A Pyoverdin from the Antarctica Strain 51W of *Pseudomonas fluorescens**

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Dedicated to Professor Lothar Jaenicke on the occasion of his 75th birthday

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From the strain 51W of *Pseudomonas fluorescens* living under extreme conditions at the Schirmacher Oasis (Antarctica) a pyoverdin was obtained. Its structure was elucidated by chemical degradation and spectroscopic methods. The NMR data of the pyoverdin and of its Ga(III) complex were compared. Appreciable influences of the metal on the chemical shifts of the atoms at its binding sites were observed. Thus the structural elements involved in the complexation can be identified and coinciding signals of amino acids occurring more than once in the peptide chain can be separated.

Introduction

Many bacteria respond to iron deprivation by the synthesis and excretion of low-molecular-mass iron chelating compounds (siderophores). These molecules convert polymeric ferric oxyhydroxides, present in aerobic environments, into soluble chelats suitable for bacterial transport mechanisms. A large number of structurally diverse compounds were identified ranging from simple molecules as salicylic acid to complex chromopeptides. *Pseudomonas* bacteria of the fluorescent group produce pyoverdin siderophores. Many structures of pyoverdins have been determined (see Budzikiewicz, 1993 and 1997; Kilz *et al.*, 1998). They all comprise a yellow-green fluorescent chromophore bound by its 1-carboxyl group to the N-terminus of a cyclic or linear peptide of six to twelve amino acids, both D and L. The peptide contains two amino acids that function as ferric-specific bidentate ligands, usually either two hydroxamate units derived from Orn or one hydroxamate and one α-hydroxy carboxylate. The high affinity chelation of Fe^{3+} occurs octahedrally through these bidantate groups and the chromophore catechol unit. Cross-feeding experiments have shown that the various fluorescent *Pseudomonas* spp. usually only accept their own pyoverdin characterized by the peptide's amino acid composition, sequence and configuration.

Shivaji *et al.* (1989) isolated and identified ten cultures of *Pseudomonas* spp. from soil samples from an oasis region in Antarctica. These bacteria appeared to possess atypical characteristics by which they differ from corresponding mesophilic species such as the ability to grow at low temperatures (4 °C) and their sensitivity to antibiotics like streptomycin and tetracycline. The pyoverdin-mediated iron uptake was systematically investigated by iron-uptake studies, cross feeding experiments as well as isoelectric focussing. The expression of iron-repressible outer membrane proteins (IROMPS) was also analyzed. These studies showed that one of the species, *Pseudomonas fluorescens* 51W, produces unique pyoverdins (Meyer *et al.* 1998). In this paper we describe the isolation and structure elucidation of two pyover-

Abbreviations: Common amino acid, 3-letter code; OHAsp, β-hydroxy aspartic acid; c-(OH)Orn, cyclo-N^5-hydroxyornithine (3-amino-1-hydroxy-piperidone-2); DSS, 2,2-dimethyl-5-silapentane-5-sulfonate; ESI, electrospray ionization; FAB-MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatograph coupled with a mass spectrometer; NMR-techniques: COSY, correlation spectroscopy; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlated spectroscopy; HMBC, heteronuclear multiple bond correlation: HMQC, heteronuclear multiple quantum coherence; RP-HPLC, reversed phase high perfomance liquid chromatography; TAP, trifluoroacetyl (amino acid) O-2-propyl ester; TMS, tetramethylsilane.

* Part LXXX of the series "Bacterial Constituents". For part LXXIX see Kilz *et al.* (1998).

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dins from *P. fluorescens* 51W and the analysis of the Ga(III) complex of one of them (pyoverdin Suc–Pyo51W).

Experimental Procedures

Spectroscopy

Mass spectrometer: Finnigan MAT Incos 500 (GC/MS) with Varian 3400 gas-chromatograph, Finnigan MAT HS-Q 30 Model 11 NF (FAB; matrix thioglycerol, dithiodiethanol) with Ion Tech FAB-gun, gas Xe, CID experiments (Ar, 30 eV); Finnigan-MAT 900 ST (ESI; 50 nM/ml solution of methanol/water/1% acetic acid (50/50/1, v/v/v). Sample preparation: removal of inorganic salts with water by adsorption on Sep-Pak RP_{18} cartridges, desorption with methanol/water (1/1, v/v).

