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Synthesis of Zn (II)-Oxazoline/Pyridine Derivative Complex as a Molecular Sensing Ensemble for Aspartate and Histidine

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ABSTRACT

Synthetic receptors can readily be converted to optical chemosensors using a displacement assay and common fluorophores and/or chromophores. Most of them are designed with covalent attachment between a receptor and a reporter moiety. We now describe the design of spectroscopic chemosensors bearing Zn (II) chelates with oxazoline/pyridine sites. The current progress of our use of noncovalently attached indicators to signal binding of analytes are demonstrated. With these systems, analyte binding which we focus on the selected amino acids, aspartate and histidine, leads to indicator displacement from the binding cavity, which in turn yields a spectroscopic signal modulation. On the basis of ^{1}H NMR studies, and preliminary x-ray diffraction analysis, it is clear that Zn (II)-oxazoline/pyridine derivative receptor cooperatively act to bind a carboxylate site of amino acids. Good agreement of the binding affinity revealed that our receptor can colorimetrically sense aspartate higher than histidine. In addition, it is demonstrated that careful choice of a chelating indicator with tuned affinity toward the receptor, lower than that of the envisaged analyte, higher than that of the interferent, can provide discrimination in sensing of a desired substrate.

Key words: synthetic receptor, zinc (II)complex, colorimetric assay, aspartic acid, histidine

INTRODUCTION

Complexes of pyridine-derived ligands have attracted much interest in recent years (Aït-Haddon *et al*., 2001; Evans, 2004; Ojida *et al*., 2004; Kojima *et al*., 2005), because of the ability of the delocalized electrons from pyridine macrocyclic ring to stabilize compounds in which the metal ion, e.g. zinc (II) are held at the center to react with small molecules or facilitate spectroscopic and magnetic exchange. Derivatives of these nitrogen heterocyclic ligands may find applications in the synthesis of new catalytic systems (Evans, 2004), molecular sensing ensembles for neurotransmitters aspartate and glutamate (Aït-Haddon *et al*., 2001), and fluorescent chemosensors for some amino acids (Huaglang, 1992-1994; Fabbrizzi *et al*., 1997; Zheng *et al*., 2001; Hortalá *et al*., 2003; Ojida *et al*., 2004;). The design of multicomponent fluorescent systems able to detect the presence and monitor concentration changes of biologically active small molecules, in particular natural amino acids, is highly desirable.

A receptor capable of recognizing histidine in the presence of any other natural amino acid has been recently developed (Fabbrizzi *et al*., 1997). Selective binding of histidine requires a

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receptor capable of interacting with the imidazole residue rather than with the carboxylate group, which is common to all the amino acids and which cannot, therefore, induce any selectivity. The high sensitivity and abundance of fluorophores makes fluorescence technique among one of the most promising tools for chemo-and biosensor development. However, the selectivity of fluorescent chemosensors for histidine remains a significant challenge. A number of currently available fluorescent probes for histidine actually change their fluorescent properties upon binding with some fluorescent indicators e.g. fluorescein, coumarine 343 and cosine Y (Hortalá *et al*., 2003). These receptor/indicator adducts will quench the proximate fluorophore through either an electron- or energy-transfer process. At this state, each receptor/indicator pair was titrated with some representative L-amino acids, especially histidine. In some cases, the amino acid was able to displace the indicator from the receptor, an event signaled by full fluorescence revival. In other cases, however, histidine was not able to dislodge the indicator, with no restoration of fluorescence detected by HPLC-fluorescent responses which have a high sensitivity and resolution by using fluorescence detector (Panpae *et al*., 2006).

Colorimetric assays are currently being sought for practical applications in single analyte and multianalyte sensing (Aït-Haddon *et al*., 2001). The vast majority of these sensing systems have the chromophore covalently attached to the recognition moiety. Unlike fluorescence, it may be difficult to perturb the microenvironment sufficiently to cause a spectral modulation when absorbance spectroscopy is used. However, one excellent method for dramatically changing the microenvironment around a chromophore is its complete displacement from the receptor (Aït-Haddon *et al*., 2001; Feuster and Glass, 2003). By virtue of making ionic or hydrogen bonding interactions to a charged receptor, colorimetric indicators are particularly useful in this regard. They have different protonation states and colors when bound to the receptor or free in solution. The use of a metal ion to coordinate with the indicator would not only change the indicators ionization state, but also give metal-ligand visual transitions that lead to larger changes in color.

