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An isopyoverdin from *Pseudomonas putida* CFML 90-33☆

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Abstract—From *Pseudomonas putida* CFML 90-33, an isopyoverdin, was isolated. Its structure could be elucidated by chemical degradation and spectroscopic data.

1. 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.[2] It is potentially human pathogenic, i.e. it may infect only persons whose immune system is severely impaired.[3] *Pseudomonas putida* belongs to the so-called fluorescent group of its genus which generally produces siderophores named pyoverdins. These are 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 (**3**, see the numbering in **1**).[4,5] In one case, a siderophore was isolated from a *P. putida* strain (BTP1) where the carboxyl group carrying the peptide chain was located at C-3 of the chromophore,[6] thus differing from the rest of the about 50 known pyoverdins.[7] The only other example of this 'iso'-chromophore was found in the siderophore of *Azomonas macrocytogenes* ATCC 12334.[8] We wish now to report the structure elucidation of a second *Pseudomonas* isopyoverdin from *P. putida* CFML 90-33.

Pseudomonas putida CFML 90-33, is a hospital isolate from an antiseptic solution (sic!). It was classified according to its phenotype as belonging to cluster II, subcluster IIa and according to its ribotype to cluster A.[9,10] When subjected to siderotyping[11] *P. putida* CFML 90-33 revealed two

Table 1. ^{1}H NMR data (δ [ppm]) of **1** (pH 4.3; 25°C; H_2O/D_2O 9:1)[a]

Glu	2′	3′	4′	NH_2						
	3.88	2.27	2.76	NO[b]						
Chr	1a	1b	2a	2b	3	$4NH^+$	6	7	10	5-NH
	3.85	4.42	2.40	2.68	4.51	8.57[c]	7.94	7.08	7.08	NO[b]
	α-NH	α	β	γ	δ	ϵ	ϵ-NH_2			
Asp	8.19	4.59	2.73							
			2.81							
Lys	8.14	4.03	1.31	1.15	1.49	2.91	7.59[c]			
			1.49							
Thr[1]	8.23	4.41	4.27	1.14						
OHAsp	8.11	4.85	4.49							
Thr[2]	8.08	4.42	4.30	1.21						
aThr	8.18	4.34	4.17	1.24						
cOHOrn	8.41	4.48	1.79	1.99	3.64					
			2.00		3.68					

[a] Based on COSY and TOCSY correlations.
[b] Not observed.
[c] 5°C.

☆ Part IC of the series 'Bacterial Constituents'.[1]

Keywords: *Pseudomonas putida*; isopyoverdin; siderophore.
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Table 2. ^{13}C NMR data (δ [ppm]) of **1** (pH 4.3; 25°C; D_2O)[a]

Glu	COOH	2′	3′	4′	5′-CO		
	175.1	55.0	26.5	32.2	177.1		
Chr	CO	1	2	3	4a	5	6
	172.5	43.8	22.0	51.9	148.8	118.3	139.3
	6a	7	8	9	10	10a	
	116.3	113.6	145.6	153.8	101.8	133.3	
	CO	α	β	γ	δ	ϵ	
Asp	173.6	53.2	38.4	176.7			
Lys	175.0	54.8	31.4	22.9	27.4	40.2	
Thr[1]	172.9	59.6	68.1	19.6			
OHAsp	172.9	57.7	72.5	177.7			
Thr[2]	172.9	60.3	68.1	19.9			
aThr	172.1	60.3	68.3	19.4			
cOHOrn	167.6	51.5	27.7	21.1	52.7		

[a] Based on HMBC and HMQC spectra.

pyoverdin isoforms with pI 5.0 and 3.9, an isoelectrophoretic pattern not found for any other known pyoverdin. Moreover, the strain was unable to use any of 35 foreign pyoverdins (cf. Ref. 12) as well as that of the strain BTP1 (the only other isopyoverdin producer, see above) for iron transport. This suggests a new structure.

