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Zinc(II) Complexation Behaviour of Sulfonamide-Based Enzyme Inhibitors

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Sulfonamide derivatives containing extrafunctional groups, such as hydroxamic acids, hydroxypyrimidinones and carboxylic acids, have been recently identified as inhibitors towards several zinc-containing enzymes, such as the matrix metalloproteinases (MMPs) and/or carbonic anhydrases (CAs). Since these inhibitors are supposed to bind the zinc ion at the active site, it was decided to study the zinc(II) complexation with a set of representative compounds in order to identify the most probable coordination modes and to find eventual relationships with the inhibitory properties. These studies were performed in aqueous solution, by potentiometry and ^{1}H NMR spectroscopy, and in the solid phase, by infrared spectroscopy. The solution equilibrium studies indicate that these compounds present similar affinity for zinc (pZn ≈ 6). Under stoichiometric conditions, the formation of 1:1 metal complex species involves a preferential (*O*,*O*) coordination via the hydroxamic or hydroxypyrimidinone moieties, while the coordination via the sulfonamide groups could mainly be achieved under zinc excess conditions.

1. Introduction

Matrix metalloproteinases (MMPs) and some carbonic anhydrase (CA) isoenzymes are known to be involved in carcinogenesis and tumour invasion processes.[1–3] Therefore, both types of zinc-containing enzymes became the targets for drug design and specific inhibitors for each type of enzymes are well known. In particular, many inhibitors of CAs containing aromatic/heteroaromatic sulfonamides have been used in the treatment of several diseases; concerning the MMP inhibitors, hydroxamate derivatives have been widely developed as possible anti-tumour/metastatic agents.[1,4]

Since these two families of inhibitors bind the zinc at the enzyme active sites, efforts have already been made to explore connections between CA and MMP inhibitors and eventual "cross-reactivity" or synergic effects.[5,6] Following the current trend of exploring potential dual inhibitors for those metalloenzymes,[7] we have recently identified a set of potent enzyme inhibitors,[8,9] some of them containing sulfonamide moieties inserted in diverse scaffolds bearing the usual zinc-binding groups in matrix metalloproteinase inhibitors (MMPi), namely: carboxylic and primary hydroxamic groups as well as, more recently, the hydroxypyrimidinones (heterocyclic hydroxamic acids).[10]

Direct comparison of the previously described compounds showed a clear trend towards an increased inhibitory activity of the sulfonamide derivatives. In the absence of X-ray structures of the enzyme-inhibitor adducts, some modelling studies have already been performed to simulate the inhibition of some MMPs and to aid the understanding of those results.[8] Aimed at shedding some extra light on the rationalization of the enzyme inhibition results, a set of four representative compounds (H_iL^n, n = 1–4, see Scheme 1) was selected and their Zn complexation behaviour was studied to identify the 1:1 zinc-binding mode preferences of the diverse bifunctional compounds, in order to establish eventual zinc-binding affinity/inhibitory activity relationships. Results with some model compounds (e.g. H_2L^5 and HL^6) were also analysed for comparison purposes.

The zinc complexation studies were mainly performed in aqueous solution, using potentiometric and ^{1}H NMR spectroscopic methods, although a brief analysis of solid zinc complexes was also carried out by infrared spectroscopy.

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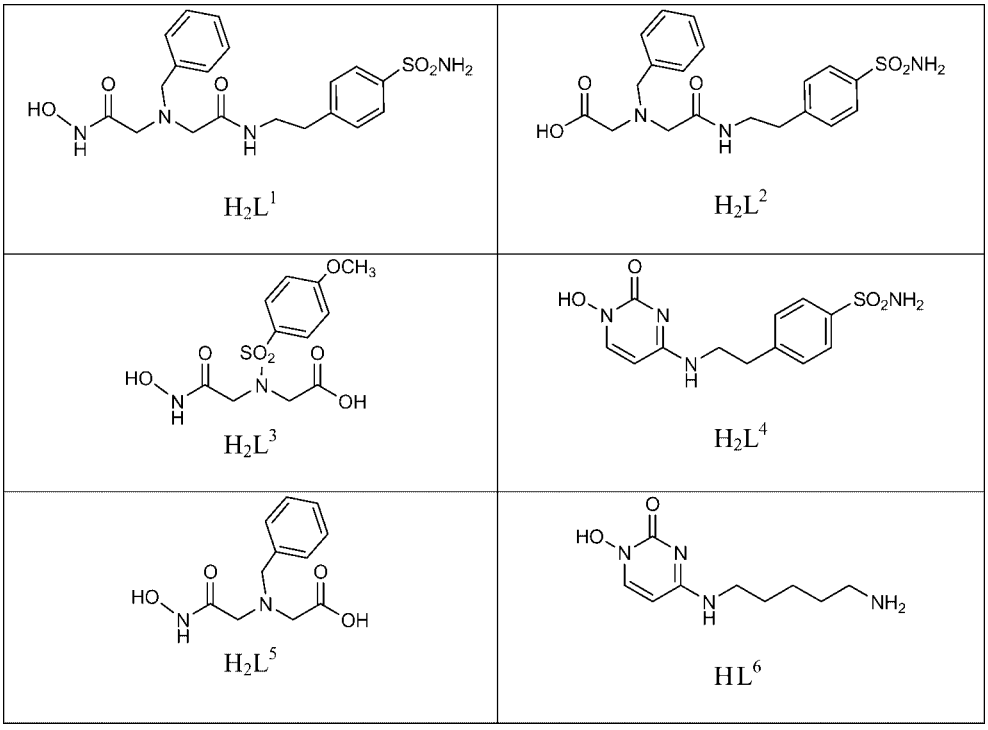

Scheme 1.

