Mansonone E from *Mansonia gagei* Inhibited α-MSH-Induced Melanogenesis in B16 Cells by Inhibiting CREB Expression and Phosphorylation in the PI3K/Akt Pathway

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Many natural products that inhibit melanogenesis, freckles, and hyperpigmentation have been selectively used in cosmetics because melanogenesis is linked to the multiple biogenesis cascades of melanin synthesis. However, some of these compounds have side effects that may result in their restriction in the future. We report here the isolation and structural elucidation of compounds extracted from *Mansonia gagei* and evaluate their activity on melanogenesis inhibition. We isolated five known compounds from *M. gagei* and identified them as mansonone E (1), mansorin I (2), populene F (3), mansonone G (4), and mansorin B (5). After evaluating the five compounds for cytotoxicity against B16 cells and inhibitory activity on α-melanocyste-stimulating hormone (α-MSH) induced melanogenesis, we determined that the cytotoxicity and melanogenesis-inhibitory effect of 1 were relatively low and high, respectively. Next, the effect of 1 on the expression of melanogenesis-related proteins was assessed; it was confirmed that 1 dose-dependently inhibited the expression levels of tyrosinase, tyrosinase-related protein 1 (TRP-1), TRP-2, cAMP response element binding protein (CREB), and microphthalmia-associated transcription factor (MITF) which were increased after stimulation by α-MSH. Furthermore, the effects of 1 on the phosphorylation levels of intracellular signaling pathway-related proteins were evaluated, and it was found that 1 dose-dependently rescued the phosphorylation of Akt and p38 mitogen-activated protein kinases (MAPK), which were up- or down-regulated after stimulation by α-MSH. In contrast, treatment with the phosphoinositide 3-kinase (PI3K)/Akt inhibitor wortmannin enhanced melanogenesis inhibition by mansonone E. Cumulatively, the data suggest that 1 suppresses α-MSH-induced melanogenesis in B16 cells by inhibiting both phosphorylation in the PI3K/Akt pathway and the expression of melanogenesis-related proteins.

**Key words** *Mansonia gagei*; melanogenesis; B16 cell; mansonone E; cAMP response element binding protein (CREB); Akt

Recent studies showed that cosmetics with whitening effects occupy a large fraction of the market, particularly in Asia. Therefore, many efforts have been devoted to elucidate novel and effective whitening compounds. As a result of studies on melanin biosynthesis, kojic acid, 2) arbutin, 3) and many other natural products were discovered to inhibit melanogenesis, 4) freckles, and hyperpigmentation. However, the side effects of these compounds (e.g., the induction of a skin allergy by kojic acid 5) and mutagenicity of arbutin 6) have recently been reported, and the use of these chemical substances may be restricted in the future. Furthermore, it is also necessary to improve the stability and cost of such compounds. Therefore, it is necessary to develop new, safer, and more effective whitening materials.

Melanin is a dark pigment, mainly controlled by the expression and activity of tyrosinase, which determines the color of human hair, skin, and eyes. 7) This bio-compound is synthesized in melanosomes and migrates to the keratinocytes. 8) Thus, tyrosinase is known to be the most important enzyme involved in melanogenesis. 9) Tyrosinase also regulates the formation of L-3,4-dihydroxyphenylalanine (L-DOPA) by the hydrolysis of tyrosine, as well as the formation of L-DOPA by oxidation of DOPA. 10) Additionally, microphthalmia-associated transcription factor (MITF) is known to regulate the expression of tyrosinase and melanogenesis-related enzymes such as tyrosinase-related protein 1 (TRP1) and TRP2. 11)

Recently, it was reported that melanogenesis is controlled by various intracellular signaling molecules such as cAMP/protein kinase A (PKA), phosphoinositide 3-kinase (PI3K)/Akt, and ras/mitogen-activated protein kinases (MAPK). 12) cAMP/PKA regulates the expression level of MITF, 13) and ras/MAPK enhances transcriptional activity by promoting phosphorylation of MITF. 14) Experiments using inhibitors suggested that the PI3K/Akt pathway is also involved in melanogenesis. 15)

It has been shown that *Mansonia gagei* DRUMM, a traditional medicinal plant of Myanmar, can be used as a cardiac stimulant, antiemetic, antidepressant, and tonic reagent. 16) However, to the best of our knowledge, no investigation has been made into melanogenesis inhibitory compounds from *M. gagei*. Therefore, we report in this study the isolation and structural elucidation of constituents from *M. gagei* and evaluation of their activity on melanogenesis inhibition.

