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Synthesis and in vitro biological activity of retinyl retinoate, a novel hybrid retinoid derivative

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ABSTRACT

A new hybrid, retinyl retinoate **1**, was synthesized with a condensing reaction between retinol and retinoic acid to improve the photo-stability, and the in vitro biological activity of the hybrid was analyzed. This retinol derivative had enhanced thermal stability and decreased photosensitivity, and exhibited decreased cell toxicity compared to that of retinol. In addition, RAR activity analysis showed that retinyl retinoate **1** had higher inhibitory activity against c-Jun than retinol and showed superior effects on collagen synthesis compared to retinol. Thus, retinyl retinoate **1** may have the potential to be conveniently used as an additive in cosmetics for prevention and improvement of skin aging and medicines for the treatment of skin troubles due to its excellent stability under severe and accelerated conditions.

1. Introduction

Photoaged skin is characterized by an increase of wrinkles, thickening, inelasticity, dryness, roughness, shallowness, and pigmentary mottling[1,2] and is of considerable importance for skin aging. This irreversible process results largely from exposure to UV radiation, which acts as a broad activator of cell surface growth factors and cytokine receptors. More specifically, UV irradiation stimulates the transcription factor activator protein 1 (AP-1), which regulates the expression of matrix-metalloproteinase (MMP) to degrade skin collagen. Thus, photoaging is associated with increased AP-1 activity, increased MMP expression, enhanced collagen degradation, and decreased collagen synthesis, all of which result in changes within the matrix of the dermis and at the dermal–epidermal junction.[3–7]

Retinoids are natural and synthetic vitamin A derivatives, and biologically active forms of retinoids are capable of modulating gene expression by binding to nuclear receptors to activate transcription of specific DNA sequences. Because of their ability to modulate genes involved in cellular differentiation and proliferation, retinoids are thought of as good candidates for both treating and preventing the photoaging process. Indeed, biologically active retinoids such as all-trans-retinoic acid (RA) have been shown to repair skin damaged by chronological aging or photoaging,[8–11] and it has been reported that photo-aging of skin may be deterred by using a cream containing all-trans-retinoic acid (RA)[12–14]; however, RA is fat-soluble and a skin-irritant, and causes a number of side-effects such as skin dryness, wounds, and scraping during the latent period,[15,16] thereby limiting the application of RA as a main component for medicines and cosmetics.[17,18] Because the side effects of RA are caused by the carboxyl end group in RA,[19–21] many efforts have been made to substitute the carboxyl end group with other functional groups. One such retinoid derivative is retinol, which has a hydroxyl group substituted in place of the carboxy end group, but has the same biological activity group of RA by maintaining the cyclic end group (ring) and polyene side chain. Retinol, also known as vitamin A, has received a great deal of attention as an alternative anti-aging agent for long-term treatment[22]; however, it can hardly be used in general cosmetic formulations, as it becomes unstable when exposed to light, oxygen, heat, lipid-peroxidation, or water.[23] For this reason, it is important to develop new retinol derivatives that have not only the same activity for deterring skin aging as vitamin A, but also reduced skin-irritant properties and enhanced photo-stability. To this end, many derivatives have been developed to improve the stability of retinol, or by using with anti-oxidants such as butylated hydroxytoluene (BHT), di-α-tocopherol, butylated hydroxyanisole (BHA), ascorbic acid, and tocopheryl linolate, or by adopting encapsulations for preventing exposure effects altogether.[24,25] Nonetheless, most of the derivatives developed to date have been designed to change the carboxy end group of retinol molecules, thus remaining unstable when exposed to light. The instability of retinol results in its quick conversion from a crystalline substance to a viscous one, which is then further decomposed to produce color change and formation of peroxides[23] as well as some toxic substances that give

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undesirable effects, making the use of these derivatives limited.[26] These results imply that the substitution method is not enough to achieve the ideal for retinoid derivatives, which should have higher activity and stability, causing less local irritation and toxicity.

In this study, we applied a condensing reaction to form an ester bond between retinol (which has a hydroxyl end group) and retinoic acid (which has a carboxyl end group), thereby synthesizing the novel hybrid vitamin derivative, retinyl retinoate **1**. This newly synthesized compound is an ester of all-trans-retinoic acid (RA) and all-trans-retinol (ROL), and has following features: (i) reduced toxicity due to blocking of the carboxyl end group of RA, (ii) retention of both the cyclic end group (ring) and polyene side chain, which are responsible for the biological activity of the two parent molecule, and (iii) enhanced photo-stability versus that of retinol. In this paper, we propose the novel anti-aging agent, retinyl retinoate **1**, which has better chemical stability, reduced skin irritant properties, and higher skin regeneration activity than the previous retinol or retinoic acid derivatives.

2. Results and discussion

2.1. Chemistry

Retinyl retinoate **1** was synthesized from the reaction of retinol with retinoic acid (Scheme 1). The starting material for the retinyl retinoate **1** is retinol **2**, which is synthesized from retinyl acetate **4**[27] (Scheme 2). In Scheme 1, retinol **2** is reacted with a retinoic acid **3** in a solvent and in the presence of a condensing agent, such as *N*,*N*-carbonyldiimidazole (CDI), *N*,*N*-dicyclohexylcarbodiimide (DCC), or ethylchloroformate, along with a catalyst to facilitate the condensation reaction, such as *N*,*N*-dimethylaminopyridine (DMAP) or 1-hydroxybenzotriazole (HOBT). In Scheme 2, retinol **2** is synthesized from retinyl acetate **4** with potassium carbonate in a solvent.

