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Isolation and Identification of a Cytotoxic Principle from *Chrysosplenium grayanum* Maxim. (Saxifragaceae) and Its Antitumor Activities[1)]

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A cytotoxic principle was newly isolated from *Chrysosplenium grayanum* Maxim. (Saxifragaceae) and identified as β-peltoboykinolic acid (1) on the basis of spectral data. Cytotoxicity of compound 1 was tested against various human cancer cell lines *in vitro*, and antitumor effect of this compound was demonstrated on Meth·A mouse fibrosarcoma. The experiment of combined treatment with compound 1, mitomycin C, and OK-432 resulted in enhancing the antitumor effect against B16-BL6 mouse melanoma in C57BL/6 mice.

Keywords β-peltoboykinolic acid; hydroxyoleanen-27-oic acid; *Chrysosplenium grayanum*; cytotoxicity; antitumor effect; B16-BL6 mouse melanoma; Meth·A mouse fibrosarcoma; combination therapy

Introduction

As part of a study on cytotoxic constituents from natural resources, we previously isolated some flavonols from *Chrysosplenium grayanum* Maxim. (Saxifragaceae) and tested for their cytotoxic activities against KB cells.[2)] However, the activities and yields of the flavonols were not significant enough to explain the cytotoxicity of the MeOH extract of the plant.

In this work, we have isolated other cytotoxic constituents from the same plant and evaluated their cytotoxicity. One newly isolated constituent was identified as β-peltoboykinolic acid (compound **1**) by chemical and spectral analysis, which has activity sufficient to explain the cytotoxicity of the MeOH extract. Then, compound **1** and its derivatives were examined for growth-inhibiting activities against various human cancer cell lines *in vitro*, and antitumor effect on mouse Meth·A fibrosarcoma *in vivo*. The effect of combined use of compound **1** in chemoimmunotherapy with mitomycin C (MMC) and OK-432 was also examined on B16-BL6 mouse melanoma.

Results and Discussion

Isolation and Identification of Compound 1 The MeOH extract of the whole plants was prepared as described previously.[2)] The 1—2%MeOH/$CHCl_3$ eluent from the silica gel column was further separated on a silica gel column by elution with an EtOAc/*n*-hexane mixture. The 25% EtOAc/*n*-hexane eluent afforded the constituent designated as compound **1**.

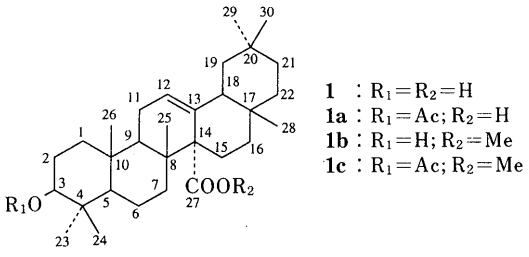
The molecular formula of compound **1** was determined to be $C_{30}H_{48}O_3$ by high resolution electron impact mass (HREI-MS) analysis, and **1** gave a positive Liebermann–Burchard (LB) reaction. Prominent peaks at *m/z* 248 and 208 due to a retro-Diels–Alder fragmentation for an oleanen skeleton[3,4)] were observed in its EI-MS spectrum. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, seven singlet methyl signals, a methine proton signal attributed to 3α-H, a hydroxy proton signal (which disappeared with D_2O) and an olefin proton signal attributed to 12-H were observed. Acetylation of compound **1** gave monoacetate (**1a**), EI-MS *m/z* 498 (M^+), mp 227—230 °C, and methylation with diazomethane afforded methyl ester (**1b**), EI-MS *m/z* 470 (M^+), mp 129—133 °C.

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of compound **1** showed seven methyl signals, ten methylene signals, five methine signals including an oxymethine carbon and an olefin carbon, and eight singlet carbon signals including an olefin carbon and a carbonyl carbon. In the comparison of the ^{13}C-NMR spectrum of **1b** with oleanolic acid methyl ester,[5)] the signals of C-14 and C-17 on **1b** were respectively shifted by −14.2 and +13.6 ppm. Accordingly, compound **1** was assumed to be a 27-oic acid.

