Tetrahedron 69 (2013) 9898–9905

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Ferrocenyl-contained dendritic-like antioxidants with dihydropyrazole and pyrazole as the core: investigations into the role of ferrocenyl group and structure–activity relationship on scavenging radical and protecting DNA

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ARTICLE INFO

Article history:
Received 5 June 2013
Received in revised form 1 August 2013
Accepted 20 August 2013
Available online 27 August 2013

Keywords:
Dendritic-like antioxidant
Ferrocene
Free radical
Oxidation of DNA

ABSTRACT

Eight ferrocenyl and three corresponding phenyl dendritic-like antioxidants with dihydropyrazole or pyrazole as the core were synthesized and their antioxidant abilities to scavenge 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$) and to protect DNA against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced oxidation were evaluated. It was found that the antioxidant abilities of almost all of the ferrocenyl dendritic-like antioxidants are greater than those of the corresponding phenyl dendritic-like antioxidants remarkably. Moreover, the structure–activity relationships of all these dendritic-like antioxidants were investigated in detail. The quantitative contributions of ferrocenyl group, hydroxyl group, and dihydro-structure in the heterocyclic core to the values of n, n_{app} and $n+n_{app}$ in scavenging $ABTS^{+\bullet}$ and the values of n in protecting DNA against AAPH-induced oxidation were also calculated.

1. Introduction

It is proved that the oxidation of lipids, DNA, membranes, and proteins caused by reactive oxygen species (ROS) incorporating free radicals in vivo is associated with a variety of chronic health problems, such as cancers, atherosclerosis,[1] neurodegenerative processes like Alzheimer's and Parkinson's diseases.[2] Free radicals are single-electron species produced in metabolism[3] or absorbed from environment,[4] which can attack many kinds of cells and tissues.[5] Therefore, supplementation with antioxidants becomes a therapeutic strategy for some diseases[6–8] as the antioxidants can provide hydrogen atoms or electrons to neutralize the single electron.[9] For this reason, exploring effective antioxidants is of importance in medicine and chemistry. Dendrimers are widely used in drug design because of their special affinities toward DNA, membranes, and other biological species.[10] In recent years, some antioxidants with analogous structures called *dendritic antioxidant* have also been synthesized and their abilities to protect DNA against 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)- and Cu^{2+}-induced oxidation were found greater than those of some polyphenols.[11] As a promising sort of dendritic antioxidants, structures with pyrazole as the core were investigated freshly.[12] Nevertheless, these pyrazoles do not possess rigid dendritic structures, which is suitable to be called dendritic-like antioxidants and their antioxidant abilities are still lower than that of our expectation.

Ferrocene, as an effective phenyl bioisostere, is becoming an applicable platform for drug design by virtue of its redox properties, high liphophilicity and three-dimensional metallocene unit, which may lead to some changes in selectivity toward biological targets compared with a phenyl or alkyl group.[13–15] A mass of drugs have possessed higher activities by introducing ferrocene moiety into molecules, such as antitumor drugs,[16] steroidal drugs,[17] antitubercular drugs,[18] etc. The same strategy was also used to develop antioxidants and the results were satisfactory.[19–21]

Therefore, such useful strategy of introducing ferrocene moiety into molecules was also used in this work to design eight ferrocenyl and three corresponding phenyl dendritic-like antioxidants with dihydropyrazole or pyrazole as the core. The aim of this work is trying to improve the antioxidant abilities of dendritic-like antioxidants with pyrazole as the core aforementioned in literature[12] and investigate the structure–activity relationship, quantitative contributions of ferrocenyl group, dihydro-structure in the heterocyclic core, and hydroxyl group to the antioxidant activities of such species of dendritic-like antioxidants in detail. The synthetic route and the names of these dendritic-like antioxidants are shown in Scheme 1.

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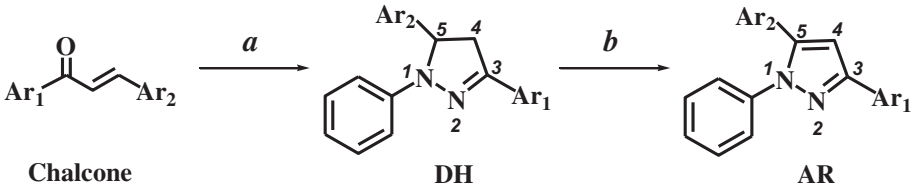
0040-4020/$ – see front matter
http://dx.doi.org/10.1016/j.tet.2013.08.053

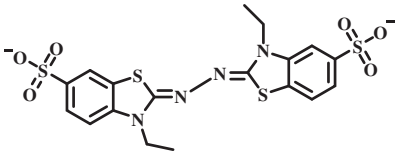


Reaction Conditions:
a) $phNHNH_2$, CH_3COOH-EtOH-H_2O, reflux.
b) The datas of **1AR** to **3AR** are used directly accroding to literatue[12], so they were not synthesized in this work; MnO_2, toluene, reflux for **4AR** and **5AR**; patial spontaneous oxidation during the reaction for **6AR** and **7AR**.

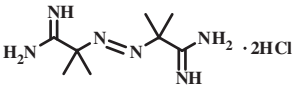
Ar_1	Ar_2	Chalcone	DH	AR
		1	1DH	1AR
HO		2	2DH	2AR
	HO	3	3DH	3AR
	Fe	4	4DH	4AR
Fe		5	5DH	5AR
HO	Fe	6	6DH	6AR
Fe	HO	7	7DH	7AR

Scheme 1. The synthetic route and the structures of the dendritic-like antioxidants with dihydropyrazole and pyrazole as the core.

Their antioxidant abilities were evaluated via scavenging 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$), which is an oxidant-type radical commonly used to test the redox capacities of antioxidants,[22] and protecting DNA against AAPH-induced oxidation, which is commonly used to mimic the oxidation of DNA caused by peroxyl radicals ($ROO^{\bullet}$) in vivo.[23] The structures of $ABTS^{+\bullet}$ and AAPH are shown in Scheme 2.



2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (**ABTS**)



2,2'-azobis(2-amidinopropane hydrochloride) (**AAPH**)

Scheme 2. Structures of AAPH and $ABTS^{+\bullet}$ used in this work.

2. Results

2.1. Radical-scavenging ability

The radical-scavenging ability is commonly regarded as the basic property of an antioxidant[24] and evaluated by trapping $ABTS^{+\bullet}$ in this work. As shown in Fig. 1, the absorbance of $ABTS^{+\bullet}$ decreases in the presence of dendritic-like antioxidants.

