Ferribactins – the Biogenetic Precursors of Pyoverdins [1]

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Z. Naturforsch. **46c,** 527–533 (1991); received February 28, 1991

Pyoverdins, Ferribactins, Biogenesis, NMR Analysis

From ferribactins which accompany pyoverdins in iron deficient cultures of fluorescent pseudomonads a subunit has been obtained formed by condensation of D-tyrosin with L-2,4-diaminobutyric acid to give 2-(1-*R*-amino-2-*p*-hydroxyphenylethyl)-1,4,5,6-tetrahydropyrimidine-4-*S*-carboxylic acid (**1**). Evidence is presented that **1** is the precursor of the typical pyoverdin chromophore **7a.**

Introduction

The so-called fluorescent group of *Pseudomonas* produces peptidic siderophores when grown in an iron deficient medium. They all contain (1*S*)-5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido-[1,2a]-quinoline-1-carboxylic acid (**7a**) or its 5,6-dihydro derivative as a chromophore. The 5-amino group is bound amidically to a small dicarboxylic acid (a member of the citric acid cycle) while the carboxylic group is linked to a peptide residue (not necessarily *via* its N-terminus). Nothing is known so far about the biogenesis of the chromophore. In 1967 Maurer *et al.* [2] isolated a peptidic iron complexing pigment from cultures of *Pseudomonas fluorescens* ATCC 13525 which they named ferribactin and which lacked the typical pyoverdin chromophore. The amino acid composition determined by Maurer [2] was corrected in 1981 by Philson and Llinas [3, 4] to be

> 1 D-Tyr, 1 L-Glu, 1 Gly, 1 D-Ser, 1 L-Ser, 1 N^5-formyl-N^5-hydroxy-D-Orn, 1 N^5-formyl-N^5-hydroxy-L-Orn and 2 L-Lys.

The *Pseudomonas* strain produces also a pyoverdin which except for Tyr and Glu possesses the same amino acid composition as ferribactin. It was suggested that ferribactin might be a precursor [3–5] or a degradation product [6] of the pyoverdin or an independent siderophore [7]. In the meantime in two strains of *Pseudomonas aptata* we discovered analogous pairs of pyoverdins and ferribactins. Thus it seemed worthwhile to investigate the relationship of these two classes of compounds somewhat closer.

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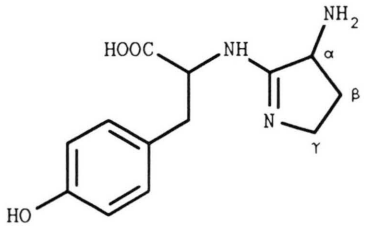
Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0700–0527 $ 01.30/0

Results and Discussion

From cultures of *Pseudomonas fluorescens* ATCC 13525 ferribactin was re-isolated by procedures we commonly apply to the isolation of pyoverdins [8]. The compound thus obtained matches all the data reported for ferribactin including the amino acid composition with one exception: After hydrolysis of deferri-ferribactin with 6 N HCl (21 h, 110 °C) and GC/MS analysis of the N/O-trifluoroacetyl *n*-butyl ester (TAB) derivatives only traces of Tyr were found, but a component with an M^+ *m/z* 589 was formed. This species disappeared when the crude acid hydrolysis product was treated with 1 N NaOH (15 h, 110 °C). Instead, larger amounts of Tyr and in addition 2,4-diaminobutyric acid (Dab) – which neither had been found by Maurer [2] or by Philson [3, 4] – could now be detected. Apparently Tyr and Dab (the configuration of which could be determined as L in agreement with the *S*-configuration of the chromophore **7a,** v. infra) form an acid-stable subunit of ferribactin. This subunit (Tyr/Dab) proved to be the crucial part of all ferribactins and makes evident, as will be shown, that ferribactins are actually the precursors of the corresponding pyoverdins. This communication discusses the structure elucidation of Tyr/Dab; the complete structures of the various ferribactins will be reported elsewhere.

