TRENPYPOLS: A New Water-Soluble Iron Chelator (both Fe^{III} and Fe^{II}) Involving Six-Membered Coordination Rings

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An eight-step synthesis of a new water-soluble tripodal ligand TRENPYPOLS (tris[2-(2-hydroxy-3-(2-pyridinyl)-5-sulfobenzamido)ethyl]amine) is described. TRENPYPOLS is built by connecting the TREN spacer through a tris-amide moiety to the *ortho* phenolic position of three 2-(2-hydroxyphenyl)pyridine chelating subunits, (followed by the sulfonation of the 5-positions). The three [O,N_{pyr}] bidentate subunits of TRENPYPOLS involve six-membered coordination rings. The solution coordination chemistry of the ligand and its iron complexes have been studied by means of pH potentiometric, spectrophotometric and voltammetric methods. The stability constants, log β_{110}, are 30.1 for the ferric complex and 17.7 for the ferrous complex (pFe^{III} and pFe^{II} values are 23.6 and 11.2 respectively at pH = 7.4, $[L]_{tot} = 10^{-5}$ M, $[Fe]_{tot} = 10^{-6}$ M). Comparison with the data of O-TRENSOX (8-hydroxyquinoline subunits with a 5-membered chelate ring) corroborates the usual rules concerning the influence of the chelate ring size on complexation ability. Fe^{III}-TRENPYPOLS has been tested as a single source of iron in nutritional experiments with *Arabidopsis thaliana* plant cells. The efficiency towards growth and resistance to iron chlorosis is good, comparable to that of Fe^{III}-EDTA, the most commonly used complex for in vitro cell cultures.

Introduction

Siderophores are iron-chelating agents that are excreted by microorganisms. They render iron soluble in the environment and favour the uptake of this metal.[1,2] Iron chelation, by some natural or abiotic chelators, can be used in the study of human diseases characterized by iron overload. Water-soluble iron complexes can be used to alleviate iron deficiency in plants, preventing and even reversing iron chlorosis. Natural siderophores, and most of their synthetic models, contain three catecholate or hydroxamate groups (hard donor groups) which bind the hard ferric ions, giving stable Fe^{III} octahedral complexes (Figure 1). As there has been an intense interest in the complexation of both ferrous and ferric iron for chelation therapy (Fe^{II} is hazardous and redox recycling of iron may occur in the cells[3]), for plant nutrition, and also for the development of tools for the study of iron metabolism, we have developed a promising water-soluble chelator O-TRENSOX (Figure 1).[4−7] This ligand was synthesized by connecting the TREN spacer through a tris-amide moiety to the 7 position of 8-hydroxyquinoline-5-sulfonic acid, a bidentate ligand containing both a hard phenolate oxygen and a softer pyridine nitrogen as donor groups. O-TRENSOX exhibits strong complexing ability towards the ferric cation, of the same order of magnitude as the triscatecholate (TRENCAMS)[8] and the trishydroxamate (TRENDROX)[9] analogs (Figure 1). Moreover, O-TRENSOX exhibits strong complexing ability towards the ferrous cation: as expected, [O,N_{pyr}] instead of [O,O] donor sets may allow strong complexation of both Fe^{3+} and Fe^{2+} ions. The pFe^{III} values[10] (calculated for $[Fe^{III}] = 1$ μM, [L] = 10μM at pH =7.4) shown in Figure 1 provide a direct comparison of the affinities of the ligands for Fe^{3+}. It must be emphasized that all the bidentate subunits of the usual iron chelators (and particularly those depicted in Figure 1) involve 5-membered chelate rings but few subunits involve 6- membered chelate rings. Several 1,3,5-tris(5-substituted salicylamino)cyclohexanes, which involve the unusual 6-membered coordination ring, have been synthesized and their Fe^{III} complexes structurally characterized, but no thermodynamic or kinetic data have been reported.[11]

The new water soluble ligand TRENPYPOLS incorporates three [O,N_{pyr}] bidentate subunits involving six-membered coordination rings. The solution coordination chemistry of the ligand and its iron complexes have been studied by means of pH potentiometric, spectrophotometric and voltammetric methods. The complexing ability of TRENPYPOLS has been compared to ligands involving 5-membered chelate ring. TRENPYPOLS has been tested as a single source of iron in nutritional experiments with *Arabidopsis thaliana* plant cells.

We have synthesized the first synthetic water-soluble tris bidentate iron chelator where the [O,N_{pyr}] type chelating units have been retained and incorporate a six-membered chelate ring. The so-called TRENPYPOLS (the full name of the

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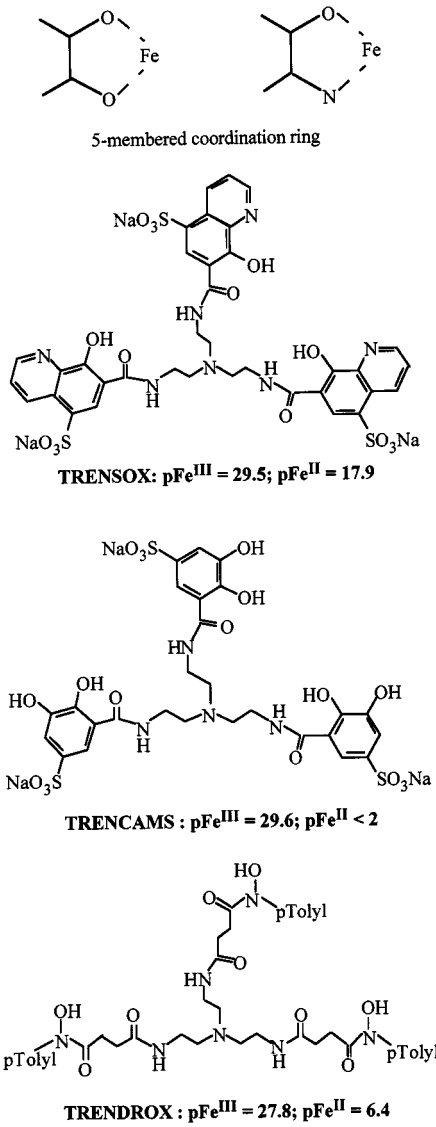

Figure 1. Iron chelators with 5-membered coordination rings

acidic form is tris[2-(2-hydroxy-3-(2-pyridinyl)-5-sulfobenzamido)ethyl]amine) is depicted in Figure 2. As in the chelators given in Figure 1, it is built by connecting the TREN spacer through a tris-amide moiety to the chelating subunits.Only a few studies of the coordination chemistry of the 2-(2-hydroxyphenyl)pyridine ligand have been reported, probably because they are difficult to prepare. Pd^{II}, Co^{III}, Ru^{II}, Ru^{III} and Cr^{III} mononuclear complexes have been described.[12−14] This ligand may be considered as an hydrolytically stable analog of salicylaldimines. It was therefore of great interest to synthesize a tripodal ligand involving three bidentate subunits of this type. This had never been realized before this work. It must be emphasized that in previous studies of the octahedral coordination involving three independent bidentate 2-(2-hydroxyphenyl)pyridine ligands, the three oxygen and the three nitrogen atoms of the coordination sphere are respectively in a *mer* arrangement, as shown in the crystal structures.[12,13] In the complex of a tripodal ligand such as TRENPYPOLS, these atoms are forced

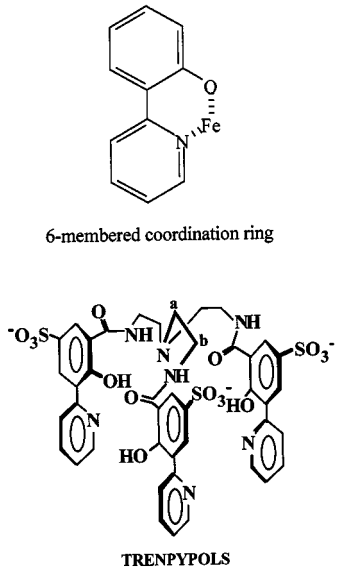

Figure 2. Structural formula of TRENPYPOLS

to be *fac*. In this paper, we report the synthesis, the iron binding properties (thermodynamic and spectroscopic studies) and the nutritional behavior for plant cells of this ligand.