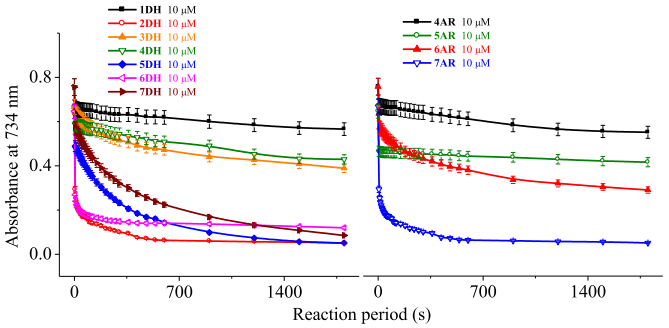


Fig. 1. The decrease in the absorbance of $ABTS^{+\bullet}$ (734 nm) in the presence of 10.0 μM dihydropyrazole and pyrazole derivatives.

The decay of the absorbance of $ABTS^{+\bullet}$ in the presence of an antioxidant was studied by means of chemical kinetics expressed by two equations eventually.[25] The difference between the concentration of $ABTS^{+\bullet}$ at the beginning ($[ABTS^{+\bullet}]_0$) and the end ($[ABTS^{+\bullet}]_\infty$) of the reaction is divided by the concentration of the antioxidant to express the number of $ABTS^{+\bullet}$ (n) trapped by the antioxidant in the primary period, as shown in Eq. 1.

$$n = \frac{[ABTS^{+\bullet}]_0 - [ABTS^{+\bullet}]_\infty}{[\text{antioxidant}]_0} \quad (1)$$

If the oxidized product of the antioxidant can also trap $ABTS^{+\bullet}$, the reaction between the antioxidant and $ABTS^{+\bullet}$ will undergo the secondary stage. Thus, the decay of the concentration of $ABTS^{+\bullet}$ can be expressed by the Eq. 2, which shows the relationship between $[ABTS^{+\bullet}]$ and the corresponding reaction time (t). In this equation, $a=n_{app}[\text{antioxidant}]_0$ and $b=1/(k_{app}[\text{antioxidant}]_0)$.

$$[ABTS^{+\bullet}] = [ABTS^{+\bullet}]_{\infty} + \frac{ab}{b+t} \quad (2)$$

Apparently, if the values of a and b are ciphered out, the values of n_{app} and k_{app} will be obtained easily. Therefore, in order to calculate the values of a and b practically, the Eq. 2 should be changed to Eq. 3, in which the intercept ($1/a$) and the slope ($1/ab$) can be obtained by linear regression analysis in Origin 6.0 Professional software.

$$\frac{1}{[ABTS^{+\bullet}] - [ABTS^{+\bullet}]_{\infty}} = \frac{1}{a} + \frac{1}{ab}t \quad (3)$$

n_{app} refers to the apparent number of $ABTS^{+\bullet}$ trapped by the oxidized products of the antioxidant, while k_{app} is the apparent rate constant in this period but it is not our interest because it can't act as an index of antioxidant ability. Therefore, $n+n_{app}$ is the total number of $ABTS^{+\bullet}$ trapped by the antioxidant. However, it should be noted that the secondary period can't be completely separated from the primary one because the oxidized products generated from the primary period may react with $ABTS^{+\bullet}$ immediately. Meanwhile, the fresh antioxidant molecules still undergo the primary reaction with $ABTS^{+\bullet}$. Therefore, the reaction between the antioxidant and $ABTS^{+\bullet}$ is artificially divided into two periods in this chemical kinetic model. All of the dendritic-like antioxidants reacting with $ABTS^{+\bullet}$ were treated with this method in this work. Moreover, in order to investigate the structure–activity relationship of all these dendritic-like antioxidants and quantitative contributions of ferrocenyl group, dihydro-structure in the heterocyclic core, and hydroxyl group to the values of n, n_{app}, and $n+n_{app}$ in detail, $n_{DH,n}$, $n_{DH,app}$, $n_{DH,t}$, $n_{OH,n}$, $n_{OH,app}$, $n_{OH,t}$, Δn, Δn_{app} and $\Delta(n+n_{app})$ were defined in addition as the contribution of dihydro-structure, hydroxyl group, and ferrocenyl group, respectively. The values of $n_{DH,n}$, $n_{DH,app}$, and $n_{DH,t}$ can be calculated by the n, n_{app}, and $n+n_{app}$ values of dihydropyrazole derivatives minus those of the corresponding pyrazole derivatives, respectively, while the values of $n_{OH,n}$, $n_{OH,app}$, and $n_{OH,t}$ can be calculated by the n, n_{app}, and $n+n_{app}$ values of hydroxylpyrazole derivatives minus those of the corresponding non-hydroxylpyrazole derivatives, respectively. The values of Δn, Δn_{app}, and $\Delta(n+n_{app})$ can be calculated by the n, n_{app}, and $n+n_{app}$ values of ferrocenyl dihydropyrazole and pyrazole derivatives minus those of the corresponding phenyl dihydropyrazole and pyrazole derivatives. All these parameters are listed in Tables 1 and 2.

Table 2
The contributions of ferrocenyl group to the incremental values of n, n_{app}, and $n+n_{app}$ in scavenging $ABTS^{+\bullet}$ radical and n in protecting DNA against AAPH-induced oxidation

Antioxidant	Δn	Δn_{app}	$\Delta(n+n_{app})$[a]	Δn_{DNA}[b]
4DH	1.42±0.07	0.83±0.04	2.25±0.11	—
5DH	3.10±0.16	3.03±0.15	6.13±0.31	2.22±0.11
6DH	−0.56±0.03	−0.66±0.03	−1.22±0.06	0.88±0.04
7DH	2.46±0.12	1.50±0.08	3.96±0.20	14.72±0.74
4AR[c]	1.04±0.05	0.57±0.03	1.61±0.08	—
5AR	1.19±0.06	0.08±0.00	1.27±0.06	—
6AR	3.00±0.15	0.61±0.03	3.61±0.18	0.80±0.04
7AR	2.67±0.13	1.72±0.09	4.39±0.22	7.40±0.37

