Anti-inflammatory Constituents and New Pterocarpanoid of *Crotalaria pallida*

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One new pterocarpanoid, crotafuran E (**1**), and three known compounds were isolated from the bark of *Crotalaria pallida*. The structure of **1** was determined by spectral methods. Two pterocarpanoids, crotafurans A (**2**) and B (**3**), previously isolated from this plant, showed significant concentration-dependent inhibitory effects on the NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage-like cells, with IC_{50} values of 23.0 ± 1.0 and 19.0 ± 0.2 μM, respectively. Compound **3** also inhibited the LPS/interferon-γ (IFN-γ)-stimulated NO production in N9 microglial cells with an IC_{50} value of 9.4 ± 0.9 μM. Moreover, compound **2** produced a concentration-dependent inhibition of the release of β-glucuronidase and lysozyme from rat neutrophils in response to formyl-Met-Leu-Phe/cytochalasin B (fMLP/CB) with IC_{50} values of 7.8 ± 1.4 and 9.5 ± 2.1 μM, respectively.

The genus *Crotalaria* has 300 species worldwide with only about 19 species reported in Taiwan and produces mainly pyrrolizidine alkaloids.[1] As a part of our ongoing search for anti-inflammatory constituents from natural sources, we have investigated the anti-inflammatory activity of different extracts of the barks of *Crotalaria pallida* (CP) and the seeds of *C. assamica* (CA) using in vitro inhibitory effects on chemical mediators released from mast cells and neutrophils. MeOH extract (CP) strongly inhibited the release of chemical mediators from rat neutrophils stimulated with fMLP/CB (Table 2). On the basis of this result (Table 2), we have isolated and characterized four pterocarpanoids from the MeOH extracts of *C. pallida* and *C. assamica*.[2] In a continuing search for anti-inflammatory constituents from *C. pallida*, a new pterocarpanoid, crotafuran E (**1**), and three known compounds, apigenin,[3] genistein,[4] and alpinum isoflavone,[5] were isolated from the bark of this plant. In the present paper, the structure elucidation of the new pterocarpanoid, **1**, and anti-inflammatory activity of crotafurans A (**2**) and B (**3**), previously isolated from this plant,[2] are reported. We cannot report the anti-inflammatory effects of compound **1** and crotafurans C and D[2] because these compounds are unstable in culture medium.

The molecular formula of crotafuran E (**1**) was determined to be $C_{20}H_{18}O_6$ by HREIMS (*m*/*z* 354.1110 $[M]^+$), which was also consistent with the ^1H and ^{13}C NMR data (Table 1). The IR absorption of **1** implied the presence of OH (3468 cm^{-1}) and aromatic ring (1620 cm^{-1}) moieties. The ^1H NMR spectrum of **1** shows signals for one tertiary methyl group at δ 1.54 (3H, s, Me-16), two sets of methylene protons at δ 3.71 (1H, m, H_β-6) and 4.32 (1H, dd, J = 9.6, 3.2 Hz, H_α-6), and 3.71 (1H, m, H_α-14) and 3.75 (1H, m, H_β-14), two methine protons at δ 3.75 (1H, m, H_α-6a) and 5.68 (1H, d, J = 6.8 Hz, H_α-11a), an olefinic proton at δ 6.64 (1H, s, H-12), five aromatic protons at δ 6.37 (1H, d, J = 2.4 Hz, H-4), 6.57 (1H, dd, J = 8.4, 2.4 Hz, H-2), 6.99 (1H, d, J = 8.0 Hz, H-8), 7.22 (1H, d, J = 8.0 Hz, H-7), and 7.38 (1H, d, J = 8.0 Hz, H-1), an alcoholic OH at δ 4.16 (1H, s, OH-14), and a phenolic OH at δ 8.32 (1H, s, OH-3).[2,6] In the ^{13}C NMR spectrum of **1**, the chemical shift values of C-1 to C-12 are similar to corresponding data of **2** (Table 1) except for C-13 to C-16.[2] The above data suggests that **1** possesses a pterocarpan (6a,11a-dihydro-6*H*-benzofuran[3,2-*c*]benzopyran) moiety. The methylene signal at δ 3.71 and 4.32 and the two methine signals at δ 3.75 and 5.68 were assigned to the pterocarpan H_β-6, H_α-6, H-6a, and H-11a protons, respectively, suggesting a *cis* arrangement of H-6a and H-11a.[2,7] The HMBC correlations between H-8/C-10, H-12/C-10, and H-12/C-13 established the connectivity between C-12 and C-13 (Table 1). The HMBC correlations between Me-16/C-13, Me-16/C-14, and Me-16/C-15 established the connectivities between C-13 and C-14, C-15 and C-14, and C-16 and C-14 (Table 1).