2. Results

2.1. Characterization of 1

The UV/Vis spectrum of **1** is characteristic for the isopyoverdin chromophore:[8] 398 nm at pH 7.0, split band at 370 and 374 nm at pH 3.0; ferri-**1** 395 nm and broad charge-transfer bands at ~475 and 550 nm. The molecular mass of **1** was determined by FAB-MS as 1193 u. The absolute configuration of C-3 of the chromophore of **1** could be determined as *S* from the CD-spectrum (Cotton effect +242 nm, −290 nm, +358 nm) of the 5-hydroxy chromophore obtained by hydrolysis (exchange of the 5-NH_2 for an OH group). It is identical with the CD-spectra reported for the 5-hydroxy chromophore from *P. putida* BTP1 and *Azomonas macrocytogenes*.[13,14]

2.2. Identification of the iso-chromophore of 1

The NMR characteristics of the isopyoverdin (iPyo) as compared with those of the pyoverdin (Pyo) chromophore have been discussed in detail.[6,8] In the ^1H NMR spectrum, the shifts of the protons of the 1-CH and the 3-CH_2 groups of Pyo, and the 3-CH and 1-CH_2 groups of iPyo show significant differences because of their respective relative proximity to the aromatic part of the chromophore: Pyo-CH-1 5.63, iPyo-CH-3 4.39 ppm, Pyo-CH_2-3 3.17/3.61, iPyo CH_2-1 3.96/4.50 ppm in DMSO-d_6.[8] Because of the specific structures of the compounds under investigation and the experimental conditions (i.e. the solvent used) the shift values observed here differ slightly from those given above (cf. Table 1), but the correlations remain obvious, especially since the localization of the CH and of the CH_2 group (C-1 or C-3) follows from two-dimensional correlations. In the case of **1** the two protons of C-1 exhibit NOE cross peaks to the H of C-10, and the one at C-3 to

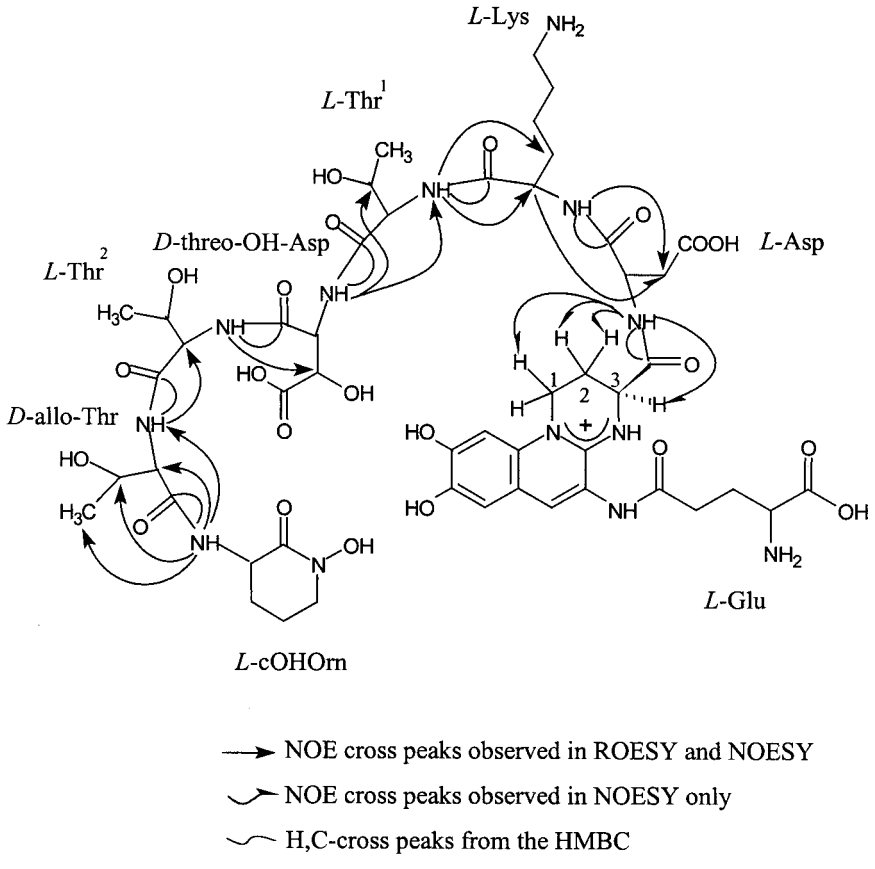


Figure 1. NMR sequential information for **1**.

