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Synthesis and antioxidant activity of hydroxylated phenanthrenes as *cis*-restricted resveratrol analogues

De-Jun Ding, Xiao-Yan Cao, Fang Dai, Xiu-Zhuang Li, Guo-Yun Liu, Dong Lin, Xing Fu, Xiao-Ling Jin, Bo Zhou *

State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou, Gansu 730000, China

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ABSTRACT

Five hydroxylated phenanthrenes as "*cis*-configuration-fixed" resveratrol analogues differing in the number and position of the hydroxyl groups were designed and synthesized. Their antioxidant activity was studied by ferric reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging, and DNA strand breakage-inhibiting assays, corresponding to their electron-donating, hydrogen-transfer and DNA-protecting abilities, respectively. In the above assays, their activity depends significantly on the number and position of the hydroxyl groups, and most of them are more effective than resveratrol. Noticeably, compound **9b** (2,4,6-trihydroxyl phenanthrene) with the same hydroxyl group substitutions as resveratrol, is superior to the reference compound, highlighting the importance of extension of the conjugation over multiple aromatic-rings. Similar activity sequences were obtained in different experimental models, but the appreciable differences could contribute detailed insights into antioxidant mechanisms. Based on these results, the hydroxylated phenanthrenes may be considered as a novel type of resveratrol-directed antioxidants.

1. Introduction

Converging evidence indicates excessive production of reactive oxygen species (ROS) have been considered responsible for a variety of diseases and pathophysiological events, including inflammation, cancer and neurodegenerative disorders (Ames, Shigenaga, & Hagen, 1993; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Consequently, there is considerable potential for the applications of antioxidant in prevention or control of these conditions. Efforts to discover potent antioxidants from the vast reserves of native components as useful drug candidates are continuously going on. Among these naturally-occurring antioxidants, resveratrol (3,5,4′-trihydroxy-stilbene), a triphenolic phytoalexin found in grapes and other food products (Burns, Yokota, Ashihara, Lean, & Crozier, 2002), has received widespread attention. This molecule has been proposed to be active in cancer prevention, cardiovascular protection, anti-ageing, and so on (de la Lastra & Villegas, 2005; Howitz et al., 2003; Jang et al., 1997; Smoliga, Baur, & Hausenblas, 2011).

Considerable efforts have devoted to study antioxidant activity of resveratrol (Amorati et al., 2004; Cao et al., 2003; Caruso, Tanski, Villegas-Estrada, & Rossi, 2004; Fukuhara et al., 2008; Murias et al., 2005; Queiroz, Gomes, Moraes, & Borges, 2009; Stojanović & Brede, 2002; Stojanović, Sprinz, & Brede, 2001; Wang, Jin, & Ho, 1999), and have established that the 4′-OH in the stilbene scaffold is responsible for the activity (Cao et al., 2003; Caruso et al., 2004; Leopoldini, Russo, & Toscano, 2011; Queiroz et al., 2009; Stojanović & Brede, 2002; Stojanović et al., 2001; Wang et al., 1999). The structural simplicity of this molecule has prompted interest in designing novel analogues with improved antioxidant potency (Amorati et al., 2004; Fukuhara et al., 2008; Murias et al., 2005). In this connection, we have also demonstrated that the introduction of electron-donating groups at positions *ortho* and *para* to the 4-OH or 4′-OH group in the stilbene scaffold (Fang & Zhou, 2008; Shang et al., 2009), construction of the hydroxylated stilbene-chroman hybrids (Yang et al., 2010), the insertion of additional double bonds between two aromatic rings (Tang et al., 2011) and the substitution of 4-SH for 4-OH in the stilbene scaffold (Cao et al., 2012) could effectively improve antioxidant activity of this parent molecule.

Resveratrol presents two isomeric forms (*trans* and *cis*). Although *cis*-resveratrol was proved to be less efficient as an antioxidant than *trans*-resveratrol (Belguendouz, Fremont, & Linard, 1997; Mikulski, Górniak, & Molski, 2010), many other beneficial properties of the former and the related *cis*-stilbenes were also reported (Leiro et al., 2005; Pettit et al., 2002; Siemann, Chaplin, & Walicke, 2009). For example, Leiro's studies (Leiro et al., 2005) showed *cis*-resveratrol has anti-inflammatory properties through a significant modulatory effect on the nuclear factor κB related genes. In a structure-activity relationship study of the potential

* Corresponding author. Fax: +86 931 8915557.
E-mail address: bozhou@lzu.edu.cn (B. Zhou).

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antineoplastic activity of various stilbene derivatives, Pettit et al. (2002) likewise reported that the *cis*-stilbenes exhibit significant inhibitory effects on a number of cancer cell lines. Of the *cis*-stilbenes, combretastatin A4 stands out as the molecule with the most potential in cancer therapy, and has now entered clinical trials for both solid and liquid tumours (Siemann et al., 2009).

Nonetheless, the *cis*-resveratrol is unstable, and during storage and on the condition of low pH, it can be easily isomerized into *trans*-resveratrol, a sterically more stable form (Trela & Waterhouse, 1996). Considering the above remarks, our interest is to design resveratrol-based derivatives by introducing a single bond linkage to "lock" the rotation of olefinic bridge and construct a phenanthrene ring (Scheme 1). This strategy not only maintains the *cis*-configuration, but also extends the conjugation over multiple aromatic-rings, resulting in the high resonance stabilization of the phenoxyl radical formed in antioxidant reaction of the hydroxylated phenanthrenes. Therefore, it should be a promising strategy to improve antioxidant activity of resveratrol analogues. Moreover, numerous phenanthrene-containing plants have been used in traditional medicine and phenanthrenes have been identified as the active constituents from phytochemical–pharmacological investigations (Kovács, Vasas, & Hohmann, 2008). For example, moscatin (4-methoxyphenanthrene-2,5-diol) obtained from *Bulbophyllum odoratissimum*, was demonstrated to induce cell death in several cancer cell lines (Chen et al., 2008). However, research on their antioxidant and structure-activity relationships (SAR) is still rare, and we believe they merit further investigation.

Herein, we report the designed synthesis of five hydroxylated phenanthrene derivatives (Scheme 1) differing in the number and position of the hydroxyl groups, aiming at finding more effective antioxidants than resveratrol. Of the compounds, **9a** had a similar hydroxyl position to 4- or 4′-OH on the phenyl rings of the stilbene, and **9b** retained the same hydroxyl group substitutions as resveratrol, while **9c**, **9d** and **8e** contained *ortho*-hydroxy-methoxy groups and *ortho*-dihydroxyl groups based on the consideration that the introduction of electron-donating groups such as methoxy and hydroxyl in the *ortho* position of phenolic hydroxyl group, helps increase its antioxidant activity (Wright, Johnson, & DiLabio, 2001). In view of the fact that antioxidant activity of *cis*-resveratrol is relatively weak compared with that of *trans*-resveratrol (Belguendouz et al., 1997; Mikulski et al., 2010), we used *trans*-resveratrol as a reference compound in the following antioxidant activity assays.

