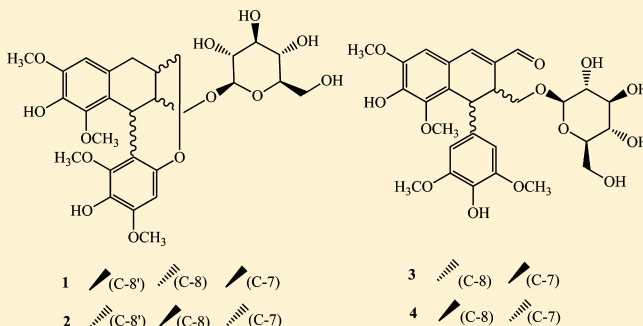


Antioxidant Lignans and Chromone Glycosides from *Eurya japonica*Li-Ming Yang Kuo,^{†,‡,∇} Li-Jie Zhang,^{†,∇} Hung-Tse Huang,[†] Zhi-Hu Lin,[†] Chia-Ching Liaw,[§] Hui-Ling Cheng,[†] Kuo-Hsiung Lee,[‡] Susan L. Morris-Natschke,[‡] Yao-Haur Kuo,^{*,†,||} and Hsiu-O Ho^{*,‡}[†]National Research Institute of Chinese Medicine, Taipei 112, Taiwan, Republic of China[‡]School of Pharmacy, Taipei Medical University, Taipei 110, Taiwan, Republic of China[§]StarSci Biotech Co. Ltd, Taipei 112, Taiwan, Republic of China[‡]Natural Products Research Laboratories, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599-7568, United States^{||}Graduate Institute of Integrated Medicine, China Medical University, Taichung 404, Taiwan, Republic of China

S Supporting Information

ABSTRACT: Four new 8,8',7,2'-lignans, (+)-ovafolinin B-9'-O- β -D-glucopyranoside (**1**), (–)-ovafolinin B-9'-O- β -D-glucopyranoside (**2**), (+)-ovafolinin E-9'-O- β -D-glucopyranoside (**3**), and (–)-ovafolinin E-9'-O- β -D-glucopyranoside (**4**), two neolignans, eusiderin N (**5**) and (7S,8R)-3,5,5'-trimethoxy-4',7-epoxy-8,3'-neolignan-9,9'-diol-4-O- β -D-xylopyranoside (**6**), and two new chromone glycosides, 5,7-dihydroxy-4H-chromen-4-one-3-O- β -D-glucopyranoside (**7**) and 5,7-dihydroxy-4H-chromen-4-one-3-O- β -D-xylopyranoside (**8**), together with 25 known compounds, were isolated from the stems of *Eurya japonica*. Structural elucidation of compounds **1**–**8** was established by spectroscopic methods, especially 2D NMR techniques, electronic circular dichroism data, and comparison with reported data. The isolates were evaluated for antioxidant and anti-NO production activities. Compounds **1**, **2**, **12**–**20**, and **29** (ED₅₀ 23.40 μ M for **1**) demonstrated potent antioxidant activity compared to the positive control α -tocopherol (ED₅₀ 27.21 μ M). On the other hand, compounds **1**, **2**, **7**–**9**, **12**–**20**, and **32** showed only weak anti-NO production activity when compared to the positive control quercetin.



The fruits and leaves of *Eurya japonica* Thunberg (Theaceae) are used in the Chinese traditional medicine “Lingmu” for the treatment of rheumatoid arthritis, tympanites, hemostasis of injuries, etc.¹ The components of the leaves² and berries³ of this plant include halleridone, cornoside, and three flavonoids; however, phytochemical research on the components in the plant stem is rare. We here report the isolation and characterization of six new lignans (**1**–**6**) and two new chromone glycosides (**7**, **8**) (Figure 1), along with 25 known compounds, including one chromone, 5,7-dihydroxy-4H-chromen-4-one-3-O- α -L-arabinopyranoside (**9**);⁴ five lignans, tortoside E (**10**),⁵ sakuraesinol (**11**),⁶ (–)-2a-O-(β -D-glucopyranosyl)lyoniresinol (**12**),⁷ (+)-3a-O-(β -D-glucopyranosyl)lyoniresinol (**13**),⁷ and aviculin (**14**);⁸ six flavonoids, (+)-epitaxifolin 3-O- β -D-xylopyranoside (**15**),⁹ (–)-epitaxifolin 3-O- β -D-xylopyranoside (**16**),⁹ (+)-taxifolin 3-O- β -D-xylopyranoside (**17**),⁹ (–)-taxifolin 3-O- β -D-xylopyranoside (**18**),⁹ (2R,3R)-(+)-glucodistylin (**19**),¹⁰ and (2S,3S)-(–)-glucodistylin (**20**);¹⁰ 11 phenyl glycosides, di-O-methylcresatin (**21**),¹¹ 2,6-dimethoxy-4-(2-hydroxyethyl)phenol 1-O- β -D-glucopyranoside (**22**),¹² dihydrosyringin (**23**),¹³ 3,4,5-trimethoxyphenyl- β -D-glucopyranoside (**24**),¹⁴ 3,4-dimethoxyphenyl- β -D-glucopyranoside (**25**),¹⁵ 3-hydroxy-4,5-dimethoxyphenyl- β -D-glucopyranoside (**26**),¹⁶ cremanthodioside (**27**),¹⁷ junipetriolo-

sides A (**28**),¹⁸ 6'-O-coumaroyl-1'-O-[2-(3,4-dihydroxyphenyl)ethyl]- β -D-glucopyranoside (**29**),¹⁹ neocalceolarioside D (**30**),²⁰ and norbergenin (**31**);²¹ one triterpene, betulinic acid (**32**);²² and one steroid, β -sitosterol glucopyranoside (**33**),²³ from the ethanol extract of *E. japonica*. The structures of the new compounds were elucidated by analysis of spectroscopic data and comparisons with reported data. Some of these compounds were also evaluated for antioxidant and anti-NO production activities.

RESULTS AND DISCUSSION

A 95% aqueous EtOH extract of the stems of *E. japonica* was suspended in H₂O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc extract was chromatographed on a silica gel column and then on a Sephadex LH-20 column. The bioactive fractions were subjected to semipreparative HPLC, using a reverse-phase (ODS) column, to yield six new lignan glycosides (**1**–**6**), two new chromone glycosides (**7**, **8**), and 25 known compounds.

