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Taxuspines A ~ C, New Taxoids from Japanese Yew *Taxus Cuspidata* Inhibiting Drug Transport Activity of P-glycoprotein in Multidrug-Resistant Cells

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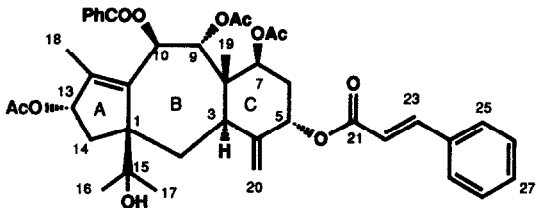
Abstract: Three new taxoids, taxuspines A ~ C (**1** ~ **3**), possessing different skeletons from usual taxane diterpenoids consisting of a 6/8/6-membered ring system have been isolated together with known taxoids (**4** ~ **12**) from stems of the Japanese yew *Taxus cuspidata* Sieb. et Zucc. and the structures elucidated on the basis of spectroscopic data. Most of the taxoids increased cellular accumulation of vincristine in multidrug-resistant tumor cells, while taxol (**12**) did not show such an activity. The taxoids (**1** ~ **11**) showed no or weak cytotoxicity and among them, **2** and **6** reduced appreciably $CaCl_2$-induced depolymerization of microtubules. These results suggest that some taxoids may be useful for overcoming multidrug resistance in tumor cells.

Since the discovery of anticancer activity of taxol, much attention has been paid for the isolation of new taxane diterpenoids from various species of yews.[1] In our continuing search for bioactive natural products,[2] we examined extracts of the Japanese yew *Taxus cuspidata* Sieb. et Zucc. and obtained three new rearranged taxane diterpenoids, named taxuspines A ~ C (**1** ~ **3**), together with known taxoids (**4** ~ **12**). Most of the taxoids increased cellular accumulation of vincristine (VCR) in multidrug-resistant (MDR) tumor cells, while taxol (**12**) did not show such an activity. The taxoids (**1** ~ **11**) showed no or weak cytotoxicity, and compounds **2** and **6** exhibited appreciable taxol-like activity to reduce $CaCl_2$-induced depolymerization of microtubules among the taxoids. In this paper the isolation and structure determination of taxuspines A ~ C (**1** ~ **3**) and the structure-activity relationships of the taxoids (**1** ~ **12**) are described.

Results and Discussion

Isolation and Structure Determination. The methanolic extract of stems of the yew collected at Sapporo was partitioned between toluene and water, and the water layer was extracted with chloroform. The toluene soluble portions were subjected to a silica gel column followed by reversed-phase and silica gel



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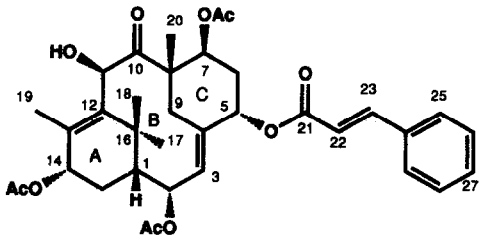

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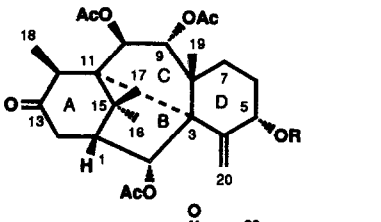
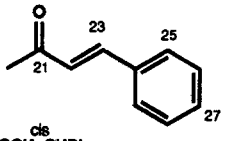
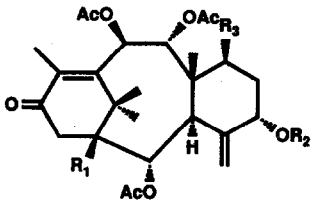


3 R =



13 R = COCH=CHPh (cis)



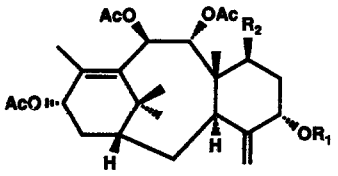
4 R_1=R_3=H, R_2=COCH=CHPh (trans)

5 R_1=R_2=R_3=H

6 R_1=H, R_2=COCH=CHPh (trans), R_3=OAc

7 R_1=OH, R_2=COCH=CHPh (trans), R_3=H

11 R_1=H, R_2=$COCH_2CH(NMe_2)Ph$, R_3=H



8 R_1=$COCH_2CH(NMe_2)Ph$, R_2=OAc

9 R_1=COCH=CHPh (trans), R_2=H

10 R_1=COCH=CHPh (trans), R_2=OAc

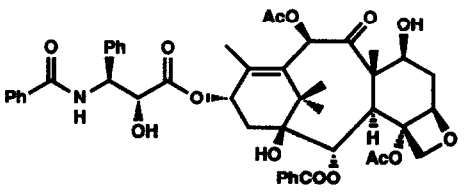


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Table 1. ^{1}H and ^{13}C NMR Data of Taxuspine A (1) in $CDCl_3$

position	$^{1}H^{a}$		J(Hz)	$^{13}C^{a}$		H coupled with C^{b}
1				62.8	s	H-2, H-2', H-3, H-10, H-14, H-14', H-16, H-17
2	2.39	m		29.5	t	H-3, H-14, H-14'
2'	1.42	m				
3	2.78	d	8.3	39.2	d	H-2, H-2', H-19, H-20, H-20'
4				145.4	s	H-3
5	5.56	t	2.7	74.7	d	H-3, H-20, H-20'
6	2.04	m		34.5	t	H-7
6'	2.07	m				
7	5.72	dd	10.8, 5.4	69.9	d	H-3, H-5, H-19
8				44.7	s	H-3, H-7, H-10, H-19
9	6.15	brd	10.3	77.3	d	H-3, H-10, H-19
10	6.70	d	10.3	69.9	d	
11				136.8	s	H-2, H-10, H-13, H-14, H-14', H-18
12				147.2	s	H-13, H-14, H-18
13	5.41	m		79.6	d	H-14, H-14'
14	1.20	dd	14.2, 5.4	45.0	t	H-2, H-2'
14'	2.50	dd	14.2, 6.8			
15				75.8	s	H-2, H-2', H-14, H-14', H-16, H-17
15-OH	2.68	brs				
16	1.34	s		25.2	q	H-17
17	1.10	s		27.3	q	H-16
18	2.07	s		12.2	q	
19	0.96	s		13.1	q	H-3, H-7
20	5.38	s		114.8	t	H-3, H-5
20'	4.96	s				
21				165.7	s	H-5, H-23
22	6.41	d	15.6	118.3	d	H-23
23	7.71	d	15.6	145.4	d	H-22
24				134.6	s	H-22, H-26
25	7.52[c]	m		128.3	d	H-23
26	7.40[c]	m		128.9	d	
27	7.40	m		129.5	d	H-25
PhCOO				164.4	s	H-10
				130.8	s	
	7.88[c]	d	7.8	129.0	d	
	7.43[c]	t	7.8	129.8	d	
	7.56	t	7.8	133.6	d	
7-AcO	2.08	s		20.6	q	
				170.0	s	H-7
9-AcO	1.76	s		20.8	q	
				170.0	s	
13-Aco	1.52	s		21.8	q	
				170.8	s	H-13

