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Short Communication

Chemical Constituents from the Sclerotia of *Inonotus obliquus*

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Six triterpenoids, two sterols, and two phenolic constituents were isolated from the methanol extract of sclerotia of *Inonotus obliquus* (Hymenochaetaceae). Their chemical structures were identified as lanosterol (1), 3β-hydroxylanosta-8,24-dien-21-al (3), inotodiol (5), trametenolic acid (6), lanosta-8,25-dien-3,22,24-triol (7), β-sitosterol (2), ergosterolperoxide (4), betulin (8), 2,5-dihydroxy-benzaldehyde (9), and 3,4-dihydroxybenzalacetone (10) on the basis of spectroscopic methods and comparison with the literature. Among these compounds, 9 was isolated for the first time from this mushroom. To the best of our knowledge, the present study marks the first chemical investigation of *I. obliquus* from Alaska. Among the isolated compounds, compounds 1, 3, 4, 5, 6, 8, 9, and 10 were evaluated for *in vitro* cytotoxic activity against A 549 (human alveolar basal epithelial carcinoma cells), L1210 (mouse lymphocytic leukemia cells), COLO 205 (colorectal adenocarcinoma cells), MCF-7 (breast cancer cells), and HL-60 (human leukemia) cancer cell lines. 3β-Hydroxylanosta-8,24-dien-21-al (3) and tramentenolic acid (6) exhibited modest cytotoxic effects against L1210 cells with IC_{50}s of 62.5 and 34.4 μM, and betulin (8) showed weak cytotoxic effects against A549 and HL-60 with IC_{50}s of 81.2 and 87.5 μM, respectively. In addition, 3,4-dihydroxybenzalacetone (10) exhibited the strongest cytotoxic activities against A549 and HL-60 cells with IC_{50} values of 23.6 and 21.7 μM, respectively.

Key words: cytotoxicity, *Inonotus obliquus*, lanostane-type triterpenoids, phenolics, sterols

Inonotus obliquus (Pers.: Fr.) Pil. [=*Fuscoporia oblique* (Pers.: Fr.) Aoshima], known as kabanoanatake in Japan and chaga or tchaga in Russia, is a white-rot fungus belonging to the family Hymenochaetaceae Donk [Hawksworth *et al*., 1995] and is distributed in Europe, Asia, and North America [Ellis and Ellis, 1990]. This mushroom, which typically lives on tree trunks, usually on *Betula* (birch) but rarely on *Ulmus*, *Alnus*, and *Fraxinus*, has been used as a folk medicine for cancer treatment in Russia and western Siberia and has also been used to prevent and treat heart, liver, and stomach diseases as well as tuberculosis. Moreover, it has been traditionally used for the treatment of gastrointestinal cancer, cardiovascular disease, and diabetes since the 16th century in Russia, Poland, and many Baltic countries [Maret, 1991; Huang, 2002].

In previous investigations on the chemical constituents of this mushroom, lanostane-type triterpenoids, such as inotodiol [De Reinach-Hirtzbach and Ourission, 1972], trametenolic acid [Kempska *et al*., 1962], and inonotsuoxides [Nakata *et al*., 2007] were reported to have antitumor and antifungal activities. Some phenolic compounds were also found in this mushroom [Lee *et al*., 2007; Nakajima *et al*., 2007].

Recently, several biological studies of *I. obliquus* have been published. For example, the extracts of this fungus possess anti-tumor [Kim *et al*., 2006; Park *et al*., 2006; Song *et al*., 2008], anti-tumor promoting [Nakata *et al*., 2007], antioxidative [Lee *et al*., 2007], and anti-inflammatory properties [Kim *et al*., 2007]. Extracts from the sclerotium of *I. obliquus* are known to have positive effects on controlling cancer, human immunodeficiency virus 1 (HIV-1), and digestive disease [Park *et al*., 2005]. Anti-tumor experiments with *n*-hexane extracts of *I. obliquus* have been conducted, and inotodiol has been found to have a significant anticancer effect on Walker 256 carcinosarcoma and MCF-7 human mammary adenocarcinoma [Kahlos *et al*., 1987]. In addition, inotodiol increases the catalase activity in HeLa S3 tumor cells [Rzymowska, 1998].

To the best of our knowledge, all *I. obliquus* used in

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chemical and biological researches to date were from Russia. However, in the present study, fungus (chaga) from *Betula plathylla* found in Alaska was used. Here, the isolation and identification of five lanostane-type triterpenoids, two steroids, and two phenolic constituents from the Alaskan chaga, as well as their cytotoxic activities against A 549 (human alveolar basal epithelial carcinoma cells), L1210 (mouse lymphocytic leukemia cells), COLO 205 (colorectal adenocarcinoma cells), and MCF-7 (breast cancer cells) are described.

The sclerotia (1.3 kg) of *I. obliquus* purchased from Alaska was extracted six times with methanol (MeOH) to give extracts (67.5 g), which were successively partitioned with *n*-hexane, CH_2Cl_2, and ethyl acetate (EtOAc). The CH_2Cl_2 fraction (24 g) was subjected to silica gel column chromatography and eluted with $CHCl_3$/EtOAc gradient system (50:1→1:1) to afford thirteen fractions (Fr. MC1~13). Compound **1** (105.2 mg) was recrystallized from fraction MC3 under $CHCl_3$ and MeOH solvents. Fraction MC5 was subjected to repeated column chromatography with a gradient elution of *n*-hexane/ EtOAc (10:1→1:1), resulting in 10 subfractions (Fr. MC5-1~MC5-10). Compound **2** (9.1 mg) was recrystallized from fraction MC5-5 under $CHCl_3$ and MeOH solvents to yield white crystals.

