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Structure determination of inonotsuoxides A and B and in vivo anti-tumor promoting activity of inotodiol from the sclerotia of *Inonotus obliquus*

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Abstract—Two new lanostane-type triterpenoids, inonotsuoxides A (**1**) and B (**2**) along with three known lanostane-type triterpenoids, inotodiol (**3**), trametenolic acid (**4**), and lanosterol (**5**), were isolated from the sclerotia of *Inonotus obliquus* (Pers.: Fr.) (Japanese name: Kabanoanakake) (Russian name: Chaga). Their structures were determined to be 22*R*,25-epoxylanost-8-ene-3β,24*S*-diol (**1**) and 22*S*,25-epoxylanost-8-ene-3β,24*S*-diol (**2**) on the basis of spectral data including single crystal X-ray analysis. These compounds except for **2** were tested for their inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as a test for potential cancer chemopreventive agents. The most abundant triterpene, inotodiol (**3**), was investigated for the inhibitory effect in a two-stage carcinogenesis test on mouse skin using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter. Compound **3** was found to exhibit the potent anti-tumor promoting activity in the in vivo carcinogenesis test.

1. Introduction

In recent years, the development of more effective and safer agents is intensively required for chemoprevention of human cancer and natural products from plants and their synthetic derivatives are expected to play an important role in creating new and better chemopreventive agents.[1,2] In particular, there is a need for chemopreventive agents targeted at the promoting stage of carcinogenesis in the two- or multi-stage theory[3] since it is difficult to avoid unfavorable hits by tumor-initiating agents in human life. Some natural products, for example, glycyrrhizin,[4] curcumine,[5] EGCg,[6] garlic,[7] and anthocyanidin,[8] have been examined as candidates for chemopreventive agents for clinical use.

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*,[9] and the distribution of this mushroom is recognized in Europe, Asia, and North America. The imperfect form of *I. obliquus* occurs parasitically on trunks, usually of *Betula* (birch), and more rarely also on *Ulmus*, *Alnus*, and *Fraxinus*. Only after the tree dies is the perfect form with pores and basidia produced under the bark.[10] *I. obliquus* is widely distributed in Hokkaido forests of *Betula platyphylla* var. *japonica* (Japanese name: shirakaba) in Japan.[11,12] In Eastern Europe, especially in Russia, the sclerotia of this mushroom have been used as a folk medicine for cancer since the 16th or 17th century.[13,14] Also the Khanty of West Siberia uses this mushroom to prevent and treat heart, liver, and stomach diseases and tuberculosis.[15] Inotodiol was first isolated by Ludwiczak et al.[16] Kempska et al. isolated 3β-hydroxylanosta-8,24-diene-21-oic acid (trametenolic acid) and lanosta-8,24-diene-3β,21-diol,[17] and Kahlos et al. isolated 3β,22,25-trihydroxylanosta-8,23-diene[18] and 3β,22-dihydroxylanosta-8,24-diene-7-one.[19] Anti-tumor experiments with *n*-hexane extracts of *I. obliquus* found that

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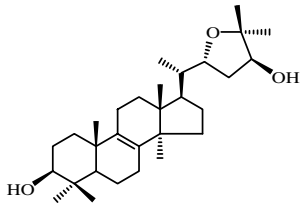
triterpenoids, especially inotodiol, have a significant anticancer effect against Walker 256 carcinosarcoma and MCF-7 human mammary adenocarcinoma in vitro, and leukemia P388 in vivo.[20] Kahlos and Tikka reported antifungal activity of C-21 oxygenated lanosterol derivatives, that are 3β-hydroxylanosta-8,24-diene-21-al, 3β,21-dihydroxylanosta-8,24-diene, and trametenolic acid.[21] On the other hand, Mizuno et al. reported the extracts from the sclerotia of *I. obliquus* have a positive effect in controlling cancer, HIV-1, and stomach ulcers[22,23] and Burczyk et al. reported that the aqueous extracts of *I. obliquus* inhibited the cell growth of human cervical and uterine cancer cells (HeLa S3) at a concentration of 10–2000 μg/ml.[24] Recently, Shin et al. isolated 3β-hydroxylanosta-8,24-diene-21,23-lactone, 21,24-cyclopentalanost-8-en-3β,21,25-triol, and lanost-8-ene-3β,22,25-triol from the sclerotia of *I. obliquus*.[25,26]

