Pergamon

Biochemical Pharmacology, Vol. 48, No. 11, pp. 2097–2102, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00350-5

INTERACTION OF SANGUINARINE IMINIUM AND ALKANOLAMINE FORM WITH CALF THYMUS DNA

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(*Received* 6 *April* 1994; *accepted* 26 *July* 1994)

Abstract—The interaction of sanguinarine iminium form (structure I) and sanguinarine alkanolamine form (structure II) with calf thymus DNA has been studied in buffer of pH 5.2 and pH 10.5, respectively, where the physicochemical properties of DNA remain unchanged. The binding of sanguinarine iminium form to DNA is characterized by hypochromism and bathochromism in the absorption band, quenching of fluorescence intensity, increase in fluorescence polarization anisotropy, increase in positive and negative ellipticity of DNA, sign and magnitude of the thermodynamic parameters and increase in contour length of sonicated rodlike duplex DNA indicating that it binds to DNA by a mechanism of intercalation. In contrast, sanguinarine alkanolamine form does not show (i) any significant change in fluorescence polarization anisotropy, (ii) alteration of B form structure of DNA and (iii) increase in contour length of DNA indicating that it does not bind to DNA. But at a very high concentration of DNA, the alkanolamine form is influenced to form an iminium–DNA complex.

Key words: alkaloid–DNA interactions; spectrophotometry; spectrofluorimetry; spectropolarimetry; thermodynamics; viscometry

The biological activity of many small molecules is believed to be due to their interaction with DNA and the elucidation of the physico-chemical characteristics of such interactions is of considerable interest [1–3]. Among the biologically active molecules, naturally occurring alkaloids occupy an important position because of their extensive medical value. Most of the alkaloids have promising antimicrobial, antitumour and a host of other biological activities [4, 5 and references therein].

Sanguinarine (Fig. 1), a benzophenanthridine alkaloid, has been reported to possess antitumour, antimicrobial and various other biological activities [4–6]. It exhibits pH dependent structural equilibrium between the iminium form (structure I) and alkanolamine form (structure II) with pK value of 7.4 as revealed by spectrophotometric and spectrofluorimetric measurements [7]. The stability of two structural forms has further been confirmed by Jones *et al.* [8]. Conversion from iminium ion (structure I) to alkanolamine form (structure II) may enhance antimicrobial activity by increasing cellular availability of the alkaloid due to its greater lipophilicity [8]. Use of the sanguinarine alkanolamine form as a prodrug in humans has been reported [9]. Sanguinarine has also been shown to form a molecular complex with DNA, by intercalation [10–13]. All previous studies were performed in a buffer of neutral pH where both the iminium and alkanolamine forms were present and some of the data are misleading. The present study is an attempt to investigate the interaction of the two forms separately under identical conditions in order to find out exclusively the exact mode of binding of these forms to calf thymus DNA using spectrophotometric, spectrofluorimetric, spectropolarimetric, thermodynamic and viscometric techniques.

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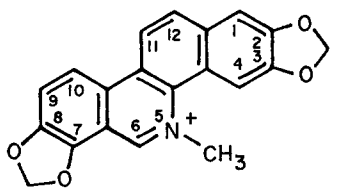
† Abbreviations: CT DNA, calf thymus deoxyribonucleic acid; CPE, citrate phosphate EDTA buffer; CBCE, carbonate bicarbonate EDTA buffer; CD, circular dichroism.

MATERIALS AND METHODS

Sanguinarine chloride was purchased from the Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.) and used without further purification. The alkaloid concentration was determined spectrophotometrically using molar extinction coefficient (ε) of 30,700 M^{-1} cm^{-1} at 327 nm in acidic buffer. CT DNA† (type I, 42 mole % GC) was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and was used as such. CT DNA stock solution was prepared in BPE buffer (0.0162 M NaH_2PO_4, $2H_2O$, 0.04165 M Na_2HPO_4 anhydrous purified, 0.25 mM EDTA, pH 7.2 ± 0.05). DNA concentration in terms of nucleotide phosphate was determined spectrophotometrically using ε value of 6600 M^{-1} cm^{-1} at 260 nm. Deionized glass distilled water and analytical grade reagents were used throughout. The DNA binding experiments were performed in CPE buffer (4.254 mM citric acid-1-hydrate, 9.75 mM Na_2HPO_4 anhydrous purified, 0.25 mM EDTA, pH 5.2 ± 0.05) and CBCE buffer (8.727 mM anhydrous Na_2CO_3, 2.047 mM $NaHCO_3$, 0.25 mM EDTA, pH 10.5 ± 0.05), respectively.

