Phthalides from *Pittosporum illicioides* var. *illicioides* with Inhibitory Activity on Superoxide Generation and Elastase Release by Neutrophils

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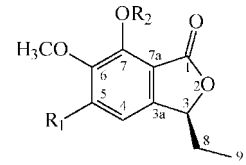
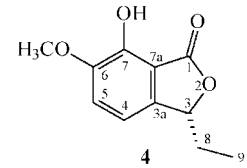
Six new phthalides, (*S*)-3-ethyl-7-hydroxy-6-methoxyphthalide (**1**), (*S*)-3-ethyl-7-hydroxy-5,6-dimethoxyphthalide (**2**), (*S*)-3-ethyl-5,6,7-trimethoxyphthalide (**3**), (*R*)-3-ethyl-7-hydroxy-6-methoxyphthalide (**4**), (*Z*)-3-ethylidene-7-hydroxy-6-methoxyphthalide (**5**), and (*Z*)-3-ethylidene-6,7-dimethoxyphthalide (**6**), have been isolated from the root of *Pittosporum illicioides* var. *illicioides*, together with seven known compounds. The structures of these new compounds were determined through spectroscopic and MS analyses. Compounds **1**−**4** exhibited inhibition ($IC_{50} \leq 29.8$ μM) of superoxide anion generation by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB). Compounds **5** and **6** inhibited fMLP/CB-induced elastase release with IC_{50} values of 38.6 ± 4.3 and 33.9 ± 3.9 μM, respectively.

Human neutrophils play significant roles in host defense against microorganisms and in pathogenesis of diseases such as rheumatoid arthritis, ischemia-reperfusion injury, chronic obstructive pulmonary disease (COPD), and asthma.[1−5] In response to different stimuli, activated neutrophils secrete cytotoxins, such as the superoxide anion radical ($O_2^{\bullet-}$), a precursor to other reactive oxygen species (ROS), granule proteases, and bioactive lipids.[2,6,7] Suppression of the extensive or inappropriate activation of neutrophils by drugs has been proposed as a way to ameliorate inflammatory diseases. Despite this, there are only a few currently available agents that directly modulate neutrophil pro-inflammatory responses in clinical practice. *Pittosporum illicioides* var. *illicioides* (Pittosporaceae) is an evergreen shrub found in medium to high altitude in forests of China and Taiwan.[8] Carotenoids,[9,10] sesquiterpene glycosides,[11] farnesyl glycosides,[12] triterpenoid saponins,[13−15] and their derivatives are widely distributed in plants of the genus *Pittosporum*. Many of these compounds exhibit biological activities, including cytotoxic[12,13] and molluscicidal[14] activities. However, the chemical constituents and biological activities of this plant have never been studied. In our studies on the anti-inflammatory constituents of Formosan plants, many species have been screened for *in vitro* inhibitory activity on neutrophil pro-inflammatory responses, and *P. illicioides* var. *illicioides* has been found to be an active species. Six new phthalides, (*S*)-3-ethyl-7-hydroxy-6-methoxyphthalide (**1**), (*S*)-3-ethyl-7-hydroxy-5,6-dimethoxyphthalide (**2**), (*S*)-3-ethyl-5,6,7-trimethoxyphthalide (**3**), (*R*)-3-ethyl-7-hydroxy-6-methoxyphthalide (**4**), (*Z*)-3-ethylidene-7-hydroxy-6-methoxyphthalide (**5**), and (*Z*)-3-ethylidene-6,7-dimethoxyphthalide (**6**), and seven known compounds have been isolated and identified from roots of *P. illicioides* var. *illicioides.* This paper describes the structural elucidation of **1**−**6** and their inhibitory activity on superoxide generation and elastase release by neutrophils.



1 $R_1 = R_2 = H$
2 $R_1 = OCH_3$, $R_2 = H$
3 $R_1 = OCH_3$, $R_2 = CH_3$



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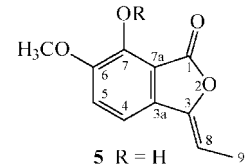

5 R = H
6 R = CH_3

Results and Discussion

Chromatographic purification of the EtOAc-soluble fraction of a MeOH extract of roots of *P. illicioides* var. *illicioides* on a silica gel column (CC) and preparative thin-layer chromatography (TLC) afforded six new (**1**−**6**) and seven known compounds (**7**−**13**).

