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PYOVERDINE TYPE SIDEROPHORES FROM *Pseudomonas fluorescens*

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Abstract - The structure of pyoverdine I, II and III isolated from the culture medium of *Pseudomonas fluorescens* have been elucidated by spectroscopic methods and degradation studies. The pyoverdines consist of a chromophore which could be identified as 5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido[1,2-a]quinoline-1-R-carboxylic acid substituted at the amino group with a 4-carboxy-4-oxobutanoyl (I), a carboxymethanoyl (II) or a succinamoyl residue (III) and at the carboxyl group with the N-terminus of L-Asn-D-Orn(N^5-OH-N^5-CHO)-L-Lys-L-Thr-L(or D)-Ala-D(or L)-Ala-D-Orn(N^5-OH-N^5-CHO)-L-Lys

From a strain of *Pseudomonas fluorescens* isolated from Rhine water three siderophores of the pyoverdine type could be obtained by a procedure described earlier [2], the peptide part of which contains a hitherto undescribed cyclodepsipeptide substructure. For siderophores produced by *Ps. fluorescens* strains so far only partial structures have been reported [3-5] which are, however, detailed enough to show that none of them can be identical with our compounds, *viz.* pyoverdine I, II and III the structure elucidation of which will be reported here.

Characterization of the pyoverdines I, II and III and of their Fe^{3+} complexes (= ferri-pyoverdines)

The reddish-brown Fe^{3+} complexes exhibit charge transfer bands at 470 (log ε 3.74) and 555 nm (log ε 3.40), an absorption maximum at 400 nm (log ε 4.23) accompanied by two shoulders at 320 and 260 nm (exitation of the quinoline system) and a maximum at 230 nm (log ε 4.56) (amide absorption). In the UV spectra of the ironfree compounds obviously the charge transfer bands are missing. The position of the absorption maxima and their pH dependence (405 nm with log ε 4.17 at pH 7.2 and increasing hypsochromic shift with decreasing pH) correspond to the data given in the literature [2,3] for the dihydroxy quinoline chromophore typical for pyoverdines and related compounds.

The electrophoretic mobility (relative to desferal with glucose as zero point) of ferri-III at pH 6.5 is twice as big as that of ferri-I or ferri-II. The charge of ferri-III at pH 6.5 could be calculated by the method of Offord [6] as +2; consequently that of ferri-I and -II has to be +1. At pH 3 ferri-III has still the same mobility as at pH 6.5. The mobility of -II at this pH value approaches that of -III, suggesting the presence of a free carboxyl group in -II with a pKa between 4 and 5. The mobility of ferri-I reaches that of -III only at pH 2, hence ferri-I must possess a strongly acidic carboxyl group (pKa between 2 and 3, *cf. infra* and [2]).

The FAB mass spectra of pyoverdines exhibit $[M + H]^+$ ions at *m/z* 1315 for I, 1287 for II and 1286 for III (nominal masses; the exact masses are 0.6 u higher). Interestingly, the masses of the $[M + H]^+$ ions obtained for the ferri-compounds are not 53 u (+ $^{56}Fe^{3+}$ - 3 H^+) but 54 u higher than those of the free pyoverdines. This unexpected behaviour can be explained plausibly by assuming a reduction of $Fe^{3+} \rightarrow Fe^{2+}$ (+ $^{56}Fe^{2+}$ - 2 H^+ = 54 u) in the ion source which has also been observed

for other Fe^{3+} complexes [7]. The structurally significant fragment ion of mass *m/z* 985 present in the mass spectra of all three free pyoverdines is formed by the loss of the chromophore together with the attached acid residues, a process typical for pyoverdines [2,8]. This suggests that I, II and III differ only in the latter and possess identical peptide parts.

For an amino acid analysis the ferri-pyoverdines I, II and III were hydrolyzed with 48% HI (which avoids Fe^{3+} catalyzed side reactions of hydroxamic acids [9]), the free amino acids were transformed into their N-trifluoroacetyl-*n*-butyl esters (TAB derivatives) and identified [10] by GC/MS and quantified [11,12] by GC. The results were the same for all three compounds (2 Ala, 1 Thr, 1 Asp, 2 Orn, 2 Lys) and could be verified by quantitative ion exchange chromatography. From II and III in addition 1 mol succinic acid was obtained. As HI is known to reduce hydroxamic acids [9] the free pyoverdines were additionally subjected to a hydrolysis with 6N HCl under Ar. In this case instead of Orn N^5-hydroxy Orn could be identified as its N,N',O-tris-trifluoroacetyl butyl ester by GC/MS. It should be noted here that none of the pyoverdines [2-5,8,13,14] and pseudobactines [15-17,30] thus far described in the literature show an identical amino acid pattern.

Nature of the chromophore

It has been mentioned above that UV and mass spectral data suggest the presence of the dihydroxyquinoline chromophore typical for pyoverdines and related compounds. This chromophore could be isolated after hydrolysis (2N HCl, 100°C, 168 h) of ferri-I and identified as 2,3-dihydro-5,8,9-trihydroxy-1*H*-pyrimido[1,2-a]quinoline-1-carboxylic acid (**1**) by its NMR and EI mass spectra. $M^{+\cdot}$ occurs at *m/z* 276. The fragmentation behaviour is indicated schematically in Fig. 1. The presence of the unsubstituted N-4 and of the C-5 hydroxyl group (rather than an NH_2, cf. discussion below) was corroborated by the DEI-mass spectrum of the N,O,O,O-tetraacetyl derivative of **1** ($[M + H]^+$ *m/z* 446, $[M + H - {}^{\cdot}COOH - CH_3CO^{\cdot}]^+$ *m/z* 358, which in turn loses three times ketene: *m/z* 316, 274, and 232), as well as by the electrophoretic behaviour (**1** has at pH 6.5 and pH 3 a mobility of zero which excludes a protonable basic functionality). The ^1H- and ^{13}C-NMR data of **1** may be found in Table 1. The measured and the simulated ^1H-NMR spectra are identical with those of "compound F" (skeleton as for **1**, but substitution on N-4 and C-5 uncertain) obtained by hydrolysis of fluorescent pigments of *Ps. aeruginosa* ATCC 9027 [18] as well as of the chromophore of azotobactine which possesses at C-5 an NH group linked by CO to N-4 (urea moiety). The structure of the latter has been determined by X-ray analysis [19]. Apparently - as it had been suggested earlier [21] - under the condition of hydrolysis a C-5 amino group can be transformed readily into a hydroxyl function. The presence of a C-5 amide group in the original chromophore could be demonstrated indirectly by its stability towards $NaBH_4$ (*v. infra*) and by the fact that the hydrolysis product

