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Bacterial Iron Transport: Structure Elucidation by FAB-MS and by 2D NMR (^{1}H, ^{13}C, ^{15}N) of Pyoverdin G4R, a Peptidic Siderophore Produced by a Nitrogen-Fixing Strain of *Pseudomonas putida*

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Abstract: *The structures of the pyoverdins excreted in iron-deficient conditions by Pseudomonas putida G4R, a nitrogen-fixing Pseudomonas have been established using FAB-MS and 2D ^{1}H, ^{13}C & ^{15}N NMR on both the unlabelled and the ^{15}N-labelled molecules. They are chromopeptides possessing the following linear heptapeptide: (L)-Asp-(L)-Orn-(D)-threo-β-OHAsp-(L)-CTHPMD-Gly-(L)-Ser-(L)-cyclo-OHOrn, bearing a new natural amino acid which is the result of the condensation of one mole of (D) β-threo-hydroxyaspartic acid and one mole of (L) 2,4-diaminobutyric acid forming a tetrahydropyrimidine ring.* © 1997 Elsevier Science Ltd.

INTRODUCTION

Among the fluorescent *Pseudomonas* species, *Pseudomonas putida* G4R is a rare case of nitrogen-fixing bacterium that transforms nitrogen into ammonia. From the cultures of this bacterium grown in iron-deficient conditions, two major pyoverdins were isolated: pyoverdin G4R A **1a** and pyoverdin G4R **1b**. In similar conditions, most microorganisms synthesize very powerful iron-sequestering compounds of low molecular weight called siderophores[1]. Their function is to chelate iron from the environment and to solubilize it, making it available to the cell for assimilation[2,3]. While certain functional aspects of siderophore structure are constant, the chemical structure of these molecules varies widely depending on the microorganisms which produce them[4-6]. Pyoverdins have a molecular mass of 1000 to 1500 Daltons and are constituted of a chromophore, structurally based on 2,3-diamino-6,7-dihydroxyquinoline, bound to a peptide of 6 to 10 amino acids *via* its N-terminus[7-15]. In addition, the 3-amino moiety of the chromophore is substituted with various acyl groups derived from succinic, malic, or α-ketoglutaric acids[7,9,14,16] but also from many other diacids from the Krebs cycle[17]. In azotobactin, a "pyoverdin-like" siderophore produced by *Azotobacter vinelandii*, the chromophore has a somewhat different structure, and the acyl group is replaced by a carbonyl group forming an extra imidazolone ring[8,18,19].

Pyoverdins have a strong affinity for ferric iron[20,21] giving very stable octahedral complexes. The three functional bidentate groups involved in the complexation are: the catechol function of the chromophore and either two hydroxamate functions or one hydroxy acid and one hydroxamate, present in the peptide moiety. Generally, the N^{δ}-hydroxyornithine (formylated or acetylated) or the β-*threo*-hydroxyaspartic acid located in the middle of the peptide chain, supplies one bidentate group whereas the second is provided either by a C-terminal cyclized N^{δ}-hydroxyornithine[7,9,10,22], or by an N^{δ}-acyl, N^{δ}-hydroxyornithine belonging to a larger ring[7,12,16,23].

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Structural elucidation of several pyoverdins and of their precursors has shown the presence in the peptide chain of new amino acids derived from 4-carboxy-3,4,5,6-tetrahydropyrimidine, as the result of the condensation of one molecule of 2,4-diaminobutyric acid with an other amino acid such as serine[9], glutamine[24], tyrosine[25,26], or homoserine[27].

In this paper, we report the structure elucidation of pyoverdins G4R (Figure 1), established using a combination of FAB-MS and NMR techniques performed on both the unlabelled and the fully ^{15}N-labelled pyoverdin G4R A **1a**.

We also report the complete assignment of the ^{15}N NMR spectrum of this siderophore showing as well that its peptide chain contains the same new type of tetrahydropyrimidine-based amino acids previously reported and compare it to other amidines found in other pyoverdins or pyoverdin-like peptides[9,24,25,27].

RESULTS AND DISCUSSION

Isolation and purification of the unlabelled and fully ^{15}N-labelled pyoverdins G4R from the cultures of *Pseudomonas putida*.

The effects of various nitrogen sources and of the cultivation time in view of production of ^{15}N-labelled pyoverdins G4R A **1a** were investigated. A 24 hours growth in presence of ammonium sulfate $(^{15}NH_4)SO_4$ (0.4 g/l) resulted in a 95% labelling of pyoverdins with ^{15}N, as determined by FAB-MS (see below), the remaining 5% of unlabelled pyoverdin originating from the residual unlabelled ammonium sulfate of the inoculum. We can conclude that the method described here represents a general technique[28]. It is based on the optimized assimilation of inorganic source of nitrogen and is useful for the ^{15}N-labelling of related siderophores produced by other-nitrogen fixing bacteria.

The general purification procedure we had previously described [8,9,21] was successfully applied here for the isolation and purification of the two major pyoverdins, pyoverdin G4R A **1a** and pyoverdin G4R **1b**. It involves successively centrifugation, ultra-filtration, hydrophobic chromatography on octadecylsilane, ion exchange chromatography on CM-Sephadex of the crude free pyoverdins. This is followed by complexation of each ligand fraction with ferric chloride, hydrophobic chromatography on octadecylsilane of the corresponding ferric complexes, ion exchange chromatography on CM-Sephadex and HPLC on octadecylsilane. The final steps are successively decomplexation by EDTA, hydrophobic chromatography on octadecylsilane and CM-Sephadex chromatography of the pure ligand. During the HPLC purification step of the ferripyoverdins, we could in some instances, isolate 5-chloropyoverdins G4R A **1c** and G4R **1d** (unlabelled or ^{15}N-labelled), as a result of the addition of an excess of $FeCl_3$ in the complexation step[29].

Spectrophotometric properties of pyoverdin G4R A 1a.

