JOURNAL OF NATURAL PRODUCTS

ARTICLE

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Thymol, Benzofuranoid, and Phenylpropanoid Derivatives: Anti-inflammatory Constituents from *Eupatorium cannabinum*

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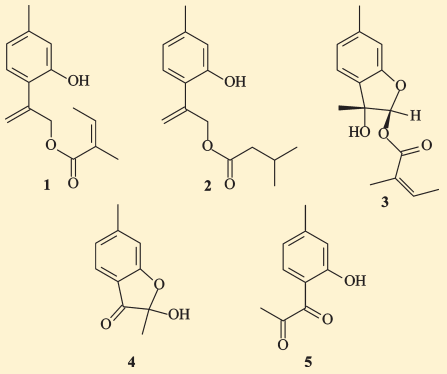
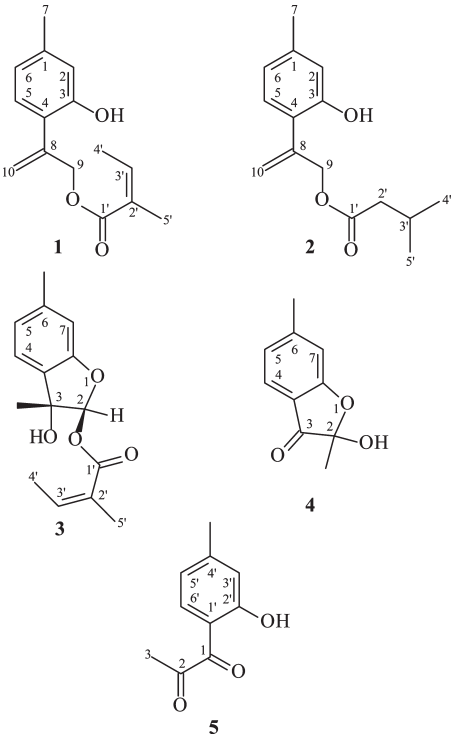
Supporting Information

ABSTRACT: Five new compounds, 9-*O*-angeloyl-8,10-dehydrothymol (**1**), 9-(3-methylbutanoyl)-8,10-dehydrothymol (**2**), eupatobenzofuran (**3**), 2-hydroxy-2,6-dimethylbenzofuran-3(2*H*)-one (**4**), and 1-(2-hydroxy-4-methylphenyl)propan-1,2-dione (**5**), have been isolated from the aerial part of *Eupatorium cannabinum* subsp. *asiaticum*, together with 16 known compounds (**6**−**21**). Compounds **6**−**8**, **11**, **13**, and **15** exhibited inhibition (IC_{50} values ≤ 18.4 μM) of superoxide anion generation by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (fMLP/CB). Compounds **2**, **3**, **10**, **13**, and **15** inhibited fMLP/CB-induced elastase release with IC_{50} values ≤ 18.3 μM.



Eupatorium cannabinum L. subsp. *asiaticum* Kitam. (Compositae)[1] is a perennial herb distributed in the Himalaya mountain range, China, and Taiwan. *E. cannabinum*, locally called Taiwan ze-lan or liu-yue-xue, has been used as a folk medicine to treat hepatitis, headache, diarrhea, hypertension, and diabetes mellitus in Taiwan.[2,3] Sesquiterpene lactones,[4−11] diterpenoids,[12,13] flavonoids,[14−16] pyrrolizidine alkaloids,[17,18] thymols,[19,20] benzofurans,[21] and their derivatives are widely distributed in plants of the genus *Eupatorium*. Many of these compounds were found to exhibit cytotoxic,[4−11,14] antimicrobial,[12,13] and anti-inflammatory[16] activities. 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 *E. cannabinum* has been found to be an active species. Five new compounds, 9-*O*-angeloyl-8,10-dehydrothymol (**1**), 9-(3-methylbutanoyl)-8,10-dehydrothymol (**2**), eupatobenzofuran (**3**), 2-hydroxy-2,6-dimethylbenzofuran-3(2*H*)-one (**4**), and 1-(2-hydroxy-4-methylphenyl)propan-1,2-dione (**5**) and 16 known compounds have been isolated and identified from the aerial part of *E. cannabinum* subsp. *asiaticum*. This paper describes the structural elucidation of **1**−**5** and the anti-inflammatory activities of the isolates.



RESULTS AND DISCUSSION

Chromatographic purification of the *n*-hexane-soluble fraction of an MeOH extract of the aerial part of *E. cannabinum* on a silica gel column and preparative TLC afforded five new (**1**−**5**) and 16 known compounds (**6**−**21**).

