JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

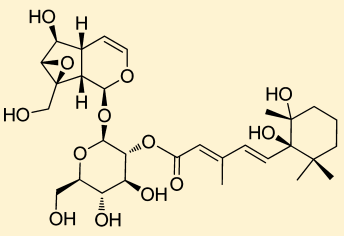
Bioactive Iridoid Glycosides from the Whole Plants of *Rehmannia chingii*

Yan-Fei Liu, Guo-Ru Shi, Xin Wang, Chun-Lei Zhang, Yan Wang, Ruo-Yun Chen, and De-Quan Yu*

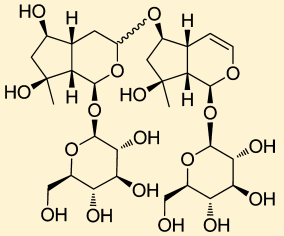
State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, People's Republic of China

Supporting Information

ABSTRACT: Nine new iridoid glycosides, rehmachingiiosides A–I (**1**–**9**), together with 16 known analogues, were isolated from the whole plants of *Rehmannia chingii*. The structures of compounds **1**–**9** were elucidated on the basis of spectroscopic data analysis and from chemical evidence. Furthermore, in two vitro assays, compounds **5** and **10** showed an inhibitory effect on LPS-induced NO production with IC_{50} values of 2.5 and 7.3 μM, and compounds **4**, **6**, and **10**–**12** (when evaluated at 10 μM) exhibited evidence of hepatoprotective effects against APAP-induced HepG2 cell damage.

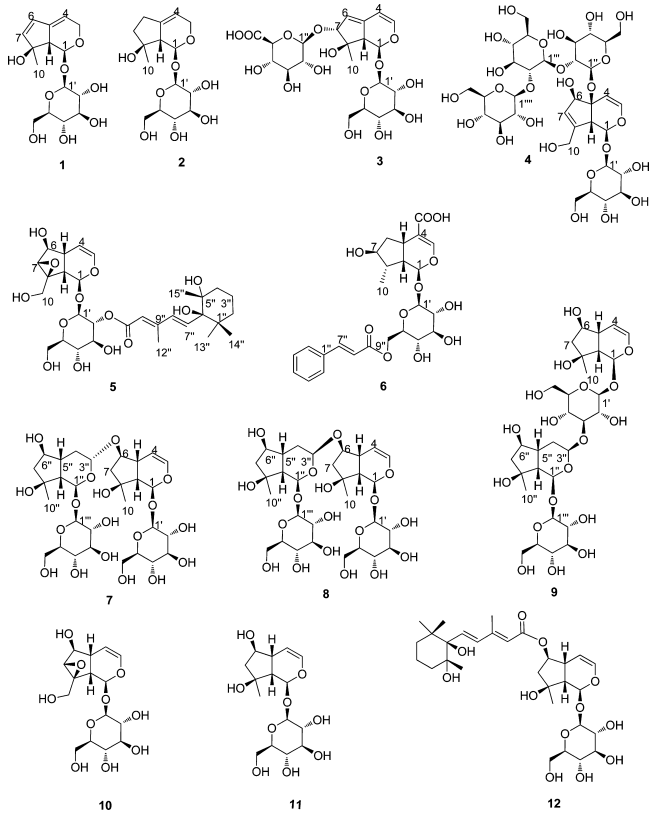


5



7, **8**

The genus *Rehmannia* belongs to the family Scrophulariaceae and consists of six species distributed mainly in eastern Asia. *Rehmannia glutinosa* is the most widely used species in the genus, and its rhizomes are known as "Dihuang" and used for treatment of a variety disorders in traditional Chinese medicine.[1] Previous phytochemical investigations on this genus have led to the isolation and identification of iridoid glycosides, ionone glycosides, phenethyl alcohol glycosides, and several other components.[2−5] *Rehmannia chingii* H. L. Li, an endemic species, is mainly distributed in Zhejiang Province in mainland China and has been used as a folk medicine for the alleviation of fever and bleeding.[6] However, chemical and biological studies of this plant have not yet been investigated. As part of ongoing research to identify bioactive substances from the genus *Rehmannia*, an aqueous extract of the whole plants of *R. chingii* has been examined. Described herein are the isolation, structure elucidation, and biological testing of nine new iridoid glycosides, rehmachingiiosides A–I (**1**–**9**), and 16 known analogues.



1 **2** **3** **4** **5** **6** **7** **8** **9** **10** **11** **12**

RESULTS AND DISCUSSION

Compound **1** (rehmachingiioside A) was isolated as a white, amorphous powder. A $C_{15}H_{22}O_8$ molecular formula was deduced from the sodiated molecular ion peak at m/z 353.1211 (calcd 353.1207) observed in the HRESIMS, in conjunction with the ^{13}C NMR spectroscopic data. The ^1H NMR spectrum displayed resonances for a tertiary methyl group at δ_H 1.30, a methine proton at δ_H 2.72, an oxygen-bearing methylene group at δ_H 4.27 and 4.42, two coupled olefinic protons as doublets at δ_H 5.93 and 6.21, an olefinic proton at δ_H 5.56, and an acetal proton at δ_H 5.06. The ^1H NMR data of **1** also showed the presence of a β-glucopyranose moiety, the anomeric proton of which resonated at δ_H 4.73 (1H, d, J = 8.4 Hz, H-1′). Consistent with these observations, the ^{13}C NMR spectrum showed 15 carbon resonances, including one methyl, one oxygenated methylene, one methine, one oxygenated quaternary carbon, four olefinic carbons (δ_C

Received: December 18, 2015

ACS Publications © XXXX American Chemical Society and American Society of Pharmacognosy

