# Revised structures of the pyoverdins from *Pseudomonas putida* CFBP 2461 and from *Pseudomonas fluorescens* CFBP 2392

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## Abstract

Several suggestions for structures of the siderophores (pyoverdins) from *Pseudomonas* spp. can be found in the literature which are based on a FAB mass spectrometric analysis only. Availability of two original strains of two *Pseudomonas* spp. allowed to re-investigate the structure of their pyoverdins. In both cases the amino acid sequence had to be corrected. In addition, D- and L-amino acids could be identified and located in the peptide chain. The knowledge of the correct structures is important in view of an ongoing study to establish relationships between the nature of the peptide chains of pyoverdins and their recognition by outer membrane proteins.

*Abbreviations:* Common amino acids – 3-letter code; AcOHOrn – $N^5$-acetyl-$N^5$-hydroxy-Orn; OHAsp – *threo*-ß-hydroxy-Asp; cOHOrn – *cyclo*-$N^5$-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); aThr – *allo*-Thr; Chr – pyoverdin chromophore; Mal (Mala) – malic acid (malamide) residue; Suc (Suca) – succinic acid (succinamide) residue; TAP derivatives – N/O-trifluoroacetyl-(amino acid)-isopropyl esters; HPTLC – high performance thin layer chromatography; MS – mass spectrometry; CID – collision induced decomposition; FAB – fast atom bombardment; COSY – correlation spectroscopy; HMBC – heteronuclear multiple bond correlation; HMQC – heteronuclear multiple quantum coherence; NOESY – nuclear Overhauser and exchange spectroscopy; ROESY – rotating frame Overhauser and exchange spectroscopy; TOCSY – total correlated spectroscopy.

Many microorganisms, when living under conditions where the supply of soluble iron compounds is limited, produce $Fe^{3+}$ chelators, so-called siderophores. Those of several species of the bacterial genus *Pseudomonas* named pyoverdins, were recognized as fluorescent pigments more than a century ago (according to a review article by Gessard (1892), the description of fluorescent pseudomonads – probably *P. fluorescens* and *P. putida* – can be traced back at least till 1886), but the first complete structure of a pyoverdin was not established before 1981 (Teintze *et al.* 1981).

Pyoverdins consist of three subunits, viz. a dihydroxyquinoline chromophore responsible for the fluorescence, a small dicarboxylic acid (usually several pyoverdins differing only in the nature of these side chains are found to co-occur), and a peptidic chain comprising 6 to 12 amino acids (both L and D). So far the structure of about 30 representatives differing in their peptide chain have been elucidated, and there are about 20 more where only the amino acid composition was established or where the amino acids present and their sequences were deduced from FAB mass spectrometry only (Kilz *et al.* 1999). To know as many correct structures as possible is of importance for two reasons: (a) The classification of the genus *Pseudomonas* and of related genera is under revision, and the strain-specific structures of the pyoverdins as chemical classifiers can be of assistance (cf. Budzikiewicz 1997a), and (b) the cellular uptake mechanisms by outer membrane proteins require a specific three-dimensional arrangement of a certain portions of the amino acids ('key-function') (e.g.,

*Table 1.* ^{1}H-NMR data of **1a** (solvent 100 mM $KH_2PO_4/D_2O$, pH 4.3); $\delta$ ppm relative to TMS, internal standard DSS, $\delta$(TMS) = $\delta$(DSS).

