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Original article

Antioxidant effectiveness generated by one or two phenolic hydroxyl groups in coumarin-substituted dihydropyrazoles

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ABSTRACT

A cascade operation was designed to synthesize nine coumarin-substituted dihydropyrazoles with only one or two phenolic hydroxyl groups contained. Antioxidant abilities of the obtained compounds were evaluated by inhibiting 2,2′-azobis(2-amidinopropanehydrochloride) (AAPH)-, Cu^{2+}/glutathione (GSH)-, and ·OH-induced oxidation of DNA. It was found that less phenolic hydroxyl groups can enhance the abilities of coumarin-substituted dihydropyrazoles to protect DNA against the oxidation. Moreover, these coumarin-substituted dihydropyrazoles were employed to scavenge 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\cdot}$), 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH), and galvinoxyl radical, respectively. It was found that double phenolic hydroxyl groups were more beneficial for enhancing the abilities of coumarin-substituted dihydropyrazoles to quench the aforementioned radicals. Therefore, dihydropyrazole linked with coumarin exhibited powerful antioxidant effectiveness even in the case of less phenolic hydroxyl groups involved.

1. Introduction

The *in vivo* oxidative stress of DNA is regarded as the pathogenesis of many fatal diseases [1]. The free radicals from the metabolism [2] or the environment [3] may abstract hydrogen atom from membrane, lipid, protein, and DNA, and consequently, lead to damages of many biological species [4]. The supplementation of antioxidants is beneficial for avoiding oxidative damages [5], keeping redox balance *in vivo* [6], and designing novel medicines [7]. The natural antioxidants attract much research attentions because of their high capacities and low toxicities. Natural polyphenols are antioxidants widely used to scavenge radicals [8]. On the other hand, many efforts are contributed to synthesize antioxidants containing phenolic hydroxyl groups [9]. Increasing the amount of phenolic hydroxyl groups may enhance the antioxidant effectiveness according to traditional concept. However, it was recently found that more phenolic hydroxyl groups contained may cause prooxidative effect and improve the risk in the biological systems. For example, hydroxycinnamic acids containing more than two phenolic hydroxyl groups accelerated the oxidation of DNA in the presence of Cu(II) ions. This was because phenolic hydroxyl groups can reduce Cu(II) to form Cu(I), and Cu(I) can cause the decomposition of DNA [10]. Hence, it is worthy to explore whether a compound containing less than two phenolic hydroxyl groups can also function as an antioxidant.

2. Chemistry

The modification on the structure of natural antioxidants [11] and the recombination of known antioxidative structures [12] are the efficient ways to increase the antioxidant ability. For example, polyphenols were used to link with 4-amino methylbenzylamine or tris(2-aminoethyl)amine to form dendritic structure. As a result, the abilities of the obtained antioxidants to scavenge 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) [13] and to inhibit 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH, R–N=N–R, R = $-CMe_2C(=NH)NH_2$)- and Cu^{2+}-induced oxidations of low-density lipoprotein and DNA were improved markedly [14]. Moreover, we applied pyrazole [15] and 1,2,4-oxadiazole [16] to bridge with phenols. It has been found that single phenolic hydroxyl group in these compounds exhibited high abilities to inhibit AAPH-induced oxidation of DNA and to scavenge radicals. This result reveals that a suitable arrangement of every structural feature can lead to high antioxidant effectiveness even in the case of less phenolic hydroxyl group involved. On the other hand, coumarin is an important skeleton widely used to construct anticancer [17], antimicrobial [18], antiviral [19], and anti-inflammatory drugs [20]. The aim of this work is to synthesize coumarin-related compounds with less than two phenolic

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hydroxyl groups contained, and to screen the antioxidant effectiveness. Thus, as shown in Scheme 1, we herein design a series of compounds with dihydropyrazole linking with coumarin and two benzene rings for comparing the antioxidant effectiveness.

3. Pharmacology

The antioxidant effectiveness can be estimated by many methods [21]. We herein evaluated the abilities of the obtained compounds to inhibit the oxidation of DNA induced by AAPH [22], Cu^{2+}/glutathione (GSH) [23], and hydroxyl radical (·OH) [24] and to scavenge 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cation ic radical ($ABTS^{+\cdot}$), DPPH, and galvinoxyl radical, respectively.

4. Results and discussion

4.1. Synthesis and spectral interpretation

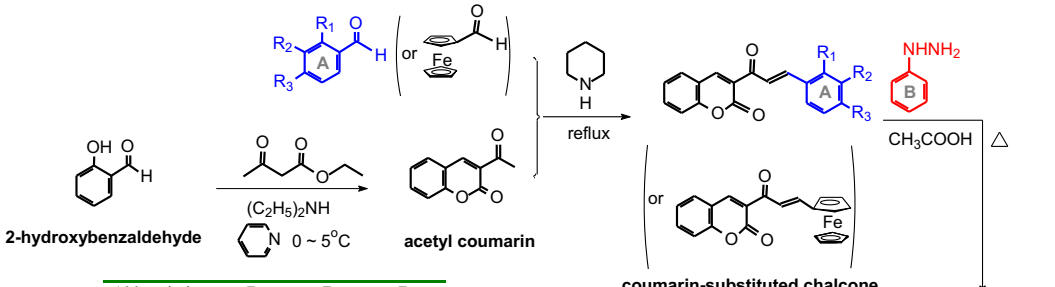
Acetyl coumarin was prepared by the reaction of 2-hydroxybenzaldehyde (10.0 mmol) with the same amount of ethyl acetoacetate (10.0 mmol) in the presence of pyridine and diethylamine as the catalysts. This step was carried out at 0–10 °C for 3 h, and the yield was >95%. The following reaction was Claisen–Schmidt condensation taking place between acetyl coumarin (2.0 mmol) and benzaldehyde (2.2 mmol) in the presence of piperidine (0.25 mL) as the catalyst and ethanol as the solvent. The catalyst used in the condensation between acetyl coumarin and benzaldehyde was the same as in the synthesis of acetyl coumarin. The difference was only on the operation. The preparation of acetyl coumarin was performed at 0–10 °C, and the Claisen–Schmidt condensation was carried out under refluxing for 6 h. The yield was also >95%. Finally, excess phenylhydrazine was added, followed by the addition of the catalyst (acetic acid) and refluxing for 3 h. Thus, the isolation after the final step was to remove phenylhydrazine. The cascade operation was just to change the catalyst from bases (pyridine, diethylamine, and piperidine) to acid (acetic acid). The yields of every step were so high that all the reagents were almost exhausted. Thus, the amount of excess reagents in the first and the second step was too little to influence the following reaction.

The peaks in ^{1}H NMR spectra of these compounds can be generally divided into three parts. The first part located around 8–10 ppm, which can be assigned to the hydrogen atom in –OH. The second part ranged from 6 to 8 ppm, which was contributed from the hydrogen atoms in the aromatic rings. The last part ranged from 3 to 6 ppm, which was donated to CH_2 and CH in dihydropyrazole. Accordingly, the peaks in ^{13}C NMR spectra of these compounds can be divided into two parts. The first part ranged from 100 to 160 ppm, which can be assigned to the carbon atoms at aromatic rings. Other peaks were lower than 100 ppm, which was contributed from CH_2 and CH in dihydropyrazole.