Careful examination of the sclerotia of *I. obliquus* has led to the isolation of two new lanostane-type triterpenes named inonotsuoxides A (**1**) and B (**2**), besides three known triterpenes (**3**–**5**). The structures of the new compounds **1** and **2** were determined on the basis of spectroscopic data including X-ray analysis. The known compounds were identified as inotodiol (**3**),[16] trametenolic acid (**4**),[25] and lanosterol (**5**)[25] by comparison of their spectral data with those reported in the literature. This report deals with the structure determination of inonotsuoxides A (**1**) and B (**2**) and results of in vitro and in vivo anti-tumor promoting activity of **1**, acetate of **1** (**1a**), **3**, **4**, and acetate of **4** (**4a**) and **5**. The assay methods employed were an in vitro assay estimating the inhibitory effect on Epstein–Barr virus early antigen (EBV-EA) activation induced by TPA[27] and an in vivo two-stage mouse-skin carcinogenesis assay using DMBA as an initiator and TPA as a promoter.[28]



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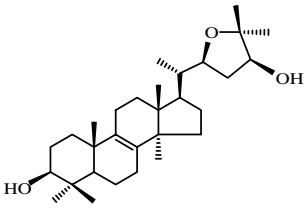

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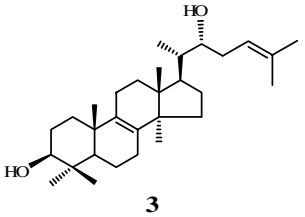

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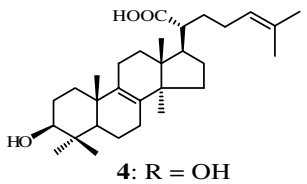

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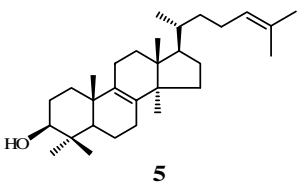

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2. Results and discussion

Sclerotia of *I. obliquus* were extracted with $CHCl_3$, and the extract was separated by silica-gel column chromatography, medium-pressure liquid chromatography (MPLC), and high medium-pressure liquid chromatography (HPLC). Two new (**1**, **2**) and three known (**3**–**5**) triterpenes were obtained. Compounds **3**–**5** were confirmed as inotodiol (lanosta-8,24-diene-3β,22*R*-diol) (**3**), trametenolic acid (3β-hydroxylanosta-8,24-diene-21-oic acid) (**4**), and lanosterol (**5**) because their physical and spectral data showed good agreement with those already published.

The molecular formula of compound **1** was assigned as $C_{30}H_{50}O_3$ (M^+; *m/z* 458.3759) by HREIMS. The IR spectrum showed a hydroxyl group (ν_{max} 3505, 3359 cm^{-1}). The ^1H and ^{13}C NMR spectra ($CDCl_3$) of **1** (Table 1) exhibited seven tertiary methyl groups, a secondary methyl group [δ_H 0.88 (3H, d, J = 6.7 Hz)], nine methylenes, six sp^3 methines including three oxymethines [δ_H 3.23 (1H, dd, J = 11.7, 4.6 Hz); δ_C 79.0 (d)]; [δ_H 3.92 (1H, dd, J = 6.4, 4.1 Hz); δ_C 78.5 (d)]; [δ_H 4.26 (1H, ddd, J = 10.3, 6.7, 3.7 Hz); δ_C 78.1 (d)], five sp^3 quarternary carbons including one oxycarbon [δ_C 81.7 (s)], and a tetrasubstituted double bond [δ_C 134.2 (s), 134.5 (s)]. The planner structure of **1** was determined by HMBC and ^1H–^1H COSY spectra. The HMBC spectrum of **1** (Table 1) indicated a long-range correlation between Me-18 (δ_H 0.71) with C-12, C-13, C-14, and C-17, between Me-19 (δ_H 0.98) with C-1, C-5, C-9, and C-10, between Me-21 (δ_H 0.88) with C-17, C-20, and C-22, between Me-26 (δ_H 1.23) with C-24, C-25, and C-27, between Me-27 (δ_H 1.22) with C-24, C-25, and C-26, between Me-28 (δ_H 1.00) with C-3, C-4, and C-5, between Me-29 (δ_H 0.81) with C-3, C-4, and C-5, and between Me-30 (δ_H 0.86) with C-8, C-13, C-14, and C-15, respectively. In the ^1H–^1H COSY spectrum (Table 1), H_2-23 (δ_H 1.65, 2.01) correlated with H-22 (δ_H 4.26) and H-24 (δ_H 3.92). Acetylation of **1** with Ac_2O/pyridine gave a diacetate (**1a**), $C_{34}H_{54}O_5$ (*m/z* 542.3962), in which the acetoxymethine proton signal appeared at δ_H 4.50 (dd) and δ_H 4.93 (dd), although one oxymethine at δ_H 4.26 (1H, ddd, H-22) resisted acetylation. The above data suggested that the structure of **1** was 22,25-epoxylanost-8-ene-3,24-diol. The configuration of C-22 in the tetrahydrofuran ring was established as C-22*R* because significant NOEs were observed for H-20β/H-22; H-22/H-23β; H-23α/H-17α. One of the hydroxyl groups was C-3β as shown by the chemical shift and the coupling constants [δ_H 3.23 (1H, dd, $J_{3,2\alpha}$ = 4.6 Hz and $J_{3,2\beta}$ = 11.7 Hz); δ_C 79.0 (d)]. The absolute configuration of C-24 in **1** was determined to be *S* because the significant NOEs for H-23α/H-24; H-24/Me-27, and the coupling constants [δ_H 3.92 (1H, dd, $J_{24,23\alpha}$ = 6.4 Hz and $J_{24,23\beta}$ = 4.1 Hz)] were observed. Another NOEs were shown from Me-29 to H-2β and Me-19, 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-30, from H-11β to Me-18, and from Me-18 to H-20β. Therefore, A, B, and C rings in **1** adopted

