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# Synthesis of Small Molecule-Peptide Conjugates as Potential Whitening Agents

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Small molecule conjugated peptides were prepared by solid-phase synthesis as potential novel whitening agents, and their melanogenesis inhibitory activities were investigated. The conjugated small molecules were well-known materials as tyrosinase inhibitors, and peptides were selected from the sequences that are known to antagonize melanocortin receptor 1 (MC1R). Most of small molecules-peptide conjugates showed superior melanin inhibition activity to kojic acid and arbutin. Among these, almost all compounds have -AR- sequence. From this study, we concluded that the small molecule conjugated peptides containing -AR- sequence have melanogenesis inhibitory activities and have potential to be used as novel whitening agents.

**Key Words :** Peptide, ASP, Tyrosinase, MC1R, Whitening agent

## Introduction

Hyperpigmentation which usually presents as age spots, uneven color, freckles and sometimes melasma has become an object of public concern.[1] Hyperpigmentation was resulted from over-production of melanin by melanocytes within the skin, and this process was called melanogenesis.

Alpha-melanocyte stimulating hormone (α-MSH) is the major melanocortin for skin pigmentation (Figure 1). On binding to the melanocortin receptor 1 (MC1R), α-MSH activates adenylate cyclase, which, in turn, causes an increase in intracellular cyclic adenosine monophosphate (cAMP).[2] cAMP activates protein kinase A (PKA), and PKA phosphorylates cAMP responsive element binding protein (CREB). Activated CREB protein binds to the cAMP responsive element (CRE) domain present in the Microphthalmia promoter, thereby upregulating its transcription. Microphthalmia transcription factor (MITF) protein increases the expression of tyrosinase which converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPA to DOPAquinone.[3]

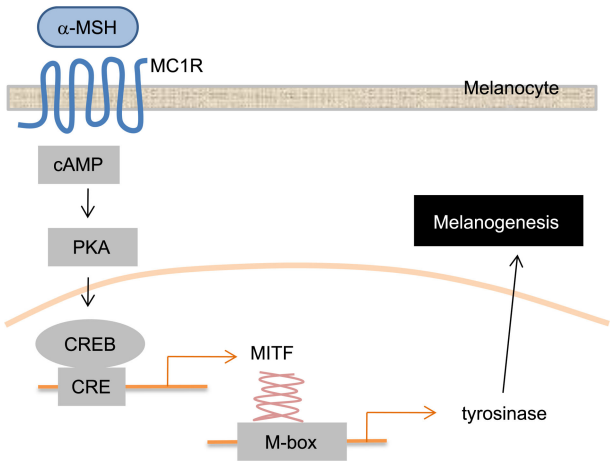



**Figure 1.** Schematic representation of the signaling pathway of α-MSH on human melanocytes.

Tyrosinase is key enzyme in melanogenesis. Therefore, tyrosinase inhibitors such as arbutin and kojic acid were usually used as skin whitening agents. However, the inhibitory activity of presently used tyrosinase inhibitors is not sufficiently potent for it to be used. So, various mechanisms or approaches for skin whitening agents are studying such as inhibition of melanosome transfers, acceleration of epidermal turnover and desquamation, antioxidants, and so on.

In this study, we prepared small molecule-peptide conjugates as potential novel whitening agents, to improve melanogenesis inhibitory activity. Peptides generally have biocompatibility and high activity, but also have very low stability. Therefore, C-terminal amidation or N-terminal capping using small organic material was used to prevent peptide degradation[4]. We designed small molecule conjugated peptide, and small molecule was used for capping N-terminal of the peptide.

Small molecules were selected from natural compound which could inhibit tyrosinase activity, and peptides were selected from the sequences that are known to antagonize melanocortin receptor 1 (MC1R). By conjugating tyrosinase inhibitory molecule and MC1R antagonist peptide, we could expect a synergy effect. Small molecules-peptide conjugates were prepared effectively by the solid-phase method and more than 95% of purities are also obtained.