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

The Cu(II) may oxidize GSH to form GSH radical ($GS^{\cdot}$), while the produced Cu(I) combines with DNA to form a complex, DNA-Cu(I)–OOH. The complex can be degraded in the presence of $GS^{\cdot}$, leading to the formation of the oxidative products of DNA [23].

The oxidative products of DNA can be detected after colorized by thiobarbituric acid [25]. As shown as the control line in Fig. 1 (the upper line), the continuous increase of the absorbance reveals much more oxidative products were produced from the mixture of DNA, Cu^{2+}, and GSH with the increase of the reaction period. However, all the absorbance lines locate below that of the control experiment, indicating that the addition of all these coumarin-substituted dihydropyrazoles can decrease the amount of oxidative products of DNA, and thus, these dihydropyrazoles act as antioxidants to protect DNA against Cu^{2+}/GSH-induced oxidation. It is worthy to note that **1**, **2**, and **3** also function as antioxidants even without phenolic hydroxyl group attaching. This finding is in agreement with our previous result that 1,3,5-triphenyl-1*H*-pyrazole, a pyrazole derivative without phenolic hydroxyl group involved, can inhibit Cu^{2+}/GSH-induced oxidation of DNA [15]. Moreover, the absorbance lines in the presence of other coumarin-substituted dihydropyrazoles locate at lower positions than those of non-hydroxyl-involving dihydropyrazoles, demonstrating that the phenolic hydroxyl group enhances the antioxidant effects of coumarin-substituted dihydropyrazoles. Especially, the additions of



Abbreviation	R_1	R_2	R_3
1	H	H	H
2	H	H	CH_3O
4	OH	H	H
5	H	OH	H
6	H	H	OH
7	H	CH_3O	OH
8	H	OH	OH
9	OH	H	OH

Scheme 1. Synthetic routine of coumarin-substituted dihydropyrazoles.

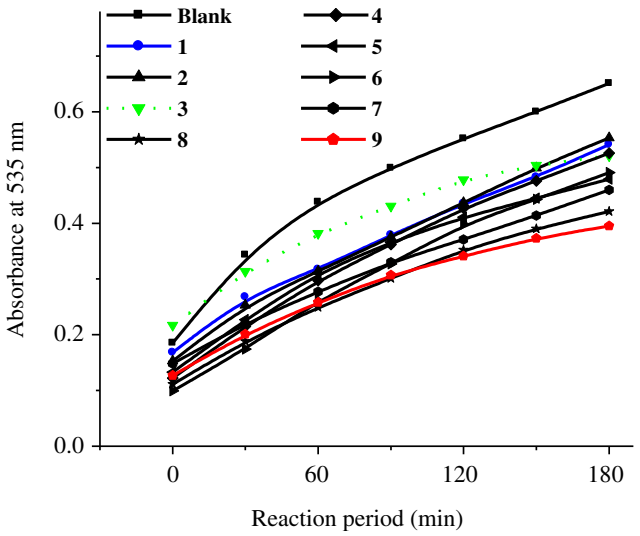


Fig. 1. The increase of the absorbance at 535 nm in the absence and presence of 0.20 mM coumarin-substituted dihydropyrazoles in the mixture of 2.0 mg/mL DNA, 5.0 mM Cu^{2+}, and 3.0 mM GSH.

8 and **9** make the absorbance lines locate at the lowest position, demonstrating that double phenolic hydroxyl groups at either *ortho*-, *para*- or *meta*-, *para*-positions are able to increase the abilities of coumarin-substituted dihydropyrazoles to inhibit Cu^{2+}/GSH-induced oxidation of DNA.

4.3. Effects of coumarin-substituted dihydropyrazoles on ·OH-induced oxidation of DNA

·OH is produced by the reaction between H_2O_2 and tetrachlorohydroquinone (TCHQ) [24] and can abstract hydrogen atom from the ribosyl moiety in DNA [26]. As can be seen from Fig. 2, the absorbance of TBARS was assigned as 100% when 2.0 mg/mL DNA was incubated with 4.0 mM TCHQ and 2.0 mM H_2O_2 at 37 °C for 30 min. Other absorbances of TBARS in the presence of 0.20 mM coumarin-substituted dihydropyrazoles were compared with that of the control experiment and listed in Fig. 2 as well.

The attractive results were also from the additions of **1**, **2**, and **3**, which do not involve phenolic hydroxyl groups but can decrease the TBARS percentage to 67.8%, 75.3%, and 91.8%, respectively. **1**, **2**, and **3** still play the antioxidant role in this case. The TBARS percentage of **1** is as low as 67.8%, demonstrating that the structural feature of dihydropyrazole with the aid of coumarin can generate

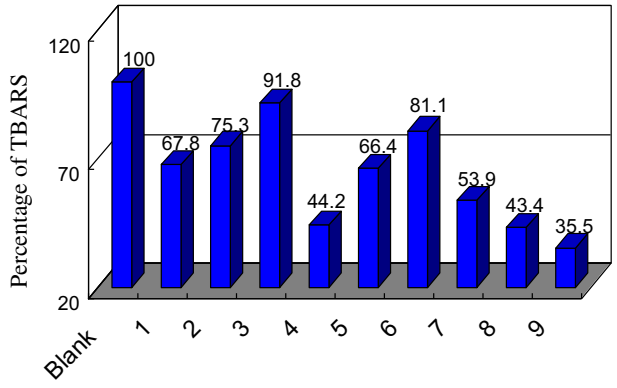


Fig. 2. The percentages of TBARS in the mixture of 2.0 mg/mL DNA, 4.0 mM H_2O_2, 2.0 mM TCHQ, and 0.20 mM coumarin-substituted dihydropyrazoles at 37 °C for 30 min.

relative high antioxidant ability to inhibit ·OH-induced oxidation of DNA. On the other hand, the relative high percentage of TBARS in the presence of **3** may be due to the Fe(II) in ferrocene moiety can induce the decomposition of H_2O_2 and generate additional ·OH. But the additional ·OH does not make **3** exhibit prooxidant effect because the antioxidant effect deriving from the structural feature of dihydropyrazole is high enough and can depress the side effect of ·OH. The methoxyl group in **2** is an electron-donating substituent, the increase of the TBARS percentage of **2** (75.3%) implies that electron-donating group is not beneficial for enhancing the ability of coumarin-substituted dihydropyrazoles to prohibit ·OH-caused oxidation of DNA. This deduction is proved by the increase of the TBARS percentage of **6** (81.1%) because hydroxyl group in **6** is also an electron-donating substituent. Moreover, it can be found that the electron-donating substituent at *para*-position (R_3 position) does not benefit for the antioxidant capacity, and contrarily, the hydroxyl group at *ortho*-position (R_1 position) as in **4** and **9** (44.2% and 35.5%, respectively) can improve the antioxidant effectiveness markedly.

4.4. Effects of coumarin-substituted dihydropyrazoles on AAPH-induced oxidation of DNA

AAPH can provide initiating peroxyl radical [27] for abstracting hydrogen atom from the guanine bases in DNA [28]. The oxidative process can be followed by measuring TBARS. As shown in Fig. 3, the absorbance line of the blank experiment indicates that the amount of TBARS increases with the reaction period of AAPH-induced oxidation of DNA.