Received: December 16, 2010
Published: March 10, 2011

Table 1. ^{1}H NMR, NOESY, and HMBC Data of 1 and 2[a]

	1			2		
position	δ_H (J in Hz)	NOESY	HMBC	δ_H (J in Hz)	NOESY	HMBC
2	6.76 br s	7	4, 6, 7	6.76 br s	7	3, 4, 6, 7
5	6.99 d (8.0)	6, 10a	1, 3, 8	6.97 d (8.0)	6, 9, 10a	1, 3, 8
6	6.70 br d (8.0)	5, 7	2, 4, 7	6.69 br d (8.0)	5, 7	2, 4, 5, 7
7	2.31 s	2, 6	1, 2, 6	2.31 s	2, 6	1, 2, 6
9	4.81 dd (1.2, 1.2)	10b	4, 10, 1′	4.75 dd (1.4, 1.4)	5, 10b, OH-3	4, 8, 10, 1′
10a	5.27 d (1.2)	5, 10b	4, 9	5.28 dt (1.4, 1.4)	5, 10b	4, 9
10b	5.47 d (1.2)	9, 10a	4, 8	5.48 dt (1.4, 1.4)	9, 10a	4, 8, 9
2′				2.27 d (7.0)	3′, 4′, 5′	1′, 3′, 4′, 5′
3′	6.17 qq (7.2, 1.6)	4′, 5′	1′, 5′	2.13 m	2′, 4′, 5′	1′, 4′, 5′
4′	2.00 dq (7.2, 1.6)	3′	2′, 3′	0.96 d (6.5)	2′, 3′	2′, 3′, 5′
5′	1.93 dq (1.6, 1.6)	3′	1′, 2′, 3′	0.96 d (6.5)	2′, 3′	2′, 3′, 4′
OH-3	6.93 br s		3, 4	6.70 s	9	3, 4

[a] Recorded in $CDCl_3$ at 400 (**1**) and 500 (**2**) MHz. Values in ppm (δ). J (in Hz) in parentheses.

9-*O*-Angeloyl-8,10-dehydrothymol (**1**) was isolated as a colorless oil. Its molecular formula, $C_{15}H_{18}O_3$, was determined on the basis of the positive HRESIMS at m/z 269.1156 $[M + Na]^+$ (calcd 269.1154), and this was supported by the ^{1}H, ^{13}C, and DEPT NMR data. The IR spectrum showed the presence of OH (3391 cm^{-1}) and carbonyl (1697 cm^{-1}) groups. Comparison of the ^{1}H NMR data of **1** with those of 9-isobutyryloxy-8,10-dehydrothymol (**10**)[22] suggested that their structures were closely related, except that the 9-*O*-angeloyl group [δ 1.93 (3H, dq, J = 1.6, 1.6 Hz, H-5′), 2.00 (3H, dq, J = 7.2, 1.6 Hz, H-4′), and 6.17 (1H, qq, J = 7.2, 1.6 Hz, H-3′)] of **1** replaced the 9-isobutyryloxy group [δ 1.19 (6H, d, J = 6.8 Hz, H-3′ and H-4′) and 2.63 (1H, m, H-2′)] of **10**. This was supported by the HMBC correlations observed between H-9 (δ 4.81)/C-1′ (δ 168.4), H-3′ (δ 6.17)/C-1′ (δ 168.4), and H-5′ (δ 1.93)/C-1′ (δ 168.4) and by the NOESY correlations observed between H-3′ (δ 6.17) and both H-4′ (δ 2.00) and H-5′ (δ 1.93). In addition, the *Z*-configuration of the angeloyl moiety of **1** was established by the NOESY correlations between H-3′ (δ 6.17) and Me-5′ (δ 1.93). On the basis of the above data, the structure of **1** was elucidated as (*Z*)-2-(2-hydroxy-4-methylphenyl)allyl 2-methylbut-2-enoate, named 9-*O*-angeloyl-8,10-dehydrothymol, which was further confirmed by ^{1}H−^{1}H COSY and NOESY (Table 1) experiments. The assignment of ^{13}C NMR resonances was confirmed by DEPT, HSQC, and HMBC (Table 1) techniques.

9-(3-Methylbutanoyl)-8,10-dehydrothymol (**2**) was isolated as a colorless oil with the molecular formula $C_{15}H_{20}O_3$, as determined by positive-ion HRESIMS, showing an $[M + Na]^+$ ion at m/z 271.1307 (calcd for $C_{15}H_{20}O_3Na$, 271.1310). The presence of a carbonyl group was revealed by a band at 1715 cm^{-1} in the IR spectrum and was confirmed by the resonance at δ 173.8 in the ^{13}C NMR spectrum. The ^{1}H NMR data of **2** were similar to 9-isobutyryloxy-8,10-dehydrothymol (**10**),[22] except that the 3-methylbutanoyl group [δ 0.96 (6H, d, J = 6.5 Hz, H-4′ and H-5′), 2.13 (1H, m, H-3′), 2.27 (2H, d, J = 7.0 Hz, H-2′)] at C-9 of **2** replaced the C-9 isobutyryloxy group of **10**. This was supported by the NOESY correlations between H-2′ (δ 2.27)/H-3′ (δ 2.13), H-2′ (δ 2.27)/H-4′ (δ 0.96), and H-3′ (δ 2.13)/H-5′ (δ 0.96) and by the HMBC correlations between H-9 (δ 4.75)/C-1′ (δ 173.8), H-2′ (δ 2.27)/C-1′ (δ 173.8), H-3′ (δ 2.13)/C-1′ (δ 173.8), H-4′ (δ 0.96)/C-2′ (δ 43.3), and H-5′ (δ 0.96)/C-3′ (δ 25.7). The full assignment of ^{1}H and ^{13}C NMR resonances was confirmed by ^{1}H−^{1}H COSY, NOESY (Table 1), DEPT, HSQC, and HMBC (Table 1) techniques. According to the evidence above, the structure of **2** was elucidated as 2-(2-hydroxy-4-methylphenyl)allyl 3-methylbutanoate, named 9-(3-methylbutanoyl)-8,10-dehydrothymol.

