New Lignan Glycosides with Potent Antiinflammatory Effect, Isolated from *Justicia ciliata*

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Two new lignan glycosides, 4-*O*-[α-L-arabinopyranosyl-(1‴→2″)-*β*-D-xylopyranosyl-(1⁗→5″)-*β*-D-apiofuranosyl]diphyllin (**1**), named ciliatoside A (**1**), and 4-*O*-{[*β*-D-apiofuranosyl-(1⁗→3‴)-α-L-arabinopyranosyl-(1‴→2″)][*β*-D-xylopyranosyl-(1⁗→5″)]-*β*-D-apiofuranosyl}diphyllin (**2**), named ciliatoside B (**2**), were isolated from the whole plant of *Justicia ciliata*. The structures of **1** and **2** were determined by spectral and chemical methods. Compounds **1** and **2** strongly inhibited the accumulation of NO_2^- in lipopolysaccharide-stimulated RAW 264.7 cells in a concentration-dependent manner with IC_{50} values of 27.1 ± 1.6 and 29.4 ± 1.4 μM, respectively.

Previously, we have isolated and characterized two new lignans, cilinaphthalides A and B, along with eight known lignans and a known lignan glycoside, tuberculatin, from the whole plant of *Justicia ciliata* (Yamamoto) Hsieh & Hung (Acanthaceae).[1] In a continued search for novel bioactive constituents from plants, two new naturally occurring 1-aryl-2,3-naphthalide glycosides, ciliatosides A (**1**) and B (**2**), were isolated from this plant. In the present paper the structure elucidation and antiinflammatory activity of **1** and **2** are reported.

Column chromatography of the methanol extract of the air-dried whole plant of *J. ciliata* afforded two new lignan glycosides. The spectral properties (UV, ^{1}H NMR, IR, and MS) of the aromatic region of these glycosides were found to correspond closely to those of diphyllin and justicidin A.[2,3] The ^{1}H NMR spectra of **1** and **2** indicated the presence of three protons in an ABX system, two one-proton signals and two protons of a dioxygenated methylene proton. The *γ*-lactone methylene protons of **1** and **2** are nonequivalent, coupling with each other (J_{gem} = 15.0 Hz) in a manner similar to that observed for the protons of tuberculatin. The UV spectra of **1** and **2** are similar to that of diphyllin. Their IR spectra showed absorption bands for hydroxy groups (3391 cm^{-1}), a *γ*-lactone ring (1749 cm^{-1}), and an aromatic ring (1623 and 1507 cm^{-1}). The positive FABMS revealed a significant ion at *m*/*z* 380, which corresponded to molecular formula $C_{21}H_{16}O_7$ of the diphyllin moiety. On acid hydrolysis with HCl/MeOH, both glycosides **1** and **2** gave diphyllin, apiose, xylose, and arabinose, identified by direct TLC comparison with authentic samples.