Results and Discussion

Synthesis

The ligand TRENPYPOLS was prepared by sulfonation (oleum) of TRENPYPOL **9**. The methoxylated podand **8** was obtained by coupling of tris(2-aminoethylamine) (TREN) and acid chloride **7**. The key step in the preparation of **7** was the heteroaromatic coupling[15] of 2-bromopyridine and the Grignard derivative **4** of 2-bromo-6-methyl-anisole **3**. The brominated precursors **2** and **3** were prepared by slight modifications of reported procedures (Figure 3).[16,17]

Ligand Deprotonation Constants

The ligand TRENPYPOLS possesses seven deprotonation sites (three pyridinium nitrogen atoms, one tertiary ammonium nitrogen atom and three hydroxy oxygen atoms) and is denoted LH_7^+, taking into account the negative charges of the three sulfonate groups. The deprotonation constants, pK_{an}, of TRENPYPOLS were determined by potentiometric titrations of the fully protonated ligand. Analysis of the potentiometric titration curve (Figure 4a) by the SUPERQUAD program[18] yielded the pK_{an} values (σ_{fit} = 4.8) defined by Equation (1) and Equation (2) (charges omitted for clarity):

$$LH_n \rightleftarrows LH_{n-1} + H^+ \quad (1)$$

$$K_{an} = [LH_{n-1}][H^+]/[LH_n] \quad (2)$$

pK_{a1} = 10.32 ± 0.03, pK_{a2} = 10.07 ± 0.04, pK_{a3} = 9.23 ± 0.06,
pK_{a4} = 5.25 ± 0.08, pK_{a5} = 4.38 ± 0.08, pK_{a6} = 3.21 ± 0.09 and pK_{a7} = 2.77 ± 0.12

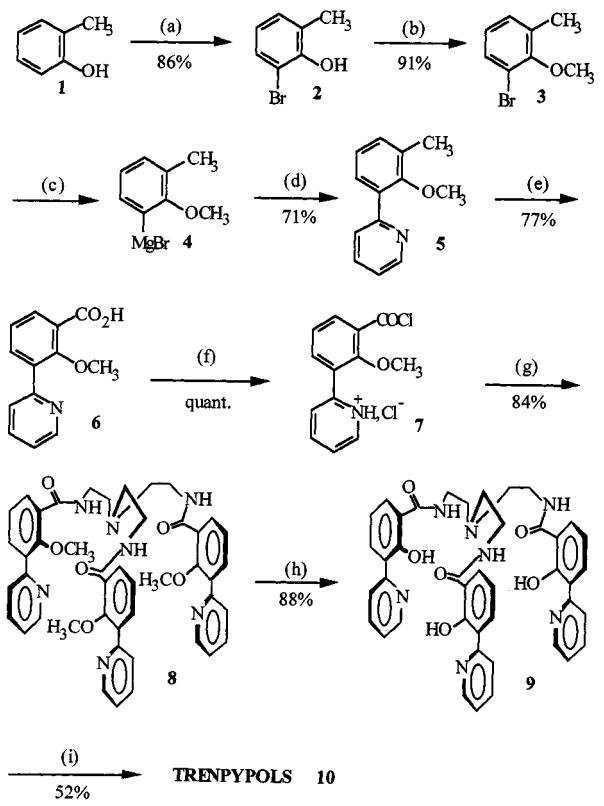

Figure 3. Synthesis of TRENPYPOLS: (a) Br_2, tBuNH$_2$, toluene, −78 °C; (b) $(CH_3)_2SO_4$, K_2CO_3, acetone reflux; (c) Mg, THF, reflux; (d) 2-bromopyridine, Ni(dppe)Cl_2 cat., THF; (e) $KMnO_4$, $NaHCO_3$, H_2O then HCl 4 N; (f) $SOCl_2$; (g) TREN, Et_3N, THF, 25 °C; (h) BBr_3, CH_2Cl_2, 0 °C; (i) H_2SO_4, oleum, 25 °C

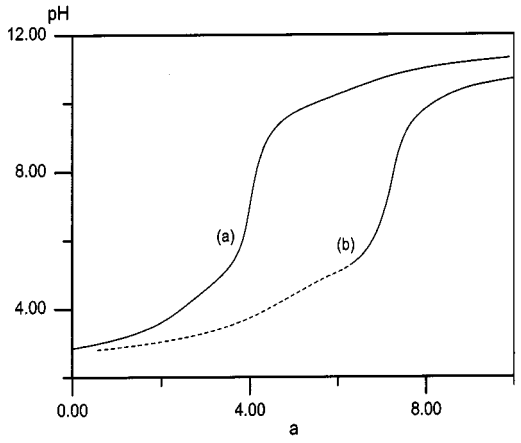

Figure 4. Potentiometric titration curves for (a) 0.5 mM ligand TRENPYPOLS; (b) TRENPYPOLS + Fe^{III} 1:1 0.5 mM (the dashed line indicates precipitation of the complex); a = mol of base added per mol of ligand. All solutions were at 25 °C and ionic strength I = 0.1 M ($NaClO_4$)

The pK_a of the tertiary ammonium nitrogen could be assigned from an ^{1}H NMR spectroscopic titration, by measuring the chemical shifts of the methylene protons H_a and H_b (see assignments in Figure 2), since they undergo a significant shift related to the deprotonation of this nitrogen. An upfield shift, as expected for a deprotonation, was observed over the pD range 4 to 6 (Figure 5) with an inflection

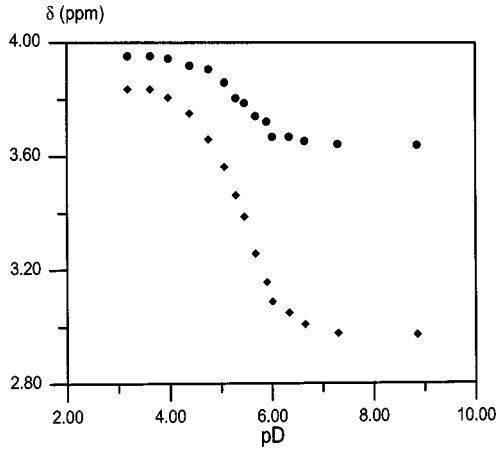

Figure 5. ^{1}H NMR chemical shifts δ (ppm) for the methylene protons H_a (◆) and H_b (●) of TRENPYPOLS in D_2O as a function of pD

point at pD 5 indicating that the value of 5.25 determined from potentiometric titration can be assigned to the tertiary amine. However, it should be noted that the pK_a values which are determined potentiometrically are macroconstants, they cannot be directly assigned to a single functional group when the pK$_a$s differ by less than 2 log units. Thus pK_{a4} is partly produced by ionization of the pyridinium nitrogens. The three lower pK_as corresponded to the pyridinium nitrogens and the three higher ones to the hydroxy groups. The range over which the determined stepwise deprotonation constants for the pyridinium nitrogens (2.77, 3.21 and 4.38) and the hydroxy groups (9.23, 10.07 and 10.32) occurs, is slightly different from the statistical factor of log 3 (0.48). This indicates that there is some cooperativity between the three arms of the tripod.