On the basis of the above evidence and the observation that carbon signals of C-14 and C-15 are for quarternary and secondary carbons, respectively, **1** was characterized as 5′-(1-methyl-1,2-dihydroxyethyl)-furo[2′,3′:9,10]pterocarpan-3-ol (**1**). A combination of 2D NMR techniques, such as $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, HMQC, HMBC, and NOESY experiments, enabled us to make complete ^1H and ^{13}C NMR assignments for **1** (Table 1).

The presence of a characteristic peak at *m*/*z* 336 $[M - H_2O]^+$, 279 $[M - a]^+$, and 221 $[M - b - H]^+$ in the EI mass spectra of **1** also supported its characterization (Figure 1). The NOESY experiment of **1** showed cross-peaks as shown in Figure 1. The relative configurations at C-6a, C-11a, and C-14 were further supported by observation of NOESY cross-peaks between H_α-6/H-6a, H-6a/H-11a, and H_α-15/OH-14, while H-6a, H-11a, and OH-14 adopted the α-configuration.

Compounds **2** and **3** were evaluated for their in vitro inhibitory effects on chemical mediators released from mast cells, neutrophils, macrophages, and microglial cells. Compound 48/80 (10 μg/mL), a poly-*p*-methoxyphenylacylmethylamine, stimulates the release of histamine and β-glucuronidase from rat peritoneal mast cells.[8] Compounds **2** and **3** (up to 30 μM) did not cause any significant inhibitory effect on compound 48/80-induced mast cell degranulation (data not shown). Formyl-Met-Leu-Phe (fMLP, 0.3 μM)/cytochalasin B (CB, 5 μg/mL) and phorbol-12-myristate-13-acetate (PMA) (3 nM) induce superoxide anion generation in rat neutrophils.[9] Compounds **2** and **3** (up to 30 μM) were not found to have appreciable inhibitory effect on superoxide anion generation in response to fMLP/CB or PMA (data not shown). As shown in Table 2, compound **2** strongly inhibited the release of β-glucuronidase and

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Table 1. ^{1}H and ^{13}C NMR Data of **1** in $(CD_3)_2CO$[a]

	δH (*J*, Hz)	δC	HMBC (^{1}H)[b]		δH (*J*, Hz)	δC	HMBC (^{1}H)[b]
1	7.38 (1H, d, 8.0)	133.2	5.68 (H-11a)	9		153.1	7.22 (H-7)
1a		112.9	6.37 (H-4), 6.57 (H-2)	10		114.5	6.64 (H-12), 6.99 (H-8)
2	6.57 (1H, dd, 8.4, 2.4)	110.6	6.37 (H-4)				
OH-3	8.32 (1H, s)			10a		157.8	6.64 (H-12), 7.22 (H-7)
3		159.8	6.37 (H-4), 7.38 (H-1)	11a-α	5.68 (1H, d, 6.8)	80.3	3.71 (H_β-6), 4.32 (H_α-6), 7.38 (H-1)
4	6.37 (1H, d, 2.4)	104.0	6.57 (H-2)	12	6.64 (1H, s)	99.5	
4a		157.9	3.71 (H_β-6), 4.32 (H_α-6), 6.37 (H-4), 7.38 (H-1)	13		163.0	1.54 (Me-16), 6.64 (H-12)
6α	4.32 (dd, 9.6, 3.2)	67.3	5.68 (H-11a)	OH-14	4.16 (1H, s)		
6*β*	3.71 (1H, m)			14		72.6	1.54 (Me-16)
6a-α	3.75 (1H, m)	41.0	3.71 (H_β-6)	15α	3.71 (1H, m)	69.7	1.54 (Me-16)
7	7.22 (1H,d, 8.0)	120.5	6.99 (H-8)	15*β*	3.75 (1H, m)		1.54 (Me-16)
7a		120.9		16	1.54 (3H, s)	24.0	
8	6.99 (1H, d, 8.0)	104.0	7.22 (H-7)				

[a]Arbitrary numbering. See Figure 1. [b] Only key interactions.

