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An unusual lanostane-type triterpenoid, spiroinonotsuoxodiol, and other triterpenoids from *Inonotus obliquus*

Noriko Handa, Takeshi Yamada, Reiko Tanaka *

Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

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ABSTRACT

An unusual lanostane-type triterpenoid, spiroinonotsuoxodiol (**1**), and two lanostane-type triterpenoids, inonotsudiol A (**2**) and inonotsuoxodiol A (**3**), were isolated from the sclerotia of *Inonotus obliquus*. Their structures were determined to be (3*S*,7*S*,9*R*)-3,7-dihydroxy-7(8 → 9)*abeo*-lanost-24-en-8-one (**1**), lanosta-8,24-dien-3β,11β-diol (**2**), and (22*R*)-3β,22-dihydroxylanosta-8,24-dien-11-one (**3**) on the basis of NMR spectroscopy, including 1D and 2D (^{1}H–^{1}H COSY, NOESY, HMQC, HMBC) NMR, and FABMS. Compounds **1**–**3** showed moderate activity against cultured P388, L1210, HL-60 and KB cells.

1. Introduction

Inonotus obliquus (PERS.: Fr.) Pil. (=*Fuscoporia obliqua* (PERS.: Fr.) Aoshima), called 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 thrives in Europe, Asia, and North America (Ellis and Ellis, 1990). It is widely distributed in *Betula platyphylla* var. *japonica* (Japanese name: shirakaba) forests in Hokkaido, Japan (Mizuno et al., 1999). In Eastern Europe, particularly Russia, the sclerotia of this mushroom have been used as folk medicine for cancer treatments since the 16th or 17th century (Shivrina, 1965). Recently, the extract of this fungus was reported to possess anti-tumor (Nakajima et al., 2009; Song et al., 2008), anti-oxidant (Kim et al., 2008; Ham et al., 2009; Zheng et al., 2009; Hu et al., 2009), anti-inflammatory (Kim et al., 2007), and anti-diabetic (Lee et al., 2006) activities. Previously, we reported the structures of new lanostane-type triterpenoids isolated from the sclerotia of this mushroom: inonotsuoxides A and B (Nakata et al., 2007), inonotsulides A, B, and C (Taji et al., 2007), inonotsutriols A, B, and C (Taji et al., 2008a), lanosta-8,23*E*-diene-3β,22*R*,25-triol, and lanosta-7:9(11),23*E*-triene-3β,22*R*,25-triol (Taji et al., 2008b), as well as the anti-tumor promoting activities of the most abundant triterpene, inotodiol, and 3β-hydroxylanosta-8,24-dien-21-al. In addition, we reported that inotodiol inhibits cell proliferation through caspase-3-dependent apoptosis (Nomura et al., 2008). Careful examination of the sclerotia of *I. obliquus* has led to the isolation of an unusual lanostane-type triterpene named spiroinonotsuoxodiol (**1**) and two new lanostane-type triterpenoids, inonotsudiol A (**2**) and inonotsuoxodiol A (**3**). The structures of new compounds **1–3** were determined on the basis of NMR spectroscopy, including 1D and 2D (^{1}H, ^{1}H COSY, NOESY, HMQC, HMBC) NMR, and FABMS. The cytotoxicity of these compounds was examined by using the murine P388 leukemia, murine L1210 leukemia, human HL-60 leukemia, and human KB epidermoid carcinoma cell lines. In this paper, the structures and biological activities of the compounds **1**–**3** are described.

2. Results and discussion

The sclerotia of *I. obliquus* were extracted with $CHCl_3$ and the extract was subjected to silica gel column chromatography, medium-pressure liquid chromatography (MPLC), and C_{18} reversed-phase high-pressure liquid chromatography (HPLC) to yield three new triterpenoids (**1**–**3**).

The molecular formula of spiroinonotsuoxodiol (**1**) was determined to be $C_{30}H_{50}O_3$ (M^+; *m/z* 458.3757) on the basis of HRFABMS. The IR spectrum showed bands assignable to a hydroxy group (ν_{max} 3448 cm^{-1}) and a six-membered ring ketone (ν_{max} 1687 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **1** (Tables 1 and 2)

* Corresponding author. Tel./fax: +81 72 690 1084.
E-mail address: tanakar@gly.oups.ac.jp (R. Tanaka).

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Table 1
^{1}H NMR spectroscopic data for compounds **1**, **1a**, **2**, and **3** (500 MHz, $CHCl_3$).[a]

