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Synthesis of new 13-diphenylalkyl analogues of berberine and elucidation of their base pair specificity and energetics of DNA binding†

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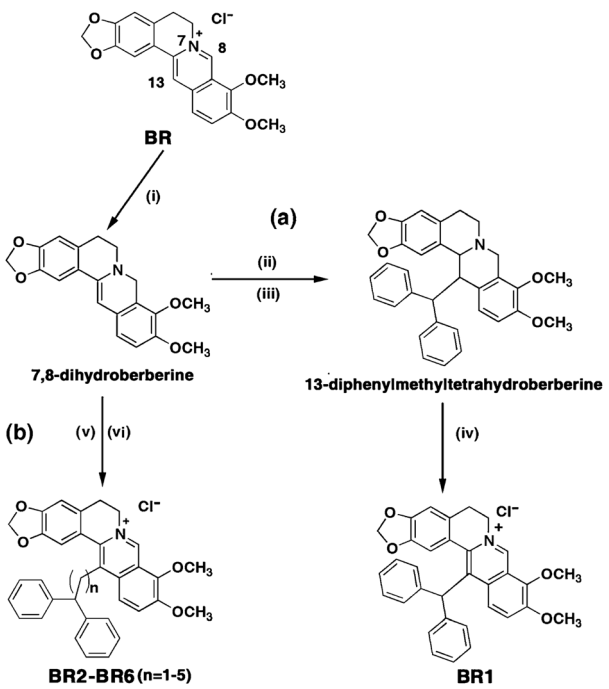
A series of 13-diphenylalkyl berberine derivatives were designed and synthesized, and their base specificity and energetics of DNA binding were evaluated using one natural and two synthetic DNA polynucleotides. Biophysical evaluation demonstrated that the addition of the diphenylalkyl chain at the 13-position of berberine significantly improved the binding ability to DNA. The binding clearly revealed the high preference of the analogues to the alternating AT sequences compared to the alternating GC sequences. The binding affinity was enhanced with the increase in chain length up to a critical length of $(CH_2)_3$ in all the cases, after which the binding affinity decreased. Analogue BR4 had the best affinity for DNA, which corresponds to a length of $(CH_2)_3$. The results also suggested the adenine-thymine (AT) base specificity of these berberine analogues and that the length of the side chain at the 13-position of the isoquinoline chromophore is critical in modulating the binding affinity.

Berberine (**BR**; Scheme 1), a natural quaternary protoberberine isoquinoline alkaloid isolated from the Chinese herb *Rhizoma coptidis*, has attracted remarkable recent attention for its extensive multiple pharmacological effects and usefulness in biomedical applications.[1,2] The high medicinal value of berberine is exemplified from its use in the Indian Ayurvedic, Unani and Chinese systems of medicine from time immemorial.[3,4] The alkaloid has been reported to exhibit antiproliferative activity *in vitro* and induced apoptosis/necrosis in several cell lines tested.[5,6] The ability of berberine to form strong complexes with nucleic acids, inflict deoxyribonucleic acid (DNA) damage, inhibit telomerase, poison topoisomerase and inhibit gene transcription have often been linked to its anticancer activities.[7] Extensive studies on the DNA and RNA binding of berberine, its adenine-thymine (AT) specificity, and the binding of many related molecules have been reported from our laboratory[8–10] and many other laboratories[11,12] including the crystal structure of the berberine–oligonucleotide duplex and G-quadruplex complex solved recently.[13] Based on the result of these studies, berberine has emerged as an attractive lead compound for the development of functional DNA and RNA targeted drugs.



Scheme 1 Synthesis of (a) 13-diphenylmethylberberine (BR1): (i) $NaBH_4$, Py, rt, 1 h; (ii) Ph_2CHBr, CH_3CN, NaI, 90 °C, 12 h, then (iii) $NaBH_4$, EtOH, 0 °C to rt, 12 h; (iv) *N*-chlorosuccinimide, $CHCl_3$, rt, 12 h; and (b) higher 13-diphenylalkylberberine (BR2–BR6): (v) $Ph_2CH(CH_2)_{0-4}CHO$, 80% aq. EtOH, AcOH, 85 °C, 6 h, then (vi) 2 M HCl, rt, 2 h.

