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5 α ,8 α -Epidioxy-22 E -ergosta-6,9(11),22-trien-3 β -ol from an Edible Mushroom Suppresses Growth of HL60 Leukemia and HT29 Colon Adenocarcinoma Cells

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We purified a sterol with antitumor activity from the edible mushroom *Sarcodon aspratus* (BERK.) S. ITO and identified it as 5 α ,8 α -epidioxy-22 E -ergosta-6,9(11),22-trien-3 β -ol (9,11-dehydroergosterol peroxide (9(11)-DHEP)). Purified 9(11)-DHEP was a more effective inhibitor of HL60 leukemia cell growth and stronger apoptosis-inducer than 5 α ,8 α -epidioxy-22 E -ergosta-6,22-dien-3 β -ol (ergosterol peroxide (EP)) that we had previously identified as an apoptosis inducer. Moreover, 9(11)-DHEP selectively suppressed the growth of HT29 human colon adenocarcinoma cells but not WI38 normal human fibroblasts. After 5 d incubation of HT29 with 7 μ M 9(11)-DHEP, the number of S phase cells decreased from 23 to 15% of total diploid cells and 17% became hypodiploid. Expression of the cyclin-dependent kinase inhibitor 1A (p21, WAF1, Cip1) (CDKN1A), which has been shown to cause cell cycle arrest and apoptosis in HT29 cells, was induced by 9(11)-DHEP. These results suggest that 9(11)-DHEP inhibits HT29 cell growth by inducing CDKN1A expression, thus causing cell cycle arrest and apoptosis.

Key words 9,11-dehydroergosterol peroxide; *Sarcodon aspratus*; HT29 cells; proliferation; cyclin-dependent kinase inhibitor 1A (p21, WAF1, Cip1)

Mushrooms are widely consumed not only as foods but also as alternative medicines. The fungus-specific constituents of mushrooms are thought to be effective against cancer and other lifestyle-related diseases. Mushroom polysaccharides belonging to the β -glucans possess the antitumor and immunomodulating actions *in vivo*, and some clinical trials have been carried out with these agents.^{1,2)} Steroids are abundantly present in mushrooms^{3,4)} and were reported to have diverse physiological functions, such as anti-oxidant effects, anti-inflammatory activity and inhibitory effects on cancer cell growth.^{5–7)} One major mushroom steroid is the provitamin D₂ ergosterol. Vitamin D₂ was shown to contribute to the prevention of prostate and colon cancer.^{8,9)} Peroxide of ergosterol, 5 α ,8 α -epidioxy-22 E -ergosta-6,22-dien-3 β -ol (**1**) is also widely distributed in mushrooms such as *Hypsizygus marmoreus*, *Pleurotus eringii* and *Ganoderma lipsiense*.^{4,6,7,10,11)} Ergosterol peroxide (EP) inhibits the growth of some cancer cells and decreases lipid peroxidation of rat liver microsomes.^{7,10)} These constituents may decrease the risk of cancer and other lifestyle diseases through dietary intake of mushrooms.

In our previous study, the suppressive effects of extracts of Japanese edible mushrooms were screened on HL60 leukemia cell growth, and an apoptosis-inducing property of

Sarcodon aspratus (BERK.) S. ITO (*S. aspratus*) was found.¹²⁾ We have isolated EP as a major apoptosis inducing component from the *S. aspratus* acetone extract.¹²⁾ However, EP was not the only apoptosis inducing component in the active fraction. In the present study, we purified another antitumor sterol from *S. aspratus* using preparative HPLC and subsequently identified it as 5 α ,8 α -epidioxy-22 E -ergosta-6,9(11),22-trien-3 β -ol (9,11-dehydroergosterol peroxide (9(11)-DHEP)) (**2**). The effects of this compound on apoptosis in HL60 cells and growth of HT29 human colon adenocarcinoma cells and WI38 normal embryonic human fibroblasts were investigated.

