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Bacterial Constituents CXIII [1]. Structure Revision of Several Pyoverdins Produced by Plant-Growth Promoting and Plant-Deleterious *Pseudomonas* Species

Diana Uría Fernández[1], **Valérie Geoffroy**[2], **Mathias Schäfer**[1], **Jean-Marie Meyer**[2], and **Herbert Budzikiewicz**[1,*]

[1] Institut für Organische Chemie der Universität zu Köln, D-50939 Köln, Germany
[2] Laboratoire de Microbiologie et Génétique, Université Louis Pasteur/CNRS FRE 2326, F-67083 Strasbourg, France

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Summary. The structural revision on the basis of spectroscopic and degradation results of several pyoverdins from *Pseudomonas* spp. is reported. Siderotyping studies by the isoelectrofocusing technique and by ferri-pyoverdin uptake experiments had prompted a re-investigation of some structures proposed in the literature.

Keywords. Mass spectroscopy; Natural products; *Pseudomonas*; Siderophore; Structure elucidation.

Introduction

Pyoverdins are the typical siderophores of the fluorescent members of the bacterial genus *Pseudomonas sensu stricto* (*rRNA* homology group I according to the original classification [2]). They 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 its carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to the NH_2-group (*Suca* and *Mala* in the examples below). Usually several pyoverdins are found in the culture broth of a *Pseudomonas* strain which have the same peptide chain, but differ in the nature of the dicarboxylic acid side chain. So far the structures of over fifty pyoverdins differing in their peptide chains have been reported [3]. The techniques employed for structure elucidation have been the same over the years (degradation by total or partial hydrolysis, mass spectrometric and NMR analysis), but

* Corresponding author. E-mail: h.budzikiewicz@uni-koeln.de

over-confidence in allegedly established structural patterns, interpretational errors in the evaluation of spectroscopic data *etc*. have lead occasionally to partially incorrect structures – a phenomenon generally not unknown in natural products chemistry.

There are many reasons to start a structural re-investigation. In the present case it was an extended study of *Pseudomonas* strains by pyoverdin-siderotyping in context with our investigation into the structural prerequisites for a cross-recognition of ferri-pyoverdins differing in their peptide chains by the receptor proteins of the respective producing strains. Pyoverdin-siderotyping consists in comparing the pyoverdins of fluorescent *Pseudomonas spp*. by their isoelectrofocusing (IEF) patterns and their capacity in bacterial iron transport. It has proved to be an efficient methodology to quickly recognize *Pseudomonas* strains producing identical pyoverdins [4].

IEF consists in the determination of the isoelectric points (*pH*i values) of the so-called isoforms of a pyoverdin differing in the dicarboxylic acid side chain. The *pH*i values are determined by the various acidic and basic functionalities of the pyoverdin. It is also likely that the secondary structure of the molecules plays a role as *e.g.* intramolecular hydrogen bridges will influence the character of the functionalities. There are only rare examples that pyoverdins differing in their peptide chains give identical IEF patterns. IEF allows *i.a.* the detection of structurally different pyoverdins showing a similar iron uptake behavior which can be explained by the presence of a common motif in the peptide chains of these pyoverdins, expected to represent the recognition site of the ferri-pyoverdins by their respective outer membrane receptors [5–13].

During the course of our investigations it became evident that some pyoverdins, although being described in the literature as different in structure, presented identical IEF patterns and showed a very similar iron uptake behavior, thus behaving like pyoverdins of identical structure. This suggested a re-examination of their structures. The results of two groups of strains will be reported here. They are rhizospheric isolates and had been investigated for their growth-promoting or deleterious effects on plants.

Results and Discussion

Electrospray Mass Spectrometric Analysis of Pyoverdins

The fragmentation of pyoverdins after electrospray ionization (ESI) with subsequent decomposition by collision activation (CA) has been discussed in detail [3].

To get a maximum of structural information CA under different experimental conditions has to be used. Thus $m/z = 204$, A^1 and B^1 prevail in the CA spectrum of $[M + H]^+$ in the quadrupole part of the mass spectrometer (see Experimental), while the remaining B-series is observed best by CA of $[M + 2H]^{2+}$ in the ion trap. Additional CA in the ion trap of selected fragment ions can confirm sequence conclusions.