Table 2. Inhibitory Effects of **2** and **3** on the Release of *β*-Glucuronidase and Lysozyme from Rat Neutrophils Stimulated with fMLP/CB[a]

		β-glucuronidase		lysozyme	
compound		(% release)	(% inhibition)	(% release)	(% inhibition)
MeOH extract (CP)	(3 μg/mL)	12.7 ± 0.6[c]	(40.7 ± 1.2)	48.7 ± 4.7	(11.7 ± 4.5)
	(10 μg/mL)	6.5 ± 0.6[c]	(70.0 ± 2.2)	38.6 ± 3.3[b]	(29.6 ± 3.6)
	(30 μg/mL)	−1.02 ± 0.3[c]	(104.9 ± 2.0)	28.0 ± 2.4[c]	(48.8 ± 2.8)
IC_{50}		5.0 ± 0.6 μg/mL			
MeOH extract (CA)	(10 μg/mL)	18.5 ± 0.2[c]	(14.2 ± 3.5)	49.5 ± 4.7	(10.3 ± 6.2)
	(30 μg/mL)	13.5 ± 0.5[c]	(37.4 ± 1.8)	46.2 ± 2.6	(15.4 ± 2.5)
	(100 μg/mL)	6.8 ± 0.1[c]	(68.4 ± 0.9)	42.5 ± 3.7[c]	(22.5 ± 4.7)
IC_{50}		63.3 ± 2.2 μg/mL			
2	(1 μM)	34.7 ± 1.6	(6.9 ± 1.6)	54.2 ± 0.8	(0.9 ± 4.0)
	(3 μM)	24.8 ± 0.7[b]	(33.4 ± 2.0)	46.9 ± 1.5	(14.0 ± 5.5)
	(10 μM)	16.5 ± 2.8[c]	(55.8 ± 6.6)	23.6 ± 3.4[c]	(56.2 ± 7.8)
IC_{50}		7.8 ± 1.4 μM		9.5 ± 2.1 μM	
3	(10 μM)	37.7 ± 1.6	(−0.9 ± 0.8)	58.9 ± 0.4	(−7.8 ± 5.1)
	(30 μM)	35.3 ± 0.1	(5.1 ± 4.5)	55.1 ± 3.3	(−0.4 ± 5.2)
TFP[d]					
IC_{50}		12.2 ± 0.3 μM		13.2 ± 0.7 μM	

[a] Results are presented as means ± SEM (n = 3). 21.6 ± 0.6 and 54.7 ± 1.6, and 37.3 ± 1.8 and 54.9 ± 2.3, percent release of *β*-glucuronidase and lysozyme, respectively, are the control values for MeOH extracts (CP and CA), and **2** and **3**, respectively. [b] $P < 0.05$. [c] $P < 0.01$ as compared with the corresponding control values. [d] Trifluoperazine (TFP) as positive control.

