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Taxuspine D, a new taxane diterpene from *Taxus cuspidata* with potent inhibitory activity against Ca^{2+}-induced depolymerization of microtubules

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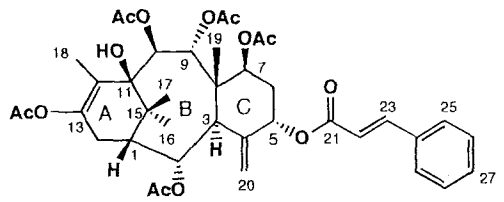
Abstract. A new taxane diterpenoid, taxuspine D (**1**), possessing an enolacetate moiety, has been isolated from stems of the Japanese yew *Taxus cuspidata* Sieb. et Zucc., and the structure elucidated on the basis of spectroscopic data. Taxuspine D (**1**) markedly inhibited Ca^{2+}-induced depolymerization of microtubules.
Key words. Taxoid; taxuspine D; yew; *Taxus cuspidata*; microtubule depolymerization.

Taxol (**2**) is an important anticancer agent with clinically useful activity against several human cancers[1]. It has a unique mechanism of action associated with cellular microtubule formation. In contrast to vinca alkaloids, taxol (**2**) increases polymerization of microtubules, stabilizes them once polymerized, and slows depolymerization[2]. Since the discovery of the anticancer activity of taxol (**2**), much attention has been paid to the isolation of new taxane diterpenoids from various species of yews[3]. In our continuing search for bioactive natural products[4], we isolated three new taxoids, taxuspines A–C (**3–5**), from the Japanese yew *Taxus cuspidata* Sieb. et Zucc.[5]. Further investigation of extracts of this yew led to isolation of a new taxoid, named taxu-



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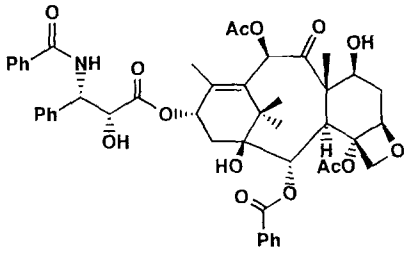

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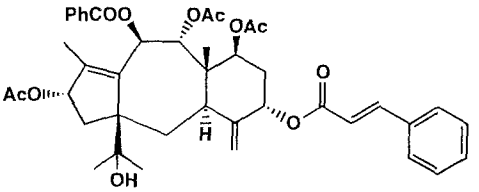

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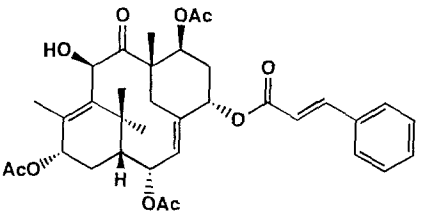

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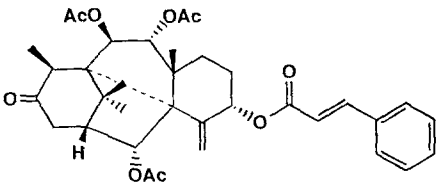

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Scheme 1.

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spine D (**1**). Taxuspine D (**1**) exhibited taxol-like activity, markedly inhibiting Ca^{2+}-induced depolymerization of microtubules, although it lacks the oxetane ring and *N*-acylphenylisoserine moiety which are associated with this unique activity in taxol (**2**). This paper describes the isolation and structure determination of taxuspine D (**1**) and its inhibitory activity against microtubule depolymerization.

