Fluorescent Small Molecules Are BIG Enough To Sense Biomacromolecule: Synthesis of Aromatic Thioesters and Understanding Their Interactions with ctDNA

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Supporting Information

ABSTRACT: The visible fluorescent chromophoric moiety present in the water-soluble photoactive yellow protein (PYP) of Ectothiorhodospira halophila is p-hydroxycinnamic acid linked to the cysteine residue (Cys-69) by a thioester bond and it controls the key photoinduced biological processes of the host organism. In the present work, we have synthesized and characterized three structurally different thiophenyl esters [viz., p-hydroxycinnamic-thiophenyl ester (1), p-N,N-dimethylaminocinnamic-thiophenyl ester (2), and S-phenyl-3-(4-chlorophenyl)-3-(phenylthio)-propanethioate (3)] in addition to a novel (to the best of our knowledge) stilbene-type olefinic compound, N,N,N,N-tetramethyl-1,2-bis(phenylthio)ethene-1,2-diamine (4), under the same reaction condition. All of these four compounds showed characteristic and distinguishable chromophoric/fluorophoric behavior in ethanol and also at pH 7.4. However, we have observed that the intrinsic chromophoric/fluorophoric activities of (1) and (2) were greatly influenced during their interactions with calf-thymus DNA, studied by a range of spectroscopic and physicochemical measurements. We have also applied density functional theory [B3LYP, 6-311G(d,p)]-based method to get optimized structures of (1) and (2), which were explored further for molecular docking studies to understand their mode of interaction with DNA. The present study opens up their possible applications as fluorescence probes for biomacromolecules like DNA in future.

1. INTRODUCTION

Small molecules have wide spectrum of biological activities. As a cell signaling chemical entity or as a drug, a small molecule can regulate a specific biological process by binding to a particular biotarget, like a specific protein or nucleic acid. Small molecules, e.g., organic dyes or drugs having fluorophoric/chromophoric activity show intense change in their spectral behavior when they bind to a specific biomacromolecule, which can be a protein, enzyme, or DNA. This change of spectral pattern of the small molecules is often indicative of their mode of interaction with that particular biomacromolecule. Hence, this type of small molecules are being extensively explored as important research tools to probe biotarget (i.e., biomacromolecule regulating a particular biological process) and also for the development of novel therapeutic drugs as well as effective diagnostic protocols. It is a highly cherished research goal for the scientists to develop novel, nontoxic, photostable, and easily synthesizable small-molecule-based fluoroprobe for a specific biotarget. Plethora of organic compounds is put into trials for this, and in this context, fluorescent thioesters, especially αβ-unsaturated thioesters, are highly relevant.

In living cells, several enzymatic reactions are triggered by such thioester derivatives. On the other hand, thioesters are often considered as better choices over oxoesters and used as vital synthetic intermediates in various important organic transformations, viz., biosynthesis of natural products. Apart from this, αβ-unsaturated thioesters are found in the photoactive yellow protein (PYP) of Ectothiorhodospira halophila. The visible fluorescent chromophoric moiety present in this water-soluble small chromoprotein is p-hydroxycinnamic acid, which is linked to the cysteine residue (Cys-69) by a thioester bond and controls the key photo-induced biological processes of the host organism.
Moreover, photochromic $\alpha,\beta$-unsaturated thioesters (e.g., $p$-hydroxycinnamic-thiophenyl ester) are studied extensively as model compounds to understand the mechanism of various photochemical reactions, and such compounds are explored nowadays for the development of memory and optical devices.\textsuperscript{16}

In the present work, we have synthesized three structurally different thioester derivatives, $p$-hydroxycinnamic-thiophenyl ester

![Figure 1. Single-crystal X-ray diffraction (XRD) structures of the synthesized compounds, $p$-hydroxycinnamic-thiophenyl ester (1), $p$-$N,N$-dimethylaminocinnamic-thiophenyl ester (2), and $N^{1},N^{1},N^{2},N^{2}$-tetramethyl-1,2-bis(phenylthio)ethene-1,2-diamine (4).]

Scheme 1. Synthesis of (1−4)\textsuperscript{a}

\textsuperscript{a}$p$-Hydroxycinnamic-thiophenyl ester (1), $p$-$N,N$-dimethylaminocinnamic-thiophenyl ester (2), $S$-phenyl-3-(4-chlorophenyl)-3-(phenylthio)propanethioate (3), and $N^{1},N^{1},N^{2},N^{2}$-tetramethyl-1,2-bis(phenylthio)ethene-1,2-diamine (4).
ester [or (E)-S-phenyl-3-(4-hydroxyphenyl)prop-2-enethioate (1), p-N,N-dimethylaminocinnamic-thiophenyl ester [or (E)-S-phenyl-3-(4-(dimethylamino)phenyl)prop-2-enethioate (2), and S-phenyl-3-(4-chlorophenyl)-3-(phenylthio)propanethioate (3) in addition to a novel (to the best of our knowledge) olefinic type of compound, substituted with amine and thiophenyl groups, i.e., N1,N2,N4,N5-tetramethyl-1,2-bis-(phenylthio)ethene-1,2-diamine (4) under the same reaction condition (Figure 1 and Scheme 1). Each of these compounds showed characteristic chromophoric/fluorophoric behavior in ethanol and also at pH 7.4 (physiological pH) (Tris–HCl buffer medium) [results are shown in Figure S1 (Supporting Information, pp S3 and S4)].

