Can the Peptide Chain of a Pyoverdin be Bound by an Ester Bond to the Chromophore? – The Old Problem of Pseudobactin 7SR1[§]

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The structure which had been proposed for the pyoverdin named pseudobactin 7SR1 (Yang and Leong, 1984) differed from those of all other pyoverdins investigated sofar: its peptide chain was supposedly linked to the chromophore not by an amide bond originating from its N-terminal amino acid, but rather by an ester bond involving one of the three Ser. It will be shown that the peptide chain of pseudobactin 7SR1 is actually bound to the chromophore amidically by its N-terminal Ser and that it comprises a *cyclo*depsipeptidic substructure with an ester bond between the C-terminal Thr and the OH-group of the second Ser in the chain.

Introduction

In 1892 Gessard summarized the earlier observations on a fluorescent compound that characterized certain strains of the bacterial genus named today *Pseudomonas.* The original name "bacterial fluorescin" or "bacterial fluorescein" was later abandoned (Elliot, 1958) for "pyoverdin" coined by Turfreijer (1941). The interest in the chemical nature of this substance continued over the years: In the thirties first attempts were made to isolate the fluorescent constituent and to get some information about its chemical nature (Giral, 1936; Turfitt, 1937; Turfreijer *et al.*, 1938). Elemental compositions (as $C_4H_7O_2N$ or $C_{32}H_{41}O_8N_7$) were suggested, but well into the sixties structural proposals resulted only in rather wild guesses – alloxazines (Giral, 1936), flavins (Birkofer and Birkofer, 1948), pyrrol derivatives (Lenhoff, 1963), pteridines (Chakrabarty and Roy, 1964). Finally, in 1981 Teintze *et al.* published the structure of the pyoverdin of a "growth-promoting *Pseudomonas*" (most likely *P. fluorescens*; Budzikiewicz *et al.,* 1998) which he named "pseudobactin". Subsequently it turned out that "pyoverdin" is a generic term for a series of related compounds having in common the (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2a]chinolin-1-carboxylic acid chromophore (see **1**) responsible for the color and the fluorescence, but differing in the composition of the peptide chain attached to the carboxyl group of the chromophore by its N-terminus (Budzikiewicz, 1997a). The structure proposed for the second representative isolated from *Pseudomonas* sp. 7SR1 (Yang and Leong, 1984) differed remarkably from that of pseudobactin: The entire peptide chain formed a cyclopeptidic ring connected to the carboxyl group of the chromophore as an *ester* by one of the three Ser present in the chain (**4**). Today the structures of over thirty pyoverdins are known and except for 7SR1 the peptide chain in every case is bound *amidically* (by the α-amino group of the N-terminal amino acid or occasionally by the ε-amino group of N-terminal Lys) to the chromophore (Kilz *et al.,* 1999). Pseudobactin 7SR1 was considered to be a curiosity until first doubts regarding the correctness of the proposed structure were raised in a recent publication of the van der Helm group (Khalil-Rizvi *et al.*, 1997) where it was suggested that the published experimental data would fit as well a

Abbreviations: Common amino acids, 3-letter code; OHAsp, β-hydroxy Asp; AcOHOrn, N^5-acetyl-N^5-hydroxy Orn; ESI, electrospray ionization; FAB, fast atom bombardment; MS, mass spectrometry; CA, collision activation; HMBC, heteronuclear multiple bond correlation; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; TAP-derivatives, N/O-trifluoroacetyl amino acid isopropyl esters.

§ Part LXXXVII of the series "Bacterial Constituents". For part LXXXVI see Lenz *et al.* (2000).

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*cyclo*depsipeptidic structure between the carboxyl group of the C-terminal Thr and a Ser in the peptide chain, which would then be bound by the N-terminal Ser amidically to the chromophore. From the strain collection of J.-M. Meyer (Strasbourg) *Pseudomonas fluorescens* P19 was obtained whose siderophore shows a remarkable relationship to that proposed for pyoverdin 7SR1: The amino acid sequence in the peptide chain is the same and it contains a *cyclo*depsipeptidic substructure compatible with the data reported for the pyoverdin 7SR1. The cyclic pyoverdin is accompanied by an acyclic one where the ester bond is opened. The structure elucidation of the two pyoverdins (**1** and **2**) and of an azotobactin (**3**) will be reported.

Materials and Methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT HSQ-30 (FAB, matrix thioglycerol/dithiodiethanol) with FAB gun 11 NF, 8 kV, FAB gas Xe (Ion Tech Ltd., Teddington, GB), Finnigan-MAT 900 ST (ESI; 50 μM solutions in CH_3OH/H_2O 1:1, v/v); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale, CA, USA) GC 3400.

NMR: AC 300, DPX 300 and DRX 500 (all Bruker, Karlsruhe). Chemical shifts are given relative to TMS with the internal standard DSS using the correlation $\delta(TMS) = \delta(DSS)$ for ^1H and $\delta(TMS) = \delta(DSS) - 1.61$ for ^{13}C.

UV/Vis: Perkin-Elmer Lambda 7 (Perkin-Elmer, Überlingen).

