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Interaction of monohydrogensulfide with a family of fluorescent pyridoxal-based Zn(II) receptors.

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Abstract

H_2S and its conjugated base HS^- have recently gained increasing attention for their reactivity with bioinorganic targets. However, so far, stable adducts of bioinorganic compounds with H_2S/HS^- are still scarce due to the propensity of sulfide to form insoluble metal sulfides. In this work we studied the reactivity of HS^- with a family of fluorescent zinc complexes *via* a variety of spectroscopical techniques. The complexes selected for this study feature a pyridoxal moiety with different substituent groups on the ligand framework. Interaction of the complexes under investigation with HS^- results in the displacement of the coordinated ligand from the Zn center with the concomitant precipitation of ZnS in the case of complexes **1** and **3** whereas for complex **2** our data points to the coordination of HS^- to the metal center likely assisted by hydrogen bondings with the OH of the pyridoxal moiety. In the presence of HS^- fluorescence emission of complex **2** is enhanced whereas fluorescence emission of complexes **1** and **3** results quenched. The results highlight the potential of complex **2** to be implemented as HS^- fluorescence sensor *via* a coordinative-based approach.

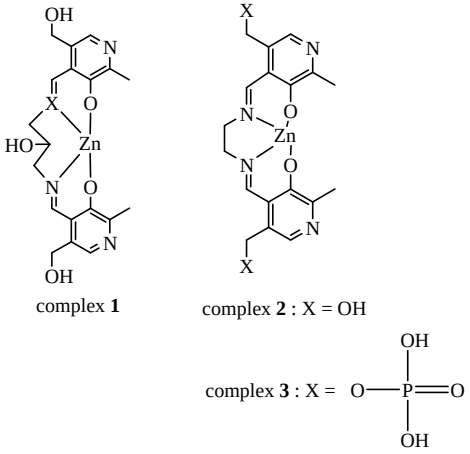
Introduction

In the last decades emerged that along with the traditional toxic action of H_2S,[1;2] this molecule exhibits a large variety of biological roles, most of which are not completely understood yet.[3] Its role as gaseous signaling molecule together with nitric oxide (NO) and carbon monoxide (CO) has been recently acknowledged.[4-6] While biological reactivity of NO[7-24] and CO[25-29] has been extensively studied and clarified by exploring their coordination chemistry to bio-inspired metal

complexes,[30-32] the understanding of the H_2S reactivity is still ongoing due to the fewer studies involving this molecule. An aspect to be considered is the fact that H_2S is a weak acid and in aqueous solutions dissociates, forming hydrogen sulfide anions (HS^-) and sulfide (S^{2-}) ions (pK_{a1} = *6.88 and* pK_{a2} *= 14.15)*. Thus, at physiological pH both the neutral (H_2S) and monoanionic (HS^-) forms are present. Of course this renders the studies on H_2S reactivity in biological media more difficult and the specific chemistry associated with its specific protonation state is still controversial. In light of these considerations finding a way to differentiate the reactivity of H_2S from that of HS^- is a challenging task.

Heme centers represent the most logical targets of H_2S/HS^- metal-based reactivity;[33-46] thus most of the studies on its reactivity focus on iron-based systems. To date, however, papers reporting the coordination of H_2S to bio-inspired transition-metal scaffolds remain still limited. This is probably due to the redox activity of sulfide and to its propensity to form insoluble metal sulfides which inhibit isolation of metal hydrosulfido adducts.[47;48] Harnessing this characteristic, over the years a large number of copper and zinc complexes have been reported in the literature as 'one-pot' H_2S sensors by exploiting the metal displacement approach.[49] However, examples of transition metal complexes (with the metal center different than iron) featuring H_2S or HS^- binding to the metal centre have also been isolated and reported.[47;50-56]In particular stable zinc hydrosulfide complexes remain not numerous.[47;48;53;57-61]

In this work we explore the propensity of a family of zinc complexes (see scheme 1) to bind HS^- and investigate their potential application as fluorescence-based sensors *via* a coordinative-based approach.[62] The modifications of the ligand skeleton were designed to enhance water solubility of the resulting complexes and to study the possible effect of different substituents on the metal hydrosulfido stabilization. More specifically, we selected complex **1** (see scheme 1) as a starting point featuring a potentially bridging OH group which could eventually stabilize HS^- coordination to the zinc center. However, in the course of our experiments, we found out that this complex is totally insoluble in water. This characteristic is a limitation for practical measurements of HS^- in biological fluids. Thus we designed complex **2** featuring a different bridge between the two coordinating nitrogens and complex **3** with phosphate groups on the pyridines (see scheme 1) to explore whether these new substituents would enhance water solubility of the resulting complexes and would influence somehow HS^- binding to the zinc center.



