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Original article

Thiaflavan scavenges radicals and inhibits DNA oxidation: A story from the ferrocene modification

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ABSTRACT

4-Thiaflavan is a sulfur-substituted flavonoid with a benzoxathiin scaffold. The aim of this work is to compare abilities of sulfur and oxygen atom, hydroxyl groups, and ferrocene moiety at different positions of 4-thiaflavan to trap radicals and to inhibit DNA oxidation. It is found that abilities of thiaflavans to trap radicals and to inhibit DNA oxidation are increased in the presence of ferrocene moiety and are further improved by the electron-donating group attaching to thiaflavan skeleton. It can be concluded that the ferrocene moiety plays the major role for thiaflavans to be antioxidants even in the absence of phenolic hydroxyl groups. On the other hand, the antioxidant effectiveness of phenolic hydroxyl groups in thiaflavans can be improved by the electron-donating group. The influences of sulfur and oxygen atoms in thiaflavans on the antioxidant property of *para*-hydroxyl group exhibit different manners when the thiaflavans are used to trap radicals and to inhibit DNA oxidation.

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1. Introduction

Flavonoid (structure shown in Scheme 1) is an important compound for natural polyphenols and is widely found in plants [1]. The biological and pharmacological properties of flavonoids are attributed to phenolic hydroxyl groups at different positions in flavonoid [2]. It has been proved that the bond dissociation energy (BDE) of O–H in hydroxyl group at B ring is about 10 kcal mol^{-1} lower than that at A ring. This result indicates that hydroxyl groups at B ring play the key role in scavenging radicals and exhibiting antioxidant effectiveness [3]. Some flavonoid derivatives are also shown in Scheme 1 [4]. For example, when the double bond between C-2 and C-3 in flavonoid is converted into single bond, the generated flavan is also natural polyphenol [5]. The antioxidant properties of flavans have been estimated in various experimental systems [6]. In addition, ferrocene-appended naphthoquinones exhibit high antiplasmodial activities [7], and copper (II) complexes of curcumin and *N*-ferrocenylmethyl-*L*-amino acids possess high photocytotoxicities [8]. The aforementioned results reveal that ferrocenyl group is able to improve the biological activities of natural compounds, and this encourages us to explore whether ferrocenyl group can also varies the antioxidant effects of thiaflavans and to investigate positions of ferrocenyl group and sulfur atom for being a mutual antioxidant.

2. Chemistry

Scheme 1 outlines that sulfur atom can be applied to replace CH_2 at 4-position in flavan, producing sulfur-contained benzoxathiin. This sulfur-involved flavan is also called as 4-thiaflavan, which behaves as a heterocyclic nucleus for designing many drugs [9].

The sulfur atom in thiaflavan can decrease the BDE of O–H attaching to its *para*-position. As shown as the structure **I** in Scheme 1, the single electron at the oxygen atom in –OH may be transferred to sulfur atom in order to use the sulfur atom for accommodating the single electron and to stabilize the peroxyl radical [10]. Similarly, the structure **II** in Scheme 1 outlines that the oxygen atom at 1-position can also stabilize the phenoxyl radical at 6-position. Therefore, it is worthy to compare the ability of hydroxyl group at 6- and 7-position to scavenge radicals and to inhibit the oxidation of biological species. Therefore, thiaflavan is an appropriate model compound for the investigation on the effect of sulfur atom on phenoxyl radical at ring A [11]. Although abilities of hydroxyl group at 5-position to quench 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) [12] and to inhibit glutathione/ferric ion-induced oxidation of DNA have been reported [13], the study on antioxidant abilities of hydroxyl groups at 6- and 7-position are not usually found. A straight carbon chain is used to attach to ring A of thiaflavan,

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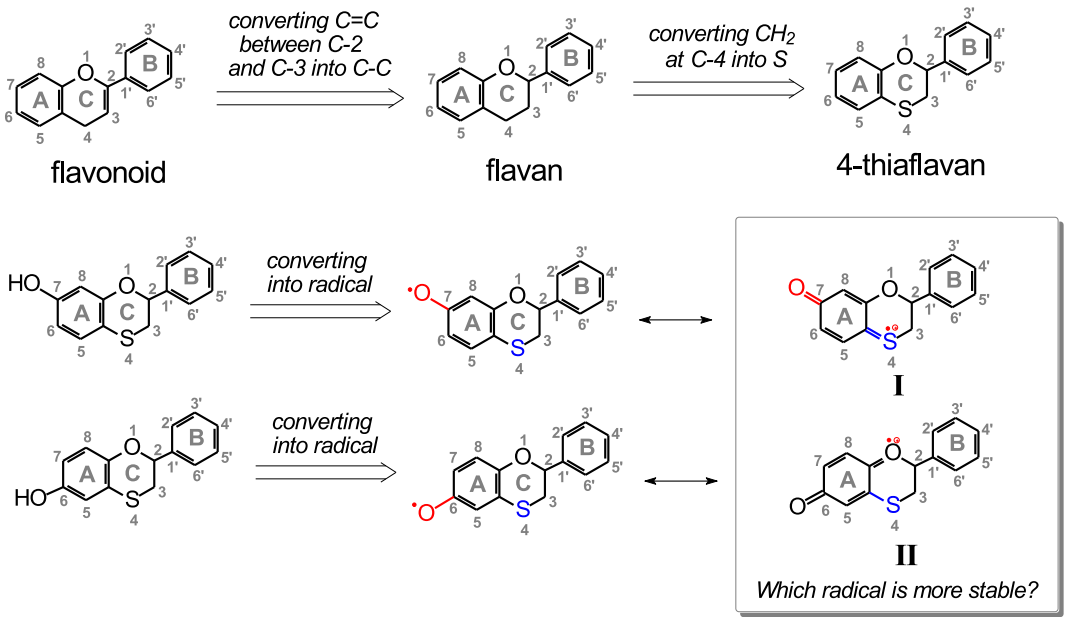


Scheme 1. Structures of flavonoid-related compounds and radicals deriving from 4-thiaflavan.

forming an amphiphilic antioxidant [14], and the carbon chain is employed to attach to ring C, producing a tocopherol-like antioxidant [15]. These results encourage us to apply an organometallic group to attach ring B and to estimate the antioxidant property of thiaflavan.

3. Pharmacology

The inhibition effect on DNA oxidation and the ability to scavenge radicals are characteristic properties of an antioxidant. So, the aim of this work is to clarify effects of hydroxyl groups at ring A and B and ferrocene moiety at ring B on the antioxidant effectiveness of thiaflavans. Therefore, as shown in Scheme 2, thiaflavans **1** to **11** are synthesized following a description in a literature (protocol A) [16], and thiaflavans **12** and **13** are prepared following the synthetic protocol B [17]. Then, the radical-scavenging properties are compared by quenching 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+\bullet}$), DPPH, and galvinoxyl radical, respectively [18]. The antioxidant effectiveness of these thiaflavans are also estimated inhibiting 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)-induced oxidation of DNA [19].

4. Results and discussion

4.1. Scavenging radicals

$ABTS^{+\bullet}$, DPPH, and galvinoxyl radical are radical resources, which are usually employed to evaluate the property of an antioxidant in scavenging radicals. The reaction of an antioxidant with $ABTS^{+\bullet}$, DPPH, and galvinoxyl radical reveals the ability of the antioxidant to reduce radical and to donate its hydrogen to N- or O-centered radicals, respectively. Some methods are designed to express the ability of the antioxidant to quench these radicals [20]. But kinetic methods are not usually found because the operation to measure the rate constant ($\boldsymbol{k}$) of the antioxidant to quench these radicals are so complicated [21]. But Figs. 1S, 2S and 3S show that some thiaflavans are able to decrease the concentrations of these radicals rapidly, while other thiaflavans cannot react with these radicals. Hence, it is necessary to find a convenient way to express the results from the aforementioned figures.

