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DNA interaction and biological activities of Copper(II) complexes of alkylamidio-O-methylurea

Ng. Lingthoingambi[1*], N. Rajen Singh[1] and M. Damayanti[2]

[1]*Department of Chemistry, Manipur University, Imphal (India)*
[2]*Department of Life Science, Manipur University, Imphal (India)*

ABSTRACT

*Two of copper(II)complexes,bis(1-methylamidino-O-methylurea)copper(II)chloride(**1**) and bis(1-ethylamidino-O-methyl urea)copper(II)chloride(**2**)were prepared. The complexes were characterized by analytical analysis, IR and UV-Vis spectroscopy. The interaction of calf thymus DNA with copper(II) complex has been investigated by using the absorption titration method and thermal denaturation method. The experimental results showed that the mode of binding is non-intercalative.The synthesized complexes were also screened for their antibacterial activity against isolated strains of Escherichia coli, Klebsiellapneumoniae and Proteus mirabilisby using disc diffusion method. Both the complexes inhibits the bacterial growth.*

Keywords: Bis(1-methyl amidino-O-methyl urea)copper (II) chloride, Bis(1-ethyl amidino-O-methyl urea) copper(II)chloride, DNA binding constant, Non-intercalative binding mode, Antibacterial activity.

INTRODUCTION

The chemistry of transition metal complexes has received much attention in recent years on account of their rational design and synthesis in coordination chemistry, also because of their potential applications as functional materials [1], enzymatic reaction mechanism [2] and in bioinorganic chemistry [3]. The transition metal complexes are applied in various activities such as anticancer,antitubercular, antibiotic, antimicrobial and antifungal agents [4-8].In biological systems, most metal ions are co-ordinated to ligands with biological functions rather than free ions. The interactions in biological systems, such as intercalation, hydrogen bonds, electrostatic , van der Waals force and so on widely exist in the biological metal complexes and are involved in various processes of life.It has been reported that co-ordinated amidino-O- alkylurea acts as a versatile hydrogen bonding ligand [9-11] and hydrogen bonding interaction are of great

importance in biological system [12-15].Literature survey revealed the absence of any report of a systematic interaction of DNA with Copper(II) complexes of alkylamidino-O-alkylurea.

In View of all of the above, it was thought worthwhile to studyDNA binding and the biological activities of these two complexes-bis(1-methylamidino-O-methylurea)copper(II)Chloride (**1**) and bis(1-ethylamidino-O-methylurea)copper(II)Chloride (**2**).Their interactions with calf thymus DNA (CT DNA) were investigated by electronic absorption and thermal denaturation measurements. Furthermore, we have tested the biological activities of the complexes using bacteria such as *Escherichia coli* (MTCC 729), *Klebsiellapneumoniae*sub sp.*pneumoniae* (MTCC 432) and *Proteus mirabilis* (MTCC 729).

EXPERIMENTAL SECTION

Materials and measurements:

Sodium dicyanamide purchased from Acros was used without further purification for the preparation of ligand ,methyldicyandiamide and ethyldicyandiamide. Metal chlorides were purchased from Qualigens and Calf thymus DNA(fibrous) from Sigma. All the other chemicals used in this study were of Analytical grade.Tris–HCl buffer solution containing 50 mMNaCl/5 mMTris–HCl (pH 7.2) in double-distilled water was used to prepare all stock solutions for DNA binding experiments.

Infrared spectra were obtained on a Shimadzu-8400S, FTIR spectrometer as KBr pressed pellets. UV-Vis spectra were obtained on a Shimadzu 2450 UV–Vis spectrophotometer. Carbon, nitrogen and hydrogen analyses were determined using a Perkin–Elmer-2400 Series ll, CHNS/O elemental analyzer and the amount of copper was determined by decomposing the complexes with a mixture of HNO_3 and H_2SO_4 and finally by performing an iodometric titration.

