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Original article

Synthesis of 4-methylcoumarin derivatives containing 4,5-dihydropyrazole moiety to scavenge radicals and to protect DNA

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ABSTRACT

A series of 4-methylcoumarin derivatives containing 4,5-dihydropyrazole moiety were synthesized and their antioxidant activities were evaluated in AAPH (2,2′-azobis(2-amidinopropane hydrochloride))-induced oxidation of DNA, and in trapping DPPH (2,2′-diphenyl-1-picrylhydrazyl) and $ABTS^{+\bullet}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical), respectively. Among coumarin derivatives, **3a**–**d** and **4a**–**c** exhibited the termination of radical propagation-chains in AAPH-induced oxidation of DNA. The *ortho* dihydroxyphenyl substitution at 5 position and 1-unsubstitution of the 4,5-dihydroxylpyrazole was found enhancing the antioxidant activities of these coumarin derivatives.

1. Introduction

The importance of free radicals, especially reactive oxygen species (ROS) in the pathogenicity of various diseases, has of late received greater attention. Because a large body of evidence has revealed the correlation of the *in vivo* oxidative stress with aging [1] and some fatal diseases [2,3], many works devoted to the synthesis of novel antioxidants and the evaluation of their activities in various experimental systems [4,5].

Coumarins, a wide family of compounds present in remarkable amounts in the nature, have been found to exhibit different biological and pharmacological significance, including anticancer [6,7], antioxidant [8,9], anti-inflammatory [10], antimicrobial [11], antiviral [12], and enzymatic inhibitory activities [13]. Compared to the coumarins, 4-methylcoumarins not only possess similar various biological activities, but known to be less toxic. In particular, 4-methylcoumarins have been studied as novel antioxidants recently [14–18]. On the other hand, pyrazole, has been found in numerous pharmaceutically active compounds [19–21], and a large number of pyrazoles have shown free radical scavenging ability [22–25].

Since the combination of two pharmacophores on the same scaffold is a well established approach to more potent drugs [26–28], some coumarin derivatives containing pyrazole moiety have been synthesized. Although these compounds exhibits various biological activities [29–32], only very a few report focused on the synthetic methodology and systematic evaluation of antioxidant activities. Our group [33] has recently reported on the antioxidant activity of coumarin derivatives xanthotoxol and methyl-substituted xanthotoxol (Fig. 1). As an following study, 4,5-dihydropyrazole ring was considered introducing into the parent 7-hydroxy-4-methyl coumarin skeleton to design novel structures with enhanced antioxidant activities. Herein, a series of 4-methylcoumarin derivatives containing 4,5-dihydropyrazole moiety were synthesized (Scheme 1). Then these 4,5-dihydropyrazole coumarins were employed to protect DNA against the oxidation damage induced by AAPH, and radical scavenging properties were screened by trapping $ABTS^{+\bullet}$ and DPPH.

2. Results and discussion

2.1. Chemistry

The title compound, 4-methylcoumarin derivatives containing 4,5-dihydropyrazole moiety, was derived from 8-acetyl-7-hydroxy-4-methylcoumrain **1** employed as the reagent (Scheme 1). The α,β-unsaturated ketone **2** was accomplished by Claisen–Schmidt

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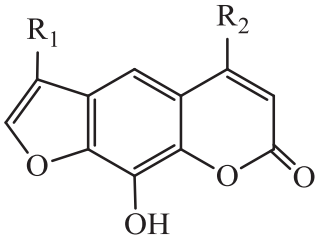


xanthotoxol, R_1=R_2=H

8-hydroxy-4-methylpsoralen, R_1=H, R_2=CH_3

8-hydroxy-4,9-dimethylpsoralen, R_1=R_2=CH_3

Fig. 1. Xanthotoxol and methyl-substituted xanthotoxol.

condensation of **1** and substituted-benzaldehyde in the presence of mild catalyst piperidine, and simply obtained as precipitate. Further purification by recrystallization was performed when necessary. The treatment of **2** with hydrazine hydrate in refluxing ethanol or acetic acid afforded 8-(4,5-dihydro-5-aryl-1-*H*-pyrazol-3-yl)-7-hydroxy-4-methyl-2*H*-1-benzopyran-2-ones **3a**, **3b** and 8-(1-acetyl-4,5-dihydro-5-aryl-1-*H*-pyrazol-3-yl)-7-hydroxy-4-methyl-2*H*-1-benzopyran-2-ones **3c**–**f**, respectively. Finally, compound **4a**–**c** were obtained from the deprotection of **3d**–**f**. We also examined the viability of a protecting-group-free route for compound **4a**–**c** (Scheme 2). Unfortunately, the Claisen–Schmidt condensation between **1** and benzaldehyde partners with free hydroxyl at *ortho* or *meta* positions led to complex mixtures. The desired products could not be isolated as pure form.

