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Study of Skin Anti-Ageing and Anti-Inflammatory Effects of Dihydroquercetin, Natural Triterpenoinds, and Their Synthetic Derivatives[1]

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Abstract—Accessible triterpenoids of ursane and lupane series, the flavonoid dihydroquercetin and their synthetic derivatives with polar substituents were tested in vitro for inhibition of collagenase 1 (MMP-1) in UVB irradiation assay. Ursolic acid and uvaol disuccinate were the most active inhibitors in the ursane series. In the lupane series, the best inhibition was manifested by carboxymethyl ester of betulonic acid and betulin succinates. Down- regulation of MMP-1 by dihydroquercetin and its synthetic derivatives surpassed the activity of a standard (retinoic acid).

Keywords: collagenase-1, triterpenoids, dihydroquercetin, photoageing, interleukin-8

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INTRODUCTION

Human skin photoageing caused by exposure to sun results in origination of coarse wrinkles, discoloration, abnormal dermal pigmentation and loss of skin elasticity. Tissue connective components are degradable by the family of proteolytic enzymes, matrix metalloproteinases including MMP-1, interstitial collagenase. MMP-1 is found in a variety of cells, including fibroblasts, endothelial cells, smooth muscle cells, chondrocytes, and multiple tumor types [1]. The synergistic action of MMP-1 with other MMP enzymes is responsible for degrading of skin collagen and elastin. In human skin, MMP-1, but not MMP-14 or MMP-15, is highly induced by UV irradiation and appears to be responsible for collagen fragmentation associated with chronic sun exposure [2].

MMP-1 treatment of young skin in organ culture causes fragmentation of collagen fibrils, which reduces fibroblast stretch, consistent with reduced mechanical tension, as observed in aged human skin [3–5]. With aging the skin, collagen synthesis becomes lower and MMP-1 levels become higher even in sun-protected human skin [6].

Treatment of photo damaged skin with certain natural chemicals, e.g., topical retinoids improves skin clinical appearance [7]. Retinoic acid (RA) is known to be a good suppressor of MMP-1 [8]. Terpenoids, flavonoids, and their accessible synthetic derivatives are of interest as components for human skin protection. Derivatives of the lupane (betulin, betulinic acid), ursane (ursolic acid, UA), and oleanane series [9] were tested as suppressors of proteinases. Certain synthetic derivatives of triterpenoids were also examined for MMP-1 inhibition [10]. Cytostatic and antiproliferative activity was discovered for ursolic acid [11, 12], and more interestingly, it showed a membrane stabilizing effect [13]. Betulin derivatives were reported to have an ability to suppress UVB-induced MMP-1 expression [14].

Betulinic acid was found to inhibit stromelysin and collagenase effectively, while its analog, C-28 alcohol betulin, demonstrated lesser inhibition potency against these proteases [15]. Low toxicity and cytotoxicity levels, characteristic of betulin and its esters [16, 17], make them prospective as additives for skin protection. Certain flavonoids (sumaflavone and amentoflavone) have been demonstrated to reduce effectively the MMP-1 levels [18].

[1] The article is published in the original.
Abbreviations: MMP-1, matrix metalloproteinase-1; collagenase-1; IL-8, Interleukin-8; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; HaCaT cells, Immortalized human Keratinocytes.