In this study, therefore, we have employed to synthesize a novel Zn (II)-oxazoline/ pyridine derivative complexes and have targeted amino acids as analytes, which are well- known to bind metals by cooperative chelation between the carboxylate and the amine. Specifically, we targeted L-aspartic acid and histidine by combining organic and inorganic molecular recognition motifs. Selecting binding of histidine requires a receptor capable of interacting with the imidazole residue rather than with the carboxylate group; which is common to all the amino acids. There were two goals for our study: (1) demonstrating a method for achieving large color changes in indicator displacement assays and (2) studying the extent to which cooperativity between coordination chemistry and organic molecular recognition could control selectivity. Moreover, zinc is the second most abundant heavy metal ion after iron in the human body. Zn (II) is an essential component of many enzymes (e.g. carbonic anhydrase), and also plays critical roles in maintaining key structural features of gene transcription proteins. So far, chelatable Zn (II) regulates neuronal transmission in excitatory nerve terminal (Hanaoka *et al*., 2004; Ryn *et al*., 2005; Salter *et al*., 2005). Therefore, there is considerable interest in detecting some amino acids by chelatable zinc (II) in aqueous solution.

2, 6-bis (4-isopropyl-2-oxazolin-2-yl) pyridine is one of the pyridine derivatives that are known to be universal ligands for many transition metals. We took advantage of the facile synthetic access to such structures to develop receptor ***(1)***. This Zn (II)-oxazoline/pyridine derivative complex of the ligand was used since such complexes have very little color to start.

Pyrocatechol violet **(*2*)** was chosen for the displacement assay due to its ability to chelate the Zn (II) in **(*1*)**.

In this paper, we describe synthesis and characterization of Zn (II)-oxazoline/pyridine derivative complex and its application to molecular recognition toward L-aspartic acid compared with histidine by achieving large color changes in response to the presence of the amino acid.

MATERIALS AND METHODS

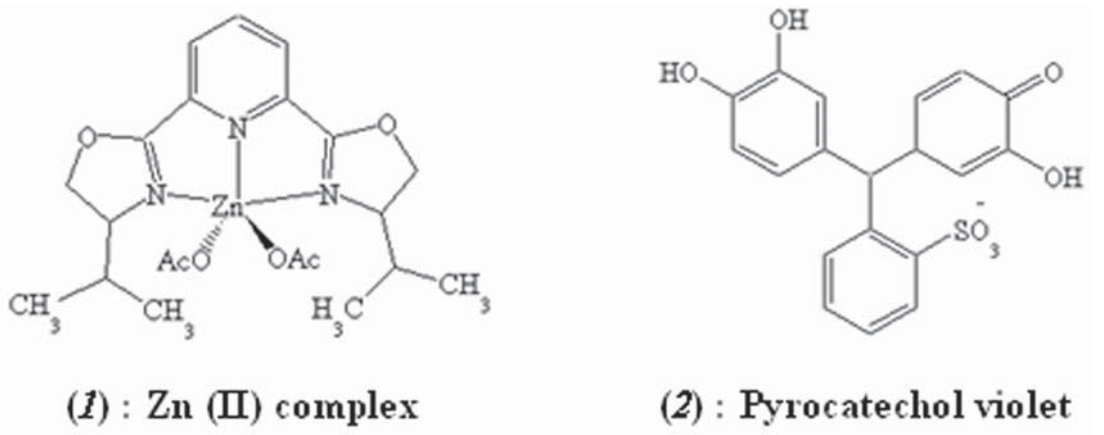
Chemicals and reagents

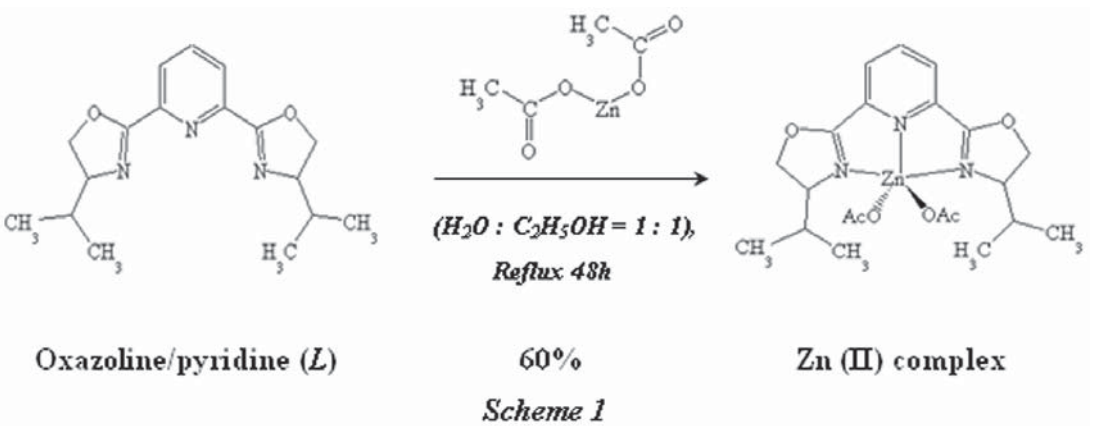
Zinc acetate dihydrate (AR grade); 2, 6-bis (4-isopropyl-2-oxazolin-2-yl) pyridine (AR grade); HEPES ($C_7H_{16}N_2O_4S$) buffer saline; pyrocatechol violet ($C_{19}H_{15}O_7S$); potassium bromide (KBr) and methanol were purchased from Aldrich-Fluka. L-aspartic acid and L-histidine were bought from Hi Media Laboratories Pvt. Limited, India. All of reagents were used without further purification.

