Note

Synthesis and ABTS Radical, MMP-1 Inhibitory Activity of CAPE Analogues

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In the photoaging process of skin, the ultraviolet (UV)-induced reactive oxygen species (ROS) is the key regulator of matrix metalloproteinase (MMPs) expression. In this study, a series of Caffeic acid phenethyl ester (CAPE) analogues were synthesized by conjugating the group VI elements (selenium, sulfur, oxygen)-containing aliphatic alcohols to polyphenolic acids. Their biological activities were evaluated by *in vitro* testing of their radical scavenging activity the of ABTS [2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] radical and inhibitory effect against the matrix metalloproteinase-1 (MMP-1) activity of collagen degradation and cytotoxicity of a human dermal fibroblast skin cell. Our results suggest these compounds displayed moderate anti-free radical, potent MMP-1 inhibitory, and low cytotoxic activities.

Keywords: CAPE; ABTS radical; MMP-1 inhibitor.

INTRODUCTION

The matrix metalloproteinases (MMPs) comprise a relatively large and ever growing family. There are now more than 28 enzymes that are classified as MMPs.[1] These enzymes have both a descriptive name (e.g. interstitial collagenase, an enzyme found in the interstitial space, which degrades fibrillar collagens) and an MMP number. The MMPs' structure related zinc-dependent endopeptidases can degrade a wide variety of extracellullar matrix components and play important roles in tissue remodeling during developmental morphogenesis, angiogenesis, tissue repair, arthritic inflammation, tumor invasion and metastasis.[2,3] Recent studies showed the development of epidermal hyperplasia, the formation of skin wrinkles and significant enhancement of MMPs' (i.e. MMP-1, MMP-2 and MMP-9) activities when human skin is exposed to UV.[4-6] Inhibition of MMPs' activity by a specific MMP inhibitor suppressed UV-induced epidermal thickness enhancement and wrinkle formation.[7-9] These results suggest that MMPs may be directly involved in the skin photoaging process. Thus, inhibition of MMP activity either directly (by means of a specific inhibitor) or indirectly (by reducing MMP expression) may provide an effective therapeutic method for counteracting photoaging.[10,11]

Oxidative stress has been reported to play an important role in the development of the various detrimental effects of UV-radiation. UV was found to increase the level of hydrogen peroxides and other reactive oxygen species (ROS) in skin tissues.[12-14] The ROS may cause oxidative damage to lipids, proteins, and DNA directly, but may also modulate their expressions through signal transduction pathways and ultimately, lead to skin damage.

CAPE (Fig. 1), a natural flavonoid-like and polyphenolic ester compound, is one of the major components of honeybee propolis.[15] It has been reported that CAPE has various biological activities, such as antiviral, antiflam-

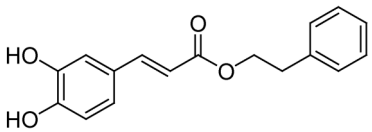

Fig. 1. Structure of CAPE.

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matory, anticarcinogenic and immunomodulatory properties.[16] Likewise, CAPE was shown[17] to inhibit lipooxygenase activities, suppress ROS-induced lipid peroxide (LPO) in tissues, and display antioxidant activity.[18] To identify potential antioxidants and anti-UV-induced photo damaging agents, it is important to study various CAPE analogues on their ROS scavenging and anti-MMP-1 activities.

Recently, studies in our laboratory have been directed at synthetic antioxidants with diverse anti-oxidative functionalities. Based on the similar chemical properties of the group VI elements (oxygen, sulfur, selenium), we speculated that ester analogues of an aliphatic alcohol containing group VI elements and polyphenolic acid might provide synergistic antioxidative activity. In the present study, a series of oxygen, sulfur, and selenium-containing polyphenolic acid esters was prepared. Their in vitro efficacy as radical scavengers, inhibition against MMP-1, and cytotoxicity in human dermal fibroblasts was investigated.

