공학석사학위논문

Caffeic acid-MHIRL derived Peptide Conjugates as Antioxidant and Tyrosinase Inhibitor

항산화 활성과 타이로시나제 억제 활성을 갖는 카페익산-우유단백질 유래 펩타이드에 관한 연구

2016년 2월

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이 논문을 공학석사학위논문으로 제출함

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ABSTRACT

Caffeic acid-MHIRL derived Peptide Conjugates as Antioxidant and Tyrosinase Inhibitor

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Anti-aging and depigmentation has been the subject of much attention in pharmaceutical and cosmetic industries. Antioxidants distributed in various natural plants such as fruits, tea and coffee have been widely studied as an agent against reactive oxygen species (ROS)-related diseases as well as cosmetic ingredients. Especially, phenolic antioxidant family, such as caffeic acid (CA), shows strong antioxidant activity via stabilization of phenoxy radical from its fully conjugated structure after scavenging of ROS. Skin-lightening agents have been also found in various natural source including milk-derived protein, which suppress melanin production by means of tyrosinase inhibition

activity. We prepared dual effector molecules consisting of CA and MHIRL derived peptides (MDPs: MHIR, HIRL, HIR), which shows both activities of antioxidant and tyrosinase inhibition. CA-MHIRL derived peptides (CA-MDPs) were synthesized by conventional solid-phase peptide synthesis (SPPS), and characterized by RP-HPLC and ESI-MS. Synthetic efficiency was improved through the protection of reactive hydroxyl groups of CA and the optimization of cleavage condition for successful separation of the product from solid support without any side reaction. As-synthesized CA-MDPs, especially CA-MHIR, showed good antioxidant activity and highly enhanced tyrosinase inhibitory activity in various assay systems. In mushroom tyrosinase inhibition test, CA-MHIR exhibited about 4 times lower IC_{50} values (48 μM) than kojic acid, one of the most popular tyrosinase inhibitor (201 μM). Kinetic study of the inhibition by CA-MHIR revealed that CA-MHIR is regarded as a non-competitive inhibitor of mushroom tyrosinase. In addition, CA-MDPs exhibited no-cytotoxicity in melanoma cell, B16-F1. We considered that CA-MHIR dual effector could be applied in the fields of cosmetic industry.

Keywords: Antioxidant, tyrosinase inhibitor, caffeic acid, milk protein, solid-phase peptide synthesis

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BHA	butylated hydroxyanisole
BOP	benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophsphate
CA	caffeic acid
DCM	dichloromethane
DIPEA	*N,N'*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMP	2,2-dimethoxypropane
DODT	3,6-dioxa-1,8-octanedithiol
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI-MS	electrospray ionization mass spectrometer
EA	ethyl acetate
EtOH	ethanol
Fmoc	9-fluorenylmethyloxycarbonyl
HOBt	*N*-hydroxybenzotriazole
RP-HPLC	reverse phase-high-performance liquid chromatography
KA	kojic acid
L-DOPA	3,4-dihydroxyphenylalanine
LC	liquid chromatography
MeCN	methyl cyanide
HEX	hexane
NMR	nuclear magnetic resonance
SPPS	solid-phase peptide synthesis
TFA	trifluoroacetic acid
THF	tetrahyrdofuran
TIPS	triisopropylsilane
UV	ultraviolet

Caffeic acid-MHIRL derived Peptide Conjugates as Antioxidant and Tyrosinase Inhibitor

Chapter 1 Introduction

1. 1 Reactive Oxygen Species and Antioxidant

1. 1. 1 Reactive Oxygen Species and Aging

Oxygen is an essential element to keep our bodies alive. However, oxygen can also produce toxic and mutagenic molecules, reactive oxygen species (ROS) during aerobic respiration.[1] When unpaired electrons are generated from molecular oxygen by high energy exposure or electron transfer, highly reactive ROS are produced. ROS include free radicals such as superoxide ($O_2^{\bullet-}$), peroxyl ($ROO^{\bullet}$) and hydroxyl ($^{\bullet}OH$) radicals, and nonradicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet oxygen.[2] These unstable ROS can react with whatever next to it, resulting in the production of toxic molecules. In healthy bodies, the ROS molecules are scavenged by various antioxidant-mediated self-defense systems. Maintaining the equilibrium between the production and the scavenging of ROS by natural antioxidant defense systems is normal physiological processes for desired cellular responses.[3,4] In pathological condition, however, ROS are overproduced and resulted in oxidative stress to various

biomolecules including lipids, proteins, DNAs, and RNAs by disrupting the balance of the cellular oxidation state.[3,4] In addition, these damages can lead to the destruction of cell membrane, acceleration of the aging process, and mediation of acute diseases such as diabetes, neurodegenerative disorders and stroke (Figure 1).[1,5] Therefore, antioxidants have been broadly studied as a protective agent against ROS-related diseases as well as cosmetic ingredients to suppress skin aging process.[2,3]

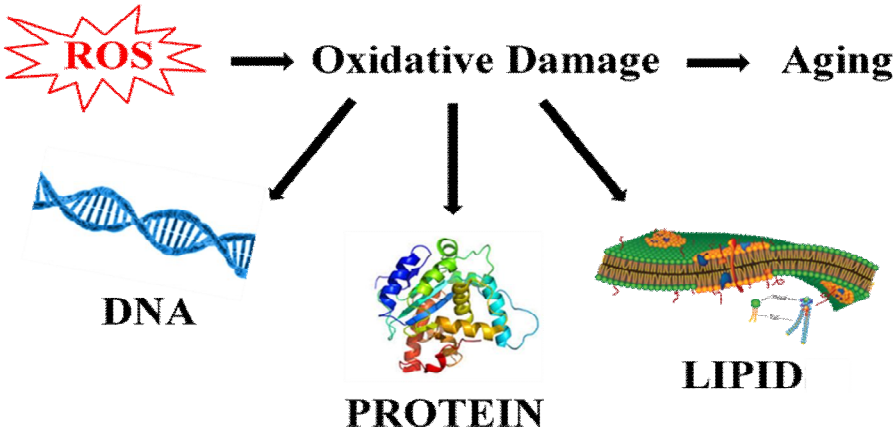


Figure 1. Effect of reactive oxygen species (ROS).

1. 1. 2 Phenolic Antioxidant and Peptide Antioxidant

Phenolic compounds such as flavonoids and phenolic acids found naturally in various plant have attracted much attentions due to their strong antioxidant activity.[6] These phenolic antioxidant family has the general structure of least one aromatic ring bearing one or more hydroxyl groups. The presence, number and position of additional hydroxyl groups are critical in determining antioxidant activity.[7] Also, it is known that the presence of alkyl and/or alkoxy groups at the *ortho* and/or *para* positions provides higher stability for antioxidants.[8]

Hydroxycinnamic acids (HCA) are a representative example of phenolic antioxidant family that includes *para*-coumaric acid (pCoA; 4-hydroxycinnamic acid), ferulic acid (FA; 3-methoxy-4-hydroxy cinnamic acid), sinapic acid (SA; 3,5-dimethoxy-4-hydroxycinnamic acid) and caffeic acid (CA; 3,4-dihydroxycinnamic acid).[7,9] These antioxidants are distributed in many plants such as fruits, tea, coffee and wine. Strong antioxidant activity of HCA family arises from its fully conjugated structure, which enables stabilization of phenoxy radical after quenching free radicals by donating a hydrogen radical (Figure 2).[10] Among them, CA has attracted much attention due to its various biological functions such as pharmacological activities; antioxidative[7,9],

antibacterial[11], antiinflammatory[12], antiviral[13], and neuroprotective[14] properties. However, despite of these biological activities, the use of CA as bioactive agent has been restricted due to both poor solubility in aqueous solution[15] and low stability under the biological condition.[16] Hence, various approaches have be explored to improve bioavailability of CA.[17,18]

Peptide or amino acid conjugation to bioactive molecules is one of the most promising strategies for modulating the activity of bioactive compounds as well as their chemical and physical properties without safety concerns.[17,19,20] In our previous works, CA conjugated with proline-histidine dipeptide (CA-PH) showed highly enhanced antioxidant activity.[21,22,23]

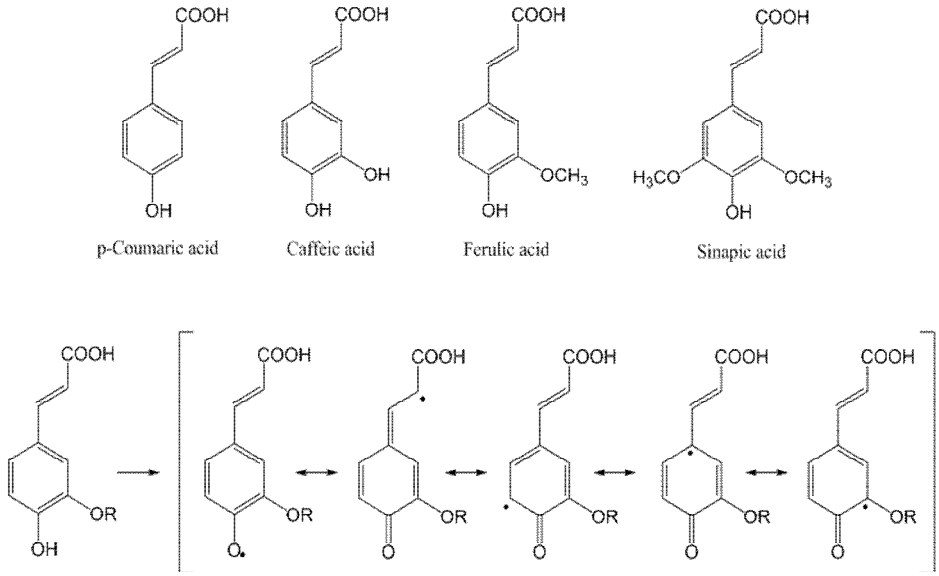


Figure 2. Structure of hydroxycinnamic acid (HCA) compounds and resonance structure of HCA phenoxy radical.

1. 2 Melanogenesis and Tyrosinase Inhibition

1. 2. 1 Melanogenesis

The skin color ranges in a variety from the darkest brown to lightest pinkish-white. Although variety of skin color comes from many substances, the most important compound is melanin. Melanin is a complex polyphenol-like biopolymer produced within a skin cells called melanocytes. Through the biosynthetic pathway called melanogenesis, two types of melanin are produced; Eumelanin (brown-black polymer) and Pheomelanin (yellow-red polymer) (Figure 3).[24] Exposure to UV radiation initiate the melanogenesis by activating the key enzyme, tyrosinase. Tyrosinase catalyzes the first two oxidation steps of melanogenesis: hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequent oxidation of L-DOPA to L-dopaquinone. After these steps, two kinds of complex molecular structures are formed depending on the existence of cysteine. Eumelanogenesis produces 5,6-dehydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) from reddish-brown dopachrome. These molecules are formed from two different reaction pathways and turn into the same product, eumelanin.

