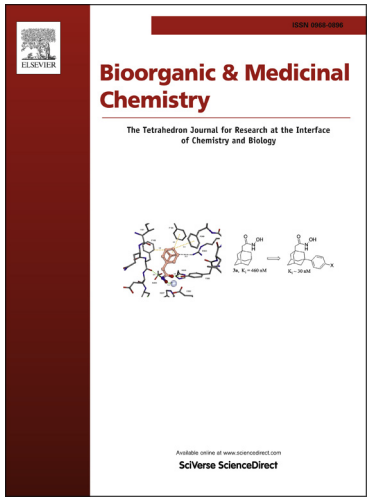

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Synthesis and antioxidant activity of novel Mannich base of 1,3,4-oxadiazole derivatives possessing 1,4-benzodioxan

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Abstract

A new series of Mannich base of 1,3,4-oxadiazole derivatives possessing 1,4-benzodioxan (**6a** – **6ae**) were synthesized and characterized by ^{1}H-NMR, ESI-MS and elemental analysis. The structure of **6b** was further confirmed by single crystal X-ray diffraction. All these novel compounds were screened for their *in vitro* antioxidant activity employing 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+}$•) and ferric reducing antioxidant power (FRAP) scavenging assays. Due to the combination of 1,4-benzodioxan, 1,3,4-oxadiazoles and substituted phenyl ring, most of them exhibited nice antioxidant activities. In all of these three assays mentioned above, compounds **6f** and **6e** showed significant radical scavenging ability comparable to the commonly used antioxidants, BHT and Trolox. Seven compounds with representative substituents or activities were selected for further assays in chemical simulation biological systems – inhibition of microsomal lipid peroxidation (LPO) and protection against 2,2'-azobis (2-amidinopropane hydrochloride) (AAPH) induced DNA strand breakage, in which, **6f** and **6e** were demonstrated to be of the most potent antioxidant activities.

Keywords

Mannich base; 1,3,4-oxadiazole; 1,4-benzodioxan; antioxidant activity

1. Introduction

Reactive oxygen species (ROS), known as mediators of intracellular signaling cascades, are chemically reactive molecules containing oxygen. Most living organisms can produce ROS and metabolize excessive ones by normal physiological processes. However, sometimes these efficient protecting systems could be disrupted by external factors (smoke, alcohol, diet and some drugs) or aging.[1] Accumulation of excessive ROS can damage lipids, proteins, carbohydrates, and DNA in cells, leading to oxidative stress, loss of cell function, and ultimate apoptosis or necrosis. And all of the above biochemical processes are the common problems in various diseases such as cancer, inflammation, atherosclerosis and cardiovascular disease.[2] Therefore, exploring antioxidant chemicals that scavenge ROS may be of great value in preventing the onset and propagation of oxidative stress.

Mannich bases derived from various heterocycles exhibit unique biological activities, such as antitubercular,[3] antimalarial,[4] anticancer[5] and analgesic[6] properties. Moreover, they were recognized as a useful pharmacophore group against thrombosis caused by antioxidants.[7] As an important class of heterocyclic compounds, 1,3,4-oxadiazoles are associated with a broad spectrum of biological activities including anti-inflammatory,[8,9] analgesic,[8] antimicrobial,[10-13] anticancer[14] and antioxidant[10,15,16] activities. And those heterocyclic derivatives bearing 1,4-benzodioxan moiety have been proved to be of pharmacological value on antitumor[17-19] and immunosuppressive,[20] anti-inflammatory[21] and antifungal[22] activities.

The combination of different pharmacophores in the same unit is an attracting approach to discover novel potent drugs, due to the possible synergistic effect.[23] In this study, a series of 1,3,4-oxadiazole derivatives possessing a 1,4-benzodioxan moiety were synthesized through

Mannich reaction. The radical scavenging properties of these novel compounds were screened by three quick assays first: 1) trapping 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH), 2) 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) cationic radical ($ABTS^{+}$•), 3) and ferric reducing antioxidant power (FRAP) methods. Then seven compounds with representative substituents or activities were selected to evaluate their antioxidant effects against lipid peroxidation (LPO) of mice liver microsomes and 2,2'-azobis (2-amidinopropane hydrochloride) (AAPH) induced supercoiled DNA breakage, respectively.

2. Results and discussion

2.1. Chemistry

A total of 30 novel Mannich base of 1,3,4-oxadiazole derivatives possessing 1,4-benzodioxan (**6a** – **6ae**) were synthesized from 5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2-thiol (**4**) and different kinds of amine derivatives (**5**) by Mannich condensation reaction as depicted in Scheme 1. The key intermediate **4** was prepared in three steps according to a previously reported method.[24] Esterification of the 2,3-dihydrobenzo[*b*]-[1,4]dioxine-6-carboxylic acid (**1**) with methanol and concentrated sulfuric acid produced the corresponding ester **2**. The aroyl hydrazide **3** was obtained by reacting **2** with 85% hydrazine monohydrate in ethanol. Treatment of the hydrazide **3** with carbon disulfide in the presence of KOH and 95% ethanol under refluxing gave the key intermediate **4**. After dissolved in ethanol, compound **4** was reacted with formalin and appropriate amines (substituted aniline, cyclohexylamine or hexadecylamine) to afford the target compounds **6a** – **6ac**, **6ad** and **6ae**, respectively. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their

depicted structures. Additionally, the structure of compound **6b** was further confirmed by X-ray diffraction. Its perspective view with the atomic labeling system was shown in Figure 1, and the crystallographic data was presented in Table S1 as supplementary material.[25]

In order to set off the antioxidant property of 1,4-benzodioxan, 5-phenyl-1,3,4-oxadiazole-2-thione (**7**) was synthesized by the same synthetic route as compound **4** (Scheme 2). And its ^{1}H-NMR and ESI-MS data were consistent with the ones in previous report.[26]

2.2. In vitro antioxidant activity profiling

2.2.1 DPPH radical scavenging activity

The DPPH radical scavenging activity assay is a simple method for measuring the antioxidant ability to trap free radicals. The scavenging effects of compounds **6a-6ae** were shown in Table 1 along with butylated hydroxytoluene (BHT) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as two positive controls. More than half of the tested compounds showed higher DPPH radical scavenging capacities than BHT (IC_{50} = 43.84 μM), including seven of them exhibited stronger activity than Trolox (IC_{50} = 30.20 μM). Compounds **6f** and **6e** exhibited the highest activity with the IC_{50} values of 19.62 and 21.80 μM, while **6ae** and **6ad** presented lowest activity with IC_{50} values of 107.5 and 94.40 μM, respectively.