2. Materials and methods

2.1. General experimental procedures

The melting points were measured with an SGWX-4 binocular microscope melting-point apparatus (Shanghai Precision & Scientific Instrument Co. Ltd., Shanghai, China) and were not corrected by the standard sample. ^{1}H NMR spectra and ^{13}C NMR were recorded by using a Bruker AV 400 (400 MHz) spectrometer with $CDCl_3$, CD_3OD or CD_3COCD_3 as a solvent. Chemical shifts (δ) are reported in parts per million (ppm) using the solvent peak. Mass spectra were recorded on a Bruker Daltonics Esquire 6000 spectrometer (ESI-MS) or a VG ZAB-HS spectrometer (EI-MS).

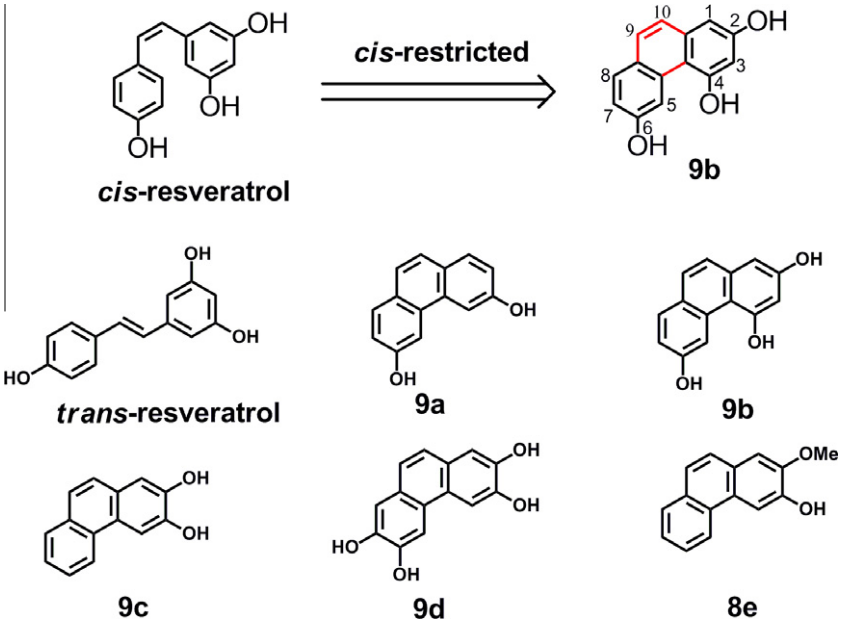
2.2. Materials

2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH·), pBR322 DNA, 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH), were purchased from Sigma–Aldrich. 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) was from Alfa Aesar. Other chemicals used were of analytical grade. All anhydrous solvents were dried and purified by standard techniques just before use. Starting materials **3**–**6** (Cui & Wang, 2009) and **7** (Tron, Pagliai, Del Grosso, Genazzani, & Sorba, 2005) were synthesized according to the available procedures.

2.3. Synthesis

2.3.1. General procedure for the synthesis of *8c*–*8e*

An appropriate amount of **6c**–**6e**, (5.56 mmol) was added to powdered copper (28.8 mmol) in 20 ml quinoline, and the resulting mixture was heated at 200 °C for 2 h. Upon cooling, acetic ether was added, and the copper was filtered off through celite. The filtrate was washed with 1 M hydrochloric acid, and the aqueous layer was separated and extracted with acetic ether. The combined



Scheme 1. Molecular structures of the hydroxylated phenanthrenes and resveratrol.

organic layers were washed in turn with saturated aqueous sodium carbonate, water, brine, dried over $MgSO_4$. The solvent was evaporated and the resultant solid was purified by silica gel chromatography (petroleum/EtOAc, 8:1) to give pure compounds.

*2.3.1.1. 2,3-Dimethoxy phenanthrene (**8c**).* A canary yellow solid; m.p. 129–130 °C; yield: 52%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.53 (d, J = 8.0 Hz, 1H, H5), 8.00 (s, 1H, H4), 7.87 (d, J = 8.0 Hz, 1H, H8), 7.61 (t, J = 8.0 Hz, 3H, H6, H7, H9), 7.52 (t, J = 8.0 Hz, 1H, H10), 7.22 (s, 1H, H1), 4.10 (s, 3H, –OCH_3), 4.03 (s, 3H, –OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 149.3, 149.2, 131.3, 129.7, 128.6, 127.1, 126.1, 125.9, 125.5, 125.2, 124.8, 122.1, 108.3, 103.1, 56.0, 55.9; MS (EI): (m/z) = 238 [M^+].

*2.3.1.2. 2,3,6,7-Tetramethoxy phenanthrene (**8d**).* A white solid; m.p. 179–181 °C; yield: 65%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (s, 2H, H4, H5), 7.53 (s, 2H, H9, H10), 7.19 (s, 2H, H1, H8), 4.10 (s, 6H, –OCH_3), 4.02 (s, 6H, –OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 149.1, 148.7, 126.3, 124.3, 124.2, 108.3, 102.7, 56.0, 55.8; MS (EI): (m/z) = 298 [M^+].

*2.3.1.3. 2-Methoxy-3-hydroxy phenanthrene (**8e**).* A canary yellow solid; m.p. 145–146 °C; yield: 45%; ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 8.60 (d, J = 8.0 Hz, 1H, H5), 8.14 (s, 1H, H4), 8.08 (s, 1H, OH), 7.89 (d, J = 8.0 Hz, 1H, H8), 7.71 (d, J = 8.0 Hz, 1H, H9), 7.63 (d, J = 8.0 Hz, 1H, H10), 7.60 (t, J = 8.0 Hz, 1H, H6), 7.52 (t, J = 8.0 Hz, 1H, H7), 7.44 (s, 1H, H1), 4.02 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 149.4, 149.2, 132.5, 130.8, 129.4, 127.8, 127.3, 127.1, 126.5, 125.3, 123.5, 109.4, 107.9, 56.4; MS (EI): (m/z) = 224 [M^+].

*2.3.2. General procedure for the synthesis of **8a**, **8b***

Anhydrous N_2 was passed through a solution containing stilbene **7a**, **7b** (0.16 mmol), iodine (0.02 g, 0.16 mmol), propylene oxide (6.3 ml, 90 mmol) and cyclohexane (500 ml) for 20 min. The solution was irradiated using a 500 W Hg lamp for approximately 3 d until the reaction was completed. The solution was washed with 15% aqueous $Na_2S_2O_3$ (50 ml), H_2O (50 ml) and brine (50 ml). The organic extract was dried ($MgSO_4$) and evaporated. Silica chromatography (petroleum/EtOAc, 10:1) provided the desired products.