It can be seen from Fig. 3 that the additions of **1** and **2** did not affect the increase of the TBARS absorbance even with the concentration increasing to 400 μM, revealing that the structural feature of dihydropyrazole cannot inhibit AAPH-induced oxidation of DNA. On the other hand, the addition of **3** retarded the increase of the absorbance line for a period, and then the absorbance line increased as in the control experiment. This inhibition period (t_{inh}) can be measured by the cross-point from the tangent lines for the inhibition and oxidation period. The antioxidant effectiveness of **3** is ascribed to the introduction of ferrocene moiety. The additions of other coumarin-substituted dihydropyrazoles exhibit the antioxidant effectiveness, and t_{inh} can be measured even in the case of low concentration employed. Fig. 4 outlines the linear relationship between the concentration of coumarin-substituted dihydropyrazole and t_{inh}, and Table 1 lists the quantitative equations of these linear relationships.

As shown as equation (1), chemical kinetics demonstrates that t_{inh} correlates proportionally with the concentration of the antioxidant [29].

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

In equation (1) n refers to the stoichiometric factor, revealing the number of the radical-propagation terminated by one molecule of the antioxidant, and R_i stands for the initiation rate of the radical-induced reaction. When equation (1) is employed to treat the results from AAPH-induced oxidation of DNA, R_i can be regarded as the radical-generation rate (R_g), $R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] s^{-1} [29], because both sodium salt of DNA and AAPH are dissolved in water, and radicals generated from AAPH attack DNA at the same phase [30]. Thus, n of coumarin-substituted dihydropyrazole is the product of the coefficient in the equation and $R_i = R_g = 1.4 \times 10^{-6} \times 40$ mM $s^{-1} = 3.36$ μM min^{-1} (see Table 1). For example, the coefficient in $t_{inh} \sim$ [**3**] is 0.87, the n of **3** is 0.87 × 3.36 μM $min^{-1} = 2.92$, implying that **3** can terminate 2.92 radical-propagation in AAPH-induced oxidation of DNA.

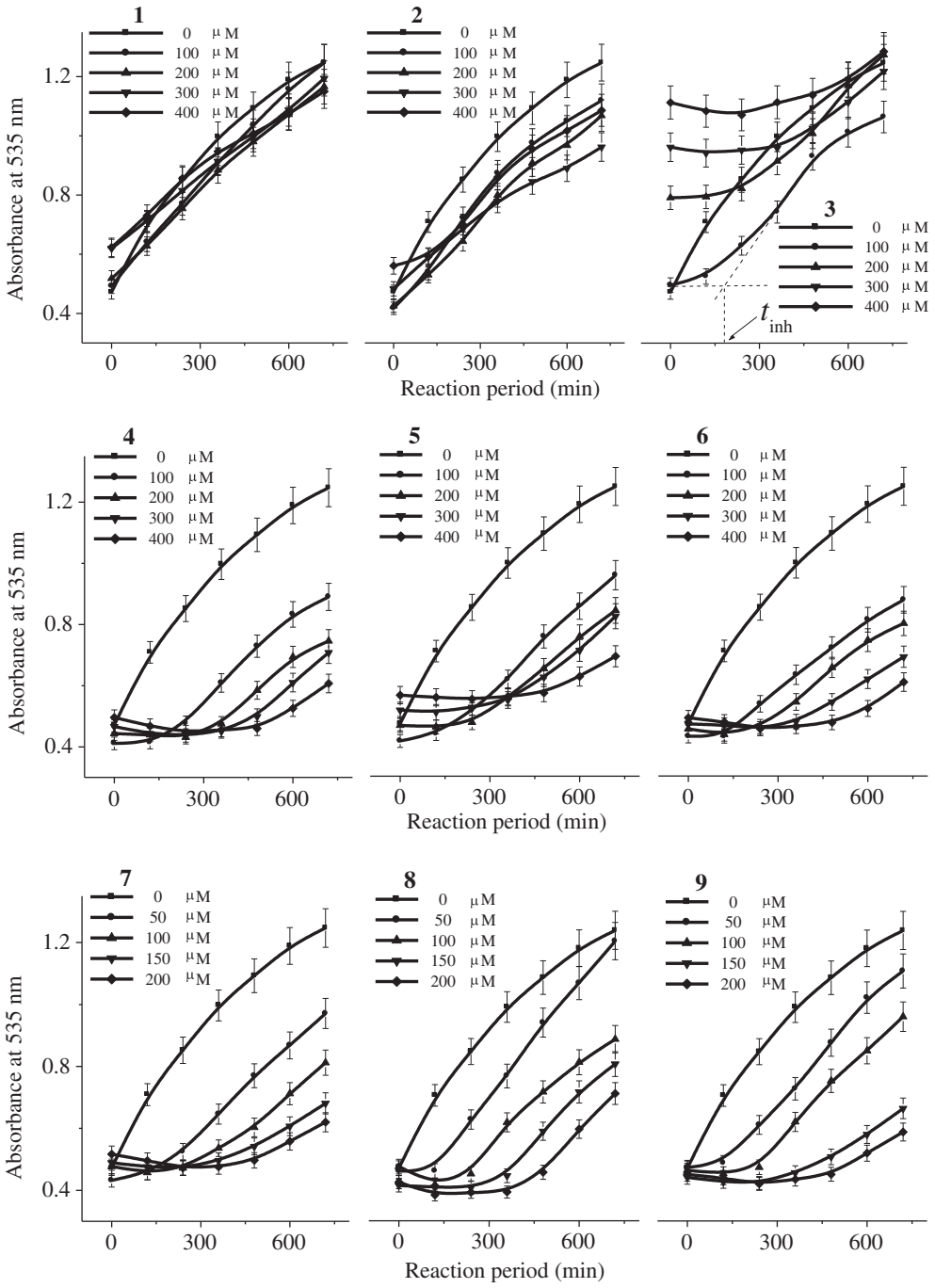


Fig. 3. The variation of the absorbance of TBARS in the mixture of 2.0 mg/mL DNA, 40 mM AAPH, and various concentrations of coumarin-substituted dihydropyrazoles at 37 °C.

Accordingly, the *n* of other coumarin-substituted dihydropyrazoles are calculated and listed in Table 1. It can be found in Table 1 that the antioxidant effectiveness of coumarin-substituted dihydropyrazoles can be divided into two parts, **7**, **8**, and **9** can terminate >6 radical-propagation because of double phenolic hydroxyl groups contained, and **4**, **5**, and **6** can terminate >3 radical-propagation because of only one phenolic hydroxyl group contained. This result indicates that double phenolic hydroxyl groups, especially, at *ortho*- and *para*-position as in **9**, are beneficial for increasing the antioxidant effectiveness, while single phenolic hydroxyl group at *ortho*-position as in **4** can enhance the antioxidant effectiveness. The antioxidant effectiveness is almost proportionally related to the number of phenolic hydroxyl group. Moreover, the *n* of **3** (2.93) is close to that of **5**, revealing that the antioxidant effectiveness of a ferrocene moiety is the same as a *meta*-phenolic hydroxyl group. As shown in Scheme 2, we have measured the *n* of dihydropyrazoles under the same experimental condition [31].