Compound **1** possesses the molecular formula $C_{36}H_{19}O_{40}$ as determined from positive FABMS ([M + 1]$^+$ at *m*/*z* 777) and from ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR of **1** shows three anomeric proton signals at *δ* 4.34 (d, *J* = 7.3 Hz), 4.66 (d, *J* = 7.6 Hz), and 5.60 (d, *J* = 3.0 Hz) and three anomeric carbon signals at *δ* 105.7(2C) and 111.6. These data support the presence of *β*-D-xylose, α-L-arabinose, and *β*-D-apiose in **1**. In the ^{13}C NMR spectrum of **1** (Experimental Section), the chemical shift values of C-1″ to C-5″ (except for C-2″ and C-5″, which were downfield by about 10 ppm), C-1‴ to C-5‴, and C-1⁗ to C-5⁗ were almost identical to those of apigenin 7-apiosyl(1→6) glucoside, methyl α-L-arabinopyranoside, and methyl *β*-D-xylopyranoside, respectively.[4,5] The connectivity of the sugar residues with each other and to the diphyllin nucleus was deduced from HMBC, which showed a correlation between the apiose anomeric proton (*δ* 5.60, H-1″) and the diphyllin C-4 (*δ* 146.1) (Figure 1). Furthermore, correlations between H-2″ and C-1″, Ha-5″ and C-3″, and Ha-4″ (*δ* 3.94) and C-3″ in the HMBC and interactions between H-2″ and H-1‴, and H$_2$-5″ and H-1⁗, in the NOESY clearly indicated 1→2 and 1→5 linkages between arabinose and apiose, and xylose and apiose, respectively (Figure 1). Therefore, cilitoside A (**1**) was deduced to be 4-*O*-[α-L-arabinopyranosyl-(1‴→2″)-*β*-D-xylopyranosyl-(1⁗→5″)-*β*-D-apiofuranosyl]-diphyllin (**1**). The information obtained from 2D NMR and ^{13}C NMR data, and a comparison with previously reported data, further supported the characterization of **1** and allowed the assignment of NMR signals as shown in the Experimental Section.[1,4,6,7]

Compound **2** has molecular formula $C_{41}H_{23}O_{48}$ as determined from positive FABMS ([M + Na]$^+$ at *m*/*z* 931) and from ^{1}H and ^{13}C NMR spectra. The spectra of **2** show four anomeric proton signals at *δ* 4.36 (d, *J* = 6.8 Hz), 4.65 (d, *J* = 8.0 Hz), 5.32 (d, *J* = 2.8 Hz), and 5.51 (d, *J* = 2.8 Hz) and four anomeric carbon signals at *δ* 105.6(2C), 111.2, and 111.6. These data indicate the presence of four sugar units: two *β*-D-apiose, one *β*-D-xylose, and one α-L-arabinose.

In the ^{13}C NMR spectrum of **2**, the chemical shift values of C-1″ to C-5⁗ (except for C-3‴, indicating a downfield shift of about 10 ppm) were identical to corresponding data of **1**. The connectivity of the sugar residues with each other and to the diphyllin nucleus was deduced from HMBC, which showed a correlation between the apiose anomeric proton (*δ* 5.50, H-1″) and diphyllin C-4 (*δ* 145.9) (Figure 1). Furthermore, correlations between H-1‴ and C-2″, Ha-5″ and C-2″, C-3″, and C-1⁗, Ha-4″ and C-3″, and H-1⁗ and C-3‴ in the HMBC clearly indicated 1→2, 1→5, and 1→3 linkages between arabinose and apiose, xylose and apiose, and apiose and arabinose, respectively (Figure 1). Therefore, cilitoside B (**2**) was characterized as 4-*O*-{[*β*-D-

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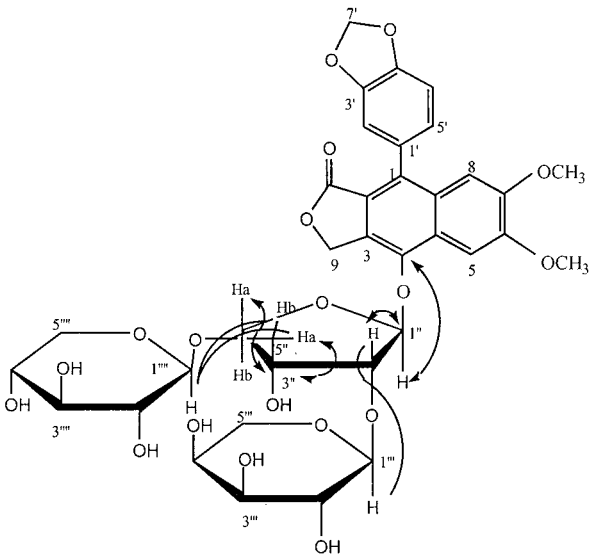

