Production of Biologically Active Taxoids by a Callus Culture of *Taxus cuspidata*

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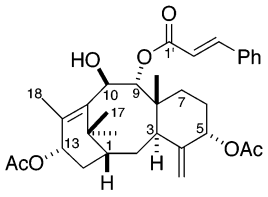
Ten known taxoids, paclitaxel, 7-*epi*-taxol, taxol C, baccatin VI, taxayuntin C, taxuyunnanine C and its analogues (**2**−**5**), and yunnanxane (**6**), and an abietane, taxamairin A, were produced in the callus culture of *Taxus cuspidata* cultivated on a modified Gamborg's B5 medium in the presence of 0.5 mg/L NAA. After stimulation with 100 μM methyl jasmonate, five more taxoids, cephalomannine, 1β-dehydroxybaccatin VI, taxinine NN-11 (**1**), baccatin I, and 2α-acetoxytaxusin, and one more abietane, taxamairin C, were found in addition to the above-mentioned compounds. It was also observed that the content of the products increased over three times. Taxinine NN-11 (**1**) is a new taxane whose structure was assigned as 5α,13α-diacetoxy-9α-cinnamoyloxy-4(20),11-taxadien-10β-ol by analysis of its spectral data. Taxinine NN-11 (**1**) exhibited significant MDR reversal activity toward 2780 AD tumor cells. The results of primary screening based on 39 human cancer cell lines suggest that **1** also belongs to a new mechanistic class. Efficient production of **1** was investigated using the callus culture of *T. cuspidata*.

Since paclitaxel (Taxol) has been found to exhibit significant antitumor activity against various cancers, great attention to its supply by efficient methods has been made. Plant cell culture of *Taxus* species is considered as one of the most promising approaches to obtain paclitaxel and related taxanes. In the past decade, there have been some successful reports and patents on the production of paclitaxel by callus or cell culture of various species of yew, but the paclitaxel content was markedly different according to the particular *Taxus* species being investigated.[1−5] However, for most of these methods, only paclitaxel accumulation was reported, and its content was generally low. A few years ago, we established a stable and fast-growing callus culture induced from the Japanese yew (*Taxus cuspidata*) through optimization of the culture conditions.[6] In a previous paper,[7] we have reported that the callus culture produced not only taxane derivatives but also abietane derivatives. Moreover, the callus culture of this species contained significant amounts of taxuyunnanine C (**2**) and its analogues (**3**−**6**) (Figure 1, Supporting Information) with an acyloxy group at C-14, which were reported to be the major products of suspension cultures of *Taxus chinensis*.[8,9]

As a part of our ongoing studies on increasing the accumulation of paclitaxel and some other bioactive taxoids in the callus culture of *T. cuspidata*, we have investigated the effect of methyl jasmonate, which is known to strongly enhance the paclitaxel content in cell cultures of *T. media* and *T. baccata*.[9−14] We wish to report the structure elucidation of a new taxane, taxinine NN-11 (**1**), isolated from the callus after stimulation with methyl jasmonate.



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We also report the activities of **1**−**6** on vincristine (VCR) accumulation in multidrug-resistant (MDR) cancer cells and the anticancer activities of **1** and **5** based on a panel of 39 human cancer cell lines.[15] Finally, we report the results of efforts to increase the yield of taxinine NN-11 (**1**) in the callus culture.

Results and Discussion

The callus culture was induced from young stems of *T. cuspidata* collected in Sendai, Japan, on modified Gamborg's B5 solid medium[16] in the presence of 0.5 mg/L NAA. This callus culture (code-named CR-5) has the ability to grow continuously on the medium containing 0.5 mg/L NAA and shows fast growth compared with the callus cultures established on media with higher concentrations of NAA or other plant growth regulators such as 2,4-D and 4-Cl IAA.[7] The CR-5 callus line was subcultured every 60 days. After reaching a stable state, a part of CR-5 was cultivated on the medium supplemented with 100 μM methyl jasmonate. The calluses harvested for analysis were freeze-dried and extracted with hexane, EtOAc, and methanol, successively. The methanol extracts were diluted with water and further extracted with $CHCl_3$. The hexane, EtOAc, and $CHCl_3$ extracts were separated by combinations of flash column chromatography and normal- and reversed-phase HPLC. The yields of the compounds isolated from the calluses are summarized in Table S1 in the Supporting Information. From CR-5, 10 known taxoids, paclitaxel,[17−19] 7-*epi*-taxol,[18,20] taxol C,[4,21] baccatin VI,[22−24]

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Table 1. NMR Data of Taxinine NN-11 (**1**) in C_6D_6

