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Terpenes from *Juniperus przewalskii* and their antitumor activities

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Two new diterpenes were isolated from *Juniperus przewalskii*, together with 17 known terpenes. Their structures were elucidated by spectroscopic methods (IR, MS, ^{1}H, ^{13}C and 2DNMR). In addition, 3α-hinokiol (**3**) and 3α-hydroxymannol (**9**) exhibited effective antitumor activities to cervical carcinoma (HeLa) and human ovaria carcinoma (HO-8910) cell lines.

1. Introduction

Fifteen species of *Juniperus* are widely distributed in China from the north to the south [1]. Among them, *Juniperus przewalskii* grows in the lower parts of the Qinzang plateau, at an altitude of 3000 m above sea level. Its leaf has long been used in China in antitussive and haemostatic drugs [2]. A series of diterpenes and sesquiterpenes have been isolated from species of *Juniperus* as described in previous papers [3–9]. In addition, the diterpenes showed biological activities such as inhibition of PAF [10], antitumor, antileukaemic, antibiotic [11, 12] and insecticidal activity [3]. From the fruits of *J. przewalskii*, a new labdane 15-oxolaba-13(14)-epoxy-8(17)-en-19-oic acid **(2)** and a new abietane 3α-hinokiol **(3)** were has been obtained, in addition to such known diterpenes as 13-epitorulosal **(4)** [13]; 19-acetoxy-13(s)-hydroxylabda-8(17),14-diene **(5)** [13]; 13-epi cupressic acid **(6)** [13]; 3α-hydroxy-labda-8(17),12E,14-trien-19oic acid **(7)** [10]; 3α-hydroxy-labda-8(17),12E,14-triene **(8)** [10]; 3α-hydroxymannol **(9)** [10]; 3α,15-dihydroxy-labda-8(17),13E-diene **(10)** [10]; 3α-acetoxyisocupressic acid **(11)** [10]; 13,14-epoxyimbricatolic acid **(12)** [9]; agatholate **(13)** [14] 4-hydroperoxide-of nor-torulosol **(14)** [15]; isopimara 8(14),15-diene-2α,18-diol **(15)** [16] which is reported as a natural compound for the first time; and sugiol **(16)** [5], and four known sesquiterpenes (+)-8α-acetoxyelemol **(17)** [17]; eudesm-5-ene-1β,4α-diol (**18**) [18]; cryptomeridiol **(19)** [19] and germacra-5,10(14)-dien-1α,4β-diol **(1)** [20]. Their structures were elucidated by spectroscopic methods. The antitumor activities of compounds **3**, **9** and **16** were tested on cervical carcinoma (HeLa) and human ovarian carcinoma (HO-8910) cell lines, only compound **3** and compound **9** showing strong and moderate cytotoxicities to the two kinds of cancer cells.

2. Investigations, results and discussion

The air-dried powdered fruits of *J. przewalskii* were extracted with petroleum ether (60–90 °C) at room temperature. The extract was chromatographed on a silica-gel column with a petroleum ether (60–90 °C)–EtOAc gradient in developing ratio. This resulted in two novel diterpenes, 15-oxolaba-13(14)-epoxy-8(17)-en-19-oic acid **(2)** and 3α-hinokiol **(3)**, together with 17 known compounds.

The molecular formula of compound **2** was determined as $C_{20}H_{30}O_4$ by the molecular ion peak $[M]^+ = 334$ in the EIMS spectrum and the ^{13}C NMR and DEPT data (Table 1). Its IR spectrum include absorption peaks at 1720 cm^{-1} (CHO) and 3320 cm^{-1} (COOH). In ^{1}H NMR (Table 2) three methyl groups appeared at δ 0.60 (s, 3 H) δ 1.24 (s, 3 H) and δ 1.44 (s, 3 H) and characteristic methylene signals appeared at δ 4.48 (brs, 1 H), δ 4.87 (brs, 1 H), indicating that **2** had a labdane skeleton, like the 8(17)-ene labdanes reported in the literature [9, 10, 13]. The ^{13}C NMR spectrum showed an epoxy group δ 56.3 (CH) δ 64.5 (C), corresponding to the signal δ 3.15 (d, J = 5.2 Hz 1 H) and δ 9.36 (d, J = 5.2 Hz, 1 H) in the ^{1}H NMR. Comparing its ^{1}H NMR spectra with those of 13(14) epoxy-diterpenes in the literature [21], its structure deduced as 15-oxolaba-13(14)-epoxy-8(17)-en-19-oic acid.

