ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT

Bioorganic & Medicinal Chemistry 14 (2006) 1437–1443

Bioorganic & Medicinal Chemistry

Structural basis for DNA-cleaving activity of resveratrol in the presence of Cu(II)

Kiyoshi Fukuhara,[a,*] Maki Nagakawa,[b] Ikuo Nakanishi,[c,d] Kei Ohkubo,[d] Kohei Imai,[e] Shiro Urano,[e] Shunichi Fukuzumi,[d] Toshihiko Ozawa,[c] Nobuo Ikota,[c] Masataka Mochizuki,[b] Naoki Miyata[f] and Haruhiro Okuda[a]

[a]*Division of Organic Chemistry, National Institute of Health Sciences, 1-18-1 Setagaya-ku, Tokyo 158-8501, Japan*
[b]*Division of Organic and Bioorganic Chemistry, Kyoritsu University of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan*
[c]*Redox Regulation Research Group, Research Center for Radiation Safety, National Institute of Radiological Sciences (NIRS), 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan*
[d]*Department of Material and Life Science, Graduate School of Engineering, Osaka University, SORST, Japan Science and Technology Agency (JST), 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan*
[e]*Department of Applied Chemistry, Shibaura Institute of Technology, Minato-ku, Tokyo 108-8548, Japan*
[f]*Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan*

Received 31 August 2005; revised 27 September 2005; accepted 27 September 2005
Available online 24 October 2005

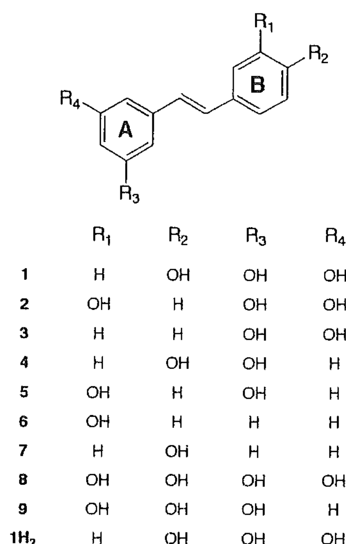
Abstract—Resveratrol (**1**, 3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol found in grapes and other food products, is known as an antioxidant and cancer chemopreventive agent. However, **1** was shown to induce genotoxicity through a high frequency of micronucleus and sister chromatid exchange in vitro and DNA-cleaving activity in the presence of Cu(II). The present study was designed to explore the structure–activity relationship of **1** in DNA strand scission and to characterize the substrate specificity for Cu(II) and DNA binding. When pBR322DNA was incubated with **1** or its analogues differing in the number and positions of hydroxyl groups in the presence of Cu(II), the ability of 4-hydroxystilbene analogues to induce DNA strand scission is much stronger than that of 3-hydroxy analogues. The high binding affinity with both Cu(II) and DNA was also observed by 4-hydroxystilbene analogues. The reduction of Cu(II) which is essential for activation of molecular oxygen proceeded by addition of **1** to the solution of the Cu(II)–DNA complex, while such reduction was not observed with the addition of isoresveratrol, in which the 4-hydroxy group of **1** is changed to the 3-position. The results show that the 4-hydroxystilbene structure of **1** is a major determinant of generation of reactive oxygen species that was responsible for DNA strand scission.
© 2005 Elsevier Ltd. All rights reserved.

1. Introduction

Natural polyphenols, including catechin, epicatechin, quercetin, and resveratrol, are natural antioxidants that are found in a wide range of plant species. Polyphenols inhibit the oxidation of human low-density lipoprotein (LDL),[1] which is responsible for promoting atherogenesis,[2,3] and the intake of foods and beverages that contain polyphenols may protect against atherosclerosis.[4] The polyphenol resveratrol (3,5,4′-trihydroxy-*trans*-stilbene; **1**) is found in grapes, where it serves as a phytoalexin that protects against fungal infection.[5] Although its biosynthesis is not well defined, **1** is thought to be synthesized in response to infection or injury.[6] Resveratrol **1** (Fig. 1) has some therapeutic effects that are due to its antioxidant potential and originate from the inhibition of the oxidation of human LDL and the reduced propensity of human plasma and LDL to undergo lipid peroxidation.[7,8] In addition to its antioxidant potential, it has also been reported to have a variety of anti-inflammatory, anti-platelet, and anti-carcinogenic effects.[9,10] Therefore, due to its high concentration in grape skin, the beneficial effects of the consumption of red wine at reducing the risk of cardiovascular disease have been attributed to the multiple effects of **1**.[11] Recently, **1** was shown to inhibit cellular events associated with

Keywords: Resveratrol; Polyphenol; Antioxidant; DNA oxidative damage.
* Corresponding author. Tel.: +81-3-3700-1141; fax: +81-3-3707-6950; e-mail: fukuhara@nihs.go.jp