We have provided a method for expressing the relationship between the concentration of a radical and the reaction period [22]. Briefly, when $ABTS^{+\bullet}$ is taken as the example, the rate constant ($\boldsymbol{k}$) of the reaction between an antioxidant and $ABTS^{+\bullet}$ can be calculated by equation (1) when [$ABTS^{+\bullet}$], [antioxidant], and the corresponding reaction rate ($\boldsymbol{r}$) are known.

$$-\frac{\mathrm{d}[\mathrm{ABTS}^{+\bullet}]}{\mathrm{d}t} = \boldsymbol{r} = \boldsymbol{k}[\mathrm{ABTS}^{+\bullet}][\mathrm{antioxidant}] \quad (1)$$

The chemical kinetic equation (1) is also available at $t = 0$ and can be expressed as equation (2), in which $[\mathrm{ABTS}^{+\bullet}]_{t=0}$ and $[\mathrm{antioxidant}]_{t=0}$ refer to concentrations of the antioxidant and $ABTS^{+\bullet}$ at the beginning of the reaction.

$$\boldsymbol{r}_0 = \boldsymbol{k}[\mathrm{ABTS}^{+\bullet}]_{t=0}[\mathrm{antioxidant}]_{t=0} \quad (2)$$

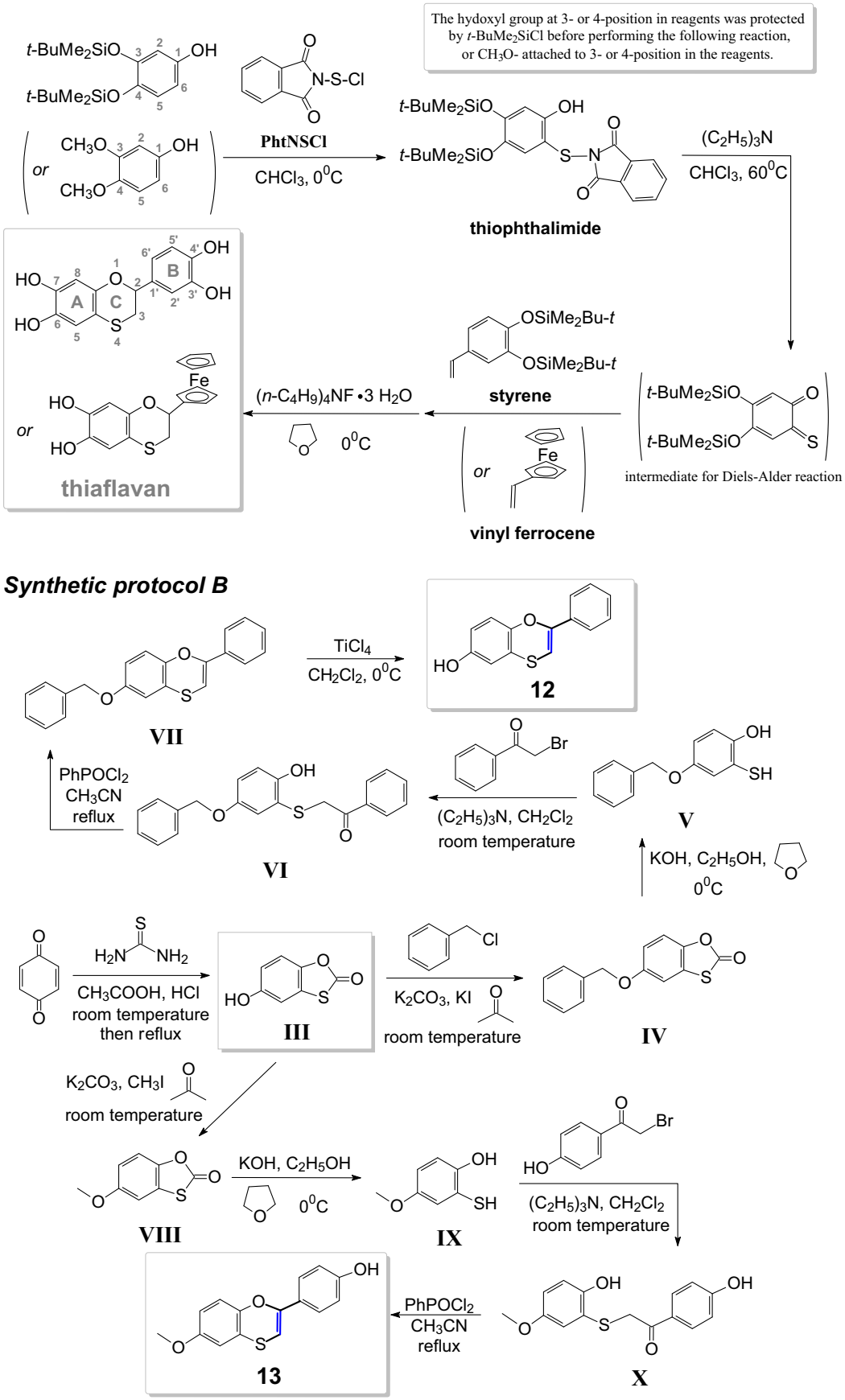
In view of the treatment of the experimental data in Fig. 1S, the concentration of $ABTS^{+\bullet}$ and the corresponding time-point (t) are input into a statistical software in order to find quantitative relationship of [$ABTS^{+\bullet}$] $\sim t$. As a result, the double exponential function is the most suitable function for expressing the relationship of [$ABTS^{+\bullet}$] $\sim t$ and listed in Table 1S.

$$[\mathrm{ABTS}^{+\bullet}] = A\boldsymbol{e}^{-\frac{t}{a}} + B\boldsymbol{e}^{-\frac{t}{b}} + C \quad (3)$$

Moreover, the differential operation is performed to equation (3) for expressing the relationship between the reaction rate ($\boldsymbol{r}$) and the time (t), $-\mathrm{d}[\mathrm{ABTS}^{+\bullet}]/\mathrm{d}t \sim t$ (equation (4)).