a) δ in ppm b) HMBC correlations c) 2H

column chromatographies to afford taxuspines A (**1**, 0.0017%), B (**2**, 0.00097%), and C (**3**, 0.0017%) together with known taxane diterpenoids, taxinine[3,4] (**4**, 0.041%), taxinines A[3] (**5**, 0.0067%) and B[4,5] (**6**, 0.0079%), *O*-cinnamoyltaxicin I triacetate[3] (**7**, 0.0085%), 2-desacetoxyaustrospicatine[6] (**8**, 0.0025%), 2-desacetoxytaxinine E[7] (**9**, 0.0013%), and 2-desacetoxytaxinine J[8] (**10**, 0.0062%). The $CHCl_3$ soluble

portions were subjected to silica gel and reversed-phase column chromatographies to give other known taxane diterpenes, taxine II[9] (**11**, 0.0052%) and taxol[10] (**12**, 0.0012%).

Taxuspine A (**1**), a colorless amorphous solid, showed a fragment ion peak at *m/z* 607 in the FABMS spectrum, and the molecular formula, $C_{42}H_{48}O_{11}$, was determined by HRFABMS [*m/z* 607.2939 (M^+-PhCOOH+H), Δ +3.2 mmu]. IR absorptions at 3550, 1730, and 1710 cm^{-1} indicated the presence of hydroxy, ester and α,β-unsaturated ester groups, respectively. The ^{13}C NMR (Table 1) spectrum of **1** showed signals due to seven primary, four secondary, eighteen tertiary, and thirteen quaternary carbons. The ^1H NMR (Table 1) spectrum of **1** in $CDCl_3$ showed proton signals due to four methyls (δ_H 0.96, 1.10, 1.34, and 2.07), three acetyl methyls (δ_H 1.52, 1.76, and 2.08), an exomethylene (δ_H 4.96 and 5.38), and a deuterium-exchangeable proton (δ_H 2.68). Proton signals due to a benzoyl group appeared at δ_H 7.88 (2H, d, J = 7.8 Hz), 7.56 (1H, t, J = 7.8 Hz), and 7.43 (2H, t, J = 7.8 Hz), while those of a cinnamoyl group were observed at δ_H 7.52 (2H, m), 7.40 (3H, m), 6.41 (1H, d, J = 15.6 Hz), and 7.71 (1H, d, J = 15.6 Hz; *trans*-oriented). UV absorption at 277 nm also supported the presence of a cinnamoyl group. Prominent fragment peaks at *m/z* 131 (C_9H_7O) and 105 (C_7H_5O) in EIMS corresponded to fission of a cinnamoyl and a benzoyl groups from **1**, respectively. The ^1H and ^{13}C NMR spectra of **1** were assigned on the basis of several types of 2D NMR data including ^1H-^1H COSY, HSQC,[11] and HMBC[12] spectra. Detailed analysis of the ^1H-^1H COSY spectrum of **1** revealed connectivities of C-2 to C-3, C-5 to C-7, C-9 to C-10, C-13 to C-14, C-22 to C-23, and C-25 to C-27. In the HMBC (Table 1) spectrum of **1** H_2-14 showed long-range ^1H-^{13}C correlations with C-1 and C-11, and H-13 showed cross peaks with C-11 and C-12, indicating the presence of a cyclopentene moiety (ring A). HMBC correlations of H_3-18 to C-11 and C-12 revealed that Me-18 was attached at C-12. The presence of a seven-membered ring (ring B) was deduced from cross peaks of H_2-2 to C-1 and C-8, and H-10 to C-8 and C-11 in the HMBC spectrum. HMBC correlations of H-20 and H-20' to C-3 and C-5 indicated that an exomethylene (C-20) was attached

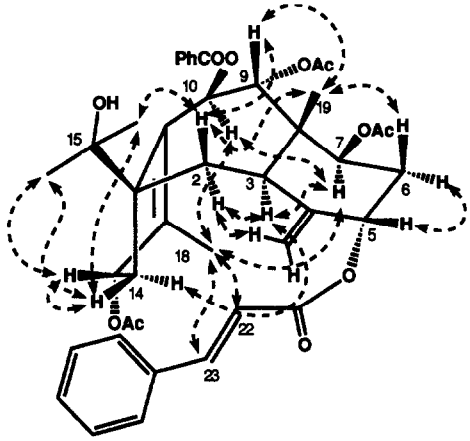

Figure 1. Relative Stereochemistry of Taxuspine A (**1**).
Dotted arrows denote NOESY correlations.

