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Tremulane sesquiterpenes from cultures of the fungus *Phellinus igniarius* and their vascular-relaxing activities

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ABSTRACT

Five new tremulane sesquiterpenes (1–5), together with seven known ones (6–12), were isolated from cultures of the fungus *Phellinus igniarius*. The new compounds were established as 6β,11,12-trihydroxy-tremul-1(10)-ene (1), 11,12-dihydroxyl-7β-peroxy-hydroxyl-tremul-1(10)-ene (2), 6β,12-dihydroxy-tremulene (3), 10β,12-dihydroxy-tremulene (4), and 12,15-dihydroxy-tremulene (5) on the basis of their MS and NMR data. Compounds **1**–**6** were evaluated for their vascular-relaxing activities. In preliminary *in vitro* assays, at 3×10^{-4} M, they showed vascular-relaxing activities against phenylephrine-induced vasoconstriction with relaxing rates of 29.9%, 39.9%, 48.5%, 78.7%, 32.3%, and 59.3%, respectively. In addition, compounds **1**, **4**, and **6** exhibited different levels of vascular-relaxing activities against KCl-induced vasoconstriction with relaxing rates of 31.3%, 57.7%, and 14.0%, as compared with the blank control.

1. Introduction

The fungus *Phellinus igniarius*, belonging to the family of Polyporaceae, is widely distributed in Yunnan and Sichuan Province of China (Mao, 2000). It mainly hosts on stems of aspen, robur, and birch with horseshoe-shaped, big and somehow stiff fruiting bodies (Mao, 2000). The species is quite popular for treating fester, abdominalgia, bloody gonorrhea and antidiarrheal as traditional Chinese medicine (Mo et al., 2003). Chemical investigations on both fruiting bodies and cultures of this fungus reported a wide range of chemically diverse and biologically intriguing small molecules (Mo et al., 2004; Wang et al., 2005a,b, 2007). Phelligridins D and E showed selective cytotoxicity against a human lung cancer cell line (A549) and a liver cancer cell line (Bel7402) (Mo et al., 2004), while phelligridins H–J, being pyrano[4,3-c][2]benzopyran-1,6-dione and furo[3,2-c]pyran-4-one derivatives, showed cytotoxic activity against human cancer cell lines and protein tyrosine phosphatase 1B inhibition (Wang et al., 2007). A pyrano[4,3-c][2]benzopyran-1,6-dione derivative and a novel 26-membered macrocyclic metabolite phelligridimer A with antioxidant activities were also isolated from the fruiting bodies (Wang et al., 2005a,b). Moreover, several tremulane sesquiterpenes were obtained from the cultures of this fungus, some of which showed significant vascular-relaxing activities against phenylephrine-induced vasoconstriction (Wu et al., 2010). These results gave a conclusion that *P. igniarius* is a creative species and the chemical constituents of the cultures were significantly different from those of the fruiting bodies. In addition, previous studies on different sesquiterpenoids showed different levels of vascular-relaxing effect (Masami et al., 1988; Masayuki et al., 1998; Hisashi et al., 2001; Fabio et al., 2008). In this study, to search for more active constituents, chemical investigation on cultures of *P. igniarius* was carried out, which resulted in the isolation and characterization of five new tremulane sesquiterpenes **1**–**5**, as well as seven known compounds (**6**–**12**) (Fig. 1). Compounds **1**–**6** were evaluated for their vascular-relaxing activities.