MATERIALS AND METHODS

Instruments Measurements of ¹H-NMR spectra, including two-dimensional NMR experiments, *i.e.* double quantum filtered-correlation spectroscopy (DQF-COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC), were performed on an Avance 800 spectrometer (Bruker, Karlsruhe, Germany) at 800.30 MHz at 298 K. ¹³C-NMR spectra including DEPT were recorded on a DRX 600 (Bruker) or Avance 500 (Bruker) spectrometers at 150.92 or 125.77 MHz at 298 K. Tetramethylsilane (TMS) was used as the standard for chemical shift calculations. Mass spectral measurement was performed on a JMS-HX/HX-110A (JEOL, Tokyo Japan) mass spectrometer by electron ionization (EI).

Material *Sarcodon aspratus* (BERK.) S. ITO grown in the South Aizu area, Fukushima, Japan was purchased from a local market as fresh or air-dried foodstuffs.

Isolation of Ergosterol Peroxide and 9,11-Dehydroergosterol Peroxide from *S. aspratus* Dried fruiting bodies of *S. aspratus* (20 g) were extracted with acetone. The ace-

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tone extract was subjected to solid phase extraction using an OASIS HLB column (20 ml cartridge (1 g), Waters, Milford, MA, U.S.A.), and the fraction eluted with 60–80% ethanol was further fractionated by silica gel column chromatography as previously described.¹²⁾ An active fraction, eluted with *n*-hexane/ethyl acetate 3 : 1, was then applied to a preparative HPLC column (Inertsil ODS-3 column, 250 mm×20 mm i.d., 3 μ m, GL Sciences Inc., Tokyo, Japan) and eluted with 97% methanol/3% H₂O (v/v) at a flow rate of 6 ml/min. The constituents were detected at 218 nm using a UV/VIS detector (UV-970, JASCO Co., Tokyo, Japan). EP and 9(11)-DHEP were eluted at a retention time of 38.3 and 32.7 min, respectively. The purities of the isolated sterols were determined by analytical HPLC using a YMC-Pack Pro C18 RS column (250 mm×4.6 mm i.d., 5 μ m, YMC Co., Ltd. Kyoto, Japan). Fresh fruiting bodies of *S. aspratus* (290 g) were lyophilized and milled using a vibration mill. The powdered *S. aspratus* (39 g) was extracted with acetone, and then EP and 9(11)-DHEP were isolated as described above.

Cell Lines and Culture HL60 human promyelocytic leukemia cells (JCRB0085) were provided by the Human Science Research Resources Bank (Osaka, Japan). HT29 human colorectal adenocarcinoma cells (ATCC HTB38) were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). WI38 human embryonic lung fibroblasts (RCB702) were provided by the RIKEN Cell Bank (Tsukuba, Japan). HL60 was maintained in RPMI1640 medium (Invitrogen Corp., Carlsbad, CA, U.S.A.). HT29 was grown in McCoy 5A medium (Invitrogen) and WI38 was maintained in MEM (Invitrogen). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air in medium supplemented with 10% heat-inactivated fetal calf serum (ICN Biomedicals, Inc., OH, U.S.A.). To analyze nuclear morphology, cells were stained with 1 mM bisbenzimidazole (Hoechst) 33258 in PBS.

Measurement of Effects of the Sterol Peroxides on Cell Viability EP and 9(11)-DHEP were dissolved in dimethyl sulfoxide and added to the cell culture media. HL60 cells (2×10⁴ cells/ml) were incubated with sterols for 24 h. The cells were then harvested, and viability was determined using WST-1 reagent (Cell Counting Kit, Dojin, Osaka, Japan). HT29 and WI38 cells (1×10⁴ cell/ml) were incubated for 24 h to allow adherence to the plate. The cells were then treated with 9(11)-DHEP for 5 d, and cell viability was determined using WST-1 reagent. The numbers of viable control cells were counted with a hemocytometer by trypan blue exclusion. Data are expressed as arithmetic mean±standard deviation (S.D.) of triplicate determinations. Statistical analysis was performed using Student's *t* test.