Compound **1** was isolated as colorless needles. Its molecular formula, $C_{11}H_{12}O_4$, was determined on the basis of the positive HRESIMS at *m/z* 231.0632 $[M + Na]^+$ (calcd 231.0633), and this was supported by the ^1H, ^{13}C, and DEPT NMR data. The IR spectrum showed the presence of OH (3372 cm^{-1}) and carbonyl (1731 cm^{-1}) groups. The ^1H NMR spectrum of **1** showed the presence of a methoxy group, a hydroxy group, two ortho-coupled aromatic protons, an oxymethine proton, and an ethyl group. On the basis of the NOESY correlations (Figure 1) between H-5 and H-4 and OMe-6, the OH group was assigned to C-7. The 3-ethyl group was placed by HMBC correlations (Figure 1) between H-9 and H-4 and C-3. Compound **1** was levorotatory ($[\alpha]^{25}_D$ −71.5) as in the case of (*S*)-3-ethylphthalide ($[\alpha]^{23}_D$ −73.5),[16] and the absolute configuration at C-3 in **1** has to be *S*.[16] The structure of **1** was thus elucidated as (*S*)-3-ethyl-7-hydroxy-6-methoxyphthalide. This struc-

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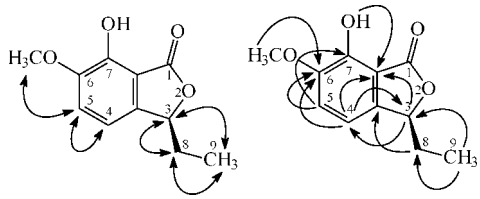

Figure 1. NOESY (a) and HMBC (b) correlations of **1**.

ture was supported by $^1H-^1H$ COSY and NOESY experiments, and ^{13}C NMR assignments were confirmed by DEPT, HSQC, and HMBC techniques (Figure 1).

Compound **2** was isolated as optically active colorless needles ($[\alpha]^{25}_D$ −73.6), and HREIMS gave an $[M]^+$ ion at *m/z* 238.0842, consistent with the molecular formula $C_{12}H_{14}O_5$. The IR spectrum indicated that OH (3380 cm^{-1}) and carbonyl (1739 cm^{-1}) groups were present. Comparison of the ^1H NMR data (Table 1) of **2** with those of **1** suggested that their structures were closely related, except that OMe-5 [δ 3.82 (3H, s)] of **2** replaced H-5 [δ 7.04 (1H, d, J = 8.8 Hz)] of **1**. This was supported by both HMBC correlations between OMe-5 (δ 3.82) and C-5 (δ 150.2) and NOESY correlations between OMe-5 (δ 3.82) and H-4 (6.78). Compound **2** was levorotatory, $[\alpha]^{25}_D$ −73.6, similar to that of (*S*)-3-ethylphthalide ($[\alpha]^{25}_D$ −73.5),[16] and the absolute configuration of C-3 in **2** must be *S*.[16] Thus, **2** was (*S*)-3-ethyl-7-hydroxy-5,6-dimethoxyphthalide.

Compound **3** was an amorphous powder, and the sodium adduct ion $[M + Na]^+$ (*m/z* 275.0893) in the HRESIMS was consistent with the formula $C_{13}H_{16}O_5Na$. The presence of a carbonyl group was revealed by a band at 1730 cm^{-1} in the IR spectrum and was confirmed by the resonance at δ 167.6 in the ^{13}C NMR spectrum. The ^1H NMR data (Table 1) of **3** were similar to those of **2**, except that the 7-methoxy group [δ 3.98 (3H, s)] of **3** replaced the 7-hydroxy group [δ 6.08 (1H, br s, D_2O exchangeable)] of **2**. This was supported by HMBC correlations observed between 7-OMe (δ 3.98) and C-7 (δ 155.8). The absolute configuration of **3** was assigned as *S* from the levorotatory optical activity ($[\alpha]^{25}_D$ −72.4) by analogy with previous observations.[16,17] Thus, **3** was (*S*)-3-ethyl-5,6,7-trimethoxyphthalide. This is the first isolation of the *S* enantiomer of **3** from a natural source, although (±)-3-ethyl-5,6,7-trimethoxyphthalide has been synthesized by Mali et al.[18]

