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Coumarin moiety can enhance abilities of chalcones to inhibit DNA oxidation and to scavenge radicals

Gao-Lei Xi, Zai-Qun Liu *

Department of Organic Chemistry, College of Chemistry, Jilin University, Changchun 130021, China

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ABSTRACT

Coumarin and chalcone are naturally occurring compounds, and coumarin as a functional group was combined with chalcone in this work, aiming to test the inhibitory effects of coumarin-substituted chalcones on the oxidation of DNA and on scavenging radicals. It was found that the antioxidant activity of hydroxyl group attaching to coumarin can be increased by hydroxyl groups attaching to chalcone. The double hydroxyl groups at adjacent position exhibited high abilities to inhibit Cu^{2+}/glutathione-induced oxidation of DNA and to trap 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$) as well as 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH). Especially, the double hydroxyl groups in chalcone were able to protect DNA against 2,2′-azobis(2-amidinopropanehydrochloride) (AAPH)-induced oxidation significantly. On the other hand, the hydroxyl group attaching to coumarin exhibited high ability to inhibit $^{\bullet}OH$-induced oxidation of DNA. Therefore, coumarin-appended chalcones exhibited higher antioxidant effectiveness with only single or double phenolic hydroxyl groups contained.

1. Introduction

Coumarin, a natural compound,[1] exhibits various biological properties, such as anticancer,[2,3] antimicrobial,[4,5] antiviral,[6] antioxidant,[7,8] anti-inflammatory,[9] and enzymatic inhibitor,[10] and are thereby used as food additives and medicines. Especially, 4-methylcoumarin is a popular scaffold for constructing novel antioxidants.[11–13] On the other hand, chalcones also possess various biological properties[14] including antioxidant[15] and anticancer activities,[16] and are usually applied as a basic structure for preparing novel antioxidants.[17] In our previous work, we found that the inhibitory effect of 4-methylcoumarin on the radical-mediated oxidation of DNA can be increased by the moiety of 4,5-dihydropyrazole.[18,19] The aforementioned backgrounds motivate us to explore whether the combination of chalcone with coumarin can be a novel antioxidant with high activity. Hence, as shown in Scheme 1, 4-methylcoumarin was prepared by the reaction of resorcinol with ethyl acetoacetate, followed by acetylizing to afford chalcones via Claisen–Schmidt condensation. The obtained coumarin-substituted chalcones contain only one or two hydroxyl groups in order to evaluate the antioxidant properties of coumarin-modified chalcones in the case of few hydroxyl groups contained.

A large body of clinic evidences elucidates that the pathogenesis for many fatal diseases correlates with the free radical-initiated oxidations of membrane, lipid, protein, and DNA.[20,21] Hence, the inhibitory effect on the oxidation of DNA and on radicals are major index for evaluating the antioxidant capacity.[22] The oxidations of DNA caused by 2,2′-azobis(2-amidino propane hydrochloride) (AAPH, R–N=N–R, R=–$CMe_2C(=NH)NH_2$),[23] Cu^{2+}/glutathione (GSH),[24] and hydroxyl radical ($^{\bullet}OH$)[25] are usually used as the experimental systems for estimating the antioxidant ability. In addition, the radical-scavenging capacities of antioxidants can be estimated by reacting with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$) and 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH). Presented here is a study on the inhibitory effects of seven coumarin-substituted chalcones on Cu^{2+}/GSH-, $^{\bullet}OH$-, and AAPH-induced oxidation of DNA and on trapping $ABTS^{+\bullet}$ and DPPH.

2. Results and discussion

2.1. Effects of coumarin-substituted chalcones on Cu^{2+}/GSH-induced oxidation of DNA

The intracellular GSH and Cu(II) may destroy DNA because the produced GSH radical (GS•) can oxidize DNA to form carbonyl species, which can be detected after reacting with TBA. The formed

* Corresponding author. Tel.: +86 431 88499174; fax: +86 431 88499159; e-mail address: zaiqun-liu@jlu.edu.cn (Z.-Q. Liu).

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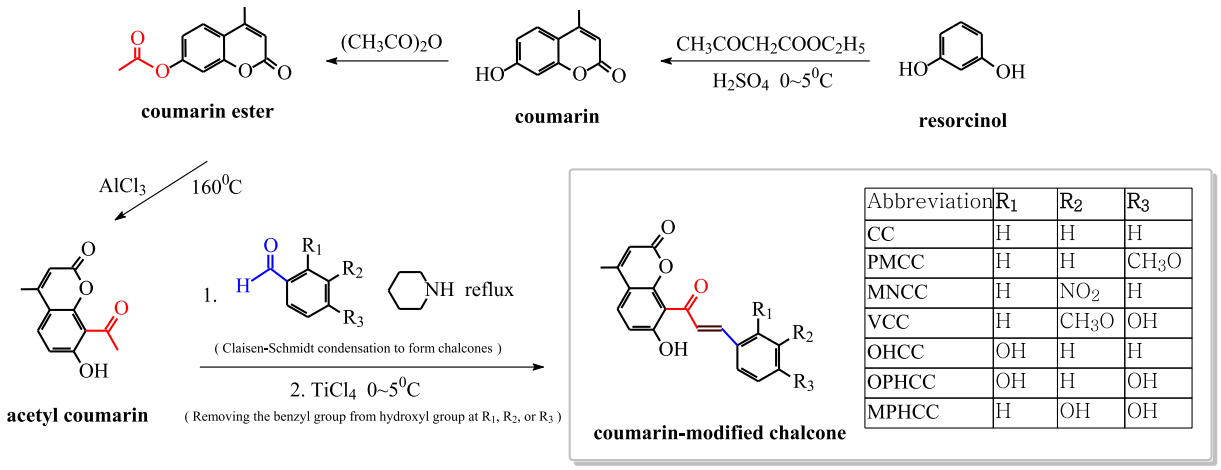


Abbreviation	R_1	R_2	R_3
CC	H	H	H
PMCC	H	H	CH_3O
MNCC	H	NO_2	H
VCC	H	CH_3O	OH
OHCC	OH	H	H
OPHCC	OH	H	OH
MPHCC	H	OH	OH

Scheme 1. Synthetic routine of coumarin-substituted chalcones.

