145. Detection of Noncovalent Complexes of Hydroxamic-Acid Derivatives by Means of Electrospray Mass Spectrometry

by **Laurent Bigler**[1]), **Andreas Baumeler**[2]), **Christa Werner**, and **Manfred Hesse***

Organisch-chemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich

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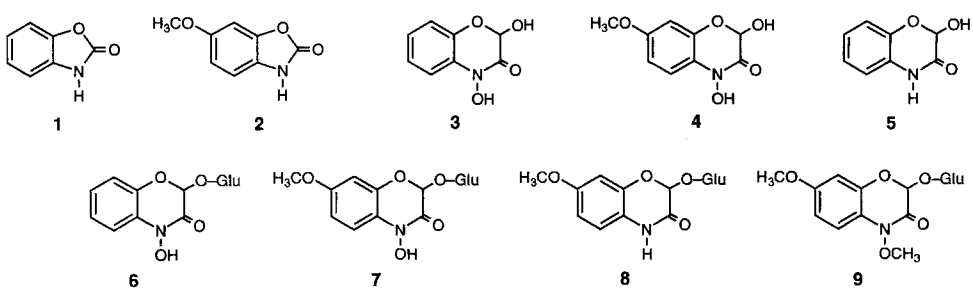
The complexes of isolated benzoxazinone derivatives with Fe^{III} were investigated by means of electrospray ionization mass spectrometry. The hydroxamic-acid derivatives could be differentiated from the lactam or the methyl-hydroxamate derivatives by the formation of Fe^{III} complexes with two or three ligands. These complexes or adducts of them have been identified in the mass spectra. Moreover, the mass-spectral behavior of these complexes was not markedly influenced by the presence of a β-D-glucopyranosyloxy substituent.

1. Introduction. – During our work on isolation and biosynthesis of macrocyclic polyamine alkaloids from the roots of several species of the genus *Aphelandra* (Acanthaceae), we detected an additional group of heterocyclic compounds which were identified as 2,3-dihydrobenzoxazol-2-one (**1**) and 2,3-dihydro-6-methoxybenzoxazol-2-one (**2**) [1] [2]. Investigations by *Niemeyer* [3] showed that these compounds are only detected in the plant material, when the integrity of the cells is disrupted, and enzymatic hydrolysis and chemical rearrangement can occur. Dihydrobenzoxazoles are decomposition products of naturally occurring hydroxamic acids such as 3,4-dihydro-2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one (**3**) of 3,4-dihydro-2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (**4**)[3]). They are accumulated in the plant cells mainly as the correspond-



[1]) Part of the Ph. D. Thesis of *L. B.*, University of Zürich, in preparation.

[2]) Part of the Diploma Thesis of *A. B.*, University of Zürich, 1996.

[3]) The natural benzoxazolinone and benzoxazinone derivatives used in this paper have abbreviations in the phytochemical literature: **1** (BOA), **2** (MBOA), **3** (DIBOA), **4** (DIMBOA), 3,4-dihydro-2-hydroxy-2*H*-1,4-benzoxazin-3-one (**5**, HBOA), **6** (DIBOA-Glu), **7** (DIMBOA-Glu), 2-(β-D-glucopyranosyloxy)-3,4-dihydro-7-methoxy-2*H*-1,4-benzoxazin-3-one (**8**, HMBOA-Glu), 2-(β-D-glucopyranosyloxy)-3,4-dihydro-4,7-dimethoxy-2*H*-1,4-benzoxazin-3-one (**9**, HDMBOA-Glu).

ing glucosides 2-(β-D-glucopyranosyloxy)-3,4-dihydro-4-hydroxy-2*H*-1,4-benzoxazin-3-one (**6**) and 2-(β-D-glucopyranosyloxy)-3,4-dihydro-4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (**7**).

Derivatives with a 3,4-dihydro-2-hydroxy-2*H*-1,4-benzoxazin-3-one skeleton were found in many species of Gramineae (Monocotyledons), and they are one of the most extensively studied group of secondary metabolites with respect to plant resistance to pests and diseases [3]. Some groups have reported their occurrence in the plant families of Scrophulariaceae, Ranunculaceae, and Acanthaceae (Dicotyledons) [4–7].