We have explored the characteristic chromophoric/fluorophoric behavior of compounds (1) and (2) for probing the biomacromolecule, DNA, the fundamental genetic material of life processes. The intrinsic fluorescence activities of p-hydroxycinnamic-thiophenyl ester (1) and p-N,N-dimethylaminocinnamic-thiophenyl ester (2) were found to be strongly dependent on their interactions with calf-thymus DNA (ctDNA). We have optimized the structures of (1) and (2) by applying a density functional theory (DFT) [B3LYP, 6-31G(d,p)] method, and their mode of interaction was studied with the help of various spectroscopic and physicochemical techniques and molecular docking studies, using DFT-based optimized structures of (1) and (2).

2. GENERAL EXPERIMENTAL SECTION

2.1. Materials. All of the reagents used for the synthesis of compounds (1–4) were of AR grade (E Merck). Solvents were dried and distilled prior to their use. In all of the experiments, Milli-Q (Milli-Q, academic with 0.22 mm Milli pack-40) water was used as per the requirement. Calf-thymus DNA (ctDNA) of molecular weight 8.4 MDa (SRL, India) was used as the model DNA for studying DNA-binding interactions of the synthesized compounds (1) and (2). Reagents used for the biocompatibility assay are of molecular biology (MB) grade, and details of these reagents are given in the Supporting Information (p S21).

For this study, a stock solution of ctDNA was prepared by dissolving solid ctDNA (sodium salt) in Tris–HCl buffer (10 mM, pH 7.4) (SRL, India) containing 0.1 mol L−1 NaCl solution and stored at 4 °C for further use. The purity of ctDNA was checked spectrophotometrically by measuring the ratio of its maximum absorbance at λmax values 260 and 280 nm, i.e., A260/A280. A value of A260/A280 ≈ 1.87 was observed, which denoted that ctDNA used was sufficiently free from protein. The concentration of ctDNA stock solution calculated from its maximum absorbance at 260 nm (A260) and e260 = (6600 L mol−1 cm−1) data was found to be 3.95 × 10−3 M.[7,8]

All other reagents for the DNA-binding studies were of molecular biology (MB) grade (SRL, India), and these reagents were used as they were purchased without any further purification. However, purity of all of the reagents used for the present study was routinely checked either spectrophoto metrically or by thin-layer chromatography (TLC) on silica gel GF254 precoated plates using appropriate eluting solvent mixtures.

2.2. Method of Synthesis of Thioester Derivatives (1–4). p-Hydroxycinnamic acid (600 mg, 1 equiv, 3.65 mmol) was dissolved in 5 mL of dichloromethane (DMC). The temperature of this reaction mixture was maintained at 0 °C, and oxalyl chloride (0.93 mL, 3 equiv, 10.97 mmol) was added to it in a dropwise manner. To this reaction mixture, dimethylformamide (DMF) (0.28 mL, 1 equiv, 3.65 mmol) was added at 0 °C and mixed thoroughly. After that, the reaction mixture was stirred at room temperature (r.t.) for 2 h. The progress of the reaction was monitored by TLC of the reaction mixture at regular intervals of time. After the completion of the reaction, the reaction mixture was subjected to evaporation under reduced pressure to get a concentrated mass, which was diluted with a little amount (~1.5 mL) of DCM, and triethylamine (Et3N) (1.53 mL, 3 equiv, 11.85 mmol) and thiophenol (PhSH) (0.807 mL, 2 equiv, 7.90 mmol) were added to it simultaneously and mixed properly by keeping the temperature of the reaction mixture at 0 °C. This reaction mixture was stirred for 12 h at r.t. After the completion of the reaction (as monitored by TLC), the reaction mixture was diluted with DCM and workup was done with aqueous NaHCO3 solution. The organic part was separated, and the solvent was evaporated under reduced pressure, whereby a gummy mass was obtained. This was subjected to column chromatography over silica gel (mesh 100–200), and the column was eluted with ethyl acetate (EA) and petroleum ether (PE, 60–80 °C) solvent mixture, whereby we have got four products: p-hydroxycinnamic-thiophenyl ester (1) (30–40% EA/PE), p-N,N-dimethylaminocinnamic-thiophenyl ester (2) (15–20% EA/PE), S-phenyl-3-(4-chlorophenyl)-3-(phenylthio)propanethioate (3) (75–90% EA/PE), and N1,N2,N4,N5-tetramethyl-1,2-bis-(phenylthio)ethene-1,2-diamine (4) (2–5% EA/PE).

Structures of (1)–(4) were confirmed on the basis of physicochemical studies, spectroscopy (1H and 13C NMR, IR, liquid chromatography–mass spectrometry (LC–MS)), and single-crystal X-ray diffraction (XRD) [for (1), (2), and (4) only] data. Single-crystal XRD patterns of (1), (2), and (4) are shown in Figure 1. NMR spectra were recorded on a 400 MHz Bruker AVANCE-400 NMR spectrometer with CDCl3 or dimethyl sulfoxide (DMSO) as solvents and tetramethylsilane as the internal standard. XRD experiments were done as per standard procedure, and relevant experimental details are given in Supporting Information (pp SS–S9).