The pyoverdin chromophore is characterized by an ion at $m/z = 204$ (loss of the peptide chain by a *retro-Diels-Alder* process in the tetrahydropyrimidine ring and loss of the side chain). Otherwise fragmentation especially in the ion trap of

Table 1. IEF and $^{59}Fe^{3+}$ cross-uptake data for the pyoverdins of *Pseudomonas sp*. A214, *P. fluorescens* A225, *P. putida* ATCC 39167, and *P. sp*. 7SR1

Pyoverdine	*pH*i-values	Pyoverdin mediated iron incorporation in strain				Amino acid sequence (open form)[a]
		A214	A225	39167	7SR1	
A214	5.1/4.5/4.0	100	103	89	96	*Ser*-*Ac*OH*Orn*-*Ala*-*Gly*-*Ser*-*Ala*-OH*Asp*-*Thr* (*Ser*-*Ala*-*Gly*-*Ser*-*Ala*-OH*Asp*-*Thr*-*Ac*OH*Orn*)
39167	5.1/4.5/4.0	90	112	100	105	(*Ser*-*Ala*-*Ac*OH*Orn*-*Gly*-*Ala*-OH*Asp*-*Ser*-*Thr*)
7SR1	5.1/4.5/4.0	111	110	97	100	*Ser*-*Ac*OH*Orn*-*Ala*-*Gly*-*Ser*-*Ser*-OH*Asp*-*Thr*
A225	5.1/4.5/4.0	97	100	64	96	(*Ser*-*Ala*-*Ac*OH*Orn*-*Gly*-*Ser*-OH*Asp*-*Ser*-*Thr*)

[a] *D*-amino acids are underlined for A214 and 7SR1; literature sequences are given in parentheses

the mass spectrometer occurs by cleavage of the peptide bonds (for the designation of fragment ions see Ref. [14]). The prevalent species are the N-terminal A- and B-ions, formally $\sim$–NH–CHR^+ and $\sim$–NH–CHR–CO^+, which allow to determine the amino acid sequence. Fragmentation processes characteristic for specific structural features will be mentioned in the discussion.

Pyoverdins Group 1

The first group comprises the pyoverdins of *Pseudomonas sp*. A214, *P. fluorescens* A225, *P. putida* ATCC 39167 and *P. sp*. 7SR1. They belong to the sub-group of pyoverdins characterized by a lactone bond between the C-terminal carboxyl group and an in-chain hydroxy-amino acid. They are usually accompanied by their hydrolysis products with a free C-terminal carboxyl group. Table 1 shows that all four have identical IEF patterns (*pH*i values of the isoforms) and high rates of cross-uptakes of their respective ferri-pyoverdins.

Pseudobactin A214

Amongst the first pyoverdins for which structural formulas have been published was pseudobactin A214, the siderophore isolated from the plant pathogenic *Pseudomonas sp*. strain A214 deleterious to beans [15]. The proposed amino acid sequence was based on partial hydrolysis which established the sequence *Ala*-*Gly*-*Ser*-*Ala*-OH*Asp*. NMR studies showed that *Ser* was the amino acid linked to the chromophore. N*O*ESY cross peaks were observed between α-CH- and NH-signals for Ser^1/Ala^1, Ala^1/Ser^2, Ala^2/OH*Asp*, and OH*Asp*/*Thr*. No cross peaks were found for *Ac*OH*Orn* which was placed at the C-terminus. Based on these data structure **1** was proposed for pseudobactin A214.