DOI: 10.1021/acs.jnatprod.5b01126
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. ^{1}H NMR Spectroscopic Data of Compounds 1−9[a]

position	1	2	3	4[b]	5[c]	6	7	8	9
1	5.06 d (7.8)	5.02 d (6.0)	5.22 d (9.0)	5.32 d (6.5)	5.01 d (9.6)	5.30 d (5.4)	5.45 d (1.5)	5.47 d (2.5)	5.46 d (2.0)
3α	4.27 dt (16.8, 2.4)	4.08 m	6.55 d (5.5)	6.48 d (6.0)	6.30 dd (6.0, 1.8)	7.38 s	6.18 dd (6.5, 2.0)	6.18 dd (6.0, 2.0)	6.16 dd (6.0, 2.0)
3β	4.42 dt (16.8, 2.4)	4.27 m							
4	5.56 dd (5.4, 2.4)	5.52 m	5.55 d (5.5)	5.22 d (6.5)	5.04 dd (6.0, 4.8)		5.01 dd (6.5, 2.5)	4.83 m	4.86 dd (6.0, 3.0)
5					3.23 m	3.02 m	2.91 dd (10.0, 2.5)	2.85 dd (9.5, 2.5)	2.71 dd (9.5, 2.5)
6α	6.21 d (6.0)	2.22 m	5.41 br s	4.51 br s	3.89 d (7.8)	1.76 dd (13.8, 6.6)	3.99 m	4.08 m	3.92 m
6β		2.45 m				2.01 m			
7α	5.93 d (6.0)	1.67 m	4.56 br s	5.78 br s	3.42 br s	3.79 m	2.11 dd (14.0, 6.0)	2.10 m	2.03 m
7β		1.83 m					1.84 m	1.75 m	1.80 m
8						2.09 m			
9	2.72 ddd (7.8, 5.4, 2.4)	2.46 m	2.66 d (9.0)	3.35 d (6.5)	2.40 dd (9.6, 7.8)	2.48 dt (5.4, 7.8)	2.49 br d (10.0)	2.49 dd (9.5, 2.0)	2.54 dd (9.5, 2.0)
10a	1.30 s	1.16 s	1.27 s	4.25 d (16.0)	4.21 d (13.2)	1.02 d (7.2)	1.32 s	1.33 s	1.32 s
10b				4.16 d (16.0)	3.72 d (13.2)				
1′	4.73 d (8.4)	4.69 d (7.8)	4.80 d (7.5)	4.64 d (7.0)	4.96 d (7.8)	4.70 d (7.8)	4.65 d (8.0)	4.65 d (8.0)	4.68 d (8.0)
2′	3.30 m	3.27 t (7.8)	3.36 m	3.24 dd (9.0, 8.0)	4.80 dd (9.6, 7.8)	3.24 t (8.4)	3.22 m	3.20 m	3.27 m
3′	3.40 t (8.4)	3.40 m	3.44 m	3.40 m	3.62 m	3.41 m	3.35 m	3.30 m	3.64 m
4′	3.35 t (8.4)	3.35 m	3.35 m	3.29 m	3.37 m	3.40 m	3.28 m	3.30 m	3.31 m
5′	3.30 m	3.29 m	3.33 m	3.40 m	3.38 m	3.57 m	3.29 m	3.31 m	3.34 m
6′a	3.85 dd (12.0, 1.8)	3.85 dd (12.0, 2.4)	3.87 d (11.0)	3.94 d (12.0)	3.93 d (11.4)	4.55 dd (12.0, 2.4)	3.89 m	3.90 m	3.88 dd (12.0, 2.0)
6′b	3.68 dd (12.0, 5.4)	3.70 dd (12.0, 5.4)	3.69 m	3.70 m	3.68 m	4.43 dd (12.0, 6.0)	3.65 m	3.65 m	3.66 m
1″			4.42 d (6.5)	4.76 d (8.0)			5.43 d (3.5)	5.13 d (6.0)	5.51 d (4.0)
2″α			3.33 m	3.41 m	1.17 m	7.60 m			
2″β					1.69 m				
3″α			3.52 m	3.60 m	1.88 m	7.40 t (7.8)	5.07 dd (8.0, 3.5)	4.93 dd (8.4, 4.0)	5.30 dd (8.0, 3.0)
3″β					1.37 m				
4″α			3.54 m	3.40 m	1.81 m	7.40 t (7.8)	1.38 m	1.93 m	2.03 m
4″β					1.48 m		1.90 m	1.30 m	1.40 m
5″			3.76 m	3.29 m		7.40 t (7.8)	2.47 m	2.60 m	2.49 m
6″a				3.87 d (12.0)		7.60 m	3.99 m	4.03 m	4.03 m
6″b				3.66 m					
7″α					6.74 d (15.6)	7.72 d (16.2)	2.15 m	2.14 m	2.17 m
7″β							1.81 m	2.04 m	1.80 m
8″					6.44 d (15.6)	6.56 d (16.2)			
9″							2.27 m	2.15 m	2.28 dd (8.5, 3.5)
10″					5.87 s		1.36 s	1.32 s	1.36 s
1‴				4.73 d (8.0)			4.71 d (8.0)	4.74 d (8.0)	4.77 d (8.0)
2‴				3.45 m			3.24 m	3.21 m	3.23 dd (9.0, 8.0)
3‴				3.70 m			3.35 m	3.39 m	3.39 t (9.0)
4‴				3.40 m			3.28 m	3.25 m	3.25 m
5‴				3.29 m			3.29 m	3.30 m	3.34 m
6‴a				3.81 d (12.0)			3.89 m	3.90 m	3.88 dd (12.0, 2.0)
6‴b				3.70 m			3.65 m	3.65 m	3.66 m

[a] ^{1}H NMR data (δ) were measured in methanol-d_4 at 600 MHz for **1**, **2**, **5**, and **6** and at 500 MHz for **3**, **4**, and **7**−**9**. Coupling constants (*J*) in Hz are given in parentheses. The assignments were based on ^{1}H−^{1}H COSY, NOESY (ROESY), HSQC, and HMBC experiments. [b] Data for Glc-4‴ δ_H 4.64 d (*J* = 7.0 Hz, H-1‴), 3.29 (m, H-2‴), 3.40 (m, H-3‴), 3.29 (m, H-4‴), 3.40 (m, H-5‴), 3.87 d (*J* = 12.0 Hz, H-6‴a), 3.70 (m, H-6‴b). [c] Data for H_{12}−H_{15}: δ_H 2.34 s (H-12″), 1.22 s (H-13″), 0.80 s (H-14″), 1.07 s (H-15″).