A small amount (1.6 mg) of Tyr/Dab could be isolated from the HCl hydrolysate of deferri-ferri-

bactin by sample collection following analytical HPLC. FAB-MS established an $[M + H]^+$ m/z 264 which corresponds to a dipeptide consisting of Tyr and Dab minus one H_2O. Tyr/Dab possesses a free carboxyl group as shown by the formation of monoesters (*i*-Pr, *n*-Bu) and loss of the respective



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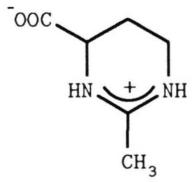

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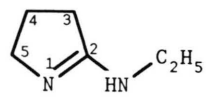

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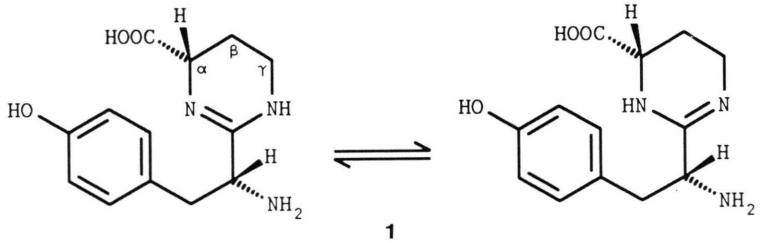

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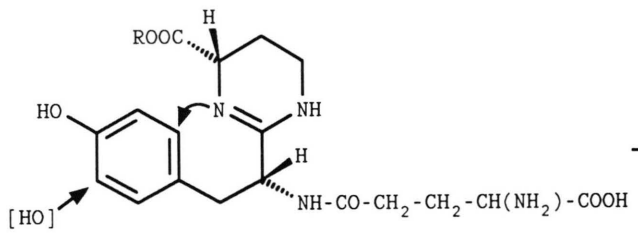

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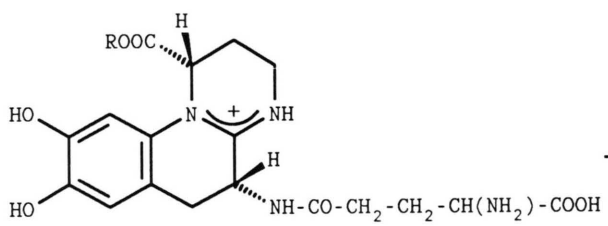

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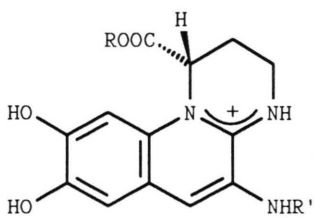

7a: R = OH, R' = H
7b: R = peptidchain, R' = acid residue

'COOR in their EI mass spectra. Three acyl groups can be introduced, one of which occupies the phenolic OH ($X-O-C_6H_4-CH_2^+$, *m/z* 203 for trifluoroacetyl and *m/z* 253 for pentafluoropropionyl). Hence H_2O cannot have been lost by formation of a second amide bond. An alternative is the formation of an amidine structure by condensation of the free amino group of Dab with the amide carbonyl of the dipeptide. Starting from Tyr-Dab structure **1,** and starting from Dab-Tyr structure **2** is obtained.

It should be possible to distinguish between **1** and **2** by NMR spectroscopy. The ^1H and ^{13}C data of Tyr/Dab are compiled in Tables I and II. The shifts and multiplicities of the Tyr part correspond to values reported for Tyr random-coil peptides [9]. The values of the H and C atoms of the Dab part agree well with those obtained for ectoin (**3**) [10, 11]. An equally suitable model compound for **2** could not be obtained, but for comparison purposes the data for **4** are given in Table III.

At least to the extent a comparison is possible (**4** lacks an NH_2 group at C-3) the differences in the chemical shifts and the $^1J_{CH}$ coupling constants from those observed for Tyr/Dab are obvious. Correlation of the ^1H-resonances of Tyr/Dab was achieved by a HOHAHA experiment [12] in DMSO-d_6 solution at 25 °C (Fig. 1). The proton of the amidine system is apparently located on the N neighboring the γ-CH_2 group of Dab because only a cross peak with one of the γ-CH_2 signals was observed. In contrast, the ^1H NMR spectrum of the deferri-ferribactin *C* (v. infra) in H_2O/D_2O (9:1, pH 5) shows two broad signals at 9.50 und 9.60 ppm which could be attributed to the amidine system (γ-NH and α-NH, resp.) by HOHAHA and NOESY at 5 °C using the jump-and-return method [13] for water suppression. These amidine protons exchange rapidly with water as evidenced by the significant broadening of the signals. Probably the following equilibrium exists:

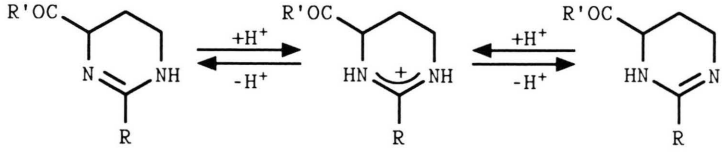

Table I. ^1H NMR data[1].

