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Two new dimeric abietane-type diterpenoids from the bark of *Cryptomeria japonica* and their enzyme inhibitory activity

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ABSTRACT

Two new dimeric abietane-type diterpenoids, 12-hydroxyabieta-8,11,13-trien-7α-yl 7β-hydroxyisopimara-8(14),15-diene-18-peroxoate (trivial name japonicinol A, **1**) and 6α,7α-*O*-(7,8-secoabieta-9(11),13-diene-8,12-dioxo-7-ylidene)ferruginol (trivial name japonicinol B, **3**), together with three known dimeric abietane-type diterpenoids, sugikurojin J (**2**), sugikurojin B (**4**) and formosaninol (**5**), were isolated from the bark of *Cryptomeria japonica* D. Don. Structure elucidation was accomplished by spectroscopic methods, mainly 1D and 2D NMR and HREIMS, as well as by comparison of their NMR data with those of related known analogues. At the concentration of 50 μM, compounds **1–5** inhibited xanthine oxidase activity by 31.3, 23.4, 18.7, 17.3 and 24.4%, respectively. In addition, compound **4** also inhibited angiotensin-converting enzyme (ACE) activity by 19.2%.

1. Introduction

Cryptomeria japonica D. Don (Cupressaceae) belongs to the monospecific genus *Cryptomeria* and is endemic to Japan, known as sugi (Japanese cedar) in Japanese (Gan, 1958). It is a massive evergreen coniferous tree, growing up to 50 meters in height and has been widely cultivated as an important plantation coniferous tree species in Taiwan since 1906. Its reddish-pink wood exhibits the aromatic, soft, lightweight but strong, and waterproof properties and has been used as a building material for Japanese-style houses and other wood products. Previous phytochemical and pharmaceutical investigations revealed that the leaves, heartwood, and barks of this plant contained diverse terpenoids, including monoterpenoids, sesquiterpenoids, and diterpenoids (Arihara et al., 2004; Chen et al., 2001; Kofujita et al., 2001, 2002; Morita et al., 1995; Nagahama et al., 1993, 1998; Narita et al., 2006; Shibuya, 1992; Shieh et al., 1981; Shimizu et al., 1988; Su et al., 1996; Morisawa et al., 2002; Yoshikawa et al., 2006), some of which showed antibacterial (Li et al., 2008), antifungal (Kofujita et al., 2001), cytotoxic (Kofujita et al., 2002), anti-inflammatory (Shyur et al., 2008), anti-androgenic (Tu et al., 2007), and insect antifeedant (Wu et al., 2008), and repellent (Morisawa et al., 2002) properties. In our continuous efforts to discover chemical ingredients of the bark of *C. japonica*, three sesquarterpenes (Chen et al., 2010; Chang et al., 2017c) and thirteen abietane-type diterpenoids (Chang et al., 2016, 2017a; Chang et al., 2017b, 2018) have been reported. Herein, we describe the isolation, structural elucidation, and enzyme inhibitory activity of **1–5** (Fig. 1).

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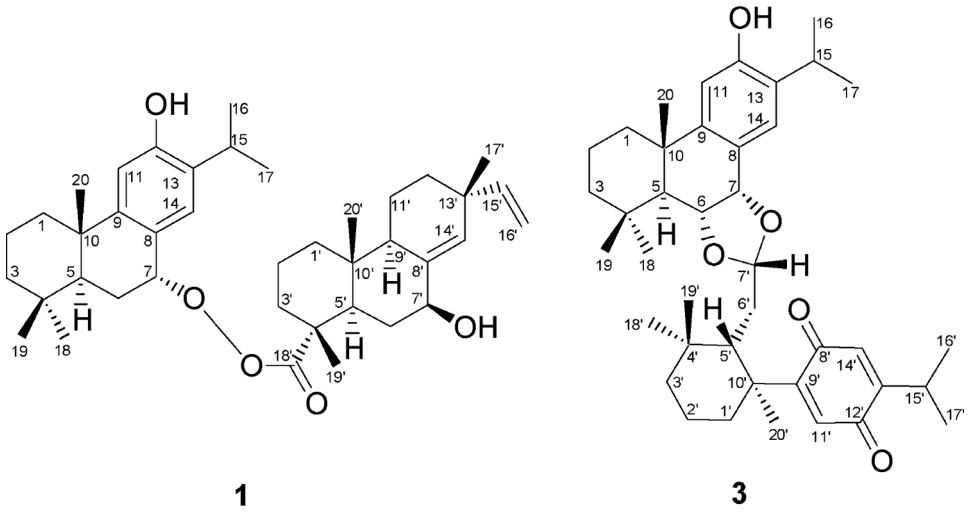