[a] The values of Δn, Δn_{app}, and $\Delta(n+n_{app})$ are calculated by the n, n_{app}, and $n+n_{app}$ values of ferrocenyl dihydropyrazole and pyrazole derivatives minus those of the corresponding phenyl dihydropyrazole and pyrazole derivatives in scavenging $ABTS^{+\bullet}$ radical.
[b] The values of Δn_{DNA} are calculated by the n values of ferrocenyl dihydropyrazole and pyrazole derivatives minus those of the corresponding phenyl dihydropyrazole and pyrazole derivatives in protecting DNA.
[c] The data of **1AR**, **2AR**, and **3AR** used in calculating Δn, Δn_{app}, $\Delta(n+n_{app})$, and Δn_{DNA} of **4AR**–**7AR** are gotten from the literature.[12]

2.2. Antioxidant effect on AAPH-induced oxidation of DNA

The guanine bases in DNA can be oxidized by radicals generated from the decomposition of AAPH.[26] In this process, the oxidation of linear DNA generates more than 20 carbonyl species, which can react with TBA to form colorful TBARS (λ_{max}=535 nm).[27] Thus, the oxidation process can be examined by detecting TBARS using ultraviolet–visible spectroscopy. As shown in Fig. 2A, in the blank experiment, the increase of the absorbance of TBARS indicates that more carbonyl species will generate as the reaction period increases. The slope of the line can be regarded as the formation rate of the carbonyl species during the oxidation of DNA. However, if addition of an antioxidant can bend the increase line of TBARS at the beginning of the reaction period, that is, to say the formation of TBARS is lagged for a period, it will imply that the antioxidant added is able to inhibit the generation of carbonyl species from the oxidation of DNA.

For the antioxidants, which possess such inhibited effect as some of the dendritic-like antioxidants with dihydropyrazole and pyrazole as the core used in this work, the time corresponding to the cross point of two tangents (short dot lines) can be regarded as the inhibition period (t_{inh}), as shown in Fig. 2A. Furthermore, Fig. 3

Table 1
Characteristic parameters of dendritic antioxidants with dihydropyrazole and pyrazole as the core reacting with $ABTS^{+\bullet}$

Antioxidant	n[a]	n_{app}[b]	$n+n_{app}$	$n_{DH,n}$	$n_{DH,app}$	$n_{DH,t}$	$n_{OH,n}$	$n_{OH,app}$	$n_{OH,t}$[c]
1DH	0.62±0.03	0.58±0.03	1.20±0.06	0.37±0.02	0.36±0.02	0.73±0.04	—	—	—
2DH	3.85±0.19	1.32±0.07	5.17±0.26	3.57±0.18	1.20±0.06	4.77±0.24	3.23±0.16	0.74±0.04	3.97±0.20
3DH	1.73±0.09	1.82±0.09	3.55±0.18	1.48±0.07	1.66±0.08	3.14±0.16	1.11±0.06	1.24±0.06	2.35±0.12
4DH	2.04±0.10	1.05±0.05	3.09±0.15	0.75±0.04	0.26±0.01	1.01±0.05	—	—	—
5DH	3.72±0.19	3.25±0.16	6.97±0.35	2.28±0.11	2.95±0.15	5.23±0.26	—	—	—
6DH	3.29±0.16	0.66±0.03	3.95±0.20	0.01±0.00	−0.07±0.00	−0.06±0.00	1.25±0.06	−0.39±0.02	0.86±0.08
7DH	4.19±0.21	3.32±0.17	7.51±0.38	1.27±0.06	1.44±0.07	2.71±0.14	0.47±0.02	0.07±0.00	0.54±0.03
1AR	0.25±0.01	0.22±0.01	0.47±0.02	—	—	—	—	—	—
2AR	0.28±0.01	0.12±0.01	0.40±0.02	—	—	—	0.03±0.00	−0.10±0.01	−0.07±0.01
3AR[d]	0.25±0.01	0.16±0.01	0.41±0.02	—	—	—	0.00±0.00	−0.06±0.00	−0.06±0.00
4AR	1.29±0.06	0.79±0.04	2.08±0.10	—	—	—	—	—	—
5AR	1.44±0.07	0.30±0.02	1.74±0.09	—	—	—	—	—	—
6AR	3.28±0.16	0.73±0.04	4.01±0.20	—	—	—	1.99±0.10	−0.06±0.00	1.93±0.10
7AR	2.92±0.15	1.88±0.09	4.80±0.24	—	—	—	1.48±0.07	1.58±0.08	3.06±0.15

[a] The values of n are calculated by Eq. 1.
[b] The values of n_{app} are calculated by Eq. 3.
[c] The values of $n_{DH,n}$, $n_{DH,app}$, and $n_{DH,t}$ are calculated by the n, n_{app}, and $n+n_{app}$ values of dihydropyrazole derivatives minus those of the corresponding pyrazole derivatives, respectively, while the values of $n_{OH,n}$, $n_{OH,app}$, and $n_{OH,t}$ are calculated by the n, n_{app}, and $n+n_{app}$ values of hydroxylpyrazole derivatives minus those of the corresponding non-hydroxylpyrazole derivatives, respectively.
[d] All of the values of n, n_{app}, and $n+n_{app}$ of **1AR**, **2AR**, and **3AR** are gotten from the literature.[12]