RESULTS AND DISCUSSION

Synthesis of compounds

The synthesis of compounds used in this study[19] was carried out according to the Scheme outlined in Fig. 2. Selenium-containing aliphatic alcohol (2-phenylselenoethanol; **1**) was prepared from 2-chloroethanol with phenylselenol produced *in situ* by a reduction of diphenyl diselenide with sodium borohydride. Thus, polyphenolic acids (**2**, **3**) were reacted directly with 2-phenylselenoethanol (**1**) or commercially obtained 2-phenythiol-ethanol (**4**) and 2-phenoxy-ethanol (**5**), respectively, in the presence of triphenylphosphine (TPP), diisopropyl azodicarboxylate (DIAD), and tetrahydrofuran to give the target compounds (**6a-6c, 7a-7c**).

Free radical ABTS assay

The model of scavenging ABTS free radicals is a convenient method[20] to evaluate the *in vitro* antioxidative activity of antioxidants. As shown in Table 1, these compounds displayed various degrees of free radical scavenging activity, with decreasing activity in the following order: CAPE > Trolox > **7b** > **6c** > **6b** > **7c** > **7a** > **6a**. Among them, compounds **7b** and **6c**, were the most potent, having antiradical effects comparable to that of Trolox. Unfortunately, all modified CAPE analogues showed less free radical scavenging activity than CAPE. These results indicated

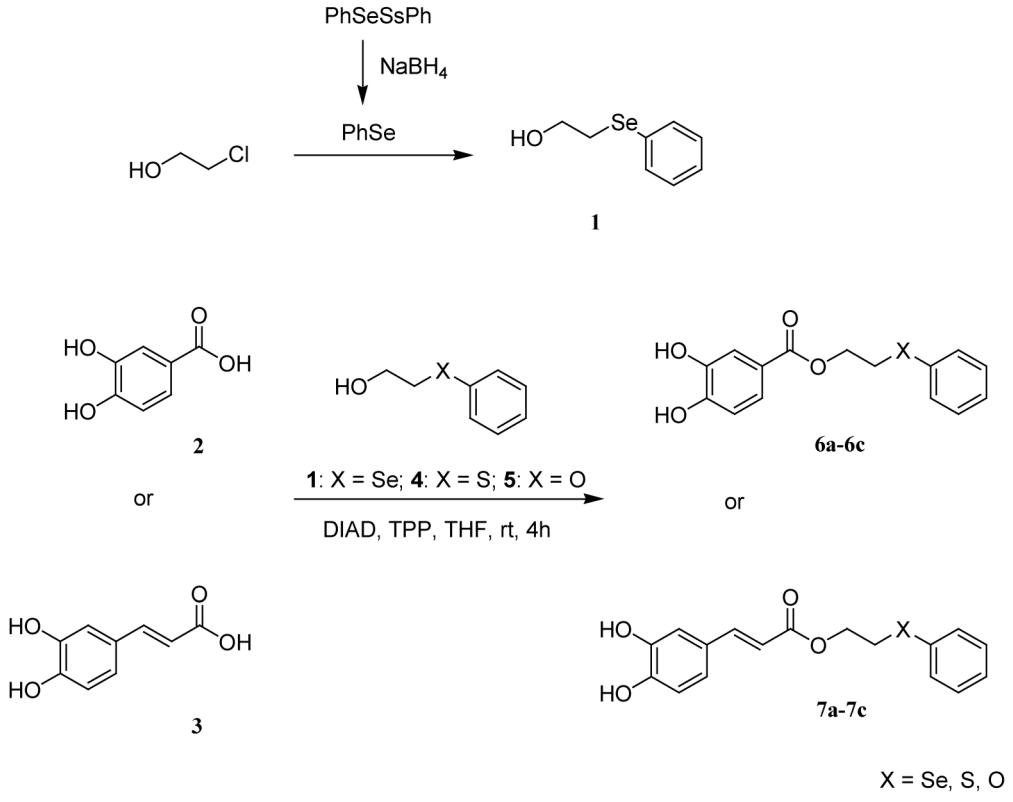

Fig. 2. Synthesis pathway of target compounds.

