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PAPER

Biological activity of Fe(III) *aquo*-complexes towards ferric chelate reductase (FCR)†

Rosa Escudero,[a] Mar Gómez-Gallego,*[a] Santiago Romano,[a] Israel Fernández,[a] Ángel Gutiérrez-Alonso,[b] Miguel A. Sierra,*[a] Sandra López-Rayo,[c] Paloma Nadal[c] and Juan J. Lucena[c]

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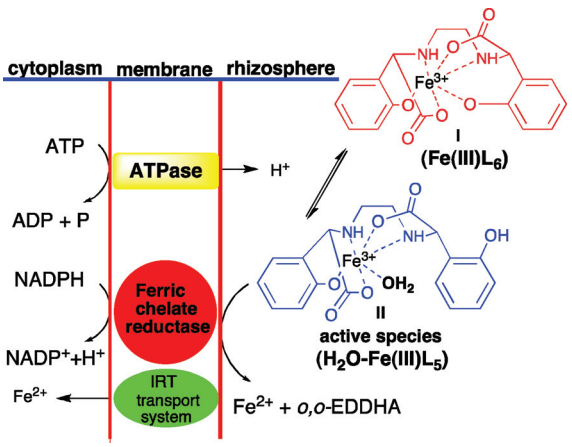
In this study we have obtained experimental evidence that confirms the high activity of *aquo* complexes **III** and **IV** towards the enzyme FCR, responsible for the reduction of Fe(III) to Fe(II) in the process of iron acquisition by plants. The *in vivo* FCR assays in roots of stressed cucumber plants have shown a higher efficiency of the family of complexes **III** and a striking structure–activity relationship with the nature of the substituent placed in a phenyl group far away from the metal center. The results obtained in this work demonstrate that all the *aquo* compounds tested interact efficiently with the enzyme FCR and hence constitute a new concept of iron chelates that could be of great use in agronomy.

Introduction

Iron is an essential element for the nutrition of plants and thence they have evolved strategies to efficiently acquire this element from the soil.[1] In particular, dicots and non-grass monocots obtain the Fe(II) they need by increasing the reduction of the low-soluble Fe(III), generally present in soils in the form of insoluble oxyhydroxide polymers. The process known as *Strategy I*, occurs in the rhizosphere and is mediated by the FRO2, a ferric chelate reductase (FCR) enzyme, NADPH-dependent, located in the root-cell membrane.[1–3] Once reduced, the Fe(II) is taken up into the cells by the IRT1, a specific transport system.[4] The FCR is able to effectively reduce synthetic ferric chelates, which are octahedral Fe(III) complexes derived from polyaminocarboxylic acids.[5] However, although the general aspects of the mechanism of iron transferring to plants are known, the way in which the process occurs in the presence of a synthetic iron chelate still remains uncertain in many ways.

The high efficiency of Fe(III)-*o*,*o*-EDDHA **I** (ethylenediamine-bis(*o*-hydroxyphenyl)acetic acid) in transferring iron to plants is well known.[6] In a previous study about the insights of the process,[7,8] we proposed that this complex ($E_{1/2}$ = −0.56 V, pH = 7) could not be directly reduced by the enzyme FCR ($E_{NADPH/NADP+}$ = −0.32 V),[9] at least not in the form of the octahedral complex depicted in Scheme 1. Based on electrochemical measurements and speciation studies of complex **I** in solution[10] we hypothesized that a new *aquo* complex **II**, of lower reduction potential, should be formed prior to the enzymatic reduction. In our model, this species **II** would be the active substrate for the FCR (Scheme 1).[11]

Overall, the process requires the generation of a coordination vacancy in the Fe(III)L_6 complex **I** and the incorporation of a water molecule. To test the validity of our proposal, in this article we have prepared a series of *aquo* complexes structurally related to the active species **II** in Scheme 1, studied their electrochemical properties and tested their activity towards enzymatic reduction in root cucumber stressed plants. In parallel, we have performed a theoretical–experimental study of the geometry and



Scheme 1

[a]*Departamento de Química Orgánica, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain. E-mail: margg@quim.ucm.es, sierraor@quim.ucm.es; Fax: (+) 34-91-3944310*
[b]*Departamento de Química Inorgánica, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain*
[c]*Departamento de Química Agrícola, Facultad de Ciencias, 10. Universidad Autónoma, 28049 Madrid, Spain*

†Electronic supplementary information (ESI) available: Titration curves, UV-vis spectra, spectroscopic and MS data, cyclic voltammograms of all compounds studied, Cartesian coordinates (in Å) and total energies (in au, non corrected zero-point vibrational energies included). See DOI: 10.1039/c2ob06754d

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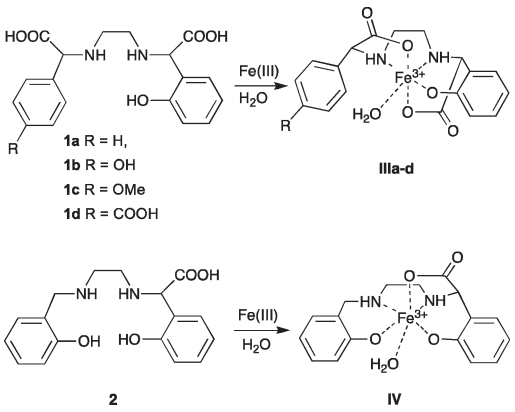


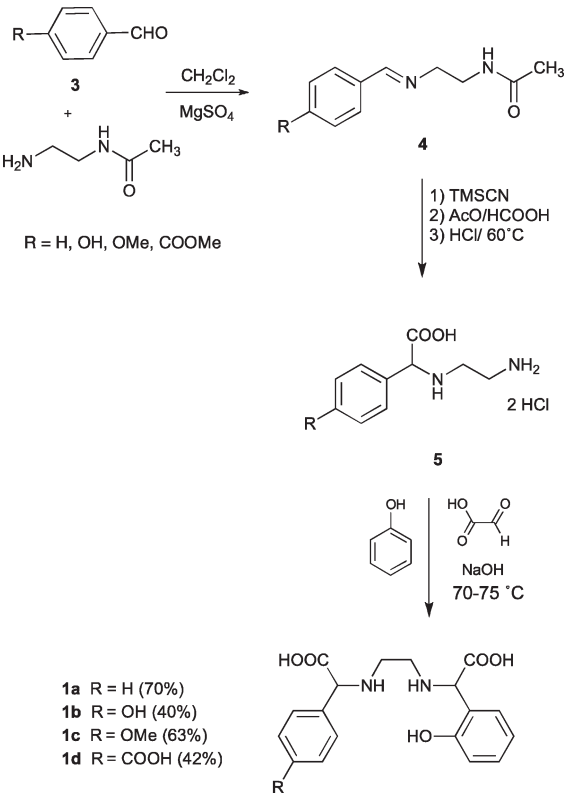
Scheme 2

spin state of the *aquo* complexes prepared. The results obtained would provide more information about the iron transfer process to the plants and also will be of great value in the design of new iron chelates, more active for the remediation of iron deficiency in cultures.

Results and discussion

Using the structure of *aquo* complex **II** in Scheme 1 as a model, an efficient synthesis for ethylenediamine carboxylate complexes **IIIa–d** was designed (Scheme 2). Complexes **IIIa–d** should be readily obtained in water solution by treatment of the corresponding pentacoordinate ligands **1a–d** (*one phenolate*, *two carboxylates*, *two amino groups*) with an Fe(III) salt. The incorporation of substituents with different polarities in the phenyl group that is not directly involved in the coordination with the metal would be useful to obtain preliminary information about possible structure–activity relationships during the enzymatic process (see below). To broaden the scope of the study, the structurally related *aquo* complex **IV** was also considered. This complex can be readily obtained from ligand **2** (*two phenolates*, *one carboxylate*, *two amino groups*) (Scheme 2).[12] Although there have been reported different coordination modes for Fe(III) *aquo* complexes,[13] our speciation studies in solution indicate that in the pH range 3–10, the Fe(III) complexes of ligands **1b**[14] and **2**[12] are hexacoordinated. In both cases, the most stable species in solution at pH < 7 incorporate a single water molecule to achieve the octahedral environment around the metal (as depicted in Scheme 2). At higher pH values, the complexes are still hexacoordinated but the water molecule can be replaced by an HO^- (see below).