		3	**1** (a)	(b)	*A*	*B*	*C*[4]
Dab	α	4.09	4.24[2]	4.37		4.3[3]	4.35
	β	2.11	2.04[2]	1.97	2.1[2,3]	2.1[2,3]	2.00/2.20
	γ	3.28/3.42	3.31[2]	3.20/3.35	3.3[2,3]	3.38[2]	3.25/3.35
	NH_α		9.50				9.60[5]
	NH_γ		9.60	9.37			9.50[5]
Tyr	α		4.38	4.02	4.72	4.7[2,3]	4.65
	β		3.19	2.85/2.97	3.19	3.12[3]	3.07/3.18
	2, 6		7.18	7.03	7.16	7.18	7.18
	3, 5		6.90	6.70	6.88	6.87	6.88
	NH						8.61
Glu	α			3.67[2]		3.7[2,3]	3.72
	β			2.3/2.13[2]		2.1[2,3]	2.07
	γ			2.48[2]		2.49[2]	2.44/2.50

[1] D_2O; **3,** *A* and *B* buffered pH 3, *C* pH 5, **1** in unbuffered D_2O (a) and in DMSO-d_6 (b); chemical shifts relative to DSS.
[2] Connectivity established by double resonance experiments.
[3] Embedded in a peak group.
[4] Identified by HOHAHA (25 °C).
[5] Identified by HOHAHA (5 °C).

Table II. ^{13}C NMR data[1].

		3[3]	**1**(a)	(b)	*A*[2]	*B*	*C*[3,5,6]
Dbu	CO	177.4, s	174.4	170.7		172.0	171.2, s[4]
	α	53.9, d, 145	53.4	50.3		51.2	52.2, d, 149
	β	22.3, t, 133	21.3	20.4		22.4	21.8, t, 134
	γ	38.2, t, 143	38.6	36.7		37.5	37.5, t, 145
	N–C=N	161.6, s	159.2	158.2		164.5	164.5, s[4]
Tyr	α		54.4	52.9	56.9	54.7	55.0, d[4]
	β		36.7	38.5	38.5	37.7	37.4, t, 133[4]
	1		125.1	126.5	127.1	127.5	127.3, s[4]
	2, 6		131.8	130.7	131.7	131.8	131.8, d, 158[4]
	3, 5		117.2	115.3	116.7	116.8	116.8, d, 159[4]
	4		156.6	156.6	156.1	156.1	156.1, s[4]
Glu	1-CO				176.4	175.0	175.1, s[4]
	α					54.9	54.9, d, 145[4]
	β					27.4	26.9, t, 131[4]
	γ					32.0	31.7, t, 129[4]
	5-CO					176.6	176.7, s[4]

[1] D_2O; **3**, *A* and *B* buffered pH 3, *C* pH 5, **1** in unbuffered D_2O (a) and in d_6-DMSO (b); chemical shifts relative to TMS (δ(TMS) = δ(DSS) − 1,61 ppm).
[2] Because of the small amount of sample available not all signals could be identified unambiguously.
[3] Multiplicity and $^1J_{CH}$ determined by gated-decoupled ^{13}C NMR.
[4] Identification by reverse ^1H, ^{13}C long range correlation.
[5] *C* possesses a second tetrahydropyridine system in the peptide chain. The set of lower values has been presented here arbitrarily. The second one consists of 22.1; 37.5; 52.5; 165.1; 172.5 ppm.
[6] Shifts obtained at pH 3 differ by 0.3 ppm or less.

Table III. NMR data of **4** in D_2O.