H	1	1a	2	3
1α	1.97 (*m*)	1.66 (*m*)	1.39 (*m*)	1.06 (*m*)
1β	1.64 (*m*)	2.00 (*m*)	2.14 (*m*)	3.01 (*dt*)
2α	1.70 (*m*)	1.73 (*m*)	1.66 (*m*)	1.68 (*m*)
2β	1.79 (*m*)	1.82 (*m*)	1.72 (*m*)	1.63 (*m*)
3	3.22 (*dd*)	4.46 (*dd*)	3.26 (*dd*)	3.24 (*dd*)
5	1.36 (*dd*)	1.45 (*m*)	0.95 (*m*)	0.90 (*d*)
6α	2.25 (*m*)	2.25 (*dt*)	1.70 (*m*)	1.75 (*m*)
6β	1.58 (*dt*)	1.57 (*m*)	1.56 (*m*)	1.46 (*m*)
7α	4.30 (*br s*)	4.31 (*m*)	2.09 (*m*)	2.27 (*dd*)
7β	–	–	2.12 (*m*)	2.36 (*dd*)
11α	2.00 (*m*)	1.56 (*m*)	4.65 (*dt*)	–
11β	1.50 (*m*)	1.26 (*m*)	–	–
12α	1.58 (*m*)	2.01 (*m*)	1.92 (*m*)	2.66 (*m*)
12β	1.75 (*m*)	1.78 (*m*)	2.32 (*d*)	2.48 (*d*)
15α	1.20 (*m*)	1.20 (*m*)	1.21 (*m*)	1.37 (*m*)
15β	1.85 (*m*)	1.86 (*m*)	1.58 (*m*)	1.80 (*m*)
16α	1.93 (*m*)	1.93 (*m*)	1.95 (*m*)	1.87 (*m*)
16β	1.30 (*m*)	1.30 (*m*)	1.37 (*m*)	1.50 (*m*)
17	1.63 (*m*)	1.63 (*m*)	1.52 (*m*)	1.81 (*m*)
18	0.66 (*s*)	0.65 (*s*)	0.86 (*s*)	0.86 (*s*)
19	1.46 (*s*)	1.49 (*s*)	1.11 (*s*)	1.13 (*s*)
20	1.41 (*m*)	1.41 (*m*)	1.42 (*m*)	1.81 (*m*)
21	0.91 (*d*)	0.92 (*d*)	0.99 (*d*)	0.92 (*d*)
22A	1.05 (*m*)	1.05 (*m*)	1.03 (*m*)	3.65 (*dt*)
22B	1.43 (*m*)	1.41 (*m*)	1.45 (*m*)	–
23A	1.86 (*m*)	1.89 (*m*)	1.87 (*m*)	2.05 (*m*)
23B	2.03 (*m*)	2.03 (*m*)	2.04 (*m*)	2.08 (*m*)
24	5.09 (*m*)	5.09 (*m*)	5.10 (*t*)	5.17 (*m*)
26	1.69 (*s*)	1.69 (*s*)	1.69 (*s*)	1.75 (*s*)
27	1.60 (*s*)	1.60 (*s*)	1.61 (*s*)	1.66 (*s*)
28	0.95 (*s*)	0.84 (*s*)	1.00 (*s*)	1.02 (*s*)
29	0.95 (*s*)	1.01 (*s*)	0.84 (*s*)	0.83 (*s*)
30	1.20 (*s*)	1.20 (*s*)	0.87 (*s*)	1.10 (*s*)

[a] Assignments were based on ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY experiments.

Table 2
^{13}C NMR spectroscopic data for compounds **1**, **1a**, **2** and **3** (125 MHz, $CHCl_3$).[a]

Position	1	1a	2	3
1	30.6 (*t*)	30.3 (*t*)	34.7 (*t*)	34.3 (*t*)
2	28.2 (*t*)	24.3 (*t*)	27.7 (*t*)	28.0 (*t*)
3	79.7 (*d*)	81.0 (*d*)	79.2 (*d*)	78.7 (*d*)
4	38.2 (*s*)	37.3 (*s*)	39.0 (*s*)	39.0 (*s*)
5	50.2 (*d*)	50.2 (*d*)	50.9 (*d*)	51.8 (*d*)
6	34.0 (*t*)	33.9 (*t*)	18.0 (*t*)	17.3 (*t*)
7	80.6 (*d*)	80.4 (*d*)	26.6 (*t*)	29.9 (*t*)
8	215.6 (*s*)	215.5 (*s*)	142.8 (*s*)	163.9 (*s*)
9	64.1 (*s*)	64.1 (*s*)	133.8 (*s*)	139.6 (*s*)
10	48.9 (*s*)	48.6 (*s*)	37.3 (*s*)	37.7 (*s*)
11	29.9 (*t*)	29.9 (*t*)	81.6 (*d*)	199.0 (*s*)
12	30.7 (*t*)	30.7 (*t*)	36.5 (*t*)	51.8 (*t*)
13	47.5 (*s*)	47.6 (*s*)	42.3 (*s*)	47.4 (*s*)
14	61.2 (*s*)	61.2 (*s*)	51.0 (*s*)	51.2 (*s*)
15	29.6 (*t*)	29.6 (*t*)	30.7 (*t*)	31.1 (*t*)
16	27.0 (*t*)	27.0 (*t*)	28.1 (*t*)	26.1 (*t*)
17	50.3 (*d*)	50.3 (*d*)	49.9 (*d*)	46.9 (*d*)
18	17.0 (*q*)	16.9 (*q*)	16.8 (*q*)	16.6 (*q*)
19	18.4 (*q*)	18.5 (*q*)	21.8 (*q*)	19.0 (*q*)
20	35.4 (*d*)	35.3 (*d*)	36.1 (*d*)	41.3 (*d*)
21	18.6 (*q*)	18.7 (*q*)	18.7 (*q*)	12.5 (*q*)
22	36.0 (*t*)	36.0 (*t*)	36.2 (*t*)	73.0 (*d*)
23	24.8 (*t*)	24.8 (*t*)	24.9 (*t*)	29.3 (*t*)
24	124.9 (*d*)	124.9 (*d*)	125.1 (*d*)	120.9 (*d*)
25	131.2 (*s*)	131.2 (*s*)	131.0 (*s*)	135.5 (*s*)
26	25.7 (*q*)	25.7 (*q*)	25.7 (*q*)	26.0 (*q*)
27	17.6 (*q*)	17.6 (*q*)	17.6 (*q*)	18.0 (*q*)
28	29.6 (*q*)	29.5 (*q*)	28.3 (*q*)	28.3 (*q*)
29	16.2 (*q*)	17.3 (*q*)	15.4 (*q*)	15.6 (*q*)
30	19.6 (*q*)	19.6 (*q*)	24.7 (*q*)	25.8 (*q*)
3-OCO*CH*$_3$		21.2 (*q*)		
3-OCOCH$_3$		171.1 (*s*)		