116.5, 130.1, 141.5, and 145.1), and resonances for a glucopyranosyl group. Analysis of the 2D NMR (including ^{1}H−^{1}H COSY, HSQC, and HMBC) data permitted the structure of compound **1** to be determined. The ROESY correlation of H-1/CH_3-10, together with the $^3J_{H1,H9}$ coupling constant (*J* = 7.8 Hz), confirmed that H-1, H-9, and CH_3-10 are α-, β-, and α-oriented, respectively. Acid hydrolysis of **1** afforded D-glucose, which was identified by TLC and optical rotation comparison with an authentic sample. Thus, rehmachingiioside A (**1**) was characterized as shown.

DOI: 10.1021/acs.jnatprod.5b01126
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 2. ^{13}C NMR Spectroscopic Data of Compounds 1−9[a]

position	1	2	3	4[b]	5[c]	6	7	8	9
1	98.5	97.9	98.0	96.9	95.1	96.1	93.6	93.5	93.8
3	66.4	64.2	147.4	144.7	141.6	152.3	140.4	140.6	140.4
4	116.5	119.2	102.2	105.6	104.1	114.0	105.9	105.7	105.9
5	141.5	139.2	134.9	82.3	38.9	31.8	39.0	38.3	41.3
6	130.1	26.9	120.7	82.1	79.5	41.4	84.6	83.8	77.8
7	145.1	39.8	95.4	128.7	62.2	78.9	48.3	49.2	50.0
8	82.5	78.9	86.3	146.2	66.1	45.5	78.8	79,0	79,4
9	55.7	54.5	52.8	53.0	43.5	43.2	51.5	51.6	51.8
10	23.0	22.2	17.7	61.3	61.9	14.4	25.3	25.3	25.2
1′	100.0	99.9	99.8	99.1	97.9	99.7	99.4	99.2	99.4
2′	74.7	74.6	74.4	74.8	74.4	74.7	74.6	74.8	74.7
3′	77.8	77.9	77.5	77.4	75.6	77.8	78.0	78.1	85.6
4′	71.5	71.3	71.3	70.9	71.8	71.7	71.6	71.6	70.2
5′	78.1	78.0	78.1	78.9	78.7	75.6	78.2	78.1	78.1
6′	62.7	62.4	62.6	62.3	62.7	64.4	62.8	62.8	62.8
1″			104.1	97.9	39.6	135.6	94.9	95.9	96.0
2″			74.7	85.3	37.4	129.3			
3″			77.5	77.5	18.9	130.1	97.3	97.4	98.9
4″			73.2	70.6	36.7	131.6	32.3	30.6	32.0
5″			74.2	77.9	75.7	130.1	43.9	43.8	44.1
6″			176.8	62.4	80.7	129.3	77.1	75.6	77.1
7″					140.5	146.6	50.3	49.8	50.4
8″					134.1	118.6	79.6	79.3	79.6
9″					154.8	168.3	52.1	53.4	52.0
10″					118.7		26.9	26.1	27.0
1‴				104.7			98.9	99.0	99.5
2‴				85.2			74.9	75.0	75.0
3‴				77.5			78.0	77.9	78.0
4‴				70.2			71.7	71.7	71.8
5‴				78.0			78.1	78.5	78.2
6‴				62.1			62.9	62.9	63.0

[a] ^{13}C NMR data (δ) were measured in methanol-d_4 at 150 MHz for **1**, **2**, **5**, and **6** and at 125 MHz for **3**, **4**, and **7**−**9**. The assignments were based on ^{1}H−^{1}H COSY, NOESY (ROESY), HSQC, and HMBC experiments. [b] Data for Glc-4‴ δ_C 106.4 (C-1‴), 76.3 (C-2‴), 77.6 (C-3‴), 71.5 (C-4‴), 78.3 (C-5‴), 62.7 (C-6‴). [c] Data for C_{11}−C_{15}: δ_C 168.3 (C-11″), 14.4 (C-12″), 25.7 (C-13″), 27.5 (C-14″), 27.3 (C-15″).

Compound **2** (rehmachingiioside B) gave a molecular formula of $C_{15}H_{24}O_8$ by HRESIMS at m/z 355.1360 [M + Na]$^+$, two mass units higher than that of **1**. The spectroscopic data of **2** (Tables 1 and 2) were very similar to those of **1** except that signals for another two methylenes (δ_C 26.9, C-6; δ_C 39.8, C-7) were observed instead of resonances for two olefinic carbons (δ_C 130.1, C-6; δ_C 145.1, C-7). The structure of this compound was confirmed by detailed analysis of the 2D NMR data including the ^{1}H−^{1}H COSY, HSQC, HMBC, and ROESY spectra. Accordingly, rehmachingiioside B (**2**) was characterized as shown.

Compound **3** (rehmachingiioside C) was assigned a molecular formula of $C_{21}H_{30}O_{15}$ from its HRESIMS (m/z 545.1480 [M + Na]$^+$). The spectroscopic data of **3** (Tables 1 and 2) were similar to those of 7-hydroxytomentoside,[7] except for the presence of a glucuronopyranosyl group located at C-7. This was confirmed by HMBC correlations from H-7 to C-1″ and from H-1″ to C-7. ROESY correlations of H-9/H-7 and CH_3-10/H-1, together with the $^3J_{H1,H9}$ coupling constant (J = 9.0 Hz) indicated that the H-1, H-7, H-9, and CH_3-10 substituents are α-, β-, β-, and α-oriented, respectively. On the basis of a reported hydrolysis procedure,[8] the absolute configurations of the glucose and glucuronic acid moieties were determined as D-glucose and D-glucuronic acid, respectively. Consequently, rehmachingiioside C (**3**) was characterized as shown.