It can be seen from Scheme 2 that the *n* of **8** (6.60) is similar to that of **4a** (6.15). The *n* of **4a** is contributed from three phenolic hydroxyl groups, while double phenolic hydroxyl groups increases the *n* value of **8** to the same level, implying that *meta*-, *para*-dihydroxyl groups play the major role in the generation of *n* value. On the other hand, when the hydrogen atom in N–H of **3b** is replaced by a benzene ring to form **7**, the *n* value increases

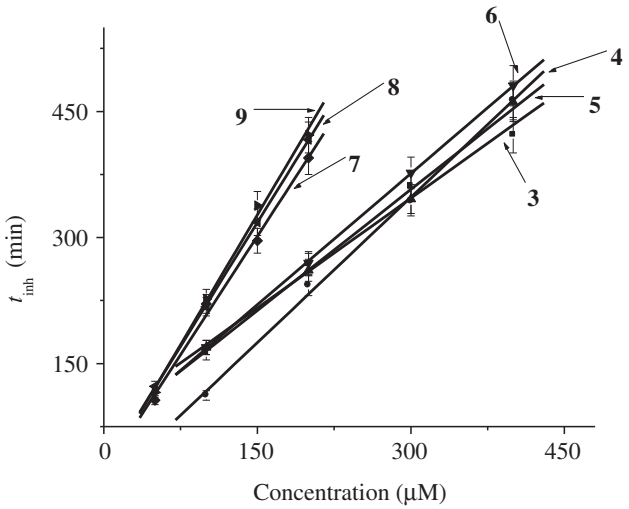


Fig. 4. The linear relationship between the concentrations of coumarin-substituted dihydropyrazoles and inhibition period (t_{inh}) in protecting DNA against AAPH-induced oxidation. The $p < 0.001$ indicated a significance difference of these equations.

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

Antioxidant	t_{inh} (min) = (n/R_i) [antioxidant (μM)] + constant[b]	n
3	t_{inh} = 0.87 (±0.04) [**3**] + 85.50 (±4.28)	2.93(±0.15)
4	t_{inh} = 1.15 (±0.06) [**4**] + 2.05 (±0.10)	3.87(±0.19)
5	t_{inh} = 0.96 (±0.05) [**5**] + 69.10 (±3.46)	3.23(±0.16)
6	t_{inh} = 1.04 (±0.05) [**6**] + 63.85 (±3.19)	3.50(±0.17)
7	t_{inh} = 1.88 (±0.09) [**7**] + 39.70 (±1.99)	6.31(±0.32)
8	t_{inh} = 1.96 (±0.10) [**8**] + 23.45 (±1.17)	6.60(±0.33)
9	t_{inh} = 2.06 (±0.10) [**9**] + 18.25 (±0.91)	6.92(±0.35)

[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, and $p < 0.001$ indicated a significance difference of these equations.

remarkably, indicating that benzene ring is beneficial for increasing the antioxidant effectiveness. Furthermore, when single phenolic hydroxyl group attaches to the skeleton of 1,3,5-triphenyl-1*H*-pyrazole, the n value cannot exceed 2 under the same experimental condition [15]. The aforementioned comparison indicates that the molecular structure containing coumarin and double benzene rings is suitable for the less phenolic hydroxyl groups to generate high antioxidant effectiveness. The antioxidant mechanism can be further elucidated by the measurement of the rate constant (***k***) when these coumarin-substituted dihydropyrazoles are applied to trap radicals.

4.5. Rate constant of coumarin-substituted dihydropyrazoles to scavenge ABTS$^{+\cdot}$ And DPPH

ABTS$^{+\cdot}$, DPPH, and galvinoxyl radical are usually used to test the ability of an antioxidant to trap radicals [32–34]. As shown in Figs. 5–7, the additions of these coumarin-substituted dihydropyrazoles except **1**, **2**, and **3** can lead to the decay of the concentrations of these radicals. This fact demonstrates that the antioxidant abilities of **1**, **2**, and **3** to inhibit Cu^{2+}/GSH and ·OH-induced oxidation of DNA are not derived from the radical-scavenging property but from redox behavior. Other coumarin-substituted dihydropyrazoles are able to trap radicals directly.

We have developed a method to calculate ***k*** of antioxidants in trapping radicals [35]. The first step is to input the data in Figs. 5–7 into statistical software. Consequently, the concentrations of these radicals ([*radical*]) and reaction period (t) is suitable for the double exponential function (equation (2)), and the results are listed in Tables 2–4.

$$[\text{radical}] = Ae^{-(t/a)} + Be^{-(t/b)} + C \quad (2)$$

Then, the equation (2) is carried out by differential operation to convert into equation (3), which expresses the relationship between the reaction rate ($\boldsymbol{r} = -d[radical]/dt$) and the reaction period (t).

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

The equation (3) can be used to calculate the reaction rate when the reaction time is assigned. Hence, when t is assigned to 0, the reaction rate at the beginning of the reaction ($\boldsymbol{r_0}$) can be obtained and listed in Tables 2–4.

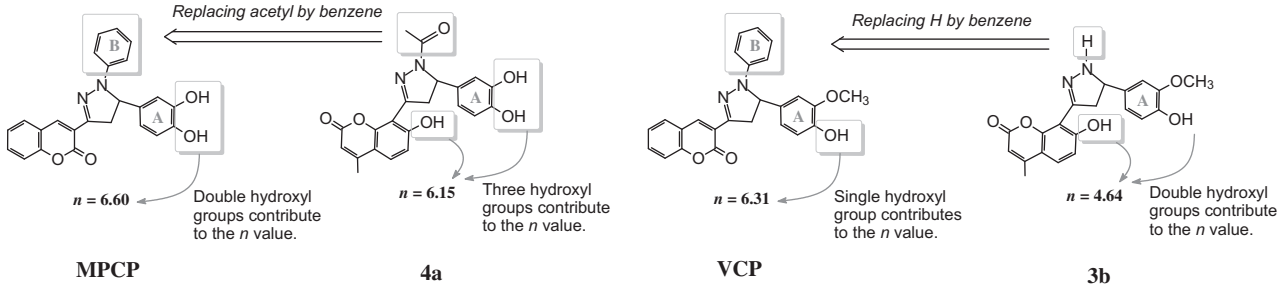
The equation (4) reveals that the reaction rate (***r***) is related to the concentrations of the radical and the antioxidant, in which the coefficient is the rate constant (***k***).

$$\boldsymbol{r} = \boldsymbol{k}[\text{radical}][\text{antioxidant}] \quad (4)$$

If the concentrations of the radical and the antioxidant at a certain time are known, and the reaction rate (***r***) is also measured, the rate constant (***k***) can be calculated. At present, the reaction rate at $t = 0$ ($\boldsymbol{r_0}$) is obtained, and the concentrations of the radical and the antioxidant at the beginning of the reaction are known, the rate constant (***k***) can be calculated by equation (5), and the results are listed in Tables 2–4.

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

The reaction between an antioxidant and ABTS$^{+\cdot}$ reveals the ability of the antioxidant to reduce the radical, and all sorts of phenolic hydroxyl group can reduce ABTS$^{+\cdot}$. **1**, **2**, and **3** cannot react with these radicals because of no hydroxyl groups attached. This



Scheme 2. The comparison of n values among structural analogs.

