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ARTICLE

Effects of Selected Organo-sulfur Compounds on Melanin Formation

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The effect of organo-sulfur compounds, including 1-propylmercaptan (PM), dimethyl disulfide (DMDS), diallyl disulfide (DADS), propyl disulfide (PDS), and 2,5-dimethylthiophene (DMT), on melanin formation was investigated. Among the selected five organo-sulfur compounds, PM displayed a significant inhibitory effect on tyrosinase activity (IC_{50} = 0.5 mM) and the highest inhibitory action on *o*-quinone formation. In the B16 intracellular model system, the inhibitory action of selected five organo-sulfur compounds on tyrosinase activity and melanin formation may be, in part, attributed to the reduction of the reactive oxygen species (ROS) formation and positive modulation of the GSH/GSSG ratio in B16 cells. Among the five organo-sulfur compounds, PM appeared to be the most potent inhibitor of melanin formation. The analysis of inhibitory kinetics revealed that PM is a mixed-type inhibitor. This is the first study indicating that organo-sulfur compounds tested may play an important role in the regulation of melanin formation, making them the potent candidates for skin-whitening agents.

KEYWORDS: Organo-sulfur compounds; melanin; tyrosinase; reactive oxygen species

INTRODUCTION

Melanin, the major pigment present on the surface of vertebrates, is a natural pigment present in the animal, microorganism, and plant kingdoms (*1*, *2*). This pigment synthesis, occurring in melanocytes located in the basal layer between the dermis and epidermis, is stored in granules termed melanosomes (*3*). With regard to melanin formation, three major enzymes of the tyrosinase gene family are involved, including tyrosinase, tyrosinase-related protein 1, and tyrosinase-related protein 2 (*4*). Among the three enzymes, tyrosinase is known to be a key enzyme for melanin biosynthesis and is responsible for melanization in melanocytes (*5*). In other words, the melanin formation because of tyrosinase activity after sunlight exposure causes dermatological disorders associated with freckles, melasma, age spots, and senile lentigines (*6*). Many research reports have shown that melanin formation may be inhibited by avoiding ultraviolet exposure, by inhibition of tyrosinase activity, by inhibition of melanocyte metabolism and proliferation, as well as by the removal of melanin by corneal ablation (*7*, *8*). Along with avoiding ultraviolet exposure, the use of tyrosinase inhibitors may be a simple and effective way to prevent melanin formation (*7*). Similarly, the inhibition of tyrosinase activity is one of the major strategies in developing new whitening agents (*9*). Therefore, investigations on tyrosinase inhibitors have received much attention (*6*, *10*, *11*).

Kojic acid and sulfite are well-known potent tyrosinase inhibitors; however, adverse side effects, such as high toxicity toward cells and low stability toward oxygen and water, limit their application (*12*). In addition, some skin-whitening products, e.g., hydroquinone, corticosteroids, and mercury-containing products, have been prohibited because of serious health concerns (*5*). With regard to the food and cosmetic application, safety is an essential consideration for tyrosinase inhibitors. Recently, a broad spectrum of natural resources, such as kaemperol, aloesin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, oxyreveratrol, and cinnamaldehyde, present in higher plants that have been reported to inhibit tyrosinase activity, have been considered to be safe and largely free of any harmful side effects (*10*−*13*).

Allium species are fascinating plants that have been consumed for many centuries (*14*). Among *Allium* species, garlic and onion are two well-known food ingredients widely used in our gastronomy (*15*). Apart from these, evidence from several investigations suggested that the biological and medical effects of garlic and onion are mainly attributed to their high organo-sulfur compound content (*15*). Organo-sulfur compounds, such as dially sulfide, dially disulfide, and others, provide, in part, to garlic and onion a unique and characteristic odor and flavor as well as biological properties (*15*−*19*). Although organo-sulfur compounds from *Allium* species showed biological and medical effects, whether they have any effects on melanin formation as well as inhibiting tyrosinase activity remains unclear. Therefore, the aim of the present study was to explore selected organo-sulfur compounds, including thiol [e.g., 1-propylmercaptan (PM)], disulfide [e.g., dimethyl disulfide (DMDS), diallyl disulfide (DADS), and propyl disulfide (PDS)], and heterocyclic sulfide [e.g., 2,5-dimethylthiophene (DMT)], commonly found in *Allium* species (**Figure 1**) (*20*−*22*), on melanin formation, and the mechanism of action was also elucidated.

