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Two new flavonols, including one flavan dimer, from the roots of *Indigofera stachyodes*

Lu Qiu [a,b], Yan Liang [a], Gui-Hua Tang [b], Chun-Mao Yuan [b], Yu Zhang [b], Xiao-Yan Hao [a,*], Xiao-Jiang Hao [b], Hong-Ping He [b,**]

[a] School of Pharmacy, Guiyang Medical College, Guiyang 550004, Guizhou, People's Republic of China
[b] State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, People's Republic of China

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ABSTRACT

A new flavan dimer, 2α,3α-epoxyflavan-5,7,3′,4′-tetraol-(4β→8)-flavan-5″,7″,4‴-triol (**1**), and a new flavonol, 3-*O*-(3-nitropropanoyl)-2,3-*cis*-5,7,3′,4′-tetrahydroxyflavan (**2**), together with a known compound, 2α,3α-epoxy-5,7,3′,4′-tetrahydroxyflavan-(4β→8)-epicatechin (**3**), were isolated from the roots of *Indigofera stachyodes*. Their structures were elucidated by spectroscopic techniques including MS, 1D NMR, and 2D NMR. Compounds **2** and **3** were evaluated to determine their protective effects against carbon tetrachloride (CCl_4)-induced hepatotoxicity in the human liver cell line HL-7702. The results showed that **2** and **3** could protect HL-7702 cells from injury induced by CCl_4, with cell survival rates of 122.0% and 72.5%, respectively.

1. Introduction

The plant *Indigofera stachyodes* Lindl. belongs to the Papilionoideae family and is distributed mainly in Miaoxiang in the midwestern region of Guizhou province, Yunnan province, and the Guangxi Zhuang National Autonomous Region. Its roots, known as Xue-Ren-Sheng (in Chinese), are used as a traditional Chinese medicine for the treatment of wounds, dysentery, cirrhosis, and rheumatism (Editorial Committee of Chinese Bencao, 1999). Recently, it was found that the EtOAc and *n*-BuOH fractions of the methanol extract of the roots of this plant can effectively prevent and treat diabetes and its complications (Li et al., 2011). To date, there has been no phytochemical investigation of the roots of *I. stachyodes*. Thus, we analyzed the chemical constituents of its roots to identify potential active natural products. This paper reports the isolation and structural elucidation of a new flavan dimer (**1**) and a new flavonol (**2**), together with a known flavan dimer (**3**), obtained from this medicinal plant. Compound **2** features a 3-nitropropanoyl unit, which is very rare in flavonols. In addition, compounds **2** and **3** were evaluated to assess their protective effects against liver cell damage induced by CCl_4.

2. Results and discussion

The roots of *I. stachyodes* were extracted with 95% EtOH. After removal of the EtOH by evaporation, the residue was suspended in H_2O and then partitioned successively with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc fraction was subjected to column chromatography over silica gel, Rp-C_{18}, and Sephadex LH-20 to obtain compounds **1**–**3** (Fig. 1).

Compound **1** was obtained as a pink amorphous power. Its positive HR-TOF-MS peak at *m*/*z* 561.1342 $[M+H]^+$ (calcd. 561.1391) indicated that it has a molecular formula of $C_{30}H_{24}O_{11}$. The IR spectrum of **1** indicated the presence of an OH group (3431 cm^{-1}) and aromatic moieties (1695, 1624, 1518, and 1452 cm^{-1}). Analysis of the 1D NMR data (Table 1) suggested that **1** contains 30 carbons, including one methylene carbon, 14 methine carbons (ten aromatic and three oxygenated), and 15 quaternary carbons (nine oxygenated). A comparison of the NMR (Table 1) and MS data of **1** with those of **3** (Ma et al., 2007; Wang et al., 2011) suggested that the former had two of the same flavan structures as the latter, with the exception that one OH group in ring B of the lower flavan part in **3** was missing in **1**. The signals (δ_H 6.84, 2H, dd, J = 8.3 and 1.9 Hz, and δ_H 7.51, 2H, dd, J = 8.3 Hz) present in the ^1H NMR spectrum of **1** indicated the presence of a 1,4-disubstituted aromatic ring. As shown in Fig. 2, the HMBC and ^1H–^1H COSY correlations confirmed that the 1,4-disubstituted aromatic ring was ring B of the lower flavan part in **1**. In addition, the 2D NMR correlations shown in Fig. 2 revealed the structure of