The average deprotonation constants p$K_{av(5-7)}$ for the pyridinium nitrogens is 3.4. The average deprotonation constants p$K_{av(1-3)}$ for the hydroxy groups is 9.87. In comparison with the pK_a of phenol (9.9), one would expect that the pK_a for the hydroxy groups should be less than that of phenols by about one log unit owing to the electron-withdrawing effect of the amide and sulfonate substituents. The high values for the hydroxy pK_as can be ascribed to the formation of an intramolecular hydrogen bond with the pyridyl nitrogen in each arm, which favors the protonated form and thus enhances the pK_a.

It is interesting to compare the pK_a value of 5.25 of the tertiary ammonium nitrogen with those of similar ligands based on the TREN backbone connected to the amide group and containing three bidentate units, such as 8-hydroxyquinoline (O-TRENSOX), pK_a = 6.36[7] ; 2,2′-dihydroxybiphenyl (TBPAS), pK_a = 7.1;[19] catechol (TRENCAMS), pK_a = 8.03[8]; methoxycatechol (TREN(ME)SAM), pK_a = 6.3;[20] and phenol (TRENSAM), pK_a = 8.9.[20] It has been observed that the basicity of the tertiary amine is related to its ability to form a hydrogen bond with the amide hydrogen depending on the conformation of the amide group and on the hydrogen bond networks available in the molecule. Two hydrogen bond networks may be envisaged according to Figure 6: (i) the tertiary amine deprotonates first (scheme 1) as in O-TRENSOX, TREN(ME)SAM and TRENPYPOLS, (ii) the o-hydroxy

deprotonates first as in TRENCAMS and TRENSAM (scheme 2). Scheme 1 appears more favorable for TRENPYPOLS since the hydroxy group can establish H-bonds with both the carbonyl oxygen and the second donor atom (N) leading to one five-membered ring and to two six-membered rings.

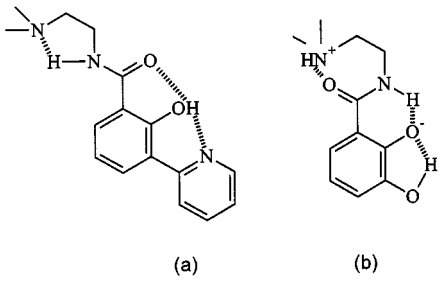

Figure 6. Deprotonation schemes of TRENPYPOLS (scheme 1) and TRENCAMS (scheme 2) showing the hydrogen bond networks

Stability Constants of the Ferric Complex

Potentiometric titrations of 1:1 solutions of ferric ion and ligand were carried out over the pH range 2.5 to 10 (Figure 4b). The titration curve has a buffer region from a (mol base per mol ligand) = 5−7 which covers a pH range of 4−5.5, indicating the formation of the FeL complex from about pH 6. However, the precipitation of the complex below pH 5 precluded the use of the titration data in the refinement program.

The spectrophotometric titration of the ferric complexes at a 1:1 metal-to-ligand molar ratio was carried out from pH 1 to 10 in order to study the Fe^{III}-TRENPYPOLS complexation equilibria as a function of pH. In addition, the equilibrium constant β_{110} was determined by spectrophotometric competition experiments. The spectra are shown in Figure 7. An increase in the pH from 1 to 3 resulted in the appearance of a charge-transfer band at λ_{max} = 470 nm (Figure 7a). The absorbance data were processed with the LETAGROP-SPEFO program.[21,22] The best fit ($\Sigma(A_{exp} - A_{calc})^2 = 2 \times 10^{-4}$) was obtained by considering the formation of the $[FeLH_5]^{2+}$ species. The values log K_{FeLH5} = 1.79 ± 0.05 and ε_{max} = 3900 M^{-1} cm^{-1} (λ_{max} = 470 nm) were obtained, where K_{FeLH5} is defined by Equation (3) and Equation (4):

$$Fe^{3+} + LH_7^+ \rightleftarrows FeLH_5^{2+} + 2\,H^+ \quad (3)$$

$$K_{FeLH5} = [FeLH_5^{2+}]\,[H^+]^2/[Fe^{3+}]\,[LH_7^+] \quad (4)$$

When the pH is raised beyond 3, the spectra exhibited one buffer region in the pH range 3.2−6 with an isosbestic point (Figure 7b), indicating the presence of only two species with different absorbance. These absorbance data were also refined with the LETAGROP-SPEFO program. The best fit [$\Sigma(A_{exp} - A_{calcd.})^2 = 3 \times 10^{-5}$] showed that deprotonation occurs via a one-proton step yielding a pK_a value of 4.50 ± 0.05 and an absorbance maximum at λ = 495 nm with ε = 2500 M^{-1} cm^{-1} for the $FeLH_{n-1}$ species according to the equilibrium shown in Equation (5):

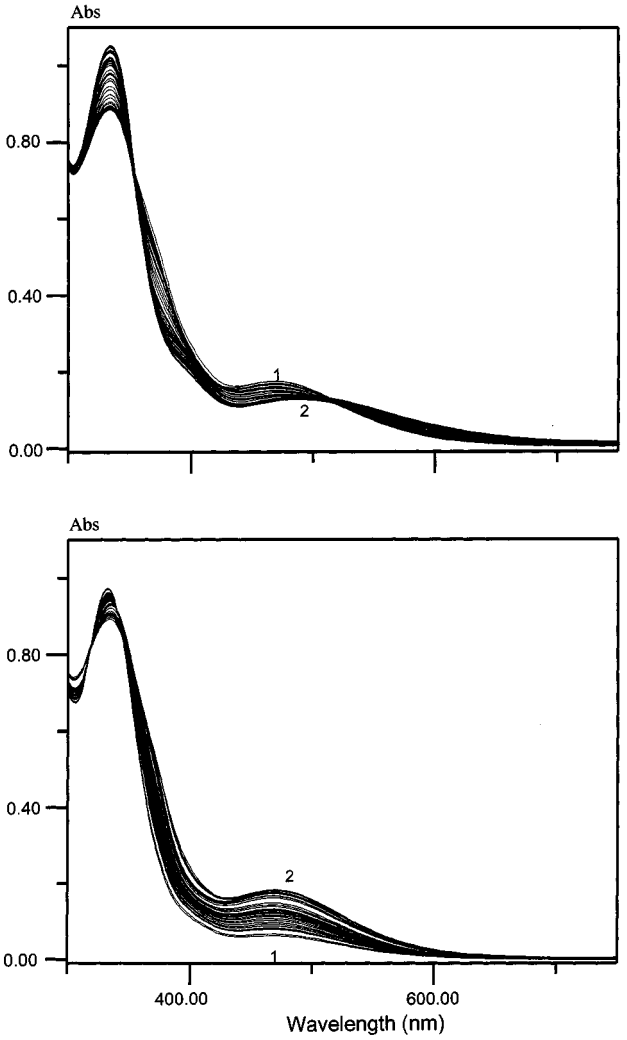

Figure 7. UV/Vis absorbance spectra of Fe^{III} -TRENPYPOLS as a function of pH; (bottom) 1: pH = 1; 2: pH = 2.5; (top) 1: pH = 3.2; 2: pH = 6; $[Fe^{3+}]_{tot}$ = $[TRENPYPOLS]_{tot}$ = 0.24 mM, I = 0.1 M ($NaClO_4$)

