An Isopyoverdin from *Pseudomonas putida* CFML 90–44

Razia Sultana[a], Bina Shaheen Siddiqui[b], Kambiz Taraz[a], Herbert Budzikiewicz[a,*] and Jean-Marie Meyer[c]

[a] Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, 50939 Köln, Germany. Fax: +49-221-470-5057. E-mail: h.budzikiewicz@uni-koeln.de
[b] HEJ Research Institute, University of Karachi, Karachi-75270, Pakistan
[c] Laboratoire de Microbiologie et Génétique, Université Louis Pasteur, UPRES-A 7010 du CNRS, 28 rue Goethe, 67000 Strasbourg, France

* Author for correspondence and reprint requests

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From *Pseudomonas putida* CFML 90–44 an isopyoverdin was isolated. Its structure could be elucidated by chemical degradation and spectroscopic data.

Introduction

Pseudomonas putida is a bacterium commonly found in soil and water. It is able to use almost any carbon source and to degrade even polycyclic and chlorinated aromatic compounds (Silver *et al.*, 1990). It is only potentially human pathogenic, i. e., it may infect only persons whose immune system is severely impaired (Graevenitz and Weinstein, 1971). *Pseudomonas putida* belongs to the so-called fluorescent pseudomonads which commonly produce siderophores named pyoverdins, chromopeptides consisting of a dihydroxyquinoline chromophore bound amidically to the N-terminus of a peptide chain by its carboxyl group at C-1, and to a small dicarboxylic acid or its amide by the amino group at C-5 (Budzikiewicz, 1997a and 1997b). In two instances a siderophore was isolated from a *P. putida* strain where the carboxyl group carrying the peptide chain is located at C-3 of the chromophore (Jacques *et al.,* 1995; Sultana *et al.*, 2001) thus differing from the rest of the about 50 pyoverdins described so far (Kilz *et al.*, 1999). The only other example of this "iso"-chromophore has been reported for the siderophore of *Azomonas macrocytogenes* ATCC 12334 (Michalke *et al.*, 1996). We wish now to report the structure elucidation of a further example from *P. putida* CFML 90–44.

Materials and Methods

Pseudomonas putida CFML 90–44 is a hospital isolate from sputum. It was classified according to its phenotype as belonging to cluster II, subcluster IIb and according to its ribotype to cluster E (Elomari *et al.*, 1994 and 1997). The strain was grown in a succinate minimal medium (Budzikiewicz *et al.*, 1997). For the work-up of the culture and isolation of the ferri-complex by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* (1999). The Biogel fraction was subjected to ion-exchange chromatography on CM-Sephadex A-25 with a pyridinium acetate buffer (pH 5.0, 0.02 M). The second (major) fraction was pure as checked by analytical RP-HPLC with CH_3OH/CH_3COONH_4 buffer (pH 6.2) and was decomplexed with 8-hydroxyquinoline (Briskot *et al.,* 1986); **1** was checked for purity by analytical RP-HPLC with a CH_3OH/0.1 M CH_3COONH_4/1 mM Na_2EDTA solution.

For the qualitative and quantitative analysis of the amino acids, the determination of their configuration by GC/MS of their TAP-derivatives on a chiral column and the dansyl derivatization see Briskot *et al.* (1986) and Mohn *et al.* (1990). For instrumental details see Sultana *et al.* (2000).

Siderotyping analysis of the strain was performed through pyoverdin isoelectrofocusing and $^{59}Fe^{3+}$ incorporation as described previously (Meyer *et al.*, 1998; Munsch *et al.*, 2000). The following strains were tested for comparison (isopyoverdins in **bold**): *Pseudomonas* sp. E8; *P. syrin-*

* Part CII of the series "Bacterial constituents". For part CI see Budzikiewicz (2001), for part C Sultana *et al.* (2001).

Abbreviations: Common amino acids, 3-letter code; AcOHOrn, N-acetyl-N-hydroxy Orn; cOHOrn, 4-N-hydroxy-*cyclo*Orn; TAP, N/O-trifluoroacetyl-(amino acid)-isopropyl ester; RP-HPLC, reversed phase high performance liquid chromatography; ESI, electrospray ionization; FAB, fast atom bombardment; MS, mass spectrometry; CA, collision activation; CFML, Collection de la Faculté de Médecine de Lille.

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gae ATCC 19310; *P. fluorescens* 9AW; *P. putida* ATCC 12633; *P. fluorescens* 51W; *P. aeruginosa* Pa6; *P. fluorescens* CCM 2798; *P. fluorescens* CHA0; *P. tolaasii* NCPPB 2192; *P. aeruginosa* ATCC 27853; *P. fluorescens* ii; *P. fluorescens* SB8.3; *P. fluorescens* ATCC 17400; *P. fluorescens* 1.3; *P.* sp. 267; *P. chlororaphis* ATCC 9446; *P. aeruginosa* ATCC 15692; *P. fluorescens* 18.1; *P. fluorescens* 12; *P. fluorescens* CFBP 2392; *Pseudomonas putida* CFBP 2461; *P.* sp. ATCC 15915; *P. putida* WCS358; *P. 'mosselii'* CFML 90–77; *P. rhodesiae* CFML 92–104; *P. veronii* CFML 92–124; *P. mandelii* CFML 96–72; *P.* sp. 2908; *P.* sp. A214; ***P. putida* CFML 90–33**; *P.* sp. CFML 90–51; ***P. putida* BTP1**; *P.*sp. CFML 95–275; *P.* sp. CFML 96–188.