Table 2. ^{1}H and ^{13}C NMR Data of Taxuspine B (2) in $CDCl_3$

position	$^{1}H^{a}$		J(Hz)	$^{13}C^{a}$		H coupled with C^{b}
1	1.66	dl	8.2, 2.2	47.4	d	H-2, H-15, H-15', H-17, H-18
2	5.73	dl	9.8, 2.2	71.0	d	H-15, H-15'
3	5.47	d	9.8	125.2	d	H-5, H-9'
4				132.3	s	H-2, H-5, H-9'
5	5.75	d	6.5	69.7	d	H-3, H-9'
6	2.02	dl	14.7, 3.0	32.7	t	
6'	2.26	ddl	14.7, 12.5, 6.5			
7	5.21	dl	12.5, 3.1	71.1	d	H-5, H-6, H-9', H-20
8				53.2	s	H-6, H-9', H-20
9	2.00	d	15.4	35.5	t	H-3, H-20
9'	2.82	d	15.4			
10				213.1	s	H-7, H-9, H-9', H-11, OH-11
11	5.41	d	2.7	76.8	d	OH-11
11-OH	4.22	s				
12				132.4	s	H-1, H-11, H-14, H-17, H-18, H-19
13				135.9	s	H-11, H-14, H-15, H-19
14	5.40	dl	10.7, 2.7	69.8	d	H-15'
15	2.72	ddl	16.2, 10.7, 8.2	27.6	t	H-1, H-2, H-14
15'	1.81	dl	16.2, 2.7			
16				37.7	s	H-11, H-15', H-17, H-18
17	1.20	s		24.7	q	H-18
18	1.18	s		32.6	q	H-17
19	1.93	d	1.8	16.9	q	
20	1.35	s		20.7	q	
21				166.7	s	H-5, H-22, H-23
22	6.61	d	16.1	117.9	d	H-23
23	7.84	d	16.1	146.4	d	H-25
24				134.1	s	H-22, H-23, H-25, H-26
25	7.52[c]	m		128.2	d	H-23, H-26, H-27
26	7.42[c]	m		129.2	d	H-25, H-27
27	7.42	m		130.9	d	H-25, H-26
2-AcO	2.02	s		21.0	q	
				169.9	s	H-2
7-AcO	2.05	s		21.4	q	
				170.0	s	H-7
14-AcO	1.99	s		21.5	q	
				170.8	s	H-14

a) δ in ppm b) HMBC correlations c) 2H

at C-4. Cross peaks from H-3 to C-4 and C-5, and H-7 to C-8 in the HMBC spectrum showed the presence of a cyclohexane moiety (ring C). Me-19 was attached at C-8 from HMBC correlations of H_3-19 to C-8. The chemical shift of C-15 (δ_C 75.8) indicated that an oxygen atom was attached the carbon. NOESY correlations of a deuterium-exchangeable proton (HO-15) with H_3-16 and H_3-17 and HMBC correlations of H_3-16 and H_3-17 to C-1 and C-15 revealed the presence of a dimethyl carbinol group at C-1. A carbonyl carbon at δ_C 164.4 showed correlations for H-10 and aromatic protons *ortho* to the carbonyl group in the HMBC spectrum, indicating the presence of a benzoate group at C-10 (δ_C 69.9). Two acetoxy (δ_C 170.0 and 170.8) and a cinnamate (δ_C 165.7) carbonyl carbons showed HMBC correlations for H-7, H-13, and H-5, respectively, indicating that the two acetoxy and the cinnamate groups were attached at C-7, C-13, and C-5, respectively. A remaining acetoxy group (δ_C 170.0), therefore, must be connected to C-

9. Thus the structure of taxuspine A was assigned to be **1**, having a rearranged taxane skeleton consisting of a 5/7/6-membered ring system. Relative stereochemistry of **1** (Fig. 1) was elucidated by the NOESY spectrum. The NOESY correlations of H-13/H-14', H-13/H_3-17, H-14'/H_3-16, and H-14'/H_3-17 in **1** revealed that H-13 and the dimethylcarbinol group were both β-oriented on ring A. A boat-like conformation of ring B was elucidated from the coupling constant (10.3 Hz) between H-9 and H-10 and NOESY correlations of H-2'/H-9 and H-3/H-14, while a chair conformation of ring C was assigned from NOESY correlations of H-3/H-7, H-5/H-6, and H-6'/H_3-19 and the following coupling constants; $J_{5,6}$ = $J_{5,6'}$ = 2.7 Hz, $J_{6,7}$ = 5.4 Hz, and $J_{6',7}$ = 10.8 Hz. The NOESY spectrum showed cross peaks of H-2/H-3, H-2'/H-9, H-2'/H_3-19, H-3/H-7, and H-9/H_3-19, indicating *trans* junction between rings B and C. The β-orientation of H-9 was assigned by NOESY correlations of H-2'/H-9 and H-9/H_3-19. The α-orientation of a cinnamate group at C-5 was deduced from NOESY correlations of H_3-18/H-22 and H_3-18/H-23, while α-orientation of H-7 and H-10 was assigned on the basis of those of H-7/H-3, H-7/H-10, H-7/H_3-18, and H-10/H_3-18. Thus the relative stereochemistry of **1** was concluded as shown in Fig. 1.

Taxuspine B (**2**) was obtained as a colorless amorphous solid and showed the molecular ion at *m/z* 622 in the EIMS spectrum. HREIMS analysis revealed the molecular formula to be $C_{35}H_{42}O_{10}$ [*m/z* 622.2779 (M^+), Δ +0.1 mmu]. IR absorptions at 3440, 1730, and 1710 cm^{-1} implied that **2** possessed hydroxy, ester, and ketone groups, respectively. The ^1H NMR (Table 2) spectrum of **2** showed proton signals due to four methyls (δ_H 1.18, 1.20, 1.35, and 1.93), three acetyl methyls (δ_H 1.99, 2.02, and 2.05), an olefin (δ_H 5.47), and a deuterium-exchangeable proton (δ_H 4.22). The ^{13}C NMR (Table 2) spectrum showed signals due to seven primary, three secondary, fourteen tertiary, and eleven quaternary carbons containing a ketone carbonyl (δ_C 213.1). Proton signals due to a cinnamoyl group appeared at δ_H 7.52 (2H, m), 7.42 (3H, m), 6.61 (1H, d, J = 16.1 Hz), and 7.84 (1H, d, J = 16.1 Hz; *trans*-oriented). UV absorption at 279 nm also supported the presence of a cinnamoyl group. Prominent fragment peak at *m/z* 131 (C_9H_7O) was characteristic for fission of cinnamoyl group from **2**. The protonated carbons were all assigned by HMQC[13] experiment. Detailed analysis of the ^1H-^1H COSY spectrum revealed

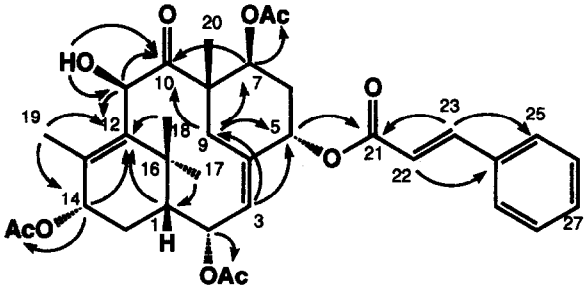

Figure 2. HMBC Correlations of Taxuspine B (**2**).