Table 1. NMR spectral data of Inonotusoxide A (**1**) in $CDCl_3$

Position		δ_H[a]		*J*/Hz	^1H–^1H COSY	NOE	δ_C		HMBC (C)[b]
1	α	1.23	td	13.5 (1β, 2β), 4.2 (2α)	1β, 2α, 2β	1β, 2α, 3, 5	35.6	(t)	
	β	1.73	dt	13.5 (1α), 4.2 (2α, 2β)	1α, 2α, 2β	1α, 2α, 11, 19			
2	α	1.66	m		1α, 1β, 2β, 3	1α, 1β, 3	27.8	(t)	
	β	1.58	tdd	13.5 (1α, 2α), 11.7 (3), 4.2 (1β)	1α, 1β, 2α, 3	2α, 19, 29			
3		3.23	dd	11.7 (2β), 4.6 (2α)	2α, 2β	1α, 2α, 5, 28	79.0	(d)	
4							38.9	(s)	
5		1.05	dd	12.8 (6β), 3.0 (6α)	6α, 6β	1α, 3, 7, 28	50.4	(d)	6, 7, 10
6	α	1.67	m		5, 6β, 7	6β, 7, 28	18.2	(t)	
	β	1.5	m		5, 6α, 7	6α, 7, 19, 29			
7		2.04	m		6α, 6β	5, 6α, 6β, 30	26.5	(t)	
8							134.2	(s)	
9							134.5	(s)	
10							37.0	(s)	
11		2.01	m		12	1β, 12, 18, 19	21.0	(t)	
12		1.7	m		11	11, 17, 18, 21, 30	30.9	(t)	
13							45.0	(s)	
14							49.3	(s)	
15	α	1.19	m		15β, 16α, 16β	15β, 16α, 30	31.0	(t)	
	β	1.62	m		15, 16α, 16β	15α, 18			
16	α	1.83	m		15α, 15β, 16β, 17	15α, 16β, 17, 30	27.3	(t)	
	β	1.48	m		15α, 15β, 16α, 17	16α, 18, 22			
17		1.40	ddd	12.0 (20), 10.8 (16α), 7.2 (16β)	16α, 16β, 20	12, 16α, 21, 23α, 23β, 30	47.8	(d)	
18		0.71	s			11, 12, 15β, 16β, 19, 20, 21	15.7	(q)	12, 13, 14, 17
19		0.98	s			1β, 2β, 6β, 11, 18, 29	19.1	(q)	1, 5, 9, 10
20		1.83	d quint	12.0 (17), 6.7 (21, 22)	17, 21, 22	18, 21, 22	38.5	(d)	
21		0.88	d	6.7 (20)	20	12, 17, 18, 20, 27	12.3	(q)	17, 20, 22
22		4.26	ddd	10.3 (23α), 6.7 (20), 3.7 (23β)	20, 23α, 23β	16β, 20, 23β, 26	78.1	(d)	
23	α	2.01	m		22, 23β, 24	17, 23β, 24	33.3	(t)	
	β	1.65	m		22, 23α, 24	17, 22, 23α, 24			
24		3.92	dd	6.4 (23α), 4.1 (23β)	23α, 23β	23α, 23β, 26	78.5	(d)	
25							81.7	(s)	
26		1.23	s			21, 24	21.2	(q)	24, 25, 27
27		1.22	s			22	27.5	(q)	24, 25, 26
28		1.00	s			3, 5, 6α, 29	28.0	(q)	3, 4, 5, 29
29		0.81	s			2β, 6β, 19, 28	15.4	(q)	3, 4, 5, 28
30		0.86	s			7, 12, 15α, 16α, 17	24.3	(q)	8, 13, 14, 15

[a] ^1H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J*/Hz). Figures in parentheses indicate the proton coupling with that position.

[b] Long range ^1H–^{13}C correlations from H to C observed in the HMBC experiments.