2.2.2. ABTS radical cation scavenging activity

The ABTS assay is another widely used method for measuring the *in vitro* antioxidant ability.[27] The ABTS radical cation scavenging capacities of the synthesized compounds **6a-6ae** were also shown in Table 1. A total of seven compounds displayed higher activity than Trolox (IC_{50} = 6.19

μM), in which, **6f** and **6e** exhibited the highest activities with the IC_{50} values of 2.92 and 3.32 μM, respectively. Meanwhile, **6ae** (IC_{50} = 22.90 μM) and **6ad** (IC_{50} = 18.41 μM) presented significant weak ABTS radical cation scavenging capacities compared to those compounds with substituted aniline (**6a** – **6ac**).

2.2.3. Ferric reducing antioxidant power (FRAP)

In the ferric to ferrous reduction assay, the electron donation capacity (reflecting the electron transfer ability) of the compounds was assessed.[28] FRAP values of compounds **6a - 6ae** were presented in Table 1. Compounds **6f** ($C_{0.5}$FRAP = 239.7 μM), **6e** ($C_{0.5}$FRAP = 268.3 μM) and **6x** ($C_{0.5}$FRAP = 291.7 μM) displayed better activities than Trolox ($C_{0.5}$FRAP = 293.7 μM). And another seven compounds were also more potent than BHT ($C_{0.5}$FRAP = 546.2 μM).

In summary of the *in vitro* antioxidant activities of the synthesized **6a** – **6ae**, more than half of the compounds exhibited higher antioxidant activity than BHT and few of them even exhibited higher activity than Trolox, which is a better antioxidant than BHT. Introducing arylamine (**6a** – **6ac**) enhanced antioxidant activity of Mannich bases, but alkyl amines had no significant effect (**6ad**, **6ae**).

In order to demonstrate the combination of 1,4-benzodioxan and 1,3,4-oxadiazole could enhance overall activity as an antioxidant, **2** with only 1,4-benzodioxan, **7** with only 1,3,4-oxadiazole and **4** with both moieties were also evaluated their antioxidant capacities by the above-mentioned assays. As shown in Table 1, compound **4** exhibited better activity than both **2** and **7** in all three methods, but less than most of the **6a** – **6ac**. The results suggested 1,4-benzodioxan, 1,3,4-oxadiazoles and Mannich base with aromatic ring all contribute to the

antioxidant activity.

Compounds **6e** and **6f** presented the best radical scavenging activity in all of the above three assays, indicating that multi-fluoro substituent on the benzene improved the antioxidant activity. Compounds **6x** – **6ac** with methyl-, methoxy- or ethyoxyl- substituent on the benzene exhibited moderate antioxidant activities, while compounds **6t**, **6u**, **6v** and **6w** with nitro substituent demonstrated weak radical scavenging activity. The results are partially consistent with Sergio and Mónica,[29] Babasaheb[30] and Kotaiah's[1] work that electron donating groups contribute more radical scavenging activity than electron withdrawing groups attached to the phenyl ring.

In order to verify whether those Mannich base derivatives were applicable in various oxidation environments, seven compounds with representative structure or activity were selected to be further assayed their inhibition of mice liver microsomal LPO and protective effect for DNA damaged induced by AAPH.

2.3. Inhibition of microsomal LPO

As shown in Figure 2, compounds **6a**, **6e**, **6f**, and **6x** were more potent than Trolox (183.2 μM), in inhibiting the LPO of mice liver microsomes, especially **6f** showed the most potent biological activity (IC_{50} = 110.3 μM). As expected, compounds **6t**, **6u** and **6w** were less active than the compounds mentioned above. The rank of these compounds matches the results of *in vitro* antioxidant assays.

2.4. DNA protective effect

DNA can be damaged by free radical-mediated oxidant as reported by many investigations.[31-33] The antioxidant activities of seven selected compounds (**6a, 6e, 6f, 6x, 6w, 6t, 6u**) were evaluated against AAPH-induced plasmid pBR322 DNA strand breakage using agarose gel electrophoresis

analysis (Figure 3A). The native DNA was mostly in its supercoiled form, which would be transformed to open-circular form completely in the presence of AAPH. Most of the newly synthetic compounds showed better activity than Trolox regarding protection against AAPH-induced DNA strand breakage. On the basis of the relative amount of intact supercoiled DNA (Figure 3B), the activity of the selected compounds followed the order: **6a** ≈ **6e** ≈ **6f** ≈ **6x** > **6w** > **6t** ≈ **6u**, which was the same order as our previous results. Compounds **6u**, **6v** and **6t** appeared less active than the others, suggesting the introduction of electron-withdrawing substituents such as nitro decrease the activity significantly.

3. Conclusion

In conclusion, a new class of Mannich base of 1,3,4-oxadiazole derivatives possessing 1,4-benzodioxan were prepared from simple starting materials and investigated for their antioxidant activities *in vitro*. In the DPPH, ABTS and FRAP chemical models, most of the compounds with substituted phenyl exhibited better antioxidant activity than the standard antioxidant agents – Trolox and BHT, in which, **6f** and **6e** with multi-fluoro substituent on the benzene displayed the most potent activity. Four high-active compounds, **6a**, **6e**, **6f** and **6x**, and three less-active compounds, **6t**, **6u** and **6w**, with distinct representative substituents were selected to be assessed using inhibition of microsomal LPO and AAPH-induced oxidation of DNA models. Highly consistent results were obtained and the SAR of these synthetic compounds was further defined. This conclusion may lead to the development of novel drugs for treating the diseases caused by oxidative stress.

4. Experimental protocols

4.1. Chemistry general

All chemicals and reagents used in current study were of analytical grade. The reactions were monitored by thin layer chromatography (TLC) using pre-coated silica gel GF254 plates from Qingdao Haiyang Chemical, China. Melting points (uncorrected) were determined on a SPSIC WRS-1B digital melting-point apparatus (Shanghai, China). IR spectra were recorded as KBr disks on a Bruker Tensor 27 IR spectrophotometer. ESI-MS spectra were obtained on a Mariner Mass 5304 instrument. ^{1}H-NMR spectra were collected on a Bruker Avance III 400 NMR spectrometer with $CDCl_3$ as solvent at room temperature, The chemical shifts(δ) were reported in ppm with reference to internal TMS and coupling constants (*J*) were given in Hz. Elemental analyzes were performed on a CHN-O-Rapid instrument and were within ± 0.4 % of the theoretical values.

*4.2. General procedure for synthesis of the target compounds (**6a ~ 6ae**)*

Formalin (150 μL, 2 mmol) was added to a stirred solution of 5-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**4**) (0.236g, 1 mmol) in ethanol (10 mL). An ethanolic solution (5 mL) of the appropriate amine (1 mmol) was added portionwise to the reaction mixture, stirred for 3 h at room temperature and left overnight in a 4 °C refrigerator. The precipitate was filtered, washed with cold ethanol, dried, and crystallized from the suitable solvent.