Syntheses

Metalloreceptor **(*1*)** was synthesized in 11% yield according to the reaction in *Scheme 1*.

Zn-receptor **(*1*)**: The solution of 0.300 g (1.107 mmol) of ***L*** and 1.25 equivalent of Zn $(CH_3COO)_2 \cdot 2H_2O$ in water/ethanol (1:1) was refluxed for 48 hours. After concentration under reduced pressure; the product was precepited from CH_3OH/CH_3CN mixture. 0.0878 g. (11% yield) of white solid was obtained; mp: 440 (decomposition). CHN analysis, by CHNS/O





Scheme 1

Analyzer (Perkin Elmer Series II, Model 2400), were found: %C, H, N, O = 54.94, 9.16, 10.35 and 19.0, respectively. (Calcd: 52.02 (C); 6.00 (H); 8.67 (N) and 19.02 (O)).

Instrumentation

The UV-visible absorption measurements were recorded on Perkin Elmer (Lambda 35) spectrometer. ^{1}H NMR spectra were obtained in CD_2-Cl_2 and CD_3OD (300 MHz) used as purchased. Spectra were recorded on a Bruker ADVANCE 300 and Varian Gemini 2000 instruments. FTIR spectra were recorded on Bio-Rad FTS 175 spectrometer in the 4000-400 cm^{-1} range with KBr pellet. Isothermal magnetic susceptibility measurements were performed by using Evan's method. R and R_0 values were reported with reference to $MnCl_6 \cdot 6H_2O$ standard solution and were measured on Sherwood Scientific MKI version 1.40 Magnetic Susceptibility Balance (MSB). χ_g in cgs. unit, χ_M and μ_{eff} were derived in the same manner as for the traditional Gouy method and were calculated from

$$\chi_g = \frac{l}{m} [c \times (R - R_0) + \chi_{vair} \times A] \quad \text{------①}$$

where C is a constant of proportionality, R is the reading obtained for tube plus sample R_0 is the empty tube reading (normally a negative value), l is the sample length (cm), m is the sample mass (g), A is the cross-sectional area of the tube (cm^2) and χ_{vair} is the volume susceptibility of the displaced air. The effective magnetic moment, μ_{eff}; of the ion in Bohr magnetons (β ; BM) is related with χ_g by the expression :

$$\mu_{eff}^2 = \frac{3kT\chi_g}{N\beta^2} \quad \text{------②}$$

where N is Avogadro's number, β is the Bohr magnetons, k is the Boltzmann 's constant and T is the absolute temperature (K). We decided to use Evans' method for determination of paramagnetic species (if any) in an expected diamagnetic Zn (II)-oxazoline/pyridine derivative complexes. This method is based on that of the Evans NMR method derived by Piguet (Piguet, 1997) and Grant (Grant, 1995) with several modifications.

The x-ray diffraction data for compound ***(1)*** were collected on a Rigaku Miniflex diffractometer using Cu Kα radiation (λ = 1.542 A° ; 30 kV/15 mA). The data were integrated and scaled using a Savitzkey-Golay's method.

Molecular recognition studies

Pyrocatechol violet ***(2)*** was chosen for the displacement assay due to its ability to chelate the Zn (II) in ***(1)***. This indicator has been previously used to signal the presence of the neurotransmitters aspartate and glutamate by combining organic and inorganic molecular recognition motifs (Aït-Haddon *et al*., 2001). The Zn (II) receptor ***(1)*** solution was prepared by dissolving 0.100 g (0.19 mmol) of ***(1)*** in water and was added a solution of the indication ***(2)*** prepared by dissolving 0.015 g (10^{-3} mmol) of ***(2)*** in a water/methanol mixture (1 : 1; buffered with 10 mM HEPES at pH 7.4). Determination of molecular sensing of Zn (II) receptor ***(1)*** was performed by adding a series of ***(1)*** and the indicator ***(2)*** solutions to a series of aqueous solution, buffered at pH 7.4 with 10 mM HEPES containing each standard aspartic acid and histidine solution (0.06 to 0.75 mM). The UV-vis spectra of these chemosensing ensemble solutions for competition assays were then recorded in the 350-700 nm range.

Binding studies

Determination of apparent 1 : 1 binding constants of our receptor and histidine were performed in a degassed water solution buffered at pH = 7.4 with HEPES 10 mM; concentration of indicators was 10^{-3} mM. Aliquots of a fresh solution of ***(1)*** and indicator were added in the standard histidine solution (1.92 mM). All of potentiometric titrations were carried out at constant temperature 25 ± 0.1°C by using a Fisher

scientific stirred digital bath Isotemp 228 model. Potentiometric measurements were made with a Mettler Toledo MPC 227 model equipped with a Radiometer PMG 201-8 glass electrode coupled to a calomel electrode. The study of pH effect was determined by varying the pH of the assay buffer, i.e. HEPES 10 mM, from 6.0 to 10.0.