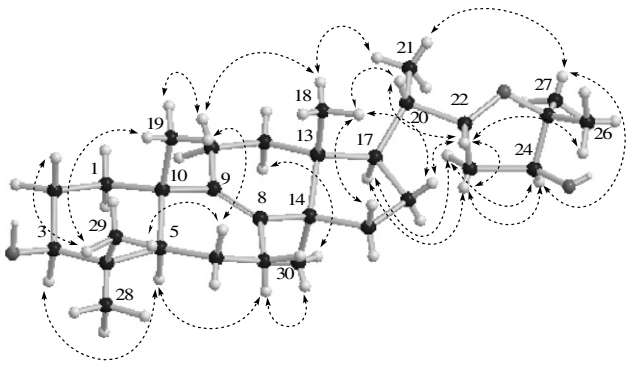

Figure 1. Observed NOEs for compound **1** (graphical representation using the program Chem 3D).

chair/twist, chair/twist, and chair conformation (Fig. 1). These data established the structure of inonotsuoxide A (**1**) as 22*R*,25-epoxylanost-8-ene-3β,24*S*-diol. To confirm the structure based on spectroscopic methods, a single crystal X-ray analysis of **1a** was performed. Figure 2 shows the ORTEP view of **1a**, and the stereostructure was established.

The minor compound **2** had the same molecular formula $C_{30}H_{50}O_3$ (M^+; *m/z* 458.3766) as **1** by HREIMS. The IR, ^1H and ^{13}C NMR spectra (Table 2) resembled those of **1** except for Me-21 (δ_H 0.93), H-22 (δ_H 4.05), and H-23α (δ_H 2.12) in ^1H NMR spectrum and C-22 (δ_C 76.7), C-26 (δ_C 25.5), and C-27 (δ_C 22.5) in ^{13}C NMR spectrum. The HMBC and ^1H–^1H COSY spectra closely resembled those of **1**. The *S* configuration at C-22 in **2** was deduced from coupling constants [δ_H 4.05 (1H, dd d, $J_{20\beta,22}$ = 3.8 Hz, $J_{22,23\alpha}$ = 6.8 Hz, $J_{22,23\beta}$ = 9.0 Hz)], and NOEs from H-20β/H-22; H-22/H-23α; Me-21/H-23β. The observed NOEs from H-23α to H-24 and Me-27, from H-24 to Me-26 and Me-27, from Me-26 to Me-21 showed the *S* configuration at C-24, which is

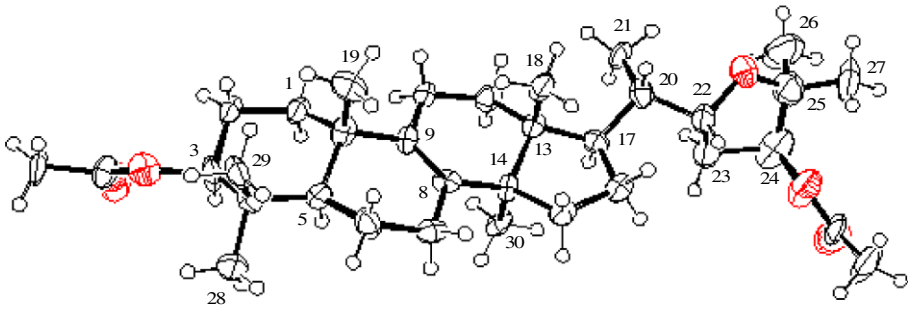

Figure 2. ORTEP drawing of compound **1a**.

