Antityrosinase and Antioxidant Effects of *ent*-Kaurane Diterpenes from Leaves of *Broussonetia papyrifera*

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Received September 9, 2008

Three new *ent*-kaurane type diterpenes, broussonetones A−C (**1**−**3**), were isolated from leaves of *Broussonetia papyrifera*, together with seven known compounds, and their structures determined by 1D and 2D NMR and MS methods. Compounds **1**−**3** were marginal inhibitors of tyrosinase. Antioxidant assays showed them also to be inhibitors of xanthine oxidase. The mild inhibition of tyrosinase and significant inhibition of xanthine oxidase suggests that **1**−**3** could be useful ingredients in the development of skin-protecting cosmetics.

Photoactivation of reactive oxygen species (ROS) plays an important role in free radical related skin-damaging ailments and aging.[1,2] In addition, uncontrolled melanin formation such as hyperpigmentation, melasma, postinflammatory melanoderma, solar lentigo, and even skin cancer are usual outcomes of excessive solar exposure.[3,4] These unfavorable skin conditions or ailments can be attributed to both excessive melanin formation and free radical damaging of dermal cells. The application of antioxidants to protect skin from the damage of UV-induced ROS is of great popularity and efficacy.[4,5] Furthermore, cosmetics are commercial products with great economic potential employed to improve the appearance of skin in modern human society. Accordingly, research and development of new and effective ingredients for skin protection in cosmetics has been pursued for decades.

Since the flavonoids isolated from *Broussonetia papyrifera* L. (Moraceae) have been demonstrated to be effective mushroom tyrosinase inhibitors[6] and the methanolic extract of ground leaves of this plant displayed a greater mushroom tyrosinase inhibitory effect than kojic acid (IC_{50} 104.2 μg/mL vs >500 μg/mL after 2 h) together with DPPH free radical scavenging activity (IC_{50} = 40.72 μg/mL) in our preliminary screening, isolation of the active principles proceeded. Three new *ent*-kaurane diterpenes (**1**−**3**), apigenin (**4**),[7] vitexin (**5**),[8] apigenin-7-*O*-glucuronide methyl ester (**6**),[9] apigenin-7-*O*-glucoside (**7**),[10] amentoflavone (**8**),[11] taraxerol acetate,[12] and glyceric acid methyl ester[13] were isolated and identified using NMR and other spectroscopic methods. The *ent*-kauranes **1**−**3** are the first diterpenoids isolated from *B. papyrifera* and from Moraceous plants. Compounds **1**−**5** were tested using DPPH free radical scavenging, SOD-like, and mushroom tyrosinase inhibitory assays. Herein, we report the structural elucidation of **1**−**3** and discuss their potential for development as new ingredients of cosmetics based on the assays mentioned.