Pheomelanogenesis produces cysteinyldopa, which leads to the production of phenomelanin.[25]

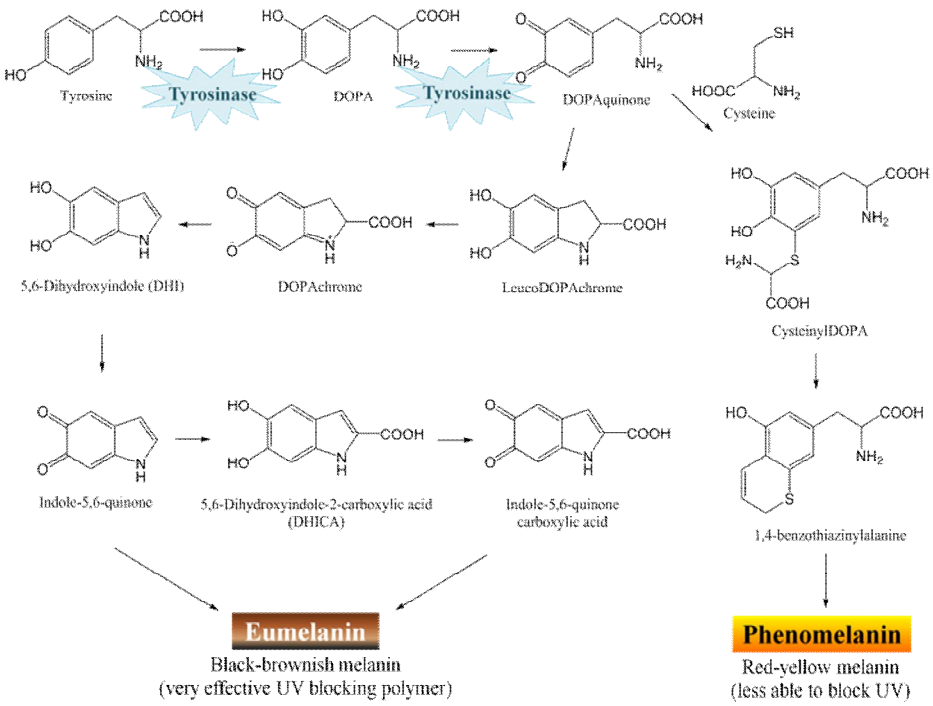


Figure 3. Biosynthetic pathway of melanin.

1. 2. 2 Peptides as Tyrosinase Inhibitor

Melanin exhibits distinct photoprotective properties by converting the harmful UV radiation to harmless heat. However, overproduction and abnormal accumulation of melanin results in dermatological disorders such as melisma, age spot, freckles and postinflammatory melanoderma.[26] In an effort to treat skin disorders associated with melanin contents, tyrosinase inhibitors have been broadly studied and applied to cosmeceutical industry as a skin whitening agent.[27] A number of tyrosinase inhibitors from natural source have been reported. For example, kojic acid (5-hydroxy-2-(hydroxymethyl)-gamma-pyrone), a fungal metabolite produced by *Aspergillus niger* and *Aspergillus penicillium*, is one of the most well-known tyrosinase inhibitor chelating copper atoms at the active site of tyrosinase.[28] In addition, other fungal metabolites, plant polyphenols and aldehyde derivatives including hydroquinone, arbutin and cinnamaldehyde have been reported.[29] However, these inhibitors have suffered from safety when used in excess.

Proteins and peptides have attracted increasing attention as tyrosinase inhibitors without safety concerns. Among them, milk protein such as k-casein and ß-lactoglobulin have been reported to

suppress melanogenesis in melanocyte.[30,31] Furthermore, peptide derived from these proteins, YFYPEL, WYSLAMAA, YVEEL and MHIRL, showed strong tyrosinase inhibitory activity.[32,33] In our previous work, we have synthesized tetrapeptide fragments of the above-mentioned peptides and screened their tyrosinase inhibitory activity. We found that MHIRL family, MHIR and HIRL, showed the highest tyrosinase inhibitory activity compared to other derivatives.

1. 3 Research Objectives

We have designed dual effector molecules which contains two properties, antioxidant activity and tyrosinase inhibitory activity (Figure 4). Caffeic acid (CA) and MHIRL fragments were chosen as antioxidative and depigmenting moieties, respectively. We expected that conjugation of the two individual moieties could induce synergy effect in both activities because antioxidant can influence skin pigmentation pathway by scavenging ROS or interacting with the active site of tyrosinase[24], and MHIRL showed antioxidant activity in our previous work. In addition, peptide conjugation could improve water solubility of CA, which is essential for bioapplication.

CA-MHIRL derived peptides were synthesized by conventional solid-phase peptide synthesis (SPPS), and the synthetic efficiency was improved through the use of acetonide protected caffeic acid and the optimization of cleavage condition. We then evaluated antioxidative activity of the dual effector molecule by DPPH radical scavenging test and lipid peroxidation inhibition test, and tyrosinase inhibitory activity. From these assay, we found that CA-MHIR exhibited highly enhanced tyrosinase inhibitory activity with strong antioxidant activity compared to CA alone and the original peptide. Kinetics for tyrosinase inhibition

of CA-MHIR was investigated. Furthermore, CA-MDPs showed no-cytoxocity when treated in B16 melanoma cells.

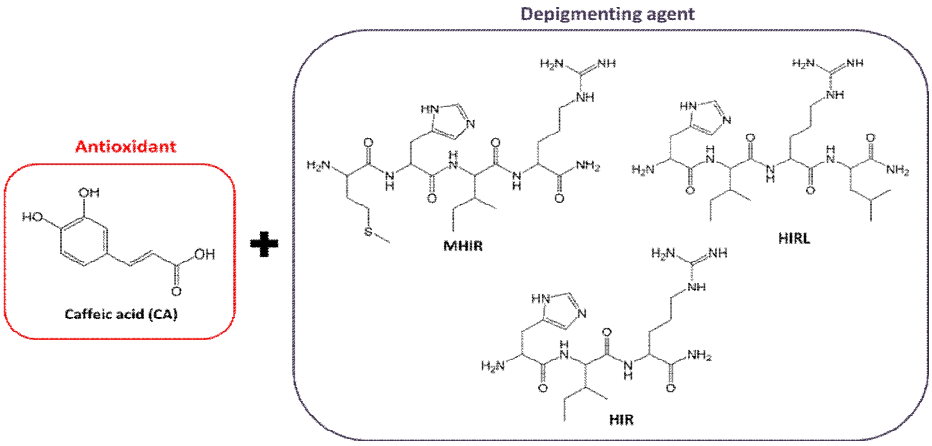


Figure 4. Dual effector molecules consisting of CA and MHIRL derived peptides (MDPs).

Chapter 2 Experiments

2. 1 General

2. 1. 1 Materials

Fmoc-Rink amide linker coupled aminomethyl polystyrene (Rink amide AM) resin (0.47 mmol/g), fritted polypropylene tube reactors (5 mL, or 15 mL Libra tube RT-20M), Fmoc-protected amino acids, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), and hydroxylbenzotriazole (HOBt) were obtained from BeadTech (Seoul, Korea). *N,N*-Diisopropylethylamine (DIPEA) was bought from Alfa Aesar (Massachusetts, USA). Caffeic acid (CA), ninhydrin, linoleic acid (~99 %), 2,2-diphenyl-1-picrylhydrazyl (DPPH), mushroom tyrosinase and 3,4-dihydroxyphenylalanine (L-DOPA) were bought from Sigma (St. Louis, MO, USA). Ammonium thiocyanate (NH_4SCN), ferrous chloride (FeCl2), polyoxyethylenesorbitan monolaurate (Tween 20), butylated hydroxyanisole (BHA), kojic acid (KA), p-toluenesulfonic acid (TsOH), lithium hydroxide monohydrate (LiOH), dimethylaminopyridine (DMAP), 2,2-dimethoxypropane (DMP), 1,2-

ethanedithiol (EDT), anisole, triisopropylsilane (TIPS), 3,6-dioxa-1,8-octanedithiol (DODT), thiazolyl blue tetrazolium bromide (MTT) and α-melanocyte stimulating hormone (α-MSH) were bought from Aldrich (St. Louis, MO, USA). *N*-Methyl-2-pyrrolidone (NMP), piperidine, dichloromethane (DCM), tetrahydrofuran (THF), thionyl chloride ($SOCl_2$), benzene, diethyl ether, ethanol and methanol were bought from Dae-Jung Chemicals (Korea). Trifluloroacetic acid (TFA) was bought from Acros Organics (Morris Plains, NJ, USA). All other solvents were used without further purification.

2. 1. 2 Apparatus

Synthesized product were identified by high performance liquid chromatography (HPLC, Thermo Scientific Spectra System AS3000; Thermo-Fisher, Waltham, MA, USA), using an AAPPTec Spirit Peptide C18 reverse phase column (120 Å, 5 μm, 4.6 x 250 mm; AAPPTec, Louisville, KY, USA). Mass Spectroscopy (Electrospray Ionization Mass Spectrometry, ESI-MS, LCQ; Thermo Finnigan, Waltham, USA) were used to determine the mass of peptides. The color reaction for linoleic acid peroxidation, DPPH and enzyme inhibition tests were measured by UV/Visible spectrophotometry

(Optizen 2120 UV, Mecasys Co. Ltd., Daejeon, Korea). Cytotoxicity assay was followed by an ELISA Reader (Infinite M200, Tecan, Austria). ^{1}H-NMR spectra were acquired by a Bruker AVANCE-400 spectrometer (Bruker, Rheinstetten, Germany) operating at 400 MHz.