NMR: Bruker AM 300 (^1H 300, ^{13}C 75.5 MHz). Sample preparation: samples were dissolved in 0.6 ml 0.1 M phosphate buffer (pH 4,3), brought to dryness. For ^1H, ^{13}C, DEPT, ^1H,^1H-COSY, HMQC and HMBC the residue was dissolved in D_2O, brought to dryness (oil pump vacuum) and redissolved in 0.6 ml D_2O for measurement. For TOCSY and ROESY the residue was redissolved in 0.6 ml D_2O/H_2O (1/9, v/v). ^1H- and ^{13}C-chemical shifts are given relative to TMS with the internal standard DSS using the correlation $\delta(TMS) = \delta(DSS)$ for ^1H and $\delta(TMS) = \delta(DSS) - 1.61$ ppm for ^{13}C.

UV/Vis: Perkin-Elmer Lambda 7, substances were dissolved in aqueous buffers pH 7 and pH 3.

Circular dichroism: Jasco J-715, 1 mg samples were dissolved in 1 N HCl, measurements were performed at 20 °C.

Chromatography

Serva Servachrom-XAD-4 (0.3–1.0 mm), Waters Sep-Pak RP_{18} cartridges; Bio-Rad-Lab Bio-Gel P-2 (200–400 mesh); Pharmacia CM-Sephadex C-25; GC: Chrompack Chirasil-L-Val; HPLC: Knauer Nucleosil-100 C_{18} – 5 μm, Knauer Polygosil-60 C_{18} – 7 μm.

Bacterial growth

Pseudomonas fluorescens 51W was grown in an artificial medium (4 g succinic acid, 6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$ and 0.2 g $MgSO_4$ in 1 l H_2O; pH adjusted to 6.5 with 40% KOH) in 500 ml Erlenmeyer flasks containing 250 ml culture medium. After 65 h the culture medium was acidified to pH 6 with 6 M HCl. 20 ml of a 5% Fe(III) citrate solution were added per l culture. The removal of cell material was performed by tangential filtration.

Isolation

The filtrate was passed through a XAD-4 resin, washed with 10 l H_2O and subsequently eluted with 3 l CH_3OH/H_2O (1/1, v/v). The eluate was then brought to dryness. The dried eluate was chromatographed on Bio-Gel P-2 using a 0.2 M pyridinium acetate buffer (pH 5). The main fractions were separated twice on CM Sephadex C-25 with the same pyridinium acetate buffer as mentioned before.

Decomplexation

Aqueous solution of ferric-pyoverdins were adsorbed on Sep-Pak RP_{18} cartridges and treated with 5 ml of a 5% potassium oxalate buffer and washed with 15 ml H_2O. The pyoverdins were eluted with 5 to 10 ml CH_3OH/H_2O (1/1, v/v). A final chromatography on CM-Sephadex C 25 with 0.2 M pyridinium acetate buffer (pH 5) gave pure isolates as shown by RP-HPLC on Polygosil 60-C_{18} with a 0.02 M ammonium acetate buffer (pH 6.2) containing 2 mM EDTA/l and with CH_3OH.

Total hydrolysis and TAP derivatisation

1 mg pyoverdin was hydrolyzed with 6 N HCl for 21 hours at 110 °C. The hydrolysis product was evaporated to dryness and treated for 60 minutes with 2-propanol and acetyl chloride (5/1, v/v) at 110 °C. After evaporation of the solvent the residue was dissolved in dichloromethane and treated with 0.3 ml trifluoroacetic acid anhydride for 5 minutes at 150 °C. The reaction products were carefully brought to dryness once again, dissolved in 0.2 ml dichloromethane and subjected to GC/MS analysis.

