Diarylheptanoids and a Monoterpenoid from the Rhizomes of *Zingiber officinale*: Antioxidant and Cytoprotective Properties

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Three new diarylheptanoids and one new monoterpenoid were isolated from the rhizomes of *Zingiber officinale* together with four known diarylheptanoids, **5**–**8**. Their structures were elucidated mainly by spectroscopic methods, and they were deduced as 5-[4-hydroxy-6-(4-hydroxyphenethyl)tetrahydro-2*H*-pyran-2-yl]-3-methoxybenzene-1,2-diol (**1**), sodium (*E*)-7-hydroxy-1,7-bis(4-hydroxyphenyl)hept-5-ene-3*S*-sulfonate (**2**), sodium (*E*)-7-hydroxy-1,7-bis(4-hydroxyphenyl)hept-5-ene-3*R*-sulfonate (**3**), and hydroxycineole-10-*O*-*β*-D-glucopyranoside (**4**), respectively. Among the isolated compounds, compounds **1**, **5**, and **8** exhibited strong superoxide anion radical scavenging activities in a phenazine methosulfate−NADH system. In a more biological system, these compounds were demonstrated to exhibit potent protection against lipid peroxidation in mouse liver microsomes exposed to oxidative conditions. These compounds were subsequently tested on primary cultures of rat hepatocytes exposed to oxidative damage, and definitive cytoprotective actions were found.

Ginger, the rhizomes of *Zingiber officinale* Roscoe (Zingiberaceae), has a long history of use in China for its tonic actions. It is frequently listed in prescriptions of traditional Chinese medicine for the treatment of rheumatism, vomiting, and duodenum and gastric ulcers.[1] Previous investigations have led to the characterization of a number of bioactive principles from this species[2] including the diarylheptanoids, exhibiting a variety of biological activities including inhibition of the biosynthesis of prostaglandins and leukotrienes[3] as well as antifungal,[4] antioxidative,[5] and cancer chemopreventive activities.[6] Chemical studies of this plant species have led to the isolation and identification of numerous biologically active compounds such as gingerols, gingerones, and shogaols.[7–12] In the course of searching for structurally novel compounds from Zingiberaceae species, three new diarylheptanoids (**1**–**3**), one new monoterpenoid (**4**), and four known diarylheptanoids (**5**–**8**) were isolated from the MeOH extract of *Z. officinale* collected in Yunnan Province, Southwestern China (Figure 1). The isolation and the structure determination of the new compounds (**1**–**4**) are reported herein.

Furthermore, the antioxidant activities of the isolated diarylheptanoids (**1**, **5**, **8**) available in sufficient quantities were evaluated by the *in vitro* models of scavenging superoxide anion radicals and inhibiting the formation of lipid peroxides in liver microsomes. To investigate the antioxidant properties of these compounds under more physiological conditions, the compounds were further tested in a primary culture of hepatocytes isolated from rat liver against the oxidative damage of *tert*-butyl hydroperoxide. The diarylheptanoids were found to possess potent antioxidant properties in all the above assays. On the other hand, these compounds did not possess any appreciable cytotoxiciy on KB cells and on rat liver hepatocytes.

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Results and Discussion

Compound **1** was obtained as a yellowish oil. Its ^{1}H NMR spectrum displayed two doublets at *δ* 7.01 (2H, d, *J* = 8.4 Hz, H-2″ and H-6″) and 6.72 (2H, d, *J* = 8.4 Hz, H-3″ and H-5″) as well as two *meta*-coupled aromatic protons at *δ* 6.57 (1H, d, *J* = 1.5 Hz, H-4) and 6.55 (1H, d, *J* = 1.5 Hz, H-6), indicating the presence of a 1,4-disubstituted phenyl moiety and a 1,2,3,5-tetrasubstituted benzene ring, respectively. This observation, along with three oxygenated methine protons at *δ* 4.21 (1H, dd, *J* = 11.2, 1.4 Hz, H-2′), 3.90 (1H, dddd, *J* = 5.0, 5.0, 12.0, and 12.0 Hz, H-4′), and 3.52 (m, H-6′) disclosed that **1** was most likely a cyclic diarylheptanoid.[10] This deduction was supported by its ^{13}C NMR and DEPT data (Table 1). In the ^{13}C NMR spectrum of **1**, the two oxygenated aromatic rings were evidenced from the resonances at *δ* 156.2, 148.6, 146.0, 135.1, 133.8 (×2), 130.1 (×2), 115.9 (×2), 107.4, and 102.1 ppm (Table 1).