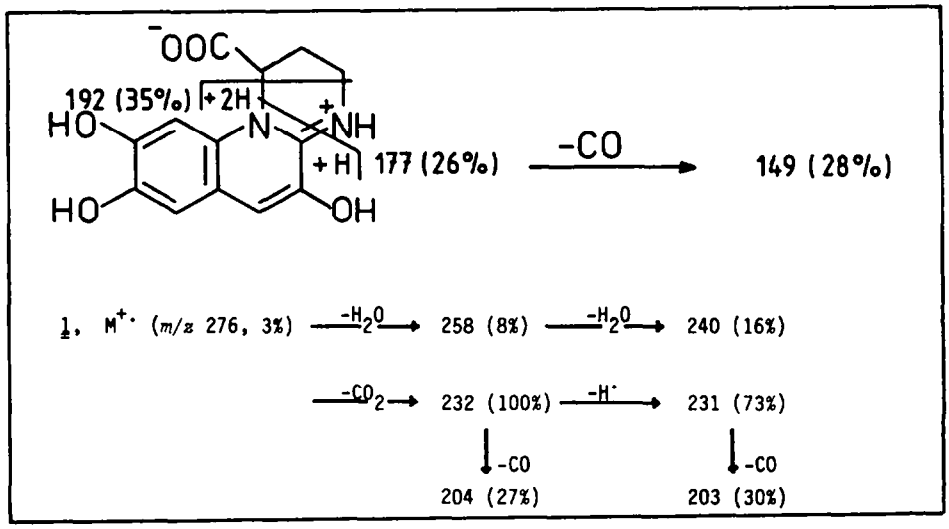

Fig. 1 EI-MS fragmentation of **1**

Chr-Asp (see Table 3) at pH 6.8 carries one negative charge (NH^+ and 2 COO^-). To determine the absolute configuration at C-1 CD spectra of ferri-I and -II have been measured in aqueous and 0.1 buffered (pH 7.2) solution. Ferric pseudobactin, the configuration of which has been determined as S by X-ray analysis [15]shows a strong CD-band at 400 nm ($\Delta\varepsilon$ +2.0). Also for pyoverdine Pa a positive CD-band at 400 nm has been mentioned [8]. In contrast, ferri-I and -II show CD bands at 400 nm with $\Delta\varepsilon$ -2.0 which suggests R-configuration at C-1. At this point it can only be speculated why optical antipodes are produced by closely related species of bacteria. An explanation may be that this readily inversible center adjusts itself to the least hindered configuration determined by the peptide part of the molecule.

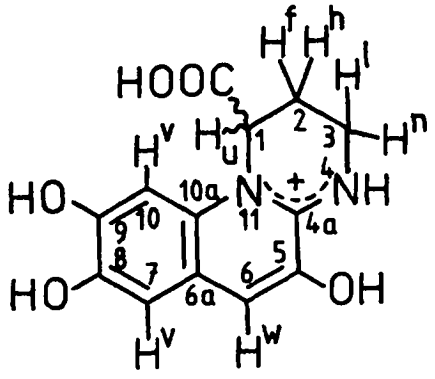

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Table 1. ^{1}H-NMR (D_2O, DDS) and ^{13}C (0.1 N DCl) NMR data of 1

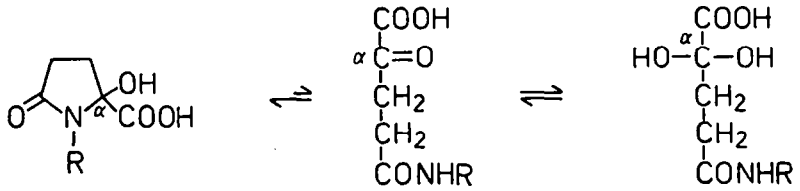
C	H	$\delta(^{13}C)$ppm [b]	$\delta(^1H)$ppm
COOH		173.8	
1	u	57.7	5.70
2	f	22.5	2.38
	h		2.83
3	l	36.5	3.46
	n		3.77
4a		148.6	
5		128.7	
6	w	117.8	7.20
6a		117.2	
7	v	101.7	6.99 [a]
8		144.7	
9		147.7	
10	v	113.7	6.99 [a]
10a		138.9	

a) Separated in 2N DCl (6.94 and 6.85 ppm), b) the assignments were made by comparison with the data reported in [20] for the azotobactine chromophore. They are, however, not unambiguous for the aromatic portion because of the differing substitution (see text) of the two compounds in the N-4/C-5 region. Regarding the racemization at C-1 cf. [21].

Carboxylic acids bound to the chromophore

It has been mentioned above that from pyoverdines II and III upon acid hydrolysis 1 mol of succinic acid is obtained. The presence of a free carboxyl group in the case of II and of an amide group in that of III accounts for the difference of 1 u in the molecular weights and is also in accordance with the electrophoretic behaviour. The dd pattern of the CH_2-CH_2 moiety (g and j in Fig. 3) in the ^{1}H-NMR spectrum of III can be found at 2.80 and 2.74 ppm, in that of II at 2.73

