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Coumarin-Fused Coumarin: Antioxidant Story from *N,N*-Dimethylamino and Hydroxyl Groups

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Supporting Information

ABSTRACT: Two coumarin skeletons can form chromeno[3,4-*c*]chromene-6,7-dione by sharing with the C═C in lactone. The aim of the present work was to explore the antioxidant effectiveness of the coumarin-fused coumarin via six synthetic compounds containing hydroxyl and *N,N*-dimethylamino as the functional groups. The abilities to quench 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$), 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), and galvinoxyl radical revealed that the rate constant for scavenging radicals was related to the amount of hydroxyl group in the scaffold of coumarin-fused coumarin. But coumarin-fused coumarin was able to inhibit DNA oxidations caused by $^{\bullet}OH$, Cu^{2+}/glutathione (GSH), and 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) even in the absence of hydroxyl group. In particular, a hydroxyl and an *N,N*-dimethylamino group locating at different benzene rings increased the inhibitory effect of coumarin-fused coumarin on AAPH-induced oxidation of DNA about 3 times higher than a single hydroxyl group, whereas *N,N*-dimethylamino-substituted coumarin-fused coumarin possessed high activity toward $^{\bullet}OH$-induced oxidation of DNA without the hydroxyl group contained. Therefore, the hydroxyl group together with *N,N*-dimethylamino group may be a novel combination for the design of coumarin-fused heterocyclic antioxidants.

KEYWORDS: *coumarin-fused coumarin, antioxidant, free radical, DNA oxidation*

INTRODUCTION

As a naturally occurring skeleton, coumarin attracts many researchers' attention due to its wide bioactivities including antioxidant, anti-inflammatory, anticancer, MAO-B inhibitory, and antimicrobial activities.[1] Because of the high bioactivities of coumarins,[2] some efforts focus on the development of novel coumarin-based therapeutic agents.[3] In addition to the exploration on the bioactivity of single coumarin,[4] fused coumarins exhibit a large scale of biological, pharmacological, photoelectronic, and pigment activities.[5] In the fused coumarin, the single coumarin skeleton shares C═C in the lactone with other organic motifs,[6] leading to the formation of coumarin-fused heterocycles. The fused heterocycle is beneficial for transferring electrons,[7] and the abundance of electrons in coumarin-fused heterocycles implies that the fused coumarins may be potential antioxidants.[8] We have synthesized coumestan (a coumarin moiety in combination with a benzofuran) and found that coumestan can inhibit radical-induced oxidation of DNA even in the absence of phenolic hydroxyl group.[9] This result encourages us to investigate the antioxidant effectiveness of coumarin-fused coumarins, in which double coumarins share with C═C in the lactone.[10] The scaffold of coumarin-fused coumarin may be beneficial for accommodating an electron from radicals and thus may exhibit high antioxidant effectiveness than traditional antioxidant such as Trolox, a structural analogue of coumarin.[11] Moreover, it was reported that some natural coumarin-fused coumarins exhibit high bioactivity *in vivo*[12] and low cytotoxicity.[13] Therefore, as shown in Scheme 1, six coumarin-fused coumarins are prepared following a reported method,[7] aiming at revealing abilities of coumarin-fused coumarins to trap radicals and to inhibit radical-induced DNA oxidation.

Because the oxidation of DNA correlates with many fatal diseases such as cancer, aging, and atherosclerosis,[14] it is urgent to find enough candidate molecules for screening inhibitory effects on DNA oxidation. We herein apply the DNA oxidation caused by $^{\bullet}OH$, Cu^{2+}/glutathione (GSH), or 2,2′-azobis(2-amidinopropanehydro chloride) (AAPH) as biologically experimental systems, as well as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$), 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), or galvinoxyl radical as chemically experimental systems for evaluating the antioxidant effectiveness of coumarin-fused coumarins.

MATERIALS AND METHODS

Materials and Instrumentation. Diammonium salt of ABTS, DPPH, and galvinoxyl radicals were purchased from Fluka Chemie GmbH, Switzerland, and AAPH and naked DNA sodium salt were purchased from Acros Organics, Belgium, and dissolved in the corresponding phosphate-buffered solution (PBS, the components were shown in every test) as the stock solution. Other agents were of analytical grade and used directly. The structures of products were identified by 1D ^{1}H and ^{13}C NMR (Bruker Avance III 400 MHz spectrometer) and high-resolution mass spectra (HRMS) equipped with ESI as the ionization mode (Agilent 1290-micrOTOF Q II), and the spectra were included in Supporting Information.

Synthesis of Coumarin-3-carboxylic Acids. A mixture of a salicylaldehyde (20 mmol) and Meldrum's acid (3.2 g, 22