connectivities of C-14 to C-1, C-1 to C-3, C-5 to C-7, C-22 to C-23, and C-25 to C-27. HMBC correlations (Table 2 and Fig. 2) of H-1 to C-16 and C-12 and H-14 to C-12 and C-13 indicated the presence of a cyclohexene moiety (ring A). In the HMBC spectrum cross peaks of H_3-17 and H_3-18 to C-1, C-12, and C-16 revealed that Me-17 and Me-18 were attached at C-16. HMBC correlations of H_3-19 to C-12 and C-13 revealed that Me-19 was attached at C-13. Cross peaks of H-3 to C-9, H-9' to C-4, C-8, and C-10 (δ_C 213.1), and H-11 to C-10 and C-12 in the HMBC spectrum indicated the presence of a ten-membered ring (ring B). A deuterium-exchangeable proton (δ_H 4.22, OH-11) was attached to an oxygen on C-11 by HMBC correlations of OH-11 to C-10 and C-11. ^1H-^{13}C long-range correlations of H-9' to C-5 and C-7 indicated the presence of a cyclohexane moiety (ring C). HMBC correlations of H_3-20 to C-7, C-8, and C-9 implied that Me-20 was attached at C-8. A carbonyl carbon at δ_C 166.7 (C-21) showed a correlation with H-5 in the HMBC spectrum, indicating the presence of a cinnamate group at C-5. Three acetoxy carbonyl carbons (δ_C 169.9, 170.0, and 170.8) showed HMBC correlations with H-2, H-7, and H-14, respectively, indicating that the three acetoxy groups were attached at C-2, C-7, and C-14, respectively. Thus the structure of taxuspine B was assigned to be **2**, which is a compound related to taxine A[14] consisting of a 6/10/6-membered ring system. Relative stereochemistry of **2** (Fig. 3) was elucidated by the NOESY spectrum. NOESY correlations of H-14/H-15', H-14/H_3-18, H-5/H-6', H-6'/H_3-20, and H-6/H-7 indicated both boat conformations of rings A and C.

The molecular formula, $C_{35}H_{42}O_9$, of taxuspine C (**3**) was established by the HREIMS [*m/z* 606.2878 (M^+), Δ +4.9 mmu]. IR absorptions at 1740 and 1700 cm^{-1} implied that **3** possessed ester and ketone groups, respectively. The ^1H NMR (Table 3) spectrum of **3** showed proton signals due to four methyl groups (δ_H 1.22, 1.29, 1.32, and 1.70), three acetyl groups (δ_H 2.05, 2.05, and 2.07), and an exomethylene (δ_H 5.71 and 5.85). The presence of a ketone group (δ_C 214.8) was implied by the ^{13}C NMR data (Table 3). Proton signals due to a cinnamoyl group appeared at δ_H 7.56 (2H, m), 7.38 (3H,

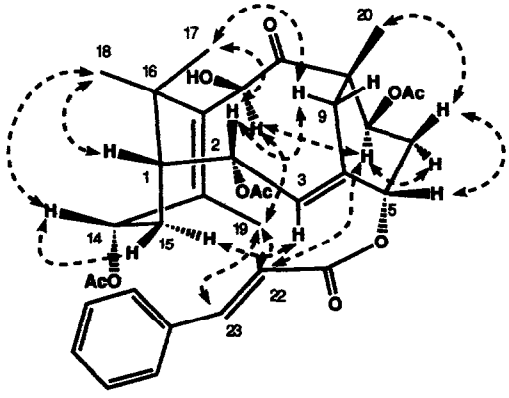

Figure 3. Relative Stereochemistry of Taxuspine B (**2**).
Dotted arrows denote NOESY correlations.

Table 3. ^{1}H and ^{13}C NMR Data of Taxuspine C (3) in $CDCl_3$

position	$^{1}H^{a}$		*J*(Hz)	$^{13}C^{a}$		H coupled with C[b]
1	2.18	m		47.8	d	H-2, H-14, H-14', H-16, H-17
2	6.14	d	5.3	76.5	d	H-1, H-14, H-14'
3				65.9	s	H-2, H-5, H-7, H-9, H-12, H-19,
4				142.2	s	H-2, H-5, H-20
5	5.64	m		77.2	d	H-20
6	1.78[c]	m		25.8	t	H-5
7	1.80[c]	m		31.2	t	H-9, H-19
8				44.5	s	H-2, H-7, H-9, H-19
9	5.70	d	8.8	79.5	d	H-10, H-19
10	5.68	d	8.8	82.3	d	H-9
11				57.8	s	H-1, H-10, H-12, H-16, H-17,
12	3.55	q	6.8	52.3	d	H-10, H-18
13				214.8	s	H-1, H-12, H-14, H-14', H-18
14	2.59	d	20.5	38.8	t	H-2
14'	2.50	dd	20.5, 6.8			
15				42.6	s	H-1, H-10, H-12, H-14, H-16,
16	1.70	s		28.8	q	H-17
17	1.22	s		26.5	q	H-16
18	1.29	d	6.8	15.7	q	H-12
19	1.32	s		26.7	q	H-9
20	5.85	s		129.4	t	H-5
20'	5.71	s				
21				165.7	s	H-5, H-22, H-23
22	6.39	d	16.1	117.8	d	H-23
23	7.67	d	16.1	145.3	d	
24				134.3	s	H-22, H-26
25	7.56[c]	m		128.2	d	H-26
26	7.38[c]	m		128.8	d	H-23, H-25
27	7.38	m		130.3	d	H-25
2-AcO	2.07	s		20.9	q	
				169.5	s	H-2
9-AcO	2.05	s		21.4	q	
				170.9	s	H-9
10-AcO	2.05	s		21.1	q	
				170.0	s	H-10

a) δ in ppm b) HMBC correlations c) 2H

m), 6.39 (1H, d, J = 16.1 Hz), and 7.67 (1H, d, J = 16.1 Hz; *trans*-oriented). UV absorption at 280 nm also supported the presence of a cinnamoyl group. Prominent fragment peak at *m/z* 131 (C_9H_7O) in EIMS corresponded to fission of a cinnamoyl group from **3**. Detailed analysis of the ^{1}H-^{1}H COSYspectrum of **3** revealed connectivities of C-1 to C-2, C-5 to C-7, C-9 to C-10, C-14 to C-1, C-12 to C-18, C-22 to C-23, and C-25 to C-27. In the HMBC (Table 3) spectrum ^{1}H-^{13}C long-range correlations were observed for H-1 to C-11 and C-15, H-12 to C-11 and C-13, and H_2-14 to C-13, indicating the presence of a cyclohexanone moiety (ring A). HMBC correlations 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. Cross peaks from H-2 to C-3 and H-12 to C-3 in the HMBC spectrum showed the presence of a cyclopentane moiety (ring B). HMBC correlations of H-9 to C-3 and C-8 and H-10 to C-11 indicated the presence of another cyclopentane moiety (ring C). Cross peaks from