Hydroxamic acids form very stable complexes with Fe^{III} and several other bivalent cations. These complexes have very high stability constants, and this lead to the consideration that they could play a role in the mineral nutrition of plants [3].

To compete successfully for iron, organisms including plants, bacteria, and fungi have developed specific mechanisms. Fe^{III} is solubilized by binding to high-affinity chelators called siderophores. In Gramineae, the known siderophores are mugineic and avenic acid as well as related compounds [8]. These Fe^{III} complexes are recognized by specific membrane receptors. In Dicotyledons, nicotianamide is considered to be a possible siderophore [9]. More recently, it was postulated that the hydroxamic-acid derivative **3** may also play a role as a siderophore. In maize and several other cereal plants, a correlation could be shown between iron deficiency and the secretion of this compound by the roots [10] [11].

The compounds produced by plants and their identification as siderophores is not always obvious by means of biological studies. Many extracted substances with various functional groups are able to complex with metal ions, and the concentrations of the interesting ones are probably low because of their high specificity. We have, therefore, tried to apply a different analytical method which permits the study of the interactions between small amounts of a substance and a metal cation.

Electrospray ionization mass spectrometry (ESI-MS) is an analytical method that is becoming increasingly important for the study of polar compounds in solution, including noncovalent complexes [12] [13]. This soft ionization method yields molecular ions with very little mass-spectral fragmentation. The substrates are transferred from the solution to the gas phase *via* a charged droplet. This procedure produces species with a small internal energy from ions which are normally preformed in the solution. The intensity of ions in the ESI-MS of solutions containing metal ions and organic molecules allows an estimation of the concentrations of the different types of complexes present in equilibrium in solution.

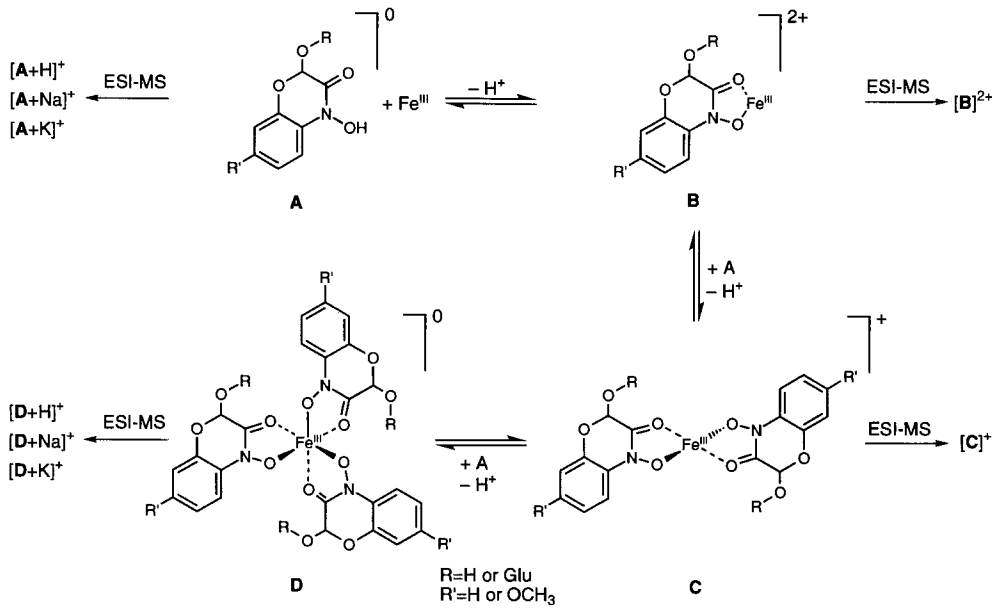
In this paper, we present the results of an ESI-MS study of the Fe^{III} complexes formed by secondary metabolites isolated from *Aphelandra* species and *Zea mays*. We tried to differentiate the hydroxamic-acid derivatives from other plant products by mass spectrometry.

2. Results and Discussion. – The hydroxamic-acid derivative **3**, its β-D-glucopyranosyloxy derivative **6**, and 2-hydroxy-benzoxazinone **5** were isolated from *Aphelandra tetragona* (VAHL) NEES, *A. fuscopunctata* MARKGRAF, and *A. squarrosa* NEES. Because the 7-MeO derivatives **7**, **8**, and **9** are detected in small quantities in *Aphelandra* plants, maize (*Zea mays*) plants were also cultivated in order to get enough material. The isolation was performed under conditions which exclude the possibility of any enzymatic degradation or chemical transformation [14].