Chromatography: HPLC Knauer with column Nucleosil-100 C_{18} 7 μm (Knauer, Berlin); low pressure chromatography: Servachrom XAD-2 and XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond, CA, USA), QAE-Sephadex A-25 (Pharmacia, Uppsala, S); GC: column Chirasil-L-Val (Macherey-Nagel, Düren); Sep-Pak RP_{18} cartouche (Waters, Milford, MA, USA).

Chemicals: Water was desalted and distilled twice in a quartz apparatus; for HPLC it was further purified on XAD-4 resin and filtered through a sterile filter. Organic solvents were distilled over a column. Reagents (Aldrich-Sigma, Deideshofen; Fluka, Neu-Ulm; Merck, Darmstadt; Riedel de Haen, Seelze) were p. a. quality.

Production, isolation and derivatization of **1a**

The strain *Pseudomonas fluorescens* P19 came from the collection of Dr. J.-M. Meyer, Strasbourg. The culture in a succinate medium, the isolation of a XAD-2 extract of the culture medium (iron-free work-up) and the first purification step by chromatography on Biogel P-2 were performed as described earlier (Georgias *et al.*, 1999). In order to get a maximum yield of **1** the pH of the culture was allowed to rise above 9. In this way the alkali labile ester bond of **2** was completely hydrolysed. The main fraction showed at 366 nm a yellow fluorescence typical for iron-free pyoverdins.

The main Biogel fraction was brought to dryness i. v., redissolved in 0.02 M pyridinium acetate buffer (pH 5.0) and chromatographed on QAE-Sephadex A-25 with a buffer gradient (detection 254 nm). The fraction containing **1a** which eluted with 0.02 M buffer was brought to dryness i. v. and rechromatographed twice on QAE-Sephadex A-25 with a 0.1 M NaCl solution. For the removal of salt the eluate was adsorbed on a Sep-Pak RP_{18} cartouche, washed with 10 ml H_2O, desorbed with acetonitrile/H_2O 3:1 (v/v) and subsequently chromatographed twice on Biogel-P2 with H_2O. The purity of **1a** was checked with RP-HPLC, solvent 50 mM CH_3COOH with 1 mM H_2Na_2EDTA/CH_3OH gradient (3 to 78% CH_3OH).

For qualitative and quantitative analysis of the amino acids and the determination of their configuration see Briskot *et al.* (1986) and Mohn *et al.* (1990).

Production, isolation and derivatisation of **2** *and* **3**

In order to avoid hydrolysis of the ester bond of **2** the pH of the culture was kept below 8.5 by repeated addition of HCl. To further stabilize the siderophores before the removal of the cell material 10 ml 5% Fe(III)-citrate solution per liter culture were added. Work-up with XAD-4 resin and Biogel P-2 was effected as above. The main Biogel fraction was brought to dryness i. v., redissolved in 3 ml 0.2 M CH_3COOH and decomplexed with 8-hydroxyquinoline in $CHCl_3$ (Briskot *et al.*, 1986). Excess 8-hydroxyquinoline was removed by chromatography on Biogel P-2 with 0.1 M CH_3COOH. The fraction containing iron-free pyoverdins (detection at 254 nm) was brought to dryness i. v. at 30 °C, redissolved in

H_2O and again brought to dryness. This process was repeated several times.

The pyoverdin fraction showing a yellowish-green fluorescence at 366 nm was followed by one with a strong green fluorescence typical for azotobactins (Hohlneicher *et al.,* 1994), which contained **3**.

Reduction of the ester bond of **2**. A solution of 2 mg of a mixture of **2a** and **2b** and 20 mg $NaBH_4$ in 0.8 ml CH_3OH/H_2O 13:3 (v/v) was stirred at room temp. for 4 days. After addition of a few drops of glacial acetic acid for quenching the reaction, the mixture was brought to dryness i. v. at 30 °C, twice dissolved in H_2O and again brought to dryness. Total hydrolysis etc. was effected as for the pyoverdin. The mass spectrum of the tris-trifluoroacetyl derivative of threoninol (CF_3CONH-$CH(CH_2OCOCF_3)$-$CH(CH_3)$-$OCOCF_3$) obtained by TAP derivatization shows in addition to CF_3^+ (*m/z* 69) the following fragment ions: *m/z* 280 (M – CF_3CONH_2), 266 (M – CF_3COOCH_2·), 252 (M – $CF_3COOCH{\cdot}CH_3$) followed by losses of CF_3COOH (*m/z* 166, 152 and 138).

Oxidation of **2**. A solution of 3 mg of a mixture of **2a** and **2b** in 0.5 ml of a pyridinium acetate buffer containing 17 mg CrO_3 in 0.5 ml glacial acetic acid and 0.017 ml pyridine was stirred at room temp. for 3 days. The mixture was brought to dryness i. v. at 30 °C, twice dissolved in H_2O and again brought to dryness. Redissolved in 1 ml H_2O it was extracted 5 times with 0.3 ml *n*-butanol each. The aqueous phase was brought to dryness and stirred with 1 ml CH_3OH. Undissolved material was removed and the solvent was evaporated. The amino acid composition was determined as described above.

