Studies on Differentiation-Inducing Activities of Triterpenes

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Differentiation-inducing activity of over 180 extracts of crude drugs and plants was tested using mouse myeloid leukemia cell line (M1). The methanol extracts of clove (*Syzygium aromaticum* MERRILL *et* PERRY, Myrtaceae) showed remarkable induction of differentiation of M1 cells into macrophage-like cells. From the extract, oleanolic acid (1) and crategolic acid (2) were isolated as the active components. We also tested other triterpenes, such as oleananes, ursanes and dammaranes, to investigate the structure–activity relationship. Some triterpene aglycones showed differentiation-inducing activity, but triterpene glycosides showed little activity. Futhermore, the differentiation-inducing activity of these triterpene compounds was tested against human acute promyelocytic leukemia cell line (HL-60).

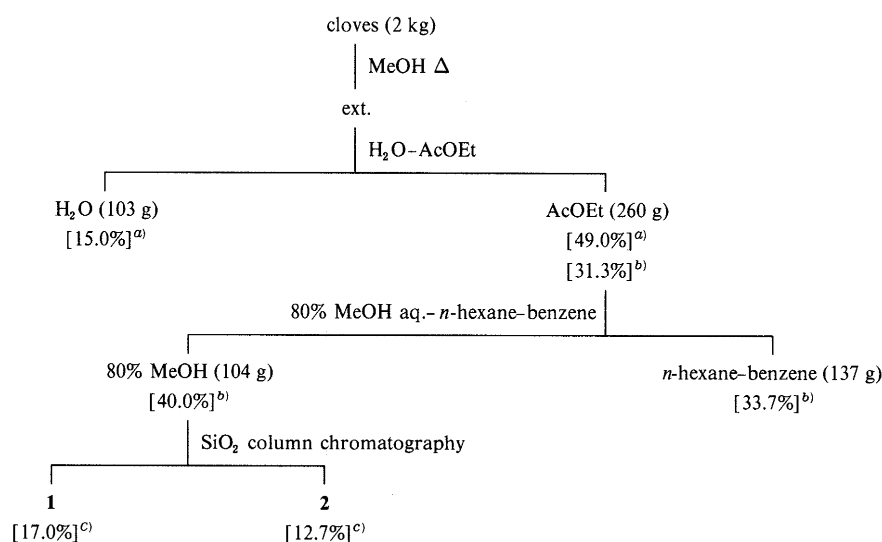
Keywords clove; triterpene; differentiation-inducing activity; phagocytosis; macrophage; M1 cell; HL-60 cell

Some myeloid leukemia cell lines are known to differentiate into granulocytes and macrophages on treatment with various inducers.[1] The differentiated cells possess some properties that are expressed in normal granulocytes and macrophages, such as Fc receptors, motility, phagocytosis, lysosomal enzymes and morphologic changes, and show decreased transplantability in syngeneic animals.[2] M1 cells were originally established by Ichikawa from SL mouse with myeloid leukemia, and are known to differentiate into macrophages and granulocytes *in vitro*.[3] Human promyelocytic leukemia cell line (HL-60), established from a patient with acute promyelocytic leukemia, has also been reported to differentiate into macrophages and granulocytes *in vitro*.[4] Differentiation inducers are expected to be a new type of antitumor agent, but only a few studies have been reported on inducers from plant sources.[5,6] Therefore, we have searched for naturally occurring substances which induce differentiation of leukemia cells. As a screening assay for differentiation inducers, the inducibility of phagocytosis was employed.

Over 180 extracts of crude drugs and plants were tested and methanol extract of clove, one of the most effective extracts, was selected as a source for the isolation of differentiation inducers. This paper deals with the isolation of the inducers in clove, and an investigation of the structure–activity relationship of several types of triterpenes using M1 and HL-60 cells.

