Novel Phthalide Compounds from *Sparassis crispa* (Hanabiratake), Hanabiratakelide A—C, Exhibiting Anti-cancer Related Activity

Kazuko YOSHIKAWA,^a Naoki KOKUDO,^a Toshihiro HASHIMOTO,^a Kyosuke YAMAMOTO,^b Toshiaki INOSE,^b and Takashi KIMURA^{*,b}

^aFaculty of Pharmaceutical Sciences, Tokushima Bunri University; Yamashiro-cho, Tokushima 770–8514, Japan: and
^bResearch & Development Center, Unitika Ltd.; 23 Uji-Kozakura, Uji, Kyoto 611–0021, Japan.

Received November 5, 2009; accepted May 6, 2010

Sparassis crispa (SC), known as Hanabiratake in Japanese, is an edible mushroom with various medicinal properties. We isolated 3 novel phthalides, designated hanabiratakelide A (1), B (2), and C (3), from the SC fruit body. In this investigation, 3 known phthalides (4–6), ubiquinone-9, and 2 known unsaturated fatty acids were also isolated. Their structures were elucidated primarily through extensive NMR experiments. The isolated compounds 1–6 were tested for their anti-oxidant activity. The *in vitro* superoxide dismutase-like activity of the 3 hanabiratakelides was stronger than that of vitamin C. The compounds also exerted inhibitory effects on lipopolysaccharide-stimulated nitric oxide and prostaglandin E2 production by a murine macrophage cell line, RAW264. In addition, the growth of the colon cancer cell lines Caco-2 and colon-26 was significantly inhibited by treatment with the 3 hanabiratakelides. *In vivo*, the frequency of azoxymethane-induced aberrant crypt foci was reduced in SC-fed F344/N rats compared to rats fed a standard diet. In conclusion, 3 novel phthalides, hanabiratakelides, derived from SC were shown to possess anti-oxidant, anti-inflammatory, and anti-tumor activity.

Key words *Sparassis crispa*; hanabiratakelide; anti-oxidant; anti-inflammatory; anti-tumor

Sparassis crispa (SC), known as Hanabiratake in Japanese, is an edible mushroom with various medicinal properties that has recently become cultivable in Japan. SC grows primarily on the stumps of coniferous trees and is abundant in northern temperate zones throughout the world.¹⁾ More than 40% of SC is composed of β -D-glucan, a polysaccharide of D-glucose comprising a β -(1→3)-D-glucan backbone with a single β -(1→6)- or β -(1→2)-D-glucosyl side branch at every third residue.^{2–4)} SC has been reported to have many biological effects such as tumor-suppressing effects,^{2,4,5)} effects in improving natural killer cell activity,⁵⁾ anti-angiogenic effects,⁴⁾ anti-allergic effects,⁶⁾ wound-healing effects,⁷⁾ and effects in enhancing hematopoietic responses.^{2,8)} In addition, a recent study suggested that the administration of β -D-glucan from SC could be an effective treatment strategy for cancer patients.⁹⁾

In our continuing search for biologically active compounds from fungi,¹⁰⁾ we have initiated chemical studies of SC. Previous studies have reported the isolation of a sesquiterpenoid¹¹⁾ 2 maleic acid derivatives,¹²⁾ and a benzoate derivative–sparasol.¹³⁾ In this study, we describe the isolation and structural elucidation of hanabiratakelide A—C (1–3), primarily by extensive NMR experiments, along with 3 known phthalides (4–6),^{14,15)} 2 unsaturated fatty acids,^{16,17)} and ubiquinone-9.¹⁸⁾ The biological activity of the isolated compounds, especially 1–3, are also indicated.

