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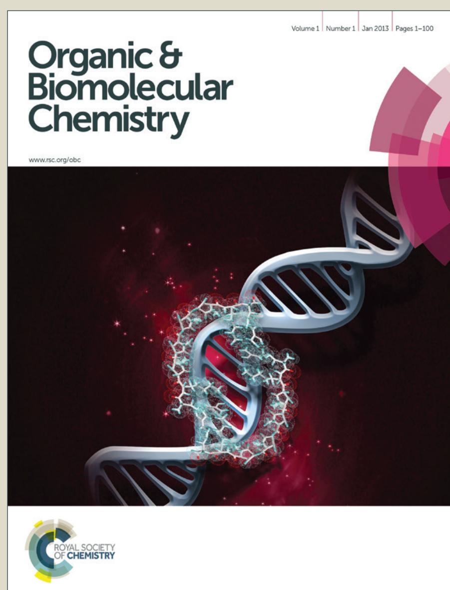

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ARTICLE TYPE

Coumarin-Pterocarpan Conjugate- A Natural Product Inspired Hybrid Molecular Probe for DNA Recognition

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Thermally induced cascade sigmatropic rearrangement of a butynyloxycoumarin derivative has led to a quick access of coumarin-pterocarpan hybrid molecule. Biophysical studies together with molecular modeling show that this nature-inspired hybrid molecule is capable of binding to minor groove of DNA as a non-conventional entity.

Introduction

Development of small molecules for the recognition of DNA is of profound importance and has attracted chemists and biologists over the last few decades.[1] Small molecules interact with DNA through mainly three different modes *viz.* intercalation,[2] groove binding[3] and covalent binding.[4] A number of heterocyclic molecules[5] have been utilized for the design of molecular sensors of nucleic acids.. Commonly, DNA-binding molecules have been designed as cations to take advantage of electrostatic attraction with negatively charged DNA. However, neutral molecular probes for nucleic acids are also known.[6] H-bonding and hydrophobic interaction play the key role for the affinity of these molecules towards DNA.[7] Hybrid or conjugate molecules have also been investigated in this regard.[8] On the other hand, natural products or derivatives thereof have found limited utility for the design of probes for nucleic acid recognition.[9] We have recently reported, a new scaffold involving a hybrid molecule of a naturally occurring pterocarpan with an important fluorophore acridone for such purpose.[10] Several coumarin derivatives fused with another heterocyclic ring have found use in molecular recognition because of very advantageous photophysical properties.[11] Pterocarpans are an important class of natural products which show significant and diverse range of biological activity.[12] Thus we became interested to synthesize a hybrid of coumarin and pterocarpan and study its DNA binding property.

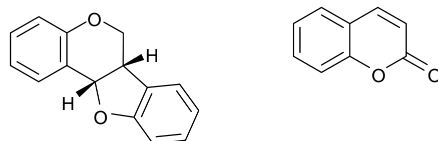
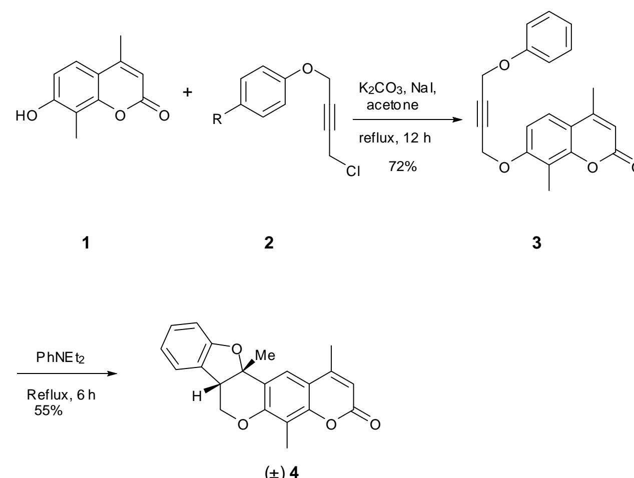


Fig.1 Pterocarpan and coumarin scaffolds

Results and discussion

We have previously described[10,13] application of cascade sigmatropic rearrangement[14] for the synthesis of hetero-fused pterocarpans on carbazole, and acridone templates. We followed a similar tactics for the synthesis of coumarin-annulated pterocarpan derivatives.

