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RESEARCH NOTE

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Synthesis of a Novel Compound from Gallic Acid and Linoleic Acid and its Biological Functions

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Abstract Octadeca-9,12-dienyl-3,4,5-hydroxybenzoate (GA-LA) was chemically synthesized from gallic acid and linoleic acid ester, and its biological functions were tested. Radical-scavenging activity of GA-LA was comparable to those of gallic and ascorbic acids at 0.24 mM, and tyrosinase inhibition effect was higher than that of ascorbic acid. Gallic and linoleic acids did not show any tyrosinase activity. Results of cyclooxygenase (COX) inhibition effect indicate GA-LA has higher selectivity in COX-1 inhibition. GA-LA from gallic and linoleic acids could be used as functional reagent for antioxidative, skin-whitening, and anti-inflammatory effects in food, pharmaceutrical, and cosmetic industries

Key words: octadeca-9,12-dienyl-3,4,5-hydroxybenzoate, gallic acid, linoleic acid, NMR, biological function

Introduction

Gallic acid and its dimeric derivative, known as ellagic acid, exist either in a free form or bound as gallo- and ellgitannins, respectively (1). These hydrolysable tannins are present in a rich variety of plants such as tea, red wine, fruits, beverages, and various medicinal plants (2). Gallic acid has a wide range of medicinal and industrial applications including as an antioxidant, antiplatelet agents, and a mediator in the modulation of genotoxicity of food carcinogens (3, 4), and a starting material in the mesomorphic material engineering (5), as well as possesses anti-carcinogenic, antioxidative, antimutagenic, anti-allergic, and anti-inflammatory activities (6). It has also been used as a building block of choice for different pharmaceutical leads due to the presence of this moiety in several bioactive natural products (7). However, in spite of these advantages, the cytotoxic and skin-trouble effects of gallic acid such as the prooxidant effect caused by copper-dependent DNA damage have also been reported (8).

Propyl gallate has been used as an antioxidant and preservative in foods, drugs, cosmetics, and pesticide products since 1948. This compound has been graded as Generally Recognized As Safe (GRAS) by the US FDA, and is listed in the Everything Added to Food in the United States (EAFUS) database and Food Chemicals Codex. The Joint Food and Agricultural Organization/ World Health Organization Expert Committee on Food Additives has established an acceptable daily intake of 0-1.4 mg/kg/day for this compound. This value is 1/100 of the 'no observed effect' level determined from a 90-day feeding study of rats (9). Recently, Wacher and Benet (10) developed a new formulation for pharmaceutical compounds, gallic acid esters, to enhance the bioavailability of the active ingredient of the pharmaceutical compounds. The authors used medium chain fatty acids (C6 to C14) and not the long chain fatty acids such as linoleic acid (C18:2). Negi *et al.* (6) also synthesized a novel plant growth promoter by modifying gallic acid into naphtophenone derivatives with esterified fatty acid side chains. Linoleic acid, α-linoleic acid, and docosahexaenoic acid showed anti-inflammatory effect by decreasing the secretion of interleukin (IL)-6 and -1β, and the tumor necrosis factor-α (11). Recently, Liu *et al.* (12) reported that hydrogel nanoparticles can be prepared using a linoleic acid-modified chitosan as a carrier for biomolecules in the fields of biotechnology and pharmaceutical sciences.

The objective of this study was to synthesize a novel compound, octadeca-9,12-dienyl-3,4,5-hydroxybenzoate (GA-LA), from gallic acid and linoleic acid ester and to investigate its biological functions to evaluate its potential use in the food, pharmaceutical, and cosmetic industries.

Materials and Methods

Chemicals Linoleic acid ethyl ester, gallic acid, diisobutylaluminum hydride (DIBAL-H), tetrahydrofuran (THF), and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Synthesis The general scheme of the synthesis is shown in Fig. 1.

Octadeca-9,12-diene-ol To a solution of linoleic acid ethyl ester (3 mM, compound **1**) in CH_2Cl_2 (10 mL) cooled at -78°C, DIBAL-H (6–9 mM) was slowly added under a N_2 atmosphere. After stirring the solution for 30 min, an aqueous $NaHCO_3$ was slowly added. The reaction mixture was diluted with methyl alcohol and filtered. The resulting filtrate was then evaporated under a reduced pressure and purified by column chromatography (SiO_2; elution with hexane/ethanol, 10:1). The structure was established by spectroscopic methods (FT-IR and NMR).