The formula of compound **3** was deduced as being $C_{20}H_{30}O_2$ from the molecular ion peak $[M]^+ = 302$ in the EIMS spectrum. While the ^{13}C NMR and DEPT data showed 5 × CH_3, 4 × CH_2; 5 × CH; and 6 × C (Table 1), δ 6.63 (s, 1 H) and δ 6.83 (s, 1 H) in the ^{1}H NMR demonstrated 1,2,4,5-tetra benzene. There were also five methyl groups in the ^{1}H NMR δ 0.95 (s, 3 H); δ 1.03 (s, 3 H); δ 1.19 (s, 3 H) and δ 1.23 (d, J = 7.2 Hz, 6 H) and an oxygenated proton δ 3.49 (t, J = 2.8 Hz, 1 H). The ^{1}H-

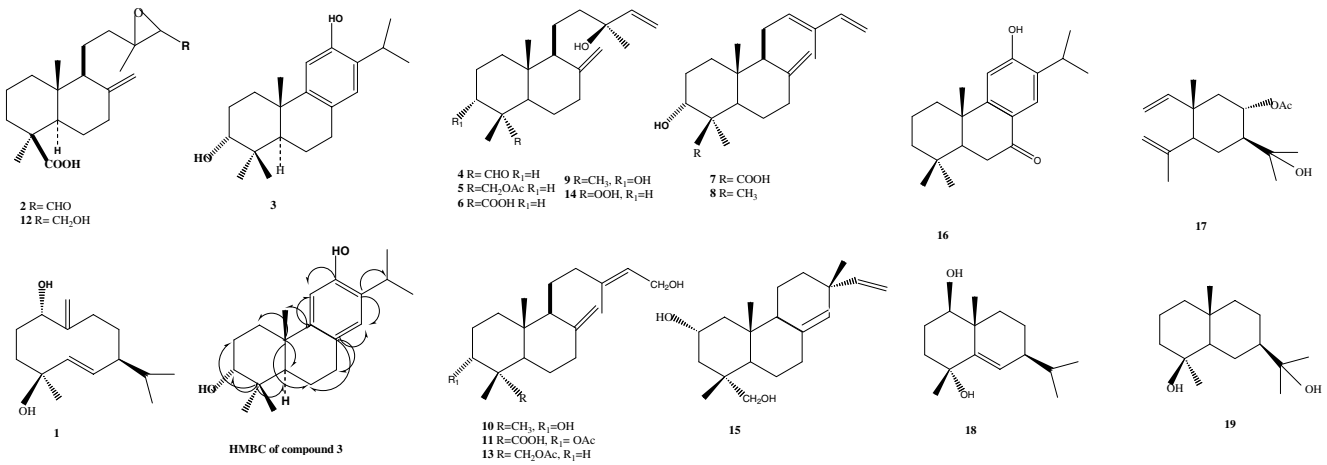

Table 1: ^{13}C NMR data of compounds 2 and 3[a]

C	2	3	C	2	3
1	39.2 (CH_2)	31.6 (CH_2)	11	32.1 (CH_2)	110.9 (CH)
2	19.9 (CH_2)	25.9 (CH_2)	12	32.6 (CH_2)	150.7 (C)
3	37.9 (CH_2)	75.8 (CH)	13	64.5 (C)	131.5 (C)
4	44.2 (C)	37.5 (C)	14	56.3 (CH)	126.6 (CH)
5	55.5 (CH)	43.6 (CH)	15	199.8 (CH)	26.8 (CH)
6	26.1 (CH_2)	18.8 (CH_2)	16	17.4 (CH_3)	22.5 (CH_3)
7	38.6 (CH_2)	29.5 (CH_2)	17	106.9 (CH_2)	22.8 (CH_3)
8	147.5 (C)	127.2 (C)	18	22.2 (CH_3)	22.1 (CH_3)
9	55.9 (CH)	148.3 (C)	19	182.2 (C)	28.1 (CH_3)
10	40.6 (C)	37.7 (C)	20	12.7 (CH_3)	24.6 (CH_3)