and 2.67 ppm at pH 6, the latter being shifted to 2.80 ppm at pH 1 due to the protonation of the carboxylate group. II can be esterified with CH_3OH/H^+. Reduction of the ester with $NaBH_4$ and subsequent hydrolysis yields γ-butyrolactone, which is formed neither by an analogous treatment of III nor of unesterified II as amides and free carboxylic acids are stable towards $NaBH_4$. The resistance against $NaBH_4$ reduction proves also that succinic acid is bound as an amide to the chromophore 1 (*cf. supra*). II and III thus parallel the results obtained with pyoverdine D and E from *Ps. aeruginosa* [2]. Pyoverdine C was shown to contain α-ketoglutaric acid, the keto group neighbouring the free carboxyl. The electrophoretic behaviour and the mass of the molecular ion indicate the presence of the same acid residue in pyoverdine I. Its ^{13}C- and especially its ^1H-NMR spectra show the same anomalies as have been discussed in detail in Ref. [2] for pyoverdine C and which are due to the an equilibrium of the keto form with hydrated and cyclized forms (cf. 2). As in the case of ferri-pyoverdine C reduction of ferri-I with $NaBH_4$ in CH_3OH/H_2O and subsequent hydrolysis with 6N HCl yields α-hydroxyglutaric acid (identified as TAB derivative by comparison with authentic material), while HI hydrolysis gives glutaric acid (identified as its dibutyl ester). Oxidative decarboxylation of ferri-I with $Pb(OCOCH_3)_4$ [22] and hydrolysis yields 1 mol succinic acid, and by oxidative decarboxylation with alkaline H_2O_2 [23] ferri-I can be transformed into ferri-II. The last reaction proves not only that the keto group is located next to the free carboxyl group, but also that -I and -II differ only in their dicarboxylic acids bound to the chromophore.



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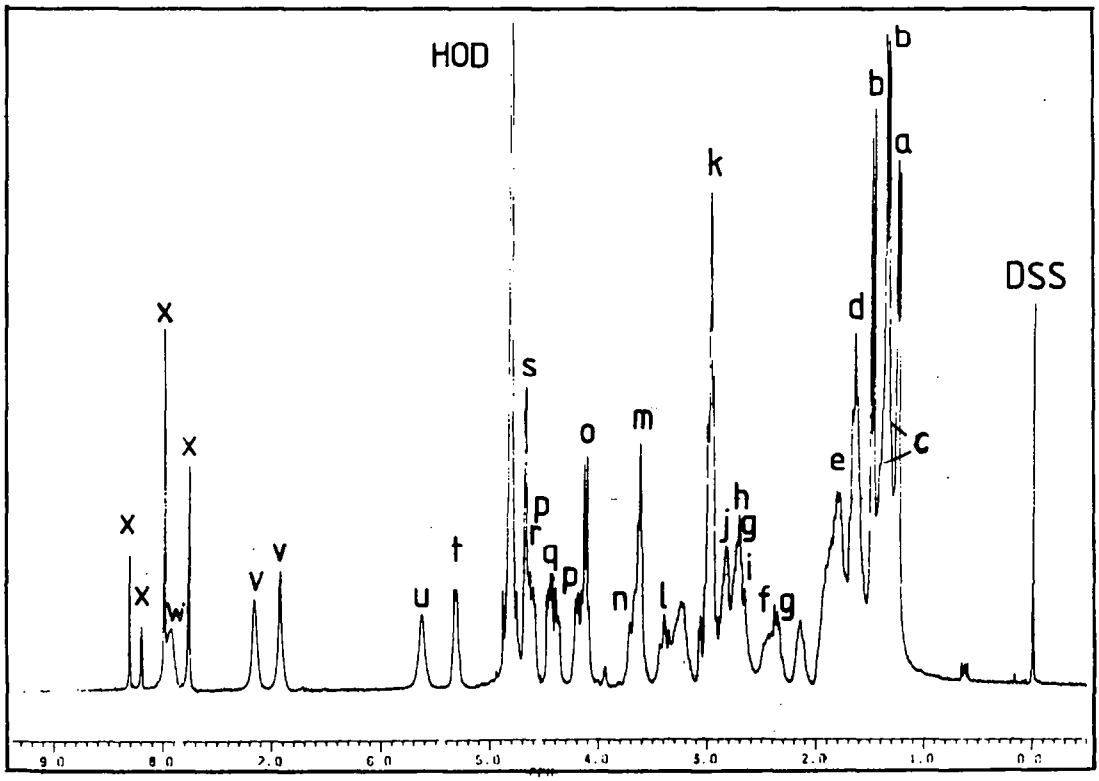


Fig. 2 300 MHz ^1H-NMR spectrum of pyoverdine I

Discussion of the ^{1}H-NMR spectra

The 300 MHz spectrum of pyoverdine I is reproduced in Fig. 2. The various signals are marked by letters which may also be found in the structural formula Fig. 3.

From the signals belonging to the chromophore (cf. l and the discussion above) (f, h, l, n, u, v and w) the last three are broadened because of the presence of the various equilibrium forms of the α-ketoglutaric acid (see above and the discussion in [2]; for II and III these signals are sharp).

Between 7.7 and 8.3 ppm four sharp singlets (x) can be found with intensity ratios 3:1 (together representing 2 H) which stem from the two hydroxamic acid formyl groups. The splitting is due to the *cis/trans* orientation of the amide system and has also been observed with other pyoverdines [2, 24].

Signals belonging to the various amino acids have been identified by decoupling experiments and by comparison with literature data [25]; in particular: Thr: CH_3 (a; d, 1.24 ppm, 6.2 Hz), β-CH(t; m, 5.32 ppm), α-CH(s; d, 4.68 ppm, 2.5 Hz). Regarding the chemical shifts *v. infra.* - Ala: CH_3 (b; d, 1.35 ppm, 8 Hz), CH (o; d, 4.19 ppm). - Ala: CH_3 (b; d, 1.48 ppm, 7.3 Hz), CH (o; d, 4.13 ppm).- Asn: CH (r; dd, 4.61 ppm), CH_2(i; ∿ 2.7 ppm, coincides in part with g, h and j).- 2 Lys: γ-CH_2(c; ∿ 1.3 ppm, coincides in part with b), β- and δ-CH_2 (d; ∿ 1.6 ppm), ε-CH_2 (k; t, 2.97 ppm) CH (p; t, 4.65 and 4.17 ppm).- 2 Orn: CH (q; m, 4.40 and 4.45 ppm), β-CH_2 + γ-CH_2 (e; m, ∿ 1.8 ppm), δ-CH_2 (n; m, 3.62 ppm, 5 Hz).