Thus, the desired butynyl ether 3 was easily prepared by alkylation of 7-hydroxy-4,8-dimethyl coumarin **1** with 1-phenoxy-4-chloro-but-2-yne **2** in the presence of K_2CO_3 and catalytic amount of NaI. When compound **3** was heated in *N,N*-diethyl aniline under reflux for 6 hours, compound **4** was formed in a moderate yield of 55%. We assigned the ring junction to be *cis* based on precedence.[10,13] Moreover, nOe correlation between the ring-junction methyl protons and the benzylic proton at the other end of the junction further corroborated such assignment.



Scheme 1 Synthesis of coumarin pterocarpan hybrid **4** by thermal cascade sigmatropic rearrangement of the alkynyl ether **3**.

The formation of **4** from **3** could be mechanistically explained by invoking preferential [3,3]-sigmatropic rearrangement of the coumarinyloxypropyne part followed by enolisation to the corresponding 2-allenyl phenol **5** (Scheme 2). The latter may undergo a [1,5]-prototropic shift to form the dienenone derivative **6**. Electrocyclic ring-closure of the latter then would lead to the pyranocoumarin derivative **7**. A second [3,3]-sigmatropic shift of the allyl vinyl ether part in **7** followed by

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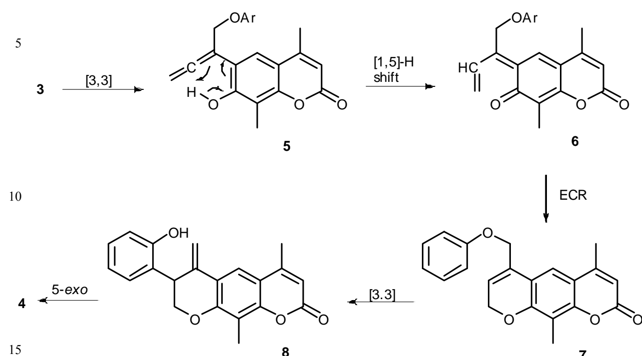
enolisation would explain the formation of the phenol **8** which then undergoes ring-closure in a 5-*exo* fashion to give rise to the observed product 4.



Scheme 2. Mechanistic rational for the formation of **4**

Biophysical studies

The absorption spectra of **4** were recorded in the absence and presence of increasing amounts of ct-DNA. Compound **4** shows absorption maxima at 331 nm (Figure 2A). Addition of ct-DNA to the solution of compound in cacodylate buffer results in gradual decrease in the absorption intensity (Figure 2 A and B) and reached saturation. Molar extinction co-efficient of compound **4** in aqueous buffered solution (sodium cacodylate) was determined from the slope of the plot of absorbance vs. concentration and averaged from the values obtained in three different experiments; the value was found to be 1.32×10^4 $M^{-1}cm^{-1}$.

We then studied the emission behavior of the hybrid compound **4**. Compound **4** shows emission maximum at 395 nm (λ_{ex}= 331 nm) in aqueous sodium cacodylate buffer solution. Quantum yield of **4** in aqueous buffer solution was determined to be 0.17. It was observed that with decrease of solvent polarity emission intensity (Figure 3A) and quantum yield of **4** increases (Table 1).[15] In presence of increasing amount of ct-DNA (0-10 μM) emission intensity of the ligand gradually increased (Figure 3B) and reached saturation at [DNA] of 8-10 μM. Quantum yield of **4** also increased about three times to 0.53 in the presence of

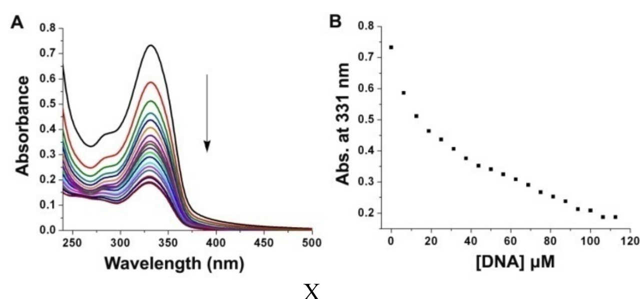


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Figure 2 Absorption spectra of compound **4** (50 μM) in presence of increasing concentration of ct-DNA (0-115 μM).