Based on these findings, compound **1** was thought to be 3β-hydroxyolean-12-en-27-oic acid, that is, β-peltoboykinolic acid (**1**) (Fig. 1), and was identified by comparison with published data.[6,7)]

Antitumor Activities of Compound 1 and Its Derivatives. a) Growth Inhibitory Effect *in Vitro* Compound **1** and its chemically modified derivatives, **1a**, **1b**, and acetoxy methyl ester (**1c**), were tested for their cytotoxic activity against KB cells *in vitro*. Although **1b** and **1c** were inactive, **1a** was active as well as original compound **1**. (see Table I.)

Then, compound **1** was further tested for growth inhibiting activities *in vitro* against various human cultured cell lines: 8 stomach cancers, 6 lung cancers, 3 osteo-



1 : $R_1=R_2=H$
1a : $R_1=Ac$; $R_2=H$
1b : $R_1=H$; $R_2=Me$
1c : $R_1=Ac$; $R_2=Me$

Fig. 1

TABLE I. Growth Inhibition by Compound **1** and Its Derivatives against KB Cells *in Vitro*

Compound	1	1a	1b	1c
IC_{50} (μg/ml)	3.06±0.20[a)]	2.40±0.23[a)]	>25	>10

Each experiment was replicated 3 times. *a)* Mean±S.E.

Table II. Growth Inhibitory Effects of Compound **1** on Various Human Cancer Cells *in Vitro*

Origin	Cell line	IC_{50} (μg/ml)
Stomach adenocarcinoma	ST-KM	2.8
	Nakajima	6.9
	KATO-III	2.2
	NKPS	3.8
	KKLS	2.6
	NUGC-4	10.0
	MKN-45	5.1
	STSA-1	4.2
Lung adenocarcinoma	PC-3	3.9
	PC-8	2.9
	PC-9	4.8
	PC-13	3.9
Lung epidermoid carcinoma	QG-56	5.5
	QG-95	3.9
Osteosarcoma	KHOS-321H	8.8
	MG-63	3.2
	OST	4.0
Colon adenocarcinoma	SW-480	3.5
	Colo-320DM	4.1
Bladder carcinoma	KK-47	2.5
Melanoma	A 375	3.7
Fibrosarcoma	HT-1080	>10
Larynx carcinoma	Hep-2	>10
Normal amnion	FL	>10

Table III. Effect of Compound **1** on Meth·A Mouse Fibrosarcoma (Solid Form) *in Vivo*

Chemical	Daily dose (mg/kg, i.p.)	Tumor size	
		Mean±S.E. (mm^3)	*T/C* (%)
None (PBS)	—	2634±276	—
Compound **1**	28	1462±337	55.5
	84	920±305	34.9

Male 7-week-old BALB/cA mice ($n=5$) were implanted with 1×10^6 Meth·A cells s.c. (midpoint of back at left). Compound **1** suspended in PBS was administered for 5 consecutive days from 5 after tumor implantation, and tumor size was measured on day 18.

sarcomas, 2 colon cancers, 1 melanoma, 1 fibrosarcoma, 1 larynx carcinoma, and 1 bladder cancer.

As shown in Table II, this compound was found to strongly inhibit the growth of several cancer cells: 4 stomach cancers (ST-KM, KATO-III, NKPS, KKLS), 4 lung cancers (PC-3, PC-8, PC-13, QG-95), 2 osteosarcomas (MG-63, OST) and others.

b) Antitumor Effect of Compound 1 *in Vivo* The antitumor effect of compound **1** against Meth·A mouse fibrosarcoma is summarized in Table III. The compound was effective in inhibiting the growth of subcutaneous solid tumors at a daily dose of 84 mg/kg, without decrease of body weight or diarrhea in the treated mice.

Many kinds of conventional anticancer drugs have been used in clinical treatment, however, it is well known that cancer cells have gradually developed the resistance to these drugs. The increase and fixing of drug-resistant cancer cells are a very challenging problem in the field of cancer chemotherapy. To resolve it, various combinations of anticancer drugs with different action mechanisms have been applied to the treatment of resistant cancer cells. Control of malignant cell proliferation by biological response modifiers including differentiation inducers has also attempted with some success *in vitro* and *in vivo*. It was recently demonstrated that pigment production in cultured B16-BL6 melanoma cells was elevated in the presence of compound **1** (unpublished data). The experiment of combination therapy with MMC as a chemotherapeutic agent, compound **1** as a differentiation-inducing agent[8,9] and OK-432 as an immunotherapeutic agent was thus undertaken. Table IV shows the results indicating the effectiveness of this combination treatment.

