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PAPER

Biologically active thiosemicarbazone Fe chelators and their reactions with ferrioxamine B and ferric EDTA; a kinetic study†

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The Fe^{III} abstraction from Fe^{III}/DFO and Fe^{III}/EDTA complex systems by thiosemicarbazone ligands derived from 2-acetylpyridine has been studied from a kinetico-mechanistic perspective at relevant pH conditions and at varying temperatures and buffer solutions. The reactions have been found to be extremely dependent on the dominant *E*/*Z* isomeric form of the TSC ligands present in the reaction medium. Consequently the isomerisation processes occurring on the free ligands have also been monitored under equivalent conditions. The isomerisation process is found to be acid dependent, despite the absence of protonation under the conditions used, and presumably proceeds *via* an azo-type tautomer of the ligand. In all cases the existence of outer-sphere interaction processes has been established, both promoting the reactions and producing dead-end complexes. The better oriented forms of the ligands (*EZ thiolate*) have been found to react faster with the $[Fe(HDFO)]^+$ complex, although for mono-N^4 substituted thiosemicarbazones the process is retarded by the formation of a dead-end outer-sphere complex. A comparison with the abstraction of Fe^{III} from $[Fe(EDTA)(H_2O)]^-$ has also been conducted with significant differences in the kinetic features that implicate keystone outer-sphere interactions which dominate reactivity, even with isomeric forms that are not the best suited for direct complexation.

Introduction

Heterocyclic thiosemicarbazones (TSC) are potentially tridentate chelating agents bearing both N and S donor centres including an aromatic heterocycle, usually pyridine. Although TSC complexes of many metal ions have been reported,[1] their iron coordination chemistry in particular is biologically important.[2,3] In previous papers it has been shown that TSC ligands derived from di-2-pyridyl ketone,[4] 2-benzoylpyridine[5] and 2-acetylpyridine[6] exhibit remarkable anti-proliferative activity against a variety of tumor cells and also that they are well tolerated by healthy cells. There has also been considerable interest in the related TSC Triapine (3-amino-2-pyridinecarbaldehyde thiosemicarbazone)[7,8] and this compound has progressed to the clinic as a potential anticancer drug.[9,10] Triapine inhibits ribonucleotide reductase (the rate limiting process in DNA replication) and its action is linked with the depletion of the iron within the cell and also to redox activity, as the iron complex of Triapine is more active than the free ligand.[2,11,12] So it has emerged that iron is an important target and that the ligands appear to act through a dual mechanism that involves sequestering intracellular Fe and also catalysing Fenton chemistry (eqn (1)) as their Fe complex, leading to intracellular oxidative stress.

$$[Fe^{II}L_2] + H_2O_2 \rightarrow [Fe^{III}L_2]^+ + OH^{\bullet} + OH^- \quad (1)$$

The complexity and importance of intracellular Fe chelation by small molecules should not be underestimated from an inorganic mechanistic point of view.[13] In this respect some studies on Fe(III) speciation between different biologically relevant molecules have already been conducted.[14,15] The Fe chelator desferrioxamine B (DFO) has been the 'gold standard' in therapeutic Fe chelation for the treatment of severe transfusion-linked Fe overload for more than 30 years. The effectiveness of DFO as an Fe chelator relies upon the high stability of its Fe(III) complex, ferrioxamine B (Scheme 1), *in vivo*, so any biologically active chelator that is capable of removing Fe from ferrioxamine B must have sufficient thermodynamic stability to also be competitive for labile intracellular Fe. Iron uptake from ferrioxamine B has been observed by some of the thiosemicarbazone ligands discussed in this paper.[16–18]

An often neglected issue in TSC coordination chemistry is the possibility of *E*/*Z* isomerism about the C=N double bond of the Schiff base. No systematic study has been reported on the effect of *E*/*Z* isomerisation on complexation behaviour in a biologically relevant medium. In this paper we report the study of the solution behaviour of a series of 2-acetylpyridine TSC ligands (HApT, HAp4mT, HAp4pT and HAp44mT; Scheme 1), which show significant changes in their isomeric distribution in different

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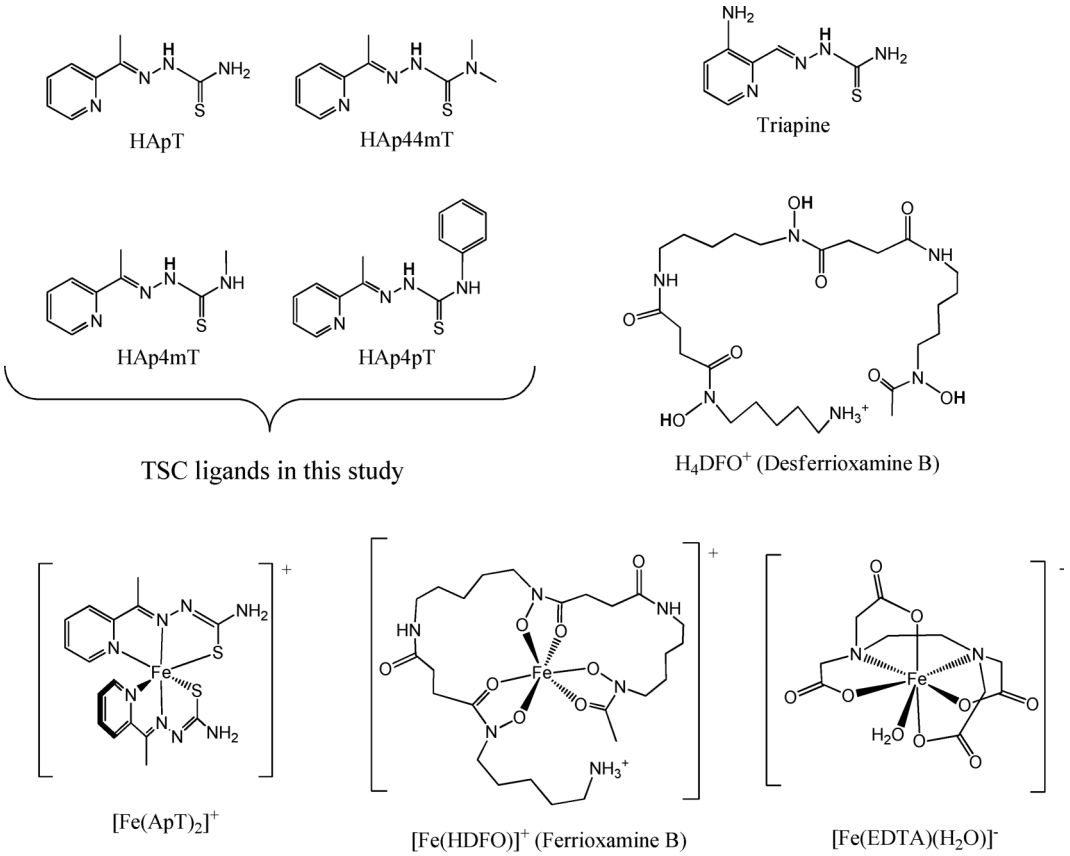