2.2. Photosensitivity and thermal stability test

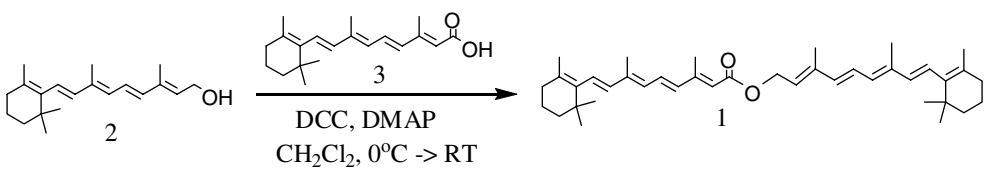
Natural retinol is very unstable when exposed to sunlight, a property that makes its application as a material for general cosmetics difficult. The newly synthesized hybrid retinoid derivative, retinyl retinoate **1**, was designed to overcome the photosensitivity of retinol, and thus the photosensitivity and thermal stability of retinyl retinoate **1** were analyzed using qualitative ^{1}H NMR and quantitative HPLC and compared with retinol and retinyl palmitate, which is an ester of retinol that is widely used in medication and cosmetics.[28–30] For NMR analysis, retinyl retinoate **1** was irradiated by UV-A light (wavelength 356 nm) using a Spectroline apparatus (Model CM-10; Fluorescence analysis cabinet; Spectronics Corporation, Westbury, NY, USA) at room temperature. The fluence rate was 8.6 mW/cm^2 and delivered for 2, 12, 24, or 48 h, to achieve total energy doses of 62, 372, 743, and 1488 J/cm^2, respectively. The results were qualitatively identified by ^{1}H NMR. Specifically, photosensitivity was analyzed based on the changes in the ^{1}H NMR of the cyclic ring group, polyene chain group, as well as the ester group of retinyl retinoate **1**. As shown in Figure 1, although multiple peaks were observed after 24 h irradiation due to the trans–cis transition between polyene chain protons, there was no decomposition or degradation observed in either the polyene chain group, ester group, or cyclic ring group after 48 h irradiation in retinyl retinoate **1**. In contrast, retinyl palmitate exhibited both the trans–cis transition between polyene chain protons and carbon decomposition of the cyclic ring group after 2 h of irradiation. After 12 h of irradiation the ester bond of retinyl palmitate was almost non-existent and only the protons of the fatty acid were observed after 24 h of irradiation. In retinol, almost all of the polyene chain group and cyclic ring group were decomposed after 2 h irradiation, and no further qualitative analysis was possible after 12 h irradiation. Retinyl retinoate **1** has the longer wavelength of maximum absorption (wavelength 333 nm) than retinol (wavelength 323 nm) and the absorbance at 356 nm was 0.800 and 0.296, respectively. Although the more energy was absorbed to retinyl retinoate **1** by UVA at 356 nm, retinyl retinoate **1** was very stable when exposed to UV light, while retinol was easily decomposed following photo irradiation.

In order to compare the time dependent thermal stability of the retinyl retinoate **1** with retinol **2**, the phase stability for 1 month in a thermohygrostat (humidity: 58%) at room temperature, 40 °C, and 4 °C was examined by quantitative HPLC analysis. As shown in Figure 2, retinyl retinoate **1** and retinol **2** remained mostly intact until week 4 when left at 4 °C. However, retinol was decomposed rapidly at room temperature and 40 °C, while the relative stability of retinyl retinoate **1** under the same conditions was higher. Together, these results indicated that the new hybrid retinyl retinoate **1** was much more stable than retinol.

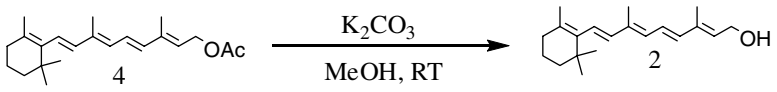
We also tested the possibility of the hydrolysis of retinyl retinoate **1** by HPLC. The retention time for fresh retinol, retinoic acid, and retinyl retinoate **1** was 8.39, 8.53, and 18.83 min, respectively. The retention time for retinyl retinoate **1** was 18.94 min after incubation with normal human fibroblasts for 2 days, and no additional peak at the position of retinol and retinoic acid was observed. This clearly indicated that the biological effect of retinyl retinoate **1** was caused by the compound itself, and not by the products of the hydrolysis of retinyl retinoate **1**.