Eupatobenzofuran (**3**) was obtained as an amorphous powder. The molecular formula $C_{15}H_{18}O_4$ was deduced from a molecular ion at m/z 262.1211 $[M]^+$ (calcd 262.1205) in the HREI mass spectrum. The presence of hydroxy and carbonyl groups was revealed by the bands at 3449 and 1719 cm^{-1}, respectively, in the IR spectrum. The ^{1}H NMR data of **3** were similar to those of 3-methyl-2,3-dihydrobenzofuran-2,3-diol,[23] except that the 6-methyl [δ 2.35 (3H, s)] and 2-*O*-angeloyl groups [δ 1.87 (3H, d, J = 1.0 Hz, H-5′), 2.01 (3H, d, J = 7.3 Hz, H-4′), and 6.17 (1H, br q, J = 7.3 Hz, H-3′)] of **3** replaced H-6 and the 2-hydroxy group of 3-methyl-2,3-dihydrobenzofuran-2,3-diol.[23] This was supported by the following NOESY and HMBC correlations (Table 2): (a) NOESY correlations between Me-6 (δ 2.35)/H-5 (δ 6.84), Me-6 (δ 2.35)/H-7 (δ 6.76), and H-4 (δ 7.22)/H-5 (δ 6.84); (b) NOESY correlations between H-5′ (δ 1.87) and Me-3 (δ 1.65); (c) HMBC correlations between H-2 (δ 6.56)/C-1′ (δ 166.1), H-3′ (δ 6.17)/C-1′ (δ 166.1), H-4′ (δ 2.01)/C-2′ (δ 126.8), H-5′ (δ 1.87)/C-1′ (δ 166.1), and H-5′ (δ 1.87)/C-3′ (δ 141.1); (d) HMBC correlations between Me-6 (δ 2.35) and C-5 (δ 123.0), C-6 (δ 141.5), and C-7 (δ 111.6). To further clarify the relative configuration of **3**, a computer-assisted 3D structure (Figure 1) was obtained by using the molecular modeling program CS CHEM 3D Ultra 11.0, with MM2 force-field calculations for energy minimization. The calculated distances between H-5′/Me-3 (2.752 Å), Me-3/H-4 (2.473 Å), and H-2/Me-3 (3.345 Å) are all less than 4 Å; this is consistent with the well-defined NOESY observed for each of these proton pairs. Thus, the structure of **3** was elucidated as (*Z*)-[(2*S**,3*R**)-3-hydroxy-3,6-dimethyl-2,3-dihydrobenzofuran-2-yl] 2-methylbut-2-enoate, named eupatobenzofuran. This structure was supported by ^{1}H−^{1}H COSY and NOESY (Table 2) experiments, and ^{13}C NMR assignments were confirmed by DEPT, HSQC, and HMBC (Table 2) techniques.

2-Hydroxy-2,6-dimethylbenzofuran-3(2*H*)-one (**4**) had the molecular formula $C_{10}H_{10}O_3$ as indicated by the sodiated

Table 2. ^{1}H NMR, NOESY, and HMBC Data of 3 and 4[a]

	3			4		
position	δ_H (J in Hz)	NOESY	HMBC	δ_H (J in Hz)	NOESY	HMBC
2	6.56 s	Me-3	1′, 7a			
4	7.22 d (7.5)	5, Me-3	3, 6, 7a	7.55 d (8.0)	5	3, 6, 7a
5	6.84 br d (7.5)	4, Me-6	3a, 7	6.91 br d (8.0)	4, Me-6	3a, 7, Me-6
7	6.76 br s	Me-6	3a, 5	6.86 br s	Me-6	3a, 5
3′	6.17 br q (7.3)	4′, 5′	1′, 2′, 5′			
4′	2.01 d (7.3)	3′	2′, 3′			
5′	1.87 d (1.0)	3′, Me-3	1′, 2′, 3′			
Me-2				1.65 s	OH-2	2, 3
Me-3	1.65 s	2, 5′	2, 3, 3a			
Me-6	2.35 s	5, 7	5, 6, 7	2.44 s	5, 7	5, 6, 7
OH-2				3.35 br s	Me-2	
OH-3	2.15 br s					

[a] Recorded in $CDCl_3$ at 500 MHz. Values in ppm (δ). J (in Hz) in parentheses.

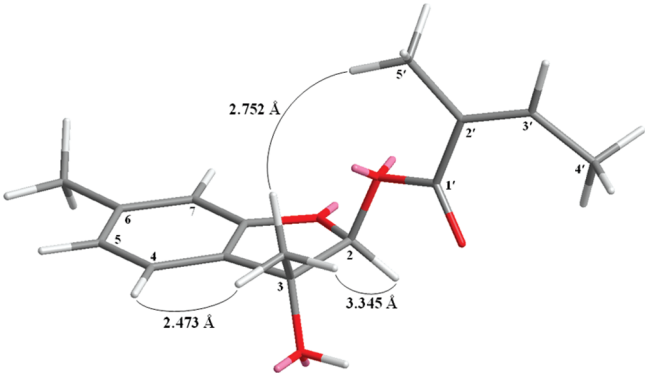


Figure 1. Selected NOESY correlations and relative configuration of 3.