Reactive oxygen and nitrogen oxide species (RONS), such as hydroxyl radical (OH), superoxide anion (O₂^{•−}), hydrogen peroxide (H₂O₂), nitric oxide (NO), and peroxynitrite (ONOO[−]), are generated by respiring cells and act as signal transduction mediators within cells. They play important roles in numerous biological processes, such as cell differentiation, immune activation, and apoptosis. However, excess amounts of these molecules can be harmful to cells and are associated with the pathogenesis of cancer, atherosclerosis, inflammation, and other such disorders.^{19,20)} Prostaglandin

E2 (PGE2) is a bioactive compound synthesized in the arachidonic acid cascade. It has been reported that overproduction of PGE2 contributes to tumor progression by activating signaling pathways that stimulate cell proliferation, migration, and angiogenesis.²¹⁾

Most RONS and PGE2 are generated by activated immune leukocytes. Therefore, suppression of over-activated leukocytes is considered beneficial for preventing oxidation- and inflammation-related diseases, including cancer.^{19–22)} Several phytochemicals have been shown to have anti-oxidant or anti-inflammatory effects. Some of these, such as curcumin and epigallocatechin gallate, are being investigated in clinical trials for cancer prevention.²³⁾

So far, no compounds in SC have been reported to have anti-oxidant or anti-inflammatory activity. In this study, we purified 3 novel phthalides and tested them for anti-oxidant, anti-inflammatory, and anti-tumor properties *in vitro*. Since compounds possessing these properties commonly also have cancer-preventive activity,^{19–23)} we also assessed SC for possible anti-carcinogenic activity *in vivo* in rats.

MATERIALS AND METHODS

Fungus The mycelia of SC were grown in a solid medium containing coniferous sawdust as a base material, and the fruit bodies were harvested in Unitika Ltd., Aichi, Japan, in 2007.

Extraction and Isolation The dry powder of the fruit body of SC (1.0 kg) was exhaustively extracted with MeOH at room temperature for 3 months. The MeOH extract residue was partitioned between EtOAc and H₂O. The EtOAc-soluble portion (41.6 g) was subjected to silica gel column chromatography with hexane–AcOEt–MeOH (9:1:0→0:9:1) to provide fractions 1–9. Fraction 1 (13.7 g) was subjected to preparative TLC [hexane–AcOEt (7.5:2.5)] to provide ubiquinone-9 (102.8 mg).¹⁸⁾ Fraction 6 (1.37 g)

* To whom correspondence should be addressed. e-mail: takashi-kimura@unitika.co.jp

and fraction 7 (1.47 g) were purified by preparative HPLC (15–30% MeOH) to provide hanabiratakelide C (**3**, 94.1 mg); compound **4** (9.5 mg)¹⁴ was isolated from fraction 6, and compound **5** (6.0 mg),¹⁵ from fraction 7. Fraction 8 (1.32 g) was purified by preparative HPLC (70–100% MeOH) to yield filoboletic acid (61.1 mg)¹⁶ and 8,9-dihydroxy-10*E*,12*Z*-octadecadienoic acid (42.0 mg).¹⁷ The residue of fraction 8 was further purified by preparative HPLC (30–50% MeOH) to provide hanabiratakelides A (**1**, 19.0 mg) and B (**2**, 47.3 mg) and compound **6** (11.5 mg).¹⁵

Hanabiratakelide A (1) A colorless amorphous solid, with mp 236–238 °C. FT-IR (dry film) cm^{-1} : 3165 (OH), 1716 (C=O), 1622, 1455 (aromatic). UV λ_{max} (MeOH) nm (log ϵ): 260 (3.78), 309 (3.66). $^1\text{H-NMR}$ (600 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 3.78 (3H, s, OMe), 5.40 (2H, brs, H_2 -3), 6.85 (1H, brs, H-7). $^{13}\text{C-NMR}$ (150 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 56.1 (q, OMe), 67.3 (t, C-3), 101.5 (d, C-7), 104.7 (s, C-7a), 134.5 (s, C-3a), 136.7 (s, C-4), 153.4 (s, C-6), 154.6 (s, C-5), 169.3 (s, C-1). High-resolution chemical ionization mass spectrum (HR-CI-MS) m/z : 197.0431 (Calcd for $\text{C}_9\text{H}_9\text{O}_5$; 197.0422).