[a] Assignments were based on ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY experiments.

exhibited signals assignable to five tertiary methyls, one secondary methyl, two vinyl methyls, nine CH_2 groups, six methine groups, including two oxymethines (δ_H 3.22 (1H, dd); 4.30 (1H, brs)), one trisubstituted olefin (δ_H 5.09 (1H, m), and seven quaternary carbons, including an sp^2-carbon and a saturated ketone (δ_C 215.6 (s)). The ^{1}H and ^{13}C NMR spectra showed that the compound had one more methine and quaternary sp^3 carbons but lacked two methylene groups in comparison with that of the corresponding lanostane-type triterpenoid. Therefore, **1** seems to be a migrated lanostane-type triterpenoid. The structure of **1** was determined from the HMBC and ^{1}H–^{1}H COSY spectra (Fig. 1). The HMBC spectrum of **1** showed correlations between: Me-19 (δ 1.46) and two sp^3 quaternary carbons, C-9 and C-10, in addition to C-1 and C-5; H-7 (δ 4.30) and C-5, C-6, C-8, C-9, and C-10; H-3 (δ 3.22) and C-1, C-2, C-4, and C-5; Me-18 (δ 0.66) and C-12, C-13, C-14, and C-17; Me-21 (δ_H 0.91) and C-17, C-20, and C-22; Me-26 (δ_H 1.69) and C-24, C-25, and C-27; Me-27 (δ_H 1.60) and C-24, C-25, and C-26; Me-28 (δ_H 0.95) and C-3, C-4, C-5, and C-29; Me-29 (δ_H 0.95) and C-3, C-4, C-5, and C-28; and Me-30 (δ_H 1.20) and C-8, C-13, C-14, and C-15. In the ^{1}H–^{1}H COSY spectrum, H-7 correlated with H_2-6, and H_2-11 correlated with only H_2-12; therefore, C-9 is a quaternary sp^3 carbon. The FABMS of **1** displayed two characteristic peaks of spiro-lanostane-type triterpenoid (Tanaka et al., 1992) at 305.2481 [$C_{20}H_{33}O_2$] and 205.1961 [$C_{15}H_{25}$] (Fig. 1) along with *m/z* 440 [M−H_2O]$^+$, 422 [M−2H_2O]$^+$, and 327. When **1** was acetylated in the usual manner, monoacetate (**1a**) was obtained in quantitative yield and the H-3 proton signal was shifted downfield to δ 4.46, indicating that the C-7 hydroxyl group is located in a considerably sterically hindered position. Based on the molecular formula of $C_{30}H_{50}O_3$ and the spectroscopic data mentioned above, it was concluded that **1** possesses a rare 7(8 → 9)*abeo*-lanostane skeleton

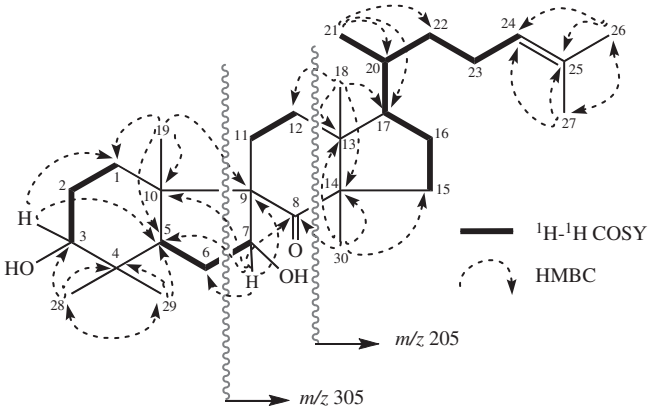


Fig. 1. Selected ^{1}H–^{1}H COSY and HMBC correlations and EIMS of **1**.

