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ORIGINAL ARTICLE

Acetylenes and fatty acids from *Codonopsis pilosula*

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Campanulaceae;
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Octenynoic acids;
Unsaturated ω-hydroxy fatty acid

Abstract Four new acetylenes (**1**–**4**) and one new unsaturated ω-hydroxy fatty acid (**5**), together with 5 known analogues, were isolated from an aqueous extract of *Codonopsis pilosula* roots. Their structures were determined by spectroscopic and chemical methods. The new acetylenes are categorized as an unusual cyclotetradecatrienynone (**1**), tetradecenynetriol (**2**), and rare octenynoic acids (**3** and **4**), respectively, and **3** and **4** are possibly derived from oxidative metabolic degradation of **1** and/or **2**. The absolute configuration of **1** was assigned by comparison of the experimental circular dichroism (CD) spectrum with the calculated electronic circular dichroism (ECD) spectra of stereoisomers based on the quantum-mechanical time-dependent density functional theory, while the configuration of **2** was assigned by using modified Mosher's method based on the MPA determination rule of $\Delta\delta_{RS}$ values for diols.

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1. Introduction

Codonopsis pilosula (Franch.) Nannf., a perennial plant of the Campanulaceae family, is widely cultivated in the northwest provinces of China to meet the demand of pharmaceutical and food industries. The root of this plant, known as "Dang shen" in Chinese, is one of the most common traditional Chinese medicines used for the treatment of body weakness, poor appetite, thirsty, indigestion, chronic diarrhea, archoptoma, chronic anemia and leukemia[1]. It exhibits similar therapeutic effects of *Panax ginseng* as tonic agents and is used in many cases as a substitute of the much more costly *Panax ginseng*[1]. Previous studies showed that extracts of the *C. pilosula* roots exhibited pharmacological effects in protecting against peptic ulceration and promoting its healing, enhancing immunity, and improving learning and memory behavior, as well as inhibiting inducible NO synthase and protein oxidation and attenuating the cardiac-impaired insulin-like growth factor II receptor pathway[2–6]. Meanwhile, different types of chemical constituents were isolated from the extracts, such as phytosteroids, sesquiterpenes, triterpenes, alkaloids, alkyl alcohol glycosides, phenylpropanoid glycosides, polyacetylene glycosides and polysaccharides[7–12], of which only polysaccharides were biologically evaluated[13–17]. As part of a program to systematically study the chemical diversity of traditional Chinese medicines and their biological effects, focusing on minor small molecules[18–25], an aqueous decoction of the *C. pilosula* roots was investigated since a variety of formulations containing "Dang shen" are practically used by decocting with water. Herein, reported are the isolation and structural elucidation of four new acetylenes (**1**–**4**), a new unsaturated ω-hydroxy fatty acid (**5**) (Fig. 1), and five known analogues, as well as their preliminary bioassays.

