The Structures of the Pyoverdins from Two *Pseudomonas fluorescens* Strains Accepted Mutually by Their Respective Producers

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From *Pseudomonas fluorescens* PL7 and PL8 structurally related pyoverdins were isolated and their primary structures were elucidated by spectroscopic methods and degradation reactions. Despite of some structural differences both Fe(III) complexes are taken up by either strain with a high rate. The implications regarding the recognition at the cell surface are discussed.

Introduction

P. fluorescens is a member of the fluorescent species in the rRNA homology group I of the family Pseudomonadaceae and it produces siderophores ("pyoverdins") with high complexing constants. Today about 50 complete or fairly complete structures were elucidated and from preliminary studies it appears that many more are to be expected (Fuchs and Budzikiewicz, 2001; Fuchs *et al.*, 2001). Pyoverdins 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 (cf. **1**). Usually several pyoverdins co-occur having the same peptide chain, but differing in the nature of the dicarboxylic acid. 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 their Fe^{3+} complexes 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-pyoverdin is available only to the producing strain because of a highly specific interaction between a ferri-pyoverdin and its receptor outer membrane protein (Hohnadel and Meyer, 1988). However, the number of examples increases where *Pseudomonas* spp. are found to accept pyoverdins 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 pyoverdin of *P. fluorescens* ATCC 13525 (Hohnadel and Meyer, 1988; Kinzel *et al.*, 1998), several pyoverdins without (Georgias *et al.*, 1999) and several with a cyclic C-terminal part (Amann *et al.*, 2000; Weber *et al.*, 2001). For each group some

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Abbreviations: Common amino acids, 3-letter code; AcOHOrn, δ-N-acetyl-N-hydroxy Orn; FoOHOrn, δ-N-formyl-N-hydroxy Orn; cOHOrn, *cyclo*-N-hydroxy Orn (3-amino-1-hydroxy-piperidone-2); aThr, *allo*-threonine; Suca, succinamide residue; Suc, succinic acid residue; Chr, pyoverdin chromophore (see Fig. 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; HOHAHA, homonuclear Hartmann Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; WATERGATE, water suppression by gradient-tailored excitation; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane.

structural similarities were recognized. The interpretation of the results, however, is complicated by the observation that cross-uptake is not strictly reciprocal. This may be due to peculiarities of the respective receptor proteins or – probably less likely when structurally closely related pyoverdins are concerned – by the ability of a strain to develop a new receptor (Koster *et al.*, 1993). The pyoverdins from *P. fluorescens* PL7 (**1**) and PL8 (**2**) are taken up mutually by the two strains with an almost equal rate. As will be shown they have extended structural elements in common. Additional uptake studies including pyoverdins which partial structures related to those of **1** and **2** will be reported elsewhere (Meyer *et al.*, 2002).

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 (^1H 500, ^{13}C 125 MHz) (both Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS) = δ(DSS) for ^1H, 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 J-715 (Jasco, Tokyo, Japan).

CD: J-715 spectropolarimeter (Jasco, Tokyo, Japan), 1 mg ferripyoverdin in 1 ml phosphate buffer (pH 6.8), 25 °C.

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 pyoverdin-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 pyoverdins

Strains PL7 and PL8 isolated from plant roots and identified phenotypically as belonging to the *Pseudomonas fluorescens* biovar VI (biovar VI was introduced by Latour *et al.* (1996) as a miscellaneous group for strains not showing the characteristics of the classical biovars I–V) were grown in a succinate minimal medium (Budzikiewicz *et al.*, 1997). For the work-up of the culture and isolation of the ferri-pyoverdins by chromatography on XAD-4 and Biogel P-2 see Georgias *et al.* (1999). From both strains two fractions were obtained which were 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-pyoverdins on a Sep-Pak cartridge and washing with a 6.5% Na oxalate solution (pH 4.3). After removing all salt residues with water the free pyoverdins were eluted with methanol/water 1:1 (v/v). The solutions were brought to dryness i.v. and the samples were stored at −25 °C.

For qualitative and quantitative analysis of the amino acids by total hydrolysis, determination of their configuration by GC/MS of their TAP derivatives on a chiral column and dansyl derivatization of the free amino groups see Briskot *et al.* (1986) and Mohn *et al.* (1990). Partial hydrolysis was effected with 6 N HCl at 110 °C for 15 min. The peptide fragments were separated by chromatography on Bio-Gel P-2 with 0.1 M acetic acid. Subsequently they were subjected to total hydrolysis, TAP derivatization and GC-analysis as above. The 5-hydroxy chromophore from **1** and **2** for CD analysis was obtained as described by Michels *et al.* (1991).