*4.2.1. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-((phenylamino)methyl)-1,3,4-oxadiazole-2(3H) -thione (**6a**)*

Light white crystal, yield: 63 %, mp: 147.3-147.9 ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.44

(m, 2H,), 7.27 (t, *J* = 8.0 Hz, 2H), 6.94 – 7.02 (m, 3H), 6.87 (t, 1H, *J* = 7.3 Hz), 5.59 (s, 2H), 5.18 (br s, 1H), 4.33 – 4.36 (m, 4H). MS (ESI): 342.08 ($C_{17}H_{15}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{15}N_3O_3S$: C, 59.81; H, 4.43; N, 12.31; Found: C, 59.65; H, 4.15; N, 12.38.

*4.2.2. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((2-fluorophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6b**)*

Colorless crystal, yield: 77 %, mp: 171.4-171.6 ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.42 (m, 2H), 7.22 (td, 1H, *J* = 8.0, 1.3 Hz), 7.02 – 7.04 (d, 1H, *J* = 8.6 Hz), 6.97 – 7.00 (m, 1H), 6.93 (d, 1H, *J* = 8.4 Hz), 6.73 – 6.78 (m, 1H), 5.55 (s, 2H), 5.32 (br s, 1H), 4.28 – 4.32 (m, 4H). MS (ESI): 359.07 ($C_{17}H_{14}FN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}FN_3O_3S$: C, 56.82; H, 3.93; N, 11.69; Found: C, 56.83; H, 3.54; N, 11.85.

*4.2.3. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((3-fluorophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6c**)*

Colorless podwer, yield: 54 %, mp: 149.5-150.4 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.44 (m, 2H), 7.15 (dd, 1H, *J* = 15.0 7.8 Hz), 6.93 (d, *J* = 8.5 Hz, 1H), 6.67 (dd, 1H, *J* = 9.7 1.32 Hz), 6.51 (m, 1H), 5.48 (s, 2H), 5.22 (br s, 1H), 4.28 – 4.33 (m, 4H). MS (ESI): 359.07 ($C_{17}H_{14}FN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}FN_3O_3S$: C, 56.82; H, 3.93; N, 11.69; Found: C, 56.43; H, 3.99; N, 11.88.

*4.2.4. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((4-fluorophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6d**)*

Colorless powder, yield: 54 %, mp: 189.8-190.6 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.40 (m, 3H), 6.89 – 6.95 (m, 2H), 6.84 – 6.87 (m, 2H), 6.01 (s, 1H), 5.47 (s, 2H), 4.23 – 4.37 (m, 4H). MS (ESI): 359.07 ($C_{17}H_{14}FN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}FN_3O_3S$: C, 56.82; H, 3.93;

N, 11.69; Found: C, 56.17; H, 3.98; N, 11.55.

*4.2.5. 3-(((2,6-difluorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6e**)*

White powder, yield: 50 %, mp: 148.5-148.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.39 (m, 2H), 6.94 – 6.98 (m, 2H), 6.86 – 6.89 (m, 2H), 5.62 (s, 2H), 5.09 (br s, 1H), 4.32 – 4.37 (m, 4H). MS (ESI): 378.07 ($C_{17}H_{13}F_2N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{13}F_2N_3O_3S$: C, 54.11; H, 3.47; N, 11.14; Found: C, 54.01; H, 3.39; N, 11.23.

*4.2.6. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((3,4,5-trifluorophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6f**)*

White powder, yield: 67 %, mp: 203.1-204.2 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.49 (m, 3H, H-), 6.99(d, 1H, *J* = 8.8), 5.71 (t, 1H, *J* = 7.5), 5.57 (d, 2H, *J* = 7.6), 4.33 – 4.38 (m, 4H). MS (ESI): 396.05 ($C_{17}H_{13}F_2N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{12}F_3N_3O_3S$: C, 51.65; H, 3.06; N, 10.63; Found: C, 51.78; H, 3.01; N, 10.02.

*4.2.7. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((2-(trifluoromethyl)phenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6g**)*

White crystal, yield: 80 %, mp: 185.4-186.2 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.40 (m, 3H), 6.90 – 6.94 (m, 2H), 6.81 – 6.85 (m, 3H), 5.58 (s, 2H), 5.05 (br s, 1H), 4.33 – 4.37 (m, 4H). MS (ESI): 410.07 ($C_{18}H_{14}F_3N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{14}F_3N_3O_3S$: C, 52.81; H, 3.45; N, 10.26; Found: C, 52.86; H, 3.21; N, 10.11.

*4.2.8. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((3-(trifluoromethyl)phenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6h**)*

White crystal, yield: 85 %, mp: 200.4-201.0 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.40 (m,

3H), 7.32 (t, 1H, *J* = 7.90 Hz), 7.20(s, 1H), 7.08 (t, 1H, *J* = 8.6 Hz), 6.93 (dd, 1H, *J* = 7.90, 0.90 Hz), 5.53 (s, 2H), 5.31 (br s, 1H), 4.28 - 4.32(m, 4H). MS (ESI): 410.07 ($C_{18}H_{14}F_3N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{14}F_3N_3O_3S$: C, 52.81; H, 3.45; N, 10.26; Found: C,52.59; H, 3.21; N, 10.20.

*4.2.9. 3-(((2,5-bis(trifluoromethyl)phenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl) -1,3,4-oxadiazole-2(3H)-thione (**6i**)*

White powder, yield: 77 %, mp: 177.3-178.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.40 (m, 1H), 7.33 – 7.35 (m, 2H), 6.91 (dd, 1H, *J* = 8.6, 0.8 Hz), 6.81 – 6.85 (m, 2H) 5.58 (s, 2H), 5.05 (br s, 1H), 4.27 – 4.33(m,4H). MS (ESI): 478.08 ($C_{19}H_{13}F_6N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{19}H_{13}F_6N_3O_3S$: C, 47.80; H, 2.74; N, 8.80; Found: C, 47.38; H, 2.61; N, 8.54.

*4.2.10. 3-(((2-chlorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6j**)*

White powder, yield: 61 %, mp: 179.6-180.4 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.41 (m, 2H), 7.24 (dd, 1H, *J* = 7.9, 1.6 Hz), 7.19 – 7.20 (m, 1H), 6.93 (d, 1H, *J* = 8.6 Hz), 6.76 (td, 1H, *J* = 7.9, 1,7 Hz) 5.70 (br s, 1H), 5.58 (s, 2H), 4.27 – 4.33(m,4H). MS (ESI): 375.04 ($C_{17}H_{14}ClN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}ClN_3O_3S$: C, 54.33; H, 3.75; N, 11.18; Found: C, 54.12; H, 3.71; N, 11.22.

