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THE STRUCTURE OF PYOVERDINE *Pa*, THE SIDEROPHORE OF *PSEUDOMONAS AERUGINOSA*

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The structure of Pyoverdine *Pa*, the siderophore of *Pseudomonas aeruginosa*, was elucidated by degradation, by FAB mass spectrometry and by NMR; it consists of a linear octapeptide bound to a 2,3-diamino-6,7-dihydroxyquinoline derived chromophore.

Pseudomonads are widespread bacteria, which are divided into five groups of genetic homology according to their rRNA-DNA hybridization (1). The fluorescent pseudomonads, which belong to group 1, release, in iron-deficient conditions, yellow-green water-soluble fluorescent pigments, strongly chelating Fe(III) ions, which are the siderophores of these bacteria (2-4).

Among the fluorescent pseudomonads, *Pseudomonas aeruginosa* is of particular importance since it is pathogenic for weakened organisms (deeply burnt people, for instance).

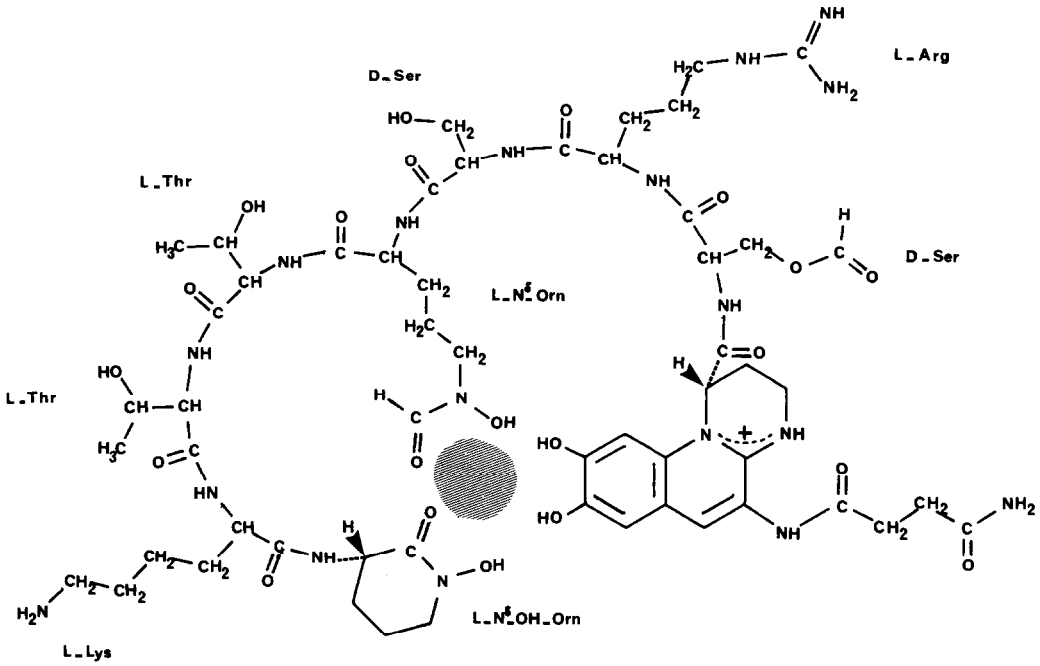
In iron-deficient conditions, *P.aeruginosa* (ATCC 15692, PAO 1) produces large amounts (*ca.* 40 mg/l) of a pigment, which we call "Pyoverdine *Pa*", and which was shown to be the siderophore of this bacterium (5). This substance was isolated according to published procedures (2) and further purified by HPLC on reverse-phase octadecylsilane columns as its Al(III) or Fe(III) complex. Its spectral data as well as those of its metal complexes are very similar to those reported for pseudobactin, the siderophore of *Pseudomonas B-10* (4).

The structure of Pyoverdine *Pa*, 1, has been elucidated as follows.

Fast Atom Bombardment mass spectrometry (FAB) (6) gave a peak at M^+= 1333 m.u.for 1 itself, shifted to 1386 m.u. for its iron complex and 1357 for the aluminum complex (which proves the 1:1 stoichiometry of these metal complexes).

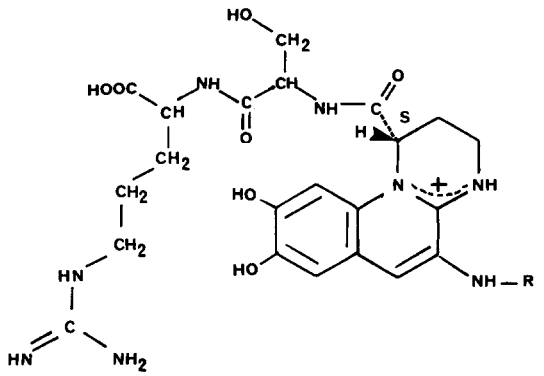
Acid hydrolysis of the pigment (24 h, 110°C, 6 M HCl or 6 M HI), followed by quantitative amino-acid analysis,showed the presence of a peptide chain containing serine (2 moles), lysine (1 mole), threonine (2 moles), arginine (1 mole) and δ-N-hydroxyornithine (OHorn, 2 moles), bound to a fluorescent chromophore. One mole of succinic acid could also be isolated from the hydrolysate. No free N- or C-terminal amino-acid could be detected, and all attempts to hydrolyse the peptide enzymatically failed.

