Taxuspinananes A and B, New Taxoids from *Taxus cuspidata* var. *nana*

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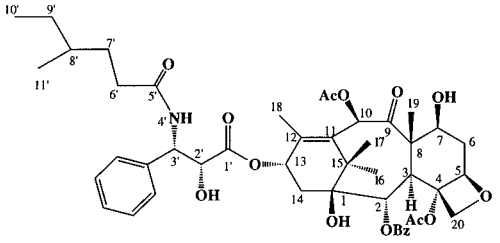
New taxoids, taxuspinanane A (**1**), showing potent cytotoxic activity, and taxuspinanane B (**2**), have been isolated from the stems of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder. Their structures were elucidated by extensive 2D NMR and MS spectroscopic analysis.

Various species of the genus *Taxus* (Taxaceae) contain many different kinds of taxoids,[1] of which paclitaxel (Taxol) has emerged as a highly promising anticancer agent approved for the treatment of advanced ovarian cancer.[2] This diterpene has a unique mechanism of action that results from specific binding to polymerized tubulin and consequent inhibition of mitosis.[3]

As a part of a research program aimed at developing new bioactive taxoids, we investigated various taxoids contained in the stems of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder. (Taxaceae). Chromatographic purification of constituents of the stems of *T. cuspidata* var. *nana* with guidance by a cytotoxic assay resulted in the isolation of two new taxoids, named as taxuspinananes A (**1**) and B (**2**), which showed potent cytotoxic activity. We report here the isolation and structure elucidation of these new taxoids by extensive 2D NMR and mass spectroscopic methods.



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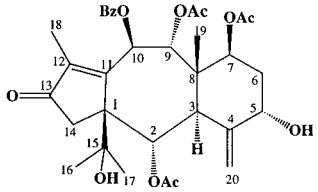

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The MeOH extract of the stems of *T. cuspidata* Sieb. et. Zucc. var. *nana* Rehder. was partitioned between toluene and H_2O. The toluene-soluble fraction, showing cytotoxicity against P-388 lymphocytic leukemia cells, was subjected to Si gel column chromatography ($CHCl_3$−MeOH), and the fraction eluted with 5% MeOH was chromatographed on a Si gel column (toluene−EtOAc−MeOH), followed by MPLC and HPLC on ODS to yield two new taxoids, named taxuspinananes A and B (**1**, 0.002% and **2**, 0.003%). Compounds **1** and **2** and paclitaxel showed cell growth-inhibitory activity against P-388 lymphocytic leukemia cells (IC_{50} **1**, 0.01 μg/mL; **2**, 10.0 μg/mL; paclitaxel, 0.04 μg/mL).