RESULTS AND DISCUSSION

1. Molecular design and characterization of (1)

The artificial receptor ***(1)*** was assembled via the reaction sequences depicted in *scheme 1*. Characterization of this compound was accomplished using ^{1}H NMR, FTIR, x-ray diffraction spectrometry and magnetic susceptibility analysis. Figure 1 shows the selected ^{1}H NMR in CD_3OD/D_2O (1 : 1) buffered at pH 7.4 with HEPES of ***(1)*** (Figure 1 (a)) and the chelated ***(1)*** with ***(2)***, ***(3)*** (Figure 1 (b)) depicted in *scheme 2*. A ^{1}H NMR study of ***(3)*** allowed us elucidation of the structure of ***(1)*** complexed with ***(2)*** in aqueous solution. ^{1}H NMR studies are summarized for ***(1)***: δ 8.73 (s, NH, 2H), 5.04 (s,

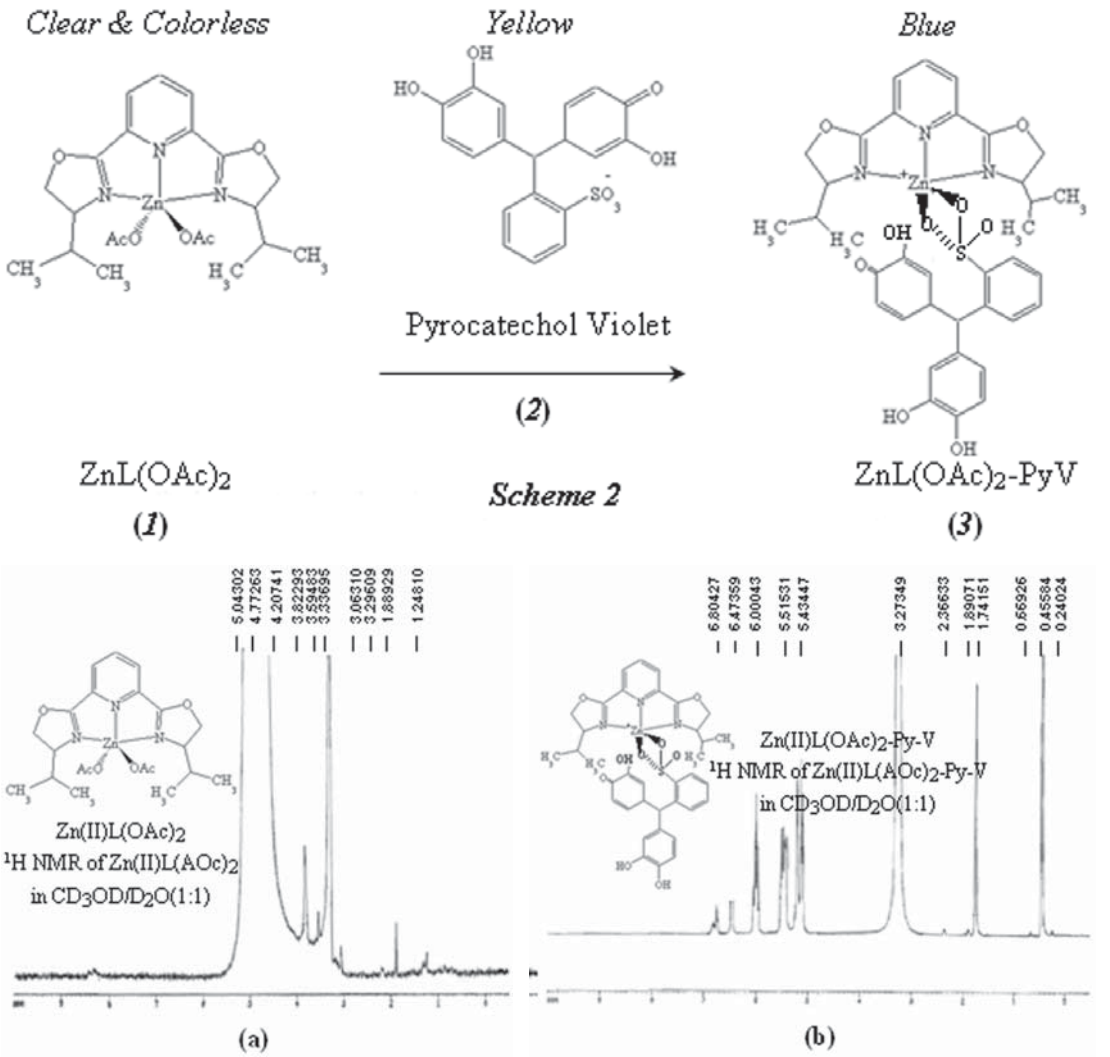


Figure 1 ^{1}H NMR spectra of ***(1)*** (0.1 mM) in the absence **(a)** and presence **(b)** of ***(2)*** measured in CD_3OD/D_2O.