position	^{13}C[a]	connected ^1H[b]	H−H COSY[c]	HMBC[d]	NOESY[e,f]
1	40.8 (d)	1.50 (m)	H-2α,*β*, 14α,*β*	H-2α,*β*, 3, 14α,*β*, 16, 17	H-2*β* (m), 14*β* (s), 16 (m), 17 (m)
2	28.5 (t)	α: 1.45 (br dd, 15.5, 5.5)	H-1, 3	H-1, 3, 14α,*β*	H-2*β* (s), 3 (w), 20b (s)
		β: 1.65 (ddd, 15.5, 6.0, 1.5)	H-1, 3		H-1 (m), 2α (s), 9 (s)
3	38.4 (d)	3.10 (br d, 6.0)	H-2α,*β*, 20a,b	H-1, 2α,*β*, 5, 7*β*, 9, 19, 20a,b	H-2α (w), 7α (m), 10 (w), 14α (s)
4	149.6 (s)			H-2α, 3, 5, 6α, 20a,b	
5	76.3 (d)	5.53 (t, 2.5)	H-6α,*β*	H-3, 6α, 7α,*β*, 20a,b	H-6α (m), 6*β* (m), 20a (m)
6	27.9 (t)	α: 1.78 (m)	H-5, 6*β*, 7α,*β*	H-5, 7α,*β*	H-5 (m)
		β: 1.57 (m)	H-5, 6α, 7α,*β*		H-5 (m), 7*β* (m)
7	27.9 (t)	α: 1.84 (m)	H-6α,*β*, 7*β*	H-5, 6α,*β*, 9,19	H-3 (m), 7*β* (s), 10 (m)
		β: 1.92 (m)	H-6α,*β*, 7α		H-6*β* (m), 7α (s), 19 (m)
8	43.5 (s)			H-2α,*β*, 3, 6α, 7α,*β*, 9, 19	
9	80.6 (d)	6.18 (d, 10.5)	H-10	H-7α,*β*, 10, 19	H-2*β* (s), 17 (s), 19 (m)
10	70.8 (d)	5.04 (d, 10.5)	H-9	H-9	H-3 (w), 7α (m), 18 (s)
11	139.0 (s)			H-1, 10, 13, 16, 17, 18	
12	133.6 (s)			H-10, 13, 14*β*, 18	
13	70.7 (d)	6.10 (ddd, 10.0, 6.5, 1.2)	H-14α,*β*, 18	H-1, 14α,*β*, 18	H-14α (m), 14*β* (s), 16 (m), 18 (m)
14	32.2 (t)	α: 1.12 (br dd, 14.5, 6.5)	H-1, 13, 14*β*	H-1, 2*β*, 13	H-3 (s), 13 (m), 14*β* (s)
		β: 2.62 (ddd, 14.5, 10.0, 10.0)	H-1, 13, 14α		H-1 (s), 13 (s), 14α (s)
15	39.6 (s)			H-1, 2α,*β*, 10, 14*β*, 16, 17	
16	31.4 (q)	1.14 (s)	H-17	H-1, 17	H-1 (m), 13 (m), 17 (m)
17	27.8 (q)	1.79 (s)	H-16	H-1, 16	H-1 (m), 9 (s), 16 (m)
18	14.9 (q)	1.86 (d, 1.2)			H-10 (s), 13 (m)
19	18.1 (q)	0.86 (s)		H-3, 7α, 9	H-7*β* (m), 9 (m)
20	113.8 (t)	a: 5.09 (br s)	H-3, 20b	H-3, 5	H-5 (m), 20b (s)
		b: 4.64 (d, 1.1)	H-3, 20a		H-2α (s), 20a (s)
OAc	169.1 (s)			H-5, OAc-5 (Me)	
	169.9 (s)			H-13, OAc-13 (Me)	
	20.9 (q)	1.85 (s)			
	21.5 (q)	1.57 (s)			
1′	167.2 (s)			H-9, 2′, 3′	
2′	118.8 (d)	6.55 (d, 16.0)	H-3′	H-3′	*o*-Ph (m)
3′	145.4 (d)	7.91 (d, 16.0)	H-2′	H-2′, *o*-Ph	*o*-Ph (m)
q-Ph	134.8 (s)			H-2′, 3′, *m*-, *o*-Ph	
o-	128.3 (d)	7.16 (m)		H-3′, *m*-, *p*-Ph	H-2′ (m), 3′ (m)
m-	129.0 (d)	6.99 (m)		*o*-, *p*-Ph	
p-	130.3 (d)	6.99 (m)		*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. [f] s = strong; m = medium; w = weak.

taxayuntin C,[25] taxuyunnanine C (**2**) and its analogues (**3**−**5**),[8,26] and yunnanxane (**6**),[8,27] and one known abietane, taxamairin A,[28,29] were isolated. Taxuyunnanine C (**2**) and its analogues (**3**−**6**) were obtained as major products in 0.486% yield based on the weight of dry callus. Paclitaxel was produced in 0.0104% yield. The yield of paclitaxel in CR-5 is almost the same as that in the bark of the Pacific yew (*Taxus brevifolia*).[30]

When 100 μM methyl jasmonate was added as an elicitor into the medium, five more taxoids, cephalomannine,[18,19] 1*β*-dehydroxybaccatin VI,[24] taxinine NN-11 (**1**), baccatin I,[22,31] and 2α-acetoxytaxusin,[32] and one more abietane, taxamairin C,[29] were found in addition to the above-mentioned compounds [paclitaxel, taxol C, baccatin VI, taxayuntin C, taxamairin A, taxuyunnanine C (**2**) and its analogues (**3**−**6**)]. It was also observed that levels of taxuyunnanine C (**2**) and its analogues (**3**−**6**) increased 3.1-fold, and paclitaxel and its analogues increased 5.2-fold compared with those in CR-5. The production of phenolic abietane derivatives, taxamairin A and taxamairin C, was also promoted.