Scheme 1

media. The reaction mechanism operating for isomerisation has been studied and the actuation of proton-assisted outer-sphere interactions has been established for the process. The existence of this solvent-assisted recognition is indicative of what may be occurring during the complexation processes *in vivo*.

The iron abstraction from ferrioxamine B (Scheme 1) has also been studied kinetically with the same HAp4mT and HAp44mT TSC ligands at different pH and temperatures. The results indicate that the more favourably oriented isomer/tautomer of each TSC ligand reacts faster with ferrioxamine B. Nevertheless, a proton-assisted mechanism is also found for the process, indicating the involvement of important outer-sphere recognition reactions *en route* to the final product. Dead-end outer-sphere complexes appear important for incorrectly oriented isomeric forms of the partially terminal amine substituted HAp4mT ligand. Finally the study has been extended to include reactions with $[Fe(EDTA)(H_2O)]^-$ (Scheme 1) with the most active TSC HAp44mT ligand. Again, the existence of outer-sphere recognition reactions between the TSC ligands and the iron complex has been established.

Results and discussion

Ligand isomeric distribution in solution

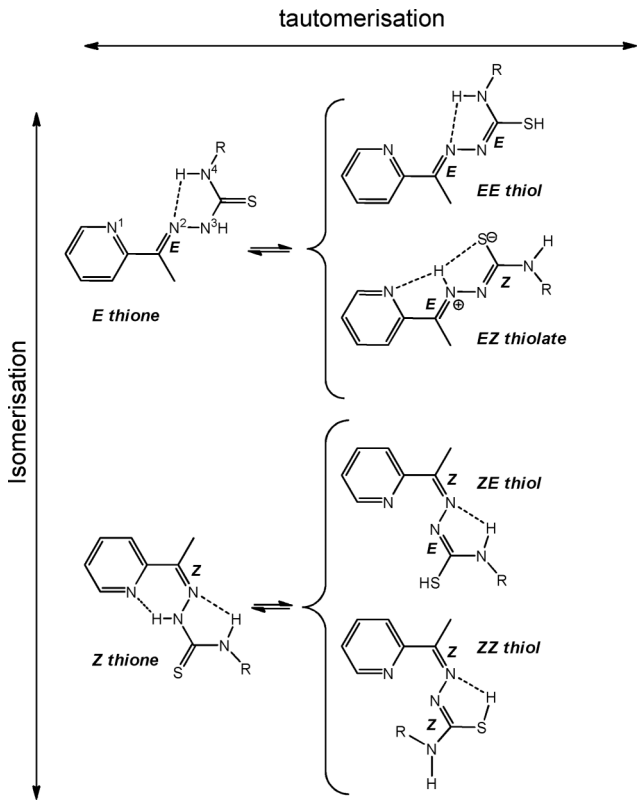
Given the large amount of literature data concerning the possible isomers of tridentate heterocyclic TSC ligands in this study[2,17,19–24] (see Scheme 2 for definitions) we have investigated the *E*/*Z* isomeric distribution and interconversion at biologically relevant pH values (where all ligands exist as neutral molecules).[4] In all cases the solutions were made up in water/DMSO mixtures (from 3.3 : 1 to 0.65 : 1 ratios depending on the ligand) to ensure total solubility of the compounds. The stability and time evolution of this *E*/*Z* isomeric distribution was also studied, this being especially relevant due to the rather slow kinetics of iron removal from ferrioxamine B and $[Fe(EDTA)(H_2O)]^-$ by the TSC ligands in Scheme 1 (see next section).[16]

The deprotonated *EZ thiolate* isomer is the one present in most of the metal complexes of TSC ligands (see Scheme 1),[25,26] especially those bearing a pyridine thiosemicarbazone tridentate *NNS* donor set.[6,27] Complexation is accompanied by deprotonation (pK_a in the 9.5–11 range for the TSC free ligand).[4,17] The same thiolate tautomeric form can also be present in the free ligand, being stabilised in solution by hydrogen bonding between the same set of donors.[20] For N^4-disubstituted TSC ligands the *E thione* and *Z thione* tautomers are disfavoured (there being no N^4-H present for H-bonding *e.g.* HAp44mT), and instead the unusual *EZ thiolate* zwitterionic tautomer (Scheme 2) is found in TSC ligands derived from 2-acetylpyridine.[6] For ligands bearing at least one proton on N^4 (*e.g.* HApT, HAp4mT, HAp4pT) intramolecular H-bonding stabilises both the *E thione* and *Z thione* forms; $N^1 \cdots HN^3$ for the *Z* form and $N^2 \cdots HN^4$ in all cases.[6,19] The corresponding tautomers, *ZE thiol* and *ZZ thiol*, are expected to be unimportant once the $N^1 \cdots HN^3$ H-bonding is lost, despite the possible existence of a new $N^2 \cdots HS$ interaction. Summarising, even though six different forms of the

Table 1 Isomeric and tautomeric distribution (%) as from Scheme 2 for the different forms possible for the thiosemicarbazone ligands indicated in Scheme 1