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MATERIALS AND METHODS

Reagents 3-(4,5-Dimethylthiazol-2-yi)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Hexane, ethyl acetate, methanol, butanol, and wortmannin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). p38 MAPK inhibitor was purchased from Calbiochem (San Diego, CA, U.S.A.).

Solvent Fractionation Raw barks of M. gagei were gifted by Pathein University in Myanmar and identified by Prof. Nyunt Phay. The voucher specimen has been deposited in College of Science and Technology, Nihon University. Powder of M. gagei barks was immersed in methanol for 24 h at room temperature. The solvent containing the extracts was filtrated through a filter paper (No. 2; Advantec, Tokyo, Japan) and the filtrate was evaporated to dryness to prepare the crude methanol extract. The crude methanol extract was fractionated by ethyl acetate and water. Then ethyl acetate- or water-soluble phase were re-fractionated by hexane and methanol/water (95/5) or n-butanol and water, respectively to prepare hexane, methanol/water, n-butanol, and water fraction.

Refining of Active Components Among four fractions, hexane and methanol/water (95/5) fraction showed relatively high melanogenesis inhibition. The hexane fraction was concentrated and then separated by silica gel column chromatography eluted with hexane/ethyl acetate and fractions were applied on octadecyl-silica (ODS) chromatography eluted with methanol/water and then purified by HPLC with silica gel column eluted with chloroform/methanol. The methanol/water (95/5) fraction was concentrated and then separated by Diaion HP-20 (Mitsubishi Chemical Co., Ltd., Tokyo, Japan) column chromatography eluted with methanol/water and fractions were applied on silica gel column chromatography eluted with hexane/ethyl acetate and then purified by HPLC with ODS eluted with methanol/water.

Analytical Instrument of Active Component 1H (400MHz) and 13C (100MHz) NMR spectra were recorded with a JEOL ECX 400 spectrometer with tetramethylsilane as an internal standard. The high-resolution MS were obtained using a LC/MSD TOF-G1969A (Agilent Technologies, Santa Clara, CA, U.S.A.).

Cell Culture B16 murine melanoma cells, purchased from Riken Cell Bank (Tsukuba, Japan) were cultured as described previously. 21 In brief, cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, U.S.A.), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified CO2 incubator.21

Measurement of Cytotoxicity The B16 cells were seeded at 5×103 cells/well onto 24-well plates (Corning Life Sciences, Lowell, MA, U.S.A.) for 1 d at 37°C in an atmosphere of 95% air/5% CO2. Cell viability was determined by the MTT reduction assay.22) The cells were incubated with MTT (59 kDa), CREB (43 kDa), and MTIF (52 kDa) (Santa Cruz Bio-
technology, Santa Cruz, CA), phospha- and total-Akt (60kDa), ERK1/2 (42/44kDa), p38MAPK (43kDa), JNK (46/54kDa) (Cell Signaling Technology, Lake Placid, NY, U.S.A.) and GAPDH (37kDa) (Signaway Antibody, College Park, ME, U.S.A.) as the primary antibodies, followed by reaction with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies from Sigma-Aldrich (St. Louis, MO, U.S.A.) as the secondary antibody. Primary and secondary antibodies were diluted 1000 or 3000 times for use, respectively. The blots were developed by the enhanced chemiluminescence method (Western Lightning ECL Pro, PerkinElmer, Inc., Waltham, MA, U.S.A.).

**Isolation of Active Constituents by Chromatography**

The hexane extract (10.0 g) was refined by silica gel column chromatography and eluted with hexane/ethanol acetate (1/0 to 0/1; v/v) in 24 fractions (Fr. 1 to Fr. 24). Among these 24 fractions, melanogenesis was inhibited by Fr. 13–15. Therefore, Fr. 13–15 (1571 mg) was subjected to ODS column chromatography and eluted with water/methanol (1/0 to 0/1; v/v) in 10 fractions (Fr. A to Fr. J). Among these 10 fractions, Fr. C most strongly inhibited melanogenesis. Thus, Fr. C (160 mg) was separated by silica gel column chromatography and eluted with chloroform/acetone (1/0 to 0/1; v/v) in 7 fractions (Fr. CA to Fr. CG). By HPLC-ODS using methanol/water (1/1, v/v), compound (1) mansorin E (3.2 mg) was extracted from Fr. CC (16.1 mg) and, compound (2) (3.3 mg) mansorin I, (3) (3.2 mg) populene F, and (4) (5.2 mg) mansorone G were isolated from Fr. CE (27.2 mg).

