New candidate for skin depigmentation : The inhibitory effect and cytotoxicity of small molecule compounds at *in vitro* cell culture

H.S, Rho, K.J, Kim, J.S, Hwang, H.J, Shin, H.K, Chang, I.S, Chang and O.S, Lee
R&D center, AmorePacific Corporation, Yongin-si 449-729, Korea,

Abstrcat

To obtain effective and safe topical depigmenting agents, we synthesized hydroxybenzoates, alkoxybenzoates, and 3,4,5-trimethoxycinnamate containing a thymol moiety and screened then for high-level inhibitory activity against melanin synthesis. Among them, 5-methyl-2-(methylethyl)phenyl (*2E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate (Melasolv)^{TM} **4h**, showed the most potent depigmenting effect (IC_{50} = 10μM) with low cytotoxicity (IC_{50} = 200μM). To find the inhibition mechanism of our candidate, various *in vitro* tests were performed such as DPPH assay, tyrosinase activity in mushroom or in culture cell and expression of tyrosinase, TRP-1 and TRP-2. The result of this study suggested that **4h** inhibited melanin synthesis by reducing the expression of tyrosinase and TRP-1 at the transcriptional level in melan-a melanocytes.

Key word alkoxy cinnamate, thymol, depigmenting effect, low cytotoxicity

Introduction

Melanogenesis is the process of production of melanin by melanocytes within the skin and hair follicles and is mediated by several enzymes such as tyrosinase, TRP-1, and TRP-2.[1-3] Since tyrosinase[4] is known to be the enzyme responsible for the oxidation of tyrosine, the first and rate-limiting step in melanogenesis, many efforts have been focused on the regulation of tyrosinase activity using small molecular compounds, for example, hydroquinone,[5,6] resorcinol,[7,8] catechol,[9] gentisic acid,[10,11] and gallic acid.[12] The low molecular weight of depigmenting agents is one requirement for efficient delivery into the skin. Their depigmenting effect is closely related to the antioxidant properties of the phenolic hydroxyl group and the cytotoxicity of their intermediates within melanocytes.[13-17] Many compounds and derivatives have been developed but there is still a need to find potent small molecular compounds for depigmentation without compromising cytotoxicity. To meet this need with another avenue of approach, benzoates and cinnamate containing a thymol moiety were synthesized and their depigmenting effects and cytotoxicity were determined in a murine melanocyte cell line. After screening these compounds, alkoxybenzoates and 3,4,5-trimethoxycinnamate

showed an unexpectedly strong depigmenting effect with low cytotoxicity, whereas hydroxybenzoates showed a simultaneous depigmenting effect and cytotoxicity, as expected. In this study, a novel way of developing depigmenting agents is presented in which depigmentation is not related to the antioxidant properties of phenolic hydroxyl groups and cytotoxicity within melanocytes. To discover the reason for inhibition activity of our candidates, various *in vitro* tests were performed such as DPPH assay, tyrosinase activity in mushroom or in culture cell and expression of tyrosinase, TRP-1 and TRP-2.

5-Methyl-2-(methylethyl)phenyl (*2E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate (Melasolv)^{TM} **4h**, selected through *in vitro* test, showed most potent inhibitory effect with low cytotoxicity.

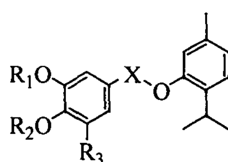
Experimental

Materials

All reagents were used without further purification. Gallic acid, thymol, hydroquinone, kojic acid were purchased from Sigma Chemical Co (USA)