*2.3.2.1. 3,6-Dimethoxy phenanthrene (**8a**).* A white solid; m.p. 104–105 °C; yield: 46%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.95 (d, J = 2.4 Hz, 2H, H4, H5), 7.80 (d, J = 8.8 Hz, 2H, H8, H1), 7.55 (s, 2H, H9, H10), 7.25 (dd, J = 8.8 Hz, 2.4 Hz, 2H, H2, H7), 4.01 (s, 6H, –OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.2, 131.0, 130.0, 127.1, 124.2, 116.5, 104.4, 55.5; MS (EI): (m/z) = 238 [M^+].

*2.3.2.2. 2,4,6-Trimethoxy phenanthrene (**8b**).* A white solid; m.p. 109–110 °C; yield: 40%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.09 (d, J = 2.4 Hz, 1H, H5), 7.78 (d, J = 8.8 Hz, 1H, H9), 7.67 (d, J = 8.8 Hz, 1H, H8), 7.51 (d, J = 8.8 Hz, 1H, H10), 7.20 (dd, J = 8.8 Hz, 2.8 Hz 1H, H7), 6.90 (d, J = 2.8 Hz, 1H, H1), 6.77 (d, J = 2.8 Hz, 1H, H3), 4.08 (s, 3H, –OCH_3), 3.98 (s, 3H, –OCH_3), 3.93 (s, 3H, –OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 160.0, 158.2, 158.1, 136.0, 131.6, 129.3, 128.1, 126.5, 124.5, 115.4, 114.6, 109.6, 101.3, 99.0, 55.8, 55.3, 55.2; MS (EI): (m/z) = 268 [M^+].

*2.3.3. General procedure for the synthesis of **9a**–**9d***

To a solution of **8a**–**8d** (0.84 mmol) in CH_2Cl_2 (5 ml) was added BBr_3 (1.0 M solution in CH_2Cl_2 1.6 ml, 1.6 mmol). The deep purple solution obtained was stirred for 24 h and then poured into ice-water (250 ml). The suspended solid formed was extracted into Et_2O (3 × 150 ml), and then the combined organic fractions were re-extracted with aqueous NaOH (2 M, 2 × 150 ml). The basic solution was acidified by dropwise addition of concentrated HCl to pH 2, and then extracted with Et_2O (3 × 150 ml). These organic fractions were dried over $MgSO_4$ and the solvent was evaporated to produce a red-brown solid. The crude residue was chromatographed with a mixture of petroleum/EtOAc (2:1) as eluent.

*2.3.3.1. 3,6-Dihydroxy phenanthrene (**9a**).* A white solid; m.p. 221–222 °C; yield: 86%; ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 8.77 (bs, 2H, –OH), 7.98 (d, J = 2.4 Hz, 2H, H4, H5), 7.78 (d, J = 8.8 Hz, 2H, H8, H1), 7.51 (s, 2H, H9, H10), 7.22 (dd, J = 8.8 Hz, 2.4 Hz, 2H, H2, H7); ^{13}C NMR (100 MHz, CD_3COCD_3): δ = 157.1, 132.4, 131.1, 127.6, 124.7, 118.3, 107.6; MS (ESI): (m/z)=211 [M+H].

*2.3.3.2. 2,4,6-Trihydroxy phenanthrene (**9b**).* A white solid; m.p. >300 °C; yield: 70%; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.99 (d, J = 2.4 Hz, 1H, H5), 7.51 (d, J = 8.4 Hz, 1H, H9), 7.38 (d, J = 8.8 Hz, 1H, H8), 7. 18 (d, J = 8.4 Hz, 1H, H10), 6.87 (dd, J = 8.8 Hz, 2.4 Hz 1H, H7), 6.59 (d, J = 2.4 Hz, 1H, H1), 6.50 (d, J = 2.4 Hz, 1H, H3); ^{13}C NMR (100 MHz, CD_3OD): δ = 159.2, 157.1, 156.7, 137.8, 133.9, 130.1, 128.6, 126.8, 124.7, 115.2, 114.3, 113.2, 104.7, 103.1; MS (ESI): (m/z) = 227 [M+H].

*2.3.3.3. 2,3-Dihydroxy phenanthrene (**9c**).* A white solid; m.p. 158–159 °C; yield: 50%; ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 8.59 (s, 1H, –OH), 8.51–8.54 (m, 2H, –OH, H5), 8.16 (s, 1H, H4), 7.84–7.86 (m, 1H, H8), 7.50–7.63 (m, 3H, H6, H9, H10), 7.47–7.51 (m, 1H, H7), 7.25 (s, 1H, H1); ^{13}C NMR (100 MHz, CD_3COCD_3): δ = 147.3, 147.0, 132.2, 130.8, 129.3, 128.1, 127.0, 126.9, 126.2, 125.8, 125.1, 123.1, 122.9, 108.0; MS (EI): (m/z) = 210 [M^+].

*2.3.3.4. 2,3,6,7-Tetrahydroxy phenanthrene (**9d**).* A white solid; m.p. >300 °C; yield: 65%; ^{1}H NMR (400 MHz, CD_3COCD_3): δ = 8.41 (bs, 4H, –OH), 7.87 (s, 2H, H4, H5), 7.36 (s, 2H, H9, H10), 7.23 (s, 2H, H1, H8); ^{13}C NMR (100 MHz, CD_3COCD_3): δ = 149. 8, 146.0, 127.1, 125.5, 124.3, 112.7, 107.4; MS (ESI): (m/z) = 241 [M−H].