The UV-Visible spectra of the free ligand and of its iron complex are similar to those determined for other pyoverdins, with two maxima at 365 nm and 380 nm (at pH 5.0) for the pH-dependent spectrum of the free ligand (ε = 16000 M^{-1} x cm^{-1} at pH 5.0 in a buffered aqueous solution). With the iron complex there is only one maximum at 400 nm (ε = 19500 M^{-1} x cm^{-1}), and two shoulders at 460 nm (ε = 6000 M^{-1} x cm^{-1}) and 540 nm (ε = 3000 M^{-1} x cm^{-1}), and these values are pH independent in a wide pH range (2.0 to 10.0). Pyoverdin G4R A **1a** and its iron complex possess therefore a chromophore with the same spectral characteristics as pyoverdins[7], suggesting that pyoverdin G4R A **1a** has a pyoverdin-like structure.

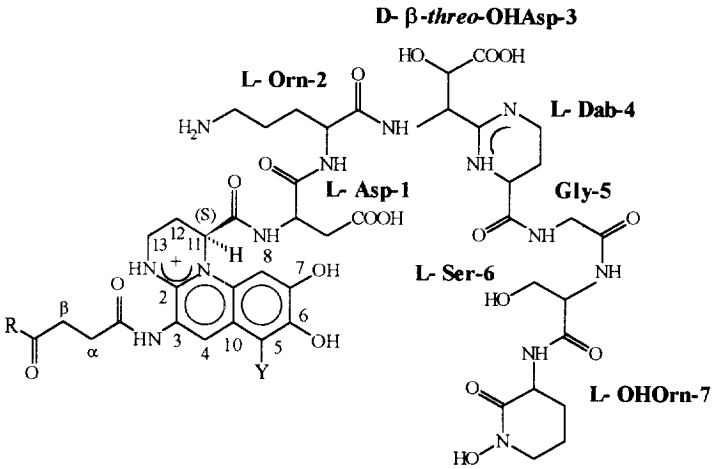
Amino acids analysis. This was performed by HPLC after derivatization of the amino acids obtained from a total hydrolysis of pure pyoverdin G4R A **1a**, into their corresponding phenylthiohydantoins using phenylthioisocyanate (with norleucine as an internal standard). Known amounts of ornithine, 2,4-diaminobutyric acid and β-hydroxyaspartic acid were also submitted to the same hydrolysis conditions in order

to quantify them in the hydrolysates of pyoverdin G4R A **1a** and to monitor the variations obtained as a function of time. It was found that pyoverdin G4R A **1a** has a peptidic moiety of 7 amino acids: serine (1), aspartic acid (1), ornithine (1), 2,4-diaminobutyric acid (1), hydroxyaspartic acid (1), glycine (1) and N^{δ}-hydroxyornithine (1). The presence of 2 moles of ornithine in the reductive HI hydrolysate and of only one mole of the same amino acid in the HCl hydrolysate indicated that pyoverdin G4R contains one mole of ornithine and one mole of N^{δ}-hydroxyornithine[30].

FAB mass spectrometry

a) Unlabelled pyoverdin G4R A 1a

The molecular mass of pyoverdin G4R A **1a** and its iron (III) complex were determined by FAB mass spectrometry. Molecular ions (M^-) were observed respectively at m/z 1074 for the free ligand and at m/z 1127 for the corresponding iron complex. The difference of 53 m.u. shows that the stoichiometry of the complex is 1:1. The signals show fragmentation patterns, typical of what is expected for pyoverdins[7,8,9,23] and give the N- and C-terminal ions assigned in Tables 1 and 2. The major signals of the C-terminal fragmentation at m/z 974 and m/z 771 can be interpreted as the loss of succinic acid (M^+ - 100), and the loss of succinic acid plus the loss of part of the chromophore due to its typical retro-Diels-Alder cleavage, simultaneously or consecutively (M^+ - 100 - 203). The N-terminal fragmentation gives the usual amide ions with the fragments at 43,44 and 45 m.u. below these ions.



1a	**pyoverdin G4R A**	**R = OH**	**Y = H**	**1c**	**5-chloropyoverdin G4R A**	**R = OH**	**Y = Cl**
1b	**pyoverdin G4R**	**R = NH_2**	**Y = H**	**1d**	**5-chloropyoverdin G4R**	**R = NH_2**	**Y = Cl**

Figure 1

b) ^{15}N-labelled pyoverdin G4R A 1a.

^{15}N-labelled pyoverdin G4R A **1a** shows electrophoretic and HPLC characteristics identical to those of the unlabelled compound. ^{15}N-labelled pyoverdin G4R A **1a** gave a molecular ion at m/z 1087 consistent with the incorporation of 13 ^{15}N atoms (Tables 1 & 2). The cleaved fragments are fully consistent with the data obtained on unlabelled pyoverdin G4R A **1a**, the molecular ion at m/z 781 showing that 10 ^{15}N nitrogen atoms are present in this peptide and only 3 ^{15}N nitrogen atoms in the chromophore moiety. From this results we concluded that the peptide moiety is linear and consistent with the sequence :

Asp-Orn-(OHAsp,Dab)-Gly-Ser-*cyclo*-OHOrn

The structure of pyoverdin G4R A as deduced from these data is shown in Figure 1.

Table 1 : N-Terminal fragments in the FAB-MS spectrum of the unlabelled and the ^{15}N-labelled pyoverdin G4R A **1a** and their interpretation. Chr is for chromophore and Succ for succinic acid.

Unlabelled pyoverdin		^{15}N-labelled pyoverdin		N-terminal fragments
Cα Ions	Amide	Cα Ions	Amide	
445, 446; 447	490	449, 450, 451	495	Succ-Chr-Asp
559, 560, 561	604	565, 566, 567	611	Succ-Chr-Asp-Orn
772, 773, 774	817	781, 782, 783	827	Succ-Chr-Asp-Orn-(OHAsp-Dab)
829, 830, 831	874	839, 840, 841	885	Succ-Chr-Asp-Orn-(OHAsp-Dab)-Gly
916, 917, 918	961	927, 928, 929	973	Succ-Chr-Asp-Orn-(OHAsp-Dab)-Gly-Ser
	1074 (M^+)		1087 (M^+)	Succ-Chr-Asp-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn

Table 2 : C-Terminal fragments in the FAB-MS spectrum of the unlabelled and the ^{15}N-labelled pyoverdin G4R A **1a** and their interpretation. C*yclo*-N^{δ}-OHOrn is for *cyclo*-N^{δ}-hydroxyornithine.