Table 1. ABTS Radical cation quenching activity of compounds **6a-7c** Versus Trolox and CAPE

Compound (20 μM)	ABTS radical Scavenging activity (%)
Trolox	35.36 ± 0.98
CAPE	67.17 ± 0.48
6a	21.52 ± 2.87
6b	29.70 ± 1.21
6c	31.10 ± 3.67
7a	23.69 ± 2.26
7b	31.26 ± 1.98
7c	28.34 ± 1.77

Results represent mean ± SD (n = 3).

Table 2. Relative MMP-1 inhibitory efficiency of compounds **6a-7c** *versus* **CAPE** and **GM-1489**

Compound (66 μM)	MMP-1 inhibit Activity (%)
DMSO	1.2 ± 3.52
CAPE	19.0 ± 8.25
6a	58.3 ± 39.18
6b	70.2 ± 27.04
6c	31.0 ± 45.22
7a	59.8 ± 48.92
7b	33.3 ± 23.78
7c	21.0 ± 15.90
GM-1489	101.2 ± 8.99

Data are calculated from the relative band intensity of Fig. 3. Lane-Collagen represents 0% MMP-1 activity and Lane DMSO represents 100% of MMP-1 activity. The relative MMP-1 inhibitory efficiency (%) of each compound = [1-(collagen-compound)/(collagen-DMSO)] × 100.

that the design of an introduction of group VI elements into CAPE failed to enhance free radical scavenging activity.

Collagen degradation assay

In the collagen degradation assay,[14,21] MMP-1 enzyme and rat tail collagen were incubated with synthetic compounds and the potency of these compounds on inhibition of MMP-1 activity was assessed by SDS-PAGE gel electrophoresis. As shown in Fig. 3, in the absence of MMP-1, the original form of the collagen sample has two major bands. In the presence of MMP-1 but with no inhibitory compound (lane-DMSO), the major collagen bands were digested into smaller fragments after 18 h of incubation. Lane-DMSO, with no apparent MMP-1 inhibitory activity, was used as a negative control for this assay. The known MMP-1 inhibitor, GM-1489, was used as a positive control for protection of collagen from further degradation (lane-GM-1489). To evaluate the efficacy of these compounds as MMP-1 inhbitiors, the density of the second band from the top of the gel was quantitated (marked with an arrow, Fig. 3). The relative MMP-1 inhibitory efficiency of these compounds is in the following order: GM-1489 > **6b** > **7a** > **6a** > **7b** > **6c** > **7c** > CAPE > DMSO (Table 2). Luckily, all modified CAPE analogues showed more effective collagen degradation activity than CAPE. These results indicated that the introduction of group VI elements into CAPE may be beneficial on collagen degradation against MMP-1. It is of interest to note that the modified selenium and sulfur analogues of CAPE showed very potent activity.

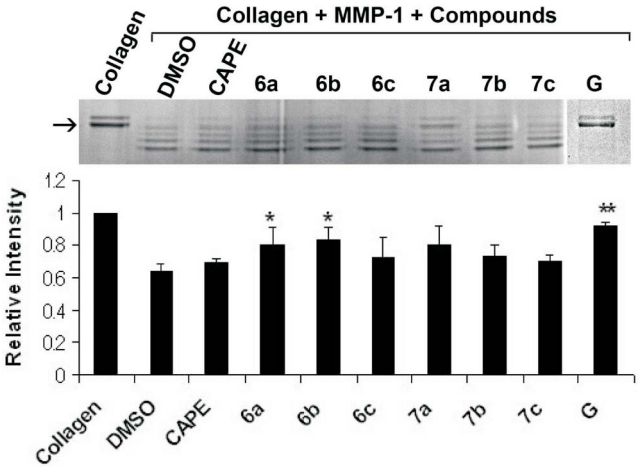

Fig. 3. Inhibition of MMP-1-mediated collagen degradation by compounds **6a-7c**. Collagen degradation. Type I collagen was cleavaged by purified MMP-1 in the presence of various compounds. G: GM-1489 (broad-spectrum inhibitor of MMPs). The relative inhibitory potency of test compounds on MMP-1 activity are expressed as relative intensity of the second bands from top of the gel (indicated by arrow). Each bar is the mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$ compared with lane-DMSO.

