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Two symmetrical unsaturated acids isolated from *Viscum album*

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[ABSTRACT] In the present study, two new acetylene conjugate compounds, dibutyl (2*Z*, 6*Z*)-octa-2, 6-dien-4-yne dioate **(1)**, and dibutyl (2*E*, 6*E*)- octa-2, 6-dien-4-yne dioate (**2**), were isolated from the dry stem leaves of *Viscum album*, along with nine known compounds (**3**–**11**). Their structures were confirmed on the basis of spectroscopic data. Compounds **1** and **8** showed antioxidant activity against xanthine oxidase (XOD) and 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydroxyl (DPPH), with the IC_{50} of 1.22 and 1.33 μmol·L^{-1}, and the SC_{50} of 4.34 and 8.22 μmol·L^{-1}, respectively.

[KEY WORDS] *Viscum album*; Symmetrical unsaturated acid; Antioxidant activity

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Introduction

As a traditional Chinese medicine, *Viscum album* (*Loranthaceae*), commonly named mistletoe, is widely used for the treatment of cardiovascular diseases. Intravenous injection of ethanol extract of mistletoe in dogs and rabbits can maintain antihypertensive effect for 3 min, which could be extended to 1 h with intraperitoneal injection, indicating that the ethanol extract of mistletoe has a significant antihypertensive effect [1]. A crude ethanolic extract of mistletoe has been found to have vasomotor reactivity of superfused rat aortic rings (with or without a functional endothelium) [2]. These results indicate that the ethanolic extract of mistletoe induces predominantly an endothelium-dependent relaxation, which seems to be mediated by the synthesis/release of nitric oxide [2]. However, the active compounds are not clearly identified. In the present study, we carried out a detailed phytochemical investigation on the aerial part of *Viscum album*, leading to the isolation of two new acetylene conjugate compounds, dibutyl (2*Z*, 6*Z*)-octa-2, 6-dien-4-yne dioate **(1)**, dibutyl (2*E*, 6*E*)-octa-2, 6-dien-4-yne dioate (**2**), as well as nine known compounds **3**–**11**. The antioxidant activities of these compounds were also evaluated to explore the pharmacological mechanism of action of these compounds.

Results and Discussion

Compound **1** had a molecular formula of $C_{16}H_{22}O_4$ from the quasimolecular ion peak in positive HR-ESI-MS at *m/z* 279.0937 $[M + H]^+$ (Calcd. for $C_{16}H_{23}O_4$, 279.15). Its IR spectrum showed absorption band for alkynyl (2300 cm^{-1}) and carbonyl (1728.4 cm^{-1}) groups. The ^1H NMR spectrum revealed one methyl group at δ_H 0.91 (3H, t), three methylene groups at 1.35 (2H, m), 1.65 (2H, m) and 4.23 (2H, t), and two protons of *cis* alkene at 7.68 (1H, d, 10 Hz) and 7.70 (1H, d, 10 Hz). The ^{13}C NMR spectrum DEPT revealed eight carbon signals for C-atoms from, one methyl (δ_C 13.6), three methylene (δ_C 18.7, 30.0 and 65.1), two alkenyls (128.7 and 131.6), one alkynyl (δ_C 91.7) and one carbonyl (δ_C 167.0), indicating the existence of symmetric skeleton. The assignments of the ^{13}C NMR data were confirmed by 2D NMR experiments. The HMBC spectrum showed correlations of

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H-2 to C-1, C-3 and C-4; of H-3 to C-2 and C-4; of H-6 to C-5 and C-7; and of H-7 to C-5, C-6 and C-8 (Fig. 1). Based on the above evidences, the structure of compound **1** was elucidated as (2*Z*, 6*Z*)-dibutyl octa-2, 6-dien-4-yne dioate (Fig. 3).