2.3. Analysis of cytotoxicity

In order to verify the primary stability of retinyl retinoate **1** as a material for medicines and cosmetics, its cellular toxicity was determined using the MTT method[31] with normal human fibroblasts (Newborn). Figure 3 shows the level of cell viability following exposure to various retinoid derivatives such as retinoic acid, retinol, and retinyl retinoate **1**. A viability of 100% was determined by treating cells with medium only. As can be seen from the results of Figure 3, retinyl retinoate **1** exhibited a lower intracellular toxicity than retinol and retinoic acid. The IC_{50}, which is the concentration where growth is inhibited by 50%, was 40 μM for retinyl retinoate **1**, which is 60% higher than that of retinol (25 μM). In addition, the cytotoxicity of retinyl retinoate **1** was lower than that of retinol. Taken together with the photosensitivity and thermal sensitivity data, our results suggest that hybrid retinyl retinoate **1**, which was synthesized via esterification, has tremendous potential as a powerful anti-wrinkle agent.



Scheme 1.



Scheme 2.

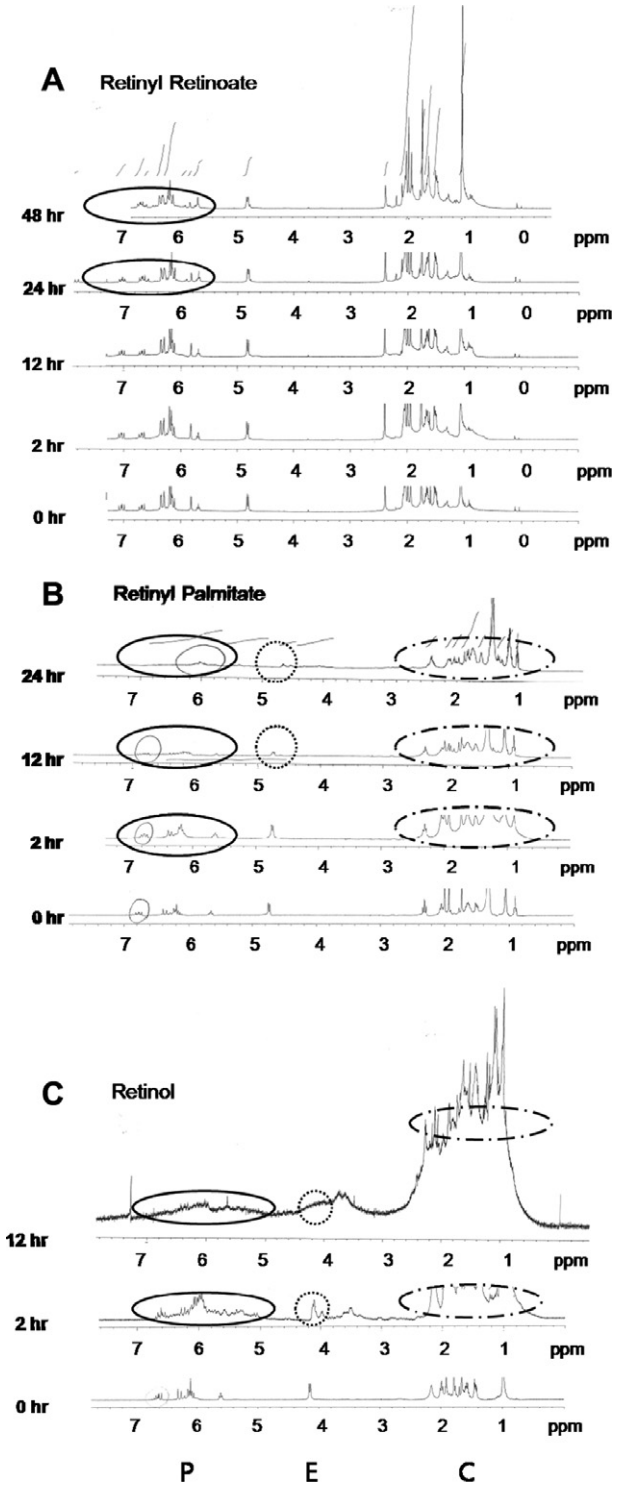


Figure 1. Effect of UVA exposure on the photo-stability of retinyl retinoate (A), retinyl palmitate (B), and retinol (C). Qualitative assays were performed by ^{1}H NMR. Circle indicates the observed decomposition or degradation in P, polyene chain group (—); E, ester group (·····); and C, cyclic ring group (— · —) in retinoids.

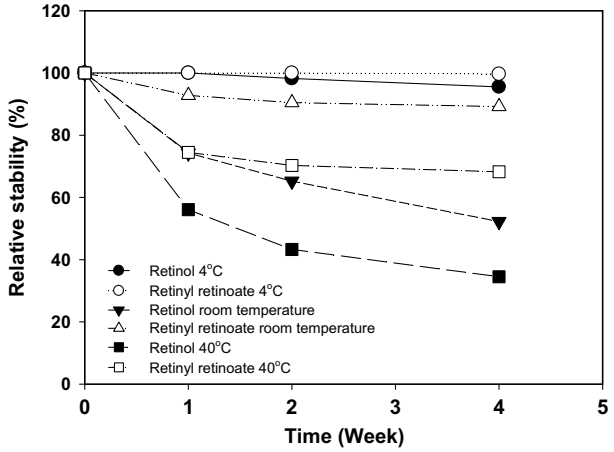


Figure 2. Comparison of the thermal stability of retinyl retinoate and retinol for one month at 4 °C, room temperature, and 40 °C. Stability (%) indicates relative purity of each compound as determined by HPLC analysis.

