Structure and Characterization of Isopyoverdin from *Pseudomonas putida* BTP1 and Its Relation to the Biogenetic Pathway Leading to Pyoverdins*

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Pyoverdin type siderophores produced by six fluorescent *Pseudomonas* strains isolated from different rhizospheres were purified and characterized. The purified ferri-pyoverdins were tested for their ability to promote the growth of other strains grown under iron deficiency conditions. Only the one obtained from *Pseudomonas putida* BTP1 did not act as a growth promoter.

The structure of the BTP1 siderophore was elucidated by spectroscopic methods and degradation studies. It turned out that it contains a chromophore which differs from the one typical for pyoverdins insofar as it carries the carboxyl group in 3- rather than in 1-position ((3*S*)-5-amino-1,2-dihydro-8,9-dihydroxy-3H-pyrimido[1,2a]quinoline-3-carboxylic acid). The amino group of the chromophore is substituted with the 5-carboxyl group of L-glutamic acid and its carboxyl group with the N-terminus of the peptide L-Asp–L-Ala–L-Asp–D-N^5–Ac–N^5–OH–Orn–L-Ser–L-c–N^5–OH–Orn. This isopyoverdin fits into the biogenetic scheme which postulates ferribactins as the precursors of pyoverdins.

Introduction

Although iron is one of the most abundant elements on earth, its bioavailability is very low under aerobic conditions due to the low dissociation constant of the various oxide hydrates of Fe^{3+} ($K_L \approx 10^{-37}$ mol$^4 \cdot$l^{-4} for $Fe(OH)_3$; Crighton, 1991). To cope with this limited supply of soluble iron in the soil most aerobic and facultatively anaerobic microorganisms produce iron chelating compounds called siderophores (Neilands and Leong, 1986) which are transferred into the cell by specific membrane receptors (Leong *et al.*, 1991). Fluorescent *Pseudomonas* spp. produce different types of siderophores (Budzikiewicz, 1993). The most common ones are fluorescent yellow-green water-soluble pigments called pyoverdins. They consist of the chromophore (1*S*)-5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido[1,2a]quinoline-1-carboxylic acid (**1a**) substituted at the amino group with a dicarboxylic acid from the citric acid cycle and at the carboxyl group with the N-terminus of a small peptide. The catechol group of the chromophore is one of the binding sites for Fe^{3+}, the other two are hydroxamic or α-hydroxy acids derived from Orn and/or threo–β-OH–Asp. More than 20 pyoverdins are described in the literature (Budzikiewicz, 1993). As probable precursors of the pyoverdins ferribactins (Budzikiewicz *et al.*, 1992; Hohlneicher *et al.*, 1992; Taraz *et al.*, 1991) were also identified in the culture broths of fluorescent *Pseudomonas* spp. which can chelate Fe^{3+} as well. Competition for iron with the help of the siderophores is one of the most cited reason for the prevalence of fluorescent *Pseudomonas* spp. in the soil (O'Sullivan and O'Gara, 1992; Jacques *et al.*, 1993). Two traits of the fluorescent *Pseudomonas* spp. contribute to their efficiency in the competition for iron, *viz.* the high affinity for Fe^{3+} of their siderophores and their ability to transport exogenous chelators into the cell (Leong *et al.*, 1991). Pyoverdin type compounds produced

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by six strains isolated from different rhizospheres were purified and characterized. The ability of the ferri-pyoverdins to stimulate the growth of other strains was determined. In this way one siderophore (from *Pseudomonas putida* BTP1) was singled out which differed in several respects from the other ones. Its complete structure was determined and its special properties will be discussed in view of its structure.