TBA reactive substance (TBARS) can be measured at 535 nm.[24] As shown in Fig. 1, the increase of the absorbance line in the blank experiment indicates that the TBARS is produced successively in the mixture of DNA, Cu^{2+}, and GSH with the reaction period increasing. Although the increasing tendency of the absorbance line cannot be inhibited completely by adding 0.4 mM coumarin-substituted chalcones, all the absorbance lines locate below that of the blank experiment. So, all the coumarin-substituted chalcones used herein can retard GS•-induced destroy of DNA. The potential antioxidant group in CC is the hydroxyl group at coumarin moiety, thus, hydroxyl group at coumarin just exhibits a weak activity in this case. The absorbance lines of PMCC and MNCC approach to that of CC, indicating that *para*-methoxyl group in PMCC together with *meta*-nitro group in MNCC are not active groups, while the hydroxyl group attaching to coumarin moiety in PMCC and MNCC still plays the inhibitory role in GS•-induced destroy of DNA. Moreover, the additions of VCC and OPHCC do not obviously vary the position of the absorbance lines, indicating that *para*-hydroxyl group at chalcone moiety cannot ameliorate the inhibition effects on GS•-induced degradation of DNA. On the other hand, relative low position of the absorbance line of MPHCC reveals that MPHCC possesses relative high activity in this case, implying that *meta*-, *para*-dihydroxyl groups are beneficial for MPHCC to chelate Cu^{2+}, and therefore, inhibit the reaction between Cu^{2+} and GSH to form GS•.

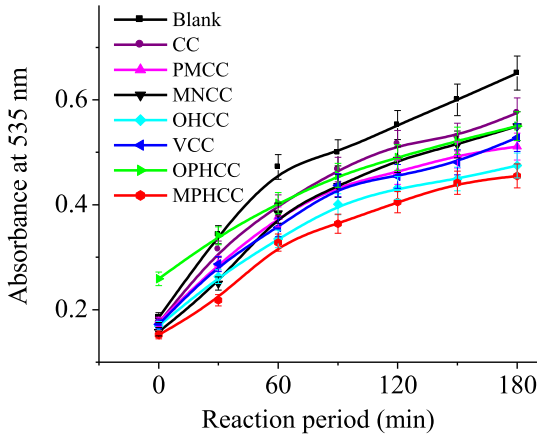


Fig. 1. The increase of the absorbance at 535 nm in the absence and presence of 0.40 mM coumarin-substituted chalcones in the mixture of 2.0 mg/mL DNA, 5.0 mM Cu^{2+}, and 3.0 mM GSH.

In addition, according to the classic concept on the antioxidant effectiveness, *ortho*-dihydroxyl benzene can be easily oxidized to form *ortho*-benzoquinone, during which the antioxidative activity is markedly enhanced.

2.2. Effects of coumarin-substituted chalcones on ·OH-induced oxidation of DNA

·OH is produced from H_2O_2 in the presence of tetrachlorohydroquinone (TCHQ).[25] The ribosyl moiety in DNA is the target for ·OH-induced oxidation, and the oxidative products can be detected as TBARS.[26] The absorbance of TBARS in the blank experiment of ·OH-induced oxidation of DNA is assigned as 100%. Fig. 2 outlines the percentages of TBARS in the presence of 0.40 mM coumarin-substituted chalcones, which can exhibit inhibition effects on ·OH-induced oxidation of DNA because the TBARS percentages are lower than 100%. The activities of CC, MNCC, and OHCC are higher than other compounds, especially, CC can decrease the percentage of TBARS to 64.0%, the lowest TBARS percentage reveals that hydroxyl group attaching to coumarin moiety is beneficial for inhibiting ·OH-induced oxidation of DNA. The inhibitory effect on ·OH is generally regarded as ·OH adding to benzene ring.[27] CC contains few functional groups and can provide much more positions for the addition of ·OH, as a result, CC exhibits relative high activity in inhibiting ·OH-induced oxidation of DNA, while more hydroxyl groups contained do not increase the activities of OPHCC and MPHCC.

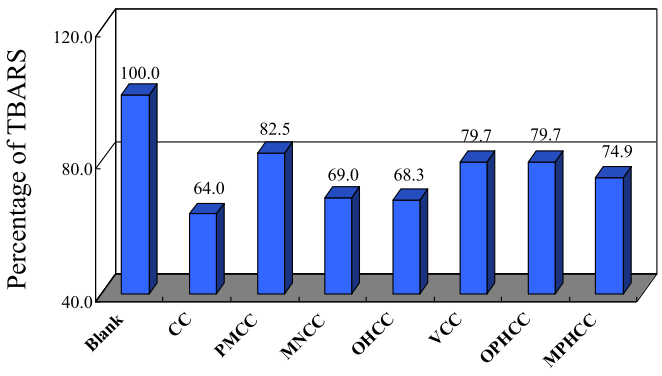
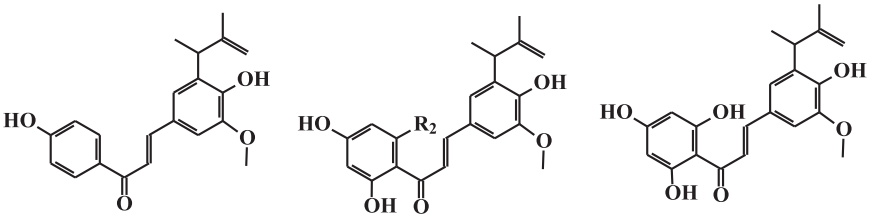


Fig. 2. The percentages of TBARS in the mixture of 2.0 mg/mL DNA, 4.0 mM H_2O_2, 2.0 mM TCHQ, and 0.40 mM coumarin-substituted chalcones at 37 °C for 30 min.