2. 1. 3 Analysis Methods

Fmoc Quantitation

Dry resin (30 mg) containing Fmoc-groups was suspended in 20% piperidine/NMP (v/v) (3 mL) in a shaking incubator at RT for 50 min. Supernatant solution (0.1 mL) was withdrawn and diluted to 10 mL with NMP. The absorbance of the diluted solution was measured at 290 nm. Loading level of the resin was calculated by the following formula:

$$\text{Loading level (mmol/g)} = (55.206 \times Abs_{290\,nm} - 1.0223) / 30$$

Ninhydrin Color Test (Kaiser Test)

To confirm the completion of amino acid coupling and Fmoc deprotection by identifying the presence of free primary amino groups, potassium cyanide solution (1 mL of 0.01 M KCN aqueous solution diluted in 100 mL of pyridine), ninhydrin solution (0.5 g of ninhydrin

solved in 10 mL of ethanol), and phenol (80 g of phenol dissolved in 20 mL of ethanol) were prepared. After each amino acid coupling reaction and Fmoc deprotection, 1~2 mg of the resin were tested by two drops of the each stock solution within a test tube, and the color reaction was carried out at 100 ℃ for 5 min. The reaction of ninhydrin with primary amines on resin gives a dark blue color when amino groups remain uncoupled.

2. 2 Synthesis

Solid-phase synthesis method

Milk-derived peptides were synthesized on Rink AM resin (0.47 mmol/g) by solid-phase peptide synthesis method. For coupling of amino acid, Fmoc-amino acid (3 equiv), BOP (3 equiv) and HOBt (3 equiv) in NMP were added to the resin with DIPEA (6 equiv) at RT, and the resin mixture was shaken for 3.5 h. To deprotect Fmoc group on Fmoc-amino acid, the deprotection was carried out using 20% piperidine/NMP in a shaking incubator for 50 min at RT. Completion of each coupling reaction was determined by Kaiser's ninhydrin color test. After repeating peptide coupling and Fmoc deprotection, caffeic acid (CA) was coupled: CA (3 equiv), BOP (3 equiv) and HOBt (3 equiv) were dissolved in NMP, and this mixture was added to the peptide anchored resin with DIPEA (6 equiv), and shaken for 3.5 h at RT. The product was detached from the resin by treating with cleavage cocktail (TFA/Anisole/TIPS/DODT = 9.5/0.3/0.1/0.1) for 2.5 h at RT. The final product was filtered under vacuum and the filtrate was precipitated with cold diethyl ether to remove impurities, yielding white powders. The white powder was washed with cold diethyl ether (5 x), and dried with nitrogen gas. The final products were identified by electrospray

ionization mass spectrometry (ESI-MS). Peptides and peptide derivatives were analyzed by reverse phase-high performance liquid chromatography (RP-HPLC) under the following conditions: AAPPTec Spirit Peptide C18 reverse phase column; gradient elution with A: 0.1 % TFA in water, B: 0.1 % TFA in acetonitrile; from 10 % to 90 % B over 30 min, at a flow rate of 1.0 mL/min; detection: UV, 230 and 326 nm. Compounds were purified by RP-HPLC (A: 0.1 % TFA in water, B: 0.1 % TFA in acetonitrile; from 10 % to 90 % B over 30 min, at a flow rate of 4.0 mL/min).

Modified synthetic procedure

A. Protection

Caffeic acid methyl ester [CA-OMe]

Caffeic acid (CA) was dissolved in methanol (20 mL) in a 100 mL round-bottem flask, and cooled in an acetonitrile/dry ice bath. Thionyl chloride (2 equiv) was added dropwise, followed by the addition of DMAP (0.2 equiv). The cooling bath was removed and the mixture was stirred overnight at RT. After the reaction, methanol was evaporated and the residue was precipitated in cold chloroform/hexane (1:3) solution for overnight. Precipitate was filtered and washed with

chloroform/hexane (1:3) several times, and dried in vaccum. Crude caffeic acid methyl ester, CA-OMe, was obtained as white powder, and purified further by silica gel chromatography (71 %) (elution with EA:Hex = 1:2).

^{1}H NMR (400 MHz, $(CD_3)_2CO$, 297K) δ 3.69 (3H, s), 6.27 (1H, d, J=16), 6.758 (1H, d, J=8), 6.99-7.0 (1H, m), 7.01-7.05 (1H, m), 7.46-7.50 (1H, m), 9.13 (1H, s), 9.6 (1H, s)

Methyl(*E*)-3-(2,2-dimethylbenzo[*d*][1,3]dioxol-5-yl)acrylate [CA(acetonide)-OMe]

Purified CA-OMe (50 mmol) dispersed in anhydrous benzene was reacted with DMP (4 equiv) in a two-necked 250 mL flask. One neck of the flask was connected to a Soxhlet extractor and the thimble was filled with anhydrous $CaCl_2$ to trap methanol and DI water. The other neck was sealed with a rubber septum for sampling. Argon gas was injected to the flask for 5 min and the solution was heated at reflux for 5 min, followed by addition of TsOH (0.05 equiv). During the reaction, ferric chloride test was performed to check the degree of reaction. After 3 h, reaction mixture was cooled to RT and benzene was removed by rotary evaporation. The crude product (deep yellowish liquid) was

purified by silica gel chromatography: elution with EA:Hex = 1:9, and methyl (*E*)-3-(2,2-dimethylbenzo[*d*][1,3]dioxol-5-yl)acrylate, acetonide protected caffeic acid methyl ester (CA(acetonide)-OMe), was obtained as white powder.

^{1}H NMR (400 MHz, $(CD_3)_2CO$, 297K) δ 1.68-1.77 (6H, s), 3.7 (3H, s), 6.478 (1H, d, J=16), 6.86-6.88 (1H, m), 7.14-7.17 (1H, m), 7.32 (1H, d, J=1.6), 7.563 (1H, d, J=16)

(*E*)-3-(2,2-dimethylbenzo[*d*][1,3]dioxol-5-yl)acrylic acid [CA(acetonide)-OH]

Purified CA(acetonide)-OMe (2 mmol) was dissolved in THF/DI water (2:1) and cooled in an ice bath, followed by addition of lithium hydroxide (3 equiv). The mixture was stirred in an ice bath overnight. (*E*)-3-(2,2-dimethylbenzo[*d*][1,3]dioxol-5-yl)acrylic acid, acetonide protected caffeic acid (CA(acetonide)-OH) was purified by silica gel chromatography (21 %): elution with EA:Hex = 1:2.

^{1}H NMR (400 MHz, $(CD_3)_2CO$, 297K) δ 1.68 (6H, s), 6.36 (1H, d, J=15.6), 6.81 (1H, d, J=7.6), 7.1-7.12 (1H, m), 7.13-7.18 (1H, s), 7.58 (1H, d, J=16)

B. Solid phase synthesis

The MDPs anchored resin was reacted with CA(acetonide)-OH (2 equiv), BOP (2 equiv), HOBt (2 equiv), and DIPEA (4 equiv) in NMP for 3 h at RT. The product was separated from the resin by using two types of cleavage cocktail; (a) TFA/Anisole/TIPS/DODT = 9.5/0.3/0.1/0.1 for 2.5 h at RT and (b) TFA/DI water/TIPS = 95/2.5/2.5 for 1.5 h at RT. Following the same procedure as above, the compound was washed, and analyzed by ESI-MS and RP-HPLC.

CA-MHIR (m/z calcd: 717.34 $[M+H]^+$; found: 717.5), MHIR (m/z calcd: 555.31 $[M+H]^+$; found: 555.5), CA-HIRL (m/z calcd: 699.7 $[M+H]^+$; found: 699.7), HIRL (m/z calcd: 537.35 $[M+H]^+$; found: 537.5) CA-HIR (m/z calcd: 586.6 $[M+H]^+$; found: 586.6) HIR (m/z calcd: 424.27 $[M+H]^+$; found: 424.4)

2. 3 Evaluation of Biological Activity

2. 3. 1 In Vitro Assay

Measurement of Free Radical Scavenging Activity

Antioxidant activity of CA-MHIRL derived peptide conjugates were evaluated by the percentage of DPPH radical scavenging activity (% RSA), which corresponds to the decreasing amount of the absorbance of the DPPH solution after adding the antioxidant.

Each CA-MHRIL derived peptides was dissolved in methanol in various concentrations, followed by addition of 25 μL of the sample solution to DPPH methanolic solution (0.1 mM, 1475 μL). After incubation for 10 min at RT, the absorbance of solution was measured at 516 nm. The percentage of RSA was calculated by the following equation:

$$\%RSA = (1 - Abs_{516nm} \text{ of sample} / Abs_{516nm} \text{ of control}) \times 100$$

Control solution contained DPPH solution and 25 μL of methanol

instead of antioxidant. Each experiment was performed in triplicate.

Measurement of Lipid Peroxidation Inhibitory Activity

The lipid peroxidation inhibitory activity was measured to find out the antioxidant activity of CA- MHIRL derived peptide conjugates. Firstly, linoleic acid emulsion (50 mM) was prepared by dissolving 0.284 g of linoleic acid and 0.284 g of Tween 20 in phosphate buffer (50 mL, 0.1 M, pH 7.0). Next, the emulsion samples for the antioxidation test were prepared by mixing 0.5 mL of DI water, 2.5 mL of the linoleic acid emulsion, 2.0 mL of phosphate buffer (0.1 M, pH 7.0), and 0.5 mL of test samples in methanol. The total reaction volumn was 5.5 mL, and the final concentration of CA-milk derived MHIRL peptides was 100 μM. Each emulsion samples in a glass vial (10 mL) was capped with rubber septa, and kept at 50 ℃ under dark conditions for 50 h. Emulsion sample with methanol instead of test samples was also prepared as a negative control.

The antioxidant activity was monitored by using modified ferric thiocyanate (FTC) method with 100 μL portion of emulsion samples withdrawn at specific time intervals. In the FTC analysis, emulsion samples (25 μL) were mixed with 1.175 mL of 75 % ethanol and 25

μL of 30 % of aq. NH4SCN. After the addition of 25 μL of 20 mM $FeCl_2$/ 10 % HCl aqueous solution, the reaction mixtures were incubated for 3 min at RT. After then, the absorbance of sample solution was measured at 500 nm.

Lipid was oxidized fast enough to be able to measure the degree of oxidation at 3 h intervals at the initial phase of oxidation. Meanwhile, at the late stage of oxidation, the absorbance were measured at 12 h intervals because the oxidation rate of lipid became slower than the early stage. Seven test results were performed in triplicate, respectively.

