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# Bacterial Siderophores: The Structure of a desferriferribactin produced by *Pseudomonas fluorescens* ATCC 13525

**Caroline Linget[a], Despina G. Stylianou[b], Anne Dell[b], Robert E. Wolff[a], Yves Piémont[c] and Mohamed A. Abdallah[a]***

[a] Laboratoire de Chimie Microbienne, Associé au C.N.R.S., Université Louis Pasteur, 1 rue Blaise Pascal, F-67008-Strasbourg, France.

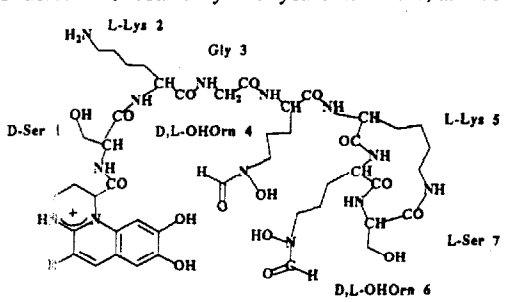
[b] Department of Biochemistry, Imperial College, London SW7 2AZ, United Kingdom.

[c] Laboratoire de Bactériologie, Université Louis Pasteur, 3 rue Koeberlé, F-67000 Strasbourg.

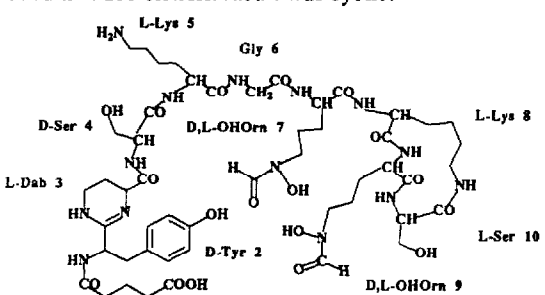
*Key Words: Pseudomonas; Pyoverdins; Siderophores; Peptides; Desferriferribactin*

*Abstract: The structure of a desferriferribactin a postulated precursor of pyoverdins produced in iron-deficient cultures of Pseudomonas fluorescens ATCC 13525 was elucidated using FAB-MS and 2D NMR techniques. It is a partly cyclic peptide containing a thirteen-membered ring, as well as an amidine type amino-acid built up on tyrosine and 2,4-diaminobutyric acid.*

When *Pseudomonas fluorescens* ATCC 13525 grows in iron deficient conditions, it excretes a large number of pyoverdins **1**[1] possessing the same type of fluorescent chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline, together with a desferriferribactin which does not have a chromophore but which also chelates iron(III). Maurer *et al.*[2] proposed a peptide structure of ten amino acids [Ser(2), Lys(3), $N^{\delta}$-OHOrn(2), Tyr(1), Gly(1), Glu(1)] and two acetyl groups for the desferriferribactin molecule. After reaction of the peptide with dinitrofluorobenzene and hydrolysis, they characterized only DNP-glutamic acid and the NHε DNP-derivative from only one lysine molecule, and concluded that desferriferribactin was cyclic.




**1**




**2**

Philson and Lliñas [3] corrected some of these data showing that desferriferribactin has only two lysines, and that the two acetyl groups were in fact formyl groups. More recently Budziekiewicz and his group have reported some NMR data on four desferriferribactins from different strains of fluorescent *Pseudomonas* [4,5] as well as the structure of a desferriferribactin excreted by a strain of *Pseudomonas aptata* [5]. They found that all contained 2,4-diaminobutyric acid (Dab) combined to tyrosine forming a cyclic amidine as in the pyoverdins

excreted by two strains of fluorescent *Pseudomonas*, namely pyoverdins ATCC 17400[6] and pyoverdins CCM 2798 (and 2799)[7] which respectively possess Gln/Dab (or GlnCTHPMD) and Ser/Dab (or SerCTHPMD). They concluded by dansylation followed by hydrolysis and by NMR that glutamic acid was at the N-terminus in the desferriferribactins and that it is bound to tyrosine by its γ carboxyl group. However they did not report any structure nor any sequence for desferriferribactin ATCC 13525 and postulated that desferriferribactins were the biosynthetic precursors of pyoverdins[4,5].

Using the purification procedure we have previously described for pyoverdins, azotobactins and azoverdin[1,6-11] we were able to separate and isolate in pure state more than fifteen pyoverdins from the cultures of *Pseudomonas fluorescens* ATCC 13525 together with one desferriferribactin[1]. Using FAB-MS as well as 2D NMR techniques, we establish here the structure of this latter as structure **2**.

