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Chemical Constituents of *Inonotus obliquus* I.

– A new triterpene, 3 β -hydroxy-8,24-dien-lanosta-21,23-lactone from sclerotium –

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Abstract

A new lanostane type triterpene with a lactone partial structure in the side chain was isolated from the sclerotium of the wood rotting fungus *Inonotus obliquus* together with three known compounds; lanosterol, inotodiol and trametenolic acid. The new compound was determined to be 3 β -hydroxy-8,24-dien-lanosta-21,23-lactone by spectroscopic analyses. Biogenesis of the new triterpene is proposed in relation to the compounds found in the fungus so far.

Key words: antitumor, *Inonotus obliquus*, lactone, lanostane type, triterpenes

Introduction

Inonotus obliquus (Kabano-anatake in Japanese, Charga in Russian, *Fuscoporia obliquus* in alternate taxon) is a white-rot fungus belonging to Hymenochaetaceae. The external appearance of the sclerotium of *I.obliquus* is a crusty and black surface, split up into large scales, with a brittle charcoal-like consistency (Photo. 1). The internal appearance of the sclerotium is dark brown, because the pigments produced by hyphae dye the wood tissues (Campbell and Davidson 1938 and Robert 1976). *I.obliquus* is found in Hokkaido and in a part of an alpine belt in mainland Japan (Takehiro 1989). It is known that this fungus invades mainly birch trees causing decay and eventually the death of the tree (Takehiro 1992).

In Eastern Europe, the sclerotium of this fungus has been used since the 16th or 17th century as a folk medicine (Kahlos 1983). Also, the Khanty of West-Siberia uses this fungus to prevent and treat heart disease, liver disease, stomach disease and tuberculosis (Saar 1991).

The extractives of the sclerotium of *I.obliquus* have been known to have a positive effect in controlling cancer, HIV-1 and stomach ulcers (Mizuno 1996). Antitumor experiments, concerning *n*-hexane extractives of *I.obliquus*, have been conducted and it has been reported that triterpenoids have a strong anticancer effect on walker 256 carcinosarcoma and MCF-7 human mammary adenocarcinoma (Kahlos 1986, 1987, 1988 and 1990).

In this paper, we describe the isolation of a new triterpenes, lactone (4), along with the known compounds, lanosterol (1), inotodiol (2) and trametenolic acid (3) from the sclerotium of *I.obliquus*. Biogenesis of the compound 4 is discussed in relation to the compounds isolated so far.

Material and Methods

Material

Some sclerotium (1kg) of *Inonotus obliquus* was obtained from the Hidaka local forestry office, Hokkaido, Japan, in 1996.

Extraction and Isolation

Powered sclerotium (900g) was extracted five times with 95% EtOH at room temperature for 24h. The EtOH extracts were combined and concentrated under reduced pressure. The concentrated extracts (30.2g) were successively separated on a silicagel column chromatograph (CC, Wakogel C-200) with solvents; HEA, *n*-hexane, *n*-hexane:EtOAc (25:1, v/v), EtOAc, EtOAc saturated with H₂O and EtOH, successively. By monitoring with TLC using the developing solvent (SGIII), the extractives were fractionated into 7 fractions.

Crude lanosterol (1) was obtained from Fr.3 and Fr.4 by using a silicagel column (Wakogel C-200) with HEA (10:1, v/v) solvent. Finally, the purified lanosterol (1) (76mg) was isolated using a silicagel column with HEA (15:1, v/v). Inotodiol (2) and trametenolic acid (3) were obtained from Fr.5, and purified by silicagel column chromatography using HEA (8:1 and 7:3, v/v). The yields of inotodiol (2) and trametenolic acid (3) were 56mg and 48mg, respectively. Compound 4 was isolated from Fr.6 by silicagel column chromatography using HEA (1:1,v/v). The yield of compound 4 was 6mg.

Acetylation of the isolated compounds was conducted with acetic anhydride and pyridine at 55°C for 24h.