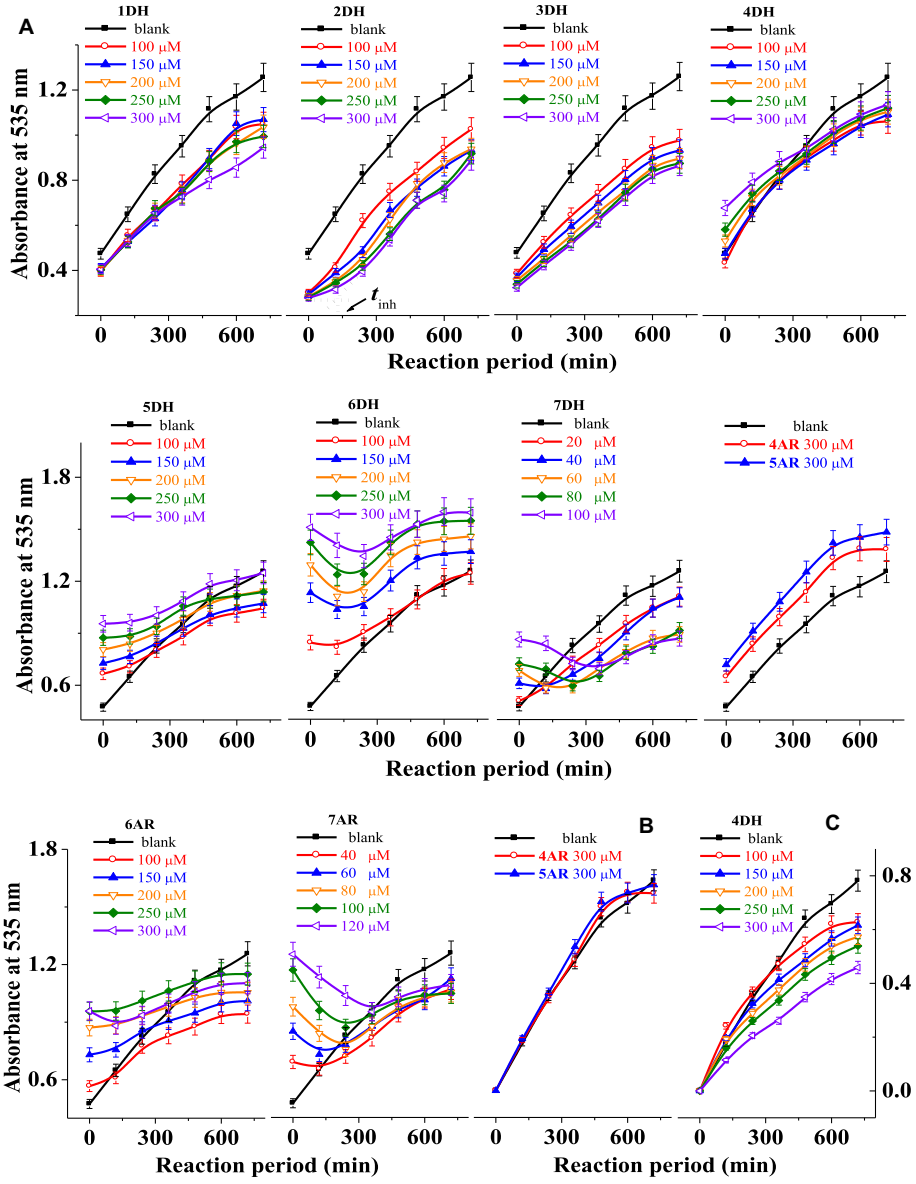


Fig. 2. (A) The range of the absorbance of TBARS in the mixture of DNA (2.0 mg/ml) and 40 mM AAPH with various concentrations of dihydropyrazole and pyrazole derivatives added, and (B) the incremental values of the absorbance of TBARS with **4AR** and **5AR** (300 μM) added, and (C) the incremental values of the absorbance of TBARS with various concentrations of **4DH** added.

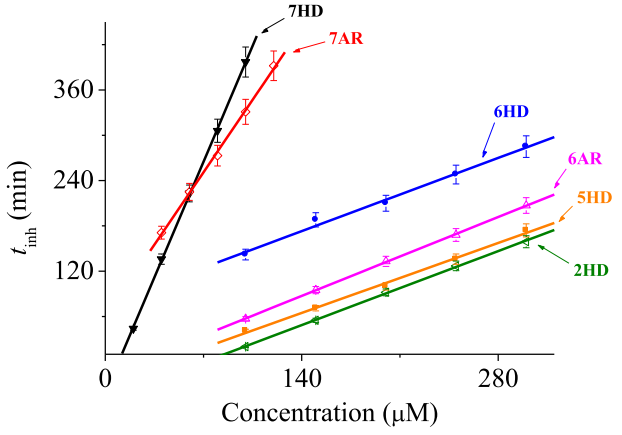


Fig. 3. The relationships between t_{inh} and the concentrations of dihydropyrazole and pyrazole derivatives in AAPH-induced oxidation of DNA.

illustrates that t_{inh} also changes with the concentrations of antioxidant employed. The quantitative equations between t_{inh} and the concentrations of these dendritic-like antioxidants can be obtained by linear regression analysis and listed in Table 3.

Chemical kinetics demonstrates that t_{inh} generated by an antioxidant (AH) is linearly related to its concentration as expressed as equation $t_{inh}=(n/R_i)$ [AH].[28] R_i is the initiation rate of a radical-induced reaction. The *stoichiometric factor* (n) implies the number of radicals trapped by one molecule of the antioxidant, and can be used as a quantitative index to express the antioxidant ability. This equation has been applied to treat the protective effects of some antioxidants on AAPH-induced oxidation of DNA.[12,19,29] R_i is assumed to be equal to the rate of the radical generated from the decomposition of AAPH ($R_g=(1.4\pm0.2)\times10^{-6}$ [AAPH] s^{-1})[30] because AAPH and DNA are both dissolved in PBS_0, and $R_i=R_g=1.4\times10^{-6}\times40$ mM $s^{-1}=3.36$ μM min^{-1} when 40 mM AAPH is employed.[29] The n values of these dendritic-like antioxidants were calculated by the equation mentioned above and the results

Table 3
The equations of t_{inh} ~ [dendritic antioxidants with dihydropyrazole and pyrazole as the core] and n, n_{DH}, and n_{OH} of dendritic antioxidants with dihydropyrazole and pyrazole as the core in protecting DNA against AAPH-induced oxidation

Antioxidant	t_{inh}(min)=(n/R_i)[a] [AH(μM)]+constant[b]	n	n_{DH}	n_{OH}[c]
1DH	—	—	0.00±0.00	—
2DH	t_{inh}=0.70 (±0.04) [**2DH**]−49.4 (±2.5)	2.35±0.12	0.64±0.03	2.35±0.12
3DH	—	—	−1.81±0.09	0.00±0.00
4DH	—	—	0.00±0.00	—
5DH	t_{inh}=0.66 (±0.03) [**5DH**]−28.0 (±1.4)	2.22±0.11	2.22±0.11	—
6DH	t_{inh}=0.69 (±0.03) [**6DH**]+76.2 (±3.8)	3.23±0.16	0.72±0.04	3.23±0.16
7DH	t_{inh}=4.40 (±0.22) [**7DH**]−41.6 (±2.1)	14.72±0.74	5.51±0.28	12.50±0.63
1AR	—	—	—	—
2AR	t_{inh}=0.51 (±0.03) [**2AR**]+1.4 (±0.1)	1.71±0.09	—	1.71±0.09
3AR[d]	t_{inh}=0.54 (±0.03) [**3AR**]+9.4 (±0.3)	1.81±0.09	—	1.81±0.09
4AR	—	—	—	—
5AR	—	—	—	—
6AR	t_{inh}=0.75 (±0.04) [**6AR**]−17.2 (±0.9)	2.51±0.13	—	2.51±0.13
7AR	t_{inh}=2.74 (±0.14) [**7AR**]+59.2 (±3.0)	9.21±0.46	—	9.21±0.46