2. Results and discussion

Compound **1** was obtained as a yellowish amorphous powder with $[\alpha]_D^{20}$ +10.8 (*c* 0.07, MeOH). Its IR spectrum showed absorption bands for hydroxyl (3395 cm^{-1}), double bond (3011 cm^{-1}), triple bond (2191 and 2062 cm^{-1}), and conjugated carbonyl (1720, 1685 and 1647 cm^{-1}) functionalities. The molecular formula of **1**, $C_{14}H_{16}O_3$, was indicated by HR-ESI-MS at 255.0999 $[M+Na]^+$ (Calcd. for $C_{14}H_{16}O_3Na$, 255.0992) and NMR data (Table 1). The ^1H NMR spectrum of **1** in CD_3OD showed signals attributed to a conjugated *cis*-disubstituted double bond at δ_H 7.13 (ddd, J=9.6, 6.0 and 2.4 Hz, H-4) and 6.07 (ddd, J=9.6, 3.0 and 0.6 Hz, H-5); a *trans*-disubstituted double bonds at δ_H 6.19 (d, J=15.6 Hz, H-8) and 5.99 (dd, J=15.6 and 2.4 Hz, H-9); and a terminal *trans*-propenyl at δ_H 5.61 (dq, J=15.6 and 1.8 Hz, H-12), 6.11 (dq, J=15.6 and 6.6 Hz, H-13), and 1.78 (dd, J=6.6 and 1.8 Hz, H_3-14). In addition, the ^1H NMR spectrum displayed signals assigned to an oxygen-bearing methylene at δ_H 3.86 (dd, J=10.8 and 4.8 Hz, H-1a) and 3.45 (dd, J=10.8 and 8.4 Hz, H-1b) and an aliphatic methylene at δ_H 2.73 (ddd, J=10.8, 6.0 and 1.0 Hz, H-3a) and 2.35 (ddd, J=10.8, 6.0 and 2.4 Hz, H-3b), as well as a multiplet due to a methine at δ_H 2.28 (m, H-2), which is vicinal to both the methylenes based on the splitting patterns and coupling constants. The ^{13}C NMR and DEPT spectra of **1** exhibited 14 resonances corresponding to proton-bearing carbons of the above units and four quaternary carbons including a conjugated carbonyl (δ_C 199.6, C-6), a triple bond (δ_C 86.4 (C-10) and 91.2 (C-11)), and an oxygen-bearing (δ_C 80.2, C-7) carbon (Table 1). Together, the above spectroscopic data indicated that **1** is a monocyclic C_{14} hydroxytrienynone with an unusual structure feature, which was further elucidated by 2D NMR experimental data analysis. The assignments of proton and proton-bearing carbon signals in the NMR spectra were confirmed by cross-peaks in the ^1H-^1H COSY and HSQC spectra. The vicinal coupling cross-peaks H_2-1 ↔ H-2 ↔ H_2-3 ↔ H-4 ↔ H-5 in the ^1H-^1H COSY spectrum and three-bond hetero nuclear correlations (Fig. 2) from H_2-1 to C-3 and C-7; from H-2 to C-4 and C-7; from H_2-3 to C-1, C-5, and C-7; from H-4 to C-2 and C-6; and from H-5 to C-3 and C-7 in the HMBC spectrum, together with the chemical shifts of these proton and carbon resonances, revealed that the oxygen-bearing quaternary carbon (C-7) was connected with both the methine (CH-2) and the carbonyl (C-6) to form a cyclohexenone moiety in **1**. The ^1H-^1H COSY cross-peaks H-8 ↔ H-9 and H-12 ↔ H-13 ↔ H_3-14, together with the HMBC correlations from H-8 to C-10, from H-9 to C-11, from H-12 to C-14, from H-13 to C-11, and from H_3-14 to C-12, in combination with their chemical shifts, confirmed the presence of a 8,12-dien-10-yn-8-yl chain moiety in **1**. In addition, the HMBC correlations from H-2 to C-8, from H-8 to C-6 and C-7, and from H-9 to C-7 indicated a C-7–C-8 bond connection between the two moieties. To match requirement of the molecular composition, two hydroxyl groups must occur at the oxygen-bearing carbons (C-1 and C-7) in **1**. Accordingly the planar structure of **1** is elucidated as shown in Fig. 2. In the NOE difference spectrum of **1**, irradiation of H-3b enhanced H-2 and H-8 (Fig. 3), indicated that these protons were cofacial and that the chain moiety had a quasi-axial orientation on the cyclohexenone ring, which was supported by conformational analysis with the MMFF94 molecular mechanics force field using molecular operating environment (MOE) software package[26]. The circular dichroism (CD) spectrum of **1** displayed a positive Cotton effect at 299 nm ($\Delta\varepsilon$ +1.41) for $n \rightarrow \pi^*$ and a negative Cotton effect at 256 nm ($\Delta\varepsilon$ –4.71) for $\pi \rightarrow \pi^*$. Based on the octant rule for cyclohexenones[27], these Cotton effects suggest that **1** possesses the 2*R*,7*S*-configuration (Fig. 4). The suggestion was further supported by comparing the experimental CD spectrum with the electronic circular dichroism (ECD) spectrum predicted from the quantum-mechanical time-dependent density functional theory (TDDFT) calculations[28] (Fig. 5). Therefore, compound **1** was determined as (+)-(2*R*,7*S*)-1,7-dihyrdroxy-2,7-cyclotetradeca-4,8,12-trien-10-yn-6-one.

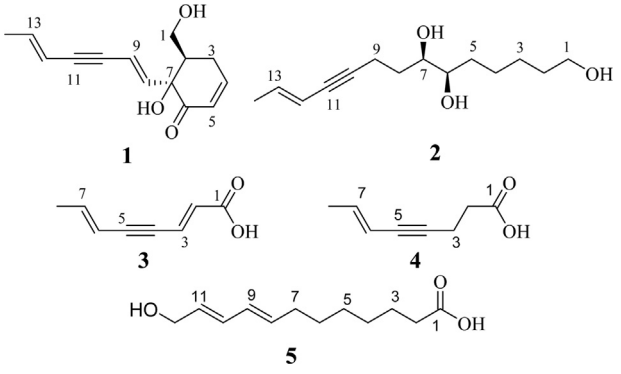

Figure 1 The structures of compounds **1**–**5**.