A new compound, taxinine NN-11 (**1**), was isolated under these conditions as colorless microcrystals in a yield of 0.0259%, mp 228−231 °C. An elemental composition of $C_{33}H_{42}O_7$ was determined by a combination of HREIMS and its ^1H and ^{13}C NMR spectra. The IR spectrum of **1** showed the absorption of a hydroxyl group at 3620 cm^{-1}, two acetyl carbonyl groups at 1732 cm^{-1}, and an α,*β*-unsaturated ester carbonyl group at 1712 cm^{-1}. Since the resolution of the ^1H NMR signals of **1** in C_6D_6 was much better than that in $CDCl_3$, especially for the H-9 and H-13 signals, the NMR data were measured in C_6D_6 to elucidate the structure of **1**. The ^1H NMR spectrum indicated the presence of a taxane skeleton with four C-Me groups at 0.86, 1.14, 1.79, and 1.86 ppm, two acetyl Me groups at 1.57 and 1.85 ppm, and a cinnamoyl group [δ 6.55 (1H, d, J = 16.0 Hz), 7.91 (1H, d, J = 16.0 Hz), 7.16 (2H, *o*-Ph), 6.99 (3H, *m*- and *p*-Ph)]. The $^1H-^1H$ COSYcorrelations of vicinal protons were observed. The assignment of all protonated carbons was determined by DEPT and HMQC experiments. The HMBC experiment was used for the assignment of the attachment of functional groups. A correlation of the signal due to the cinnamoyl carbonyl (C-1′) at 167.2 ppm with those of H-9 (6.18 ppm), H-2′ (6.55 ppm), and H-3′ (7.91 ppm) indicated the location of the cinnamoyl group at C-9. Correlation of the signals due to two acetyl carbonyls at 169.1 and 169.9 ppm with those of H-5 (5.53 ppm) and H-13 (6.10 ppm) showed the location of two acetoxyl groups of **1** at C-5 and C-13. The location of a hydroxyl group was determined at C-10 by the HMBC correlations of the hydroxymethine proton at 5.04 ppm to C-9, C-11, C-12, and C-15. The multiple-bond $^1H-^{13}C$ correlations of the remaining five nonprotonated carbons of **1** were assigned from the HMBC experiment and allowed the unambiguous carbon skeletal connectivities to be made. The stereochemistry of the taxane skeleton of **1** was determined by 1D-NOE and NOESY experiments as well as by a consideration of vicinal coupling constants. The full NMR data in C_6D_6 of **1** are summarized in Table 1. The ^1H and ^{13}C NMR data in $CDCl_3$ are shown in the Experi-

Table 2. Effects of Compounds **1**−**6** on the Accumulation of Vincristine (VCR) in Multidrug-Resistant Cells 2780AD

	VCR accumulation[a]					
compound	concentration (μg/mL)	average[b] (dpm/well)	% of control[c]	activities[d]	verapamil (%)[e]	evaluation max. verapamil % conc
1	0.1	370	162	+	139	P[f]
	1	909	399	++	191	191%
	10	1478	648	+++	122	1 μg/mL
3	0.1	272	119	+	102	P[f]
	1	673	295	+	134	145%
	10	1755	770	+++	145	10 μg/mL
4	0.1	218	96	±	82	N[f]
	1	445	195	+	95	98%
	10	1190	522	+++	98	10 μg/mL
5	0.1	252	111	+	95	P[f]
	1	584	256	+	123	132%
	10	1599	701	+++	132	10 μg/mL
verapamil	0 (control)	228	100			
	0.1	266	117	+	100	
	1	473	207	+	100	
	10	1211	531	+++	100	
2	0.1	448	105	±	91	N[f]
	1	658	155	+	97	97%
	10	1039	244	+	78	1 μg/mL
6	0.1	477	112	+	97	N[f]
	1	637	150	+	94	97%
	10	995	234	+	75	0.1 μg/mL
verapamil	0 (control)	425	100			
	0.1	490	115	+	100	
	1	680	160	+	100	
	10	1335	314	++	100	

[a] The amount of VCR accumulated in multidrug-resistant human ovarian cancer 2780AD cells was determined with the control 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 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%; ++++, >1000−2000%; +++, 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%); N, negative: the activity is weaker than that of verapamil (verapamil % <100%).

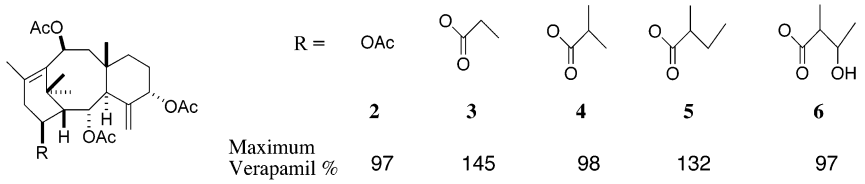

Figure 1. Structure−activity relationship of the 14-acyloxy taxoids **2**−**6**.

mental Section. Accordingly, compound **1** was assigned as 5α,13α-diacetoxy-9α-cinnamoyloxy-4(20),11-taxadien-10β-ol.

The known compounds (Supporting Information) were also identified by the analyses of their spectral data compared with those in the corresponding literature.

At the same time, an additional callus line code-named CR-6 was induced and cultivated from the same explants on the medium in the presence of 1.0 mg/L NAA and 0.3 mg/L KTOS. KTOS[33] is a mixture of oligosaccharides that can promote cell growth. It was found that CR-6 showed even faster growth than CR-5, and the color degree was lower than that of CR-5. However, in this fast-growing callus culture, although the yield of taxuyunnanine C increased, the content of paclitaxel and its analogues was lower than that in CR-5, as shown in Table S1.