Cytotoxicity of synthetic test compounds 6a-7c

Concentration-dependent cytotoxicity[22] of **6a-7c** was determined by a CCK-8 assay on human derma fibroblast (HDF) cells. Results presented in Fig. 4 show cell viability upon treatment with increasing concentrations of test compounds after 48 h. With less than 25 μM, these compounds displayed no apparent cytotoxicity as compared with the

control cells that were treated with 0.01% DMSO. Significant cytotoxicity was observed in cells treated with over 50 μM of these compounds.

In summary, we designed and synthesized a series of CAPE analogues and evaluated these new compounds for inhibition of free radical and collagenase (MMP-1) activity. In addition, the cytotoxicity of these compounds in HDF cells was also determined. Our results suggest that these compounds displayed moderate anti-free radical, potent MMP-1 inhibitory, and low cytotoxic activities and may have the potential of being used as functional cosmetic agents. It should be noted that these compounds can be easily prepared by a single step; therefore, industrial mass production may be possible. In our further studies, the effect of these newly synthesized CAPE analogues of polyphenol derivatives on UV-induced MMP-1 activity and the regulatory mechanism will be examined in human dermal fibroblasts.

EXPERIMENTAL SECTION

Instrumentation

Melting points (mp) were taken on a BUCHI 530 apparatus and are uncorrected. Merck Art No. 105554 plates precoated with Silica gel 60 containing a fluorescent indicator were used for thin-layer chromatography, and Silica gel 60 (Merck Art No 109385, 230-400 mesh) was employed for column chromatography. Evaporations were carried out at < 50 °C using a rotary evaporator at reduced pressure (water aspirator). ^{1}H- and ^{13}C-NMR spectra were obtained with a Varian 300 NMR spectrometer at 300 and 75 MHz, respectively. Where necessary, deuterium exchange experiments were used to obtain proton shift assignments. Mass spectra were recorded on a JEOL J.M.S-300 spectrophotometer. Analytical samples were dried under reduced pressure at 78 °C in the presence of P_2O_5 for at least 12 h unless otherwise specified. Elemental analyses were obtained using a Perkin-Elmer 2400 Elemental Analyzer.

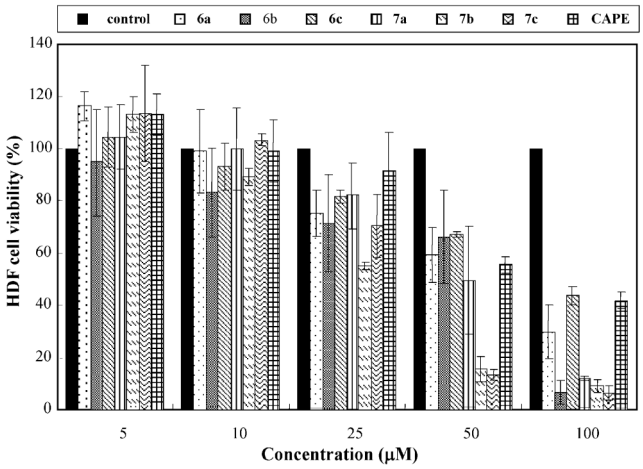

Fig. 4. Viability of HDF cells treated with different concentrations of **6a-7c**. Results represent mean ± SD (n = 3).

