Pyoverdins with a Lys ε-Amino Link in the Peptide Chain?[§]

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For a pyoverdin isolated from Pseudomonas putida it could be shown that Lys is incorporated into the peptide chain by its ε-amine group in contrast to the normally observed connection by the α-amino group. The structure elucidation of the pyoverdin by chemical degradation and spectroscopic methods is reported and the criteria for the distinction between α- and ε-connection in the case of Lys are discussed.

Introduction

Fluorescent members of the rRNA homology group I of the Pseudomonadaceae are characterized by the production of so-called pyoverdins, i.e., siderophores consisting of a dihydroxyquinoline chromophore bound amidically to the N-terminus of a peptide chain consisting of 6 to 12 amino acids, L as well as D and partially modified (Budzikiewicz, 1997). By now about 50 pyoverdins are known, for more than 30 complete or fairly complete structures were established (Kilz *et al.*, 1999). Consistently, the amino acids of the peptide chain are connected by amide bonds between the carboxyl group of one amino acid and the α-amino group of the following one (typical peptide bonds). In two cases it was suggested that a Lys formed an amide bond with its ε- rather than with its α-amino group (Wang *et al.*, 1990; Persmark *et al.*, 1990), but doubts remained regarding the conclusions (see **Discussion**). We will now present the structure elucidation of a pyoverdin (**1**) where the ε-connection of Lys in the peptide chain could be established unambiguously.

Materials and Methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT 900 ST (ESI; 50 μM solutions in CH_3OH/H_2O 1:1, v/v); GC/MS Incos 500 (Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: DRX 500 (Bruker, Karlsruhe). Chemical shifts are given relative to TMS with the internal standard DSS using the correlation δ(TMS) = δ(DSS) for ^1H and δ(TMS) = δ(DSS) + 1.61 for ^{13}C. H_2O signals were suppressed either by the WATERGATE pulse sequence (solvent D_2O) or by presaturation (solvent H_2O/D_2O 9:1 v/v).

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

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

[§] Part LXXXV of the series "Bacterial Constituents". For Part LXXXIV see Münzinger *et al.* (1999).

Abbreviations: Common amino acids, 3-letter code; OHAsp, *threo*-β-hydroxy Asp; cOHOrn, *cyclo*-N^5-hydroxy Orn (3-amino-1-hydroxy-piperidone-2); TAP-derivatives, N/O-trifluoroacteyl amino acid isopropyl esters; ESI, electrospray ionization; FAB, fast atom bombardment; MS, mass spectrometry; CA, collision activation; COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser and exchange spectroscopy; TOCSY, total correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; CAA, casamino acid; CFBP, Collection Française de bacteries phytopathogènes.

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Chemicals: Water was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column. Reagents (Aldrich-Sigma, Deideshofen; Fluka, Buchs, CH; Merck, Darmstadt; Riedel de Haen, Seelze) were p. a. quality. Culture medium 5 g Casamino Acids (CAA; a casein hydrolysate) (Diffco, Augsburg) dissolved in 100 ml H_2O with 0.25 g $MgSO_4.7H_2O$ and 300 ml buffer (1.18 g $KH_2PO_4.3H_2O$ plus 1.3 g KH_2PO_4, pH 6.8).

Production, isolation and derivatisation of **1**

The pyoverdin **1** was obtained first from a *Pseudomonas* sp. strain isolated from dried termite soil collected in the province Udornthani (Thailand) by the Dept. of Microbiology, Faculty of Sciences, Khon Kaen University. Isolation of **1** was effected by a procedure reported earlier (Glennon *et al.*, 1994). Subsequently, **1** could also be isolated from *Pseudomonas putida* biovar B CFBP 11370 grown in a CAA medium. Preparation of a XAD-2 extract of the culture medium (iron-free work-up) is described elsewhere (Georgias *et al.*, 1999). After addition of Fe(III) citrate the Fe^{3+} complex was chromatographed on Biogel P-2 with a 0.2 M pyridinium acetate buffer (pH 5.0). The fraction showing the typical ferri-pyoverdin absorption (Budzikiewicz, 1997) was collected and rechromatographed on Sephadex with the same buffer system. The ferric complex was adsorbed on a Sep-Pak cartouche and treated with a 4% potassium oxalate buffer. After washing with H_2O the free pyoverdin was desorbed with CH_3OH/H_2O (2:1, v/v). The purity of the eluate was checked by HPLC on Nucleosil.