Ligands **1a–d** were prepared as shown in Scheme 3. Condensation of *N*-acetylethylenediamine with benzaldehydes **3** resulted in the formation of imines **4** in nearly quantitative yields. The imines **4** were subsequently treated with trimethylsilyl cyanide (TMSCN), to form the corresponding α-aminonitriles, which were *in situ* formylated ($HCOOH/Ac_2O$)[15] and then hydrolysed, by treatment with hydrochloric acid, to give amines **5** as hydrochlorides. Finally, a Mannich-type reaction between amines **5**, phenol and glyoxylic acid, yielded the desired ligands **1a–d**, which were obtained from the reaction crudes by precipitation in the isoelectric point, as 1 : 1 diastereomeric mixtures and in



Scheme 3

Table 1 Purity of the ligands determined as the iron chelating ability using photometric titration and molar absorptivities (ε) recorded at 480 nm as the slopes in the titration curves[a]

Ligand	Purity[b]	ε (480 nm)
1a	86.7 ± 0.5	2024 ± 12
1b	92.3 ± 0.4	2129 ± 7[14]
1c	77.9 ± 1.0	2438 ± 41
1d	78.4 ± 0.3	2291 ± 31
2	85.4 ± 0.4	4617 ± 20[12]

[a] μ = 0.1 M (NaCl); t = 25 °C. [b] Expressed as % of the free acid (excluding other ions, mainly Na^+, and water).

yields ranging from 40% to 70%. The sequence in Scheme 3 is a simple, new, general procedure for non-symmetrical ligands **1**. This procedure highly improves, in both yields and in the notable reduction of the number of steps, the reported synthetic method for **1b**.[16]

The iron complexing ability of ligands **1** and **2** was determined using photometric titration.[10] The Fe(III) chelates **III** and **IV** derived from ligands **1** and **2** were prepared in water from $FeCl_3{\cdot}6H_2O$ in neutral medium. Their purity was adequate for the FCR activity experiments described below, since the impurities present were mainly inorganic salts (NaCl) formed during the precipitation of the ligands. From the titration curves, the molar absorptivities (ε) of the chelates **III** at 480 nm (corresponding to the Fe(III)–phenolate bond) could be obtained (Table 1). Having two phenolates, the molar absorptivity of complex **IV** is similar to that of Fe(III)-*o*,*o*-EDDHA **I** (4721 ± 16),[10] and almost double those of complexes **IIIa–IIId** having

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only one phenolate–Fe(III) bond. The titration curves and the UV-vis spectra obtained are provided as supporting information.†

Investigation of the structure of *aquo* complexes in solution is rather difficult because various species could be present in the medium. That is why our next concern was to gain more information about the structure of complexes **III** and **IV**. The optimization of the geometries was approached by quantum chemical calculations (uB3LYP/def2-SVP level)[17] of complexes **IIIa** and **IV**, considering all the possible hexacoordinate arrangements of the ligand and the water molecule around the metal and both high ($S = 5/2$) and low ($S = 1/2$) spin states for Fe(III). In the case of the diastereomeric complexes **IIIa**, the calculations revealed that high spin ($S = 5/2$) geometries were more stable than the corresponding low spin ($S = 1/2$) ones. The geometries and energies of high spin ($S = 5/2$) complexes **IIIa** are collected in Fig. 1, whereas the analogous low spin ($S = 1/2$) complexes **IIIa** are included as supporting information.†

As shown in Fig. 1, the lowest energy species corresponds to diastereomeric complexes **IIIa3** and **IIIa7**, having the water molecule placed *cis* to the O atoms of the carboxylates and phenolate groups and *trans* to one of the NH groups. Complexes **IIIa3** and **IIIa7** are epimers in the carbon bearing the free phenyl group and their energies differ only by 0.1 kcal mol^{-1}. Both structures exhibit a distorted octahedral environment and, as expected, the Fe–OH_2 bond length is longer than the rest of the Fe–O bond distances due to the lower donor strength of the OH_2 ligand compared to the phenolate or carboxylate ligands. As an example, this is reflected in the computed Wiberg–NBO bond orders of the most stable **IIIa3**: 0.22 au for the Fe–OH_2 bond, 0.45 au and 0.32 au for the Fe–O carboxylate and 0.53 au for the Fe–O phenolate bonds, respectively. Finally, there is less than 2 kcal mol^{-1} between complex **IIIa3** and **IIIa1** having the water molecule *trans* to the carboxylate group, with the subsequent elongation of the Fe–OH_2 bond.

The computed uB3LYP/def2-SVP geometries of diastereomeric complexes **IV** also show a preference for the high spin state ($S = 5/2$) in the metal and are collected in Fig. 2 (see the supporting information for the corresponding low spin ($S = 1/2$) complexes **IV**). All are distorted octahedral arrangements and the energy minimum corresponds to **IV4**, placing the water molecule *cis* to the O atoms of both phenolates and carboxylate, and *trans* to one of the NH groups. The computed Wiberg–NBO bond orders are in this case 0.22 au for the Fe–OH_2 bond, 0.51 au and 0.50 au for the Fe–O phenolate and 0.30 au for the Fe–O carboxylate bonds, respectively. Now, **IV4** is only 1 kcal mol^{-1} more stable than its diastereoisomer **IV1**, placing the water molecule *cis* to NH and *trans* to the O-carboxylate group. The small difference in energy would indicate that both types of complexes could be present in solutions of complex **IV** in water.

The average calculated Fe–OH_2 bond distance in complexes **IIIa3**, **IIIa7** and **IV4** is 2.19 Å, in the range of the data reported for high spin Fe(III) aminocarboxylate complexes (2.02–2.19 Å).[18]

Electrochemical studies

The electrochemical study was key to test the ability of the *aquo* complexes **III** and **IV** towards the reduction, and hence to probe the validity of our initial design premises. The measurements were made in water solutions of the corresponding ligands **1a–d**, **2** (10^{-2} M) and $FeCl_3 \cdot 6\ H_2O$ (10^{-3} M) at room temperature in 0.1 M phosphate buffer solutions, and at pH values ranging from 5.0 to 9 (Table 2). The electrochemical study of the free ligands was also made prior to the study of the complexes. The experimental data, together with the cyclic voltammograms of all compounds studied are included in the supporting information.†

On the whole, and in spite of the particularities found, all the chelates **IIIa–d** and **IV** studied were more easily reduced in water than the reference complex Fe(III)-*o*,*o*-EDDHA **I** (Table 2). Complexes **IIIa–d** showed quasi-reversible electrochemistry [$E_{1/2} = (E_{pa}+E_{pc})/2$] and the reduction potentials become more negative as the pH of the medium increased (Fig. 3). This trend is in agreement with the gradual deprotonation of the species [**Fe(III)L_5H_2O**] to become the negatively charged species [**Fe(III)L_5OH**]$^-$ (L = **1a–c**),[14] less prone to reduction. In this regard, the reduction potentials of **IIId** (R = CO_2H) are noticeably higher than the rest at intermediate pH values, which is not surprising considering that the presence of the *p*-carboxylate group makes this compound negatively charged above pH 5.

The electrochemical behaviour of the complex **IV** is rather different. In general, and in the pH range studied, the $E_{1/2}$ values are more negative than those of complexes **IIIa–d**, and closer to the values recorded for the reference compound Fe(III)-*o*,*o*-EDDHA **I**. At acid pH values (pH 5-6) the reduction is irreversible and a single wave is observed. However, at pH 7, two reduction waves ($E_{pc} = -0.46$ V and $E_{pc} = -0.70$ V) are formed in a reversible process, which suggests the coexistence of both species **[Fe(III)L_5H_2O]** and **[Fe(III)L_5OH]**$^-$ (L = **2**) in the solution. The two waves turn into a single one from pH 8 ($E_{pc} = -0.71$ V), as should be expected if the species **[Fe(III)L_5OH]**$^-$ were predominant in solution (Fig. 3).[19]

Overall, the electrochemical results confirm our hypothesis about the higher reduction ability of the *aquo* species, giving support to our initial proposal about their possible involvement in the process of transferring iron to plants. Additionally, for all complexes **IIIa–d** and **IV** tested, the lowest reduction potential was obtained at pH 5, which is biologically significant, as the pH in the acidified rhizosphere is about 5.5–6.[20]

Ferric chelate reductase (FCR) assays

The ability of iron chelates **IIIa–d** and **IV** to act as substrates in the enzymatic reduction was evaluated next. Their activity towards the enzyme FCR was measured in roots of stressed cucumber plants by determining Fe(III)/Fe(II) reduction rates. Two different assays were performed with freshly prepared water solutions of complexes **III**, **IV** and Fe(III)-*o*,*o*-EDDHA **I**. The FCR reduction data of this latter chelate were used as normalizing parameters. The experiments were carried out at pH 6, to simulate the reduction conditions in the rhizosphere.