*4.2.11. 3-(((3-chlorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6k**)*

White powder, yield: 61 %, mp: 139.9-140.0 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.54 (m, 4H), 7.34 (d, 1H, *J* = 8.2 Hz), 6.98 (d, 1H, *J* = 8.0 Hz), 6.93 (t, 1H, *J* = 7.6 Hz), 5.74 (t, 1H, *J* = 7.2 Hz), 5.61 (d, 2H, *J* = 7.6 Hz), 4.33 – 4.37(m, 4H). MS (ESI): 375.04 ($C_{17}H_{14}ClN_3O_3S$,

$[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}ClN_3O_3S$: C, 54.33; H, 3.75; N, 11.18; Found: C, 54.09; H, 3.63; N, 11.05.

*4.2.12. 3-(((4-chlorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6l**)*

White powder, yield: 71 %, mp: 216.1-216.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.43 (m, 2H), 7.15 (t, 1H, *J* = 8.0 Hz), 6.95 – 6.97 (m, 2H), 6.80 – 6.83 (m, 2H), 5.50 (s, 2H), 5.22 (br s, 1H), 4.31 – 4.35 (m, 4H). MS (ESI): 375.04 ($C_{17}H_{14}ClN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}ClN_3O_3S$: C, 54.33; H, 3.75; N, 11.18; Found: C, 54.25; H, 3.70; N, 10.99.

*4.2.13. 3-(((2,4-dichlorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6m**)*

White powder, yield: 89 %, mp: 155.9-156.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.40 (m, 2H), 7.28 (d, 1H, *J* = 1.6 Hz), 7.14 – 7.20 (m, 2H), 6.93 (d, 1H, *J* = 8.1 Hz), 5.65 (t, 1H, *J* = 7.7 Hz), 5.54 (d, 2H, *J* = 7.6 Hz), 4.28 – 4.32 (m, 4H). MS (ESI): 410.01 ($C_{17}H_{13}Cl_2N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{13}Cl_2N_3O_3S$: C, 49.77; H, 3.19; N, 10.24; Found: C, 49.61; H, 3.10; N, 10.19.

*4.2.14. 3-(((2,5-dichlorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6n**)*

White powder, yield: 61 %, mp: 166.3-167.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.42 (m, 2H), 7.32 (d, 1H, *J* = 2.5 Hz), 7.18 (d, 1H, *J* = 8.4 Hz), 6.95 (d, 1H, *J* = 8.5 Hz), 6.74 (dd, 1H, *J* = 8.3 2.2 Hz), 5.72 (t, 1H, *J* = 7.6, Hz), 5.54 (d, 2H, *J* = 7.7 Hz), 4.29 – 4.33 (m, 4H). MS (ESI): 410.01 ($C_{17}H_{13}Cl_2N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{13}Cl_2N_3O_3S$: C, 49.77; H, 3.19; N, 10.24; Found: C, 49.53; H, 3.09; N, 10.17.

*4.2.15. 3-(((3,4-dichlorophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6o**)*

White powder, yield: 51 %, mp: 168.3-168.9 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.40 (m, 2H), 7.24 (s, 1H), 7.06 (d, 1H, *J* = 2.7 Hz), 6.94 (d, 1H, *J* = 4.6 Hz), 6.78 (dd, 1H, *J* = 4.7 2.8 Hz), 5.46 (s, 2H), 5.18 (br s, 1H), 4.29 – 4.33 (m, 4H). MS (ESI): 410.01 ($C_{17}H_{13}Cl_2N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{13}Cl_2N_3O_3S$: C, 49.77; H, 3.19; N, 10.24; Found: C, 49.37; H, 3.01; N, 10.02.

*4.2.16. 3-(((2-bromophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6p**)*

Light yellow powder, yield: 81 %, mp: 158.8-159.6 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.45 (m, 3H), 7.22 – 7.24 (m, 2H), 6.93 (d, 1H, *J* = 8.4 Hz), 6.68 – 6.72 (m, 1H), 5.69 (br s, 1H), 5.57 (s, 2H), 4.28 – 4.32 (m, 4H). MS (ESI): 420.00 ($C_{17}H_{14}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}BrN_3O_3S$: C, 48.58; H, 3.36; N, 10.00; Found: C, 48.11; H, 3.40; N, 9.98.

*4.2.17. 3-(((3-bromophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6q**)*

White powder, yield: 78 %, mp: 150.0-150.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.41 (m, 2H), 7.05 – 7.11 (m, 2H), 6.93 – 6.95 (m, 2H), 6.83 (dd, 1H, *J* = 4.0 1.6 Hz), 5.47 (d, 2H, *J* = 4.0 Hz), 5.17 (br s, 1H), 4.29 – 4.33 (m, 4H). MS (ESI): 420.00 ($C_{17}H_{14}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}BrN_3O_3S$: C, 48.58; H, 3.36; N, 10.00; Found: C, 48.62; H, 3.09; N, 9.92.

*4.2.18. 3-(((4-bromophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6r**)*

White crystal, yield: 59 %, mp: 205.3-206.2 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.40 (m,

2H), 7.05 – 7.11 (m, 2H), 6.93 – 6.94 (m, 2H), 6.84 (dd, 1H, *J* = 4.1 1.7 Hz), 5.47 (d, 2H, *J* = 5.2 Hz), 5.18 (br s, 1H), 4.28 – 4.32 (m, 4H). MS (ESI): 420.00 ($C_{17}H_{14}BrN_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}BrN_3O_3S$: C, 48.58; H, 3.36; N, 10.00; Found: C, 48.71; H, 3.02; N, 9.85.

*4.2.19. 3-(((2,4-dibromophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6s**)*

White crystal, yield: 76 %, mp: 187.6-188.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, 1H, *J* = 2.2 Hz), 7.42 – 7.45 (m, 2H), 7.38 (dd, 1H, *J* = 8.7 2.1 Hz), 7.16 (d, 1H, *J* = 8.8 Hz), 6.98 (dd, 1H, *J* = 8.2 0.4 Hz), 5.72 (t, 1H, *J* = 7.7 Hz), 5.58 (d, 2H, *J* = 7.8 Hz), 4.33 – 4.37 (m, 4H). MS (ESI): 497.90 ($C_{17}H_{13}Br_2N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{13}Br_2N_3O_3S$: C, 40.90; H, 2.62; N, 8.42; Found: C, 40.53; H, 2.54; N, 8.52.