Furthermore, the six-membered cyclic ether, a pyrane skeleton, was confirmed by the five aliphatic carbon resonances at *δ* 78.1, 75.4, 68.4, 44.4, and 42.1 (Table 1). In addition, the presence of two methylenes and a methoxy group attached to the aromatic ring was substantiated by resonances at *δ* 39.1, 31.5, and 56.4 (Table 1). These features showed close resemblance to those of 5-[4-hydroxy-6-(3-methoxy-4-hydroxyphenethyl)tetrahydro-2*H*-pyran-2-yl]-3-methoxybenzene-1,2-diol, particularly for the striking similarity of the 3-methoxybenzene-1,2-diol and the six-membered cyclic ether moieties.[10] Thus, compound **1** was determined to be 5-[4-hydroxy-6-(4-hydroxyphenethyl)tetrahydro-2*H*-pyran-2-yl]-3-methoxybenzene-1,2-diol, which was further confirmed by its HRESIMS spectrum, giving an $[M + H]^+$ ion at *m/z* 361.1647, corresponding to a molecular formula of $C_{20}H_{24}O_6$ (calc for $[M + H]^+$: 361.1651). The 4′-hydroxy group was shown to be in an equatorial orientation by both the coupling constants of H-4′ in the ^{1}H NMR and the chemical shift value of C-4′ (Table 1) in the ^{13}C NMR spectrum.[10] The ^{1}H–^{1}H COSY measurement of **1** allowed the assignment of the aliphatic protons, shown by bold lines in Figure 2. In addition, the NOESY experiment of **1** indicated the correlations of H-6′ (*δ* 3.53, m) with H-2′ and H-4′, thus suggesting the axial orientation of H-6′. Meanwhile, the W-type H−H long-range coupling displayed between H-3′eq (*δ* 2.07) and H-5′eq (*δ*

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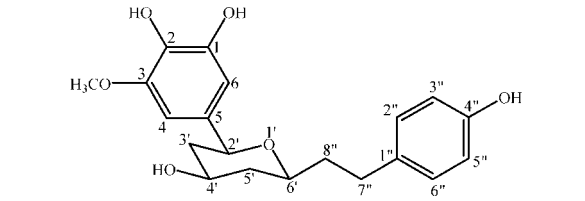

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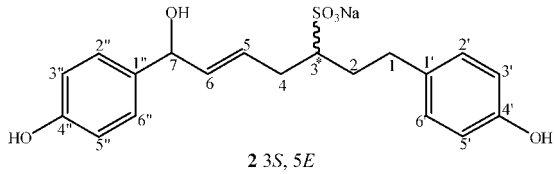
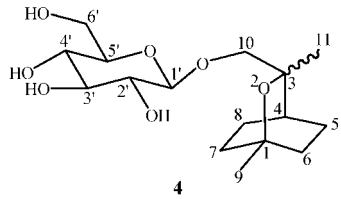


2 3*S*, 5*E*

3 3*R*, 5*E*



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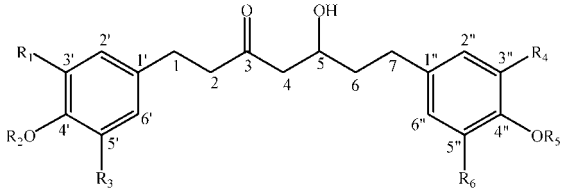

	R_1	R_2	R_3	R_4	R_5	R_6
5	OCH_3	H	H	OH	H	H
6	OCH_3	H	H	OH	H	OCH_3
7	OH	H	OCH_3	OCH_3	H	H

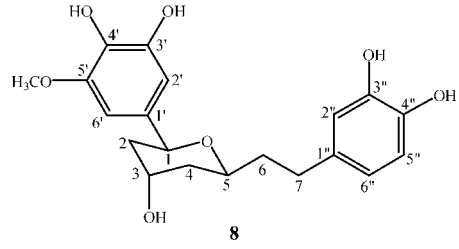

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Figure 1. Compounds isolated from the rhizomes of *Z. officinale*.

1.94) confirmed the chair form of the six-membered ring. Furthermore, the observed HMBC correlations demonstrated with curved arrows in Figure 2 are also in agreement with the elucidated structure of **1**. The correlations between H-2′ and C-4, H-3′ and C-5, and H-7″ and C-2″/C-6″ suggested that the 3-methoxybenzene-1,2-diol and 4-hydroxyphenyl groups should be attached to C-2′ and C-7″, respectively. Furthermore, the presence of a stable fragment ion peak at *m/z* 107 $[CH_2C_6H_4(OH)]^+$ supported the attachment of a 4-hydroxyphenyl group to C-7″. On the basis of these data, the structure of compound **1** was confirmed.