[a] R_i=R_g=1.4×10^{-6} [AAPH] s^{-1}=3.36 μM min^{-1} when [AAPH]=40 mM in protecting DNA, thus, n=coefficient×3.36 μM min^{-1}.
[b] The constant was generated from the linear regression analysis.
[c] The values of n_{DH} are calculated by the n values of dihydropyrazole derivatives minus those of the corresponding pyrazole derivatives, while the values of n_{OH} are calculated by the n values of hydroxylpyrazole derivatives minus those of the corresponding non-hydroxylpyrazole derivatives.
[d] The n values of **1AR**, **2AR**, and **3AR** are gotten from the literature.[12]

are also listed in Table 2. Moreover, in order to investigate the structure–activity relationship of these dendritic-like antioxidants and quantitative contributions of ferrocenyl group, dihydro-structure in the heterocyclic core, and hydroxyl group to the values of n, as what has been done in radical scavenging, n_{DH}, n_{OH}, and Δn_{DNA} were analogously defined in addition as the contribution of dihydro-structure, hydroxyl group, and ferrocenyl group, respectively. The values of n_{DH} can be calculated by the n values of dihydropyrazole derivatives minus those of the corresponding pyrazole derivatives, while the values of n_{OH} can be calculated by the n values of hydroxylpyrazole derivatives minus those of the corresponding non-hydroxylpyrazole derivatives. The values of Δn_{DNA} can be calculated by the n values of ferrocenyl dihydropyrazole and pyrazole derivatives minus those of the corresponding phenyl derivatives. Apparently, the strategy to calculate the values of n_{DH}, n_{OH}, and Δn_{DNA} is similar with that in radical scavenging and the results are listed in Tables 2 and 3.

3. Discussion

3.1. Radical-scavenging ability

As shown in Table 1, the n+n_{app} values of **1AR**, **4AR**, and **5AR** are larger than zero, in which no common effective group exists, indicating that the electron pair in the N atom of pyrazole is able to trap ABTS radicals by donating its electron. Dihydro-structure is an effective group as all of the $n_{DH,t}$ values are positive except **6DH**. Hydroxyl group is another effective group for the same reason by seeing about the values of $n_{OH,t}$ similarly, only with the exception of **2AR** and **3AR**. Compared **1AR** with **2AR** and **3AR**, it can be found that the n+n_{app} value of **1AR** without hydroxyl group is 0.47, larger than those of **2AR** (0.40) and **3AR** (0.41) with hydroxyl group, showing a special negative effect of hydroxyl group only in **2AR** and **3AR**. The result of the $n_{OH,t}$ value of **3AR** larger than that of **2AR** indicates hydroxyl group connected to the phenyl at 5-position of the pyrazole ring is advantageous. Moreover, such phenomenon is prevalent, which can be summarized as the contribution of hydroxyl group to the antioxidant ability depends on its position in the molecule. It is greater when the hydroxyl group is on the phenyl at 5-position of the pyrazole ring while it is weaker when the hydroxyl group is on the phenyl at 3-position of the pyrazole ring. However, it is opposite for those with dihydropyrazole as the core. It should be noted that nearly all of the $\Delta(n+n_{app})$ values are much higher than zero, as shown in Table 2, demonstrating that the replacement of phenyl by ferrocenyl can improve the antioxidant abilities of this kind of dendritic-like antioxidants remarkably, except the same change in **6DH** exhibiting a negative effect. Such positive result may be due to the higher electron cloud density of π_5^6 in ferrocenyl group than that of π_6^6 in phenyl group. Another reason may be the ferrocenyl ring in these dihydropyrazole and pyrazole derivatives can form a conjugation system with the dihydropyrazole or pyrazole ring more completely than phenyl due to its special three-dimensional metallocene unit. Thus, the antioxidant radicals generated by reacting with ABTS radical may be stabilized by the whole conjugation system. Nevertheless, such promotive effect of ferrocenyl group does not operate independently. Compared **6AR** and **7AR** with **4AR** or compared **4DH** and **5DH** with **5AR**, it can be found that only hydroxyl group or dihydro-structure one exists with ferrocenyl group together in the molecule can improve the effect of ferrocenyl group. But compared **6AR** and **7AR** with **6DH** or compared **4DH** and **5DH** with **7DH**, the effect of ferrocenyl group becomes weaker as both hydroxyl group and dihydro-structure exist in the same time with ferrocenyl group in the molecule. The same phenomena when observing the effect of hydroxyl group or dihydro-structure can also be found by seeing about $n_{OH,t}$ and $n_{DH,t}$. Such interesting structure–activity relationship can be concluded in brief that there is a greater contribution of any one of the three factors (ferrocenyl group, dihydro-structure, and hydroxyl group) to the antioxidant ability if only one of the other two factors exists with it in the same time, or else there will be a weaker contribution. Fig. 4 offers a visual expression.

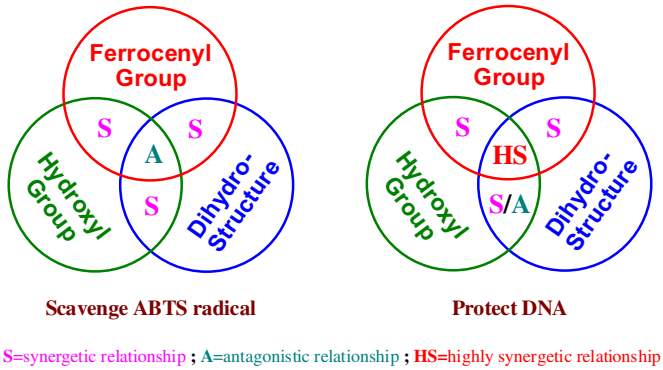


Fig. 4. The relationships of ferrocenyl group, hydroxyl group, and dihydro-structure in their contributions to the antioxidant abilities of dendritic-like antioxidants in scavenging ABTS$^{+\cdot}$ and protecting DNA.

