Three New C-14 Oxygenated Taxanes from the Wood of *Taxus yunnanensis*

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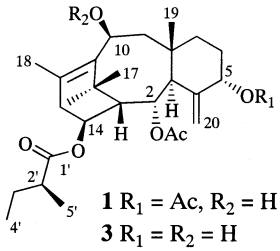
Three new C-14 oxygenated taxane-type diterpenes, hongdoushans A−C (**1**−**3**), were isolated from the wood of *Taxus yunnanensis* together with four known diterpenes and two lignans. The absolute stereochemistry of the 2-methylbutyryloxy group attached at C-14 of the taxane skeleton was determined to be *S* by GC analysis of the methyl ester of 2-methylbutyric acid obtained after alkaline hydrolysis of **1** and **4** followed by treatment with CH_2N_2. The complete stereostructure of the known compound 2α,5α,-10β-triacetoxy-14β-[(*S*)-2-methylbutyryloxy]-4(20),11-taxadiene (**4**) was established for the first time. The isolates obtained were evaluated for their antiproliferative activity toward murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cell lines.

The discovery of paclitaxel (Taxol) as a potent anticancer drug, initially isolated from *Taxus brevifolia*, has encouraged several groups all over the world to conduct research work on other *Taxus* species, to isolate potentially more effective paclitaxel derivatives for the treatment of various cancers or as starting materials for semisynthesis.[1] As a consequence, more than 350 taxane-type diterpenoids have been isolated from various *Taxus* plants, and some of them possess interesting anticancer activity.[1] *Taxus yunnanensis* Cheng et L. K. Fu (Taxaceae), an evergreen tree commonly known as "Hongdoushan" and distributed mainly in Yunnan Province of the People's Republic of China,[2] is considered as a promising source of taxane-type diterpenoids. Several taxol derivatives together with rearranged taxanes were reported from the leaves, roots, seeds, bark, and stems of this plant.[3] The wood of *T. yunnanensis* is used as a traditional Chinese medicine by several ethnic groups in Yunnan Province. In the present study, we have investigated the chemical constituents of the wood of *T. yunnanensis* and subjected these isolates to biological evaluation against two cancer cell lines.

The dried powder of the wood was successively extracted with H_2O, MeOH/H_2O (1:1), and MeOH under reflux for 30 min. The H_2O extract of the wood contained lignans, secoisolariciresional,[4] taxiresinol,[5] and isotaxiresinol[5,6] as major constituents. The combined CH_2Cl_2-soluble fractions of the MeOH and MeOH/H_2O (1:1) extracts, on the other hand, led to the isolation of three new diterpenes, which were given the trivial names hongdoushans A−C (**1**−**3**) according to the local name of the title plant, together with 2α,5α,10β-triacetoxy-14β-[(*S*)-2-methylbutyryloxy]-4(20),-11-taxadiene (**4**),[7] whose complete stereostructure has been confirmed for the first time. Three taxanes, taxusin,[8] 10-desacetyltaxuyunnanine C,[9] and taxuyunnanine E,[10] were also isolated from the CH_2Cl_2-soluble fractions, together with two lignans, α-conidendrin[11] and secoisolariciresinol (**5**).

2α,5α,10β-Triacetoxy-14β-[(*S*)-2-methylbutyryloxy]-4(20),-11-taxadiene (**4**) was isolated as a colorless amorphous solid with the molecular formula $C_{31}H_{46}O_8$ calculated from HRFABMS. Both the ^1H and ^{13}C NMR data of **4** were found to be identical to those of 2α,5α,10β-triacetoxy-14β-(2-methylbutyryloxy)-4(20),11-taxadiene, whose absolute stereochemistry at C-2 of the 2-methylbutyryloxy moiety still remains unsolved.[7] In the present study, the absolute stereochemistry of 2-methylbutyric acid obtained after alkaline hydrolysis of **4** was determined to be *S* by GC analysis of its methyl ester using a chiral column. The t_R of the hydrolyzed product was 12.3 min, the same as that of the standard methyl ester of (*S*)-2-methylbutyric acid. Co-injection of the sample with a standard sample was also performed for confirmation of the peak identity.