Compounds **2** and **3** were obtained as a mixture, in a yellow amorphous powder form, and could not be further separated even by HPLC. Both the ESIMS [*m/z* 377 ($[M - Na]^-$)] and HRESIMS data [*m/z* 423.0849 ($[M + Na]^+$, $C_{19}H_{21}O_6Na_2S$)] of **2** and **3** revealed the molecular formula as $C_{19}H_{21}O_6SNa$. The ^1H and ^{13}C NMR spectra (Table 1) of **2** and **3** demonstrated similar resonances due to diarylheptanoid moieties with nearly identical intensities except for those of H-3 and H-1. The ^1H and ^{13}C NMR spectra of **2** and **3** also indicated the presence of a *trans*-olefinic moiety [δ 6.08 (1H, dd, J = 15.2, 8.8 Hz, H-6), 5.73 (1H, m, H-5)], two *para*-substituted aromatic rings [δ 6.99, 6.69 (2H each, both d, J = 8.4 Hz, H-2″,6″ and H-3″,5″, respectively), δ 7.31, 6.77 (2H each, both d, J = 8.8 Hz, H-2′,6′ and H-3′,5′, respectively)], one oxygen-bearing methine [δ 4.52 (1H, d, J = 8.8 Hz)], and the other methine substituted by a sodium sulfonate group [δ 3.62 (1H, m)]. This observation is in agreement with the molecular formula, which revealed that **2** and **3** are a pair of both hydroxylated and sodium sulfonate derivatives of a diarylheptanoid. Furthermore, the spectroscopic data disclosed that the pair of sodium sulfonate diarylheptanoid isomers (**2** and **3**) possess partially similar structural characteristics to those of shogasulfonic acids, while they were also partially similar to those of (1*S*,5*R*,2*E*)-1,7-diphenylhept-2-ene-1,5-diol.[13,14] As shown by the HMBC experiment (Figure 2), long-range correlations were observed between the proton and carbon pairs of **2** and **3**, e.g., H-6/C-4, C-1″, H-7/C-2″, C-5, H-3/C-5, C-1, H-1/C-2′. All of the data including the diagnostic olefinic chemical shifts of C-6 and C-5 evidenced this pair of isomers containing an allyl alcohol moiety and a nonconjugated sulfonyl group. Taking into account the fact that the sulfonyl functionality usually appears at C-3 for diarylheptanoids (e.g., shogasulfonic acids), it would be logical to assume that the attachment of the sulfonyl group of **2** and **3** should be at C-3, which appeared at δ 71.3 and 71.4, respectively (Table 1). The postulated biogenetic pathway of this pair of enantiomers is illustrated in Figure 3. The pair of diarylheptanoid enantiomers was therefore identified as sodium (*E*)-7-hydroxy-1,7-bis(4-hydroxyphenyl)hept-5-ene-3*S*-sulfonate (**2**) and sodium (*E*)-7-hydroxy-1,7-bis(4-hydroxyphenyl)hept-5-ene-3 *R*-sulfonate (**3**).

The HRESIMS spectrum of compound **4** exhibited an $[M + Na]^+$ ion peak at *m/z* 355.1719, corresponding to the molecular formula $C_{16}H_{28}O_7$. Its NMR data exhibited close similarities to those of the monoterpene hydroxycineole.[15,16] The C-10 resonance of **4** was downfield shifted to 6.2 ppm, while the C-3 resonance of **4** was upfield shifted to 1.5 ppm when compared to the ^{13}C NMR data of hydroxycineole. This suggested that the glycosidation of **4** occurred at C-10. Furthermore, the ^1H NMR and ^{13}C NMR data as well as the acid hydrolysis and TLC characteristics of **4** revealed that the sugar moiety is a β-glucopyranosyl functionality. The optical rotation measurement of the sugar part indicated the presence of a D-glucose in the case of **4**. The results of the HMBC study (Figure 2) were in agreement with the deduction that **4** is a C-10 glucoside of hydroxycineole. Accordingly, **4** was characterized as hydroxycineole-10-*O*-β-D-glucopyranoside.

The structures of the known compounds were identified by comparing their ^1H NMR, ^{13}C NMR, and MS data with those reported: 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)heptan-3-one (**5**),[17] 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(4,5-dihydroxy-3-methoxyphenyl)heptan-3-one (**6**),[11] 5-hydroxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-dihydroxy-3-methoxyphenyl)heptan-3-one (**7**),[11] and 1,5-epoxy-3-hydroxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)heptane (**8**).[12]

The *in vitro* cytotoxicities of all the above compounds against KB cells were tested using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) colorimetric assay[18] with etoposide (VP-16) as a positive control. None of the isolated natural products showed significant cytotoxicities. The cytotoxic effects against hepatocytes were also checked by incubating a primary culture of rat hepatocytes with the compounds up to 100 μM for 48 h, and no cytotoxicities were observed.