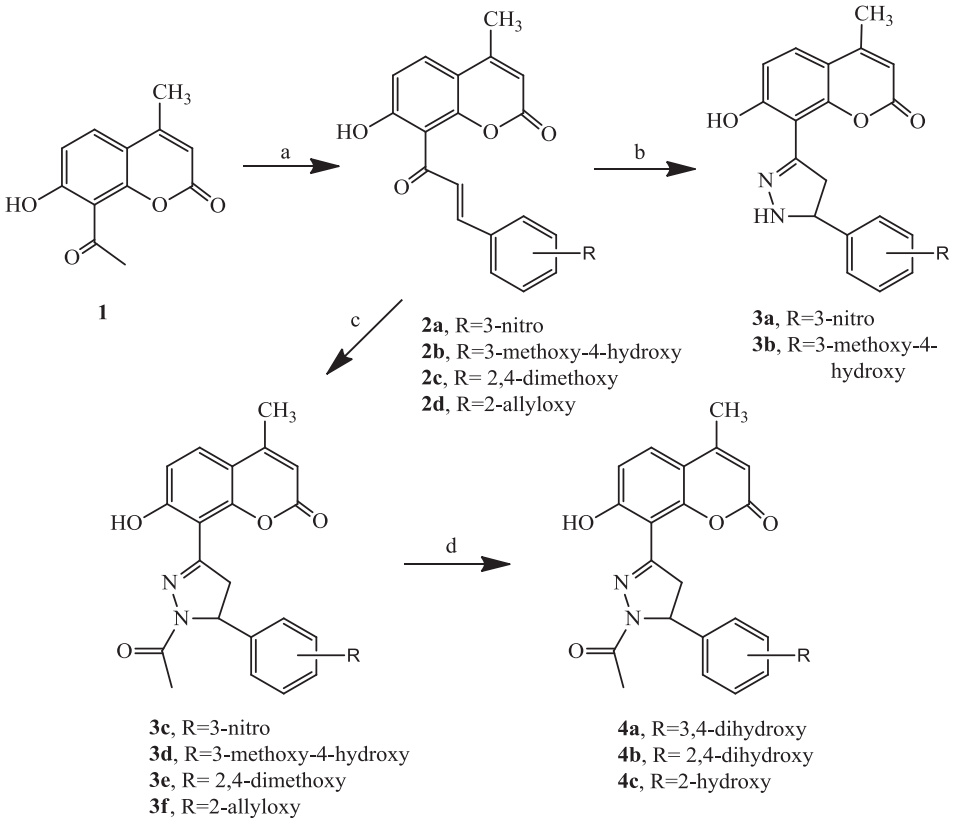
2.2. Antioxidant profiling

2.2.1. Antioxidant effects on AAPH-induced oxidation of DNA

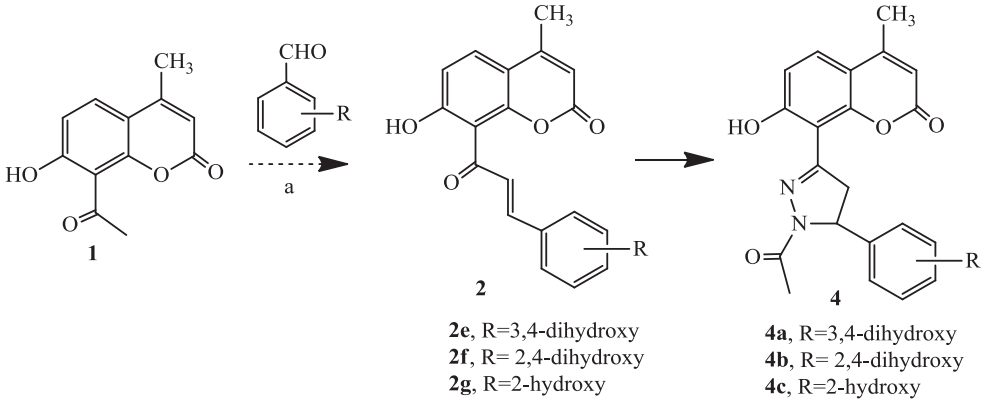
The peroxyl radical (ROO·) generated from the decomposition of AAPH is able to abstract H atom from the C-4′ atom of DNA, resulting in strand breaks and further generating more than 20 carbonyl species called thiobarbituric acid reactive substance (TBARS, λ_{max} = 535 nm) [34]. The oxidation of aforementioned biological samples can also be detected by measuring TBARS [35]. Hence, we herein also applied this method in the *in vitro* assays of the inhibitory activities of synthetic compounds **3a**–**d** and **4a**–**c** on DNA oxidation (Fig. 2). xanthotoxol, a good natural coumarin antioxidant [36,37], was used as positive control.

As shown in Fig. 2(A), the negtive control shows a continual increase of the absorbance of TBARS, indicating that much more carbonyl species are produced in AAPH-induced oxidation of DNA with the incubation period increasing. However, the additions of various concentrations of coumarin derivatives (**3a**–**d**, **4a**–**c**) and xanthotoxol remarkably suppress the increase of the absorbance of TBARS at the beginning of the incubation period, indicating that these compounds were able to inhibit the oxidation of DNA for a period. This period could be designated as *inhibition period* (t_{inh}) being related proportionally to the concentration of 4,5-dihydropyrazole coumarins (**3a**–**d** and **4a**–**c**) and xanthotoxol as shown in Fig. 2(B). Moreover, the quantitative equations of t_{inh} *versus* the concentration of **3a**–**d**, **4a**–**c** and xanthotoxol were calculated and listed in Table 1. The application of the linear correlation between t_{inh} and the concentration of the antioxidant for the evaluation of the antioxidant efficacy was shown as Eq. (1) [38].

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



Scheme 1. Synthesis of title compounds Reagent and conditions: (a) substituted-benzaldehyde, piperdine, ethanol, reflux; (b) 80% hydrazine monohydrate, ethanol, reflux, 12 h; (c) 80% hydrazine monohydrate, acetic acid, reflux, 2h; (d) $AlCl_3$, toluene, pyridine, reflux, 6 h; or BBr_3, dichloromethane, −20–0 °C; or $Pd(PPh_3)_4$, K_2CO_3, methanol, reflux, 6 h.



Scheme 2. Attempt to synthesize compound **4** reagent and conditions: (a) piperdine, ethanol, reflux.

The *n* refers to the stoichiometric factor, expressing the number of the radical propagation terminated by one molecule of the antioxidant, and R_i is the initiation rate of the radical-induced reaction. This equation has been employed in the study of the protection of DNA against AAPH-induced oxidation by homoisoflavonoids [39]. R_i is equal to the generation rate (R_g) from the decomposition of AAPH ($R_g = (1.4 \pm 0.2) \times 10 - 6$ [AAPH] s^{-1} [38]). This is because both sodium salt of DNA and AAPH are dissolved in the same water phase, and radicals generated from AAPH attack DNA at the water phase. Thus, the *n* values of **3a**–**d**, **4a**–**c** and xanthotoxol are the products of coefficients in the corresponding equation multiplied by $R_i = R_g = 1.4 \times 10{-}6 \times 40$ mM $s^{-1} = 3.36$ μM min^{-1} and listed in Table 1 as well.

Table 1 shows an order of the inhibitory effect of assayed compounds on the oxidation of DNA as **4a** ≥ **4b** ≥ **3b** ≥ **3d** ≈ **4c** ≥ **3a** ≥ **3c** ≥ xanthotoxol. This reveals all of our synthesized 4,5-dihydropyrazole coumarins have higher activity than xanthotoxol against DNA damage induced by AAPH.

Among the N-acetylation compounds **3c**, **3d**, and **4a**–**c**, the order of the antioxidant capacities is **4a** > **4b** > **3d** ≈ **4c** > **3c**. Apparently, the *n* value of compound **4a** (6.15) and **4b** (5.55), both bearing with more two hydroxyl groups on the phenyl rings, are much higher than compound **3c** (2.15). This indicates that the antioxidant activity is largely depended on the number of hydroxyl group on the phenyl ring. The protective activity of **4a** ($n = 6.15$) is higher than **4b** ($n = 5.55$), indicating the *ortho* dihydroxyl groups on the benzene ring could enhance the antioxidant activities of these 4,5-dihydropyrazole coumarin derivatives. This could be rationalized by the increased stabilization of the semiquinone radical with intramolecular hydrogen bond (Scheme 3). The intramolecular hydrogen bond, in fact, is stronger than in the parent phenol [40,41].

