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The pyoverdine from *Pseudomonas chlororaphis* D-TR133 showing mutual acceptance with the pyoverdine of *Pseudomonas fluorescens* CHA0

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Abstract

From *Pseudomonas chlororaphis* D-TR133 a pyoverdine was isolated and its primary structure were elucidated by spectroscopic methods and degradation reactions. Despite some structural differences, its Fe(III) complex and that of the pyoverdine from *Pseudomonas fluorescens* CHA0 were taken up by either strain with a high rate. This is explained by a structural similarity between the two pyoverdines which were shown to differ in their structures only by the replacement of Lys by Ala in the C-terminal part of the molecules. An unexpected feature is that the main pyoverdine of *P. chlororaphis* D-TR133 is accompanied by a minor one where specifically one Ala is replaced by Gly. So far amino acid variations in the peptide chain of pyoverdines produced by a given strain had not been observed amongst the producers of the about fifty pyoverdines reported in the literature.

Abbreviations: Common amino acids – 3-letter code; FoOHOrn – δ-N-formyl-N-hydroxy Orn; Suc – succinic acid residue; Chr – pyoverdine chromophore (see Figure 1); TAP – N/O-trifluoroacetyl (amino acid) isopropyl ester; RP-HPLC – reversed phase high performance liquid chromatography; GC – gas chromatography; ESI – electrospray ionization; FAB – fast atom bombardment; CA – collision activation; COSY – correlated spectroscopy; DEPT – distortionless enhancement by polarization transfer; HMBC – heteronuclear multiple bond correlation; HMQC – heteronuclear multiple quantum coherence; NOE – nuclear Overhauser effect; NOESY – nuclear Overhauser and exchange spectroscopy; ROESY – rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY – total correlation spectroscopy; WATERGATE – water suppression by gradient-tailored excitation; DSS – 2,2-dimethyl-2-silapentane-5-sulfonate; TMS – tetramethylsilane.

Introduction

Pseudomonas chlororaphis is a member of the fluorescent species in the rRNA homology group I of the family Pseudomonadaceae and it produces siderophores ('pyoverdines') with high Fe^{3+} complexing constants. Today about 50 complete or fairly complete pyoverdine structures have been elucidated and from preliminary studies it appears that many more are to be expected (Fuchs & Budzikiewicz 2001; Fuchs *et al.*2001). Pyoverdines consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6 to 12 amino acids bound to the chromophore carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to its NH_2-group (cf. **1**). The peptide chains have a twofold function. They provide two of the ligand sites for Fe^{3+}, and they are responsible for the recognition of the ferri-pyoverdines by specific receptors located at the surface of the producing cell (Budzikiewicz 1997a). The variability of the peptide chain is closely connected with the second function: It safeguards that a given ferri-pyoverdine is available only to the producing strain because of the usually highly specific interaction between the ferri-pyoverdine and its re-

ceptor outer membrane protein (Hohnadel & Meyer 1988). However, the number of examples increases where *Pseudomonas* spp. are found to accept pyoverdines differing in the peptide chain from the one produced by themselves: certain *P. fluorescens* and *P. putida* strains (Jacques *et al.* 1995), *P. aeruginosa* ATCC 15692 which recognizes the pyoverdine of *P. fluorescens* ATCC 13525 (Hohnadel & Meyer 1988; Kinzel *et al.* 1998), several pyoverdines without (Georgias *et al.*, 1999; Barelmann *et al.* 2002; Meyer *et al.* 2002) and several with a cyclic C-terminal part (Amann *et al.* 2000; Weber *et al.* 2001). For each group some structural similarities were recognized. The interpretation of the results, however, is complicated by the observation that cross-uptake may not be strictly reciprocal. This may be due to peculiarities of the respective receptor proteins or – probably less likely when structurally closely related pyoverdines are concerned – by the ability of a strain to develop receptors for siderophores of foreign origin (Koster *et al.* 1993, 1995). As shown here, the pyoverdines from *P. chlororaphis* D-TR133 (**1**) and *P. fluorescens* CHA0 (Won-Lun-Sang *et al.* 1996) are taken up mutually by the two strains. In view of our interest to find 'key portions' of the peptide chains of pyoverdines which may explain the cross-uptake (e.g., Meyer *et al.* 2002), we decided to determine the structure of that of *P. chlororaphis* D-TR133. As will be shown the two pyoverdines have extended structural elements in common.