Partial hydrolysis of **2**. 15 mg of a mixture of **2a** and **2b** were hydrolyzed with 4 M HCl at 57 °C for 30 min. After evaporation to dryness i. v. at 40 °C the residue was dissolved in a small amount of 0.1 M acetic acid and chromatographed on Bio-Gel P-2 with 0.1 M acetic acid (detection at 214 and 254 nm). Several fluorescent fractions were collected and brought to dryness i. v. at room temp. Their composition was determined by ESI-MS, and the one containing the chromophore together with the first four amino acids was hydrolized and analyzed after TAP derivatization.

Results

Characterization of pyoverdin Pf P19 (**1**)

The UV/Vis spectra of **1** (pH 6.8: 405 nm, pH 3.0: splitting of the band to 379 and 371 nm) correspond to those typically observed for pyoverdins (Budzikiewicz, 1997b). The molecular mass was determined by ESI- and FAB-MS as 1168. After total hydrolysis the following amino acids could be identified: 1 L-Ala, D-*threo*-3-OH-Asp, 1 Gly, 1 D-Orn, 2 D- and 1 L-Ser, 1 L-Thr plus succinic acid.

Sequence determination by NMR

For a detailed discussion of the NMR techniques see Evans (1995). TOCSY allows to detect ^4J- and ^5J-coupling within one amino acid residue, ROESY establishes correlations between NH-protons and spacially close α- and β-H's of the preceding amino acid (C**H**-C**H**-CO-N**H**-). ^2J- and ^3J-coupling can be detected by HMBC. The experiments were performed in 15 mM solutions in H_2O/D_2O 9:1 (v/v) at pH 4.3 (phosphate buffer) to minimize the exchange rate of the amide protons. Suppression of the H_2O signal was effected by presaturation. The best separation of the NH-signals was achieved at 25 °C.

The ^1H- and ^{13}C-data are assembled in Tables I and II. Those of the chromophore and of the succinic acid side chain correspond to the ones observed for other pyoverdins (Budzikiewicz, 1997b). The signals of the amino acids could be identified by TOCSY experiments and comparison with literature data. The low-field resonance of one Ser-NH (Ser_1, 9.37 ppm) is in agreement with the direct connection by an amide bond with the chromophore. The shift values of the CH_2-groups of the 3 Ser (3.85–3.97 ppm) as well as that of the β-CH-group of Thr (4.37 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected; cf. the discussion for **2** below). For N^5-acetyl-N^5-hydroxy-Orn two sharp methyl signals in a ratio 4:1 are observed for the acetyl CH_3-group caused by the *cis, trans*-isomeric structures typical for hydroxamic acids (Budzikiewicz, 1997b). Since all amide protons of the peptide chain could be identified (see Table I) the sequence could be determined from the ROESY

spectrum as depicted in Fig. 1 (full arrows). The result is confirmed by the observed coupling of several carbonyl-C signals of the amide bonds with the NH-signals of the following amino acid (half arrows).

Sequence determination by mass spectrometry

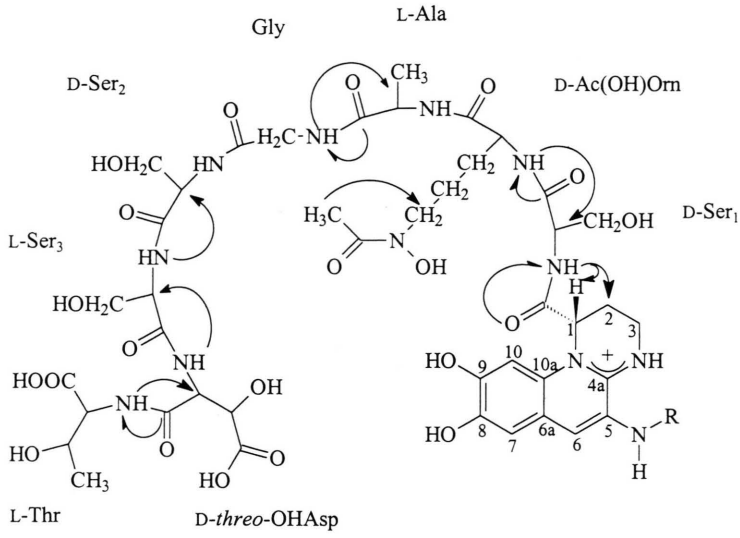
When ions are collided in a mass spectrometer with the molecules of an auxiliary gas, part of their translational energy is transformed into vibrational energy and fragmentation is induced in this way ("collision activation", CA). Peptides fragment primarily by cleavage of the amide bonds. When the charge is retained at the N-terminal part according to the current nomenclature the following fragments may be observed: NH_2-CHR$^+$ (A), NH_2-CHR-CO$^+$ (B) and NH_2-CHR-CONH$^+$ (C), a subscript indicating the position of the amino acid in the chain, and hyphens the number of attached hydrogen atoms (NH_2-CHR-CONH_2+ ... C_1'). In the Finnigan-MAT 900 ST mass spectrometer CA can be effected at various segments

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

Suc	2′	3′								
	2.86	2.82								
Chr	1	2a	2b	3a	3b	4NH$^+$	6	7	10	5-NH
	5.69	2.51	2.75	3.42	3.76	8.64	7.87	7.04	6.98	9.76
	NH	α	β	γ	δ	CH_3 *cis*	CH_3 *trans*			
Ser_1	9.37	4.53	3.97							
Ac(OH)Orn	8.55	4.35	1.61	1.44	3.31	2.05	1.86			
			1.74		3.41					
Ala	8.28	4.30	1.35							
Gly	8.32	3.99								
Ser_2	8.29	4.52	3.92							
Ser_3	8.40	4.59	3.94							
			3.85							
OHAsp	8.31	5.01	4.75							
Thr	7.99	4.46	4.37	1.21						

Table II. ^{13}C NMR data (δ [ppm]) of **1a** (pH 4.3; 25 °C; H_2O/D_2O).