Preparation ofmethyldicyandiamide and ethyldicyandiamide

These ligands were prepared by following the method of Curd and Rose [16].Methylaminehydrochloride (6.8gm) / ethylaminehydrochloride(8.2gm), sodium dicyanamide (8.9gm) and butanol (50ml) were refluxed for three hours and kept in a refrigerator for an overnight. The cooled suspension was filtered and the filtrate was evaporated. The residual syrup was solidified on treatment with dioxane and the compound was recrystallized from the same solvent in colourless prisms. The compound methyldicyandiamide / ethyldicyandiamidewas dried for 48 hours over sodium hydroxide and paraffin wax in a vacuum. The analytical data are presented in table -1

Preparation of Complexes

The following complexes were prepared following the reported procedures [17].Complex (**1**) was prepared by refluxing cupric chloride dihydrate(1.7gm) and methyldicyandiamide(2gm) in methanol in a 1:2 stoichiometric ratio on a water bath for 3hrs. The light violet colour complex was filtered off immediately after keeping overnight in a refrigerator. The complex was recrystallized from methanol and dried in air.

Complex (**2**) was prepared like its methyl analogue by refluxing ethyldicyandiamide (1.12gm) and 0.8gm of cupric chloride dihydrate in methanol for about 6 hours until the colour changes from blue to red violet. The compound was recrystallized from methanol and dried in air. The analytical data are presented in table -1

Table 1. Analytical data of methyldicyandiamide and ethyldicyandiamide

Compound	m.p(°C) found(Literature)	Analytical found (calcd.) % C	H	N
Methyldicyandiamide $C_3H_6N_4$	92 (93)	36.5 (36.7)	6.2 (6.1)	56.9 (57.1)
Ethyldicyandiamide $C_4H_8N_4$	71 (72)	42.3 (42.8)	6.9 (7.1)	49.6 (50)

Table 2. Analytical data of Bis(1-methylamidino-O-methylurea)copper(II)chloride and Bis(1-ethylamidino-O-methylurea)copper(II)chloride

Compound	Colour	m.p(°C)	Analytical found (calcd.) % Cu	N	Anion	H_2O
$[Cu(MAMUH)_2]Cl_2.H_20$	Violet-Red	165	15.3 (15.39)	27.1 (27.15)	17.18 (17.21)	4.30 (4.36)
$[Cu(EAMUH)_2]Cl_2.2H_20$	Red-Violet	88	13.8 (13.85)	24.4 (24.42)	15.4 (15.48)	7.9 (7.85)

DNA binding studies:

Absorption titration experiments:

All experiments involving CT-DNA were performed in Tris buffer solution (50 mMNaCl/5mM Tris–HCl, pH 7.2) at 25^0C.Double distilled H_2O was used to prepare the buffer. The concentrationof DNA was determined from the band intensity at 260 nm with a known extinction coefficient value (ε_{260} = 6600M^{-1} cm^{-1}) [18]. The purity (freedom from bound protein) was assessed from the ratio of the absorbance at 260 nm and 280 nm[19]. In general, the commercial DNA preparation was found to be free of protein ($A_{260}nm/A_{280}nm$ = 1.9) according to this criterion and no further purification was attempted. The stock solution of DNA was used up to a maximum of 4 days. Absorption titration measurements were carried out by varyingthe concentration of CT DNA from 0 to $44x10^{-6}$M, while keeping themetal complex concentration constant at $25x10^{-6}$M in the buffer solution.Samples were incubated at 25°C for 24 hours before recording each spectrum. The data were then fitted to Eq. (1) to obtain the intrinsic binding constant, K_b [20]

$$[DNA]/ (\varepsilon_a - \varepsilon_f) = [DNA]/ (\varepsilon_b - \varepsilon_f) + 1/ K_b (\varepsilon_a - \varepsilon_f) \quad (1)$$

Where [DNA] is the concentration of DNA, the apparent absorption coefficients ε_a, $\varepsilon_f$and ε_b correspond to A_{obsd}/[Cu], the extinction coefficient for the free copper (II) complex and the extinction coefficient for the copper (II) complex in the fully bound form, respectively. A plot of [DNA]/ (ε_a-ε_f) versus [DNA] gave a slope of 1/(ε_b-ε_f) and a Y-intercept equal to 1/K_b (ε_b-ε_f), K_b is the ratio of the slope to the Y-intercept.