So far with the many pyoverdines investigated (Fuchs & Budzikiewicz 2001) all evidence pointed to the conclusion that for a given strain the peptide part of the molecule has a constant composition. Variations occur only in the chromophore part and in the dicarboxylic acid side chain in agreement with a biogenetic process which implies that an amino acid sequence is formed with the ferribactin terminus, which then is transformed to a pyoverdine with its various acid side chains (Hohlneicher *et al.* 2001). Here for the first time a minor pyoverdine was found to be formed together with 1 where specifically one of the two Ala is replaced by Gly.

Materials and methods

Instruments and chemicals

Mass spectrometry. Finnigan-MAT H-SQ 30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI, CH_3OH/H_2O 1:1); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR. DRX 500 (^{1}H 500, ^{13}C 125 MHz) (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).

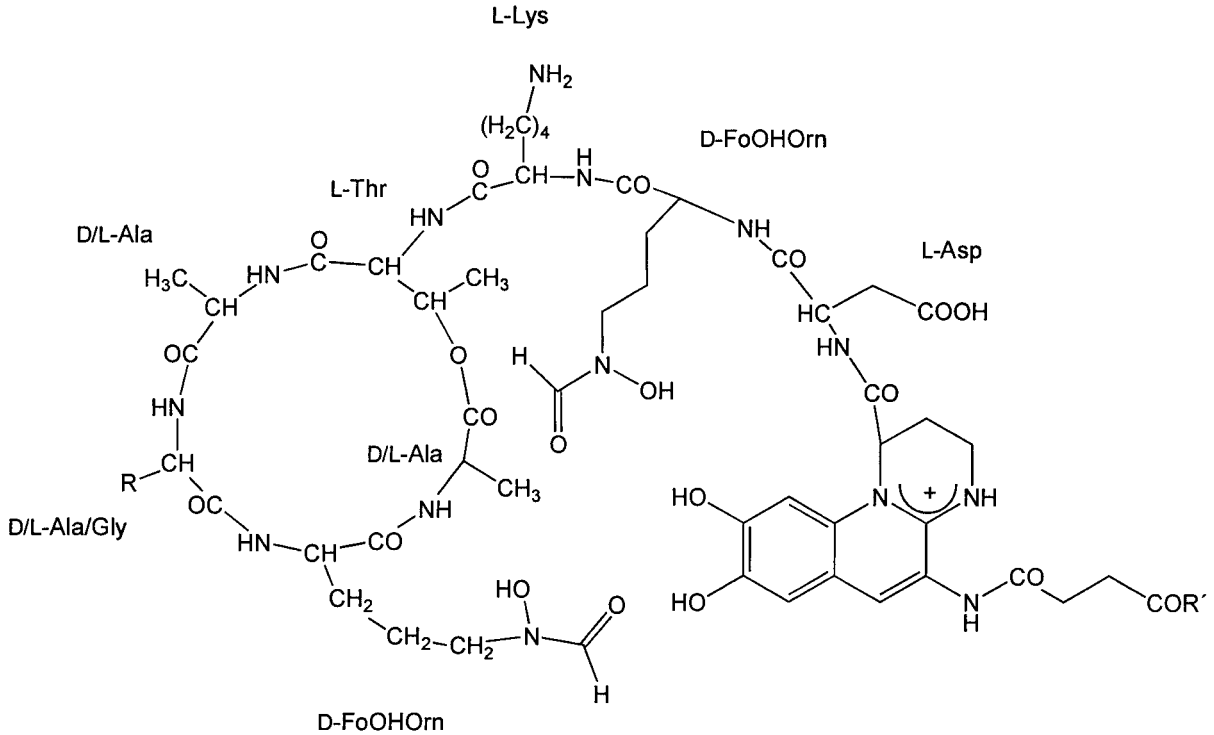
Chromatography. RP-HPLC columns Nucleosil 100-C_{18} (5 μm) and Eurospher 100-C_{18} (7 μm) (Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex C-25 (Pharmacia, Uppsala, S), Sep-Pak RP_{18} cartridges (Waters Millipore, Milford MA, USA); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt).

Siderotyping. Isoelectrofocussing and pyoverdine-mediated ^{59}Fe uptake were executed as described earlier (Meyer *et al.* 1998; Munsch *et al.* 2000).

Chemicals. Water was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column. Reagents were of p. a. quality.