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.09.070



	R_1	R_2	R_3	R_4
1	H	OH	OH	OH
2	OH	H	OH	OH
3	H	H	OH	OH
4	H	OH	OH	H
5	OH	H	OH	H
6	OH	H	H	H
7	H	OH	H	H
8	OH	OH	OH	OH
9	OH	OH	OH	H
$1H_2$	H	OH	OH	OH

Figure 1. Structure of resveratrol (1), its analogues 2–9, and dihydroresveratrol ($1H_2$).

tumor initiation, promotion, and progression.[12] Furthermore, it has been reported that 1 has the potential to inhibit DNA polymerase and cyclooxygenase,[13] and also has a direct antiproliferative effect on human breast epithelial cells.[14] Based on its antimutagenic activity,[15,16] it has been suggested that 1 should be effective as a cancer chemopreventive agent in humans.

Meanwhile, 5-alkyl-1,3-dihydroxybenzenes (5-alkylresorcinol) have long been recognized to have potential as therapeutic agents, since natural resorcinols have a wide variety of biological activities, including fungicidal and bactericidal activities against numerous pathogens.[17] Hecht and co-workers were the first to demonstrate that 5-alkylresorcinol induced Cu(II)-dependent DNA strand scission under alkaline pH.[18] This DNA cleavage requires the initial oxygenation of the benzene nucleus, a process that occurs readily at an alkaline pH in the presence of Cu(II) and O_2. The resulting trihydroxylated benzene mediates DNA cleavage in a reaction that depends on the presence of Cu(II) and O_2. Recently, based on the similar structures of 5-alkylresorcinol and resveratrol, we suggested that 1 may be able to mediate Cu(II)-dependent DNA strand scission under neutral conditions.[19] Interestingly, DNA strand scission occurred at neutral pH, indicating that 1 can induce DNA cleavage without the oxygenation of benzene nuclei to the catechol moiety, which is a requisite intermediate in resorcinol-induced DNA cleavage. It has also been shown that DNA cleavage is more likely caused by a copper–peroxide complex as the reactive species rather than by a freely diffusible oxygen species that mediates DNA degradation by resorcinol in the presence of Cu(II). However, instead of the catechol structure, the structural feature of 1 that is effective for DNA cleavage is still unknown. To address this question, the present study was designed to explore the structure–activity relationship of synthesized hydroxystilbene derivatives (Fig. 1) in DNA strand 4 scission and also to characterize the substrate specificity for Cu(II) and DNA binding. The results show that the 4-hydroxy group of 1 is a major determinant of DNA cleaving ability and confirm that the stilbene structure is also important for this ability.

2. Result and discussion

2.1. DNA-cleaving activity

The ability of 1 and its analogues to induce DNA-cleaving activity was examined using pBR322, a supercoiled, covalently closed circular DNA (Form I), and analyzed by agarose gel electrophoresis. Consistent with the fact that Cu(II) is required for potent DNA-cleaving activity of 1, the individual hydroxylated stilbenes induced DNA cleavage only when the reaction was carried out in the presence of Cu(II); in the absence of Cu(II), no DNA cleavage was observed (data not shown). Figure 2 shows the results of the analysis in the presence of Cu(II). Replacement of the internal double bond in the stilbene moiety by a saturated one ($1H_2$) resulted in a marked reduction in potency, suggesting that the structure of stilbene is important for mediating DNA relaxation. In a series of hydroxylated stilbene analogues, Cu(II)-dependent DNA-cleaving activity was greatly affected by the number and positions of hydroxyl groups attached to the stilbene structure. For a given structural series (i.e., all 4-OH analogues: 1, 4, 7; 3-OH analogues: 2, 3, 5, 6; or 3,4-(OH) analogues: 8, 9), the DNA-cleaving activity seemed to increase with an increase in the number of hydroxyl groups. Densitometric analysis of agarose gel indicated that the DNA-cleaving ability of 1 resulted not only in the complete conversion of substrate DNA (Form I) into open circular DNA (Form II) but also the further conversion of Form II into linear

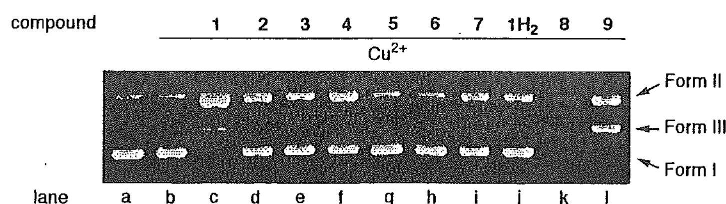


Figure 2. Gel electrophoretic analysis of single- and double-strand breaks generated in pBR322DNA with 1 or its analogues in the presence of Cu(II). Assays were performed in 50 mM sodium cacodylate buffer, pH 7.2, containing 45 μbp pBR322DNA and 10 μM of samples (lane c–l) in the presence of 10 μM Cu(II) (lane b–l), for 1 h at 37 °C. The samples in lane c–l are 1–9, and $1H_2$, respectively.