The percentage of lipid peroxidation inhibition (%Pi) was determined at 12 h when the absorbance of negative control reaches approximately 1. The percentage of %Pi was calculated by the following equation:

$$\%Pi = (1\text{-}Abs_{500nm} \text{ of test sample} / Abs_{500nm} \text{ of control}) \times 100$$

Measurement of Tyrosinase Inhibition Activity

Depigmenting activity of CA-MHIRL derived peptides was evaluated via tyrosinase inhibition assay. The degree of convertion of

L-DOPA, the tyrosinase substrate, to dopaquinone was measured by monitoring the absorbance value at 475 nm. The assay samples were prepared by mixing 250 μL of phosphate buffer (0.1 M, pH 6.8), 250 μL of 2.5 mM L-DOPA, 200 μL of DI water, and 25 μL of inhibitor in methanol. After the addition of 25 μL of aqueous mushroom tyrosinase solution (400 U/mL), the mixture was incubated for 10 min at RT. After 10 min, the absorbance of mixture was measured at 475 nm. The percentage of tyrosinase inhibition activity was calculated by the following equation:

$$\%\text{Inhibition} = (1 - \text{Abs}_{475\ \text{nm}}\ \text{of test sample} / \text{Abs}_{475\ \text{nm}}\ \text{of control}) \times 100$$

Blank solution contained the same components but not the enzyme and inhibitor. In negative control, methanol was added instead of inhibitor. Each experiment was performed in triplicate.

Measurement of Storage Stability

To evaluate storage stability, 20 μM of CA or CA-MHIR dissolved in DI water/DMSO (1:1) were stored at RT, and DPPH radical scavenging activities were evaluated for 2 months at 10 days intervals.

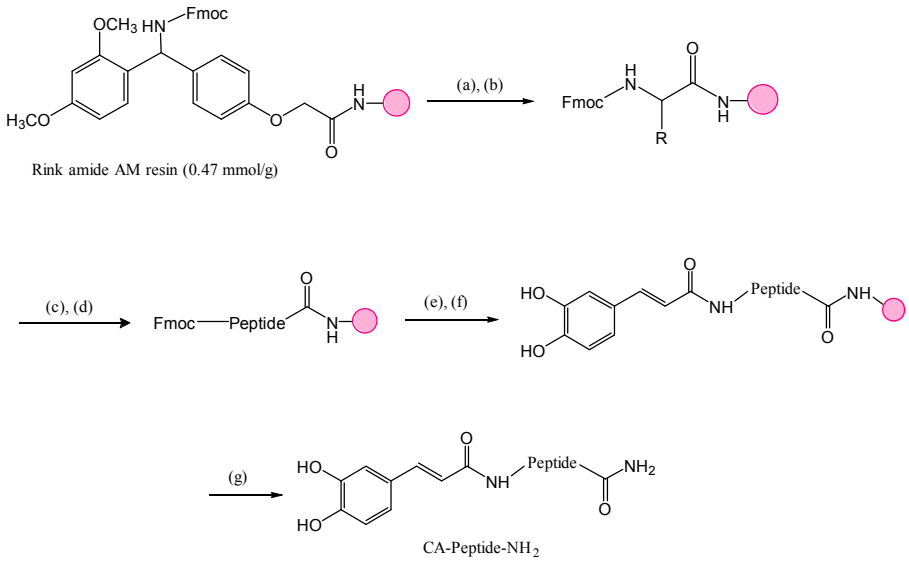
2. 3. 2 Cellular Assay

Mouse melanoma cell line (B16-F1) was inculated in serum-free medium for 24 h to measure the cytotoxicity. B16-F1 cells were seeded into 6-well plates. After culture media were replaced with α-melanocyte stimulating hormone (α-MSH) containing media, cell were incubated with test samples for 72 h. The cell was washed with PBS, and thiazolyl blue tetrazolium bromide (MTT) solution was added and incubated for 4 h. Isopropyl alcohol was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at RT to ensure that all crystals were dissolved, the amount of water-soluble formazan was measured by optical density at 570 nm using enzyme-linked immunosorbent assay (ELISA) reader.

Chapter 3 Results and Discussions

3. 1 Synthesis and Characterization of Caffeoyl Peptides

MHIRL derived peptides (MDPs) and their caffeic acid (CA) conjugates were synthesized by solid-phase peptide synthesis (SPPS) method on Rink amide AM resin with Fmoc strategy (Scheme 1). Crude products were purified using preparative HPLC. The purity of both crude and purified caffeoyl peptide conjugates were determined by HPLC and characterized by ESI-MS.



Scheme 1. Solid phase synthesis of caffeoyl peptide conjugate. (a) 20 % Piperidine/NMP (v/v) for 50 min, (b) Fmoc-L-amino acid (3 equiv), BOP (3 equiv), HOBt (3 equiv) and DIPEA (6 equiv) in NMP for 3.5 h, (c) repeat (a), (d) repeat (b), (e) repeat (a), (f) Caffeic acid (3 equiv), BOP (3 equiv), HOBt (3 equiv) and DIPEA (6 equiv) in NMP for 3.5 h, (g) cleavage cocktail: 95 % TFA, 3 % anisole, 1 % TIPS, 1 % DODT/DCM (v/v) for 2.5 h, and diethyl ether precipitation.

3. 2 Profile of the Product and Impurities

The purities of MHIRL derived peptides (MDPs) and CA-MHIRL derived peptides (CA-MDPs) were confirmed by HPLC and ESI-MS analysis. MHIR is one of MDPs, which exhibited a single peak in HPLC corresponding to its own mass. On the other hand, five peaks were observed in HPLC of crude CA-MHIR. From the MS analysis, we found that peaks at 13.9, 14.5 min (red star) corresponded to the desired product CA-MHIR. Major peak at 11.4 min (blue inverted triangle) was an impurity and peaks at 15.6, 16.2 min (green spade) were the mixture of product and impurities (Figure 5). Analysis of other MDPs and CA-MDPs corresponded to each spectrum of MHIR and CA-MHIR. As the conventional synthetic protocol gave poor performance, we tried to modify the synthetic scheme.

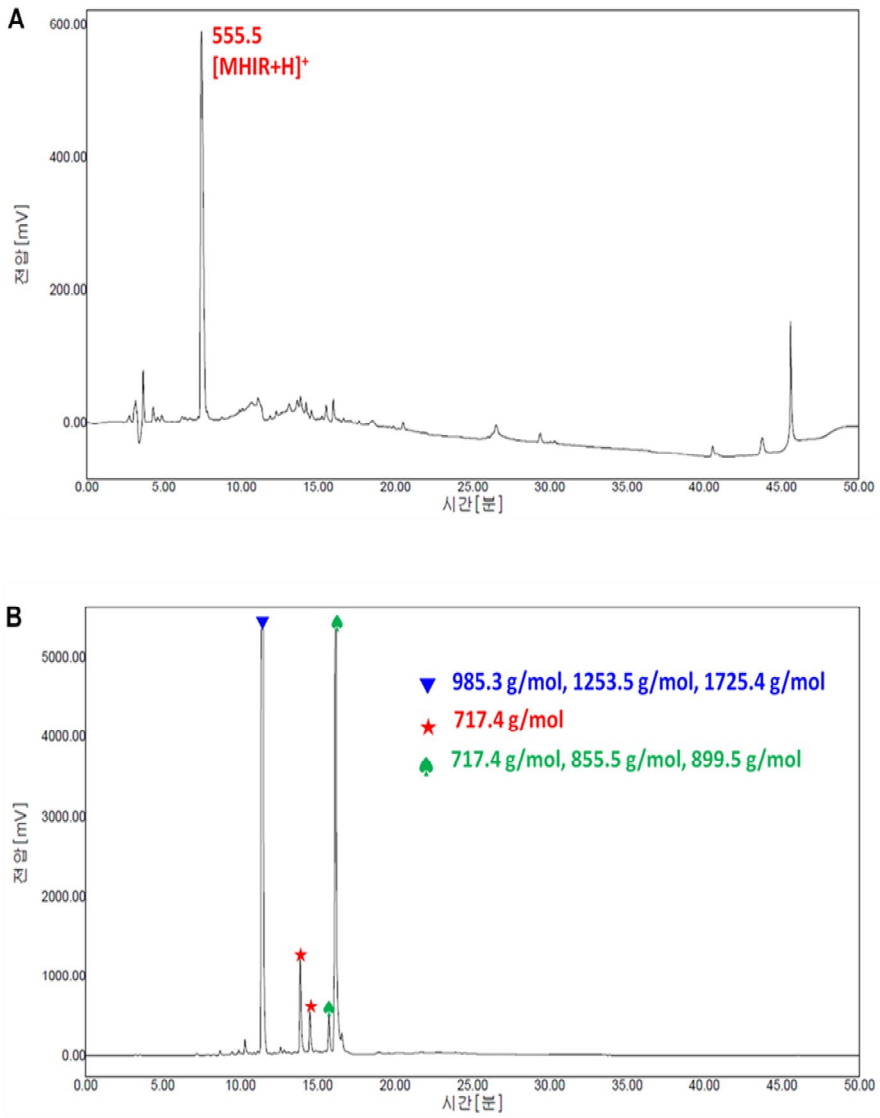


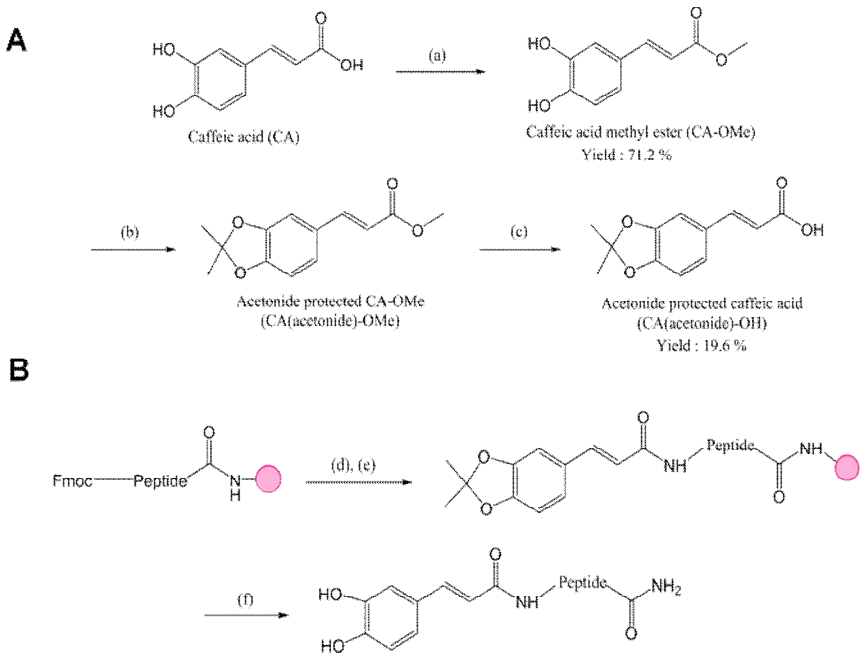
Figure 5. Profiling of synthesized product and impurities. (a) HPLC and ESI-MS data of MHIR. Single peak corresponding to MHIR ($C_{23}H_{42}N_{10}O_4S$) was observed; (b) HPLC and ESI-MS data of CA-MHIR. Five peaks were observed; Peak eluted at 11.4 min (blue

inverted triangle) was characterized as impurities; Mass = 985.3, 1253.5, and 1725.4. Peaks elected at 13.9, 14.5 min (red star) was characterized as product; calculated mass for $C_{32}H_{48}N_{10}O_7S$ (CA-MHIR). Peak eluted at 15.6, 16.2 min (green spade) were a mixture of product and impurities; Mass for CA-MHIR $[M+H]^+$ 717.4, and impurity mass 855.5 and 899.5.

