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## Chemical Constituents of *Inonotus obliquus* IV.

### — Triterpene and Steroids from Cultured Mycelia —

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#### Abstract

The four compounds, lanosterol, glucositol, ergosterol and ergosterol peroxide were isolated from cultured mycelia of *Inonotus obliquus*, of which components show certain antitumor activities. Ergosterol peroxide was found in different species of fungi, lichen and plants, but it was firstly found in cultured mycelia of *Inonotus obliquus*.

*Key words:* antitumor, ergosterol peroxide, *Inonotus obliquus*, lanosterol

#### Introduction

*Inonotus obliquus* is a white-rot fungus belonging to Hymenochaetaceae. This fungus is found in Hokkaido and in various alpine regions in Honshu (Yamaguchi 1989). In Eastern Europe, the sclerotium of this fungus has been used since the 16<sup>th</sup> or 17<sup>th</sup> century as a folk medicine for cancer (Kahlos 1983). Also, the Khanty of West-Siberia use this fungus to prevent and treat heart disease, liver disease, stomach disease and tuberculosis (Saar 1991).

In the previous reports (Shin *et al.* 2000 I, II, III), six triterpenes including three new triterpenes from extractives of sclerotia of *I. obliquus* have been reported. They were lanosterol (1), inotodiol (2), trametenolic acid (3), 3 $\beta$ -hydroxy-8,24-dien-lanosta-21,23-lactone (4), 21,24-cyclopenta-lanosta-3 $\beta$ ,21,25-triol-8-ene (5) and 3 $\beta$ ,22,25-trihydroxy-lanosta-8-ene (6). In the previous reports, we discussed the antitumor activity of the lanostane type triterpenoids by reviewing previous literatures dealing with antitumor activity of lanostane type triterpenoids. Compounds, such as inotodiol (2), substituted at C-22 of lanosterol (1) increased the antitumor activity, whereas compounds such as trametenolic acid (3), substituted at C-21 of lanosterol (1) decreased the activity.

Ergosterol peroxide (8)(5 $\alpha$ ,8 $\alpha$ -epidioxy-ergosta-6,22-dien-3 $\beta$ -ol) is a natural steroid that has been found in a variety of fungi, yeast, lichens and sponges (Kahlos 1996 and Diana *et al.* 1997). In fungi, ergosterol peroxide synthesized by the conversion of ergosterol to its epidioxide by the H<sub>2</sub>O<sub>2</sub>-dependent enzymatic oxidation of ergosterol. This reaction may play a role in the detoxication reaction of reactive oxygen species H<sub>2</sub>O<sub>2</sub> in neoplastic cells, which has shown tumoricidal properties (Michael 1976 and Diana *et al.* 1997).

In this paper, we describe the isolation of

ergosterol peroxide (8) with known compounds, lanosterol (1), ergosterol (7) and glucositol (9) from cultured mycelia of *I. obliquus*. Lanosterol is precursor compound of lanostane type triterpenoids and ergosterol peroxide has not previously been isolated from *I. obliquus*.

#### Materials and methods

##### 1. Cultures and growth conditions

*Inonotus obliquus* strain IO-BIHU isolated from the natural fungus was stored in PDA (potato dextrose agar) medium at 4°C. The mycelium was cultivated in MYG (malt extract: yeast: glucose, 0.5%:0.5%:2%) medium with shaking at 25°C for two months. After two months, the mycelia and culture media were separated using filter paper (Advantec No. 5C 125 mm). The mycelia were freeze-dried for 2 days. The yield of mycelia was 23.9 g.

##### 2. Extraction and isolation

The mycelia (23.9 g) were extracted five times with ethanol (EtOH) (500ml) at room temperature for 24h. The extracts were combined and concentrated under reduced pressure. The concentrated extracts (3.5 g) were successively separated on a silica gel column chromatography (CC, Wakogel C-200) with developing solvents of *n*-hexane, *n*-hexane (H): ethyl acetate (EtOAc) (HEA, 4:1, v/v), EtOAc and EtOH, successively. By monitoring with TLC using the developing solvent (SGIII), the extractive was separated into 4 fractions. Crude lanosterol, ergosterol and ergosterol peroxide were obtained from Fr.2 by using a silicagel column (Wakogel C-200) with developing solvent (HEA, 9:1, v/v).