Fraction MC5-6 (58 mg) was subjected to MPLC with *n*-hexane/EtOAc gradient (EtOAc in *n*-hexane 5-100% over 70 min, 100% over 10 min) to afford compound **3** (7.1 mg), and fraction MC5-10 (13 mg) was subjected to semi-preparative HPLC with 0.1% *aq* trifluoroacetic acid/MeOH gradient (MeOH in water 50-80% over 5 min, 80-100% over 15 min, 100% over 25 min) to afford compound **4** (2.2 mg). Compounds **5** (301.5 mg) and **6** (8.7 mg) were recrystallized from fractions MC7 and MC10, respectively, under $CHCl_3$ and MeOH solvents. Fraction MC9 was subjected to silica gel column chromatography with a gradient elution of *n*-hexane/ EtOAc (15:1→1:1), resulting in 17 subfractions (Fr. MC9-1~MC9-17). Fraction MC9-14 was subjected to semi-preparative High Performance Liquid Chromatography (HPLC) with 0.1% *aq* trifluoroacetic acid/MeOH gradient (MeOH in water 50-80% over 5 min, 80-100% over 15 min, 100% over 25 min) to afford compounds **7** (2.7 mg) and **8** (3.2 mg).

The EtOAc fraction (12.4 g) was subjected to Sephadex LH-20 column chromatography and eluted with 100% MeOH to afford ten fractions (Fr. E1~10). Fraction E4 (750 mg) was subjected to silica gel column chromatography with a gradient elution of *n*-hexane/ EtOAc (8:5→1:2), resulting in 10 subfractions (Fr. E4-1~E4-10). Compound **9** (2.4 mg) was isolated from fraction E4-3, and fraction E4-5 was subjected to semi-preparative HPLC with a water/MeOH gradient (MeOH in water 20-80% over 30 min, 80-100% over 5 min, 100% over 5 min) to afford compound **10** (1.2 mg). The chemical structures of the isolated compounds (**1-10**) and ^{13}C-Nuclear Magnetic Resonance (NMR) data of compounds 1-8 are respectively shown in Fig. 1 and Table 1.

Lanosterol (1). white powder; Positive FAB-MS: *m/z* 449 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.08 (1H, t, *J*=6.9 Hz, H-24), 3.21 (1H, m, H-3), 1.66 (3H, s, H_3-26), 1.58 (3H, s, H_3-27), 0.98 (3H, s, H_3-28), 0.96 (3H, s, H_3-19), 0.89 (3H, d, *J*=6.3 Hz, H_3-21), 0.85 (3H, s, H_3-30), 0.79 (3H, s, H_3-29), 0.66 (3H, s, H_3-18); ^{13}C-NMR (75 MHz, $CDCl_3$).

β-Sitosterol (2). white needles. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.33 (1H, d, *J*=5.1 Hz, H-6), 3.50 (1H, m, H-3), 0.98 (3H, s, H_3-19), 0.89 (3H, d, *J*=6.6 Hz, H_3-21), 0.82 (3H, t, *J*=6.9 Hz, H_3-29), 0.81 (3H, d, *J*=6.6 Hz, H_3-27), 0.78 (3H, d, *J*=6.9 Hz, H_3-26), 0.65 (3H, s, H_3-18); ^{13}C-NMR (75 MHz, $CDCl_3$).

3β-Hydroxylanosta-8,24-dien-21-al (3). white crystals. Positive FAB-MS: *m/z* 463 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 9.44 (1H, d, *J*=5.7 Hz, H-21), 5.02 (1H, t, *J*=6.9 Hz, H-24), 3.20 (1H, dd, *J*=4.5, 11 Hz, H-3), 1.65 (3H, s, H_3-26), 1.53 (3H, s, H_3-27), 0.97 (3H, s, H_3-19), 0.93 (3H, s, H_3-29), 0.87 (3H, s, H_3-30), 0.78 (3H, s, H_3-28), 0.66 (3H, s, H_3-18); ^{13}C-NMR (75 MHz, $CDCl_3$).

Ergosterol peroxide (4). white powder. Positive FAB-MS: *m/z* 451 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.48 (1H, d, *J*=8.4 Hz, H-6), 6.22 (1H, d, *J*=8.4 Hz, H-7), 5.15 (2H, m, H-22, 23), 3.94 (1H, m, H-3), 0.97 (3H, d, *J*=6.6 Hz, H_3-27), 0.88 (3H, d, *J*=6.6 Hz, H_3-26), 0.86 (3H, s, H_3-19), 0.80 (3H, d, *J*=7.2 Hz, H_3-21), 0.79 (3H, s, H_3-18), 0.79 (3H, d, *J*=6.6 Hz, H_3-28); ^{13}C-NMR (75 MHz, $CDCl_3$).