Ligand	Solvent	*E thione*	*EZ thiolate*	*Z thione*
HApT	DMSO(d_6)	95	5	—
HAp4mT	DMSO(d_6)	90	10	—
	D_2O/DMSO(d_6)	85	15	—
HAp4pT	DMSO(d_6)	90	10	—
	D_2O/DMSO(d_6)	90	10	—
	after 4 h at 60 °C	80	10	10
	H_2O/DMSO(d_6), $NaClO_4$ 0.30 M	90	10	—
	H_2O/DMSO(d_6), $(Bu_4P)Br$ 0.30 M	85	15	—
	H_2O/DMSO(d_6), pH[a] = 5.4 (PIPPS)	85	15	—
	H_2O/DMSO(d_6), pH[a] = 5.5 (MES)	90	10	—
	after 24 h at 60 °C	75	5	20
	H_2O/DMSO(d_6), pH[a] = 6.5 (MES)	85	15	—
	H_2O/DMSO(d_6), pH[a] = 7.5 (TRIS)	90	10	—
HAp44mT	DMSO(d_6)	55	30	15
	D_2O/DMSO(d_6)	55	40	5
	after 24 h at 50 °C	50	40	10
	H_2O/DMSO(d_6), pH[a] = 5.5 (MES)	55	40	5
	after 24 h at 60 °C	25	20	55

[a] As measured in the buffer aqueous component of the solvent mixture.



Scheme 2

TSC ligands studied could be present in solution, only some of them are expected to be observable once thermodynamic equilibrium conditions are attained. For the HApT, HAp4mT and HAp4pT (each bearing at least one H-atom on N^4), the preferred forms should be *E thione*/*EE thiol* and *Z thione*/*ZE thiol*, while for the fully substituted HAp44mT the *EZ thiolate* is expected to be dominant. The rigidity caused by the H-bonding interactions makes the tautomeric forms distinguishable by ^{1}H NMR,[6] while the slow $E \leftrightarrows Z$ process should make these isomeric forms fully distinguishable also.[28–30]

^{1}H NMR of freshly prepared DMSO-d_6 solutions of HApT, HAp4mT and HAp4pT confirmed, as reported in the literature,[6,19,21,22] that the *E thione* isomer is dominant. The key resonances for the HApT, HAp4mT and HAp4pT ligands are found at 10.3, 10.3 and 10.7 ppm (N^3**H**) and 8.1, 8.6 and 10.2 ppm (N^4**H**), respectively. Minor downfield signals were associated with the presence of small amounts of *EZ thiolate* tautomers; 14.1, 14.2 and 14.6 ppm for the N^2**H** proton for the same ligands, respectively (Table 1). For the disubstituted HAp44mT ligand the *E thione*, *EZ thiolate* and *Z thione* forms were all present (9.6 ppm and 15.4 ppm for the N^3**H** proton of the *E thione* and *Z thione* forms respectively, and 14.7 ppm for the N^2**H** proton of the *EZ thiolate* form).[20] The relative proportions are in fair agreement with the literature,[6,19–21] but only partially support the H-bonding considerations discussed above.

On dissolution of the ligands in aqueous DMSO (with different DMSO contents, see above) only minor changes are seen in the *thione*/*thiolate* tautomeric distribution (Table 1), as determined from the associated ^{1}H NMR signals of the aromatic (non-water exchangeable) protons. The *thione*/*thiolate* ratios in Table 1 were time-independent in this solvent. However, the relative amount of *thiolate* tautomer increased slightly at more acidic pH values.[17] The *E*/*Z* isomeric ratio of these solutions was found to be time dependent. The amount of the *Z* form increases gradually to a final thermodynamic ratio which is in much better agreement with the distribution expected from the hydrogen bonding interactions discussed above.[17] Clearly the *E*/*Z* isomeric equilibration reaction appears to be promoted by protons.

In view of the marked changes in the final equilibrium distribution of the different forms occurring in aqueous acidic solutions of these ligands (relative to pure DMSO), a detailed kinetic study of the $E \leftrightarrows Z$ isomerisation process ($k_{E\to Z}$+$k_{Z\to E}$) was pursued at varying temperature, pressure, pH and ionic strength conditions. Time-resolved UV-Vis spectroscopy of the TSC ligands in water/DMSO indicates a neat single step process

Table 2 Relevant kinetic and activation parameters (pH = 5.5, 0.08 M MES) for the $E \leftrightarrows Z$ isomerisation process observed for the HAp44mT, HAp4mT and HAp4pT TSC ligands under different conditions; [TSC] = (1–3)×10^{-5} M, I = 0.27–1.0 M $NaClO_4$

Ligand	T/°C	$10^4 \times k_{obs}$/s^{-1}	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J $K^{-1}mol^{-1}$	$\Delta V^{\ddagger}$/cm^3 mol^{-1}	$[H^+]$-dependence
HAp44mT	60	2.1[a]	78 ± 2	−85 ± 7	1.2 ± 0.4	$k = (61 \pm 4) \times [H^+]$
HAp4mT	60	0.073[a]	not measured	not measured	not measured	not measured
HAp4pT	62	0.093[b]	122 ± 5	16 ± 15	not measured	$k = (0.8 \pm 0.1) \times [H^+]$

[a] 23% DMSO; [b] 62% DMSO.