Taxuspinanane A (**1**), colorless powder, $[\alpha]_D$ −40.2° (*c* 0.37, MeOH), showed a HRFABMS spectral quasi-molecular ion peak at *m/z* 862.4014 [$(M + H)^+$, Δ 0.0 mmu], corresponding to the molecular formula $C_{47}H_{59}NO_{14}$. The IR absorptions at 3423, 1719, and 1655 cm^{-1} were attributed to hydroxyl, ester, and amide carbonyl groups, respectively. Complete assignments of the ^{1}H- and ^{13}C-signals were achieved by using various NMR measurements such as ^{1}H−^{1}H COSY, HMQC[4] for direct $^1J_{H-C}$ connectivities, and HMBC[5] for long-range $^2J_{H-C}$ and $^3J_{H-C}$ ones (Table 1). The results of the assignments are shown in Table 1, being similar to those of paclitaxel.[6] Deuterobenzene (C_6D_6) was selected as the NMR solvent because of ^{1}H signal overlapping in $CDCl_3$. The presence of two acetyl groups, one benzoyl group, and an oxetane ring was indicated by ^{1}H signals at δ 1.80, 2.18 (acetyl methyl: each, 3H, s), at 8.33, 7.22, and 7.18 (benzoyl) attached to the ^{13}C signals at δ 130.68, 128.89, and 133.42, and at 4.35 and 4.36, mutually coupled with a coupling constant of 8.6 Hz (oxetane). In addition, the presence of a side chain similar to the C-13 side chain of paclitaxel was suggested by ^{1}H signals at δ 6.51 (H-13), 4.63 (H-2′), 5.82 (H-3′), 6.09 (H-4′), 7.39, 7.16, 7.11 (Phe at C-3′). However, aliphatic signals were observed in place of signals for the *N*-benzoyl group of paclitaxel. The coupling connectivity analyzed by ^{1}H−^{1}H COSY and TOSCY spectra proved the *N*-acyl group to be a 4-methyl hexanoyl group. These results were also implied by the MS fragmentation ions (*m/z* 569, 294, 276, 248, 207, 113, and 85) and ^{13}C signals corresponding to the 4-methylhexanoyl group (δ_C 172.99, 34.10, 32.51, 34.10, 29.46, 11.38, 18.84). The configurations at C-2′ and C-3′ were concluded to be 2′*R*, 3′*S* by the ^{1}H vicinal coupling constants ($J_{2'3'}$ 2.0 Hz, $J_{3'4'}$ 9.2 Hz in C_6D_6; $J_{2'3'}$ 2.6 Hz, $J_{3'4'}$ 9.0 Hz in $CDCl_3$) compared with those of paclitaxel ($J_{2'3'}$ 2.7 Hz, $J_{3'4'}$ 8.9 Hz in $CDCl_3$);[6] this conclusion was also verified by the lack of an NOE enhancement between H-3′ and Me-18.[7] The relative stereochemistry of **1** was confirmed by a phase-sensitive ROESY spectrum as shown in Figure 1. The ROE correlations in the taxane skeleton indicated that **1** possessed the same configurations as those of paclitaxel. In addition, intramolecular hydrophobic interactions between the C-4 acetoxyl group and the C-13 side chain were indicated

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Table 1. ^{1}H- and ^{13}C-NMR Signal Assignments and HMBC Correlations of Taxuspinananes A and B (**1** and **2**)