DNA (Form III) in 8% yield. Compounds **8** and **9** were much more efficient at mediating DNA relaxation than **1**; especially, **8** induced the complete degradation of linear DNA (Form III) as indicated by the smearing of the band. The high potency of **8** and **9** can be attributed to their *ortho*-hydroquinone (catechol) structure, which logically improves their ability to cleave DNA by facilitating the generation of oxygen radicals through the formation of *ortho*-hydroquinone–Cu(II)–O_2 complex. Of particular interest is the difference in the potency between **1** and its 3-hydroxy isomer **2**. A change in the placement of the 4-hydroxy group of **1** to the 3-position resulted in a significant decrease in its ability to cleave DNA. Similar differences were noted in comparing the dihydroxy (**4** vs **5**) and monohydroxy (**6** vs **7**) stilbenes, which is consistent with the suggestion that the 4-hydroxy group is essential for effecting DNA cleavage. Since oxygen radical is believed to be the active species responsible for Cu(II)-dependent DNA cleavage, the 4-hydroxy group in combination of O_2 and Cu(II) may serve to facilitate the generation of oxygen radical. However, **3** was found to be quite efficient at mediating DNA relaxation, which suggests that the 3,5-dihydroxybenzene structure, which is distinct from 3-hydroxybenzene, is also essential for potentiating DNA strand scission.

2.2. Cu(II)-binding ability

It has been shown that several xenobiotics that contain a catechol moiety undergo Cu(II)-mediated oxidation to form reactive oxygen species (ROS) that are capable of causing DNA strand breaks. Sotomatsu et al. demonstrated that Cu(II) and Fe(III) had affinity for the hydroquinone moiety of 3,4-dihydroxyphenylalanine (dopa) and, after coordinating with dopa, promoted the peroxidative cleavage of unsaturated phospholipids.[20] Since **1** can coordinate Cu(II), its binding ability has been observed as a change in UV absorption spectra using a Cu(II) titration experiment, whereas no such binding has been observed in the case of Fe(III).[8] Considering the unique specificity of Cu(II), which may induce DNA strand scission of **1**, the ability of **1** to bind to Cu(II) might be advantageous for generating ROS and inducing Cu(II)-dependent DNA scission. Therefore, to elucidate the structural component of **1** that is responsible for its Cu(II)-binding ability, the UV spectra of **1** and its analogues (**2**–**7** and **1H₂**) in various concentrations of Cu(II) were observed, and their Cu(II)-binding abilities were compared. As shown in Figure 3, the incremental addition of Cu(II) to 20 μM of **1** resulted in a blueshift of the peak from 220 to 210 nm with a concomitant increase in absorbance and a decrease in the absorbance at 308 nm, consistent with the binding of **1** with Cu(II). This spectral change reflects a 1:1 stoichiometry for the complex between **1** and Cu(II), and the binding constant was determined to be $1.75 \times 10^7\ M^{-1}$. Figure 4 shows the effect of the Cu(II) concentration on the absorbance of **1** and its analogues in the range 270–330 nm. A decrease in absorbance, similar to that of **1** at 308 nm, was observed for 3,4- and 4-hydroxy analogues of **4** and **7** at 324 and 304 nm, respectively, indicating that the change is due to its coordination with

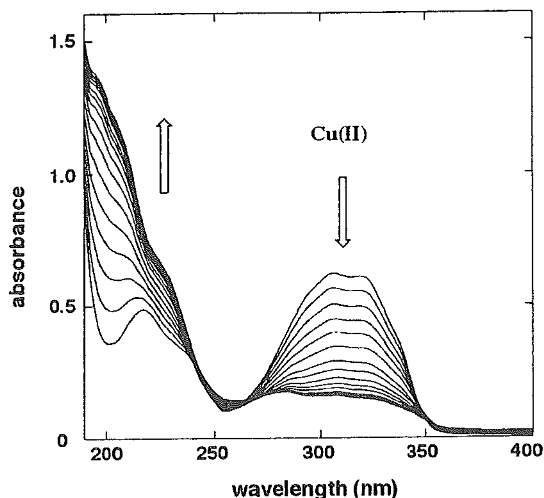


Figure 3. Spectral changes observed upon addition of $CuCl_2$ (0–30 μM) to a sodium cacodylate buffer (pH 7.1)/CH_3CN mixed solution of **1** (20 μM).

