# Bioactive Constituents of the Roots of *Cynanchum atratum*

Shiow-Hwa Day,[†,‡] Jih-Pyang Wang,[§] Shen-Jeu Won,[⊥] and Chun-Nan Lin*[,†]

*School of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan 807, Republic of China, Ta-Jen Institute of Technology, Ping Tung Hsieng, Taiwan 907, Republic of China, Department of Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan 407, Republic of China, and Department of Microbiology and Immunology, National Cheng Kung University, Tainan, Taiwan 701, Republic of China*

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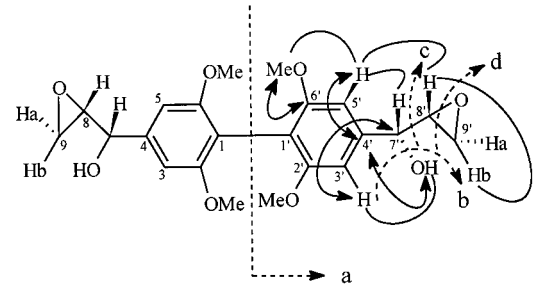
A novel biphenylneolignan, 2,6,2′,6′-tetramethoxy-4,4′-bis(2,3-epoxy-1-hydroxypropyl)biphenyl (**1**), and two new glycosides named atratoglaucosides A (**2**) and B (**3**), were isolated from the roots of *Cynanchum atratum*, and their structures were determined on the basis of chemical and spectroscopic evidence. The aglycons of **2** and **3** were identified as glaucogenin C and 7-desoxyneocynapanogenin A, a new disecopregnane. A known compound, glaucogenin C 3-*O*-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-thevetopyranoside (**4**), isolated from the same source, showed a significant cytotoxic effect against 212 cells. This substance also gave a significant inhibitory effect on TNF-α (tumor necrosis factor-α) formation from the RAW 264.7 mouse macrophage-like cell line stimulated with LPS (lipopolysaccharide) and on the N9 microglial cell line stimulated with LPS/IFN-*γ* (interferon-*γ*).

The Chinese drug "Pai-Wei", the dried roots of *Cynanchum atratum* Bunge (Asclepiadaceae), has been used as an antifebrilitic and diuretic in mainland China.[1] Previously, several new $C_{21}$-substituted steroidal glycosides have been isolated and identified from this plant.[1] In a continued search for novel bioactive constituents from plants, a novel biphenylneolignan (**1**) and two new glycosides, atratoglaucosides A (**2**) and B (**3**), with aglycons possessing a 13,14: 14,15 disecopregnane type skeleton, and five known compounds, glaucogenin C 3-*O*-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-thevetopyranoside (**4**), glaucogenin C 3-*O*-*β*-D-thevetopyranoside (**5**), acetovanillone (**6**), 3,4-dihydroxyacetophenone (**7**), and *p*-hydroxyacetophenone, were isolated from this plant. In the present paper, the structure elucidation of **1**−**3** and the cytotoxic and/or antiinflammatory activities of **4**−**7** are reported (see Chart 1 for structures).

