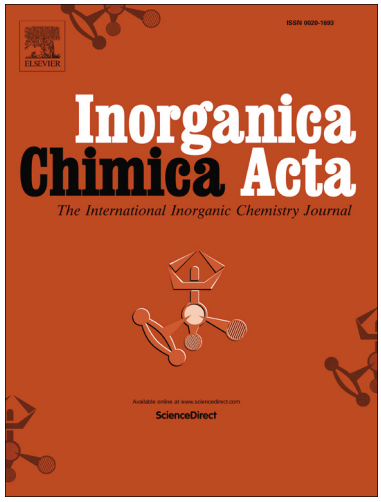

Research paper

Synthesis, Structure and DNA Interaction Studies of Bisphosphoramides: Theoretical and Experimental Insights

E. Veerashekhar Goud, Akella Sivaramakrishna, Kari Vijayakrishna, C.V.S. Brahmmananda Rao, Vijay M. Khedkar, Prakash C. Jha

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Synthesis, Structure and DNA Interaction Studies of Bisphosphoramides: Theoretical and Experimental Insights

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Abstract

New bisphosphoramides (having phenyl ($EDAPOPh_2$) and ethoxy (EDADEP) substituents attached to phosphoryl groups bridged with ethylenediamine spacer) are synthesized and structurally characterized by spectroscopic techniques as well as elemental analysis. The molecular structure of $EDAPOPh_2$ was determined by single crystal X-ray diffraction technique. The interaction of these bisphosphoramides with calf thymus DNA (*ct*-DNA) is investigated using UV-Visible absorption and fluorescence spectral data as well as the DFT calculations. These studies reveal that $EDAPOPh_2$ and EDADEP interact with DNA in a partial intercalation mode. The intrinsic binding constants K_b of two different bisphosphoramides with *ct*-DNA were determined by fluorescence spectroscopy as 2.08×10^4 and 3.86×10^4 M^{-1} respectively. The results indicated that the two compounds bind to *ct*-DNA with different binding affinities, *i.e.* $EDAPOPh_2$ > EDADEP. The binding mechanism of these bisphosphoramides to *ct*-DNA is also discussed.

Key words: Bisphosphoramides, crystal structure, molecular docking, DNA interaction.

1. Introduction

Deoxyribonucleic acid (DNA) is an important genetic material for the transfer of genetic information over generations with a double stranded helical structure in living organisms. It plays a vital role in the transcription, replication and regulation of genes which are important steps for living organisms. When small organic molecules bind to DNA that leads to a significant change in the DNA sequence and further damages the growth of cancer cells [1-2]. Among the molecules reported, the importance of organophosphorus reagents was elucidated in terms of anti-bacterial, anti-viral, anti-oxidant activity [3-4], anti-cancer [5], anti-inflammatory agents [6], pesticides, herbicides, insecticides [7-8] and anti HIV agents [9]. There are three types of possible interactions *i.e.* non-covalent connections such as intercalative binding between DNA base pairs and ligand, groove binding and static electronic effects [10]. Ligand planarity is the key factor in intercalative binding [11]. The intercalative is the most important binding which is similar to the anti-tumor activity of drug molecules. Various researchers have been working on the intercalative binding which involves antibiotics, transition metal complexes and *N*-donor containing molecules [12]. Since the investigations on interactions of smaller organic molecules and DNA is of fundamental importance [1], there is an increasing demand for synthesis of new molecules in molecular biology as well as in medical field to act as sequence selective DNA binding agents and DNA targeting anti-tumor drugs. As shown in the literature, various heterocyclic compounds containing phosphorus derivatives with more electron donating groups exhibited significant antitumor activity [13]. The successful history of cisplatin and platinum derivatives as anticancer drugs in clinical chemistry is due to their covalent binding with DNA even though it has some draw backs such as serious cellular side effects, toxicity and acquired drug resistance [14]. In order to minimize these drawbacks of platinum based drugs, the research is intensified based on replacing them with other molecules or complexes having covalent binding [15]. In this connection, we have studied earlier the DNA interactions with various poly(ionic liquids), hydazide and semicarbazide derivatives as well as some selected quinine derivatives [16]. The research in the past is mainly focused on the involvement of various transition metal complexes, heterocyclic compounds, and dye molecules in the interaction of DNA, but the information on binding of phosphoramides, phosphine oxides and phosphates with DNA is very meager [17]. As it has been shown earlier that phosphoryl groups are very good electron donors [18], it would be interesting to investigate the influence of aminophosphine oxides with multi donor sites on DNA interaction. The present work deals with the

synthesis and the structural characterization of two different bisphoshoramides and their involvement in DNA interactions.

2. Experimental part

2.1. Materials and methods

All synthetic procedures were carried out under inert atmosphere using Schlenk line techniques. The commercially available solvents were distilled from Na/benzophenone before the usage. The reagents including magnesium turnings, HCl, diethyl phosphite, isopropyl alcohol, ethyl acetate and cyclohexyl bromide (Merck, India) were used as received. Ethylene diamine, Ethidium bromide, NaOH, TRIS-HCl buffer and sodium salt of calf thymus DNA (*ct*-DNA) were procured from Sigma-Aldrich.

Diphenylphosphine oxide was prepared from diethylphosphite using Mg turnings and bromobenzene as per the modified literature procedure [19].

The spectrophotometric titrations were performed by maintaining the concentration of bisphosphoramide (10.0 μM) throughout by varying the concentration of *ct*-DNA from 10.0 to 100 μM at room temperature.

2.3. Instrumentation

^{1}H, ^{13}C, and $^{31}P\{^{1}H\}$ NMR spectra were recorded on a Bruker DMX-400 spectrometer and all 1H chemical shifts were reported relative to the residual proton resonance in deuterated solvents (all at 298 K, $CDCl_3$). Infrared spectra were recorded on a Shimadzu Affinity 1 FT-IR Spectrometer. UV-Visible absorption spectrum was recorded on UNICAM UV4-100 type double-beam spectrophotometer (ATI UNICAM, Cambridge, UK) controlled by software VISION 3.4 and instrument equipped with 1.0 cm quartz cuvettes. Blank buffer was placed in the reference cell and sample was placed in the sample cell. The fluorescence quenching studies were carried out using the HITACHI F-700 fluorescence spectrophotometer. Infrared spectra were recorded on a Shimadzu Affinity 1 FT-IR Spectrometer. Heraeus CHNS rapid micro analyzer was used for elemental analysis. GC analyses were recorded on a Perkin Elmer Clarus 680 (GC) and Clarus 600 (EI, mass) equipped with an FID and a 30 m x 0.32 mm CP-Wax 52 CB column (0.25 μm film thickness). The carrier gas was helium at 5.0 psi. The oven was programmed to hold at 32°C for 4 min and then to ramp to 200°C at 10 deg/min and hold 5 min.