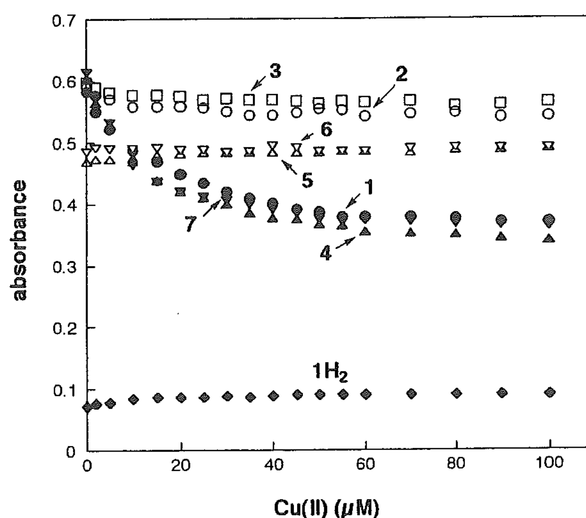


Figure 4. Changes in the absorbance (λ_{max}) of **1** and its analogues (20 μM) upon the addition of $CuCl_2$ (0–100 μM).

Cu(II). In contrast, with an increase in the concentration of Cu(II), there was little or no effect on the absorbance of **2**, **3**, **5**, and **6**, which lacked a 4-hydroxy group on the stilbene moiety, at 306, 300, 298, and 298 nm, respectively, suggesting that ligation of these compounds to Cu(II) did not occur. Further, only a slight effect was observed for dihydroresveratrol **1H₂**. These results constitute strong evidence that the 4-hydroxy group of **1** is essential for Cu(II) coordination and the ability of the 4-hydroxy group to bind with Cu(II) depends on the structure of stilbene.

2.3. DNA-binding ability

Since **1** is capable of binding to DNA,[19] ROS produced by **1** in combination with Cu(II) might be effective at mediating DNA relaxation. Therefore, to

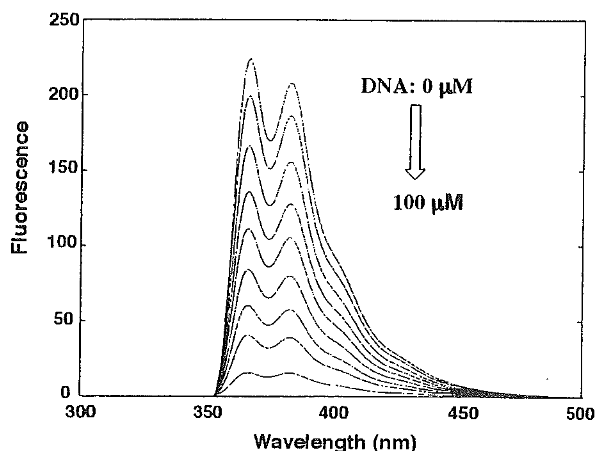


Figure 5. Effect of calf thymus DNA on the fluorescence emission (excitation wavelength 260 nm) of **1**. Trace 1 is the emission spectrum of **1** alone (20 μM); traces 2–9 are emission spectra of **1** in the presence of 2, 5, 10, 15, 20, 30, 50, and 100 μM DNA bp, respectively.

characterize the interaction of **1** with DNA, the ability of **1** and its analogues to bind DNA was estimated by fluorescence titration. The fluorescence emission spectra of **1** in the presence of calf thymus DNA are shown in Figure 5. As indicated, the addition of DNA to **1** causes a decrease in fluorescence emission, and, without any modification of the spectral shape, a decrease in the degree of fluorescence is seen with an increasing concentration of DNA, suggesting that **1** binds to duplex DNA not via groove binding but rather through significant intercalation. In fact, denatured DNA does not appreciably quench the fluorescence of **1** (data not shown). Stern–Volmer plots of the quenching of the fluorescence of **1** and its analogues (**2**–**7** and **1H$_2$**) by calf thymus DNA are shown in Figure 6. Native DNA quenches the fluorescence of **1** five times more efficiently than it quenches **1H$_2$**,

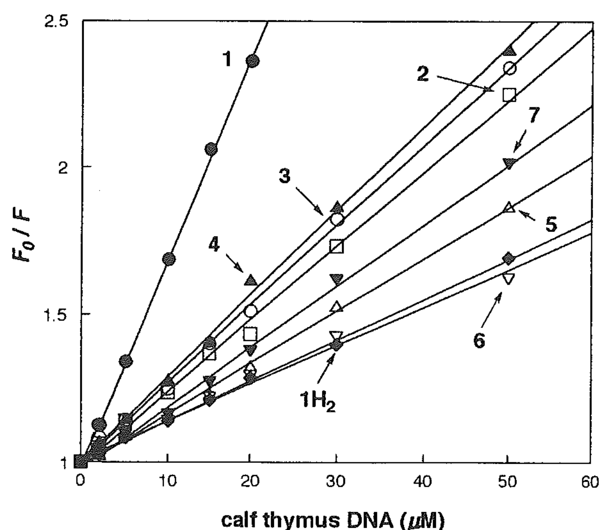


Figure 6. Stern–Volmer plot of quenching of the fluorescence of **1** and its analogues by calf thymus DNA.