As shown in Scheme 2, we have synthesized some natural licochalcones and evaluated their abilities to inhibit ·OH-induced oxidation of DNA.[28] It was found that HMP, DHM, and HMT cannot prohibit ·OH-induced oxidation of DNA as efficiently as coumarin-substituted chalcones because the TBARS percentages of licochalcones (all the percentages of TBARS are higher than 90.0%) are higher than those of coumarin-substituted chalcones (all the percentages of TBARS are lower than 85%). So, 7-hydroxylcoumarin is able to enhance the inhibitory effects on ·OH-induced oxidation of DNA even in the case of few hydroxyl group contained (as CC, whose percentage of TBARS is 64.0%).



	HMP	DHM	HMT
Percentage of TBARS in inhibiting ·OH-induced oxidation of DNA	91.3%	105.8%	98.2%
Stoichiometric factor (n) in inhibiting AAPH-induced oxidation of DNA	1.68	2.42	5.61

Scheme 2. Some natural licochalcones applied to inhibit ·OH- and AAPH-induced oxidation of DNA as reported in our previous work.[28]

2.3. Effects of coumarin-substituted chalcones on AAPH-induced oxidation of DNA

The guanine bases in DNA can be oxidized by peroxyl radicals generated from AAPH,[29] and the oxidative process can also be followed by measuring TBARS. The absorbance line in the blank experiment indicates that the amount of TBARS increases with the reaction period (see Fig. 3).

The additions of CC and MNCC cannot inhibit the increase of TBARS even the concentration increases to 400 μM. Hence, the hydroxyl group at coumarin moiety is not active in this case. But the addition of 400 μM PMCC retards the increase of the absorbance line for a period, and then the absorbance line recovers as the blank experiment. The inhibition period (t_{inh}) can be measured by the cross-point from the tangent lines for the inhibition and oxidation period. Although the *para*-methoxyl group in PMCC cannot form a conjugation system with the hydroxyl group at coumarin moiety, it is still beneficial for enhancing the antioxidant ability of PMCC. This intramolecular synergistic effect among hydroxyl groups via a long distance was found in our previous work, in which ferrocene group can enhance the antioxidant effect of hydroxyl group in ailanthoidol on AAPH-induced oxidation of DNA.[30] The additions of VCC, OHCC, OPHCC, and MPHCC can generate t_{inh} (see Fig. 3), which is measured and plotted versus the concentration as shown in Fig. 4. The lines in Fig. 4 are expressed by the quantitative equations as listed in Table 1.

The t_{inh} is proved to be proportionally related to the concentration of the antioxidant as shown as Eq. 1.[31]

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

The stoichiometric factor (n) means the number of the radical-propagation terminated by one molecule of the antioxidant. R_i, the initiation rate of the radical-induced reaction, is assumed to be equal to the generation rate (R_g) of radicals ($R_g=(1.4\pm0.2)\times10^{-6}$ [AAPH] s^{-1})[31] because both sodium salt of DNA and AAPH are dissolved in water, and radicals resulting from AAPH can attack DNA at the same phase.[32] The values of n of coumarin-substituted chalcones are the product of the coefficients in the equation and $R_i=R_g=1.4\times10^{-6}\times40$ mM $s^{-1}=3.36$ μM min^{-1} (see Table 1). For example, the coefficient in $t_{inh}\sim$[OHCC] is 0.61, which multiplies 3.36 μM min^{-1} to give the n of OHCC, 2.07. So, OHCC can trap ~2 radicals in protecting DNA against AAPH-induced oxidation. Accordingly, the n values of MPHCC, VCC, and OPHCC are 2.92, 2.96, and 3.86, implying that MPHCC, VCC, and OPHCC can trap ~3, ~3, and ~4 radicals, respectively. The antioxidant effectiveness follows the order of OPHCC>VCC~MPHCC>OHCC. The characteristic structure of the aforementioned compounds is to contain hydroxyl group at chalcone moiety. The antioxidant activity of *ortho*-hydroxyl group (in OHCC) is not as high as *para*-hydroxyl group (in VCC and MPHCC). But *ortho*-, *para*-dihydroxyl groups (in OPHCC) increase the antioxidant activity more markedly than *meta*-, *ortho*-dihydroxyl groups (in MPHCC). This is not in agreement with previous report that dihydroxyl groups at adjacent position are beneficial for increasing the antioxidant activity.[33] However, as reported in our previous study on licochalcones, the n values of HMP, DHM and HMT are 1.68, 2.42, and 5.61, respectively (see Scheme 2),[28] while the n value of OPHCC is 3.86, indicating that double hydroxyl groups at *meta*-position may increase the inhibitory effect on AAPH-induced oxidation, and *ortho*-, *para*-dihydroxyl groups may interact with the hydroxyl group at coumarin moiety via intramolecular synergistic effect to enhance the antioxidant activity.

The inhibitory effects of coumarin-substituted chalcones on Cu^{2+}/GSH-, ·OH-, and AAPH-induced oxidation of DNA can be summarized by Scheme 3. The peroxyl radical resulting from AAPH is able to abstract hydrogen atom at C′-4 position of DNA, and the produced single electron at DNA can be recovered by the hydroxyl group of CC (taken as the example compound). The hydroxyl group and/or carbonyl group are able to chelate copper ion in inhibiting Cu^{2+}/GSH-mediated oxidation of DNA, while CC can quench ·OH by donating its hydrogen atom or by accepting ·OH as an electrophile.

2.4. Rate constant of coumarin-substituted chalcones to scavenge $ABTS^{+\cdot}$ and DPPH

$ABTS^{+\cdot}$ and DPPH are usually used to test the ability of an antioxidant to trap radicals.[34,35] The rate constants (***k***) in trapping $ABTS^{+\cdot}$ and DPPH are important index for antioxidative ability. The additions of VCC, OHCC, OPHCC, and MPHCC lead to the decay of