HRESIMS ion peak at m/z = 201.0529 $[M + Na]^+$ (calcd for $C_{10}H_{10}O_3Na$, 201.0528). Hydroxy and carbonyl groups were revealed by IR bands at 3395 and 1705 cm^{-1}, respectively. The ^{1}H NMR spectrum showed the presence of two methyl groups [δ 1.65 (3H, s, Me-2), 2.44 (3H, s, Me-6)], a hydroxy group [δ 3.35 (1H, br s, D_2O exchangeable, OH-2)], and three ABX-coupled aromatic protons [δ 6.86 (1H, br s, H-7), 6.91 (1H, br d, J = 8.0 Hz, H-5), and 7.55 (1H, d, J = 8.0 Hz, H-4)], similar to resonances described for 2,5-dimethyl-2-hydroxy-3(2*H*)-benzofuranone,[24] except that the 6-methyl group [δ 2.44 (3H, s)] of **4** replaced the 5-methyl group [δ 2.30 (3H, s)] of 2,5-dimethyl-2-hydroxy-3(2*H*)-benzofuranone. This was supported by the HMBC correlations between Me-6 (δ 2.44) and C-5 (δ 124.0), C-6 (δ 151.5), and C-7 (113.5) and by the NOESY correlations between H-4 (δ 7.55)/H-5 (δ 6.91), H-5 (δ 6.91)/Me-6 (δ 2.44), and Me-6 (δ 2.44)/H-7 (δ 6.86). Furthermore, the full assignment of ^{1}H and ^{13}C NMR resonances was confirmed by ^{1}H−^{1}H COSY, NOESY (Table 2), DEPT, HSQC, and HMBC (Table 2) techniques. On the basis of the evidence above, the structure of **4** was elucidated as 2-hydroxy-2,6-dimethylbenzofuran-3(2*H*)-one.

1-(2-Hydroxy-4-methylphenyl)propan-1,2-dione (**5**) was isolated as a colorless oil. The ESIMS of **5** afforded an $[M + Na]^+$ ion at m/z 201, implying a molecular formula of $C_{10}H_{10}O_3$, which was

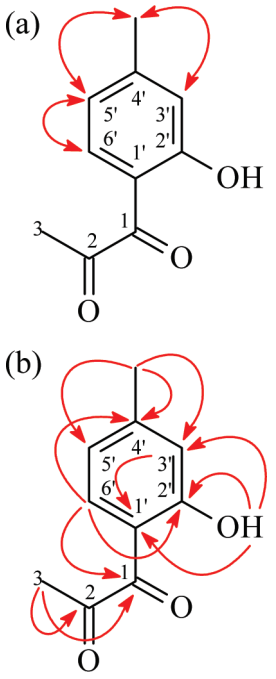


Figure 2. NOESY (a) and HMBC (b) correlations of 5.

confirmed by HRESIMS (m/z 201.0532 $[M + Na]^+$, calcd for $C_{10}H_{10}O_3Na$, 201.0528). The IR spectrum showed the presence of OH (3383 cm^{-1}) and carbonyl (1714 cm^{-1}) groups. Comparison of the ^{1}H NMR data of **5** with those of 1-(2-hydroxy-5-methylphenyl)propan-1,2-dione[24] suggested that their structures were closely related, except that the 4′-methyl group [δ 2.38 (3H, s)] of **5** replaced the 5′-methyl group [δ 2.25 (3H, s)] of 1-(2-hydroxy-5-methylphenyl)propan-1,2-dione. This was supported by the NOESY correlations between Me-4′ (δ 2.38) and both H-3′ (δ 6.85) and H-5′ (δ 6.74) and by the HMBC correlations between Me-4′ (δ 2.38) and C-3′ (δ 118.6), C-4′ (δ 150.3), and C-5′ (121.1). The structure of **5** was thus elucidated as 1-(2-hydroxy-4-methylphenyl)propan-1,2-dione. This was further confirmed by ^{1}H−^{1}H COSY and NOESY (Figure 2) experiments. The assignment of ^{13}C NMR resonances was confirmed by DEPT, HSQC, and HMBC (Figure 2) techniques.