1 R_1 = Ac, R_2 = H
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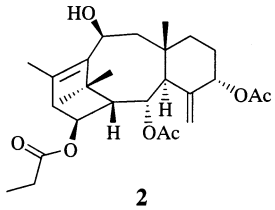
4 R_1 = R_2 = Ac



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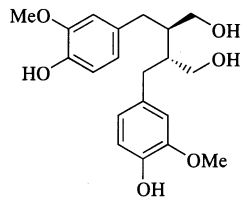

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Hongdoushan A (**1**) was also isolated as a colorless amorphous solid with $[\alpha]^{25}_D$ +81.3° (*c* 0.06, $CHCl_3$). The molecular formula of **1** was determined to be $C_{29}H_{44}O_7$ by HRFABMS. The ^1H NMR spectrum of **1** displayed the signals of two acetyl methyls (δ 2.17, 2.01) together with four quaternary methyls (δ 1.98, 1.73, 1.19, 0.85), four oxygenated methines (δ 5.36, dd, J = 6.3, 2.2 Hz, H-2; δ 5.10, dd, J = 11.7, 5.6 Hz, H-10; δ 5.28, t, J = 3.0 Hz, H-5; δ 4.99, dd, J = 9.2, 4.7 Hz, H-14), two exo-olefinic protons (δ 5.25 and 4.81, br s, H_2-20), and a 2-methylbutyryl group (δ 2.35,1H, m, H-2′; δ 1.63 and 1.46 each 1H, m, H-3′; δ 1.12, 3H, d, J = 6.9 Hz, H_3-5′; δ 0.88, 3H, t, J = 7.3 Hz,

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Table 1. ^{1}H and ^{13}C NMR Data of Hongdousans A−C (**1**−**3**)[a]

	1			**2**			**3**		
position	δ_H	δ_C	HMBC[b]	δ_H	δ_C	HMBC[b]	δ_H	δ_C	HMBC[b]
1	1.86 d (2.2)	59.4	3, 13, 16, 17	1.87 d (2.2)	59.3	2, 3, 13, 14, 16, 17	1.81 d (2.2)	59.5	3, 13, 16, 17
2	5.36 dd (6.3, 2.2)	70.6	3, 14	5.36 dd (6.3, 2.2)	70.7	1, 3, 14	5.35 dd (6.3, 2.2)	71.1	3, 14
3	2.94 d (6.3)	41.9	1, 5, 19, 20	2.93 d (6.3)	41.9	2, 19, 20	3.21 d (6.3)	39.8	2, 20
4		142.3	3, 20		142.4	3, 20		147.8	3
5	5.28 t (3.0)	78.2	3, 20	5.28 t (3.0)	78.4	3, 6, 7, 20	4.17 t (3.0)	76.4	3, 20
6	1.80 m 1.76 m	28.9	7	1.80 m 1.76 m	29.7	7	1.70 m 1.63 m	30.9	7
7	1.92 m 1.21 m	33.9	3, 5, 9, 19	1.92 m 1.21 m	33.9	3, 5, 6, 9	2.05 m 1.63 m	33.2	5, 9, 19
8		39.5	2, 3, 9		39.5	2, 3, 6, 7, 9, 19		40.0	3, 9, 19
9	2.35 m 1.64 dd (15.1, 5.9)	47.1	10, 19	2.35 dd (15.1, 11.9) 1.67 dd (15.1, 5.9)	47.2	10, 19	2.35 m 1.63	47.1	10, 19
10	5.10 dd (11.7, 5.6)	67.2	9	5.11 dd (11.7, 5.6)	67.4	9	5.14 dd (11.7, 5.6)	67.6	9
11		138.7	1, 10, 13, 16, 17, 18		138.7	10, 13, 16, 17, 18		137.9	1, 9, 13, 16, 17, 18
12		132.1	10, 13, 18		132.4	10, 13, 14, 18		133.7	10, 13, 18
13	2.83 dd (19.1, 9.2) 2.35 m	39.6	18	2.81 dd (19.1, 9.2) 2.40 dd (19.1, 4.7)	39.6	1, 14, 18	2.77 dd (18.7, 9.2) 2.35 m	39.5	1, 18
14	4.99 dd (9.2, 4.7)	70.2	2, 13	5.00 dd (9.2, 4.7)	70.6	1, 2, 13	5.03 dd (9.2, 4.7)	70.4	13
15		37.3	10, 14, 16, 17		37.4	1, 10, 14, 16, 17		37.5	1, 10, 14, 16, 17
16	1.73 s	25.3	17	1.72 s	25.4	17	1.71 s	25.4	17
17	1.19 s	31.9	16	1.18 s	32.1	16	1.19 s	32.1	16
18	1.98 s	21.0	13	1.98 s	21.1	13	1.97 s	21.0	13
19	0.85 s	22.5	3	0.84 s	22.5	3, 9	0.81 s	22.3	3
20	5.25 brs 4.81 brs	116.6	3	5.25 brs 4.88 brs	116.8	3, 5	5.09 brs 4.75 brs	113.5	3
1′		175.6	14, 2′, 3′, 5′		173.4	14, 2′, 3′		175.9	14, 2′, 3′, 5′
2′	2.35 m	41.0	3′, 4′, 5′	2.28 q (7.5)	28.0	3′	2.35 m	41.1	3′, 4′, 5′
3′	1.63 m 1.46 m	26.6	2′, 4′, 5′	1.10 t (7.5)	9.2	2′	1.63 m 1.46 m	26.8	2′, 4′, 5′
4′	0.88 t (7.3)	11.5	2′, 3′				0.89 t (7.3)	11.5	2′, 3′
5′	1.12 d (6.9)	16.5	2′, 3′				1.12 d (7.1)	16.6	2′, 3′
OAc-2	2.17 s	21.3[c] 169.8	2, −$COCH_3$	2.17 s	21.8 170.0	2, −$COCH_3$	2.00 s	21.3 169.8	2, −$COCH_3$
OAc-5	2.01 s	21.4[c] 169.8	-$COCH_3$	2.04 s	21.4 169.7	5, −$COCH_3$			

