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Two new dimeric abietanoid peroxides with xanthine oxidase and ACE inhibitory activities from the bark of *Cryptomeria japonica*

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ABSTRACT

Two new dimeric abietane-type diterpenoids, 12-hydroxyabieta-8,11,13-trien-7-α-yl 7-oxoabieta-8,11,13-trien-12-yl peroxide (trivial name japonicinol C, **1**) and 12-hydroxyabieta-8,11,13-trien-7-α-yl 7-oxoabieta-5,8,11,13-tetraen-12-yl peroxide (trivial name japonicinol D, **3**), together with two known dimeric abietane-type diterpenoids, obtusanol A (**2**) and 7-α-(2-butoxyethoxy)-12-hydroxyabieta-6-yl 6,7-dehydroabieta-8,11,13-trien-12-yl ether (**4**), were isolated from the methanol extract of the bark of *Cryptomeria japonica*. Their structures were established by mean of spectroscopic analysis and comparison of NMR data with those of known analogues. At a concentration of 50 μM, compounds **1**–**4** inhibited xanthine oxidase activity by 29.8, 39.3, 27.1, and 14.7 %, respectively. In addition, compounds **1** and **4** also showed 13.9 and 20.5 % inhibition toward angiotensin-converting enzyme (ACE), respectively.

1. Introduction

Cryptomeria japonica D. Don, belonging to monotypic genus in the Cupressaceae, is endemic to Japan, known as sugi (Japanese cedar) in Japanese (Gan, 1958). It is a massive evergreen coniferous tree, growing up to in height and has been cultivated in large quantities in Taiwan since 1906. Its wood exhibited the aromatic, redidsh-pink in color, soft, lightweight but strong, and waterproof properties and is used as a construction material. Previous investigations revealed the presence of 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 et al., 1992; Shieh et al., 1981; Shimizu et al., 1988; Su et al., 1996; Morisawa et al., 2002; Yoshikawa et al., 2006) in the leaves, heartwood, and barks of *C. japonica*. The crude extracts and secondary metabolites from this species demonstrated a broad spectrum of biological activities including 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. A continuous search for bioactive compounds from the bark of *C. japonica*, we have already reported the isolation of three sesquarterpenes (Chen et al.,

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2010; Chang, et al. 2017), thirteen abietane-type diterpenoids (Chang et al., 2016; Chang, et al. 2017, 2017; Chang et al., 2018) and five dimeric abietane-type diterpenoids (Chang et al., 2019). Herein, the isolation and structure elucidation of two new and two known dimeric abietane-type diterpenoids, and their enzyme inhibitory activity are described.

2. Results and discussion

The MeOH extract of the bark of *C. japonica* was suspended in H_2O and partitioned with EtOAc and n-BuOH, successively. The combined EtOAc-soluble fraction was purified by repeated silica gel column chromatography and normal phase semipreparative HPLC. Two new dimeric abietane-type diterpenoids, 12-hydroxyabieta-8,11,13-trien-7-α-yl 7-oxoabieta-8,11,13-trien-12-yl peroxide (**1**) and 12-hydroxyabieta-8,11,13-trien-7-α-yl 7-oxoabieta-5,8,11,13-tetraen-12-yl peroxide (**3**), together with two known dimeric abietane-type diterpenoids, obtusanol A (**2**) (Kuo et al., 2002) and 7-α-(2-butoxyethoxy)-12-hydroxyabieta-6-yl 6,7-dehydroabieta-8,11,13-trien-12-yl ether (**4**) (Hsieh et al., 2005) were obtained (Fig. 1).