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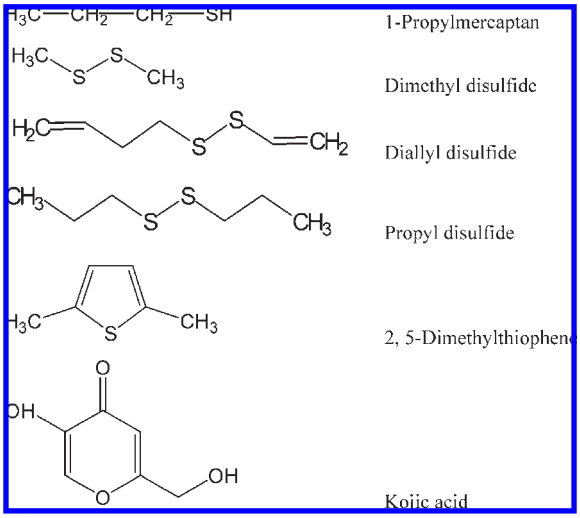

Figure 1. Chemical structures of the employed organo-sulfur compounds and kojic acid.

MATERIALS AND METHODS

Chemicals. PM, DMDS, DADS, PDS, DMT, kojic acid, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), synthetic melanin, and mushroom tyrosinase were purchased from Sigma Chemical Co. (St. Louis, MO). L-3,4-Dihydroxyphenylalanin (L-DOPA) was obtained from Acros Organic (Geel, Belgium). Dimethyl sulfoxide (DMSO) was purchased from Aldrich Chemical (Milwaukee, WI). *tert*-Butyl catechol (*t*-BC) was obtained from E. Merck (Darmstadt, Germany).

Cell Culture. B16 mouse melanoma cells were obtained from the Culture Collection and Research (CCRC, Hsinchu, Taiwan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin and at 37 °C in a humidified 5% CO_2/95% air-controlled incubator (*23*).

Effect of Organo-sulfur Compounds on Tyrosinase Activity: Mushroom Tyrosinase Activity. The mushroom tyrosinase was used for the bioassay. The tyrosinase inhibitory activity was determined with the degree of inhibition on tyrosinase-catalyzed oxidation of L-DOPA, as previously described (*23*). Diphenolase inhibitory activity was determined by measuring the dopachrome accumulation at 475 nm using a spectrophotometer. All of the experiments were performed in sodium phosphate buffer (pH 6.8). The reaction mixture consisting of 0.1 mL of test compound (10 mM), 0.1 mL of mushroom tyrosinase (1000 unit/mL), and L-DOPA (3.8 mM) was added in this order to read the absorbance at 475 nm for 5 min. The reaction was performed at 25 °C. The value in the absence of the test compound was represented as the control. The percent inhibition of tyrosinase activity was calculated with the following formula:

$$\text{inhibition (\%)} = (1 - (\text{OD}_{475} \text{ in the sample}/\text{OD}_{475} \text{ in the control}))$$

$$m_x d7; 100\%$$

IC_{50} is the concentration of a test compound that inhibits a standard reaction by 50%. The value of IC_{50} is derived from the x axis. It is determined from the alignment of the standard reaction on the dependent y axis. We determined the linear curves and their equations based on the inhibition percentages at five doses for each experiment. Then, we calculated individual IC_{50} values when the y axis showed 50% of the inhibition percentage.

