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Spirocyclic Nortriterpenoids with NGF-Potentiating Activity from the Fruits of *Leonurus heterophyllus*

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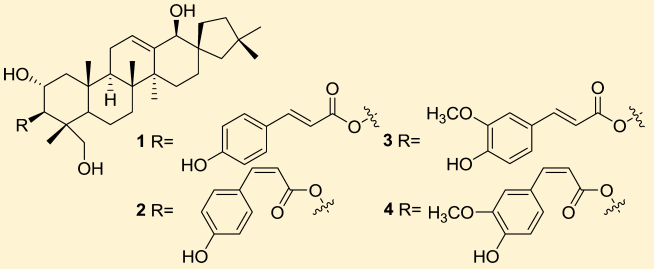
Supporting Information

ABSTRACT: Four new spirocyclic nortriterpenoids, leonurusoleanolide A (**1**), leonurusoleanolide B (**2**), leonurusoleanolide C (**3**), and leonurusoleanolide D (**4**), were isolated from the MeOH extract of the fruits of *Leonurus heterophyllus*. Compounds **1** and **2**, and compounds **3** and **4**, were found to exist as equilibrium mixtures of *trans* and *cis* isomers. Mixtures of **1** and **2**, and **3** and **4**, significantly enhanced the neurite outgrowth of nerve growth factor-treated PC12 cells at concentrations ranging from 1 to 30 μM. Compound **8** was also found to have a neurite outgrowth-promoting effect at concentrations of 1 and 10 μM. The structure−activity relationship of these compounds is discussed.

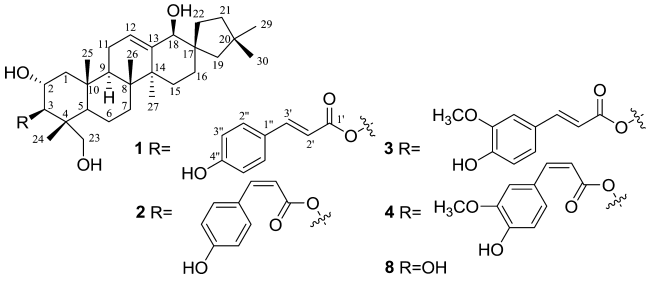


Leonurus heterophyllus Sweet, which belongs to the family Lamiaceae, is an herbaceous plant that is distributed throughout China. The aerial parts of this plant are used as a famous traditional Chinese medicine named Yimucao, while the ripe fruits, which are also known as Chongweizi (Fructus leonuri), are used as a traditional medicine for invigorating blood circulation, regulating menstrual disturbance, and dispelling edema.[1] Alkaloids,[2−4] labdane-type diterpenoids,[5−10] flavonoids,[7] and sterols,[7,11] with neuroprotective[12] and inhibitory effects against platelet activating factor[5,6] and cholinesterase,[10] have recently been isolated from the aerial parts. However, except for data regarding several cyclic peptides,[13−15] little is known about the chemistry and active ingredients of the fruits of *L. heterophyllus*. As part of our search for neurotrophic natural products,[16] we investigated the MeOH extract of the fruits of *L. heterophyllus*, which exhibited neurite outgrowth-promoting activity in nerve growth factor (NGF)-treated PC12 cells at 50 μg/mL, resulting in the isolation of four new nortriterpenoids, leonurusoleanolide A (**1**), leonurusoleanolide B (**2**), leonurusoleanolide C (**3**), and leonurusoleanolide D (**4**), together with three known compounds, β-sitosterol glucopyranoside (**5**),[17] aurantiamide acetate (**6**),[18] and auraptenol (**7**).[19] The new compounds displayed an unusual carbon skeleton, i.e., a 28-noroleanane-type spirocyclic framework. In this paper, we report the structures of **1**−**4** and their NGF-potentiating activity in PC12 cells.

RESULTS AND DISCUSSION

The air-dried and powdered fruits of *L. heterophyllus* were extracted with MeOH at room temperature. Fractionation of the MeOH extract afforded compounds **1**−**7**.