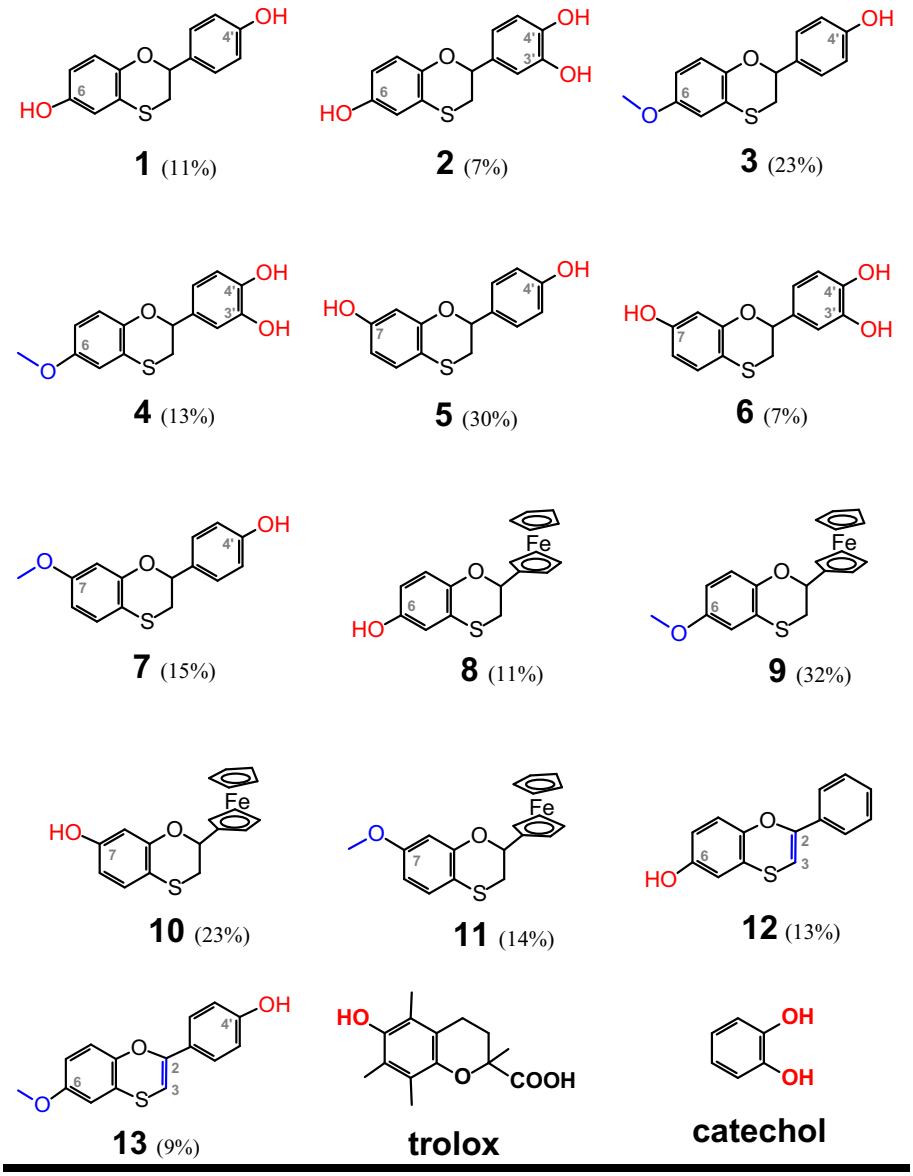
$$-\frac{\mathrm{d}[\mathrm{ABTS}^{+\bullet}]}{\mathrm{d}t} = \boldsymbol{r} = \frac{A}{a}\boldsymbol{e}^{-\frac{t}{a}} + \frac{B}{b}\boldsymbol{e}^{-\frac{t}{b}} \quad (4)$$

The reaction rate at $t = 0$ ($\boldsymbol{r}_0$) can be calculated following equation (4) when the reaction time (t) is assigned to 0. The rate constant ($\boldsymbol{k}$) is thereby calculated by using equation (2) and collected in Table 1. This method is also used to treat the



Scheme 2. Synthesis and structures of thiaflavans in the present work.

Compounds **1** to **11** are produced following the synthetic protocol A, and compounds **12** and **13** are produced following the synthetic protocol B. The total yields are included in parentheses. **Trolox** and **catechol** are the reference compounds.



Scheme 2. (*continued*).

experimental results from thiaflavan quenching other radicals, and results are listed in Table 1 as well.

The data in Table 1 reveal that ***k*** values of all the thiaflavans in trapping $ABTS^{+\bullet}$ are higher than those in trapping DPPH and galvinoxyl radical. So, abilities of thiaflavans to reduce radicals are higher than to donate hydrogen atom to N- and O-centered radicals. Compounds **9** and **11** cannot react with DPPH and galvinoxyl radical because of no phenolic hydroxyl group contained. Scheme 3 illustrates the comparison of ***k*** and their structures.

The low ***k*** values of compounds **3** and **7** in trapping $ABTS^{+\bullet}$ indicate that a single hydroxyl group at 4′-position in ring B only exhibits weak ability to reduce radical and even cannot donate its hydrogen atom to N- and O-centered radicals. On the contrary, compounds **9** and **11** can react with $ABTS^{+\bullet}$ even in the absence of hydroxyl group. The ***k*** values of compounds **8** and **10** are higher than that of compound **12**. In compounds **8** and **10**, only one hydroxyl group attaches to 6- and 7-position, respectively, while the ring B is a phenyl group in compound **12**. These results reveal that the ferrocene moiety at ring B remarkably enhances the ability of thiaflavan to quench radicals.

In trapping $ABTS^{+\bullet}$, the ***k*** value of compound **12** is similar to those of compounds **9** and **11**. This result reveals that the single hydroxyl group at 6-position in ring A is able to react with $ABTS^{+\bullet}$ and DPPH, but cannot react with galvinoxyl radical. It can be found by comparing ***k*** values of compounds **13** and **3** that the C=C bond between 2- and 3-position in ring C is beneficial for the hydroxyl group at 4′-position in ring B of compound **13** to quench $ABTS^{+\bullet}$ and DPPH. Moreover, the ***k*** value of compound **5** is higher than that of compound **1** in trapping $ABTS^{+\bullet}$, indicating that the hydroxyl group at 7-poisiton in ring A (the *para*-position of sulfur atom) exhibits higher ability to scavenge radical than that at 6-position (the *para*-position of oxygen atom). This phenomenon is also

Table 1
The rate constant (***k***) for thiaflavans, trolox, and catechol in scavenging $ABTS^{+\bullet}$, DPPH and galvinoxyl radicals.

Compound	Rate constant for thiaflavans to scavenge radicals, ***k*** ($\times 10^3$ M^{-1} s^{-1})		
	$ABTS^{+\bullet}$	DPPH	Galvinoxyl radical
1	8.92	0.043	0.025
2	11.7	1.18	0.52
3	0.202	–	–
4	390	2.20	2.54
5	10.8	0.069	0.034
6	16.7	1.61	0.57
7	0.168	–	–
8	22.3	0.070	0.039
9	5.54	–	–
10	22.9	0.124	0.084
11	5.03	–	–
12	4.89	0.0071	–
13	8.14	0.031	–
Trolox	29.2	0.353	1.70
Catechol	2.58	1.30	1.78

found in the comparison of ***k*** values of compounds **5** and **1** and of compounds **6** and **2**. As shown in Scheme 1, it can be concluded that the radical resonance structure **I** is more beneficial for stabilizing the single electronic species than structure **II** because the sulfur atom accommodates single electron more sufficiently than oxygen atom. This finding is in agreement with a previous report, in which hydroxyl group at *para*-position of sulfur atom inhibits the oxidation of styrene more efficiently than that at *para*-position of oxygen atom [23].

Scheme 3 also illustrates that the ***k*** values of compound **6** in trapping three radicals are higher than the corresponding values of compound **5**, revealing that *ortho*-dihydroxyl groups in ring B of compound **6** play the key role in scavenging radicals [24]. But it is not implied that the catechol structure feature itself can function as the efficient radical-scavenger because the ***k*** value of catechol in trapping $ABTS^{+\bullet}$ is lower than that of compounds **2** and **6**. In thiaflavan, the *ortho*-dihydroxyl groups in ring B are the active site for trapping radicals only in the case of the electron-donating group attaching to ring A. Hence, when methoxyl group (a strong electron-donating group) attaches to ring A, the radical-scavenging ability of compound **4** increases to the highest level among thiaflavans employed herein. It can be concluded that single hydroxyl group at *para*-position of sulfur atom in ring A, adjacent double hydroxyl groups in ring B with the aid of the electron-donating group in ring A, and ferrocene moiety as ring B in thiaflavan play the key role in scavenging radicals.

4.2. Inhibiting AAPH-induced oxidation of DNA

In the presence of oxygen at 37 °C, the decomposition of AAPH leads to the formation of peroxyl radical, which is able to oxidize the guanine bases in DNA [19]. The products deriving from the oxidation of DNA can be colorized after they react with thiobarbituric acid. Thus, the oxidative products of DNA are also called as thiobarbituric acid reactive species (TBARS) and can be determined by measuring the absorbance of at 535 nm (ε_{TBARS} = 1.56×10^5 M^{-1} cm^{-1}). The blank experiment in Fig. 1 shows a continual increase of the absorbance, indicating that much more TBARS are generated along with the increases of the reaction times. The relationship between the concentration of TBARS and the reaction period (t) is fitted by linear equation (5).

$$[\text{TBARS (nM)}] = 7.1\ (\pm 0.3)\ t\ (\text{min}) + 3073\ (\pm 137) \quad (5)$$

The differential operation on the equation (5) reveals the formation rate of TBARS as $d[TBARS]/dt = 7.1$ nM min^{-1}.