Detailed biological evaluation of the isolated compounds could be performed only on compounds **1**, **5** and **8** but not on others because of the limited yields. The abilities of compounds **1**, **5** and **8** to scavenge superoxide anion radicals were studied in a phenazine methosulfate−NADH system by following the reduction of nitroblue tetrazolium.[19] The inhibitory actions of these compounds on free radical-induced lipid peroxidation in mouse liver microsomes were also evaluated.[20] Compounds **1**, **5**, and **8** were

Table 1. NMR Data for Compounds **1**–**3** [400 MHz (^{1}H) and 100 MHz (^{13}C)]

	1[a]		**2**[b]	**3**[b]	**2**, **3**[b]
position	δ_C	δ_H (mult, J in Hz)	δ_C[c]	δ_C[c]	δ_H (mult, J in Hz)
1	148.6		115.9	115.9	6.69 (d, $J = 8.4$)
2	133.8		156.2	156.2	
3	146.0		115.9	115.9	6.69 (d, $J = 8.4$)
4	107.4	6.57 (d, $J = 1.5$)	130.3	130.3	6.99 (d, $J = 8.4$)
5	135.1		134.4	134.4	
6	102.1	6.55 (d, $J = 1.5$)	130.3	130.3	6.99 (d, $J = 8.4$)
2′	78.1	4.21 (dd, $J = 11.2$, 1.4)	70.6	70.6	4.52 (d, $J = 8.8$)
3′	44.4	1.35 (ddd, $J = 10.0$, 10.0, 12.0), 2.07 (m)	132.5	132.5	6.08 (dd, $J = 15.2$, 8.8)
4′	68.4	3.90 (dddd, $J = 5.0$, 5.0, 12.0, 12.0)	129.8	129.8	5.73 (m)
5′	42.1	1.21 (ddd, $J = 10.0$, 10.0, 12.0), 1.94 (br d, $J = 12.0$)	42.0	41.6	2.29 (m)
6′	75.4	3.53 (m)	71.3	71.4	3.62 (m)
1″	133.8		130.3	130.3	
2″, 6″	130.1	7.01 (d, $J = 8.4$)	131.4	131.4	7.31 (d, $J = 8.8$)
3″, 5″	115.9	6.72 (d, $J = 8.4$)	116.0	116.0	6.77 (d, $J = 8.8$)
4″	156.2		157.7	157.7	
7″	31.5	2.65 (m)	31.9	32.0	2.55 (m), 2.66 (m)
8″	39.1	1.71 (m), 1.82 (m)	39.9	39.9	1.64 (m)
OMe	56.4	3.79 (s)			

[a] Measured in CD_3COCD_3. [b] Measured in CD_3OD. [c] Assignments for each carbon of the **2** and **3** are interchangeable.

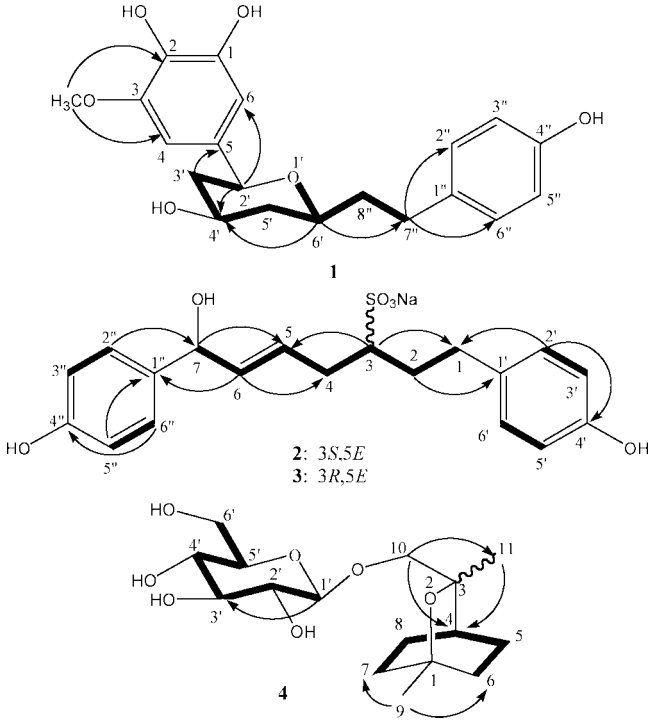

Figure 2. Structures and key ^{1}H–^{1}H COSY (bold lines) and selected HMBC correlations (arrows) of compounds **1**–**4**.

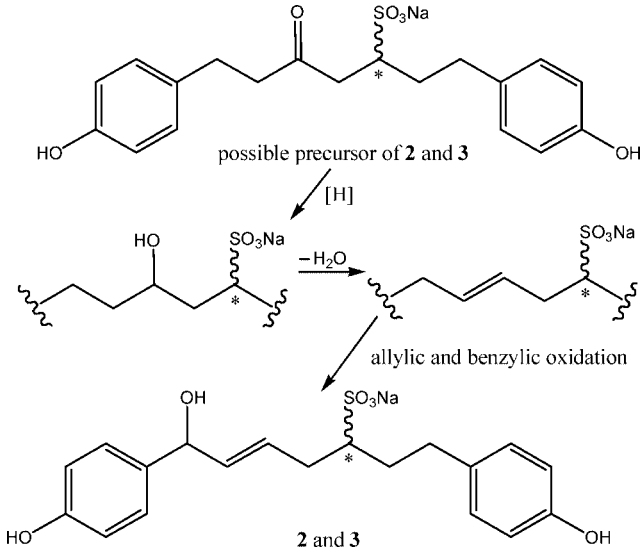

Figure 3. Possible biosynthetic pathway to compounds **2** and **3**.

capable of scavenging superoxide anion radicals and inhibiting formation of lipid peroxides in the liver microsomes.

