Iridoids from *Neopicrorhiza scrophulariiflora* and Their Hepatoprotective Activities *in Vitro*

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Four new non-glycosidic iridoids, piscrocins D (1), E (2), F (6), and G (7), as well as two new iridoid glycosides, piscrosides A (8) and B (9), were isolated from the roots of *Neopicrorhiza scrophulariiflora* (Scrophulariaceae), together with seven known iridoids. The structures of the isolated compounds were established by means of 1D and 2D NMR spectroscopy and chemical methods. The hepatoprotective activities of these compounds were evaluated by measuring their effects on CCl_4-induced hepatocytes damage *in vitro*, and the structure–activity relationships were also discussed.

Key words *Neopicrorhiza scrophulariiflora*; Scrophulariaceae; iridoid; hepatoprotective activity

The plant *Neopicrorhiza scrophulariiflora* (PENNELL) HONG (basionym. *Picrorhiza scrophulariiflora* PENNEL) grows in the high altitude region (over 4400 m) in the southeast of Tibet and the northwest of Yunnan in China. The root part of this plant is used in traditional Chinese medicine for the treatment of damp-heat dysentery, jaundice, and steaming of bone.[1)] *Picrorhiza kurrooa*, another unique species of the same genus, is also widely used in India for the treatment of jaundice, indigestion, common fever, acute viral hepatitis, and bronchial asthma.[2)] From the roots of *N. scrophulariiflora*, iridoid glycosides, triterpenoids, phenol glycosides and phenylenthanoid glycosides had been isolated.[3—7)] The ethanol extract of *N. scrophulariiflora* had been shown to possess hepatoprotective activities against carbon tetrachloride (CCl_4), thioacetamide, and acetaminophen-induced liver damage in mice.[8)] In our continuing investigation of hepatoprotective constituents of *N. scrophulariiflora*, the *n*-BuOH portion from the ethanol extract of this plant showed potent hepatoprotective activity evaluated on its effect on CCl_4-induced hepatocytes damage *in vitro*, with the concentration exhibiting 50% inhibition (IC_{50}) being 2.9 ± 0.7 mg/ml, further bioassay-guided analysis led to the isolation of thirteen non-glycosidic iridoids and iridoid glycosides. In this paper, we describe the isolation and structure elucidation of four new non-glycosidic iridoids, piscrocins D (**1**), E (**2**), F (**6**), and G (**7**), two new iridoid glycosides, piscrosides A (**8**) and B (**9**), along with seven known compounds, obtained from this plant. In addition, the hepatoprotective activities of these thirteen iridoids were evaluated on their effects on CCl_4-induced hepatocytes damage *in vitro*.

Results and Discussion

The ethanol extract of the plant was suspended in water and then successively extracted with petroleum ether (60—90 °C), EtOAc, and *n*-BuOH. The *n*-BuOH portion was chromatographed on silica gel, Sephadex LH-20, and C_{18} repeatedly to afford compounds **1**—**13**. The other seven known compounds were subsequently identified as rehmaglutin A (**3**),[9)] rehmaglutin D (**4**),[10)] (−)-3′-methoxy specinonin (**5**),[11)] pikuroside (**10**),[2)] picroside II (**11**),[4)] picroside I (**12**),[12)] picroside III (**13**),[13)] by comparison of spectral data with literature data values. The purity of these compounds was proven by TLC and HPLC (purity >90% for all compounds).

Piscrosin D (**1**) was obtained as pale yellow amorphous power. The HR-ESI-MS of **1** showed a quasimolecular ion $[M-H]^-$ at *m/z* 201.0773, consistent with a molecular for-

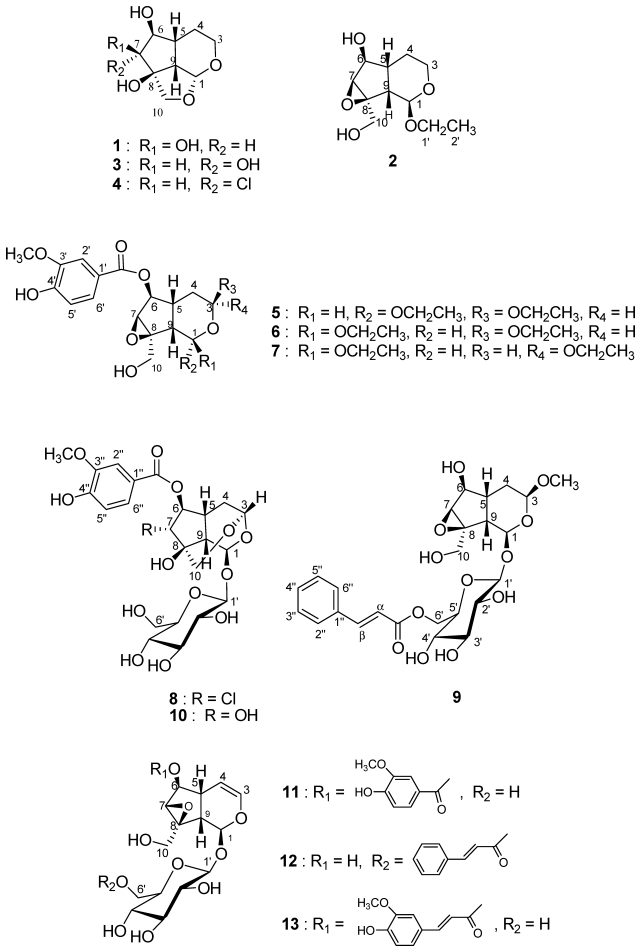

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Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **2**[a)]