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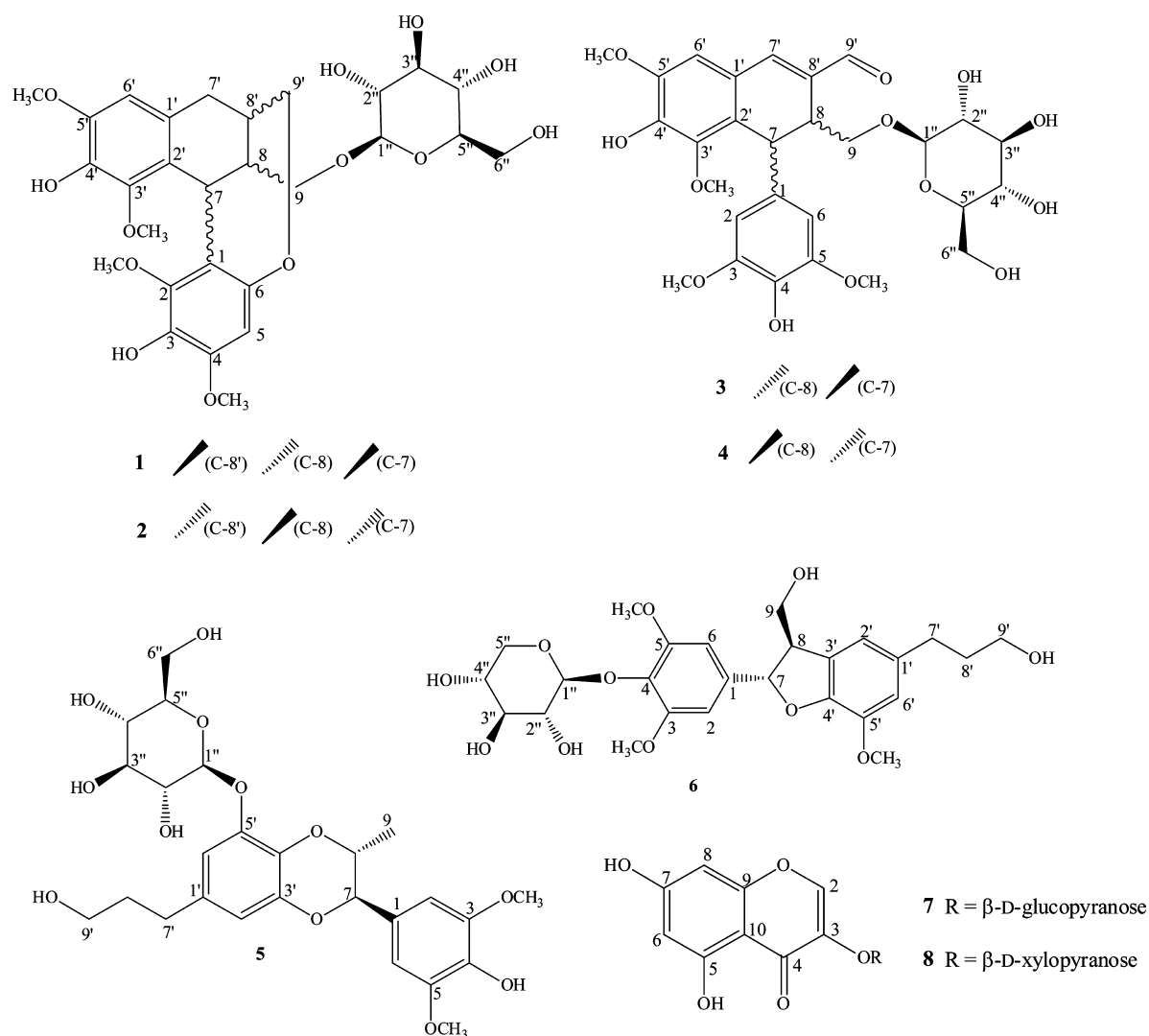


Figure 1. Compounds isolated from *E. japonica*.

Compounds **1** and **2** were obtained as pale yellow, amorphous powders and had similar HRESIMS, UV, IR, and NMR spectra, suggesting that they are stereoisomers. The elemental formula was $C_{28}H_{36}O_{13}$ from HRESIMS (corresponding to 11 degrees of unsaturation). Absorptions for hydroxy (3382 cm^{-1}) and aromatic ($1616, 1497, 1457, \text{ or } 1461\text{ cm}^{-1}$) groups were found in the IR spectra of **1** and **2**, and their UV spectra showed absorption maxima at 283 nm. In the ^1H and ^{13}C NMR spectra, the two compounds possessed the same resonances for two benzene rings, four aromatic methoxy groups, three aliphatic methines, three aliphatic methylenes, and a hexose moiety. The foregoing data accounted for nine of the 11 required degrees of unsaturation and suggested that both **1** and **2** possessed a tetracyclic skeleton with a sugar moiety. Their structures and the locations of the attached groups were determined from ^1H – ^1H COSY, HMQC, and HMBC data (Figure 2). In the ^1H – ^1H COSY spectrum, cross-peaks were found between H-7/H-8/H-9, H-8/H-8', and H-7'/H-8'/H-9'. In the HMBC spectrum, long-range correlations were observed between H-7/C-2, C-6, C-1', C-2', and C-3' and between H-7'/C-1', C-2', and C-6'. These facts suggested an 8,8',7,2'-lignan skeleton. The positions of the four methoxy groups were determined at C-2, C-4, C-3', and C-5' based on the HMBC correlations. The long-range correlation between H-9' and C-6

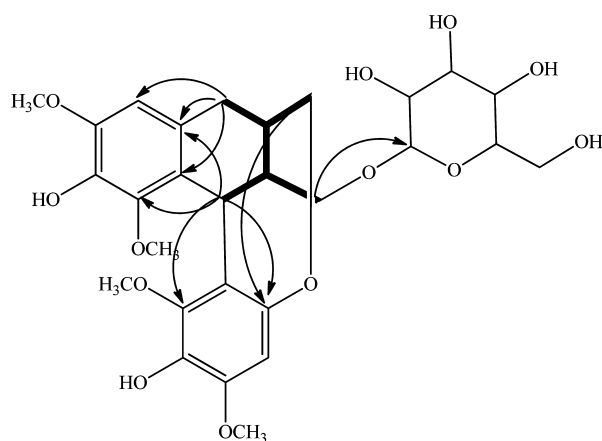


Figure 2. Key HMBC (\rightarrow) and COSY ($-$) correlations of **1** and **2**.

and the ^{13}C NMR chemical shifts (δ_{C} 81.3 C-9', 153.8 C-6) indicated the presence of an ether bridge between C-9' and C-6. Furthermore, a correlation between the anomeric proton (H-1'') and C-9 established the hexose at C-9. The hexose moiety was confirmed as β -glucose,²⁴ and the D-configuration was identified by acid hydrolysis of compounds **1** and **2**. Thus, the

gross structures of **1** and **2** were determined as shown in Figure 1 and are similar to that of ovafolinin B,²⁵ except for the glucose moiety.

