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ARISTOLOLACTAM-β-D-GLUCOSIDE

A NEW DNA BINDING MONOFUNCTIONAL INTERCALATING ALKALOID

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Abstract—The binding of aristololactam-β-D-glucoside to DNA is characterized by hypochromism and bathochromism in the absorption band, quenching of the fluorescence intensity, increase in the positive and negative ellipticity of DNA, enhancement of thermal transition temperature, sign and magnitude of thermodynamic parameters, increase of the contour length of sonicated rod-like DNA and induction of the unwinding–rewinding process of covalently closed superhelical DNA. Binding parameters determined from absorbance and fluorescence titration by Scatchard analysis, according to an excluded-site model, indicate a very high affinity towards DNA. The binding of the alkaloid is an exothermic process with Gibbs free energy of −7.4 kcal/mol, van't Hoff enthalpy of −13.8 kcal/mol and entropy of −21.5 cal/degree/mol at 25°. On the basis of these observations it is concluded that aristololactam-β-D-glucoside binds to DNA by a mechanism of intercalation.

Biological activities of many naturally occurring and synthetic compounds are presumed to be due to their interaction with DNA, interfering with its expression in the cells [1, 2]. The binding of these compounds to DNA is a topic of wide interest and investigation in order to establish a structure–function relationship. One important class of these compounds is comprised of those that bind to DNA by intercalation. Such compounds are important tools in molecular biology, and some are used for the treatment of cancer in man [1–3].

Aristolochia group of alkaloids and their glucoside derivatives has attracted recent attention for their antimicrobial, antitumor and various other biological properties [4]. This group of compounds has been successively tested for the therapy of tuberculosis, chronic bronchitis, pneumococcal disease and in the treatment of cancer [4]. Although there are reports for their clinical and pharmacological uses, the physical and molecular basis of their interaction with DNA are still unexplored. Among this group of alkaloids, aristololactam-β-D-glucoside (ADG)† or 6-β-D-glucopyranosyl-8-methoxy benzo (*f*)-1,3-benzodioxolo(6,5,4-*cd*) indol-5(6H)-one (Fig. 1), a phenanthrenic lactam derivative is of particular interest to us for its attached glucoside ring in the structure. As a part of our investigation on the interaction of alkaloids with nucleic acids [5–11], we report here, for the first time, the binding properties of ADG to DNA from the measurements of spectrophotometry, spectrofluorimetry, spectropolarimetry, thermal melting profiles, thermodynamic parameters and viscometric studies.

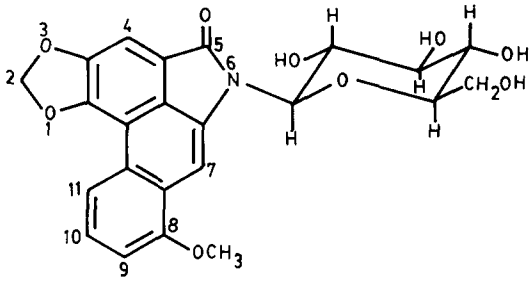

Fig. 1. Chemical structure of aristololactam-β-D-glucoside.

MATERIALS AND METHODS

ADG was extracted from *Aristolochia indica* and crystallized from ethanol. Its purity was checked by thin layer chromatography, melting point, and it was characterized by mass and NMR spectra respectively [12, 13]. It is slightly yellowish in colour, freely soluble in DMSO but sparingly soluble in water. EB was obtained from Sigma Chemical Co. (St Louis, MO) and was used without further purification. The ligand concentrations were determined spectrophotometrically using a molar extinction coefficient (ε) of 10930/M/cm at 398 nm for ADG in DMSO and of 5680/M/cm at 480 nm for EB in water.

CT DNA (type I, 42 mole % GC), CCS Col El DNA (53 mole % GC) and DMSO were obtained from Sigma Chemical Co. and were used as such. DNA concentrations in terms of nucleotide phos-

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† Abbreviations: ADG, aristololactam-β-D-glucoside; CT, calf thymus; CD, circular dichroism; CCS Col E1, covalently closed superhelical Col E1 plasmid; DMSO, dimethyl sulphoxide; EB, ethidium bromide; GC, guanine-cytosine content; T_m, thermal melting temperature.

