(4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine, a Nitrogen Analog of Stilbene as a Potent Inhibitor of Melanin Production

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The current study was carried out to investigate *in vitro* the effects of (4-methoxy-benzylidene)-(3-methoxy-phenyl)-amine on melanin biosynthesis which is closely related to hyper-pigmentation. (4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine, a nitrogen analog of stilbene, was synthesized by a single step process. This compound inhibited the tyrosinase activity, which converts dopa to dopachrome in the biosynthetic process of melanin, and showed a UV-blocking effect at UV-B band. The compound also exhibited SOD-like activity, which is involved in the protection against auto-oxidation and inhibited melanin production in melan-a cell line. Our results suggest the possibility that (4-methoxy-benzylidene)-(3-methoxy-phenyl)-amine might be used as a skin whitening agent.

Key words oxyresveratrol; stilbene; whitening agent

Melanogenesis results from melanin delivered to keratinocytes and stored in the epidermis, increasing melanin in melanocyte.[1)] The over-pigmentation of skin, facial discoloration, freckles and melanogenesis caused by dermatitis and geriatric pigment spots can affect beauty and have a negative effect on an individual's state of mind. Thus various bleaching cosmetics and medicines are being developed to control melanogenesis.

Oxyresveratrol, (Fig. 1) one of the ingredients of Mori cortex,[2)] has recently been reported to exert a potent inhibitory effect on dopa oxidase activity of tyrosinase which catalyzes rate-limiting steps of melanin biosynthesis. Its inhibitory effects were stronger than kojic acid which is currently widely used in cosmetics.[3)] At present, the production of bleaching cosmetics based on oxyresveratrol is dependent on Mori cortex extract, because the quantity of the ingredient is limited and an effective method of synthesis has yet to be established.[4,5)] Therefore, an attempt to search for alternative materials like stilbene derivatives, which have high bioactivity and can be easily obtained, is warranted. This study was designed to examine the melanogenesis inhibiting activity of oxyresveratrol derivatives with a transformed connection chain between the two benzene rings and lower polarity for the development of optimal functional cosmetics. A new compound I, (4-methoxy-benzylidene)-(3-methoxy-phenyl)-amine, was prepared by a single step synthetic process. The examining methods involved the inhibitory effects on dopa oxidase activity of tyrosinase, UV-blocking effects, superoxide dismutase (SOD)-like activities, and decreasing effect of melanin production in melan-a cell line.

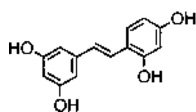

Fig. 1. Structure of Oxyresveratrol

Experimental

Instrumentation and General Techniques UV spectra were obtained with a Hewlett Packard 8453 UV/VIS spectrophotometer and the Molecular Devices E09090 microplate reader. ^{1}H- (300 MHz) and ^{13}C- (75 MHz) NMR spectra were run on a Gemini-2000 spectrometer. FAB-MS spectra were measured on a Hewlett Packard GC-mass spectrometer. TLC and column chromatography were carried out on precoated silica gel F254 plates (Merck) and Si gel 60 (Merck, 70—230 mesh). Oxyresveratrol was obtained from organic synthesis in our laboratory. All other chemicals and solvents were analytical grade and used without further purification.

Synthesis of (4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine (Compound I) *p*-Anisaldehyde (269 μl, 2.2 mmol) and *m*-anisidine (247 μl, 2.2 mmol) was dissolved in toluene (11 ml). The reaction mixture was equipped with the dean-stark and refluxed for 12 h. The toluene was evaporated and the products were recrystallized with 30 ml of methanol at -20 °C. Yield of the compound was 53 mg (10%).

(4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine (Compound I): Yellowish solid; mp=152 °C decomposed. ^{1}H-NMR ($CDCl_3$) δ: 8.39 (1H, s), 7.86 (1H, t), 7.83 (1H, t), 7.28 (1H, m), 7.00 (1H, t), 6.97 (1H, t), 6.79 (1H, t), 6.76 (2H, m), 3.88 (3H, s), 3.84 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 159.83, 131.99, 130.56, 129.83, 129.15, 114.19, 112.87, 111.49, 107.90, 106.60, 101.07, 55.43, 55.32. IR ν_{max} cm^{-1}: 2994, 2936, 2836, 1593, 1512, 1464, 1309, 1252, 1165, 1142, 1034, 939, 833, 774. MS (EI^{+}) *m/z* 241 (M^{+}, 100), 210 (3), 121 (2), 108 (7), 92 (12), 77 (22).