NMR analysis

The NMR spectra were measured on a Bruker AMX-500 (¹H:500MHz; ¹³C:125MHz) using deuterated chloroform (CDCl₃), CDCl₃:CD₃OD (20:1, v/v), pentadeuterated pyridine



Photo. 1. Sclerotium of *Inonotus obliquus* on a Japanese white birch, *Betula platyphylla* var. *japonica*

(C_5D_5N) as solvents, and tetramethylsilane (TMS) as an internal standard. A Two-Dimensional (2D)-NMR was performed with 1H - 1H COSY, HMQC and HMBC. A FD-MS was obtained by a JEOL JMS-SX102A mass spectrometer. An EI-MS and an EI-HR-MS were obtained by a JEOL JMS-AX500 mass spectrometer. A thin-layer chromatography (TLC) was performed on a Wakogel B-10 and a Silicagel 70 plate-wako, using the solvents; *n*-hexane: EtOAc (HEA, 1:1, v/v), $CHCl_3$: CH_3OH (CM, 10:1, v/v) and toluene:formic acid:ethyl formate (SGIII, 5:1:4, v/v).

Physico-chemical properties

Compound 1 (*lanosterol*) isolated as white powder: Rf value on TLC(SGIII) was 0.81; FD-MS m/z 426; EI-HR-MS m/z 426.3881, $C_{30}H_{50}O$; EI-MS m/z(rel.int): M^+ 426(35), 412(31), 411(100)[$M^+ - CH_3$], 394(17), 393(55) [$M^+ - (CH_3 + H_2O)$], 273(7), 255(9), 241(13), 109(44), 69(66), 55(25), 41(21); 1H -NMR (in $CDCl_3$) δ = 0.691 (s, 3H, 18-Me), 0.812 (s, 3H, 29-Me), 0.876 (s, 3H, 30-Me), 0.913 (d, 3H, 21-Me), 0.980 (s, 3H, 19-Me), 1.001 (s, 3H, 28-Me), 1.050 (m, 1H, 5-H), 1.400 (m, 1H, 20-H), 1.480 (m, 1H, 17-H), 1.603 (s, 3H, 27-Me), 1.683 (s, 3H, 26-Me), 3.24, 3.22 (dd, J = 4.43, 1H, 3-CHOH), 5.10 (t, 1H, 24-H); ^{13}C -NMR (in $CDCl_3$): see Table 1.

Acetate of compound 1: FD-MS m/z 468; 1H -NMR (in $CDCl_3$) δ = 0.69 (s, Me), 0.87 (s, Me), 0.88 (s, 2Me), 0.91 (d, Me), 1.00 (s, Me), 1.13 (m, 1H), 1.43 (m, 1H), 1.49 (m, 1H), 1.60 (s, Me), 1.68 (s, Me), 4.51, 4.49 (dd, J = 4.43, 1H, CHOAc), 5.10 (t, 1H).

Compound 2 (*Inotodiol*) isolated as white powder: Rf value on TLC(SGIII) was 0.72; FD-

MS m/z 442; EI-HR-MS m/z 442.3817, $C_{30}H_{50}O_2$; EI-MS m/z(rel.int): M^+ 442(35), 427(32) [$M^+ - CH_3$], 409(21) [$M^+ - (CH_3 + H_2O)$], 372(28), 357(50) [$M^+ - (CH_3 + H_2O + C_8H_{13}O_2 + H^+)$], 339(17), 273(9), 255(11), 109(39), 99(100), 69(63), 55(34), 41(33); 1H -NMR (in $CDCl_3$: CD_3OD , 20:1, v/v) δ = 0.72 (s, 3H, 18-Me), 0.81 (s, 3H, 29-Me), 0.88 (s, 3H, 30-Me), 0.94 (d, 3H, 21-Me), 0.98 (s, 3H, 28-Me), 0.99 (s, 3H, 19-Me), 1.050 (m, 1H, 5-H), 1.57 (m, 1H, 17-H), 1.57 (s, 3H, 26-Me), 1.66 (s, 3H, 27-Me), 1.80 (m, 1H, 20-H), 3.24, 3.21 (dd, J = 4.43, 1H, 3-CHOH), 3.65 (m, 1H, 22-CHOH), 5.19 (t, 1H, 24-H); ^{13}C -NMR (in $CDCl_3$): see Table 1.

Acetate of compound 2: FD-MS m/z 526; 1H -NMR (in $CDCl_3$) δ = 0.68 (s, Me), 0.86 (s, Me), 0.88 (s, 2Me), 0.94 (d, Me), 1.00 (s, Me), 1.15 (m, 1H), 1.34 (m, 1H), 1.51 (m, 1H), 1.62 (s, Me), 1.68 (s, Me), 4.51, 4.49 (dd, J = 4.43, 1H), 4.91 (m, 1H, CHOAc), 5.10 (t, 1H).