## Results and Discussion

The HREIMS of **1** revealed a molecular ion at *m*/*z* 418.1627, which corresponds to the molecular formula $C_{22}H_{26}O_8$. The IR spectrum of **1** showed absorption bands for a hydroxyl group (3444 $cm^{-1}$) and an aromatic ring (1614 and 1517 $cm^{-1}$). The $^1H$ NMR spectrum of **1** suggested the presence of two substituted benzene rings which are symmetrical, two methoxyl groups, and an allylic alcohol.[2] The presence of a secondary and a tertiary carbon signal at *δ*71.8 and 54.3, respectively, in the $^{13}C$ NMR spectrum of **1** indicated the presence of an epoxide as a terminal moiety. A hydroxyl group was deduced to be substituted at C-7′ from the $^1H$ NMR coupling pattern. The 2,3-epoxy-1-hydroxypropyl moiety in **1** was also clearly revealed from the COSY spectrum and was located at C-4′, by HMBC cross-peaks for H-5′ or H-3′/C-7′, OH-7′/C-4′, H-5′ or H-3′/C-4′, and Me-6′/C-6′ or Me-2′/C-2′, and NOESY cross-peaks for OH-7′/H-3′ or OH-7′/H-5′ and H-3′/MeO-2′ or H-5′/MeO-6′ (Figure 1). The above evidence suggested the presence of a 2′,6′-dimethoxy-4′-(2,3-epoxy-1-hydroxypropyl)phenyl moiety in the structure of **1**. The EIMS of **1** (Figure 1) gave a molecular ion at *m*/*z* 418 and significant fragment ion peaks at *m*/*z* 210 $[M - a + H]^+$, 193 $[210 - b]^+$, 181 $[210 - d + H]^+$, and 167 $[210 - c]^+$. The EIMS fragmentation pattern revealed the presence of two 2,6-dimethoxy-4-(2,3-epoxy-1-hydroxypropyl)phenyl monomers connected by a C-1 to C-1′ bond. In the NOESY experiment on **1**, correlations between H-3′/H-8′ or H-5′/H-8′, H-3′/H-7′ or H-5′/H-7′, and a strong cross-peak between H-8′/$H_b$-9′ suggested the *β*-configuration for H-7′ and H-8′ and an α-configuration for OH-7′. A combination of 2D NMR techniques, such as $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, HMBC, and NOESY experiments enabled us to make complete $^1H$ and $^{13}C$ NMR assignments for **1** (Experimental Section).



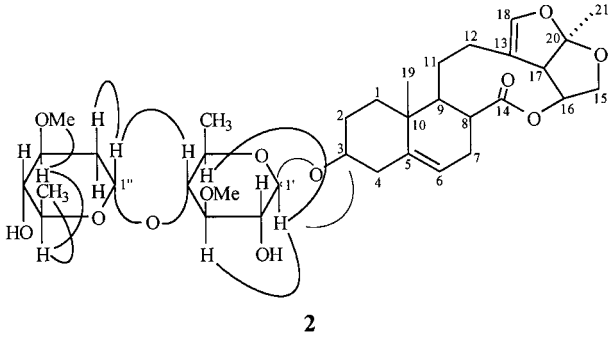




**Figure 1.** H/H and C/H long-range correlation of **1** and **2** obtained from NOESY experiements (—) and HMBC spectra (↔), respectively, and the EIMS fragmentation pattern of **1**.

* To whom correspondence should be addressed. Tel: +886 7 3121101, ext 2163. Fax: +886 7 5562365. E-mail: lincna@cc.kmu.edu.tw.
† Kaohsiung Medical University.
‡ Ta-Jen Institute of Technology.
§ Taichung Veterans General Hospital.
⊥ National Cheng Kung University.

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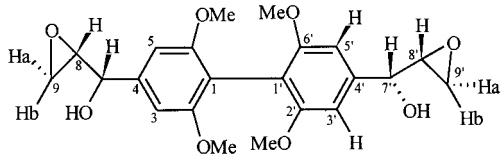
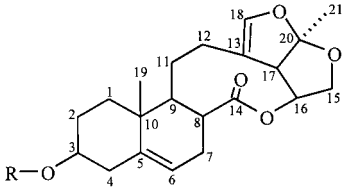
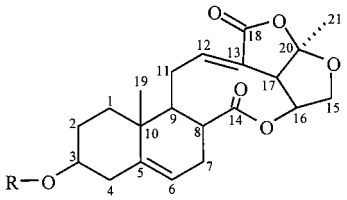
**Chart 1**



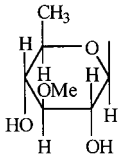
**1**



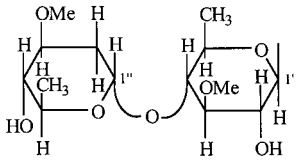
**2** : R = $S_2$
**4** : R = $S_3$
**5** : R = $S_1$



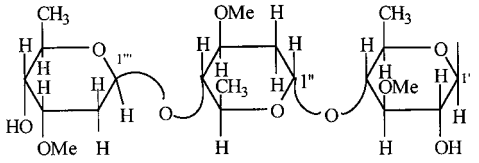
**3** : R = $S_3$



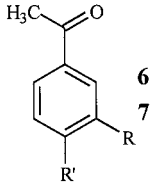
$S_1$



$S_2$



$S_3$



**6** : R = $OCH_3$, R' = OH
**7** : R = OH, R' = OH

Consequently, **1** was characterized as 2,6,2′,6′-tetramethoxy-4,4′-bis(2,3-epoxy-1-hydroxypropyl)biphenyl (**1**).