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Structurally, berberine has an extended π delocalized system with a positively charged nitrogen atom. However, serious limitations with berberine are its low aqueous solubility and moderate nucleic acid binding affinity [$(1–2) \times 10^5$ M^{-1}].[14] To circumvent these inherent problems several synthetic efforts have been made to derivatise key positions on the isoquinoline moiety.[15,16] These studies have suggested that positions 9 and 13 are critical for topoisomerase inhibition and quadruplex structure binding.[17,18] Analogues with substitutions at these positions have also exhibited better anticancer activity[19] and other biological activities,[20] and higher DNA and RNA binding efficacy.[14,16] We recently showed the enhanced DNA binding affinity of 13-phenylalkyl-substituted analogues.[21] In continuation of these studies we synthesized six new 13-diphenylalkyl analogues (Scheme 1) to see how these analogues bind and exhibit specificity to DNA. Sequence selectivity of the interaction of these alkaloid analogues and the related energetics of interaction are essential for drug development and DNA-based therapeutics. In order to gain a better insight into these aspects we probed the binding affinity, base sequence specificity and thermodynamics of binding of the analogues to DNA through spectroscopy, competition dialysis assay and micro calorimetry experiments with calf thymus (CT) DNA, poly(dG-dC)·poly(dG-dC), and poly(dA-dT)·poly(dA-dT).[22]

The details of the synthesis and characterization of the 13-diphenylalkyl berberine analogues (Scheme 1) are outlined in the ESI.†[23] We at first probed the mode of binding of these analogues with CT DNA in comparison with berberine using ferrocyanide quenching experiments.[24] The basis of this study is that in a drug–nucleic acid complex the molecules that are free or bound weakly on the surface may be readily available to an anionic quencher, whereas those bound between bases or inside the polynucleotide may be shielded from quenching. The electrostatic barrier of the negatively charged phosphate groups limits the penetration of an anionic quencher into the interior of the helix. Hence, very little or no quenching may be observed in the presence of an anionic quencher like $K_4[Fe(CN)_6]$ if the binding involves intercalation and/or deep-stacking interactions. The results revealed that more quenching was observed in the case of the free analogues and less quenching for the bound analogues (ESI, Fig. 1a†). This suggested that the bound analogues, like BR, are located in a relatively more protected environment compared to the free molecules. The quenching constants (K_{sv}) calculated were in the range of 216–230 M^{-1} for the unbound BR and analogues and 92, 152, 74, 34, 50 and 66 M^{-1}, respectively, for bound BR, BR2, BR3, BR4, BR5 and BR6. In BR4, the phenyl group is separated by four carbon atoms from the isoquinoline chromophore and appears to be flexible, resulting in better intercalation geometry compared to all the other analogues. The critical length of $(CH_2)_3$ for the best binding intercalator, was also apparent from these results.

Fluorescence polarization anisotropy experiments were performed[25] that gave anisotropy values of 0.17, 0.18, 0.19, 0.17, respectively, for BR3, BR4, BR5, and BR6 (ESI, Fig. 1b†) against a value of 0.14 for BR, confirming the intercalated binding and the higher binding strength of BR3–BR6 over BR.

In viscosity experiments[26] we observed β values of 0.78, 0.85, 0.80 and 0.78, respectively, for BR3, BR4, BR5 and BR6 in comparison to the theoretical value of 1.0 for a classical intercalator, suggesting a better intercalation geometry for the analogues compared to BR that gave a value of 0.70.

Optical thermal melting and differential scanning calorimetry (DSC) experiments[27] suggested that BR stabilized the DNA by about 13.0 °C whereas BR2–BR5 stabilized the DNA by 7.0 °C, 16.0 °C, 19 °C and 17.0 °C, respectively (ESI, Fig. 2†) at saturating condition ($D/P = 1.0$). The binding constant (K_{uv}) values calculated for 20 °C from these data[27] are depicted in Table S1 (ESI†). The higher stabilization of the DNA by the analogues essentially is due to the stronger binding. This result further confirms the stronger binding of these analogues to DNA, better intercalation geometry compared to BR and the critical length of $(CH_2)_3$ of the side chain in the binding process.