indicating that the planarity of the stilbene structure is effective for binding with the duplex DNA structure, probably by taking advantage of its stacking against the base pair. Figure 6 also indicates that phenolic hydroxyl groups attached to the stilbene structure greatly affect the DNA-binding affinity. An increase in the number of hydroxyl groups tends to increase the DNA-binding affinity, which is consistent with the suggestion that the number of phenolic hydroxyl groups is important for its DNA-binding affinity. However, the binding affinity is also determined by the combination of the number and localization of phenolic hydroxyl groups. Thus, the fluorescence of isoresveratrol **2**, in which the 4-hydroxy group of resveratrol **1** is changed to the 3-position, was quenched by DNA with low efficiency ($K_{sv} = 2.40 \times 10^4$ M^{-1}) compared to **1** ($K_{sv} = 6.80 \times 10^4$ M^{-1}), and the same results were also observed with dihydroxyl (**4** vs **5**) and monohydroxyl (**7** vs **6**) stilbenes, suggesting that the 4-hydroxy group may be the essential component for binding DNA and plays an important role in specific hydrogen bond interactions with DNA.

2.4. ESR analysis

To confirm the electrostatic interaction of hydroxylated stilbenes with both Cu(II) and DNA, ESR signals of Cu(II) were observed in the presence of **1** or its analogues together with calf thymus DNA. Once the ternary complex of Cu(II)-**1**-DNA, which is due to the efficient binding affinities of **1** with both Cu(II) and DNA, is formed, the complex may result due to its high DNA-cleaving ability. Figure 7 shows that an ESR signal of Cu(II) became multiple upon the addition of DNA, consistent with the fact that Cu(II) complexes DNA. In fact, the decrease in the peak height of Cu(II) in a solution of calf thymus DNA is due to the intercalation of Cu(II) with a large molecule of DNA, which limits the mobility of Cu(II). When **1** was added to the solution of Cu(II)–DNA complex, the peak height of the ESR signal was reduced to one-half of that of the Cu(II)–DNA complex and the resonance was weakened, suggesting that **1** was bound to Cu(II)–DNA complex and thus induced the reduction of Cu(II), which was converted to an ESR-silent species, very likely Cu(I). If the binding of Cu(II) to DNA decreases with the addition of **1**, the signal of Cu(II) should increase to the height of unbound Cu(II). An increase in peak height was also not observed for other resveratrol analogues, suggesting that Cu(II) remains in a complex with DNA even after the addition of **1** and its analogues. Compared to the reduction of Cu(II) to Cu(I) by **1**, an efficient reduction of the peak height of Cu(II)–DNA complex was not observed with the addition of isoresveratrol **2**. It is possible that the insufficient binding affinity of **2** with both DNA and Cu(II) may impair the highly efficient reduction of Cu(II) to Cu(I). Similarly, **7** affected the spectra of Cu(II)–DNA with efficient reducing and broadening, whereas there was no effect on the spectra of Cu(II)–DNA upon the addition of **6**, indicating that the reductive activation of Cu(II) is accelerated by electrostatic interaction of a hydroxyl group at the 4-position.