The known isolates were readily identified by a comparison of physical and spectroscopic data (UV, IR, ^{1}H NMR, $[\alpha]_D$, and MS) with corresponding authentic samples or literature values, and this included eight thymol derivaties, 9-acetoxy-8,10-epoxythymol 3-*O*-tiglate (**6**),[25] 9-acetoxy-8,10-dehydrothymol 3-*O*-tiglate (**7**),[20] 9-acetoxythymol 3-*O*-tiglate (**8**),[20] 9-hydroxy-8,10-dehydrothymol (**9**),[26] 9-isobutyryloxy-8,10-dehydrothymol (**10**),[22] 8-methoxy-9-*O*-isobutyrylthymol (**11**),[20] 8-methoxy-9-*O*-angeloylthymol (**12**),[20] 10-acetoxy-8-hydroxy-9-*O*-angeloylthymol (**13**),[20] a dimeric thymol derivative, 3′,4′,4a′,9a′-tetrahydro-6,7′-dimethylspiro[benzofuran-3(2*H*),2′-pyrano-[2,3-*b*]benzofuran]-2,4a′-diol (**14**),[27] an acetophenone, 1-[2-hydroxy-4-(hydroxymethyl)phenyl]ethan-1-one (**15**),[28] a 1,4-diphenyl-butane-1,4-dione, hofmeisterin II (**16**),[29] a benzofuran, euparin (**17**),[30] a coumarin, 2*H*-chromen-2-one (**18**),[31] a triterpene, taraxasterol acetate (**19**),[32] and a mixture of β-sitosterol (**20**)[33] and stigmasterol (**21**).[33]

Neutrophils are known to play crucial roles in host defense against microorganisms and in pathogenesis of various diseases such as asthma, rheumatoid arthritis, chronic obstructive

Table 3. In Vitro Inhibitory Effects of Compounds 1−21 from the Aerial Part of *E. cannabinum* on Superoxide Radical Anion Generation and Elastase Release by Human Neutrophils in Response to fMet-Leu-Phe/Cytochalasin B[a]

compound	IC_{50} (μM)[b] or (Inh %)[c]	
	superoxide anion generation	elastase release
9-*O*-angeloyl-8,10-dehydrothymol (**1**)	(20.3 ± 5.6)[e]	(23.0 ± 11.3)
9-(3-methylbutanoyl)-8,10-dehydrothymol (**2**)	(39.6 ± 8.3)[f]	18.3 ± 2.0
eupatobenzofuran (**3**)	(37.1 ± 3.9)[g]	11.3 ± 2.9[e]
2-hydroxy-2,6-dimethylbenzofuran-3(2*H*)-one (**4**)	(0.02 ± 3.53)	(17.3 ± 10.7)
1-(2-hydroxy-4-methylphenyl)propan-1,2-dione (**5**)	(10.1 ± 6.9)	(37.3 ± 9.3)[e]
9-acetoxy-8,10-epoxythymol 3-*O*-tiglate (**6**)	8.32 ± 1.02[e]	25.8 ± 1.8
9-acetoxy-8,10-dehydrothymol 3-*O*-tiglate (**7**)	8.50 ± 1.63[e]	(36.6 ± 2.1)[g]
9-acetoxythymol 3-*O*-tiglate (**8**)	18.4 ± 6.4	(21.6 ± 8.6)
9-hydroxy-8,10-dehydrothymol (**9**)	(2.67 ± 4.75)	(1.86 ± 2.38)
9-isobutyryloxy-8,10-dehydrothymol (**10**)	(32.7 ± 3.8)[g]	16.1 ± 3.3
8-methoxy-9-*O*-isobutyrylthymol (**11**)	8.19 ± 0.71[e]	(30.0 ± 2.2)[g]
8-methoxy-9-*O*-angeloylthymol (**12**)	(14.7 ± 6.1)	(13.6 ± 9.1)
10-acetoxy-8-hydroxy-9-*O*-angeloylthmol (**13**)	13.1 ± 0.4	6.27 ± 0.68[e]
3′,4′,4a′,9a′-tetrahydro-6,7′-dimethylspiro[benzofuran-3(2*H*),2′-pyrano[2,3-*b*]benzofuran]-2,4a′-diol (**14**)	(4.37 ± 3.37)	(13.0 ± 6.5)
1-[2-hydroxy-4-(hydroxymethyl)phenyl]ethan-1-one (**15**)	8.13 ± 2.34[e]	6.38 ± 1.86[e]
hofmeisterin II (**16**)	(7.34 ± 6.64)	(1.77 ± 8.14)
euparin (**17**)	(13.7 ± 2.6)	(12.6 ± 6.6)
2*H*-chromen-2-one (**18**)	(8.50 ± 3.60)	(23.8 ± 3.7)[f]
taraxasterol acetate (**19**)	(0.68 ± 2.62)	(4.73 ± 1.58)
mixture of β-sitosterol (**20**) and stigmasterol (**21**)	(24.3 ± 5.7)	(13.2 ± 5.0)
LY294002[d]	1.09 ± 0.11	1.98 ± 0.25

[a] Results are presented as average ± SEM (n = 3−4). [b] Concentration necessary for 50% inhibition (IC_{50}). [c] Percentage of inhibition (Inh %) at 10 μg/mL. [d] LY294002, a phosphatidylinositol-3-kinase inhibitor, was used as a positive control for superoxide anion generation and elastase release. [e] $p < 0.05$ compared with the control. [f] $p < 0.01$ compared with the control. [g] $p < 0.001$ compared with the control.