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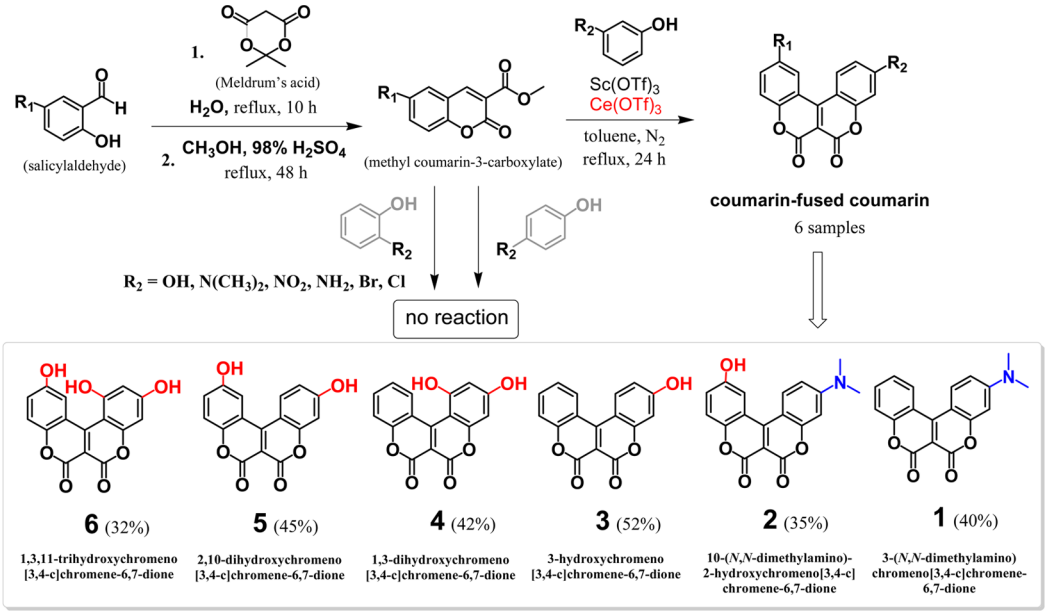
Scheme 1. Synthesis and Structures of Coumarin-Fused Coumarins Employed in This Work



mmol) was refluxed in water (20 mL) under stirring for 10 h. Then, the mixture was cooled by ice water, and the precipitate was filtered and washed with ice water to obtain white or green product. The yields were higher than 93%.

Esterification of Coumarin-3-carboxylic Acids. To 20 mL of methanolic solution of coumarin-3-carboxylic acid (10 mmol), H_2SO_4 (98%, 0.6 mL) was added dropwisely under stirring. The above mixture was refluxed for 48 h and cooled to room temperature. After the solvent was evaporated under vacuum, the residue was purified by silica chromatography with ethyl acetate and petroleum ether (1:3, v/v) being eluent to afford a white product, yield >90%.

Oxidative Annulation for the Formation of Chromeno[3,4-*c*]chromene-6,7-diones. $Ce(OTf)_3$ (0.05 g), $Sc(OTf)_3$ (0.05 g), methyl coumarin-3-carboxylate (4 mmol), and phenol (2 mmol) were mixed in 10 mL of toluene and heated at 110 °C for 24 h under a protective atmosphere of N_2. Then, the mixture was cooled to room temperature by ice water, and the precipitate was purified by silica chromatography with methanol and dichloromethane being eluent to afford the product, yield 32%∼ 52%.

*3-(N,N-Dimethylamino)chromeno[3,4-c]chromene-6,7-dione (**1**).* R_f = 0.58 (CH_3OH:CH_2Cl_2 = 1:20, v/v), 0.25 g of red product, yield 40%. mp: 185−187 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.21 (d, J = 8.0 Hz, 1H), 8.07 (d, J = 9.6 Hz, 1H), 7.66 (t, J = 8.0 Hz, 1H), 7.37−7.40 (m, 2H), 6.72 (dd, J = 2.4 Hz, J = 9.2 Hz, 1H), 6.51 (d, J = 10.4 Hz, 1H), 3.15 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 157.8, 157.0, 156.7, 154.9, 152.8, 134.1, 130.0, 128.7, 124.2, 118.1, 109.9, 104.5, 100.6, 98.1, 40.1. MS: m/z 308.0925 [M+H$^+$].

*10-(N,N-Dimethylamino)-2-hydroxychromeno[3,4-c]-chromene-6,7-dione (**2**).* R_f = 0.21 (CH_3OH:CH_2Cl_2 = 1:20, v/v), 0.23 g of red product, yield 35%. mp: > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.94 (s, 1H), 8.13 (d, J = 9.6 Hz, 1H), 7.67 (s, 1H), 7.20−7.34 (m, 2H), 6.90 (d, J = 9.6 Hz, 1H), 6.65 (s, 1H), 3.14 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 157.6, 156.4, 154.9, 152.1, 147.8, 130.3, 128.2, 122.9, 118.9, 116.3, 113.6, 110.5, 104.1, 100.2, 97.8, 42.0. MS: m/z 324.0877 [M + H$^+$].

*3-Hydroxychromeno[3,4-c]chromene-6,7-dione (**3**).* R_f = 0.63 (CH_3OH:CH_2Cl_2 = 1:10, v/v), 0.29 g of yellow product, yield 52%. mp: > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.30 (s, 1H), 8.37 (d, J = 8.0 Hz, 1H), 8.29 (d, J = 9.2 Hz, 1H), 7.82 (t, J = 7.6 Hz, 1H), 7.47−7.51 (m, 2H), 6.96 (dd, J = 2.4 Hz, J = 9.2 Hz, 1H), 6.82 (d, J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 164.5, 157.3, 155.7, 154.6, 152.7, 135.1, 131.5, 129.7, 125.1, 117.9, 115.6, 114.6, 107.6, 103.4. MS: m/z 281.0459 [M + H$^+$].