2. Results and discussion

Compound **1** was isolated as a colorless oil. Its molecular formula was established as $C_{15}H_{26}O_3$, on the basis of the HREIMS at *m/z* 254.1976 (calcd for $C_{15}H_{26}O_3$, $[M]^+$, 254.1882), which implied the presence of three degrees of unsaturation. The IR spectrum of **1**

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1874-3900/

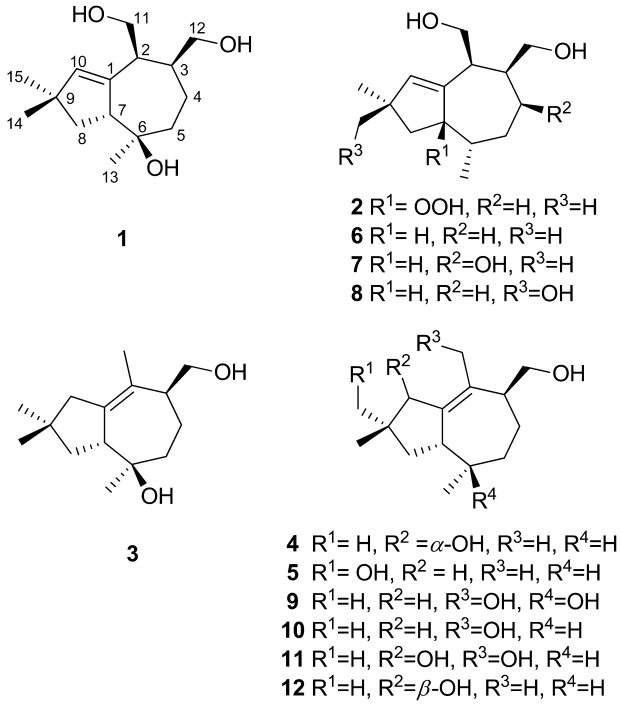

Fig. 1. Structures of compounds **1**–**12**.

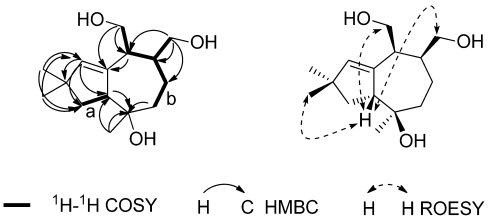

Fig. 2. Key ^{1}H–^{1}H COSY, HMBC and ROESY correlations of compound **1**.

showed absorption bands at 3423 and 1631 cm^{-1}, indicating the presence of hydroxy and double bond groups, respectively. The ^{13}C NMR (Table 2) and DEPT spectra of **1** indicated 15 carbon resonances, including three methyls, five methylenes (two oxygenated at δ_C 63.2 and 64.1), four methines comprising of three sp^3 carbons resonating at δ_C 47.2, 43.8, 58.6 and one sp^2 carbon at δ_C 140.1, and three quaternary carbons (one sp^2 carbon at δ_C 143.7; one oxygenated at δ_C 74.9; one sp^3 quaternary carbon at δ_C 42.5). In consideration of one degree of unsaturation occupied by one double bond, compound **1** was suggested to possess a two-ring system. Inspection of ^{1}H–^{1}H COSY correlations (Fig. 2) resulted in the fragment a (C-7–C-8) and fragment b (C-2–C-3–C-4–C-5). In the HMBC spectrum, correlations from H-14 to C-9, C-8 and C-10, H-15 to C-9, C-8 and C-10, H-14 to C-15 and H-15 to C-14 (Fig. 2) revealed the fragment of C-8–C-9–C-10, while C-14 and C-15 connecting to the same quaternary carbon C-9. C-10 and C-1 were connected by the double bond. Herein, a five-member ring (C-1–C-7–C-8–C-9–C-10) was established. Likewise, in the HMBC spectrum, the correlations of H-11 to C-1 and C-2 established the connection of C-1–C-2, while the HMBC correlations from H-13 to C-5, C-6, C-7, and from H-7 and H-5 to C-6 indicated the presence of a seven-member ring (C-1–C-2–C-3–C-4–C-5–C-6–C-7). Finally, compound **1** possessed a backbone of a 5/7 ring system related to that of tremulenediol B (Ayer and Cruz, 1993). In the tremulane skeleton, H-7 and H-14 were usually in β-orientation, while H-13 and H-15 were in α-orientation (Ying et al., 2013). In the ROESY spectrum of compound **1**, correlations of H-7/H-11 and H-7/H-12 (Fig. 2) demonstrated that H-11 and H-12 both occupied β-orientation. Thus, compound **1** was elucidated as 6β,11,12-trihydroxy-tremul-1(10)-ene.