Absorption study. The absorption spectra of sanguinarine mixed with or without DNA were obtained using Shimadzu UV-260 spectrophotometer (Shimadzu Corporation, Japan) against an appropriately prepared reference or control in quartz cell



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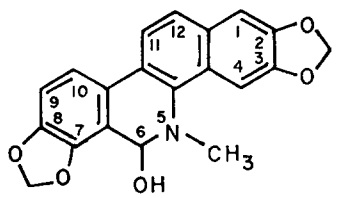

II

Fig. 1. Chemical structure of sanguinarine iminium form (structure I) and sanguinarine alkanolamine form (structure II).

of 1 cm path length. The spectrophotometric titrations were performed by keeping the concentration of sanguinarine constant while varying the CT DNA concentration, as described in [14, 15].

The titration plot was obtained from the absorbance at 327 nm for each sanguinarine/DNA (D/P) ratio. Binding data obtained were cast into the form of Scatchard Plot [16] as r/C vs r, where r is the number of moles of ligand bound per mole of nucleotide and C is the concentration of free ligand and were fitted to the neighbour exclusion model [17, 18].

$$\frac{r}{C} = \mathrm{K}(1 - nr)\,[(1 - nr)/(1 - (n-1)r)]^{n-1} \quad (1)$$

where K is the intrinsic binding constant to an isolated DNA binding site and n is the exclusion parameter.

Fluorescence study. The fluorescence measurements were recorded in a Hitachi F4010 spectrofluorimeter (Hitachi Ltd, Tokyo, Japan), as described in Refs. 14, 15. When a fixed concentration of sanguinarine was titrated by increasing concentration of DNA, the relative fluorescence intensity of emission spectra at 577 nm with excitation at 475 nm was used in titration plot. Binding isotherms were obtained and fitted as described above. In fluorescence quenching experiment, the data were also plotted according to the Stern–Volmer equation,

$$I_0/I = 1 + \mathrm{K_{sv}}[\mathrm{DNA}] \quad (2)$$

where I_0 and I are the fluorescence intensities in the absence and in the presence of DNA, respectively. $\mathrm{K_{sv}}$ is the Stern–Volmer quenching constant, which is a measure of the efficiency of quenching by DNA.

Fluorescence polarization anisotropy was calculated from the following expression,

$$\mathrm{A} = \frac{I_{11} - I_\perp}{I_{11} + 2I_\perp} \quad (3)$$

where I_{11} is the relative fluorescence intensity with polarization parallel to the excitation light and $I_\perp$ is the relative fluorescence intensity with polarization perpendicular to the excitation light.

Thermodynamic study. Temperature dependent fluorescence studies of sanguinarine structure I—CT DNA binding were performed at 15°, 20°, 30° and 40° either by a complete titration at the given temperature or by increasing the temperature of a sample containing a fixed ratio of alkaloid-DNA as described in Ref. 19, allowing an equilibrium period of 5 min before each spectrum was recorded. Thermodynamic parameters were estimated by the analysis of a LnK vs $1/T$ plot (van't Hoff plot) obtained over the temperature range of the study which enables the calculation of ΔH°, since the gradient is equal to $-\Delta H°/R$. ΔG° and ΔS° were calculated in turn from the relationships,

$$\Delta G° = -RT\,\mathrm{Ln}K \quad (4)$$

and

$$\Delta G° = \Delta H° - T\Delta S° \quad (5)$$

Circular dichroism study. CD spectra were recorded on a Jasco J-20A spectropolarimeter equipped with a data processor attachment, Model J-DPY (Japan Spectroscopic Ltd, Japan), in a cylindrical quartz cell of 1 cm path-length as described in Refs. 20, 21. The CD unit was routinely calibrated by using a solution of D-10-camphorsulfonic acid in water. Molar ellipticity [θ] is expressed in deg. cm^2/dmole.