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

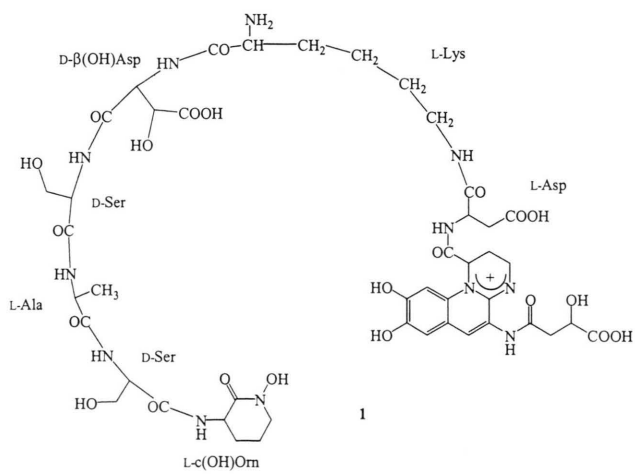
Results

Characterization of **1**

The UV/Vis spectrum of **1** is characteristic for a pyoverdin (Budzikiewicz, 1997). Its molecular mass as determined by ESI-MS amounts to 1121 Da. Gas chromatographic analysis of the TAP derivatives after total hydrolysis showed the presence of L-Ala, L-Asp, D-OHAsp, L-Lys, L-Orn and D-Ser. By total hydrolysis after dansylation α-dansyl Lys was obtained as could be shown by chromatograhic comparison with samples of authentic α- and ε-dansyl Lys. Hence in **1** the α-amino group is free.

Sequence determination by MS and NMR

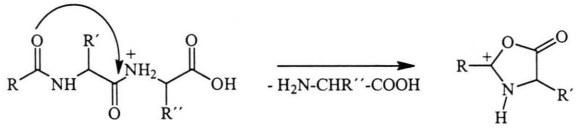
The characteristic fragment ions of **1** upon collision activation (CA) of $[M + 2H]^{2+}$ are assembled in Table I. Of sequence importance are the so-called B-ions (formally H_2N-CHR-CO^+ etc., for pyoverdins the N-terminus is bound to the chromophore) accompanied by $[B - H_2O]$-ions down to B_3 (loss of H_2O from Ser and OHAsp) and – with lower abundance due to a less efficient charge stabilization at the C-terminus – the Y″-ions (H_3N^+-CHR-COOH etc.) (Roepstorff, 1984).



1

Table I. Fragment ions of **1** with a succinamide side chain observed in the mass spectra after collision activation (B-ions N-terminal, Y"-ions C-terminal).

Amino acid	n	B_n	Y_n''	n
Asp	1	472	–	7
Lys	2	–	635	6
OHAsp	3	731	–	5
Ser	4	818	376	4
Ala	5	889	289	3
Ser	6	976	218	2
cOHOrn	7	–	131	1



R = peptide chain
R', R'' = side chains

Fig. 1. Formation of B-fragments.

The facile formation of the B-Ions has been explained by a protonation of the amide nitrogen of a peptide bond and subsequent cleavage aided by anchimeric assistance given by the carbonyl oxygen of the preceding bond (Fig. 1) (Yalcin *et al.*, 1995). It is evident from Table I that the ion B_2 (cleavage after Lys) is essentially missing (in the original spectra its abundance does not exceed that of background ions): Due to the ε-linkage (see below) the carbonyl group of the preceding amino acid is too far away to be available for an interaction with the next peptide bond. The same peculiarity is observed for another pyoverdin with an ε-Lys linkage in the peptide chain (unpublished). For the two examples from the literature (see **Discussion**) only FAB spectral data were reported where generally B-ions are hardly noticeable.