As indicated in Figure 4, compounds **1**, **5**, and **8** were capable of scavenging superoxide anion radicals in the *in vitro* assay. Superoxide anion radicals generated by a phenazine methosulfate−NADH system were effectively removed in a dose-dependent manner by the compounds. The amount of the reduced nitroblue tetrazolium produced was thus decreased, giving rise to a diminished absorbance at 560 nm. As shown in Figure 4, the IC_{50} values were 48.14, 36.35, and 39.89 μM for compounds **1**, **5**, and **8**, respectively. It can be seen that compounds **5** and **8** were of similar potency in this assay, whereas compound **1** is less potent. The antioxidant properties of the compounds were thus further evaluated in another system in which free radical-induced lipid peroxidation of liver microsomes was employed. As shown in Figure 5, all three compounds were capable of inhibiting the formation of lipid peroxides in the liver microsomes. As shown in Figure 5, the IC_{50} values were 27.90, 17.08, and 21.86 μM for compounds **1**, **5**, and **8**, respectively. Among the three compounds, compound **5** is clearly the most potent in this assay. Also, both compounds **1** and **8** afforded complete protection at 40 μM, whereas a lower concentration of 20 μM gave complete protection by compound **5**.

In order to investigate the antioxidant properties of these compounds in a more physiological system, the compounds were tested in a primary culture of rat hepatocytes against the oxidative damage of *tert*-butyl hydroperoxide. *tert*-Butyl hydroperoxide, an analogue of lipid peroxide, is widely used to induce oxidative stress in rat hepatocytes.[21–23] It is metabolized by the microsomal cytochrome P450 system to generate reactive oxygen species,[24,25] which subsequently initiate lipid peroxidation[26] and deplete cellular reduced glutathione levels.[27] As a result, the cells are damaged, leading to the leakage of cellular contents into the culture medium. The amount of the housekeeping enzyme lactate dehydrogenase in the culture medium is generally used to indicate the extent of cellular damage.[28] As shown in Figure 6, the oxidative stress induced by *tert*-butyl hydroperoxide caused a 100% increase in the enzyme leakage. Pretreatment of the primary hepatocytes in culture with the compounds for 24 h significantly protected the cells from oxidative damage in a dose-dependent manner. However, it is only at 100 μM of the compounds that statistically significant protective effects could be observed. The effectiveness of the pretreatment

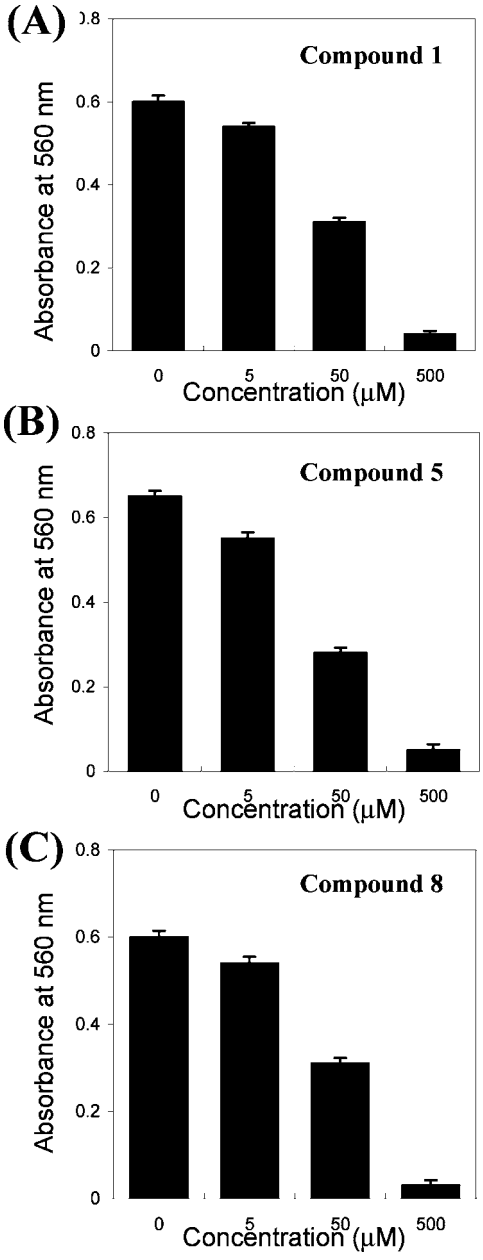

Figure 4. Superoxide anion radical scavenging activities of compounds **1**, **5**, and **8**. Data represent mean ± SEM ($n = 4$).

