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Neutral taxoids from *Taxus cuspidata* as modulators of multidrug-resistant tumor cells ☆

Katsuhiko Kosugi [a], Jun-ichi Sakai [b], Shujun Zhang [b], Yusuke Watanabe [a], Hirofumi Sasaki [a], Toshio Suzuki [a], Hisahiro Hagiwara [a], Naonori Hirata [c], Katutoshi Hirose [c], Masayoshi Ando [b,*], Akihiro Tomida [d], Takashi Tsuruo [d]

[a] *Graduate School of Science and Technology, Niigata University, Ikarashi 2-8050, Niigata 950-2181, Japan*
[b] *Department of Applied Chemistry, Faculty of Engineering, Niigata University, Ikarashi 2-8050, Niigata 950-2181, Japan*
[c] *Kobe Natural Products and Chemicals Co., Ltd., 527 Nishikawanabe Ichikawa-cho Kanzaki-Gun Hyogo 679-2315, Japan*
[d] *Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032, Japan*

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Abstract

Two taxoids, taxinine NN-7 (**1**) and 3,11-cyclotaxinine NN-2 (**2**), were isolated from the neutral fraction of the EtOAc extract of a mixture of needles and young stems of *Taxus cuspidata*. The structures were determined by spectroscopic analysis. Both compounds showed some activity as modulators of multidrug-resistant tumor cells. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Taxus cuspidata*; Taxaceae; Taxinine NN-7; Cyclotaxinine NN-2; Modulators of multidrug-resistant tumor cells

1. Introduction

Since the discovery of the anticancer activity of paclitaxel against ovarian and breast cancer, much attention has been paid to the isolation of new taxane diterpenoids from various species of yew (Kingston et al., 1993). Current interest in the Japanese yew, *Taxus cuspidata* Sieb. et Zucc. (Taxaceae), focuses on the nonalkaloid diterpenoids from the needles, stems, heartwood, and bark of this plant, for the purpose of finding improved biological sources for paclitaxel analogues and for precursors for the practical synthesis of paclitaxel (Kobayashi et al., 1994, 1995a, 1995b).

The needles and young stems of *T. cuspidata* contain an impressive array of taxane diterpenoids. However, the paclitaxel content in the nonalkaloid diterpenoid of this species was reported to be generally low. Taxane derivatives occurring in consistently large amounts are taxinine (Shiro et al., 1966; Dukes et al., 1965; Eyre et al., 1967), and the alkaloid diterpenoids 2′-hydroxytaxine II (taxine NA-1) (Ando et al., 1997) and taxine II (Ando et al., 1997; Yoshizaki et al., 1986). The needles and young stems of *Taxus* varieties with a constant content of paclitaxel and its synthetic precursors have practical importance as reproducible sources of paclitaxel. Recently, we found that the needles and young stems of *T. cuspidata* contain significant amounts of paclitaxel, 7-epipaclitaxel, cephalomanine, 7-epicephalomanine, baccatin III, and 10-desacetyl-7-epipaclitaxel (Ando et al., 1998a, 1998b).

As part of an ongoing study on the constituents of *T. cuspidata*, we report here the structure elucidation of two biologically active nontaxol-type taxanes, taxinine NN-7 (**1**) and 3,11-cyclotaxinine NN-2 (**2**) isolated from a mixture of needles and young stems of this species. We also report activity of **1** and **2** on vin-

☆ Studies on diterpenoids from *Taxus cuspidata* 2.

* Corresponding author. Tel.: +81-25-262-7326; fax: +81-25-262-7326.

E-mail address: mando@eng.niigata-u.ac.jp (M. Ando).

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cristine (VCR) accumulation in multidrug-resistant (MDR) cancer cells.

2. Results and discussion

The neutral fraction of ethyl acetate extracts of the fresh needles and the young stems of *T. cuspidata* collected at Wakkanai in Hokkaido, Japan was purified by flash chromatography on a silica gel column followed by normal- and reversed-phase HPLC to afford **1** (0.0005%) and **2** (0.0004%). The yields were based on the weight of fresh plant material. This is the first isolation of **1** and **2** from natural sources.