Compound 3 (*Trametenolic acid*) isolated as white powder: Rf value in SGIII was 0.72; FD-MS m/z 456; EI-HR-MS m/z 456.3593, $C_{30}H_{48}O_3$;

EI-MS m/z(rel.int): M^+ 456(56), 441(68) [$M^+ - CH_3$], 423(100) [$M^+ - (CH_3 + H_2O)$], 395(12), 299(11), 281(18), 273(6), 255(7), 109(20), 95(32), 69(34), 55(25), 41(26); 1H -NMR (in $CDCl_3$: CD_3OD , 20:1, v/v) δ = 0.76 (s, 3H, 18-Me), 0.80 (s, 3H, 29-Me), 0.89 (s, 3H, 30-Me), 0.97 (s, 3H, 19-Me), 0.99 (s, 3H, 28-Me), 1.04 (m, 1H, 5-H), 1.40 (m, 1H, 17-H), 1.59 (s, 3H, 26-Me), 1.68 (s, 3H, 27-Me), 3.21, 3.19 (dd, J = 0.01, 1H, 3-CHOH), 5.19 (t, 1H, 24-H); ^{13}C -NMR (in $CDCl_3$): see Table 1.

Acetate of compound 3: FD-MS m/z 498; 1H -NMR (in $CDCl_3$) δ = 0.88 (s, 2Me), 0.89 (s, Me),

0.94(s,Me), 1.13(s,Me), 1.07(m,1H), 1.11(m,1H), 1.38(m,1H), 1.58(s,Me), 1.67(s,Me), 4.52, 4.50(dd, $J=4.43, 1\text{H}, \text{CHOAc}$), 5.08(t,1H)

Compound 4 (*3 β -hydroxy-8,24-dien-lanosta-21,23-lactone*) isolated as white powder; Rf value on SGIII was 0.44; FD-MS m/z 454; EI-HR-MS m/z 454.3442, $\text{C}_{30}\text{H}_{46}\text{O}_3$; $^1\text{H-NMR}$ (in CDCl_3) δ = 0.79(s,3H,Me), 0.81(s,3H,Me), 0.93(s,3H,Me), 0.98(s,3H,Me), 1.00(s,3H,Me), 1.74(s,3H,Me), 1.76(s,3H,Me), 2.05(m,2H), 2.25(m,1H), 2.68(m,1H), 3.22, 3.24(dd, $J=4.43, 1\text{H}, 3\text{-CHOH}$), 5.18(m,1H), 5.21(t,1H); $^{13}\text{C-NMR}$ (in CDCl_3): see Table 1.

Acetate of compound 4: FD-MS m/z 496.

Results and Discussion

The compounds were examined by FD-MS, EI-MS, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, DEPT, $^1\text{H-}^1\text{H COSY}$, HMQC and HMBC spectrometry. Spectral data of the compounds 1, 2, 3 and 4 suggest that these compounds are lanostane type triterpenes.

Compound 1

(*3 β -hydroxy-lanosta-8,24-diene, lanosterol*)

Compound 1 exhibited a molecular ion peak at m/z 426 in a FD-MS spectrum. Its

formula was $\text{C}_{30}\text{H}_{50}\text{O}$ by an EI-HR-MS, implying that compound 1 is a triterpene. The molecular ion of the acetate compound 1 was m/z 468. The difference in the molecular ions between compound 1 (426) and its acetate (468) was m/z 42, which corresponded to one acetyl unit, indicating that compound 1 has a hydroxyl group in its structure.

On the EI-MS spectrum of compound 1, the parent ion appeared at m/z 426 and the fragment ions of m/z 411, 408 and 393 were assigned as $[\text{M}^+ - \text{CH}_3]$, $[\text{M}^+ - \text{H}_2\text{O}]$ and $[\text{M}^+ - (\text{CH}_3 + \text{H}_2\text{O})]$, respectively. The appearance of an ion at m/z 69 revealed the presence of an isopentenyl group derived by allylic cleavage. An ion at m/z 109 was due to the formation of a diene fragment after the cleavage of the side chain. Fragment ions m/z 255 and 273 were specific ions derived from lanostane type triterpenes due to the cleavage of ring D, with the side chain bearing a hydroxyl group. The fragmentation pattern of compound 1 in EI-MS is shown in Fig.1.