3. 3 Synthesis and Characterization of Acetonide Protected Caffeoyl peptide

We modified the original synthetic method as shown in Sheme 2. We assumed that two free hydroxyl groups of CA might induce undesired side reactions during the coupling of CA to MHIR anchored resin. Actually, it is necessary to properly protect the reactive functional groups to ensure a pure product. As an acetonide protecting group for diol moiety has a problem to be compatible with Fmoc strategy in SPPS method,[34] we tried to synthesize acetonide protected caffeic acid via three steps (Scheme 2A). First, CA was converted to CA-methyl ester (CA-OMe) by $SOCl_2$ in methanol with catalytic amount of DMAP (yield ca. 71%). CA-OMe was converted to refluxing with DMP in anhydrous benzene in the presence of TsOH as a catalyst. Since the reaction of acetonide protection is controlled by equilibria, it is necessary to remove water from the reaction system. For this purpose, the reaction flask was equipped with a Soxhlet extractor, the thimble filled with $CaCl_2$ to remove water produced during the reaction. The reaction was completed within 3 h, which was monitored by $FeCl_3$ test. After removal of the solvents and purification with silica gel chromatography, CA(acetonide)-OMe was obtained as

white powder. Finally, alkaline hydrolysis of CA(acetonide)-OMe by lithium hydroxide in THF/DI water provided CA(acetonide)-OH. The overall yield was 20 %. The modified CA was characterized by ^{1}H-NMR spectroscopy. CA-MDPs were then synthesized with acetonide protected CA by SPPS method (Scheme 2B).



Scheme 2. Modified synthetic method. A) Synthesis of CA(acetonide)-OH from CA. (a) CA (1 equiv), $SOCl_2$ (2 equiv), DMAP (0.2 equiv) in anhydrous methanol for overnight at RT, (b) DMP (4 equiv), TsOH (0.05 equiv) in benzene, refluxing for 3 h, (c) LiOH (3 equiv) in THF/DI water (2:1) in ice bath for 24 h. Each step was purified by column chromatography. B) Synthesis of CA-MDPs by using CA(acetonide)-OH. (d) 20 % Piperidine/NMP (v/v) for 50 min, (e) CA(acetonide)-OH (2 eq), BOP (2 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in NMP for 3.5 h, (f) cleavage cocktail: 95 % TFA, 3 % TIPS, 1 % DODT, 1 % anisole for 2.5 h, and diethyl ether precipitation.

Characterization of CA-MHIRL derived Peptides (CA-MDPs) Synthesized with CA(acetonide)-OH

After separation of the product from the resin by DODT containing cleavage cocktail, CA-MHIR was isolated and characterized with HPLC and ESI-MS. A single peak was eluted at 16.3 min in HPLC spectrum, however, this peak was proved to be as DODT adduct of CA-MHIR by ESI-MS data and the previously reported result, which described about 1,4-addition of thiol compound to α,ß–unsaturated ketones (Figure 6A).[35,36] Therefore, DI water was used instead of DODT in the cleavage cocktail. As a result, CA-MHIRL was obtained with high purity as shown in HPLC spectrum and ESI-MS data (Figure 6). From these results, we found that thiol containing scavenger should be excluded in the cleavage solution for release of CA-peptide conjugate from solid support. As-synthesized CA-MDPs and MDPs was characterized by HPLC and ESI-MS (Table 1).

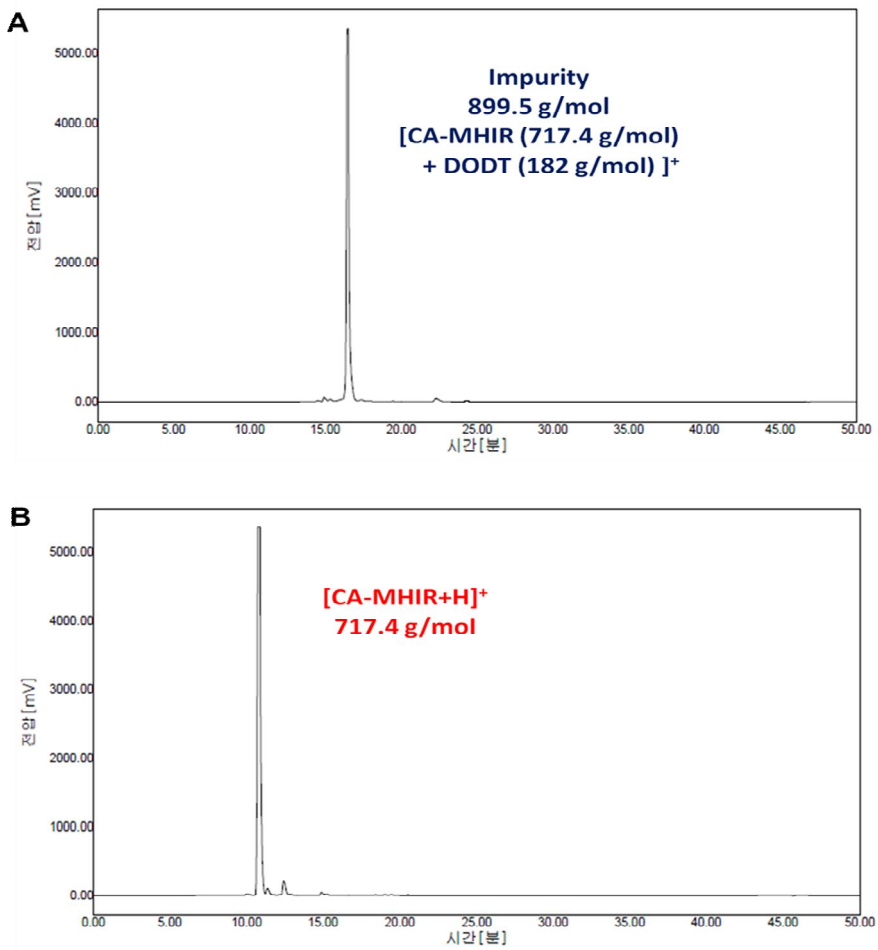


Figure 6. Profiling of products synthesized by modified synthetic strategy. (a) HPLC and ESI-MS data of CA-MHIR using DODT contains cleavage solution. A single peak was observed as an impurity at 16.3 min; Mass of impurity at 15.3 min = 899.5, Calculated Mass for CA-MHIR+DODT $[M+H]^+$. (b) HPLC and ESI-MS data of CA-MDP (CA-MHIR) using DI water as cleavage solution. Product

emerged from a single peak at 11.5 min; found product mass = 717.3 for CA-MHIR $[M+H]^+$.

Table 1. Characterization of CA-MDPs and MDPs

Compounds	Purity (%)	Mass ([M+H]+)	
		Calculated	Found
CA-MHIR	>99	717.34	717.5
MHIR	>99	555.31	555.5
CA-HIRL	95	699.7	699.7
HIRL	94	537.35	537.5
CA-HIR	94	586.6	586.6
HIR	>99	424.27	424.4

3.4 Antioxidant Activity of Caffeoyl Peptide Derivatives

Antioxidant activities of CA-MHIRL derived peptides (CA-MDPs) were investigated by the DPPH radical scavenging test and the linoleic acid oxidation assay with FTC method. CA was used as a positive control.

Free Radical Scavenging Activity of CA-MDPs and MDPs

The free radical scavenging activity (RSA) of CA-MDPs and MDPs were evaluated with DPPH. DPPH which is an unstable free radical becomes a stable molecule by accepting an electron or hydrogen radical. Figure 7 showed the results of %RSA. CA showed higher %RSA than that of CA-MDPs. CA-MDPs showed much higher %RSA than MDPs. Among CA-MDPs, CA-MHIR showed the highest %RSA. The order of %RSA was as follows:

CA (76±4) > CA-MHIR (61±2) > CA-HIR (56±1) > CA-HIRL (50±2) >> MHIR (8±1) > HIRL (5±1) > HIR (3±1)

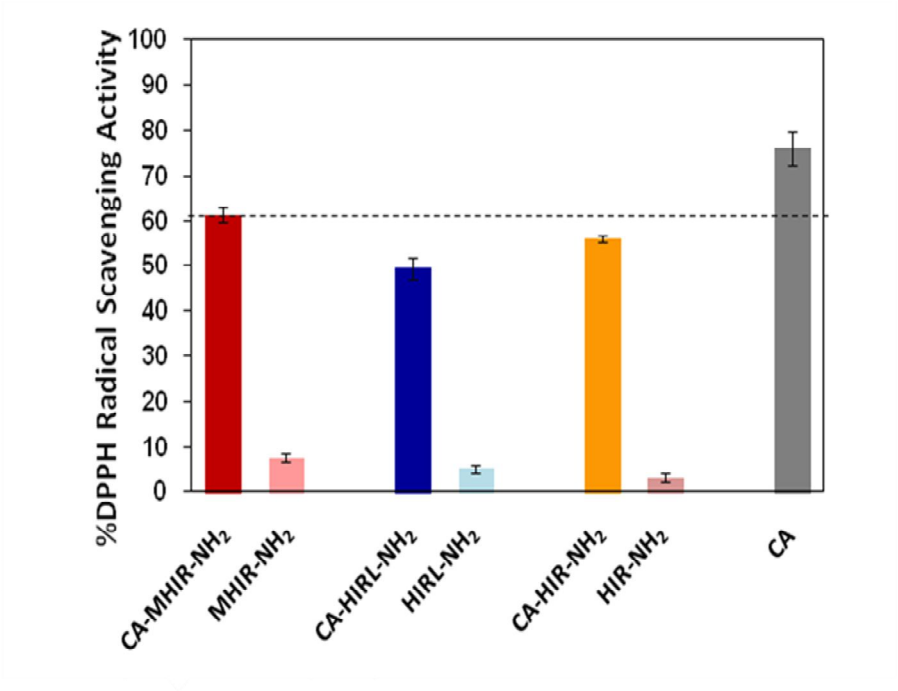


Figure 7. DPPH radical scavenging activity (%RSA) of CA-MDPs and MDPs. [Antioxidant]/[DPPH] (mol/mol) = 0.25. The absorbance was measured at 516 nm. Each experiment was performed in triplicate and repeat three times. The values are given as the mean ± standard error.