The methanol extract of clove showed potent differentiation-inducing activity toward M1 cells. The methanol extract was fractionated into ethyl acetate-soluble and water-soluble fractions. The ethyl acetate layer showed significant activity against M1 cells (Chart 1). The 80%-MeOH soluble part of the ethyl acetate layer was chromatographed on a silica gel column to give oleanolic acid (**1**) and crategolic acid (**2**) as active compounds (shown in Chart 1). Oleanolic acid (**1**) was identified by direct comparison with an authentic sample. Crategolic acid (**2**) was identified by comparison of various data with reported values.[7,8] Compounds **1** and **2** were also isolated from the *n*-hexane–benzene layer as active components.

As these active compounds are triterpenoids having an oleanane skeleton, we examined the differentiation-inducing



asterisks indicate that the fraction exhibits activity at: *a*) 100 μg/ml; *b*) 50 μg/ml; *c*) 10 μg/ml
( ), yield; [%], phagocytosis of M1 cells

Chart 1

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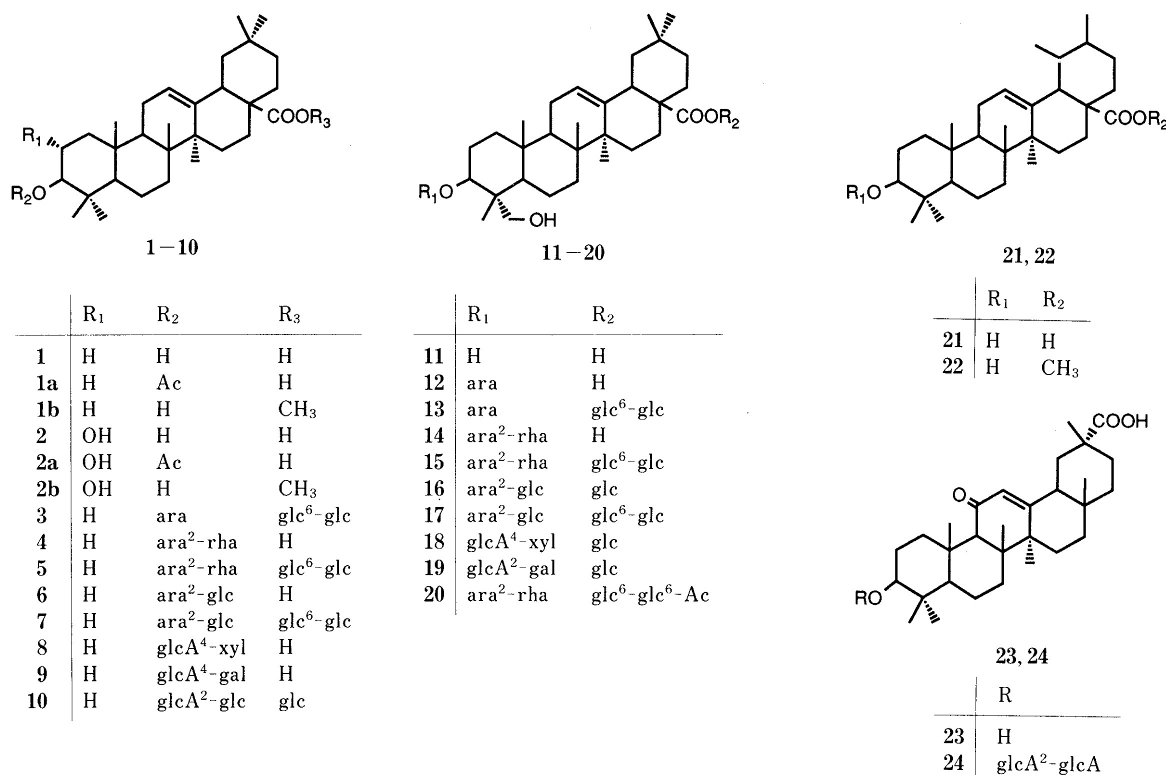
activity of other triterpenes. Besides **1** and **2**, 38 triterpenes (**3**—**40**) were tested for differentiation-inducing activity to investigate their structure–activity relationship (Charts 2, 3, Table I). Among oleanane compounds, oleanolic acid (**1**) and oleanolic acid acetate (**1a**) showed activity at 5×10^{-5} M and 2×10^{-5} M, respectively. Crategolic acid (**2**) and crategolic acid diacetate (**2a**) exhibited the activity at the concentration of 2×10^{-5} M. Methyl oleanolate (**1b**) and methyl crategolate (**2b**) had no differentiation-inducing activity, but casued growth inhibition. The mono desmosides at the C-3 position (**4**, **6**, **8**, **9**, **12** and **14**) showed enhanced cytotoxicity against M1 cells. Compounds **8** and **12** showed slight differentiation-inducing activity, but other compounds could not be evaluated because of their cytotoxicity. Of these triterpene compounds, the bisdesmosides (**3**, **5**, **7**, **10**, **13**, **15** and **20**) showed no differentiation-inducing activity or cytotoxicity. Glycosidation at the C-3 position weakened the activity of the aglycones. Ursane compounds (**21** and **22**) showed remarkable cytotoxicity but no differentiation-inducing activity. Dammarane compounds (**25**—**40**) showed no cytotoxicity, but some bisdesmosides (**29**, **31** and **36**) showed



