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Phthalide derivatives from *Ligusticum chuanxiong*†

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Eleven new phthalide derivatives (**1–11**) have been isolated from the rhizome of *Ligusticum chuanxiong*. In particular, **1** and **2** contain a mercaptopropionic acid moiety in the phthalide derivatives. All the structures, including their absolute configurations, were determined by UV, IR, HRESIMS, 1D and 2D NMR spectroscopic measurements, and by a comparison of the experimental and calculated electronic circular dichroism (ECD) spectra. Results of a bioassay showed that compound **4** has a moderate neuroprotective activity on human neuroblastoma SH-SY5Y cell injury induced by H_2O_2 and oxygen glucose deprivation (OGD).

Introduction

The rhizome of *Ligusticum chuanxiong* Hort. (syn. *Ligusticum striatum* DC.) (Apiaceae), named Chuanxiong in traditional Chinese medicine, is commonly used for treating atherosclerosis, cardiovascular disease, anemia and hypertension.[1] This plant is mainly distributed in the Sichuan province but also in Yunnan, Guizhou, and Guangxi provinces in China. So far, the phenolic acids, alkaloids, and phthalides[2–16] contained in it have been investigated. Among these compounds, tetramethylpyrazine and ferulic acid were considered to be the active constituents.[17–24] The phthalides from *L. chuanxiong* have attracted particular attention due to their vasodilative,[17,25,26] neuroprotective,[27] antiproliferative,[28,29] anti-inflammatory[24] and antibacterial effects.[30] In particular, *Z*-ligustilide, a phthalide isolated from *L. chuanxiong*, displays various pharmacological activities including neuroprotective, anti-inflammatory, antiproliferative and vasorelaxation effects.[31–34] It is considered a major bioactive compound relevant to the therapeutic effects and has attracted great interest in recent years. Overall, more than 40 phthalide lactones have been obtained from the rhizome of *L. chuanxiong*,[35,36] but most of them were isolated from the liposoluble fraction. In consideration of the bioavailability *in vivo*, the phthalide derivatives from the water-soluble extract of this plant deserve to be the primary focus of further studies. In the course of our study, eleven new phthalide derivatives (**1–11**) have been isolated and their structures were elucidated by various spectroscopic methods (Fig. 1). Subsequently, their neuroprotective activities on SH-SY5Y cell injury induced by H_2O_2 and OGD were evaluated at a concentration of 10 μM.

Results and discussion

Compound **1** was obtained as a white amorphous powder, and its molecular formula was established as $C_{15}H_{20}O_7S$ by HRESIMS at *m*/*z* 345.0999 $[M + H]^+$ (calcd 345.1003). The IR spectrum peaks at 3387 cm^{-1}, 1741 cm^{-1} and 1682 cm^{-1} suggest the presence of hydroxy, γ-lactone and diene groups, respectively. In the ^1H NMR spectra (Table 1), a double bond at δ_H 5.48 (1H, t, J = 7.5 Hz, H-8), six methylene resonances at δ_H 2.43 (2H, m, H-4), 1.81 (2H, m, H-5), 2.31 (2H, q, J = 7.5 Hz, H-9), 1.56 (2H, m, H-10), 3.41 (2H, t, J = 6.0 Hz, H-11), 2.89 (1H, dd, J = 7.0, 13.5 Hz, H-1′a), 3.02 (1H, dd, J = 4.5, 13.5 Hz, H-1′b), one methine resonance at δ_H 3.50 (1H, brs, H-7), and two oxygen-methine resonances at δ_H 4.10 (1H, brs, H-6) and 4.17 (1H, dd, J = 4.5, 7.0 Hz, H-2′) were presented. The ^{13}C NMR spectrum (Table 1) shows 15 carbon resonances, including two carboxyl carbons at δ_C 173.9 (C-3′) and 168.1 (C-1), four olefinic carbons, five methylene carbons, a methine carbon resonance at δ_C 41.8 (C-7), an oxygen-methylene carbon resonance at δ_C 60.2 (C-11), and two oxygen-methine resonances at δ_C 67.5 (C-6) and 70.4 (C-2′). In the HMBC experiment (Fig. 2), the correlations from H-8 (δ_H 5.48) to C-3 (δ_C 147.7) and C-3a (δ_C 151.6) suggest the existence of a 1,3-butadiene moiety. Furthermore, it was confirmed to be connected to a 3-hydroxypropyl on the basis of the correlations of the ^1H–^1H COSY experiment (Fig. 2), including those between H-8 and H-9, H-9 and H-10, and H-10 and H-11. In addition, the ^1H–^1H COSY correlations from H-4 to H-5, from H-5 to H-6, and from H-6 to H-7 together with the HMBC correlations from H-6 (δ_H 4.10) to C-7a, from H-7 (δ_H 3.50) to C-7a and C-3a, from H-5 (δ_H 1.81) to C-3a, and from H-4 (δ_H 2.43) to C-3a and C-7a indicate the existence of a cyclohexene moiety. The 2-carboxyl-2-hydroxy-ethylthio moiety on position C-7 was confirmed by the ^1H–^1H COSY correlations

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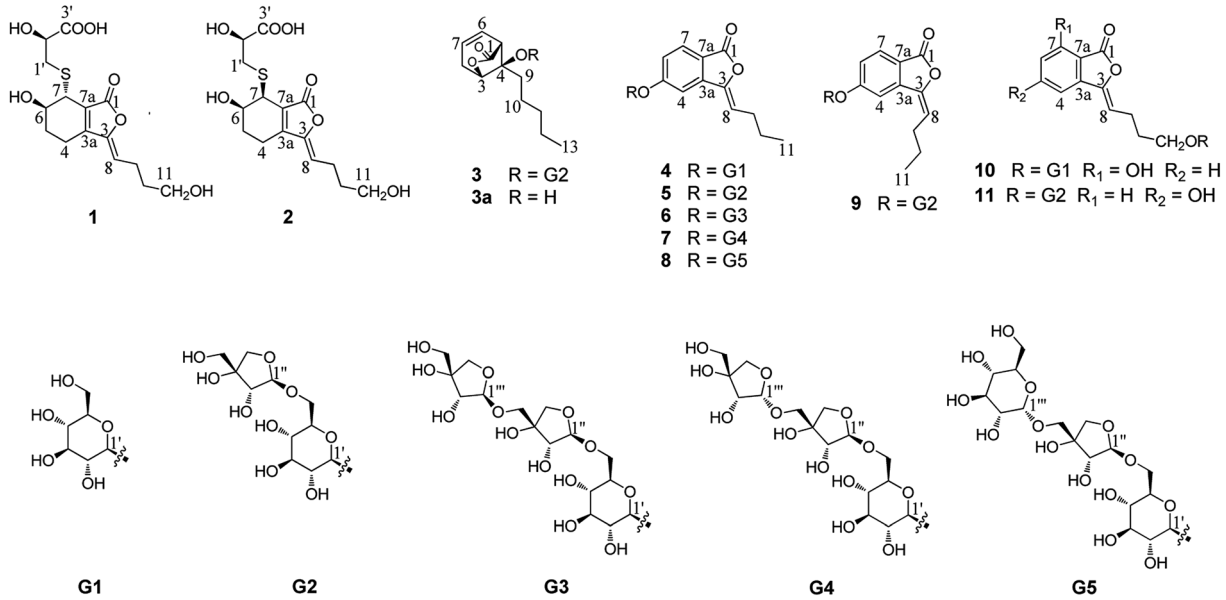

