Ferribactins as the Biosynthetic Precursors of the *Pseudomonas* Siderophores Pyoverdins[§]

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By a feeding experiment with a ^{15}N-labelled precursor it is shown that ferribactins are transformed into pyoverdins and thus are their biogenetic precursors.

Pyoverdins are the typical siderophores of the fluorescent members of the *Pseudomonas* spp. belonging to the rRNA homology group I. They are characterized by the chromophore (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido[1,2a]chinolin-1-carboxylic acid, to the carboxyl group of which a peptide chain is bound by its N-terminus. (Budzikiewicz, 1997a, b). Pyoverdins are usually accompanied by congeners having the same peptide chain but differing in the nature of the chromophore. Most notable are 5,6-dihydropyoverdins and ferribactins. While pyoverdins may have Glu, α-ketoglutaric, malic or succinic acid bound to the amino group of the chromophore, ferribactins are found only with Glu. Ferribactins were shown to be condensation products of D-Tyr and L-Dab and were considered to be precursors of the pyoverdins (for a detailed discussion see Böckmann *et al.*, 1997), but it had also been surmised that they are side products of the pyoverdin biosynthesis (Meyer and Stintzi, 1998). To clarify this point and thus to further the evidence for the biogenetic chain leading to the pyoverdins the incorporation experiments described below were performed.

The pyoverdin **1** (Fig. 1., with a succinic acid side chain) and the corresponding ferribactin **2** (with a Glu side chain) labeled with ^{15}N in every position were obtained by growing *Pseudomonas chlororaphis* ATCC 9446 (this strain produces considerable amounts of **2**) in an artificial medium (Hohlneicher *et al.*, 1995) containing $(^{15}NH_4)_2SO_4$ as the only nitrogen source. $(^{15}N_{14})$-**1** was characterized by a detailed mass spectrometric analysis in comparison with unlabelled **1** using collision induced decomposition after electrospray ionization of $[M+2H]^{2+}$ (the doubly charged ions are much more abundant than $[M+H]^+$ and for reasons discussed in the cited references give more characteristic fragments) and of selected fragment ions (Fuchs, 2000; Fuchs and Budzikiewicz, 2000). This technique is necessary to obtain a large number of fragments which can be correlated with the structure of the pyoverdin and thus be used for identification.

Assuming that in case of a transformation of **2** into **1** (Fig. 2) the best results for an incorporation of labeled **2** would be obtained when the enzyme apparatus is already activated, the following procedure was employed. *Pseudomonas chlororaphis* was grown in an iron deficient medium containing $(^{14}NH_4)_2SO_4$ for 48 hrs. At this time the production of (unlabelled) **1** had started. The cell material was removed by centrifugation and washed several times with fresh culture medium. Even then the cells showed yellowish fluorescence due to the presence of **1** at the cell surface or in the cell. Cell material from a 200 ml culture (Hohlneicher *et al.*, 1995) was suspended in a solution of 25 mg $(^{15}N_{14})$-**2** in 50 ml KH_2PO_4 buffer and 150 ml culture medium was added. The culture was shaken at 25 °C for 2 hrs, kept at 4 °C for 15 hrs and than again at 25 °C for 36 hrs. The metabolites were isolated by adsorption on XAD resin and purified by chromatography on biogel (Hohlneicher *et al.*, 1995). In one of the pyoverdin fractions besides large amounts of unlabelled **1** ($[M+2H]^{2+}$ *m/z* 581) a few percent of $(^{15}N_{14})$-**1** (*m/z* 588) could be detected. The fragmentation pattern after collision activation corresponded exactly to that of the authentic $(^{15}N_{14})$-**1**. The fact that a completely ^{15}N-labelled pyoverdin is obtained de-

[§] Part CIV of the series "Bacterial Constituents". For part CIII see Uría Fernández *et al.* (2001), for part CII Sultana R. *et al.*(2001).