3.2. Antioxidant effect on AAPH-induced oxidation of DNA

As shown in Fig. 2A, the slopes of the absorbance line of **4AR** and **5AR** are the same as that of the blank, indicating that the N atom in pyrazole does not possess the inhibited effect in the oxidation of DNA as the representation of **1AR** in the literature,[12] which is different from that in ABTS radical scavenging. Introducing ferrocenyl group is still not able to change the result in this case. The phenomenon that the absorbance of **4AR** and **5AR** is higher than that of the blank may be attributed to the impact of their color on absorbance, not prooxidant effect. Thus, in order to differentiate no effect from prooxidant effect, the impact of color should be eliminated. Observing the incremental values of absorbance related to that at the beginning of the reaction period instead of the absolute values of absorbance is an available method and the results of using this method are shown in Fig. 2B. From Fig. 2B, it is found that the incremental values of **4AR** and **5AR** are nearly the same as that of the blank, so it can be confirmed that it has no effect for **4AR** and **5AR**, not prooxidant effect. In the presence of **1DH** and **4DH**, the rates of oxidation of DNA are lower than that of the blank though the oxidation process is not inhibited completely. As **1DH** and **4DH** do not have the hydroxyl group, the results of their antioxidant effect are due to the dihydro-structure. However, in the presence of **5DH**, the absorbance of TBARS does not increase at the beginning of the reaction period, indicating that the dihydro-structure in **5DH** can inhibit the oxidation of DNA for a certain period (t_{inh}), and so it possesses n_{DH} value of 2.22($>$0). Other dihydropyrazole derivatives except **3DH** also possess positive n_{DH} values as shown in Table 2, demonstrating the dihydro-structure is also the effective group in protecting DNA as that in scavenging ABTS radical. To all appearances, hydroxyl group is alike according to the positive n_{OH} values, also with the exception of **3DH**. Compared **2AR** with **3AR** first, the n and n_{OH} values of **3AR** are larger than those of **2AR** indicating the hydroxyl group connected to the phenyl at 5-position of the pyrazole ring is advantageous. Furthermore, such phenomenon is prevalent like that in ABTS radical scavenging as the effect of hydroxyl group still depends on its position in the molecule. There will be a greater contribution when it on the phenyl at 5-position of the heterocyclic core while there will be a weaker contribution at 3-position, only opposite for **2DH** and **3DH**. Compound **3DH** is a special case, though it has both dihydro-structure and hydroxyl group, its activity is very weak and the relationship between the oxidation rates of DNA and its concentrations is also non-significant as that of **1DH** without hydroxyl group. But compared with **1DH** and **3DH**, there is an evident relationship between concentrations and oxidation rates for **4DH** by observing the incremental values of absorbance as shown in Fig. 2C, exhibiting the promotive effect of ferrocenyl group. As shown in Table 2, nearly all of the values of Δn_{DNA} are higher than zero, especially it is 14.72 and 7.40 for **7DH** and **7AR**. The absolute n values of the two dendritic-like antioxidants are also much higher than those of the traditional antioxidants, such as curcumin 8.40 and feruloylacetone 4.80.[19] Such results indicate that replacement of phenyl by ferrocenyl can also improve the antioxidant ability of these dendritic-like antioxidants on protecting DNA remarkably as that in scavenging ABTS radical. Moreover, those of ferrocenyl group at 3-position will offer stronger activity than those at 5-position. Like in ABTS radical scavenging, the promotive effect of ferrocenyl group in protecting DNA is also not independent but different from that in ABTS radical scavenging. The situations of the other two factors (hydroxyl group and dihydro-structure) are alike. In brief, the relationship between any two factors is synergetic only except what between hydroxyl group and dihydro-structure is dubious because it is synergetic for **7DH** but antagonistic for **6DH**. If all of the three factors exist in the molecule together, it will be a highly synergetic result, which is quite different from that in ABTS radical scavenging. The visual expression of the structure–activity relationship between the three factors in protecting DNA is also shown in Fig. 4.

4. Conclusion

In conclusion, N atom in pyrazole is able to react with ABTS radical but cannot react with peroxyl radical. Both hydroxyl group and dihydro-structure are effective groups. The effect of hydroxyl group is depending on its position in the molecule. For most dendritic-like antioxidants with dihydropyrazole or pyrazole as the core, they will possess much greater antioxidant abilities both in protecting DNA and ABTS radical scavenging if phenyl is replaced by ferrocenyl. This result exhibits the significant role of the ferrocenyl group. However, such role of ferrocenyl group is also related to its position in molecule. In ABTS radical scavenging, the relationship between any two factors of ferrocenyl group, dihydro-structure, and hydroxyl group is synergetic but it is antagonistic when all of the three factors exist in the molecule together. In protecting DNA, the relationship between any two factors is synergetic only except what between hydroxyl group and dihydro-structure is dubious. Especially, if all of the three factors exist in the molecule together, it will give out a highly synergetic result, which is quite different from that in ABTS radical scavenging.

5. Experimental section

5.1. Materials and instrumentations

ABTS radical was purchased from Fluka Chemie GmbH, Switzerland. AAPH and naked DNA sodium salt were purchased from Acros Organics, Belgium. Other reagents were of analytical grade and used without further purification. Structures of ferrocenyl and phenyl dendritic-like antioxidants with dihydropyrazole or pyrazole as the core were identified by ^{1}H and ^{13}C NMR (Varian Mercury 300 NMR spectrometer).

5.2. Synthesis and structural characterization of dendritic-like antioxidants

All of the ferrocenyl and phenyl dendritic-like antioxidants with dihydropyrazole or pyrazole as the core were synthesized according to the literature[31,32] with some modifications as the following general procedure:

One of chalcones **1**–**7** (1.0 equiv) and phenylhydrazine (3.5 equiv) were added to a mixture of ethanol/water/acetic acid (v/v/v=3:1:1). The reaction mixture was refluxed by protecting from light and monitored by TLC. After the reaction had finished, the mixture was evaporated to give a dark brown solid or syrup. (A) The solid was purified by recrystallization in suitable solvent to give a pure product (**1DH**–**5DH**). (B) The syrup was dissolved in ethyl acetate and washed with saturated brine. The organic phase was dried with Na_2SO_4 and evaporated to give oily-solid crude product, which was purified by column chromatography on silica gel using petroleum ether/ethyl acetate or benzene as eluant to give a pure product as oil (**6DH** and **7DH**), which can be crystallized slowly in petroleum ether/ethyl acetate or methanol to give a crystal.

Compound **4DH** or **5DH** (1.0 equiv) was dissolved in toluene and MnO_2 (1.5 equiv) was added. The reaction mixture was refluxed for 10 min before the solvent was evaporated under reduced pressure. The crude product as reddish-brown syrup with residual solid was purified by column chromatography on silica gel using dichloromethane as eluant to give a pure product as oil (**4AR** or **5AR**), which can be crystallized slowly in ether or methanol to give crystal of **4AR** or **5AR**. Compounds **6AR** and **7AR** can be obtained by partial spontaneous oxidation of **6DH** and **7DH** during the reactions and

also can be separated from **6DH** and **7DH** by column chromatography at the same time.