2.3.1. Single crystal X-ray diffraction analysis

X-Ray diffraction intensity data was collected at room temperature (293K) on a Brukeraxs SMART APEXII single crystal X-ray diffractometer equipped with graphite monochromatic Mo*Kα* (λ=0.71073 Å) radiation and CCD detector. A crystal of dimensions 0.30 X 0.25 X 0.20 mm^3 was mounted on a glass fiber using cyanoacrylate adhesive. The unit cell parameters were determined from 36 frames measured (0.5° phi-scan) from three different crystallographic zones using the method of difference vectors. The intensity data were collected with an average four-fold redundancy per reflection and optimum resolution (0.80 Å). The intensity data collection, frames integration, Lorentz and polarization corrections and decay correction were carried out using *SAINT-NT* (version 7.06a) software. An empirical absorption correction (multi-scan) was performed using the *SADABS* program. The crystal structure was solved by direct methods using *SHELXS-97* and refined by full-matrix least-squares using *SHELXL-97*. Molecular geometry was calculated using PARST. All non-hydrogen atoms were refined using anisotropic thermal parameters. The hydrogen atoms were included in the structure factor calculation at idealized positions by using a riding model, but not refined. Images were created with the ORTEP-PLATON program. The CCDC deposition number is 1486557.

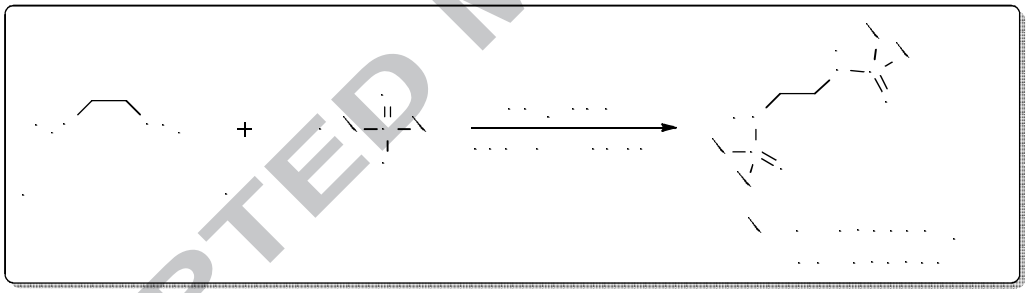
2.3.2. Molecular Docking studies

The experimental results clearly showed the interaction of DNA with two different phosphoramides (EDADEP and $EDAPOPh_2$) at varied concentrations. These observations further prompted us to investigate the mechanistic steps involved through the molecular docking, which is generally used to predict the orientation of ligands to their protein active sites in order to predict their efficiency and activity in the drug designing. An interactive molecular graphics program Glide (Grid-Based Ligand Docking With Energetic) incorporated in the Schrödinger molecular modeling package (Schrödinger, Inc., USA, version- 2015) installed on a Windows workstation with an Intel (R) xenon 2.8 GHz processor and 32 GB physical memory was used to predict the mode of binding and the associated interactions of the bisphosphoramide derivatives with DNA. The crystal structure of DNA duplex receptor (B-DNA) used for docking was retrieved from the Protein Data Bank (www.rcsb.org) (PDB code 425D) [20]. The B-DNA crystal structure was preprocessed for docking calculation by deleting the crystallographically observed water molecules since no water molecule was found to be conserved and adding hydrogen atoms corresponding to pH 7.0. Following the assignment of appropriate charge and protonation state, the structure was energy minimized (to

relieve the steric clashes introduced due to addition of hydrogen atoms) using the Optimized Potentials for Liquid Simulations (OPLS-2005) force field until an root mean square deviation (RMSD) value of 0.30 Å was reached. The 3D-strucutres of bisphosphoramide derivatives were sketched using the build panel in Maestro and their geometries were optimized using *LigPrep* module. The partial charges were ascribed using the OPLS-2005 force-field and the structures were then subjected to energy minimization until their RMSD reached 0.01 Å. The shape and properties of the binding site of B-DNA were defined for docking using the *receptor grid generation* panel. This grid was defined by a 14x14x14 Å box that was centered on the geometric centroid of the B-DNA structure and was sufficiently large to include a significant part of the B-DNA. The bisphosphoramide derivatives were docked flexibly into the B-DNA structures using with extra precision (*i.e.* with GlideXP) scoring function to rank the docking poses and to estimate their binding affinities.

3. Synthesis of Bisphosphoramides

3.1. General procedure



Scheme 1. Synthesis of bisphosphoramides

3.1.1. *Synthesis of bis(diphenyloxophosphino)ethylenediamine (EDAPOPh$_2$)*

To a 2-neck 500 mL RB flask, 6.72 g (33.0 mmol) of diphenylphosphine oxide in 150 mL of THF and 6.42 mL (66.0 mmol) of carbon tetrachloride were transferred under N_2 atm. To this, 1.1 mL (16.6 mmol) of ethylenediamine and 9.26 mL (66.0 mmol) of triethylamine (dissolving in 100 ml of THF) were added drop wise over a period of 30-60 minutes at ice cold temperature. There after the temperature of the vessel was allowed to attain the room temperature and contents of the reaction vessel were stirred for 12 h. The reaction mixture was filtered to remove hydrochloride salt of triethylamine and solvent traces were removed under high vacuum. The desired compound was obtained as crystalline solid in quantitative yields. Further the recrystallization of product was carried out using a solvent mixture of dichloromethane and *n*-hexane (1:1). The yield of the product

was 90% and melting point is 154–156 °C. FT-IR (KBr, cm^{-1}): 1180, ^{1}H NMR (400 MHz, $CDCl_3$, 300 K, relative to TMS): δ 3.15-3.18 (q, 4H, $–CH_2–N$), 7.86-7.91 (d, 8H, o-CH), 7.50-7.52 (t, 4H, p–CH), 7.43-7.47 (t, 8H, m–CH). ^{31}P NMR (162 MHz ppm relative to H_3PO_4 in $CDCl_3$): δ 30.85. ^{13}C NMR (100 MHz): δ 42.13, 128.33-128.45, 131.48-131.56, 131.65-131.23, 132.80, 134.06. Mass: Positive-ion EI (*m/z*): 460.16. Anal. Calcd. for $C_{26}H_{26}N_2O_2P_2$: C, 67.82; H, 5.69; N, 6.08. Found-: C, 68.12; H, 5.75; N, 6.11. The characterization data of this product closely agrees with the literature report as they have followed a different procedure [21].