4H), 4.20 (s, H, oxazoline-H), 3.76 (s, H, CH_2), 1.88 (s,-C(CH_3)$_2$-,12H); of **(*3*)**: δ 6.80-6.76 (d, 2H), 6.47-6.44 (t, 2H), 6.04-5.94 (s, H), 5.20 (s, O-H).

2. FTIR spectra

FTIR spectra for ***L*** compound with **(*1*)** are shown in Figures 2 and 3, respectively and are summarized as the followings : ***L*** 3543-3118 [ν (CH_3 , NH)], 1652 [ν (C=N, aromatic)], 1570 [ν (C=C)], 1071 [ν (C-O)] ; **(*1*)** 3543-3118 [ν (CH_3 , NH)], 2354 [ν (N-CO)], 1652 [ν (C=N, aromatic)], 1570 [ν (C=C)], 1024 [ν (C-O)].

3. X-ray crystallography

X-ray diffraction data of **(*1*)** were collected and compared with Zn $(OAc)_2 \cdot 2H_2O$. The structure of **(*1*)** was solved and refined to confirm the covalent coordination bonds of Zn (II)-N of pyridine derivative. This tendency has been observed on the spectra shown in Figures 4 (a) and (b) which indicate that the coordination environments around the metal center are considerably changed and distorted from starting molecules. This expected structure (**(*1*)** from *scheme 1*) is similar to Zn (II)-oxazoline/pyridine derivative receptor (Aït-Haddon *et al*., 2001) showed the Zn (II) ion adopts a distorted trigonal bipyramidal geometry which the guanidinium groups are roughly located in the same plane as the ligand.

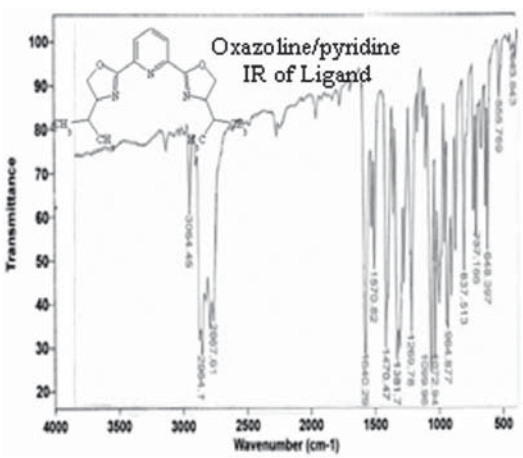


Figure 2 IR spectrum of ***L*** at 300 K.

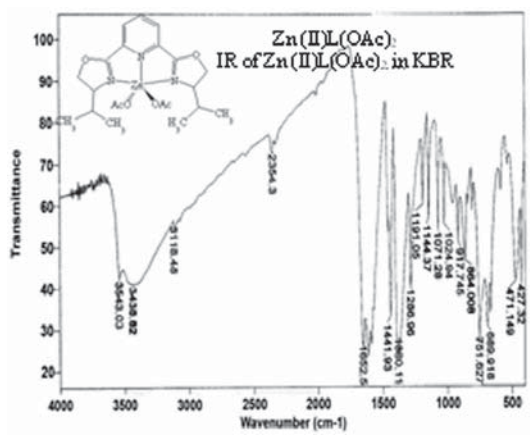
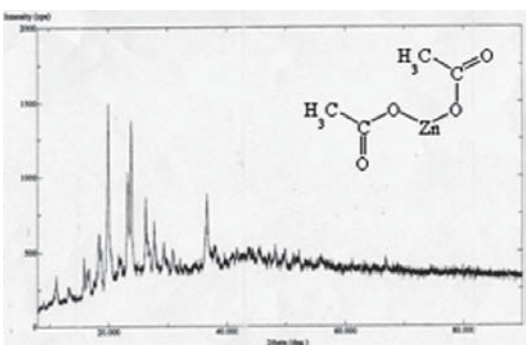


Figure 3 IR spectrum of **(*1*)** at 300 K.



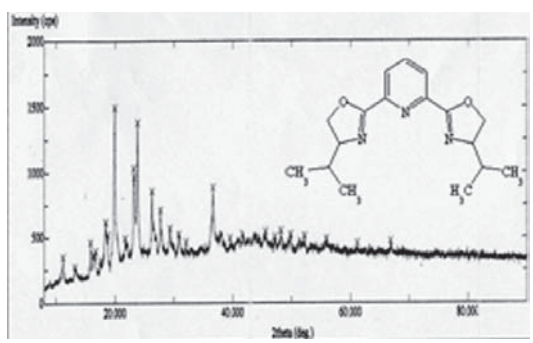


Figure 4 XRD patterns of **(a)** Zn $(OAc)_2 \cdot 2H_2O$ and **(b)** receptor **(*1*)**.