having a ketone at C-8 and two hydroxyl groups at C-3 and C-7. The relative structure of **1** was established from the NOESY spectrum (Fig. 2): significant NOE correlations were observed between: Me-19 and H-1β, H-2β, H-6β, and Me-18; H-7 and H-5α, H-11α, H-15α, and Me-30; and H-3 and H-1α, H-5α, and Me-28. Therefore, the configuration of C-9 was *R* and those of the hydroxyl groups at C-3 and C-7 were *S* and *S*. In addition, correlations were found between H-5α and H-3α, and H-11β; H-17α and Me-30; H-20 and H-16, and Me-18; and H-12β and H-1α, Me-18, and Me-19 which led us to deduce that the C-ring adopted a boat conformation. Therefore, structure **1** was determined (3*S*,7*S*,9*R*)-3,7-dihydroxy-7(8 → 9)*abeo*-lanost-24-en-8-one (**1**). 3,7-Dihydroxy-7(8 → 9)*abeo*-lanostane-type triterpenoids, spiroveitchionolide (Tanaka et al., 1992), and spiromarienonols A and B (Tanaka et al., 2004) were isolated from *Abies veitchii* and *Abies*

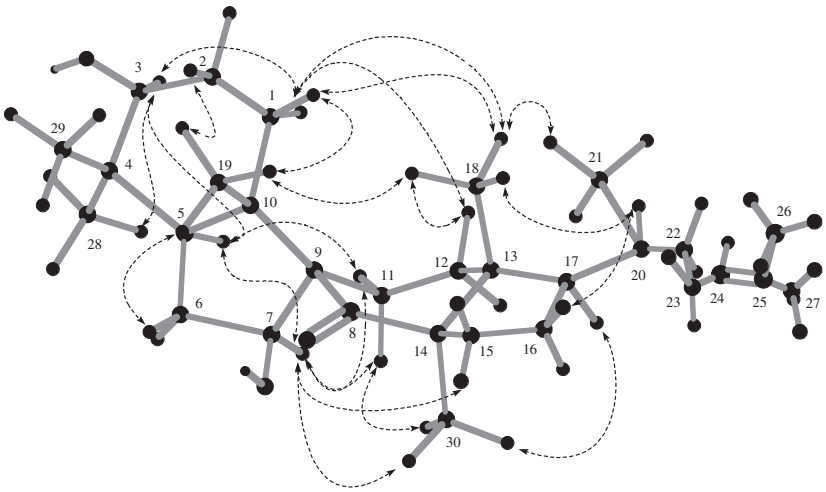
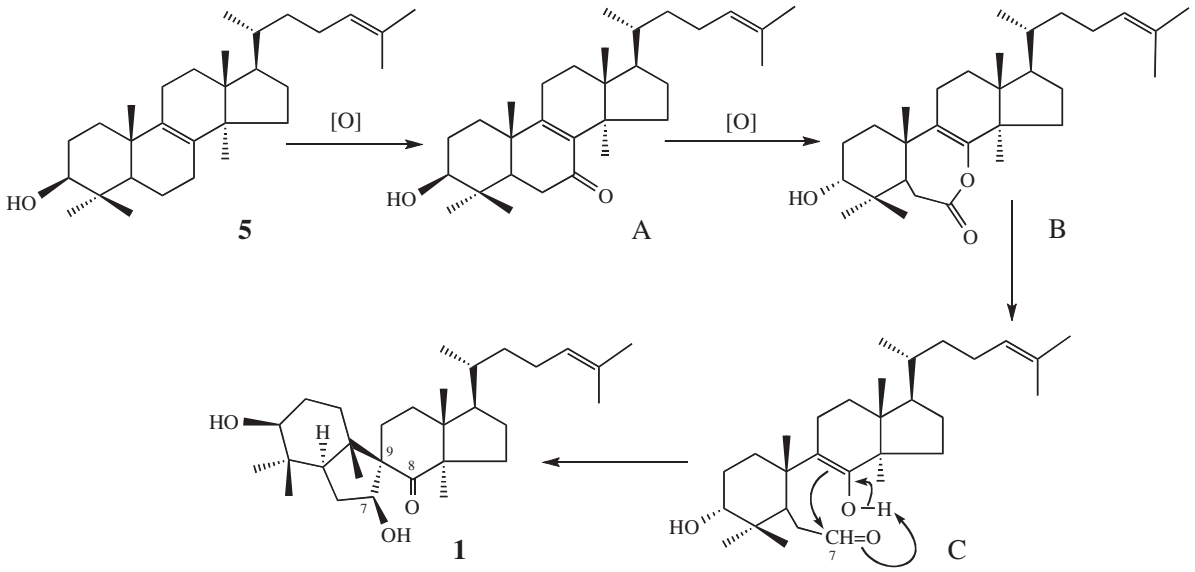


Fig. 2. Key NOEs correlations in **1** (graphical representation using the program CHEM 3D).

mariesii by our group, but those compounds are abieslactone derivatives. Compound **1** seems to be biosynthesized from lanosterol (**5**), one of the major components in the sclerotium (Nakata et al., 2007). Compound **5** is oxidized to give 7-oxo-lanosterol (A), and this is followed by the Baeyer–Villiger oxidation to furnish intermediate (B). Lactone B is reduced and rearranged to give **1** (Scheme 1).