2. Results and Discussion

2.1. Acid Base Properties of the Ligands

The fully protonated form of the ligands under study has three (H_2L^n, n = 1, 2, 4) or two (H_2L^3) dissociable protons. The determination of the corresponding protonation constants was mainly performed by potentiometry but, for H_2L^2, $\log K_3$ (<2) was calculated by ^{1}H NMR spectroscopy. Table 1 shows the stepwise protonation constants ($\log K_i$) calculated for the sulfonamide derivatives under study (H_2L^n, n = 1–4) and also the values previously determined for H_2L^5 and HL^6 (used for comparison purposes).

^{1}H NMR titration was also used to determine the protonation sequence for compounds containing a primary sulfonamide group (H_2L^n, n = 1, 2, 4). In fact, for H_2L^1 and H_2L^4, the protonation sequence is more difficult to predict than for the remaining compounds, because both the sulfonamide anions and the hydroxamate hydroxy groups are protonated at nearby high pH ranges. H_2L^2 was also analysed by ^{1}H NMR titration due to its analogy to H_2L^1, to aid the discussion of the coordination modes for the corresponding zinc complexes. Analysis of the titration curve profiles (Figure 1) suggests that, for ligands H_2L^1 and H_2L^2, the sulfonamide anion (SO_2NH^-) is the first group to be protonated. In fact, for both ligands, at pD ca. 10–11, peaks 1, 5 and 6 are downfield shifted. Regarding H_2L^1, the chemical shift profiles of the nonlabile protons nearby the sulfonamide group, indicate that the protonation successively occurs on the hydroxamate group (downfield shifts of peaks 3 and 4 at pD ≈ 8.5) and the *N*-amine group (at pD ca. 2 large deshielding effect on peaks 2, 3, 4).

Table 1. Stepwise protonation constants ($\log K_i$) of H_iL^n (i = 1, 2, n = 1–6) as well as the global formation constants ($\log\beta$) of the corresponding Zn^{2+} complexes (I = 0.1 M KCl, t = 25.0±0.1 °C).

Ligand	H_iL $\log K_i$	Protonated centre	$Zn_mH_hL_l$ (m,h,l) $\log\beta$
H_2L^1	10.28(2)	[b]	(1,1,1) 15.14(5)
	8.75(3)	[b]	(1,2,2) 30.09(7)
	3.21(4)	amine	
pZn[a]			6.04
H_2L^2	9.64(1)	sulfonamide	(1,1,1) 13.52(2)
	5.86(2)	amine	(1,2,2) 26.61(6)
	1.8(1)[c]	carboxylate	
pZn			6.05
H_2L^3	8.47(1)	hydroxamate	(1,0,1) 4.96(1)
	2.99(2)	carboxylate	(1,0,2) 8.68(3)
pZn			6.05
H_2L^4 [d]	10.16(1)	sulfonamide	(1,1,1) 14.74(3)
	7.09(2)	hydroxamate	(1,2,2) 28.95(4)
	2.90(2)	*N*-imine	
pZn			6.12
H_2L^5 [e]	9.11(3)	hydroxamate	(1,0,1) 6.63(5)
	5.75(5)	amine	(1,1,1) 12.84(4)
	1.6	carboxylate	(1,0,2) 11.06(7)
			(1,1,2) 18.6(1)
			(1,2,2) 24.51(5)
pZn			6.27
HL^6 [d]	10.45(1)	amine	(1,1,1) 15.30(1)
	7.11(2)	hydroxamate	(1,2,2) 29.59(2)
	2.99(3)	*N*-imine	
pZn			6.18

[a] pZn is defined as –log[Zn] calculated for C_L/C_M = 10, C_M = 10^{-6} M at pH 7.4. [b] Overlap of the protonation processes of hydroxamate and sulfonamidate groups. [c] Determined by ^{1}H NMR titration. [d] In 5% DMSO solution. [e] Ref.[12]