Cell Cycle Analysis HT29 cells were treated with 9(11)-DHEP for 5 d. The cells were then harvested, stained with propidium iodide using a Cycle Test Plus DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.), and analyzed by flow cytometry (FACS Sort, Becton Dickinson Immunocytometry Systems) with ModFit LT Ver. 2.0 (Verity Software House, Topsham, ME, U.S.A.) software. The distribution of DNA content is expressed as a percentage of total diploid cells. The proportion of hypodiploid cells is expressed as a percentage of the total cell population.

Real-Time PCR (RT-PCR) Analysis Total RNA was extracted using TRIzol reagent and reverse transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems,

Foster City, CA, U.S.A.). Quantitative Real-time PCR (RT-PCR) was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's specifications. The TaqMan primers and probes for CDKN1A (assay identification number Hs00355782_m1) were TaqMan Gene Expression Assay products (Applied Biosystems). The human GAPDH gene was used as an endogenous control (Human-GAPDH MGB, Applied Biosystems). The thermal cycler conditions were as follows: 2 min at 50 °C and then 10 min at 95 °C, followed by two-step PCR for 40 cycles consisting of 95 °C for 15 s followed by 60 °C for 1 min. All samples were performed in triplicate. The results are expressed relative to the GAPDH internal control.

RESULTS

Purification and Identification of Ergosterol Peroxide and 9,11-Dehydroergosterol Peroxide We obtained a fraction mediating apoptosis-induction in HL60 leukemia cells from *S. aspratus* acetone extract by solid phase extraction on an OASIS HLB column and silica gel column chromatography as previously described.¹²⁾ The active fraction, which eluted with *n*-hexane/ethyl acetate 3 : 1, was applied to a C18 column for preparative HPLC and eluted with 97% methanol. An apoptosis inducing component was separated from EP; the purities of these isolated active components measured by analytical HPLC were 98–100%.

The mass spectrum of the newly isolated compound could not be obtained by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) nor FAB other than EI. The molecular ion was observed at *m/z*: 426 ((M)⁺, 42%) accompanied by fragment ions at *m/z*: 408 (M–H₂O)⁺ (27%), 394 (M–O₂)⁺ (43%), 376 (M–O₂–H₂O)⁺ (27%), 299 (29%), 251 (41%) and 249 (28%). The pattern of the mass spectrum was similar to that of EP (428 (M)⁺ (52%), 410 (M–H₂O)⁺ (45%), 396 (M–O₂)⁺ (82%), 392 (82%), 376 (96%), 374 (88%), 266 (54%), 251 (68%) and 249 (99%)) but the molecular weight of the compound was lower by 2 mass units. The results suggested the presence of an additional double bond in the molecule. NMR spectra of the component were compared with those of EP (Table 1). As for EP we assigned C5 and C10 based on HMBC from H19, and C11 based on HMBC from H9 and H12, resulting in a minor difference compared to reported assignments.^{7,13,14)} The second component exhibited a proton signal at 5.49 ppm in addition to coupled H6–H7 and H22–H23 pairs in the lower field region, which also suggested the presence of an additional double bond (Table 1). Coupling of this signal with H12 and the lower field shift of C9 to 144.0 ppm, assignment of which was confirmed by HMBC from H7, H12 α , H12 β , and H19, revealed that the position of the extra double bond was at C9–C11. The second antitumor component in the *S. aspratus* extract was thus identified as 5 α ,8 α -epidioxy-22 E -ergosta-6,9(11),22-trien-3 β -ol, (9,11-dehydroergosterol peroxide (9(11)-DHEP)) (2). The yields of EP and 9(11)-DHEP were ca. 11 mg and 6 mg, respectively, from 20 g of *S. aspratus* dried fruiting bodies.