Melanocortin receptor 1 (MC1R) is a G-protein-coupled receptor (GPCR) with seven transmembrane domains. MC1R is the high affinity receptor for α-MSH that is expressed specifically on melanocytes.[5] It is well known that α-MSH stimulates *de novo* melanin synthesis in mammalian melanocytes, and induces eumelanin synthesis. To investigate the most effective sequence for MC1R antagonist, melanogenesis is induced by α-MSH in melanocytes. Also, we used B16F1 murine melanoma cells for *in vitro* assay because these cells naturally express MC1R.[6]

Agouti signaling protein (ASP) is known to block the binding of α-MSH to the MC1R and inhibit the effects of α-MSH on melanocytes. It acts as an inverse agonist.[7] Human

ASP consists of 132 amino acids and is 80% identical in amino acid sequence to its mouse homolog.[8] Among these amino acid sequences, five-residue motif (KVARP) is known as bioactive region.[9] Various types of 2 to 4 amino acid sequences from five-residue motif (KVARP) are used for small molecules-peptide conjugates synthesis, and their melanogenesis inhibitory activities were investigated.

## Experimental

**Materials.** Fmoc-amino acid was purchased from Bead-Tech (Seoul, Korea). Benzotriazole-1-yl-oxy-tris-(dimethyl-amino)-phosphonium hexafluorophosphate (BOP reagent), hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), caffeic acid, and *p*-coumaric acid were purchased from TCI (Tokyo, Japan). Rink amide resin and 2-chlorotrityl (CTC) resin were obtained from GLS (shanghai, China). Mushroom tyrosinase (EC 1.14.18.1), L-tyrosine, α-melanocyte stimulating hormone (α-MSH), synthetic melanin, and albumin from bovine serum (BSA) were purchased from Sigma (St. Louis, MO, USA). Protein assay reagent was obtained from Cytoskeleton (Denver, USA).

**Amide Type Peptide Conjugates Synthesis.** Fmoc-amino acid (1.5 equiv.), BOP (1.5 equiv.), HOBt (1.5 equiv.) and DIPEA (1.5 equiv.) were added to a swollen Rink amide resin (0.5 mmol of $NH_2$/g, 1 g, 0.5 mmol) in NMP (10 mL) and the reaction mixtures were stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. The loading levels of the resulting resins were 0.45-0.47 mmol/g, which were confirmed by Fmoc titration. After deprotection of Fmoc with 20% piperidine in NMP for 30 min, the next amino acids were coupled to the resin by the general BOP-mediated solid-phase peptide synthesis protocol. Then, caffeic acid or *p*-coumaric acid (2 equiv.) was added to the resin-bound tripeptide in NMP with BOP (2 equiv.), DIPEA (2 equiv.) and the mixture was stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. Finally, the resin was treated with reagent K (82.5% TFA: 5% phenol: 5% $H_2O$: 5% thioanisole: 2.5% EDT) for 10 min.[10] The resin was filtered and washed. The collected filtrate was evaporated and precipitated with cold ether to give a product. The product was filtered, washed with diethyl ether, and dried under a high vacuum to give the desired small molecule-peptide conjugates. Small molecule-peptide conjugates were obtained in overall yield 72-93%. The purity and molecular weight of synthesized peptide conjugates were analyzed using HPLC (Shimadzu LC-10Avp system, Shimadzu, Japan) and MALDI-TOF-MS (Kratos Kompact matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Shimadzu, Japan).