Production and isolation of the pyoverdines

The strain D-TR133 isolated from soil and identified phenotypically as a *Pseudomonas chlororaphis* (Latour *et al.* 1996) was grown in a succinate minimal medium (Budzikiewicz *et al.* 1997). For the work-up of the culture after addition of ferric citrate and the isolation of the ferri-pyoverdines by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* 1999. One main fraction was obtained which was further purified by chromatography on CM-Sephadex C-25 with a pyridinium acetate buffer (pH 5.0, gradient 0.02 to 0.2 M); final purification by RP-HPLC on Nucleosil-100 with 50 mM acetic acid/methanol (gradient 3 to 30% acetic acid). Decomplexation was achieved by adsorption of the ferri-pyoverdine on a Sep-Pak cartridge and washing with a 6.5% K oxalate solution (pH 4.3). After removing all salt residues with water the free pyoverdine was eluted with methanol/water 1:1 (v/v). The solutions were brought to dryness i.v. and the samples were stored at -25 °C. The pyoverdine of *P. fluorescens* CHA0 was purified analogously.



1a: R = CH_3, R‘ = OH
1b: R = CH_3, R‘ = NH_2
2a: R = H, R‘ = OH
2b: R = H, R‘ = NH_2

Fig. 1. The pyoverdines of *Pseudomonas chlororaphis* D-TR133.

For qualitative and quantitative analysis of the amino acids by total hydrolysis and determination of their configuration by GC/MS of their TAP derivatives on a chiral column see Briskot *et al.* 1986 and Mohn *et al.* 1990.

Results

Siderotyping of P. chlororaphis D-TR133

When analyzed for its pyoverdine-mediated iron uptake specificity, strain D-TR133 demonstrated the capacity of using at an efficiency close to its own pyoverdine, the pyoverdine of *P. fluorescens* CHA0 (Table 1). On the contrary, all the other 34 pyoverdines of foreign origin which were tested (listed in Weber *et al.* 2001) were inefficient (data not shown). Reciprocally, it was controlled that strain CHA0 was exclusively able to well incorporate iron complexed by its own pyoverdine and by the pyoverdine of D-TR133 (Table 1). The pyoverdine isoelectrophoretic patterns of the two strains were, however, not identical (Table 1). These behaviors suggested that the structures of the two pyoverdines, although different according to the isoelectrophoretic patterns, should be closely related because of their functional relationships.

Characterization of **1a**

The UV/Vis spectrum of **1a** is characteristic for a pyoverdine, especially the split band (368 and 381 nm) at pH 3.0 shifted to 410 nm for the Fe^{3+} complex which in addition shows a broad charge-transfer band at ca. 500 nm (Budzikiewicz 1997a, b). The molecular mass of **1a** was determined by FAB- and ESI-MS as 1230u. *retro*-Diels-Alder fragmentation of the chromophore (Michels *et al.* 1991) (loss of the dihydroquinoline part with the side chain, 303u with subsequent loss of the succinic acid giving 203u) is in agreement with a pyoverdine chromophore (in contrast to isopyoverdines) with a succinic acid side chain. GC analysis of the TAP derivatives on a chiral column after total hydrolysis gave D- and L-Ala (1:2), L-Asp, L-Lys, D-Orn, and

Table 1. Cross-uptake of $^{59}Fe^{3+}$-pyoverdines (100% corresponds to the uptake of the own ferri-pyoverdine) and pHi values.

Bacterial strain	$^{59}Fe^{3+}$ incorporation (%) as mediated by the pyoverdines of strain		pHi values of the pyoverdine isoforms
	D-TR133	CHA0	
D-TR133	100	91	7.5; 5.4; 4.1
CHA0	63	100	8.5; 7.5; 5.3

Table 2. B-ions in the MS-CA spectrum of **1a** and **2a**.

n		**1a**, B	+ CO	+ H_2O	**2a**, B	+ CO	+ H_2O
1	Asp	473			473		
2	FoOHOrn	631			631		
3	Lys	759	787		759	787	
4	Thr – H_2O	842[a]	870		842[a]	870	
5	Ala – H_2O	913	941		913	941	
6	Ala/Gly – H_2O	984	1012	1002[c]	970	998	988[c]
7	FoOHOrn – H_2O	1142[b]		1160	1128[b]	1160	
8	Ala $[M+H]^+$	1231			1217		

[a] Accompanied by an abundant ion m/z 224 (- H_2O)
[b] Of very low abundance, but pronounced as doubly protonated ion (m/z 571.5) accompanied by one due to the loss of CO (m/z 557.5) for **1a** and m/z 564.5/550.5 for **2a**.
[c] $[B_6 - H_2O] + CO + H_2O$ m/z 1030 (**1a**) and 1016 (**2a**).