Fig. 1 also illustrates that the addition of 100 μM compound **5** hinders the increase of the absorbance of TBARS, and equations of [TBARS] ~ t together with $d[TBARS]/dt$ in the presence of compound **5** are listed in Fig. 1. Fig. 4S outlines the [TBARS] *vs.* t in the presence of the same concentration of compounds **3**, **12**, and **13**, and Table 2 gives $d[TBARS]/dt$ in the presence of compounds **3**, **5**, **12**, and **13**.

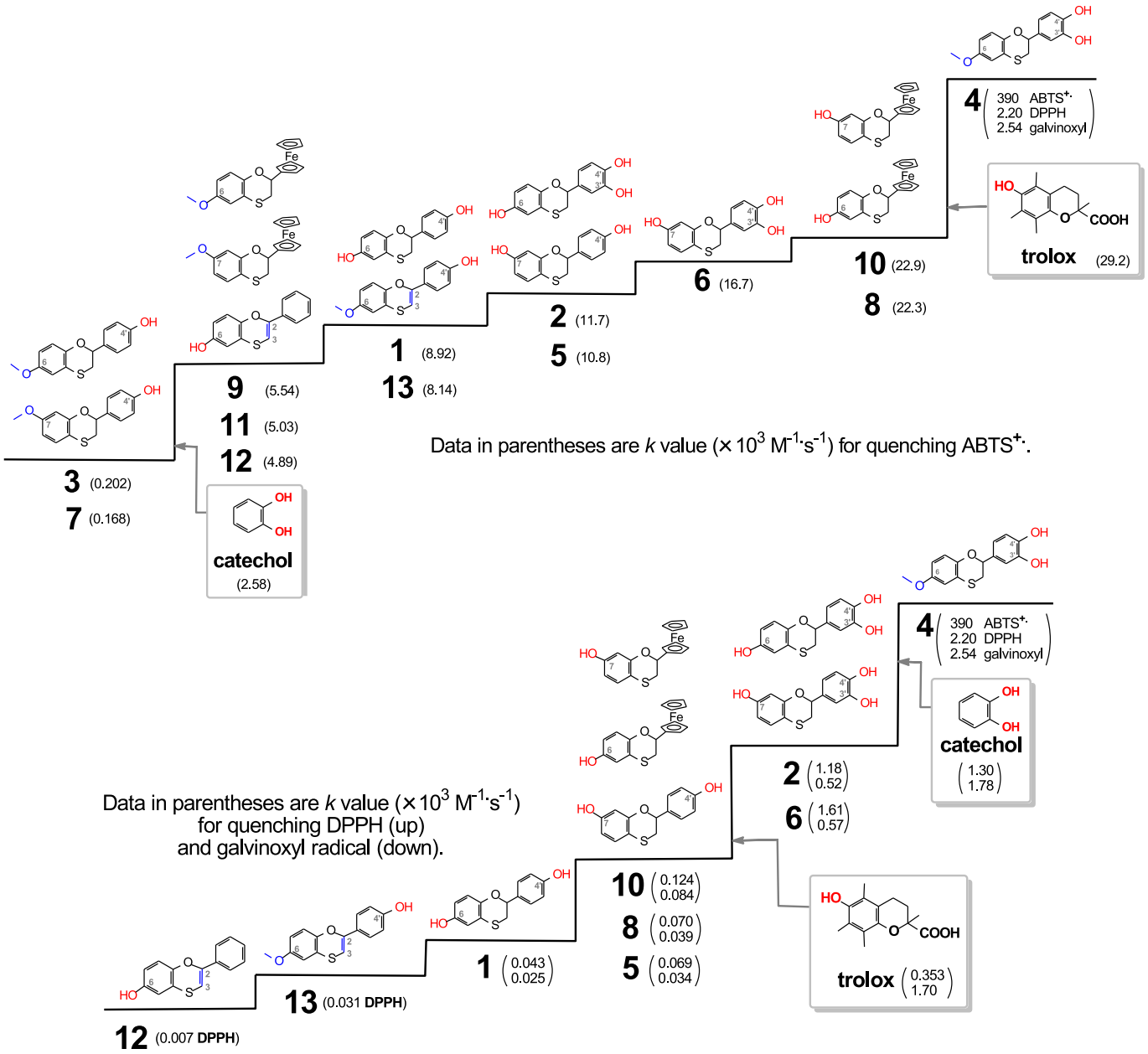
The $d[TBARS]/dt$ value of compound **3** is lower than that of compound **13**, indicating that the C=C between 2- and 3-position is not beneficial for enhancing the inhibitive effect of compound **13** on DNA oxidation. The $d[TBARS]/dt$ value of compound **13** approaches to that of compound **12**, implying that the inhibitive effect of hydroxyl group in ring B of compound **13** is similar to that in ring A of compound **12**. On the other hand, compound **3** contains a single hydroxyl at 4′-position in ring B, while compound **5** contains two hydroxyl groups at 7-position in ring A and 4′-position in ring B. But similar $d[TBARS]/dt$ values of compounds **3** and **5** indicate that hydroxyl group at 7-position does not increase the inhibitive effect of compound **5** on DNA oxidation. So, hydroxyl group at ring B plays the major role in hindering the formation of TBARS.

As shown in the panel A of Fig. 2, the addition of compound **1** hinders the increase of the absorbance of TBARS for a period, and then the absorbance of TBARS increases as the blank experiment. The *inhibition period* (t_{inh}) can be measured from the beginning of the reaction to the cross point of tangents for the inhibition and the increase period in the absorbance line of TBARS. Then, as shown in the panel B of Fig. 2, t_{inh} increases with the concentration of compound **1**, and the linear relationship between t_{inh} and the concentration of compound **1** is given in the panel B of Fig. 2. Fig. 5S shows the relationships between the absorbance of TBARS and the concentrations of compounds **1**, **2**, **4**, **6**, **7**, **8**, **9**, **10**, and **11**, while Fig. 6S outlines the linear relationships between t_{inh} and the concentrations of the aforementioned compounds. The equations of t_{inh} ~ [thiaflavans] are given in Table 3.

As shown in equation (6), it has been demonstrated by chemical kinetics that t_{inh} correlates proportionally with the concentration of an antioxidant [25].

$$t_{inh} = (n/R_i)\ [\text{antioxidant}] \quad (6)$$

R_i refers to the initiation rate of the radical-induced reaction, and the *stoichiometric factor* (n) is the number of radicals trapped by one molecule of the antioxidant. It has been assumed that R_i is equal to the generation rate (R_g) of radicals ($R_g = (1.4 \pm 0.2) \times 10^{-6}$ [AAPH] s^{-1} [25]) because the water-soluble AAPH and the sodium salt of DNA locate at the same phase, and radicals generated from AAPH attack DNA directly. Hence, $R_i = R_g = 1.4 \times 10^{-6} \times 40$ mM $s^{-1} = 3.36$ μM min^{-1} in this experimental system. The n value is the product of the coefficient in the equation of t_{inh} ~ [thiaflavans] and R_i. So, n provides a quantitative index for comparing the effect of an antioxidant on DNA oxidation. Accordingly, the n values of thiaflavans are calculated and listed in Table 3, in which the data are schematized in Scheme 4. We have measured the t_{inh} ~ [isoflavonoids] under the same experimental condition [26]. The isoflavonoids include 3-(2′-hydroxybenzylidene)-7-methoxychroman-4-one (*o*-HBMC), 3-(3′-hydroxybenzylidene)-7-methoxychroman-4-one (*m*-HBMC), and 3-(4′-hydroxybenzylidene)-7-methoxychroman-4-one (*p*-HBMC). These isoflavonoids have the similar structures with thiaflavans, but the coefficients of t_{inh} ~ [*o*-HBMC or *p*-HBMC] are only 0.82 and 0.15, respectively, lower than those of thiaflavans. So, the introduction of sulfur atom into flavans improves the sensitivity of t_{inh} towards the variation of concentrations of thiaflavans.



