Siderotyping of Fluorescent Pseudomonads - Problems in the Determination of Molecular Masses by Mass Spectrometry

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Abstract: Species and even subspecies of fluorescent members of the Pseudomonadaceae can be identified by determining the nature of their main siderophores (iron chelating metabolites), the pyoverdins. This can be done by establishing their electrophoretic behavior and by uptake studies using $^{59}Fe^{3+}$ labeled comparison compounds ("siderotyping"). Siderotyping requires a large collection of comparison material. Search could be facilitated and siderotyping results could be substantiated by a mass spectrometric determination of the molecular mass of the respective pyoverdin. Problems and strategies will be discussed.

Key words. Pseudomonas, siderophore, pyoverdin, siderotyping, mass spectrometry.

INTRODUCTION

Pseudomonas spp. belonging to the fluorescent species in the rRNA homology group I of the family Pseudomonadaceae produce siderophores ("pyoverdins") with high Fe^{3+} complexing constants. Pyoverdins [1] consist of three distinct structural parts, viz. a dihydroxyquinoline chromophore, a peptide chain commonly comprising 6 to 12 amino acids bound to the carboxyl group of the chromophore (Fig. (**2**), **a**) and a small dicarboxylic acid (or its monoamide) connected amidically to the NH_2-group of the chromophore (Fig. (**1**)). Pyoverdins may be accompanied by compounds containing chromophores derived from precursors of **a** (Fig. (**2**), **e - h**) or from transformation products (Fig. (**2**), **c** and **d**).

The identification of specific pyoverdins has been used as a tool for a quick characterization of *Pseudomonas* species or even sub-species ("siderovars") by "siderotyping" (short for siderophore typing) [2], i.e. by the comparison of isoelectrofocusing (IEF) patterns and iron transport capacities by the isolated siderophor mixture. IEF reflects the migrational aptitude of a given siderophore mixture in a pH gradient. The IEF pattern arises from the arrangement of basic (Lys, Orn, Dab, Arg) and acidic (Asp, Glu) amino acids in the peptide part as well as the type of dicarboxylic acids present in the siderophore sample obtained under strictly defined growth conditions. Comparison material must be available. Incubation of cell material with radioactively labeled Fe^{3+} pyoverdin complexes demonstrates whether a specific ferri-pyoverdin is recognized by the receptor protein at the cell surface of the bacterial strain under investigation.

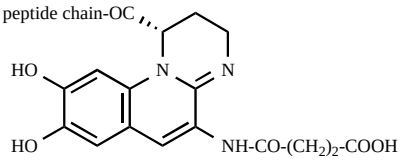

Fig. (1). General structure of a pyoverdin with succinic acid side chain.

PROBLEMS IN MOLCULAR MASS DETERMINATION

The siderotyping results could be confirmed and the search for comparison material could be facilitated by the determination of the molecular mass of the isolated pyoverdin by mass spectrometric

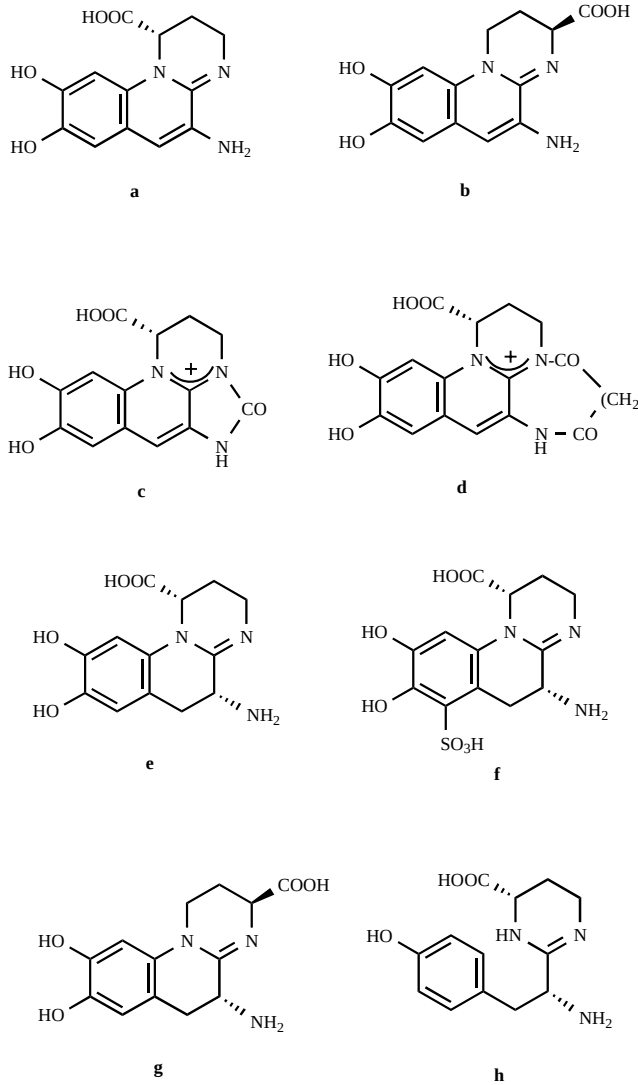

Fig. (2). Types of chromophores with residue masses in parentheses. **a**: pyoverdin (**257**), **b**: isopyoverdin (**257**), **c**: azotobactin (**285**), **d**: succino-pyoverdin (**341**), **e**: dihydropyoverdin (**259**), **f**: dihydropyoverdin-6-sulfonic acid (**339**), **g**: dihydro-isopyoverdin (**259**), **h**: ferribactin (**245**). In parentheses are the masses of the units ~CO-chromophore-NH~.

