Microbial Transformations of Two Lupane-Type Triterpenes and Anti-Tumor-Promoting Effects of the Transformation Products

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Microbial transformation of betulin (**1**), a lupane-type triterpene obtained from the bark extract of white birch (*Betula platyphylla* Sukatshev var. *japonica*), and its chemical oxidation product, betulonic acid (**2**), by the fungus *Chaetomium longirostre* yielded 4,28-dihydroxy-3,4-*seco*-lup-20(29)-en-3-oic acid (**3**) and 4-hydroxy-3,4-*seco*-lup-20(29)-ene-3,28-dioic acid (**4**) from **1**, and 4,7β,17-trihydroxy-3,4-*seco*-28-norlup-20(29)-en-3-oic acid (**5**) and 7β,15α-dihydoxy-3-oxolup-20(29)-en-28-oic acid (**6**) from **2**. The four metabolites, **3**−**6**, along with **1** and **2**, were evaluated for their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells as a primary screening test for inhibitors of tumor promotion. All of the triterpenes tested showed potent inhibitory effects, with the four metabolites **3**−**6** exhibiting the more potent effects.

Betulin [**1**; lup-20(29)-ene-3β,28-diol] is a naturally occurring lupane-type triterpene abundantly available from birch bark.[1] Lupane-type triterpenes including **1**, betulinic acid [3β-hydroxylup-20(29)-en-28-oic acid], and their derivatives have been reported to exhibit a variety of biological activities, such as anti-inflammatory activity,[2−4] and inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation,[5] tumor-promoting activity in two-stage carcinogenesis in mouse skin promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA),[2,6] human immunodeficiency virus (HIV)-1,[7] and HIV-1 reverse transcriptase.[8] Betulinic acid has also been reported to exhibit anti-tumor activity against human melanoma.[9] We were especially interested in preparing various structurally modified lupane-type triterpenes from **1** in order to find compounds with more potent biological activities. Thus, we undertook the microbial transformation of **1** and its chemically oxidized product, betulonic acid [**2**; 3-oxolup-20(29)-en-28-oic acid], by the filamentous fungus *Chaetomium longirostre*[10] in order to obtain oxygenated derivatives.[10−12] This paper reports the microbial transformations of **1** and **2** to metabolites that exhibited more potent inhibition of tumor-promoting activities than the parent compounds.

Results and Discussion

Since *C. longirostre* has been reported to efficiently transform oleanonic acid (3-oxo-olean-12-en-28-oic acid), a pentacyclic triterpene, into ring-A cleaved and hydroxylated compounds,[10] we opted to use this fungus for transformation of **1** and **2**.

To evaluate the ability of *C. longirostre* to metabolize compounds **1** and **2**, preliminary experiments were conducted in 500 mL flasks containing a four-day-old cultures of the fungus. After addition of the substrates, the fermentation was continued for two more weeks, after which
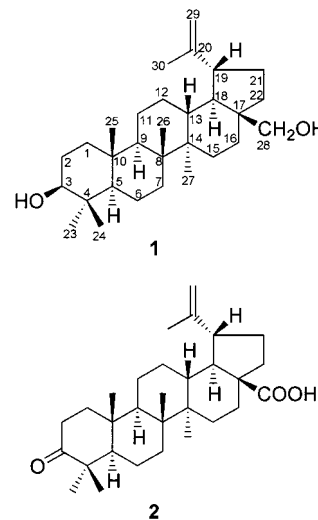
metabolites were detected in the broth. The metabolites were not present in the controls undertaken without either the mycelium or the substrate.

Assignments of all of the ¹³C and ¹H NMR spectral data for **3**−**6** (Table 1) described in this paper have been done with the aid of DEPT, ¹H−¹H COSY, HMQC, HMBC, and phase-sensitive NOESY experiments.

Incubation of **1** with *C. longirostre* on a preparative scale resulted in formation of **3** in 3% yield based on weight relative to starting material, along with two other minor uncharacterized metabolites and unmetabolized **1**. The molecular formula of **3** was determined as $C_{30}H_{50}O_4$ from its HREIMS ([M − H₂O]⁺, *m/z* 456.3603), positive ESIMS ([M + Na]⁺, *m/z* 497), and negative ESIMS ([M − H]⁻, *m/z* 473) as well as from its DEPT. The compound had a carboxyl group [ν_{max} 1710, 1640 cm⁻¹; δ_C 177.4 (s)] and a 1-hydroxy-1-methylethyl group [ν_{max} 3410 cm⁻¹; δ_C 28.0 (q), 34.0 (q), and 74.8 (s); δ_H 1.45 and 1.50 (each 3H and s)] but showed no evidence for the secondary hydroxyl group at C-3 and the geminal dimethyl group at C-4 present in **1**.[13] These data, in combination with the diagnostic EIMS