Assay of Tyrosinase Activity Dopa oxidase activity of tyrosinase was spectrophotometrically determined as described previously with minor modification.[6)] Each concentration (1 mM, 500, 100, 10 μM) of test substance was dissolved in MeOH. Forty microliters of L-dopa (15 mM), 80 μl of 67 mM phosphate buffer (pH 6.8) and 40 μl of either the same buffer or test sample were added to a 96-well microplate, and then mixed with 40 μl of mushroom

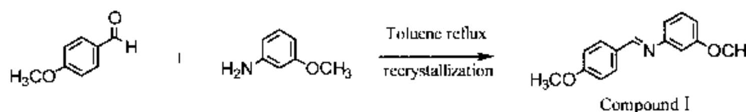

Fig. 2. Synthesis Pathway of (4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine (Compound I)

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tyrosinase (125 U). After incubation at 37 °C for 30 min, the amount of dopachrome in the reaction mixture was determined. Based on the optical density at 490 nm (OD_{490}), the inhibitory activity of the sample was expressed as the concentration which inhibited 50% of the enzyme activity (IC_{50}). Kojic acid and oxyresveratrol were used as reference.

Absoption of UV-A and UV-B The UV spectrum of sample, and of oxyresveratrol and kojic acid as a reference, was measured at the concentration of 33.4 μM in methanol.[7]

Superoxide Dismutase-Like Activity SOD-like activity was measured according to the methods of Oyanagui.[8] Thirty-two microliters of sample in methanol was mixed with a mixture of 0.2 ml of reagent A solution (0.5 mM hypoxanthine, 10 mM hydroxylamine hydrochloride) and 0.77 ml of EDTA-phosphate buffer (pH 8.2), and then preincubated at 37 °C for 10 min. Five mU/ml xanthine oxidase solution (0.2 ml) was added to the above solution, and the mixture was incubated for 30 min. Reagent B (2 ml coloring reagent to a final concentration of 20 μM *N*-1-naphthylethylene diamine, 2 mM sulfanilic acid and 16.7% acetic acid) was added to the reaction mixture. The resulting mixture was allowed to stand for 30 min at room temperature, and the OD was then measured at 550 nm. SOD-like activity was expressed as an inhibitory percentage against the production of a superoxide anion from hypoxanthine oxidase. Kojic acid was used as a positive control.

Cell Line and Culture Procedures Melan-a cells were kindly donated by Dr. B. Lee at the Skin Research Institute, Pacific Co., Korea. The cells were cultured in RPMI1640 medium under 10% FBS and 200 nm Phobol 12-Myristate 13-Acetate (TPA) conditions. To 100 π tissue culture dish, 10 ml of medium was added and seeded with 5×10^5 cells. If the cells were grown confluently after 3 to 4 d at 37 °C and 5% CO_2, they were seeded with 10^5 cells/well in a 24 well plate and then incubated for 24 h. Each well was changed with 990 μl of medium everyday and treated with 10 μl of sample (solvent (v/v): propylene glycol/EtOH/H_2O=5/3/2) for 3 d, then incubated for 1 d.

Cell Viability The percentage of viable cells was determined by staining the cell population with crystal violet. After removal of medium from each well, the well was washed with PBS and 200 μl of crystal violet (CV 0.1%, 10% EtOH, the rest was PBS) was added. The mixture was incubated at room temperature for 5 min and washed with water two times. After adding 1 ml of EtOH, the mixture was shaken at room temperature for 10 min. The UV absorption was measured at 590 nm.

Determination of Melanin Contents in Melan-a Cells After each well media removal, the well was washed with PBS followed by the addition of 1 ml of 1 N NaOH to dissolve the melanin. The UV absorption was measured at 400 nm. Phenylthiourea (PTU) was used as a positive control.

Statistical Analysis Data were presented as mean$\pm$S.D. from three independent experiments. Statistical comparison between different treatments was done by Stunt's *t*-test.