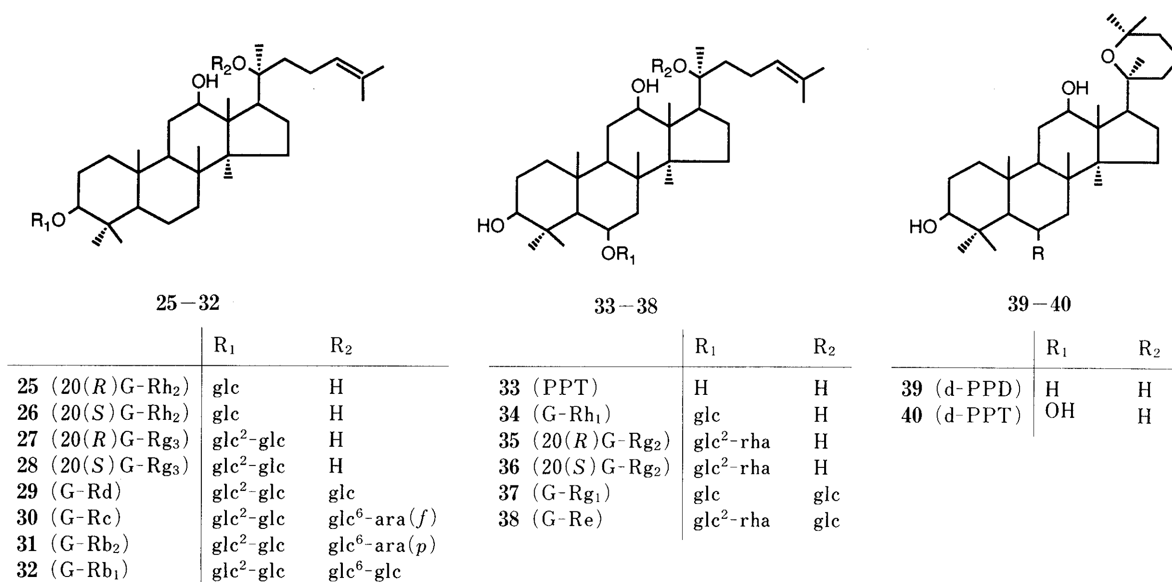
	R1	R2	R3
1	H	H	H
1a	H	Ac	H
1b	H	H	CH_3
2	OH	H	H
2a	OH	Ac	H
2b	OH	H	CH_3
3	H	ara	glc^6-glc
4	H	ara^2-rha	H
5	H	ara^2-rha	glc^6-glc
6	H	ara^2-glc	H
7	H	ara^2-glc	glc^6-glc
8	H	glcA^4-xyl	H
9	H	glcA^4-gal	H
10	H	glcA^2-glc	glc