1: *Suca*-Chr-Ser^1-Ala^1-*Gly*-Ser^2-Ala^2-OH*Asp*-*Thr*-*Ac*OH*Orn*

The position of *Ac*OH*Orn* at the C-terminus was assigned because N*O*ESY cross peaks were observed between Ser^1/Ala^1 and OH*Asp*/*Thr* from which it was concluded that the respective amino acids followed each other in the sequence. What was overlooked was that *Overhauser* correlations determine spatial and not

Table 2. Sequence characteristic fragment ions observed for pyoverdin A214 in its open (**2**) and lactonic form (**3**)

Ion	Amino acid	**2**		**3**	
		m/z	$m/z + H_2O$	m/z	$m/z + H_2O$
B_1	*Ser*[1]	444		444	
B_2	*Ac*OH*Orn*	616		616	
B_3	*Ala*[1]	687		687	
B_4	*Gly*	744	762	744	762
B_5	*Ser*[2]	831			
B_6	*Ala*[2]	902	920	884	902
B_7	OH*Asp*	1033	1051	1015	
M + H	*Thr*	1152		1134	

sequentional vicinities as it had been observed between *Ala*[1] and *Ser*[2] in the sequence *Ala-Gly-Ser*. From the many pyoverdin structures established by now it is also obvious that a C-terminal OH-*Orn* is always a *c*OH*Orn*.

A mass spectral analysis revealed that the structure of pseudobactin A214 has to be revised as far as the location of *Ac*OH*Orn* is concerned: the *Suca*-Chr-*Ser* sequence follows from the ions $m/z = 204$ (RDA-decomposition of the Chr and loss of *Suca*), 416 (A_1) and 317 (A_1 – *Suca*). The sequence of B-ions (Table 2) clearly shows that *Ac*OH*Orn* is the second amino acid. The [$B + H_2O$] ions indicated in Table 2 are characteristic rearrangement ions when a OH-containing amino acid (*Ser*, OH*Asp*, *Thr*) follows the amino acid after which cleavage is observed [3]. The sequence ions expected for **1** starting from B_2, *viz.* *Ala*, $m/z = 515$, *Gly*, 572, *Ser*, 659, *Ala*, 730, OH*Asp*, 861, and *Thr*, 962 are missing. The chiralities of the amino acids of pseudobactin A214 had not been determined with the exception of *L-aThr*. Experimental details were not given except that a procedure modified from the one reported in Ref. [16] was applied. Our analysis gave *L-Ala*, *L*-OH*Asp*, *D-Orn*, *D-Ser*, *L-Thr*. The amino acid sequence of pseudobactin A214 has to be revised as follows (Fig. 1):

2: *Suca*-Chr-*D-Ser-D-Ac*OH*Orn-L-Ala-Gly-D-Ser-L-Ala-L*-OH*Asp-L-Thr*

2 is accompanied by a second pyoverdin with a molecular mass 16u higher, which suggests a Mala instead of a Suca side chain since A_1 and all B-ions are also shifted by 16u.

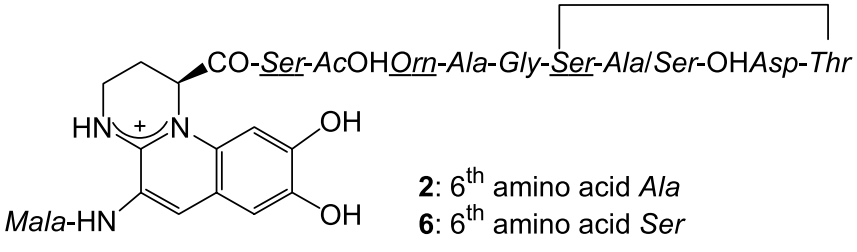

Fig. 1. Pyoverdins **2** and **6** lactone form (*D*-amino acids underlined)

The amino acid sequence of **2** corresponds to that of the revised structure [17] of the hydrolyzed form of pseudobactin 7SR1 (see **6** below), with the exception that *Ala*^{2} is replaced there by *Ser*. There is evidence that the pyoverdins with a free C-terminal carboxyl group are actually hydrolysis products of precursors with a C-terminal cyclic substructure formed by a lactone bond between the C-terminal carboxyl group and an in-chain hydroxy-amino acid. **2** is accompanied by a species with a mass 18u lower ($[M+H]^+$ $m/z = 1134$). Its CA-spectrum shows B-ions up to B_4 with the same mass as observed for **2**. Starting from B_5 they occur 18u lower (elimination from the esterified *Ser* results in the formation of dehydro-*Ala*). This suggests that as in pseudobactin 7SR1 a lactone cycle is formed between the C-terminal *Thr* and *Ser*^{2} (**3**).