L-Thr plus succinic acid. In addition a small amount of Gly could be detected (see below).

MS and NMR analysis and determination of the amino acid sequence

The amino acid sequences of **1a** can be deduced from the B-fragment ions (cleavage of the amide bonds with charge retention at the C-terminal fragment, Roepstorff & Fohlman 1984) obtained after ESI by CA of $[M+2H]^{2+}$ in the quadrupole region and in the ion trap (Table 2). The fragmentation patterns show however some peculiarities. Typically for pyoverdines with Asp as the amino acid bound to the chromophore the ion B_1 is of high abundance. In the ion trap spectrum the B-ions up to B_3 (Lys) are observed with there appropriate masses. B_4 and all subsequent B-ions are found 18u too low. This is characteristic for Thr forming an ester bond which is opened by a McLafferty rearrangement to give dehydro-Abu (-NH-CH($CHORCH_3$)-CO- → -NH-(C=$CHCH_3$)-CO-). Starting from B_3 (Lys) all B-ions up to B_6 are accompanied by satellites 28u heavier. These species are formed by a transfer of the formyl group from the second FoOHOrn to the free ε-NH_2 group of Lys with back-transfer of one H (transacylation). B_7 contains the second FoOHOrn; here it makes no difference whether the formyl group is located on Orn or on Lys, hence no +28u ion is observed. In addition, B_6 and B_7 have accompanying ions 18u higher formed by an OH-transfer from the second FoOHOrn (B_6) and from the C-terminal COOH-group formed by the lactone opening mentioned above (B_7), respectively. For a detailed discussion of these rearrangement processes see Fuchs & Budzikiewicz 2001. The high abundance of the B_3-ion is an indication that Lys is incorporated into the peptide chain by its α-rather than its ε-amino group. The latter would make the formation of B_3^+ less likely: B^+-ion formation in normal peptides is fostered by nucleophilic attack of the CO group of the preceding amino acid yielding a five membered cycle (Schlosser and Lehmann 2000). This is not possible due to the larger distance when Lys is bound by its ε-amino group to FoOHOrn (Budzikiewicz *et al.* 1999; Fuchs & Budzikiewicz 2000). The ESI-CA fragmentation pattern of $[M+2H]^{2+}$ up to B_7+H_2O (Table 2) matches that of the pyoverdine from the strain CHA0 obtained by us for reference. This confirms the identity of the peptide chains of the two pyoverdines up to the seventh amino acid.

The pyoverdine with a molecular mass of 1330u is accompanied by a species with a mass of 1329u corresponding to a pyoverdine with a Suca side chain (**1b**). All B-fragments occur one mass units lower. Pyoverdines with a Suca side chain are the original bacterial products suffering saponification in the cultural medium and during work-up (Schäfer *et al.* 1991).

Basis for the sequence determination by NMR is the unambiguous identification of all ^{1}H- and ^{13}C-signals (Tables 3 and 4) by a combination of homo- and heteronuclear one- and two-dimensional experiments: COSY allows to detect the ^{3}J-, TOCSY higher H,H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). HMQC identifies ^{1}J-C,H, HMBC ^{2}J- and ^{3}J-coupling and allows thus to identify also quaternary C-atoms. Sequence information is obtained by NOESY/ROESY which correlates NH-protons (sharp signals are obtained with ca. 20 mM solutions in an aqueous phosphate buffer pH 4.3, suppression of the H_2O signal by presaturation or by the WATERGATE method) 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, DEPT allows to differentiate between CH/CH_3 and CH_2 groups. The ^{1}H- and ^{13}C-data of **1a** are compiled in Tables 2 and 3.

The ^{1}H- and ^{13}C-shifts of **1a** correspond to those observed with the pyoverdines from *P. fluorescens* CHA0 (Wong-Lun-Sang *et al.* 1996) and from *P. fluorescens* II (Poppe *et al.* 1987). These two differ from each other only by the replacement of Asp by Asn and from **1a** by the exchange of the C-terminal Lys by Ala, as will be shown. The following signals in the spectrum of **1a** deserve a comment: the NH-signal of Asp bound directly to the carboxyl group of the chromophore are typically shifted downfield. The shift values of the β-CH of Thr (m, 5.30 ppm, identified by COSY cross peaks with the β-C- and the α- and γ-H-signals) shows that the OH-group is esterified (downfield shift of about 1 ppm; Budzikiewicz 1997b). The presence of a succinic acid side chain can be deduced from the shift values and especially from the appropriate cross signals. For the formyl signals the typical Z/E-splitting is observed. The ^{1}H shift values of the CH-1 and of CH_2-5 do not allow a distinction between an incorporation in the peptide chain of Lys by its α-rather than its ε-amino group (Budzikiewicz *et al.* 1999). Peptidic α-linkage can however be established by COSY and TOCSY cross signals between the NH-signal at 8.29 ppm and the Lys-α-CH (4.45 ppm) and -β-CH_2 (1.76 ppm) and a NOESY cross peak with the α-CH signal of FoOHOrn (4.36 ppm).