Viscometric study. For viscometric experiments, samples of linear duplex native DNAs were sonicated in a Labsonic 2000 sonicator (B. Braun Swiss, West Germany) by using a needle probe of 4 mm diameter as described [15]. The sonicated DNA samples had a molecular mass of the order of 2–3 $\times$ 10^5 Da [22] with an intrinsic viscosity of 3.1 dL/g. Viscosity measurements were performed with a Cannon-Manning Type 75 semimicroviscometer, mounted vertically in a constant-temperature-bath (Cannon Instruments Co., State College, PA, U.S.A.) maintained at 25 $\pm$ 0.05°. Flow times of sonicated rod-like DNA alone and DNA-alkaloid complexes were measured by an electronic stopwatch (Fisher Scientific, Pittsburgh, PA, U.S.A.) with an accuracy of 0.01 sec. The increase in helix contour length of sheared DNA was calculated by using the expression [23],

$$L/L_0 = [(t_2 - t_0)/(t_1 - t_0)]^{1/3} = 1 + \beta r \quad (6)$$

where L is the contour length in the presence of the alkaloid, L_0 is the contour length of free DNA, t_2 is the flow time in presence of the alkaloid, t_1 is the flow time for pure DNA, t_0 is the flow time for buffer at a given volume in the viscometer and β is the slope when L/L_0 is plotted against r, as described in Refs. 10, 15.

RESULTS

Absorption spectra

The characteristic electronic absorption spectrum of sanguinarine iminium form shows the peaks at 327, 400 and 475 nm, respectively and a kink at

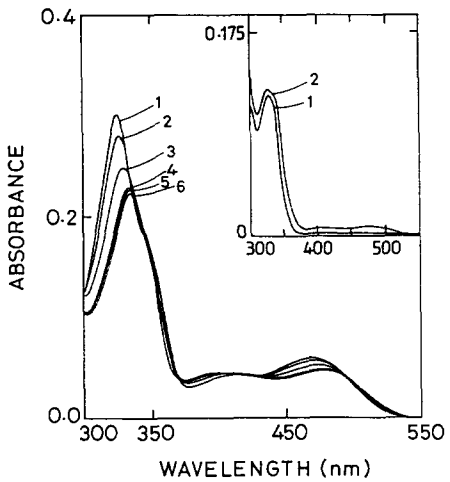

Fig. 2. Influence of CT DNA on the absorption spectrum of structure I (9.86 μM, curve 1) treated with 9.9, 29.7, 49.4, 69.1 and 98.7 μM of CT DNA, respectively, denoted by curves 2–6 in CPE buffer, $[Na^+] = 20$ mM, pH 5.2. Inset: Structure II (9.86 μM, curve 1) treated with 59.8 μM CT DNA (curve 2) in CBCE buffer, $[Na^+] = 20$ mM, pH 10.5.

351 nm; while that for sanguinarine alkanolamine form shows one peak at 327 nm only, in the range of 300 to 550 nm. The spectral characteristics indicate the existence of sanguinarine iminium form (structure I) and sanguinarine alkanolamine form (structure II) in almost 100 per cent at pH 5.2 and 10.5, respectively. The effect of progressively increasing concentration of CT DNA on the absorption spectrum of sanguinarine iminium form is depicted in Fig. 2. The spectral changes involve hypochromism and bathochromism in all the three bands with clear isosbestic points observed at 346, 370 and 492 nm, respectively. Isosbestic points observed in this spectra are indicative of equilibrium between bound and free molecules. The spectral data shows a gradual red shift of about 8 to 10 nm until saturation was reached at a P/D value of about 6 and onwards. On the other hand, the spectral characteristics of structure II did not show any significant change at P/D value of 6 (Fig. 2, inset). The results of absorption titration were expressed in the form of Scatchard plots and were analysed using equation 1. The quantitative data of the binding parameters thus evaluated are presented in Table 1.

Fluorescence spectra

It has been observed that fluorescence characteristic pattern of structure I and structure II are quite different. The characteristic emission spectrum of structure I in the region of 500–650 nm has an emission maximum at 577 nm when excited either at 327 or at 475 nm; while characteristic emission spectrum of structure II in the region of 350–500 nm has a maximum at 418 nm only when excited at 327 nm. This difference is correlated with the characteristic excitation spectra of the two structures. The excitation spectrum of structure I has three peaks at 327, 400 and 475 nm, respectively, while that of structure II has only one peak at 327 nm in the region of 300–550 nm. It is interesting to note that according to our previous study [7] the relative quantum yields of structure I and structure II are 0.0036 and 0.134, respectively.

