Biogenesis of the Pyoverdin Chromophore

M. Böckmann, K. Taraz and H. Budzikiewicz*

Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, D-50939 Köln, Germany

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After growing *Pseudomonas aeruginosa* in the presence of 2,4-[4-^{15}N]-diaminobutyric acid (Dab) its incorporation into the quinoline chromophore of the pyoverdin produced by this bacterium could be shown by mass and NMR-spectroscopic techniques. In combination with earlier results it can thus be stated that the precursor of the chromophore is a condensation product of L-Dab and D-Phe or D-Tyr. A synthesis for [4-^{15}N]-Dab is described.

Introduction

When growing in a surrounding lacking a sufficient quantity of soluble iron many microorganisms give off so-called siderophores, i.e. substances with a high complexing constant for Fe^{3+}. The typical siderophores of the so-called fluorescent group of the genus *Pseudomonas*, the pyoverdins (Budzikiewicz, 1993) comprise a peptide chain consisting of 6 to 12 partially modified D- and L-amino acids bound with its N-terminus to the chromophore (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2a]quinoline-1-carboxylic acid (**1**). The peptide chain is probably synthesized through a multi-enzyme thiotemplate mechanism involving peptide synthetases. They activate the constituent amino acids as their adenylates (Georges and Meyer, 1995; Menhart and Viswanatha, 1990) and are probably also responsible for the L/D-isomeration (Kreil, 1994) as had been shown for the peptide antibiotics (Kleinkauf and Döhren, 1987).

Regarding the biosynthesis of the chromophore two schemes were advanced. The first (Fig. 2) is based on the isolation of compounds accompanying the pyoverdins in the culture broth which possess the same peptide chain but differ in the chromophore, as there are ferribactins (**2**), 5,6-dihydropyoverdins (**3**) and their 7-sulfonic acids (**4**) (Schröder *et al.*, 1995). **2** comprises all structural elements for the formation of **1** by ring closure and introduction of a second hydroxyl group and also explains the formation of isopyoverdins (**5**) (Jacques *et al.*, 1995). For the ring closure a mechanism corresponding to a Bucherer reaction leading to **3** can be assumed.

Feeding experiments with isotope labelled Phe or Tyr established their incorporation into **1** for different *Pseudomonas* spp. (Maksimova *et al.*, 1992; Stintzi and Meyer, 1993; Novak-Thompson and Gould, 1994). However, 3,4-dihydroxy-Phe (DOPA) was not accepted in agreement with the presence of only one hydroxyl group in **2**. Apparently the second hydroxyl group of **1** (and the third one in the hypothetical intermediate **6** - note the formation of **7** by a genetically modified non-fluorescent *Pseudomonas aeruginosa*: Longerich *et al.*, 1993) is introduced at a later stage. Condensation of D-Tyr and L-Dab would lead directly to **2** (similar condensation products of L-Dab with other amino acids were also observed in the peptide chain of pyoverdins, (e.g., Demange *et al.*,1990; Gipp *et al.*, 1991).

Based on the observation that for non-fluorescent mutants of *Pseudomonas putida* M whose pyrimidine biosynthesis was blocked at different stages the production of the pyoverdin was restored by auxotrophic dihydroorotate, it was concluded that the latter (and, therefore, Asp rather than Dab) is a precursor of the tetrahydropyrimidine part of **1** and that the carboxyl group of Phe is lost. The reaction sequence depicted in Fig. 5 was proposed (Maksimova *et al.*, 1993; Blazhevich

Abbreviations: Common amino acids, 3-letter code; FAB-MS, fast atom bombardement mass spectrometry; TMS, tetramethylsilane; DSS, 2,2-dimethyl-5-silapentan-5-sulfonate.

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Reprint requests to Prof. Dr. H. Budzikiewicz.
Telefax: +49-221-470-5057.

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and Maksimova, 1994). In order to clarify this point [4-^{15}N]-Dab was synthesized and added to the culture medium of *Pseudomonas aeruginosa* ATCC 15692 (Briskot *et al.*, 1989; Demange *et al.*, 1990) and pyoverdin D (=PaA) (succinic acid side chain) was isolated.

Material and Methods

Spectroscopy

Mass: Finnigan MAT 900 ST (FINNIGAN, Bremen), matrix: Thioglycerol/dithioethanol.

