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TAXOIDS FROM *TAXUS CUSPIDATA* VAR. *NANA*

HIROSHI MORITA, AKIRA GONDA, LAN WEI, YUKINORI YAMAMURA, HIDEKI WAKABAYASHI, KOICHI TAKEYA and HIDEJI ITOKAWA*

Department of Pharmacognosy, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

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Key Word Index—*Taxus cuspida* var. *nana*; Taxaceae; taxuspinananes H–K; taxoid.

Abstract—Four new taxoids, taxuspinananes H–K have been isolated from the stems of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder. Their structures were elucidated by spectroscopic analysis. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Paclitaxel (Taxol®), isolated from various species of the genus *Taxus* and docetaxel (Taxotere®), a semisynthetic analog, are a new class of anticancer agents especially effective for patients with advanced ovarian and breast cancers [1–3]. Paclitaxel is considered a leading compound in cancer chemotherapy, and is currently intensively investigated from a chemical, biological, pharmacological, and clinical point of view. The mechanism of action of these diterpenes involves the facilitated assembly and stabilization of microtubules [4].

As a part of research program aimed at developing new bioactive taxoids, we have investigated the taxoids contained in the stems of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder, which is a popular garden shrub in Japan [5–7]. Further chromatographic purification of constituents of the stems of *T. cuspidata* var. *nana* with guidance by a cytotoxic assay resulted in the isolation of four new taxoids, named as taxuspinananes H (**1**)–K (**4**). We report here the isolation and structure elucidation of these new taxoids by extensive 2D NMR methods.

RESULTS AND DISCUSSION

The toluene extract obtained by solvent partition of a methanol extract of the stems of *T. cuspidata* var. *nana*, showed cytotoxicity against P-388 lymphocytic leukemia cells, and were successively subjected to bioassay-guided fractionation using silica gel to afford cytotoxic fractions. Further separation of these fractions by reversed-phase HPLC using ODS silica gel yielded four new taxoids, named taxuspinanane H (**1**: 0.00006%), I (**2**: 0.00008%), J (**3**: 0.00001%), and K (**4**: 0.0001%).

Taxuspinanane H (deaminoacyl cinnamoyltaxine A: **1**), an amorphous powder, showed a high-resolution FAB-mass spectral quasimolecular ion peak at *m/z* 603.2565 [$(M+Na)^+$, Δ −0.5 mmu], corresponding to molecular formula, $C_{33}H_{40}O_9$. The IR absorptions at 3423, 1719 and 1638 cm^{-1} were attributed to hydroxyl, ester and α,β-unsaturated ketone groups, respectively. In the NMR spectra, the presence of two acetyl (δ_H 1.98 and 2.03, δ_C 21.42 × 2) and one cinnamoyl [δ_H 6.51 (1H, *d*, 16.0 Hz), 7.82 (1H, *d*, 16.0 Hz), 7.51, 7.40, 7.42, δ_C 166.24, 117.60, 146.31, 134.02, 128.07, 129.11, 130.87] groups was suggested. In the ^{13}C NMR spectrum, the presence of signals at 122.96 (*d*) and 133.19, 133.02, and 134.73 (*s*) was diagnostic of the 6–10–6 ring system of taxine A [8] and taxuspine B [9]. This skeleton was further verified by the complete assignments of all ^1H and ^{13}C signals (Tables 1 and 2), which was done using 2D measurements (^1H–^1H COSY, TOCSY, HMQC, and HMBC). The HMBC correlations: H-20/C-3, C-4, C-5, H-19/C-7, C-8, C-9, H-1/C-11, C-13, C-20, H-10/C-9, C-12, C-15 were especially diagnostic. The location of the acetyl groups at C-2 and C-13, and the cinnamoyl group at C-5, were verified by the HMBC correlations (Fig. 1) between H-2 and H-13 and acetyl carbonyls, and between H-5 and the cinnamoyl carbonyl carbon. In addition, the stereostructure was elucidated by NOE correlations observed by a phase sensitive ROESY spectrum (Fig. 1).

Taxuspinanane I (*N*-methyl paclitaxel: **2**) was obtained as an amorphous powder and a high-resolution FAB-mass spectrum gave a quasimolecular ion peak at *m/z* 890.3346 [$(M+Na)^+$, Δ −1.8 mmu], corresponding to the molecular formula, $C_{48}H_{53}NO_{14}$.