Table 2. NMR spectral data of **2** in $CDCl_3$

Position		δ_H[a]		*J*/Hz	^1H–^1H COSY	NOE	δ_C		HMBC (C)[b]
1	α	1.23	m		1β, 2α, 2β	1β, 2α, 3, 5	35.6	(t)	
	β	1.74	dt	13.0 (1α), 3.6 (2α, 2β)	1α, 2α, 2β	1α, 2α, 11, 19			
2	α	1.68	m		1α, 1β, 2β, 3	1α, 2β, 3	27.8	(t)	1, 4, 10
	β	1.58	tdd	13.2 (1α, 2α), 12.0 (3), 2.5 (1β)	1α, 1β, 2α, 3	2α, 19, 29			
3		3.23	dd	12.0 (2β), 4.5 (2α)	2α, 2β	1α, 2α, 5	79.0	(d)	2, 28, 29
							38.9	(s)	
		1.04	dd	12.8 (6β), 3.0 (6α)	6α, 6β	1α, 3, 7, 28	50.4	(d)	4, 6, 7, 10, 19, 28, 29
6	α	1.67	m		5, 6β, 7	5, 6β, 7, 28	18.2	(t)	8
	β	1.51	m		5, 6α, 7	6α, 7, 19, 29			5, 7, 10
7		2.04	m		6α, 6β	5, 6α, 6β, 30	26.5	(t)	5
8							134.2	(s)	
9							134.6	(s)	
10							37.0	(s)	
11		2.02	m		12	1β, 12, 18, 19	21.0	(t)	8, 9, 12
12		1.72	m		11	11, 17, 18, 21, 30	30.9	(t)	11, 13, 18
13							45.0	(s)	
14							49.3	(s)	
15	α	1.20	m		15β, 16α, 16β	15β, 16α, 30	31.0	(t)	
	β	1.63	m		15, 16α, 16β	15α, 18			14, 16, 30
16	α	1.79	m		15α, 15β, 16β, 17	15α, 16β, 17, 22, 30	27.3	(t)	15
	β	1.47	m		15α, 15β, 16α, 17	16α, 18, 20, 22			14, 15, 17, 20
17		1.41	ddd	12.3 (20), 9.0 (16β), 2.8 (16α)	16α, 16β, 20	12, 16α, 21, 22, 30	47.9	(d)	12, 13, 15, 16, 18, 20, 21
18		0.72	s			11, 12, 15β, 16β, 19, 20, 21	15.8	(q)	12, 13, 14, 17
19		0.98	s			1β, 2β, 6β, 11, 18, 29	19.1	(q)	1, 5, 9, 10
20		1.86	dqd	12.3 (17), 6.3 (21), 3.8 (22)	17, 21, 22	16β, 18, 21, 22	38.2	(d)	
21		0.93	d	6.3 (20)	20	12, 17, 18, 20, 23β, 26	12.5	(q)	17, 20, 22
22		4.05	ddd	9.0 (23β), 6.8 (23α), 3.8 (20)	20, 23α, 23β	16α, 16β, 17, 20, 23α, 27	76.7	(d)	21
23	α	2.12	dt	13.2 (23β), 6.8 (22, 24)	22, 23β, 24	22, 23β, 24, 26	34.1	(t)	20, 24, 25
	β	1.64	m		22, 23α, 24	21, 23α			
24		3.97	dd	6.8 (23α), 4.9 (23β)	23α, 23β	26, 27	78.3	(d)	22, 26
25							81.4	(s)	
26		1.20	s			22, 23α, 24	25.5	(q)	24, 25, 27
27		1.23	s			21, 24	22.5	(q)	24, 25, 26
28		1.00	s			3, 5, 6α, 29	28.0	(q)	3, 4, 5, 29
29		0.81	s			2β, 6β, 19, 28	15.4	(q)	3, 4, 5, 28
30		0.87	s			7, 12, 15α, 16α, 17	24.3	(q)	8, 13, 14, 15

[a] ^1H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J*/Hz). Figures in parentheses indicate the proton coupling with that position.
[b] Long range ^1H–^{13}C correlations from H to C observed in the HMBC experiments.

same as that of **1** (Fig. 3). These data established the structure of inonotsuoxide B (**2**) as 22*S*,25-epoxylanost-8-ene-3β,24*S*-diol which is the C-22 epimer of **1**.

Compounds **1**, **1a**, **3**, **4**, **4a**, and **5** were evaluated for in vitro inhibitory activity against EBV-EA activation induced by TPA. Compound **2** was not tested in this assay because it was obtained only as a minor product. As shown in Table 3, the inhibitory activities of all compounds were almost similar to that of oleanolic acid[20] known as a representative anti-tumor promoting agent. The relative ratios of compound **3** with respect to TPA (100%) were 2.4%, 37.6%, 66.1%, and 93.1% at the concentrations of 1000, 500, 100, and 10 mol ratio/TPA,

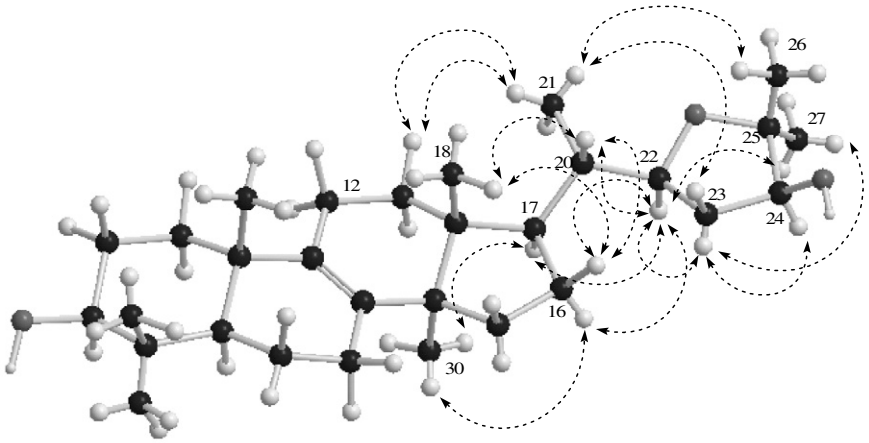

Figure 3. Observed NOEs for compound **2** (graphical representation using the program Chem 3D).