Octadeca-9,12-diene-ol **2** was obtained at a 90% yield

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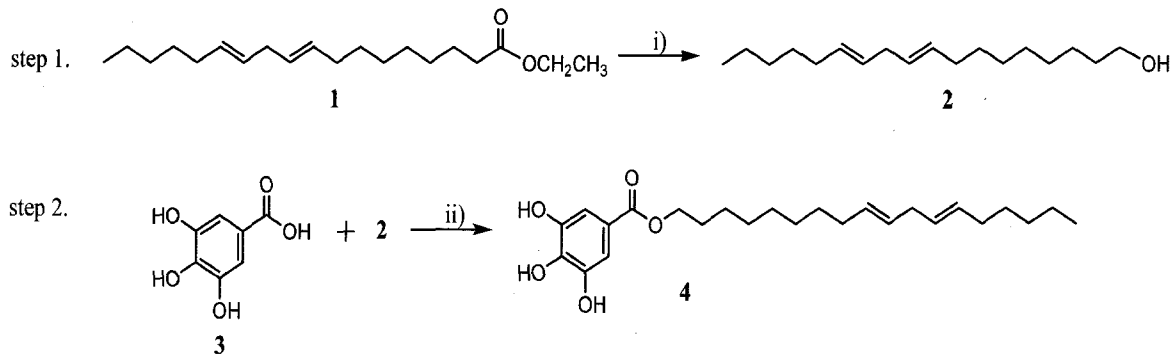

Fig. 1. Scheme for a chemical synthesis of octadeca-9,12-dienyl-3,4,5-hydroxybenzoate. Compounds: **1**, linoleic acid ethyl ester; **2**, octdeca-9,12-diene-ol; **3**, gallic acid; **4**, octadeca-9,12-dienyl-3,4,5-hydroxybenzoate. Reagent and conditions: i) diisobutylaluminum hydride (2-3 M eq. of compound **1**) $CHCl_2$ at -78°C, ii) *N,N'*-dicyclohexylcarbodiimide (3 M eq.), tetrahydrofuran at 0°C to room temperature for 20 hr.

as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (t, J = 7.5 Hz, 3H, -CH_3), 1.31 (m, 18H, H2-7, H15-17), 1.53 (m, 2H, H14), 2.06 (dd, J = 5.7, 9.4 Hz, 2H, H11), 3.58 (t, J = 6.6Hz, -CH_2OH), 5.35 (m, 4H, H9, H10, H12, H13); IR ($CHCl_3$) 3332, 3009, 2962, 2856, 2362, 1649, 1459, 1056 cm^{-1}.

Octadeca-9,12-dienyl 3,4,5-trihydroxybenzoate (GA-LA) To a solution of gallic acid (5.5 mM) and compound **2** (5.5 mM) in tetrahydrofuran THF (15 mL) cooled at 0°C, a solution of DCC (16.5 mM) in THF (7 mL) was added. After stirring the solution at room temperature for 20 hr, the solvent was removed under a reduced pressure. The residue was extracted with ethyl acetate and filtered. The filtrate was washed successively with a diluted aqueous citric acid solution (5%), saturated aqueous $NaHCO_3$ solution, and water, dried by $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography (SiO_2; elution with $CHCl_3$/MeOH, 95:2). The final product was confirmed by FT-IR and NMR spectroscopies. The yield of GA-LA was approximately 80±0.27%.

GA-LA **4** was obtained at an 85% yield as a pale yellow powder: ^{1}H NMR (300 MHz, $CDCl_3$)δ 0.91 (t, J = 6.8 Hz, 3H, H18), 1.31 (m, 16H, H3-7, H15-17), 1.72 (m, 2H, H2), 2.06 (m, 4H, H8, H14), 2.79 (dd, J = 5.9, 5.9 Hz, 2H, H11), 4.26 (t, J = 6.4 Hz, 2H, -OCH_2), 5.37 (m, 4H), 7.28 (s, 1H, aromatic), 7.30 (s, 1H, aromatic); IR ($CHCl_3$) 3356, 3008, 2928, 2855, 1686, 1613, 1465, 1395, 1242, 1184 cm^{-1}.