The initial analysis of the data obtained in the enzymatic essays (Fig. 4) indicated that iron complexes **III** and **IV** not only were active substrates of the FCR, but also showed much higher activities than the reference compound Fe(III)-*o*,*o*-EDDHA **I**. These results nicely agree with our design premise (Scheme 1)

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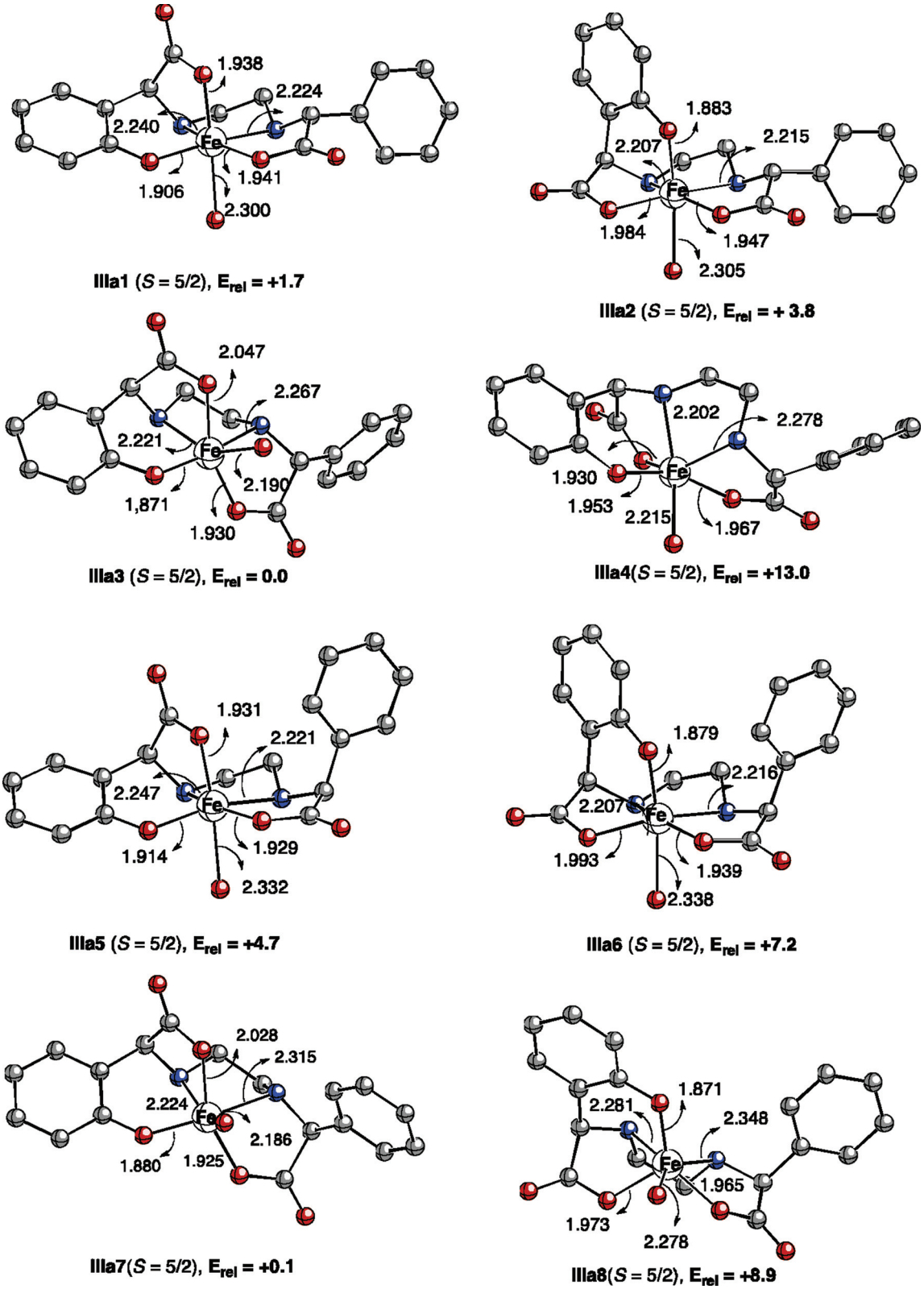

Fig. 1 Fully optimized geometries of high spin (S = 5/2) complexes **IIIa**. Bond lengths are given in Å and relative energies in kcal mol^{-1}. All data have been computed at the uB3LYP/def2-SVP level.

and confirm that all this new class of *aquo* iron chelates could have interesting agronomical properties.

The huge differences found in the FCR activities in Fig. 4 deserve special attention. Complexes **IIIa** (R = H) and **IV** derive

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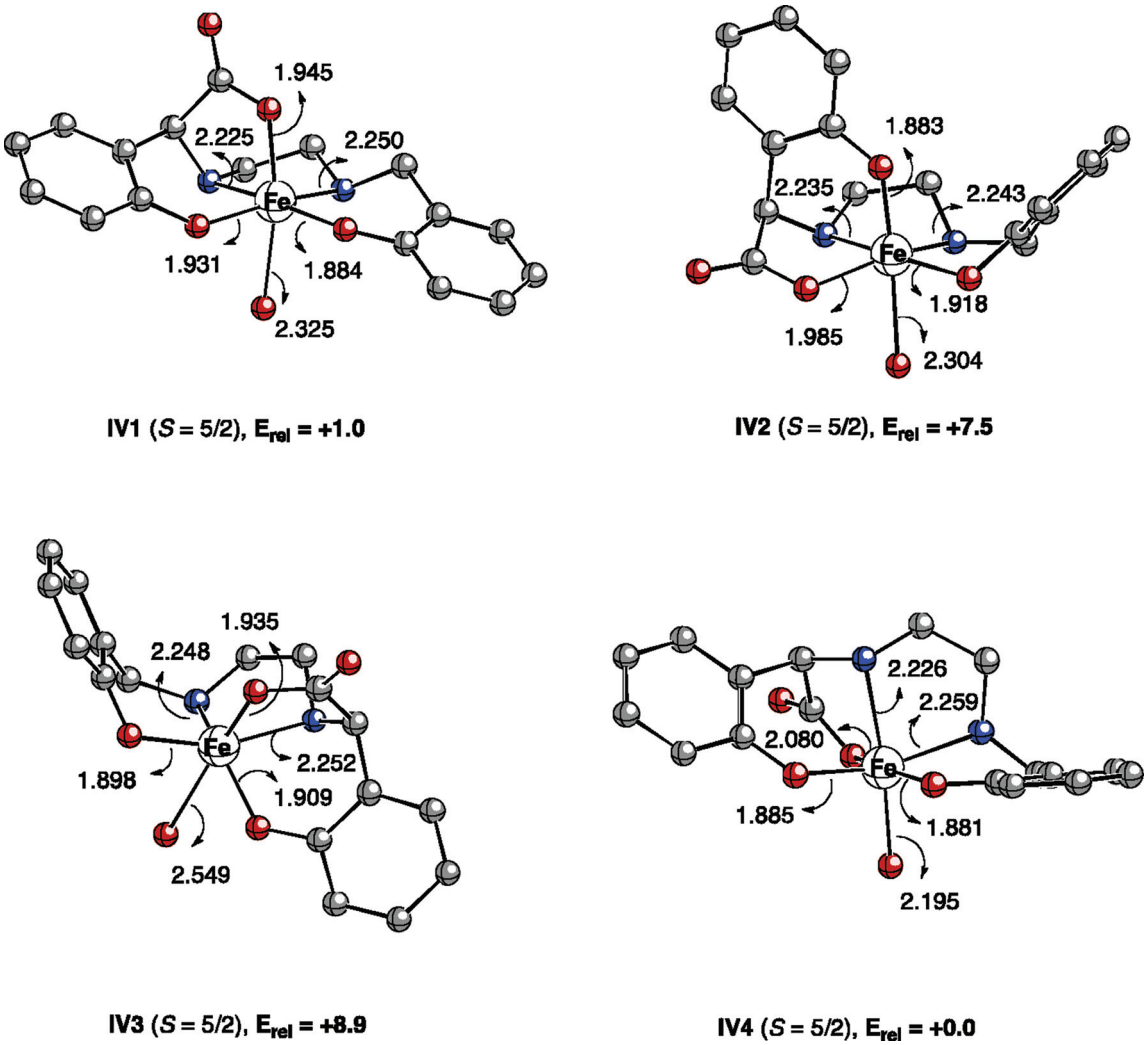

Fig. 2 Fully optimized geometries of high spin (S = 5/2) complexes **IV**. Bond lengths are given in Å and relative energies in kcal mol^{-1}. All data have been computed at the uB3LYP/def2-SVP level.