Unlabelled pyoverdin m/z	^{15}N-labelled pyoverdin m/z	C-terminal fragments $(M+H)^+$
488	495	H_2N-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn
602	611	H_2N-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn
717	727	H_2N-Asp-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn
771	781	CH_2=CH-CO-NH-Asp-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn
974	987	H_2N-Chr-Asp-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn
1074 (M^+)	1087 (M^+)	Succ-Chr-Asp-Orn-(OHAsp-Dab)-Gly-Ser-*cyclo*-N^{δ}-OHOrn

NMR studies on unlabelled and ^{15}N-labelled pyoverdin G4R A 1a.

^1H and ^{13}C NMR spectroscopy confirmed the sequence of the peptide chain (HOHAHA, ROESY and ^1H-^{13}C heteronuclear correlation, HMBC, HMQC). ^{15}N-labelled pyoverdin G4R A **1a** showed ^1H and ^{13}C NMR chemical shifts identical to those of the unlabelled pyoverdin G4R A **1a**.

^1H NMR studies: The ^1H NMR spectra of unlabelled pyoverdin G4R A **1a** and ^{15}N-labelled pyoverdin G4R A **1a** (H_2O/[$^2H_{10}$] t-butanol 99:1, 300K pH = 4.0) show three singlets at 7.90 ppm, 7.15 ppm and 6.95 ppm. The values of these chemical shifts are very close to those reported for pyoverdin Pa A[23]. 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 (Table 3).

HOHAHA NMR spectroscopy on the same samples[23,31] performed at the same temperature, allowed the assignment of the NH protons and the protons of the amino acid side chains. The chemical shifts are in agreement with the values reported in the literature[32-34].

ROESY NMR experiments[35,36] were performed as for pyoverdin Pa A[23]. The presence of CHα(i)-NH(i+1) ROE connectivities between neighbouring residues was used for sequential assignment of pyoverdin G4R A **1a**, independently of the FAB-MS determination.

A cross-peak was observed between H-8 of the chromophore (6.95 ppm) and H-11 of the chromophoric tetrahydropyrimidine ring (5.60 ppm). Another cross-peak was observed between H-11 and NH (Asp-1) (9.04 ppm) indicating that Asp-1 is bound directly to the chromophore. The CHα (Asp-1) - NH (Orn-2) cross-peak,

and the following CHα (Orn-2) - NH(OHAsp-3) cross-peak confirm the sequence **Asp-Orn-OHAsp**. Similarly CHα (Dab-4) -NH (Gly-5), CH_2 (Gly-5)-NH (Ser-6) and CHα (Ser-6)-NH (*cyclo*-OHOrn-7) cross-peaks confirm the sequence **Dab-Gly-Ser-OHOrn**. No $CH_2\alpha$ (Succ)-NH (3-Chr) and CHα (OHAsp-3)-NH (Dab-4) cross-peaks were observed, however the binding of OHAsp-3 to Dab-4 was established by ^1H - ^{13}C HMBC (see below).

From these results, the sequence of the peptide chain of pyoverdin G4R A **1a**:
Chromophore-Asp-Orn-OHAsp/Dab-Gly-Ser-*cyclo*-OHOrn
as determined by FAB-MS, could be confirmed.

The chemical shift of the resonance of the β-CH_2OH group of serine in the ^1H NMR spectrum (δ =3.86 ppm) indicated that the hydroxyl function is not esterified, by comparison with its values in esterified[12,14] or non-esterified[9,23,27] reference compounds and non-esterified serine[32]. In the case of esterification, it would be shifted by approximately 0.6 ppm to lower field.

^{15}N NMR studies. At pH 4.0, the proton decoupled ^{15}N spectrum of pyoverdin G4R A **1a** presents 13 signals between 88 ppm and 170 ppm (Table 4). An additional signal at 25.00 ppm corresponds to ammonia resulting from the partial hydrolysis of the succinamide group of pyoverdin G4R **1b** into succinic acid, as was observed for pyoverdin Pa.[23]

Table 3: Assignment of the protons of ^{15}N-labelled pyoverdin G4R A **1a** at 300K and pH 4.0 in H_2O/deuterated t-butanol 99:1, as an internal standard.

Residue	NH	α	β	γ	δ		
Asp-1	9.04	4.65	2.78-2.86				
Orn-2	8.44	4.27	1.71	1.59	2.95		
OHAsp-3	8.63	5.12	4.55				
Dab-4		4.37	2.09-2.19	3.24-3.52			
Gly-5	8.36	3.95					
Ser-6	8.43	4.44	3.86				
c-OHOrn-7	8.51	4.42	1.97	1.78	3.61-3.67		
Chromophore	NH 8.57	H4 7.90	H5 7.15	H8 6.95	H11 5.60	H12-12' 2.36-2.71	H13-13' 3.38-3.68
Succinate		2.61	2.69				

By comparison of the proton undecoupled ^{15}N spectrum and Distorsionless Enhancement Polarisation Transfer (DEPT 90 and 135) spectra (Table 4), it was possible to show the presence of :

-one signal corresponding to a $^{15}NH_2$ group at 106.87 ppm, occurring as a triplet ($^1J\,^1H$-^{15}N = 90.0 Hz) and assigned as the free amino group of Orn-2;

-seven signals, between 105.80 ppm and 121.44 ppm corresponding to the CONH amide groups of the peptidic chain which were unambiguously assigned using the HOHAHA and the 2D ^1H-^{15}N heteronuclear spectra of pyoverdin G4R A **1a**.

The signal at 88.77 ppm was assigned to the ^{15}NH of the chromophoric tetrahydropyrimidine moiety. Its tertiary ^{15}N partner is located at 129.16 ppm. The latter is the only ^{15}N nucleus in pyoverdin G4R A **1a** to show a positive NOE effect. The two ^{15}N atoms of the peptidic tetrahydropyrimidine behaved like two ^{15}NH, in agreement with the results of Inbar and Lapidot[37]. These data show that the ^{15}N of the two tetrahydropyrimidine systems behave differently: the chromophoric tetrahydropyrimidine shows no positive charge delocalisation between the two nitrogen atoms, as observed for azoverdin[27]. On the contrary, the peptidic tetrahydropyrimidine shows an important proton delocalization between the two nitrogen atoms, resulting in the observation of two very distinct ^{15}NH signals[27,37]. These findings were further corroborated by the ^{13}C NMR spectrum of the ^{15}N-labelled pyoverdin G4R A **1a**.