The binding affinity and the base specificity of the analogues were evaluated using absorbance spectroscopy,[28] competition dialysis[29] and isothermal titration calorimetry (ITC) experiments.[30] Hypochromic and bathochromic effects were observed in the visible absorption bands of the berberine analogues with three sharp isosbestic points providing unambiguous evidence for the formation of stable complexes with an equilibrium between the free and bound alkaloid molecules. Such spectral changes have been ascribed to a strong interaction between the π electron cloud of the alkaloid molecule and the base pairs of DNA resulting from strong intercalative interaction.[31] Representative absorption spectra of the free and fully bound BR3 with the three polynucleotides are presented in Fig. 1. The binding affinities of the alkaloid analogues were estimated from Scatchard plots fitted to appropriate McGhee and von Hippel analysis.[28] The binding of berberine was cooperative as evidenced by the positive slope in the Scatchard plot at low r values while the binding of the analogues studied was found to be non-cooperative (negative slope) (Fig. 1 inset). The intrinsic binding affinity (K) values obtained from absorption spectral analysis are presented in Table 1. From the data it is clear that the binding of the analogues to poly(dA-dT)·poly(dA-dT) was remarkably stronger as compared to that with CT DNA and poly(dG-dC)·poly(dG-dC). The binding affinities varied as BR < BR1 < BR2 < BR3 < BR4 > BR5> BR6, suggesting that the alkyl chain length influenced the binding remarkably.

Competition dialysis is an effective tool based on the fundamental thermodynamic principle of equilibrium dialysis developed by Chaires and co-workers[29] for the discovery of ligands that can bind with sequence specificity to DNA. We included additionally two natural DNAs, one of high GC and one of high AT content in this assay. The results of competition dialysis assay are presented in Fig. 2 as bar graphs showing the concentration of alkaloid bound to each of the polynucleotides. The highest binding in terms of more accumulation of the analogues was found to be with poly(dA-dT)·poly(dA-dT); poly(dG-dC)·poly(dG-dC) had the least preference as revealed by the lowest amount of bound analogues. The apparent binding affinities (K_{app}) have been calculated from the concentrations of the bound alkaloids[32] (Fig. 2) and the magnitude for the analogues varied in the order poly(dA-dT)·poly(dA-dT) >

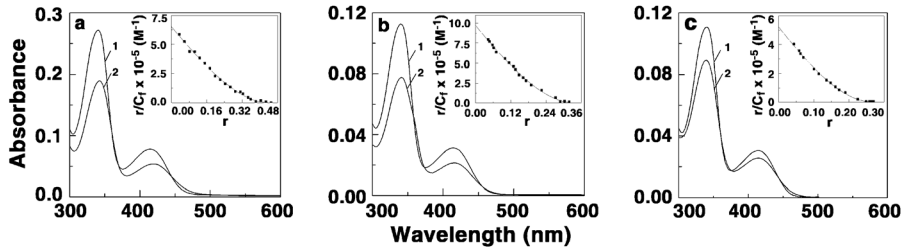

Fig. 1 Absorption spectra of free (curve 1) and bound (curve 2) BR3 in the presence of saturating concentrations of (a) CT DNA, (b) poly(dA-dT)·poly(dA-dT), and (c) poly(dG-dC)·poly(dG-dC). Inset: respective non-cooperative Scatchard isotherms of the binding.

Clostridium perfringens (CP) DNA (72% AT) > calf thymus (CT) DNA (58% AT) > *Micrococcus lysodeikticus* (ML) DNA (28% AT) > poly(dG-dC)·poly(dG-dC).

The conformational aspects of the binding were evaluated by circular dichroism (CD) spectroscopy.[33] The circular dichroism spectral pattern of the DNA and polynucleotides displayed a B-form conformation characterized by a large positive band in the 270–280 nm region and a negative band around 245 nm, although there are differences in the ellipticity and wavelength maxima. In the presence of the BR analogues, the positive ellipticity of the CT DNA decreased with a concomitant red shift of the maximum (ESI, Fig. 3a†) which is in contrast to the behaviour of BR.[21] From the comparative CD curves it is clear that the binding-induced CD perturbations were clearly different for the analogues compared to BR. An induced CD band with negative ellipticity appeared in the 320–380 nm region with a maximum around 360 nm for the analogues. From the nature of the induced CD bands it can be inferred that the