Compounds **1** and **2**, which existed as a 2:1 equilibrium mixture, were obtained as a white, amorphous powder. They



could be separated by normal-phase HPLC but readily interconverted and reached equilibrium within 2 h. The ^{1}H NMR spectrum of compounds **1** and **2** contained two sets of well-separated signals at δ_H 6.0−8.5. One gave resonances for conjugated olefinic protons at δ_H 6.61 (1H, d, J = 15.9 Hz), 7.79 (1H, d, J = 15.9 Hz) and aromatic protons at δ_H 7.16 (2H, d, J = 8.7 Hz), 7.56 (2H, d, J = 8.7 Hz). The 15.9 Hz coupling constant indicated an *E*-olefinic geometry, while the number of aromatic protons and their coupling constant revealed the presence of a 1,4-disubstituted benzenoid system. These data suggested the presence of a *p*-coumaroyl moiety, which was further supported by ^{13}C NMR (Table 1). The other set of resonances corresponded to conjugated olefinic protons at δ_H 6.09 (d, J = 12.9 Hz), 6.91 (d, J = 12.9 Hz) and aromatic protons at δ_H 7.11 (d, J = 8.7 Hz), 8.12 (d, J = 8.7 Hz). The 12.9 Hz coupling constant of the olefinic protons indicated the presence of a *cis-p*-coumaroyl moiety. These NMR data suggested compounds **1** and **2** exist in an equilibrium mixture of *trans*- and *cis-p*-coumaroyl moieties.

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds 1−4 (600 MHz, Pyridine-d_5)

	1		2[a]		3[a]		4[a]	
position	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)
1	48.8	α 1.44 m		α 1.42 m		α 1.44 m		α 1.42 m
		β 2.44 dd (12.6, 4.6)		β 2.42 dd (12.6, 4.5)		β 2.44 dd (12.6, 4.5)		β 2.41 dd (12.7, 4.5)
2	66.7	4.49 td (9.9, 4.6)		4.44 m		4.49 m		4.43 m
3	79.9	5.83 d (9.9)	79.7	5.82 d (10.9)		5.84 d (10.6)	79.7	5.82 d (10.0)
4	43.8		43.7				43.7	
5	47.4	2.00 m	47.3	1.97−2.03[b]		1.99 m	47.3	1.98−2.03[b]
6	18.2	α 1.77 m		α 1.74 −1.83[b]		α 1.77 m		α 1.72−1.83[b]
		β 1.46 m		β 1.42−1.52[b]		β 1.46 m		β 1.40−1.50[b]
7	34.0	α 1.47 m		α 1.42−1.51[b]		α 1.47 m		α 1.42−1.52[b]
		β 1.77 m		β 1.74−1.82[b]		β 1.77 m		β 1.72−1.82[b]
8	39.8							
9	47.8	1.74 m		1.72 m		1.74 m		1.72 m
10	38.0							
11	23.4	2.07 m		2.05 m		2.08 m		2.05 m
12	118.4	6.29 m		6.28 m		6.30 m		6.28−6.35[b]
13	143.2							
14	44.2							
15	27.6	α 1.80 m		α 1.76−1.84[b]		α 1.80 m		α 1.74 −1.84[b]
		β 1.01 m		β 0.96−1.03[b]		β 1.01 m		β 1.59−1.68[b]
16	36.2	1.63 m		1.61 m		1.63 m		1.59−1.68[b]
17	50.4							
18	74.8	4.20 brs[c]		4.19 brs[c]		4.21 brs[c]		4.19 brs[c]
19	52.5	α 2.35 d (12.7)		α 2.34 d (12.5)		α 2.35 d (12.6)		α 2.34 d (12.4)
		β 1.27 d (12.7)		β 1.26 d (12.5)		β 1.27 d (12.6)		β 1.26 d (12.4)
20	39.1							
21	42.4	α 1.82 m		α 1.78−1.86[b]		α 1.82 m		α 1.78−1.87[b]
		β 1.49 m		β 1.45−1.53[b]		β 1.50 m		β 1.46−1.54[b]
22	29.2	α 1.45 m		α 1.40−1.50[b]		α 1.45 m		α 1.40 −1.49[b]
		β 2.01 m		β 1.98−2.06[b]		β 2.01 m		β 1.97−2.05[b]
23	64.8	3.52 d (11.6)	64.7	3.54 d (11.2)		3.53 d (10.9)		3.51−3.55[b]
		3.66 d (11.6)		3.64 d (11.2)		3.66 d (10.9)		3.64 d (10.4)
24	14.9	1.00 s		0.94 s		0.99 s		0.95 s
25	17.8	1.18 s		1.14 s	17.9	1.18 s		1.15 s
26	17.9	1.08 s		1.06 s		1.08 s		1.07 s
27	23.1	1.09 s		1.04 s		1.09 s		1.07 s
29	30.2	1.10 s		1.09 s		1.10 s		1.09 s
30	30.1	1.19 s		1.18 s		1.19 s		1.18 s
1′	168.4		167.5		168.4		167.4	
2′	115.8	6.61 d (15.9)	116.9	6.09 d (12.9)	116.0	6.72 d (15.8)	116.8	6.10 d (12.9)
3′	145.0	7.79 d (15.9)	143.8	6.91 d (12.9)	145.4	7.98 d (15.8)	144.2	6.92 d (12.9)
1″	126.2		126.6		126.6		126.8	
2″	130.6	7.56 d (8.7)	133.8	8.12 d (8.7)	111.4	7.28 d (1.5)	115.2	8.33 d (2.0)
3″	116.8	7.16 d (8.7)	115.8	7.11 d (8.7)	149.3		148.9	
4″	161.4		160.5		151.0		149.5	
5″	116.8	7.16 d (8.7)	115.8	7.11 d (8.7)	116.8	7.16 d (8.1)	116.0	7.17 (in solvent)
6″	130.6	7.56 d (8.7)	133.8	8.12 d (8.7)	123.4	7.20 (in solvent)	126.6	7.48 dd (8.4, 2.0)
−OMe					55.9	3.78 s	55.9	3.82 s