Table 4: Assignment of the nitrogen atoms in pyoverdin G4R A **1a** at 300K and pH 4.0. The chemical shifts were determined using $(^{15}NH_4)_2SO_4$ as an external reference.

	Assignment	Chemical shift δ (ppm)	$^1J\,^1H$-^{15}N (Hz)
NH_4^+	ammonium	25.00	(m)
NH	chromophoric tetrahydropyrimidine	88.77	95.0 (d)
NH	peptidic tetrahydropyrimidine (Dab-4)	101.92	93.0 (d)
NH	peptidic tetrahydropyrimidine (Dab-4)	103.10	93.5 (d)
NH	Gly-5	105.80	93.0 (d)
NH_2	Orn-2	106.87	90.0 (t)
NH	OHAsp-3	107.18	93.0 (d)
NH	Succinate	107.68	93.5 (d)
NH	Ser-6	111.24	93.0 (d)
NH	c-OHOrn-7	118.87	92.5 (d)
NH	Orn-2	121.18	93.0 (d)
NH	Asp-1	121.44	93.0 (d)
N	chromophoric tetrahydropyrimidine	129.16	(s)
N	c-OHOrn-7	168.22	(s)

^{13}C NMR spectra. The ^{13}C NMR spectrum of pyoverdin G4R A **1a** is characteristic of a chromopeptide, and can be divided into three regions: the aliphatic region (from 20 to 75 ppm, 23 signals), the aromatic region (from 100 to 156 ppm, 9 signals) and the carbonyl region (from 160 to 185 ppm, 12 signals). The assignment

Table 5: Assignment of the carbon atoms of the aliphatic region of pyoverdin G4R A **1a** in 1% deuterated t-butanol in H_2O at 300K and pH4.0.

Assignment	Chemical shift δ (ppm)	$^1J\,^{13}C$-^{15}N (Hz)
Cβ OHAsp-3	72.18	
Cβ Ser-6	62.02	
C11 Chr	57.68	9.0 (d)
Cα Ser-6	56.38	7.5 (d)
Cα OHAsp-3	55.10	11.5 (d)
Cα Orn-2	54.21	9.5 (d)
Cα Asp-1	53.16	11.0 (d)
Cα Dab-4	52.44	9.0 (d)
Cδ c-OHOrn-7	52.33	8.5 (d)
Cα c-OHOrn-7	51.20	7.5 (d)
CH_2 Gly-5	43.34	10.5 (d)
Cδ Orn-2	39.75	5.5 (d)
Cβ Asp-1	38.32	
Cγ Dab-4	37.54	7.0 (d)
C-13 Chr	35.99	9.5 (d)
Cβ Succ	33.23	
Cα Succ	32.80	
Cβ c-OHOrn-7	28.27	
Cβ Orn-2	27.25	
Cγ Orn-2	23.59	
C-12 Chr	22.49	
Cβ Dab-4	21.59	
Cγ c-OHOrn-7	20.80	

of each carbon atom was performed using the ^{1}H-^{13}C heteronuclear correlations and by measuring the $J\,^{1}H$-^{13}C coupling constants of the proton undecoupled spectrum and the $^{1}J\,^{13}C$-^{15}N coupling constants of the fully ^{15}N labelled pyoverdin G4R A **1a**.

The aliphatic region presents 23 signals. These were assigned by Distortionless Enhancement Polarization Transfer, by heteronuclear 2D ^{1}H-^{13}C correlation and by inverse heteronuclear correlation ^{1}H-^{13}C at 300K, in agreement with literature data[38] (Table 5). The multiplicity of each signal in addition to the $^{1}J^{13}C$-^{15}N coupling constants obtained from the ^{13}C NMR spectrum of the fully ^{15}N-labelled pyoverdin G4R A, confirmed these assignments.

The assignments of the aromatic carbon atoms were readily obtained by comparison with those in pyoverdin Pa A, and the chemical shifts were in complete agreement with those reported earlier[9]. In particular the quaternary carbons C-3 and C-9 which are both in the vicinity of one ^{15}N atom occur as doublets at 118.05 ppm ($^{1}J\,^{13}C$-^{15}N = 16.0 Hz) and 132.85 ppm $^{1}J\,^{13}C$-^{15}N = 15.0 Hz) respectively, whereas carbon C-2, at 149.98 ppm occurs as a triplet ($^{1}J\,^{13}C$-^{15}N = 19.0 Hz) (Table 6).

The carbonyl region presents 12 signals, 8 signals occurring as doublets, 3 signals occurring as a singlet and one at 162.50 ppm as a triplet ($^{1}J\,^{13}C$-^{15}N = 18.50 Hz). This second triplet supports the amidine structure of 2,4 -diaminobutyric acid in pyoverdin G4R A **1a** (Table 7).

The assignments of most of the signals of the carbonyl region of the spectra were obtained using ^{1}H-^{13}C HMBC correlations (Table 7).

Table 6: Assignment of the chromophoric carbon atoms of ^{15}N-labelled pyoverdin G4R A **1a** in 1% deuterated t-butanol in H_2O at pH 4.0.

Assignment	Chemical shift δ (ppm)	$^{1}J\,^{13}C$-^{15}N (Hz)
Chr C-7	153.58	
Chr C-2	149.98	19.0 (t)
Chr C-6	144.81	
Chr C-4	139.48	
Chr C-9	132.85	15.0 (d)
Chr C-3	118.05	16.0 (d)
Chr C-10	115.09	
Chr C-5	114.28	
Chr C-8	101.32	

Cross peaks were observed between $(^{15}N\underline{H})_i$ and $(\underline{C}O)_{i-1}$, between $(C\underline{H}\alpha)_i$ and $(\underline{C}O)_i$ (they were all present), and finally between the $(C\underline{H}\beta)_i$ and $(\underline{C}O)_i$. Other correlations between ^{1}H and ^{13}C of the side chain in each residue of pyoverdin G4R A **1a** were also observed. From all these correlations it was possible to assign completely the carbonyl signals and confirm the sequence of the peptide moiety in pyoverdin G4R A **1a**.