*5.2.1. 1,3,5-Triphenyl-4,5-dihydro-1H-pyrazole (**1DH**).* Yellow needles, yield 70%, mp 134–135 °C. Elemental analysis: calcd for $C_{21}H_{18}N_2$ (%): C 84.53, H 6.08, N 9.39, found: C 84.86, H 6.09, N 9.53. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.74–7.71 (m, 2H), 7.47–7.32 (m, 5H), 7.27–7.15 (m, 5H), 7.07 (dd, J=9.0 Hz, J=1.2 Hz, 2H), 6.84–6.75 (m, 1H), 5.27 (dd, J=12.3 Hz, J=7.2 Hz, 1H), 3.84 (dd, J=17.1 Hz, J=12.3 Hz, 1H), 3.14 (dd, J=17.1 Hz, J=7.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 146.65, 144.79, 142.53, 132.67, 129.08, 128.85, 128.53, 128.48, 127.50, 125.81, 125.68, 119.03, 113.32, 64.42, 43.50.

*5.2.2. 4-(1,5-Diphenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (**2DH**).* Khaki sheets, yield 40%, mp 132–133 °C. Elemental analysis: calcd for $C_{21}H_{18}N_2O$ (%): C 80.23, H 5.77, N 8.91, found: C 78.18, H 5.78, N 8.73. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.79 (s, 1H), 7.58 (d, J=8.4 Hz, 2H), 7.36–7.21 (m, 5H), 7.12 (t, J=8.1 Hz, 2H), 6.95 (d, J=8.1 Hz, 2H), 6.81 (d, J=8.4 Hz, 2H), 6.67 (t, J=7.5 Hz, 1H), 5.38 (dd, J=12.0 Hz, J=6.3 Hz, 1H), 3.86 (dd, J=17.4 Hz, J=12.0 Hz, 1H), 3.03 (dd, J=17.4 Hz, J=6.3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 158.24, 147.49, 144.69, 142.70, 128.82, 128.67, 127.29, 127.19, 125.76, 123.28, 118.02, 115.44, 112.68, 63.04, 43.27.

*5.2.3. 4-(1,3-Diphenyl-4,5-dihydro-1H-pyrazol-5-yl)phenol (**3DH**).* Light olivine needles, yield 60%, mp 146–147 °C. Elemental analysis: calcd for $C_{21}H_{18}N_2O$ (%): C 80.23, H 5.77, N 8.91, found: C 79.52, H 5.54, N 8.86. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.39 (s, 1H), 7.74 (dd, J=7.8 Hz, J=1.5 Hz, 2H), 7.45–7.34 (m, 3H), 7.17–7.08 (m, 4H), 7.01 (d, J=7.8 Hz, 2H), 6.72–6.68 (m, 3H), 5.35 (dd, J=12.0 Hz, J=6.3 Hz, 1H), 3.86 (dd, J=17.4 Hz, J=12.0 Hz, 1H), 3.06 (dd, J=17.4 Hz, J=6.3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 156.68, 147.14, 144.39, 132.83, 132.47, 128.86, 128.71, 128.68, 127.13, 125.69, 118.57, 115.74, 113.08, 62.93, 43.10.

*5.2.4. 5-Ferrocenyl-1,3-diphenyl-4,5-dihydro-1H-pyrazole (**4DH**).* Brown needles, yield 81%, mp 158–159 °C. Elemental analysis: calcd for $C_{25}H_{12}FeN_2$ (%): C 73.90, H 5.46, N 6.89, found: C 73.94, H 5.53, N 7.00. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.822 (d, J=7.2 Hz, 2H), 7.458–7.329 (m, 3H), 7.234–7.160 (m, 4H), 6.834–6.780 (m, 1H), 5.045 (dd, J=11.1 Hz, J=6.0 Hz, 1H), 4.223 (s, 1H), 4.148 (s, 8H), 3.856–3.674 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 147.36, 145.39, 132.90, 128.83, 128.68, 128.61, 125.77, 119.23, 114.12, 90.59, 68.73, 68.46, 68.14, 67.74, 67.01, 59.34, 42.13.

*5.2.5. 5-Ferrocenyl-1,3-diphenyl-1H-pyrazole (**4AR**).* Red grains, yield 60%, mp 100–101 °C. Elemental analysis: calcd for $C_{25}H_{20}FeN_2$ (%): C 74.27, H 4.99, N 6.93, found: C 74.27, H 4.98, N 6.97. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.931–7.902 (m, 2H), 7.453–7.331 (m, 8H), 6.821 (s, 1H), 4.197 (s, 4H), 4.091 (s, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 151.65, 143.09, 140.43, 133.21, 128.84, 128.62, 128.11, 127.90, 126.32, 125.80, 103.79, 74.87, 69.88, 68.73, 68.67.

*5.2.6. 3-Ferrocenyl-1,5-diphenyl-4,5-dihydro-1H-pyrazole (**5DH**).* Brown grains, yield 56%, mp 244–245 °C. Elemental analysis: calcd for $C_{25}H_{12}FeN_2$ (%): C 73.90, H 5.46, N 6.89, found: C 73.79, H 5.45, N 6.81. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.36–7.34 (m, 4H), 7.29–7.27 (m, 1H), 7.17 (dd, J=8.4 Hz, J=7.5 Hz, 2H), 6.96 (d, J=7.8 Hz, 2H), 6.71 (t, J=7.2 Hz, 1H), 5.26 (dd, J=11.4 Hz, J=6.3 Hz, 1H), 4.70 (s, 1H), 4.56 (s, 1H), 4.35 (s, 2H), 4.11 (s, 5H), 3.76 (dd, J=16.8 Hz, J=12.0 Hz, 1H), 2.99 (dd, J=16.8 Hz, J=6.3 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 147.93, 145.08, 142.84, 129.06, 128.78, 127.45, 125.75, 118.47, 113.03, 69.63, 69.47, 69.18, 66.78, 66.51, 63.81, 45.02.

*5.2.7. 3-Ferrocenyl-1,5-diphenyl-1H-pyrazole (**5AR**).* Yellow needles, yield 58%, mp 175–176 °C. Elemental analysis: calcd for $C_{25}H_{20}FeN_2$ (%): C 74.27, H 4.99, N 6.93, found: C 74.63, H 4.91, N 7.05. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.346–7.273 (m, 10H), 6.552 (s, 1H), 4.797 (s, 2H), 4.326 (s, 2H), 4.160 (s, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 151.49, 143.66, 140.18, 130.71, 128.88, 128.73, 128.43, 128.17, 127.17, 125.30, 105.83, 78.47, 69.69, 68.76, 66.96.