The molecular formula of inonotsudiol A (**2**) was determined to be $C_{30}H_{48}O_2$ (M^+; *m/z* 440.3643) on the basis of HRFABMS. The IR spectrum indicated the presence of a hydroxy group (ν_{max} 3436 cm^{-1}). The ^1H and ^{13}C NMR spectra of **2** (Tables 1 and 2) exhibited signals assignable to five tertiary methyls, one secondary methyl, two vinyl methyls, nine CH_2 groups, five methine groups including two oxymethines [δ_H 3.26 (1H, dd); 4.65 (1H, dt)], one trisubstituted olefin [δ_H 5.10 (1H, t)], four quaternary carbons, and one tetrasubstituted olefin. In the HMBC spectrum of **2**, long-range correlations were observed between: Me-18 (δ 0.86) and C-12, C-13, C-14, and C-17; H_2-12 (δ 1.92, 2.32) and C-9, C-11, C-13, and C-14; Me-19 (δ_H 1.11) and C-1, C-5, C-9 and C-10; Me-21 (δ_H 0.99) and C-17, C-20, and C-22; Me-26 (δ_H 1.69) and C-24, C-25, and C-27; Me-27 (δ_H 1.61) and C-24, C-25, and C-26; Me-28 (δ_H 1.00) and C-3, C-4, C-5, and C-29; Me-29 (δ_H 0.84) and C-3, C-4, C-5, and C-28; Me-30 (δ_H 0.87) and C-8, C-13, C-14, and C-15; and H-24 (δ_H 5.10) and C-22, C-23, C-25, C-26, and C-27. In the ^1H–^1H COSY spectrum, H_2-12 correlated with an oxymethine proton at H-11 (δ_H 4.65). On the basis of the above spectroscopic data, structure **2** was suggested to be lanosta-8,24-dien-3,11-diol, and the relative stereostructure was established by NOESY experiment. In the NOESY spectrum (Fig. 3), significant NOE correlations were observed between: H-3α and H-5α; between H-11 and H-1α and H-1β; Me-30 and H-7α, H-12α, H-15α, and H-17α; and Me-21 and H-12β and H-23. Therefore, the configurations of the hydroxyl groups of **2** were established as 3β and 11β. Compound **2** was synthesized from lanosterol (Woodward et al., 1957), and this is its first report in nature.

Inonotsuoxodiol A (**3**) was assigned the molecular formula $C_{30}H_{48}O_3$ (M^+; *m/z* 456.3602) on the basis of HRFABMS. The UV and IR spectra showed bands indicating the presence of a hydroxy group (ν_{max} 3398 cm^{-1}) and an α,β-unsaturated six-membered ring ketone (ν_{max} 1655 cm^{-1}; λ_{max} 256 nm). The ^1H and ^{13}C NMR spectra of **3** (Tables 1 and 2) exhibited signals assignable to five tertiary methyls, one secondary methyl, two vinyl methyls, eight



Scheme 1. Proposed mechanism for bioenergies of **1** from **5**.

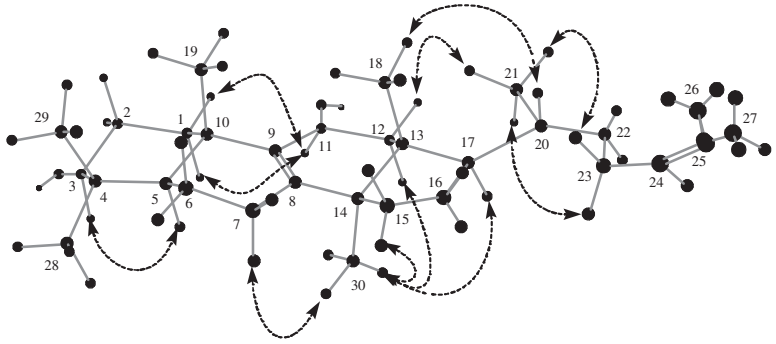


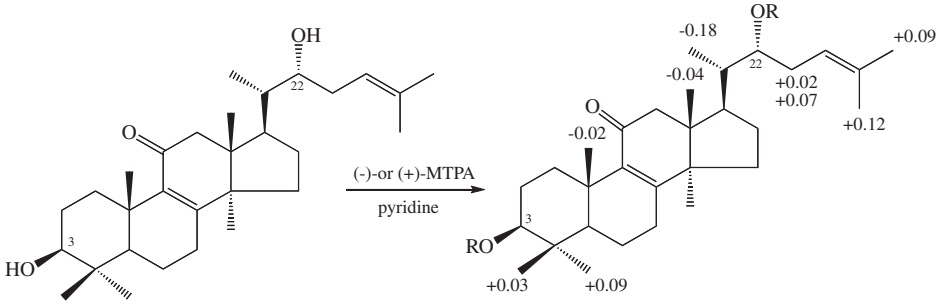
Fig. 3. Key NOEs correlations in **2** (graphical representation using the program CHEM 3D).