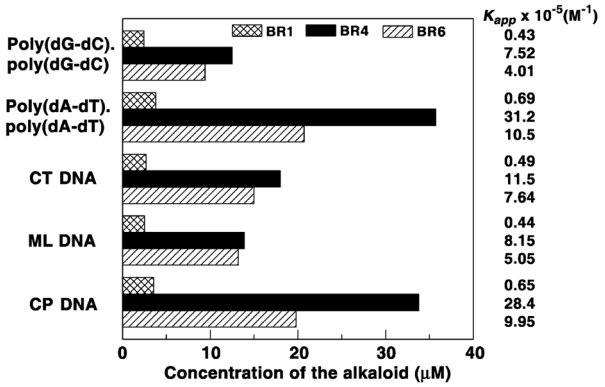

Fig. 2 Results of competition dialysis assay of BR1, BR4 and BR6 binding to various polynucleotides at 20 °C in 10 mM CP buffer, pH 7.0. The concentration of analogues bound each polynucleotide sample is depicted as a bar graph. Values on the right are the apparent binding constants (K_{app}) for the polynucleotides calculated as described by Chaires and co-workers.[29]

Table 1 Association constant and thermodynamic parameters for the binding of berberine analogues with DNA polynucleotides at 20 °C[a]

			Isothermal titration calorimetry				
Polynucleotide	Berberine analogue	Spectrophotometry $K \times 10^{-5}/M^{-1}$	$K_a \times 10^{-5}/M^{-1}$	n	ΔG^o/kcal mol^{-1}	ΔH^o/kcal mol^{-1}	$T\Delta S^o$/kcal mol^{-1}
CT DNA	BR 1	0.48 ± 0.018	0.48 ± 0.04	6.2	−6.32 ± 0.11	+1.46 ± 0.02	7.77
	BR 2	0.51 ± 0.028	0.51 ± 0.06	6.0	−6.35 ± 0.10	+1.31 ± 0.04	7.65
	BR 3	7.07 ± 0.571	6.89 ± 0.15	4.0	−7.87 ± 0.21	−1.71 ± 0.06	6.17
	BR 4	10.04 ± 1.12	11.2 ± 0.55	4.1	−8.16 ± 0.35	−1.65 ± 0.05	6.51
	BR 5	8.90 ± 1.281	8.58 ± 0.45	4.7	−8.01 ± 0.29	−1.26 ± 0.03	6.75
	BR 6	7.48 ± 0.611	7.36 ± 0.41	6.0	−7.92 ± 0.32	−0.97 ± 0.03	6.95
Poly(dA-dT)·poly(dA-dT)	BR 1	0.75 ± 0.02	0.71 ± 0.04	6.3	−6.55 ± 0.14	+0.85 ± 0.01	7.40
	BR 2	0.89 ± 0.04	0.80 ± 0.08	6.3	−6.61 ± 0.19	+0.69 ± 0.03	7.31
	BR 3	9.3 ± 0.21	9.58 ± 0.21	2.4	−8.70 ± 0.29	−1.82 ± 0.05	6.25
	BR 4	31.1 ± 0.59	30.1 ± 0.81	2.1	−8.74 ± 0.25	−1.71 ± 0.07	7.26
	BR 5	16.8 ± 0.46	15.5 ± 0.55	2.8	−8.35 ± 0.31	−1.43 ± 0.08	6.92
	BR 6	11.2 ± 0.39	10.9 ± 0.35	3.0	−8.15 ± 0.32	−1.29 ± 0.06	6.86
Poly(dG-dC)·poly(dG-dC)	BR 1	0.41 ± 0.03	0.44 ± 0.03	6.2	−6.26 ± 0.20	+1.40 ± 0.04	7.66
	BR 2	0.48 ± 0.02	0.48 ± 0.05	6.0	−6.32 ± 0.22	+1.35 ± 0.03	7.67
	BR 3	5.42 ± 0.11	5.05 ± 0.12	3.3	−7.69 ± 0.23	−1.54 ± 0.05	6.15
	BR 4	7.44 ± 0.16	7.14 ± 0.22	3.7	−7.89 ± 0.29	−1.29 ± 0.07	6.60
	BR 5	5.90 ± 0.22	5.50 ± 0.14	3.8	−7.74 ± 0.31	−1.16 ± 0.02	6.58
	BR 6	4.23 ± 0.19	4.14 ± 0.26	3.0	−7.54 ± 0.35	−0.95 ± 0.02	6.59