Compound **2**, a yellowish amorphous powder with $[\alpha]_D^{20}$ +41.5 (*c* 0.06, MeOH), has the molecular formula $C_{14}H_{24}O_3$ as indicated by HR-ESI-MS at *m/z* 241.1807 $[M+H]^+$ (Calcd. for $C_{14}H_{25}O_3$, 241.1798) and 263.1620 $[M+Na]^+$ (Calcd. for $C_{14}H_{24}O_3Na$, 263.1618). The IR and NMR spectroscopic data of **2** (Section 4

and Table 1) demonstrated that it was a C_{14} enynetriol derivative with the same terminal unit *trans*-12-en-10-ynyl as that of **1**. Comparison of the NMR spectroscopic data between **2** and **1** indicated replacement of the 2,7-cyclo-4,8-dien-6-one moiety in **1** by a linear diol moiety consisting of two hydroxyl-bearing methines δ_H 3.34 (q, J=4.0 Hz, H-6) and 3.46 (q, J=4.0 Hz, H-7), δ_C 75.3 (C-6) and 74.2 (C-7) and six aliphatic methylenes δ_H1.34–1.48 (8H, partially overlapped, H_2-2–H_2-5), 1.62 (m, H-8a), 1.57 (m, H-8b), and 2.34 (m, H_2-9); δ_C 16.9 (C-9), 27.1 (C-4), 27.3 (C-3), 33.6 (C-8), 33.9 (C-2), and 34.2 (C-5) in **2**. In addition, the two double doublets of inequivalent H-1a and H-1b in **1** were replaced by a hydroxymethylene triplet at δ_H 3.49 (J=6.5 Hz, H_2-1) in **2**, while the triple bond carbon resonances were shifted from δ_C 86.4 (C-10) and 91.2 (C-11) in **1** to δ_C 88.8 (C-10) and 80.6 (C-11) in **2**. These data suggest that **2** differs from **1** in the 2,7-cyclo-4,8-dien-6-one moiety with cleavage of the C-2–C-7 bond and saturation of the 4,8-dien-6-one. This was further confirmed by 2D NMR data analysis, especially by the ^{1}H-^{1}H COSY cross-peaks H-12↔H-13↔H_3-14 and the HMBC correlations from H_2-8 to C-6 and C-10; from H_2-9 to C-7 and C-11; from H-13 to C-11 and C-14; and from H_2-14 to C-11, C-12, and C-13, combined with their chemical shifts. The absolute configuration the 6,7-diol moiety in **2** was determined using the modified Mosher's method[29] since the primary alcohol at C-1 is away from and has less shielding/deshielding effects on protons around the chiral centers. Treatment of **2** with (−)-(*R*)- and (+)-(*S*)-α-methoxyphenylacetic acid (*R*- and *S*-MPA) in anhydrous CH_2Cl_2 afforded the tri-(*R*)-and tri-(*S*)-MPA esters of **2**. The $\Delta\delta_{RS}$ values of H-6 and H-7 were calculated by NMR data measurement of the esters. According to the Mosher's model of vicinal diols, the $\Delta\delta_{RS}$ values predict that **2** has the 6*R*,7*R*-configuration (Fig. 6). This was supported by $Mo_2(OAc)_4$-induced circular dichroism (ICD) spectrum of **2**, which displayed a negative Cotton effect at 316 nm (Fig. S17, Supporting information). On the basis of the empirical rule proposed by Snatzke[30,31], the Cotton effect indicate that the O–C–C–O torsion angle of the diol moiety in **2** is negative and also predicts 6*R*,7*R* configuration. Thus, compound **2** was determined as (+)-(6*R*,7*R*,12*E*)-tetradeca-12-en-10-yne-1,6,7-triol.

Compound **3**, a yellowish amorphous powder, has the molecular formula $C_8H_8O_2$ as indicated by HR-ESI-MS *m/z* 137.0595 $[M+H]^+$ (Calcd. for $C_8H_9O_2$, 137.0597) and 159.0413 $[M+Na]^+$ (Calcd. for $C_8H_8O_2Na$, 159.0417). Its IR spectrum showed the presence of OH (3395 cm^{-1}), C=C (3011 cm^{-1}), C≡C (2185 cm^{-1}), and carbonyl (1698 cm^{-1}, sh) functionalities. The ^{1}H NMR data of **3** (Table 1) demonstrated occurrence of a terminal *trans*-propenyl (δ_H 6.23 (dq, J=16.0 and 7.0 Hz, H-7), 5.66 (d, J=16.0 Hz, H-6), and 1.77 (d, J=7.0 Hz, H-8)) and a *trans*-disubstituted double bond (δ_H 6.08 (d, J=16.0 Hz, H-2) and 6.74 (d, J=16.0 Hz, H-3)). Besides the resonances corresponding to the proton-bearing carbons, the ^{13}C NMR and DEPT spectral data confirmed the presence of the triple-bond (δ_C 85.9 (C-4) and 98.3 (C-5)) and carbonyl (δ_C 170.1 (C-1)) units. These spectroscopic data reveal that **3** is (2*E*,6*E*)-octa-2,6-dien-4-ynoic acid, which was further confirmed by 2D NMR data analysis, particularly by HMBC correlations from H-2 to C-1 and C-4 and from H-3 to C-1 and C-5 combined with their chemical shifts. Thus, compound **3** was determined as (2*E*,6*E*)-octa-2,6-dien-4-ynoic acid.