Fig. 1. Structures of compounds **1**and **3**.

2. Results and discussion

The EtOAc soluble portion partitioned from methanol extract of the bark of *C. japonica* was subjected to repeated chromatography on silica gel followed by semipreparative NP-HPLC. Two new dimeric abietane-type diterpenoids, 12-hydroxyabieta-8,11,13-trien-7α-yl 7β-hydroxyisopimara-8(14),15-diene-18-peroxoate (**1**) and 6α,7α-*O*-(7,8-secoabieta-9(11),13-diene-8,12-dioxo-7-ylidene)ferruginol (**3**), together with three known dimeric abietane abietane-type diterpenoids, sugikurogin J (**2**) (Yoshikawa et al., 2006), sugikurojin B (**4**) (Arihara et al., 2004), and formosaninol (**5**) (Kuo and Yu, 1996) were obtained (Fig. 1 and S19).

The HR-EI-MS of **1** gave a molecular ion at *m/z* 618.4280, consistent with the molecular formula of $C_{40}H_{58}O_5$, implying twelve degrees of unsaturation. The UV maxima (227 and 281 nm) and IR absorptions (3429, 1646, and 1467 cm^{-1}) of **1** indicated the presence of hydroxyl and aromatic functionalities. The IR absorptions for a peroxy ester carbonyl group (1719, 1228, 1169 cm^{-1}) were also observed. The EI-MS fragmental ions at *m/z* 300 $[C_{20}H_{28}O_2]^+$ and 318 $[C_{20}H_{30}O_3]^+$ (Fig. 2) indicated that **1** is composed of constituent monomer-1 and constituent monomer-2. The ^1H and NMR data of **1** (Table 1) exhibited signals for constituent monomer-1: three tertiary methyl groups [δ_H 0.89, 0.94, and 1.30 (each 3H, s, Me-18, Me-19, and Me-20], an isopropyl group attached to a phenyl group [δ_H 1.17 (3H × 2, d, J = 7.0 Hz, Me-16 and Me-17) and 3.02 (1H, sept, J = 7.0 Hz, H-15)], two para aromatic protons [δ_H 6.64 (1H, s, H-14) and 6.72 (1H, s, H-11)], and a typical dehydroabeitane type diterpene downshifted H_β-1 signal [δ_H 1.93 (1H, br d, J = 12.0 Hz). The ^1H NMR signal of a carbinol proton [δ_H 6.27 (1H, t, J = 3.0 Hz)] was assigned to H-7, showing the ^1H-^1H COSY correlations with the H_2-6 methylene protons [δ_H 2.05 (1H, dt, J = 14.5, 3.0 Hz), 2.25 (1H, td, J = 14.5, 3.0 Hz)] and HMBC correlations with C-5 (δ_C 43.5) and C-8 (δ_C 146.0). The lower field signal of H-7 appeared at δ_H 6.27 with a small coupling constant (J = 3.0 Hz) in comparison with that of 7-hydroxyferruginol analogues (Dellar et al., 1996; Arihara et al., 2004; Kuo et al., 2002), and showed the NOESY correlations with H-6$_\beta$ (δ_H 2.25) and H-14 (δ_H 6.64), which suggested that the peroxyester group was attached on C-7 in α-axial orientation. The above spectral data of **1** is very similar to that of compound **2** (Arihara et al., 2004) (Fig. 1). Thus, the structure of constituent monomer-1 was related to 7α-hydroxyferruginol. The ^1H

