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TETRAHEDRON

Taxezopidines J, K, and L, New Taxoids from *Taxus cuspidata* Inhibiting Ca^{2+}-induced Depolymerization of Microtubules

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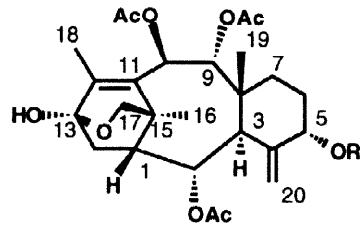
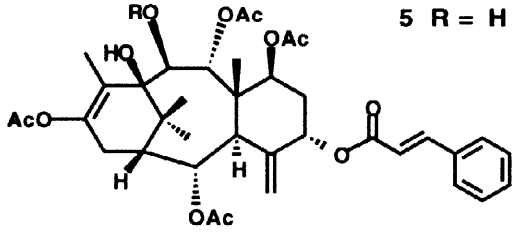
Abstract: Three new taxoids, taxezopidines J (**1**), K (**2**), and L (**3**), have been isolated from seeds of the Japanese yew *Taxus cuspidata* Sieb. et Zucc. and the structures were elucidated on the basis of spectroscopic data and chemical means. Taxezopidines K (**2**) and L (**3**) markedly inhibited Ca^{2+}-induced depolymerization of microtubules, while taxuspine D (**4**), a known taxoid from the yew, induced unusual shape change of mitotic spindles like taxol.

Keywords: biologically active compounds; proteins; taxoids

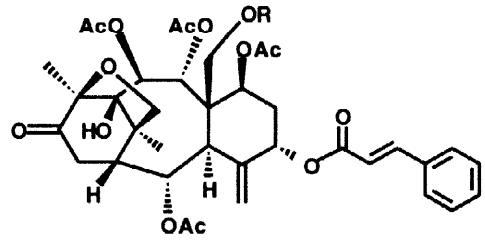
Numerous taxane-type diterpenoids have been found in yew trees of various *Taxus* species, and some of them exhibit interesting biological activities.[1,2] In our continuing search for bioactive taxoids, we previously isolated taxuspines A ~ Z[3] and taxezopidines E ~ H[4] from stems and leaves of the Japanese yew *Taxus cuspidata* Sieb. et Zucc and taxezopidines A ~ D[4,5] from seeds of this yew. Further investigation on extract of seeds of this yew led to the isolation of three new taxoids, named taxezopidines J (**1**), K (**2**), and L (**3**). In this



1 R = COCH=CHPh (*E*, 22, 23)
5 R = H



2 R = H
4 R = Ac



3 R = Ac
6 R = H

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Table 1. ^{1}H and ^{13}C NMR Data of Taxezopidines J (**1**), K (**2**), and L (**3**) in $CDCl_3$