	R1	R2
11	H	H
12	ara	H
13	ara	glc^6-glc
14	ara^2-rha	H
15	ara^2-rha	glc^6-glc
16	ara^2-glc	glc
17	ara^2-glc	glc^6-glc
18	glcA^4-xyl	glc
19	glcA^2-gal	glc
20	ara^2-rha	glc^6-glc^6-Ac

	R1	R2
21	H	H
22	H	CH_3

	R
23	H
24	glcA^2-glcA

Chart 2



	R1	R2
25 (20(*R*)G-Rh2)	glc	H
26 (20(*S*)G-Rh2)	glc	H
27 (20(*R*)G-Rg3)	glc^2-glc	H
28 (20(*S*)G-Rg3)	glc^2-glc	H
29 (G-Rd)	glc^2-glc	glc
30 (G-Rc)	glc^2-glc	glc^6-ara(*f*)
31 (G-Rb2)	glc^2-glc	glc^6-ara(*p*)
32 (G-Rb1)	glc^2-glc	glc^6-glc

	R1	R2
33 (PPT)	H	H
34 (G-Rh1)	glc	H
35 (20(*R*)G-Rg2)	glc^2-rha	H
36 (20(*S*)G-Rg2)	glc^2-rha	H
37 (G-Rg1)	glc	glc
38 (G-Re)	glc^2-rha	glc

	R1	R2
39 (d-PPD)	H	H
40 (d-PPT)	OH	H

G, ginsenoside; PPD, protopanaxadiol; PPT, protopanaxatriol; d, dehydroxy

Chart 3

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TABLE I. Cell Growth and Phagocytosis of M1 Cells Treated with Triterpenes

Compound[a]	Concentration (M)	Growth rate (%)	Phagocytic[b] activity
Cont.		100	—
Dex.	10^{-6}	88	+ + +
1	10^{-4}	14	+ + +
1	5×10^{-5}	56	+
1a	10^{-4}	5	+ +
1a	2×10^{-5}	82	+
1b	10^{-4}	0	uc
1b	2×10^{-5}	0	uc
3	5×10^{-5}	100	—
4	5×10^{-5}	1	uc
5	5×10^{-5}	65	—
6	5×10^{-5}	0	uc
6	2×10^{-5}	1	uc
7	5×10^{-5}	90	—
8	5×10^{-5}	11	+ +
9	5×10^{-5}	59	—
10	5×10^{-5}	55	—
Cont.		100	—
Dex.	10^{-6}	58	+ + +
2	10^{-4}	0	uc
2	2×10^{-5}	85	+
2	2×10^{-6}	75	—
2a	10^{-4}	2	uc
2a	2×10^{-5}	42	+ +
2a	2×10^{-6}	86	—
2b	10^{-4}	0	uc
2b	2×10^{-5}	15	—
2b	2×10^{-6}	69	—
Cont.		100	—
Dex.	10^{-6}	58	+ +
21	10^{-4}	0	uc
21	2×10^{-5}	1	uc
21	2×10^{-6}	73	—
22	10^{-4}	0	uc
22	2×10^{-5}	29	—
22	2×10^{-6}	72	—
23	5×10^{-5}	92	—
24	5×10^{-5}	71	—
Cont.		100	—
Dex.	10^{-6}	88	+ + +
11	5×10^{-5}	39	+ +
11	2×10^{-5}	59	—
12	5×10^{-5}	0	uc
12	2×10^{-5}	59	+
12	1×10^{-5}	59	—
13	5×10^{-5}	100	—
14	5×10^{-5}	0	uc
14	2×10^{-5}	1	uc
14	1×10^{-5}	63	—
15	5×10^{-5}	95	—
16	5×10^{-5}	100	—
17	5×10^{-5}	91	—
18	5×10^{-5}	55	—
19	5×10^{-5}	65	—
20	5×10^{-5}	73	—
Cont.		100	—
Dex.	10^{-6}	67	+ +
25	5×10^{-5}	70	—
26	5×10^{-5}	49	—
27	5×10^{-5}	94	—
28	5×10^{-5}	89	—
29	5×10^{-5}	93	+
30	5×10^{-5}	79	—
31	5×10^{-5}	71	+
32	5×10^{-5}	100	—
33	5×10^{-5}	66	—
34	5×10^{-5}	82	—
35	5×10^{-5}	82	—
36	5×10^{-5}	100	+
37	5×10^{-5}	100	—
38	5×10^{-5}	92	—
39	5×10^{-5}	91	—
40	5×10^{-5}	55	—