Table 2 Electrochemical properties of complexes **III** and **IV**[a]

	IIIa	**IIIb**	**IIIc**	**IIId**	**IV**			**I**	
pH	$E_{1/2}$	$E_{1/2}$	$E_{1/2}$	$E_{1/2}$	E_{pc}	E_{pa}	$E_{1/2}$	E_{pc}	$E_{1/2}$
5	−0.25	−0.22[b]	−0.23	−0.32	−0.40	—	—	−0.54[b]	−0.37[b]
6	−0.33	−0.33	−0.31	−0.38	−0.47	—	—	−0.57[b]	−0.48[b]
7	−0.36	−0.39	−0.38	− 0.43	−0.46 −0.70	−0.38	—	−0.61[b]	−0.56[b]
8	−0.37	—	−0.38	− 0.43	−0.71	−0.48	−0.59	−0.62	−0.59
9	−0.45	−0.49	−0.41	− 0.49	−0.70	−0.51	−0.60	−0.62	−0.60

[a] Values given in V. [b] Data obtained from ref. 7 **IIIa**, R = H, **IIIb**, R = OH, **IIIc**, R = OMe, **IIId**, R = COOH.

from structurally different ligands, but show comparable FCR activities. However, and being both more active than the reference complex **I**, their reduction rates are noticeably lower than those of complexes **IIIb–d**. In fact, the enzymatic reduction of **IIId** (R = COOH) is about eight times faster than that of the reference complex **I**, and those of **IIIb** (R = OH) and **IIIc** (R = OMe) are about five and three times faster respectively.

These results cannot be easily explained just considering the E_{pc} data obtained in the electrochemical study of the complexes. The reduction potentials in Table 2 reflect the reduction ability of the Fe(III) within the complex, a process in which the metal and its environment are relevant. In fact, the general trend of data in Table 2 (at any pH value), is that complexes **III** are in general *easier to reduce* than complex **IV** (having a different

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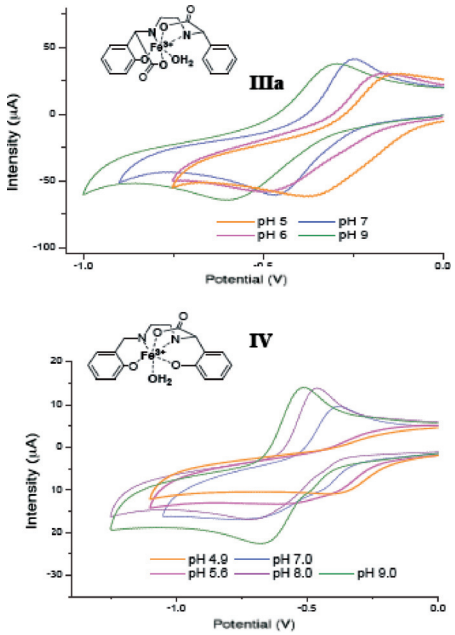

Fig. 3 Electrochemical study of **IIIa** and **IV**.

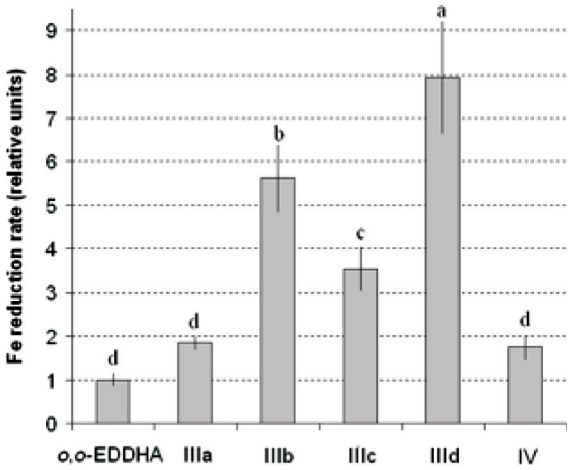

Fig. 4 FCR root activity measured in cucumber stressed plants using the Fe(III) chelates **IIIa–d** and **IV** as substrates. Units are relative to Fe (III)-*o*,*o*-EDDHA **I** and were obtained in fresh weight basis. Different letters denote significant differences among treatments according to the Duncan test ($P > 0.1$).

metal environment), but the differences found in $E_{1/2}$ among the series **IIIa–IIId** are very small. This behaviour contrasts with the results obtained in the FCR essays, showing noticeable differences within **IIIa–IIId**. The main structural change made on these family of compounds is the substituent placed in the *para* position of the free phenyl group, indeed located rather far away from the metal site, as shown in Fig. 2.

To observe structure–activity relationships is not rare in the study of an enzymatic process. The FCR is a NADPH-dependent transmembrane enzyme whose topology has been reported,[21] and the enzymatic reduction of any Fe(III) complex must require some kind of interaction with the FCR active site for the electron transfer process.[22] Probably in this event the influence of structural changes in remote positions of **IIIa–IIId** is relevant and, considering the experimental results, the polarity of the substituent located on the free phenyl group and the rate of enzymatic reduction, are related. To go further with only these data would be speculative, but we believe that the results obtained are a good starting point for an in depth study about the insights of the reaction.

Conclusions

In this study we have obtained experimental evidence that confirms the high activity of *aquo* complexes **III** and **IV** towards the enzyme FCR, responsible for the reduction of Fe(III) to Fe(II) in the process of plant nutrition. The *in vivo* FCR essays in roots of stressed cucumber plants have shown a higher efficiency of the family of complexes **III** and a striking structure–activity relationship with the nature of the substituent placed in a phenyl group far away from the metal center.

The study (DFT) of the geometry of complexes **III** has shown a preference for a *quasi*-symmetrical octahedral arrangement around the metal, placing the water molecule *trans* to one of the NH groups and *cis* to the oxygen atoms. For complex **IV** this type of arrangement is also compatible with the *cis* to NH–*trans* to carboxylate placement of the water molecule. The results obtained in this work confirm the interesting agronomical properties of all this new class of *aquo* iron chelates and will be used as the starting point for a benchmark study of a process of high biological relevance. Further work directed to establish the details of this fascinating enzymatic ET reaction is currently in progress in our laboratories.

Experimental section

General

Reactions requiring an inert atmosphere were conducted under argon and the glassware was flame dried under vacuum (<0.2 mmHg). Tetrahydrofuran (THF) was distilled from sodium and benzophenone immediately prior to use. ^{1}H NMR and ^{13}C NMR spectra were recorded at 22 °C on a Bruker Avance 300 (300.1 and 75.4 MHz) spectrometer. Chemical shifts are given in ppm relative to TMS (^{1}H, 0.0 ppm), $CDCl_3$ (^{13}C, 77.0 ppm), D_2O, and D_2O/Na_2CO_3 (^{1}H, 4.78 ppm), D_2O/Na_2CO_3 (^{13}C, 165.7 ppm), DMSO-d_6 (^{1}H, 2.5 ppm; ^{13}C, 40.6 ppm). IR spectra were taken on a Perkin-Elmer 781 spectrometer. ESI-MS spectra were carried out in MeOH, using an ESQUIRE-LC (Bruker Daltonic, Bremen, Germany) ion trap spectrometer in negative mode of detection. The stainless-steel capillary was held at a potential of 5.0 kV. Nitrogen was used as nebulizer gas at a flow-rate of 3.98 L min^{-1} (nebulizer pressure 11 psi) at 150 °C.

General procedure for the synthesis of imines 4. Imines **4a**, **4c** and **4d** were obtained in quantitative yield by reaction of the corresponding aldehydes with *N*-acetylethylenediamine at room temperature (2 h) in CH_2Cl_2 and in the presence of $MgSO_4$. Imine **4b** was prepared by refluxing equimolar amounts of *p*-hydroxybenzaldehyde and *N*-acetylethylenediamine in EtOH for 2 h.

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Imine 4a. From 1.8 g (16.9 mmol) of benzaldehyde, 8.14 g (67.7 mmol) of $MgSO_4$ and 1.72 g (16.9 mmol) of *N*-acetylethylenediamine in 30 mL of dry CH_2Cl_2 were obtained 3.7 g (99%) of imine **4a** as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 8.24 (s, 1H, CH═N), 7.69–7.66 (m, 2H, ArH), 7.39–7.34 (m, 3H, ArH), 3.68–3.64 (m, 2H, CH_2), 3.54–3.48 (m, 2H, CH_2), 1.91 (s, 1H, CH_3).^{13}C NMR ($CDCl_3$) δ 170.2 (C═O), 162.6 (CH═N), 135.6, 130.7, 128.4, 127.92 (ArC), 60.1 (CH_2), 40.1 (CH_2), 22.9 (CH_3). IR (film) ν 3300, 1648, 1551, 754 cm^{-1}. HRMS (ESI): calc for $C_{11}H_{15}N_2O$ ($[M + H]^+$): 191.1181; found 191.1177.