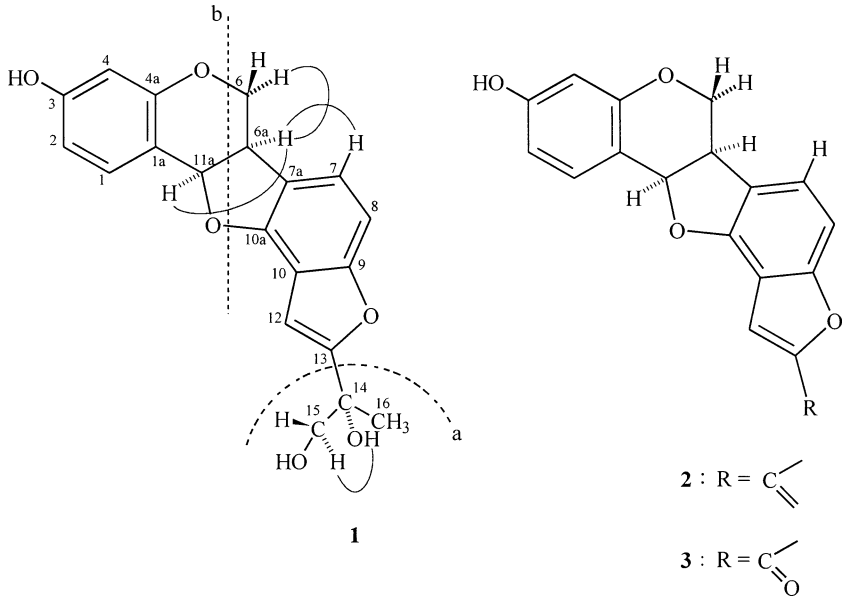

Figure 1. Structures of **1**−**3** and key interactions and EIMS fragmentation patterns of **1**.

lysozyme from rat neutrophils stimulated with fMLP (1 μM)/CB (5 μg/mL). In contrast, compound **3** did not cause significant inhibitory effects on these aspects.

The effects of pterocarpanoids on the production of NO and tumor-necrosis factor-α (TNF-α) were determined in the macrophage-like cell line RAW 264.7 and microglial cell line N9, which were activated by lipopolysaccharide (LPS) and LPS/interferon-*γ* (IFN-*γ*), respectively.[10−12] Compounds **2** and **3** did not indicate significant inhibitory effects on TNF-α production in LPS (1 μg/mL)-stimulated

Table 3. Inhibitory Effects of **2** and **3** on the NO Production in RAW 264.7 Cells Stimulated with LPS and in N9 Cells Stimulated with LPS/IFN-γ[a]

		RAW 264.7 cells		N9 cells	
compound	(μM)	nitrite (μM)	(% inhibition)	nitrite (μM)	(% inhibition)
control		53.9 ± 0.2		36.6 ± 0.16	
2	(3)	47.5 ± 3.1	(−5.1 ± 7.0)	31.0 ± 0.1[c]	(15.4 ± 0.4)
	(10)	35.8 ± 0.5[b]	(20.6 ± 1.2)	19.9 ± 0.2[b]	(45.6 ± 0.8)
	(30)	18.2 ± 0.5[b]	(59.5 ± 1.2)	*d*	
	IC_{50}	23.0 ± 1.0 μM			
3	(1)	N.D.[f]		35.3 ± 2.0	(4.2 ± 1.7)
	(3)	43.7 ± 1.8	(3.2 ± 4.0)	26.4 ± 3.0[b]	(29.0 ± 3.0)
	(10)	33.5 ± 1.5[b]	(25.6 ± 3.3)	18.3 ± 1.6[b]	(50.4 ± 1.2)
	(30)	16.1 ± 0.1[b]	(64.4 ± 0.1)	*d*	
	IC_{50}	19.0 ± 0.2 μM		9.4 ± 0.9 μM	
1400 W[e]					
	IC_{50}	2.3 ± 1.0 μM		3.6 ± 0.1 μM	

[a] Results are presented as means ± SEM ($n = 3$). [b] $P < 0.01$. [c] $P < 0.05$ as compared with the corresponding control values. [d] Cytotoxic to the tested cell lines. [e] *N*-(3-Aminomethyl)benzylacetamidine (1400 W) as positive control. [f]N.D. not determined.

RAW 264.7 cells and LPS (10 ng/mL)/IFN-γ (10 unit/mL)-stimulated N9 cells (data not shown). As shown in Table 3, compounds **2** and **3** produced a concentration-dependent inhibition of NO_2^- accumulation in RAW 264.7 cell culture medium in response to LPS (1 μg/mL) with IC_{50} values of 23.0 ± 1.0 and 19.0 ± 0.2 μM, respectively. Compound **3** also showed potent inhibitory effects on NO_2^- accumulation in N9 cell culture medium in response to LPS (10 ng/mL)/IFN-γ (10 unit/mL) with an IC_{50} value of 9.4 ± 0.9 μM. Compounds **2** and **3**, at high concentration (30 μM), showed cytotoxic effects against both cell lines, as assessed by LDH (lactate dehydrogenase) release assay and trypan blue exclusion (data not shown). The present study demonstrated that the inhibition by **2** and **3** of neutrophil degranulation and NO production in macrophages and microglial cells may have therapeutic value in the treatment or prevention of central and peripheral inflammatory diseases.