Compound **1** was obtained as colorless prisms, mp 98–100°C ($CHCl_3$), and had a molecular formula of $C_{33}H_{40}O_8$ as determined by HR-EIMS, and analysis of the ^{1}H- and ^{13}C-NMR spectra. The IR spectrum of **1** showed the existence of a hydroxyl group (3610 cm^{-1}), ester carbonyl groups (1745 cm^{-1}), an α,β-unsaturated ester carbonyl group (1714 cm^{-1}), and an α,β-unsaturated carbonyl group (1672 cm^{-1}). Its ^{1}H-NMR spectrum showed the presence of a taxane skeleton with four C–Me groups (0.97, 1.18, 1.71, and 2.27 ppm), two acetyl Me groups (2.06 and 2.08 ppm), and a cinnamoyl group [6.41 (1 H, *d*, $J = 16.1$ Hz), 7.66 (1 H, *d*, $J = 16.1$ Hz), 7.40 (3 H, *m*- and *p*-Ph), and 7.76 (2 H, *o*-Ph)].

The ^{1}H−^{1}H correlations were determined by analysis of a ^{1}H−^{1}H COSY spectrum; the assignments of all

Table 1
NMR spectral data of taxinine NN-7(**1**) in $CDCl_3$

Position	^{13}C[a]	Connected ^{1}H[b]	H–H COSY[c]	HMBC[d]	NOE[e]
1	51.39 (*d*)	2.39 (*dd*, 7.0, 2.2)	H2, 14β	H3, 14α,β, 16, 17	H2, 16, 17
2	68.13 (*d*)	4.32 (*br t*, 6.3)	H1, 3, 2-OH	H1, 3, 14α,β	H1, 9, 17, 19
3	45.08 (*d*)	3.27 (*br d*, 6.3)	H2, 20b	H1, 5, 7α, 19, 20a,b	H7α, 14α
4	143.57 (*s*)	–	–	H3, 5, 20a,b	–
5	78.00 (*d*)	5.35 (*br t*, 3.0)	H6α,β	H3, 6α,β, 20a,b	H6α,β
6	28.68 (*t*)	(β) 2.02 (*m*)	H5, 6α, 7α,β		H5, 6α
		(α) 1.74 (*m*)	H5, 6β, 7α,β	H7α	H5, 6β
7	27.71 (*t*)	(β) 2.05 (*m*)	H6α,β		H7α
		(α) 1.76 (*m*)	H6α,β	H3, 5, 9, 19	H3, 7β, 10
8	44.67 (*s*)	–	–	H3, 7α,β, 9, 19	–
9	75.89 (*d*)	5.83 (*d*, 10.3)	H10	H10, 19	H2, 17, 19
10	73.48 (*d*)	6.04 (*d*, 10.3)	H9	H9	H7α, 18
11	150.65 (*s*)	–	–	H1, 10, 16, 17, 18	–
12	137.55 (*s*)	–	–	H10, 14α,β, 18	–
13	199.67 (*s*)		–	H1, 14α,β, 18	–
14	35.72 (*t*)	(β) 2.86 (*dd*, 20.0, 7.0)	H1, 14α		H1, 14α, 16
		(α) 2.27 (*d*, 20.0)	H14β	H1	H3, 14β
15	37.76 (*s*)		–	H1, 10, 14α, 16, 17	–
16	37.59 (*q*)	1.18 (*s*)	–	H1, 17	H1, 14β, 17
17	25.43 (*q*)	1.71 (*s*)	–	H16	H1, 2, 9, 16
18	13.90 (*q*)	2.27 (*br s*)	–	–	H3, 10
19	17.59 (*q*)	0.97 (*s*)	–	H3, 7β, 9	H2, 7β, 9
20	118.06 (*t*)	(a) 5.45 (*s*)	H20b		H5, 20b
		(b) 5.43 (*t*, 1.1)	H3, 20a	H3, 5	H20a
2-OH		1.85 (*br d*, 7.5)	H2	–	
9-OAc	170.16 (*s*)	–	–	H9	–
10-OAc	169.67 (*s*)	–	–	H10	–
	20.91 (*q*)	2.08 (*s*)	–	–	–
	20.75 (*q*)	2.06 (*s*)	–	–	–
1′	166.32 (*s*)	–	–	H5, 2′, 3′	–
2′	117.70 (*d*)	6.41 (*d*, 16.1)	H3′	H3′	–
3′	145.65 (*d*)	7.66 (*d*, 16.1)	H2′	H2′, *o*-Ph	–
q-Ph	134.48 (*s*)	–	–	H2′, *m*,*p*-Ph	–
o-Ph	128.94 (*d*)	7.76 (*m*)	*m*-Ph	*m*,*p*-Ph	–
m-Ph	128.49 (*d*)	7.40 (*m*)	*o*,*p*-Ph	*o*,*p*-Ph	–
p-Ph	130.39 (*d*)	7.40 (*m*)	*m*-Ph	*o*-Ph	–