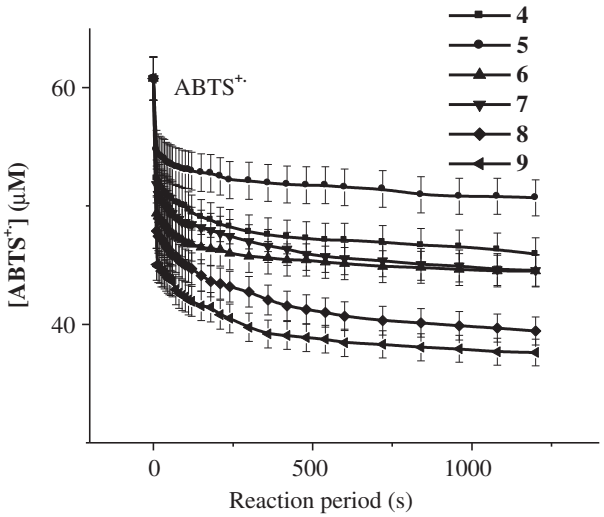


Fig. 5. Decay of 60.75 μM $ABTS^{+\bullet}$ in the presence of 5 μM coumarin-substituted dihydropyrazoles.

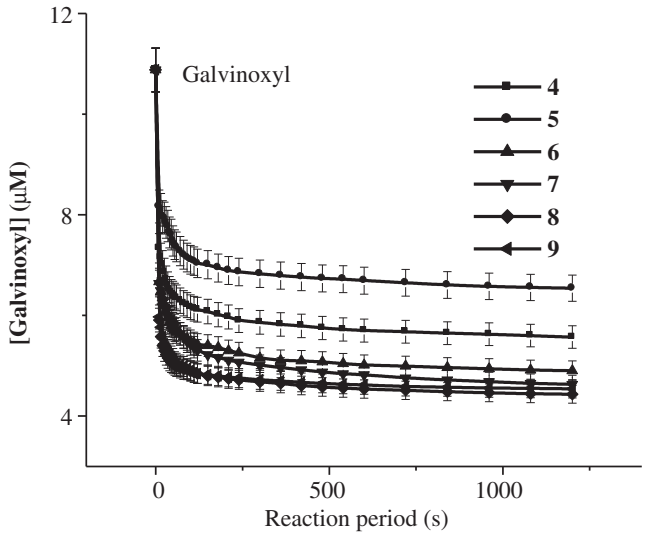


Fig. 7. Decay of 10.88 μM galvinoxyl in the presence of 15 μM coumarin-substituted dihydropyrazoles.

fact indicates that phenolic hydroxyl group plays the role in trapping radicals, while dihydropyrazole is not active to quench radicals. The rate constants (***k***) of **4**, **5**, and **7** are lower than that of other dihydropyrazoles, revealing that the ability of single phenolic hydroxyl group to reduce radical is lower than that of double phenolic hydroxyl groups. Especially, **9** has the highest ***k*** in trapping $ABTS^{+\cdot}$, indicating that *ortho*-, *para*-dihydroxyl groups exhibit the strongest ability to reduce $ABTS^{+\cdot}$. The reactions between an antioxidant and DPPH and galvinoxyl radical reveal the abilities of the antioxidant to donate its hydrogen atom to N-centered and O-centered radicals, respectively. It can be seen from Table 3 that the ***k*** values of **4** and **5** are lower than that of other dihydropyrazoles, indicating that single phenolic hydroxyl group cannot donate its hydrogen atom to N-centered radical as fast as the dihydropyrazoles containing double phenolic hydroxyl groups. In particular, **8** possesses the highest ***k*** value, revealing that dihydroxyl groups at *meta*-, *para*-positions are beneficial for donating hydrogen atoms to N-centered radical. But, it can be found in Table 4 that the difference of ***k*** value among these dihydropyrazoles in trapping galvinoxyl radical is not as large as in trapping DPPH. The hydrogen atom in phenolic hydroxyl groups of dihydropyrazoles is transferred to O-centered radical, forming a hydroxyl group in galvinoxyl radical. There is no variation in the bond energy in this process. One O–H bond is broken and one N–H bond is formed in the reaction between an antioxidant and DPPH, the bond energy changes in this process. Therefore, the ***k*** value from the reaction of an antioxidant and galvinoxyl radical reveals the real rate of the transfer of hydrogen atom.

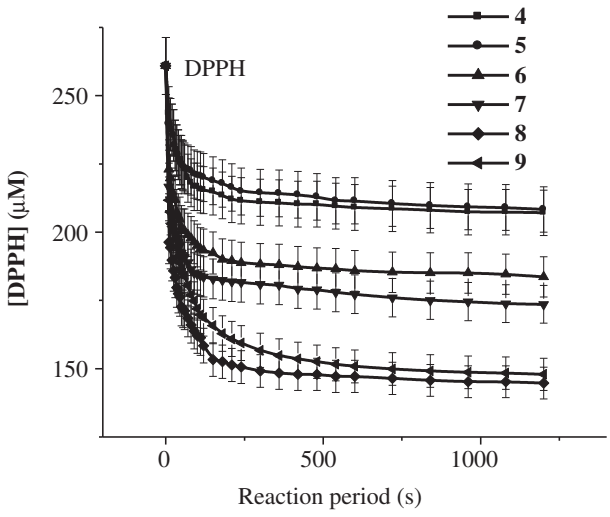


Fig. 6. Decay of 260.88 μM DPPH in the presence of 10 μM coumarin-substituted dihydropyrazoles.

5. Conclusion

A cascade method is designed to prepare coumarin-substituted dihydropyrazoles. The continuous operation without the isolation of the intermediate products increases the efficiency of the synthesis. The coumarin moiety is an important structural feature for enhancing the antioxidant effectiveness of single or double phenolic hydroxyl groups in dihydropyrazole. Hence, the structural style of coumarin and two benzene rings attaching to dihydropyrazole exhibits powerful antioxidant potential to inhibit DNA oxidation. This structural style also exhibits the ability to protect DNA against Cu^{2+}/GSH and ·OH-induced oxidation even in the absence of phenolic hydroxyl group. In particular, **3**, a dihydropyrazole derivative without any phenolic hydroxyl groups involving, still exhibit the antioxidant effectiveness in inhibiting Cu^{2+}/GSH-induced oxidation of DNA. This result shows the potential application of ferrocene moiety for designing antioxidant molecules. The skeleton of coumarin-substituted dihydropyrazole can be widely applied in the design of novel antioxidants.

6. Experimental section

6.1. Materials and instrumentation

Diammonium salt of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS salt), DPPH, and galvinoxyl radical 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-substituted dihydropyrazoles were identified by ^{1}H and ^{13}C NMR (Varian Mercury 300 NMR spectrometer) and high solution mass spectra equipped with ESI as the ionization mode (Agilent1290-micrOTOF Q II).