Position	**1**		Position	**2**	
	^{1}H	^{13}C		^{1}H	^{13}C
1	5.29 d (5.9)	101.9	1	4.24 d (9.0)	102.4
3	3.51 ddd (2.1, 5.1, 11.8) 3.92 dd (2.7, 11.8)	56.4	3	3.44 dd (2.7, 12.7) 3.84 dd (7.8, 12.7)	62.8
4	1.62 br dd (2.0, 14.1) 1.78 m	22.3	4	1.54 br d (14.1) 1.74 m	24.1
5	2.45 m	37.1	5	2.00 m	38.4
6	3.95 dd (3.7, 8.5)	74.1	6	4.09 br d (9.0)	73.4
7	3.70 d (3.7)	79.4	7	3.44 br s	61.9
8		85.8	8		66.0
9	2.18 dd (5.9, 10.3)	46.8	9	2.14 dd (7.5, 9.0)	44.2
10	3.62 d (10.1) 3.78 d (10.1)	75.4	10	3.63 d (13.2) 3.92 d (13.2)	61.2
			1′	3.53 dt (2.4, 7.1) 3.95 m	65.5
			2′	1.21 3H t (7.1)	15.5

a) Measured in CD_3OD. Assignments were established by DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. *J* values (in Hz) are in parentheses.