The HR-EI-MS of **1** gave a molecular ion at *m/z* 600.4188, establishing the molecular formula of **1** as $C_{40}H_{56}O_4$, with thirteen degrees of unsaturation. The base peak of EI-MS at *m/z* 300 $[C_{20}H_{28}O_2]^+$, as well as forty carbon signals found in the ^{13}C NMR spectrum hinted that **1** was a dimeric diterpenoid (Fig. 2). The UV maxima at 223 and 278 nm suggested the presence of a benzoyl functionality. The absorption bands for hydroxyl (3390 cm^{-1}), conjugated carbonyl (1666 cm^{-1}) and aromatic (3051, 1593, and 1487 cm^{-1}) groups were observed in its IR spectrum. The ^1H and ^{13}C NMR data of **1** (Table 1) exhibited signals for constituent upper monomer-1: three tertiary methyl groups [δ_H 0.96, 0.98, and 1.34 (each 3H, s, Me-18, Me-19, and Me-20], an isopropyl group attached to a phenyl group [δ_H 0.68 (3H, d, J =7.0 Hz, Me-16), 0.97 (3H, d, J =7.0 Hz, Me-17) and 2.90 (1H, sept, J =7.0 Hz, H-15)], two *para* aromatic protons [δ_H 6.28 (1H, s, H-14) and 6.74 (1H, s, H-11)], and a typical downshifted H_β-1 signal of a dehydroabietane diterpene [δ_H 1.91 (1H, br d, J =12.4 Hz)] (Kuo and Yu, 1996). A downshifted benzyl proton connected with a peroxyl group [δ_H 5.97 (1H, br s)], instead of a hydroxyl group (Arihara et al., 2004; Dellar et al., 1996; Kuo et al., 2002), was assigned as H-7, showing the H^1-H^1 COSY correlations to the two methylene protons of H-6 [δ_H 2.24 (1H, br d, J =14.5 Hz, H_α-6), δ_H 2.34 (1H, br t, J =14.5 Hz, H_β-6)] and HMBC correlations with C-5 (δ_C 43.5) and C-8 (δ_C 144.7) (Fig. 3). Additionally, the proton signal of H-7 was a broad triplet peak with a small constant coupling constant, J =3.1 Hz, and showed the NOESY correlations to both H-6_α and H-6_β, suggesting that the peroxyl group was attached on C-7 in α-axial orientation (Kuo et al., 2002; Yoshikawa et al., 2006) (Fig. 3). Those data proved that the structure of constituent upper monomer-1 was related to 7α-peroxyferruginol. The ^1H and ^{13}C NMR data of **1** (Table 1) also exhibited another set of signals for constituent lower monomer-2: three tertiary methyl groups [δ_H 0.90, 0.99, and 1.37 (each 3H, s, Me-18′, Me-19′, and Me-20′], an isopropyl group attached to a phenyl group [δ_H 0.76 (3H, d, J =7.0 Hz, Me-16′), 0.98 (3H, d, J =7.0 Hz, Me-17′) and 2.80 (1H, sept, J =7.0 Hz, H-15′)], two *para* aromatic protons [δ_H 7.14 (1H, s, H-11′) and 7.78 (1H, s, H-14′)], one set of ABX coupling system neighboring to the carbonyl group [δ_H 1.85 (1H, dd, J = 13.5, 4.5 Hz, H-5′), 2.61 (1H, dd, J = 14.5, 13.5 Hz, H_β-6′), and 2.69 (1H, dd, J = 14.5, 4.5 Hz, H_α-6)], and a typical downshifted H_β-1 signal of a dehydroabietane diterpene [δ_H 1.91 (1H, br d, J =12.4 Hz)]. The above NMR spectroscopic data of constituent lower monomer-2 is very similar to that of sugiol (Yang et al., 2016; Zhao et al., 2017), suggesting constituent lower monomer-2 was a sugiol-derivative. Thus, the gross structure of **1** is composed of 7α-peroxyferruginol and sugiol. The C-7 and C-12′ linkage of the two monomers in **1** was through a peroxyl functionality, judged by its molecular formula containing four oxygen atoms and the downfield shift of H-7 (δ_H 5.97) in constituent monomer-1, comparing to that of the 7α-hydroxyferruginol analogues (Arihara et al., 2004; Dellar et al., 1996). The conformation of compound **1** is very interesting. The phenol functionality of upper 7α-peroxyferruginol derivative is an electron-rich site, while the benzoyl group of lower sugiol derivative is electron-poor site. The two aromatic functionalities will attract each other to form the most stable conformer as shown in Fig. 3. Therefore, the chemical shifts of H-14, H-15, H-16, H-17, H-14′, H-15′, H-16′ and H-17′ upshifted to the high field region due to receiving the anisotropic effect of the opposite phenyl group. Meanwhile, the NOESY correlation between H-7 and H-11′ was also found. The above evidences further confirmed the peroxide linkage between C-7 and C-12′ was in α-axial orientation (Fig. 3). Complete ^1H and ^{13}C NMR chemical shifts were established by ^1H- ^1H COSY, HMQC, HMBC, and NOESY spectra. Based on these above evidences, compound **1** was elucidated as 12-hydroxyabieta-8,11,13-trien-7-yl 7-oxoabieta-8,11,13-trien-12-yl peroxide, namely japonicinol C.