Compound **2** was obtained as amorphous powder. A molecular formula of $C_{15}H_{26}O_4$ was determined for compound **2** on the basis of its HREIMS data at *m/z* 270.1839 (calcd for $C_{15}H_{26}O_4$, $[M]^+$, 270.1831), 16 mass units higher than that of **1**. The NMR spectroscopic data (Tables 1 and 2) were similar to those of compound **1**. One difference was that the oxygenated quaternary carbon of C-6 in **1** changed into a methine in that of **2**, which resulted in a doublet for Me-13 at δ_H 0.93 (3H, d, J = 7.0 Hz) in ^{1}H NMR spectrum of **2**. In addition, a significant downfield signal at δ_C 102.7 in ^{13}C NMR spectrum of **2** was assigned to a hydroperoxy group at C-7, which was supported by HMBC correlations from H-13 and H-10 to C-7 (at δ_C 102.7), as well as analysis of mass data. Detailed analysis of 1D and 2D NMR data (HSQC, HMBC, ^{1}H–^{1}H COSY, ROESY) suggested that the other parts of **2** were the same as those of **1**. Therefore, structure of compound **2** was identified as 11,12-dihydroxyl-7β-peroxy-hydroxyl-tremul-1(10)-ene.

Compound **3** possessed a molecular formula $C_{15}H_{26}O_2$ as established by HREIMS at *m/z* 238.1938 (calcd for $C_{15}H_{26}O_2$, $[M]^+$, 238.1933). The ^{13}C NMR (Table 2) and DEPT spectra of **3** indicated 15 carbon resonances, including four methyls, five methylenes (one oxygenated at δ_C 60.5), two methines, and four quaternary carbons (two sp^2 carbons at δ_C 139.2 and 130.6; one oxygenated at δ_C 73.6; one sp^3 quaternary carbon at δ_C 36.8). The above data indicated that compound **3** possessed the same tremulane sesquiterpene skeleton to that of **1**. The main differences were that an oxygenated methylene in **1** was reduced to a methyl in **3** and the trisubstituted double bond in **1** was changed as a tetrasubstituted double bond. The HMBC correlations from H-11 to C-1 and C-2, as well as from H-14 to C-10, indicated that the methyl should be assigned as Me-11, while the tetrasubstituted double bond was supposed to locate between C-1 and C-2 in **3**. Further analysis of 1D and 2D NMR data suggested that the other parts of **3** were the same as those of **1**. Finally, the structure of compound **3** was established as 6β,12-dihydroxy-tremulene.

Compound **4** was isolated as a colorless oil. Its molecular formula of $C_{15}H_{26}O_2$, the same as that of compound **3**, was concluded from HREIMS at *m/z* 238.1933 (calcd for $C_{15}H_{26}O_2$, $[M]^+$, 238.1933). The 1D NMR data (Tables 1 and 2) were also quite similar to those of **3**. The main differences were the missing of an oxygenated quaternary carbon signal and the presence of an oxygenated methine, which suggested the change of substitution location of a hydroxy group. In the HMBC spectrum, the correlations from H-14 to C-10 and from H-10 to C-1 indicated that the hydroxy group should be located at C-10. These results suggested that the planar structure of compound **4** was the same as tremulenediol C (Ayer and Cruz, 1993). However, the ROESY correlations of H-7/H-12 and H-7/H-10 of compound **4** indicated that the hydroxy group at C-10 was in a β-orientation. Therefore, the structure of compound **4** was established as 10β,12-dihydroxy-tremulene.