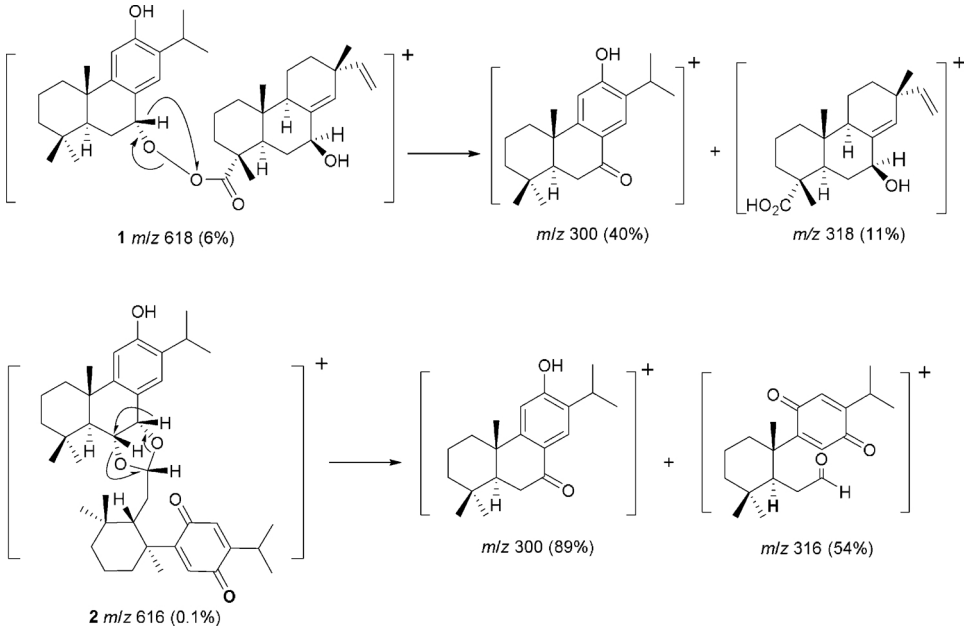

Fig. 2. Some key EI-Mass fragmentations of **1** and **3**.

Table 1
^{1}H NMR data for compounds **1** and **3**. ($CDCl_3$, δin ppm, J in Hz, 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR).

	1		3	
No.	δ_C	δ_H	δ_C	δ_H
1	40.6	1.80[a] α, 1.93 br d (12.0)[b] β	38.4	1.39[a] α, 2.11 br d (11.5) β
2	18.6	1.59[a], 1.69 m	19.0	1.57[a], 1.64 m
3	42.8	1.28[a] α, 1.49[a] β	43.0	1.26[a], 1.40[a]
4	34.5		33.1	
5	43.5	1.63[a]	45.7	2.11 d (11.5)
6	32.9	2.05 dt (14.5, 3.0) α, 2.25 td (14.5, 3.0) β	78.7	4.17 br d (11.5)
7	94.0	6.27 t (3.0)	73.4	4.55 br s
8	146.0		124.3	
9	141.8		147.9	
10	40.9		40.0	
11	113.2	6.72 s	110.2	6.56 s
12	148.8		153.5	
13	132.2		132.6	
14	121.5	6.64 s	129.1	7.00 s
15	26.7	3.02 sept (7.0)	27.1	3.11 sept (7.0)
16	22.3	1.17 d (7.0)	22.4	1.29 d (7.0)
17	22.4	1.17 d (7.0)	22.2	1.30 d (7.0)
18	33.8	0.89 s	35.5	1.11 s
19	23.1	0.94 s	22.0	1.01 s
20	21.3	1.30 s	26.6	1.07 s
12-OH				4.90 s
1’	37.7	0.88 m, 1.64 m	36.8	1.21 β, 2.37 m α,
2’	17.9	1.41[a]	18.7	1.50[a], 1.56[a]
3’	36.7	1.30[a], 1.37[a]	40.8	1.43[a], 1.47[a]
4’	46.9		34.0	
5’	45.4	1.66[a]	42.2	2.43 dd (7.0, 4.5)
6’	34.8	1.29[a], 1.55[a]	31.1	1.35[a], 1.60[a]
7’	71.8	3.84 dd (9.5, 4.0)	103.2	5.08 br s
8’	138.5		187.7	
9’	48.6	1.54[a]	155.8	
10’	37.9		43.2	
11’	18.4	1.49[a] a, 1.52[a] b	133.8	6.57 s
12’	33.9	1.34[a], 1.43[a]	188.2	
13’	36.9		151.9	
14’	125.8	5.59 s	133.1	6.19 s
15’	148.4	5.77 dd (17.5, 11.0)	26.0	2.92 sept (7.0)
16’	110.5	4.90 d (11.0), 4.91 d (17.5)	21.4	1.03 d (7.0)
17’	26.2	1.02 s	21.0	1.05 d (7.0)
18’	176.1		22.8	0.87 s
19’	16.9	0.99 s	33.8	0.90 s
20’	15.2	0.74 s	20.2	1.19 s

