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NOTE

Synthesis of Dilinoleoyl-D-Glyceric Acid and Evaluation of Its Cytotoxicity to Human Dermal Fibroblast and Endothelial Cells

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Abstract: A novel derivative of glyceric acid (GA), dilinoleoyl-D-glyceric acid (LA_2-DGA), was synthesized from D-GA calcium salt and linoleoyl chloride and evaluated for cytotoxicity. The D-GA calcium salt was first reacted with 4-methoxybezylchloride, and the resulting compound was esterified with linoleoyl chloride. This reaction was followed by hydrolysis of the 4-methoxybenzyl moiety, yielding LA_2-DGA. LA_2-DGA was then subjected to cytotoxicity testing using normal human dermal fibroblasts and primary normal human dermal microvascular endothelial cells. LA_2-DGA showed no significant toxic effects in either type of cell.

Key words: glyceric acid, linoleoyl glyceric acid, cytotoxicity, biodiesel fuel, glycerol use

1 INTRODUCTION

Biodiesel fuel is now widely produced via transesterification of alcohols and triacylglycerols such as vegetable oils and animal fats under alkaline conditions. This reaction yields glycerol as a by-product in amounts equivalent to approximately 10 wt% of the initial triacylglycerols. The production of biodiesel fuel is rapidly increasing, and the development of efficient applications for this residual glycerol is an urgent issue from both industrial and fundamental viewpoints.

We have developed a biotechnological method for converting glycerol to glyceric acid (GA) using acetic acid bacteria as biocatalysts[1-7]. Enantioselective formation of D-GA with 99% enantio excess can also be achieved by *Acetobacter tropicalis* at more than 100 g/L in culture broth. D-GA is found in a variety of plants[8-10], and phosphoesters of D-GA are also common intermediates of sugar metabolism. Additionally, D-GA itself has some biological properties, including diruetic[9], liver-stimulating, and cholesterolytic activities[11] and the ability to accelerate ethanol and acetaldehyde oxidation[12]. Because D-GA has potential use not only as a starting material for fine chemicals but also as a biologically active ingredient in foods and cosmetics, we are currently researching the synthesis and properties of D-GA derivatives.

Linoleic acid (LA), a dominant constituent of safflower and sunflower oils, is a polyunsaturated fatty acid with *cis*-double bonds at C9 and C12. LA is the most abundant fatty acid *in vivo*[13] and is found in the lipids of the cell membrane[14]. LA is used as a substrate for the biosynthesis of eicosanoids such as prostaglandins, leukotrienes, and thromboxane via arachidonic acid, and LA has been reported to influence various physiological activities, such as trypsin inhibition, cell signaling, and cell surface effects. In particular, LA is sensitive to oxidative stress, because the aryl proton between the double bonds in the acyl chain undergoes oxidation induced by reactive oxygen species. This feature may make LA useful for evaluating antioxidant activity, and several methods have been developed for this purpose[15, 16].

LA has already been used as an ingredient in skin care and hair tonic preparations[17, 18], and it activates peroxisome proliferator-activated receptors, which could accelerate epidermal development *in vitro*[19]. Because D-GA is also a biological molecule, conjugated compounds of D-GA and LA are of interest for their functions relating to skin

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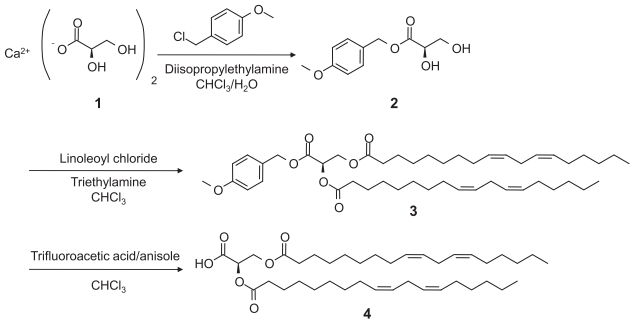

Scheme 1 Synthesis of dilinoleoyl-D-glyceric acid.

care.

In this study, a novel derivative of D-GA esterified with LA (dilinoleoyl-D-glyceric acid [LA_2-DGA]; Scheme 1) was synthesized and its cytotoxicity in two kinds of human dermal cells was evaluated.