Figure 3A shows that the intensity of the emission band of structure I decreased progressively with increasing concentration of DNA. A maximum blue shift of about 10 nm was observed at saturation corresponding to P/D value of about 6. The binding parameters K and n and Stern–Volmer quenching constant K_{sv} evaluated from spectrofluorimetric titration data as described in the Materials and Methods are presented in Table 1. The binding constants obtained from the two methods are consistent within the experimental error of two different methods and lend credibility to these measurements. The binding of structure I to the DNA helix was found to quench very strongly which is revealed from the high value of K_{sv} (Table 1).

An interesting emission spectral characteristic of structure II with increasing concentration of DNA is illustrated in Fig. 3B, when excited at 475 nm. Figure 3B shows that upto P/D value of about 6 no change in the spectral pattern was noticed. Thereafter as the DNA concentration was increased the

Table 1. Binding parameters for the interaction of sanguinarine with CT DNA in CPE ($[Na^+] = 20$ mM, pH 5.2 ± 0.05)*

Parameters	Methods	Values
K, the intrinsic binding constant (M^{-1})	Spectrophotometry†	$(9.35 \pm 0.2) \times 10^5$
	Spectrofluorimetry†	$(9.5 \pm 0.3) \times 10^5$
n, the number of nucleotide occluded	Spectrophotometry	3.7 ± 0.05
	Spectrofluorimetry	3 ± 0.05
K_{sv}, Stern–Volmer quenching constant (M^{-1})	Spectrofluorimetry	9.0×10^4
$\Delta G°$ (20°) (Kcal/mol)	Spectrofluorimetry	−8.03 ± 0.04
$\Delta H°$ (Kcal/mol)	Spectrofluorimetry	−5.37 ± 0.2
$\Delta S°$ (20°) (Cal/deg-mol)	Spectrofluorimetry	+9.07 ± 0.55
β, the slope of L/L_0 vs r plot	Viscometry	2 ± 0.05

* Average from three determinations.
† Concentration of sanguinarine is 9.86 μM for both the methods.

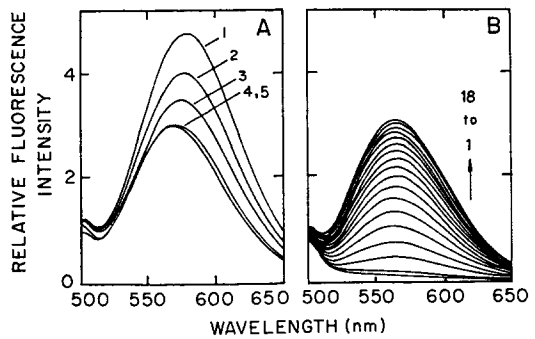

Fig. 3. (A) Influence of CT DNA on the fluorescence emission spectrum of structure I (2.11 μM, curve 1) treated with 2.2, 4.3, 9.7 and 15.2 μM of CT DNA, respectively, denoted by curves 2–5. The excitation was fixed at 475 nm. (B) Influence of CT DNA on the fluorescence emission spectrum of structure II (2.11 μM, curve 1) treated with 5.4, 16.3, 27.1, 37.9, 48.8, 59.7, 70.5, 81.4, 92.3, 103.1, 113.9, 124.8, 135.7, 146.5, 157.4, 168.2 and 179.1 μM of CT DNA, respectively, denoted by curves 2–18. The excitation was fixed at 475 nm.