Compound **4**, colorless needles, had the molecular formula $C_{11}H_{12}O_4$ as deduced from a sodium adduct ion peak at *m/z* = 231.0633 (HRESIMS). IR absorptions for OH (3375 cm^{-1}) and carbonyl functions (1732 cm^{-1}) were observed. Comparison of the ^1H and ^{13}C NMR data of **4** with those of **1** suggested that their structures are closely related. The absolute configurations of **4** ($[\alpha]^{25}_D$ +72.9) at C-3 were determined as *R* by comparison with the analogous phthalide, (*R*)-3-ethylphthalide ($[\alpha]_D$ +77.3).[19] Thus, the structure of **4** was elucidated as (*R*)-3-ethyl-7-hydroxy-6-methoxyphthalide, which was confirmed by $^1H-^1H$ COSY, NOESY, DEPT, HSQC, and HMBC experiments (Figure 2).

Compound **5** had a molecular formula of $C_{11}H_{10}O_4$ as determined by positive-ion HRESIMS. Hydroxy and carbonyl groups were revealed by IR bands at 3310 and 1744 cm^{-1}, respectively. The ^1H NMR spectrum of **5** showed the presence of a methoxy group, a hydroxy group, two ortho-coupled aromatic protons, and an ethylidene group, similar to signals described previously for **4**, except for the resonance of the 3-ethylidene group [δ 1.96 (3H, d, J = 7.2 Hz, H-9) and 5.50 (1H, q, J = 7.2 Hz, H-8)] in the spectrum of **5** that replaced the 3-ethyl group of **4**. This was supported by HMBC correlations (Figure 3) between H-9 (δ 1.96) and C-3 (δ 145.9) and C-8 (δ 101.8). The configurations of the double bond at C-3 and C-8 were both determined as *Z* by NOESY correlations between H-4 (δ 7.25) and H-8 (δ 5.50). According to the above data, **5** was determined to be (*Z*)-3-ethylidene-7-hydroxy-6-methoxyphthalide.

Compound **6** had the molecular formula $C_{12}H_{12}O_4$ by HRESIMS. The ^1H and ^{13}C NMR data of **6** were similar to those of **5**, except that a 7-methoxy group [δ 4.13 (3H, s)] of **6** replaced the 7-hydroxy group [δ 7.42 (1H, s, D_2O exchangeable)] of **5**. This was supported by HMBC correlations between OMe-7 (δ 4.13) and C-7 (δ 148.1). NOESY correlations (Table 2) of **6** were observed between H-4 (δ 7.26) and H-8 (δ 5.50). Moreover, NOESY correlations could not be detected between H-4 and H-9 (δ 1.97). Thus, the *Z*-configuration of **6** was established. On the basis of the above data, the structure of **6** was elucidated as (*Z*)-3-ethylidene-6,7-dimethoxyphthalide.

The known isolates were readily identified by a comparison of physical and spectroscopic data (UV, IR, ^1H NMR, $[\alpha]_D$, and MS) with corresponding authentic samples or literature values, and this included two coumarins, aesculetin dimethyl ether[20] and 6,7,8-trimethoxycoumarin,[21] a lignan, (−)-syringaresinol,[22] a benzenoid, syringic acid,[23] two steroids, stigmasterol[24] and chondrillasterol,[25] and a fatty acid, stearic acid.[26]

The effects on neutrophil pro-inflammatory responses of compounds isolated from the root of *P. illicioides* var. *illicioides* were evaluated by suppressing formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB)-induced superoxide radical anion ($O_2^{\bullet-}$) generation and elastase release by human neutrophils. The inhibitory activity data on neutrophil pro-inflammatory responses are shown in Table 3. Diphenyleneiodonium and phenylmethylsulfonyl fluoride were used as positive controls for $O_2^{\bullet-}$ generation and elastase release, respectively. From the results of our biological tests, the following conclusions can be drawn: (a) Compounds **1**−**4** exhibited inhibitory activities ($IC_{50} \leq 29.8$ μM) on human neutrophil $O_2^{\bullet-}$ generation. (b) Compounds **5** and **6** inhibited fMLP/CB-induced elastase release with IC_{50} values ≤ 38.64 μM. (c) Among the phthalide analogues (**1**−**6**), compounds **1**−**4**, with a 3-ethyl group, exhibited more effective inhibition than analogues **5** and **6**, with a 3-ethylidene substituent, against fMLP-induced $O_2^{\bullet-}$ generation. (d) Compounds **5** and **6**, with a 3-ethylidene group, showed stronger inhibition than the analogous phthalides **1**−**4**, with a 3-ethyl group, against fMLP-induced elastase release. (e) Compound **2** was the most effective among these compounds, with an IC_{50} value of 13.7 ± 2.6 μM against fMLP-induced superoxide anion generation. (f) Compound **6** exhibited the most effective inhibition among the isolates, with an IC_{50} value of 33.9 ± 3.9 μM against fMLP-induced elastase release.