As to the 1-*N*-H compound **3a**, **3b** and their *N*-acetylated derivatives **3c**, **3d**, the order of the antioxidant capacities was **3a** (3.36) ≥ **3c** (2.15) and **3b** (4.64) ≥ **3d** (3.99), indicating that the N–H in position 1-*N*-unsubstituted 4,5-dihydropyrazole ring could also enhance ability to protect DNA against AAPH-induced oxidation. The reason for the antioxidant activity of the N–H in 4,5-dihydropazole coumarins can be concluded that the hydrogen atom can be abstracted by radicals and the resulting single electron can be stabilized via resonance (Scheme 4). In Table 1, the *n* value of **3b** and **3d** is higher than **3a** and **3c**, respectively, due to the hydroxyl group on benzene ring (5 position of the 4,5-dihydropazole ring).

2.2.2. Radical scavenging ability

The radical scavenging ability of an antioxidant is usually evaluated by trapping $ABTS^{+\bullet}$ and DPPH [42]. Fig. 3 indicates the variation of the absorbance of $ABTS^{+\bullet}$ and DPPH decreases in the presence of 4,5-dihydropyrazole coumarins and xanthotoxol. Recently, the interaction between an antioxidant with $ABTS^{+\bullet}$ was investigated by chemical kinetics [43]. As shown in Eq. (2), $[ABTS^{+\bullet}]_0$ and $[ABTS^{+\bullet}]_\infty$ stand for the concentration of $ABTS^{+\bullet}$ at the beginning and the end of the reaction, respectively. Thus, $[ABTS^{+\bullet}]_0 - [ABTS^{+\bullet}]_\infty$ means the concentration of $ABTS^{+\bullet}$ depleted by a certain concentration of the antioxidant. The depleted concentration of $ABTS^{+\bullet}$ is divided by the concentration of the antioxidant to indicate the number of $ABTS^{+\bullet}$ (n) trapped by the antioxidant at the primary period:

$$n = \frac{[ABTS^{+\cdot}]_0 - [ABTS^{+\cdot}]_\infty}{[\text{antioxidant}]} \quad (2)$$

If the oxidized product of the antioxidant can trap $ABTS^{+\bullet}$, this period is called the secondary period for the antioxidant to trap $ABTS^{+\bullet}$. In the secondary period of the reaction between the antioxidant and $ABTS^{+\bullet}$, the variation of the concentration of $ABTS^{+\bullet}$ ($[ABTS^{+\bullet}]$) follows:

$$\left[ABTS^{+\cdot}\right] = \left[ABTS^{+\cdot}\right]_\infty + \frac{ab}{b+t} \quad (3)$$

where $a = n_{app}[\text{antioxidant}]_0$, $b = 1/(k_{app}[\text{antioxidant}]_0)$, and t is the reaction time. In Eq. (3), n_{app} refers to the apparent number of $ABTS^{+\bullet}$ trapped by the oxidized products of the antioxidant, and k_{app} is the apparent rate constant in this period. Therefore, $n + n_{app}$ is the total number of $ABTS^{+\bullet}$ trapped by the antioxidant [44]. In this work we apply this method to study the reaction between 4,5-dihydropyrazole coumarins, xanthotoxol and DPPH as well, and the n and n_{app} in $ABTS^{+\bullet}$ and DPPH trapping reactions were listed in Table 2.

Firstly, in the evaluations of the *N*-acetylated compund **3c**, **3d** and **4a**–**c**, the order of the total numbers of electrons donated in trapping $ABTS^{+\bullet}$ and DPPH was found in the order: **4b** > **4a** > **4c** > **3d** > **3c** and **4a** > **3d** > **4b** ≈ **4c** > **3c**, respectively (Table 2). With the phenol moieties, the capacities of 4,5-dihydropyrazole coumarins **3d**, and **4a**–**c** to trap $ABTS^{+\bullet}$ ($n + n_{app}$ ranges from 1.52 to 2.48) and DPPH ($n + n_{app}$ ranges from 1.01 to 4.53) are remarkably higher than the *p*-nitrophenyl substituted analog **3c** ($n + n_{app}$ in trapping $ABTS^{+\bullet}$ and DPPH were 0.18 and 0.79, respectively).

On the other hand, among the compounds with phenol substituents, the total number of electrons donated to DPPH by **4a** ($n + n_{app} = 4.53$) was also found much higher than **4b** ($n + n_{app} = 1.04$). This indicated that the *ortho* dihydroxyl on the benzene ring are more efficient to donate hydrogen atom in the