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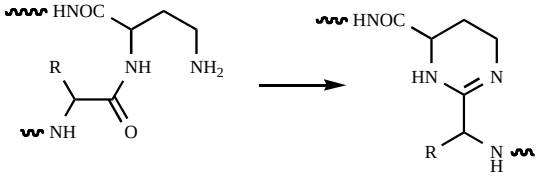
means. Molecular mass determination is a straightforward procedure with many natural products, but not so with pyoverdins. Here the following points have to be considered:

- The species selected for the tabulation of their molecular masses must be defined. In Table **1** compounds with the chromophore **a** (for pyoverdins), **b** (for isopyoverdins) or **c** (for azotobactins) (Fig. (**2**)) with a succinic acid side chain have been chosen as standard forms.
- The ions observed in the $[M + H]^+$ or $[M + 2H]^{2+}$ region of the mass spectrum must be identified. Rarely only one molecular species will be present, clusters of ions are more frequent. Besides or instead of the pyoverdin chromophore **a** one of the other chromophores depicted in Fig. (**2**) may be encountered (masses of the various chromophore residues are given there), in addition species varying in the dicarboxylic acid side chains (succinic acid/amide - the latter can cyclize by loss of NH_3 to a succinimide derivative -, malic acid/amide, α-ketoglutaric and glutamic acid) may be present (see, e.g., Fig. (**3**)). Even if the sample had been desalted and CF_3COOH had been added to the solvent for measurement (see Experimental) $[M + Na/K]^+$ as well as $[M + H + Na/K]^{2+}$ and $[M + 2Na, NaK$ or $2K]^{2+}$ may exceed the protonated species $[M + H]^+$ and $[M + 2H]^{2+}$. Replacement of H by Na results in a mass increase of 22 Da, by K of 38 Da for singly charged ions, and by 11 or 19 Da, respectively, for doubly charged ones. Mono-alkaliated doubly charged molecular species upon collision activation (CA) mainly lose an alkali ion to give a singly charged protonated molecular species. Thus, CA of *m/z* 642 ($[M + H + Na]^{2+}$ (Fig. (**4**)) yields *m/z* 1261 ($[M + H]^+$ (Fig. (**5**)).
- The problem "plus or minus H_2O" (18 Da). There are three types of peptide chains to be found in pyoverdins, (a) linear ones ending with *cyclo*-hydroxy-Orn, (b) cyclic ones where the C-terminal amino acid forms an amide bond with the free amino group of an in-chain Lys or Orn giving a tri- or tetra-cyclopeptidic substructure, and (c) cyclic ones where the C-terminal amino acid forms an ester bond with the hydroxyl group of an in-chain Ser or Thr giving a cyclodepsipeptidic substructure. While the amide bonds are stable the ester bonds can be hydrolyzed more or less readily during work-up by taking up one molecule of H_2O. The cyclic and the hydrolyzed form may be encountered side by side or only one of them may be isolated. The two species differ in mass by 18 Da. Further, Dab may condense with the preceding amino acid to give a tetrahydropyrimidine ring (Scheme **1**). Also here both forms or more frequently only one of them may be found (see Fig. (**3**)).



Scheme 1. Ring closure of Dab with the preceding amino acid giving a tetrahydropyrimidine ring.

- Variations in the peptide chain. In some cases minor components of the siderophore mixture have been identified with slight variations in the peptide chain, *viz*. Gly instead of Ala (nos. 17 and 45 in Table **1**) or acetyl instead of formyl bound to hydroxy-Orn (no. 32). They result in a mass difference of 14 Da. See also Fig. (**4**).
- In interpreting the signal patterns and in calculating the relative amounts of the species present the contributions of ^{13}C must be kept in mind. For a compound with 50 C-atoms the peak $^{12}C_{49}{}^{13}C_1$ following in mass $^{12}C_{50}$ has an intensity of 55% of $^{12}C_{50}$, and $^{12}C_{48}{}^{13}C_2$ of 14% (for the calculations see [3] or any other textbook on mass spectrometry). An M+2 ion too high in abundance may, however, be caused partially by the presence of the corresponding dihydropyoverdin (**a**/**e**, **b**/**g**). For mass calibration purposes it should also be kept in mind the exact masses of pyoverdins are about 0.5 Da higher than their nominal masses.

Two examples will serve as illustration.

Fig. (**3**) shows the doubly charged molecular ion region ($[M + 2H]^{2+}$) of the sidereophore mixture from *Pseudomonas fluorescens*

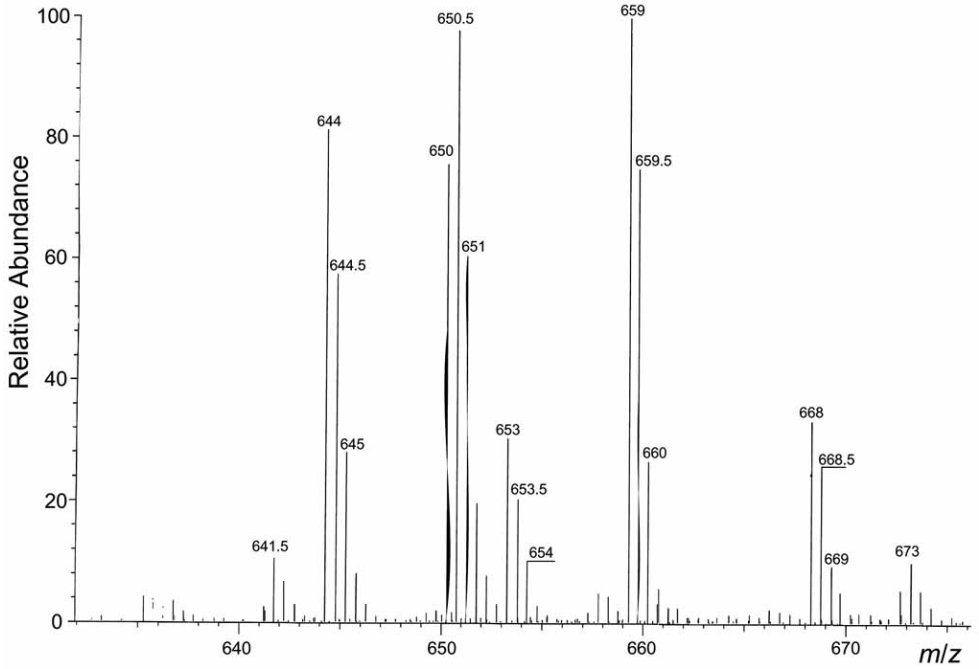


Fig. (3). Doubly charged molecular ion region of the siderophore mixture from *Pseudomonas fluorescens* 17400 (No. 20 in Table **1**).