Table 2
Equation of $[ABTS^{+\cdot}] \sim t$ and its differential style ($-d[ABTS^{+\cdot}]/dt \sim t$), reaction rate at $t = 0$ ($\boldsymbol{r}_0$), and rate constant ($\boldsymbol{k}$).[a]

Antioxidant	Equation of $[ABTS^{+\cdot}]$ (μM) $\sim t$ (s)	Equation of $-d[ABTS^{+\cdot}]/dt \sim t$	$\boldsymbol{r}_0$(μM·s^{-1})	$\boldsymbol{k}$ (mM^{-1} s^{-1})
4	$[ABTS^{+\cdot}] = 8.66e^{-\frac{t}{3.88}} + 5.55e^{-\frac{t}{203.16}} + 46.53$	$-\frac{d[ABTS^{+\cdot}]}{dt} = 2.23e^{-\frac{t}{3.88}} + 0.03e^{-\frac{t}{203.16}}$	2.26	7.44
5	$[ABTS^{+\cdot}] = 6.64e^{-\frac{t}{5.11}} + 3.30e^{-\frac{t}{328.90}} + 50.80$	$-\frac{d[ABTS^{+\cdot}]}{dt} = 1.30e^{-\frac{t}{5.11}} + 0.01e^{-\frac{t}{328.90}}$	1.31	4.31
6	$[ABTS^{+\cdot}] = 11.67e^{-\frac{t}{3.47}} + 4.23e^{-\frac{t}{167.98}} + 44.85$	$-\frac{d[ABTS^{+\cdot}]}{dt} = 3.36e^{-\frac{t}{3.47}} + 0.03e^{-\frac{t}{167.98}}$	3.39	11.16
7	$[ABTS^{+\cdot}] = 10.22e^{-\frac{t}{5.69}} + 5.85e^{-\frac{t}{308.25}} + 44.66$	$-\frac{d[ABTS^{+\cdot}]}{dt} = 1.80e^{-\frac{t}{5.69}} + 0.02e^{-\frac{t}{308.25}}$	1.82	5.99
8	$[ABTS^{+\cdot}] = 13.20e^{-\frac{t}{3.39}} + 7.92e^{-\frac{t}{286.21}} + 39.63$	$-\frac{d[ABTS^{+\cdot}]}{dt} = 3.89e^{-\frac{t}{3.39}} + 0.03e^{-\frac{t}{286.21}}$	3.92	12.91
9	$[ABTS^{+\cdot}] = 15.47e^{-\frac{t}{2.08}} + 7.39e^{-\frac{t}{216.43}} + 37.89$	$-\frac{d[ABTS^{+\cdot}]}{dt} = 7.44e^{-\frac{t}{2.08}} + 0.03e^{-\frac{t}{316.43}}$	7.47	24.59

[a] The concentrations of coumarin-substituted dihydropyrazoles is 5 μM, and the concentration of $ABTS^{+\cdot}$ is 60.75 μM **1**, **2**, and **3** cannot react with $ABTS^{+\cdot}$ radical. The $p < 0.001$ indicated a significance difference of these equations.

6.2. A cascade synthetic operation of coumarin-substituted dihydropyrazoles

The hydroxyl group in aldehyde A was protected by etherizing with benzyl chloride before used in the following synthesis. Briefly, hydroxyl-substituted benzaldehyde (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 benzoxyl-substituted benzaldehyde (>90%).

The following synthesis was carried out as a cascade operation without purification. 2-Hydroxylbenzaldehyde (1.22 g, 10.0 mmol) was mixed with ethyl acetoacetate (1.30 g, 10.0 mmol) and stirred at 0–10 °C for 0.5 h. Then, pyridine (0.05 mL) and $(C_2H_5)_2NH$ (0.05 mL) were added dropwisely and stirred at 0–10 °C for 3 h. To above mixture, 40 mL of ethanol, the corresponding aldehyde (12.0 mmol), and piperidine (0.5 mL) were added and refluxed for 6 h. Finally, phenylhydrazine (50.0 mmol) and acetic acid (15 mL) were added and refluxed for 3 h. The reaction mixture was poured into ice-cold water and extracted with ethyl acetate at room temperature. The organic phase was washed with brine and dried over Na_2SO_4. The solvent was evaporated under vacuum pressure. The crude product was purified by silica chromatography with ethyl acetate/petroleum ether (1:4, *v*:*v*) being eluent to afford the hydroxyl-protected coumarin-substituted dihydropyrazoles.

The benzyl group was removed by $TiCl_4$. In brief, hydroxyl-protected coumarin-substituted dihydropyrazoles (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/petroleum ether (1:1, *v*:*v*) being eluent. The NMR data of coumarin-substituted dihydropyrazoles were listed as following.

6.2.1. 3-(1,5-Diphenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**1**)

Yield 78%. m. p.: 204–206 °C ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.42 (s, 1H, H_{vinyl}), 7.57–7.59 (m, 1H, phenyl), 7.48–7.54 (m, 1H, phenyl), 7.27–7.33 (m, 6H, phenyl), 7.23 (*t*, 1H, phenyl), 7.17–7.20 (m, 2H, phenyl), 7.07–7.10 (m, 2H, phenyl), 6.83 (*t*, 1H, phenyl), 5.34 (dd, $J = 12.9$ Hz, $J = 7.2$ Hz, 1H, CH), 4.10 (dd, $J = 18.6$ Hz, $J = 12.9$ Hz, 1H, CH_2), 3.39 (dd, $J = 18.3$ Hz, $J = 7.2$ Hz, 1H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 159.7, 153.5, 144.0, 142.9, 142.0, 137.6, 131.5, 129.1, 129.0, 128.2, 127.6, 125.7, 124.7, 120.8, 119.8, 119.5, 116.4, 113.6, 64.8, 45.2. MS: *m*/*z* 367.1476 [M + H$^+$].

6.2.2. 3-(5-(4-Methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**2**)

Yield 82%. m. p.: 131–133 °C ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.40 (s, 1H, H_{vinyl}), 7.48–7.60 (m, 2H, phenyl), 7.27–7.39 (m, 3H phenyl), 7.08–7.23 (m, 5H, phenyl), 6.83–6.87 (m, 2H, phenyl), 5.30 (dd, $J = 12.6$ Hz, $J = 7.2$ Hz, 1H, CH), 4.07 (dd, $J = 18.3$ Hz, $J = 12.6$ Hz, 1H, CH_2), 3.78 (s, 3H, OCH_3), 3.36 (dd, $J = 18.3$ Hz, $J = 7.2$ Hz, 1H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 159.6, 159.0, 153.5, 144.0, 142.9, 137.4, 134.1, 131.4, 130.0, 129.0, 128.9, 128.1, 126.9, 125.4, 124.6, 120.8, 119.7, 119.5, 116.3, 114.4, 113.9, 113.6, 64.4, 55.2, 45.2. MS: *m*/*z* 397.1576 [M + H$^+$].

6.2.3. 3-(5-Ferrocenyl-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl) chromen-2-one (**3**)

Yield 73%. m. p.: 193–195 °C ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.42 (s, 1H, H_{vinyl}), 7.50–7.60 (m, 2H, phenyl), 7.30–7.37 (m, 3H, phenyl), 7.18–7.22 (m, 3H, phenyl), 6.85 (*t*, 1H, phenyl), 5.20 (dd, $J = 10.8$ Hz, $J = 6.0$ Hz, 1H, CH), 4.24–4.28 (m, 1H, CH_2), 4.19 (s, 5H, ferrocenoyl),

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]

