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# A mechanistic study on the nuclease activities of some hydroxystilbenes

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**Abstract**—The nuclease activities of a series of hydroxystilbeneoids have been studied so as to establish a structure–activity correlation and deduce the mechanistic pathway of the process. Although the test compounds could nick plasmid DNA, only three of these including resveratrol produced double strand breaks in DNA. Amongst these new stilbenes, compound 2e containing a partially methylated catechol and a C-4 hydroxy moieties was equally potent as resveratrol. The activities of the unprotected catechol-derivatives were less than those of the resorcinol-derivatives, which were, however, compensated by partial methylation of the former. The presence of $Cu^{2+}$ and $O_2$ and the participation of a $Cu^{+}$-oxo intermediate were obligatory in the process which did not require addition of any external reducing agent. Overall, the differential nuclease activities of the compounds could be explained primarily with their superoxide anion generation abilities, and to a lesser extent with their DNA binding and $Cu^{2+}$ reducing capacities. The amount of superoxide anion produced by the compounds depended strongly on their $Cu^{+}$-complexation abilities, which again, was decided by the pattern and nature of the oxygenated substituents in the aromatic rings.


## 1. Introduction

The hydroxystilbenoids, abundant[1,2] in the plant kingdom, are of recent interest due to their diverse biological profiles. Amongst these, resveratrol, present in grapes and other food products is valued for its preventive potential against atherosclerosis and carcinogenesis.[3,4] Likewise, its congeners like piceatanol, piceid etc., are also known to possess a wide range of bioactivities.[5] Several other related compounds known as combretastatins are promising anti-neoplastic agents.[6] The phenolic group in these compounds can scavenge the reactive oxygen species (ROS) and the associated antioxidant properties may, partly, explain their beneficial physiological roles. However, polyphenolic compounds are also known to behave as pro-oxidants because of their easy oxidization by metal ions such as $Fe^{3+}$, $Cu^{2+}$ etc.,[7] which trigger the Fenton-mediated ROS generation. Thus, some of these compounds might exhibit nuclease activity in the presence of a suitable redox active metal ion. Indeed, the DNA-cleaving property of resveratrol in the presence of $Cu^{2+}$ ion has recently been reported.[8] This prompted us to assess the potential of a series of hydroxystilbenes as the DNA-cleaving agents to gain further insight in the mechanism of their action which might help in designing simpler and more potent analogues. Herein, we report the results of our study.

To account for the DNA-cleaving activity of the hydroxystilbenoids, structural features such as number of hydroxyl/methoxyl substituents and their relative positions as well as the requirement of the olefin function were considered. We envisaged that the anti- or prooxidant activities of the hydroxystilbenes would not be affected by their olefin geometry, which is also supported by some earlier observations from our group and others.[9a,b] Consequently, a series of hydrostilbenoids **2a–g** (Fig. 1.) were synthesized[9a,c] as well-defined *E*/*Z*-mixtures by the low-valent titanium ($TiCl_3$-Zn-THF)-mediated McMurry coupling[10] of suitably protected phenolic aldehydes, and their ability to mediate cleavage of supercoiled plasmid pBR 322 DNA (Form I) was assessed using agarose gel electrophoresis.

The preliminary screening carried out with all the test compounds revealed that amongst the metals tested viz. $Cu^{2+}$, $Fe^{3+}$, $Ni^{2+}$ and $Zn^{2+}$, the DNA cleavage was induced only in the presence of $Cu^{2+}$. The gel electrophoresis pattern (Fig. 2) shows the DNA nicking ability

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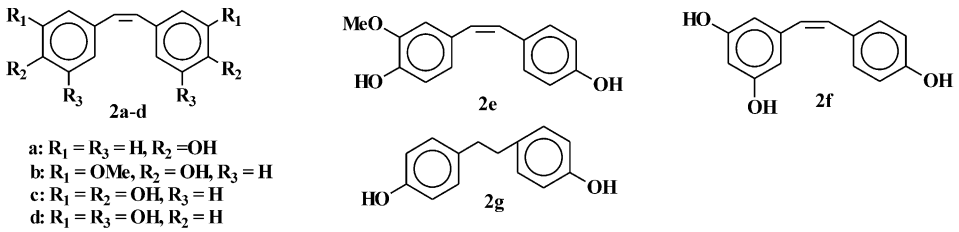


