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Research Article

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Reduced Schiff base zinc complexes as proposed models of the active site of the dinuclear zinc enzyme *A.* Aminopeptidase

Khaleel A. Abu-Sbeih* and Abdel Aziz Abu-Yamin

Department of Chemistry, College of Sciences, Al-Hussein Bin Talal University, Ma'an, Jordan

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ABSTRACT

Complexes of zinc with ligands prepared through the reduction of Schiff bases derived from salicylaldehyde and the primary aliphatic amines 1,4-diaminobutane (H_2Salbn), 1,6-diaminohexane (H_2Salhx), and tris(hydroxymethyl) aminomethane (H_2Saltris) have been prepared and proposed as models of the active site of the zinc enzyme Aeromonas Proteolytica aminopeptidase. The complexes were characterized by ^{1}H-NMR, FT-IR spectroscopy, and mass spectrometry. In complexes of both H_2Salbn and H_2Salhx, the 5-ccordinate zinc atoms are present in a N,O environment with bridging carboxylate ligands thus providing close analogues to the enzyme's active site. On the other hand, the complex of H_2Saltris contains one zinc atom with an all-oxygen environment and one zinc atom bound to one N and five O atoms.

Key words: dinuclear zinc complexes, Reduced Schiff bases, models of enzymes, amino alcohol complexes, Aeromonas aminopeptidase.

INTRODUCTION

Zinc plays important roles in biological systems that can be either catalytic or structural. Zinc is mostly mononuclear in these systems, although there are several dinuclear zinc enzymes [1-4]. The enzymes that incorporate two zinc centers include metallo β-lactamases, alkaline phosphatases, and aminopeptidases [5]. Metallo β-lactamase, which hydrolyzes β-lactam antibiotics, contains two zinc ions bridged by a hydroxide [6]. The zinc ions are present in asymmetric nitrogen-rich environments. Alkaline phosphatase of *E. coli*, which cleaves phosphate monoesters, has two Zn^{2+} ions and one Mg^{2+} ion in the active site of each of its two subunits [7]. Zn1 is coordinated by two His, Asp and one oxygen of phosphate and Zn2 by His, two Asp, and one oxygen of the phosphate. There are no bridging ligands between the zinc ions.

Aminopeptidases, which remove the N-terminal amino acid from proteins, have two zinc atoms, typically linked by bridging carboxylate ligands, at their active sites. Bovine lens leucine aminopeptidase (LAP) contains two Zn^{2+} ions that are bridged by Glu (bidentate), Asp (monodentate), and a water molecule, Figure 1(a). Each zinc ion is five coordinated in a near square pyramid geometry [8]. Zn1 is additionally bonded to Asp and Zn2 to Lys and Asp. *Aeromonas proteolytica* aminopeptidase has two Zn^{2+} ions bridged by a water molecule and the carboxylate group of Asp, Figure 1(b). Both Zn^{2+} ions are present in very similar five-coordinate environments [9]. Carboxypeptidase G2 from *Pseudomonas sp*. and human Aminoacylase-1 both contain a dizinc center similar to that in *Aeromonas* aminopeptidase [10,11].

Many complexes have been prepared as models of dinuclear zinc enzymes. A number of multidentate lignads have been used to prepare the zinc dimers. These ligands are mostly N,O-donor ligands with varying numbers of N- and O- donating atoms. Ligands that have been used to bridge two zinc atoms together include phenolates [12-14] and carboxylates [15], or both types of bridges [16,17] among other biologically less relevant ligand bridges. The zinc complex $[(bomp)Zn_2(CO_2Me)_2]^+$ (Figure 2) with an $O\{NO_2\}_2$ heptadentate ligand (bomp) and two bridging acetate ligands has been prepared as a model for the aminopeptidase active site [13]. The zinc complex $[(bipy)_2Zn_2(O_2CMe)_3]^+$ has both monodentate and bidentate bridging carboxylates [18], in a situation similar to that found in the active site of leucine aminopeptidase.