H-5 to C-3 and C-4 and H-7 to C-3 and C-8 in the HMBC spectrum showed the presence of a cyclohexane moiety (ring D). HMBC correlations of H-20 and H-20' to C-3 and C-5 indicated that an exomethylene (C-20) was attached at C-4. Me-19 was attached at C-8 from HMBC correlations of H_3-19 to C-3, C-7, C-8, and C-9. Three acetoxy (δ_C 169.5, 170.9, and 170.0) and a cinnamoyl (δ_C 165.7) carbonyl carbon showed HMBC correlations with H-2, H-9, H-10, and H-5, respectively, indicating that the three acetoxy and the cinnamate group were attached at C-2, C-9, C-10, and C-5, respectively. Thus the structure of taxuspine C was elucidated to be **3**. Relative stereochemistry of **3** (Fig. 4) was elucidated by the NOESY spectrum. NOESY correlations of H-1/H-14' and H-14'/H_3-17 indicated a boat conformations of ring A, while a chair conformation of ring C was assigned from NOESY correlations of H-5/H-7' and H-7'/H_3-19. The absolute stereochemistry of **3** was established by the following photochemical reaction; irradiation of taxinine (**4**), of which absolute stereochemitry has been determined by X-ray analysis,[15] in dioxane with a Hg-lamp gave rise to compounds **3** and **13**, the latter is 22-Z-form of **3**.[16] All spectral data ($[\alpha]_D$, ^1H and ^{13}C NMR, IR, UV, and EIMS) of **3** derived from **4** were identical with those of taxuspine C. Thus the absolute stereochemistry of taxuspine C (**3**) was concluded to be the same as that of taxinine (**4**).

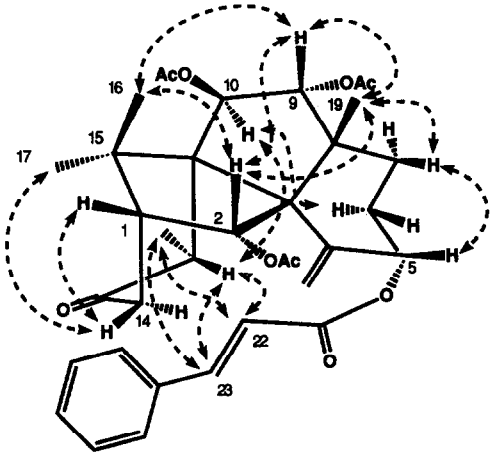

Figure 4. Relative Stereochemistry of Taxuspine C (**3**).
Dotted arrows denote NOESY correlations.

Taxuspines A ~ C (**1** ~ **3**) are new taxane diterpenoids isolated from stems of the Japanese yew *Taxus cuspidata* Sieb. et Zucc. These compounds possess different skeletons from usual taxoids consisting of a 6/8/6-membered ring system. Taxuspine A (**1**) is a rare example of taxoids involving a 5/7/6-membered ring system, of which the skeleton has been more recently reported for taxchinins.[17] Taxuspine B (**2**) is the second example of taxane diterpenoid involving a 6/10/6-membered ring system, although the first one is taxine A.[14] On the other hand, taxuspine C (**3**) possesses an unusual 6/5/5/6-membered ring system.[5]

Table 4. Cytotoxicity of Taxoids (**1** ~ **13**) against L1210 Murine Leukemia Cells and KB Human Epidermoid Carcinoma Cells

	L1210		KB	
compound	10 μg/mL (%)[a]	IC_{50} (μg/mL)	10 μg/mL (%)[a]	IC_{50} (μg/mL)
1	85.4	4.2	37.2	
2	42.3	18	11.8	
3	72.6	5.8	8.9	
4	10.5		4.8	
5	55.0	8.9	30.7	
6	45.9		28.8	
7	82.3	4.6	70.1	6.9
8	58.7	7.2	13.9	
9	51.1	9.5	65.6	8.2
10	79.5	4.9	27.6	
11	46.6		12.6	
12	86.8	0.33	92.0	0.0088
13	41.0		5.3	

a) inhibition (%) at 10 μg/mL

Though taxuspine C (**3**) is generated from taxinine (**4**) through photochemical reaction described above, **3** is considered to be a natural constituent of the yew, judging from the presence of **3** and the absence of **13** in the fresh extract of the yew. This is the first isolation of 2-desacetoxyaustrospicatine (**8**) and 2-desacetoxytaxinine E (**9**) from *Taxus cuspidata* Sieb. et Zucc., although **8** and **9** were isolated previously from *Austrotaxus spica* and *Taxus mairei*, respectively.[6,7]

Cytotoxicity Studies. Cytotoxic activity of taxoids (**1** ~ **13**) against murine lymphoma L1210 and human epidermoid carcinoma KB cells is shown in Table 4. Taxuspines A (**1**) and C (**3**) exhibited modest cytotoxicity against L1210 cells with IC_{50} values of 4.2 and 5.8 μg/mL, respectively, while taxuspine B (**2**) showed weak cytotoxicity. Nine known taxoids (**4** ~ **11** and **13**) also showed modest or weak cytotoxicity although taxol (**12**) was most cytotoxic among the taxoids examined.

Effect of Taxoids on the Ca^{2+} Induced Microtubule Depolymerization. Taxol is unique among antimitotic drugs in that it enhances the assembly of tubulin into stable microtubules, whereas other drugs, such as colchicine and VCR, inhibit the assembly. Microtubules polymerized in the presence of taxol are resistant to depolymerization by Ca^{2+} ions.[18] The effect of taxoids **1** ~ **13** was thus examined against the $CaCl_2$-induced depolymerization of microtubules.