The HR-EI-MS of **3** showed a molecular ion at *m/z* 598.4029, which corresponded to the molecular formula, $C_{40}H_{54}O_4$, indicating fourteen degrees of unsaturation. The EI-MS fragmental ions of **3** at *m/z* 300 $[C_{20}H_{28}O_2]^+$ and 298 $[C_{20}H_{26}O_3]^+$ (Fig. 2) indicated that **3** was also a dimeric diterpenoid. The IR spectrum indicated the presence of hydroxyl (3363 cm^{-1}), conjugated carbonyl (1646 cm^{-1}) and aromatic (3040, 1593, and 1493 cm^{-1}) groups. The UV maxima at 218, 244, 289 and 309 nm indicated a benzoyl functionality. Comparison of ^1H and ^{13}C NMR

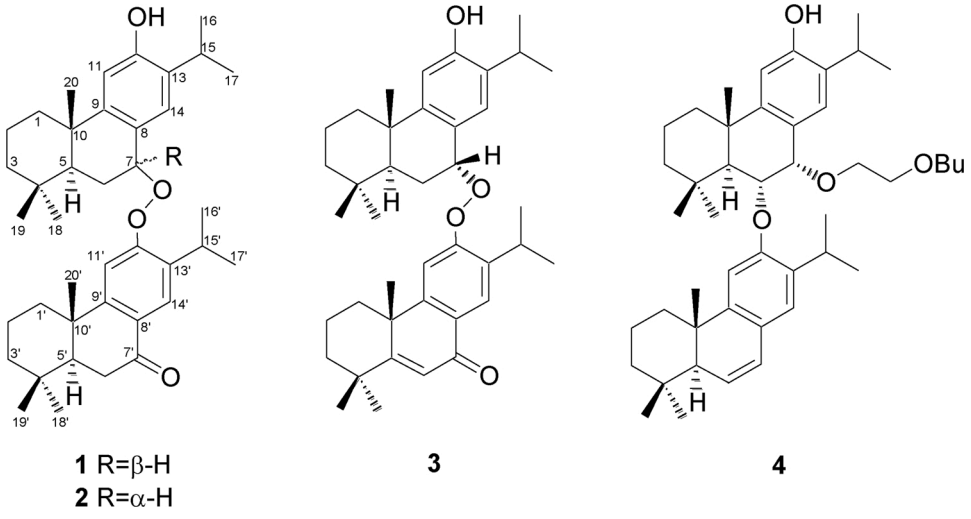

Fig. 1. Structures of compounds **1**–**4**.