The multidrug resistance (MDR) in cancer chemotherapy is attributed to overexpression of P-glycoprotein (P-gp), which has been widely observed in various MDR cell lines. P-gp transports antitumor agents outside the cells in a manner analogous to the ATP-driven ion pumps, involving the conformational change of P-gp induced by ATP hydrolysis. 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 in MDR cells and overcomes multidrug resistance.[34] The effects of taxinine NN-11 (**1**) and 14-acyloxy taxoids **2**−**6** on the cellular accumulation of VCR in human ovarian cancer 2780 AD cells were examined, and the results are summarized in Table 2. Compounds **1**, **3**, and **5** showed more potent activities than verapamil toward VCR accumulation in MDR tumor cells, and **1** exhibited about 2 times the activity of verapamil.

As shown in Figure 1, the alkyl moiety of the 14-acyloxy group at C-14 influenced the MDR-reversing activities of the compounds significantly. A polar alkyl group decreased the activity as in the case of **6**. Elongation of an unbranched alkyl side chain increased the activity as shown in the

Table 3. Summary of Evaluation of Compound **1** Based on the 39 Human Cancer Cell Lines[a]

Parameters of Effective Concentrations			
	GI_{50}	TGI	LC_{50}
MG-MID	−4.65	−4.01	−4

Results of the COMPARE Analysis			
rank	compound	r^b	molecular targets/drug type
1	interferon-α	0.335	
2	ICRF-193	0.318	topoisomerase
3	W80	0.313	tubulin

[a] The mean graph of compound **1** was compared with those of 200 standard compounds using the COMPARE analysis. Drugs were ordered according to the correlation coefficient. Drugs with correlation coefficients higher than 0.5 ($P < 0.001$) were included. [b] Peason correlation coefficient.

Table 4. Summary of Evaluation of Compound **5** Based on the 39 Human Cancer Cell Lines[a]

Parameters of Effective Concentrations			
	GI_{50}	TGI	LC_{50}
MG-MID	−4.53	−4.03	−4

Results of the COMPARE Analysis			
rank	compound	r^b	molecular targets/drug type
1	W80	0.506	tubulin
2	ICRF-154	0.447	topoisomerase
3	W80	0.437	tubulin

[a] The mean graph of compound **5** was compared with those of 200 standard compounds using the COMPARE analysis. Drugs were ordered according to the correlation coefficient. Drugs with correlation coefficients higher than 0.5 ($P < 0.001$) were included. [b] Peason correlation coefficient.

changes from **2** to **3**, and in **3** and **4**. These results suggest that compounds with a hydrophobic less-hindered alkyl side chain of the C-14 acyloxy group bind preferentially to P-gp to block the efflux channel, allowing the anticancer agent to penetrate into the cancer cells.

It is noted that the potent taxoids **1**, **3**, and **5** possess a structure different from those of previously reported MDR-reversing taxoids, with a cinnamoyl group at C-5 or a oxetane ring.[35−38] The results of biological activities of **1**, **3**, and **5** suggest that these taxoids isolated from the callus culture of *T. cuspidata* might be useful for overcoming multidrug resistance and that cell culture would be a promising approach to produce the taxoids as modulators of MDR tumor cells.

The cytotoxicity of taxinine NN-11 (**1**) and 2α,5α,10β-triacetoxy-14β-(2′-methyl)butyryloxy-4(20),11-taxadiene (**5**) was examined. The results of in vitro primary screening of **1** and **5** based on the 39 human cancer cell lines[15,39] are shown in Tables 3 and 4, respectively. Although the effective concentration of **1** is rather high, differential growth inhibition is recognized. Since the result of COMPARE analysis of **1** was negative ($r < 0.5$), it possibly belongs to a new mechanistic class and a new member of anticancer agents. On the other hand, the cell growth inhibition effect (MG-MID of $GI_{50} = -4.53$) and differential growth inhibition of compound **5** were weak. The results suggest that compound **5** is not an effective anticancer agent per se.

Taxinine NN-11 (**1**) exhibited such marked bioactivities that it was thought necessary to produce it in further quantity for additional bioassays. Therefore, we investigated the production by the callus cultures in several conditions. As shown in Table 5, **1** was not produced in culture CR-5, although it was obtained from CR-5 in 0.0259% yield by addition of 100 μM methyl jasmonate into the medium, the growth rate became slower, and the callus turned dark brown. Thus, it was not easy to improve the yield of **1** by further addition of methyl jasmonate. Since it was found that the callus culture (CR-6) cultivated on the medium containing 0.3 mg/L KTOS and 1.0 mg/L NAA showed faster growth than CR-5, we attempted to use callus culture CR-6 instead of CR-5. When methyl jasmonate was added at a concentration of either 100 or 200 μM to the medium of CR-6, the growth of the callus was markedly improved in comparison with that of the callus of CR-5 stimulated with 100 μM methyl jasmonate. The maximum content of taxinine NN-11 (**1**) was obtained when the concentration of methyl jasmonate in the medium was 200 μM. However, when the concentration was increased to 300 μM, the callus turned completely dark brown and no **1** was found.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured using a Horiba SEPA-200 polarimeter. IR spectra were recorded in $CHCl_3$ on a Hitachi 270-30 spectrometer. ^1H NMR (499.87 MHz) and ^{13}C NMR (125.70 MHz) spectra were recorded on a Varian UNITY-PS 500 spectrometer. HREIMS spectra were obtained using a JEOL JMS HX-110 spectrometer. The flow rate for HPLC was 5 mL/min, and the solvent used for reversed-phase HPLC was MeOH−0.05 M ammonium acetate buffer (pH 4.8)−MeCN (1:2:2). Inertsil Prep-sil (GL Science), 25 × 1 cm and 25 × 2 cm i.d., stainless columns were used for normal-phase HPLC, and Inertsil Prep-ODS (GL Science), 25 × 1 cm i.d., stainless columns were used for reversed-phase HPLC, unless otherwise stated. Silica gel (70−230 mesh) was employed for column chromatography, and silica gel (230−400 mesh) was employed for flash column chromatography.