Ferribactin has a $MH^+$ at m/z 1231 (FAB-MS). Its corresponding free ligand, desferriferribactin, studied here by FAB-MS has the corresponding $MH^+$ at m/z 1178 (All masses were assigned by counting and are therefore nominal).

Acid hydrolysis (6 M HCl, 1 min, 80°C) resulted in two main fragments at m/z 1122 (hydrolysis of two formyl groups) and m/z 993 (loss of glutamic acid), this latter being consistent with a terminal position of this acid.

Peracylation with trifluoroacetic anhydride and acetic acid yielded a mixture of monotrifluoroacetyl derivatives having up to five acetyl groups (m/z 1484, 1442 and 1400) as well as hexaacetyl and pentaacetyl derivatives (m/z 1430, 1388). No significant loss of formyl groups or formyl/acetyl exchange could be detected. Fragments were also found, which could be interpreted as a indicating that the trifluoroacetyl group (TFA) most probably acylated the glutamic acid amino group, and b (Table 1).

Mild methanolysis with a 1:1 mixture of deuterated and undeuterated methanol, and HCl, followed by peracetylation showed that deformylation took place, and that one free carboxyl group was then esterified (pentaacetyl, m/z 1349-1346); tetraacetyl, m/z 1307-1304). Fragments c to f (Table 1) were also found, none of them containing the esterified carboxyl group, and they are consistent with the sequence **Ser-Lys-Gly**.

Reaction with phenylisothiocyanate yielded a mono- (m/z 1313) and a di- (m/z 1448) phenylthiourea derivatives, indicating the presence of two amino groups of differing reactivity, in agreement with the previous results of Maurer *et al.*[2], and leading to the conclusion that 2,4-diaminobutyric acid does not possess a free amine group in the peptide.

**Table 1** : Assignment of ions in FAB-MS of peracyl desferriferribactin and the peracetylated products of very mild methanolysis

| Code | m/z | Assignment |
|---|---|---|
| a | 617, 659 | [Glu-Tyr-Dab-Ser$NH_2$ - $H_2O$ + H] + 1 TFA + 1 and 2 Acetyls |
| b | 801, 843 | [Lys-Gly-Nformyl,NOHOrn-Lys-Nformyl,NOHOrn-Ser -$H_2O$ + H] + 2 and 3 Acetyls |
| c | 874, 916 | [Ser-Lys-Gly-OHOrn-Lys-OHOrn-Ser -$H_2O$ + H] + 3 and 4 Acetyls |
| d | 787, 829 | [Lys-Gly-OHOrn-Lys-OHOrn-Ser -$H_2O$ + H] + 3 and 4 Acetyls |
| e | 659, 701 | [Gly-OHOrn-Lys-OHOrn-Ser -$H_2O$ + H] + 3 and 4 Acetyls |
| f | 602, 644 | [OHOrn-Lys-OHOrn-Ser -$H_2O$ + H] + 3 and 4 Acetyls |

Upon acidification under the Edman conditions, the loss of glutamic acid hydantoin from the di-derivative (m/z 1448) is, at most, very minor (very weak peak at m/z 1183): together with the loss of water to mass 1430, this is consistent with the proposed desferriferribactin sequence.

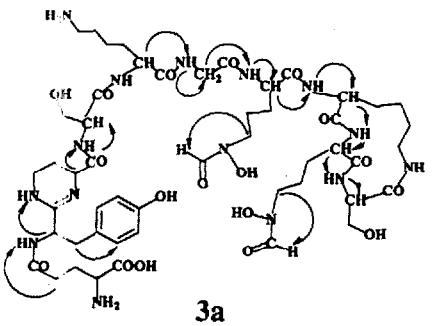
From these data it could be concluded that desferriferribactin has the following sequence : Glu-(Tyr/Dab)-Ser-Lys-Gly-(OHOrn,Lys,OHOrn,Ser).

2D NMR spectra showed that all the amino acids common to pyoverdin Pf ATCC 13525 and desferriferribactin have similar chemical shifts[1]. 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-5 is linked via its α amino group to the peptide chain, and Lys-8, via both its α and ε amino groups [1,7,10].