$$FeLH_n \rightleftarrows FeLH_{n-1} + H^+ \quad (5)$$

No spectral change was observed over the pH range 6−8.5. Beyond pH 8.5, a slight decrease in absorbance was observed suggesting the formation of hydroxo complexes.

The UV/Vis spectrum of the $[FeLH_5]^{2+}$ species exhibited features (λ_{max} = 470 nm, ε_{max} = 3900 M^{-1} cm^{-1}) that suggest a bis-salicylate coordination, involving the amide oxygen and the hydroxy oxygen atoms, as opposed to the tris-salicylate complex Fe^{III}-TRENSAM (λ_{max} = 455 nm, ε = 4800 M^{-1} cm^{-1}).[20] On raising the pH from 3 to 6, an increase of λ_{max} from 470 to 495 nm with a decrease in the molar extinction coefficient (ε = 2500 M^{-1} cm^{-1}) was observed. These parameters are consistent with a phenolato−Fe^{III} charge-transfer band. This result indicates a shift of the coordination from a salicylate to a phenolate coordination and suggests the replacement of the O(=C) → Fe^{III} bonding mode by the N_{pyr} → Fe^{III} bonding mode.

However, it should be noted that the UV/Vis absorbance dependence on the pH can only give information on the

deprotonation of the phenol group. The deprotonation constants of the $[FeLH_5]^{2+}$ complex corresponding to the ammonium nitrogen and to the three pyridinium nitrogens cannot be determined since these deprotonations do not change the phenolato−Fe^{III} charge-transfer band. It is reasonable to predict that the pK_a values for the pyridinium nitrogens are lower than that of the free ligand (i.e. < about 4). Furthermore, the potentiometric titration clearly showed that there are two deprotonation constants that are close to one another between pH 4.5 and 5. We may attribute one deprotonation to the ammonium nitrogen and the other to the phenol oxygen when compared with the value of 4.5 determined from visible spectrum.

The stability constant β_{110} of the ferric complex FeL was determined by spectrophotometric competition experiments. The constant was determined by competition against EDTA (noted Y) over the pH range 7−8. The competition equilibrium can be expressed by Equation (6) and Equation (7) (charges omitted for clarity):

$$FeL + YH + 2\,H^+ \rightleftarrows FeY + LH_3 \quad (6)$$

$$K = [FeY][LH_3]/[FeL][HY][H^+]^2 = \beta_{110}(FeY)\,K'_{a1}/\beta_{110}(FeL)\,K_{a1}\,K_{a2}\,K_{a3} \quad (7)$$

where L represents the deprotonated form of the ligand TRENPYPOLS; K_{a1}, K_{a2}, K_{a3}, the deprotonation constants of LH_3 and K'_{a1} the deprotonation constant of YH ($10^{-10.2}$).[23] The concentration of FeL was calculated from the absorbance at 500 nm where FeL is the only absorbing species. The concentrations of the other species in Equation (6) were calculated from the pH and mass balance Equations (8)−(10):

$$[Fe]_{tot} = \alpha_{FeL}\,[FeL] + \alpha_{FeY}\,[FeY] \quad (8)$$

$$[L]_{tot} = \alpha_{FeL}\,[FeL] + \alpha_{L}\,[L] \quad (9)$$

$$[Y]_{tot} = \alpha_{FeY}\,[FeY] + \alpha_{Y}\,[Y] \quad (10)$$

where α represents the usual Ringbom′s coefficients.[24] Using the known formation constants of FeY (log β_{110} = 25.0),[23] the average formation constant of the Fe^{III}-TRENPYPOLS complex was determined to be log β_{110} = 30.1 ± 0.15. The pFe^{III} values have been calculated over the pH range 3−9 (or 4−9 for TRENPYPOLS and TRENCAM since the stability constants for their protonated complexes existing at pH<4 are unknown) and reported on the plot pFe = f(pH) presented in Figure 8, together with the plots calculated for other tripodal ligands based on the TREN backbone. In addition, the pFe^{III} value for TRENPYPOLS (under biologically reasonable conditions, i.e. pH = 7.4, $[L]_{tot}$ = 10^{-5} M, $[Fe]_{tot}$ = 10^{-6} M) has been calculated to be 23.6. This value is lower than those for other tripodal ligands such as TRENCAM: 27.8,[25] TRENCAMS: 29.6,[8] TRENDROX: 27.8,[9] O-TRENSOX: 29.5[7] The pFe^{III} values for the complexes involving five-membered chelate rings are significantly higher than the values for the complexes having six-membered chelate rings as in Fe^{III}-TRENPYPOLS. This result is in accordance with a general rule: an increase in the size of the chelate ring usually leads to a decrease in complex stability.[26] However, this rule had never been experimentally confirmed for octahedral complexes with tris-bidentate tripodal ligands.

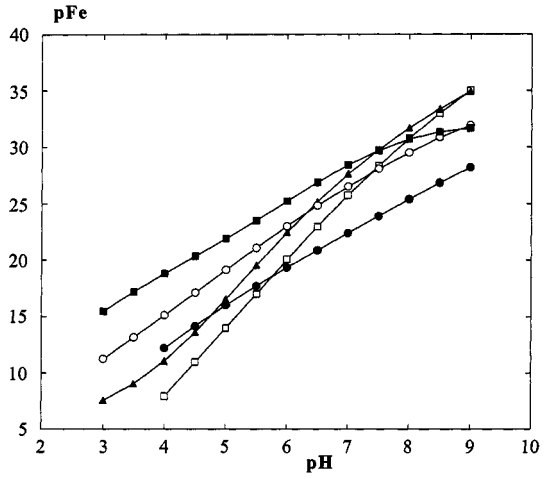

Figure 8. Plot of pFe^{III} *vs* pH for various tripodal ligands. pFe^{III} was calculated for $[L]_{tot}$ = 10^{-5} M, $[Fe^{3+}]_{tot}$ = 10^{-6} M using the known constants of TRENPYPOLS (●), O-TRENSOX[7] (■), TRENCAM[25] (□) TRENCAMS[8] (▲) and TRENDROX[9] (O).