Antioxidant	Equation of [DPPH (μM)] $\sim t$ (s)	Equation of $-d$[DPPH]/$dt \sim t$	$\boldsymbol{r}_0$ (μM s^{-1})	$\boldsymbol{k}$ (mM^{-1} s^{-1})
4	$[DPPH] = 37.89e^{-\frac{t}{28.03}} + 12.79e^{-\frac{t}{229.76}} + 207.69$	$-\frac{d[DPPH]}{dt} = 1.35e^{-\frac{t}{28.03}} + 0.06e^{-\frac{t}{229.76}}$	1.41	0.54
5	$[DPPH] = 33.01e^{-\frac{t}{18.64}} + 17.11e^{-\frac{t}{281.95}} + 208.90$	$-\frac{d[DPPH]}{dt} = 1.77e^{-\frac{t}{18.64}} + 0.06e^{-\frac{t}{281.95}}$	1.83	0.70
6	$[DPPH] = 41.36e^{-\frac{t}{6.96}} + 33.19e^{-\frac{t}{77.70}} + 186.10$	$-\frac{d[DPPH]}{dt} = 5.94e^{-\frac{t}{6.96}} + 0.43e^{-\frac{t}{77.70}}$	6.37	2.44
7	$[DPPH] = 57.00e^{-\frac{t}{8.99}} + 26.45e^{-\frac{t}{95.08}} + 176.98$	$-\frac{d[DPPH]}{dt} = 6.34e^{-\frac{t}{8.99}} + 0.28e^{-\frac{t}{95.08}}$	6.62	2.54
8	$[DPPH] = 80.69e^{-\frac{t}{3.61}} + 53.36e^{-\frac{t}{77.63}} + 146.80$	$-\frac{d[DPPH]}{dt} = 22.35e^{-\frac{t}{3.61}} + 0.69e^{-\frac{t}{77.63}}$	23.04	8.83
9	$[DPPH] = 49.50e^{-\frac{t}{5.43}} + 60.50e^{-\frac{t}{102.60}} + 150.75$	$-\frac{d[DPPH]}{dt} = 9.12e^{-\frac{t}{5.43}} + 0.59e^{-\frac{t}{102.60}}$	9.71	3.72

[a] The concentrations of coumarin-substituted dihydropyrazoles is 10 μM, and the concentration of DPPH is 260.88 μM **1**, **2**, and **3** cannot react with DPPH radical. The $p < 0.001$ indicated a significance difference of these equations.

Table 4
Equation of [galvinoxyl] ~ t and its differential style ($-d$[galvinoxyl]/dt ~ t), reaction rate at $t = 0$ ($\boldsymbol{r}_0$), and rate constant ($\boldsymbol{k}$).[a]

Antioxidant	Equation of [galvinoxyl (μM)] ~ t (s)	Equation of $-d$[galvinoxyl]/dt ~ t	$\boldsymbol{r}_0$ (μM·s^{-1})	$\boldsymbol{k}$ (mM^{-1} s^{-1})
4	$[\text{galvinoxyl}] = 3.94e^{-\frac{t}{5.59}} + 1.24e^{-\frac{t}{113.48}} + 5.69$	$-\frac{d[\text{galvinoxyl}]}{dt} = 0.70e^{-\frac{t}{5.59}} + 0.01e^{-\frac{t}{113.48}}$	0.71	4.35
5	$[\text{galvinoxyl}] = 2.52e^{-\frac{t}{2.17}} + 1.66e^{-\frac{t}{74.39}} + 6.69$	$-\frac{d[\text{galvinoxyl}]}{dt} = 1.16e^{-\frac{t}{2.17}} + 0.02e^{-\frac{t}{74.39}}$	1.18	7.23
6	$[\text{galvinoxyl}] = 4.53e^{-\frac{t}{4.82}} + 1.33e^{-\frac{t}{103.69}} + 5.02$	$-\frac{d[\text{galvinoxyl}]}{dt} = 0.94e^{-\frac{t}{4.82}} + 0.01e^{-\frac{t}{103.69}}$	0.95	5.82
7	$[\text{galvinoxyl}] = 4.41e^{-\frac{t}{4.10}} + 1.69e^{-\frac{t}{112.21}} + 4.78$	$-\frac{d[\text{galvinoxyl}]}{dt} = 1.08e^{-\frac{t}{4.10}} + 0.02e^{-\frac{t}{112.21}}$	1.10	6.74
8	$[\text{galvinoxyl}] = 5.58e^{-\frac{t}{4.98}} + 0.79e^{-\frac{t}{158.45}} + 4.50$	$-\frac{d[\text{galvinoxyl}]}{dt} = 1.12e^{-\frac{t}{4.98}} + 0.01e^{-\frac{t}{158.45}}$	1.13	6.92
9	$[\text{galvinoxyl}] = 5.13e^{-\frac{t}{4.04}} + 1.11e^{-\frac{t}{70.16}} + 4.63$	$-\frac{d[\text{galvinoxyl}]}{dt} = 1.27e^{-\frac{t}{4.04}} + 0.02e^{-\frac{t}{70.16}}$	1.29	7.90

[a] The concentrations of coumarin-substituted dihydropyrazoles is 15 μM, and the concentration of galvinoxyl is 10.88 μM **1**, **2**, and **3** cannot react with galvinoxyl radical. The $p < 0.001$ indicated a significance difference of these equations.

4.12–4.15 (m, 4H, ferrocenoyl), 4.00–4.03(m, 1H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 159.9, 153.5, 144.3, 143.5, 137.5, 131.4, 128.8, 128.1, 124.6, 120.8, 119.8, 119.5, 116.3, 114.3, 89.8, 68.7, 68.6, 68.1, 67.6, 66.7, 59.8, 43.5. MS: m/z 475.1116 [M + H$^+$].

*6.2.4. 3-(5-(2-Hydroxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**4**)*

Yield 85%. m. p.: 216–218 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.89 (s, 1H, OH), 8.47 (s, 1H, H_{vinyl}), 7.83 (dd, J = 7.8 Hz, J = 1.2 Hz, 1H, phenyl), 7.57–7.63 (m, 1H, phenyl), 7.34–7.42 (m, 2H, phenyl), 7.16–7.21 (m, 2H, phenyl), 7.00–7.10 (m, 3H, phenyl), 6.84–6.91 (m, 2H, phenyl), 6.66–6.78 (m, 2H, phenyl), 5.62 (dd, J = 12.3 Hz, J = 6.3 Hz, 1H, CH), 3.95 (dd, J = 18.0 Hz, J = 12.6 Hz, 1H, CH_2), 3.16 (dd, J = 18.0 Hz, J = 6.0 Hz, 1H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.6, 159.5, 158.3, 149.0, 148.9, 143.6, 137.2, 134.4, 134.2, 133.8, 132.9, 131.6, 130.1, 125.5, 124.7, 124.5, 121.3, 121.0, 118.4, 63.2, 48.7. MS: m/z 383.1421 [M + H$^+$].

*6.2.5. 3-(5-(3-Hydroxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**5**)*

Yield 80%. m. p.: 197–198 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.43 (s, 1H, OH), 8.47 (s, 1H, H_{vinyl}), 7.82 (d, J = 6.6 Hz, 1H, phenyl), 7.58–7.63 (m, 1H, phenyl), 7.35–7.42 (m, 2H, phenyl), 7.04–7.22 (m, 5H, phenyl), 6.63–6.79 (m, 4H, phenyl), 5.42 (dd, J = 12.0 Hz, J = 6.0 Hz, 1H, CH), 3.96 (dd, J = 18.0 Hz, J = 12.6 Hz, 1H, CH_2), 3.20 (dd, J = 18.0 Hz, J = 6.3 Hz, 1H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 158.1, 157.9, 152.9, 152.8, 143.8, 143.5, 143.1, 138.3, 131.8, 130.1, 128.9, 128.7, 124.7, 119.8, 119.2, 116.3, 115.8, 114.5, 113.2, 112.1, 63.0, 44.5. MS: m/z 383.1425 [M + H$^+$].