Fig. 1 Chemical structures of **1–11**.

Table 1 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **1–3** in DMSO-d_6

	1		**2**		**3**	
Position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		168.1		167.1		174.7
3		147.7		147.4	4.58, brs	78.9
3a		151.6		150.9		
4	2.43, m	16.1	2.41, m	19.6		84.2
			2.55, m			
5	1.81, m	22.9	1.74, m	25.8	3.21, dd (1.5, 7.5)	47.7
6	4.10, brs	67.5	3.95, dd (3.5, 10.5)	68.2	5.87, m	123.9
7	3.50, brs	41.8	3.81, d (3.5)	43.6	5.77, m	127.9
7a		124.2		126.1		
8	5.48, t (7.5)	112.7	5.42, t (7.5)	112.5	2.46, m	31.3
9	2.31, q (7.5)	22.4	2.29, q (7.5)	22.4	1.67, m	26.1
10	1.56, m	31.8	1.55, m	31.7	1.38, m	21.7
11	3.41, t (6.0)	60.2	3.40, t (6.5)	60.2	1.19, m	31.3
12					1.25, m	21.7
13					0.85, t (7.5)	13.7
1′	2.89, dd (7.0, 13.5)	36.9	2.93, dd (7.0, 13.5)	37.9	4.38, d (8.0)	97.2
	3.02, dd (4.5, 13.5)		3.07, dd (4.5, 13.5)			
2′	4.17, dd (4.5, 7.0)	70.4	4.12, dd (4.5, 7.0)	70.7	2.94, m	73.2
3′		173.9		174.1	3.28, m	75.2
4′					2.99, m	70.1
5′					3.14, m	76.9
6′					3.35, m	67.9
					3.81, dd (1.5, 10.5)	
1″					4.84, d (3.0)	109.4
2″					3.74, brs	75.9
3″						78.6
4″					3.58, d (9.5)	73.4
					3.85, d (9.5)	
5″					3.35, m	63.4

from H-1′ (δ_H 2.89, 3.02) to H-2′ (δ_H 4.17) and the HMBC correlations from H-1′ and H-2′ to the carbonyl carbon at δ_C 173.9 and from H-7 to C-1′ at δ_C 36.9 in consideration of the changes of the chemical shifts of C-7 and C-1′. The HMBC correlation from H-7 to the carbonyl at δ_C 168.1 indicates that C-7a connected with the carbonyl. Thus, the planar structure was

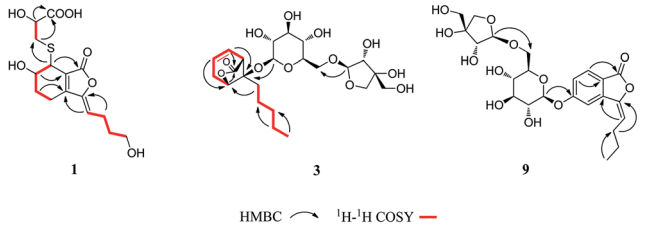

Fig. 2 Key HMBC and ^{1}H–^{1}H COSY correlations of **1**, **3** and **9**.

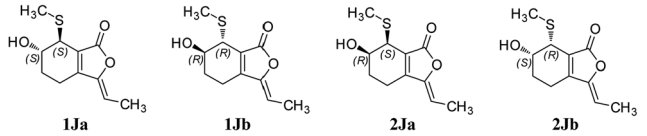

Fig. 4 The structures of **1Ja**, **1Jb**, **2Ja** and **2Jb**.

determined to be 6-hydroxy-7-(2-carboxyl-2-hydroxy-ethylthio)-3-(4-hydroxybutylidene)-4,5,6,7-tetrahydrophthalide. The absolute configuration of C-2′ in **1** was established by a dimolybdenum tetraacetate [$Mo_2(AcO)_4$]-induced circular dichroism procedure.[37] The diagnostic Cotton effect approximately 344.5 nm was negative, so the absolute configuration of C-2′ in **1** was assigned as *S*. In the ROESY spectra (Fig. 3), the correlation between H-8 and H-4 proved the *Z*-configuration of the double bond on position C-3, and the correlation between H-6 and H-1′ suggested that their relative configuration was *trans*. Their absolute configuration was determined by comparing the experimental and calculated ECD data based on the time-dependent density functional theory (TD-DFT) method at the B3LYP/6-31+G (d,p) level. Considering that the variations in the side chain resulted in too many conformations and had little effect on the CD spectrum of the tetrahydrophthalide core in **1**, the selected conformers **1Ja** (6*S*,7*S*) and **1Jb** (6*R*,7*R*) were calculated after optimization (Fig. 4). A comparison of the theoretically calculated and experimental ECD curves (Fig. 5) permitted the assignment of the absolute configuration of **1** as 6*R*,7*R*. Finally, the structure of **1** was determined to be (3*Z*,3a*E*)-(6*R*,7*R*,2′*S*)-6-hydroxy-7-(2-carboxyl-2-hydroxyethylthio)-3-(4-hydroxybutylidene)-4,5,6,7-tetrahydrophthalide, named thiosenkyunolide A.