Compound **4** was isolated as a yellowish amorphous powder. The IR and HR-ESI-MS data (Section 4) indicated that **4** was a dihydro analogue of **3**. Comparison of the NMR spectral data of **4** and **3** (Tables 1 and 2) demonstrated that the 2-en in **3** was saturated in **4**. Therefore, compound **4** was determined as (*E*)-oct-6-en-4-ynoic acid, which was also verified by 2D NMR data analysis.

Compound **5** has the molecular formula $C_{12}H_{20}O_3$ as indicated by HR-ESI-MS at *m/z* 235.1296 $[M+Na]^+$ (Calcd. for $C_{14}H_{16}O_3Na$, 235.1305). The IR spectrum showed the presence of OH (3200 cm^{-1}), C=C (3017 cm^{-1}), and C=O (1723 cm^{-1}) functionalities. The ^{1}H NMR spectral data of **5** (Table 2) revealed the presence of two double bonds, an oxygen-bearing methylene, and six aliphatic methylenes. These spectroscopic data suggested that **5** is (8*E*,10*E*)-12-hydroxydodeca-8,10-dienoic acid though an expected carboxylic carbon resonance was extremely diminished and almost not recognized in the ^{13}C NMR spectrum. The suggestion was confirmed by 2D NMR experiments. Especially, the HMBC spectrum exhibited correlations from H_2-2 and H_2-3 to a carbon resonated at δ_C 178.9, leading to an ambiguous assignment of the carboxylic carbon in **5**. In addition, the HMBC correlations from H-10 to C-8 and C-12 and from H_2-12 to C-10 and C-11 confirmed positions of the hydroxyl and double bonds. Therefore, compound **5** was determined as (8*E*,10*E*)-12-hydroxydodeca-8,10-dienoic acid.

The known compounds were identified by comparing their spectroscopic data with the reported data as (6*R*,7*R*,4*E*,8*E*,12*E*)-tetradeca-4,8,12-trien-10-yn-1,6,7-triol[32], (10*E*)-12-hydroxydodeca-10-enoic acid[33], hexadecanoicacid-2′,3′-dihydroxy propyl ester[34], fulgidic acid[35], and pinellic acid[36].

Various biological activities of acetylene and unsaturated fatty acid derivatives were reported, such as adjuvant activity[36], cytotoxicity[38] and antidiabetic activity[39]. This indicates that these types of compounds might play some biological roles in clinical effects of this herbal medicine. In the preliminary *in vitro* assays carried out in this study, the isolates were assessed for inhibitory activity against LPS-induced NO production in BV2-cells[40], protein tyrosine phosphatase 1B (PTP1B) [41], HIV-1 replication[42] and several human cancer cell lines[43], as well as antioxidant activity[44], but all were inactive at a concentration of 10 μmol/L. Therefore, their potential biological activity is still expected from a further in depth evaluation on other biological models.

3. Conclusions

From the aqueous extract of *C. pilosula* roots, four new acetylenes (**1**–**4**) and one new unsaturated ω-hydroxy fatty acid (**5**), together with five known analogues, were isolated. The new acetylenes are categorized as an unusual cyclotetradecatrienynone (**1**), tetradecenynetriol (**2**), and rare octenynoic acids (**3** and **4**), respectively, of which **3** and **4** are possibly derived from oxidative metabolic degradation of **1** and/or **2**. Although the new compounds were inactive in the assays carried out in this study, the results provide a clue for further studies of synthesis, chemical transformation, structural modification and biosynthesis, as well as biological evaluations on other pharmacological models, of the diverse ployacetylene derivatives from the *C. pilosula*.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on P-2000 polarimeter (JASCO, Tokyo, Japan). UV spectra were measured on a V-650 spectrometer (JASCO, Tokyo, Japan). IR spectra were recorded on a

Table 1 ^{1}H and ^{13}C NMR spectral data (δ) for compounds **1–5**.[a]

No.	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1a	3.86 dd (10.8, 4.8)	62.8	3.49 t (6.5)	63.2		170.1
1b	3.45 dd (10.8, 8.4)					
2	2.28 m	48.2	1.48 m	33.9	6.08 d (16.0)	131.9
3a	2.73 ddd (10.8, 6.0, 1.0)	29.9	1.34 m	27.3	6.74 d (16.0)	126.2
3b	2.35 ddd (10.8, 6.0, 2.4)		1.47 m			
4	7.13 ddd (9.6, 6.0, 2.4)	152.4		27.1		85.9
5	6.07 ddd (9.6, 3.0, 0.6)	127.8	1.39 m	34.2		98.3
6		199.6	3.34 q (8.0)	75.3	5.66 d (16.0)	111.7
7		80.2	3.46 m	74.2	6.23 dq (16.0, 7.0)	144.0
8a	6.19 d (15.6)	138.2	1.62 m	33.6	1.77 d (7.0)	19.2
8b			1.57 m			
9	5.99 dd (15.6, 2.4)	113.6	2.34 m	16.9		
10		86.4		88.8		
11		91.2		80.6		
12	5.61 dq (15.6, 1.8)	111.9	5.39 dd (16.5, 1.5)	112.6		
13	6.11 dq (15.6, 6.6)	140.8	5.93 dq (16.5, 7.0)	138.9		
14	1.78 dd (6.6, 1.8)	18.7	1.67 dd (7.0, 1.5)	18.7		

[a]NMR data (δ) were measured in MeOH-d_4 for **2**, **3**, and **4** at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR and **1**, **5** at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on DEPT, ^{1}H-^{1}H COSY, HSQC, and HMBC experiments.