Scheme 3. The comparison of ***k*** values in trapping radicals with thiaflavan structures.

Similar *n* values of compounds **4** and **7** reveal that one or two hydroxyl groups in ring B exhibit similar antioxidant effect on DNA oxidation. But compound **1** contains two hydroxyl groups, which locate at ring A and B, respectively, while compound **7** contains only one hydroxyl group in ring B. The *n* value of compound **1** is higher than that of compound **7**, indicating that two hydroxyl groups in ring A and B, respectively, increase the ability of thiaflavan to inhibit DNA oxidation. Moreover, the *n* value of compound **2** is much higher than that of compound **1**, revealing that double hydroxyl groups at adjacent position in ring B together with one hydroxyl group in ring A are beneficial for enhancing the antioxidant effect of thiaflavan. The comparison of *n* values of compounds **2** and **6** reveals that hydroxyl group in ring A at the *para*-position of oxygen atom exhibits higher antioxidant effectiveness than that at the *para*-position of sulfur atom. In compound **8**, the hydroxyl group in ring A locates at the *para*-position of oxygen atom and possesses higher *n* value than that of compound **10**, in which the hydroxyl group in ring A locates at the *para*-position of sulfur atom. Thus, hydroxyl group at *para*-position of oxygen atom exhibits higher antioxidant effectiveness than that at the *para*-position of sulfur atom.

At present, modifying traditional drugs by organometallic groups becomes a popular way to improve the pharmacological properties [27], while ferrocene is usually applied to link with the other organic structural features [28]. The cyclopentadiene moiety in ferrocene can be used to enlarge the conjugation system of an organic structure, and ferrocene moiety can also be a substituent in an organic species [29]. We have explored the antioxidant effects of ferrocene-appended curcumin on AAPH-induced oxidation of DNA. It is found that the *n* value of ferrocene-appended curcumin is 9.5 [30], while that of curcumin is 8.2 [31]. So, the conjugation system formed between carbon chain in curcumin and cyclopentadiene moiety in ferrocene actually increases the antioxidant

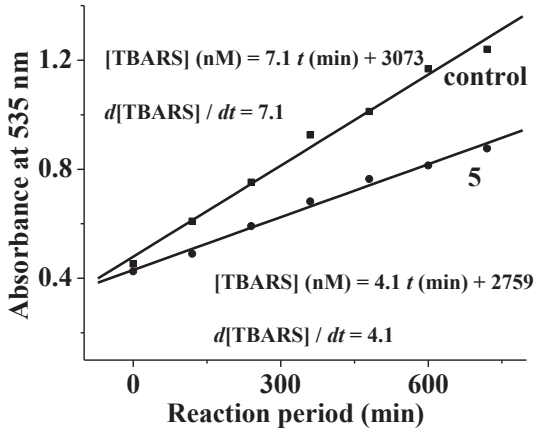


Fig. 1. The increase of the absorbance of TBARS in the mixture of 40 mM AAPH and 2.0 mg/mL DNA and in the presence of 100 μM compound **5**.

Table 2
The formation rate of TBARS (d[TBARS]/d*t*) in the control experiment and in the presence of compounds **3**, **5**, **12**, and **13**.

Compound	d[TBARS]/d*t* (nM min^{-1})
Control	7.1
3	4.2
5	4.1
12	5.2
13	5.8

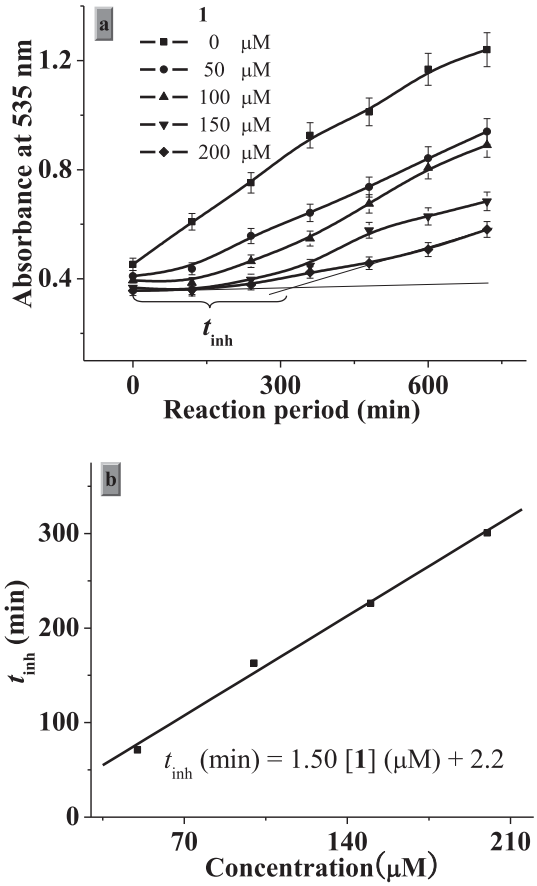


Fig. 2. (a) The variation of the absorbance of TBARS in the mixture of DNA (2.0 mg/mL) and 40 mM AAPH at 37 °C in the presence of various concentrations of compound **1**. **(b)** The relationship between t_{inh} and the concentration of compound **1**.

Table 3
The equations between inhibition period (t_{inh}) and concentrations of thiaflavans in AAPH-induced oxidation of DNA and *n* values of thiaflavans.[a]

Compound	t_{inh} (min) = (n/R_i) [concentration (μM)]	*n*
1	t_{inh} = 1.50 (±0.08) [**1**] + 2.2 (±10.9)	5.04 (±0.29)
2	t_{inh} = 2.57 (±0.03) [**2**] − 1.5 (±0.4)	8.64 (±0.01)
4	t_{inh} = 1.15 (±0.12) [**4**] + 62.1 (±21.7)	3.86 (±0.40)
6	t_{inh} = 2.17 (±0.16) [**6**] + 15.2 (±21.5)	7.29 (±0.54)
7	t_{inh} = 1.18 (±0.06) [**7**] + 43.6 (±1.5)	3.96 (±0.02)
8	t_{inh} = 2.30 (±0.17) [**8**] + 63.2 (±11.9)	7.73 (±0.57)
9	t_{inh} = 3.76 (±0.34) [**9**] + 40.9 (±31.5)	12.64 (±1.14)
10	t_{inh} = 1.99 (±0.01) [**10**] + 63.9 (±1.1)	6.69 (±0.03)
11	t_{inh} = 3.74 (±0.23) [**11**] + 21.1 (±21.1)	12.57 (±0.77)

[a] The value of *n* is the product of the coefficient of t_{inh} ~ [thiaflavans] and $R_i = 1.4 \times 10^{-6} \times 40$ mM s^{-1} = 3.36 μM min^{-1} when 40 mM AAPH is employed.

effectiveness. Moreover, synthetic methods are recently reported to prepare ferrocenyl-substituted flavones and flavonols [32]. This encourages us to replace ring B of thiaflavan by ferrocene moiety and to detect their antioxidant effects on DNA oxidation. It is found herein that ferrocene moiety as B ring indeed enhances the *n* values of compounds **8** and **10** although cyclopentadiene moiety in ferrocene does not form a conjugation system with ring C of thiaflavans. When an electron-donating group such as methoxyl group attaches to ring A, compounds **9** and **11** possess the highest *n* values and thus exhibit the strongest antioxidant effects on DNA oxidation even in the absence of hydroxyl group. Hence, ferrocene group at ring B together with electron-donating group at ring A remarkably promote antioxidant effect of thiaflavans.