[a] The ^{1}H and ^{13}C NMR spectra were measured at 400 and 100 MHz, respectively, in $CDCl_3$, and coupling constants (parentheses) are in hertz. [b] ^{1}H correlating with ^{13}C resonance. [c] Values are interchangeable within the same column.

H_3-4′). These signals are almost identical to those of **4** except for the absence of one acetyl group, which was confirmed by the molecular weight being 42 amu less than **4** and by the 29 carbon signals in the ^{13}C NMR spectrum. The high-field shift of H-10 and C-10 signals in **1** (δ_H 5.10, δ_C 67.2) in comparison with those of **4** (δ_H 6.06, δ_C 70.1) suggested that **1** is a 10-desacetyl derivative of **4**, which was substantiated by long-range correlations observed in the HMBC spectrum (Table 1). The stereochemistry of the taxane unit was determined to be 2α,5α-diacetoxy, 10*β*-hydroxy, and 14*β*-2-(methylbutyryloxy), on the basis of coupling constants and NOE correlations observed in NOE difference experiments; NOEs were observed from H_3-16 to H-2, from H_3-18 to H-10, and from H-3 to H-14. The stereochemistry on the 2-methylbutyryloxy group was determined to be *S* by GC analysis of the hydrolyzed product as described above for **4**. Thus, the structure of hongdoushan A was determined to be **1**. Hongdoushan C (**3**) also displayed spectral data similar to that of **1** except for the absence of the acetyl group at C-5, which was confirmed by a detailed spectral analysis. Moreover, the molecular weight of **3** was 42 amu less than **1**, in accord with the loss of an acetyl group. Although due to the meager amount obtained, the absolute stereochemistry of the 2-methylbutyryloxy group of **3** could not determined, and the ^{1}H NMR signals of 2-methylbutyryloxy group of **3** were found to be identical to those of **1**. Thus, the structure of hongdoushan C was concluded to be **3**.