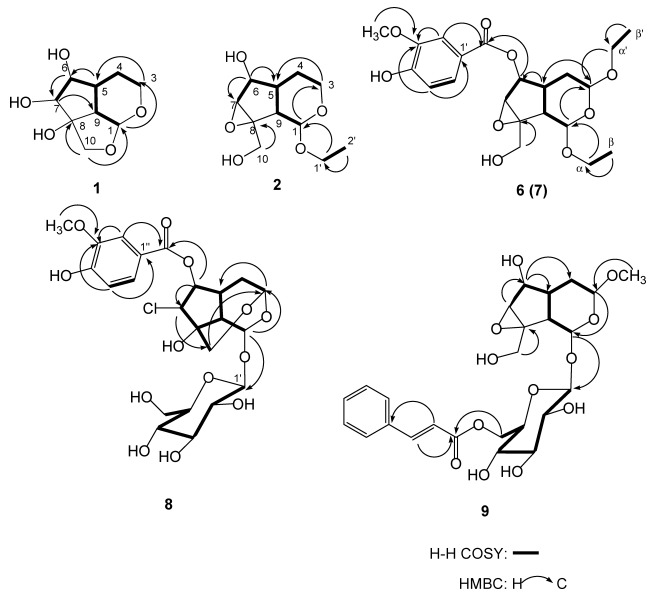

Fig. 1. H–H COSY and Key HMBC Correlations of **1**, **2**, **6**—**9**

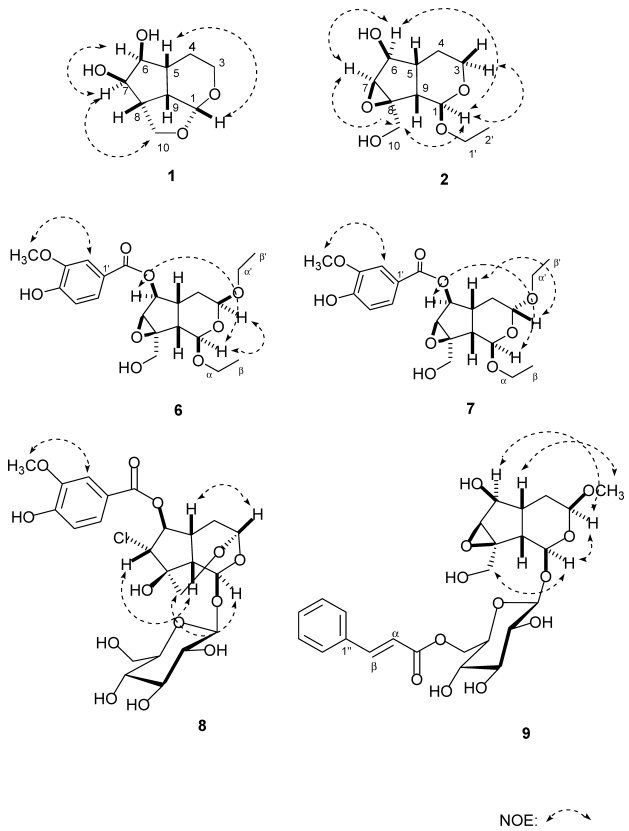

Fig. 2. Key NOE Correlations of **1**, **2**, **6**—**9**

mula of $C_9H_{14}O_5$. The ^{13}C- and DEPT NMR spectra of **1** indicated the presence of nine carbon signals including three methylenes, five methines and one quaternary carbon. The ^{1}H-NMR spectrum showed the presence of one acetalic proton signal [δ 5.29 (1H, d, J=5.5 Hz, H-1)], two hydroxylated methine signals [δ 3.95 (1H, dd, J=3.7, 8.5 Hz, H-6), 3.70 (1H, d, J=3.7 Hz, H-7)], two pairs of hydroxylated methylene signals (see Table 1). With the aid of ^{1}H–^{1}H COSY, HMQC and HMBC experiments, all the ^{1}H- and ^{13}C-NMR signals of **1** were assigned as shown in Table 1. Both the ^{1}H- and ^{13}C-NMR data were similar to those of rehmaglutin A (**3**),[9)] except for the signals due to C-6 and C-7 methines, which led us to the assumption that **1** might be a C-7 epimer of **3**. As shown in Fig. 1, the ^{1}H–^{1}H COSY experiment on **1** indicated the presence of partial structures written in bold lines. The NOESY spectrum (Fig. 2) showed the significant correlations between H-7 (δ 3.70) and H-10b (δ 3.78). However, no correlation was observed between H-7 (δ 3.70) and β-positioned H-9 (δ 2.18). The coupling constants for H-6 and H-5 (8.5 Hz), H-7 and H-6 (3.7 Hz), also indicated that the relative disposition of protons from H-5 to H-7 could be β, α, and α positions. In addition, the $[\alpha]_D^{20}$ value of **1** (+15.2°) was different with that of **3** (+43.6°). Hence, the structure of **1** was elucidated as 6β,7β,8β-trihydroxy-2,10-dioxatricyclo-[6.2.1.0^{5,11}]undecane.

Piscrosin E (**2**) was isolated as colorless oil. The HR-ESI-MS of **2** showed a quasimolecular ion $[M+Na]^+$ at *m/z* 253.1064, consistent with a molecular formula of $C_{11}H_{18}O_5$. The ^{1}H- and ^{13}C-NMR spectra (Table 1), coupled with the detailed analysis of the ^{1}H–^{1}H COSY (Fig. 1) and HMQC

Table 2. ^{1}H- and ^{13}C-NMR Data of Compounds **6** and **7**[a)]

Position	**5**		**6**		**7**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	5.10 d (3.8)	97.8	4.76 d (7.8)	99.4	4.68 d (7.8)	100.0
3	4.90 dd (2.6, 6.3)	95.0	4.99 d (3.4)	95.3	4.82 m	98.7
4	1.83 dd (6.9, 14.0)	30.4	1.76 br d (14.7)	27.8	1.64 m	29.8
	1.96 ddd (2.7, 5.4, 13.9)		1.85 m		1.81 td (2.3, 16.2)	
5	2.35 m	34.2	2.40 m	34.6	2.42 m	36.4
6	5.33 dd (1.0, 8.3)	81.0	5.60 dd (1.1, 9.1)	79.3	5.36 dd (1.1, 8.9)	77.5
7	3.62 br s	61.5	3.75 br s	59.6	3.71 br s	59.9
8		67.5		66.9		66.7
9	2.80 dd (3.8, 8.2)	48.0	2.30 t (8.8)	42.8	2.34 t (7.8)	43.9
10	3.54 d (12.6)	61.3	3.72 d (13.1)	61.0	3.69 d (13.1)	60.9
	4.04 d (12.7)		3.98 d (13.1)		3.99 d (13.1)	
α	3.54 dd (3.1, 7.2)	64.8	3.59 ddd (2.2, 7.1, 14.2)	65.6	3.62 dd (2.3, 7.1)	65.5
	3.88 dd (2.4, 7.4)		3.79 dd (2.5, 7.1)		3.97 dd (2.4, 7.1)	
β	1.25 3H, t (7.1)	15.6	1.25 3H, t (7.1)	15.6	1.25 3H t (7.1)	15.6
α'	3.48 dd (2.7, 7.1)	64.1	3.49 ddd (2.6, 7.1, 14.1)	64.4	3.57 dd (2.3, 7.1)	65.5
	3.83 dd (2.2, 7.2)		3.96 dd (2.2, 12.1)		3.92 dd (2.5, 7.1)	
β'	1.18 3H t (7.1)	15.5	1.22 3H t (7.1)	15.4	1.20 3H t (7.1)	15.6
1′		122.3		122.3		122.2
2′	7.56 br s	113.8	7.55 br s	125.3	7.56 br s	125.4
3′		153.3		153.3		153.4
4′		148.9		149.0		149.0
5′	6.85 d (8.1)	116.1	6.84 d (8.4)	113.8	6.85 d (8.1)	113.8
6′	7.58 dd (1.9, 8.0)	125.4	7.56 dd (2.0, 7.7)	116.1	7.59 dd (2.0, 8.0)	116.1
C=O		168.3		168.4		168.4
OMe	3.91 3H s	56.6	3.89 3H s	56.6	3.90 3H s	56.6

a) Measured in CD_3OD. Assignments were established by DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. *J* values (in Hz) are in parentheses.

spectra, revealed the presence of an iridoid skeleton, and a hydroxylated ethyl group [δ_H 1.21 (3H, t, J=7.1 Hz, H_3-2′), 3.53 (1H, dt, J=2.4, 7.1 Hz, H-1′a), 3.95 (1H, m, H-1′b); δ_C 15.5 (C-2′), 65.5 (C-1′)]. These data were closely correlated with those of picroside II (**11**) isolated from the same plant,[4)] and characterized that **2** was a bicyclic C_9-type non-glycosidic iridoid containing an alcohol group at C-1. As shown in Fig. 1, the long-range correlations between H-1′a, b (δ 3.53, 3.95) and C-1 (δ 102.4) in the HMBC spectrum suggested that the hydroxylated ethyl group should be located at C-1 of the iridoid aglycone. The relative stereochemistry of **2** was elucidated by means of a NOESY spectrum (Fig. 2), which showed the significant correlations between H-1 (δ 4.24) and H-6 (δ 4.09), and also between H-1 and H-10a, b (δ 3.63, 3.92). However, no correlation was observed between H-1 and H-5 (δ 2.00). These findings indicated that H-1 is α-oriented, which are in consistency with the coupling constant between H-1 and β-positioned H-9 (9.0 Hz). Hence, the structure of piscrosin E was determined to be **2**.