| | HN | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\epsilon$ | $H_2N$ | | |
|---|---|---|---|---|---|---|---|---|---|
| Ala | 8.38 | 4.34 | 1.35 | | | | | | |
| Asp | 8.97 | 4.50 | 2.8/2.68 | | | | | | |
| OH Asp | 8.78 | 4.94 | 4.56 | | | | | | |
| Lys | 8.59 | 4.34 | 1.89 | 1.42 | 1.66 | 3.1/2.75 | 8.14 | | |
| Lys | 8.59 | 4.34 | 1.89 | 1.42 | 1.66 | 3.1/2.75 | 8.14 | | |
| Ser | 8.58 | 4.57 | 3.84 | | | | | | |
| Thr1 | 8.24 | 4.32 | 4.22 | 1.20 | | | | | |
| Thr2 | 8.33 | 4.22 | 4.07 | 1.24 | | | | | |
| cOH-Orn | 8.33 | 4.40 | 1.73 | 1.95 | 3.63 | | | | |
| Mala | $CONH_2$ | 2 | 3 | NH-Chr | | | | | |
| | (-) | 2.90 | 4.66 | (-) | | | | | |
| Chromophore | 1 | 2a | 2b | 3a | 3b | $4HN^+$ | 6 | 7 | 10 |
| | 5.54 | 2.7 | 2.4 | 3.4 | 3.7 | (-) | 7.8 | 6.97 | 6.89 |

Georgias *et al.* 1999). The knowledge of which part of the peptide chain is essential for this purpose may be used for constructing compounds antibiotically active against human and animal infective species (Kinzel *et al.* 1998; Kinzel & Budzikiewicz 1999).

In the following article we wish to report the revised structures of two pyoverdins from *Pseudomonas* strains of the Strasbourg University collection where structural proposals were based on FAB mass spectral data (not reported in detail) only.

## Materials and methods

### Bacterial cultures and workup

The bacteria were grown in succinate medium (Budzikiewicz *et al.* 1997). Bacterial culture, work up and isolation of the ferric siderophores was performed as described earlier (Geisen *et al.* 1992). Decomplexation was achieved with 8-hydroxyquinoline (Briskot *et al.* 1986).

### Separation techniques and spectroscopic methods

Chromatographic materials: Biogel-P-2 (200-400 mesh; Bio-Rad, Richmond, CA, USA); Sephadex G-10 (Pharmacia, Uppsala, S); Chirasil-L-Val (Chrompack, Frankfurt); chiral HPTLC plates (Merck, Darmstadt).

Mass spectrometer: MAT 900 ST with EBT geometry, CID in the ion trap section (Finnigan-MAT, Bremen). NMR: DRX 500 (Bruker, Karlsruhe).

## Results and discussion

### The pyoverdins of Pseudomonas putida CFBP 2461 (identical with Pseudomonas L 1)

For the main pyoverdin an amino acid composition of 1 Ala, 1 OHAsp (the presence of an additional unsubstituted Asp was not realized), 2 Lys, 1 OHOrn, 1 Ser and 2 Thr was reported (Demange *et al.* 1986). The chirality of the amino acids was not determined. Later on the sequence

Chr-Asp-Lys-OHAsp-Ser-Thr-Ala-Lys-Thr-cOHOrn

was suggested based on FAB-MS measurements (Demange *et al.* 1989).

After decomplexation and chromatographic separation two fractions were obtained the molecular masses of which were determined by FAB-MS as 1364 (**1a**) and 1348 u (**1b**), the mass difference of 16 u suggesting the presence of a malic and a succinic acid side chain or of their amides, resp. (Budzikiewicz 1997b). Amino acid analysis by total hydrolysis (6N HCl, 12 h, 110 °C) and GC analysis of the TAP derivatives on a Chirasil-L-Val column established the presence of D-Ala, L-Asp, D-OHAsp, D-Lys, L-Lys, L-Orn, L-Ser, L-Thr and D-aThr, in agreement with the ^{1}H- and ^{13}C-NMR spectra (Tables 1 and 2). Identification of the signals was achieved by comparison with literature data (Budzikiewicz 1997a and 1997b), as well as by H,H-COSY- and ^{1}H,[13]C-correlations. In this way the

*Table 2.* $^{13}$C-NMR data of **1a** (solvent and standard as for Table 1); $\delta$(TMS) = $\delta$(DSS) − 1.61 ppm.