Structure and synthesis of thymol ester

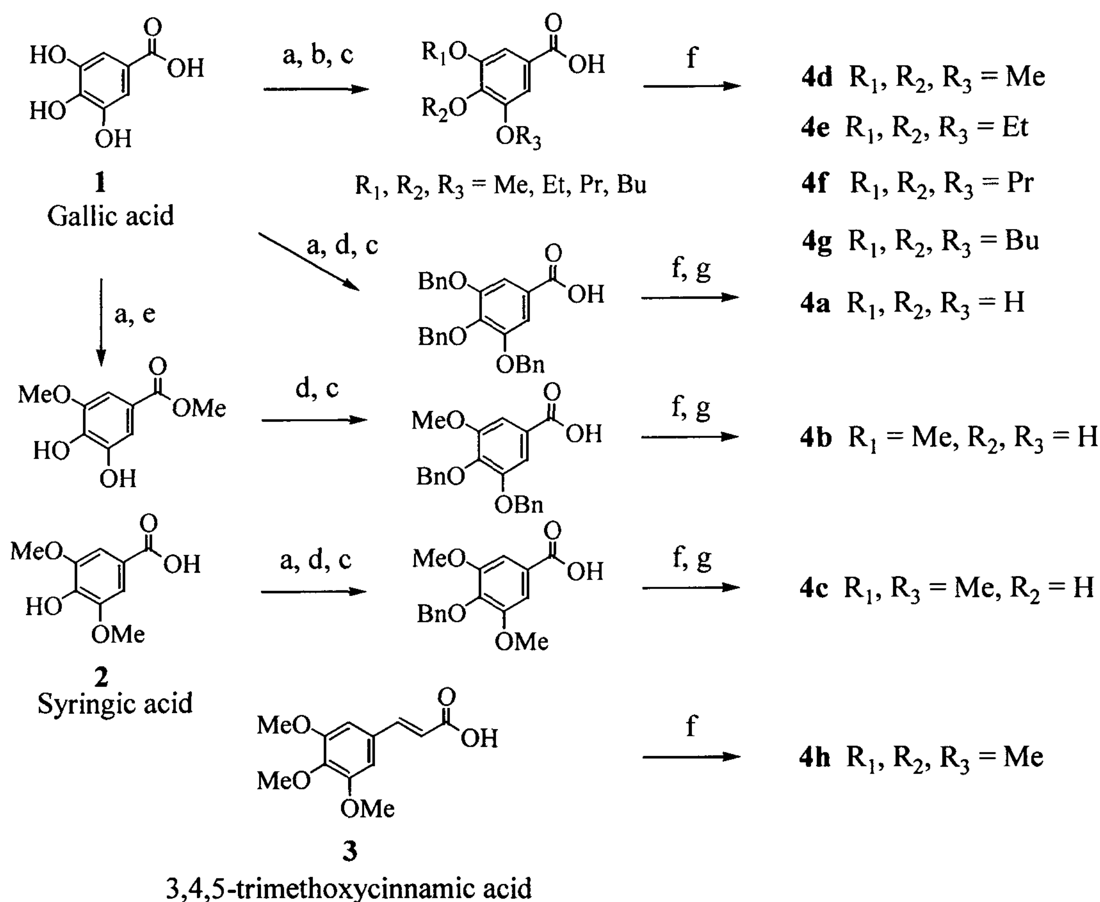
In the synthesis of **4a**, **4b**, and **4c**, the phenolic hydroxyl groups were protected by a benzyl group and deprotected. All acids were coupled with thymol using the mixed anhydride method.



4

R_1, R_2, R_3 = H, Me, Et, Pr, Bu. X = CO, CH_2=CH_2CO (*E*)

Fig. 1. Structure of thymol esters



(a) MeOH, TsOH, toluene; (b) alkyl bromide, K_2CO_3, DMF; (c) KOH, H_2O;
(d) benzyl bromide, K_2CO_3, DMF; (e) DMS, borax, H_2O; (f) benzenesulfonyl chloride, pyridine, thymol; (g) Pd/C, H_2, EtOAc

Fig. 2. Synthetic pathway of thymol esters (4a – 4h)

Cell culture

Melan-a melanocytes are a highly pigmented, immortalized normal murine melanocyte cell line derived from C57BL/6 mice. The melan-a melanocytes used in this study were obtained from Dr. Dorothy Bennett (St. George's Hospital, London, UK). Cells were grown and maintained at 37°C in an atmosphere of 95% air, 5% CO_2 in RPMI-1640 (Bio Whittaker, Walkersville, MA, USA) supplemented to a final concentration of 10% heat-inactivated fetal bovine serum, penicillin 5 units/ml, streptomycin 5 μg/ml and 200 nM phorbol 12-myristate 13-acetate. Cells were passaged every 3 days with a maximal passage number of 33. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM EDTA (Gibco BRL, Grand Island, NY, USA).

