A New Taxoid from a Callus Culture of *Taxus cuspidata* as an MDR Reversal Agent

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A new taxoid, 5α,13α-diacetoxy-10β-cinnamoyloxy-4(20),11-taxadien-9α-ol (1) along with its 9,10-isomer, taxinine NN-11 (2) were isolated from the callus cultures of *Taxus cuspidata*. The structures were identified by the analyses of the spectral data and chemical method. Their *in vitro* cytotoxicity against 3 cell lines (HepG2, WI-38 and VA-13) and multidrug resistance (MDR) reversal activity toward 2780AD tumor cells were preliminarily evaluated, the low cytotoxicities and potent MDR reversal activities suggested that they might be good lead compounds of tumor MDR reversal agent.

Key words taxoid; cell culture; multidrug resistance; cytotoxicity; *Taxus cuspidata*

Since the discovery of paclitaxel (taxol®) as an important anticancer drug,[1,2] a number of bioactive paclitaxel derivatives have been isolated from the plants and the cell cultures of *Taxus* species[3—6] and prepared by chemical syntheses.[7—11] Interestingly, some non-taxol-type taxoids have been reported to possess significant reversal activity against multidrug-resistance (MDR) tumor cells.[12—16] Therefore, for increasing the production of paclitaxel and its related taxanes and sighting new biologically active taxoids, we established a stable and fast-growing callus strain of *T. cuspidata* SIEB. *et* ZUCC. (taxaceae)[17,18] and investigated the secondary metabolites in several different culture conditions,[19,20] among these compounds, taxinine NN-11 (**2**) showed significant MDR reversal activity.[19] Further chemical investigation on the callus cultures of *T. cuspidata* has led to the isolation of a new taxoid (**1**, Chart 1), the 9,10-isomer of taxinine NN-11 (**2**). Herein, the isolation, structure elucidation, evaluations of cytotoxicity and MDR reversal activities of **1** in comparison with those of **2** are described.

Results and Discussion

Compound **1** was obtained in the yield of 0.0018% (dry weight) and had the composition of $C_{33}H_{42}O_7$, which was determined by the combination of HR-FAB-MS (High Resolution Fast Atom Bombardment Mass Spectrum) and ^{1}H- and ^{13}C-NMR (Nuclear Magnetic Resonance) spectra. The IR (Infrared) spectrum of **1** showed the existence of a hydroxyl group (3616 cm^{-1}), ester carbonyl group (1728 cm^{-1}), and an α,β-unsaturated ester carbonyl group (1715 cm^{-1}). The ^{1}H-NMR spectrum similar to that of **2**[19] indicated the presence of a taxane skeleton with four C–Me groups (δ 0.96, 1.10, 1.55, 2.16), two acetyl Me groups (δ 2.18, 2.07), and a cinnamoyl group [δ 6.47 (1H, d, J=16.1 Hz), 7.72 (1H, d, J=16.1 Hz), 7.54 (2H, *o*-Ph), 7.40 (3H, *m*-, *p*-Ph)]. The ^{1}H–^{1}H correlation, H-1 to H-2α,β and H-14β; H-2α,β to H-3; H-3 to H-20a,b; H-5 to H-6α,β; H-6α,β to H-7α,β; H-9 to H-10; H-13 to H-14α,β and H-18; H-2′ to H-3′, was determined by the analysis of the ^{1}H–^{1}H COSY spectrum. The ^{13}C-NMR spectrum displayed 31 carbon resonances including two pairs of equivalent ones (δ 128.2, 128.9) due to *ortho*- and *meta*-carbons of the phenyl ring. Signals for four oxygenated carbon (δ 77.3, 76.7, 76.4, 70.8) were observed. The assignments of all protonated carbons were determined by DEPT (Distortionless Enhancement by Polarization Transfer) and HMQC (Heteronuclear Multiple-Quantum Coherence) experiments. An HMBC (Heteronuclear Multiple Bond Coherence) experiment was used for the assignments of the quaternary carbons and attachment of functional groups. A correlation of the signal due to the cinnamoyl carbonyl (δ 166.4) with those of H-10 (δ 6.02), H-2′ (δ 6.47), and H-3′ (δ 7.72) indicated the location of the cinnamoyl group at C-10. Correlations of the signals due to two acetyl carbonyls (δ 170.4, 170.0) with those of H-13 (δ 5.88) and H-5 (δ 5.37) showed the location of two acetoxyl groups of **1** at C-13 and C-5. The location of a hydroxyl group at C-9 was determined by the HMBC correlation of the hydroxymethine proton (δ 4.36) to C-7, C-8, C-10, and C-19. The multiple-bond ^{1}H–^{13}C correlations of the remaining five non-protonated carbons of **1**, H-2α,β, H-3, H-6β and H-20b to C-4; H-2α,β, H-3, H-6β, H-7α, H-9, H-19 to C-8; H-10, H-13, H-16, H-17, H-18 to C-11; H-10, H-13, H-14β, H-18 to C-12;