The fruiting bodies of *S. aspratus* are usually air-dried before cooking to strengthen the typical smell and for long

Table 1. ^1H and ^{13}C Spectral Data of Ergosterol Peroxides

Position	1 (in CDCl_3)			2 (in pyridine- d_6)		
	^1H (δ , multiplicity, J (Hz))		^{13}C (δ)	^1H (δ , multiplicity, J (Hz))		^{13}C (δ)
1	1.70	ddd, 13.5, 3.4, 3.4	34.7	1.77	ddd, 13.3, 3.0, 3.0	33.3
	1.95	m		2.40	ddd, 14.1, 13.3, 3.5	
2	1.54	m	30.1	1.89	m	31.7
	1.84	m		2.16	m	
3	3.97	m	66.5	4.42	m	65.7
4	1.91	dd, 13.7, 11.8	37.0	2.29	dd, 13.6, 11.8	37.4
	2.11	ddd, 13.7, 5.0, 2.0		2.46	ddd, 13.6, 3.5, 0.4	
5			82.2			83.0
6	6.24	d, 8.5	135.4	6.40	d, 8.4	136.4
7	6.51	d, 8.5	130.8	6.70	d, 8.4	131.0
8			79.4			78.4
9	1.50	m	51.1			144.0
10			37.0			38.6
11	1.23	m	23.4	5.49	dd, 6.1, 1.8	119.2
	1.53	m				
12	1.23	m	39.4	2.02	m	41.4
	1.96	m		2.20	dd, 17.0, 6.1	
13			44.6			43.8
14	1.56	m	51.7	2.01	m	48.7
15	1.41	m	20.6	1.34	m	29.0
	1.60	m		1.7	m	
16	1.35	m	28.7	1.7	m	21.3
	1.75	m		1.7	m	
17	1.22	m	56.2	1.27	m	55.9
18	0.82	s	12.9	0.76	s	13.1
19	0.89	s	18.2	1.17	s	25.7
20	2.02	ddq, 9.9, 8.6, 6.6	39.7	2.01	m	40.2
21	1.00	d, 6.6	20.9	1.00	d, 6.6	20.9
22	5.14	ddd, 15.2, 8.6, 0.7	135.2	5.16	dd, 15.2, 8.9	136.0
23	5.22	dd, 15.2, 7.8	132.3	5.26	dd, 15.2, 7.8	132.4
24	1.85	ddq, 13.5, 7.8, 6.8	42.8	1.88	m	43.0
25	1.47	dq, 13.5, 6.8	33.1	1.46	dq, 13.1, 6.6	33.3
26 or 27	0.82	d, 6.8	19.7	0.86	d, 6.8	19.8
26 or 27	0.83	d, 6.8	20.0	0.85	d, 6.8	20.1
28	0.91	d, 6.8	17.6	0.95	d, 6.8	17.8

term preservation. Thus, EP and 9(11)-DHEP could theoretically have been produced from ergosterol and 9,11-dehydroergosterol during the air-drying process. To confirm that these peroxides were already present in the fresh fruiting bodies, we isolated EP and 9(11)-DHEP from fresh fruiting bodies of *S. aspratus*, which were lyophilized and extracted with acetone. EP and 9(11)-DHEP were both purified from the acetone extract by solid phase extraction using an OASIS HLB column, silica gel column chromatography and preparative HPLC as for the air-dried samples. The yields of EP and 9(11)-DHEP were *ca.* 5.3 mg and 0.7 mg, respectively, from 39 g lyophilized *S. aspratus*.