[a] Multiplicities were determined by DEPT.
[b] Connections were determined by HMQC and multiplicities and coupling constants (*J*) in Hz are in parentheses.
[c] Determined by PFG-COSY.
[d] Correlations from C to the indicated protons.
[e] NOESY cross peaks.

protonated carbons were determined by DEPT and HMQC experiments. The assignment of the quaternary carbons and the attachment of ester functions were determined by HMBC experiment, allowing unambiguous carbon skeletal connection assignments. The stereostructure of taxane skeleton of **1** was determined by CYCLENOE and NOESY experiments, as well as by consideration of vicinal coupling constants. The full NMR spectral data for **1** are summarized in Table 1.

Recently, Sako and co-workers reported the partial synthesis of **1** by the hydride reduction of taxinine with diisobutylaluminum hydride in 30% yield (Sako et al., 1998b). Although the assignment of the ^{13}C-NMR spectrum was not included in this report, the chemical shift values reported there are in good agreement with those shown in Table 1. However, the assignment of H-5 and H-20 in that report should be interchanged as shown in Table 1.

Compound **2** was isolated as colorless microcrystals, mp 95–96°C ($CHCl_3$), and had the molecular formula $C_{35}H_{42}O_{10}$ as determined by HR-EIMS, and ^{1}H and ^{13}C-NMR spectra. The IR spectrum of compound **2** showed the presence of a hydroxyl group (3600 cm^{-1}), acetyl carbonyl groups (1742 cm^{-1}), and α,β-unsaturated ester carbonyl and saturated carbonyl groups (1712 cm^{-1}). Its ^{1}H-NMR spectrum showed the pre-

Table 2
NMR spectral data of 3,11-cyclotaxinine NN-2 (**2**) in $CDCl_3$

Position	^{13}C[a]	Connected ^{1}H[b]	H–H COSY[c]	HMBC[d]	NOE[e]
1	79.12 (*s*)	–	–	H2, 14α,β, 16, 17, 1-OH	–
2	79.19 (*d*)	6.14 (*d*, 2.0)	H14β	H14α,β	H17, 19
3	61.45 (*d*)	–	–	H2, 5, 7β, 12, 20a,b, 19	–
4	140.92 (*s*)	–	–	H2, 5, 20a,b	–
5	76.31 (*d*)	5.64 (*br t*, 9.0)	H6α,β	H6α, 20a,b	H6β, 20b
6	25.77 (*t*)	(β) 2.20 (*m*)	H5, 6α, 7α,β		H5, 7β
		(α) 1.82 (*m*)	H5, 6β, 7α,β	H5	H7α
7	31.10 (*t*)	(β) 1.78 (*m*)	H6α,β, 7α		H6β, 7α, 9
		(α) 1.16 (*m*)	H6α,β, 7β	H9, 19	H6α, 7β
8	45.20 (*s*)	–	–	H2, 9, 10, 19	–
9	82.20 (*d*)	5.76 (*d*, 10.0)	H10	H10, 19	H7β, 17, 19
10	79.59 (*d*)	5.74 (*br d*, 10.0)	H9	H9	–
11	56.12 (*s*)	–	–	H9, 10, 12, 16, 17, 18	–
12	51.58 (*d*)	3.35 (*q*, 7.1)	H18	H10, 18	H18
13	212.89 (*s*)	–	–	H12, 14α,β, 18	–
14	46.23 (*t*)	(α) 2.89 (*d*, 20.5)	H14β		H14β, 20a
		(β) 2.43 (*br d*, 20.5)	H2, 14α	H2, 1-OH	H14α, 16
15	45.11 (*s*)	–	–	H10, 12, 14α, 16, 17	–
16	23.30 (*q*)	1.11 (*s*)	–	H17	H14β, 17, 18
17	22.53 (*q*)	1.64 (*s*)	–	H16	H2, 9, 16, 19
18	15.79 (*q*)	1.30 (*d*, 7.1)	H12	H12	H12, 16
19	26.39 (*q*)	1.29 (*s*)	–	H9	H9, 17
20	129.75 (*t*)	(a) 5.88 (*s*)	–		H20b
		(b) 5.72 (d, 0.7)	–	H5	H5, 20a
1-OH	–	2.54 (*s*)	–	–	–
2-OAc	171.99 (*s*)	–	–	H2	–
9-OAc	169.96 (*s*)	–	–	H9	–
10-OAc	170.93 (*s*)	–	–	H10	–
	21.41 (*q*)	2.16 (*s*)	–	–	–
	21.12 (*q*)	2.07 (*s*)	–	–	–
	21.94 (*q*)	2.06 (*s*)	–	–	–
1′	165.66 (*s*)	–	–	H2′,3′,5	–
2′	117.68 (*d*)	6.39 (*d*, 16.1)	H3′	*m*-Ph	H3′
3′	145.50 (*d*)	7.68 (*d*, 16.1)	H2′	H2′	H2′
q-Ph	134.24 (*s*)	–	–	H2′, *o*,*m*-Ph	–
o-Ph	128,24 (*d*)	7.57 (*m*)	*m*-Ph	–	–
m-Ph	128.90 (*d*)	7.39 (*m*)	*o*,*p*-Ph	–	–
p-Ph	130.44 (*d*)	7.39 (*m*)	*m*-Ph	–	–