2.3. Characterization Data for the Synthesized Products (1–4).

2.3.1. p-Hydroxycinnamic-thiophenyl Ester or (E)-S-Phenyl-3-(4-hydroxyphenyl)prop-2-enethioate (1). Light yellow crystal, 264 mg, 28.16% yield, mp 134–135 °C. 1H NMR (400 MHz, CDCl3): δ (ppm) 7.60 (d, 1H, J = 15.6 Hz), 7.48–7.51 (m, 2H), 7.42–7.44 (m, 5H), 6.80 (d, 2H, J = 8.8 Hz), 6.65 (d, 1H, J = 15.6 Hz); 13C NMR (100 MHz, CDCl3): δ (ppm) 193.30, 158.62, 141.93, 134.86, 130.70, 129.64, 129.38, 127.69, 126.56, 121.56, 116.21; IR (KBr, cm−1): 3371, 3058, 2936, 1649, 1579, 1511, 1435, 1277, 1040; LC–MS (M – H+) 255.12,16

2.3.1.1. Single-Crystal X-ray Crystallography for (1). Empirical formula C15H12O2S, FW 256.31, T 296 K, monoclinic, space group = P21/n, α = 16.6461(6) Å, b = 7.6854(3) Å, c = 20.8254(8) Å, α = 90°, β = 98.583(2)°, γ = 90°, V = 2634.40(17) Å3, Z = 8, λ (Mo Kα) = 0.71073 Å, μ = 0.236 mm−1; μmin = 1.6950, μmax = 28.4522, R = 0.0663(5065), wR2 = 0.2219(6619). Crystallographic data for the structures of 1 reported in the present work have been deposited at the Cambridge Crystallographic Data Centre with CCDC No. 1523138.

2.3.2. p-N,N-Dimethylaminocinnamic-thiophenyl Ester or (E)-S-Phenyl-3-(4-(dimethylamino)phenyl)prop-2-enethioate (2). Yellow crystal, 88 mg, 8.5% yield, mp 166–168 °C, 1H NMR (400 MHz, CDCl3): δ (ppm) 7.61 (d, 1H, J = 15.6 Hz), 7.51 (m, 2H), 7.42 (m, 5H), 6.80 (d, 2H, J = 8.8 Hz), 6.65 (d, 1H, J = 15.6 Hz); 13C NMR (100 MHz, CDCl3): δ (ppm) 195.12,16

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336
Molecular docking studies were done to (1) and (2) with ctDNA, the lowest energy docked conformation according to the AutoDock scoring function was selected as the binding mode. Python Molecular Viewer software (Stefano Forli, Olson Molecular Graphics Laboratory, The Scripps Research Institute, CA) was used to observe the output of the AutoDock studies.

2.4.2. Steady-State UV–Vis Absorption Spectroscopic Studies. UV–vis absorption spectra of all of the synthesized compounds (1–4) in ethanol and Tris–HCl buffer (10 mM, pH 7.4) medium were recorded on a dual-beam UV–vis spectrophotometer (PerkinElmer, Lambda 35) in the wavelength range of 200–600 nm. The interactions of either (1) or (2) with ctDNA were also studied with the help of UV–vis spectroscopy. Tris–HCl buffer (10 mM, pH 7.4) was used as the reference solution in all of the studies of ctDNA–compound interaction. In each case of the test compounds [(1) or (2)], the stock solution (1 mM) was prepared in EtOH. In a typical interaction study, specific volume of this stock solution [0.04 and 0.16 mL for (1) and (2), respectively] was added to 2.5 mL of Tris–HCl buffer in a 1.0 cm quartz cuvette and mixed thoroughly. In each case of (1) and (2), this test solution was allowed to stand for 5 min. After this time interval, the respective test solution was titrated spectrophotometrically by the successive addition of 3.95 mM of ctDNA solution. In case of (1), the volume of ctDNA solution added each time was 2 μL, whereas 5 μL of ctDNA was added each time to (2). The final concentration of ctDNA in test solution varied from 0.07 mM for (1) and 0.08 mM for (2). The concentrations of (1) and (2) or ctDNA used for the present study were optimized after several trials. In all of the cases, the volume effect was considered to be negligible. All of the UV–vis absorption measurements were performed at 25 °C. The final concentration of EtOH in the test solution was negligible (<1%).

2.4.3. Steady-State Fluorescence Spectroscopic Measurements. Fluorescence spectra of compounds (1) and (2) in both EtOH and Tris–HCl buffer (10 mM, pH 7.4) media were recorded using PerkinElmer spectrophotometer of model no. LS55. Fluorescence emission spectra of all of the test solutions remained unchanged at 10 nm for both excitation and emission beams. We have also used steady-state fluorescence spectroscopic method to study the interaction between ctDNA and the thioester derivative (1) or (2). For this study, we have used the same concentrations of ctDNA, (1) or (2) and Tris–HCl buffer, as discussed in the previous section. All of the fluorescence measurements were done at 25 °C, and we have observed that spectra of all of the test solutions remained unchanged for a long time during which the experiments were done. Hence, we can safely rule out the possibility of photodecomposition of the experimental samples, which may give errors in the results.
2.4.4. Viscometric Studies. Complexation/interaction of ctDNA with a binding ligand [like thioester derivatives (1) and (2)] changes its viscosity. Hence, we have done viscometric measurements of ctDNA solution in the presence of (1) or (2) to study the interaction of ctDNA with this type of ligands.

A Brookfield DV-II+ Pro viscometer, thermostated at 25 °C was used for the present study. Tris–HCl buffer (2.5 mL, 10 mM, pH 7.4) was used as reference solution. Different volumes of (1) or (2) were added separately into the viscometer by keeping ctDNA concentration fixed at 0.08 mM, and for each addition, viscosity of the experimental solution was measured.

Scheme 2. Plausible Mechanism for the Formation of (1–4)*

*p-Hydroxycinnamic-thiophenyl ester (1), p,N,N-dimethylaminocinnamic-thiophenyl ester (2), S-phenyl-3-(4-chlorophenyl)-3-(phenylthio)propanethioate (3), and N1,N1,N2,N2-tetramethyl-1,2-bis(phenylthio)ethene-1,2-diamine (4).
The concentration of (1) was varied within the range of 0.01–0.08 mM in the experimental solution. The same procedure was followed for (2).