Table 2. ^{13}C-NMR data of pyoverdine I in phosphate buffered D_2O solution (pH 6.8). The nature of the C-atoms (p=primary etc.) has been determined by DEPT experiments.

identification	δ(lit.)	δ(obs.)	DEPT	identification	δ(lit.)	δ(obs.)	DEPT
β-C-Ala	17.7 (2) b)	16.2	p	α-C-Thr	60.2 (1) b)	56.3	t
γ-C-Thr	20.0 (1) f)	16.5	p	C-1 -Chr a)	57.7 (1) e)	57.8	t
C-2-Chr a)	22.5 (1) e)	22.4	s	β-C-Thr	68.3 (1) b)	74.2	t
		22.6	s	CO-Glu	94.2 f)	94.6	q
γ-C-Lys	23.1 (2) b)	23.1	s		95.2 d)	95.2	q
γ-C-Orn	23.9 (2) c)	23.4	s	C-7-Chr a)	100.9 (1) d)	101.3	t
		24.1	s	arom. C-Chr a)	114.2 (1) d)	115.3	t
β-C-Lys	27.5 (2) b)	27.2	s		114.4 (1) d)	115.6	q
		27.4	s		115.9 (1) d)	117.8	q
		27.6	s		133.9 (1) d)	133.4	q
β-C-Orn	29.3 (2) b)	28.7	s		141.8 (1) d)	142.4	t
		30.3	s		145.8 (1) d)	144.6	q
δ-C-Lys	31.8 (2) b)	30.8	s	C-4a-Chr a)	150.5 (1) d)	152.9	q
		30.9	s	CHO (cis)	160.2 (2) c)	160.2	t
C-3-Chr a)	31.8 (1) e)	36.1	s			160.5	t
β-C-Asn	37.7 (1) b)	39.2	s	CHO (trans)	164.7 c)	164.4	t
ε-C-Lys	40.5 (2) b)	40.2	s			164.7	t
δ-Orn-trans	46.7 (2) c)	46.5	s			169.8	q
		46.7	s			171.1	q
δ-Orn-cis	50.6 (2) c)	50.3	s			171.9	q
		50.6	s	CO-Thr	172.7 (1) b)	173.3	q
α-C-Asn	51.5 (1) b)	51.1	t	CO-Asn	173.1 (1) b)	173.5	q
α-C-Ala	50.8 (2) b)	52.1	t	CO-Lys	174.8 (2) b)	174.7	q
		52.3	t	CO-Orn	174.8 (2) b)	174.9	q
		53.3	t	NH-CO-Glu	176.2 (1) d)	176.3	q
α-C-Orn	55.1 (2) b)	53.6	t	γ-C-Asn	175.6 (1) b)	176.7	q
α-C-Lys	54.5 (2) b)	54.6	t	CO-ALa	175.8 (2) b)	176.7	q
		54.7	t			178.2	q
				COOH-Glu	180.2 d)	180.3	q

a) Chr.....Chromophore, b) Ref. [25], c) Ref. [24], d) Ref. [2], e) Ref. [20], f) see text

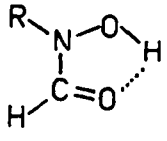
Discussion of the ^{13}C-NMR spectra

The ^{13}C-NMR spectra of the three pyoverdines are very similar. Differences are to be found only regarding the signals of the chromophore and of the dicarboxylic acids attached to it. Thus, pyoverdine II shows two sharp pH dependent signals (32.1 and 32.3 ppm at pH∿6) for the succinic acid CH_2 groups, which are shifted to 30.6 and 31.5 ppm for III. The corresponding CO signals occur at 177.6 and 180.3 (II) and 177.1 and 178.5 ppm (III), resp., in agreement with those re-

ported for other pyoverdines [2, 16]. In the case of I for reasons discussed earlier (equilibrium structures of the α-ketoglutaric acid) low intensity signals at 30.0, 31.5, 32.8 and 33.9 ppm are observed while a CO signal in the vicinity of 200 ppm is missing. For the same reason some of the aromatic signals are broadened (cf. [2]). The equilibrium depicted for 2 explains the presence of two signals of quaternary C-atoms at 94.2 and 95.3 ppm [28] as well as that of CO signals at 176.3, 180.2 and 180.3 ppm. - The ^{13}C-NMR data for pyoverdine I have been summarized in Table 2.

Additional experiments regarding the peptide portion of the pyoverdines

N^5-formyl-N^5-hydroxy ornithine. The ^{1}H-NMR signals of the formyl groups have been mentioned. Upon acidification (pH∿1) these signals disappear with an half-life of about 1.5 h at 20°C with a concomitant increase of a signal at 8.22 ppm attributable to formic acid. In order to exclude the N^6-formyl Lys alternative (as, e.g., encountered in pyoverdine Pa [8]) the ^{1}H-NMR spectrum of N^6-formyl Lys have been analyzed. The two signals of the formyl group appear at 7.96 and 8.02 with an intensity ration of 1:8 which reflects the higher stability of the *trans* configuration [26, cf. also 31] (in contrast to the pyoverdines where the *cis* form 3 is more stable probably due to hydrogen bonding). In accordance with the literature data [24] for N-formyl Gly N^6-formyl Lys is relatively stable towards hydrolysis (half-life (pH∿1) about 168 h at 20°C). The ^{1}H-NMR (which agrees well with the values given in [24]) as well as the facile hydrolysis allow a decision in favour of the N^5-formyl-N^5-hydroxy Orn structure (note that N^6-formyl-N^6-hydroxy Lys can be excluded by the isolation of N^5-hydroxy Orn upon hydrolysis, *v. supra*).