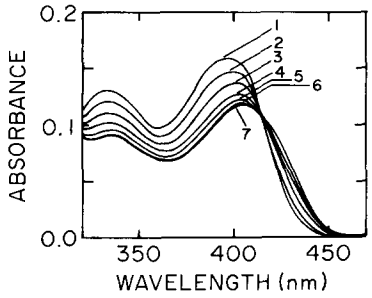

Fig. 2. Effect of CT DNA on the absorption spectrum of ADG. Curves (1–7) denote 14.27 μM of ADG treated with 0, 27.12, 76.88, 124.12, 176.38, 273.23, 320.60 μM of DNA, respectively.

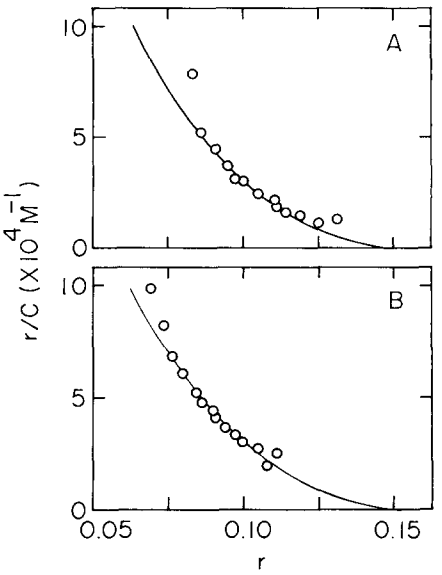

Fig. 3. Scatchard plots from spectrophotometric (A), spectrofluorimetric (B) titrations for the binding of ADG to CT DNA with correlation coefficient of 0.883 and 0.933, respectively. Binding data are limited to ADG/DNA ratios corresponding to percentages of bound ADG ranging from 35–45% to 80–90%, as the lower and upper limits, respectively.

phate were determined spectrophotometrically using ε values of 6600/M/cm and 6550/M/cm at 260 nm for CT DNA and CCS Col E1 DNA, respectively. Deionized glass distilled water and analytical grade reagents were used throughout. Except T_m measurements, all DNA binding experiments were performed in a buffer BPES–DMSO, pH 6.9, containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA, 16 mM NaCl, 240 mM DMSO. T_m measurements were conducted in BPE–DMSO buffer, pH 6.9, containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA and 240 mM DMSO. The properties of DNA remained unaltered in these buffers as revealed by absorbance, T_m, viscosity and CD data.

Absorption spectra of ADG mixed with and without DNA were obtained at 25° using the Shimadzu UV-260 automatic recording spectrophotometer (Shimadzu Corporation, Japan) against an appropriately prepared reference sample in a 1 cm quartz cuvette. Spectrophotometric titrations of the alkaloid–DNA mixtures were carried out at 25° generally following the method described earlier [6, 9, 10].

Fluorescence measurements were recorded at 25° in a Farrand System 3 spectrofluorimeter (Farrand Optical Co., U.S.A.), using a 1 cm quartz cuvette. Emission spectra of the alkaloid alone and in the presence of increasing concentration of DNA were measured. The concentrations of free and bound alkaloid were calculated from the fluorescence titrations as previously described [6, 10]. Uncorrected fluorescence was recorded. The alkaloid obeyed Beer's law in the concentration range used in this study.

To estimate the thermodynamic parameters, the binding studies were done at 15°, 25° and 40° from the measurements of absorption spectrophotometry and the binding constants were determined either from 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 [14].

Thermal melting profiles of DNA and alkaloid–DNA complex were determined using the Shimadzu UV-260 spectrophotometer, equipped with a thermoelectric cell temperature controller S-260/SPR-5 and temperature programmer KPC-5 at the rate of 1° per min in 1 cm quartz cuvette.

CD spectra were recorded at 25° in a cylindrical cell of 1 cm path length on a Jasco J-20A spectropolarimeter (Japan Spectroscopic Ltd, Japan) with a data processor attachment model J-DPY as stated in [8, 11]. CD results were derived from the mean values of at least three determinations and were expressed as molar ellipticity $[\theta]$ in units of degree $cm^2/dmol$.

For viscometric studies, linear duplex CT DNA was sonicated in a Labsonic 2000 (B. Braun, Swiss) sonicator with a needle probe as described in [6, 9]. The estimated molecular weight of CT DNA was found to be of about 3.5×10^5 [9]. The measurements of viscosity of linear sonicated CT DNA (300 μM), CCS Col E1 DNA (150 μM) and their complexes were done with a type 75 Cannon Manning Semimicro viscometer using the method as described previously [8, 9]. Temperature was maintained at 25 ± 0.1° in a Cannon constant temperature bath model M1 (Cannon Instrument Co., U.S.A.).