Electron Spin Resonance

The ESR spectra of frozen aqueous solutions (with 10% glycerol) of Fe^{III}-TRENPYPOLS (Figure 9, spectra 1−5) were recorded at 100 K over the pH range 2−8. Below pH 4.8, the spectrum exhibited one single signal at g = 4.3 characteristic of a high-spin octahedral ligand field. The isotropic signal reflects a rhombic symmetry and represents a transition within the intermediate Kramer doublet (for an E/D ratio of 1/3 and a D parameter of about 0.5 cm^{-1} for this type of complex), and is large relative to the quantum of energy used in the EPR experiment. At pH above 5.8, a dissymmetric signal at g = 4.3 was observed indicating a slightly distorted rhombic geometry. The spectra resemble those of the ferric complexes with O-TRENSOX (Figure 9) in acidic (pH = 1.5, spectrum 6) and in neutral media (pH = 7.1, spectrum 7) in which the ferric ion is coordinated through a tris-salicylate mode [O(=C),O)][27] and a tris-oxinate mode $[O,N_{pyr}]^7$, respectively. These results suggest similar change of coordination for Fe^{III}-TRENPYPOLS complexes on raising the pH and are consistent with the change observed in the UV/Vis spectra.

Electrochemistry

The electrochemical behavior of the Fe^{III}-TRENPYPOLS complex, studied by cyclic voltammetry, in an aqueous electrolyte (0.1 M $NaClO_4$) buffered at pH 7 is characterized by a well-defined pair of peaks at $E^c_{1/2}$ = −0.18 V vs Ag/AgCl (0.04 V vs NHE) (ΔE_p = 0.11 V, υ = 0.1 V s^{-1}) corresponding to the quasi-reversible complexed Fe^{II}/Fe^{III} redox couple (Figure 10). Taking into account a value of $E^f_{1/2}$= 0.55 V (i.e. 0.77 V vs NHE) for the free Fe^{II}/Fe^{III} redox couple, this result shows that the complexation with TREN-

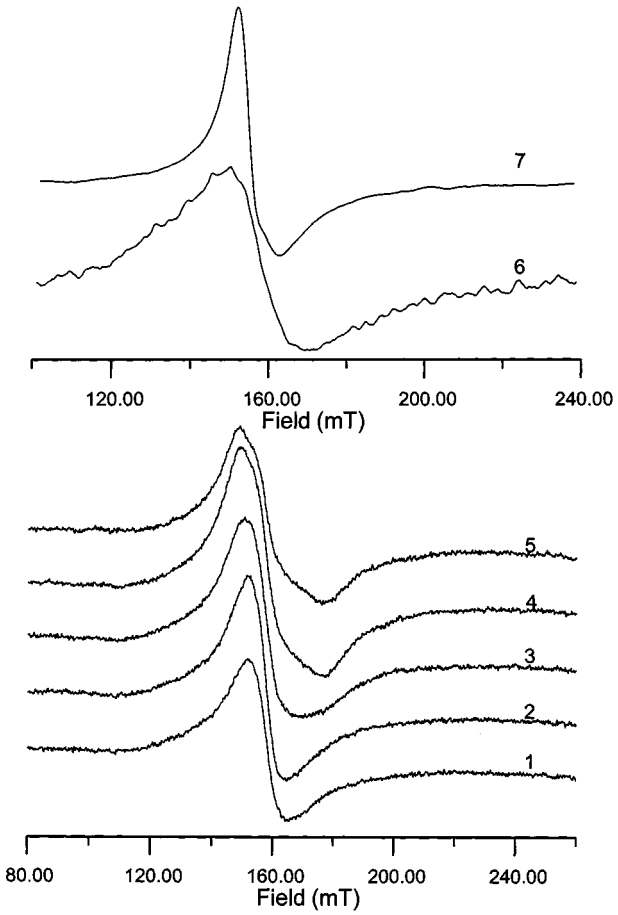

Figure 9. ESR spectra of (bottom) Fe^{III}-TRENPYPOLS (0.001 M) in frozen water-glycerol (90:10) solution at pH 4.0 (1), 4.8 (2), 5.4 (3), 5.8 (4) and 7.0 (5); (top) Fe^{III}-O-TRENSOX (0.001 M) in frozen water-glycerol (90:10) solution a pH 1.5 (6) and 7 (7).

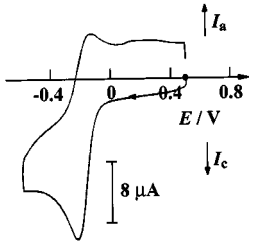

Figure 10. Cyclic voltammogram of Fe^{III}-TRENPYPOLS complex in aqueous solution (1 mM) at pH 7 with 0.1 M $NaClO_4$; v = 0.1 V s^{-1}; working electrode: glassy carbon disc (5 mm diameter).

PYPOLS leads to a strong stabilization by ca 0.73 V of the oxidized form of the redox couple. The ratio $\beta_{110}(Fe^{II}L)/\beta_{110}(Fe^{III}L)$ can be calculated from Equation (11):

$$E^c_{1/2} = E^f_{1/2} + 0.059 \log \beta_{110}(Fe^{II}L)/\beta_{110}(Fe^{III}L) \quad (11)$$

and gives $\beta_{110}(Fe^{II}L) = 17.7$ leading to a pFe^{II} value of 11.2. TRENPYPOLS appears to be a stronger ferrous ion chelator than the catecholate and hydroxamate ligands. This is a characteristic property of the $[O,N_{pyr}]$ donor set as previously observed for the ligand O-TRENSOX ($E^c_{1/2} = 0.087$ V vs NHE).[7] The difference in pFe^{II} between O-TRENSOX and

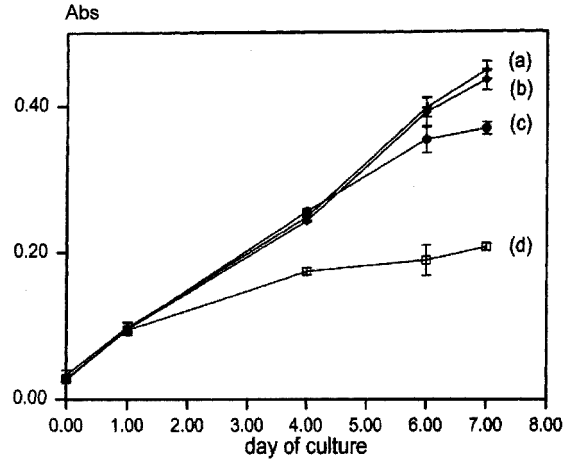

Figure 11. Growth of cells of *Arabidopsis thaliana* using Fe^{III} -TRENPYPOLS or Fe^{III} -EDTA as siderophores. A: 50 μM Fe^{III}-TRENPYPOLS; B: 50 μM Fe^{III}-EDTA; C: 5 μM Fe^{III} -TRENPYPOLS; D: without iron; 25 °C, 220 rpm, 18 hours light per day. The inoculum originated from a previous culture with 2.0 μM Fe^{III} -EDTA presenting slight symptoms of chlorosis.

TRENPYPOLS (6.7) is of the same order of magnitude as the difference between their pFe^{III} (5.9). The electrochemical behavior of the Fe^{III}-TRENPYPOLS complex has also been studied at pH 2.5 where the major species is $[FeLH_5]^{2+}$. Reduction of the complex could not be observed on the voltammograms in the accessible potential range at pH 2.5 i.e. the reduction of the complex occurs at a potential lower than −0.6 V vs Ag/AgCl (−0.38 V vs NHE). The lower reduction potential at pH 2.5 indicates that the Fe^{III} complex is more stable relative to the Fe^{II} complex at pH 2.5 than at pH 7. This suggests a change in coordination of iron as previously deduced from the UV/Vis and EPR spectra.