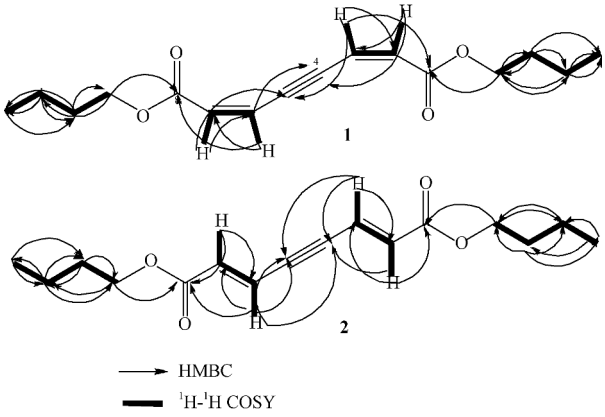

Fig. 1 Key ^{1}H-^{1}H COSY and HMBC correlations of 1 and 2

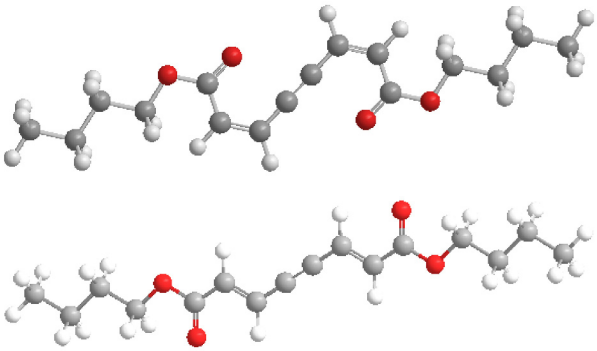

Fig. 2 ORTEP view of compounds 1 and 2

Compound **2** was isolated as a colorless amorphous solid, the HR-ESI-MS spectrum (*m/z* 279.1402 $[M-H]^-$), suggested the molecular formula $C_{16}H_{22}O_4$. The ^{1}H and ^{13}C NMR spectrum (Table 1) of Compound **2** showed that its structure was similar to that of **1,** except for the relative configuration of the vinyl group. In the ^{1}H NMR spectrum of **2**, two hydrogen groups of alkenes at 7.66 (1H, d, 15.6 Hz) and 7.69 (1H, d, 15.6 Hz) were shown, indicating the presence of trans alkene. Thus, the structure of compound 2 was determined to be (2*E*, 6*E*)-dibutyl octa-2, 6-dien-4-yne dioate (Fig. 3).

According to the reported data, the nine known compounds (Fig. **3**) were identified as rhamnazine-3-*O*-*β*-D-apiofuranosyl(1→2)-[6''-(3-oxhydryl-3-methylgluaryl)]-*O*-*β*-D-glucopyranoside(**3**) [5], liquidamboside (**4**) [3-4],homeriodictyol-7-*O*-*β*-*D*-glucopyranoside-4′-*O*-*β*-*D*-apioside (**5**) [5], homeriodictyol-7-*O*-*β*-*D*-apiofuranosyl (1 → 2)-*O*-*β*-*D*-glucopyranoside (**6**) [6-7], rhamnazin-3-*O*-*β*-*D*-glucopyranoside (**7**) [8-9], rhamnazine (**8**) [7-8], rhamnazin-3-*O*-*β*-*D*-(6″-acetyl)-*O*-*β*-*D*-glucopyranoside (**9**) [8-9] homeriodictyol-7-*O*-*β*-*D*-glucopyranoside (**10**) [5, 8], and eriodictyol-7-*O*-*β*-*D*-glucopyranoside (**11**) [5].

Compounds **1**–**11** were evaluated for their antioxidant activity against xanthine oxidase (XOD) and 1,1-Diphenyl-2-picrylhydrazyl radical2,2-Diphenyl-1-(2,4,6- trinitrophenols) hydroxyl (DPPH). Compounds **1** and **8** demonstrated significant inhibitory activities against DPPH, with the SC_{50} of 4.34 and 8.22 μmol·L^{-1} respectively. They also displayed moderated inhibitory effects on XOD, with the IC_{50} of 1.22 and 1.33 μmol·L^{-1}, respectively (Table 2). All these compounds showed antioxidant activity against XOD and DPPH at 12 μmol·L^{-1}.