Compound **4**, a white powder, gave the molecular formula $C_{33}H_{52}O_{25}$ from its positive-mode HRESIMS (m/z 871.2694 [M + Na]$^+$). The ^{13}C NMR spectrum showed 33 carbon signals including 24 signals for four glucopyranosyl units and the remaining nine for an iridoid skeleton. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **4** displayed signals characteristic of an iridoid glycoside, which were similar to those reported for rehmannioside D.[2] The only evident difference was that **4** showed resonances due to an additional glucopyranosyl unit. Furthermore, an HMBC correlation of H-2‴ (δ 3.45) with C-1‴ (δ 106.4) was used to locate the additional glucopyranosyl unit at C-2‴ in compound **4**. The ROESY correlation of H-1/H-6, together with the $^3J_{H1,H9}$ coupling constant (J = 6.5 Hz), confirmed that H-1, H-6, and H-9 are α-, α-, and β-oriented. Acid hydrolysis of **4** afforded D-glucose, which was identified by TLC comparison with an authentic sample, and the β-anomeric configuration was judged from its large $^3J_{H1,H2}$ coupling constant (J = 7.0−8.0 Hz). From these data, compound **4** (rehmachingiioside D) was characterized as shown.

Compound **5** was isolated as an amorphous powder, and its molecular formula was established as $C_{30}H_{44}O_{13}$ on the basis of the HRESIMS data (m/z 635.2680 [M + Na]$^+$). The ^{13}C NMR spectrum of **5** showed 30 signals, of which six could be

DOI: 10.1021/acs.jnatprod.5b01126
J. Nat. Prod. XXXX, XXX, XXX−XXX

attributed to a glucopyranosyl unit, nine for an iridoid skeleton, and the other 15 to a norcarotenoid moiety.[4] The ^{1}H and ^{13}C NMR data of **5** displayed signals characteristic of an iridoid glycoside, which were similar to those reported for catalpol.[2] The ^{1}H NMR spectrum of the norcarotenoid moiety of **5** showed an olefinic proton signal at δ 5.87 (1H, s), *trans*-olefinic proton signals at δ 6.44 and 6.74 (each 1H, d, J = 15.6 Hz), and an olefinic methyl proton signal at δ 2.34 (3H, s), which were in good agreement with those of aeginetic acid.[4] The ^{1}H NMR spectrum of the cyclohexyl end group of **5** exhibited three singlet methyl signals at δ_H 0.80, 1.07, and 1.22 (each 3H, s), and its ^{13}C NMR spectrum showed two quaternary carbon signals carrying hydroxy groups at δ_C 75.7 and 80.7. Detailed 2D NMR analysis was used to assign the norcarotenoid moiety as aeginetic acid.[4] In the HMBC spectrum, a correlation from H-2′ (δ_H 4.80) to the ester carbonyl at δ_C 168.3 demonstrated that the norcarotenoid ester group is present at C-2′ of the catalpol moiety. The ROESY correlations of H-4″β/H-3″β and CH_3-15″ and of H-13″α/H-3″α and H-7″ indicated that the hydroxy groups at C-5″ and C-6″ are α- and β-oriented, respectively. The CD spectrum of **5** exhibited similar Cotton effects (positive at 223 nm and negative at 264 nm, Supporting Information, Figure S45) to that of *sec*-hydroxyaeginetic acid,[4] indicating that the asymmetric centers at C-5″ and C-6″ are both in the *R* configuration. From these data, compound **5** (rehmachingiioside E) was characterized as shown.

Compound **6** (rehmachingiioside F) gave a molecular formula of $C_{25}H_{30}O_{11}$, as established by HRESIMS (m/z 529.1676 [M + Na]$^+$). The ^{13}C NMR spectrum of **6** showed 25 carbon signals including six for a glucopyranosyl unit, nine for a cinnamoyl group, and the remaining 10 for an iridoid skeleton. The ^{1}H and ^{13}C NMR data of **6** displayed signals characteristic of an iridoid glycoside, which were similar to those reported for 8-*epi*-loganic acid.[3] The only evident difference was that **6** showed resonances due to an additional cinnamoyl group. In the HMBC spectrum, correlations from H-6′a/H-6′b (δ 4.55, 4.43) to the ester carbonyl at δ 168.3 indicated that the cinnamoyl group is present at C-6′ of the 8-*epi*-loganic acid moiety. The relative configuration of **6** was established by analysis of the NOESY spectrum and coupling constants. The cinnamoyl group was assigned an *E* configuration from the large coupling constant between H-7″ and H-8″ (J = 16.2 Hz). In the NOESY spectrum, correlations of H-9/H-5, H-6β, and H-8 indicated that H-5, H-8, and H-9 are all β-oriented. Moreover, NOESY correlations of CH_3-10/H-7, H-6α, and H-1 confirmed that H-1, H-7, and CH_3-10 are, in turn, all α-oriented. Analysis of the ^{1}H−^{1}H COSY, HMBC, and HSQC spectra led to the complete assignments of the proton and carbon signals in compound **6** (Tables 1 and 2). On the basis of the above data, rehmachingiioside F (**6**) was characterized as shown.