5. Conclusion

Both radical-scavenging property and inhibition effect on DNA oxidation are changed markedly when ferrocene moiety is applied to modify the structure of thiaflavan. The ferrocene moiety at ring B is beneficial for enhancing abilities of thiaflavans to trap radicals and to inhibit DNA oxidation. The antioxidant effectiveness generated by hydroxyl groups or ferrocene moiety at ring B is enhanced by the electron-donating group attaching to ring A. In addition, sulfur and oxygen atom at ring C exhibit different effects on the antioxidant property of hydroxyl group locating at their *para*-positions in ring A. The sulfur atom is beneficial for the hydroxyl group in ring A to quench radicals, while the oxygen atom is beneficial for the hydroxyl group in ring A to inhibit DNA oxidation. The structure–activity relationship obtained from thiaflavans is useful for the study on designing novel antioxidants.

6. Experimental section

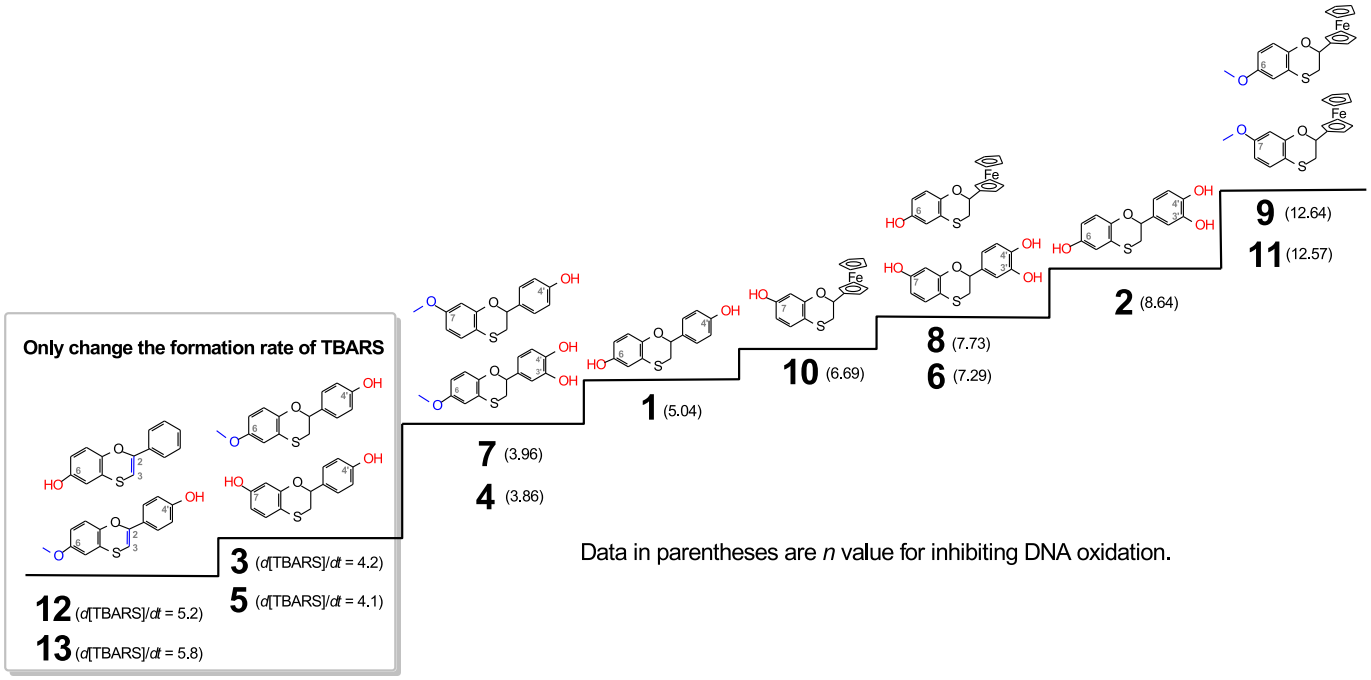
6.1. Materials and instrumentation

ABTS, DPPH, and galvinoxyl radical were purchased from Fluka Chemie GmbH, Switzerland, and AAPH and naked DNA sodium salt were purchased from Acros Organics, Belgium. Other agents were of analytical grade and used directly. The structures of the obtained products were identified by ^{1}H and ^{13}C NMR (Varian Mercury 300 MHz NMR spectrometer and Bruker Avance III 400 MHz spectrometer), and the spectral data were included in Supporting Information.

6.2. Synthesis and structural characterization of thiaflavans

6.2.1. Thiaflavans prepared by the synthetic protocol A

As shown as the synthetic protocol A in Scheme 2, compounds **1**–**11** were synthesized following literature description [16] with *m*-methoxyphenol, *p*-methoxyphenol, resorcinol, and



Scheme 4. The d[TBARS]/dt and n values of thiaflavans in inhibiting DNA oxidation.

hydroquinone as reagents. One hydroxyl group in resorcinol and hydroquinone was protected by *tert*-butyldimethylsilyl chloride. Then, 17 mL of dry $CHCl_3$ solution containing excess PhtNSCl was added dropwisely to 8 mL of dry $CHCl_3$ solution containing mono-protected hydroquinone or resorcinol and stirred for 16 h at 0 °C until phenols cannot be detected by thin layer chromatography (TLC). The mixture was diluted with CH_2Cl_2 and washed by saturated $NaHCO_3$ and water. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. The residue was purified by column chromatography with CH_2Cl_2 as the eluent to afford thiophthalimides as colorless solid. The following cycloaddition reactions were carried out in dry $CHCl_3$ solution of thiophthalimides (~0.1 M) and styrenes (2 equiv.) or vinyl ferrocene (2 equiv.) and freshly distilled $(C_2H_5)_3N$ (2 equiv.) at 60 °C. The reaction was finished with thiophthalimides not detected by TLC. Then, the solvent was evaporated under vacuum pressure, and the residual solid was purified with column chromatography to afford silylated adducts. The desilylation operation was performed in dry tetrahydrofuran (THF) solution containing 0.04 M aforementioned adducts at 0 °C, to which a solution of $(n\text{-}C_4H_9)_4NF{\cdot}3H_2O$ in THF (1 equiv. for each protective group) was added. The reaction was finished with the reagent not detected by TLC, and then the mixture was diluted with ethyl acetate and washed with saturated NH_4Cl and water. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum pressure. The residue was purified with column chromatography to afford thiaflavans.