Results

Siderotyping behavior of P. putida CFML 90–44

P. putida CFML 90–44 is characterized by a pyoverdin-mediated iron uptake system specifically restricted to its own pyoverdin. None of 34 tested pyoverdins (see above) of foreign origin was able to facilitate iron incorporation into the strain. The isoelectrophoretic pattern developed by the pyoverdin isoforms (i.e. differing in the chromophore side chains) present in the growth culture supernatant (CAA growth medium, see Munsch *et al.*, 2000) revealed a major band at pI 4.85 and three minor bands at pI 7.10, 4.25 and 3.90. This pattern was unique compared with those developed by the 34 pyoverdins mentioned above. Thus, both siderotyping methods suggested a novel structure for the pyoverdin of *P. putida* CFML 90–44.

Characterization of **1**

The UV/Vis spectrum of **1** is characteristic for the isopyoverdin chromophore (Michalke *et al.*, 1996): 402 nm at pH 7.0, split band at 366 and 376 nm at pH 3.0; ferri-**1** 400 nm and broad charge-transfer bands at ~475 and 550 nm. The molecular mass of **1** was determined by FAB-MS as 1437 u.

Identification of the iso-chromophore and the amino acids of **1**

The NMR characteristics of the isopyoverdin (iPyo) as compared with those of the pyoverdin (Pyo) chromophore were discussed in detail before (Jacques *et al.*, 1995; Michalke *et al.*, 1996; Sultana *et al.*, 2001). In the ^{1}H spectrum the shifts of the protons of the 1-CH- and of the 3-CH_2-group of Pyo and of the 3-CH- and of the 1-CH_2-group of iPyo show significant differences due to their respective relative proximity to the aromatic part of the chromophore. Especially the downfield-shifted signal of the CH-1 of Pyo (ca. 5.7 ppm) is missing in the iPyo spectrum. The presence of a CH_2-group at C-1 of **1** follows from two-dimensional correlations: the two protons of C-1 (3.88 and 4.50 ppm) exhibit NOE cross peaks to the H of C-10 at 7.10 ppm. Also the ^{13}C shifts of C-1 and C-3 (43.8 and 51.9 ppm, identified in the HMQC spectrum which offers ^{1}J-CH correlations) differ from those of a pyoverdin chromophore in correspondence with the literature data. Typically the NH resonance value of the amino acid (Asp) attached to the carboxyl group of the chromophore is not shifted downfield relative to the other NH signals as it is observed for pyoverdins: it is not in the influence sphere of the aromatic part of the chromophore.

After total hydrolysis and GC-MS analysis of the TAP-derivatives on a chiral column the following amino acids could be identified: L-Asp, L-Glu, Gly, L-Lys, D-Orn, D- and L-Ser (1:1) and L-Thr. Which of the Ser are D- and which are L-configurated remains open. Hydrolysis after dansylation yielded ε-dansyl Lys as shown by chromatographic comparison with authentic α- and ε-dansyl Lys. Hence, in **1** the ε-amino group of Lys is free.

Determination of the amino acid sequence

For a discussion of the various NMR techniques applied for the identification of the ^{1}H- and ^{13}C-signals see e.g. Sultana *et al.* (2000 and 2001). Those of the peptide chain and the Glu side chain correspond to those observed with other pyoverdins (Budzikiewicz, 1997a and 1997b). Only the following ones deserve a comment: the shift values of the β-CH-groups of Thr (4.29 ppm) and of the four Ser (3.90 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected) (Budzikiewicz, 1997b). The shift values of Asp correspond to literature data (e.g., Jacques *et al.*, 1995) in ac-