**Acid Type Peptide Conjugates Synthesis.** Fmoc-amino acid (1.5 equiv.) and DIEA (1.5 equiv.) were added to a swollen 2-chlorotrityl chloride (CTC) resin 140 (1.0 mmol of Cl/g, 1 g, 1.0 mmol) in NMP (10 mL) and the reaction mixtures were stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. The loading levels of the resulting resins were 0.89-0.93 mmol/g, which were confirmed by Fmoc titration. After deprotection of Fmoc with 20% piperidine in NMP for 30 min, the next amino acids were coupled to the resin by the general BOP-mediated solid-phase peptide synthesis protocol. Then, caffeic acid or *p*-coumaric acid (2 equiv.) was added to the resin-bound tripeptide in NMP with BOP (2 equiv.), DIPEA (2 equiv.) and the mixture was stirred for 3 h. The resins were filtered, washed, and dried under high vacuum. Finally, the resin was treated with reagent K (82.5% TFA: 5% phenol: 5% $H_2O$: 5% thioanisole: 2.5% EDT) for 10 min.[8] The resin was filtered and washed. The collected filtrate was evaporated and precipitated with cold ether to give a product. The product was filtered, washed with diethyl ether, and dried under a high vacuum to give the desired small molecule-peptide conjugates. Small molecule-peptide conjugates were obtained in overall yield 78-95%. The purity and molecular weight of synthesized peptide conjugates were analyzed using HPLC (Shimadzu LC-10Avp system, Shimadzu, Japan) and MALDI-TOF-MS (Kratos Kompact matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Shimadzu, Japan).

**Mushroom Tyrosinase Assay.** Tyrosinase activity was determined using Jang's method with some modification.[11,12] 100 μL of 10 mM L-tyrosine and 50 μL of test sample solution were mixed. Then 50 μL of mushroom tyrosinase (2400 units/mL) was added. Control was replaced 50 μL of L-tyrosine with 50 μL of 0.1 M sodium phosphate buffer. The test mixture was incubated for 10 min at 37 °C, and the amount of dopachrome produced in the reaction was measured at 475 nm using microplate reader (SpectraMax 340PC, Molecular Devices, USA). $IC_{50}$ represents the concentration of test sample that is required for 50% tyrosinase activity inhibition.

**Cell Culture.** B16F1 murine melanoma cell lines used in this study were purchased from Korea Cell Line Bank (KCLB). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Walkersville, MA) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and maintained in 5% of $CO_2$ at 37 °C. 80% confluent monolayer cells were harvested with 0.05% trypsin-EDTA (Gibco BRL, Grand Island, NY).

**Measurement of Melanin Contents.** B16F1 murine melanoma cells were seeded into each well of 6-well plate at the concentration of $1 \times 10^5$ cells/well. Culture media were changed after 24 h, and samples were treated in each well. After 3 days of culture, cells were washed using PBS buffer and obtained using 0.25 M trypsin-EDTA solution. Cell mixtures were centrifuged in the condition of 2500 rpm, 4 °C for 10 min. The supernatant solution was removed, and pellets were dried under 60 °C oven. Dried pellets were solubilized in 200 μL of 1 N NaOH + 10% DMSO solution, and then sonicated at 60 °C for 1 h to dissolve melanins. The relative quantity of melanin was estimated by the absorbance at 490 nm and correlated to the quantity of proteins. The quantity of proteins was estimated by the standard curve of bovine serum albumin (BSA). Protein assay reagent was utilized to assay and absorbance was measured at 595 nm using micro-

plate reader (SpectraMax 340PC, Molecular Devices, USA). All experiments were performed in triplicate.

Melanin inhibition rate (%) = [(C−S)/C] × 100
C : Absorbance of α-MSH(+) group
S : Absorbance of test compound group

## Results and Discussion

To select small molecules having tyrosinase inhibitory activity, the effectiveness of 17 compounds were evaluated and compared with standard tyrosinase inhibitors, kojic acid and arbutin. Table 1 shows the result of mushroom tyrosinase assay.[13] $IC_{50}$ of kojic acid and arbutin were 0.118 mM and 0.183 mM. Among 17 compounds of Table 1, *p*-coumaric acid showed the lowest $IC_{50}$, 0.005 mM. Next, $IC_{50}$ of caffeic acid was 0.037 mM. They represented more effective tyrosinase inhibitory activity than kojic acid or arbutin. Thus, one of the *p*-coumaric acid and caffeic acid which were selected from natural origin compounds for their high tyrosinase inhibitory activity was used as small molecule. And peptide sequences of small molecule-peptide conjugates were selected from five-residue motif (KVARP) of agouti signaling protein (ASP). ASP acts as inverse agonist to MC1R, and KVARP are the sequences that are known to antagonize melanocortin receptor 1 (MC1R). In this study, we used 2 to 4 amino acid sequences from this motif.