Our goal was to study the solution behavior of the three hydroxamic-acid derivatives **3**, **6**, and **7**, the methyl hydroxamate **9**, and the two corresponding lactams **5** and **8** in the presence of Fe^{III} ions in order to obtain structural information on complex formation.

It is known that the hydroxamic acids have very high formation constants with bivalent or trivalent metal cations [15–17], and that the complexes are stable over a large pH range [18] [19]. The *Scheme* shows the stoichiometry of the studied complexes and the ions detected by ESI-MS. Species **A** represents the uncharged compounds which can form adducts with the cations H^+, Na^+, and K^+, as well as with Fe^{III}. The complexes containing one molecule of hydroxamic acid and one Fe^{III}, species **B**, could not be recognized. However, complex **C** (containing two molecules of hydroxamic acid and one Fe^{III} with one positive charge and the neutral **D** are present in solution and can be recognized in the ESI-MS. Complex **D**, formed by three molecules of the hydroxamic acid and one Fe^{III} ion, behaves in a similar manner to **A**, and forms adducts with H^+, Na^+, and K^+ ions.

Scheme. *Equilibria of Hydroxamic-Acid Derivatives* **3**, **6**, *and* **7** *with* $FeCl_3$ *in MeOH*[a])



[a]) The octahedral complex has different geometrical isomers [15] [17], and only one is given. The choice of the representation is arbitrary.

Firstly, we checked the behavior of different ligands in equimolar methanolic solutions of $FeCl_3$. *Fig. 1* represents the ESI-MS of **3** (*Fig. 1, a*), and the corresponding lactam **5** (*Fig. 1, b*). The mass spectrum of **3** in $FeCl_3$ solution clearly shows that iron complexes are formed, and because of the ubiquitous presence of H^+, Na^+, and K^+ ions, additional adduct ions are present (m/z 182, 204, and 220, resp.). The ions of type **C** represent the base peak of the spectrum at m/z 416 (*Scheme*). The additional signal at m/z 448 corresponds to the ion $[\mathbf{C} + MeOH]^+$ and shows the additional solvent ligand. Finally, all three **D** signals at m/z 597 ($[\mathbf{D} + H]^+$), 619 ($[\mathbf{D} + Na]^+$), and 635 ($[\mathbf{D} + K]^+$) can be recognized in the spectrum. Undoubtedly, the hydroxamic-acid derivative **3** forms stable iron complexes formed from $FeCl_3$ and **3**.

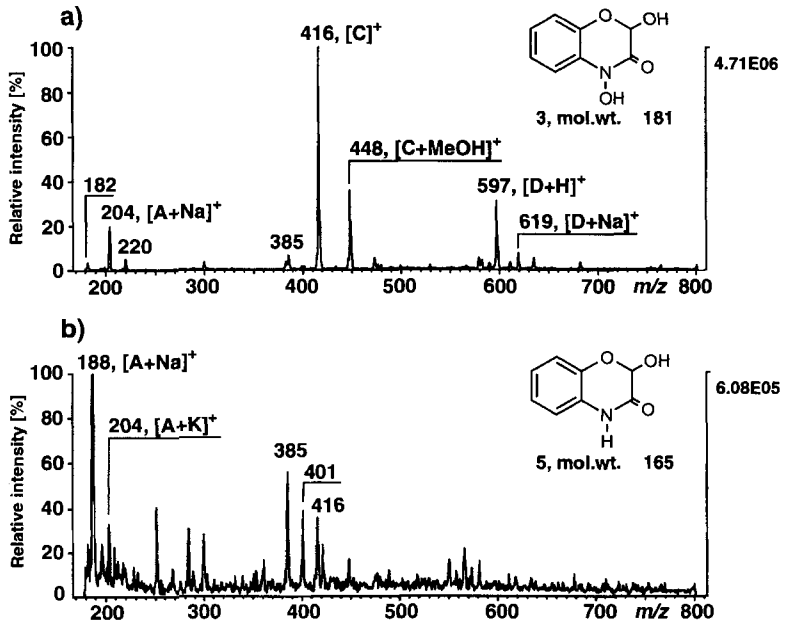


Fig. 1. *ESI-MS of* a) *the hydroxamic-acid derivative* **3**, *and* b) *the lactam* **5**, *at* 10^{-3} M *in a methanolic solution of* 10^{-3} M $FeCl_3$

Reduction of the N–OH group in **3** to the N–H in **5** gives an ESI-MS of completely different character. The signals are of low intensity, and the only ions present are those formed by cationization of the lactam with H^+, Na^+, and K^+, respectively (see *Table 1*)[4]).