Plant Material. Young stems of *T. cuspidata* Sieb. et Zucc., collected in Sendai, Japan, in January 1997, were used as explants in the experiments. The stems were stripped of needles, washed with distilled water, immersed for 3 min in 70% ethanol, and surface sterilized by immersion in saturated calcium hypochlorite solution for 10 min. After sterilization, the stems were rinsed three times with sterile distilled water and aseptically dissected into explants 5−8 mm in length.

Induction and Culture Conditions. Gamborg's B5 medium supplemented with 2 × vitamins and 20 g/L sucrose was used with either 0.5 mg/L NAA for CR-5 or a combination of 1.0 mg/L NAA and 0.3 mg/L KTOS for CR-6. (All medium components except KTOS were purchased from Wako Pure Chemicals, Co., Ltd., Osaka, Japan.) After the pH of the media was adjusted to 5.8 with 0.1 M sodium hydroxide, 10 g/L agar was added. The media were autoclaved at 120 °C for 15 min. The explants were placed on the solidified nutrient media and incubated in the dark at 25 °C for 30−40 days. Then the initiated calluses were subcultured continuously every 60 days under the same conditions.

After the callus of CR-5 was subcultured eight times, one part of this callus line was harvested for analysis. The other part was subcultured subsequently on the medium supplemented with 100 μM methyl jasmonate (Wako Pure Chemicals, Co., Ltd.) and incubated for another 60 days.

After CR-6 was subcultured 13 times, a part of this callus line was harvested for analysis. The remaining callus was divided into three parts, which were subcultured subsequently on the media supplemented with methyl jasmonate at concentrations of 100, 200, and 300 μM, respectively, and incubated for another 60 days.

Extraction and Isolation of the Products from CR-5. The fresh callus (2678.5 g) was freeze-dried for 2 days to give dry callus (206.6 g). It was extracted with hexane (3 × 1.3 L)

Table 5. Production of **1** Using Callus Culture of CR-6 by Addition of Methyl Jasmonate

parameter	CR-5	CR-5 + methyl jasmonate 100 μM	CR-6	CR-6 + methyl jasmonate 100 μM	CR-6 + methyl jasmonate 200 μM	CR-6 + methyl jasmonate 300 μM
dry callus (g)	206.6	74.1	49.0	87.0	93.6	12.9
growth rate[a]	1	0.68	1.36	1.06	0.98	0.67
color degree[b]	1.9	3.5	1.6	2	2.2	5
isolated yield of **1** (mg)	no	19.2	no	22.3	59.1	no
content of **1** (%)	no	0.0259	no	0.0256	0.0631	no

[a] The relative values were compared to those of CR-5. [b] The color of the callus cultures was evaluated in five degrees: 1 (colorless) to 5 (dark brown).

for 3 h to give a crude hexane extract (523.5 mg). The remaining cell mass was successively extracted with EtOAc (3 × 1.3 L) for 3 h to give a crude EtOAc extract (1396.8 mg). The remaining cell mass was further extracted with MeOH (3 × 1.3 L) for 3 h. After removal of MeOH, the extract was diluted with a saturated aqueous solution of NaCl (150 mL) and extracted with $CHCl_3$ (3 × 100 mL) to give a crude $CHCl_3$ extract (2655.0 mg). Subsequently, the crude $CHCl_3$ extract was dissolved in a mixture of MeOH−EtOAc (1:4, 25 mL) and extracted with a 0.5 M aqueous solution of H_2SO_4 (3 × 5 mL). The organic layer was successively extracted with a 29% aqueous solution of NH_4OH until the pH value of the aqueous solution became 9.0, washed with a saturated aqueous solution of NaCl, dried over Na_2SO_4, and concentrated to give a crude neutral fraction of $CHCl_3$ extract (1362.0 mg).

The crude hexane extract (523.5 mg) was separated into four fractions by column chromatography using a gradient elution from hexane−EtOAc (1:1) to EtOAc (100%). Fractions 2 (254.6 mg) and 3 (116.1 mg) were purified further by normal-phase HPLC [hexane−EtOAc (8:2)] to give compounds **5** (165.1 mg), **4** (8.5 mg), **3** (9.4 mg), and **2** (77.6 mg).

The crude EtOAc extract (1396.8 mg) was separated into four fractions (EF1−EF4) by column chromatography using a gradient elution from hexane−EtOAc (1:1) to MeOH (100%). EF1 (609.3 mg) was further purified by normal-phase HPLC [hexane−EtOAc (8:2)] to give compounds **5** (202.4 mg), **4** (9.8 mg), **3** (18.1 mg), and **2** (80.3 mg). EF2 (199.0 mg) was separated by reversed-phase HPLC [MeOH−H_2O−MeCN (1:1:2)] to give two subfractions, EF2-1 (53.9 mg) and EF2-2 (109.6 mg). EF2-1 was separated by a combination of reversed-phase HPLC and normal-phase HPLC [hexane−EtOAc (4:6)] to give taxamairin A (2.9 mg) and paclitaxel (0.9 mg). EF2-2 was purified by normal-phase HPLC [hexane−EtOAc (1:1)] to give compounds **2** (13.4 mg), **6** (47.2 mg), and taxayuntin C (4.9 mg). EF3 (111.7 mg) was separated by reversed-phase HPLC to give paclitaxel (11.6 mg) and a subfraction that was further purified by normal-phase HPLC [hexane−EtOAc (1:1)] to give 7-*epi*-taxol (0.4 mg) and taxol C (1.2 mg).