Piscrosin G (**6**) was obtained as white amorphous powder. The HR-ESI-MS of **6** showed a quasimolecular ion $[M-H]^-$ at *m/z* 423.1662, consistent with a molecular formula of $C_{21}H_{28}O_9$. The IR spectrum of **6** displayed absorption bands of hydroxyl group at 3357 cm^{-1} and α,β-unsaturated ester carbonyl at 1710 cm^{-1}. The ^{13}C and DEPT spectra of **6** exhibited 21 carbon signals, of which nine were assigned to the C_9-type iridoid aglycone moiety, the remaining 12 signals corresponded to two ethoxyl groups and a vanillic acid group. The ^{1}H-NMR spectrum showed the presence of an ABX system assigned to 1,2,4-trisubstituted aromatic ring [δ 7.55 (1H, br s, H-2′), 6.84 (1H, d, J=8.4 Hz, H-5′), 7.56 (1H, dd, J=2.0, 7.7 Hz, H-6′)], one methoxyl group [δ 3.89 (3H, s)], two acetal signals [δ 4.76 (1H, d, J=7.8 Hz, H-1), 4.99 (1H, d, J=3.4 Hz, H-3)], along with two pairs of ethoxyl groups (see Table 2). With the aid of ^{1}H–^{1}H COSY, HMQC, and HMBC experiments (Fig. 1), all the ^{1}H- and ^{13}C-NMR signals of **6** were assigned as shown in Table 2. The ^{1}H- and ^{13}C-NMR data of **6** were similar to those of (−)-3′-methoxyspecinonin (**5**) (see Table 2), except for the signal assignable to H-1 and C-1, which led us to the assumption that **6** might be a C-1 epimer of **5**. The NOESY spectrum (Fig. 2) showed the significant correlations between H-1 (δ 4.76) and α-positioned H-6 (δ 5.60). However, no correlation was observed between H-1 (δ 4.76) and β-positioned H-5 (δ 2.40). The coupling constant for H-1 and β-positioned H-9 (8.8 Hz), also suggested that the relative configuration of H-1 could be assigned as α position. In addition, the $[\alpha]_D^{20}$ value of **6** (−115.0°) was different with that of **5** (−43.3°). Thus, the structure of piscrosin G was determined to be **6**.

Piscrosin H (**7**) was isolated as white amorphous powder. The HR-ESI-MS of **7** showed a quasimolecular ion $[M-H]^-$ at *m/z* 423.1660, consistent with a molecular formula of $C_{21}H_{28}O_9$. The IR spectrum of **7** displayed absorption bands of hydroxyl group at 3358 cm^{-1} and α,β-unsaturated ester carbonyl at 1711 cm^{-1}. Both the ^{1}H- and ^{13}C-NMR spectra of **7** were very similar to those of compound **6**. However, the C-3 signal (δ 98.7) showed a downfield shift by 3.4 ppm, compared with that of **6**. These observations indicate that compound **7** might be the C-3 epimer of **6**. With the aid of ^{1}H–^{1}H COSY, HMQC and HMBC experiments (Fig. 1), all the ^{1}H- and ^{13}C-NMR signals of **7** were assigned as shown in Table 2. The NOESY spectrum of **7** (Fig. 2) showed the significant correlations between H-1 (δ 4.68) and α-positioned H-6 (δ 5.36), and also between H-3 (δ 4.82) and β-positioned H-5 (δ 2.42), indicating that the orientations of H-1, and H-3 were in α and β positions, respectively. The coupling con-

Table 3. ^{1}H- and ^{13}C-NMR Data of Compounds **8** and **9**[a)]

Position	**8**		Position	**9**	
	^{1}H	^{13}C		^{1}H	^{13}C
1	5.61 d (2.2)	93.2	1	4.90 d (8.6)	95.9
3	5.27 d (2.8)	96.0	3	4.54 dd (3.0, 9.0)	100.5
4	2.07 dd (3.2, 13.4)	34.5	4	1.59 m	29.7
	2.31 dd (8.6, 13.4)			1.81 br d (13.9)	
5	2.33 ddd (2.5, 7.9, 9.5)	34.9	5	2.03 m	38.2
6	5.06 dd (2.6, 8.2)	88.1	6	3.90 br d (9.1)	75.1
7	4.46 dd (1.0, 8.2)	70.5	7	3.39 br s	62.3
8		80.0	8		66.0
9	2.59 br d (9.5)	S.O.	9	2.37 t (8.1)	43.4
10	3.64 dd (1.3, 12.2)	62.2	10	3.63 d (13.1)	61.5
	4.00 dd (1.0, 12.2)			4.05 d (13.1)	
1′	4.63 d (7.9)	99.0	1′	4.75 d (7.8)	99.6
2′	3.08 dd (8.0, 8.9)	74.8	2′	3.27 m	74.9
3′	3.30 t (8.8)	78.2	3′	S.O.	77.8
4′	S.O.	71.7	4′	S.O.	71.7
5′	3.28 m	78.2	5′	3.53 m	76.0
6′	3.60 dd (2.2, 12.0)	62.8	6′	4.44 dd (5.5, 11.9)	64.4
	3.81 m			4.53 dd (2.4, 11.5)	
1″		122.1	1″		135.7
2″	7.49 d (1.8)	113.8	2″, 6″	7.62 2H m	130.2
3″		149.0	3″, 5″	7.41 2H m	129.4
4″		153.4	4″	7.41 m	131.7
5″	6.80 d (8.2)	116.2	α	6.57 d (16.0)	118.8
6″	7.52 dd (1.8, 8.2)	125.4	β	7.72 d (16.0)	146.7
C=O		167.9	C=O		168.4
OMe	3.84 3H s	56.6	OMe	3.45 3H s	56.6