**Figure 1.** Structures of the hydroxystilbenoids.

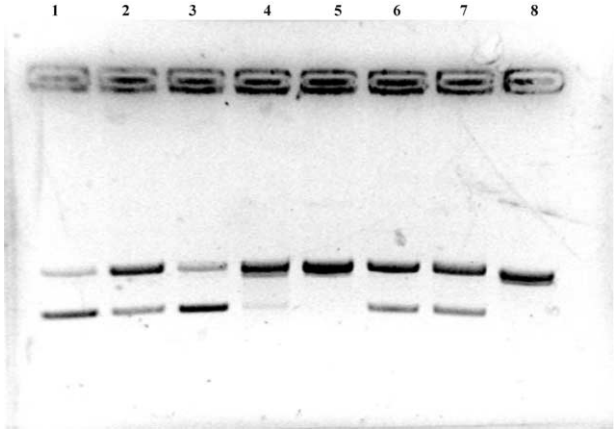


**Figure 2.** Gel electrophoresis pattern of supercoiled plasmid DNA (form I) cleavage by **2a–g** + $Cu^{2+}$. Reaction mixture (20 μL, total volume) contained 200 ng of form I DNA in 10 mM HEPES buffer, pH 7.2 and $Cu(OAc)_2$ (50 μM). Other conditions were as described in the Experimental. Lane 1, without any test compound; lane 2, **2a**; lane 3, **2g**; lane 4, **2b**; lane 5, **2e**; lane 6, **2c**; lane 7, **2d**; lane 8, **2f**.

of **2a–g** in the presence of $Cu^{2+}$ under oxic condition, the quantification of the results being presented in Table 1.

Compound **2f** (resveratrol) was by far the most active candidate producing 76% DNA nicking. The extent of DNA nicking by **2e** was almost similar (73%), while that by **2b** was marginally less but still significant (62%). The cleavage capacity of the simple *para*-hydroxylated stilbene **2a** was moderate (27%) although its dihydro analogue, **2g**, could not nick DNA. Thus, the importance of the olefinic function in imparting the cleavage activity was apparent. The bicatechol compound **2c** and the biresorcinol compound **2d** showed almost equal capacity for the DNA cleavage, the former being marginally more potent. Thus, the tetraphenolic stilbenes (**2c** and **d**) behaved similarly irrespective of the relative positions (1,2- or 1,3) of their hydroxyl groups. Comparison of the results for **2c** *vis-à-vis* those of **2e** and **2b** revealed that partial methylation of the catechol bearing stilbenes improved their activity considerably. Given that the dihydroxyl stilbene, **2a** showed similar cleavage capacity as those of tetraphenolics (**2c** and **d**), the number of phenolic groups present in stilbenes seems to have little bearing on their nuclease activity. In comparison, the presence of a 4-hydroxy group appeared most crucial for ensuring the DNA cleavage capacity of the hydroxystilbenes. This is also apparent from the impressive and almost similar nuclease activities of **2e** and **f**, both possessing the 4′-hydroxyl group. In comparison, the partially methylated catechol (in **2e**) and unprotected resorcinol (in **2f**) moieties, possibly contributed much less in the nuclease activity.

**Table 1.** $Cu^{2+}$-Mediated nuclease activities of **2a–g**

| Compound | Nicked DNA (%) | Ds-DNA |
|---|---|---|
| **2a** | 27.18 ± 11.27 | Absent |
| **2b** | 62.65 ± 7.48 | Present |
| **2c** | 28.68 ± 12.82 | Absent |
| **2d** | 24.70 ± 12.83 | Absent |
| **2e** | 72.78 ± 3.93 | Present |
| **2f** | 76.15 ± 2.19 | Present |
| **2g** | 1.8 ± 1.09 | Absent |

More interestingly, the above study revealed that amongst all the test compounds, only **2b**, **e** and **f** could cause double strand breaks (dsb)in DNA. Considering that dsb in DNA is more difficult for the cells to repair than single strand breaks (ssb), compounds that facilitate this, are promising candidates as cytotoxic and anti-tumour agents.[11a–c] Hence, in all the subsequent studies, major emphasis were given to the nuclease properties **2b** and **e**, as **f** (resveratrol) is already known to show nuclease activity.[8]