A second example of a complexation equilibrium is shown in *Fig. 2*. The hydroxamic-acid derivative **6** and Fe^{III} ions in methanolic solution form complexes of type **C** (*m/z* 740) and **D** (*m/z* 1083 ($[D + H]^+$) and 1105 ($[D + Na]^+$)) as well as adducts of **A** type (see *Table 1*).

Table 1. *Summary of the Observed ESI-MS Ions* (*m/z*) *Arising from the Complexation Equilibria of the Compounds* **3** *and* **5–9** *Described in the* Scheme

Ligand	$[A+H]^+$	$[A+Na]^+$	$[A+K]^+$	$[2A+Na]^+$	$[2A+K]^+$	$[C]^+$	$[C+MeOH]^+$	$[D+H]^+$	$[D+Na]^+$	$[D+K]^+$
3 (*Fig. 1,a*)	182	204	220	385	401	416	448	597	619	635
5 (*Fig. 5,b*)		188	204							
6 (*Fig. 2*)		366	382	709	725	740		1083	1105	
3/**6** (*Fig. 4*)						578[a])				
7 (*Fig. 3,a*)		396	412	769		800	832	1173	1196	1212
8 (*Fig. 3,b*)		380	396	737	753					
9 (*Fig. 3,c*)	388	410	426	797	813					

[a]) Ions of the iron complex with the two different ligands **3** and **6**.

[4]) Compound **5** was difficult to purify by high-performance liquid chromatography. Impurities of **3** are present in the solution of **5**. Therefore, in the spectrum of **5** shown in *Fig. 1,b*, traces of **3** (*Fig. 1,a*, *m/z* 385 $[2 \cdot 181 + Na]^+$, 401 $[2 \cdot 181 + K]^+$, and 416 $[2 \cdot 181 + Fe^{III}–H]^+$) are present. Other intense impurity signals are at *m/z* 252, 284, and 299.

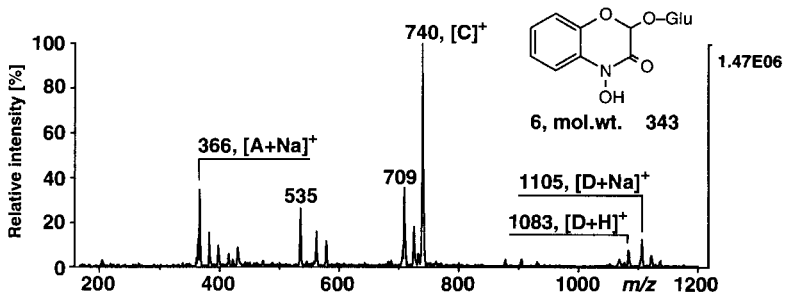


Fig. 2. *ESI-MS of* **6** *at* 10^{-3} M *in a methanolic solution of* 10^{-3} M $FeCl_3$

The hydroxamic-acid derivative **7**, its reduction product, the lactam **8**, and the methyl hydroxamate **9** behave analogously to the previously mentioned compounds **3**, **5**, and **6**. Of the β-D-glucopyranosyloxy derivatives, only **7** forms the complex ions of type **C** (*m/z* 800, *Fig. 3, a*).

The lactam **8** and the methyl hydroxamate **9** can be recognized by their **A**-type adducts. They do not form any iron complex which could be detected by ESI-MS (*Fig. 3, b* and *c*). This shows that the Glu group does not have any property to complex with Fe^{III} cations, and that the presence of the hydroxamic-acid function is a prerequisite for complex formation. The only difference results in the stronger interactions of the OH groups of the Glu residue toward alkali-metal ions. This is shown in an increased intensity of the $[\mathbf{A} + Na]^+$ and $[\mathbf{A} + K]^+$ signals in the spectra of **8** and **9** compared to those of **5**.