Hanabiratakelide B (2) A colorless amorphous solid, with mp 202–204 °C. FT-IR (dry film) cm^{-1} : 3235 (OH), 1708 (C=O), 1603, 1473 (aromatic). UV λ_{max} (MeOH) nm (log ϵ): 235 (3.41), 264.5 (3.41), 300 (3.13). $^1\text{H-NMR}$ (600 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 4.17 (3H, s, OMe), 5.14 (2H, brs, H_2 -3), 6.98 (1H, brs, H-4). $^{13}\text{C-NMR}$ (150 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 62.0 (q, OMe), 69.0 (t, C-3), 104.8 (d, C-4), C-109.1 (s, C-7a), 140.0 (s, C-5), 140.8 (s, C-3a), 147.0 (s, C-6), 155.9 (s, C-7), 169.8 (s, C-1). HR-CI-MS m/z : 197.0411 (Calcd for $\text{C}_9\text{H}_9\text{O}_5$; 197.0422).

Hanabiratakelide C (3) An amorphous solid, with mp 122–124 °C. FT-IR (dry film) cm^{-1} : 3350 (OH), 1729 (C=O), 1624, 1518 (aromatic). UV λ_{max} (MeOH) nm (log ϵ): 275 (4.19), 302 (3.83). $^1\text{H-NMR}$ (600 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 4.11 (3H, s, OMe), 5.41 (2H, s, H_2 -3). $^{13}\text{C-NMR}$ (150 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 62.2 (q, OMe), 67.7 (t, C-3), 108.0 (s, C-7a), 126.1 (s, C-3a), 137.6 (s, C-4), 140.5 (s, C-6), 140.9 (s, C-5), 143.3 (s, C-7), 170.2 (s, C-1). HR-CI-MS m/z : 213.0411 (Calcd for $\text{C}_9\text{H}_9\text{O}_6$; 213.0422).

General Experimental Procedures Melting points were determined with a Yanagimoto micro melting point apparatus. IR spectra were measured on a Jasco FT/IR-5300 instrument, and UV spectra were recorded using a Shimadzu UV-6000 spectrophotometer. NMR spectra were recorded on a Varian Unity 600 spectrometer. The chemical shifts are given in δ (ppm) in $\text{C}_5\text{D}_5\text{N}$ solution, using tetramethylsilane as an internal standard. NMR experiments included correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), and rotating-frame Overhauser enhancement spectroscopy (ROESY). Coupling constants (J values) are given in hertz (Hz). HR-CI-MS were measured on a JEOL JMS-700 MS station.

Materials Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were obtained from Wako (Tokyo, Japan). Antibiotic–antimycotic solution (100 $\times$) containing 10000 units/ml penicillin, 10 mg/ml streptomycin and 25 $\mu\text{g}/\text{ml}$ amphotericin B in phosphate-buffered saline (PBS) was purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Gibco BRL (Auckland, New Zealand). Lipopolysaccharide (LPS) was purchased from

Sigma (St. Louis, MO, U.S.A.).

Cells The human colon cancer Caco-2 cells, kindly provided by Dr. Shimizu from the University of Tokyo, were maintained in DMEM supplemented with 10% (v/v) FBS and 1 $\times$ antibiotic–antimycotic solution. The murine colon cancer colon-26 cells and mouse macrophage-like RAW264 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer of Tohoku University, and the RIKEN BioResource Center, respectively. These cell lines were maintained in RPMI-1640 supplemented with 10% (v/v) FBS and 1 $\times$ Antibiotic–Antimycotic solution.

Superoxide Dismutase (SOD)-Like Activity SOD-like activity was determined according to the method of Ukeda²⁴ using a SOD Assay Kit-WST (Dojindo Laboratory, Kumamoto). A test sample was dissolved in dimethyl sulfoxide (DMSO) to a final DMSO concentration of 0.8% (v/v).