NMR: Bruker AM 300 (BRUKER, Karlsruhe). For ^{13}C-experiments samples of 20 mg and for ^{15}N-experiments samples of 50 mg were dissolved in 0.6 ml 0.1 M phosphate buffer (pH 4.3), brought to dryness and redissolved in 0.6 ml D_2O/H_2O 1:9 (v/v). ^{13}C chemical shifts are given relatively to TMS with the internal standard DSS using the correlation δ(TMS) = δ(DSS)-1.61 ppm. For ^{15}N experiments $^{15}NH_4Cl$ was used as external standard. The chemical shifts are given in relation to $CH_3{}^{15}NO_2$ using the relation $\delta(CH_3{}^{15}NO_2) = \delta(^{15}NH_4Cl)$-352.9 ppm.

Bacterial Growth

Pseudomonas aeruginosa was grown in 250 ml culture medium in 500 ml Erlenmeyer flasks with passiv aeration, rotary shaking (100 rpm) and light. The bacteria were grown in two steps. The first 500 ml culture medium consisted of 6.5 g Na-D-gluconate, 2.0 g KH_2PO_4, 2.5 g $(NH_4)_2SO_4$ and 0.25 g $MgSO_4 \cdot 7\,H_2O$, pH adjusted to 7.1 with NaOH. After 36 hours 1.5 l culture medium containing 19.5 g Na-D-gluconate, 6 g KH_2PO_4, 0.75 g $MgSO_4 \cdot 7\,H_2O$, and 0.5 g D/L-[4-^{15}N]-Dab were added and the bacteria grown for further 48 hours. Isolation and purification is described elsewhere (Demange *et al.*, 1990).

Chemicals

For the experiments comercial chemicals of p.a. quality were used. The potassium[^{15}N]phthalimide was of 98% isotopic purity (CAMBRIDGE ISOTOPE LABORATORIES, Cambridge MA/USA).

Synthesis of 2,4-[4-^{15}N]-diaminobutyric acid dihydrochloride

Methyl 2,4-[4-^{15}N]-diphthalimidobutyrate:

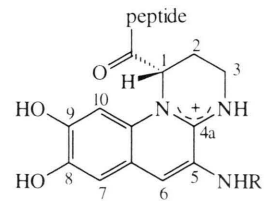
Methyl 4-bromo-2-phthalimidobutyrate (12.65 g) (synthesized according to Logusch, 1986), potassium[^{15}N]phthalimide (5 g) and dimethylformamide (80 ml) were kept at 100 °C for 24 hours. After cooling to room temperature, the reaction mixture was treated with aqueous acetic acid (0.1 M, 150 ml) and the resulting mixture was extracted with three 50 ml portions of $CHCl_3$. The $CHCl_3$ was evaporated under reduced pressure. The product was chromatographed on silicagel with ether:hexane 1:1 (v:v). In doing so the unreacted part of [^{15}N]phthalimide could be recovered. Yield 7.40 g (70%), m.p. 160 °C.

2,4-[4-^{15}N]-diaminobutyric acid dihydrochloride:

A mixture of methyl 2,4-[4-^{15}N]-diphthalimidobutyrate (7.4 g), conc. hydrochloric acid (35 ml) and glacial acetic acid (15 ml) was heated to 100 °C for 36 hours. After the reaction mixture was set aside at -18 °C overnight, phthalic acid was filtered of and the filtrate was evaporated to dryness. Absolute ethanol was added to the residue and after two days 2,4-[4-^{15}N]-diaminobutyric acid dihydrochloride crystallized from the solution. The product was collected and washed with acetone and ethanol. Yield 1.81 g (51 %). ^{15}N-NMR: -342.5 ppm (sample: 20 mg, 0.6 ml D_2O/H_2O 1:9 (v/v), pH 1.5).

Results

[^{15}N]pyoverdin D isolated from the bacteria grown with [4-^{15}N]-Dab showed in FAB mass spectrometry a prominent ion $[M+H]^+$ at *m/z* 1335 shifted by 1 u with respect to unlabelled pyoverdin D. The incorporation of labelled Dab into the



R: dicarboxylic acid side chain

1

Fig. 1. Structure of pyoverdins.