a) Measured in CD_3OD. Assignments were established by DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC experiments. *J* values (in Hz) are in parentheses. S.O., Signal obscured by the solvent or the other signals.

stant for H-1 and H-9 (7.8 Hz) indicated that H-1 is in the α form. Hence, the structure of piscrosin H was concluded to be **7**.

Piscroside A (**8**) was obtained as white amorphous powder, which was positive in the Beilstein test. The HR-ESI-MS of **8** showed a quasimolecular ion $[M+Na]^+$ at *m/z* 571.1141, consistent with a molecular formula of $C_{23}H_{29}O_{13}Cl$. The IR spectrum of **8** displayed absorption bands of hydroxyl group at 3430 cm^{-1} and α,β-unsaturated ester carbonyl at 1695 cm^{-1}. The ^{13}C and DEPT spectra of **8** displayed 23 signals, of which nine were assigned to the C_9-type iridoid aglycone moiety, the remaining 14 signals corresponded to a hexose sugar residue and a vanillic acid group. The ^{1}H-NMR spectrum of **8** displayed an ABX system assigned to a 1,2,4-trisubstituted aromatic ring [δ 7.49 (1H, d, J=1.8 Hz, H-2″), 6.80 (1H, d, J=8.2 Hz, H-5″), 7.52 (1H, dd, J=1.8, 8.2 Hz, H-6″)], a methoxyl signal [δ 3.84 (3H, s)], two acetal protons [δ 5.61 (1H, d, J=2.2 Hz, H-1), 5.27 (1H, d, J=2.8 Hz, H-3)], and an anomeric proton [δ 4.63 (1H, d, J=7.9 Hz, H-1′)]. Acid hydrolysis of **8** afforded D-glucose, which was identified by direct comparison with authentic sample using HPLC and optical rotation measurement. As shown in Fig. 1, the ^{1}H–^{1}H COSY spectrum showed obvious W form long range coupling (J^4=1.3 Hz) between H-7 (δ 4.46) and H-10a (δ 3.64) and also a coupling (J^4=1.0 Hz) between H-9 (δ 2.59) and H-10b (δ 4.00), as observed in the case of pikuroside (**7**),[2)] indicating that H-7 could be in β position. The acetal linkage between C-3 and C-10 was confirmed by HMBC correlations (Fig. 1) between H-10a, b (δ 3.64, 4.00) and C-3 (δ 96.0). Moreover, the HMBC correlations between H-1 (δ 5.61) and C-1′ (δ 99.0) of glucose, as well as between H-6 (δ 5.06) and C=O (δ 167.9), indicated that the glucose should be attached to C-1 and the vanillic acid group to C-6 of the aglycone. The NOESY spectrum of **8** (Fig. 2) showed the significant correlations between H-7 (δ 4.46) and β-positioned H-9 (δ 2.59), and also between H-3 (δ 5.27) and β-positioned H-5 (δ 2.33), indicating that the orientations of H-3 and H-7 were both in β positions. Furthermore, the NOESY experiment showed a strong cross peak between the methoxyl group (δ 3.84) and H-2″ (δ 7.49), suggesting that the methyl group should be located at C-3″ of the aromatic ring. The ^{1}H and ^{13}C spectra of **8** were quite similar to those of pikuroside (**10**),[2)] except for the C-7 methine carbon at higher field (δ 70.5) in **8** than the hydroxylated one (δ 82.7) in **10**. This indicated that the hydroxyl group at C-7 in **10** was replaced by a chlorine atom in **8**, which was further verified by HR-ESI-MS data. Accordingly, the structure of **8** was elucidated as 7-deoxy-7α-Cl-pikuroside.

Piscroside B (**9**) was obtained as white amorphous powder. The HR-ESI-MS of **9** showed a quasimolecular ion $[M+Na]^+$ at *m/z* 547.1809, consistent with a molecular formula of $C_{25}H_{32}O_{12}$. The IR spectrum of **9** displayed absorption bands of hydroxyl group at 3433 cm^{-1} and α,β-unsaturated ester carbonyl at 1712 and 1635 cm^{-1}. The ^{13}C and DEPT spectra of **9** displayed 25 carbon signals, of which ten were assigned to the C_9-type iridoid aglycone and one methoxyl group, the remaining 15 signals corresponded to a hexose sugar residue and a cinnamoyl group. Acid hydrolysis of **9** afforded D-glucose, which was also identified by direct comparison with authentic sample using HPLC and optical rotation measurement. The ^{1}H- and ^{13}C-NMR spectral data of **9** were closely related to those of picroside I (**12**),[12)] which

