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Bacterial Siderophores: Structure Elucidation, 2D ^{1}H and ^{13}C NMR Assignments of Pyoverdins Produced by *Pseudomonas fluorescens* CHAO

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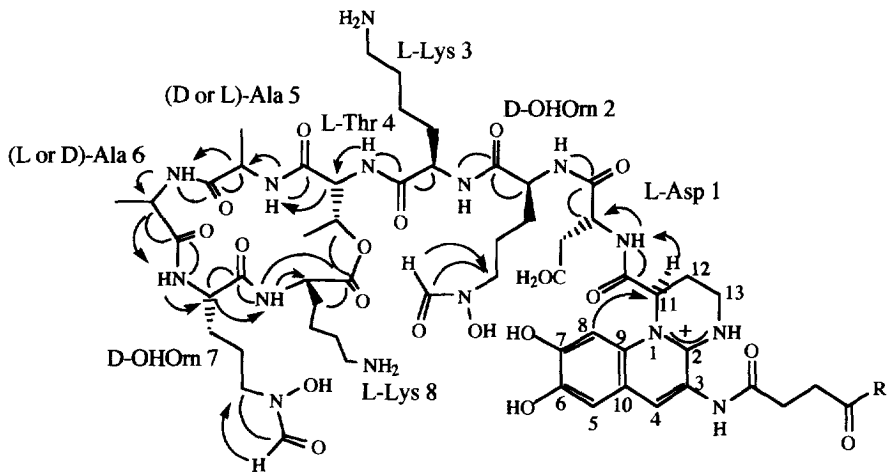
Abstract: The structure of two pyoverdins produced in iron-deficient cultures of *Pseudomonas fluorescens* CHAO, a bacterium responsible for suppression of fungal diseases in plant roots, was elucidated using essentially FAB-MS and homonuclear and heteronuclear 2D NMR techniques. It is a partly cyclic chromopeptide: chromophore-Asp-OHOrn-Lys-*cyclo*-(Thr-Ala-Ala-OHOrn-Lys) forming a sixteen-membered lactone ring. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Pseudomonas fluorescens CHAO is a bacterium occurring in suppressive soils and excreting several metabolites such as antibiotics and siderophores which prevent fungal plant diseases[1,2]. *Pseudomonas fluorescens* CHAO exhibits two different high affinity iron-transport systems when grown under iron-deficient conditions and synthesizes two types of siderophores: salicylic acid[3,4] and pyoverdins. The purification and the structure elucidation of two major pyoverdins, CHAO **1a** and CHAO A **1b**, produced by *Pseudomonas fluorescens* CHAO are reported here.



Pyoverdins of *Pseudomonas fluorescens* CHAO **1a** R = OH
CHAO A **1b** R = NH_2

ROESY correlations
HMBC correlations

Because the iron complexes of these pyoverdins are relatively insoluble we have modified the purification procedure we had previously described for a number of pyoverdins and azotobactins[5-8]. We are able, using this new purification procedure, to separate and isolate in pure state these two major pyoverdins from the cultures of *Pseudomonas fluorescens* CHAO[9].

Preliminary hydrolysis experiments performed on pyoverdin CHAO **1a** indicates a peptide chain containing the following amino-acids: Asp(1), Lys(2), Nδ-OHOrn (2), Ala (2), Thr (1). FAB-MS[10]shows a molecular peak at m/z 1288 (M^+). When submitted to hydrolysis in 6N HCl at 95°C pyoverdin CHAO **1a** rapidly loses two formyl groups to give a major product of m/z 1232 which is able to add or lose 18 mass units (m/z 1250 and 1214) presumably due to addition of water and ring opening of the lactone or cyclization of the succinic acid. Further hydrolysis trims off the C-terminal residues to give signals at m/z 992 (corresponding to a fragment having lost lysine and Nδ-hydroxyornithine), and at m/z 921 (which corresponds to the loss of an alanine). These two fragments are still able to lose 18 mass units by cyclization of the succinic acid residue giving respectively signals at m/z 974 and m/z 903. Mild methanolysis with a mixture of deuterated/undeuterated methanol (1:1) and HCl, splits the molecule and gives major signals as follows: three triplets respectively at m/z 549, 552, 555; 677, 680, 683; 778, 781, 784; and two doublets at m/z 276, 279; 534, 537 (Table 1). The triplets fit for chromophore-containing fragments which have lost the succinyl moiety but have two esterifiable groups *i.e.* aspartic acid and the C-terminal residue. From these results it was possible to suggest the following sequence for pyoverdin CHAO **1a**:

(Succinic acid)-Chromophore-(Asp, OHOrn)-Lys-*cyclo*-[Thr-Ala-Ala-(OHOrn, Lys)]

with a partly cyclized peptide involving a bond between one of the C-terminal amino acids (Nδ-hydroxyornithine or lysine) and possibly the hydroxyl group of threonine. This type of structure can take into account the ready addition of water observed in the hydrolysis experiments, and is completely in agreement with the NMR data (see below).

Table 1: Interpretation of the FAB-MS data from the methanolysis products of pyoverdin CHAO **1a** with a mixture of methanol/deuterated methanol (1:1) and HCl.

m/z	Chromophoric fragments assignment	m/z	Peptidic fragments assignment
549, 552, 555	Chromophore-(Asp,OHOrn)	276, 279	Thr-Ala-Ala
677, 680, 683	Chromophore-(Asp,OHOrn)-Lys	534, 537	Thr-Ala-Ala-(OHOrn-Lys)
778, 781, 784	Chromophore-(Asp,OHOrn)-Lys-Thr		

2D ^{1}H NMR spectra[11] show that the chromophore is identical to the chromophore of pyoverdin Pa[7,8]. The HOHAHA assignments of all the amino acids signals show identical chemical shifts of the protons of the hydroxyornithines and of the Hε protons of the two lysines respectively (Table 2). This suggests, by comparison with previously reported data, that the side chain of the hydroxyornithines are not cyclized and that both lysines are bound *via* their α amino group to the peptide chain[7,8,12,13].

2D ROESY NMR confirmed partly the FAB-MS sequence, showing that aspartic acid is bound to the chromophore, assigned without ambiguity the signals of the lysines, provided evidence for the partial sequence Thr 4-Ala 5-Ala 6-OHOrn 7-Lys 8 and established that the formyl groups are located on the Nδ-hydroxyornithines (arrows in structures **1a** and **1b**).

Using 2D ^{1}H-^{13}C heteronuclear and HMBC correlations, the carbon atoms could all be assigned (Tables 3 and 4), except for the two hydroxyornithines which show identical proton shifts. However the Cα carbon atoms of these two amino acids could be differenciated by HMBC, their other carbon atoms being undistinguishable. 1D and 2D ^{13}C NMR spectra show that the chromophore of pyoverdin CHAO **1a** is identical to that of all the pyoverdins so far investigated, confirming the ^{1}H NMR spectra data (Table 4)[7].

2D HMBC NMR spectra completed without ambiguity the proof of the total sequence of pyoverdin CHAO **1a**, confirming the partial determination so far obtained. A correlation between the carboxyl group of Lys 8 and the CHβ of Thr 4 gave evidence for the presence of an ester bond between these two amino acids, hence the lactone ring in the molecule, and consistent with the unusual chemical shifts presented for Cβ and Hβ by Thr4

(respectively at 74.20 ppm instead of 69.00 ppm, and at 5.31 ppm instead of 4.31 ppm) which are in agreement with a similar but not identical reported structure[14] (arcs in structures **1a** and **1b**).

Table 2: Assignment of the protons of pyoverdin CHAO **1a** in H_2O/deuterated *t*-butanol (2%) using HOHAHA correlation.

Residue	NHα	CHα	CHβ	CHγ	CHδ	CHε	Others
Asp-1	8.95	4.58	2.71-3.02				
OHOrn-2	7.62*	4.38*	1.86*	2.13*	3.61*		7.99* *cis* 8.31* *trans* formyls
Lys-3	8.30	4.17	1.76	1.44	1.56	2.99	7.80 NHε
Thr-4	8.13	4.67	5.31	1.25			
Ala-5	8.71	4.19	1.36				
Ala-6	9.10	4.11	1.47				
OHOrn-7	7.62*	4.38*	1.86*	2.13*	3.61*		7.99* *cis* 8.31* *trans* formyls
Lys-8	7.49	4.66	1.63	1.93	1.33	2.99	7.80 NHε
Chromophore		H-4 7.87	H-5 7.09	H-8 6.91	H-11 5.60	H-12,12' 2.41/2.73	H-13,13' 3.39/3.72
Succinate		2.68	2.61				

* Proton shifts are identical for OHOrn-2 and OHOrn-7.