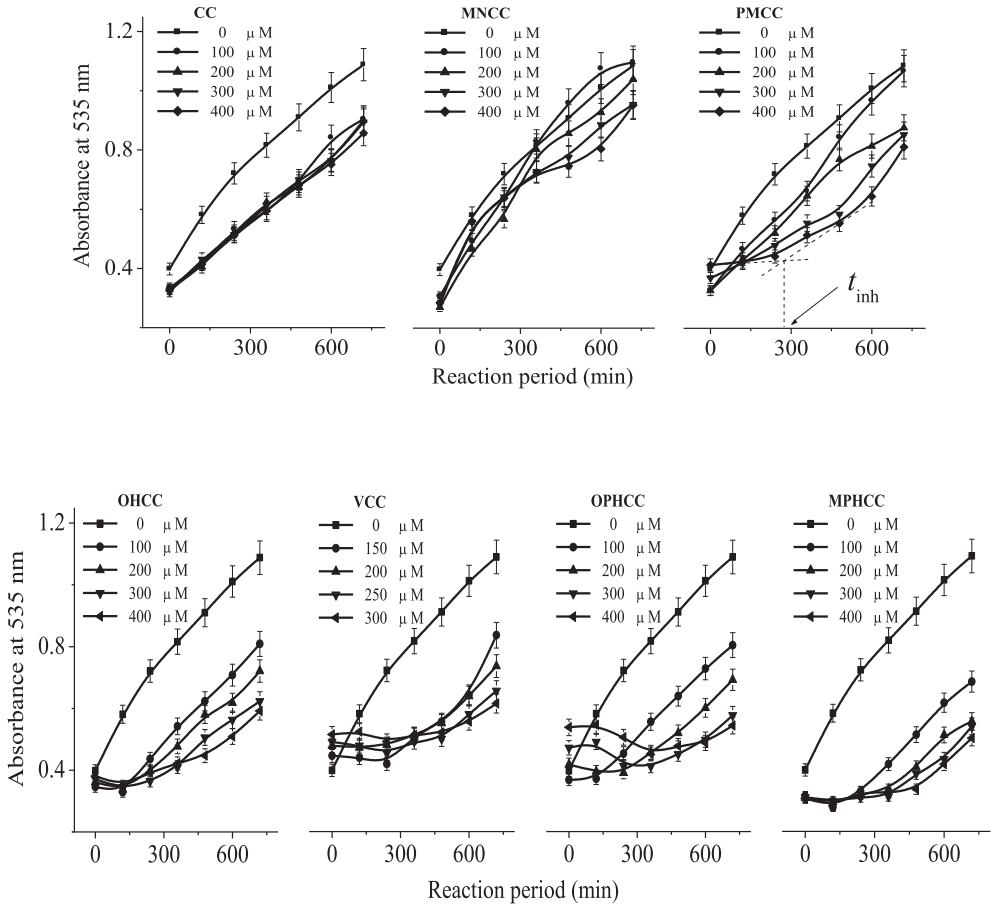


Fig. 3. The variation of the absorbance of TBARS in the mixture of 2.0 mg/mL DNA, 40 mM AAPH, and various concentrations of coumarin-substituted chalcones at 37 °C.

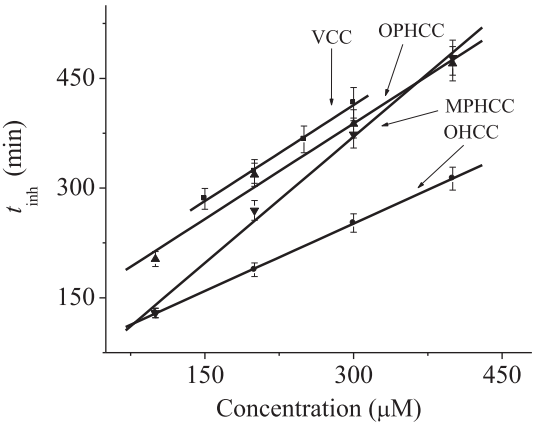


Fig. 4. The linear relationship between the concentrations of coumarin-modified chalcones and inhibition period (t_{inh}) in protecting DNA against AAPH-induced oxidation.

Table 1
The equations of t_{inh} ~[coumarin-substituted chalcones] and n of coumarin-substituted chalcones in protecting DNA against AAPH-induced oxidation[a]

Antioxidant	t_{inh} (min)=(n/R_i) [coumarin-substituted chalcones (μM)]+constant[b]	n
OHCC	t_{inh}=0.61 (±0.03) [OHCC]+66.96 (±3.35)	2.07(±0.10)
MPHCC	t_{inh}=0.87 (±0.04) [MPHCC]+127.01 (±6.35)	2.92(±0.15)
VCC	t_{inh}=0.88 (±0.04) [VCC]+150.94 (±7.55)	2.96(±0.15)
OPHCC	t_{inh}=1.15 (±0.06) [OPHCC]+24.85 (±1.24)	3.86(±0.19)

[a] R_i=R_g=1.4×10^{-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.

the concentrations of ABTS$^{+\bullet}$ and DPPH (see Fig. 5), indicating that these chalcones can quench radicals, while others cannot. Therefore, the hydroxyl group at coumarin moiety does not exhibit radical-scavenging property, and the hydroxyl groups at chalcone moiety play the major role in this case.

After the data in Fig. 5 are input into statistical software, the relationship between the concentrations of ABTS$^{+\bullet}$ and DPPH ([*radical*]) and reaction period (t) fits for a double exponential function (Eq. 2), and the results are listed in Tables 2 and 3.

$$[radical] = A\,e^{-(t/a)} + B\,e^{-(t/b)} + C \quad (2)$$

Moreover, as shown as Eq. 3, the differential style of Eq. 2 reveals the variation of the reaction rate ($\boldsymbol{r}$=$-d[radical]/dt$) with the reaction period (t). The results are contained in Tables 2 and 3 as well.

$$-d[radical]/dt = \boldsymbol{r} = (A/a)e^{-(t/a)} + (B/b)e^{-(t/b)} \quad (3)$$