Materials and methods

The Japanese yew *Taxus cuspidata* Sieb. et Zucc. was collected at Sapporo, Hokkaido. The stems (0.5 kg) of the yew were extracted with MeOH (2 l × 3). The MeOH extract was partitioned between toluene (750 ml × 3) and H_2O (750 ml). The toluene-soluble portions were evaporated under reduced pressure to give a residue (8.3 g). Part of this residue (4.3 g) was loaded onto silica gel column (4.0 × 37 cm) and eluted with hexane/acetone [7:1 (800 ml) → 3:1 (800 ml) → 1:3 (800 ml)]. The fraction eluted from 1650 to 2250 ml was separated by a reversed-phase column (Develosil Lop ODS 24S, Nomura Chemical, 2.5 × 50 cm; flow rate 3.0 ml/min; UV detection at 278 nm; eluent: CH_3CN/H_2O, 80:20) to give a fraction (24.6 mg, t_R 80 min) which was purified by a silica gel column (0.6 × 16 cm, eluent: $CHCl_3$/acetone, 8:1) to give taxuspine D (**1**, 11.2 mg, 0.0044%, 12–17 ml).

Effect of taxoids on the Ca^{2+}-induced microtubule depolymerization. Tubulin polymerization to microtubules is promoted by GTP, whereas $CaCl_2$ causes depolymerization of microtubules back to tubulin[2]. Taxol (**2**) is unique among antimitotic drugs in that it enhances the assembly of tubulin into stable microtubules, whereas other drugs, such as colchicine and vincristine, inhibit the assembly. The effect of taxuspine D (**1**) on the $CaCl_2$-induced depolymerization of microtubules was therefore examined. Microtubule proteins were polymerized under normal polymerization condition[6] in the absence and the presence of taxol (**2**) or taxuspine D (**1**). After 30 min incubation, $CaCl_2$ was added. Microtubule polymerization and depolymerization were monitored by the increase and the decrease in turbidity. The results are summarized in the figure as