Scheme 1

Results and discussion

Our ligands of choice were obtained by condensation of pyridoxal and the appropriate diamine;[63-65] one reason to select pyridoxal-based ligands for the present study is that pyridoxal is a form of vitamin B6, a known cofactor required by many enzymes and a nontoxic metabolite: positive requirements when foreseeing (bio)sensing applications. Complexes **1**-**3** were obtained in good yields by allowing to react an equimolar amount of the deprotonated ligands and $Zn(CH_3COO)_2$ in pure methanol, by following a literature procedure.[66]

Characterization of the complexes was achieved by ESI-MS (figure S1-S3), ^{1}H and ^{13}C NMR analysis (figures S4-S9).

*1.1 Interaction of HS^- with complexes **1**-**3** studied via NMR spectroscopy*

Interaction of HS^- with complexes **1**-**3** and its possible binding to the metal centers was first studied *via* NMR. Figure 1 displays the shift in the ^{1}H NMR spectrum of complex **2** upon addition of an excess of NaSH to a DMSO-*d6* solution of the complex. Figures S10 and S11 display the ^{1}H NMR spectra of complexes **1** and **3** before and after addition of NaSH (see also figs S12-S14).

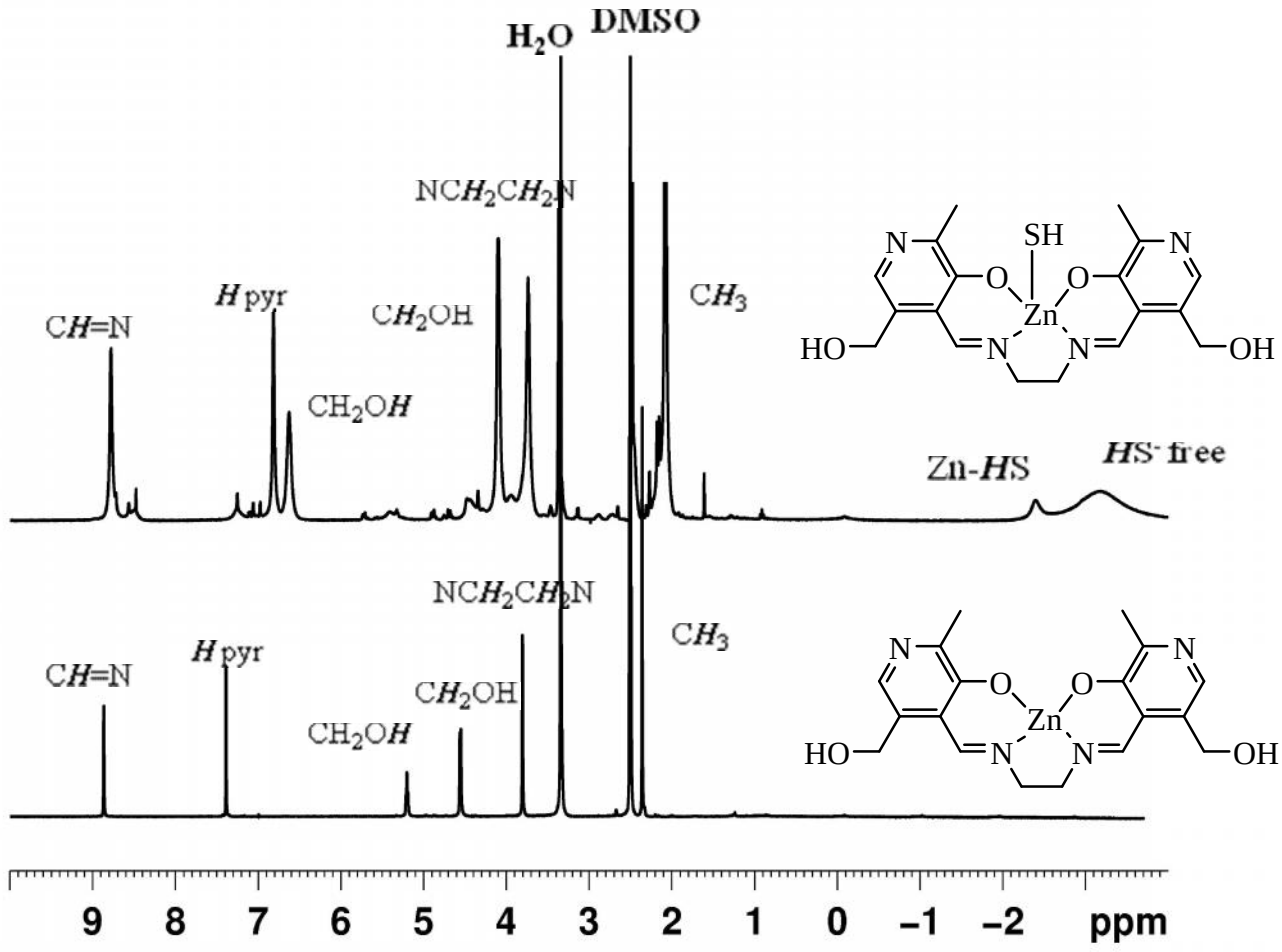


Figure 1 . ^{1}H NMR spectrum of complex **2** in DMSO-*d6* (lower trace) and after addition of an excess of HS^- (upper trace). [complex **2**] = $50*10^{-3}$ M; [NaSH] = 0,5 M.