Hongdoushan B (**2**), with $[\alpha]^{25}_D$ +68.9° (*c* 0.08, $CHCl_3$), showed a sodiated molecular ion at *m*/*z* 499.2690 in the HRFABMS, suggesting the molecular formula of **2** to be $C_{27}H_{40}O_7$. The ^{1}H NMR spectrum of **2** showed signals corresponding to two acetyl methyls, four quaternary methyls, four oxygenated methines, and two exo-olefinic protons, identical to those of **1** (Table 1), indicating that **2** also bears 2α,5α-diacetoxy and 10*β*-hydroxy groups. Instead of the signals for a 2-methylbutyryloxy group as in **1**, **2** showed signals corresponding to a propanoyloxy group (δ_H 2.28, 3H, q, J = 7.5 Hz, H_3-2′; δ_H 1.10, 3H, t, J = 7.5 Hz, H_3-3′; δ_C 173.4, C-1′; δ_C 28.0, C-2′; δ_C 9.2, C-3′). The position of the propanoyloxy group was confirmed to be at C-14 of the taxane skeleton on the basis of long-range correlations between H-14 and C-1′ in the HMBC spectrum; accordingly the planar structure of **2** was determined. The stereochemistry of **2** was determined to be 2α,5α-diacetoxy, 10*β*-hydroxy, and 14*β*-propanoyloxy by coupling constant analysis and an NOE experiment, as in the case of **1**. Thus, the structure of hongdoushan B was concluded to be **2**.

All of the compounds isolated were tested for their antiproliferative activity against the murine colon 26-L5

Table 2. Antiproliferative Activity of Isolated Compounds from the Wood of *T. yunnanensis* (EC_{50} values are in μg/mL)[a]

compound	HT-1080	colon 26-L5
hongdoushan A (**1**)	61.0	40.1
hongdoushan B (**2**)	>100	70.4
hongdoushan C (**3**)	61.1	3.8
2α,5α,10β-triacetoxy-14β-[(*S*)-2-methylbutyryloxy]-4(20),11-taxadiene (**4**)	84.9	84.1
secoisolariciresinol (**5**)	5.9	60.2
10-desacetyltaxuyunnanine C	76.1	53.8
taxusin	61.4	51.7
5-fluorouracil	0.29	0.07

[a] EC_{50} values were calculated from the mean of data from four determinations; an EC_{50} value > 10 μg/mL was considered as inactive. α-Conidendrin and taxuyunnanine E exhibited an EC_{50} value > 100 μg/mL for both cell lines.

carcinoma and human HT-1080 fibrosarcoma cell lines. The results are summarized in Table 2 in terms of their EC_{50} values. Almost all compounds including the taxanes possessed weak antiproliferative activity toward the tested cell lines except for secoisolariciresinol (**5**), having an EC_{50} value of 5.9 μg/mL toward the HT-1080 fibrosarcoma cell line, and hongdoushan C (**3**), with an EC_{50} value of 3.8 μg/mL toward the colon 26-L5 carcinoma cell line.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solution. Both 1D and 2D NMR data were taken on a JEOL LA-400 spectrometer with tetramethylsilane (TMS) as an internal standard. HRFABMS measurements were carried out on a JEOL JMS-700T spectrometer with glycerol as the matrix. Column chromatography was performed with normal-phase silica gel (Fuji Silysia, BW-820 MH). Analytical and preparative TLC were carried out on precoated Merck Kieselgel $60F_{254}$ plates (0.25 or 0.50 mm thickness). Gas chromatography was performed on a Shimadzu GC-14AH gas chromatograph using a Chiraldex G-TA column (ASTEC, Whippany, NJ) with nitrogen as carrier gas. Authentic (*S*)- and (*R*)-2-methylbutyric acids were purchased from Aldrich, Milwaukee, WI.