The HRESIMS of **2** gave the same molecular formula as **1**, $C_{15}H_{20}O_7S$, based on the $[M + Na]^+$ ion peak at *m*/*z* 367.0827 (calcd 367.0822). The ^{1}H and ^{13}C NMR spectra of **2** (Table 1) were closely comparable to those of **1**, with the minor differences occurring in the signals in the cyclohexene moiety, that is, the ^{13}C NMR data were shifted downfield, except that of C-3a (Table 1). Further 2D NMR analysis, including HSQC, HMBC, and ^{1}H–^{1}H COSY experiments, confirmed that compound **2** was the optical isomer of compound **1**. Using the same method, the absolute configuration of C-2′ in **2** was established as *S* by a dimolybdenum tetraacetate [$Mo_2(AcO)_4$]-induced circular dichroism procedure,[37] whose diagnostic Cotton effect at approximately 345 nm was also negative. In the ROESY spectra, the correlation between H-8 and H-4 proved the *Z*-configuration of the double bond at position C-3, but the correlation between H-6 and H-1′ was not observed in **2** compared with **1**, which suggested that the relative configuration between them was *cis*. The absolute configurations of 6*R*,7*S* were further confirmed by a comparison of the experimental ECD spectrum and calculated ECD data using the aforementioned methods (Fig. 5). Thus, the structure of **2** was determined to be (3*Z*,3a*E*)-(6*R*,7*S*,2′*S*)-6-hydroxy-7-(2-carboxyl-2-hydroxyethylthio)-3-(4-hydroxylbutylidene)-4,5,6,7-tetrahydrophthalide, named thiosenkyunolide B.

Compound **3** was obtained as a white amorphous powder. Its molecular formula was identified as $C_{23}H_{36}O_{12}$ (*m*/*z* 503.2148 $[M - H]^-$) by negative HRESIMS. The IR absorptions at 3336 cm^{-1}, 1774 cm^{-1} and 1049 cm^{-1} represented a hydroxyl group, carbonyl group and alkenyl group, respectively. The ^{1}H NMR and ^{13}C NMR spectra (Table 1) of **3** displayed an amyl side chain that produced a methyl resonances [δ_H 0.85 (3H, t, $J = 7.5$ Hz, H-13); δ_C 13.7] and four methylene resonances [δ_H 1.25 (2H, m, H-12), 1.19 (2H, m, H-11), 1.38 (2H, m, H-10), 1.67 (2H, m, H-9); δ_C 21.7, 31.3, 21.7, 26.1, respectively]; two olefinic [δ_H 5.77 (1H, m, H-7), 5.87 (1H, m, H-6); δ_C 127.9, 123.9]; an oxymethine [δ_H 4.58 (1H, brs, H-3); δ_C 78.9] and a methine [δ_H 3.21 (1H, dd, $J = 1.5$, 7.5 Hz, H-5); δ_C 47.7]; and one methylene [2.46 (2H, m, H-8); δ_C 31.3]. Furthermore, a carbonyl carbon at δ_C 174.7 (C-1) and a quaternary carbon δ_C 84.2 (C-4) were observed. The presence of two anomeric protons at δ_H 4.38 (1H, d, $J = 8.0$ Hz, H-1′) and 4.84 (1H, d, $J = 3.0$ Hz, H-1″) and eleven carbons proved that compound **3** contained two sugar moieties. Comparison of the NMR data of **3** with those of the known compound ligusticoside A[16] suggested that these two compounds shared a similar skeleton except sugar moieties. In the HMBC spectrum, the correlations H-1′/C-4 and H-1″/C-6′ proved that the glucose moiety was attached at C-4 and the pentose was attached at the C-6′ of glucose (Fig. 2). The configurations of the apiose and glucose were determined to be D-configurations by GC analysis after acidic hydrolysis and chiral derivatization (retention times at 18.26, 29.32 min). The β-anomeric configurations were deduced on the basis of their coupling constants (Glc: $J = 8.0$ Hz; Api: $J = 3.0$ Hz). In the ROESY spectrum of **3**, the correlations of H-8/H-9, H-8/H-10, H-5/H-9, H-5/H-10 and H-3/H-9 indicated that H-3, H-5 and amyl chain were located on the same side (Fig. 3).

The absolute configuration of **3** was determined by comparing the experimental and calculated ECD data based on the TD-DFT method at the B3LYP/6-31+G (d,p) level. Considering the numerous conformations of sugar, we used the aglycone **3a**

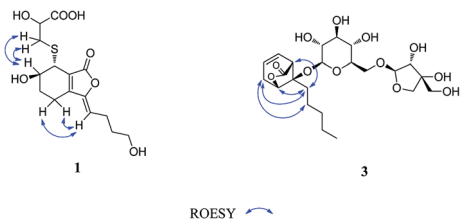

Fig. 3 Key ROESY correlations of **1** and **3**.

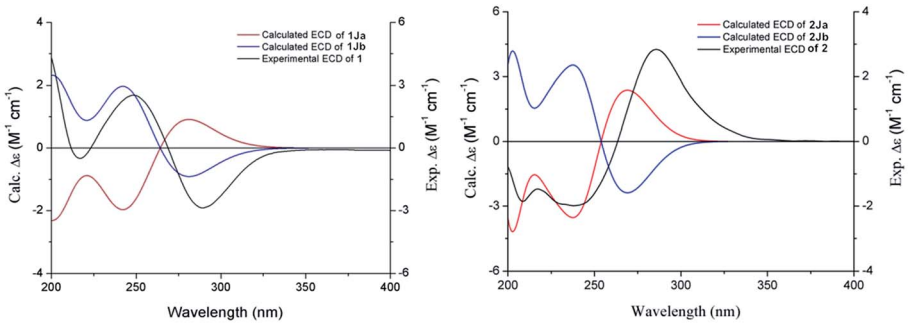

Fig. 5 Experimental ECD and calculated ECD spectrum of **1** and **2** in MeOH.

which was obtained from the acidic hydrolysis for ECD calculations. Through all wavelengths, the absolute configuration of 3*S*,4*R*,5*R* was confirmed by matching the calculated spectrum with the experimental ECD data (Fig. 6). Thus, the structure of **3** was confirmed and named ligusticoside B.