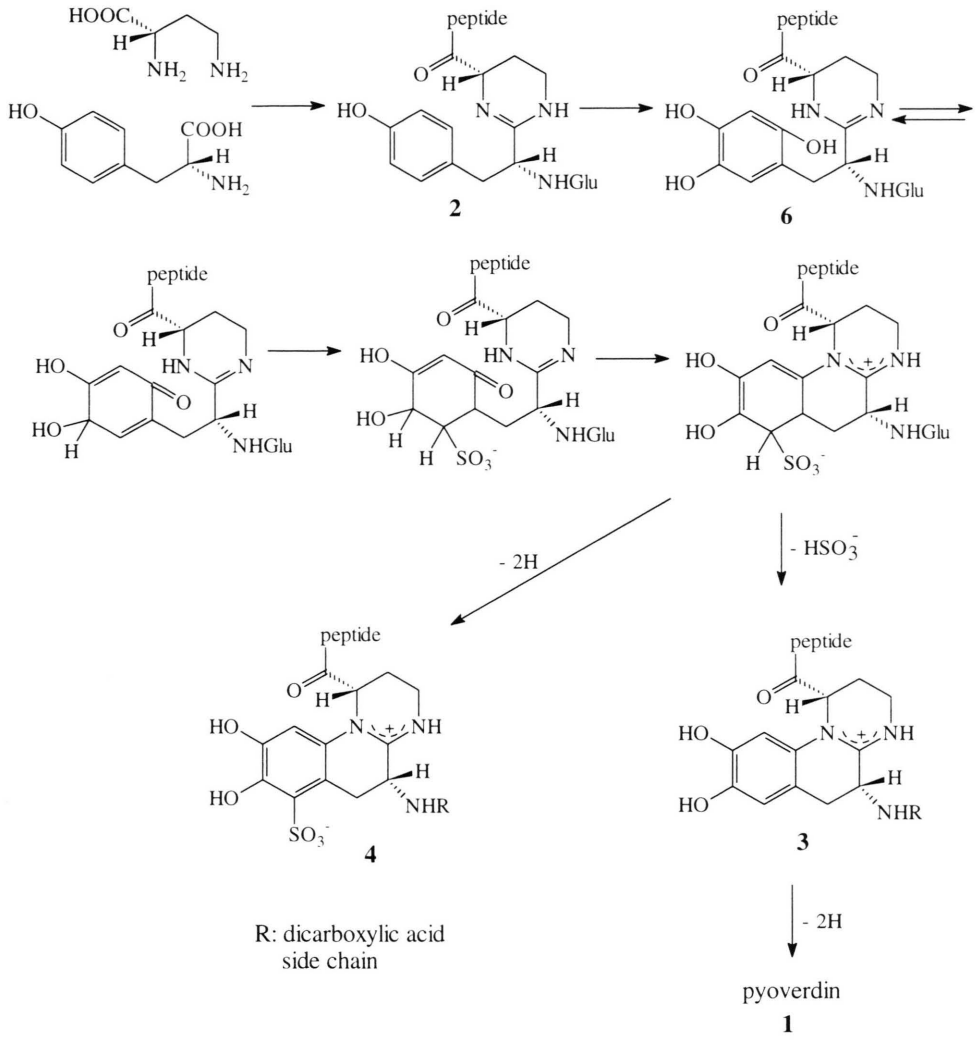


Fig. 2. Proposed biogenesis of pyoverdine starting from L-Dab and D-Tyr.

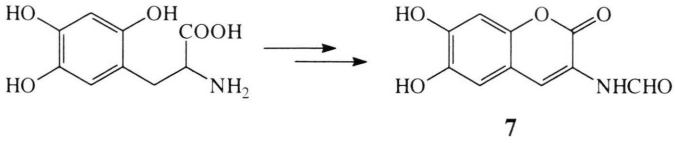


Fig. 3. Proposed biogenesis of pseudoverdin.

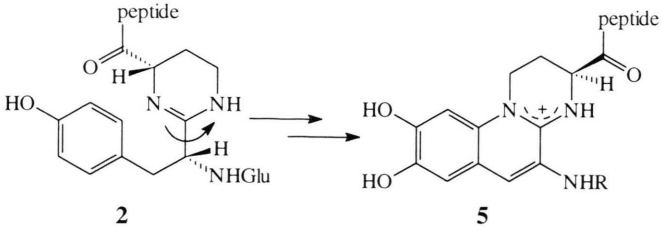


Fig. 4. Proposed biogenesis of isopyoverdins.