*1,3-Dihydroxychromeno[3,4-c]chromene-6,7-dione (**4**).* R_f = 0.52 (CH_3OH:CH_2Cl_2 = 1:10, v/v), 0.25 g of orange product, yield 42%. mp: > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.23 (s, 1H), 11.02 (s, 1H), 7.98 (dd, J = 1.2 Hz, J = 8.4 Hz, 1H), 7.70 (td, J = 1.6 Hz, J = 8.4 Hz, 1H), 7.38 (dd, J = 1.2 Hz, J = 8.0 Hz, 1H), 7.32 (td, J = 1.2 Hz, J = 8.4 Hz, 1H), 6.42 (d, J = 1.2 Hz, 1H), 6.30 (d, J = 2.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 164.8, 158.3, 157.2, 155.7, 154.3, 153.7, 134.3, 132.0, 122.7, 116.1, 115.8, 102.9, 100.2, 98.2, 94.8. MS: m/z 297.0405 [M + H$^+$].

*2,10-Dihydroxychromeno[3,4-c]chromene-6,7-dione (**5**).* R_f = 0.52 (CH_3OH:CH_2Cl_2 = 1:10, v/v), 0.25 g of orange product, yield 45%. mp: > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.27 (s, 1H), 9.97 (s, 1H), 8.26 (d, J = 9.2 Hz, 1H), 7.70 (s, 1H), 7.24−7.38 (m, 2H), 6.98 (d, J = 8.4 Hz, 1H), 6.82 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 163.7, 157.3, 156.2, 154.3, 149.3, 147.9, 131.0, 123.3, 122.7, 119.5, 118.8, 114.2, 113.5, 107.4, 106.3, 103.4. MS: m/z 297.0403 [M + H$^+$].

*1,3,11-Trihydroxychromeno[3,4-c]chromene-6,7-dione (**6**).* R_f = 0.37 (CH_3OH:CH_2Cl_2 = 1:5, *v*:*v*), 0.20 g of red product, yield 32%. mp: > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.63 (s, 1H), 9.37 (s, 1H), 9.33 (s, 1H), 7.31 (d, J = 8.8 Hz, 1H), 7.05 (dd, J = 2.8 Hz, J = 8.8 Hz, 1H), 6.62 (d, J = 2.8 Hz, 1H), 5.90 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 165.0, 160.0, 158.4, 156.3, 154.3, 151.3, 146.4, 125.9, 122.7, 121.2, 120.6, 119.5, 117.6, 112.6, 99.5. MS: m/z 313.0357 [M + H$^+$].

Cu^{2+}/GSH- and $^{\bullet}$OH-Induced Oxidations of DNA. Cu^{2+}/GSH-induced oxidation of DNA was carried out in a mixture of DNA (2.0 mg/mL), $CuSO_4$ (5.0 mM), and glutathione (GSH,

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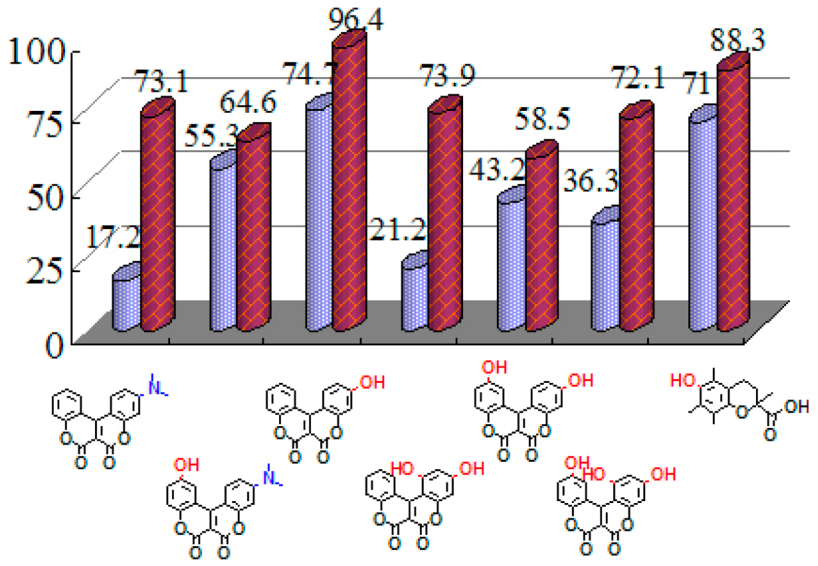
Scheme 2. TBARS Percentages (Data on the Column) in the Presence of 0.10 mM Coumarin-Fused Coumarins When the Oxidation of DNA (2.0 mg/mL) Is Caused by 5.0 mM Cu^{2+} and 3.0 mM GSH for 90 min (Right Column for Every Compound) or 4.0 mM H_2O_2 and 2.0 mM TCHQ for 30 min (Left Column for Every Compound)



3.0 mM), which were dissolved in phosphate-buffered solution (PBS_1: 6.1 mM Na_2HPO_4, 3.9 mM NaH_2PO_4, pH = 7.0). The coumarin-fused coumarin was dissolved in dimethyl sulfoxide (DMSO) as the stock solution and added to the aforementioned solution. The final concentration of tested compound was 0.10 mM, and the volume of DMSO was less than 0.5% in the total volume of the solution. The mixture of DNA, Cu^{2+}, GSH, and coumarin-fused coumarin was delivered into test tubes in a total volume of 2.0 mL. The control experiment contained the same volume of DMSO without the tested compounds dissolved. The test tubes were incubated at 37 °C for initiating the DNA oxidation, and three of them were taken out at 90 min and cooled immediately. PBS_1 solution of EDTA (1.0 mL, 30.0 mM) was added to chelate Cu^{2+}, followed by adding 1.0 mL of thiobarbituric acid (TBA) solution (1.00 g of TBA and 0.40 g of NaOH dissolved in 100 mL of PBS_1) and 1.0 mL of 3.0% aqueous solution of trichloroacetic acid. The test tubes were heated in boiling water for 30 min and cooled to room temperature by ice water; subsequently, 1.5 mL of *n*-butanol was added and shaken vigorously to extract thiobarbituric acid reactive substance (TBARS) for measuring the absorbance at 535 nm.