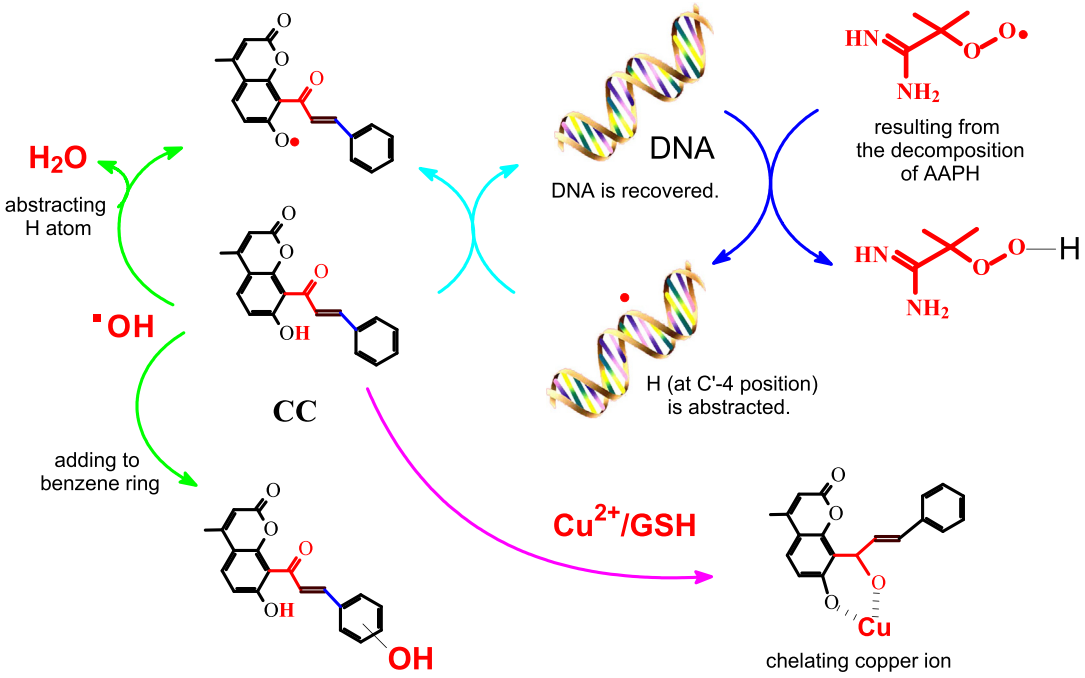
The reaction rate at t=0 ($\boldsymbol{r_0}$) can be calculated by Eq. 3 when t is assigned to be 0 (see Tables 2 and 3). According to the kinetic equation (Eq. 4), the reaction rate at t=0 ($\boldsymbol{r_0}$) is related to the concentrations of radical and antioxidant at the beginning of the reaction.

$$\boldsymbol{r_0} = \boldsymbol{k}[radical]_0[antioxidant]_0 \quad (4)$$

The concentrations of radical and antioxidant at t=0 together with $\boldsymbol{r_0}$ are known, and rate constant ($\boldsymbol{k}$) can be calculated by Eq. 5.

$$\boldsymbol{k} = \frac{\boldsymbol{r_0}}{[radical]_0[antioxidant]_0} \quad (5)$$

This method has been applied to calculate the rate constant ($\boldsymbol{k}$) of dihydropyrimidine in trapping ABTS$^{+\bullet}$ and DPPH,[36] and herein,



Scheme 3. A plausible mechanism for the oxidative modes of DNA by different initiators.

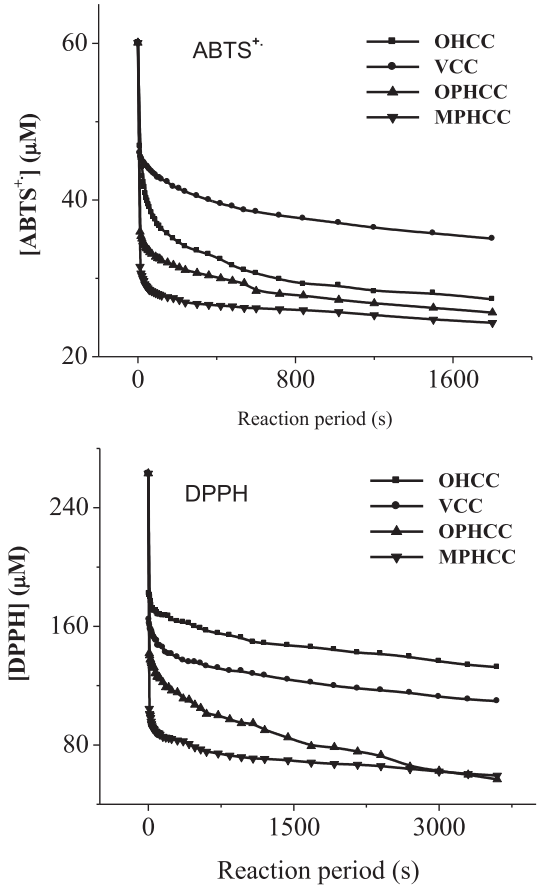


Fig. 5. Decay of 60 μM $ABTS^{+\cdot}$ in the presence of 10 μM coumarin-substituted chalcones, and decay of 263 μM DPPH in the presence of 15 μM coumarin-substituted chalcones.

the ***k*** values of VCC, OHCC, OPHCC, and MPHCC are also calculated and listed in Tables 2 and 3. Only one hydroxyl group at chalcone moiety (in VCC and OHCC) exhibits a weak radical-scavenging ability since the ***k*** values of VCC and OHCC are lower than those of OPHCC and MPHCC in trapping either $ABTS^{+\cdot}$ or DPPH. *ortho*-, *para*-Dihydroxyl groups increase the ability of OPHCC to trap these two radicals. In particular, *meta*-, *para*-dihydroxyl groups increase the ***k*** of MPHCC to the highest value, especially, in trapping $ABTS^{+\cdot}$. Hence, dihydroxyl groups at adjacent position in chalcone moiety possess high abilities to donate its hydrogen atom to *N*-centered radical (as DPPH) and to reduce radical directly (as $ABTS^{+\cdot}$).

The present result can be employed to justify the function of coumarin moiety. The compound CC with a hydroxyl group attaching to coumarin cannot trap $ABTS^{+\cdot}$ and DPPH, indicating that the coumarin moiety is not active group for trapping radicals. This phenomenon is also found in PMCC and MNCC, in which only one hydroxyl group is contained at coumarin moiety. On the contrary, OHCC contains a hydroxyl group at *ortho*-position of benzene ring and is able to trap $ABTS^{+\cdot}$ and DPPH with the rate constants (***k***) being 2.63 and 4.23 mM^{-1} s^{-1}, respectively. Thus, the hydroxyl group at benzene ring plays the major role in trapping radicals. Furthermore, MPHCC traps $ABTS^{+\cdot}$ and DPPH with the rate constants (***k***) being 148.0 and 10.70 mM^{-1} s^{-1}, respectively, while, as reported in our previous work, the ***k*** values of catechol are only 2.58 and 1.30 mM^{-1} s^{-1} to trap $ABTS^{+\cdot}$ and DPPH, respectively.[37] As the major functional group for trapping radicals, the activity of the catechol moiety in MPHCC is markedly enhanced by the coumarin moiety, and an intermolecular synergistic interaction is proved to be existed in coumarin-substituted chalcones.