10 μM ct-DNA. Such increase in the emission intensity may result from change in the local polarity of ligand **4** due to inclusion of the molecule in the hydrophobic binding sites of DNA. Binding constant K_a was determined from the fluorescence titration data using Benesi-Hildebrand[16] equation and was found to be 3.33×10^5 M^{-1}. The detection limit of the probe was measured to be 0.125 nm (S/N 3) (see ESI).

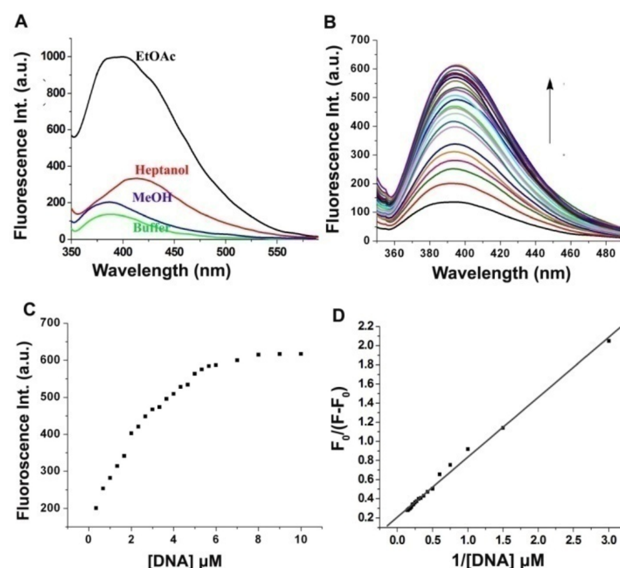

Figure 3 (a) Emission spectra of ligand **4** (10 nM) in different solvents. (b) Emission spectra of ligand **4** (10 nM) (λ_{ex}= 331 nm) with increasing concentration of ct-DNA (from 0-10 μM). (c) Plot of emission intensity of **4** at 395 nm vs. ct-DNA concentration (in μM). (d) Plot of F_0/(F-F_0) vs. 1/[DNA] for ligand **4** in presence of different concentration of ct-DNA.

Table 1 Quantum yield (φ) of compound **4** in different solvents

Solvent	Quantum yield (φ)
Water	0.17
Methanol	0.27
Heptanol	0.63
Ethyl Acetate	0.86

We then studied the interaction of the ligand with four different monomeric nucleotides. Emission spectra of ligand **4** showed small interaction with AMP and GMP but almost no interaction was found for TMP and CMP (see ESI). However this interaction is nearly negligible (~ 20-30% change in intensity) when compared to that with duplex DNA (ct-DNA). This observation may indicate the requirement of groove cleft of duplex-DNA for binding of ligand **4**.

We became interested to study the interaction of the ligand **4** with a DNA sequence having complementary GC and AT content to that of ct-DNA. Thus, emission spectra of compound **4** (20 nM) in presence of increasing concentration of d(5'-CATGGCCATG-3')$_2$ up to 10 μM were recorded (supporting information) and the binding constant was determined (Table 2) and compared. This study revealed more or less similar kind of interaction with the two types of DNA sequence.