Experimental Section

General Experimental Procedures. All melting points were determined on a Yanaco micromelting point apparatus and were uncorrected. Optical rotations were measured using a Jasco DIP-370 polarimeter in $CHCl_3$. UV spectra were obtained on a Jasco UV-240 spectrophotometer. IR spectra (KBr or neat) were recorded on a Perkin-Elmer 2000 FT-IR spectrometer. NMR spectra, including COSY, NOESY, HMBC, and HSQC experiments, were recorded on a Varian Unity 400 or a Varian Inova 500 spectrometer operating at 400 and 500 MHz (^1H) and 100 and 125 MHz (^{13}C), respectively, with chemical shifts given in ppm (δ) using TMS as an internal standard. EI, ESI, and HRESI mass spectra were recorded on a Bruker APEX II mass spectrometer. HREI mass spectra were recorded on a JEOL JMX-HX 110 mass spectrometer. Silica gel (70−230, 230−400 mesh) (Merck) was used for CC. Silica gel 60 F-254 (Merck) was used for TLC and PTLC.

Plant Material. Roots of *P. illicioides* var. *illicioides* were collected from Wutai, Pingtung County, Taiwan, in October 2007 and identified by one of the authors (I.S.C.). A voucher specimen (Chen 5652) was deposited in the Faculty of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Separation. The dried root of *P. illicioides* var. *illicioides* (12.3 kg) was pulverized and extracted three times with MeOH (32 L each) for 3 days. The MeOH extracts were concentrated under reduced pressure at 35 °C, and the residue (315 g) was partitioned between EtOAc and H_2O (1:1). The EtOAc layer was concentrated to give a residue (fraction A, 70.5 g). The water layer was further extracted with *n*-BuOH, and the *n*-BuOH-soluble part (fraction B, 86 g) and

Table 1. ^{1}H NMR Data of **1**−**3**[a]

	1	2			3		
position	δ_H *J* (Hz)	δ_H *J* (Hz)	NOE	HMBC	δ_H *J* (Hz)	NOE	HMBC
3	5.51 ddd (7.2, 3.4, 0.6)	5.37 dd (7.2, 3.2)	8, 9	1, 4, 7a, 9	5.42 dd (6.8, 3.2)	8, 9	1, 4, 7a, 8, 9
4	6.88 dd (8.8, 0.6)	6.78 s	OMe-5	3, 6, 7a	6.47 s	OMe-5	3, 3a, 6, 7a
5	7.04 d (8.8)						
8	1.83 dqd (14.8, 7.4, 7.2)	1.76 dqd (14.6, 7.4, 7.2)	3, 9	3, 3a, 9	1.81 dqd (14.4, 7.2, 6.8)	3, 9	3, 3a, 9
	2.30 dqd (14.8, 7.4, 3.4)	2.25 dqd (14.6, 7.4, 3.2)	3, 9	3, 3a, 9	2.25 dqd (14.4, 7.2, 3.2)	3, 9	3, 3a, 9
9	0.95 t (7.4)	0.91 t (7.4)	3, 8	3, 8	0.94 t (7.2)	3, 8	3, 8
OMe-5		3.82 s	4	5	3.98 s	4	5
OMe-6	3.84 s	4.10 s		6	3.83 s		6
OH-7	7.37 s	6.08 br s		7, 7a			
OMe-7					3.98 s		7

[a] Recorded in $CDCl_3$ at 400 MHz. Values in ppm (δ). *J* (in Hz) in parentheses.

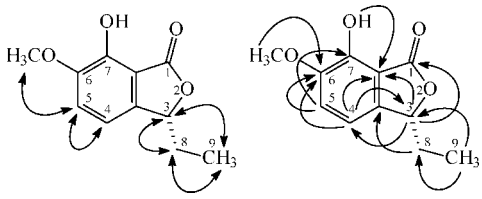

Figure 2. NOESY (a) and HMBC (b) correlations of **4**.