Radical-scavenging activity To observe the antioxidative activity of the newly synthesized compound, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging effect was evaluated. The sample was dissolved in ethanol to achieve 0.024 and 0.24 mM concentrations, and the free radical-scavenging effect was estimated according to the method of Blois (13). A sample (1 mL) was added to 0.2 mM DPPH (1 mL), and the mixture was shaken and placed at room temperature for 30 min. The reaction mixture was measured at 517 nm using a spectrophotometer (UV 1600 PC; Shimadzu, Tokyo, Japan). Ascorbic acid was used to compare the activity with the new compound.

Tyrosinase inhibition activity To observe the skin-whitening effect of the sample for an evaluation of its possible use in the cosmetic industry, a tyrosinase inhibition effect was evaluated. The sample was dissolved in ethanol (1.2 mM), and 0.2 mL was added to the reaction mixture containing 10 mM L-3,4-dihydroxyphenyl-alanine (L-DOPA, Sigma Chemical Co.) solution, 1/15 M sodium phosphate buffer (pH 6.8), and mushroom tyrosinase (100 unit/mL, Sigma-Aldrich Co.). The reaction mixture was incubated at 25°C for 15 min. The amount of dopachrome produced in the reaction mixture was determined at 475 nm (14) by a spectrophotometer (UV 1600 PC; Shimadzu).

Cyclooxygenase (COX) assay To investigate the anti-inflammatory activity of the sample, the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitions were tested by measuring the prostaglandin E_2 (PGE_2) using a COX Inhibitor Screening Kit (Catalog No 560131; Cayman Chemicals, Ann Arbor, MI, USA) following the method of Murias *et al.* (15). Reaction mixtures were prepared in 100 mM Tris-HCl buffer, pH 8.0, containing 1 μM heme and COX-1 (ovine) or COX-2 (human recombinant), and pre-incubated for 10 min in a water bath (37°C). The reaction was initiated by the addition of 10 μL arachidonic acid (final concentration in reaction mixture 100 μM). After 2 min the reaction was terminated by adding 1 M HCl, and PGE_2 was quantified by an enzyme-linked immunosorbent assay (ELISA) method. The test compounds were dissolved in dimethylsufoxide (DMSO) and diluted to the desired concentration with a 100 mM potassium phosphate buffer (pH 7.4). Following a transfer to 96-well plates coated with a mouse anti-rabbit IgG, the tracer prostaglandin acetylcholine esterase and primary antibody (mouse anti PGE_2) were added. Plates were then incubated at room temperature overnight, reaction mixtures were removed, and the wells were washed with a 10 mM postassium phosphate buffer containing 0.05% Tween 20. Ellman's reagent (200 μL) was added to each well, and the plate was incubated at room temperature in darkness for 60 min until the control wells yielded an O.D.=0.3-0.8 at 412 nm. A standard curve with PGE_2 was generated from the same plate, which was used to quantify the PGE_2 levels produced in the presence of the test samples. Results were

Table 1. Radical scavenging and tyrosinase inhibition activity of octadeca-9,12-dienyl-3,4,5-hydroxybenzoate

	Conc. (mM)	GA-LA[1)]	Gallic acid	Linoleic acid	Ascorbic acid
Radical scavenging (%)	0.24	95.7±0.58[a3)]	95.2±0.63[a]	-[b2)]	96.2±0.42[a]
	0.024	69.6±0.28[c]	82.8±0.24[b]	-	86.5±0.06[a]
Tyrosinase inhibition (%)	1.20	90.9±0.33[a]	-[c]	-[c]	63.7±0.17[b]

[1)]Octadeca-9, 12-dienyl-3,4,5-hydroxybenzoate.
[2)]Activity was not detected.
[3)]Different letters within the same column differ significantly ($p<0.05$).

expressed as percentages relative to a control (solvent-treated samples). All determinations were duplicated, and the values generally agreed to within 10%.

Statistical analysis All experiments were duplicated with three observation numbers adapted for each experiment. Analysis of the variance was performed using the raw data, and the mean values and standard deviation were calculated by the Statistical Analysis System (16). Differences among the mean values were determined by the Duncan's multiple range test with a significance defined at $p<0.05$.