Measurements of melanin content and cell viability

Melanin content and cell number were measured in melan-a melanocytes. One hundred thousand cells were seeded into each well of 24-well plates and compounds were added to triplicate cultures. Medium was changed daily, and after 4 days of culture, the cells were lysed with 1 N NaOH 1ml and pipetted repeatedly to homogenize. For analysis, 200 μl of each crude cell extract was transferred into 96-well plates. The relative melanin content was measured at 400 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tex Instruments). Cell viability was determined using the crystal violet assay. The culture medium was removed from the 24-well culture plates and replaced with 0.5 ml of 0.1% crystal violet in 10% ethanol per well. The plates were stained for 5 min at room temperature and rinsed four times. The crystal violet retained by adherent cells was extracted with 1 ml of 95% ethanol. Absorbance was determined at 540 nm using an ELISA reader.

Mushroom tyrosinase assay

Mushroom tyrosinase, L-tyrosine, and L-DOPA were purchased from Sigma Chemical (St. Louis, MO, USA). Tyrosinase activity was determined using the method of Pomerantz[20)] with minor modification. Twenty-five microliters of 0.5 mM L-DOPA, 25 μl of 10 mM L-tyrosine, 875 μl of 50 mM phosphate buffer (pH 6.5), and 25 ml of test sample solution were mixed. Then 50 μl of mushroom tyrosinase (1600 U/ml) was added. The amount of dopachrome produced in the reaction mixture was determined against a blank (solution without enzyme) at 475 nm (OD_{475}) using a spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

DPPH assay

DPPH reagent was prepared at a DPPH concentration of 80 μg/ml in MeOH. A test sample (50 μl) was dissolved in DMSO and mixed with 100 mM Tris-HCl buffer (pH 7.4, 50 μl), distilled water, and 400 ml of DPPH ethanolic solution (50 μl). The mixture was shaken well and allowed to stand for 20 min in the dark. The absorbance was measured at 515 nm using an Elx800 microtiter plate reader (Bio-Tek Instruments, Vermont, USA).

***In Situ* Tyrosinase Assay**

The early rate limiting step of the biosynthetic pathway of melanin (hydroxylation of tyrosine) was estimated during the last day of treatment from the amount of $^{3}H_2O$ released into the medium during the conversion of L-[ring-3,5-^{3}H]tyrosine to dihydroxyphenylalanine according to an adaptation of the methods of

Pomerantz and Oikawa et tal as described previously.[18] Cells were seeded into 48well culture plate $2x10^5$ cells per well and allowed to attach overnight. The medium was then exchanged for growth medium supplemented with compounds under investigation 24h before the termination of the experiment, medium was supplemented with 2μCi[^{3}H] tyrosine per ml. At the end of the experiment the radiolabeled medium was assayed for the presence of ^3H_2O.

Western blot analysis

The levels of tyrosinase, TRP-1, TRP-2 were determined by immunoblot analysis as described previously. Melan-a melanocytes were seeded into $60mm^2$ culture dishes and treated with 15μM and 30μM 4h or vehicle for 72hrs. The cells were removed from the dishes and cell extracts electrophoresed on NuPAGE gells and the proteins electroblotted onto nitrocellulose membrane and detected by chemiluminescence as described in materials and methods. Equal protein loading was checked using actin antibody.

RT-PCR analysis (*Tyrosinase, TRP-1, TRP-2*)

Melan-a melanocytes were treated with 15 and 30μM of compounds for 48hrs. RT-PCR analysis was done on tyrosinase, TRP-1 and TRP-2. Total cellular RNA was extracted from melan-a melanocytes using Trizol Reagent (Gibcol BRL) according to the manufacturer's instruction

Result and discussion

In hibition activity and cytotoxicity

To evaluate synthetic compounds for their potency in melanogenesis inhibition, we compared their activities and cytotoxicity with known depigmenting agents such as hydroquinone, gallic acid, and kojic acid.[19] The results are shown in table 1.