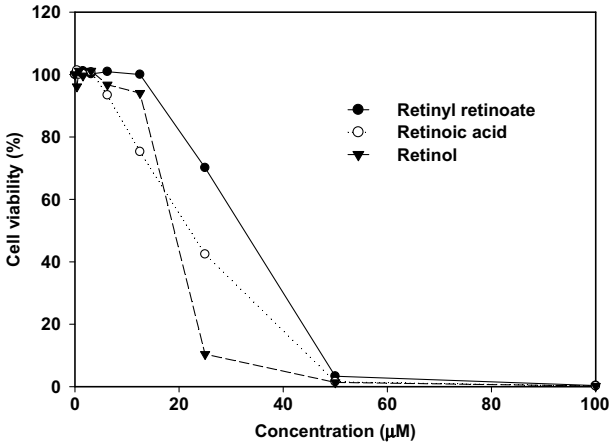


Figure 3. Cytotoxic effect of retinol derivatives. Normal human fibroblast cells cultured with Modified Eagle Medium (DMEM, GIBCO™) were used for the cell toxicity test. After the cells were treated with each different concentrations (0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 μM) for 1 day, the IC_{50} values of the derivatives were determined by MTT assay. A viability of 100% was determined from cells treated with medium only.

2.4. Analysis of activity for RAR (retinoic acid receptor)

The effect of the retinol derivatives on the activity of RAR was examined using skin keratinocyte derived HaCaT cells. Recombinant genes DR5-tk-CAT, which contain either the DR1 response element of RAR, a thymine kinase promoter, and CAT (chloramphenicol acetyl transferase) were co-transfected with a plasmid DNA that expresses RAR α, β, or γ in the HaCaT skin cancer cell line using Lipofectamine (GibcoBRL). Following transfection, the cells were incubated with various retinoid derivatives, and proteins were extracted from each cell. Next, the activity of β-galactosidase was assayed, and the amount of total protein was measured to determine transfection efficiency. The level of transcription for RAR was analyzed by CAT ELISA (Roche Molecular Biochemicals,

Mannheim, Germany). As is shown in Figure 4, the dose-dependent assays confirmed that retinyl retinoate **1** exhibited partial properties of both retinol and retinoic acid, although the activity of retinyl retinoate **1** was closer to the activity of retinol for all three RARs. That is, retinyl retinoate **1** showed much lower activity than retinoic acid but slightly higher activity than retinol. Also, retinyl retinoate **1** exhibited more specific activity against RAR α among the three RAR subtypes in a manner similar to retinol and retinoic acid. The dose-dependent data shown in Figure 4D confirmed that while retinyl retinoate **1** was less active than retinoic acid, it was just as active as retinol for RAR α. It has been reported that greater RAR activity and lack of subtype-specificity are indicative of side effects such as skin irritation,[32–34] and although retinyl retinoate **1** is a hybrid of retinol and retinoic acid, the RAR activity of retinyl retinoate **1** was much closer to retinol, and thus appears to be less likely to cause skin irritation compared to retinoic acid.

2.5. Inhibition of AP-1 protein (activation protein-1)

Upon binding a ligand such as retinoid derivatives, activated RARs can functionally interact with the transcription factor c-Jun to inhibit its activity.[35,36] AP-1 (comprising c-Jun protein) is a transcription factor that induces the expression of collagenase, a major cause of skin wrinkles. If retinol derivatives exhibit an inhibitory activity against AP-1, a factor inducing skin wrinkles, they may eventually provide an effective prevention and treatment of skin wrinkles. Collagenase CAT reporter (Coll-CAT) gene, which contains a AP-1 response element, was transfected into the HaCaT cells using liposomes and the degree of inhibition against the activity of AP-1 by retinol derivatives was measured through CAT ELISA in a manner similar to the RAR activity analysis. Further, the effect of retinol derivatives on the transcriptional activity of c-Jun (a protein that induces metastasis of cancer, skin aging, and inflammation, and consists of either homologous or heterologous isomers), a constitutive element of AP-1, was examined by co-transfecting c-Jun and RAR α expression vectors. As shown in Table 1, the expression of collagenase increased as much as 8.6 times when c-Jun was expressed; however, when cells were treated with retinol and RAR α, the expression of collagenase decreased more so than in treatment with retinol alone. With the RAR α expression vector present, retinol, retinoic acid, and retinyl retinoate **1** inhibited the collagenase expression by about 33%, 64%, and 52%, respectively. It is important to note that retinyl retinoate **1** inhibited collagenase expression in-between that of retinol and retinoic acid when cells were treated in