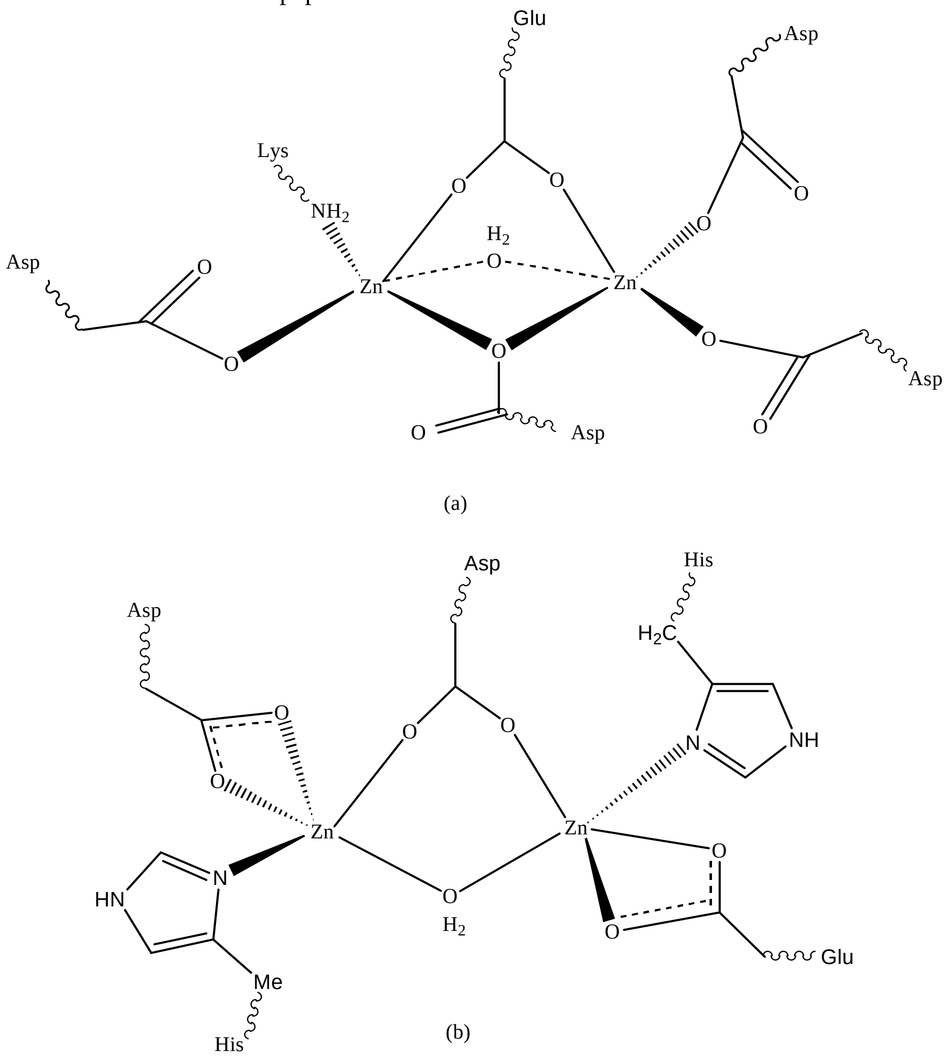


Figure 1. Drawings of the active sites of some aminopeptidases. (a) leucine aminopeptidase, (b) *aeromonas proteolytica* aminopeptidase.

Schiff bases containing the functionality –RC=N– are usually formed by the condensation of a primary amine with an active carbonyl. These ligands play an important role in inorganic chemistry as they form stable complexes with most transition metal ions including zinc [19,20]. Reduced Schiff base complexes with zinc as well as the other metals are much less studied, however [21-26]. Herein, zinc complexes with a group of reduced Schiff bases derived from salicylaldehyde and primary amines will be prepared and studied as structural models for the active site of the enzyme *Aeromonas* aminopeptidase. The structures of the reduced Schiff bases used in the current study are shown in Figure 3.

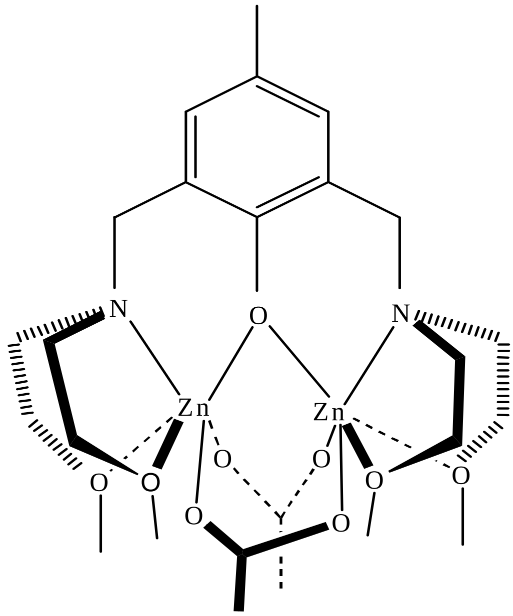


Figure 2. Zinc dimer with phenolate bridging ligands

EXPERIMENTAL SECTION

Materials and Instruments
Sodium borohydride was supplied by Lancaster, salicylaldehyde by Schuchardt Hohenbrunn, Germany, tris(hydroxymethyl)aminomethane by Merck, Germany, 1,6-hexanediamine by Fluka, Switzerland, 1,4-butanediamine by Acros, U.S.A., zinc chloride by Riedel-de Haën, sodium acetate by Loba chemie, India, and the solvents by Avonchem, U.K.