a) Overlapping with other signals. b) Coupling constants are presented in Hz.

and NMR data of **1** (Table 1) also exhibited another set of signals for constituent monomer-2: three tertiary methyl groups [δ_H 0.74, 0.99, and 1.02 (each 3H, s, Me-20′, Me-19′, and Me-17′], a vinyl group [δ_H 4.90 (1H, d, J = 11.0 Hz, H-16$_a$’), 4.91 (1H, d, J = 17.5 Hz, H-16$_b$’), and 5.77 (1H, dd, J = 17.5, 11.0 Hz, H-15′) ; δ_C 110.5 (C-16′), 148.4 (C-15′)], a trisubstituted double bond [δ_H 5.59 (1H, s, H-14′) ; δ_C 138.5 (C-8′), 125.8 (C-14′)], and a peroxy ester carbonyl carbon [δ_C 176.1 (C-18′)]. In addition, the ^{1}H NMR signal for a carbinol proton [δ_H 3.84 (1H, dd, J = 9.5, 4.0 Hz) was assigned to H-7′, showing the H^1-H^1 COSY correlations with the H_2-6′ methylene protons [δ_H 1.55 (1H, m, H-6$_a$’) and 1.29 (1H, m, H-6$_b$’)] and HMBC correlations with C-6′ (δ_C 34.8). The ^{1}H NMR signal of H-7′ exhibited a doublet of doublet splitting pattern with a large constant coupling constant (J = 9.5 Hz) and also showed the NOESY correlation with H-5′ (δ_H 1.66), H-6′$_\alpha$ (δ_H 1.55) and H-9′ (δ_H 1.54), suggesting that the hydroxyl group was attached on C-7′ (δ_C 71.8) in β-equatorial orientation (Fig. 3). The NMR spectroscopic data of constituent monomer-2 is very similar to that of methyl 7β-hydroxyisopimara-8(14),15-dien-18-oate (Taran and Delmond, 1988; Mihashi et al., 1969). Thus, the structure of constituent monomer-2 was related to 7β-hydroxyisopimara-8(14),15-dien-18-oic acid. The linkage of constituent monomer-1 and constituent monomer-2 through a peroxy ester was confirmed by the downfield shift of H-7 [δ_H 6.27 (1H, t, J = 3.0 Hz; δ_C 94.0)] and total five oxygen atoms existing in the molecular formula of **1**, $C_{40}H_{58}O_5$. Comparison of the physical data of compound **1** with those of compound **2** revealed that the only difference between two compounds was a carbinol group at C-7′ in **1**, instead of a carbonyl group in **2**. From the above evidences, compound **1** was thus elucidated as 12-hydroxyabieta-8,11,13-trien-7α-yl 7β-hydroxyisopimara-8(14),15-diene-18-peroxoate, for which the trivial name japonicinol A is given.