* Author to whom correspondence should be addressed.

Table 1. ^{1}H NMR signal assignments of taxuspinananes H–K (**1**–**4**) in $CDCl_3$

Position	**1**	**2**	**3**	**4**
1	1.65 (1H, *dd*, 2.0, 8.3)			2.16 (1H, *br t*)
2	5.72 (1H, *dd*, 2.0, 9.8)	5.67 (1H, *d*, 7.0)	5.69 (1H, *d*, 7.0)	1.88 (1H, *m*, α)
				1.66 (1H, *br d*, 5.0 β)
3	1.91 (1H, *d*, 15.4, α)	3.80 (1H, *d*, 7.0)	3.88 (1H, *d*, 7.0)	3.20 (1H, *d*, 5.0)
	2.81 (1H, *d*, 15.4, β)			
5	5.60 (1H, *br d*, 7.4)	4.93 (1H, *dd*, 9.4, 1.9)	4.99 (1H, *br d*, 9.5)	4.26 (1H, *br s*)
6α	1.70 (1H, *m*)	2.54 (1H, *m*)	2.59 (1H, *m*)	1.82 (1H, *m*)
6β	2.40 (1H, *m*)	1.86 (1H, *m*)	1.87 (1H, *m*)	1.65 (1H, *m*)
7	4.20 (1H, *br s*)	4.41 (1H, *m*)	4.47 (1H, *m*)	5.59 (1H, *dd*, 5.2, 11.5)
9				5.88 (1H, *d*, 10.8)
10	5.33 (1H, *d*, 2.5)	6.29 (1H, *s*)	6.35 (1H, *s*)	6.29 (1H, *d*, 10.8)
13	5.45 (1H, *br d*, 10.0)	6.24 (1H, *br t*, 8.2)	6.21 (1H, *br t*, 9.1)	
14α	1.81 (1H, *dd*, 16.2, 3.0)	2.34 (2H, *m*)	2.42 (1H, *m*)	1.90 (1H, *m*)
14β	2.72 (1H, *ddd*, 8.3, 10.0, 16.2)		2.29 (1H, *m*)	2.88 (1H, *dd*, 7.1, 19.4)
16	1.23 (3H, *s*)	1.14 (3H, *s*)	1.28 (3H, *s*)	1.61 (3H, *s*)
17	1.19 (3H, *s*)	1.26 (3H, *s*)	1.67 (3H, *s*)	1.07 (3H, *s*)
18	1.93 (3H, *s*)	1.88 (3H, *s*)	2.02 (3H, *d*, 1.4)	2.27 (3H, *s*)
19	1.30 (3H, *s*)	1.67 (3H, *s*)	1.17 (3H, *s*)	0.82 (3H, *s*)
20	5.43 (1H, *br d*, 9.8)	4.17 (1H, *d*, 8.3)	4.30 (1H, *d*, 8.3)	5.09 (1H, *s*)
		4.28 (1H, *d*, 8.3)	4.19 (1H, *d*, 8.3)	4.77 (1H, *s*)
2′		4.99 (1H, *m*)		
3′		5.88 (1H, *br s*)		
3′-Ph		7.46 (2H, *m*)*		
		7.39 (2H, *m*)†		
		7.39 (1H, *m*)†		
5′-Ph		7.46 (2H, *m*)*		
		7.39 (2H, *m*)†		
		7.39 (1H, *m*)†		
N-Me		2.82 (3H, *br s*)		
2-Ac	1.98 (3H, *s*)			
4-Ac		2.25 (3H, *s*)	2.30 (3H, *s*)	
7-Ac				2.04 (3H, *s*)
9-Ac				2.04 (3H, *s*)
10-Ac		2.24 (3H, *s*)	2.26 (3H, *s*)	1.99 (3H, *s*)
13-Ac	2.03 (3H, *s*)			
2-Bz		8.08 (2H, *dd*, 1.4, 7.3)	8.07 (2H, *dd*, 1.4, 8.4)	
		7.51 (2H, *m*)*	7.46 (2H, *m*)	
		7.60 (1H, *t*, 7.3)	7.60 (1H, *m*)	
5-cinnamoyl			13-cinnamoyl	
2′	6.51 (1H, *d*, 16.0)		6.51 (1H, *d*, 16.0)	
3′	7.82 (1H, *d*, 16.0)		7.85 (1H, *d*, 16.0)	
5′	7.51 (2H, *m*)		7.63 (2H, *m*)	
6′	7.40 (2H, *m*)		7.48 (2H, *m*)	
7′	7.42 (1H, *m*)		7.48 (1H, *m*)	
1-OH		1.89 (1H, *br s*)		
5-OH				1.86 (1H, *br s*)
7-OH		2.43 (1H, *d*, 4.1)		
2′-OH		4.32 (1H, *br d*, 7.5)		

