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The pyoverdin of *Pseudomonas fluorescens* BTP2, a novel structural type†

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Abstract—From *Pseudomonas fluorescens* BTP2 a pyoverdin was isolated which contained the so far not encountered Val in its peptide chain. Its structure could be elucidated by chemical degradation and spectroscopic data.

Pseudomonas fluorescens belongs to the so-called fluorescent group of its genus which 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, and to a small dicarboxylic acid or its amide by the amino group (cf. Fig. 1).[2,3] Pyoverdins are produced in order to form water-

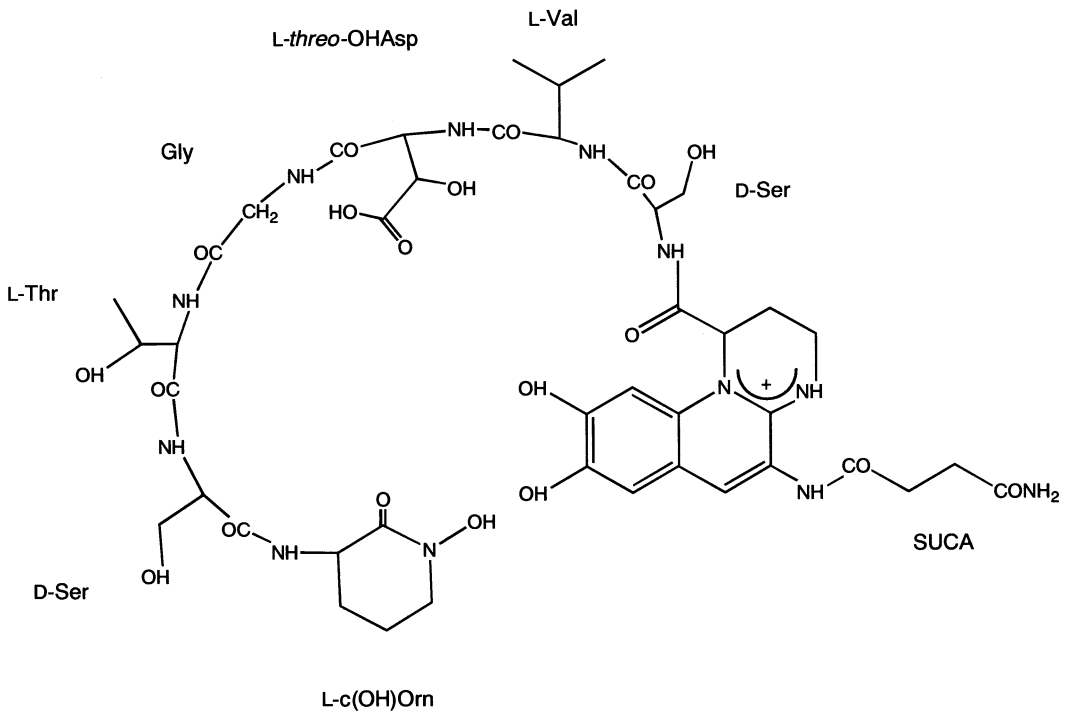

Figure 1. Structure of pyoverdin **1**.

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soluble Fe^{3+}-chelates. Accordingly none of the about 50 representatives whose structures have been elucidated[4] contain amino acid residues with lipophilic side chains. Non-functionalized amino acids encountered so far are just Gly and Ala. We wish now to report the structure elucidation of the pyoverdin from *P. fluorescens* BTP2 isolated from the rhizosphere of tomato plants[5] which contains Val in its peptide chain.‡

Bacterial culture isolation of the ferri-pyoverdins from the culture supernatant, purification and decomplexation was effected as described earlier.[5] After total hydrolysis and quantitative GC–MS analysis of the TAP-derivatives on a chiral column, the following amino acids could be identified: L-*threo*-OHAsp, Gly, L-Orn, 2 D-Ser, L-Thr and L-Val. NMR analysis (see below) shows that Orn is present as cOHOrn. In the ESI-MS (Finnigan-MAT 900 ST) an $[M+H]^+$ ion of the main component was observed at m/z 1049 (nominal mass). This amounts to a peptide chain consisting of the amino acid residues mentioned plus the pyoverdin chromophore with a succinamide side chain (**1**). In addition derivatives with a succinic acid (m/z 1050), a succinic acid methyl ester (m/z 1064) and a succinimide side chain (m/z 1032) are observed. They are formed during the work-up as artifacts. The stability constants of the Fe^{3+} complex of **1** were determined as 20.3 at pH 5.0 and as 26.2 at pH 7.0, in agreement with other pyoverdins.[3]

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. Using these techniques the ^1H and ^{13}C

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

Suca	2′	3′					
	2.79	2.73					
Chr	1	2	3	$4NH^+$	6	7	10
	5.73	2.71/2.79	3.41/3.72	9.23	7.92	7.28	7.08
Amino acids	α-NH	α	β	γ	δ		
Ser[1]	9.94	4.49	3.95				
Val	8.17	4.23	2.08	0.81			
OHAsp	8.40	4.81	4.48				
Gly	8.35	3.86					
Thr	8.21	4.38	4.24	1.19			
Ser[2]	8.57	4.47	3.88				
cOHOrn	8.46	4.51	1.94	1.74/1.85	3.65		

[a] Based on COSY and TOCSY correlations.