^{13}C NMR, 100 Hz, $CDCL_3$, TMS, δ, ppm; [a] DEPT data in parentheses

^{1}H COSY and HMQC studies indicated fragments such as: $-CH_2CH_2CHOH-$; $-CHCH_2CH_2-$ and CH_3CHCH_3 and two aromatic CH groups. HMBC was then used to join these fragments and groups into an abietane skeleton, whose planar structure is the same as hinokiol [22]. However in the ^{1}H- ^{1}H COSY, H-3 in this compound coupled with H-2α, H-2β only in 2.8 Hz, showing that H-3β was in equitol orientation contrary to δ 3.35 (dd, $J_{2\beta,3\alpha} = 8.5$, $J_{2\alpha,3\alpha} = 5$ Hz, 1 H) of H-3α in hinokiol. Finally, CH_3-20 showed a cross peak with H-7β in the NOESY spectrum, but no cross peak with H-5, indicating trans orientation of the A and B rings with H-5α. Thus this compound was deduced as being 3α-hinokiol.

3. Experimental

3.1. Equipment

Optical rotation: Perkin-Elmer 241 polarimeter solvent MeOH, IR spectra were taken on a Nicolet 170sx FT-IR spectrometer. ^{1}H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$) spectra and 2D NMR spectra (HMQC, HMBC) were recorded on a Bruker AM 400FT-NMR spectrometer with TMS as internal standard. EIMS data were obtained on a HP-5988 MS spectrometer. Silica gel (200–300 mesh) was used for CC and silica GF_{254} for TLC. Spots were detected on TLC under UV or by heating after spraying with 5% H_2SO_4 in C_2H_5OH.

3.2. Plant material

The fruit of *Juniperus przewalskii* was collected in Sept. 1999, in Luqu County, Gansu Province, People's Republic of China, and was identified by Prof. Y. J. Zhang, Department of Biology, Lanzhou University. A voucher specimen (No. 990923) is deposited in Department of Chemistry Lanzhou University.

3.3. Extraction and isolation

Air dried and powdered fruits of *J. przewalskii* (1.0 kg) were extracted with petroleum ether (60–90 °C) to give a residue of 73.0 g. The residue was put on a silica-gel column with petroleum ether (60–90 °C)–EtOAc as a developing gradient yielding five fractions. Compound **8** (5 mg) was purified by PTLC ($CHCl_3$–acetone 40 : 1) from Fr. 1 (petroleum ether (60–90 °C)–EtOAc 20 : 1). From Fr. 2 (petroleum ether (60–90 °C)–EtOAc 10 : 1) compounds **4** (10 mg), **14** (5 mg), **5** (7 mg), **16** (30 mg) and **17** (5 mg) were obtained by repeated silica-gel column chromatography with petroleum ether (60–90 °C)–EtOAc as eluant. When rechromatographed on a silica-gel column with petroleum ether (60–90 °C)–EtOAc then $CHCl_3$–acetone, compounds **3** (80 mg), **7** (6 mg), **6** (5 mg) and **13** (4 mg) were obtained from Fr. 3 (petroleum ether (60–90 °C)–EtOAc 8 : 1). Compound **2** (2 mg) was obtained on a silica-gel column with petroleum ether (60–90 °C)–EtOAc 6 : 1 from Fr. 4 (petroleum ether (60–90 °C)–EtOAc 5 : 1), from which compound **12** (3 mg), **9** (70 mg), **18** (8 mg) and **11** (7 mg) were also obtained. Fr. 5 (petroleum ether (60–90 °C)–EtOAc 3 : 1) was again chromatographed on a silica-gel column to give compound **10** (8 mg), and then crude **1** was purified by silica CC with $CHCl_3$–acetone 40 : 1 to give **1** (8 mg), while compounds **19** (3 mg) and **15** (4 mg) were also obtained from Fr. 5 by repeated chromatography on silica CC with petroleum ether (60–90 °C)–EtOAc 3 : 1 and $CHCl_3$–acetone 5 : 1.