$^{\bullet}OH$-induced oxidation of DNA was performed in a mixture of DNA (2.0 mg/mL), tetrachlorohydroquinone (TCHQ, 4.0 mM, dissolved in DMSO as the stock solution), and H_2O_2 (2.0 mM), which were dissolved in phosphate-buffered solution (PBS_2: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA, pH = 7.4). The coumarin-fused coumarin was dissolved in DMSO as the stock solution and added to the aforementioned solution with a final concentration being 0.10 mM, while the volume of DMSO was less than 0.5% in the total volume of the solution. The solution of DNA, TCHQ, H_2O_2, and coumarin-fused coumarin was delivered into test tubes in a total volume of 2.0 mL. The control experimental system contained the same volume of DMSO without the tested compounds dissolved. The test tubes were incubated at 37 °C for initiating the oxidation of DNA, and three of them were taken out at 30 min and cooled immediately. Then, 1.0 mL of TBA solution (1.00 g of TBA and 0.40 g of NaOH dissolved in 100 mL of PBS_2) and 1.0 mL of 3.0% aqueous solution of trichloroacetic acid were added and heated in boiling water for 30 min. After the test tubes were cooled to room temperature by ice water, 1.5 mL of *n*-butanol was added and shaken vigorously to extract TBARS for measuring the absorbance at 535 nm. In the aforementioned two tests, the absorbances in the control experiment and in the presence of coumarin-fused coumarins were assigned as A_0 and A_{detect}, respectively. The effects of coumarin-fused coumarins on the oxidation of DNA were expressed by $A_{detect}/A_0 \times 100$, called TBARS percentage.

AAPH-Induced Oxidation of DNA Test. AAPH-induced oxidation of DNA was performed in a mixture of DNA (2.0 mg/mL, dissolved in PBS_2) and AAPH (40.0 mM, dissolved in PBS_2). The solution of DNA, AAPH, and a certain concentration of coumarin-fused coumarin (dissolved in DMSO as the stock solution) was delivered into test tubes in a total volume of 2.0 mL. The control experimental system contained the same volume of DMSO (0.5% of the total volume) without the tested compounds dissolved. The test tubes were incubated at 37 °C for initiating the oxidation of DNA, and three of them were taken out at every 2 h and cooled by ice water. Then, 1.0 mL of TBA solution (1.00 g of TBA and 0.40 g of NaOH dissolved in 100 mL of PBS_2) and 1.0 mL of 3.0% aqueous solution of trichloroacetic acid were added and heated in boiling water for 15 min. After the test tubes were cooled to room temperature, 1.5 mL of *n*-butanol was added and shaken vigorously to extract TBARS for measuring the absorbance at 535 nm. The absorbance of TBARS was plotted versus the reaction period.

Scavenging $ABTS^{+\bullet}$, DPPH, and Galvinoxyl Radicals. A 2.0 mL aqueous solution containing 4.0 mM ABTS salt and 1.41 mM $K_2S_2O_8$ was kept for 20 h to form $ABTS^{+\bullet}$ and then diluted by 100 mL of ethanol. The absorbance of $ABTS^{+\bullet}$ was around 1.00 at 734 nm ($\varepsilon_{ABTS^{+\bullet}} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$). DPPH and galvinoxyl radicals were dissolved in ethanol, respectively. The absorbances of DPPH and galvinoxyl radicals were around 1.00 at 517 nm ($\varepsilon_{DPPH} = 4.09 \times 10^3\ M^{-1}cm^{-1}$) and 428 nm ($\varepsilon_{galvinoxyl} = 1.4 \times 10^5\ M^{-1}cm^{-1}$), respectively. A certain concentration of DMSO solution of coumarin-fused coumarin