3. Conclusion

Integrating coumarin with chalcone is a useful way to construct novel antioxidant. The antioxidant activity of hydroxyl group at chalcone can be enhanced by coumarin even in the absence of a conjugation system. The hydroxyl groups at different positions display different activities in inhibiting the oxidation of DNA.

Table 2
Equation of $[ABTS^{+\bullet}]\sim t$ and its differential style ($-d[ABTS^{+\bullet}]/dt\sim t$), reaction rate at t=0 ($\boldsymbol{r_0}$), and rate constant ($\boldsymbol{k}$)[a]

Compound	Equation of $[ABTS^{+\bullet}]$ (μM)$\sim t$ (s)	Equation of $-d[ABTS^{+\bullet}]/dt\sim t$	$\boldsymbol{r_0}$ (μM s^{-1})	$\boldsymbol{k}$ (mM^{-1} s^{-1})
OHCC	$[ABTS^{+\bullet}] = 18.83e^{-\frac{t}{12.24}} + 12.73e^{-\frac{t}{340.11}} + 28.11$	$-\frac{d[ABTS^{+\bullet}]}{dt} = 1.54e^{-\frac{t}{12.24}} + 0.04e^{-\frac{t}{340.11}}$	1.58	2.63
VCC	$[ABTS^{+\bullet}] = 15.03e^{-\frac{t}{4.27}} + 9.50e^{-\frac{t}{480.74}} + 35.53$	$-\frac{d[ABTS^{+\bullet}]}{dt} = 3.52e^{-\frac{t}{4.27}} + 0.02e^{-\frac{t}{480.74}}$	3.54	5.89
OPHCC	$[ABTS^{+\bullet}] = 25.56e^{-\frac{t}{3.93}} + 8.67e^{-\frac{t}{523.51}} + 25.82$	$-\frac{d[ABTS^{+\bullet}]}{dt} = 6.50e^{-\frac{t}{3.93}} + 0.02e^{-\frac{t}{523.51}}$	6.52	10.86
MPHCC	$[ABTS^{+\bullet}] = 21.21e^{-\frac{t}{0.24}} + 10.53e^{-\frac{t}{20.06}} + 26.96$	$-\frac{d[ABTS^{+\bullet}]}{dt} = 88.38e^{-\frac{t}{0.24}} + 0.52e^{-\frac{t}{20.06}}$	88.90	148.0

[a] The concentration of coumarin-substituted chalcone is 10 μM, and the concentration of $ABTS^{+\bullet}$ is 60.06 μM.

Table 3
Equation of $[DPPH]\sim t$ and its differential style ($-d[DPPH]/dt\sim t$), reaction rate at t=0 ($\boldsymbol{r_0}$), and rate constant ($\boldsymbol{k}$)[a]

Compound	Equation of [DPPH (μM)]$\sim t$	Equation of $-d[DPPH]/dt\sim t$	$\boldsymbol{r_0}$ (μM s^{-1})	$\boldsymbol{k}$ (mM^{-1} s^{-1})
OHCC	$[DPPH] = 89.35e^{-\frac{t}{5.36}} + 39.78e^{-\frac{t}{1235.41}} + 133.60$	$-\frac{d[DPPH]}{dt} = 16.67e^{-\frac{t}{5.36}} + 0.03e^{-\frac{t}{1235.41}}$	16.70	4.23
VCC	$[DPPH] = 107.89e^{-\frac{t}{4.79}} + 41.39e^{-\frac{t}{827.33}} + 133.61$	$-\frac{d[DPPH]}{dt} = 22.52e^{-\frac{t}{4.79}} + 0.05e^{-\frac{t}{827.33}}$	22.57	5.72
OPHCC	$[DPPH] = 129.47e^{-\frac{t}{4.21}} + 76.45e^{-\frac{t}{1293.15}} + 57.04$	$-\frac{d[DPPH]}{dt} = 30.75e^{-\frac{t}{4.21}} + 0.06e^{-\frac{t}{1293.15}}$	30.81	7.81
MPHCC	$[DPPH] = 169.14e^{-\frac{t}{4.01}} + 30.67e^{-\frac{t}{720.02}} + 63.16$	$-\frac{d[DPPH]}{dt} = 42.18e^{-\frac{t}{4.01}} + 0.04e^{-\frac{t}{720.02}}$	42.22	10.70

[a] The concentration of coumarin-substituted chalcone is 15 μM, and concentration of DPPH is 263.08 μM.

Dihydroxyl groups at adjacent position can inhibit Cu^{2+}/GSH-induced oxidation of DNA and trap radicals efficiently, while dihydroxyl groups at *ortho*-, *para*-position are useful for protecting DNA against AAPH-induced oxidation. The hydroxyl group at coumarin moiety plays the major role in inhibiting $^{\bullet}OH$-induced oxidation of DNA. Therefore, coumarin-substituted chalcones can be widely applied as antioxidants to inhibit the various styles of the oxidation.

4. Materials and methods

4.1. Materials and instrumentation

Diammonium salt of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS salt) and DPPH were purchased from Fluka Chemie GmbH, Buchs, Switzerland. AAPH, GSH, and the naked DNA sodium salt were purchased from Acros Organics, Geel, Belgium. Other agents were of analytical grade and used directly. The structures of coumarin-modified chalcones were identified by ^1H and ^{13}C NMR (Varian Mercury 300 NMR spectrometer).