Tyrosinase Activity in B16 Cells. Tyrosinase activity in B16 cells was assessed as previously described (*23*). Cells were plated in 6-well dishes at a density of 10^5 cells/mL. B16 cells were incubated in the presence or absence of organo-sulfur compounds for 72 h, washed with ice-cold phosphate-buffered saline (PBS), and then treated with lysis buffer [PBS containing 1% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS), and 0.02% proteinase inhibitor cocktail, Roche, Germany]. Cellular lysates were centrifuged at 10000*g* at 4 °C for 20 min. The supernatants were collected, and the protein contents were determined using the BCA protein assay kit (Pierce, Rockford, IL). The remaining protein contents for the subsequent determination of intracellular tyrosinase inhibitory activity were stored at −80 °C. The reaction mixture consisting of cell extract supernatant (0.1 mL) and 0.9 mL of L-DOPA (3.8 mM) in 25 mM phosphate buffer (pH 6.8) were added, and the tyrosinase activity was read at 475 nm for 30 min. The reaction was performed at 25 °C.

Determination of *o*-Quinone. The levels of *o*-quinone were determined according to the method by Waite (*24*). The *t*-BC was oxidized by $NaIO_4$, and then 4-*tert*-butyl-*o*-benzoquinone (*t*-BQ) was formed and determined spectrophotometrically by measuring the accumulation of its corresponding *o*-quinone at 400 nm (*24*). The reaction mixture consisting of 0.1 mM $NaIO_4$ and 0.1 mM *t*-BQ in the absence and presence of test compound was added, and the levels of *o*-quinones were immediately measured at optical density (OD) at 400 nm.

Cell Viability Assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed as previously described (*25*). MTT is a tetrazolium salt and is converted to insoluble formazan by mitochondrial dehydrogenase of living cells. Briefly, cells were dispensed into 96-well plates, and organo-sulfur compounds were added and cultured for 24 h. Then, 20 μL of MTT (5 mg/mL stock solution) was added to each well. After 1 h, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by the addition of dimethyl sulfoxide. The OD of each well was measured with an Anthos 2010 microplate reader at 570 nm.

Melanin Assay. The melanin assay was performed using procedures described previously (*26*), with slight modification. Organo-sulfur compounds were added to the cell culture at a final concentration of 500 μM in all experiments and cultured for 72 h. After washing 2 times in PBS, cells were harvested. An aliquot was used for cell counting, and the remaining cells were centrifuged at 10000*g* for 15 min. The cells were collected, and the protein contents were determined using the BCA protein assay kit (Pierce, Rockford, IL). The remaining cells were lysed in 500 μL of 5 N NaOH at 100 °C for 1 h, and 200 μL portions of crude cell extract were transferred to 96-well plates. Melanin concentrations were calculated by a comparison of the OD at 405 nm of unknown samples with a standard curve obtained with synthetic melanin (0−200 μg/mL), run in triplicate, and averaged.

Evaluation of Reactive Oxygen Species (ROS) in B16 Cells. To determine the generation of ROS in B16 cells, DCFH-DA was used when it penetrates the cell membranes and was hydrolyzed by intracellular esterase to form dichlorofluorescin (DCFH) (*27*). Subsequently, DCFH reacted with ROS generated by intracellular stress to produce highly fluorescent DCF, which emitted fluorescence when excited at 485 nm. B16 cells were pretreated with DCFH-DA (50 μM) for 30 min, and then various organo-sulfur compounds were added to the medium and then incubated at 37 °C for 30 h. After incubation, ROS produced from intracellular stress was detected using a Bio-Tek FLx800 microplate fluorescence reader with excitation and emission wavelengths of 485 and 530 nm, respectively.

Evaluation of Glutathione (GSH) and GSSG in B16 Cells. GSH and GSSG levels were determined by the method of Pandey and Katiyar (*28*, *29*), with slight modification. A total of 10% of the trichloroacetic acid was added to cell pellets and centrifuged at 12000*g* for 10 min, and the supernatants were then taken for subsequent determination. For GSH, 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM phosphate buffer were added to the supernatants followed by *o*-phthaladehyde. After 20 min at room temperature, the GSH levels were estimated in fluorescence at 360 nm excitation/460 nm emission. GSSG was estimated after preincubation with *N*-ethylmaleimide for 20 min, and 0.1 M NaOH replaced 1 mM EDTA and 50 mM phosphate buffer.