* Corresponding author. Tel.: +86 0851 6908508; fax: +86 0851 6908568.
** Corresponding author. Tel.: +86 0871 65223263; fax: +86 0871 65223070.
E-mail addresses: haoxiaoyan@vip.163.com (X.-Y. Hao), hehongping@mail.kib.ac.cn (H.-P. He).

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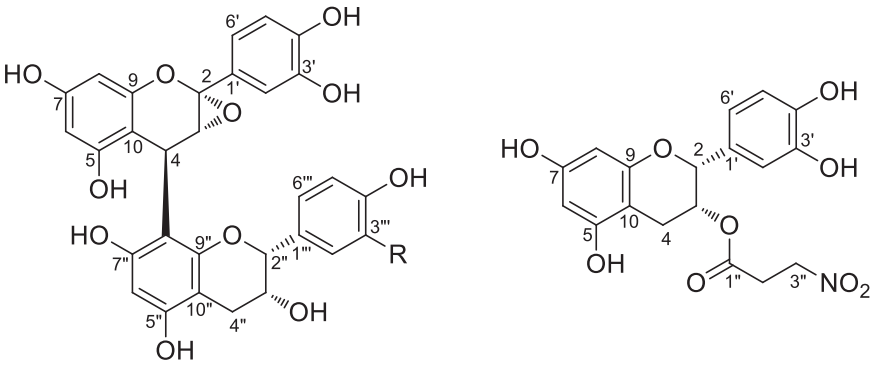

Fig. 1. Structures of compounds **1**–**3**.

the upper flavan part and the other moiety of the lower flavan part. The key HMBC correlation between H-4 and C-8″ connected the two flavan parts. Thus, the planar structure of **1** was established as shown. The relative configuration of **1** was determined to be the same as that of **3** through the analysis of the ^{1}H NMR chemical shifts, the ^{1}H–^{1}H coupling values, and the ROESY correlations. In addition, the CD spectrum of **1** showed a positive Cotton effect (Wang et al., 2011), indicating that the interflavan linkage at C-4 has a β-orientation. Thus, compound **1** was established as **2**α,**3**α-epoxyflavan-5,7,3′,4′-tetraol-(4β→8)-flavan-5″,7″,4‴-triol.

Table 1
NMR data of compound **1** and **3** (in CD_3OD; δ in ppm, J in Hz).

No.	**1**		**3**	
	δ_H[a]	δ_C[a,c]	δ_H[b]	δ_C[b,c]
2		100.3		100.2
3	4.05 d (3.4)	68.3	4.07 d (3.5)	68.0
4	4.41 d (3.4)	29.4	4.42 d (3.5)	29.2
5		152.5		152.2
6	6.07 d (2.3)	96.6	6.08 d (2.0)	96.6
7		158.3		158.1
8	6.01 d (2.3)	98.3	6.02 d (2.0)	98.3
9		157.3		156.5
10		107.4		107.2
1′		132.6		132.4
2′	7.13 d (2.2)	115.6	7.15 d (2.0)	115.7
3′		145.8		145.6
4′		146.9		146.2
5′	6.81 d (8.3)	116.0	6.82 d (8.0)	116.0
6′	7.01 dd (8.3, 2.2)	119.9	7.03 dd (8.0, 2.0)	119.8
2″	4.99 (s)	81.8	4.91 (s)	81.7
3″	4.23 (s)	67.2	4.23 (s)	66.9
4″	2.77 (m) 2.96 dd (17.0, 4.9)	30.1	2.76 (m) 2.94 dd (17.0, 5.0)	29.8
5″		154.4		154.2
6″	6.09 (s)	96.7	6.10 (s)	96.5
7″		156.8		156.9
8″		104.3		104.3
9″		152.4		152.1
10″		102.5		102.5
1‴		130.8		131.2
2‴	7.51 dd (8.6, 1.9)	130.1	7.17 d (2.0)	115.7
3‴	6.84 dd (8.6, 1.9)	116.0		145.9
4‴		158.5		146.7
5‴	6.84 dd (8.6, 1.9)	116.0	6.81 d (8.0)	116.1
6‴	7.51 dd (8.6, 1.9)	130.1	6.98 dd (8.0, 2.0)	120.4