*4.2.20. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((2-nitrophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6t**)*

Yellow powder, yield: 84 %, mp: 191.9-193.2 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (t, 1H, *J* = 6.6 Hz), 8.20 (d, 1H, *J* = 8.4 Hz), 7.55 (t, 1H, *J* = 7.1 Hz), 7.48 (d, 1H, *J* = 8.5 Hz), 7.39 – 7.42 (m, 2H), 6.94 (d, 1H, *J* = 8.4 Hz), 6.85 (t, 1H, *J* = 8.1 Hz), 5.66 (d, 2H, *J* = 7.8 Hz), 4.33 – 4.37 (m, 4H). MS (ESI): 387.07 ($C_{17}H_{14}N_4O_5S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}N_4O_5S$: C, 52.84; H, 3.65; N, 14.50; 8.42; Found: C, 51.99; H, 3.55; N, 14.12.

*4.2.21. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((3-nitrophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6u**)*

Yellow powder, yield: 85.0%, mp: 181.4-181.4 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (t, 1H, *J* = 2.1 Hz), 7.65 (dd, 1H, *J* = 10.5 0.6 Hz), 7.39 – 7.41 (m, 2H), 7.35 (d, 1H, *J* = 8.0 Hz), 7.22 (dd, 1H, *J* = 7.6 0.8 Hz), 5.56 (d, 2H, *J* = 7.9 Hz), 5.44 (t, 1H, *J* = 4.2 Hz), 4.28 – 4.32 (m, 4H). MS

(ESI): 387.07 ($C_{17}H_{14}N_4O_5S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}N_4O_5S$: C, 52.84; H, 3.65; N, 14.50; 8.42; Found: C, 52.37; H, 3.60; N, 14.31.

*4.2.22. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((4-nitrophenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6v**)*

Light yellow powder, yield: 83 %, mp: 233.7-234.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.42 (m, 2H), 7.32 (d, 1H, *J* = 2.2 Hz), 7.18 (d, 1H, *J* = 8.4 Hz), 6.96 (d, 1H, *J* = 8.8 Hz), 6.73 (dd, 1H, *J* = 8.4 2.2 Hz), 5.71 (t, 1H, *J* = 7.6 Hz), 5.54 (d, 2H, *J* = 7.7 Hz), 4.29 – 4.33 (m, 4H). MS (ESI): 387.07 ($C_{17}H_{14}N_4O_5S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{14}N_4O_5S$: C, 52.84; H, 3.65; N, 14.50; Found: C, 52.29; H, 3.42; N, 14.34.

*4.2.23. 3-(((2-chloro-4-nitrophenyl)amino)methyl)-5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1,3,4-oxadiazole-2(3H)-thione (**6w**)*

Light yellow powder, yield: 59 %, mp: 167.2-168.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, 1H, *J* = 2.5 Hz), 8.14 (dd, 1H, *J* = 8.3 2.5 Hz), 7.39 – 7.41 (m, 2H), 7.35 (d, 1H, *J* = 9.4 Hz), 6.95 (d, 1H, *J* = 8.0 Hz), 6.21 (t, 1H, *J* = 7.8 Hz), 5.64 (d, 2H, *J* = 7.5 Hz), 4.29 – 4.33 (m, 4H). MS (ESI): 421.03 ($C_{17}H_{13}ClN_4O_5S$, $[M+H]^+$). Anal. Calcd. For $C_{17}H_{13}ClN_4O_5S$:C, 48.52; H, 3.11; N, 13.31; Found: C, 48.43; H, 3.02; N, 13.07.

*4.2.24. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-((o-tolylamino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6x**)*

Colorless crystal, yield: 69 %, mp: 147.2-147.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.44 (m, 2H), 7.19 (d, 1H, *J* = 7.4 Hz), 7.11 – 7.15 (m, 2H), 6.97 (d, 1H, *J* = 8.2 Hz), 6.81 (t, 1H, *J* = 5.1 Hz), 5.62 (d, 2H, *J* = 5.2 Hz), 5.15 (br s, 1H), 4.23 – 4.37 (m, 4H), 2.27 (s, 3H). MS (ESI): 356.10 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 60.83; H, 4.82; N, 11.82;

Found: C, 60.41; H, 4.32; N, 11.56.

*4.2.25. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-((p-tolylamino)methyl)-1,3,4-oxadiazole-2(3H) –thione (**6y**)*

Colorless crystal, yield: 51 %, mp: 161.3-161.3 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (dd, 1H, *J* = 8.5 2.00 Hz), 7.27 (d, 1H, *J* = 2.0 Hz), 7.05 (d, 1H, *J* = 8.5 Hz), 6.93 – 6.95 (m, 2H), 6.78 (d, 2H, *J* = 8.4 Hz), 5.43 (d, 2H, *J* = 7.4 Hz), 4.28 – 4.34 (m, 4H), 2.14 (s, 3H). MS (ESI): 356.10 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 60.83; H, 4.82; N, 11.82; Found: C, 60.73; H, 4.55; N, 11.88.

*4.2.26. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((2-methoxyphenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6z**)*

Light yellow powder, yield: 44 %, mp: 156.3-157.5 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (dd, 1H, *J* = 8.3 2.0 Hz), 7.27 (d, 1H, *J* = 2.0 Hz), 6.96 (dd, 1H, *J* = 7.9 1.1 Hz), 6.87 (d, 1H, *J* = 7.9 Hz), 6.79 (t, 1H, *J* = 7.6 Hz), 6.68 (td, 1H, *J* = 7.7 1.2 Hz), 6.37 (t, 1H, *J* = 7.4 Hz), 5.50 (d, 2H, *J* = 7.3 Hz), 4.30 – 4.40 (m, 4H), 3.80 (s, 3H). MS (ESI): 372.09 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 58.21; H, 4.61; N, 11.31; Found: C, 58.29; H, 4.72; N, 11.11.

*4.2.27. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((4-methoxyphenyl)amino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6ab**)*

White powder, yield: 80 %, mp: 191.0-191.1 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.28 – 7.37 (m, 2H), 7.06 (d, 1H, *J* = 8.5 Hz), 6.96 – 6.97 (m, 1H), 6.74 – 6.84 (m, 3H), 5.42 (d, 2H, *J* = 7.4 Hz), 4.32 – 4.35 (m, 4H), 3.64 (s, 3H). MS (ESI): 372.09 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 58.21; H, 4.61; N, 11.31; Found: C, 58.03; H, 4.69; N, 11.27.