	1		**2**		**3**	
position	^{1}H[a]	^{13}C[a]	^{1}H	^{13}C	^{1}H	^{13}C
1	2.18, m	48.30, d	1.89, m	50.60, d	2.40, dd, 11.2,2.4	48.79, d
2	5.48, dd, 5.6,1.5	70.00, d	5.76, d, 6.8	68.74, d	6.18, dd, 10.0,2.4	70.57, d
3	3.13, d, 5.6	42.78, d	3.61, d, 6.8	41.06, d	3.46, d, 10.0	39.87, d
4		146.63, s		142.96, s		140.66, s
5	5.41, brs	76.37, d	5.24, m	67.58, d	5.50, m	73.87, d
6 (a)	1.81, m	28.45, t	2.03, m	32.70, t	2.28, dd, 14.8,6.0	37.15, t
(b)	1.66, m				1.73, m	
7 (a)	1.87, m	27.90, t	4.80, m	70.65, d	5.52, m	68.48, d
(b)	1.83, m					
8		43.74, s		43.03, s		49.79, s
9	5.71, d, 10.8	76.37, d	5.09, d, 4.4	76.36, d	5.48, d, 3.2	70.57, d
10	6.09, d, 10.8	70.00, d	4.33, d, 4.4	78.55, d	5.36, d, 3.2	64.01, d
11		128.96, s		78.41, s		80.43, s
12		141.40, s		124.28, s		91.58, s
13		96.25, s		144.29, s		203.97, s
14 (a)	2.05, m	35.35, t	2.50, dd, 18.6,8.0	25.89, t	2.97, dd, 18.4,11.2	34.29, t
(b)	1.73, d, 16.0		2.30, d, 18.6		2.54, d, 18.4	
15		37.62, s		41.17, s		49.06, s
16	1.52, s	17.36, q	1.52, s	24.46, q	1.59, s	15.29, q
17 (a)	3.48, d, 8.1	74.54, t	1.20, s	31.70, q	4.20, d, 8.0	82.24, t
(b)	3.09, d, 8.1				3.69, d, 8.0	
18	2.23, s	11.90, q	1.50, s	11.44, q	1.22, s	12.35, q
19 (a)	0.92, s	17.36, q	1.57, s	14.80, q	4.44, d, 12.4	61.65, t
(b)					4.34, d, 12.4	
20 (a)	5.37, s	118.03, t	5.20, s	110.59, t	5.51, s	115.45, t
(b)	4.93, s		5.04, s		4.59, s	
21		166.34, s		165.14, s		165.91, s
22	6.70, d, 16.0	118.08, d	6.45, d, 16.0	117.84, d	6.80, d, 16.0	117.72, d
23	7.70, d, 16.0	145.65, d	7.70, d, 16.0	145.08, d	7.92, d, 16.0	146.24, d
24		134.45, s		134.29, s		134.71, s
25,29	7.60, m	128.30, d	7.53, m	128.14, d	7.81, m	128.77, d
26,28	7.37, m	128.95, d	7.39, m	128.95, d	7.40, m	128.81, d
27	7.37, m	130.41, d	7.39, m	130.43, d	7.40, m	130.27, d
2-AcO	2.06, s	21.11, q	1.96, s	21.65, q	1.97, s	21.50, q
		169.51, s		170.58, s		169.80, s
7-AcO			2.19, s	21.31, q	1.97, s	21.50, q
				170.31, s		172.14, s
9-AcO	2.04, s	20.91, q	2.02, s	21.04, q	2.12, s	20.97, q
		169.97, s		170.45, s		168.45, s
10-AcO	1.98, s	21.60, q			2.15, s	20.97, q
		169.51, s				167.90, s
13-AcO			2.20, s	20.86, q		
				168.55, s		
19-AcO					2.20, s	20.29, q
						170.45, s

a) in ppm

paper we describe the isolation and structure elucidation of taxezopidines J (**1**), K (**2**), and L (**3**), and potent inhibitory activity of taxezopidines K (**2**) and L (**3**) against microtubule depolymerization as well as unusual shape change of mitotic spindles by taxuspine D (**4**).

The methanolic extract of seeds of the yew collected at Sapporo was partitioned between toluene and water. The toluene-soluble portions were subjected to silica gel column chromatography followed by reversed-

phase column chromatographies to afford taxezopidines J (**1**, 0.0016%), K (**2**, 0.00078%), and L (**3**, 0.00046%) together with a known taxoid, taxuspine D (**4**).[6]