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Esterification of the OH-group of threonine. As compared with the literature value for random coil proteins [25] (4.3 ppm) the position of the signal of the β-CH (t) is shifted downfield by 1 ppm (5.32 ppm). This shift can be accounted for by an esterification of the hydroxyl group [26]. An analogous effect can be noted in the ^{13}C-NMR spectra: The CH signal (74.2 ppm) has been shifted by 6 ppm to a lower and that of the CH_3 group (16.5 ppm) by 4 ppm to a higher field as compared with literature [25] values (cf. [26]). The presence of the ester bond could be verified by mild hydrolysis conditions (10% aqueous K_2CO_3 + CH_3OH 1:1, 3 h, 20°C) which are known to saponify esters selectively in the presence of amides and hydroxamic acids [17]. In the ^{1}H-NMR spectrum of pyoverdine II treated as described the signal at 5.32 ppm has disappeared, while a new signal could be localized at 4.3 ppm underneath the o, p multiplet by irradiation of the methyl doublet at 1.24 ppm (a).

Proof of a cyclic ester structure. As has been pointed out earlier $NaBH_4$ reduces selectively ester bonds. Depending on the structure of pyoverdine II, in the case of a cyclic depsipeptide treatment with $NaBH_4$ should yield a reduction product containing the entire peptide chain, in the case of a non-cyclic ester, however, two smaller molecules would be formed. The isolation by gel chromatography of a product giving in the FAB spectrum an $[M + H]^+$ ion of the (nominal) mass *m/z* 1291 demonstrates that a transformation -O-CO- ⟶ -OH + $HOCH_2$- (mass increase of 4 u) had occured and that, therefore, pyoverdine II comprises a cyclodepsipeptide element.

Lysine as acid part of the ester function. The hydrolysate of any of the three pyoverdines obtained after reduction with $NaBH_4$ contained only one mol of Lys (determined as TAB derivative by quantitative GC/MS, *v. supra*) and in addition one mole of 2,6-diamino-1-hexanol (lysinol) which was identified by comparison with authentic material (GC and GC/MS). Hence the carboxyl group of one Lys must be part of an ester function. It also follows that Lys has to be the C-terminus of the peptidic part of pyoverdines I - III (see foregoing discussion). As an additional proof that Lys does not provide a free terminal carboxyl group $NaBH_4$ reduction was performed after esterification of pyoverdine II. Again only one of the two Lys was transformed to lysinol.

Absence of a free N-terminus. All attempts to perform an Edman degradation proved unsuccessful which indicate that the N-terminus must be blocked.

Proof of the free N^6-amino groups of the two lysines. The presence of two free amino groups in pyoverdine I could be demonstrated by treatment with maleic anhydride [27] which at pH 9 reacts selectively with NH_2, but not with OH or SH groups. The increase in molecular weight ($[M + H]^+$ *m/z* 1513 as determined by FAB, *i.e.* 1315 + 2 x 98) reveals the presence of two free amino groups. To show that these were actually the N^6-amino groups of Lys the pyoverdines were treated with dansyl chloride and then hydrolized. By co-chromatography in several solvent systems with authentic N^2- and N^6-dansyl Lys only the latter could be identified.

Proof of the presence of asparagine (C^4ONH_2-group). The presence of Asn (rather than Asp) follows from the molecular weights of the three pyoverdines taking into account all the other structural increments as well as from the electrophoresis experiments (*v. supra*): ferri-pyoverdine III is charged + 2 at pH 6.5 (1 x protonated chromophore, 2 Lys-NH_3^+, Fe^{3+}, 4 complexing $-O^-$, *viz.*, 2 phenolic groups and 2 hydroxamic acids, cf. [2]). The presence of a free carboxyl group (at pH 6.5 $-COO^-$) would require a global charge of +1 (cf. [30]).

From all these data it follows that the peptide portions of the three pyoverdines do not contain a free N-terminus, that the C-terminus (Lys) is esterified with the hydroxyl group of Thr to form a cyclic depsipeptide, that the N^6-amino group of the two Lys are free, that the two Orn are present as their N^5-formyl-N^5-hydroxy derivatives and Asp as its amide (Asn).

Amino acid sequence

Owing to the blocked N-terminus Edman degradation and related methods could not be applied to the pyoverdines I-III. Sequentional information could, however, be obtained from time dependant partial hydrolysis (analysis by FAB mass spectrometry and isolation of the cleavage products).

FAB analysis of the hydrolysate (6 N HCl, 105°C, salt and acid free lyophilized concentrated solutions (10 µg/µl), parallel measurements with glycerol and thioglycerol matrices) after 10 min allowed to identify the degradation products listed in Table 3. With increasing time the amounts of smaller peptides increase proportionally (higher intensities of the corresponding ions in the mass spectra). It should be mentioned that the $[M + H]^+$ ions of the hydrolysis products are frequently accompanied by $[M + H - H_2O]^+$ ions.

Table 3. Hydrolysis products of pyoverdine II

product [a]	$[M + H]^+$ [amu]		hydrolysis min 10	30	HPLC [d]	GC [e]	Edman [f]
Chr-Asp	391	b) c)	x	x	x	x	
Chr-Asp-Orn(N^5-OH) - H_2O	503	b) c)	x	x	x	x	
Chr-Asp-Orn(N^5-OH)	521	b) c)	x	x	x	x	
Chr(Suc)-Asp-Orn(N^5-OH)			x	x	x	x	
Chr-Asp-Orn(N^5-OH)-Lys - H_2O	631	b) c)	x	x	x	x	
Chr-Asp-Orn(N^5-OH)-Lys	649	b) c)	x	x	x	x	
Chr(Suc)-Asp-Orn(N^5-OH)-Lys	749	c)	x		x	x	
Chr-Asp-Orn(N^5-OH)-Lys-Thr	750	b)	x				
Chr-Asp-Orn(N^5-OH)-Lys-Thr-Ala	821	b)	x				
Chr-Asp-Orn(N^5-OH)-Lys-Thr-Ala-Ala	892	b)	x		x	x	
Desformylpyoverdine II	1231	c)	x			x	
Orn(N^5-OH)-Lys-Thr-Ala-Ala-Orn(N^5-OH)			x				x
Thr-Ala	191	b) c)		x			x
Thr-Ala-Ala	262	b) c)		x			x
Orn(N^5-OH)-Lys	277	b) c)		x			x
Ala-Orn(N^5-OH)-Lys	348	b)					
Thr-Ala-Ala-Orn(N^5-OH)-Lys-H_2O	520	b)					

a) Chr .... chromophore with a free C-5 NH_2; Chr(Suc) .... chromophore with succinic acid bound as an amide , b) determined by FAB in the unseparated hydrolysate, c) determined by FAB of the pure compound, d) after separation by HPLC, e) amino acid composition additionally verified by total hydrolysis and GC/MS of the TAB derivatives, f) sequence determined by Edman degradation.

