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Characterization of the chromophores of pyoverdins and related siderophores by electrospray tandem mass spectrometry

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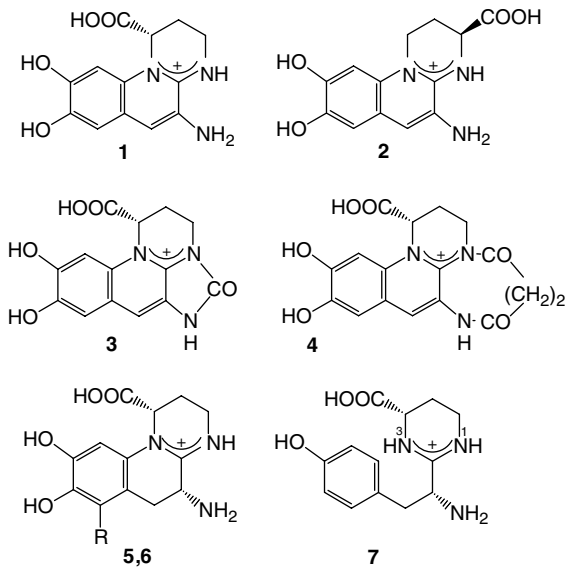
Abstract

Characteristic fragment ions of the various chromophores of the pyoverdin siderophore family obtained by collision activated dissociation of the $[M+2H]^{2+}$ ions are reported allowing unambiguous identification. Tandem mass spectrometrical studies revealed the existence of the first example of a ferribactin with a succinamide side chain, and they add some information to the problem in which way a malic acid side chain is attached to the chromophore.

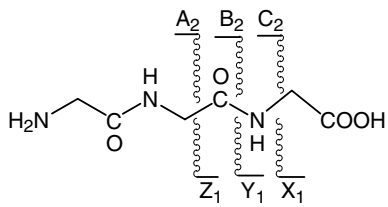
Introduction

Members of the fluorescent species in the rRNA homology group I of the family Pseudomonadaceae produce siderophores with high complexing constants. The most important representatives are the pyoverdins. They comprise three distinct structural parts, viz. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6–12 amino acids (partially modified) bound to the carboxyl group, and a small dicarboxylic acid (succinic and malic acid or their monoamides, glutamic and α-ketoglutaric acid) connected amidically to the NH_2-group of the chromophore. Frequently several pyoverdins co-occur differing only in the nature of this acid side chain. The pyoverdins may be accompanied by related compounds which are considered as their biosynthetic precursors or later modifications (see Scheme 1). These compounds possess the same peptide chain as the pyoverdin but differ in the nature of the chromophore (Budzikiewicz 2004). For a rapid partial characterization of pyoverdins and their congeners electrospray mass spectrometry with fragmentation of the molecular ion species, especially of $[M+2H]^{2+}$, by collision activation (CA) has been proven to be highly efficient (Fuchs & Budzikiewicz 2001a).

It is reasonable to assume that in $[M+2H]^{2+}$ ions one proton is located in the chromophore due to the distinct ability of the latter to delocalize the positive charge. This leads to a series of abundant *N*-terminal fragments. The second proton according to the "mobile proton" model (Harrison & Yalcin 1997) can reside at the various amide groups of the peptide backbone facilitating cleavages in the near vicinity, giving thus amino acid sequence information (for the nomenclature of peptide fragments (see Scheme 2). In order to have a starting point for the identification of the peptide fragments it is necessary to recognize the nature of the chromophore. In the following discussion, characteristic fragmentation processes for the various chromophore types will be described. Additional amino acid sequence information by MS^n experiments in the ion trap (Fuchs & Budzikiewicz 2000, 2001a) and possible



Scheme 1. Chromophores: **1**: pyoverdin, **2**: isopyoverdin, **3**: azotobactin, **4**: succinopyoverdin, **5**: R = H, dihydropyoverdin, **6**: R = SO_3H, dihydropyoverdin-7-sulfonic acid, **7**: ferribactin.



Scheme 2. Nomenclature of peptide fragments obtained by mass spectrometry. Apostrophs indicate the number of transferred hydrogen atoms.

pitfalls due to rearrangement reactions (Fuchs & Budzikiewicz 2001b; Schäfer *et al*. 2006) have been discussed elsewhere. Wherever possible the characteristic processes discussed below were confirmed by comparing the fragmentation behavior of several structurally different pyoverdins and related siderophores.

Experimental

Mass spectra were obtained with a MAT 900 ST instrument with an EB-QIT (quadrupol ion trap) geometry and equipped with an ESI II ion source (Finnigan MAT, Bremen, Germany); spray voltage 3.4–3.6 kV, capillary temperature 230 °C. The samples were dissolved in water, methanol, and acetic acid 50:50:0.1 (v/v). Selection and isolation of the monoisotopic signal of $[M+2H]^+$ precursor ions was effected in the double focussing (EB) sector part of the mass spectrometer. Collision activation (CA) in the octapole unit in front of the QIT with He diffusing from the QIT as collision gas was followed by product ion analysis in the QIT.