[a] Multiplicities were determined by DEPT.
[b] Connections were determined by HMQC and multiplicities and coupling constants (*J*) in Hz are in parentheses.
[c] Determined by PFG-COSY.
[d] Correlations from C to the indicated protons.
[e] NOESY cross peaks.

Table 3
Effects of taxinine NN-7 (**1**) and 3,11-cyxlotaxinine NN-2 (**2**) on the accumulation of vincristine (VCR) in multidrug-resistant 2780AD cells

Compound	VCR accumulation with taxoids[a]					Evaluation maximum verapamil% concentration
	Concentration (μg/ml)	Average[b] (dpm/well)	% of control[c]	Activities[d]	Verapamil%[e]	
Taxinine NN-7 (**1**)	0.1	283	124	+	106	P[f]
	1	732	321	++	155	191
	10	2318	1017	+++	191	10 μg/ml
3,11-Cyclotaxinine NN-2 (**2**)	0.1	327	143	+	123	P
	1	966	424	++	204	204
	10	1887	828	+++	156	1 μg/ml
	0	228	100			
Verapamil	0.1	266	117	+	100	
	1	473	207	++	100	
	10	1211	531	+++	100	

[a] The amount of VCR accumulated 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 (%): +++++, >2001%; ++++, 1001–2000%; +++, 501–1000%; ++, 301–500%; +, 111–300%; ±, 91–110%; −, <90%.
[e] The values are expressed as the relative amount of 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%).

sence of a 3,11-cyclotaxane skeleton with four C–Me groups (1.11, 1.29, 1.30, and 1.64 ppm), three acetyl Me groups (2.06, 2.07, and 2.16 ppm), and a cinnamoyl group [6.39 (1 H, *d*, $J = 16.1$ Hz), 7.68 (1 H, *d*, $J = 16.1$ Hz), 7.39 (3 H, *m*- and *p*-Ph), and 7.57 (2 H, *o*-Ph)].

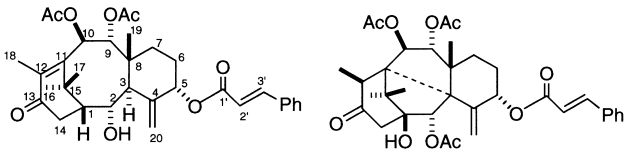
The $^1H-^1H$ correlations of **2** were determined by analysis of its $^1H-^1H$ COSY spectrum, and assignment of all protonated carbons was accomplished by analysis of DEPT and HMQC experiments. The assignment of the quaternary carbons and the attachment of ester functions were determined by an HMBC experiment, which allowed unambiguous carbon skeletal connections. The full stereostructure of the cyclotaxane skeleton of **2** was determined by CYCLENOE and NOESY experiments, and by consideration of vicinal coupling constants. The full NMR spectral data for compound **2** are summarized in Table 2.