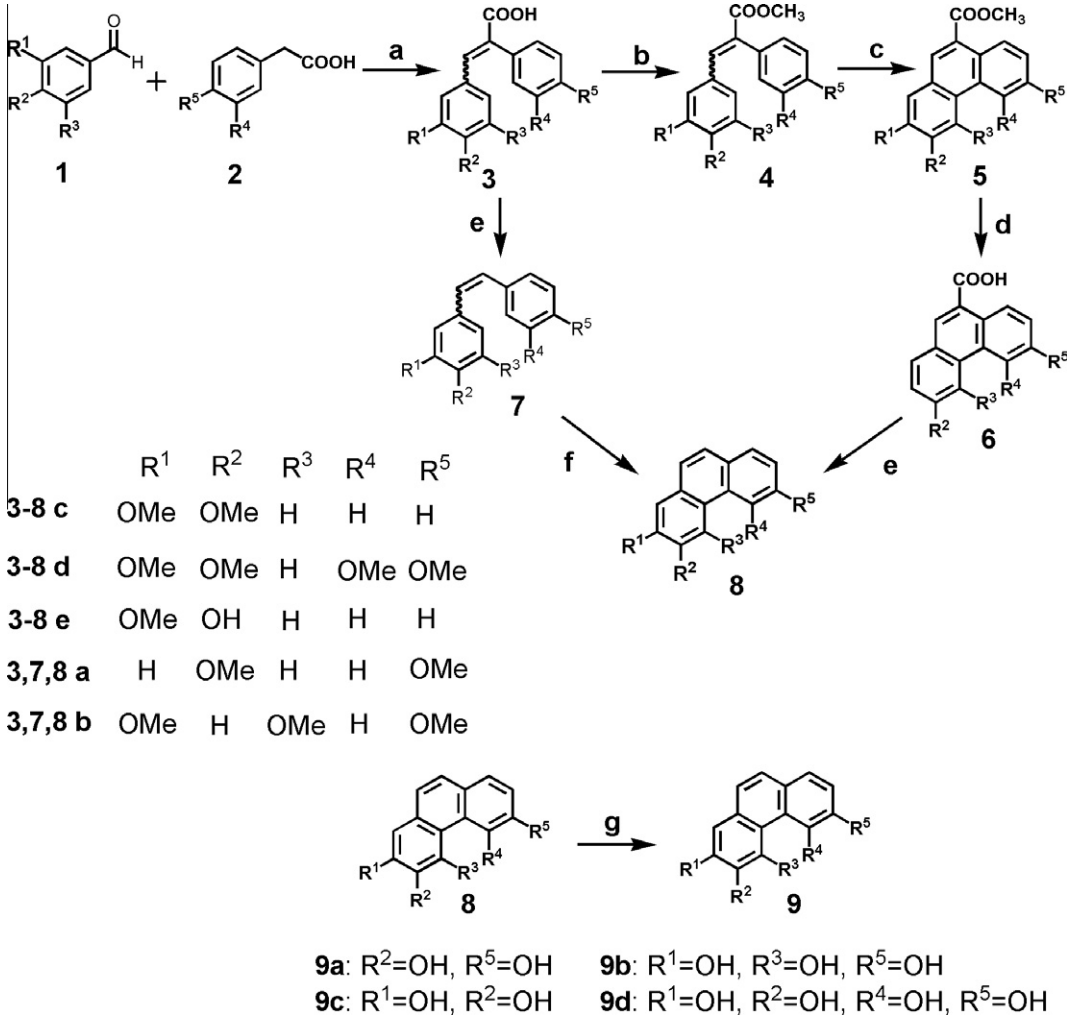
2.4. Assay for ferric reducing/antioxidant power (FRAP)

FRAP assay (Benzie & Strain, 1996) was used to evaluate the reducing capacity of hydroxylated phenanthrenes and the related procedure was described in our previous work (Tang et al., 2011).

2.5. Assay for DPPH·-scavenging activity

The EC_{50} value of hydroxylated phenanthrenes in the scavenging of DPPH· was determined by monitoring the absorbance of DPPH· (60 μmol) at 517 nm in methanol during a 120 min observation. The stoichiometry n was calculated by the equation, n = 1/(EC_{50} × 2) (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007).

The rates of hydroxylated phenanthrenes with DPPH· in methanol were determined by monitoring the absorbance change at 517 nm, using a Varian Cary 300 Spectrophotometer equipped with a quartz cell (optical path length, 1 cm) and using the second-order kinetics with the ratio of [hydroxylated phenanthrenes]/[DPPH·] being 1/1. The rates of **9b** in the presence of acetic acid were determined in the same manner. Temperature in the cell was kept at 25 °C by means of a thermostatted bath. The concentrations of DPPH· in methanol were measured from its molar extinction coefficient value, ε, of 1.023×10^4 M^{-1} cm^{-1}. As the DPPH·-scavenging rates of **9c** and **9d** in methanol were very fast, their rates were measured using the stopped-flow technique with a SFA-20 accessory.



Scheme 2. Synthesis of the hydroxylated phenanthrenes. Reagents and conditions: (a) Ac_2O/Et_3N; (b) CH_3OH/conc. H_2SO_4; (c) $FeCl_3/CH_2Cl_2$; (d) NaOH/methanol:H_2O = 1:1; (e) Cu/quinoline; (f) I_2, propylene oxide/cyclohexane, UV; (g) BBr_3/CH_2Cl_2.

2.6. Assay for oxidative DNA strand breakage induced by AAPH

The inhibition of AAPH-induced DNA strand breakage by ArOH was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA following the procedure described previously (Qian et al., 2011).

3. Results

3.1. Synthesis of the hydroxylated phenanthrenes

The overall strategy for synthesis of the hydroxylated phenanthrenes is outlined in Scheme 2. The E- and Z-2,3-diphenylacrylic acids **3** were easily available by Perkin condensation of the appropriate aromatic aldehyde **1** with the corresponding benzeneacetic acid **2**. Esterification of acids **3** with methanol in the presence of concentrated sulphuric acid gave the corresponding methyl esters **4** in almost quantitative yields. Construction of the phenanthrene unit is the key step of the overall synthesis. To the best of our knowledge, metal-based intramolecular oxidative coupling by using oxidative coupling reagents, such as thallium(III) trifluoroacetate (TTFA) (Bringmann, Walter, & Weirich, 1990), vanadium oxytrifluoride (VOF_3) (Halton, Maidment, Officer, & Warnes, 1984), manganese(IV) dioxides (MnO_2) (Wang et al., 2010) and iron(III) chloride ($FeCl_3$) (Wang, Lü, Wang, & Huang, 2008), is the most convenient way to construct the phenanthrene ring system. Thus, a facile synthesis of the phenanthrene unit by using $FeCl_3$ at room temperature was chosen. Interestingly, only the coupling of ester **4** with *ortho*-dimethoxy or hydroxyl substituted 2,3-diphenylacrylate (for **4c**, **4d** and **4e**) gave the oxidative coupling product **5** in an excellent yield, whereas no oxidative coupling product was formed from **4a** and **4b**. This indicated substituted *ortho*-dimethoxy or *ortho*-hydroxy-methoxy groups on phenyl is necessary for this type of intramolecular oxidative coupling. Ester **5** was converted to its corresponding acids **6** with methanol in the presence of sodium hydroxide. Decarboxylation reactions of **6** were carried out in the presence of Cu/quinoline at 220 °C under the protection of N_2 to give the methoxyl phenanthrene **8c**–**8e**, followed by demethylation with BBr_3 in dichloromethane to furnish **9c**–**9d**. For **4a** and **4b**, we also tried MnO_2 as an oxidative coupling reagent. Unfortunately, we also failed to construct the phenanthrene ring. We then turned to photochemical cyclisation of stilbenes with iodine as a catalyst, which was reported to efficiently construct the phenanthrene ring system (Scheme 2) (Lawrence et al., 1999). Decarboxylation of **3a** and **3b** provided E- and Z-stilbenes **7a** and **7b**, which were used in the next stage without separation. Photolysis of a diluted solution of stilbene **7** in the presence of iodine using a 500 W Hg lamp led to the production of phenanthrene **8a** and **8b**. Hydroxyl-substituted phenanthrene **9a** and **9b** were