The ferribactin **9** was isolated from a pyoverdin-negative transposon of *Pseudomonas fluorescens* ATCC 17400 (unpublished data). The strain was grown for 48 hours in a succinate medium and the supernatant was passed on a C-18 column which was equilibrated with methanol and subsequently rinsed with water. After removing inorganic material with water the siderophore was eluted with 80% methanol. The fraction giving a large halo on CAS medium (Schwyn & Neilands 1987) was analyzed. Detailed descriptions of the work-up of siderophore mixtures obtained from *Pseudomonas* strains had been published earlier (e.g., Mohn *et al*. 1990); for decomplexation see Schröder *et al*.(1995).

Results

*The pyoverdin chromophore (***1***)*

For a large number of pyoverdins the first amino acid after the chromophore is serine, as for pyoverdin **8** (Scheme 3) (Uría Fernández *et al*. 2003). In this case CA of $[M+2H]^{2+}$ results in the loss H_2O (*m*/*z* 579), and an abundant A_1 ion is formed for a Suca (*m*/*z* 416) or Suc side chain (*m*/*z* 417) (for abbreviations see Scheme 3) which loses NH_3 or H_2O, respectively, (*m*/*z* 399) as well as the complete Suca/Suc residue leaving the free NH_2 group (*m*/*z* 317). For a Mala/Mal side chain analogous processes are observed leading to *m*/*z* 432/433 and 317. Opening of the tetrahydropyrimidine ring of the chromophore by a *retro*-Diels-Alder (RDA) process should lead to *m*/*z* 303/304 for Suca/Suc and *m*/*z* 319/320 for Mala/Mal, but only *m*/*z* 304/320 can be observed, while NH_3 is lost readily (possibly under formation of a succinimide structure), giving *m*/*z* 286/302, respectively. Elimination of the acid residue as described above results in the ion *m*/*z* 204, most characteristic for the pyoverdin chromophore (Scheme 4).

Additional ions which are observed with varying abundance (*m*/*z* values are given here for Ser

8: RCO-Chr-Ser-Ala-Aho-[Orn-Ser-Aho-Asp]

9: RCO-Chr-Ala-Lys-Gly-Gly-OHAsp-[Gln-Dab]-Ser-Ala-cOHOrn

10: RCO-Chr-Ser-Lys-Gly-Fho-[Lys-Fho-Ser]

11: RCO-Chr- Lys-OHAsp-Thr-[Thr-Ser-OHAsp-Ser]

12: RCO-Chr-Asp- Lys-OHAsp-Ser-Thr-Ala-Glu Ser-cOHOrn

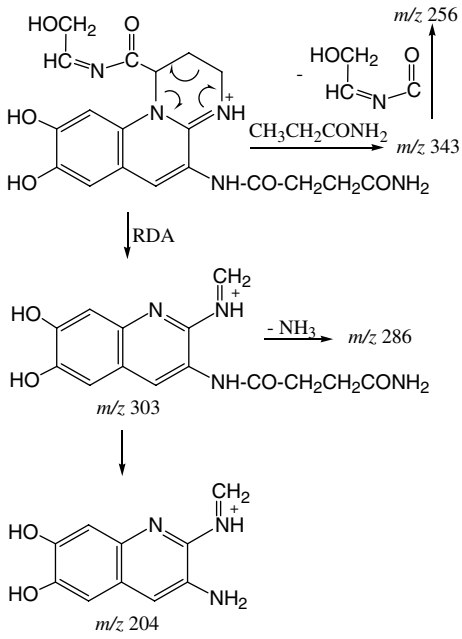
13: RCO-Chr-Asp-Lys-Thr-OHAsp-Thr-Thr-cOHOrn

Scheme 3. Structures of the siderophores mentioned in the text. Chr stands for the respective chromophores depicted in Scheme 1; R = HOOC–CH_2–CH_2– (Suc), H_2NOC–CH_2–CH_2– (Suca), HOOC–CHOH–CH_2– (Mal), H_2NOC–CHOH–CH_2– (Mala), HOOC–$CHNH_2$–CH_2–CH_2– (Glu), HOOC–CO–CH_2–CH_2– (Kgl); Fho, *δ–N*-formyl-*δ*-*N*-hydroxy Orn; εLys, Lys bound amidically with its ε amino group; OHAsp, 3-hydroxy Asp, cOHOrn *cyclo*-*N*-hydroxy Orn; Chr, chromophore (see text and Scheme 1), [...] indicates a cyclic substructure.

and Suca) are *m/z* 343 (loss from A_1 of $[CH_2CH_2CONH_2 + H]$ for Suca possibly giving an azotobactin structure) and *m/z* 256 (loss of $CONH=CHCH_2OH$ from *m/z* 343). Comparison with the spectrum of a ^{15}N labeled pyoverdin with the Ser/Suca pattern established the expected number of N atoms for all fragments discussed above.