Experimental Procedures

Strains

The strains BTP1, 2, 7, 9, 14 and 16 were isolated by the Phytopathology Dept., Agricultural Fac. of Gembloux (Belgium) from the rhizospheres of barley (BTP1) and tomatoes (BTP2, 7 and 9) and from compost by using barley roots as a trap (BTP14 and 16). BTP1, 14 and 16 are strains of *Pseudomonas putida,* BTP2, 9 and 17 of *Ps. fluorescens.* They are deposited in the bacteria collection of the Centre Wallon de Biologie Industrielle, Liège, Belgium.

Culture conditions

For the siderophore production cultures were grown at 30 °C in casamino acids (CAA: casamino acids 5, $MgSO_4 \cdot 7H_2O$ 0.25, K_2HPO_4 0.9 g/l) or in a succinate medium (succinic acid 4, $(NH_4)_2SO_4$ 1, $MgSO_4 \cdot 7H_2O$ 0.2, KH_2PO_4 3, K_2HPO_4 6 g/l, pH adjusted to 7.0). The cultures were grown in 1 l shaked flasks (150 rpm) or in 2 or 20 l fermentors (with pH regulation to 7.0). The aeration rate was 1 vvm and the stirring rate 250 rpm. The cultures were stopped after 48 (flask cultures) or 24 h (fermentors). The bacterial growth was estimated turbidimetrically at 540 nm, 1 unit of optical density corresponding to 5×10^8 C.F.U. The biomass of the strains investigated lay between 11 and 14×10^8 C.F.U./ml after 48 h in flask cultures. For determining the siderophore concentration the bacteria were removed by centrifugation and by measuring the absorbance of the supernatant liquid at 400 nm for BTP1 (ε = 27,000 $cm^2 \cdot mmol^{-1}$, *v. infra*) a concentration of 31.8 μmol/l (34 mg/l) was thus determined. Assuming similar ε values for the other pyoverdins their yields range from 17 to 140 μmol/l.

Siderophore purification

Ferric citrate was added to the cultures in order to avoid a degradation of the siderophores since their iron complexes are more stable. Then two different techniques were used for the purification: (a) The cells were removed by centrifugation (11,400×*g*, 20 min) and the supernatant liquid was subjected to ultrafiltration through a membrane (Amicon cell, Sartorius, Göttingen) with a cut-off at 5000 Da and subsequently through a membrane with a cut-off at 1000 Da. This "semi-purified ferri-pyoverdin solution" was then brought on a preparative RPC column HR 5/5 (Pharmacia, Uppsala) and the siderophores were eluted with an acetonitrile gradient (0 to 70%) with 0.1% trifluoroacetic acid. (b) This technique was only used for the purification of larger amounts of the BTP1 siderophore. The cells were removed by centrifugation (4950×*g*, 30 min). The supernatant liquid was freeze-dried, the resulting powder was suspended in water and brought onto a XAD-4 column. The ferri-siderophore was eluted with 50% methanol. The solution was brought to dryness, dissolved in a 0.2 molar pyridine/acetic acid buffer (pH 5.2) and brought onto a Bio-Gel P-2 column (l = 22, d = 7 cm). The fraction containing the ferri-siderophore was rechromatographed on a DEAE Sephadex A-25 column with the same pyridine/acetic acid buffer. For the elution a pyridine gradient (0.02 to 1.0 M) was used. In both cases the solutions containing the siderophores were brought to dryness, the residue was suspended in a 1% citrate solution and decomplexed with a 5% solution of 8-hydroxyquinoline in chloroform. The extraction of Fe^{3+} was repeated at least three times, the aqueous phase was then brought to dryness and for further purification chromatographed on a Bio-Gel P-2 column (l = 52, d = 3.2 cm) with a 0.1 M acetate buffer.

