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Bacterial Siderophores: The Structures of the Pyoverdins of *Pseudomonas fluorescens* ATCC 13525

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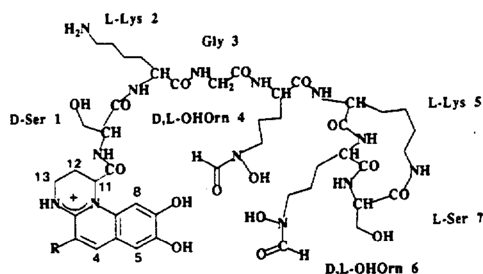
Abstract: The structures of five pyoverdins occurring in iron-deficient cultures of Pseudomonas fluorescens ATCC 13525 were elucidated using FAB-MS and 2D NMR techniques; they contain a common partly cyclic peptide containing a thirteen-membered ring bound to differently substituted chromophores derived from 2,3-diamino-6,7-dihydroxyquinoline.

We define the structure of five of the pyoverdins of *Pseudomonas fluorescens* ATCC 13525 as **1**.



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When *Pseudomonas fluorescens* ATCC 13525 grows in iron deficient conditions, it excretes a large number of pyoverdins possessing the same type of fluorescent chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline, together with deferriferribactin which does not have a chromophore but which also chelates iron(III). Philson and Lliñas [1] have reported an extensive study on one of the major siderophores excreted by the bacteria. They characterized by amino acid analysis and by NMR spectroscopy the nature of the amino acids composing its peptide chain, and have analyzed the nature of its chromophore using UV-visible and NMR spectroscopy. They concluded that the compound was a pyoverdin type of siderophore but did not report any structure[1]. In an early study on deferriferribactin, an orange-red compound when complexing iron(III) and colorless as a free ligand, Maurer et al. [2] proposed a peptide structure of ten amino acids [Ser(2), Lys(3), N^{δ}-OHOrn(2), Tyr(1), Gly(1), Glu(1)] and two acetyl groups. Philson and Lliñas [1] corrected these data showing that deferriferribactin has only two lysines, and that the two acetyl groups were in fact formyl groups. Although the structures of these molecules have not been fully defined, these preliminary structural studies

stimulated research into iron transport [3], production of antibodies [4] and improvement of the pyoverdin yield in the culture of *Pseudomonas fluorescens* ATCC 13525 as a function of the nitrogen source of the medium[5].

Using the purification procedure we have previously described for pyoverdins and azotobactins [6-9] we were able to separate and isolate in pure state more than fifteen pyoverdins from the cultures of *Pseudomonas fluorescens* ATCC 13525. The three numbers which characterize each compound refer to their order of elution in the successive chromatographic steps involved in their purification process (CM-Sephadex chromatography of the crude free ligand, CM-Sephadex chromatography of the iron-complex followed by HPLC on octadecylsilane). We describe now the structures of pyoverdin Pf 3/8/2 ATCC 13525, and of its four companions pyoverdins Pf 1/1/1, 2/2/1, 3/4/3 and 3/8/1.

Preliminary experiments performed on pyoverdin Pf 3/8/2 ATCC 13525, one of the major pyoverdins, confirmed the results reported by Philson and Lliñas [1] giving a peptide chain containing [Ser(2), Lys(2), N^{δ}-OHOrn(2), Gly(1)]. Two formyl groups and one succinamidyl residue were found to be bound to the peptide chain and to the chromophore respectively. FAB-MS gave a molecular peak at m/z 1160 (M^+) with a main fragment at m/z 858, the mass difference of 302 corresponding to loss of the chromophoric fragment: chromophore + succinamide [7-9]. Mild acid hydrolysis afforded after five minutes major ions due to loss of two formyl groups (m/z 1105) followed by H_2O (m/z 1087) or succinic acid (m/z 1005) [10]. After 15 minutes hydrolysis, ions at lower mass appeared corresponding to C-terminal and N-terminal fragments which are interpreted in Table 1.

From these results, the sequence

(Succinamide)-Chromophore-Ser-Lys-Gly-N^{δ} OHOrn-(Lys,N^{δ} OHOrn,Ser-H_2 O),

could be deduced.