Although Appendino and co-workers reported **2** as an *E* and *Z* mixture of cinnamoyl double bond isomers following photo-reaction of the corresponding taxadiene derivative, detailed spectroscopic and physical data of **2** were not included in their report (Appendino et al., 1992). The only spectroscopic evidence of photo-product **2** comes from ^1H-NMR chemical shifts, but the values are not clear and some of them are apparently incorrect as compared with those of natural product **2** shown in Table 2.

The cellular accumulation of VCR is reduced in 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 (Turuo et al., 1986). The effect of taxoids **1** and **2** 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 (Kobayashi et al., 1994, 1997, 1998; Kobayashi and Shigemori, 1998; Sako et al., 1998a, 1998b; Hosoyama et al., 1999; Ojima et al., 1998).

The values of VCR accumulation with taxinine NN-7 (**1**) (321% of control and 155% of verapamil at 1 μg/ml) and 3,11-cyclotaxinine NN-2 (**2**) (424% of control and 204% of verapamil at 1 μg/ml) shown in Table 3 are almost the same as or stronger than the maximum value of those reported by Kobayashi and Tsuruo. The most efficient MDR-reversing agent reported by Kobayashi et al. (1994, 1997, 1998), Kobayashi and Shigemori (1998), Sako et al. (1998a, 1998b), and Hosoyama et al. (1999) is compound **33** by Hosoyama et al. (1999) (401% of control and 158% of verapamil at 1 μg/ml). The activity of taxinine NN-7 (**1**) is comparable with that of **33** and the activity of 3,11-cyclotaxinine NN-2 (**2**) is stronger than that of **33**. We employed the same assay system with those employed by Kobayashi and exact comparison of activity is possible by the comparison of the verapamil%. The results suggest that cinnamoyloxy and BOM groups at C-5 effectively increases the cellular accumulation of VCR in MDR tumor cells.

Direct comparison of our results with those by Ojima is impossible because of differences in the assay system, including the MDR tumor cell-line used (2780AD vs. MCF7R or MDA/LCC6-MDR), the anticancer reagent used (VCR vs. Paclitaxel), and the expression of activity (increase of VCR accumulation of control vs. IC_{50} and IC_{50} reduction% of control). But that study examined modulation of tritiated paclitaxel accumulation by a MDR reversal taxane in MCF7 cell and the value of ca. 700% of control at ca. 1 μg/ml by **2** (the compound appeared in Ojima et al. (1998)) is almost comparable with those of 3,11-cyclotaxinine NN-2.



1 **2**

3. Experimental

3.1. General

Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were recorded in $CHCl_3$ on a Hitachi 270-30 spectrometer. Optical rotations were measured using a Horiba Polarimeter SEPA-200. HR-EIMS spectra were obtained using a JEOL JMS HX-110 spectrometer. ^1H-NMR (499.87 MHz) and ^{13}C-NMR (125.70 MHz) spectra were recorded on a Varian UNITY-PS 500 spectrometer. To describe HPLC conditions, we designate column, solvent, flow rate (ml/min), and retention time (R_t in min) in this order. The column codes are as follows: (A) INERTSIL PREP-SIL (GL Science), 25 × 1 cm i.d. stainless column; (B) INERTSIL PREP-SIL (GL Science), 25 × 2 cm i.d. stainless column; (C) INERTSIL PREP-ODS (GL Science), 25 × 1 cm i.d. stainless column.

3.2. Plant material

A mixture of needles and young stems of *T. cuspidata* was collected from two female trees of 2-m height

grown in Wakkanai, northern Japan, on 15 October 1995.

3.3. Extraction

A mixture of fresh needles and young stems (1248 g) was defatted by extraction with hexane (8 l) for 1 week. The remaining plant material was soaked in EtOAc (8 l) for 1 week. The residue (19.69 g) remaining after removal of the solvent was dissolved in a mixture of MeOH–EtOAc (1:3, 400 ml) and extracted with 0.5 M aqueous solution of H_2SO_4 (3 × 100 ml). Subsequently, the combined acid solution (pH 1.0) was brought to pH 9.0 by addition of a 29% aqueous solution of NH_4OH (200 ml) and then extracted with $CHCl_3$ (3 × 200 ml). The combined extracts were dried (Na_2SO_4), filtered, and concentrated to give a crude basic taxoid fraction (3.65 g; 0.292% from fresh plant material, 18.537% from defatted EtOAc extract).