Compound **1** was obtained as colorless needles (MeOH), mp 180−182 °C, $[\alpha]^{25}_D$ −8.0 (*c* 0.05, MeOH), with OH and carbonyl absorption bands at ν_{max} 3447 and 1711 cm^{-1} in the IR spectrum. The EIMS of **1** showed the molecular ion at *m*/*z* 336, and HRFABMS had a quasi-molecular ion at *m*/*z* 359.2190 $[M + Na]^+$ (calcd for $C_{20}H_{32}O_4Na$, 359.2198), consistent with the molecular formula $C_{20}H_{32}O_4$ and suggesting that **1** was a tetracyclic diterpene. In the ^1H NMR spectrum of **1** (Table 1) there were three singlet methyl signals (δ_H 1.27, 1.01, and 0.90) and a pair of geminal methylene signals [δ_H 3.74, 3.68 (each 1H, d, J = 11.0 Hz)] similar to *ent*-kaurane diterpenes having a 16,17-diol group.[14−16] The ^1H NMR spectrum of **1** also showed an oxygenated methine signal at δ_H 4.07 (1H, s) (Table 1). The ^{13}C, DEPT-135, and HMQC NMR spectra of **1** displayed 20 carbon signals including a carbonyl (δ_C 211.3), an oxygenated quaternary carbon (δ_C 81.2), an oxygenated methine (δ_C 79.1), and an oxygenated methylene (δ_C 66.2) as shown in Table 1. The above information all indicated that **1** was an *ent*-kaurane ketone 16,17-diol[14−16] with an additional OH group. On the basis of the HMQC spectrum (Table 1), both of the singlet signals at δ_H 2.16 (H-5) and 4.07 (H-7) showed cross-peaks ($^2J_{CH}$) with the carbonyl (δ_C 211.3) and revealed the 6-oxo-7-hydroxy-*ent*-kaurane partial structure of **1**. In addition, the hydroxylated methylene signals at δ_H 3.74 and 3.68 showed cross-peaks with δ_C 81.2 (C-15, $^2J_{CH}$), 44.7 (C-13, $^3J_{CH}$), and 49.9 (C-15, $^3J_{CH}$), each indicating the structure of **1** to be 7,16,17-trihydroxy-*ent*-kauran-6-one. However, the NOESY interaction (Figure 1) shown by H-5 and H-7 with respective signals in accord with the MM2 energy minimized structure of **1** confirmed the conformations of the C-7 and C-16 OH groups. Thus, the structure of **1** was characterized as 7α,16α,17-trihydroxy-*ent*-kauran-6-one, and it was named broussonetone A.

Compound **2** was also obtained as colorless needles (MeOH), mp 179−181 °C, $[\alpha]^{25}_D$ −12.3 (*c* 0.05, MeOH), with OH and carbonyl absorption bands at ν_{max} 3446 and 1706 cm^{-1} in the IR spectrum similar to those of **1**. Quasi-molecular ions in both the ESIMS at *m*/*z* 359 $[M + Na]^+$ and HRFABMS at *m*/*z* 337.2390 (calcd for $C_{20}H_{37}O_4$, 337.2679) confirmed the molecular formula to be $C_{20}H_{32}O_4$, an isomer of **1**. The ^1H NMR spectrum of **2** (Table 1) displayed three methyl signals [δ_H 1.24, 1.00, and 0.84 (H-18, H-20, H-19)], a set of hydroxylated methine signals [δ_H 4.90 (1H, d, J = 4.2 Hz, C-7 OH) and 3.39 (1H, d, J = 4.2 Hz, H-7)], a set of hydroxylated methylene signals [δ_H 3.70, 3.57 (each 1 H, dd, J = 10.4, 4.0 Hz) and 3.63 (1H, br t, C-17 OH)], and a tertiary OH [δ_H 3.26 (1H, br s, C-16 OH)], also indicating that **2** was an isomer of **1**.[14−16] The ^{13}C, DEPT-135, and HMQC NMR spectra of **2** (Table 1) displayed 20 carbon signals similar to those of **1**. The key cross-peak of δ_H 3.08 (H-5) with δ_C 214.1 (C-6 carbonyl) in the HMBC spectrum (Table 1) indicated that **2** was also a 7,16,17-trihydroxy-*ent*-kauran-6-one. The NOESY spectrum of **2** (Figure 2) showed an identical interaction of H-7 (δ_H 3.39) with H-14 (δ_H 1.66), indicating that the conformation of the C-7 hydroxy group was β oriented and confirmed **2** to be a steric isomer of **1**. The structure of **2** was thus elucidated as 7β,16α,17-trihydroxy-*ent*-kauran-6-one (**2**), namely, broussonetone B.