The peptide sequence as derived from MS, ROESY/NOESY and HMBC correlations is given in Figure 1. The molecular masses of **1a** correspond to the structural details discussed above (amino acids, one cyclic substructure, succinic acid side chain). Mass spectrometric analysis of crude cultural extracts shows in varying amounts masses at m/z 1248/1249 stemming from admixtures where the ester bond of **1** was hydrolyzed; they are probably artifacts of the work-up (cf. Voßen *et al.* 2000).

In addition, MS analysis shows in addition to the masses of the $[M+H]^+$ ions at m/z 1231 and 1230 mentioned above (**1a/b**) ions at m/z 1217/1216, also a Suc/Suca pair but lacking a CH_2 group. CA fragmentation of the [M+2H]2+ ion derived from the Suc species (m/z 1217) gave a pattern differing from that of **1a** in the following way: while up to B_5 (m/z 913/941, see Table 2) the fragment masses coincide, B_6 and B_7 and their derived species including the doubly charged ones have masses 14u lower. This suggests that the second Ala (amino acid 6) is replaced by Gly (**2a**). The conclusion is confirmed by the C-terminal Y″ ions. Y″ ions of appreciable abundance are formed only when an easily protonated amino acid such as Lys is present in the respective fragment. Y''_6 (cleavage after Lys) occurs for **1a** at m/z 601 and for **2a** at m/z 587. Y''_7 from **1a** (m/z 759) coincides in mass with B_3, but for **2a** it can be recognized with appreciable abundance at m/z 745. The co-occurrence of **2** explains the detection of a minor amount of Gly in the total hydrolysate. The presence of Gly is further confirmed by the observation of a weak singlet at 3.75 ppm belonging to a CH_2 group as confirmed by DEPT which shows a CH-COSY cross peak with a signal at 44.0 ppm, values typical for Gly. The B ion masses of **2b** are 1u lower than those of **2a**.

The co-occurrence of **1** and **2** at about the same 4:1 ratio was verified for three cultures which were grown from single cells, and also for cultures in succinate medium supplemented with 1 mM glycine or 1 mM alanine.

Discussion

The structures of the pyoverdines from *P. fluorescens* CHA0 and from *P. chlororaphis* D-TR133 differ only

Table 3. ^{1}H-NMR data (δ[ppm]) of **1a** (pH 4.3; 5 °C; H_2O/D_2O 9:1)[a]

Suc	2′	3′									
	2.69	2.63									
Chr	1	2a	2b	3a	3b	$4NH^+$	6	7	10	5-NH	
	5.49	2.27	2.56	3.26	3.54	9.37	7.76	7.14	6.97	8.91	
Amino acid	NH	α	β		γ	δ	ε	NH_2		CHO_Z	CHO_E
Asp	9.06	4.50	2.67								
			3.01								
FoOHOrn	8.17	4.36	2.11		1.45	3.60				7.57	8.20
Lys	8.29	4.45	1.76		1.39	1.65	2.98	7.86			
Thr	8.20	4.65	5.30		1.27						
Ala[b]	7.85	4.17	1.31								
Ala[b]	9.06	4.11	1.46								
FoOHOrn	8.31	4.35	1.79		1.43	3.27				7.57	8.20
Ala[b]	8.31	4.30	1.37								

[a]Based on COSY and TOCSY correlations.
[b]A correlation of the respective values with specific Ala residues is not possible.

by the replacement of the Lys in the C-terminal cyclic part by Ala. It is therefore not astonishing that uptake studies with the $^{59}Fe^{3+}$-pyoverdines show that both ferri-pyoverdines are accepted by the producing strain and by the one producing the other pyoverdine (Table 1). From the otherwise identical structures it is not possible to suggest where the critical part for the recognition by the receptor lies. However, the heterologous uptakes being not strictly reciprocal, it could be postulated that the amino acid affected by the modification belongs to the recognition site. It was observed earlier that especially parts of a C-terminal cyclic substructure are likely candidates for a cross-recognition (Amann *et al.* 2000).

