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TWO TAXOIDS FROM *TAXUS CUSPIDATA* AS MODULATORS OF MULTIDRAG RESISTANT TUMOR CELLS[†, 1]

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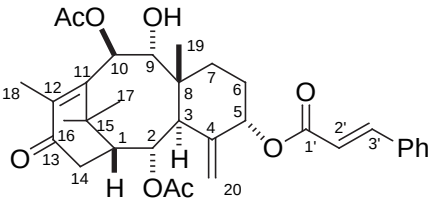
Abstract – Two new taxoids taxinine NN-3 (**1**) (9-deacetyl taxinine) and taxinine NN-4 (**2**) (taxinine 11,12-epoxide) isolated from *Taxus cuspidata* showed high activity as modulators of multidrug-resistant tumor cells.

Since the discovery of the anticancer activity of taxol® against ovarian and breast cancer, much attention has been paid to the isolation of new taxane diterpenoids from various species of yews.[2] Current interest in the Japanese yew, *Taxus cuspidata*, Sieb. *et* Zucc. (Taxaceae), focuses on the nonalkaloidal diterpenoids from the needles, stems, heartwood, and bark of this plant for the purpose of finding improved biological sources for taxol analogues and for precursors for the practical synthesis of taxol.[3—5] The needles and the young stems of *T. cuspidata* contain an impressive array of taxane diterpenoids. However, the content of the antitumor taxol in the needles and the young stems of this species was reported to be generally low. Taxane derivatives occurring in consistently large amounts are taxinine[6] as

† This paper is warmly dedicated to Professor Sho Ito on the occasion of his 77th birthday.

a nonalkaloidal diterpenoid, and 2'-hydroxytaxine II (taxine NA-1)[7] and taxine II[7,8] as alkaloidal diterpenoids. The needles and the young stems of *Taxus* variety with a constant content of taxol and its synthetic precursors are practically important as a reproducible source of taxol. Recently we found that the needles and the young stems of *T. cuspidata* contain constantly a significant amount of taxol, 7-epitaxol, cephalomanine, 7-epicephalomanine, baccatin III, and 10-desacetyl-7-epitaxol.[9]

As a part of on going study on the constitute of *T. cuspidata*, we report here the structure elucidation of two new nonalkaloidal taxanes, taxinine NN-3 (**1**) (9-deacetoxy taxinine) and taxinine NN-4 (**2**) (taxinine 11,12-epoxide) isolated from a mixture of needles and young stems of *T. cuspidata.* We also report activity of **1** and **2** on vincristine (VCR) accumulation in multidrug-resistant cancer cells.



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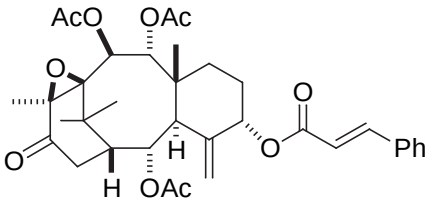

2

The neutral fraction of ethyl acetate extracts of the fresh needles and the young stems of *T. cuspidata* collected at Matumoto in Nagano, Japan was purified by flash chromatography on a silica gel column followed by normal- and reversed-phase HPLC to afford taxinine NN-3 (**1**, 0.0005%),[10] and taxinine NN-4 (**2**, 0.0006%).[10]

Taxinine NN-3 (**1**) was obtained as a colorless microcrystals, mp 58—60 ˚C. Compound (**1**) had the composition of $C_{33}H_{40}O_8$, which was determined by the combination of HREIMS, and ^{1}H- and ^{13}C-NMR spectra. The IR spectrum of **1** showed the existence of a hydroxyl group (3628 cm^{-1}), ester carbonyl group (1734 cm^{-1}), an α,β–unsaturated ester carbonyl group (1718 cm^{-1}), and an α,β–unsaturated carbonyl group (1672 cm^{-1}). The UV absorption at 217 and 278 nm implied that **1** possessed α,β–unsaturated carbonyl group and cinnamoylester groupes. Its ^{1}H-NMR showed the presence of a taxane skeleton with four C-Me groups (1.14, 1.15, 1.65, and 2.29 ppm), two acetyl Me groups (2.07 and 2.16 ppm), and a cinnamoyl group [6.44 (1 H, d, *J* =15.9 Hz), 7.66 (1 H, d, *J* = 15.9 Hz), 7.76 (2 H, *o*-Ph),