Table 1. *In vitro* Assessment of Putative Depigmenting

Compound	Inhibition IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
Hydroquinone	9	25
Gallic acid	35	40
Kojic acid	>2mM	>2mM

4a, R_1, R_2, R_3=H ; X = CO	10	33
4b, R_1=Me, R_2, R_3=H ; X = CO	32	71
4c, R_1, R_3=Me, R_2=H ; X = CO	60	300
4d, R_1, R_2, R_3=Me ; X = CO	25	120
Thymol	> 300	> 300
3,4,5-Trimethoxybenzoic acid	> 80	> 80
Mixture	> 80	> 80
4e, R_1, R_2, R_3 = Et ; X = CO	30	> 200
4f, R_1, R_2, R_3 = Pr ; X = CO	12	> 200
4g, R_1, R_2, R_3 = Bu ; X = CO	8	> 200
4h, R_1, R_2, R_3 = Me ; X = CH_2 = CH_2CO	10	> 200

Compounds **4a** - **4d**, hydroquinone, gallic acid, and kojic acid were examined for their inhibitory effects against melanin synthesis and cytotoxicity. Hydroquinone showed a potent inhibitory effect (IC_{50} = 9 μM) and severe cytotoxicity (IC_{50} = 25 μM). Gallic acid showed mild inhibitory activity and cytotoxicity compared with those of hydroquinone. However, kojic acid did not inhibit pigmentation at concentrations up to 2 mM and showed no cytoxicity at this concentration. Compound **4a**, containing three phenolic hydroxyl groups, exhibited potent inhibitory activity (IC_{50} = 10 μM) with cytotoxicity (IC_{50} = 33 μM). The inhibitory activity appeared to be related to the cytotoxicity of the phenolic hydroxyl groups. Compound **4b**, containing two phenolic hydroxyl groups, showed similar behavior. However, compound **4c**, containing phenolic hydroxyl group at the *para*-position showed moderate activity (IC_{50} = 60 μM) with low cytotoxicity (IC_{50} = 300 μM). The reason for this low cytotoxicity might be due to the absence of an oxidation process to yield reactive quinone. Two neighboring methoxyl groups interfere with the transformation of **4c** to its reactive cytotoxic intermediate. Compound **4d**, which three hydroxyl groups are replaced by methoxyl groups, showed unexpectedly favorable results. The IC_{50} of pigmentation was about 25 μM, but it was not cytotoxic (IC_{50} = 120 μM). To expand our understanding of the scope of its activity, we adjusted the thymol moiety and modified the chain length of the alkoxyl group. Thymol showed no depigmenting activity and no cytotoxicity, but trimethoxybenzoic acid showed moderate activity (IC_{50} = 80 μM) and cytotoxicity (IC_{50} = 80 μM). The activity and cytotoxicity of a simple mixture of thymol and 3,4,5-trimethoxybenzoic

acid were similar to those of 3,4,5-trimethoxybenzoic acid. Although the simple mixture was not effective, two components connected with an ester bond in general showed strong depigmenting effects with low cytotoxicity. Analysis in terms of cytotoxicity IC_{50}/pigmentation inhibition IC_{50} indicated that the increase in chain length of the alkoxyl group distinctly enhanced depigmentation and safety. Compound **4h**, containing an α,β-unsaturated carbonyl group, showed the most potent depigmenting effect (IC_{50} = 10 μM) with low cytoxicity (IC_{50} = 200 μM). The activity was similar to that of hydroquinone.

Tyrosinase and DPPH assay

Compounds **4d**, **4e**, **4f**, **4g** and **4h**, which three hydroxy groups are replaced by alkoxy groups, there was no effect in the tyrosinase assay and DPPH assay. The reason for no effect is probably due to the lack of a phenolic hydroxyl group responsible for the antioxidation and radical scavenging activity (Table 2).

Table 2. Mushroom Tyrosinase Inhibition and Radical-scavenging Effects

Compound	Mushroom tyrosinase inhibition IC_{50} (μM)	DPPH IC_{50} (μM)
Hydroquinone	1.1	38.4
Gallic acid	—	12.8
Kojic acid	21.1	—
4a, R_1, R_2, R_3=H ; X = CO	0.8<	22.4
4b, R_1=Me, R_2, R_3=H ; X = CO	0.8<	38.3
4c, R_1, R_3=Me, R_2=H ; X = CO	—	—
4d, R_1, R_2, R_3=Me ; X = CO	—	—
4e, R_1, R_2, R_3 = Et ; X = CO	—	—
4f, R_1, R_2, R_3 = Pr ; X = CO	—	—
4g, R_1, R_2, R_3 = Bu ; X = CO	—	—
4h, R_1, R_2, R_3 = Me ; X = CH_2 = CH_2CO	—	—

— ; Not effective.