Table 3. Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in the presence of **1**, **1a**, **3**, **4**, **4a**, and **5**

Compound	Concentration (mol ratio/TPA)			
	1000 mol ratio/TPA[b]	500 ratio/TPA[b]	100 ratio/TPA[b]	10 ratio/TPA[b]
1	6.3 (70)[c]	42.0	71.3	94.3
1a	7.9 (70)	47.0	78.1	96.7
3	2.4 (70)	37.6	66.1	93.1
4	9.2 (70)	45.0	75.4	98.6
4a	12.7 (70)	47.8	79	100
5	4.9 (70)	44.7	74.3	96.3

[a] Values represent percentages relative to the positive control value (100%).
[b] TPA concentration was 20 ng/ml (32 pmol/ml).
[c] Values in parentheses are the viability percentages of Raji cells.

respectively; meaning 97.6%, 62.4%, 33.9%, and 6.9% inhibition of the EBV-EA activation by TPA, respectively. The inhibitory activities of compounds **1**, **1a**, **4**, **4a**, and **5** were a little weaker (42.0–47.8% at the concentration of 500 mol ratio/TPA) as compared with that of compound **3**. The viability percentages of Raji cells treated with the test compounds were 70% at the highest concentration of 1000 mol ratio/TPA; suggesting that the cytotoxicities of all compounds were considerably moderate against in vitro cell lines (Table 3).

From the results and consideration of yield, we selected inotodiol (**3**) to examine the effect on the in vivo two-stage mouse-skin carcinogenesis with DMBA and TPA. During the in vivo assay, the body-weight gains of the mice were not influenced by the treatment with the test compounds and no toxic effects, such as lesional damages and inflammation on the areas of mouse skin topically treated with the test compounds, were observed. Figure 4A and B demonstrate the results of the papilloma formation in the skin of mice. The papilloma bearers in the standard group treated with DMBA (390 nmol) and TPA (1.7 nmol, twice/week) appeared as early as at week 6, and the percentages of the papillo-

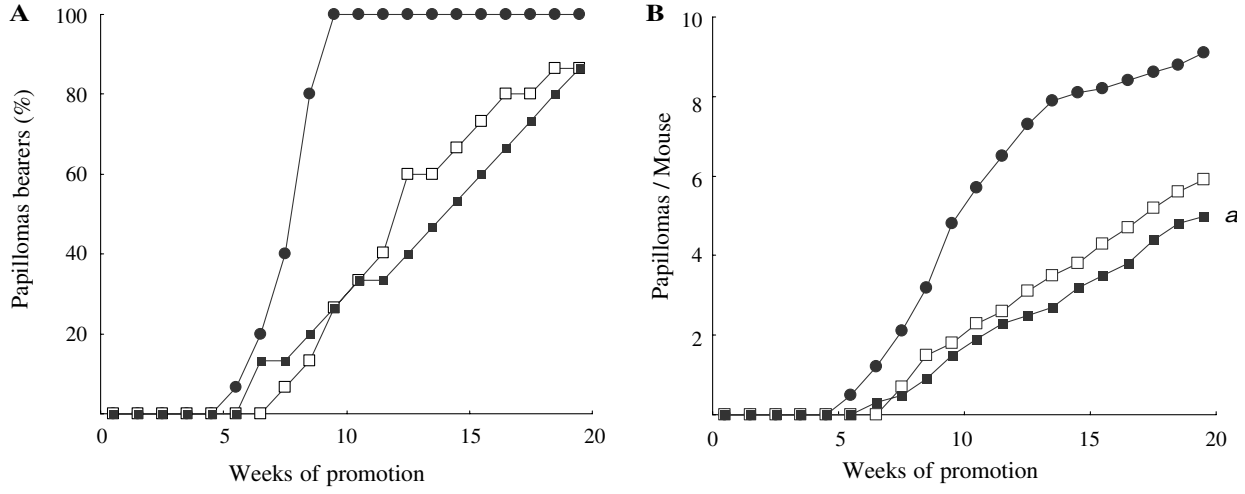

Figure 4. Inhibition effects of rosin on DMBA–TPA mouse skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly, starting 1 week after initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas per mouse. ●, control (TPA alone); □, TPA + 85 nmol of positive control (oleanolic acid); ■, TPA + 85 nmol of **3**. [a]Statistically different from the positive control ($P < 0.05$).

ma-bearers increased rapidly to reach 100% after week 10. On the other hand, the treatment with compound **3** or with positive control, oleanolic acid (85 nmol), along with DMBA/TPA suppressed 30% formation of papillomas down to less than 30% until week 10. As shown in Figure 4A, the percentage of papilloma-bearers of these groups were approximately 86% over the period until week 20.[29] As shown in Figure 4B, in the positive control group with DMBA/TPA, the number of papillomas per mouse formed increased rapidly after week 6 to reach 9.0 papillomas/mouse at week 20, whereas the mice treated with compound **3** bore only 5.0 papillomas even at week 20 ($P > 0.05$). This in vivo result showed the inhibitory activity of compound **3** was almost equal to or a little stronger than that of positive control, oleanolic acid. Inotodiol (**3**) constituted 20% of the extract from the sclerotia of *I. obliquus*. *Inonotus obliquus* were harvested for daily use in many countries including Japan. It is used widely in Russia in particular. One of the authors cultivates this mushroom for business. We plan to further clarify its ingredients and to examine carcinoma prophylactic action of each individual compound.