Table 2. Binding constant estimated for interaction of **4a** with different DNAs of varying base pair content

DNA	**% GC**	**% AT**	**K** (L mol^{-1})
ct-DNA	42	58	$3.33 \pm 0.04 \times 10^5$
d(5'-CATGGCCATG-3')$_2$	60	40	$1.41 \pm 0.05 \times 10^4$

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To evaluate the possible mode of binding of ligand **4** with DNA, thermal denaturation study was undertaken (Figure 4). Significant stabilization of double helix is commonly observed for intercalating ligands, whereas groove binders may lead to stabilization or disstabilization or negligible change in the melting temperature (T_m) of double standard DNA.[17] It was found that ligand **4** disstabilizes DNA double helix by 10 degrees. For ct-DNA in 1 mM sodium cacodylate buffer the melting temperature (T_m) was measured to be at 56 °C where as for 1:1 DNA ligand (**4**) complex the T_m was found to be 46 °C. This study indicated the binding mode to be other than intercalation.

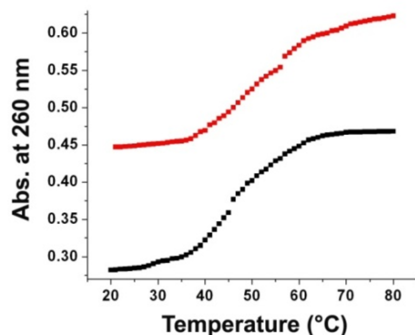

Figure 4 Thermal denaturation profile of ct-DNA in absence (red) and in presence (black) of the ligand **4** ([DNA]/[ligand] = 1:1).

Dye displacement assay

A dye displacement assay was undertaken to understand more in this regard (Figure 5).[18] Ethidium bromide is a well known intercalator that exhibits strong enhancement of emission intensity upon binding with DNA. Addition of Ethidium bromide to a solution of ct-DNA (25 μM) resulted in the increase of emission intensity of ethidium till saturation (Figure 5A). Then we gradually added the ligand **4** to this solution but it did not produced significant reduction of emission intensity of DNA bound ethidium bromide (Figure 5B). Thus it can be said that the ligand could not replace ethidium bromide bound to DNA. So the possibility of intercalative binding of the ligand is less.

On the other hand, a similar experiment was performed with a minor groove binding dye Hoechst 33258. Addition of Hoechst 33258 to a solution of ct-DNA resulted in increase of emission intensity of Hoechst (Figure 5C). With gradual addition of the ligand the emission intensity of Hoechst was reduced to a significant level (Figure 5D), thus indicating removal of Hoechst dye from the minor groove of DNA by the ligand

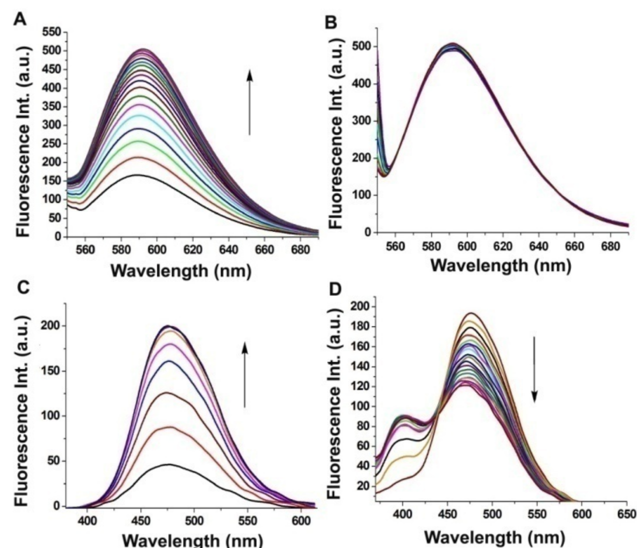

Fig. 5 (A) Extrinsic fluorescence spectra of ethidium bromide (0.5 to 8.0 μM) bound ct-DNA (25 μM). (B) Gradual addition of compound **4** from 4-80 μM shows no significant change in the fluorescence intensity of ethidium bromide bound ct-DNA. (C) Extrinsic fluorescence spectra of Hoechst (0.2 to 1.6 μM) bound ct-DNA (100 μM). (D) Gradual addition of compound **4** from 4-80 μM shows a decrease in the fluorescence intensity of Hoechst bound ct-DNA.