and 7.43 (3 H, *m*- and *p*-Ph)]. The ^{1}H-^{1}H correlations, H-1 and H-2; H-1 and H-14β; H–2 and H-3; H-3 and H-20a,b; H-5 and H-6α,β; H-6α and H-6β; H-6α,β and H-7α,β; H-7α and H-7β; H-9 and H-10; H-14α and H-14β; H-16 and H-17; H-20a and H-20b; H-2' and H-3', were determined by the analysis of a ^{1}H-^{1}H COSY spectrum. The assignment of all protonated carbons were assigned by DEPT and HMQC experiments. An HMBC experiment was used for the assignment of the quaternary carbons and the attachment of ester functions. A correlation of the signal due to the cinnamoyl carbonyl (C-1') at 166.40 ppm with those of H-5 (5.35 ppm), H-2' (6.44 ppm), and H-3' (7.66 ppm) indicated the location of the cinnamoyl group at C-5. Correlations of the signals due to two acetyl carbonyls at 170.12 and 169.73 ppm with those of H-2 (5.49 ppm) and H-10 (5.83 ppm) showed the location of two acetoxy groups of **1** at C-2 and C-10. The location of a hydroxy group was determined by the oxymethine resonance at C-9 (4.32 ppm) and HMBC correlations of H-10 (5.83 ppm) and H-19 (1.14 ppm) to C-9 (75.70 ppm). The multiple-bond ^{1}H-^{13}C correlations of the remaining six nonprotonated carbons of **1**, C-4 with H-3, 20a,b; C-8 with H-2, 3, 19; C-11 with H-1, 10, 16, 17, 18; C-12 with H-10, 14β, 18; C-13 with H-1, 14α,β, 18; C-15 with H-1, 10, 14α, 16, 17, were assigned by HMBC experiment and allowed unambiguous carbon skeletal connection. The full stereostructure of taxane skeleton of **1** was determined by 1D-NOE and NOESY experiments as well as by a consideration of vicinal coupling constants (Figure 1). The full NMR data of **1** were summarized in Table 1.

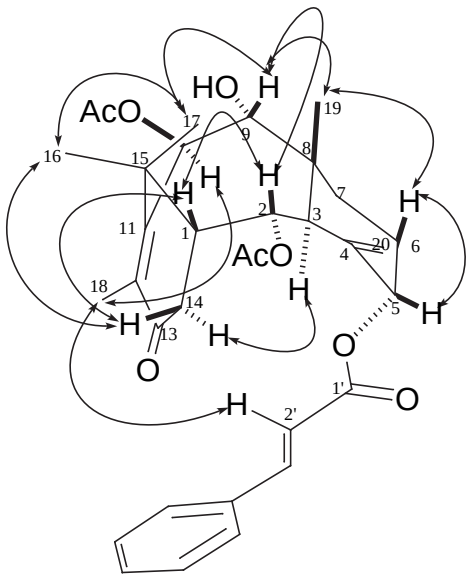

Figure 1. Key NOE cross peaks of taxinine NN-3 (**1**)

Taxinine NN-4 (**2**) was isolated as a colorless microcrystals, mp 216—218 °C. Compound (**2**) had the composition $C_{35}H_{42}O_{10}$, which was determined by a combination of HREIMS, and ^{1}H- and ^{13}C-NMR