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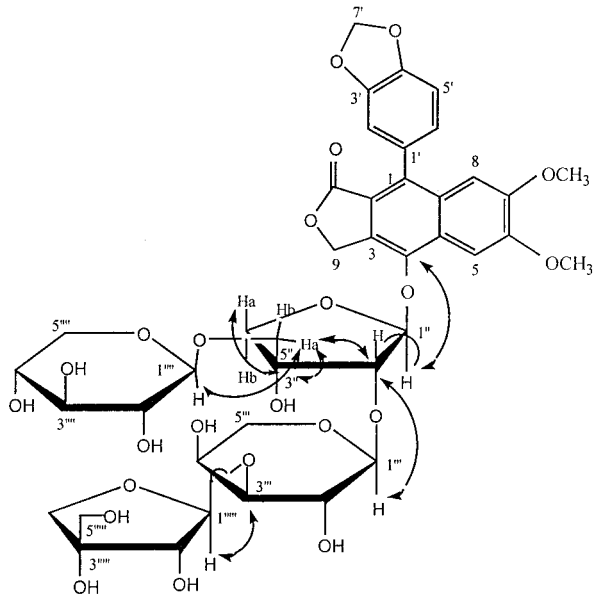

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Figure 1. Some key C/H and H/H long-range correlations of **1** and **2** obtained from HMBC (↔) and NOESY (—) spectra.

apiofuranosyl-(1‴‴→3‴)-α-L-arabinopyranopyranosyl-(1‴→2″)][β-D-xylopyranosyl-(1‴‴→5″)]-β-D-apiofuranosyl}-diphyllin (**2**). The information obtained from 2D NMR and ^{13}C NMR data, and a comparison with previously reported data, further supported the characterization of **2** and allowed the assignment of NMR signals as shown in the Experimental Section.[1,4,6,7]

The antiinflammatory activities of **1** and **2** were determined in vitro by examining their effects on the chemical mediators released from mast cells, neutrophils, macrophages, and microglial cells. Compounds **1** and **2** did not cause significant inhibition of mast cell degranulation stimulated with compound 48/80 and neutrophil degranulation stimulated with formyl-Met-Leu-Phe (fMLP) (1 μM)/cytochalasin B (CB) (5 μg/mL) (data not shown).[8−10] FMLP (0.3 μM)/CB (5 μg/mL) or Phorbol-12-myristate-13-acetate (PMA) (3 nM) induced superoxide anion formation from rat neutrophils.[11] Compounds **1** and **2** did not cause significant inhibition of superoxide anion formation from rat neutrophils stimulated with fMLP/CB or PMA (data not shown).

Table 1. Inhibitory Effects of **1** and **2** on the Accumulation of NO_2^- (μM) from RAW 264.7 Cells Stimulated with LPS and N9 Cells Stimulated with LPS/IFN-γ[a]

		% inhibition	
compound	(μM)	RAW 264.7 cells	N9 cells
control		25.1 ± 0.9	51.3 ± 0.4
1	(3)	22.0 ± 1.3[e]	nd[e]
	(10)	18.7 ± 1.0[d]	46.7 ± 0.1[d]
	(30)	11.9 ± 0.3[d]	37.9 ± 0.8[d]
IC_{50}		27.1 ± 1.6 μM	
2	(3)	20.7 ± 0.5[d]	nd[e]
	(10)	20.5 ± 0.3[d]	42.8 ± 0.4[d]
	(30)	12.4 ± 0.4[d]	36.5 ± 0.5[d]
IC_{50}		29.4 ± 1.4 μM	
L-NAME[c]	(0.1 mM)	16.8 ± 0.4[d]	44.2 ± 0.7[d]
	(0.3 mM)	15.6 ± 0.5[d]	32.2 ± 0.9[d]
	(1 mM)	6.6 ± 0.5[d]	15.7 ± 0.01[d]
IC_{50}		0.51 ± 0.02 mM	0.63 ± 0.009 mM

[a] Results are presented as average ± s.e.m. ($n = 3$). [b] nd, not determined. [c] L-NAME (N^{ω}-nitro-L-arginine methyl ether): positive control. [d] $P < 0.01$. [e] $P < 0.05$ as compared with control values.