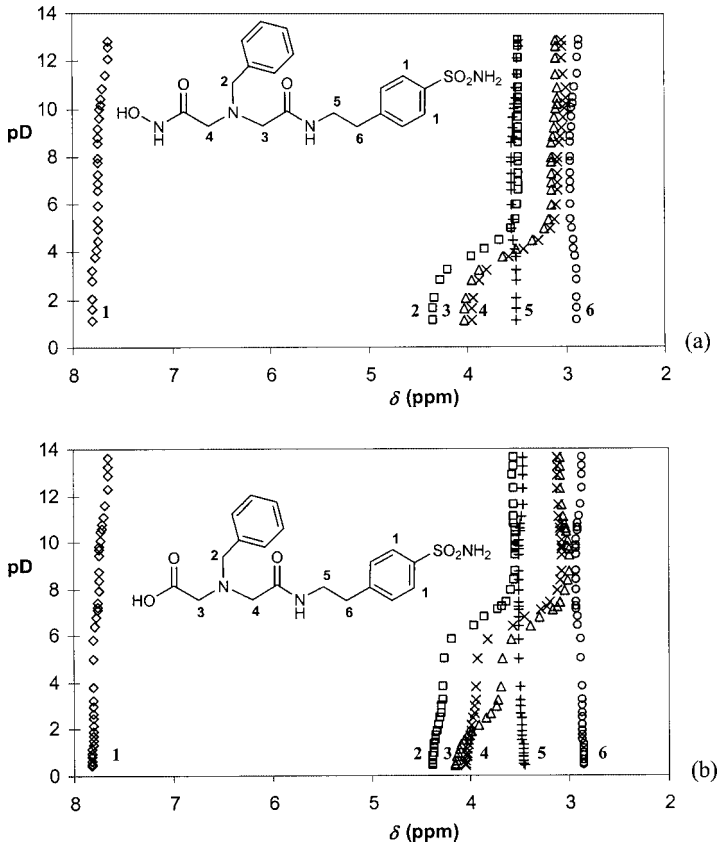

Figure 1. ^{1}H NMR titration curves of (a) H_2L^1 and (b) H_2L^2.

For H_2L^2, Figure 1, b) illustrates that the second and third protonation occur in the *N*-amine (large downfield shifts of peaks 2, 3 and 4 at pD ≈ 5) and carboxylate (at pD ≈ 1 large downfield shift of peak 3) groups, respectively.

Concerning the hydroxypyrimidinone derivative, H_2L^4, Figure 2 shows the following protonation sequence: sulfonamide group (at pD ca. 11 downfield shift of peaks 2 and 3); hydroxy group (downfield shift of peaks 1 and 4 at pD ca. 7); *N*-imine group (downfield shift of all peaks at pD ca. 2). As found for the HL^6 analogue,[11] all the protons feel the protonation of the *N*-imine group, probably due to the enamine-imine equilibrium.

A brief analysis of the protonation constants summarized in Table 1 indicates that the $\log K_1$ (9.6, 10.2) values of ligands H_2L^n (n = 2, 4) should be attributed to the protonation of the sulfonamide group. The lower value of $\log K_1$ found for H_2L^2, as compared to H_2L^1, is probably due to an electron donation effect that maybe slightly higher for the hydroxamate than the carboxylate group. The values calculated for the hydroxamate protonation constants of ligands H_2L^1 ($\log K_2$ = 8.75) and H_2L^3 ($\log K_1$ = 8.47) are also slightly lower than the previously calculated value for H_2L^5 ($\log K_1$ = 9.11[12]) due to a possible cumulative effect of the carboxylate group on the stabilisation of the H_2L^5 protonated form through a bifurcated hydrogen bond network with the *N*-amine. For H_2L^1, the protonation processes of the hydroxamate and the sulfonamidate groups seem to overlap and so the corresponding values of $\log K_1$ and $\log K_2$ presented in Table 1 cannot be accurately assigned to each individual group.

Regarding the acid-base properties of the hydroxypyrimidinone derivatives, the protonation constants calculated for H_2L^4 and HL^6 present very similar values, namely for the hydroxy ($\log K_2$ 7.09 and 7.11) and the *N*-imine groups ($\log K_3$ 2.90 and 2.99). The fact that the amine group of H_2L^1 ($\log K_3$ = 3.21) presents a lower basicity than those of H_2L^2 ($\log K_2$ = 5.86) and H_2L^5 ($\log K_2$ = 5.75) must be due to the presence of the adjacent negatively charged carboxylate groups in H_2L^2 and H_2L^5, which stabilise the formation of the positively charged ammonium group. Finally, analysis of the protonation constants obtained for the carboxylate groups shows that lower values were obtained for H_2L^2 ($\log K_3$ = 1.76) and H_2L^5 ($\log K_3$ = 1.6), than for H_2L^3 ($\log K_2$ = 2.99); that difference can be rationalized in terms of an extra stabilization of the conjugate base due to the electrostatic interaction between the positively charged amine and the negatively charged carboxylate groups, as well as to hydrogen bonding interaction between the ammonium proton and the carboxylate, involving 5-member ring intermediates.