*–† Assignment may be interchanged.

The IR absorptions indicated the presence of hydroxyl (3449 cm^{-1}), ester (1720 cm^{-1}) and amide (1619 cm^{-1}) groups. The NMR signals of **2** (Tables 1 and 2) assigned by 2D measurements (^{1}H–^{1}H COSY, HMQC, and HMBC) were very close to those of paclitaxel [10], indicating the presence of two acetyl at δ_H 2.24 and 2.25, one benzoyl at δ_H 8.08, 7.60, and 7.51, an oxetane ring at δ_H 4.17 and 4.28, mutually coupled with a coupling constant of 8.3 Hz and a side chain at C-13 at δ 6.24 (H-13), 4.99 (H-2′), 5.88 (H-3′), 7.39, 7.46 (Ph at C-3′). The major different point is due to the presence of *N*-methyl signal (δ_H 2.82 and δ_C 37.53). The NOE correlations (Fig. 2) in the taxane skeleton indicated that **2** possessed the same configurations as those of paclitaxel. Thus, compound **2** is *N*-methyl paclitaxel.

Table 2. ^{13}C NMR signal assignments of taxuspinananes H–K (**1**–**4**) in $CDCl_3$

Position	**1**	**2**	**3**	**4**
1	47.31	79.06	79.31	40.96
2	70.98	75.09	74.95	25.64
3	35.09	45.62	45.94	34.80
4	133.19	80.99	81.16	151.90
5	70.52	84.48	84.40	72.57
6	36.70	35.61	35.70	36.06
7	68.06	72.18	72.36	69.81
8	53.26	58.63	58.77	46.83
9	213.87	203.85	203.83	75.86
10	77.07	75.70	75.82	72.86
11	133.02	132.97	132.86	138.59
12	134.73	142.65	143.22	151.53
13	69.55	72.18	70.11	200.74
14	27.22	35.82	36.24	39.64
15	37.74	43.26	43.07	39.81
16	24.51	21.96	21.38	25.58
17	32.58	26.86	26.85	37.07
18	16.66	14.90	15.53	14.11
19	18.45	9.59	9.51	12.56
20	122.96	76.50	76.51	111.41
1′		173.53		
2′		72.85		
3′		61.46		
4′		173.38		
3′-Ph		129.08*		
		126.82†		
		130.01‡		
		136.18`		
5′-Ph		128.65*		
		126.82†		
		128.47‡		
		136.02`		
N-Me		37.53		
2-Ac	21.42			
	169.92			
4-Ac		22.34	22.63	
		170.21	169.84	
7-Ac				21.43¶
				169.86
9-Ac				20.90¶
				170.24
10-Ac		20.88	20.89	20.78
		171.28	171.33	169.13
13-Ac	21.42			
	170.48			
2-Bz		130.21*	128.67	
		128.47†	130.08	
		133.64‡	133.74	
		129.31	129.25	
		166.90	167.01	
5-cinnamoyl			13-cinnamoyl	
1′	166.24		166.27	
2′	117.60		116.85	
3′	146.31		146.82	
4′	134.02		133.95	
5′	128.07		128.24	
6′	129.11		129.17	
7′	130.87		130.97	

*–¶ Assignment may be interchanged.