a) Cont., control; Dex., dexamethasone. *b*) +, >10%; + +, >25%; + + +, >50%; uc, uncountable.

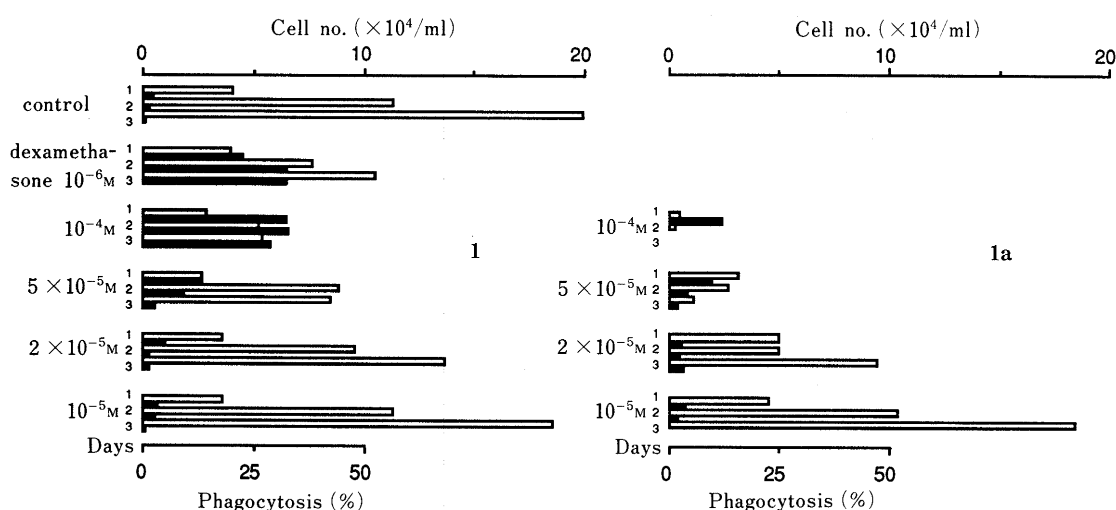

Fig. 1. Time Course of Cell Growth and Phagocytosis of M1 Cells Treated with **1** and **1a**

▭, cell no.; ■, phagocytosis.

slight differentiation-inducing activity. Compounds **36** having 20 (*S*) configuration and **31** which has arabinopyranose in the sugar chain exhibited differentiation-inducing activity, but **35** having 20 (*R*) configuration and **30** which has arabinofuranose in the sugar chain did not. It was reported that ginsenoside Rh_1 showed differentiation-

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TABLE II. Cell Growth and Phagocytosis of HL-60 Cells Treated with Triterpenes