Compound **2** possesses the molecular formula $C_{35}H_{52}O_{12}$ as determined from FABMS ($[M + Na]^+$ at *m/z* 687) and from the $^1H$ and $^{13}C$ NMR spectra. IR absorptions at 3444 and 1733 $cm^{-1}$ showed the presence of hydroxyl and ester groups. The $^1H$ NMR spectrum showed proton signals characteristic of glaucogenin C mono-D-thevetoside (**5**).[3] The $^{13}C$ NMR spectrum displayed four olefinic carbon signals, one lactone carbonyl signal, and one dioxygenated carbon signal. These carbon signals were almost identical to the corresponding carbon signals of **5**.[3] The $^1H$ NMR spectrum of **2** also indicated two anomeric proton signals at $\delta$ 4.36 (d, $J$ = 7.6 Hz) and 4.98 (d, $J$ = 3.2 Hz), two secondary methyl proton signals at $\delta$ 1.18 (d, $J$ = 7.2 Hz) and 1.23 (d, $J$ = 6.4 Hz), and two methoxy proton signals at $\delta$ 3.31 and 3.54. Mild acid hydrolysis of **2** afforded **5** and diginose.[3,4] Therefore, **2** was found to possess a $\beta$-linked D-thevetopyranose unit and an α-linked L-diginopyranose unit.[3,4]

In the $^{13}C$ NMR spectrum of **2** (Table 1), the chemical shift values of C-1′ to C-6′ (except for C-4′) and C-1″ to C-6″ were almost identical to the corresponding data of glaucogenin C $\beta$-D-thevetopyranoside (**5**) and methyl α-L-diginopyranose, respectively.[3,4] The NOESY experiment of **2** indicated correlations between H-3/H-1′, H-1′/H-3′, H-1′/H-5′, H-4′/H-1″, and H-1″/H-2″. In the positive FABMS of **2**, significant peaks at *m/z* 521 $[M - 145 + 2H]^+$ and 343 $[521-160 - H_2O]^+$ were attributable to those derived by initial loss of the terminal diginose and finally the thevetose linked to the aglycon. Consequently, atratoglaucoside

**Table 1.** $^{13}C$ NMR Chemical Shifts of Compounds **2** and **3** in $C_5D_5N$[a]

| carbon | **2** | **3** | carbon | **2** | **3** |
|---|---|---|---|---|---|
| 1 | 36.4 | 37.3 | 1′ | 102.4 | 102.3 |
| 2 | 30.0 | 30.0 | 2′ | 75.3 | 75.5 |
| 3 | 78.3 | 77.5 | 3′ | 85.5 | 85.6 |
| 4 | 39.0 | 38.8 | 4′ | 80.2 | 80.2 |
| 5 | 140.6 | 140.1 | 5′ | 72.0 | 72.0 |
| 6 | 120.4 | 119.8 | 6′ | 17.6 | 17.7 |
| 7 | 30.7 | 30.1 | OMe | 60.1 | 60.3 |
| 8 | 53.2 | 52.2 | 1″ | 98.9 | 98.8 |
| 9 | 40.6 | 41.0 | 2″ | 30.0 | 32.3 |
| 10 | 38.6 | 38.0 | 3″ | 76.0 | 74.2 |
| 11 | 23.9 | 27.0 | 4″ | 67.8 | 74.8 |
| 12 | 28.4 | 146.9 | 5″ | 67.5 | 67.4 |
| 13 | 118.5 | 130.0 | 6″ | 17.9 | 18.8 |
| 14 | 174.5 | 179.3 | OMe | 55.0 | 55.4 |
| 15 | 67.8 | 71.3 | 1‴ | | 99.5 |
| 16 | 75.5 | 77.4 | 2‴ | | 35.3 |
| 17 | 56.1 | 54.5 | 3‴ | | 79.0 |
| 18 | 143.8 | 167.5 | 4‴ | | 74.2 |
| 19 | 18.8 | 19.5 | 5‴ | | 71.7 |
| 20 | 114.3 | 113.4 | 6‴ | | 18.9 |
| 21 | 24.8 | 23.5 | OMe | | 57.9 |