was also isolated from the same plant. However, two olefinic protons located at C-3 and C-4 of the iridoid aglycone as found in **11**—**13** were absent, and instead, an acetal proton [δ 4.54 (1H, dd, J=3.0, 9.0 Hz, H-3)], two geminal methylene protons [δ 1.59 (1H, m, H-4a), 1.81 (1H, br d, J=13.9 Hz, H-4b)], and a methoxyl group [δ 3.45 (3H, s)], were observed. As shown in Fig. 1, the sequences from H-3 to H-9 were firmly established by ^{1}H–^{1}H COSY experiment. The location of the methoxyl group at C-3 of the iridoid aglycone was confirmed by an HMBC correlation (Fig. 1) between the protons of the methoxyl group (δ 3.45) and C-3 (δ 100.5). In addition, the attachment of *E*-cinnamoyl group through an ester linkage to the C-6′ position of the glucose was determined by the HMBC correlations between the H-6′a, b (δ 4.44, 4.53) and the carbonyl carbon (δ 168.4). The NOESY spectrum (Fig. 2) showed the significant correlations between the methoxyl group (δ 3.45) and β-positioned H-5, as well as between H-3 and α-positioned H-6, indicating that the methoxyl group at C-3 of the aglycone must be in the β orientation. Thus, the structure of **9** was elucidated as 6′-*O*-*E*-cinnamoyl-3-β-methoxy-3,4-dihydro-catalpol.

Compounds **1**—**13** were isolated from the active *n*-BuOH fraction of the ethanol extract of the roots of *N. scrophulariiflora*. Catalpol derivatives (**9**, **11**—**13**) were the major iridoid glycosides in the ethanol extract of *N. scrophulariiflora* and these non-glycosidic iridoids (**1**—**7**) were present in minor quantities. Compounds **2**, **5**—**7**, bearing one or two ethoxy groups in the molecular, might be artifacts of catalpol derivative (**11**) during the ethanol extraction process, which was proposed in the case of specionin.[14] However, piscrosins E (**2**), D (**6**), and G (**7**) was detected from the MeOH extract of this plant on the basis of TLC analysis. Thus, these 3 compounds bearing one or two ethoxy groups were confirmed to be naturally occurring compounds. In the iridoid series, a few compounds possessing a chlorine atom, for instance, glutinoside, rehmaglutin D,[10] had been reported. Since piscroside A (**5**) was detected from the EtOH extract of this plant on the basis of HPLC analysis, compared with authentic sample, piscroside A was also confirmed to be a naturally occurring substance. The hepatoprotective activities of these compounds were assessed by measuring their effects on the release of alanine transaminase (ALT) from the primary cultures of mice hepatocytes injured by CCl_4 *in vitro* and the results are summarized in Table 4. Seven non-glycosidic iridoids (**1**—**7**), displayed potent activities with IC_{50} values ranging from 1.7 to 3.9 μM, comparable to that of well-known hepatoprotective glycyrrhizic acid with an IC_{50} value of 4.7 μM. In a references survey, no more reports about the hepatoprotective activity of non-glycosidic iridoid have been found so far. Among these iridoid glycosides, compounds **9**, **11**, **12**, and **13** exhibited potent hepatoprotective effects *in vitro* with IC_{50} values of 7.3, 4.7, 8.3, and 5.9 μM, respectively. However, compounds **8** and **10**, possessing acetal linkage between C-3 and C-10, were found to lack the inhibitory activity. It is likely that iridoids with a glycosidic linkage at C-1 are less active than those non-glycosidic iridoids. It is known that iridoid glycosides are instable in the gastrointestinal tract and could be transformed to their corresponding non-glycosidic iridoids. These observations seem to be in agreement to the hypothesis that these major iridoid glycosides could be considered as pro-drugs and those non-glycosidic iridoids derived from the parent compounds contain the pharmacophores.[15] These results also suggest the hepatoprotective activities of *N. scrophulariiflora* are partly attributed to these iridoid derivatives.

Table 4. Hepatoprotective Effects of Compounds **1**—**13** on CCl_4-Induced Hepatocytes Damage *in Vitro*

Compound	IC_{50}±S.D. (μM)
Piscrocin D (**1**)	3.5±1.2
Piscrocin E (**2**)	3.9±0.9
Rehmaglutin A (**3**)	2.6±1.5
Rehmaglutin D (**4**)	2.0±0.2
(−)-3′-Methoxyspecinonin (**5**)	1.7±1.1
Piscrocin F (**6**)	2.5±0.1
Piscrocin G (**7**)	2.6±0.4
Piscroside A (**8**)	—
Piscroside B (**9**)	7.3±4.0
Pikuroside (**10**)	—
Picroside II (**11**)	4.7±1.5
Picroside I (**12**)	8.3±1.7
Picroside III (**13**)	5.9±2.9
Glycyrrhizic acid	4.7±2.2