[a]The chemical shifts of the carbon atoms were the same as in **1** unless otherwise mentioned in the table. [b]Not assigned due to signal overlapping. [c]Displayed long-range coupling in $^{1}H-^{1}H$ COSY.

The molecular formula of these compounds was deduced to be $C_{38}H_{54}O_6$ from the HRFABMS peak at m/z 629.3849 $[M + Na]^+$ ($C_{38}H_{54}O_6Na$ calcd for 629.3818). In the IR spectrum of **1** and **2**, absorption bands were detected at 3379 (OH), 1685 (C═O), and 1604 and 1515 cm^{-1} (aromatic ring). The ^{1}H NMR spectrum of **1** and **2** revealed six methyl singlets at δ_H 1.00, 1.08, 1.09, 1.10, 1.18, and 1.19, one olefinic proton at δ_H 6.29 (1H, m), three oxygenated methine protons at δ_H 4.49 (1H, td, J = 9.9, 4.6 Hz), 5.83 (1H, d, J = 9.9 Hz), and 4.20 (1H, brs), and a hydroxymethylene group (CH_2OH) at δ_H 3.52, 3.66 (each 1H, d, J = 11.6 Hz), which was confirmed by a HMQC experiment (δ_C 64.8). These data suggested that **1** and **2** were pentacyclic nortriterpenoids with coumaroyl ester substituents.

The EIMS data of compounds **1** and **2** exhibited an ion peak at m/z 147, confirming the presence of a coumaroyl moiety. The characteristic RDA fragment at m/z 202 [D/E ring − H_2O]$^+$ indicated that a double bond was located between C-12

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and C-13 and that there was a hydroxy substituent in the D/E ring.[20] The correlations between the methyl singlet at δ_H 1.09 (H_3-27) and the olefinic carbon at δ_C 143.2 (C-13) detected by HMBC also proved the position of the olefinic moiety, while the long-range coupling between the oxymethine proton at δ_H 4.20 and the olefinic proton at δ_H 6.29 (H-12) observed in the ^{1}H−^{1}H COSY indicated the presence of an 18-OH moiety in the D ring. The two oxymethine protons at δ_H 4.49 and 5.83 and two hydroxymethylene protons (δ_H 3.52 and 3.66) were assigned to H-2 and H-3, and H_2-23, respectively, based on the HMBC correlations of H-3 to C-2 (δ_C 66.7), C-4 (δ_C 43.8), C-23 (δ_C 64.8), and C-24 (δ_C 14.9) and of H_3-24 (δ_H 1.00) to C-3 (δ_C 79.9), C-4 (δ_C 43.8), C-5 (δ_C 47.4), and C-23 (δ_C 64.8). The downfield shift of H-3 suggested that the 3-hydroxy group was esterified with a coumaroyl moiety, which was confirmed by the HMBC correlations between H-3 and C-1′ (δ_C 168.4).