RESULTS AND DISCUSSION

When the DNA–ADG complex was precipitated with ethanol, the supernatant had an absorbance spectrum resembling that of free ADG, while the redissolved precipitate had no absorbance above 300 nm. These results indicate that DNA–ADG binding is fully reversible and that spectral changes are due to physical interaction with DNA.

The effect of progressive increasing concentrations of CT DNA on the absorption spectrum of ADG is illustrated in Fig. 2. The spectral changes involved essentially a gradual red shift and hypochromicity in

Table 1. Binding parameters for the interaction of ADG with DNA in BPES–DMSO buffer, pH 6.9*

Parameters	Methods	Values
K, the binding constant (/M)	Spectrophotometry	$(2.6 \pm 0.2) \times 10^5$
	Spectrofluorimetry	$(2.5 \pm 0.4) \times 10^5$
n, the number of nucleotide occluded	Spectrophotometry	6.1 ± 0.15
	Spectrofluorimetry	6.1 ± 0.4
$-\Delta G$ (25°) (kcal/mol)	Spectrophotometry	7.43 ± 0.04
	Spectrofluorimetry	7.4 ± 0.08
$-\Delta H$ (kcal/mol)	Spectrophotometry	13.8 ± 0.25
$-\Delta S$ (25°) (cal/degree/mol)	Spectrophotometry	21.5 ± 0.8
β, the slope of L/Lo *v.* r plot	Viscometry	1.95 ± 0.05
Helix length extension per bound alkaloid (nm)	Viscometry	0.315 ± 0.009
θ, unwinding angle (degree)	Viscometry	14 ± 1.0

* Average from three determinations.

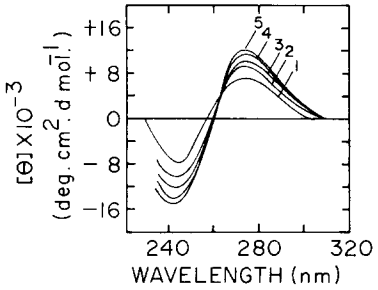

Fig. 4. CD spectra resulting from interaction of ADG with CT DNA. Curve (1), CT DNA (150 μM) treated with varying concentration of ADG as shown by curves (2–5), denoting 11.8, 23.6, 35.5, 47.4 μM, respectively. The expressed molar ellipticity is based upon the DNA concentration.

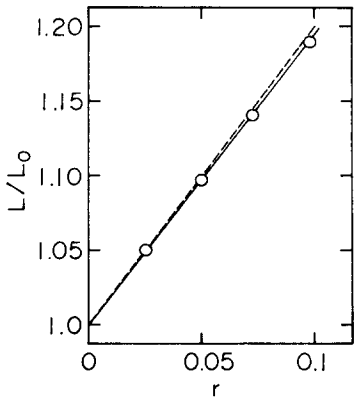

Fig. 5. Length enhancement of sonicated CT DNA (0–0) upon interaction with ADG. Theoretical line (. . . .) represents a slope (β) of 2 predicted for perfect monofunctional intercalator.

the complex until saturation was reached, indicating possible intercalative type of interaction between ADG chromophore and DNA. The results are comparable with intercalated anthracycline [15] or phenanthridine compounds [1, 6]. A maximum red shift of 12 nm was observed at a DNA–ADG ratio of ≥16. The series of spectra exhibited a sharp isosbestic point at 416 nm indicating the existence of an equilibrium between bound and free form of alkaloid [5].

The fluorescence spectrum of ADG excited at 400 nm showed an emission maximum at 485 nm. The quenching of fluorescence spectrum of ADG with increasing concentrations of DNA is observed.

The spectrophotometric and spectrofluorimetric titration data were expressed in the form of Scatchard plot, r vs r/C, where r is the moles of bound ligand/moles of nucleotide, C is the molar concentration of free ligand. For concave binding isotherm, experimental data (Fig. 3) were fitted to the excluded-site model [16, 17].

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

where K is the intrinsic binding constant to an isolated site and n is the exclusion parameter as described in [9, 10]. The best-fit values for K and n are displayed graphically (Fig. 3). The K and n values obtained from the two independent methods (Table 1) are comparable to the data reported for daunomycin [18], Adriamycin® [19] and ellipticin [20].