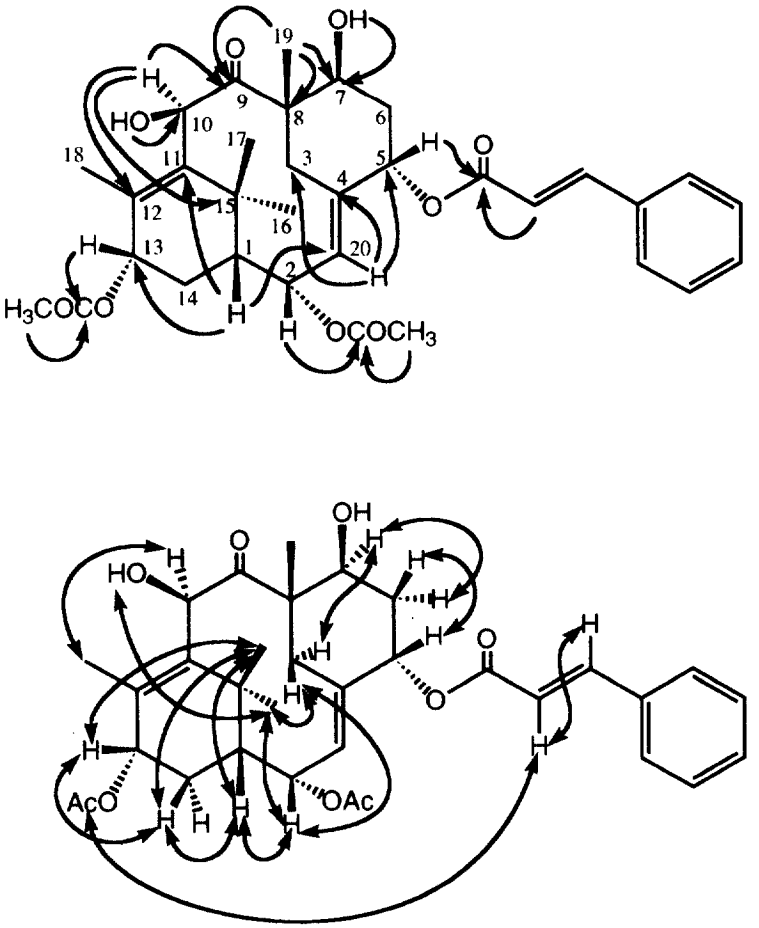

Fig. 1.

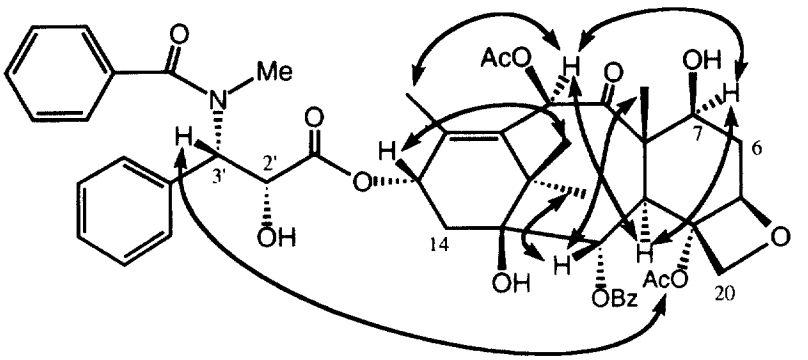

Fig. 2.

Taxuspinanane J (deaminoacyl cinnamoyltaxol: **3**), $C_{40}H_{44}O_{12}$, -43.8 (c 0.14, $CHCl_3$), was isolated as an amorphous powder. The NMR spectra showed the presence of two acetyl (δ_H 2.26 and 2.30; δ_C 20.89 and 22.63), one benzoyl (δ_H 7.46, 7.60 and 8.07; δ_C 128.67, 129.25, 130.08, 133.74) and one cinnamoyl groups [δ_H 6.51 (1H, *d*, 16.0 Hz), 7.85 (1H, *d*, 16.0 Hz), 7.48 and 7.63, δ_C 166.27, 116.85, 146.82, 128.24, 129.17, 130.97, 133.95]. The presence of an oxetane ring was implied by signals at δ_H 4.19, 4.30 and δ_C 76.51. The ^1H and ^{13}C signals (Tables 1 and 2), which were assigned by using ^1H–^1H COSY, TOCSY, HMQC, and HMBC, closely resembled those of paclitaxel [10] except for the *N*-acylisoserine moiety at C-13. The substituent at C-13 was disclosed to be a cinnamoyl group by the HMBC correlations of H-13/C-1′ and H-2′/C-1′ and the structure to be as shown in Fig. 3. The stereostructure of its skeleton was elucidated by NOE correlations as in Fig. 3 to be the same as in paclitaxel.