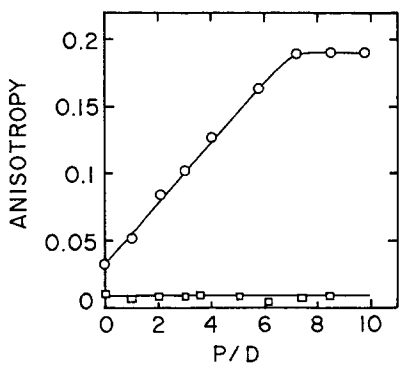

Fig. 4. Plot of fluorescence polarization anisotropy vs P/D ratio for structure I (○—○) and structure II (□—□). The concentration of structure I and structure II is the same, i.e. 2.11 μM.

progressive appearance of characteristic emission band for bound iminium–DNA complex was observed. At P/D of about 100, the spectral pattern is superimposable with that of bound iminium–DNA complex corresponding to P/D ratio 6 (spectrum No. 4 of Fig. 3A). It is likely that the strong increase in emission intensity at 577 nm in presence of very high concentration of DNA may be owing to the fact that high concentration of DNA can influence uncharged structure II to form charged structure I and then it binds to DNA.

Further evidence for the strong interaction of structure I is obtained from fluorescence polarization method. When the benzophenanthrene chromophore of sanguinarine intercalates into the DNA-helix, its rotational motion should be affected and hence fluorescence from the bound chromophore should

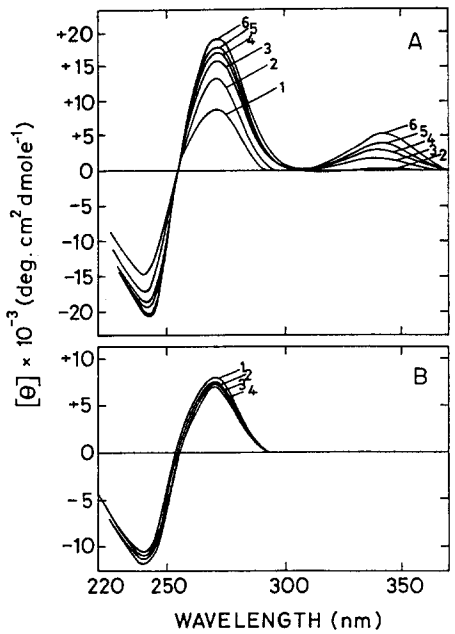

Fig. 5. CD spectra resulting from (A) 100 μM of CT DNA (curve 1) treated with 10.2, 20.5, 30.1, 40.0 and 50.6 μM of structure I (curves 2–6), respectively, in CPE buffer, [Na^+] = 20 mM, pH 5.2 and (B) 100 μM of CT DNA (curve 1) treated with 10.3, 29.9 and 47.9 μM of structure II (curves 2–4), respectively, in CBCE buffer, [Na^+] = 20 mM, pH 10.5.

be polarized. The fluorescence forms of sanguinarine iminium and alkanolamine in solution are weakly polarized and their values are 0.0332 and 0.0118, respectively, due to the rapid tumbling motion of the chromophores in the aqueous medium. Figure 4 shows the anisotropy data for both forms at various P/D values. The large increase in polarization anisotropy of structure I upon binding to DNA suggests intercalation of this structure into the helix, while no significant change in polarization anisotropy of structure II with increasing concentration of DNA indicates that this structure does not bind to DNA.

Thermodynamics

The values of thermodynamic parameters of structure I–DNA complexation are presented in Table 1. The negative ΔH° is indicative of the exothermic process of binding and the positive value of entropy change implies that the binding of structure I to DNA is an entropy-driven intercalation process, as reported for several other intercalators [24–26].

CD spectra. Other evidence for strong interaction of structure I with DNA helix comes from circular dichroism studies. Both the structure I and structure II are optically inactive and they do not have CD spectrum in the region of 220 nm to 350 nm. In presence of structure I the B form CD spectrum of DNA was remarkably perturbed and the results are illustrated in Fig. 5. The intensity of 270 nm band and 240 nm band were increased significantly on

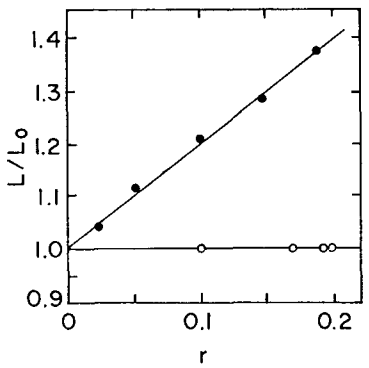

Fig. 6. A plot of L/L_0 versus r for sonicated CT DNA with increasing concentration of structure I (●—●) and structure II (○—○), respectively, in CPE buffer, $[Na^+] = 20$ mM, pH 5.2 and CBCE buffer, $[Na^+] = 20$ mM, pH 10.5.

progressive addition of structure I, with the appearance of a new peak at a wavelength of 340 nm. An isoelliptic point appeared at 255 nm, indicating the equilibrium between the free and bound form of structure I. The peak intensity at 340 nm also increases with increasing concentration of structure I due to the asymmetric arrangement of alkaloids in the DNA helix. Structure II did not cause any alteration to the B form structure of DNA at the same D/P ratio as that of iminium–DNA.