Analysis of the ^{1}H−^{1}H COSY spectrum revealed five spin−spin coupling systems, H-2′/H-3′, H-2″/H-3″, H_2-1/H-2/H-3, H-5/H_2-6, and H-9/H_2-11/H-12/H-18, as indicated with bold bonds in Figure 1. The HMBC data (Figure 1) indicated that

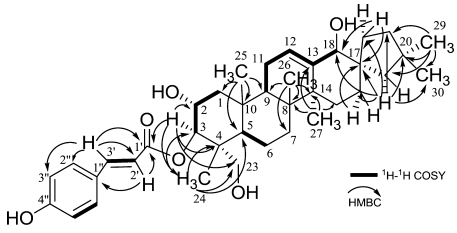

Figure 1. Selected ^{1}H−^{1}H COSY and HMBC correlations of **1**.

the quaternary carbon C-17 (δ_C 50.4) showed strong cross-peaks to H-19β (δ_H 1.27), H-19α (δ_H 2.35), H-22β (δ_H 2.01), H-22α (δ_H 1.45), and H_2-16 (δ_H 1.63). This observation indicated that a spiro system existed between the D/E ring. All of the carbon signals were assigned on the basis of an analysis of the HMBC, COSY, and HMQC data (Table 1).

The relative configuration of **1** was deduced by a NOESY experiment (Figure 2). The 2-OH group and 3-coumaroyl ester

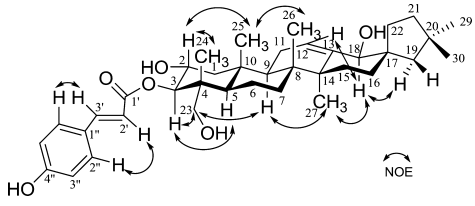

Figure 2. Selected NOESY correlations of **1**.

moiety were considered to take α and β orientations, respectively, because according to their large J values (both 9.9 Hz) H-2 was in a β axial position and H-3 was an α axially disposed proton. The NOESY correlations from H-2 to H_3-24 and H_3-25 and from H_3-25 to H_3-26 suggested that three methyl groups were β-oriented, whereas the NOESY correlations from H-3 to H-5 and H_2-23, H_2-23 to H-9, H-9 to H_3-27, and H_3-27 to H-18 indicated that the H-5, H-9, H-18, 23-hydroxymethylene group, and the C-27 methyl group were α-oriented. The additional NOESY correlation from H-18 (δ_H 4.20) to H-19α (δ_H 2.35) suggested that the C-17−C-19 bond in the spirocyclic E ring was α-oriented, indicating an R* configuration for C-17.

On the basis of the spectroscopic data, compounds **1** and **2** were considered to be 3β-*E*-*p*-coumaroyloxy-(2α,17*R**,18β)-19(18→17)-abeo-28-norolean-12-ene-2,18,23-triol and 3β-*Z*-*p*-coumaroyloxy-(2α,17*R**,18β)-19(18→17)-abeo-28-norolean-12-ene-2,18,23-triol and were named leonurusoleanolides A and B, respectively.

Compounds **3** and **4**, another pair of *trans* and *cis* isomers (2.5:1 ratio), were obtained as a white, amorphous powder. Their molecular formula was determined to be $C_{39}H_{56}O_7$ by the HRFABMS peak at m/z 659.3956 $[M + Na]^+$ (calcd as 659.3924 for $C_{39}H_{56}O_7Na$). The IR spectrum of **3** and **4** indicated the presence of a hydroxy group (3372 cm^{-1}), a phenyl group (1600, 1515 cm^{-1}), and a conjugated ester carbonyl (1685 cm^{-1}). Comparison of the NMR spectrum of **3** and **4** with that of **1** and **2** detected differences in the aromatic ring and the presence of an additional methoxy group. Analysis of the ^{1}H NMR and ^{1}H−^{1}H COSY spectra of **3** and **4** revealed two aromatic ABX systems: δ_H 7.28 (1H, d, J = 1.5 Hz), 7.16 (1H, d, J = 8.1 Hz), and 7.20 (1H, merged with a pyridine signal) for the *E*-isomer; δ_H 8.33 (d, J = 2.0 Hz), 7.17 (merged with a pyridine signal), and 7.48 (dd, J = 8.4, 2.0 Hz) for the *Z*-isomer. These data suggested the presence of a feruloyl moiety, which was confirmed by the ion at m/z 177. The structure of compound **3** was therefore assigned as 3β-*E*-feruloyloxy-(2α,17*R**,18β)-19(18→17)-abeo-28-norolean-12-ene-2,18,23-triol, and that of compound **4** was assigned as 3β-*Z*-feruloyloxy-(2α,17*R**,18β)-9(18→17)-abeo-28-norolean-2-ene-2,18,23-triol; the two compounds were named leonurusoleanolides C and D, respectively. The complete NMR assignments of compounds **3** and **4** are listed in Table 1.