Inotodiol (5). white powder. Positive FAB-MS: *m/z* 465 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.16 (1H, t, *J*=7.5 Hz ,H-24), 3.64 (1H, m, H-22), 3.21 (1H, m, H-3), 1.63 (3H, s, H_3-27), 1.53 (3H, s, H_3-26), 0.98 (3H, s, H_3-19), 0.96 (3H, s, H_3-28), 0.92 (3H, d, *J*=6.6 Hz, H_3-21), 0.85 (3H, s, H_3-30), 0.79 (3H, s, H_3-29), 0.70 (3H, s, H_3-18); ^{13}C-NMR (75 MHz, $CDCl_3$).

Trametenolic acid (6). white powder. Positive FAB-MS: *m/z* 479 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$+ CD_3OD) δ: 5.00 (1H, t, *J*=5.4 Hz, H-24), 3.11 (1H, dd, *J*=5.4, 11 Hz, H-3), 1.58 (3H, s, H_3-27), 1.49 (3H, s, H_3-26), 0.89 (3H, s, H_3-28), 0.87 (3H, s, H_3-19), 0.79 (3H, s, H_3-30), 0.70 (3H, s, H_3-29), 0.65 (3H, s, H_3-18); ^{13}C-NMR (75 MHz, $CDCl_3$+CD_3OD).

Lanosta-8,25-dien-3,22,24-triol (7). white powder. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.08 (1H, s, H-26B), 4.92 (1H, s, H-26A), 4.34 (1H, br s, H-24), 3.95 (1H, br d, *J*=9.9 Hz, H-22), 3.21 (1H, dd, *J*=5.1, 12 Hz, H-3), 1.70

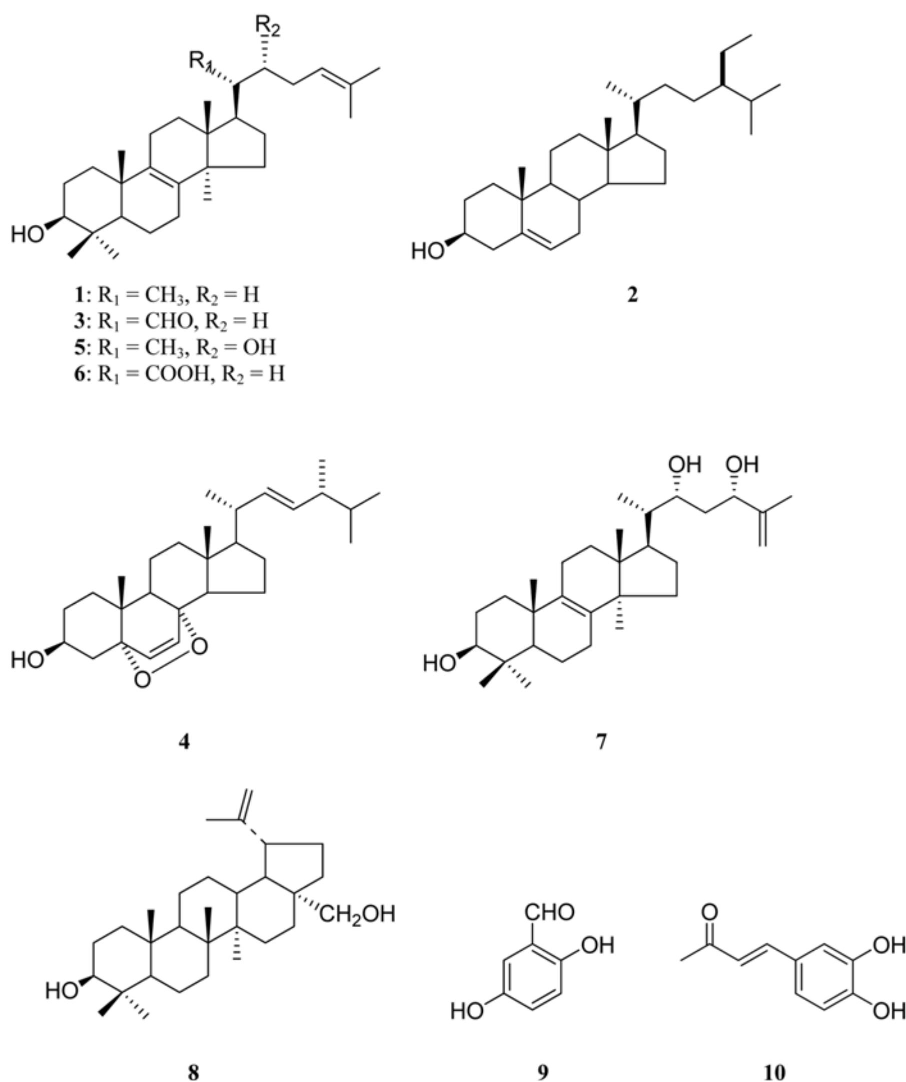

Fig. 1. Chemical structures of compounds 1-10 from *I. obliquus*.