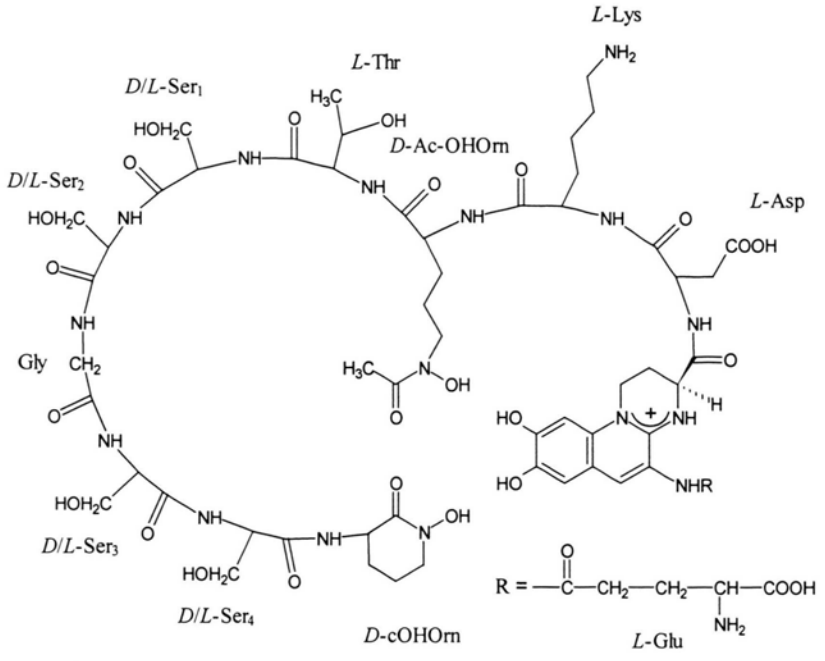

Fig. 1. Structure of the isopyoverdin **1** (2 D-Ser and 2 L-Ser).

cordance with a free β-carboxyl group. The C-terminal cOHOrn is characterized by the CO-resonance at 167.5 ppm (for peptidically bound Orn the CO resonance is about 174.5 ppm) and by the lower shift values for the ring protons (for reference values see Georgias *et al.* 1999 – cyclic – and Hohlneicher *et al.*, 1995 – open). Due to the *cis/trans*-arrangement of the acetyl groups of AcOHOrn two CH_3- (2.18 and 2.15 ppm) and two CO-signals (174.8 and 170.0 ppm) are observed. Glu could be identified as the side chain dicarboxylic acid because no NOE cross peaks were evident with other amino acids. Its connection to the NH_2 group of the chromophore by its γ-carboxyl group follows from the ^1H- and ^{13}C- shifts (Budzikiewicz, 1997b) (the values are identical with those reported by Sultana *et al.*, 2001). The peptide sequence was derived from ROESY/NOESY (correlation of amide NH protons with spatially close α- andβ-H's of the preceding amino acid, C**H**-C**H**-CO-N**H**) and HMBC (correlating amide CO with the α-H if the following amino acid, **C**O-NH-C**H**) (cf. Sultana *et al.*, 2000 and 2001).

The amino acid sequence deduced from NMR data is confirmed by the fragment ions obtained after ESI by ion trap CA of $[M\text{-}H_2O\text{+}2H]^{2+}$ (Table I): The N-terminal B-ions (Roepstorff and Fohlman, 1984), viz. X-NH-CHR-CO^+ after loss of H_2O as well as the C-terminal Y″ions, cOHOrn-NH_3^+ etc. are present for the entire sequence and confirm the results obtained by NMR.

Discussion

According to the current biosynthesis scheme the chromophore of pyoverdins originates from a condensation product of D-Tyr and L-Dab found

Table I. MS-CA spectrum of **1**, $[B\text{-}H_2O]$ and Y″-ions[a].

Amino acid	n	$B_n\text{-}H_2O$	Y_n''	n
Glu-Chr	0			
Asp	1	484		10
Lys	2	612	937	9
AcOHOrn	3	784	809	8
Thr	4	885		7
Ser^1	5	972	636	6
Ser^2	6	1059	449	5
Gly	7	1116	362	4
Ser^3	8	1203	305	3
Ser^4	9		218	2
cOHOrn			131[b]	1

[a] CA of $[M\text{-}H_2O\text{+}2H]^{2+}$ in the ion trap, [b] CA of $[M\text{+}2H]^{2+}$ in the octapole.

in the ferribactins (Böckmann *et al.*, 1997). Connection of the α-N of Dab with the 2-position of the phenyl ring of Tyr results in the isoquinoline ring of the pyoverdins. An analogous reaction sequence with the γ-N of Dab leads to the isopyoverdins. *Pseudomonas* isopyoverdins were found to be produced so far only by *P. putida* strains and they exhibit some structural analogies (L-Asp as the first amino acid, Glu as side chain). How and why isopyoverdins rather than pyoverdins are produced is still a moot point.

It is important to note that *P. putida* CFML 90–33 and *P. putida* BTP1 are unable to mediate iron uptake in *P. putida* 90–44, neither does *P. putida* CFML 90–33 accept the ferri-pyoverdin of *P. putida* BTP1 (Sultana *et al.*, 2001). Obviously the isochromophore is not involved primarily in the recognition at the receptor site and differences in the peptide chains, viz.

L-Asp-L-Lys-D-AcOHOrn-L-Thr-Ser-Ser-Gly-Ser-Ser-D-cOHOrn (90–44)
L-Asp-L-Lys-L-Thr-D-OHAsp-L-Thr-D-aThr-L-cOHOrn (90–33)
L-Asp-L-Ala-L-Asp-D-AcOHOrn-L-Ser-L-cOHOrn (BTP1)

are responsible for the negative cross-uptake results.

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