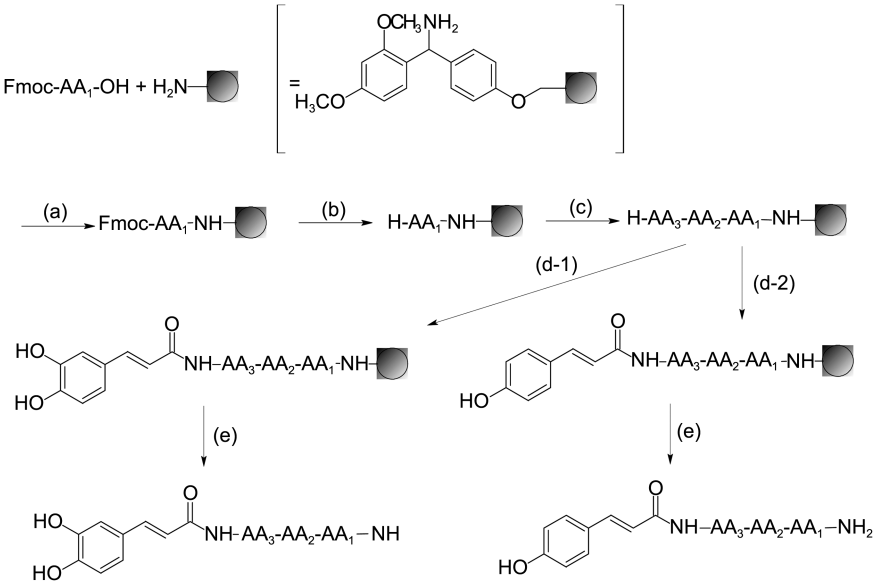
Two types of small molecule-peptide conjugates were synthesized with two distinct resins. Amide type peptide conjugates were prepared with Rink-amide resin (Scheme 1), and acid type peptide conjugates were prepared with 2-chlorotrityl resin (Scheme 2).

In Scheme 1, Fmoc-amino acid was quantitatively introduced to the Rink amid (0.3-0.5 mmol/g) resin using the general protocol of BOP-mediated coupling method, and then introducing 1-3 more amino acid with same method afforded resin-bound peptides. Selected small molecule was introduced to free amine of above resin-bound peptide, and small molecule-peptide conjugates (amide type) were obtained in overall yield 72-93% by cleavage solution (reagent K).

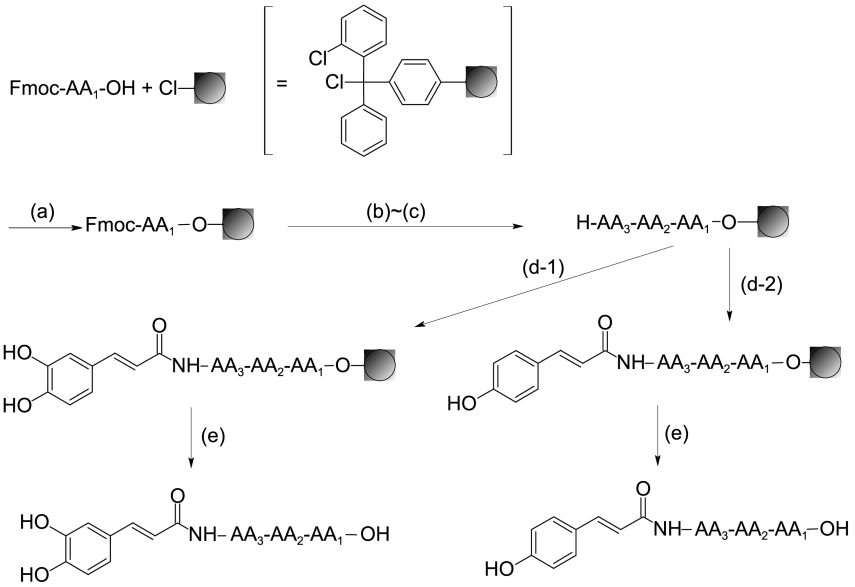
In Scheme 2, Fmoc-amino acid was quantitatively introduced to the resin under DIPEA in *N*-methylpyrrolidone