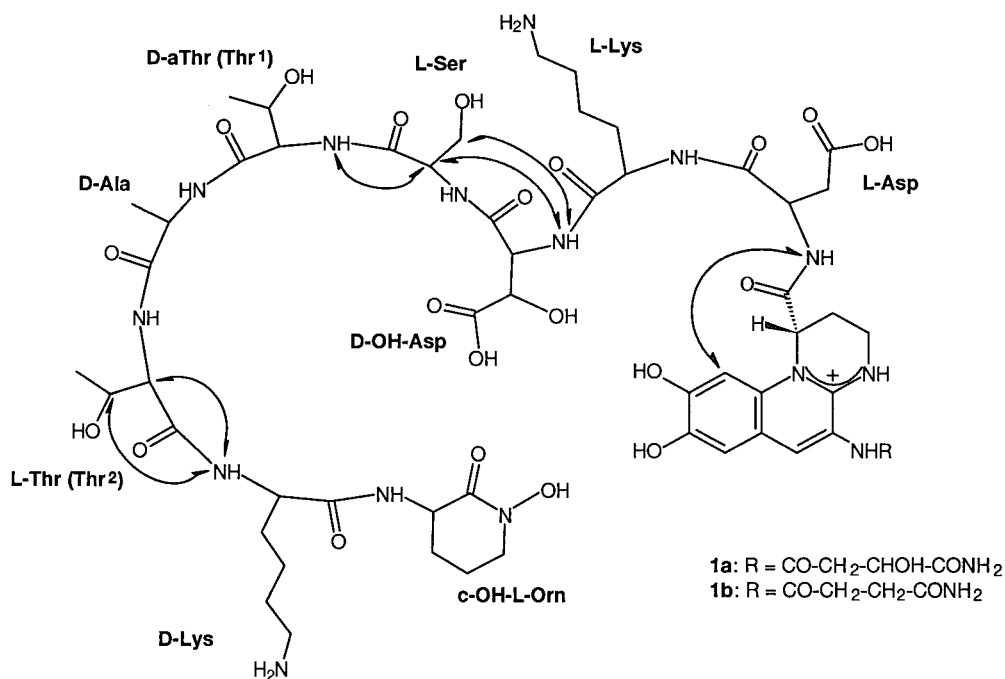|  | CO | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\epsilon$ |  |
|---|---|---|---|---|---|---|---|
| Ala | 175.8 | 50.6 | 17.2 |  |  |  |  |
| Asp | 173.0 | 54.0 | 39.5 | 177.4 |  |  |  |
| OH Asp | 171.4 | 57.7 | 73.3 | 172.9 |  |  |  |
| Lys | 172.8 | 54.0 | 31.0 | 23.1 | 27.1 | 40.1 |  |
| Lys | 174.0 | 54.0 | 31.0 | 23.1 | 27.1 | 40.1 |  |
| Ser | 173.4 | 56.9 | 61.9 |  |  |  |  |
| Thr 1 | 173.1 | 60.4 | 68.2 | 19.8 |  |  |  |
| Thr 2 | 173.1 | 60.4 | 68.2 | 22.1 |  |  |  |
| cOH-Orn | 167.3 | 51.3 | 31.0 | 21.1 | 52.5 |  |  |
| Mala | CONH$_2$ | C2 | C3 | CONH-Chr |  |  |  |
|  | 177.9 | 40.3 | 69.2 | 176.1 |  |  |  |
| Chromophore | CO | 1 | 2 | 3 | 4a | 5 |  |
|  | 171.9 | 57.6 | 22.0 | 365.0 | 149.0 | 118.0 |  |
|  | 6 | 6a | 7 | 8 | 9 | 10 | 10a |
|  | 139.0 | 115.6 | 114.5 | 144.6 | 152.4 | 101.4 | 136.3 |



*Figure 1.* Pyoverdin from *Pseudomonas putida* CFBP 2461 including sequence specific signals in the NOESY spectrum.