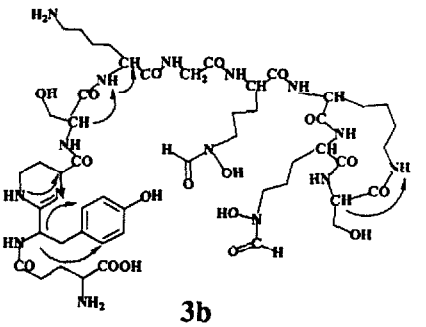
**Table 2:** Assignment of the protons of desferriferribactin Pf ATCC 13525 at 280°K in $H_2O/([^2H]_3C)_3CO[^2H]$, acidified to pH 3.0 by addition of $CF_3COOH$ and using sodium $[^2H_2]_6$-trimethylsilylpropane sulfonate as an internal standard.

| | NHα | CHα | CHβ | CHγ | CHδ | CHε | NHω |
|---|---|---|---|---|---|---|---|
| Glu-1 | 7.95 | 3.68 | 2.09 | 2.46 | | | |
| Tyr-2 | 8.68 | 4.67 | 3.11 | 7.17 (H3,H5) 6.86 (H2,H6) | | | |
| Dab-3 | | 4.41 | 2.09 | 3.33 | | | 9.41-9.56 |
| Ser-4 | 8.96 | 4.34 | 3.87 | | | | |
| Lys-5 | 8.83 | 4.38 | 1.89 | 1.43 | 1.66-1.73 | 2.97 | 7.60 |
| Gly-6 | 8.54 | 3.94 | | | | | |
| $N^{\delta}$OHOrn-7 | 8.17 | 4.39 | 1.74 | 1.58 | 3.55 | | |
| Lys-8 | 8.34 | 4.11 | 1.90 | 0.98-1.34 | 1.47-1.56 | 3.16-3.28 | 7.38 |
| $N^{\delta}$OHOrn-9 | 8.41 | 4.21 | 1.75 | 1.64 | 3.55 | | |
| Ser-10 | 8.49 | 4.44 | 3.87 | | | | |
| Formyl | 7.95 (*cis*) and 8.25 (*trans*) 0.5:1.5 | | | | | | |

2D ROESY NMR techniques completed these findings: after having assigned all the protons of the amino acids constituting the peptide, the spectra were determined in two different conditions and gave a number of cross peaks which yielded the final sequence of desferriferribactin Pf ATCC 13525. At 300°K using a $^2H_2O/H_2O$ 1:9 mixture and a 300 ms mixing time, the connectivities between the protons $CH_i$, $NH_i$ and $NH_{i+1}$ of the amino acids represented by arrows in solid lines in **3a**, gave the following partial sequences: **Glu(1)-Tyr(2)/Dab(3)-Ser(4)** and **Lys(5)-Gly(6)-OHOrn(9)-Lys(8)-OHOrn(9)-Ser(10).** In addition the two formyl groups which resonate as a set of two signals of unequal intensity at 7.90-7.91 and 8.26-8.28 ppm (*cis* and *trans* with a ratio of 1.5:0.5) are each connected to the CHδ protons of the corresponding $N^{\delta}$-hydroxyornithine.



**3a**



**3b**

At 280°K, in a mixture of $H_2O/([^2H]_3C)_3C\text{-}O[^2H]$ 9:1 acidified with $CF_3CO_2H$ to pH 3.0 and a mixing time of 400 ms most of the connectivities were the same. The new cross peaks occurring in these conditions (represented by solid arrows in **3b**) show in addition that in desferriferribactin, Ser(10) is bound to Lys(8) through its NHε in a similar fashion as in pyoverdins Pf ATCC 13525[1] and confirm the presence of the same 13 membered ring. The complete sequence of desferriferribactin ATCC 13525 is

**Glu(1)-Tyr(2)/Dab(3)-Ser(4)-Lys(5)-Gly(6)-OHOrn(7)-Lys(8) — Ser(10).**
**OHOrn(9)**

GC-MS of the O-methyl, N-heptafluorobutyryl esters on a L-Chirasil-Val column of a total hydrolyzate of desferriferribactin Pf ATCC 13525 confirmed the results already reported [2-4] and showed that all the amino acids common to this compound and pyoverdins ATCC 13525[1] have the same stereochemistry.

The structure of desferriferribactin Pf ATCC 13525 is reported in **2**. It is very similar to the structure of pyoverdins Pf ATCC 13525 **1**[1] and is compatible with the plausible role of desferriferribactins as biogenetic precursors in the biosynthesis of the siderophores, tyrosine being oxidized to dopa which together with the tetrahydropyrimidine ring built up between tyrosine and 2,4-diaminobutyric acid yields the fluorescent chromophore of pyoverdins.

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