(3H, s, H_3-27), 0.97 (3H, s, H_3-28), 0.95 (3H, s, H_3-19), 0.90 (3H, d, *J*=6.6 Hz, H_3-21), 0.84 (3H, s, H_3-30), 0.78 (3H, s, H_3-29), 0.69 (3H, s, H_3-18); ^{1}H-NMR (300 MHz, CD_3OD) δ: 5.05 (1H, s, H-26B), 4.87 (1H, s, H-26A), 4.29 (1H, t, H-24), 4.02 (1H, bs, H-22), 3.22 (1H, t, H-3), 1.79 (3H, s, H_3-27), 1.08 (3H, s, H_3-28), 1.04 (3H, s, H_3-19), 0.97 (3H, s, H_3-30), 0.96 (3H, d, overlapped, H_3-21), 0.87 (3H, s, H_3-29), 0.83 (3H, s, H_3-18); ^{13}C-NMR (75 MHz, CD_3OD).

Betulin (8). white powder. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 4.66 (1H, s, H-29B), 4.56 (1H, s, H-29A), 3.77 (1H, d, *J*=10 Hz, H-28B), 3.31 (1H, d, *J*=10 Hz, H-28A), 3.16 (1H, dd, *J*=4.2, 11 Hz, H-3), 1.66 (3H, s, H_3-30), 1.00 (3H, s, H_3-27), 0.96 (3H, s, H_3-26), 0.95 (3H, s, H_3-24), 0.80 (3H, s, H_3-25), 0.74 (3H, s, H_3-23), 0.66 (1H, d, *J*=10 Hz, H-5); ^{13}C-NMR (150 MHz, $CDCl_3$).

2,5-Dihydroxybenzaldehyde (9). yellow needles. ^{1}H-NMR (300 MHz, CD_3OD) δ: 9.70 (1H, s, CHO), 7.32 (1H, dd, *J*=7.8, 2.1 Hz, H-4), 7.30 (1H, s, H-6), 6.91 (1H, d, *J*=7.8 Hz, H-3).

3,4-Dihydroxybenzalacetone (10). yellow needles. ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.54 (1H, d, *J*=15.9 Hz, H-4), 7.10 (1H, d, *J*=2.1 Hz, H-2'), 7.02 (1H, dd, *J*=8.1, 2.1 Hz, H-6'), 6.81 (1H, d, *J*=8.1 Hz, H-5'), 6.57 (1H, d, *J*=15.9 Hz, H-3), 2.36 (3H, s, H_3-1).

The HL-60 (human promyelocytic leukemia), LLC (mouse lewis lung carcinoma), A549 (human lung carcinoma), and MCF-7 (human breast adenocarcinoma cell lines) were maintained in RPMI (Roswell Park Memorial Institute) and/or IMDM (Iscove's Modified Dulbecco's Medium) containing L-glutamine (GIBCO, Rockville, MD) with 10% FBS (GIBCO) and 2% penicillin-streptomycin (GIBCO). Cells were cultured at 37°C in a 5% CO_2 incubator. Cytotoxic activity was measured using a modified 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay [Van

Table 1. ^{13}**C-NMR data of compounds 1-8 from the sclerotia of *I. obliquus***

No. of Carbon	1	2	3	4	5	6	7	8
1	35.7	37.4	35.7	37.1	35.8	35.3	36.9	38.7
2	28.0	31.8	28.1	30.3	28.0	28.3	28.4	27.3
3	79.1	72.0	79.1	66.6	79.2	78.1	79.6	78.9
4	39.0	42.4	39.1	51.2	39.1	38.4	39.9	38.8
5	50.5	140.9	50.5	79.6	50.6	50.1	51.9	55.2
6	18.4	121.9	19.3	135.6	19.3	17.8	18.9	18.2
7	26.6	32.0	29.4	130.9	26.7	26.8	27.6	34.2
8	134.5	32.0	134.9	82.3	134.8	134.2	136.0	40.9
9	134.5	50.3	134.1	34.8	134.4	133.7	135.6	50.4
10	37.2	36.7	37.2	37.1	37.2	36.6	38.2	37.1
11	21.1	21.1	20.9	21.0	21.2	20.3	22.0	20.8
12	31.1	39.9	26.6	39.5	29.3	29.9	32.0	25.2
13	44.6	42.4	44.5	44.7	45.0	43.8	45.9	37.3
14	49.9	56.9	49.6	51.8	49.6	49.0	50.6	42.7
15	31.0	24.5	30.8	28.8	31.1	32.1	32.2	27.0
16	28.4	28.4	29.8	23.6	31.1	26.6	28.3	29.1
17	50.5	56.2	45.5	56.3	47.4	47.7	48.4	47.7
18	15.6	12.0	17.0	13.0	15.9	15.2	16.2	48.7
19	19.3	19.6	18.4	18.3	18.2	18.4	19.4	47.7
20	36.5	36.3	55.6	39.9	41.9	46.7	44.1	150.3
21	18.8	18.9	206.4	19.8	12.8	179.1	13.0	29.7
22	36.4	34.1	27.0	135.3	73.5	26.0	70.8	33.9
23	25.1	26.2	25.9	132.4	27.4	25.4	36.3	27.9
24	125.4	46.0	123.7	42.9	121.5	123.3	73.1	15.3
25	131.1	29.3	132.6	33.2	135.3	131.3	149.8	16.0
26	25.9	19.2	25.9	20.1	26.2	24.8	110.1	16.0
27	17.8	20.0	17.9	20.8	18.4	16.7	19.6	14.7
28	28.1	23.2	15.6	17.7	28.1	27.2	28.6	60.5
29	15.9	12.1	28.0	-	15.6	14.8	16.1	109.6
30	24.4		24.4	-	24.5	23.5	24.6	19.0