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Table 1. ^{13}C (125 MHz) and ^1H (500 MHz) NMR Spectral Data CDCl$_3$) for Four Lupane-type Triterpenoids (**3**–**6**)

C no.	**3** δ_C	$\delta_H{}^a$	HMBC (H→C)^b	**4** δ_C	$\delta_H{}^a$	**5** δ_C	$\delta_H{}^a$	**6** δ_C	$\delta_H{}^a$
1	35.3 t	2.12, 3.10		35.3 t	2.13, 3.10 br t (10.0)	35.5 t	2.16, 3.18 br t (10.0)	39.8 t	1.32, 1.80
2	30.1 t	2.62, 3.01		30.1 t	2.62, 3.02	30.2 t	2.59, 2.96	34.4 t	2.50 (2H)
3	177.4 s			177.4 s		177.4 s		216.1 s	
4	74.8 s			74.8 s		74.4 s		47.0 s	
5	52.1 d	1.70	4, 6, 10, 23	52.1 d	1.70	50.1 d	1.95	52.1 d	1.54 dd (2.4, 12.4)
6	11.8 t	1.59, 1.69	7	22.8 t	1.60 (2H)	34.1 t	2.02, 2.07	29.8 t	1.82, 1.92
7	33.9 t	1.34, 1.67	5, 6	34.1 t	1.40, 1.68	74.3 d	4.07 dd (3.9, 10.7)	72.2 d	4.17 dd (4.6, 10.7)
8	41.1 s			41.0 s		46.4 s		49.6 s	
9	42.4 d	1.80	10, 11, 14, 26	42.5 d	1.86	44.2 d	1.75	50.3 d	1.39
10	41.6 s			41.7 s		41.9 s		37.3 s	
11	21.1 t	1.21, 1.68		21.3 t	1.26, 1.71	21.8 t	1.33, 1.80	21.7 t	1.42 (2H)
12	26.0 t	1.29, 1.83		26.3 t	1.31, 1.99 br d (11.5)	27.8 t	1.03, 1.89	26.2 t	1.38, 1.99
13	37.8 d	1.84	14, 18	38.8 d	2.82 br t (11.5)	47.6 d	1.35	38.3 d	2.71
14	43.4 s			43.3 s		42.7 s		48.6 s	
15	27.7 t	1.12, 1.92	14, 16, 27	30.4 t	1.32, 1.88	32.3 t	1.81, 2.84 dt (13.6, 3.9)	69.1 d	4.69 br d (9.2)
16	30.0 t	1.33, 2.45	14, 17, 28	32.8 t	1.62, 2.63	34.5 t	2.05, 2.22	42.3 t	1.94, 3.18 br d (10.7)
17	48.6 s			56.7 s		79.0 s		55.3 s	
18	49.1 d	1.73	13, 16, 17, 19, 28	49.7 d	1.80	53.1 d	1.88	49.6 d	1.85
19	48.3 d	2.63 ddd (5.6, 11.0, 11.2)	13, 18, 20, 21, 29, 30	47.7 d	3.54 br t (12.4)	55.4 d	2.59	47.7 d	3.55
20	151.2 s			151.2 s		151.5 s		151.1 s	
21	30.5 t	1.50, 2.17	17	31.2 t	1.53, 2.26	30.5 t	1.92, 2.23	31.9 t	1.59, 2.36 br t (9.1)
22	34.8 t	1.16, 2.41	17, 18, 21, 28	37.5 t	1.57, 2.28	38.6 t	1.69, 2.04	37.7 t	1.83, 2.25 br t (9.3)
23	28.5 s	1.45 s	4, 5, 24	28.4 a	1.43 s	28.6 q	1.48 s	26.5 q	1.14 s
24	34.0 q	1.49 s	4, 5, 6, 23	34.0 q	1.48 s	33.7 q	1.48 s	21.2 q	1.02 s
25	21.0 q	1.17 s	1, 5, 9, 10	21.0 q	1.14 s	21.3 q	1.26 s	15.6 q	0.88 s
26	16.4 q	1.09 s	7, 8, 9, 14	16.6 q	1.14 s	10.1 q	1.37 s	11.3 q	1.36 s
27	14.9 q	1.13 s	8, 13, 14, 15	14.8 q	1.14 s	14.4 q	1.28 s	9.1 q	1.38 s
28	59.5 t	3.69 d (10.7), 4.12 d (10.7)	16, 22	178.9 s		79.0 s		178.7 s	
29	110.0 t	4.75 s, 4.91 d (1.2)	19, 30	110.0 t	4.76 s, 4.95 d (1.2)	109.6 t	4.87 t (1.5), 5.14 d (2.4)	110.1 t	4.82 s, 4.98 d (1.2)
30	19.3 q	1.74 s	19, 20, 29	19.5 q	1.77 s	20.8 q	2.04 s	19.7 q	1.83 s