On the basis of the NOE correlations between H-7 and H-9 and between H-8' and H-9, the relative configuration was assigned as 7*R**, 8*R**, and 8'*R** (Figure 3). The stereochemical

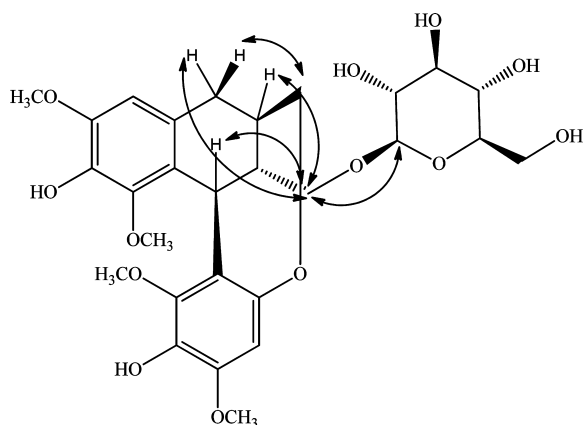


Figure 3. NOE correlations of **1**.

difference between the two compounds was further determined from electronic circular dichroic (ECD) spectra. Compound **1** and ovafolinin B (**1a**), the acid hydrolysis product of **1**, had similar ECD spectra, indicating that the presence of the glucose moiety did not influence the Cotton effects (Figure 4). Comparison of the ECD spectra **1** and **2** showed their Cotton effects at 284 nm were negative and positive, respectively. The negative Cotton effect indicates a 7*R* configuration, while the positive one indicates 7*S*.^{26–28} Therefore, the absolute configurations of compounds **1** and **2** are 7*R*, 8*R*, 8'*R* and 7*S*, 8*S*, 8'*S*, respectively. Accordingly, the structures of **1** and **2** are (+)-ovafolinin B-9'-*O*- β -D-glucopyranoside and (–)-ovafolinin B-9'-*O*- β -D-glucopyranoside, respectively, as shown in Figure 1.

Like compounds **1** and **2**, compounds **3** and **4** also possessed similar NMR, UV, IR, and HRESIMS spectra. The HRESIMS of **3** and **4** suggested an elemental formula of C₂₈H₃₄O₁₃ based on the quasi-molecular ions at *m/z* 577.1935 (**3**) and 577.1932 (**4**) [*M* – H][–]. The IR spectra displayed absorption bands for hydroxy and aromatic groups, and the UV spectra showed absorption maxima at 354, 320 (sh), 270 (sh), and 254 nm. The ¹H and ¹³C NMR spectra suggested that **3** and **4** have the same lignan skeleton. From the HMQC and HMBC spectra, the aglycone moieties of **3** and **4** were determined as ovafolinin

E,²⁵ and the β -D-glucose moiety was assigned at C-9. The stereochemical difference between the two compounds was also determined from the ECD spectra. The ECD spectrum of **3** (Figure 5), which is identical with that of dimethyl (1*S*,2*R*)-1,2-dihydro-1-(3,4-dimethoxyphenyl)-6,7-dimethoxynaphthalene-2,3-dicarboxylate, showed a positive Cotton effect at 357 nm, and that of **4** a negative Cotton effect at 357 nm.²⁸ Thus, the absolute configurations of compounds **3** and **4** were confirmed as 7*S*, 8*R* and 7*R*, 8*S*, respectively. From the above findings, the structures of **3** and **4** were identified as (+)-ovafolinin E-9'-*O*- β -D-glucopyranoside and (–)-ovafolinin E-9'-*O*- β -D-glucopyranoside, respectively.

Compound **5** was isolated as a white powder. The molecular formula was established as C₂₆H₃₄O₁₂ from the HRESIMS (*m/z* 561.1964 [*M* + Na]⁺). The IR spectrum displayed bands for hydroxy (3407 cm^{–1}) and aromatic (1596, 1560, 1505 cm^{–1}) moieties. The UV spectrum showed absorption maxima at 273 and 230 (sh) nm. The ¹H NMR spectrum showed resonances at δ_H 6.49 (d, *J* = 2.4 Hz) and 6.65 (d, *J* = 2.4 Hz), corresponding to two *meta*-coupled aromatic protons, one two-proton singlet at δ_H 6.68, and a singlet at δ_H 3.85 (6H, s) corresponding to two aromatic methoxy groups. In addition, resonances were present for a 1,3,4,5-tetrasubstituted aromatic ring: resonances at δ_H 3.54 (t, *J* = 6.6 Hz, 2H), 2.56 (m, 2H), and 1.80 (m, 2H) corresponding to a 1-propanol moiety; resonances at δ_H 4.56 (d, *J* = 7.8 Hz), 4.14 (m), and 1.19 (d, *J* = 6.6 Hz, 3H) corresponding to a 1,2-propylene glycol unit, and a resonance at δ_H 4.91 (d, *J* = 7.8 Hz, 1H) corresponding to the anomeric proton of a β -D-glucopyranose moiety. The ¹³C NMR spectrum also exhibited the corresponding carbon resonances for the above structural units, confirming that **5** was a lignan glucoside. In the HMBC spectrum, a correlation was observed between H-1'' and C-5' indicating that the glucose moiety was linked at C-5'. The lignan portion was assigned as 1,4-benzodioxane-type, according to the molecular formula and NMR data [δ_H 4.56 (d, *J* = 7.8 Hz, H-7), 4.14 (m, H-8), and 1.19 (d, *J* = 6.6 Hz, 3H, H-9); δ_C 82.3 (C-7), 75.5 (C-8), and 17.4 (C-9)], and the relative configuration of H-7 and H-8 was *trans*.²⁹ The ECD spectrum of **5** showed a negative Cotton effect at 236 nm, indicating an 8*R* absolute configuration.²⁹ Thus, the absolute configuration of **5** was determined as 7*R*, 8*R*. On the basis of the above data, the structure of **5**, a eusiderin derivative,²⁹ was defined and assigned the trivial name eusiderin N.

Compound **6**, a white powder, has a molecular formula of C₂₆H₃₄O₁₁ determined from the HRESIMS spectrum (*m/z* 545.1996 [*M* + Na]⁺). The IR spectrum displayed bands for hydroxy (3437 cm^{–1}), double-bond (1640 cm^{–1}), and aromatic

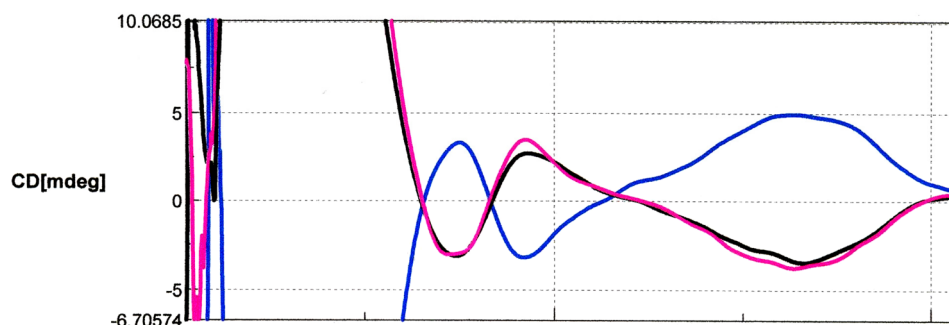


Figure 4. ECD spectra of compounds **1** (red), **1a** (blue), and **2** (black).