Growth promotion experiments

The bacteria were grown under stirring at 30 °C for 24 h in 100 ml flasks containing 50 ml succinate or CAA medium. 2 ml portions of the cell suspensions (optical density at 540 nm 0.3 to 0.5) were spread on petri plates containing 20 ml succinate or CAA medium solidified with 1.5% agar containing 100 mg/l ethylene diamine di(*o*-hydroxyphenyl acetic acid) (EDDHA), a potent Fe^{3+} che-

lator. In holes (d = 5 mm) in the center of the plates 10 µl of "semi-purified ferri-pyoverdin solutions" (1.5 mmol) were deposited. The plates were incubated at 30 °C for 8 h and then checked for the bacterial growth around the holes.

Amino acid analysis of the BTP1 siderophore

0.5 mg of the purified siderophore were hydrolyzed with 6 N HCl or 6 N HI for 24 h at 110 °C. The amino acids were transformed into their N/O-trifluoroacetyl isopropyl esters (TAP derivatives) by treatment with 2-propanol and trifluoroacetic acid anhydride and analyzed by GC/MS with a Kratos MS 25RF mass spectrometer with a CARLO ERBA HRGC MFC 500 gas chromatograph (capillary column SE-54, Chrompack, l = 25 m, d = 0.25 mm, 0.32 µm film thickness). The absolute configuration of the amino acids was determined by GC (CARLO ERBA HRGC 4160 gas chromatograph, FID detector, integrator Shimadzu C-R3A) on a Chirasil L-Val column (Chrompack, l = 25 m, d = 0.25 mm, 0.12 µm film thickness).

Peptide sequence of the BTP1 siderophore

2 mg of the purified siderophores were submitted to a partial hydrolysis (6 N HCl, 90 °C, 5 and 10 min). The resulting mixture of peptides was analyzed by FAB-MS. When necessary peptide fragments were purified by RP-HPLC (Polygosil 60-C18, acetonitrile gradient 0 to 100% with 0.2% trifluoroacetic acid) and their amino acid compositions were determined as described above.

Purification of the chromophore of the BTP1 siderophore

300 mg of the purified siderophore were hydrolyzed with 3 N HCl for 7 days at 110 °C. The chromophore was isolated by RP-HPLC (Polygosil 60-C18, isocratic elution with water/methanol/trifluoroacetic acid 800/200/1).

FAB-mass spectrometry

Fast atom bombardment mass spectroscopic measurements were performed with a MAT HSQ 30 or a Varian MAT 731 instrument equipped with a FAB gun (Ion-Tech Ltd., Teddington, U.K.). FAB gas Xe, matrix thioglycerol. The siderophores were dissolved in water, the isolated chromophore in dimethylsulfoxide.

Isoelectrofocussing

Samples of the pure siderophore (5 nmol in 10 µl water) were deposited on an Ampholine PAG plate (Pharmacia, Uppsala), pH 3.5 to 9.5. Isoelectrofocussing was performed with a Multiphor II Electrophoresis system equipped with a Multidrive XL (Pharmacia). The starting conditions were: 300 V, 50 A, 30 W. The electrophoresis was stopped after 75 min when the current was stabilized at 10 A.

NMR analyses

For NMR spectroscopy samples of the BTP1 siderophore were dissolved in a D_2O phosphate buffer solution (pH 4.0 and 6.8). ^1H and ^{13}C measurements as well as correlation experiments (COSY, TOCSY, ROESY, HMQC, DEPT-HMQC, HMBC) were performed with a Bruker AMX 500 instrument with H_2O as reference. The chromophore **2b** was dissolved in DMSO-d_6 and spectra (^1H, homonuclear decoupling, NOE experiments, ^{13}C, H,C and H,C-long range correlations) were obtained with a Bruker AM 300 instrument.

Results

Siderophore production and growth promotion ability

Six strains of fluorescent *Pseudomonas* spp. isolated from different rhizospheres (BTP1, 2, 7, 9, 14 and 16) were cultivated in a succinate medium. The biomass and siderophore production was evaluated for each strain by measuring the optical density at 540 nm and after centrifugation at 400 nm *(v. supra)*. Small quantities of each siderophore were purified by using ultrafiltration/reversed phase chromatography. Table I gives the results of the growth promoting tests. It should be noted that only the ferri-siderophore of BTP1 does not cause growth promotion for anyone of the other strains while it can utilize all the other ferri-siderophores.