Compound **7** (rehmachingiioside G) was isolated as an amorphous powder, and its molecular formula was established as $C_{30}H_{48}O_{18}$ on the basis of HRESIMS (m/z 719.2746 [M + Na]$^+$). The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of **7** exhibited signals for two sets of C_9-iridoid glycosides, with one unit determined as ajugol[3] and the other as an analogue of ajugol. The NMR data of the latter unit were very similar to those of ajugol except for an additional acetal carbon (δ_C 97.3, C-3″) and a methylene (δ_C 32.3, C-4″) instead of an olefinic carbon (δ_C 140.4, C-3; δ_C 105.9, C-4). The NMR signal of C-6 of **7** was deshielded significantly by comparison with those of ajugol.[3] This indicated that the second unit is located at C-6 through the connectivity of C-6−*O*−C-3″, which was verified by correlations from H-6 to C-3″ and from H-3″ to C-6 in the HMBC spectrum of **7**. In the ROESY spectrum, correlations of H-5″/H-9″ and H-4″β indicated that H-5″ and H-9″ are both β-oriented. In turn, ROESY correlations of CH_3-10″/H-7″α, H-6″, and H-1″ confirmed that H-1″, H-6″, and CH_3-10″ are all α-oriented. For the determination of the configuration of H-3″, a ROESY correlation of H-3″/H-5″ implied that H-3″ is β-oriented. Analysis of the 2D NMR data permitted the construction of the structure of compound **7** (Tables 1 and 2). Therefore, rehmachingiioside G (**7**) was characterized as shown.

Compound **8** was found to have the same molecular formula, $C_{30}H_{48}O_{18}$, as **7**, as established by its HRESIMS (m/z 719.2728 [M + Na]$^+$). The spectroscopic data of **8** (Tables 1 and 2) indicated that it is an epimer of **7**. Regarding the configuration of H-3″, a ROESY correlation of H-3″/H-1″ implied that H-3″ is α-oriented. Thus, the structure of **8** (rehmachingiioside H) was therefore established as shown.

The molecular formula of compound **9** (rehmachingiioside I) was determined as $C_{30}H_{48}O_{18}$ from the HRESIMS (m/z 719.2750 [M + Na]$^+$). The NMR spectroscopic data of **9** were very similar to those of **8** except that the position of the second unit is located at C-3′ through the connectivity of C-3′−*O*−C-3″, which was verified by correlations from H-3′ to C-3″ and from H-3″ to C-3′ in the HMBC spectrum of **9**. A ROESY correlation of H-3″/H-1″ indicated that H-3″ is α-oriented. Hence, rehmachingiioside I was characterized as **9**.

The known iridoid glycosides were identified as catalpol (**10**),[9] ajugol (**11**),[3] rehmaglutoside F (**12**),[5] jioglutoside A (**13**),[9] rehmaglutoside K (**14**),[5] mellittoside (**15**),[2] rehmannioside A (**16**),[2] rehmannioside B (**17**),[2] rehmannioside D (**18**),[2] 6-*O*-β-D-glucopyranosyl ajugol (**19**),[3] 7-hydroxytomentoside (**20**),[7] harpagide (**21**),[10] 6-*O*-*p*-hydroxybenzoyl catalpol (**22**),[11] 6-*O*-*E*-feruloyl catalpol (**23**),[12] aucubin (**24**),[13] and aeginetoyl ajugol 5″-*O*-β-D-quinovoside (**25**),[4] by NMR analysis and comparison with literature data.

Compounds **1** and **2** are based on a rare iridoid skeleton with a 4,5-double bond, and compound **3** contains a 5,6-double bond. Compounds **5** and **12** are interesting examples composed of iridoid glycoside and sesquiterpenoid units. Furthermore, compounds **7**−**9** are of interest in being iridoid glycoside dimers, although similar compounds were identified previously from *R. glutinosa*.[5]

Compounds **1**−**25** were tested for their cytotoxicity against five human tumor cell lines (A549, HT-29, Bel-7402, BGC-823, and A2780). However, all were inactive for all cell lines used (IC_{50} > 10 μM). These compounds were also evaluated for their inhibitory activity against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine microglia BV2 cells. As shown in Table 3, compounds **5** and **10** exhibited inhibitory activities, and no influence on cell viability was observed using the MTT method. They were also bioassayed for their hepatoprotective activities against *N*-acetyl-*p*-aminophenol (APAP)-induced toxicity in HepG2 cells, using the hepatoprotective activity drug bicyclol as the positive control. As shown in Table 4, compounds **4**, **6**, and **10**−**12** exhibited evidence of hepatoprotective effects.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-2000 polarimeter, and UV spectra with a JASCO V-650 spectrophotometer. IR spectra were recorded on a

DOI: 10.1021/acs.jnatprod.5b01126
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 3. Inhibitory Effects of Compounds 5 and 10 against LPS-Induced NO Production in Murine Microglia BV2 Cells[a]

compound	IC_{50} (μM)	cell viability[b]
5	2.5 ± 0.38	91.7 ± 1.8
10	7.3 ± 0.53	86.1 ± 1.6
curcumin[c]	0.52 ± 0.08	93.4 ± 3.0

[a]Results are reported as means ± SD based on three independent experiments. [b]Cell viability is expressed as a percentage (%) of the LPS-only treatment group. [c]Positive control.

Table 4. Hepatoprotective Effects of Compounds 4, 6, and 10−12 (10 μM) against APAP-Induced Toxicity in HepG2 Cells[a]

compound	cell survival rate (% of normal)
normal	100 ± 1.9
control	58.5 ± 3.7
bicyclol	66.5 ± 1.8[b]
4	64.0 ± 3.3[d]
6	65.9 ± 1.8[c]
10	69.1 ± 0.7[b]
11	69.9 ± 3.6[b]
12	63.4 ± 0.8[d]

[a]Results are expressed as means ± SD (n = 3; for normal and control, n = 6); bicyclol was used as positive control (10 μM). [b]$p < 0.001$. [c]$p < 0.01$. [d]$p < 0.05$.