By mild hydrolysis (30 min, 100°C, 6 M HCl), two dipeptides, Ser-OHorn and Lys-OHorn, as well as a tetrapeptide Thr-Thr-Lys-OHorn could be isolated and purified by electrochromatography on cellulose sheets. The N-termini of these latter peptides were identified by dansylation.



1

Two fluorescent molecules, one containing serine and the other containing serine and arginine were also isolated by electrochromatography. However the main product of this hydrolysis was shown to be compound 2 after purification on CM-25 Sephadex column. Small amounts of 3 were also isolated.

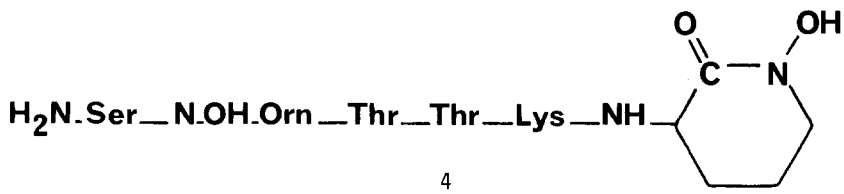


2 R = H

3 R = CO-CH_2-CH_2-COOH

The molecular masses of 2 (519 m.u.) and 3 (619 m.u.) were determined by FAB mass spectrometry. Furthermore 3 was shown to contain 2 acid functions because its mass shifted by 28 m.u. after methanol-HCl esterification.

The time course of mild acid hydrolysis of the siderophore was monitored by FAB mass spectrometry of aliquots of the hydrolysis mixture. A considerable proportion of the peptide sequence and the presence of the two formyl groups were established in this manner. Very mild acid treatment (acidified methanol at pH 1.0, 20°C, 30s) results in consecutive loss of the two formyl groups. More vigorous conditions (6M HCl, 90°C, time course up to 30 minutes) rapidly converted the succinamide moiety to succinic acid. This was followed by hydrolysis of the Arg-Ser bond and loss of succinic acid giving two major components having molecular masses consistent with compound 2 and the peptide 4 . The latter was accompanied by a signal 18 m.u. higher, showing that partial opening of the N-hydroxy-piperidone ring had taken place. Further hydrolysis removed arginine from compound 2, and serine followed by δ-N-hydroxyornithine from compound 4.



4

Pyoverdine *Pa*, its metal complexes and the deformylated derivative, exhibit a specific FAB cleavage accross the saturated ring of the chromophore, giving a major fragment ion 302 m.u. below the molecular ion. The presence of this ion is therefore diagnostic of the chromophore present in pyoverdine and related siderophores.

The chromophore of pyoverdine was found to be identical to that of pseudobactin (4) by comparison of the corresponding ^{1}H NMR data of 1 and 3 and the ^{13}C NMR data of 1 with pseudobactin (4 singlets at 5.75, 7.09, 7.20 and 7.97 ppm, 9 carbon resonances consisting of 3 doublets and 6 singlets having the same chemical shifts as in pseudobactin).

The formyl groups produce 2 signals differing in intensity at 7.95 and 8.30 ppm. Upon acid addition, they give one singlet at 8.22 ppm, corresponding to the resonance of formic acid, in agreement with what has been reported by Llinas et al.(7) for the fluorescent pigment synthesized by *P.fluorescens* ATCC 13525.

One of the formyl groups is very likely located on the Nδ nitrogen atom of the hydroxy-ornithine in the middle of the peptide chain, forming a hydroxamate which is the third group participating to the very strong complexation of Fe(III) by pyoverdine (the two others being the catechol group of the chromophore and the hydroxamate group of the terminal N-hydroxy-piperidone ring) thus forming a very stable 1:1 octahedric Fe(III)-pyoverdine complex (with an association constant $K = 10^{32}$ as measured by competition with EDTA (2)). The other formyl group is probably located on the serine next to the chromophore.

The stereochemistry of the different chiral groups of the molecule was also investigated. The comparison of the CD spectra of pseudobactin and pyoverdin *Pa*, which both exhibit a positive Cotton effect in the 400 nm region, shows that their chirality centers vicinal to the

chromophores have the same absolute configuration which is S. In addition, the stereochemistry of the amino-acids was determined either after purification and measurement of their CD spectra, or by derivatization (O-n-propyl-N-heptafluorobutyryl esters of the amino-acids) followed by gas chromatography on a chiral capillary column whose stationary phase was made of a bound derivative of L-valine (8).

Only the two serines were found to have an R configuration, all the other amino-acids having the S configuration, including the terminal hydroxyornithine in its cyclic form (which was found to have the R configuration in pseudobactin).

Both pseudobactin and pyoverdine are constituted with a peptide chain bound to the same chromophore. However, they differ in the length of the peptide chain (6 vs 8 amino-acids), in most of the amino-acids and their absolute configuration (presence of D-serine, L-lysine, L-threonine, L-arginine and L-hydroxyornithine in pyoverdine *Pa*, instead of L-alanine, D-allothreonine, D-threohydroxyaspartic acid, L-ε-lysine and D-hydroxyornithine in pseudobactin).

A CPK molecular model built in our laboratory according to structure 1 shows how pyoverdine *Pa* can chelate Fe(III) ions without constraint, forming a cavity where iron is held by the three bidentate complexing groups, and which is hydrophobic enough on its outer surface to allow iron transport through hydrophobic membranes.

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