Compound **4** was obtained as a white amorphous powder. The positive HRESIMS gave the $[M + H]^+$ ion peak at *m*/*z* 367.1399, in accordance with an empirical molecular formula of $C_{18}H_{22}O_8$. The ^{1}H NMR spectrum (Table 2) of **4** presented a methyl resonance at δ_H 0.95 (3H, t, J = 7.5 Hz, H-11), two methylene resonances at δ_H 1.51 (2H, m, H-10) and 2.36 (2H, q, J = 7.5 Hz, H-9) and an olefinic proton at δ_H 5.96 (1H, t, J = 8.0 Hz, H-8). An ABX system at δ_H 7.80 (1H, d, J = 8.5 Hz, H-7), 7.20 (1H, dd, J = 8.5, 2.0 Hz, H-6) and 7.59 (1H, d, J = 2.0 Hz, H-4) was presented. Additionally, the presence of multiple protons between δ_H 3.20 and 3.70 and the presence of a doublet at δ_H 5.12 (1H, d, J = 6.5 Hz, H-1′) suggested the occurrence of a glycose moiety. The ^{13}C NMR spectrum (Table 2) of **4** showed 18 carbons, including a carbonyl carbon at δ_C 165.9 (C-1), a tertiary olefinic carbon at δ_C 109.5 (C-8), a quaternary olefinic carbon at δ_C 145.0 (C-3), six aromatic carbons at δ_C 141.4 (C-3a), 106.4 (C-4), 162.8 (C-5), 119.4 (C-6), 126.6 (C-7), and 116.9 (C-7a), and two methylene carbon resonances at δ_C 27.4 (C-9) and 22.0 (C-10). In addition, six oxygenated carbons that contributed to a glycose moiety were also observed at δ_C 99.9 (C-1′), 73.1 (C-2′), 76.6 (C-3′), 69.5 (C-4′), 77.2 (C-5′), and 60.5 (C-6′).

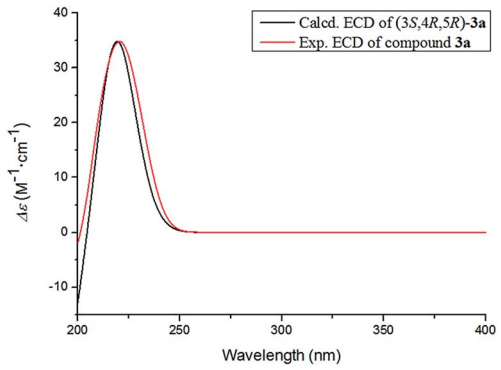

Fig. 6 Experimental ECD and calculated ECD spectrum of **3a** in MeOH.

In the HMBC spectrum of **4**, the correlations H-7/C-1, H-6/C-7a, H-4/C-3, H-8/C-3a, H-9/C-3, H-10/C-8 and H-11/C-9 confirmed the presence of a butenylphthalide moiety. The glucose moiety was attached at C-5 by the correlation of H-1′/C-5. In the NOE experiment, when irradiating H-4, H-8 generated a gain, and when irradiating the H-8, H-4 also generated gain signal (ESI†). Thus the geometric configuration of the double bond was confirmed as *Z*. The glucose was confirmed as having a D-configuration through the same method as used for **3**. The relatively large coupling constant (J = 6.5 Hz) of the anomeric proton suggested that the glucose moiety was β-configured. Therefore, the structure of **4** was established as (*Z*)-3-butylidene-5-*O*-β-D-glucopyranosyl phthalide, named ligusticumside A.

The molecular formula of compound **5** was confirmed as $C_{23}H_{30}O_{12}$ by the $[M - H]^-$ ion peak at *m*/*z* 497.1664 in HRESIMS. The ^{1}H NMR spectrum (Table 2) of **5** presented a methyl resonance at δ_H 0.95 (3H, t, J = 7.5 Hz, H-11), two methylene resonances at δ_H 1.52 (2H, m, H-10) and 2.35 (2H, m, H-9) and an olefinic proton at δ_H 5.95 (1H, t, J = 8.0 Hz, H-8). An ABX system at δ_H 7.83 (1H, d, J = 8.5 Hz, H-7), 7.23 (1H, dd, J = 8.5, 2.0 Hz, H-6) and 7.54 (1H, d, J = 2.0 Hz, H-4) was presented. According to the above ^{1}H NMR spectroscopic data, compound **5** had a similar skeleton to **4** except for the glycose moiety. The presence of multiple protons between δ_H 3.10 and δ_H 3.89 and the presence of two doublets at δ_H 5.10 (1H, d, J = 7.0 Hz, H-1′) and 4.80 (1H, d, J = 3.0 Hz, H-1″) suggested the occurrence of two sugar moieties. Compared with the ^{13}C NMR spectroscopic data of **4**, compound **5** had an additional five carbons whose resonances at δ_C 109.2 (C-1″), 75.9 (C-2″), 78.8 (C-3″), 73.3 (C-4″), and 63.1 (C-5″) were observed, and combined with the coupling constant (J = 3.0 Hz), the pentose was confirmed to be β-apiose. The apiose and glucose moieties were further determined to have D-configurations by the above method. The β-anomeric configuration was deduced on the basis of the coupling constant (Glc: J = 7.0 Hz). The connective position of apiose was identified by the correlation of H-6′/C-1″, which confirmed that the C-1″ of the apiofuranosyl was located at the C-6′ of the glucopyranosyl in the HMBC spectrum. The geometric configuration of the double bond was confirmed as *Z* by comparison with the data of ^{1}H NMR and ^{13}C NMR in **4**. Thus, compound **5** was established as (*Z*)-3-butylidene-5-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl phthalide, named ligusticumside B.