Le *et al.*, 2009]. Viable cells were seeded in the growth medium (180 μL) in 96-well microtiter plates ($1{\times}10^4$ cells per well) and incubated at 37°C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5 to 100 μg/mL by dilution with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was <0.1%. After standing for 4 h, 20 μL of the test sample was added to each well. The same volume of 0.1% DMSO in growth medium was added to the control wells. After 48-h incubation, 20 μL of MTT (5 μg/mL) was added to each well. Four hours after the addition, the plate was centrifuged for 5 min at 1500 rpm, and the medium was removed. The resulting formazan crystals were dissolved in 150 μL DMSO. The optical density (O.D.) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow; Tokyo, Japan). The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

Compound **1** exhibited a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 449 in a FAB-MS spectrum. In the ^{1}H-NMR spectrum of compound **1**, five angular methyl groups and one secondary methyl group appeared at ä 0.66, 0.79, 0.85, 0.96, 0.98, and 0.89, respectively. In the ^{13}C-NMR spectrum of **1**, thirty carbons appeared, indicating that compound **1** was a triterpenoid. Two characteristic nonprotonated sp^2 carbon signals at δ_C 134.5, suggestive of an olefinic group at C-8 and C-9, indicated that this compound is a lanostane-type triterpenoid. In addition, two methyl singlets at δ_H 1.58 and 1.66 and an olefinic triplet signal at δ_H 5.08 (corresponding to δ_C 125.4) indicated that **1** has an isopentenyl group in its side chain. On the basis of the above evidence, compound **1** was assigned as lanosterol, which has been reported to be a major compound from *I.*

obliquus. The spectral data of **1** were in good agreement with previously reported data for lanosterol [Kahlos and Hiltunen, 1983; Shin *et al*., 2000].

Compound **2** was identified as β-sitosterol by comparison with the spectral data in the literature [Goad, 1991]. The identification of 2 was further confirmed by direct comparison of TLC pattern with an authentic sample.

Compound **3** exhibited a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 463 in a positive FAB-MS spectrum. The NMR spectrum of compound **3** showed that it had five angular methyls at δ_H 0.66, 0.78, 0.87, 0.93, and 0.97, an olefinic group at C-8 and C-9 at δ_C 134.9 and 134.1, and an isopentenyl group at δ_H 1.65 (3H, s), 1.53 (3H, s), and 5.02 (1H, t, *J*=6.9 Hz), suggesting that **3** had a lanostane skeleton. Comparing the spectral data of compound **3** with those of **1**, **3** had an aldehyde group at δ_H 9.44 (d, *J*=5.7 Hz), corresponding to δ_C 206.4, instead of the secondary methyl group found in **1**. These data suggested that **3** did not have a methyl group but an aldehyde group at C-20. Thus, compound **3** was assigned as 3β-hydroxylanosta-8,24-dien-21-al. The above spectral data were in good agreement with the literature values [Kahlos *et al*., 1984].

Compound **4** exhibited a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 451 in a positive FAB-MS spectrum. In the ^{1}H-NMR spectrum of compound **4**, two angular methyl groups at δ_H 0.79 and 0.86 and four secondary methyl groups at δ 0.79, 0.80, 0.88 and 0.97 indicated that **4** had an ergostane-type steroid skeleton. The olefinic multiplet signals at δ_H 5.15 (corresponding to δ_C 135.3 and 132.4) revealed the presence of a double bond in the side chain. Furthermore, two downfield-shifted olefinic doublet signals at δ_H 6.22 and 6.48 (*J*=8.4 Hz) suggested that a peroxy group existed around the olefinic group. On the basis of the spectral data, compound **4** was identified as ergosterol peroxide, which is predominant in fungus, and confirmed by direct comparison with the authentic sample [Jin *et al*., 1999; Takei *et al*., 2005].

Compound **5** exhibited a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 465 in a positive FAB-MS spectrum. The ^{1}H-NMR spectrum of **5** also exhibited characteristic signals for lanostane-type triterpenoid: five angular methyl groups at δ_H 0.70, 0.79, 0.85, 0.96, and 0.98, one secondary methyl group at δ_H 0.92 in the side chain, an olefinic group between C-8 and C-9 at δ_C 134.8 and 134.4, and an isopentenyl group at δ_H 1.53 (3H, s, H_3-26), 1.63 (3H, s, H_3-27), and 5.16 (1H, t, *J*=7.5 Hz). Comparing the spectral data of compound **5** with those of **1**, **5** had an oxygenated-methine group at δ_H 3.64 corresponding to δ_C 73.5. The position of the oxymethine group was deduced by comparison of the splitting pattern and chemical shifts of H-22 with those in the literature [Kahlos and Hiltunen, 1983; Shin *et al*., 2000]. On the basis of the above evidence, the structure of **5** was assigned as inotodiol. Its spectral data were in good agreement with the previously reported data [Kahlos and Hiltunen, 1983; Shin *et al*., 2000].