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 and TOCSY allows to detect the H-couplings within one amino acid residue (amide bonds interrupt the scalar H,H-coupling). Quaternery C-atoms can be identified with HMBC optimized for ^{2}J- and ^{3}J-coupling. Sequence information is obtained by NOESY/ROESY which allows a correlation of an NH-proton with spacially close α- and β-H's of the preceding amino acid (C**H**-C**H**-CO-

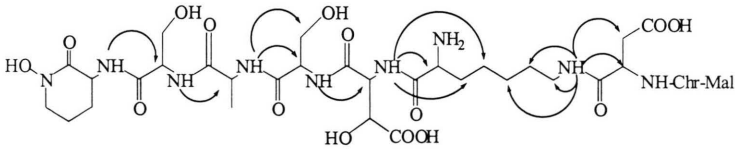


Fig. 2. Sequence relevant NOE-cross peaks of **1**.

Table II. ^{1}H-NMR data of **1** (H_2O/D_2O 9:1, pH 4.5, 5 °C).

Amino acid	α-NH	α	β	γ	δ	ε	ε-NH
Ala	8.47	4.40	1.42				
Asp	9.11	4.68	2.71/2.93				
OHAsp	8.78	4.94	4.77				
Lys	–	4.09	1.73	1.21	1.29	2.88/3.11	8.22
cOHOrn	8.53	4.48	1.42/1.78	1.98/2.01	3.65		
Ser-1	8.58	4,51	3.91				
Ser-2	8.47	4.45	3.87				
Mal	2	3					
	2.90	4.68					
Chr	1	2	3	6	7	10	
	5.70	2.48/2.80	3.40/3.72	8.00	7.31	7.07	

Table III. ^{13}C-data of **1** (D_2O).

Amino acid	α	β	γ	δ	ε	CO
Ala	51.2	17.6				176.5
Asp	51.8	69.5	172.5*			171.9*
OHAsp	57.1	72.5	178.5*			176.3*
Lys	54.3	31.5	22.3	28.8	40.3	171.8
cOHOrn	51.1	20.6	23.0	53.0		176.4
Ser-1	57.0	62.2				172.8
Ser-2	57.2	62.4				172.6
Mal	CO	2	3	COOH		
	173.7	40.2	69.5	177.2		
Chr	1	2	3	4a	5	6
	57.8	32.5	36.3	150.5	118.2	140.7
	7	8	9	10	10a	CO
6a	133.1	144.9	152.7	101.6	116.2	170.5
115.5						

N**H**) and by HMBC correlating amide-CO with the α-H of the following amino acid (see Fig. 1). The ^{1}H- and ^{13}C-data of **1** are compiled in Tables II and III. They correspond to those observed with other pyoverdins (Budzikiewicz, 1997). The following ones deserve a comment: The NH-signal of Asp bound directly to the carboxyl group is typically shifted downfield. The shift values of the CH_2-groups of the two Ser (3.87 and 3.91 ppm) show that the OH-groups are not esterified (otherwise a downfield shift of about 0.5 ppm would have been expected). The signals of the α-CH (4.09 ppm), the ε-CH_2 (2.88/3.11 ppm) and the NH (8.22 ppm) of Lys suggest that Lys is not α-peptidically, but rather ε-amidically connected to the preceding amino acid (see **Discussion** below). This is confirmed by NOESY cross peaks of the NH-signal (identified by TOCSY and NOESY cross peaks with the γ-, δ- and ε-CH_2 signals of Lys) with the α- and β-protons of Asp. A signal of the α-NH_2 group of Lys could not be detected due to the faster exchange of amine as compared with amide protons.

Discussion

Incorporation of a Lys into the peptide chain of a pyoverdin via its ε- rather than its α-amino bond was claimed twice in the literature: For the pyoverdin from *Pseudomonas fluorescens* 244 it is stated in a preliminary report (Wang *et al.*, 1990) that Lys is connected by its ε-amino group to a Ser carboxyl group "as (NMR) studies on the Ga-chelated material suggest", but no data were given and the claim was not repeated (and substantiated) in the subsequent publication (Hancock and Reeder, 1993). From *P. fluorescens* 9AW a pyoverdin was obtained which most likely is identical with the one mentioned before (Budzikiewicz *et al.*, 1997) and where the α-connection of Lys was established beyond doubt. The second example is the pyoverdin from *P. putida* 589A (Persmark *et al.*, 1990). Here chemical (ninhydrin reaction, ε-dansyl derivative; see below) did not agree with the NMR evidence (especially that the Lys-NH which shows a NOE crosspeak to one of the β-protons of Asp is a triplet and not a doublet; hence its neighbor is a CH_2- and not a CH-group).