Imine 4b. From 2 g (16.4 mmol) of *p*-hydroxybenzaldehyde, 7.8 g (65.6 mmol) of $MgSO_4$ and 1.7 g (16.4 mmol) of *N*-acetylethylenediamine in 20 mL absolute EtOH, were obtained 3.19 g (94%) of imine **4b** as an orange solid (mp 115–116 °C, EtOH). ^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H, CH═N), 7.55 (d, J = 8.61 Hz, 2H, ArH), 6.84 (d, J = 8.61 Hz, 2H, ArH), 3.70–3.66 (m, 2H, CH_2), 3.59–355 (m, 2H, CH_2), 1.97 (s, 1H, CH_3).^{13}C NMR ($CDCl_3$) δ 170.6 (C═O), 162.9 (CH═N), 160.1, 129.9, 127.1, 115.8 (ArC), 59.8 (CH_2), 40.4 (CH_2), 23.2 (CH_3). IR (KBr) ν 3309, 1641, 1606, 1583, 1552, 1512, 896 cm^{-1}. HRMS (ESI): calc for $C_{11}H_{15}N_2O_2$ ($[M + H]^+$): 207.1130; found 207.1133.

Imine 4c. From 2 g (14.7 mmol) of *p*-anisaldehyde, 7.10 g (58.8 mmol) of $MgSO_4$, and 1.49 g (14.7 mmol) of *N*-acetylethylenediamine in 30 mL of dry CH_2Cl_2 were obtained 3.25 g (100%) of imine **4c** as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 8.18 (s, 1H, CH═N), 7.63 (d, J = 8.8 Hz, 2H, ArH), 6.43 (d, J = 8.8 Hz, 2H, ArH), 3.80 (s, 3H, CH_3O), 3.64–3.61 (m, 2H, CH_2), 3.54–3.50 (m, 2H, CH_2), 1.97 (s, 1H, CH_3).^{13}C NMR ($CDCl_3$) δ 170.2 (C═O), 162.0 (CH═N), 161.8, 129.7, 128.7, 113.9 (ArC), 60.1 (CH_2), 55.3 (O–CH_3), 40.4 (CH_2), 23.1 (CH_3). IR (film) ν 3314, 1648, 1606, 1578, 1513, 755 cm^{-1}. HRMS (ESI): calc for $C_{12}H_{17}N_2O_2$ ($[M + H]^+$): 221.1286; found 221.1289.

Imine 4d. From 1 g (6.1 mmol) of 4-formyl methyl benzoate, 3.6 g (30 mmol) of $MgSO_4$ and 0.62 g (6.1 mmol) of *N*-acetylethylenediamine in 30 mL of dry CH_2Cl_2 were obtained 1.43 g (96%) of imine **4d** as a solid (mp 113–114 °C, EtOH). ^{1}H NMR ($CDCl_3$) δ 8.32 (s, CH═N), 7.98 (d, J = 6.7 Hz, 2H, ArH), 7.74 (d, J = 6.7 Hz, 2H, ArH), 3.93 (s, 3H, CH_3O), 3.72–3.69 (m, 2H, CH_2), 3.61–3.52 (m, 2H, CH_2), 1.96 (s, 3H CH_3).^{13}C NMR ($CDCl_3$) δ 170.6 (C═O), 166.9 (C═O), 162.2 (CH═N), 140.0, 132.4, 130.3, 128.4 (ArC), 60.9 (CH_2), 52.7 (CH_3), 40.6 (CH_2), 23.7 (CH_3O). IR (KBr) ν 3293, 1721, 1648, 1581, 818 cm^{-1}. HRMS (ESI): calc for $C_{13}H_{16}N_2NaO_3$ ($[M + Na]^+$): 271.1053; found 271.1055.

General procedure for the synthesis of amines 5. To a solution of the corresponding imine **4** in anhydrous THF, under argon atmosphere, and at 0 °C, TMSCN was added (1 : 1.5 molar ratio). The reaction was stirred at room temperature for 20 h, then quenched at 0 °C with NH_4Cl (sat. soln.) and extracted with Et_2O (3 × 100 mL). The combined organic extracts were washed with water, dried over $MgSO_4$ and concentrated under reduced pressure. The aminonitrile so formed was obtained in nearly quantitative yield and was not isolated. Formylation of the aminonitrile was carried out with acetic formic anhydride, freshly prepared by heating a 1 : 1.1 mixture of Ac_2O and formic acid at 60 °C for 1 h.[15] The reagent was added over the neat nitrile at 0 °C and the mixture was stirred at 0 °C for 30 min, and then for 1 h at room temperature. 12 M HCl was then poured over the solution that was refluxed for 2 h. The mixture was allowed to cool to room temperature, and after removing the solvent under vacuum the amine hydrochloride was obtained as a solid.

Amine 5a. From 3.17 g (16.67 mmol) of imine **4a** and 3.2 mL (25 mmol) of TMSCN. The formylation of the aminonitrile was carried out with acetic formic anhydride (prepared from a mixture of 8.2 mL (0.217 mol) HCO_2H and 20.6 mL (0.22 mol) Ac_2O) at 0 °C. The mixture was stirred at 0 °C for 30 min and then for 1 h at room temperature before the addition of (42 mL, 0.5 mol) 12 M HCl. After 2 h reflux and work-up, amine **5a** (2.03 g, 46%) was obtained as the hydrochloride. ^{1}H NMR (D_2O) δ 7.54–7.47 (m, 5H, ArH), 5.04 (s, 1H, CH), 3.43–3.29 (m, 4H, $2CH_2$).^{13}C NMR (D_2O) δ 170.9 (C═O), 131.1, 130.4, 130.2, 129.0 (ArC), 64.9 (CH), 43.1 (CH_2), 35.8 (CH_2). IR (KBr) ν 3424, 3009, 1741, 1723, 1488, 1327, 1218, 914, 710 cm^{-1}.

Amine 5b. From 4 g (19.41 mmol) of imine **4b** and 3.88 mL (22.14 mmol) of TMSCN. The formylation of the aminonitrile was carried out with acetic formic anhydride (prepared from a mixture of 9.52 mL (0.252 mol) HCO_2H and 24.1 mL (0.254 mol) Ac_2O) at 0 °C. The mixture was stirred at 0 °C for 30 min and then for 1 h at room temperature before the addition of 48 mL (0.58 mol) of 12 M HCl. After 2 h reflux and work-up, amine **5b** (2.96 g, 54%) was obtained as the hydrochloride. ^{1}H NMR (D_2O) δ 7.03 (d, J = 8.67 Hz, 2H, ArH), 6.64 (d, J = 8.67 Hz, 2H, ArH), 4.81 (s, 1H, CH), 3.07–2.97 (m, 4H, $2CH_2$).^{13}C NMR (D_2O) δ 171.3 (C═O), 157.9, 130.9, 122.1, 116.87 (ArC), 64.58 (CH), 42.91 (CH_2), 35.85 (CH_2). IR (KBr) ν 3300, 3071, 1737, 1613, 1443, 1393, 1272, 910, 820 cm^{-1}.

Amine 5c. From 3.25 g (14.7 mmol) of imine **4c** and 2.8 mL (22.14 mmol) of TMSCN. The formylation of the aminonitrile was carried out with acetic formic anhydride (prepared from a mixture of 7.2 mL (0.192 mol) HCO_2H and 18.2 mL (0.193 mol) Ac_2O) at 0 °C. The mixture was stirred at 0 °C for 30 min and then for 1 h at room temperature before the addition of 37 mL (0.46 mol) 12 M HCl. After 2 h reflux and work-up, amine **5c** (1.84 g, 42%) was obtained as the hydrochloride. ^{1}H NMR (D_2O) δ 7.46 (d, J = 8.82 Hz, 2H, ArH), 7.09 (d, J = 8.82 Hz, 2H, ArH), 5.06 (s, 1H, CH), 3.85 (s, 3H, CH_3O), 3.41–3.39 (m, 4H, $2CH_2$).^{13}C NMR (D_2O) δ 171.0 (C═O), 160.9, 130.8, 122.5, 115.6, (ArC), 64.3 (CH), 55.9 (CH_3O), 42.9 (CH_2), 35.9 (CH_2). IR (KBr) ν 3408, 3057, 1734, 1444, 1368, 1260, 1071, 956, 830 cm^{-1}.