[a] The number of protons directly attached to each carbon was verified by DEPT experiments and with the $^1H-^1H$, COSY, HMQC, HMBC, and NOESY techniques.

A (**2**) was characterized as glaucogenin C 3-*O*-α-L-diginopyranosyl-(1→4)-*β*-D-thevetopyranoside (**2**). The $^{13}C$ NMR assignments of **2** (Table 1) were made by performing $^{1}H$-decoupled, DEPT, 2D NMR correlation experiments and by comparing the corresponding data for glaucogenin C 3-*O*-*β*-D-thevetopyranoside and methyl α-L-diginopyranoside,[3] respectively.

The HRFABMS of **3** revealed a $[M + Na]^+$ ion at *m/z* 845.3959, which corresponded to the molecular formula $C_{42}H_{62}O_{16}$. Two olefinic bonds and two lactone carbonyl groups were apparent from the $^{13}C$ NMR spectrum. The IR spectrum of **1** showed absorption bands characteristic of one or more hydroxyl groups, a nine-membered lactone, and an α,*β*-unsaturated five-membered lactone. The $^{1}H$ NMR spectrum of **3** showed two tertiary methyl signals at *δ*1.10 (s) and 1.64 (s), four proton signals adjacent to oxygen at *δ* 3.80 (m), 4.10 (dd, $J$ = 10.4, 5.0 Hz), 4.43 (dd, $J$ = 10.4, 7.3 Hz), and 5.75 (ddd, $J$ = 8.0, 7.3, 5.0 Hz), an olefinic proton signal at *δ* 6.00 (bs), and a proton signal of the α,*β*-unsaturated lactone at *δ* 6.14 (dd, $J$ = 12.0, 4.5 Hz). In the $^{13}C$ NMR spectrum of **3** (Table 1), the chemical shift values of C-1 to C-10 and C-19 were similar to the corresponding data of **2** and the chemical shift values of C-11 to C-18 and C-20 to C-21 were similar to the corresponding data of neocynapanoside A,[5] which is a 13,14:14,15 disecopregnane with five- and nine-membered lactone rings. Therefore, the structure of the aglycon of **3** was deduced to be 15,20*β*:18,20*β*-diepoxy-13,14:14,15-disecopregna-5,12-dien-14(16),18(20*β*])-dioic acid dilactone, a new disecopregnane, named 7-desoxyneocynapanogenin A.[5]

The $^{13}C$ NMR chemical shift values due to the sugar moiety of **3** coincide with those of **4**,[5] which contains a *β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl -(1→4)-*β*-D-thevetopyranosyl sugar moiety. The positive ion FABMS fragment peaks at *m/z* 845 $[M + Na]^+$, 663 $[M - 161 + 2H]^+$, 535 $[663 - 128]^+$, and 375 $[535 - 160]^+$ indicated the stepwise cleavage of each sugar unit from the chain. The NOESY experiment showed correlations between H-3/H-1′, H-1′/H-3′, H-1′/H-5′, H-4″/H-1‴, H-1‴/H-5‴, and H-1‴/OMe-3‴. The above evidence provided further support that the sugar sequence was cymarose, diginose, and finally thevetose linked to the aglycon. These results led to the assignment of atraglaucoside B (**3**) as 7-desoxyneocynapanogenin A 3-*O*-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-thevetopyranoside (**3**). The $^{13}C$ NMR assignments of **3** (Table 1) were made by performing $^{1}H$-decoupled, DEPT, and 2D NMR correlation experiments and by comparison with the corresponding data of **2**, **4**, and neocynapanoside A.[4,5] The FABMS fragment ion pattern and $^{13}C$ NMR spectrum also supported the structure proposed for **3**.