In the $^1\text{H-NMR}$ spectrum of compound 1, five singlets and one doublet representing the protons of six methyl groups appeared at δ 0.69 ~ 1.00. Two singlets at δ 1.60 and 1.68 corresponded to the protons of two methyl groups of an isopentenyl group. Three multiplets

Table 1. $^{13}\text{C-NMR}$ spectral data of the isolated compounds (in CDCl_3)

Carbon	Lanosterol(1)	Inotodiol(2)	Trametenolic acid(3)	Compound 4
1	36.0	35.6	35.9	35.6
2	28.3	27.7	28.9	28.5
3	79.4	79.0	78.9	78.9
4	39.3	38.9	39.0	38.9
5	50.8	50.5	50.6	50.4
6	18.7	19.2	18.4	18.2
7	26.9	26.6	27.6	27.8
8	134.8	134.7	134.7	135.0
9	134.8	134.3	134.3	133.8
10	37.4	37.1	37.2	37.1
11	21.4	21.0	21.0	20.8
12	31.4	29.1	30.6	30.6
13	44.9	44.9	44.4	44.7
14	50.2	49.5	49.6	49.5
15	31.3	31.0	32.8	29.7
16	28.6	31.0	27.3	26.5
17	50.8	47.3	48.1	41.4
18	15.8	15.7	16.0	17.0
19	19.5	18.3	19.2	19.1
20	36.7	41.8	47.3	44.7
21	19.0	12.6	179.6	178.9
22	36.7	73.5	26.6	34.4
23	25.3	27.3	26.1	75.0
24	125.7	121.4	124.0	123.4
25	131.3	135.0	132.2	139.3
26	26.1	26.0	25.7	25.7
27	17.9	18.0	17.6	18.4
28	28.4	28.0	28.0	28.0
29	16.2	15.5	15.5	15.4
30	24.6	24.3	24.4	24.4

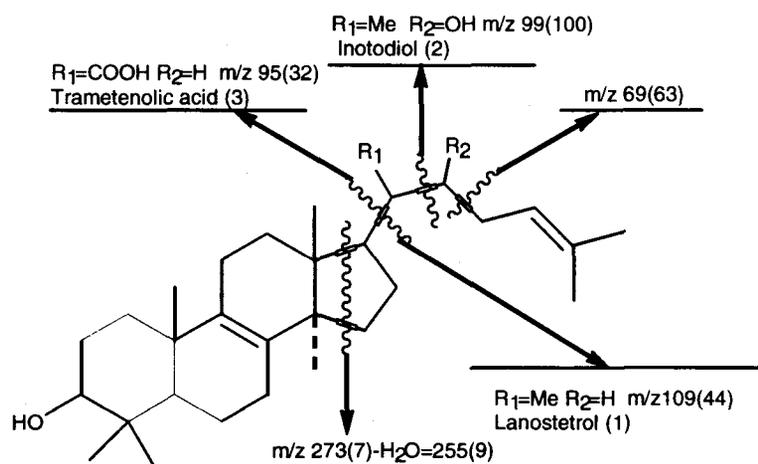


Fig.1. Fragmentation pattern of lanosterol (1), inotodiol (2) and trametenolic acid (3) in EI-MS.

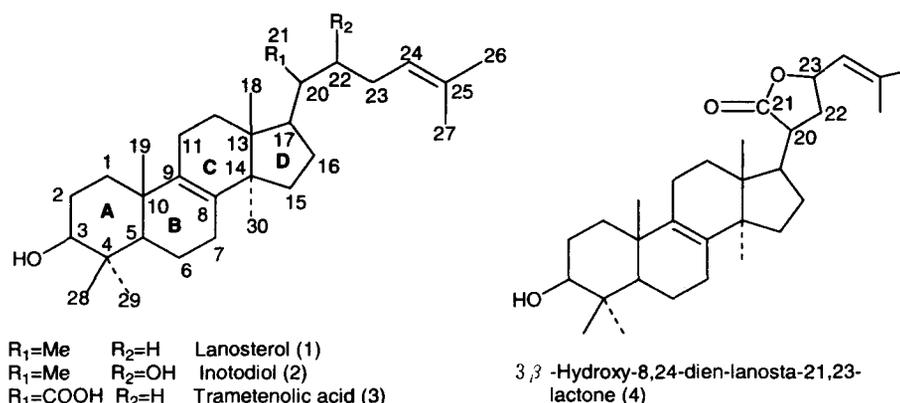


Fig. 2. The structures of the isolated compounds, 1, 2, 3 and 4.

at δ 1.05(m), 1.40(m) and 1.48(m) were assigned to three methine groups. One doublet at δ 3.23 ($J=4.43$) bearing a hydroxyl group was assigned to one methine group. One triplet that appeared at δ 5.10 showed the presence of an olefinic proton displaying one methylene group at its adjacent carbon.