Inhibition of Growth and Induction of Apoptosis of HL60 Human Leukemia Cells by 9,11-Dehydroergosterol Peroxide Purified ergosterol, EP or 9(11)-DHEP (>98% pure by HPLC) was added to the medium for HL60 cell culture (2×10^4 cells/ml). After 24 h incubation, 9(11)-DHEP at a concentration of $6 \mu\text{M}$ had reduced cell viability to 25% of control (Fig. 1A). The growth inhibitory effect of 9(11)-DHEP was greater than that of EP (Fig. 1A). Ergosterol did not affect cell viability at concentrations up to $25 \mu\text{M}$. Next, to determine the apoptosis inducing effect of 9(11)-DHEP, HL60 cells (2×10^5 cells/ml) were treated with 9(11)-DHEP for 24 h and then stained with Hoechst33258. Apoptosis was

found to be induced in most of the cells treated with $7 \mu\text{M}$ 9(11)-DHEP for 24 h (Fig. 1B).

Suppression of HT29 Human Colon Adenocarcinoma Cell Growth by 9,11-Dehydroergosterol Peroxide Next, we examined the effect of 9(11)-DHEP on the growth of HT29 human colon adenocarcinoma cells. We treated these cells as well as WI38 human fibroblasts with 9(11)-DHEP and determined cell viability after 5 d. 9(11)-DHEP significantly reduced the viability of HT29 but not WI38 cells at concentrations of 7–12 μM (Fig. 2A). During this time, control HT29 cells seeded at 1×10^4 cells/ml had increased to $6.3 \pm 1.0 \times 10^5$ cells/ml. The effect of 9(11)-DHEP was not cytotoxic but cytostatic for HT29 cells. Control WI38 cells expanded to $3.2 \pm 1.5 \times 10^4$ cell/ml over this time period. Cell cycle analysis showed that $7 \mu\text{M}$ 9(11)-DHEP decreased the number of S phase cells and increased the number of cells in G1/G0 after 3–5 d (Fig. 2B). 9(11)-DHEP decreased the number of S phase cells from 23 to 15% of total diploid cells and induced 17% hypodiploid cells after 5 d incubation (Fig. 2B). We examined the effect of 9(11)-DHEP on the expression of cyclin-dependent kinase inhibitor 1A (p21, WAF1, Cip1) (CDKN1A), which is known to cause cell cycle arrest and to induce apoptosis in HT29 cells. We found that 9(11)-DHEP induced CDKN1A mRNA expression in HT29 cells

Fig. 1. 9,11-Dehydroergosterol Peroxide Isolated from *S. aspratus* Inhibits Growth and Induces Apoptosis of HL60 Cells

(A) Effect of EP and 9(11)-DHEP on the growth of HL60 cells. Cells (2×10^4 cells/ml) were incubated with ergosterol (●), EP (▲) or 9(11)-DHEP (■) for 24 h. The cell viability was quantified using WST-1 reagent (Cell Counting Kit, Dojin). Each value is the mean $\pm$ S.D. of triplicate cultures. * $p < 0.01$ (two-sided), significantly different from the group treated with the same dose of EP by Student's *t* test. (B) Induction of nuclear fragmentation in HL60 cells. Cells (2×10^5 cells/ml) were untreated or treated with $7 \mu\text{M}$ 9(11)-DHEP for 24 h and then stained with Hoechst 33258. a, control cells; b, cells treated with 9(11)-DHEP.

at a concentration of $7 \mu\text{M}$ after 3 d incubation (Fig. 3).

DISCUSSION

We isolated an apoptosis inducing component from *S. aspratus* and identified it as 9(11)-DHEP. This sterol possesses one more double bond than EP, which was previously identified as a major apoptosis inducer in *S. aspratus* extract. 9(11)-DHEP was found in several edible mushrooms and fungi used in medicine.^{15–20} EP was reported to be synthesized by biological oxidation of ergosterol²¹ while it can also be formed by photo-oxidation with singlet oxygen.²² The yields of these peroxides from fresh and dried mushroom samples could not be compared directly in this study due to different lots. However, isolation of a significant amount of the peroxides from the fresh sample strongly suggested enzymatic formation of these peroxides in the fresh fruiting bodies. The amount of peroxides in the mushroom may increase during the air-drying process.