For pyoverdin **9** (Demange *et al*. 1990) (first amino acid alanine and Mala as side chain) shifts to lower *m/z* values by 16 u for parts of the molecule containing Ala (*m/z* 327, 301), and to higher *m/z* values by 16 u for parts containing Mala are recorded (Figures 1 and 2 and Table 1). For A_1 and $[A_1 - NH_3]$ (*m/z* 416 and 399) the shifts cancel each other.

If a glutamic acid side chain is present (e.g., pyoverdin **9**, $[M+2H–Glu]^{2+}$ gives *m/z* 600.5), A_1 (*m/z* 430) is of low abundance, but pronounced $[A_1 - H_2O]^+$ (*m/z* 412) and $[A_1–Glu]^+$ (*m/z* 301) ions are observed. The ion (*m/z* 204) is clearly



Scheme 4. Fragmentation of the pyoverdin chromophore.

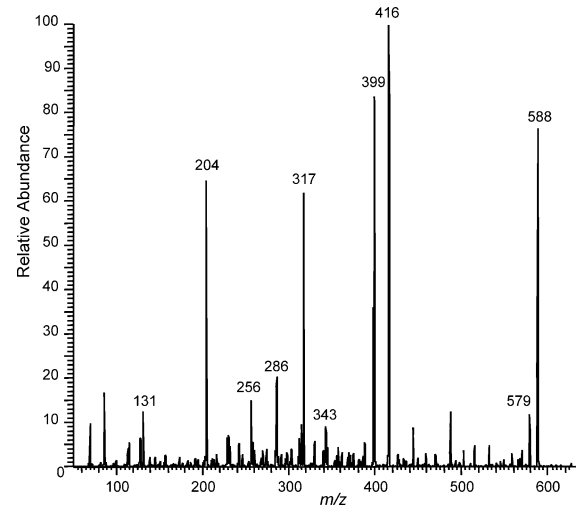


Figure 1. Octapole CID spectrum of $[M+2H]^{2+}$ (*m/z* 588) of pyoverdin **8** (R = Suca).

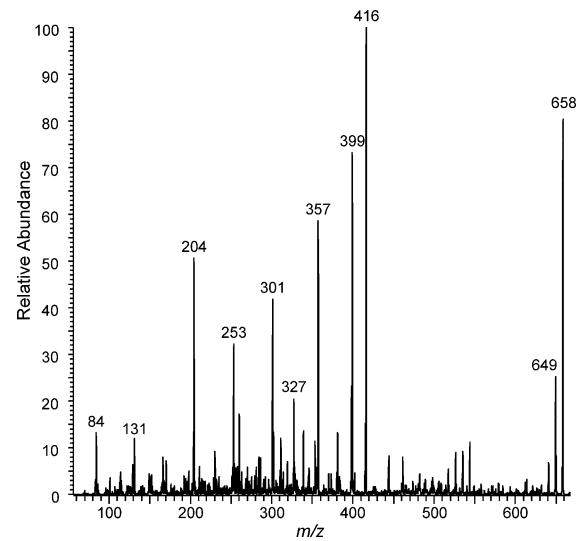


Figure 2. Octapole CID spectrum of $[M+2H]^{2+}$ (*m/z* 658) of pyoverdin **9** (R = Mala).

Table 1. Pyoverdin fragments (*m/z* values in parentheses weak to negligible)

Figure	1		2	
1st amino acid	Ser	Ser	Ala	Ala
Side chain	Suca	Mala	Suc	Mala
A_1	416	432	400	416
-NH_3	399	415	–	399
-CH_2=COH-NH_2	–	373	–	357
-NH_2-CO-CHR-CH_3	(343)	343	(327)	327
-COSer	256	256	–	–
-acid residue	317	317	301	301
RDA	(303)	(319)	(303)	(319)
-NH_3	286	302	286	302
-acid residue	204	204	204	204

recognizable (Figure 3). The presence of a Kgl side chain (e.g., pyoverdin **10**) (Hohlneicher *et al.* 1995) is evidenced by the loss of $[H_2O + CO_2]$ from an A_1 ion of low abundance (*m/z* 445) yielding a pronounced (*m/z* 383). Loss of $[H_2O + CO_2]$ (i.e. 62 Da) is typical for pyoverdins with a Kgl side chain and it is observed starting from the doubly charged molecular ion species (*m/z* 595 → 564; 62/2 Da) as well as from all fragment ions containing the Kgl residue. An abundant ion at *m/z* 270 could be explained by elimination of COOH from the RDA fragment, while *m/z* 204 is of low abundance (Figure 4).

When the first amino acid is lysine bound with its ε-amino group to the chromophore (pyoverdin **11**) (Bultreys *et al.* 2004) the A_1 fragment (*m/z* 457, - NH_3 *m/z* 440, B_o *m/z* 357) is of medium abundance, otherwise the fragmentation corresponds to that described above for Suca/Suc side chains, (RDA–NH_3 *m/z* 286; *m/z* 256) resulting in an abundant *m/z* 204 ion (Fig. 5). For aspartic acid as the first amino acid as in pyoverdin **12** (Persmark *et al.* 1990) B_1 (*m/z* 472, -NH_3 *m/z* 455) instead of A_1 was observed, at least when the second amino acid is lysine bound by its ε-amino group (the only examples available). B_1 loses the acid side chain leaving the free NH_2 group as described above (-Suca *m/z* 373). The RDA fragment (*m/z* 302/286 for a Mala/Suca side chain) as well as an abundant *m/z* 204 ion are observed. The abundant ion *m/z* 131 stems from the *C*-terminal cOHOrn (Figure 6).