	1[a]				**2**[b]				
position	δ_H [int mult, J(Hz)]		δ_C	HMBC		δ_H		δ_C	HMBC
1			79.45	H-2, H-3, Me-16, Me-17, Me-18	1			62.66	H-2, H-14a, H-14b, Me-16, Me-17
2	5.95	(1H, d, 7.0)	75.69	H-3	2	6.04	(1H, d, 9.2)	68.92	H-3
3	4.02	(1H, d, 7.0)	46.26	H-2, Me-19	3	2.79	(1H, d, 9.2)	45.40	H-9, Me-19, H-20a, H-20b
4		81.64		H-3, H-5, H-20a, H-20b	4			146.37	H-3, H-20a, H-20b
5	4.89	(1H, dd, 1.8, 9.5)	84.55	H-6α, H-6β	5	4.72	(1H, br t)	65.67	H-3, H-20a, H-20b, HO-5
6α	2.58	(1H, m)	36.19	HO-7	6α	1.89	(1H, m)	35.78	H-7
6β	2.11	(1H, m)			6β	2.06	(1H, m)		
7	4.68	(1H, m)	72.71	H-3, H-5, H-6α, H-6β, HO-7, Me-19	7	4.82	(1H, t, 9.0)	70.57	Me-19
8		59.15		H-2, H-3, H-6α, H-6β, Me-19	8			43.77	H-3, H-9, H-10, HO-5, Me-19
9			203.52	H-3, H-10, Me-19	9	5.11	(1H, d, 2.8)	74.34	H-3, H-10, Me-19
10	6.61	(1H, s)	76.03		10	6.30	(1H, d, 2.8)	70.51	H-9
11		133.96		H-10, H-13	11			148.11	H-10, H-14β, Me-18
12		142.23		H-10, H-13, H-14, Me-18	12			160.99	H-9, H-10, H-14β, Me-18
13	6.51	(1H, br dd)	72.71	H-14, Me-18	13			206.87	H-14α, H-14β, Me-18
14	2.58	(2H, m)	36.33	HO-1, H-13	14α	2.75	(1H, d, 18.6)	44.66	H-2
					14β	2.45	(1H, d, 18.6)		
15			43.68	H-10, Me-16, Me-17, Me-18	15			75.60	H-2, H-14α, H-14β, HO-15, Me-16, −17
16	1.15	(3H, s)	22.11	Me-17	16	1.14	(3H, s)	28.46	
17	1.20	(3H, s)	26.92	Me-16	17	0.81	(3H, s)	27.56	
18	1.97	(3H, s)	14.82		18	1.95	(3H, s)	9.39	
19	1.98	(3H, s)	10.07	H-3	19	1.83	(3H, s)	14.45	H-3, H-7
20a	4.35	(1H, d, 8.6)	76.54	H-3	20a	4.78	(1H, s)	114.14	H-3
20b	4.36	(1H, d, 8.6)			20b	5.48	(1H, s)		
1′			173.63	H-13, H-2′, HO-2′					
2′	4.63	(1H, br dd)	73.11	H-3′, HO-2′					
3′	5.82	(1H, dd, 2.0, 9.2)	54.51	H-2′, H-4′, H-3′Ph (o)					
4′	6.09	(1H, d, 9.2)							
5′			172.99	H-3′, H-4′, H-6′					
6′	1.82	(2H, m)	34.10	H-7′					
7′	1.27	(1H, m)	32.51	H-6′, H-8′, H-9′					
	1.55	(1H, m)							
8′	1.14	(1H, m)	34.10	H-6′, H-7′, H-9′, H-10′, H-11′					
9′	0.95	(1H, m)	29.46	H-7′					
	1.12	(1H, m)							
10′	0.75	(1H, t, 7.1)	11.38	H-9′, H-11′					
11′	0.70	(1H, d, 6.4)	18.84	H-10′, H-7′, H-9′					
1-OH	2.04	(1H, s)			5-OH	1.89	(1H, s)		
7-OH	2.97	(1H, d, 4.2)			15-OH	1.17	(1H, br d)		
2′-OH	3.76	(1H, br d)							
4-OAc	2.18	(3H, s)	22.55		2-OAc	1.97	(3H, s)	21.67	
			170.46	Me-4Ac				170.26	H-2, Me-2Ac
10-OAc	1.80	(3H, s)	20.42		7-OAc	1.95	(3H, s)	21.01	
			171.23	H-10, Me-10Ac				170.40	H-7, Me-7Ac
2-OBz	8.33	(2H, d, 1.3, 8.0)	130.68	H-2 OBz (p)	9-OAc	1.99	(3H, s)	20.65	
	7.22	(2H, m)	128.89[c]					169.53	H-9, Me-9Ac
	7.18	(1H, m)	133.42	H-2 OBz (o)	10-OBz	8.10	(2H, d, 7.2)	133.64	
			130.35	H-2 OBz (m)		7.47	(2H, t, 7.2)	128.82	
			167.13	H-2, H-2OBz(o)		7.59	(1H, t, 7.2)	129.74	H-10OBz(o)
3′-Ph	7.39	(2H, d, 7.3)	127.60	H-3′, H-3′Ph (p)				129.32	H-10OBz(m)
	7.16	(2H, m)	128.98[c]					165.34	H-10, H-10 OBz (o)
	7.11	(1H, m)	128.15	H-3′Ph(o)					
			139.31	H-3′, H-3′Ph(m)					

[a] Measured at 400 MHz in benzene-d_6. [b] Measured at 400 MHz in chloroform-d. [c] Assignment may be interchanged.

by ROE enhancements between the methyl protons of the C-4 acetoxyl group and the H-2′ and H-3′ protons. This kind of solution conformation has also been reported in the case of paclitaxel.[6] Taxuspinanane A showed more potent cell growth-inhibitory activity against P-388 cells (IC_{50} 0.01 μg/mL) than paclitaxel (IC_{50} 0.03 μg/mL).