To gain further insight into the mechanism of the DNA cleavage by **2b** and **e**, the dependence of various factors and the involvement of the exact reactive oxygen species (ROS) were studied. Compound **2b** alone did not to lead to any DNA strand scission (data not shown). As shown in Figure 3a, $Cu^{2+}$ also did not cleave DNA (lane 1) which could be effected only when both are present (lane 2). The cleavage was complete with equimolar concentration of $Cu^{2+}$, while lowering the $Cu^{2+}$ concentration progressively reduced the extent of DNA strand scission (data not shown). The cleavage was very fast and significant dsDNA cleavage was noticed within 15 min. The metal chelator EDTA and the $Cu^{+}$-specific chelator, bathocuproine could prevent the cleavage (lanes 4 and 7 respectively). No cleavage was observed under anoxic conditions (lane 8).

Superoxide dismutase (4000 U/mL) had a little effect on the extent of DNA cleavage (lane 5) but prevented the nicking at a high concentration (10,000 U/mL). The hydroxyl radical scavenger, DMSO also could not prevent the DNA cleavage (lane 3), indicating non-participation of this freely diffusible radical in the reaction. Likewise, the singlet oxygen quencher,[12] sodium azide could not prevent the DNA scission. Catalase, the enzyme responsible for the disproportionation of $H_2O_2$, completely inhibited the cleavage (lane 6).

$$HST + Cu^{2+} \longrightarrow HST^{\cdot+} + Cu^{+}$$

$$Cu^{+} + O_2 \longrightarrow Cu^{2+} + O_2^{\cdot-}$$

$$O_2^{\cdot-} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{+}\text{-oxo species}$$

$$Cu^{+}\text{-oxo species} \xrightarrow{DNA} \text{Damaged DNA}$$

**Scheme 1.**

The results obtained with **2e** were also identical (Fig. 3b) as obtained with **2b** and was in conformity with those reported for **2f**.[8]

Based on these result, it was clear that the primary requirement of the DNA cleaving properties of **2b** and **2e** was the presence of $Cu^{2+}$ and oxygen. The cleavage was found to be oxidative and did not require any external reducing agent. The data also indicated that $Cu^{+}$ was an obligatory intermediate of the process which appears to proceed via a $Cu^{+}$-peroxo-complex as is reported with **2f**[8] and some polypyrroles.[13] Mechanistically the entire process can be depicted as shown in Scheme 1. In short, $Cu^{2+}$ gets reduced with the hydroxystilbenes (HST) to $Cu^{+}$, which on reaction with oxygen produces the superoxide ($O_2^{\cdot-}$) radical and $Cu^{2+}$ is regenerated. Disproportionation of the $O_2^{\cdot-}$ radical furnishes $H_2O_2$ which on subsequent reaction with $Cu^{+}$ produces the $Cu^{+}$-peroxo complex as the DNA cleaving agent.

In the light of the above mechanism, the variations in the nuclease activity of the test compounds **2a–g** may possibly be attributed to their differential (i) binding properties with DNA, (ii) abilities to reduce $Cu^{2+}$, and (iii) abilities to generate superoxide radicals, which, in turn, produces the $H_2O_2$. Hence, all these factors were studied so as to assess their roles individually or in combination in deciding the DNA cleavage properties of **2a–g**. The results are presented below.

### 1.1. DNA-binding properties of 2b and 2e

The DNA-binding properties of compounds are known to enhance their capacity to cleave DNA. Given that only **b**, **e** and **f** produced double-strand DNA cleavage, their binding capacities with calf thymus (CT)-DNA were investigated by absorption spectroscopy. Compounds **2b** and **e** have strong absorption bands at 331 nm, while **2f** showed absorption maximum at 283 nm which were well suited for the DNA binding studies. Addition of CT-DNA in increasing amounts to a fixed concentration (50 μM) of **2b**, **e** and **f** led to gradual reductions in the intensity of their respective absorption maxima confirming their efficient binding with DNA. Based on the site-exclusion model,[14] the equilibrium binding constants (K) in respective cases were derived by quantitative analysis of the UV–visible data. The DNA–titration curve with **2b** and **e** are shown in Figures 4a and 4b respectively. The binding constant values of **2e** and **f** were similar, and 2.5 times that of **2b**. The specificity of their bindings if any, with the DNA segments was subsequently assessed by individual titration of **2b** and **e** (50 μM) with poly[G–C]$_2$ and poly[A-T]$_2$. In both the cases, a slightly more hypochromicity with poly[G–C]$_2$ was seen which reflected a marginal G–C specific binding by these compounds. The respective binding constants also indicated ~10 times stronger binding by **2b** and **e** with poly[G–C]$_2$. In these cases also, the binding by **2e** was stronger than by **2b**, which was in tune with their DNA-cleaving capacity. The magnitudes of the K-values are summarized in Table 2. The binding constants for the compounds that intercalate with DNA are usually of the order[15a] of $10^5$–$10^7$ $M^{-1}$ and show[15b,c] hypochromism on binding. Similar hypochromism was also observed with the compounds