CH_2 groups, five methine groups, including two oxymethines (δ_H 3.24 (1H, dd); 3.65 (1H, dt)) and one trisubstituted olefin (δ_H 5.17 (1H, m), four sp^3 quaternary carbons, one tetrasubstituted olefin, and one unsaturated ketone (δ_C 199.0 (s)). The structure of **3** was determined from analysis of the HMBC and ^1H–^1H COSY spectra. The HMBC spectrum of **3** indicated long-range correlations between: Me-18 (δ_H 0.86) and each of C-12, C-13, C-14, and C-17; Me-19 (δ_H 1.13) and C-1, C-5, C-9, and C-10; Me-21 (δ_H 0.92) and C-17, C-20, and C-22; Me-26 (δ_H 1.75) and C-24, C-25, and C-27; Me-27 (δ_H 1.66) and C-24, C-25, and C-26; Me-28 (δ_H 1.02) and C-3, C-4, C-5, and C-29; Me-29 (δ_H 0.83) and C-3, C-4, C-5, and C-28; Me-30 (δ_H 1.10) and C-8, C-13, C-14, and C-15; H_2-12 (δ_H 2.48, 2.66) and C-9, C-11, C-13, and C-14; and H-24 (δ_H 5.17) and C-22, C-23, C-25, C-26, and C-27. In the ^1H–^1H COSY spectrum, the H-20 (δ_H 1.81) and H_2-23 (δ_H 2.05, 2.08) correlated with H-22 (δ_H 3.65), and H_2-12 proton correlated with only the geminal proton. The above spectroscopic data suggested that structure 3 was 3,22-dihydroxylanosta-8,24-diene-11-one. The absolute configuration at C-20 of **3** was established as C-20β because of the significant NOEs for H-20 to Me-18, Me-21, and H-16β. One of the hydroxyl groups was C-3β, as shown by the chemical-shift and the coupling constants [δ_H 3.24 (1H, dd, $J_{3,2\alpha}$ = 5.0 Hz and $J_{3,2\beta}$ = 11.4 Hz); δ_c 78.7 (d)]. The absolute configuration at C-22 of **3** was deduced to be *R* because coupling constants were observed [δ_H 3.65 (1H, dt, $J_{22,20\beta}$ = 9.8 Hz and $J_{22,23}$ = 3.0 Hz); δ_c 73.0 (d)] and significant NOEs were noted from H-22 to H-16α; from H-22 to H-24; from H-22 to H-17α; and from H-17α to Me-21. The absolute configuration at C-22 was determined by the modified Mosher's method (Ohtani et al., 1991). Compound **3** gave 3,22-di-(*S*)-MTPA ester (**3a**) on treatment with [(−)-MTPACl] in pyridine, and 3,22-di-(*R*)-MTPA ester (**3b**) on treatment with [(+)-MTPACl] in pyridine. As shown in Fig. 4, signals due to protons attached to the C-19 position of 3,22-di-(*S*)-MTPA ester (**3a**) were observed at a higher field than those of the 3,22-di-(*R*)-MTPA ester (**3b**) [Δδ: negative], while signals due to protons attached to the C-28 and C-29-positions of **3a** were observed at lower fields than those of **3b** [Δδ: positive]. Therefore, the absolute configuration at the C-3 position of **3** was determined to be *S*. On the other hand, signals due to protons attached to the C-23, C-26, and C-27 positions of 3,22-di-(*S*)-MTPA ester (**3a**) were observed at lower fields than those of 3,22-di-(*R*)-MTPA ester (**3b**) [Δδ: positive], while the resonance due to the proton attached to the C-21 position in **3a** was observed at a higher field than that of **3b [Δδ: negative]**. Hence, the absolute configuration at the C-22 position of **3** was determined to be *R* and the total stereostructure of inonotsuoxodiol A (**3**) was determined to be (22*R*)-3β,22-dihydroxylanosta-8,24-dien-11-one (**3**). NOEs were observed from Me-19 to H-2β and Me-29; from H-5α to H-7α; from H-6β to Me-19 and Me-29; from H-7α to Me-30; from H-12α to Me-21 and Me-30; and from H-11β to Me-18. Therefore, the C rings in **3** adopted a boat conformation.

As a primary screen for anti-tumor activity, the cancer cell growth inhibitory properties of spiroinonotsuoxodiol (**1**), inonotsudiol A (**2**), and inonotsuoxodiol A (**3**) were examined using murine P388 leukemia, murine L1210 leukemia, and human HL-60 leukemia cell lines. All compounds exhibited moderate cytotoxic activity against the cancer cell lines (Table 3).

2.1. Concluding remarks

In this study, an unusual lanostane-type triterpenoid, spiroinonotsuoxodiol (**1**), and two new lanostane-type triterpenoids, inonotsudiol A (**2**) and inonotsuoxodiol A (**3**) were isolated from the sclerotia of *I. obliquus*. Their structures were determined to be (3*S*,7*S*,9*R*)-3,7-dihydroxy-7(8 → 9)*abeo*-lanost-24-en-8-one (**1**), lanosta-8,24-dien-3β,11β-diol (**2**), and (22*R*)-3β,22-dihydroxylanosta-8,24-dien-11-one (**3**).



3

3a: R=(*S*)-MTPA
3b: R=(*R*)-MTPA

Fig. 4. ^1H chemical-shift differences ($\Delta\delta = \delta_S - \delta_R$) between the (*R*)- and (*S*)-MTPA esters (**3a** and **3b**).

Table 3
Cytotoxity of compounds **1–3** against P388, L1210, HL-60, and KB cell lines.