Taxuspinanane K (13-dehydro-5,13-deacetyl-2-

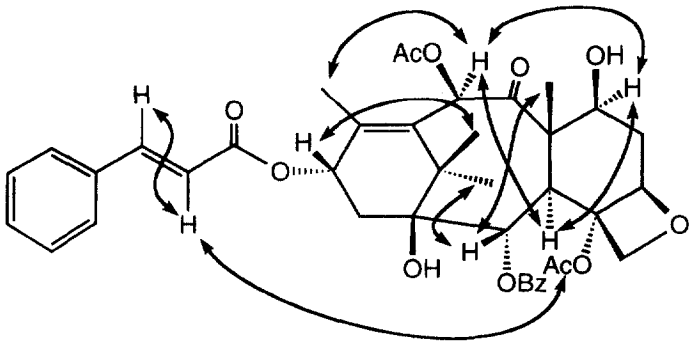

Fig. 3.

deacetoxydecinnamoyltaxinine J: **4**), $C_{26}H_{36}O_8$. $[\alpha]_D$ +95.2° (*c* 0.29, $CHCl_3$), was isolated as an amorphous powder. The presence of a taxane skeleton with three acetyl [δ_H 1.99 (3H, *s*), 2.04 (6H, *s*)] and an exomethylene [δ_H 4.77 and 5.09 (each 1H, *s*); δ_C 111.41] groups was suggested by the ^1H and ^{13}C NMR signals. Further evidence indicating the presence of hydroxyl and α,β-unsaturated ketone was provided by an IR absorption band at 3450 cm^{-1} and diagnostic NMR signals (δ_C 138.59, 151.53, 200.74). The assignments, performed using ^1H–^1H COSY, TOCSY, HMQC and HMBC measurements, are shown in Tables 1 and 2. The acetyl groups were located at C-7, C-9 and C-10 by detection of HMBC correlations between the corresponding proton and the acetyl carbonyl. The relative stereochemistry was confirmed by a phase sensitive NOESY spectrum as shown in Fig. 4.

Taxuspinananes H (**1**)–K (**4**) showed moderate cytotoxic activity against P-388 lymphocytic cells (IC_{50}, **1**: 21 $\mu g\ ml^{-1}$, **2**: 0.17 $\mu g\ ml^{-1}$, **3**: 2.8 $\mu g\ ml^{-1}$, **4**: 82 $\mu g\ ml^{-1}$, paclitaxel: 0.02 $\mu g\ ml^{-1}$). It was found that the introduction of the *N*-methyl substituent of paclitaxel greatly reduced cytotoxicity.

EXPERIMENTAL

General

IR and UV spectra were recorded on JASCO A-302 spectrometer and Hitachi 557 spectrophotometer, respectively. Optical rotation was measured with a JASCO DIP-4 spectrometer and $[\alpha]_D$ values are given in 10^{-1} deg $cm^2\ g^{-1}$. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying with 10% H_2SO_4. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm and 30 mm i.d. × 250 mm, GL Science Inc.) packed with 10 μm ODS. All NMR spectroscopy were carried out on Bruker AM400, AM500, and Varian Unity 400 spectrometer. The spectra were recorded at 300 K in $CDCl_3$. The phase sensitive ROESY and NOESY experiments were acquired with mixing times of 300 and 600 ms, respectively. The values of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 150 ms and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 ms.

Plant material

The stems without leaves of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder. were collected in Saitama, Japan in October 1995. The plant was identified by Dr Zhi-Sheng Qiao, Department of Pharmacognosy, College of Pharmacy, Second Military Medical University, Shanghai, China. A voucher specimen has been deposited in the Herbarium of the Tokyo University of Pharmacy and Life Science.