^{a} Figures in parentheses denote *J* values (hertz). *J* values not included were not determined. ^{b} HMBC spectral data.

fragmentations observed at m/z 398 [M − C$_3$H$_6$O]$^+$, formed from cleavage of the C-4−C-5 bond with 1H transfer, and 367 (m/z 398-CH$_2$OH), and m/z 383 [M − CH$_2$CH$_2$COOH − H$_2$O]$^+$, corresponding to the loss of C-1−C-3 and H$_2$O, suggested a 4-hydroxy-3,4-*seco*-3-oic acid partial structure for **3**. This was further supported from the ^{13}C and ^1H NMR data (Table 1) and from spectral comparison with **1**.[13] Thus, metabolite **3** was characterized as 4,28-dihydroxy-3,4-*seco*-lup-20(29)-en-3-oic acid. The proposed stereochemistry of **3** was supported from NOE correlations observed between [H-24−H-25 (10β-Me)−H-26 (8β-Me)−H-13β−H-19β and H-28 (17β-CH$_2$OH)] on the β-face and [H-5α−H-9α−H-27 (14α-Me)−H-18α] on the α-face of the molecule (Figure 1).

Incubation of **2** afforded three metabolites, **4**–**6**, in 6%, 12%, and 4% yield, respectively, based on weight relative to starting material. The molecular formula of **4** was determined as C$_{30}$H$_{48}$O$_5$ from its HREIMS ([M − H$_2$O]$^+$, m/z 470.3396), positive ESIMS ([M + Na]$^+$, m/z 511), and

3

4

5

6
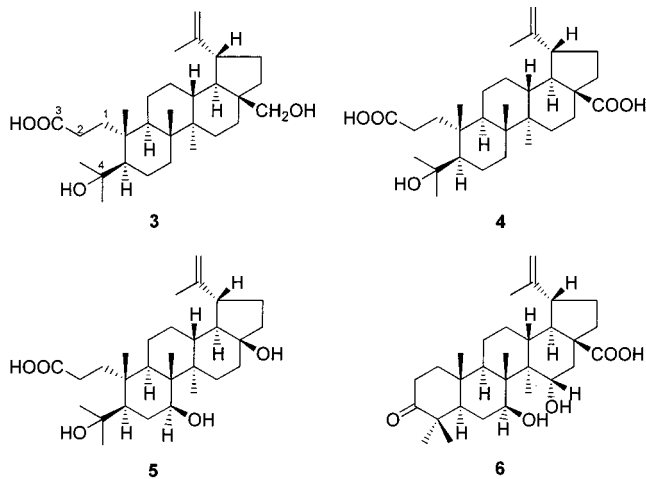
negative ESIMS ([M − H]$^-$, m/z 487) as well as from its DEPT. Compared to **2**,[14] metabolite **4** had new carboxyl [δ$_C$ 177.4 (s)] and 1-hydroxy-1-methylethyl [ν$_{max}$ 3420 cm^{-1}; δ$_C$ 28.4 (q), 34.0 (q), and 74.8 (s); δ$_H$ 1.43 and 1.58 (each 3H and s)] groups with the disappearance of the oxo group at C-3. The diagnostic EIMS fragmentations were observed for **4** at m/z 430 [M − C$_3$H$_6$O]$^+$ and m/z 415 [M − CH$_2$CH$_2$-COOH]$^+$, which, in combination with the data mentioned above, suggested a partial structure of 4-hydroxy-3,4-*seco*-3-oic acid for **4**. Furthermore, comparison of the ^{13}C and ^1H NMR data (Table 1) of **4** with those of **2**[14] and **3** allowed characterization of **4** as 4-hydroxy-3,4-*seco*-lup-20(29)-ene-3,28-dioic acid. The NOE correlations observed for **4** (Figure 1) were consistent with the proposed structure.