Table 1: Interpretation of the FAB-MS data from hydrolyzates of pyoverdin Pf 3/8/2 ATCC 13525

m/z	Assignment
346	(Lys, N^{δ}OHOrn, Ser)
363	Chromophore-Ser
476	N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser)
491	Chromophore-Ser-Lys
533	Gly-N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser)
548	Chromophore-Ser-Lys-Gly
573	(Succinimide)-Chromophore-Ser-Lys
591	(Succinic Acid)-Chromophore-Ser-Lys
630	(Succinimide)-Chromophore-Ser-Lys-Gly
648	(Succinic Acid)-Chromophore-Ser-Lys-Gly
661	Lys-Gly-N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser)
678	Chromophore-Ser-Lys-Gly-N^{δ}OHOrn
1005	Chromophore-Ser-Lys-Gly-N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser - H_2O)
1023	Chromophore-Ser-Lys-Gly-N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser)
1087	(Succinimide)-Chromophore-Ser-Lys-Gly-N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser - H_2O)
1105	(Succinic Acid)-Chromophore-Ser-Lys-Gly-N^{δ}OHOrn-(Lys, N^{δ}OHOrn, Ser - H_2O)

2D NMR spectra showed that the chromophore was identical to the chromophore of pyoverdin Pa [8,9]. The HOHAHA assignment of all the amino acids signals showed a marked difference in the chemical shifts of the Hε protons of the lysines (Table 2), which suggest, by comparison with previously reported data that Lys-2 is linked via its α amino group to the peptide chain, and Lys-5, via both its α and ε amino groups [7-9].

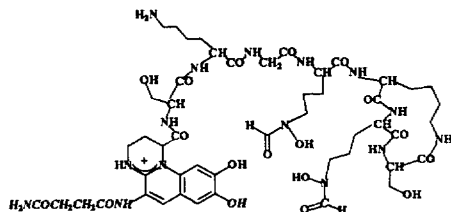
2D ROESY NMR confirmed the FAB-MS sequence, and provided evidence for Lys-2 being located between Ser-1 and Gly-3 and linked to the peptide chain via its α amino group, for Lys-5 (the C-terminal lysine) being bound respectively to OHOrn-4 via its α amino group, to Ser-7 via its ε amino group and to OHOrn-6 via its carboxyl group, and finally for Ser-7 and OHOrn-6 being bound together. It also showed that the protons of the formyl groups are correlated with the δ protons of both hydroxyornithines. In the free ligand, they resonate as a set of two signals of unequal intensity at 7.91-7.92 and 8.28-8.30 ppm (*cis* and *trans*), and merge into one doublet at 8.04-8.08 (*cis*) in the corresponding gallium(III) complex, similar to the pyoverdins Pa from *Pseudomonas aeruginosa* ATCC 15692 [8]. These results are consistent with the formyl groups being located on the two hydroxyornithines forming two hydroxamate groups and the peptidic moiety being partly cyclic with a thirteen-membered ring comprising Lys-5, OHOrn-6 and Ser-7. Consequently the earlier assignment of a linear peptide structure has to be modified [11].

Circular dichroism showed that the stereochemistry of the chromophore is (S) as in all the pyoverdins so far investigated [7-9]. GC-MS of the O-methyl, N-pentafluoropropionyl esters on a chiral column of a total hydrolyzate of pyoverdin Pf 3/8/2 ATCC 13525 showed that both lysines are L while the two serines have both configurations and the two hydroxyornithines both are present as one D and one L enantiomer. The same stereochemical analysis performed on a purified hydrolytic fragment, Chromophore-Ser-Lys, showed that the serine bound to the chromophore is D.

Table 2: Assignment of the protons of pyoverdin Pf 3/8/2 ATCC 13525

	NHα	CHα	CHβ	CHγ	CHδ	CHε	NHε
Ser-1	8.59	4.39	3.88				
Ser-7	8.91	4.48	3.95				
Lys-2	8.31	4.34	1.82	1.27	1.54-1.65	2.80-2.89	
Lys-5	8.04	4.20	1.91	1.03-1.27	1.49-1.58	3.2-3.25	7.23
Gly-3	8.12	3.65-3.78					
N^{δ}OHOrn-4	7.85	4.41	1.64	1.74		3.53	
N^{δ}OHOrn-6	7.98	4.27	1.68	1.80		3.58	
Chromo-	H-4	H-5	H-8	H-11	H-12,12'	H-13,13'	
phore	7.92	7.21	7.05	5.73	2.5-2.73	3.42-3.78	
Succinyl	2.73-2.75	2.67-2.69					
Formyl	7.91-7.92 & 8.28-8.30						

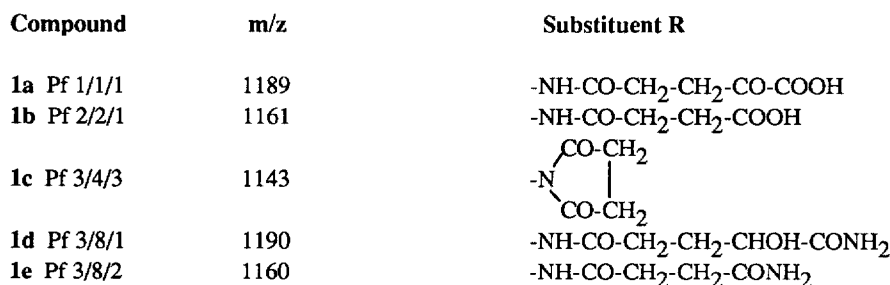
The structure of pyoverdin Pf 3/8/2 ATCC 13525 is reported in **1e**.