Compounds	P388 IC_{50} (μM)[a]	L1210 IC_{50} (μM)[a]	HL-60 IC_{50} (μM)[a]	KB IC_{50} (μM)[a]
1	29.5	12.5	30.1	21.2
2	23.8	23.8	27.2	14.5
3	15.2	19.7	17.7	89.8
5-Fluorouracil[b]	2.5	2.1	3.7	7.7

[a] DMSO was used for vehicle.
[b] Positive control.

3. Experimental

3.1. General

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-1000 digital polarimeter. IR spectra were recorded using a Perkin–Elmer 1720X FTIR spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively. $CDCl_3$ was used as the solvent and TMS, as the internal standard. NOESY spectrum was represented based on Chem 3D program: CS Chem 3D Pro 2000, version 5.0, Cambridge, MA 02140-237, USA. FABMS were recorded on a Hitachi 4000H double-focusing mass spectrometer (70 eV). Column chromatography (CC) was carried out over silica gel (70–230 mesh, Merck) and MPLC was carried out with silica gel (230–400 mesh, Merck). HPLC was run on a JASCO PU-1586 instrument equipped with a differential refractometer (RI 1531). CC fractions were monitored by TLC (silica gel 60 F_{254}, Merck). Preparative TLC was carried out on Merck silica gel F_{254} plates (20 × 20 cm, 0.5 mm thick).

3.2. Material

Sclerotia (4 kg) of *I. obliquus* (PERS.: Fr.) Pil. were purchased from Salad Melon Co., Ltd. (Nayoro City, Hokkaido, Japan) in April, 2005, and the extraction and preliminary separation procedures were as previously mentioned (Nakata et al., 2007). A voucher specimen (IO-02) is deposited at the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

3.3. Isolation procedure

Preliminary silica gel CC was performed to separate the $CHCl_3$ extract (153.9 g) of the sclerotia of *I. obliquus* into five fractions (residues A–E) Residue C was further purified using silica gel CC as reported previously (Taji et al., 2007, 2008a, 2008b). Residue C1 (Fr. nos. 21–47 (4.98 g) of residue C) was applied to a silica gel (70–230 mesh, 500 g) column using hexane:EtOAc = 5:1–3:1 to give residues C1-1 (Fr. nos. 68–74, 128.37 mg), C1-2 (Fr. nos. 75–77, 97.57 mg), C1-3 (Fr. nos. 78–81), C1-4 (Fr. nos. 82–83), C1-5 (Fr. nos. 84–88), and C1-6 (Fr. nos. 89–92). Residue C1-1 was separated by HPLC (ODS, MeOH–H_2O (95:5, v/v)) to give compounds **1** (22.8 mg), **4** (15.4 mg), and **2** (11.8 mg). Residue C1-6 was separated by HPLC (ODS, MeOH–H_2O (95:5, v/v)) to give compound **3** (17.4 mg). Compound **4** was identified as lanosta-8,23-diene-3β,25-diol on the basis of published data (Leong and Harrison, 1999).

3.4. Spiroinonotsuoxodiol (*1*)

Colorless crystals; m.p. 133–135 °C (from MeOH–$CHCl_3$); $[\alpha]_D^{16}$ −66.3 (*c* 0.101, $CHCl_3$); HRFABMS *m/z*: 458.3757 $[M]^+$ ($C_{30}H_{50}O_3$, calcd for 458.3764); IR (KBr) ν_{max} cm^{-1}; 3448 (OH), 2962, 1687 (C=O), 1459, 1383, 1019; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. FABMS *m/z* (rel. int.): 458 (28) $[M]^+$, 443 (7), 440 (37) $[M-H_2O]^+$, 369 (25), 327 (20), 318 (5), 305.2481 (100) [$C_{20}H_{33}O_2$, calcd for 305.2480]$^+$, 205.1961 (14) [$C_{15}H_{25}$, calcd for 205.1956]$^+$, 179 (22), 161 (39), 136 (35).

3.5. Spiroinonotsuoxodiol monoacetate (*1a*)

A mixture of compound **1** (8.39 mg) and Ac_2O (1 mL) in pyridine (1 mL) was kept at room temperature overnight, which following work-up furnished a residue (9.01 mg) that was subjected to HPLC (ODS, 95% MeOH) to give compound **1a** (5.21 mg). M.p. 141–143 °C; $[\alpha]_D^{20}$ −171.2 (*c* 0.079, $CHCl_3$); HRFABMS *m/z*: 500.3864 $[M]^+$ ($C_{32}H_{52}O_4$, calcd for 500.3858); IR (KBr) ν_{max} cm^{-1}: 3506, 2957, 1724 (OAc), 1459, 1377, 1261, 1153, 1030; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. FABMS *m/z* (rel. int.): 500 (22) $[M]^+$, 440 (13) $[M-HOAc]^+$, 422 (14) $[M-HOAc-H_2O]^+$, 327 (58), 305 (100), 205 (9), 161 (29), 136 (25).