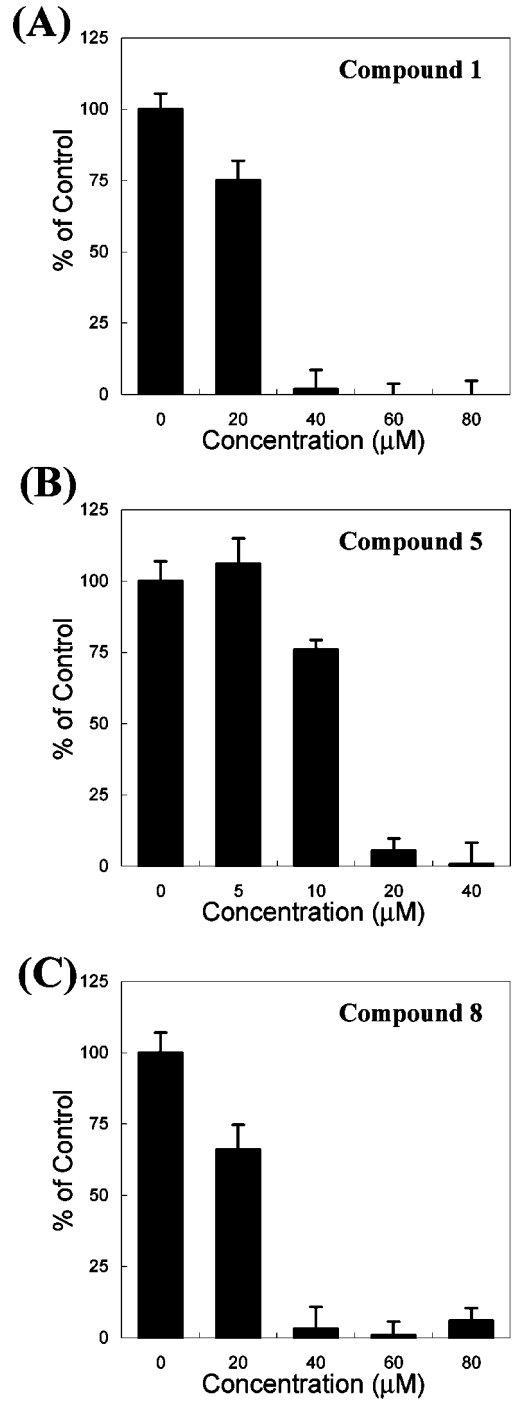

Figure 5. Protection against lipid peroxidation in mouse liver microsomes by compounds **1**, **5**, and **8**. Data represent mean ± SEM ($n = 4$).

protocol and the subsequent removal of the compounds prior to the oxidative stress indicated that the compounds must have accumulated inside the hepatocytes to exert their protective effects intracellularly, rather than extracellularly by reacting with the *tert*-butyl hydroperoxide in the culture medium. In this assay system, compounds **1** and **8** were again shown to be rather similar in potency, whereas compound **5** was more active. At a concentration of 100 μM, compound **5** could afford full protection of the hepatocytes, while compound **1** and compound **8** could provide only partial protection at this concentration. Taken together with the *in vitro* antioxidant activities of the compounds, the protective actions afforded by the compounds on the cultured hepatocytes are most likely mediated through effective removal of the reactive oxygen species formed.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Polax-2L polarimeter. Melting points were obtained on an X-4 digital melting point instrument and are uncorrected. IR spectra were recorded as KBr disks on a Bruker Vector-22 spectrometer. 1D and 2D NMR spectra were obtained at 400 and 100 MHz for ^{1}H and ^{13}C, respectively, on an INOVA NMR spectrometer with TMS as internal standard. MS data were measured on a Bruker Esquire 3000+ instrument, and HRESIMS were recorded on a Bruker Bio Apex 70 eV FT-ICR (Bruker Daltronics). Column chromatography was performed with Si gel (200–300 mesh), Sephadex LH-20, and RP-18. TLC was carried out with glass precoated Si gel GF_{254} plates. Spots were visualized under UV light or by spraying with 5% H_2SO_4 in EtOH followed by heating.

Plant Material. The rhizomes of *Z. officinale* were obtained from Yunnan Province, China, in March 2000. The plant was authenticated by Dr. Hua Peng at the Kunming Institute of Botany, Chinese Academy