Nicolet 5700 spectrometer using an FT-IR microscope transmission method. NMR measurements were performed on Bruker AV500-III and Bruker AV600 IIIHD spectrometers in methanol-d_4. HRESIMS were obtained using an Agilent 1100 series LC/MSD ion trap mass spectrometer. Silica gel (200−300 mesh, Qingdao Marine Chemical Factory, Qingdao, People's Republic of China), Sephadex LH-20 (GE), and ODS (50 μm, YMC, Kyoto, Japan) were used for column chromatography. Analytical thin-layer chromatography (TLC) was carried out with GF254 plates (Qingdao Marine Chemical Factory). For visualization of TLC plates, sulfuric acid reagent was used. All procedures were carried out at room temperature using solvents purchased from commercial sources and employed without further purification.

Plant Material. The whole plants of *R. chingii* were collected in Hangzhou, Zhejiang Province, People's Republic of China, in July 2013 and identified by Professor Lin Ma (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College). A voucher specimen (ID-S-2577) has been deposited at the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing.

Extraction and Isolation. The air-dried whole plants (15 kg) of *R. chingii* were extracted with H_2O (3 × 45 L) under reflux two times (2 h each). The combined extracts were concentrated under reduced pressure to dryness. The residue was suspended in H_2O and applied to a Diaion HP20 column that was eluted using a stepwise gradient of EtOH−H_2O (0:100, 45:55, and 95:5, v/v), to afford three fractions. After removing the solvent, the EtOH−H_2O (45:55) eluate (297 g) was separated subsequently by silica gel chromatography eluting with $CHCl_3$−MeOH (6:1 to 0:1, v/v) to afford three major fractions (F_1−F_3) based on TLC analysis. The EtOH−H_2O (95:5) eluate (251 g) was subjected to passage over a silica gel column, and successive elution with a gradient of increasing methanol (0−100%) in chloroform afforded four fractions (F_4−F_7). Fraction F_1 (91.3 g) was chromatographed on a reversed-phase C_{18} silica gel column (7.5 × 47 cm), eluting with a MeOH−H_2O (1:99 to 70:30) gradient, to give subfractions $F_{1\text{-}1}$−$F_{1\text{-}27}$. Fraction $F_{1\text{-}3}$ (120 mg) was subjected to a column of reversed-phase silica gel (1.8 × 35 cm), eluting with MeOH−H_2O (3:97), to yield **7** (9 mg) and **8** (11 mg). $F_{1\text{-}5}$ (90 mg) was chromatographed over Sephadex LH-20 eluted with MeOH as mobile phase to give **9** (8 mg) and **13** (9 mg). Fraction $F_{1\text{-}20}$ (30 mg) was further separated by repeated Sephadex LH-20 column chromatography to afford **1** (10 mg). Fraction F_3 (93.9 g) was separated using a reversed-phase C_{18} silica gel column (7.5 × 47 cm), eluted with a MeOH−H_2O (1:99 to 70:30) gradient, to yield subfractions $F_{3\text{-}1}$−$F_{3\text{-}25}$. Fraction $F_{3\text{-}2}$ (1.2 g) was chromatographed over a silica column and eluted with gradient mixtures of $CHCl_3$−MeOH (4:1) to afford **10** (550 mg), **11** (30 mg), and **19** (8 mg). Fraction $F_{3\text{-}6}$ (28 mg) and fraction $F_{3\text{-}7}$ (150 mg) were subjected separately to separation over Sephadex LH-20 (MeOH−H_2O, 1:1) to yield **3** (10 mg) from $F_{3\text{-}6}$ and **16** (12 mg) and **18** (94 mg) from $F_{3\text{-}7}$.

Fractions $F_{3\text{-}13}$ (33 mg), $F_{3\text{-}14}$ (120 mg), $F_{3\text{-}23}$ (22 mg), and $F_{3\text{-}24}$ (25 mg) were subjected separately to separation over Sephadex LH-20 (MeOH−H_2O, 1:1) to yield **4** (12 mg), **15** (98 mg), **14** (10 mg), and **17** (9 mg), respectively. Eluting with a stepwise gradient of MeOH−H_2O (5:95 to 50:50), fraction F_5 (58.5 g) was chromatographed on a reversed-phase C_{18} silica gel column (7.5 × 47 cm), to give subfractions $F_{5\text{-}1}$−$F_{5\text{-}26}$. Fraction $F_{5\text{-}4}$ (1.2 g) was purified through repeated C_{18} and Sephadex LH-20 (MeOH−H_2O, 1:1) columns to afford **2** (20 mg). Fractions $F_{5\text{-}5}$ (21 mg), $F_{5\text{-}9}$ (41 mg), $F_{5\text{-}15}$ (20 mg), and $F_{5\text{-}17}$ (34 mg) were subjected separately to separation over Sephadex LH-20 (MeOH−H_2O, 1:1) to yield **22** (7 mg), **23** (20 mg), **25** (13 mg), and **12** (10 mg). Fraction $F_{5\text{-}19}$ (1.5 g) was fractionated via repeated silica gel (EtOAc−EtOH−H_2O, 12:2:1; 9:2:1; 7:2:1) and Sephadex LH-20 (MeOH−H_2O, 1:1) to yield **5** (8 mg) and **6** (9 mg). Fractions F_6 (51.7 g) and F_7 (53.7 g) were separated using a reversed-phase C_{18} silica gel column (7.5 × 47 cm), eluted with a MeOH−H_2O (10:90 to 90:10) gradient, to yield subfractions $F_{6\text{-}1}$−$F_{6\text{-}42}$ and $F_{7\text{-}1}$−$F_{7\text{-}37}$. Fractions $F_{6\text{-}2}$ (61 mg), $F_{6\text{-}4}$ (38 mg), and $F_{7\text{-}5}$ (14 mg) were subjected separately to separation over Sephadex LH-20 (MeOH−H_2O, 1:1) to yield **24** (30 mg), **20** (20 mg), and **21** (5 mg), respectively.