3.1.2. *Synthesis of bis(diethylphosphino)ethylenediamine (EDADEP)*

N,N-Bis(diethylphosphoryl)ethylenediamine was synthesized according to the literature report by modifying the synthetic procedure [22]. The title compound was synthesized by taking the 7.25 g (52.5 mmol) of diethylphosphite, 8.08 g (52.4 mmol) of carbon tetrachloride, 2.001 g (26.2 mmol) of ethylenediamine and 5.30 g (52.4 mmol) of triethylamine in THF. The colorless crystalline compound was obtained in 98% yield with a melting point of 80-82 °C. ^{1}H NMR (400 MHz, chloroform-d, 300 K, relative to TMS): δ 1.29–1.32 (t, 12H, CH_3), 2.98–3.01 (q, 4H, $–CH_2–N$), 4.01-4.08 (p, 8H, $–CH_2–O–$), 3.53 (s, 2H, –NH). ^{31}P NMR (162 MHz, relative to H_3PO_3): δ 9.25. ^{13}CNMR (100 MHz, D_2O): δ 15.3 (CH_3), 41.8 (–CH2–NH), 63.7 ($–CH_2–O–P$). 135DEPT (100 MHz, D2O): δ 15.3 (CH_3), 41.7 ($-CH_2$-NH), 63.7 ($-CH_2$-O-P). Mass: Positive-ion EI (*m/z*): 332.21.Anal.Calcd. for $C_{10}H_{26}N_2O_6P_2$: C, 36.15; H, 7.89; N, 8.43. Found: C, 36.18; H, 7.92; N, 8.47.

4. General procedure used for DNA interaction studies

The 10 mM TRIS-HCl buffer was prepared using double distilled deionized water, and the pH was adjusted to 7.4 using 0.1 M NaOH solution. *ct*-DNA (1% w/w) in buffer was prepared and stored at 5 °C. The concentration of *ct*-DNA was estimated by using its known molar extinction coefficient at 260 nm (ε_{260} = 6600 M^{-1} cm^{-1}) and the absorbance was recorded as (λ_{max} = 0.42) followed by concentration of 63.8 μM. Then the final concentration of *ct*-DNA stock solution was obtained as 12.7 mM. The *ct*- DNA used in this study was sufficiently free from protein which is determined from the absorbance ratio (A_{260}/A_{280}) = 1.8.45 [21]. EDADEP ligand was prepared in phosphate buffer and $EDAPOPh_2$ was prepared in 10% DMSO in phosphate buffer.

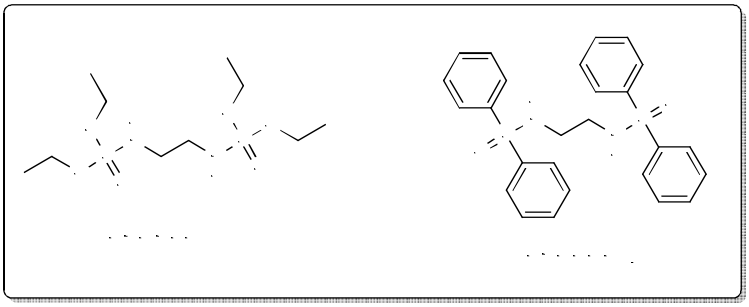


Chart 1. Bisphosphoramides employed in DNA interaction studies

The EB-DNA complex was titrated against various concentrations (0-100 μM) of phosphoramide ligands by keeping the concentration of *ct*-DNA (10 μM) constant. The prepared solutions were mixed thoroughly and incubated for 5 min, following by measuring the fluorescence intensities at room temperature. EDADEP has an emission maximum at 604 nm when excited at 530 nm and for $EDAPOPh_2$ was excited at 500 nm followed by emission at 606 nm.

4. Results and Discussion

4.1. Synthesis and structural characterization of bisphosphoramides

The bisphosphoramides ($EDAPOPh_2$ and EDADEP) were prepared by the reaction of two moles of either diphenylphosphine oxide or diethylphosphite with ethylenediamine in the presence of triethylamine. The purity of the products was assessed by ^{31}P-NMR spectral data based on the formation of a single peak due to the presence of two equivalent phosphorus atoms. These compounds were characterized by UV/Vis, FT-IR, ^{1}H, ^{13}C, ^{31}P NMR and mass spectral data and further the composition was confirmed by elemental analysis.

All the absorption spectra were recorded using a matrix containing phosphate buffer and 10% DMSO as a blank to nullify the influence of the matrix on absorption spectra. The $EDAPOPh_2$ and EDADEP ligands showed an intense absorption bands at 205 nm and 209 nm respectively due to intra-ligand π–π* transition of the coordinated groups (NH, P=O) [23].

The compounds $EDAPOPh_2$ and EDADEP showed strong infrared absorptions at 1180 and 1172 $cm^{-1}$for their phosphoryl groups respectively. ^{31}P-NMR spectra exhibited a single peak for each compound at δ 30.85 ($EDAPOPh_2$) and δ 9.25 (EDADEP). The mass spectra showed the formation of fragmented species $(EtO)_2P(H)=O$ of *m/z* 138.27 and 202.19 for $Ph_2P(H)=O$.

Single crystals of $EDAPOPh_2$ were obtained from the solvent mixture of dichloromethane and *n*-hexane (1:1). The molecular structure of $EDAPOPh_2$ was determined by single crystal X-ray analysis. The perspective view of $EDAPOPh_2$ is shown in Fig.1.

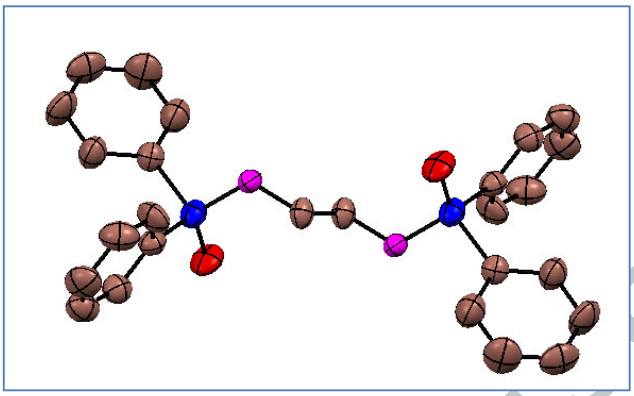


Fig.1. Molecular structure of $EDAPOPh_2$: Hydrogen atoms were removed for clarity. Some selected bond lengths (Å): P=O, 1.486; P-N, 1.626; P-C, 1.797 and bond angles (°): O-P-C, 109.8; O-P-N, 112.8; O-P-C, 114.9; C-P-N, 111.1 and C-P-C, 104.8.