Amine 5d. From 2.5 g (10.08 mmol) of imine **4d** and 2 mL (15.12 mmol) of TMSCN. The formylation of the aminonitrile was carried out with acetic formic anhydride (prepared from a mixture of 5 mL (0.131 mol) HCO_2H and 12.53 mL (0.132 mol) Ac_2O) at 0 °C. The mixture was stirred at 0 °C for 30 min and then for 1 h at room temperature before the addition of 25 mL (0.3 mol) of 12 M HCl. After 2 h reflux and work-up, amine **5d** (2.12 g, 68%) was obtained as the hydrochloride. ^{1}H NMR (D_2O) δ 7.97 (dd, J_1 = 6.68, J_2 = 1.79 Hz, 2H, ArH), 7.47 (d, J = 6.66 Hz, 2H, ArH), 4.93 (s, 1H, CH), 3.51–3.16 (m, 4H, $2CH_2$).^{13}C NMR (D_2O) δ 170.6 (C═O), 169.9 (C═O), 136.1,

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131.9, 131.2, 129.2 (ArC), 64.9 (CH), 43.4 (CH_2), 35.8 (CH_2). IR (KBr) ν 3429, 3074, 1741, 1723, 1488, 1327, 1218, 914, 710 cm^{-1}.

General procedure for the synthesis of amino acids 1a–d. To a mixture of melted phenol and 3 eq of a NaOH (50% w/w in water), at 35 °C, was added slowly 1 eq of the corresponding amine hydrochloride **5** and 1 eq of 50% glyoxylic acid, keeping the temperature below 40 °C during the addition. The mixture was heated at 70–75 °C for 2 h, then left to reach room temperature and quenched with a mixture of water (20 mL) and CH_2Cl_2 (20 mL). The reaction mixture was stirred for 20 min, the organic phase separated, and the aqueous phase extracted with 3 × 20 mL of CH_2Cl_2 to remove all the excess phenol. The volume of the aqueous phase was reduced under vacuum and the amino acids **1** were obtained by addition of 1 M HCl to the solution until precipitation at the isoelectric point. All compounds decompose before melting, between 220 and 260 °C.

Amino acid 1a. From 10.56 g (112.36 mmol) 95% phenol, 1.08 g (13.47 mmol) NaOH (50% w/w in water), 1.2 g (4.49 mmol) of amine hydrochloride **5a** and 0.66 g (4.49 mmol) of 50% glyoxylic acid. After work-up and precipitation at pH 3.5, amino acid **1a** (1.14 g, 70%) was obtained as an orange solid and as a 1 : 1 diastereomeric mixture. ^{1}H NMR (D_2O/Na_2CO_3) δ 7.5–7.3 (m, 5H, ArH), 7.29–7.10 (m, 2H, ArH), 6.8–6.7 (m, 2H, ArH), 4.42 (s, 1/2H, CH), 4.33 (s, 1/2H, CH), 4.11 (s, 1/2H, CH), 4.10 (s, 1/2H, CH), 2.80–2.59 (m, 4H, $2CH_2$). ^{13}C NMR (D_2O/Na_2CO_3) δ 180.7 (C═O), 179.9 (C═O), 159.8, 140.8, 131.5, 131.4, 130.9, 130.8, 130.5, 129.6, 129.5, 129.1, 129.0, 127.7, 124.4, 122.6, 120.2, 118.9, 118.7, 118.4, 118.2 (ArC), 68.9 (CH), 68.0 (CH), 67.96 (CH), 65.9 (CH), 46.9 (CH_2), 46.9 (CH_2), 46.7 (CH_2), 46.4 (CH_2). IR (KBr) ν 3419–2400 (broad), 1613 (C═O), 753, 700 cm^{-1}. ESI-MS *m/z* 343 $[M-H]^-$, 325 $[M-H-18]^-$, 399 $[M-H-44]^-$, 255 $[M-H-88]^-$. HRMS (ESI): calc for $C_{18}H_{21}N_2O_5$ ($[M + H]^+$): 345.1445; found 345.1447.

Amino acid 1b (*o,p*-EDDHA).[16] From 8.3 g (88.3 mmol) 95% phenol, 0.84 g (10.65 mmol) of NaOH (50% w/w in water), 1 g (3.55 mmol) of amine hydrochloride **5b** and 0.52 g (3.55 mmol) of 50% glyoxylic acid. After work-up and precipitation at pH 3.5, amino acid **1b** (500 mg, 40%) was obtained as an orange solid, and as a 1 : 1 diastereomeric mixture. ^{1}H NMR (D_2O/Na_2CO_3) δ 7.09–6.96 (m, 4H, ArH), 6.69–6.61 (m, 2H, ArH), 6.53 (d, J = 8.61 Hz, 2H, ArH), 4.30 (s, 1/2H, CH), 4.28 (s, 1/2H, CH), 3.9 (s, 1/2H, CH), 3.89 (s, 1/2H, CH), 2.73–2.51 (m, 4H, $2CH_2$). ^{13}C NMR (D_2O/Na_2CO_3) δ 178 (C═O), 176.8 (C═O), 175.7 (C═O), 160.8, 158.5, 131.1, 130.7, 130.2, 129.4, 129.33, 127.3, 121.97, 119.1, 117.45, 117.41, 117.0 (ArC), 66.77 (CH), 65.24 (CH), 64. 39 (CH), 45.23 (CH_2), 45.04 (CH2), 44.82 (CH2). IR (KBr) ν 3416–2500 (broad), 1613, 1517, 1460, 1383, 1261, 1178, 1178, 831, 752, 568 cm^{-1}. ESI-MS *m/z* 359 $[M-H]^-$, 315 $[M-H-44]^-$, 209 $[M-H-150]^-$. HRMS (ESI): calc for $C_{18}H_{21}N_2O_6$ ($[M + H]^+$): 361.1394; found 361.1387.

Amino acid 1c. From 7.92 g (84.25 mmol) 95% phenol, 0.8 g (10.1 mmol) NaOH (50% w/w in water), 1 g (3.37 mmol) of amine hydrochloride **5c** and 0.5 g (3.37 mmol) of 50% glyoxylic acid. After work-up and precipitation at pH 7.5, amino acid **1c** (800 mg, 63%) was obtained as a colorless solid, and as a 1 : 1 diastereomeric mixture. ^{1}H NMR (D_2O/Na_2CO_3) δ 7.28—7.12 (m, 4H, ArH), 6.94 (d, J = 8.7 Hz, 2H, ArH), 6.85–6.75 (m, 2H, ArH), 4.46 (s, 1/2H, CH), 4.37 (s, 1/2H, CH), 4.11 (s, 1/2H, CH), 4.09 (s, 1/2 H, CH), 3.79 (s, 3H, CH_3O), 2.76–2.67 (m, 4H, $2CH_2$). ^{13}C NMR (D_2O/Na_2CO_3) δ 181.5 (C═O), 179.3 (C═O), 162.0, 160.0, 134.2, 133.9, 132.5, 132.3, 131.4, 131.1, 125.5, 119.8, 119.0, 116.8, (ArC), 69.1 (CH), 67.1 (CH), 66.9 (CH), 58.0 (CH_3O), 47.9 (CH_2), 47.7 (CH_2), 47.3 (CH_2), 47.1 (CH_2). IR (KBr) ν 3427–2400 (broad) 1616, 1256, 1032, 832, 800, 755 cm^{-1}. ESI-MS *m/z* 373 $[M-H]^-$, 329 $[M-H-44]^-$, 285 $[M-H-88]^-$. HRMS (ESI): calc for $C_{19}H_{23}N_2O_6$ ($[M + H]^+$): 375.1551; found 375.1560.