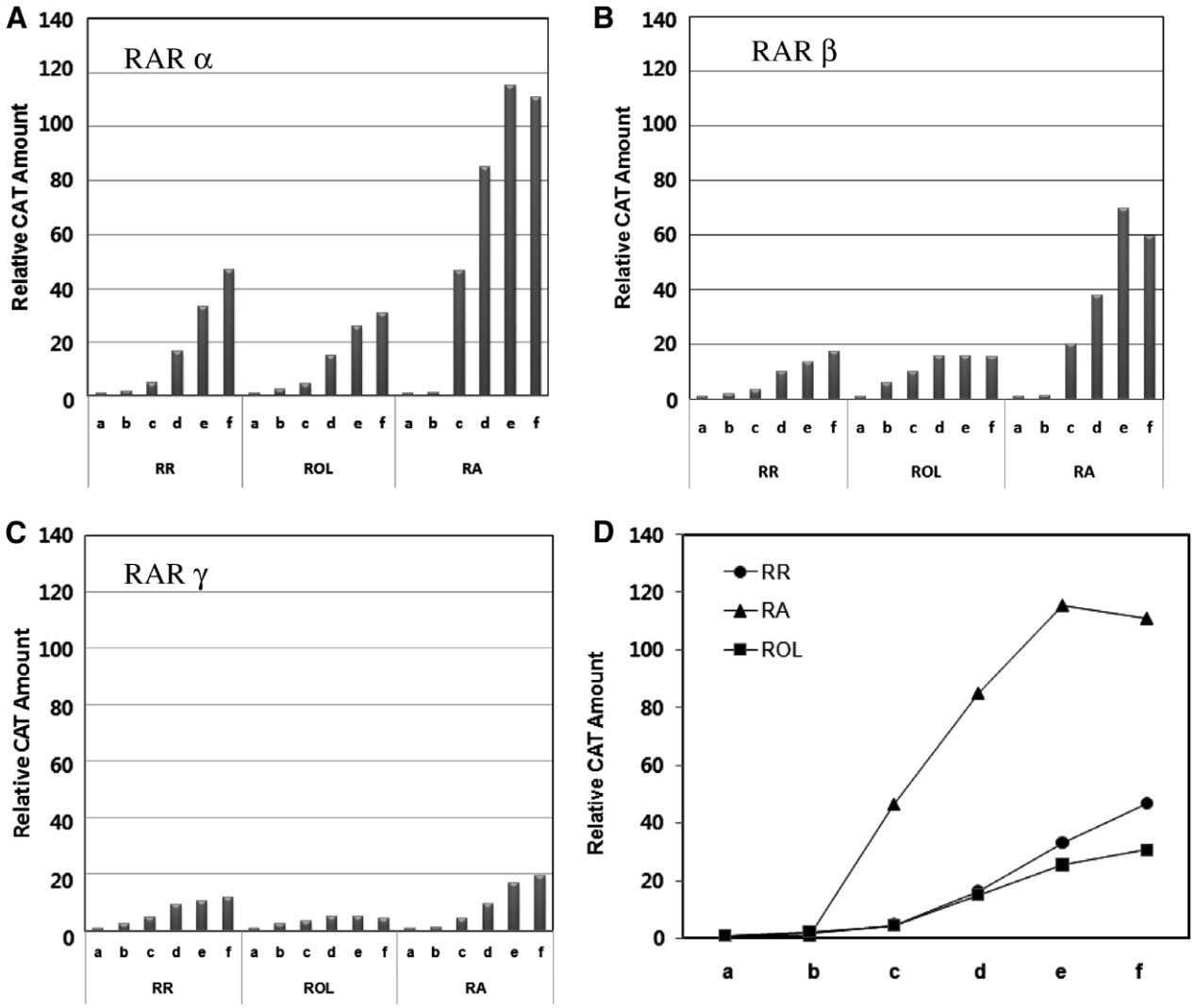


Figure 4. Effect of retinol derivatives on the transcriptional activity of RAR α (A), RAR β (B), and RAR γ (C). Recombinant DR5-tk-CAT reporter, which contains the one copy of the retinoic acid-responsive element DR5 (direct repeat 5) bound to a herpes simplex thymidine kinase promoter upstream of a chloramphenicol acetyl transferase (CAT) reporter gene, was co-transfected with plasmid DNA expressing either RAR α, β, or γ into the HaCaT cells. The effects of DMSO (a), 1 nM (b), 10 nM (c), 100 nM (d), 1 μM (e), and 10 μM (f) for retinol, retinoic acid, and retinyl retinoate were examined. (D) RAR α subtype specificity for each retinoid derivatives according to increasing concentrations. RA, retinoic acid; ROL, retinol, and RR, retinyl retinoate.

Table 1
Relative inhibition against the expression of collagenase

Condition of expression	—	Retinol	Retinoic acid	Retinyl retinoate
—	1.0	0.8	0.6	0.7
c-Jun	8.6	7.3	5.4	6.7
c-Jun + RAR α	8.6	5.8	3.1	4.1

the same manner. Similar to our RAR activity analysis, these results on the inhibition of AP-1 activity also show that the retinyl retinoate **1** exhibited lower inhibitory activity against c-Jun than retinoic acid, but higher than retinol.

2.6. Comparison of CRABPII mRNA expression

Upon entry into cells RA binds to lipid binding proteins such as cellular retinoic acid binding protein (CRABPs) and translocates to the nucleus where it binds to either RAR or RXR.[37] There are two types of CRABPs: CRABP I is expressed throughout the body, while CRABP II is expressed only in the skin, spermary, ovary, or leucocyte[38,39]; especially, CRABP II, but not CRABP I, is induced by retinoic acids in adult skin.[40] Thus, the expression of CRABP II helps retinoic acids to bind receptors such as RAR or RXR in the nucleus and enhances its transcriptional activity.[41] There have been several reports showing that the expression of CRABP II mRNA is increased by retinoids.[42,43] Figure 5 shows a Western blot for CRABP II and GAPDH (a housekeeping gene used as an equal protein loading control). As shown in this figure, the expression of CRABP II mRNA increased following exposure to retinoic acid, retinyl retinoate **1**, retinol, and retinyl palmitate in a concentration dependant manner. Thus, retinyl retinoate **1** enhanced the expression of CRABP II mRNA, and CRABPII acts as mediator for the derivative of retinoids to increase collagen expression.