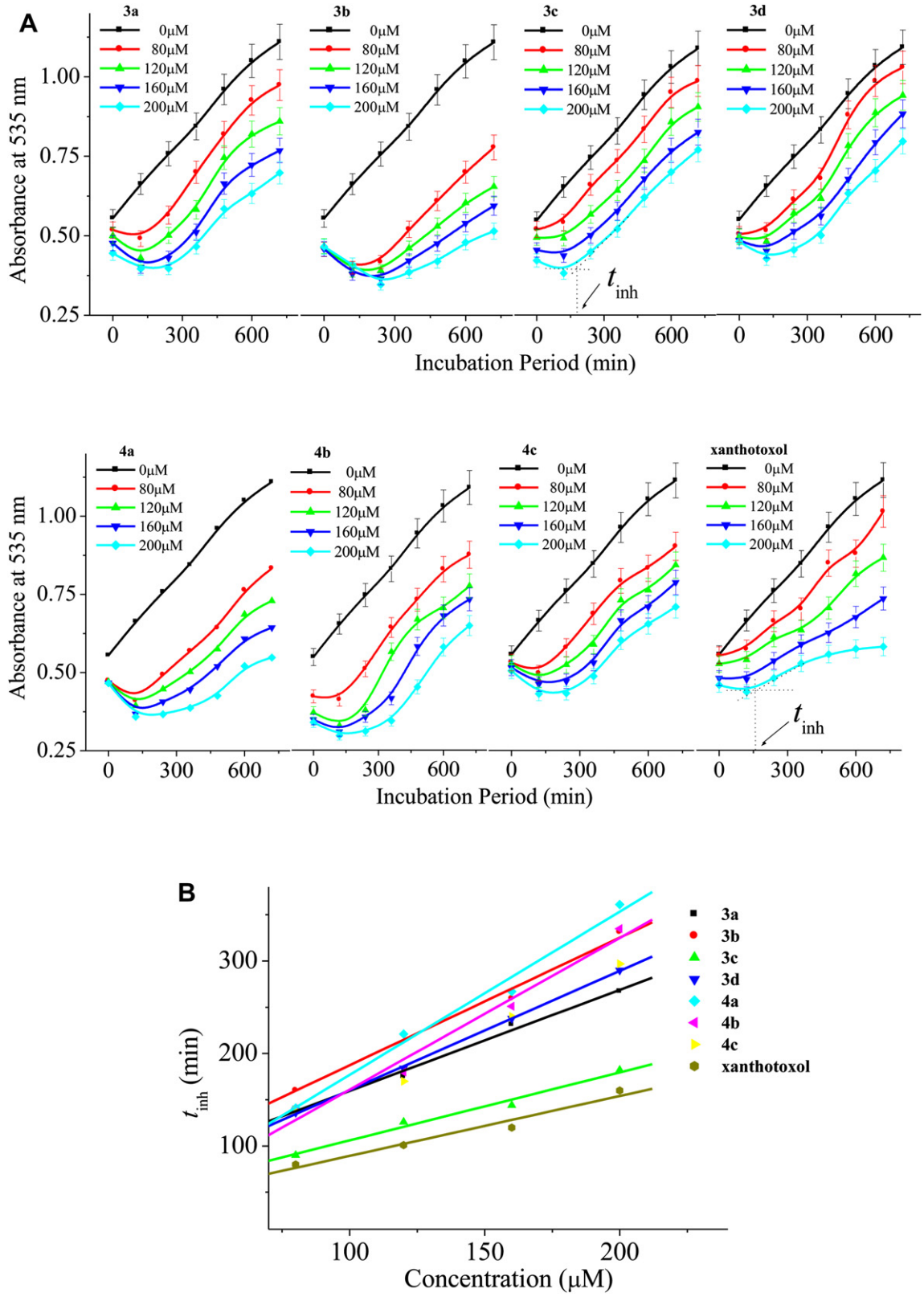


Fig. 2. (A) The variation of the absorbance of TBARS in the mixture of DNA (2.0 mg/ml) and 40 mM AAPH with various concentrations of 4,5-dihydropyrazole coumarins (**3a**–**d** and **4a**–**c**) and xanthotoxol added. (B) The relationships between t_{inh} and the concentrations of 4,5-dihydropyrazole coumarins and xanthotoxol.

hydroxyl group to *N*-centered radicals compared with *meta* dihydroxyl counterpart. On the contrary, **4b** is more efficient to reduce radicals than **4a** since $n + n_{app}$ of **4b** (2.48) is higher than those of **4a** (1.68) in trapping $ABTS^{+\bullet}$.

Secondly, in the survey of the effect of the N-substituents on the pyrazole core, we found that the total numbers of electrons transferred in $ABTS^{+\bullet}$ and DPPH trapping by 1-*N*-H compunds **3a** (2.54/3.35) and **3b** (4.41/5.13) are much higher than those of

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

Compound	t_{inh} (min) = (n/R_i) [Antioxidant (μM)] + constant[b]	n
3a	t_{inh} = 1.09 [**3a**] + 50.3	3.36
3b	t_{inh} = 1.38 [**3b**] + 49.3	4.64
3c	t_{inh} = 0.64 [**3c**] + 40.6	2.15
3d	t_{inh} = 1.19 [**3d**] + 31.4	3.99
4a	t_{inh} = 1.83 [**4a**] + 8.4	6.15
4b	t_{inh} = 1.64 [**4b**]−3.1	5.55
4c	t_{inh} = 1.18 [**4c**] + 50.3	3.96
xanthotoxol[c]	t_{inh} = 0.59 [**3c**] + 24.6	2.00

[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 is generated from the linear regression analysis.
[c] Xanthotoxol was used as standard.

their 1-*N*-acetylated derivatives **3c**(0.18/0.79) and **3d** (1.52/2.09). This results suggest that the N–H in position 1-*N*-unsubstituted 4,5-dihydropyrazole ring could trap $ABTS^{+\bullet}$ and DPPH more efficiently, given the more stable resonance structures of radicals of **3b** (Scheme 4). As shown in Table 2, the higher radical scavenging activities of **3b** and **3d** than **3a** and **3c**, respectively, is also attributed to the presence of phenol substituents at the 5 position of 4,5-dihydropazole core.

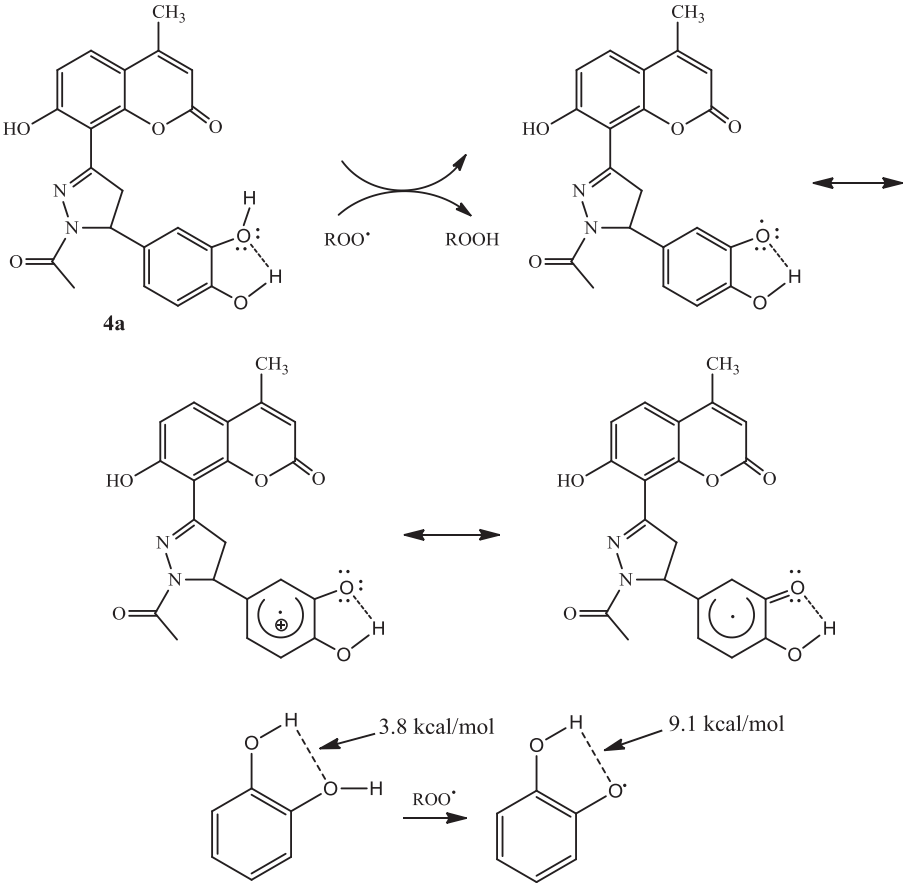
3. Conclusion

A novel and concise synthetic entry to the 4-methylcoumarin derivatives containing 4,5-dihydropyrazole moiety from 8-acetyl-7-hydroxy-4-methylcoumrain and substituted-benzaldehyde has been developed. Introducing hydroxyl groups onto the benzene ring at the 5 position of 4,5-dihydropyrazole and leave the 1 position of the 4,5-dihydropyrazole core unsubstituted improve the DNA protection activity against AAPH-induced oxidation via $ABTS^{+\bullet}$ and DPPH trapping. Especially, with two hydroxyl groups *ortho* to each other in the benzene ring of 4,5-dihydropyrazole coumarins possess stronger antioxidant capacity. Compound **3b** and **4a** are the two novel and promising lead compounds suitable for further development for designing antioxidants.