[a] Recorded at 600 MHz.
[b] Recorded at 500 MHz.
[c] Multiplicities inferred from DEPT and HMQC experiments.

Compound **2** was obtained as a yellow amorphous power. Its HR-ESI-MS spectrum contained a $[M+Na]^+$ peak at m/z 414.0796 (calcd. 414.0801), consistent with the molecular formula $C_{18}H_{17}NO_9$. The IR spectrum of **2** indicated the presence of an OH group (3426 cm^{-1}). The ^{1}H NMR data displayed five aromatic signals, suggesting the presence of a 1,2,3,5-tetrasubstituted aromatic ring (δ_H 5.92, 1H, d, J = 1.8 Hz and 5.95, 1H, d, J = 1.8 Hz) and a 1,3,4-trisubstituted aromatic ring (δ_H 6.75, 6.75, and 6.91, each 1H, brs). Compound **2** was compared with (−)-epicatechin (Morimoto et al., 1986), and **2** had 15 carbons with the chemical shifts similar to those of the known compound, which indicated that **2** contains a flavan-3-ol moiety of the epicatechin type with a different substituent. The characteristic signals (δ_C 171.0, 70.5, 31.8; δ_H 4.54, 1H, t, J = 5.9 Hz, δ_H 2.81, 1H, m, and δ_H 2.89, 1H, m) in its 1D NMR spectra indicated the presence of a 3-nitropropanoyl substituent (Su et al., 2008). As shown in Fig. 2, the ^{1}H–^{1}H COSY correlations of H-2″/H-3″, the HMBC correlation between H-3″ and C-1″, and the analysis of the MS data confirmed the substituent. Furthermore, the HMBC correlation between H-3 (δ_H 5.40, s) and C-1″ (δ_C 171.0) indicated that the 3-nitropropanoyl unit is linked at C-3. The relative configuration of H-2 and H-3 in **2** was determined to be the *cis*-configuration because the proton signal attributable to H-2 (δ_H 4.95, s) appeared as a singlet. Thus, compound **2** was elucidated as 3-*O*-(3-nitropropanoyl)-2,3-*cis*-5,7,3′,4′-tetrahydroxyflavan.

The biological activities of compounds **2** and **3** were tested *in vitro* to determine their protective effects against damage to the human liver cell line HL-7702. Bicyclol tablets and bifendate pills were used as positive controls. The results (Table 2) indicated that compound **2** exhibited very significant protective effects against liver cell damage compared with the positive controls (bicyclol tablets and bifendate pills), whereas compound **3** showed moderate activity.

3. Experiment

3.1. General comments

Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were detected on a Shimadzu UV 2401 spectrometer. IR spectra were determined on a Bruker tensor-27 infrared spectrophotometer with KBr disks. 1D and 2D NMR spectra were recorded on Bruker AM-400, Bruker DRX-500 and Bruker Avance III 600 spectrometers with TMS as internal standard. MS and HR-ESI-MS analyses were carried out on an API Qstar Pulsar 1 instrument. Silica gel (80–100 and 300–400