In addition to the cross-peaks confirming the sequences **Chr-Asp-Orn-OHAsp** and **Dab-Gly-Ser-OHOrn** identified above from ^{1}H spectra, two cross-peaks between the triplet at 162.50 ppm (which corresponds to a cyclic amidine function formed by the condensation of 2,4-diaminobutyric acid with β-hydroxyaspartic acid), and the $C\underline{H}\alpha$ (5.12 ppm) of β-hydroxyaspartic acid-3 and $C\underline{H}\alpha$ (4.37 ppm) of 2,4-diaminobutyric acid, were observed.

Furthermore, the signal at 172.44 ppm (assigned to the carbonyl of 2,4-diaminobutyric acid), gives two cross-peaks with $^{15}N\underline{H}$ (8.36 pmm) and with $C\underline{H}_2$ (3.95 ppm) of glycine-5 respectively.

The N^{δ}-hydroxyornithine was found to be cyclic: its $\underline{C}H\alpha$ resonance (51.20 ppm) shows a high field shift of about 3 ppm compared to ornithine and N^{δ}-acyl, N^{δ}-hydroxyornithine[39].

This shift indicates that N^{δ}-hydroxyornithine-7 is cyclic and is in agreement with the N-terminal fragmentation found in the FAB-MS spectrum of the molecule[9].

These results unambiguously confirm the sequence :
Succinic acid-Chr-Asp-Orn-OHOAspCTHPMD-Gly-Ser-*cyclo*-OHOrn.

Table 7: Assignment of the carbonyls of ^{15}N-labelled pyoverdin G4R A **1a** in 1% deuterated t-butanol in H_2O at pH 4.0.

Assignment	Chemical shift δ (ppm)	$^1J^{13}C$-^{15}N (Hz)
COOH Succ	181.46	
CO Succ	178.15	13.0 (d)
COOH OHAsp	177.10	
COOH Asp	175.65	
CO Orn	175.21	13.0 (d)
CO Asp	173.39	13.0 (d)
CO Dab	172.44	15.0 (d)
CO Ser	172.01	15.0 (d)
CO Gly	171.88	15.0 (d)
CO Chr	169.90	15.0 (d)
CO c-OHOrn	167.09	15.0 (d)
N-C=N OHAsp	162.50	18.5 (t)

Configuration of the chromophore and of the amino acids.

The circular dichroism spectrum of pyoverdin G4R A **1a** - Fe(III) complex has been measured at three pH values namely 4.0, 5.0 and 7.2. No difference was found between the three spectra which show the same positive Cotton effect at 400 nm ($\Delta\varepsilon$ = + 1.7 M^{-1} x cm^{-1}) and the same two negative Cotton effects at 460 nm ($\Delta\varepsilon$ = -0.8 M^{-1} x cm^{-1}) and at 540 nm ($\Delta\varepsilon$ = -0.3 M^{-1} x cm^{-1}). On the contrary, the circular dichroism spectrum of the free ligand is very pH-sensitive, presenting at pH 4.0 a positive Cotton effect at 365 nm ($\Delta\varepsilon$ = +3.15 M^{-1} x cm^{-1}) and at 380 nm ($\Delta\varepsilon$ = +2.68 M^{-1} x cm^{-1}). At pH 5.0 the Cotton effects remain positive showing a decrease in their intensities ($\Delta\varepsilon_{365}$= +1.20 M^{-1} x cm^{-1}, $\Delta\varepsilon_{380}$ = +1.10 M^{-1} x cm^{-1}), while at pH 7.2 the maxima are shifted to higher wavelengths ($\Delta\varepsilon_{380}$= +0.60 M^{-1} x cm^{-1}, $\Delta\varepsilon_{400}$ = +0.70 M^{-1} x cm^{-1}). From these results which are comparable to those reported for pseudobactin B10[10] and other pyoverdins[9,23], we could conclude that the configuration of the asymmetric carbon atom C-11 bound to the chromophore is (S).

The configuration of the amino acids forming the peptide moiety was determined by gas chromatography on an (R)-Chirasil Val stationary phase capillary column of the O-methyl, N-pentafluoropropionyl (PFP) derivatives of the amino acids after total hydrolysis of pyoverdin G4R A **1a**. For OHAsp a modified procedure was used[40]. It was found that all the amino acids have the same configurations (S) except OHAsp which has the (R,R) configuration (D-*threo*).The complete structure of pyoverdins G4R is reported in Figure 1.

Structures of the other pyoverdins isolated from the cultures of *Pseudomonas putida* G4R

The structures of the other siderophores isolated from the culture supernatants were deduced by FAB-MS. They all possess the same peptide chain as pyoverdin G4R A **1a** and differ in the chromophoric part of the molecule. Pyoverdin G4R **1b** has a mass of 1073 and its ^{15}N-labelled homologue shows the same molecular ion at m/z 1087 as ^{15}N-labelled pyoverdin G4R A **1a**, consistent with an extra amide function on the chromophore.

Two other pyoverdins occurring in small amounts were also isolated during the HPLC purification step: 5-chloropyoverdin G4R A **1c** and 5-chloropyoverdin G4R **1d**. FAB-MS on the free ligands shows a molecular peak (M^+) respectively at m/z 1108 and 1107 which are 34 m.u. higher than the molecular peaks of pyoverdin

G4R A **1a** and pyoverdin G4R **1b**, consistent with the presence of a chlorine atom on the molecule. They respectively lose 337 m.u. and 336 m.u. giving the same peptidic fragment at m/z 771 as pyoverdin G4R A **1a** and pyoverdin G4R **1b**, indicating that the chlorine atom is on the chromophore. The same results were found with ^{15}N-labelled 5-chloropyoverdin G4R A **1c** and 5-chloropyoverdin G4R **1d** which present both the same molecular ion peak at m/z 1121 m.u. and give the same fragment peak at m/z 781, indicating the presence of the succinamide moiety on the chromophore of 5-chloropyoverdin G4R **1d**.

The ^{1}H NMR spectra of both 5-chloropyoverdins G4R A **1c** and G4R **1d** show the presence of only two aromatic protons on the chromophore: H-4 and H-8, H-5 having been substituted by a chlorine atom[29].