Compound **6** exhibited a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 479 in a positive FAB-MS spectrum. Its ^{1}H and ^{13}C-NMR spectra indicated that **6** also had a lanostane skeleton. Compared with the ^{1}H-NMR data of compound **1**, **6** was missing one secondary methyl group in the side chain. In the ^{13}C-NMR spectrum of **6**, one carboxylic acid carbon signal appeared at δ_C 179.1 in place of the methyl carbon in **1**, suggesting that compound **6** had a carboxylic acid substituent at C-20. On the basis of the above spectral data, **6** was assigned as tramentenolic acid. The spectral data showed good agreement with those in the literature [Kahlos and Hiltunen, 1983; Shin *et al*., 2000].

The ^{1}H and ^{13}C-NMR spectra of **7** were similar to those of **1**, in that five angular methyl groups at δ_H 0.69, 0.78, 0.84, 0.95, and 0.97, one secondary methyl group appeared at δ_H 0.90, and characteristic nonprotonated sp^2 carbon signals appeared at δ_C 135.6 and 136.0, indicating that **7** was also a lanostane-type triterpenoid. However, the signals for an isopentenyl group in the side chain were not observed in compound **7**. Instead, a signal for a methyl group attached to an sp^2 carbon was observed at δ_H 1.79, and the presence of an exomethylene group was confirmed at δ_H 4.92 and δ_H 5.08 (each 1H, br s). In addition, the signals of an oxymethine group appeared at δ_H 4.34 (in $CDCl_3$) and δ_C 73.1 (in CD_3OD). The positions of the oxymethine group and exomethylene group were presumed to be C-24 and C-26, respectively, by comparison with the literature values [Trofimova *et al*., 1998; Huang *et al*., 2008]. The 22*S* and 24*S* stereochemistry of **7** was also established by comparison of the spectral data with the literature values [Trofimova *et al*., 1998; Huang *et al*., 2008]. The structure of **7** was identified as lanosta-8,25-dien-3,22,24-triol.

The NMR spectra of **8** displayed signals for an isopropylene group (δ_H 4.56 and 4.66, 1H each, br s and 1.66, 3h, s), five angular methyls (δ_H 0.74, 0.80, 0.95, 0.96, and 1.00), a set of germinal protons at δ_H 3.31 and 3.77 (1H each, d, *J*=10 Hz, H_2-28), in addition to an oxymethine group at δ_H 3.16 (1H, dd, *J*=4.2, 11 Hz, H-3), indicating that **8** was a lupane-type triterpenoid. The structure of **8** was identified as betulin by comparison of the spectral data with the literature values [Patra *et al*., 1988].

In the ^{1}H-NMR spectrum of compound **9**, characteristic signals for a 1,2,5-trisubstituted benzene ring system appeared at: 6.91 (1H, d, *J*=7.8 Hz, H-3), 7.30 (1H, s, H-

Table 2. Cytotoxicity of compounds 1 and 3-6 against human cancer cell lines (IC_{50}, μM)[a]

Compounds	IC_{50} (μM)				
	A549	L1210	COLO205	MCF-7	HL-60
Lanosterol (**1**)	>200	>200	>200	>200	NT
3β-Hydroxylanosta-8,24-dien-21-al (**3**)	>200	62.5	>200	>200	NT
Ergosterol peroxide (**4**)	>200	>200	>200	>200	NT
Inotodiol (**5**)	>200	110	171	>200	NT
Trametenolic acid (**6**)	>200	34.4	>200	>200	NT
Betulin (**8**)	81.2	NT	NT	NT	87.5
2,5-Dihydroxybenzaldehyde (**9**)	>200	NT	NT	NT	>200
3,4-Dihydroxybenzalacetone (**10**)	23.6	NT	NT	NT	21.8
Adriamycin	0.75	1.6	0.42	1.2	2.1

[a]IC_{50}: concentration (μM) at which 50% inhibition of cell growth occurs *in vitro*.

6), and 7.32 (1H, dd, *J*=7.8, 2.1 Hz, H-4). In addition, a signal for an aldehyde appeared at δ_H 9.70 as a singlet. On the basis of the above spectral data, the structure of **9** was assigned as 2,5-dihydroxybenzaldehyde. Its spectral data showed good agreement with the literature [Charles, 1983].

The ^{1}H-NMR spectrum of compound **10** was similar to that of **9**, suggesting that **10** had a 1,2,5-trisubstituted benzene ring system at: 6.81 (1H, d, *J*=8.1 Hz, H-5'), 7.02 (1H, dd, *J*=8.1, 2.1 Hz, H-6'), and 7.10 (1H, d, *J*=2.1 Hz, H-2'). Two *trans*-coupling proton signals at δ_H 6.57 and 7.54 (each d, *J*=15.9 Hz) indicated that one olefinic group was attached to a benzene ring. In addition, a methyl singlet signal at δ_H 2.36 indicated that the methyl group was attached to a nonprotonated sp^2 carbon. On the basis of the above spectral data, **10** was assigned as 3,4-dihydroxybenzalacetone. Its spectral data showed good agreement with the literature [Nakajima *et al*., 2007]. Among the isolated compounds, **9** was isolated from this fungus for the first time. Although several chemical and biological studies on this fungus have been carried out, the fungus investigated was always from Russia. To the best of our knowledge, the chemical and biological study of the Alaskan fungus was conducted for the first time in the present study.