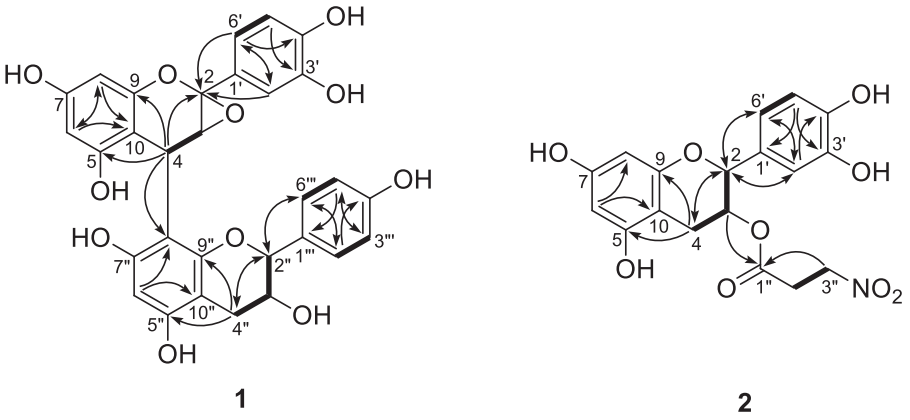

Fig. 2. Key HMBC (H → C) and ^{1}H–^{1}H COSY correlations of compounds **1** and **2**.

mesh, Qingdao Makall Group Co., Ltd.), Rp-C_{18} silica gel (40–75 μm, Fuji Silysia Chemical Ltd.), and Sephadex LH-20 (GE Healthcare Bio-Xciences AB) were used for column chromatography (CC). TLC spots were visualized under UV light and by dipping into 5% H_2SO_4 in alcohol followed by heating. RPMI-1640 medium (The Fisher's Biochemical Products Co., Ltd.), fetal bovine serum (Zhejiang Tianhang BioteChnology Co., Ltd.), green streptomycin mixture, pancreatic enzymes (Beijing Solarbio Science & Technology Co., Ltd.), MTS Cell Proliferation and Cytotoxicity Assay Kit (Genmed Scientifics Inc., USA), CCl_4 (AR, Chongqing Beibei Fine Chemical Factory), DMSO (Chongqing Beibei Fine Chemical Factory), bicyclol tablets (Beijing Union Pharmaceutical Factory), bifendate pills (IMC Zhejiang Pharmaceutical Co., Ltd.).

3.2. Plant material

The roots of *I. stachyodes* were collected from Liuzhi, Guizhou Province, People's Republic of China, in October 2010. And the plant was identified by Prof. Deyuan Chen, Guiyang College of Traditional Chinese Medicine. And a voucher specimen (HIS20101023) was deposited at School of Pharmacy, Guiyang Medical College.

3.3. Extraction and isolation

Dried and finely powdered roots of *I. stachyodes* (10 kg) were extracted with 95% EtOH three times (4, 3, and 3 h, respectively). After removal of the EtOH by evaporation, the residue was suspended in H_2O and then partitioned successively with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc fraction (290 g) was then applied to a silica gel column with $CHCl_3$:MeOH (95:5 → 50:50) to afford eight fractions (A → H). Fraction C (16.5 g) was applied to an Rp-C_{18} silica gel column with MeOH:H_2O (10%, 30%, 50%, 70%, 90%) to obtained 12 fractions (C1 → C12). Fraction C4 was applied to a Sephadex LH-20 column with MeOH and then to a silica gel column with $CHCl_3$:acetone:formic acid (8:2:0.1) to obtain **2** (23.2 mg). Fraction E (12.1 g) was then applied to a silica gel column with $CHCl_3$:acetone:formic acid (65:35:2) to afford seven fractions (E1 → E7). In a similar manner, fraction E3 was applied to a silica gel column with $CHCl_3$:acetone:formic acid (7:3:0.2) and then to a Sephadex LH-20 column with MeOH to afford **1** (16.9 mg) and **3** (181.3 mg).

Table 2
Protective effects against liver cell damage.

Group	Dose (μg/μl)	Cell survival rate (%)
Normal group[a]	–	100
CCl_4 model group[b]	–	31.7
2	0.2	122
3	0.6	72.5
Bicyclol tablets[c]	0.5	105.5
Bifendate pills[c]	0.5	94.7

[a] Without any treatment.
[b] Without the treatment of drugs.
[c] Positive control.