An interesting fragmentation process of pyoverdins with a Mala side chain should be mentioned which had not been discussed before, viz. the loss of 59 Da from A_1 ions (see *m/z* 357 in Figure 2, which is shifted to *m/z* 373 when the first amino acid is Ser as in **1** with a Mala side chain). The B_1 ion *m/z* 488 of a pyoverdin with Asp as the first amino acid gives *m/z* 429 of high abundance. Obviously this fragmentation process does not occur in the peptide residue. An explanation would be an elimination of CH_2=COH–NH_2 by a McLafferty rearrangement provided the hydoxyl group is located on the carbon atom closer to the chromophore (Scheme 5) in contrast to the for-

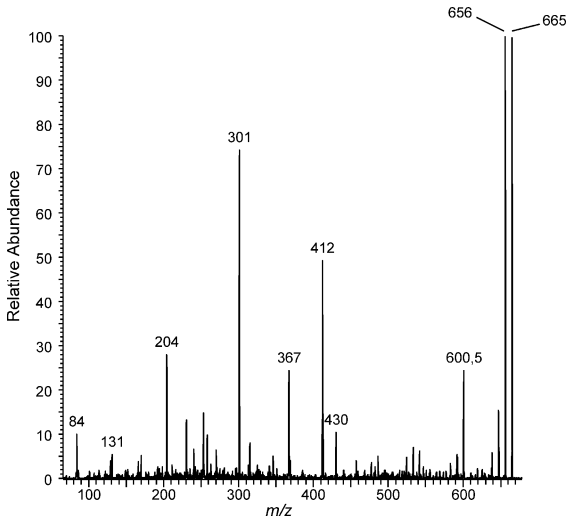


Figure 3. Octapole CID spectrum of $[M + 2H]^{2+}$ (*m/z* 665) of pyoverdin **9** (R = Glu).

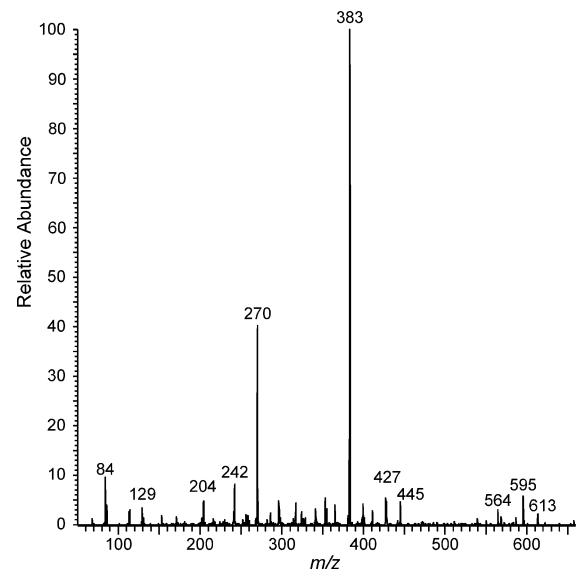


Figure 4. Octapole CID spectrum of $[M + 2H]^{2+}$ (*m/z* 595) of pyoverdin **10** (R = Kgl).

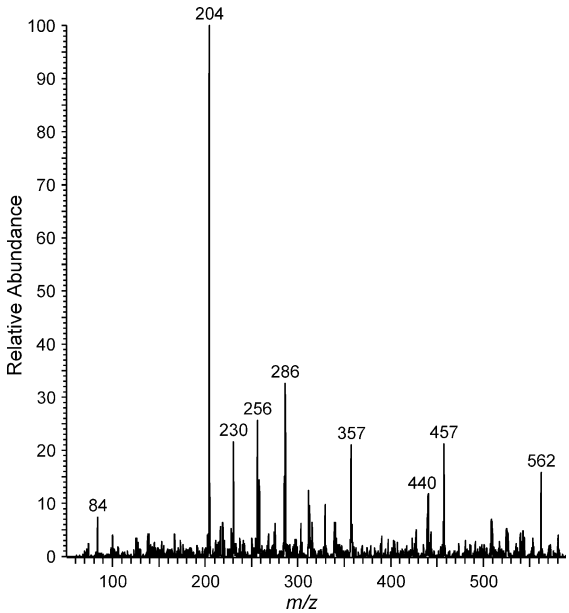


Figure 5. Octapole CID spectrum of $[M+2H]^{2+}$ (m/z 562) of pyoverdin **11** (R = Suca).