Table 1. NMR data of taxinine NN-3 (**1**) in $CDCl_3$

position	^{13}C[a]	connected ^{1}H[b]	H-H COSY[c]	HMBC[d]
1	48.68 (d)	2.16 (m)	H2, 14β	H3, 14α,β, 16, 17
2	69.76 (d)	5.49 (dd, 6.2, 2.0)	H1, 3	H1, 3, 14α,β
3	43.13 (d)	3.39 (br d, 6.2)	H2, 20a,b	H1, 19, 20a,b
4	142.34 (s)	-	-	H3, 20a,b
5	78.55 (d)	5.35 (br t, 2.7)	H6α,β	H3, 20a,b
6	28.51 (t)	α) 1.99 (br ddd, 13.7, 2.7, 2.7)	H5, 6β, 7α,β	H7α,β
		β) 1.75 (br dddd, 13.7, 11.0, 4.9, 2.7)	H5, 6α, 7α,β	
7	26.11 (t)	α) 1.56 (m)	H6α,β, 7β	H3, 5, 6α,β, 9
		β) 1.88 (br ddd, 13.3, 4.9, 2.7)	H6α,β, 7α	-
8	44.84 (s)	-	-	H2, 3, 19
9	75.70 (d)	4.32 (br d, 9.6)	H10	H10, 19
10	76.95 (d)	5.83 (d, 9.6)	H9	H9
11	151.25 (s)	-	-	H1, 10, 16, 17, 18
12	137.71 (s)	-	-	H10, 14β, 18
13	199.53 (s)	-	-	H1, 14α,β, 18
14	36.08 (t)	α) 2.43 (d, 19.8)	H14β	H2
		β) 2.83 (dd, 19.8, 7.1)	H1,14α	-
15	37.77 (s)	-	-	H1, 10, 14α, 16, 17
16	37.14 (q)	1.15 (s)	H17	H1, 17
17	25.58 (q)	1.65 (s)	H16	H1, 16
18	14.00 (q)	2.29 (s)	-	-
19	17.67 (q)	1.14 (s)	-	H3, 9
20	116.78 (t)	a) 5.33 (br s)	H3, 20b	H3, 5
		b) 4.87 (br s)	H3, 20a	-
OAc	170.12 (s)	-	-	H2, 2-OAc(Me)
	169.73 (s)	-	-	H10, 10-OAc(Me)
	21.18 (q)	2.16 (s)	-	-
	21.45 (q)	2.07 (s)	-	-
1'	166.40 (s)	-	-	H5, 2', 3'
2'	117.98 (d)	6.44 (d, 15.9)	H3'	H3'
3'	145.62 (d)	7.66 (d,15.9)	H2'	*o*-Ph
q-Ph	134.57 (s)	-	-	H2', *m*-Ph
o-	128.48 (d)	7.76 (m)	*m*-Ph	H3', *p*-Ph
m-	128.94 (d)	7.43 (m)	*p*-Ph	*o*-Ph, *p*-Ph
p-	130.32 (d)	7.43 (m)	*m*-Ph	*o*-Ph

a Multiplicities were determined by DEPT. b Connections were determined by HMQC and multiplicities and coupling constants in Hz are in parentheses.
c Determined by PFG-COSY. d Correlations from C to the indicated protones.

spectra. The IR spectrum of compound (**2**) showed the existence of acetyl carbonyl group (1750 cm^{-1}), and α,β-unsaturated ester carbonyl and α,β-epoxy carbonyl group (1718 cm^{-1}). Its ^{1}H-NMR spectra showed the presence of a taxane skeleton with four C-Me groups (0.83, 0.99, 1.85, and 2.00 ppm), three acetyl Me groups (2.05, 2.07, and 2.08 ppm), and a cinnamoyl group [6.27 (1H, d, *J* = 15.9 Hz), 7.67 (1H,

d, J = 15.9 Hz), 7.43 (*m*- and *p*-Ph), and 7.62 (*o*-Ph)]. The ^{1}H-^{1}H correlations were determined by the analysis of a ^{1}H-^{1}H COSY spectrum; the assignment of all protonated carbons were established by DEPT and HMQC experiments. The assignment of the quaternary carbons and the attachment of ester functions were determined by HMBC experiment and allowed unambiguous carbon skeletal connections. In comparison with compound (**1**), the 11,12-epoxy taxane skeleton was suggested by the fact that the up field shift was observed at C-11 (64.29 ppm) and C-12 (59.33 ppm). In addition, the 11,12-epoxide stood for β-configuration because the ^{13}C-shift of C-16 changed to the up field shift from 37.14 ppm to 28.87 ppm^{13}C-shift of C-16 changed to the up field shift from 37.14 ppm to 28.87 ppm by steric compression, γ-effect between C-16 methyl group and the 11,12-epoxide. The full stereostructure of the 11,12-β-epoxy taxane skeleton of **2** was determined by 1D-NOE and NOESY experiments as well as by a consideration of vicinal coupling constants (Figure 2). The ^{1}H and ^{13}C NMR data of compound (**2**) were summarized in Table 2.