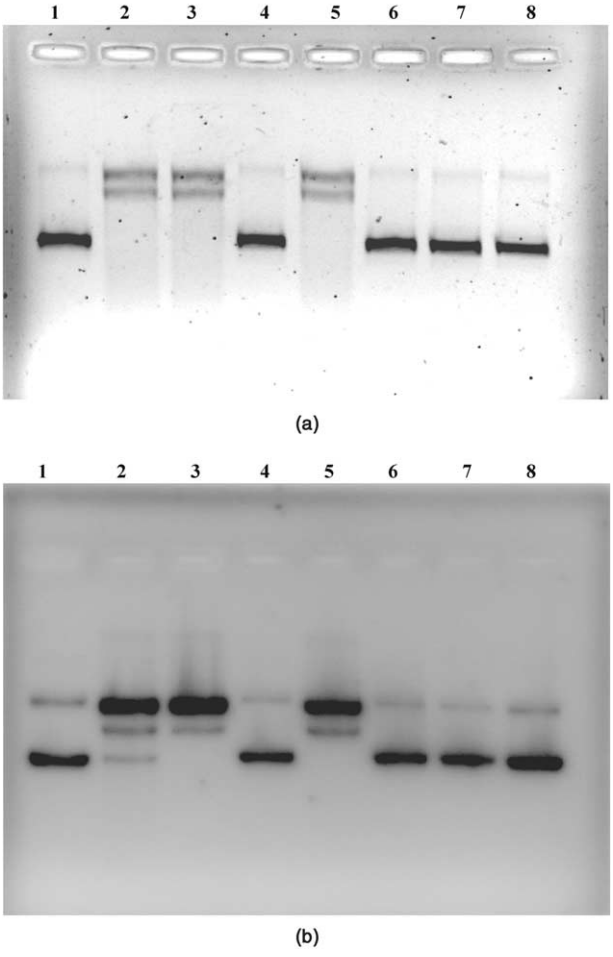


**Figure 3.** (a) Effect of different inhibitors on supercoiled plasmid DNA (form I) cleavage by **2e** + Cu(OAc)$_2$. Reaction mixture (20 μL, total volume) contained 200 ng of form I DNA in 10 mM HEPES buffer, pH 7.2 and Cu(OAc)$_2$ (50 μM). Other conditions were as described in the Experimental. Lane 1, without any inhibitor and **2e**; lane 2, with **2e** (50 μM); lane 3, + DMSO (500 μM); lane 4, EDTA (1mM), lane 5, + SOD (1000 U/mL); lane 6, + catalase (1000 U/mL); lane 7, bathocuproine (500 μM); lane 8, without $O_2$; (b) Effect of different inhibitors on supercoiled plasmid DNA (form I) cleavage by **2b** + Cu(OAc)$_2$. Reaction mixture (20 μL, total volume) contained 200 ng of form I DNA in 10 mM HEPES buffer, pH 7.2 and Cu(OAc)$_2$ (50 μM). Other conditions were as described in the Experimental. Lane 1, without any inhibitor and **2b**; lane 2, with **2b** (50 μM); lane 3, + DMSO (500 μM); lane 4, EDTA (1mM), lane 5, + SOD (1000 U/mL); lane 6, + catalase (1000 U/mL); lane 7, bathocuproine (500 μM); lane 8, without $O_2$.