Molecular modelling

To rationalize the incorporation of ligand **4** in the DNA minor groove, docking studies with the geometry optimized structure of one of the enantiomers of ligand **4** was carried out. The width of the DNA minor groove is known to be sequence dependent;[19] thus, one may see different alignments of the ligand **4** in the minor groove. For this purpose we have used two DNA crystal structures from PDB. Docking studies were carried out with one enantiomer of **4**. The dodecameric DNA d(5'-CGCAAATTTGCG-3')$_2$ (PDB identifier 1D65)[20] when used as the receptor shows alignment of coumarin moiety within the minor groove (Figure 6A) with estimated binding energy of - 8.0 Kcal mol^{-1}. For another receptor DNA i.e. decameric d(5'-CGATATCG-3')$_2$ (PDB identifier 1D56)[21] we observed slightly different alignment of **4** in the minor groove where the coumarin moiety is directed outward from the minor groove (Figure 6B). Estimated binding energy for this alignment was -8.4 Kcal mol^{-1}.

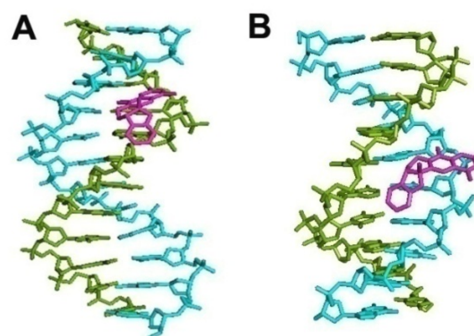

Fig.6 AutoDock generated lowest energy docking pose of **4** (magenta) in complex with the minor groove region of the dodecameric DNA d(5'-CGCAAATTTGCG-3')$_2$ (A) and decameric DNA d(5'-CGATATCG-3')$_2$ (B).

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Conclusion

We have identified that a coumarin-pterocarpan hybrid molecule is capable of binding duplex DNA, probably through minor groove binding. Compound **4** differs significantly from the crescent shape of classical minor groove binders.[22] Moreover, it is non-nitrogenous and neutral. Therefore its ability to bind in the minor groove region is noteworthy. Since the probe **4** is hydrophobic in nature it may prefer to bind between hydrophobic walls of the minor groove rather than to stay in aqueous environment. Such type of interaction is possibly supported by hydrophobic and van der Waals forces, which play key roles in minor groove recognition.[23] Moreover, this process is expected to be entropically favored due to removal of ordered water molecules from the minor groove.[24] Several novel cationic derivatives are known to display stronger binding than this new entity. They are also associated with somewhat better photo- physical properties.[25] But the presently developed molecular probe may provide new platform in the design of molecules for DNA recognition.

Experimental

Infrared spectra were recorded with Perkin-Elmer Infrared Spectrometer model No L120-000A purchased through DST-FIST grant. ^{1}H- and ^{13}C-NMR spectra were recorded with Bruker 400 MHz Ultrashield NMR spectrometer purchased through DST-FIST Grant. Mass spectra were recorded in a JEOL-JNM Spectrometer at IICB, Kolkata and were obtained as a paid service. Elemental analysis was performed with Perkin Elmer 2400 series II Instrument. All solvents obtained from commercial sources were dried with appropriate drying agents and were immediately distilled before use. Column chromatography was performed with silica gel (230-400) purchased from Spectrochem India Pvt. Ltd. Thin layer chromatography was done in pre-coated silica plates and were visualized through UV lamp or iodine spray. Melting points were determined in open capillaries and are uncorrected.