**Table 1.** Results of mushroom tyrosinase assay. $IC_{50}$ represents the concentration of test sample that is required for 50 % tyrosinase activity inhibition

| No. | Compound | Tyrosinase |
|---|---|---|
| | | $IC_{50}$ (mM) |
| Control | Kojic acid | 0.118 |
| Control | Arbutin | 0.183 |
| 1 | D-(−)-Salicin | > 25 |
| 2 | 2,5-Dihydroxybenzoate | 2.938 |
| 3 | Methyl-2,5-dihydroxybenzoic acid | > 25 |
| 4 | *trans*-Cinnamic aldehyde | 18.085 |
| 5 | 4-Hydroxybenzoic acid | 2.733 |
| 6 | *trans*-*p*-Coumaric acid | 0.012 |
| 7 | 2-Feraldehyde | > 25 |
| 8 | Caffeic acid | 0.037 |
| 9 | *p*-Coumaric acid | 0.005 |
| 10 | 3-(4-Hydroxyphenyl)-propionic acid | 2.757 |
| 11 | *trans*-Ferulic acid | 0.559 |
| 12 | 4-Hydroxyphenyl acetic acid | 2.537 |
| 13 | *p*-Anisaldehyde | 4.170 |
| 14 | Genistein | 0.147 |
| 15 | Quercetin | 0.109 |
| 16 | Galangin | 0.107 |
| 17 | Psoralene | > 2.5 |




**Scheme 1.** Small molecule-peptide conjugates (amide type) synthesis. Reagents and conditions: (a) BOP (benzotriazole-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate), 1-hydroxybenzotriazole (HOBt), DIPEA, NMP; (b) 20% piperidine in NMP; (c) repeat (a) and (b) two times with Fmoc amino acid; (d-1) caffeic acid, BOP, HOBt, NMP; (d-2) *p*-coumaric acid, BOP, HOBt, DIPEA; (e) Reagent K (82.5% TFA: 5% phenol: 5% $H_2O$: 5% thioanisole: 2.5% EDT).




**Scheme 2.** Small molecule-peptide conjugates (acid type) synthesis. Reagents and conditions: (a) DIPEA, NMP; (b) 20% piperidine in NMP; (c) Fmoc-AA-OH, BOP, HOBt, NMP [repeat (b) and (c) two times]; (d-1) caffeic acid, BOP, HOBt, NMP; (d-2) *p*-coumaric acid, BOP, HOBt, DIPEA; (e) Reagent K (82.5% TFA: 5% phenol: 5% $H_2O$: 5% thioanisole: 2.5% EDT).

(NMP) and then the general protocol of BOP-mediated coupling method afforded resin-bound peptides, which were further reacted with small molecule. Small molecule-peptide conjugates (acid type) were obtained in overall yield 78-95% by cleavage solution (reagent K).

We synthesized 28 compounds using these methods, and all products were purified with cold ether precipitation method. Small compound-peptide conjugates prepared by above method are listed in Table S1 (Supporting Information). Table S1 also shows the purity and molecular weight of synthesized small molecule-peptide conjugates. HPLC and MALDI-TOF-MS were used to analyze these compounds, and more than 95% of purities were obtained. Figure S1 (Supporting Information) shows the example of HPLC chromatogram and MALDI-TOF-MS spectrum.

To investigate melanogenesis inhibitory activity of synthesized compounds, B16F1 melanoma cell lines were used to quantify melanin production. To find out the most effective sequence for MC1R antagonist, melanogenesis was induced by α-MSH on B16F1 cells because α-MSH is the high affinity ligand for MC1R and induces melanin synthesis.