Suc	1′CO	2′CH_2	3′CH_2	4′COOH				
	177.3	31.6	30.4	178.6				
Chr	CO	1	2	3	4a	5	6	
	171.4	58.0	23.2	36.4	150.5	118.8	139.9	
	6a	7	8	9	10	10a		
	115.0	115.9	144.7	152.4	101.2	132.7		
	CO	α	β	γ	δ	$CH_{3\,Ac}$	CO_{Ac} *cis*	CO_{Ac} *trans*
Ser_1	172.9	56.8	61.9					
Ac(OH)Orn	174.7	54.6	29.1	23.4	48.1	20.5	174.6	170.0
Ala	176.7	51.2	17.6					
Gly	172.8	43.8						
Ser_2	173.1	57.2	62.3					
Ser_3	173.2	56.9	62.5					
OHAsp	172.1	57.6	72.0	176.6				
Thr	175.4	60.1	68.5	20.2				



1a R = $CO\text{-}CH_2\text{-}CH_2\text{-}COOH$
1b R = $CO\text{-}CH_2\text{-}CHOH\text{-}COOH$

Fig. 1. NMR connectivities in the NMR spectra of **1a** (ROESY full arrows, HMBC half arrows).

of the instrument, *i. a.*, in the octapole before the ion trap and in the ion trap. CA in the octapole region yielded essentially only the A_1 fragment (*m/z* 417), i. e., [chromophore + $NH\text{-}CHCH_2OH$]$^+$ confirming that Ser is the first amino acid. CA in the trap allows to recognize the complete B series (Fig. 3) in addition to A_1, several ions formed by additional loss of H_2O (*m/z* 399 or 1032) and [A_7 – CO_2]$^+$ (*m/z* 978). This confirms the amino acid sequence determined by NMR.

The CA spectra of **1a** deserve a comment. The prevalent B fragments are formed according to the current opinion (Yalcin *et al.*, 1995) from molecular ions protonated at the amide nitrogen by anchimeric assistance of the preceding amide carbonyl oxygen (Scheme 1). For peptides where protonation occurs predominantly at a basic amino acid (e.g., Arg) and the NH_2-leaving group is not preformed, an alternative mechanism is observed where the OH-group of the C-terminal amino acid is back-transferred during its loss resulting in a [B_{n-1}' + OH]$^+$ (= [B_{n-1} + H_2O]$^+$) ion (Thorne *et al.*, 1990; Gonzales *et al.*, 1996). In the trap CA spectrum of **1a** (Fig. 2) [B + H_2O]$^+$ ions can be seen for B_4 (*m/z* 763) and B_6 (*m/z* 937) and with low abundance for B_3 (*m/z* 708) and B_5 (*m/z* 850). Assuming protonation of the chromophore rather than of an amide nitrogen an analogous cyclic transition (depicted in Scheme 2 for B_6) can be formulated. What kind of preferred conformations of a peptide chain may foster this process will have to await further investigations.

Pyoverdins with other side chains

Commonly several pyoverdins are found to cooccur having the same peptide chain but differing in the dicarboxylic acid side chain (Budzikiewicz, 1997a and 1997b). Screening the Biogel fraction by HPLC/UV-Vis/MS (Kilz *et al.*, 1999) the congeners with succinamide, glutamate and malamide (**1b**, see also below) side chains could be detected.

Structure of pyoverdin cyclo-Pf P19 (**2**)

The cyclic pyoverdin could be isolated with a succinamide (**2a**) ([M + H]$^+$ as determined by FAB-MS (*m/z* 1150)) and a malamide side chain (**2b**) ([M + H]$^+$ *m/z* 1166). The ^{1}H- and ^{13}C-NMR analysis including the determination of the connectivities was effected as described for **1a**. The chemical shifts of **2b** differ from those of **1a** by more than 0.1 ppm only in the terminal part of the