Table 2. Cyclooxygenase inhibition rate of the synthesized octadeca-9,12-dienyl-3,4,5-hydroxybenzoate

Concentration (μM)	Inhibition rate (%)	
	COX-1	COX-2
0.01	55±1.2	10±0.8
0.1	64±1.5	21±0.9
1	67±1.3	28±0.9
10	74±2.1	32±1.1

Results and Discussion

GA-LA was synthesized using standard chemical methodologies. Firstly, octadeca-9,12-diene-ol was obtained, and gallic acid was esterified using this compound. The procedures afforded a yield of around 80%. The synthesized compound was identified by FT-IR, and ^{1}H and ^{13}C NMR.

Free radicals and reactive oxygen species have been proposed to induce cellular damage and to be involved in several human diseases such as cancer and arteriosclerosis inflammatory disorders, as well as in aging processes (17,18). Thus, the DPPH radical-scavenging ability of our novel compound, GA-LA, was measured (Table 1). The novel compound showed obvious scavenging activity on DPPH radical compared with gallic acid, however, its synergistic effect was not observed at each concentration (0.024 and 0.24 mM). The inhibitory effect of tyrosinase, a key enzyme in the melanin biosynthesis, was also examined to investigate its application in cosmetic materials. The tyrosinase inhibitory activity of GA-LA was higher (90.9%) than that of ascorbic acid (63.7%) at 1.2 mM (Table 1), whereas gallic acid and linoleic acid alone did not show any tyrosinase inhibitory activity. An *et al.* (19) reported that green tea polyphenol at 200 ppm showed more than 60% tyrosinase inhibition and demonstrated that the activity was higher than those of green laver, codiaceae, and bundles (30, 11, and 24%, respectively) at 1,000 ppm (20). The 50% tyrosinase inhibitory concentration (IC_{50}) of kojic acid (IC50 = 1.1 μg/mL) showed greatest activity when compared with arbutin (65 mg/mL), mulberry root powder (9.2 μg/mL), and epigallocatechin gallate (80 μg/mL) (21).

Prostagladins (PGs) are known to be involved in many physiological and pathological processes including inflammation (22), bone resorption (23), and ovulation (24). The biosynthesis of PGs was catalyzed from arachidonic acid by COX enzyme. The most well known COX isoforms are COX-1 and COX-2. COX-1 is constitutively expressed and is responsible for maintaining normal physiologic function. The PGs produced by this enzyme play a protective role. On the other hand, COX-2 is the principal isoform that participates in inflammatory and other chronic diseases including cancer (25) and rheumatic arthritis (26). Since the recognition of these facts, numerous studies have focused on the development of COX-2 inhibitors (27). However, recent findings that the inflammatory response can be maintained by COX-2 knock-out mice have raised questions (28). A COX-1 inhibitor, indolmethacin, inhibited human breast cancer, which was transplanted into nude mice (29). Therefore, dual inhibition of COX-1 and COX-2 can have beneficial effect on most of the chronic inflammatory diseases, and could be more effective and safe in respect to the selective COX-2 inhibitor (30). Anti-inflammatory effects of non-steroidal anti-inflammatory drugs are attributable to their ability to inhibit COX-1 and COX-2 enzymes (31).

Our novel compound, GA-LA, was tested on COX-1 and COX-2 enzymes. The compound showed a dose-dependant inhibition response on both COX-1 and COX-2. Inhibition rates of COX-1 and COX-2 at 0.01 μM of minimum concentration of the compound were 55 and 10%, respectively, and increased considerably up to 10 μM (maximum concentration tested). IC_{50} value for COX-1 of GA-LA was found to be less than 0.01 μM, whereas much higher for COX-2. The result indicates that this compound has more selective inhibition response on COX-1 enzyme. Murias *et al.* (15) reported that IC_{50} of COX-1 was 0.53 μM by resveratrol, while approximately 0.01 μM by synthesized hydroxylated resveratrol analogues. On the other hand, celecoxib, a commercial selective COX-2 inhibitor currently used in the clinics, showed IC_{50} of 19.0 and 0.04 μM for COX-1 and COX-2 inhibitions, respectively.

These results indicate the newly synthesized compound, GA-LA, from gallic acid and linoleic acid can be used as a functional reagent for antioxidative, skin-whitening, and anti-inflammatory effects in the food, pharmaceutical, and cosmetic industries. Additionally, various fatty acids with different chain lengths can be applied to alter its hydro-

phobicity.

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