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 CH₂N₂. The complete stereostructure of the known compound 2α , 5α ,-10 β -triacetoxy-14 β -[(*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 H₂O, MeOH/H₂O (1:1), and MeOH under reflux for 30 min. The H₂O extract of the wood contained lignans, secoisolariciresional,⁴ taxiresinol,⁵ and isotaxiresinol^{5,6} as major constituents. The combined CH₂Cl₂-soluble fractions of the MeOH and MeOH/H₂O (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 10desacetyltaxuyunnanine C,9 and taxuyunnanine E,10 were also isolated from the CH₂Cl₂-soluble fractions, together with two lignans, α -conidendrin¹¹ and secoisolariciresinol **(5)**.

 $2\alpha,5\alpha,10\beta$ -Triacetoxy-14 β -[(*S*)-2-methylbutyryloxy]-4(20),-11-taxadiene (**4**) was isolated as a colorless amorphous solid with the molecular formula C₃₁H₄₆O₈ calculated from HRFABMS. Both the ¹H and ¹³C NMR data of **4** were found to be identical to those of $2\alpha,5\alpha,10\beta$ -triacetoxy-14 β -(2methylbutyryloxy)-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 $t_{\rm R}$ 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.

Hongdoushan A (1) was also isolated as a colorless amorphous solid with $[\alpha]^{25}{}_{\rm D}$ +81.3° (*c* 0.06, CHCl₃). The molecular formula of 1 was determined to be C₂₉H₄₄O₇ 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, H₂-20), 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, H₃-5'; δ 0.88, 3H, t, J = 7.3 Hz,

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	1			2			3		
position	$\delta_{ m H}$	$\delta_{\rm C}$	HMBC ^b	$\delta_{ m H}$	$\delta_{\rm C}$	HMBC ^b	$\delta_{ m H}$	$\delta_{\rm C}$	$HMBC^{b}$
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	28.9	7	1.80 m	29.7	7	1.70 m	30.9	7
	1.76 m			1.76 m			1.63 m		
7	1.92 m	33.9	3, 5, 9, 19	1.92 m	33.9	3, 5, 6, 9	2.05 m	33.2	5, 9, 19
	1.21 m			1.21 m			1.63 m		
8		39.5	2, 3, 9		39.5	2, 3, 6, 7, 9, 19		40.0	3, 9, 19
9	2.35 m	47.1	10, 19	2.35 dd (15.1, 11.9)	47.2	10, 19	2.35 m	47.1	10, 19
	1.64 dd (15.1,			1.67 dd (15.1,			1.63		
	5.9)			5.9)					
10	5.10 dd (11.7,	67.2	9	5.11 dd (11.7,	67.4	9	5.14 dd (11.7,	67.6	9
	5.6)			5.6)			5.6)		
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	39.6	1, 14, 18	2.77 dd (18.7, 9.2) 2.35 m	39.5	1, 18
	2.00 111			4 7)			2.00 11		
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	1)	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}		2.17 s	21.8		2.00 s	21.3	
	0.04	169.8	2, -COCH ₃	0.04	170.0	2, -COCH ₃		169.8	2, -COCH ₃
UAc-5	2.01 s	21.4 ^c 169.8	-COCH ₃	z.04 s	21.4 169.7	5, -COCH ₃			

Table 1. ¹H and ¹³C NMR Data of Hongdousans A–C (1–3)^a

^{*a*} The ¹H and ¹³C NMR spectra were measured at 400 and 100 MHz, respectively, in CDCl₃, and coupling constants (parentheses) are in hertz. ^{*b*} ¹H correlating with ¹³C 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 ¹³C NMR spectrum. The high-field shift of H-10 and C-10 signals in **1** ($\delta_{\rm H}$ 5.10, $\delta_{\rm C}$ 67.2) in comparison with those of **4** ($\delta_{\rm H}$ 6.06, $\delta_{\rm C}$ 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 $2\alpha, 5\alpha$ -diacetoxy, 10β hydroxy, and 14β -2-(methylbutyryloxy), on the basis of coupling constants and NOE correlations observed in NOE difference experiments; NOEs were observed from H₃-16 to H-2, from H₃-18 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 ¹H 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, CHCl₃), 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 ¹H 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 10β -hydroxy groups. Instead of the signals for a 2-methylbutyryloxy group as in 1, 2 showed signals corresponding to a propanoyloxy group $(\delta_{\rm H} 2.28, 3H, q, J = 7.5 \text{ Hz}, H_3-2'; \delta_{\rm H} 1.10, 3H, t, J = 7.5$ Hz, H₃-3'; 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* (EC₅₀ values are in μ g/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
$2\alpha, 5\alpha, 10\beta$ -triacetoxy- 14β -[(<i>S</i>)-2-methyl-	84.9	84.1
butyryloxy]-4(20),11-taxadiene (4)		
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 EC₅₀ values were calculated from the mean of data from four determinations; an EC₅₀ value > 10 μ g/mL was considered as inactive. α -Conidendrin and taxuyunnanine E exhibited an EC₅₀ value > 100 μ g/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 secoisolaricity (5), having an EC_{50} value of 5.9 µg/mL toward the HT-1080 fibrosarcoma cell line, and hongdoushan C (3), with an EC₅₀ value of $3.8 \,\mu g/$ 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 CHCl₃ 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 60F₂₅₄ 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 \times 3) under reflux for 30 min to yield a H₂O extract (52.2 g). The residue was further extracted with MeOH/H₂O (1:1) (4 L \times 3) and MeOH (4 L \times 3) to give the MeOH/H2O 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 CHCl₃ and MeOH. Both the CH₂Cl₂-soluble fractions of the MeOH/H₂O 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 imes 3.5 cm) with a gradient mixture of CHCl₃ and MeOH to give seven fractions [fraction 1, CHCl₃, 100 mg; fraction 2, 1% MeOH-CHCl₃, 1.88 g; fraction 3, 1% MeOH/CHCl₃, 580 mg; fraction 4, 1% MeOH/CHCl₃, 260 mg; fraction 5, 2% MeOH/ CHCl₃, 515 mg; fraction 6, 5% MeOH/CHCl₃, 1.58 g; fraction 7, 5-30% MeOH/CHCl₃, 2.03 g]. Further silica gel column chromatography and reversed-phase preparative TLC (CH₃-

CN/MeOH/H₂O, 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; $[\alpha]^{25}_{D}$ $+81.3^{\circ}$ (c 0.06, CHCl₃); IR (CHCl₃) ν_{max} 3600, 1780, 1370, 1210, 1020 cm⁻¹; ¹H and ¹³C 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, CHCl₃); IR (CHCl₃) v_{max} 3600, 1730, 1230, 1020 cm⁻¹; ¹H and ¹³C 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; $[\alpha]^{25}$ +77.4° (c 0.14, CHCl₃); IR (CHCl₃) v_{max} 3600, 1750, 1460, 1370, 1250 cm⁻¹; ¹H and ¹³C 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 \times 2). The EtOAc layer was evaporated, and the residue was dissolved in MeOH (1 mL) and treated with excess CH₂N₂. After evaporation, the residue was dissolved in CHCl₃ (5.0 mL) and filtered. An aliquot of the filtrate was analyzed by GC (column, Astec Chiraldex G-TA G0012-08, 30 m \times 0.25 mm; column temperature, 50 °C; detector temperature, 250 °C; injection temperature, 250 °C), to give a peak at $t_{\rm R}$ 12.3 min. The standard methyl ester of (R)- or (S)-2-methylbutyric acid gave a peak at $t_{\rm R}$ 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 μ g/mL are considered inactive.

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