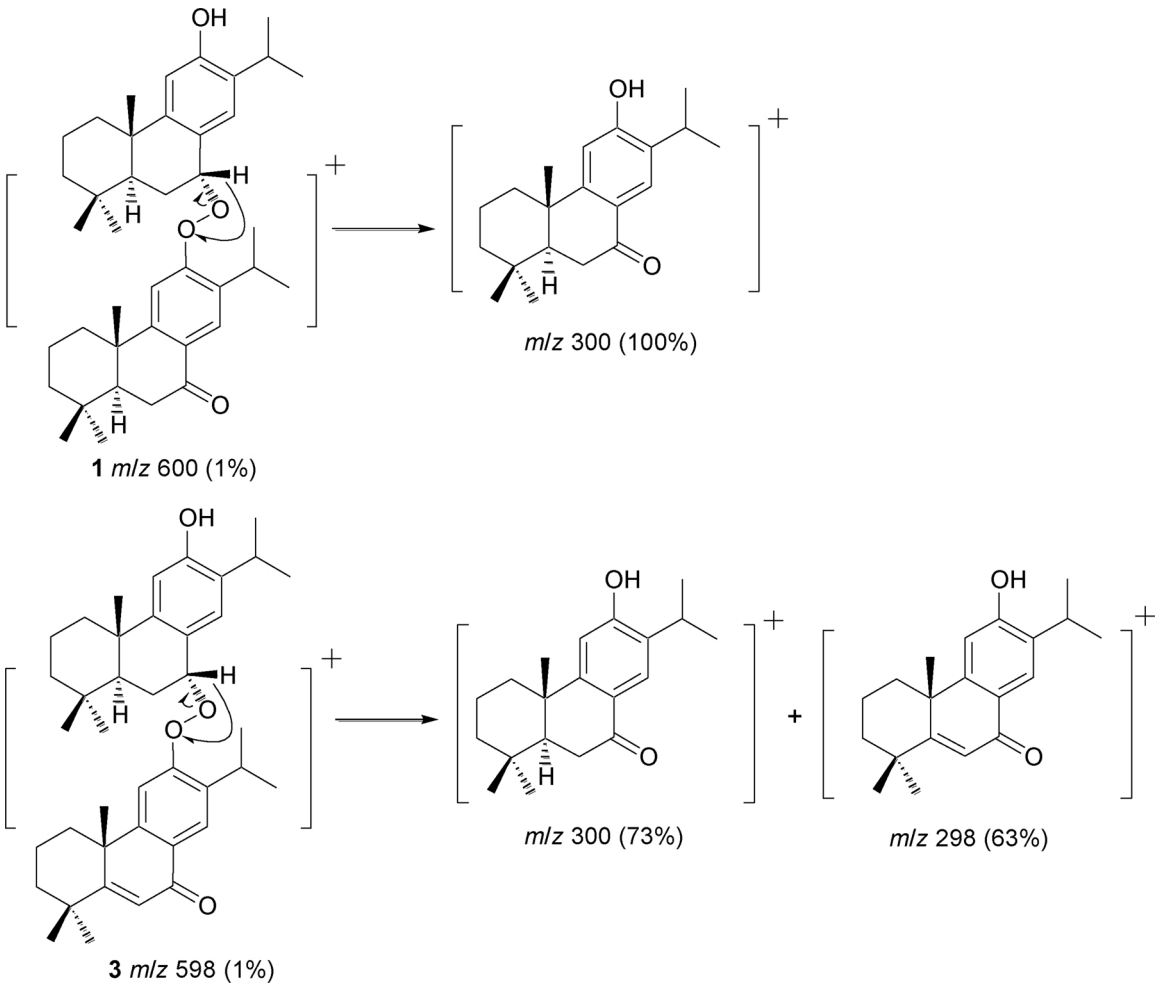

Fig. 2. Some key EI-Mass fragmentations of **1** and **3**.

data of **1** and **3** (Table 1) showed that the signals of constituent upper monomer-1 of **3** were almost the same as those of **1**, thus, the structure of constituent upper monomer-1 was related to 7α-peroxyferruginol. The ^{1}H and ^{13}C NMR signals for constituent lower monomer-2 as follows: three tertiary methyl groups [δ_H 1.25, 1.36, and 1.68 (each 3H, s, Me-18′, Me-19′, and Me-20′], an isopropyl group attached to a phenyl group [δ_H 0.76 (3H, d, J =7.0 Hz, Me-16′), 1.01 (3H, d, J =7.0 Hz, Me-17′) and 2.81 (1H, sept, J =7.0 Hz, H-15′)], two *para* aromatic protons [δ_H 7.29 (1H, s, H-11′) and 7.87 (1H, s, H-14′)], one trisubstituted double bond [δ_H 6.47 (1H, s, H-6′) ; δ_C 124.5 (d, C-6′), 173.0 (s, C-5′)], and a typical downshifted H_β-1 signal of a dehydroabietane diterpene [δ_H 2.23 (1H, br d, J =13.5 Hz). The NMR spectroscopic data of constituent lower monomer-2 showed a close structural resemblance to that of 5,6-dehydrosugiol (Yang et al., 2016). Thus, the structure of constituent lower monomer-2 was tentatively determined as 5,6-dehydrosugiol-related abietane. H-7 of constituent upper monomer-1 showed a downfield proton signal at δ_H 5.98, comparing to that of the 7-hydroxyferruginol analogues (Arihara et al., 2004; Dellar et al., 1996; Yoshikawa et al., 2006). and exhibited the NOESY correlation to H-11′ (δ_H 7.29) (Fig. 3) suggested the connectivity of two monomers between C-7 and C-12′ through a peroxyl functionality. The most stable conformation of compound **3** is the same as that of compound **1** (Fig. 3). Thus, the structure of **3** was identified as 12-hydroxyabieta-8,11,13-trien-7-yl 7-oxoabieta-5,8,11,13-tetraen-12-yl peroxide, namely japonicinol D.