PGE2 and NO-Induction in RAW264 Cells Analyses of the anti-inflammatory potentials of the 3 compounds were according to the methods of Han *et al.* and Lee *et al.*^{25,26} Briefly, RAW264 cells were cultured in a 96-multiwell plate at 5×10^5 cells/ml in the presence of LPS (0.1 $\mu\text{g}/\text{ml}$) and the purified compounds. Culture supernatants were collected from the wells at 24 h after seeding cells. PGE2 and NO were measured by ELISA (R&D Systems, Minneapolis, MN, U.S.A.) and the Griess reagent system (Promega, Madison, WI, U.S.A.), respectively.

Colon Cancer Cell Growth Assay *in Vitro* Colon cancer cell lines were seeded into a 96-multiwell culture plate at 3×10^4 cells/ml, and the isolated compounds were added. Cell proliferation was determined using a cell counting kit (CCK-8, Dojindo Laboratory) at 72 h post-seeding.

Animals Female F344/N rats (5 weeks old) were obtained from Japan SLC (Shizuoka, Japan). They were housed in a room maintained at 24 ± 1 °C with a 12 h light–dark cycle. Powdered SC was mixed with AIN-76 diet (Japan Clea, Osaka) at 0.3, 1.0, and 3.0% (w/w) of the total diet and fed to rats. Control rats were fed AIN-76 diet only. The animals were treated according to the ethical guidelines prescribed by the Animal Study committee of Unitika Ltd.

Induction of Aberrant Crypt Foci (ACF) by Azoxymethane (AOM)²⁷ AOM solution (15 mg/kg) was subcutaneously injected at 1 and 2 weeks after beginning the feeding with the experimental or control diet. Three weeks after the second AOM injection, each rat was sacrificed and its colon was excised. Excised colons were cut like a sheet, washed with PBS, and fixed with 20% formaldehyde solution for more than 72 h. The numbers of ACF and total aberrant crypts (ACs) per colon were counted using a stereomicroscope after staining with 0.2% methylene blue.

Statistical Analysis All results are given as means $\pm$ S.D. Comparisons between means were analyzed by Dunnett's test. Differences were considered significant at $p < 0.05$.

RESULTS

Chemical Characterizations of Isolated Compounds The chemical structures of the isolated compounds (**1**–**6**) are shown in Fig. 1.

Hanabiratakelide A (**1**) was obtained as a colorless amorphous solid and gave a $[\text{M} + \text{H}]^+$ peak at m/z 197.0431 on its

Fig. 1. Chemical Structures of Compounds (1–6) Isolated from SC

HR-Cl-MS. This corresponded to the molecular formula $C_9H_8O_5$, which required 6 unsaturation equivalents. The IR spectrum of **1** contained absorption bands at 3165 (OH), 1716 (C=O), and at 1622 and 1455 (aromatic) cm^{-1} . Also, the presence of an aromatic ring was supported by UV data (λ_{max} 260, 3.09 nm). In the ^{13}C -NMR spectrum of **1**, 9 resonances attributable to 1 methoxy (δ 56.1), 1 oxymethylene (δ 67.3), 6 olefinic [δ 101.5 (d), 104.7 (s), 134.5 (s), 136.7 (s), 153.4 (s), 154.6 (s)] and 1 carbonyl carbon (δ 169.3) were evident. The 1H -NMR spectrum of **1** clearly indicated signals corresponding to methoxyl at δ 3.78 (3H, s), oxymethylene at δ 5.40 (2H, brs), and an aromatic proton at δ 6.85 (1H, brs). As the data could account for 6 of the hydrogen atoms within **1**, it was evident that the remaining 2 must be present as parts of OH groups, a deduction that was also supported by the IR spectrum. Further, the remaining 2 unsaturation equivalents of **1** could be satisfied by constructing 2 rings.