***In Situ* Tyrosinase Assay**

After screening through a melan-a assay, 5-methyl-2-(methylethyl)phenyl (*2E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoate **4h** was selected as a desirable candidate for depigmenting agent. It showed the most potent depigmenting effect (IC_{50} = 10 μM) with low cytotoxicity (IC_{50} = 200 μM). Although there was no effect in mushroom tyrosinase, the inhibition activity was detected *in situ* tyrosinase assay. When 30μM of **4h** was treated for 72hrs, inhibition activity was decreased about 80% without affecting cell growth.

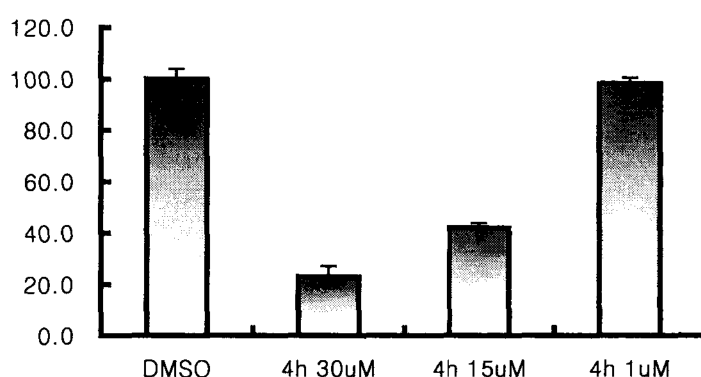
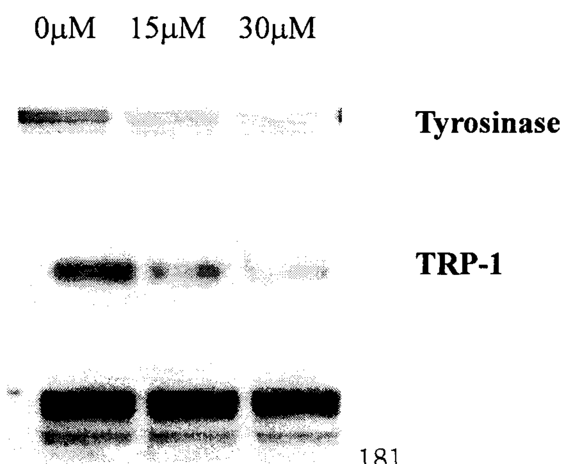


Fig 3. Effects of 4h on tyrosinase activity in melan-a melanocytes.

Expression of Tyrosinase, TRP-1 and TRP-2 assay

To discover effects of **4h** on tyrosinase or other melanogenic enzymes like a TRP-1 and TRP-2, western blot analysis was performed onto these proteins. From western blot analysis, it is clear that the reduced tyrosinase activity was due to lowered tyrosinase protein level (Fig 4). In order to examine effects of **4h** at the transcriptional levels, RT-PCR analysis was done. From RT-PCR analysis, tyrosinase was controlled by **4h** (Fig 5). The mRNA level of tyrosinase and TRP-1 was reduced by the treatment of **4h**. However, TRP-2 was not changed.