[a] All the data in the table are averages of four determinations conducted in CP buffer, pH 7.0. The values of ΔH^o and $T\Delta S^o$ were determined using $\Delta G^o = -RT \ln K_a$ and $T\Delta S^o = \Delta H^o - \Delta G^o$. All the ITC data were fit to a model of single binding site.

orientation of the bound BR analogues on the DNA helix is significantly different from that of BR, reflecting the altered interaction of the transition moments of the bound analogues with those of the base pairs. In poly(dA-dT)·poly(dA-dT), the positive 275 nm band ellipticity was lowered significantly along with a small decrease in the 248 nm negative band (ESI, Fig. 3b†). In poly(dG-dC)·poly(dG-dC), there was a decrease in the ellipticity of both the 275 and 250 nm bands, but the change was much less pronounced compared to the other polynucleotides (ESI, Fig. 3c†). Overall, the changes were more pronounced in the AT polynucleotide compared to the GC polynucleotide.

The energetics of the binding of the BR analogues to the DNA were elucidated by ITC.[34] Fig. 3 presents the representative primary data from the calorimetric titration of the BR3 analogue into a solution of the three different DNAs at 20 °C. The binding was characterized by exothermic heats. The fitting to a one-site model gave excellent fitting of the data, yielding the standard molar binding enthalpies (ΔH^{o}) and equilibrium binding constants K_a. Additionally, the standard molar entropy change (ΔS^{o}) and standard molar Gibbs energy change (ΔG^{o}) of the association were calculated using standard thermodynamic relations.[29b,35] The results of the ITC experiments are summarized in Table 1. We find a large positive enthalpy change for the binding of BR1 and BR2 to the DNAs whereas the binding of the other analogues were driven by negative enthalpy changes. For BR1 and BR2, the phenyl moiety is close to the isoquinoline chromophore and appears to create hindrance to intercalation between the base pairs resulting lower binding affinity for the analogues. As the length of the side chain increased beyond $(CH_2)_2$, the standard molar Gibbs energy change enhanced, revealing more favourable contacts to the binding from the side chain. The strong and dominating positive entropy term in each of the analogues compared to BR is suggestive of the disruption and release of water molecules upon intercalation into the DNA double helix. BR binding to DNA has been shown to be enthalpy-driven.[21] A large entropy contribution for the binding of the analogues may be interpreted in terms of the binding-induced release of bound water and condensed sodium ions. It may be observed that the binding affinity values obtained from ITC are in the order of 10^5 M^{-1} for BR3, BR5, and BR6 and of the order of 10^6 M^{-1} for BR4 and follow the same trend as that obtained from spectroscopic studies, being the highest for poly(dA-dT)·poly(dA-dT) and varying in the order poly(dG-dC)·poly(dG-dC) < CT DNA < poly(dA-dT)·poly(dA-dT) for all of the analogues.

In summary, we have successfully designed and synthesized six new 13-substituted diphenylalkyl analogues of berberine that exhibited higher AT base pair specificity and enhanced DNA binding affinities compared to berberine. These properties were dependent on the length of the side chain as revealed from multifaceted experiments. The best affinity to DNA corresponded to a length of $(CH_2)_3$ for the side chain. The base pair preference of these analogues was revealed from their highest affinity to poly(dA-dT)·poly(dA-dT) and lowest affinity to poly(dG-dC)·poly(dG-dC). In a comparative study, the 13-phenylalkyl berberine analogues were also revealed to have a higher preference for the AT sequence (ESI, Fig. 4†). Thus, the base preference of berberine is found to be faithfully propagated in these analogues and also this may be better exploited for sequence-selective drug targeting. This study advances our knowledge on the interaction of small molecules with DNA and may be useful for designing DNA intercalating AT base pair-specific therapeutic agents.