2-Phenylselenoethanol (1)

Sodium borohydride (1.2 g, 15 mmol) was added in portions to a stirred solution of diphenyl diselenide (3.5 g, 11 mmol) in absolute ethanol at 0 °C. To the resulting colorless solution was added a solution of the appropriate 2-chloroethanol (1.8 g, 22 mmol) dissolved in the minimum quantity of ethanol. The mixture was stirred under reflux for 3 h. Solvent was filtered and removed in a vacuum. The residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (3/1) to give 2-phenylselenoethanol (4.1 g). Yield 92%, as pale yellow oil, IR (KBr) ν (cm^{-1}): 3452 (OH). ^{1}H-NMR (DMSO-d_6) δ: 7.487.21 (m, 5H, ArH), 4.95 (s, 1H, OH), 3.60 (t, 2H, J = 6.4 Hz, CH_2), 3.00 (t, 2H, J = 14.2 Hz, $SeCH_2$); ^{13}C-NMR (DMSO-d_6) δ: 131.2, 130.2, 129.2, 126.4, 60.7, 29.4. FAB-MS, *m/z*: 202 (Ca1cd for $C_8H_{10}OSe$: 200.13). Anal. Ca1cd for $C_8H_{10}OSe$: C, 47.77; H, 5.01. Found: C, 47.67; H, 5.03.

Mitsunobu Esterification of Polyphenolic Acids General Procedure

To a solution of polyphenolic acids (2.3 g, 15 mmol) and 2-phenylselenoethanol (2 g, 10 mmol) in dry tetrahydrofuran (35 mL) were added TPP (15 mmol) and DIAP (15 mmol) at 0 °C. After stirring at room temperature for 48 h, the reaction was worked up by removal of the solvent, and the residue was partitioned between ethyl acetate and saturated $NaHCO_3$. The organic phase was washed with brine and then dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by flash chromatography on a silica gel column with 1:1 n-hexane/ethyl acetate as eluent to give **6a** (1.9 g, 55%). The compounds **6b-7c** were obtained under the same conditions.

4-Dihydroxy-benzoic acid-(2-phenylseleno-ethyl ester) (6a)

Yield 55%, mp. 90-92 °C (*n*-hexane/ethyl acetate); IR (KBr) ν (cm^{-1}): 3253 (ArC-OH), 3023 (ArC-H), 1738 (C=O), 1119 (C-O). ^{1}H-NMR ($CDCl_3$) δ: 7.62-6.90 (m, 8H, ArH), 6.41 (s, 2H, OH), 4.53 (t, J = 14.2 Hz, 2H, $COOCH_2$), 3.24 (t, J = 13.2 Hz, 2H, $SeCH_2$). ^{13}C-NMR ($CDCl_3$) δ: 165.95, 157.08, 156.41, 133.03, 132.00, 126.18, 108.98,

107.526, 70.31, 64.53, 25.45, 21.86; FAB-MS *m/z*: 338 (Ca1cd for $C_{15}H_{14}O_4Se$: 337.24). Anal. Ca1cd for $C_{15}H_{14}O_4Se$: C, 53.42; H, 4.18. Found: C, 53.46; H, 4.22.

3,4-Dihydroxy-benzoic acid-(2-thiolphenoxy-ethyl ester) (6b)

Yield 42%, mp. 116-118 °C (*n*-hexane/ethyl acetate); IR (KBr) ν (cm^{-1}): 3296 (ArC-OH), 2927 (ArC-H), 1683 (C=O), 1126 (C-O). ^{1}H-NMR ($CDCl_3$) δ: 7.55-7.30 (m, 7H, ArH), 6.93 (d, *J* = 8.7 Hz, 2H, ArH), 6.68 (s, 2H, OH), 4.47 (t, *J* = 7.2 Hz, 2H, OCH_2), 3.29 (t, *J* = 7.2 Hz, 2H, SCH_2). ^{13}C-NMR (DMSO-d_6) δ: 169.30, 155.89, 147.68, 135.09, 130.27, 129.38, 129.12, 129.07, 126.84, 126.65, 125.13, 114.69, 111.89, 64.07, 32.57. FAB MS *m/z*: 291 (Ca1cd for $C_{15}H_{14}O_5S$: 290.34). Anal. Ca1cd for $C_{15}H_{14}O_5S$: C, 62.05; H, 4.86. Found: C, 63.23; H, 4.98.