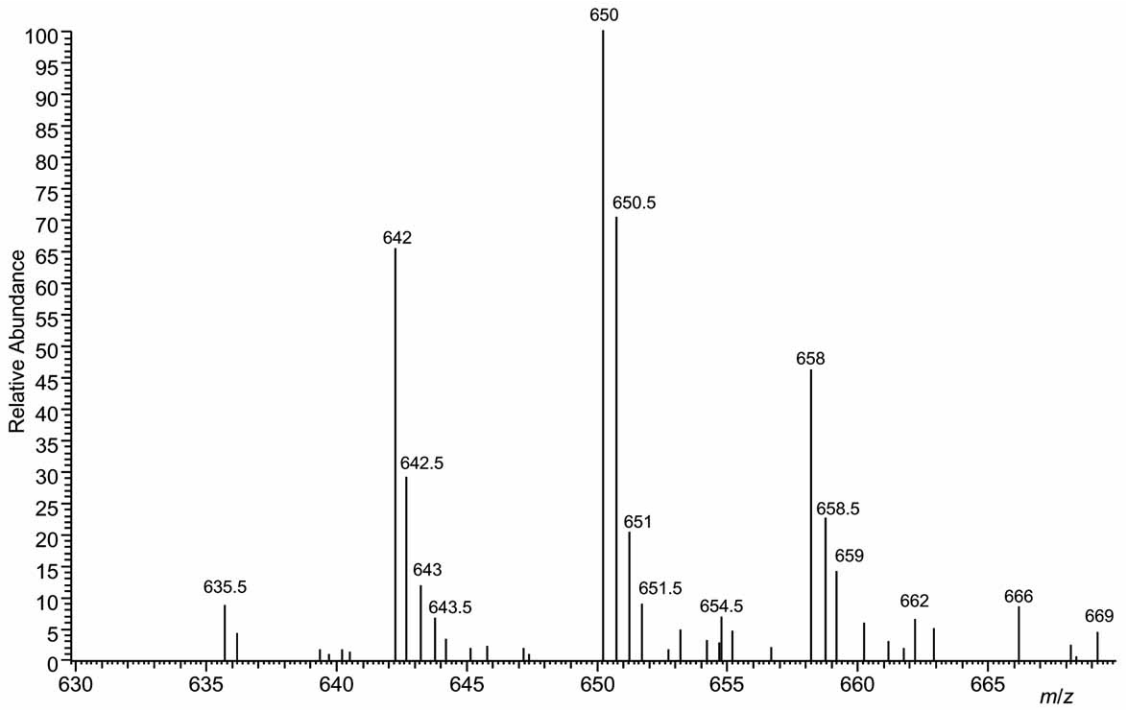


Fig. (4). Doubly charged molecular ion region of the siderophore mixture from *Pseudomonas thievervalensis* ML 45.

17400 (no. 20 in Table **1**). The pyoverdin fraction contains 54 carbon atoms. The contributions of the ^{13}C satellites is 60 and 17%, respectively. Nominal masses are given.

m/z 641.5: pyoverdin **a** with succinimide

m/z 650 : pyoverdin **a** with succinamide

m/z 650.5: pyoverdin **a** with succinic acid (about 50% of the peak)

m/z 644 : ferribactin **h** with succinamide

m/z 644.5: ferribactin **h** with succinic acid (about 15% of the peak)

m/z 659 : ferribactin **h** with glutamic acid

m/z 653 : ferribactin **h** (hydrolyzed form) with succinamide

m/z 668 : ferribactin **h** (hydrolyzed form) with glutamic acid.

Fig. (**4**) shows the doubly charged molecular ion region of the siderophore mixture from *Pseudomonas thievervalensis* ML 45 of unknown structure (see also below).

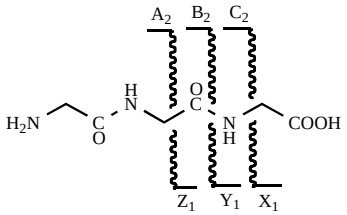
m/z 642: $[M + H + Na]^{2+}$ of pyoverdin **a** with succinamide

m/z 650: $[M + H + K]^{2+}$ of pyoverdin **a** with succinamide

$[M + H + Na]^{2+}$ of pyoverdin **a** with malamide

m/z 658: $[M + H + K]^{2+}$ of pyoverdin **a** with malamide.

The ions *m/z* 642.5, 650.5 and 658.5 are mainly ^{13}C satellites of the preceding ions with small amounts of the succinic/malic acid analogs. The ions *m/z* 635, 635.5, 643, 643.5, 651 and 651.5 stem from a lower homolog of the pyoverdin (in addition to some $^{13}C_2$ satellites).



Scheme 2. Designation of peptide fragments.

It must be pointed out that from the observed masses in the molecular ion region an identification of the various species as listed above is not possible. Further information is needed which may be obtained by CA fragmentation.