Table 2 ^{1}H and ^{13}C NMR spectral data (δ) for compounds **4** and **5**.[a]

No.	**4**		**5**	
	δ_H	δ_C	δ_H	δ_C
1a		176.8		178.9
1b				
2	2.40 t (7.5)	35.4	2.25 t (7.2)	35.7
3a	2.47 t (7.5)	16.4	1.59 quin (7.2)	26.3
3b			1.33 m	
4		87.6		29.9
5		80.7	1.33 m	30.1
6	5.39 dd (16.0, 1.5)	112.4	1.39 quin (7.2)	30.3
7	5.95 dq (16.0, 6.5)	139.4	2.07 q (7.2)	33.5
8a	1.67 dd (6.5, 1.5)	18.7	5.65 dt (15.0, 7.2)	131.1
8b				
9			6.04 dd (15.0, 4.2)	131.0
10			6.18 dd (15.0, 4.2)	132.5
11			5.68 dt (15.0, 6.0)	135.6
12			4.05 d (6.0)	63.4
13				
14				

[a]NMR data (δ) were measured in MeOH-d_4 for **4** at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR and **5** at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on DEPT, ^{1}H-^{1}H COSY, HSQC and HMBC experiments.

Nicolet 5700 FT-IR microscope instrument (FT-IR microscope transmission) (Thermo Electron Corporation, Madison, USA). NMR spectra were obtained at 500 MHz or 600 MHz for ^{1}H NMR, and 125 MHz or 150 MHz for ^{13}C NMR, respectively, on Inova 500 or SYS 600 (Varian Associates Inc., Palo Alto, USA) or Bruker 600 NMR spectrometers (Bruker Corp. Switzerland) in MeOH-d_4 with solvent peak used as references. ESI-MS and HR-ESI-MS data were measured using an AccuToFCS JMS-T100CS spectrometer (Agilent Technologies, Ltd., Santa Clara, USA). Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), and CHP 20P (Mitsubishi Chemical Inc., Tokyo, Japan). HPLC separation was performed on an instrument consisting of an Agilent ChemStation for LC system, an Agilent 1200 pump, and an Agilent 1100 single-wavelength absorbance detector (Agilent Technologies, Ltd.) with a YMC-Pack Ph (250 mm × 10 mm, i.d.) column packed with Phenyl-silica gels (5 μm) (YMC Co., Ltd.,

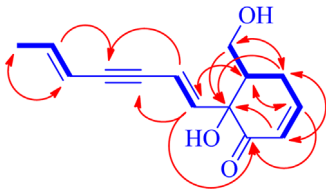

Figure 2 The ^{1}H-^{1}H COSY (thick line) and key HMBC (arrows) correlations of compound **1**.

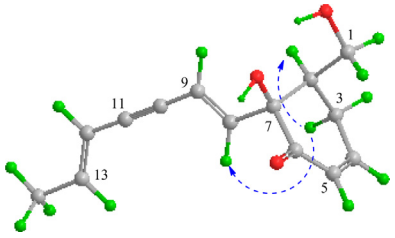

Figure 3 The NOE enhancements induced by irradiation of H-3b (dashed arrows) for compound **1**.

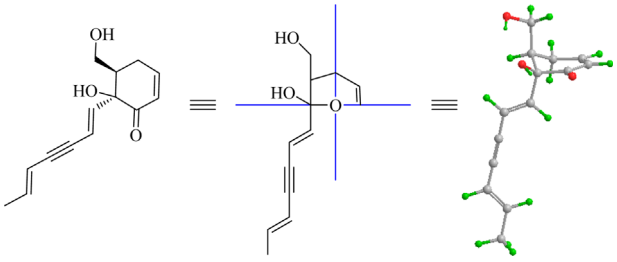

Figure 4 Absolute configuration of compound **1**.

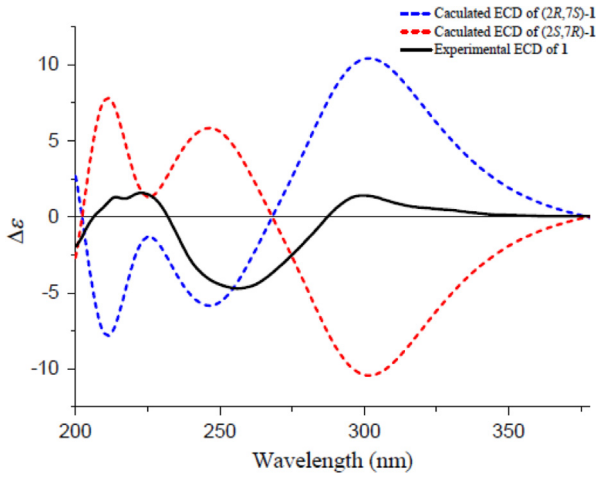

Figure 5 The experimental CD spectrum of **1** (black) and the calculated ECD spectra of (2*R*,7*S*)-**1** (dashed blue) and (2*S*,7*R*)-**1** (dashed red).