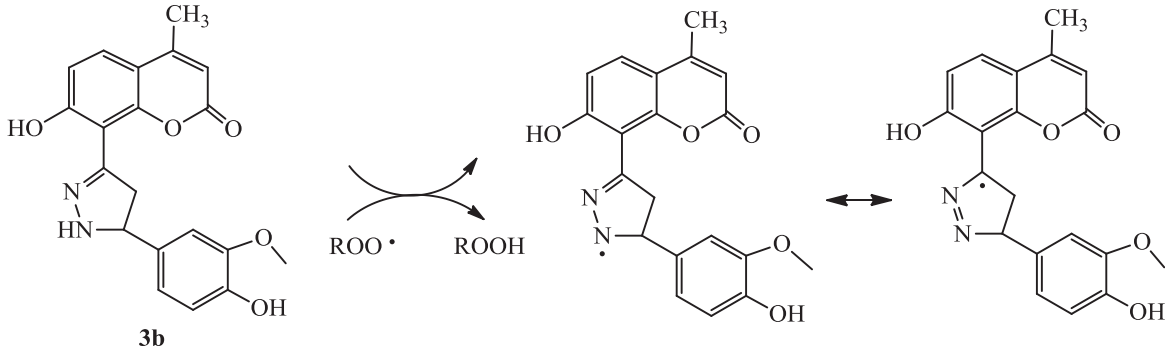
4. Experiment

4.1. Materials and instrumentation

Diammonium salt of 2, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), DPPH and galvinoxyl radicals were purchased from Fluka Chemie GmbH, Buchs, Switzerland. AAPH, the naked DNA sodium salt, and GSH were purchased from ACROS ORGANICS, Geel, Belgium. Other agents were of analytical grade and used directly. Other reagents were of analytical grade, and purchased from Beijing Chemical Reagent Co., China. Flash column chromatography was performed on a silica gel (300–400 mesh) and thin layer chromatography (TLC) inspections were carried out on a silica gel GF254 plates. The ^1H and ^{13}C NMR data were recorded with a Varian Mercury 300 NMR spectrometer, using TMS as an internal standard. Chemical shifts (δ) were given in parts per million and coupling constants were given as absolute values expressed in Hertz. IR spectra were obtained with a 360 FT-IR spectrophotometer of Nicolet. Mass spectra were obtained using LC/MS 1100 of Agilent Technology Corporation and Alltech ELSD 2000 instrument. Melting points were determined by an X-4 digital microscope. The synthetic



Scheme 3. Stability of phenoxy radical via hydrogen bonding.



Scheme 4. The N–H as antioxidative group in 4,5-dihydropyrazole coumarins.

compounds were analyzed by high performance liquid chromatography, and the purities of these compounds were larger than 98.0%.

4.2. Synthesis of title compound *3a–d* and *4a–c*

4.2.1. General procedure A for the synthesis of 7-hydroxy-8-((2E)-3-(3-aryl)-1-oxo-2-propen-1-yl)-4-methyl-2H-1-benzopyran-2-ones (*2a–d*)

To 8-acetyl-7-hydroxy-4-methylcoumrain **1** (0.218 g, 1.0 mmol) and substituted-benzaldehyde (1.1 mmol) in ethanol (10 mL) was added piperidine (0.1 mL). The reaction mixture was refluxed for 12 h or until completion of the reaction indicated by TLC. After the reaction mixture was cooled to room temperature, the precipitates were filtered to give the product. Further purification was performed by recrystallization from 95% ethanol.

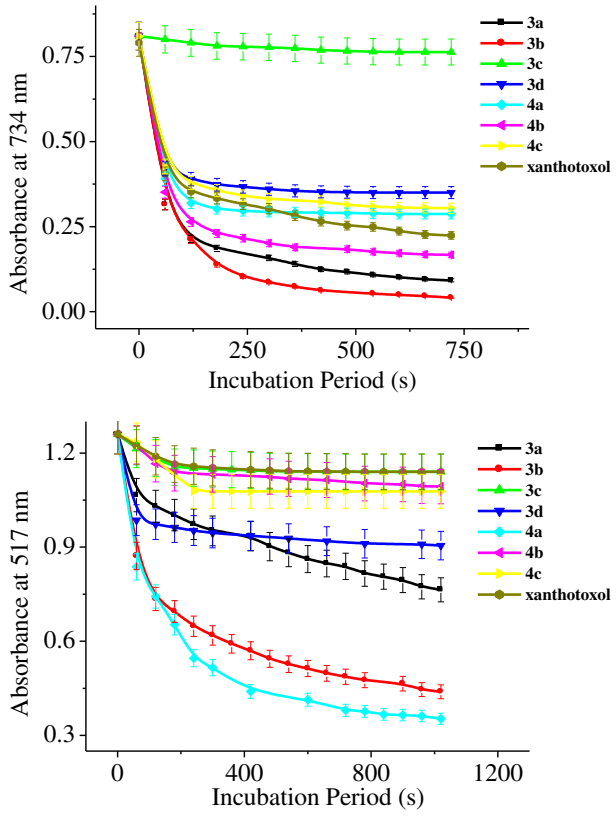


Fig. 3. The decrease of the absorbance of $ABTS^{+\bullet}$ (734 nm) in the presence of 0.04 μM 4,5-dihydropyrazole coumarins and xanthotoxol, and the decrease of the absorbance of DPPH (517 nm) in the presence of 0.40 μM 4,5-dihydropyrazole coumarins and xanthotoxol.