The crude neutral fraction of $CHCl_3$ extract (1362.0 mg) was separated into six fractions (CF1−CF6) by column chromatography using a gradient elution from hexane−EtOAc (6:4) to MeOH (100%). CF1 (655.0 mg) was purified by normal-phase HPLC [hexane−EtOAc (8:2)] to give compounds **5** (190.4 mg) and **2** (71.7 mg). CF2 (176.0 mg) was separated by two steps of normal-phase HPLC [hexane−EtOAc (1:1 and 8:2)] to give compounds **5** (9.3 mg), **4** (1.1 mg), **3** (2.2 mg), **2** (36.0 mg), and **6** (53.0 mg). CF3 (51.0 mg) was separated by reversed-phase HPLC to give compound **6** (8.5 mg). CF4 (154.8 mg) was separated by reversed-phase HPLC and normal-phase HPLC [hexane−EtOAc (1:1)] to give 7-*epi*-taxol (2.9 mg), baccatin VI (1.1 mg), paclitaxel (9.0 mg), taxol C (0.6 mg), **6** (0.6 mg), and taxayuntin C (1.0 mg).

Extaction and Isolation of Compound 1 and the Other Products from CR-5 Stimulated with 100 μM Methyl Jasmonate. The fresh callus (721.1 g) was freeze-dried for 2 days to give dry callus (74.1 g). It was extracted with hexane (3 × 1 L) for 3 h to give a crude hexane extract (1126.1 mg). The remaining cell mass was successively extracted with EtOAc (3 × 1 L) for 3 h to give a crude EtOAc extract (1673.0 mg). The remaining cell mass was further extracted with MeOH (3 × 1 L) for 3 h. After removal of MeOH, the extract was diluted with a saturated aqueous solution of NaCl (150 mL) and extracted with $CHCl_3$ (3 × 100 mL) to give a crude $CHCl_3$ extract (1445.5 mg).

The crude EtOAc extract (1673.0 mg) was separated into five fractions by column chromatography over silica gel using a gradient elution from hexane−EtOAc (1:1) to MeOH (100%)]. A part (200 mg) of fraction 2 (549.3 mg) was separated by normal-phase HPLC on a Inertsil Prep-sil (GL Science) 25 × 2 cm i.d. column [hexane−EtOAc (1:1); flow rate 10.0 mL/min] to give a subfraction (t_R 5.0−11.0 min, 73.6 mg), which was further purified by normal-phase HPLC [hexane−EtOAc (8:2)] to give compound **1** (t_R 39.7 min, 7.0 mg).

Similarly, the isolation of the other products was performed by the same operation mentioned above, and their yields are shown in Table S1 (Supporting Information).

Extraction and Isolation from CR-6. The fresh callus (648.0 g) was freeze-dried to give dry callus (49.0 g). The extraction and isolation were carried out according to the method described above. The products and their yields are also shown in Table S1 (Supporting Information).

Extraction and Isolation of Compound 1 from CR-6 Stimulated with Methyl Jasmonate. The freeze-dried calluses were defatted with hexane (3 × 20 mL/g dry callus) for 3 h. The remaining cell masses were successively extracted with EtOAc (3 × 20 mL/g dry callus) for 3 h to give the crude EtOAc extracts. The crude EtOAc extracts were separated by the method described above, and compound **1** was obtained in the yields shown in Table 5.

5α,13α-Diacetoxy-9α-cinnamoyloxy-4(20),11-taxadien-10*β*-ol (Taxinine NN-11) (1): colorless microcrystals; mp 228−231 °C; $[\alpha]^{20}_D$ +99.8° (*c* 0.538, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3620, 1732, 1712 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.76 (1H, d, J = 16.0 Hz, H-3′), 7.58−7.53 (2H, m, *o*-Ph), 7.42−7.38 (3H, m, *m*- and *p*-Ph), 6.52 (1H, d, J = 16.0 Hz, H-2′), 5.92 (1H, d, J = 10.3 Hz, H-9), 5.92 (1H, m, H-13), 5.37 (1H, br t, J = 2.7 Hz, H-5), 5.21 (1H, d, J = 1.1 Hz, H-20a), 5.05 (1H, br d, J = 10.3 Hz, H-10), 4.86 (1H, d, J = 1.1 Hz, H-20b), 3.04 (1H, br d, J = 6.1 Hz, H-3), 2.71 (1H, dt, J = 15.0, 9.8 Hz, H-14*β*), 2.17, 2.08 (each 3H, s, acetyl methyl), 2.00 (3H, d, J = 1.5 Hz, H-18), 1.92−1.68 (7H, m, H-1, 2α, 2*β*, 6α, 6*β*, 7α, and 7*β*), 1.72 (3H, s, H-17), 1.22 (3H, s, H-16), 1.09 (1H, br dd, J = 15.0, 8.0 Hz, H-14α), 0.79 (3H, s, H-19); ^{13}C NMR ($CDCl_3$) δ 170.5 (s, 13-OAc), 170.0 (s, 5-OAc), 167.7 (s, C-1′), 148.9 (s, C-4), 145.8 (d, C-3′), 138.2 (s, C-11), 134.3 (d, *q*-Ph), 134.1 (s, C-12), 130.5 (d, *p*-Ph), 128.9 (d, *o*-Ph), 128.2 (d, *m*-Ph), 117.7 (d, C-2′), 114.1 (t, C-20), 80.6 (d, C-9), 76.4 (d, C-5), 70.9 (d, C-13), 70.6 (d, C-10), 43.2 (s, C-8), 40.5 (d, C-1), 39.4 (s, C-15), 38.1 (d, C-3), 32.0 (t, C-14), 31.4 (q, C-16), 28.3 (t, C-2), 27.5 (t, C-6), 27.4 (t, C-7), 27.4 (q, C-17), 21.8 (q, 5-OAc or 13-OAc), 21.5 (q, 5-OAc or 13-OAc), 17.8 (q, C-19), 15.0 (q, C-18); HREIMS *m*/*z* 550.2936 (calcd for $C_{33}H_{42}O_7$, 550.2931).