Microtubule proteins were polymerized under normal polymerization condition[19] in the absence and the presence of taxol (**12**) or taxoids **1** ~ **11** and **13**, and, after 30 minutes incubation, $CaCl_2$ was added. Microtubule polymerization and depolymerization were monitored by the increase and the decrease in turbidity. The results are summarized in Fig. 5 as the changes in the relative absorbance at 400 nm.

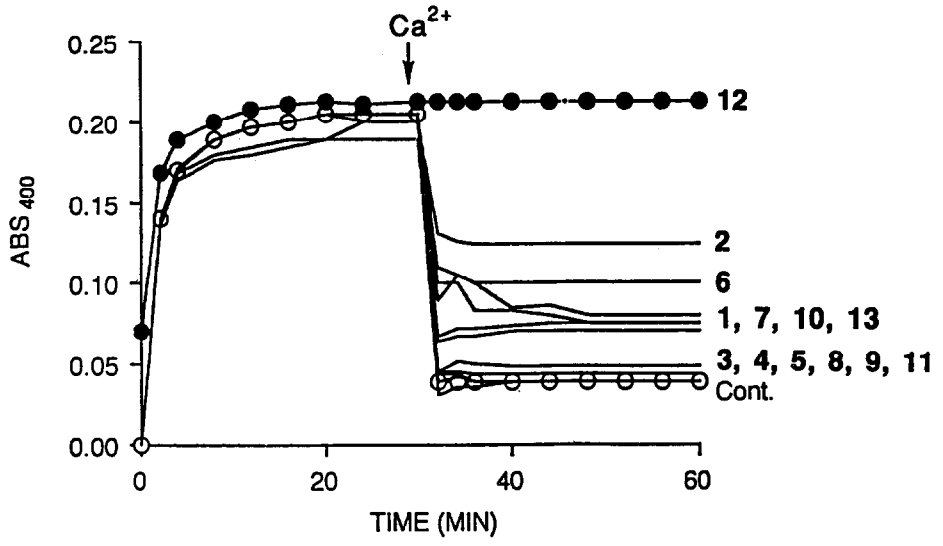

Figure 5. Effect of Taxoids (**1** ~ **13**) on Ca^{2+} Induced Microtubule Depolymerization.

The $CaCl_2$-induced depolymerization of microtubules (shown as control) was completely inhibited by 10 μM of taxol (**12**). Among the tested taxoids, compounds **2** and **6** appreciably reduced the depolymerization process, suggesting that these compounds have taxol-like activity to microtubule systems. The other compounds showed little or no such effect.

Increased Cellular Accumulation of Vincristine in Multidrug-Resistant Cells by Taxoids. The cellular accumulation of vincristine is reduced in multidrug-resistant tumor cells as compared with the parental cells.[20] MDR-reversing agents such as verapamil increase the reduced accumulation of antitumor agents in MDR cells and overcome drug resistance.[21,22] The effect of taxoids (**1** ~ **13**) on the cellular accumulation of VCR in multidrug-resistant 2780AD cells is shown in Table 5. Verapamil at 1 and 10 μg/mL increased the VCR accumulation in a dose dependent manner. Compounds **2**, **3**, **8**, **10**, **11**, and **13** increased the VCR accumulation as well as verapamil. Compounds **1**, **4**, **5**, **6**, **7**, and **9** increased the accumulation moderately, while taxol (**12**) decreased the VCR accumulation in 2780AD cells. These results imply that the taxoids could be substrates of P-glycoprotein and the compounds could be useful for overcoming of multidrug-resistance. Further studies are needed on mechanism of the action.

Table 5. Effects of Taxoids (**1** ~ **13**) on the Accumulation of Vincristine in Multidrug-Resistant Cells

Compound	VCR accumulation (% of control) with a taxoid concentration of	
	1 μg/mL	10 μg/mL
1	142[a]	246
2	219	713
3	246	768
4	195	571
5	153	461
6	116	438
7	167	436
8	233	841
9	102	517
10	266	798
11	177	704
12	83	56
13	253	782
Verapamil	254	739

a) The amounts of VCR accumulated in multidrug-resistant 2780AD cells were determined in the presence of 1 and 10 μg/mL of taxoids as described in Experimental Section. The values represent means of triplicate determinations, and are expressed as the relative amounts of VCR accumulated in the cells as compared with the control experiment. The accumulation of VCR in 2780AD cells without taxoids (control values) ranged from 27.4 to 44.3 fmol/10^6 cells.

In summary we have isolated three new taxoids together with nine known taxoids from the Japanese yew *Taxus cuspidata* Sieb. et Zucc. Some of the taxoids increased the vincristine accumulation in multidrug-resistant tumor cells as potent as verapamil. These taxoids may be useful for overcoming multidrug-resistance in tumor cells. The present results are interesting in cancer chemotherapy, and mechanisms of the action are currently under investigation.

Experimental Section

General Methods. Optical rotations were determined on a JASCO DIP-370 polarimeter. ^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 77.1 ppm of $CDCl_3$ were used as internal references, respectively. EIMS was obtained on a JEOL DX-303 spectrometer operating at 70 eV. FABMS was measured on an HX-110 spectrometer by using glycerol matrix. Wako C-300 silica gel (Wako Pure Chemical) was used for glass column chromatography. TLC was carried out on Merck silica gel GF_{254}.