As clearly visible from figure 1, in the presence of an excess of NaSH the whole pattern of the signals of complex **2** undergoes a shift. In addition, in the spectrum of the complex **2**-HS adduct (see figure 1, upper trace), a high field signal appears at -2 ppm which, by comparison with literature data, indicate binding of SH^- to the zinc center.[57-59] Differently when performing the same experiment in the case of complexes **1** and **3,** despite the visible shifts of the initial NMR patterns (figs S10 and S11), no high shielded signals appeared. Furthermore the resulting species feature ^{1}H NMR spectra superimposable with those of the related starting ligand. These findings suggest that in the case of complex **1** and complex **3** interaction with HS^- results in the release of the organic ligands with the concomitant precipitation of ZnS.

As control experiment (i.e. to exclude that the observed changes in the NMR spectrum upon HS^- addition were simply due to deprotonation of the oxidrilic groups or of the phosphate groups on the

ligand frameworks) we added a strong base (NaOH) to the NMR samples of complexes **1**-**3** and no visible changes in the initial ^{1}H NMR spectra could be detected.

In a different experiment we observed that when bubbling H_2S gas through the NMR samples of each complex under investigation no shifts in the NMR pattern could be detected. This suggests that the treatment of complexes **1**-**3** with neutral H_2S fails to produce any reaction.

*1.2 Interaction of HS^- with complexes **1**-**3** studied via UV-visible spectroscopy*

Next, we started a study *via* UV-vis spectroscopy in DMSO to gain further insights on the mechanism by which the target analyte interacts with complexes **1** and **2** (as said before complex **3** resulted insoluble in DMSO). In the presence of an excess of NaSH (~ 10 equivalents) the electronic absorption spectra of both complexes **1** and **2** underwent sizeable modifications (see figure 1) pointing to the formation of new species. More precisely, in the case of complex **1** we obtained a spectrum which closely resembles that of the starting ligand which suggests decomplexation of the zinc center from the organic ligand with concomitant precipitation of ZnS (see figure S15 where is displayed also the spectrum of ligand **1** in the absence and in the presence of HS^-). As for complex **2** the intense band centered at 378 nm is red-shifted to 400 nm and a less intense band centered at 604 nm appears.

Upon addition of HS^- a concomitant and immediate color change (visible to the naked eye) from yellow to dark orange (which is the color of the free ligand) occurred in the case of complex **1** whereas for complex **2** addition of HS^- resulted in a color change from yellow to light yellow.

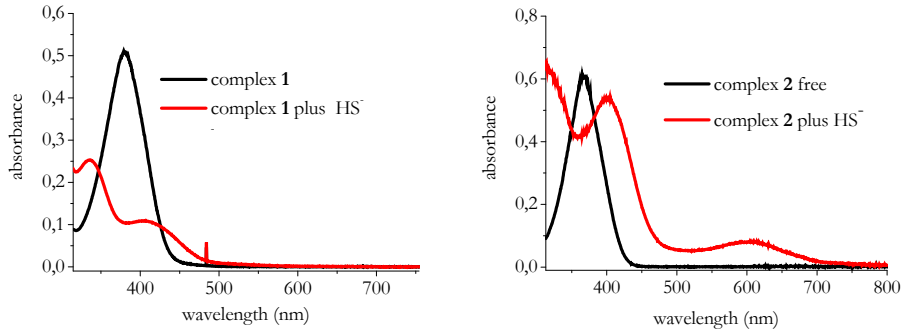


Figure 1. Electronic absorption spectra of complexes **1** and **2** free and upon addition of 500µM of NaSH. Spectra of complexes **1** and **2** measured in DMSO (rt). [complexes] = 50µM**.**

Since complex **3** is insoluble in DMSO, we studied its interaction with HS^- *via* UV-vis in aqueous buffered solution. For comparative purposes we registered also the spectrum of complex **2** in buffer solution (see figure 2) . Complex **1** resulted insoluble in aqueous buffered solution.