The ^{1}H NMR spectrum of chloropyoverdin G4R A **1c** shows that either proton H-8 or proton H-5 is replaced by another atom since in chloropyoverdin G4R A **1c** only one proton is present between 7.5 and 6.5 ppm, at precisely 6.58 ppm. The ^{13}C NMR spectrum confirms these results, and the DEPT 135 ^{13}C NMR spectrum shows in addition that it is indeed the position C-5 which is substituted by the chlorine atom.

The 2D NMR ^{1}H-^{13}C long distance correlation spectrum confirms this result showing predominantly the ^{3}J correlations given by H-4 (at 8.23 ppm) with C-5 (at 116.56 ppm) , C-9 (at 136.70 ppm) and C-2 (at 151.28 ppm) , and by H-8 (at 6.58 ppm) with C-6 (at 148.32 ppm) and C-10 (at 111.91 ppm). The chemical shifts of the signals for carbon atoms C-5, C-6 and C-10, shifting respectively from 114.28, 144.81 and 115.09 ppm in pyoverdin G4R A **1a** to 116.56, 148.32 and 111.91 ppm in 5-chloropyoverdin G4R A **1c**, are also consistent with the replacement of H-5 in pyoverdin G4R A **1a** by a chlorine atom.

The 5-chloropyoverdin G4R A **1c** and 5-chloropyoverdin G4R **1d** were isolated in fairly large amounts (22% each) when the complexation step was performed using 20 equivalents of $FeCl_3$, and the reactants kept 30 minutes at room temperature. Longer reaction times[29] result in much higher yields of 5-chloropyoverdins. Addition of only 5 equivalents resulted here in only 4% of each chlorinated siderophore.

The mechanism we have already suggested for this reaction[29] is a two step electron transfer oxidation with a homolytic cleavage of a hydrogen at the bisallylic position of the tautomeric form of the chromophore, namely C-5, resulting in the formation of both an $H^{\bullet}$ radical and a bisallylic aromatic radical. Both radicals would reduce ferric halide to ferrous halide and give respectively a halide anion and the halogenated chromophore. This mechanism illustrates the general character of this reaction: when applied to pyoverdins, it yields readily 5-halopyoverdins, independently of the peptidic moieties and independently of the side chains attached to the chromophore. It also explains the occurrence of 5-chloropyoverdins during the usual purification steps of pyoverdins which involve a step of complexation with iron(III) chloride, illustrating how easily artefactual 5-substituted pyoverdins can sometimes be isolated from bacterial cultures.

Comparison of OHAsp-CTHPMD with other tetrahydropyrimidines occurring in pyoverdin-like siderophores.

Similar 4-carboxy-3,4,5,6-tetrahydropyrimidine structures were already reported by us in pyoverdins from *Pseudomonas fluorescens* CCM 2298 and 2299 (Ser-CTHPMD)[9], in pyoverdin from *Pseudomonas fluorescens* ATCC 17400 (Gln-CTHPMD)[24], in *Azomonas macrocytogenes* ATCC 12334 (Hse-CTHPMD)[27] and in desferriferribactin from *Pseudomonas fluorescens* ATCC 13525 (Tyr-CTHPMD)[25], a possible biogenic precursor of the homologous pyoverdins.

The ^{13}C NMR shifts of the tetrahydropyrimidine moieties common to these compounds are reported in Table 8 together with the corresponding shifts occurring in pyoverdin G4R A **1a**, illustrating the close similarities of all these differently 2-substituted tetrahydropyrimidine rings.

In all the pyoverdins so far investigated, the configuration of the Dab moiety has always been found to be (S), whereas the configuration of the amino acid bound to it (homoserine, glutamine, tyrosine, β-hydroxyaspartic acid and serine) was found to be (R). All these 4-carboxy-3,4,5,6-tetrahydropyrimidines were readily cleaved by acid hydrolysis except Ser-CTHPMD which appeared to be particularly resistant, and could only be cleaved in drastic conditions with racemization of both chiral centers[9]. However the racemization is

considerably slowed down in milder hydrolytic conditions involving prolonged hydrolysis times at lower temperature, confirming the configuration (R) for serine and (S) for Dab in pyoverdin Pf 2798 [9].

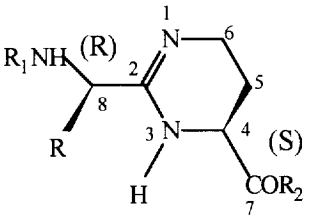


Figure 2

Table 8: ^{13}C NMR shifts (ppm) of the tetrahydropyrimidine moieties in the natural pyoverdins and pyoverdin-like siderophores. For the numbering see Figure 2.

Structure	C2	C4	C5	C6	C7	C8	Reference
Ser-CTHPMD (pyoverdin Pf 2798)	165.10	54.40	23.80	39.40	173.50	57.20	9
Gln-CTHPMD (pyoverdin Pf 17400)	164.60	52.30	22.20	37.60	172.30	52.70	23
Tyr-CTHPMD (desferriferribactin)	164.49	52.21	22.28	37.58	172.02	55.11	24
Hse-CTHPMD (Azoverdin)	167.74	54.97	26.54	40.21	184.10	55.77	26
OHAsp-CTHPMD (pyoverdin G4R)	162.50	52.44	21.59	37.54	172.44	55.10	This work

CONCLUSION

The purification and the complete structure elucidation of the two major peptidic siderophores, pyoverdin G4R A **1a** and pyoverdin G4R **1b** isolated in iron-deficient cultures of *Pseudomonas putida* has been achieved. The sequence of the peptide was determined using essentially FAB-MS and 2D ^{1}H, ^{13}C and ^{15}N NMR spectroscopy.

Comparison of the FAB-MS spectra of the ^{15}N -labelled pyoverdin G4R A **1a** and of the unlabelled siderophore afforded unambiguously the sequence of the peptide chain, while the NMR spectra of the ^{15}N-labelled siderophore confirmed this sequence and gave evidence for the presence of two tetrahydropyrimidine rings on the molecule, one on the chromophore and the other in the middle of the peptide chain. This latter is the result of the condensation of one mole of β-hydroxyaspartic acid and one mole of 2,4-diaminobutyric acid.