Collection, Extraction, and Separation. The Japanese yew *Taxus cuspidata* Sieb. et Zucc. was collected at Sapporo, Hokkaido. The stems (0.5 kg) of the yew was extracted with MeOH (2 L x 3). The MeOH extract was partitioned between toluene (750 mL x 3) and H_2O (750 mL). The aqueous layer was extracted with $CHCl_3$ (750 mL x 3). The toluene soluble portions were evaporated under reduced pressure to give a residue (8.3 g), part of which (4.3 g) was subjected to a silica gel column (4.0 x 37 cm) eluted with hexane/acetone [7:1 (800 mL) → 3:1 (800 mL) → 1:3 (800 mL)] to afford four fractions **I** (650 ~ 1100 mL), **II** (1100 ~ 1350 mL), **III** (1350 ~ 1650 mL), and **IV** (1650 ~ 2250 mL). Fraction **IV** was applied to a reversed-phase column (Develosil Lop ODS 24S, Nomura Chemical, 2.5 x 50 cm; flow rate 3.0 mL/min; UV detection at 278 nm; eluent: CH_3CN/H_2O, 80:20) to give fractions **a** (13.7 mg, t_R 79 min) and **b** (22.6 mg, t_R 48 min), taxinine A (**5**, 11.4 mg, t_R 30 min), taxinine B (**6**, 18.6 mg, t_R 70 min), *O*-cinnamoyltaxicin I triacetate (**7**, 8.5 mg, t_R 64 min), and 2-desacetoxyaustrospicatine (**8**, 6.4 mg, t_R 88 min). The fraction **a** was purified by a silica gel column (1.0 x 10 cm; eluent: $CHCl_3$/EtOAc, 9:1) to give taxuspine A (**1**, 4.5 mg). The fraction **b** was separated by a silica gel column (1.0 x 10 cm, eluent: $CHCl_3$/EtOAc, 95:5), of which the fraction eluting from 4 to 6 mL was subjected to a reversed-phase column (Develosil ODS-5, Nomura Chemical, 1.0 x 25 cm; flow rate 3.0 mL/min; UV detection at 278 nm; eluent: $MeOH/H_2O$, 80:20) to give taxuspine B (**2**, 2.5 mg, t_R 18.0 min). Fraction **III** was separated by a reversed-phase column (Develosil Lop ODS 24S; flow rate 3.0 mL/min; eluent: CH_3CN/H_2O, 80:20) to give fractions **c** (6.3 mg, t_R 70 min) and **d** (34 mg, t_R 100 min). The fraction **c** was subjected to a silica gel column (1.2 x 20 cm) eluted with $CHCl_3$/acetone (98:2) to give taxuspine C (**3**, 4.4 mg). The fraction **d** was purified by preparative silica gel TLC with $CHCl_3$/EtOAc (8:1) to afford 2-desacetoxytaxinine J (**10**, 16 mg). Fraction **II** was purified by a reversed-phase column (YMC-GEL ODS-60A, YMC Co, Ltd., 2.4 x 5 cm, eluent: CH_3CN/H_2O, 80:20) to give taxinine (**4**, 106 mg). Fraction **I** was subjected to gel filtration on a Sephadex LH-20 column (Pharmacia Fine Chemical, 2.0 x 100 cm) with $CHCl_3$/MeOH (1:1) to give 2-desacetoxytaxinine E (**9**, 3.4 mg). The $CHCl_3$ soluble portions were evaporated under reduced pressure to give a residue (1.9 g), part of which (1.0 g) was subjected to a silica gel column (2.4 x 40 cm) eluted with $CHCl_3$/MeOH (98:2), of which the fraction eluting from 160 to 240 mL was separated by a reversed-phase column (Develosil Lop ODS 24S, 2.5 x 50 cm, flow rate: 3.0 mL/min, eluent: $MeOH/H_2O$, 85:15) to give taxine II (**11**, 14.2 mg, t_R 60 min) and taxol (**12**, 3.4 mg, t_R 35 min).

Taxuspine A (1): A colorless amorphous solid; $[\alpha]^{28}_D$ -3.4° (*c* 0.75, $CHCl_3$); IR (film) ν_{max} 3550, 1730, 1710, 1630, and 1240 cm^{-1}; UV (MeOH) λ_{max} 218 (ε 19700), 224 (19100), and 277 nm (17800); ^1H and ^{13}C NMR (Table 1); EIMS *m/z* (%) 606 (M^+-PhCOOH, 2), 550 (3), 488 (10), 446 (9), 428 (8), 400 (3), 340 (6), 298 (8), 238 (23), 220 (43), 131 (100), and 105 (85); FABMS (positive ion, glycerol matrix) *m/z* 607 (M^+-PhCOOH+H); HRFABMS *m/z* 607.2939 (M^+-PhCOOH+H) calcd for $C_{35}H_{43}O_9$, 607.2907; ^1H-^1H COSY correlations ($CDCl_3$, H/H): 2/3, 5/6, 6/7, 9/10, 13/14, 22/23, 25/26, and 26/27; NOESY correlations ($CDCl_3$, H/H): 2/2', 2/3, 2/14, 2/16, 2/20', 2'/9, 2'/16, 2'/19, 3/7, 3/14, 5/6', 6/6', 6'/19, 7/10, 7/18, 9/19, 10/18, 13/14', 13/17, 14/14', 14'/16, 14'/17, 15-OH/16, 15-OH/17, 16/17, 18/22, 18/23, and 20/20'.

Taxuspine B (2): A colorless amorphous solid; $[\alpha]^{23}_D$ -40.6° (*c* 0.43, $CHCl_3$); IR (film) ν_{max} 3440, 1730, 1710, 1630, 1570, and 1230 cm^{-1}; UV (MeOH) λ_{max} 218 (ε 17500) and 279 nm (18200); ^1H and ^{13}C NMR (Table 2); EIMS *m/z* (%) 622 (M^+, 0.1), 621 (0.2), 561 (0.3), 501 (0.4), 441 (0.9), 265 (7), and 131 (100); HREIMS *m/z* 622.2779 (M^+) calcd for $C_{35}H_{42}O_{10}$, 622.2778; ^1H-^1H COSY correlations ($CDCl_3$, H/H): 1/2, 2/3, 5/6, 6/7, 14/15, 15/1, 22/23, 25/26, and 26/27; NOESY correlations ($CDCl_3$, H/H): 1/2, 1/15, 1/15', 1/18, 2/3, 2/9', 2/17, 3/15, 5/6', 6/6', 6/7, 6'/20, 7/11, 7/22, 9/9', 9'/17, 11/19, 11-OH/11, 11-OH/17, 14/15, 14/15', 14/18, 15/15', 15'/18, 19/22, 19/23, and 22/25.