Pyoverdin ATCC 39167

The structure elucidation [18] of the pyoverdin from *P. putida* ATCC 39167 was based on amino acid analysis without a determination of the chiralities, one-dimensional ^{1}H, ^{13}C and H,H-COSY NMR spectra, and sequence analysis by CA of the $[M+H]^+$ ion. Both a lactonic and a hydrolized open chain form were isolated. For the latter the sequence **4** was suggested. Which of the two *Ser* formed the lactone ring had not been determined.

4: *Mala*-Chr-*Ser-Ala-Ac*OH*Orn-Gly-Ala*-OH*Asp-Ser-Thr*

Re-investigation of the ESI CA mass spectrum of the cyclic form (mainly present in the culture isolate) indicated a sequence with lactone formation between the C-terminal *Thr* and *Ser*^{2} (Table 3). The *Mala*-Chr-*Ser* sequence follows from the ions $m/z = 204$ (RDA-decomposition of the Chr and loss of *Mala*), 432 (A_1) and 317 (A_1 – *Mala*) and 460 (B_1) obtained from CA of $[M+H]^+$ in agreement with the structure proposal for the N-terminus suggested for **4** [17]. But the masses of the subsequent B-ions obtained by CA of $[M+2H]^{2+}$ show that some of the remaining amino acids have to be rearranged. Thus, ions calculated for the further sequence proposed for **4** where the lactone ring had to be formed either with *Ser*^{1} or with *Ser*^{2} are missing (see Table 3).

Table 3. Sequence characteristic fragment ions observed for pyoverdin ATCCC 39167 (lactone form) and those calculated for the published structure **4** with a lactone ring either to *Ser*^{1} or to *Ser*^{2}

Ion	39167 (lactone)		**4** (lactone)	*Ser*^{1}	*Ser*^{2}
	Amino acid	m/z	Amino acid	m/z	m/z
B_1	*Ser*^{1}	460	*Ser*^{1} (-H_2O)	442	460
B_2	*Ac*OH*Orn*	632	*Ala*^{1}	513	531
B_3	*Ala*^{1}	703	*Ac*OH*Orn*	685	703
B_4	*Gly*	760	*Gly*	742	760
B_5	*Ser*^{2}-H_2O	829	*Ala*^{2}	813	831
B_6	*Ala*^{2}	900	OH*Asp*	944	962
B_7	OH*Asp*	1031	*Ser*^{2} (-H_2O)	1031	1031
M + H	*Thr*	1150	*Thr*	1150	1150

The mass difference between B^4 and B^5 (Table 3, 69u) corresponds to [*Ser*-H_2O] showing that the lactone ring is formed with *Ser*[2]. The chiral amino acid analysis gave the same results as had been obtained for the pyoverdin A214. Hence the pyoverdin ATCC 39167 is identical with A214 (revised structure **2** with a Mala side chain, Fig. 1).

Inspection of the original spectra [18] which had been obtained by CA of $[M+H]^+$ yielding A-ions shows that *Ala* as the third amino acid was deduced from a rather minor ion at $m/z = 503$ while an abundant one at $m/z = 605$ indicative of *Ac*OH*Orn* as the third amino acid was ignored. Starting from about $m/z = 800$ the peaks are broad, ill-resolved, and imbedded in much background noise so that an erroneous assignment is understandable.

Pyoverdin A225

The structure **5** of the pyoverdin from *P. fluorescens* A225 (lactonic and open form) was obtained in the same way as that of pyoverdin ATCC 39167 [18].

5: *Mala*-Chr-*Ser-Ala-Ac*OH*Orn-Gly-Ser*-OH*Asp-Ser-Thr*

Re-investigation as described above by CA of $[M+H]^+$ and $[M+2H]^{2+}$ (ions at $m/z = 204$, 317, 432, and 460, B-ions listed in Table 4) resulted in sequence **6** for the open form prevailing in the culture isolate identical with that of pyoverdin 7SR1. Also chiral amino acid analysis gave the same results as had been obtained for this pyoverdin. The structure of pyoverdin A225 is therefore identical with that of the revised structure of pyoverdin 7SR1 (**6**) (Fig. 1) [17].