Xanthine oxidase, an olybdoflavoprotein, is a critical enzyme that catalyzes hypoxanthine to xanthine then to uric acid in purine metabolic pathway. Hyperuricemia caused by the high uric acid level in the blood leads to gout and cardiovascular diseases (Cos et al., 1998). Additionally, ACE is defined as dipeptidyl carboxypeptidase that plays a key physiological role in in controlling and regulating blood pressure through the renin–angiotensin system due to its action in the formation of angiotensin II, a potent vasoconstrictor, and in the degradation of bradykinin, a vasodilator (Soffer, 1976). Compounds **1**–**4** were evaluated the above two enzyme inhibitory activities (Chen et al., 2009; Cushman and Cheung, 1971). At a concentration of 50 μM, compounds **1**–**4** exhibited 29.8, 39.3, 27.1, and 14.7 % inhibition toward xanthine oxidase, respectively. The positive control, quercetin, showed enzyme inhibitory activity by 34.6 % under the same concentration. In addition, compounds **1** and **4** also showed 13.9 and 20.5 % inhibition toward angiotensin-converting enzyme (ACE), respectively, whereas the positive control, captopril, inhibited angiotensin-converting enzyme activity by 63.9 % at a low concentration of 10 nM.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-180 digital polarimeter. UV and IR spectra were obtained on a Shimadzu UV-1601PC and a Perkin-Elmer 983 G spectrophotometer, respectively. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at room temperature on a Varian-Unity-Plus-400 spectrometer with residual solvent signals as internal reference. Chemical shifts are given in δ values and coupling constants (J) are given in hertz (Hz). EI-MS and HR-EI-MS were run on a Jeol-JMS-HX300 mass spectrometer. Column chromatography (CC) was performed on silica gel (230–400 mesh; Merck & Co., Inc.) and TLC analysis was

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	41.1	1.76[a], 1.91 br d (12.4)[b]	41.0	1.82[a], 1.93 br d (12.5)
2	18.7	1.69 m, 1.76[a]	18.6	1.61 m, 1.74[a]
3	42.8	1.27 m,1.49 m	42.8	1.31 m, 1.51 br d (13.0)
4	34.4		34.5	
5	43.5	1.90[a]	43.6	1.92 dd (15.0, 2.0)
6	33.8	2.24 br d (14.5), 2.34 br t (14.5)	33.9	2.25 br d (15.0), 2.36 br t (15.0)
7	96.6	5.97 br t (3.1)	96.4	5.98 br t (3.1)
8	144.7		144.8	
9	142.0		142.2	
10	40.5		41.5	
11	113.3	6.74 s	113.3	6.73 s
12	149.0		148.8	
13	132.2		132.0	
14	121.0	6.28 s	121.2	6.28 s
15	26.0	2.90 sept (7.0)	26.0	2.82 sept (7.0)
16	22.3	0.68 d (7.0)	22.4	0.65 d (7.0)
17	21.7	0.97 d (7.0)	22.1	0.94 d (7.0)
18	33.4	0.96 s	33.7	0.97 s
19	23.1	0.98 s	23.1	0.99 s
20	21.6	1.34 s	21.4	1.36 s
1′	38.0	1.50 m, 2.23 br d (13.5)	37.8	1.62[a], 2.23 br d (13.5)
2′	18.8	1.59 m, 1.61 m	18.6	2.04 m, 1.72[a]
3′	41.3	1.24 m, 1.52 m	40.3	1.42 td (13.5, 4.0), 1.68[a]
4′	33.2		37.5	
5′	49.6	1.85 dd (13.5, 4.5)	173.0	
6′	36.0	2.61 dd (14.5, 13.5), 2.69 dd (14.5, 4.5)	124.5	6.47 s
7′	198.9		185.2	
8′	124.4		123.9	
9′	156.0		153.4	
10′	38.2		41.4	
11′	107.8	7.14 s	108.9	7.29 s
12′	158.3		157.4	
13′	135.0		135.7	
14′	125.8	7.78 s	124.3	7.87 s
15′	27.2	2.80 sept (7.0)	27.4	2.81 sept (7.0)
16′	21.6	0.76 d (7.0)	21.6	0.76 d (7.0)
17′	22.1	0.98 d (7.0)	21.6	1.01 d (7.0)
18′	32.5	0.90 s	32.6	1.25 s
19′	21.3	0.99 s	29.1	1.36 s
20′	23.4	1.37 s	32.4	