2 EXPERIMENTAL PROCEDURES

2.1 Chemicals

All reagents and solvents were of the highest purity commercially available. D-GA calcium salts were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution (PSS), and 0.25% trypsin-ethylenediaminetetraacetic acid solution were purchased from nacalai tesque (Kyoto, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). Dulbecco's phosphate-buffered saline was purchased from Nissui Pharmaceutical (Tokyo, Japan). A CS-C medium kit R consisting of CSC medium, CultureBoost-R (containing human recombinant growth factors) and Attachment Factor™ was purchased from Cell Systems (Kirkland, WA).

2.2 Synthesis of 4-methoxybenzyl-D-glycerate (2)

To a dispersion of GA calcium salt (1; 1.00 g, 8.0 mmol) in 10 mL of chloroform and 2 mL of distilled water was added diisopropylethylamine (2.0 mL, 12 mmol) and 4-methoxybezylchloride (1.56 g, 10 mmol) at room temperature with stirring. After heating at 60℃ for 4 h, 2 mL of distilled water, 2.0 mL of diisopropylethylamine (12 mmol), and 1.56 g of 4-methoxybenzyl-chloride (10 mmol) were added to the mixture, which was kept at 60℃ with stirring for an additional 3 h. Then 2.0 mL of diisopropylethylamine (12 mmol) and 1.56 g of 4-methoxybenzylchloride (10 mmol) were added, and the mixture was stirred overnight at room temperature. In total, 3.75 equivalents of 4-methoxybezylchloride to GA calcium salt were used to increase the reaction yield. After the reaction, 6 mL of chloroform and 5 mL of brine were added to the reaction mixture. The water phase was washed twice with 15 mL of chloroform. The chloroform phases were combined, dried over magnesium sulfate, and evaporated. Approximately 6.5 g of the resulting yellowish oily material were purified with silica gel column chromatography using elution of 2:1, 1:1, and 0:1 *n*-hexane:ethyl acetate. The fractions containing the target compound were collected and evaporated. The colorless crystalline compound was obtained with a yield of 0.26 g (14%).

2.3 Synthesis of dilinoleoyl-(4-methoxybenzyl)-D-glycerate (3)

To a mixture of 2 (0.26 g, 1.15 mmol) and chloroform (7 mL) was added triethylamine (0.6 mL, 4 mmol) and a linoleoyl chloride (0.86 g, 2.9 mmol)/chloroform (3 mL) solution. The mixture was stirred for 1.5 h at room temperature. Then 0.6 mL of triethylamine (4 mmol) and 0.86 g of linoleate chloride (2.9 mmol)/chloroform (3 mL) solution were added to the mixture, which was stirred for an additional 2 h. After the reaction, 50 mL of ethyl acetate and 30 mL of water were added to the reaction mixture. The ethyl acetate phase was extracted, washed twice with brine, dried over magnesium sulfate, and evaporated. Approximately 2.9 g of the resulting yellowish oily material was purified with silica gel column chromatography using elution with *n*-hexane:ethyl acetate (10:1). Fractions containing the target compound were collected and evaporated. A colorless oily compound was obtained in a yield of 0.67 g (78%).

2.4 Synthesis of dilinoleoyl-D-glyceric acid (4)

To a mixture of 3 (0.67 g, 0.9 mmol), anisole (0.2 mL), and chloroform (10 mL) was added 2.0 mL of trifluoroacetic acid (27 mmol). The mixture was stirred for 20 min at room temperature. Then 20 mL of trifluoroacetic acid was added, and the mixture was stirred for 1 h. After concentration under vacuum, the reaction mixture was washed with 50 mL of ethyl acetate, 50 mL of water, and 5 mL of saturated disodium carbonate aq., and the organic layer was extracted. The water phase was washed with 15 mL of ethyl acetate, and all of the ethyl acetate fractions were combined and washed with 30 mL of brine. After drying with magnesium sulfate, the organic layer was concentrated under vacuum. Approximately 1.0 g of yellowish oily material was purified with silica gel chromatography using elution with 10:1 and 5:1 of *n*-hexane:ethyl acetate and 5:1 of ethyl acetate:methanol. The fractions containing the target compound were collected and evaporated. A colorless oily compound was obtained in a yield of 0.29 g (52%). The assignments in the ^{1}H NMR spectrum of 4 are shown in Table 1.