The methanol/water (95/5) (30 g) extract was separated by Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan) column chromatography and eluted with methanol/water (4/6 to 0/1; v/v) in 8 fractions (Fr. 1 to Fr. 8). Among the eight fractions, melanogenesis was inhibited by Fr. 5. Therefore, Fr. 5 (5.0 g) was subjected to silica gel column chromatography and eluted with hexane/ethanol acetate (1/0 to 0/1; v/v) in 28 fractions (Fr. 5–1 to Fr. 5–28). Among the 28 fractions, Fr. 5–8 most strongly inhibited melanogenesis. Thus, Fr. 5–8 (132 mg) was purified by HPLC-ODS and eluted with methanol/water (7/3, v/v) to afford compound (5) mansorin B (7.1 mg).

**Characterization of the Isolated Compounds**

All the compounds were identified by comparison of their spectral data, shown in supplementary material, with the literature. Mansorine E (1), mansorin I (2), and mansorin B (5) were previously isolated from *M. gagei*.[17,20] Compounds 3 and 4 were also isolated from Thespesia populnea and were determined to be Populene F (3) and mansorone G (4).[20] (Fig. 1).

The molecular conformation of mansorin I and populene F was specified in the previous studies.[17,26] Since the data of MS and NMR measured in the present study were almost identical to them, the molecular conformations of mansorin I and populene F could be determined as shown in Fig. 1.

**Cytotoxicity and Melanogenesis Inhibition Effects of Five Compounds Isolated from *M. gagei***

The cytotoxicity and melanogenesis inhibition of the five compounds isolated from *M. gagei* are shown in Table 2. The potency of mela-

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### Table 1. The Cytotoxicity and Melanogenesis Inhibitory Activities of Four Extracts from *M. gagei* in B16 Cells

| Treatment | Melanin content (Cell survivability) (%) | IC50 (g/mL) |
|-----------|-----------------------------------------|-------------|
|          | 10 µg/mL | 30 µg/mL | 100 µg/mL | µg/mL |
| Hexane    | 80.8±2.8* | 41.0±4.4* | 11.6±1.9* | 26 |
| Methanol/H2O | 73.3±0.8* | 43.1±4.6* | 8.2±3.2* | 26 |
| Butanol   | 89.7±3.4  | 94.7±2.3  | 22.7±1.5* | 73 |
| H2O       | 105.5±3.6 | 118.9±4.1 | 86.3±43.5 | >100 |
| Arbutin   | 98.3±0.5  | 88.1±0.5  | 58.8±0.9* | >100 |

Values in parentheses indicate cell viability (%) in MTT assay. Asterisks indicate significant differences from the control at p<0.05 (n=3).

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Fig. 1. Isolated Compounds from *M. gagei*
nogenesis inhibition decreased in the order of mansonone G > mansorin B > mansorin I > mansonone E > populene F, and the activity of all five compounds was stronger than that of arbutin, which was used as a reference compound. In addition, the level of cytotoxicity against B16 cells decreased in the order of mansonone G > mansorin I / uni2267 mansorin B > populene F > mansonone E. Thus, cytotoxicity mostly correlated with melanogenesis inhibition.

It was deduced that the influence of the presence or absence of the ether binding site between C-4 and C-13 on biological activities (cytotoxicity and melanogenesis inhibition) was small since the biological activities of mansorin I and mansorin B are similar. The difference in chemical structure between mansonone G, which has the highest biological activities and mansonone E which is almost weakest, is presence or absence of a hydroxy group bonded to C-6. The biological activities of mansorin G, mansorin I, and mansorin B which has hydroxy group at C-6, were rather strong among the compounds isolated in the present study. Thus, it can be presumed that presence or absence of a hydroxy group at C-6 influences cytotoxicity and melanogenesis inhibition. On the other hand, in order to obtain a compound with low cytotoxicity for cosmetic raw materials, it is necessary to search for a compound having no hydroxy group at C-6.