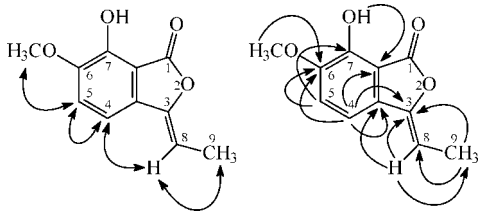

Figure 3. NOESY (a) and HMBC (b) correlations of **5**.

Table 2. ^{1}H NMR Data of **5** and **6**[a]

	5	6		
position	δ_H *J* (Hz)	δ_H *J* (Hz)	NOE	HMBC
4	7.25 d (8.0)	7.26 d (8.0)	5, 8	3, 6, 3a, 7a
5	7.20 d (8.0)	7.22 d (8.0)	4, OMe-6	3a, 4, 7
8	5.50 q (7.2)	5.50 q (7.2)	4, 9	3, 3a, 9
9	1.96 d (7.2)	1.97 d (7.2)	8	3, 8
OMe-6	3.92 s	3.92 s	5	6
OH-7	7.42 s			
OMe-7		4.13 s		7

[a] Recorded in $CDCl_3$ at 400 MHz. Values in ppm (δ). *J* (in Hz) in parentheses.

the water-solubles (fraction C, 136 g) were separated. Fraction A (70.5 g) was chromatographed on silica gel (70−230 mesh, 3.1 kg), eluting with CH_2Cl_2, gradually increasing the polarity with MeOH to give 11 fractions: A1 (10 L, CH_2Cl_2), A2 (20 L, CH_2Cl_2), A3 (11 L, CH_2Cl_2/MeOH, 20:1), A4 (8 L, CH_2Cl_2/MeOH, 20:1), A5 (10 L, CH_2Cl_2/MeOH, 10:1), A6 (8 L, CH_2Cl_2/MeOH, 5:1), A7 (9 L, CH_2Cl_2/MeOH, 4:1), A8 (8 L, CH_2Cl_2/MeOH, 3:1), A9 (6 L, CH_2Cl_2/MeOH, 2:1), A10 (8 L, CH_2Cl_2/MeOH, 1:1), A11 (12 L, MeOH). Fraction A1 (11.6 g) was chromatographed further on silica gel (230−400 mesh, 410 g) eluting with *n*-hexane/acetone (20:1) to give 12 fractions (each 1.5 L, A1-1−A1-12). Fraction A1-8 (1.3 g) was washed with MeOH and filtered to obtain chondrillasterol (685 mg) after recrystallization (MeOH). The MeOH washing (510 mg) was subjected to CC (20 g silica gel, 230−400 mesh; *n*-hexane/EtOAc, 3:1, 700 mL fractions) to give 10 subfractions: A1-8-1−A1-8-10. Fraction A1-8-5 (46 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 3:1) to obtain **1** (3.2 mg). Fraction A1-8-6 (51 mg) was purified further by preparative TLC (CH_2Cl_2/acetone, 40:1) to provide **2** (4.1 mg). Fraction A2 (10.3 g) was chromatographed further on silica gel (230−400 mesh, 425 g) eluting with *n*-hexane/EtOAc (15:1) to give 10 fractions (each 1.5 L, A2-1−A2-10). Fraction A2-7 (2.8 g) was subjected to CC (135 g silica gel, 230−400 mesh; *n*-hexane/EtOAc, 7:1, 800 mL fractions) to give 13 subfractions: A2-7-1−A2-7-13. Fraction A2-7-2 (205 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/MeOH, 30:1) to provide 6,7,8-trimethoxycoumarin (11.2 mg). Fraction A2-7-3 (188 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/MeOH, 40:1) to yield **5** (11.2 mg). Fraction A2-7-6 (174 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/MeOH, 40:1) to provide **4** (2.8 mg). Fraction A2-8 (198 mg) was purified further by PTLC (silica gel, CH_2Cl_2/MeOH, 30:1) to obtain **3** (3.7 mg) and **6** (2.2 mg). Fraction A3-4-12 (7.5 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 1:1) to obtain aesculetin dimethyl ether (3.6 mg).