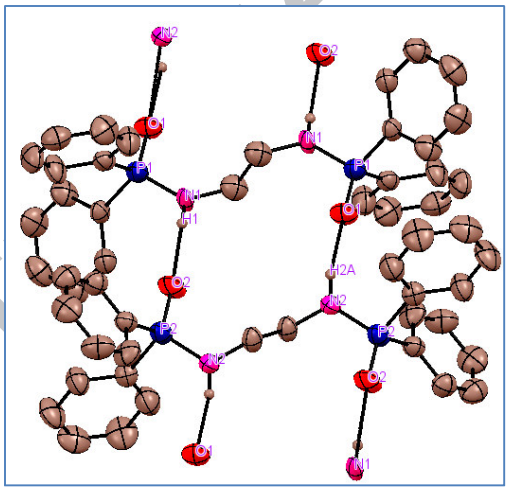


Fig. 2. A drawing of the molecular structure of $EDAPOPh_2$ with hetero atoms and intermolecular hydrogen bonds. Hydrogen atoms are omitted for clarity; Brown color ellipsoids are carbons, pink color indicates nitrogen atom, royal blue indicates phosphorous atom and red indicates oxygen atom.

As shown in the molecular structure of $EDAPOPh_2$ (Fig.1), the phosphorous centers are present in tetrahedral environment and four phenyl groups exist in the same plane. The P=O bond length was 1.486 Å and in good agreement with the literature reports. Interestingly, the monomers are connected to each other via two P=O-----H-N bonds through prominent hydrogen bonds in the solid state structure and the bond lengths were observed as H(2)-N(2)------O(1)-P(1) 2.094, H(1)-N(1)----

--O(2)-P(2) 1.959 Å. The strong hydrogen bonds are formed due to the presence of highly electronegative oxygen atom (Fig. 2). The packing arrangement of molecules in crystal lattice is given in Fig. 3.

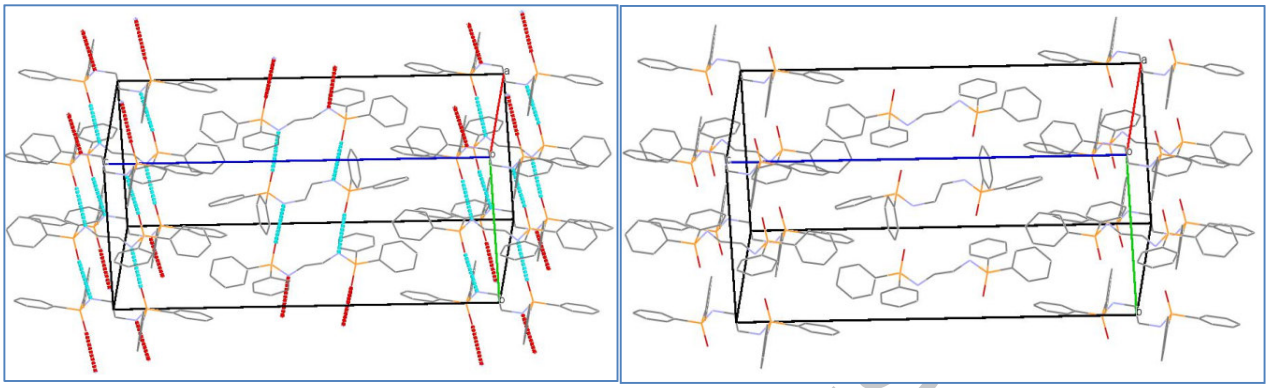


Fig. 3. Crystal packing of $EDAPOPh_2$ molecules in the lattice

4.2. DNA interaction studies

UV-Visible absorption spectroscopy is an important tool for DNA binding studies. The interaction of EDADEP and $EDAPOPh_2$ with *ct*-DNA was investigated in vitro by UV spectroscopy and indirectly by the evaluation of the EB-displacing ability of these multi-dentate molecules examined by fluorescence emission spectroscopy. The absorption studies of EDADEP in the presence and in absence of DNA are shown in Fig. 4 and Fig. 5. EDADEP and $EDAPOPh_2$ ligands showed an intense absorption bands at 205 and 209 nm respectively due to intra-ligand π–π* transition of the coordinated groups [24,25]. These bands were considered to characterize the DNA interaction studies in phosphate buffer.

A decrease in the absorption was observed by the addition of *ct*-DNA with varying concentration (0-100 μM) of ligands. The hypochromic shifts (or hypsochromic shift) in the wavelength of the bands suggest that there was a strong intercalative binding of bisphosphoramide ligands with DNA base pairs, indicating the direct formation of a new complex-DNA [24]. The DNA binding affinities with $EDAPOPh_2$ or EDADEP show strong hypochromism and a prominent red-shift, which is in accordance with earlier report [25] In order to compare the binding affinities quantitatively, the intrinsic binding constant K_b was calculated by using the following equation [26]:

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f)+ 1/K_b(\varepsilon_b-\varepsilon_f)\text{.....Eq. 1}$$

Where ε_a refers to apparent extinction coefficient calculated by using A_{obs}/[complex], ε_b refers to extinction coefficient of the complex in the bound form, ε_f refers to the extinction coefficient of the free form of complex and [DNA] refers to the concentration of DNA in base-pairs.

By substituting the obtained values in the above equation gives a plot of [DNA]/(ε_a-ε_f) *vs.* [DNA] with a straight line with a slope of 1/(ε_b-ε_f) and a *y*-intercept of 1/K_b(ε_b-ε_f) and K_b is obtained from the ratio of the slope to the *y*-intercept. K_b is calculated from the graphs for EDADEP and EDAPOPh$_2$ are 2.08 x 10^4 and 3.86 x 10^4 M^{-1} respectively (Fig. 4 and 5)**.**