6: *Mala*-Chr-*D-Ser-D-Ac*OH*Orn-L-Ala-Gly-D-Ser-L-Ser-L*-OH*Asp-L-Thr*

Pyoverdin Group 2

The second group comprises the pyoverdins of *P. putida* L1 (= *P. putida* CFBP 2461) and *P. putida* WCS358. They belong to the sub-group of pyoverdins characterized by a C-terminal *c*OH*Orn*. The *pH*i values and the cross-uptake rates are identical (Table 5).

Table 4. Sequence characteristic fragment ions observed for pyoverdin A225 (open form, **6**) and those calculated for the published structure **5**

Ion	**6**		**5**	
	Amino acid	m/z	Amino acid	m/z
B_1	*Ser*[1]	460	*Ser*[1]	460
B_2	*Ac*OH*Orn*	632	*Ala*	531
B_3	*Ala*	703	*Ac*OH*Orn*	703
B_4	*Gly*	760	*Gly*	760
B_5	*Ser*[2]	847	*Ser*[2]	847
B_6	*Ser*[3]	934	OH*Asp*	978
B_7	OH*Asp*	1065	*Ser*[3]	1065
$M+H$	*Thr*	1184	*Thr*	1184

Table 5. IEF and cross-uptake data for the pyoverdins of *P. putida* L1, *P. P. putida* WCS358, and *P. putida* ATCC 12633

Pyoverdine	*pH*i-values	Pyoverdin mediated iron incorporation in strain		Amino acid sequence[a]
		L1	WCS358	
L1	7.4/5.2/5.0/4.5	100	106	*Asp-ε-Lys*-OH*Asp-Ser*-a*Thr-Ala-Thr-Lys-c*OH*Orn*
WCS358	7.4/5.2/5.0/4.5	103	100	literature: **7** or **8**

[a] *D*-amino acids are underlined

Pseudobactin WCS 358 and pyoverdin CFBP 2461 (L1)

P. putida WCS 358 has been well used for studying iron metabolism and it is one of the few *Pseudomonas spp*. for which the amino acid sequence of the ferri-pyoverdin receptor was deduced from the nucleotide sequence of the encoding *pupA* structural gene [19]. Yet, there has been a problem regarding the structure of its pyoverdin. In 1986 the sequence **7** was deduced [20] from an amino acid analysis (no *D*/*L*-identification) and from a stepwise Edman degradation with some uncertainties in the identification of the Edman products (1st, 7th, and 9th amino acid)**.** The α-amino group of *Lys*[1] had to be free (otherwise an Edman degradation would not have been possible) and hence *Lys*[1] had to be linked by its ε-amino group to the chromophore.

7: *Suca*-Chr-*ε-Lys*[1]*-Ser-Asp-Thr-Ala-Thr*-OH*Asp-Lys*[2]*-c*OH*Orn*

In a subsequent publication [21] it was stated that ''the structure of pseudobactin 358 produced by *P. putida* WCS358 has recently been elucidated (*GAJM van der Hofstad*, personal communication)'' as **8**, without giving any further details or explanation.

8: *Suca*-Chr-*ε-Lys*-OH*Asp-Ser-Thr-Ala-aThr-Lys-Asp-c*OH*Orn*

Our mass spectral analysis by CA of the $[M + 2H]^{2+}$ ion indicated still another sequence with the same amino acids, changing only the position of *Asp* (see Table 6; the mass spectrum does not distinguish between *Thr* and *aThr*), *viz.* **9**.

9: *Suca*-Chr-*Asp-ε-Lys*-OH*Asp-Ser-Thr-Ala-Thr-Lys-c*OH*Orn*

The absence of the ion B_2 is characteristic for an ε-amino-*Lys* linkage as a consequence of the fragmentation mechanism yielding B-ions [22]. The presence of a Lys residue as the second amino acid is however evidenced by the Y''_8 ion at $m/z = 878$ of high abundance (only basic amino acids as *Arg* and *Lys* can compete with the chromophore for the ionizing proton yielding H_3N^+–CH*R*–CO–$\sim$ ions). Chiral amino acid analysis gave the following result: *D-Ala*, *L-Asp*, *D*-OH*Asp*, *D*- and *L-Lys*, *L-Orn*, *L-Ser*, *L-Thr*, and *D-aThr*. Hydrolysis of the pyoverdin after dansylation and chromatographic comparison with authentic material showed the