Taxezopidine J (**1**) was obtained as colorless amorphous solid and showed the molecular ion peak at *m/z* 622 in the EIMS spectrum. The molecular formula, $C_{35}H_{42}O_{10}$, was deduced from HREIMS [*m/z* 622.2770 (M^+), Δ-0.8 mmu]. The IR spectrum indicated the presence of hydroxy (3420 cm^{-1}), ester (1740 cm^{-1}), and unsaturated ester (1716 cm^{-1}) groups. The UV absorption at 281nm and a prominent fragment ion peak at *m/z* 131 (C_9H_7O) in EIMS suggested the presence of a cinnamoyl group. Proton signals due to the cinnamoyl group were observed at δ_H 7.60 (2H, m), 7.37 (3H, m), 6.70 (1H, d, J = 16.0 Hz), and 7.70 (1H, d, J = 16.0 Hz; *trans*-oriented). Analyses of the ^1H and ^{13}C NMR data and HMQC spectrum provided three acetyls, one tetrasubstituted olefin, one exomethylene, one ketal carbon, four oxymethines, two methines, one oxymethylene, five methylenes, two quaternary carbons, three methyl groups, and six aromatic carbons. NMR data of **1** were similar to those of taxezopidine A (**5**).[5] Connectivities of C-1 to C-3, C-3 to C-20, C-9 to C-10, C-14 to C-1, and C-22 to C-23 were deduced from the ^1H-^1H COSY spectrum. HMBC correlations of H_3-18 to C-11, C-12, and C-13 and H_3-16 to C-1, C-11, and C-15 revealed that Me-18 and Me-16 were attached at C-12 and C-15, respectively. Three acetoxy groups were attached at C-2, C-9, and C-10 on the basis of HMBC correlations, while a hydroxy group was attached at C-13 from comparison of the ^{13}C NMR chemical shift of C-13 (δ 96.25) with those of hemiketal carbons (δ 96-98).[7] Treatment of **1** with hydroxylamine sulfate gave the 5-*O*-decinnamoyl derivative, of which all spectral data were identical with those of taxezopidine A (**5**).[5] Thus the structure of taxezopidine J was assigned as **1**.

Taxezopidine K (**2**) was shown to have the molecular formula, $C_{37}H_{46}O_{12}$, by HRFABMS [*m/z* 683.3080 $(M+H)^+$, Δ +1.2 mmu]. The UV absorption at 279 nm and a prominent fragment ion peak at *m/z* 131 (C_9H_7O) in EIMS suggested the presence of a cinnamoyl group. Proton signals due to the cinnamoyl group were observed at δ_H 7.53 (2H, m), 7.39 (3H, m), 6.45 (1H, d, J = 16.0 Hz), and 7.70 (1H, d, J = 16.0 Hz; *trans*-oriented). Analyses of the ^1H and ^{13}C NMR data and HMQC spectrum provided four acetyls, one tetrasubstituted olefin, five oxymethines, one oxygenated quaternary carbon, and four methyl groups. The carbon chemical shift of C-11 (δ_C 78.41) indicated that a hydroxy group was attached at C-11. The chemical shifts of two olefin carbons (δ_C 124.28, C-12; δ_C 144.29, C-13) implied the presence of an enolacetate moiety in ring A.[6] HMBC correlations of H_3-18 to C-12 and C-13 revealed that Me-18 was attached at C-12. HMBC correlations of H_3-16 and H_3-17 to C-1, C-11, and C-15 indicated that Me-16 and Me-17 were attached at C-15. Cross-peaks of H-2 to C-1, H-3 to C-8, H-10 to C-8, H-10 to C-11, H-3 to C-4, and H-7 to C-8 in the HMBC spectrum revealed the presence of an eight-membered ring (ring B) and a cyclohexane moiety (ring C). Three acetoxy groups were attached at C-2, C-7, and C-9, judging from the chemical shift of each oxymethine proton (δ_H 5.76, 4.80, and 5.09, respectively) and HMBC correlations. The cinnamoyl group was connected to C-5 by the HMBC correlation between H-5 (δ_H 5.24) and C-21 (δ_C 165.14). All spectral data of the acetate of **2**, which was obtained by treatment of **2** with Ac_2O in pyridine, were identical with those of taxuspine D (**4**).[6] Thus the structure of taxezopidine K was assigned as **2**.

The molecular formula, $C_{39}H_{46}O_{15}$, of taxezopidine L (**3**) was established by HREIMS [*m/z* 754.2836 (M^+), Δ 0.0 mmu]. The ^1H and ^{13}C NMR spectra of **3** resembled those of taxuspine S (**6**).[8] An HMBC correlation between H-17a and C-15 and proton signals (δ_H 4.20 and 3.69, d, J = 8.0 Hz; H-17a and H-17b) revealed the presence of a tetrahydrofuran ring fused to ring A. The olefin proton signals of a cinnamoyl group appeared at δ_H 6.80 (1H, d, J = 16.0 Hz) and 7.92 (1H, d, J = 16.0 Hz, *trans*-oriented). The cinnamoyl

