Potent Cytotoxic Lignans from *Justicia procumbens* and Their Effects on Nitric Oxide and Tumor Necrosis Factor-α Production in Mouse Macrophages

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Received March 29, 2001

A new lignan glycoside, 4-*O*-α-L-arabinopyranosyl-(1‴→2″)-*β*-D-apiofuranosyldiphyllin (**2**), named procumbenoside A, and 11 known compounds were isolated from the whole plant of *Justicia procumbens*. The structure of **2** was established by spectral analysis and chemical methods. The known compounds justicidin A (**1**), diphyllin (**3**), and tuberculatin (**4**) showed potent cytotoxic effects against a number of cancer cells in vitro. Compounds **1** and **4** also strongly enhanced tumor-necrosis factor-α (TNF-α) generation from mouse macrophage-like RAW 264.7 cells stimulated with lipopolysaccharide (LPS).

The whole plants of *Justicia procumbens* L. (Acanthaceae) are used as an herbal remedy for the treatment of fever, pain, and cancer in China.[1] Previous phytochemical studies on this plant have afforded lignans.[1–6] In a continued search for novel cytotoxic constituents from plants, a new lignan glycoside, procumbenoside A (**2**), and 11 known compounds were isolated from *J. procumbens*. In the present paper we report the structure elucidation of the new compound, **2**, the cytotoxicity of **1**–**6** against several cell lines, and the effects of **1**, **3**, and **4** on nitric oxide formation and tumor-necrosis factor-α (TNF-α) generation from mouse macrophage-like RAW264.7 cells stimulated with lipopolysaccharide (LPS).

Compound **2** was assigned the molecular formula $C_{31}H_{32}O_{15}$ as determined from its negative-ion HRFABMS ($[M - 1]^-$ at *m/z* 643.1659) and ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR spectra of **2** showed two anomeric proton signals at δ 4.44 (d, $J = 7.5$ Hz) and 5.45 (d, $J = 3.0$ Hz) and two anomeric carbon signals at δ 106.0 and 111.4. These data indicated the presence of two sugar units, namely, *β*-apiose and α-arabinose.[7,8] The ^{1}H NMR spectrum of **2** indicated the presence of three protons in an ABX system, two one-proton signals, two protons of a dioxygenated methylene group, and two methoxyl protons. The γ-lactone methylene protons of **2** were nonequivalent, coupling with each other ($J_{gem} = 15.0$ Hz) in a manner similar to that observed for the protons of tuberculatin (**4**). The UV spectrum of **2** was similar to that of diphyllin (**3**). The IR spectrum showed hydroxyl (3402 cm^{-1}), γ-lactone ring (1750 cm^{-1}), and aromatic ring (1623 cm^{-1}) absorption. The negative-ion FABMS revealed a significant ion at *m/z* 379, which indicated the presence of a diphyllin (**3**) moiety. On acid hydrolysis with HCl/MeOH, **2** gave **3**, apiose, and arabinose, as identified by direct TLC comparison with authentic samples.

In the ^{13}C NMR spectrum of **2**, the chemical shift values of C-1″ to 4″ were identical to corresponding data for ciliatoside A (**5**).[9] The connectivity of the sugar residues with each other and to the diphyllin nucleus was deduced from the HMBC spectrum, which showed a correlation between the apiose anomeric proton (δ 5.45, H-1″) resonance and the diphyllin C-4 (δ 145.8) signal. Furthermore, a HMBC correlation between H-1‴ and C-2″ clearly indicated a 1→2 linkage between arabinose and apiose. Therefore, procumbenoside A (**2**) was characterized as 4-*O*-α-L-arabinopyranosyl-(1‴→2″)-*β*-D-apiofuranoyldiphyllin (**2**).

The cytotoxicity of **2**–**6** isolated from this plant and *J. ciliata* was studied against a number of cancer cell types. The results are listed in Table 1. Compound **1** has shown significant cytotoxic activities against the Hep 3B, Hep G2, MCF-7, MCF-7-*ras*, and other cell lines, as reported previously.[10] Compound **4** showed almost the same cytotoxic potencies against Hep 3B, Hep G2, MCF-7, and MCF-7-*ras* as did **1**, but showed stronger cytotoxic activities against the Hep 3B, SiHa, Hep G2, HT-29, HCT 116, MCF-7, and MCF-7-*ras* than those of its aglycon (i.e., **3**). Compound **4** showed significant cytotoxic activities against the HT-29, HCT 116, MCF-7, and MCF-7-*ras* cell lines. Compound **2** showed significant cytotoxic activity against the 212, Hep 3B, and Hep G2 cell lines, while **5** and **6** (data not shown) did not show significant cytotoxic activity against the cell lines shown in Table 1. It was apparent, therefore, that *O*-methylation or *O*-glycosylation at C-4 of **3** enhanced the cytotoxic activities against several of the cell lines used, while *O*-glycosylation with more than one sugar unit at C-4 of **3** led to less cytotoxic activity (Table 1).