Table I. Growth promoting activity of ferri-pyoverdins in cross experiments (+, growth stimulation; –, no stimulation; ±, not reproducible results).

Ferri-siderophore from strain BTP	Strain BTP 1	2	7	9	14	16
1	+	–	–	–	–	–
2	+	+	–	–	+	±
7	±	+	+	+	–	–
9	±	+	+	+	–	–
14	+	–	+	–	+	+
16	+	–	–	+	+	+

Structure of the BTP1 siderophore

Larger amounts were purified by using the 2nd technique described above.

Spectral characteristics and isoelectric point

The UV/Vis spectrum of the BTP1 siderophore shows the typical absorption behavior of pyoverdins (Meyer and Abdallah, 1978), *viz.* a maximum at 400 nm (ε = 27,200 $cm^2 \cdot mmol^{-1}$ at pH 7.0) and at 375 nm (ε = 24,100 $cm^2 \cdot mmol^{-1}$ at pH 5.2). The ferri-siderophore shows a pH-independent position of the absorption maximum at 400 nm (ε = 34,300 $cm^2 \cdot mmol^{-1}$). Isoelectrofocussing of the purified siderophore gave one band with an isoelectric point lower than pH 3.5.

Amino acid analysis

Quantitative amino acid analysis after total hydrolysis with 6 N HI (which reduces N-hydroxy-Orn to Orn) by GC and GC/MS of their TAP derivatives (Schurig, 1984) and subsequent determination of their configurations by GC on a Chirasil-L-Val column gave the following result: 1 L-Ala, 2 L-Asx, 1 L-Glx, 1 D- and 1 L-Orn and 1 L-Ser. Hydrolysis with 6 N HCl yields D- and L-N^5-hydroxy-Orn in addition to Orn (Orn is typically formed as a decomposition product of N-hydroxy-Orn by non-reductive acid hydrolysis of Orn-hydroxamic acids (Emery and Neilands, 1961)). These two hydroxy-Orn form hydroxamic acids *(v. infra)* involved in the iron complexation.

Except for Ala all amino acids present are bifunctional and hence the nature of the second functional group (free or derivatized) has to be accounted for. That the two Asx units are actually Asp with free β-carboxyl groups follows from the molecular mass of the siderophore, the low pI value and from the pH dependence of the β-CH_2 signals in the ^{13}C NMR spectrum (δ = 37.53 and 38.13 ppm at pH 4.0; 38.40 and 39.20 ppm at pH 6.8 (Seinsche *et al.*, 1993)). That Asp is not bound amidically *via* its β-carboxyl group could be established by the method of Holcomb (1968) as modified by Williams (1983): After dissolving the siderophore in D_2O pyridine and acetic acid anhydride were added and the mixture was stirred for 3 h at room temperature. After evaporation to dryness, washing with H_2O, total hydrolysis and TAP derivatization no deuterated Asp could be detected by GC/MS (CH_2/CD_2 exchange would have been expected if Asp were bound amidically by its β-COOH group rather than peptidically). The CH_2OH resonance of Ser in the ^1H NMR spectrum (δ = 3.87 ppm) demonstrates that the hydroxyl function is not esterified (as the resonances of the corresponding esters are in the vicinity of 4.4 to 4.6 ppm (Yang and Leong, 1984; Isogai *et al.*, 1990). That both N-hydroxy-Orn form hydroxamic acids follows from the non-reductive acid cleavage giving N^5-hydroxy-Orn, and from the reductive cleavage giving Orn. One of the hydroxamic acids is N^5-acetyl-N^5-hydroxy-Orn and the other one N^5-hydroxy-c-Orn (3-amino-1-hydroxypiperidone-2). This can be deduced from the characteristic ^1H and ^{13}C NMR data (Tables II and III) (Budzikiewicz *et al.*, 1992; Mohn *et al.*, 1990). Glu has a free α-COOH and an amidically bound γ-COOH group. This follows from the ^{13}C NMR data (Table III) (*cf.* Taraz *et al.*, 1991; Geisen

Table II. ^1H NMR data (relative to DSS δ = 0) of **3** in D_2O at pH 4.0.