The cytotoxicities of **3** and **4** were studied against various cancer cell types, namely, T-24, CaSki, SiHa, HT-3, PLC/PRF/5, and 212. Compound **4** showed a significant cytotoxic effect against 212 cells with an $ED_{50}$ value of 0.96 *μ*g/mL, while **3** did not show significant activity in all of the cancer cell lines used ($ED_{50}$ > 4 *μ*g/mL).

The antiinflammatory activities of compounds **4**−**7** were studied for inhibitory effects against mast cells, neutrophils, microglial cells, and macrophages, stimulated with various inducers. The results indicated that compounds **4**−**7** did not show significant inhibitory effects on mast cells and neutrophils stimulated with various inducers (data not shown).[6−9] Compounds **5**−**7** also did not indicate significant inhibitory effects on TNF-α formation from RAW 264.7 stimulated with LPS and N9 cells stimulated with LPS/INF-*γ* (data not shown), but **4** showed significant inhibitory effects on TNF-α formation from cell lines RAW 264.7 stimulated with LPS and N9 stimulated with LPS/IFN-*γ* (Table 2). These results clearly indicate that the antiinflammatory effect of **4** is mediated through suppression of TNF-α formation in RAW 264.7 and N9 cells. However, further experiments are needed to determine the exact mechanism of action.

**Table 2.** Inhibitory Effects of Compounds **4**−**7** on TNF-α Formation from RAW 264.7 Cells Stimulated with LPS and N9 Cells Stimulated with LPS/IFN-*γ*[a]

| | | inhibition of release (%) | |
|---|---|---|---|
| compound | (*μ*M) | LPS | LPS/IFN-*γ* |
| **4** | 30 | 33.7 ± 6.2[b] | 30.9 ± 4.3[b] |
| **5** | 10 | 29.5 ± 10.3 | 3.2 ± 14.3 |
| **6** | 30 | −19.8 ± 6.3 | −22.6 ± 2.2 |
| **7** | 30 | −29.2 ± 13.6 | −21.5 ± 3.0 |
| dexamethasone | 10 | 49.9 ± 1.7[b] | 82.0 ± 3.8[b] |

[a] Average ±sem ($n$ = 3−5). [b] $P$ < 0.01 compared with corresponding control values (73.2 ± 10.5 ng/mL for LPS and 1.4 ± 0.2 ng/mL for LPS/IFN-*γ*).

## Experimental Section

**General Experimental Procedures.** Melting points are reported uncorrected. The optical rotations were obtained on a Jasco model DIP-370 digital polarimeter. IR spectra were recorded on a Hitachi model 260-30 spectrophotometer. $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer, and MS were obtained on a JMS-HX 100 mass spectrometer.

**Plant Material**. Whole plants of *C. atratum* (Asclepiadaceae) were collected at Kunming, Yunnan, People's Republic of China, in July 1997, and identified by Dr. M. H. Yen, School of Pharmacy, Kaohsiung Medical University. A voucher specimen (No. 9703) is on deposit at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical University.

**Extraction and Isolation**. The $CH_2Cl_2$ extract of the roots of *C. atratum* (2 kg) was evaporated, and the residue (230 g) was chromatographed over a Si gel column. Elution with cyclohexane−$CH_2Cl_2$ (1:1) yielded acetovanillone (**6**) (200 mg), 3,4-dihydroxyacetophenone (**7**) (225 mg), and *p*-hydroxyacetophenone (135 mg), while elution with cyclohexane−$Me_2$-CO (7:3) afforded **1** (30 mg), **2** (30 mg), **3** (35 mg), glaucogenin C 3-*O*-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-thevetopyranoside (**4**) (60 mg), and glaucogenin C 3-*O*-*β*-D-thevetopyranoside (**5**) (110 mg). The known compounds **4**−**7** and *p*-hydroxyacetophenone were identified by spectroscopic methods and by comparison with authentic samples or reported data.[1−5]