Viscometric titration

An increase in the viscosity of native rod-like duplex DNA is regarded as a diagnostic feature of intercalation process [1, 10 and references therein]. The viscometric measurements of sonicated duplex DNA with increasing concentration of structure I and structure II are shown in Fig. 6. The L/L_0 value increases with r for structure I while no significant change or enhancement was observed for structure II. The β value of about 2 (Table 1) is the same as that predicted for monofunctional intercalators [1, 15]. Thus, viscometric data shows quite concrete evidence that structure II does not bind to DNA.

DISCUSSION

The strong interaction of structure I with CT DNA is evident from the observation of typical bathochromic and hypochromic effects in the absorption spectra, the extensive fluorescence quenching, increase in fluorescence polarization anisotropy, significant perturbation of B form DNA helix, sign and magnitude of thermodynamic parameters and increase in viscosity of sonicated DNA. The bathochromic and hypochromic effects are similar to that observed for other intercalators [1, 23] and are also in agreement with earlier studies [11, 12, 27, 28]. Again, the order and magnitude of intrinsic binding constants obtained from spectrophotometric and spectrofluorimetric studies are the same as that of other intercalators [1, 15, 19, 23]. It can be seen from Table 1 that the binding process is exothermic and the intercalation of structure I into the DNA helix is entropy-driven [24–26]. Accordingly, entropy-driven binding reaction in aqueous solution generally reflects solvent effects such as binding-induced disruption of both DNA-bound water [29] and hydrophobically restricted water surrounding the drug in solution [26]. The most likely candidate for such positive entropy changes would be related to the Frank and Evans type of "iceberg" or saturated regions of water around nonpolar solute [30]. The $\Delta S°$ value presented in Table 1 thus provides strong evidence for the importance of water–structure effect in promoting stacking type association of intercalating alkaloids with the DNA bases. The same features were previously observed with other intercalating drugs actinomycin D, ellipticine, proflavin, ethidium whose binding is also entropy-driven [26].

The study on the interaction of structure II with DNA reveals that it did not alter (i) the absorption and fluorescence spectra in presence of DNA upto a P/D value of 6, (ii) B form CD spectrum of DNA helix, (iii) fluorescence polarization anisotropy and also (iv) it did not increase the length of the sonicated DNA. The study has, thus, clearly demonstrated that structure II does not bind to DNA. However, the present data are contradictory to the work reported by Smekal *et al.* [12] where they found that both the cationic (structure I) and neutral (structure II) form intercalate completely and partially, respectively, to DNA helix. Their observations were mainly from spectrophotometric studies. Again, it is pertinent to point out that the structure II has a partial saturation in the chemical structure and in contrast to that, structure I has a planar and aromatic ring system like ethidium. This structural difference may cause the drastic difference in the process of binding. In this respect, it may be noted that both the cationic and the base form of ellipticine and phenanthridine derivative can bind to DNA by the process of intercalation [31, 32], where both the cationic and base form are planar structure. It is likely that structure II is unable to bind to DNA due to its structural nonplanarity. Furthermore, the emission spectral characteristics of structure II (Fig. 3B) show that emission spectra for bound structure I–DNA complex appears at a very high concentration of DNA. This indicates that DNA influences structure II to form structure I, though the reason is not known.

In conclusion, the results presented here indicate that (i) sanguinarine iminium form binds to DNA by a mechanism of intercalation, (ii) sanguinarine alkanolamine form does not bind to DNA, and (iii) at a very high DNA/alkanolamine ratio the bound spectral pattern is the same as that of bound-iminium–DNA complex.

Acknowledgements—A.S. is indebted to the University Grants Commission, Government of India for the award of Senior Research Fellowship. Partial grant from DST, Government of India (No. SP/SO/D21/91) is acknowledged.

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