13** gave only aspartic acid.

The complete sequence of azotobactin D, deduced from the FAB results, is given below (**14**).

(*a*) *Relationship between Azotobactin D and Azotobactin δ.* The second major compound isolated during the purification procedure was called azotobactin δ. Both siderophores gave the same electronic spectral data and very similar NMR spectra. The sequence of its peptide chain is identical, and the stereochemistry of the amino acids is the same. Moreover, the circular dichroism spectra of both compounds are identical.

The FAB spectrum of azotobactin D shows the same fragment ions of azotobactin δ up to and including the signal at m/z 1309. The only difference resides in the molecular ions (m/z 1411 for azotobactin D and m/z 1393 for azotobactin δ) and is probably due to the conversion of the C-terminal homoserine to its lactone.

To confirm this hypothesis, the number of free acid groups in azotobactin δ was established by FAB-MS monitoring of its esterification products: Treatment of azotobactin δ with an equimolar mixture of methanol and deuteriated methanol containing anhydrous hydrochloric acid gave rise to a 1:2:1 triplet at m/z 1421, 1424, and 1427, consistent with the formation of a diester.

Despite relatively mild esterification conditions, some methanolysis of the amide bonds occurred, affording the products whose molecular ions are present at m/z 1148, 1151, 1154 (1:2:1); 821, 824 (1:1); and 601, 604 (1:1). These correspond to the diester of peptide **10**, the monoester of peptide **12**, and the monoester of peptide **11**, respectively. The results clearly showed that azotobactin δ contains only two free acid groups, both of which are present in its cleavage product **10**, and neither of which, therefore, can be at the C-terminus. From the sequence, it can be seen that aspartic acid and hydroxyaspartic acid are the two residues carrying free carboxyl groups. Three free acid functions were expected for the siderophore containing a C-terminal carboxyl group. We concluded, therefore, that azotobactin δ is lactonized at the C-terminus.

This was also corroborated with both ^{13}C and ^{1}H NMR spectra of azotobactin δ. The ^{13}C NMR spectrum showed four differences with the spectrum of azotobactin D, corresponding to the difference introduced by the lactonization of the terminal homoserine.

The signal of one carbonyl carbon shifts from 179.9 ppm in azotobactin D to 180.9 ppm in azotobactin δ. The three other carbons shifting are respectively C-o (OCH_2, from 61.1 to 70.3 ppm), C-u ($NHCHCO$, from 54.4 to 52.2 ppm), and finally C-f ($CHCH_2CH_2O$, from 36.2 to 30.6 ppm).

In the ^{1}H NMR spectrum of azotobactin δ there is an additional ABCDX system where the five protons are centered at 4.47 ppm ($NHCHCO$), 2.11 and 2.41 ppm ($CHCH_2CH_2O$), and 4.11 and 4.25 ppm ($CHCH_2CH_2O$) (see Table II).

(*b*) *Stereochemistry of the Chromophore and the Amino Acids of Azotobactin D.* The stereochemistry of the amino acids was determined by derivatization of acid hydrolysates (HCl and HI) of azotobactin D or its partial hydrolytic fragments, followed by gas chromatography on a capillary Chirasyl column (Frank et al., 1977). The derivatives were *N*-pentafluoropropionyl *O*-methyl esters.

As standards, L and DL amino acids constituting the peptide chain were separately treated the same way as the hydrolysates of azotobactin.

It was found that all the homoserines and aspartic acid were L and citrulline and N^{δ}-hydroxyornithine were D. β-Hydroxyaspartic acid was found to be D-*threo* and easily distinguishable from its stereoisomers (P. Demange, M. A. Abdallah, and H. Frank, unpublished results).

The two serines had opposite configurations. The same treatment performed on compound **11** established that the configuration of the serine on the chromophore side was D.

The stereochemistry of the chromophore of azotobactin D was established by using circular dichroism techniques by comparison with the chromophore of pyoverdin Pa, which is the closest compound of known stereochemistry we had available. Azotobactin D had a negative Cotton effect, while pyoverdin Pa showed a positive one in the 300–400-nm range with a CD spectrum identical with that reported for pseudobactin (Teintze et al., 1981).