The results were expressed as relative viscosity ($\eta / \eta_0$) versus the ratio of concentrations of ligand [(1) or (2)] to ctDNA (i.e., [(1)]/[ctDNA] or [(2)]/[ctDNA]). Here, $\eta$ and $\eta_0$ denote the viscosities of ctDNA solution in the presence and absence of ligand [either (1) or (2)], respectively.

2.4.5. Circular Dichroism (CD) Studies on the Binding Interaction of ctDNA with $p$-Hydroxycinnamic-thiophenyl Ester (1) or $p$-N,N-Dimethylaminocinnamic-thiophenyl Ester (2). A JASCO J-815 spectropolarimeter (Jasco International Co., Ltd., Hachioji, Japan) was used to record the CD spectra of all of the sample solutions. A rectangular quartz cuvette of path length 1.0 cm was used in all of the measurements. All of the CD measurements were done under inert and dry atmosphere, and for this, the optical chamber of the CD spectropolarimeter was kept in the dry and nitrogen atmosphere. The concentration of ctDNA was kept constant at 0.15 mM, and the concentration of test compound [either (1) or (2)] was varied. For both (1) and (2), the concentration range was 0–0.037 mM. CD spectra were recorded in the wavelength range of 200–320 nm at a scan speed of 100 nm min$^{-1}$ and a band width of 1.0 nm. The scans were collected and automatically averaged by the instrument. All experiments were performed in Tris–HCl buffer (10 mM, pH 7.4) medium at 25 °C.

2.4.6. Cytotoxicity Assays of (1) and (2). [[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assays of (1) and (2) were done to determine their cytotoxicity in human embryonic kidney cells (HEK293). Furthermore, we have also tested the in vivo cytotoxicity of these compounds using *Saccharomyces cerevisiae* 699 (MATa ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11, 15 cel1-100) cells. Detailed methods for these assays are discussed in Supporting Information (p S21).

3. RESULTS AND DISCUSSION

3.1. Synthesis of $p$-Hydroxycinnamic-thiophenyl Ester (1), $p$-N,N-Dimethylaminocinnamic-thiophenyl Ester (2), $S$-Phenyl-3-(4-chlorophenyl)-3-(phenylthio)propanethioate (3) and $N^1,N^2,N^3,N^4$-Tetramethyl-1,2-bis(phenylthio)ethene-1,2-diamine (4) and Studies on Their Spectral Behavior. Usefulness of thioesters either as synthetic intermediates or photoactive compounds is very much dependent on their structures and therefore the synthetic organic chemists are engaged in the development of synthetic protocols for the thioesters having task-specific structures. One of the most common methods for the synthesis of thioesters involves the reaction of carboxylic acid or acid chloride with thiol in the presence of a coupling agent, like dicyclohexyl carbodiimide (DCC)/$N,N$-dimethylaminopyridine. Various Lewis acid-catalyzed acylations of thiols are also common methods for the synthesis of thioesters. Bandgar et al. reported Dess–Martin periodinane (DMP)-mediated reaction of aldehyde and thiol to produce corresponding thioester derivatives. "TIOH-catalyzed direct thioesterification of carboxylic acids using thiol was done by limura et al. Synthesis of thioesters via ruthenium-catalyzed olefin cross-metathesis with thioacrylate was reported by van Zijl et al. These two methods were also effective in the synthesis of $\alpha$,$\beta$-unsaturated thioesters. However, the synthesis of $\alpha$,$\beta$-unsaturated thioesters by the direct thioesterification of the $\alpha$,$\beta$-unsaturated acid, e.g., $p$-hydroxycinnamic acid, which itself has functional groups susceptible toward the self-esterification, is troublesome and protection of such functional group(s) (e.g., $-\text{OH}$ group) is required to get better yield.

PYP chromophore analogue having an $\alpha$,$\beta$-unsaturated thioester moiety, i.e., $p$-hydroxycinnamic-thiophenyl ester (1), was prepared by the coupling of $p$-hydroxycinnamic acid with thiophenol in the presence of DCC as the coupling agent in DMF medium. Yoya et al. synthesized $p$-hydroxycinnamic-thiophenyl ester (1) by reacting (COCl)$_2$/DMF-treated $p$-hydroxycinnamic ester with thiophenolate lithium salt at $-30$ °C in tetrahydrofuran medium. Duran et al. have also reported similar synthetic protocol for thioester derivatives. Unlike these methods, the present method is very simple and cost-effective, and most importantly, we have obtained four structurally and photochromically different S compounds under the same reaction condition (Scheme 1). Among these, three are thioester analogues [$p$-hydroxycinnamic-thiophenyl ester (1), $p$-N,N-dimethylaminocinnamic-thiophenyl ester (2), and $S$-phenyl-3-(4-chlorophenyl)-3-(phenylthio)propanethioate (3)] and the fourth one, $N^1,N^2,N^3,N^4$-tetramethyl-1,2-bis(phenylthio)ethene-1,2-diamine (4), is not a thioester compound but dimethylamine and thiophenyl group-substituted olefinic (stibene-type) compound (Scheme 1 and Figure 1).

Single-crystal XRD patterns of (1), (2), and (4) are shown in Figure 1, and Tables S1–S3 (Supporting Information, pp S5–S9) represent the corresponding single-crystal XRD data and related experimental details for (1), (2), and (4). Selected bond lengths and bond angles of the single-crystal XRD structures of (1), (2), and (4) are shown in Tables S4–S6, respectively (Supporting Information, pp S10–S12).