For further identification the 10 and 30 min hydrolysates were separated by gel chromatography and where necessary by HPLC. Whereever possible the pure fractions (controlled i.a. by FAB) were subjected to total hydrolysis with HI. The amino acids thus obtained were identified by GC/MS of their TAB derivatives. The free N-terminus and the sequence were determined by automatic Edman degradation. The results of these analyses have been assembled in Table 3.

It should be noted that sequential information can also be extracted from the FAB spectra of the three genuine pyoverdines, *viz.*, the sequence Asn-Orn(N^5-OH, N^5-CHO)-Lys-Thr-Ala-Ala. However, as encountered frequently with FAB spectra of peptides, there are other fragment ions whose abundances are comparable with those characteristic for the amino acid sequence and hence the spectra analysis should primarily be taken as a corroboration of the degradation experiments [29].

Configuration of the amino acids.

The determination of the configuration was performed by gas chromatographic analysis of the isopropyl esters of the N-heptafluorobutyryl amino acids obtained by complete hydrolysis of the pyoverdines with HI using a chiral capillary column (Chirasil-Val®). The results indicate D-Ala and L-Ala 1:1, L-Thr, L-Asp, D-Orn and L-Lys. The high percentage of D-amino acids explain the resistance of the pyoverdines against enzymatic cleavage which has been reported also for other pyoverdines [8, 16, 30].

The structure elucidation studies reported here allow to assign to pyoverdines I, II and III the structures depicted in Fig. 3. The only remaining ambiguity is the localization of D- and L-Ala in the Ala-Ala sequence.

Fluorescence of pyoverdine II metal complexes.

In Table 4 UV and fluorescence spectra of a series of metal complexes of pyoverdine II are given. Only Fe^{3+} complexes possess the charge transfer bands at long wave length responsible for the brown colour.

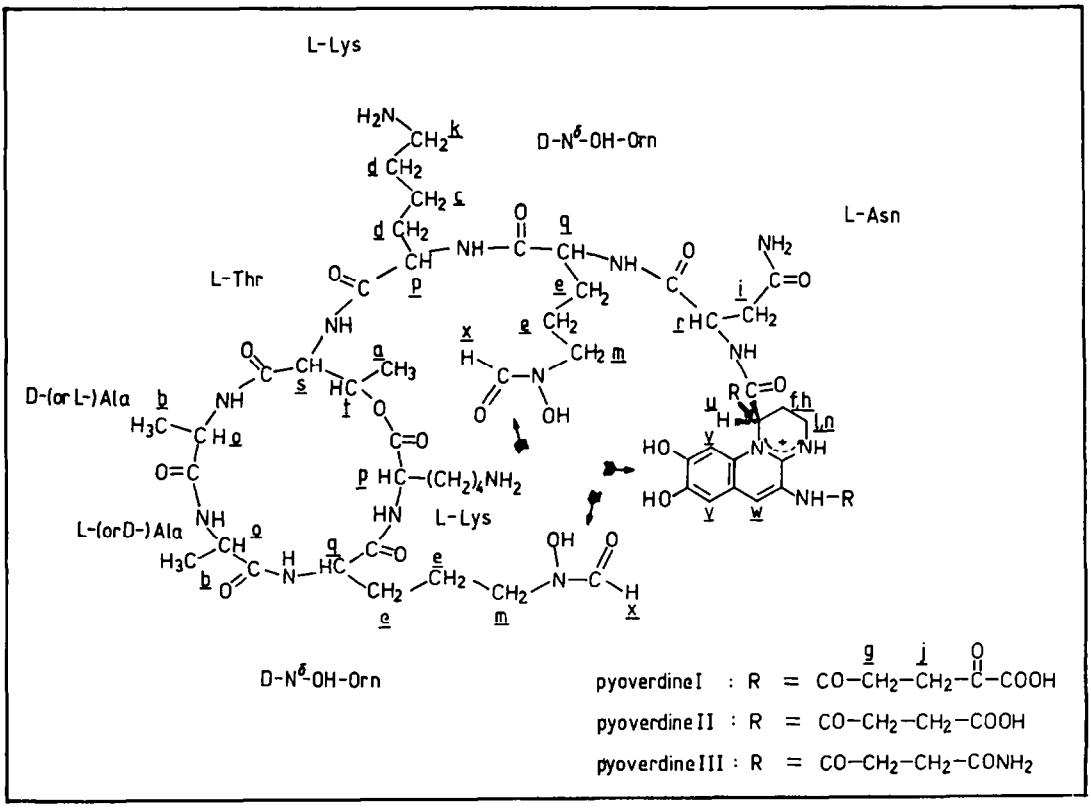

Fig. 3. Structural formulae of pyoverdine I, II and III. The letters refer to the ^{1}H-NMR spectrum (Fig. 2), cf. also text; the arrows indicate the sites of complexation.