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

carried out on pre-coated silica gel plates (60 F-254; Merck & Co., Inc.). Semi-preparative 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 × 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 with MeOH (100 L × 3) by maceration at room temperature for 7 days each time. After filtration and solvent evaporation, the crude extract (480 g) was suspended in H_2O (1 L), and then partitioned with EtOAc and BuOH, successively. The EtOAc soluble fraction (430 g) was chromatographed over silica gel, 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 *n*-hexane/EtOAc (19:1) elution was further purified through a silica gel column (5 × 45 cm), eluted with *n*-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) using *n*-hexane/EtOAc (19:1). Fr. 3 from *n*-hexane/EtOAc (9:1) elution was further purified through a silica gel column (7 × 60 cm), eluted with *n*-hexane/CH_2Cl_2 (1:0 – 0:1) to obtain nine fractions, 3A – 3I. Further purification of subfraction 3H by HPLC gave **2** (6.8 mg) and **3** (2.2 mg), using *n*-hexane/EtOAc (9:1). Fr. 4 from *n*-hexane–EtOAc (4:1) elution 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 **1** (7.2 mg) using *n*-hexane–EtOAc (4:1).

3.3.1. 12-Hydroxyabieta-8,11,13-trien-7α-yl 7-oxoabieta-8,11,13-trien-12-yl peroxide (1)

Gum; [α]25 *D*–7.2 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 223 (4.64), 278 (4.58) nm; IR (KBr) ν_{max} 3390, 3051, 1666, 1593, 1487, 1414, 1367, 1261, 1162, 1036, 996, 903, 850, 731, 645, 579 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 600 (2) $[M]^+$, 300 (100), 285 (73), 243 (10), 217 (18), 203 (14), 189 (28); HR-EI-MS *m/z* $[M]^+$ 600.4188 (calcd for $C_{40}H_{56}O_4$ 600.4181).

3.3.2. 12-Hydroxyabieta-8,11,13-trien-7α-yl 7-oxoabieta-5,8,11,13-tetraen-12-yl peroxide (3)

Gum; [α]25 *D*–11.5 (*c* 0.61, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 218 (3.55), 244 (3.42), 289 (3.26), 309 (3.28) nm; IR (KBr) ν_{max} 3363, 3040, 1646, 1593, 1493, 1467, 1414, 1288, 1228, 1169, 996, 897, 731 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 598 (1) $[M]^+$, 300 (73), 298 (63), 283 (32), 255 (28), 229 (82), 213 (61), 189 (100); HR-EI-MS *m/z* $[M]^+$ 598.4029 (calcd for $C_{40}H_{54}O_4$ 598.4024).

3.4. Xanthine oxidase inhibition assay

The XO inhibition assay was determined according to the method of Chen et al. with some modifications (Chen et al., 2009). Briefly, 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) were mixed and pre-incubated at 25 °C for 15 min, 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 25 °C. 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

ACE assay was performed using the method that was described by Cushman and Cheung with some modifications (Cushman and Cheung, 1971). The assay mixture contained 30 μL of 2.5 mM Hippuryl-L-histidyl-L-leucine (HHL), 10 μL of testing sample at a certain concentration, and 20 μL of ACE (0.05 mU/μL) in 200 mM borate buffer containing 300 mM NaCl (adjusted to pH 8.3). The reaction was terminated after incubation at 37 °C for 60 min, through the addition of 30 μL of 2 N HCl. The HHL and ACE′s hydrolyzed product, hippuric acid (HA), were determined by HPLC equipped with a Hypersil GOLD C-18 analytical column (250 × 4.6 mm, 5 μm). To analyze HA, an isocratic elution was conducted for 15 min in 23 % ACN containing 0.1 % TFA with a flow rate of 1 mL/min and the absorbance was monitored at 228 nm. The ACE inhibition (%) was calculated using the following