Effects on the generation of nitric oxide and TNF-α were determined in RAW 264.7 cells activated by LPS.[11–13] As shown in Table 2, **1** and **4** weakly inhibited the accumulation of NO_2^-, while **3** significantly enhanced the accumulation of NO_2^- in LPS-stimulated RAW 264.7 cells. Previously, we have reported that **5** and **6** strongly inhibited the formation of nitric oxide in this same cell line.[9] It was evident that *O*-methylation and *O*-glycosylation at C-4 of **3** enhanced the inhibitory effects on LPS stimulation of nitric oxide formation. Compounds **1** and **4** strongly enhanced TNF-α generation in LPS-stimulated RAW 264.7 cells, while **3** and **6** showed only slight enhancement. As shown in Table 2, *O*-methylation and *O*-glycosylation at C-4 of **3** strongly enhanced the TNF-α generation, while

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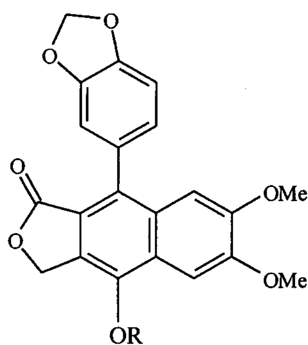
10.1021/np0101651 CCC: $22.00 © 2002 American Chemical Society and American Society of Pharmacognosy
Published on Web 02/07/2002

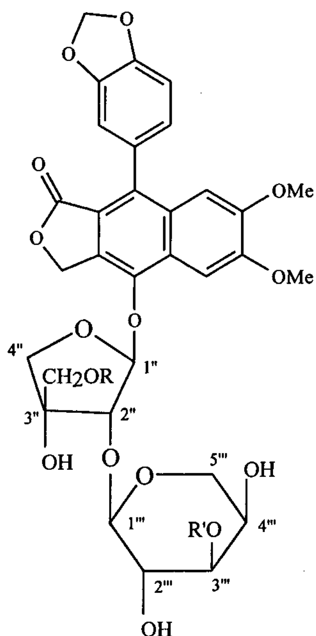
Table 1. Cytotoxicity of Lignans Isolated from *Justicia procumbens*[a]

compound	cell line								
	212	CaSKi	Hep3B	SiHa	Hep G2	HT-29	HCT116	MCF-7	MCF-7-*ras*
1	22.7×10^{-3} [b]	3.0×10^{-3} [b]	2.9×10^{-2}	7.4×10^{-3} [b]	2.0×10^{-2}	*c*	*c*	3.9×10^{-1}	7.4×10^{-2}
2	3.1	*c*	3.1	*c*	3.9	6.7	*d*	*c*	*c*
3	2.7	*d*	3.6	*d*	0.4	2.5	0.8	*d*	*d*
4	*c*	*c*	1.4×10^{-2}	1.2×10^{-1}	4.0×10^{-2}	2.9×10^{-1}	2.8×10^{-1}	9.7×10^{-1}	9.0×10^{-2}
cisplatin	1.3	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
actinomycin D	*c*	1.9×10^{-3}	*c*	8.1×10^{-4}	*c*	*c*	*c*	*c*	*c*
5-fluorouracil	*c*	*c*	7.15×10^{-2}	*c*	3.3×10^{-2}	7.4×10^{-2}	0.48	*c*	*c*

[a] Data are presented as ED_{50} values in μg/mL. For significant activity of the pure compounds, an $ED_{50} \leq 4.0$ μg/mL is required. [b] Data obtained from ref 11. [c] Not determined. [d] No significant activity.



1 R = Me
3 R = H
4 R = β-D-apiofuranosyl



2 R = R' = H
5 R = β-D-xylopyranosyl, R' = H
6 R = β-D-xylopyranosyl, R' = β-D-apiofuranosyl

glycosidation with three or four sugar units reduced this effect. The potent cytotoxic lignan, **1**, showed potent proinflammatory effects through the enhancement of TNF-α generation.