Thermal denaturation

DNA melting temperature were carried out by monitoring the absorption intensity of CT DNA(100µM) at 260nm in the temperature range from 25 to 100°C both in the absence and presence of copper(II) complex. Measurements were performed with Perkin Elmer Lambda - 35UV-Visible spectrophotometer. The melting temperature(Tm) of DNA is defined as the temperature at which 50% of double strand becomes single stranded. It was determined as the

mid point of the optically detected transition curves. The ΔTm value was defined as the difference between Tm of the free DNA and Tm of the bound DNA.

Biological activity

In vitro antimicrobial assay of the parent salt $CuCl_2.2H_2O$ and the complexes (**1**) and (**2**) were performed by the standard disc diffusion method [21]. The in vitro antimicrobial activities were performed againstovernight grown cultures of three selected bacteria, namely *Escherichia coli* (MTCC 729), *Proteus mirabilis* (MTCC 729) and *Klebsiellapneumonia* sub sp. *Pneumonia* (MTCC 432)on blood-agar media. Overnight grown bacteria (1 O.D.) were spread on blood-agar petriplates and kept for about half an hour. Filter discs (about 6mm in diameter) were placed on the inoculated plates into which 0.01 cm^3 each of the test solution was loaded. Dilutions were made in dimethyl sulphoxide (DMSO) to obtain final concentrations of 2 mg/ml, 4 mg/ml and 8mg/ml. After addition of each of the test solutions, the inoculated plates were kept at room temperature for about one hour to enable diffusion of the test solutions and subsequently, incubated at 37^{o}C. Microbial growth inhibition was determined by measuring the diameter of the zone of inhibition which was assessed at the end of 24hrs incubation.

RESULTS AND DISCUSSION

Infra red study

Methyldicyandiamide and ethyldicyandiamide have strong nitrile (C=N) bandat 2145 and 2155 cm-1, respectively. The IR spectra of the complexes show the absence of these bands and have a very strong ν_{asy}(C-O-C) stretch at *ca.* 1225-1205 cm-1 and ν_{sym}(C-O-C) at *ca.* 972-965 cm-1. The IR spectra of the ligands have a band at *ca.* 1610 cm-1 for the alkylamidino(azomethine) C=N stretching. The appearance of a strong band at *ca.* 1585 cm-1 and a band at *ca.* 1662 cm-1 have been assigned for ν(C=N) of the alkylamidino part and for ν(C=N) of the N=C-O-C fragment of 1-methyl/ ethylamidino-O-methyl/ethylurea [22,23].

UV–Vis Spectra

The UV- Vis spectra of the Cu(II) complexes exhibited n→π*or π→π* charge transfer bands at 209. Another broad bands, observed at the range (550 - 560) nm were attributed to d - d transitions ($^2B_{1g} \rightarrow {}^2A_{1g}$), typical for Cu(ll) in square planar CuN_4 chromospheres.

Absorption titration experiments

The binding mode of the complexes**1** and **2** with CT DNA was investigated using the absorption spectra. Complex binding with DNA via intercalation generally results in hypochromism and a red shift (bathochromism) of the absorption band due to strong interaction between the ligand and the base pairs of the DNA[24-26]. On the other hand, the absorption intensity of a complex is increased (hyperchromism) upon increasing the concentration of CT DNA due to degradation of the DNA double - helix structure[27]. The extent of the hyperchromism is indicative of the partial or non- intercalative binding modes, such as electrostatic forces, vander Waals interaction, dative bonds, hydrogen bonds and hydrophobic interaction. The electronic spectra of the complex**1** and **2**in the absence and presence of CT-DNA are given in fig 3and4respectively. In the presence of increasing concentration of CT-DNA, the complex exhibited hyperchromism with almost no shifts at 260 nm, and at the 209 nm, (n→π*or π→π*) charge transfer bands. These results suggest that the interaction of the complex and CT DNA are partial or non intercalative binding modes which would possibly by hydrogen bonds of the –NH groups and the oxygen atoms on the ligand with DNA nucleobases or electrostatic interaction among the $[CuL_2]^{2+}$ cationic species and the negatively charged phosphate groups on the DNA backbone.The observed results suggested that the binding nature of the copper complexes **1** and