We have carried out the thermodynamic study of the interaction between ADG and DNA, based on the temperature-dependence of the binding constant, using the neighbour exclusion model [17] to fit the experimental data. The Gibbs free energy was determined from the binding constant according to the relationship

$$\Delta G = -RT \ln K. \quad (2)$$

The binding enthalpy ΔH was determined from the plots of the temperature dependence of the binding constant according to the van't Hoff relationship

$$[\delta \ln K/\delta(1/T)] = -\Delta H/R. \quad (3)$$

The entropy was estimated from the Gibbs free energy and the enthalpy as

$$\Delta S = -(\Delta G - \Delta H)/T. \quad (4)$$

The results of thermodynamic parameters are pre-

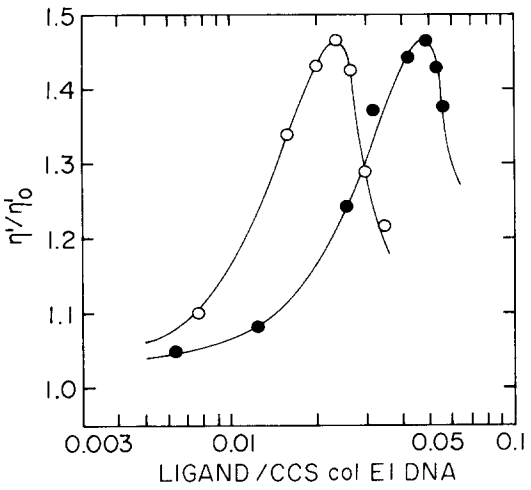

Fig. 6. Viscometric titration of CCS Col E1 DNA (150 μM) by ADG (●—●) or EB (○—○). [Viscosity expressed by the specific viscosity of ligand bound CCS Col E1 DNA solution (η') relative to that of respective CCS Col E1 DNA solution (η'_0).]

sented in Table 1. It can be seen from Table 1 that our values of $\Delta H = -13.8$ kcal/mol and ΔS at 25° = −21.5 cal/degree/mol are of the same sign as and a similar magnitude to values obtained for other intercalators [14, 21]. Taking analogy with other intercalators, the possible contributions for negative enthalpy are the molecular interactions at the intercalation site, while the negative entropy value may result from decreased flexibility in the DNA helix following intercalation.

ADG enhanced the thermal stability of DNA and the T_m of CT DNA in BPE–DMSO buffer, pH 6.9 was raised from 56° to 63° at $r = 0.13$. An elevation of T_m on interaction of ADG indicates that the hydrophobic chromophore of ADG contributes to the stabilization of the DNA double helix, implying a probable intercalative mode of interaction [1, 5, 8].

Although ADG is an optically active compound, it does not have any CD peak in the region 230 nm to 600 nm. CD spectra of ADG–DNA complexes (Fig. 4) showed a regular increase in magnitude of positive and negative ellipticity of DNA with increasing values of ADG/DNA, indicating the conformational change induced in DNA by ADG without alteration in the inclination of bases [11]. It has also been observed that DNA–ADG complexes did not produce any extrinsic CD in the wavelength range between 300 and 600 nm under the conditions studied here.

The increase of sheared DNA length upon interaction of ADG was calculated from the experimental results of the intrinsic viscosity according to the approximation as described in [6, 9].

$$L/Lo = [\eta/\eta_0]^{1/3} = 1 + \beta r. \quad (5)$$

where, L and Lo are the contour lengths of DNA in the presence and absence of ligand respectively, η and η_0 are the corresponding values of intrinsic viscosity (approximated by the reduced viscosity) of the solution. The intercalation model of Lerman [22] predicts that for perfect monofunctional and bifunctional intercalators the β values should be 2 and 4, respectively.

Figure 5 shows a plot of L/Lo vs r. The β value obtained from the slope was found to be 1.95 (Table 1) suggesting perfect monofunctional intercalation for linear rod-like DNA molecules. The calculated value for helix length extension per bound ligand of 0.315 nm (Table 1) lies well within the range of values reported for other monofunctional intercalating agents, i.e. between 0.18 and 0.45 nm [1].

The observation of a viscosity enhancement on binding to linear duplex DNA is an insufficient evidence by itself to establish the mechanism of intercalation [1, 6, 20]. Combined with evidence of helix unwinding from the CCS DNA binding test [1, 8, 18] the conclusion is more secured. The viscometric titrations of CCS Col E1 DNA with ADG and EB were done and the results are presented in Fig. 6. Considering an unwinding angle of 26° for EB, the unwinding angle of ADG was found to be 14° (Table 1), well within the range for other intercalators [1] and comparable to daunomycin [18]. Figure 6 also shows that ADG is capable of inducing an unwinding-rewinding process of CCS Col E1 DNA. This type of viscosity change is a characteristic property induced by a variety of DNA intercalating agents [1, 8, 18].