Lipid Peroxidation Inhibition Assay of CA-MDPs

A common method to evaluate antioxidant activity in hydrophobic environment is lipid peroxidation assay using linoleic acid emulsion. The percentage of lipid peroxidation inhibition (%Pi) was calculated when the absorbance of the negative control reached approximately 1. The absorbance was measured at 12 h after the reaction was started. Unlike the %RSA, all of CA-MDPs exhibited higher inhibition activity than CA due to the differences in the hydrophobic environment of the assay system. CA-MDPs showed highly enhanced antioxidant activity than MDPs, and %Pi of MHIR even showed a negative value. CA-MHIR exhibited the highest %Pi among other CA-MDPs by a small margin and the activity was similar to a synthetic antioxidant BHA (Figure 8A). The degree of %Pi value was in the following order:

BHA (86±1) > CA-MHIR (79±0.2) > CA-HIR (78±0.8) > CA-HIRL (76±0.3) > CA (73±1) >> HIRL (32±6) > HIR (26±5) > MHIR (-23 ±4)

This result is different from that of %RSA. CA which has two hydroxyl groups and a carboxyl group performed the highest activity in hydrophilic environment. However, in relatively hydrophobic environment such as for the lipid peroxidation inhibition test, CA exhibited the lowest antioxidant activity due to its hydrophilicity.

We evaluated the antioxidant activity of MDPs and CA-MDPs in lipid peroxidation by measuring UV absorbance for 35 h at 50 ℃ under dark condition at certain time intervals (Figure 8B,C,D). At 35 h, the negative control reached its maximum value of approximately 2.0.

MDPs revealed rather poor antioxidant activity on its own. However, the lipid peroxidation inhibition activity of CA and CA-MDPs did not show any big difference up to 12 h (Figure 8B,C,D). This demonstrated that all the CA-MDPs inhanced to stability of CA and showed more excellent antioxidant activity.

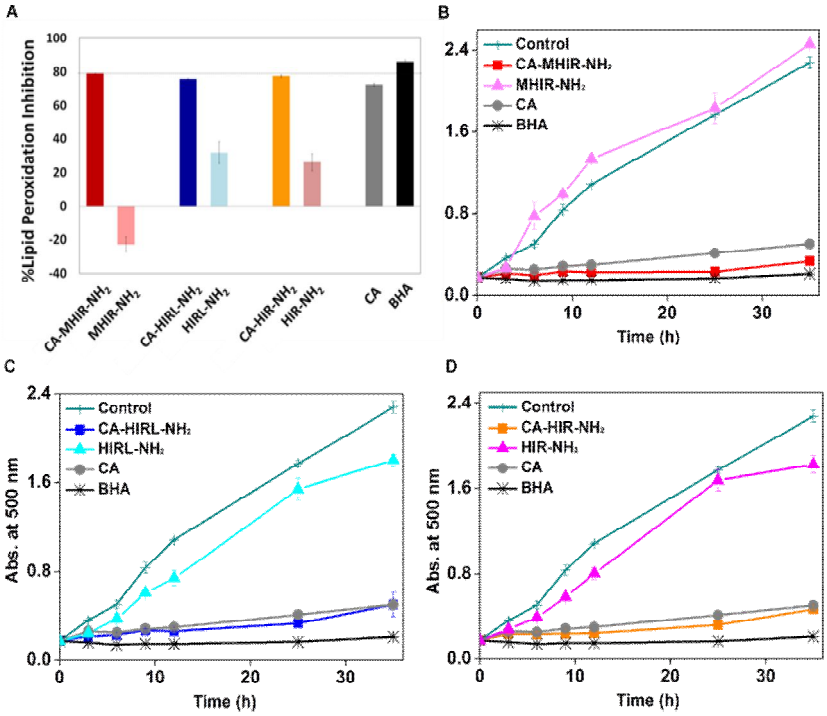


Figure 8. Lipid peroxidation inhibitory activity of CA-MDPs and MDPs. The absorbance was determined at 500 nm. The final concentration of antioxidant was 100 μM. A) The absorbance was measured at 12 h after the reaction was started. B), C) and D) Reaction was performed for 35 h at 50 ℃ in the dark condition. The values are given as the mean ± standard error.

3. 5 Evaluation of Tyrosinase Inhibitory Activity

Tyrosinase inhibition assay was carried out to evaluate the tyrosinase inhibitory activity of MDPs and CA-MDPs. The tyrosinase inhibitory activity was measured by UV absorbance of the reaction media. The concentration of each inhibitor was 100 μM and L-DOPA as a substrate was 2.5 mM (Figure 9). To compare tyrosinase inhibition activities, kojic acid (KA) and arbutin were selected as reference compounds. As expected, each CA-MDPs exhibited better tyrosinase inhibitory activity than each MDPs. Among them, CA-MHIR showed the highest tyrosinase inhibitory activity. These results demonstrated that conjugation of MDPs to CA gave better tyrosinase inhibitory activity than the milk-derived peptides. Tyrosinase inhibitory activity(%) decreased in the following order:

CA-MHIR (75±7) > Kojic acid(KA) (52±6) > CA-HIRL (35±3) > MHIR (27±5) > CA-HIR (24±7) > HIRL (14±6) > HIR (4±8) > Arbutin (-15±2)

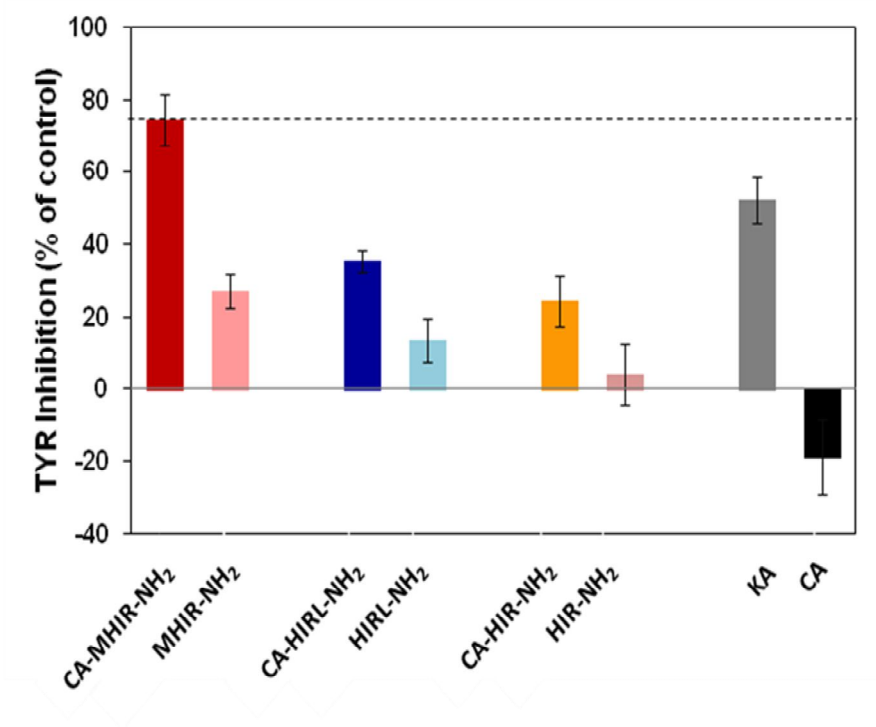


Figure 9. Tyrosinase inhibition activity of CA-MDPs and MDPs. Tyrosinase inhibitory activity(%) was determined at 100 μM of each inhibitor; substrate was 2.5 mM L-DOPA; reaction was performed at RT for 10 min. The UV absorbance of the colored reaction solution was measured at 475 nm respectively. The values are given as the mean ± standard error.

To determine the most efficient tyrosinase inhibitor, half inhibition concentrations (IC_{50}) of these compounds was calculated (Table 2). IC_{50} values were obtained by measuring the tyrosinase inhibitory activity of MDPs and CA-MDPs at different concentrations (Figure 10). CA-MHIR remarkably showed the lowest IC_{50} values and its tyrosinase inhibitory activity toward mushroom tyrosinase was significantly increased to over 90 % at 100 μM.

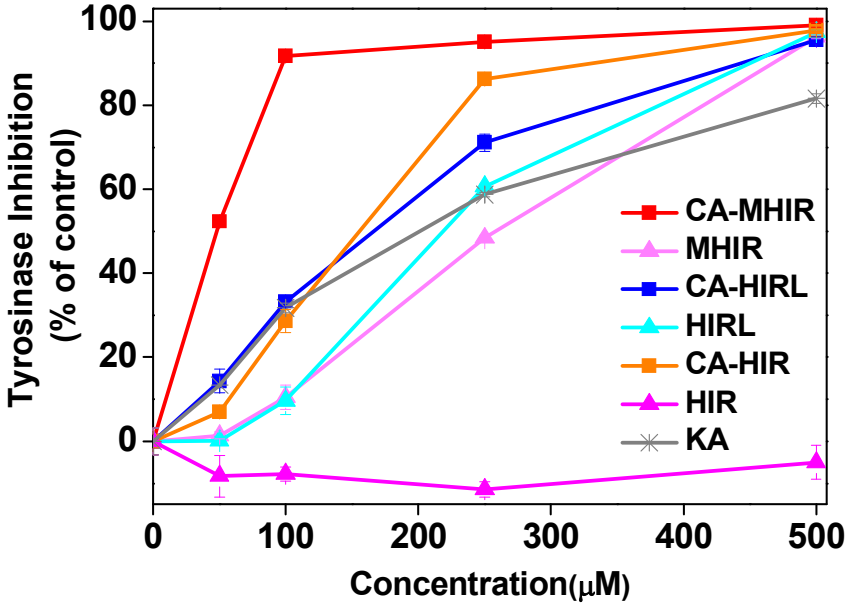


Figure 10. Tyrosinase inhibitory activity of CA-MDPs and MDPs at different concentrations. Tyrosinase inhibitory activity(%) was evaluated after treating 50, 100, 250, 500 μM of each inhibitor; substrate was 2.5 mM L-DOPA; reaction was performed at RT for 10 min. The UV absorbance of the colored reaction solution was measured at 475 nm respectively. The values are given as the mean ± standard error.