The gross structure of **1** was determined by analyzing 2D NMR data, including COSY, HMQC, HMBC, and ROESY. The COSY spectrum revealed a homoallylic coupling between H_2 -3 (δ 5.40) and H_7 (δ 6.85). The HMBC spectrum of **1** contained cross peaks from H_2 -3 to C-1 (δ 169.3), C-3a (δ 134.5), C-4 (δ 136.7), and C-7a (δ 104.7); from H_7 (δ 6.85) to C-1, C-3a, C-5 (δ 154.6), and C-6 (δ 153.4); and from OMe (δ 3.78) to C-6. Furthermore, the nuclear Overhauser effect (NOE) was observed between H_7 and the methoxy signal at C-6. These findings enabled us to construct the structure of **1** as a phthalide compound with hydroxyl groups at C-4 and C-5, and a methoxy group at C-6 as its substituents. Thus, the structure of hanabiratakeli A was established as **1**.

Hanabiratakeli B (**2**) was obtained as a colorless amorphous solid and showed a $[M+H]^+$ peak at m/z 197.0411 in its HR-Cl-MS, indicative of a molecular formula $C_9H_8O_5$ and requiring 6 unsaturation equivalents. The IR spectrum of **2** exhibited absorption bands due to hydroxyl, carbonyl, and aromatic functions. NMR data of **2** showed the presence of the same structure fragments as those of **1**, such as 1 benzylic methylene, 1 penta-substituted benzene ring, 1 carbonyl and 1 downfield shifted methoxy group (δ_H 4.17, δ_C 62.0), suggesting that it was sandwiched between the oxygen groups. The upfield shift of H_2 -3 (δ 5.14) by comparison with that of **1** (δ 5.40) suggested that C-4 was not substituted with hydroxyl or methoxy groups. The COSY spectrum revealed the connectivity of an allyl coupling between H_2 -3

Table 1. SOD-Like Activity of Compounds 1–6

Compound	1	2	3	4	5	6	Vitamin C
IC ₅₀ (μ M)	15.7	49.0	3.2	324.9	123.0	66.8	71.0

(δ 5.14, brs) and aromatic proton H_4 (δ 6.98, brs). HMBC showed long-range correlations from H_2 -3 to C-1 (δ 169.8), C-3a, C-4 (δ 104.8), and C-7a (δ 109.1); from H_4 (δ 6.98) to C-5 (δ 140.0), C-6 (δ 147.0), and C-7a; and from OMe (δ 4.17) to C-6. These data aided with deriving the structure of **2** as a phthalide compound with hydroxyl groups at C-5 and C-7 and a methoxy group at C-6 as its substituents, which was further confirmed by the observation of NOE between H_2 -3 and H_4 . Thus, the structure of hanabiratakeli B was established as **2**.

Hanabiratakeli C (**3**) had the molecular formula $C_9H_8O_6$ in its HR-Cl-MS, requiring 6 unsaturation equivalents like those of **1** and **2**. 1H -NMR data of **3** were indicative of the presence of a benzylic methylene at δ 5.41 (2H, s), and a downfield shifted methoxy group at δ 4.11 (3H, s). However, the characteristic aromatic proton in the downfield was absent. ^{13}C -NMR data of **3** contained structure fragments including 1 methoxy, 1 benzylic methylene, 1 carbonyl carbon and a fully substituted benzene ring. The HMBC spectrum of **3** contained cross peaks from H_2 -3 (δ 5.41) to C-1 (δ 170.2), C-3a (δ 126.1), C-4 (δ 137.6), C-7 (δ 143.3), and C-7a (δ 108.0); and from OMe (δ 4.11) to C-7 (δ 143.3). However, in the ROESY spectrum, NOE was not observed. On the basis of these findings and the reported NMR data for analogous compounds,^{14,15} the structure of hanabiratakeli C was formulated as **3**.

SOD-Like Activity Six phthalide compounds, **1**–**6**, isolated from SC were evaluated for their SOD-like activity by using SOD water soluble tetrazolium (WST)-1 methods²⁴ and vitamin C as a positive control. Among these, compounds **1** and **3** showed much stronger activity than vitamin C. The IC₅₀ values are shown in Table 1.