^{1}H-NMR spectra were recorded on an AV1 ultra shield 300 MHz Bruker NMR spectrometer. IR spectra were recorded on a Unicam (Mattson 5000) FTIR spectrophotometer using KBr pellets. Molecular masses were obtained using an API 3200^{TM} LC/MS/MS AB SCIEX mass spectrometer.

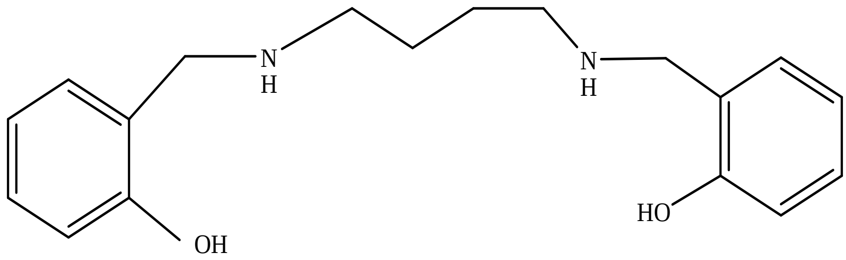
Synthesis of the Schiff bases The Schiff bases were synthesized according to published procedures [27].

Salbn: 1,4-diaminobutane (0.025 mol) was added (0.025 mol) to salicylaldehyde (0.05 mol) in 25 cm^3 of absolute ethanol. The mixture was refluxed for two hours, and then allowed to cool to room temperature. **Salbn** was filtered and recrystallized from ethanol. The yellow product was then washed with cold ethanol and diethyl ether ($C_{18}H_{20}N_2O_2$, 62% yield). ^{1}H-NMR ($CDCl_3$) δ (ppm): 13.55 (s, 2H, Ph-OH), 8.56 (s, 2H, CH=N), 6.8-7.5 (m, 4H, Ar-H), 3.64 (m, 4H, NCH_2), 1.63 (m, 4H, CH_2-CH_2). IR (KBr, cm^{-1}): 1635 (s, C=N), 1354 and 1284 (s, C-O(Ar) and O-H def), 2500-3000 (br, s, OH), 1604 (s, C=C), 2930 and 2860 (s, R-H), 3020 (w, Ar-H).

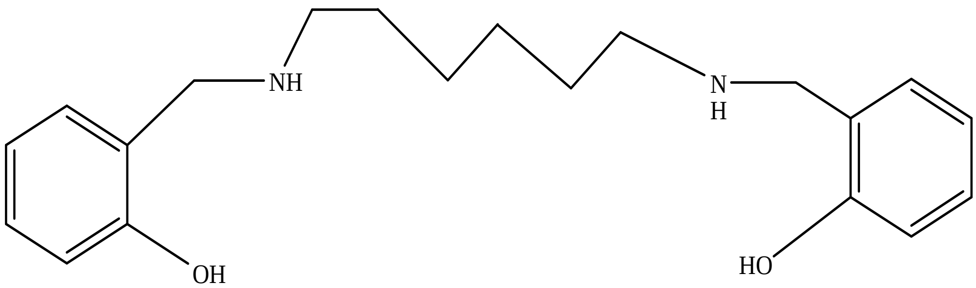
Salhx: **Salhx** was synthesized from 1,6-diaminohexane and salicylaldehyde similar to **Salbn** (yellow, $C_{20}H_{24}N_2O_2$, 84% yield). ^{1}H-NMR ($CDCl_3$) δ (ppm): 13.61 (br s, 2H, Ph-OH), 8.50 (s, 2H, CH=N), 6.7-7.5 (m, 4H, Ar-H), 3.72 (t, 4H, NCH_2), 1.40 and 1.63 (m, 8H, CH_2-CH_2). IR (KBr, cm^{-1}): 1635 (s, C=N), 1357 and 1280 (s, C-O(Ar) and O-H def), 2400-3050 (s, br, OH), 1612 (s, C=C), 2931 and 2885 (s, R-H), 3054 (m, Ar-H).

Saltris: Tris(hydroxymethyl)aminomethane (0.05 mol) was added to salicylaldehyde (0.05 mol) to produce **Saltris** using the same procedure as **Salbn**. (yellow, $C_{11}H_{15}NO_4$, 67% yield). ^{1}H-NMR (DMSO-d_6) δ (ppm): 14.53 (s, 2H, Ph-OH), 8.56 (s, 2H, CH=N), 6.7-7.45 (m, 4H, Ar-H), 3.62 (s, 6H, CH_2O), 4.74 (s, 3H, ROH). IR (KBr, cm^{-1}): 1635 (s, C=N), 1338 and 1281 (w, C-O(Ar) and O-H def), 2300-3300 (s, br, OH), 1608 (s, C=C), 2935 and 2885 (s, R-H), 3032 (w, Ar-H).