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10.1021/np800564z CCC: $40.75
Published on Web 11/06/2008

Table 1. NMR Data (500 MHz) of Broussonetones A−C (**1**−**3**)[d]

	1 ($CDCl_3$)			**2** (acetone-d_6)			**3** ($CDCl_3$)		
no.	δ_C, mult	δ_H (*J*)	HMBC	δ_C, mult	δ_H (*J*)	HMBC	δ_C, mult	δ_H (*J*)	HMBC
1	40.7, CH_2	1.87, br d (12.5); 0.97, dd (12.5, 4.2)		41.5, CH_2	1.84, br d (12); 1.04, dd (12, 4)	9	40.6, CH_2	1.97, dd (12, 6); 0.97, td (12, 4)	3, 5
2	18.3, CH_2	1.51, m; 1.36, dm (13)		19.1, CH_2	1.61, m; 1.44, dm (12)		18.4, CH_2	1.72, dd, (15, 6); 1.55 (m)	
3	42.2, CH_2	1.36, dm (13); 1.07, td (13, 3.8)		43.2, CH_2	1.28, dm (12); 1.12, td (12, 4)		42.2, CH_2	1.38, dm (14); 1.07, td (14, 4)	
4	32.3, qC			32.4, qC			32.3, qC		
5	64.1, CH	2.16, s	1, 4, 6, 9, 10, 18, 20	58.7, CH	3.08, s	4, 6, 10, 18, 20	64.1, CH	2.15, s	1, 4, 6, 9, 10, 18, 20,
6	211.3, qC			214.1, qC			211.6, qC		
7	79.1, CH	4.07, s	6, 8, 14, 15	86.2, CH	3.39, d (4.2)[c]		78.9, CH	4.03 s	8, 14
8	55.6, qC			53.5, qC			55.6, qC		
9	54.8, CH	1.52, m[a]		51.7, CH	1.83, d (12.6)	7	54.4, CH	1.64, m	1, 8, 10, 15
10	46.2, qC			46.0, qC[b]			46.2, qC		
11	17.9, CH_2	1.64, m; 1.51, m[a]		18.8, CH_2	1.62, m[e]		18.3, CH_2	1.65, dt (14.5); 1.45, dd (14.5, 6)	
12	26.1, CH_2	1.54, d (8.1); 1.62 (m)		26.8, CH_2	1.52, m; 1.62, m[e]		27.1, CH_2	1.56, d (8); 1.62, m	11, 14
13	44.7, CH	2.10, br s		46.0, CH[b]	2.04[f]	7, 9, 15, 16	45.0, CH	2.17, br s	8
14	28.1, CH_2	1.26, dd (13, 1.6); 2.03, dd (13, 4)	15, 16	35.2, CH_2	1.66, m; 1.56, d (12)	15, 16	29.3, CH_2	1.26, dd (13, 1.6); 1.97, dd (13, 4)	7, 15
15	49.9, CH_2	1.77, d (13); 2.04, d (13)	14, 16, 17	49.7, CH_2	1.80, d (15.2); 1.66, d (15.2)	9, 14, 16, 17	52.4, CH_2	2.49, d (14.3); 1.79, dd (14.3, 2.1)	7, 8, 9, 14, 16, 17
16	81.2, qC			81.2, qC			88.2, qC		
17	66.2, CH_2	3.74, 3.68, d (11)	13, 15, 16	66.3, CH_2	3.57, 3.70[g] dd (10.4, 4)		69.7, CH_2	3.94, 4.06, d (8.6)	13, 15, ketal
18	22.0, CH_3	1.27, s	3, 4, 5, 19	22.4, CH_3	1.24, s	3, 4, 5, 19	22.0, CH_3	1.27, s	3, 5, 19
19	32.4, CH_3	0.90, s	3, 5, 18	32.7, CH_3	0.84, s	3, 4, 5	32.5, CH_3	0.90, s	3, 5, 18
20	18.8, CH_3	1.01, s	1, 6, 9, 10	19.0, CH_3	1.00, s	1, 5, 9, 10	18.8, CH_3	1.00, s	1, 5, 9
Me							26.7, CH_3	1.40, s	ketal
Me							26.9, CH_3	1.34, s	ketal
ketal							108.8, qC		

[a] These signals were overlapped in each column. [b] These signals were overlapped in each column. [c] This signal coupled with OH signals. [d] Proton−carbon attached signals were verified by DEPT technique and HMQC cross-peaks; HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon. [e] These signals were overlapped in each column. [f] This signal was overlapped with the signals of acetone-d_6. [g] This signal coupled with OH signals.