4. Exchange interactions

Magnetic susceptibility data were collected at 298 K and corrected for diamagnetism. It is found that the χ_g , χ_M and μ_{eff} of **(*1*)** are 5.93 $\times$ 10^{-7} cgs., 3.09 $\times$ 10^{-4} cgs. and 0.85 BM., respectively (values based on ten consecutive analyses). This temperature independence of the magnetic susceptibility of a pair of exchanged-coupled S = 0 and $^1/_2$ ions may be occurred, assuming an isotropic Zeeman effect, has been given elsewhere (Grant, 1995) and takes the form

$$\chi_g = \frac{[N g^2 \beta^2/kT] \times 2\exp(A/kT) + 10\exp(\beta/kT) + 28\exp(C/kT)}{1 + 3\exp(A/kT) + 5\exp(\beta/kT) + 7\exp(C/kT)}$$

with the following abbreviations : A = 2J-6.5j ; B = 6J-13.5j ; C = 12J-9.0j. The exchange integral J was evaluated by fitting this equation to the experimental susceptibility data. The fitting procedure presents some difficulties due to paramagnetic impurities, which is not unexpected in view of the preparation conditions. A change in magnetic signals upon coordinating can result from resonance energy transfer, or simple microenvironment changes. However, in this case a very low value of μ_{eff} indicate that the diamagnetic Zn II)-complexes are remained although a mechanism to communicate between metalloreceptor, containing “binding site” and a “signaling site”, and the coordinating groups established a micro- environment modulation that slightly perturbed the magnetic properties of Zn (II).

5. Spectroscopic characterization

Table 1 and Figure 5 summarize the spectroscopic properties for the **(*1*), (*2*)** together with the receptor **(*3*)** developed in our laboratory for comparison.

Table 1 Spectroscopic Data for **(*1*), (*2*)** and **(*3*)** at pH 7.4 with HEPES Buffer.

	Visible absorption	
	λ (nm)	ε ($M^{-1}cm^{-1}$)
1 (0.19 mM)	-	-
2 (0.10 mM)	444	1.70
3 (0.10 mM)	616	0.59

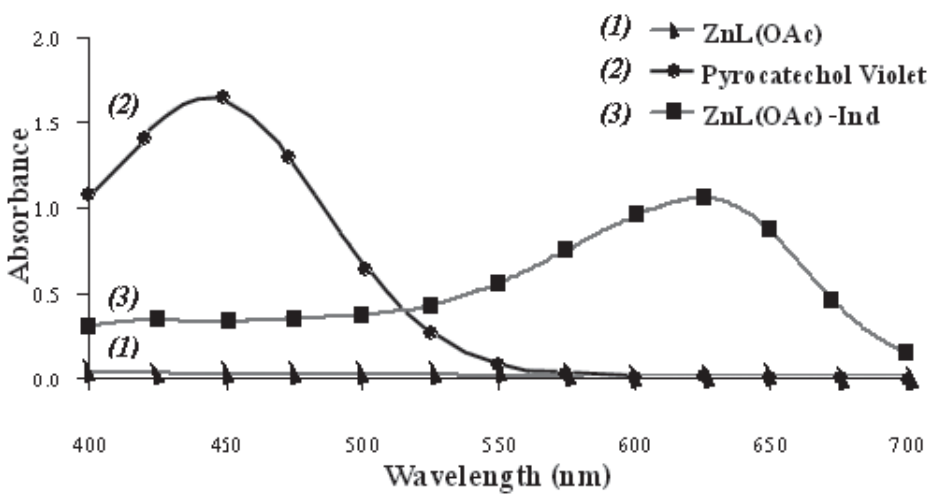


Figure 5 UV-vis absorption spectra of **(*1*)** (0.19 mM), **(*2*)** (0.10 mM) and **(*3*)** (0.10 mM) at pH 7.4 (10 mM HEPES), 300 K.

Titration of the indicator **(*2*)** with the receptor **(*1*)** at pH 7.4 buffered with 10 mM HEPES resulted in a complete quenching of the absorbance, while nonlinear least-squares fitting of the titration profiles (Absorbance activity, A vs. equiv. of (Evans, 2004)) indicated formation of 1:1 adducts. Formation of a 1:2 adducts had to be ruled out, due to the very poor fitting of titration data. It is suggested that, in the receptor/indicator 1:1 adduct, the two oxygen atoms of SO_3^- group coordinate with zinc metal center which quench due to proximate chromophore of dye through either an electron–or energy transfer process. Moreover, analysis of the ^{1}H NMR of **(*3*)** showed resonances that were indicative of an intact Zn (II) complex, confirming that the indicator did not simply strip the metal from the ligand.

6. Binding mode of the Zn (II)-pyridine based receptors to aspartic acid and histidine species

Figure 6 shows the ^{1}H NMR signals of **(*3*)** binded to aspartate Figure 6 (a) and histidine Figure 6 (b) in aqueous solution. Upon the addition of 1equid of aspartate to receptor ***(3)***, the proton signals of the pyridine ring of ***L*** slightly upfield-shifted (0.09-0.16 ppm) whereas histidine addition made little lowfield-shifted (~0.02). In addition, the proton signals of CH_2 in oxazoline groups slightly upfield-shifted (~0.04-0.10 ppm) upon adding both of aspartate and histidine. The corresponding proton signals, however, were not distinguished among the pyridine and two oxazoline rings of ***L***. Furthermore, two sets of methylene protons connected to the tertiary nitrogen become sharper upon the amino acid binding. There observations suggest that receptor **(*1*)** directly interacts with aspartate and histidine sites in aqueous solutions.