Taxuspinanane B (**2**), $C_{33}H_{40}O_{11}$, $[\alpha]_D$ +26.6° (c 0.34, MeOH), was isolated as an amorphous powder. The ^{1}H- and ^{13}C-NMR spectra showed the presence of three acetyl groups and one benzoyl group. The presence of an *exo*-methylene group was implied by signals at δ_H 4.78 and 5.48 and of an α,β-unsaturated carbonyl group by signals at δ_C 206.87, 160.99, and 148.11, together with a UV absorption band at 241 nm (ϵ 8570). The unusual downfield ^{13}C shift for C-15 (δ 75.60) suggested that **2** possesses the rearranged 11(15→1)abeotaxane skeleton.[8] Complete ^{1}H and ^{13}C assignments using ^{1}H−^{1}H COSY, TOCSY, HMQC, and HMBC spectra are shown in Table 1. The position of the ester linkage was elucidated by ^{1}H−^{13}C long-range correlations by HMBC spectrum (Table 1). The small coupling constant (2.8 Hz) between H-9 and H-10 suggested a chair/boat conformation for the B/C ring.[9] The stereochemistry of **2** was elucidated by ROE correlations (Figure 2) observed in a phase-sensitive ROESY spectrum. Taxus-

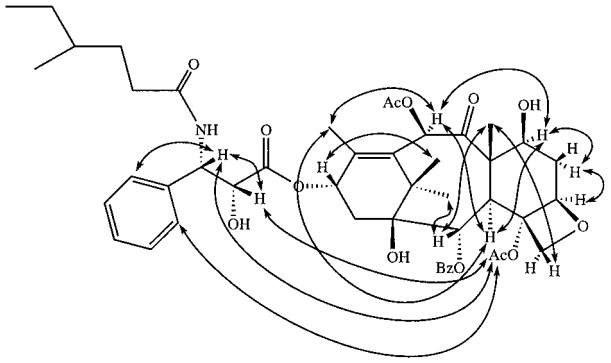

Figure 1. ROE correlations (arrows) for taxuspinanane A (**1**) in C_6D_6.

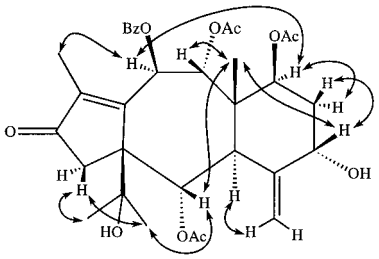

Figure 2. ROE correlations (arrows) for taxuspinanane B (**2**) in $CDCl_3$.

pinanane B (**2**) showed moderate cytotoxic activity against P-388 cells (IC_{50} 10 μg/mL).

The investigation of other new taxoids and the stereochemistry of the 4-methylhexanoyl side chain is ongoing.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a JASCO DIP-4 polarimeter. The IR spectrum (KBr) was obtained on a Perkin-Elmer 1710 spectrophotometer. Mass spectra were recorded on a VG Autospec instrument. HPLC was performed on an Inertsil PREP-ODS packed with 10 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (art. 5715; Merck), and the spots were detected by spraying with 10% H_2SO_4. ^{1}H- and ^{13}C-NMR spectra were run in C_6D_6 and $CDCl_3$ using a Bruker AM-500 and Varian Unity 400 instruments, respectively with chemical shifts (δ) reported in ppm. The spectra were recorded at 300 °K. A phase-sensitive ROESY NMR experiment was acquired with mixing times of 200 msec. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 Hz, and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec.

Plant Material. The stems of *T. cuspidata* Sieb. et. Zucc. var. *nana* Rehder. were collected in Saitama, Japan, in November 1995. The botanical identification was made by Dr. Zhi-Sheng Qiao, Department of Pharmacognosy, College of Pharmacy, Second Military Medical University, Shanghai, China. A voucher specimen has been deposited in the herbarium of Tokyo University of Pharmacy & Life Science.