*Rehmachingiioside A (**1**):* amorphous powder, $[\alpha]_D^{20}$ −108.7 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.19) nm; CD (MeOH) 206 ($\Delta\varepsilon$ 3.52) nm; IR ν_{max} 3373, 2927, 1703, 1643, 1375, 1261, 1151, 1078 cm^{-1}; ^1H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR (methanol-d_4, 150 MHz), see Tables 1 and 2; (+)-HRESIMS *m/z* 353.1211 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_8Na$, 353.1207).

*Rehmachingiioside B (**2**):* amorphous powder, $[\alpha]_D^{20}$ −113.8 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.49) nm; CD (MeOH) 236 ($\Delta\varepsilon$ 1.02) nm; IR ν_{max} 3391, 2930, 1656, 1373, 1077 cm^{-1}; ^1H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR (methanol-d_4, 150 MHz), see Tables 1 and 2; (+)-HRESIMS *m/z* 355.1360 $[M + Na]^+$ (calcd for $C_{15}H_{24}O_8Na$, 355.1363).

*Rehmachingiioside C (**3**):* amorphous powder, $[\alpha]_D^{20}$ −26.3 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 248 (4.46) nm; CD (MeOH) 239 ($\Delta\varepsilon$ −0.13) nm; IR ν_{max} 3381, 2909, 1613, 1425, 1376, 1229, 1160, 1077, 1023 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (methanol-d_4, 125 MHz), see Tables 1 and 2; (+)-HRESIMS *m/z* 545.1480 $[M + Na]^+$ (calcd for $C_{21}H_{30}O_{15}Na$, 545.1477).

*Rehmachingiioside D (**4**):* amorphous powder, $[\alpha]_D^{20}$ +3.2 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.55) nm; CD (MeOH) 203 ($\Delta\varepsilon$ 47.21) nm; IR ν_{max} 3397, 2920, 1650, 1368, 1075, 1032 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (methanol-d_4, 125 MHz), see Tables 1 and 2; (+)-HRESIMS *m/z* 871.2694 $[M + Na]^+$ (calcd for $C_{33}H_{52}O_{25}Na$, 871.2690).

*Rehmachingiioside E (**5**):* amorphous powder, $[\alpha]_D^{20}$ −75.8 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 271 (4.66) nm; CD (MeOH) 264 ($\Delta\varepsilon$ −5.93) nm; IR ν_{max} 3393, 2928, 1707, 1652, 1609, 1446, 1079 cm^{-1}; ^1H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR (methanol-d_4, 150 MHz), see Tables 1 and 2; (+)-HRESIMS *m/z* 635.2680 $[M + Na]^+$ (calcd for $C_{30}H_{44}O_{13}Na$, 635.2674).

*Rehmachingiioside F (**6**):* amorphous powder, $[\alpha]_D^{20}$ −49.9 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.77), 278 (4.63) nm; CD (MeOH) 225 ($\Delta\varepsilon$ −7.05) nm; IR ν_{max} 3384, 2932, 1699, 1637, 1451, 1281, 1182, 1076 cm^{-1}; ^1H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR (methanol-d_4, 150 MHz), see Tables 1 and 2; (+)-HRESIMS *m/z* 529.1676 $[M + Na]^+$ (calcd for $C_{25}H_{30}O_{11}Na$, 529.1680).

DOI: 10.1021/acs.jnatprod.5b01126
J. Nat. Prod. XXXX, XXX, XXX−XXX

Rehmachingiioside G (7): amorphous powder, $[\alpha]_D^{20}$ −123.0 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.56) nm; CD (MeOH) 200 ($\Delta\varepsilon$ −17.27) nm; IR ν_{max} 3374, 2931, 1658, 1374, 1178, 1047, 952 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (methanol-d_4, 125 MHz), see Tables 1 and 2; (+)-HRESIMS *m*/*z* 719.2746 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_{18}Na$, 719.2733).

Rehmachingiioside H (8): amorphous powder, $[\alpha]_D^{20}$ −91.6 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.59) nm; CD (MeOH) 200 ($\Delta\varepsilon$ −18.31) nm; IR ν_{max} 3387, 2919, 1657, 1371, 1080, 1051, 950 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (methanol-d_4, 125 MHz), see Tables 1 and 2; (+)-HRESIMS *m*/*z* 719.2750 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_{18}Na$, 719.2733).

Rehmachingiioside I (9): amorphous powder, $[\alpha]_D^{20}$ −135.3 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.67) nm; CD (MeOH) 200 ($\Delta\varepsilon$ −24.49) nm; IR ν_{max} 3379, 2928, 1657, 1375, 1079, 1048, 952 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) and ^{13}C NMR (methanol-d_4, 125 MHz), see Tables 1 and 2; (+)-HRESIMS *m*/*z* 719.2741 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_{18}Na$, 719.2733).

Acid Hydrolysis of 1−9. Each compound (6 mg) was refluxed individually in 6% HCl (5.0 mL) at 80 °C for 2 h. Then, each reaction mixture was extracted with $CHCl_3$ (3 × 6 mL), and the H_2O phase was dried using a N_2 stream. The residues were separately subjected to column chromatography over silica gel with $CHCl_3$−MeOH−H_2O (7:4:1) as eluent to yield D-glucose and D-glucuronic acid, respectively, with D-glucose exhibiting $[\alpha]_D^{20}$ +41.3 to +58.9 (lit. $[\alpha]_D^{25}$ +43.2, H_2O)[17] and D-glucuronic acid, $[\alpha]_D^{20}$ +15.9 (*c* 0.08, H_2O). The sugars were confirmed as D-glucose and D-glucuronic acid by comparison with an authentic sample on TLC ($CHCl_3$−MeOH−H_2O, 6:4:1, R_f 0.45 and R_f 0.05) and by measuring their optical rotations as shown above.