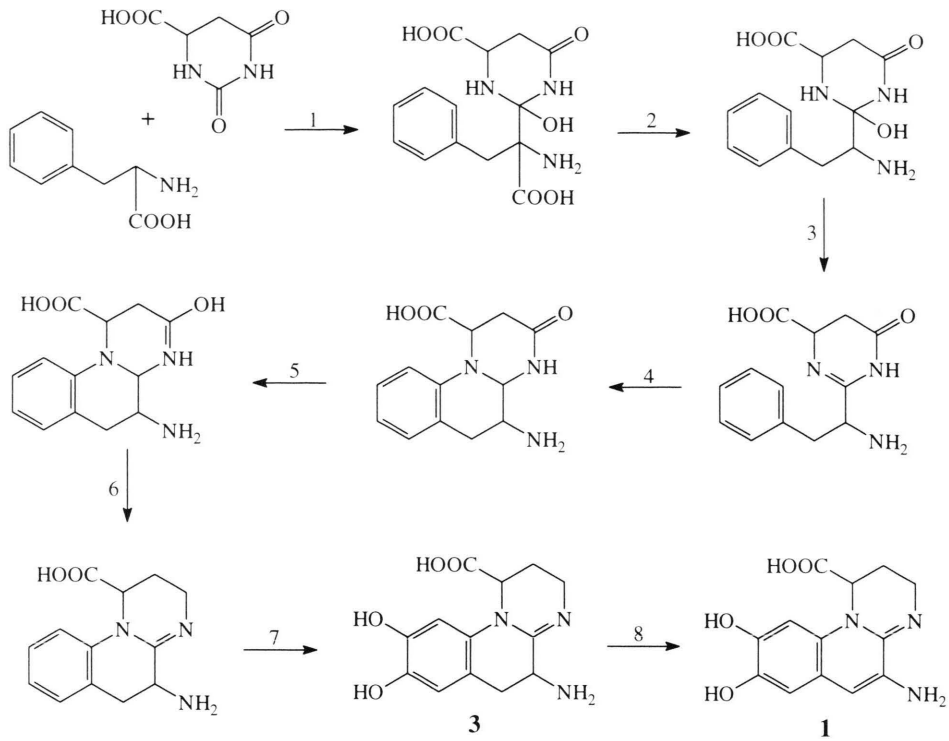


Fig. 5. Proposed biogenesis of the pyoverdin chromophore **1** starting from dihydroorotic acid and Phe.

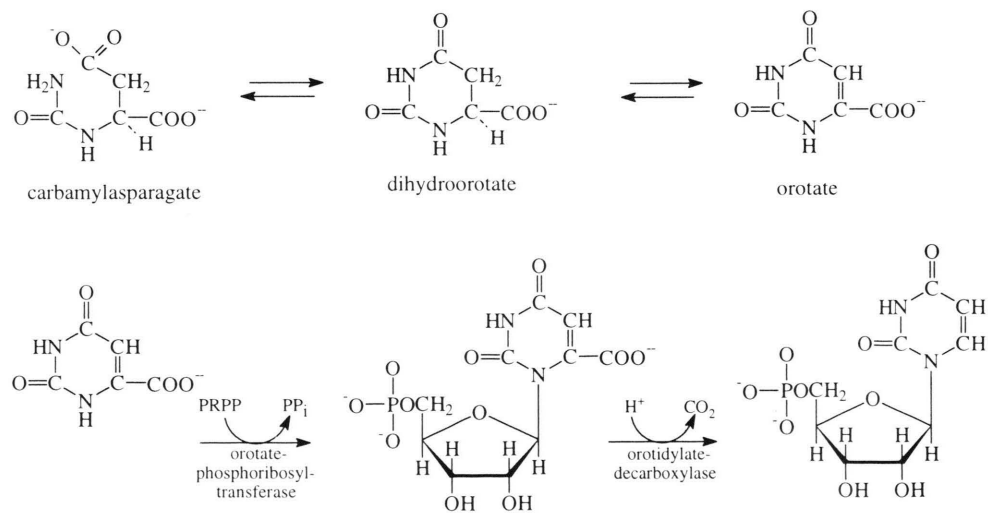


Fig. 6. Role of dihydroorotate and aspartate in the pyrimidine biosynthesis.

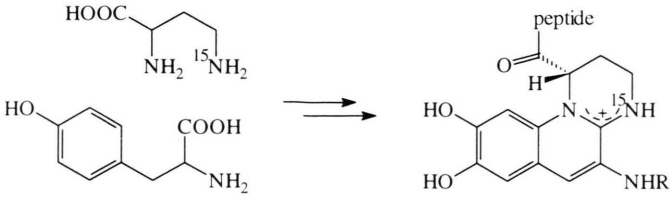


Fig. 7. [^{15}N]pyoverdin isolated after growing with [4-^{15}N]-Dab.