	C-2	-3	-4	-5	-1′	-2′	
^1H	–	2.55	1.99	3.52	3.19	1.15	ppm
^{13}C	171.2	32.9	23.3	53.5	38.7	14.6	ppm
$^1J_{CH}$	–	130	130	139	138	127	Hz

Also cross peaks (**1**, DMSO-d_6) were observed which allowed to connect the resonances attributed to the butyric acid side chain in agreement with double resonance experiments (D_2O) which upon irradiation at 2.04 ppm resulted in a simplification of the signals at 3.31 and 4.24 ppm. From a comparison with **3** as well as with literature data [15] the ^{13}C-signal in the region of 160–165 ppm has to be attributed to the amidine-C. The ^{13}C-resonances of Tyr/Dab were identified by reverse heteronuclear ^1H, ^{13}C-correlation experiments in DMSO-d_6 at 45 °C using one-, two- and three-

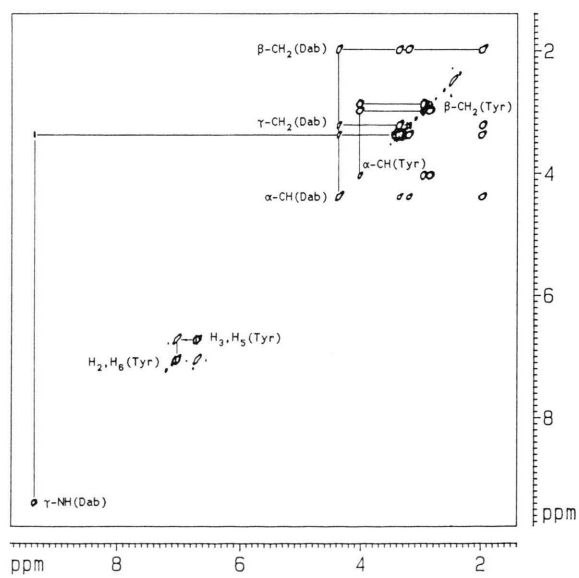

Fig. 1. 500 MHz 2D-HOHAHA spectrum of **1** in DMSO-d_6 (25 °C).

bond scalar coupling. The main observation was that the CO-signal at 172 ppm was shown to correspond to the Dab part of Tyr/Dab (cf. [14]). However, because of the short relaxation time ($T_2 = 24$ ms) of Tyr-H_α no crosspeak could be observed between this proton and the amidine-C. Further information came from an NMR investigation of the intact deferri-ferribactins from *Pseudomonas fluorescens* ATCC 13525 (*A*), *Pseudomonas aptata 4b* (*B*) and especially *Pseudomonas aptata 3b* (*C*) for which the most complete set of experiments is available and to which the subsequent discussion refers. For comparison purposes the pertinent data have been compiled in Table I and II.

The unambiguous identification of the various H resonances allowed the crucial experiment to differentiate between **1** and **2**: In an inverse heteronuclear ^1H, ^{13}C shift correlation experiment [16] for *C* in D_2O the 2D-map clearly shows $^2J_{CH}$ coupling of the amidine-C at 164.5 ppm with the H_α (4.65 ppm) and $^3J_{CH}$ coupling with the H_β protons (3.07 and 3.18 ppm) for Tyr, while the H_α of Dab at 4.35 ppm couples with the CO-signal at 171.2 ppm. If structure **2** were correct one would have observed $^2J_{CH}$ coupling of the amidine-C with the α- and $^3J_{CH}$ coupling with the H_β of Dab. These experiments allow an unambiguous decision in favor of **1.** In a recent publication [17] analogous condensation products of Dab and Ser or Gln have been postulated to form parts of the peptide chains of two pyoverdins from *Pseudomonas fluorescens* strains. The basis for the structure assignments analogous to **1** are the masses as determined by FAB-MS and ^1H and ^{13}C NMR data "very close to these of Dab, Ser and Gln, resp.". Alternative structures analogous to **2** have not been considered and – as it just has been shown – could not have been excluded by the data presented. Unless much more comparison material becomes available in future the crucial information will always have to come from a heteronuclear correlation of the amidine carbon with the protons of the attached amino acid.

For all three ferribactins *A*, *B* and *C* L-glutamic acid is bound amidically by its 5-carboxyl group to the amino group of **1** giving **5.** This has been shown, *e.g.*, for *C* by 2D-NOESY (5 °C). The observation of a cross peak of the Tyr-NH signal and the Glu-H_γ signals (identified by 2D-HOHAHA, v. supra) together with the absence of a NOE for Tyr-NH with Glu-H_α proved the linkage *via* the 5-COOH group. This conclusion is confirmed by the absence of a pH influence on the shifts of Glu-H_γ and Glu-C-5. The free amino group of Glu has been confirmed by isolation and identification of 2-dansylamino-glutaric acid after dansylation and HCl-hydrolysis as described earlier [18–20].