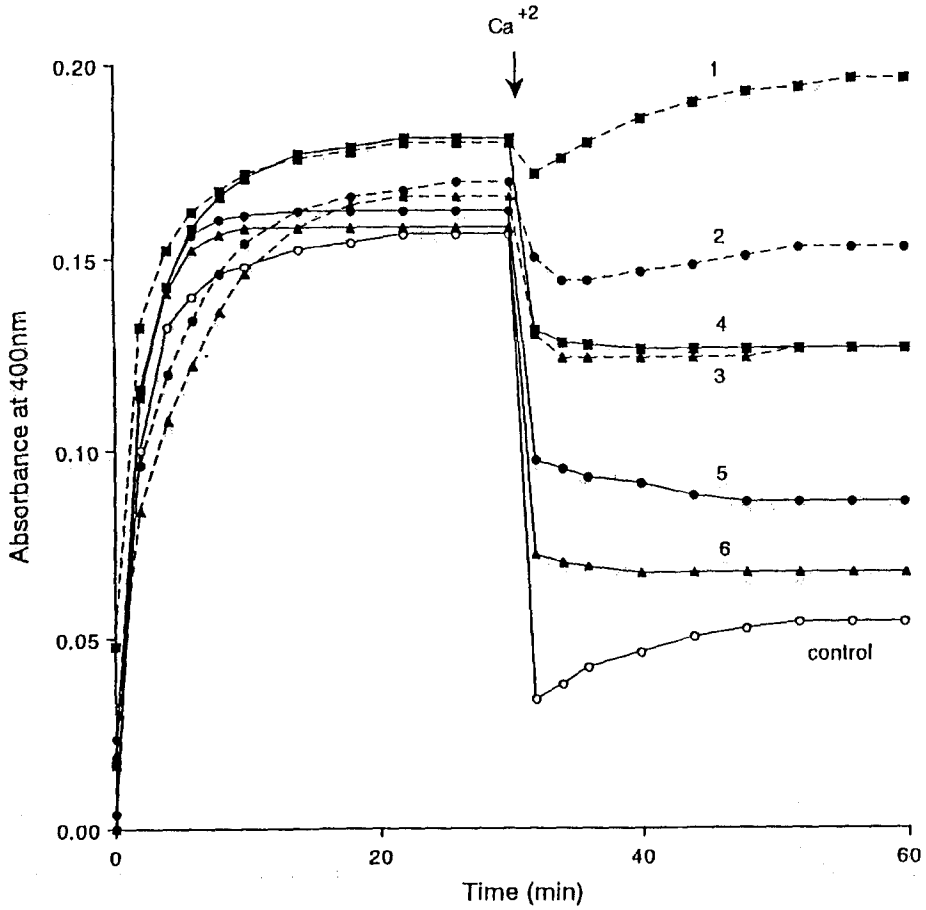

Figure. Effect of taxuspine D (**1**) and taxol (**2**) on Ca^{2+}-induced microtubule depolymerization. Each 1 ml reaction mixture contained 1 mg/ml of microtubule proteins prepared from porcine brain, and drug in DMSO as follows: curves 1–3 (dashes), 5, 2, and 1 μM taxol, respectively; curves 4–6 (solid), 5, 2, and 1 μM taxuspine D, respectively; curve (control), none. After 30 min incubation, 4 mM $CaCl_2$ was added. Absorbance measured at 400 nm.

the changes in the relative absorbance at 400 nm. The $CaCl_2$-induced depolymerization of in vitro polymerized microtubules (shown as control) was inhibited completely by 5 μM taxol (**2**), and about 80% by 2 μM and about 60% by 1 μM taxol (**2**), whereas other taxoids[5] showed no activity towards microtubule systems. On the other hand, taxuspine D (**1**) at 5 μM and 2 μM concentrations markedly inhibited the depolymerization process. The potency of **1** in the inhibition of the depolymerization process corresponded to half to one third of that of taxol (**2**).

Cytotoxicity studies. Taxuspine D (**1**) exhibited cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells in vitro with IC_{50} values of 3.0 and 1.8 μg/ml, respectively.

The structure of taxuspine D (1). Taxuspine D (**1**)[7], a colorless amorphous solid, showed a fragment ion peak at *m/z* 707 in the EIMS spectrum, and the molecular formula, $C_{39}H_{48}O_{13}$, was deduced by HREIMS [*m/z* 707.3073 (M^+-OH), Δ + 0.6 mmu]. IR absorptions at 3540, 1750, and 1710 cm^{-1} indicated the presence of hydroxy, ester, and α,β-unsaturated ester groups, respectively. UV absorption at 277 nm and a prominent fragment ion peak at *m/z* 131 (C_9H_7O) in EIMS supported the presence of a cinnamoyl group. Proton signals due to a cinnamoyl group were observed at δ_H 7.54 (2H, m), 7.40 (3H, m), 6.46 (1H, d, J = 16.0 Hz), and 7.70 (1H, d, J = 16.0 Hz; *trans*-oriented). The ^1H and ^{13}C NMR (table) spectra of **1** were assigned on the basis of several types of 2D NMR data including ^1H-^1H COSY, HMQC[8], and HMBC[9] spectra. Detailed analysis of the ^1H-^1H COSY spectrum[10] revealed connectivities of C-1 to C-3, C-5 to C-7, C-9 to C-10, C-14 to C-1, C-22 to C-23, and C-25 to C-27. HMBC correlations (table) of H-1 to C-11, C-13, and C-15 and H_2-14 to C-12 indicated the presence of a cyclohexene moiety (ring A). The carbon chemical shift (δ 78.7, C-11) indicated that a hydroxy group was attached at C-11 [11].