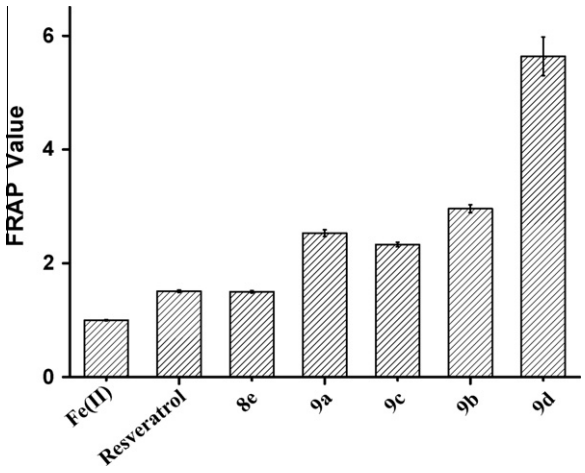


Fig. 1. Ferric reducing antioxidant power of the hydroxylated phenanthrenes and resveratrol. Data are the average of three determinations, which were reproducible with a deviation of less than ±10%.

obtained by demethylation with BBr_3. All the phenanthrene compounds were characterised with ^1H and ^{13}C NMR and MS.

3.2. Ferric reducing antioxidant power of the hydroxylated phenanthrenes

Reducing power of the hydroxylated phenanthrenes were investigated by FRAP assay (Benzie & Strain, 1996), a method that depends on the reduction of a ferric tripyridyltriazine $Fe(TPTZ)_2(III)$ complex to the ferrous tripyridyltriazine $Fe(TPTZ)_2(II)$ in the presence of antioxidants or reductants (ArOHs) (Eq. (1)):

$$ArOH + Fe^{3+} \rightarrow ArOH^{\cdot+} + Fe^{2+} \quad (1)$$

The results are expressed as the number of donated electrons per molecule and illustrated in Fig. 1. It is seen from the figure that the reducing capacity depends significantly on the degree of hydroxylation, and decreases in the order of **9d** (four hydroxyl groups) > **9b** (three hydroxyl groups) > **9c** ~ **9a** (two hydroxyl groups) > **8e** (one hydroxyl group) ~ resveratrol.

3.3. DPPH·-scavenging activity of the hydroxylated phenanthrenes

It is well-known that one of the main characteristics responsible for antioxidant activity of a phenolic compound is its ability to scavenge free radicals, and the DPPH·-scavenging assay is the most commonly used method. Therefore, the method was employed to evaluate antioxidant activity of the hydroxylated phenanthrenes in methanol, and their values of EC_{50} (concentration able to scavenge 50% of DPPH·) and stoichiometric factor (number of DPPH· reduced by one molecule of antioxidant), n, are presented in Table 1. Based on the EC_{50} and n values, their DPPH·-scavenging activity follows the order: **9d** > **9b** > **9c** ~ **9a** > **8e** ~ resveratrol, which is in accordance with the result determined by the FRAP method. Among the compounds tested, compound **9d** with two catechol moieties had the lowest EC_{50} value (5.76 μM) and highest n value (8.67) and hence possessed the highest DPPH·-scavenging ability. Compound **9b**, with the same hydroxyl group substitution mode as resveratrol, ranks the second place, and shows more than 3-fold DPPH·-scavenging efficiency than resveratrol, suggesting the importance for construction of a phenanthrene ring. Compounds **9a** and **9c** with two hydroxyl groups are almost equally effective,

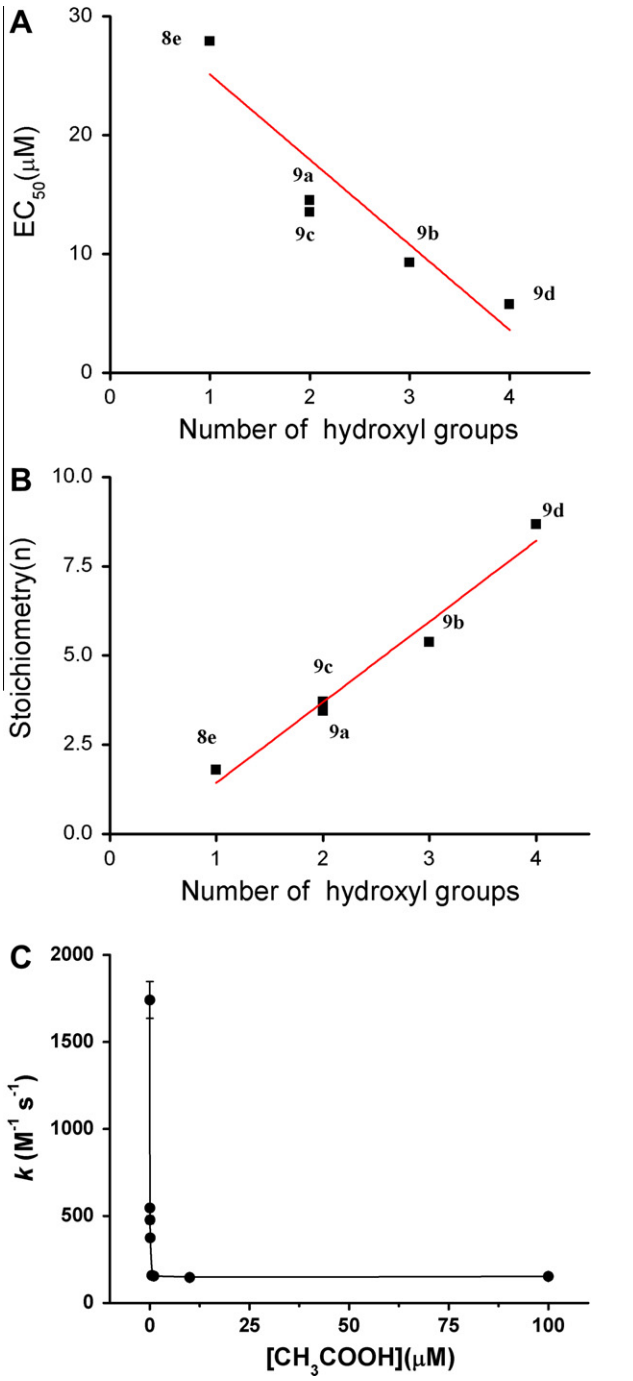


Fig. 2. Relationship of the number of hydroxyl groups of the hydroxylated phenanthrenes with their EC_{50} (A) or *n* values (B) in the DPPH·-scavenging reaction. (C) Plot of *k vs.* $[CH_3COOH]$ for the reaction of **9b** with DPPH· in methanol at 298 K.