The aromatic substitution by a MeO group that increases the electron density of the hydroxamic acid has no detectable influence on the formation of the complex.

To compare the affinity of compounds **3**, **5**, and **6** with respect to their behavior towards Fe^{III} ions, solution of all three compounds were mixed together at the same concentration in a $FeCl_3$ solution. The ESI-MS (*Fig. 4*) confirms the inability of lactam **5** to form complexes of type **B**, **C**, or **D**. In addition, complexes of mixed composition, *e.g.* of one lactam ligand and one hydroxamic-acid ligand together with Fe^{III}, are absent. In contrast, the two hydroxamic-acid derivatives **3** and **6** give ESI-MS signals for type-**C** complexes in a ratio of nearly 1:2:1 (for **3** + **3**, **3** + **6**, and **6** + **6**). The base peak (*m/z* 578) corresponds to the mixed **3** + **6** complex. This behavior confirms that the Glu group has a negligible effect on the Fe^{III} complexation ability of the hydroxamic acids.

The 7-methoxy derivatives of **3** and **6** are **4** and **7**, respectively. *Tripton* and *Buell* have measured the complex formation constants of complexes of **4** and **7** with Fe^{III} ions by potentiometric titration in H_2O [20]. The value found for **4** (21.3) is very close to that found for **7** (19.4) and shows that the inclusion of a Glu substituent, as in **7**, has very little effect on the complex-formation constants. The results observed from ESI-MS are thus in a good agreement with the nearly equal stability observed by titration of **4** and **7**.

The spectra given in *Fig. 5* represent the time-dependent complexation of **7**. The equilibrium described in the *Scheme* only reaches a 'steady state' after a few minutes. The first spectrum (*Fig. 5, a*) was taken *ca.* 30 s after mixing the two components. The Na^+ and the K^+ ion adducts give very intense signals compared to those of complex ions. After