With the introduction of the ^{15}N nucleus in pyoverdin G4R A **1a** for extensive NMR studies, it was possible to perform a complete assignment of all the nitrogen atoms including the amidine nitrogen atoms of the two tetrahydropyrimidine moieties of the chromopeptidic siderophore, which were in agreement with literature data[37,41]. From the HOHAHA and the 2D ^{1}H-^{15}N correlations, it was possible to assign all the peptidic nitrogen atoms, while it was also possible to assign all the carbonyl groups of pyoverdin G4R A **1a** from the $C\underline{H}\alpha$, $^{15}N\underline{H}$, $^{13}\underline{C}O$ correlations.

The configuration of the amino acids was established by GC-MS using a capillary (R)-Chirasil-Val chiral column after derivatization of acid hydrolyzates (HCl and HI). The configuration of the chromophore was found to be (S), as in all pyoverdins investigated so far, from the circular dichroism spectra of pyoverdin G4R A **1a** and by comparison with the spectra of other pyoverdins[7] and pseudobactin B10, the structure of the latter having been determined by X-ray diffraction[10].

The pyoverdins G4R differ only by a substituent bound to the nitrogen on C-3 of the chromophore: pyoverdin G4R **1b**, the most abundant possesses a succinamide moiety, whereas pyoverdin G4R A **1a** bears a succinic acid moiety. 5-chloropyoverdins G4R A **1c** and G4R **1d** could also be isolated in the course of the purification step. Their occurrence which is artefactually due to an excess of iron trichloride generating a free radical reaction[29], could be considerably diminished using controlled amounts of the reagent (less than 4%).

EXPERIMENTAL

Instruments and materials:

FAB Mass spectrometry: FAB mass spectra were 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.

GC-MS was performed on a Carlo Erba (Milano, Italy) Model MEGA instrument. The (R)-Chirasil-Val capillary column was connected to an MS-QMD 1000 instrument (Carlo Erba, Milano, Italy).

NMR Spectroscopy: ^{1}H, ^{13}C, and ^{15}N NMR spectra were determined either on a Bruker ARX 500 or a Bruker DRX 600 spectrometer (Bruker Spectrospin, Wissembourg, France). 1% fully deuterated [$^{2}H_{10}$]-*t*-butanol (CEA-Saclay, France) was used as an internal standard in the ^{1}H and ^{13}C spectra while $(^{15}NH_4)_2SO_4$ (Aldrich Chemicals, St Quentin Fallavier, France) was used as an external standard for ^{15}N NMR spectra.

^{1}H coupled ^{15}N NMR spectra, ^{1}H-decoupled ^{15}N NMR spectra with no NOE effect were measured using the inverse gated pulse programme. ^{1}H decoupled ^{15}N NMR spectra with NOE effect were determined using the broad-band decoupling programme.

The $^{15}NH_2$ and ^{15}NH resonances were assigned from Distorsionless Enhancement Polarization Transfer (DEPT) experiments as well as from 2D Heteronuclear ^{1}H-^{15}N correlations.

2D Homonuclear Hartmann Hahn Spectroscopy (HOHAHA) NMR spectra, 2D Rotating Frame Overhauser Effect Spectroscopy (ROESY) and 2D Heteronuclear Multiple Quantum Coherence ^{1}H-^{13}C (HMQC) correlations were recorded at 300K[42]. Gradient Spectroscopy Heteronuclear Multiple Bound Coherence (HMBC-gs) spectra were determined either at 300 K or 310 K with a Z-gradient [43-45]. All spectra were measured in the phase-sensitive absorption mode with quadrature detection in both dimensions, using States TPPI. The through bond connectivities were obtained from a HOHAHA spectrum determined with the MLEV-17 sequence[31]. A mixing time of 70 ms was used with a spin-locking radiofrequency field strength of 25 kHz. In the ROESY experiments[35,36], the spin-lock time was of 300 ms duration, using a 2.3 kHz radiofrequency field. In all experiments using spin-lock, the carrier frequency was centered on the water resonance and a relaxation delay of 2.0 s was used. The water signal was suppressed by Watergate in ROESY and HOHAHA[46], and by a 2.0 s presaturation pulse in HMBC-gs.

The assignment of the carbon signals was performed using 2D ^{1}H-^{13}C HMQC[47,48] and 2D ^{1}H-^{13}C HMBC. These spectra were determined with 512 t1 points and 2048 t2 points (512 experiments with 32 and 64 scans of 2048 complex points in t2 were collected in both cases).

Amino acid analyses: Total acid hydrolyses of purified pyoverdin G4R A **1a** were performed with HCl 6N and with HI 6N at 110°C for 24, 48 and 72 hours. For each analysis 250 picomoles of norleucine were added

as an internal standard. Simultaneously these hydrolyses were performed on known amounts of ornithine, 2,4-diaminobutyric acid and β-hydroxyaspartic acid in order to quantify them in the hydrolysates of pyoverdin G4R A and to monitor the variations obtained as a function of time.The samples were derivatized in triplicate with phenylthioisocyanate (PITC) on a 420A derivatizer coupled with a 920A Applied Biosystems Analyzer (Perkin Elmer Division, Roissy-en-France, France). The results are expressed in picomoles after integration with respect to the standard, and the number of residues by integration with respect to the different molecular masses.

Electrophoresis: Film electrophoreses were performed using a Consort Bioblock E 455, cellulose acetate film Biomidi instrument. Samples of approximately 0.05 mg were applied to the midpoint of cellulose acetate membranes (5.6 cm x 14 cm), 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% iron trichloride (w/v) in distilled H_2O to detect iron-binding compounds.

UV-visible spectrophotometry: The absorption spectra were determined using an Uvikon 930 spectrophotometer. The extinction coefficient of ferripyoverdin G4R A was calculated from the absorbance of a solution whose iron content was measured by atomic absorption using either a Techtron 1200 or a Jobin-Yvon ICP 2500 instrument. The extinction coefficient of the free ligand was deduced after titration with a 10^{-2} M ferric chloride solution in 0.05 M pyridine / acetic acid buffer pH 5.0.

Circular Dichroism: the spectra were measured on a Jobin-Yvon CD6 instrument. The solvents were 0.1 M acetate buffer pH 4.0 or 5.0, and 0.1 M phosphate buffer pH 7.2.