Table 3. MS-CA spectrum of **1**, B and Y″ ions

Amino acid	*n*	B_n	$-H_2O$	Y''_n	*n*
Glu–Chr	0	387			
Asp	1		484		7
Lys	2		612	693	6
Thr[1]	3	731	713		5
OHAsp	4		844		4
Thr[2]	5		945	333	3
aThr	6		1046	232	2
cOHOrn					1

NH-4. Also the ^{13}C shifts (Table 2) of C-1 and C-3 (identified in the HMQC spectrum which offers ^1J-CH correlations) differ from those of a pyoverdin chromophore in correspondance with the literature data. Typically also the NH resonance value of the amino acid 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.

2.3. Identification of the amino acids

After total hydrolysis and GC–MS analysis of the TAP-derivatives on a chiral column the following amino acids could be identified: L-Asp, D-*threo*-OHAsp, L-Glu, L-Lys, L-Orn, L-Thr and D-aThr. 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.

Table 4. $[M+H]^+$ Ions after partial hydrolysis of **1** as determined by FAB-MS

m/z	Assignment
501[a]	Chr–Asp–Lys
519	Chr–Asp–Lys
648	Glu–Chr–Asp–Lys
749	Glu–Chr–Asp–Lys–Thr
751	Chr–Asp–Lys–Thr–OHAsp
852	Chr–Asp–Lys–Thr–OHAsp–Thr
880	Glu–Chr–Asp–Lys–Thr–OHAsp
981	Glu–Chr–Asp–Lys–Thr–OHAsp–Thr
1065[a]	Chr–Asp–Lys–Thr–OHAsp–Thr–aThr–cOHOrn
1082	Glu–Chr–Asp–Lys–Thr–OHAsp–Thr–aThr
565	Thr–OHAsp–Thr–aThr–cOHOrn
583	Thr–OHAsp–Thr–aThr–OHOrn
711	Lys–Thr–OHAsp–Thr–aThr–OHOrn

[a] After loss of H_2O.

2.4. Determination of the amino acid sequence by NMR spectroscopy

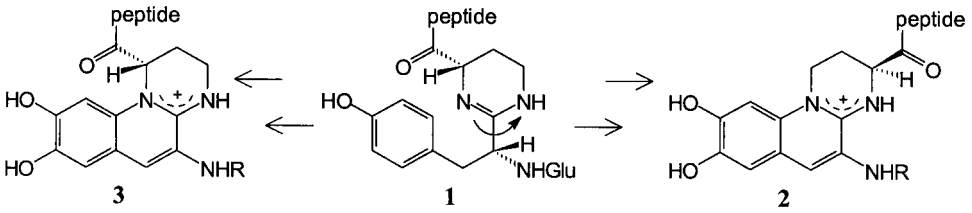
The basis for the sequence determination by NMR spectroscopy is the unambiguous identification of all ^1H and ^{13}C signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: COSY and TOCSY allows the detection of the H couplings within one amino acid residue (amide bonds interrupt the scalar H,H coupling). Quaternary C-atoms can be identified with HMBC optimized for ^2J- and ^3J-coupling. Sequence information is obtained by NOESY/ROESY, which allows a correlation of an NH proton with spatially close α- and β-H's of the preceding amino acid (C**H**–C**H**–CO–N**H**), and by HMBC correlating amide-CO with the α-H of the following amino acid (see Fig. 1). The ^1H and ^{13}C NMR data of **1** are compiled in Tables 1 and 2. They correspond to those observed with other pyoverdins.[4,5] The following ones deserve a comment: the shift values of the β-CH groups of Thr (4.27 and 4.30 ppm) and of aThr (4.34 ppm) show that the OH groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected).[5] The C-terminal cOHOrn is characterized by the CO resonance at 167.6 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 Ref. 15 for cyclic and Ref. 16 for open structures). Glu could be identified as the side chain dicarboxylic acid because no NOE cross peaks with other amino acids were observed. Its connection to the NH_2 group of the chromophore follows from the ^1H and ^{13}C shifts. The peptide sequence as derived from ROESY/NOESY and HMBC correlations is given in Fig. 1.