*Table 3.* Fragments observed from **1a** in a CID-experiment.

| Structure unit | B-ions | $m/z$ | (Y+2)−ions | $m/z$ |
|---|---|---|---|---|
| Mala-Chr-CO | $B_0$ | 374 | $Y_9$ | – |
| Asp | $B_1$ | 488 | $Y_8$ | 878 |
| Lys | $B_2$ | – | $Y_7$ | – |
| OHAsp | $B_3$ | 747 | $Y_6$ | 619 |
| Ser | $B_4$ | 834 | $Y_5$ | – |
| aThr | $B_5$ | 935 | $Y_4$ | – |
| Ala | $B_6$ | 1006 | $Y_3$ | 360 |
| Thr | $B_7$ | 1107 −$H_2O$ | $Y_2$ | 259 |
| Lys | $B_8$ | – | $Y_1$ | – |



*Figure 2.* Azotobactin chromophore.



*Figure 3.* Retro–Diels–Alder-fragmentation of **2**.

presence of two Lys residues could also be established, where all [1]H- and with the exception of the CO- all ^{13}C-signals coincide: the $\alpha$-CH-signal at 4.34 ppm gave cross peaks with two CO-signals (172.8 and 174.0 ppm). A TOCSY experiment showed crosspeaks between the amide-NH- and the side chain protons of the respective amino acids as well as between the $\epsilon$-NH$_2$- and the $\epsilon$-CH$_2$-protons of the two Lys residues demonstrating connections via their $\alpha$-NH$_2$ groups. Two shift values deserve special attention: The amide proton of Asp exhibits a pronounced downfield shift as compared with the other amide protons caused by the connection with the chromophore (see below). The position of the CO-signal of Orn at 167.3 ppm is a clear indication that this amino acid is cyclized to give cOHOrn. The CO-signal of the malic acid residue at 177.9 ppm suggests that it is present as its amide (Chr-NH-CO-CH$_2$-CHOH-CONH$_2$). From the NMR data it could, however, not be decided whether Asp and OHAsp have free COOH-groups or also form amides. The molecular mass (1364 u) and the mass differences in the CID-spectrum (see below) confirm the presence of the malamide side chain and demonstrate the presence of free carboxyl groups for Asp and OHAsp.

The amino acid sequence of the peptide chain could be established by a CID-MS experiment. The pertinent fragment ions (Biemann *et al.* 1987) are listed in Table 3. As can be seen the B-series (cleavage of the -CO-NH-bonds with charge retention on the carbonyl group, i.e. R-C=O$^+$) is complete with exception of the cleavage between the two Lys residues and the following amino acids. The series of the complementary (Y+2)-ions (cleavage of the CO-NH-bonds with transfer of two H to give H$_3$N$^+$-R ions) is less complete. It can b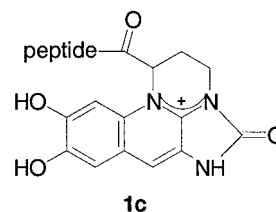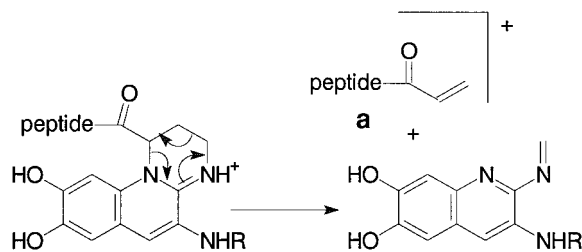e seen that the sequence of the amino acids (Figure 1) differs from that originally deduced from a FAB-MS spectrum in that at the C-terminal end Lys and Thr are interchanged. The sequence deduced from the CID-MS spectrum is confirmed by NMR data: cross peaks between the Lys-NH and the $\alpha$- and the $\beta$-CH of Thr were established by a NOESY experiment (mix time 140 ms, solvent H$_2$O/D$_2$O 9:1, pH 4.2, suppression of the water signal by presaturation).