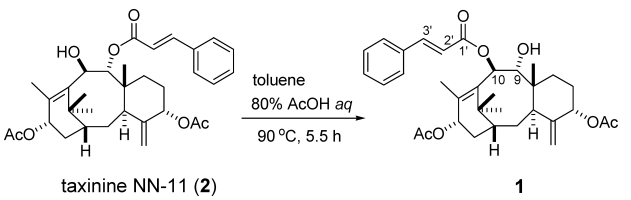

Chart 1. Chemical Conversion of Taxinine NN-11 (**2**) to **1**

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Table 1. NMR Data of Compound **1** in $CDCl_3$ (500 MHz for ^{1}H-NMR, 125 MHz for ^{13}C-NMR)

Position	$^{13}C^{a)}$	Connected $^1H^{b)}$	H–H COSY$^{c)}$	HMBC$^{d)}$	NOESY$^{e)}$
1	40.5 (d)	1.81 (m)	H2α,β, 14β	H3, 16, 17	H14β, 17
2	28.3 (t)	α) 1.68 (m), β) 1.75 (m)	H1, 2β, 3	H3, 14α,β	H2β, 3, 10, 20b
			H1, 2α, 3		H2a, 9, 19
3	38.0 (d)	3.02 (br d, 6.0)	H2α,β, 20a,b	H2α,β, 5, 19, 20a,b	H2α, 14α, 18
4	149.4 (s)			H2α,β, 3, 6β, 20b	
5	76.7 (d)	5.37 (t, 2.5)	H6α,β	H20a,b	H6α,β, 20a
6	27.7 (t)	α) 1.88 (m)	H5, 6β, 7α,β	H7α,β	H5, 6β
		β) 1.71 (m)	H5, 6α, 7α,β		H5, 6α, 7β
7	26.0 (t)	α) 1.64 (m)	H6α,β, 7β	H5, 9, 19	H3, 7β
		β) 1.91 (m)	H6α,β, 7α		H6β, 7α, 19
8	43.2 (s)			H2α,β, 3, 6β, 7α, 9, 19	
9	77.3 (d)	4.36 (d, 10.1)	H10	H10, 19	H2β, 17, 19
10	76.4 (d)	6.02 (d, 10.1)	H9	H9	H7α, 18
11	135.3 (s)			H10, 13, 16, 17, 18	
12	136.6 (s)			H10, 13, 14β, 18	
13	70.8 (d)	5.88 (br dd, 9.8, 7.8)	H14α,β, 18	H14α,β, 18	H14β, 16
14	31.9 (t)	α) 1.08 (br dd, 14.4, 7.8)	H13, 14β	H2α,β	H3, 14β
		β) 2.69 (dt, 14.4, 9.8)	H1, 13, 14α		H1, 13, 14α
15	39.4 (s)			H1, 2α,β, 10, 16, 17	
16	31.0 (q)	1.10 (s)	H17	H17	H1, 13, 17
17	27.6 (q)	1.55 (s)	H16	H16	H1, 9, 16
18	14.9 (q)	2.16 (d, 1.2)			H3, 10, 13
19	18.1 (q)	0.96 (s)		H3, 7α, 9	H2β, 7β, 9
20	113.6 (t)	a) 5.20 (br d, 1.0)	H3, 20b	H5	H5, 20b
		b) 4.84 (br d, 1.0)	H3, 20a		H2α, 20a
OAc	170.4 (s)			H13, 13-OAc (Me)	
	170.0 (s)			H5, 5-OAc (Me)	
	21.4 (q)	2.07 (s)			
	21.8 (q)	2.18 (s)			
1′	166.4 (s)			H9, 2′, 3′	
2′	117.9 (d)	6.47 (d, 16.1)	H3′	H3′	
3′	145.4 (d)	7.72 (d, 16.1)	H2′	H2′, *o*-Ph	
q-Ph	134.2 (s)			H2′, *m*,*o*-Ph	
o-	128.2 (d)	7.54 (m)	*p*,*m*-Ph	H3′, *m*,*p*-Ph	
m-	128.9 (d)	7.40 (m)	*o*,*p*-Ph	*o*,*p*-Ph	
p-	130.5 (d)	7.40 (m)	*m*,*o*-Ph	*o*,*m*-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 protons. *e*) NOESY cross peaks.