7-(4-phenoxybut-2-ynyloxy)-4,8-dimethyl coumarin (3)

A heterogeneous mixture of 4,8-dimethyl-7-hydroxy coumarin **1** (200 mg, 1.05 mmol), anhydrous potassium carbonate (852 mg, 6.17 mmol), 1-aryloxy-4-chlorobut-2-yne **2** (267 mg, 1.48 mmol) and a pinch of NaI in dry acetone (15 ml) was heated to reflux for 12 h. It was allowed to cool to room temperature, filtered and the filtrate was concentrated in vacuo. The residue was diluted with water (20 ml) and the aqueous layer was extracted with ethyl acetate (2×25 ml). The combined extract was washed successively with saturated aqueous $NaHCO_3$ (2×25 ml), water (2×25 m) and brine (30 ml), and then dried over $MgSO_4$. The solvent was evaporated in *vacuo* to leave a crude mass which was purified by column chromatograph over silica gel using 10 % ethyl acetate in petroleum ether as eluent. The product **3** was obtained as colourless solid. Yield: 253 mg (72 %). Mp. 118-120°C. IR: 2985, 2860, 1985, 1873, 1705, 1607, 1494, 1370 cm^{-1}. ^{1}H NMR (500MHz, $CDCl_3$): ∂ 7.33(1H, d, *J* = 8.5 Hz), 7.26-7.23(2H, m), 6.97(1H, t, *J* = 7.0 Hz), 6.91(1H, d, *J* = 8.0 Hz), 6.88(2H, d, *J* = 9.0 Hz), 6.15(1H, s), 4.84(2H, s), 4.72(2H, s), 2.39(3H, s), 2.30 (3H, s) ppm. ^{13}C NMR (100MHz, $CDCl_3$): ∂ 161.4, 158.1, 157.4, 152.7, 152.6, 129.4, 122.3, 121.5, 115.0, 114.8, 114.3, 112.3, 108.3, 83.0, 81.9, 56.6, 55.8, 18.7, 8.3 ppm. Elemental Analysis: Calculated for $C_{21}H_{18}O_4$: C, 75.43; H, 5.43. Found: C, 75.56; H, 5.37. MS (QTOF ESMS+): *m/z* 357 (M^+ + Na).

Compound 4

A solution of **3** (200 mg, 0.6 mmol) in *N,N*-diethylaniline (6 mL) was heated to reflux for 12 h under nitrogen atmosphere. It was allowed to cool to room temperature and then poured into ice-cold 2 (N) HCl (50 ml) while stirring with a glass rod. The aqueous portion was extracted repeatedly with ethyl acetate (3×25 ml) and the combined organic extract was washed successively with saturated aqueous $NaHCO_3$ (2×20 ml), water (20 ml), brine (25 ml) and then dried ($MgSO_4$). It was then filtered and the filtrate was concentrated under reduce pressure to leave a crude solid mass which was purified by column chromatography (silica gel) using mixture of petroleum ether and ethyl acetate (50:1) as eluent to afford the benzofuropyranocoumarin **4** as colourless solid.

Yield: 109 mg (55 %). Mp. 198-200 °C. IR: 3051, 2972, 2926, 2871, 2324, 1711, 1614, 1585, 1345, 1243 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): ∂ 7.72(1H, s), 7.28(1H, d, *J* = 7.6 Hz), 7.19(1H, t, *J* = 7.6 Hz), 6.92(1H, t, *J* = 7.6 Hz), 6.81(1H, d, *J* = 8.0 Hz), 6.17(1H, s), 4.43(1H, dd, *J* = 11.6, 4.8 Hz), 3.95(1H, dd, *J* = 11.6, 9.2 Hz), 3.56(1H, dd, *J* = 8.8, 4.8 Hz), 2.47(3H, s), 2.28(3H, s), 1.79(3H, s) ppm. ^{13}C NMR (100MHz, $CDCl_3$): ∂ 161.2, 156.9, 155.4, 152.5, 152.1, 129.3, 128.0, 125.7, 125.0, 121.6, 114.8, 114.2, 112.8, 111.4, 84.3, 66.5, 47.5, 28.1, 18.9, 8.4 ppm. Elemental Analysis: Calculated for $C_{21}H_{18}O_4$: C, 75.43; H, 5.43. Found: C, 75.65; H, 5.31. HRMS (QTOF MSES+): found: 357.1126; calcd. For $C_{21}H_{18}NaO_4$: 357.1103.