1 e

The structures of the other pyoverdins isolated were deduced by FAB-MS. They all contain the same peptidic moiety and differ in the acyl substituent bound to the chromophore. Their structures are summarized in Table 3. These pyoverdins are structurally identical to those excreted by *Pseudomonas chlororaphis* ATCC 9446 (they show the same FAB-MS data, the same ^{1}H and ^{13}C NMR data and give the same stereochemical analysis), and very close to pyoverdins Pa excreted by *Pseudomonas aeruginosa* ATCC 15692[9]: they have the same type of chromophore with its catechol group, a basic amino acid at position 2 of the peptide chain, the first formylhydroxyornithine at position 4, and the second as part of a large ring. These similarities can explain the lack of specificity shown by the corresponding strains in cross-binding and cross-feeding experiments [3] and suggest some similarities in the binding sites of their pyoverdin-Fe(III) complexes receptors.

Table 3: Structure of the major pyoverdins isolated from the cultures of *Pseudomonas fluorescens* ATCC 13525 as determined by FAB-MS



Compound	m/z	Substituent R
1a Pf 1/1/1	1189	$\text{-NH-CO-CH}_2\text{-CH}_2\text{-CO-COOH}$
1b Pf 2/2/1	1161	$\text{-NH-CO-CH}_2\text{-CH}_2\text{-COOH}$
1c Pf 3/4/3	1143	$\text{-N}(\text{CO-CH}_2)(\text{CO-CH}_2)$
1d Pf 3/8/1	1190	$\text{-NH-CO-CH}_2\text{-CH}_2\text{-CHOH-CONH}_2$
1e Pf 3/8/2	1160	$\text{-NH-CO-CH}_2\text{-CH}_2\text{-CONH}_2$

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References

1. Philson, S. B.; Lliñas, M. *J. Biol. Chem.*, **1982**, ***257***, 8081-8085. Philson, S. B.; Lliñas, M. *J. Biol. Chem.*, **1982**, ***257***, 8086-8090
2. Maurer, B.; Müller, A.; Keller-Schierlein, W.; Zähner, H. *Arch. Mikrobiol.*, **1969**, *60*, 326-339
3. Hohnadel, D.; Meyer, J-M. *J. Bacteriol.*, **1988**, *170*, 4865-4873
4. Buyer, J. S.; Sikora, L. J.; Kratzke, M. G. *Appl. Environm. Microbiol.*, **1990**, *56*, 419-424.
5. Albesa, I.; Barberis, L. I.; Pajaro, M. C.; Eraso, A. J. *J. Gen. Microbiol.*, **1985**, *131*, 3251-3254.
6. Demange, P.; Bateman, A.; Dell, A.; Abdallah, M. A. *Biochemistry*, **1988**, *27*, 2745-2752
7. Demange, P.; Bateman, A.; Mertz, C.; Dell, A.; Piémont, Y.; Abdallah, M. A. *Biochemistry*, **1990**, *29*, 11041-11051
8. Abdallah, M. A. : Pyoverdins and Pseudobactins. In *Handbook of Microbial Iron Chelates*; Winkelmann, G. Ed., CRC Press Inc., Boca Raton, Florida, U.S.A., 1991; pp. 139-153.
9. Demange, P.; Wendenbaum, S.; Linget, C.; Mertz, C.; Cung, M. T.; Dell, A.; Abdallah, M. A. *Biol. Metals*, **1990**, *3*, 155-170.
10. In the conditions used, the succinamidyl group on the chromophore is readily hydrolyzed to a succinyl group.
11. Demange, P.; Wendenbaum, S.; Bateman, A.; Dell, A.; Abdallah, M. A.: Bacterial Siderophores Structure and Physicochemical Properties of Pyoverdins and related Compounds. In *Iron Transport in Microbes, Plants and Animals*. Winkelmann, G.; van der Helm, D.; Neilands, J. B. Eds., VCH Publishers, Weinheim, West Germany, 1987; pp. 167-187.

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