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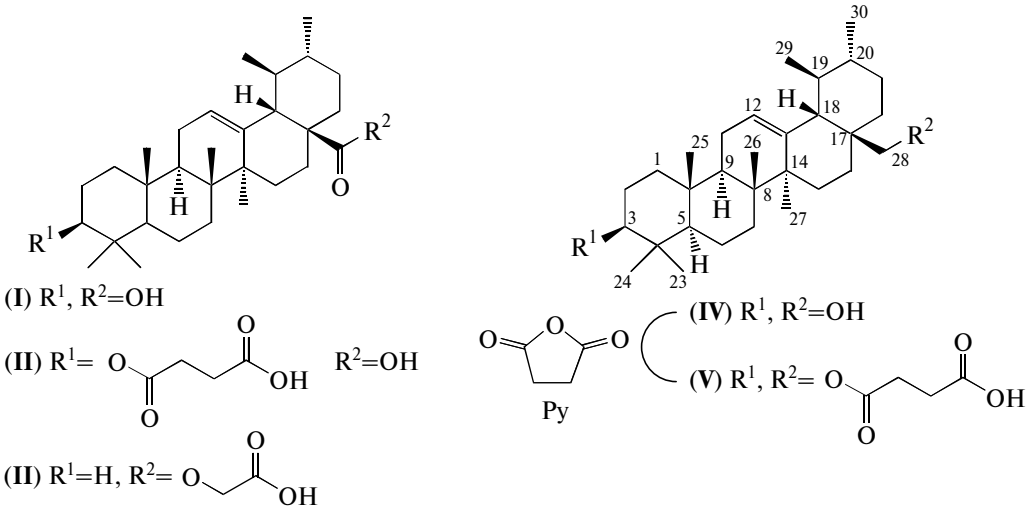
To develop the derivatives of natural products with effective suppression of MMP-1 expression, in this study we examined the accessible triterpenoids and their derivatives as potential inhibitors of UV-induced damage of human fibroblasts. Modification of triterpenoids, achieved by introduction of polar functions to their molecules, can enhance the efficacy of preparations (which can be due to, e.g., the increase in solubility). Betulonic acid, the component of *Betula* species extracts, which is available as betulin oxidation product, is also of interest as a suppressor of MMPs.

We prepared several polar esters of betulin, uvaol, ursolic acid, and betulonic acid and tested their inhibition effect on collagenase-1 (MMP-1). The flavonoid dihydroquercetin and its Mannich adducts [19] were also examined as possible inhibitors of MMP-1 (we did not succeed to find the data on down-regulation of MMP-1 with dihydroquercetin).

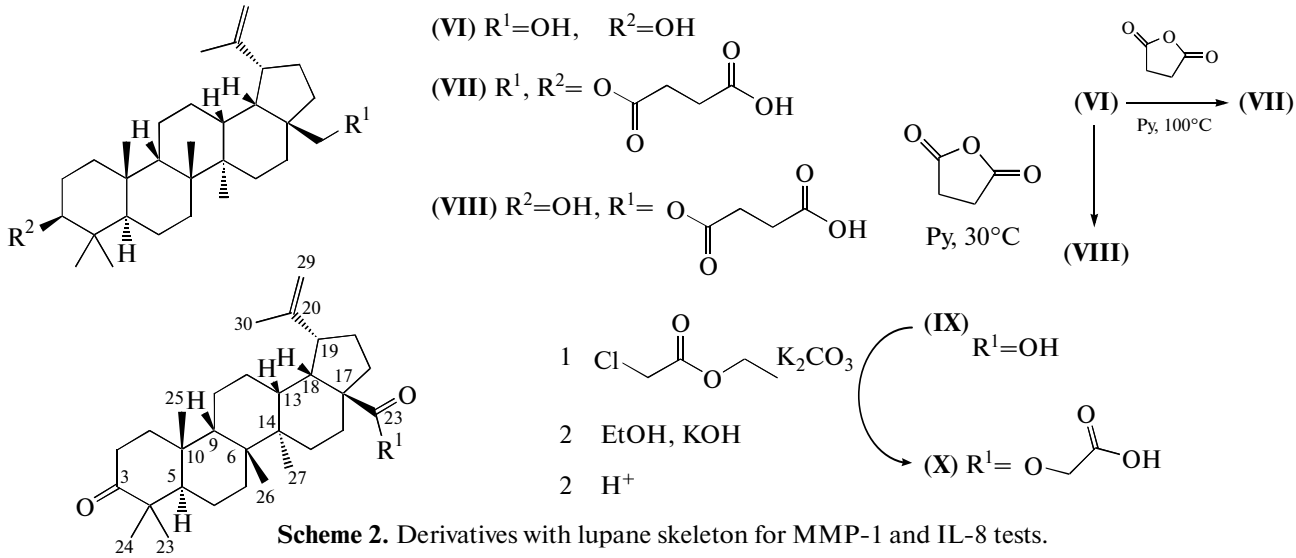
Anti-inflammatory activity of natural compounds and their synthetic derivatives were evaluated in interleukin-8 inhibition tests. Interleukin-8, causing recruitment of inflammatory cells and inducing a further increase in oxidant stress mediators, is known as a key parameter in localized inflammation [20].