H-1, H-2α,β, H-10, H-16, H-17 to C-15, were assigned by HMBC experiment and allowed unambiguous carbon skeletal connection. The stereochemistry of taxane skeleton of **1** was determined by NOESY (Nuclear Overhauser Effect Spectroscopy) experiments as well as by a consideration of vicinal coupling constants. The ^{1}H- and ^{13}C-NMR data in $CDCl_3$ were shown in Table 1.

The known compound **2** was also obtained in the yield of 0.0022%. Treatment of **2** with 80% aqueous solution of acetic acid using toluene as a solvent at 90 °C gave compound **1** (Chart 1). The ^{1}H-, ^{13}C-NMR, IR, HR-MS spectral data including $[\alpha]_D$ value of **1** prepared from **2** were identical with those of natural taxoid **1**, which further confirmed the structural elucidation of **1**.

Many reports revealed that non-cytotoxic MDR modulator is desired.[23,24] In this context, both the cytotoxicities and MDR reversal activities of **1** and **2** were evaluated.

Cytotoxicities of **1** and **2** were investigated by using fibroblast cell line (WI-38) induced from human normal lung cells as a normal human lung cell model, malignant tumor cell line (VA-13) induced from WI-38 by infection with SV-40 virus as a malignant lung tumor model, and human liver tumor cell line (HepG2) as a human liver cancer model. As shown in Table 2, both compound **1** and **2** showed very weak cytotoxicities (IC_{50}>50 μM) against WI-38, VA-13 and HepG2 cell lines.

Table 2. Cytotoxicities of Compounds **1** and **2** against WI-38, VA-13, and HepG2 Cells

Compounds	IC_{50} (μM, n=3)$^{a)}$		
	WI-38	VA-13	HepG2
Paclitaxel	0.034	0.0043	6.9
Adriamycin	0.38	0.22	0.69
1	>100	>100	93±10
2	>100	>100	>100

a) The IC_{50} values were the means of triplicates±standard deviations.

The mechanism of MDR in tumor cells is very complex, one of the mechanisms is that the cellular accumulation of calcein cleaved from calcein AM by endogenous esterase is reduced in MDR tumor cells as compared with the parental sensitive cells.[25,26] The typical MDR reversal agent, verapamil, increases the accumulation of calcein in MDR cells and overcomes MDR.[21,22] The effects of compounds **1** and **2** on the cellular accumulation of calcein in MDR human ovarian cancer 2780AD cells were examined by comparison with that of verapamil. As shown in Table 3, compound **1** showed

Table 3. Effects of Compounds **1** and **2** on the Accumulation of Calcein in MDR 2780AD Cells

Compound	Concentration[a] (μg/ml)	Average of fluorescence /well[b]	% of control[c]	Equivalent verapamil %[d]
Control	0	3653±176		
	0.25	3743±159	102	100
Verapamil	2.5	4161±161	114	100
	25	5556±81	152	100
1	0.25	3460±360	95	92
	2.5	3608±55	99	87
	25	3716±158	102	67
2	0.25	3675±159	101	98
	2.5	4243±836	116	102
	25	4730±832	130	85

a) The amount of calcein accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control in the presence of 0.25, 2.5, and 25 μg/ml of taxoid. *b*) The values represent means of triplicate±standard deviations. *c*) The values are the relative amount of calcein accumulated in the cell compared with the control experiment. *d*) The values are expressed as the relative amount of calcein accumulation in the cell as compared with that of verapamil.

102% of control at 25 μg/ml and 92% activity of verapamil at 0.25 μg/ml. Compound **2** showed 130% of control at 25 μg/ml and 102% activity of verapamil at 2.5 μg/ml. The MDR reversal activity of **1** was lower than that of taxinine NN-11 (**2**). As cytotoxicity is not a desirable feature of an MDR reversal agent,[23,24] compounds **1** and **2** might become the promising lead compounds for further investigation of non-cytotoxic taxane analogues as a modulator that mediates MDR.