### Effects of Mansonone E on Melanogenesis-Related Proteins in B16 Cells

One of the aims of this study is to develop new cosmetic materials. Mansonone E did not show significant cytotoxicity at less than 100 µM, and melanogenesis inhibitory effects of it were significantly higher than arbutin, a practically used melanogenesis inhibitor. We deduced that

| Treatment          | Melanin content (Cell survivability) (%) | IC50 |
|--------------------|-----------------------------------------|------|
|                    | 5 µM         | 10 µM      | 30 µM      | 100 µM     | µM  |
| Mansonone G        | 56.6±4.7*   | 29.6±4.1*  | 9.5±0.7*   | 37.2±7.0*  | 7   |
|                    | (106.2±4.4) | (73.6±1.4)*| (44.1±2.5)*| (4.3±1.7)* | (26) |
| Mansorine I        | 73.7±0.9*   | 65.2±1.4*  | 32.9±8.9*  | 24.3±1.6*  | 48  |
|                    | (106.3±10.1)| (104.1±5.7)| (104.8±6.1)| (3.7±0.2)* | (56) |
| Populene F         | 80.8±2.8*   | 71.3±6.0*  | 31.9±8.9*  | 68         |
|                    | (104.1±5.7) | (104.8±6.1)| (3.7±0.2)* | (68)       |
| Mansorin B         | 74.6±4.3    | 74.6±4.9*  | 42.1±4.5*  | 83         |
|                    | (104.1±5.6) | (90.0±3.7) | (69.6±8.2)*| (>100)     |
| Arbutin            | 90.8±8.7    | 87.0±6.3   | 76.7±7.2*  | 100        |
|                    | (108.9±2.1) | (107.3±6.1)| (107.6±5.8)| (>100)     |

Values in parentheses indicate cell viability (%) in MTT assay. Asterisks indicate significant differences from the control at p>0.05 (n=3).

Fig. 2. Effects of Mansonone E on the Level of Melanogenesis Related Protein in B16 Cells

B16 cells were cultured for 2 d. They were treated or not treated with α-MSH and/or mansonone E. After culturing for 48 h, the level of each protein were evaluated by immunoblotting. The intensity of each band of protein was measured densitometrically by Image J. Values were expressed as the ratio of each protein to GAPDH, and the mean±S.E.M. of the value of the three separate experiments are shown. Significant difference of the value from the value of the corresponding control group were determined by ANOVA, followed by Tukey test. The same letters indicate that there are no differences between those groups, and different letters indicate significant differences (p<0.05).
the mechanism of melanogenesis inhibitory effects could be clarified without interference of cytotoxicity if a nontoxic compound was treated. Therefore, we decided to clarify the mechanism of melanogenesis inhibition using mansonone E, which has low cytotoxicity and shows significantly higher melanogenesis inhibition than arbutin.

The expression levels of proteins related to melanogenesis 24 or 48 h after the addition of mansonone E and a-MSH are shown in Fig. 2. First, 24 h after cell stimulation by only a-MSH, the expression levels of the five proteins shown in Fig. 2 were significantly increased. After 48 h, the expression levels of tyrosinase and MITF increased sequentially, the expression level of CREB decreased, and the expression levels of the remaining two proteins were equivalent to those after 24 h. Addition of mansonone E significantly suppressed the increase in protein expression caused by a-MSH stimulation. Therefore, it was presumed that mansonone E suppresses melanogenesis by reducing the expression levels of all five melanogenesis-related proteins.

**Effects of Mansonone E on the Phosphorylation Levels of Intracellular Signal Transduction-Related Proteins**

Melanogenesis-related proteins are known to be regulated by intracellular signaling proteins such as Akt and MAPKs, which are activated by phosphorylation. Therefore, phosphorylation levels of intracellular signaling-related proteins were measured 30 min after addition of a-MSH and mansonone E.

As shown in Fig. 3, phosphorylation of p38 MAPK significantly decreased 30 min after the addition of a-MSH. In addition, phosphorylation of Akt increased after the addition of a-MSH. On the other hand, mansonone E dose-dependently inhibited the effects of a-MSH on the phosphorylation of p38 MAPK and Akt.