Results

Characterization of **1** and **2**

The UV/Vis spectra of **1** and **2** (Table I) are characteristic for pyoverdins, especially the pH dependence of those of the free siderophores (Budzikiewicz, 1997a and 1997b) and the broad charge-

Table I. UV-Vis data of **1** and **2**.

Compound	log ε	λ_{max}	Compound	λ_{max}	log ε
1 (pH 3.0)	4.03	244	**2** (pH 3.0)	245	4.27
	3.88	365		364	4.11
	3.87	375		376	4.09
1 (pH 7.1)	4.32	225	**2** (pH 6.8)	224	4.49
	4.03	400		400	4.20
Fe-**1** (pH 6.8)	4.58	229	Fe-**2** (pH 6.8)	228	4.27
	4.14	266		266	3.83
	4.21	402		401	3.88
	3.73	469		469	3.44
	3.32	558		558	3.13

transfer bands of the Fe^{3+}-complexes. The molecular mass of **1** was determined by FAB- and ESI-MS as 1046u, that of **2** as 1103u. *retro*-Diels-Alder fragmentation of the chromophore (Michels *et al.*, 1991) (loss of the dihydroquinoline part with the side chain, 303 u) is in agreement with a succinic acid side chains in both compounds. The accompanying fractions mentioned above contain the respective pyoverdins with a succinamide side chain which can be hydrolyzed to give **1** and **2**, respectively. Gas chromatographic analysis of the TAP derivatives on a chiral column after total hydrolysis gave for

1: L-Ala, Gly, D- and L-Orn, D-Ser and D-aThr plus succinic acid

2: L-Ala, Gly, D-Lys, D- and L-Orn, L-Ser and D-aThr plus succinic acid.

The location of D- and of L-Orn in the peptide chains of **1** and **2** was determined by partial hydrolysis (6 N HCl, 110 °C, 20 min). From **1** fragments could be isolated by adsorption on a SepPak cartridge which contained the chromophore and only one Orn. After total hydrolysis, TAP derivatisation and GC analysis D-Ser, D-Orn, L-Ala and Gly could be identified. Hence the Orn closer to the chromophore is D- and the C-terminal cOHOrn is L-configurated. In the same way the first four amino acids of **2** were determined as D-Lys, D-Orn, L-Ala and Gly. Also here the C-terminal cOHOrn is L-configurated.

By total hydrolysis after dansylation only ε-dansyl Lys was obtained from **2** as could be shown by chromatograhic comparison with samples of authentic α- and ε-dansyl Lys; so in **2** the ε-amino group of Lys is free. For **1** no dansylated amino acid was detected. Therefore in both cases neither of the amino groups of Orn is free. The absolute configuration of C-1 of the chromophore from **1** and **2** could be determined as *S* from the CD-spectra (Cotton effect neg. 298 nm, pos. ca.370 nm) of the 4-hydroxy chromophore (exchange of the NH_2- by an OH-group) obtained by hydrolysis (Michels *et al.*, 1991).

MS and NMR analysis and determination of the amino acid sequence

The amino acid sequences of **1** and **2** can be deduced from the fragment ions obtained after ESI by CA in the ion trap (Table II): In both cases all of the so-called B-ions (Roepstorff and Fohlman, 1984), viz. X-NH-CHR-CO^+ can be identified and are accompanied in several cases by ions formed by the loss of H_2O (- 18u). **1** does not contain a basic amino acid which could be protonated and thus stabilize the positive charge in competition with the chromophore. Therefore C-terminal Y"-ions are missing. For **2** the first amino acid is Lys and in addition to the B-ions Y_7" (cleavage of the ChrCO-NH-bond after protonation of Lys) is formed (*m/z* 747). For both compounds several A-ions (B – CO) can be observed; of especially high abundance is A_1 from **1** (typical for Ser as the first amino acid; Fuchs and Budzikiewicz, 2001).

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

n	**1**	*m/z*	**2**	*m/z*
1	Ser	445	Lys	486
2	AcOHOrn	617	AcOHOrn	658
3	Ala	688	Ala	729
4	Gly	745	Gly	786
5	aThr	846	aThr	887
6	Ala	917	Ser	974
7	cOHOrn	–	cOHOrn	–

Basis for the sequence determination by NMR is the unambiguous identification of all ^{1}H- and ^{13}C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: COSY allows to detect the ^3J-, HOHAHA higher H,H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). HMQC identifies ^1J-C,H, HMBC ^2J- and ^3J-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, sup-

pression 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. The ^1H- and ^{13}C-data of **1** and **2** are compiled in Tables III-VI. They correspond to those observed with other pyoverdins (Budzikiewicz, 1997a and 1997b).