In the ^{13}C -NMR and DEPT spectra of compound 1, the signals of eight methyl groups appeared in δ 15.8 ~ 28.4, supporting the results obtained by ^1H -NMR. Also, the signals appeared in a slightly lower field (18.7, 36.7) than those of methyl groups that contained ten methylene groups. The five signals at δ 36.7, 50.8, 50.8, 79.4 and 125.7 showed the presence of five methine groups. The signal at δ 125.7 was derived from olefin carbon, corresponding to the signal of the olefinic proton found at δ 5.10(t) in ^1H -NMR. And also the signal at δ 79.4 corresponded to a methine group bearing a hydroxyl group. The four signals of quaternary carbons appeared at δ 50.2, 44.9, 39.3 and 37.4, respectively. The three signals, which

appeared in the low field at δ 131.3, 134.8 and 134.8, were derived from two double bonds.

From the data above, compound 1 was determined to be 3 β -hydroxy-lanosta-8,24-diene. By comparing spectral data from the literature on lanosterol type triterpenes (Kahlos 1983), compound 1 was identified to be lanosterol (1) (Fig. 2).

The chemical shift of δ 79.4 in ^{13}C -NMR suggested that the hydroxyl group at C-3 is located on β -position because the chemical shift of C-3 of 3 α -OH derivatives (δ 76.0) shielded lower field 2.8ppm more than that of C-3 of 3 β -OH derivatives (δ 78.8) (Asakawa 1977).

Compound 2

(3 β -hydroxy-lanosta-8,24-diene, lanosterol)

Compound 2 showed a molecular ion peak at m/z 442 in a FD-MS spectrum. Its formula was $\text{C}_{30}\text{H}_{50}\text{O}_2$ by an EI-HR-MS. The molecular ion of the acetate of compound 2 was m/z 526.

The difference in the molecular ions between compound 2 (442) and its acetate (526) was m/z 84, which corresponded to two acetyl groups, showing that compound 2 has two hydroxyl groups in its structure.

Comparing spectral data of compound 2 with those of compound 1, suggested that compound 2 has a lanosterol type partial structure, except at the side chain (Table 1). In the EI-MS spectrum of compound 2, a fragment ion at m/z 99(100), which did not appear in compound 1, did appear in compound 2. This result supported our belief that the compound has a partial structure bearing a hydroxyl group at the side chain.

In the $^1\text{H-NMR}$ spectrum of compound 2, proton signals due to two methyl groups of an isopentenyl group (C-26 and C-27, s), an olefinic (C-24, t), one methine (C-20, m) and one methyl group (C-21, dd) were observed. One methine group bearing a hydroxyl group, which did not appear in compound 1, did appear at δ 3.65(m) in compound 2. The data suggested that the compound has a hydroxyl group at C-22, which was supported by $^{13}\text{C-NMR}$ spectral data (Table 1).

From the data above, compound 2 was determined to be 3β ,22-dihydroxy-lanosta-8,24-diene. By comparing the spectral data of compound 2 with those from the literature on lanosterol type triterpenes (Kahlos 1983), compound 2 was identified as inotodiol (2) (Fig. 2). The fragmentation pattern of compound 2 in EI-MS is shown in Fig.1. The chemical shift of δ 79.0 in $^{13}\text{C-NMR}$ indicated that the hydroxyl group at C-3 is a β -substitution (Asakawa 1977).

Compound 3

(3 β -hydroxy-lanosta-8,24-dien-21-oic acid, trametenolic acid)

Compound 3 exhibited a molecular ion peak at m/z 456 in a FD-MS spectrum. Its formula was $\text{C}_{30}\text{H}_{48}\text{O}_3$ by an EI-HR-MS. The molecular ion of the acetate of compound 3 was m/z 498 and the difference in the molecular ions of compound 3 (456) and its acetate (498) was m/z 42, which corresponds to one acetyl group, showing that compound 3 has a hydroxyl group in its structure.

Comparing the spectral data of compound 3 with those of compound 1 suggested that compound 3 has a lanostane type partial structure, except at the side chain (Table 1). In the EI-MS spectrum, a fragment ion of m/z 95 (32), which did not appear in compound 1, did appear in compound 3, suggesting that it has a partial structure bearing a carboxyl group at the side chain.