7. Binding selectivity and colorimetric sensing toward aspartic acid and histidine

Subsequently, we tested the sensing capacity of our receptor for aspartic acid and histidine by detecting the color changed from deep blue (λ_{max} = 616 nm) to a yellow (λ_{max} = 444 nm) upon addition of each amino acid to a solution of **(*3*)** buffered with 10 mM HEPES at 7.4 (Figure 7)

The addition of various concentrations of amino acids to an ensemble of **(*3*)** resulted in a complete color change from deep blue to yellow,

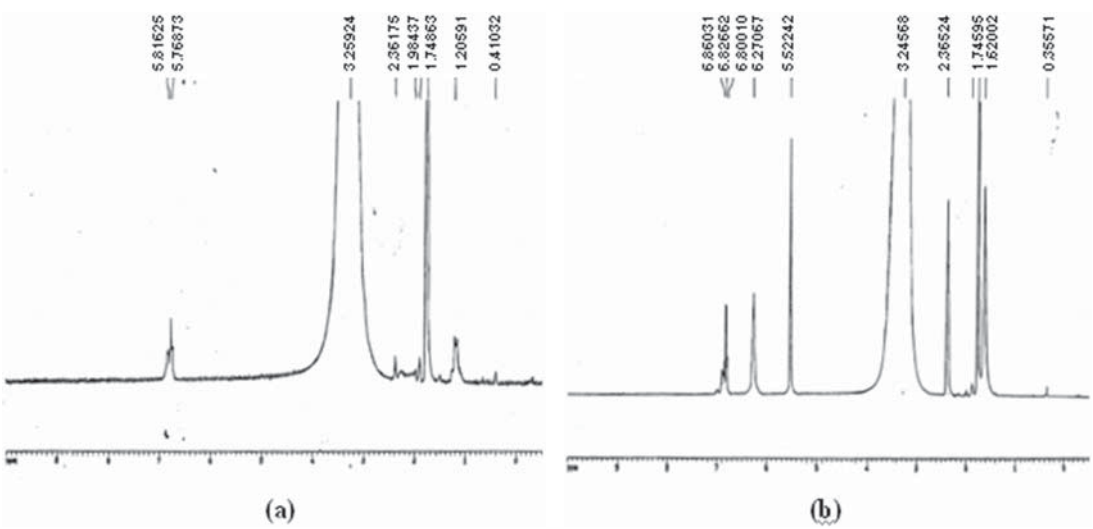


Figure 6 **(a)** ^{1}H NMR spectra of **(*3*)** (0.1 mM) in the presense of ***L***-aspartic acid. **(b)** ***L***-histidine measured in CD_3OD/D_2O (1 : 1), pH 7.4 1 equiv. of with HEPES

indicating the expected displacement of the indicator by each amino acid. Now, a sharp isosbestic point was found, pointing out a simple competition between complexes both having 1:1 stoichiometries. The change in absorbance depended on amino acid concentrations can be analyzed by using isothermal standard measurement for competitive binding to exact affinity constants for aspartic acid and histidine. By following the absorption at 616 and 444 nm (Figure 7) and analyzing the data with a 1:1 binding algorithrm (Aït-Haddon *et al*., 2001; Hortalá *et al*., 2003; Ojida *et al*., 2004) (Figure 8) the estimated binding constants (K_b, M^{-1}) of 4.70×10^{-2} M^{-1} and 4.0 $\times10^{-3}$ M^{-1} for aspartate and histidine were obtained, respectively. Upon ligation of an amino acid to the metal ion via a carboxylate and an amine, two freely rotating methyl groups on each size of ligand are placed in proximity to the aspartate or histidine chain. In comparison, the receptor **(*1*)** showed an higher affinity of binding constant toward the aspartate than histidine. These observations suggest that histidine, by immidazolate residue, does not bind as well as aspartate which possesses less steric repulsive effects exerted by the ***R*** substituent. Moreover, the oxazoline groups can deprotonate carboxylate and amine groups of aspartate, such that the hydrogen bonding interactions are probable whereas the immidazole ring of histidine