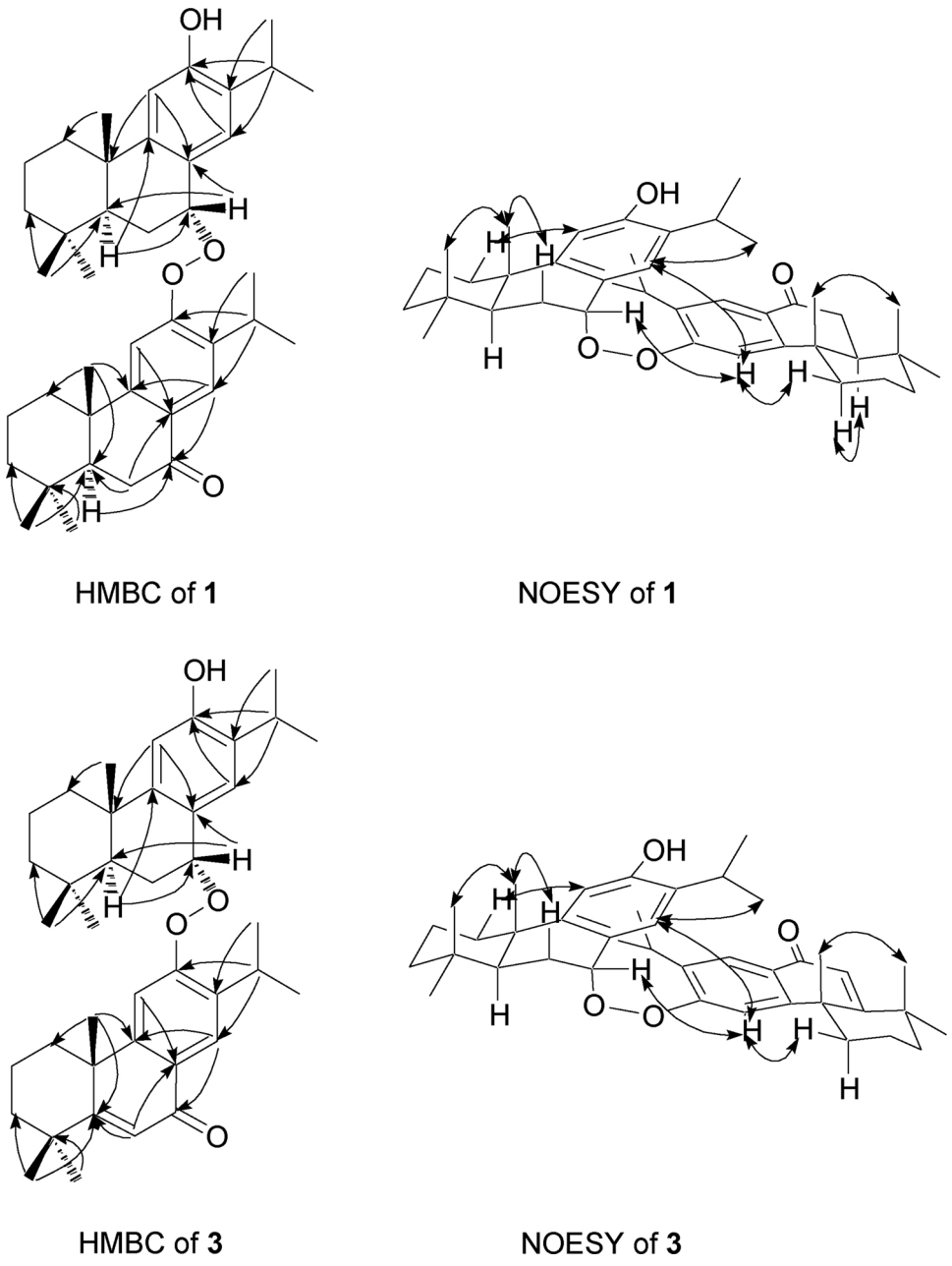

Fig. 3. Selected HMBC and NOE correlations of **1** and **3**.

equation: ACE Inhibition (%) = $[1 - (\Delta A_{Inhibitor}/\Delta A_{Blank})] \times 100$, 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.

Declaration of Competing Interest

The authors declare no conflict of interest

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