Table 1
DPPH·-scavenging activity of the hydroxylated phenanthrenes and resveratrol.

Compounds	EC_{50} (μM)[a]	*n*[b]	*k* (M^{-1} s^{-1})[a]
8e	27.90 ± 0.03	1.79	(1.05 ± 0.03) × 10^2
9a	14.53 ± 0.18	3.44	(0.99 ± 0.03) × 10^2
9c	13.53 ± 0.63	3.70	(1.14 ± 0.01) × 10^{4c}
9b	9.28 ± 0.55	5.38	(1.74 ± 0.11) × 10^3
9d	5.76 ± 0.16	8.68	(6.74 ± 0.13) × 10^{4c}
trans-Resveratrol	26.23 ± 2.12	1.90	(2.09 ± 0.01) × 10^{2d}

[a] Data are expressed as the mean ± SD for three determinations.
[b] Calculated by the equation, $n = 1/(EC_{50} \times 2)$.
[c] Determined by the stopped-flow technique.
[d] Cited from Ref. Cao et al. (2012).

and more active than **8e** with one hydroxyl group. Furthermore, a good linear relationship between the number of hydroxyl groups and the EC_{50} (Fig. 2A) or *n* (Fig. 2B) values was also obtained.

However, both the EC_{50} and *n* value assays are rather static, and do not reflect the kinetic details, namely radical-scavenging rate. Thus, we monitored the reaction rates of the hydroxylated phenanthrenes with DPPH· in methanol at 25 °C by following the second-order decay of the absorbance at 517 nm due to the radical. For **9c** and **9d**, their rates were very high and were therefore measured using the stopped-flow technique. These kinetic data (second-order rate constants, *k*) are given in Table 1. By comparing the *k* values, DPPH·-scavenging activity follows the sequence of **9d** > **9c** > **9b** > resveratrol > **8e** ~ **9a**. Compound **9b** is still more active than resveratrol and its *k* value is about 9-fold larger than that of this reference compound. Noticeably, a clear difference appears in this assay, that is, compound **9c** with a catechol moiety exhibits significantly higher activity than compound **9b**, while the former has relatively weak activity in comparison with the latter in the FRAP, EC_{50} and *n* values assays.

To rationalise the reaction mechanism, the effect of adding acetic acid on DPPH·-scavenging reaction of **9b** in methanol was investigated. As shown in Fig. 2C, the rate constant ($1.74 \times 10^3\ M^{-1}\ s^{-1}$) decreased remarkably with increasing concentration of acetic acid to reach a limiting value ($1.46 \times 10^2\ M^{-1}\ s^{-1}$), unambiguously supporting that the reaction occurs via a sequential proton loss electron transfer mechanism and the actual electron donor is the phenolate anion formed by deprotonation of **9b** (Litwinienko & Ingold, 2007) (see below).

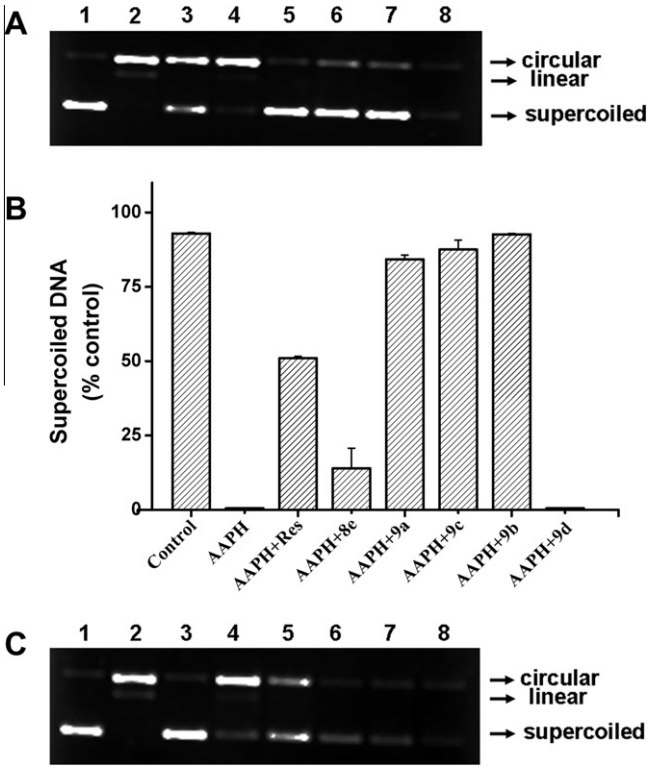


Fig. 3. Agarose gel electrophoresis pattern of pBR332 DNA (100 ng/25 μl) after incubation with AAPH (5 mM) and/or the hydroxylated phenanthrenes (20 μM) in PBS (pH 7.4) at 37 °C for 60 min. (A) Effect of the hydroxylated phenanthrenes on AAPH-induced DNA strand breakage. Lane 1, control; lane 2, 5 mM AAPH alone; lanes 3–8, resveratrol, **8e**, **9a**, **9c**, **9b** and **9d**, respectively. (B) Histogram of supercoiled DNA percentages relative to native DNA for the hydroxylated phenanthrenes. (C) Promoting effect of **9d** on AAPH-induced DNA strand breakage. Lane 1, control; lane 2, 5 mM AAPH alone; lane 3, 20 μM **9d** alone; lanes 4–8, 5 mM AAPH and 2.5, 5, 10, 15, 20 μM **9d**, respectively.