Synthesis of the reduced Schiff bases The reduced Schiff bases were synthesized according to published procedures [28]. ^{1}H-NMR and IR data for the reduced Schiff bases are given in Tables 2 and 4, respectively.



(a)



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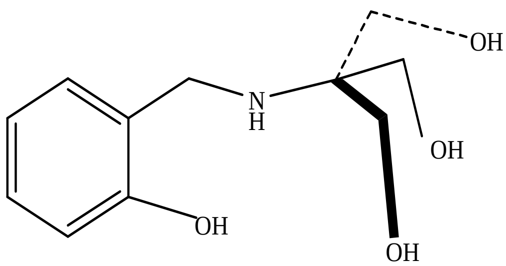


(c)

Figure 3. Compounds 1b (a), 2b (b), and 3b (c).

H_2Salbn (1b) The reduced Schiff base **1b** was prepared by the slow addition of excess sodium borohydride (1: 3 ratio) to **Salbn** in methanol. The solution was then refluxed for 2 hrs, filtered, and the white precipitate washed with ethanol and diethyl ether then dried (white, m.p. 145 ºC, $C_{18}H_{24}N_2O_2$, 53% yield).

H_2Salhx (2b) was prepared from **Salhx** in a procedure similar to that of **1b** (white, m.p. 129 ºC, $C_{20}H_{28}N_2O_2$**,** 88% yield).

H_2Saltris (3b) The reduced Schiff base **3b** was prepared by the same procedure with 1: 1.5 molar ratio of **Saltris** (6 g) to $NaBH_4$ (3.022 g). (white, m.p. 187 ºC decompose, $C_{11}H_{17}NO_4$, 50% yield).

Synthesis of the zinc complexes

Reduced Schiff base metal complexes were prepared by adding $ZnCl_2$ (2 mmol) dissolved in absolute ethanol (30 mL) to a stirred solution of 1 mmol of the reduced Schiff base (**1b**, **2b**, or **3b**) and sodium acetate (4 mmol) in ethanol (30 mL). The reaction mixture was heated for about 4 hours at 55 ºC. The solutions were filtered while still hot then cooled to room temperature. Following partial evaporation of the solvent, the product was filtered off, washed with ethanol and diethyl ether then dried in air to give solid products. Analytical data are tabulated in Table 1. ^{1}H-NMR and IR data are given in Tables 3 and 4, respectively.

Table 1. Physical and analytical data of the reduced Schiff base complexes

Compound	color	m. p. (ºC)	yield	Calculated Molar Mass (Formula)	Found Mass* (g/ mol)
1bZn	White	247[d]	81%	639.4 ($C_{26}H_{40}N_2O_8Zn_2$)	640.1
2bZn	White	296[d]	82%	667.5 ($C_{28}H_{44}N_2O_8Zn_2$)	666.9
3bZn	White	229[d]	79%	552.2 ($C_{19}H_{35}NO_9Zn_2$)	551.8

*d; decomposed, * molecular ion mass.*

RESULTS AND DISCUSSION

Synthesis

Three Schiff bases were prepared through the condensation of salicylaldehyde with the primary amines 1,4-diaminobutane (**Salbn**), 1,6-diaminohexane (**Salhx**), and tris(hydroxymethyl)aminomethane (**Saltris**) in ethanol. Reduction of the Schiff bases with sodium borohydride in methanol afforded the corresponding reduced Schiff bases as evidenced by the change of color from yellow to white for all three Schiff bases and the characteristic changes in the ^{1}H-NMR and IR spectra as will be discussed below. Reaction of the reduced Schiff bases with zinc chloride in a 1:2 molar ratio in ethanol afforded the dimeric products. Acetate was used in excess and acts as a base for proton abstraction from the reduced Schiff bases as well as a ligand to Zn^{2+}. The compounds were characterized by ^{1}H-NMR, IR spectroscopy, and mass spectrometry.

^{1}H-NMR Spectra of the Schiff bases

Generally, the spectra of the Schiff bases show multiplet signals for the aromatic protons at δ= 6.7- 7.5 ppm, and signals at δ= 13.55–14.53 ppm for enolic hydroxyl protons [29]. The signals at δ= 8.50-8.56 ppm can be assigned to the azomethine protons, thus proving the formation of the Schiff bases [30]. The chemical shifts of CH_2-N groups in **Salbn** and **Salhx** appear shifted down field between 3.64-3.72 ppm, while the CH_2O protons of **Saltris** appear at 3.62 ppm [31].