Plant Nutrition

TRENPYPOLS was tested as single source of iron in nutritional experiments. This was performed by comparing the growth and greening of *Arabidopdis thaliana* plant cells during cell suspension cultures. In the control experiment, iron was provided as a complex Fe^{III} -EDTA (50μM). An iron free control was cultivated in the iron free nutrient solution (MS−Fe). The assays were performed in MS−Fe supplemented with Fe^{III}-TRENPYPOLS at 5 and 50μM using 8 independent replications. The results are shown in Figure 11. Growth and resistance to chlorosis were equivalent when Fe^{III} -TRENPYPOLS was used instead of Fe^{III} -EDTA. Symptoms of chlorosis appeared when 5 μM Fe^{III} -TRENPYPOLS was used and the growth was slowed down. The efficiency of Fe^{III}-TRENPYPOLS is good, comparable to that of Fe^{III}-EDTA, the most commonly used complex for in vitro cell cultures.

Conclusion

The iron-complexing agent TRENPYPOLS is the first water-soluble tris-bidentate ligand involving three $[O,N_{pyr}]$ donor sets with 6-membered chelate rings. The solution physicochemical data (thermodynamic) of this new chelator have

been established, allowing comparisons with the usual iron chelators, with a special focus on O-TRENSOX which involves three [O,N_{pyr}] donor sets with 5-membered coordination rings. TRENPYPOLS and O-TRENSOX form a new class of Fe chelators with an affinity for both Fe^{III} and Fe^{II}. There are two important distinctions in the metal binding cavity between TRENPYPOLS and O-TRENSOX that influence the affinity of these ligands for Fe^{III} and Fe^{II}. The first, is the flexibility of the binding cavity. TRENPYPOLS provides a more flexible cavity by forming six-membered chelate rings and allowing free rotation of the pyridine ring relative to the phenol ring. O-TRENSOX forms five-membered chelate rings and prevents pyridine ring rotation through a fused ring structure. The second, is a difference in ligand pK_a values which results in a lower pFe^{III} and pFe^{II} for TRENPYPOLS. These features influence the relative Fe^{III} and Fe^{II} affinity of the two ligands as determined by their pFe values. Consequently, TRENPYPOLS has a lower Fe^{III} and Fe^{II} affinity than O-TRENSOX, but the same relative Fe^{III}/Fe^{II} selectivity ($\Delta pFe = pFe^{III} - pFe^{II}$), making it more suitable for use as a biological Fe sensor. There is always a need for biological sensors for the study of iron metabolism. They have to be able to measure the labile iron pool and the concentration of cellular free iron.[28] For this purpose, ligands with complexing abilities that are too high must be avoided since they induce perturbation in iron metabolism (uptake of iron from proteins). The pFe^{III} values of TRENPYPOLS are in a suitable range. It is envisaged that a fluorescent probe could be grafted on the ligand in order to build a new sensor, well suited to be used in conjunction with calcein, the most frequently used probe for iron to date.[28]

Experimental Section

Materials and Equipment: Solvents were distilled prior to use. The amine TREN (tris-2-aminoethyl)amine) was distilled over sodium. All other compounds were of reagent grade and were used without further purification. Fe^{III} stock solutions were prepared by dissolving appropriate amounts of ferric perchlorate hydrate (Aldrich) in standardized $HClO_4/NaClO_4$ solutions. The solutions were standardized spectrophotometrically for ferric ion by using a molar extinction coefficient of 4160 M^{-1} cm^{-1} at 240 nm.[29] − IR spectra were collected using a Perkin−Elmer 397 or Nicolet Impact 400 spectrometer. − UV/Vis absorbance spectra were recorded using 1.000 cm path length quartz cells housed in a Perkin−Elmer Lambda 2 spectrometer connected to a microcomputer. − Mass spectra were recorded on a NERMAG R 10 1 C mass spectrometer. − Microanalyses were performed by the Central Service of CNRS, Solaize (France). − Melting points were determined with a Büchi apparatus and are not corrected. − ^1H and ^{13}C NMR spectra were obtained in 5 mm tubes at 25 °C with a Bruker AM 300 or a Bruker AM 400 spectrometer. For ^1H NMR spectroscopic titrations, the ligand TRENPYPOLS was dissolved in D_2O. The pD was adjusted with DCl or NaOD solutions. pH measurements were performed with a Tacussel PHN 850 apparatus equipped with a microelectrode Radiometer XC61. pD values were calculated according to $pD = pH_{meas} + 0.4$.[30] − EPR experiments were conducted using a ESP 300E Bruker apparatus with a variable temperature unit. Spectra were treated by using the winEPR software. Each aqueous Fe^{III}-TRENPYPOLS sample (200 μL, 10^{-3} M) contained 10% glycerol. The field was scanned from 0.1 to 0.5 Tesla.

Synthesis of TRENPYPOLS (Figure 3)

2-(2-Methoxy-3-methylphenyl)pyridine (5): The Grignard reagent **4** was prepared under Argon at 0 °C, from **3** (5.05 g, 25.0 mmol) and Mg (0.65 g, 27.0 mmol) in dry THF (30 mL). It was transferred, *via* canula, to a mixture of 2-bromopyridine (3.79 g, 24.0 mmol) and $Ni(dppe)_2Cl_2$ (0.55 g, 1.00 mmol) in THF (20 mL). The mixture was then stirred overnight at room temperature. After quenching with aqueous NH_4Cl and acidification with HCl (4 M) to pH ≈ 1, THF was evaporated under vacuum. Extraction with CH_2Cl_2 (3 × 100 mL), drying on $MgSO_4$ and evaporation of the solvent gave a brown oil. Column chromatography (silica eluted with a CH_2Cl_2 + MeOH mixture (1% to 5% vol)) afforded pure **5** (3.54 g, 17.7 mmol). Yield 71%. − ^1H NMR (250 MHz, $CDCl_3$): δ = 2.35 (s, 3 H), 3.46 (s, 3 H, OCH_3), 7.12 (t, 1 H, *J* = 7.5 Hz), 7.18−7.26 (m, 2 H), 7.54 (dd, 1 H, *J* = 7.5/1.7 Hz), 7.71 (td, 1 H, *J* = 7.5 Hz, *J* = 0.7 Hz), 7.84 (broad d, 1 H, *J* = 7.5 Hz), 8.70 (ddd, 1 H, *J* = 4.9 Hz, *J* = 1.7 Hz, *J* = 0.7 Hz). − ^{13}C NMR (50 MHz, $CDCl_3$): δ = 16.1 (CH_3), 60.5 (OCH_3), 121.8 (CH), 124.2 (CH), 124.5 (CH), 128.9 (CH), 131.5 (C*q*), 131.6 (CH), 133.3 (C*q*), 136.1 (CH), 149.4 (CH), 156.3 (C*q*), 156.6 (C*q*). − MS (EI): 199 (M^+), 184, 181, 168, 154. − $C_{13}H_{13}NO$ (199.3): calcd. C 78.30, H 6.58, N 7.03; found C 77.90, H 6.57, N 6.91.