Determination of Inhibition Type. Taking various concentrations of L-DOPA as the substrate, the tyrosinase inhibitory activity was measured according to the method described above. Inhibitory kinitics of the tested compounds was analyzed by Lineweaver−Burk plots. The kinetic data were plotted as 1/activity ($1/V$) versus 1/substrate concentration ($1/S$), according to the method of Lineweaver−Burk, and the Michaelis−Menten constant (K_m) and maximum velocity (V_{max}) were determined with variable substrate concentrations in the standard reaction mixture (*30*).

Statistical Analysis. All data were presented as means ± standard deviation (SD). Statistical analysis involved use of the Statistical Analysis

System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Effect of Organo-sulfur Compounds on Tyrosinase Activity. To investigate the inhibitory effect of different classes of organo-sulfur compounds in *Allium* species on mushroom tyrosinase activity, PM, DMDS, DADS, PDS, and DMT were selected by the structure as tested compounds in the current study. The effect of the five organo-sulfur compounds on mushroom tyrosinase activity was shown in **Table 1**. The IC_{50} value of PM was 0.5 mM, which was lower than other organo-sulfur compounds and not significantly different from kojic acid (IC_{50} = 0.5 mM). Apparently, the inhibitory effect of PM on mushroom tyrosinase activity was higher than the others; DMDS showed mild inhibitory action on mushroom tyrosinase activity (IC_{50} = 6.5 mM), followed by DADS, PDS, and DMT. The molecular structure of the selected organo-sulfur compounds shows PM with a sulfhydryl group (SH group), a disulfide group present in DMDS, DADS, and PDS, and DMT containing a sulfide bond. Structural analysis of these compounds reveals that the presence of a SH group in compounds tested is crucial and superior to a disulfide group and a sulfide group in compounds tested for the inhibitory effect on mushroom tyrosinase activity. This observation is in agreement with the reports that showed that SH-containing compounds are effective inhibitors of polyphenol oxidase (*7*). In this regard, PM with a SH group demonstrated higher tyrosinase inhibitory activity than other organo-sulfur compounds tested, possibly as a result of increasing hydrophilicity of PM. With regard to the structural characteristics of these compounds, DMDS, PDS, and DADS with disulfides contain dimethyl, diporpyl, and diallyl groups, respectively. DMT is a hetercyclic sulfide. DMDS showed inhibitory activity on mushroom tyrosinase activity, followed by PDS, DADS, and DMT, which possibly resulted from more lipophilicity or steric hindrance (*7*).

Effect of Organo-sulfur Compounds on *o*-Quinone Formation. To obtain evidence of the response of the selected five organo-sulfur compounds to inhibition of *o*-quinone formation, the interaction between organo-sulfur compounds and *tert*-butylquinone was determined. **Table 2** shows the effect of the five organo-sulfur compounds on *o*-quinone formation. The sample at 400 nm showed inhibitory action on *o*-quinone formation, ranging from 25.5 to 32.7%. Obviously, PM showed a higher inhibitory effect on *o*-quinone than others and kojic acid. Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-DOPA and oxidation of L-DOPA to dopaquinone. The *o*-quinones then undergo further oxidation to melanin in a series of enzymatic and non-enzymatic reactions (*31*). In addition, quinones being powerful electrophiles can interact with amino acids, peptides, and proteins to form Michael addition products and therefore lower the digestibility of the protein and the bioavailability of essential amino acids, such as cysteine and lysine (*10*). Thus, any effort that destroys these processes could reduce melanin formation on the skin and an undesirable change in color, off-flavor, off-odor, and nutritive values of food. In the present study, although the selected five organo-sulfur compounds showed weak inhibition of *o*-quinone formation, they virtually affect the intermediates and thereby prevent the further transformation of *o*-quinone to melanin (*32*).