Suppression of LPS-Induced PGE2 and NO Production by RAW264 Cells We also assessed isolated compounds **1**–**3** for other anti-inflammatory properties. These compounds significantly suppressed LPS-induced PGE2 production by RAW264 cells in a dose-dependent manner. NO production by these cells was also suppressed by treatments with compounds **1** and **3** (Fig. 2).

Inhibition of Colon Cancer Cell Growth To evaluate the anti-tumor activity of compounds **1**–**3**, we tested their growth inhibitory activity on colon cancer cells. Hanabiratakeli A–C significantly inhibited the growth of Caco-2 and colon-26 cells. Each compound showed dose-dependent activity, except for compound **2** in Caco-2 cells (Fig. 3). The IC₅₀ values of hanabiratakeli A and hanabiratakeli C in Caco-2 cells were 342 and 535 μ M, respectively. Also, the IC₅₀ values of hanabiratakeli A, B, and C in colon-26 cells were 96, 18, and 49 μ M, respectively.

Preventive Effects of SC on AOM-Induced ACF We investigated the possible preventive effects of SC on AOM-induced colon ACF in rats. SC feeding suppressed the malignant changes of ACF dose-dependently by 54% (0.3% group), 64% (1.0% group), and 75% (3.0% group) (Table 2).

DISCUSSION

SC (Hanabiratake in Japanese) is an edible mushroom with various medicinal properties and contains large amounts of β -1,3-D-glucan. Previous research on SC has focused on its β -glucan-dependent properties.^{2,4-7)} Only a few studies have investigated the low-molecular weight bioactive compounds of SC.²⁸⁾ In this study, we isolated and identified 3 novel phthalides, 3 known phthalides, ubiquinone-9, and 2 unsaturated fatty-acids from the SC fruit body. We named the 3 novel phthalides hanabiratakelide A—C and investigated their bioactive effects.

First, we found SOD-like activity in the 3 novel and 3 known phthalide compounds *in vitro*. Second, we showed inhibitory effects of the 3 hanabiratakelides on the production of NO and PGE2 by LPS-stimulated RAW264 cells. These results suggest that the 3 novel phthalides act as inhibitors of oxidative and inflammatory stress. Furthermore, cytotoxic effects of the hanabiratakelides against 2 colon cancer cell lines were demonstrated.

In general, the potential for anti-oxidant activity is often characterized by the number of phenolic hydroxyl groups in each substance. Hanabiratakelide C, having 3 hydroxyl groups, exhibited the most significant SOD-like activity,

while the activity of the other hanabiratakelides with 2 hydroxyl groups were not as strong as hanabiratakelide C. Hanabiratakelide A showed stronger activity than hanabiratakelide B. This may indicate that a phenolic hydroxyl group at C-4 enhances anti-oxidant activity. In the cell-based assay, NO appeared to be scavenged by hanabiratakelides since the patterns of inhibition of NO production were similar to the anti-oxidant effects. On the other hand, patterns of PGE2 inhibition were different from the former assays. Two other phthalides, senkyunolide A and Z-ligustilide isolated from *Ligusticum chuanxiong* or *Angelica sinensis*, are also reported to have suppressive effects on LPS-activated immune cells.²⁹⁾ These compounds also caused inhibition of tumor necrosis factor (TNF)- α secretion. NO and PGE2 levels were not examined in that study, however, TNF- α secretion was inhibited at a dose in the order of μ M. While further investigation is required to clarify the mode of action of hanabiratakelides, one possibility is that the hanabiratakelides act by inhibiting the activity of cyclooxygenase-2, a rate-limiting enzyme in the arachidonic acid cascade.³⁰⁾

Excess amounts of RONS and PGE2 *in vivo* are thought to

Fig. 2. Anti-inflammatory Activity of Hanabiratakelides

LPS-stimulated NO production (A) and PGE2 release (B) are shown for RAW264 cells treated with hanabiratakelides as normalized to a control (untreated) group; values per cell were obtained by dividing by the cell number (which was determined using a cell counting kit). (a) Hanabiratakelide A, (b) Hanabiratakelide B, (c) Hanabiratakelide C, mean $\pm$ S.D., $n=3$, * $p<0.05$, ** $p<0.01$.