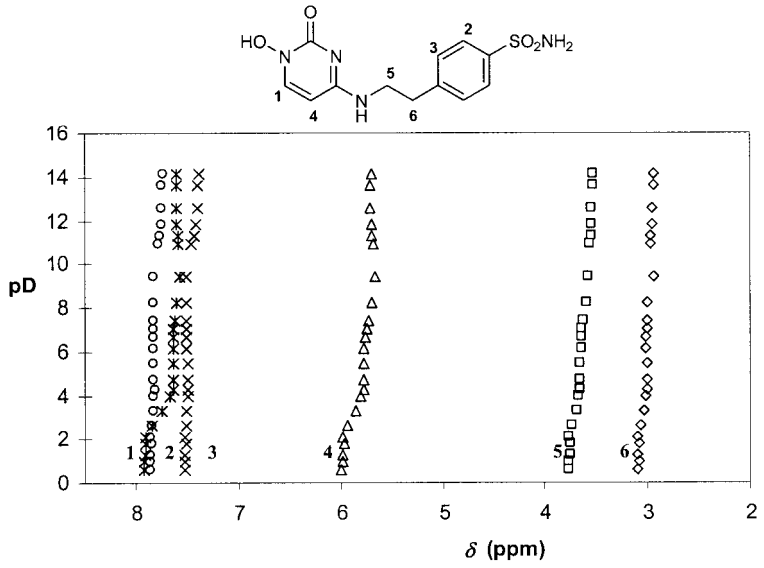

Figure 2. ^{1}H NMR titration curves of H_2L^4.

2.2. Zinc Complexation Studies

In this study, the chelating ability of four sulfonamide derivatives towards Zn^{II} in aqueous solution was evaluated. Although the sulfonamides, in their neutral form, are expected to be poor ligands due to the withdrawal of electron density from the nitrogen atom onto the electronegative oxygen atoms, if this *N*-atom has a dissociable proton, the electron-withdrawing effect increases its acidity making the sulfonamide anions effective sigma-donor ligands.

The global formation constants of the H_iL^n (n = 1–4) zinc complexes were determined by potentiometry at conditions of 1:1, 1:2 and 2:1 metal-to-ligand molar ratios and they are collected in Table 1, together with the reported values for two analogues (H_2L^5 and HL^6).

Figure 3 shows representative potentiometric titration curves for one of the ligands (H_2L^1), alone and in the presence of Zn^{II}. Analysis of the titration curve profiles (Figure 3) indicates the noninvolvement of the *N*-amine group in the zinc coordination and that the hydroxamic acid deprotonation feels the presence of the metal ion. For pH values above 6.5–7.0, precipitation occurred and the potentiometric titrations were stopped.

Regarding the other ligands under study, the potentiometric titration curves also showed the noninvolvement of specific groups in the coordination to the Zn^{II} ion, namely: the carboxylate group for H_2L^3 and the *N*-imine group for H_2L^4. In the case of H_2L^2, the potentiometric curves cannot give information about the involvement of the carboxylate group in the zinc coordination since the determined value of $\log K_3$ is extremely low (<2).

Aimed at getting some further information about coordination modes, ^{1}H NMR titrations of solutions containing the systems Zn^{II}/H_2L^n (n = 1, 2, 4) were performed and the corresponding titration curves compared to those of the respective ligand alone. However, for some of those solution zinc systems, precipitation occurred due to the eventual formation of neutral zinc complexes, namely: for H_2L^1 (at pH above 7), for H_2L^2 (at pH ca. 4.5–9) and for H_2L^4 (at pH 6–10). Therefore, conclusions can be only drawn for pH ranges outside the precipitation zone. ^{1}H NMR studies of the Zn^{II} complexes (C_L/C_M = 1) with H_2L^n (n = 2, 4) suggested the noninterference of the sulfonamide group in the coordination mode of these complexes, which is in accordance with the formation of more soluble zinc-hydroxo species for high pH values. In fact, the NMR titration curves of the ligands and respective zinc complexes are superimposed at pD > 11. In the case of H_2L^1, it is possible to confirm the noninvolvement of the amine group in the zinc coordination (Figure S1 in the Supporting Information). Moreover, the binding of compounds H_2L^1 and H_2L^4 to Zn^{II}, via the two oxygen atoms of the hydroxamic moiety, was also somehow supported by the absence of significant modifications of the ^{1}H NMR spectrum of these ligands due to the presence of the metal ion.[13]

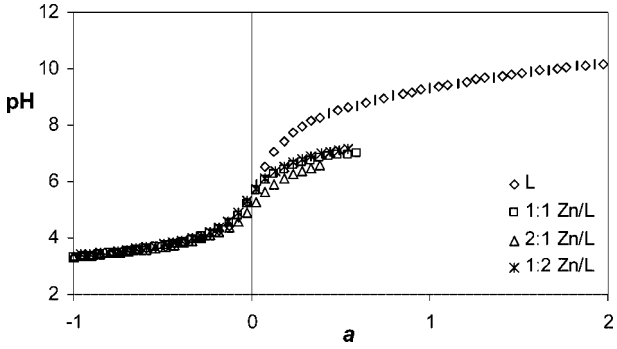

Figure 3. Potentiometric titration curves of H_2L^1 and 1:1, 2:1 and 1:2 Zn^{II}/H_2L^1 systems (C_L = 0.67 mM).