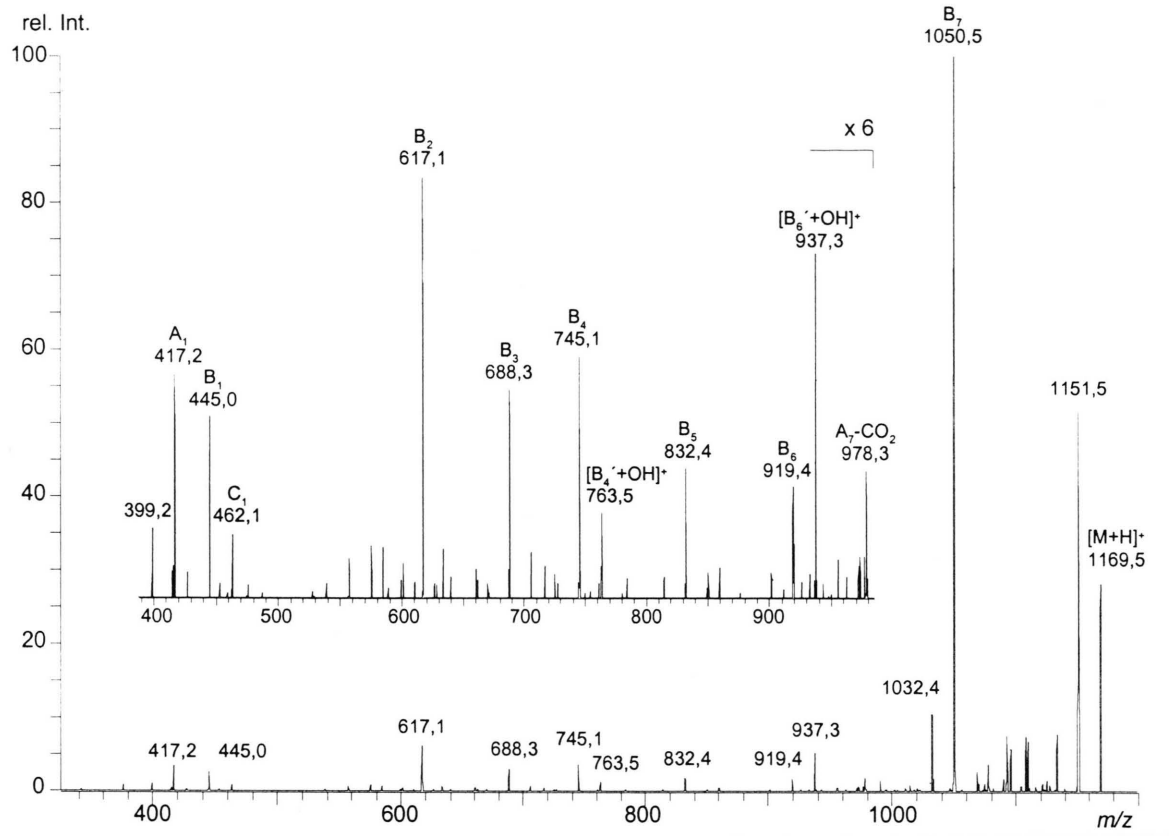
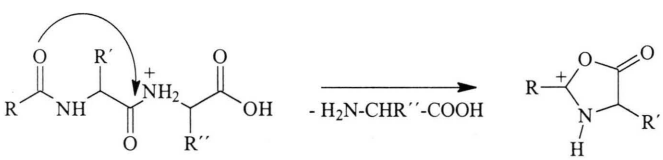


Fig. 2. Ion trap MS-CA spectrum of the $[M + H]^+$ ion of **1a**.

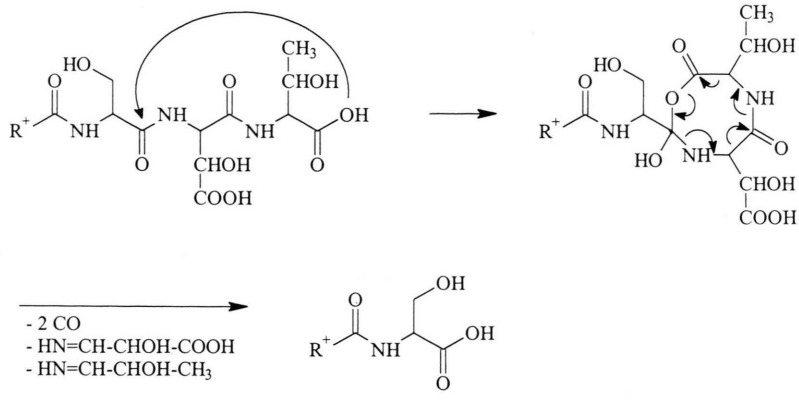


R = peptide chain
R′, R″ = side chains

Scheme 1. Formation of B-fragments.

peptide chain (see Table III). Especially remarkable is the shift of the β-protons of Ser_2 by 0.43 ppm to 4.35 which suggests the formation of an ester bond. In the ^{13}C spectrum the high field shift of the Thr-CO signal by 4.1 ppm to 171.3 indicates the participation in the ester bond. Lactone formation had occured, therefore, between the C-terminal Thr and Ser_2.

This conclusion is confirmed by the mass spectra obtained by CA of the $[M + H]^+$ ion of **2b**. As for **1** in the octapole spectrum only the A_1 ion at *m/z* 432 prevails in agreement with a structure that does not let participate Ser_1 in the lactone formation. In the trap-CA spectrum of the $[M + H]^+$ ion the sequence of the B-ions can be seen (Table IV). For a comparison with the open chain analog the data obtained for **1b** with a malamide side chain are given to allow a direct comparison. From B_1 to B_4 the two series give fragments with the same mass, but starting from B_5 (corresponding to Ser_2)



Scheme 2. Formation of the $[B_6' + OH]^+$ ion.

Table III. ^1H NMR data (δ [ppm]) of **2b** (pH 4.3; 25 °C; H_2O/D_2O).