Effects on NO_2^- and tumor-necrosis factor-α (TNF-α) were determined in the mouse macrophage-like cell line RAW 264.7 and the murine microglial cell line N9, which were activated by lipopolysacharide (LPS) and LPS/interferon-γ (IFN-γ), respectively.[12−14] As shown in Table 1, **1** and **2** strongly inhibited the accumulation of NO_2^- in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner, with IC_{50} values of 27.1 ± 1.6 and 29.4 ± 1.4 μM, respectively. Compounds **1** and **2** showed weak inhibitory effects on NO_2^- accumulation in LPS/IFN-γ-stimulated N9 cells. Both **1** and **2** had no inhibitory effect on TNF-α generation from RAW 264.7 and N9 cells (data not shown). Because NO and its metabolites have been demonstrated in the pathology of central neurologic disease and implicated in the pathogenesis of the peripheral tissue damage associated with acute and chronic inflammation,[15,16] the present study suggests that the inhibition of NO_2^- generation by **1** and **2** in macrophages and microglial cells may have value in the therapeutic treatment or prevention of certain central as well as peripheral diseases associated with increased NO production.

Experimental Section

General Experimental Procedures. Mps are reported uncorrected. UV spectra were obtained on a JASCO model 7800 UV−vis spectrophotometer, and IR spectra were recorded on a Hitachi model 260-30 spectrophotometer. ^1H (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 *J. ciliata* (Yamamoto) Hsieh & Huang (Acanthaceae) were collected in Pong Hwu Hsein, Taiwan, in July 1996, and a voucher specimen has been deposited at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical University.

Extraction and Isolation. The air-dried whole plant of *J. ciliata* (1.6 kg) was chipped and extracted with methanol at room temperature. The MeOH extract (150 g) was chromatographed on silica gel to yield seven fractions. Fraction 7 was rechromatographed on Si gel and eluted with CH_2Cl_2−MeOH (4:1) to yield **1** (50 mg) and **2** (30 mg).

Ciliatoside A (1): colorless oil; $[\alpha]_D^{25}$ −272° (*c* 0.15, MeOH); UV (MeOH) λ_{max}(log ε) 262 (4.78), 288 (4.22), 313 (sh) (4.17), 350 (sh) (3.87) nm; IR (KBr) ν_{max} 3391 (OH), 1749 (CO), 1623, 1507 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 3.24 (1H, dd, J = 10.0, 7.6 Hz, H-2‴), 3.28 (1H, dd, J = 10.0, 7.3 Hz, H-2⁗), 3.36 (1H, dd, J = 10.0, 4.5 Hz, H-3⁗), 3.38 (1H, dd, J = 10.0, 3.0 Hz, H-3‴), 3.49 (1H, m, H-4⁗), 3.52 (1H, m, H-4‴), 3.73 (1H, d, J = 10.3 Hz, H_b-5″), 3.73 (3H, s, OMe-6), 3.80 (2H, dd,