For most investigations the mass spectrometer described in Experimental has been used. For structure elucidation the precursor ions $[M + H]^+$ or $[M + 2H]^{2+}$ (they give better results than the alkaliated ions) are excited by CA both in the octapole region and in the ion trap of the mass spectrometer. In the octapole several collisions may occur and ions resulting from consecutive fragmentation processes will be observed. In the ion trap a single ion species is selected and only single-step fragmentation processes are possible, however, two or more not structurally connected neutral particles may be lost within the time frame of excitation, and the relatively long lived ions in the trap may partially undergo rearrangement processes. CA of $[M + H]^+$ mainly yields fragments containing the protonated chromophore, while in the CA spectra of $[M + 2H]^{2+}$ also ions comprising parts of the C-terminus of the peptide chain (Y"-ions; for the nomenclature of peptide fragments see Scheme **2**; hyphens as in Y" indicate the number of additional H atoms [4]) are observed, since the second proton can induce fragmentation anywhere in the chain [5,6]. For other instrumental arrangements little information is available (e.g., ESI-four sector instrument (ZAB) with collision cell [7], ESI-ICR [8], MALDI-TOF-PSD [9]).

CA fragmentation of $[M + 2H]^{2+}$ in the octapole region of the mass spectrometer frequently allows to recognize the species with a pyoverdin chromophore by the appearance of an abundant ion at *m/z* 204. This ion is formed by loss of the dicarboxylic acid unit from the *retro*-Diels-Alder (RDA) fragment (Scheme **3**). The mass difference between the latter and the ion *m/z* 204 would allow to determine the nature of the dicarboxylic acid unit, but the RDA fragment is usually of low abundance. With 2-ketoglutaric acid as a side chain ion *m/z* 204 can be of low abundance. Instead an ion *m/z* 270 has been reported. The presence of 2-ketoglutaric acid is evidenced by the loss of 62 Da ($CO_2 + H_2O$) from $[M + H]^+$ and from all fragments containing this residue, as well as from $[M + 2H]^{2+}$ (loss of 62/2 = 31 Da). Isopyoverdins may be recognized by an abundant ion *m/z* 230

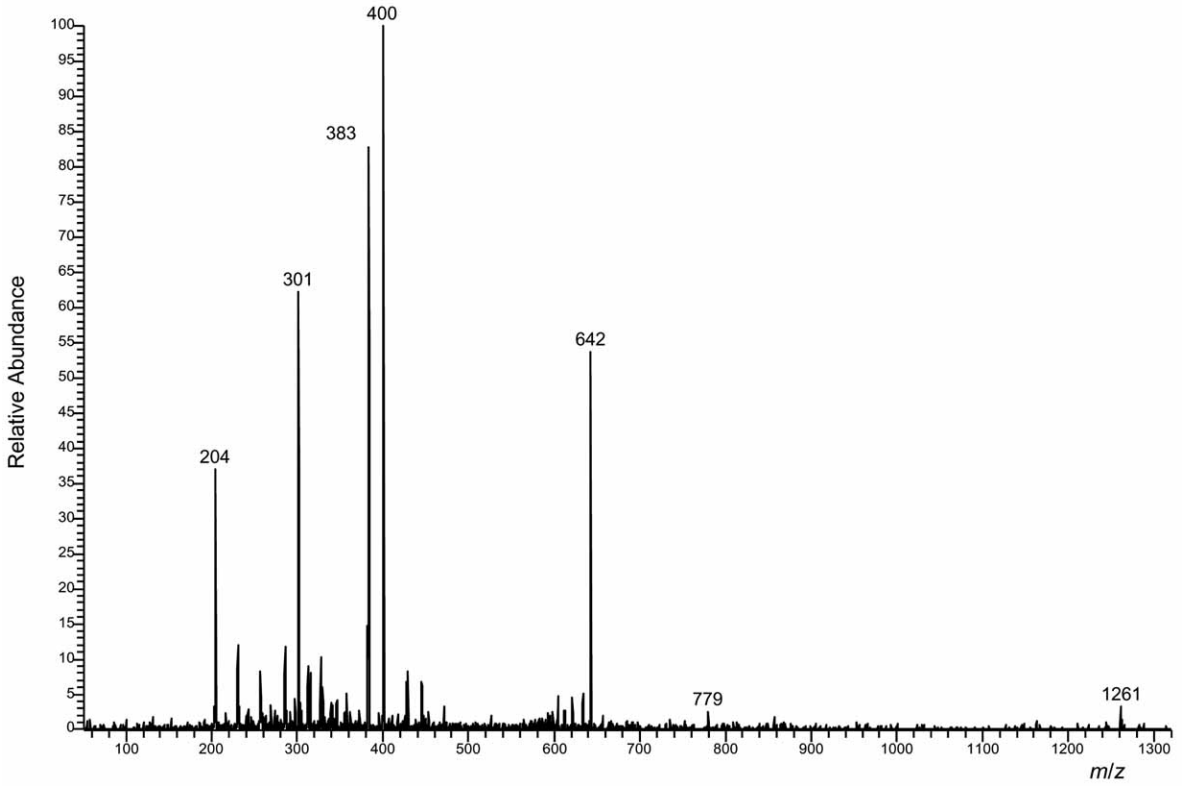
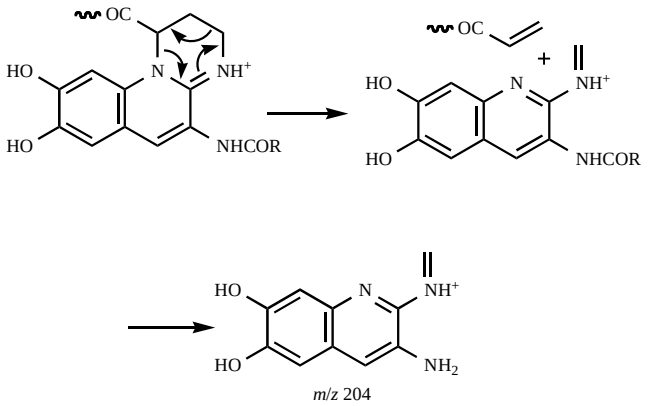


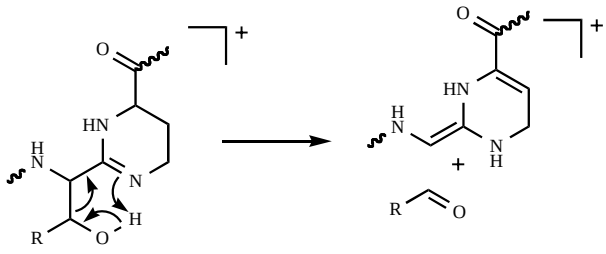
Fig. (5). CA spectrum (octapole) of *m/z* 642 from Fig. (**4**).