**2,6,2′,6′-Tetramethoxy-4,4′-bis(2,3-epoxy-1-hydroxypropyl)biphenyl (1)**: colorless needles ($CHCl_3$); mp 175−176 °C; $[\alpha]^{25}_D$ 75° (*c* 0.11, $CHCl_3$); IR (KBr) $\nu_{max}$ 3444, 1614, 1517 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 400 MHz) *δ* 3.09 (2H, m, H-8, H-8′), 3.90 (12H, s, OMe-2, OMe-2′, OMe-6, OMe-6′), 3.89 (2H, m, H-9a, H-9a′), 4.26 (2H, m, H-9b, H-9b′), 4.78 (2H, d, $J$ = 7.0 Hz, H-7, H-7′), 5.54 (2H, bs, OH-7, OH-7′), 6.57 (4H, s, H-3, H-3′, H-5, H-5′); $^{13}C$ NMR ($CDCl_3$, 100 MHz) *δ* 54.3 (2C, C-8, C-8′), 56.3 (4C, OMe-3, OMe-3′, OMe-5, OMe-5′), 71.8 (2H, C-9, C-9′), 86.0 (2C, C-7, C-7′), 102.7 (4C, C-3, C-3′, C-5, C-5′), 132.0 (2C, C-1, C-1′), 134.3 (2C, C-4, C-4′), 147.1 (4C, C-2, C-2′, C-6, C-6′); EIMS *m/z* 418 $[M]^+$ (29), 387 (2), 210 (13), 193 (22), 167 (67), 181 (100); HREIMS *m/z* $[M]^+$ 418.1627 (calcd for $C_{22}H_{26}O_8$, 418.1627).

**Atratoglaucoside A (2):** colorless oil; $[\alpha]^{25}_D$ −21° (*c* 0.16, $CHCl_3$); IR (KBr) $\nu_{max}$ 3444, 1733 $cm^{-1}$; $^{1}H$ NMR ($(CD_3)_2CO$, 400 MHz) *δ* 0.93 (3H, s, Me-19), 1.18 (3H, d, $J$ = 7.2 Hz, Me-6′), 1.23 (3H, d, $J$ = 7.2 Hz, Me-6″), 1.45 (3H, s, Me-21), 3.39 (1H, dd, $J$ = 8.0, 2.0 Hz, H-17), 3.54, 3.31 (each 3H, s, OMe-3′, OMe-3″), 3.71 (1H, dd, $J$ = 10.0, 9.0 Hz, H-15), 4.14 (1H, dd, $J$ = 9.0, 7.0 Hz, H-15), 4.36 (1H, d, $J$ = 7.6 Hz, H-1′), 4.98 (1H, d, $J$ = 3.2 Hz, H-1″), 5.27 (1H, ddd, $J$ = 10.0, 8.0, 7.0 Hz,

H-16), 5.40 (1H, d, $J$ = 5.6 Hz, H-6), 6.32 (1H, s, H-18); $^{13}$C NMR ($C_5D_5N$, 100 MHz) data, see Table 1; FABMS $m/z$ 687 $[M + Na]^+$ (0.5), 663 $[M - H]^+$ (0.2), 521 $[M - 144 + H]^+$ (0.2), 359 $[521 - 160]^+$ (0.3).

**Atratoglaucoside B (3):** colorless oil; $[\alpha]^{25}_D$ −60° (*c* 1.25, $CHCl_3$); IR (KBr) $\nu_{max}$ 3420, 3400, 1745, 1715, 1350, 1030 cm$^{-1}$; $^1$H NMR ($C_5D_5N$, 400 MHz) $\delta$ 1.10 (3H, s, Me-19), 1.64 (3H, s, Me-21), 3.43, 3.55, 3.90 (each 3H, s, OMe-3′, OMe-3″, OMe-3‴), 3.80 (1H, m, H-3), 4.10 (1H, dd, $J$ = 10.4, 5.0 Hz, H-15), 4.43 (1H, dd, $J$ = 10.4, 7.3 Hz, H-15), 4.78 (1H, d, $J$ = 7.6 Hz, H-1′), 5.13 (1H, dd, $J$ = 9.8, 1.6 Hz, H-1‴), 5.29 (1H, d, $J$ = 3.2 Hz, H-1″), 5.75 (1H, ddd, $J$ = 8.0, 7.3, 5.0 Hz, H-16), 6.00 (1H, bs, H-6), 6.14 (1H, dd, $J$ = 12.0, 4.5 Hz, H-12); $^{13}$C NMR ($C_5D_5N$, 100 MHz) data, see Table 1; FABMS $m/z$ 845 $[M + Na]^+$ (3), 663 $[M - 161 + 2H]^+$ (5), 535 $[663 - 128]^+$ (100), 375 $[535 - 160]^+$ (75), 357 $[359 - 16 - 2H]^+$ (97); HRFABMS $m/z$ $[M + Na]^+$ 845.3959 (calcd for $C_{42}H_{62}O_{16}$, 845.3936).