carbonyl carbon (δ_C 165.91) showed an HMBC correlation for H-5, indicating that the cinnamoyl group was connected to C-5. Five acetoxy groups were attached at C-2, C-7, C-9, C-10, and C-19 based on the HMBC correlations and oxymethine and oxymethylene proton resonances (δ_H 6.18, H-2; 5.52, H-7; 5.48, H-9; 5.36, H-10; and 4.44 and 4.34, H-19a and H-19b). The presence of a ketone (δ_C 203.97) at C-13 was elucidated by HMBC correlations of H_3-18, H-14a, and H-14b to C-13. HMBC correlations of H-20b to C-3 and C-5 indicated the presence of an exomethylene at C-4. Thus the structure of taxezopidine L was assigned as **3**. The relative stereochemistry of **3** was elucidated by NOESY data and ^1H-^1H coupling constants.

Inhibitory Activity of Ca^{2+}-Induced Microtubule Depolymerization by Taxoids (1 ~ 3).

Microtubule proteins were polymerized under normal polymerization conditions in the absence and the presence of taxol® or taxezopidines J, K, and L (**1** ~ **3**) and, after 30 minutes incubation, $CaCl_2$ was added. The $CaCl_2$-induced depolymerization of microtubules (shown as control) was completely inhibited by 10 μM of taxol, while the inhibitory activities of taxezopidines K (**2**) and L (**3**) corresponded to half to one third of that of taxol (Figure 1).[3,6] On the other hand, taxezopidine J (**1**) showed a weak inhibitory activity.

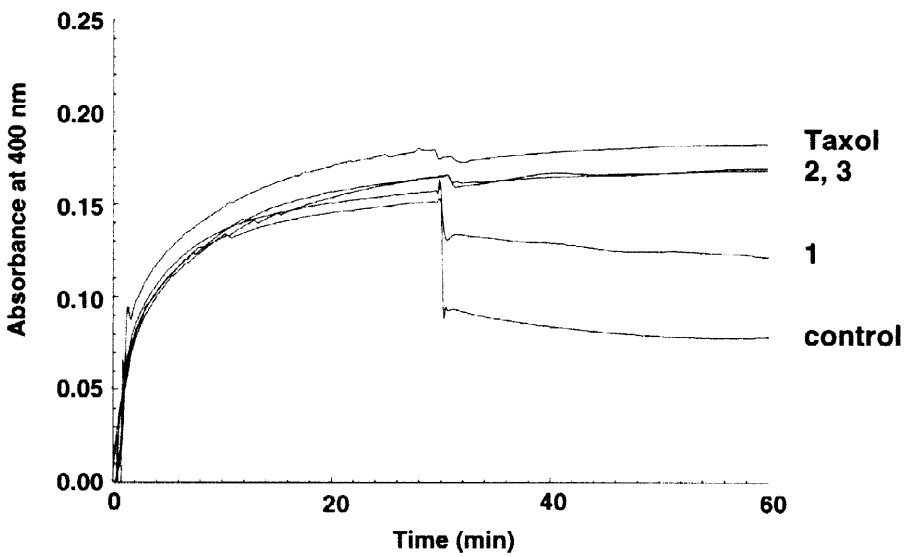

Figure 1. Effects of Taxoids (**1** ~ **3**) on Ca^{2+}-Induced Microtubule Depolymerization
Each 1 mL reaction mixture contained 1 mg/mL of microtubule proteins prepared from porcine brain, and each 10 μM of taxezopidines J (**1**), K (**2**), and L (**3**) in DMSO.

Effect of Taxuspine D (4) on Mitotic Spindle Formation in Sea Urchin Embryo.