Amino acid 1d. From 4.9 g (52 mmol) 95% phenol, 0.7 g (8.36 mmol) NaOH (50% w/w in water), 0.65 g (2.09 mmol) of amine hydrochloride **5d** and 0.3 g (2.09 mmol) of 50% glyoxylic acid. After work-up and precipitation at pH 4, amino acid **1d** (350 mg, 42%) was obtained as a brown solid and as a 1 : 1 diastereomeric mixture. ^{1}H NMR (D_2O/Na_2CO_3) δ 7.67 (d, J = 7.6 Hz, 2H, ArH), 7.23 (d, J = 7.21 Hz, 2H, ArH), 7.07–6.97 (m, 2H, ArH), 6.66–6.61 (m, 2H, ArH), 4.33 (s, 1/2H, CH), 4.27 (s, 1/2H, CH), 4.03 (s, 1/2H, CH), 4.01 (s, 1/2H, CH), 2.69–2.48 (m, 4H, $2CH_2$). ^{13}C NMR (D_2O/Na_2CO_3) δ 179.5 (C═O), 179.3 (C═O), 177.3 (C═O), 175.8 (C═O), 159.0, 158.9, 143.0, 142.8, 135.83, 135.81, 130.1, 130.0, 129.6, 127.6, 127.5, 123.7, 118.7, 118.6, 117.74, 117.72 (ArC), 67.55 (CH), 67.49 (CH), 64.6 (CH), 64.5 (CH), 45.7 (CH_2), 45.6 (CH_2), 45.3 (CH_2), 45.0 (CH_2). IR (KBr) ν 3441–2400 (broad), 1587 (C═O), 1383 (C═O), 750, 708 cm^{-1}. ESI-MS *m/z* 387 $[M-H]^-$, 342 $[M-H-45]^-$. HRMS (ESI): calc for $C_{19}H_{21}N_2O_7$ ($[M + H]^+$): 389.1343; found 389.1347.

Complexing ability determination

Ligand solutions 1 × 10^{-4} M were prepared by dissolving the appropriate amount of NaOH (calculated to be three times the molar amount of the ligand), and the pH was fixed at 6, with the exception of **1d** (for which the pH was fixed at 4.5), by addition of 2mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid]. Ionic strength was adjusted to 0.1 M by addition of NaCl. The experimental solution was photometrically titrated at 25.0 ± 0.5 °C during the addition of 4.58 × 10^{-4}M Fe(III) standard solution under N_2 atmosphere, until the absorbance at 480 nm presented no changes. Finally, the purity of the chelating agent was calculated following the mathematical procedure previously described.[10] Purity data are expressed as % of the free acid. Other ions and hydration water are not considered in the calculations.

Electrochemical measurements

Cyclic voltammetric experiments were performed in water at room temperature in 0.1 M phosphate buffer in solutions of the corresponding ligand **1a–d**, **2** (10^{-2} M) and $FeCl_3 \cdot 6\ H_2O$ (10^{-3} M) at the different pH values. A Metrohm 6.084.010 glassy carbon electrode (GCE) was used as the working electrode. A BAS MF 2063 Ag/AgCl 3 M reference and a Pt wire counter

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electrode were employed. All voltammetric measurements were carried out using a PGSTAT 12 potentiostat from Autolab. The electrochemical software was the General Purpose Electrochemical System (GPES) (EcoChemie B.V.).

Computational details

All the calculations reported in this paper were obtained with the GAUSSIAN 09 suite of programs.[17] They were performed using the three-parameter exchange functional of Becke in conjunction with the gradient corrected correlation functional of Lee, Yang, and Parr[23] in its unrestricted formulation (uB3LYP) using the double-ζ valence plus polarization basis set def2-SVP[24] for all atoms. This protocol is designated uB3LYP/def2-SVP. Zero point vibrational energy (ZPVE) corrections have been computed at the same level (uB3LYP/def2-SVP) and have not been corrected. Stationary points were characterized by frequency calculations,[25] and have positive defined Hessian matrices indicating that all complexes are minima on the potential energy surface. Wiberg-bond orders have been computed using the Natural Bond Order (NBO)[26] method. Cartesian coordinates (in Å) and total energies (in au, noncorrected zero-point vibrational energies included) of all the complexes discussed in the text are included as supplementary material.†

Ferric chelate reductase (FCR) essays

The solutions of the iron chelates were prepared by dissolving the corresponding ligand, with NaOH (ligand : NaOH, 1 : 3 molar ratio) followed by addition of a solution of $FeCl_3 \cdot 6H_2O$ (Merck) (5% iron excess of the molar amount of the ligand in a pH stat system).[30] In these studies, cucumber plants were used since they are efficient and induce the FCR when iron is limited. Cucumber seeds (*Cucumis sativus* L. cv. Ashley) were germinated on standard seed germination papers moistened with macronutrient solution in diffuse light in a growth chamber for 7 days. Uniform seedlings were selected and stems of two individual plants were wrapped together with polyurethane foam, and placed in a 12 L polypropylene bucket (12 pair of plants per bucket) containing a continuously aerated EDTA buffered nutrient solution with the following composition: macronutrients (mM): 1.0 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, 0.1 KH_2PO_4; cationic micronutrients (μM): 5.0 EDTA/Fe^{3+}, 2.5 $MnSO_4$, 1.0 $CuSO_4$, 10 $ZnSO_4$, 1.0 $CoSO_4$, 1.0 $NiCl_2$, 115.5 $EDTANa_2$; anionic micronutrients (μM): 35 NaCl, 10 H_3BO_3, 0.05 Na_2MoO_4; 0.1 mM HEPES and 1g L^{-1} of $CaCO_3$ to buffer pH at 7.5 to simulate conditions in a calcareous soil. Plants were grown for 14 days in this nutrient solution in a Dycometal type CCK growth chamber provided with fluorescent and sodium vapour lamps with a 16 h/30 °C and 50% humidity day and 8 h/25 °C and 70% humidity night regime. Water was added every 2 days and the nutrient solution was renewed every 7 days. The amount of iron added (5 μM) was found as the most adequate to produce green cucumber plants but with a high FCR activity (stressed plants) in an assay with similar experimental conditions.[27] For the measurement of FCR activity, 300 mL beakers, wrapped up with tin foil to avoid light exposure, were placed in the growth chamber. Each beaker contained 200 mL of reduction assay solution consisting of macronutrient solution as in the growth period, 100 μM Fe chelates (Fe(III)-*o*,*o*-EDDHA **I**, **IIIa**, **IIIb** and **IIIc** in the first experiment and Fe(III)-*o*,*o*-EDDHA **I**, **IIIb**, **IIId** and **IV** in the second), 2 mM MES to buffer the pH at 6 and 300 μM Na_2BPDS as Fe(II) trapping and colorimetric reagent. Each solution was continuously aerated. The roots of 21 day old plants were washed three times in macronutrient solution containing 37.5 μM Na_2BPDS before each individual pair of plants were transferred to one beaker. Aliquots of 3 mL were withdrawn at 0, 10, 20, and 60 min after transfer for absorbance measurement. Six replicates were prepared for each treatment and also two replicate blanks per chelate, consisting of solutions without plants, were used. $Fe(II)(BPDS)_3$ concentration was calculated after the determination of the absorbances at 535 nm (maximum absorbance of the $Fe(II)(BPDS)_3$) and at 480 nm (near the maximum absorbance of Fe(III)-*o*,*o*-EDDHA **I**, **IIIa–d** and **IV**).

The slope of the plots of Fe(II) (μmol g^{-1} fresh root) produced *versus* time (h) was used as the Fe(II) reduction rate for each pair of plants. Data were expressed as the mean reduction rate respect the reduction observed for Fe(III)-*o*,*o*-EDDHA **I** in order to normalize the data obtained from both experiments and the standard error, corresponding to six plant replications for each treatment and experiment. Data were processed using the SPSS 16.0 statistical package. Duncan's Multiple Range Test ($p > 0.1$) was used to test for differences among means.

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Notes and references

1 (*a*) H. F. Bienfait, *J. Plant Nutr.*, 1988, **11**, 605; (*b*) V. D. Jolley and J. C. Brown, *J. Plant Nutr.*, 1989, **12**, 423; (*c*) D. Staiger, *Angew. Chem., Int. Ed.*, 2002, **41**, 2259; (*d*) J. Morrissey and M. L. Guerinot, *Chem. Rev.*, 2009, **109**, 4553.
2 (*a*) N. J. Robinson, C. M. Procter, E. L. Connolly and M. L. Guerinot, *Nature*, 1999, **397**, 694; (*b*) S. Tandy, K. Bossart, R. Mueller, J. Ritschel, L. Hauser, R. Schulin and B. Nowack, *Environ. Sci. Technol.*, 2004, **38**, 937; (*c*) J. M. Ma, *Crit. Rev. Plant Sci.*, 2005, **24**, 267.
3 E. L. Connolly, M. L. Guerinot in *Plasma membrane redox systems and their role in biological stress and disease*, ed. H. Asard, A. Berczi and R. J. Caubergs, Kluwer Academic, Dordrecht, 1998, p. 179.
4 (*a*) D. Eide, M. Broderius, J. Fett and M. L. Guerinot, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 5624; (*b*) T. Fox and M. L. Guerinot, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1998, **49**, 669; (*c*) G. Vert, J.-F. Briat and C. Curie, *Plant J.*, 2001, **26**, 181; (*d*) M. Séguéla, J-F. Briat, G. Vert, M. Barberon, E. Zelany and C. Curie, *Planta*, 2009, **229**, 1171; (*e*) G. Vert, N. Grotz, F. Dédaldéchamp, F. Gaymard, M. L. Guerinot, J-F. Briat and C. Curie, *Plant Cell*, 2002, **14**, 1223.
5 R. L. Chaney, *J. Plant Nutr.*, 1984, **7**, 47.
6 (*a*) K. Mengel, E. A. Kirby, H. Kosegarten, T. Appel, Iron, in *Principles of Plant Nutrition*, Kluwer Academic Pubs, Dordrecht, 2001; (*b*) Y. Chen and P. Barak, *Adv. Agron.*, 1982, **35**, 217.
7 M. Gómez Gallego, D. Pellico, P. Ramírez López, M. J. Mancheño, S. Romano, M. C. de la Torre and M. A. Sierra, *Chem.–Eur. J.*, 2005, **11**, 5997.