Plant Material. The wood of *T. yunnanensis* was collected from Mt. Laojunshan at an altitute of 3800 m, 100 km west of Lijiang City, Yunnan Province, People's Republic of China, in October 2000. A voucher sample (TMPW 21495) is preserved in the Museum for Materia and Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and Isolation. The wood of *T. yunnanensis* was chopped into small pieces and crushed into a powder form. The dried powder (850 g) was extracted with H_2O (4 L × 3) under reflux for 30 min to yield a H_2O extract (52.2 g). The residue was further extracted with MeOH/H_2O (1:1) (4 L × 3) and MeOH (4 L × 3) to give the MeOH/H_2O extract (32.2 g) and the MeOH extract (7.2 g), respectively. The H_2O extract was divided into EtOAc-soluble (34.1 g) and -insoluble (16.1 g) parts, and the EtOAc-soluble fraction yielded three lignans, secoisolariciresional (**5**, 3.62 g),[4] taxiresinol (0.84 g),[5] and isotaxiresinol (7.8 g),[5,6] as major constituents by silica gel column chromatography eluted with a gradient mixture of $CHCl_3$ and MeOH. Both the CH_2Cl_2-soluble fractions of the MeOH/H_2O extract (1:1) (5.5 g) and the MeOH extract (2.7 g) showed comparable TLC patterns. Thus, they were combined (7.0 g) and subjected to silica gel column chromatography (60 × 3.5 cm) with a gradient mixture of $CHCl_3$ and MeOH to give seven fractions [fraction 1, $CHCl_3$, 100 mg; fraction 2, 1% MeOH−$CHCl_3$, 1.88 g; fraction 3, 1% MeOH/$CHCl_3$, 580 mg; fraction 4, 1% MeOH/$CHCl_3$, 260 mg; fraction 5, 2% MeOH/$CHCl_3$, 515 mg; fraction 6, 5% MeOH/$CHCl_3$, 1.58 g; fraction 7, 5−30% MeOH/$CHCl_3$, 2.03 g]. Further silica gel column chromatography and reversed-phase preparative TLC (CH_3CN/MeOH/H_2O, 1:1:2) of fractions 2 and 3 yielded the following compounds: fraction 2, **1** (15.5 mg), **4** (68.0 mg),[7] taxusin (95.6 mg),[8] α-conidendrin (37.1 mg),[11] taxuyunnanine E (1.9 mg);[10] fraction 3, **2** (35.6 mg), **3** (2.9 mg), and 10-desactyltaxuyunnanine C (2.6 mg).[9] Secoisolariciresional (**5**)[4] was obtained from fractions 6 (938 mg) and 7 (600 mg) by fractional crystallization. The physical as well as spectral data of the known compounds were found to be identical to those published in the literature.

Hongdoushan A (1): colorless amorphous solid; $[\alpha]^{25}_D$ +81.3° (*c* 0.06, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3600, 1780, 1370, 1210, 1020 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 527.2950 [calcd for $C_{29}H_{44}O_7Na$ $(M + Na)^+$, 527.2984].

Hongdoushan B (2): colorless amorphous solid; $[\alpha]^{25}_D$ +68.9° (*c* 0.08, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3600, 1730, 1230, 1020 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 499.2690 [calcd for $C_{27}H_{40}O_7Na$ $(M + Na)^+$, 499.2672].

Hongdoushan C (3): colorless amorphous solid; $[\alpha]^{25}_D$ +77.4° (*c* 0.14, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3600, 1750, 1460, 1370, 1250 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 485.2858 [calcd for $C_{27}H_{42}O_6Na$ $(M + Na)^+$, 485.2879].

Alkaline Hydrolysis of 1 and 4. A solution of either **1** or **4** (5.0 mg) in MeOH (2.0 mL) and 1 N KOH (2.0 mL) was stirred overnight at room temperature. The reaction mixture was neutralized with 1 N HCl and extracted with EtOAc (10 mL × 2). The EtOAc layer was evaporated, and the residue was dissolved in MeOH (1 mL) and treated with excess CH_2N_2. After evaporation, the residue was dissolved in $CHCl_3$ (5.0 mL) and filtered. An aliquot of the filtrate was analyzed by GC (column, Astec Chiraldex G-TA G0012-08, 30 m × 0.25 mm; column temperature, 50 °C; detector temperature, 250 °C; injection temperature, 250 °C), to give a peak at t_R 12.3 min. The standard methyl ester of (*R*)- or (*S*)-2-methylbutyric acid gave a peak at t_R 11.1 and 12.3 min, respectively.

Antiproliferative Activity. Cellular viability in the presence and absence of experimental agents was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5- dimethyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assays as described previously.[12] Compounds with an EC_{50} value of >10 μg/mL are considered inactive.

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