The mitotic spindle formation of the taxuspine D (**4**)-treated sea urchin eggs was followed under a polarization microscope. When the fertilized eggs were treated with 2.5 μg/mL of **4**, the normal spindles were not seen at the metaphase and overstabilized spindles with high birefringence density were observed (Figure 2), and the following egg divisions were completely suppressed. This mode of operation was almost identical to that of taxol (10 μg/mL).[9]

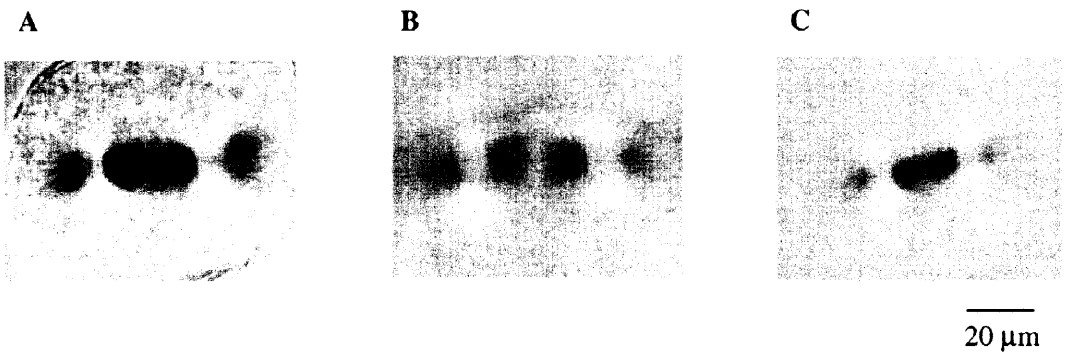

Figure 2. Effects of Taxuspine D (**4**) and Taxol on Mitotic Spindle Formation in Sea Urchin Embryo. A; treated with taxuspine D (**4**, 2.5 μg/mL), B; treated with taxol (10 μg/mL) at 35 min after fertilization, C; control. Polarization microscopy.

Taxezopidines J (**1**), K (**2**), and L (**3**) exhibited cytotoxicity against murine lymphoma L1210 cells with IC_{50} values of 4.2, 1.7, and 2.1 μg/mL, respectively, and human epidermoid carcinoma KB cells with IC_{50} values of 2.4, 4.8, and 2.0 μg/mL, respectively.

Taxuspine J (**1**) is the second example of taxane diterpenes containing an oxabicyclo[2.2.2]octene moiety[5], while taxezopidine K (**2**) is a rare taxoid having an enolacetate moiety in ring A like taxuspine D (**4**).[6] On the other hand, taxezopidine L (**3**) is a taxoid possessing a tetrahydrofuran ring fused to ring A. It is noted that the non-taxol-type taxoids, taxezopidines K (**2**) and L (**3**), exhibited potent inhibitory activity against Ca^{2+}-induced depolymerization of microtubules, while another non-taxol-type taxoid, taxuspine D (**4**), induced spindles with strong birefringence like taxol.

Experimental Section

General Methods. Optical rotations were determined on JASCO DIP-370 polarimeter. UV and IR spectra were obtained on JASCO Ubest-35 and JASCO IR report-100 spectrometers, respectively. ^1H and ^{13}C NMR spectra were recorded on a JEOL EX-400, Bruker ARX-500 and AMX-600 spectrometers. The 7.26 ppm resonance of residual $CHCl_3$ and 77.0 ppm of $CDCl_3$ were used as internal references, respectively. EIMS was obtained on a JEOL DX-303 spectrometer operating at 70 eV.

Collection, Extraction, and Separation. The Japanese yew *Taxus cuspidata* Sieb. et Zucc. was collected at Sapporo, Hokkaido. The MeOH extract (87.7 g) of seeds (401 g) of the yew was partitioned between toluene (0.5 L x 4) and H_2O (0.5 L). The toluene layer (21 g) was subjected to activated charcoal chromatography ($CHCl_3$) and then silica gel column chromatography ($CHCl_3$/acetone, 9:1) to afford a fraction (1.35 g), which was purified by silica gel column chromatography (hexane/EtOAc, 1:1) to give fractions **a** (341 mg) and **b** (144 mg). Fraction **a** was applied to reversed-phase HPLC (Develosil ODS-5, Nomura Chemical, 1.0 x 25 cm; flow rate 2.5 mL/min; UV detection at 279 nm; eluent: MeOH/H_2O, 70:30) to give taxezopidine J (**1**, 8.2 mg, t_R 37 min) and a fraction (18.4 mg, t_R 56 min), which was subjected to silica gel column chromatography (CH_2Cl_2/MeOH, 99:1) to afford taxezopidine K (**2**, 3.9 mg). Fraction **b** was purified under the same separation conditions as fraction **a** to afford taxezopidine L (**3**, 2.3 mg, t_R 16 min).