Compound **5** was obtained as colorless oil. It also possessed the same molecular formula $C_{15}H_{26}O_2$ with those of compounds **3** and **4**, on the basis of HREIMS data at *m/z* 238.1935 (calcd for $C_{15}H_{26}O_2$, $[M]^+$, 238.1933). Detailed comparison of the ^{1}H NMR data (Table 1) between compounds **5** and **4** indicated that one hydroxy group should be substituted at C-15 in **5** rather than at C-10 in **4**, which was suggested by the HMBC correlations from H-15 to C-14, C-8, C-9 and C-10. The ROESY correlations of H-7/H$_2$-12 and H-7/H$_3$-14 revealed that H-7, H-14 and H-12 were in the same side. Thus, the structure of compound **5** was established as 12,15-dihydroxy-tremulene.

In addition to the five new compounds described above, seven known ones were identified by comparing their spectral data with

Table 1
^{1}H NMR data for compounds **1–5** (δ in ppm, J in Hz).[a]

No.	1	2	3	4	5
2	2.64 (td, 5.8, 1.1)	3.13 (dt, 11.4, 4,4)			
3	1.99 (m)	1.62 (m)	2.22 (td, 9.7, 5.1)	2.11 (td, 9.6, 4.8)	2.22 (dt, 13.9, 4.8)
4	1.55 (overlap)	1.23 (overlap)	1.88 (overlap)	1.62 (overlap)	1.75 (overlap)
	1.90 (m)	1.75 (m)	1.66 (overlap)		1.71 (overlap)
5	1.72 (overlap)	1.24 (overlap)	1.96 (td, 13.9, 2.6)	1.81 (m)	1.94 (m)
		1.38 (dd, 12.8, 6.3)	1.68 (overlap)	1.45 (m)	1.53 (overlap)
6		1.92 (m)		1.67 (m)	1.73 (overlap)
7	3.00 (m)		2.98 (m)	2.78 (m)	2.99 (m)
8	1.52 (overlap)	1.87 (overlap)	1.60 (overlap)	1.57 (dd, 11.4, 11.2)	1.56 (overlap)
	1.82 (m)		1.54 (m)	1.22 (dd, 11.4, 7.8)	1.36 (ddd, 12.2, 8.2, 2.1)
10	5.36 (s)	5.60 (s)	2.14 (dd, 14.7, 2.5)	3.72 (d, 3.0)	2.04 (overlap)
			1.90 (overlap)		2.02 (overlap)
11	3.70 (dd, 10.8, 7.0)	3.73 (overlap)	1.69 (s)	1.74 (d, 2.0)	1.68 (dd, 1.8, 1.7)
	3.83 (dd, 10.8, 6.0)	3.96 (m)			
12	3.53 (d, 2.0)	3.44 (overlap)	3.78 (t, 10.5)	3.35 (overlap)	3.75 (td, 10.5, 6.0)
	3.54 (d, 2.0)		3.55 (dd, 10.8, 5.6)	3.54 (td, 10.2, 5.8)	3.55 (dt, 10.5, 5.3)
13	1.12 (s)	0.93 (d, 7.0)	1.06 (s)	0.79 (d, 6.9)	0.82 (d, 6.9)
14	1.05 (s)	1.17 (s)	1.08 (s)	0.71 (s)	0.85 (s)
15	0.99 (s)	1.03 (s)	0.85 (s)	0.92 (s)	3.38 (overlap)
					3.38 (overlap)

[a] Data (δ) were measured in methanol-d_4 for **1** and **3**, in DMSO-d_6 for **4** and in acetone-d_6 for **2** and **5** at 600 MHz. The assignments were based on DEPT, ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

Table 2
^{13}C NMR data compounds **1–5** (δ in ppm).[a]