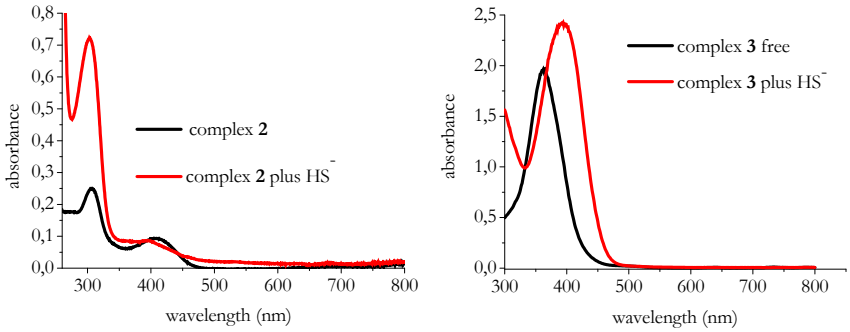


Figure 2. Electronic absorption spectra of complexes **2** and **3** free and upon addition of 500µM of NaSH. Spectra registered in (MOPS buffer (20 mM). [complexes] = 50µM**.**

The initial spectrum of complex **2** in these experimental conditions appears quite different from that in DMSO featuring absorption bands at 315 nm and 410 nm due to π-π* and n-π* intraligand transitions. In the presence of HS^- the 315 nm band increases. In the case of complex **3** the interaction with HS^- resulted in the red-shift (to 392 nm) of the band centered at 378 nm. The latter spectrum closely resembles that of the organic ligand in the presence of HS^- (figure S16). This finding suggests that also in the case of complex **3** interaction with HS^- results in the release of the ligand (with the concomitant precipitation of ZnS).

*1.3 Interaction of HS^- with complexes **1-3** studied via mass spectrometry*

To obtain further indications on the mechanism by which HS^- interacts with the complexes **1-3** we examined the reactions by mass spectrometry. Figure 4 shows an enlargement of a region of the MALDI spectrum of complex **2** in the presence of 10 equivs. of NaSH in methanol (see figure S17 for the full MALDI spectrum) in which appears a peak at 453.05 (this peak is consistent with the [complex **2**-HS]$^-$ species ($C_{18}H_{21}N_4O_4SZn$)). As clearly visible in figure 4, the peak at 453.05 in the spectrum of the complex **2**-HS adduct matches the expected mass (453.05 m/z) and isotope pattern distribution of the theoretical trace.