Analysis of Table 1, containing the results of the solution complexation studies of the diverse compounds (H_2L^n, n = 1–4) with Zn^{II}, indicates the existence of 1:1 (ZnHL and ZnL) and 1:2 (ZnH_2L_2 and ZnL_2) complex species in the

adopted equilibrium model. As expected, the Zn^{II} complexes with typical (*O*,*O*) hydroxamate coordination, as those formed with H_2L^1, H_2L^4 and HL[6], have analogous $\log\beta_{MHL}$ and $\log\beta_{MH_2L_2}$ values. For the complexes with H_2L^1 or H_2L^4, the sulfonamide groups are protonated and the net charges are +1 and 0 for the MHL and the MH_2L_2 species, respectively. The speciation at different pH conditions for the zinc binary systems under study (H_2L^n, n = 1–4) is illustrated in Figure S2 of the Supporting Information.

Concerning H_2L^2, the NMR spectral changes with pD showed the noninvolvement of the sulfonamide group in the metal coordination, in agreement with the potentiometric results that indicated a coordination via amine group. The carboxylate group may also be coordinated to zinc, although the estimated value of $\log\beta_{ZnL}$ (ca. 3.6, $\log\beta_{ZnHL} - \log K_1$) is lower than the reported one for glycine (4.96 with α-aminocarboxylate coordination mode[14]) probably due to the electron-withdrawing effect of the amide group in its vicinity. With regard to H_2L^3, a comparative analysis of the potentiometric curve profiles of the ligand, alone and in the presence of Zn^{II}, showed a superimposition of the two curves in the buffer region corresponding to the deprotonation of the carboxylic group, thus indicating that the carboxylate group is not in the coordination sphere of the metal ion, according to our expectations. Furthermore, the global formation constants calculated for the zinc complexes with this ligand ($\log\beta_{ZnL}$ = 4.96 and $\log\beta_{ZnL_2}$ = 8.68) are quite similar to the reported values for the corresponding complexes with glycinehydroxamic acid (5.38, 10.07[15]), thus suggesting that for H_2L^3 the complexation with zinc should also involve a (*O*,*O*) hydroxamate coordination mode (the small differences found for the stability constants of those two ligands are ascribed to the electron-withdrawing effect of the sulfonamide group). So, the coordination mode for the 1:1 zinc complex with H_2L^3 is different from that previously presented for H_2L^5 (see Figure 4), in which the species ZnL evidenced an (*N*,*N*,*O*) coordination type with the involvement of the hydroxamate-*N* atom, but also the amine-*N* atom and the carboxylate group.[12]

To make a comparative analysis of the affinity of the studied compounds towards Zn^{II}, the corresponding pM values at the physiological pH (pM = –log[M], C_L/C_M = 10 and $C_L = 10^{-5}$ M, pH = 7.4) were calculated, in order to account for the distinct proton concentration dependence of the compounds. Table 1 shows that, in spite of the possible different coordination modes, all the compounds present quite similar affinity for zinc (pZn ≈ 6).

The infrared (IR) spectra of the solid-state samples for the ligands H_2L^n (n = 1–4) and their corresponding zinc species were also performed to aid the identification of the metal coordination modes, namely the eventual involvement of the sulfonamide group. IR data for the ligands and respective zinc species are listed in Table 2 with provisional assignments of the selected vibrational modes.

The IR spectra of ligands H_2L^n (n = 1, 2, 4) present two bands (near 1330 and 1160 cm^{-1}), attributable to the asymmetric (as) and symmetric (sy) S=O stretching (st) frequencies; the spectra of the corresponding zinc complexes

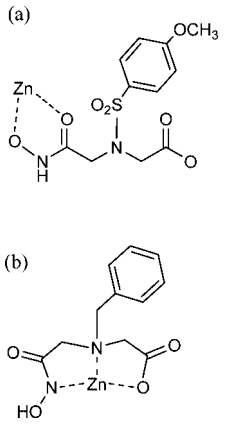

Figure 4. Proposed structures for 1:1 Zn^{II} complexes with the following ligands: (a) H_2L^3 and (b) H_2L^5[12] (charges and coordinated water molecules are omitted).

show these bands with lower intensity and two new bands appear at lower wave numbers (1260 and 1130 cm^{-1}), the intensity of which increases with Zn^{II} excess, namely from 1:1 to 2:1 (M:L) stoichiometric conditions (see Figure 5). The blue shifts of these IR bands, upon complexation,[16] suggest the involvement of the sulfonamide group on the zinc complexation, at least in the solid phase, for the compounds H_2L^n (n = 1, 2, 4).

On the contrary, the IR spectra obtained for H_2L^3 and the corresponding zinc complex reveal that the sulfonamide group is not coordinated to the metal ion, even in conditions of metal ion excess, since there is no shift of the S=O stretching frequencies (see Table 2).

The spectra of H_2L^n (n = 1, 3) also present a strong band near 1650 cm^{-1}, which should be attributed to the C=O st of the amide groups; upon zinc complexation, this band is shifted to lower wave numbers, thus suggesting that the coordination of these ligands to Zn^{II} occurs via the hydroxamate group. The corresponding C=O st band for the hydroxypyrimidinone derivative (H_2L^4) appears at a higher wave number (1722 cm^{-1}) than the primary hydroxamic acids, caused by the higher double character of this carbonyl group, and it is considerably shifted to lower energy upon complexation with zinc, thus giving support to the expected hydroxypyrimidinone (*O*,*O*) coordination mode.