J = 11.0, 5.3 Hz, H_2-5‴), 3.91 (2H, dd, J = 11.0, 5.3 Hz, H_2-5‴), 3.94 (1H, d, J = 9.8 Hz, Ha-4″), 4.02 (3H, s, OMe-7), 4.06 (1H, d, J = 10.3, Ha-5″), 4.30 (1H, d, J = 9.8 Hz, H_b-4″), 4.34 (1H, d, J = 7.3 Hz, H-1⁗), 4.66 (1H, d, J = 7.6 Hz, H-1‴), 4.81 (1H, d, J = 3.0 Hz, H-2″), 5.46 (1H, d, J = 15.0 Hz, H-9), 5.52 (1H, d, J = 15.0 Hz, H-9), 5.60 (1H, d, J = 3.0 Hz, H-1″), 6.04 (1H, s, H-7′), 6.06 (1H, s, H-7′), 6.77 (1H, dd, J = 8.0, 1.5 Hz, H-6′), 6.81 (1H, d, J = 1.5 Hz, H-2′), 6.95 (1H, d, J = 8.0 Hz, H-5′), 7.05 (1H, s, H-8), 7.64 (1H, s, H-5); ^{13}C NMR (CD_3-OD, 100 MHz) δ 56.0 (OMe-7), 57.0 (OMe-6), 67.2 (C-5‴), 67.3 (C-5⁗), 68.8 (C-9), 71.1 (C-4‴), 71.2 (C-4⁗), 71.9 (C-5″), 74.9 (C-2‴), 75.4 (C-2⁗), 75.3 (C-3‴), 75.7 (C-4″), 77.8 (C-3⁗), 80.1 (C-3″), 86.9 (C-2″), 102.0 (C-8), 102.6 (C-7′), 105.7 (C-1‴ and −1⁗), 107.1 (C-5), 109.0 (C-5′), 111.6 (C-1″), 111.9 (C-2′), 120.1 (C-3), 124.7 (C-6′), 128.3 (C-1a), 130.0 (C-1′), 130.6 (C-2), 131.8 (C-4a), 137.2 (C-1), 146.1 (C-4), 149.0 (C-3′ and −4′), 151.7 (C-6), 153.4 (C-7), 172.1 (C-10); FABMS (postitive) m/z $[M + Na]^+$ 799 (2), $[M + 1]^+$ 777 (1), 412 (7), 380 (16), 307 (10), 154 (100), 136 (80). Acid hydrolysis (2 N HCl−MeOH) of **1** yielded diphyllin, pale yellow needles, mp 280−283 °C; the mmp, IR, NMR, and MS were identical with those of authentic diphyllin and sugars, examined by TLC [CH_2Cl_2−MeOH (4:1) on silica gel], R_f 0.71 (apiose), methyl α,β-D-xylopyranoside (R_f 0.36, 0.45), and methyl α,β-L-arabinopyranoside (R_f 0.08, 0.12).