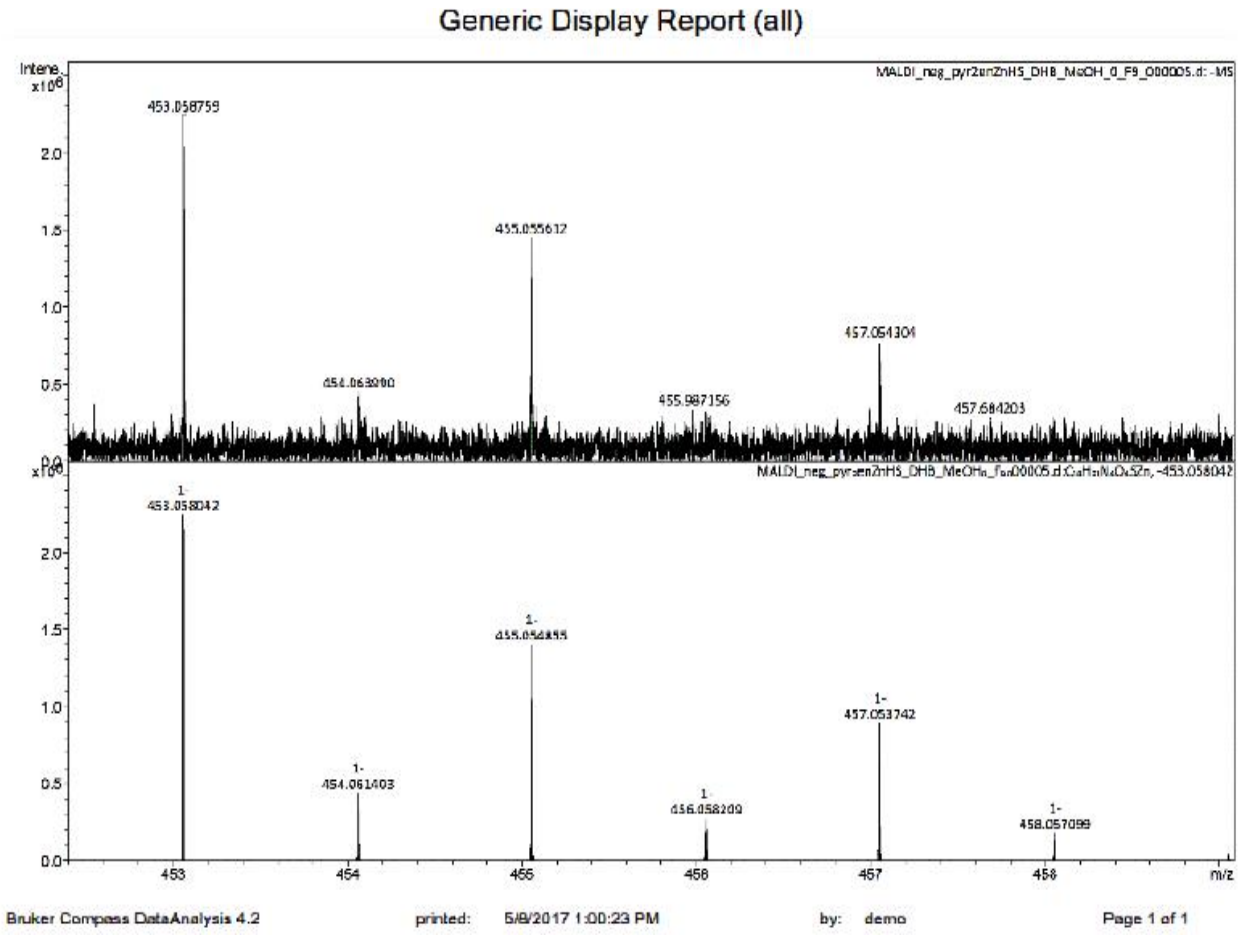


Figure 4. Enlargement of a region of the MALDI spectrum of complex **2** in the presence of 10 equiv. of NaSH in methanol (ionizing the sample in the negative ion mode). The upper trace is the experimental trace whereas the lower is the theoretical one.

This finding corroborates the binding of the analyte to the zinc complex. In the case of complexes **1** and **3** we did not observe the formation of the HS^- adducts. The ESI spectrum of complex **1** in the presence of 10 equivs. of NaSH in methanol indicates that there is the release of the organic ligand (figure S18). As for complex **3**, the MALDI spectrum in the presence of NaSH (figure S19) results in the same spectrum which we obtained when measuring the MALDI spectrum of ligand **3** with 10 equivs of NaSH (figure S20). However, the latter spectrum is clearly different from that of ligand **3** without NaSH (figure S21). This last finding suggests that ligand **3** tends to react with the HS^- anion.

*1.3 Interaction of HS^- with complexes **1-3** studied via fluorescence spectroscopy*

In an effort to study whether one of the synthesized complexes can efficiently function as HS^- sensor, we studied the interaction of HS^- with complexes **1-3** *via* fluorescence spectroscopy. In the presence of an excess of NaSH sizeable modifications of the initial fluorescence emission spectra of each complex under investigation were observed (see figure 5).

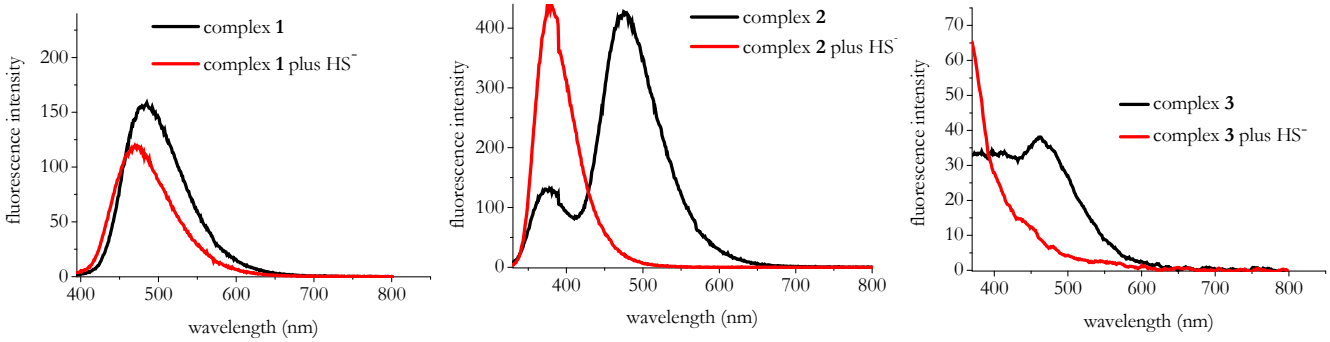


Figure 5. Emission spectra of complexes **1**-**3** free and upon addition of 60 equivs of NaSH. [complexes **1**-**3**] = $1 \cdot 10^{-5}$M; [NaSH] = $60 \cdot 10^{-5}$M. complex **1** (λ_{exc} = 380 nm, DMSO); complex **2** (λ_{exc} = 316 nm, MOPS 20 mM, pH = 7.4); complex **3** (λ_{exc} = 365 nm, MOPS buffer 20 mM, pH 10).

In detail, fluorescence emission of complex **1** is quenched and blue shifted; complex **2** undergoes a quenching of the emission band centered at 500 nm whereas the 380 nm band is sizeably enhanced. As for complex **3** the emission band centered at 480 nm is quenched. Zinc complexes functioning as H_2S sensors via the metal displacement approach and exhibiting quenching of the initial fluorescence intensity have been already reported in the literature.[49;67-69]

In the next set of experiments we focused on complex **2**, which can behave as a 'turn-on' sensor for HS^- detection (for practical measurements a 'turn-on' sensor is inherently more sensitive than a 'turn-off 'one).