Table 3: Assignment of the aliphatic carbon atoms and carbonyls of pyoverdin CHAO **1a** in H_2O/deuterated *t*-butanol (2%) using 2D ^1H-^{13}C heteronuclear correlation.

Residue	Cα	Cβ	Cγ	Cδ	Cε	α Carbonyls	Other Carbonyls
Asp 1	51.29	39.20				176.91	γ 173.63
OHOrn 2	53.69	30.37*	23.61*	*trans** 46.52; 46.80 *cis** 50.49; 50.66		174.96	*trans** 164.40; 164.55 *cis** 160.30; 160.60
Lys 3	54.70	27.53	24.11	31.02	40.49	174.79	
Thr 4	56.42	74.20	16.29			171.17	
Ala 5	52.13	16.36				178.19	
Ala 6	52.43	16.66				176.94	
OHOrn 7	53.83	28.62*	23.51*	*trans** 46.52; 46.80 *cis** 50.49; 50.66		173.40	*trans** 164.40; 164.55 *cis** 160.30; 160.60
Lys 8	53.51	27.35	23.17	30.87	40.49	171.88	
Chromophore	57.44	22.86	36.28			170.02	
Succinate	32.88	32.37				NHCO 178.28	COOH 181.56

* ^{13}C shifts can not be clearly distinguished since the ^1H shifts of both OHOrn are identical.

Table 4: Assignment of the chromophoric carbon atoms of pyoverdin CHAO **1a**.

	C2	C3	C4	C5	C6	C7	C8	C9	C10
δ(ppm)	150.32	118.78	139.57	114.80	144.80	152.64	101.32	132.67	115.62

Circular dichroism shows that the configuration of the chromophore in position 11 is (S), as in all the pyoverdins so far investigated[7]. GC-MS on a D-chirasil-Val chiral column of the O-methyl, N-pentafluoropropionyl esters of a total hydrolyzate of pyoverdin CHAO showed that aspartic acid, threonine and

both lysines are L while the two alanines have both configurations. The two hydroxyornithines are present as D enantiomers[15].

The structure of pyoverdin CHAO A **1b** was deduced from FAB-MS. It contains the same peptidic moiety and differs only in the acyl substituent bound to the chromophore which is succinamide instead of succinic acid. From the FAB-MS results it was possible to propose a partly cyclized structure for the peptidic moiety of these siderophores, but 2D NMR gives complete evidence for a cyclization between threonine-4 and lysine-8 *via* an ester bound. The presence of this lactone ring is fully compatible with the ready addition of water in the FAB-MS hydrolysis experiments where a lactam ring would not open but would be cleaved as a whole. Thus all the FAB-MS and NMR data fit well with the proposed partly cyclic structure.

Two very similar pyoverdins possessing a lactone ring were formerly purified from a *Pseudomonas fluorescens* strain isolated from Rhine water. The structures of these pyoverdins are very similar but not identical to CHAO **1a** and CHAO A **1b**: the only difference is that they were found to have an asparagine residue instead of aspartic acid[14].

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9. The different purification steps were: centrifugation, ultra-filtration, octadecylsilane chromatography, CM-Sephadex chromatography of the crude free ligand, HPLC on octadecylsilane in the presence of 0.1M EDTA followed by a final CM-Sephadex chromatography.
10. Mass spectrometry measurements were performed on a VG Analytical (Manchester,UK) ZAB-HF mass spectrometer fitted with a FAB gun manufactured by M-Scan (Ascot, UK).
11. ^{1}H and ^{13}C NMR spectra were determined at pH 5.60 on a Bruker ARX 500 spectrometer (Bruker Spectrospin, Wissembourg, France), using as solvent, water containing 2% of fully deuterated $[^{2}H_{10}]$ *t*-butanol (CEA-Saclay, France) as an internal standard.
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