The plausible mechanism of formation of these four products (1–4) is shown in Scheme 2. Reaction of oxalyl chloride with DMF generates $N,N$-dimethylchloromethylene iminium chloride (b), which on reaction with $p$-hydroxycinnamic acid produces carboxymethylene iminium chloride intermediate (c). The thiophenolate anion (d) (may be generated by the reaction of thiophenol and triethylamine) reacts with (e) to give (1) (Scheme 2). However, (1) may also react with (b) at its phenolic-OH nucleophilic center to give intermediate (e). This intermediate (e) may be decomposed by path (A) (shown by red line) or by path (B) (shown by blue line) to give (f) or (2), respectively. There is another possibility that, (f) on reaction with another thiophenolate moiety (d) gives (3). During these conversions, DMF may be regenerated, which on reaction with excess oxalyl chloride present in the reaction medium produces carboxymethylene iminium chloride intermediate (b) again. This iminium chloride (b) besides reacting with $p$-hydroxycinnamic acid can also react with unreacted (d) ions present in the reaction medium to generate (g), which on self-coupling may produce (4).

Thus, this present simple and low-cost method was found to be useful in the synthesis and isolation of three different thioester analogues with an S- and N-substituted olefinic compound simultaneously under the same reaction condition. In future, this method may be suitability scaled up to get better yield of each individual compound (1–4).

Among compounds (1–3), (1) and (2) were $\alpha$,$\beta$-unsaturated thiophenyl esters having electron-releasing groups ($-\text{OH}$ and $-\text{NMe}_2$, respectively) at p-position of the
Figure 2. (a) Docking models of \( p \)-hydroxycinnamic-thiophenyl ester (1) with ctDNA showing its binding at the minor groove of the DNA: (i) atomic sphere model, (ii) ribbon model, and (iii) close-up view (shown in line model); (iv) DFT [B3LYP 6-311G\(^+(d,p)\)]-optimized structure of (1).

(b) Docking models of \( p \)-N,N-dimethylaminocinnamic-thiophenyl ester (2) with ctDNA showing its binding at the minor groove of the DNA: (i) atomic sphere model, (ii) ribbon model, and (iii) close-up view (shown in line model); (iv) DFT [B3LYP 6-311G\(^+(d,p)\)]-optimized structure of (2).
However, we did not get any significant results with compounds (3) and (4). So, these two compounds were not further selected for other experimental studies on the ctDNA binding interaction (discussed in a later section).

Results of the docking study between (1) and ctDNA or (2) and ctDNA are shown in Figure 2a,b respectively. The present molecular docking study gives an idea about the two important parameters involved in this binding interaction of either (1) and ctDNA or (2) and ctDNA: (i) structure of the binding ligand [i.e., (1) or (2)] and the binding site(s) of the ligand and (ii) binding sites of the biomacromolecule (i.e., ctDNA). These parameters not only show strong influence on the binding profile and activity of (1) and (2), but also control the binding nature of the biomacromolecule, DNA. These are actually the factors determining the efficacy of (1) or (2) as fluorescent probe for biomacromolecules like DNA or as bioactive compounds (e.g., drugs).

Results of docking studies indicate huge deviation of structural geometry of both thiophenyl esters (1) and (2) on interaction with DNA from their respective crystal structures and also from their DFT-optimized structures, and the corresponding molecular docking profiles are shown in Figure 2.

Docking studies revealed that both thiophenyl esters (1) and (2) adopted fully nonplanar geometry to accommodate itself into the G–C-rich region of ctDNA and thus covalent interaction, e.g., hydrogen-bonding interaction, played a vital role in the binding of ctDNA and the thioster analogue (1) or (2), as shown in Figure 2.

We have also observed drastic changes in some of the dihedral angles and slight changes in bond lengths and bond angles in the ctDNA-docked structures of (1) and (2) compared to their free/unbound structures (Scheme 1 and Figure 1). These data are summarized in Tables S7a,b [for (1)] and S8a,b [for (2)] (Supporting Information, pp S17–S20).

The binding free energies (−ΔG) of the ctDNA-docked structures of both (1) and (2) were calculated as 6.4 kcal mol−1. These results support the existence of strong binding (possibly via noncovalent interactions) between (1) and ctDNA and also between (2) and ctDNA, as shown in Figure 2 and Tables S7a,b, and S8a,b, respectively (Supporting Information, pp S17–S20). Although, it is apparent from Figure 2a,b that these thiophenyl esters, either (1) or (2), bind to the minor groove of the DNA, but there is an inherent drawback associated with AutoDock software, which identifies most of the small molecules as minor groove binders for DNA. Hence, to...
further understand the binding interaction between the synthesized thiophenyl esters [(1) or (2)] and ctDNA, we have done a series of experiments and the results are discussed below.

3.3. UV−Vis Spectral Responses of p-Hydroxycinnamic-thiophenyl Ester (1) and p-N,N-Dimethylamino-cinnamic-thiophenyl Ester (2) in the Presence of ctDNA. Chromophoric/fluorophoric small molecules show intense change in the spectral behavior when their microenvironment is changed, and this can happen when biomacromolecules, like proteins and DNA, are present in their vicinity. These spectral changes of small molecules can be monitored to explore their usefulness as molecular probe for that biomacromolecule (in the present case, it is ctDNA). Hence, to explore the possibility of the use of thiophenyl esters (1) and (2) as probe for DNA, we have studied their UV−vis spectral responses individually in the presence of different concentrations of ctDNA.