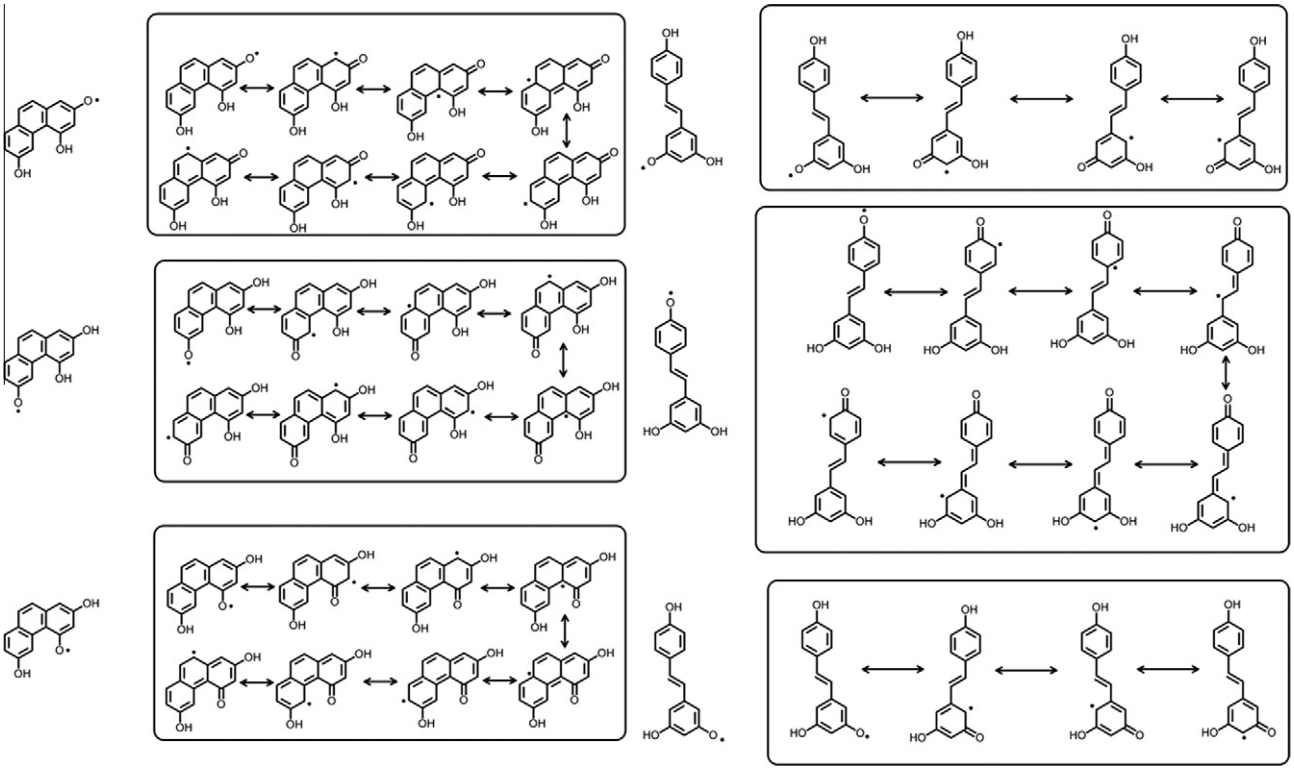
3.4. Inhibition of AAPH-initiated DNA strand breakage by the hydroxylated phenanthrenes

DNA is a primary target for free radical-mediated oxidative damage, and this damage is widely believed to be a causative factor in cancer, chronic inflammation and ageing (Ames et al., 1993; Reuter et al., 2010). Consequently, ability of the hydroxylated phenanthrenes to inhibit AAPH-induced oxidative damage of DNA was also assessed *in vitro* by using agarose gel electrophoresis analysis to measure the conversion of supercoiled pBR322 plasmid DNA to the open circular and linear forms, indicating of single- and double-strand breakage, respectively. As shown lane 2 in Fig. 3A, the supercoiled DNA was completely converted into its circular and linear forms by addition of 5 mM AAPH. These compounds (20 μM) except **9d** inhibited significantly the DNA strand breakage (lanes 3–7) with the activity sequence of **9b** > **9c** ~ **9a** > resveratrol > **8e** based on the percentage of intact supercoiled DNA (Fig. 3B). Interestingly, **9d**, the most active compound in the FRAP and DPPH·-scavenging assays, did not provide any protection effect against the DNA strand breakage (lane 8 in Fig. 3A). Instead, it converted completely the supercoiled DNA into a fragmental form in the presence AAPH, further intensifying AAPH-induced DNA damage. On the other hand, there was no such breaking effect in the absence of AAPH (lane 3 in Fig. 3C), whereas in the presence of AAPH, the degree of the DNA strand breakage increased with increasing concentrations of **9d** (lanes 4–8 in Fig. 3C). This clearly suggests the involvement of *ortho*-quinone, an oxidative product of **9d**. The interesting prooxidant phenomenon will be further discussed in the following section.

4. Discussion

Antioxidants are compounds that, at low concentrations compared to those of oxidizable substrates, including proteins, DNA, polyunsaturated lipids and so on, can significantly prevent their oxidative damage (Halliwell & Gutteridge, 2007), by direct scavenging of free radicals or ROS (Leopoldini, Chiodo, Russo, & Toscano, 2011), induction of phase 2 enzymes (Dinkova-Kostova & Talalay, 2008) and chelation of metal ions (Leopoldini, Russo, Chiodo, & Toscano, 2006). Free radical-scavenging activity of polyphenolic antioxidants depends significantly on their molecular structure such as number and position of hydroxyl groups, as well as conjugation and resonance effects (Leopoldini, Marino, Russo, & Toscano, 2004; Leopoldini et al., 2011). This work describes a strategy in structural modifications of resveratrol based on extension of conjugation and alternation of number and position of hydroxyl groups on the aromatic rings.

As we expected, compound **9b**, with the same hydroxyl group substitutions as resveratrol, displays significantly increased antioxidant activity as compared to this parent molecule in FRAP (Fig. 1), DPPH·-scavenging (Table 1) and DNA strand breakage-inhibiting (Fig. 3) assays, highlighting that construction of a phenanthrene ring is a successful strategy to improve antioxidant activity of resveratrol. This could be understood because the presence of the single bound linkage, between the benzene rings and the olefinic bridge of **9b**, extends delocalization and conjugation of the π electrons over the entire molecule and thus increases stability of neutral radical species. As shown in Scheme 3, only the 4′-OH phenoxyl radical of resveratrol could be completely delocalized over the whole molecule due to the *para* position of the olefinic bridge to the OH, and this contribution is missing in the 3- or 5-OH radical since the unpaired electron cannot travel across the olefinic bridge. However, such a delocalization could be found for all the possible hydroxyl-derived phenoxyl radical in the phenanthrene ring.



Scheme 3. Resonance structures of phenoxyl radicals derived from **9b** and *trans*-resveratrol.

We turn now to a consideration of the three different experimental models that correspond to different mechanisms and targets. The first experiment, FRAP assay is relevant to electron-donating ability (reducing capacity) of these compounds and stability of the corresponding radical cations (Eq. (1)), which are characterised by their oxidative potentials. In general, the lower the oxidation potential, the stronger the electron-donating ability. Therefore, our FRAP results indicate that increasing degree of hydroxylation and conjugation help decrease their oxidative potentials, and hence increase their electron-donating ability and stability of the corresponding radical cations.