### 3. Spectral analyses

The NMR spectra were measured on a Bruker AMX-500 ( $^1\text{H}$ :500 MHz;  $^{13}\text{C}$ :125 MHz) using deuterated chloroform ( $\text{CDCl}_3$ ) as a solvent, and tetramethylsilane (TMS) as an internal standard. Two-Dimensional (2D)-NMR was performed with  $^1\text{H}$ - $^1\text{H}$ ,  $^1\text{H}$ - $^{13}\text{C}$  COSY, HMQC and HMBC. FD-MS data were obtained by a JEOL JMS-SX102A mass spectrometer. EI-MS and EI-HR-MS spectra were obtained by a JEOL JMS-AX500 mass spectrometer. Thin-layer chromatography (TLC) was performed on a Wakogel B-10 and a Silica gel 70 plate-wako, using the developing solvents; *n*-hexane: ethyl acetate (HEA, 10:1 and 4:1, v/v) and toluene: formic acid: ethyl formate (SGIII, 5:1:4, v/v).

## Results and Discussion

### 1. Yields of isolated compounds

The yields of the isolated compounds, lanosterol (1), ergosterol (7) and compound 8 (ergosterol peroxide) were 5 mg, 12 mg and 8 mg, respectively. The glucositol was isolated as deposit from Fr.3. The yield of glucositol (9) was 35 mg.

### 2. Structure determination

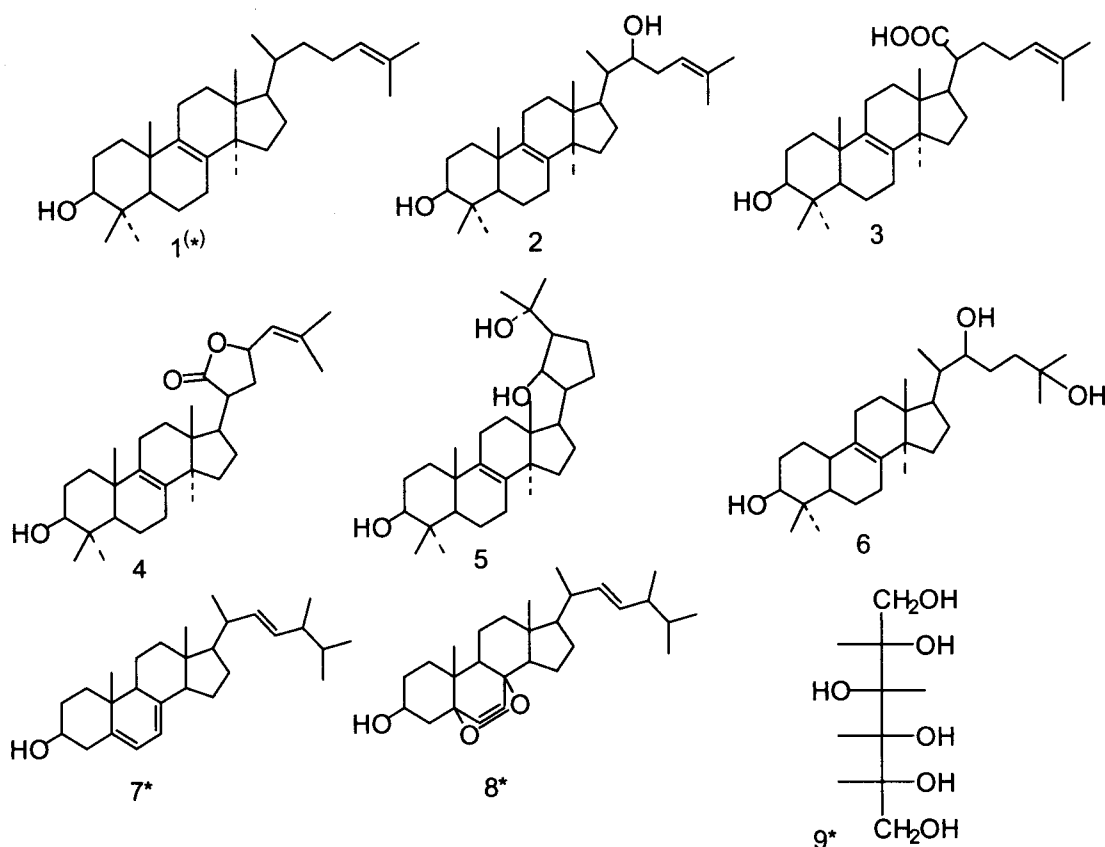
The three isolated compounds, lanosterol (1), ergosterol (7) and glucositol (9) were identified in comparison with FD-MS, EI-MS, and  $^1\text{H}$ -NMR, respectively along with  $^{13}\text{C}$ -NMR spectral data of published earlier. Compound 8 seemed to have an ergosterol type steroid structure by comparing its NMR spectral data with those of ergosterol (7). Compound 8 exhibited a molecular ion peak at  $m/z$  428 in an FD-MS spectrum and it was formulated as  $\text{C}_{28}\text{H}_{44}\text{O}_3$  by an EI-HR-MS. Ergosterol (7) has a molecular formula of  $\text{C}_{28}\text{H}_{44}\text{O}$ . The difference in molecular formula between ergosterol (7) and compound 8 is two oxygens, implying that compound 8 is a peroxidated derivative of ergosterol (7).