Quantitative analysis

The number of the amino acids was determined by molare response factors using 1 ml 1×10^{-6} M norleucine as an internal standard.

Partial hydrolysis

40 mg pyoverdin were hydrolyzed for 10 minutes in 6 ml 6 N HCl at 90 °C. After evaporating to dryness the residue was dissolved in 0.1 M acetic acid and chromatographed on Bio-Gel P-2, detection at 254 nm. Each fraction was subjected to PI-FAB-MS analysis.

Dansyl derivatisation

For the dansyl derivatisation of free amino groups see Briskot *et al.* (1989).

Isolation of the chromophore

14 mg pyoverdin were hydrolyzed for 7 days in 3 ml of 3 N HCl at 110 °C. The hydrolysate was brought to dryness and dissolved in 3 ml 1 N HCl. 3 ml H_2O were added and the mixture was retarded on an activated Sep-Pak catritidge. The cartridge was washed with H_2O. The methanol/HCl-eluate (100/1, v/v) was brought almost to dryness and chromatographed on Polygosil 60-C18 with 0.1% aqueous CF_3COOH/CH_3OH (85/15, v/v), detection at 254 nm.

Synthesis of Ga(III)-pyoverdin

25 mg pyoverdin were treated with Ga(III) nitrate-hydrate in 5 ml 0.2 M pyridinium acetate (pH 5) for 60 minutes at room temperature. The reaction product was evaporated to dryness and chromatographed on Bio-Gel P-2 using the same buffer, detection at 254 nm. The main fraction was brought to dryness and subjected to NMR and MS analysis.

Results and Discussion

Characterization

The UV/Vis-spectra of the pyoverdins Suca–Pyo51W and Suc–Pyo51W as well as the ferri-pyoverdins showed the characteristic maxima at 405 nm. Two broad charge-transfer bands at about 470 nm and 560 nm were identified for the ferric complexes. The molecular masses for Suca–Pyo51W and Suc–Pyo51W were determined by positive FAB-MS. $[M+H]^+$-ions in the spectra were Suc: *m/z* 1376, Suca: *m/z* 1375. The retro-Diels-Alder fragment at *m/z* 1073 was present in both spectra. The mass differences of 302 and 303 u were a hint to Suca and Suc as chromophore side chains. The exact mass of Suc–Pyo51W as determined by ESI-MS was 1375.564 u.

The derivatized amino acids were analyzed by GC/MS using a chiral Chirasil-L-Val-column. The following amino acids were identified: D-Ala, L-Ala, Gly, D-Ser, D-*allo*Thr, D-*threo*(OH)Asp, D-Glu, L-Orn, D-Lys and succinic acid. Due to the instability of hydroxamic acids, hydroxyornithine often gives Orn under the hydrolysis condition (6 N HCl).

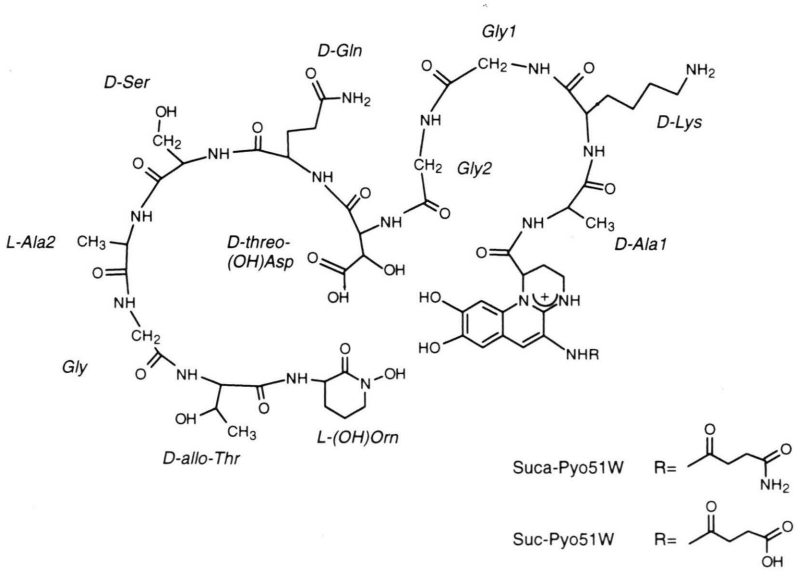

Fig. 1: Structure of pyoverdins Suca–Pyo51W and Suc–Pyo51W.