Total acid hydrolysis (6 M HCl, 40 h, 110 °C) of both siderophores cleaved the peptide chain, leading to the unchanged chromophore **1a** for azotobactin D and to the modified hydroxylated chromophore **2b** in the case of pyoverdin Pa.

However, when the chromophore of azotobactin was subjected to prolonged acid hydrolysis with dilute acid (2 M HCl, 150 °C, 170 h) or when it was successively treated with ammonia (7 M NH_4OH, 150 °C, 3 h) and then with hydrochloric acid (6 M HCl, 150 °C, 24 h), it yielded a compound shown to be identical with the trihydroxylated chromophore **2b** derived from pyoverdin Pa and having the same CD spectrum.

This established clearly that azotobactin D and pyoverdin Pa have chromophores with the same configuration, *S*.

The complete structure of azotobactin δ is reported in Figure 7.

Conclusion

The structure of azotobactin D, the yellow-green water-soluble peptide produced by *Azotobacter vinelandii* strain D (CCM 289) in iron-deficient conditions, was established unambiguously by using essentially FAB mass spectrometry in combination with NMR as well as CD techniques.

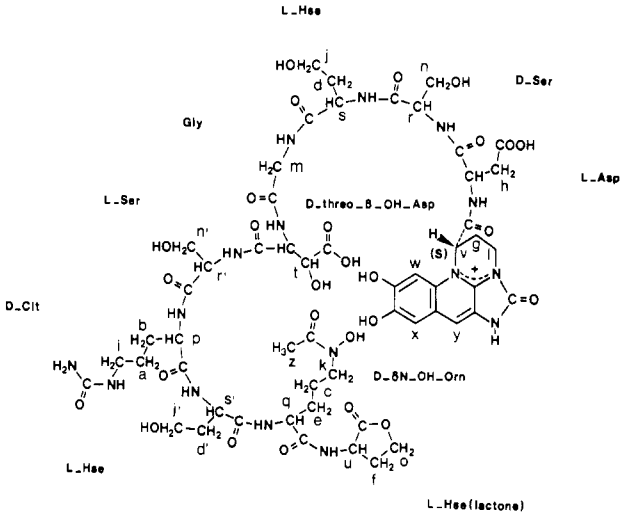


FIGURE 7: Structure of azotobactin δ.

During the culture (at pH 7), *A. vinelandii* strain D produces mainly one fluorescent peptide type siderophore, azotobactin D. However, during the purification steps that take place at more acidic pHs, this compound is partly converted into azotobactin δ, which is the lactonized form of azotobactin D at its terminal homoserine.

Azotobactin D presents striking similarities with the compound described by Fukasawa et al. (1972): they both have the same chromophore, and most of their amino acids are the same. However, azotobactin D possesses three homoserine residues (instead of two for azotobactin O) and in addition one N^{δ}-hydroxyornithine (acetylated on its N^{δ} nitrogen atom). Moreover, the differences are more pronounced in the sequence of both peptides, which are chromophore-Asp-Ser-Hse-Gly-OHAsp-Ser-Cit-Hse-N^{δ}-OHOrn-Hse (azotobactin D) and chromophore-Asx-Hse-Ser-Hse-Cit-Ser-Gly-OHAsp for azotobactin O.

In azotobactin D, the peptide chain contains two amino acids capable of chelating iron(III): one hydroxy acid group from D-*threo*-β-hydroxyaspartic acid and one hydroxamic acid group from D-N^{δ}-acetyl-N^{δ}-hydroxyornithine.

Together with the catechol group of the chromophore, these two amino acids form the three groups that bind firmly metal cations such as iron(III) to give very stable octahedral complexes.

In this respect, azotobactin D is closer to pyoverdin Pa (Wendenbaum et al., 1983) and pseudobactin (Teintze et al., 1981), which also give 1:1 metal complexes and which possess as well a short peptide chain containing *R* and *S* amino acids and a very similar chromophore.

All these compounds can be considered as belonging to the same molecular family, namely, the pyoverdins, which are the siderophores of the microorganisms that they originate from but which can also act as growth factors or as antagonists for other microorganisms.

ACKNOWLEDGMENTS

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Registry No. **1a**, 113380-06-6; **1c**, 113380-07-7; **2b**, 113380-08-8; azotobactin D, 109336-19-8; azotobactin δ, 113471-71-9.

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