Ciliatoside B (2): colorless oil; $[\alpha]_D^{25}$ −40°(c 0.10, MeOH); UV (MeOH) λ_{max}(log ϵ) 262 (4.87), 294 (4.23), 319 (sh) (4.18), 352 (sh) (3.98) nm; IR (KBr) ν_{max} 3391 (OH), 1749 (CO), 1623, 1507 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 3.32 (2H, m, H_2-5⁗), 3.38 (1H, d, J = 8.0 Hz, H-2⁗), 3.47 (1H, bd, J = 6.8 Hz, H-2⁗), 3.49 (1H, bd, J = 8.0 Hz, H-3⁗), 3.62 (2H, m, H_2-5‴), 3.62 (2H, s, H_2-5⁗), 3.64 (3H, s, OMe-7), 3.73 (1H, m, H-4⁗), 3.75 (1H, d, J = 10.4 Hz, H_b-5″), 3.80 (1H, m, H-4‴), 3.81 (1H, d, J = 9.6 Hz, Ha-4⁗), 3.95 (1H, d, J = 9.6 Hz, Ha-4″), 3.96 (3H, s, OMe-6), 4.02 (1H, d, J = 2.8 Hz, H-2⁗), 4.04 (1H, t, J = 6.8 Hz, H-3⁗), 4.05 (1H, d, J = 10.4 Hz, Ha-5″), 4.15 (1H, d, J = 9.6 Hz, H_b-4⁗), 4.26 (1H, d, J = 9.6 Hz, H_b-4″), 4.36 (1H, d, J = 6.8 Hz, H-1⁗), 4.64 (1H, d, J = 8.0 Hz, H-1‴), 4.78 (1H, d, J = 2.8 Hz, H-2″), 5.30 (1H, d, J = 2.8 Hz, H-1⁗), 5.38 (1H, d, J = 15.0 Hz, H-9), 5.44 (1H, d, J = 15.0 Hz, H-9), 5.50 (1H, d, J = 2.8 Hz, H-2″), 6.03 (1H, s, H-7′), 6.04 (1H, s, H-7′), 6.71 (1H, d, J = 1.2 Hz, H-2′), 6.76 (1H, dd, J = 8.0, 1.2 Hz, H-6′), 6.89 (1H, d, J = 8.0 Hz, H-5′), 6.91 (1H, s, H-8), 7.47 (1H, s, H-5); ^{13}C NMR (CD_3OD, 100 MHz) δ 56.0 (OMe-7), 56.9 (OMe-6), 65.2 (C-5⁗), 66.8 (C-5‴), 67.3 (C-5⁗), 68.8 (C-9), 71.1 (C-4‴), 71.2 (C-4⁗), 72.0 (C-5″), 74.9 (C-4⁗), 75.3 (C-2‴ and −2⁗), 75.7 (C-4″), 77.8 (C-2⁗), 77.9 (C-3⁗), 80.1 (C-3″), 80.5 (C-3⁗), 85.0 (C-3‴), 86.8 (C-2″), 101.9 (C-8), 102.6 (C-7′), 105.5 (C-1‴), 105.6 (C-1⁗), 107.1 (C-5), 110.0 (C-5′), 111.2 (C-1″), 111.6 (C-1⁗), 111.9 (C-2′), 119.9 (C-3), 121.6 (C-6′), 128.1 (C-1a), 129.8 (C-1′), 130.6 (C-2), 131.6 (C-4a), 136.8 (C-1), 145.9 (C-4), 148.8 (C-4′), 148.9 (C-3′), 151.5 (C-6), 153.2 (C-7), 172.0 (C-10); FABMS (postive) m/z $[M + Na]^+$ 931 (5), 799 (2), 777 (5), 645 (4), 460 (9), 413 (14), 380 (100), 363 (6). The results of acid hydrolysis were the same as for **1**.

Macrophage Culture Assay. RAW 264.7 mouse macrophage-like cells (American Type Culture Collection) were plated in 96-well tissue-culture plates in Dulbeco's Modified Eagle medium supplemented with 5% fetal calf serum (FCS), 100 units mL^{-1} of penicillin, and streptomycin at 2 × 10^5 cells per 200 μL per well. Cells were allowed to adhere overnight. Cells were pretreated with 0.5% DMSO or test compounds at 37 °C for 1 h before stimulation with 1 μg 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. The final concentration of test compound in DMSO was fixed at 0.5%.

Microglial Cell Culture Assay. Murine microglial cells (cell line N9)[17] (kindly provided by Dr. P. Ricciardi-Castagnoli, CNR Cellular and Molecular Pharmacology Center, Italy) were 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 per 200 μL per well. Cells were pretreated with test compounds at 37 °C for 1 h before stimulation with LPS (10 μg mL^{-1}) for 24 h, and then the medium was collected and stored at −70 °C until used. The final concentration of test compound in DMSO was fixed at 0.5%.

NO Determination. The production of NO in the cell medium was determined by measuring the content of nitrite using the Griess reaction.[18] Briefly, 40 μL of 5 mM sulfanilamide, 10 μL of 2 M HCl, and 20 μL of 40 mM naphthylethylenediamine were added to 150 μL of culture medium. After 10 min of incubation at room temperature, absorbance was measured at 550 nm in a microplate reader. A standard nitrite curve was generated in the same fashion using $NaNO_2$. The final concentration of test compound in DMSO was fixed at 0.5%.

Statistical Analysis. Data are presented as the means ± s.e.m. Statistical analyses were performed using the least significant difference test method after analysis of variance. P values < 0.05 were considered to be significant. Analysis of the regression line was used to calculate IC_{50} values.

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