2.7. Collagen synthesis test

The effect of retinyl retinoate **1** on collagen synthesis was compared to that of retinol, retinoic acid, and retinyl palmitate to confirm its utility as inhibitors for skin aging. The increasing ratio for collagen synthesis of the test group over the control group, which was treated with DMEM only, is depicted in Figure 6. Retinol derivatives such as retinol palmitate showed a much lower effect on collagen synthesis than retinol and other retinoic acid derivatives. In contrast, retinyl retinoate **1** exhibited either a superior or an equivalent effect on collagen synthesis at low concentrations when compared to retinol or retinoic acid, respectively. These results indicate that retinyl retinoate **1** has the potential to be an effective anti-wrinkle agent for cosmetic formulations.

3. Conclusions

We have synthesized the novel hybrid retinyl retinoate **1** by applying a condensation reaction between retinol (which has a hydroxyl end group) and retinoic acid (which has a carboxyl end group), and analyzed whether this compound is suitable for use as an anti-wrinkle agent in cosmetics based on in vitro biological assays. The hybrid retinol derivative had enhanced thermal stability and decreased photosensitivity, and exhibited decreased cell toxicity compared to that of retinol. Although the biological activity of retinyl retinoate **1** for RAR α was in between the properties of retinol and retinoic acid, the overall activity was far less than that of retinoic acid and much closer to retinol, suggesting that retinyl retinoate **1** may have side effect similar to that of retinol, and not retinoic acid. Retinyl retinoate **1** showed higher biological activity than that of retinol. Specifically, RAR activity analysis showed that Retinyl retinoate **1** exhibited lower inhibitory activity against c-Jun than retinoic acid, but higher than retinol. Also, retinyl retinoate **1** showed either superior or equivalent effects on collagen synthesis at low concentration compared to retinol or retinoic acid, respectively.

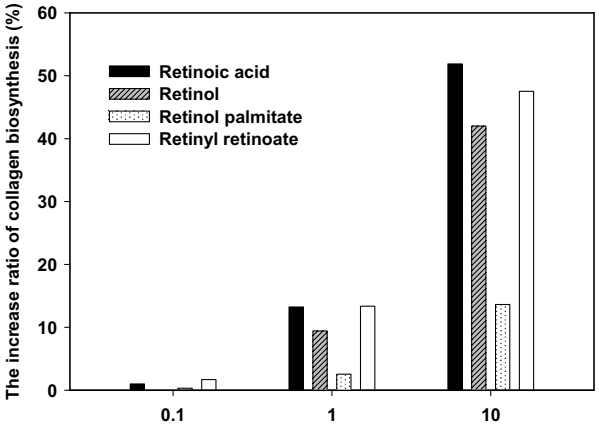


Figure 6. The effect of retinoic acid, retinol, retinol palmitate, and retinyl retinoate on the increase ratio of collagen synthesis (%).

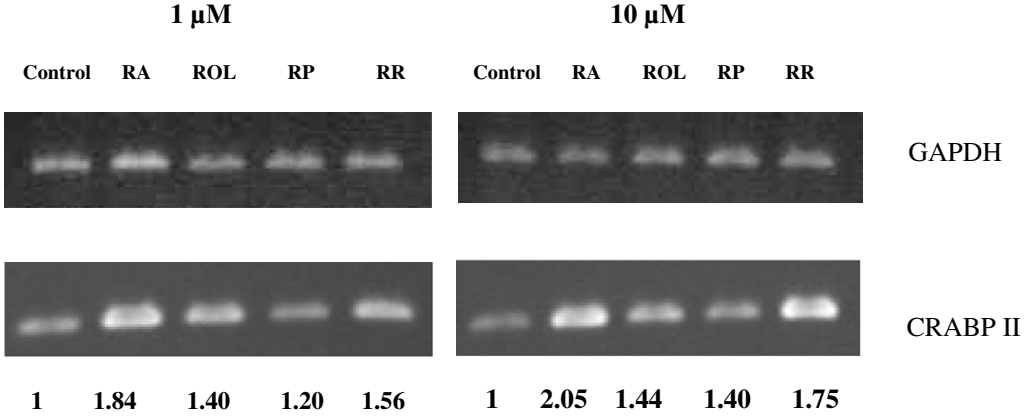


Figure 5. Effects of retinol derivatives on the expression of GAPDH and CRABP II. RA, retinoic acid; ROL, retinol, RP, retinyl palmitate, and RR, retinyl retinoate. The numbers depicted on the Western blot indicate the relative amount of expressed GRABP II based on densitometry.