The second study on DPPH·-scavenging activity is utilised to estimate hydrogen-transfer ability of these compounds. In term of the static parameters (EC_{50} or n values), their hydrogen-transfer ability also relies on the degree of hydroxylation (Table 1, Fig. 2A and B). Noticeably, the n values of all compounds except resveratrol are larger than the available number of hydroxyl groups (Table 1). This could be due to the involvement of their oxidative products in the radical-scavenging reaction (Dangles, Fargeix, & Dufour, 1999, 2000; Villaño et al., 2007). Moreover, a methanol molecule (solvent) can regenerate the catechol moiety of phenol by nucleophilic attack to the corresponding *ortho*-quinone (Dangles et al., 1999), and hence enlarge the n values. A comparison of the activity sequences derived from the static and kinetic (k values) parameters clearly shows an activity reversal between compounds **9c** and **9b**. This implies that in addition to the number of the hydroxyl groups, the position of the hydroxyl groups is also of great importance for improving the hydrogen-transfer ability of the hydroxylated phenanthrenes. Obviously, the significantly high k values of **9c** with a catechol moiety relative to that of **9b** due to the intramolecular hydrogen-bonding interaction of the oxidative intermediate, the *ortho*-hydroxy-phenoxyl radical, (Leopoldini et al., 2004, 2011; Wright et al., 2001), is easier to further oxidise to form the *ortho*-quinone. This also leads to **9d** with two catechol moieties, which is not only the strongest electron-donating donor, but also the best hydrogen atom donor among all compounds investigated. The important role of the catechol moiety in antioxidant activity has also been elucidated in the case of caffeic acid (Leopoldini et al., 2011), catechin and its planar analogue (Leopoldini, Russo, & Toscano, 2007) and quercetin (Chiodo, Leopoldini, Russo, & Toscano, 2010).

Currently, at least four different chemical pathways was used to describe the formal hydrogen-transfer from a phenol antioxidant (ArOH) to free radical (X·), that is, direct hydrogen atom transfer (HAT, Eq. (2)), proton-coupled electron transfer (PCET), sequential proton loss electron transfer (SPLET, Eq. (3)), and electron transfer then proton transfer (ETPT) (Litwinienko & Ingold, 2007). Although the four different pathways ultimately result in generation of the same ArO· radical, their occurrence and contribution depend significantly on the bond dissociation enthalpy (BDE) of the phenolic O–H bond, the oxidation (ionisation) potential of ArOH and its anion (ArO^-), as well as the acid dissociation constant (pK_a) of ArOH. Generally, in HAT or PCET reaction, the BDE of the O–H bond is an important parameter in determining the reaction rate; the lower the BDE value, the faster the HAT process (Leopoldini et al., 2004, 2011; Wright et al., 2001). However, in the ETPT reaction, the oxidation (ionisation) potential of ArOH controls the ration rate; the lower the oxidation (ionisation) potential value, the easier the electron abstraction (Leopoldini et al., 2004, 2011; Wright et al., 2001). For the SPLET reaction, the pK_a of ArOH and oxidation (ionisation) potential of ArO^- are the most significant parameters (Litwinienko & Ingold, 2007). Moreover, occurrence and contribution of these reactions are also controlled by the nature of the attacking radical and the solvent used (Litwinienko & Ingold, 2007). In alcoholic solvents that support ionisation, ArOH first loses a proton to form the corresponding anion (ArO^-), which is a much stronger electron donor than the parent ArOH, followed by rapid electron transfer to an electron-deficient radical such as DPPH·, an SPLET mechanism well demonstrated by Litwinienko and Ingold based on acidified-kinetic analysis (Litwinienko & Ingold, 2007).

We have used the same method to find that the DPPH·-scavenging reaction of resveratrol and its hydroxylated analogues in ethanol proceed mainly via a SPLET mechanism (Shang et al., 2009). Similarly, the DPPH·-scavenging rate of **9b** in methanol was decreased approximately 12-fold by the addition of acetic acid (Fig. 2C). This result supports that this unacidified-kinetic in methanol is mostly governed by SPLET (Eq. (3)), whereas the addition of acetic acid suppresses the ionisation of **9b** and hence eliminates SPLET to leave a limiting rate corresponding to HAT (Eq. (2)). Due to the crucial role of the amount and oxidation potential of ArO^- in the SPLET reaction (Litwinienko & Ingold, 2007), the significant high DPPH·-scavenging rates of **9b**–**9d** relative to resveratrol in methanol, also confirm that construction of a phenanthrene helps to decrease pK_a value of ArOH or oxidation potential of its anion. As a matter of fact, the existence of the phenanthrene ring could promote the proton releasing process, due to the resonance stabilization of the corresponding proton releasing product (ArO^-) by the larger aromatic system (Zhu, Wang, & Liang, 2010):

$$ArOH + X^{\cdot} \rightarrow ArO^{\cdot} + XH \quad (2)$$

$$ArOH \xrightarrow{-H^+} ArO^- \xrightarrow{X^{\cdot}} ArO^{\cdot} + X^- \xrightarrow{H^+} ArO^{\cdot} + XH \quad (3)$$

Finally, we turned to another target, DNA and employed AAPH-induced plasmid pBR322 DNA strand breakage as a simple model to evaluate the protecting ability of these compounds against the oxidative damage. Although a similar activity sequence to that of FRAP and DPPH·-scavenging experiments were obtained in this assay, interestingly, **9d** with two catechol moieties further intensified AAPH-induced DNA strand breakage as a prooxidant rather than an antioxidant (Fig. 3). The prooxidant action may be accounted for by the following two factors: (1) this molecule could directly react with molecular oxygen to generate superoxide, thereby causing DNA strand breakage, and (2) the formation of *ortho*-quinone oxidation product in the presence of AAPH may involve in DNA strand breakage by forming covalent adducts with DNA via a Michael addition reaction (Samuni et al., 2003). The fact that **9d** itself could not effectively induced DNA strand breakage (Fig. 3C) excludes the first factor and demonstrates the involvement of *ortho*-quinone in the process. Taken together, these results imply that *ortho*-quinone plays an important role in not only antioxidant, but also prooxidant action, of catechol type compounds. From an antioxidant point of view, this prooxidant action is a drawback, but the prooxidant-induced DNA strand breakage is believed to be a promising strategy for killing cancer cells (Bai et al., 2010; Fan et al., 2009). Zhou and co-workers have recently reported that bis(catechol) derivatives could selectively suppress melanoma cells by inducing DNA cross-link via an *ortho*-quinone intermediate (Bai et al., 2010). Therefore, the prooxidant action and mechanism of compound **9d** probably deserves further investigation.

5. Conclusion

In summary, structural modification of resveratrol by construction of a phenanthrene ring to extend the conjugation over multiple aromatic-rings, leads to the enhancement of antioxidant activity, as exemplified in **9b**, which is more active in the FRAP, DPPH·-scavenging and DNA strand breakage-inhibiting assays than resveratrol.

On the basis of the obtained results in this work, we can draw the following conclusions:

(i) Construction of a phenanthrene ring is valuable in the development of a novel type of resveratrol-inspired antioxidants.
(ii) The number and position of the hydroxyl groups exert significant influence on antioxidant activity of the hydroxylated phenanthrenes.
(iii) Although similar activity sequences were obtained in the FRAP, DPPH·-scavenging and DNA strand breakage-inhibiting assays, the appreciable differences could contribute detailed insights into antioxidant mechanisms.
(iv) Compound **9d** has emerged as a prooxidant in AAPH-induced DNA strand breakage, suggesting a potential application as a DNA cleaving or cross-linking agent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.05.074.

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