Metabolite **5** was determined to have the molecular formula of C$_{29}$H$_{48}$O$_5$, which is one carbon less than that of **2**, from its HREIMS ([M]$^+$, m/z 476.3504), positive ESIMS ([M + Na]$^+$, m/z 499), negative ESIMS ([M − H]$^-$, m/z 475), and DEPT. The presence of a 4-hydroxy-3,4-*seco*-3-oic acid structural moiety in the molecule, similar to metabolites **3** and **4**, was indicated from the ^{13}C NMR signals at δ$_C$ 177.4 (s; carboxyl carbon at C-3) and δ$_C$ 28.6 (q; C-23), 33.7 (q; C-24), and 74.4 (s; C-4), arising from the 1-hydroxy-1-methylethyl group at C-5, and from EIMS fragmentations observed at m/z 418 [M − C$_3$H$_6$O]$^+$ and m/z 400 [M − CH$_2$-CH$_2$COOH − H]$^+$. The two other oxygen atoms were shown to be present as a secondary hydroxyl [δ$_C$ 74.3 (d); δ$_H$ 4.07 (dd; *J* = 3.9, 10.7 Hz, axially oriented methine)] and a tertiary β-hydroxyl at C-17 [δ$_C$ 79.0 (s)], which replaced the carboxyl group at C-17β, accounting for the loss of a carbon unit. The axially oriented hydroxymethine (>C*H*OH; δ$_H$ 4.07; dd) was linked with H-5α (δ$_H$ 1.95) via a methylene group (δ$_H$ 2.02 and 2.07), as revealed from the ^1H−^1H COSY spectrum, suggesting that the secondary hydroxyl group was located at C-7 with a β-orientation. From these data, in combination with the ^{13}C and ^1H NMR data (Table
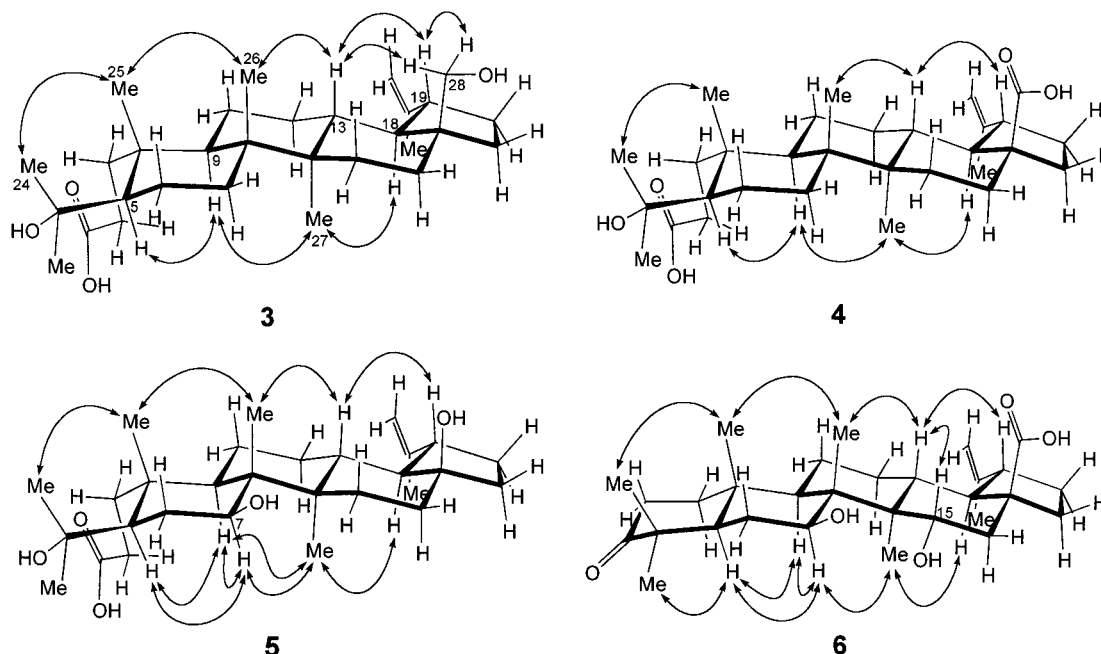
Figure 1. Major NOE correlations (←→) for **3**−**6**. Drawings correspond to energy-minimized conformations of triterpenes. Calculations were performed using MacroModel Ver. 6.0 with extended MM3 parameters. The conformation with minimum steric energy was obtained through a Metropolis Monte Carlo procedure.[20]

1), metabolite **5** was characterized as 4,7β,17-trihydroxy-3,4-*seco*-28-norlup-20(29)-en-3-oic acid. This was supported from significant NOE correlations observed between [H-24−H-25 (10β-Me)−H-26 (8β-Me)−H-13β−H-19β] on the β-face and [H-5α−H-7α and H-9α−H-27 (14α-Me)−H-18α] on the α-face of the molecule (Figure 1).