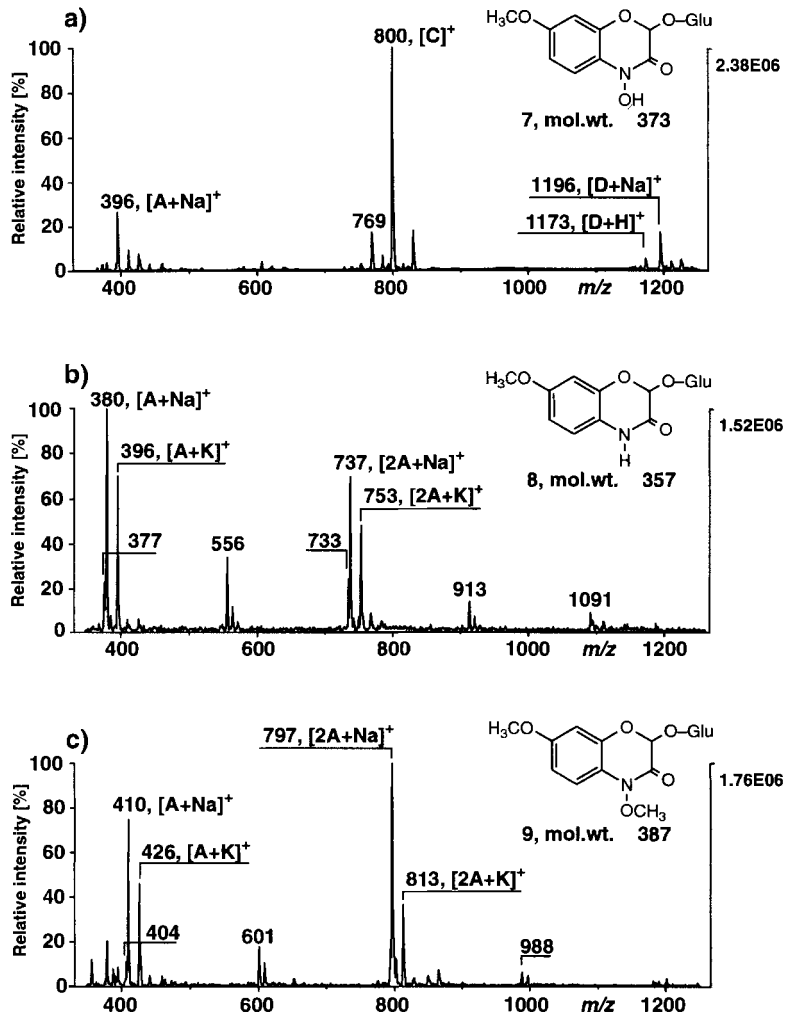


Fig. 3. *ESI-MS of* a) 7, b) **8**, *and* c) **9** *at* 10^{-3} M *in a methanolic solution of* 10^{-3} M $FeCl_3$

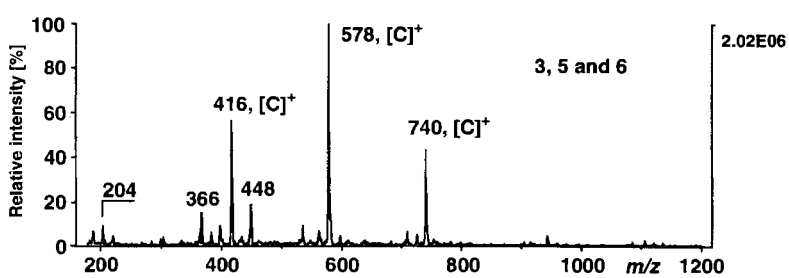


Fig. 4. *ESI-MS of the mixture of* **3**, **5**, *and* **6** (all 10^{-3} M) *in a methanolic solution of* $3 \cdot 10^{-3}$ M $FeCl_3$

300 s, the signal of complex **C** is the base peak (see *Fig. 5, b*). Interestingly, the absolute intensity of the alkali-metal adducts remains more or less stable, as opposed to those of **C**. The 'steady state' is reached after *ca.* 600 s[5]) (*Fig. 3, a*).

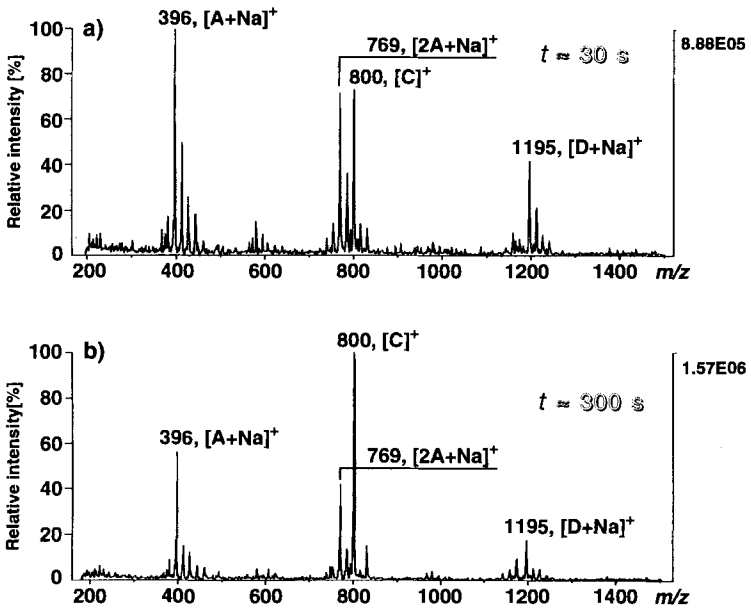


Fig. 5. *ESI-MS of a solution containing* **7** (10^{-3} M in MeOH) *and an equimolar concentration of* $FeCl_3$: a) ca. *30 s after mixing*, b) *300 s after mixing*. See *Fig. 3, a* for the steady state (*ca.* 600 s).

For this reason, the mass-spectral analyses are only performed, when the relative intensities of the signals remain constant over a period of several minutes.

Besides the ions discussed, several spectra show a number of signals which result from multiply charged species. The distribution of these peaks in the spectra of different compounds and their good agreement with calculated values permit their identification as 4+ ions (*Table 2*). These ions correspond to the interaction of the organic compounds with Fe^{III} and with Na^+. The molecules are not deprotonated as in the real Fe^{III} complexes but form clusters probably by electrostatic interactions [21][6]). The hydroxamic-acid derivative **6** shows these peaks too but with lower intensities (*m/z* 535; *Fig. 2*).