4.2. Synthesis and structural characterization of coumarin-substituted chalcones

4.2.1. Preparation of coumarin. A mixture of resorcinol (3.30 g, 30 mmol) and ethyl acetoacetate (3.90 g, 30 mmol) was added to 98% H_2SO_4 under stirring at 0–10 °C for 2 h and at 25 °C for 2 days. Then the reaction mixture was poured into ice-cold water to obtain the solid, which was recrystallized by 95% ethanol to afford coumarin at 80% yield. Mp 181–183 °C.

4.2.2. Esterification of coumarin. Coumarin (4.36 g, 20 mmol) was refluxed in acetic anhydride (10 mL) for 2 h. Then the reaction mixture was poured into ice-cold water to obtain the solid, which was recrystallized by 95% ethanol to afford coumarin acetate at 95% yield. Mp 133–134 °C.

4.2.3. Formation of acetyl coumarin. Coumarin acetate (2.18 g, 10 mmol) was mixed with $AlCl_3$ (2 g, 15 mmol) and heated at 160 °C for 2 h. The reaction mixture was then cooled to room temperature and diluted by adding HCl aqueous solution. The obtained solid was recrystallized by 95% ethanol to afford acetyl coumarin at 72% yield. Mp 161–163 °C.

4.2.4. Protection of hydroxyl group in benzaldehyde. Before the hydroxyl-substituted benzaldehydes were employed as the reagents, the hydroxyl group was etherized by reacting with benzyl chloride. In brief, the preparation of 2-phenylmethoxyl benzaldehyde was taken as an example. 2-Hydroxybenzaldehyde (1.22 g, 10 mmol) and K_2CO_3 (2.07 g, 15 mmol) were refluxed in 10 mL of 95% ethanol for 0.5 h, then benzyl chloride (1.55 g, 12 mmol) was added and refluxed for 6 h. The reaction mixture was cooled to room temperature and filtered to remove inorganic salt. The organic solvent was removed under vacuum to yield 2-phenylmethoxylbenzaldehyde. The yields of this reaction were generally >90%.

4.2.5. Claisen–Schmidt condensation. Acetyl coumarin (0.436 g, 2.0 mmol), hydroxyl protected-benzaldehyde (2.5 mmol), and piperidine (0.25 mL) were mixed in 15 mL of ethanol and refluxed for 6 h. The reaction mixture was then cooled to room temperature, and the precipitates were filtered to obtained crude product, followed by recrystallization with 95% ethanol to afford hydroxyl-protected coumarin-modified chalcones.

4.2.6. Debenzylation from hydroxyl group. The protective group for the hydroxyl group was removed by $TiCl_4$. In brief, hydroxyl-protected coumarin-modified chalcone (1.0 mmol, dissolved in 10 mL of anhydrous CH_2Cl_2) was added dropwisely to 10 mL of 2.0 M CH_2Cl_2 solution of $TiCl_4$ within 30 min at 0 °C and stirred overnight at room temperature. The reaction mixture was poured into ice-cold water and extracted with ethyl acetate. The organic phase was washed with brine and dried over Na_2SO_4. The solvent was evaporated under vacuum. The crude product was purified by silica chromatography with ethyl acetate/chloroform (1:1, v:v) being eluent. The NMR data of coumarin-modified chalcones were listed as following.

CC, yield 76%. Mp 150–151 °C. ^1H NMR (300 MHz, $CDCl_3$) δ: 13.81 (s, 1H), 8.24–8.29 (m, 1H), 7.95–8.00 (m, 1H), 7.67–7.75 (m, 3H), 7.44 (s, 3H), 6.96 (d, J=9 Hz, 1H), 6.20 (s, 1H), 2.44 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 193.4, 167.3, 159.3, 154.8, 153.1, 145.9, 134.8, 131.1, 131.0, 130.2, 129.1, 128.2, 126.1, 125.3, 115.2, 112.0, 111.1, 109.6, 19.3.

PMCC, yield 84%. Mp 176–178 °C. ^1H NMR (300 MHz, $CDCl_3$) δ: 14.01 (s, 1H), 8.15–8.20 (m, 1H), 7.95–8.00 (m, 1H), 7.66–7.72 (m, 3H), 6.93–6.98 (m, 3H), 6.19 (s, 1H), 3.87 (s, 3H), 2.44 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 193.1, 167.3, 162.1, 159.3, 154.7, 153.1, 146.0, 130.9, 130.7, 127.6, 123.6, 115.1, 114.5, 111.9, 110.9, 109.7, 55.4, 19.2.

MNCC, yield 56%. Mp 224–226 °C. ^1H NMR (300 MHz, DMSO-d_6) δ: 10.98 (s, 1H), 8.58 (s, 1H), 8.26 (d, J=7.8 Hz, 2H), 7.69–7.76 (m, 2H), 7.55–7.60 (m, 1H), 7.34–7.40 (m, 1H), 6.97 (d, J=8.7 Hz, 1H), 6.20 (s, 1H), 2.42 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.3,

159.5, 158.2, 153.5, 151.6, 148.3, 143.1, 136.1, 134.2, 130.3, 127.4, 124.9, 123.7, 115.0, 112.8, 112.1, 110.6, 18.3.

VCC, yield 81%. M.p 240–241 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.92 (s, 1H), 9.74 (s, 1H), 7.71 (d, J=8.7 Hz, 1H), 7.32 (d, J=1.8 Hz, 1H), 7.20–7.25 (m, 1H), 7.13 (dd, J=8.4, 2.1 Hz, 1H), 7.00–7.05 (m, 1H), 6.95 (d, J=8.7 Hz, 1H), 6.78 (d, J=8.1 Hz, 1H), 6.18 (d, J=1.2 Hz, 1H), 3.80 (s, 3H), 2.41 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.2, 159.7, 158.2, 153.6, 151.5, 149.9, 148.0, 146.9, 127.0, 125.6, 125.2, 123.7, 115.6, 115.5, 112.8, 112.0, 111.8, 110.5, 55.7, 18.3.