In the EI-MS spectrum of compound 8, the ions of  $m/z$  428(19), 396(100), 363(35) and 337(16) were assigned as  $\text{M}^+$ ,  $[\text{M}^+-\text{O}_2]$ ,  $[\text{M}^+-(\text{O}_2+\text{CH}_3+\text{H}_2\text{O})]$  and  $[\text{M}^+-(\text{O}_2+\text{C}_3\text{H}_5+\text{H}_2\text{O})]$ , respectively. The fragmentations supported the supposition that the compound 8 has a peroxy and a hydroxyl group in the molecular.

From the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data, compound 8 was identified as ergosterol peroxide (8) (Table 1). The ergosterol peroxide is first found in *Inonotus obliquus*. The chemical structure of isolated compounds is shown in Fig.1.

Table 1.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data of ergosterol peroxide (8) ( $\text{CDCl}_3$ )

	Yu et al. (1994)		Kim et al. (1997)		Compound 8	
	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$
1	-	34.7	1.71,dd, $J=13.5$ , 3.1	35.1	-	34.7
2	-	30.2	-	30.5	-	30.1
3	3.97,m	66.5	3.98,m	66.8	3.96,m	66.5
4	-	37.0	-	37.3	-	36.9
5	-	79.5	-	83.1	-	82.1
6	6.24,d, $J=8.4$	130.8	6.51,d, $J=8.6$	135.8	6.51,d, $J=8.6$	135.4
7	6.50,d, $J=8.4$	135.5	6.25,d, $J=8.6$	131.1	6.24,d, $J=8.6$	130.7
8	-	82.2	-	79.8	-	79.4
9	-	51.7	-	51.4	-	51.7
10	-	37.0	-	37.3	-	36.9
11	-	20.7	1.22,m, 1.53,m	23.8	-	20.9
12	-	39.4	1.25,m, 1.96,m	39.7	-	39.3
13	-	44.6	-	44.9	-	44.5
14	-	51.1	1.57,m	52.0	-	51.1
15	-	23.4	1.40,m, 1.65,m	21.0	-	23.4
16	-	28.7	1.35,m 1.80,m	29.0	-	28.6
17	-	56.2	1.24,m	56.5	-	56.2
18	0.82,s	12.9	0.83,s	13.3	0.82,s	12.9
19	0.88,s	18.2	0.89,s	18.6	0.88,s	18.2
20	-	39.8	2.03,m	40.1	-	39.7
21	1.00,d, $J=6.6$	20.9	1.00,d, $J=6.6$	21.3	1.00,d, $J=6.6$	19.6
22	5.23,dd, $J=15.3$ , 7.0	135.2	5.15,dd, $J=15.2$ , 7.7	135.6	5.16,d, $J=7.6$	135.2
23	5.14,dd, $J=15.3$ , 7.8	132.3	5.22,dd, $J=15.2$ , 8.2	132.4	5.20,d, $J=6.8$	132.3
24	-	42.8	1.85,m	43.1	-	42.8
25	-	33.1	1.50,m	33.4	-	33.0
26	0.82,d, $J=6.3$	19.7	0.82,d, $J=6.7$	20.0	0.82,d, $J=6.7$	19.9
27	0.83,d, $J=6.5$	20.0	0.84,d, $J=6.7$	20.3	0.84,d, $J=6.7$	20.6
28	0.91,d, $J=6.8$	17.6	0.91,d, $J=6.7$	17.9	0.91,d, $J=6.9$	17.5