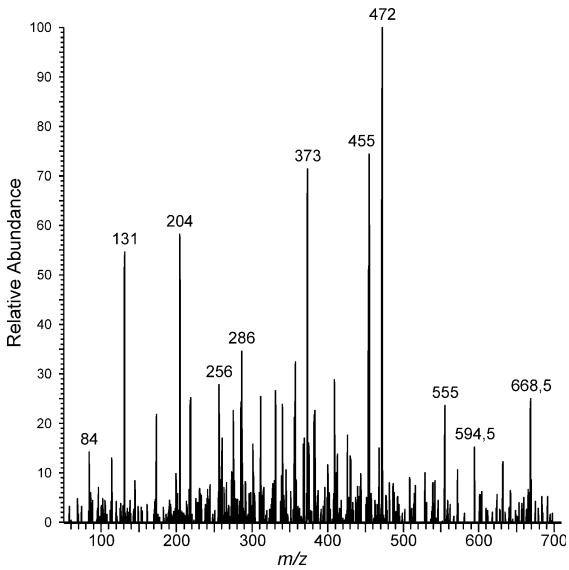
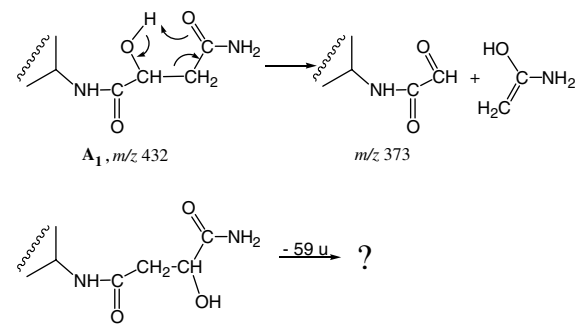


Figure 6. Octapole CID spectrum of $[M+2H]^{2+}$ (m/z 668.5) of pyoverdin **12** (R = Suca).

mulation usually found in literature (see Scheme 3). This issue is further discussed below.

*The azotobactin (**3**) and succinopyoverdin (**4**) chromophores*

Azotobactins are actually the typical siderophores of the species *Azotobacter vinelandii*, but



Scheme 5. Proposed McLafferty rearrangement of a malamide side chain.

occasionally they co-occur with pyoverdins (Hohlneicher *et al*. 1995). With serine as the first amino acid A_1 dominates the CA spectrum (**8**). The RDA fragment (m/z 230) is of rather low abundance (Figure 7). For the corresponding succinopyoverdin (**8**) A_1 is of high and the RDA fragment (m/z 286) of appreciable abundance. A small (m/z 204) ion can be observed (Scheme 6 and Figure 8).

*The dihydropyoverdin chromophore (**5**)*

While azotobactins and succinopyoverdins are probably late transformation products in the biosynthetic chain, dihydropyoverdins are the

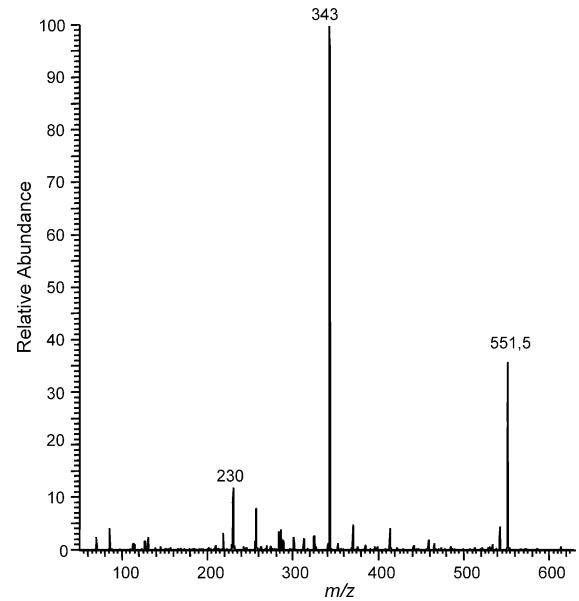
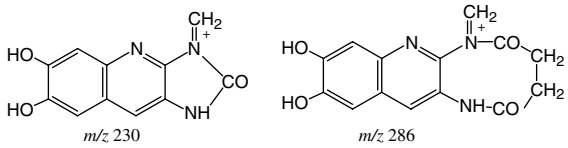


Figure 7. Octapole CID spectrum of $[M+2H]^{2+}$ (m/z 551.5) of azotobactin **8**.



Scheme 6. RDA fragments of azotobactins and succinopyoverdins.