(loss of the entire peptide chain and the dicarboxylic acid. Characteristic features for the other chromophores (Fig. (**2**)) can be found in a recent survey [10].



Scheme 3. *retro*-Diels-Alder (RDA) fragmentation of the protonated pyoverdin chromophore and loss of the acid side chain leading to the characteristic ion *m/z* 204.

The nature of the dicarboxylic acid side chain can frequently be deduced from a fragment containing the pyoverdin chromophore with the first amino acid. If the first amino acid is a small neutral one (Ala, Ser) and succcinic acid (amide) or malic acid (amide) is the side chain an abundant A_1 ion is observed (*m/z* 400 in Fig. (**5**)); for Lys bound with its ε-amino group to the chromophore it is of moderate abundance. If the side chain in Glu (its loss from $[M + 2H]^{2+}$ can be observed) A_1 is of low abundance, but $[A_1 - H_2O]^+$ is rather abundant. From the A_1 species in addition to the loss of NH_3 (from amides, *m/z* 383 in Fig. **5**) or H_2O (from acids) the dicarboxylic acid side chain is lost with back-transfer of one H (giving an NH_2-group) yielding the ions with varying abundance (*m/z* 301 in Fig. (**5**)). For a 2-ketoglutaric acid side chain an ion $[A_1 - 62]^+$ (see above) is more pronounced than A_1. When the first amino acid is Asp B_1 (instead of A_1) is observed (at least for the few examples where mass spectra are available) which fragments further as described above for A_1.



amino acid	R	Da
Ser	H	30
Thr	CH_3	44
OHAsp	COOH	74
OHHis	4-imidazoyl	96

Scheme 4. McLafferty-degradation of ß-hydroxy amino acids incorporated in a tetrahydropyrimidine ring.

Fig. (**5**) shows the octapole CA spectrum of *m/z* 642 ($[M + H + Na]^{2+}$ from Fig. (**4**). The ion *m/z* 204 demonstrates the presence of the pyoverdin chromophore **a** (Fig. (**2**)), *m/z* 400 is the A_1 ion with a succinamide side chain (losses of NH_3 and of CH_2=CH-$CONH_2$, *m/z* 383 and 301) and alanine as the first amino acid in the peptide chain. The ion *m/z* 658 yields the A_1 ion with *m/z* 416 due to the presence of malamide, for *m/z* 650 both ions from species containing succinamide and malamide (*m/z* 400 and 416), respectively, are observed. Loss of the acid residue yields in all cases *m/z* 301.

Table 1. Pyoverdins, Isopyoverdins and Azotobactins

a) Complete or nearly complete (esp. missing stereochemistry of the amino acids) structures

With a C-termnal cOHOrn

No.	P.[b]	Name	Peptide Chain[a,b,c,d]	Mass[e]	Refs.[f]
6 amino acids					
1	f	Ps (= B10[h])	ε–Lys-OHAsp-Ala-aThr-Ala-cOHOrn	989	[12]
2	f	Py 9AW[n]	Ser-Lys-OHHis-aThr-Ser-cOHOrn	1043	[13]
3	ap	Py 4a[i] (= Py SB83)	Ala-Lys-Thr-Ser-AcOHOrn-cOHOrn	1046	[14]
4	p	iPy BTP1	Asp-Ala-Asp-AcOHOrn-Ser-cOHOrn	1047	[15]
7 amino acids					
5	f	Py PL7	Ser-AcOHOrn-Ala-Gly-aThr-Ala-cOHOrn	1046	[16]
6	f	Py BTP2	Ser-Val-OHAsp-Gly-Thr-Ser-cOHOrn	1049	[17]
7	p	Py G4R	Asp-Orn-(OHAsp-Dab)-Gly-Ser-cOHOrn[i]	1073	[9,18]
8		Py 2908	Ser-Orn-OHAsp-Ser-Ser-Ser-cOHOrn	1088	[19]
9	ae	Py T II[g] (=27853)	Ser-FoOHOrn-Orn-Gly-aThr-Ser-cOHOrn	1091	[20]
10	f	Py PL8	Lys-AcOHOrn-Ala-Gly-aThr-Ser-cOHOrn	1103	[16]
11	p	Py 11370	Asp–ε-Lys-OHAsp-Ser-Ala-Ser-cOHOrn	1106	[21]
12	p	iPy 90-33	Asp-Lys-Thr-OHAsp-Thr-aThr-cOHOrn	1164	[22]
8 amino acids					
13	p	Py 90-51	Asp-ε-Lys-OHAsp-Ser-Gly-aThr-Lys-cOHOrn	1234	[23]
9 amino acids					
14	c,au	Py Pau[u]	Ser-AcOHOrn-Gly-aThr-Thr-Gln-Gly-Ser cOHOrn	1277	[24]
15	f	Py 2392 (= A6[h])	Lys-AcOHOrn-Gly-aThr-Thr-Gln-Gly-Ser-cOHOrn	1318	[25]
16	p	Ps 589A[o]	Asp-ε-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOrn	1336	[26]
17	p	Py 2461 (=L1[h], WCS358[h])	Asp-ε-Lys-OHAsp-Ser-aThr-Ala-Thr-Lys-cOHOrn[ff]	1349	[27]
18	ap	Py 3b[t]	Asp-(AcOHOrn-Dab)-Thr-Ala-Thr-Thr-Gln-cOHOrn	1358	[28]
10 amino acids					
19	f	Py 2798[dd]	(Ser-Dab)-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-cOHOrn	1187	[29]
20	f	Py 17400	Ala-Lys-Gly-Gly-OHAsp-(Gln-Dab)-Ser-Ala-cOHOrn[i]	1299	[30,31]
21	p	Py 1,2	Ser-Thr-Ser-Orn-OHAsp-(Gln-Dab)-Ser-aThr-cOHOrn	1405	[32]
22	f	Py 1.3	Ala-Lys-Gly-Gly-OHAsp-(Gln-Dab)-Gly-Ser-cOHOrn	1285	[33]
23	t	Py 2192	Ser-Lys-Ser-Ser-Thr-Ser-AcOHOrn-Thr-Ser-cOHOrn	1424	[29]
24	p	iPy 90-44	Asp-Lys-AcOHOrn-Thr-Ser-Ser-Gly-Ser-Ser-cOHOrn[cc]	1408	[34]
11 amino acids					
25	f	Py 51W	Ala-Lys-Gly-Gly-OHAsp-Gln-Ser-Ala-Gly-aThr-cOHOrn	1375	[35]
12 amino acids					
26	f	Py GM	Ala-Lys-Gly-Gly-OHAsp-Gln-Ser-Ala-Ala-Ala-Ala-cOHOrn	1430	[36]
27	f	Py 1547	Ser-Lys-Ala-AcOHOrn-Thr-Ala-Gly-Gln-Ala-Ser-Ser-cOHOrn	1547	[37]