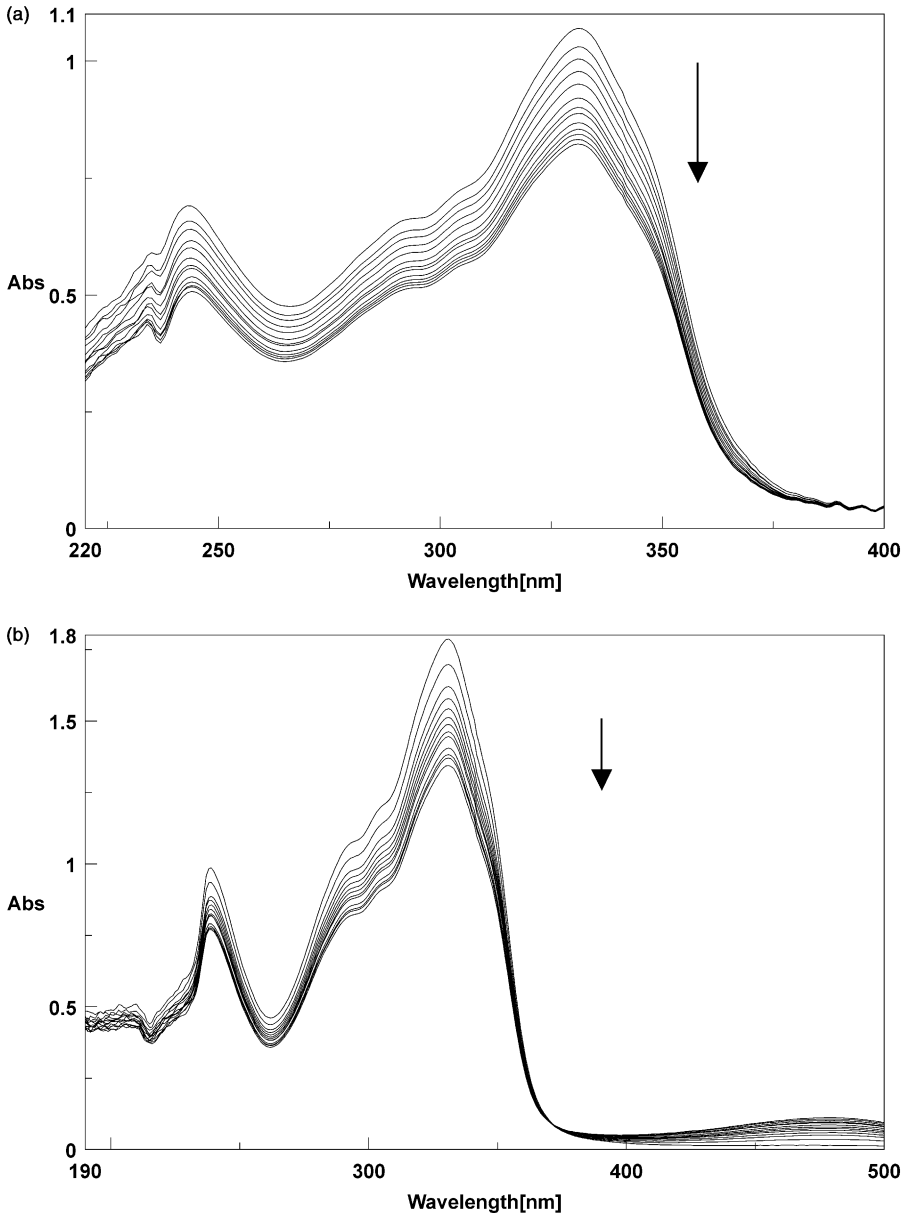


**Figure 4.** (a) The UV–vis titration of **2b** (50 μM) with CT-DNA was carried in 10 mM HEPES buffer, pH 7.2 at 25 °C. The CT DNA was added in 0.164 μM bp aliquots, other details are provided in the Experimental. (b) The UV–Vis titration of **2e** (50 μM) with CT-DNA was carried in 10 mM HEPES buffer, pH 7.2 at 25 °C. The CT DNA was added in 0.164 μM bp aliquots, other details are provided in the Experimental.

**2b**, **e** and **f**, while their binding constants with CT-DNA were $\sim 10^5$–$10^6$ $M^{-1}$. Based on these, intercalative binding by both **2b** and **e** was inferred. The reduction of their binding constants with CT-DNA by $\sim 100$ fold in the presence of a large excess of the classical intercalator, ethidium bromide provided further credence to our inference. The regression analysis in all the cases showed linear fits indicative of a single mode binding of them with DNA. Thus, the DNA binding properties of **2b**, **e** and **f** could partly explain their nuclease activities.