2-Methoxy-3-(2-pyridyl)benzoic Acid (6): A mixture of **5** (2 g, 10.0 mmol), $KMnO_4$ (6 g, 38.0 mmol) and $NaHCO_3$ (0.78 g, 9.3 mmol) in water (120 mL) was stirred under reflux for 3 h. MnO_2 was removed by filtration and the solution extracted with diethyl ether. The aqueous solution was acidified to pH ≈ 5 with dilute HCl and the water was evaporated under vacuum. The solid residue was treated by methanol, and inorganic salts were removed by filtration. Evaporation of methanol gave **6** as a white powder (1.77 g, 7.7 mmol), pure enough for the following step. Yield 77%. − ^1H NMR (250 MHz, CD_3OD): δ = 3.58 (s, 3 H, OCH_3), 7.22 (t, 1 H, *J* = 7.7 Hz), 7.39 (td, 1 H, *J* = 5.0 Hz, *J* = 1.6 Hz), 7.56−7.67 (m, 2 H), 7.79−7.90 (m, 2 H), 8.61 (broad d, 1 H, *J* = 4.8 Hz). − ^{13}C NMR (50 MHz, D_2O−NaOD): δ = 62.0 (OCH_3), 123.2 (CH), 124.4 (CH), 125.2 (CH), 128.8 (CH), 131.3 (CH), 133.2 (C*q*), 134.0 (C*q*), 138.0 (CH), 148.7 (CH), 153.5 (C*q*), 155.3 (C*q*), 176.3 (CO). − MS (DCI, NH_3+isobutane): 230 [M^+ + 1].

2-(3-Chloroformyl-2-methoxyphenyl)pyridinium Chloride (7): A mixture of **6** (1 g, 4.4 mmol) and distilled thionyl chloride (50 mL) was stirred under nitrogen for 4 h at room temperature. Excess $SOCl_2$ was removed under vacuum, giving **7** as a yellow powder (1.24 g, quant. yield). − ^1H NMR (200 MHz, $CDCl_3$): δ = 3.72 (s, 3 H, OCH_3), 7.56 (t, 1 H, *J* = 7.9 Hz), 8.07 (t, 1 H, *J* = 7.9 Hz), 8.25−8.35 (m, 3 H), 8.58 (t, 1 H, *J* = 7.6 Hz), 9.05 (d, 1 H, *J* = 5.1 Hz). − ^{13}C NMR (50 MHz, $CDCl_3$): δ = 63.8 (OCH_3), 125.07 (CH), 125.08 (C*q*), 125.5 (C*q*), 125.7 (CH), 128.6 (CH), 137.0 (CH), 137.7 (CH), 142.3 (CH), 145.3 (CH), 148.7 (C*q*), 157.6 (C*q*), 164.4 (CO).

Methoxylated Podand 8: A mixture of TREN (tris-2-aminoethyl)amine) (0.24 g, 16.4 mmol) and triethylamine (2.9 g, 29.0 mmol) in dry THF (10 mL) was added dropwise, under nitrogen, to a solution of acid chloride **7** (1.24 g, 4.4 mmol) in THF (50 mL). The mixture was stirred at room temperature for 12 h. Filtration and evaporation of THF afforded an orange oil which was mixed with CH_2C1_2. The solution was washed with brine then dried on Na_2SO_4. Removal of the solvent under vacuum gave a foamy material which was added to pentane. Tripodand **8** was recovered by filtration as a yellow powder (0.8 g). Yield 70%. M.p. 62−63 °C. − IR (KBr):

$\tilde{\nu}$ = 1650, 1523 cm^{-1} . − ^1H NMR (200 MHz, $CDCl_3$): δ = 2.92 (t, 6 H, *J* = 6.5 Hz, CH_2N), 3.48 (s, 9 H, OCH_3), 3.65 (td, 6 H, *J* = 6.5 Hz, $CH_2-NH-CO$), 7.26 (td, 6 H, *J* = 5.5 Hz, *J* = 3.1 Hz), 7.64−7.72 (m, 6 H), 7.75 (dd, 3 H, *J* = 7.5 Hz, *J* = 2.0 Hz), 8.03 (dd, 3 H, *J* = 7.9 Hz, *J* = 1.7 Hz), 8.16 (broad t, 3 H, NH), 8.70 (broad d, 3 H, *J* = 4.8 Hz). − ^{13}C NMR (50 MHz, $CDCl_3$): δ = 37.8 (CH_2), 53.4 ($N-CH_2$), 62.2 (OCH_3), 122.3 (CH), 124.4 (CH), 124.8 (CH), 126.9 (C*q*), 131.9 (CH), 134.1 (C*q*), 134.6 (CH), 136.3 (CH), 149.6 (CH), 155.4 (C*q*), 156.0 (C*q*), 165.6 (CO). − MS (DCI, NH_3+isobutane): 780 [M^+ + 1]. − $C_{45}H_{45}N_7O_6{\cdot}1\ H_2O$ (797.9): calcd. C 67.74, H 5.94, N 12.29; found C 67.42, H 5.83, N 12.03.

TRENPYPOL (9): Podand **8** (1.09 g, 1.4 mmol) was dissolved in dry CH_2Cl_2 (100 mL), under nitrogen at 0 °C, and BBr_3 (15 mL of a solution with 1 M CH_2Cl_2, excess) was added dropwise. The mixture was stirred for 12 h at room temperature and then concentrated under vacuum. Methanol (50 mL) was added and the mixture was stirred for 1 h and then repeatedly evaporated with methanol (5 × 100 mL). The resulting yellow precipitate was dissolved in HCl 4N and the solution was extracted with CH_2Cl_2. The aqueous solution was adjusted to pH 7−8 with $NaHCO_3$ and extracted with CH_2Cl_2. Washing with brine, drying on $MgSO_4$, evaporation of the solvent and treatment with pentane-ether gave **9**·2HCl as a yellow powder (0.964 g). Yield 85%. − IR (KBr): $\tilde{\nu}$ = 3356, 1644 cm^{-1}. − $C_{42}H_{39}N_7O_6$, $1H_2O$ (755.8): calcd. C 66.74, H 5.47, N 12.97; found C 66.68, H 5.54, N 12.66. − ^1H NMR (250 MHz, D_2O): δ = 3.77 (broad s, 6 H, CH_2N), 3.85 (broad s, 6 H, $CH_2-NH-CO$), 6.71 (t, 3 H, *J* = 7.8 Hz), 7.49 (d, 3 H, *J* = 7.6 Hz), 7.64 (m, 6 H), 7.71 (broad t, 3 H, *J* = 7.6 Hz), 8.30 (broad t, 3 H, *J* = 7.8 Hz), 8.49 (broad d, 3 H, *J* = 5.6 Hz). − ^{13}C NMR (50 MHz, DMSO): δ = 37.3 (CH_2), 52.8 ($N-CH_2$), 117.9 (CH), 119.7 (C*q*), 120.5 (C*q*), 122.3 (CH), 130.3 (CH), 131.9 (CH), 138.5 (CH), 145.2 (CH), 155.7 (C*q*), 158.9 (C*q*), 165.6 (CO). − MS (DCI, NH_3+isobutane): 738 [M^+ + 1], 567, 541, 524, 510.