In this paper, we first show that 9(11)-DHEP inhibits the growth of HL60 leukemia cells through the induction of apoptosis (Figs. 1A, B). The growth inhibitory effect of 9(11)-DHEP was stronger than that of EP. Therefore we further examined the anti-cancer effects of 9(11)-DHEP.

It has been reported that most of orally administered ergosterol is excreted in the feces and only a small fraction absorbed through the intestine in rats.²³ Therefore, orally consumed ergosterol derivative 9(11)-DHEP would most likely impact on colon cancer, which is known to be related to diet. Therefore, we investigated the effect of 9(11)-DHEP on the growth of HT29 human colon adenocarcinoma cells. Although it did not affect the growth of these cells within the

Fig. 2. 9,11-Dehydroergosterol Peroxide Inhibits the Growth and Increases the Number of Hypodiploid Cells in the HT29 Human Colon Carcinoma Cell Lines

(A) Effect of 9(11)-DHEP on the growth of HT29 cells and WI38 human lung fibroblasts. Cells (1×10^4 cells/ml) were incubated for 24 h and then treated with 9(11)-DHEP for 5 d. Cell viability was determined using WST-1 reagent (Cell Counting Kit, Dojin). Each value is the mean $\pm$ S.D. of triplicate cultures. * $p < 0.01$ (two-sided), significantly different from the untreated group ($0 \mu\text{M}$) by Student's *t* test. (B) Effect of 9(11)-DHEP on the cell cycle and DNA content of HT29 cells. Cells (1×10^4 cells/ml) were incubated for 24 h and then treated with 9(11)-DHEP for 2, 3 or 5 d. The cell cycle (■, G1/G0; ■, G2/M; □, S) and the ratio of hypodiploid cells (▨) was determined using Cycle Test Plus DNA Reagent Kit (Becton Dickinson Immunocytometry Systems). The distribution of DNA content is expressed as a percentage of total diploid cells and the hypodiploid cells as a percentage of the total cell population.

first 24 h (data not shown), a cytostatic effect was observed after longer periods in the presence of $7 \mu\text{M}$ of this agent (Fig. 2). In contrast, at concentrations up to $12 \mu\text{M}$, 9(11)-DHEP did not significantly inhibit growth of WI38 normal embryonic human fibroblasts. Exposure to $7 \mu\text{M}$ 9(11)-DHEP reduced the proportion of S phase cells and increased the number of hypodiploid cells in the HT29 colon adenocarcinoma cell line. Induction of CDKN1A has been reported to cause cell cycle arrest and apoptosis in HT29 colon cancer cells.^{24,25} Here, we found that 9(11)-DHEP induced CDKN1A expression in HT29 cells but not in WI38 fibroblasts (Fig. 3). Dietary antitumor flavonoids have been shown

Fig. 3. 9,11-Dehydroergosterol Peroxide Induces CDKN1A Gene Expression in HT29 Human Colon Carcinoma Cells but Not in WI38 Human Fibroblasts

Cells (1×10^4 cells/ml) were incubated with 7 or 12 μ M ergosterol peroxide for 3 d. RT-PCR was then performed and CDKN1A expression levels normalized to GPDH and plotted relative to those of control cells. Values shown are the means $\pm$ S.D. of triplicate cultures.

to induce apoptosis by elevating levels of intracellular reactive oxygen species (ROS) with subsequent induction of CDKN1A.²⁶⁾ We determined intracellular ROS using the ROS-sensitive fluorescent dye, CM-H₂DCFDA, but failed to detect accumulation of ROS in HT29 cells treated with 2 or 7 μ M 9(11)-DHEP (data not shown). Triggering mechanisms for the 9(11)-DHEP-induced CDKN1A expression are currently under investigation. Thus, 9(11)-DHEP is suggested to inhibit HT29 cell growth due to cell cycle arrest and apoptosis caused by CDKN1A induction. This may be the mechanism by which 9(11)-DHEP contributes to the antitumor effects of *S. aspratus* extract.

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