Kyoto, Japan) or a Grace (250 mm × 10 mm, i.d.) semipreparative column packed with C18 reversed phase silica gel (5 μm) (W.R Grace & Co., USA). TLC was carried out with glass precoated silica gel GF_{254} plates (Qingdao Marine Chemical Inc.). Spots were visualized under UV light or by spraying with 7% H_2SO_4 in 95% EtOH followed by heating. Unless otherwise noted, all chemicals were obtained from commercially available sources and were used without further purification.

4.2. *Plant material*

The roots of *C. pilosula* were collected in October 2012 from the culture field in Weiyuan, Gansu Province, China. Plant identity was verified by Mr. Lin Ma (Institute of Materia Medica, Beijing, China). A voucher specimen (No. ID-S-2503) was deposited at the herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Beijing, China.

4.3. *Extraction and isolation*

The dried and minced roots of *C. pilosula* (50 kg) were extracted with H_2O (150 L, 3 × 1 h). The aqueous extracts were evaporated under reduced pressure to yield a dark brown residue (26 kg). The residue was dissolved in H_2O (100 L), loaded on a macroporous adsorbent resin (HPD-110, 20 L) column (20 cm × 200 cm), and eluted successively with H_2O (100 L), 50% EtOH (120 L), and 95% EtOH (80 L) to yield three corresponding fractions, A, B and C. Fraction C (31.0 g) was subjected to CC over silica gel, with elution by a gradient of increasing acetone concentration (0–100%) in petroleum ether, to yield fractions C_1–C_{18} based on TLC analysis. Fraction C_{10} (1.0 g) was separated by CC over Sephadex LH-20, eluted with petroleum ether–$CHCl_3$–MeOH (5:5:1, *v*/*v*/*v*), to give C_{10}-1–C_{10}-8. Hexadecanoicacid-2′-3′-dihydroxy propyl ester (7.0 mg) was precipitated from C_{10}-3 (MeOH). Fraction C_{10}-6 (80 mg) was separated by reversed-phase (Phenyl-silica gel) semipreparative HPLC, using MeOH–H_2O (40:60, *v*/*v*) as the mobile phase (1.5 mL/min, UV 254 nm), to yield **1** (1.0 mg, t_R = 36 min). Fraction C_{12} (0.8 g) was separated by CC over Sephadex LH-20, eluted with petroleum ether-$CHCl_3$-MeOH (5:5:1, *v*/*v*/*v*), to give C_{12}-1–C_{12}-8, of which C_{12}-6 (100 mg) was separated by reversed-phase (phenyl-silica gel) semipreparative HPLC, using MeOH–H_2O (42:58, *v*/*v*) as the mobile phase (1.5 mL/min, UV 254 nm), to yield **4** (1.1 mg, t_R = 23 min). Fraction C_{12}-8 (60 mg) was isolated by preparative TLC, using petroleum ether-ethyl acetate (1:1, *v*/*v*) containing 0.1% HOAc as the mobile phase, to give (10*E*)-12-hydroxydodeca-10-enoic acid (6.9 mg). Fraction C_{14} (1.0 g) was separated by CC over Sephadex LH-20, eluted with petroleum ether-$CHCl_3$-MeOH (5:5:1, *v*/*v*/*v*), to give C_{14}-1–C_{14}-8. Fraction C_{14}-4 (80 mg) was separated by reversed-phase (C18) semipreparative HPLC, using MeOH-H_2O (62:38, *v*/*v*) as the mobile phase (1.5 mL/min, UV 220 nm), to

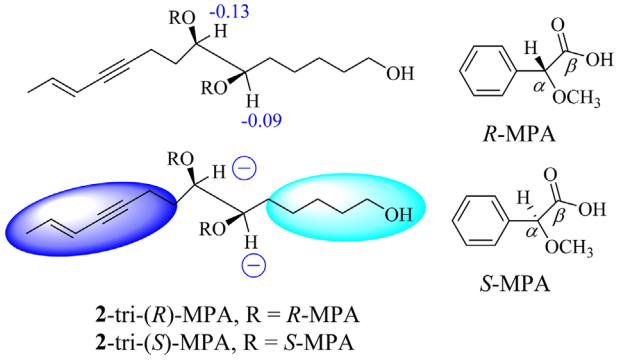

Figure 6 Diagnostic $\Delta\delta_{RS}$ values ($\delta_R - \delta_S$, blue data in ppm, left upper) and applied model for tri-MPA esters of compound **2**.