pulmonary disease (COPD), and ischemia-reperfusion injury.[34−37] In response to different stimuli, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion radical ($O_2^{\bullet-}$), a precursor to other reactive oxygen species (ROS), bioactive lipids, granule proteases, and neutrophil elastase, a major contributor to destruction of tissue in chronic inflammatory disease.[37−39] Suppression of the extensive or inappropriate activation of neutrophils by drugs has been proposed as a way to ameliorate inflammatory diseases. The in vitro effects on neutrophil pro-inflammatory responses of compounds isolated from the aerial part of *E. cannabinum* were evaluated by suppressing fMet-Leu-Phe/cytochalasin B (fMLP/CB)-induced superoxide anion ($O_2^{\bullet-}$) generation and elastase release by human neutrophils. The inhibitory activity data on neutrophil pro-inflammatory responses are summarized in Table 3. LY294002 (Sigma, St. Louis, MO, USA), a phosphatidylinositol-3-kinase inhibitor, was used as a positive control for $O_2^{\bullet-}$ generation and elastase release, respectively.[40,41] From the results of our biological tests, the following conclusions can be drawn: (a) 9-Acetoxy-8,10-epoxythymol 3-*O*-tiglate (**6**), 9-acetoxy-8,10-dehydrothymol 3-*O*-tiglate (**7**), 9-acetoxythymol 3-*O*-tiglate (**8**), 8-methoxy-9-*O*-isobutyrylthymol (**11**), 10-acetoxy-8-hydroxy-9-*O*-angeloylthymol (**13**), and 1-[2-hydroxy-4-(hydroxymethyl)phenyl]ethan-1-one (**15**) exhibited potent inhibition (IC_{50} values ≤ 18.4 μM) of superoxide anion ($O_2^{\bullet-}$) generation by human neutrophils in response to fMLP/CB. (b) 9-(3-Methylbutanoyl)-8,10-dehydrothymol (**2**), eupatobenzofuran (**3**), 9-isobutyryloxy-8,10-dehydrothymol (**10**), 10-acetoxy-8-hydroxy-9-*O*-angeloylthymol (**13**), and 1-[2-hydroxy-4-(hydroxymethyl)phenyl]ethan-1-one (**15**) inhibited fMLP/CB-induced elastase release with IC_{50} values ≤ 18.3 μM. (c) Among the 9-acetoxythymol 3-*O*-tiglate analogues (**6**−**8**), compounds **6** (with an 8,10-epoxy group) and **7** (with a C-8−C-10 double bond) exhibited more effective inhibition than analogue **8** (with a C-8−C-10 single bond) against fMLP-induced $O_2^{\bullet-}$ generation and elastase release. (d) Among the 3-hydroxy-8,10-dehydrothymol analogues (**1**, **2**, **9**, and **10**), compounds **2** (with a 3-methylbutanoyl group at C-9) and **10** (with a 9-isobutyryloxy group) exhibited more effective inhibition than analogues **1** (with a 9-*O*-angeloyl group) and **9** (with a 9-hydroxy group) against fMLP-induced $O_2^{\bullet-}$ generation and elastase release. (e) 8-Methoxy-9-*O*-isobutyrylthymol (**11**) (with a 9-*O*-isobutyryl group) exhibited more effective inhibition than its analogue, 8-methoxy-9-*O*-angeloylthymol (**12**) (with a 9-*O*-angeloyl substituent), against fMLP-induced $O_2^{\bullet-}$ generation and elastase release. (f) 10-Acetoxy-8-hydroxy-9-*O*-angeloylthmol (**13**) and 1-[2-hydroxy-4-(hydroxymethyl)phenyl]ethan-1-one (**15**) were the most effective among the isolated compounds, with IC_{50} values of 6.27 ± 0.68 and 8.13 ± 2.34 μM, respectively, against fMLP-induced elastase release and superoxide anion generation in vitro.

EXPERIMENTAL SECTION

General Experimental Procedures. 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 (neat or KBr) 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 500 spectrometer

operating at 400 or 500 MHz (^{1}H) and 100 or 125 MHz (^{13}C), respectively, with chemical shifts given in ppm (δ), using TMS as an internal standard. ESI and HRESI-mass spectra were recorded on a Bruker APEX II mass spectrometer. EI and HREI-mass spectra were obtained on a Finnigan/Thermo Quest MAT 95XL 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 preparative TLC.

Plant Material. The aerial part of *E. cannabinum* subsp. *asiaticum* was collected from Shuei-Shang, Chiayi County, Taiwan, in November 2009, and identified by J.-H. Huang. A voucher specimen (No. 251800) was deposited in the Herbarium of the Institute of Ecology and Evolutionary Biology, College of Life Science, National Taiwan University, Taiwan.