HPLC: They were performed on a Kontron instrument with a diode array detector 440, with Kontron Instruments 325 pumps. For preparative reverse-phase HPLC, an octadecylsilane column was used (ODS, 2.25 cm x 25 cm, 10μm particle size; Nucleosil, Shandon, Neuilly sur Marne, France). For analytical HPLC, another column (ODS, 0.5 cm x 26 cm, 5μm particle size; Nucleosil, SFCC, Neuilly sur Marne, France) was used

Column chromatography: C-18 octadecylsilane (ODS) (LiChroprep RP-18) was from Merck (Darmstardt, Germany) and CM-Sephadex C-25 from Pharmacia Fine Chemicals (Saint Quentin en Yvelines, France).

Reagents: pyridine, acetonitrile, and acetic acid were from SDS (Peypin, France); formic acid, from Acros Organics (Noisy-Le-Grand, France), HCl from Sigma (Saint Quentin Fallavier, France); HI from Fluka (Saint Quentin Fallavier, France); Ferric trichloride solution 27.5% from Prolabo (Paris, France).

Strain and culture medium: *Pseudomonas putida* G4R was isolated from the rhizosphere of sorghum grown on sandy soils of Ismailia (Egypt). The culture medium had the following composition per liter: K_2HPO_4, 6.0 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; succinic acid, 4.0 g. It was adjusted to pH 7.0 before sterilization. For fully ^{15}N-labelled pyoverdins G4R, ammonium sulfate was replaced by 0.40 g 98.9% $(^{15}NH_4)_2SO_4$[28].

Isolation and purification of the pyoverdins:

^{15}N-labelled pyoverdin: The bacteria were grown aerobically at 25°C in a laboratory fermentor with a working volume of 1.25 l, at 26°C, with a stirring of 400 rpm, an aeration of 1.5 l of air per minute, a culturing time of 24 hours. The inoculum was grown for 24 h and diluted 50 times for the batch culture in the fermentor. The same medium was used for the inoculum and for the batch culture in the fermentor.

Unlabelled pyoverdin: the same medium was used with higher concentration of ammonium sulfate 1.0 g per liter. The batch culture was performed in an air-lifted column with a working volume of 18 liters, at 26 °C and an aeration of 4 l/min. The culturing time was 48 hours. For the inoculum, a total volume of 500 ml was cultured at 28 °C for 24 h, and used entirely for inoculation.

The bacterial supernatant was filtered through a 0.22 μm membrane (Millipore, Molsheim, France), and the resulting filtrate was acidified to pH 3.5 with formic acid and applied to a column (2.0 cm x 30 cm) of octadecylsilane[8,9]. The fluorescent material retained by the ODS was rinsed with acidified distilled water (adjusted to pH 3.5 with acetic acid) then eluted from the column with 500 ml of 50% (v/v) acetonitrile in 0.05 M-pyridine/acetic acid pH 5.0. This fluorescent eluate was concentrated and lyophilized (430 mg). The corresponding pyoverdins were chromatographed on a CM-Sephadex C-25 column (2 cm x 30 cm) made up in 0.05 M pyridine/acetic acid pH 5.0. The column was first eluted isocratically with the same buffer (0.4 l), then with a linear gradient of 0.05 to 2 M pyridine/acetic acid pH 5.0 (2 x 1 l). The fractions (5 ml) were monitored by spectrophotometry at 380 nm. Three fractions were separeted: the first (20 mg) was discarded, the second (120 mg) was called pyoverdin G4R A **1a** and the third (220 mg) pyoverdin G4R **1b**.

The second fraction recovered from this column was treated with 20 equivalents of Fe^{3+} (added as a 2M solution of ferric chloride) for the preparation of 5-chloropyoverdin G4R A **1c**, the pH of the solution was adjusted to 3.5 before it was chromatographed on an ODS column (1.5 cm x 15 cm). The column was washed firstly with acidified distilled water (adjusted to pH 3.5 with acetic acid) in order to remove the excess of iron chloride, then with a 1:1 mixture of acetonitrile-0.05 M pyridine/acetic acid buffer pH 5.0 which eluted the ferripyoverdin G4R A **1a** (110 mg). The bulk of the complex was applied on a CM-Sephadex C-25 column (2.0 cm x 20 cm) eluted firstly isocratically with 250 ml 0.05 M pyridine/acetic acid pH 5.0, then with a linear gradient of pyridine/acetic acid pH 5.0 (0.05 to 2 M, 2 x 300 ml) to separate the complex pyoverdin G4R A **1a**-Fe(III) (90 mg).

This pyoverdin G4R A **1a**-Fe(III) complex was purified by preparative reverse-phase HPLC, monitored spectrophotometrically at 400 nm, and eluted with 0.025 pyridine/acetic acid pH 5.0 containing 4% (v/v) acetonitrile at flow rate of 8 ml x min^{-1} at 30°C. Pure pyoverdin G4R A **1a**-Fe(III) complex, the major compound (67 mg), and pure 5-chloropyoverdinG4R A **1c**-Fe(III) (15 mg) were obtained after lyophylization.

The purity of the ferripyoverdins was monitored by analytical HPLC, samples of approximately 0.025 mg were eluted using the above conditions at a flow rate of 0.8 ml x min^{-1}.

Decomplexation of the pyoverdin G4R A-Fe(III) complex: This was performed according to a procedure previously described by us for the purification of pyoverdin Pa[21] and azoverdin[27]. The solution was stirred for 1h, diluted twice with water at pH 3.5 and chromatographed on a reverse-phase column ODS (2 cm x 15 cm) made up in water pH 3.5. The column was firstly washed with 50 ml of a 0.1 M solution of EDTA, then with 300 ml water at pH 3.5. Pyoverdin G4R A **1a** was eluted as its free ligand with a 1:1 mixture (v/v) of acetonitrile in 0.05 M pyridine/acetic acid pH 5.0, lyophilized, applied to and eluted from a CM-Sephadex C-25 column with a linear gradient of pyridine/acetic acid pH 5.0 (0.05 M to 0.5 M, 2 x 0.3 l). Pure pyoverdin G4R A was lyophilized and stored desiccated in the dark at -20°C.

The isolation and purification of ^{15}N-labelled pyoverdin G4R A **1a**, unlabelled and ^{15}N-labelled pyoverdin G4R **1b**,were performed as described above for the unlabelled pyoverdin G4R A **1a**.

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