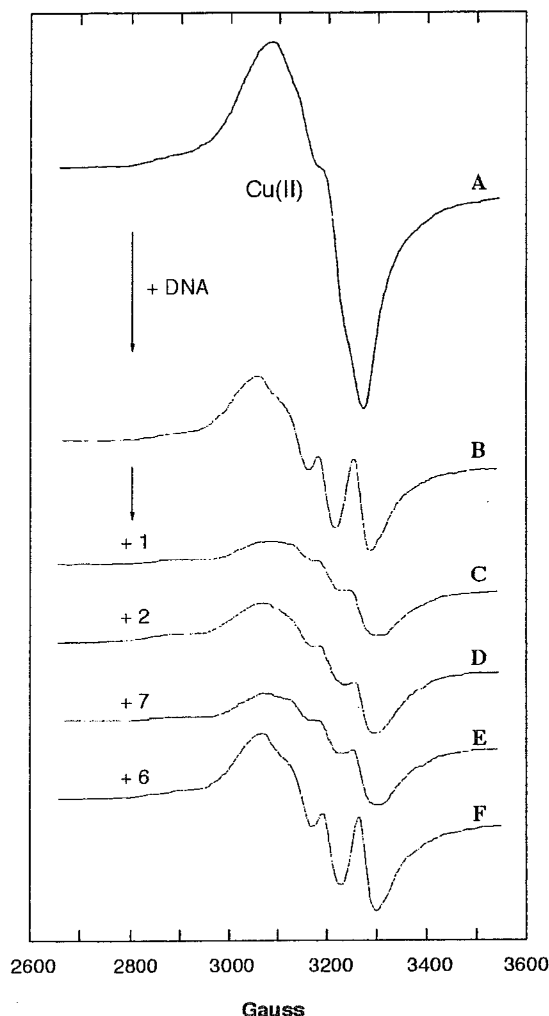


Figure 7. Effect of 1 and its analogues on the ESR spectra of Cu(II) in the presence of calf thymus DNA. Spectra A is 1 mM $CuCl_2$ and spectra B–F show after the addition of 2 mM NP of calf thymus DNA in the absence (B) or presence of 1 mM chemicals (C: **1**; D: **2**; E: **7**; F: **6**). All spectra were recorded after incubation for 30 min at room temperature.

3. Conclusion

In general, polyphenols, which are responsible for reactive oxygen-associated toxicity, appear to play an important role in the reductive activation of molecular oxygen by its autooxidation, which, in most cases, is coupled with the formation of redox active *ortho*- or *para*-quinones. Catechol is the typical polyphenol that is essential for generating oxygen radical in the presence of Cu(II).[21] It is formed as an activated metabolite of polycyclic aromatic hydrocarbons, which are known to be ubiquitous environmental pollutants. Although **1** is a polyphenol that is known to be an antioxidant and a potential cancer chemopreventive agent, it cleaved DNA strongly without oxidative transformation to the catechol structure in the presence of Cu(II). The DNA cleavage is attributed to the generation of copper-peroxide complex that is formed by electron transfer from **1** to molecular oxygen.[19] The oxidative product of **1** is a

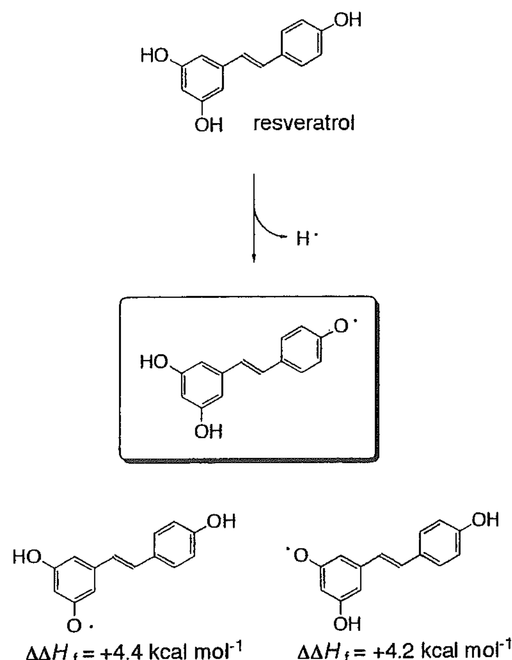


Figure 8. Relative energy values ($\Delta\Delta H_f$) of three types of resveratrol radicals calculated by the DFT calculation, B3LYP/6-31G* basis set.

dimer[22] and formation of the catechol structure has not been reported. Therefore, the oxidative dimer might be formed by dimerization of resveratrol radical as a result of the reductive activation of molecular oxygen. In the present work, the number and positions of the hydroxyl groups in the stilbene structure were associated with DNA-cleaving activity and the 4-hydroxy group of stilbene played an especially critical role in DNA cleavage. The high binding affinity of a hydroxyl group at the 4-position with both Cu(II) and DNA makes it possible to form a ternary complex and therefore cleave DNA efficiently. When the heats of formation of three types of resveratrol radicals are compared, as shown in Figure 8, the 4-oxyl radical of **1** is the most stable, indicating that the hydroxyl group at the 4-position is much more subjected to oxidation than other hydroxyl groups. In fact, the efficient reduction of Cu(II) to Cu(I) was seen with **1**, which has a hydroxyl group at the 4-position, while there was no effect on Cu(II) reduction in the presence of **2** in which the 4-hydroxy group in **1** is moved to the 3-position. The decrease in the DNA-cleaving ability of $\mathbf{1H_2}$ compared to that of **1** also indicated the importance of the stilbene structure, which might be effective not only for DNA binding for the planarity of the overall structure but also for the stability of the 4-oxyl radical. These results suggest that the ability of **1** to induce oxidative DNA damage in the presence of Cu(II) can be attributed to the structure of 4-hydroxystilbene which is comparable to that of catechol.