3.3.1. 2α,3α-Epoxyflavan-5,7,3′,4′-tetraol-(4β→8)-flavan-5″,7″,4‴-triol (**1**)

Pink amorphous power; $[\alpha]_D^{20}$ + 31.4 (*c* 0.13, MeOH); IR (KBr): 1695, 1624, 1518, 1452, 3431 cm^{-1}; UV (MeOH) λ_{max} (log ε): 205 (4.81), 278 (3.70) nm; ^{1}H and ^{13}C NMR see Table 1; HR-TOF-MS at *m*/*z* 561.1342 ($[M+H]^+$, 561.1391 calcd. for $C_{30}H_{25}O_{11}$).

3.3.2. 3-O-(3-nitropropanoyl)-2,3-cis-5,7,3′,4′-tetrahydroxyflavan (**2**)

Red oil; $[\alpha]_D^{20}$ − 48.4 (*c* 0.14, MeOH); IR (KBr): 3426, 1609, 1557, 1518, 1466 cm^{-1}; UV (MeOH) λ_{max}(log ε): 204(4.65), 281 (3.48) nm; ^{1}H NMR (400 MHz, CD_3OD) δ: 4.95 (1H, s, H-2), 5.40 (1H, s, H-3), 2.93 (1H, m, H-4a), 2.79 (1H, m, H-4b), 5.92 (1H, d, *J* = 1.8 Hz, H-6), 5.95 (1H, d, *J* = 1.8 Hz, H-8), 6.75 (1H, brs, H-2′), 6.75 (1H, brs, H-5′), 6.91 (1H, brs, H-6′), 2.89 (1H, m, H-2″a), 2.81 (1H, m, H-2″b), 4.54 (1H, t, *J* = 5.9 Hz, H-3″); ^{13}C NMR (100 MHz, CD_3OD) δ: 78.0 (C-2), 70.7 (C-3), 26.5 (C-4), 157.8 (C-5), 95.7 (C-6), 157.9 (C-7), 96.5 (C-8), 157.0 (C-9), 99.0 (C-10), 131.1 (C-1′), 119.0 (C-2′), 146.0 (C-3′), 146.0 (C-4′), 116.0 (C-5′), 114.8 (C-6′), 171.0 (C-1″), 31.8 (C-2″), 70.5 (C-3″); HR-ESI-MS at *m*/*z* 414.0796 ($[M+Na]^+$, 414.0801 calcd. for $C_{18}H_{17}NO_9Na$).

3.3.3. 2α,3α-Epoxy-5,7,3′,4′-tetrahydroxyflavan-(4β→8)-epicatechin (**3**)

Pink amorphous power; $[\alpha]_D^{20}$ + 44.0 (*c* 0.33, MeOH); IR (KBr): 3417, 1521, 1503 cm^{-1}; UV (MeOH) λ_{max}(log ε): 205 (4.94), 286 (3.97) nm; ^{1}H and ^{13}C NMR see Table 1; HR-ESI-MS at *m*/*z* 599.1166 ($[M+Na]^+$, 599.1165 calcd. for $C_{30}H_{24}O_{12}$ Na).

3.4. Biological assays

The human liver cell line HL-7702, cultured in RPMI-1640 medium with 10% fetal bovine serum in 5% CO_2 at 37 °C, was used in the biological assays. The protection assay was performed using the MTS [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium, inner salt] method (Baltrop and Owen, 1991). In brief, HL-7702 cells in the log phase of

their growth cycle were seeded into 96-well plates with 90 μl per well. After the cells were grown in adherent cultures for 24 h, 10 μl of RPMI-1640 medium with or without different concentrations of drugs was added to each well, and then the cells were cultured for another 16 h. Afterwards, 3 μl of CCl_4 was added to each well except for the control wells to induce cell damage. After treatment for 3 h, assays were carried out by adding 10 μl of MTS directly to each well. The plates were incubated for 3 h, and then, the absorbance at 490 nm was recorded with a 96-well plate reader. The cell survival rate of the treated cells was calculated using the formula $OD_{test}/OD_{control} \times 100\%$. In these experiments, bicyclol tablets and bifendate pills were used as positive controls.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2013.04.010.

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