RESULTS AND DISCUSSION

We were interested in preparation of polar nontoxic derivatives of accessible triterpenoids and dihydroquercetin (Schemes 1–3). Treatment of triterpenoids (**I**), (**IV**), (**VI**) with succinic anhydride in the presence of pyridine resulted in hydrogen succinates of ursolic acid (**II**), uvaol (**V**), and betulin (**VII**, **VIII**). Alkylation of ursolic acid (**I**) and betulonic acid (**IX**) with ethyl 2-chloroacetate followed with saponification with ethanolic KOH afforded the corresponding carboxymethyl esters (**III**), (**X**). Accessible Mannich adducts (**XII**), (**XIII**) were prepared by reaction of dihydroquercetin (**XI**) with formaldehyde and morpholine or diethylamine [19].



Scheme 1. Derivatives with ursane skeleton for MMP-1 and IL-8 tests.



Scheme 2. Derivatives with lupane skeleton for MMP-1 and IL-8 tests.

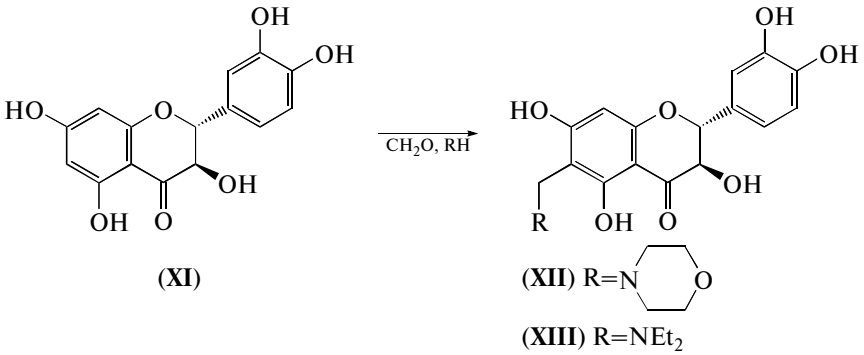
The effect of compounds (**I**)–(**XIII**) on the production of MMP-1 by human dermal fibroblasts

	Control	UVB (40 mJ)	RA				(I)		(II)		(III)		(IV)	
Concentration of tested compound, ppm	–	–	0.3	0.75	1.5	3.0	0.1	1	0.1	1	0.1	1	0.1	1
MMP-1, % to control	100.0	189.8	145.0	118.3	103.1	95.2	125.9	84.8	147.0	127.4	125.9	142.4	129.6	119.5
Standard deviation, %	11.31	17.36	5.81	14.85	4.62	21.52	12.81	26.04	8.88	17.60	17.01	8.73	14.08	11.59

	(V)		(VI)			(VII)			(VIII)			(IX)		
Concentration of tested compound, ppm	0.1	1	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10
MMP-1, % to control	140.3	91.2	193.8	142.9	139.2	173.2	95.7	3.0	113.3	110.6	17.6	92.1	97.1	73.9
Standard deviation, %	8.77	15.10	16.14	9.79	8.8	19.61	35.71	2.5	17.94	5.08	5.9	10.52	22.05	16.2

	(X)		(XI)			(XII)			(XIII)		
Concentration of tested compound, ppm	0.1	1	0.1	1	10	0.1	1	10	0.1	1	10
MMP-1, % to control	88.1	41.9	106.3	94.7	31.0	124.3	94.0	6.9	105.2	88.4	13.9
Standard deviation, %	9.85	3.87	8.92	9.69	18.7	6.17	4.26	6.9	13.37	2.19	11.1

The results are obtained in three independent experiments.