Extraction and Separation. The shade-dried aerial part of *E. cannabinum* (4.1 kg) was pulverized and extracted three times with MeOH (15 L each) for 3 days. The MeOH extracts were concentrated under reduced pressure at 35 °C, and the residue (413 g) was partitioned between *n*-hexane and H_2O (1:1, each 4 L). The *n*-hexane layer was concentrated to give a residue (fraction A, 116 g). The water layer was further extracted three times with EtOAc (3 L each), and the EtOAc-soluble part (fraction B, 90 g) and the water-solubles (fraction C, 188 g) were separated. Fraction A (116 g) was chromatographed on a 60 cm × 10 cm i.d. silica gel column (70−230 mesh, 4.7 kg), eluting with *n*-hexane, gradually increasing the polarity with EtOAc and MeOH to give 11 fractions: A1 (3 L, *n*-hexane), A2 (5 L, *n*-hexane/EtOAc, 50:1), A3 (6 L, *n*-hexane/EtOAc, 30:1), A4 (5 L, *n*-hexane/EtOAc, 20:1), A5 (6 L, *n*-hexane/EtOAc, 10:1), A6 (6 L, *n*-hexane/EtOAc, 5:1), A7 (6 L, *n*-hexane/EtOAc, 3:1), A8 (6 L, *n*-hexane/EtOAc, 1:1), A9 (7 L, EtOAc), A10 (7 L, EtOAc/MeOH, 1:1), and A11 (4 L, MeOH). Fraction A2 (9.3 g) was chromatographed on silica gel (230−400 mesh, 372 g), eluting with *n*-hexane/EtOAc (20:1), to give 12 fractions (each 1.5 L, A2-1−A2-12). Fraction A2-6 (154 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 20:1) to obtain **19** (5.4 mg) (R_f = 0.70). Fraction A2-7 (146 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 20:1) to yield **17** (4.2 mg) (R_f = 0.54). Fraction A3 (12.7 g) was chromatographed on silica gel (230−400 mesh, 568 g), eluting with *n*-hexane/acetone (10:1), to give 13 fractions (each 1.6 L, A3-1−A3-13). Fraction A3-2 (703 mg) was washed with MeOH and filtered to obtain a mixture of **20** and **21** (194 mg) after recrystallization (CH_2Cl_2/MeOH, 1:5). Fraction A3-4 (947 mg) was separated by MPLC (42 g silica gel, 230−400 mesh, *n*-hexane/EtOAc, 10:1, 220 mL fraction) to give nine subfractions: A3-4-1−A3-4-9. Fraction A3-4-2 (128 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 10:1) to obtain **8** (6.5 mg) (R_f = 0.5). Fraction A3-4-5 (139 mg) was purified further by preparative TLC (silica gel, *n*-hexane/CH_2Cl_2, 2:3) to yield **7** (9.7 mg) (R_f = 0.55) and **11** (13.2 mg) (R_f = 0.53). Fraction A3-4-9 (133 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 10:1) to afford **6** (26 mg) (R_f = 0.49). Fraction A3-7 (145 mg) was purified further by preparative TLC (silica gel, *n*-hexane/$CHCl_3$, 1:3) to obtain **10** (7.1 mg) (R_f = 0.41). Fraction A3-9 (164 mg) was purified further by preparative TLC (silica gel, *n*-hexane/$CHCl_3$, 1:3) to yield **2** (5.7 mg) (R_f = 0.39). Fraction A3-12 (176 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 3:1) to afford **3** (10.2 mg) (R_f = 0.38). Fraction A6 (10.3 g) was chromatographed on silica gel (230−400 mesh, 425 g), eluting with *n*-hexane/acetone (7:1), to give 10 fractions (each 950 mL, A6-1−A6-10). Fraction A6-2 (196 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 10:1) to obtain **12** (7.5 mg) (R_f = 0.62). Fraction A6-5 (166 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 10:1) to yield **16** (7.1 mg) (R_f = 0.56). Fraction A6-7 (185 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 10:1) to afford **18** (19.7 mg) (R_f = 0.26). Fraction A6-9 (157 mg) was purified further by preparative TLC (silica gel, *n*-hexane/acetone, 7:1) to yield **1** (5.1 mg) (R_f = 0.33). Fraction A7 (9.2 g) was chromatographed on silica gel (403 g silica gel, 230−400 mesh, *n*-hexane/acetone, 6:1, 1.3 L fraction) to give 13 subfractions: A7-1−A7-13. Fraction A7-3 (182 mg) was purified further by preparative TLC (silica gel, CH_2Cl_2/acetone, 15:1) to obtain **13** (6.4 mg) (R_f = 0.69) and **15** (4.6 mg) (R_f = 0.61). Fraction A7-6 (160 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/acetone, 15:1) to yield **5** (7.4 mg) (R_f = 0.43). Fraction A7-9 (148 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/EtOAc, 15:1) to afford **4** (4.1 mg) (R_f = 0.31). Fraction A7-10 (156 mg) was purified further by preparative TLC (silica gel, *n*-hexane/EtOAc, 15:1) to obtain **9** (6.5 mg) (R_f = 0.23). Fraction A7-11 (145 mg) was purified further by preparative TLC (silica gel, $CHCl_3$/acetone, 15:1) to yield **14** (9.6 mg) (R_f = 0.50).

Biological Assay. The effects of the isolated compounds on neutrophil pro-inflammatory responses were evaluated by monitoring the inhibition of superoxide anion generation and elastase release in fMLP/CB-activated human neutrophils in a concentration-dependent manner.[42]

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.[43] Purified neutrophils, containing >98% viable cells, as determined by the trypan blue exclusion method,[44] were resuspended in a Ca^{2+}-free HBSS buffer at pH 7.4 and were maintained at 4 °C prior to use.

In Vitro Measurement of $O_2^{\bullet-}$ Generation. Measurement of superoxide anion generation was based on the SOD-inhibitable reduction of ferricytochrome *c*.[42] In brief, after supplementation with 0.5 mg/mL ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5/mL) were equilibrated at 37 °C for 2 min and incubated with different concentrations of compounds or DMSO (as control) for 5 min. Cells were incubated with cytochalasin B (1 μg/mL) for 3 min prior to the activation with 100 nM formyl-L-methionyl-L-leucyl-L-phenylalanine for 10 min. 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).