C-atom	1	2	3	4	5
1	143.7 (s)	142.9 (s)	139.2 (s)	140.9 (s)	138.7 (s)
2	47.3 (d)	46.3 (d)	130.6 (s)	134.0 (s)	129.8 (s)
3	44.0 (d)	50.0 (d)	48.7 (d)	48.5 (d)	49.9 (d)
4	26.7 (t)	31.2 (t)	24.1 (t)	19.7 (t)	20.8 (t)
5	41.0 (t)	32.9 (t)	41.8 (t)	31.5 (t)	32.4 (t)
6	74.9 (s)	41.3 (d)	73.6 (s)	30.8 (d)	32.8 (d)
7	58.7 (d)	102.7 (s)	53.1 (d)	44.8 (d)	46.2 (d)
8	44.6 (t)	47.0 (t)	44.3 (t)	41.7 (t)	41.3 (t)
9	42.5 (s)	41.2 (s)	36.8 (s)	40.6 (s)	43.2 (s)
10	140.1 (s)	147.5 (d)	49.1 (t)	79.8 (d)	44.4 (t)
11	63.2 (t)	60.5 (t)	24.1 (q)	23.3 (q)	23.8 (q)
12	64.1 (t)	66.1 (t)	60.5 (t)	58.2 (t)	60.1 (t)
13	23.7 (q)	18.6 (q)	20.0 (q)	12.8 (q)	12.2 (q)
14	29.9 (q)	30.9 (q)	29.1 (q)	25.9 (q)	22.9 (q)
15	27.5 (q)	27.7 (q)	27.3 (q)	22.8 (q)	71.1 (t)

[a] Data (δ) were measured in methanol-d_4 for **1** and **3**, in DMSO-d_6 for **4** and in acetone-d_6 for **2** and **5** at 150 MHz. The assignments were based on DEPT, ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

those in literature as tremulenediol B (**6**) (Ayer and Cruz, 1993), (−)-(2*S*,3*S*,4*S*,6*S*,7*S*)-tremul-1(10)-ene-4,11,12-triol (**7**) (Wu et al., 2010), (−)-(2*S*,3*S*,6*S*,7*S*,9*R*)-tremul-1(10)-ene-11,12,14-triol (**8**) (Wu et al., 2010), (+)-(3*S*,6*S*,7*R*)-tremulene-6,11,12-triol (**9**) (Wu et al., 2010), tremulenediol A (**10**) (Ayer and Cruz, 1993), (+)-(3*S*,6*S*,7*S*,10*S*)-tremulene-10,11,12-triol (**11**) (Wu et al., 2010), and tremulenediol C (**12**) (Ayer and Cruz, 1993).

Compounds **1–6** were tested for their vascular-relaxing activities. In preliminary *in vitro* assays, at 3×10^{-4} M, **1–6** showed vascular-relaxing activities against phenylephrine-induced vasoconstriction with relaxing rates of 29.9%, 39.9%, 48.5%, 78.7%, 32.3%, and 59.3%, respectively. In addition, compounds **1**, **4**, and **6** exhibited vascular-relaxing activities against KCl-induced vasoconstriction with relaxing rates of 31.3%, 57.7%, and 14.0%, as compared with the blank control.

Five new tremulane sesquiterpenes (**1–5**) were isolated from cultures of the fungus *P. igniarius*. Until now, 17 sesquiterpenoids of this type have been obtained from this fungus. It is worth mentioning that the hydroperoxy group in compound **2** was rare in natural products and reported for the first time in tremulane sesquiterpenoids. In addition, the vascular-relaxing activities of the five new ones were tested. Hence, this research not only enriched the structure diversity of this fungus, but also provided some potential active compounds.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco-P-1020 polarimeter (Horiba, Japan). IR spectra were obtained using a Bruker Tensor 27 FT-IR spectrometer with KBr pellets (Bruker, Germany). NMR spectra were acquired with instrument of a Bruker Avance III with deuterated solvent signals used as internal standards at room temperature (Bruker, Germany). HREIMS were measured on a Waters Auto Premier P776 spectrometer (Waters, USA). Silica gel (200–300 mesh and 80–100 mesh, Qingdao Marine Chemical Inc., China), Sephadex LH-20 (Amersham Biosciences, Sweden) and RP-18 gel (20–45 μm, Fuji Silysia Chemical Ltd., Japan) were used for column chromatography (CC). Preparative HPLC was performed on an Agilent 1100 series with a Zorbax SB-C18 (5 μm, 9.4 mm × 150 mm) column (Agilent Technologies, USA). Fractions were monitored by TLC (thin layer chromatography) (Qingdao Marine Chemical Inc., China) and spots were visualized by heating silica gel plates immersed in H_2SO_4 in EtOH, in combination with the Agilent 1200 series HPLC system (Eclipse XDB-C18 column, 5 μm, 4.6 mm × 150 mm) (Agilent Technologies, USA).