Cellular Accumulation of [^{3}H]-VCR. The MDR 2780AD cells were maintained in PRMI-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 nonradioactive 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).

Measurements of Cell Growth Inhibition and Data Analysis. The details of measuring cancer cell growth inhibition are described elsewhere.[15,40,41] Briefly, the cells were plated at proper density in 96-well plates in RPMI-1640 medium with 5% fetal bovine serum and allowed to attach overnight. The cells were exposed to drugs for 48 h. Then, the cell growth was determined according to the sulforhodamine B assay, described by Skehan et al.[42] Three dose response parameters were calculated for each experimental agent according to the method described previously:[40] growth inhibition of 50% (GI_{50}), which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation, the drug concentration resulting in total growth inhibition (TGI), and the drug concentration resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC_{50}), which indicated a net loss of cells following treatment.[40] The mean graph, which shows the differential growth inhibition of the drug in the cell line panel, was drawn based on a calculation using a set of GI_{50}.[43,44] To analyze the correlation between the mean graphs of drugs A and B, the COMPARE computer algorithm was developed according to the method described by Paull et al.[43] Peason correlation coefficients were calculated using the following formula: $r = (\sum(x_i - x_m)(y_i - y_m))/(\sum(x_i - x_m)^2\sum(y_i - y_m)^2)^{1/2}$, where x_i and y_i are log GI_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