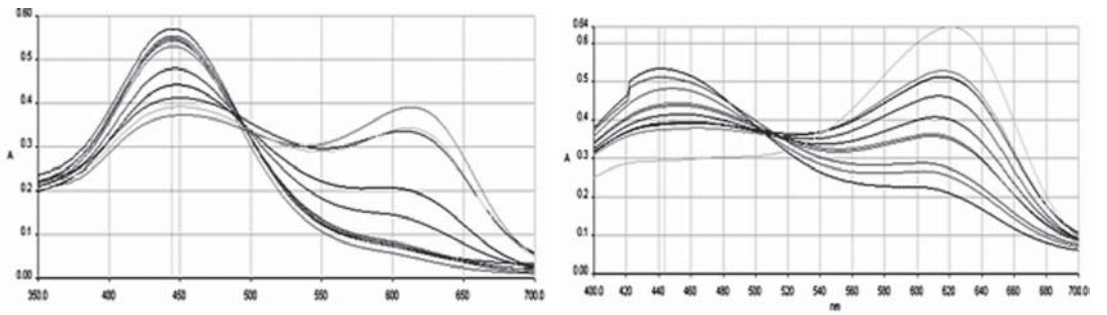


Figure 7 Addition of selected amino acids to a constant concentration of **(*3*)** (0.1 mM). **(a)** aspartic acid (0.08 – 0.68 mM). **(b)** histidine (0.19 – 0.58 mM)

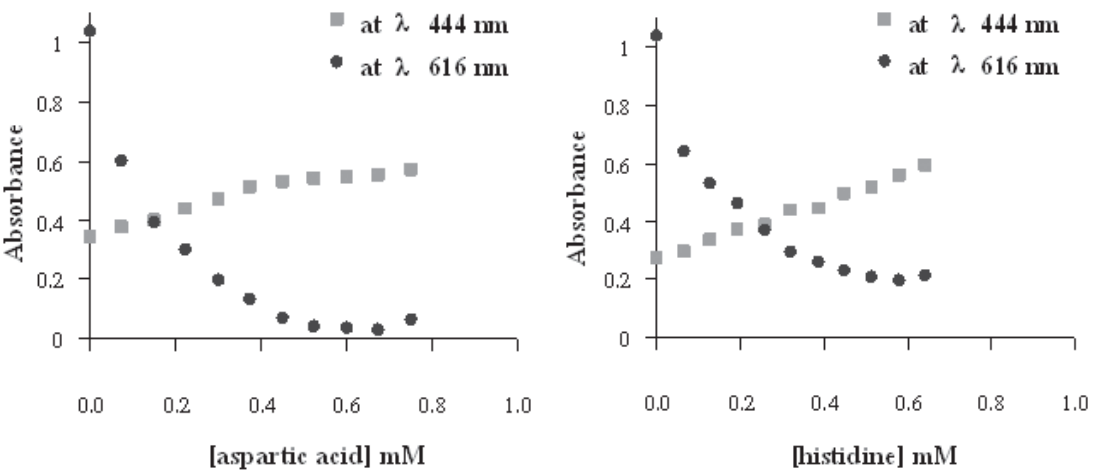


Figure 8 UV-vis calibration curve at λ = 444 and 606 nm of chemosensing ensemble mixture **(*3*)** with selected amino acids buffered with 10 mM HEPES, pH 7.4. **(a)** aspartic acid. **(b)** histidine.

cannot interact with them. All there studies, therefore, confirm a contribution of the oxazoline sides in the observed selectivity for aspartic acid. This binding selectivity and colorimetric sensing toward aspartic acid is good agreement with those obtained by Zn (II) coordination to the terpyridine derivatives as previously shown by Aït-Haddon (Aït-Haddon *et al*., 2001) and Wiskur (Wiskur *et al*., 2001).

CONCLUSIONS

In the development of chemosensors, one important feature is the mechanism through which the receptor signals the binding of the analyte. A colorimetric indicator needs to be associated with the receptor, and the most common way is to noncovalently attach the indicator. In this study, our both Zn (II)-complex receptor and pyrocatechol violet indicator match these requirements. We presented the molecular recognition and colorimetric sensing features of the Zn (II)-oxazoline/pyridine complex toward the aspartic acid and histidine. Our experimental results clearly indicates that receptors **(*1*)** can effectively bind to the selected amino acids under neutral aqueous conditions by the cooperative use of the metal-ligand interaction of the Zn (II)-oxazoline site. It is also clear that receptor **(*1*)** not only discriminate the amino acids but also show the sequence-dependent affinity toward the decreasing of steric repulsive effects. Thus, it can be suggested that the other natural amino acids not investigated here, which present more hindering substituents, should have a binding affinity lower than aspartate and could be discriminated when using this chemosensing ensemble. Thus, this receptor presented here in our a novel type of chemosensor that can directly bind and sense amino acids. Although the binding selectivity of the receptor between aspartate and histidine is not probably satisfactory, elaborated structural modification could provide artificial chemoreceptor with a reasonable recognition selectivity toward some amino acids. Furthermore, such artificial receptor may serve a potential regulator of all natural amino acids as well as a fluorescent chemosensor (Huaglang, 1992-1994; Fabbrizzi *et al*., 1997; Wiskur, 2001; Zheng *et al*., 2001; Hortalá *et al*., 2003; Ojida *et al*., 2004; Ugalde, 2005), since the hydrogen bond interactions between oxazoline sites and amino acid side chain residues significantly contribute to the stability of the complex. We are now underway along this line.

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