Scheme 3. Dihydroquercetin and its derivatives for MMP-1 and IL-8 tests.

The effects of compounds (**I**–**XIII**) on human dermal fibroblasts are given in table.

MMP-1 Regulation by Ursane Derivatives (Fig. 1)

UVB irradiation caused MMP-1 expression growth up to 189% as compared to control. The addition of retinoic acid (RA) (0.3 ppm) down-regulated MMP-1 expression to 145%.

The increase in RA concentration caused the further reduction of MMP-1 expression. Ursane derivatives in concentrations of 0.1 ppm decreased MMP-1 to 128–149%.

The best indices were found for ursolic acid (**I**) and uvaol bis(hydrogen succinate) (**V**). Uvaol (**IV**) activity (concentration ~1.0 ppm) was slightly inferior to that of RA.

At low concentration levels of UA derivatives (0.1 ppm), MMP-1 inhibition was comparable or slightly exceeded that of the standard (RA).

Among UA derivatives, the best results were observed for ursolic acid (**I**): the inhibition activity of (**I**) at 0.1 and 1 ppm was superior over the standard

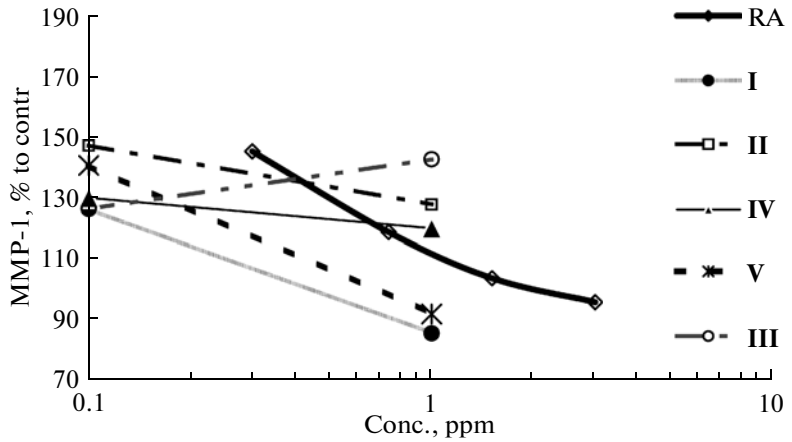


Fig. 1. Effect of ursane derivatives on MMP-1 expression.

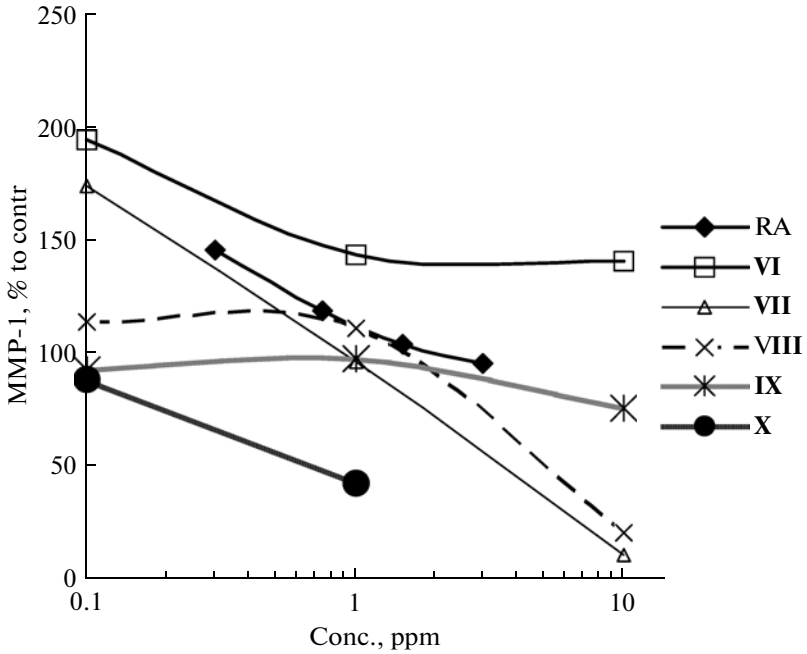


Fig. 2. Effect of lupane derivatives on MMP-1 expression.