Table 2. IC_{50} Values of CA-MHIRL derived peptides (CA-MDPs)

Compound	IC_{50} (μM)
CA-MHIR	47.98
MHIR	257.17
CA-HIRL	166.24
HIRL	218.81
CA-HIR	154.87
HIR	-
KA	201.76

Inhibition kinetics of tyrosinase-mediated L-DOPA oxidation by CA-MHIR was explored. The K_m and V_{max} values can be determined by algebraic manipulation of the basic Michaelis-Menten equation, which is transformed into a straight-ine plot, *Lineweaver-Burk plot*. The V_{max} value was 0.10 nm min^{-1} at 25 μM, 0.073 nm min^{-1} at 50 μM and 0.056 nm min^{-1} at 75 μM of inhibitor. The K_m value was constant as 2.0 mM with various concentrations of CA-MHIR. The V_{max} values of tyrosinase activity was decreased without changing the K_m values when concentration of inhibitors was increased, which means that CA-MHIR can be regarded as a non-competitive inhibitor (Figure 11). These results indicate that CA-MHIR binds to tyrosinase other than the active site, accompanied by changing three dimensional conformation of tyrosinase. Active site of tyrosinase can still hold the specific substrate, L-DOPA. However, it is no longer in the optimal arrangement to catalyze the reaction.

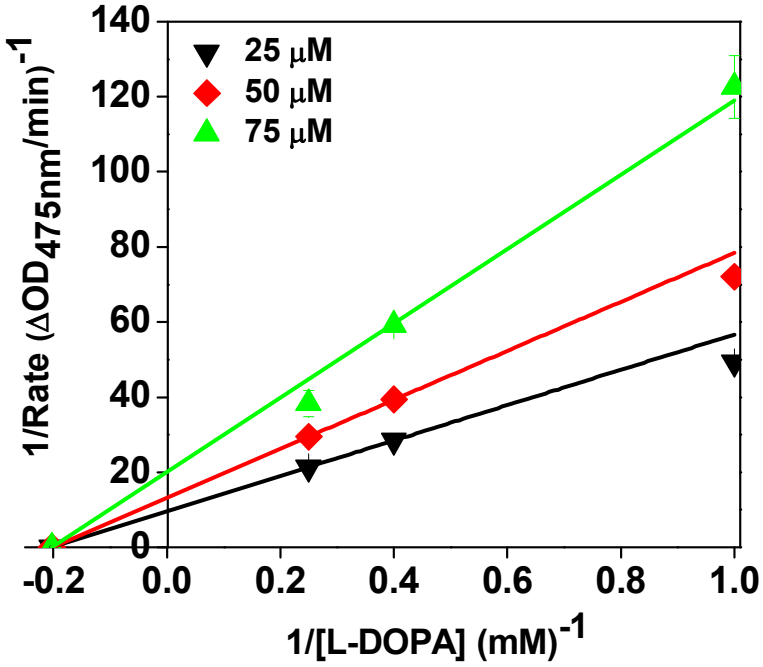


Figure 11. Lineweaver-burk plots of mushroom tyrosinase with L-DOPA as a substrate in the presence of CA-MHIR. Tyrosinase inhibitory activity(%) was determined at 25, 50, 75 μM of each inhibitor. The concentration of L-DOPA was 1.0, 2.5, 4.0 mM in each reaction. The reaction was performed for 0, 1, 2.5, 5, 10 min to evaluate the enzyme kinetics at RT. The UV absorbance of the reaction solution was measured at 475 nm respectively. The values are given as the mean ± standard error.

3. 6 Cytotoxicity of CA-MDPs in Cell System

The cytotoxicity of all the MDPs and CA-MDPs was examined by MTT assay in B16-F1 melanoma cell. There was no significant cytotoxic at 100 μM of all the compounds (Figure 12). Kojic acid and arbutin were used as controls, and they did not show any cytotoxicity in the cells.

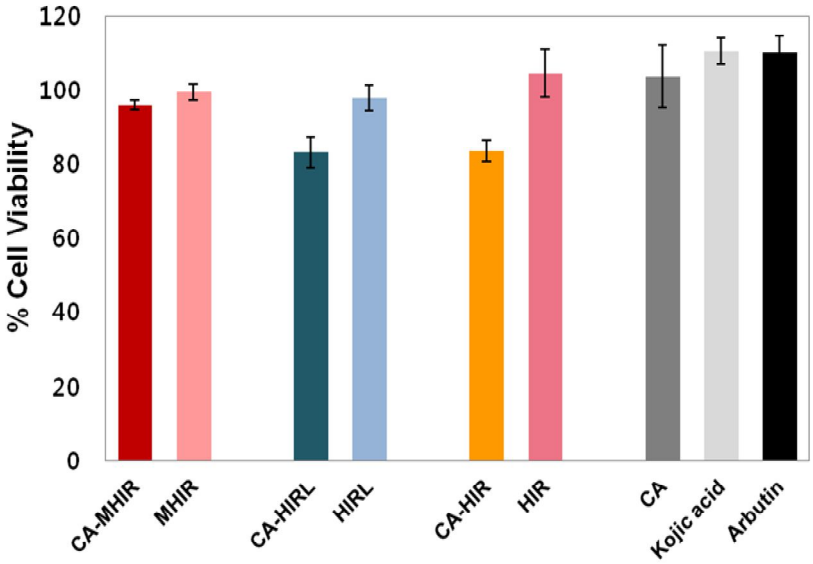


Figure 12. Cytotoxicity of CA-MDPs and MDPs in B16-F1 melanoma cells. The concentration of each sample was 100 μM.

Conclusion

Dual effector molecules which contain two properties, antioxidant activity and tyrosinase inhibitory activity, were synthesized. Coupling of caffeic acid (CA) to MHIRL derived peptide (MDPs: MHIR, HIRL, HIR) induced synergy effect in both activities. CA-MHIRL derived peptides (CA-MDPs) were synthesized by conventional solid-phase peptide synthesis (SPPS), and characterized by RP-HPLC and ESI-MS. Synthetic method was improved through the use of acetonide protected CA and the optimization of cleavage condition. Five peaks were observed from HPLC spectrum of the compounds synthesized by conventional method with the product and unknown impurities. However, a single peak of CA-MHIR was obtained by modified synthetic strategy. Antioxidant activity and tyrosinase inhibitory activity of dual effector molecules were evaluated by DPPH radical scavenging test, lipid peroxidation inhibition test, and mushroom tyrosinase inhibition test. From these assay result, we demonstrated that CA-MHIR exhibited highly enhanced tyrosinase inhibitory activity with better antioxidant activity than CA and the natural milk peptides. In mushroom tyrosinase inhibition test, CA-MHIR showed about 4 times lower IC_{50} value than kojic acid (KA). In addition, CA-MHIR was regarded as a non-competitive inhibitor by kinetic study of tyrosinase

inhibition. No cytotoxicity of CA-MDPs was observed in B16 melanoma cells.

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Abstract in Korea

인간의 몸 속에서 산화반응을 통해 생성되는 과량의 활성산소는 다양한 질병과 노화를 일으키는 중요한 원인이기에 이를 방지하는 항산화제에 대한 연구가 중요하다. 또, 인간의 피부와 눈의 색을 결정하는 멜라닌의 과량 생성은 피부질환과 신경변성과 관련된 질환이 생기므로 이에 관여하는 효소인 타이로시나제의 억제제가 개발되고 있다. 그 중에 우유에 있는 단백질 내 특정 펩타이드 서열이 멜라닌 생성을 억제한다는 논문이 발표된 바 있다.

본 연구에서는 항산화제인 카페익산 (CA) 과 우유 유래 미백제인 MHIRL 유래 펩타이드 (MHIR, HIRL, HIR) 를 결합시켜 항산화 활성과 미백 활성이 모두 보이는 물질을 개발했다. CA-MHIRL 유래 펩타이드는 고체상 펩타이드 합성으로 만들었고 고성능 액체크로마토그래피와 질량분석법으로 확인했다. 그러나 카페익산의 두 개의 하이드록시기가 합성과정에서 부반응을 일으켜 카페익산에 아세토나이드를 보호기로 붙이는 단계를 추가함으로써 합성 효율을 높였다. 또한, 고체상 펩타이드 합성 단계 중 Cleavage 용액 내에 있는 스캐빈저인 DODT 대신 물을 사용하여 부반응없이 99 %의 순도로 CA-MHIR을 합성하였다.

합성한 CA-MHIRL 유래 펩타이드의 능력을 DPPH 라디칼 제거, 지질의 과산화 억제, 타이로시나제의 저해의 3가지 분석법으로 활성을 평가했다. 3가지 분석법에서 가장 좋은 항산화 활성과 미백 활성을 갖는 물질은 CA-MHIR이었다. 타이로시나제 억제 실험과 반응속도록적 연구를 통해서 CA-MHIR의 IC_{50}값이 코직산보다도 약 4배 낮았으며 비경쟁적으로 작용하는 매우 효율적인 타이로시나제 억제제라는 것을 알 수 있었다.

CA-MHIRL 유래 펩타이드와 MHIRL 유래 펩타이드는 B16-F1 세포에서 세포 독성이 거의 없었다.