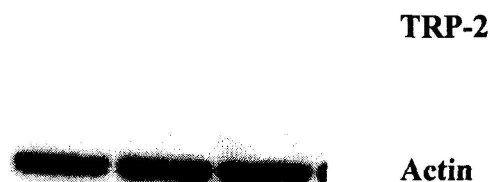


Fig 4. Western blot analysis of tyrosinase, TRP-1 and TRP-2 on melan-a cell

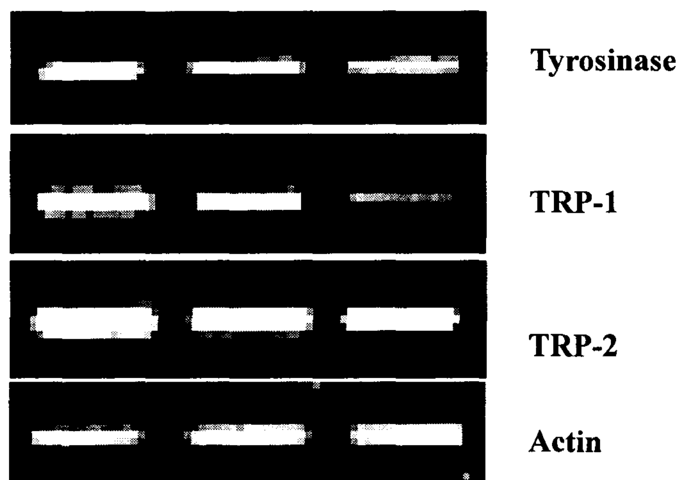


Fig 5. Semi-quantitative RT-PCR results of tyrosinase, TRP-1 and TRP-2

Conclusion

After screening hydroxybenzoates, alkoxybenzoates, and 3,4,5-trimethoxycinnamate containing a thymol moiety to determine their depigmenting activity using the melan-a assay, we propose that compounds **4e** - **4h** are a class of desirable candidates for depigmenting agents. Them showed potent inhibition activity with low cytotoxicity. The reason for their low cytotoxicity must be the absence of phenolic hydroxyl groups in their structure. Compound **4h**, a synthetic ester of 3,4,5-trimethoxycinnamic acid and thymol, showed potent inhibitory activity similar to that of hydroquinone, with much less cytotoxicity. We selected compound **4h** as a candidate and performed various *in vitro* tests to discover inhibition mechanism. The reason for this inhibition is considered to be regulation of transcriptional level. Compound **4h** reduced the expression of tyrosinase and TRP-1 in melan-a melanocytes. Further studies about its activity on reconstitute human epidermis and UVB-induced hyperpigmentation animal model are underway.

Reference

1) Sturm R. A., *Mutat. Res.*, **422**, 69-76 (1998).

2) Marmol V. D., Ito S., Jackson I., Vachtenheim J., Berr P., Ghanem G., Morandini R., Wakamatsu K., Huez G., *FEBS Lett.*, **327**, 307-310 (1993).

3) Amae S., Yasumoto K., Takeda K., Udono T., Takahashi K., Shibahara S., *Biochim. Biophys. Acta*, **1492**, 505-508 (2000).

4) Marmol V. D., Beermann F., *FEBS Lett.*, **381**, 165-68 (1996).

5) Jimbow K., Obata H., Pathak M. A., Fitzpatrick T. B., *J. Invest. Dermatol.*, **62**, 436-449 (1974).

6) Patick E., Juberg D. R., O'Donoghue J., Maibach H. I., *Food Chem. Toxicol.*, **37**, 169-175 (1999).

7) Coupvray A. L, Sevran H., *US Patent*, 5,468,472 (1995).

8) Katagiri T., Okubo T., Oyobikawa M., Futaki K., Sagaku M., *Proceedings of the 20th IFSCC Congress*, Cannes, volume 1, 93-102 (1998).

9) Ito S., Kato T., Ishikawa K., Kasuga T., Jimbow K., *Biochem. Pharmacol.*, **36**, 2007-2011 (1987).

10) Dooley T. P., Gadwood R. C., Kilgore K., Thomasco L. M., *Skin Pharmacol.*, **7**, 188-200 (1994).

11) Curto E. V., Kwong C., Hermersdorfer H., Glatt H., Santis C., Virador V., Hearing V. J., Dooley T. P., *Biochem. Pharmacol.*, **57**, 663-672 (1999).

12) Tadashi H., Takatoshi M., Jusuke S., Ichiro T., Yoshinori N., *Japanese Patent*, 08,283,137 (1996).

13) Riley P. A., Sawyer B., Wolff M. A., *J. Invest. Dermatol.*, **64**, 86-89 (1975).

14) Alena F., Jimbow K., Ito S., *Cancer Res.*, **50**, 3743-3747 (1990).

15) Passi S., Picardo M., Nazzaro-Porro N., *Biochem. J.*, **245**, 537-542 (1987).

16) Thorneby-Addersson K., Sterner O., Hansson C., *Pigment Cell Res.*, **13**, 33-38 (2000).

17) Tayama K., Takahama M., *Pigment Cell Res.*, **15**, 447-453 (2002).

18) Pomerantz S. H. *J. Biol. Chem.*, **238**, 2351-2357 (1963).

19) Yasuaki O., Yudaka M., *Fragrance J.*, **6**, 53-58 (1990).