The cytotoxic activities of compounds **1**, **3**, **4**, **5**, **6**, **8**, **9**, and **10** were evaluated against four human tumor cell lines (A549, L1210, COLO 205, MCF-7, and HL-60) with adriamycin as the positive control (Table 2). Compounds **3** and **6** exhibited modest cytotoxic effects against L1210 cells with IC_{50}s of 62.5 and 34.4 μM, respectively, and **8** showed weak cytotoxic effects against A549 and HL-60 with IC_{50}s of 81.2 and 87.5 μM, respectively. In addition, compound **10** exhibited the strongest cytotoxic activities against A549 and HL-60 cells with IC_{50} values of 23.6 and 21.7 ìM, respectively.

Nakajima *et al*. [2009] reported on the cancer cell cytotoxicity of extracts and some phenolic compounds from chaga; however, among the compounds isolated in the present study, only 3,4-dihydroxybenzalacetone (**10**) was evaluated for the cytotoxicity by the above-mentioned group. Although 2,5-dihydroxybenzaldehyde (**9**) was first isolated from this fungus in the present study, its cytotoxic activity has been previously reported by Tseng *et al*. [2001]. It exhibited weak cytotoxic activity against HL-60 cell lines, which agreed with our result. There are several reports on the inhibition of cancer cell proliferation by triterpenoids from *I. obliquus* [Nomura *et al*., 2008; Surowiak *et al*., 2009; Chung *et al*., 2010]. Inotodiol was reported to inhibit cell proliferation through caspase-3-dependent apoptosis [Nomura *et al*., 2008]. Chung *et al*. [2010] report on cytotoxicity of triterpenoids isolated from this fungus including lanosterol, 3β-hydroxylanosta-8,24-dien-21-al, and inotodiol against various cancer cell lines including A549 and MCF-7. Though the inhibition ratios of the compounds were in accordance with the results of the present study, they were not comparable to the NCI guidelines. Betulin, which was first isolated from birch (*Betula platyphylla*), showed potent anticancer as well as anti-inflammatory and anti-HIV activities [Surowiak *et al*., 2009]. Tramentenolic acid has been reported to have no antiproliferative effects against P388 cells *in vitro* as well as *in vivo* [Kahlos *et al*., 1987; Nomura *et al*., 2008]. To the best of our knowledge, this is the first report that tramentenolic acid exhibited cytotoxic activity against L1210 cell lines.

In conclusion, chemical and biological investigations of *I. obliquus* collected from Alaska were carried out in the present study for the first time. Among the 10 compounds isolated from the sclerotia of this fungus, 2,5-dihydroxybenzaldehyde (**9**) was first isolated from the present study. In addition, some of the isolated compounds exhibited weak to strong cytotoxic activities against a various cancer cell lines.

References

Charles JP (1983) In *The Aldrich Library of NMR spectra*, (2nd ed.), p. 117D, Aldrich Chemical Company, Inc., Milwaukee, WI, USA.

Chung MJ, Chung CK, Jeong Y, and Ham S (2010) Anticancer activity of subfractions containing pure compounds of Chaga mushroom (*Inonotus obliquus*) extract in human cancer cells and in Balbc/c mice bearing sarcoma-180 cells. *Nutr Res Pract* **4**, 177-182.

De Reinach-Hirtzbach F and Ourisson G (1972) Structure of inotodiol (obliquol), tetracyclic triterpene. *Tetrahedron* **28**, 2259-2266.

Ellis MB and Ellis PJ (1990) In *An Identification Handbook,* p. 329. Chapman and Hall, London, UK.

Goad LJ (1991) Phytosterols. In *Methods in Plant Biochemistry*, Bryant J, pp. 369-434. Academic Press, London, UK.

Hawksworth DL, Kirk PM, Sutton BC, and Pegler DN (1995) In *Ainsworth and Bisby's Dictionary of the Fungi,* Kirk PM, Cannon PF, David JC, and Stalpers JA (8th ed.), p. 616, CAB International, University Press, Cambridge, UK.

Huang J, Tang X, Ikejima T, Sun X, Wang X, Xi R, and Wu L (2008) A new triterpenoid from *Panax ginseng* exhibits cytotoxicity through p53 and the caspase signaling pathway in the HepG2 cell line. *Arch Pharm Res* **31**, 323-329.

Huang NL (2002) *Inonotus obliquus*. *Edible Fungi of China* **21**, 7-8.

Jin WB, Lermer L, Chilton J, Klingeman H, and Towers GH (1999) Antitumor sterols from the mycelia of *Cordyceps sinensis*. *Phytochemistry* **51**, 891-898.

Kahlos K and Hiltunen R (1983) Identification of some lanostane type triterpenes from *Inonotus obliquus*. *Acta Pharm Fenn* **92**, 220-224.

Kahlos K, Hiltunen R, and Schantz MV (1984) 3β-Hydroxylanosta-8, 24-dien-21-al, a new triterpene from *Inonotus obliquus*. *Planta Med* **50**, 197-198.

Kahlos K, Kangas L, and Hiltunen R (1987) Antitumor activity of some compounds and fractions from an *n*-hexane extract of *Inonotus obliquus*. *Acta Pharm Fenn* **96**, 33-40.