OHCC, yield 88%. Mp 204–206 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.96 (s, 1H), 10.21 (s, 1H), 7.71 (d, J=8.7 Hz, 1H), 7.65 (dd, J=7.8, 1.2 Hz, 1H), 7.56–7.61 (m, 1H), 7.27 (td, J=7.5, 1.5 Hz, 1H), 7.12–7.17 (m, 1H), 6.96 (d, J=8.7 Hz, 1H), 6.89 (d, J=8.1 Hz, 1H), 6.85 (t, J=7.5 Hz, 1H), 6.19 (d, J=1.2 Hz, 1H), 2.42 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.6, 159.6, 158.2, 157.0, 153.7, 151.5, 141.4, 132.4, 128.8, 127.3, 127.2, 120.8, 119.6, 116.3, 115.4, 112.8, 112.0, 110.6, 18.3.

OPHCC, yield 88%. Mp 197–198 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.91 (s, 1H), 10.11 (s, 1H), 10.00 (s, 1H), 7.69 (d, J=8.7 Hz, 1H), 7.49 (d, J=5.4 Hz, 1H), 7.45 (d, J=1.8 Hz, 1H), 6.96 (d, J=3.3 Hz, 1H), 6.92 (d, J=3.9 Hz, 1H), 6.33 (d, J=2.4 Hz, 1H), 6.29 (dd, J=8.7, 2.4 Hz, 1H), 6.18 (s, 1H), 2.41 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 192.3, 161.8, 159.7, 158.9, 158.2, 153.7, 151.5, 142.3, 130.5, 127.0, 123.9, 115.7, 112.8, 112.7, 112.0, 110.5, 108.3, 102.5, 18.3.

MPHCC, yield 81%. Mp >266 °C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.93 (s, 1H), 9.72 (s, 1H), 9.17 (s, 1H), 7.70 (d, J=8.7 Hz, 1H), 7.14–7.19 (m, 1H), 7.06 (s, 1H), 6.92–6.99 (m, 2H), 6.74–6.85 (m, 2H), 6.17 (s, 1H), 2.40 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 191.9, 159.6, 158.2, 153.6, 151.5, 149.0, 146.8, 145.7, 127.1, 125.5, 124.6, 122.4, 115.8, 115.4, 114.9, 112.8, 112.0, 110.5, 18.3.

4.3. Cu^{2+}/GSH-induced oxidation of DNA test

Cu^{2+}/GSH-induced oxidation of DNA was carried out following a previous report[24] with a slight modification. Briefly, DNA, $CuSO_4$, and GSH were dissolved in phosphate buffered solution (PBS_1: 6.1 mM Na_2HPO_4, 3.9 mM NaH_2PO_4), and coumarin-substituted chalcones were dissolved in dimethyl sulfoxide (DMSO). Then, 2.0 mg/mL DNA, 5.0 mM Cu^{2+}, 3.0 mM GSH, and 0.4 mM coumarin-substituted chalcones were mixed to form a solution. The solution was poured into test tubes, and each test tube contained 2.0 mL. The test tubes were incubated at 37 °C to initiate the oxidation of DNA, and three of them were taken out at every 30 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% trichloroacetic acid aqueous solution. The test tubes were heated in boiling water for 30 min and cooled to room temperature, 1.5 mL of n-butanol was added and shaken vigorously to extract thiobarbituric acid reactive substance (TBARS) whose absorbance was measured at 535 nm.

4.4. ·OH-induced oxidation of DNA test

·OH was generated by mixing H_2O_2 with tetrachlorohy droquinone (TCHQ, dissolved in DMSO as the stock solution) as the description in a literature.[25] DNA and H_2O_2 were dissolved in phosphate buffered solution (PBS_2: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA). DNA (2.0 mg/mL), 4.0 mM TCHQ, 2.0 mM H_2O_2, and 0.4 mM coumarin-substituted chalcones (dissolved in DMSO as the stock solution) were mixed to form a solution. The solution was poured into test tubes, and each test tube contained 2.0 mL. The test tubes were incubated at 37 °C for 30 min and cooled immediately. The following operation was the same as in Cu^{2+}/GSH-induced oxidation of DNA except EDTA was not added. The absorbances in the control experiment and in the presence of coumarin-substituted chalcones were assigned as A_0 and A_{detect}, respectively. The effects of coumarin-substituted chalcones on ·OH-induced oxidation of DNA were expressed by $A_{detect}/A_0\times100$.

4.5. AAPH-induced oxidation of DNA test

The experiment of AAPH-induced oxidation of DNA was performed as the description in a literature.[23] Briefly, 2.0 mg/mL DNA, 40 mM AAPH, and a certain concentration of coumarin-substituted chalcones (dissolved in DMSO as the stock solution) were mixed to form a solution. The solution was poured into test tubes, and each test tube contained 2.0 mL. The test tubes were incubated at 37 °C to initiate the oxidation of DNA, and three of them were taken out at every 2 h and cooled immediately. The following operation was the same as in ·OH-induced oxidation of DNA except the heating period was 15 min after TBA and trichloroacetic acid were added. The absorbance of TBARS was plotted versus the incubation period.

4.6. Scavenging DPPH and $ABTS^{+\cdot}$

DPPH was dissolved in 50 mL of ethanol to make the absorbance around 1.00 at 517 nm ($\varepsilon_{DPPH}=4.09\times10^3$ M^{-1} cm^{-1}). $ABTS^{+\cdot}$ was produced from 2.0 mL of a mixture containing 4.0 mM ABTS aqueous solution and 1.41 mM $K_2S_2O_8$ after kept for 16 h and diluted by 100 mL of ethanol. The absorbance of $ABTS^{+\cdot}$ solution was around 1.00 at 734 nm ($\varepsilon_{ABTS^{+\cdot}}=1.6\times10^4$ M^{-1} cm^{-1}). The DMSO solutions of coumarin-modified chalcones (0.1 mL) were added to 1.9 mL of DPPH or $ABTS^{+\cdot}$ solution. The final concentrations of coumarin-substituted chalcones were 10 and 15 μM in trapping $ABTS^{+\cdot}$ and DPPH, respectively. The decreases of the absorbance of these radicals were recorded at 25 °C at a certain time interval.

4.7. Statistical analysis

All the data were the average value from at least three independent measurements with the experimental error within 10%. The equations were analyzed by one-way ANOVA in Origin 8 professional software, and $p<0.001$ indicated a significant difference.

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Supplementary data

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