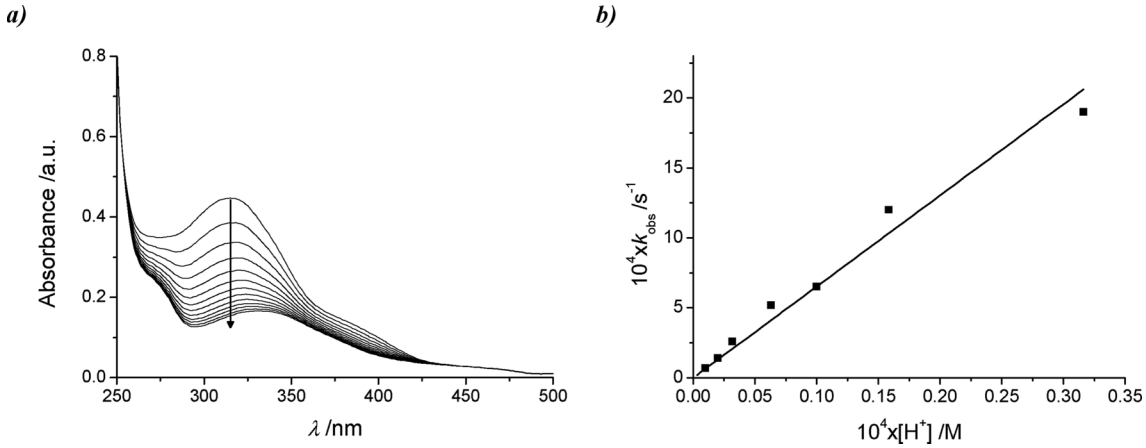
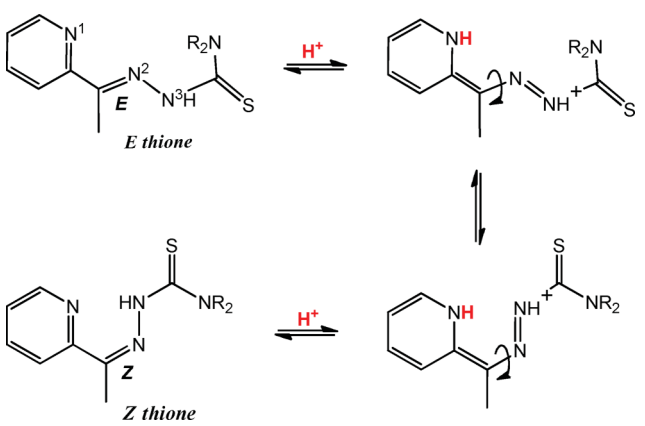


Fig. 1 a) UV-Vis spectral changes obtained on a 2.5 × 10^{-5} M aqueous (23% DMSO) solution of HAp44mT at 40 °C (pH = 5.5, MES); total time 24 h. b) Plot of the rate constants observed for the same $E \leftrightarrows Z$ isomerisation process *versus* $[H^+]$ (T = 62 °C, I = 0.27 M ($NaClO_4$), MES 0.08 M).

(Fig. 1a) that produces well-behaved first order absorbance/time kinetic traces. Table 2 collects the relevant kinetic and activation parameters for the process; the values for the observed rate constants under the conditions used for the study are collected in Table S1, ESI.†

From the data in Table S1, ESI,† it is clear that the equilibration rate constant ($k_{E\to Z}+k_{Z\to E}$) is independent, within experimental error, of the ionic strength and the buffer concentrations used in the study, although it is definitively accelerated by increasing $[H^+]$ of the solution (Fig. 1b). The latter agrees with the fact that the $E \leftrightarrows Z$ isomerisation process is only observed when slightly acidic or polar solutions are used (Table 1),[17] and effectively indicates that the process must occur *via* a proton interaction with the TSC in the transition state. The pH range investigated here is well above the published pyridyl ring protonation constants of the HApT series (*ca.* pK_a 3.5),[17] so no significant protonated TSC will be present in the low pH region of this study. The proton interaction should enable the above mentioned loss of double bond C=N^2 character and produce an increased rotation reaction rate, as observed in other $E \leftrightarrows Z$ or related isomerisation processes.[28–31] The proton interaction in the transition state evidently should be linked with any of the available neighbouring nitrogen donors. A simple interaction with the pyridine N^1 or thioamide N^3 donors would inductively stabilise a $C^{\delta-}$–$N^{\delta+}$ charge separation of the double bond generated on a transition state;[31,32] the alternative direct interaction with the imine N^2 donor would stabilise a $C^{\delta+}$–$N^{\delta-}$ charge separation in the same manner. Nevertheless, the most plausible explanation for the process involves the acid catalysed azo N^2–N^3 tautomerisation. The tautomerisation produces an increase of the negative charge on the pyridine nitrogen (N^1) that can be easily stabilised with external protons; a similar mechanism has been recently proposed for hydrazone rotary switches (Scheme 3).[33]



Scheme 3

The fact that all the coordination compounds prepared from these TSC compounds show the *EZ thiolate* tridentate arrangement, despite the presence of other forms of the ligand in solution, can thus be rationalised. Attachment of the metal ion to the most accessible pyridyl N^1 donor is likely as a prelude to eventual tridentate coordination in a sort of zipper motion, as found for other types of complexation processes;[34–37] on doing so the facile conversion of the isomeric mixture to the final preferred *EZ thiolate* arrangement will be attained (Scheme 3).

Interestingly, the thermal and pressure activation parameters for the isomerisation reaction indicated in Table 2 are very different between the two pyridine thiosemicarbazone ligands

studied at variable temperature. For the HAp44mT ligand, the thermal activation parameters are similar to those found for $E \leftrightarrows Z$ isomerisation processes involving a loss of double bond character by coordination and charge separation stabilisation *via* a rotational transition state.[29,30,32,33] Although the value determined for $\Delta V^{\ddagger}$ is rather surprising (expected to be negative in any of the two highly polar solvents used, or in their mixtures), the reactivity indicated in Scheme 3 can easily explain the opposite trends of the entropy and volume of activation. The existence of a hydrogen bonded solvent aggregation due to the presence of a protonated nitrogen in the isomerising species has been consistently held responsible for these opposite trends.[38]

For the HAp4pT ligand the process is *ca.* two orders of magnitude slower (as the value for HAp4mT), and the value for $\Delta H^{\ddagger}$ is much more positive while that for $\Delta S^{\ddagger}$ is practically zero ($\Delta V^{\ddagger}$ has not been determined for this very slow process). The data clearly indicates some changes in the mechanistic characteristics of the reaction, which is also shown by the *ca.* two orders of magnitude difference in the $[H^+]$-dependence of the rate constant. Although an alternative direct inversion process, as indicated for comprehensive studies on azo-derivatives,[31,32,39,40] would agree with the measured thermal activation parameters for the HAp4pT molecule, the $[H^+]$-dependence of the rate constant data in Table 2 does not. The partially substituted character of the N^4 centre should be related to the overall change in reactivity; the very stable dominant initial *E thione* form of the ligand encumbers the formation of the azo transition state indicated in Scheme 3 for the $E \leftrightarrows Z$ isomerisation *via* N^4–N^2 hydrogen bonding.