In the $^1\text{H-NMR}$ spectral data of compound 3, proton signals due to two methyl groups of an isopentenyl group (C-26 and C-27, s), an olefinic (C-24, t), one methine (C-20, m) and two

methylene groups (C-22 and C-23, m) were observed. One methyl group (C-21, dd), which appeared in compound 1, did not appear in compound 3. The data suggested that the compound substitutes a structure at C-21. In the $^{13}\text{C-NMR}$ spectral data of compound 3, one chemical shift representing a carboxyl group appeared at δ 179.6. The result supported our belief that compound 3 has a carboxyl group at C-21 in the side chain.

From the data above, compound 3 was determined to be 3β -hydroxy-lanosta-8,24-dien-21-oic acid. By comparing the spectral data of compound 3 with those from the literature on lanosterol type triterpenes (Kahlos 1983), compound 3 was identified to be trametenolic acid (3), which has a carboxyl group at C-21 in the side chain (Fig. 2). The EI-MS fragmentation pattern of trametenolic acid (3) is shown in Fig.1. The chemical shift of δ 78.9 in $^{13}\text{C-NMR}$ indicated that the hydroxyl group at C-3 is a β -substitution.

Compound 4

(a new triterpene, 3 β -hydroxy-8,24-dien-lanosta-21,23-lactone)

Compound 4 was also a lanosterol type triterpene which was determined by comparing its spectral data with those of trametenolic acid (3) (Table 1). The compound exhibited a molecular ion peak at m/z 454 in a FD-MS spectrum and its formula is $\text{C}_{30}\text{H}_{46}\text{O}_3$ by an EI-HR-MS. Trametenolic acid (3) had a molecular formula of $\text{C}_{30}\text{H}_{48}\text{O}_3$.

The difference in molecular weight between trametenolic acid (3) and compound 4 was 2H, implying that compound 4 is a lactone of trametenolic acid (3). The molecular ion peak of the acetate of compound 4 was m/z 496. The difference in the molecular weight of the compound 4 and its acetate was 42, which corresponded to one acetyl group (42), indicating that compound 4 has a hydroxyl group.

In the $^1\text{H-NMR}$ spectrum of compound 4, six protons of two methyl groups of isopentenyl group (C-26 and C-27) and a olefinic proton (C-24) appeared in a slightly lower field (δ 1.74, 1.76 and 5.21) than those of trametenolic acid (3) (δ 1.59, 1.68 and δ 5.10). A peak derived from one methine proton(C-20) and peaks derived from methylene protons(C-22) appeared at δ 2.23 and δ 1.90-2.1, respectively. The data was considered with those of trametenolic acid (3). The peak corresponding to the other single methine proton(C-23) appeared at a lower field of δ 2.68(m) in compound 4 than that of trametenolic acid (3). The one methylene proton (δ 1.40-1.60,m), which existed in the spectrum of the trametenolic acid (3), was not observed in the spectrum of compound 4. Instead, one methine proton appeared at a lower field of δ 5.18(m).