Table IV. Antitumor Effect of Combined Treatment with Compound **1**, MMC and OK-432 on B16-BL6 Mouse Melanoma *in Vivo*

Group	Treatment	Tumor size[a]	
		Mean±S.E. (mm^3)	*T/C* (%)
I	None	6613±1312	—
II	MMC+OK-432	3641± 525	55.0
III	MMC+OK-432+compound **1**	2103± 461	31.8[b]

a) Measured on day 23 ($n=6$). *b*) Group II *vs.* group III; $p<0.05$ (Student's *t*-test).

Experimental

General Procedures for Chemical and Spectral Analysis All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectrum was recorded on a Hitachi 260-10 IR spectrometer with polystyrene calibration at 1601 cm^{-1}. Specific rotation was determined on a JASCO DIP-400 digital polarimeter. ^1H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 270 spectrometer at 270 and 67.9 MHz, respectively, with tetramethylsilane as an internal standard. The chemical shifts are recorded in δ (ppm) values. Multiplicity of the ^{13}C-NMR data was determined by distorsionless enhancement by polarization transfer (DEPT) method. EI-MS were obtained on a JEOL JMS-D-200 mass spectrometer operating at 70 eV.

Plant Materials The collection and identification of *Chrysosplenium grayanum* (Saxifragaceae) have been described previously.[2]

Extraction and Isolation The $CHCl_3$-soluble fraction of the MeOH extract, which was prepared from fresh whole plants of *C. grayanum*, was previously partitioned and separated.[2] The 1—2% MeOH/$CHCl_3$ eluent from one silica gel column was further separated on another silica gel column by elution with an EtOAc/*n*-hexane mixture. The 25% EtOAc/*n*-hexane eluent afforded colorless needles (**1**), 103 mg (2.45×10^{-3}% from fresh material).

Identification of Compound 1 Colorless needles, mp 249—252 °C, $[\alpha]_D^{20}$ +105.5° ($c=1.0$), positive to LB reaction. HREI-MS measurement *m/z*: 456.3606 ($C_{30}H_{48}O_3$ requires 456.3601). *Anal.* Calcd for $C_{30}H_{48}O_3$: C, 78.89; H, 10.60. Found: 79.03; H, 10.42. IR ν_{max}^{KBr} cm^{-1}: 3470, 2940, 1680, 1620, 1380, 1220, 1210, 1040. EI-MS *m/z*: 456 (M^+), 438, 412, 410, 397, 395, 258, 248, 208, 207, 203, 190. ^1H- and ^{13}C-NMR: identical with the published values.[6,7] Compound **1** was identified as β-peltoboykinolic acid.

Acetylation of Compound 1 Compound **1** was reacted with pyridine and Ac_2O at room temperature overnight. The reaction mixture was treated in the usual way to afford monoacetate (**1a**), colorless needles, mp 227—230 °C. EI-MS *m/z*: 498 (M^+). ^1H- and ^{13}C-NMR: identical with the published values for acetyl β-peltoboykinolic acid.[6,7]

Methylation of Compound 1 To an Et_2O solution of compound **1**, Et_2O solution of diazomethane was added and the reaction mixture was treated in the usual way to afford methyl ester (**1b**), colorless needles, mp 129—133 °C. EI-MS *m/z*: 470 (M^+). ^1H- and ^{13}C-NMR: identical with the published values for β-peltoboykinolic acid methyl ester.[6,7]

Methylation of Compound 1-Acetate Compound **1a** was reacted as described for compound **1** to afford **1c** as colorless needles, mp 205—209 °C. EI-MS *m/z*: 512 (M^+). ^1H- and ^{13}C-NMR: identical with the published values for acetyl β-peltoboykinolic acid methyl ester.[6]

Animals Male 7-week-old BALB/cA mice and C57BL/6 mice which were purchased from Clea Japan Co., Ltd., Tokyo were used. The mice were kept under specific pathogen-free conditions.