Absorption measurements of the TAP-derivatized amino acids were employed to quantify the amino acids. The relative response factors were obtained from analysis of standards normalized to leucine. Three mole equivalent of Gly were found for one pyoverdin, each of the other amino acids giving only one equivalent. The combination of amino acid analysis and mass spectrometry showed that Gln and c-(OH)Orn were hydrolyzed during amino acid analysis to give Glu and Orn (also see NMR).

The assignment of free amino groups was accomplished by reaction with dansyl chloride. The hydrolyzed dansylation products were analyzed by RP-HPLC. Co-elution of standards gave ε-DNS-lysine as the only reaction product.

NMR spectroscopy

a) Assignment of Resonances

The signals of the amino acids, the side chain and the chromophore in the ^{1}H-NMR-spectra were identified by H,H-COSY and TOCSY at two different temperatures as well as by comparison with literature data. ^{13}C-NMR signals were assigned by HMQC and HMBC. The ^{1}H- and ^{13}C-chemical shifts are given in Table I and Table II. Those of the chromophore and the side chains correspond other pyoverdins (Budzikiewicz, 1993 and 1997). The low-field shift of the NH–Ala^1-proton (9.52 ppm) is caused by the deshielding effects of the aromatic part of the chromophore. A connectivity other than by the α-NH can be excluded by the chemical shifts observed for multi-functional amino acids such as Lys, (OH)Asp, Gln, Ser, and *allo*Thr. Gln and the C-terminal amino acid N-hydroxy-cyclo-Orn were identified by their typical spin systems.

b) Sequence determination by NMR

Sequential assignments are based on $NH_i/H_{i-1}\alpha$, $NH_i/H_{i-1}\beta$ and NH_i/NH_{i-1}-NOEs obtained by ROESY-spectra of Suc–Pyo51W at T = 278 K, T = 283 K and T = 288 K. The sequence information is shown in Fig. 2.

C(OH)Orn can only be the C-terminus of the peptide chain so that the partial sequences Gly–Gly–(OH)Asp–Gln and Ser–Ala–Gly–*allo*Thr–c(OH)Orn could only be combined as

Table I. ^{1}H-NMR-data of the pyoverdins Suca–Pyo51W and Suc–Pyo51W.

Amino acid	HN 278 K	HN 288 K	α	β	γ	δ	ε	NH_2
Ala^1	9.52	9.45	4.36	1.46				
Lys	8.69	8.58	4.28	1.64	1.17	1.45	2.69	7.46
Gly^1	8.40	8.38	3.96					
Gly^2	8.39	8.34	3.99					
			4.11					
(OH)Asp	8.36	8.29	4.83	4.56				
Gln	8.57	8.49	4.41	2.02	2.35			6.90
				2.17				7.59
Ser	8.49	8.44	4.50	3.92				
Ala^2	8.54	8.46	4.38	1.43				
Gly^3	8.51	8.45	4.00					
*allo*Thr	8.22	8.17	4.39	4.16	1.24			
c(OH)Orn	8.68	8.57	4.54	1.82	2.03	3.66		
				2.05				

Chromophore	HN5	1	2a/2b	3a/3b	$HN^{+}4$	6	7	10
	9.97	5.65	2.95	3.41	8.93	7.78	7.23	7.03
			3.29	3.75				

Side chain	2′	3′	H_2N 298 K
Suc	2.76	2.69	
Suca	2.80	2.65	7.55
			6.80

Table II. ^{13}C-NMR data of the pyoverdins Suc–Pyo51W and Suca–Pyo51W.