*4.2.1.1. 7-Hydroxy-8-((2E)-3-(3-nitrophenyl)-1-oxo-2-propen-1-yl)-4-methyl-2H-1-benzopyran-2-one (**2a**).* The reaction was conducted according to the general procedure A and the product 2a was obtained as an orange yellow solid (0.175 g, 50% yield). m. p. 220–221 °C. IR (KBr): 3428, 1724, 1638, 1596, 1525, 1390 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 13.52 (s, 1 H, –OH), 8.51 (d, 1 H), 8.26–8.34 (m, 2 H), 8.11–8.13 (m, 1 H), 7.90–7.95 (m, 1 H), 7.65–7.75 (m, 2 H), 6.97–7.01 (m, 1 H), 6.22 (d, 1 H), 2.46 (s, 3 H).

*4.2.1.2. 7-Hydroxy-8-((2E)-3-(3-methoxy-4-hydroxy)-1-oxo-2-propen-1-yl)-4-methyl-2H-1-benzopyran-2-one (**2b**).* The reaction was conducted according to the general procedure A to afford **2b** as an orange yellow solid (0.282 g, 80% yield). m. p. 240–241 °C. IR (KBr): 3419, 1723, 1629, 1596, 1509, 1385 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 14.18 (s, 1 H, –OH), 8.28 (d, J = 15.5 Hz, 1 H), 7.94 (d, J = 15.5 Hz, 1 H), 7.69 (d, J = 8.7 Hz, 1 H), 7.43 (d, 2 H), 6.96 (d, J = 8.7 Hz, 1 H), 6.18 (s, 1 H), 5.97 (s, 1 H), 4.03 (s, 3 H), 2.45 (s, 3 H).

*4.2.1.3. 7-Hydroxy-8-((2E)-3-(2,4-dimethoxy)-1-oxo-2-propen-1-yl)-4-methyl-2H-1-benzopyran-2-one (**2c**).* The reaction was conducted according to the general procedure A to afford **2c** as an orange solid (0.30 g, 82% yield). m. p. 212–214 °C. IR (KBr): 3421, 1720, 1631, 1600, 1512, 1385 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 14.29 (s, 1 H, –OH), 8.39 (d, J = 15.6 Hz, 1 H), 8.25 (d, J = 15.6 Hz, 1 H), 7.65 (d, 2 H), 6.93 (d, 1 H), 6.48–6.58 (m, 2 H), 6.17 (s, 1 H), 3.98 (s, 3 H), 3.87 (s, 3 H), 2.43 (s, 3 H).

*4.2.1.4. 7-Hydroxy-8-((2E)-3-(2-allyloxy)-1-oxo-2-propen-1-yl)-4-methyl-2H-1-benzopyran-2-one (**2d**).* The reaction was conducted according to the general procedure A to afford **2d** as an orange solid (0.25 g, 70% yield). m. p. 122–124 °C. IR (KBr): 3427, 1722, 1630, 1599, 1509, 1387 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 13.90 (s, 1 H, –OH), 8.28–8.42 (m, 2 H), 7.79–8.82 (m, 1 H), 7.67 (d, 1 H), 7.34–7.40 (m, 1 H), 7.00–7.05 (m, 1 H), 6.92–6.96 (m, 2 H), 6.18 (s, 1 H), 6.07–6.16 (m, 1 H), 5.44 (dd, J = 17.3, 1.2 Hz, 1 H), 5.31 (dd, J = 10.5, 1.2 Hz, 1 H), 4.72 (d, 1 H), 2.44 (s, 3 H).

4.2.2. General procedure B for the synthesis of 8-(4,5-dihydro-5-aryl-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-ones (*3a*, *3b*)

To a suspension of α,β-unsaturated ketone **2** (0.5 mmol) in ethanol (8 mL) was added hydrazine monohydrate (1 mmol), and the reaction mixture was refluxed for 2 h. After the reaction mixture was cooled, the precipitates were collected by filtration. It was then washed by ethanol and dried to give the product.

*4.2.2.1. 8-(4,5-Dihydro-5-(3-nitrophenyl)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**3a**).* The reaction was conducted according to the general procedure B to compound **3a** as

Table 2
The numbers of $ABTS^{+\bullet}$ and DPPH trapped by **3a**–**d**, **4a**–**c** and xanthotoxol in the primary stage (n), the secondary stage (n_{app}), and the total number of trapped radicals ($n + n_{app}$).

Compound	$ABTS^{+\bullet}$			DPPH		
	n	n_{app}	$n + n_{app}$	n	n_{app}	$n + n_{app}$
3a	2.24 ± 0.11	0.30 ± 0.02	2.54 ± 0.13	3.05 ± 0.15	0.30 ± 0.02	3.35 ± 0.17
3b	3.84 ± 0.19	0.30 ± 0.02	4.14 ± 0.21	5.01 ± 0.25	0.12 ± 0.01	5.13 ± 0.26
3c	0.14 ± 0.01	0.04 ± 0.01	0.18 ± 0.01	0.73 ± 0.04	0.06 ± 0.01	0.79 ± 0.04
3d	1.44 ± 0.07	0.08 ± 0.01	1.52 ± 0.08	2.07 ± 0.10	0.02 ± 0.01	2.09 ± 0.10
4a	1.64 ± 0.08	0.04 ± 0.01	1.68 ± 0.08	4.43 ± 0.22	0.10 ± 0.01	4.53 ± 0.23
4b	2.00 ± 0.10	0.48 ± 0.02	2.48 ± 0.12	1.02 ± 0.05	0.02 ± 0.01	1.04 ± 0.05
4c	1.58 ± 0.08	0.02 ± 0.01	1.60 ± 0.08	0.95 ± 0.05	0.06 ± 0.01	1.01 ± 0.05
xanthotoxol[a]	0.80 ± 0.04	0.16 ± 0.01	0.96 ± 0.05	0.73 ± 0.04	0.02 ± 0.01	0.75 ± 0.04

[a] Xanthotoxol was used as standard.

a yellow–white solid (0.173 g, 95% yield). m. p. 230–232 °C. IR (KBr): 3430, 3339, 1729, 1632, 1599, 1532, 1383 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 12.48 (s, 1 H, –OH), 8.33 (s, 1 H), 8.19 (d, J = 8.4 Hz, 1 H), 7.77–7.79 (m, 1 H), 7.49–7.60 (m, 2 H), 6.99 (d, J = 8.7 Hz, 1 H), 6.10 (s, 1 H), 5.03–5.10 (m, 1 H), 4.22–4.31 (m, 1 H), 3.53–3.63 (m, 1 H), 2.41 (s, 3 H). MS (ESI): m/z 364.3 [M-H^+].