^{1}H-NMR Spectra of the reduced Schiff bases

For the reduced Schiff bases **H_2Salbn (1b) and H_2Salhx (2b)**, the appearance of the ArCH_2–N signal at 3.97-3.98 ppm simultaneously with the disappearance of the CH=N signal around 8.50-8.56 ppm can be considered as indications for the reduction of the imine bond, Table 2 [22-24]. Moreover, the NCH_2 protons appear shifted up-field in the reduced Schiff bases further proving that the CH=N bond is reduced. The newly formed NH protons appear in the spectrum as a broad peak at 5.54 and 4.7 in **1b** and **2b**, respectively, due to hydrogen-bonding. This peak can be probably assigned to both NH and OH protons [24]. This interpretation is supported by the integration of the peak which suggests that it represents 4 protons not just 2 as expected for the 2 NH protons.

For **3b**, the reduced Schiff base **H_2Saltris**, the peak of the azomethine proton of the corresponding Schiff base at 8.56 ppm disappeared and a new peak at 3.2 ppm appears instead. The new peak can be assigned to CH_2N formed after reduction. Finally a new peak for NH at 7.1 ppm appears as an extra evidence for the reduction of CH=N. The ROH protons appear at 1.1 ppm, Table 2.

Table 2: ^{1}H-NMR analysis for the reduced Schiff bases in DMSO.

Compound	Chemical shifts (δ) in ppm						
	ArCH_2N	NH	Ar-H	OH	NCH_2	CH_2O	CH_2CH_2
1b	3.97 (s)	5.54 (br)	6.7-7.2 (m)	-	2.67 (m)	-	1.57 (m)
2b	3.98 (s)	4.7 (br)	6.7-7.2(m)	-	2.65 (t)	-	1.35 (m) 1.53 (m)
3b	3.2 (d)	7.1 (br)	6.0-6.9 (m)	1.1 (m)	-	3.39 (m)	-

s, singlet; m, multiplet, t, triplet; d, doublet, br, broad

^{1}H-NMR Spectra of the zinc complexes

1bZn and 2bZn

Evidence for zinc binding to the nitrogen atoms of **1b** and **2b** comes from the significant up-field shifts of the PhCH_2N and NCH_2 protons upon zinc complexation [24], Table 3. In both complexes **1bZn** and **2bZn** the PhCH_2N protons are shifted from 3.97 and 3.98 ppm to 3.35 and 3.19, respectively. Meanwhile, NCH_2 protons are shifted from 2.67 and 2.65 to 2.56 and 2.25, respectively. Additional proof for N-binding comes from the shift of the NH protons from 5.54 and 4.7 up to 4.36 and 3.7, respectively. The appearance of shifted and broadened peaks for NH protons indicates that Zn is bound to NH not N^-.

The slight up-filed shifting of the aromatic protons as well as the disappearance of the phenolic OH protons can be takes as indications, though not conclusive, for the binding of Zn to O^- in both **1b** and **2b**.

Completing the coordination sphere of Zn are two extra ligands, acetate and ethanol, as evidenced by the appearance of a peak for the acetate methyl group at 1.85 ppm for **1bZn** and 1.6 ppm for **2bZn** as well as the appearance of ethanol methyl groups at 1.05 for **1bZn** and 0.78 for **2bZn**. The CH_2O protons of ethanol appear at 3.45 ppm for both complexes.

The ^{1}H-NMR spectrum of 2bZn is shown in Figure 4(a).

3bZn

Evidence for zinc binding to the nitrogen atoms of **3b** comes from the down-field shift of the $PhCH_2N$ protons from 3.2 to 3.32 upon zinc complexation, Table 3. Additional proof for N-binding comes from the shift of the NH protons from 7.1 up to 3.91. The appearance of shifted and broadened peaks for NH protons indicates that Zn is bound to NH not N^-. In addition, the slight down-field shifting of the aromatic protons as well as the disappearance of the phenolic OH protons can be takes as indications, though not conclusive, for the binding of Zn to phenolic O^-. The CH_2O protons of **3b** are not shifted in the complex, however.

Completing the coordination sphere of Zn are two extra ligands, acetate and ethanol, as evidenced by the appearance of a peak for the acetate methyl group at 1.73 ppm as well as the appearance of ethanol methyl groups at 1.06. The CH_2O protons of ethanol appear at 3.39 ppm together with the CH_2O protons of the **3b**. The broad peak at 4.4 ppm is probably due to the ethanolic OH proton and the OH protons of **3b,** Figure 4(b).