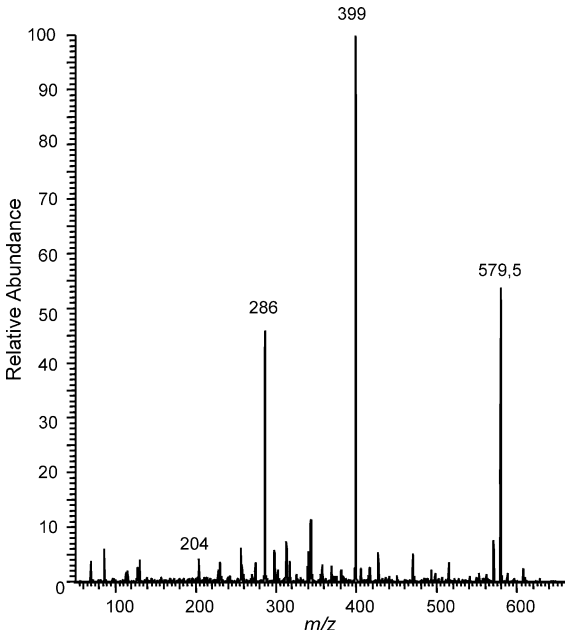
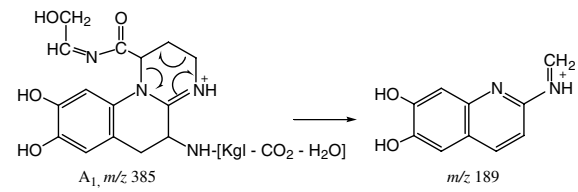


Figure 8. Octapole CID spectrum of $[M+2H]^{2+}$ (m/z 579.5) of succinopyoverdin **8**.

immediate precursors of the pyoverdins. With serine as the first amino acid (**10**) they yield A_1 (m/z 447) and its degradation products as discussed above, e.g., loss of $[H_2O+CO_2]$ for a Kgl side chain (m/z385, Figure 9). The RDA can not be discerned; dihydropyoverdins lose readily the [H_2N-acid residue] group yielding m/z 189 (Scheme 7). An ion (m/z 189) can also be observed for dihydropyoverdin-7-sulfonic acids (**6**) due to the additional loss of SO_3.

*The ferribactin chromophore (**7**)*

Ferribactins are considered to be the peptidic precursors of the dihydropyoverdins (Hohlneicher *et al.* 2001) whose tricyclic chromophore is formed by a complex sequence of steps (Budzikiewicz 2004). Until recently only ferribactins with a glutamic acid side chain had been reported (**8**), but recently a representative with Suca side chain (**9**) was found. Comparison of the fragmen-



Scheme 7. Characteristic fragment of a dihydropyoverdin.

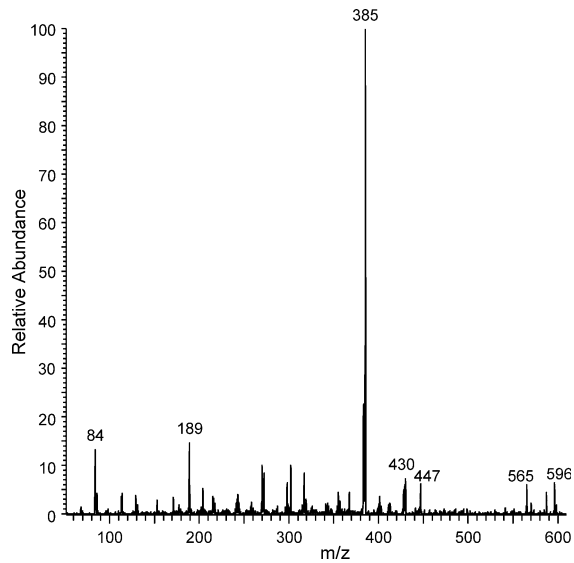


Figure 9. Octapole CID spectrum of $[M+2H]^{2+}$ (m/z 596) of dihydropyoverdin **10** (R = Kgl).

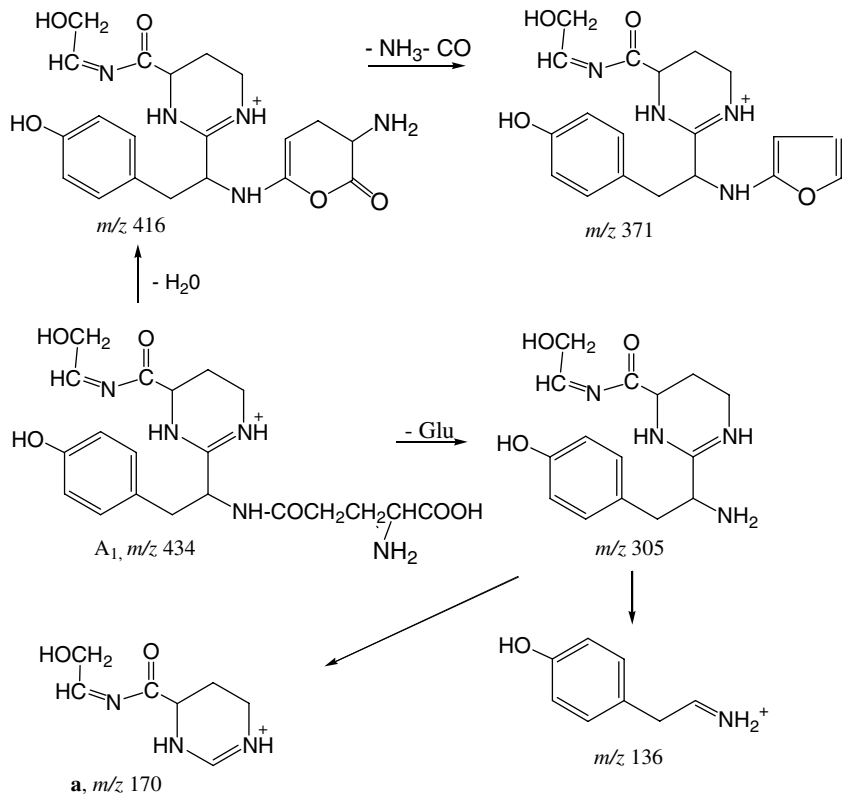
tation patterns allows to confirm the proposed sequences by recording the mass differences due to the replacement of Glu by Suca and Ser by Ala (Table 2). A_1 is observed only when the acid side chain is Suca; if Glu is present as side chain ready loss of H_2O is observed: for the product ion a dihydro-α-pyrone structure is proposed. In agreement with this formulation is the subsequent loss of NH_3 and CO resulting in a furan ring. Loss of Glu/Suca from A_1 as above is followed by the elimination of methylphenol (*m*/*z* 170) and by the formation of **a** (Scheme 8 and Figures 10 and 11).