**Table 2.** Comparative DNA binding constants (K) of **2b**, **e** and **f**

| DNA | 2b | 2e | 2f |
|---|---|---|---|
| CT-DNA | $6.15\times10^5$ | $1.23\times10^6$ | $1.48\times10^6$ |
| Poly $[A–T]_2$ | $3.63\times10^4$ | $1.26\times10^5$ | |
| Poly $[G–C]_2$ | $3.8\times10^5$ | $1.26\times10^6$ | |

### 1.2. $Cu^{2+}$-reducing properties of 2a–g

The $Cu^{2+}$ reducing abilities of **2a–g** at same concentrations were assayed by the bathocuproine (a $Cu^+$-specific ligand) method[16] and the results are presented in Table 3. The method is based on measuring the absorbance at 480 nm of the complex formed between bathocuproine and $Cu^+$, produced during the reduction of $Cu^{2+}$ by the test compounds. Expectedly, minimum amount of $Cu^+$ was formed with **2g** which produced insignificant DNA damage. The amounts of $Cu^+$ produced by **2a, c** and **d** were similar and commensurate with their DNA

damaging abilities. Compounds **2b** and **e** produced significantly higher amounts of $Cu^{+}$, the latter being the stronger reductant. Thus, overall the DNA cleaving capacities of **2a–e** and **g** correlated well with their $Cu^{2+}$ reducing abilities. However, no such correlation was evident with **2f** which reduced lesser amount of $Cu^{2+}$ than even **2a, c** and **d**, but produced almost equal amount of DNA cleavage as **2e**.

### 1.3. Superoxide generating capacities of 2a–g

In order to explain the anomalous DNA cleavage capacity of **2f** vis-à-vis those of **2e** and **b**, the amounts of superoxide generated by the same concentrations of **2a–g** were quantified (Table 4) via the nitro blue tetrazolium (NBT) reduction assay.[17] The assay is based on measuring the absorbance at 560 nm of the chromogen formed by the reduction of NBT by any reducing agent, in this case, $O_2^{\cdot-}$ which, is generated by the test compounds. It was gratifying to note that amongst the test compounds, **2f** produced the maximum amount of superoxide radical, commensurate with its highest DNA cleavage capacity. In general, the order of superoxide generating capacity **2f** > **2e** > **2b** > **2d** ~ **2g** ~ **2a**–**2c** matched with that of their DNA cleaving activities. Evidently, the catechol-containing compounds **2b**, **e** and **c** produced lesser amounts of the superoxide radicals compared to the resorcinol-stilbene, **2f**. Possibly the complexes formed by the catechol compounds with $Cu^{+}$ are more stable and have lower reactivity with $O_2$, accounting for the result. In comparison, the $Cu^{+}$ complex with compound **2f** is comparatively weaker and hence its reaction with $O_2$ was more facile furnishing more superoxide radicals. The other resorcinol compound **2d** has an additional site of $Cu^{+}$ chelation due to its 3,3′-dihydroxy moiety which provides extra stability to the $Cu^{+}$ complex compared to **2f**. This, in turn, hampers its superoxide generating capacity. As a representative example, the copper complexation ability of **2d** is shown in Figure 5. Between the partially methylated catechol compounds **2e** and **b**, the poorer capacity of the latter to produce the superoxide radical can be explained on the basis of its bicatechol ligand, which leads to a more stable and less reactive complex. Again between **2b** and **c**, the $Cu^{+}$ complex of **2c** is expected to be stronger than that of **2b**, as the former contains free phenolic groups which can form covalent bonds with the metal ion. The complex of **2b**, on the other hand, contains one coordinate bond and hence, would be more reactive with oxygen producing more superoxide radicals.