Table 2. Suppression of AOM-Induced ACF Formation in F344/N Rats

Experimental diet	Number of ACF/colon	Number of ACs/colon	Number of ACs/focus	Number of ACF>3ACs/colon
AIN-76	29.8 $\pm$ 11.4	51.4 $\pm$ 21.0	1.7 $\pm$ 0.1	5.0 $\pm$ 2.5
0.3% SC ^{a)}	13.7 $\pm$ 3.5*	22.3 $\pm$ 5.7**	1.6 $\pm$ 0.1	1.7 $\pm$ 0.6*
1.0% SC ^{a)}	10.8 $\pm$ 6.2**	17.5 $\pm$ 8.7**	1.7 $\pm$ 0.3	1.2 $\pm$ 1.0**
3.0% SC ^{a)}	7.4 $\pm$ 0.6**	8.7 $\pm$ 0.6**	1.2 $\pm$ 0.1*	0.3 $\pm$ 0.6**

a) Powder of SC was mixed with AIN-76 diet to give the concentrations indicated before feeding to AOM-treated rats. Mean $\pm$ S.D., $n=3-6$, * $p<0.05$, ** $p<0.01$.

Fig. 3. Cytotoxic Effects of Hanabiratakelides on Colon Cancer Cell Lines

Survival rates of the human colon cancer cell Caco-2 (A) and the murine colon cancer cell colon-26 (B), following treatment with the hanabiratakelides, as normalized to a control (untreated) group. (a) Hanabiratakelide A, (b) Hanabiratakelide B, (c) Hanabiratakelide C, mean $\pm$ S.D., $n=4$, * $p<0.05$, ** $p<0.01$.

be one of the risk factors for the initiation and progression of cancer. Several studies have indicated that factors suppressing the generation of RONS and PGE2 could be effective for cancer prevention.^{19–23,31,32} The 3 novel hanabiratakelides inhibited the growth of 2 colon cancer cell lines, exhibiting IC₅₀ values of 342 μ M (hanabiratakelide A) and 535 μ M (hanabiratakelide C) in Caco-2 cells and 96 μ M (hanabiratakelide A), 18 μ M (hanabiratakelide B), and 49 μ M (hanabiratakelide C) in colon-26. *N*-Butyridenephthalide, senkyunolide A, and *Z*-ligustilide are also reported to inhibit growth of the human colon cancer cell line, HT-29. The inhibitory activity (IC₅₀) of those compounds was 236.9, 54.2, and 60.6 μ M, respectively, in the same order of magnitude as those observed for hanabiratakelides.³³⁾

ACF are early morphological changes or hyperproliferative lesions found in the colon of humans and carcinogen-treated rodents. They are also considered to be putative preneoplastic lesions for colon cancer.³⁰⁾ Epidemiological study indicated that the frequency of colon cancer is related to dietary consumption of vegetables and fruits.³⁴⁾ As described above, plant or food constituents possessing anti-oxidative or anti-inflammatory compounds are involved in chemoprevention of cancer. In our studies, feeding of SC suppressed malignant changes of ACF in AOM-treated rats. It is also likely that hanabiratakelides in SC might be involved in the preventive effects on carcinogenesis either directly or indirectly.

In conclusion, our results indicate that the 3 novel phthalides (hanabiratakelides) we characterized for the first time showed anti-oxidant and anti-inflammatory activity along with an inhibitory effect on tumor cell growth. Therefore, these hanabiratakelides may contribute to the cancer-preventive activity of SC.

Acknowledgments We thank Dr. Makoto Shimizu from the University of Tokyo for providing Caco-2 cells, and Dr. Nariaki Matsuura from Osaka University and Dr. Akio Sugitachi from Takamatsu Hospital for their experimental suggestions.

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