주요어 : 항산화제, 타이로시나제, 카페익산, 우유 단백질, 합성, 항산화 활성, 미백 활성
학 번 : 2014-20570

Appendix

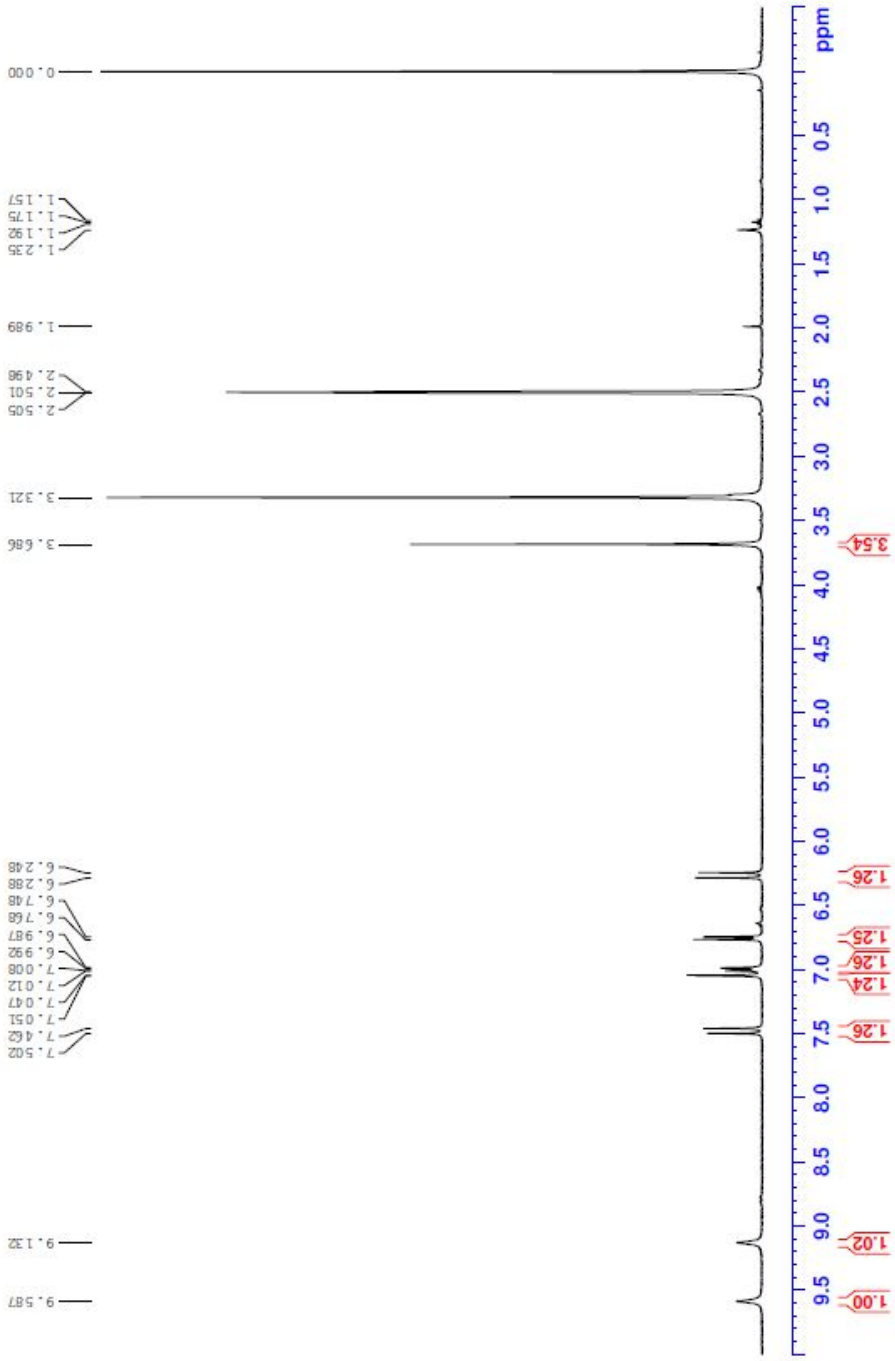

Figure A. 1 ^{1}H-NMR spectra of Caffeic acid methyl ester [CA-OMe].

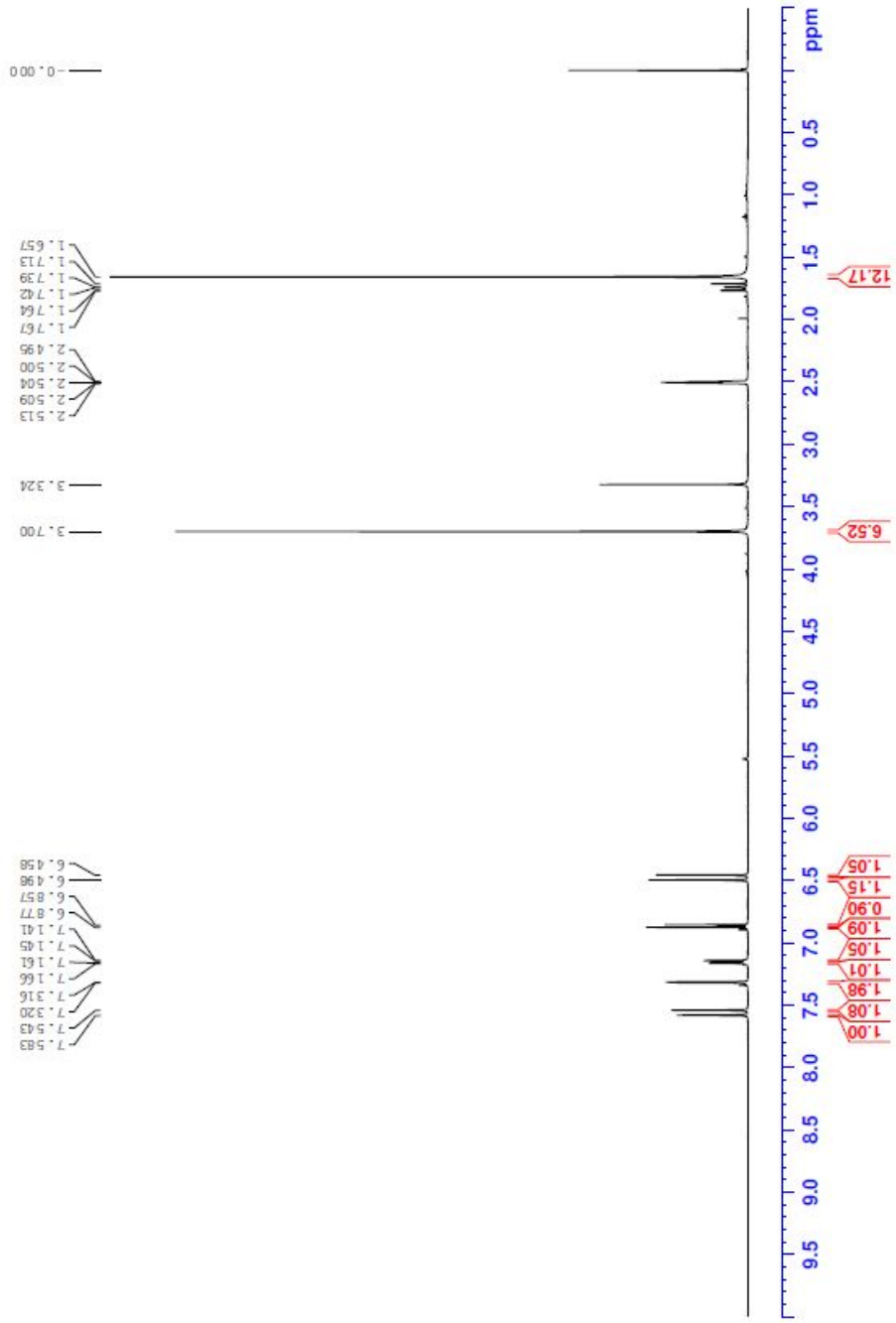

Figure A. 2 ^{1}H-NMR spectra of acetonide protected CA-OMe [CA(acetonide)-OMe].

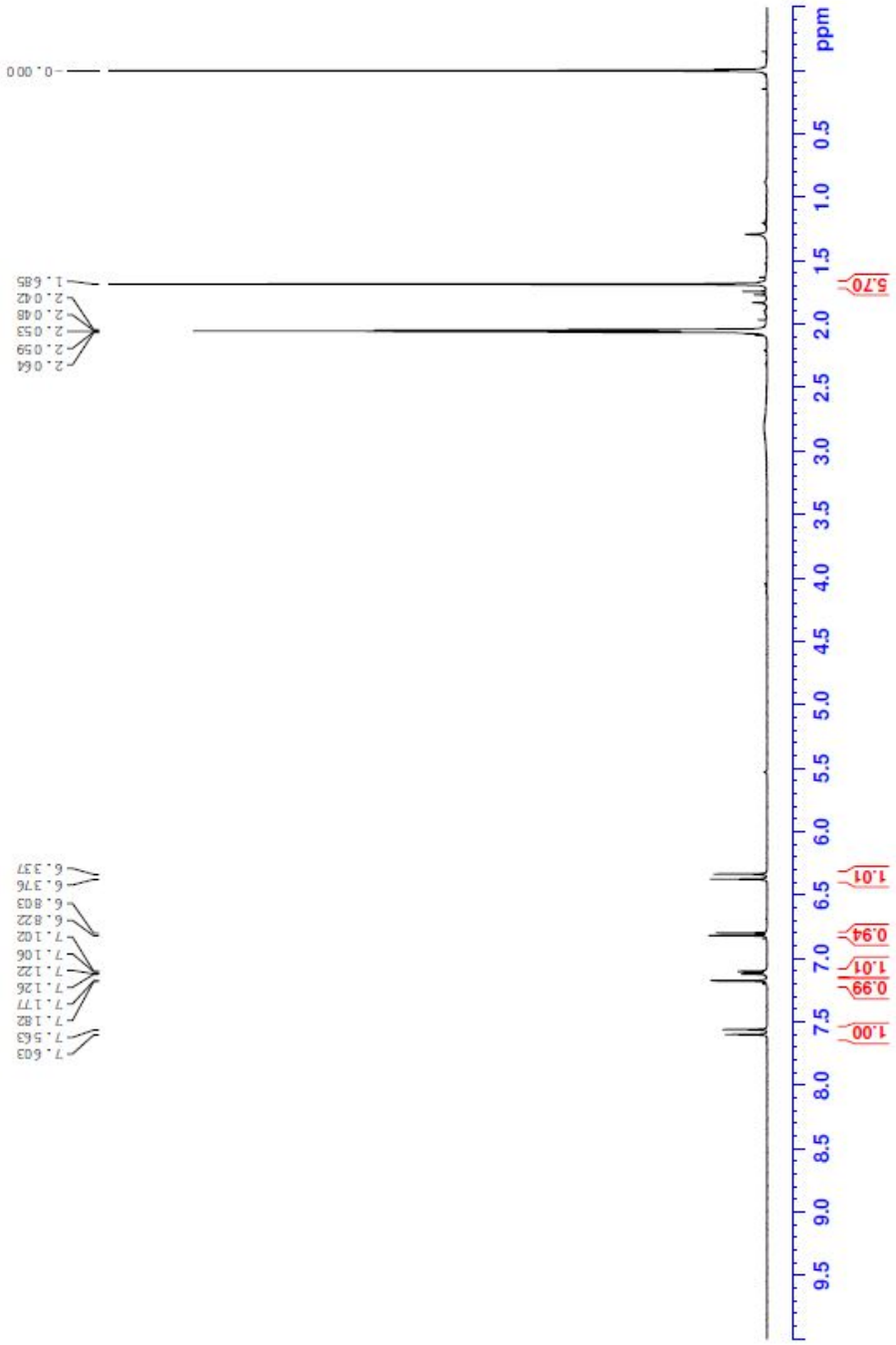

Figure A. 3 ^{1}H-NMR spectra of acetonide protected caffeic acid [CA(acetonide)-OH].