Metabolite **6**, determined to have the molecular formula $C_{30}H_{46}O_5$ from its HREIMS ([M − H_2O]$^+$, m/z 468.3237), positive ESIMS ([M + Na]$^+$, m/z 509), and negative ESIMS ([M − H]$^-$, m/z 485) and from its DEPT, possesses two oxygen atoms more than substrate **2**. The two oxygen atoms were shown to be present as secondary hydroxyl groups at C-7 and C-15. One of the hydroxyls at C-7 [δ_C 72.2 (d)] with a β (equatorial)-orientation was located from the ^{1}H−^{1}H COSY spectrum in which the diagnostic one-bond cross-correlations were observed between the ^{1}H signal of the axially oriented hydroxymethine [>C*H*OH; δ_H 4.17 (dd; J = 4.6, 10.7 Hz); H-7α] and H-5α (δ_H 1.54) via a methylene group (δ_H 1.82 and 1.92; H-6). The other hydroxyl group was located at C-15 with an α (equatorial)-orientation based on the HMBC spectrum (see Experimental Section) in which a three-bond correlation was observed between H-27 [14α-Me; δ_H 1.38 (s)] and the ^{13}C signal of the hydroxyl carbon [δ_C 69.1 (d)] possessing an axially oriented hydroxymethine [>C*H*OH; δ_H 4.69 (br d; J = 9.2 Hz); H-15β]. On the basis of these observations, in combination with the ^{13}C and ^{1}H NMR data (Table 1), metabolite **6** was characterized as 7β,15α-dihydoxy-3-oxolup-20(29)-en-28-oic acid. This structure was supported by NOE correlations observed between [H-24 (4β-Me)−H-25 (10β-Me)−H-26 (8β-Me)−H-13β−H-15β and H-19β] on the β-face and [H-23 (4α-Me)−H-5α−H-7α (and H-9α)−H-27 (14α-Me)−H-18α] on the α-face of the molecule (Figure 1). The ^{13}C NMR data of **6** (Table 1) were in accord with those reported for 3β,7β,15α-trihydroxylup-20(29)-en-28-oic acid,[12] except for the expected differences in the ^{13}C signals of C-3 and adjacent carbons, further supporting the proposed structure of **6**. A fragmentation ion at m/z 271 ($C_{19}H_{27}O$) observed as a base peak in the EIMS of **6** could be accounted for by the loss of D- and E-rings resulting from

cleavage of bonds C-13−C18 and C-14−C-15 with concomitant loss of H_2O and 1H transfer.

In summary, oxidative ring cleavage, hydroxylation, and decarboxylation were observed in this study of the transformation of **1** and **2** with the fungus *C. longirostre* IFO 9873. The microbial oxidative cleavage of ring-A of a triterpene to give 4-hydroxy-3,4-*seco*-3-oic acid has previously been observed in the transformation of eburicoic acid [3β-hydroxy-24-methyllanosta-8,24(24^1)-dien-21-oic acid] by the fungus *Glomerella fusarioides* ATCC 9552[15] and oleanonic acid by *C. longirostre* RF-1095[10] and has been suggested to arise by a Baeyer−Villiger type oxidation of the 3-ketone followed by hydrolysis of the resulting seven-membered ring lactone.[10,15] Formation of metabolite **5** requires decarboxylation followed by hydroxylation at C-17 of substrate **2**. Although dealkylation−hydroxylation of the sterol cycloartenol (9β,19-cycloart-24-en-3β-ol) to afford 17β-hydroxyandrosta-4,8(14)-dien-3-one by *Mycobacterium* sp. (NRRL B-3805) has been previously reported,[16] this appears to be the first report of such a biotransformation of a tritepene. 7β,15α-Dihydroxylation has recently been reported also in the transformation of betulinic acid by *Bacillus megaterium* ATCC 13368.[12]

The inhibitory effects of the substrates **1** and **2**, their microbial metabolites **3**−**6**, and the reference substance β-carotene on EBV-EA activation induced by TPA were examined as a preliminary evaluation of their potential to inhibit tumor-promotion, and the results are shown in Table 2. All compounds evaluated showed inhibitory effects on EBV-EA activation at 1×10^2 mol ratio concentration (compound/TPA) and exhibited significant activity at a high concentration (90−100% inhibition at 1×10^3 mol ratio concentration) while not adversely affecting the viability of Raji cells. The inhibitory effects of all compounds tested were equivalent to or more potent than those of β-carotene, a vitamin A precursor that has been intensively studied in cancer prevention using animal models.[17] Among the compounds tested, the biotransformation products that underwent ring-opening and hydroxylation exhibited more potent activity than their corresponding precursors. Since

Table 2. Percentage of Epstein–Barr Virus Early Antigen Induction in the Presence of Compounds **1**–**6** with Respect to a Positive Control (100%)[a]

compound	mol ratio concentration (compound/TPA)			
	1000	500	100	10
1	4.7 (70)	33.3	74.2	96.8
2	9.9 (70)	45.7	79.3	100
3	0 (60)	24.7	71.6	94.7
4	0 (60)	24.7	71.2	94.1
5	0 (60)	20.0	66.4	89.1
6	3.5 (60)	42.6	76.5	96.9
glycyrrhetic acid[b]	15.6 (>80)	54.3	100	100