**Effects of Specific Inhibitors on Melanogenesis in B16 Cells**

The effects of a PI3K inhibitor (wortmannin) and p38 MAPK inhibitor on a-MSH-induced melanogenesis inhibition by mansonone E were measured in B16 cells. Melanin production with mansonone E (IC₅₀: 55 μM) either in the absence of inhibitor, with wortmannin, or with the p38 MAPK inhibitor was 48.3±3.2, 40.3±5.9 or 47.7±6.9%, respectively. When compared to the values in Table 2, the melanogenesis-inhibitory effect of mansonone E was improved after treatment with the PI3K/Akt inhibitor.

**DISCUSSION**

Although all the compounds in this study have been previously isolated and identified, it has not yet been reported that *M. gagei* contains populene F. In addition, there has been no report on melanogenesis inhibition by extracts or compounds from *M. gagei*. Since three kinds of solvent extracts, excluding the water extract, from *M. gagei* inhibited melanogenesis, *M. gagei* must contain melanogenesis inhibitors of various polarities. All of the 5 compounds isolated and identified in this study [mansonone E (1), mansorin I (2), populene F (3), mansonone G (4), and mansorin B (5)] inhibited melanogenesis. However, since all compounds showed relatively high cytotoxicity, we focused attention on compound 1, which had low cytotoxicity and a relatively strong inhibitory effect on melanogenesis. 1 has been reported as an anti-cancer or anti-obesity agent. However, there has been no report on melanogenesis inhibition of 1.

Tyrosinase, TRP-1, and TRP-2 are closely involved in the mechanism of melanogenesis inhibitory effects could be clarified without interference of cytotoxicity if a nontoxic compound was treated. Therefore, we decided to clarify the mechanism of melanogenesis inhibition using mansonone E, which has low cytotoxicity and shows significantly higher melanogenesis inhibition than arbutin.

**Effects of Specific Inhibitors on Melanogenesis in B16 Cells**

B16 cells were cultured for 2 d. They were treated or not treated with a-MSH and/or mansonone E. After culturing for 30 min, the phosphorylation level of each protein was measured densitometrically by Image J. Values were expressed as the ratio of each phosphorylated- to total-protein, and the mean±S.E.M. of the value of the three separate experiments are shown. Significant difference of the value from the value of the corresponding control group were determined by ANOVA, followed by Tukey test. The same letters indicate that there are no differences between those groups, and different letters indicate significant differences (p<0.05).
melanin biosynthesis. The mechanisms underlying the effectiveness of skin whitening agents are the suppression of melanogenesis by lowering tyrosinase activity. The expression of tyrosinase, TRP-1 and TRP-2 genes is up-regulated by micro-ocular tissue-associated transcription factor (MITF). Additionally, CREB is a transcription factor that promotes MITF expression. MITF plays an important role in inducing hyperpigmentation by inhibiting ERK and PI3K/Akt signaling. Furthermore, it is also reported that melanin production is regulated by the signaling protein p38 MAPK.

In the present study, 1 dose dependently inhibited the expression levels of MITF, CREB, tyrosinase, TRP-1, and TRP-2, which were increased after stimulation with α-MSH in B16 cells. Because the expression of CREB decreased after the addition of 1 when compared to before the addition of α-MSH (Fig. 2), 1 down-regulated the expression of MITF by suppressing the expression of CREB, followed by suppressing tyrosinase, TRP-1, and TRP-2 genes. Stimulation by α-MSH tended to promote the phosphorylation of Akt while significantly inhibiting the phosphorylation of p38MAPK. By stimulation of B16 cells by 1 with α-MSH, both the up-regulation of phosphorylation of Akt and the down-regulation of phosphorylation of p38MAPK were dose-dependently recovered. Therefore, 1 may suppress melanogenesis via down- or up-regulation of phosphorylation of Akt and/or p38 MAPK.

Furthermore, in the present experimental conditions in which B16 cells were treated with PI3K/Akt inhibitor (wortmannin) or p38 MAPK inhibitor followed by α-MSH and 1 treatment, wortmannin and 1 synergistically inhibited melanogenesis, while melanogenesis was not affected by the p38 MAPK inhibitor. Therefore, in addition to suppressing the expression of CREB, 1 may suppress α-MSH-induced melanogenesis in B16 cells by down-regulation of phosphorylation in the PI3K/Akt pathway.

In conclusion, melanogenesis inhibition by compound 1 was significantly strong when compared to arbutin (actually used as a cosmetic material), which was used as a reference compound, (Table 2) and cytotoxicity was low. Therefore, further examination, including animal studies with 1, could bring us a step closer to commercialization of 1 for cosmetics and drugs.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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