2 with DNA goes in the similar fashions. The changes in the alkyl groups of the copper complexes **1** and **2** does not alter in their DNA binding nature. The changes in absorbance in the increasing amount of CT-DNA were used to evaluate the intrinsic binding constant K_b for the copper complexes.The intrinsic binding constants K_b were calculated by using Eq. (1)(see figure 3 inset which is obtained by plotting [DNA/(ε_a-ε_f)] vs [DNA]) monitoring the changes in the π-π^* or n- π^* charge transfer bands with increasing concentration of

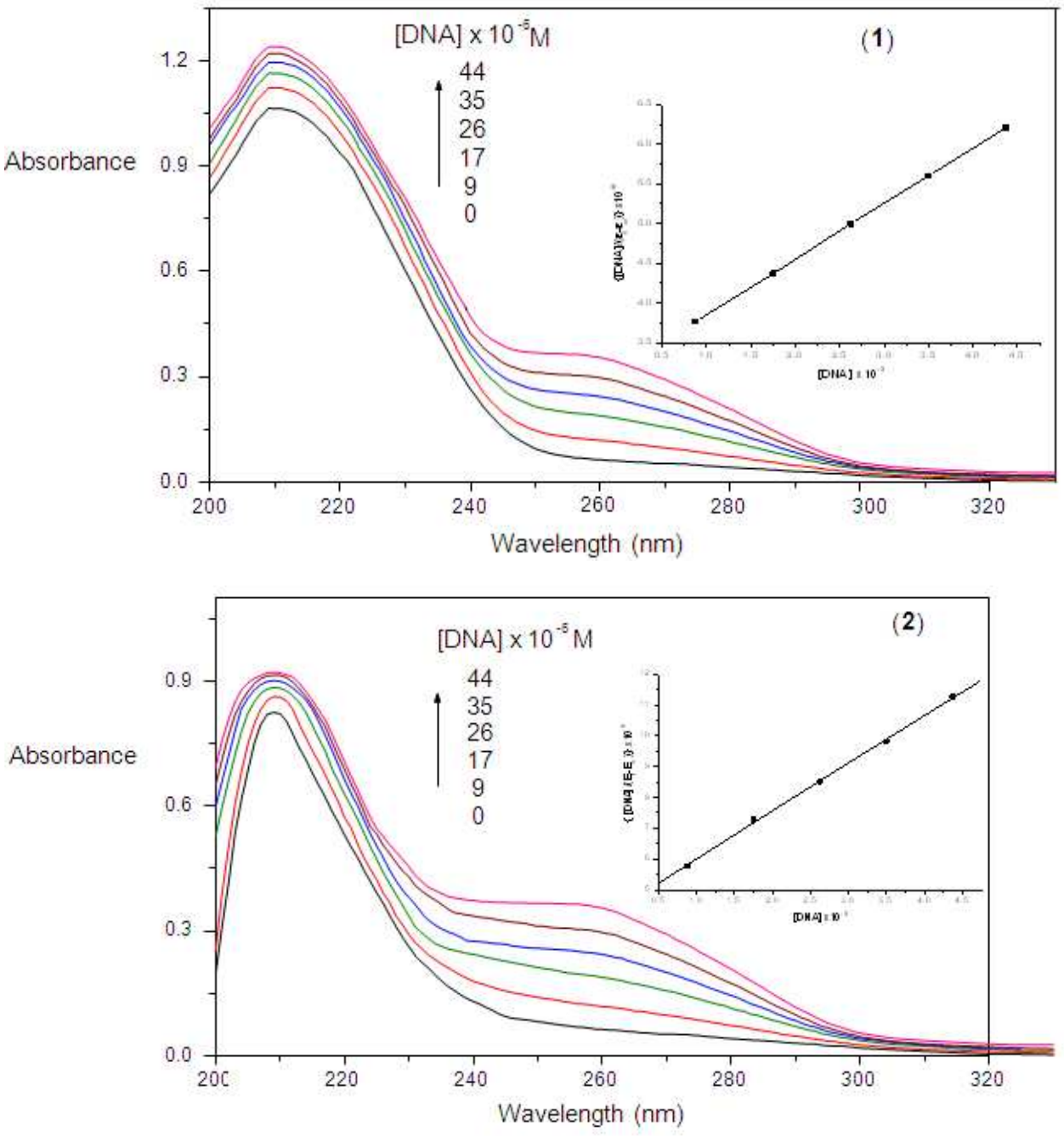

Fig. 3 Absorption titration spectra of Copper(II)-complexes (1) and (2) in Tris- HCl buffer at pH-7.2 in the absence and presence of increasing amounts of CT DNA from 0 to 44µM. [Complex]=25µM.