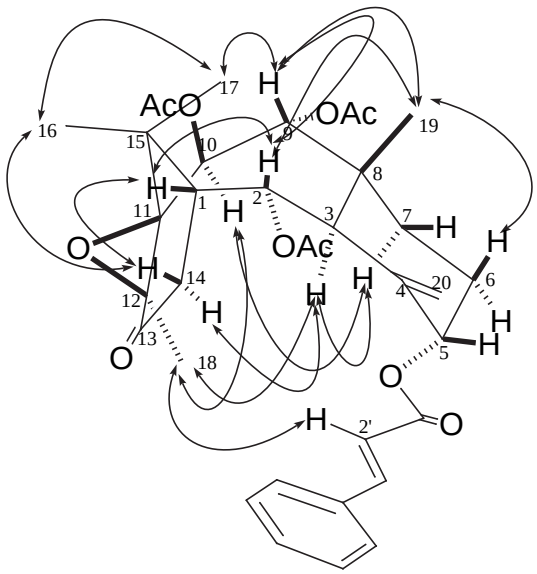

Figure 2. Key NOE cross peaks of taxinine NN-4 (**2**)

Recently Zamir and his co-workers reported the isolation of **2** from Taxus canadensis.,[11a] but we presented the preliminary report of **2** at the 74th Annual Meeting of the Chemical Society of Japan (1998)[1c] earlier than their report. Our preliminary report of **2** also appeared earlier than that of taxinine A 11,12-epoxide reported by Oritani and his co-workers.[11b] Thus the compound (**2**) is the second example of 11,12-epoxy taxoids. [11c]

The cellular accumulation of vincristine (VCR) is reduced in multidrug-resistant (MDR) tumor cells as compared with the parental cells. The MDR-reversing agent, verapamil, increases the accumulation of antitumor agents in MDR cells and overcomes multidrug resistance.[12] The effect of taxoids (**1**) and (**2**)

Table 2. NMR data of taxinine NN-4 (**2**) in $CDCl_3$

position	$^{13}C^a$	connected $^1H^b$	position	$^{13}C^a$	connected $^1H^b$
1	51.07 (d)	1.95 (br d, 8.4, 1.6)	17	25.27 (q)	1.85 (s)
2	69.87 (d)	5.75 (br dd, 5.2, 1.6)	18	15.75 (q)	2.00 (s)
3	43.11 (d)	3.12 (br d, 5.2)	19	17.97 (q)	0.99 (s)
4	140.72 (s)	-	20	119.90 (t)	a) 5.51 (br s)
5	78.45 (d)	5.50 (br s)			b) 5.22 (br s)
6	27.72 (t)	α) 2.01 (m)	OAc	169.25 (s)	-
		β) 1.84 (m)		169.76 (s)	-
7	26.95 (t)	α) 1.84 (m)		169.52 (s)	-
		β) 1.84 (m)		20.83 (q)	2.08 (s)
8	43.76 (s)	-		20.59 (q)	2.05 (s)
9	77.00 (d)	5.98 (d, 10.7)		21.41 (q)	2.07 (s)
10	71.86 (d)	5.39 (d, 10.7)	1'	165.99 (s)	-
11	64.29 (s)	-	2'	117.02 (d)	6.27 (d, 15.9)
12	59.33 (s)	-	3'	146.39 (d)	7.67 (d, 15.9)
13	208.21 (s)	-	*q*-Ph	134.09 (s)	-
14	38.06 (t)	α) 2.43 (d, 20.4)	*o*-	128.34 (d)	7.62 (m)
		β) 2.69 (dd, 20.4, 8.4)	*m*-	129.03 (d)	7.43 (m)
15	38.34 (s)	-	*p*-	130.67 (d)	7.43 (m)
16	28.87 (q)	0.83 (s)			

a Multiplicities were determined by DEPT. *b* Connections were determined by HMQC and multiplicities and coupling constants in Hz are in parentheses.