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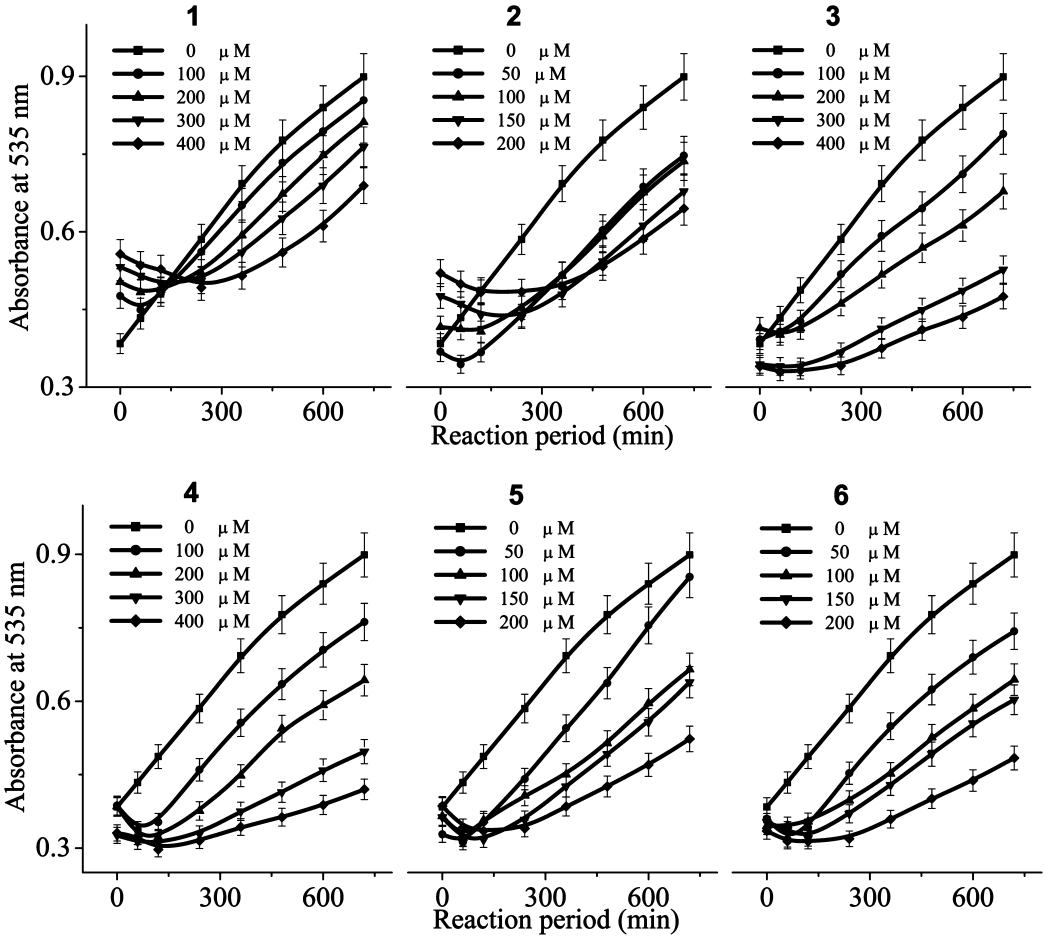

Figure 1. Variation of the absorbance of TBARS in the mixture of 2.0 mg/mL DNA, 40 mM AAPH, and various concentrations of coumarin-fused coumarins at 37 °C.

(0.1 mL) were added to 1.9 mL of $ABTS^{+\bullet}$, DPPH, and galvinoxyl radicals solution, respectively. The decreases of the absorbance of these radicals were recorded at 25 °C at a certain time interval.

Statistical Analysis. All the data were the average values from at least three independent measurements with the experimental error within 10%. The equations were analyzed by one-way ANOVA in Origin 6.0 professional software, and $p < 0.001$ indicated a significance difference.

RESULTS AND DISCUSSION

Synthesis of Coumarin-Fused Coumarins. Many efforts are contributed to develop synthetic protocols for constructing coumarin-fused heterocycles. The C═C in the lactone of coumarin possesses high reactivity for driving nucleophilic addition, leading to the formation of the fused heterocyclic[15] or spirocyclic coumarins.[16] For example, the C═C in the lactone can be shared with the C−C in cycloheptanone, forming a tricyclic fused coumarin as NO inhibitor.[17] In the synthetic operation, some novel techniques such as ultrasound vibration[18] and solvent-free heating[19] are widely used, but the formation of the lactone for coumarin-fused coumarin scaffold still depends upon appropriate catalysts. For example, the usage of cationic ruthenium complex catalyzes Diels−Alder reaction of enediynes can produce coumarin-fused polycycles.[20] The Cu(II) and Pb(II) are able to drive the cyclization of lactone,[21] and $Sc(OTf)_3$ can lead to the formation of chromeno[3,4-*c*]chromene- 6,7-dione, called coumarin-fused coumarin (see Scheme 1) in the yield of 33% with nitrobenzene being oxidant.[7] In this work, we follow our previously reported method[22] to apply $Ce(OTf)_3$ as the oxidant (instead of nitrobenzene) for catalyzing the annulation of methyl coumarin-3-carboxylate and *m*-substituted phenol. The mild oxidizability of $Ce(OTf)_3$ cannot oxidize the phenolic hydroxyl groups when resorcinol and phloroglucinol act as reactants in the synthesis of **3** ∼ **6**, and $Ce(OTf)_3$ can be conveniently removed in the purification of products via silica chromatography. The methyl 6-hydroxy-coumarin-3-carboxylate (a single coumarin scaffold) is prepared following a reported method,[23] and toluene is applied for the annulation between the single

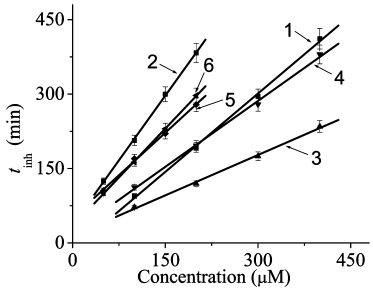

Figure 2. Linear relationships between concentrations of coumarin-fused coumarins and inhibition period (t_{inh}) in protecting DNA against AAPH-induced oxidation.

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Table 1. Equations of t_{inh} ~ [Coumarin-Fused Coumarin] and *n* of Coumarin-Fused Coumarin in Protecting DNA against AAPH-Induced Oxidation[a]

compd	t_{inh} (min) = (n/R_i) [coumarin-fused coumarin (μM)] + constant[b]	*n*
1	t_{inh} = 1.05 (±0.05) [**1**] + 14.73 (±0.74)	3.53(±0.18)
2	t_{inh} = 1.74 (±0.09) [**2**] + 36.35 (±1.82)	5.85(±0.29)
3	t_{inh} = 0.54 (±0.03) [**3**] + 14.45 (±0.72)	1.81(±0.09)
4	t_{inh} = 0.89 (±0.04) [**4**] + 20.40(±1.02)	2.99(±0.15)
5	t_{inh} = 1.14 (±0.06) [**5**] + 50.73 (±2.54)	3.83(±0.19)
6	t_{inh} = 1.31 (±0.07) [**6**] + 33.52 (±1.68)	4.40(±0.22)

[a] $R_i = R_g = 1.4 \times 10^{-6}$ [AAPH] s^{-1} = 3.36 μM·min^{-1} when 40 mM AAPH was employed, thus, *n* = coefficient ×3.36 μM·min^{-1}. [b] The constant was generated from the linear regression analysis.