The HR-EI-MS of **3** gave a molecular ion at m/z 616.4137, establishing the molecular formula of **3** as $C_{40}H_{56}O_5$, with thirteen degrees of unsaturation. IR absorptions (3476, 1610, and 1520 cm^{-1}) of **3** indicated the presence of the hydroxyl and aromatic functionalities. The EI-MS fragmental ions of **3** at m/z 300 $[C_{20}H_{28}O_2]^+$ and 316 $[C_{20}H_{28}O_3]^+$ (Fig. 2) hinted that **3** was also a dimeric diterpenoid. The ^{1}H and NMR data of **3** (Table 1) exhibited signals for constituent monomer-1: three tertiary methyl groups [δ_H 1.01, 1.07, and 1.11 (each 3H, s, Me-19, Me-20, and Me-18], an isopropyl group attached to a phenyl group [δ_H 1.29 (3H, d, J = 7.0 Hz), 1.30 (3H, d, J = 7.0 Hz), and 3.11 (1H, sept, J = 7.0 Hz)], two para aromatic protons [δ_H 6.56 (1H, s) and 7.00 (1H, s)], and a typical dehydroabietane type downshifted H$_\beta$-1 signal [δ_H 2.11 (1H, br d, J = 11.5 Hz)]. The ^{1}H NMR signals of three consecutive protons including one aliphatic methine [δ_H 2.11 (1H, d, J = 11.5 Hz, H-5)] and two carbinol protons [δ_H 4.17 (1H, br d, J = 11.5 Hz, H-6) and 4.55 (1H, br s, H-7)] were assigned to H-5, H-6 and H-7, respectively, on the basis of the H^1-H^1 COSY correlations between H-5/H-6 and H-6/H-7 and the HMBC correlations between H-5/C-9, H-6/C-10, and H-7/C-14. A 1,3-dioxolidene group located at C-6 and C-7 were in α-equatorial and α-axial orientations, respectively, judging from the splitting pattern of H-6 and H-7, a broad doublet signal with a constant coupling constant of 11.5 Hz for H-6 and a broad singlet signal for H-7 (Hirasawa et al., 2007). Thus, the gross structure of constituent monomer-1 was related to abieta-8,11,13-triene-6α,7α,12-triol. IR absorptions (1639, and 1620 cm^{-1}), together with the ^{1}H and ^{13}C NMR signals of **3** for two conjugated ketone carbonyl groups at δ_C 187.7 (s) and 188.2 (s) and two trisubstituted double bonds at δ_H 6.19 (s) and 6.57 (s) and δ_C133.1 (d), 133.8 (d), 151.9 (s), and 155.8 (s) hinted the presence of a di-substituted *p*-quinone moiety (Yoshikawa et al., 2006). Moreover, an isopropyl group was located at C-13′, which was confirmed by the HMBC correlations between H-15′/C-12′ and C-14′. The H^1-H^1 COSY correlations between H-1′/H-2′ and H-2′/H-3′ suggested the presence of the portion of the consecutive protons H-1′–3′. The HMBC correlations between H-5′/C-4′, C-6′, C-9′, C-10′, C-18′, and C-19′, Me-18′/C-3′, C-4′ and C-5′, Me-19′/C-3′, C-4′ and C-5′, and Me-20′/C-1′, C-5′, C-9′, and C-10′ further helped to construct the structure of A ring shown in Fig. 3. The Me-18′, 19′ and 20′ were located at C-4′,4′, and 10′, respectively. The dioxoethylidene and di-substituted *p*-quinone moiety were attached on C-5′, and C-10′, respectively, supporting by the HMBC correlations between H’-6/C-4′, C-5′, and C-10′ and H’-11/C-10′. The Me-20′ (δ_H 1.19) showed the NOESY correlation with Me-18′ (δ_H 0.87) and H-6′, which confirmed the Me-20′, Me-18′ and H-6′ being in same phase. From the above information, the gross structure of constituent monomer-2 was related to 7,8-secoabieta-9(11),13-dien-8,12-dioxo-7-ol. Compound **3** exhibited thirteen degrees of unsaturation. The monomer-1 has a total of 6 degrees of unsaturation, while there are 6 degrees of unsaturation in monomer-2. Then, it must have one additional degree of unsaturation deriving from the 1,3-dioxolane functionality (Fig. 3), which was further supported by the EI-MS fragmental ions of compound **3** at m/z 300 $[C_{20}H_{28}O_2]^+$ and 316 $[C_{20}H_{28}O_3]^+$. The above evidences suggested that compound **3** was a combination of a sugiol and a 7,8-secoabieta-9(11),13-dien-8,12-dioxo-7-al. Furthermore, the HMBC correlations between H’-7 and C-7 confirmed the 7′-*O*-6 and 7′-*O*-7 linkages of constituent monomer-1 and constituent monomer-2 through an acetal functionality. Thus, the structure of **3** was identified as 6α,7α-*O*-(7,8-secoabieta-9,13-diene-8,12-dioxo-7-ylidene)ferruginol, namely japonicinol B.