Effects of Organo-sulfur Compounds on Melanin and Tyrosinase Activity in B16 Cells. To obtain further information about the inhibiting potency against melanin formation in the intracellular model, the selected five organo-sulfur compounds were assessed for their cytotoxicity and the inhibitory effect on melanin formation in the cultured B16 cells. The cytotoxicity of selected organo-sulfur compounds in B16 cells was evaluated using the MTT test. Each organo-sulfur compound in the present study did not show any cytotoxic effect at the concentration ranging from 0.1 to 1.0 mM (data not shown). Thus, each sample was used for testing their possible inhibition against melanin formation. The effects of selected five organo-sulfur compounds on melanin formation and tyrosinase activity in B16 cells were summarized in **Table 3**. In comparison, PM (24.15%) showed the greatest inhibitory effect on melanin formation, followed by DMDS (20.77%), DMT (15.61%), DADS (15.61%), and PDS (14.62%), indicating that PM displayed a significant inhibitory effect on melanin

Table 1. Inhibitory Effects of Organo-sulfur Compounds on Mushroom Tyrosinase Activity[a]

compound	inhibitory activity (%)	IC_{50} (mM)
PM	100.0 ± 0.1 a	0.5 ± 0.1 a
DMDS	50.3 ± 0.3 b	6.5 ± 0.0 b
DADS	6.5 ± 0.4 d	ND
PDS	8.4 ± 0.3 c	ND
DMT	3.9 ± 0.4 e	ND
kojic acid	100.0 ± 0.5 a	0.5 ± 0.0 a

[a] The concentration of each sample was 10 mM. The data were displayed with mean ± SD for (n = 3). Mean values with different letters in each column are significantly different ($p < 0.05$). Inhibition (%) = $(1 - B/A) \times 100\%$, where A is the value of absorbance in the control and B is the value of absorbance in the sample. ND, not detectable; PM, 1-propylmercaptan; DMDS, dimethyl disulfide; DADS, diallyl disulfide; PDS, propyl disulfide; DMT, 2,5-dimethylthiophene.

Table 2. Inhibitory Effect of Organo-sulfur Compounds on *o*-Quinone Formation[a]

compound	inhibition (%)
PM	32.7 ± 0.7 a
DMDS	30.8 ± 0.4 b
DADS	27.8 ± 0.6 c
PDS	25.5 ± 0.4 d
DMT	25.5 ± 0.5 d
kojic acid	19.6 ± 0.5 e

[a] The concentration of each sample was 500 μM. Results are expressed as a percentage of the control, and the data were displayed with mean ± SD (n = 3). Mean values with different letters are significantly different ($p < 0.05$). Inhibition (%) = $(1 - B/A) \times 100\%$, where A is the value of absorbance in the control and B is the value of absorbance in the sample. PM, 1-propylmercaptan; DMDS, dimethyl disulfide; DADS, diallyl disulfide; PDS, propyl disulfide; DMT, 2,5-dimethylthiophene.

Table 3. Inhibitory Effects of Organo-sulfur Compounds on Tyrosinase Activity and Melanin Formation in B16 Cells[a]

	inhibition (%)	
compound	melanin formation	tyrosinase activity
PM	24.15 ± 0.19 b	46.89 ± 1.70 a
DMDS	20.77 ± 0.24 c	40.57 ± 1.86 b
DADS	15.61 ± 0.19 d	24.35 ± 1.67 d
PDS	14.62 ± 0.34 d	24.79 ± 2.87 d
DMT	15.61 ± 0.11 d	35.77 ± 1.92 c
kojic acid	28.32 ± 0.20 a	47.16 ± 1.45 a