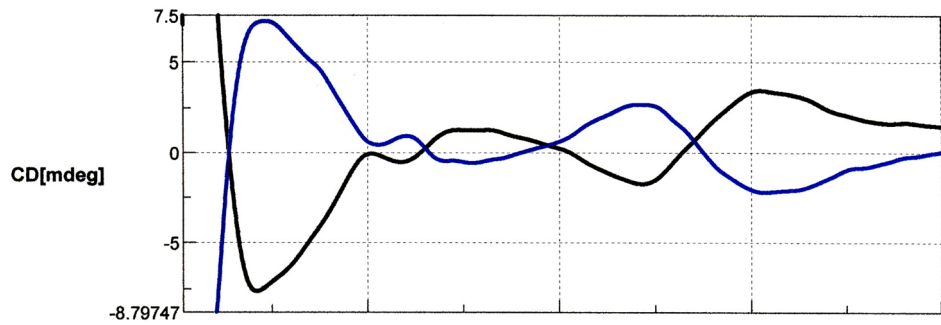


Figure 5. ECD spectra of compounds 3 (blue) and 4 (black).

Table 1. ^1H NMR Spectroscopic Data (δ) of 1–6 and 1a (J in Hz)^a

position	1	1a	2	3	4	5	6
2				6.31, s	6.29, s	6.68, s	6.72, s
5	6.22, s	6.24, s	6.24, s				
6				6.31, s	6.29, s	6.68, s	6.72, s
7	4.70, s	4.56, s	4.59, s	4.90, overlapped	4.81, overlapped	4.56, d (7.8)	5.55, d (6.0)
8	2.29, brt (4.2)	2.15, brt (4.2)	2.35, t (6.8)	3.36, m	3.42, m	4.14, m	3.45, m
9	3.86, brd (9.2)	3.61, m	3.89, m	3.51, m	3.72, dd (10.2, 4.8)	1.19, d (6.6)	3.86, m
	3.58, dd (9.6, 6.8)	3.52, dd (10.8, 7.6)	3.63, m	3.36, m	3.20, m		3.75, dd (11.2, 8.0)
2'						6.65, d (2.4)	6.74, brs
6'	6.42, s	6.44, s	6.45, s	6.98, s	6.98, s	6.49, d (2.4)	6.71, brs
7'	3.00, dd (17.6, 7.2)	3.10, dd (17.2, 7.2)	3.11, dd (17.6, 7.6)	7.52, s	7.51, s	2.56, m, 2H	2.62, m, 2H
	2.80, d (17.6)	2.83, d (17.2)	2.82, d (17.6)				
8'	2.22, brd (5.2)	2.21, brd (6.4)	2.24, brd (6.4)			1.80, m, 2H	1.80, m, 2H
9'	4.33, dd (12.0, 2.4)	4.38, dd (12.0, 2.8)	4.36, dd (12.0, 2.8)	9.45, s	9.46, s	3.54, t (6.6), 2H	3.55, m, 2H
	3.74, m	3.74, dd (12.0, 8.0)	3.56, m				
1''	4.24, d (7.6)		4.26, d (7.6)	4.36, d (7.8)	4.23, d (7.8)	4.91, d (7.8)	4.98, d (8.4)
2''	3.20, m		3.20, m	3.27, t (8.4)	3.25, m	3.50, t (8.4)	3.54, m
3''	3.34, m		3.33, m	3.37, m	3.33, m	3.46, t (8.4)	3.45, t (8.0)
4''	3.35, m		3.27, m	3.33, m	3.28, m	3.38, t (8.4)	3.57, m
5''	3.15, m		3.25, m	3.23, m	3.25, m	3.41, m	3.95, dd (11.6, 4.8)
							3.16, dd (12.0, 8.4)
6''	3.77, m		3.83, m	3.77, dd (12.0, 2.4)	3.82, dd (12.0, 3.6)	3.88, dd (12.0, 2.4)	
	3.63, m		3.63, m	3.63, dd (12.0, 5.4)	3.64, dd (12.0, 6.0)	3.69, dd (12.6, 5.4)	
2-OMe	3.89, s, 3H	3.89, s, 3H	3.90, s, 3H				
3-OMe				3.69, s, 3H	3.69, s, 3H	3.85, s, 3H	3.80, s, 3H
4-OMe	3.67, s, 3H	3.71, s, 3H	3.70, s, 3H				
5-OMe				3.69, s, 3H	3.69, s, 3H	3.85, s, 3H	3.80, s, 3H
3'-OMe	3.32, s, 3H	3.36, s, 3H	3.35, s, 3H	3.54, s, 3H	3.59, s, 3H		3.87, s, 3H
5'-OMe	3.74, s, 3H	3.78, s, 3H	3.77, s, 3H	3.93, s, 3H	3.93, s, 3H		

^a1, 1a, 2, and 6, 400 MHz, 3–5, 600 MHz, all in methanol-*d*₄.

(796 cm^{-1}) groups. The UV spectrum showed absorption maxima at 280 and 230 (sh) nm. The ^1H and ^{13}C NMR spectra showed the presence of two 1,3,4,5-tetrasubstituted benzene rings, a 1-propanol and a 1,3-propanediol fragment, one pentose moiety, and three aromatic methoxy groups. These data also corresponded to a lignan glycoside. The pentose was determined as β -D-xylose by the acid hydrolysis method. In the HMBC spectrum, correlations between H-7/C-4', H-7/C-3', H-8/C-2', H-8/C-3', H-8/C-4', and H-1''/C-4 indicated that the 1-phenylpropane-1,3-diol and 3-phenylpropan-1-ol fragments were linked via 7-O-4' and 8–3', which constitutes a benzofuran neolignan, and the β -D-xylose moiety was connected at C-4. The relative configuration of H-7 and H-8 was determined as *trans*, based on association of H-9 and H-7 in the NOE spectrum. The ECD spectrum of 6 showed a positive Cotton effect at 232 nm and a negative effect at 217

nm, indicating a 7*S*,8*R* absolute configuration.³⁰ Consequently, compound 6 was determined as (7*S*,8*R*)-3,5,5'-trimethoxy-4',7-epoxy-8,3'-neolignan-4,9,9'-triol-4-*O*- β -D-xylopyranoside.