With a C-terminal cyclo-tetra- or -tripeptide

No.	P.[b]	Name	Peptide Chain[a,b,c,d]	Mass[e]	Refs.[f]
cyclo-tetrapeptide					
28	f	Py G173	Ser-Ala-AcOHOrn-(Orn-Asp-AcOHOrn-Ser)	1175	[38]
29		Py 96-312	Ser-Ser-FoOHOrn-(Lys-FoOHOrn-Lys-Ser)	1190	[39]
30		Py 96.188	Ser-Lys-FoOHOrn-(Lys-FoOHOrn-Glu-Ser)	1232	[40]
31	ae	Py C-E (= PAO1[h],ATCC 15692, Pa)	Ser-Arg-Ser-FoOHOrn-(Lys-FoOHOrn-Thr-Thr)	1333	[41,42]
32		Py 95-275 (= BTP7[h])	Ser-Ser-FoOHOrn-Ser-Ser-(Lys-FoOHOrn-Lys-Ser)[ee]	1364	[43]
33	f	Py 12	Ser-Lys-Gly-FoOHOrn-Ser-Ser-Gly-(Lys-FoOHOrn-Glu-Ser)	1520	[44]
cyclo-tripeptide					
34	f	Py 13525[m]	Ser-Lys-Gly-FoOHOrn-(Lys-FoOHOrn-Ser)	1160	[8,45,46]
35	pa	Py 96-318	Ser-Orn-FoOHOrn-Ser-Ser-(Lys-FoOHOrn-Ser)	1263	[39]
36	f	Py 18-1	Ser-Lys-Gly-FoOHOrn-Ser-Ser-Gly-(Lys-FoOHOrn-Ser)	1391	[47]

(Table 1) contd…..

With a C-terminal cyclodepsipeptide or a free carboxyl group

No.	P.[b]	Name	Peptide Chain[a,b,c,d]	Mass[e]	Refs.[f]
6 amino acids					
37		iPy azoverdin[h,s]	Hse-(Hse-Dab)-AcOHOrn-Ser-AcOHOrn	1090	[48]
38		PS 6.10	Ala-Orn-OHAsp-Dab-AcOHOrn-Lys	1091	[49]
7 amino acids					
39	ae	Py R'	(Ser-Dab)-FoOHOrn-Gln-FoOHOrn-Gly	1045	[50]
40	ci	PaB	ε-Lys-OHAsp-Thr-(Thr-Gly-OHAsp-Ser)[j]	1093	[51]
41	s	Py 19310	ε-Lys-OHAsp-Thr-(Thr-Ser-OHAsp-Ser)[j]	1123	[51,52]
42	ae	Py R (=Pa6)	(Ser-Dab)-FoOHOrn-Gln-Gln-FoOHOrn-Gly	1173	[53]
8 amino acids					
43	p	Ps A214 (= Ps 39167)	Ser-AcOHOrn-Ala-Gly-(Ser-Ala-OHAsp-Thr)[j]	1134	[27]
44	f	Py P19 (= Ps SR1[h], Ps A 225)	Ser-AcOHOrn-Ala-Gly-(Ser-Ser-OHAsp- Thr)[j]	1150	[27,54]
45	ch	Py D-TR133	Asp-FoOHOrn-Lys-(Thr-Ala-Ala-FoOHOrn-Ala)[j,x]	1230	[55]
46	f	Py I-III	Asn-FoOHOrn-Lys-(Thr-Ala-Ala-FoOHOrn-Lys)	1286	[56]
47	f	CHAO	Asp-FoOHOrn-Lys-(Thr-Ala-Ala-FoOHOrn-Lys)	1287	[57]
9 amino acids					
48	p	Py C	Asp-BuOHOrn-Dab-Thr-Gly-Ser-Ser-OHAsp-Thr	1370	[58]
49	p	Py BTP16	Asp-BuOHOrn-Dab-Thr-Gly-Ser-Ser-OHAsp-Thr[v]	1370	[59]
10 amino acids					
50		azotobactin 87[y]	Ser-Ser-Hse-Gly-OHAsp-Hse-Hse-Hse- BuOHOrn-Hse[z]	1385	[60]
51		azotobactin D[y]	Asp-Ser-Hse-Gly-OHAsp-Ser -Cit-Hse-AcOHOrn-Hse	1411	[61]

b) Partial or tentative structures

With a C-terminal cOHOrn

No.	P.[b]	Name	Peptide Chain[a,b,c,d]	Mass[e]	Refs.[f]
52	*p*	Thai	(Ser-Dab)-Thr-Ser-AcOHOrn-cOHOrn	1016	[62]
53	*f*	Py 244[n]	Ser-ε-Lys-OHHis-aThr-Ser-cOHOrn[k]	1043	[63,64]
54	*f*	Py 2392[q]	OHOrn-Lys-Gly-Thr-Thr-Gly-Gln-Ser-cOHOrn	1277	[65,66]
55	*p*	Py 12633[o]	Asp-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOrn	1336	[65]