Table 4. UV and fluorescence data of pyoverdine II metal complexes

metal	pH	λ_{max}[nm] UV	fluor.	colour
Fe^{3+}	3.0	361, 380	-	quenched
	5.9	367, 382	-	quenched
	7.2	386, 405	-	quenched
Ga^{3+}	3.1	407	490	blue
Al^{3+}	3.4	400	475	blue
Cu^{2+}	7.4	396	-	quenched
	3.1	360, 375	-	quenched
Mg^{2+}	7.7	403	495	green
	3.1	360, 375	560	turquoise
	10.0	425	510	green
Ca^{2+}	6.7	400	478	turquoise
	3.4	360, 375	510	turquoise
Ba^{2+}	3.4	404	488	turquoise
Zn^{2+}	6.7	414	510	turquoise
Pb^{2+}	7.2	409	(509)	quenched

EXPERIMENTAL PART

Instruments and materials

Mass spectra: GC/MS: Varian-MAT CH7A with gas chromatograph Varian 2700 (capillary column BP-1, 25 m); Finnigan 3200 with gas chromatograph 9100 (column as above). DEI: Varian-MAT 212. FAB: Varian MAT 731 with FAB gun Ion Tech. Ltd., Xe, glycerol and thioglycerol matrix, pos. ions; VG 7070E, FAB as above. FD: Varian-MAT 731. NMR: Bruker AM 300 (^1H 300 MHz, ^{13}C 75.4 MHz), internal standard $(CH_3)_3SiCD_2CD_2CD_2SO_3Na$ = DSS. UV: Beckman spectrometer 25. CD: Jasko J-41A spectropolarimeter. Fluorescence spectra: Polrisationsgrad-Spektrometer Dörr [32]. Electrophoresis: CAMAG HVE System 606000. GC: Carlo Erba HRGC 4160, capillary column SE-52, 25 m. FID. Integrator: Shimadzu Chromatopak C-R3A.- Ion exchange and gel chromatography: pumps: LKB 12000 Varioperpex and Pharmacia P-3; gradient former: LKB ultrograd 11300; detectors: LKB uvicord II and III; ISCO-UA-5 absorbance monitor. - HPLC: Kontron Uvikon 725; detector: DuPont ultraviolet spectrophotometer; pumps: DuPont chromatographic pump module 8800; Knauer FR 30; column material; Nucleosil 5 C-18 (Macherey & Nagel). - Column chromatography: Servachrom XAD-4 p.a. (0.3 - 1.0 mm): Serva; Polyamid (0.06 - 0.16) and CM-Cellulose MN 2100: Macherey & Nagel; CM-Sephadex C-25, DEAE-Sephadex A-25 and Sephadex G-10, G-25: Pharmacia Fine Chemicals; Bio-Gel P-2 (200-400 mesh): Bio-Rad Lab. - TLC: Kieselgel 60 F-254: Merck; Polygram CEL 300 CM and Polygram Polyamid-6: Macherey & Nagel.

Reagents: Pyridine was distilled from chlorosulfonic acid (ca. 5 ml/l) and then from KOH and kept over a molecular sieve. HI was distilled twice; HCl (12 N) Merck p.a.; both acids were diluted immediately before the hydrolysis to 6 N. Amino acids: Merck, EGA or Sigma, α-hydroxyglutaric acid: EGA, trifluoroacetic anhydride: EGA; all p.a. quality.

Bacterial culture; *Pseudomonas fluorescens* "ng" was isolated from Rhine water and identified by Dr. H. Korth (Hygiene-Institut der Universität zu Köln). Culture medium (g/l): Na gluconate (12.0), $KH_2PO_4.3H_2O$ (4.0), $MgSO_4 \cdot 7H_2O$ (0.5), $(NH_4)_2SO_4$ (5.0), pH adjusted to 7.4 - 7.6. For solid cultures 2 - 3 ml 0.5% Fe^{3+} citrate solution and 2% agar were added. 200 ml of liquid culture medium was inoculated with bacteria from agar cultures, incubated for 48 h and then added to 2 l culture medium in a small fermenter aerated with sterile compressed air saturated with H_2O (2 l/min). After bacterial growth and pyoverdine concentration (monitored at 650 and 400 nm, resp.) had reached a constant value (2 - 3 days) the cultures were worked up except for 200 ml which was recycled to a fresh medium.

Isolation of pyoverdines I-III. To the centrifuged culture medium 1 g/l Fe^{3+} citrate was added and the pH adjusted to 5 - 6. The solution was chromatographed through an XAD-4 column (6 x 80 cm are sufficient for 4 - 5 l). After washing with 1 - 2 l H_2O (until the filtrate was clear) the ferri-pyoverdines were desorbed with acetone/H_2O 1:4 (ca. 400 ml). (The red Fe-complex of pyocheline can be desorbed subsequently with acetone/H_2O 1:4 + 0.5% (vol.) conc. HCl; pyocheline was identified by its mass spectrum). The pyoverdine solution was concentrated at 40°C/10 Torr (residue 400 - 600 mg/l culture medium). 700 - 800 mg of this residue was brought onto a CM-cellulose column (5.4 x 23 cm, pyridinium form, pH 5) and eluted with pyridinium acetate buffer: 0.05 M buffer yielded a brown fraction which was discarded, 0.2 M buffer eluted ferri-pyoverdine I and II, and 0.5 M buffer ferri-pyoverdine III. Finally, with 2.0 M buffer two green and red iron complex could be obtained. After removal of the buffer i.v. the pyoverdine containing fractions (together *ca.* 500 - 700 mg) were united and chromatographed on a CM-Sephadex C-25 column (2.6 x 38 cm, pyridinium form, pH 5) with pyridinium acetate buffer which eluted several small unidentified fractions (0.05 M) and then I (0.25 M), II (0.40 M) and III (0.55 M). Purification was achieved by rechromatographing. The temperature during the concentration of the solution should be kept below 20°C to avoid partial transformation of ferri-III into -II. From 1 l culture medium thus ca. 40 mg ferri-I, ca. 70 mg ferri-II and ca. 50 mg ferri-III could be obtained.

Decomplexation of the ferri-pyoverdines. *Ca.* 50 - 100 mg ferri-pyoverdine was dissolved in 10 ml H_2O and acidified with citric acid (pH 2.5). This solution was stirred several times with a freshly prepared solution of 8-hydroxy quinoline (5% w/v in $CHCl_3$) which resulted in a removal of more than 90% of Fe^{3+}. After removal of the excessive 8-hydroxy quinoline by extraction with $CHCl_3$ the solution was concentrated. The iron-free pyoverdines were obtained in pure form by gel chromatography (Bio-Gel P-2, column 2.6 x 60 cm, 0.2 M pyridinium acetate, pH 6.5) and removal of the solvent and the buffer i.v. The pyoverdine acetates were kept in the dark at -15°C. For NMR acetate was exchanged for chloride by filtering through a DEAE Sephadex column in aqueous solution (1.6 x 10 cm, Cl^- -form).