Compound 7 was obtained as a white powder. The HRESIMS indicated a molecular formula of $\text{C}_{15}\text{H}_{16}\text{O}_{10}$ (m/z 379.0656 [$\text{M} + \text{Na}]^+$). The IR spectrum displayed bands for hydroxy (3384 cm^{-1}), carbonyl (1657 cm^{-1}), double-bond (1618 cm^{-1}), and aromatic (1592, 1509, 1451 cm^{-1}) groups, and the UV spectrum showed absorption maxima at 330 (sh), 300, 262 (sh), and 252 nm. In the ^1H NMR spectrum, resonances were observed for two *meta*-coupled aromatic protons at δ_{H} 6.34 (d, $J = 2.0$ Hz) and 6.21 (d, $J = 2.0$ Hz), one double-bond proton at δ_{H} 8.23 (s), and a series of proton resonances for a β -glucopyranose moiety. In the ^{13}C NMR spectrum, the corresponding resonances together with a conjugated carbonyl carbon at δ_{C} 178.4 were found. From

Table 2. ¹³C NMR Spectroscopic Data (δ_C, mult.) for 1–6 and 1a^a

C	1	1a	2	3	4	5	6
1	125.5, qC	125.6	125.4	136.3, qC	136.0	129.2, qC	147.4, qC
2	146.7, qC	146.8	146.8	106.2, CH	106.2	105.9, CH	103.9, CH
3	136.8, qC	136.7	136.7	149.0, qC	149.0	149.4, qC	154.5, qC
4	147.8, qC	147.8	147.9	135.0, qC	135.1	137.2, qC	134.7, qC
5	102.3, CH	102.2	102.3	149.0, qC	149.0	149.4, qC	154.5, qC
6	153.9, qC	153.9	153.9	106.2, CH	106.2	105.9, CH	103.9, CH
7	31.3, CH	31.3	31.4	38.6, CH	38.7	82.3, CH	88.6, CH
8	42.9, CH	45.1	42.5	40.9, CH	41.4	75.5, CH	55.8, CH
9	73.5, CH ₂	65.2	72.5	69.2, CH ₂	70.6	17.4, CH ₃	65.1, CH ₂
1'	127.4, qC	127.5	127.6	124.2, qC	124.2	135.7, qC	137.2, qC
2'	124.0, qC	124.0	123.9	127.1, qC	127.1	110.8, CH	114.2, CH
3'	147.3, qC	147.4	147.3	147.8, qC	147.8	147.1, qC	129.5, qC
4'	138.2, qC	138.2	138.2	135.7, qC	136.0	133.3, qC	140.3, qC
5'	148.4, qC	148.5	148.5	149.4, qC	149.4	146.0, qC	145.3, qC
6'	107.4, CH	107.3	107.5	109.8, CH	109.8	112.0, CH	117.9, CH
7'	30.2, CH ₂	30.0	30.0	149.5, CH	149.6	32.7, CH ₂	32.9, CH ₂
8'	35.6, CH	35.3	35.3	136.3, qC	136.2	35.4, CH ₂	35.8, CH ₂
9'	81.3, CH ₂	81.5	81.4	194.3, CH	194.4	62.6, CH ₂	62.2, CH ₂
1''	104.6, CH		104.3	103.9, CH	105.3	102.8, CH	104.8, CH
2''	75.3, CH		75.2	75.1, CH	75.2	74.9, CH	74.3, CH
3''	78.0, CH		78.2	78.3, CH	78.0	77.9, CH	76.1, CH
4''	71.2, CH		71.6	71.5, CH	71.7	71.4, CH	71.0, CH
5''	77.5, CH		77.9	77.9, CH	78.0	78.3, CH	66.3, CH ₂
6''	62.3, CH ₂		62.8	62.6, CH ₂	62.9	62.1, CH ₂	
2-OMe	62.1, CH ₃	61.7	62.0				
3-OMe				56.7, CH ₃	56.7	56.8, CH ₃	56.7, CH ₃
4-OMe	56.5, CH ₃	56.5	56.5				
5-OMe				56.7, CH ₃	56.7	56.8, CH ₃	56.7, CH ₃
3'-OMe	60.0, CH ₃	60.0	60.1	61.0, CH ₃	61.0		56.8, CH ₃
5'-OMe	56.4, CH ₃	56.5	56.5	56.8, CH ₃	56.8		

^aCompounds 1, 1a, 2, and 6, 100 MHz, 3, 4, and 5 150 MHz, all in methanol-*d*₄.

the HMBC spectrum, correlations of H-6/C-5, C-7, and C-10; H-8/C-7, C-9, and C-10; H-2/C-3, C-4, and C-9; and H-1' (glucose)/C-3 were observed. Thus, the skeleton is 3,5,7-trihydroxy-4*H*-chromen-4-one, and the glucose moiety is located at C-3. Accordingly, the structure of 7 was determined as 5,7-dihydroxy-4*H*-chromen-4-one-3-*O*-β-*D*-glucopyranoside.

Compound 8 was obtained as a white powder, and its molecular formula is C₁₄H₁₄O₉ (HRESIMS *m/z* 349.0559 [M + Na]⁺). The UV and IR spectra of 8 were similar to those of compound 7. A comparison of the NMR spectra of 7 and 8 showed that resonances for the glucose moiety in 7 were replaced by those for a pentose moiety in 8. Furthermore, the pentose moiety was determined as β-*D*-xylopyranose. Thus, the structure of compound 8 was defined as 5,7-dihydroxy-4*H*-chromen-4-one-3-*O*-β-*D*-xylopyranoside.

Moreover, 25 known compounds were also isolated from the 95% aqueous EtOH extract of *E. japonica*. The structures of isolates 9–33 were identified by comparing their NMR and MS data with reported analytical data.

Compounds 1, 2, 7–9, 12–26, and 29–33 were evaluated for antioxidant activity by using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method. As shown in Table 4, compounds 1, 2, 12–20, and 29 (ED₅₀ = 23.40 ± 0.46 to 44.80 ± 0.38 μM) had potent antioxidant activities compared with the positive control α-tocopherol (ED₅₀ = 27.21 ± 0.02 μM).

Fifteen compounds (1, 2, 7–9, 12–20, and 32) were also evaluated for inhibitory effects on LPS-induced NO production in RAW 264.7 macrophages, using quercetin as the positive

Table 3. ¹H and ¹³C NMR Spectroscopic Data (δ in ppm, in methanol-*d*₄, 400 MHz for ¹H, 100 MHz for ¹³C) for 7 and 8

position	7		8	
	δ _C , type	δ _H (J in Hz)	δ _C , type	δ _H (J in Hz)
2	147.4, CH	8.23, s	147.4, CH	8.13, s
3	141.5, qC		141.2, qC	
4	178.4, qC		178.4, qC	
5	163.4, qC		163.4, qC	
6	100.2, CH	6.21, d (2.0)	100.2, CH	6.22, d (2.0)
7	166.5, qC		166.4, qC	
8	95.0, CH	6.34, d (2.0)	95.0, CH	6.33, d (2.0)
9	159.4, qC		159.3, qC	
10	106.1, qC		106.2, qC	
1'	104.2, CH	4.73, d (7.6)	104.6, CH	4.73, d (7.2)
2'	74.8, CH	3.42, m	74.6, CH	3.40, m
3'	77.4, CH	3.42, m	77.1, CH	3.40, m
4'	71.3, CH	3.30, m	70.9, CH	3.54, m
5'	78.5, CH	3.38, m	67.1, CH ₂	3.93, dd (11.2, 5.2)
				3.30, overlapped
6'	62.6, CH ₂	3.90, dd (12.4, 2.0)		
		3.67, dd (12.0, 6.4)		

control (IC₅₀ = 1.30 ± 0.44 μM). As shown in Table 5, the inhibitory percentages of compounds 7 and 32 exceeded 50%, while those of the other tested compounds were below 50%

Table 4. Antioxidant Activity of Compounds 1, 2, 7–9, 12–26, and 29–33^a

compound	ED ₅₀ (μM)	compound	ED ₅₀ (μM)	compound	ED ₅₀ (μM)
1	23.40 ± 0.46	16	26.47 ± 0.71	25	—
2	24.70 ± 0.26	17	30.39 ± 0.64	26	—
7	—	18	25.23 ± 1.26	29	32.95 ± 0.69
8	—	19	33.55 ± 0.79	30	—
9	133.34 ± 4.54	20	36.21 ± 0.08	31	—
12	37.65 ± 0.09	21	—	32	—
13	26.59 ± 1.65	22	—	33	—
14	44.80 ± 0.38	23	—		
15	26.61 ± 0.96	24	—		

^a“—” means the ED₅₀ (μg/mL) value is >100. Positive control: (±)-α-tocopherol ED₅₀ = 27.21 ± 0.02 μM.