[a] Values represent relative percentages to the positive control value. Lower values reflect higher potency. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells. [b] Reference compound.

the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel inhibition of tumor promotion in vivo,[18] the lupane-type triterpenes, and especially the biotransformation products obtained in this study, may be valuable inhibitors of promotion (potential cancer chemopreventive agents). Thus, this study has established that microbial transformation of natural products has a potential value in developing more potent inhibitors of tumor promotion.

Experimental Section

General Experimental Procedures. Crystallizations were performed in MeOH, and melting points reported are uncorrected. IR spectra were recorded in KBr disks. NMR spectra were recorded at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in C_5D_5N with tetramethylsilane (TMS) as internal standard. EIMS and HREIMS were recorded at 70 eV. Positive and negative ESIMS were recorded on an Agilent Technologies 1100 LC/MSD SL instrument. Column chromatography was carried out on silica gel (Si gel 60, 220–400 mesh; Merck). Reversed-phase HPLC was carried out on an a reversed-phase (C_{18}) column (Pegasil ODS II column, 25 cm × 10 mm i.d.; Senshu Scientific Co., Ltd., Tokyo, Japan) at 25 °C with a refractive index detector and MeOH–H_2O–AcOH (80:20:1, v/v/v; 3.0 mL/min) as a mobile phase. Betulin (**1**; retention time 52 min) was the standard for the determination of relative retention time (Rt_R) in HPLC of the triterpenes described in this paper.

Materials. A tree of *Betula platyphylla* Sukatchev var. *japonica* (Miq.) Hara was collected in September 1998, at Bifuka-cho, Hokkaido (Japan), and kindly donated by Mr. Ryoji Hamatani, The Bifuka-cho Forest Owner's Cooperative (Bifuka-cho, Hokkaido, Japan). Identification of the plant material was done by Mr. R. Hamatani. A voucher specimen has been deposited at College of Science and Technology, Nihon University. Compound **1** used as the authentic specimen was described previously.[2]

Isolation of Betulin (1). The outer bark (124 g) separated from the birch tree (3.6 kg) was powdered and oven-dried (98 g) and was then extracted by soaking in MeOH at room temperature for 3 days each for three times to give an extract (54.7 g). The extract was crystallized from acetone–MeOH, which gave a crystallized portion (13.9 g), consisting most exclusively of compound **1**, which upon column chromatography on a Si gel (700 g) column with a stepwise gradient of *n*-hexane–EtOAc [9:1 (2.4 L), 6:1 (2.1 L), 4:1 (3.0 L), 1:1 (5.1 L); v/v] as eluant yielded purified compound **1** (12.2 g; Rt_R 1.00 in HPLC). The physical and spectral data of **1** were in agreement with the reported data for betulin.[13]

Preparation of Betulonic Acid (2) from Betulin (1). To a solution of compound **1** (1.0 g) in acetone (50 mL) was added dropwise freshly prepared Jones' reagent at 0 °C.[19] The solution was stirred for 1.5 h at 0 °C, quenched with MeOH (25 mL), and stirred for 5 min, and H_2O (40 mL) was added.

The organic solvent was removed in vacuo, and the aqueous residue was extracted with EtOAc (2 × 40 mL). The EtOAc layer was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. Preparative TLC (Si gel G; developing solvent: *n*-hexanes–EtOAc–HOAc = 85:15:1, v/v/v; 2 developments) of the residue gave betulonic acid (**2**; 316 mg; Rt_R 1.40 in HPLC). The physical and spectral data of **2** were in agreement with the reported data for betulonic acid.[14,19]

Fungus and Culture Conditions. Stock culture of the fungus *Chaetomium longirostre* IFO 9873 obtained from the Institute for Fermentation (IFO), Osaka, was stored on potato-dextrose agar medium (PDA, Nissui Pharmaceutical Co., Ltd., Tokyo) at 24 °C. A seed culture was grown in a 500 mL flask containing 300 mL of potato-dextrose broth medium (PDB; 39.5 g of Nissui PDA powder was suspended in 1 L of H_2O and the agar was removed by filtration). After incubation at room temperature under stirring with a magnetic stirrer for 4 days, the whole culture was transferred into a 5 L culture flask containing 2.3 L of PDB and incubated for 2 days under aeration by bubbling and stirring. The cells were harvested and washed with H_2O by filtration, obtaining 70 g fresh weight of mycelium.