Qualitative amino acid analysis. 2 - 3 mg ferri-pyoverdine or pyoverdine was hydrolyzed under Ar with 2 ml 6N HCl or 48% HI at 105°C for 18 - 21 h. The acids (and I_2) were removed by distilling twice i.v. with 2 ml H_2O. To the residue were added at 0°C 2 ml *n*-butanol and 0.4 ml acetyl chloride, the mixture was homogenized for 1 min in an ultrasonic bath and then heated to 100°C for 30 min. The excess of reagents was removed i.v., the residue dissolved in 2 ml CH_2Cl_2, added to 0.4 ml trifluoroacetic anhydride and heated to 150°C for 5 min. After removal of the excess of reagent (20° i.v.) the TAB derivatives were dissolved in 200 µl CH_2Cl_2 and analyzed by GC/MS.

Quantitative GC amino acid analysis. The method was tested with standard solutions of amino acids with known concentrations. Leu and adipic acid were used as internal standards. In addition, the amino acids obtained from hydrolysis experiments were quantified by ion exchange chromatography, (Durrum-DC-4A resin, column 0.9 x 25 cm, Na^+ citrate buffers in steps pH 3.25 - 4.75).

Isolation of $\underline{\underline{1}}$. 178 mg ferri-pyoverdine I were dissolved in 20 ml 2N HCl and heated (105°C) under Ar for 7 days. After removal of HCl by distillation the blackish brown residue was chromatographed on polyamide (column 1.8 x 30 cm) with H_2O as solvent. After removal of a yellowish fluorescent fraction $\underline{\underline{1}}$ was desorbed with 30% CH_3OH/H_2O. Rechromatography yielded a yellow oil which was finally purified by HPLC (Nucleosil 5 C-18, CH_3OH/0.1% HCOOH in H_2O (pH 2.7) 2:8).- Yield: 3.7 mg. Acetylation of $\underline{\underline{1}}$. 0.5 mg $\underline{\underline{1}}$ in a mixture of pyridine and acetic anhydride were left for 24 h at room temperature and worked up as usual.

Oxidation of ferri-pyoverdine I with $Pb(OCOCH_3)_4$. To a mixture of a CH_3OH solution of 5 mg ferri-pyoverdine I and a CH_3OH solution of 6.9 mg freshly recrystallized $Pb(OCOCH_3)_4$ H_2O was added. The gas formed in this reaction was driven over by a stream of N_2 into a solution of $Ba(OH)_2$ and the precipitate formed was identified as $BaCO_3$. After 20 h the brown solution was decanted and evaported i.v. to dryness. Hydrolysis as described above yielded i.a. succinic acid identified after derivatization by GC/MS.

Oxidation of ferri-pyoverdine I with H_2O_2/KOH. 2 mg ferri-pyoverdine I was dissolved at 0°C in an alkaline (0.01 N KOH) 10% H_2O_2 solution and then kept for 68 h at 20°C. After neutralization the solution was evaporated to dryness i.v. at 40°C. The reaction product was identified as ferri-

pyoverdine II by comparison of the chromatographic behaviour (silica gel, CH_3OH/H_2O 7:3; Polygram CEL 300 CM, 0.1 M pyridinium acetate buffer, pH 5) and electrophoresis at pH 3 and 6.5. After total hydrolysis succinic acid was identified as described above.

Reduction of ferri-pyoverdines with $NaBH_4$. A solution of 4 mg ferri-pyoverdine I in 5 ml CH_3OH/H_2O 10:1 at 0°C was treated with 50 mg $NaBH_4$ and kept for 4 days at 5°C. After addition of HCl the H_3BO_3 formed was removed as $B(OCH_3)_3$ by adding 6 times 1 ml 3N methanolic HCl and distillation. The reaction product was hydrolyzed and analyzed for amino acids as described above. Glutaric acid (HI hydrolysis) or 2-hydroxyglutaric acid (HCl hydrolysis) as well as lysinol (also obtained in the same way from ferri-pyoverdine II and III) were identified after derivatization by GC/MS.

Ester cleavage with K_2CO_3/CH_3OH. 5.3 mg pyoverdine II was dissolved in 200 µl of a mixture of CH_3OH/10% aqueous K_2CO_3 1:1 and kept for 3 h at 35°C. After acidification with CD_3COOD to pH 4 - 5 and removal of the solvent the residue was investigated by NMR.

Maleylation of the free NH_2-functions. 10.8 mg pyoverdine I was dissolved in 10 ml 0.1 M $Na_4P_2O_7$ buffer (pH 9) and treated in small portions with 300 µl of a solution of 10.8 mg maleic anhydride in 430 µl dioxan at °C. The pH was kept constant at 8.3 - 8.5 by addition of 1 N KOH solution (pH-meter control). After stirring for 1 h at 20°C the reaction product was isolated by gel chromatography (Bio-Gel P-2, 0.01 N NH_3).

Dansylation of pyoverdine I. 7 ng pyoverdine I was dissolved in 10 µl 0.2 M $NaHCO_3$ solution, evaporated to dryness, dissolved in 10 µl H_2O and treated with 10 µl of a fresh solution of dansyl chloride (2.5 mg/ml acetone) for 1 h at 37°C. After total hydrolysis (50 µl 6 N HCl, 19 h, 105°C) and removal of HCl the residue was dissolved in 10 µl 50% pyridine and chromatographed by twodimensional TLC on polyamide (1.5% formic acid and benzene/acetic acid 9:1). N^6-Dansyl Lys was identified by co-chromatography with authentic N^2 - and N^6-dansyl Lys.

Partial hydrolyses. Portions of 6 mg pyoverdine II were hydrolyzed with 6 N HCl at 105°C for 10, 20 or 30 min, diluted with 4 ml H_2O, cooled to 0°C and evaporated to dryness. Separation was achieved by gel chromatography (Bio-Gel P-2, column 1.6 x 90 cm, 0.1 N CH_3COOH (pH 3)) and HPLC (RP C-18, 14 ... 18% CH_3OH in 0.1% TFA buffer). For automatic sequencing the instrument Beckman Model 890 C was used.

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