Table 2. Ferribactin fragments (*m*/*z* values in parentheses – weak to negligible).

Figure	10	11	
1st amino acid	Ser	Ala	Ala
Side chain	Glu	Glu	Suca
A_1	(434)	(418)	388
- H_2O	416	400	370
- NH_3- CO	371	355	–
- NH_3	–	–	371
- Glu/Suca	305	289	289
- CH_3-C_6H_4OH	198	182	182
a	170	154	154
HOC_6H_4-CH_2-CH = NH_2^+	136	136	136

*The isopyoverdin chromophore (***2***)*

Ring closure between *N*-3 of the dihydropyrimidine ring of the ferribactin chromophore and the phenol ring yields the pyoverdin chromophore. Alternate reaction with *N*-1 leads to the isomeric isopyoverdin structure produced by some *Pseudomonas putida* strains. CA spectra are available for two representatives (e.g. **13**) (Sultana *et al*. 2001) which have aspartic acid as the first amino acid followed by lysine, and Glu as side chain. As observed for corresponding pyoverdins (see above) B_1 (*m*/*z* 502) is formed though with low abundance, but ions due to the loss of H_2O (*m*/*z* 484) and of Glu (*m*/*z* 373), respectively, are clearly recognizable. RDA as for pyoverdins is not observed. Instead, loss of the entire peptide chain and of the glutamic acid residue results in *m*/*z* 230 giving a conjugated dihydropyrimidinium system (Scheme 9 and Figure 12). With



Scheme 8. Fragmentation of ferribactins.

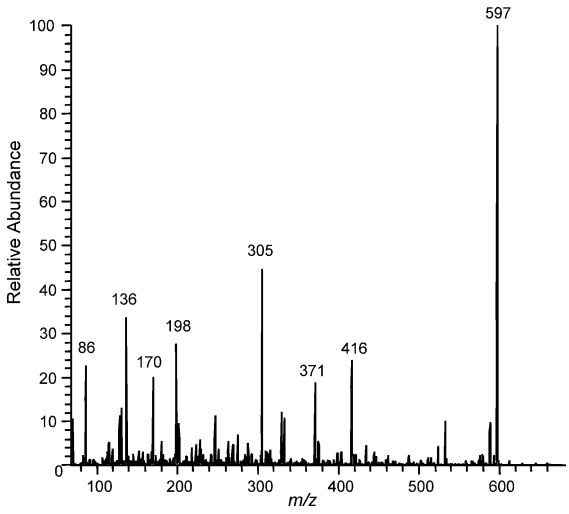


Figure 10. Octapole CID spectrum of $[M+2H]^{2+}$ (*m*/*z* 597) of ferribactin **8** (R = Glu).

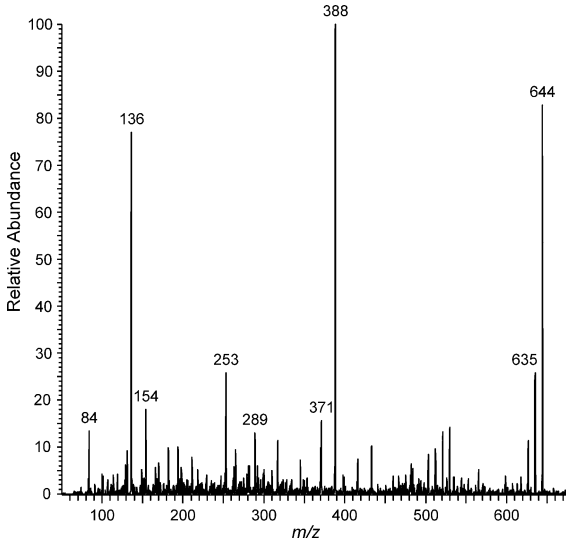
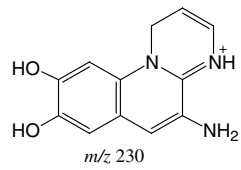


Figure 11. Octapole CID spectrum of $[M+2H]^{2+}$ (*m*/*z* 644) of ferribactin **9** (R = Suca).

pyoverdins *m*/*z* 230 is observed occasionally with negligible abundance because RDA prevails.