Biophysical Studies

General remarks

10 mM sodium cacodylate (pH 7.4) buffer solution was prepared with solution of freshly autoclaved Milli-Q water (resistivity 18.2 MΩ). This buffer was used throughout the experiments. Compounds were dissolved in DMSO to produce 5 mM stock solutions and diluted with buffer immediately before experiments to working solutions. ct-DNA was purchased from Sigma-Aldrich and used without further purification. Concentration of DNA solution was determined from absorbance intensity at 260 nm (ε_{260} = 6600 M^{-1} cm^{-1}) and mentioned in terms of base pairs (bp). Compound **4** obeyed Beer's law in the range of concentrations used in the experimental study.

UV-Vis spectra of compound 4 in presence of increasing concentration of ct-DNA

All the spectra were recorded at ambient temperature (300 K). UV-Vis spectra were recorded on a Shimadzu-2401 PC spectrometer with a 1 cm cuvette. Any background buffer signal was electronically subtracted. Absorption titrations were performed with constant ligand concentration of 50 µM and increasing ct-DNA concentration (up to 0.115 mM). Control experiment was done by adding equal volumes of buffer solution instead of DNA solution to the same concentration of compound. The control experiment shows no significant

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change in the absorption spectra of the compound.

Molar extinction coefficient was determined from a plot of absorbance vs. concentration of **4** in 1 mM sodium cacodylate buffer and averaged from the values obtained in three different experiments.

Fluorescence spectra of ligand 4 in presence of ct-DNA

Emission spectra were recorded with a Perkin Elmer LS 55 fluorescence spectrometer. Fluorescence titration spectra were obtained with constant ligand concentration (10 nM) and increasing amount of ct-DNA (up to 10 μM). Binding constant K_a for ligand-DNA complex was estimated from fluorescence titration data using Benesi-Hildebrand equation. K_a was determined from the ratio of intercept to slope obtained from the linear fit of the plot of $F_0/(F-F_0)$ vs. 1/[DNA]. Where F_0 and F are the emission intensities of the ligand in absence and presence of DNA respectively. Control experiment was done by adding equal volumes of buffer solution instead of DNA solution to the same concentration of compound. No significant change in the emission spectra was observed in that control experiment.

Determination of quantum yield

Quantum yield of **4** in 1 mM sodium cacodylate solution was determined by relative comparison method taking anthracene in cyclohexane as standard (quantum yield of anthracene in cyclohexane = 0.36).[26] To determine the quantum yield by this method, the following equation was used.

$\varphi_u = (\varphi_s \times F_u \times A_s \times \lambda_s \times n_u^2)/ (F_s \times A_u \times \lambda_u \times n_s^2)$

Where φ is the quantum yield, F is the integrated area under the emission spectrum, A is the absorbance at excitation wavelength, λ is the excitation wavelength and n is the refractive index of the respective solutions. Here u and s stands for unknown and standard respectively.

Thermal denaturation study

Thermal melting curves were measured in a Shimadzu-2401 PC spectrometer equipped with a Peltier temperature controller system (± 0.1 °C). ct-DNA was treated with compound **4** (ratio of DNA to compound = 1:1) and kept at 25 °C for 5 minutes. All the solutions were degassed before the experiment. Absorbance of ct-DNA (50 μM) in 1 mM cacodylate buffer (pH 7.4) was measured at 260 nm. The samples were heated from 20°C-90°C and absorbance was measured after attaining thermal equilibrium at a particular temperature with an interval of 1 °C. Absorbance of the compound was subtracted at each point. T_m was determined from the 1st derivative of the absorbance vs. temperature curve.