Table 1 ^{1}H NMR(600 MHz) and ^{13}C NMR(150 MHz) spectroscopic data of compound 1 and 2 in DMSO-d_6 (*J* in Hz)

Pos.	1		2	
	δ_C	δ_H	δ_C	δ_H
1	167.0	-	167.1	-
2	128.7	7.68 (1H, d, 10 Hz)	128.8	7.66 (1H, d, 15.6 Hz)
3	131.6	7.70 (1H, d, 10 Hz)	131.6	7.69 (1H, dd, 15.6 Hz)
4	91.7	-	91.5	-
5	91.7	-	91.5	-
6	128.7	7.70 (1H, d, 10 Hz)	128.8	7.69 (1H, d, 15.6 Hz)
7	131.7	7.68 (1H, d, 10 Hz)	131.6	7.66 (1H, d, 15.6 Hz)
8	167.0	-	167.1	-
9	65.1	4.23 (2H, t)	65.0	4.23 (2H, t)
10	30.0	1.65 (2H, m)	29.9	1.65 (2H, m)
11	18.7	1.35 (2H, m)	18.7	1.35 (2H, m)
12	13.6	0.91 (3H, t)	13.4	0.91 (3H, t)
13	65.1	4.23 (2H, t)	65.0	4.23 (2H, t)
14	30.0	1.65 (2H, m)	29.9	1.65 (2H, m)
15	18.7	1.35 (2H, m)	18.7	1.35 (2H, m)
16	13.6	0.91 (3H, t)	13.4	0.91 (3H, t)

Experimental

General

IR spectra were recorded on a Bruker Vector 22 spectrometer (Bruker Beijing Technology Co., Ltd., Beijing, China) with KBr disks. NMR spectra were measured on a Bruker AV-600 spectrometer with TMS as the internal standard. UV spectra were recorded on a Shimadzu UV-2550 spectrophotometer. ESI-MS were acquired on an Agilent LC-MSD Trap XCT mass spectrometer, whereas HR-ESI-MS were measured using a Waters Q-TOF micro mass spectrometer. Analytical HPLC was carried out with a Waters 515/2487 instrument and a Chiralpak Semi-preparative HPLC was conducted on a Waters 510/484 instrument with a YMC-Pack ODS-A column (5 μm, 10.0 mm × 250 mm). Materials for column chromatography were silica gel (200–300 and 300–400 mesh; Huiyou Silical Gel Development Co.), Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech), and RP-18 silica gel (Greenherbs Sci & Tech Development Co.). All other chemicals used in the present study were of analytical grade.

Plant materials

The dry stem and leaves of *Viscum album* were collected in Anhui Province, China, in June 2014, and were identified by Dr. WU Tong, Department of Traditional Chinese Medicine, Shanghai Institute of Pharmaceutical Industry, Shanghai, China. A voucher specimen has been deposited in the Department of Traditional Chinese Medicine, Shanghai Institute of Pharmaceutical Industry (#14031901).