With a C-terminal cyclo-tetra- or -tripeptide

No.	P.[b]	Name	Peptide Chain[a,b,c,d]	Mass[e]	Refs.[f]
cyclo-tetrapeptide					
56	f	D47	Ser-Orn-FoOHOrn-(Lys-FoOHOrn-Glu-Ser)	1218	[8]
57	r	L25	Ser-Lys-FoOHOrn-Ser-Ser-Gly-(Lys-FoOHOrn-Ser-Ser)	1421	
cyclo-tripeptide					
58	m	G 76	Ser-Ser-FoOHOrn-Ser-Ser-(Lys-FoOHOrn-Ser)	1236	[67]
59		DSM 50106	Ser-Lys-Gly-FoOHOrn-Ser-Ser-Gly-(Orn- FoOHOrn-Ser)	1377	[67]

Various pyoverdins

No.	P.[b]	Name	Peptide Chain[a,b,c,d]	Mass[e]	Refs.[f]
60	*ae*	Py UNK[p]	Ser-Thr-Ser-Gly-Orn-Orn		[68]
61	*p*	Py Pm	OHAsp, Lys, OHOrn, 2 Ser, 3 Thr		[69]
62	*s*	Py Ps	Lys, OHOrn, 3 Ser, 3 Thr		[70]
63	*s*	Py PSS[bb]	2 OHAsp, Lys, 2 Ser, 2 Thr		[71]
64		*P. mildenbergii*	Glu, Lys, Ser, Thr[w]		[72]
65	*p*	Py A1	Asx, Glx, 3 Gly, His, Lys, 4 Ser, Thr, Val[r]		[73]
66	*f*	BTP9[aa]	2 Lys, 2 FoOHOrn, 5 Ser		[74]
67	*p*	BTP14[aa]	Asx, Dab, Glx, Gly, Orn, 2 Ser, Thr, aThr		[74]
68	*as*	Py *fuscovaginae*	Ala, 2 OHAsp, 2 Gly, Lys, 2 Thr, Dab	1316	[75]

(Table 1) contd…..

a	In part (a) D-amino acids are underlined; a broken line indicates either that the stereochemistry of the amino acid has not been determined or that a specific amino acid occurs both in the D- and the L-form, but a localization of the two enantiomers has not been effected. In part (b) D-amino acids are indicated only when data are available from the literature.
b	Abbreviations: P., *Pseudomonas*; ae, *aeruginosa*; ap, *aptata*; as, *asplenii*; au, *aureofaciens*; c, *costantinii*; ci, *cichoriae*; f, *fluorescens*; m, *marginalis*; p, *putida*; pa, *palleroniana*; r, *rhodesiae*; s, *syringae*; t, *tolaasii*; Ps, pseudobactin; Py, pyoverdin; iPy, isopyoverdin; amino acids: 3-letter code - in addition: OHAsp, threo-β-hydroxy-Asp; OHHis, threo-β-hydroxy-His; OHOrn, N^4-hydroxy-Orn; Ac(Fo,Bu)OHOrn, N^4-acetyl (formyl, R-β-hydroxy-butyryl) OHOrn; cOHOrn, cyclo-OHOrn (3-amino-1-hydroxy-piperidone-2); aThr, allo-Thr.
c	Amino acids are bound to the chromophore or to the preceding amino acid by their α-amino group or in the case of Lys occasionally by its ε-amino group (indicated as ε-Lys).
d	Parentheses indicate either a cycle formed by an amide or ester bond between the carboxyl group of the C-terminal amino acid and a side chain functionality of another amino acid or the condensation product of the NH_2 groups of Dab with the amide carbonyl group of the preceding amino acid giving a tetrahydropyrimidine ring.
e	Nominal molecular mass for a pyoverdin, isopyoverdin or azotobactin chromophore (**a**,**b** or **c**) with a succinic acid side chain; the exact mass is about 0.5 Da higher.
f	References refer to publications where the structure was reported. Additional literature may be found in [1].
g	Probably identical with the pyoverdin of *Pseudomonas aeruginosa* ATCC 9027 [76].
h	The structure published originally had to be corrected or amended; literature references to the originally proposed structures are given in [1].
i	Accompanied by the not cyclized Dab form (M + 18 Da).
j	Accompanied by a non-cyclic pyoverdin with the same amino acid sequence (M + 18 Da).
k	For this pyoverdin an ε-amino Lys linkage was claimed but not substantiated. It is probably identical with the pyoverdin from *P. putida* 9AW (No. 2) where a α-amino Lys linkage was established.
l	*P. aptata* is a pathovar of *P. syringae*. The same pyoverdin was found produced by *P. fluorescens* SB83 [66]. The identification of *P. aptata* may, therefore, be questioned (cf. also [52]).
m	The same pyoverdin was isolated from *P. chlororaphis* ATCC 9446 [45]. The reported isolation from *P. putida* KT2440 [77] is the result of a mix-up of strains.
n	The pyoverdins 9AW (No. 2) from *P. fluorescens and* 9BW from *P. putida* [12] are probably identical with pyoverdin 244 (no. 53) from *P. fluorescens.*
o	The Py 589a (No. 16) is probably identical with the pyoverdin Py Pp 12633 (No. 55).
p	Either the preliminary structural work or the identification of the strains may be questioned since screening of a large number of *P. aeruginosa* strains revealed the existence of only three siderovars characterized by the production of the pyoverdins Py TII, C-E and Py R (Nos. 9, 31 and 42) [78] plus probably of a mutant of Py R (R', No. 39).
q	Re-investigation of the strain revealed the presence of L-Gln, 2 Gly, D-AcOHOrn, D-cOHOrn, D-Lys, D-Ser, L-Thr and D-aThr in agreement with the structure proposal based on FAB mass spectrometric sequencing. However, the pronounced downfield shift by ca. 1 ppm of the amide proton of Lys (9.61 ppm) suggests that Lys is the first amino acid in the sequence in agreement with the observation that AcylOHOrn for steric reasons (Fe^{3+} complexation site) is never bound directly to the chromophore.
r	The reported amino acid composition can not be correct. The minimum molecular mass calculated from it is about 120 u higher than the molecular mass determined by mass spectrometry. Also the amino acids acting as ligands for Fe^{3+} are missing.
s	From *Azomonas macrocytogenes*.
t	Contains 2 Thr and one aThr. The amino acid analysis of the corresponding ferribactin gave D-Ala, L-Asp, L-Dab, D- and L-Glu, L-Orn, D-aThr, L-Thr and D-Tyr.
u	The same pyoverdin was isolated from *P. tolaasii* NCBBP 2192 (*P. constantinii*); the fact that the strain designated as *P. aureofaciens* does not produce phenazines casts doubts on the correct identification [79].
v	1 Thr, 1 aThr.
w	Ratios of 1:1:2:4 and 1:2:3:5 are reported for the pyoverdins from two strains of *P. mildenbergii*; for the second one a blocked N-terminus was demonstrated.
x	1 D-, 2L-Ala; the pyoverdin D-TR 133 is accompanied by a small amount of a pyoverdin where the second Ala is replaced by Gly.
y	From Azotobacter vinelandii strains.
z	Hse^2-Hse^3-Hse^4 1 D and 2 L.
aa	Probably identical with the pyoverdins of BTP7 and BTP16, respectively (private communication Dr. M. Ogena, Liège).
bb	Probably identical with the pyoverdin Py 19310 (no. 41).
cc	2 D-, 2 L-Ser
dd	Identical with pyoverdin W mentioned in [80].
ee	Accompanied by a variety with one AcOHOrn (unpublished).
ff	Accompanied by a small amount of a pyoverdin where Ala is replaced by Gly.