Table. ^1H and ^{13}C NMR data of taxuspine D (**1**) in $CDCl_3$

Position	^1H*		J (Hz)	^{13}C*		H coupled with C**
		mult.			mult.	
1	1.89	m		50.4	d	H-3, H-14a, H-16, H-17
2	5.80	d	6.8	68.3	d	H-1, H-14a, H-14b
3	3.69	d	6.8	40.8	d	H-1, H-2, H-7, H-9, H-10, H-19
4				142.6	s	H-3, H-5, H-20a
5	5.34	dd	9.4, 6.2	67.4	d	H-3, H-6, H-20a
6	2.06***	m		32.7	t	H-5
7	4.84	m		70.4	d	H-6, H-9, H-19
8				43.1	s	H-2, H-3, H-6, H-7, H-9, H-10, H-19
9	4.84	d	4.8	74.6	d	H-3, H-10, H-19
10	5.60	d	4.8	78.7	d	H-9
11				77.3	s	H-1, H-9, H-10, H-16, H-17, H-18
12				124.3	s	H-10, H-14a, H-14b, H-18
13	5.41	m		144.2	s	H-1, H-14a, H-14b, H-18
14(a)	2.36	d	17.8	25.8	t	H-1, H-2
14(b)	2.57	dd	17.8, 6.8			
15				41.3	s	H-1, H-2, H-10, H-14a, H-16, H-17
16	1.49	s		24.1	q	H-17
17	1.17	s		31.3	q	H-1, H-16
18	1.57	s		11.6	q	
19	1.53	s		14.8	q	H-3, H-7, H-9
20(a)	5.20	s		110.9	t	H-3, H-5
20(b)	5.08	s				
21				165.2	s	H-5, H-22, H-23
22	6.46	d	16.0	117.8	d	H-23
23	7.70	d	16.0	145.1	d	H-25
24				134.2	s	H-22
25	7.54***	m		128.1	d	H-23, H-26, H-27
26	7.40***	m		128.9	d	H-25
27	7.40	m		130.4	d	H-25
2-AcO	1.96	s		21.4	q	
				170.6	s	H-2
7-AcO	2.19	s		21.2	q	
				170.2	s	H-7
9-AcO	2.00	s		20.7	q	
				169.3	s	H-9
10-AcO	2.02	s		21.2	q	
				170.4	s	H-10
13-AcO	2.20	s		20.7	q	
				168.5	s	

*δ in ppm. **HMBC correlations. ***2H.

In the HMBC spectrum cross peaks of H_3-16 and H_3-17 to C-1, C-11, and C-15 revealed that Me-16 and Me-17 were attached at C-15. HMBC correlations of H_3-18 to C-11, C-12, and C-13 revealed that Me-18 was attached at C-12. The chemical shifts of two olefin carbons (δ_C 124.3, C-12; δ_C 144.2, C-13) implied that an acetoxy group was attached at C-13, as compared with those of 2-acetoxy-1-cyclohexene[12]. The presence of an eight-membered ring (ring B) and a cyclohexane moiety (ring C) was deduced from HMBC correlations. HMBC correlations of H-20a and H-20b to C-3 and C-5 indicated that an exomethylene (C-20) was attached at C-4. A carbonyl carbon at δ_C 165.2 (C-21) showed correlation for H-5 in the HMBC spectrum, indicating the presence of a cinnamate group at C-5. Four acetoxy carbonyl carbons (δ_C 170.6, 170.2, 169.3, and 170.4) showed HMBC correlations for H-2, H-7, H-9, and H-10, respectively, indicating that the four acetoxy groups were attached at C-2, C-7, C-9, and C-10, respectively. A remaining acetoxy group (δ_C 168.5), therefore, must be attached at C-13. Thus the structure of taxuspine D was assigned to be **1**, with an enolacetate moiety in ring A. Relative stereochemistry of **1** was elucidated by the NOESY spectrum[13]. A boat-like conformation of ring B was elucidated from NOESY correlations of H-2/H_3-16 and H-2/H_3-19, and a boat conformation of ring C was also assigned from NOESY correlations of H-2/H-5 and H-5/H_3-19.

Discussion

It has been reported that taxol (**2**) has two key binding regions on tubulin, one involving an *N*-acylphenylisoserine group at C-13 and the other involving an oxetane ring[14]. In this study, a new taxoid, taxuspine D (**1**), possessing an enolacetate moiety, was found to inhibit remarkably the microtubule depolymerization, although **1** has neither the C-13 side chain nor the oxetane ring, and the spatial structure of **1** elucidated by NOESY data is considerably different from that of taxol (**2**)[15]. This result suggests that in the case of **1** the other functional groups in the position of the two binding regions in taxol (**2**) may play an important role in enhancing the assembly of tubulin into stable microtubules. Taxuspine D (**1**) also moderately increased cellular accumulation of vincristine in multidrug-resistant tumor cells.

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