Results

Inhibitory Effects of Compounds against Tyrosinase Activity Compound I, oxyresveratrol and kojic acid were examined for their inhibitory effect on dopa oxidase activity of tyrosinase. Compound I (IC_{50}=192.1 μM) and oxyresveratrol (IC_{50}=53.7 μM) showed a strong inhibitory activity against tyrosinase (Table 1). Compound I at a concentration of 200 μM exhibited 57.1% inhibition on dopa oxidase activity of mushroom tyrosinase, where as 50% inhibition was shown at the concentration of 192.1 μM. Dose-dependent inhibitions on the enzyme activity were exhibited by compound I and oxyresveratrol at concentrations of 2 to 200 μM. Oxyresveratrol exhibited about a 3-fold more potent inhibitory effect than compound I. Tyrosinase is the key enzyme for melanin biosynthesis, playing a role in oxidation from tyrosine to L-dopa and dopa to dopaquinone.[9,10] This process is a determinant of animal skin color, and is involved in local hyperpigmentations such as melanoma, ephelides, and lentigo.[11]

SOD-Like Activity of Compounds Skin-whitening is caused by the reduction of melanin as antioxidant. SOD is an enzyme preventing skin damage with oxygen radical. The SOD-like activity was expressed as percent inhibition against the production of superoxide anion from hypoxanthin–xanthin oxidase. As shown in Table 2, compound I at 50 μM showed higher activity (18.8%) than oxyresveratrol (10.6%) at the same concentration. SOD-like activity of compound I was comparable to kojic acid (21.9%). Their effects were not increased at concentrations above 50 μM of a compound.

UV-Absorption The absorption at UV-A area (350—370 nm) and UV-B area (270—290 nm) was measured for compound I, oxyresveratrol and kojic acid at a concentration of 33 μM. Compound I at a concentration of 33 μM showed a maximum absorption band at 285 nm related to the UV-B area, in spite of a weak absorption at 362 and 385 nm, corre-

Table 1. Inhibitory Effects of (4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine (Compound I), Oxyresveratrol and Kojic Acid against Mushroom Tyrosinase

Sample	Concentration (μM)	Inhibition[a] (%)	IC_{50}[b] (μM)
Compound I	2	14.3±1.1	192.1
	20	18.3±1.2	
	100	43.0±3.3	
	200	57.1±1.4	
Kojic acid	2	8.3±6.2	275.6
	20	10.9±2.3	
	100	23.4±1.5	
	200	38.4±1.5	
Oxyresveratrol	2	17.8±5.1	53.7
	20	39.3±4.3	
	100	73.4±1.8	
	200	77.8±1.1	

a) Tyrosinase was preincubated with test substances at 25 °C for 10 min prior to incubation with dopa for 30 min, and the absorbance was read at 490 nm. Each value represents the mean±S.E. of three experiments. *b*) 50% inhibitory concentration.

Table 2. SOD-Like Activity of (4-Methoxy-benzylidene)-(3-methoxy-phenyl)-amine (Compound I), Oxyresveratrol and Kojic Acid

Sample	Concentration (μM)	Inhibition[a] (%)
Compound I	10	15.6
	50	18.8
Kojic acid	10	18.8
	50	21.9
Oxyresveratrol	10	3.9
	50	10.6

a) SOD-like activity was expressed as percent inhibition against the production of superoxide anion from hypoxanthin-xanthin oxidase.

Table 3. Activity of PTU, Kojic Acid, Oxyresveratrol and Compound I on Cell Growth and Melanin Production of Melan-a Cells

Sample	Concentration (μM)	Melanin production (%)	Cell viability (%)
Phenylthiourea	10	41.4±9.3	80.1±9.3
	100	25.3±8.4	72.3±9.4
Kojic acid	10	96.0±2.6	98.6±6.4
	100	91.9±4.0	84.3±5.8
Oxyresveratrol	10	88.1±7.3	94.4±8.4
	100	20.1±8.4	16.8±5.0
Compound I	10	100.8±4.7	94.6±2.8
	100	57.8±5.5	71.3±4.2

Melan-a cells were grown to confluence in 24-well culture plates overnight. Test samples were added to the plates and incubated for 3 d. And the medium was renewed everyday. After 3 d incubations, cell viability and melanin contents in melan-a cells were determined by an ELISA reader. Each value represents the mean±S.E. of three experiments.