Kempska K, Ludwiczak RS, and Wrzeciono U (1962) Investigation of chemical components of *Inonotus obliquus*. VI. 3β-Hydroxylanosta-8,24-dien-21-oic acid. *Roczniki Chem* **36**, 1453-1457.

Kim HG, Yoon DH, Kim CH, Shrestha B, Chang WC, Lim SY, Lee WH, Han SG, Lee JO, Lim MH, Kim GY, Choi S, Song WO, Sung JM, Hwang KC, and Kim TW (2007) Ethanol extract of *Inonotus obliquus* inhibits lipopolysaccharide-induced inflammation in RAW 264.7 Macrophage Cells. *J Med Food* **10**, 80-89.

Kim YO, Park HW, Kim JH, Lee JY, Moon SH, and Shin CS (2006) Anti-cancer effect and structural characterization of endo-polysaccharide from cultivated mycelia of *Inonotus obliquus*. *Life Sci* **79**, 72-80.

Lee IK, Kim YS, Jang YW, Jung JY, and Yun BS (2007) New antioxidant polyphenols from the medicinal mushroom *Inonotus obliquus*. *Bioorg Med Chem Lett* **17**, 6678-6681.

Maret S (1991) Fungi in khanty folk medicine. *J Ethnopharmacol* **31**, 175-179.

Nakajima Y, Nishida H, Matsugo S, and Konishi T (2009) Cancer cell cytotoxicity of extracts and some phenolic compounds from Chaga (*Inonotus obliquus* (persoon) Pilat). *J Med Food* **12**, 501-507.

Nakajima Y, Sato Y, and Konishi T (2007) Antioxidant small phenolic ingredients in *Inonotus obliquus* (persoon) Pilat (Chaga). *Chem Pharm Bull* **55**, 1222-1226.

Nakata T, Yamada T, Taji S, Ohishi H, Wada S, Tokuda H, Sakuma K, and Tanaka R (2007) Structure determination of inonotsuoxides A and B and in vivo anti-tumor promoting activity of inotodiol from the sclerotia of *Inonotus obliquus*. *Bioorg Med Chem* **15**, 257-264.

Nomura M, Takahashi T, Uesugi A, Tanaka R, and Kobayashi S (2008) Inotodiol, a lanostane triterpenoid, from *Inonotus obliquus* inhibits cell proliferation through caspase-3-dependent apoptosis. *Anticancer Res* **28**, 2691-2696.

Park JR, Park JS, Jo EH, Hwang JW, Kim SJ, Ra JC, Aruoma OI, Lee YS, and Kang KS (2006) Reversal of the TPA-induced inhibition of gap junctional intercellular communication by Chaga mushroom (*Inonotus obliquus*) extracts: Effects on MAP kinases. *BioFactors* **27**, 147-155.

Park YM, Won JH, Kim YH, Choi JW, Park HJ, and Lee KT (2005) In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of *Inonotus obliquus*. *J Ethnopharmacol* **101**, 120-128.

Patra A, Chaudhuri SK, and Panda SK (1988) Betulin-3-caffeate from *Quercus suber*. 13C-NMR spectra of some lupenes. *J Nat Prod* **51**, 217-220.

Rzymowska J (1998) The effect of aqueous extracts from *Inonotus obliquus* on the mitotic index and enzyme activities. *Boll Chim Farm* **137**, 13-15.

Shin Y, Tamai Y, and Terazawa M (2000) Chemical constituents of *Inonotus obliquus* I. A new triterpene, 3-hydroxy-8,24-dien-lanosta- 21,23-lactone from sclerotium. *Eurasian J For Res* **1**, 43-50.

Song Y, Hui J, Kou W, Xin R, Jia F, Wang N, Hu F, Zhang H, and Liu H (2008) Identification of *Inonotus obliquus* and analysis of antioxidation and antitumor activities of polysaccharides. *Curr Microbiol* **57**, 454-462.

Surowiak P, Drag M, Materna V, Dietel M, and Lage H (2009) Betulinic acid exhibits stronger cytotoxic activity on the normal melanocyte NHEM-neo cell line than on drug-resistant and drug-sensitive MeWo melanoma cell lines. *Mol Med Rep* **2**, 543-548.

Takei T, Yoshida M, Ohnishi-Kameyama M, and Kobori M (2005) Ergosterol peroxide, an apoptosis-inducing component isolated from *Sarcodon aspratus* (Berk.) S. Ito. *Biosci Biotechnol Biochem* **69**, 212-215.

Trofimova NN, Gromova AS, Lutsky YI, Semenov AA, Avilov SA, Kalinovsky AI, Li D, and Owen NL (1998) New triterpenoid glycosides from *Thalictrum minus* L. 10. Structure of thalicosides G1 and G2. *Russ Chem Bul* **47**, 1396-1398.

Tseng T, Tseng N, and Lee Y (2001) Cytotoxicity effects of di-

and tri-hydroxybenz aldehydes as a chemopreventive potential agent on tumor cells. *Toxicology* **161**, 179-187.
Van Le TK, Hung TM, Thuong PT, Ngoc TM, Kim JC, Jang HS, Cai XF, Oh SR, Min BS, Woo MH, Choi JS, Lee HK, and Bae K (2009) Oleanane-type triterpenoids from *Aceriphyllum rossii* and their cytotoxic activity. *J Nat Prod* **72**, 1419-1423.