1 comprises all the structural elements necessary for the formation of the pyoverdin chromophor **7a:** Ring closure between N-3 and the benzene ring yields a dihydroquinoline skeleton. Introduction of the second hydroxyl group to form the catechol unit (**6**) seems to occur at this stage since only dihydropyoverdins with both hydroxyl groups have been found to accompany the pyoverdins. Glutamic acid forms a transamination equilibrium with α-ketoglutaric acid which by ways of the citric acid cycle leads to the other dicarboxylic acids encountered as bound to the pyoverdin chromophore [21]. **5** seems to be a separate building block common to all fluorescent pseudomonads to which the strain-specific peptide residue is attached subsequently by peptide, amide or ester bonds (**7b**).

Experimental

The general procedures for the bacterial cultures, the isolation and purification of the siderophores, the formation of the desferri compounds, the amino acid analysis and the determination of their configurations, the dansylation procedure etc. may be found in refs. [8, 18–20]. Further details will be presented in connection with the report of the complete structure of the various ferribactins.

Isolation of **1**

Portions of 1 mg of deferri-ferribaction *A* were dissolved in 1 ml 6 N HCl and hydrolysed at 110 °C for 21 h. The reaction product was evaporated to dryness i.v. at 60 °C, redissolved in H_2O and brought to dryness three times in order to remove all HCl. Pure **1** was obtained by repeated HPLC on Polygosil 60-C 18 (7 μ) with 6% CH_3OH and 0.1% trifluoroacetic acid in H_2O for the separation, 3% CH_3OH and 0.1% trifluoroacetic acid for the purification.

Alkaline hydrolysis of A

The dry residue obtained after acid hydrolysis of *A* was dissolved in 1 ml 1 N NaOH and heated to 110 °C for 15 h. After cooling to room temperature 1 ml 6 N HCl was added to the solution and an excess of HCl was removed as described above. The free amino acids were transformed into their TAB derivatives and subjected to GC/MS analysis.

Configuration of Tyr and Dab

Since alkaline hydrolysis causes racemization the three deferri-ferribactins were hydrolysed with 6 N HI at 110 °C for 144 h. The mixture of amino acids was transformed into their N/O-trifluoroacetyl isopropyl esters and subjected to gas-chromatography on a Chirasil-Val-L column using D- and L-standards for comparison [19, 20].

Mass spectrometry

GC-MS: Kratos MS 25 RF with a Carlo Erba HRGC MFC 500, capillary column SE-54; FAB-MS: Finnigan-MAT HSQ-30, Xe, Matrix thioglycerol.

NMR spectroscopy

1 D NMR experiments were performed with a Bruker AM 300, all 2 D experiments with a Bruker AMX 500 spectrometer. Data processing was achieved with an Aspect X32 computer using UXNMR software.

The measurements were performed with 10 mM solutions in DMSO-d_6 or phosphate buffered D_2O or H_2O/D_2O 9:1 (pH 3 and pH 5) at 5, 25 or 45 °C. The H_2O-resonance was suppressed using the jump-and-return method [13] or by presaturation during the relaxation delay.

NOESY and MLEV 17 HOHAHA data were acquired in the phase sensitive mode using the time proportional phase incrementation scheme (TPPI) [22]. The spectral width in F_1 and F_2 was 5150 Hz. 512 experiments with 8 or 16 scans of 1024 complex data points in t_2 were collected. The time domain data were zero filled in F_1. Resolution enhancement was obtained by apodization of the time domain data with a $\pi/3$ shifted squared sine bell.

In HOHAHA-experiments the mixing time was 40 ms. For NOESY a mixing time of 200 ms yielded the best results.

Reverse ^1H, ^{13}C-correlation data were acquired with a proton spectral width of 5150 Hz and a carbon spectral width of 25,000 Hz as described by Bax *et al.* [23] and Bax and Summers [16]. The delay for the evolution of long range couplings was 80 ms. For the observation of $^1J_{CH}$-couplings a delay of 3.33 ms was used. 256 (**1**) or 1024 (for *C*, in order to increase resolution in F_1) experiments with 16 scans of 1024 complex points were accumulated. The time domain data were zero filled in F_1. Resolution enhancement in F_1 and F_2 was obtained by apodization of the time domain data with $\pi/2$ shifted sine or squared sine bell.

Acknowledgements

We wish to thank Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie for financial assistance.

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