Table 2 ^{1}H and ^{13}C NMR data of compounds **4–7** in DMSO-d_6

	4[a]		**5**[a]		**6**[b]		**7**[b]	
Position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		165.9		165.9		165.8		165.8
3		145.0		144.9		144.9		144.9
3a		141.4		141.3		141.2		141.2
4	7.59, d (2.0)	106.4	7.54, d (2.0)	106.7	7.54, d (1.8)	106.6	7.54, d (1.8)	106.6
5		162.8		162.7		162.6		162.6
6	7.20, dd (2.0, 8.5)	119.4	7.23, dd (2.0, 8.5)	119.1	7.23, dd (1.8, 9.0)	119.0	7.23, dd (1.8, 8.4)	119.0
7	7.80, d (8.5)	126.6	7.83, d (8.5)	126.7	7.82, d (9.0)	126.6	7.82, d (8.4)	126.6
7a		116.9		117.0		117.0		117.0
8	5.96, t (8.0)	109.5	5.95, t (8.0)	109.6	5.95, t (7.8)	109.4	5.95, t (7.8)	109.4
9	2.36, q (7.5)	27.4	2.35, m	27.4	2.36, m	27.4	2.35, m	27.3
10	1.51, m	22.0	1.52, m	22.0	1.52, m	21.9	1.52, m	21.9
11	0.95, t (7.5)	13.8	0.95, t (7.5)	13.8	0.95, t (7.8)	13.7	0.95, t (7.2)	13.7
Glc								
1′	5.12, d (6.5)	99.9	5.10, d (7.0)	99.9	5.12, d (7.2)	99.8	5.11, d (7.2)	99.8
2′	3.20, m	73.1	3.13, m	73.1	3.29, m	73.0	3.29, m	73.0
3′	3.29, m	76.6	3.30, m	75.6	3.30, m	75.6	3.28, m	75.6
4′	3.29, m	69.5	3.29, m	69.8	3.16, m	69.6	3.14, m	69.6
5′	3.47, m	77.2	3.63, m	76.5	3.63, m	76.4	3.62, m	76.4
6′	3.47, m	60.5	3.43, m	67.4	3.45, dd (6.6, 10.8)	67.3	3.45, dd (6.6, 10.8)	67.2
	3.70, m		3.88, m		3.88, d (10.8)		3.88, m	
Api-1								
1″			4.80, d (3.0)	109.2	4.80, d (3.0)	108.7	4.80, d (3.0)	108.9
2″			3.73, dd (3.0, 6.5)	75.9	3.73, dd (3.0, 6.6)	76.1	3.69, dd (3.0, 6.6)	76.8
3″				78.8		78.7		77.6
4″			3.59, d (9.0)	73.3	3.58, d (9.6)	73.4	3.64, m	73.6
			3.86, m		3.84, d (9.0)		3.86, m	
5″			3.33, m	63.1	3.33, m	69.1	3.36, d (9.6)	69.8
					3.50, d (9.6)		3.62, m	
Api-2								
1‴					4.81, d (3.6)	109.0	4.85, d (4.8)	102.1
2‴					3.76, dd (3.6, 6.0)	75.6	3.76, dd (4.8, 9.6)	71.4
3‴						77.4		76.1
4‴					3.60, m	73.3	3.65, m	73.8
					3.84, d (9.0)		3.90, d (9.6)	
5‴					3.30, m	63.1	3.23, dd (5.4, 9.6)	63.0
					3.33, m		3.28, m	

[a] 500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR. [b] 600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR.

The molecular formula of **6** was assigned as $C_{28}H_{38}O_{16}$ based on the $[M + Na]^+$ ion peak at m/z 653.2046 (calcd 653.2052) in the HRESIMS. By carefully comparing the related data, **6** had similar structural characteristics to **4** and **5**, with the main difference that **6** had a three-monosaccharide unit. In the ^{1}H NMR spectrum (Table 2) of **6**, the presence of multiple protons between δ_H 3.15 and δ_H 3.89 and the presence of three anomeric protons at δ_H 5.12 (1H, d, J = 7.2 Hz, H-1′), 4.80 (1H, d, J = 3.0 Hz, H-1″) and 4.81 (1H, d, J = 3.6 Hz, H-1‴) suggested the occurrence of three monosaccharide units. The ^{13}C NMR resonances (Table 2) at δ_C 99.8, 108.7, and 109.0 indicated that **6** had a pyranose and two furanoses. The apiose and glucose were determined to have D-configurations through the same method as used above. The β-anomeric configurations were confirmed on the basis of their coupling constants (Glc: J = 7.2 Hz; Api-inner: J = 3.0 Hz; Api-outer: J = 3.6 Hz). In the HMBC spectrum, the glucose moiety was also attached at C-5 by the correlation of H-1′/C-5. The correlation H-6′/C-1″ confirmed that the apiofuranosyl was located at the glucopyranosyl. The correlation H-5‴/C-1‴ confirmed that the outer apiofuranosyl was located at the inner apiofuranosyl. Thus, compound **6** was established as (*Z*)-3-butylidene-5-*O*-β-D-apiofuranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl phthalide, named ligusticumside C.

Compound **7** had the same molecular formula ($C_{28}H_{38}O_{16}$) as **6** through the HRESIMS at m/z 653.2066 $[M + Na]^+$. A careful comparison of the IR, UV and NMR data of **6** and **7** suggested that they had the same planar structure. The main difference was the chemical shifts of C-1‴ and C-2‴ of the outer apiose at δ_C 102.1 and 71.4 in **7**, instead of δ_C 109.0 and 75.6 in **6**, respectively. This was confirmed by the coupling constant of H-1‴ (J = 4.8 Hz in **7** *vs.* J = 3.6 Hz in **6**). Clearly, the outer sugar unit had an α configuration instead of the β configuration in **6**.

The sugar moieties were determined to be D-configurations of apiose and glucose through the same method as used above. Therefore, the structure of **7** was confirmed as (*Z*)-3-butylidene-5-*O*-α-D-apiofuranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl phthalide, named ligusticumside D.

According to the $[M + Na]^+$ ion peak at *m*/*z* 683.2164 in the HRESIMS, the molecular formula of **8** was assigned as $C_{29}H_{40}O_{17}$. Comparing its NMR data with those of **7**, both of them are trisaccharides of butylidenephthalide with the major difference between them being the type of the outer sugar. The presence of an anomeric proton at δ_H 4.64 (1H, d, $J = 3.5$ Hz, H-1‴) and carbons at δ_C 99.2, 72.8, 72.1, 70.0, 73.3, and 60.8 suggested that the outer sugar of **8** was α-glucose instead of α-apiose in **7**. The apiose and glucose were determined to have D-configurations by the same method as used above. Thus, the structure of **8** was confirmed as (*Z*)-3-butylidene-5-*O*-α-D-glucopyranosyl-(1 → 5)-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl phthalide, named ligusticumside E.

The molecular formula of compound **9** was determined to be $C_{18}H_{22}O_8$ through HRESIMS ($[M + Na]^+$ 521.1629). The identical data of HRESIMS, IR, and UV suggested that **5** and **9** were isomers, with the difference being the geometric configuration of the double bond. In the ^{1}H NMR spectrum of **9** (Table 3), an olefinic proton was presented at δ_H 5.90 (1H, t, $J = 7.8$ Hz, H-8). In the NOE experiment, when irradiating H-4, H-8 did not generate a gain, and when irradiating H-8, H-4 also did not generate a gain signal, which was entirely different from the results for **5** (ESI†). Additionally, the C-4 (δ_C 110.1) and C-8 (δ_C 114.2) were shifted significantly downfield in the ^{13}C NMR spectrum when compared to those of **5** (C-4 at δ_C 106.7 and C-8 at δ_C 109.6). Thus, the double bond was proven to have an *E* configuration, and compound **9** was confirmed as (*E*)-3-butylidene-5-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl phthalide, named ligusticumside F.