3. Conclusions. – The hydroxamic-acid derivatives **3**, **6**, and **7** form metal complexes which are shown in the *Scheme*. The behavior of these compounds is different from that of the lactams **5** and **8** and the methyl hydroxamate **9**. Even the presence of the polar Glu

[5]) The kinetics of the complexation can be observed in this experiment, but the accuracy of the mass spectra does not allow quantitative results (the addition of the signals of the different species identified in the mass spectra is not exact enough).

6) A reason for the formation of cluster ions by compounds **6** (*Fig. 2*), **8**, and **9** (*Fig. 4* and *Table 2*) could be the polarisability of the Glu groups. It is remarkable than an even number of ligands is registered in the mass spectra of these complexes. This could be explained by strong ion-dipole interactions and by π-interactions between the Ph rings.

Table 2. *Composition,* m/z *Values Detected by Means of ESI-MS, and Calculated* m/z *Values of the 4+ Cluster Ions*

Compound	*Fig.*	m/z	$m/z_{calc.}$	Composition
6	*2*	535	534	$[4\cdot343 + Fe + Na]^{4+}$
8	*3,b*	377	377	$[4\cdot357 + Fe + Na]^{4+}$
		556	555	$[6\cdot357 + Fe + Na]^{4+}$
		564	563	$[6\cdot357 + Fe + Na + MeOH]^{4+}$
		733	734	$[8\cdot357 + Fe + Na]^{4+}$
		913	912	$[10\cdot357 + Fe + Na]^{4+}$
		1091	1091	$[12\cdot357 + Fe + Na]^{4+}$
9	*3,c*	407	407	$[4\cdot387 + Fe + Na]^{4+}$
		601	601	$[6\cdot387 + Fe + Na]^{4+}$
		609	609	$[6\cdot387 + Fe + Na + MeOH]^{4+}$
		802	802	$[8\cdot387 + Fe + Na + MeOH]^{4+}$
		988	988	$[10\cdot387 + Fe + Na]^{4+}$

group did not significantly affect the iron affinities, as shown by their mass spectra. It, therefore, seems possible to use this mass-spectral method to characterize compounds as siderophores.

It was also demonstrated that, if the 'steady state' is not reached within a short time (less than 30 s), the kinetics of the complex formation could be observed by ESI-MS. Nevertheless, for the quantification of the results, a modification of the inlet system would be necessary, because the time between sample preparation and injection into the ion source should be reduced in order to get a good reproducibility.

Finally, the formation of complexes like types **C** or **D** and the simultanous detection of aggregates formed by several molecules, Fe^{III}, and Na^+ ions are of interest, too. The reason is that complexes **C** and **D** should represent the species preformed in solution, while the aggregates should only be formed during the ionization process. Such processes are consistent with the problem associated with the ionization mechanisms and the detection of non-covalent complexes [21–26].

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Experimental Part

Materials. The free ligands were isolated and characterized by ^{1}H-NMR, ^{13}C-NMR, IR, ESI-MS, and elemental analysis [14]. Anh. $FeCl_3$ (*Fluka, purum*) was used without purification. The sample solutions (1 mM of the isolated compound and 1 mM of $FeCl_3$) were prepared in MeOH (HPLC-grade, from *Scharlau*, Spain). Such high concentrations were necessary to minimize the impurity effect derived from the ubiquitous Na^+ cations and to obtain reproducible mass spectra.

Mass Spectrometry. The ESI-MS were obtained on a *Finnigan TSQ 700* triple-stage quadrupole mass spectrometer (San Jose, CA, USA) fitted with the ion source from *Analytica®* of *Brandford, Inc.* Samples were continously introduced through the electrospray interface (N_2 drying gas at 110°) at a rate of 2 µl/min by biasing the electrospray probe to a voltage of 3.0–3.2 kV. The ions were detected by scanning the third quadrupole, and the scans were monitored over 3 s in the range m/z 100–800 (for the aglucone derivatives) or m/z 180–1500 (for the glucosyl derivatives). 15–20 scans were averaged to obtain representative spectra. The abundances of the ions are reported as relative intensity with respect to the base peak.

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