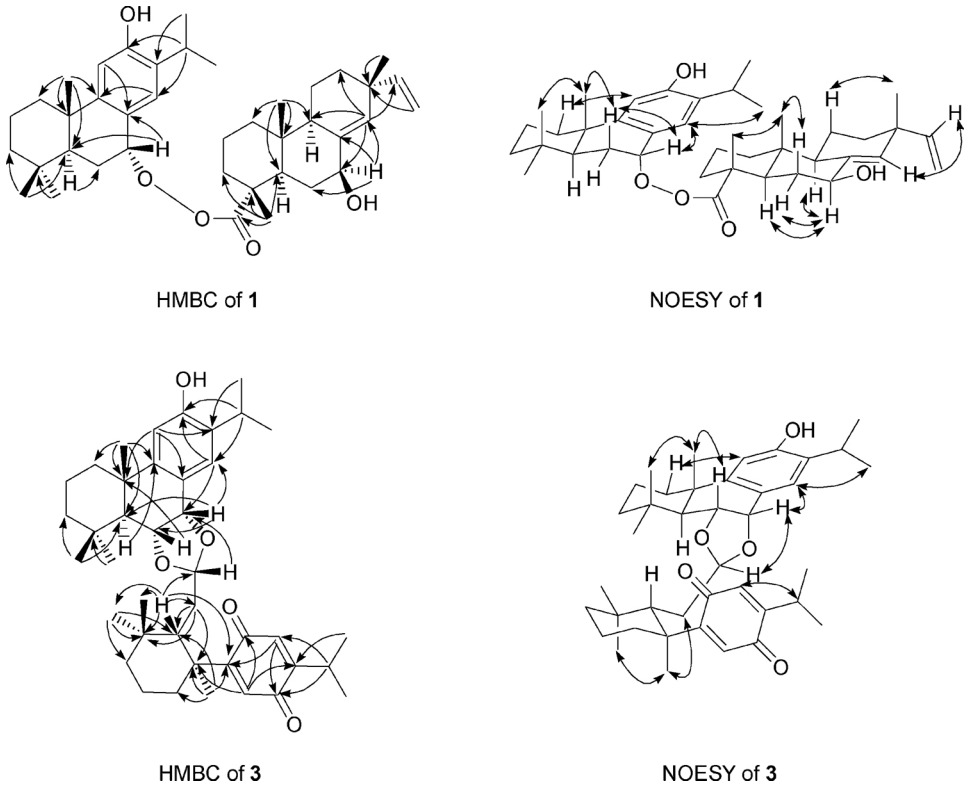

Fig. 3. Selected HMBC and NOE correlations of **1** and **3**.