Overall, the novel hybrid retinyl retinoate **1** may be useful for the prevention, improvement, or treatment of skin aging including wrinkles and freckles that may be caused by skin cancer or other skin conditions such as acne and psoriasis. In addition, retinyl retinoate **1** was effective at low concentrations in our in vitro biological assays, and thus may be effective at a low concentrations in vivo. Further, retinyl retinoate **1** exhibited excellent stability and extremely low cell toxicity. In conclusion, retinyl retinoate **1** may have the potential to be conveniently used as an additive for cosmetics for prevention and improvement of skin aging and medicines for the treatment of skin troubles such as acne and psoriasis, due to its excellent stability under severe and accelerated conditions.

4. Experimental

4.1. Chemistry

4.1.1. Retinyl retinoate 1

Retinoic acid (1.26 g, 0.0042 mol, Basf Co.), dicyclohexylcarbodiimide (DCC) (0.87 g, 0.0042 mol) and a catalytic amount of dimethylaminopyridine (DMAP) were added to retinol (1.00 g, 0.0035 mol) and dissolved in anhydrous dichloromethane (50 ml) at 0 °C. The reaction solution was slowly warmed to room temperature and stirred for 12 h under a nitrogen atmosphere in the absence of exposure to light or moisture. The reaction solution was then filtered, and the solvent was removed by distillation under reduced pressure. The residue was purified by column chromatography (silica gel 60 for cc part, Merk Co., mesh size 270 400, hexane/diethylether = 19:1, v/v) to give 1.55 g (Yield 78%) of the pale yellow title compound.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.03 (dd, 1H, J = 26.4 Hz, 11.4 Hz, C_{20H}), 6.67 (dd, 1H, J = 26.4 Hz, 11.4 Hz, C_{11H}), 6.34 (d, 2H, J = 2.1 Hz, C_{19H}, C_{12H}), 6.29 (d, 2H, J = 2.1 Hz, C_{7H}, C_{24H}), 6.18 (d, 2H, J = 9.9 Hz, C_{23H}, C8H), 6.12 (d, 2H, J = 10.2 Hz, C_{10H}, C_{21H}), 5.82 (s, 1H, C_{7H}), 5.69 (t, 1H, J = 7.2 Hz, C_{14H}), 4.81 (d, 2H, J = 6.9 Hz, $C_{5H.sub.2}$), 2.39 (s, 3H, $C18CH_3$), 2.05 (t, 4H, J = 5.1 Hz, $C_{4H.sub.2}$, $C_{27H.sub.2}$), 2.03 (s, 3H, $C_{13CH.sub.3}$), 1.99 (s, 3H, $C_{22CH.sub.3}$), 1.94 (s, 3H, $C_{9CH.sub.3}$), 1.74 (s, 6H, $C_{5CH.sub.3}$, $C_{26CH.sub.3}$), 1.65 (m, 4H, $C_{3H.sub.2}$, $C_{28H.sub.2}$), 1.50 (m, 4H, $C_{2H.sub.2}$, $C_{29H.sub.2}$), 1.06 (s, 12H, $C1(CH_3)_2$, $C_{30}(CH_3)_2$).

4.2. Stability test

In order to compare the photostability of the retinyl retinoate **1** with retinol and retinyl palmitate, each sample was dissolved in $CDCl_3$ (50 mg/mL) and irradiated by UV-A light (wavelength 356 nm) using a Spectroline apparatus (Model CM-10; Fluorescence analysis cabinet; Spectronics Corporation, Westbury, NY, USA) for 0, 2, 12, 24, and 48 h at room temperature. Changes in the ^{1}H NMR were observed, especially that of the cyclic ring group, polyene chain group, and ester group of the retinoid molecular structure.

4.3. Thermal stability test

In order to compare thermal stability of the retinyl retinoate **1** and retinol, the phase stability for 1 month in a thermohygrostat (humidity: 58%) of room temperature, 40 °C, and 4 °C was examined by a quantitative HPLC analysis. The HPLC was performed using a Capcellpak UG 120 (5 μm, 4.6 mm × 150 mm, Shiseido, Japan) column, followed by elution with dilute acetic acid (1–100): acetonitrile:methanol:dichloromethane (2:10:60:28, v/v; retinyl retinoate) or methanol/water (90:10, v/v; retinol) for 15 min at a linear gradient of 1.5 ml/min. Absorption spectra were recorded at 326 nm.

4.4. Analysis of cytotoxicity

Normal human fibroblasts (Newborn, passage 5) cells were grown in Modified Eagle Medium (DMEM, GIBCO^{TM}) 5% CO_2 and 37 °C. The cytotoxic effects of retinoids were monitored by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. Cells were seeded in 96-well plates at a density of 5×10^3 cell per plate. After cell attachment, fresh retinoid derivatives such as retinol, retinyl palmitate, retinoic acid, and retinyl retinoate **1** were added to medium at different concentrations ranging from 0.4 μM to 100 μM. Dimethylsulphoxide (DMSO), which did not affect cell proliferation, was used as control with a final concentration of 0.1%. After incubation for 1 day, 50 μL of MTT solution (2 mg/mL in PBS) was added to cell culture medium and incubated at 37 °C for 4 h. The optical density was measured using a spectrophotometer at 570 nm (ELISA Reader). An IC_{50} value, which indicates 50% growth inhibition, was determined for each retinoid derivative.