Apparently, the above results seem to be contradictory to some of the previous reports[18a,b] wherein catechols

**Table 3.** The absorbance of the $Cu^{+}$-bathocuproine complexes formed by **2a–g**

| Compd | $Abs_{480}$ |
|---|---|
| **2a** | 0.372±0.02 |
| **2b** | 0.636±0.13 |
| **2c** | 0.325±0.04 |
| **2d** | 0.379±0.01 |
| **2e** | 0.846±0.03 |
| **2f** | 0.235±0.01 |
| **2g** | 0.088±0.01 |

**Table 4.** The absorbance of the reduced NBT formed with **2a–g**

| Compd | $Abs_{560}$ |
|---|---|
| **2a** | 0.009±0.001 |
| **2b** | 0.050±0.003 |
| **2c** | 0.008±0.003 |
| **2d** | 0.015±0.003 |
| **2e** | 0.068±0.009 |
| **2f** | 0.071±0.017 |
| **2g** | 0.008±0.001 |

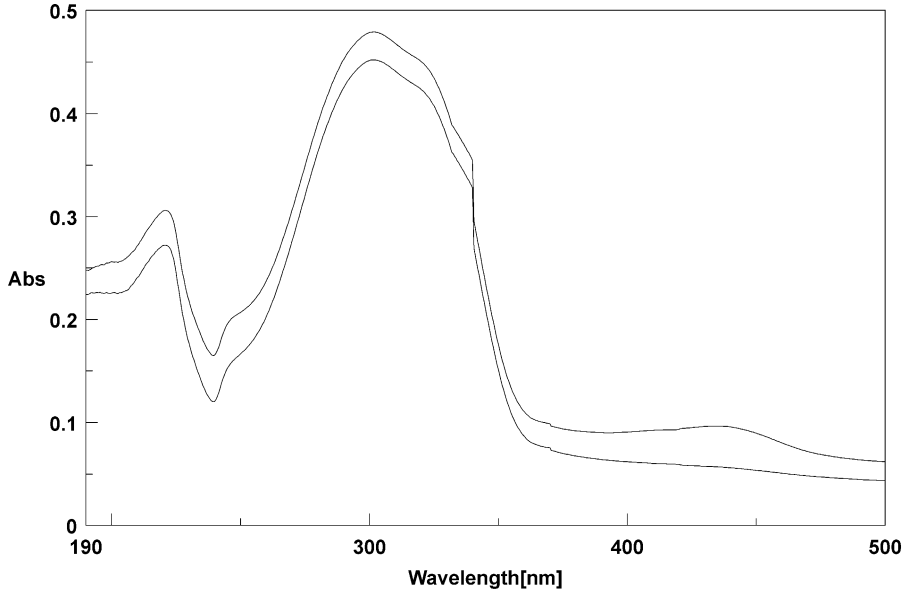


**Figure 5.** The absorption spectroscopic studies on copper chelation by **2d** (50 μM) was carried in 10 mM HEPES buffer, pH 7.2 at 25 °C. The spectra of **2d** were recorded as such, and 5 min after addition of cupric acetate (50 μM), other details are provided in the Experimental.

were found to possess higher metal-dependent nuclease activity amongst the benzene diols. However, it is worth noting that the catechols showed[18b] higher nuclease activity than 1,4-quinols in the presence of the reducing agent NADH, while the activity trend was reversed when NADH was absent. In our system also, the additional reducing agent was not included. Further, the previous study revealed[18b] that the amounts of superoxide radicals produced by the catechols were significantly less than those generated by the 1,4-quinols. These results are in tune with those observed presently. Interestingly, benzene diols without a catechol or partially methylated catechol moiety are well known[18c,d] to cleave DNA, with equal efficiency as the free catechols. Thus, our results are in conformity with all the previous reports, although to the best of our knowledge, we have proposed a new mechanistic rationale to explain the activity.

There is a burgeoning interest in small organic molecules that are capable of binding and cleaving DNA as these can be used in the design and development of new drugs, synthetic restriction enzymes, DNA footprinting agents etc.[19a,b] Although, the redox potentials of a variety of metal ions have been exploited for the development of DNA cleaving agents, the organic compounds possibly play more important roles in this regard, as they provide multitude binding interactions with the target DNA, while ensuring the required electron transfer via their intrinsic chemical, electrochemical and photochemical properties.[20a,b] Considering that compounds that facilitate dsb in DNA are known to possess cytotoxic and anti-tumour properties, the compounds **2b** and **e** appear to be promising anti-cancer compounds. Furthermore, although a large number A–T specific DNA binders are known, designing compounds to recognize G–C sequence is more challenging. To this end, the better G–C specificity shown by these compounds is very significant. Overall, the present study revealed that compounds **2b** and especially **e** can be able substitutes for resveratrol with nuclease property and the compounds might provide unique probes of nucleic acid sequence-dependent molecular recognition.