Taxezopidine J (1): A colorless amorphous solid: $[\alpha]^{22}_D$ +48° (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} 281 (ε 17300) and 218 (15300) nm; IR (film) ν_{max} 3420, 1740, 1716, 1370, and 1020 cm^{-1}; ^1H and ^{13}C NMR (Table 1); EIMS *m/z* 622 (M^+), 562 (M-AcOH)$^+$, 502 (M-2AcOH)$^+$, and 131 (PhCH=CHCO)$^+$; HREIMS *m/z* 622.2770 (M^+), calcd for $C_{35}H_{42}O_{10}$, 622.2778; HMBC correlations ($CDCl_3$, C/H) 1/3, 1/16, 1/17, 2/3, 2/14, 3/2, 3/20a, 3/20b, 4/6, 4/20a, 7/5, 7/9, 8/5, 8/19, 9/19, 10/9, 11/1, 11/16, 11/18, 12/10, 12/16, 12/18, 13/14, 13/18, 14/2, 15/10, 15/16, 17/16, 19/3, 19/9, 20/5, 21/23, 23/22, 24/23, 24/25, 25/23, 25/26, 25/27, 26/23, 26/25, 27/25, 2-AcO/2, 9-AcO/9, and 10-AcO/10; NOESY correlations ($CDCl_3$, H/H) 1/2, 1/17a, 2/9, 2/16, 2/19, 3/18, 5/20b, 9/16, 9/19, 10/18, 17a/16, 17b/16, 18/22, and 22/25.

Taxezopidine K (2): A colorless amorphous solid: $[\alpha]^{23}_D$ +53° (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} 279 (ε 11500) and 218 (7900) nm; IR (film) ν_{max} 3502, 1731, and 1370 cm^{-1}; ^1H and ^{13}C NMR (Table 1);

EIMS *m/z* 622 $(M-AcOH)^+$, 562 $(M-2AcOH)^+$, and 131 $(PhCH=CHCO)^+$; FABMS *m/z* 683 $(M+H)^+$, 665 $(M-H_2O+H)^+$, and 605 $(M-AcOH-H_2O+H)^+$; HRFABMS *m/z* 683.3080 $(M+H)^+$, calcd for $C_{37}H_{47}O_{12}$, 683.3068; HMBC correlations (C/H, $CDCl_3$) 1/3, 1/14a, 1/16, 1/17, 2/1, 2/14a, 2/14b, 2/3, 3/1, 3/2, 3/10, 3/19, 4/3, 4/5, 5/3, 7/19, 8/2, 8/3, 8/7, 8/9, 8/10, 8/19, 9/3, 9/10, 9/19, 10/9, 11/1, 11/10, 11/16, 11/17, 11/18, 12/14a, 12/14b, 12/18, 13/14a, 13/14b, 13/18, 14/1, 14/2, 15/1, 15/2, 15/10, 15/14a, 15/16, 15/17, 16/17, 17/16, 19/3, 19/7, 20/3, 21/5, 21/22, 21/23, 22/23, 23/25, 24/22, 25/23, 25/26, 25/27, 26/25, 27/25, 2-$CH_3\underline{C}O$/2, 7-$CH_3\underline{C}O$/7, and 9-$CH_3\underline{C}O$/9; NOESY correlations ($CDCl_3$, H/H) 1/14b, 1/17, 2/1, 2/5, 2/16, 2/19, 3/7, 3/14a, 5/19, 9/19, 10/18, and 14/20.