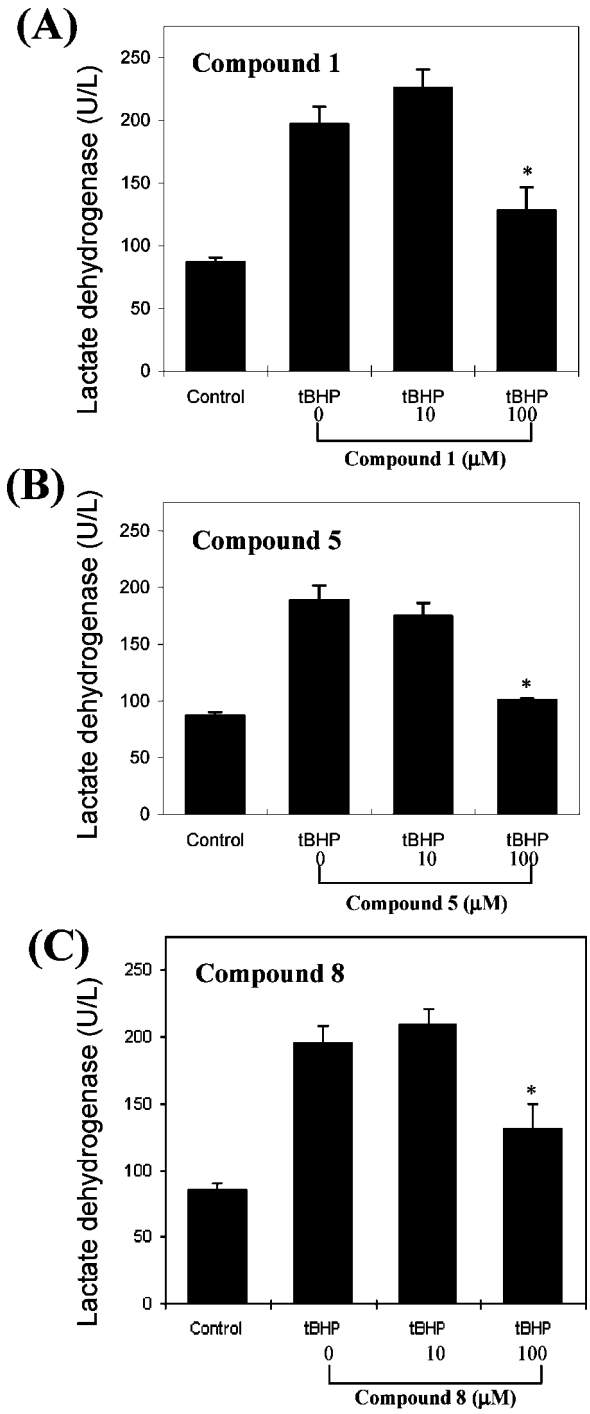

Figure 6. Protection of primary rat hepatocytes against *tert*-butyl hydroperoxide (*t*BHP)-induced oxidative stress by compounds **1**, **5**, and **8**. Data represent mean ± SEM ($n = 4$). Statistical analysis of the data by ANOVA indicates a P value of <0.01 (*) when compared to the control.

of Sciences, and a voucher specimen (No. 20-03002) has been deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. Dried and powdered rhizomes of *Z. officinale* (5.5 kg) were extracted with MeOH (×4) using an ultrasonic apparatus for 3 h, and the solvent was removed under reduced pressure. The residue was suspended in H_2O and partitioned with petroleum ether, EtOAc, and *n*-BuOH successively. The EtOAc extract was subjected to column chromatography on Si gel and eluted with a petroleum ether−acetone gradient (10:1 → 0:1) followed by an acetone−MeOH gradient (10:1 → 0:1). According to TLC monitoring, six fractions were collected (F-1 to F-6). Column chromatography of F-4 over Si gel with a CH_2Cl_2−Me_2CO gradient (50:1 → 10:1) followed by repeated gel filtration on Sephadex LH-20 eluted by $CHCl_3$−MeOH (1:1) yielded **1** (32 mg), **5** (26 mg), and **8** (28 mg). F-5 was rechromatographed over a Si gel column using stepwise elution with $CHCl_3$−MeOH (100:1 → 5:1) and purified by gel filtration over Sephadex LH-20 eluted with $CHCl_3$−MeOH (1:1) to afford **8** (7 mg), **6** (11 mg), and **7** (10 mg). The *n*-BuOH-soluble portion was subjected to repeated column chromatography on RP-18 and Sephadex LH-20 to yield mixtures of **2** and **3** (26 mg) and **4** (19 mg).

Table 2. NMR Data for Compound **4** [400 MHz (^{1}H) and 100 MHz (^{13}C) in CD_3OD]

position	δ_C	δ_H (mult, J in Hz)
1	71.5	
3	77.1	
4	30.5	1.62 (m)
5	23.2	2.05 (m)
6	32.7	1.62 (m)
7	32.9	1.65 (m)
8	23.2	2.05 (m)
9	27.5	1.00 (s)
10	76.4	3.31 (d, $J = 10.0$), 3.91 (d, $J = 10.0$)
11	24.3	1.28 (s)
1′	104.9	4.23 (d, $J = 7.3$)
2′	75.0	} 3.2−3.4 (m)
3′	77.9	
4′	71.8	
5′	77.9	
6′	62.6	3.64 (m), 3.82 (dd, $J = 7.6, 12.9$)

1,5-Epoxy-1-(4,5-dihydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)heptan-3-ol (1): yellowish oil; $[\alpha]^{25}_D$ –32.5 (*c* 0.75, MeOH); ^{1}H NMR (Me_2CO-d_6, 400 MHz) and ^{13}C NMR (Me_2CO-d_6, 100 MHz), see Table 1; FABMS *m/z*361 $[M + H]^+$; HRESIMS *m/z* 361.1647 $[M + H]^+$ (calcd for $C_{20}H_{24}O_6$, 361.1651).