Table 2. Effects of **1**, **3**, and **4** on the Accumulation of NO_2^- (μM) (A) and TNF-α (ng/mL) Formation (B) from RAW 264.7 Cells Stimulated with LPS[a]

compound	(μM)	% inhibition	
		A	B
1	(0.1)		-6.0 ± 13.1
	(0.3)	20.3 ± 0.5	-157.5 ± 14.2[c]
	(1.0)	19.8 ± 2.5	-340.5 ± 19.4[c]
3	(10)	-28.4 ± 3.6[b]	
	(30)	-0.5 ± 13.5	-22.2 ± 12.7
4	(3)	16.9 ± 1.1	-225.8 ± 16.4[c]
5	(30)		18.7 ± 11.7
6	(30)		-26.0 ± 7.9
1400 W[d]		(IC_{50} 3.1 ± 0.1 μM)	
dexamethasone			(IC_{50} 0.7 ± 0.2 μM)

[a] Results are presented as means ± SEM ($n = 3$), with 36.7 ± 0.5 μM and 73.2 ± 10.5 ng/mL being the control values for **1** on A and B, respectively, 67.8 ± 1.1 μM and 57.2 ± 1.5 ng/mL being the control values for **3** and **4** on A and B, respectively, 52.4 ± 3.4 ng/mL being the control values for **5** and **6** on B, and 68.7 ± 1.1 μM and 57.2 ± 1.5 ng/mL being the control values for 1400 W and dexamethasone on A and B, respectively. [b] $p < 0.05$. [c] $p < 0.01$ as compared with the respective control values. [d] 1400 W [*N*-(3-aminomethyl)benzylactamidine].

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanaco micro melting point apparatus and are reported uncorrectly. The optical rotations were obtained on a JASCO model DIP-370 digital polarimeter. UV spectra were obtained on a JASCO model 7800 UV–vis spectrophotometer, and 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. Mass spectra were obtained on a JMS-HX 100 mass spectrometer.

Plant Material. Whole plants of *J. procumbens* were collected at Chu-Shan, Nantu Hsein, Taiwan, in October 1997, and a voucher specimen is on deposit at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical University.

Extraction and Isolation. The air-dried whole plants of *J. procumbens* (5 kg) were chipped and extracted with MeOH at room temperature. The MeOH extract (150 g) was chromatographed on a Si gel column, and elution with cyclohexane–EtOAc (4:1) yielded justicidins D and E (30 mg). In turn, elution with cyclohexane–EtOAc (7:3) yielded taiwan E methyl ether (23 mg), **1** (0.2 g), and justicidin C (35 mg), while elution with CH_2Cl_2–EtOAc (20:1) yielded chinensinaphthol methyl ether (16 mg) and secoisolariciresinol (56 mg). Elution with CH_2Cl_2–MeOH (10:1) yielded stigmasterol-3-*O*-*β*-D-glucoside (15 mg) and **2** (15 mg), and elution with CH_2Cl_2–MeOH (9:1) yielded **3** (12 mg). Finally, elution with CH_2Cl_2–MeOH (4:1) yielded **4** (20 mg), and elution with CH_2Cl_2–MeOH (3:1) yielded **5** (5 mg). The known compounds were identified by spectroscopic methods and comparison with reported data or authentic samples.[2–4,9]

Procumbenoside A (2): colorless powder (MeOH); $[\alpha]^{25}_{D}$ $-16°$ (*c*, 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (4.30), 225