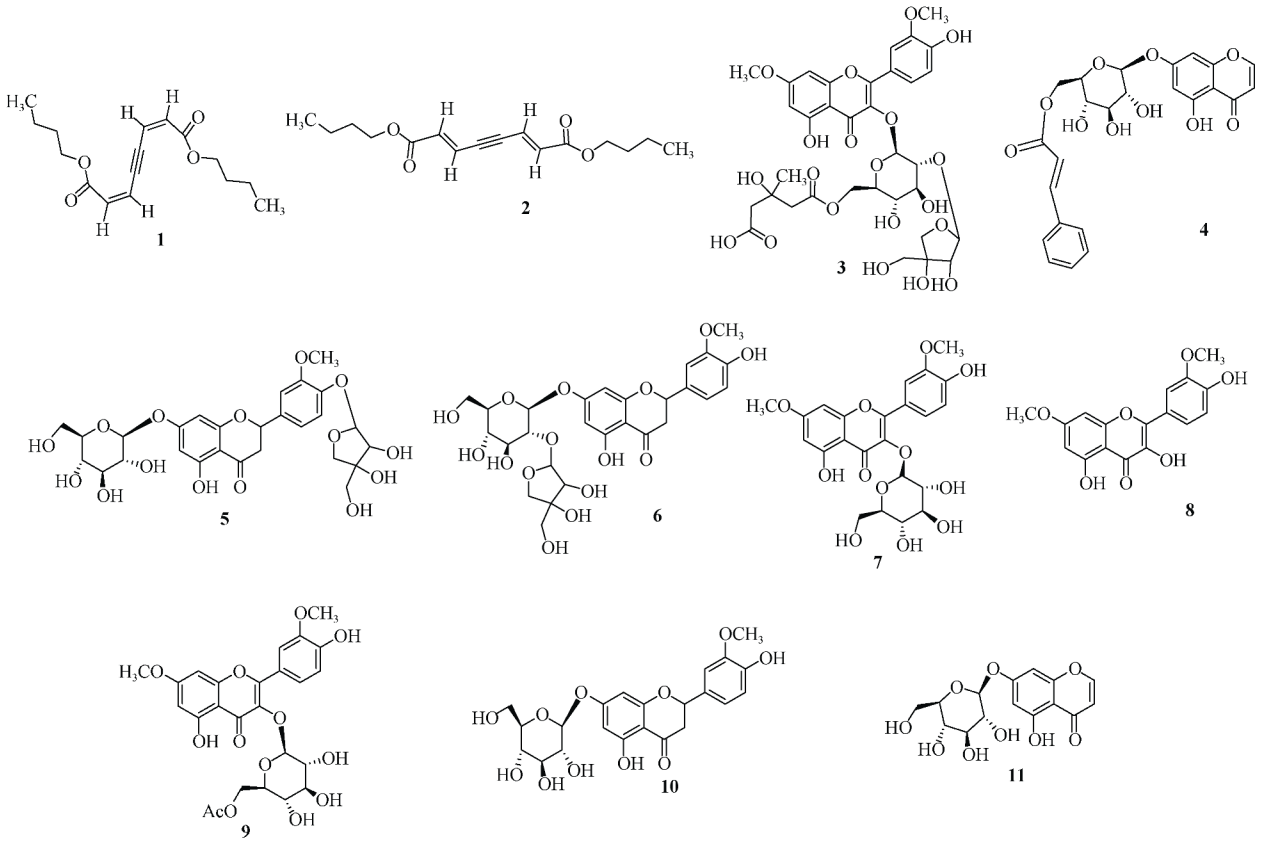

Fig. 3 The chemical structures of compounds 1–11 obtained from *Viscum album*

Extraction and isolation

The dry stem and leaves of *Viscum album* were extracted with 95% ethanol under refluxing four times, 2 h each. After evaporation of the solvent, the extract was diluted with water (4.0 L) and then partitioned three times with $CHCl_3$ (1.8 L). The fluid extract of water diluted by 3.8 L of water, was subjected to column chromatography (CC) over macroporous resin (D101) and eluted with a mixture of water–ethanol (0%, 10%, 30%, 50%, 70%, and 95% ethanol) to give 6 major fractions on the basis of TLC (water, 10%, 30%, 50%, 70%, 95% ethanol). The fraction of 50% ethanol eluted was subjected to column chromatography (CC) over silica gel (200–300 mesh, 2.0 kg, 10 cm × 120 cm) eluted with a mixture of ethyl acetate–MeOH (100: 0 to 0: 100) to afford 5 subfractions on the basis of TLC (Frs. B1–B5). Fr. B3 (23.0 g) was re-chromatographed on silica gel CC (200–300 mesh, 600 g, 5 cm × 80 cm) eluted with a mixture of EAOAc–MeOH (50: 1 to 1: 1) to afford 7 subfractions on the basis of TLC (Frs. B 3-1–B3-7). Fr. B3-4 (7 g) was further separated by repeated silica gel (300–400 mesh, 120 g, 2.5 cm × 60 cm) chromatogry ($CHCl_3$–MeOH, 18: 1 to 1: 1) and purified by Sephadex LH-20 (100 g, 3.0 cm × 100 cm, MeOH, 800 ml) to give **5** (18.3 mg), **6** (23.7 mg), **8** (20.6 mg), and **11**(13.5 mg). Fr. B3-5 (5.8 g) was subjected to an RP-18 column (180 g, 5.0 cm × 60 cm) eluted with H_2O–MeOH (100% to 30%, 1800 mL) to obtain **3** (8.9 mg, 200–300 mL), **4** (14.3 mg, 600–900 mL), and **7** (17.4 mg, 1200–1400 mL). Fr. B3-7 (8.2 g) was subjected to silica gel CC (300–400 mesh, 200 g, 2.5 cm × 120 cm) eluted with acetate–MeOH (30 : 1 to 1 : 1), and finally purified by semi-preparative HPLC (MeOH–H_2O, 40 : 60, 3.0 mL·min^{-1}) to give **1** (6.0 mg, t_R = 12.5 min), **2** (8.2 mg, t_R = 18.9 min), **9** (7.9 mg, t_R = 26.7 min), and **10** (6.3 mg, t_R = 33.5 min).

Antioxidant activity