(RA) at 0.3 and 1.5–3.0 ppm, respectively, and significantly better than the corresponding results obtained for uvaol. Ursolic acid hydrogen succinate (**II**) and carboxymethyl ester (**III**) were inferior in MMP-1 inhibition to UA (**I**).

Uvaol 3,28-bis(hydrogen succinate) (**V**) demonstrated better activity than the parent uvaol (**IV**), whereas for (**IV**) the notable MMP-1 up-regulation at 10 ppm was observed, while inhibition at 1 ppm for (**V**) was comparable with that of UA (**I**).

MMP-1 Regulation by Lupane Derivatives (Fig. 2)

The betulin esterification products with succinic anhydride demonstrated MMP-1 suppression enhancement (the similar pattern was observed for uvaol (**IV**) and its bis(hydrogen succinate) (**V**), (see above).

Betulin (**VI**) demonstrated insignificant inhibition level, inferior to RA.

Betulin bis(hydrogen succinate) and mono(hydrogen succinate) (**VII**, **VIII**) were found comparable with the standard (RA) showing similiar inhibition at 1 ppm, and close-up to that of RA at 0.75–3 ppm. At 10 ppm (**VII**, **VIII**) showed very good MMP-1 suppression (about 90 and 80% down-regulation, respectively.

Betulonic acid (**X**), which is closely structurally related to betulinic acid, known for its good collagenase inhibition [15], was comparable or superior to standard (RA) within the test concentration range. Surprisingly, betulonic acid carboxymethyl ester (**X**) manifested ~60% inhibition of MMP-1 at 1 ppm concentration—the best results among the tested compounds.

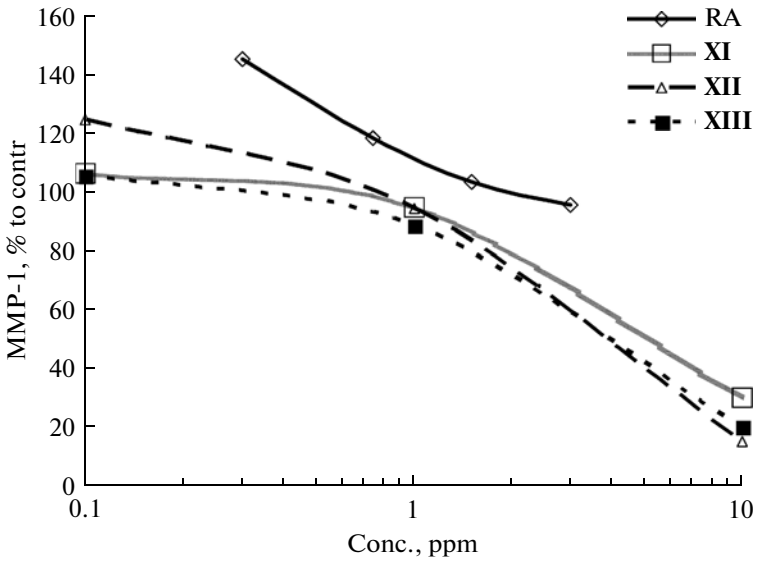


Fig. 3. Effect of dihydroquercetin derivatives on MMP-1 expression.

MMP-1 Regulation by Dihydroquercetin Derivatives (Fig. 3)

Dihydroquercetin (**XI**) and its Mannich condensation products (**XII**, **XIII**) down-regulated MMP-1 activity better than the standard. Dihydroquercetin (**XI**) and its synthetic derivatives (**XII**, **XIII**) possessed similar activity level within the range of tested concentrations.

Results of IL-8 Experiments

None of the tested compounds demonstrated notable levels of activity in IL-8 experiments.