TSC iron uptake from ferrioxamine B

In view of preliminary observations that indicate that iron is extracted from ferrioxamine B[13,41] by similar TSC ligands,[16] and the fact that the process is so slow that equilibration studies are not practical, the kinetics of the iron uptake from ferrioxamine B by the ligands indicated in Scheme 1 was pursued at pH values between 5.5 and 7.5. Lower values were considered biologically irrelevant and higher (pH 8.0–9.0) values produced $[Fe^{II}(Ap\mathit{X}T)_2]$ complexes (presumably due to the ferric complexes oxidising water being facilitated by alkaline pH).[4,6,42]

Despite their structural similarity, there was variation in reactivity across the series of TSC ligands. The unsubstituted ligand HApT showed no significant reactivity with $[Fe(HDFO)]^+$ relative to HAp4pT, HAp4mT and HAp44mT on the same time-scale. Nevertheless, for the HAp4pT ligand the spectral changes indicated that, even in the slightly acidic pH range used, reduction of $[Fe(Ap4pT)_2]^+$ takes place, which is only prevented at higher acidities (pH < 5.5). This observation is consistent with $[Fe(Ap4pT)_2]^+$ having the highest $Fe^{III/II}$ redox potential of this series;[6] no further studies were conducted on the $[Fe(HDFO)]^+$/HAp4pT system as a consequence of this behaviour. For the HAp4mT and HAp44mT systems the reactivity observed leads to final UV-Vis spectra that agree with those expected from the characterized $[Fe^{III}(Ap4\mathit{X}T)_2]^+$ complexes. The bulk process observed by UV-Vis is complex and shows a kinetic profile that depends on the chosen TSC ligand (Fig. 2).

No dependence of the spectral changes on the concentration of TSC was found, indicating a non-equilibrium reaction under the conditions of the study. In this respect the recent stability constants evaluated for similar systems agree with the complete formation of the $[Fe^{III}(Ap4\mathit{X}T)_2]^+$ complexes under the conditions of the study.[17] The data are found independent of ionic strength in the 0.1–0.6 M range. Similarly no dependence on the concentration (0.01–0.16 M) of the buffer was found. The overall transmetallation reaction is faster than the isomerisation process (see above), thus indicating that the reaction is occurring on the initial non-equilibrated isomeric distribution of the TSC ligands used in the study.

For both systems studied (>20-fold excess of ligand HAp4mT or HAp44mT), the changes in the initial spectrum of $[Fe(HDFO)]^+$ in the visible region (where the ligand isomerisation processes do not interfere) can be modelled by a set of two first order reactions (once the induction period observed for the HAp4mT ligand system has finished). The values of pseudo-first order rate constants derived (k_{obs1} and k_{obs2}, Table S1, ESI†) follow the same trends with respect to the ligand concentration for the two systems. While the values of k_{obs1} increase linearly with the concentration of the TSC with no significant intercept, the values for k_{obs2} are independent of the concentration of the ligand; Fig. 3a and Fig. S1a, ESI,† collect these respective data for the HAp44mT and HAp4mT ligand systems. From the slopes of the plots of k_{obs1} *versus* [TSC] a value

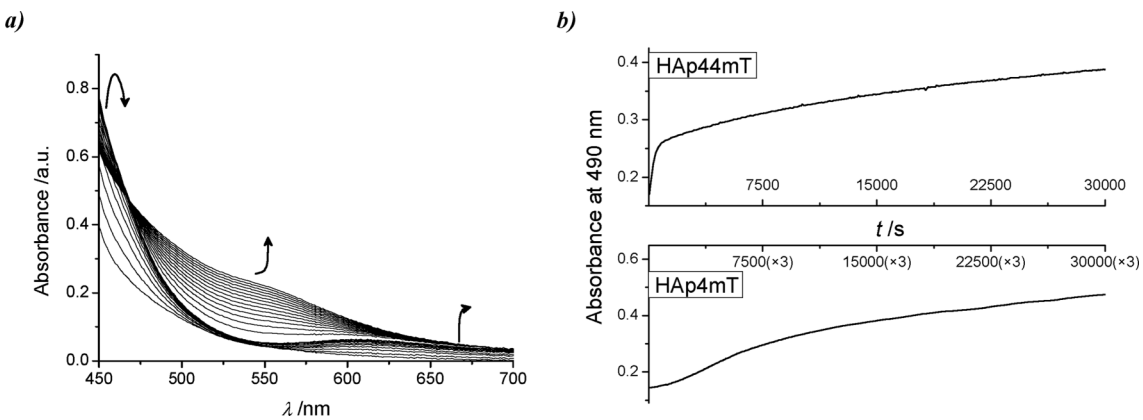


Fig. 2 a) Spectral changes upon the reaction of an aqueous solution of Fe^{III}/DFO with HAp44mT; b) Absorbance *versus* time spectral changes obtained from aqueous solutions of Fe^{III}/DFO upon addition of the TSC ligands studied (note the different time-scales). $[[Fe(HDFO)]^+] = 5 \times 10^{-5}$ M, [TSC] = 5×10^{-3} M, pH = 5.5, T = 60 °C. 23% DMSO in aqueous solution.