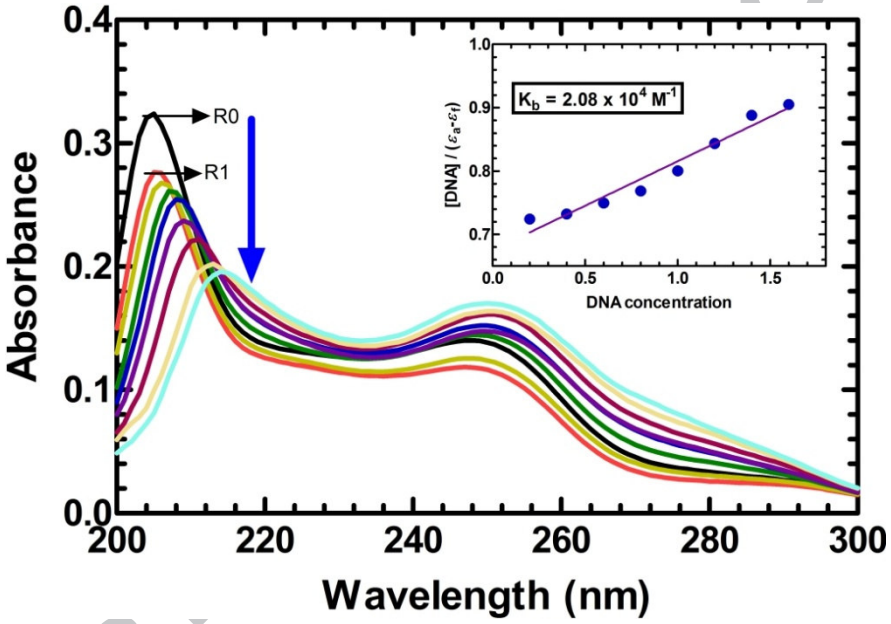


Fig. 4. UV spectrum of EDADEP (1x10^{-3} M) in the absence (R0) and presence of (R1-R9) *ct*-DNA (0-100 μM) in 10 mM phosphate buffer (pH = 7.2). Inset: the plot of [DNA]/(ε_a-ε_f) *vs.* [DNA] for EDADEP. The arrow depicts the decrease in the absorption with increasing the *ct*-DNA concentration.

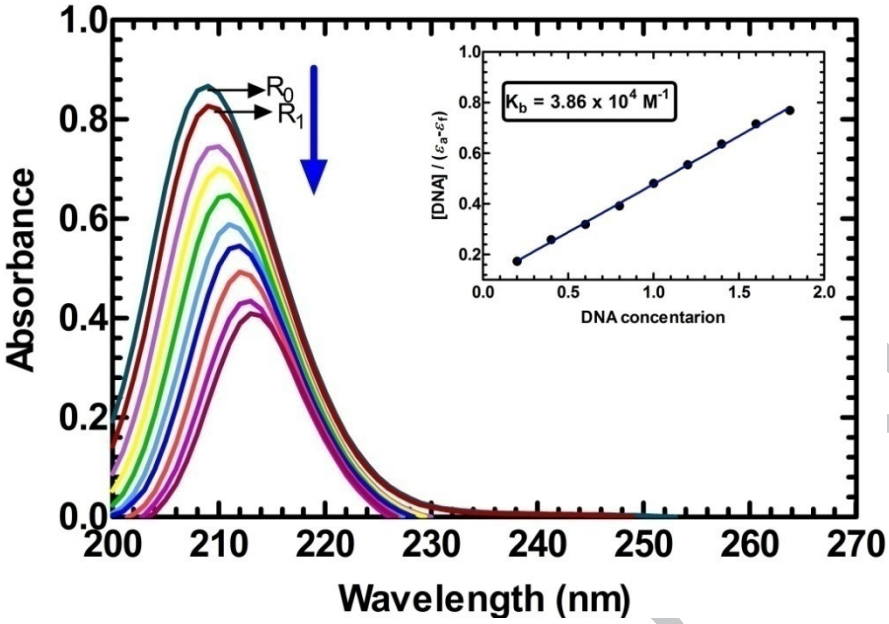


Fig. 5. UV-Vis spectrum of $EDAPOPh_2$ ($1x10^{-3}$ M) in the absence (R0) and presence of (R1-R9) *ct*-DNA (0-100 μM) in 10 mM phosphate buffer (pH = 7.2). Inset: the plot of [DNA]/(ε_a-ε_f)vs. [DNA] for $EDAPOPh_2$. The decrease in the absorption with increasing the *ct*-DNA concentration was shown with arrow mark.

The FTIR analysis shows the binding of EDADEP to *ct*-DNA, which is evident by shifting the characteristic bands at 1209, 1174 and 1092 cm^{-1} are due to the increase in the intensity of the phosphate stretching vibrations as a result of its interaction with EDADEP ligand. The sharp DNA band at 978 cm^{-1} is a characteristic of C-C and C-O vibration stretch of deoxyribose sugar and gets shifted to 964 cm^{-1} due to the interaction of EDADEP with deoxyribose of *ct*-DNA (Fig. 6).

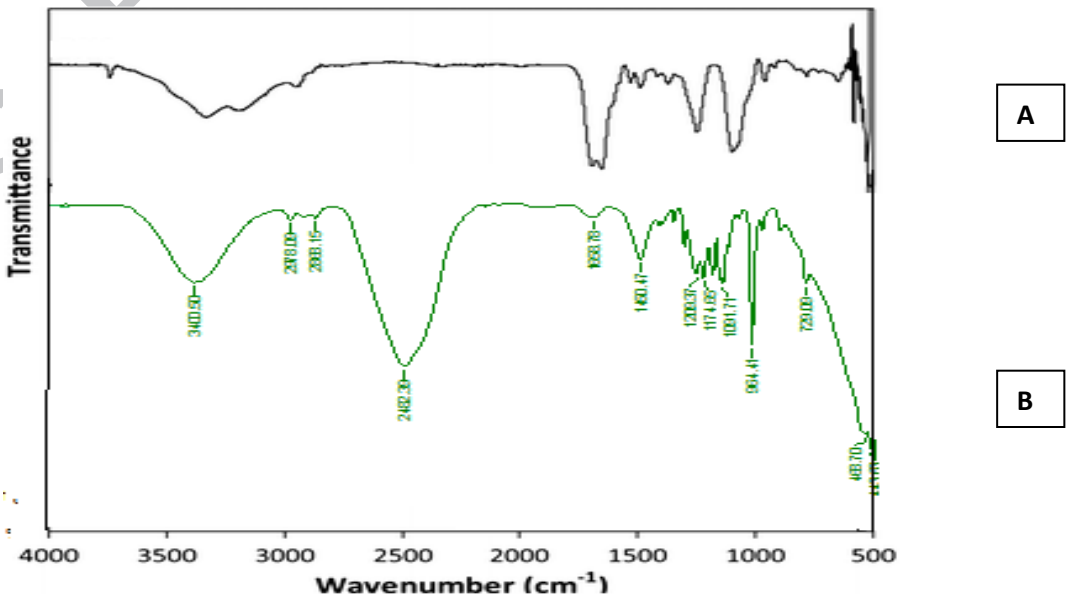


Fig. 6: The infrared spectral data of *ct*-DNA (**A**) and a combination of *ct*-DNA-EDADEP (**B**)

4.3. Fluorescence quenching studies

The fluorescence titration is an important task in binding studies to investigate the mode of EDADEP and $EDAPOPh_2$ binding to the *ct*-DNA. For this purpose competitive binding experiment was carried out using very well-known ethidium bromide (EB) (20 μM) as an intercalative probe. It is a known that the most sensitive fluoresce is due to its planar structure that binds to DNA in intercalative mode followed by weak emission of fluorescence in aqueous medium. In spite of this the fluorescence ability will increase in presence of DNA due to intercalative mode bind between DNA base pairs and EB led to formation of EB-DNA complex [27]. The enhancement of EB-DNA complex fluorescence can be quenched by adding other ligand molecule. If the added ligand intercalates into DNA, there was a clear diminution in the fluorescence spectrum because of decrease in the binding sites of DNA available for EB [28].