yield **2** (1.0 mg, t_R=26 min) and **3** (20 mg, t_R=23 min). Fraction C_{14}-6 (60 mg) was purified by reversed-phase (C18) semipreparative HPLC, using MeOH-H_2O (63:37, *v/v*) containing 0.1% HOAc as the mobile phase (1.5 mL/min, UV 220 nm), to yield **5** (1.7 mg, t_R=42 min). Fraction C_{17} (1.1 g) was separated by CC over Sephadex LH-20, eluted with $CHCl_3$-MeOH (1:1, *v/v*), giving C_{17}-1–C_{17}-6. Fraction C_{17}-4 (300 mg) was separated by CC over silica gel, eluted by a gradient of increasing MeOH concentration (0–100%) in $CHCl_3$, yielding C_{17}-4-1–C_{17}-4-8 based on TLC analysis. Fulgidic acid (13.0 mg) was precipitated from C_{17}-4-7 (MeOH). Pinellic acid (15.0 mg) was precipitated from C_{17}-6 (MeOH). Purification of B_3-2-1–2 (100 mg) by RP HPLC (C18) (49% CH_3OH in H_2O), afforded (6*R*,7*R*,4*E*,8*E*,12*E*)-tetradeca-4,8,12-trien-10-yn-1,6,7-triol (2.0 mg, 1.5 mL/min, UV 220 nm, t_R=51 min).

4.3.1. (+)-(2R,7S)-1,7-dihyrdroxy-2,7-cyclotetradeca-4,8,12-trien-10-yn-6-one (*1*)

Yellowish amorphous powder; $[\alpha]_D^{20}$ +10.8 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (logε): 270 (3.22) nm; CD (MeOH) 256 ($\Delta\varepsilon$ −4.71), 299 ($\Delta\varepsilon$ +1.41) nm; IR ν_{max} 3395, 3189, 2921, 2850, 2191, 2062, 1721, 1684, 1647, 1469, 1420, 1134, 1120, 1092, 957, 648 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) data, see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 1; (+)-ESI-MS *m/z* 255 $[M+Na]^+$, 271 $[M+K]^+$; HR-ESI-MS *m/z* 255.0999 $[M+Na]^+$(Calcd. for $C_{14}H_{16}O_3Na$, 255.0992).

4.3.2. (+)-(6R,7R,12E)-tetradeca-12-en-10-yn-1,6,7-triol (*2*)

Yellowish amorphous powder; $[\alpha]_D^{20}$ +41.5 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (logε): 227 (3.60), 265 (2.28 sh), 279 (2.10) nm; IR ν_{max} 3389, 3294, 3197, 2926, 2851, 2217, 1646, 1468, 1419, 1112, 1073, 1045, 954, 723, 654 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) data, see Table 1; ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 1; (+)-ESI-MS *m/z* 241 $[M+H]^+$, 263 $[M+Na]^+$; HR-ESI-MS *m/z* 241.1807 $[M+H]^+$ (Calcd. for $C_{14}H_{25}O_3$, 241.1798), 263.1620 $[M+Na]^+$ (Calcd. for $C_{14}H_{24}O_3Na$, 263.1618).

4.3.3. (2E,6E)-octa-2,6-dien-4-ynoic acid (*3*)

Yellowish amorphous powder; UV (MeOH) λ_{max} (logε) 207 (3.73), 284 (3.97) nm; IR ν_{max} 3395, 3187, 3011, 2922, 2850, 2185, 1647, 1468, 1420, 1302, 1273, 1201, 1120, 971, 946, 722, 648 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) data, see Table 1; ^{13}C NMR (CD_3OD,125 MHz) data, see Table 1; (+)-ESI-MS *m/z* 137 $[M+H]^+$, 159 $[M+Na]^+$, 175 $[M+K]^+$; (−)-ESI-MS *m/z* 135 $[M-H]^-$; HR-ESI-MS *m/z* 137.0595 $[M+H]^+$(Calcd. for $C_8H_9O_2$, 137.0597), 159.0413 $[M+Na]^+$ (Calcd. for $C_8H_8O_2Na$, 159.0417).

4.3.4. (E)-oct-6-en-4-ynoic acid (*4*)

Yellowish amorphous powder; UV (MeOH) λ_{max} (logε): 190 (3.56), 226 (4.02) nm; IR ν_{max} 3393, 3031, 2962, 2852, 2216, 1722, 1558, 1489, 1435, 1413, 1356, 1301, 1020, 955, 837 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) data, see Table 2; ^{13}C NMR (CD_3OD, 125 MHz) data, see Table 2; (+)-ESI-MS *m/z*161 $[M+Na]^+$, 177 $[M+K]^+$; (–)-ESI-MS *m/z* 173 $[M+Cl]^-$; HR-ESI-MS *m/z* 137.0601 $[M-H]^-$ (Calcd. for $C_8H_9O_2$, 137.0608).