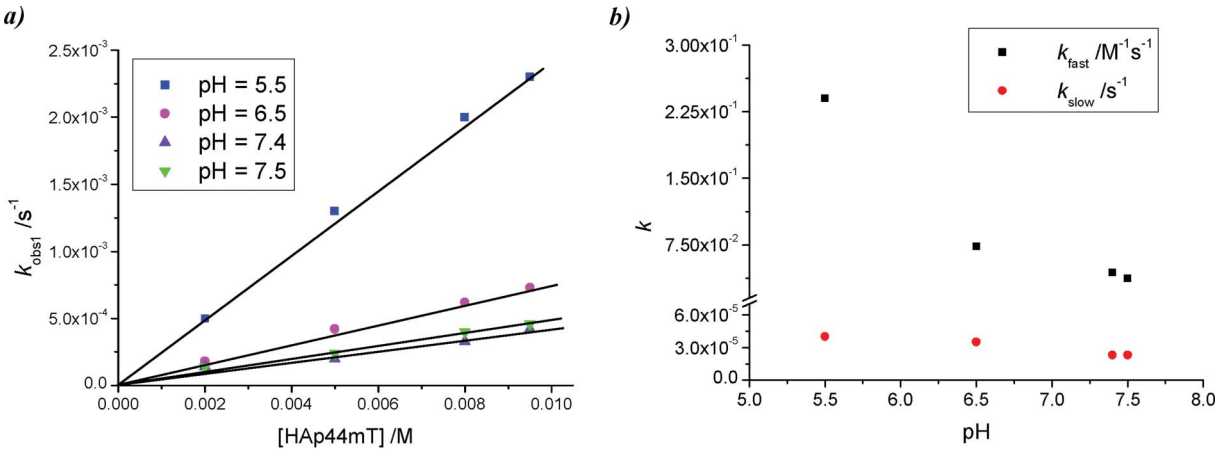


Fig. 3 a) Dependence on the concentration of HAp44mT for the faster process observed on its reaction with [Fe(HDFO)]$^+$ at 55 °C; b) pH profile of the values derived from k_{fast} and k_{slow} for the same reaction. 23% DMSO in aqueous solution.

Table 3 Kinetic data for the two processes observed upon reaction of different TSC ligands with [Fe(HDFO)]$^+$ at various pH values. The values of k_{slow} indicated correspond to the average for the [TSC] used. 23% DMSO in aqueous solution

Ligand	T/°C	pH	$10^3 \times k_{fast}$/M^{-1}s^{-1}	$10^5 \times k_{slow}$/s^{-1}
HAp44mT	55	5.5	240 ± 9	4.0 ± 0.5
		6.5	73 ± 2	3.5 ± 0.5
		7.4	44 ± 3	2.4 ± 0.1
		7.5	37 ± 5	2.4 ± 0.1
	60	5.5	580 ± 50	5.5 ± 0.5
HAp4mT	70	5.5	26 ± 4	0.030[a]
		6.0	16 ± 1	not determined
		6.5	10 ± 1	0.060[a]
		7.5	5.0 ± 0.1	0.050[a]

[a] Large errors are involved for such a slow process.

of k_{fast} can be derived, which shows a decrease upon increasing the pH value of the medium, as indicated in Fig. 3b and Fig. S1b, ESI.† The values of $k_{obs2} = k_{slow}$ are seen to be independent of pH in the same plots. Table 3 summarises the data collected for these systems as a function of the different variables used.

It is clear that while the value of k_{fast} should correspond to a coordination of the ligand to Fe^{III} ion, the process associated with k_{slow} does not correspond to a ligand concentration dependent entry of a TSC to the coordination sphere. An intramolecular process involving the final thiosemicarbazone chelation appears responsible for this second slower step; the alternative DFO final dissociation would be expected to show an increasing rate on acidity. The two-fold increase of k_{obs1} obtained on increasing the DMSO content (not observed for k_{obs2}), agrees with the formation of a relevant {[Fe(HDFO)]$^+$;TSC^0} outer-sphere complex prior to the initial substitution step, favoured in less polar medium.

The pH profile (Fig. 3b and Fig. S1b, ESI†) for the values of k_{fast} indicate that a significant acceleration of the process is observed on increasing the acidity of the medium. This is in good agreement with what had been observed for the exchange process between [Fe(HDFO)]$^+$ and ferrichrome A,[14] and was associated with a partial protonation of the Fe^{III}/DFO system (despite a pK_a value for [Fe(HDFO)]$^+$ of *ca.* 1.0),[43] thus labilising the iron centre.[44] If this is so, the [Fe(H$_2$DFO)]$^{2+}$ species should be extremely reactive in comparison with the major [Fe(HDFO)]$^+$ component of the system in this pH range; the alternative existence of an outer-sphere complex between the neutral TSC ligand and the [Fe(HDFO)]$^+$ species, very sensitive to [H$^+$] as in the studies of the previous section, can also explain the data much more reasonably.

As for the values of k_{slow}, they are smaller than those found for the $E \leftrightarrows Z$ isomerisation process indicated in the previous section (Table 2) (and this isomerisation cannot be observed at the wavelengths shown in Fig. 2a), which definitively agrees with the slow step observed being the final chelation of the TSC ligands. Summarising, the substitution of DFO by the incoming TSC ligand seems to be governed by the sequential entry, as found for similar systems,[45] and partial coordination (k_{fast}) followed by the complete tridentate coordination (k_{slow}) of one of the TSC ligands. After the first initial coordination, DFO dissociates from the ternary Fe^{III}/TSC/DFO complex and fast entry of a second TSC ligand completes the reaction.

In this respect, the dramatic differences in the values of the rate constants determined for the two studied ligands could be tentatively attributed to the different amounts of the *EZ thiolate* (Scheme 2) form present in solution for the two ligand systems; this form being the reactive one for formation of the final [Fe(TSC)$_2$]$^+$ complexes. Effectively, a definite decrease in the reaction rate constant for the fast step (k_{obs1}) is obtained for the reaction of [Fe(HDFO)]$^+$ with HAp44mT at pH 5.5 after isomeric equilibration (according to the data collected in the previous section), which is fully compensated once the change from a 40% to a 20% *EZ thiolate* form of the ligand is considered (Table 1, Fig. S2, ESI†). Nevertheless, this can only explain a factor of three in the rate constant increase from HAp4mT to HAp44mT, and by no means the induction period observed for the spectral changes on HAp4mT transmetallation (Fig. 2b, 2.5 h at 60 °C and pH = 5.5). Given the fact that the basicities of the pyridine rings in HAp4mT and HAp44mT are similar, and should not lead to any significant differences in reactivity according to the facts indicated in the previous section,[46,47] the degree of substitution on the N^4 atom, has to be considered as responsible. The fast initial formation of a very favourable outer-sphere aggregate of HAp4mT with the [Fe(HDFO)]$^+$ complex, that does not evolve directly to the substituted complex (*i.e.* a dead-end species), can easily explain the