The following signals deserve a comment: the NH-signals of Ser (**1**) and Lys (**2**), resp., bound directly to the carboxyl group of the chromophore are typically shifted downfield. The shift values of the CH_2-groups of Ser (3.95 -**1**- and 3.91 -**2**- ppm) and of the β-CH of aThr (4.17 ppm -**2**) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected; Budzikiewicz, 1997b). The C-terminal cOHOrn's are characterized by the CO-resonance at 167.4 and 167.5 ppm, respectively (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). The presence of a succinic acid side chain can be deduced from the shift values and from the appropriate cross signals. It is worth mentioning that for **1** and **2** only one CH_3 and one CO signal

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

Suc	2′	3′								
	2.75	2.70								
Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.62	2.52	2.72	3.40	3.74	8.88	7.80	6.93	6.93	9.94
Amino acid	NH	α	β		γ	δ	CH_3CO			
Ser	9.58	4.50	3.95							
AcOHOrn	8.78	4.37	1.61		1.43	3.28	2.04			
			1.73			3.37				
Ala[1]	8.44	4.30	1.34							
Gly	8.46	3.95								
aThr	8.20	4.32	4.14		1.24					
Ala[2]	8.68	4.35	1.42							
cOHOrn	8.45	4.44	1.79		1.92	3.63				
			1.99		1.99	3.69				

[a] Based on COSY and TOCSY correlations.

Table IV. ^1H-NMR data (δ [ppm]) of **2** (pH 4.3; 5 °C; H_2O/D_2O 9:1)[a].

Suc	2′	3′								
	2.75	2.70								
Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.34	2.50	2.69	3.40	3.74	8.93	7.85	7.01	6.92	9.99
Amino acid	NH	α	β		γ	δ	ε	NH_2	CH_3CO	
Lys	9.55	4.37	1.86		1.40	1.70	2.99	7.65		
AcOHOrn	8.71	4.30	1.58		1.38	3.24			2.05	
			1.66			3.33				
Ala	8.51	4.26	1.33							
Gly	8.47	3.95								
aThr	8.33	4.39	4.17		1.24					
Ser	8.65	4.49	3.91							
cOHOrn	8.54	4.48	1.81		1.92	3.63				
			2.00		2.00	3.69				

[a] Based on COSY and TOCSY correlations.

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

Suc	1′CO	2′CH_2	3′CH_2	4′COOH			
	178.1	32.4	32.5	180.7			
Chr	CO	1	2	3	4a	5	6
	171.4	58.0	23.0	36.3	150.5	118.8	139.9
	6a	7	8	9	10	10a	
	115.9	115.1	144.8	152.5	101.2	132.7	
Amino acid	CO	α	β	γ	δ	$\underline{CH_3}CO$	$CH_3\underline{CO}$
Ser	172.8	57.1	61.8				
AcOHOrn	174.7	54.5	29.0	23,3	48.0	20.3	174.6
Ala[1]	176.5	51.2	17.5				
Gly	172.5	43.6					
aThr	172.5	60.4	68.1	19.7			
Ala[2]	175.5	51.0	17.7				
cOHOrn	167.5	51.4	27.7	21.1	52.7		

[a] Based on HMBC and HMQC spectra.

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

Suc	1′CO	2′CH_2	3′CH_2	4′COOH				
	178.2	32.7	32.7	181.0				
Chr	CO	1	2	3	4a	5	6	
	171.2	58.1	23.3	36.3	150.5	118.8	140.1	
	6a	7	8	9	10	10a		
	116.0	115.1	144.7	152.4	101.3	132.8		
Amino acid	CO	α	β	γ	δ	ε	$\underline{CH_3}CO$	$CH_3\underline{CO}$
Lys	172.7	55.4	30.9	23.3	27.7	40.3		
AcOHOrn	174.7	54.4	29.2	23.3	48.0		20.3	174.6
Ala	176.5	51.0	17.5					
Gly	172.5	43.6						
aThr	172.9	60.3	68.1	19.7				
Ser	172.3	56.9	62.2					
cOHOrn	167.5	51.5	27.5	21.1	52.7			