The EtOAc phase was then extracted with a 2 M aqueous solution of NaOH (2 × 100 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 (260 ml) and then extracted with $CHCl_3$ (3 × 200 ml). The combined extracts were dried (Na_2SO_4), filtered, and concentrated to give a crude phenolic fraction (1.08 g; 0.087% from fresh plant material; 5.485% from defatted EtOAc extract).

The remaining EtOAc phase was washed with a saturated salt solution (4 × 200 ml), dried (Na_2SO_4), and concentrated to give a crude neutral fraction (7.49 g; 0.600% from fresh plant material; 38.040% from defatted EtOAc extract).

3.4. Isolation

The crude fraction (7.49 g) was divided into 10 fractions (fraction 1, 830 mg; fraction 2, 2233 mg; fraction 3, 722 mg; fraction 4, 405 mg; fraction 5, 311 mg; fraction 6, 208 mg; fraction 7, 177 mg; fraction 8, 144 mg; fraction 9, 237 mg; fraction 10, 2051 mg) by flash column chromatography [silica gel (230–400 mesh), 749 g; solvent, EtOAc–hexane (6:4) for fractions 1–8, EtOAc for fraction 9, MeOH for fraction 10].

Fraction 2 from flash chromatography (F2) was separated by HPLC [column, A; solvent, EtOAc–hexane (3:7); flow rate 5 ml/min]. The third region (F2–3, R_t 12.0–45.2 min) gave 962 mg of a crude viscous oil. F 2–3 was further separated by HPLC under the same conditions. The second fraction (F 2-3-2, R_t 14.0–21.8 min, 479.2 mg) was subjected to further reversed phase HPLC [column, C; solvent, MeOH–0.05 M NH_4OAc buffer (pH 4.8)–CH_3CN (1:1:2)] to give F2-3-2-4 (R_t 26.4 min, 14.5 mg) as the fourth peak, which was further purified by reversed phase HPLC under the same conditions except the solvent ratio [MeOH–0.05 M NH_4OAc buffer (pH 4.8)–CH_3CN (1:2:2)] to give taxinine NN-7 (**1**) (R_t 40.3 min, 6.2 mg, 0.08% based on the crude neutral fraction and 0.0005% based on fresh plant material).

Fraction 3 of flash chromatography (F3) was separated by HPLC [column B; solvent, EtOAc–hexane (1:1), flow rate 15 ml/min]. The third region (R_t 18.0–22.0 min) gave a crude viscous oil (F3-3, 164.7 mg), which was further separated by HPLC in the same conditions. The fifth peak (F3-3-5, R_t 17.5 min) gave a colorless viscous oil (154.4 mg). Finally, the separation of F3-3-5 by reversed phase HPLC [column, C; solvent, MeOH–0.05 M NH_4OAc buffer (pH 4.8)–CH_3CN (1:1:2)] gave 3,11-cyclotaxinine NN-2 (**2**) (R_t 9.8 min, 4.4 mg, 0.06% based on crude neutral fraction and 0.0004% based on fresh plant material).

3.5. Identification of compounds *1* and *2*

Taxinine NN-7 (**1**): prisms, mp 98–100°C ($CHCl_3$); $[\alpha]_D^{20}$ + 97.3° (*c*, 0.45, $CHCl_3$); HR-EIMS *m/z*: 564.2720, calcd. for $C_{33}H_{40}O_8$ 564.2723.

3,11-Cyclotaxinine NN-2 (**2**): microcrystals, mp 95–96°C ($CHCl_3$); $[\alpha]_D^{20}$ +22.1° (*c*, 0.29, $CHCl_3$); HR-EIMS *m/z*: 622.2775, calcd. for $C_{35}H_{42}O_{10}$ 622.2778.

3.6. Cellular accumulation of [^{3}H]-VCR

The MDR 2780AD cells were maintained in RPMI 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 treated 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 taxoids. 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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