**Tumor Cell Growth Inhibition Assays**. A microassay for cytotoxicity was performed using a MTT procedure.[10,11] Briefly, (1−3) × 10$^3$ cells/100 $\mu$L were seeded in 96-well microplates (Nunck, Roskilde, Denmark) and preincubated for 6 h in order to allow cell attachment. This medium was then aspirated, and 100 $\mu$L of fresh medium containing various concentrations of test drug were added to the cultures. The cells were incubated with each drug for 6 days. Cell survival was evaluated by adding 10 $\mu$L of tetrazolium salt solution (1 mg MTT/mL in phosphate-buffered saline (PBS). After 4 h of incubation at 37 °C, 100 $\mu$L of DMSO were added to dissolve the precipitate of reduced MTT. Microplates were then shaken for 15 min, and the absorbance was determined at 550 nm with a multiwell scanning spectrophotometer (Dynex MR 5000, Chantilly, VA).

PLC/PRF/5 cells were established from a human hepatoma and are known to produce hepatitis B surface antigen (HBs Ag) continuously in culture fluids.[8] Human hepatoma PLC/PRF/5, T24 cells, human cervical carcinoma, HT-3, SiHa, and CaSki cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY),[8,9] containing 10% fetal bovine serum (FBS; Gibco BRL), 2 mM 1-glutamine, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin. The 212 cells (an inducible Ha-*ras* oncogene transformed NIH/3T3 cell line) were maintained in minimum essential alpha medium (MEM; Gibco BRL), containing 10% calf serum (Gibco BRL) and antibiotics.[9] For the microassay, the growth medium was supplemented with 10 mM HEPES buffer pH 7.3 and incubated at 37 °C in a $CO_2$ incubator.

**Macrophage Cultures and Drugs Treatment.** The RAW 264.7 mouse macrophage-like cell line (American Type Culture Collection) was plated in 96-well tissue-culture plates in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (FCS) and 100 units mL$^{-1}$ of penicillin and streptomycin at 2 × 10$^5$ cells 200 $\mu$L$^{-1}$ per well. Cells were allowed to adhere overnight. Cells were pretreated with 0.5% DMSO, test drugs, or dexamethasone at 37 °C for 1 h before stimulation with 1 $\mu$g mL$^{-1}$ of LPS (*Escherichia coli*, serotype 0111:B4) for 24 h, and then the medium was collected and stored at −70 °C until used.

**Microglial Cell Cultures and Drugs Treatment.** The murine microglial cell line N9[12] (kindly provided by Dr. P. Ricciardi-Castagnoli, CNR Cellular and Molecular Pharmacology Center, Milan, Italy) was plated in 96-well tissue-culture plates in Iscove's modified Dulbecco's medium containing 2% heat-inactivated FCS and antibiotics at 8 × 10$^4$ cells 200 $\mu$L$^{-1}$ per well. Pretreatment of cells was carried out with 0.5% DMSO, test drugs, or dexamethasone at 37 °C for 1 h before stimulation with LPS (10 ng/mL)/IFN-$\gamma$ (10 unit/mL) for 24 h, and then the medium was collected and stored at −70 °C until used.

**TNF-α Determination.** TNF-α in medium was measured by use of an enzyme immunoassay (EIA) kit according to the procedure described by the manufacturer (Genzyme Co., Cambridge, MA).[13]

## References and Notes

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