Table 5. Anti-NO Production Activity of Compounds 1, 2, 7–9, 12–20, and 32

compound	anti-NO (%) ^a	cell viability (%) ^a	compound	anti-NO (%) ^a	cell viability (%) ^a
1	38.99 ± 1.95	106.21 ± 4.12	15	35.32 ± 5.08	107.73 ± 0.6
2	40.37 ± 1.94	110.30 ± 0.15	16	36.70 ± 0.01	102.17 ± 0.08
7	53.79 ± 1.78	105.03 ± 2.53	17	33.26 ± 4.22	105.44 ± 5.21
8	36.24 ± 1.29	102.23 ± 0.38	18	37.84 ± 6.16	107.31 ± 2.79
9	38.07 ± 1.94	106.42 ± 2.15	19	35.55 ± 2.91	101.45 ± 7.60
12	39.22 ± 1.62	100.62 ± 7.29	20	35.78 ± 5.18	104.71 ± 12.67
13	38.76 ± 2.27	104.26 ± 0.15	32	56.19 ± 2.27	60.31 ± 0.76
14	39.68 ± 2.27	112.41 ± 3.29			—

^aCells were treated with LPS (1 μg) in combination with test compound (20 μg/mL) for 24 h. Positive control: Quercetin IC₅₀ = 1.30 ± 0.44 μM.

(20 μg/mL). Yet, the cell viability with compound 32 was 60.31%, measured by the MTT assay, indicating that the anti-NO production effect resulted from cell death. Compared with the positive control, these flavonol glycosides, lignan glycosides, and chromone glycosides were weak inhibitors of LPS-induced NO production.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained on a JASCO P-2000 polarimeter. ECD spectra were measured with a JASCO J-715 spectropolarimeter. IR spectra were recorded neat, using an IR-FT Mattson Genesis II spectrometer. NMR spectra were recorded using Bruker UltraShield 400 MHz and Varian Inova-600 MHz spectrometers. HRESIMS spectra were recorded on a Shimadzu IT-TOF HR mass spectrometer. For column chromatography, silica gel 60 (70–230, 230–400 mesh, Merck) and Sephadex LH-20 (GE Healthcare) were used. Precoated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 5% H₂SO₄ followed by heating at 110 °C. MPLC was performed on a system equipped with a Buchi B-688 pump, Buchi B-684 fraction collector, and Buchi columns. HPLC separations were performed on a Hitachi L-2130 pump with an L-2450 diode array detector, equipped with a 250 × 10 mm i.d. preparative Cosmosil 5C₁₈ AR-II column (Nacalai Tesque, Inc.).

Plant Material. Stems of *E. japonica* (20 kg) were collected at Yangming Mountain, Taiwan, in April 2008, and identified by one of the authors (Y.-H.K.). A voucher specimen (No. NRICM20080410B) has been deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation. The stems of *E. japonica* (20 kg) were extracted with 95% aqueous EtOH at 55 °C (3 × 80 L) in an airtight heating extraction tank, and the extract was concentrated under reduced pressure. The EtOH extract (1500 g) was suspended in H₂O, and the suspension was extracted successively with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc layer gave 247.3 g of extract that was separated on an MPLC silica gel column, eluted with CH₂Cl₂/MeOH (10/0, 9/2, 8/2, 7/3), to yield four fractions (E1 to E4). Fraction E2 (CH₂Cl₂/MeOH, 9/2, 90 g) gave an insoluble precipitate, which was identified as compound 32 (about 40 g, 0.2%), and a soluble part,

which was subjected to column chromatography (CC) on Sephadex LH-20, eluting with CHCl₃/MeOH (1/1), to afford compound 33 (65.4 mg, 0.000327%). Fraction E3 (CH₂Cl₂/MeOH, 8/2, 52.3 g) was separated into five subfractions (E3.1 to E3.5) by CC on Sephadex LH-20, eluting with 100% MeOH. Fraction E3.2 (12.78 g) was then separated into four subfractions (E3.2.1 to E3.2.4) by CC on Sephadex LH-20, eluting with CHCl₃/MeOH (1/1). Finally, fraction E3.2.2 (3.24 g) was separated into four subfractions (E3.2.2.1 to E3.2.2.4) by Sephadex LH-20 CC, eluting with 100% MeOH. Fraction E3.2.2.2 (1.79 g) was applied to an ODS open column, eluting with gradient mobile phase (10% to 100% MeOH in H₂O, v/v), to give eight subfractions, E3.2.2.2.1 to E3.2.2.2.8. Fraction E3.2.2.2.1 (0.1296 g) was subjected to semipreparative HPLC [250 × 10 mm i.d., Cosmosil 5 C₁₈ AR-II column (also used for all HPLC experiments), MeCN/H₂O, using the concentration gradient 10–20% for 20 min, 20–30% for 10 min, 100% for 10 min, flow rate 2 mL/min] to afford compounds 21 (*t*_R: 11.9 min, 1.3 mg, 0.0000065%), 22 (*t*_R: 15.1 min, 3.9 mg, 0.0000185%), and 23 (*t*_R: 19.0 min, 0.9 mg, 0.0000045%). Fraction E3.2.2.2.2 (0.2722 g) was purified by semipreparative HPLC with MeCN/H₂O (17–30% for 30 min, 100% for 10 min, flow rate 2 mL/min) to give 11 subfractions (E3.2.2.2.2.1 to E3.2.2.2.2.11). Fraction E3.2.2.2.2.5 was purified by semipreparative HPLC (MeOH/H₂O, 4/1, flow rate 2.5 mL/min) to afford compounds 4 (*t*_R: 14.0 min, 0.7 mg, 0.0000035%) and 3 (*t*_R: 14.6 min, 1.0 mg, 0.0000050%). Fraction E3.2.2.2.3 (0.4222 g) was purified by semipreparative HPLC (MeCN/H₂O, 21% for 30 min, 100% for 10 min, flow rate 2 mL/min), to give 11 subfractions (E3.2.2.2.3.1 to E3.2.2.2.3.11). By using preparative TLC (CHCl₃/MeOH/EtOAc/acetone, 4.5/1/1/1), compound 6 (4.6 mg, 0.000023%) was obtained from fraction E3.2.2.2.3.7, and compounds 10 (0.8 mg, 0.000004%) and 5 (0.6 mg, 0.000003%) were obtained from fraction E3.2.2.2.3.8. Preparative TLC with a different mobile solvent system (CHCl₃/MeOH/EtOAc/acetone, 5/1/1/1) was used for the purification of compound 2 (23 mg, 0.000115%) from fraction E3.2.2.2.3.9 and compounds 1 (75.5 mg, 0.000378%) and 11 (2.3 mg, 0.000012%) from fraction E3.2.2.2.3.10. Fraction E3.2.3 (5.15 g) was separated into five subfractions (E3.2.3.1 to E3.2.3.5) by Sephadex LH-20 CC, eluting with 100% MeOH. Fraction E3.2.3.3 (3.45 g) was applied to an ODS open column, eluting with gradient mobile phase (10–100% MeOH in H₂O, v/v), to afford nine subfractions (E3.2.3.3.1 to E3.2.3.3.9). Fraction E3.2.3.3.2