4.2.28. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-(((2-ethoxyphenyl)amino)methyl)-1,3,4-

*oxadiazole-2(3H)-thione (**6ac**)*

White powder, yield: 79 %, mp: 178.2-179.0 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (dd, 1H, *J* = 8.4 2.0 Hz), 7.27 (d, 1H, *J* = 2.0 Hz), 6.96 (d, 1H, *J* = 8.5 1.1 Hz), 6.96 (d, 1H, *J* = 7.8 Hz), 6.85 (d, 1H, *J* = 7.5 Hz), 6.78 (t, 1H, *J* = 7.7 1.24 Hz), 6.30 (t, 1H, *J* = 7.4 Hz), 5.51 (d, 2H, *J* = 7.3 Hz), 4.30 – 4.34 (m, 4H), 4.03 (dd, 2H, *J* = 13.7 7.0 Hz), 1.38 (t, 3H, *J* = 6.9 Hz). MS (ESI): 386.11 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). MS (ESI): 386.11 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 59.21; H, 4.97; N, 10.90; Found: C, 59.11; H, 4.33; N, 10.34

*4.2.29. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-((hexadecylamino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6ad**)*

White powder, yield: 90 %, mp: 129.9-130.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.41 (m, 2H), 6.89 – 6.92 (m, 1H), 5.47 (s, 2H), 4.29 – 4.33 (m, 6H), 1.19 – 1.79 (m, 8H). MS (ESI): 348.13 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 58.77; H, 6.09; N, 12.09; Found: C, 58.43; H, 6.02; N, 12.20.

*4.2.30. 5-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-3-((hexadecylamino)methyl)-1,3,4-oxadiazole-2(3H)-thione (**6ae**)*

White powder, yield: 89 %, mp: 131.0-131.0 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.39 (m, 2H), 6.92 (d, 1H, *J* = 8.3 Hz), 5.41 (s, 2H), 4.33 – 4.37 (m, 6H), 3.09 (t, 1H, *J* = 7.2 Hz), 1.66 (s, 2H), 1.34(s, 26H), 0.88(t, 3H, *J* = 2.1 Hz). MS (ESI): 490.30 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). MS (ESI): 490.30 ($C_{18}H_{17}N_3O_3S$, $[M+H]^+$). Anal. Calcd. For $C_{18}H_{17}N_3O_3S$: C, 66.22; H, 8.85; N, 8.58; Found: C, 66.21; H, 8.80; N, 8.64

*4.3. Procedure for synthesis of the compounds **7***

Be similar with synthesis of compound **4**, benzoic acid was used as starting reactant, followed

by esterification, hydrazintion and cyclization reaction to get compound **7**.

4.4 Crystal structure determination

X-ray single-crystal diffraction data for compound **6b** were collected on a Bruker SMART APEX CCD equipped with graphitemonochromated MoKα (λ = 0.71073 Å) radiation. The structure was solved by direct methods and refined on F^2 by full-matrix least-squares methods using SHELX-97.[34] All the non-hydrogen atoms were refined anisotropically. All the hydrogen atoms were placed in calculated positions and were assigned fixed isotropic thermal parameters at 1.2 times the equivalent isotropic U of the atoms to which they are attached and allowed to ride on their respective parent atoms. The contributions of these hydrogen atoms were included in the structure-factors calculations. The crystal data and refinement parameter for compound **6b** are listed in Table S1 in supplementary material.

4.5. In vitro antioxidant activity assay

4.5.1 DPPH scavenging capacity assay

The scavenging rate of DPPH radical was carried out by the improved protocol of previously reported method by Skrede *et al*.[35] In brief, in 96-well microliter plates, appropriately serial dilution (100, 50, 25, 12.5, 6.25, 3.125 μg/mL, respectively) of each test compound (100 μL) was mixed with DPPH reagent (100 μL, 0.2 mmol/L DPPH in ethanol). Ethanol was used as a blank experiment while BHT was assayed as controls. The absorbance of the mixture was read at 515 nm after 0.5 h at 25 °C using microplate reader infinite M200 (Tecan, Switzerland). Antioxidant capacity was evaluated as IC_{50} (sample concentration that produced 50% scavenging of the DPPH radical).

4.5.2 ABTS scavenging capacity assay

The ABTS radical-scavenging capacity assay of each test compound was conducted according to the previous method reported by Re *et al*.[27] Briefly, $K_2S_2O_8$ (88 μL, 140 mmol/L) was mixed with ABTS [5 mL, 7 mmol/L in PBS (pH 7.4)]. The mixture was stored in the dark for 12-16 h and the resulting ABTS radical solution was adjusted to an absorbance of 0.700 ± 0.020 at 734 nm with the addition of PBS. Diluted sample solutions (20 μL) were mixed with ABTS radical solution (180 μL). The mixture was reacted at 37 °C under restricted light for 6 min. The decrease of absorbance at 734 nm was recorded and the results were evaluated as IC_{50} (The amount of samples required to decrease the ABTS radical concentration by 50%).

4.5.3 FRAP scavenging capacity assay

The FRAP assay was performed according to the previous procedure reported by Benzie and Strain.[36] Specifically, the same dilution described in DPPH assay (20 μL) were mixed with the freshly prepared ferric-tripyridyltriazine (TPTZ) reagent [180 μL, a mixture of 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tris(2-pyridyl)-s-triazine in 40 mmol/L HCl, and 20 mmol/L $FeCl_3$ (v/v/v: 10/1/1)]. The absorbance of the reaction mixture was measured at 593 nm after 4 min. The standard curve was established using the ferrous sulfate solutions (range 0.1-1.0 mmol/L with n = 6 concentrations), and the results were expressed as $C_{0.5FRAP}$ (the concentration of samples with the antioxidant capacity equivalent to that of ferrous sulfate at 0.5 mmol/L).

4.5.4. Inhibition of Microsomal LPO

Mouse liver microsomes used for the LPO studies were prepared adopting the method of Gao and Wang.[37] The adult male mice (8 - 12 weeks old) of KM strain (purchased from the Comparative Medicine Center of Yangzhou University) were used for the preparation of liver microsomes. The protein content of microsomes was measured using the Bradford method.[38]

Microsomes (0.67 mg protein/mL) were incubated at 37 ºC for 60 min with test compounds of varying concentrations,10 mM $FeSO_4$ and 0.1 mM ascorbic acid in 1.0 mL potassium phosphate buffer solution (0.2 M, pH 7.4). The reaction was stopped by 20 % (w/v) trichloroacetic acid (1.0 mL) and 0.67 % (w/v) 2-thiobarbituric acid (1.5 mL) in succession, and the solution was then heated to 100 ºC for 15 min. After centrifugation of precipitated protein, the color reaction of malondialdehyde (MDA)-TBA complex was detected at 535 nm.