Xanthine oxidase is a key enzyme in purine metabolic pathway, catalyzing the oxidation of oxypurines to produce uric acid and plays an important role in causing gout (Cos et al., 1998). Additionally, angiotensin-converting enzyme (ACE) also plays a key role in the control of blood pressure due to its action in the formation of angiotensin II, a potent vasoconstrictor, and in the degradation of bradykinin, a vasodilator. (Soffer, 1976). Several abieta-8,11,13-triene diterpene derivatives have been reported as potential inhibitors of xanthine oxidase (Lin et al., 2010) or angiotensin-converting enzyme (Liu et al., 2014). Therefore, the inhibitory activities of the above two enzymes of compounds **1**-**5** were evaluated (Chen et al., 2009; Cushman and Cheung, 1971). At the concentration of 50 μM, compounds **1**–**5** inhibited xanthine oxidase activity by 31.3, 23.4, 18.7, 17.3 and 24.4%, respectively, whereas the positive control, quercetin, inhibited xanthine oxidase activity by 35.1%. Additionally, compound **4** also inhibited angiotensin-converting enzyme activity by 19.2%, while compounds **1**, **2**, **3**, and **5** were inactive. The positive control, captopril, inhibited angiotensin-converting enzyme activity by 65.1% at a low concentration of 10 nM.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured in MeOH using a Jasco-DIP-180 polarimeter. UV and IR spectra were acquired on a Shimadzu UV-1601PC and a Perkin-Elmer spectrophotometer, respectively. ^{1}H and ^{13}C NMR and 2D NMR spectra were recorded in $CDCl_3$ on a Varian-Unity-Plus-400 spectrometer with residual solvent signals as internal reference. EI-MS and HR-EI-MS were acquired on a Jeol-JMS-HX300 mass spectrometer. Column chromatography (CC) was carried out on Silica gel (230–400 mesh; Merck & Co., Inc.). Thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (silica gel 60 F_{254}; Merck & Co., Inc.). HPLC was performed using a normal phase column (Purospher STAR Si, 5 μm, 250 × 10 mm; Merck & Co., Inc.) and a reversed phase column (Hypersil Gold C18, 5 μm, 250 cm × 4.6 mm; Thermo Scientific) on a LDC Analytical-III system.

3.2. Plant material

The bark of *C. japonica* D. Don was collected in Sitou, Taiwan in June 2000. The plant material was identified by Dr. Yen-Hsueh Tseng, Department of Forestry, National Chung-Hsing University (NCHU). A voucher specimen (TCF13443) has been deposited at the Herbarium of the Department of Forestry, NCHU, Taiwan.

3.3. Extraction and isolation

The air-dried bark of *C. japonica* (16.0 kg) was extracted by immersing in MeOH (100 L × 3) at room temperature for 7 days each time, and the MeOH extracts was concentrated under reduced pressure. A brown crude residue (480 g) obtained from the combined MeOH extracts, was suspended in H_2O (1 L), and then partitioned between H_2O and EtOAc (1 L) for three times. The EtOAc soluble fraction (430 g) was subjected to passage over a silica gel (4.0 kg) column, eluted with *n*-hexane–EtOAc and EtOAc–MeOH mixtures to give 11 fractions, fr. 1 (2.6 g), 2 (29.4 g), 3 (47.8 g), 4 (92.4 g), 5 (21.6 g), 6 (18.1 g), 7 (22.5 g), 8 (35.8 g), 9 (19.2 g), 10 (44.2 g), and 11 (72.2 g). Fr. 2 from hexane/EtOAc (19:1) elution (47.8 g) was further purified through a silica gel column (5 × 45 cm), eluted with hexane/CH_2Cl_2 (1:0 – 0:1) to obtain eight fractions, 2A – 2H. Further purification of subfraction 2 F by HPLC gave **4** (1.4 mg) and **5** (1.3 mg) using hexane/EtOAc (19:1). Fr. 3 from hexane/EtOAc (9:1) elution (47.8 g) was further purified through a silica gel column (7 × 60 cm), eluted with hexane/CH_2Cl_2 (1:0 – 0:1) to obtain nine fractions, 3A – 3I. Further purification of subfraction 3H by HPLC gave **1** (1.1 mg) and **2** (*n*-hexane–EtOAc (4:1) elution (92.4 g), was further purified through a silica gel column (7 × 60 cm), eluted with a gradient mixture of CH_2Cl_2–EtOAc (100:1 to 0:1) to obtain sixteen fractions, 4A - 4 P. Further purification of subfraction 4 L by HPLC afforded **3** (4.2 mg) using *n*-hexane–EtOAc (4:1).