Compound **10** was obtained as a white amorphous powder. The HRESIMS showed a positive ion at *m*/*z* 405.1156 $[M + Na]^+$ that matched the molecular formula $C_{18}H_{22}O_9$. The IR spectrum presented hydroxyl (3395 cm^{-1}) and γ-lactone (1757 cm^{-1}) peaks. The ^{1}H NMR spectrum of **10** (Table 3) showed three aromatic resonances at δ_H 7.27 (1H, d, $J = 8.0$ Hz, H-4), 7.54 (1H, t, $J = 8.0$ Hz, H-5), and 6.89 (1H, d, $J = 8.0$ Hz, H-6), which were assigned to a 1,2,3-trisubstituted benzene ring, an olefinic resonance at δ_H 5.86 (1H, t, $J = 7.5$ Hz, H-8), two methylene resonances at δ_H 2.40 (2H, q, $J = 7.5$ Hz, H-9) and 1.71 (2H, m, H-10), and a pair of oxygen-methylene resonances at δ_H 3.50 (1H, m, H-11a) and 3.83 (1H, m, H-11b), as well as the glucoside resonances. The ^{13}C NMR experiment (Table 3) presented 18 carbon resonances, including one carbonyl carbon at δ_C 164.4 (C-1), six aromatic carbons, two olefinic carbons at δ_C 145.0 (C-3) and 107.9 (C-8), two methylene carbon resonances at δ_C 22.3 (C-9) and 28.9 (C-10), one oxygen-methylene resonance at δ_C 68.1 (C-11), and a set of glucose carbon resonances, implying that **10** was still a phthalide derivative with a glucose unit. The correlation of H-1′/C-11 in the HMBC spectrum suggested that the glucose moiety was located at C-11. The glucose was confirmed as having a D-configuration by the same method as used above. The β-configuration was deduced based on the coupling constant ($J = 8.0$ Hz) of H-1′. Thus, compound **10** was determined to be (*Z*)-3-(4-*O*-β-D-glucopyranosyl-butylidene)-7-hydroxyphthalide, named ligusticumside G.

Compound **11** had the molecular formula $C_{23}H_{30}O_{13}$ by the analysis of its HRESIMS, which gave a positive ion at *m*/*z* 537.1587 $[M + Na]^+$ (calcd 537.1579). An ABX system at δ_H 7.17 (1H, d, $J = 2.0$ Hz, H-4), 6.97 (1H, dd, $J = 2.0$, 8.0 Hz, H-6) and 7.68 (1H, d, $J = 8.0$ Hz, H-7) (Table 3) in the ^{1}H NMR spectrum suggested the presence of the same trisubstituted aromatic ring as in **4** instead of that in **10**. In the HMBC experiment, H-7 (δ_H 7.68) correlated with C-5 (δ_C 163.8) and C-3a (δ_C 141.8), H-6 (δ_H 6.97) correlated with C-4 (δ_C 114.4) and C-7a (δ_C 105.6), and H-4 (δ_H 7.17) correlated with C-6 (δ_C 118.5), C-7a (δ_C 105.6), and C-3 (δ_C 144.9), indicating that the aromatic hydroxyl was located at position C-5. The additional resonances at δ_H 4.84 (1H, d, $J = 3.0$ Hz, H-1″) and from δ_H 3.84 to 3.30 were attributed to one pentose in **11**. The ^{13}C NMR spectrum showed 23 carbon resonances (Table 3), and the corresponding pentose resonances at δ_C 109.2 (C-1″), 75.8 (C-2″), 78.8 (C-3″), 73.4 (C-4″), and 63.1 (C-5″) were observed. The apiofuranosyl and glucopyranosyl were determined to have D-configurations by the same method as used above (retention times at 18.26, 29.32 min). The coupling constants of the anomeric protons indicated the β-configuration (Glc: $J = 8.0$ Hz; Api: $J = 3.0$ Hz). Moreover, the correlation between H-1″ (δ_H 4.84) and C-6′ (δ_C 68.2) confirmed that the C-1″ of the apiofuranosyl was located at the C-6′ of the glucopyranosyl. Thus, compound **11** was confirmed as (*Z*)-3-[4-*O*-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl-butylidene]-7-hydroxyphthalide, named ligusticumside H.

Compounds **1–11** were tested for their neuroprotective effects on SH-SY-5Y cell injury induced by H_2O_2 and OGD with L-NBP (L-3-*n*-butylphthalide) as a positive control. The results showed that compound **5** exhibited a weak neuroprotective effect with the increase cell viability rate of 15.60% and compound **4** exhibited a moderate neuroprotective effect with the increase cell viability rate of 24.35%, compared with the positive control L-NBP with increase cell viability rate of 18.26% on H_2O_2-induced neurotoxicity. And compound **4** exhibited a moderate neuroprotective effect with the increase cell viability rate of 13.72%, compared with the positive control L-NBP with increase cell viability rate of 4.03% on OGD-induced neurotoxicity.

Experimental

General experimental procedures

The optical rotations, UV spectra and ECD spectra were recorded with JASCO P-2000, V650 and J-815 spectrometer (JASCO, Easton, MD, USA), respectively. The Infrared spectra were measured on Nicolet 5700 spectrometer (Thermo Scientific, FL, USA). The NMR spectra were recorded with Varian 500 MHz (Bruker-Biospin, Billerica, MA, USA) and 600 MHz NMR spectrometers (Varian, Inc., Palo Alto, CA, USA). HRESIMS reports were obtained from Agilent 6520 HPLC-Q-TOF (Agilent Technologies, Waldbronn, Germany) and LCMS-IT-TOF system (Shimadzu Scientific Instruments Inc., Kyoto, Japan). Preparative HPLC was performed using a Shimadzu LC-10AT with