After treated with 50 μM of each compound and 1 μM of α-MSH for 3 days, melanins from cell pellets were obtained. The relative quantity of melanin was estimated by the absorbance at 490 nm and correlated to the quantity of proteins. Because synthesized compounds showed cytotoxicity at the concentrations higher than 50 μM, we treated compounds with maximum concentration. Also, α-MSH showed the highest melanin synthesis in 1 μM, so we treated 1 μM of α-MSH as an inducer of melanogenesis.

Through the preliminary screening of 28 compounds, we selected 18 compounds (data not shown). Table 2 shows the results of melanin inhibition rate (%). Higher melanin inhibition rate indicates higher melanin inhibitory activity.

**Table 2.** Effect of small molecule-peptide conjugates on melanin production of B16F1 murine melanoma cell lines. Test materials were treated for 3 days at the concentration of 50 μM. All experiments were performed in triplicate. An asterisk indicates values significantly different from the α-MSH(+) group as determined by t-test. $^*p < 0.05$

| No. | Compound | Melanin inhibition rate (%) |
|---|---|---|
| 1 | Caffeic acid-KVAR-$NH_2$ | 32* |
| 2 | Caffeic acid-VAR-$NH_2$ | 13 |
| 3 | Caffeic acid-AR-$NH_2$ | 23* |
| 4 | Caffeic acid-KVAR-OH | 41* |
| 5 | Caffeic acid-VAR-OH | 15 |
| 6 | Caffeic acid-AR-OH | 44* |
| 7 | Caffeic acid-VARP-OH | 23* |
| 8 | Caffeic acid-RP-OH | 27* |
| 9 | Coumaric acid-KVAR-$NH_2$ | 17 |
| 10 | Coumaric acid -VAR-$NH_2$ | 39* |
| 11 | Coumaric acid -AR-$NH_2$ | 30 |
| 12 | Coumaric acid-VARP-$NH_2$ | 21* |
| 13 | Coumaric acid-KV-$NH_2$ | 17 |
| 14 | Coumaric acid-KVAR-OH | 23* |
| 15 | Coumaric acid-VAR-OH | 39* |
| 16 | Coumaric acid-AR-OH | 36* |
| 17 | Coumaric acid-VARP-OH | 24* |
| 18 | Coumaric acid-RP-OH | 45* |
| Control | Caffeic acid | 16 |
| Control | *p*-Coumaric acid | 23 |
| Control | Kojic acid | 29* |
| Control | Arbutin | 18* |

Melanin inhibitory activities of small molecule-peptide conjugates were compared with caffeic acid, *p*-coumaric acid, and well-known whitening agents, kojic acid and arbutin.

We found that almost all small molecule-peptide conjugates showed higher melanin inhibition rate than caffeic acid (16%) or *p*-coumaric acid (23%) itself. Also, we found that 7 compounds showed superior melanin inhibitory activities than kojic acid (29%) that was known as whitening agent, and these were statistically significant. Except coumaric acid-RP-OH, these 6 compounds have -AR- sequence in common. From this result, we could estimate that AR sequence has a high affinity with active site of MC1R, and further efforts to find mechanism are underway.

## Conclusion

Small molecule-peptide conjugates were prepared successfully using the solid-phase method, and their inhibitory effects on the melanogenesis have been studied. Most of small molecules-peptide conjugates showed superior melanin inhibition activity to kojic acid and arbutin. Among these, almost all compounds have -AR- sequence in common. From this study, we concluded that the small molecule conjugated peptides containing -AR- sequence have great potential to be used as novel whitening agents due to their high melanogenesis inhibitory activities. They reduced melanin production induced by α-MSH statistically significant. Further efforts to find a mechanism that -AR- sequence has high affinity with active site of MC1R are underway. Also, studies on the inhibition pathway in melanogenesis are underway. And, we will develop the method of analyzing small molecule-peptide conjugates within emulsions, and the optimal formulation for whitening cosmetics.

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