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. ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer with standard pulse sequences, operating 500 and 125 MHz, respectively. $CDCl_3$ was used as the solvent and TMS as the internal standard. EIMS were recorded on a Hitachi 4000H double-focusing mass spectrometer (70 eV). Column chromatography was carried out over silica gel (70–230 mesh, Merck) and medium-pressure liquid chromatography (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). Fractions obtained from column chromatography 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

Inonotus obliquus is succeeded in culture in Salada Melon Co. Ltd., Nayoro City, Hokkaido, Japan. Sclerotia (4 kg) of *I. obliquus* was obtained from the above office.

3.3. Bioassays

3.3.1. Inhibition of EBV-EA activation test. EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medicinal University. The EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui). Spontaneous activation of EBV-EA in our sub-line Raji cells was less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described previously.[27] The indicator cells (Raji cells, 1×10^6/ml) were incubated at 37 °C for 48 h in 1 ml of a medium containing *n*-butyric acid (4 mmol), TPA [32 pmol = 20 ng in dimethylsulfoxide (DMSO), 2 μl] as inducer, and various amounts of test compounds in 5 μl DMSO. Smears were made from the cell suspension, and the activated cells that were stained by EBV-EA positive serum from NPC patients were detected by an indirect immunofluorescence technique.[30] In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compounds was expressed as a relative ratio to the control experiment (100%) which was carried out only with *n*-butyric acid (4 mmol) plus TPA (32 pmol). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by the Trypan blue staining method.

3.3.2. Two-stage mouse-skin carcinogenesis test. Mouse studies were approved by the Committee of Animal Experimental Center for Kyoto Prefectural University of Medicine throughout all treatments. Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC Inc., Shizuoka, Japan, and the animals were housed, five per polycarbonate cage, in a temperature-controlled room at 24 °C and given food and water and libitum throughout the experiment. Animals were divided into three experimental groups containing 15 mice each. The back of each mouse was shaved with surgical clippers and the mice were topically treated with DMBA (100 ng, 390 nmol) in acetone (0.1 ml) as an initiating treatment. One week after the initiation, papilloma formation was promoted twice weekly by the application of TPA (1 ng, 1.7 nmol) in acetone (0.1 ml) to the skin. One hour before each treatment, the mice were treated with the samples (85 nmol) in acetone (0.1 ml). The incidence of papillomas was examined weekly over a period of 20 weeks. Student's *t* test was used for all statistical analysis.

3.3.3. Extraction and isolation. The sclerotia of white-rot fungus, *I. obliquus* (pers. Fr.) Pil. (4 kg), were extracted with chloroform (10 l) employing an automatic percolator for 7 days at 50 °C. The chloroform solution was evaporated under reduced pressure, and the resulting dark brown residue (150.0 g) was subjected to silica gel (2.8 kg) column chromatography. Elution of the column with chloroform/EtOAc (10:1) afforded residues: **A** (Fr. No. 1–39, 5.1 g), **B** (Fr. No. 40–47, 30.2 g), and **C** (Fr. No. 48–64, 42.3 g). Elution was continued with chloroform:EtOAc (5:1) to give residue **D** (Fr. No. 65–79, 4.2 g) and subsequent column chromatography with chloroform/EtOAc (2:1) to give residue **E** (Fr. No. 80–105, 14.5 g), respectively. Residue **B** was rechromatographed by silica gel (70–230 mesh, 1.5 kg) column chromatography using chloroform to give compound **5**

(2.2 g) from fraction Nos. 13–17. Residue **C** was recrystallized from MeOH to give compound **3** (25.2 g), and the filtrate (17.1 g) was rechromatographed on MPLC (230–400 mesh silica gel, 500 g) using *n*-hexane/EtOAc (5:1) to afford compounds' **1** and **2** mixture (72.3 mg), which was separated with HPLC [ODS, MeOH/H_2O (85:15)] to give compounds **1** (45.7 mg) and **2** (7.8 mg), respectively. Residue **E** was recrystallized from MeOH to give compound **4** (11.8 g). Compounds **3**–**5** were identified by comparison of their spectral data with published data, and the results of in vitro EBV-EA activation test are shown in Table 3. Compounds **1**–**5** had the purity of over 99.5%.