TRENPYPOLS (10): Podand **9** (1 g, 1.35 mmol) was dissolved in oleum (15 mL) and the mixture was stirred for 12 h, at room temperature. The brown solution was poured onto ice and the pH was raised to 9−10 by cautious addition of 1 M NaOH. Methanol (100 mL) was added to precipitate Na_2SO_4. Filtration and concentration gave a yellow powder, containing again some Na_2SO_4; this salt was removed by repeated treatment (formation of a white precipitate, then concentration) with a mixture of methanol and diethyl ether. Evaporation of the resulting solution gave a yellow powder (tetrasodium salt, 0.74 g). Yield 52%. − IR (KBr): $\tilde{\nu}$ = 3440, 1640 cm^{-1}. − ^1H NMR (200 MHz, D_2O+NaOD): δ = 2.69 (broad s, 6 H, CH_2N), 3.36 (broad s, 6 H, $CH_2-NH-CO$), 7.06 (m, 3 H), 7.40−7.55 (m, 9 H), 8.05 (m, 3 H), 8.27 (m, 3 H). − ^{13}C NMR (50 MHz, D_2O+NaOD): δ = 36.8 (CH_2), 53.0 ($N-CH_2$), 119.0 (C*q*), 122.7 (CH), 125.9 (CH), 126.1 (C*q*), 128.8 (CH), 130.9 (CH), 132.8 (C*q*), 137.4 (CH), 148.8 (CH), 157.3 (C*q*), 170.2 (C*q*), 170.4 (CO). − MS (FAB positive mode M + 1, NBA matrix): 1066 ($C_{42}H_{35}N_7O_{15}S_3Na_4$ + 1). The molecular mass was also checked by potentiometric titration.

Potentiometric Titrations: All measurements were performed at 25 °C and the solutions were prepared with deionized and twice distilled water. The ionic strength was fixed at *I* = 0.1 M with sodium perchlorate (PROLABO puriss.). The potentiometric titrations were performed using an automatic titrator system DMS 716 Titrino (Metrohm) with a combined glass electrode (Metrohm, filled with saturated NaCl solution) and connected to an IBM Aptiva microcomputer. The electrodes were calibrated to read p[H] according to the classical method[31] (titration of 0.1 M $HClO_4$ with 0.1 M NaOH). The ligand and its Fe^{III} complex, ca 0.001 M, were titrated with standardized 0.025 M sodium hydroxide. Argon was bubbled through the solutions to exclude CO_2 and O_2. Sodium hydroxide was prepared from 0.1 M NaOH (Prolabo) and was standardized against potassium hydrogen phthalate. Carbonate content was checked by Gran's method. The titration data (108 points collected over the pH range 2.85−10.77 for the ligand solution) were refined by the nonlinear least-squares refinement program SUPERQUAD[18] to determine the deprotonation constants. The pK_{an} values were calculated from the cumulative constants determined with the program. The uncertainties in the pK_{an} values correspond to the added standard deviations (1σ) in the cumulative constants.

Spectrophotometric Experiments: The ferric complexes were studied by spectrophotometry. The UV/Vis spectrum of a solution containing equal amounts of ligand and Fe^{III} (10^{-4} M) was recorded as a function of pH over the range 1−9 (adjusted with $HClO_4$ or NaOH). An aliquot was taken from the solution after each adjustment of the pH and its spectrum was recorded. pH measurements were made with a 713 Metrohm digital pH meter equipped with a microelectrode. The ionic strength was fixed at *I* = 0.1 M with $NaClO_4$/$HClO_4$. The spectrophotometric data were analyzed using the LETAGROP-SPEFO program.[19,20]The program uses a nonlinear least-squares method to calculate the thermodynamic constants of the absorbing species and their corresponding electronic spectra. The calculations were performed using absorbance values from about 6-8 wavelengths (between 400 and 600 nm). The range of values for the residual-squares sum ($\Sigma(A_{exp}-A_{calc})^2$) of the fits was $10^{-4}-10^{-5}$.

Spectrophotometric competition experiments were carried out with Na_2H_2EDTA over the pH range 7−8. Typical solutions contained 10^{-4} M Fe^{III} ion, TRENPYPOLS and Na_2H_2EDTA. Five solutions were prepared. The samples were allowed to equilibrate for one week in the dark at 25 °C.

Electrochemical Measurements: Electrochemical experiments were carried out using a PAR model 273 potentiostat equipped with a Kipp−Zonen *x*-*y* recorder. All experiments were run at room temperature under argon in a glove-box. A standard three-electrode cell was used. An Ag/AgCl, 3M NaCl, aqueous reference electrode (+0.222 V *vs* NHE) was used. A glassy carbon disc electrode was used as the working electrode (5 mm diameter) and was polished with 1 μm diamond paste. The electrochemical behavior of the Fe^{III} -TRENPYPOLS complex was studied by cyclic voltammetry (CV) in an aqueous solution containing 0.1 M $NaClO_4$ as the supporting electrolyte and buffered with Tris-buffer for the experiments performed at pH 7 or acidified with $HClO_4$ for the experiments at pH 2. The Fe^{III} -TRENPYPOLS solutions were prepared by dissolving stochiometric amounts of ferric perchlorate and TRENPYPOLS in the electrolytic solutions.

Plant Cells Culture and Growth Measurements: Plant cells were grown in 24 well sterile cell culture plates (Nunc); each well contained an independent axenic culture (1.5 mL). Wells were inoculated at an absorbance of 0.003 (*ca* 1/12) with early stationary phase *Arabidopsis thaliana* (var. Columbia) cell suspensions. For axeny, plates were sealed with "Magic Scotch®" tape. 24 well plates were agitated at 220 rpm at 25 °C in a New Brunswik "innova" 4230 refrigerated incubator shaker. Light was supplied for 18 h a day by 2 growlux sylvania 15 W in the " photosynthesis " accessory.

The measurements of cell density were made daily as previously described.[28] The Biorad video camera system Geldoc 1000, with the "molecular analyst" software was used for quantification. Full frame images were captured in constant light conditions, i.e. white

light was adjusted so that just a few saturated pixels appeared in red in the empty optical field. In these light conditions, a reproducible relationship was found between the absorbance of wells in densitometric scanning profiles of recorded images and the amount of cells in the wells. Images of culture plates without cells were used as a baseline reference. They showed an uneven light background. This fact was compensated by the subtraction of an adapted baseline.

The medium (MS) was a modified Murashige and Skoog[32] medium; for 1 L of medium: 4.3 g macro and micro elements powder (provided by Duchefa, cat N° M0221, Haarlem Netherlands), 10 mL KH_2PO_4 (20 g/l), 0.5 mL Kinetin (0.1 g/l), 0.5 mL 2,4 D (0.2 g/l), 1 mL × 1000 vitamin solution (Duchefa M 0409) and 30 g saccharose. Iron free MS medium (MS−Fe) has the same content, made from reagent grade products, omitting the Fe^{III}-EDTA complex. The Fe^{III}-TRENPYPOLS complex was first prepared at low pH then raised to pH 5.8 with NaOH and a Tris maleate buffer, then mixed to the MS−Fe solution at the same pH. The complex was filtered using a 0.22 μm Millipore filter and sterilized. MS−Fe was autoclaved.

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