Chr	1	2a	2b	3a	3b	4NH+	6	7	10
	5.70	2.52	2.72	3.42	3.74	8.77	7.92	7.10	7.03
	NH	α	β	γ	δ	CH_3 *cis*	CH_3 *trans*		
Ser_1	9.43	4.55	3.97						
Ac(OH)Orn	8.56	4.36	1.62 1.73	1.44	3.37	2.05	1.85		
Ala	8.27	4.35	1.36						
Gly	8.35	4.00							
Ser_2	8.30	4.54	4.35						
Ser_3	8.39	4.44	3.94						
OHAsp	8.18	4.87	4.47						
Thr	7.86	4.65	4.44	1.25					

Table IV. MS-CA spectra of **1b**- and **2b**-B-ions.

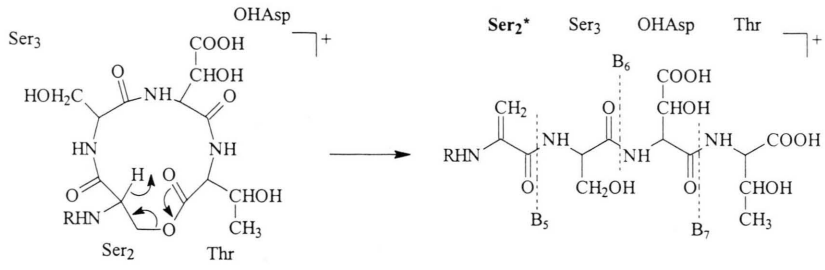
	$[M+H]^+$	B_7	B_6	B_5	B_4	B_3	B_2	B_1
2b	1166,4	1047,3	916,2	829,3	760,4	703,2	632,4	460,1
1b	1184,5	1065,5	934,4	847,4	760,3	703,3	632,2	460,1

the masses of the B-ions are by 18 u (H_2O) lower for **2b**. Accordingly, $[B_4 + H_2O]^+$ has the same mass for **1b** and **2b** (*m/z* 778), while the masses of $[B_6 + H_2O]^+$ differ by 18 u (*m/z* 952 and 934). Also A_1 has the same mass (*m/z* 432) for both compounds while for the only other member of the A series ($[A_7 - CO_2]^+$) *m/z* 993 and 975 are observed. The $[M + H]^+$ ions of *cyclo*depsipeptides are opened by a *McLafferty*-type rearrangement (cf. Roboz *et al.*, 1988) (Scheme 3) leaving Ser as (Ser – H_2O).

The final proof that the N-terminal Thr carboxyl group forms the ester bond in **2** was furnished by selective reduction with $NaBH_4$ of the ester bond (Poppe *et al.*, 1987) by which the carbonyl group is transformed to $-CH_2OH$. After total hydrolysis and TAP derivatization instead of the TAP derivative of Thr that of threoninol ($H_2NCH(CH_2OH)CH(CH_3)OH$) was found by GC/MS analysis confirmed by comparison (retention time and fragmentation pattern, see **Materials and Methods**) with an authentic sample.

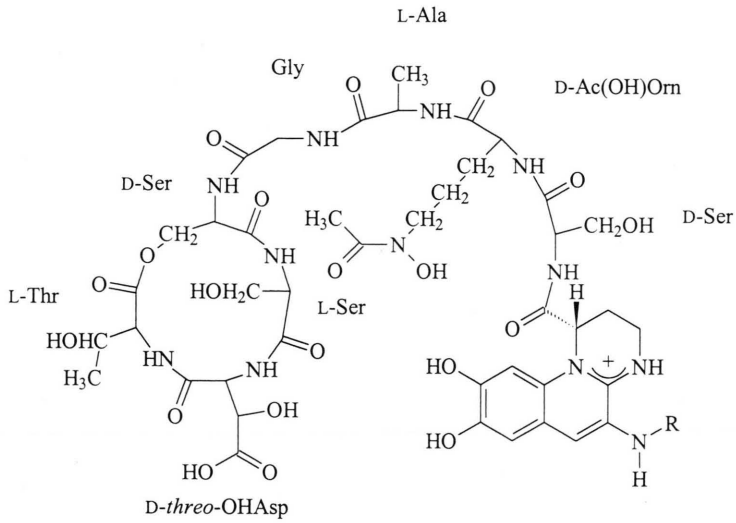
Location of the D- and L-Ser

The petide chain of **1** contains three Ser, two of them are D- and one is L-configurated. Their location was determined for a mixture of **2a** and **2b** in the following way. After partial hydrolysis a



R = Mala-Chr-Ser_1-Ac(OH)Orn-Ala-Gly

Scheme 3. Ring opening of **2** by a McLafferty rearrangement.



2a R = $CO\text{-}CH_2\text{-}CH_2\text{-}CONH_2$
2b R = $CO\text{-}CH_2\text{-}CHOH\text{-}CONH_2$

product containing the first four amino acids in addition to the chromophore and the side chains could be separated and identified by ESI-MS. Its amino acid composition was determined to be D-Ser, D-Orn, L-Ala and Gly, hence Ser_1 is D-configurated. CrO_3 oxidizes all amino acids with free hydroxyl groups. Thus, after total hydrolysis and TAP derivatization only the TAP-Ser involved in the ester bond should be detectable. By GC analysis L-Ala, Gly, D-Orn and D-Ser (and D-Glu, the oxidation product of D-Ac(OH)Orn) were found. Therefore, Ser_2 forming the ester bond is D- and consequently Ser_3 is L-configurated.