Taxezopidine L (3): A colorless amorphous solid; $[\alpha]^{24}_D$ +94° (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} 280 (ε 18000) and 218 (15000) nm; IR (film) ν_{max} 3427, 1708, 1374, and 1229 cm^{-1}; ^1H and ^{13}C NMR (Table 1); EIMS *m/z* 754 (M^+), 606 $(M-PhCH=CHCO_2H)^+$, and 131 $(PhCH=CHCO)^+$; HREIMS *m/z* 754.2836 (M^+), calcd for $C_{39}H_{46}O_{15}$; HMBC correlations (C/H, $CDCl_3$); 1/3, 1/16, 1/17, 2/1, 2/3, 2/14, 3/19b, 3/20a, 3/20b, 5/20b, 6/7, 7/19a, 8/3, 8/10, 9/19, 11/1, 11/9, 11/16, 12/18, 13/1, 13/14, 13/18, 15/16, 15/17a, 2-$CH_3\underline{C}O$/2, 7-$CH_3\underline{C}O$/7, 9-$CH_3\underline{C}O$/9, 10-$CH_3\underline{C}O$/10, 19-$CH_3\underline{C}O$/19, 21/5, 21/23, 24/23, 24/25, and 25/23 NOESY correlations ($CDCl_3$, H/H) 1/2, 1/14b, 1/17a, 2/9, 2/20a, 3/7, 3/10, 3/14a, 5/6b, 5/20b, 6a/9, 6b/19a, 7/10, 9/16, 10/18, and 25/26.

Hydrolysis of Taxezopidine J (1). To a solution of taxezopidine J (**1**, 1.0 mg) in THF (400 μL) / H_2O (400 μL) / EtOH (100 μL) was added hydroxylamine sulfate (1 mg) and triethylamine (100 μL), and the mixture was stirred at 80 °C for 21 h. The mixture was partitioned with EtOAc (5 mL x 3) and H_2O (5 mL), and the organic layer was evaporated under reduced pressure. The residue was purified by a silica gel column ($CHCl_3$/acetone, 4:1) to give taxezopidine A (**5**, 0.4 mg, 51%).

Acetylation of Taxezopidine K (2). Taxezopidine K (**2**, 0.5 mg) was dissolved in Ac_2O (30 μL) and pyridine (30 μL) containing 4-dimethylaminopyridine (cat.). After stirring at room temperature for 5 h, the mixture was extracted with EtOAc (5 mL x 2) and evaporation under reduced pressure afforded a residue, which was purified by a silica gel column ($CHCl_3$/acetone, 3:2) to give taxuspine D (**4**, 0.3 mg, 57%).

Bioassay. Sexually mature sea urchins (*Hemicentrotus pulcherrimus*) were collected during the breeding season (January ~ March) from the intertidal marsh near Ushimado Marine Laboratory in Okayama Prefecture. Eggs and sperm were obtained by 0.5 N KCl-shedding. The sperm shaded from the genital papilla was collected with the glass capillary (dry sperm) and stored in the refrigerator (4°C) until use. The eggs were placed in the filtered sea water (30 mL) and gently agitated. After 3 min the eggs near the surface and the bottom were removed by decantation and suction. This process promised better fertilization rate and synchronous development of the eggs. Throughout this experiment, more than 90% of fertilization rate was obtained. Eggs and sperm thus obtained were used for the following experiments. The eggs (*ca.* 4 x 10^3 eggs) was inseminated at 18°C with the sperm suspension (1 mL, *ca.* 1 x 10^3 x dilution of the dry sperm with sea water). The following development of the control eggs was checked under an ordinary light microscope until the swimming blastula stage (24 hr after fertilization). In the first cell cycle of the normal eggs, the metaphase was observed at 65-70 min after fertilization and the first cleavage occured at 85-90 min after fertilization.

Samples [taxuspine D (**4**) and taxol] were dissolved in a small amount of MeOH and diluted with sea water (maximum MeOH content: 1%). The fertilized eggs were treated with sample solutions (0.08 ~ 10 μg/mL) at 35 min after fertilization (shortly after the S-stage) and left for 40 min. The eggs were then collected by centrifugation (500 rpm, 1 min) and treated with PIPES stabilizing medium (10 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$, 2 M glycerol, 1% Nonidet P-40; pH 6.9). The stabilized eggs were subjected to the microscopic analysis, and the mitotic spindles of treated eggs were compared with that of control eggs at the metaphase using a polarizing microscope.

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