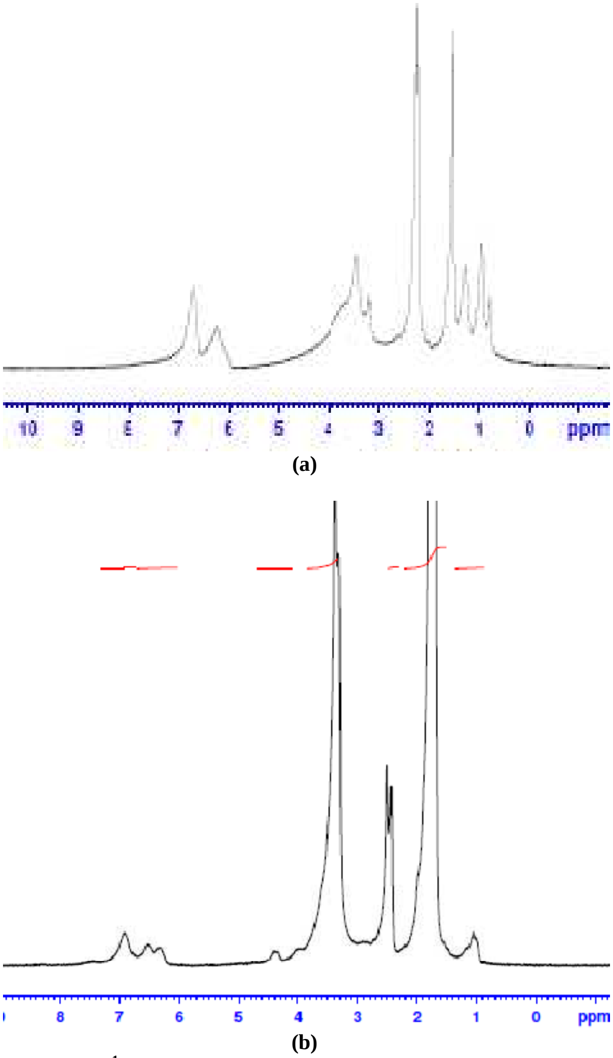


Figure 4. ^{1}H-NMR spectra of 2bZn (a) and 3bZn(b) in DMSO

Table 3. ^{1}H-NMR analysis for the reduced Schiff base Zn complexes in DMSO

Compound	Chemical shifts (δ) in ppm							
	$ArCH_2N$	NH	Ar-H	OH	NCH_2	CH_2O	CH_2CH_2	CH_3
1bZn	3.35 (s with sh)	4.36 (br,s)	6.2-7.2 (m)	-	2.56 (m)	3.45(m)	1.68, 1.74(m)	1.05, 1.85 (m)
2bZn	3.19 (m)	3.7 (br)	6.04-7.1 (m)	-	2.25 (m)	3.45(m)	0.96, 1.26(m)	0.78, 1.6(m)
3bZn	3.32 (m)	3.91 (br)	6.2-7.1 (m)	4.4 (br)	-	3.39(m)	-	1.06, 1.73(m)

IR spectra

The strong bands that appear at about 1635 cm^{-1} in the free Schiff base ligands disappear upon reduction with $NaBH_4$ as an indication of reduced Schiff base formation. This is further supported by the appearance of a new band assigned to a secondary amine, NH, at 3233-3287 cm^{-1} in all reduced Schiff bases, Table 4. This peak is broadened and shifted to lower frequencies between 3217 and 3267 cm^{-1} in all zinc complexes as an evidence of N-Zn bond formation [23-24]. N-Zn coordination is further supported by the appearance of new bands in the low frequency region between 300 and 340 cm^{-1} assigned to N-Zn stretching frequencies.

Two relatively strong bands appear at 1270-1400 cm^{-1} for all Schiff bases. These bands can be assigned to phenolic C-O stretching vibrations and O-H deformation vibrations [32]. These two peaks still appear in the reduced Schiff bases, although the lower frequency peak becomes weaker, probably because OH is involved in intramolecular OH·······N hydrogen bonding [30]. On the other hand, only one peak appears for the complexes suggesting that there is no OH group anymore, and together with a shift in this peak towards lower frequencies [23], confirms the formation of O^--Zn bond in the complexes. Further confirmation for O-Zn bond formation comes from the multiple new bands between 350- 400 cm^{-1}. Taken together with the strong broad bands above 3000 cm^{-1}, these new O-Zn bands suggest that there is at least one more ligand bound to zinc.

The broad bands around 3400 cm^{-1} which appear in the complexes are an indication to the participation of ethanolic OH in the coordination sphere of Zn. Additional bands at 1540-1590 cm^{-1} and 1400-1430 cm^{-1}, due to asymmetric stretching C=O and symmetric stretching C=O vibrations, respectively, indicate the participation of bridging acetate ligands in the binding of two zinc ions [33]. The extra bands that appear between 350- 400 cm^{-1}can thus be assigned to O-Zn bonds between zinc and both ethanol and acetate.