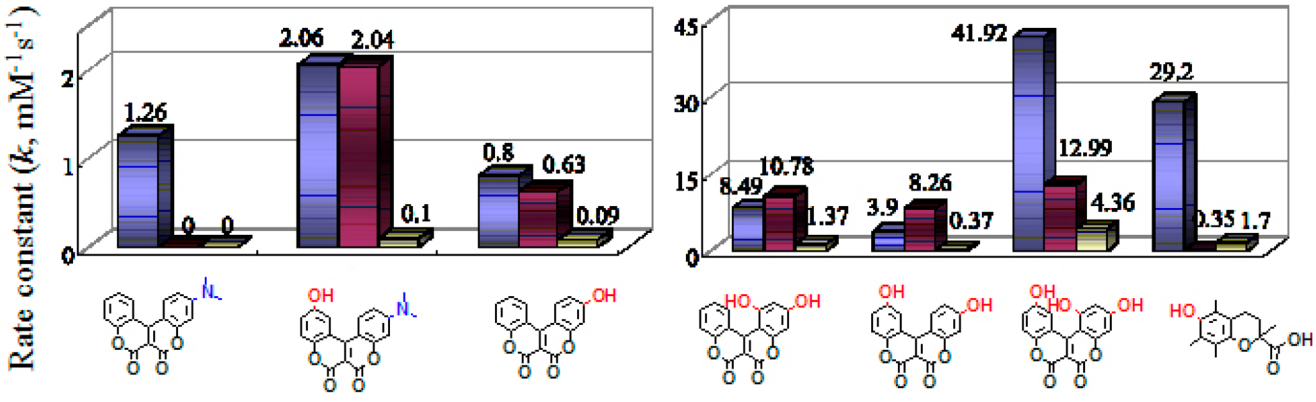
coumarin scaffold and *m*-substituted phenol because of the temperature controlled around 140 °C. We have attempted to enlarge the scale of suitable phenols for the annulation. As shown in Scheme 1, *ortho*- and *para*-substituted phenols are applied to carry out the reaction, and −OH, $-N(CH_3)_2$, and $-NH_2$ represent the electron-donating group (EDG), while $-NO_2$, −Br, and −Cl stand for the electron-withdrawing group (EWG). The annulation for forming coumarin-fused coumarin scaffold is not successful when the EDG or EWG (as R_2) locates at *ortho*- or *para*-position in the phenol because the first step in the formation of coumarin-fused coumarin is a transesterification between the methyl group in coumarin-3-carboxylate and the hydroxyl group in *meta*-substituted phenol, and then, the carbon atom at *para*-position in the phenol performs a C−C coupling reaction with the carbon atom in the lactone of coumarin. An EDG as $\mathbf{R_2}$ in the phenol is beneficial for the C−C coupling reaction, and other functional groups cannot take place in the same reaction. So, the limitation of the present synthetic protocol results in that only EDG at *meta*-position of phenol can successfully produce the coumarin-fused coumarin scaffold, and thus, we just explore the interaction of −OH (at the same or different benzene ring) and $-N(CH_3)_2$ (as a typical EDG) on the antioxidant effectiveness and do not mention coumarin-fused coumarins with other functional groups contained.

Inhibiting Cu^{2+}/GSH and •OH-Induced Oxidations of DNA. The glutathione radical (GS•) can be produced by the reaction of glutathione (GSH) and Cu^{2+},[24] while •OH can be produced by the reaction of H_2O_2 and tetrachlorohydroquinone (TCHQ).[25] DNA is susceptible to be oxidized by GS• and •OH, and the inhibitory effects on Cu^{2+}/GSH and •OH-induced oxidations of DNA are important index for an antioxidant. The oxidative products from the DNA oxidation can be readily detected after reacting with thiobarbituric acid (TBA).[26] Thus, a low percentage of thiobarbituric acid reactive substance (TBARS) (comparing with that in the control experiment as 100%) indicates that the antioxidant possesses high ability to inhibit the DNA oxidation. Scheme 2 outlines the TBARS percentage in the presence of 0.10 mM coumarin-fused coumarin added to the experimental systems of Cu^{2+}/GSH and •OH-induced oxidations of DNA with Trolox being the reference, which exhibits weak activities in inhibiting •OH- and Cu^{2+}/GSH-induced oxidation of DNA (TBARS% = 71.0 and 88.3, respectively).