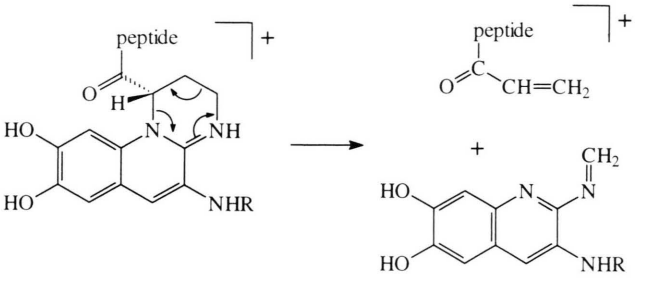


Fig. 8. retro-Diels-Alder fragmentation.

chromophore can be concluded from the observation of a retro-Diels-Alder fragment (Fig. 8) unshifted by mass at *m/z* 1031.

For the assignment of the ^{13}C-NMR signals to pyoverdin D see Briskot *et al.*, 1988. The proton decoupled ^{13}C-spectrum from [^{15}N]pyoverdin D shows ^{15}N-coupling of the signals at 36.3 ppm (C-3), 150.5 ppm (C-4a) and 118.5 ppm (C-5). The $^{1}J(^{13}C,^{15}N)$ coupling constants amount to 8 Hz (C-3) and 20 Hz (C-4a), the $^{2}J(^{13}C,^{15}N)$ coupling constant to 8 Hz (C-5). This observation excluds an incorporation of ^{15}N at a locus other than N-4 of the chromophore.

The described couplings are in agreement with only one signal at -281.2 ppm observed in the proton decoupled ^{15}N-NMR-spectrum. The assignment of this signal to N-4 could be verified by comparison with literature data (Gipp *et al.*, 1991).

Discussion

As mentioned in the introduction two hypotheses were advanced regarding the biosynthesis of the pyoverdin chromophore **1**. Both of them can explain the intermediacy of ferribactins (**2**) and dihydropyoverdins (**3**) as well as the formation of isopyoverdins (**5**), though the mechanism proposed by the Minsk group for the cyclization step would have to be modified (they assume that steps 4 and 5 in Scheme 2 are not enzyme-catalyzed and take place spontaneously; this would require a nucleophilic attack of the imine nitrogen on the benzene ring and a hydride shift, reactions not in agreement with general rules of aromatic substitution). As far as the precursor atoms incorporated into **1** are concerned the two mechanisms (Fig. 2 and 5) lead to the following differing results (Table I).

Table I. Differences in the origin of various atoms of **1** according to the biogenetic Schemes 1 and 2.

Atom of **1**	Scheme 1	Scheme 2
COOH, C-1, C-2, C-3	Dab	Asp
C-4a	COOH of Phe (Tyr)	carbamyl CO of N-carbamyl Asp
N-4	γ-NH_2 of Dab	carbamyl NH_2 of N-carbamyl Asp
N-10b	α-NH_2 of Dab	α-NH_2 of Asp

Labelling experiments so far performed with Phe and Tyr do not allow to distinguish between the two Schemes as only C-2 and C-3 (but not the carboxyl group C-1) had been marked specifically (the label from both [3-^{14}C]-Tyr (Novak-Thompson and Gould, 1994) and from [2-^{14}C]-Phe (Maksimova *et al.*, 1992) was found to be incorporated into the respective pyoverdins; 1 D from [2,3,3-$^{2}H_3$]-Tyr could be located at the C-6 of **1** (Novak-Thompson and Gould, 1994). For the present feeding experiment [4-^{15}N]-Dab was chosen for the following reasons: Even in the case dihydroorotic acid being one of the precursors of **1** the carboxyl group of Phe could be inserted into the urea portion of the latter. So labelling of C-1 of Phe would

not be conclusive. Using [2-^{15}N]-Dab might have been misleading in a different way: Dab by transamination and oxidation conceivably can be transformed into Asp, the precursor of dihydroorotic acid (Fig. 6). The incorporation of [4-^{15}N]-Dab conclusively proves that Dab is one of the precursors of **1**.

If one does not accept that the biosynthesis of **1** in different species of the closely related group of the fluorescent pseudomonads (Palleroni, 1984; Palleroni, 1992) follows alternative pathways, the results of the Minsk group, *viz.* that for non-fluorescent mutants of *Pseudomonas putida* M the pyoverdin production could be restored by auxotrophic dihydroorotate, must be explained in a different way. Dihydroorotic acid is the precursor of the pyrimidine nucleotides and its auxotrophic effect may, therefore, lie on a completely different level.

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