[a] Based on HMBC and HMQC spectra.

is observed for the acetyl group of AcOHOrn. While for the formyl-H and -CO in the case of FoOHOrn always two signals are present with an intensity ratio of about 3:1 corresponding to the *E*- and *Z*- conformations of the amide system, for the acetyl-CH_3 and -CO in some cases an analogous splitting, in other cases (as here) only one signal is observed. In these cases only the *Z*-conformer (H-bond between the OH and the CO) exists. Both for **1** and **2** no cross signals between the δ-CH_2-protons and the acetyl-CO could be detected in the HMBC experiment. Though the presence of AcOHOrn could be deduced from MS and NMR data, it was confirmed by treating **1** at 25 °C for 7 days with 0.1 M DCl. In this way the hydroxamic acid unit was cleaved. In the ^1H-NMR spectrum of the resulting OHOrn derivative of **1** the CH_3-signal of the acetyl group had disappeared, that for CH_3COOH (2.12 ppm) emerged and the signals for the δ-Orn NH_2 were shifted upfield to 3.11/3.14 ppm (Budzikiewicz, 1997b). The molecular mass as determined by FAB-MS was now 1004 u (loss of CH_2CO). For **2** the comparison of the MS and NMR data with those of **1**

was considered as sufficient to prove the presence of AcOHOrn.

The peptide sequence as derived from MS, ROESY/NOESY and HMBC correlations is given in Figs. 1 and 2 (for the positions of D- and L-Orn see above). The molecular masses of **1** and **2** correspond to the structural details discussed above (amino acids, one cyclic substructure, succinic acid side chain).

CD spectra of the ferri complexes

The CD spectra of the ferri complexes of **1** and **2** coincide as far as the extrema are concerned (the $\Delta\varepsilon$ values differ slightly) with a negative Cotton effect at ca. 500 nm indicative for a Δ-configuration (cf. the *P. fluorescens* GM-II pyoverdin with Λ-configuration giving a positive Cotton effect, Mohn *et al.*, 1994).

Siderotyping

The isoelectrofocussing analysis of the pyoverdin isolates shows three bands for both strains (Table VII). The number of the pHi bands indicates the different carboxylic acid (amides) (Fuchs *et al.*, 2001) connected to the chromophore as obtained under standardized growth conditions, the pHi

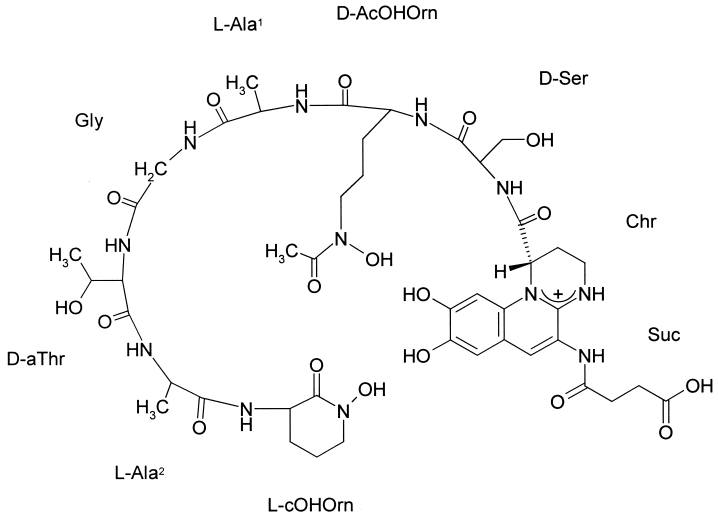

Fig. 1. Primary structure of **1**.

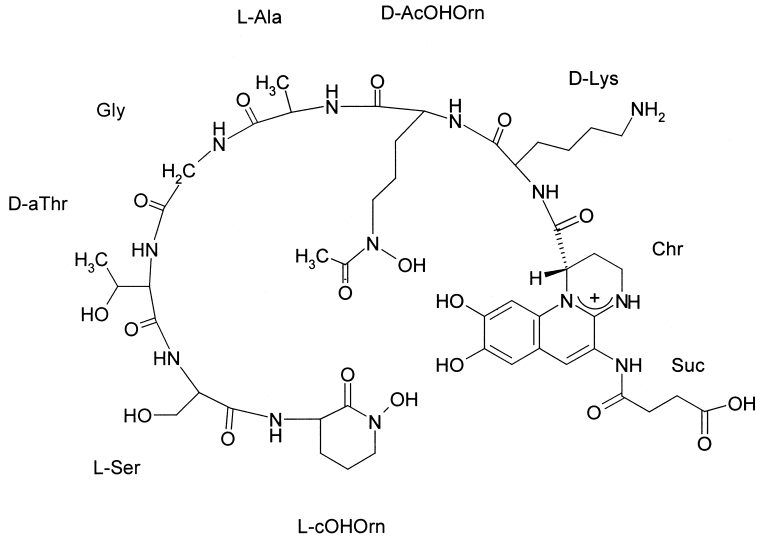

Fig. 2. Primary structure of *2*.