[a] The concentration of each sample was 500 μM. The cells were cultured and incubated for 72 h in the presence of organo-sulfur compounds and then assayed for tyrosinase activity and melanin formation, as detailed in the Materials and Methods. Results are expressed as a percentage of the control, and the data were displayed with mean ± SD (n = 3). Mean values with different letters in each column are statistically significant ($p < 0.05$), Inhibition (%) = $(1 - B/A) \times 100\%$, where A is the value of absorbance in the control and B is the value of absorbance in the sample. PM, 1-propylmercaptan; DMDS, dimethyl disulfide; DADS, diallyl disulfide; PDS, propyl disulfide; DMT, 2,5-dimethylthiophene.

formation. Melanin formation, being the most crucial factor of mammalian skin color, is synthesized in a multistep biochemical pathway that operates within a specialized intracellular organelle. After multibiosynthesis steps, further polymerization yields the melanosome (*30*). As seen in **Table 3**, selected five organo-sulfur compounds showed inhibitory action on melanin formation in B16 cells. Of the organo-sulfur compounds tested in the present study, PM showed the greatest effective inhibitory action on melanin formation. As for the cellular tyrosinase model system, PM displayed the greatest tyrosinase inhibitory activity (46.89%), with no significant difference to kojic acid (47.16%), followed in turn by DMDS (40.57%), DMT (35.77%), PDS (24.79%), and DADS (24.35%) (**Table 3**). Apparently, PM showed a higher tyrosinase inhibitory activity than the others in B16 cells. As shown in **Table 3**, more interestingly, the inhibition of melanin formation by selected organo-sulfur compounds correlated with tyrosinase inhibitory activity; in other words, the inhibition of melanin formation is well-related to tyrosinase inhibitory activity. Furthermore, PM showed the higher tyrosinase inhibitory activity on mushroom tyrosinase system (**Table 1**) as well as in the B16 cellular system (**Table 3**). Apparently, of the five organo-sulfur compounds, PM showed marked inhibitory action on tyrosinase activity. The data obtained from **Table 1** showed that PM displayed stronger inhibitory action than DMDS on tyrosinase in the mushroom tyrosinase model. However, the difference between PM and DMDS seems to be unobvious in the B16 cells model (**Table 3**). Apparently, the inhibitory effects of organo-sulfur compounds on tyrosinase varied with different model systems tested. Further, Friedman and Bautista (*32*) noted that possible inhibitory tyrosinase mechanisms are divided into three types: (1) Thio compounds have a strong affinity for copper; subsequently, they displace histidine residues linked to the copper or active site of tyrosinase and completely remove the copper from the enzyme. (2) Oxidation−reduction reactions involve thiol-mediated reduction of *o*-quinone intermediates back to hydroxyphenols. (3) Nucleophilic addition of the thiolate anions to *o*-quinone intermediates prevents the formation of melanin formation. Thus, according to the data obtained from **Tables 1**−**3**, we hypothesized that the tyrosinase inhibitory activity and the inhibition of melanin formation by PM may be mainly attributed to a SH group present in PM.