Discussion

A complete structure elucidation of a new pyoverdin is a tedious undertaking requiring multi-dimensional MS and NMR studies as well as chemical degradation. For a preliminary examination electrospray ionization tandem mass spectrometry with product analysis of fragment ions obtained by CA offers some structural evidence. In



Scheme 9. Characteristic fragment of isopyoverdins.

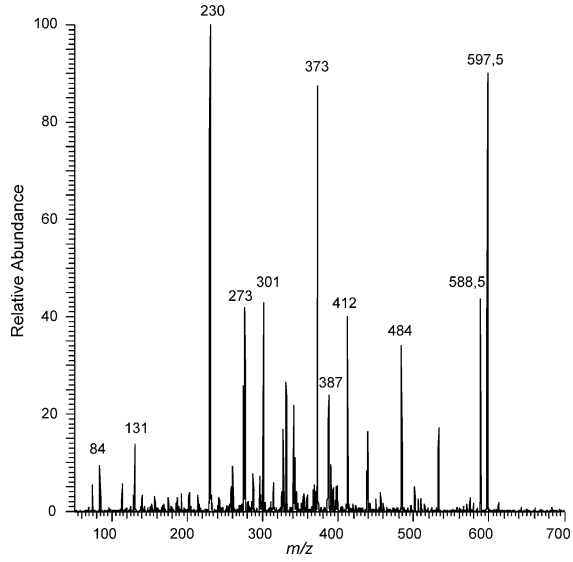


Figure 12. Octapole CID spectrum of $[M+2H]^{2+}$ (*m*/*z* 597.5) of isopyoverdin **13** (R = Glu).

general, the first amino acid bound to the chromophore can be identified unambiguously, and under optimal conditions a suggestion for the complete amino acid sequence can be made. Known representatives can be identified more reliably than by the isoelectrofocussing technique (Koedam *et al*. 1994), for new ones it is easier to decide whether it would be worth while to go into further detail. From a culture extract usually a mixture of siderophores is obtained varying in the nature of the acid side chain and/or the nature of the chromophore. A major advantage of the tandem MS approach is that mixture analysis is possible without complicated isolation of the single components. Molecular ion species are selected and subsequently fragmented by CA. Removal of admixtures by chromatography on an XAD-4 resin and decomplexation is sufficient (Fe^{3+} complexes yield less characteristic fragmentation patterns than the free chromophores). Special care should be taken that the sample is essentially free from inorganic salts which are detrimental to the

ion desorption by electrospray. It should also be kept in mind that fragments from other parts of the molecule may be found in the mass region below the masses of A_1 and m/z 204 characteristic for the pyoverdin chromophore, such as m/z 131 from Aho, Fho or cOHOrn, 84 from Lys to be seen in some of the Figures.

For structural studies by mass spectrometry it is essential to recognize the type of chromophore and the nature of the acid side chain. The typical fragment ions discussed above will be of help. Best results will be obtained by selection and monoisotopic isolation of $[M+2H]^{2+}$ precursor ions and CA fragmentation in collision multipoles, e.g. an octapole unit as described above. It should be kept in mind that the relative abundances of the various ions are influenced by the general structure of the molecule (e.g., the preferred loci for protonation), the pressure of the collision gas, and by the collision energy. For further mass spectrometrical studies it is best to select a component with a pyoverdin chromophore and a succinic acid/amide (Suc/Suca) side chain.

Two additional observations were made worth mentioning. Ferribactins had been shown to be the biosynthetic precursors of the pyoverdins (Hohlneicher *et al*. 2001). So far only ferribactins with a glutamic acid side chain had been reported. Its transformation following the citric acid cycle to ketoglutaric acid, succinamide and malamide was assumed to take place after the ring closure to the dihydropyoverdin system. The discovery reported here of a ferribactin from *P. fluorescens* ATCC 17400 with a Suca side chain shows that this process may occur even at that early stage (cf. also the transformation products of Glu in the ferribactin from *P. fluorescens* G173, Uría Fernández *et al*. 2003).

Regarding the dicarboxylic acid side chains the problem had to be solved by which of their two carboxyl groups they are attached to the amino group of the chromophore. Succinic acid is symmetrical and for glutamic acid and ketoglutaric acid the binding site had been established by chemical degradation. For malic acid some not really convincing and partially contradicting NMR arguments had been advanced (Seinsche *et al*. 1993). Commonly attachment to the chromophore NH_2–group by the carboxyl group neighboring the CH_2 is formulated. In the discussion above a fragmentation process was described which required the inverse attachment. The argument is that the loss of 59 mass units can not be explained reasonably by a degradation of Chr–NH–CO–CH_2–CHOH–$CONH_2$. So the argumentation is open anew. A clear cut chemical decision would be welcome.

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