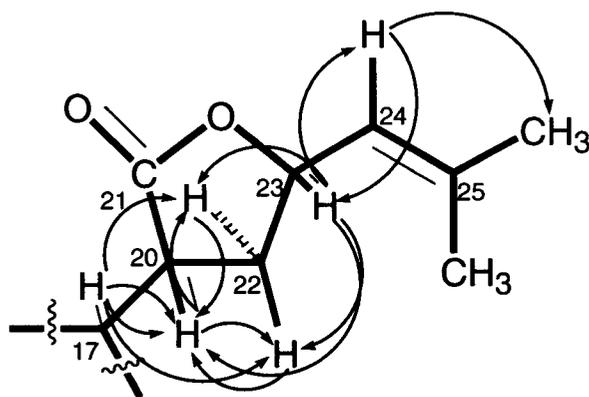
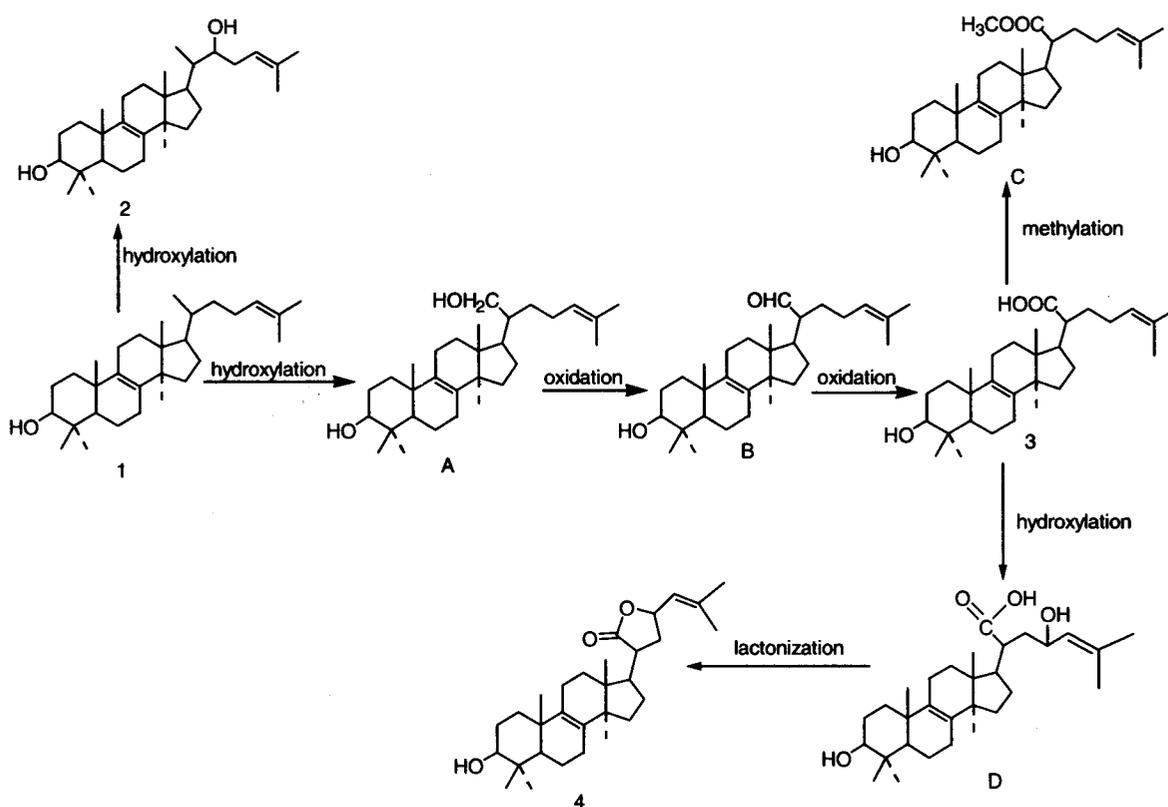


Fig. 3. The correlation of the partial structure of compound 4 in ^1H - ^1H COSY.



1. Lanosterol 2. Inotodiol 3. Trametenolic acid 4. 3β -Hydroxy-8,24-dien-lanosta-21,23-lactone
 A. $3\beta,21$ -Dihydroxy-lanosta-8,24-diene B. 3β -Hydroxy-lanosta-8,24-dien-21-al
 C. Methyl trametenolate D. Not isolated

Fig. 4. A proposed biogenesis of compound 4, a new triterpene, in relation to the compounds isolated.

In the ^{13}C -NMR, a peak representing a carboxyl group that appeared at δ 179.6 in trametenolic acid (3), was also observed at δ 178.9 in compound 4. Also, a signal of δ 75.0, representing a methine group bearing an oxygen (C-23), appeared in compound 4.

In the 2D homonuclear chemical shift correlated spectroscopy (^1H - ^1H COSY) of compound 4, a methine proton (C-23, δ 5.18, m) on the side chain correlated with the peak of an

olefinic methine proton at δ 5.21 (C-24) and with the peaks of methylene protons at δ 1.90-2.10 (C-22). Also, a correlation between the two methine protons at δ 2.23 (C-20) and δ 2.68 (C-17) was observed. One methine proton at δ 2.68 (C-17) correlated with methylene protons at δ 1.90-2.10 (C-22). Also, a correlation between the two methine protons, at δ 2.23 (C-20) and δ 5.18 (C-23), was observed. The data showed the partial structure of the lactone of compound