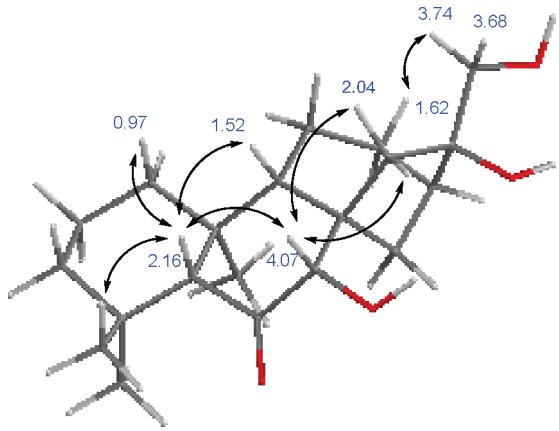

Figure 1. MM2 energy minimized steric conformation of broussonetone A (**1**). Arrows represent the major correlations in the NOESY spectrum.

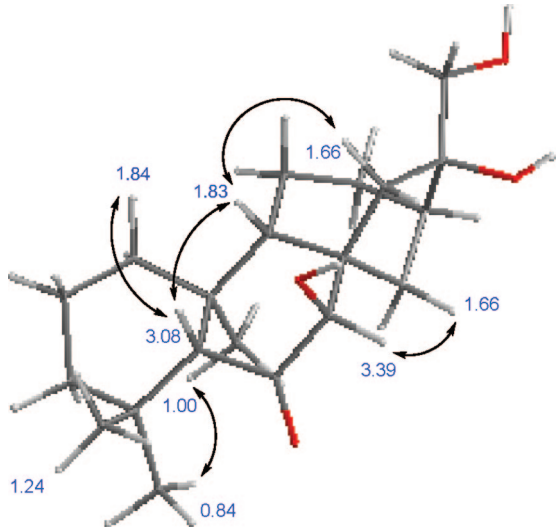

Figure 2. MM2 energy minimized steric conformation of broussonetone B (**2**). Arrows represent the major correlations in the NOESY spectrum.

Compound **3** crystallized as colorless needles (MeOH), mp 160−163 °C, $[\alpha]^{25}_{D}$ −37.1 (*c* 0.05, MeOH) and with significant IR absorption bands at ν_{max} 3396 (OH), 2935 (CH), and 1718 (C=O) cm^{-1}. The molecular formula of **3** was established as $C_{23}H_{36}O_4$ by both the quasi-molecular ion peaks found in the EIMS at *m/z* 377 and HRFABMS at *m/z* 377.2691 (calcd for $C_{23}H_{37}O_4$, 377.2692). The ^{1}H NMR spectrum (Table 1) of **3** displayed features similar to those of **1**. However, two additional methyl signals at δ_H 1.40 (3H, s) and 1.34 (3H, s) and the downfield shifted methylene signals at δ_H 4.06 and 3.94 (each 1H, d, J = 8.6 Hz) suggested that **3** could be an *ent*-kaurane 16,17-acetonide.[17] The ^{13}C, DEPT-135, and HMBC NMR spectra of **3** also displayed signals similar to those of **1** except for the identical acetonide signals at δ_C 108.8 (qC), 26.9 (CH_3), and 26.7 (CH_3) and the downfield shifted signals of C-16 and C-17 at δ_C 88.2 (qC) and 69.7 (CH_2), supporting **3** as being the 16,17-acetonide of **1**.[17] Evidence from HMQC, HMBC, and NOESY spectra confirmed **3** to be 7α,16α,17-trihydroxy-*ent*-kauran-6-one 16,17-acetonide, and it was named broussonetone C (**3**). Compound **3** could be an artifact of **1** since the fraction containing **1** and **3** was purified by column chromatography in the solvent system of acetone/hexane.