Estrogens have been reported to cause cancer through a genotoxic effect. The genotoxicity of estrogens is attributed to the accumulation of potentially carcinogenic metabolites and almost all of these are the catechol form of estrogens. Catechol estrogen metabolites are capable

of causing chromosomal aberrations and gene mutations in cultured cells. The oxidative DNA damage and/or alkylation of DNA that is responsible for the risk of developing cancer are also induced by catechol estrogen metabolites. In fact, the catechol structure, which can cause genotoxicity, is capable of inducing DNA strand scission and the oxidation of DNA bases in the presence of Cu(II). Recently, we reported the genotoxicity of **1**, which induced micronucleus, sister chromatid exchange, and S phase arrest.[23] Among the many types of hydroxylated stilbenes, 4-hydroxystilbene most effectively caused genotoxic effects. Therefore, the finding that the 4-hydroxylstilbene structure is responsible for various biological activities, especially DNA damage leading to genotoxicity, might be important for understanding the toxicity of polyphenols that do not have a catechol structure.

4. Experimental

4.1. Materials

Resveratrol **1** and calf thymus DNA were purchased from Sigma (St. Louis, MO). Supercoiled plasmid pBR322DNA was purchased from Nippon Gene (Tokyo, Japan). Analogues of **1**; 3,5,3′-trihydroxy- (**2**), 3,5-dihydroxy- (**3**), 3,4′-dihydroxy- (**4**), 3,3′-dihydroxy- (**5**), 3-hydroxy- (**6**), 4-hydroxy (**7**), 3,4,3′,5′-tetrahydroxy- (**8**), and 3,4,3′-trihydroxy-*trans*-stilbene (**9**), as shown in Figure 1, were synthesized as previously reported.[24] Saturated form of **1** (**1H$_2$**) was synthesized by hydrogenation of **1** using 10% Pd/C as catalyst. Yield: 98%. ^{1}H NMR(acetone-d_6): δ 2.73 (m, 4H), 6.19 (d, 1H, $J = 2.0$ Hz), 6.22 (d, 1H, $J = 2.0$ Hz), 6.74 (d, 2H, $J = 8.4$ Hz), 7.03 (d, 2H, $J = 8.4$ Hz). All other chemicals and solvents were of reagent grade or better.

4.2. DNA-cleaving activity

DNA strand breakage was measured in terms of the conversion of supercoiled pBR322 plasmid DNA to the open circular and linear forms. Reactions were carried out in 20 μL (total volume) of 50 mM Na cacodylate buffer (2.5% DMF), pH 7.2, containing 45 μM bp pBR322 DNA, 10 μM $CuCl_2$, and 100 μM of each stilbene derivative. The reaction mixtures were incubated at 37 °C for 1 h and then treated with 5 μL of loading buffer (100 mM TBE buffer, pH 8.3, containing 30% glycerol, 0.1% bromophenol blue) and applied to 1% agarose gel. Horizontal gel electrophoresis was carried out in 50 mM TBE buffer, pH 8.3. The gels were stained with ethidium bromide (1 μg mL^{-1}) for 30 min, destained in water for 30 min, and photographed with UV translumination.

4.3. UV–visible spectra measurements

UV-visible spectra were measured at 37 °C with a Hewlett Packard 8452A Diode Array Spectrophotometer. A solution in a final volume of 1 mL consisted of 20 μM of sample and 0–100 μM $CuCl_2$ in sodium cacodylate buffer (pH 7.1)/acetonitrile mixed solvent (1:1 v/v) was prepared and subjected to spectral analysis. The binding constant between **1** and Cu(II) was obtained according to the method described by Itoh et al.[25]

4.4. Fluorescence measurements

Fluorescence excitation and emission spectra were recorded on a Shimadzu RF-5300PC. A solution in a final volume of 1 mL, which consisted of 20 μM of sample and 0–100 μM calf thymus DNA in 10 mM sodium cacodylate buffer (pH 7.1) and DMF (10% by volume), was used for fluorescence-quenching experiments. The excitation wavelengths used were 255 nm for **1**, **3**, **6**, and **7**, and 260 nm for **2**, **4**, **5**, and **1H$_2$**, and emissions were recorded in the range of 300–500 nm. For all experiments, the sample temperature was maintained at 37 °C. The quenching data were analyzed by the Stern–Volmer equation:[26]

$$F_0/F = 1 + K_{sv}[Q],$$

where [Q] is the molar concentration of the calf thymus DNA, F_0 and F are the fluorescence intensities in the absence and in the presence of the calf thymus DNA [Q], respectively, and K_{sv} is the Stern–Volmer quenching constant.

4.5. ESR analysis

ESR spectra were recorded at room temperature on a JES-FE 2XG spectrometer (JEOL Co. Ltd., Tokyo, Japan). The sample containing 1 mM $CuCl_2$, 2 mM NP of calf thymus DNA, and 1 mM of chemical in 50 mM phosphate buffer (pH 7.2) and acetonitrile (5% by volume) was introduced into a quartz flat cell and incubated at room temperature for 30 min. The ESR spectrum was then recorded. The spectrometer settings were modulation frequency, 100 kHz; modulation amplitude, 10 G; and microwave power, 16 mW.