*6.2.6. 3-(5-(4-Hydroxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**6**)*

Yield 86%. m. p.: 230–232 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.40 (s, 1H, OH), 8.47 (s, 1H, H_{vinyl}), 7.83 (dd, J = 7.8 Hz, J = 1.5 Hz, 1H, phenyl), 7.58–7.63 (m, 1H, phenyl), 7.35–7.42 (m, 2H, phenyl), 7.15–7.20 (m, 2H, phenyl), 7.04–7.10 (m, 4H, phenyl), 6.70–6.78 (m, 3H, phenyl), 5.41 (dd, J = 12.6 Hz, J = 6.3 Hz, 1H, CH), 3.93 (dd, J = 18.0 Hz, J = 12.3 Hz, 1H, CH_2), 3.18 (dd, J = 18.0 Hz, J = 6.3 Hz, 1H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 158.0, 156.6, 152.7, 143.5, 142.9, 138.1, 132.3, 131.6, 128.7, 128.6, 126.9, 124.6, 119.9, 119.1, 119.0, 115.7, 115.6, 113.2, 62.6, 44.5. MS: m/z 383.1419 [M + H$^+$].

*6.2.7. 3-(5-(4-Hydroxy-3-methoxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**7**)*

Yield 76%. m. p.: 185–187 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.97 (s, 1H, OH), 8.48 (s, 1H, H_{vinyl}), 7.84 (d, J = 6.6 Hz, 1H, phenyl), 7.58–7.64 (m, 1H, phenyl), 7.35–7.43 (m, 2H, phenyl), 7.16–7.21 (m, 2H, phenyl), 7.06–7.09 (m, 2H, phenyl), 6.89 (d, J = 1.5 Hz, 1H, phenyl), 6.70–6.79 (m, 2H, phenyl), 6.62–6.66 (m, 1H, phenyl), 5.38 (dd, J = 12.3 Hz, J = 6.3 Hz, 1H, CH), 4.03 (dd, J = 14.4 Hz, J = 7.2 Hz, 1H, CH_2), 3.72 (s, 3H, OCH_3), 3.22 (dd, J = 18.0 Hz, J = 6.6 Hz, 1H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 158.0, 152.7, 147.8, 145.7, 143.7, 143.1, 138.0, 133.0, 131.6, 128.7, 128.6, 124.6, 119.8, 119.1, 117.8, 115.7, 113.3, 109.8, 63.1, 55.4, 44.5. MS (ESI): m/z 413.1536 [M + H$^+$].

*6.2.8. 3-(5-(3,4-Dihydroxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**8**)*

Yield 87%. m. p.: 233–234 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.93 (s, 1H, OH), 8.86 (s, 1H, OH), 8.45 (s, 1H, H_{vinyl}), 7.83 (d, J = 7.8 Hz, 1H, phenyl), 7.61 (*t*, 1H, phenyl), 7.35–7.42 (m, 2H, phenyl), 7.05–7.21 (m, 4H, phenyl), 6.58–6.78 (m, 4H, phenyl), 5.32 (dd, J = 12.3 Hz, J = 6.0 Hz, 1H, CH), 3.91 (dd, J = 18.0 Hz, J = 12.3 Hz, 1H, CH_2), 3.18 (dd, J = 18.0 Hz, J = 6.3 Hz, 1H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 157.9, 152.7, 145.5, 144.6, 143.5, 142.9, 138.0, 133.1, 131.6, 128.7, 128.5, 124.6, 119.8, 119.1, 119.0, 116.6, 115.8, 115.7, 113.2, 112.6, 62.7, 44.5. MS: m/z 399.1387 [M + H$^+$].

*6.2.9. 3-(5-(2,4-Dihydroxyphenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)chromen-2-one (**9**)*

Yield 84%. m. p.: 149–150 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.67 (s, 1H, OH), 9.21 (s, 1H, OH), 8.45 (s, 1H, H_{vinyl}), 7.83 (d, J = 7.5 Hz, 1H, phenyl), 7.57–7.62 (m, 1H, phenyl), 7.34–7.42 (m, 2H, phenyl), 7.15–7.21 (m, 2H, phenyl), 7.02–7.05 (m, 2H, phenyl), 6.64–6.77 (m, 2H, phenyl), 6.35 (d, J = 1.8 Hz, 1H, phenyl), 6.10 (dd, J = 8.4 Hz, J = 2.1 Hz, 1H, phenyl), 5.51 (dd, J = 12.3 Hz, J = 6.0 Hz, 1H, CH), 3.88 (dd, J = 18.0 Hz, J = 12.3 Hz, 1H, CH_2), 3.13 (dd, J = 18.0 Hz, J = 6.3 Hz, 1H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 157.6, 155.0, 152.8, 143.6, 143.4, 137.9, 131.7, 128.8, 128.7, 127.0, 124.7, 120.2, 119.3, 118.9, 118.2, 115.8, 113.0, 106.6, 102.7, 64.9, 43.4. MS: m/z 399.1383 [M + H$^+$].

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

Cu^{2+}/GSH-induced oxidation of DNA was carried out following a previous report [23] 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 dihydropyrazoles were dissolved in dimethyl sulfoxide (DMSO). Then, 2.0 mg/mL DNA, 5.0 mM Cu^{2+}, 3.0 mM GSH, and 0.2 mM coumarin-substituted dihydropyrazoles 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.

6.4. ·OH-induced oxidation of DNA test

•OH was generated by mixing H_2O_2 with tetrachlorohydro quinone (TCHQ, dissolved in DMSO as the stock solution) as the description in a literature [24]. 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 0.2 mM coumarin-substituted dihydropyrazoles (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 dihydropyrazoles were assigned as A_0 and A_{detect}, respectively. The effects of coumarin-substituted dihydropyrazoles on ·OH-induced oxidation of DNA were expressed by $A_{detect}/A_0 \times 100$.

6.5. AAPH-induced oxidation of DNA test

The experiment of AAPH-induced oxidation of DNA was performed as the description in a literature [22]. Briefly, 2.0 mg/mL DNA, 40 mM AAPH, and a certain concentration of coumarin-substituted dihydropyrazoles (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 *vs* the incubation period.

6.6. Scavenging DPPH, $ABTS^{+\cdot}$ and galvinoxyl radical

A 2.0 mL of solution containing 4.0 mM ABTS aqueous solution and 1.41 mM $K_2S_2O_8$ was kept for 16 h and diluted by 100 mL of ethanol to form $ABTS^{+\cdot}$, whose absorbance was around 1.00 at 734 nm ($\varepsilon ABTS^{+\bullet} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$). DPPH and galvinoxyl radical were dissolved in ethanol to make the absorbance 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. The DMSO solutions of coumarin-substituted dihydropyrazoles (0.1 mL) were added to 1.9 mL of $ABTS^{+\cdot}$, DPPH, and galvinoxyl radical solution. The final concentrations of coumarin-substituted dihydropyrazoles were 5, 10, and 15 μM in trapping $ABTS^{+\cdot}$, DPPH, and galvinoxyl radical, respectively. The decreases of the absorbance of these radicals were recorded at 25 °C at a certain time interval.

6.7. 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 6.0 professional Software, and $p < 0.001$ indicated a significance difference.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.06.059.

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