Compound[a]	Concentration (M)	Growth rate (%)	Phagocytic[b] activity	Compound[a]	Concentration (M)	Growth rate (%)	Phagocytic[b] activity
Cont.		100	−	Cont.		100	−
TPA	10^{-8}	20	++	TPA	10^{-8}	22	++
1	2×10^{-5}	27	+++	**2**	2×10^{-5}	3	uc
1	1×10^{-5}	37	+++	**2**	1×10^{-5}	41	+
1	5×10^{-6}	70	++	**2**	5×10^{-6}	100	−
1	2×10^{-6}	90	−	**2**	2×10^{-6}	97	−
1a	2×10^{-5}	23	++	**2a**	2×10^{-5}	19	+
1a	1×10^{-5}	27	+	**2a**	1×10^{-5}	22	+
1a	5×10^{-6}	40	−	**2a**	5×10^{-6}	34	+
1a	2×10^{-6}	50	−	**2a**	2×10^{-6}	62	−
1b	2×10^{-5}	3	uc	**2b**	2×10^{-5}	41	−
1b	1×10^{-5}	33	−	**2b**	1×10^{-5}	53	−
1b	5×10^{-6}	67	−	**2b**	5×10^{-6}	100	−
1b	2×10^{-6}	70	−	**2b**	2×10^{-6}	97	−

a) Cont., control; TPA, 12-*O*-tetradecanoylphorbol-13-acetate. *b*) +, >10%; ++, >25%; +++, >50%; uc, uncountable.

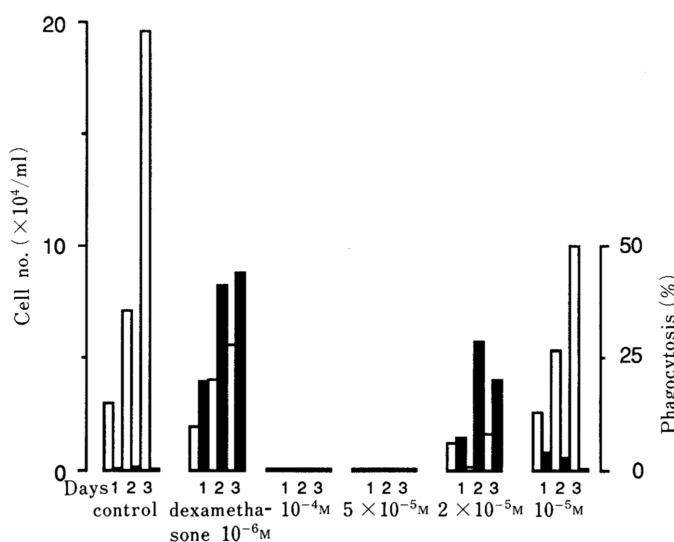

Fig. 2. Time Course of Cell Growth and Phagocytosis of M1 Cells Treated with **2**

▭, cell no.; ■, phagocytosis.

inducing activity toward melanoma B16 cells and ginsenoside Rh_2 showed growth-inhibitory activity toward B16 cells.[5] Ginsenoside Rh_2 (**26**) inhibited the growth of M1 cells by about 50% at 5×10^{-5} M, but ginsenoside Rh_1 (**34**) did not induce differentiation of M1 cells (Table I). Compounds **1**, **2**, their acetates (**1a**, **2a**) and their methyl esters (**1b**, **2b**) were tested at different incubation times and various concentrations against M1 and HL-60 cells (Figs. 1 and 2 and Table II). Dexamethasone showed maximum effect after 48 h incubation. Compound **1** showed activity even at 24 h, and suppressed cell proliferation at 5×10^{-5} M (Fig. 1). Compound **1a** was less active than **1** but marked suppression of cell proliferation was observed at 2×10^{-5} M. Compound **2** showed differentiation-inducing activity against M1 cells after 48 h incubation and cytotoxicity at 10^{-5} M (Fig. 2). HL-60 cells differentiate into macrophages on treatment of 12-*O*-tetradecanoylphorbol-13-acetate (TPA),[9] but granulocytes on treatment with dimethylsulfoxide,[10] retinoic acid[11] and so on. In this paper, TPA was used as a positive control and assay was carried out in the same way as in the case of M1 cells. Compound **1** showed remarkable activity toward HL-60 over 5×10^{-6} M, and the acetate (**1a**) also showed activity (Table II). Compound **2** was difficult to evaluate because of its cytotoxicity. Methylation of these compounds destroyed their differentiation-inducing activity toward both M1 and HL-60 cells. It was suggested that the carboxyl group at C-28 was necessary for induction of differentiation of these cells. Acetylation of these compounds increased their cytotoxicity toward M1 cells, but their activity toward HL-60 cells was retained.