Moreover, in the IR spectra of compounds containing carboxylate groups, besides the strong C=O st band corresponding to the amide groups, it is also possible to identify two bands near 1630 cm^{-1} (COO^- st, very strong) and 920 cm^{-1} (COO^- bending δ). For the systems containing H_2L^2, upon complexation, these two bands shifted to lower wave numbers, which is consistent with the participation of the carboxylate group in the zinc coordination. Nevertheless, for the H_2L^3 spectra, there is a broad band at 1628 cm^{-1}, probably arising from overlapping of C=O st (amide) and COO^- st (carboxylate), which upon complexation presents only a slight shift to lower wave numbers. Although this feature cannot give much help to the elucidation of the group involved in the coordination (hydroxamate or carboxylate), the fact that no shift was observed

Table 2. Infrared spectroscopic data of compounds H_iL^n (n = 1–4) and respective zinc(II) species (C_M/C_L = 2).

Systems	Vibrational spectra ν [cm^{-1}] S=O as st	S=O sy st	C=O st amide	COO^- as st	COO^- δ
H_2L^1	1334	1162	1653	–	–
(2:1) Zn^{2+}/H_2L^1	1259	1138	1641		
H_2L^2	1334	1160	1660	1633	912
(2:1) Zn^{2+}/H_2L^2	1265	1136	1639	1605	858
H_2L^3	1346	1151	1628	1628	943
(2:1) Zn^{2+}/H_2L^3	1342	1151	1616	1616	945
H_2L^4	1306	1167	1722	–	–
(2:1) Zn^{2+}/H_2L^4	1261	1136	1634		

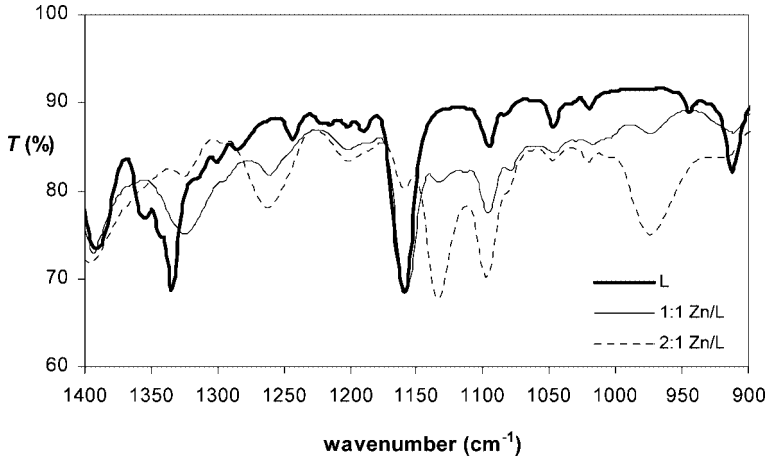

Figure 5. IR spectra of H_2L^2 and corresponding 1:1 and 2:1 Zn^{II}/H_2L^2 systems in KBr pellets.

for the COO^- bending band, supports the noninvolvement of the carboxylate group in the coordination to the metal ion.

Therefore, the solid-state IR data corroborate the coordination modes found in the solution studies for all the ligands and indicate that for the zinc H_2L^2 complex the carboxylate group may be also involved in the metal coordination. Furthermore, it also illustrated the existence of coordination via the sulfonamide group in conditions of metal ion excess.

Previous studies of the compounds H_2L^n (n = 1–6) as MMP and CA inhibitors showed that the sulfonamide derivatives present, in general, a much higher activity against these metalloenzymes than the corresponding non-sulfonamide analogues. In fact, from all the compounds under study, enzyme inhibition (IC_{50}) of nanomolar order was only detected for the primary sulfonamide derivatives (H_2L^1, H_2L^2, H_2L^4), against CA I and CA II, and the secondary sulfonamide derivative (H_2L^3) against MMP-2, MMP-7 and MMP-9.[8,9] However, the present complexation studies reveal that, in aqueous solution, the sulfonamide group does not seem to be involved in the zinc-coordination. Therefore, since the primary sulfonamides are believed to bind the zinc active site of CAs,[4] the difference between the in vivo and the aqueous solution behaviour should be due to the inaccessibility of the active site to the stronger binding moieties. Concerning the secondary sulfonamide derivative (H_2L^3), its high inhibitory activity can be rationalized in terms of extra-functional interactions between the sulfonyl groups and specific amino-acid residues, namely through hydrogen bonds and lipophilic interactions at the active site of the metalloenzymes, as suggested by molecular simulation.[8]

3. Conclusions

The complexation equilibrium studies of four sulfonamide derivatives (H_2L^n, n = 1–4), already known for their inhibitory activity towards zinc-containing enzymes, show that they have similar affinity for zinc (pZn ≈ 6). The solution and solid infrared studies proved that the 1:1 complexes with ligands H_2L^n (n = 1, 3, 4) present (O,O) hydroxamate coordination mode, while the zinc complex with H_2L^2 seems to involve both the N-amine and the carboxylate groups. Moreover, the infrared studies of the solid complexes indicate that the metal coordination via the sulfonamide groups can mainly occur under conditions of zinc excess. Therefore, the considerable improvements observed in the enzyme inhibitory activity of these sulfonamide-containing compounds, as compared to the non-sulfonamide derivatives, does not seem to be determined by their zinc coordination mode but by extra-binding or specific stereochemical requirements for the accommodation of the inhibitor into the enzyme active site.