To further confirm their structures, mixtures of **1** and **2**, and **3** and **4**, were hydrolyzed respectively under basic conditions to give phlomistetraol B (**8**),[21] which was previously isolated from *Phlomis umbrosa*.

Nortriterpenoids with a D/E spirocyclic framework have unusual carbon skeletons and have been isolated from only a few plants (*Phlomis umbrosa*, *Cleistanthus indochinensis*, *Notochaete hamosa*, and *Gomphostemma parviflorum*).[21−25] In the present study, we obtained four new such nortriterpenoids from the fruits of *L. heterophyllus*. It is also worth noting that this study is the first to report the existence of ester derivatives of the spironortriterpenoid **8**.

The effects of compounds **1**−**4** and **8** on the neurite outgrowth of PC12 cells were evaluated according to previously reported methods.[26−29] None of the compounds had any morphological effects on PC12 cells in the absence of NGF, whereas in the presence of NGF (0.5 ng/mL), mixtures of **1** and **2**, and **3** and **4**, significantly promoted neurite outgrowth from PC12 cells in a dose-dependent manner at concentrations ranging from 1 to 30 μM. In the case of compound **8**, it significantly promoted neurite outgrowth at concentrations of 1 and 10 μM, but showed toxicity against PC12 cells at concentrations higher than 30 μM. The effects of the compounds on the neurite outgrowth in PC12 cells were assessed by morphological observations (Figure 3) and a quantitative analysis of neurite length (Figure 4). All the compounds caused the mean neurite length (compounds **1** and **2**: 41.9 μm at 30 μM, 33.5 μm at 10 μM, and 27.6 μm at 1 μM; compounds **3** and **4**: 55.1 μm at 30 μM, 42.0 μm at 10 μM, and 32.1 μm at 1 μM; compound **8**: 33.1 μm at 10 μM and 22.9 μm at 1 μM) of the NGF-treated PC12 cells to increase dose-dependently, and all of these increases were significant (mean neurite length of the control: 21.1 μM) (Figure 4).

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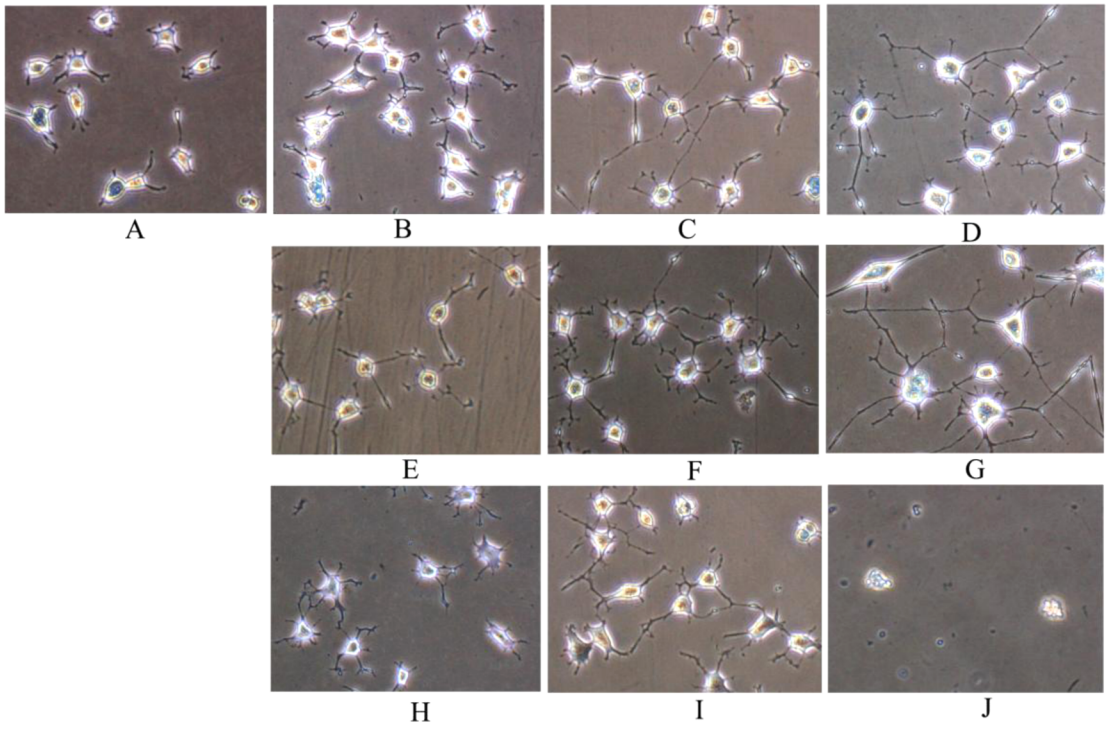