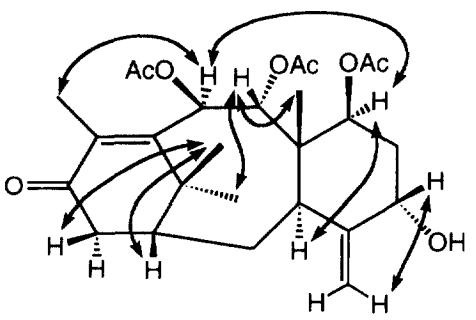

Fig. 4.

Extraction and isolation

The stems of *Taxus cuspidata* Sieb. et. Zucc. var. *nana* Rehder. (20.0 kg) were extracted with hot MeOH 3× to give a MeOH extract which was treated with toluene and H_2O. The toluene soluble fraction (230 g) was subjected to silica gel CC using a $CHCl_3$–MeOH gradient system (1:0–0:1). The fraction which eluted with 10% MeOH was further subjected to silica gel CC using a toluene–EtOAc–MeOH solvent system (12:4:1), followed by ODS HPLC with 70% MeOH, MeOH–CH_3CN–H_2O and CH_3CN–H_2O solvent sys-

tems to give taxuspinanane H (**1**: 11 mg), I (**2**: 15 mg), J (**3**: 1 mg), and K (**4**: 24 mg).

Taxuspinanane H (**1**). Amorphous powder, $[\alpha]_D$ $-42.0°$ (c 0.12, $CHCl_3$). FAB-MS m/z: 603 $[M+Na]^+$, HRFAB-MS (Found: 603.2565, Calcd for $C_{33}H_{40}O_9Na$, requires 603.2570). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3423, 1719, 1638, 1371, 1244, 1170, 1017. UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 279 (4.22), 216 (4.24), 208 (4.23).

Taxuspinanane I (**2**). Amorphous powder, $[\alpha]_D$ $-71.0°$ (c 0.04, $CHCl_3$). FAB-MS m/z: 890 $[M+Na]^+$, HRFAB-MS (Found: 890.3346, Calcd for $C_{48}H_{53}NO_{14}Na$, requires 890.3364). IR ν_{max}^{KBr} cm^{-1}: 3449, 2923, 1720, 1619, 1246. UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 282 (3.22), 272 (3.27), 218 (4.32), 204 (4.41).

Taxuspinanane J (**3**). Amorphous powder, $[\alpha]_D$ $-43.8°$ (c 0.14, $CHCl_3$). FAB-MS m/z: 739 $[M+Na]^+$, HRFAB-MS (Found: 739.2729, Calcd for $C_{40}H_{44}O_{12}Na$, requires 739.2731). IR ν_{max}^{KBr} cm^{-1}: 3424, 2974, 1718, 1631, 1370, 1266, 1178, 938, 717, UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 282 (2.94), 274 (3.02), 232 (4.10), 205 (4.05).

Taxuspinanane K (**4**). Amorphous powder, $[\alpha]_D$ $+95.2°$ (c 0.29, $CHCl_3$). FAB-MS m/z: 499 $[M+Na]^+$, HRFAB-MS (Found: 499.2315, Calcd for $C_{26}H_{36}O_8Na$, requires 499.2322). IR ν_{max}^{KBr} cm^{-1}: 3450, 2985, 1747, 1677, 1245, UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 269 (3.58), 203 (3.64).

Cytotoxic activity on P388 cells

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well plate. The blue formazan produced by the mitochondrial dehydrogenase of viable cells was measured spectrophotometrically. RPMI-1640 medium (100 μl) supplemented with 5% fetal calf serum and 100 μg ml^{-1} of kanamycin and containing mouse P388 leukemia cells (3×10^4 cells ml^{-1}) was added to each well. After overnight incubation (37°, 5% CO_2), 100, 30, 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 μg ml^{-1} of sample solutions were added to the wells and the plates were incubated for 48 h. Then, 20 μl of MTT was added to each well and the plates were incubated for 4 h. The resulting formazan was dissolved in 100 μl of 10% SDS (sodium dodecyl sulfate) containing 0.01 N HCl. Each well was mixed gently with a pipette for 1 or 2 min and the plate was read on a microplate reader (Tosoh MPR-A4i) at 540 nm. The IC_{50} (μg ml^{-1}) value was defined as the concentration of sample which achieved 50% reduction of viable cells with respect to the control.

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