on the cellular accumulation of VCR in MDR human ovarian cancer 2780AD cells was examined with the results summarized in Table 3. Compounds (**1**) and (**2**) showed strong activity toward VCR accumulation in MDR tumor cells compared with those of previously reported taxoids.[3, 13, 14]

The values of VCR accumulation with taxinine NN-3 (**1**), (980 % of control and 185 % verapamil at 10 μg/mL) and taxinine NN-4 (**2**), (295 % of control and 142 % verapamil at 1 μg/mL) shown in Table 3 are almost the comparable or stronger than the maximum value of those reported by Kobayashi and Tsuruo.[15]

EXPERIMENTAL

General Experimental Procedure. The melting point was determined with a Yanagimoto micro-melting point apparatus and is uncorrected. IR spectrum was recorded in $CHCl_3$ on a Hitachi 270-30 spectrophotometer. Optical rotation was measured by a Horiba Polarimeter SEPA-200. UV spectrum was obtained on a Jasco V-550 spectrophotometer. HREIMS was taken on a JEOL JMS HX-110

Table 3. Effects of Taxinine NN-3 (**1**) and Taxinine NN-4 (**2**) on the Accumulation of Vincristine (VCR) in Multidrug-Resistant 2780AD Cells.

compound	VCR accumulation with taxoids[a]					Evaluation
	concentration (μg / mL)	average[b] dpm / well	% of control[c]	activities[d]	Verapamil %[e]	Maximum verapamil % concentration
Taxinine NN-3 (**1**)	0.1	280	123	+	105	P[f]
	1	687	301	+ +	145	185 %
	10	2235	980	+ + +	185	10 μg / mL
Taxinine NN-4 (**2**)	0.1	276	121	+	104	P
	1	673	295	+	142	142 %
	10	1268	556	+ + +	105	1 μg / mL
Verapamil	0 (control)	228	100			
	0.1	266	117	+	100	
	1	473	207	+	100	
	10	1211	531	+ + +	100	

[a] The amount of VCR accumulation in multidrug-resistant human ovarian cancer 2780AD cells was determined in the presence of 0.1, 1 and 10 μg/mL of taxoids.

[b] The values represent means of triplicate determination.

[c] The values are expressed as the relative amount of VCR accumulated in the cell compared with the control experiment.

[d] The indices are expressed on a scale of seven by the range of the relative amount of VCR accumulation as compared with the control experiment (%);
+++, 501 ~ 1000 %; ++, 301 ~ 500 %; +, 111 ~ 300 %; ±, 91 ~ 110 %; -, < 90 %.

[e] The values are expressed as the relative amount vincristine (VCR) accumulation in the cell as compared with that of verapamil.

[f] P; positive: The activity is stronger than that of verapamil (verapamil % > 100 %).

spectrometer. ^{1}H-NMR (499.87 MHz) were run on a Varian UNITY-PS 500 spectrometer and ^{13}C-NMR (150.09 and 125.70 MHz) spectra run on a Bruker AM-600 and Varian UNITY-PS 500 spectrometer. To describe HPLC conditions, we designate column, solvent, flow rate (mL/min), and retention time (t_R in min) in this order.

Plant material. A mixture of needles and young stems of *T. cuspidate* were collected from two female trees of 2-m height grown in matumoto, Nagano prefecture, Japan, on November 15, 1997.

Extraction. A mixture of fresh needles and young stems (264 g) were defatted by extraction with hexane (1.6 L) for one week at 25 °C. The remaining plant material was soaked in EtOAc (1.6 L) for one week at 25 °C. The residue (3.61 g) remaining after removal of the solvent was dissolved in a mixture of MeOH–EtOAc (1 : 4, 50 mL) and extracted with a 0.5 M aqueous solution of H_2SO_4 (3 × 15 mL). Subsequently, the combined acid solution (pH 1.5) was brought to pH 10.0 by addition of a 29 % aqueous solution of NH_4OH (50 mL) and then extracted with $CHCl_3$ (5 × 20 mL). The combined extracts were dried (Na_2SO_4), filtered, and concentrated to give a crude basic taxoid fraction (0.36 g; 0.14 % from fresh plant material, 9.97 % from defatted EtOAc extract).