Differentiation inducers are expected to be a new type of anticancer agent, because they induce neoplasmic cells to differentiate into normal cells. Thus far, some flavonoids and ginsenosides have been reported as differentiation inducers isolated from plants. In this paper, we revealed the differentiation-inducing activity of triterpenes and the existence of additional differentiation inducers in plant materials. Differentiation-inducing activity of various compounds was tested using M1 and HL-60 cells, and some exhibited activity. The mechanism of induction of differentiation of M1 and HL-60 lines by these compounds is unknown. Further studies using these cell lines are in progress.

Experimental

Melting points were taken on Yanaco MP-500 micromelting point apparatus and are uncorrected. Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were recorded on a JEOL FX-90Q NMR spectrometer (89.55 and 22.5 MHz, respectively). Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). High performance liquid chromatogaphy (HPLC) was conducted on a JASCO model 880 PU with UVIDEC 100V systems.

Isolation Commercially available cloves (2 kg from Niiya in Shimizu) were extracted with hot MeOH under reflux. The extract was concentrated under reduced pressure and the extract was partitioned between AcOEt and water. The AcOEt layer was successively partitioned between *n*-hexane–benzene (1 : 1) and methanol–water (8 : 2) to give a lipophilic fraction (hexane–benzene fr.) (137 g) and a hydrophilic fraction (methanol–water fr.) (104 g). The former was chromatographed on a silica gel column to give active components **1** and **2**.

Cell Culture M1 cells were grown in Eagle's MEM medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml kanamycin and 2 mmol/l L-glutamine in 10% heat-inactivated calf serum (CS) at a density of 2×10^5/ml in a 5% CO_2 humidified atmosphere at 37 °C. HL-60 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml

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streptomycin, 50 μg/ml kanamycin and 2 mmol/l L-glutamine at 37 °C in a humidified 5% CO_2 incubator.

Materials Eagle's MEM, RPMI 1640 medium, Eagle's MEM amino acids and vitamins medium were purchased from Nissui Pharmaceutical Co., Ltd. CS and FBS were from Gibco. Antibiotics were from Meiji Seika Kaisha, Ltd. L-Glutamine was from Wako Pure Chemical Industries, Ltd. Dexamethasone was from Nakarai Chemicals, Ltd. TPA was from Sigma Chemical Co. Polystyrene latex particles were from The Dow Chemical Company. Triterpene compounds **3**—**7**, **11**—**17** and **20**—**22** were isolated from *Lonicera japonica*,[12] and **8**—**10** and **18**, **19** were isolated from *Araria cordata*.[13] Compounds **23** and **24** were purchased from Tokyo Kasei Kogyo Co., Ltd. Compounds **25**—**40** were isolated from Ginseng Radix.

Measurement of Phagocytosis Phagocytic activity was assayed as reported by Hayashi.[14] Cells were inoculated at a concentration of 2×10^5 cells/ml into 2 ml of culture medium and incubated with 20 μl of sample solution diluted with ethanol. After 48 h, the cells were washed and incubated for 4 h with a suspension of polystyrene latex particles (2 μl/ml of serum free culture medium). Then the cells were washed thoroughly 3 or 4 times with phosphate-buffered saline (PBS) and the percentage of phagocytic cells was calculated.

Oleanolic Acid (1) Colorless needles (10.5 g), mp 298—300 °C(MeOH). Thin layer chromatographic (TLC) and HPLC behavior and melting point were identical with those of an authentic sample.