Figure 3. Morphological changes observed in PC12 cells treated with (A) NGF 0.5 ng/mL, (B) **1** and **2** (1 μM) + NGF 0.5 ng/mL, (C) **1** and **2** (10 μM) + NGF 0.5 ng/mL, (D) **1** and **2** (30 μM) + NGF 0.5 ng/mL, (E) **3** and **4** (1 μM) + NGF 0.5 ng/mL, (F) **3** and **4** (10 μM) + NGF 0.5 ng/mL, (G) **3** and **4** (30 μM) + NGF 0.5 ng/mL, (H) **8** (1 μM) + NGF 0.5 ng/mL, (I) **8** (10 μM) + NGF 0.5 ng/mL, or (J) **8** (30 μM) + NGF 0.5 ng/mL.

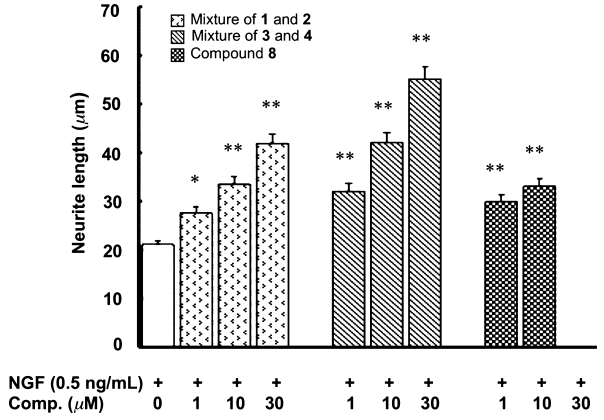

Figure 4. Quantitative analysis of neurite outgrowth promoted by a mixture of **1** and **2**, a mixture of **3** and **4**, or compound **8**. PC12 cells were cultured in a 24-well plate in DMEM + 10% HS and 5% FBS for 24 h at a density of 8×10^3 cells/cm^2, and then the medium was replaced with DMEM + 2% HS and 1% FBS with NGF (0.5 ng/mL) containing a mixture of **1** and **2**, a mixture of **3** and **4**, or **8**. After 4 days, the neurite lengths of the PC12 cells were quantified. Data are expressed as the mean ± SE (n = 100). *p < 0.05, **p < 0.01 vs control, respectively; Dunnett's t test.

The above-mentioned results suggested that the parent compound **8** possessed NGF-potentiating activity on PC12 cells. However, compounds **1**–**4** seemed to be more potent NGF potentiators than **8**, which showed toxicity at concentrations higher than 30 μM. These results mean that a coumaroyl or a feruloyl moiety at C-3 in compounds **1**–**4** contributes to increase their activities. In a comparison of the mean neurite lengths between a mixture of **1** and **2**, and a mixture of **3** and **4**, compounds **3** and **4** seemed to be more potent NGF potentiators than **1** and **2**, suggesting that the additional methoxy group on the phenyl ring in compounds **3** and **4** enhances the NGF-potentiating activity in PC12 cells.