Amino acid	CO	α	β	γ	δ	ε	CO′
Ala^1	176.0	50.7	16.6				
Lys	176.7	54.4	30.9	22.9	27.1	40.0	
Gly^1	171.7	42.8					
Gly^2	172.7	43.0					
(OH)Asp	173.4	58.1	72.8				179.1
Gln	174.3	54.4	27.4	32.1	178.3		
Ser	172.5	56.6	61.7				
Ala^2	176.3	51.0	17.1				
Gly^3	172.3	43.0					
*allo*Thr	171.7	60.0	68.1	18.9			
c(OH)Orn	167.6	51.1	27.4	20.7	52.3		

Chromophore	CO	1	2	3	4a	5	6
	171.2	57.9	23.1	35.9	156.6	117.7	140.1
	6a	7	8	9	10	10a	
	115.2	114.5	144.7	152.4	101.2	133.0	

Side chain	CO(1′)	2′	3′	CO(4′)
Suc	178.8	32.8	33.2	182.1
Suca	177.6	31.3	31.8	178.9

shown in Fig. 2. The HN shift of one Ala suggests that it is connected to the chromophore and thus forms the N-terminus. This was substantiated by MS analysis of the pyoverdin and of partial hydrolysis products.

ESI-Mass spectrometry

The sequence specific ions that were observed in the ESI-MS spectra of Suc–Pyo 51W after collision induced fragmentation either in the skimmer region or in the ion trap are illustrated in Fig. 3. The mass spectrometric experiences not only confirmed the NMR data, but enabled a full amino acid sequence determination. Note that the amino acids Gln and Lys are isobaric, but could be identified by analysis of partial hydrolysis products and NMR-spectroscopy.

Stereochemistry

The isolated 5-hydroxy chromophore (hydrolytic substitution of the original NH_2- by a OH-group) was analyzed by FAB-CID-experiments. The characteristic fragments at *m/z* 277, *m/z* 205, *m/z* 177, *m/z* 176 and *m/z* 148 were found in agreement with literature data. The CD spectrum of the chromophore gave S-configuration for C-1 as for all pyoverdins described in literature.

Ala is present in its L- and D-form. The mass spectrometric analysis of SucPyo51W as mentioned above showed that one Ala residue is directly bound to the chromophore (Chr). A partial hydrolysis gave various fractions that were analyzed by FAB-MS. One fraction contained Suc–Chr–Ala, Suc–Chr–Ala–Lys, Suc–Chr–Ala–Lys–Gly and Suc–Chr–Ala–Lys–Gly–Gly as hydrolysis products. The amino acid analysis of this fraction after total hydrolysis fraction gave D-Ala.

The Ga complex

The Fe(III) complex cannot be studied by NMR-spectroscopy since Fe(III) is paramagnetic. The diamagnetic Ga(III) forms stable complexes with pyoverdins that can undergo NMR spectroscopic analysis (Mohn *et al.*, 1994).

The UV/Vis spectrum of Ga-**1a** showed the typical maximum at 405 nm but no charge transfer bands other than the ferric-pyoverdin complex. The molecular mass of the Ga complex was determined by ESI-MS and gave *m/z* 721.6 and *m/z* 722.6 for the doubly charged $[M+2H]^{2+}$ for ^{69}Ga and ^{71}Ga.

The ^{1}H and ^{13}C resonances of the Ga(III) complex were assigned as described above. The ^{1}H chemical shifts are given in Table III, the ^{13}C shifts in Table IV. The metal binding causes strongly restricted dynamics especially in the peptide chain and in the chromophore which result in a better signal/noise ratio as compared to the free ligand. The proton chemical shifts differ from the free ligand up to 1 ppm, the ^{13}C chemical shifts up to 11 ppm. They are influenced by the metal binding as well as the shielding and deshielding effects of the aromatic part of the chromophore. The greatest effects were detected for the ligands that coordinate Ga^{3+}, namely the hydroxyaspartic acid,