(0.6815 g) was subjected to semipreparative HPLC (MeCN/H₂O, 10% for 5 min, 10–20% for 15 min, 20–30% for 10 min, 100% for 5 min, flow rate 2 mL/min) to afford 11 subfractions (E3.2.3.3.2.1 to 3.2.3.3.2.11). Fraction E3.2.3.3.2.2 was purified by semipreparative HPLC (MeOH/H₂O, 3/7, flow rate 2 mL/min) to give **28** (t_R : 32.0 min, 0.9 mg, 0.0000045%) and **27** (t_R : 33.8 min, 1.5 mg, 0.0000075%). Compounds **24** (42.0 mg, 0.00021%) and **13** (27.8 mg, 0.00014%) were obtained from fractions E3.2.3.3.3 and E3.2.3.3.4, respectively, by recrystallization with MeOH. Fractions E3.2.3.3.5 and E3.2.3.3.6 were purified by preparative TLC (CHCl₃/MeOH/EtOAc/acetone, 4.5/1/1/1) to afford **26** (14.6 mg, 0.000073%) and **25** (20 mg, 0.0001%), respectively. Fraction E3.2.3.4 was further purified by preparative TLC (CHCl₃/MeOH, 4/1) to give **29** (28.3 mg, 0.000142%) and **30** (20.1 mg, 0.000101%). Fraction E3.3 (5 g, total 22.64 g) was subjected to Sephadex LH-20 CC, eluting with 100% MeOH, to yield five fractions (E3.3.1 to E3.3.5). Fraction E3.3.2 (1.5 g) was purified by semipreparative HPLC (MeCN/H₂O, 13.5/86.5, flow rate 3 mL/min) to give **31** (t_R : 9.4 min, 28.3 mg, 0.000642%) and **7** (t_R : 15.3 min, 381 mg, 0.00861%). Fraction E3.3.3 (1.2 g) was purified by semipreparative HPLC (MeCN/H₂O, 15–17% for 20 min, 17–45% for 20 min, 100% for 10 min, 3 mL/min) to yield compounds **8** (t_R : 13.9 min, 449 mg, 0.010147%) and **9** (t_R : 18.6 min, 44.0 mg, 0.000994%). Fraction E3.3.4 (0.6 g) was purified by semipreparative HPLC (MeCN/H₂O, 17% for 20 min, 25% for 20 min, 100% for 10 min, flow rate 3 mL/min) to yield **17** (t_R : 18.1 min, 120 mg, 0.0027%), **18** (t_R : 19.7 min, 50 mg, 0.00112%), **15** (t_R : 28.6 min, 18.8 mg, 0.000424%), and **16** (t_R : 30.6 min, 45.3 mg, 0.001021%). Fraction E4 (eluted with CH₂Cl₂/MeOH, 7/3, 57 g) was subjected to Sephadex LH-20, eluting with 100% MeOH, to afford five fractions (E4.1 to E4.5). Fraction E4.4 (0.5 g, total 15.9 g) was purified by semipreparative HPLC (MeCN/H₂O, 10% for 25 min, 25% for 20 min, flow rate 3 mL/min) to afford compounds **19** (t_R : 22.1 min, 53.4 mg, 0.00849%), **20** (t_R : 26.5 min, 20.5 mg, 0.00327%), **14** (t_R : 34.1 min, 57.2 mg, 0.00909%), and **12** (t_R : 36.1 min, 23.4 mg, 0.00372%).

(+)-Ovafolinin B-9'-O-β-D-glucopyranoside (1): pale yellow, amorphous solid; $[\alpha]_D^{25}$ +193.1 (c 0.2, MeOH); ECD $[\theta]_{285}$ −12869.6, $[\theta]_{246}$ +11727, and $[\theta]_{237}$ −13010.5 (c 2.5×10^{-5} M, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 283 (3.76) nm; IR ν_{max} (neat) 3382, 2931, 1616, 1497, 1457, 1121, 1085, 807 cm^{−1}; ¹H NMR and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z 579.2099 [M − H][−] (calcd for C₂₈H₃₅O₁₃, 579.2077).

(−)-Ovafolinin B-9'-O-β-D-glucopyranoside (2): pale yellow, amorphous solid; $[\alpha]_D^{25}$ −127 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ): 283 (3.92) nm; ECD $[\theta]_{284}$ +19052.5, $[\theta]_{246}$ −12866.8, and $[\theta]_{237}$ +15282.2 (c 2.5×10^{-5} M, MeOH); IR ν_{max} (neat) 3382, 2927, 1616, 1497, 1461, 1125, 1077, 800 cm^{−1}; ¹H NMR and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z 579.2090 [M − H][−] (calcd for C₂₈H₃₅O₁₃, 579.2077).

(+)-Ovafolinin E-9'-O-β-D-glucopyranoside (3): yellow, amorphous solid; $[\alpha]_D^{25}$ +64.9 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 354 (3.66), 320 (3.56, sh), 270 (3.51, sh), and 254 (3.87) nm; ECD $[\theta]_{357}$ +6446.9, $[\theta]_{323}$ −3559.6, $[\theta]_{276}$ +2323.68, and $[\theta]_{261}$ −938.1 (c 5.0×10^{-5} M, MeOH); IR ν_{max} (neat) 3422, 2966, 2930, 1683, 1572, 1461, 1350, 1085, 796 cm^{−1}; ¹H NMR and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z 577.1935 [M − H][−] (calcd for C₂₈H₃₃O₁₃, 577.1920).

(−)-Ovafolinin E-9'-O-β-D-glucopyranoside (4): yellow, amorphous solid; $[\alpha]_D^{25}$ −5.9 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 354 (3.61), 320 (3.53, sh), 270 (3.54, sh), and 253 (3.84) nm; ECD $[\theta]_{357}$ −4589.6, $[\theta]_{324}$ +5289.7, $[\theta]_{276}$ −1080.0, and $[\theta]_{261}$ +1838.2 (c 5.0×10^{-5} M, MeOH); IR ν_{max} (neat) 3399, 2961, 2918, 1686, 1556, 1259, 1089, 800 cm^{−1}; ¹H NMR and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z 577.1932 [M − H][−] (calcd for C₂₈H₃₃O₁₃, 577.1920).