Other important bands such as the C=C and C-H (both aromatic and aliphatic) vibrations are present in the expected regions of the spectrum and are affected by zinc complexation to some degree.

A representative IR spectrum of 2bZn is shown in Figure 5.

Table 4. IR data for the reduced Schiff bases and their zinc complexes

	1b	1bZn	2b	2bZn	3b	3bZn
N-H	3287, sharp	3267, w	3287, sharp	3237, sh	3233, br	3217, sh
C-O(Ar) O-H def	1350, s 1234, m	1339, m	1367, m 1265, s	1339, m	1367, m 1273, m	1343, m
C-O(R) O-H def	-	1250, m 1053, m	-	1250, s 1045, m	1273, m 1049, m	1273, w 1049, m
O-H	3174,s, br	3441, s, br	3445, s, br	3441, s, br	3433, s, br	3445, s, br
$CO_{2\ (ass)}$ $CO_{2\ (sym)}$ Acetate	-	1601, s 1404, s	-	1574, s 1416, s	-	1578, s 1412, s
C=C	1605, s	1622, sh	1583, s	16*07, sh	1574, s	1611, sh
C-H	3050, sh 2940, w 2816, w	3013, w 2978, w 2932, w 2893, w	3071, sh 2924, m 2847, m 2816, m	3009, w 2940, w 2862, w	3000, sh 2933, sh 2874, w	3000, v w 2983, v w 2933, w 2883, v w
Zn-O	-	393, m 375, w	-	390, s 350, s	-	395, br
Zn-N	-	339, w	-	300, w	-	330, w
Unassigned peaks	752, 880, 940, 1003, 1451, 1674	676, 771, 937, 1022, 1447 (sh), 1485, 2573, 2762 (w) 3395 (sh)	752, 835, 933, 983, 1083, 1420, 1460, 2567	679, 756, 872, 937, 1022, 1443 (sh), 1481, 2767 (vw)	652, 756, 879, 945, 1099, 1416, 1478	621, 675, 768, 934, 1022, 1196, 1485, 2600, 2733 (v w)