To assess whether the modification of the fluorescence emission depends on the amount of HS^- we monitored the change of the initial fluorescence intensity after the addition of increasing concentrations of NaSH. Figure 6 displays the response of the fluorescence switching of complex **2** for a series of subsequent measurements with increased concentrations of NaSH. The changes in the emission spectra were fit to a 1:1 binding isotherm. The value of the apparent equilibrium constant of association (K_a) was quoted to be $1.3 \times 10^5\ M^{-1}$ (insert in figure 6). [70]

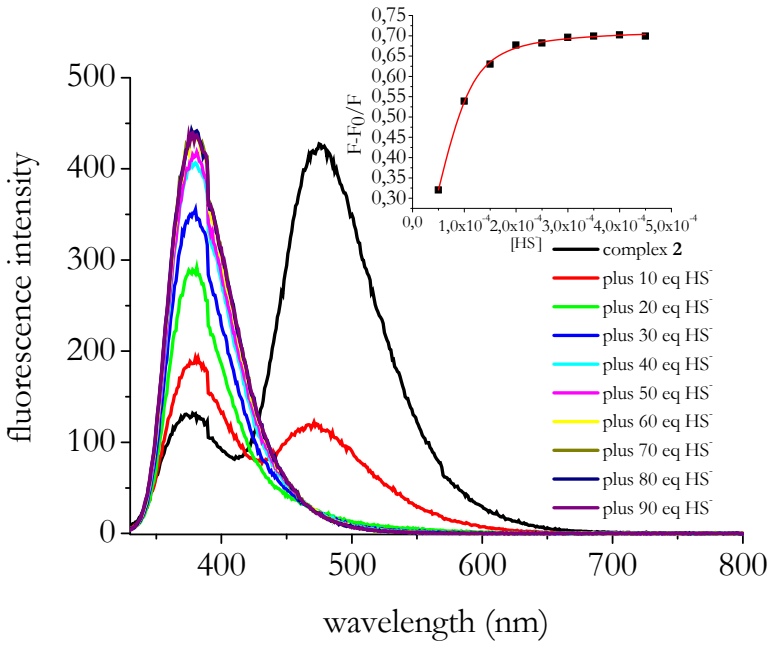


Figure 6. Emission spectra of complex **2** (λ_{exc} = 316 nm) when titrated with NaSH (rt, MOPS 20mM pH 7.4). [complex **2**] = $1*10^{-5}$M; end concentration of NaSH varied in the range (10-90) $*10^{-5}$M. **Insert**. The fluorescence titration of complex **2** with NaSH shown in this figure have been fit to a binding isotherm. The ratio $F\text{-}F_0/F$ is plotted versus the total HS^- concentration where F_0 is the fluorescence intensity of the solution without HS^-. The solid line represents the best fit to the data with a K_a of $1.3 \times 10^5\ M^{-1}$.

*1.4 Investigations into the recognition mechanism of HS^- by complexes **1-3***

As largely documented in the literature, when H_2S/HS^- interacts with a metal complex, three scenarios are possible: *i*) displacement of the metal from the ligand to generally produce fluorescence changes *via* HS^- mediated precipitation of metal sulfides, *ii*) binding of HS^- to the metal center, *iii*) HS^--mediated reduction of the metal center (in the case of redox-active metals).

In the present work NMR, UV-VIS, mass and fluorescence investigations support scenario *i*) in the case of complexes **1** and **3** whereas in the case of complex **2** point to scenario *ii*).

In an effort to understand the possible reasons of the different behavior of complex **2** we further investigated its interactions with HS^- by NMR. When carefully looking at the spectrum in figure 1 (upper trace), it is evident that the signal ascribed to the $CH_2O\boldsymbol{H}$ protons on the pyridines (at 5.198 ppm) exhibited a dowfield shift (to 6.63 ppm). In the literature downfield shifted protons are usually explained as a result of the deshielding promoted by hydrogen-bonding interactions.[48;71-74] Most likely, in the present case, the dowfield shift of the oxidrilic protons observed in the presence of HS^- may be due with their involvement in hydrogen-bonding interactions with the Zn-SH moiety. A NOESY NMR experiment revealed a NOE contact between the S***H*** and the C$\boldsymbol{H}_2$OH signals (see figure S22), supporting the latter hypothesis. Stabilization of a Zn (II) hydrosulfide complex by hydrogen-bond assistance of the ligand was previously reported.[48]

Conclusions

HS^- reactivity with a family of fluorescent zinc receptors was studied *via* NMR, UV-vis, MALDI-FT-ICR and fluorescence experiments. Complexes **1** and **3** in the presence of HS^- undergo zinc precipitation with the concomitant release of the organic ligand. Differently, complex **2** coordinates HS^- to the zinc center, as demonstrated by NMR, UV-vis and MALDI investigations. Most likely in the case of complex **2** the stabilization of the zinc hydrosulfide complex is promoted by hydrogen-bonding interactions between the CH_2OH groups on the pyridines and the Zn-SH moiety.