In conclusion, four new nortriterpenoids with an unusual carbon skeleton, leonurusoleanolides A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from the MeOH extract of the fruits of *L. heterophyllus*. A remarkable pathological symptom of neurodegenerative diseases is the progressive loss of neuronal cells in the brain. NGF, a growth-promoting protein for certain neuronal populations, exerts neurotrophic actions on neuronal cells and protects them from death.[30,31] Compounds **1**–**4** possess the ability to potentiate the activity of NGF to stimulate neurite outgrowth from PC12 cells and thus might be regarded as candidates to develop drugs for the treatment of neurodegenerative diseases such as Alzheimer's disease.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured on a JASCO P-2200 digital polarimeter. IR spectra were recorded on a JASCO FT-IR 410 infrared spectrophotometer. The NMR experiments were performed on a Varian Unity 600 MHz NMR spectrometer. Deuterated solvent peaks were used as reference for the ^{1}H and ^{13}C NMR spectra. HRFABMS and EIMS were acquired on an MStation JMS-700 and a JMX-AX 500. Silica gel column chromatography (CC) was carried out on Wako C-300, Merck silica gel 60 (70–230 and 230–400 mesh), and Kanto silica gel 60N (40–50 μm). HPLC was performed on a JASCO PU-1580 equipped with a JASCO UV-1575 detector.

Plant Material. The fruits of *L. heterophyllus* Sweet were collected in Jinan, Shandong Province, PRC, in September 2010. A voucher specimen (1810FR) was identified by Prof. Lingchuan Xu at Shandong University of Traditional Chinese Medicine and was deposited in the Institute of Pharmacognosy, Tokushima Bunri University.

Extraction and Isolation. The dried fruits of *L. heterophyllus* (1 kg) were powdered and extracted with MeOH (5 L) at room temperature for one month to give 30 g of dark green extract. The

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MeOH extract (30 g) was chromatographed on a Si gel column (0.5 kg) eluted with a step gradient of CH_2Cl_2 (A: 100%, 3 L), CH_2Cl_2–EtOAc (B: 9:1, 3 L; C: 5:5, 3 L), EtOAc (D: 100%, 3 L), EtOAc–MeOH (E: 9:1, 3 L; F: 7:3, 3 L), and MeOH (G: 100%, 3 L) to yield seven fractions (A–G).

Fraction C (1.5 g) was first subjected to Sephadex LH-20 chromatography (GE Healthcare Bio-Sciences, Uppsala, Sweden, 2.5 × 40 cm) eluting with MeOH to give fractions 1–6. Fraction 4 (57.5 mg) was further chromatographed on a Si gel column eluted with *n*-hexane–EtOAc (6:4) to give fractions 7–15. Fraction 14 (18 mg) was purified by reversed-phase HPLC (COSMOSIL πNAP, 10 × 250 mm, 5 μm) eluted with MeOH–H_2O (88:12) at a flow rate of 2 mL/min. The fractions with retention times of 24.5 and 27.8 min gave a mixture of **1** and **2** (5.8 mg) and a mixture of **3** and **4** (4.2 mg), respectively. Fraction 11 (7.3 mg) was purified by reversed-phase HPLC (COSMOSIL C18-MS-II, 10 × 250 mm) eluted with MeOH–H_2O (7:3) at a flow rate of 2 mL/min. The fraction with a retention time of 22.3 min afforded aurantiamide acetate (**6**, 1.8 mg). Fraction 13 (12.7 mg) was separated by reversed-phase HPLC (COSMOSIL C18-MS-II, 10 × 250 mm) using a MeOH–H_2O (6:4) solvent system at a flow rate of 2 mL/min. The fraction with a retention time of 22.3 min gave auraptenol (**7**, 1.3 mg).

Fraction E (1.0 g) was subjected to Si gel chromatography eluted with $CHCl_3$–MeOH–H_2O (80:20:0.2) to afford six subfractions. Subfraction 4 (57.2 mg) was recrystallized [$CHCl_3$–MeOH (1:1)] to give β-sitosterol glucopyranoside (**5**, 13 mg).