Table 6. Sequence characteristic fragment ions observed for the pyoverdin WCS 358 (**9**, **10**) and those calculated for the published structures **7** and **8**

Ion	**9**		**7**		**8**		**10**	
	Amino acid	m/z	Amino acid	m/z	Amino acid	m/z	Amino acid	m/z
B_1	*Asp*	472	*ε-Lys*	(485)	*ε-Lys*	(485)	*Asp*	472
B_2	*ε-Lys*	(600)	*Ser*	572	OH*Asp*	616	*Lys*	(600)
B_3	OH*Asp*	731	*Asp*	687	*Ser*	703	OH*Asp*	731
B_4	*Ser*	818	*Thr*	788	*Thr*	804	*Ser*	818
B_5	*Thr*	919	*Ala*	859	*Ala*	875	*Thr*	919
B_6	*Ala*	990	*Thr*	960	*Thr*	976	*Gly*	976
B_7	*Thr*	1091	OH*Asp*	1091	*Lys*	1104	*Thr*	1077
B_8	*Lys*	1219	*Lys*	1219	*Asp*	1219	*Lys*	1205
M + H	*c*OH*Orn*	1349	*c*OH*Orn*	1349	*c*OH*Orn*	1349	*c*OH*Orn*	1335

presence both of α- and of ε-dansyl *Lys* in agreement with the above conclusion that one *Lys* is incorporated amidically by its ε- and the other one by its α-amino group.

The amino acid sequence of **9** and the chirality of the amino acids correspond to that proposed for the pyoverdin CFBP 2461 [23], where, however, Lys^1 is reported to be bound into the amino acid chain by its α-amino group. The B-series of pyoverdin CFBP 2461 (shifted by 16u due to the *Mala* instead of the *Suca* side chain) agrees with that observed for **10**; also here the B_2-ion is essentially missing and the Y''_8-ion is of high abundance. This would suggest that also in pyoverdin CFBP 2461 Lys^1 is incorporated into the peptide chain by its ε-amino group. To clarify this problem the pyoverdin was subjected to hydrolysis after dansylation. Chromatographic comparison with authentic material showed the presence both of α- and of ε-dansyl *Lys* demonstrating that one *Lys* is incorporated amidically by its ε- and the other one by its α-amino group as shown above for pseudobactin WCS 358. The structure of the pyoverdin CFBP 2461 has thus to be corrected accordingly. It and pyoverdin WCS 358 are identical and should be formulated as **11** (Fig. 2).

11: *Suca*-Chr-*L-Asp-ε-L-Lys-D*-OH*Asp-L-Ser-D-aThr-D-Ala-L-Thr-D-Lys-L-c*OH*Orn*

The wrong assignment of the way of incorporation of Lys^1 into the peptide chain [23] is apparently the result of an erroneous interpretation of NMR data. Shift data of the α-CH and the ε-CH_2 groups of *Lys* incorporated into the peptide chain by the α- or the ε-amino function, respectively, do not allow a differentiation

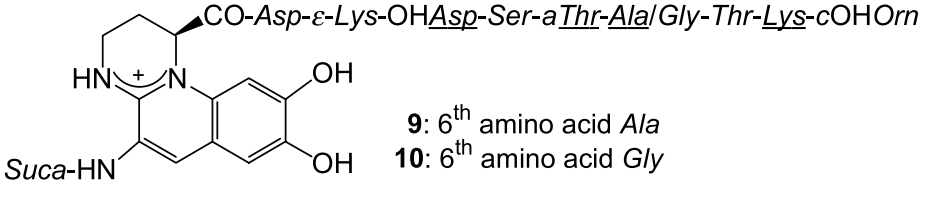

Fig. 2. Pyoverdins **9** and **10** (*D*-amino acids underlined)