From the fragments obtained by CA frequently further structural information up to a complete amino acid sequence (from a series of B-ions) may be obtained. The procedures [5, 6] and possible pitfalls [11] have been discussed in detail. It should be kept in mind that by mass spectrometry mass differences are determined and that Asn/Orn and Gln/Lys have the same nominal mass, but can be distinguished by exact mass measurements. An amino acid analysis after total hydrolysis usually helps to solve this problem. Alternatively, the presence of some amino acids can be deduced from specific ions: *m/z* 129 and 84 are characteristic for Lys, 115 and 70 for Orn, 101 for Dab (NH_2-$(CH_2)_{4/3/2}CHNH_2CO^+$ and loss of CO + NH_3 therefrom), 157 and 70 in an analogous way for Arg; *m/z* 131 and 86 indicate the presence of a C-terminal *cyclo*-hydroxy-Orn. ß-hydroxy amino acids may be recognized by the loss of R-CH=O from ions containing this unit (Scheme **4**), especially when they are incorporated into a tetrahydropyrimidine ring (Scheme **1**); however, occasionally all or any of these fragments do not occur with sufficient abundance.

In Table **1** pyoverdins are listed for which structural data were published.

EXPERIMENTAL

Sample production. *Pseudomonas* strains were grown in a Casamino Acid (CAA) medium made of Casamino Acid (Difco) 5.0 g/l, K_2HPO_4 1.18 g/l, $MgSO_4.7H_2O$ 0.25 g/l in 1 l Erlenmeyer flasks containing 500 ml medium under vigorous shaking (200 rpm) at 25°C for 24 to 40 hrs depending on the strain to reach a maximal pyoverdin production observed at the beginning of the stationary phase of growth. Bacterial cells were removed by centrifugation (12 000 rpm for 10 min). The supernatant was adjusted to pH 6.0 with 1N HCl and passed with a speed of 2 drops/sec through an Amberlite XAD-4 column (2.5 x 20 cm for 500 ml culture supernatant). The column was then washed with 100 ml distilled water and the adsorbed pyoverdin fraction was eluted with 100 ml CH_3OH/H_2O 1:1. The solution was brought to dryness i.v. and lyophilized.

Mass spectral data were obtained with a MAT 900 ST instrument providing an EB-QIT geometry and equipped with an ESI II ion source (Finnigan MAT, Bremen, Germany); spray voltage 3.4-3.6 kV, capillary temperature 230°C. Source conditions set to minimize fragmentation, resolution ca. 5000 (10% valley). The samples containing mixtures of siderophores were dissolved in water, methanol, and trifluoroacetic acid 50:50:0.1 (v/v). Fragmentation induced by low energy collision activation (CA) was effected in the QIT (~$2 \cdot 10^{-3}$ Pa He as bath gas diffusing in the collision octapole).

CONCLUSION

It has been shown that in most cases the species observed in the singly and doubly charged molecular ion region can be identified by using information derived from fragment ions obtained following CA decomposition. In this way the standard molecular mass (see above, chromophore **a** and succinic acid side chain) can be established. It is worth noting that in the large list (cf. Table **1**) of pyoverdins only in rare cases (nos. 3/5, 9/38, 48/49) identical molecular masses are

observed. In this way results obtained by siderotyping suggesting a new or a known pyoverdin under investigation can be substantiated. While for complete structural studies including NMR and chemical degradation the isolation of a single molecular species is necessary, for screening studies discussed here the analysis of the mixture obtained from the XAD extract is of advantage. Mass differences between the different molecular species give welcome hints such as to the presence of protonated and cationized ions, compounds differing by one molecule of H_2O (cyclized or not) of by H_2 (presence of pyoverdins and dihydropyoverdins) etc.

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