Assignment	Resonance	Assignment	Resonance
β-Ala	1.19	β-Ser	3.87
γ-Ac–OH–Orn	1.63/1.70	α-Glu	3.89
β-Ac–OH–Orn	1.76/1.85	Chr C-1	3.97/4.45
β-c–OH–Orn	1.80/2.02	α-Ala	4.03
γ-c–OH–Orn	1.92/1.99	α-Ac–OH–Orn	4.30
Ac	2.12	α-Ser	4.41
β-Glu	2.29	α-c–OH–Orn	4.42
Chr C-2	2.45/2.68	Chr C-3	4.57
β-Asp^2	2.78	α-Asp^2	4.58
γ-Glu	2.78	α-Asp^1	4.62
β-Asp^1	2.79/2.91	Chr C-7	6.95
δ-c–OH-Orn	3.61/3.66	Chr C-10	6.97
δ-Ac–OH–Orn	3.63	Chr C-6	7.88

Ac, acetyl; Ac–OH–Orn, N^5-acetyl-N^5-hydroxy Orn; c–OH–Orn, cyclo-N^5-hydroxy Orn; Chr, chromophore **2a.**

Table III. ^{13}C NMR data (relative to DSS $\delta(CH_3)$ −1.61) of **3** in D_2O at pH 4.0 (for abbreviations see Table II).

Assignment	Resonance	Mult.	Assignment	Resonance	Mult.
β-Ala	17.11	q	β-Ser	61.96	t
Ac	20.23	q	Chr C-10	101.50	d
γ-c-OH-Orn	20.91	t	Chr C-7	113.71	d
Chr C-2	22.15	t	Chr C-6a	116.31	s
γ-Ac-OH-Orn	23.35	t	Chr C-5	118.60	s
β-Glu	26.39	t	Chr C-10a	132.64	s
β-c-OH-Orn	27.50	t	Chr C-6	138.99	d
β-Ac-OH-Orn	28.61	t	Chr C-8	144.80	s
γ-Glu	32.19	t	Chr C-4a	148.90	s
β-Asp^2	37.53	t	Chr C-9	152.01	s
β-Asp^1	38.13	t	c-OH-Orn CO	167.21	s
Chr C-1	43.81	t	Chr CO	172.14	s
δ-Ac-OH-Orn	48.07	t	Ser CO	172.14	s
α-Ala	50.83	d	Asp^1 CO	173.94	s
α-c-OH-Orn	51.33	d	Asp^2 CO	173.94	s
Chr C-3	51.63	d	Ac CO	174.68	s
α-Asp^2	51.75	d	Ac-OH-Orn CO	174.68	s
δ-c-OH-Orn	52.45	t	Glu α-COOH	174.68	s
α-Asp^1	52.65	d	Ala CO	175.76	s
α-Ac-OH-Orn	54.77	d	Glu γ-CO	176.28	s
α-Glu	54.87	d	Asp^2 COOH	176.56	s
α-Ser	56.70	d	Asp^1 COOH	176.94	s

et al., 1992) and the pH independence of the signal for the γ-CH_2 group between pH 3.0 and 7.0) (Geisen *et al.*, 1992). For this amino acid no C,H long range correlation with any other amino acid was detected. It is, therefore, bound to the NH_2 group of the chromophore.

Molecular mass

The molecular mass was determined by FAB-MS as 1076 u corresponding to $C_{44}H_{60}N_{12}O_{20}$. The ferri complex has a mass of 1129 u (replacement of 3 H^+ by $^{56}Fe^{3+}$).