Effect of Organo-sulfur Compounds on ROS in B16 Cells. Under physiological metabolism, the excessive level of ROS could decrease the intracellular antioxidant capacity and then produce oxidative stress. In addition, ROS may induce and enhance the development of melanin formation. Therefore, removing ROS may contribute to the inhibition of melanin formation. Thus, in the present investigation, ROS determination was measured using fluorescent probe DCFH-DA with a fluorospectrophotometer. When the five organo-sulfur compounds were added to B16 cells, the fluorescence intensity decreased in comparison to the control, indicating that the generation of intracellular ROS in B16 cells treated with organo-sulfur compounds occurred. As expected, the treatment of B16 cells with organo-sulfur compounds significantly inhibited ROS generation (**Table 4**). The inhibition of ROS generation was in order of PM > DMDS > DADS ≈ PDS > DMT. Among the samples, PM displayed the greatest inhibition on ROS generation. In contrast, DMT showed nearly no inhibitory action on ROS formation with no significant difference ($p > 0.05$) to kojic acid. With regard to the structure of selected organo-sulfur compounds, we hypothesized that PM with a SH group was responsible for its higher inhibitory action on ROS formation because of marked reducing properties (*18*). Kovacic (*34*) noted that ROS are commonly formed by electron functionalities, such as quinone, metal complexes, and aromatic nitro compounds, that may undergo cyclicing with oxygen to form ROS. These ROS will cause damage to DNA, protein, and membranes. Several lines of evidence showed that ROS are believed to be involved in photodamage to dermal connective tissue cells and protein (*35*). On the other hand, 5,6-dihydroxy-indole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which are melanin precursors in melanogenesis, can be converted into the corresponding melanin pigments by the action of the lipoxygenase/H_2O_2 system or because of the generation of ROS. In addition, oxidative stress and UV light caused by excessive ROS are associated with skin disorders (*36*). ROS are considered to play significant roles in the regulation of melanocyte proliferation and melanogenesis, while ROS scavengers and an inhibitor of ROS generation may downregulate hyperpigmentation (*37*). In other words, ROS generation may enhance the melanin formation (*38*). Thus, the suppression of ROS by the selected five organo-sulfur compounds tested in the present study is, at least in part, a contributor to inhibition of melanin formation.

Table 4. Inhibitory Effects of Organo-sulfur Compounds on ROS Formation in B16 Cells[a]

compound	inhibition (%)
PM	25.8 ± 4.4 a
DMDS	23.1 ± 2.7 b
DADS	15.0 ± 1.2 c
PDS	12.9 ± 0.9 c
DMT	6.5 ± 2.0 d
kojic acid	3.9 ± 3.6 d

[a] The concentration of each sample was 500 μM. The data were displayed with mean ± SD (n = 3). Mean values with different letters are significantly different ($p < 0.05$). Inhibition (%) = (1 − B/A) × 100%, where A is the value of fluorescence without the sample and B is the value of fluorescence with the sample. PM, 1-propylmercaptan; DMDS, dimethyl disulfide; DADS, diallyl disulfide; PDS, propyl disulfide; DMT, 2,5-dimethylthiophene.

Table 5. Effects of Organo-sulfur Compounds on Levels of GSH and GSSG and the Ratio of GSH/GSSG in B16 Cells[a]

compound	GSH (%)	GSSG (%)	GSH/GSSG
PM	134.7 ± 9.7 a	13.9 ± 4.2 c	9.92 ± 2.11 a
DMDS	112.2 ± 9.0 b,c,d	21.4 ± 7.6 c	5.44 ± 1.17 b
DADS	129.8 ± 6.2 a,b	73.7 ± 8.1 a,b	1.94 ± 0.60 c
PDS	94.8 ± 11.0 d	65.1 ± 10.5 b	1.60 ± 0.68 c
DMT	117.3 ± 9.7 a,b,c	27.4 ± 2.9 c	4.23 ± 0.46 b
kojic acid	99.3 ± 9.9 d	93.1 ± 13.6 a,b	1.06 ± 0.13 c

[a] The concentration of each sample was 500 μM. The cells were cultured and incubated for 30 h in the presence of organo-sulfur compounds and then assayed for GSH and GSSG levels, as detailed in the Materials and Methods. Results are expressed as a percentage of the control, and the data were displayed with mean ± SD (n = 3). Mean values with different letters in each column are significantly different ($p < 0.05$). Inhibition (%) = (1 − B/A) × 100%, where A is the value of fluorescence without the sample and B is the value of fluorescence with the sample. PM, 1-propylmercaptan; DMDS, dimethyl disulfide; DADS, diallyl disulfide; PDS, propyl disulfide; DMT, 2,5-dimethylthiophene.