4.5. Analysis of RAR (retinoic acid receptor) activity

A recombinant gene of DR5-tk-CAT comprising DR1 as a response element of RAR, a thymine kinase promoter, and CAT (chloramphenicol acetyl transferase) was co-transfected with plasmid DNA expressing either RAR α, β, or γ into the HaCaT cell line. The cells were incubated in DMEM/10% FBS medium at 5% CO_2 and 37 °C for 1 day, at which time retinol derivatives were added at a concentration of 1 μM. The final concentration of DMSO was 0.01%. The cells were then incubated for one more day and then washed with phosphate-buffered saline (PBS). Proteins extracts were prepared from the cells, the activity of β-galactosidase was assayed, and the amount of total protein was measured to determine the transfection efficiency. The degree of transcription for RAR was analyzed by CAT ELISA (Roche Molecular Biochemicals, Mannheim, Germany).

4.6. Inhibition of AP-1 protein (activation protein-1)

The CAT reporter (Coll-CAT) contained the collagenase promoter and AP-1 response element (TRE) and was transfected into the HaCaT cell line. The degree of inhibition against the activity of AP-1 by retinol derivatives was measured using CAT ELISA (Roche Molecular Biochemicals, Mannheim, Germany). Further, the effect of retinol derivatives on the transcriptional activity of c-Jun (a protein that induces metastasis of cancer, skin aging, and inflammation, and consists of either homologous or heterologous isomers), a constitutive element of AP-1, was examined by co-transfecting c-Jun and RAR α expression vectors.

4.7. CRABPII mRNA expression

Normal skin primary fibroblasts were grown in 10 cm dishes in the absence or presence of retinol, retinyl palmitate, retinoic acid, or retinyl retinoate **1**. Total RNA from cultured human skin fibroblasts was prepared using a commercial kit (Reasy Mini kit, Qiagen) according to the manufacturer's protocol; the RNA concentration was determined with a spectrophotometer. Total RNA was subjected to reverse transcription and subsequent PCR to confirm changes in CRABP II mRNA levels observed previously. Specifically, 1 μg of total RNA was reverse-transcribed in a 50 μL reaction mixture containing reverse transcriptase, primers, dNTP mix, and RNAase free-water. PCR cycling conditions were chosen for each cDNA to ensure that measurements were performed during the exponential component of the reaction. PCR primers were purchased from Bioneer oligonucleotide synthesis service. The reaction mixture was first incubated at 50 °C for 30 min, at which time Taq DNA polymerase was activated by heating at 95 °C for 15 min using a Perkin-Elmer cycler 9700 (Perkin-Elmer Applied

biosystems, USA). Following initial denaturation the reactions were cycled 25 times at 94 °C for 1 min for denaturation, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. PCR products were visualized by ethidium bromide staining on 2% agarose gels after electrophoresis. The primers used for RT-PCR were 5′-CCC AAC TTC TCT GGC AAC TGG-3′ (sense) and 5′-CTC TGC GAC GTA GAC CCT GGT-3′ (antisense) to give 413 bp PCR fragment. GAPDH was amplified in parallel, and the results were used for normalization. Primers used for RT-PCR of GAPDH were 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′ (sense) and 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′ (antisense) to give 983 bp PCR fragment.

4.8. Collagen synthesis test

The effect of the retinyl retinoate **1** on collagen synthesis was examined and compared to that of retinol, retinoic acid, and retinyl palmitate to confirm its utility as an inhibitor of skin aging. A range of concentrations where the samples exhibited no overt cellular toxicity were selected through a cell toxicity test and was determined as 0.000001–0.00001% (w/v). Normal human fibroblasts (Newborn, passage: 6) were cultured with DMEM. A total of 1×10^5 cells/well were inoculated into each well of a 6-well plate, and cells were subsequently cultured until they were approximately 80% confluent. Cells were then treated with the various retinoid derivatives and incubated for 2 days (FBS was not added to the medium when cells were treated with samples), at which time the cell medium was removed and stored at 4 °C. After removal of the medium, a small amount of PBS was added, and cells were dislodged from the plate with a cell scraper. Next, 1 N NaOH (100 μl) and distilled water (400 μl) were added and the resulting mixture was allowed to stand overnight at room temperature, at which time protein analysis was performed (BCA Protein Assay Kit, PIERCE 23225). The stored medium was subjected to collagen analysis using an analysis kit (Sircol Collagen assay kit; a dye that specifically combines with soluble collagen). A total of 1.0 ml of Sircol Dye Reagent was introduced into each tube and mixed for 30 min, centrifuged for 10 min at 5000g or more, and the supernatant was removed. 1.0 ml of Sircol Alkali Reagent was added, slowly mixed, and the absorbance at 540 nm was measured. The amount of total protein was analyzed and converted into a value for the ratio of collagen biosynthesis (%) as follows:

$$\text{The increase ratio of collagen biosynthesis }(\%) = \left[\frac{(\text{Amount of collagen synthesized/total protein})_{\text{sample group}}}{(\text{Amount of collagen synthesized/total protein})_{\text{control group}}}\times 100\right] - 100$$

Here, amount of collagen synthesized of sample and control was analyzed after treatment with retinoid and solvent, respectively.

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