Sodium (*E*)-7-hydroxy-1,7-bis(4-hydroxyphenyl)hept-5-ene-3*S*-sulfonate (2) and sodium (*E*)-7-hydroxy-1,7-bis(4-hydroxyphenyl) hept-5-ene-3*R*-sulfonate (3): yellow, amorphous powder; $[\alpha]^{25}_D$ –16.4 (*c* 0.8, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 1; negative ESIMS *m/z* 377 $[M - Na]^-$; HRESIMS *m/z* 423.0849 $[M + Na]^+$ (calcd for $C_{19}H_{21}O_6Na_2S$, 423.0854).

Hydroxycineole-10-*O*-*β*-D-glucopyranoside (4): yellow oil; $[\alpha]^{25}_D$ –22.7 (*c* 0.6, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; HRESIMS *m/z* 355.1719 $[M + Na]^+$ (calcd for $C_{16}H_{28}O_7Na$, 355.1733). A solution of **4** (11 mg) in 2 M HCl (3 mL) was heated at 85 °C for 6 h. The neutralization by $NaHCO_3$ was carried out after cooling of the reaction mixture. $CHCl_3$ was used to extract the aglycone, while the co-TLC of the aqueous layer with the authentic sample of *β*-D-glucose (Sigma) was performed with a developing system of $CHCl_3$−MeOH−H_2O−HOAc (7:3:1:1). The water layer was further evaporated under vacuum to afford a residue, which was subsequently purified by column chromatorgraphy ($CHCl_3$−MeOH, 2:3) to give a D-form glucose with the optical rotation of $[\alpha]^{25}_D$ +26.4 (*c* 0.3, H_2O).

Determination of *in Vitro* Radical Scavenging Activity. The ability of the test compounds to scavenge superoxide anion radicals was studied in a phenazine methosulfate−NADH system by following the reduction of nitroblue tetrazolium.[19] In this assay, the superoxide anion radicals were generated in 3 mL of 16 mM Tris-HCl buffer pH 8.0 containing 78 μM NADH, 50 μM nitroblue tetrazolium, 10 μM phenazine methosulfate, and the compounds to be tested at various concentrations. The color reaction between the superoxide anion radicals and nitroblue tetrazolium was monitored at 560 nm. L-Ascorbic acid was used as the positive control for the assay. The ability of the compounds to inhibit free radical-induced lipid peroxidation in a mouse liver microsomal preparation was also examined.[20] Liver microsomes were prepared from male BALB/c mice of approximately 30 g in body weight. An aliquot of the microsome (200–300 μg/mL) was incubated at 37 °C for 60 min in the presence of $FeSO_4$ and 0.1 mM ascorbic acid in 1 mL of 10 mM potassium phosphate buffer pH 7.4, in the absence or presence of the test compounds. The extent of lipid peroxidation was assayed by the amount of malondialdehyde formed. The reaction was stopped by 1 mL trichloroacetic acid (20%), followed by the addition of 1.5 mL of 2-thiobarbituric acid (0.8%). The solution

was then boiled at 100 °C for 20 min and centrifuged at 3000 rpm for 5 min to get rid of the precipitated proteins, and the absorbance of the supernatant was measured at 532 nm. Butylated hydroxyanisole was used as a positive control.

Isolation and Culture of Rat Hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats (body weight ca. 200 g) by a two-stage collagenase liver perfusion *in situ*.[29] Only preparations with cell viability greater than 90%, as determined by the trypan blue exclusion test, were used for subsequent experiments. Cells seeded onto collagen-precoated plates were cultured in William's medium E supplemented with 0.3 μM insulin, 0.1 μM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum at 37 °C under 95% humidity and 5% CO_2. The cells were seeded onto 24-well plates at 2×10^5 cells/well for cell viability studies by monitoring the lactate dehydrogenase leakage.

Lactate Dehydrogenase Assay. After preincubation with the compounds for 24 h, the hepatocytes were washed with Hank's balanced salt solution. Oxidative stress was initiated by incubating the hepatocytes with 400 μM *tert*-butyl hydroperoxide for 3 h. The medium was then assayed for lactate dehydrogenase activity by following the absorbance change at 340 nm in an assay medium containing 1 mM pyruvate, 0.15 mM NADH, and 0.1 M sodium phosphate pH 8.0.[28] The amount of lactate dehydrogenase leaked into the culture medium reflects the extent of cell damage and thus the degree of protection afforded by the compounds.

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