Azotobactin P19 (**3**)

Actually, azotobactins are the typical siderophores of *Azotobacter vinelandii* (Demange *et al.*, 1988; Schaffner *et al.*, 1996), but occasionally azotobactins were reported to co-occur with pyoverdins having the same peptide chain (Demange *et al.*, 1990; Hohlneicher *et al.*, 1995; Beiderbeck *et al.*, 1999). Their chromophore differs from that of the pyoverdins in the way that the two amine functions (N-4 and C-5-NH_2) are connected by a CO group (urea structure). This is reflected in the green fluorescence (in contrast to the yellowish-green one of the pyoverdins) and that the main absorption band is not split at low pH values. The values for **3** are 408 nm (pH 6.8) and 378 nm

(pH 3.0) in accordance with the literature data. The mass of the M^+ ion is 1117u. The ^1H- and ^{13}C-data (Table V; the connectivities in the aliphatic part of the ring system were confirmed by a TOCSY spectrum) of the chromophore correspond to the literature data. As compared with the data for **1** most protons are characterized by a downfield shift of 0.2–0.3 ppm except for H-3b that is located in the deshielding region of the new carbonyl group and is shifted by 0.6 ppm. In the ^{13}C-spectrum the upfield shifts of several C-signals can be noted. Both, in the ^1H- and in the ^{13}C-spectrum the signals of the dicarboxylic acid side chain are missing, but an additional CO signal (C-4b) can be seen.

The sequence of the amino acids could be confirmed by the CA spectra of M^+. In the octapole spectrum the ion A_1 is shifted to lower mass by 74 u (*m/z* 343) corresponding to the replacement of H (N-4) and $COCH_2CH_2COOH$ by CO. In the

Table V. ^1H and ^{13}C NMR data (δ [ppm]) of the chromophore of **3** (pH 4.3; 25 °C; H_2O/D_2O).

	Chr-1	Chr-2	Chr-3	Chr-4a	Chr-4b	Chr-5a	Chr-6
^1H	6.03	2.70/3.00	3.72/4.36	–	–	–	7.98
^{13}C	57.3	24.4	35.5	140.1	154.2	122.2	122.1
	Chr-6a	Chr-7	Chr-8	Chr-9	Chr-10	Chr-10a	Chr-CO
^1H	–	7.30	–	–	7.30	–	–
^{13}C	121.6	113.8	147.6	149.4	100.5	129.4	172.0

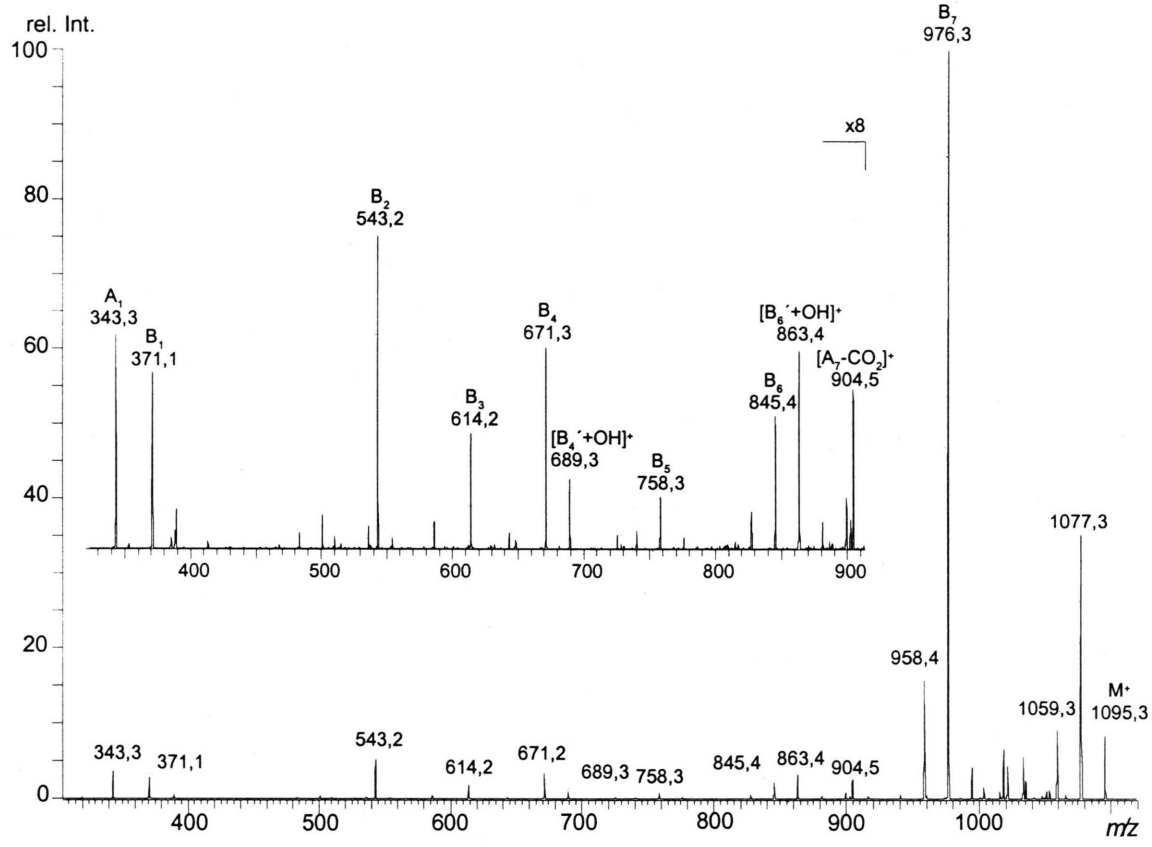
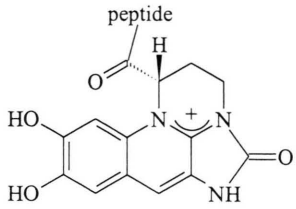


Fig. 3. Ion trap MS-CA spectrum of the M^+ ion of **3**.