3.4. 15-Oxolaba-13(14)-epoxy-8(17)-en-19-oic acid (2)

Colorless oil, $[\alpha]_D^{20}$: +46.0 (MeOH, c 0.5,); Rf. 0.30 (petroleum ether (60–90 °C)–EtOAc 5 : 1); IR (ν_{max}^{KBr}, cm^{-1}): 1720 (CHO), 3320 (COOH) 890 (C=CH_2); EIMS (m/z, %): 334 $[M]^+$ (3), 316 $[M\text{-}H_2O]^+$ (10), 287(3), 235(9), 121(57), 55(54), 43(100); ^{13}C NMR data (Table 1) and ^{1}H NMR data (Table 2).

3.5. 3α-Hinokiol (3)

Colorless needle crystals, m.p. = 214–215 °C, $[\alpha]_D^{20}$: +44.4 (MeOH, c 0.23,); Rf. 0.51 ($CHCl_3$–acetone 10 : 1); IR (ν_{max}^{KBr}, cm^{-1}): 3511, 3313, 860; EIMS (m/z, %): 302 $[M]^+$ (29), 287 $[M\text{-}H_2O]^+$ (4), 269(100), 227(5), 147(98), 43(11); ^{13}C NMR data (Table 1) and ^{1}H NMR data (Table 2).

3.6. Antitumor assays

The 50% inhibition concentration (IC_{50} μg/ml) of compounds was tested in human cervical carcinoma (HeLa) and human ovaria carcinoma (HO-8910) cell lines is shown in Table 3.

Cancer cells numbers were measured by the MTT method. IC_{50} of compounds **9** and **3** were higher than that of vincristine in HeLa cells, and IC_{50} of both compounds in HO-8910 cells were very close to that of vincristine, especially IC_{50} of compound **3** which was slightly lower than that of vincristine. It is indicated that compound **3** significantly inhibits human ovaria carcinoma cells.

Table 3: IC_{50} (μg/ml) of compounds tested

Compd.	HeLa cells	HO-8910 cells
Vincristine	83.6	67.4
9	107.6	74.7
3	121.3	63.1

Table 2: ^{1}H NMR data of compounds 2 and 3[a]

H	2	3	H	2	3
1α	1.83 (brd, 10.0)	1.05 (ddd, 11.3, 8.0, 5.0)	11	1.08 (m)	6.63 (s)
1β	1.31 (m)	1.93 (dt, 11.3, 4.0)	12	1.05 (m)	–
2α	1.59 (m)	2.09 (m)	13	–	–
2β	1.59 (m)	1.86 (m)	14	3.15 (d, 5.2)	6.83 (s)
3α	2.17 (brd, 13.6)	–	15	9.36 (d, 5.2)	3.10 (qq, 8.0)
3β	1.60 (m)	3.49 (t, 2.8)	16	1.44 (s)	1.23 (d, 8.0)
4	–	–	17	4.48 (brs), 4.87 (brs)	1.23 (d, 8.0)
5α	1.05 (dd, 13.0, 3.0)	1.74 (dd, 11.0, 4.0)	18	1.24 (s)	0.95 (s)
6α	1.54 (m)	1.77 (m)	19	–	1.03 (s)
6β	1.33 (m)	1.77 (m)	20	0.60 (s)	1.19 (s)
7α	1.96 (ddd, 10.8, 4.0, 2.8)	2.78 (ddd, 12.8, 10.3, 4.0)			
7β	2.39 (dt, 10.8, 3.8)	2.85 (ddd, 12.8, 5.6, 1.5)			
8	–	–			
9α	1.86 (t, 7.0)	–			
10	–	–			

^{1}H NMR, 400 Hz, $CDCl_3$, TMS, δ, ppm; [a] Coupling constants in parentheses in Hz

Acknowledgements: This work was supported by the NNSFC (No. 29972017) and the FMEC (No. 98073003)

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Received July 22, 2001
Accepted October 19, 2001

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