*Leonurusoleanolides A (**1**) and B (**2**):* amorphous, white powder; $[\alpha]^{20}_D$ −12.0 (*c* 0.01, MeOH); IR (film) ν_{max} 3379 (OH), 1685 (C=O), 1604 and 1515 (aromatic ring), 2946, 2861, 1203, 1169, 1045, 983, 743 cm^{-1}; ^1H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 600 MHz) data (Table 1); HRFABMS *m/z* 629.3849 [M + Na]$^+$ (calcd for $C_{38}H_{54}O_6Na$, 629.3818); EIMS *m/z* 606 [M]$^+$ (1.1), 588 (25), 442 (24), 254 (15), 202 (97), 147 (100).

*Leonurusoleanolides C (**3**) and D (**4**):* amorphous, white powder; $[\alpha]^{24}_D$ −6.8 (*c* 0.01, MeOH); IR (film) ν_{max} 3372 (OH), 1685 (C=O), 1600 and 1515 (aromatic ring), 2931, 1275, 1176, 1036, 754 cm^{-1}; ^1H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 600 MHz) data (Table 1); HRFABMS *m/z* 659.3956 [M + Na]$^+$ (calcd for $C_{39}H_{56}O_7Na$, 659.3924); EIMS *m/z* (rel int) 618 (8), 553 (19), 442 (22), 254 (12), 202 (100), 177 (98).

Hydrolysis of Compounds 1–4. A mixture of **1** and **2** (or a mixture of **3** and **4**) (3.5 mg) was dissolved in MeOH (3 mL), and 1 N NaOH was added to produce a pH of 10. The mixture was stirred at room temperature for 12 h, neutralized with 1 N HCl, and evaporated to 400 μL; then the residue was filtered and purified by RP-HPLC (COSMOSIL C18-AR-II, 10 × 250 mm, 5 μm) eluted with MeOH–H_2O (9:1) to afford phlomistetraol B (**8**):[21] $[\alpha]^{22}_D$ +12.7 (*c* 0.01, MeOH; $[\alpha]^{25}_D$ +15.4[21]); ^1H NMR (methanol-d_4, 500 MHz) δ 5.78 (1H, m, H-12), 3.89 (1H, brs, H-18), 3.72 (1H, m, H-2), 3.51 (1H, d, *J* = 11.0 Hz, H-23a), 3.36 (1H, d, *J* = 9.3 Hz, H-3), 3.27 (1H, d, *J* = 11.0 Hz, H-23b), 1.12 (3H, s, CH_3-27), 1.09 (3H, s, CH_3-26), 1.03 (3H, s, CH_3-30), 1.01 (3H, s, CH_3-29), 0.98 (3H, s, CH_3-25), 0.70 (3H, s, CH_3-24), 1.25–2.10 (methylene and methine protons); HREIMS *m/z* 460.3532 [M]$^+$ (calcd for $C_{29}H_{48}O_4$, 460.3552).

Neurite Outgrowth-Promoting Activity. PC12 (pheochromocytoma) cells were cultured in a 24-well plate at a density of 8 × 10^3 cells/cm^2 in DMEM + 10% HS, 5% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 for 24 h. The culture medium was then replaced with DMEM + 2% HS, 1% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. At the same time, different concentrations of the test samples with or without 0.5 ng/mL NGF were added. Each concentration was repeated in three wells. After being incubated with the test samples for 4 days, the cultures were fixed with 4% paraformaldehyde–PBS and stained with methylene blue. Cell morphology was observed under a phase-contrast microscope, and neurite length was quantified. Ten fields were randomly selected under a microscope for each well, and 3–5 significantly differentiated cells were selected for each field and had the length of their longest neurite measured. At least 100 cells were examined for each concentration. Statistical analyses were performed using Dunnett's *t* test. A mixture of **1** and **2**, a mixture of **3** and **4**, and compound **8** had neurite outgrowth-promoting effects on NGF-treated PC12 cells at concentrations of 1, 10, and 30 μM, 1, 10, and 30 μM, and 1 and 10 μM, respectively (Figures 3 and 4).

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR, ^1H–^1H COSY, HMQC, HMBC, NOESY, and FABMS spectra of the mixture of **1** and **2**; ^1H and ^{13}C NMR, ^1H–^1H COSY, HMQC, HMBC, NOESY, and FABMS spectra of the mixture of **3** and **4**; ^1H and ^{13}C NMR and EIMS spectra of compound **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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