Crategolic Acid (2) Colorless powder (1.5 g), mp 252—254 °C (MeOH). **2** was identified after methylation by comparison of various data (mp, ^{1}H- and ^{13}C-NMR) with reported values.[7,8]

Acetylation of 1 and 2 Compounds **1** (5 mg) and **2** (5 mg) were acetylated in the usual manner using acetic anhydride and pyridine to give the acetate **1a** (4 mg) and **2a** (4 mg) as colorless needles. **1a** was identified by direct comparison with an authentic sample and **2a** was identified by comparison of its melting point with the reported value.[7] **1a**: Colorless needles, mp 253—255 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.75, 1.13, 1.26 (each 3H, CH_3), 0.87, 0.94 (each 6H, $2 \times CH_3$), 2.05 (3H, $OCOCH_3$), 2.80 (1H, dd, J=14, 4 Hz, C_{18}-H), 4.49 (1H, t, J=8 Hz, C_3-H), 5.27 (1H, br s, C_{12}-H). **2a**: Colorless needles, mp 235—238 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.72, 1.05, 1.11 (each 3H, CH_3), 0.86 (12H, $4 \times CH_3$), 1.98, 2.05 (each 3H, $OCOCH_3$), 2.80 (1H, dd, J=14, 4 Hz, C_{18}-H), 4.76 (1H, d, J=10 Hz, C_3-H), 5.12 (1H, m, C_2-H), 5.24 (1H, br s, C_{12}-H).

Methylation of 1 and 2 Compounds **1** (5 mg) and **2** (5 mg) were methylated in the usual manner using diazomethane and methanol to give **1b** (5 mg) and **2b** (5 mg), respectively, as colorless needles. **1b** was identified by direct comparison with an authentic sample and **2b** was identified by comparison of various data (mp, ^{1}H- and ^{13}C-NMR) with reported values.[7,8] **1b**: Colorless needles, mp 204—209 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.73, 0.78, 0.99, 1.14, 1.26 (each 3H, CH_3), 0.92 (6H, $2 \times CH_3$), 2.86 (1H, dd, J=14, 4 Hz, C_{18}-H), 3.16 (1H, t, J=8 Hz, C_3-H), 3.64 (3H, s, OCH_3), 5.20 (1H, br s, C_{12}-H). ^{13}C-NMR ($CDCl_3$) δ: 38.4 (C-1), 27.1 (C-2), 78.9 (C-3), 38.7 (C-4), 55.2 (C-5), 18.3 (C-6), 32.6 (C-7), 39.2 (C-8), 47.6 (C-9), 37.0 (C-10), 23.0 (C-11), 122.2 (C-12), 143.7 (C-13), 41.6 (C-14), 27.6 (C-15), 23.4 (C-16), 46.6 (C-17), 41.2 (C-18), 45.8 (C-19), 30.6 (C-20), 33.8 (C-21), 32.3 (C-22), 28.1 (C-23), 15.5 (C-24), 15.2 (C-25), 16.8 (C-26), 25.9 (C-27), 178.1 (C-28), 33.1 (C-29), 23.6 (C-30). **2b**: Colorless needles, mp 231—233 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.72, 0.84, 0.90, 0.93, 0.97, 1.04, 1.14 (each 3H, CH_3), 2.94 (1H, br d, J=14 Hz, C_{18}-H), 3.64 (3H, s, OCH_3), 5.28 (1H, br s, C_{12}-H). ^{13}C-NMR ($CDCl_3$) δ: 46.4 (C-1), 68.9 (C-2), 83.9 (C-3), 39.1 (C-4), 55.3 (C-5), 18.3 (C-6), 32.5 (C-7), 39.3 (C-8), 47.6 (C-9), 38.3 (C-10), 23.0 (C-11), 122.1 (C-12), 143.7 (C-13), 41.7 (C-14), 27.6 (C-15), 23.6 (C-16), 46.6 (C-17), 41.2 (C-18), 45.8 (C-19), 30.6 (C-20), 33.8 (C-21), 32.3 (C-22), 28.6 (C-23), 16.8, 16.6, 16.5 (C-24, 25, 26), 25.9 (C-27), 178.1 (C-28), 33.0 (C-29), 23.4 (C-30).

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