6.2.2. *Thiaflavans prepared by the synthetic protocol B*

As shown as the synthetic protocol B in Scheme 2, compounds **12** and **13** were prepared by the following operation [17]. The glacial acetic acid (90 mL) solution of quinone (5.4 g, 50.0 mmol) was added to hydrochloride acid (100 mL) solution of thiourea (5.7 g, 75.0 mmol) and stirred at room temperature for 4 h, then refluxed for 1 h and cooled to room temperature. The solid was filtered to give crude product, which was recrystallized with ethanol-water to afford the regent **III** for the synthesis of compounds **12** and **13**. The hydroxyl group in reagent **III** was protected by methyl or benzyl group before the following operation. In brief, 50 mL of acetone was used to dissolve reagent **III** and potassium iodide, which was not needed when the methylation was carried out. The solution was cooled to 0 °C and then benzyl chloride or methyl iodide was added. The reaction mixture was stirred for 30 min, and potassium carbonate was added. Then, the mixture was stirred for 36 h in nitrogen atmosphere. After the insoluble material was filtered, the liquid was concentrated and purified by column chromatography with ethyl acetate–petroleum ether as eluent to afford compound **VIII** or **IV** as white solids. The alcoholysis of compound **VIII** or **IV** was performed in ethanol and THF solution (1:1, v:v) containing ~1.0 M compound **VIII** or **IV**. An ethanolic solution of KOH (2.5 equiv.) was added to the aforementioned solution and stirred for 6 h in nitrogen atmosphere and then 2 M hydrochloride acid was added. The mixture was extracted with ethyl acetate, and the organic layer was washed with water and dried over anhydrous Na_2SO_4. The organic layer was concentrated under vacuum pressure, and the obtained oil (compound **V** or **IX**) was used without further purification. The compound **V** and 2-bromo-1-phenylethanone (1.5 equiv.) (or compound **IX** and 2-bromo-1-(*p*-hydroxyphenyl)ethanone (1.5 equiv.)) were dissolved in 20 mL of CH_2Cl_2, followed by adding $(C_2H_5)_3N$ and stirring for 12 h. The solvent was evaporated under vacuum pressure, and the residual solid was purified with column chromatography to afford compound **VI** or **X**. The annulation of compound **VI** or **X** to afford compound **VII** or **13** was performed in a solution of CH_3CN with $PhPOCl_2$ (2.0 equiv.) as the dehydrant in nitrogen atmosphere. The reaction mixture was refluxed for 16 h and then quenched by adding ethanol. The mixture was poured into water and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum pressure. The residual solid was purified with column chromatography to afford compound **VII** or **13**. The compound **VII** (0.4 g, 1.2 mmol) was added to 5 mL of CH_2Cl_2, to which $TiCl_4$ (600 μL, 5.5 mmol, in 10 mL of CH_2Cl_2) was added dropwisely at 0 °C. After stirring overnight, methanol was added to quench the reaction. The solvent was removed under vacuum pressure, and the residual solid was

purified with column chromatography to afford compound **12** as colorless oil. The systematic nomenclature, isolation method, and total yields for various thiaflavans were given as following.

6.2.3. The characterization of structures of thiaflavans

*6.2.3.1. 2-(4-Hydroxyphenyl)-2,3-dihydro-benzo[1,4]oxathiin-6-ol (**1**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 11%. m.p. 179–182 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.49 (s, 1H, OH), 9.04 (s, 1H, OH), 7.24 (d, J = 7.6 Hz, 2H, aromatic), 6.77 (d, J = 7.6 Hz, 2H, aromatic), 6.68 (d, J = 8.4 Hz, 1H, aromatic), 6.48 (s, 1H, aromatic), 6.42 (d, J = 8.4 Hz, 1H, aromatic), 4.94 (d, J = 9.2 Hz, 1H, CH), 3.12–3.28 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 157.3, 151.5, 145.1, 130.7, 127.7, 119.0, 117.5, 115.1, 112.7, 112.1, 75.5, 30.6.

*6.2.3.2. 4-(6-Hydroxy-2,3-dihydro-benzo[1,4]oxathiin-2-yl)-benzene-1,2-diol (**2**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 7%. m.p. 172–176 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.08 (s, 1H, OH), 9.00 (br s, 2H, OH), 6.84 (s, 1H, aromatic), 6.74–6.78 (m, 2H), 6.71 (d, J = 8.8 Hz, 1H, aromatic), 6.52 (s, 1H, aromatic), 6.46 (d, J = 8.8 Hz, 1H, aromatic), 4.91 (d, J = 8.8 Hz, 1H, CH), 3.15–3.25 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 151.5, 145.2, 145.1, 145.0, 131.3, 119.0, 117.6, 117.4, 115.3, 113.8, 112.7, 112.1, 75.6, 30.8.

*6.2.3.3. 4-(6-Methoxy-2,3-dihydro-benzo[1,4]oxathiin-2-yl)-phenol (**3**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 23%. m.p. 160–163 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.50 (s, 1H, OH), 7.25 (d, J = 7.6 Hz, 2H, aromatic), 6.78 (s, 3H, aromatic), 6.69 (s, 1H, aromatic), 6.59 (d, J = 8.0 Hz, 1H, aromatic), 4.99 (d, J = 8.8 Hz, 1H, CH), 3.68 (s, 3H, OCH_3), 3.17–3.28 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 157.4, 153.6, 146.2, 130.6, 127.7, 119.1, 117.9, 115.1, 111.9, 110.8, 75.6, 55.4, 30.5.

*6.2.3.4. 4-(6-Methoxy-2,3-dihydro-benzo[1,4]oxathiin-2-yl)-benzene-1,2-diol (**4**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 13%. m.p. 134–137 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.02 (s, 1H, OH), 8.99 (s, 1H, OH), 6.85 (s, 1H, aromatic), 6.81 (s, 1H, aromatic), 6.73–6.79 (m, 3H, aromatic), 6.63 (d, J = 8.8 Hz, 1H, aromatic), 4.96 (d, J = 8.0 Hz, 1H, CH), 3.72 (s, 3H, OCH_3), 3.19–3.28 (m, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 153.6, 146.2, 145.3, 145.2, 131.2, 119.0, 117.9, 117.4, 115.3, 113.8, 111.9, 110.8, 75.6, 55.4, 30.7.

*6.2.3.5. 2-(4-Hydroxyphenyl)-2,3-dihydro-benzo[1,4]oxathiin-7-ol (**5**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 30%. Colorless oil. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.51 (s, 1H, OH), 9.32 (s, 1H, OH), 7.24 (d, J = 8.4 Hz, 2H, aromatic), 6.87 (d, J = 8.4 Hz, 1H, aromatic), 6.77 (d, J = 8.4 Hz, 2H, aromatic), 6.36 (dd, J = 2.0 Hz, 8.4 Hz, 1H, aromatic), 6.28 (d, J = 2.0 Hz, 1H, aromatic), 5.07 (d, J = 7.2 Hz, 1H, CH), 3.11–3.22 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 157.4, 155.6, 152.8, 130.6, 127.7, 127.5, 115.1, 109.7, 105.6, 105.1, 76.2, 30.3.

*6.2.3.6. 4-(7-Hydroxy-2,3-dihydro-benzo[1,4]oxathiin-2-yl)-benzene-1,2-diol (**6**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 7%. Colorless oil. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.30 (s, 1H, OH), 8.98 (s, 1H, OH), 8.96 (s, 1H, OH), 6.87 (d, J = 8.4 Hz, 1H, aromatic), 6.79 (s, 1H, aromatic), 6.73 (d, J = 8.4 Hz, 1H, aromatic), 6.69 (d, J = 8.0 Hz, 1H, aromatic), 6.36 (dd, J = 2.0 Hz, 8.4 Hz, 1H, aromatic), 6.28 (d, J = 2.0 Hz, 1H, aromatic), 5.00 (t, J = 5.2 Hz, 1H, CH), 3.12 (d, J = 5.2 Hz, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 155.6, 152.8, 145.3, 145.2, 131.2, 127.5, 117.4, 115.3, 113.7, 109.7, 105.6, 105.1, 76.2, 30.4.

*6.2.3.7. 4-(7-Methoxy-2,3-dihydro-benzo[1,4]oxathiin-2-yl)-phenol (**7**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 15%. Colorless oil. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.51 (s, 1H, OH), 7.26 (d, J = 7.2 Hz, 2H, aromatic), 7.00 (d, J = 7.2 Hz, 1H, aromatic), 6.78 (d, J = 6.0 Hz, 2H, aromatic), 6.49–6.53 (m, 2H, aromatic), 5.10 (d, J = 8.0 Hz, 1H, CH), 3.68 (s, 3H, OCH_3), 3.15–3.24 (m, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 157.6, 157.4, 152.9, 130.4, 127.8, 127.6, 115.1, 108.7, 107.7, 103.6, 76.2, 55.2, 30.2.