Table IV. $[M+H]^+$ Ions observed in the FAB mass spectra after partial hydrolysis of **3** (for abbreviations see Table II).

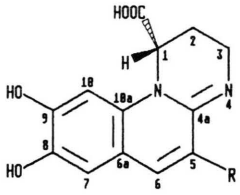
m/z	Assignment
391	H_2N-Chr-Asp
445	Asp-OHOrn-Ser-OHOrn − $2H_2O$
462	H_2N-Chr-Asp-Ala
577	H_2N-Chr-Asp-Ala-Asp
689	H_2N-Chr-Asp-Ala-Asp-OHOrn − H_2O
707	H_2N-Chr-Asp-Ala-Asp-OHOrn
776	H_2N-Chr-Asp-Ala-Asp-OHOrn-Ser − H_2O
836	Glu-Chr-Asp-Ala-Asp-OHOrn
905	Glu-Chr-Asp-Ala-Asp-OHOrn-Ser − H_2O

Sequence of the peptide chain

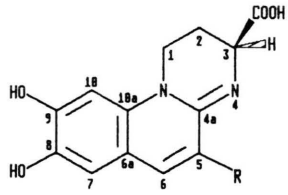
In order to determine the sequence of the peptide chain the mixture of degradation products after partial hydrolysis was analyzed by FAB-MS. The results are shown in Table IV. The sequence established in this way could be confirmed by C,H-long range correlations (HMBC) in D_2O. Significant cross peaks between the signals for the CO carbon of one amino acid and the α-CH hydrogen of the following one caused by $^3J(C,H)$ coupling were observed as indicated in **3.** That L-N-hydroxy-Orn constitutes the C-terminus of the peptide chain could be shown as follows: Purified fragments which contain the chromophore and only one N-hydroxy-Orn gave upon complete hydrolysis (HI) D-Orn only.

Structure of the chromophore

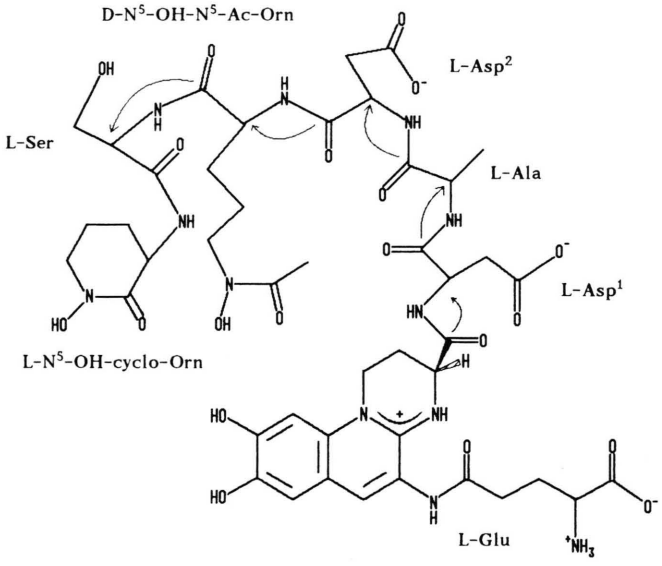
The most interesting moiety in the structure of the BTP1 is the chromophore **2a** which differs from **1a** commonly encountered in pyoverdins as can, *e.g.*, be seen from the 5 spin systems associated with the aliphatic protons of the chromophores (Table V). A ROESY spectrum revealed the reason for this anomaly: While **1a** shows **one**