3,4-Dihydroxy-benzoic acid-(2-phenoxy-ethyl ester) (6c)

Yield 58%, mp: 118-120 °C (*n*-hexane/ethyl acetate); IR (KBr) ν (cm^{-1}): 3296 (ArC-OH), 2927 (ArC-H), 1683 (C=O), 1128 (C-O). ^{1}H-NMR ($CDCl_3$) δ: 7.66-6.92 (m, 8H, ArH), 4.67 (t, *J* = 3.9 Hz, 2H, $COOCH_2$), 4.33 (t, *J* = 4.8 Hz, 2H, OCH_2); ^{13}C-NMR (DMSO-d_6) δ: 169.03, 134.46, 162.90, 158.37, 131.82, 129.34, 121.03, 114.81, 108.52, 104.05, 65.81, 63.40. FAB-MS *m/z*: 275 (Ca1cd for $C_{15}H_{14}O_5$: 274.28). Anal. Ca1cd for $C_{15}H_{14}O_5$: C, 65.69; H, 5.15. Found: C, 64.73; H, 5.19.

3,4-Dihydroxy-cinnamic acid-(2-phenylseleno-ethyl ester) (7a)

Yield 32%, mp. 70-72 °C (*n*-hexane/ethyl acetate); IR (KBr) ν (cm^{-1}): 3251 (ArC-OH), 2979 (ArC-H), 1737 (C=O), 1109 (C-O). ^{1}H-NMR ($CDCl_3$) δ: 7.62-7.01 (m, 8H, ArH), 6.92 (d, *J* = 10.2 Hz, 1H, COCH), 6.39 (s, 2H, OH), 6.25 (d, *J* = 8.1 Hz, 1H, CH), 4.45 (t, *J* = 14.7 Hz, 2H, $COOCH_2$), 3.19 (t, *J* = 14.7 Hz, 2H, $SeCH_2$). ^{13}C NMR ($CDCl_3$) δ: 166.95, 156.31, 146.42, 145.08, 144.01, 132.86, 129.13, 127.52, 127.23, 122.33, 115.40, 115.23, 114.26, 70.18, 63.76, 25.58, 21.87. FAB-MS *m/z*: 364 (Ca1cd for $C_{17}H_{16}O_4Se$: 363.27). Anal. Ca1cd for $C_{17}H_{16}O_4Se$: C, 56.21; H, 4.44. Found: C, 56.27; H, 4.51.

3,4-Dihydroxy-cinnamic acid-(2-thiolphenoxy-ethyl ester) (7b)

Yield 32%, mp. 123-126 °C (*n*-hexane/ethyl acetate); IR (KBr) ν (cm^{-1}): 3212 (ArC-OH), 2988 (ArC-H), 1720 (C=O), 1120 (C-O). ^{1}H-NMR ($CDCl_3$) δ: 7.60 (d, *J* = 15.6 Hz, 1H, COCH), 7.55-6.89 (m, 8H, ArH), 6.27 (d, *J* = 15.9 Hz, CH), 7.47-6.92 (m, 8H, ArH), 6.74 (d, *J* = 15.6 Hz, 1H, CH), 4.07 (t, *J* = 6.9 Hz, 2H, OCH_2), 3.26 (t, *J* = 7.2 Hz, 2H, SCH_2). ^{13}C-NMR (DMSO-d_6) δ: 166.31, 151.26, 149.19, 145.10, 131.75, 129.51, 129.41, 126.96, 123.07, 115.32, 111.81, 110.71, 63.35, 55.81, 55.77, 25.07. FAB-MS *m/z*: 317 (Ca1cd for $C_{17}H_{16}O_5$: 316.38). Anal. Ca1cd for $C_{17}H_{16}O_5$: C, 64.54; H, 5.1. Found: C, 65.86; H, 5.26.