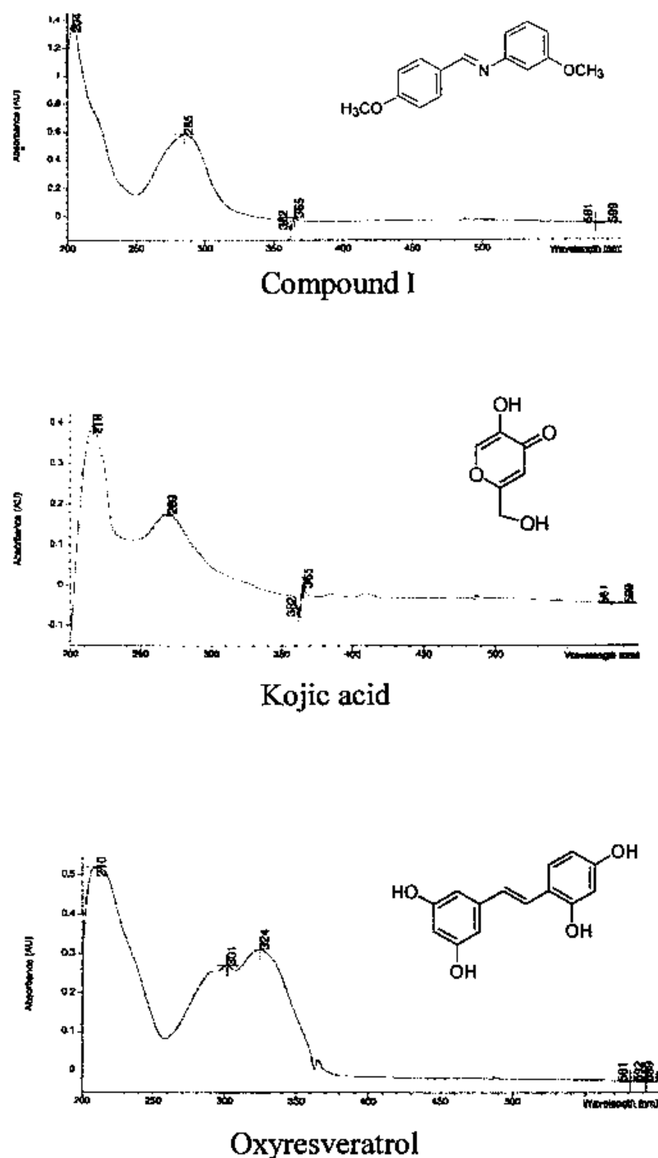

Fig. 3. Absorption of UV-A and UV-B of Each Compound

sponding to the UV-A region. Kojic acid at a concentration of 33 μM also showed a maximum absorption band at 280 nm related to the UV-B area. However, oxyresveratrol had no absorption at UV-A or UV-B. Compound I showed the most characteristic absorption band at the UV-B area (Fig. 3), indicating that it has an excellent UV-blocking effect.

Melanin Production and Cell Viability of Melan-a Cells An immortal cell line of pigmented melanocytes, "melan-a", has been derived from normal epidermal melanoblasts from embryos of inbred C57BL mice.[12] The melan-a cell line was used for testing the melanin production. Phenylthiourea, kojic acid, oxyresveratrol and compound I were tested for their melanogenic effects in cultured melan-a cells at concentrations of 10 and 100 μM. The highest concentration tested resulted in a slight decrease in cell viability. In particular, treatment of melan-a cells with oxyresveratrol of 100 μM resulted in 16.8% cell viability. Oxyresveratrol showed high toxicity, and kojic acid showed no inhibitory effect on melanin production. The results were that compound I showed 42.2% inhibiting melanin production at 100 μM, although this was not as much as PTU (1-phenyl-2-thiourea).

Discussion

Among the stilbene compounds, the connection chain between two benzene rings has a critical function in pharmacological activity.[13] Compound I is an oxyresveratrol derivative that has a lower polarity and modified connection chain between two benzene rings. In respect to organic synthesis, oxyresveratrol possesses through many synthetic steps. In contrast, compound I can be easily prepared by a single step process involving refluxing with *p*-anisaldehyde and *m*-anisidine, then recrystallization;[14] therefore, industrial mass production may be possible. Importantly, compound I not only inhibited tyrosinase activity, but also showed SOD-like activity and excellent UV blocking effect. In the present work, we demonstrated that this compound has little toxicity or inhibitory effect of melanin generation in the melan-a cell line. We have shown that compound I decreased melanogenic activity, tyrosinase activity and total melanin production at 100 μM while minimally affecting cell viability. This result may be explained by direct inhibition of melanocyte tyrosinase or by an antioxidant mechanism affecting tyrosinase expression or other cell signaling mechanism. Some phenolic compounds have been shown to decrease melanin production *in vivo* or *in vitro.* Hydroquinone, arbutin and glabridin have been reported to be potential inhibitors of tyrosinase activity.[15—17] Until now, none of these compounds has had top value. Based on these results, the compound I related analogs must be evaluated in order to determine the relationship between structure and activity. Through an experiment, compound I and its derivative might be used as functional cosmetic agents. Currently, our efforts are directed towards the synthesis of a new derivative of compound I with stronger activity.

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