w: weak, m: medium, s: strong, br: broad, sh: shoulder, v w: very weak

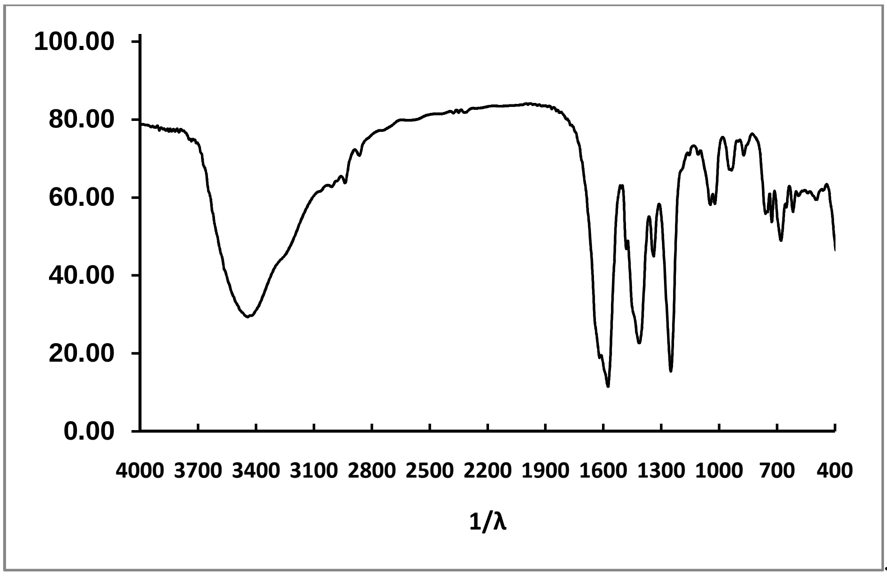


Figure 5. The FT-IR Spectrum of 2bZn

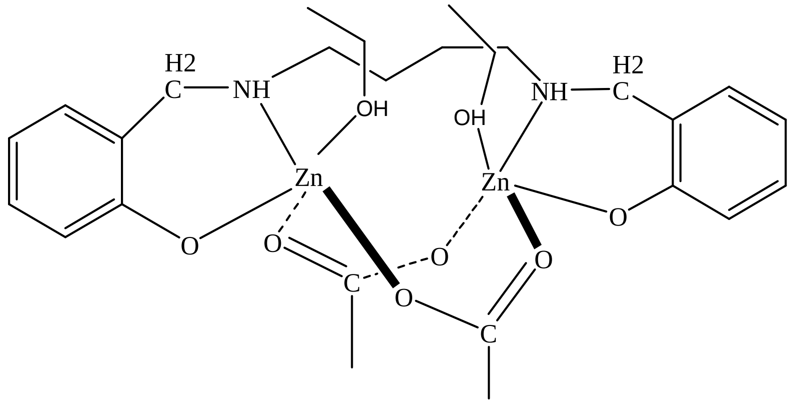
Proposed structures of the complexes

Based on the evidence obtained from ^{1}H-NMR and the IR spectra as well as the masses of the molecular ions obtained from the mass spectra we propose the structures shown in Figure 6. The masses of the proposed structures match the masses obtained from the mass spectra, Table 1, thus proving the proposed formulas of the compounds. Both in compounds **1bZn** and **2bZn**, the zinc atom is present in a distorted trigonal bipyramidal geometry composed of one N atom and four oxygen atoms, a phenolate, an oxygen from ethanol, and two from two bridging acetate ligands. The coordination number 5 is not unusual in Zn^{2+} chemistry as it often takes place when Zn^{2+} cannot form a tetrahedral geometry [27].

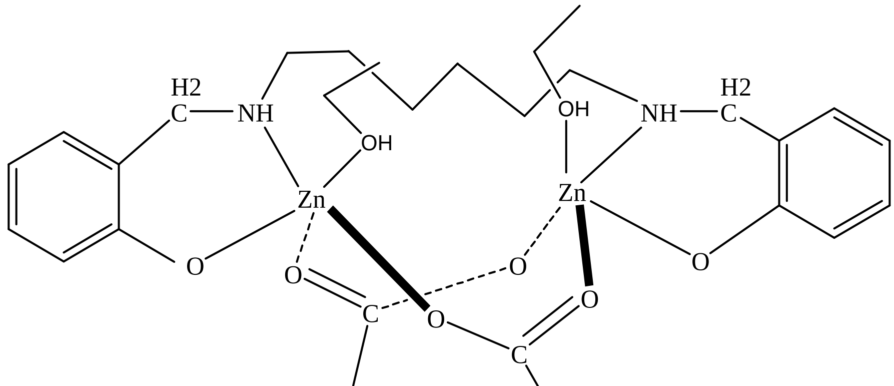
In compound **3bZn**, one zinc atom is coordinated to one N and five oxygen atoms in a distorted octahedron. Zn^{2+} is bound to the phenolate oxygen, one oxygen atom from acetate, two oxygen atoms from ethanol, and one from the tris hydroxyl groups. The second zinc is bound to the three oxygen atoms of the tris(hydroxymethyl)aminomethane part of the ligand along with one ethanolic oxygen and an oxygen atom from acetate forming a near square pyramidal geometry.

The complexes **1bZn** and **2bZn** represent many features of the active site of *A.* Aminopeptidase:
- Both complexes have Zn^{2+} in a 5-coordinate sphere.
- One nitrogen atom is bound to each Zn^{2+} along with four oxygen atoms.
- There is an acetate ligand bridging the two zinc ions together similar to the role played by Asp in *A.* Aminopeptidase.

Complex **3bZn** holds fewer similarities as Zn1 has a 6-coordinate geometry and Zn2 is not bound to any nitrogen atoms.



(a)



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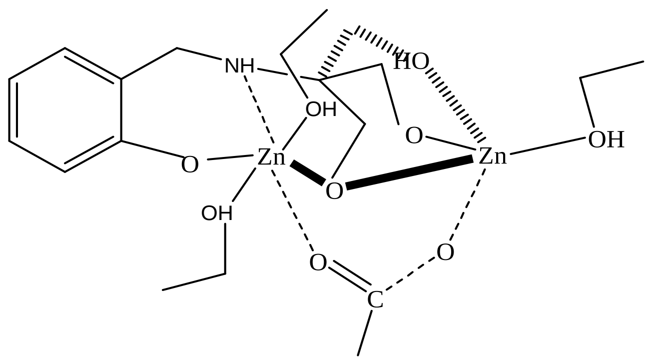

(c)

Figure 6. Proposed structures of the complexes

CONCLUSION

Three complexes of zinc with ligands prepared by the $NaBH_4$ reduction of Schiff bases derived from salicylaldehyde and the primary aliphatic amines 1,4-diaminobutane, 1,6-diaminohexane, and tris(hydroxymethyl)aminomethane have been prepared and characterized by ^{1}H-NMR, FT-IR spectroscopy, and mass spectrometry. The zinc atoms are present in a N,O environment with bridging carboxylate ligands as evidenced by both ^{1}H-NMR and IR spectroscopic methods. The proposed structures of the complexes hold similarities to the active site of the zinc enzyme *Aeromonas Proteolytica* aminopeptidase making these complexes plausible candidates as models of the enzyme. Reactivity studies of the prepared complexes with amide compounds will be conducted in order to gain further information about the mechanism of action of this enzyme.

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