Experimental

IR spectra were recorded in $CHCl_3$ on a Hitachi 270-30 spectrometer. Optical rotations were measured using a Horiba Polarimeter SEPA-200. HR-FAB-MS spectra were obtained using a JEOL JMS HX-110 spectrometer. ^{1}H- (499.87 MHz) and ^{13}C-NMR (125.70 MHz) spectra were recorded on a Varian UNITY-PS 500 spectrometer. Reaction was cried out under the Ar atmosphere. Inertsil Prep-sil (GL Science), 25×1 cm i.d. stainless column was used for HPLC and the flow rate was 5 ml/min. Silica gel (70—230 mesh) was employed for column chromatography.

Extraction and Isolation of Compounds 1 and 2 The freeze-dried callus cultures[19,20] (450 g) was extracted with hexane, EtOAc, and MeOH successively. The crude EtOAc extract (18.9 g) was fractionated into seven fractions (F1—F7) by column chromatography over silica gel using a gradient elution from hexane–acetone (9 : 1) to acetone (100%). F5 (823 mg) was further separated by normal-phase HPLC [hexane–EtOAc (2 : 1)] to give compounds **1** (8.0 mg) and **2** (10.0 mg).

Chemical Transformation of 2 to 1 A solution of **2** (11.3 mg, 0.021 mmol) in toluene (850 μl) was treated with 80% aqueous solution of acetic acid (58.7 μl, 0.82 mmol) at 90 °C for 5.5 h. The reaction was quenched by addition of saturated aqueous solution of $NaHCO_3$ and the mixture was extracted with EtOAc, then the combined extracts were washed successively with saturated aqueous solution of NaCl, dried with Na_2SO_4, and concentrated to give a crude extract (20.2 mg). The crude extract was purified by HPLC [hexane–EtOAc (7 : 3)] to give compound **1** (7.4 mg, 65.5%) and the recovered **2** (2.9 mg, 25.7%).

5α,13α-Diacetoxy-10β-cinnamoyloxy-4(20),11-taxadien-9α-ol (**1**): White amorphous powders; $[\alpha]_D^{20}$ +105.2° (c=0.08, $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 3616, 1728, 1715, 1638; HR-FAB-MS m/z 573.2838 $[M+Na]^+$ (Calcd for $C_{33}H_{42}O_7Na$: 573.2828); ^{1}H- and ^{13}C-NMR data are shown in Table 1.

Cytotoxicity of 1 and 2 WI-38 is the normal human fibroblast derived from female human lung. VA-13 is malignant tumor cells induced from WI-38 by infection of SV-40 virus. HepG2 is human liver tumor cells. These cell lines were obtained from the Institute of Physical and Chemical Research (RIKEN), Tsukuba, Ibaraki, Japan. WI-38 and VA-13 cells were maintained in Eagle's MEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) and RITC 80-7 medium (Asahi Technoglass Co., Chiba, Japan), respectively, both supplemented with 10% (v/v) fetal bovine serum (FBS) (Filtron PTY LTD., Australia) with 80 μg/ml of kanamycin. HepG2 cells were maintained in D-MEM medium (Invitrogen) supplemented with 10% (v/v) FBS (Filtron PTY LTD., Australia) with 80 μg/ml of kanamycin. A 100 μl medium containing *ca.* 5000 cells (WI-38, VA-13, HepG2) were incubated at 37 °C in humidified atmosphere of 5% CO_2 for 24 h in a 96 well microplate. Then test samples dissolved in DMSO were added to the medium and incubated further for 48 h under the same conditions and the coloration substrate, WST-8 [2-(2-methyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodiun salt] was added to the medium. The resulting formazan concentration was determined by the spectrum absorption at 450 nm. Cell viability (%) was calculated by [(experimental absorbance−background absorbance)/(control absorbance−background absorbance)]×100. Cell viability at different concentration of compounds was plotted and 50% inhibition of growth was calculated as IC_{50}.

Cellular Accumulation of Calcein MDR human ovarian cancer A2780 cells (AD10) were maintained in PRMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Filtron PTY LTD., Australia) with 80 μg/ml of kanamycin. A 100 μl medium containing *ca.* 1×10^5 cells were incubated at 37 °C in humidified atmosphere of 5% CO_2 for 24 h in 96 well microplate. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS(−)). Fifty microliters of test samples were added to the medium and incubated for 15 min. Then, 50 μl of fluorogenic dye calcein acetoxymethyl ester [1 μM in PBS(−)] was added to the medium and incubated for additional 60 min. After removal of the supernatant, each microplate was washed with 200 μl of cold PBS(−). The washing step was repeated twice and 200 μl of cold PBS(−) was added. Retention of resulting calcein was measured by calcein-specific fluorescence. The maximum absorption for calcein is 494 nm, and the maximum emission is 517 nm.

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