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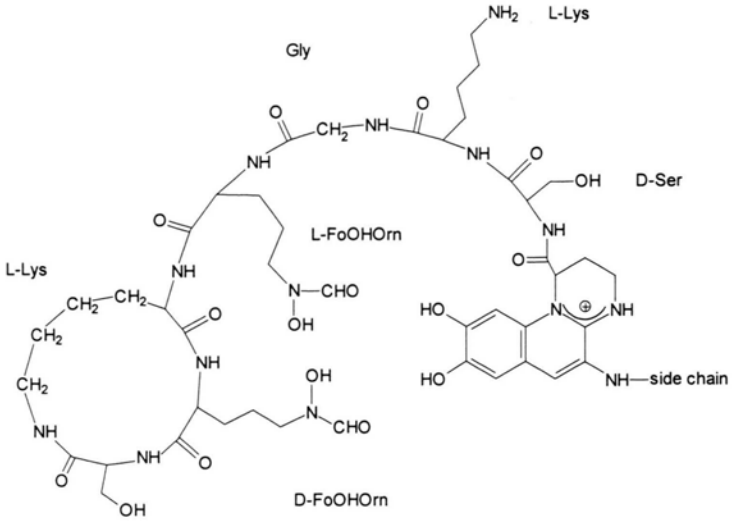

Fig. 1. Structure of the pyoverdin **1** from *Pseudomonas chlororaphis* ATCC 9446.

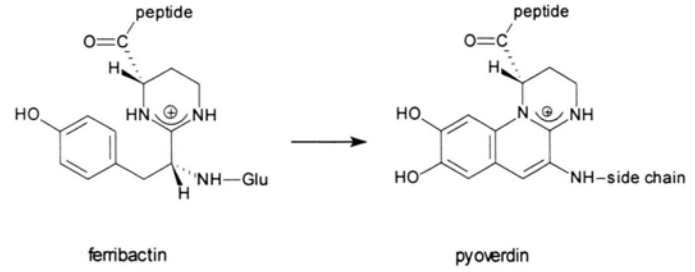

Fig. 2. Ferribactin (**2**) as the precursor of pyoverdin (**1**).

monstrates that the ferribactin is transformed *in toto* into the corresponding pyoverdin. If the ferribactin added to the bacterial culture had been degraded to the individual amino acids which in turn were used for the synthesis of the pyoverdin, only one or at best a few labelled amino acids would have been found incorporated in the pyoverdin.

A note of caution should be added here. The feeding experiments were performed in 1993 (Hohlneicher, 1993). At this time only fast atom bombardment was available as ionization technique which only allowed to determine the nominal molecular masses of components of low abundance. A mass increase of 14u is certainly indicative of the presence of fourteen ^{15}N atoms, but it could *i.a.* also be due to an additional CH_2-group or even due to the oxidation of CH_2 to CO. And indeed, in other fractions **1** with a succinic acid methyl ester side chain instead of the free carboxyl group and **1** with probably one of the N-formyl groups replaced by an N-acetyl group (cf. Kilz *et al.*, 1999) were found. It needed the electrospray ionization technique and the facilities of a tandem mass spectrometer with an ion trap as instrumental prerequisite plus the detailed study of the induced fragmentation processes of pyoverdins (Fuchs, 2000) to allow a definite identification of a compound present only with a few percent together with the unlabelled material.

Although it has not been proven definitely, there is ample evidence that the peptide chain of the pyoverdins is not formed by the ribosomal pathway, but rather by a multi-enzyme thiotemplate mechanism as demonstrated for several bacterial peptide antibiotics (see Meyer and Stintzi, 1998). These authors assume an independent formation of peptide chain, chromophore and dicarboxylic acid side chain which are linked together

towards the end of the biosynthetic sequence. The now established intermediacy of ferribactins in the biosynthesis of pyoverdins suggests that the entire pyoverdin structure is derived from a quasi-peptide chain starting N-terminally with L-Glu-D-Tyr-L-Dab. Glu is linked to Tyr by its γ-carboxyl group, but "wrong-way" incorporation of amino acids was also observed e.g. for Lys (ε- instead of the α-amino group) (Budzikiewicz *et al.*, 1999). Condensation of Dab with the preceding amino acid in the chain to give a tetrahydropyrimidine ring has been observed in several instances (listed in Kilz *et al.*, 1999). The ring closure leading from the ferribactin chromophore to the pyoverdin chromophore may than occur by a *Bucherer*-type reaction with the possible intermediacy of a sulfonic acid derivative (Böckmann *et al.*, 1997). The resulting 5,6-dihydropyoverdins are than easily dehydrogenated (e.g. by atmospheric O_2). Also the formation of isopyoverdins (Jacques *et al.*, 1995) falls into this pattern. The N-terminal Glu is transformed at a later stage via its equilibrium with α-ketoglutaric acid to the various dicarboxylic acids attached to the chromophore (Schäfer *et al.*, 1991). Starting from a single precursor which is subsequently transformed is certainly biogenetically more economic than the separate production of three parts which are then stitched together.

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