Biotransformation of Betulin (1) and Isolation of Metabolites. Betulin (**1**; 200 mg/4 mL DMSO) was dispersed to the mycelium (70 g fr. wt), suspended in 3 L of H_2O in a 5 L culture flask, and incubated for 2 weeks at room temperature under aeration by bubbling and stirring. After incubation, the mycelium was filtered off and washed with EtOAc. The broth, after adjusting the acidity at pH 3–4 by diluted HCl, was extracted three times with EtOAc, and the organic layers were combined. The extract (130 mg) was then subjected to column chromatography on Si gel (10 g). Elution with 500 mL of *n*-hexanes–EtOAc (9:1, v/v) and 600 mL of EtOAc gave fraction A (63 mg), from which unmetabolized betulin (**1**) was recovered (38 mg) after rechromatography. Subsequent elution with MeOH (1 L) gave fraction B (50 mg), from which compound **3** (6 mg; Rt_R 0.52 in HPLC) was isolated along with two minor uncharacterized compounds by reversed-phase HPLC.

4,28-Dihydroxy-3,4-*seco*-lup-20(29)-en-3-oic acid (3): fine needles, mp 171–173 °C; IR ν_{max} 3410 (OH), 1710, 1640 (COOH), 880 (C=CH_2) cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; EIMS *m/z* 456 [M − H_2O]$^+$ (7), 441 [M − Me − H_2O]$^+$ (1), 425 [M − CH_2OH − H_2O]$^+$ (12), 398 [M − C_3H_6O − H_2O]$^+$ (14), 385 (11), 383 [M − CH_2CH_2COOH − H_2O]$^+$ (11), 367 [M − C_3H_6O − CH_2OH]$^+$ (39), 357 (13), 345 (9), 327 (3), 315 (4), 271 (6), 257 (6), 245 (10), 234 [M − $C_{14}H_{22}O_2$ − H_2O]$^+$ (15), 221 (13), 215 (13), 203 (*m/z* 234 − CH_2OH) (46), 189 [M − $C_{15}H_{24}O_2$ − H_2O]$^+$ (55), 95 (100); HREIMS *m/z* 456.3603 (calcd for $C_{30}H_{48}O_3$, 456.3602); positive ESIMS *m/z* 497 [M + Na]$^+$; negative ESIMS *m/z* 473 [M − H]$^-$.

Biotransformation of Betulonic Acid (2) and Isolation of Metabolites. The procedure was similar to that described above for the transformation of **1**. From 150 mg of transformed **2** we obtained an EtOAc extract (120 mg), which was fractionated by column chromatography on Si gel. Elution with *n*-hexanes–EtOAc (9:1, v/v; 850 mL) followed by EtOAc (500 mL) gave fraction A′ (48 mg), from which unmetabolized **2** (16 mg) was recovered after rechromatography. Further elution with MeOH (1 L) yielded fraction B′ (53 mg). Reversed-phase HPLC of the fraction yielded metabolites **4** (6 mg; Rt_R 0.44 in HPLC), **5** (18 mg; Rt_R 0.18), and **6** (9 mg; Rt_R 0.28).

4-Hydroxy-3,4-*seco*-lup-20(29)-ene-3,28-dioic acid (4): fine needles, mp 197–199 °C; IR ν_{max} 3420 (OH), 1698, 1642 (COOH), 881 (C=CH_2) cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; HMBC: H-1→C-2, C-5, C-9, C-10; H-2→C-1; H-5→C-4, C-6, C-10, C-23, C-24, C-25; H-6→C-5; H-9→C-8, C-10, C-11, C-26; H-13→C-14, C-18, C-27; H-16→C-14, C-18, C-28; H-18→C-28; H-19→C-18, C-21, C-29, C-30; H-21→C-18, C-19, C-22; H-22→C-17, C-20, C-21, C-28; H-23→C-5, C-24; H-24→C-5, C-23; H-25→C-1, C-5, C-9, C-10; H-26→C-7, C-9; H-27→C-13, C-15; H-29→C-19, C-30; H-30→C-19, C-20, C-29; EIMS *m/z* 470 [M − H_2O]$^+$ (13), 455 [M − Me − H_2O]$^+$ (4), 430 [M − C_3H_6O]$^+$ (42), 424 (6), 415 [M − CH_2CH_2COOH]$^+$ (12), 412 (*m/z* 430-H_2O) (48), 397 (*m/z* 415 − H_2O) (36), 384 [M − C_3H_6O −

COOH]$^+$ (49), 369 (17), 366 (20), 357 (24), 343 (42), 325 (13), 312 (13), 259 (20), 235 (42), 234 (40), 219 (36), 203 (61), 189 (100); HREIMS m/z 470.3396 (calcd for $C_{30}H_{46}O_4$, 470.3393); positive ESIMS m/z 511 [M + Na]$^+$; negative ESIMS m/z 487 [M − H]$^-$.