3.2. Fungal material and cultivation conditions

Fruiting bodies of *P. igniarius* were collected at Changbai Mountain National Nature Reserve, Antu, Jilin Province, China in 2008 and identified by Prof. Yu-Cheng Dai (Beijing Forestry University). A specimen (No. KIB20081017) was deposited at Kunming Institute of Botany, Chinese Academy of Sciences. The culture medium was composed of glucose (5%), pork peptone (0.15%), yeast powder (0.5%), KH_2PO_4 (0.05%) and $MgSO_4$ (0.05%). The initial pH was adjusted to 6.0 and the fermentation was carried out on a shaker at 150 rpm for 25 days.

3.3. Extraction and isolation

The cultures (20 L) were filtered through cheesecloth to separate broth and mycelium. The broth was extracted four times

with EtOAc, while the mycelium was extracted three times with $CHCl_3$–MeOH (1:1, v/v). The organic layer of both parts was evaporated together to yield a crude extract (9 g). Then this residue was subjected on RP-18 gel eluted with MeOH/H_2O (30:70–100:0, v/v)). Fractions were collected and monitored by TLC. Similar fractions were pooled to give 12 sub-fractions (A–L). Sub-fraction D was isolated by CC eluted with a PE/Me_2CO gradient system (4:1, v/v) and then purified by CC (Sephadex LH-20; Me_2CO) to yield compound **1** (2.2 mg). So was sub-fraction F, which gave compound **3** (2.8 mg). Sub-fraction H was isolated by CC (Sephadex LH-20; MeOH) and prep. HPLC (MeCN/H_2O, 0–30%, 10 ml/min over 30 min) to give compounds **2** (1.5 mg), **6** (12.2 mg), **10** (8.3 mg), and **12** (2.7 mg). Sub-fraction K, isolated by CC (Sephadex LH-20; MeOH), was then purified by CC (SiO_2; PE/Me_2CO, 5:1, v/v) to afford compounds **4** (1.6 mg) and **5** (0.9 mg). Sub-fraction B was isolated by RP-18 gel eluted with MeOH/H_2O (30:70–100:0, v/v) and CC (SiO_2; PE/Me_2CO, 7:2, v/v) to afford compound **9** (3.7 mg). Sub-fraction E was purified by CC (Sephadex LH-20; MeOH), followed by CC (SiO_2; PE/Me_2CO, 5:1, v/v) to yield compounds **11** (7.7 mg) and **8** (4.0 mg). Sub-fraction I was isolated by RP-18 gel eluted with MeOH/H_2O (20:80–70:30, v/v) and CC (SiO_2; PE/Me_2CO, 5:1, v/v), then purified by CC (Sephadex LH-20; Me_2CO), followed by prep. HPLC (MeCN/H_2O, 0–35%, 10 ml/min over 35 min) to yield compound **12** (1.3 mg).

*3.3.1. 6β,11,12-Trihydroxy-tremul-1(10)-ene (**1**)*

Colorless oil. $[\alpha]_D^{22} = -16.9$ (c = 2.20, MeOH). IR (KBr) 3423, 2927, 1631, 1462, 1383, 1029 cm^{-1}. ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (methanol-d_4): see Tables 1 and 2; HREIMS: m/z 254.1976 (calcd for $C_{15}H_{26}O_3$, $[M]^+$, 254.1882).