To summarize, the flavonoid dihydroquercetin, triterpenoids of the ursane and lupane series and their synthetic derivatives with polar substituents were studied as potential inhibitors of MMP-1 in UVB irradiation tests. Antiinflammatory activity was studied in IL-8 experiments, Ursolic acid and uvaol bis(hydrogen succinate) were the most active inhibitors of MMP-1 in the ursane series. In the lupane series, the best inhibition of MMP-1 was manifested by carboxymethyl ester of betulonic acid and betulin bis(hydrogen succinate). The activity of dihydroquercetin derivatives was similar to those of the parent compound and exceeded the activity of retinoic acid.

None of the studied compounds showed notable anti-inflammatory activity in IL-8 experiments.

EXPERIMENTAL

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-300 (300.13 MHz for ^{1}H, 75.48 MHz for ^{13}C) and Bruker AM-400 (400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C) at room temperature in $CDCl_3$ or $CDCl_3$-pyridine-d_5. The chemical shifts are given in ppm relative to signals of the solvents used as the internal standards: in ^{1}H NMR spectra, δ_H 7.24 ($CHCl_3$), and in ^{13}C NMR spectra, δ_C 76.90 ($CDCl_3$). Signals in the NMR spectra were assigned by correlation with those of ursolic acid (**I**) [21, 22], ursolic acid 3-acetate (**II**) [23]; *J* values are given in Hertz.

Melting points were measured on a Kofler melting point apparatus and are uncorrected. Optical rotation was measured on a PolAAR 3005 polarimeter (Great Britain) at 589 nm in a tube 5 cm long. TLC was carried out on Sorbfil plates (Russia). Purity of the tested compounds was ≥90% (by ^{1}H NMR).

Reagents

Dulbecco's modified Eagle's medium (DMEM), Penicillin/Streptomycin, fetal bovine serum were purchased from Lonza (Switzerland), phosphate-buffered saline (PBS) was from Welgene (South Korea).

Ursolic acid (**I**) 95%+ was prepared from *Vaccinium vitis idaea* L. peels extract according to the method [24]. Ursolic acid 3-(hydrogen succinate) (**II**) was synthesized as described in [25], mp 243–244°C (lit. mp 248–249°C), α_D^{23} = +46.8 (*c*1, MeOH). Alkylation of ursolic acid (**I**) with ethyl 2-chloroacetate and subsequent hydrolysis afforded 3-hydroxyursan-12-ene-28-oic acid carboxymethyl ester (**III**) [26]. Uvaol (**IV**) was synthesized from methyl ursolate according to the published method [27], mp 228–229°C (MeOH), (lit. mp 223–224°C).

Crystallisation of the extract of *Betula pendula* bark gave betulin 95%+ [28], mp 255–257°C (EtOH), (lit. mp 256–258°C).

(3,28-Dihydroxylup-20-ene)-bis(hydrogen succinate) (**VII**), mp 112–114°C (EtOAc-hexane), (lit. mp 110–112°C) and 3,28-dihydroxylupane-20-ene 28-(hydrogen succinate) (**VIII**), mp 240–242°C (i-PrOH), (lit. mp 242–244°C) were prepared by

reaction of betulin with succinic anhydride in the presence of pyridine as described in [29, 30].

Betulonic acid (**IX**), mp 249–252°C (lit. mp 250–254°C) was prepared by oxidation of betulin (**VI**) with chromium trioxide [31]. Alkylation of betulonic acid (**IX**) with ethyl 2-chloroacetate and subsequent hydrolysis afforded 3-oxo-lupane-20-ene-28-oic acid carboxymethyl ester (**X**).

Dihydroquercetin (**XI**) 95%+ was purchased from *VedaBio* (Russia). The Mannich reaction of (**XI**) with amines and formaldehyde was the route to 6-(morpholinomethyl)-dihydroquercetin (**XII**), mp 163–165°C (lit. mp 160–162°C) and 6-(diethylaminomethyl)-dihydroquercetin (**XIII**), mp 199–203°C (lit. mp 200–202°C) [18].