Also of interest is the observation that **1a/1b** is accompanied by minor amounts of **2a/2b** (ratio about 4:1). A separation by HPLC was not possible, since the structural differences do hardly change the adsorption characteristics. However, the mass spectrometric analysis demonstrates that the two pairs differ in the sixth amino acid (Ala vs. Gly) though two Ala residues are present in the molecule.

A variation of structurally related amino acids in the biogenesis of peptidic secondary bacterial metabolites has been observed before as, e.g., for peptide antibiotics produced by *Bacillus* spp. Most comonly it is a variabilty of amino acids with alkyl chains (Val vs. Ile etc.) or of aromatic ones. This is explained by a rather broad specificity of the enzymes involved in the non-ribosomal biosynthesis which also allows to foster the production of specific variants by the addition of the respective amino acid to the culture medium. Interestingly these variations seem to be restricted to specific amino acid positions in the peptide chain (e.g., Katz & Demain 1977). For species of the genus *Pseudomonas sensu stricto* (those belonging to the rRNA group I of the Pseudomonadaceae *sensu lato*) there are rare examples in the literature for the cooccurance of bacterial peptidic secondary metabolites with an alternative incorporation of structurally similar amino acids. E.g., *Pseudomonas syringae* pv. *syringae* produces variants of the tetrapeptide syringolin where in two positions Val and Ile are found to be incorporated randomly (Wäspi *et al.* 1999). The amino acid patterns of pyoverdines have been considered so far as strictly conservative for each producing strain. The only exception suggested so far is the possibly partial replacement of a formyl- by an acetyl-hydroxy-Orn unit (Kilz *et al.* 1999), though an unambiguous confirmation has not been offered so far. A recent publication may also be mentioned describing the additional incorporation (not the exchange of a amino acid present in the peptide chain) of Ile into the pyoverdine of *P. putida* BTP16 upon addition of Ile to the culture medium (Ongena *et al.* 2001).

In the present case the single cell colony culture experiments show clearly that one is not dealing with a contamination problem, i.e., with two strains having different enzymatic systems, but that the two pyoverdine varieties are produced side by side by the strain D-TR133. In view of the preceding discussion it is remarkable that the replacement of Ala by Gly is not

Table 4. ^{13}C-NMR data (δ [ppm]) of **1a** (pH 4.3; 25 °C; D_2O)[a].

Suc	1′CO	2′CH_2	3′CH_2	4′COOH					
	177.7	32.9	31.1	181.1					
Chr	CO	1	2	3	4a	5	6		
	170.3	57.9	22.8	36.5	150.5	117.8	139.9		
	6a	7	8	9	10	10a			
	117.0	129.4	144.0	152.2	101.4	115.5			
Amino acid	CO	α	β	γ	δ	$\underline{\varepsilon}$	CHO_Z	CHO_E	COOH
Asp	172.8	57.1	61.8						173.7
FoOHOrn[b]	171.2	60.7	31.1	19.6	50.7		160.5	164.9	
Lys	171.9	54.0	27.3	23.1	31.1	40.5			
Thr	171.0	56.6	74.0	16.5					
Ala[c]	172.5	51.4	16.4						
Ala[c]	177.8	52.4	16.6						
FoOHOrn[b]	171.7	55.0	29.1	24.3	50.8		160.3	164.7	
Ala[c]	173.7	52.5	17.6						

[a]Based on HMBC and HMQC spectra.
[b]Formyl values may be exchanged.
[c]A correlation of the respective values with specific Ala residues is not possible.

random but restricted to the sixth amino acid. It is, however, interesting that an addition of Gly or Ala to the culture medium did not change the ratio of production of the two pyoverdine varieties.

The remarkable number of more than 50 different pyoverdines reported in the literature allows to raise the question why such structural variations have not been discovered before. As it was pointed out above, variations in peptidic antibiotics occur either between aromatic amino acids or between those with an alkyl chain. Aromatic amino acids have not been observed in the peptide chains of pyoverdines with the only exception of a β-hydroxy His in one case (Budzikiewicz *et al.* 1997), and amongst the aliphatic amino acids only Gly and Ala have been encountered. Small amounts of a species with a molecular mass 14u lower might have been overlooked in the past as losses of H_2O (-18u), NH_3 (-17u), O (-16u) and CH_3 (-15u) are common and a low abundance -14u ion may well disappear in the ^{13}C-satellite cluster of the other ions.

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