Compounds **1**−**5** were evaluated for mushroom tyrosinase inhibitory and antioxidant activity using DPPH free radical scavenging and SOD-like effect assays. As shown in Table 2, the brousonetones A−C (**1**−**3**) and apigenin (**4**) displayed stable, though mild, inhibitory effects on mushroom tyrosinase from 30 to 90 min of incubation rather than degradation in the manner of kojic acid, a marketed whitening agent applied as positive control. The activity of *ent*-kauranes **1**−**3** could be due to the 6-oxo-7-hydroxy carbinol moiety due to the structural similarity to kojic acid and tropolone, the most potent tyrosinase inhibitor reported by Ley et al.[18] The free radical scavenging assays revealed that **2** and **3** exhibited the greatest SOD-like effect (Table 2). However, all of the compounds failed to show any DPPH free radical scavenging activity. Xanthine oxidase inhibitors have been demonstrated to be defensive agents against oxidative stress and effective in the prevention of skin carcinogenesis.[19] The marginal but stable inhibition of tyrosinase together with significant xanthine oxidase inhibition of *ent*-kauranones **1**−**3** could make them useful as ingredients of cosmetics providing further safety evaluation is performed.

Table 2. Mushroon Tyrosinase Inhibitory and Antioxidant Effects of **1**−**3**, Apigenin (**4**), and Vitexin (**5**)[a]

	anti-tyrosinase IC_{50} (mM)		SOD-like IC_{50} (μM)		DPPH
entry	30 min	90 min	30 min	60 min	IC_{50} (μM)
1	0.336	0.322	107.90	107.70	ns
2	ns	0.317	69.65	53.90	ns
3	0.353	0.323	54.83	43.89	ns
4	0.305	0.305	88.24	81.27	ns
5	ns	ns	109.94	84.95	ns
kojic acid	0.176	0.294			
BHA			ns	ns	79.16

[a] Inhibition assays for blank, each concentration of sample and positive control $n \geq 3$; ns: no significance; 50% inhibition (IC_{50}) value expressed by mM or μM; BHA = 3-*tert*-butyl-4-hydroxyanisole.

Experimental Section

General Experimental Procedures. Melting points were recorded on a Electrothermal MEL-TEMP 3.0 apparatus. IR spectra were measured on a Perkin-Elmer 2000 FTIR spectrophotometer, UV by a Beckman DU 640 UV−visible spectrophotometer, NMR spectra with a Bruker AV-500 (500 MHz) and/or Varian Mercury-400 spectrometer (400 MHz), and mass spectra on a Bruker Daltonic Apex II mass spectrometer. Kieselgel 60 (Merck, Germany) was used for chromatography. Optical density measurements of the related bioassays were measured using an Elisa reader (DYNEX MRX). Mushroom tyrosinase, xanthine, xanthine oxidase, DPPH free radical, NBT, and positive controls kojic acid and BHA were all purchased from Sigma Company.

Plant Material. Yellow leaves (2.5 kg) of *B. papyrifera* were collected during September 2006 in Kaohsiung, Taiwan, and a voucher specimen (M-95-01-BP) was deposited in the herbarium of Kaohsiung Medial University.

Extraction and Isolation. The leaves were extracted three times (10 L each) with methanol under ambient temperature. The crude extract (120 g) was obtained after concentration under vacuum. The extract was subjected to silica gel column chromatography (CC) and eluted with a gradient solvent system of CH_2Cl_2/MeOH (100−0%). Compounds were eluted in the following order: taraxerol acetate (**9**, 96 mg),[12] **1** (63 mg), **3** (47 mg), glyceric acid methyl ester (**10**, 6 mg),[13] apigenin (**4**, 18 mg),[7] broussonetone B (**2**, 13 mg), amentoflavone (**8**, 10 mg),[11] apigenin-7-*O*-glucuronide methyl ester (**6**, 12 mg),[19] vitexin (**5**, 55 mg),[8] and apigenin-7-*O*-β-glucopyranoside (**7**, 16 mg).[10] Compounds **1**−**3** were further purified by CC with hexane/acetone (3:2−1:1) and crystallized from MeOH. The known compounds were recrystallized and identified by NMR and other spectroscopic data and by comparison with literature values.