3.3.4. Inonotsuoxide A (1). Colorless prisms; mp 290–292 °C (from MeOH–$CHCl_3$); HREIMS *m/z*: 458.3759 $[M]^+$ ($C_{30}H_{50}O_3$, calcd for 458.3759); IR (KBr) ν_{max} cm^{-1}: 3505, 3359 (OH), 2966, 2876, 1458, 1371, 1075, 1037; ^1H and ^{13}C NMR, see Table 1. EIMS *m/z* (rel. int.): 458 (52) $[M]^+$, 443 (75) $[M-Me]^+$, 425 (66), 407 (11), 339 (17), 314 (10), 311 (10), 301 (11), 283 (12), 115 (100), 71 (68).

3.3.5. Inonotsuoxide A diacetate (1a). A mixture of compound **1** (10.3 mg) and Ac_2O (1 ml) in pyridine (1 ml) was kept at room temperature over night. Usual work-up gave a residue (10.8 mg), which was recrystallized from MeOH–$CHCl_3$ to a corresponding inonotusoxide A diacetate (**1a**) (8.1 mg). Colorless prisms; mp 198–200 °C (MeOH–$CHCl_3$); $[\alpha]_D^{15}$ +45.8° (*c* 1.00, $CHCl_3$); HREIMS *m/z*: 542.3962 $[M]^+$ ($C_{34}H_{54}O_5$, calcd for 542.3971); ^1H NMR δ: 0.71 (3H, s, Me-29), 0.85 (3H, s, Me-30), 0.88 (6H, s, Me-18 and Me-28), 0.93 (3H, d, J = 6.7 Hz, Me-21), 1.20 (3H, s, Me-26), 1.26 (3H, s, Me-27), 2.05 (3H, s, C-24 OCO*CH*$_3$), 2.08 (C-3 OCO*CH*$_3$), 4.25 (1H, ddd, J = 10.3, 6.7, 3.7, H-22), 4.50 (1H, dd, J = 11.7, 4.6, H-3α), 4.93 (1H, dd, J = 6.4, 4.1 Hz, H-24). ^{13}C NMR δ: 12.2 (C-21), 15.7 (C-29), 16.5 (C-18), 18.1 (C-6), 19.1 (C-19), 21.0 (C-11), 21.1 (C-3 OCO*CH*$_3$), 21.3 (C-24 OCO*CH*$_3$), 22.0 (C-26), 24.1 (C-2), 24.3 (C-30), 26.4 (C-7), 27.3 (C-16), 27.8 (C-27), 27.9 (C-28), 30.8 (C-12), 30.9 (C-15), 31.2 (C-23), 35.2 (C-1), 36.9 (C-10), 37.8 (C-4), 38.2 (C-20), 45.0 (C-13), 47.8 (C-17), 49.3 (C-14), 50.5 (C-5), 78.8 (C-22), 79.8 (C-24), 80.9 (C-3), 81.2 (C-25), 134.2 (C-8), 134.4 (C-9), 170.6 (C-3 O*C*OCH$_3$), 171.0 (C-24 O*C*OCH$_3$).

3.3.6. Crystal data of 1a. $C_{34}H_{54}O_5$, M = 542.77, orthorhombic, space group: $P2_1$, a = 6.0345(11) Å, b = 28.669(5) Å, c = 9.0551(16) Å, β = 93.169(5)°, V = 1564.2(5) Å^3, Dx = 1.152 g/cm^3, and Z = 4. A single crystal was used for X-ray diffraction data collection on a Rigaku Rapid diffractometer employing graphite-monochromated CuKα radiation. A total of 3917 independent reflection intensities up to 2θ = 45.0° were collected in an ω scan mode and were corrected for the Lorenz and polarization factors. The structure was solved by direct method using the SHELX-97 program.[31] The non-hydrogen atoms were refined by a full-matrix least-squares method with anisotropic thermal parameters using the SHELXL-97 programs.[32] Hydrogen atoms were calculated assuming idealized geometries but not refined. The discrepancy indices R and wR are 0.1163 and 0.2450 for 2605 [$F_0 > 4\sigma$ (F_0)] reflections. All calculations were performed using teXan[33] crystallographic software package of Molecular Structure Corporation. Lists of atomic coordinates, anisotropic thermal parameters, and bond lengths and angles have been deposited at the Cambridge Crystal Crystallographic Data Centre, UK. CCDC No. 622113.

3.3.7. Inonotsuoxide B (2). Colorless prisms; mp 240–242 °C (from MeOH–$CHCl_3$); $[\alpha]_D^{15}$ +36.6° (*c* 0.47, $CHCl_3$); HREIMS *m/z*: 458.3766 $[M]^+$ ($C_{30}H_{50}O_3$, calcd for 458.3760); IR (KBr) ν_{max} cm^{-1}: 3431 (OH), 2966, 2941, 2876, 1456, 1372, 1064, 1033; ^1H and ^{13}C NMR, see Table 2. EIMS *m/z* (rel. int.): 458 (23) $[M]^+$, 443 (24) $[M-Me]^+$, 425 (19), 339 (3), 314 (3), 301 (2), 283 (2), 115 (100), 71 (44).

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