Table 3. Inhibitory Effects of **1**−**6** and Some Additional Compounds on Superoxide Radical Anion Generation and Elastase Release by Human Neutrophils in Response to fMet-Leu-Phe/Cytochalasin B[a]

	IC_{50} (μM)[b]	
compound	superoxide anion	elastase
1	29.8 ± 6.1***	>50
2	13.7 ± 2.6***	>50
3	17.6 ± 0.2***	>50
4	21.2 ± 1.8***	>50
5	45.3 ± 7.3**	38.6 ± 4.3***
6	>50	33.9 ± 3.9***
aesculetin dimethyl ether	41.4 ± 5.9**	>50
6,7,8-trimethoxycoumarin	48.6 ± 5.7***	>50
(−)-syringaresinol	40.2 ± 6.2**	>50
diphenyleneiodonium	1.7 ± 0.7***	
phenylmethylsulfonyl fluoride		202.3 ± 32.5***

[a] Diphenyleneiodonium and phenylmethylsulfonyl fluoride were used as positive controls. Results are presented as average ± SEM (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control. [b] Concentration necessary for 50% inhibition (IC_{50}).

Biological Assay. The effects of the isolated compounds on neutrophil pro-inflammatory responses were evaluated by inhibiting the generation of $O_2^{\bullet-}$ and the release of elastase in fMLP-activated human neutrophils in a concentration-dependent manner.

Preparation of Human Neutrophils. Human neutrophils from venous blood of healthy, adult volunteers (20−28 years old) were isolated using a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes.[27] Purified neutrophils containing >98% viable cells, as determined by the trypan blue exclusion method,[28] were resuspended in a Ca^{2+}-free HBSS buffer at pH 7.4 and were maintained at 4 °C prior to use.

Measurement of $O_2^{\bullet-}$ Generation. Measurement of $O_2^{\bullet-}$ generation was based on the SOD-inhibitable reduction of ferricytochrome *c*.[29,30] In brief, after supplementation with 0.5 mg/mL ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils were equilibrated at 37 °C for 2 min and incubated with compounds for 5 min. Cells were activated with 100 nM fMLP for 10 min. When fMLP was used as a stimulant, CB (1 μg/mL) was incubated for 3 min before activation by peptide (fMLP/CB). Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/mL) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ϵ = 21.1/mM/10 mm).

Measurement of Elastase Release. Degranulation of azurophilic granules was determined by measuring elastase release as described previously.[30] Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (6×10^5/mL) were equilibrated at 37 °C for 2 min and incubated with compounds for 5 min. Cells were stimulated with fMLP (100 nM)/cytochalasin B (0.5 μg/mL), and changes in absorbance at 405 nm were monitored continuously in order to assay elastase release. The results were expressed as the percent of elastase release in the fMLP/cytochalasin B-activated, drug-free control system.

Statistical Analysis. Results are expressed as the mean ± SEM, and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant. The software SigmaPlot was used for the statistical analysis.

(*S*)-3-Ethyl-7-hydroxy-6-methoxyphthalide (1): colorless needles ($CHCl_3$/MeOH); mp 107−109 °C; $[\alpha]^{25}_D$ −71.5 (*c* 0.10, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 216 (4.36), 234 (3.87), 323 (3.71) nm; IR (neat) ν_{max} 3372 (OH), 1731 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 9.1 (C-9), 25.9 (C-8), 56.2 (OMe-6), 83.3 (C-3), 112.7 (C-7a), 116.4 (C-4), 118.9 (C-5), 136.3 (C-3a), 147.5 (C-6), 150.0 (C-7), 172.3 (C-1); ESIMS *m/z* 231 $[M + Na]^+$; HRESIMS *m/z* 231.0632 $[M + Na]^+$ (calcd for $C_{11}H_{12}O_4Na$, 231.0633).

(*S*)-3-Ethyl-7-hydroxy-5,6-dimethoxyphthalide (2): colorless needles (CH_2Cl_2/MeOH); mp 111−113 °C; $[\alpha]^{25}_D$ −73.6 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 217 (4.51), 248 (sh, 3.67), 323 (3.70) nm; IR (neat) ν_{max} 3380 (OH), 1739 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 9.0 (C-9), 26.2 (C-8), 56.1 (OMe-5), 63.3 (OMe-6), 81.3 (C-3), 104.6 (C-4), 118.8 (C-7a), 129.6 (C-3a), 137.8 (C-6), 150.2 (C-5), 150.2 (C-7), 168.5 (C-1); EIMS *m/z* (rel int) 238 ($[M]^+$, 53), 209 (100), 181 (27), 165 (18), 136 (11), 95 (10), 69 (32); HREIMS *m/z* 238.0842 $[M]^+$ (calcd for $C_{12}H_{14}O_5$, 238.0841).