4.5.5. DNA nicking assay for AAPH scavenging activity

The method of AAPH induced DNA breakage in plasmid pBR322 was modified from Jie *et al*.[39]. Briefly, in an Eppendorf tube was conducted at total volume of 25μL containing pBR322DNA (purchased from Shanghai Sangon Biological Company) and AAPH in phosphate buffered solution (PBS: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 mM EDTA) with the final concentrations at 4 μg/mL and 4 mM, respectively. Samples and Trolox (standard) both at 100 μM in DMSO were added to the above mixture. After incubation at 37 ºC for 1 hour, 2 μL of electrophoresis loading buffer (0.25% bromophenol blue and 30% (w/v) glycerol) were added to 10 μL the reaction mixture, and an aliquot (10 μL) was then loaded onto a 1% agarose gel and electrophoresed in Tris-acetate-EDTA buffer at 60 V for 100 min. DNA bands (supercoiled and open circular) were stained with ethidium bromide. PBR322 DNA bands were visualized and photographed with a Gel Imaging System (ImageQuant 400, GE) under UV illuminator. One-way ANOVA was used for the comparison of the DNA damage control with the other treatments. A difference was considered statistically significant when $P < 0.05$ (*). Values represent the mean ± SD of triplicate samples.

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25. CCDC-924998 (**6b**) contains the supplementary crystallographic data, which can be obtained

from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Table 1. The *in vitro* antioxidant activity of **6a** - **6ae** in DPPH, ABTS and FRAP methods.

Figure 1. ORTEP drawing of molecular structure of compound **6b**.

Figure 2. The IC_{50} values of microsomal lipid peroxidation activity (μM).

Figure 3. Protection against AAPH-induced pBR322 DNA strand breakage by synthetic compounds.

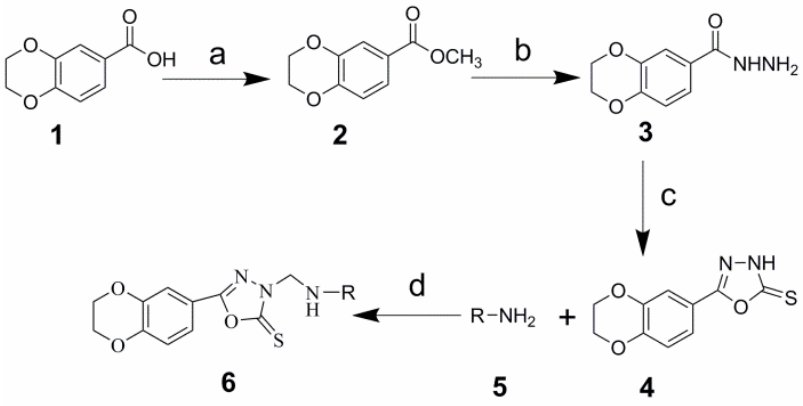
(A) Lanes1: blank, native DNA; Lanes 2-9, DNA, AAPH and test compounds (**6a**, **6e**, **6f**, **6x**, **6t**, **6u**, **6w** and Trolox, respectively).

(B) The relative amount of supercoiled (SC) DNA of test compounds and control.

Scheme 1. General synthesis of compounds (**6a**-**6ae**).

Scheme 2. Synthesis of compound **7**.

Scheme 1. General synthesis of compounds (**6a**-**6ae**).



6a = C_6H_5-

6b = 2-F-C_6H_4-

6c = 3-F-C_6H_4-

6d = 4-F-C_6H_4-

6e = 2,6-2F-C_6H_3-

6f = 3,4,5-3F-C_6H_2-

6g = 2-CF_3-C_6H_4-

6h = 3-CF_3-C_6H_4-

6i = 3,5-2CF_3-C_6H_3-

6j = 2-Cl-C_6H_4-

6k = 3-Cl-C_6H_4-

6l = 4-Cl-C_6H_4-

6m = 2,4-2Cl-C_6H_3-

6n = 2,5-2Cl-C_6H_3-

6o = 3,4-2Cl-C_6H_3-

6p = 2-Br-C_6H_4-

6q = 3-Br-C_6H_4-

6r = 4-Br-C_6H_4-

6s = 2,4-2Br-C_6H_3-

6t = 2-NO_2-C_6H_4-

6u = 3-NO_2-C_6H_4-

6v = 4-NO_2-C_6H_4-

6w = 2-Cl-4-NO_2-C_6H_3-

6x = 2-CH_3-C_6H_4-

6y = 4-CH_3-C_6H_4-

6z = 2-OCH_3-C_6H_4-

6ab = 4-OCH_3-C_6H_4-

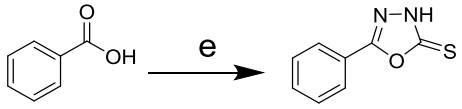
6ac = 2-OCH_2CH_3-C_6H_4-

6ad = -cyclohexane

6ae = -hexadecyl

Reagents and conditions: (a) methanol, concentrated sulfuric acid; reflux, 8-12 h; (b) $NH_2NH_2.H_2O$ (85%), ethanol; reflux, 8–12 h; (c) (1) CS_2/KOH, ethanol (95%), reflux, 24 h; (2) HCl, pH 5–6; (d) HCHO(40%), ethanol, rt, 3 h.

Scheme 2. Synthesis of compound **7**.



7

Reagents and conditions (e): (1) methanol, concentrated sulfuric acid; reflux, 8-12 h; (2) $NH_2NH_2{\cdot}H_2O$ (85%), ethanol; reflux, 8-12 h; (3-i) CS_2/KOH, ethanol (95%), reflux, 24 h; (3-ii) HCl, pH 5-6.

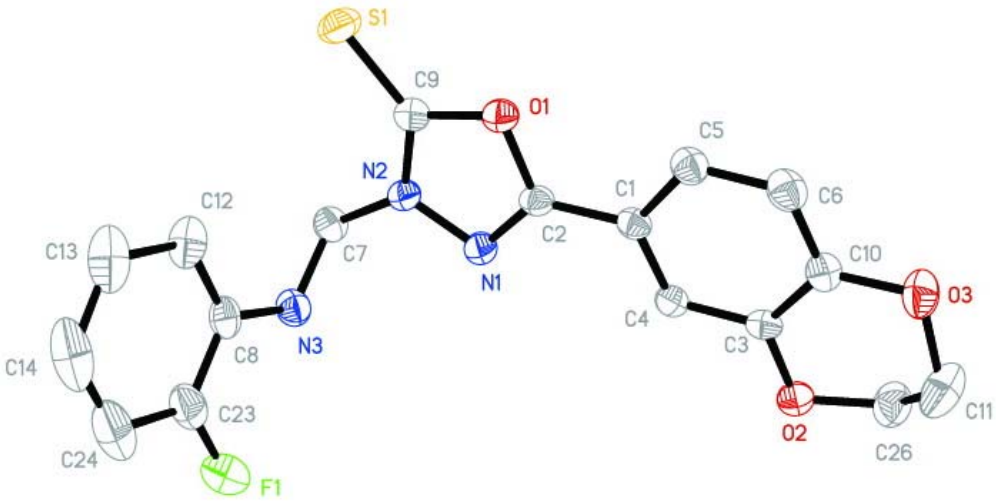


Figure 1. ORTEP drawing of molecular structure of compound **6b**.