EtOAc phase was then extracted with a 2 M aqueous solution of NaOH (2 × 15 mL). Subsequently, the combined alkali solution (pH 7.5) was brought to pH 3.0 by addition of a 0.5 M aqueous solution of H_2SO_4 (50 mL) and then extracted with $CHCl_3$ (3 × 20 mL). The combined extracts were dried (Na_2SO_4), filtered, and concentrated to give a crude phenolic fraction (0.88 g; 0.33 % from fresh plant material; 24.38 % from defatted EtOAc extract).

Finally remaining EtOAc phase was washed with a saturated salt solution (3 × 20 mL), dried (Na_2SO_4), and concentrated to give a crude neutral fraction (1.86 g; 0.70 % from fresh plant material; 51.52 % from defatted EtOAc extract).

Isolation. The crude fraction (1.86 g) was divided into ten fractions (fraction 1, 1002.5 mg; fraction 2, 320.8 mg; fraction 3, 104.3 mg; fraction 4, 30.5 mg; fraction 5, 12.9 mg; fraction 6, 27.7 mg, fraction 7, 35.5 mg; fraction 8, 278.4 mg; fraction 9, 10.1 mg; fraction 10, 4.1 mg) by the flash column chromatography [silica gel (230—400 mesh), 55.8 g; solvent, EtOAc–hexane (6 : 4) for fractions 1—5, EtOAc for fraction 6, 7, MeOH for fraction 8—10].

The fraction 1 (F 1) of flash chromatography was separated by HPLC [column, INERTSIL PREP-SIL (GL Science), 25- × 1 cm i.d.; solvent, EtOAc–hexane (3 : 7); flow rate 5 mL/min] to give crude taxinine (t_R 17.4 min, 27.2 mg) and taxinine NN-3 (**1**) (t_R 33.2 min, 1.2 mg, 0.065% based on the crude neutral

fraction and 0.0005% based on fresh plant material). Finally, crude taxinine was further purified by HPLC in the same conditions except the solvent ratio [EtOAc–hexane (2 : 8)] to give taxinine (t_R 35.0 min, 21.8 mg, 1.17% based on the crude neutral fraction and 0.0083% based on fresh plant material) and taxinine NN-4 (**2**) (t_R 40.5 min, 1.6 mg, 0.086% based on the crude neutral fraction and 0.0006% based on fresh plant material).

Identification of compound (1) and (2). Taxinine NN-3 (**1**): microcrystals ($CHCl_3$), mp 58—60 ˚C; $[\alpha]^{20}_D$ +87.0° (*c*, 0.10, $CHCl_3$); UV(MeOH) λmax nm (log ε), 217 (3.95), 278 (4.16); HREIMS *m/z* 564.2723, calcd for $C_{33}H_{40}O_8$ 564.2724. Taxinine NN-4 (**2**): microcrystals ($CHCl_3$), mp 216—218 ˚C; $[\alpha]^{20}_D$ +21.7° (*c*, 0.09, $CHCl_3$); HREIMS *m/z* 622.2783 calcd for $C_{35}H_{42}O_{10}$ 622.2779.

Cellular accumulation of [^{3}H]-VCR. The multidrug-resistant 2780AD cells were maintained in PPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 5 % heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin. 2780AD cells (1×10^6 cells/well) were seeded in a 24-well plate and cultured for 18 h before the assay. The cells were treaded with 1×10^5 dpm of [^{3}H]-VCR (222 Gbq/mmol; Amersham Pharmacia Biotech, Tokyo, Japan) in the presence or absence of verapamil or taxiods. Immediately after incubation for 2 h at 37 °C, the cells were washed five times with ice-cold phosphate-buffered saline containing 0.1 mg/mL of non-radioactive VCR and lysed with 500 μL of 0.2 M NaOH. After incubation for 45 min at 56 °C, lysates were neutralized with 2 M acetic acid, and the radioactivity was counted in ACS II (Amersham Pharmacia Biotech).

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