The fluorescence screening indicated that in the presence of HS^- complex **2** undergoes an enhancement of the initial fluorescence intensity, while complexes **1** and **3** exhibit a quenching. Complex **2** resulted soluble in physiological conditions (MOPS buffer, pH 7.4) and fluorescence measurements were performed in these conditions. Furthermore the fluorescence of complex **2** displays a concentration-dependent trend. The binding affinity constant for the HS^- to the zinc center was quoted to be $1.3\times 10^5\ M^{-1}$ when fitting the fluorescence data to a binding isotherm.

The systems under investigation did not exhibit any reactivity with neutral H_2S. The selectivity of the present systems for HS^- over H_2S foresees their potential applications in biological media to shed light on the reactivity of the different protonation states of H_2S.

We believe our findings might be helpful in the fast growing field of H_2S/HS^- sensors.

Experimental section

Materials: All chemicals used for the synthetic work were obtained from Sigma-Aldrich or Strem Chemicals and were of reagent grade. They were used without further purification. Synthesis of ligands **1-3** and of complexes **1-3** was performed by following literature procedures.[63-66]

General. HR MALDI mass spectra or ESI analyses were recorded using a Bruker SolariX XR Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a 7 T refrigerated actively-shielded superconducting magnet (Bruker Biospin, Wissembourg, France). For the ESI experiments samples were ionized in negative ion mode using the ESI ion source (Bruker Daltonik GmbH, Bremen, Germany). The mass range was set to m/z 150 – 3000. The mass spectra were calibrated externally using a NaTFA solution in negative ion mode. A linear calibration was applied. For the MALDI experiments samples were ionized in positive or negative ion mode using the MALDI ion source. The mass range was set to

m/z 150 – 2000. The laser power was 15% and 15 laser shots were used for each scan. Mass spectra were calibrated externally using a mix of peptide clusters in MALDI ionization positive ion mode. A linear calibration was applied. To improve the mass accuracy, the sample spectra were recalibrated internally by matrix ionization (2,5-DHB). The samples were dissolved in methanol at a concentration of 1 mg/mL. For the *in situ* experiments between complex **2** and NaSH the reaction was performed in methanol in a 1:10 molar ratio. The matrix used was 2,5-DHB acid and was dissolved in H_2O/CH_3CN (50:50) with 0.1% formic acid at a concentration of 30 mg/mL. Solutions of matrix and sample were mixed in a volume ratio of 1:1. The mixed solution was spotted onto a stainless steel MALDI target and left to dryness. Room temperature NMR spectra were recorded on a Bruker AVANCE 400 NMR instrument (^{1}H, 400.13 MHz; ^{13}C, 100.62 MHz) or on a 600 MHz spectrometer [600 (^{1}H) and 150 MHz (^{13}C)] using 5 mm o.d. NMR tubes. The chemical shifts were reported in ∂ (ppm) referenced to $SiMe_4$. Typically, 5 mg of the complex in 0.5 mL of solvent were used for each experiment.

Characterization of complex 1: MS (ESI methanol): m/z (%) 451.09 (100) [complex **1**-H]$^+$. ^{1}H NMR [400 MHz, DMSO-d_6]: δ = 8.60 (s, 2H, C***H***=N), 7.33 (s, 2H, pyridinio-***H***), 5.13 (s, 6H, CH_2O***H*** + NCH_2CH***OH***CH_2N), 4.53 (dd, 4H, C***H***$_2$OH), 4.0-3.3 (m, 5H, NC***H***$_2$C***H***OHC***H***$_2$N), 2.30 (s, 6H, pyridinio-C***H***$_3$), ^{13}C NMR [100 MHz, DMSO-d_6] : δ = 21.43 (***C***H_3), 60.68 (***C***H_2OH), 68.97 (N***C***H_2CHOH***C***H_2N), 69.17(NCH_2***C***HOHCH_2N), 118.53 (***C***-aromatic), 131.23 (***C***-aromatic), 134.83 (***C***-aromatic), 156.07 (***C***-aromatic), 165.18 (***C***-aromatic), 168.21 (***C***H=N).