Competitive binding study of ligand 4 with ct-DNA in presence of Hoechst 33258 and Ethidium bromide

Competitive binding of **4** with DNA in presence of classical minor groove binder Hoechst 33258 was also studied. Hoechst 33258 (Sigma) was dissolved in Milli-Q water to prepare a stock solution with 1 mM strength and diluted immediately before use. Fluorescence spectra was taken in presence of 100 μM ct-DNA with increasing amount of Hoechst 33258 (0.2 to 1.6 μM) and using excitation wavelength of 353 nm. Compound **4** was added with an increasing concentration of **4** μM (up to 80 μM), mixed thoroughly after each addition and emission spectrum was recorded with λ_{ex} = 353 nm. In a similar manner ethidium bromide (EtBr) solution was added to a solution of ct-DNA (25 μM) in such a manner to increase the concentration of Ethidium bromide by 0.5 μM at each addition. Emission spectra was recorded after each addition with λ_{ex}= 546 nm. A saturation in the fluorescence emission was observed with EtBr concentration of 8μM. Then Compound **4** was added with an increasing concentration of 4 μM (up to 80 μM), mixed thoroughly after each addition and emission spectrum was recorded with λ_{ex} = 546 nm.

Molecular modeling studies

Molecular modeling studies were performed with geometry optimized structure of **4**. Geometry optimization of **4** was carried out by DFT calculation in Gaussian 3 software. For this study the hybrid function B3LYP with 631G basis set was used and that generated the energy minimized structure for ligand **4**. Protein Data Bank was searched for the crystal structures of DNA. Structure of B-DNA oligomers were extracted from crystal structure from Protein Data Bank Identifier 1D65 d(5'-CGCAAATTTGCG-3')2 and 1D56 d(5'-CGATATCG-3')$_2$. Rotable bonds in ligand were assigned with AutoDock Tools. Ligand docking was carried out with Auto Dock Vina.[27] Docking search space on DNA was set in such a way to cover the whole DNA with 0.375 Å spacing. The output from AutoDock was rendered in PyMol.[28]

Acknowledgement

We are thankful to DST, Govt. of India for a grant (No. SR/S1/OC-92/2012). RM is thankful to CSIR, New Delhi for a fellowship. IK is thankful to UGC New, Delhi for a fellowship.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Copies of ^{1}H and ^{13}C-NMR spectra of all new compounds. See DOI: 10.1039/b000000x/

This work is dedicated with respect to Professor Richard J. K. Taylor on the occassion of his 65th birthday.

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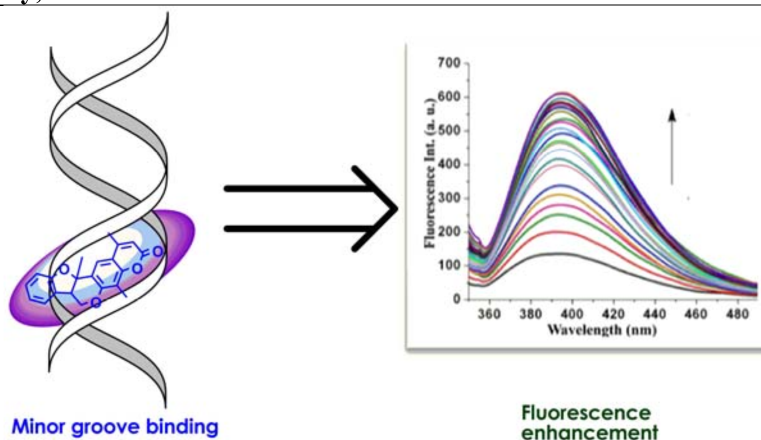
GRAPHICAL ABSTRACT

Coumarin-Pterocarpan Conjugate- A Natural Product Inspired Hybrid Molecular Probe for DNA Recognition

Shital K Chattopadhyay,* Indranil Kundu and Ratnava maitra



Thermally induced cascade sigmatropic rearrangement of a butynyloxycoumarin derivative has led to a quick access of coumarin-pterocarpan hybrid 55 molecule. Biophysical studies together with molecular modeling show that this nature-inspired hybrid molecule is capable of binding to minor groove of DNA as a non-conventional entity.

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