The absorption spectra of (1) and (2) recorded in the absence and presence of different concentrations of ctDNA are shown in Figure 3a,b respectively.

Absorption spectrum of (1) (in Tris−HCl buffer, pH 7.4) is characterized by the appearance of a prominent band at 343 nm and two overlapping small humps at 223 and 236 nm (denoted by the dotted curve in Figure 3a). In case of (2), a prominent band at 354 nm was observed in addition to another small peak at 221 nm and a broad hump at 280 nm (denoted by the dotted curve in Figure 3b). On gradual addition of ctDNA solution to (1), humps at 223 and 236 nm became more sharp and shifted to λmax values 212 and 258 nm, respectively, at saturation.

In case of (2), gradual addition of ctDNA solution changed its spectral pattern. The band at 354 nm became more broad, whereas the two overlapping bands at 221 and 280 nm became distinctly separated. At the same time, these bands became more sharp and shifted to 215 and 258 nm, respectively. The increase of absorbance value at 223 nm was associated with blue shift or hypsochromic shift for (1). A similar phenomenon was observed in case of (2). But in case of the band at λmax 236 nm, the bathochromic or red shift was observed for (1). Hypsochromic or blue shift of the λmax values (from 221 to 215 nm and from 280 to 258 nm) were observed for (2). Intensities of these bands were also gradually increased with the increasing concentration of ctDNA solution.

Small molecules can interact with DNA through two types of binding modes: intercalation and groove binding. Electrostatic mode of binding is also observed depending upon the availability of the charged structure of the small molecule, but this does not resemble the present case. In case of intercalation mode of binding, pronounced hypochromic shift associated with broadening of the band envelope and bathochromic or red shift of the λmax value are observed.

A large shifting of λmax value is generally observed in the case of groove binding small molecules.2,3,5,9,57−59,40a However, no
such prominent characteristic features of either intercalation or groove binding were observed in the present case (Figure 3). But prominent changes in the UV−vis spectral characteristics of (1) and (2) in the presence of ctDNA surely indicated the strong noncovalent intermolecular interactions between (1) or (2)−ctDNA, leading to the formation of ground-state (1)−ctDNA or (2)−ctDNA complexes, respectively.

The formation constants $K_B$ of the (1)−ctDNA or (2)−ctDNA complex were evaluated spectrophotometrically by applying the Benesi−Hildebrand equation as shown below

$$
\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{(A_\infty - A_0)K_B[DNA]} \tag{1}
$$

where $A_0$ and $A$ are the absorbance values of free and bound ligands [(1) or (2)], respectively, and $A_\infty$ is the absorbance of the final complex. The Benesi−Hildebrand plots of (1) and (2) are shown in the inset of Figure 3a,b, respectively. The straight-line trend in the Benesi−Hildebrand plot suggests the possible formation of 1:1 complex between (1) or (2) and ctDNA. From the plot of $1/(A - A_0)$ to $1/[ctDNA]$, the ratio of the intercept to the slope gives the binding constant $K_B$. The binding constants ($K_B$) were found to be $1.63 \times 10^3$ and $0.10 \times 10^3$ M$^{-1}$ for (1) and (2), respectively.

3.4. Steady-State Fluorescence Studies on the Interaction of Thiophenyl Esters [(1) or (2)] with ctDNA. The fluorescence emission spectra of (1) and (2) in the absence and presence of different concentrations of ctDNA were recorded individually and the results are shown in Figures 4a and 5a, respectively.

The distinct change of fluorophoric behavior of (1) [and also that of (2)] was observed in the presence of ctDNA. Upon photoexcitation at 280 nm ($\lambda_{ex}$), (1) showed three emission band maxima ($\lambda_{em}$) at 312, 368, and 494 nm, whereas (2) showed two emission band maxima at $\lambda_{em}$ 494 and 516 nm. On gradual addition of ctDNA solution to (1), a significant decrease in the fluorescence intensity was observed. A similar phenomenon was also observed for (2). A prominent quenching of the bands at $\lambda_{em}$ 494 and 516 nm was observed (Figure 5a), but interestingly, the $\lambda_{em}$ at 516 nm of (2) began to disappear at saturation, accompanying with a gradual blue shift of the bands at $\lambda_{em}$ 494−486 nm (Figure 5a).

3.5. Mode of Interaction between Thiophenyl Esters [p-Hydroxycinnamic-thiophenyl Ester (1) or p-N,N-
Dimethylaminocinnamic-thiophenyl Ester (2) and ctDNA. Quenching (as in the present case) or enhancement of fluorescence of a fluorophore during its interaction with either a fluorescence quencher or an enhancer may be the outcome of several processes like excited-state energy-transfer reactions or ground-state complex formation, which are mechanistically classified into two categories: dynamic and static fluorescence quenching or enhancement. To understand the mechanistic pathway involved in the case of fluorescence quenching of either (1) or (2), we have applied the classical Stern–Volmer equation for quenching, as shown below

\[ \frac{F_0}{F} = 1 + K_{SV}[E] \]

where \( F_0 \) and \( F \) represent the fluorescence intensities of (1) or (2) in the absence and presence of different concentrations of the quencher, i.e., ctDNA, respectively. The concentration of ctDNA is denoted by \([E]\), and \( K_{SV} \) represents the Stern–Volmer constant.

Stern–Volmer plots (\( F_0/F \) vs \([E]\)) for (1) and (2) are shown in Figures 4b and 5b, respectively, and we have calculated the Stern–Volmer fluorescence quenching constant \( K_{SV} \) as 8.75 × 10^3 M^{-1} for (1) and 8.49 × 10^3 M^{-1} for (2).