In order to locate D- and L-Lys as well as D-aThr and L-Thr which cannot be distinguished by MS and NMR techniques a partial hydrolysis of **1b** was performed (6N HCl, 30 min, 90 °C) and the hydrolysate was separated by chromatography on Bio-Gel P-2 with 0.1 M CH$_3$COOH. The main fraction was rechromatographed on Sephadex G-10. In this way a compound with [M + H]$^+$ at $m/z$ 1009 as determined by FAB-MS could be isolated corresponding to Suc-Chr-Asp-Lys-OHAsp-Ser-Thr-Ala which contains only one Lys and one Thr. This compound was subjected to total hydrolysis (6N HCl, 8 h, 110 °C). One half of the hydrolysate was TAP derivatized and GC analyzed on a Chirasil-L-Val column and L-Lys and D-aThr could be identified as the stereoisomers located closer to the chromophore. The other half of the hydrolysate was dansylated and separated on a chiral HPTLC plate (solvent: methanol/H$_2$O/acetontrile 50:40:20, 0.05 M KH$_2$PO$_4$). Again D-aThr was identified. From these data the structures of the pyoverdins of *Pseudomonas putida* CFBP 2461 **1a** and **1b** as given in Figure 1 could be deduced.

From the pyoverdin fraction a green fluorescent component could be separated by HPLC with a mole-

*Table 4.* $^1$H-NMR data from **2** (solvent and standard as for Table 1).

| | HN | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\epsilon$ | H$_2$N | AcCH$_3$ |
|---|---|---|---|---|---|---|---|---|
| Gln | 8.76 | 4.38 | 2.20/2.14 | 2.321 | | | – | |
| Gly 1 | 8.62 | 3.99 | | 2.37 | | | | |
| Gly 2 | 8.44 | 3.86 | | | | | | |
| AcOH-Orn | 8.73 | 4.35 | 1.99/1.84 | 1.80 | 3.57 | | | 1.98 |
| Lys | 9.61 | 4.37 | 1.86 | 1.4 | 1.68 | 2.99 | – | |
| Ser | 8.47 | 4.47 | 3.88 | | | | | |
| Thr1 | 8.57 | 4.31 | 4.11 | 1.23 | | | | |
| Thr2 | 8.24 | 4.35 | 4.18 | 1.19 | | | | |
| cOH-Orn | 8.66 | 4.49/4.44 | 2.03/1.81 | 1.93 | 3.63 | | | |
| Suc | 2 | 3 | | | | | | |
| | 2.69 | 2.61 | | | | | | |
| Chromophore | 1 | 2a | 2b | 3a | 3b | 4HN$^+$ | 6 | 7 | 10 |
| | 5.47 | 2.492 | 2.66 | 3.32 | 3.70 | – | 7.79 | 6.82 | 6.78 |
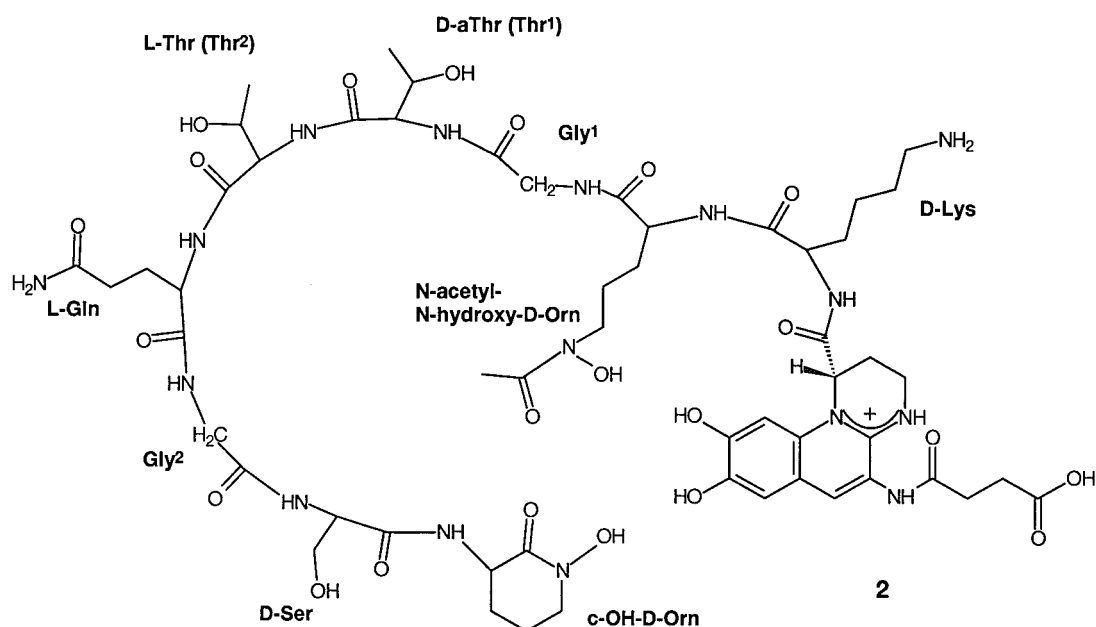