## 2. Experimental

HEPES buffer (Sigma Chem. Co.), $CuOAc_2$ (Aldrich) were used as received. NBT and bathocuproine were purchased from HiMedia, India. The pBR322 DNA, poly[A–T]$_2$ decamer and poly[G–C]$_2$ decamer were obtained from Bangalore Genei Ltd, India. Calf thymus DNA (CT-DNA, Sigma) was sonicated and phenol-extracted prior to use. The DNA concentrations were detremined using appropriate molar extinction coefficients: $\varepsilon_{260} = 12824\ M^{-1}\ cm^{-1}$ in base pair (bp) for CT-DNA, $\varepsilon_{254} = 8400\ M^{-1}\ cm^{-1}$ in bp for poly[G–C]$_2$, and $\varepsilon_{254} = 6800\ M^{-1}\ cm^{-1}$ in bp for poly[A–T]$_2$.[13a]

### 2.1. DNA nicking assay[21]

The reaction mixture (tolal volume 20 μL) contained $Cu(OAc)_2$ (50 μM) and pBR322 plasmid DNA (200 ng) in 10 mM HEPES buffer pH 7.2. The stock solutions (250 μM) of **2a–g** were prepared in 1% DMF/MilliQ water. The reactions were initiated by addition of the stock solution (4 μL) of the respective stilbenoids so that the final concentration of the test compounds were 50 μM. After addition of the compound, the tubes were incubated at 37 °C for 15 min. The reaction was terminated by addition of the DNA gel loading dye (0.25% bromophenol blue, 50% glycerol and 500 μM EDTA). The samples were loaded in an agarose gel and subjected to electrophoresis at 72 V for 2 h. The gel was removed and stained in ethidium bromide solution (0.5 μg/mL) for 30 min. The DNA was visualized under UV light. The image of the gel was quantified by Bio-rad gel documentation system.

### 2.2. Cu reduction assay[16]

The reaction mixture (total volume 0.4 mL) contained bathocuproine (500 μM) and the stilbenoids (50 μM) in 10 mM HEPES buffer pH 7. The reaction was initiated by adding $Cu(OAc)_2$ (5 μL, 50 μM) in the sample tube and the absorbance of the mixture at 480 nm was read at the end of 5 min. Appropriate blanks, without $Cu(OAc)_2$ were used in each experiment.

### 2.3. Superoxide generation assay

[17] The reaction mixture (total volume 0.5 mL) contained NBT (100 μM) and the stilbenoids (50 μM) in 10 mM HEPES buffer pH 7.2. The reaction was initiated by addition of $CuOAc_2$ (5 μL, 50 μM). The amount of reduced NBT was read spectrophotometrically from the absorbance at 560 nm at the end of 30 min.

### 2.4. DNA binding assay

Apparent equilibrium binding constants (K) for DNA binding by the stilbenoids were determined at 25 °C using a Jasco V-550 UV–Vis spectrophotometer. Wavelength scans and absorbance measurements were made in 1 mL quartz cells of 1-cm path length containing the test compound (50 μM) in 10 mM HEPES buffer pH 7.2. The spectra were recorded after baseline correction in UV spectrophotometer. 2 μL of the compound to be tested was added in to the sample cell from a 10 mM stock solution (in DMF) to achieve a final concentration of 50 μM. 2 μL DMF was added in the reference cells. The DNA samples were added to the samples and the respective spectrum was recorded. With all the samples, the DNA–titrations were carried out using 0.164 μM bp aliquots of CT-DNA and 1.42 μM bp aliquots of poly [A–T]$_2$. For titration with poly [G–C]$_2$, 0.73 μM and 0.24 μM bp aliquots were used with **2b** and **2e** respectively.

**2.4.1. Copper chelation study.** Copper chelation study was carried out by recording the UV-Visible spectra (190-600 nm) of a solution of **2d** (0.5 mL, final concentration 50 μM) in 10 mM HEPES pH 7.2 as such and after adding cupric acetate (final concentration 50 μM) solution followed by incubation for 5 min. The copper chelation was evaluated from the change in absorbance and/ or spectral shift.

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