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

Suca	CO	2′	3′	$CONH_2$			
	174.7	21.9	31.1	177.1			
Chr	CO	1	2	3	4a	5	6
	170.5	57.8	22.7	36.2	150.2	118.1	140.0
	6a	7	8	9	10	10a	
	115.1	132.7	144.7	152.6	101.3	115.7	
Amino acids	CO	α	β	γ	δ		
Ser[1]	171.9	56.8	62.0				
Val	172.6	60.7	31.1	19.6			
OHAsp	175.6	58.0	73.1	178.1			
Gly	171.7	44.0					
Thr	173.3	60.6	68.7	20.0			
Ser[2]	172.6	56.8	62.1				
cOHOrn	171.7	51.5	27.8	21.2	52.9		

[a] Based on HMBC and HMQC spectra.

‡ *Abbreviations*: Common amino acids, 3-letter code; OHAsp, β-hydroxy Asp; cOHOrn, *cyclo*-N^5-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); TAP, *N*/*O*-trifluoroacetyl (amino acid) isopropyl ester; Chr, pyoverdin chromophore; Suca, succinamide residue; MS, mass spectrum or spectrometry; ESI, electrospray ionization; CA, collision activation; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence.

NMR data of **1** compiled in Tables 1 and 2 were identified. They correspond to those observed with other pyoverdins.[2,3] The following ones deserve a comment: the shift values of the β-CH groups of Thr (4.27 and 4.30 ppm) and of Ser (4.34 ppm) show that the OH groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected).[3] The NH-signal of Ser[1] bound directly to the carboxyl group of the chromophore is typically shifted downfield. The C-terminal cOHOrn is characterized by the CO resonance at 171.7 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. 6 for cyclic and Ref. 7 for open structures). Sequence information is obtained by NOESY/ROESY which allows a correlation of an NH proton with spatially close α- and β-Hs of the preceding amino acid (CH-CH-CO-NH), and by HMBC correlating amide-CO with the α-H of the following amino acid. Connectivities were established for all peptide bonds.

The amino acid sequence deduced from NMR data is confirmed by the fragment ions obtained after ESI of **1** by ion trap CA (Table 3): Typical[4] for pyoverdins with a Suca side chain and Ser as the first amino acid is the ion A_1 [Suca-Chr-NH^+=CH(CH_2OH)] (m/z 416) which loses NH_3 (m/z 399) as well as the entire Suca residue (m/z 317), and m/z 204 (loss of C-1+C-2 of Chr together with the peptide chain and of the Suca residue). The A_2 ion at m/z 515 confirms Val as the second amino acid. The B ions,[8] X-NH-CHR-CO^+, are present up to B_6. For the pyoverdin with a succinimide side chain the A and B ions occur with masses 17 u lower, those of the pyoverdin with a Suc methyl ester side chain 15 u higher. After partial hydrolysis (6N HCl, 90°C, 15 min) the masses of the $[M+H]^+$ ions of the products assembled in Table 4 could be determined by MS. They also confirm the amino acid sequence.

The pyoverdin of *P. fluorescens* BTP2 with seven amino acids in its peptide chain belongs to the smallest representatives of its class (six amino acids is the minimal number observed so far). It is unique in its structure insofar that except for the Fe^{3+} chelating amino acids OHAsp and cOHOrn, it contains only small neutral ones, viz. Ser and Thr in addition to the unexpectedly lipophilic Val not encountered so far in pyoverdins.

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

Amino acid	n	m/z
Suca-Chr-Ser^+	1	444
$+Val^+$	2	543
$+OHAsp^+$	3	674
$+Gly^+$	4	731
$+Thr^+$	5	832
$+Ser^+$	6	919
$+cOHOrn+H^+$	7	1049

Table 4. $[M+H]^+$ ions after partial hydrolysis of **1**

m/z	Assignment
363	Chr-Ser
462	Chr-Ser-Val
593	Chr-Ser-Val-OHAsp
650	Chr-Ser-Val-OHAsp-Gly
750	Suc-Chr-Ser-Val-OHAsp-Gly
337	Thr-Ser-OHOrn
394	Gly-Thr-Ser-OHOrn

The amino acid composition of pyoverdins serves several purposes: it provides two of the ligand sites for Fe^{3+} in an appropriate position for the binding of this ion, and it is responsible for the recognition of the complex at the bacterial cell surface, frequently safeguarding that the own ferri-pyoverdin cannot be taken up by other strains. Thus, as shown by growth promotion tests,[5] from eleven strains belonging to the species *P. aeruginosa*, *P. fluorescens* and *P. putida* only three *P. putida* strains (BTP1,[5] BTP16,[9] and C,[10]) reacted positively upon addition of ferri-1 to the culture medium.[11] Apparently in competition with other microorganisms, the peculiar structure offers selectional advantages. Since rather little is known regarding this last point, the structure elucidation of pyoverdins with specific structural features may help to understand the underlying mechanisms.

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