The TBARS percentages of coumarin-fused coumarins in inhibiting •OH-induced oxidation of DNA are lower than those in inhibiting Cu^{2+}/GSH-induced oxidation of DNA. Hence, coumarin-fused coumarins possess higher abilities to inhibit •OH-induced oxidation of DNA than to inhibit Cu^{2+}/GSH-induced DNA oxidation. An antioxidant may inhibit Cu^{2+}/GSH-induced oxidation of DNA by chelating copper ions.[27] A single hydroxyl group such as Trolox (TBARS % = 88.3) and **3** (TBARS % = 96.4) cannot exhibit high activity for chelating Cu^{2+}, and antioxidant effectiveness is low in this case. With the amount of phenolic hydroxyl groups increasing, the TBARS % decreases from 73.9 of **4** to 58.5 of **5**. The comparison of TBARS % of **4** (73.9) with **6** (72.1) leads to an interesting conclusion that the hydroxyl group at benzene ring **A** seems not very active in inhibiting Cu^{2+}/GSH-induced DNA oxidation. But the lowest TBARS % of **5** (58.5) demonstrates that double hydroxyl groups locating at benzene rings **A** and **B**, respectively, exhibit the highest ability in this case. Taking **1** and **2** into consideration, it can be found that the TBARS % of **1** (73.1), similar to that of **4** (73.9), indicates that -$N(CH_3)_2$ (an EDG) at benzene ring **B** is equivalent to double hydroxyl groups for inhibiting Cu^{2+}/GSH-induced DNA oxidation. In addition, as in **2** (TBARS % = 64.6), -$N(CH_3)_2$ (at benzene ring **B**) and a hydroxyl group (at benzene ring **A**) are beneficial for chelating copper ion, and the decrease of the amount of copper ion cannot generate much more GS· for oxidizing DNA.

Scheme 2 also reveals that coumarin-fused coumarins are more active in inhibiting DNA oxidation caused by •OH than

Scheme 3. Rate Constant (*k*, Data on the Column) for Coumarin-Fused Coumarins and Trolox in Scavenging $ABTS^{+\bullet}$ (Left Column for Every Compound), DPPH (Middle Column for Every Compound) and Galvinoxyl Radical (Right Column for Every Compound)



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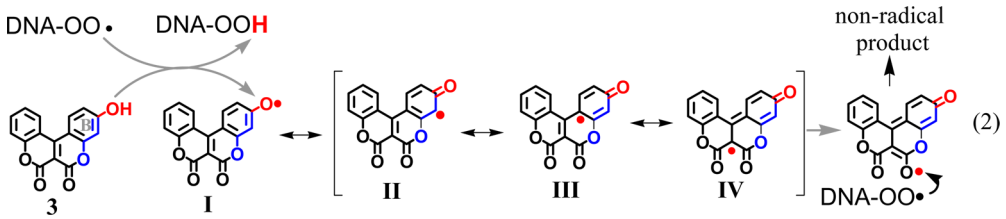

(2)

by Cu^{2+}/GSH. As we have pointed out in our previous work, the skeleton of a fused heterocycle is beneficial for the electrophilic addition by •OH.[9] Thus, the double latones in coumarin-fused coumarin may also be able to accommodate •OH, and thus, it may play antioxidative role in •OH-induced oxidation of DNA. Moreover, it can be found that more hydroxyl groups attaching to the benzene ring **B** increase the antioxidant effect of **4** (TBARS % = 21.2), and hydroxyl group at benzene ring **A** is not active because TBARS % of **6** (36.3, another hydroxyl group attaching to benzene ring **A** comparing with **4**) is higher than that of **4** (21.2). But all the efforts from phenolic hydroxyl groups on inhibiting •OH-induced oxidation of DNA cannot reach the level of the contribution from -$N(CH_3)_2$ at benzene ring **B** because **1** has the lowest TBARS % (17.2) among these compounds. On the other hand, TBARS % of **2** increases to 55.3, demonstrating again that the hydroxyl group attaching to benzene ring **A** may not be a positive factor in inhibiting •OH-induced oxidation of DNA.

Inhibiting AAPH-Induced Oxidation of DNA. The aforementioned experimental systems for evaluating antioxidant effectiveness are based on qualitative comparison. We have estimated a method for quantitatively expressing antioxidant effectiveness by using the experimental system of AAPH-induced oxidation of DNA.[9] As shown in Figure 1, an increase of the absorbance means that much more TBARS is generated for the longer reaction time than in the control experiment. But TBARS cannot be formed for a period when a coumarin-fused coumarin is added to the reaction system. Then, TBARS is produced as in the control experiment. The *inhibition period* (t_{inh}) is measured from the beginning of the reaction to the cross point of tangents for the inhibiting and the increasing period in TBARS absorbance line. Then, relationships between t_{inh} and concentrations of coumarin-fused coumarins are outlined in Figure 2 and expressed by the equation of $t_{inh} \sim$ [coumarin-fused coumarin] (see Table 1). Based on equations of $t_{inh} \sim$ [coumarin-fused coumarin], numbers of radical trapped by a coumarin-fused coumarins can be calculated by chemically kinetic method.

In view of chemical kinetics, the t_{inh} correlates proportionally with the concentration of an antioxidant as shown as eq 1.[28]

$$t_{inh} = (n/R_i)[\text{antioxidant}] \quad (1)$$

R_i refers to the initiation rate of the radical-induced reaction, and n is the *stoichiometric factor*. Both AAPH and the sodium salt of DNA are water-soluble compounds, and radicals generated from AAPH can attack DNA at water phase. R_i is thereby assumed to be equal to the radical generation rate (R_g, $R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] s^{-1}[28]). The coefficient in the equation of $t_{inh} \sim$ [coumarin-fused coumarin] (see Table 1) is equivalent to (n/R_i) in eq 1. Hence, when R_i is equal to $R_g = 1.4 \times 10^{-6} \times 40$ mM·s^{-1} = 3.36 μM·min^{-1}, the n is the product of the coefficient in the equation (see Table 1) and R_i. The obtained n values of coumarin-fused coumarins are involved in Table 1 as well.