A regular decrease in the fluorescence intensity without change in the fluorescence emission wavelength of EB-DNA complex by increasing the concentration of EDADEP and $EDAPOPh_2$ is observed as shown in Fig.7 and Fig.8. Small organic molecules can interact with DNA by intercalative when the concentration of organic molecules to DNA (cM/cDNA) is less than 100 and the fluorescence intensity of EB-DNA complex decreases by 50% [24] here, the quenching extent has been reached up to 35%. This clearly indicates that phosphoramides are competing with the ethidium bromide in *ct*-DNA binding.

There is a change in the absorbance of fluorescence spectrum of EDADEP ($1x10^{-3}$ M) and $EDAPOPh_2$ ($1x10^{-3}$ M) in 10 mM phosphate buffer at pH 7.0, in the absence and presence of phosphoramides (from top to bottom, 0-100μM). According to the classical Stern-Volmer equation [29]:

$$F_0/F = 1 + K_q[Q] \text{.....Eq. 2}$$

Here, both F_0 and F denote the fluorescence intensities in the absence and presence of the complex, respectively. K_q is a linear Stern-Volmer quenching constant, [Q] is the concentration of a quencher. The K_q value can be obtained from a linear plot drawn against F_0/F vs. [Q] as shown in the Fig. 7 and 8 insets. The K_q values for EDADEP and $EDAPOPh_2$ are 1.12 x 10^4, 7.7 x 10^4 M^{-1} respectively. These results suggest that the mode of binding is intercalative with *ct*-DNA by releasing EB from EB-DNA complex, which closely agrees with the absorption results.

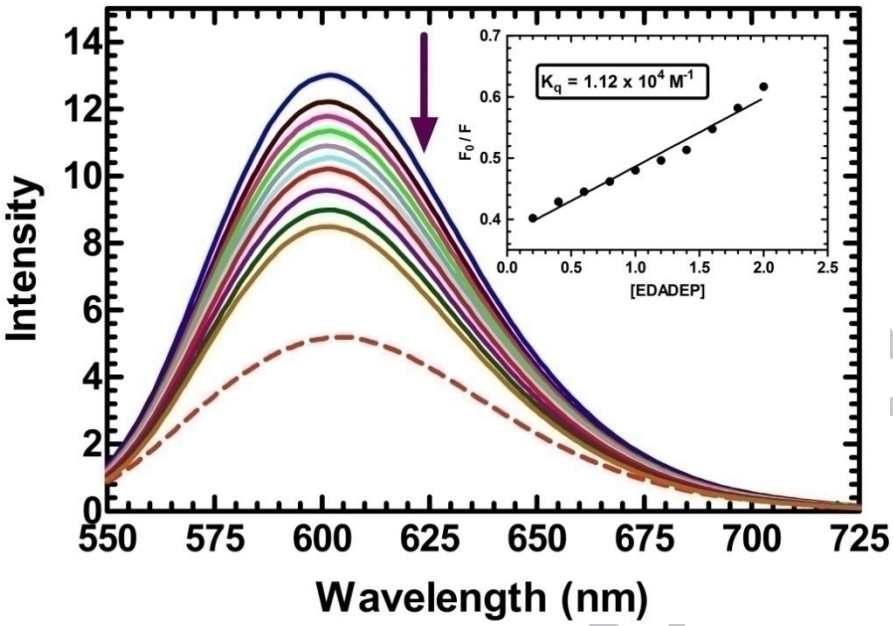


Fig.7. Fluorescence spectrum of EDADEP (1x10^{-3} M) in the absence and presence of *ct*-DNA (0-100 μM) in 10 mM phosphate buffer (at pH 7.2). The inset is Stern-Volmer quenching plot of DNA-EB system of EDADEP. A steady decrease in the emission intensity upon increasing the EDADEP concentration is observed.

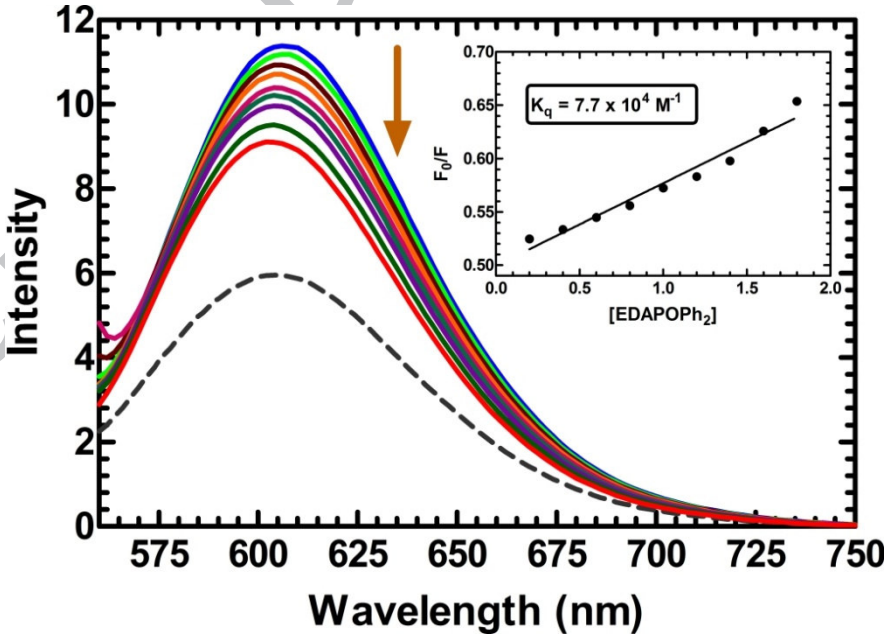


Fig.8. Fluorescence spectrum of EDAPOPh$_2$ (1x10^{-3} M) in the absence and presence of EDAPOPh$_2$ (0-100 μM) in 10 mM phosphate buffer (pH=7.2). The inset is Stern-Volmer quenching plot of DNA-EB system of EDAPOPh$_2$. With the addition of EDAPOPh$_2$, it is revealed that the emission intensity decreases gradually.

2.4. Molecular Docking studies

The molecular docking is a very well-known computational tool to predict the interaction energy between two molecules and ligand orientation with overall minimum energy. This computational method mainly requires algorithm like molecular dynamics, Monte stimulation, genetic algorithms, point complementary methods, fragment based searching methodologies, distance geometry mythologies and systematic searching [30]. The small molecules (considering them as ligands) generally fit into the protein cavities and this is predicted by the search algorithms. These protein cavities can be activated automatically when they contact with external molecules so, they are known as active sites. The docking results are analyzed by a statistical function which is converting the interacting energies into numerical values (docking scores) which are based on non-covalent interactions. The 3-D view of the bound complex can be visualized using various tools like Pymol, Rasmol etc. which is helpful in the identification of the best fit within the complex. Hence docking studies have great importance in the designing and discovery of new drugs [31].