Table 4 Relevant kinetic (average for 3–5 runs at different TSC concentration at 60 °C) and thermal activation data for the processes observed upon reaction of HAp44mT with $[Fe(EDTA)(H_2O)]^-$. 40% DMSO in aqueous solution

pH	$10^4 \times {}^{333}k_{obs1}/s^{-1}$	$10^4 \times {}^{333}k_{obs2}/s^{-1}$	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J K^{-1}mol^{-1}	$10^5 \times {}^{333}k_{obs3}/s^{-1}$	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J K^{-1}mol^{-1}
5.5	2.1 (2.1)[a]	1.6	48 ± 5	−176 ± 15	1.7	79 ± 1	−101 ± 1
6.5	0.18 (0.18)[a]	1.8	not determined	not determined	1.2	not determined	not determined

[a] In brackets are the values of the $E \leftrightarrows Z$ isomerisation process indicated in Table 2.

induction period observed. The decomposition of this aggregate to produce the final $[Fe(Ap4mT)_2]^+$ would be consequently slower than for the species directly involving the N^1 donor in iron capture. In this respect, the fact that the induction period is observed to increase with acidity seems to indicate that an involvement of a solvent- and proton-assisted outer-sphere complexation is present in this reactivity pattern.

TSC iron uptake from Fe^{III}/EDTA

In view of the data determined in the previous section and the historical importance of the EDTA competition studies to assess the quality of a chelator system,[41] the kinetics of iron removal from ferric EDTA was pursued. The reactions were studied with the HAp44mT ligand at pH values of 5.5 and 6.5, where the precursor complex exists as a heptacoordinate $[Fe(EDTA)(H_2O)]^-$ pentagonal bipyramid (Scheme 1)[48,49] and the TSC ligands exist dominantly as neutral molecules.[4] Under these conditions no Fe^{II}/TSC signature at 640 nm was observed,[4,6,42] which confirms the fact the TSC ligand removes Fe^{III} directly from the EDTA complex without any redox chemistry; the final UV-Vis spectra showed in all cases the characteristics of the $[Fe(Ap44mT)_2]^+$ complex. The time resolved spectral changes are very complex, nevertheless, the fitting of the full spectral changes in the 400–700 nm range using SPECFIT[50] produces three identifiable reaction steps with well defined spectra and concentration profiles (Fig. 4). One of the reaction rate constants, as well as the spectral changes associated, corresponds within error limits to the previously determined $E \leftrightarrows Z$ isomerisation process, k_{obs} from Table 2, while the other two, k_{obs2} and k_{obs3}, do not show any dependence on the concentration of the ligand or pH, and have spectral changes which correspond to the formation of the final substituted complex. The relevant kinetic and thermal activation parameters collected in Table 4 clearly indicate that these two (k_{obs2} and k_{obs3}) processes monitored correspond to an associative process, which is in good agreement with a simple chelation sequence from a Fe^{III}/EDTA/TSC ternary system to produce the final $[Fe(Ap44mT)_2]^+$ complex. Furthermore, the simple substitution processes on the $[Fe(EDTA)(H_2O)]^-$ complex has been established as having a well defined dissociative activation character.[48,49]

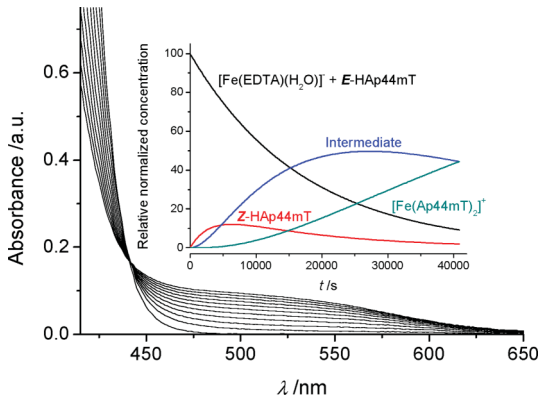


Fig. 4 UV-Vis spectral changes observed for the reactions of $[Fe(EDTA)(H_2O)]^-$ with HAp44mT. Inset: Kinetically fitted concentration profile of the different species for the same reaction. [HAp44mT] = 6×10^{-4} M, $[Fe(EDTA)(H_2O)]^-$ = 2.2×10^{-5} M, pH = 5.5, T = 70 °C, 40% DMSO in aqueous solution.

From the data it is clear that the mechanism involved in the $[Fe(EDTA)(H_2O)]^- \rightarrow [Fe(Ap44mT)_2]^+$ conversion process is very different from that observed for the Fe^{III}/DFO system. While for the substitution of $[Fe(HDFO)]^+$ the reaction clearly involves the *EZ thiolate* form of the HAp44mT ligand, this is not the case for the $[Fe(EDTA)(H_2O)]^-$ substitution process where an initial build-up (Fig. 4) of the *Z thione* isomer is observed before the reaction occurs. Experiments similar to those conducted in the previous section were run on $E \leftrightarrows Z$ equilibrated HAp44mT solutions at pH 5.5 and 6.5 and results confirmed that the reaction sequence for the HAp44mT iron complexation from $[Fe(EDTA)(H_2O)]^-$ is much more complex than expected. At pH 6.5 only two consecutive first-order kinetic steps are observed, as expected from a build up of the *Z thione* isomer of HAp44mT. Furthermore, the rate constants agree reasonably well with those measured for k_{obs2} and k_{obs3} on the initial non-equilibrated reaction mixture. At pH 5.5, nevertheless, the results are more surprising, but highly revealing. After a fast reaction (occurring on conventional thermostating time), the kinetic profiles are equivalent to those measured in standard experiments. The nature of the fast reaction involves spectral changes that are the reverse of the observed $E \leftrightarrows Z$ isomerisation reaction; the UV-Vis spectrum of the reaction mixture immediately after the complex $[Fe(EDTA)(H_2O)]^-$ is added agrees with that measured for the initial non-equilibrated HAp44mT solution. It seems thus that, under these pH conditions, the reaction of $[Fe(EDTA)(H_2O)]^-$ with the *Z thione* form present in the equilibrated reaction mixture of the HAp44mT ligand (see Table 1) competes unfavourably with a catalysed back isomerisation to the initial *E thione* and *EZ thiolate* tautomer mixture of the ligand. This catalytic effect has to be clearly related to the formation of an acid-assisted outer-sphere complex between the iron $[Fe(EDTA)(H_2O)]^-$ complex and the *Z thione* form of the TSC ligand, in line with the data reported in the first part of this section. Similar experiments carried out at different pH from 4.5 to 6.5 and at room temperature indicated that this reversion to the initial mixture of the *E thione* and *EZ thiolate* forms is effectively faster at higher acidities (from 2–3 h to 24 h at pH 4.5 and 6.5 respectively at 20 °C). Thus, the different behaviour observed at pH 5.5 and 6.5 for the iron abstraction from the Fe^{III}/EDTA system by thermodynamically equilibrated isomeric mixtures of