1. Lanosterol  
 2. Inotodiol  
 3. Trametenolic acid  
 4. 3 $\beta$ -Hydroxy-8,24-dien-lanosta-21,23-lactone  
 5. 21,24-Cyclopenta-lanosta-3 $\beta$ ,21,25-triol-8-ene  
 6. 3 $\beta$ ,22,25-Trihydroxy-lanosta-8-ene  
 7. Ergosterol  
 8. Ergosterol peroxide  
 9. Glucositol

Fig.1. The isolated compounds from the sclerotia and cultured mycelia\* of *Inonotus obliquus*  
 The compounds with \* are isolated from the cultured mycelia

### 3. Bioassay of ergosterol and ergosterol peroxide

Reports on the antitumor activity of the ergosterol type steroids have been reviewed and the results are summarized.

Ergosterol peroxide (8) was found to both inhibit the growth and in some cases killed cancer cells. Ergosterol peroxide (8) proved to be more active against Walker 256 cells after 5 days than against MCF-7 cells as shown in Table 2 (Kahlos *et al.* 1989).

In the cytotoxic activity test, ergosterol peroxide showed a strong cytotoxic activity ( $ED_{50}$  3.42-5.16) (Kwon *et al.* 1999).

Ergosterol (7) and ergosterol peroxide (8) showed strong anticomplementary activity on a classical pathway. Ergosterol (7) was shown to be effective than ergosterol peroxide (8) in 0.5 to 12  $\mu$  M, but ergosterol peroxide (8) was shown to be more effective than ergosterol (7) from 24  $\mu$  M to 96  $\mu$  M as shown in Table 3 (Kim *et al.* 1997).

Based on the summarized results described above, it could be concluded that ergosterol peroxide (8), which is peroxidated at C-5 and C-8 of ergosterol (7), have strong antitumor activity, cytotoxic activity and anticomplementary activity.

Table 2. Effect of ergosterol peroxide (8) on cancer cells *in vitro*\*

	MCF-7 human mammary adenocarcinoma				Rat Walker 256 carcinosarcoma			
	1.0	5.0	10.0	50.0( $\mu$ g/ml)	1.0	5.0	10.0	50.0( $\mu$ g/ml)
Cultivation times								
2days	25	40	36	56	0	0	20	88
5days	3	18	60	99	2	60	99	-

\* Number: the number of killed cells(%) on the control

\* Revised based on the data reported by Kahlos (Kahlos 1989)

Table 3. Anticomplementary activity of ergosterol and ergosterol peroxide.

	0.5	1	2	6	12	24	48	96( $\mu$ M)
Ergosterol	27	48	63	76	83	86	88	90
Ergosterol peroxide	-	10	33	56	78	92	96	97

Inhibition=%, Concentration= $\mu$  M

\* Revised based on the data reported by Kim et al. (Kim et al., 1997)

\* Rosmarinic acid was used as a positive control ( $IC_{50}$ =180  $\mu$  M)

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