Tumors Mouse B16-BL6 melanoma was maintained by subcutaneous passage in C57BL/6 mice, and mouse Meth·A fibrosarcoma was

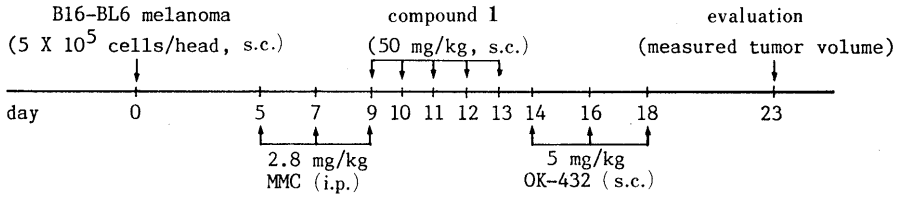


Fig. 2. Treatment Schedule for Combined Use of MMC, Compound **1** and OK-432 *in Vivo*

maintained by intra-peritoneal passage in BALB/cA mice in our laboratories. Twenty-four human cancer cell lines were used in this study. The lung adenocarcinomas, PC-3, 8, 9, and 13 were originally established by Prof. Y. Hayata (Tokyo Medical College, Tokyo, Japan). Lung epidermoid carcinomas, QG-56 and QG-95, and stomach adenocarcinomas, NUGC-4 and MKN-45, were supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan). The stomach adenocarcinoma, Nakajima, was kindly donated by Dr. S. Yanoma (Kanagawa Cancer Center, Yokohama, Japan). An osteosarcoma, OST, was donated by the Department of Orthopedic Surgery, Kanazawa University (Kanazawa, Japan). Other cell lines were supplied by the American Type Culture Collection (Rockville, MD, U.S.A.).

Drugs MMC (Kyowa Hakko Kogyo Co., Ltd., Tokyo) was purchased. OK-432 was generously supplied by Chugai Pharmaceutical Company.

Assay for Cytotoxic Activity *in Vitro* The assays employing human cancer cell lines were carried out as previously described.[10, 11] In brief: The human cancer cells were maintained on MEM media supplemented with 5% fetal calf serum. The assays were performed on tissue culture plates with flat-bottomed wells in a CO_2 incubator at 37 °C. After the incubation, MTT reagent [3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well. After another 4 h of incubation at 37 °C, dimethylsulfoxide (DMSO) was added to solubilize the resulting MTT-formazan, and then each absorbance was measured. The percentage of cell growth inhibition was calculated in the usual way, and IC_{50} values were determined graphically from the dose–response curve.

Antitumor Effect of Compound 1 *in Vivo* Meth·A cells (1×10^6 cells/head) were implanted subcutaneously (s.c.) into the left side of the midpoint of the back of BALB/cA mice. Five days after the implantation, compound **1** suspended in phosphate-buffered saline (PBS) at a given dose was administered intraperitoneally (i.p.), once a day for 5 consecutive days. The experiment was terminated on the 18th day after the tumor implantation, and the tumor size was measured.

Combination Treatment with MMC, Compound 1, and OK-432 B16-BL6 cells (5×10^5 cells/head) were implanted s.c. into the left side of the midpoint of the back of C57BL/6 mice. MMC (2.8 mg/kg) was administered 3 times i.p. once a day from day 5 to day 9 on alternate days. Then, compound **1** (50 mg/kg) was administered s.c. once a day from day 9 to day 13, consecutively. OK-432 (5 mg/kg) was administered 3 times s.c., once a day from day 14 to day 18 on alternate days. The tumor size was measured at day 23 and treated/control (T/C) ratio calculated (%) (see Fig. 2).

Evaluation of Antitumor Effect The antitumor effect was evaluated by comparing the mean tumor volume (T_v) of the mice in each group. T_v was calculated for an ellipsoid using the formula $T_v = (L \times W^2)/2$, where L and W are the data (in mm) on length and width, respectively. The criteria for effectiveness include a T/C ratio (%).

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References and Notes

1) A part of this work was presented at the 37th Annual Meeting of Japanese Society of Pharmacognosy, Chiba, November 1990.
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