Experimental

General Experimental Procedures Melting points were determined on an X-4 melting point apparatus (Beijing, China) and were uncorrected. Optical rotations were measured on a Jasco P-1030 polarimeter. IR spectra were recorded on a Nicolet Impact 410 FT-IR instrument. UV spectra were recorded using a Shimadzu UV-2501 spectrophotometer. NMR spectra were obtained on a Bruker AV 300 Avance spectrometer with CD_3OD as solvent. Electrospray ionization mass spectrometry (ESI-MS) was performed on a HP 1100 HPLC/EST instrument. High resolution electrospray ionization mass spectrometry (HR-ESI-MS) was obtained on a Micromass TOF spectrometer. Column chromatography was carried out using silica gel (200—300 mesh, 400 mesh, Qingdao Haiyang Chemical Group Co., Ltd., P. R. China), octodecyl silica gel (ODS) (C_{18}, 40—63 μm, Merck), and Sephadex LH-20 (Pharmacia) as a stationary phase. TLC was performed on precoated silica gel 60 F_{254} or RP-18 F_{254} plates (Merck) and visualized under UV light and by spraying with vanillin reagent followed by heating. HPLC was performed on a Waters 600E pump equipped with a refractive index detector.

Plant Material The roots of *N. scrophulariiflora* were collected in Zhong-Dian County (altitude 4400 m), Yunnan province, P. R. China, in October 1999, and authenticated by Dr. Ming-Jian Qin (Dept. of Pharmacognosy, China Pharmaceutical University). A voucher specimen (No. 991006) was deposited in the herbarium of China Pharmaceutical University, Nanjing.

Animals Male ICR mice (5 weeks, 20—22 g) were purchased from the Experimental Animal House of China Pharmaceutical University (Nanjing, P. R. China). All mice were placed in plastic cages and maintained with a free access to pellet food and water in a temperature controlled environment of 21±2 °C under a 12 h dark–light cycle.

Extraction and Purification The air-dried and powdered roots of *N. scrophulariiflora* (1.7 kg) were extracted with 95% EtOH (61×3) under reflux. The EtOH extract was suspended in water and then successively extracted with petroleum ether (60—90 °C) (PE), EtOAc, and *n*-BuOH. The *n*-BuOH-soluble fraction (62 g) was subjected to silica gel (200—300 mesh, 1000 g) column chromatography using $CHCl_3$–MeOH (90 : 10, 85 : 15, 70 : 30, each 5 l) as eluents to yield 6 fractions (A—F), based on silica gel TLC [$CHCl_3$–MeOH (9 : 1) and $CHCl_3$–MeOH–H_2O (6.5 : 3.5 : 1, lower layer)] results. Fraction B (2.1 g) was further subjected to silica gel (400 mesh, 50 g) column chromatography using $CHCl_3$–MeOH (95 : 5, 90 : 10, 85 : 15, each 500 ml) as eluents to yield 30 fractions (Frs. B1—B30). Fractions B12 and B13 were combined and chromatographed on silica gel (400 mesh, 30 g) column using $CHCl_3$–MeOH (92 : 8, 500 ml) as eluents to afford compounds **1** (90 mg, 110—140 ml), **3** (30 mg, 170—210 ml). Fraction B15 was chromatographed on Sephadex LH-20 (30 g) eluting with $CHCl_3$–MeOH (40 : 60) to give **2** (30 mg, 60—80 ml). Fraction B7 was further separated by silica gel (400 mesh, 20 g) column eluted with $CHCl_3$–MeOH (95 : 5, 300 ml) to yield **4** (12 mg, 80—100 ml). Fractions B8—B10 were combined and chromatographed on silica gel (400 mesh,

30 g) column eluting with a gradient of PE–EtOAc (80 : 20, 70 : 30, each 300 ml) to afford **5** (30 mg, 120—180 ml), **6** (25 mg, 350—370 ml). Fractions B16—B18 were combined and purified on Sephadex LH-20 (30 g) column chromatography eluted with $CHCl_3$–MeOH, 40 : 60) as eluent to afford **7** (25 mg, 80—90 ml). Fraction D (8.5 g) was successively chromatographed on silica gel (400 mesh, 300 g) eluted with a gradient of $CHCl_3$–MeOH (90 : 10, 85 : 15, each 1500 ml) to obtain 30 fractions (Frs. D1—D30). Fractions D5–D8 were combined and chromatographed on C_{18} (40—63 μM, 150 g) low-pressure column eluted with MeOH–H_2O (30 : 70, 40 : 60, 60 : 40, each 600 ml) and further purified by Sephadex LH-20 to afford **8** (55 mg, 200—350 ml), **10** (42 mg, 400—500 ml), **11** (210 mg, 850—1000 ml). Fractions D12—D16 were purified on C_{18} (40—63 μM, 180 g) column using MeOH–H_2O (45 : 55) as eluent to give **9** (58 mg, 450—550 ml), **12** (96 mg, 600—800 ml). Fractions D16 and D17 were combined and subjected to silica gel (400 mesh, 30 g) column chromatography using $CHCl_3$–MeOH (85 : 15) as eluent to yield **13** (64 mg, 120—180 ml).

Piscrosin D (**1**): Yellow amorphous power (MeOH); mp 104—106 °C; $[\alpha]_D^{20}$: +15.2° (c=0.1, MeOH); UV (MeOH) λ_{max}=205 nm; IR (KBr) ν_{max}=3422, 2941, 1405, 1253, 1107, 1030 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table 1. ESI-MS m/z=201 $[M-H]^-$; HR-ESI-MS m/z=201.0773 (Calcd for $[C_9H_{14}O_5-H]^-$: 201.0763).