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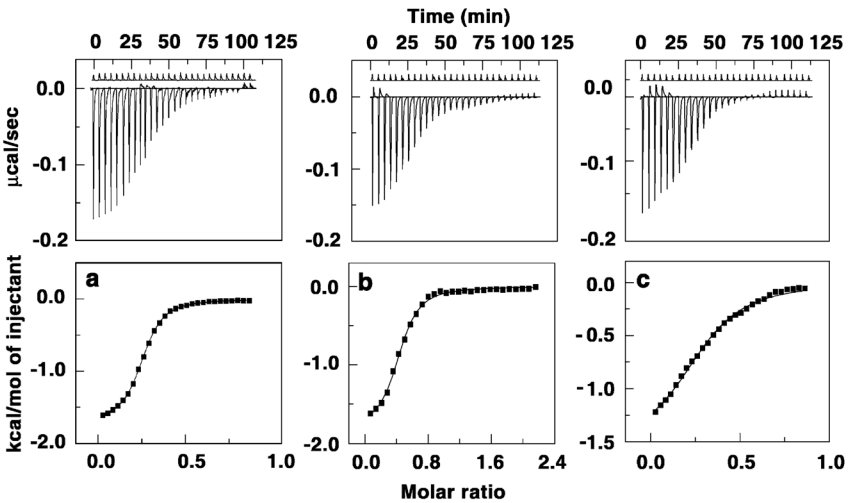

Fig. 3 Representative ITC profiles for the titration of (a) BR3 (400 µM) into a 100 µM solution of DNA, (b) BR3 (250 µM) into a 25 µM solution of poly(dA-dT)·poly(dA-dT) and (c) BR3 (100 µM) into a 25 µM solution of poly(dG-dC)·poly(dG-dC). The upper panels show the heat burst curve for the injection of BR analogues in each experiment into the buffer as control. The lower panels represent the corresponding normalized heat signals *versus* molar ratio.

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22 DNA samples from *Clostridium perfringens* (CP) DNA (Type XII, 27% GC), calf thymus (CT) DNA (Type I, 42% GC), *Micrococcus lysodeikticus* (ML) DNA (Type XI, 72% GC), poly(dG-dC)·poly(dG-dC), poly(dA-dT)·poly(dA-dT) and berberine chloride were the products of Sigma-Aldrich Corporation (St. Louis, MO, USA). DNA samples were purified by ethanol precipitation. The ratio of A_{260}/A_{280} was found to be between 1.88 and 1.92. The concentrations were estimated using extinction (ε) values of (a) 12 600 (M^{-1} cm^{-1}) at 260 nm for CP DNA, (b) 13 200 (M^{-1} cm^{-1}) at 260 nm for CT DNA, (c) 14 800 (M^{-1} cm^{-1}) at 260 nm for ML DNA, and (d) 16 800 (M^{-1} cm^{-1}) at 255 nm for poly(dG-dC)·poly(dG-dC) and 13 600 (M^{-1} cm^{-1}) at 260 nm for poly(dA-dT)·poly(dA-dT).
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24 Steady state fluorescence measurements were conducted using a Shimadzu RF-5301 PC unit (Shimadzu Corporation, Kyoto, Japan) fluorescence spectrometer in fluorescence-free quartz cuvettes of 1 cm path length. Quenching studies were carried out with the anionic quencher $[Fe(CN)_6]^{4-}$. Experiments were performed by mixing, in different ratios, two solutions, one containing KCl and the other containing $K_4[Fe(CN)_6]$, in addition to the normal buffer components, at a fixed total ionic strength. Experiments were performed at a constant *P*/*D* (DNA base pair/alkaloid molar ratio), monitoring fluorescence intensity as a function of the increasing concentration of ferrocyanide ion. For reference, see: M. M. Islam, S. Roy Chowdhury and G. Suresh Kumar, *J. Phys. Chem. B*, 2009, **113**, 1210–1224.
25 Fluorescence polarization experiments were carried out keeping the excitation and emission wavelengths fixed at 338 and 527 nm, respectively. Anisotropy was calculated using the equation $A = (I_{vv} - I_{vh}G)/(I_{vv} + 2I_{vh}G)$ where I denotes the intensity and the subscripts refer to the vertical or horizontal positioning of excitation and emission polarizers, respectively. G is the ratio I_{hv}/I_{hh}, the instrumental correction factor for correcting the polarizing effects in the emission monochromator and detector. For references, see: (*a*) P. Basu, D. Bhowmik and G. Suresh Kumar, *J. Photochem. Photobiol., B*, 2013, **129**, 57–68; (*b*) P. Giri and G. Suresh Kumar, *J. Photochem. Photobiol., A*, 2008, **194**, 111–121.
26 A Cannon-Manning semimicro dilution viscometer (Type 75) mounted vertically in a constant temperature bath (Cannon Instruments Co., State College, PA, USA) maintained at 20 ± 0.5 °C was used for flow time measurements. Flow times of DNA alone (500 μM) and sample with different ratios of the alkaloid were measured in triplicate by an electronic stopwatch with an accuracy of ±0.01 s. For detailed experimental protocol and estimation of β values, see: (*a*) M. Maiti, R. Nandi and K. Chaudhuri, *FEBS Lett.*, 1982, **142**, 280–284; (*b*) R. Sinha, M. M. Islam, K. Bhadra,