*6.2.3.8. 2-Ferrocenyl-2,3-dihydro-benzo[1,4]oxathiin-6-ol (**8**).* Purified by column chromatography with CH_2Cl_2 as eluent. Yield 11%. m.p. 170–172 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.78 (d, J = 8.8 Hz, 1H, aromatic), 6.59 (s, 1H, aromatic), 6.51 (d, J = 8.8 Hz, 1H, aromatic), 4.89 (d, J = 9.2 Hz, 1H, CH), 4.39 (s, 1H, OH), 4.32 (s, 1H, ferrocenyl), 4.29 (s, 1H, ferrocenyl), 4.22 (br, 7H, ferrocenyl), 3.15–3.26 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 153.9, 153.0, 127.9, 109.6, 108.3, 105.6, 87.5, 74.1, 69.1, 68.5, 68.3, 67.1, 66.5, 30.7.

*6.2.3.9. 6-Methoxy-2-ferrocenyl-2,3-dihydro-benzo[1,4]oxathiine (**9**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 32%. m.p. 140–142 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.81 (d, J = 8.7 Hz, 1H, aromatic), 6.63 (d, J = 3.0 Hz, 1H, aromatic), 6.58 (dd, J = 8.7 Hz, 3 Hz, 1H, aromatic), 4.84 (d, 1H, J = 9.0 Hz, CH), 4.26–4.36 (m, 9H, ferrocenyl), 3.75 (s, 3H, OCH_3), 3.16–3.29 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 154.1, 146.6, 119.2, 118.1, 112.2, 111.1, 87.7, 73.4, 69.1, 68.4, 68.2, 67.1, 66.5, 55.8, 31.0.

*6.2.3.10. 2-Ferrocenyl-2,3-dihydro-benzo[1,4]oxathiin-7-ol (**10**).* Purified by column chromatography with CH_2Cl_2 as eluent. Yield 23%. m.p. 134–136 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.78 (d, J = 8.8 Hz, 1H, aromatic), 6.58 (s, 1H, aromatic), 6.50 (d, J = 8.8 Hz, 1H, aromatic), 4.87 (d, J = 9.2 Hz, 1H, CH), 4.33 (s, 1H, ferrocenyl), 4.30 (s, 1H, ferrocenyl), 4.23 (br, 7H, ferrocenyl), 3.15–3.26 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 153.8, 153.0, 127.9, 109.5, 108.4, 105.6, 87.4, 74.1, 69.0, 68.4, 68.2, 67.0, 66.4, 30.7.

*6.2.3.11. 7-Methoxy-2-ferrocenyl-2,3-dihydro-benzo[1,4]oxathiine (**11**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 14%. m.p. 94–97 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.97 (d, J = 8.4 Hz, 1H, aromatic), 6.51 (d, J = 8.4 Hz, 1H, aromatic), 6.49 (s, 1H, aromatic), 4.99 (s, 1H, CH), 4.23–4.33 (m, 9H, ferrocenyl), 3.76 (s, 3H, OCH_3), 3.19 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 158.2, 153.0, 127.7, 108.7, 108.2, 103.8, 87.5, 74.1, 69.0, 68.4, 68.2, 67.1, 66.4, 55.5, 30.6.

*6.2.3.12. 2-Phenylbenzo[1,4]oxathiin-6-ol (**12**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 13%. Pale orange oil. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.13 (s, 1H, OH), 7.57 (s, 1H, CH), 7.55 (s, 1H, aromatic), 7.41–7.49 (m, 4H, aromatic), 6.87 (d, J = 8.0 Hz, 1H, aromatic), 6.51 (d, J = 8.0 Hz, 1H, aromatic), 6.50 (s, 1H, aromatic). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 152.4, 141.9, 140.1, 129.15, 129.07, 129.0, 126.7, 120.2, 119.0, 113.6, 112.5, 96.1.

*6.2.3.13. 4-(6-Methoxybenzo[1,4]oxathiin-2-yl)-phenol (**13**).* Purified by column chromatography with petroleum ether/ethyl acetate as eluent. Yield 9%. Pale orange oil. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.59 (s, 1H, OH), 7.58 (s, 1H, CH), 7.54 (d, J = 8.0 Hz, 2H, aromatic), 7.24 (d, J = 8.0 Hz, 2H, aromatic), 6.93 (d, J = 8.8 Hz, 1H, aromatic), 6.70 (d, J = 2.8 Hz, 1H, aromatic), 6.66 (dd, J = 8.8 Hz, J = 2.8 Hz, 1H, aromatic), 3.70 (s, 3H, OCH_3). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 159.2, 158.0, 152.5, 149.5, 128.9, 128.2, 126.0, 113.8, 112.6, 111.5, 96.2, 58.7.

6.3. Scavenging radicals

The tests for thiaflavans, trolox, and catechol to scavenge $ABTS^{+\bullet}$, DPPH, and galvinoxyl radicals were performed following previous description [22]. ABTS salt and $K_2S_2O_8$ were dissolved in 2.0 mL of water, in which the final concentration of ABTS and $K_2S_2O_8$ were 4.0 mM and 1.41 mM, respectively. The aforementioned solution was kept for 16 h and then diluted by 100 mL of ethanol to give $ABTS^{+\bullet}$ solution, whose absorbance was around 0.70 at 734 nm ($\varepsilon_{ABTS^{+\bullet}} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$). DPPH and galvinoxyl radical were dissolved in 50 mL of ethanol, and their absorbances were around 1.00 at 517 nm ($\varepsilon_{DPPH} = 1.09 \times 10^4\ M^{-1}\ cm^{-1}$) and 428 nm ($\varepsilon_{galvinoxyl} = 1.4 \times 10^5\ M^{-1}\ cm^{-1}$), respectively. Dimethyl sulfoxide (DMSO) solutions of thiaflavans, trolox, and catechol (0.1 mL) were added to 1.9 mL of radical solutions. The final concentrations of these compounds were 7.5 μM in reacting with $ABTS^{+\bullet}$. The final concentrations of **2, 4**, **6**, and catechol were 37.5 μM (75.0 μM for the other compounds) in reacting with DPPH, while the final concentrations of **2, 4**, **6**, trolox and catechol were 7.5 μM (75.0 μM for the other compounds) in reacting with galvinoxyl radical. Decays of the absorbance of radicals were recorded at 25 °C within a certain time interval.

6.4. Inhibiting AAPH-induced oxidation of DNA

AAPH-induced oxidation of DNA was performed following previous description [33]. DNA sodium salt and AAPH were dissolved in phosphate buffered solution (PBS: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 mM EDTA) with the final concentration as 2.0 mg/mL and 40 mM, respectively. The aforementioned solution also contained various concentrations of thiaflavans (dissolved in 0.1 mL of DMSO). The same volume of DMSO was also contained in the blank experiment as the control. The solution was then dispatched into test tubes with each one containing 2.0 mL. These tubes were heated at 37 °C in a water bath for initiating the oxidation of DNA. Three of them were taken out at every 2 h and cooled to room temperature, followed by adding 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 and then heating in a boiling water bath for 15 min. After the tubes were cooled to room temperature immediately, 1.5 mL of *n*-butanol was added and shaken vigorously to extract thiobarbituric acid reactive species (TBARS). Finally, the tubes were centrifuged for a few minutes to obtain *n*-butanol layer for measuring the absorbance at 535 nm ($\varepsilon_{TBARS} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) [34].

6.5. Statistical analysis

All the data were the average value from at least three independent measurements within 10% experimental error. Equations were fitted by using Origin 7.5 professional software, and $p < 0.001$ indicated a significant 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.2014.04.081.

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