4.3.5. (8E,12E)-12-hydroxydodeca-8,10-dienoic acid (*5*)

Yellowish amorphous powder; UV (MeOH) λ_{max} (logε): 195 (3.77), 229 (4.30) nm; IR ν_{max} 3200, 3017, 2922, 2847, 1723, 1571, 1462, 1439, 986, 725 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) data, see Table 2; ^{13}C NMR (CD_3OD, 150 MHz) data, see Table 2; (+)-ESI-MS *m/z* 235 $[M+Na]^+$, 251 $[M+K]^+$; (−)-ESI-MS *m/z* 211 $[M-H]^-$; HR-ESI-MS *m/z* 235.1296 $[M+Na]^+$ (Calcd. for $C_{14}H_{16}O_3Na$, 235.1305).

4.4. ECD calculation of *1*

Conformational analysis of **1** was performed with the MMFF94 molecular mechanics force field using Molecular Operating Environment (MOE) software package[26]. The lowest-energy conformers having relative energies within 2 kcal/mol were optimized with the Gaussian09 program[37]at the B3LYP/6–31+G(d) level in MeOH (Fig. S1, Supporting information). The stabilities of these conformers were confirmed by harmonic vibrational frequency calculations at the B3LYP/6–31+G(d) level. The energies, oscillator strengths, and rotational strengths of the electronicexcitations of the lowest-energy conformers were calculated using the TDDFT method at the B3LYP/6–311++ G(2d,2p) level in MeOH, and ECD spectra were then simulated by the Gaussian function. The final ECD spectrum of **1** was obtained according to Boltzmann weighting of each conformer.

4.5. Synthesis of tri-(R)- and tri-(S)-MPA esters of *2*

Compound **2** (0.3 mg) was stored in 10 mL round-bottomed flask and dried under vacuum. Anhydrous CH_2Cl_2 (3.0 mL) and *R*-MPA or *S*-MPA (5.3 mg), 1-ethyl-3-(3- dimethyllaminopropyl) carbodiimide hydrochloride (EDCI) (6.1 mg), and 4-dimethylaminopyridine (DMAP) (3.9 mg) were added to the round-bottomed flask. The reaction round-bottomed flask was permitted to stand at room temperature for 15 min. The residue obtained after evaporation of the solvent was applied to preparative TLC (petroleum ether-acetone (3:1, *v/v*)) to give **2**-tri-(*R*)-MPA and **2**-tri-(*S*)-MPA. **2**-tri-(*R*)-MPA: ^1H NMR (600 MHz, CD_3OD) δ_H: 7.28–7.37 (15H, m, aromatic H), 5.96 (1H, dq, *J*=15.6, 6.6 Hz, H-13), 5.39 (1H, d, *J*=15.6 Hz, H-12), 4.95 (1H, m, H-7), 4.80 (1H, overlapped, H-6), 4.79 (1H, s, α-H), 4.77 (1H, s, α-H), 4.74 (1H, s, α-H), 3.98 (1H, dt, *J*=4.2, 12.6 Hz, H-1a), 3.90 (1H, dt, *J*=12.6, 6.6 Hz, H-1b), 3.33 (3H, s, α-OCH_3), 3.31 (3H, s, α-OCH_3), 3.30 (3H, s, α-OCH_3), 1.95 (2H, m, H-9), 1.69 (3H, d, *J*=6.6 Hz, H-14), 1.23 (10H, m, H-2, 3, 4, 5, 8); **2**-tri-(*S*)-MPA: ^1H NMR (600 MHz, CD_3OD) δ_H 7.24–7.40 (15H, m, aromatic H), 5.95 (1H, dq, *J*=15.6, 6.6 Hz, H-13), 5.35 (1H, dd, *J*=15.6, 1.8 Hz, H-12), 5.08 (1H, m, H-7), 4.89 (1H, m, H-6), 4.78 (1H, s, α-H), 4.77 (1H, s, α-H), 4.74 (1H, s, α-H), 3.89 (1H, dt, *J*=12.6, 4.8 Hz, H-1a), 3.84(1H, dt, *J*=12.6, 6.0 Hz, H-1b), 3.33 (6H, s, α-OCH_3), 3.31 (3H, s,α-OCH_3), 1.67 (3H, dd, *J*=6.6, 1.8 Hz, H-14), 1.50 (2H, m, H-9), 1.15 (10H, m, H-2, 3, 4, 5, 8).

4.6. Measurement of $Mo_2(OAc)_4$-induced circular dichroism (ICD) spectrum of *2*

According to the published approach, a solution of **2** (0.5 mg) in dry DMSO (1 mL) was mixed with dimolybdenumtetraacetate (1.0 mg). The first CD of the mixture (*ca*. 1:1 diol/dimolybdenumtetraacetate) was recorded immediately after mixing, and its time evolution was monitored until stationary. The observed sign of the diagnostic band at 316 nm in the ICD was correlated to the 6*R*,7*R*-configuration of the 6,7-diol moiety in **2**.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2015.03.005.

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