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27 Absorbance *versus* temperature profiles (melting curves) of DNA and alkaloid–DNA complexes were measured using a Shimadzu Pharmaspec 1700 unit equipped with the Peltier controlled TMSPC-8 model accessory (Shimadzu Corporation, Kyoto, Japan). For reference, see: M. Hossain and G. Suresh Kumar, *Mol. BioSyst.*, 2009, **5**, 1311–1322. DSC experiments were performed using a Microcal VP-DSC unit. In a series of DSC scans, both the sample and reference cells were loaded with buffer solution, equilibrated at 35 °C for 15 min and scanned from 35° to 110 °C at a scan rate of 60 °C h^{-1}. The buffer scans were repeated till a reproducible base line was achieved and on cooling, the sample cell was rinsed and loaded with DNA solution and then with alkaloid–DNA complexes of different molar ratios and scanned in the same range as above. For detailed experimental protocols, see: (*a*) D. M. Crothers, *Biopolymers*, 1971, **10**, 2147–2160; (*b*) M. Hossain and G. Suresh Kumar, *Mol. BioSyst.*, 2009, **5**, 1311–1322.

28 Absorbance spectral titrations were performed using a Jasco V 660 double beam duel monochromator unit (Jasco International Co., Tokyo, Japan) at 20 ± 1.0 °C as described previously. For refs, see: (*a*) J. B. Chaires, N. Dattagupta and D. M. Crothers, *Biochemistry*, 1982, **21**, 3933–3940; (*b*) K. Bhadra, M. Maiti and G. Suresh Kumar, *Biochim. Biophys. Acta*, 2007, **1770**, 1071–1080. Scatchard plots of r/C_f *versus* r were constructed where r is the number of moles of alkaloid bound per mole of DNA base pair and C_f is the molar concentration of the free alkaloid. Wherever non-linear Scatchard plots were obtained they were further analyzed by the excluded site model for the non-linear non-cooperative ligand binding phenomenon using the McGhee and von Hippel equation to obtain K, the intrinsic binding constant to an isolated DNA binding site and n the number of base pairs excluded after the binding of a single alkaloid molecule (the exclusion parameter). Binding data were analyzed using the Origin 7.0 software to determine the best fit values of K and n by non-linear curve fitting. For refs, see ; (*c*) J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, 1974, **86**, 469–489; (*d*) K. Bhadra, M. Maiti and G. Suresh Kumar, *Chem. Biodiversity*, 2009, **6**, 1323–1342; (*e*) Ref. 21.

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30 Isothermal titration calorimetry (ITC) experiments were performed at 20 °C using a MicroCal VP-ITC unit (MicroCal, Inc.; Northampton, MA, USA). Origin 7.0 software, supplied by the manufacturer, was used for data acquisition. In a typical experiment, 10 μL aliquots of berberine analogues were injected from a 250 μL rotating syringe (290 rpm) into the isothermal sample chamber containing 1.4235 mL of DNA solution. Corresponding control experiments to determine the heat of dilution were performed by injecting 10 μL aliquots of alkaloid into a solution of buffer alone. For reference, see: M. Hossain and G. Suresh Kumar, *J. Chem. Thermodyn.*, 2009, **41**, 764–774.

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