We have further exploited the fluorescence intensity quenching data of (1) and (2) in the presence of ctDNA to measure their binding constants (\( K_B \)) and number of binding sites \((n)\). The change of fluorescence intensity at \( \lambda_{em} \) 494 nm for (1) and also for (2) was used to calculate the corresponding \( K_B \) and \( n \) values by applying the following equation:

\[ \log \left( \frac{F_0 - F}{F} \right) = \log K_B + n \log[\text{ctDNA}] \]

where \( F_0 \) and \( F \) are the fluorescence intensities of (1) and (2) at \( \lambda_{em} \) 494 nm in the absence and presence of different concentrations of ctDNA, respectively. In each case of (1) or (2), \( \log K_B \) was calculated from the linear plot of \( \log \left( \frac{F_0 - F}{F} \right) \) vs \( \log[\text{ctDNA}] \) \((r^2 = 0.99 \text{ for both (1) and (2)})\), as shown in Figures 4c and 5c, respectively. The values of \( K_B \) and \( n \) were obtained as 1.38 × 10^5 M^{-1} and 1.07 \( \approx 1.0 \) for (1) and 2.40 × 10^5 M^{-1} and 1.12 \( \approx 1.0 \) for (2), indicating the weak binding between (1) and (2) with ctDNA.

These \( K_{SV} \) values are comparable to those of the small-molecule fluorophores, such as 3-hydroxyflavones, isoxazolocurcumin, and curcumin derivatives, which showed static fluorescence enhancement/quenching phenomena associated with ground-state complex formation. These small molecules are also known to be DNA groove binders. So, in the present case of (1) and (2), we can assume a similar type of process of fluorescence quenching of these compounds in the presence of a quencher, like DNA. This may be originated due to the ground-state complex formation of (1) or (2) with DNA possibly via groove binding mode of interaction. However, to further verify these facts, we have done viscometric measurements of ctDNA in the presence of (1) or (2).

3.6. Viscosity Measurements To Elucidate the Mode of Interaction between Thiophenyl Esters [p-Hydroxycinnamic-thiophenyl Ester (1) or p-N,N-Dimethylaminocinnamic-thiophenyl Ester (2)] and ctDNA. Change of viscosity of DNA solution in the presence of various concentrations of small-molecule-based binding ligands is an indication of their intermolecular interaction/complexation with ctDNA. In the presence of a perfect intercalator ligand, the DNA base pairs became separated and thus the chain length of DNA increases, which ultimately increases the viscosity of the DNA solution. However, decrease of viscosity of DNA solution is observed when the ligand is a partial or nonclassical intercalator. In case of ligands binding at DNA grooves or ligands binding DNA through electrostatic interactions, no change of viscosity of DNA solution can be observed.

The change of relative viscosity (\( \eta/\eta_0 \)) of ctDNA solution (0.08 mM) on gradual addition of different concentration of (1) or (2) in Tris–HCl buffer (10 mM, pH = 7.4) is shown in Figure 6.

As shown in Figure 6, a gradual increase in viscosity of ctDNA solution on interaction with (1) was noticed. A similar result was also observed with (2). So, these viscosity measurement data obviously confirmed the binding activities of (1) or (2) with ctDNA through intercalation mode of intermolecular interaction. This was further established with the help of circular dichroism (CD) studies of ctDNA in the presence of (1) or (2).

3.7. Circular Dichroism (CD) Studies To Confirm the Mode of Interaction between Thiophenyl Esters [p-Hydroxycinnamic-thiophenyl Ester (1) or p-N,N-Dimethylaminocinnamic-thiophenyl Ester (2)] and ctDNA. DNA undergoes conformational changes on binding with small-molecule ligands. This can be monitored by measuring its CD spectra in the absence and presence of the respective binding ligand. These changes in the CD pattern of DNA often indicate the mode of binding of that particular ligand to DNA. For a small-molecule ligand-based classical intercalator, a significant decrease of intensities of both the positive and negative bands of CD spectrum of the bound DNA is observed, but the intensities of these two CD bands of DNA remain unchanged on its binding with a small molecule, which prefers groove binding, more precisely minor groove binding mode of interaction.

CD spectra of ctDNA in the absence and presence of different concentrations of (1) or (2) with ctDNA are shown in Figure 7.

We have used these CD spectrum data of ctDNA when it binds to (1) or (2) to confirm the mode of binding of (1) or (2) with ctDNA. Being achiral, (1) and (2) are not optically...
active and no CD spectra were observed for (1) and also for (2) in the wavelength region (200−350 nm) selected for the present experiment. CD spectrum of the free ctDNA, i.e., in the absence of (1) [or (2)] in Tris−HCl buffer medium (10 mM, pH = 7.4), showed two bands: a positive band 274 nm and a negative band 245 nm (Figure 7a,b) which originates due to π−π stacking of DNA base pairs and right-handed helicity of DNA, respectively. This type of characteristic nature of CD spectrum is often associated with the B-form of DNA.47 However, on addition of (1) [or (2)] to ctDNA, significant changes in the intensities of negative bands as well as positive bands of CD spectra of DNA were observed (Figure 7a,b). Similar conclusions can be drawn regarding the mode of binding of (2) with ctDNA (Figure 7b). This indicates the intercalative binding mode of interaction of (1) [or (2)] with ctDNA. We have further explored the CD responses of DNA at 245 nm to calculate equilibrium constant (K_B) by applying the Benesi−Hildebrand equation