2.5. Determination of the amino acid sequence by ESI mass spectrometry and by partial hydrolysis

The amino acid sequence deduced from NMR data is confirmed by the fragment ions obtained after ESI by ion trap CA (Table 3): the so-called B ions, viz.[17] $X–NH–CHR–CO^+$ are present up to B_6. After partial hydrolysis, the masses of the $[M+H]^+$ ions of the products assembled in Table 4 could be determined by FAB mass spectrometry. They confirm also the amino acid sequence.

3. Discussion

According to the current biosynthesis scheme[18] the chromophore of pyoverdins originates from a condensation product of D-Tyr and L-Dab as found in the ferribactins



Scheme 1. Ferribactin (**2**) as precursor of pyoverdins (**3**) and isopyoverdins (**1**).

(**2**). 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 (**3**). An analogous reaction sequence with the γ-N of Dab leads to the isopyoverdins (Scheme 1). The only other *Pseudomonas* isopyoverdin encountered so far stems also from a *P. putida* strain and the two isopyoverdins have some structural analogies (L-Asp as the first amino acid, Glu as the side chain), but they were not exchangeable in iron cross-uptake experiments. Any speculations as to the how and why isopyoverdins rather than pyoverdins are produced would be still too early.

4. Experimental

4.1. General

Mass spectrometry: Finnigan-MAT H-SQ 30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400. NMR: DPX 300 (^{1}H 300, ^{13}C 75.5 MHz) and DRX 500 (^{1}H 500, ^{13}C 125 MHz) (both Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS)=δ(DSS) for ^{1}H, δ(DSS)=−1.61 ppm for ^{13}C. Suppression of the H_2O signal by the WATERGATE puls sequence. UV/Vis: Lambda 7 (Perkin–Elmer, Überlingen), CD: Jasco 715 (Jasco, Tokyo, Japan), solvent 1N HCl. Chromatography: RP–HPLC column Nucleosil and Eurosphere 100-C_{18} (5 μm) (Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), DEAE-Sephadex A-25 (Pharmacia, Uppsala, S); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt). Water was desalted and distilled twice in a quartz apparatus; for HPLC it was further purified on XAD-4 resin and filtered through a sterile filter. Organic solvents were distilled over a column. Reagents were of *p. a.* quality.

Abbreviations: Common amino acids, 3-letter code; Dab, 2,4-diaminobutyric acid; OHAsp, β-hydroxy Asp; cOHOrn, 4-*N*-hydroxy-*cyclo*Orn; aThr, *allo*Thr; TAP, *N*/*O*-trifluoroacetyl-(amino acid)-isopropyl ester; RP-HPLC, reversed phase high performance liquid chromatography; ESI, electrospray ionization; CA, collision activation; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; CFML, Collection de la Faculté de Médecine de Lille.

4.2. Production and isolation of the isopyoverdin 1

The strain was grown in a succinate minimal medium.[19] For the work-up of the culture and isolation of the ferri-complex by chromatography on XAD-4 and Biogel P-2 see Ref. 12. The Biogel fraction was subjected to ion-exchange chromatography on CM-Sephadex A-25 with a pyridinium acetate buffer (pH 5.0, gradient 0.02 to 0.2 M). The first (major) fraction was rechromatographed with 0.02 M pyridinium acetate buffer (pH 5.0). The thus obtained second (main) fraction (pure as checked by analytical RP-HPLC with CH_3OH/CH_3COONH_4 buffer, pH 6.2) was decomplexed with 8-hydroxyquinoline;[20] **1** was finally purified by chromatography on Biogel P2 with 0.1 M acetic acid and checked for purity by analytical RP-HPLC as above.

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 Refs. 20, 21. Partial hydrolysis (see Table 4) was achieved with 6 N HCl at 50°C for 15 or 30 min. and at 90°C for 15 min. The 5-hydroxy chromophore of **1** was obtained by acid hydrolysis[8] and purified by HPLC on Eurosphere 100 with 0.1% aqueous CF_3COOH/CH_3OH 8:2; molecular mass (by FAB-MS) 276 u.

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