4. Experimental Section

4.1. Materials and General Information

The synthesis of the compounds has already been reported or is in the process of being published, namely the hydroxamic/acetic acid derivatives[8,9,12]: 2-(*N*-{[2-(4-sulfamoylphenyl)ethyl-carbamoyl]methyl}-*N*-benzyl-amino)acetohydroxamic acid (H_2L^1), 2-(*N*-{[2-(4-sulfamoylphenyl)ethylcarbamoyl]methyl}-*N*-benzylamino)acetic acid (H_2L^2), *N*-[(hydroxycarbamoyl)methyl]-*N*-(4-methoxyphenylsulfonyl)aminoacetic acid (H_2L^3), *N*-{(hydroxycarbamoyl)methyl}-*N*-benzylaminoacetic acid (H_2L^5), and the hydroxy-pyrimidinone derivatives:[10,11] 4-(2-(4-sulfamoylphenyl)-ethylamino)-1-hydroxy-2(1*H*)-pyrimidinone (H_2L^4) and 4-(5-aminopentylamino)-1-hydroxy-2(1*H*)-pyrimidinone (HL^6).

All ^{1}H NMR spectra were recorded with a Varian Unity 300 MHz spectrometer at probe temperature. Chemical shifts are reported in ppm (δ) from internal reference DSS (sodium 3-trimethylsilyl-[D_4]-propionate). The pD values were adjusted with DCl or CO_2-free KOD, by using a ThermoORION model 420 instrument fitted with a combined Mettler Toledo U402-M3 S7/200 microelectrode. The IR spectra were performed with a FTS3000MX Bio-Rad spectrophotometer.

4.2. Potentiometric Titrations

Solutions: The $ZnCl_2$ (0.0156 M) solution from Merck was standardized by titration with K_2H_2EDTA (EDTA = ethylenediaminetetraacetic acid). The titrant (KOH) was prepared from a carbonate-free commercial concentrate (Titrisol) and standardized by titration with a solution of potassium hydrogen phthalate, being discarded whenever the percentage of carbonate (Gran's method[17]) was higher than 0.5% of the total amount of base.

Measurements: The equipment used was as previously described.[18] All the potentiometric titrations were performed at 25.0 ± 0.1 °C and ionic strength (I) 0.10 M KCl, the atmospheric CO_2 being excluded from the cell by passing purified N_2 across the top of the experimental solution. Because of the low water-solubility of H_2L^4, this compound and HL^6 were studied in a 5% DMSO aqueous medium. For these studies, the calibration procedure was analogous to that in an aqueous medium, but in a 5% DMSO/95% water solvent mixture, and the same procedure was adopted for the parent ligand HL^6.

The cell ligand concentrations, in the absence and in the presence of the metal ion, at different stoichiometric conditions (1:1, 1:2 and 2:1 molar ratios), were ca. $(6.0–7.0)\times10^{-4}$ M, except for H_2L^3 ($C_L = 1\cdot10^{-3}$ M). The measurement of the electromotive force of the cell allowed the calculation of $[H^+]$[19] and the determined value of K_w used in the computations was $10^{-13.76}$ and $10^{-13.74}$ for the water and 5% DMSO studies, respectively.

Calculation of Equilibrium Constants: The stepwise protonation constants, $K_i = [H_iL]/[H_{i-1}L][H]$, and the overall zinc complex stability constants, $\beta_{Zn_mH_hL_l} = [Zn_mH_hL_l]/[Zn]^m[H]^h[L]^l$, were calculated by fitting analysis of the potentiometric titration curves of the ligand in the absence and in the presence of the zinc ion, respectively, with the HYPERQUAD 2003 program.[20] The stability constants determined in aqueous medium for the Zn^{2+} hydrolytic species[21] were included in all the equilibrium models proposed for the metal complex systems and the species distribution curves were plotted with the HYSS program.[20] The quoted errors are the standard deviations of the overall stability constants given by the program for the input data. The K_i standard deviations were determined by the normal propagation rules and do not represent the total experimental errors.