1a : R = NH_2
1b : R = OH



2a : R = NH_2
2b : R = OH



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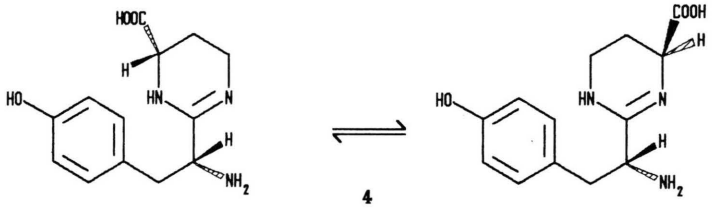

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NOESY/ROESY cross peak between H-10 and the low-field C**H**–CO peptide (H-1), **3** exhibits **two** ROESY cross peaks between H-10 (δ = 6.97 ppm) and the protons of a CH_2 group at δ = 3.97 and 4.45 ppm (H-1 a and 1 b). After hydrolysis (3 N HCl, 110 °C, 7 days) **2b** could be isolated by RP-HPLC (during hydrolysis the NH_2 group is replaced by OH; *cf.* **1a** giving **1b** (Michels and Taraz, 1991)). **2b** has the same molecular mass as **1b** (determined by FAB-MS) but differs in the ^1H (Table V) and ^{13}C NMR data from those of **1b.** The assignments were confirmed by NOE, decoupling, C,H correlation and long-range correlation experiments.

For the new siderophore which may be named isopyoverdin Pp BTP1 structure **3** can, therefore, be proposed (the absolute configuration at C-3 of **2a** and **2b** is based on the assumption that – as in the case of **1a** – it is derived from the α-C of L-Dab).

Table V. ^{1}H NMR data of the chromophore in the pyoverdin from *Pseudomonas putida* C (Py Pp) (Seinsche *et al.*, 1993), of the one in **3** as well as of **1b** and **2b.**

Compound	H-1	H-2	H-3	H-6	H-7	H-10
Py Pp[a]	5.68	2.44/2.70	3.38/3.72	7.93	7.18	6.98
3[b]	3.97/4.45	2.45/2.68	4.57	7.88	6.95	6.97
1b[c]	5.63	2.29/2.57	3.17/3.61	7.37	7.08	6.99
2b[c,d]	3.96/4.50	2.42/2.42	4.39	7.45	7.11	7.21

[a] In D_2O (pH 3.0) relative to DSS ($\delta = 0$).
[b] In D_2O (pH 4.0) relative to DSS ($\delta = 0$), confirmed by 2D-COSY and 1D- and 2D-TOCSY experiments.
[c] In d_6-DMSO ($\delta = 2.49$).
[d] Assignments confirmed by NOE and decoupling experiments.

Discussion

So far about 20 different pyoverdins have been described in the literature (Budzikiewicz, 1993), all containing the chromophore **1a.** The siderophore of *Pseudomonas putida* BTP1 differs insofar as the peptide chain is linked to a carboxyl group to C-3 rather than C-1 of the chromophore. This structural variety is probably responsible for the failure of the iron complex to act as a growth promoter for other *Pseudomonas* strains. Concerning the iron chelation molecular models show that the three binding sites for Fe^{3+} (the catecholate of the chromophore and the two hydroxamate units) can be brought into proper location. The transport of pyoverdins into the cell is normally strain specific but the level of this specificity is not well known. There is no evidence in the literature regarding the structural requirements of a pyoverdin essential for the recognition by the membrane receptor. The singular structure of the isopyoverdin chromophore may well be responsible that this siderophore is not recognized by *Pseudomonas* spp. producing "normal" pyoverdins. The discovery of isopyoverdin chromophore **2a** is of importance in view of the biogenesis of the pyoverdins: There are good reasons for assuming that their precursors are desferri-ferribactins (Taraz *et al.*, 1991) which comprise the structural unit **4** formed by condensation of D-Tyr and L-Dab. Ring closure should be possible both *via* the α- and the γ-N of Dab, but only the first possibility had been encountered with the rather large number of pyoverdins the structure of which has been elucidated. **2a** stems obviously from the other possibility thus corroborating the biogenetic intermediacy of the ferribactins.

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