*4.2.2.2. 8-(4,5-Dihydro-5-(3-methoxy-4-hydroxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**3b**).* The reaction was conducted according to the general procedure B, the product was further purified by flash column chromatography with chloroform and methanol (15:1, v:v) as eluent. 4,5-Dihydropyrazol derivative **3b** was obtained as a yellow–white solid (0.170 g, 93% yield). m. p. 228–230 °C. IR (KBr): 3433, 3418, 1723, 1625, 1598, 1514, 1385 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 12.75 (s, 1 H, –OH), 8.94 (s, 1 H, –OH), 7.92 (s, 1H, –NH), 7.15 (d, 1 H), 6.77–7.01 (m, 4 H), 6.19 (s, 1 H), 4.77–4.84 (m, 1 H), 3.83–3.93 (m, 1 H), 3.77 (s, 3 H), 3.22–3.28 (m, 1 H), 2.39 (s, 3 H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 160.69, 159.33, 153.85, 151.54, 149.03, 147.59, 132.82, 125.95, 119.17, 115.34, 113.14, 111.95, 110.83, 110.19, 105.33, 62.27, 55.56, 44.89, 18.45. MS (ESI): m/z 366.1 [M – H^+].

*4.2.3. General procedure C for the synthesis of 8-(1-acetyl-4,5-dihydro-5-aryl-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-ones (**3c**–**f**)*

To a suspension of ketone **2** (1.0 mmol) in acetic acid (2.0 mL) was added hydrazine monohydrate (4.0 mmol) and the reaction mixture was refluxed for 2 h. The mixture was then cooled to room temperature, poured into crush ice, and allowed to stand at room temperature over night. The crude product was collected by filtration and purified by flash column chromatography with chloroform and ethyl acetate (9:1, v:v) as eluent.

*4.2.3.1. 8-(1-Acetyl-4,5-dihydro-5-(3-nitrophenyl)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**3c**).* The reaction was conducted according to the general procedure C to give title compounds **3c** as a white solid (0.362 g, 89% yield). m. p. 235–237 °C. IR (KBr): 3432, 1736, 1673, 1633, 1597, 1500, 1384 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 11.68 (s, 1H, –OH), 8.08–8.17 (m, 2 H), 7.52–7.66 (m, 3 H), 7.05 (d, 1 H), 6.13 (s, 1 H), 5.60–5.65 (m, 1 H), 4.40–4.51 (m, 1 H), 3.80–3.88 (m, 1 H), 2.42 (s, 6 H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 166.86, 160.69, 158.59, 153.30, 152.20, 147.65, 142.11, 131.45, 129.02, 126.89, 122.02, 119.68, 113.28, 113.27, 111.68, 110.27, 56.95, 45.47, 21.01, 18.10.

*4.2.3.2. 8-(1-Acetyl-4,5-dihydro-5-(3-methoxy-4-hydroxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl 2H-benzopyran-2-one (**3d**).* The reaction was conducted according to the general procedure C the eluent for purification by flash column chromatography is chloroform and methanol (15:1, v:v). **3d** was obtained as a white solid (0.346 g, 85% yield). m. p. 210–213 °C. IR (KBr): 3406, 1728, 1670, 1633, 1599, 1516, 1384 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 9.70 (s, 1 H, –OH), 7.15 (d, 1 H), 6.68–6.89 (m, 4 H), 6.10 (s, 1 H), 5.38–5.43 (m, 1 H), 3.75–3.90 (m, 1 H), 3.71 (s, 3 H), 3.15–3.23 (m,1 H), 2.37 (s, 3 H), 2.21 (s, 3 H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 168.08, 162. 24, 159.84, 153.84, 151.71, 147.68, 145.81, 133.55, 127.28, 118.49, 115.24, 113.59, 111.01, 109.94, 109.44, 106.47, 58.44, 55.56, 21.93, 20.48.

*4.2.3.3. 8-(1-Acetyl-4,5-dihydro-5-(2,4-dimethoxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**3e**).* The reaction was conducted according to the general procedure C to give **3e** as a yellow–white solid (0.176 g, 87% yield). m. p. 213–214 °C. IR (KBr): 3426, 1735, 1672, 1633, 1600, 1508, 1386 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 11.97 (s, 1H, OH), 7.55 (d, 1H), 6.99–7.04 (m, 2H), 6.40–6.43 (m, 2H), 6.12 (d, 1H), 5.64–5.569 (m, 1H), 4.19–4.30 (m, 1H), 3.75 (d, 6H), 3.64–3.72 (m, 1H), 2.39 (d, 6H).

*4.2.3.4. 8-(1-Acetyl-4,5-dihydro-5-(2-allyloxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**3f**).* The reaction was conducted according to the general procedure C to give **3f** as a yellow–white solid (0.129 g, 62% yield). m. p. 209–210 °C. IR (KBr): 3437, 1739, 1671, 1632, 1599, 1384 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 12.01 (s, 1 H, –OH), 7.55 (d, J = 8.7 Hz, 1 H), 7.17–7.25 (m, 2 H), 7.00 (d, J = 9.0 Hz,1 H), 6.85–6.93 (m, 1 H), 6.11 (s, 1 H), 5.86–5.97 (m, 1 H), 5.69–5.74 (m, 1 H), 5.27 (dd, J = 1.2, 17.1 Hz, 1 H), 5.11 (dd, J = 1.2, 10.2 Hz, 1 H), 4.48–4.52 (m, 2 H), 4.24–4.34 (m, 1 H), 3.73–3.81 (m, 1 H), 2.40 (d, 6 H).

*4.2.4. 8-(1-Acetyl-4,5-dihydro-5-(3,4-dihydroxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**4a**)*

A suspension of **3d** (0.204 g, 0.5 mmol) in toluene (10 mL), at 0–5 °C, was added aluminum chloride (0.131 g, 1.0 mmol) lot wise. Then the temperature was raised to 50–60 °C, and pyridine (166 mL, 2.0 mmol) was added drop-wise. Then the reaction was heated to reflux for 6 h. Upon completion of the reaction (progress of the reaction was monitored by TLC), the reaction mixture was cooled to room temperature. Then aq. HCl (5.0%) was added drop-wise and the mixture was stirred for 30 min. The organic phase was separated and the aqueous phase was extracted with ethyl acetate, and the combined organic phase was washed with water, dried over anhydrous sodium sulfate. The solvent was removed and the crude product was purified by flash chromatography column with chloroform and methanol (9:1, v:v) as eluent. Compound **4a** was obtained as a white solid (0.138 g, 70% yield). m. p. 229–231 °C. IR (KBr): 3443, 3428, 1724, 1673, 1623, 1599, 1517, 1385 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.85 (s, 1 H, –OH), 8.84 (s, 2 H, –OH), 7.72 (d, J = 8.7 Hz, 1 H), 6.99 (d, J = 8.7 Hz, 1 H), 6.59–6.67 (m, 3 H), 6.21 (s, 1 H), 5.30–5.38 (m, 1 H), 3.89–3.99 (m, 1 H), 3.23–3.32 (m, 1 H), 2.39 (s, 3 H), 2.23 (s, 3 H). ^{13}C NMR (75 MHz, DMSO-d_6)

δ (ppm): 166.94, 160.20, 159.18, 157.52, 154.88, 153.71, 153.05, 152.72, 127.73, 126.97, 118.41, 113.16, 112.24, 110.49, 106.06, 102.74, 54.14, 45.18, 21.82, 18.39. MS (ESI): *m/z* 393.2 [M − H^+], *m/z* 787.3 [2M − H^+].