4.3. ^{1}H NMR Titrations

Measurements: ^{1}H NMR titrations of solutions containing H_2L^n (n = 1, 2, 4, $C_L \approx 10$ mM) or of the corresponding Zn^{II}/H_2L^n, 1:1 systems were performed in NMR tubes. The adopted medium was D_2O and 1:4 DMSO/D_2O solutions for ligands H_2L^n, with n = 1, 2 and n = 4, respectively. The pH* value corresponds to the reading of the pH meter calibrated with the aqueous buffers (pH 4 and 7) and it was adjusted with DCl or CO_2-free KOD. The final pD values are determined from the equation pD = pH* + 0.40.[22]

Calculation of Equilibrium Constants: The $\log K_3^D$ value for H_2L^2 was calculated by fitting analysis of the experimental data, using the PSEQUAD program[23] and then that value determined in D_2O was converted into the corresponding value in H_2O by the equation $pK^D = 0.32 + 1.044\ pK^H$.[24]

4.4. IR Measurements

The solid-state IR spectra of the compounds H_2L^n (n = 1–4) and related zinc species were performed as KBr pellets. The complexes were prepared by adding to the ligand solution the corresponding amount of 0.0156 M $ZnCl_2$ standard solution, in order to obtain a 1:1 or 2:1 metal-to-ligand molar ratio. In the cases of H_2L^1, H_2L^2 and H_2L^4, 1 M HCl solution was added to enhance the solubility. Afterwards, the pH was raised (6–8) with 0.5 M KOH solution until precipitation occurred; the solid was filtered and dried before recording the IR spectra.

Supporting Information (see also the footnote on the first page of this article): Figure S1 containing the ^{1}H NMR titration curves of H_2L^1 and 1:1 Zn^{II}/H_2L^1 systems and Figure S2 containing species distribution diagrams for the 1:2 Zn^{2+}-to-ligand systems with ligands H_2L^n (n = 1–4).

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[1] C. M. Overall, O. Kleifeld, *Nat. Rev. Cancer* **2006**, *6*, 227–239.
[2] C. T. Supuran, A. Scozzafava, "Matrix Metalloproteinases (MMPs)" in *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development* (Eds.: H. J. Smith, C. Simons), Taylor & Francis, London, New York, **2002**, p. 35–61.
[3] E. Svastova, A. Hulikova, M. Rafajova, M. Zat'ovicova, A. Gibadulinova, A. Casini, A. Cecchi, A. Scozzafava, C. T. Supuran, J. Pastorek, S. Pastorekova, *FEBS Lett.* **2004**, *577*, 439–445.
[4] C. T. Supuran, A. Casini, A. Scozzafava, *Med. Res. Rev.* **2003**, *23*, 535–558.
[5] A. Scozzafava, C. T. Supuran, *J. Med. Chem.* **2000**, *43*, 3677–3678.
[6] A. Casini, A. Scozzafava, A. Mastrolorenzo, C. T. Supuran, *Curr. Cancer Drug Targets* **2002**, *2*, 55–75.
[7] R. Morphy, Z. Rankovic, *J. Med. Chem.* **2005**, *48*, 6523–6543.
[8] M. A. Santos, S. M. Marques, T. Tuccinardi, P. Carelli, L. Panelli, A. Rossello, *Bioorg. Med. Chem.*, in press.
[9] M. A. Santos, S. M. Marques, D. Vullo, A. Innocenti, A. Scozzafava, C. T. Supuran, unpublished results.
[10] M. A. Esteves, A. Cachudo, C. Ribeiro, S. Chaves, A. Rossello, M. A. Santos, *Metal Ions in Biology and Medicine*, John Libbey Eurotext, **2006**, vol. 9, p. 35–39.
[11] M. A. Esteves, A. Cachudo, S. Chaves, M. A. Santos, *Eur. J. Inorg. Chem.* **2005**, 597–605.
[12] S. Chaves, S. Marques, M. A. Santos, *J. Inorg. Biochem.* **2003**, *97*, 345–353.

[13] B. Kurzak, H. Kozlowski, P. Decock, *J. Inorg. Biochem.* **1991**, *41*, 71–78.
[14] R. M. Smith, A. E. Martell, *Critical Stability Constants*, Plenum, New York, **1989**, vol. 6, p. 2.
[15] E. B. Paniago, S. Carvalho, *Inorg. Chim. Acta* **1987**, *136*, 159–163.
[16] G. Socrates, *Infrared and Raman Characteristic Group Frequencies, Tables and Charts*, John Wiley & Sons, Chichester, **2001**; W. P. Griffith, S. I. Mostafa, *Polyhedron* **1992**, *11*, 2997–3005.
[17] F. J. C. Rossotti, H. Rossotti, *J. Chem. Educ.* **1965**, *42*, 375–378.
[18] S. Chaves, S. Marques, L. Gano, M. A. Santos, *J. Inorg. Biochem.* **2003**, *97*, 161–172.
[19] M. A. Santos, M. Gil, S. Marques, L. Gano, G. Cantinho, S. Chaves, *J. Inorg. Biochem.* **2002**, *92*, 43–54.
[20] P. Gans, A. Sabatini, A. Vacca, *Talanta* **1996**, *43*, 1739–1753.
[21] R. M. Smith, A. E. Martell, *Critical Stability Constants*, Plenum, New York, **1976**, vol. 4, p. 9.
[22] A. K. Covington, M. Paabo, R. A. Robinson, R. G. Bates, *Anal. Chem.* **1968**, *40*, 700–706.
[23] L. Zékány, I. Nagypál, G. Peintler, *PSEQUAD* 501, **2001**.
[24] R. Delgado, J. J. R. Fraústo da Silva, M. T. S. Amorim, M. F. Cabral, S. Chaves, J. Costa, *Anal. Chim. Acta* **1991**, *245*, 271–282.

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