Broussonetone A (1): colorless needles (MeOH); mp 180−182 °C; $[\alpha]^{25}_{D}$ −8.0 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3447 (OH), 2928 (CH), 1711 (C=O) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1; EIMS *m/z* 336 $[M]^+$ (4), 318 $[M - H_2O]^+$ (57), 305 (64), 287 (52), 269 (20), 263 (21), 151 (77), 109 (74); HRFABMS (+) *m/z* 359.2190 $[M + Na]^+$ (calcd for $C_{20}H_{32}O_4Na$, 359.2198).

Broussonetone B (2): colorless needles (MeOH); mp 179−181 °C; $[\alpha]^{25}_{D}$ −12.3 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3446 (OH), 2925 (CH),

1706 (C=O) cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) see Table 1; ^{13}C NMR (125 MHz, acetone-d_6) see Table 1; ESIMS (+) *m/z* 359 $[M + Na]^+$; HRFABMS (+) *m/z* 337.2390 $[M + H]^+$ (calcd for $C_{20}H_{33}O_4$, 337.2379).

Broussonetone C (3): colorless needles (MeOH); mp 160−163 °C; $[\alpha]^{25}_D$ −37.1 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3396 (OH), 2935 (CH), 1718 (C=O) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1; EIMS *m/z* 377 $[M + H]^+$ (4), 361 $[M - Me]^+$ (100), 301 (64), 283 (77), 273 (22), 255 (22); HRFABMS *m/z* 377.2691 $[M + H]^+$ (calcd for $C_{23}H_{37}O_4$, 377.2692).

Mushroom Tyrosinase Inhibition Assay. Compounds **1**−**5** and kojic acid were dissolved in DMSO or methanol and diluted to various concentrations using potassium phosphate buffer (pH 6.8). The 96-well plate contained 80 μL of L-tyrosine (2.0 mM) and various concentrations of compounds **1**−**5** or kojic acid (20 μL), which were diluted with buffer to 200 μL after the addition of 20 μL of mushroom tyrosinase (1000 U/mL, EC 1.14.18.1) to initiate the assay. Inhibitory percentage of each test compound and positive control was determined by the optical density measured at 490 nm after 30 and 90 min of incubation according to the method of Chang et al. with modification.[20]

Superoxide Free Radical Scavenging Assay. The assay was initiated by the addition of xanthine oxidase (0.5 U, 55 μL) into the 96-well plate containing 0.1 mM xanthine (15 μL), 0.1 mM EDTA (14 μL), 25 mM NBT (16 μL), and various concentrations of compounds **1**−**5** or BHA diluted with buffer to 200 μL before being incubated at 25 °C for 30 and 60 min. The superoxide free radical scavenging activity of each compound was determined by the absorbance measured at 540 nm due to the inhibition of blue formazan formation by the reaction of NBT with the free radicals.[21,22]

DPPH Free Radical Scavenging Model. DPPH (0.1 mM) dissolved in MeOH (150 μL) and various concentrations of test samples in the same solvent (50 μL) were added and incubated for 30 min. Then absorption intensity at 517 nm was measured. Inhibition percentage was calculated by decreased absorption intensity in comparison with blank control.[23]

Acknowledgment. The authors are grateful for financial support from the National Science Council of Taiwan (NSC-96-2320-B-037-021). We are also indebted to the National Centre of High-Performance Computing for computer time and facilities, to Miss R. R. Wu, Department of Chemistry, National Cheng Kung University, Tainan, Taiwan, for 2D NMR spectra, and Miss S. C. Yu, Department of Chemistry, Nation Sun Yat-sen University, Kaohsiung, Taiwan, for mass spectra.

Supporting Information Available: The 1D NMR, 2D NMR (HMQC, HMBC, NOESY, and COSY), and MS spectra of broussonetone A−C (**1**−**3**) and spectral data of all the known compounds are available free of charge via the Internet at http://pubs.acs.org.

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NP800564Z