The usage of Trolox in AAPH-induced oxidation of DNA does not generate t_{inh}, and the n of Trolox cannot be obtained. As shown in Table 1, the n of **3** is 1.81, meaning that **3** can trap ~**2** radicals in protecting DNA against AAPH-induced oxidation. This can be understood by eq 2, in which DNA-OO• refers to the radical deriving from the DNA oxidation. DNA-OO· can abstract a hydrogen atom from hydroxyl group in **3**, producing a phenoxyl radical (**I**). The phenoxyl radical (**I**) accommodates the single electron via a series of resonance structures, **II**, **III**, and **IV**, and then to form another phenoxyl radical at C═O for quenching another DNA-OO•. So, two radicals trapped by **3** can be regarded as the contribution from the hydroxyl group and the adjacent lactone motif.

Moreover, the n values of **4**, **5**, and **6** are 2.99, 3.83, and 4.40, respectively, implying that **4**, **5**, and **6** (with 2, 2, and 3 hydroxyl groups, respectively) can trap ~3, ~4, and 4−5 radicals, respectively. The numbers of trapped radicals increase with the amount of hydroxyl group. But the n of **1** (3.53) is higher than that of **3** (1.81), indicating that -$N(CH_3)_2$ can improve the radical-scavenging property of the lactone motifs in the absence of hydroxyl group. So, the ring of lactone in the fused coumarin is an efficient radical-receptor in the presence of electron-donating group.[9] The n value of **2** is further increased to 5.85 by the hydroxyl group at benzene ring **A**, demonstrating that -$N(CH_3)_2$ in combination with hydroxyl group at different benzene rings can improve the ability to inhibit AAPH- induced oxidation of DNA.

Rate Constants in Scavenging Radicals. The radical-scavenging test reveals the direct interaction of an antioxidant with radicals, in which $ABTS^{+\bullet}$, DPPH, and galvinoxyl radicals are usually applied to evaluate the ability of the antioxidant to donate its hydrogen atom to *N*- and *O*-centered radicals, respectively. The expression on the radical-scavenging ability usually focuses on the amount of radicals trapped by the antioxidant.[29] We have developed a chemically kinetic method for measuring the rate constant (k) of the antioxidant quenching radicals.[30] Briefly, the concentrations of radicals decreases rapidly when coumarin-fused coumarins are mixed with radicals (see Supporting Information). The decay of the concentrations of radicals can be quantitatively expressed by eq 3, in which t represents the reaction time.

$$[\text{radical}] = Ae^{-(t/a)} + Be^{-(t/b)} + C \quad (3)$$

The differential operation is performed on eq 3 to obtain the reaction rate (r), −d[radical]/dt, as shown in eq 4.

$$-\text{d}[\text{radical}]/\text{d}t = r = (A/a)e^{-(t/a)} + (B/b)e^{-(t/b)} \quad (4)$$

If the reaction time is assigned to 0 (the beginning of the reaction), the reaction rate at $t = 0$ (r_0) can be calculated by eq 4. Meanwhile, [radical] and [antioxidant] at $t = 0$ are concentrations of radical and antioxidant at the beginning of the reaction. So, the rate constant (k) can be calculated by eq 5 (see Supporting Information).

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J. Agric. Food Chem. 2015, 63, 3516−3523

$$r_0 = k[\text{radical}][\text{antioxidant}] \quad (5)$$

The rate constants (k) of coumarin-fused coumarins together with Trolox are outlined in Scheme 3. It can be found that k of **3** (only one hydroxyl group contained) in reducing $ABTS^{+\bullet}$ (0.80 $mM^{-1}\cdot s^{-1}$) is only 0.80 $mM^{-1}\cdot s^{-1}$, while those of **4** (two hydroxyl groups contained) and **6** (three hydroxyl groups contained) are 8.49 $mM^{-1}\cdot s^{-1}$ and 41.92 $mM^{-1}\cdot s^{-1}$, respectively. So, the ability of coumarin-fused coumarin to trap radical is related to the amount of hydroxyl group. The high k values of **3**, **4**, and **6** in trapping DPPH (0.63, 10.78, and 12.99 $mM^{-1}\cdot s^{-1}$, respectively) and galvinoxyl radical (0.09, 1.37, and 4.36 $mM^{-1}\cdot s^{-1}$, respectively) indicate that coumarin-fused coumarin prefers to donating the hydrogen atom in −OH to *N*- and *O*-centered radicals. In particular, three hydroxyl groups in **6** increase the radical-scavenging property even higher than that of Trolox. But k values of **4** (in trapping three radicals) are higher than those of **5**, implying that two hydroxyl groups locate at the different benzene rings are not beneficial for scavenging radicals. Moreover, **1** (without hydroxyl group contained) can reduce $ABTS^{+\bullet}$, indicating that $-N(CH_3)_2$ makes the scaffold of coumarin-fused coumarin a radical-scavenger.

In conclusion, $-N(CH_3)_2$ and the hydroxyl group play synergistically antioxidative role for coumarin-fused coumarin even though they do not locate at the same benzene ring. In particular, $-N(CH_3)_2$-involved coumarin-fused coumarin shows the highest activity in inhibiting $^{\bullet}OH$-induced oxidation of DNA even in the absence of a hydroxyl group. Therefore, $-N(CH_3)_2$ is an assisting group for the antioxidant effectiveness in the coumarin-fused heterocycles.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra of the synthetic compounds, the decay of the concentration of radicals together with the calculation of the rate constant (k) in quenching radicals. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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