A linkage via the ε-amino group of Lys is not unreasonable *per se.* An N-terminal Lys was found occasionally to be linked to the chromophore by its ε-amino group (for listings see Kilz *et al.*, 1999). According to the current biogenetic scheme the precursor of the pyoverdin chromophore is the dipeptide L-Dab-D-Tyr connected to the N-terminus of the peptide chain (Böckmann *et al.*, 1997); obviously the formation of an ε-amide bond is possible.

There are three ways to confirm the presence of an ε- instead of an α-bond of Lys: (i) Dansylation and subsequent hydrolysis (α- and ε-dansyl Lys can be distinguished chromatographically, but authentic samples of both isomers should be avail-

able to test the chromatographic system); (ii) Edman degradation is possible only with a free α-amino group; a positive ninhydrin reaction is not reliable as not only α-amino acids, but also primary amines (here the ε-amino group) can give the color reaction (D. J. McCaldin, 1960; Schönberg and Singer, 1987); and (iii) NMR analysis. Evaluation of the ^{1}H-chemical shifts of pyoverdins containing Lys bound in different manners were extracted from published data (for a listing see Kilz *et al.*, 1999) and from unpublished material from this laboratory; they gave the following picture (* solitary values; in parentheses the number of examples) (values for NH-protons bound directly to the carboxyl group of the chromophore suffering a pronounced downfield shift – see above – are not included):

Lys incorporated into the peptide chain by its α-NH
α-CH 4.3–4.4 (4.7*), ε-CH_2 (2.5*) 2.7–3.1 (11), α-NH (pH ca. 4) 8.5–8.7 ppm (6)

Lys incorporated into the peptide chain by its ε-NH
α-CH 4.1, ε-CH_2 2.9–3.4 (4), ε-NH (pH ca. 4) 8.1–8.2 ppm (2)

Lys incorporated into a cyclic structure with both NH-groups
α-CH 4.0–4.3, ε-CH_2 2.9–3.5 (5), α-NH 8.1–8.2, ε-NH 7.3–7.5 (pH ca. 4) ppm (2).

For the α-CH- and ε-CH_2-shifts the tendency is clear, that amide formation causes a downfield shift, but the possibility exists that due to the location of the respective group in a shielding or deshielding region of the molecule unexpected values may be obtained. The number of shift values reported for NH-groups is too small for drawing any reliable conclusions. What can (and has) been done, is to unambiguously identify the chain protons of Lys by two-dimensional NMR techniques and than establish NOE connectivities to the α- and β-protons of the next amino acid. Whether the essential absence of a B-ion after an ε-bound Lys in the CA mass spectrum will prove to be characteristic, further examples will have to show. The present work proves by chemical and NMR evidence the existence of Lys incorporated amidically via its ε-amino group into a pyoverdin peptide chain. For the pyoverdin from *P. putida* 589A the authors probably concluded correctly that in their case the NMR evidence outweighed the chemical data (ninhydrin reaction, not having available both Lys dansyl derivatives; see above).

About half of the pyoverdins where peptide sequences were proposed have Lys as the second amino acid after the chromophore, less frequently Lys is found fartheron in the peptide chain (see Kilz *et al.*, 1999). Checking of the original literature confirmed that in most cases the α-linkage was proven by isolation of ε-dansyl Lys and/or by two-dimensional NMR techniques. The present example, however, shows that α-linkage cannot be taken for granted. FAB mass spectral and partial hydrolysis data do not give an answer, and chemical shifts of the α- and ε-Lys protons can only be taken as a hint. It is essential that all functional groups of the amino acids present in the peptide chain are being accounted for.

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