Thus our results strongly indicate that the mode of binding of ADG to DNA is consistent with the intercalation model.

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REFERENCES

1. Waring MJ, DNA modification and cancer. *A Rev Biochem* **50**: 159–192, 1981.
2. Neidle S and Waring MJ, *Molecular Aspects of Anticancer Drug Action*. MacMillan Press Ltd, U.K., 1983.
3. Pratt WB and Ruddon RW, *The Anticancer Drugs*. Oxford, New York, 1979.
4. Chen Z and Zhu D, Aristolochia alkaloids. In: *The Alkaloids* (Ed. Brossi A), Vol. 31, pp. 29–65. Academic Press, Inc Orlando, FL, 1987.
5. Maiti M and Chaudhuri K, Interaction of berberine chloride with naturally occurring deoxyribonucleic acids. *Indian J Biochem Biophys* **18**: 245–250, 1981.
6. Maiti M, Nandi R and Chaudhuri K, Sanguinarine: a monofunctional intercalating alkaloid, *FEBS Lett* **142**: 280–284, 1982.
7. Maiti M, Nandi R and Chaudhuri K, The effect of pH on the absorption and fluorescence spectra of sanguinarine. *Photochem Photobiol* **38**: 245–249, 1983.
8. Maiti M, Nandi R and Chaudhuri K, Interaction of sanguinarine with natural and synthetic deoxyribonucleic acid. *Indian J Biochem Biophys* **21**: 158–165, 1984.
9. Nandi R and Maiti M, Binding of sanguinarine to deoxyribonucleic acids of different base composition. *Biochem Pharmacol* **21**: 321–324, 1985.
10. Nandi R, Chaudhuri K and Maiti M, Effects of ionic strength and pH on the binding of sanguinarine to deoxyribonucleic acid. *Photochem Photobiol* **42**: 497–

503, 1985.
11. Maiti M and Nandi R, Circular dichroism of sanguinarine–DNA complexes: effects of base composition, pH and ionic strength. *J Biomolec Str Dyn* **5**: 159–175, 1987.
12. Pakrashi SC, Ghosh Dastidar PP, Basu S and Achari B, New phenanthrenic derivatives from *Aristolochia indica*. *Phytochemistry* **16**: 1103–1104, 1977.
13. Achari B, Bandyopadhyay S, Chakraborty AK and Pakrashi SC, Carbon-13 nmr spectra of some phenanthrenic derivatives from *Aristolochia indica*. *Org Mag Reson* **22**: 741–746, 1984.
14. Chaires JB, Thermodynamics of the daunomycin–DNA interaction: ionic strength dependence of the enthalpy and entropy. *Biopolymers* **24**: 403–419, 1985.
15. Zunino F, Gambetta R, DiMarco A and Zaccara A, Interaction of daunomycin and its derivatives with DNA. *Biochem Biophys Acta* **277**: 489–498, 1972.
16. Crothers DM, Calculation of binding isotherm for heterogeneous polymers. *Biopolymers* **6**: 575–584, 1968.
17. McGhee JD and von-Hippel PH, Theoretical aspects of DNA–protein interactions: co-operative and non-co-operative binding of large ligands to a one dimensional homogeneous lattice. *J Molec Biol* **86**: 469–489, 1974.
18. Chaires JB, Dattagupta N and Crothers DM, Studies on interaction of anthracycline antibiotics and deoxyribonucleic acid: equilibrium binding studies on interaction of daunomycin with deoxyribonucleic acid. *Biochemistry* **21**: 3933–3940, 1982.
19. Britt M, Zunino F and Chaires JB, The interaction of the β-anomer of doxorubicin and B and Z DNA. *Molec Pharmacol* **29**: 74–80, 1986.
20. Kohn KW, Waring MJ, Glaubiger D and Friedman C, Intercalative binding of ellipticin to DNA. *Cancer Res* **35**: 71–76, 1975.
21. Barcelo F, Martorell J, Gavilanes F and Gonzalez-Ros JM, Equilibrium binding of daunomycin and adriamycin to calf thymus DNA: temperature and ionic strength dependence of thermodynamic parameters. *Biochem Pharmacol* **37**: 2133–2138, 1988.
22. Lerman LS, The structure of the DNA–acridine comcomplex. *Proc Natl Acad Sci USA* **49**: 94–102, 1963.