(*S*)-3-Ethyl-5,6,7-trimethoxyphthalide (3): amorphous powder; $[\alpha]^{25}_D$ −72.4 (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 222 (4.33), 257 (3.90), 300 (3.71) nm; IR (neat) ν_{max} 1730 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.9 (C-9), 26.5 (C-8), 56.6 (OMe-5), 56.6 (OMe-7), 61.0 (OMe-6), 80.1 (C-3), 97.0 (C-4), 106.9 (C-7a), 129.7 (C-3a), 136.6 (C-6), 155.8 (C-7), 159.5 (C-5), 167.6 (C-1); ESIMS *m/z* 275 $[M + Na]^+$; HRESIMS *m/z* 275.0893 $[M + Na]^+$ (calcd for $C_{13}H_{16}O_5Na$, 275.0895).

(*R*)-3-Ethyl-7-hydroxy-6-methoxyphthalide (4): colorless needles (MeOH); mp 106−108 °C; $[\alpha]^{25}_D$ +72.9 (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 217 (4.35), 234 (3.86), 322 (3.70) nm; IR (neat) ν_{max} 3375 (OH), 1732 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.01 (3H, t, J = 7.3 Hz, H-9), 1.82 (1H, dqd, J = 14.4, 7.3, 7.2 Hz, H-8), 2.07 (1H, dqd, J = 14.4, 7.3, 4.4 Hz, H-8), 3.94 (3H, s, OMe-6), 5.42 (1H, ddd, J = 7.2, 4.4, 0.6 Hz, H-3), 6.84 (1H, dd, J = 8.0, 0.6 Hz, H-4), 7.15 (1H, d, J = 8.0 Hz, H-5), 7.60 (1H, s, D_2O exchangeable, OH-7); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 9.0 (C-9), 28.1 (C-8), 57.1 (OMe-6), 83.5 (C-3), 112.4 (C-7a), 112.8 (C-4), 119.2 (C-5), 141.2 (C-3a), 145.9 (C-7), 147.3 (C-6), 173.8 (C-1); ESIMS *m/z* 231 $[M + Na]^+$; HRESIMS *m/z* 231.0633 $[M + Na]^+$ (calcd for $C_{11}H_{12}O_4Na$, 231.0633).

(*Z*)-3-Ethylidene-7-hydroxy-6-methoxyphthalide (5): amorphous powder; UV (MeOH) λ_{max} (log ε) 213 (4.37), 233 (3.88), 310 (sh, 3.49) nm; IR (neat) ν_{max} 3310 (OH), 1744 (C═O) cm^{-1}; ^{1}H NMR data, see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.3 (C-9), 57.1 (OMe-6), 101.8 (C-8), 114.8 (C-4), 120.4 (C-5), 133.0 (C-7a), 133.8 (C-3a), 145.9 (C-3), 147.6 (C-7), 148.4 (C-6), 164.9 (C-1); ESIMS *m/z* 229 $[M + Na]^+$; HRESIMS *m/z* 229.0479 $[M + Na]^+$ (calcd for $C_{11}H_{10}O_4Na$, 229.0477).

(*Z*)-3-Ethylidene-6,7-dimethoxyphthalide (6): amorphous powder; UV (MeOH) λ_{max} (log ε) 211 (4.39), 252 (3.90), 287 (sh, 3.48) nm; IR (neat) ν_{max} 1760 (C═O) cm^{-1}; ^{1}H NMR data, see Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.4 (C-9), 57.2 (OMe-6), 101.9 (C-8), 114.5 (C-4), 116.9 (C-7a), 120.1 (C-5), 133.5 (C-3a), 145.9 (C-3), 148.1 (C-7), 152.9 (C-6), 165.0 (C-1); ESIMS *m/z* 243 $[M + Na]^+$; HRESIMS *m/z* 243.0636 $[M + Na]^+$ (calcd for $C_{12}H_{12}O_4Na$, 243.0633).

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