HAp44mT is related to the fact that, at pH = 5.5, the catalysed reversion to the *E thione* and *EZ thiolate* forms of the ligand competes favourably with the substitution of EDTA by the *Z thione* form of the HAp44mT ligand.

Conclusions

The freshly prepared thiosemicarbazones of the HApT family used in this study show a dominant presence of the isomeric *E thione* and *EZ thiolate* tautomers that equilibrates in protic medium to a mixture with increased presence of the *Z thione* form. The relative thione/thiolate tautomeric ratios are dependent on the degree of substitution on the N^4 terminal amino group. The $E \leftrightarrows Z$ isomerisation reaction has been kinetically studied at variable temperatures and pressures and indicates an acid-assisted isomerisation mechanism.

The kinetic studies carried out for the iron abstraction from the Fe^{III}/DFO system with the fully substituted HAp44mT ligand indicate a dominant activity of its *EZ thiolate* form (the one matching the conformation of the final bis-thiosemicarbazone complexes), which is acid-assisted *via* the formation of an outer-sphere complex. When the mono-N^4-methylated ligand HAp4mT is used instead, the above mentioned outer-sphere complexation leading to a dead-end complex implies a definite induction period for the formation of the final $[Fe(Ap4mT)_2]^+$ complex with consequently longer reaction times.

Unexpectedly, the HAp44mT iron uptake from the Fe^{III}/EDTA system is much more complex than that observed for the Fe^{III}/DFO. In this case the substitution-active TSC ligand isomer is found to be the *Z thione* form; the importance of outer-sphere hydrogen bonding in these reactions is a key point, as in the previous cases. The $[Fe(EDTA)(H_2O)]^-$ complex is seen to catalyse the recovery of the *E thione*/*EZ thiolate* non-equilibrated mixture after a previously conducted thermodynamic equilibration.

Experimental

Compounds

All the thiosemicarbazone ligands used in this study were prepared according to literature methods.[4] $[Fe(EDTA)(H_2O)]^-$ is commercially available and was used without any further treatment; $[Fe(HDFO)]^+$ (ferrioxamine B) was also prepared according to the literature.[14]

The buffer solutions used in this study were selected according to their inability to act as competing ligands for the Fe^{III} complexes in this study as well as their pK_a values. MES, PIPPS, TRIS, CAPS have been used in all cases at concentrations that were capable of buffering all the relevant species in the medium. The final pH was adjusted by adding perchloric acid or sodium hydroxide to the final solutions. Their final ionic strength was always adjusted to 0.25–0.30 M with $NaClO_4$ unless stated.

Instruments

^1H NMR spectra were recorded on a Varian VNMRS 400 MHz instrument at the Serveis Científico-Tècnics de la Universitat de Barcelona. pH values were measured on a Crison pH & ION-Meter GLP 22+ instrument using a standard glass combined electrode. UV-Vis spectra were recorded on Cary50, HP8453 or J&M TIDAS instruments.

Kinetics

All the solutions to be used for monitoring the kinetic processes were prepared by adding the calculated amounts of the different ligands dissolved in water or DMSO, according to their solubility in the final media and ionic strength, to stock buffered aqueous solutions. In all cases the maximum volume percentage of DMSO was 65%. The final ionic strength was set normally at 0.27 M with $NaClO_4$, although changes from 0.15 to 1.0 M do not produce any significant difference in the processes observed.

All the kinetic measurements for the different systems carried out at ambient pressure were followed by UV-Vis spectroscopy in the full 300–800 nm range inside the thermostatic multicell compartments of a Cary50 or HP8453 instrument. For the monitoring of reactions at variable pressure, an already described pressurising setup[51–53] connected to an J&M TIDAS instrument has been used. For the isomerisation studies the values of the concentrations of the thiosemicarbazone ligands were set within the $(1–3)\times10^{-5}$ M range, while for all the substitution systems the limiting $[Fe^{III}]$ values were within the $(1–5)\times10^{-5}$ M range, unless stated, and the uptaking ligands in excess were in at least 10 or 20-fold flooding factor (depending on the stoichiometry of the final complex). The general kinetic technique has already been described.[54–56] The time-dependence of the spectral changes were fitted to single (k_{obs1}) or double (k_{obs1} plus k_{obs2}) or triple (k_{obs1} plus k_{obs2} plus k_{obs3}) exponential dependence by the use of the SPECFIT software.[50] For the simpler processes, fitting was conducted on a single wavelength basis in the range where no interference of other secondary reactions was observed; in these cases no dependence of the value of the observed rate constants with the wavelength was observed. Nevertheless, for systems where the process was more complex, the fitting of the full spectral changes was also conducted with good results. Table S1, ESI† collects all the values of k_{obs} determined for the different systems as a function of temperature and pH. All post run fittings were carried out by least-squares methods using standard commercial software. The typical error limits for the values of k_{obs} are within 15% of the value indicated in Table S1, ESI.†

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