Extraction and Isolation. The stems of *T. cuspidata* Sieb. et. Zucc. var. *nana* Rehder. (20.0 kg) were extracted with hot MeOH three times to give a MeOH extract residue (1987 g) that was partitioned between toluene and H_2O. The toluene-soluble fraction (230 g) was subjected to Si gel column chromatography using a $CHCl_3$−MeOH gradient system (1:0−0:1). The fraction that eluted with 5% MeOH was further subjected to Si gel column chromatography using a toluene−EtOAc−MeOH solvent system (12:4:1), followed by ODS MPLC with 70% MeOH and ODS HPLC with MeOH−CH_3CN−H_2O (1:2:2 and 18:25:57) to give taxuspinanane A **1** (72 mg) and taxuspinanane B **2** (75 mg).

Taxuspinanane A (1): colorless powder; $[\alpha]_D$ −40.2° (*c* 0.37, MeOH); IR (KBr) ν_{max} 3423, 2961, 1719, 1655, and 1245 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 1; FABMS *m/z* 862 $[M + H]^+$, 569, 294, 276, 248, 207, 113, and 85; HRFABMS *m/z* 862.4014, calcd for $C_{47}H_{60}NO_{14}$ 862.4014; UV λ_{max} 230 (ϵ 13 300), 274 (ϵ 1220).

Taxuspinanane B (2): colorless powder; $[\alpha]_D$ +26.6° (*c* 0.34, MeOH); IR (KBr) ν_{max} 3449, 1718, 1373, 1244, and 1029 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Table 1; EIMS *m/z* $[M - 2H_2O]^+$ 576; HREIMS *m/z* 576.2359, calcd for $C_{33}H_{36}O_9$ 576.2359. UV λ_{max} 241 (ϵ 8570), 274 (ϵ 1380).

Cytotoxic Activity on P-388 Cells. The MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well plate. The blue formazan produced by the mitochondrial dehydrogenase of viable cells was measured spectrophotometrically. RPMI-1640 medium (100 μL) supplemented with 5% fetal calf serum and 100 μg/mL of kanamycin and containing mouse P-388 leukemia cells (3×10^4 cells/mL) was added to each well. After overnight incubation (37 °C, 5% CO_2), 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, and 0.001 μg/mL of sample solutions were added to the wells, and the plates were incubated for 48 h. Then, 20 μL of MTT was added to each well, and the plates were incubated for 4 h. The resulting formazan was dissolved in 100 μL of 10% SDS (sodium dodecyl sulfate) containing 0.01 N HCl. Each well was mixed gently with a pipette for 1 or 2 min, and the plate was read on a microplate reader (Tosoh MPR-A4i) at 540 nm. The IC_{50} (μg/mL) value was defined as the concentration of sample that achieved a 50% reduction of viable cells with respect to the control.

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References and Notes

(1) Kingston, D. G. I.; Molinero, A. A.; Rimoldi, J. M. *Prog. Chem. Org. Nat. Prod.* **1993**, *61*, 1−206.
(2) Swenerton, K.; Eisenhauer, E.; ten Bokkel Huinink, W. *Proc. Am. Soc. Clin. Oncol.* **1993**, *12*, 256.
(3) Schiff, P. B.; Fant, J.; Horwitz, S. B. *Nature* **1979**, *277*, 665−667.
(4) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565−569.
(5) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(6) Williams, H. J.; Scott, A. I.; Dieden, R. A.; Swindell, C. S.; Chirlian, L. E.; Francl, M. M.; Heerding, J. M.; Krauss, N. E. *Tetrahedron* **1993**, *49*, 6545−6560.
(7) Dubois, J.; Guenard, D.; Gueritte-Voegelein, F.; Guedira, N.; Potier, P.; Gillet, B.; Beloeil, J.-C. *Tetrahedron* **1993**, *49*, 6533−6544.
(8) Fuji, K.; Tanaka, K.; Li, B.; Shingu, T.; Sun, H.; Taga, T. *Tetrahedron Lett.* **1992**, *51*, 7915−7916.
(9) Fuji, K.; Tanaka, K.; Li, B.; Shingu, T.; Yokoi, T.; Sun, H.; Taga, T. *Tetrahedron* **1995**, *51*, 10 175−10 188.

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