3.6. Inonotsudiol A (*2*)

Colorless crystals; m.p. 123–125 °C; $[\alpha]_D^{20}$ +4 (*c* 0.089, $CHCl_3$); HRFABMS *m/z*: 440.3643 $[M]^+$ ($C_{30}H_{48}O_2$, calcd for 440.3643); IR (KBr) ν_{max} cm^{-1}: 3436 (OH), 2952, 1458, 1376, 1027; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. FABMS *m/z* (rel. int.): 456 (22) $[M]^+$, 441 (20) $[M-Me]^+$, 438 (27) $[M-H_2O]^+$, 371 (21), 341 (58), 311 (66), 261 (36).

3.7. Inonotsuoxodiol A (*3*)

Colorless crystals; m.p. 112–114 °C; $[\alpha]_D^{21}$ +51.7 (*c* 0.092, $CHCl_3$); HRFABMS *m/z*: 456.3602 $[M]^+$ ($C_{30}H_{48}O_3$, calcd for 456.3603); UV (EtOH) λ_{max} nm: 256.0 (log ε 3.77); IR (KBr) ν_{max} cm^{-1}: 3398 (OH), 2966, 1655 (C=C–C=O), 1459, 1376, 1288, 1031; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. FABMS *m/z* (rel. int.): 456 (48) $[M]^+$, 386 (100), 369 (10), 339 (11), 290 (17), 261 (15), 235 (35), 135 (9).

3.8. Preparation of (*S*)- and (*R*)-MTPA esters (*3a* and *3b*) from *3*

A solution of **3** (2.57 mg) in pyridine (1 mL) was treated with (−)-MTPACl (2.0 mg), and the mixture was stirred at room temp. for 24 h. The solvent was resonance off under reduced pressure and the residue was purified by HPLC (reversed-phase silica gel) [MEOH–H_2O (95:5, v/v)] to give (*S*)-MTPA ester derivative (**3a**, 2.27 mg) as an amorphous powder. Using a similar procedure, the (*R*)-MTPA ester derivative (**3b**, 2.07 mg) was obtained from **3** (3.67 mg) using (+)-MTPACl (2.0 mg).

(*S*)-MTPA ester derivative (**3a**); white powder; FABMS *m/z* (rel. int.): 889 ([M+H]$^+$, 37.7%). HRFABMS *m/z*: 889.4480 [M+H]$^+$ (calcd for $C_{50}H_{62}F_6O_7$: 888.4396). ^1H NMR δ ppm ($CDCl_3$): 0.84 (6H, s, H_3-18 and 29), 0.86 (3H, s, H_3-28), 0.94 (3H, d, *J* = 6.3 Hz, H_3-21), 1.12 (3H, s, H_3-30), 1.16 (3H, s, H_3-19), 1.50 (3H, s, H_3-27), 1.58 (3H, s, H_3-26), 4.76 (1H, dd, *J* = 11.8, 4.9 Hz, H-3), 4.91 (1H, m, H-24), 5.12 (1H, dt, *J* = 9.6, 3.3 Hz, H-22).

(*R*)-MTPA ester derivative (**3b**); white powder; FABMS *m/z* (rel. int.): 889 ([M+H]$^+$, 25.8%). HRFABMS *m/z*: 889.4472 [M+H]$^+$ (calcd for $C_{50}H_{62}F_6O_7$: 888.4396). ^1H NMR δ ppm ($CDCl_3$): 0.73 (3H, d, *J* = 6.6 Hz, H_3-21), 0.82 (3H, s, H_3-18), 0.86 (3H, s, H_3-29), 0.95 (3H, s, H_3-28), 1.11 (3H, s, H_3-30), 1.14 (3H, s, H_3-19), 1.59 (3H, s, H_3-27), 1.70 (3H, s, H_3-26), 4.73 (1H, dd, *J* = 11.8, 4.7 Hz, H-3), 5.10 (2H, m, H-22 and 24).

3.9. Assay for cytotoxicity to P388, L1210, HL-60 and KB cell lines

Cytotoxic activities of spiroinonotsuoxodiol (**1**), inonotsudiol A (**2**), and inonotsuoxodiol A (**3**) were examined with the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method. P388, L1210, HL-60 and KB cells were cultured in Eagle's Minimum Essential Medium (10% fetal calf serum) at 37 °C in 5% CO_2. The test material was dissolved in dimethyl sulfoxide (DMSO) to make a concentration of 10 μM, and the solution was diluted with the medium to yield concentrations of 200, 20, and 2 μM, respectively. Each solution was combined with each cell suspension (1×10^5 cells mL^{-1}) in the medium, respectively. After incubating at 37 °C for 72 h in 5% CO_2, the grown cells were labeled with 4 mg mL^{-1} MTT in phosphate-buffered saline (PBS), and the absorbance of formazan dissolved in 20% sodium dodecyl sulfate (SDS) in 0.1 N HCl was measured at 540 nm using a microplate reader (Model 450) (Bio-Rad Laboratories, Inc., Tokyo, Japan). Each absorbance value was expressed as percentage relative to that of the control cell suspension that was prepared without the test substance using the same procedure as that described above. All assays were performed three times, semilogarithmic plots were constructed from the averaged data, and the effective dose of the substance required to inhibit cell growth by 50% (IC_{50}) was determined.

Compounds **1**–**3** had purities of over 99%.

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