Table VII. Cross-uptake of $^{59}Fe^{3+}$-pyoverdins (100% correspond to the uptake of the own ferri-pyoverdin) and pHi values.

	Producing strain		pHi
Accepting strain	*P. f.* PL7	*P. f.* PL8	
P. f. PL7	100	91	7.7, 5.2, 4.2
P. f. PL8	85	100	9.0, 8.9, 7.6

values reflect the acidic and/or basic functionalities of the latter and of the amino acids present in the peptide chain. The more basic values for PL8 as compared with PL7 is due to the replacement of Ser by Lys. ^{59}Fe-pyoverdin uptake values are the average of three experiments.

Discussion and Results

The peptide sequences of **1** and of **2** show obvious similarities (D-amino acids are underlined):

1: Suc-Chr-Ser-AcOHOrn-Ala-Gly-aThr-Ala-cOHOrn

2: Suc-Chr-Lys-AcOHOrn-Ala-Gly-aThr-Ser-cOHOrn

As shown in Table VII uptake studies of the $^{59}Fe^{3+}$-pyoverdins show that ferri-**1** and -**2** are accepted with almost the same rate by the producing strain and by that producing the other pyoverdin (heterologous uptake). A free ε-NH_2 group of Lys close to the chromophore is not essential for the cell surface recognition as became obvious in derivatisation studies of other pyoverdins (Kinzel *et al.*, 1998). The critical part for the recognition seems to lie within the C-terminal part of the amino acid chain, an exchange of a small neutral amino acid (Ala/Ser) being of no importance (cf. Weber *et al.*, 2001).

Of importance is certainly also the three-dimensional structure of the Fe^{3+}-complexes. As mentioned above they show identical CD-extrema and they are Δ-configurated. Mohn *et al.* (1994) had developed a method to approximate the three-dimensional structures of ferri-pyoverdins in solution by NMR-studies of the isomorphic Ga^{3+}-complexes. Both Fe^{3+} and Ga^{3+} form octahedral complexes and their ion radii are almost equal (Ga^{3+} 62 and Fe^{3+} 65 pm), but Ga^{3+} is diamagnetic and its complexes are therefor amenable to NMR. Basis for the calculations are the dihedral angles obtained from the $^3J(NH,CH\alpha)$ coupling constants and proton distances derived from $^1H,^1H$-NOESY spectra. From these calculations (Tappe, 1995, Barelmann, 1998; the details will be presented elsewhere) it follows that the three-dimensional structures of the two Ga^{3+}-complexes are very similar with the metal ion lying at the surface of the complex (typical for all pyoverdin complexes from which three-dimensional structures were reported; see Atkinson *et al.*, 1998) forming a somewhat distorted octahedron.

The rather low temperature dependence (between $-6{\cdot}10^{-3}$ and 10^{-2} ppm/°C except for the 2nd to 4th amino acid Orn-Ala-Gly) of the amide NH-resonances (pH 4.3) shows that in the range 5–35 °C no major conformational changes occur (Kessler *et al.*, 1983; Ohnishi and Urry, 1969). Orn/Ala/Gly lie above the aromatic part of the chromophore which shields their NH-protons from exchange with solvent molecules. This explains the lower temperature dependence of their ^1H-resonances (ca. $-3{\cdot}10^{-3}$ ppm/°C for Orn/Ala and almost 0 for Gly). The four C-terminal amino acids form a free lying loop which could contain the "key" for the "lock" of the receptor protein.

The linear temperature dependence between 5 and 25 °C with coefficients between $-6.8{\cdot}10^{-3}$ and $-1.1{\cdot}10^{-4}$ ppm/°C for uncomplexed **1** and between $-6.4{\cdot}10^{-3}$ and $-9.4{\cdot}10^{-3}$ ppm/°C for **2** corresponds to values typical for amides and allows to exclude substantial conformational changes also here.

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