*3.3.1. 12-hydroxyabieta-8,11,13-trien-7α-yl 7β-hydroxyisopimara-8(14), 15-diene-18-peroxoate (**1**)*

Gum; $[\alpha]_D^{25}$ =–6.2∘ (*c* 0.8, $CHCl_3$)ν_{max} 3429, 1719, 1646, 1467, 1414, 1381, 1228, 1169, 990, 731 cm^{-1}; UV (MeOH) λ_{max} (log) 227 (4.65), 281 (3.71) nm; ^1H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 618 (1) $[M]^+$, 318 (11), 300 (40), 285 (20), 203 (22), 189 (100). HR-EI-MS $[M]^+$ *m/z* 618.4280 (calcd for $C_{40}H_{58}O_5$ 618.4286).

*3.3.2. 6α,7α-O-(7,8-secoabieta-9(11),13-diene-8,12-dioxo-7-ylidene) ferruginol (**3**)*

Gum; $[\alpha]_D^{25}$ =–21.6∘ (*c* 0.8, $CHCl_3$)ν_{max} 3476, 1639, 1620, 1610, 1520, 1500, 1460, 1374, 1321, 1228, 1169, 1056, 910, 857, 738 cm^{-1}; UV (MeOH) λ_{max} (log) 225 (4.45), 234 (4.51), 260 (4.63) nm; ^1H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 616 (0.1) $[M]^+$, 316 (54), 300 (89), 285 (63), 272 (74), 257 (53), 243 (39), 229 (87), 215 (59), 203 (100), 189 (47), 179 (45). HR-EI-MS $[M]^+$ *m/z* 616.4137 (calcd for $C_{40}H_{56}O_5$ 616.4130).

3.4. Xanthine oxidase inhibition assay

The XO inhibition assay was performed according to the method of Chen et al. with some modifications (Chen et al., 2009). The assay mixture consists of 35 μL of 0.1 mM phosphate buffer (pH = 7.5), 30 μL of enzyme solution (0.01 units/ml in 0.1 mM phosphate buffer, pH = 7.5), and 20 μL of the sample solution (final concentration was 50 μM). The solution was mixed thoroughly by vortexing and pre-incubated for 15 min at, and then 60 μL of substrate solution (150 mM xanthine in the same buffer) was added. The reaction mixture was incubated for further 30 min at The reaction was stopped by adding 50 μL of 2 N HCl, and the absorbance was measured at 290 nm. The percentage activity of xanthine oxidase was calculated according to the following equation: XO Inhibition (%) = (1-B/A) × 100, where A and B are the activities of the enzyme without and with test sample. Quercetin, a known inhibitor of xanthine oxidase, was used as a positive control, whereas negative control was performed without any inhibitor.

3.5. Angiotensin-converting enzyme (ACE) inhibition assay

The ACEI activity assay was performed according to the method of Cushman with some modifications (Cushman and Cheung, 1971). In brief, 30 μL 2.5 mM Hippuryl-L-histidyl-L-leucine (HHL) was used as a substrate and added by 10 μL of testing sample at a certain concentration in 200 mM borate buffer containing 300 mM NaCl (adjusted to pH 8.3). The solution was mixed thoroughly by vortexing and preincubated for 5 min at 37 ˚C, and then 20 μL of ACE (0.05 mU/μL) in 200 mM borate buffer was added. The reaction was incubated statically for 30 min at 37 ˚C and then incubated thermostatically using shaker incubation (200 rpm) for 30 min at 37 ˚C. The reaction was stopped by adding 30 μL of 2 N HCl. The HHL and ACE's hydrolyzed product, hippuric acid (HA), were was determined by an HPLC method using a Hypersil GOLD C-18 analytical column (250 × 4.6 mm, 5 μm). The reaction mixtures were separated using an isocratic elution of 23% ACN containing 0.1% TFA at a flow rate of 1 mL/min for 15 min. HA was monitored for absorbance at 228 nm. The ACE inhibition (%) was determined according to the following equation: ACE Inhibition (%) = [1 − ($\Delta A_{Inhibitor}/\Delta A_{Blank}$)] × 100, where $\Delta A_{Inhibitor}$ and ΔA_{Blank} were the peak areas of HA in testing and blank samples, respectively. Captopril, a known inhibitor of ACE, was used as a positive control, whereas negative control was performed without any inhibitor.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2019.08.001.

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