Eusiderin N (5): white powder, mp 195 °C; $[\alpha]_D^{25}$ −5.0 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 273 (3.48), 230 (4.25, sh) nm; ECD $[\theta]_{236}$ −15 926; IR ν_{max} (KBr) 3407, 2928, 1596, 1560, 1505, 1081, 800 cm^{−1}; ¹H NMR and ¹³C NMR data, Tables 1 and 2; HRESIMS m/z 561.1964 [M + Na]⁺ (calcd for C₂₆H₃₄O₁₂Na, 561.1950).

(7S,8R)-3,5,5'-Trimethoxy-4',7-epoxy-8,3'-neolignan-4,9,9'-triol-4-O-β-D-xylopyranoside (6): white powder, mp 218 °C; $[\alpha]_D^{25}$ −19.1 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 280 (2.98), 230 (3.70, sh) nm; ECD $[\theta]_{232}$ +846.8, $[\theta]_{217}$ −3021.2; IR ν_{max} (KBr) 3437, 2966, 1640, 1025, 796 cm^{−1}; ¹H NMR and ¹³C NMR data, Tables 1 and 2; HRESIMS m/z 545.1996 [M + Na]⁺ (calcd for C₂₆H₃₄O₁₁Na, 545.2001).

5,7-Dihydroxy-4H-chromen-4-one-3-O-β-D-glucopyranoside (7): white powder, mp 202 °C; $[\alpha]_D^{25}$ −68.7 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 330 (3.46, sh), 300 (3.60), 262 (3.98, sh), and 252 (3.99) nm; IR ν_{max} (KBr) 3384, 1657, 1618, 1592, 1509, 1451, 1206, 1074, 800 cm^{−1}; ¹H NMR and ¹³C NMR data, Table 3; HRESIMS m/z 379.0656 [M + Na]⁺ (calcd for C₁₅H₁₆O₁₀Na, 379.0643).

5,7-Dihydroxy-4H-chromen-4-one-3-O-β-D-xylopyranoside (8): white powder, mp 208 °C; $[\alpha]_D^{25}$ −70.6 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 330 (3.63, sh), 300 (3.84), 262 (4.20, sh), and 252 (4.22) nm; IR ν_{max} (KBr) 3368, 1655, 1616, 1588, 1460, 1204, 1038, 803 cm^{−1}; ¹H NMR and ¹³C NMR data, Table 3; HRESIMS m/z 349.0559 [M + Na]⁺ (calcd for C₁₄H₁₄O₉Na, 349.0538).

Ovafolinin B (1a): pale yellow, amorphous powder, mp 125 °C; $[\alpha]_D^{25}$ +176.8 (c 0.2, MeOH); UV λ_{max} (MeOH) nm (log ϵ) 284 (3.66) nm; ECD $[\theta]_{285}$ −13 892, and $[\theta]_{246}$ +14 826.2 (c 2.5×10^{-5} M, MeOH); ¹H NMR and ¹³C NMR data, see Tables 1 and 2.

Scavenging Activity of 1,1-Diphenyl-2-picrylhydrazyl Radical. The radical scavenging activities of the 25 compounds on the DPPH free radical were measured using the method of Rangkadilok et al.³¹ and Chung et al.³² with minor modifications. An aliquot (120 μL) of each compound (100–10 μg/mL) or (±)-α-tocopherol (40–10 μg/mL, Fluka Biochemika, ≥97.0% HPLC) was mixed with 30 μL of 0.75 mM DPPH methanol solution in a 96-well microplate. The mixture was shaken vigorously with an orbital shaker in the dark at room temperature for 30 min, and then the absorbance was measured at 517 nm with an ELISA reader. (±)-α-Tocopherol was used as the positive control. The negative control contained no test compound. The final results were reported as ED₅₀, the concentration of compound that scavenged 50% of DPPH radicals in the reaction solution.

Cell Culture and NO Measurement. The macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD, USA), cultured in DMEM containing 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and grown at 37 °C with 5% CO₂ in fully humidified air. Cells were plated at a density of 2×10^5 cells/well in 96-well culture plates and stimulated with LPS (1000 ng/mL) in the presence (20 μg/mL) or absence of test compound for 24 h. All compounds were dissolved in DMSO and further diluted with sterile PBS. Nitrite (NO₂[−]) accumulation in the medium was used as an indicator of NO production, which was measured by adding the Griess reagent (1% sulfanilamide and 0.1% naphthylendiamine in 5% phosphoric acid). NaNO₂ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm. All experiments were performed in triplicate. NO production by LPS stimulation was designated as 100% for each experiment. Quercetin (Sigma, 98.0% HPLC) was employed as a positive control.³³

Acid Hydrolysis of Glycosides. A methanol solution of compound **1** (3.0 mg) was placed on a TLC plate (20 × 10 cm), and this plate was placed in an atmosphere of concentrated HCl for 30 min. The HCl and H₂O were then evaporated in a vacuum oven. The plate was developed in CHCl₃/MeOH (3/1) (saturated with H₂O). The aglycone band was detected by UV absorption at 254 nm. To detect the glycone band, part of the TLC plate was cut off, sprayed with 5% H₂SO₄ in EtOH, and then heated at 110 °C. The aglycone and glycone bands were removed from the TLC plate. The glycone was dissolved in EtOH and subjected to HPLC (Shimadzu HPLC equipped with LC-20AT pump, RID-10A detector, and SCL-10AVP system controller; column: Chiralpak AD-H column (Daicel), 4.6 × 250 mm, 5 μm; mobile phase: EtOH/*n*-hexane:TFA, 3/7/0.05 (v/v); flow rate: 0.5 mL/min).³⁴ The sugar was identified by comparison with authentic samples: t_R (min) 14.23 (D-glucose), 14.69 (L-glucose);

18.96 (D-xylose), 18.35 (L-xylose). Compounds **2**, **6**, **7**, and **8** were treated in the same manner. All compounds produced either D-glucose or D-xylose. Therefore, although compounds **3**, **4**, and **5** were not obtained in sufficient quantity to perform the acid hydrolysis experiment, we postulated they would also contain D-glucose.

■ ASSOCIATED CONTENT

● Supporting Information

¹H and ¹³C NMR spectra of compounds **1–8** are available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +886-02-27361661, ext. 6126. E-mail: hsuoho@tmu.edu.tw (H.O.H.). Tel: +886-2-28201999, ext. 7051. Fax: +886-2-28236150. E-mail: kuoyh@nricm.edu.tw (K.Y.H.).

Author Contributions

[†]Li-Ming Yang Kuo and Li-Jie Zhang contributed equally to this paper.

Notes

The authors declare no competing financial interest.

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