4.2.5. 8-(1-Acetyl-4,5-dihydro-5-(2,4-dihydroxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**4b**)

Compound **3e** (0.211 g, 0.5 mmol) was dissolved in anhydrous CH_2Cl_2 (15 mL) under N_2, and cooled to −20 °C. Then 1 M BBr_3 in CH_2Cl_2 (1.5 mL) was added drop-wise via a syringe. The mixture was stirred at room temperature for the indicated time. The mixture was poured cautiously to icy water and extracted with ethyl acetate. The combined organic phase was washed with brine, dried over anhydrous sodium sulfate, and. concentrated under vacuum. The residue was purified by column chromatography with chloroform and methanol (9:1, *v:v*) as eluent. Compound **4b** was obtained as a white solid (0.16 g, 81% yield). m. p. 240–241 °C. IR (KBr): 3451, 3429, 1721, 1670, 1629, 1598, 1519, 1386 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.50 (s, 1 H, −OH), 9.48 (s, 1 H, −OH), 9.16 (s, 1 H, −OH), 7.71 (d, $J = 8.7$ Hz, 1 H), 6.98 (d, $J = 8.7$ Hz, 1 H), 6.77 (d, $J = 8.4$ Hz, 1 H), 6.14–6.29 (m, 3 H), 5.49–5.55 (m, 1 H), 3.91–4.02 (m, 1 H), 3.16–3.24 (m, 1 H), 2.39 (s, 3 H), 2.27 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): 166.89, 160.18, 159.15, 157.51, 154.85, 153.68, 153.00, 152.73, 127.71, 126.95, 118.39, 113.12, 112.22, 110.49, 106.04, 105.19, 102.72, 54.11, 45.14, 21.79, 18.36. MS (ESI): *m/z* 393.1 [M − H^+].

4.2.6. 8-(1-Acetyl-4,5-dihydro-5-(2-dihydroxy)-1H-pyrazol-3-yl)-7-hydroxy-4-methyl-2H-1-benzopyran-2-one (**4c**)

To a stirred suspension of compound **3f** (0.209 g, 0.5 mmol) in MeOH (8 mL) was added $Pd(PPh_3)_4$ (6.2 mg, 5.0 μmol) under a nitrogen atmosphere. The slightly yellow mixture was stirred for 5 min, and K_2CO_3 (0.204 g, 1.5 mmol) was added. The reaction was then refluxed for 6 h, before cooled to room temperature and concentrated in vacuo. The residue was treated with 2 N HCl, and extracted with ethyl acetate. The combined organic phase was washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The crude product was purified by flash column chromatography with chloroform and methanol (15:1, *v:v*) as eluent. Compound **4c** was obtained as an off-white solid (0.124 g, 68% yield). m. p. 231–234 °C. IR (KBr): 3430, 1731, 1670, 1633, 1599, 1517, 1384 cm^{-1} ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.45 (s, 1 H, −OH), 9.71 (s, 1 H, −OH), 7.72 (d, $J = 8.7$ Hz, 1 H), 7.06–7.11 (m, 1 H), 6.98 (d, $J = 8.7$ Hz, 2 H), 6.83 (d, $J = 7.8$ Hz, 2 H), 6.72–6.77 (m, 1 H), 6.21 (s, 1 H), 5.61–5.66 (m, 1 H), 3.98–4.08 (m, 1 H), 3.22–3.29 (m, 1 H), 2.39 (s, 3 H), 2.30 (s, 3 H).

4.3. AAPH-induced oxidation of DNA test

The experiment of AAPH-induced oxidation of DNA was performed as described in the literature [33,45]. Briefly, DNA and AAPH were dissolved in phosphate buffered solution (PBS: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA) with the final concentrations at 2.0 mg/mL and 4.0 mM, respectively. Various concentrations 4,5-dihydroxypyrazole coumarins **3a**–**d**, **4a**–**c** and xanthotoxol (standard) in DMSO were added to the above mixture. The solution was poured into test tubes as 2.0 ml aliquots. The test tubes were incubated in a water bath (37 °C) to initiate the oxidation of DNA. Three tubes were taken out at every 120 min and cooled immediately. After addition of 1.0 mL of thiobarbituric acid (TBA) solution (1.00 g TBA and 0.40 g NaOH dissolved in 100 mL of PBS) and 1.0 mL of 3.0% trichloroacetic acid aqueous solution, the tubes were heated in boiling water for 15 min. Upon cooling to room temperature, 1.5 mL of *n*-butanol was added and shaken vigorously to extract TBA reactive substance (TBARS). The UV-absorbance of *n*-butanol layer was measured at 535 nm, and plotted *versus* incubation time.

4.4. Antioxidant effectiveness in chemical experimental systems

The experiments of **3a**–**d** and **4a**–**c** to trap $ABTS^{+\bullet}$ and DPPH were carried out following the description in literature [46,47]. The radical scavenging activity of xanthotoxol was also assayed as positive control. Briefly, ABTS (2.00 mL, 4.0 mM) was oxidized by 1.41 mM $K_2S_2O_8$ for 16 h to generate $ABTS^{+\bullet}$, to which 100 mL of ethanol was added to make the absorbance (Absref) around 0.70 at 734 nm ($\varepsilon_{ABTS+\bullet} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$) [48]. DPPH was dissolved in ethanol to make the absorbance (Abs_{ref}) around 1.00 at 517 nm ($\varepsilon_{DPPH} = 4.09 \times 10^3\ M^{-1}\ cm^{-1}$), to which ethanol solution of **3a**–**d** or **4a**–**c** was added with 40 μM as the final concentration in the radical solutions. The decay of the absorbance for DPPH solution (Abs_{detect}) was recorded. The same operation was performed to $ABTS^{+\bullet}$ with 40 μM as the final concentration for **3a**–**d** or **4a**–**c**.

4.5. Statistical analysis

All the data were the average values from at least triple independent measurements with the experimental error within 10%. The equations were analyzed by one-way ANOVA in Origin 7.5 Professional software, and $p < 0.001$ indicated a significant difference.

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