(4.24), 257 (4.53), 292 (3.89), 307 (3.87), 350 (3.59) nm; IR (KBr) ν_{max} 3402 (OH), 1750 (CO), 1623 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 3.18 (1H, m, H-5‴), 3.21 (1H, t, J = 7.6 Hz, H-2‴), 3.28 (1H, m, H-3‴), 3.42 (1H, m, H-4‴), 3.56 (3H, s, OMe-7), 3.62 (1H, d, J = 11.5 Hz, H-5″), 3.67 (1H, d, J = 11.5 Hz, H-5″), 3.73 (1H, m, H-5‴), 3.79 (1H, d, J = 9.6 Hz, H-4″), 3.87 (3H, s, OMe-6), 4.20 (1H, d, J = 9.6 Hz, H-4″), 4.44 (1H, d, J = 7.6 Hz, H-1‴), 4.62 (1H, d, J = 3.0 Hz, H-2″), 5.30 (1H, d, J = 15.0 Hz, H-9), 5.36 (1H, d, J = 15.0 Hz, H-9), 5.45 (1H, d, J = 3.0 Hz, H-1″), 5.91 (1H, s, H-7′), 5.93 (1H, s, H-7′), 6.50 (1H, d, J = 1.5 Hz, H-2′), 6.56 (1H, dd, J = 8.4, 1.5 Hz, H-6′), 6.79 (1H, d, J = 8.4 Hz, H-5′), 6.81 (1H, s, H-8), 7.38 (1H, s, H-5); ^{13}C NMR (CD_3OD, 100 MHz) δ 56.0 (OMe-7), 56.8 (OMe-6), 64.2 (C-5″), 67.3 (C-5‴), 68.7 (C-9), 71.1 (C-4‴), 75.2 (C-2‴), 75.8 (C-4″), 77.7 (C-3‴), 81.2 (C-3″), 85.9 (C-2″), 101.8 (C-5), 102.6 (C-7′), 106.0 (C-1‴), 107.0 (C-8), 108.9 (C-5′), 111.4 (C-1″), 111.8 (C-2′), 119.9 (C-2), 124.8 (C-6′), 128.1 (C-4a), 129.8 (C-1′), 130.5 (C-3), 131.5 (C-8a), 136.7 (C-1), 145.8 (C-4), 148.8 (2C, C-3′, C-4′), 151.5 (C-7), 153.2 (C-6), 172.0 (CO); FABMS (negative-ion) m/z $[M - 1]^-$ 643 (3), 379 (100); HRFABMS (negative-ion) m/z $[M - 1]^-$ 643.1659 (calcd for $C_{31}H_{31}O_{15}$, 643.1663).

Tumor Cell Growth Inhibition Assays. A microassay for cytotoxicity was performed using MTT.[14,15] Briefly, (1−3) × 10^3 cells/100 μL were seeded in 96-well microplates (Nunck, Roskilde, Denmark) and preincubated for 6 h in order to allow cell attachment. The cells were incubated with each drug for 6 days and then pulsed with 10 μL of MTT (5 mg MTT/mL; Sigma, St. Louis, MO) and incubated for an additional 4 h at 37 °C. The microplates were read at 550 nm on a Multiskan photometer (MR5000; Dynatech, McLean, VA) after lysis of cells with 100 μL of 10% SDS in 0.01 M HCl. Control wells contained medium plus cells (total absorbance) or medium alone (background absorbance). Cell death was calculated as the percentage of MTT inhibition.

The 212 cells (an inducible Ha-*ras* oncogene transformed from the NIH/3T3 cell line) and MCF-7-*ras* (an inducible Ha-*ras* oncogene transformed from the MCF-7 cell line) were maintained in minimum essential alpha medium (MEM; Gibco BRL; Grand Island, NY) and Dulbecco's modified Eagle medium (DMEM; Gibco BRL), respectively, containing 10% fetal bovin serum (FBS; Gibco BRL, Grand Island, NY) and antibiotics.[16] Human hepatomacellular carcinoma Hep 3B and Hep G2, human cervical carcinoma SiHa and CaSki, human colorectal adenocarcinoma HT-29, human colorectal carcinoma HCT 116, and human breast cancer MCF-7 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and grown in DMEM,[15,16] containing 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. 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 Culture Assay. The RAW 264.7 mouse macrophage-like cell line ATCC (American Type Culture Collection) was plated in 96-well tissue-culture plates in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 2 ×10^5 cells per 200 μL per well. Cells were allowed to adhere overnight. Pretreatment of cells with 0.5% DMSO or test drugs at 37 °C for 1 h before stimulation with 1 μg/mL of LPS (*Escherichia coli*, serotype 0111:B4; Sigma, St. Louis, MO) for 24 h, and then the medium was collected and stored at −70 °C until used. The final concentration of drugs in DMSO was fixed at 0.5%.

NO Determination. The production of NO was determined in cell medium by measuring the content of nitrite based on the Griess reaction.[17] 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 drugs in DMSO was fixed at 0.5%.

TNF-α Determination. Tumor-necrosis factor-α (TNF-α) in DMEM was measured by use of an ELISA Kit according to the procedure described by the manufacturer (R&D Systems, Minneapolis, MN).[18]

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

Acknowledgment. This work was supported by a grant from the National Science Council of the Republic of China (NSC 89-2320-B 037-077).

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NP0101651