*3.3.2. 11,12-Dihydroxyl-7β-peroxy-hydroxyl-tremul-1(10)-ene (**2**)*

Amorphous powder. $[\alpha]_D^{27} = -10.7$ (c = 1.60, MeOH). IR (KBr) 3441, 2955, 2924, 1631, 1445, 1384, 1032, 874, 791, 583 cm^{-1}. ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (acetone-d_6): see Tables 1 and 2; HREIMS: m/z 270.1839 (calcd for $C_{15}H_{26}O_4$, $[M]^+$, 270.1831).

*3.3.3. 6β,12-Dihydroxy-tremulene (**3**)*

White powder. $[\alpha]_D^{20} = +73.9$ (c = 0.11, MeOH). IR (KBr) 3447, 2947, 2870, 1636, 1461, 1369, 1234, 1106, 1038, 693 cm^{-1}. ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (methanol-d_4): see Tables 1 and 2; HREIMS: m/z 238.1938 (calcd for $C_{15}H_{26}O_2$, $[M]^+$, 238.1933).

*3.3.4. 10β,12-Dihydroxy-tremulene (**4**)*

Colorless oil. $[\alpha]_D^{21} = +26.0$ (c = 1.60, MeOH). IR (KBr) 3441, 2927, 2867, 1631, 1448, 1382, 1030, 1002 cm^{-1}. ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (DMSO-d_6): see Tables 1 and 2; HREIMS: m/z 238.1933 (calcd for $C_{15}H_{26}O_2$, $[M]^+$, 238.1933).

*3.3.5. 12,15-Dihydroxy-tremulene (**5**)*

Colorless oil. $[\alpha]_D^{21} = +35.6$ (c = 0.03, MeOH). IR (KBr) 3442, 2925, 2856, 1631, 1446, 1384, 1033 cm^{-1}. ^1H (600 MHz) and ^{13}C NMR (150 MHz) data (methanol-d_4):

see Tables 1 and 2; HREIMS: m/z 238.1935 (calcd for $C_{15}H_{26}O_2$, $[M]^+$, 238.1933).

3.4. Vasodilating activity assays

Sprague-Dawley rats, weighing 250–350 g, were anaesthetized with pentobarbital sodium (40 mg/kg, i.p.), and the thoracic aorta was removed and placed in Krebs–Henseleit solution (KHS). An aortic ring of about 2–3 mm in length was suspended between two stainless steel hooks in a 5 mL water-jacketed bath containing KHS of the following composition (in mmol/L): NaCl, 120; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $CaCl_2 \cdot 2H_2O$, 2.5; $NaHCO_3$, 25; and glucose, 10. The bathing solution was maintained at 37 ± 0.5 °C and was bubbled with 95% O_2 and 5% CO_2 (pH 7.4) throughout the experiments. One of stainless steel hooks was then connected to a force-displacement transducer (Chengdu instrument factory, Sichuan, China). The initial tension was adjusted to 1.5 g and an equilibration period of 90 min was allowed before commencing the experiments. The resting tension acting in the artery was readjusted periodically until stabilization was achieved. After equilibration, the reactivity of the thoracic aorta was ensured by KCl (60 mmol/L)-induced contraction. When a steady contraction was reached, 10^{-5} mol/L acetylcholine (ACh) was added to induce endothelium-dependent relaxation. This step was necessary to verify the integrity of the endothelium.

In order to investigate the effects of various agents on phenylephrine hydrochloride (PE)-induced contraction, when a steady contraction induced by PE (10^{-6} mol/L) was reached, various agents (3×10^{-4} mol/L) was added to the organ bath. The resulting relaxation was expressed as a percentage (%) of the PE-induced steady contraction in the absence of treatment with various agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2014.10.019.

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