Table 3 ^{1}H and ^{13}C NMR data of compounds **8–11** in DMSO-d_6

	8[a]		**9**[b]		**10**[a]		**11**[a]	
Position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		165.9		165.6		164.4		166.1
3		144.9		144.7		145.0		144.9
3a		141.3		139.4		141.0		141.8
4	7.54, d (2.0)	106.6	7.45, d (1.8)	110.1	7.27, d (8.0)	110.3	7.17, d (2.0)	114.4
5		162.7		162.5	7.54, t (8.0)	136.6		163.8
6	7.23, dd (2.0, 8.5)	119.0	7.32, dd (1.8, 9.0)	118.7	6.89, d (8.0)	116.4	6.97, dd (2.0, 8.0)	118.5
7	7.82, d (8.5)	126.8	7.89, d (9.0)	127.0		157.2	7.68, d (8.0)	126.8
7a		117.0		118.7		109.4		105.6
8	5.96, t (8.0)	109.6	5.90, t (7.8)	114.2	5.86, t (7.5)	107.9	5.89, t (7.5)	108.7
9	2.35, m	27.4	2.52, m	27.2	2.40, q (7.5)	22.3	2.41, q (7.5)	22.3
10	1.51, m	22.0	1.57, m	22.3	1.71, m	28.9	1.72, m	28.9
11	0.95, t (7.5)	13.8	0.98, t (7.8)	13.6	3.50, m	68.1	3.51, m	67.7
					3.83, m		3.80, m	
Glc-1								
1′	5.12, d (7.5)	99.8	5.11, d (7.2)	100.2	4.12, d (8.0)	102.9	4.14, d (8.0)	102.8
2′	3.29, m	73.1	3.30, m	73.0	2.94, t (8.0)	73.4	2.95, overlap	73.2
3′	3.29, m	75.6	3.31, m	75.7	3.06, m	76.9	3.12, t (8.5)	76.6
4′	3.16, m	69.5	3.14, m	69.8	3.03, q (9.0)	70.1	2.98, overlap	70.2
5′	3.64, m	76.4	3.89, m	76.2	3.11, m	76.8	3.25, m	75.5
6′	3.46, m	67.4	3.44, dd (6.6, 10.8)	67.5	3.42, dd (5.0, 11.0)	61.1	3.39, dd (7.0, 11.0)	68.2
	3.89, m		3.87, m		4.12 d (8.0)		3.82 m	
Api								
1″	4.83, d (4.0)	108.8	4.80, d (3.0)	109.2			4.84, d (3.0)	109.2
2″	3.71, dd (4.0, 6.5)	76.8	3.75, dd (3.0, 6.6)	75.8			3.74, d (3.0)	75.8
3″		77.7		78.7				78.8
4″	3.65, m	73.7	3.57, d (9.0)	73.2			3.56, d (9.5)	73.4
	3.91, m		3.86, d (9.0)				3.84, d (9.5)	
5″	3.25, m	69.6	3.31, m	62.9			3.30, t (11.0)	63.1
	3.64, m						3.32, t (11.0)	
Glc-2								
1‴	4.64, d (3.5)	99.2						
2‴	3.29, m	72.8						
3‴	3.16, m	72.1						
4‴	3.07, m	70.0						
5‴	3.40, m	73.3						
6‴	3.45, m	60.8						
	3.58, m							

[a] 500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR. [b] 600 MHz for ^{1}H NMR, 150 MHz for ^{13}C NMR.

a ODS-A column (250 mm × 20 mm, 5 μm; YMC Corp., Kyoto, Japan). The Agilent 1200 series system was used to carry on the HPLC-DAD analysis with an Apollo C_{18} column (250 mm × 4.6 mm, 5 μm; Alltech Corp., Lexington, KY, USA). The Agilent 7890A was used to carry on the GC analysis with a capillary column, HP-5 (60 m × 0.32 mm, with a 1 μm film; Agilent Technologies Inc., CA, USA). Macroporous resin Diaion HP-20 (Mitsubishi Chemical Corp., Tokyo, Japan), RP-C_{18} (50 μm, YMC Corp.), and Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) were used to column chromatograph.

Plant material

The roots of *Ligusticum chuanxiong* Hort. were collected from Pengzhou Town, Sichuan Province in PRC, in Jun 2013 and identified by professor L. Ma. A voucher specimen (ID-S-2594) was deposited at the Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China.

Extraction and isolation

The powdered rhizome of *L. chuanxiong* Hort. (100.0 kg) was exhaustively extracted with 80% EtOH under reflux condition. The solvent was evaporated by reduced pressure and then the residue (23.1 kg) was partitioned successively with EtOAc and *n*-BuOH. The *n*-BuOH-soluble portion (1300 g) was applied on a HP-20 column to give five fractions A–E through gradient elution with H_2O, 15% ethanol, 30% ethanol, 50% ethanol, and 95% ethanol, respectively. Fraction C (103.0 g) was

chromatographed over an RP-C_{18} column, eluting with H_2O/MeOH (from 100 : 0 to 0 : 100) to give 16 fractions (C1–C16) on the basis of HPLC analyses. Fraction C5 was subjected to a Sephadex LH-20 with a gradient of increasing MeOH (0–100%) in H_2O and then separated by preparative HPLC (MeOH/H_2O, 30 : 70, V/V, HOAc, 0.01%) to give **2** (4 mg). Fraction C13 was separated by column chromatography over a Sephadex LH-20 using H_2O as the eluent and was further purified by preparative HPLC (MeOH/H_2O, 45 : 55, v/v, HOAc, 0.01%) to give **1** (64 mg). Compounds **10** (4 mg) and **11** (4 mg) were obtained from fraction C14 with the above method. Fraction D (48.0 g) was chromatographed over an RP-C_{18} column, eluting with H_2O/MeOH (from 95 : 5 to 0 : 100) to give 24 fractions (D1–D24) on the basis of HPLC and TLC analyses. Fraction D9 was purified by a Sephadex LH-20 with a gradient of increasing MeOH (0–100%) in H_2O and then separated by preparative HPLC (MeCN/H_2O, 28 : 72, V/V, HOAc, 0.02%) to give **4** (70 mg), **5** (50 mg), **6** (3 mg), **7** (4 mg), **8** (3 mg), and **9** (10 mg). Fraction D8 was chromatographed over a Sephadex LH-20 with a gradient of increasing MeOH (0–100%) in H_2O to give 19 fractions (D8-1–D8-19) on the basis of HPLC and TLC analyses. Fraction D8-2 was chromatographed over silica gel (EtOAC/MeOH/H_2O, from 120 : 1:0.5 to 1 : 1:0.5) to give **3** (120 mg).

Structure characterization

Thiosenkyunolide A, 1. White amorphous powder; UV λ_{max} (MeOH) (log ε): 201 (4.01), 277 (4.09) nm; $[\alpha]_D^{20}$ −49 (*c* 0.1 MeOH); HRESIMS *m*/*z* 345.0999 $[M + H]^+$ (calcd 345.1003), *m*/*z* 367.0823 $[M + Na]^+$ (calcd 367.0822); IR (KBr) ν_{max}: 3387, 2938, 1741, 1682, 1633, 1514, 1414, 1230, 1184, 1093, 1043, 954 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 1.