\[ 1/(\theta - \theta_0) = 1/(\theta_1 - \theta_0) + 1/[(\theta_1 - \theta_0)K_B\text{[ligand]}] \]

where \( \theta - \theta_0 \) = change of CD responses of ctDNA at 245 nm, \( \theta = \) CD response of ctDNA on gradual addition of ligand, thiophenyl ester [(1) or (2)], \( \theta_0 = \) CD response of ctDNA in the absence of either (1) or (2), and \( \theta_1 = \) final CD response of thiophenyl ester−ctDNA. From the linear plot of \( 1/(\theta - \theta_0) \) versus \( 1/[\text{ligand}] \) (Figure 7a(i),b(i)), we have calculated the binding constant \( K_B \) for (1) and (2) as 3.43 × 10^4 and 1.93 × 10^4 M^{-1}, respectively, which are in accordance with the results obtained from fluorescence spectroscopic studies. These values suggest the possibility of intercalative binding mode of interaction between (1) and ctDNA and also between (2) and ctDNA.48 However, the changes in the CD bands in case of (1) [and also for (2)] are not so drastic as it is observed for a perfect intercalator.

3.8. Biocompatibilities of (1) and (2). Previous report on (1) showed its weak activity toward M. tuberculosis.12 However, no report on bioactivity was found for (2). In the present case, we have done an elaborate study on the biocompatibilities of (1) and (2) (detailed experimental methods are discussed in Supporting Information, pp S21 and S22).

The cell cytotoxicity assay of compounds (1) and (2) performed on the normal human cell line, HEK293, using the MTT assay method showed a concentration- and time-dependent effect. More than 82% of the cells survived after 24 h of exposure to compound (1) at its 30 μM concentration (Figure S2, Supporting Information, p S22). This value was found to be approximately 70 and 45% at 48 and 72 h, respectively. A proportion of 54% of the cells survived in case of compound (2) after 24 h of its exposure at 15 μM concentration (Figure S2, Supporting Information, p S22). The effect of these compounds (at their 40 μM concentration) on S. cerevisiae cells were also evaluated for a period of up to 10 days. No toxicity of the compounds was observed after checking for viability of the cells at 0, 2, and 10 days.

In case of compound (1), cytotoxicity toward normal human cell lines (HEK293) was observed after a concentration of 30 μM, but in case of compound (2), this optimal cytotoxic
concentration was found to be 15 μM. Lower concentrations of these compounds did not significantly affect the animal cell lines. More than 70% growth of cells was observed for compound (1) up to its 30 μM concentration. However, the same growth rate of cells was noted with compound (2) when its concentration was 12 μM. Furthermore, no such toxicity was observed in case of in vivo cytotoxicity assay using S. cerevisiae cells. Even at 40 μM concentrations of compounds (1) and (2), no loss in viability was observed in cells. This difference in results between the animal cell lines and yeast cells may be due to the difference in the composition of the cell walls of these two different kinds of cells. Microbial cells may be inherently hard as they are more exposed to inclement conditions on their growth. The difference in their bioactivity may be originated from the difference in their substituent pattern (Figure 1).

4. CONCLUSIONS

Development of easy and low-cost synthetic methodologies for α/β-unsaturated thioester type of compounds having prominent photochromic behavior or having potential to be used as synthetic intermediates in various important organic transformations is a well-appreciated research problem. In this context, our work may be noteworthy as in the present work, under the same reaction condition using (COCl)₂/DMF and PhSH/Et₃N in DCM medium at ambient temperature. We have synthesized four structurally and photochromically different thiophenyl esters of p-hydroxycinnamic and (4) is a novel stilbene-type compound (to the best of our knowledge) substituted with dimethylamine and thiophenyl groups.

Furthermore, (1) and (2) showed a drastic change in their intrinsic chromophoric/photochromic activities during their interactions with ctDNA. Spectroscopic (UV–vis, fluorescence, and CD) and viscometric measurements indicated the intercalation mode of binding between these thiophenyl esters (1) or (2) and ctDNA. However, results of a preliminary molecular docking study indicate the possibility of minor groove binding mode of hydrogen-bonding interaction of (1) or (2) with ctDNA. It is interesting to note that structurally these compounds satisfy the characteristics of both intercalator and groove binders for DNA. These two compounds have single-bonded flexible parts, for which torsional rotation of molecule (1) or (2) is possible. This may help these molecules to fit into the shallow minor groove of DNA. On the other hand, (1) and (2) have planar parts (aromatic rings), which may allow them to interact with DNA through the intercalation mode. So, it is quite possible that these compounds are not perfect intercalator or perfect grooved binder of DNA.

It is also worth mentioning that the change of intrinsic fluorescence activity of (1) or (2) in the presence of biomacromolecule like ctDNA opens up the possibility for application of this type of small molecules as DNA-based fluorescence biomarkers. Moreover, the change of fluorescence activity of (1) or (2) in the presence of biomacromolecule like DNA (monitored under the physiological condition) can be considered as a model to understand the drug–DNA interaction, which is highly essential for the development of novel therapeutic agents. However, (1) will be comparatively more suitable than (2), considering its high biocompatibility.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01933.

UV–vis spectra and fluorescence spectra of compounds (1–4); single-crystal XRD data of compounds (1, 2, and 4); selected bond lengths and bond angles of compounds (1, 2, and 4); computational details; change of structural parameters of (1) and (2) on binding with ctDNA; methods of cytotoxicity assay and in vivo cell viability assay (Figure S2); and 1H and 13C NMR, LC–MS, and IR spectra of the synthesized products (1–4) (Figures S3a–S6d) (PDF)

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Notes

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