CT-DNA and wasobserved as $2.2x10^4$ M^{-1} and 3.5 $x10^4$ M^{-1} for complex **1** and **2** respectively.The increase in the binding constant value of the complex **2** may be due to the increase in the bulkiness of the complex.

Thermal denaturation

Presence of hydrogen bonding and base stacking interactions in the double- helical structure of DNA make it remarkably stable. Upon increasing the temperature, the double-helix dissociates to single strands since heat damages those chemical forces. The melting temperature(Tm) is strongly related to the stability of the double -helical structure. A change of Tm may be observed when an interaction of complexes with

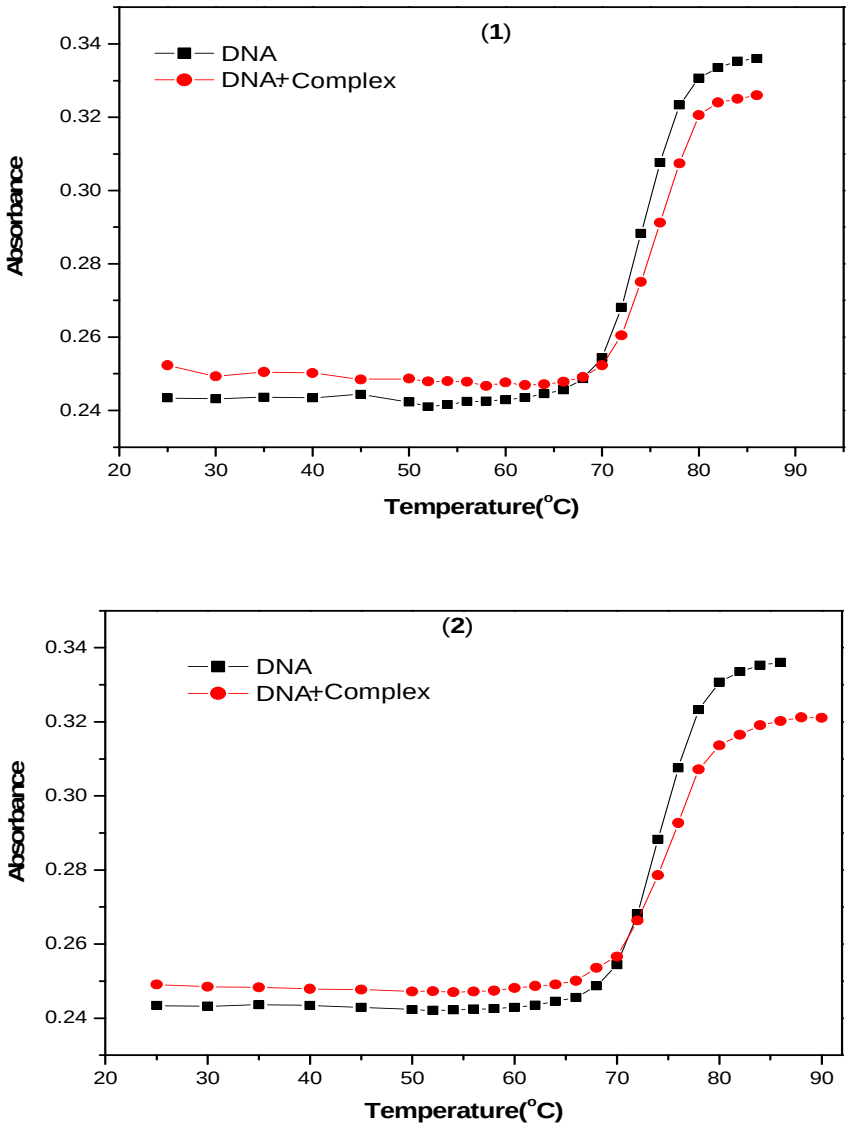

Fig. 5. DNA melting curves of Copper(II)-complexes (1) and (2) in the absence and presence of DNA