Effects of Organo-sulfur Compounds on GSH and GSSG in B16 Cells. To elucidate the mechanism of reducing ROS formation, we therefore determined the level of GSH and the ratio of GSH/GSSG in B16 cells. **Table 5** shows the effects of each organo-sulfur compound on the content of GSH and the GSH/GSSG ratio in B16 cells. Pretreatment with tested organo-sulfur compounds positively modulated GSH levels and the ratio of the GSH/GSSG compared to the control. The positive modulation in this cellular model system was in order of PM > DMDS ≈ DMT > DADS ≈ PDS. Among the organo-sulfur

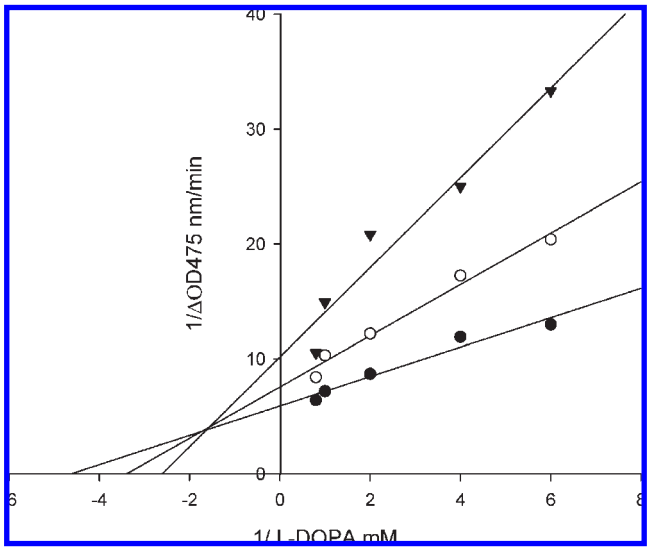

Figure 2. Lineweaver−Burk plots for the inhibition of PM on tyrosinase using L-DOPA as a substrate, in the presence of 0.4 mM PM (▼) and 0.15 mM PM (○) and in the absence of PM as a control (●).

compounds, the modulation of PM, DMDS, and DMT on the GSH/GSSG ratio is higher than that of kojic acid. The physiological role of reduced GSH is an essential reducing agent for maintaining thiol groups on intracellular protein and antioxidant molecules (*39*). Ye et al. noted that cellular GSH represents the nonprotein sulfhydryl reserve of cells, which is responsible in reducing cytotoxicity induced by the active metabolites of toxicants (*40*). In addition, GSH plays a fundamental role in intracellular defense against reactive free radical and other oxidant species as well as inhibiting melanogenesis (*39*, *41*). According to the data in **Table 5**, tested organo-sulfur compounds positively modulated the ratio of GSH/GSSG in B16 cells. Consequently, this action may be associated with reducing free radical and other oxidant species and thereby inhibiting the ROS formation, leading to the inhibition of melanin formation.

Effect of PM on the Inhibition Type of Tyrosinase. On the basis of data obtained, PM tented to show marked inhibitory action on melanin formation in various model systems. Therefore, the inhibitory kinetics of PM on tyrosinase was analyzed by a Lineweaver−Burk plot, as shown in **Figure 2**. The lines are obtained from the uninhibited enzyme and from the three different concentrations of PM. As shown in **Figure 2**, changes of both K_m and V_{max} by various concentrations of PM in the Lineweaver−Burk plot were found. The double-reciprocal plot yields a family line with different slopes and different intercepts, and they intersect one another in the first quadrant. The kinetic behavior indicates that PM worked as mixed-type inhibitors of tyrosinase activity. In other words, the result of the kinetic study suggested that PM reduced the affinity of the substrate for the enzyme, whereas it did not bind to the active site (*6*).

In conclusion, the data suggest that the selected five thio compounds seem to be effective in the inhibition of melanin formation. Of the five organo-sulfur compounds, PM demonstrated the highest inhibition on melanin formation. The mechanism of inhibitory action on melanin formation may be due to the interaction of organo-sulfur compounds with tyrosinase, to reduce the ROS generations as a result of reducing the ability as well as a positive modulation of the ratio of GSH/GSSG. Thus, these selected five organo-sulfur compounds, PM especially, may be the potent candidates for cosmetic application. Further investigations of this matter are needed.

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