Table 2. Effect of lanostane type triterpenoids on cancer cells *in vitro*

	Rat Walker 256 carcinoma* ¹			MCF-7 Human mammary adenocarcinoma* ²		
	1.0	10.0	50.0($\mu\text{g}\cdot\text{ml}^{-1}$)	1.0	10.0	50.0($\mu\text{g}\cdot\text{ml}^{-1}$)
C-22 substituted						
Lanosterol (1)	-	-	-	48	63	90
Inotodiol (2)	7	45	98	48	70	100
C-21 substituted						
3 β ,21-Dihydroxy-lanosta-8,24-diene (A)	13	18	17	-	-	-
3 β -Hydroxy-lanosta-8,24-dien-21-al (B)	-	-	-	20	46	72
Trametenolic acid (3)	-	-	-	15	14	25
Methyl trametenolate (C)	-	-	-	24	76	75

- The data were revised based on the data reported by Kahlos (Kahlos 1987). Activity of the compounds is expressed % of the killed cells.
- Compounds were dissolved in ethanol and 100 μl of cell suspension added. The concentration of the compounds in the culture medium ranged 1.0, 10.0 and 50.0 $\mu\text{g/ml}$. Ethanol concentration never exceeded 0.07%. The quantity of living cells was determined by the bioluminescence method (Kangas *et al.* 1984). The number of living cells (ATP level as mV) in the culture medium was recorded at intervals of two days(*¹) and five days(*²).

4 (Fig. 3).

On the basis of the data, we concluded that compound 4 is 3 β -hydroxy-8,24-dien-lanosta-21,23-lactone (Fig. 2). The chemical shift of δ 78.9 in ¹³C-NMR indicated that the hydroxyl group at C-3 is a β -substitution.

The proposed biogenesis of compound 4, in relation to the compounds isolated so far, is: Lanosterol (1) is hydroxylated at C-21 to yield 3 β ,21-dihydroxy-lanosta-8,24-diene (A) (Kahlos 1983). 3 β , 21-Dihydroxy-lanosta-8,24-diene (A) is oxidized to yield 3 β -hydroxy-lanosta-8,24-dien-21-al (B) (Kahlos 1984). Also, 3 β -hydroxy-lanosta-8,24-dien-21-al (B) is oxidized to yield trametenolic acid (3). Trametenolic acid (3) is methylated to yield methyl trametenolate (C) (Kahlos 1983). When trametenolic acid (3) is hydroxylated at C-23 on the side chain, it yields an intermediate (D), and successive lactonization between the carboxyl group at C-21 and the hydroxyl group at C-23 of the intermediate (D), forms the lactone partial structure of compound 4, 3 β -hydroxy-8,24-dien-lanosta-21,23-lactone (4) (Fig. 4). Although the hydroxylated intermediate (D) has not been isolated so far, the hydroxylation at C-23 might be similar to the hydroxylation at C-22 of lanosterol (1) resulting in the formation of inotodiol (2).

Antitumor activity of lanostane type triterpenoids

Reports on the antitumor activity of the lanostane type triterpenoids have been searched and the results are summarized in Table 2.

Against Walker 256 carcinoma, Inotodiol (2), which a hydroxyl group is substituted at C-22 of lanosterol (1), shows a strong activity (98%) at the highest concentration of 50.0 $\mu\text{g/ml}$. In contrast, 3 β , 21-dihydroxy-lanosta-8,24-diene (A), which a

hydroxyl group is substituted at C-21 of lanosterol (1), shows a relatively low activity (13-18%).

Against MCF-7 human mammary adenocarcinoma, lanosterol (1) is able to kill 90% of the cells at the highest concentration of 50 $\mu\text{g/ml}$. Inotodiol (2), which a hydroxyl group is substituted at C-22 of lanosterol (1), shows a strong activity (100%) at the highest concentration of 50 $\mu\text{g/ml}$. On the other hand, trametenolic acid (3), which a carboxylic group is substituted at C-21 lanosterol (1), shows a low activity (25%). However, methylation of trametenolic acid (3), increases the activity from that of the original acid (25%) to 75% at the 50 $\mu\text{g/ml}$. 3 β -Hydroxy-lanosta-8,24-dien-21-al (B) shows 72% activity.

Based on the summarized results described above, it could be concluded that substitution at C-22 of lanosterol (1) increases the activity (inotodiol 100%) and in contrast, substitution at C-21 of lanosterol (1) decreases the activity (75-25%). The degree of inactivation seems to be depending on the polarity of the substituted functional groups. Namely, substitution by polar functional groups such as hydroxyl and carboxyl groups results in the drastic inactivation (0-25%) but substitution by non-polar functional groups such as aldehyde and methylester results in retaining the activity relatively (72-75%).

The assay of the biological activity of the new triterpen, compound 4 is an interesting problem to be searched in future.

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