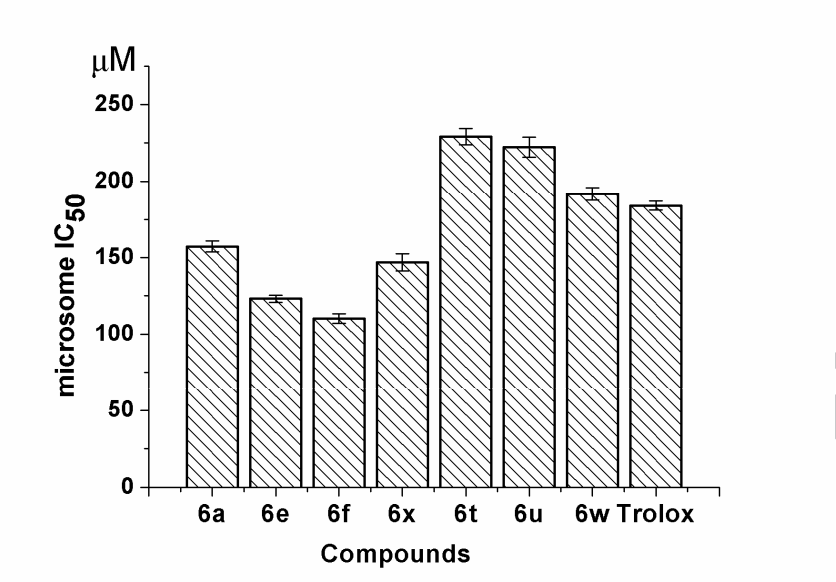
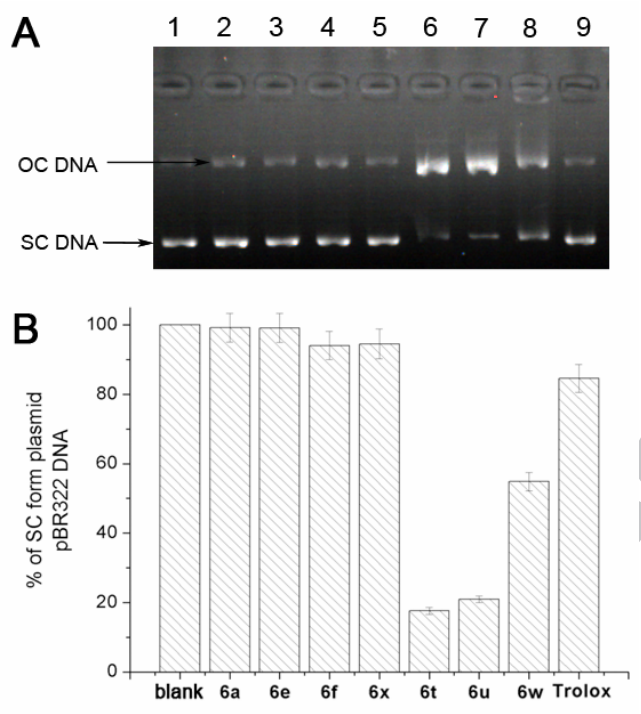


Figure 2. The IC_{50} values of microsomal lipid peroxidation activity (μM).



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(B) The relative amount of supercoiled (SC) DNA of test compounds and control.

Table 1. The *in vitro* antioxidant activity of **6a-6ae** in DPPH, ABTS and FRAP methods.

Test Comp.	DHHP (IC_{50} μM)	ABTS (IC_{50} μM)	FRAP ($C_{0.5FRAP}$ μM)	Test Comp.	DHHP (IC_{50} μM)	ABTS (IC_{50} μM)	FRAP ($C_{0.5FRAP}$ μM)
2	87.42 ± 3.14	13.81 ± 0.53	> 1000	**6p**	36.06 ± 1.74	12.68 ± 0.36	439.8 ± 33.5
4	63.87 ± 2.58	10.62 ± 0.48	611.5 ± 23.2	**6q**	50.96 ± 1.98	10.80 ± 0.56	> 1000
7	89.78 ± 3.01	10.93 ± 0.81	> 1000	**6r**	71.29 ± 1.69	14.66 ± 0.61	531.3 ± 25.4
6a	32.63 ± 1.32	5.14 ± 0.39	735.0 ± 30.0	**6s**	35.92 ± 1.75	6.41 ± 0.49	> 1000
6b	37.29 ± 1.05	6.39 ± 0.67	521.6 ± 29.6	**6t**	47.82 ± 1.62	10.86 ± 0.32	> 1000
6c	35.56 ± 1.38	6.62 ± 0.41	398.0 ± 33.1	**6u**	38.31 ± 1.70	8.87 ± 0.21	> 1000
6d	29.27 ± 1.26	6.48 ± 0.25	300.0 ± 28.1	**6v**	47.82 ± 1.54	9.95 ± 0.43	> 1000
6e	21.80 ± 1.27	3.32 ± 0.43	268.3 ± 17.7	**6w**	83.35 ± 2.37	8.66 ± 0.62	> 1000
6f	19.62 ± 1.83	2.92 ± 0.38	239.7 ± 19.2	**6x**	36.30 ± 1.28	3.45 ± 0.17	291.7 ± 23.1
6g	68.26 ± 2.10	9.22 ± 0.61	> 1000	**6y**	25.17 ± 1.93	5.70 ± 0.28	433.6 ± 29.6
6h	35.52 ± 1.34	7.62 ± 0.58	> 1000	**6z**	28.64 ± 1.44	6.30 ± 0.51	334.7 ± 18.6
6i	89.30 ± 2.52	9.32 ± 0.36	> 1000	**6ab**	31.75 ± 1.57	5.83 ± 0.42	493.0 ± 32.6
6j	30.50 ± 1.52	7.23 ± 0.64	531.7 ± 28.0	**6ac**	22.40 ± 1.01	5.28 ± 0.39	421.7 ± 37.9
6k	22.77 ± 1.63	6.22 ± 0.31	845.9 ± 35.1	**6ad**	94.40 ± 1.69	18.41 ± 0.79	> 1000
6l	31.30 ± 1.12	7.27 ± 0.52	584.3 ± 41.9	**6ae**	107.57 ± 1.72	22.90 ± 0.90	> 1000
6m	43.45 ± 1.42	8.71 ± 0.40	592.7 ± 48.6	BHT	43.84 ± 0.93	7.57 ± 0.09	546.2 ± 13.5
6n	35.06 ± 1.19	5.42 ± 0.29	899.3 ± 38.6	Trolox	30.20 ± 0.80	6.19 ± 0.13	293.7 ± 9.8
6o	36.21 ± 1.53	6.84 ± 0.31	763.1 ± 38.1				

Values are expressed as mean ± SD deviation of three replicate assays in each *in vitro* experiment.

Graphical abstract