The results obtained show that constituents **2** and **3** are active constituents in the MeOH extract. This is the first report of pterocarpanoids exhibiting anti-inflammatory activity.

Experimental Section

General Experimental Procedures. Melting points are reported uncorrected. The optical rotations were obtained on a JASCO model DIP-370 digital polarimeter. UV spectra were taken on a JASCO model 7800 UV−vis spectrophotometer. IR spectra were recorded on a Perkin-Elmer system 2000 FT-IR spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer, and MS and HRMS were obtained on a JMS-HX 100 and a MAT-95 XL mass spectrometer, respectively.

Plant Material. Whole plants of *C. pallida* (Leguminosae) were collected at Ping Tung Hsieng, Taiwan, in July 2000, and a voucher specimen (2003) has been deposited at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical University.

Extraction and Isolation. The bark (8 kg) of *C. pallida* was chipped and extracted with methanol at room temperature. The MeOH extract (85 g) was subjected to column chromatography over silica gel. Elution with cyclohexane−acetone (1:1) yielded **1** (5 mg). The isolation, purification, and identification of **2** and **3** were reported previously.[2]

5′-(1-Methyl-1,2-dihydroxyethyl)-furo[2′,3′:9,10]pterocarpan-3-ol (1): white powder; $[\alpha]^{25}_D$ −143° (*c* 0.07, acetone); mp 229 °C; UV (MeOH) λ_{max} (log ϵ) 243 (4.06), 256 (4.39), 286 (4.38) nm; IR (KBr) ν_{max} 3468, 3335, 1620, 1598 cm^{-1}; ^{1}H NMR ((CD$_3$)$_2$CO, 400 MHz), see Table 1; ^{13}C NMR ((CD$_3$)$_2$CO, 100 MHz), see Table 1; EIMS *m*/*z* 354 [M]$^+$ (36), 336 (21), 323 (71), 307 (21), 279 (5), 221 (3), 147(17); HREIMS *m*/*z* [M]$^+$ 354.1110 (calcd for $C_{20}H_{18}O_6$, 354.1103).

Neutrophil Degranulation. Blood was withdrawn from rat and mixed with EDTA. After dextran sedimentation, Ficoll-Hypaque separation, and hypotonic lysis of the residual erythrocytes, neutrophils were washed and suspended in Hanks' balanced salt solution (HBSS) to 1 × 10^7 cells/mL.[13] The cell suspension was preincubated at 37 °C with DMSO or drugs for 10 min and then challenged with fMLP (1 μM)/CB (5 μg/mL). After 45 min, the lysozyme and β-glucuronidase in the supernatant were determined.[14,15] The total content was measured after treatment of the cell suspension with Triton X-100, and the percentage released was calculated. The final volume of DMSO was ≤0.5%.

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 10% fetal calf serum (FCS) and 100 units/mL of penicillin and 100 μg/mL of streptomycin at 2 × 10^5 cells/200 μL per well. Cells were allowed to adhere overnight. Cells were pretreated with DMSO or test drugs at 37 °C for 1 h before stimulation with 1 μg/mL 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 volume of DMSO was ≤0.5%.

Microglial Cell Cultures and Drug Treatment. The murine microglial cell line N9[16] (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 5% heat-inactivated FCS and antibiotics at 8 × 10^4 cells/200 μL per well. Cells were pretreated with DMSO or test drugs at 37 °C for 1 h before stimulation with LPS (10 ng/mL)/IFN-γ (10 unit/mL) for 24 h, and then the medium was collected and stored at −70 °C until used. The final volume of DMSO was ≤0.5%.

NO Determination. The production of NO was determined in cell medium by measuring the content of nitrite in the culture medium 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 sequentially into 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 $NaNO_2$.

Statistical Analysis. Data are presented as the means ± SEM. 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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