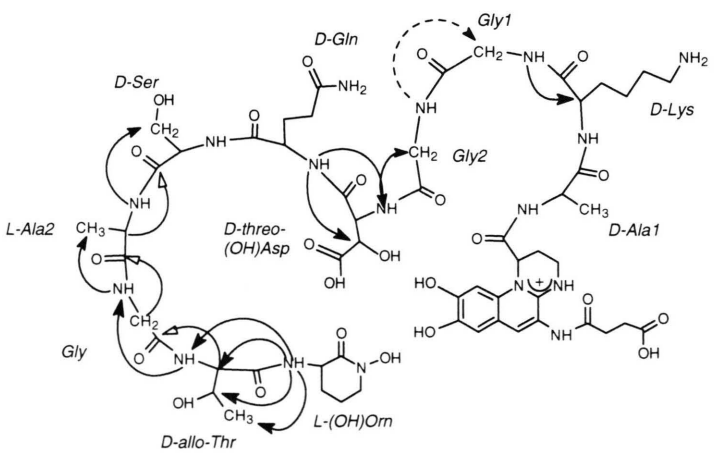

Fig. 2: Sequence specific NOE cross signals for Suc–Pyo51W (full arrows: ROESY; open arrows: HMBC; broken arrow: weak signal).

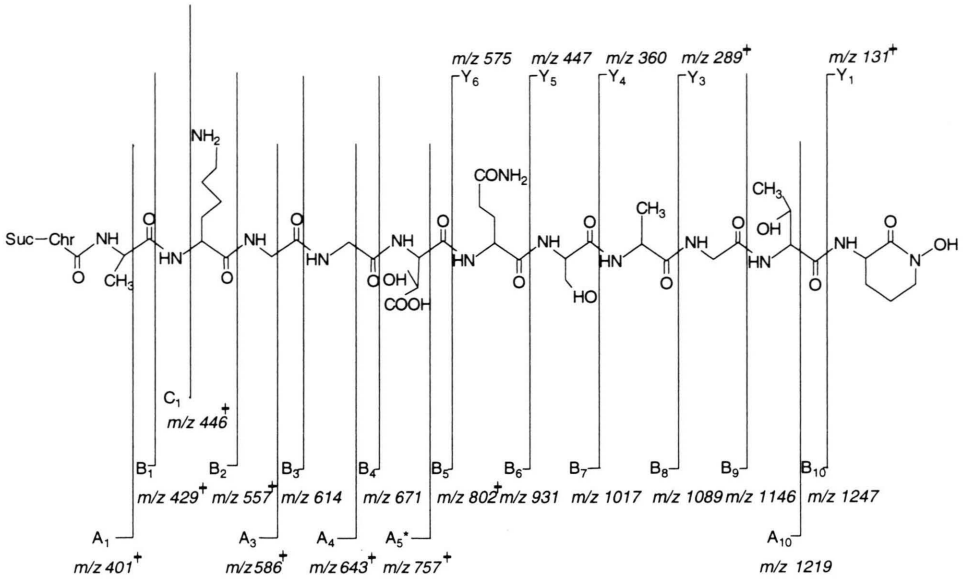

Fig. 3: Characteristic ions in the ESI mass spectrum of Suc–Pyo51W as observed by skimmer collision activated decomposition and in the ion trap (marked ions were only detected by skimmer collision activated decomposition).

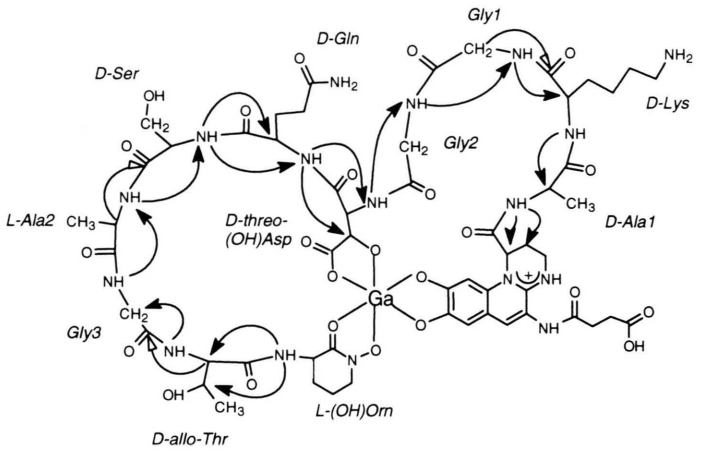

Fig. 4: Sequence specific NOE cross signals of Ga–Suc–Pyo51W.