4.6. Theoretical calculations

Density functional calculations were performed with Gaussian03 (Revision C.02, Gaussian, Inc.) using the unrestricted B3LYP functional for the open shell molecule on an 8-processor QuantumCube^{TM} developed by Parallel Quantum Solutions.

Acknowledgments

This work was supported partly by a grant (MF-16) from the Organization for Pharmaceutical Safety and Research of Japan, a grant from the Ministry of Health, Labour and Welfare, a Grant-in-Aid for Research of Health Sciences focusing on Drug Innovation (KH51058) from the Japan Health Sciences Foundation, and partly by Grants-in-Aid for Scientific Research (B) (No. 17390033) and for Young Scientist (B) (No. 17790044) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and notes

1. Hirano, R.; Sasamoto, W.; Matsumoto, A.; Itakura, H.; Igarashi, O.; Kondo, K. *J. Nutr. Sci. Vitaminol. (Tokyo)* **2001**, *47*, 357.
2. Witztum, J. L.; Steinberg, D. *J. Clin. Invest.* **1991**, *88*, 1785.
3. Steinberg, D. *J. Biol. Chem.* **1997**, *272*, 20963.
4. Hayek, T.; Fuhrman, B.; Vaya, J.; Rosenblat, M.; Belinky, P.; Coleman, R.; Elis, A.; Aviram, M. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 2744.
5. Jeandet, P.; Bessis, R.; Gautheron, B. *Am. J. Enol. Vitic.* **1991**, *42*, 41.
6. Langcake, P.; Pryce, R. *J. Physiol. Plant Pathol.* **1976**, *9*, 77.
7. Frankel, E. N.; Waterhouse, A. L.; Kinsella, J. E. *Lancet* **1993**, *341*, 1103.
8. Belguendouz, L.; Fremont, L.; Linard, A. *Biochem. Pharmacol.* **1997**, *53*, 1347.
9. Jang, M.; Pezzuto, J. M. *Drugs Exp. Clin. Res.* **1999**, *25*, 65.
10. Olas, B.; Wachowicz, B.; Stochmal, A.; Oleszek, W. *Platelets* **2002**, *13*, 167.
11. Wu, J. M.; Wang, Z. R.; Hsieh, T. C.; Bruder, K. L.; Zou, J. G.; Huang, Y. Z. *Int. J. Mol. Med.* **2001**, *8*, 3.
12. Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. *Science* **1997**, *275*, 218.
13. Jang, M.; Pezzuto, J. M. *Drug Exp. Clin. Res.* **1999**, *25*, 65.
14. Mgbonyebi, O. P.; Russo, J.; Russo, I. H. *Int. J. Oncol.* **1998**, *12*, 865.
15. Uenobe, F.; Nakamura, S.; Miyazawa, M. *Mutat. Res.* **1997**, *373*, 197.
16. Kim, H. J.; Chang, E. J.; Bae, S. J.; Shim, S. M.; Park, H. D.; Rhee, C. H.; Park, J. H.; Choi, S. W. *Arch. Pharm. Res.* **2002**, *25*, 293.
17. Lindenberg, B. A.; Massin, M. J. *J. Physiol. (Paris)* **1957**, *49*, 285.
18. Scannell, R. T.; Barr, J. R.; Murty, V. S.; Reddy, K. S.; Hecht, S. M. *J. Am. Chem. Soc.* **1988**, *110*, 3650.
19. Fukuhara, K.; Miyata, N. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3187.
20. Sotomatsu, A.; Nakano, M.; Hirai, S. *Arch. Biochem. Biophys.* **1990**, *283*, 334.
21. Seike, K.; Murata, M.; Oikawa, S.; Hiraku, Y.; Hirakawa, K.; Kawanishi, S. *Chem. Res. Toxicol.* **2003**, *16*, 1470.
22. Wang, M.; Jin, Y.; Ho, C.-T. *J. Agric. Food Chem.* **1999**, *47*, 3974.
23. Matsuoka, A.; Takeshita, K.; Furuta, A.; Ozaki, M.; Fukuhara, K.; Miyata, N. *Mutat. Res.* **2002**, *521*, 29.
24. Thakkar, K.; Geahlen, R. L.; Cushman, M. *J. Med. Chem.* **1993**, *36*, 2950.
25. Itoh, S.; Taniguchi, M.; Takada, N.; Nagatomo, S.; Kitagawa, T.; Fukuzumi, S. *J. Am. Chem. Soc.* **2000**, *122*, 12087.
26. Barcelo, F.; Barcelo, I.; Gavilanes, F.; Ferragut, J. A.; Yanovich, S.; Gonzalez-Ros, J. M. *Biochim. Biophys. Acta* **1986**, *884*, 172.