*Figure 4.* Pyoverdin from *Pseudomonas fluorescens* CFBP 2392.

cular mass of 1276 u as determined by FAB-MS. The green fluorescence (in contrast to the yellow-green one of pyoverdins) suggested an azotobactin chromophore **1c** (see Figure 2). This was confirmed by the $^1$H-NMR spectrum where the signals of the malamide side chain are missing. The signals of the chromophore protons (Table 1) are shifted downfield as compared with those of the pyoverdin chromophore (especially H-3b which is located in the deshielding space of the new carbonyl group) in accordance with literature data (Hohlneicher *et al.*, 1994; Schaffner *et al.*, 1996). The co-occurance of an azotobactin (the typical siderophore of *Azotobacter vinelandii*) with pyoverdins isolated from a *Pseudomonas* strain (both having the same peptide chain) has been reported in the literature only twice (Demange *et al.* 1990; Hohlneicher *et al.* 1994).

*Table 5.* $^{13}$C-NMR data from **2** (solvent and standard as for Table 2).

|  | CO | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\epsilon$ | AcCH$_3$ | AcCO |
|---|---|---|---|---|---|---|---|---|
| Gln | 174.7 | 54.4 | 27.4 | 31.8 | 178.8 | | | |
| Gly 1 | 172.3 | 43.7 | | | | | | |
| Gly 2 | 172.2 | 43.7 | | | | | | |
| AcOH-Orn | 174.7 | 54.5 | 27.7 | 21.2 | 53.1 | | 22.0 | 176 |
| Lys | 172.9 | 54.5 | 27.5 | 23.0 | 27.1 | 40.1 | | |
| Ser | 172.3 | 56.5 | 62.1 | | | | | |
| Thr1 | 173.1 | 60.4 | 67.8 | 19.5 | | | | |
| Thr2 | 173.2 | 59.9 | 68.3 | 19.7 | | | | |
| cOH-Orn | 167.4 | 48.9 | 31.8 | 20.9 | 52.9 | | | |

| Suc | $\gamma$-COOH | C2 | C3 | CONH-Chr | | | | |
|---|---|---|---|---|---|---|---|---|
| | 181.7 | 33.6 | 32.9 | 178.3 | | | | |

| Chromophore | CO | 1 | 2 | 3 | 4a | 5 | | |
|---|---|---|---|---|---|---|---|---|
| | 171.3 | 140.4 | 23.1 | 35.9 | 155.0 | 117.0 | | |

| | 6 | 6a | 7 | 8 | 9 | 10 | 10a | |
|---|---|---|---|---|---|---|---|---|
| | 140.4 | 114.7 | 114.4 | 145.0 | 150.0 | 101.5 | 133.0 | |

### The pyoverdin of Pseudomonas fluorescens CFBP 2392 (identical with Pseudomonas A6)

For this pyoverdin an amino acid composition of 1 Glu, 2 Gly, 1 Lys, 1 OHOrn, 1 Ser and 2 Thr was reported (Demange *et al.* 1986) and later on the sequence

Chr-OHOrn-Lys-Gly-Thr-Thr-Gly-Gln-Ser-cOHOrn

was suggested based on FAB-MS measurements (Demange *et al.* 1989).