[22]. In the present case it was even reported that the signals of the two *Lys* coincide. The argumentation for the α-incorporation of both *Lys* rested on the assumption that the observed cross peak in the TOCSY spectrum between *Lys*-ε-CH_2 and *Lys*-ε-NH_2 stemmed from both *Lys* residues. Possibly the shift values of the one α- and the one ε-NH coincided. The corollary is that NMR arguments for the α- and/or ε-incorporation of *Lys* into the peptide chain should be based on reliable long-range correlations between the neighboring amino acids only. So far an essentially missing B-ion after *Lys* in the ESI mass spectrum (see above) seems to be the most reliable indication for an incorporation of Lys via its ε-amino function. Identification of the *Lys* dansyl derivatives after hydrolysis is still highly recommended.

In the ESI mass spectra of the crude XAD extracts of WCS 358 and of CFBP 2461 an additional molecular species, *viz.* $[M+H]^+$ $m/z = 1335$ as well as the $[M+2H]^{2+}$ ion $m/z = 668$ in a ratio of about 100:20 was observed. The mass difference of 14u amounts to one CH_2 group. The masses of the B-ions obtained after CA of the $[M+2H]^{2+}$ ions up to B_5 correspond to those of **9**. As can be seen from Table 6 all ions starting from B_6 are shifted indicating that *Ala* is replaced by *Gly* (**10**). Accordingly also the mass of Y_8'' is 14u lower. An additional ion in the CA spectrum of **10** at $m/z = 990$ suggests that also a further variation with *Ala-Ser* instead of *Gly-Thr* could exist. Indeed a further molecular species of $[M+2H]^{2+}$ ($m/z = 661$) with an abundance of about one tenth of that of the higher homolog can be detected, and the fragments of it obtained after CA indicate the replacement of both amino acids of **9** (*Gly-Ser* as the sixth and seventh amino acid), but the intensity of the signals is so low that an unambiguous interpretation is not possible. This is the second example where the exchange of small neutral amino acids in the peptide part in the otherwise highly conservative peptide chains of pyoverdins has been observed ([13]; see the discussion there).

Conclusion

Identical IEF patterns and uptake results observed during screening programs for pyoverdin producing *Pseudomonas* strains are highly indicative of identical or at least closely related structures of the peptide part. The present studies show that the exchange of small neutral amino acids may have no influence on the IEF pattern. In cases where the structures reported in literature of pyoverdins showing identical results with both techniques differ considerably a structural re-investigation is advisable.

Experimental

Abbreviations: Common amino acids, 3-letter code; *Ac*OH*Orn*, δ-*N*-acetyl-*N*-hydroxy *Orn*; *c*OH*Orn*, *cyclo-N*-hydroxy *Orn* (3-amino-1-hydroxy-piperidone-2); *aThr*, *allo*-threonine; *Suca*, succinamide residue; *Mala*, malamide residue; Chr, pyoverdin chromophore; *TAP*, *N*/*O*-trifluoroacetyl (amino acid) isopropyl ester; GC, gas chromatography; ESI, electrospray ionization; CA, collision activation; NMR–COSY, correlated spectroscopy; N*O*ESY, nuclear *Overhauser* and exchange spectroscopy; TOCSY, total correlation spectroscopy.

Mass spectrometry: Finnigan-MAT 900S (Bremen) electrospray ionization (ESI), positive ion detection, solvent CH_3OH:H_2O = 1:1.

GC: Hewlett Packard series 5890 with series 6890 injector.

Chromatography: Columns XAD-4 (Serva, Heidelberg); Sep-Pak RP_{18} cartridges (Waters Millipore, Milford MA, USA); Chirasil-L-Val (Chrompack, Frankfurt).

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

Siderotyping: Isoelectrofocusing (IEF) and pyoverdin-mediated ^{59}Fe uptake were executed as described earlier [24, 25].

Production and isolation of the pyoverdins: For the work-up of the cultures after addition of ferric citrate, the isolation of the ferri-pyoverdins by chromatography on XAD-4 and for decomplexation on Sep-Pak cartridges see *Georgias* et al. [8]. For the analysis of the amino acids by total hydrolysis and determination of their configuration by GC/MS of their TAP derivatives on a chiral column and for the dansylation of free amino groups see Refs. [26, 27].

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