Molecular docking study was performed to simulate the modes of interactions between the bisphosphoramide derivatives and B-DNA which can corroborate the experimental results. Structures of the bisphosphoramide derivatives as well as B-DNA were kept flexible to explore the conformational space in order to predict the best fit orientation and the best docked complex has been analyzed. The lowest energy docked complexes revealed that the bisphosphoramide derivatives could snuggly fit into the curved contour of B-DNA engaging in intimate electrostatic and van der Waals interactions with the walls of the groove. Table-1 shows the optimal energy ranked results of EDADEP and $EDAPOPh_2$ interactions with B-DNA.

Table 1. The binding affinity data for the bisphosphoramide derivatives (EDADEP and $EDAPOPh_2$)

S. No.	Compound	Binding Constant [M^{-1}]	Docking score	Binding energy(kcal/mol)
1.	EDADEP	2.08×10^4	-7.378	-37.374
2.	$EDAPOPh_2$	3.86×10^4	-9.333	-44.752

From the ensuing docked structures it is revealed that the binding energy of $EDAPOPh_2$ is lower than that of EDADEP which indicates that $EDAPOPh_2$ binds strongly to B-DNA compared to EDADEP. Analysis of the docked complex of $EDAPOPh_2$ (Fig.9) showed that it could slide into the minor grove of B-DNA forming significant van-der Waals interactions with G22 (-4.214 kcal/mol), C21 (-6.617 kcal/mol), C20 (-8.444 kcal/mol), A19(-4.496 kcal/mol), C8(-2.323 kcal/mol), A7 (-5.870 kcal/mol), T6 (-6.261 kcal/mol), G5 (-3.632 kcal/mol), G4 (-1.170 kcal/mol) while favorable electrostatic interactions observed with G22 (-2.477 kcal/mol), C21 (-5.716 kcal/mol), C20 (-3.247 kcal/mol), A19 (-1.221 kcal/mol) residues further stabilized the complex. The per-residue interaction analysis revealed that van-der Waals interactions (-36.397 kcal/mol) surpassed electrostatic component (-8.354 kcal/mol) in the overall binding affinity (-44.752 kcal/mol) of $EDAPOPh_2$ towards B-DNA. In addition, there are two hydrogen bonding interactions observed between amide hydrogens of $EDAPOPh_2$ and oxygen atoms of C20 and C21 with a bonding distance of 2.221 Å and 1.726 Å respectively. The hydrogen bonding interactions are crucial in that they act as "anchors" guiding the 3D orientation of a ligand in its binding site, thereby facilitating the steric and electrostatic interactions. Further the interaction of amide hydrogen with DNA through hydrogen bonding is also feasible. On the other hand, docking of EDADEP (Fig. 10) revealed that the compound binds to B-DNA at the same site as $EDAPOPh_2$ with no significant difference in their binding mode but with a weaker strength. Inspection of the non-bonded interaction energies revealed that even for EDADEP, the contribution of van-der Waals interactions (-31.425 kcal/mol) dominated over the electrostatic component (-5.948 kcal/mol) in the overall binding energy (-37.374 kcal/mol). The per residue interaction analysis showed that it is stabilized within the active site through extensive Vander Waals contacts with G22 (-1.73 kcal/mol), C21 (-6.052 kcal/mol), C20 (-5.567 kcal/mol), A19 (-3.122 kcal/mol), C8 (-1.185 kcal/mol), A7 (-5.531 kcal/mol), T6 (-4.853 kcal/mol), G5 (-2.476 kcal/mol) and G4 (-0.780 kcal/mol) and favorable electrostatic interactions with G22 (-2.112 kcal/mol), C21 (-3.603 kcal/mol), C20 (-2.852 kcal/mol) and A19 (-0.835 kcal/mol). Even EDADEP was found to be anchored to the minor groove of B-DNA through two hydrogen bonding interactions observed between amide hydrogens of EDADEP and oxygen atoms of C20 and C21 with a bonding distance of 1.983Å and 1.879 Å respectively. An additional hydrogen bond was observed between the amine H of G5 and the O atom of EDADEP with a bond length of 1.832 Å. Inspection of the per-residue interaction energy break up clearly justify that significantly higher values observed for $EDAPOPh_2$ contributed to its stronger binding affinity to B-DNA over EDADEP which is in harmony with the experimentally observed data. Furthermore, analysis of the binding modes of these bisphosphoramide derivatives also revealed

that the B-form was well retained by the DNA with no significant change in the base pairs even after complexation with bisphosphoramides.