In Vitro Measurement of Elastase Release. Degranulation of azurophilic granules was determined by measuring elastase release as described previously.[42] 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)/CB (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/CB-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.

*9-O-Angeloyl-8,10-dehydrothymol (**1**):* colorless oil; UV (MeOH) λ_{max} (log ε) 240 (sh, 3.70), 283 (3.39) nm; IR (neat) ν_{max} 3391 (OH), 1697 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 15.9 (C-4′), 20.5 (C-5′), 21.2 (C-7), 65.6 (C-9), 116.3 (C-10), 116.7 (C-2), 120.9 (C-6), 122.5 (C-4), 127.1 (C-2′), 129.2 (C-5), 139.9 (C-1), 140.1 (C-3′), 142.0 (C-8), 153.4 (C-3), 168.4 (C-1′); ESIMS *m/z* 269 $[M + Na]^+$; HRESIMS *m/z* 269.1156 $[M + Na]^+$ (calcd for $C_{15}H_{18}O_3Na$, 269.1154).

*9-(3-Methylbutanoyl)-8,10-dehydrothymol (**2**):* colorless oil; UV (MeOH) λ_{max} (log ε) 209 (4.25), 238 (sh, 3.76), 284 (3.41) nm; IR (neat) ν_{max} 3420 (OH), 1715 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500

MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.2 (C-7), 22.4 (C-4′), 22.4 (C-5′), 25.7 (C-3′), 43.3 (C-2′), 65.8 (C-9), 116.3 (C-10), 116.6 (C-2), 120.9 (C-6), 122.4 (C-4), 129.1 (C-5), 140.1 (C-1), 142.0 (C-8), 153.4 (C-3), 173.8 (C-1′); ESIMS m/z 271 $[M + Na]^+$; HRESIMS m/z 271.1307 $[M + Na]^+$ (calcd for $C_{15}H_{20}O_3Na$, 271.1310).

Eupatobenzofuran (= (Z)-[(2S,3R*)-3-Hydroxy-3,6-dimethyl-2,3-dihydrobenzofuran-2-yl] 2-methylbut-2-enoate) (**3**):* amorphous powder; $[\alpha]^{25}_D$ −7.5 (*c* 0.11, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 277 (3.35), 284 (3.32) nm; IR (neat) ν_{max} 3449 (OH), 1719 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) data, see Table 2; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 15.9 (C-4′), 20.3 (C-5′), 20.5 (Me-3), 21.7 (Me-6), 79.8 (C-3), 105.2 (C-2), 111.6 (C-7), 122.7 (C-4), 123.0 (C-5), 126.8 (C-2′), 127.7 (C-3a), 141.1 (C-3′), 141.5 (C-6), 158.4 (C-7a), 166.1 (C-1′); EIMS m/z 262 $[M]^+$; HREIMS m/z 262.1211 $[M]^+$ (calcd for $C_{15}H_{18}O_4$, 262.1205).

*2-Hydroxy-2,6-dimethylbenzofuran-3(2H)-one (**4**):* colorless oil; $[\alpha]^{25}_D$ −6.7 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 256 (3.67), 341 (3.15) nm; IR (neat) ν_{max} 3395 (OH), 1705 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) data, see Table 2; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 22.0 (Me-2), 22.7 (Me-6), 103.6 (C-2), 113.5 (C-7), 116.0 (C-3a), 124.0 (C-5), 125.0 (C-4), 151.5 (C-6), 170.5 (C-7a), 198.0 (C-3); ESIMS m/z 201 $[M + Na]^+$; HRESIMS m/z 201.0529 $[M + Na]^+$ (calcd for $C_{10}H_{10}O_3Na$, 201.0528).

*1-(2-Hydroxy-4-methylphenyl)propan-1,2-dione (**5**):* colorless oil; UV (MeOH) λ_{max} (log ε) 256 (4.01), 329 (3.60) nm; IR (neat) ν_{max} 3383 (OH), 1714 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.38 (3H, s, Me-4′), 2.52 (3H, s, H-3), 6.74 (1H, br d, J = 8.5 Hz, H-5′), 6.85 (1H, br s, H-3′), 7.64 (1H, d, J = 8.5 Hz, H-6′), 11.43 (1H, s, D_2O exchangeable, OH-2′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 22.2 (Me-4′), 26.6 (C-3), 112.9 (C-1′), 118.6 (C-3′), 121.1 (C-5′), 132.2 (C-6′), 150.3 (C-4′), 164.3 (C-2′), 195.2 (C-1), 199.3 (C-2); ESIMS m/z 201 $[M + Na]^+$; HRESIMS m/z 201.0532 $[M + Na]^+$ (calcd for $C_{10}H_{10}O_3Na$, 201.0528).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This research was supported by grants from the National Science Council of the Republic of China (Nos. NSC 95-2320-B-127-001-MY3 and NSC 98-2320-B-127-001-MY3), awarded to J.-J.C. We also thank the National Center for High-Performance Computing (NCHC, Taiwan) for providing computer resources and chemical database services.

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