The antioxidant activity of compounds **1**–**11** (> 90% purity) were measured *in vitro* on XOD and DPPH. In the xanthine oxidase (XOD) inhibitory activity assay, added 100 μL of the samples to be tested, 50 μL of 0.08 U·mL^{-1} XOD solution to the 96-well plate, and used PB as a blank control, incubated for 3 min at 37 °C, and added 50 μL of 0.48 mmol·L^{-1}

XA solution to start the reaction at 295 nm. The readings were taken every 15 s and the absorbance values were recorded for a total of 10 min. Four sets of holes were arranged in parallel for each group. The XOD inhibition rate of the compound was calculated according to the following formula:

Table 2 Antioxidant activity against XOD and DPPH of compounds 1–11

Compounds	XOD inhibitory activity (%)	DPPH clearance (%)
1	85	88
2	66	54
3	11	22
4	< 0	< 0
5	8	2
6	15	8
7	12	5
8	77	95
9	6	4
10	21	14
11	13	19
allopurinol	92	-
quercertin	-	98

*Compounds **1**–**11** showed antioxidant activity against XOD and DPPH at 12 mol·L^{-1}

XOD inhibitory activity (%) = $[A_{blank}\text{-}A_{sample}] / A_{blank} \times 100\%$

A_{blank} is the absorbance value of the control group; A_{sample} is the absorbance value of the sample group

In the DPPH free radical scavenging activity assay, 100 μL of the samples to be tested, DPPH (0.1 mmol·L^{-1}) 100 μL were added to the 96-well plate, and the absorbance was measured at 517 nm after 30 min in the dark. Three replicate wells were set in parallel for each group. Methanol was used as a blank control. The DPPH free radical scavenging rate of the compound was calculated according to the following formula:

DPPH clearance (%)= $[A_{blank}\text{-}A_{sample}] / A_{blank} \times 100\%$

A_{blank} is the absorbance value of the control group; A_{sample} is the absorbance value of the sample group

According to the concentration and the correspond-ding inhibition and clearance rate, half of the inhibitory concentration (IC_{50}) and clear concentration (SC_{50}) were calculated by GraphPad Prism 6.0.2. All the assays were performed in duplicate. Quercetin and allopurinol (purity > 95%) are used as positive controls.

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