Syntheses

(31,28-Dihydroxyurs-12-en)-bis(hydrogen succinate) (V). A mixture of uvaol (**IV**) (0.44 g, 1 mmol) and succinic anhydride (1 g, 10 mmol) was refluxed in a mixture of pyridine (1 mL) and toluene (10 mL) for 10 h. The cooled reaction mixture was washed with water (50 mL), 10% H_2SO_4 (20 mL) and evaporated. The residue was washed with water and filtered to give the title compound (85%) as white crystals, m.p. ~95°C (dec) (EtOAc) $\alpha_D^{23} = +32.6$ (*c* 1.0, MeOH).

NMR ^{1}H: 10.05 (br.s, 2 $CH_2CH_2COO\underline{H}$ (2 H)), 5.10 (dd, *J* 3.0, 3.0 Hz, H12), 4.50 (dd, *J* 8.0, 8.0 Hz, H3), 4.06 (d, *J* 10.9 Hz, H28a), 3.62 (d, *J* 10.9 Hz, H28b), 2.62 (m, $2C\underline{H}_2C\underline{H}_2COOH$ (8 H)), 1.05 (s, 3 H27), 0.94 (s, 3 H25[§]), 0.93 (s, 3 H26[§]), 0.90 (d, *J* 5.7 Hz, 3 H30[#]), 0.82 (s, 3 H23[§]), 0.82 (s, 3 H24[§]), 0.78 (m, 3H 29[#]).

NMR ^{13}C: 178.00 and 178.00 (s, 2 COOH), 171.97 (s, $\underline{C}OO(C3)^{§}$), 171.73 (s, $\underline{C}OO(C28)^{§}$), 137.97 (s, C13), 125.38 (d, C12), 81.34 (d, C3), 71.45 (t, C28), 55.10 (d, C5), 54.11 (d, C18), 47.40 (d, C9), 41.78 (s, C14), 39.80 (s, C8), 39.16 (d, C19[#]), 39.04 (d, C20[#]), 38.28 (t, C1), 37.58 (s, C4), 36.79 (s, C10[‡]), 36.58 (s, C17[‡]), 35.52 (t, C22), 32.49 (t, C7), 30.32 (t, C21), 29.23 and 29.00 (t, $2\underline{C}H_2CH_2COOH^{¶}$), 29.00 and 28.93 (t, $2CH_2\underline{C}H_2COOH^{¶}$), 27.84 (q, C23), 25.82 (t, C15), 23.33 (t, C2[†]), 23.25 (t, C16[†]), 23.18 (t, C-11[†]), 23.18 (q, C27), 21.12 (q, C30), 18.01 (t, C6), 17.15 (q, C29[◇]), 16.60 (q, C26[◇]), 16.50 (q, C-24[◇]), 15.55 (q, C25).

Signals in the NMR ^{1}H and ^{13}C spectra were assigned by correlation with those of uvaol-3β-yl caffeate (in $CDCl_3$) [32]. The assignments marked with the same symbols [§, #,‡,¶,†, ◇] could be interchanged.

3-Oxo-lup-20-en-28-oic acid carboxymethyl ester (X). To a suspension of betulonic acid (**I**) (1.0 g, 2.2 mmol) in DMF (10 mL), anhydrous powdered K_2CO_3 (0.45 g, 3.3 mmol) and ethyl-2-chloroacetate (0.27 g, 2.2 mmol) were added. The mixture was stirred at ambient temperature for 4 h (TLC monitoring). The reaction mixture was poured to water and the precipitate was filtered and washed with water (3 × 20 mL). The wet residue was added to a solution of KOH (0.4 g, 7.2 mmol) in EtOH (10 mL), refluxed for 1 h and cooled to ambient temperature. The mixture was poured into 5% aq. HCl (50 mL), filtered, washed with water and dried on the open air. Yield 0.98 g (87%) as white crystals, mp ~110°C (dec), $\alpha_D^{23} = +17.8$ (*c* 1.0, MeOH).

Signals in the NMR ^{1}H and ^{13}C spectra were assigned by correlation with those of betulonic acid (in $CDCl_3$) [33].