3,4-Dihydroxy-cinnamic acid-(2-phenoxy-ethyl ester) (7c)

Yield 33%, mp. 127-128 °C (*n*-hexane/ethyl acetate); IR (KBr) ν (cm^{-1}): 3210 (ArC-OH), 2990 (ArC-H), 1720 (C=O), 1120 (C-O). ^{1}H-NMR (DMSO-d_6) δ: 7.61-6.73 (m, 8H, ArH), 6.31 (d, *J* = 15.9 Hz, 1H, CH), 4.09 (t, *J* = 4.5 Hz, 2H, $COOCH_2$), 4.21 (t, *J* = 3.9 Hz, 2H, OCH_2). ^{13}C-NMR (DMSO-d_6) δ: 167.24, 149.24, 146.33, 164.23, 132.67, 132.12, 130.21, 129.49, 129.33, 126.23, 122.08, 121.54, 116.50, 115.64, 115.35, 66.47, 63.07. FAB-MS *m/z*: 301 (Ca1cd for $C_{17}H_{16}O_5$: 300.31). Anal. Ca1cd for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37. Found: C, 68.03; H, 5.41.

Evaluation of the antioxidant activity by ABTS radical scavenging test

In vitro antioxidant activity of test compounds was evaluated using an improved $ABTS^{\cdot+}$ scavenging assay described by Re et al.[23] Briefly, the purple $ABTS^{\cdot+}$ solution (7.5 mM) was prepared by mixing ABTS solution with potassium persulfate (2.5 mM) overnight in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with ethanol or PBS (pH 7.4) to an OD_{734} of 0.70 ± 0.02 for assay. Six minutes after the 5 μL of studied compounds was added to 30 μL of $ABTS^{\cdot+}$ dilution, the amount of $ABTS^{\cdot+}$ remaining was determined at 734 nm, and the radical scavenging activity was obtained from the following equation:

$$\text{Radical scavenging activity (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100.$$

Collagen degradation assay

Rat tail collagen 5.9 g/mL (BD Biosciences, San Jose, CA USA) was diluted to 238 μg/μL in Tris-glucose (0.5 M Glucose and 0.33 M Tris). 48 μg/mL of purified MMP-1 (Calbiochem, San Diego, CA, USA) was diluted to 6×10^{-4} μg/μL with Tris-glucose. Degradation of the native collagen was carried out by mixing 19 μL of MMP-1 solution, 9 μL of collagen solution and 2 μL of 1 mM synthetic compounds and incubated for 18 h at 37 °C. Intact collagen exposed to buffer alone served as control. Collagen fragments released were resolved by SDS-PAGE (8.5% gel) and staining with Coomassic brilliant blue R-250. The relative intensity of the protein bands were measured by an image

analyzing system (Alpha Innotech, St. San Leandro, CA, USA).

Cell culture

Human skin cell HDF were cultured as monolayers in DMEM supplemented with 15 mM Hepes, 26 mM sodium bicarbonate, 2 mM L-glutamine, 100 g/mL streptomycin, 100 u/mL penicillin, and 10% fetal bovine serum with 5% CO_2 in a 37 °C humidified atmosphere. The cells in the logarithmic growth phases were used in this study. The cells were seeded into culture dishes and allowed to incubate for 24 h prior to drug treatment. Synthetic compounds or MMP inhibitors were added to the medium as a concentration stock in DMSO. Control cells were fed with medium containing the same amount of DMSO.

Cell viability assay (CCK-8 staining)

Cell viability was assessed by CCK-8 solution (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc, Kumamoto, Japan) by combining WST [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] and 1-methoxy PMS [1-methoxy-phenazine methosulfate] as described by the manufacturer. Briefly, cells were seeded in 96-well plates at the density of 1×10^4 cells/well 24 h prior to treatment. Compounds were added to the medium as a concentration stock and the control cells were fed with medium containing the same amount of drug-free vehicle. After incubation for 48 h, 10 μL of CCK-8 was added and cells were further incubated for 3 h. Relative cell viability was obtained by measuring absorbance at 450 nm using an ELISA microplate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA).

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