Piscrosin E (**2**): Colorless oil, $[\alpha]_D^{20}$: −70.7° (c=0.03, MeOH); UV (MeOH) λ_{max}=205 nm; IR (film) ν_{max}=3358, 2924, 1442, 1144, 1038 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table 1. ESI-MS m/z=231 $[M+H]^+$; HR-ESI-MS m/z=253.1064 (Calcd for $[C_{11}H_{18}O_5+Na]^+$: 253.1052).

Piscrosin F (**6**): White amorphous powder (MeOH), mp 142—145 °C; $[\alpha]_D^{20}$: −115.0° (c=0.02, MeOH); UV (MeOH) λ_{max}=205, 218, 264, 292 nm; IR (KBr) ν_{max}=3357, 2974, 1710, 1597, 1515, 1285, 1030 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table 2. ESI-MS m/z=423 $[M-H]^-$; HR-ESI-MS m/z=423.1662 (Calcd for $[C_{21}H_{28}O_9-H]^-$: 423.1660).

Piscrosin G (**7**): White amorphous powder (MeOH), mp 137—139 °C; $[\alpha]_D^{20}$: −91.8° (c=0.03, MeOH); UV (MeOH) λ_{max}=205, 218, 264, 293 nm; IR (KBr) ν_{max}=3358, 2975, 1711, 1604, 1524, 1240, 1042 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table 2. ESI-MS m/z=423 $[M-H]^-$; HR-ESI-MS m/z=423.1660 (Calcd for $[C_{21}H_{28}O_9-H]^-$: 423.1660).

Piscroside A (**8**): White amorphous powder (MeOH); mp 196—199 °C; $[\alpha]_D^{20}$: −83.2° (c=0.06, MeOH); UV (MeOH) λ_{max}=205, 218, 263, 291 nm; IR (KBr) ν_{max}=3430, 1695, 1604, 1460, 1380, 1283, 1155 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table 3. ESI-MS m/z=571 $[M+Na]^+$; HR-ESI-MS m/z=571.1141 (Calcd for $[C_{23}H_{29}O_{13}Cl+Na]^+$: 571.1194).

Piscroside B (**9**): White amorphous powder (MeOH); mp 180—184 °C; $[\alpha]_D^{20}$: −51.0° (c=0.05, MeOH); UV (MeOH) λ_{max}=215, 276 nm; IR (KBr) ν_{max}=3433, 2921, 1712, 1635, 1449, 1388, 1263, 1175 cm^{-1}; ^{1}H- and ^{13}C-NMR data see Table 3. ESI-MS m/z=523 $[M-H]^-$; HR-ESI-MS m/z=547.1809 (Calcd for $[C_{25}H_{32}O_{12}+Na]^+$: 547.1791).

Acid Hydrolysis of 8 and 9 A solution of compound **8** and **9** (each 5 mg) in 2 M HCl–MeOH (1 : 1, 5 ml) was refluxed at 90 °C for 2 h, respectively. After being neutralized with NaOH–H_2O, the solution was extracted with EtOAc (5 ml×3). The H_2O layer was concentrated and passed through an Alltech C_{18} SPE cartridge and then separated repeatedly by HPLC [LichroCART NH_2 column (5 μm, 4.6×250 mm); mobile phase: MeCN–H_2O (75 : 25); flow rate: 1.0 ml/min; detection: refractive index (RI)] to afford D-glucose [**8**: 0.9 mg, t_R: 9.5 min, $[\alpha]_D^{20}$: +50.2°; **9**: 1.1 mg, t_R: 9.5 min, $[\alpha]_D^{20}$: +45.7°].[7]

Detection of 2, 6, and 7 in the MeOH Extract Dried and powdered roots of *N. scrophulariiflora* (5 g) were extracted with MeOH (50 ml) at 90 °C under reflux for 2 h. The MeOH extract was concentrated and extracted with $CHCl_3$. The presence of **2**, **6**, and **7** in the residue was detected by silica gel TLC comparison with authentic samples, respectively [**2**: $CHCl_3$–MeOH (90 : 10), *Rf*: 0.58; **6** and **7**: Pe–EtOAc (6 : 4), *Rf*: 0.36, 0.29].

Isolation and Culture of Hepatocytes Hepatocytes were isolated from ICR male mice (5 weeks) by modified two-step perfusion method.[16] The cell suspension was diluted to 1×10^5 cells/ml in the William's Medium E supplemented with 10% (v/v) New-born-calf serum (NBS) (Gibco BRL), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Portions (0.2 ml) were seeded onto 96-well microplates (Nunclon) and cultured in a humidified incubator at 37 °C with 5% (v/v) CO_2 in air for 4 h.

CCl_4-Induced Hepatocytes Damage and Transaminase-Releasing Assay After 4 h, the hepatocytes monolayers were washed twice with WE medium, then pretreated with various concentrations of drugs (30, 10, 3, 1, 0.3, 0.1 μmol/l) or without drugs in three replicates for 2 h, respectively. After two further washings, the cultured hepatocytes were exposed to a medium (0.2 ml) containing 0.15% CCl_4 [CCl_4/EtOH (10 mM) 100 μl dissolved in 10 ml WE medium] for 2 h to induce cytotoxicity, followed by collection of the supernatant to assay alanine transaminase activity.[17] Glycyrrhizic acid was used as a positive control.

Statistical Analysis Results were expressed as mean±S.D. of three independent experiments and each experiment includes triplicate sets.

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