NMR ^{1}H: 9.22 (br.s, COOH), 4.69 (br.s, H29a), 4.60 (AB, $\Delta\nu_{AB} = 11.7$ Hz, J_{AB} 15.8 Hz, $OC\underline{H}_2COOH$ (2 H)), 4.56 (br.s., H29b), 2.95 (ddd, J 10.5, 10.5, 4.5 Hz, H19), 1.64 (s, 3 H30), 1.03 (s, 3 H23[§]), 0.98 (s, 3 H24[§]), 0.94 (s, 3 H27[§]), 0.91 (s, 3 H25[§]), 0.87 (s, 3 H26[§]).

NMR ^{13}C: 218.89 (s, C3), 175.23 (s, C28[§]), 173.23 (s, COOH[§]), 150.13 (s, C20), 109.60 (t, C29), 59.74 (t, $O\underline{C}H_2COOH$), 56.35 (s, C17), 54.76 (d, C5), 49.74 (d, C9), 49.16 (d, C18), 47.20 (s, C4), 46.59 (d, C19), 42.33 (s, C14), 40.49 (s, C8), 39.45 (t, C1), 38.10 (d, C13), 36.73 (t, C22), 36.73 (s, C10), 33.95 (t, C2), 33.47 (t, C7), 31.69 (t, C16), 30.24 (t, C21), 29.35 (t, C15), 26.49 (q, C23), 25.38 (t, C12), 21.25 (t, C11), 20.88 (q, C24), 19.50 (t, C6), 19.20 (q, C30), 15.82 (q, C26[#]), 15.51 (q, C25[#]), 14.44 (q, C27).

The assignments marked with the same symbols [§,#], could be interchanged.

Methods (MMP-1). Cell culture for MMP-1. Primary cultures of dermal fibroblasts were established from human adult foreskins of healthy volunteers in (DMEM) supplemented with 1% Penicillin/Streptomycin, and 10% fetal bovine serum (Lonza) at 37°C in a humidified atmosphere of 5% CO_2. Cells were cultured until they were 90% confluent before being passaged. Dermal fibroblasts from passages 4 to 10 were used for the experiments.

UV irradiation and MMP-1 assay. For each experiment, cells were maintained in the culture medium without FBS for 24 h. Serum-starved cells were washed twice with PBS and exposed to UVB irradiation (UVB lamp: G15TBE, Sankyo Denki, Japan). The total energy dose of UVB irradiation was 40 mJ/cm^2. After UVB exposure, serum-free DMEM containing each RA and the tested reagents at the indicated concentration was added to the cells. The culture medium was harvested 24 h later. MMP-1 sandwich ELISA assays were performed according to the manufacturer's protocols (RPN 2610, Amersham Bioscience) using precoated 96-well immunoplates, rabbit anti-human MMP-1 antibodies, and anti-rabbit horseradish peroxidase conjugate. 3,3',5,5'-Tetramethylbenzidine(TMB) was used as a peroxidase substrate. Absorbances were read at 450 nm using a microtiter plate reader.

Methods (IL-8)

(1) Cell cultures. Immortalized human Keratinocytes (HaCaT cells) were maintained at 37°C in a humidified 5% CO_2 atmosphere in DMEM medium that contained 10% FBS, and Penicillin/Streptomycin. The cells were plated in the previous day to treatment in the following day.

(2) Measurement of IL-8 (interleukin-8) secretion. Overnight plated HaCaT cells in 24-well plates were washed two times with PBS and exposed to UVB radiation (30 mJ/cm^2). Then the medium was exchanged for Phenol red-free DMEM that contained 2% FBS, and Penicillin/Streptomycin in the absence or presence of test substances for 24 h. To determine the levels of IL-8 secreted into cultured media, the supernatants were collected and analyzed for the presence of IL-8 using commercial available ELISA kits according to the instructions from the manufacturer (R&D Systems). At the same time, cell proliferation assay was performed with WST-1 reagent (Roche) and used to compensate for the substance toxicity.

(3) Statistical analysis. Experimental data were analyzed by paired-samples *t* test [34] using MINITAB® statistical software to compare the difference between each of the treatment groups and the control group.

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