In the course of our investigations by FAB-MS a molecular mass of 1318 u was determined. In addition, a fragment formed by *retro*-Diels-Alder-(RDA)-decomposition of the tetrahydropyrimidine ring of the chromophore (Michels & Taraz 1991) was observed at *m/z* 1016 (Figure 3, **a**). The mass difference of 303 u to the [M + H]$^+$-ion at *m/z* 1319 indicates the presence of a succinic acid side chain. Amino acid analysis by total hydrolysis (6N HCl, 12 h, 110 °C) and GC analysis of the TAP derivatives on a Chirasil-L-Val column established the presence of L-Glu, Gly, D-Lys, D-Orn, D-Ser, L-Thr and D-aThr in agreement with the $^1$H- and $^{13}$C-NMR data (Tables 4 and 5). The identification of the signals was achieved as described above. Several points should be emphasized: The CO-signals of one of the Orn residues occurs at 167.4 ppm in agreement with the presence of cOHOrn, one of the complexing sites. The second one in the peptide chain is N-acetyl-N-hydroxy-Orn as evidenced by the signals for an N-acetyl group. The pronounced down-field shift of the amide NH resonance of Lys as compared with those of the remaining amide protons is a strong evidence for placing Lys as the amino acid bound to the chromophore. This is not in agreement with the sequence originally proposed because the amino acid pairs OHOrn-Lys and Gly-Gln have to be reversed.

The amino acid sequence of the peptide chain was established by a CID-MS experiment starting from the RDA-fragment **a** (Table 6). It is supported by ROESY experiments confirming especially those parts of the peptide chain which differ from the sequence originally proposed from FAB measurements only. To locate L-Thr and D-aThr in the peptide chain 1 mg of the pyoverdin **2** was hydrolyzed with 400 $\mu$l 6 M HCl/glacial acetic acid 1:1 (v:v) for 15 min at 50 °C. The solution was dried i. v., FAB-MS of the residue showed that the peptide chain was hydrolyzed at the amide bond between Gly1 and Thr1 (see Figure 4). The material was dansylated without further purification and completely hydrolyzed with 1 ml of 6 M HCl at 90 °C for 8 h (Risse *et al.* 1998). Dansyl-derivatives of the amino acids were adsorbed on SepPak cartridges and further investigated by TLC on chiral plates. Only D-aThr could be identified.

*Table 6.* Fragments of **a** (see Figure 3) observed in a CID-experiment.

| Structure unit | B-ions | m/z | (Y+2)−ions | m/z |
|---|---|---|---|---|
| RDA-peptide | $B_0$ | – | $Y_9$ | – |
| Lys | $B_1$ | 183 | $Y_8$ | 832 |
| AcHOOrn | $B_2$ | 355 | $Y_7$ | 660 |
| Gly | $B_3$ | 412 | $Y_6$ | 603 |
| Thr | $B_4$ | 513 | $Y_5$ | 502 |
| Thr | $B_5$ | 614 | $Y_4$ | – |
| Gln | $B_6$ | 742 | $Y_3$ | – |
| Gly | $B_7$ | 799 | $Y_2$ | – |
| Ser | $B_8$ | 886 | $Y_1$ | – |

From the data presented follows the structure **2** of the pyoverdin from *Pseudomonas fluorescens* CFBP 2392 as presented in Figure 4. It seems worth mentioning that, besides the first amino acid next to the chromophore (Lys instead of Ser), the peptide chain of **2** is identical to the peptide chain of the pyoverdin from *P. aureofaciens* published earlier (Beiderbeck *et al.* 1999). Therefore it would be interesting to perform crossfeeding experiments between the two strains.

## Conclusion

Two pyoverdin structures reported earlier without experimental details had to be revised on the basis of degradation experiments and modern NMR and MS techniques. In this way also the D- and L-amino acids could be distinguished and located. It became evident that the FAB-MS technique of the late eighties was not reliable enough to determine pyoverdin amino acid sequences.

## Acknowledgements

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