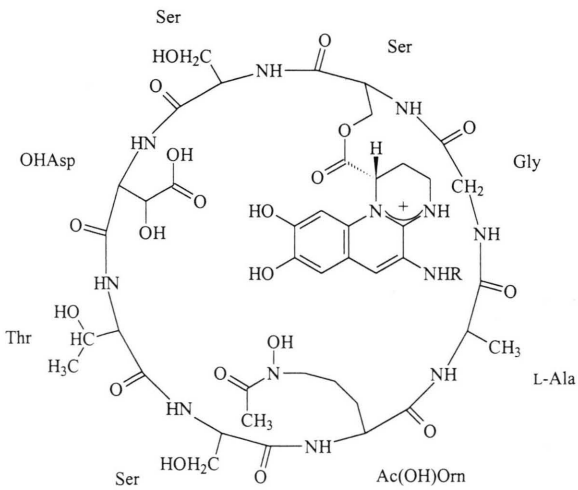
3, peptide chain as in **1**

trap spectrum (Fig. 3) the entire B-series including $[B_4 + H_2O]^+$ and $[B_6 + H_2O]^+$ as well as $[A_7 - CO_2]^+$ is shifted by 74 u.

Discussion

Co-occurance of pyoverdins with a *cyclo*depsipeptidic and with the corresponding open peptide chain was reported before by Khalil-Rizvi *et al.* (1997). Clearly the ester bond gets hydrolyzed when the pH of the culture medium is allowed to raise to 9 (see above). This suggests that the open form is an artefact. Khalil-Rizvi *et al.* reported that they obtained the open form also when the pH of the medium was readjusted repeatedly during the growth period. This does, however, not exclude local conditions where hydrolysis may occur. It is unlikely that the bacteria have two different synthetic apparatus for the pyoverdin production.

This is the fourth instance that an azotobactin was found to co-occur with the pyoverdins of a



4

Pseudomonas sp. Where it actually fits into the biogenetic scheme (Böckmann *et al.*, 1997) is not clear. It may be a sideway in the sequence of transformations of the dicarboxylic acid side chains (Schäfer *et al.*, 1991) (e.g., oxidative decarboxylation of the α-ketoglutaric acid side chain giving succinic acid, but starting from a cyclic form so that the CO_2 remains in the molecule; cf. Lenz *et al.*, 2000) or a completely different pathway near the end of the formation of the pyoverdin chromophore. Now fast screening of the culture media being possible (Kilz *et al.*, 1999) it would be interesting to check whether azotobactins can be found also in the cultures of pyoverdins producing *Pseudomonas* spp. outside the closely related *fluorescens/putida* group, but especially whether *Azotobacter vinelandii* strains also produce pyoverdins.

The actual reason for this investigation was the structure of pseudobactin 7SR1 (**4**) which does not fit into the general scheme and where doubts regarding the correct interpretation of the analytical data had been raised (see **Introduction**). Yang and Leong (1984) by NMR, amino acid analysis and partial hydrolyses had established the sequence Ser-AcOHOrn-Ala-Gly-Ser-Ser-OHAsp-Thr (though without determination of the stereochemistry of the amino acids except for L-Ala), but had offered no proof for the formation of a macrocycle by formation of an amide bond between the N-terminal Ser and the C-terminal Thr, nor for an ester bond between one of the three Ser and the chromophore. They explicitely point out that upon partial hydrolysis no fragment could be found containing Thr-Ser which would have been expected for the proposed *cyclo*octapeptidic structure.

In our attempts to find the original strain of *Pseudomonas* sp. 7SR1 for comparison we were informed by Prof. Dick van der Helm, University of Oklahoma, Norman, OK, that Dr. Jeffrey S. Buyer, USDA ARS, Beltsville, MD, could be in possession of this strain. Dr. Buyer kindly sent us a culture which in an isoelectrofocussing analysis (Koedam *et al.*, 1994) gave the same pattern as *Pseudomonas fluorescens* P19. The culture broth of this strain contained *i. a.* two compounds which were identified by ^{1}H-NMR and by their ion trap MS-CA spectra as **1b** and **2b**. This proves that the pyoverdins of *Pseudomonas fluorescens* P19 and of *Pseudomonas* sp. 7SR1 are identical belonging

to the small subgroup of pyoverdins possessing a C-terminal *cyclo*depsipeptide part (Poppe *et al.*, 1987; Won-Lung-San *et al.*, 1996; Khalil-Rizvi *et al.*, 1997; Ongena *et al.*, 1997).

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