Taxuspine C (3): A colorless amorphous solid; $[\alpha]^{20}_D$ +7.4° (*c* 0.20, $CHCl_3$); IR (film) ν_{max} 1740, 1700, 1640, and 1240 cm^{-1}; UV (EtOH) λ_{max} 218 (ε 18400), 224 (sh), and 280 nm (20500); ^1H and ^{13}C NMR (Table 3); EIMS *m/z* (%) 606 (M^+, 0.4), 546 (0.5), 486 (0.5), 458 (2), 398 (4), and 131 (100); HREIMS *m/z* 606.2878 (M^+) calcd for $C_{35}H_{42}O_9$, 606.2829; ^1H-^1H COSY correlations ($CDCl_3$, H/H): 1/2, 5/6, 6/7, 9/10, 12/18, 14/1, 22/23, 25/26, and 26/27; NOESY correlations (C_6D_6, H/H): 1/2, 1/14, 1/14', 1/16, 1/17, 2/16, 2/19, 5/7, 6/7, 5/20, 7/19, 9/16, 9/19, 10/12, 10/18, 12/18, 12/20, 12/22, 12/23, 14/14', 14/20, 14'/17, 16/17, 18/22, 18/23, 18/25, 20/23, 22/25, and 23/25.

Photochemical Reaction of 4. A solution of **4** (12.0 mg) in 3.0 mL of degassed dioxane was irradiated using a mercury lamp (500 W) housed in a water-cooled Pyrex jacket at room temperature for 17 min. Evaporation under reduced pressure afforded a residue (11.0 mg), which was purified by HPLC (Develosil ODS HG-5, Nomura Chemical, 2.0 x 25 cm; flow rate 2.5 mL/min; UV detection at 278 nm; eluent: MeOH/H_2O, 80:20) to give compounds **3** (6.0 mg, t_R 24 min) and **13**[15] (3.9 mg, t_R 22 min). **3:** A colorless amorphous solid; $[\alpha]^{20}_D$ +9.9° (*c* 0.75, $CHCl_3$); IR (film) ν_{max} 1740, 1700, 1630, and 1240 cm^{-1}; UV (EtOH) λ_{max} 218 (ε 20400), 224 (sh), and 280 nm (25400); ^1H NMR ($CDCl_3$) δ 7.67 (1H, d, *J* = 16.1 Hz, H-23), 7.55 (2H, m, H-25), 7.38 (3H, m, H-26 and H-27), 6.39 (1H, d, *J* = 16.1 Hz, H-22), 6.13 (1H, d, *J* = 5.3 Hz, H-2), 5.85 (1H, s, H-20), 5.71 (1H, s, H-20'), 5.70 (1H, d, *J* = 8.8 Hz, H-9), 5.68 (1H, d, *J* = 8.8 Hz, H-10), 5.64 (1H, m, H-5), 3.55 (1H, q, *J* = 6.8 Hz, H-12), 2.58 (1H, d, *J* = 20.5 Hz, H-14), 2.50 (1H, dd, *J* = 20.5 and 6.8 Hz, H-14'), 2.18 (1H, m, H-1), 2.07 (3H, s, AcO), 2.05 (6H, s, AcO x 2), 1.80 (2H, m, H-7), 1.78 (2H, m, H-6), 1.70 (3H, s, H-16), 1.28 (3H, d, *J* = 6.8 Hz, H-18), and 1.22 (1H, s, H-17); EIMS *m/z* (%) 606 (M^+, 0.4), 546 (0.5), 486 (0.5), 458 (2), 398 (4), and 131 (100). **13:** A colorless amorphous solid; $[\alpha]^{20}_D$ -27.1° (*c* 0.62, $CHCl_3$); IR (film) ν_{max} 1740, 1710, 1695, 1620, and 1240 cm^{-1}; UV (EtOH) λ_{max} 225 (sh) and 227 nm (ε 9400); ^1H NMR ($CDCl_3$) δ 7.62 (2H, m, H-25), 7.35 (3H, m, H-26 and H-27), 7.00 (1H, d, *J* = 12.4 Hz, H-23), 6.10 (1H, d, *J* = 5.1 Hz, H-2), 5.91 (1H, d, *J* = 12.4 Hz, H-22), 5.77 (1H, s, H-20), 5.70 (1H, d, *J* = 8.4 Hz, H-10), 5.64 (1H, s, H-20'), 5.63 (1H, m, H-5), 5.59 (1H, d, *J* = 8.4 Hz, H-9), 3.46 (1H, q, *J* = 6.9 Hz, H-12), 2.57 (1H, d, *J* = 20.5 Hz, H-14), 2.50 (1H, dd, *J* = 20.5 and 6.8 Hz, H-14'), 2.16 (1H, m, H-1), 2.05 (9H, s, AcO x 3), 1.80 (2H, m, H-7), 1.77 (2H, m, H-6), 1.68 (3H, s, H-16), 1.30 (3H, s, H-17), 1.25 (3H, d, *J* = 6.9 Hz, H-18), and 1.20 (3H, s, H-19); EIMS *m/z* 606 (M^+, 0.4), 546 (0.8), 486 (0.7), 458 (3), 398 (5), and 131 (100).

Preparation of Microtubule Proteins. Microtubule protein was prepared from porcine brain as described previously.[23] The protein concentrations were determined by the method of Lowry *et al.*[24] using bovine serum albumin as a standard. Microtubule assembly assays were carried out in MES buffer containing 100 mM 2-N-morpholino ethanesulfonic acid (MES), 1 mM ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA), 0.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 1 mM guanosine 5'-triphosphate trisodium salt (GTP) (pH 6.5).

Microtubule Assembly Assay. Microtubule assembly was monitored spectroscopically by using a spectrophotometer equipped with a thermostatically regurated liquid circulator. The temperature was held at 37 °C and changes in turbidity were monitored at 400 nm. For the drug-protein studies, 10 μM of drug dissolved in DMSO was added to 1 mL buffer solution containing 2 mg microtubule protein. The final DMSO concentration was less than 1%. After 30 minutes incubation of the test mixture, 4 mM $CaCl_2$ was added, and the mixture was further incubated for another 30 minutes. The turbidity changes were monitored throughout the incubation time.

Cellular Accumulation of Vincristine. Multidrug-resistant human ovarian cancer 2780AD cells were provided from Dr. R. Ozols (National Cancer Institute, NIH),[25] and maintained in PRMI-1640 medium supplemented with 5% fetal bovine serum and 100 μg/mL kanamycin. One million of 2780AD cells were plated in Corning six-well tissue-culture clusters and incubated for 24 h at 37°C. The medium in each well was aspirated and 0.5 mL fresh growth medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and [^{3}H]VCR (7.5 pmol, specific activity; 259 GBq/mmol) was added. Then the indicated concentrations of drugs dissolved or suspended in phosphate-buffered saline were added. After incubation at 37°C for 2 h, the intracellular VCR accumulation was determined as described previously.[22]

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