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Downloaded by State University of New York at Albany on 01 March 2012

8 M. Gómez Gallego, I. Fernández, D. Pellico, A. Gutiérrez, M. A. Sierra and J. J. Lucena, *Inorg. Chem.*, 2006, **45**, 5321.

9 J. L. Pierre, M. Fontecave and R. R. Crichton, *BioMetals*, 2002, **15**, 341.

10 F. Yunta, S. García-Marco, J. J. Lucena, M. Gómez-Gallego, R. Alcázar and M. A. Sierra, *Inorg. Chem.*, 2003, **42**, 5412. A referee pointed out the possible formation of 2 : 1 Fe(III) complexes from non-symmetrical ligands having different chelating abilities in both moieties (such as compounds **1** and **2**). However, the formation of 2 : 1 (Fe(III) complexes in solution would not be compatible with the observed absorptivities shown in Table 1. Additionally the ESI-MS analysis of the Fe(III) complex derived from ligands **1a**, **1b** and **2** show neither MS peaks suggesting the presence of 2 : 1 complexes nor multicharged peaks. For example, the ESI-MS analysis (negative mode) of the Fe(III) complex derived from **1a** and **1b** only reveals peaks corresponding to $[M - H]^-$ and $[M + Cl]^-$. Moreover the only detected peaks in the case of the Fe(III) complex derived from ligand **2** (scan ranging from 150.00 *m*/*z* to 1000.00 *m*/*z*, positive mode) were the $[M + H]^+$ (the most intense peak) and with much lower intensity, the $[M + Na]^+$ and $[M + K]^+$ (see supporting information†).

11 This model was in agreement with the high reduction rates observed when the chelate Fe(III)-*o*,*p*-EDDHA was used as substrate of the reductasein cucumber plants. See: S. García-Marco, N. Martínez, F. Yunta, L. Hernández-Apaolaza and J. J. Lucena, *Plant Soil*, 2006, **279**, 31.

12 (*a*) S. López-Rayo, D. Hernández, J. J. Lucena, R. Escudero, M. Gómez-Gallego and M. A. Sierra, *J. Agric. Food Chem.*, 2010, **58**, 7908; (*b*) M. A. Sierra, M. G. Gallego, R. M. Escudero, J. J. Lucena, S. García-Marco, *PCT Int. Appl.* (2008), WO 200877897 Al 20080703; *EP* 1939157 Al 20080702.

13 For example, seven and six-coordinate structures are known for the *aquo* complexes of Fe(III)-EDTA (ethylenediaminetetraacetic acid), Fe(III)-tmdta (trimethylenediaminetetraacetic acid) and Fe(III)-EDDDA (ethylenediamine-*N*,*N*-diacetic-*N*,*N*′-dipropionic acid). See: (*a*) J. Maigut, R. Meier, A. Zahl and R. van Eldik, *J. Am. Chem. Soc.*, 2008, **130**, 14556 , and references therein (*b*) T. Schnepensieper, S. Seibig, A. Zahl, P. Tregloan and R. van Eldik, *Inorg. Chem.*, 2001, **40**, 3670; (*c*) R. Meier and F. Heinemann, *Inorg. Chim. Acta*, 2002, **337**, 317; (*d*) H. Sakane, I. Watanabe, K. Ono, S. Ikeda, S. Kaizaki and Y. Kushi, *Inorg. Chim. Acta*, 1990, **178**, 67; (*e*) T. Mizuta, J. Wang and K. Miyoshi, *Inorg. Chim. Acta*, 1990, **175**, 121.

14 This is in agreement with the fact that compound **IIIb** is neutral up to pH 6.3; see F. Yunta, S. García-Marco and J. J. Lucena, *J. Agric. Food Chem.*, 2003, **51**, 5391.

15 (*a*) M. S. Sigman, P. Vachal and E. N. Jacobsen, *Angew. Chem., Int. Ed.*, 2000, **39**, 1279; (*b*) H. Groger, *Chem. Rev.*, 2003, **103**, 2795; (*c*) M. S. Taylor and E. N. Jacobsen, *Angew. Chem., Int. Ed.*, 2006, **45**, 1520.

16 M. Gómez-Gallego, M. A. Sierra, R. Alcázar, P. Ramírez, C. Piñar, M. J. Mancheño, S. García-Marco, F. Yunta and J. J. Lucena, *J. Agric. Food Chem.*, 2002, **50**, 6395.

17 *Gaussian 09, Revision B.01*, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, Gaussian, Inc., Wallingford CT, 2009

18 Most of the available data correspond to heptacoordinated high spin Fe (III) amino carboxylate *aquo* complexes, see: (*a*) X. Solans, M. Font-Altaba and J. García-Oricain, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 1984, **40**, 635; (*b*) X. Solans, M. Font-Altaba and J. García-Oricain, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 1985, **41**, 525; (*c*) J. M. López-Alcalá, M. C. Puerta-Vizcaino, F. González-Vilchez, E. N. F. Duesler and R. E Tapscott, *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 1984, **40**, 939; (*d*) R. Meier and F. W. Heinemann, *Inorg. Chim. Acta*, 2002, **337**, 317; (*e*) S. Seibig and R. van Eldik, *Inorg. Chim. Acta*, 1998, **279**, 37; (*f*) S. Poussereau, G. Blondin, G. Chottard, J. Guilhem, L. Tchertanov, E. Riviere and J-J. Gired, *Eur. J. Inorg. Chem.*, 2001, 1057.

19 This species should be present in solution up to pH 10 as above this pH value the reduction potential increases.

20 P. R. Moog and W. Bruggermann, *Plant Soil*, 1994, **165**, 241.

21 The topology of the FCR has been obtained from FRO2, a ferric chelate reductase from *Arabidopsis thaliana*. See: (*a*) U. Schagerlöf, G. Wilson, H. Herbert, S. Al-Karadaghi and C. Hägerhäll, *Plant Mol. Biol.*, 2006, **62**, 215; (*b*) J. Jeong and E. L. Connolly, *Plant Sci.*, 2009, **176**, 709.

22 The structural homology within the FCR and the flavocytochrome *b* family suggests that the main features of their functional mechanisms are conserved. See: S. K. Chapman, S. A. White and G. A. Reid, *Adv. Inorg. Chem.*, 1991, **36**, 257.

23 (*a*) A. D. Becke, *J. Chem. Phys.*, 1993, **98**, 5648; (*b*) C. Lee, W. Yang and R. G. Parr, *Phys. Rev. B*, 1998, **37**, 785; (*c*) S. H. Vosko, L. Wilk and M. Nusair, *Can. J. Phys.*, 1980, **58**, 1200.

24 F. Weigend and R. Ahlrichs, *Phys. Chem. Chem. Phys.*, 2005, **7**, 3297.

25 J. W. McIver and A. K. Komornicki, *J. Am. Chem. Soc.*, 1972, **94**, 2625.

26 (*a*) J. P. Foster and F. Weinhold, *J. Am. Chem. Soc.*, 1980, **102**, 7211; (*b*) A. E. Reed and F. J. Weinhold,, *J. Chem. Phys.*, 1985, **83**, 1736; (*c*) A. E. Reed, R. B. Weinstock and F. Weinhold, *J. Chem. Phys.*, 1985, **83**, 735; (*d*) A. E. Reed, L. A. Curtiss and F. Weinhold, *Chem. Rev.*, 1988, **88**, 899.

27 J. J. Lucena and R. Chaney, *J. Plant Nutr.*, 2006, **29**, 423.