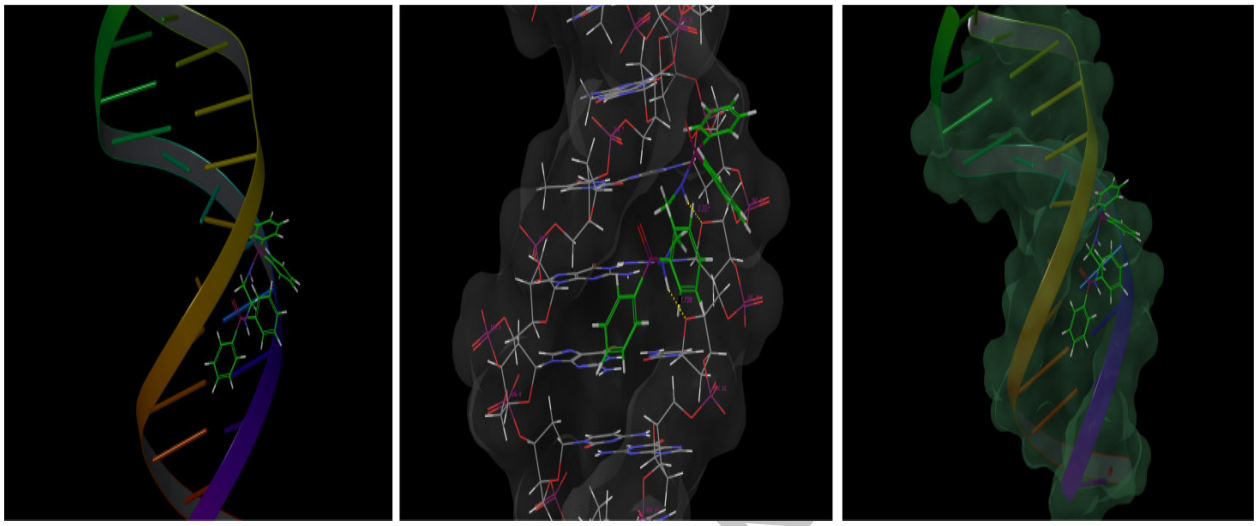


Fig. 9. Binding mode of EDAPOPh$_2$ into the groove of B-DNA

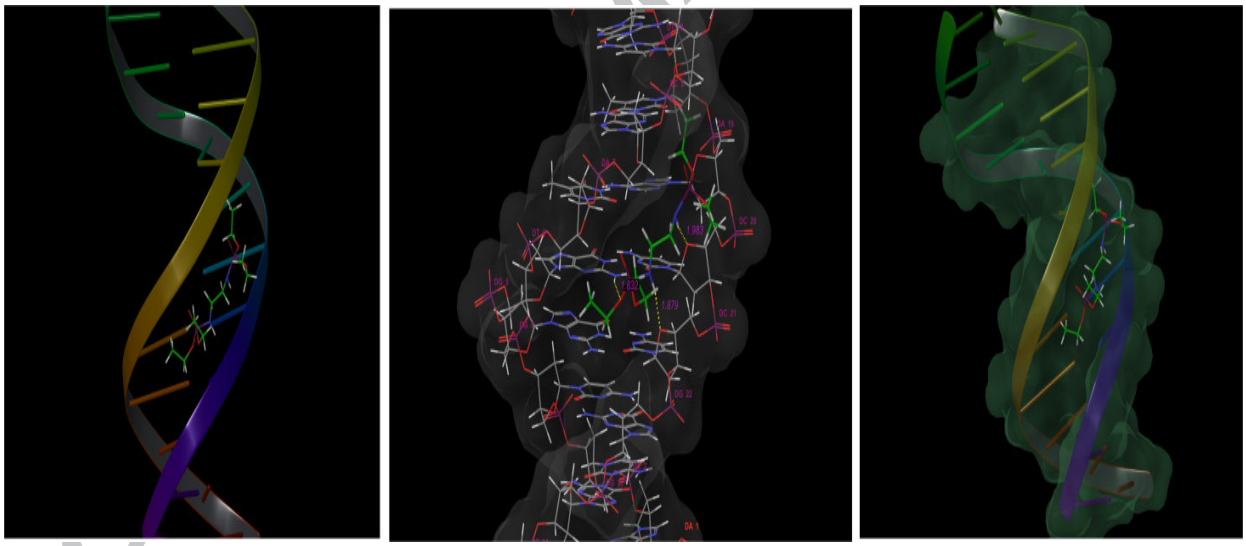


Fig. 10. Binding mode of EDADEP into the groove of B-DNA.

7. Conclusions

In this paper, the interaction of bisphosphoramides with B-DNA was studied by experimental and molecular modeling techniques. Combining the results from UV/Visible absorption spectral and molecular modeling, it is concluded that bisphosphoramides bind to B-DNA through an intercalation mode. The results of molecular modeling studies indicate that the primary driving

force for mechanical interlocking was the steric complementarities between the bisphosphoramides and B-DNA as evidenced from the relatively higher contribution of van-der Waals interaction over electrostatic components in the overall binding scores while hydrogen bonding forces may also play an essential role in the binding. The binding pattern predicted by molecular docking complemented with a detailed per residue interaction analysis provides a strong a basis for the rational design of bisphosphoramides with higher binding affinity to B-DNA.

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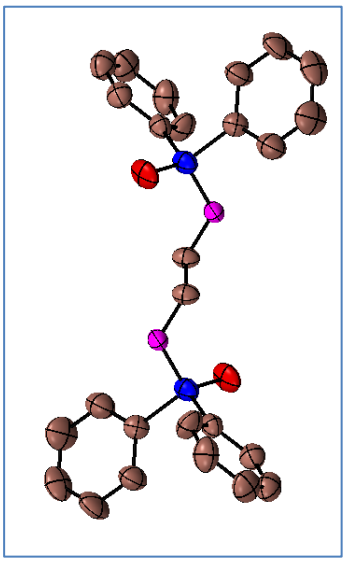
Graphical Abstract

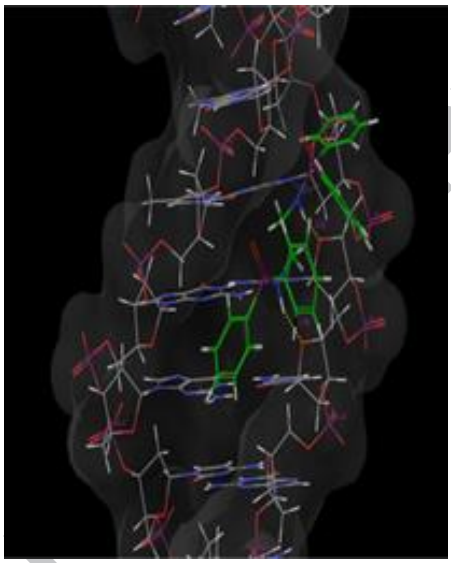
New bisphosphoramides (having phenyl ($EDAPOPh_2$) and ethoxy (EDADEP) substituents attached to phosphoryl groups bridged with ethylenediamine spacer) are synthesized and structurally characterized by IR and NMR spectroscopic techniques as well as elemental analysis. The molecular structure of $EDAPOPh_2$ was determined by single crystal X-ray diffraction technique. The interaction of these bisphosphoramides with calf thymus DNA (*ct*-DNA) is investigated using UV-Visible absorption and fluorescence spectral data as well as the DFT calculations. These studies reveal that $EDAPOPh_2$ and EDADEP interact with DNA in a partial intercalation mode. The intrinsic binding constants K_b of two different bisphosphoramides with *ct*-DNA were determined by fluorescence spectroscopy as 2.08×10^4 and 3.86×10^4 M^{-1} respectively. The results indicated that the two compounds bind to *ct*-DNA with different binding affinities, *i.e.* $EDAPOPh_2$ > EDADEP. The binding mechanism of these bisphosphoramides to *ct*-DNA is also discussed.

Graphical Pictogram



Bisphosphoramide

***ct*-DNA**



Highlights

- Two new bisphosphoramides with phenyl and ethoxy substituents attached to phosphoryl groups bridged with ethylenediamine spacer are synthesized.
- These molecules are structurally characterized by IR and NMR spectroscopic techniques as well as elemental analysis including the molecular structure of phenyl substituted bisphosphoramide.
- The interaction of these bisphosphoramides with *ct*-DNA is investigated using UV-Visible absorption and fluorescence spectral data.
- Both experimental and DFT calculations reveal that both the phosphoramides interact with DNA in a partial intercalation mode, but the binding affinity is more for the phenyl substituent than the ethoxy analogue.