Characterization of complex 2: MS (MALDI FT-ICR methanol): m/z (%) 443.066 (100) [complex **2** Na]$^+$. ^{1}H NMR [400 MHz, DMSO-d_6]: δ =8.86 (s, 2H, C***H***=N), 7.39 (s, 2H, pyridinio-***H***), 5.20 (t, 2H, CH_2O***H***) 4.56 (d, 4H, C***H***$_2$OH), 3.80 (s, 4H, NC***H***$_2$C***H***$_2$N), 2.36 (s, 6H, pyridinio-C***H***$_3$), ^{13}C NMR [100 MHz, DMSO-d_6] : δ = 20.70 (***C***H_3), 57.07 (N***C***H_2***C***H_2N), 59.98 (***C***H_2OH), 117.90 (***C***-aromatic), 130.89 (***C***-aromatic), 134.13 (***C***-aromatic), 155.32 (***C***-aromatic), 163.52 (***C***-aromatic), 165.91 (***C***H=N).

Characterization of complex 3: MS (ESI water): m/z (%) 288.998 (100) [complex **3**- 2H/2]$^{2-}$. ^{1}H NMR [400 MHz, D_2O, pH = 10]: δ =8.87 (s, 2H, C***H***=N), 7.52 (s, 2H, pyridinio-***H***), 4.78 (d, 2H, C***H***$_2$OP), 3.90 (s, 4H, NC***H***$_2$C***H***$_2$N), 2.36 (s, 6H, pyridinio-C***H***$_3$), ^{13}C NMR [100 MHz, DMSO-d_6] : δ = 20.25 (***C***H_3), 58.22 (N***C***H_2***C***H_2N), 62.83 (***C***H_2OP), 122.40 (***C***-aromatic), 129.10 (***C***-aromatic), 133.12 (***C***-aromatic), 156.35 (***C***-aromatic), 162.90 (***C***-aromatic), 165.73 (***C***H=N).

Absorbance and fluorescence measurements. Absorption spectra were recorded on a Cary-50 Spectrophotometer, using a 1 cm quartz cuvette (Hellma Benelux bv, Rijswijk, Netherlands) and a slit-width equivalent to a bandwidth of 5 nm. Fluorescence spectra were measured on a Cary

Eclipse Spectrophotometer in a 10 × 10mm^2 airtight quartz fluorescence cuvette (Hellma Benelux bv, Rijswijk, Netherlands) with an emission band-pass of 10 nm and an excitation band-pass of 5 nm. Both absorption and fluorescence measurements were performed either in DMSO or in MOPS buffer 20 mM pH 7.4 at room temperature. Fluorescence emission spectra were registered by exciting the samples at a specific wavelength (as stated in the figure captions). In the case of complex **2** H_2S titration experiments were performed as follows: the cuvette was filled with sample solutions in MOPS buffer 20 mM pH 7.4. Then µL amounts of NaSH (a commonly employed HS^- donor) solutions in MilliQ water (to the end concentrations specified in the figure captions) were injected via gas-tight syringe at intervals of 1 min between subsequent additions. The experiment ended when no changes in the fluorescence intensities could be detected upon H_2S addition.

NMR characterization of the complexes 1-3 upon addition of HS^-. The NMR tube was charged with the free complex solutions either in DMSO-d_6 or in D_2O then NaSH solid or in solution (to the end concentrations specified in the figure captions) was added and the spectra registered.

complex **1**/HS. ^{1}H NMR [400 MHz, DMSO-d_6]: δ =8.9 (s, 2H, C*H*=N), 7.86 (s, 2H, pyridinio-*H*), 4.64 (s, 2H, CH_2OH), 4.10 (m, 2H, NCH$_2$C***H***OHCH$_2$N), 3.87 (dd, 2H, NC***H***$_2$CHOHC***H***$_2$N), 3.72 (m, 2H, NC***H***$_2$CHOHC***H***$_2$N), 2.12 (s, 6H, pyridinio-CH_3).

complex **2**/HS. ^{1}H NMR [400 MHz, DMSO-d_6]: δ =8.78 (s, 2H, C***H***=N), 6.83 (s, 2H, pyridinio-***H***), 6.63 (s, 2H, CH$_2$O***H***) 4.13 (s, 4H, C***H***$_2$OH), 3.78 (s, 4H, NC***H***$_2$C***H***$_2$N), 2.12 (s, 6H, pyridinio-C***H***$_3$), -2,38 (2, 1H, SH).^{13}C NMR [100 MHz, DMSO-d_6] : δ = 20.90 (***C***H$_3$), 61.80 (N***C***H$_2$***C***H$_2$N), 61.80 (***C***H$_2$OH), 122.0 (***C***-aromatic), 125.40 (***C***-aromatic), 133.43 (***C***-aromatic), 154.30 (***C***-aromatic), 164.60 (***C***-aromatic), 167.85 (***C***H=N).

complex **3**/HS. ^{1}H NMR [400 MHz, D_2O, pH = 10]: δ =10.26 (s, 2H, C***H***=N), 7.56 (s, 2H, pyridinio-***H***), 4.93 (s, 2H, C***H***$_2$OP), 2.54 (s, 4H, NC***H***$_2$C***H***$_2$N), 2.24 (s, 6H, pyridinio-C***H***$_3$).

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