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Supporting Information Available: Compound list, Table S1, ^1H and ^{13}C NMR spectra of **1** in $CDCl_3$, ^1H, ^{13}C, H−H COSY, HMQC, and HMBC NMR spectra of **1** in C_6D_6, in vitro anticancer testing results, dose response curves, and mean graphs of **1** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Fett-Neto, A. G.; DiCosmo, F.; Reynolds, W. F.; Sakata, K. *Biotechnology* **1992**, 1572−1575.
(2) Wickremesinhe, E. R. M.; Arteca R. N. *Plant Cell, Tissue Organ Cult.* **1993**, *35*, 181−193.
(3) Banerjee, S.; Upadhyay, N.; Kukreja, A. K.; Ahuja, P. S.; Kumar, S.; Saha, G. C.; Sharma, R. P.; Chattopadhyay, S. K. *Planta Med.* **1996**, *62*, 333−335.
(4) Ma, W.; Park, G. L.; Gomez, G. A.; Nieder, M. H.; Adams, T. L.; Aynsley, J. S.; Sahai, O. P.; Smith, R. J.; Stahlhut, R. W.; Hylands, P. J.; Bitsch, F.; Shackleton, C. *J. Nat. Prod.* **1994**, *57*, 116−122.
(5) Zhang, C. H.; Wu, J. Y.; He, G. Y. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 396−402.
(6) Ando, M. Jpn. Kokai Tokkyo Koho JP 2000106893 A2, 2000.
(7) Sakai, J.; Ando, M.; Uchiyama, T.; Fujisawa, H.; Kitabatake, M.; Toyoizumi, K.; Hirose, K. *Nippon Kagaku Kaishi* **2002**, 231−238.
(8) Ma, W.; Stahlhut, R. W.; Adams, T. L.; Park, G. L.; Evans, W. A.; Blumenthal, S. G.; Gomez, G. A.; Nieder, M. H.; Hylands, P. J. *J. Nat. Prod.* **1994**, *57*, 1320−1324.
(9) Menhard, B.; Eisenreich, W.; Hylands, P. J.; Bacher, A.; Zenk, M. H. *Phytochemistry* **1998**, *49*, 113−125.
(10) Yukimune, Y.; Tabata, H.; Higashi, Y.; Hara, Y. *Nat. Biotechnol.* **1996**, *14*, 1129−1132.
(11) Furmanowa, M.; Glowniak, K.; Syklowska-Baranek, K.; Zgorka, G.; Jozefczyk, A. *Plant Cell, Tissue Organ Cult.* **1997**, *49*, 75−79.
(12) Qiu, D.; Kim, Y. S.; Williams, R. M.; Croteau, R. B. *Phytochemistry* **2003**, *62*, 901−909.
(13) Zhang, C. H.; Mei, X. G.; Liu, L.; Yu, L. J. *Biotechnol. Lett.* **2002**, *22*, 1561−1564.
(14) Baebler, S.; Camloh, M.; Kovac, M., Revnikar M.; Zel, J. *Planta Med.* **2002**, *68*, 475−476.
(15) Yamori, T.; Matsunaga, A.; Sato, S.; Yamazaki, K.; Komi, A.; Ishizu, K.; Mita, I.; Edatsugi, H.; Matsuba, Y.; Takezawa, K.; Nakanishi, O.; Kohno, H.; Nakajima, Y.; Komatsu, H.; Andoh, T.; Tsuruo, T. *Cancer Res.* **1999**, *59*, 4042−4049.
(16) Gamborg, O. L.; Miller, R. A.; Ojima, K. *Exp. Cell Res.* **1968**, *50*, 151−158.
(17) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *J. Am. Chem. Soc.* **1971**, *93*, 2325−2327.
(18) Chmurny, G. N.; Hilton, B. D.; Brobst, S.; Look, S. A.; Witherup, K. M.; Beutler, J. A. *J. Nat. Prod.* **1992**, *55*, 414−423.
(19) Miller, R. W.; Powell, R. G.; Smith, C. R., Jr.; Arnold, E.; Clardy, J. *J. Org. Chem.* **1981**, *46*, 1469−1474.
(20) Huang, C. H. O.; Kingston, D. G. I.; Magri, N. F.; Samaranayake, G.; Boettner, F. E. *J. Nat. Prod.* **1986**, *49*, 665−669.
(21) Zhang, H.; Takeda, Y.; Matsumoto, T.; Minami, Y.; Yoshida, K.; Xiang, W.; Mu, Q.; Sun, H. *Heterocycles* **1994**, *38*, 975−980.
(22) Rojas, A. C.; Marcano, D. de; Méndez, B.; Méndez, *J. Org. Magn. Reson.* **1983**, *21*, 257−260.
(23) Sénilh, V.; Blechert, S.; Colin, M.; Guénard, D.; Picot, F.; Potier, P.; Varenne, P. *J. Nat. Prod.* **1984**, *47*, 131−137.
(24) Min, Z.-D., Jiang, H., Liang J.-Y. *Acta Pharm. Sin.* **1989**, *24*, 673−677.
(25) Chen, W. M.; Zhang, P. L.; Zhou, J. Y.; Fang, Q. C. *Acta Pharm. Sin.* **1997**, *32*, 363−367.
(26) Zhang, H.; Takeda, Y.; Minami, Y.; Yoshida, K.; Matsumoto, T.; Xiang, W.; Mu, Q.; Sun, H. *Chem. Lett.* **1994**, 957−960.
(27) Chen, W. M.; Zhang, P. L.; Wu, B.; Zheng, Q. T. *Acta Pharm. Sin.* **1991**, *26*, 747−754.
(28) Liang, J.-Y.; Min, Z.-D.; Iinuma, M.; Tanaka, T.; Mizuno, M. *Chem. Pharm. Bull.* **1987**, *35*, 2613−2614.
(29) Liang, J.-Y.; Min, Z.-D.; Tanaka, T.; Mizuno, M.; Iinuma, M. *Acta Chim. Sin.* **1988**, *46*, 21−25.
(30) Vidensek, N.; Lim, P.; Campbell, A.; Carlson, C. *J. Nat. Prod.* **1990**, *53*, 1609−1610.
(31) Della Casa de Marcano, D. P.; Halsall, T. G. *J. Chem. Soc. D* (*Chem. Commun.*) **1970**, 1381−1382.
(32) Della Casa de Marcano, D. P.; Halsall, T. G. *Chem. Commun.* **1969**, 1282−1283.
(33) KTOS was provided by Kobe Natural Products and Chemicals Co., Ltd., Kobe, Japan. It is a mixture of oligosaccharides that consists of five kinds of sugar, namely, rhamnose, galactose, glucose, galacturonic acid, and glucuronic acid.
(34) Tsuruo, T.; Iida-Saito, H.; Kawabata, H.; Oh-hara, T.: Hamada, H.; Utakoji, T. *Jpn. J. Cancer Res.* **1998**, *77*, 682−692.
(35) Kosugi, K.; Sakai, J.; Zhang S., Watanabe, Y.; Sakai H.; Suzuki, T.; Hagiwara, H.; Hirata, N.; Hirose, K.; Ando, M.; Tomida, A.; Tsuruo, T. *Phytochemistry* **2000**, *54*, 839−845.
(36) Sakai, J.; Sakai, H.; Kosugi, K.; Zhang, S.; Hirata, N.; Hirose, K.; Tomida, A.; Tsuruo, T.; Ando, M. *Heterocycles* **2001**, *54*, 999−1009.
(37) (a) Kobayashi, J.; Shigemori, H. *Heterocycles* **1998**, *47*, 1111−1133. (b) Kobayashi, J.; Hosoyama, H.; Wang, X.-X.; Shigemori, H.; Sudo, Y.; Tsuruo, T. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1555−1558.
(38) Ojima, I.; Bounaud, P.-Y.; Takeuchi, C.; Pera, P.; Bernacki, R. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 189−194.
(39) Boyd, M. R.; Paull, K. D. *Drug Dev. Res.* **1995**, *34*, 91−109.
(40) Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrichi, M.; Campbell, H.; Mayo, J.; Boyd, M. *J. Natl. Cancer Inst.* **1991**, *83*, 757−766.
(41) Yamori, T.; Sato, S.; Chikazawa, H.; Kadota, T. *Jpn. J. Cancer Res.* **1997**, *88*, 1205−1210.
(42) Skenhan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.
(43) Paull, K. D.; Shoemaker, R. H.; Hodes, L.; Monks, A.; Scudiero, D. A.; Rubinstein, L.; Plowman, J.; Boyd, M. R. *J. Natl. Cancer Inst.* **1989**, *81*, 1088−1092.
(44) Boyd, M. R., Devita, V. T., Hellman, S., Jr., Rosenberg, S. A., Eds. *Cancer: Principles and Practice of Oncology Update*; Philadelphia: Lippincott, 1989; Vol. 3, pp 1−12.

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