DNA occurs. Thus the study of thermal behaviour of DNA in the presence of complexes provides information on the conformational changes and the strength of the DNA- complex interactions. The intercalation of small molecules into the double helix is known to significantly increase the helix melting temperature, at which the double helix denatures into single_stranded DNA [28-30]. The extinction coefficient of DNA bases at 260 nm in the double_helical form is much less than that in the single_stranded form [31,32] hence, the melting of the helix leads to an increase in the absorption at this wavelength. Thus, the helix_to_coiltransition temperature can be determined by monitoring the absorbance of DNA bases at 260 nm as a function of temperature (*T*m). However, the *T*m will increase lightly on the interaction of smallmolecules

with DNA through nonspecific electrostatic interactions with the phosphate backbone of DNA [33] .The melting curves of CTDNA in the absence and presence of the copper(II) complex are illustrated in fig5. The Tm value for the free CT DNA is 74.8^{o}C and Tm value in presence of complex **1** and complex **2** are 75.2^{o}C and 75^{o}C respectively. A small change (0.2 to 0.4) in the DNA melting temperature(ΔTm) on addition of the complex to CT DNA was observed. The low ΔTm values pointed that the complex possibly interact with DNA by non-intercalative interaction[34,35]**.**

Biological Activity

The in vitro antimicrobial screening effects of the Complexes**1**, **2** and $CuCl_2.2H_2O$ salt were performed against three pathogenic bacteria .The test solutions at three concentrations 2%, 4% and 8% were used for antibacterial study. Since DMSO was usedas a solvent, it was also screened against all organisms. The diameter of zone of inhibition induced by DMSO in Escherichia coli , Proteus mirabilis and Klebsiella pneumonia were found to be 6mm, 7mm and 8mm respectively. The results of the investigated samples were summarized in Table 3. The antibacterial activity results revealed that both the complex and saltshowed less activity as compared to standard Gentamycin Sulphate. The inhibition of the $CuCl_2.2H_2O$ and the synthesized metal complexes against all tested microorganisms are in the order complex **2** □ complex **1** □ $CuCl_2.2H_2O$. The antibacterial activity of the synthesized Cu(II)-complexes depends on the size of the alkyl groups present and the concentration of the test solution used. These Cu(II) complexes have greater inhibitory activity on all tested microorganisms compared to their corresponding metal salt ($CuCl_2.2H_2O$). This may probably be due to the extensive conjugation effects observed in the metal complexes than the metal salts.

Table 3.Antibacterialactivity ofcopper(II) saltand its complexes, along with standard Gentamycin Sulphate [* represents the diameter of zone after subtracting the inhibition zone induced by DMSO]

Test complex	*Diameter of zone of inhibition (mm)								
	Escherichia Coli			Proteus mirabilis			Klebsiella pneumonia		
	2mg/ml	4mg/ml	8mg/ml	2mg/ml	4mg/ml	8mg/ml	2mg/ml	4mg/ml	8mg/ml
Gentamycin Sulphate	50			42			46.7		
$CuCl_2.2H2O$	8	9.5	12.1	8.3	9.8	12.7	8.1	9.6	12.6
$[Cu(MAMUH)_2]Cl_2.H_20$	10	11.7	22	10.7	18.7	20.7	9.1	11.2	14.5
$[Cu(EAMUH)_2]Cl_2.2H_20$	10.8	12.2	23.3	11.1	19	21.4	7	9.3	12.3

CONCLUSION

In this report, bis(1-alkylamidino-O-methylurea)copper(II)Chloride where alkyl = methylor ethyl have been synthesized and characterized. The mode of binding of both the complexes to DNA has been investigated by the spectroscopic studies and thermal denaturation experiments. The UV-Vis spectral data showed hyperchromism (i.e., increase in absorbance on addition of DNA) and no shift which indicated that the complex bind to DNA by non-intercalative modes. The thermal denaturation experiment results support the non-intercalative binding nature of the complexes.The antimicrobial data show that themetal complexes to be more biological active compared to those metal saltagainst all pathogenic species. The compounds also inhibit the growth of bacteriato a greater extent as the concentration is increased.

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