*5.2.8. 4-(5-Ferrocenyl-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)phenol (**6DH**).* Khaki solid, yield 53%, mp 110–111 °C. Elemental analysis: calcd for $C_{25}H_{22}FeN_2O$ (%): C 71.10, H 5.25, N 6.63, found: C 69.99, H 5.68, N 6.10. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.780 (s, 1H), 7.687 (d, J=8.7 Hz, 2H), 7.179–7.062 (m, 4H), 6.866 (d, J=8.7 Hz, 2H), 6.680 (t, J=7.2 Hz, 1H), 5.120 (dd, J=11.1 Hz, J=5.4 Hz, 1H), 4.417 (s, 1H), 4.206 (s, 5H), 4.146 (s, 1H), 4.096 (s, 1H), 3.850 (dd, J=17.1 Hz, J=11.1 Hz, 1H), 3.689 (dd, J=17.1 Hz, J=5.4 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 163.44, 153.35, 150.21, 133.82, 132.60, 128.72, 123.27, 120.76, 118.65, 94.82, 74.40, 73.78, 73.04, 72.43, 71.23, 63.54, 46.80.

*5.2.9. 4-(5-Ferrocenyl-1-phenyl-1H-pyrazol-3-yl)phenol (**6AR**).* Orange solid, yield 6%, mp 225–226 °C. Elemental analysis: calcd for $C_{25}H_{20}FeN_2O$ (%): C 71.44, H 4.80, N 6.67, found: C 71.40, H 4.93, N 6.64. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.771 (d, J=7.2 Hz, 2H), 7.410 (s, 5H), 6.861 (d, J=7.8 Hz, 2H), 6.727 (s, 1H), 4.207 (s, 2H), 4.188 (s, 2H), 4.099 (s, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 155.57, 151.41, 143.01, 140.38, 128.74, 127.98, 127.26, 126.32, 126.02, 115.50, 103.46, 77.12, 70.11, 68.85.

*5.2.10. 4-(3-Ferrocenyl-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)phenol (**7DH**).* Orange sheets, yield 74%, mp 186–187 °C. Elemental analysis: calcd for $C_{25}H_{22}FeN_2O$ (%): C 71.10, H 5.25, N 6.63, found: C 69.33, H 5.29, N 6.62. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.368 (s, 1H), 7.133–7.080 (m, 5H), 6.896 (d, J=7.8 Hz, 2H), 6.728 (d, J=8.4 Hz, 2H), 6.646 (t, J=7.2 Hz, 2H), 5.239 (dd, J=11.7 Hz, J=5.7 Hz, 1H), 4.694 (s, 1H), 4.598 (s, 1H), 4.388 (s, 2H), 4.133 (s, 5H), 3.757 (dd, J=17.1 Hz, J=11.7 Hz, 1H), 2.909 (dd, J=17.1 Hz, J=5.7 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 161.72, 153.65, 149.86, 138.11, 133.92, 132.13, 122.84, 120.79, 117.74, 82.55, 74.67, 74.58, 74.18, 72.00, 71.49, 67.28, 49.86.

*5.2.11. 4-(3-Ferrocenyl-1-phenyl-1H-pyrazol-5-yl)phenol (**7AR**).* Yellow solid, yield 7%, mp 256–257 °C. Elemental analysis: calcd for $C_{25}H_{20}FeN_2O$ (%): C 71.44, H 4.80, N 6.67, found: C 70.80, H 4.93, N 6.14. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.316–7.276 (m, 5H), 7.083 (d, J=8.4 Hz, 2H), 6.714 (d, J=8.4 Hz, 2H), 6.485 (s, 1H), 4.767 (s, 2H), 4.297 (s, 2H), 4.125 (s, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 156.23, 151.50, 143.83, 139.90, 130.09, 128.86, 127.25, 125.42, 122.47, 115.48, 105.14, 77.21, 69.62, 68.77, 66.95.

5.3. Scavenging of $ABTS^{+\cdot}$ by dendritic-like antioxidants

$ABTS^{+\cdot}$ was formed in a mixture of 2.0 ml aqueous solution of 4.0 mM ABTS and 1.41 mM $K_2S_2O_8$ for 16 h. Then, 100 ml ethanol was added to make the absorbance of $ABTS^{+\cdot}$ around 0.800 at 734 nm ($\varepsilon^{+\cdot}_{ABTS} = 1.6\times10^4\ M^{-1}\ cm^{-1}$[33]). The ethanol solutions of ferrocenyl and phenyl dendritic-like antioxidants with dihydropyrazole or pyrazole as the core were added to the aforementioned radical solution with the final concentrations being 10.0 μM. The decay of the absorbance of $ABTS^{+\cdot}$ was recorded at room temperature.

5.4. DNA protection against AAPH-induced oxidation by dendritic-like antioxidants

The experiment of AAPH-induced oxidation of DNA was performed as described in the literature[34] with a little modification. Briefly, DNA and AAPH were dissolved in phosphate-buffered solution (PBS_0: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA). Then, 2.0 mg/ml DNA, 40 mM AAPH, and a certain concentration of

ferrocenyl and phenyl dendritic-like antioxidants with dihydropyrazole or pyrazole as the core (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. All the tubes were incubated in a water bath at 37 °C to initiate the reaction. Three tubes were taken out at appropriate interval and cooled immediately, to which 1.0 ml thiobarbituric acid (TBA) solution (1.00 g TBA and 0.40 g NaOH dissolved in 100 ml PBS_0) and 1.0 ml trichloroacetic acid (3.0% aqueous solution) were added. The tubes were heated in a boiling water bath for 15 min. After cooling to room temperature, 1.5 ml *n*-butanol was added and shaken vigorously to extract thiobarbituric acid reactive substance (TBARS, λ_{max}=535 nm). The absorbance of *n*-butanol phase was measured and plotted versus incubation time.

5.5. Statistical analysis

All the data were the average values from at least three independent measurements with the experimental error within ±5%. The equations were analyzed by one-way ANOVA in Origin 6.0 Professional software, in which $p<0.001$ indicated a significant difference.

Acknowledgements

Financial support from Jilin Provincial Science and Technology Department, China, is acknowledged gratefully (20130206075GX).

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