4,7β,17-Trihydroxy-3,4-*seco*-28-norlup-20(29)-en-3-oic acid (5): fine needles, mp 145−146 °C; IR ν_{max} 3445 (OH), 1709, 1641 (COOH), 884 (C=CH$_2$) cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; HMBC: H-1→C-3; H-5→C-4, C-6, C-7, C-10, C-23, C-24, C-25; H-6→C-5, C-7, C-8; H-7→C-5, C-6, C-8, C-14, C-26, C-27; H-9→C-1, C-5, C-8, C-10, C-11, C-25, C-26; H-11→C-9; H-12→C-9, C-13; H-13→C-12, C-18, C-19, C-27; H-15→C-13, C-14, C-16, C-17, C-27; H-16→C-14, C-15, C-17; H-18→C-12, C-17, C-19, C-22; H-19→C-13, C-18, C-20, C-21, C-29, C-30; H-21→C-17, C-18, C-19, C-20, C-22; H-22→C-17, C-18, C-19, C-21; H-23→C-4, C-5, C-24; H-24→C-4, C-5, C-23; H-25→C-1, C-5, C-9, C-10; H-26→C-7, C-8, C-9, C-14; H-27→C-8, C-14, C-15, C-16, C-26; H-29→C-19, C-20, C-30; H-30→C-19, C-20, C-29; EIMS m/z 476 [M]$^+$ (2), 458 [M − H$_2$O]$^+$ (25), 440 [M − 2H$_2$O]$^+$ (37), 429 (6), 418 [M − C$_3$H$_6$O]$^+$ (7), 400 [M − CH$_2$-CH$_2$COOH − H]$^+$ (26), 382 (m/z 400 − H$_2$O) (26), 376 (19), 367 (10), 339 (10), 205 (22), 201 (34), 189 (52), 175 (54), 161 (51), 121 (86), 81 (100); HREIMS m/z 476.3504 (calcd for $C_{29}H_{48}O_5$, 476.3502); positive ESIMS m/z 499 [M + Na]$^+$; negative ESIMS m/z 475 [M − H]$^-$.

7β,15α-Dihydroxy-3-oxolup-20(29)-en-28-oic acid (6): fine needles, mp 264−266 °C; IR ν_{max} 3454 (OH), 1706, 1641 (COOH), 1692 (C=O), 881 (C=CH$_2$) cm^{-1}; ^{13}C and ^{1}H NMR data, see Table 1; HMBC H-1→C-2, C-3, C-10, C-25; H-2→C-1, C-3, C-10; H-5→C-1, C-4, C-6, C-9, C-10, C-23, C-24, C-25; H-6→C-5, C-7, C-10; H-7→C-6, C-8, C-26; H-9→C-11, C-26; H-11→C-9, C-13; H-13→C-12; H-15→C-16; H-16→C-14, C-28; H-18→C-19, C-21, C-22; H-19→C-30; H-23→C-3, C-5, C-24; H-24→C-3, C-5, C-23; H-25→C-1, C-5, C-10; H-26→C-7, C-8, C-9, C-14; H-27→C-8, C-13, C-14, C-15; H-29→C-19, C-30; H-30→C-19, C-20, C-29; EIMS m/z 468 [M − H$_2$O]$^+$ (66), 453 (13), 450 [M − 2H$_2$O]$^+$ (18), 440 (6), 435 (12), 422 (6), 407 (5), 389 (11), 343 (3), 271 (100), 262 (12), 243 (11), 234 (8), 218 (12), 203 (18); HREIMS m/z 468.3239 (calcd for $C_{30}H_{44}O_4$, 468.3237), 271.2062 (calcd for $C_{19}H_{27}O$, 271.2061); positive ESIMS m/z 509 [M + Na]$^+$; negative ESIMS m/z 485 [M − H]$^-$.

Method of EBV-EA Induction Tests. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 10% fetal bovine serum−Roswell Park Memorial Institute (FBS RPMI) 1640 medium solution (Nacalai Tesque, Inc., Kyoto, Japan). The indicator cells (Raji) (1×10^6/mL) were incubated at 37 °C for 48 h in 1 mL of the medium containing *n*-butyric acid (4 mM, trigger), 32 pmol of TPA (20 ng/mL, inducer) in dimethyl sulfoxide (DMSO), and a known amount of test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients and were detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated twice. The average EA induction was compared with that of positive control experiments using *n*-butyric acid plus TPA, in which EA induction was ordinarily around 30%.

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