Thiosenkyunolide B, 2. White amorphous powder; UV λ_{max} (MeOH) (log ε): 201 (3.84), 277 (4.06) nm; $[\alpha]_D^{20}$ +30 (*c* 0.1 MeOH); HRESIMS *m*/*z* 345.1008 $[M + H]^+$ (calcd 345.1003), *m*/*z* 367.0827 $[M + Na]^+$ (calcd 367.0822); IR (KBr) ν_{max}: 3347, 2932, 2882, 1756, 1680, 1632, 1412, 1323, 1239, 1090, 1048, 1026, 997, 966 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 1.

Ligusticoside B, 3. White amorphous powder; UV λ_{max} (MeOH) (log ε): 203 (4.18), 216 (3.90) nm; $[\alpha]_D^{20}$ +4 (*c* 0.1 MeOH); HRESIMS *m*/*z* 503.2148 $[M - H]^-$ (calcd 503.2134); IR (KBr) ν_{max}: 3336, 2924, 1774, 1275, 1350, 1049 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 1.

Ligusticumside A, 4. White amorphous powder; UV λ_{max} (MeOH) (log ε): 253 (4.42), 273 (4.16) nm; $[\alpha]_D^{20}$ −73 (*c* 0.1 MeOH); HRESIMS *m*/*z* 367.1399 $[M + H]^+$ (calcd 367.1387); IR (KBr) ν_{max}: 3377, 2929, 1759, 1689, 1613, 1484, 1297, 1078, 991 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 2.

Ligusticumside B, 5. White amorphous powder; UV λ_{max} (MeOH) (log ε): 253 (4.35), 274 (4.07) nm; $[\alpha]_D^{20}$ −96 (*c* 0.1 MeOH); HRESIMS *m*/*z* 497.1664 $[M - H]^-$ (calcd 497.1665); IR (KBr) ν_{max}: 3406, 2931, 1763, 1611, 1482, 1293, 1066, 1011 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 2.

Ligusticumside C, 6. White amorphous powder; UV λ_{max} (MeOH) (log ε): 252 (4.19), 275 (3.92) nm; $[\alpha]_D^{20}$ −78 (*c* 0.1 MeOH); HRESIMS *m*/*z* 653.2046 $[M + Na]^+$ (calcd 653.2052); IR (KBr) ν_{max}: 3372, 2930, 1762, 1681, 1611, 1387, 1293, 1066, 930 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 2.

Ligusticumside D, 7. White amorphous powder; UV λ_{max} (MeOH) (log ε): 253 (4.27), 272 (4.03) nm; $[\alpha]_D^{20}$ −30 (*c* 0.1 MeOH); HRESIMS *m*/*z* 653.2066 $[M + Na]^+$ (calcd 653.2052); IR (KBr) ν_{max}: 3398, 2932, 1765, 1687, 1613, 1295, 1072, 930 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 2.

Ligusticumside E, 8. White amorphous powder; UV λ_{max} (MeOH) (log ε): 253 (4.38), 270 (4.16) nm; $[\alpha]_D^{20}$ −29 (*c* 0.1 MeOH); HRESIMS *m*/*z* 683.2164 $[M + Na]^+$ (calcd 683.2158); IR (KBr) ν_{max}: 3384, 2926, 1762, 1688, 1614, 1294, 1073, 928 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 3.

Ligusticumside F, 9. White amorphous powder; UV λ_{max} (MeOH) (log ε): 254 (4.08), 272 (3.91) nm; $[\alpha]_D^{20}$ −21 (*c* 0.1 MeOH); HRESIMS *m*/*z* 521.1629 $[M + Na]^+$ (calcd 521.1629); IR (KBr) ν_{max}: 3399, 2931, 1765, 1689, 1612, 1483, 1294, 1072, 953 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 3.

Ligusticumside G, 10. White amorphous powder; UV λ_{max} (MeOH) (log ε): 198 (3.96), 225 (4.14), 265 (3.95), 330 (3.66), 339 (3.70) nm; $[\alpha]_D^{20}$ −10 (*c* 0.1 MeOH); HRESIMS *m*/*z* 405.1156 $[M + Na]^+$ (calcd 405.1156); IR (KBr) ν_{max}: 3395, 2928, 2880, 1757, 1687, 1606, 1473, 1372, 1297, 1199, 1163, 1079, 1017, 895 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 3.

Ligusticumside H, 11. White amorphous powder; UV λ_{max} (MeOH) (log ε): 254 (4.54) nm; $[\alpha]_D^{20}$ −30 (*c* 0.1 MeOH); HRESIMS *m*/*z* 537.1587 $[M + Na]^+$ (calcd 537.1579); IR (KBr) ν_{max}: 3375, 2932, 2884, 1753, 1689, 1610, 1481, 1467, 1381, 1295, 1165, 1057, 999, 932 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 3.

Determination of the absolute configuration of sugar

Compounds **5** (2 mg) was dissolved in 1 mol L^{-1} CF_3COOH (14 mL) and then the mixture was heated in 70 °C for 1 h. The mixture was then extracted three times with EtOAc, and the aqueous layer was freeze-dried to obtain residue. Using the same method with the literature,[38] the residue was dissolved in anhydrous pyridine (2 mL), L-cysteine methyl ester hydrochloride (4 mg) was added, and then the mixture was heated in a water bath (60 °C) for 1 h. After the reaction solution was dried under vacuum, *N*-trimethylsilylimidazole (1 mL) was added, and the solution was heated in a water bath (60 °C) for 1 h and extracted three times with H_2O/*n*-hexane. Then, the *n*-hexane layer was analyzed using GC under conditions as follows: injection temperature, 300 °C; detector temperature (FID), 300 °C; capillary column, HP-5 (30 m × 0.32 mm, Dikma); start temperature, 200 °C, raised to 260 °C at a rate of 10 °C min^{-1}, and the final temperature maintained for 30 min; and N_2 used as the carrier gas.

Neuroprotective activities of compounds 1–11

The assay method of neuroprotective effects about compounds refer to the procedures of literature.[39]

Conclusions

In the course of a search for neuroprotective compounds from *Ligusticum chuanxiong* Hort., eleven new phthalide derivatives

were obtained. Among them, two are substituted with the rare mercaptopropionic acid. All compounds were tested for their neuroprotective effects, and it was observed that compound **4** had moderate effect against H_2O_2 and OGD-induced neurotoxicity in SH-SY5Y cells at 10 μM. These results could contribute to the better understanding of the therapeutic usage of *Ligusticum chuanxiong* Hort.

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