Cytotoxicity Assay. Compounds **1−25** were tested for cytotoxicity against HCT-8 (human colon carcinoma), Bel-7402 (human liver carcinoma), BGC-823 (human stomach carcinoma), A549 (human lung carcinoma), and A2780 (human ovarian carcinoma) by means of an MTT method described in the literature. Taxol was used as the positive control.[15]

Inhibitory Effects on NO Production in LPS-Activated Microglia. Compounds **1−25** were tested for their ability to inhibit LPS-activated NO production in the BV2 cell line. The murine microglial BV2 cells were obtained from the Cell Culture Centre at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and LPS was purchased from Sigma-Aldrich. The BV2 cells were plated into a 96-well plate. After being preincubated for 24 h, the cells were pretreated with 0.3 μg/mL LPS for an additional 24 h. Nitrite, which is a soluble oxidation product of NO, was determined in the culture supernatant using the Griess reaction. $NaNO_2$ was used as a standard to assay the NO_2^- concentration. The OD values of the samples at 550 nm were measured. Cell viability was assessed using an MTT assay. Curcumin was used as the positive control.[14]

Hepatoprotective Activity Assay. Human HepG2 hepatoma cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air. The cells were then passaged by treatment with 0.25% trypsin in 0.02% EDTA. The MTT assay was used to assess the cytotoxicity of test samples.[16] The cells were seeded in 96-well multiplates. After an overnight incubation at 37 °C with 5% CO_2, 10 μM test samples and APAP (final concentration of 8 mM) were added into the wells and incubated for another 48 h. Then, 100 μL of 0.5 mg/mL MTT was added to each well after the withdrawal of the culture medium and incubated for an additional 4 h. The resulting formazan was dissolved in 150 μL of DMSO after aspiration of the culture medium. The plates were placed on a plate shaker for 30 min and read immediately at 570 nm using a microplate reader. Bicyclol was used as the positive control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01126.

Copies of IR, MS, 1D and 2D NMR, and CD spectra for compounds **1−9** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +86-10-63165224. Fax: +86-10-63017757. E-mail: dqyu@imm.ac.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge grants from the National Natural Science Foundation of China (No. 81403076) and the State Key Laboratory of Bioactive Substance and Function of Natural Medicines (No. GTZC201201), Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College.

■ REFERENCES

(1) Chinese Pharmacopoeia Commission. *Pharmacopoeia of the People's Republic of China*; China Medical Science Press: Beijing, 2010; Vol. 2, pp 115−116.

(2) Oshio, H.; Inouye, H. *Phytochemistry* **1981**, *21*, 133−138.

(3) Nishimura, H.; Sasaki, H.; Morota, T.; Chin, M.; Mitsuhashi, H. *Phytochemistry* **1989**, *28*, 2705−2709.

(4) Sasaki, H.; Nishimura, H.; Morota, T.; Katsuhashi, T.; Chin, M.; Mitsuhashi, H. *Phytochemistry* **1991**, *30*, 1639−1644.

(5) Liu, Y. F.; Liang, D.; Luo, H.; Hao, Z. Y.; Wang, Y.; Zhang, C. L.; Zhang, Q. J.; Chen, R. Y.; Yu, D. Q. *J. Nat. Prod.* **2012**, *75*, 1625−1631.

(6) Flora of the China Editorial Committee. *Flora of China*; Science Press: Beijing, 1998; Vol. 18, pp 53−54.

(7) Damtoft, S.; Jensen, S. R.; Skaltsa, H. *Phytochemistry* **1993**, *34*, 1636−1638.

(8) Zhang, Y. L.; Gan, M. L.; Lin, S.; Liu, M. T.; Song, W. X.; Zi, J. C.; Wang, S. J.; Li, S.; Yang, Y. C.; Shi, J. G. *J. Nat. Prod.* **2008**, *71*, 905−909.

(9) Morota, T.; Sasaki, H.; Nishimura, H.; Sugama, K.; Chin, M.; Mitsuhashi, H. *Phytochemistry* **1989**, *28*, 2149−2153.

(10) Hannedouche, S.; Stanislas, E.; Moulis, C.; Fouraste, I. *Phytochemistry* **2000**, *54*, 807−809.

(11) Iwagawa, T.; Hamada, T.; Kurogi, S.; Hase, T.; Okubo, T.; Kim, M. *Phytochemistry* **1991**, *30*, 4057−4060.

(12) Wang, H.; Wu, F. H.; Xiong, F.; Wu, J. J.; Zhang, L. Y.; Ye, W. C.; Li, P.; Zhao, S. X. *Chem. Pharm. Bull.* **2006**, *54*, 4445−4450.

(13) Akdemir, Z.; Calis, I.; Junior, P. *Phytochemistry* **1991**, *30*, 2401−2402.

(14) Zhang, Y.; Liu, Y. B.; Li, Y.; Ma, S. G.; Li, L.; Qu, J.; Zhang, D.; Chen, X. G.; Jiang, J. D.; Yu, S. S. *J. Nat. Prod.* **2013**, *76*, 1058−1063.

(15) Zhang, C. L.; Wang, Y.; Liu, Y. F.; Liang, D.; Hao, Z. Y.; Luo, H.; Zhang, Q. J.; Shi, G. R.; Chen, R. Y.; Cao, Z. Y.; Yu, D. Q. *Tetrahedron* **2015**, *71*, 5579−5583.

(16) Li, Y.; Zhang, D. M.; Li, J. B.; Yu, S. S.; Li, Y.; Luo, Y. M. *J. Nat. Prod.* **2006**, *69*, 616−620.

(17) Liu, Y. X.; Young, K.; Rakotondraibe, L. H.; Brodie, P. J.; Wiley, J. D.; Cassera, M. B.; Callmander, M. W.; Rakotondrajaona, R.; Rakotobe, E.; Rasamison, V. E.; Tendyke, K.; Shen, Y. C.; Kingston, D. G. I. *J. Nat. Prod.* **2015**, *78*, 1543−1547.

DOI: 10.1021/acs.jnatprod.5b01126
J. Nat. Prod. XXXX, XXX, XXX−XXX