cyclo-hydroxyornithin and the *o*-dihydroxy part of the chromophore. The larger shifts also allowed to differentiate between the Gly residues. The sequential analysis of the Ga complex was based on the same NMR-spectroscopic experiments as described for the free pyoverdin. A greater number of NOEs was detected (as shown in Fig. 4) due to the restricted dynamics of the Ga complex. This confirmed the sequence results of the sequential NMR spectroscopic and mass spectrometric analysis of the free ligand.

Conclusions

The structure of the pyoverdin 51W was elucidated by chemical degradation and spectroscopic examination of the free pyoverdin as well as of its Fe^{3+}- and Ga^{3+}-complexes. The introduction of Ga^{3+} influenced the chemical shifts of the various amino acid residues. In this way coinciding signals as *e.g.* those of the 3 Gly could be separated and especially the binding sites of the complexed metal could be determined unambiguously.

The structure elucidation of the pyoverdin was of special interest since the producing bacteria were isolated from an extreme habitat (antarctic region) and were atypical in their growth conditions and in their behavior against antibiotics (Shivaji *et al.*, 1989). The close structural relationship of its pyoverdin to the bulk of pyoverdins isolated sofar (Kilz *et al.*, 1998) shows that at least the iron uptake mechanism remained unaltered even in the absence of a competitive selection mechanism.

Table III: ^{1}H-NMR-data of Ga–Suc–Pyo51W.

Amino acid	HN	α	β	γ	δ	ε	NH_2
Ala[1]	9.34	4.44	1.45				
Lys	8.54	3.94	1.45	1.24	1.64	2.95	7.61
			1.74	1.49			
Gly[1]	8.92	4.17					
Gly[2]	9.55	3.89					
		4.35					
(OH)Asp	9.34	4.32	4.81				
Gln	7.54	3.92	1.03	1.85			6.84
			1.43				7.39
Ser	8.29	4.04	3.81				
			4.55				
Ala[2]	7.64	4.36	1.46				
Gly[3]	8.81	3.63					
		4.36					
*allo*Thr	8.34	4.17	4.00	1.23			
c(OH)Orn	8.73	4.92	1.87	2.04	3.62		
			2.16		3.82		

Chromophore	HN5	1	2a/2b	3a/3b	$NH^{+}4$	6	7	10
	9.83	5.71	2.46	3.25	8.18	7.78	6.79	7.20
			2.67	3.63				

Side chain								
Suc	2′	3′						
	2.75	2.71						

Table IV: ^{13}C-NMR-data of Ga–Suc–Pyo51W.

Amino acid	CO	α	β	γ	δ	ε	CO′
Ala[1]	174.8	50.5	17.1				
Lys	177.2	56.6	30.3	23.3	27.6	40.0	
Gly[1]	173.2	44.2					
Gly[2]	173.2	45.7					
(OH)Asp	175.8	60.4	75.2				181.4
Gln	174.4	54.9	25.5	32.8			178.0
Ser	174.2	59.3	62.3				
Ala[2]	175.1	51.2	17.1				
Gly[3]	170.5	43.1					
*allo*Thr	173.3	60.5	68.1	19.8			
c(OH)Orn	163.1	47.9	27.5	20.8	51.4		

Chromophore	CO	1	2	3	4a	5	6
	171.2	57.9	23.1	35.9	156.6	117.7	140.1
	6a	7	8	9	10	10a	
	115.2	114.5	144.7	152.4	101.2	133.0	

Side chain	CO(1′)	2′	3′	CO(4′)
Suc	178.8	32.8	33.2	182.1
Suca	177.6	31.3	31.8	178.9

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