2.5 Analytical measurements

The purity of the compounds was checked on thin-layer chromatography using Silica gel 60 F254 (Merck, Darmstadt, Germany) with a solvent system of ethyl acetate:

Table1 ^{1}H NMR data for dilinoleoyl-D-glyceric acid ($CDCl_3$, 400 MHz)

	^{1}H-NMR Δ (ppm)	*J* (Hz)
GA		
H-2	5.29-5.42 m	
H-3a	4.43 dd	5.4, 12.0
H-3b	4.55 dd	3.3, 12.1
Acyl group		
–CO-$\underline{CH_2}$–(at C-2)	2.42 td	3.0, 7.6
–CO-$\underline{CH_2}$–(at C-3)	2.34 t	7.6
–CO-$CH_2\underline{CH_2}$–	1.60-1.67 m	
–$(CH_2)_n$–	1.23-1.39 br	
–CH=CH–	5.29-5.42 m	
–CH=CH-$\underline{CH_2}$–	2.05 q	6.8
CH=CH$\underline{CH_2}$CH=CH–	2.77 t	6.6
–CH_3	0.89 t	7.0

methanol(5:1)to develop the chromatogram, and analyzed with a GC-2010/AOC-20i(Shimadzu, Kyoto, Japan)fitted with a fused silica, chemically bonded capillary column (DB-5, 1.0 μm, 30 m; J&W Scientific, Folsom, CA). Each sample(1 μL; 1 mg/mL)was injected into the column at 100℃. The column temperature was increased by 10℃/min to 250℃. The flow rate of the helium carrier gas was 2.5 mL/min. Structural determination of the purified product dissolved in $CDCl_3$ was performed at 27℃ using ^{1}H NMR analysis with an AV-400 NMR spectrometer(400 MHz; Bruker, Karlsruhe, Germany).

2.6 Cytotoxicity testing

The cytotoxicity of dilinoleoyl-D-glyceric acid(LA_2-DGA) was evaluated using normal human dermal fibroblasts (NHDF; an original cell line provided by Applied Cell Biotechnologies, Inc., Yokohama, Japan)and primary normal human dermal microvascular endothelial cells(HME; Cell Systems). Growth media used for NHDF and HME were DMEM containing 10% FBS and 1% PSS, and CSC medium containing 10% FBS, 1% PSS, and 2% CultureBoost-R, respectively. Both cells were grown under 5% CO_2 at 37℃. A stock solution(20 g/L, 32 mM)of LA_2-DGA was prepared in dimethyl sulfoxide and diluted with the growth media for NHDF or HME to 0.002, 0.2, and 20 mg/L(0.0032, 0.32 and 32 μM, respectively). At concentrations of LA_2-DGA higher than 20 mg/L, the diluted solution became turbid. Aliquots of 100 μL of the cell solution containing 5×10^4/mL were incubated in 96-well plates for 1 d. Before the incubation of HME cells, the inner surface of each well was coated with Attachment Factor™. Then the culture was transferred to respective growth media containing defined sample concentrations and incubated for an additional 1 and 3 d. After removal of the culture, 100 μL of a 10% viability test reagent(Cell Count Reagent SF; nacalai tesque) in growth media was added to each well and the cells were incubated under 5% CO_2 at 37℃ for 3 h. During the incubation period, optical density at 450 nm(OD_{450})was measured every hour and viability was evaluated at OD_{450} after 2 h of incubation. Data are expressed as cell viability(%) relative to a control culture without supplementation.

3 RESULTS AND DISCUSSION

3.1 Synthesis of dilinoleoyl-D-glyceric acid

The low solubility of GA calcium in nonpolar organic solvents, such as tetrahydrofuran and dichloromethane, limits its use in the organic synthesis of GA derivatives. To address this, D-GA calcium salt was first reacted with 4-methoxy-benzylchloride in the presence of diisopropylethylamine, forming compound **2** with a yield of 14%. Ester **2** could be dissolved in chloroform, and successive esterification of two hydroxyl groups in **2** with linoleoyl chloride was achieved in the presence of triethylamine (yield, 78%). After removal of the 4-methoxybenzyl moiety in **3** by trifluoroacetic acid(52% yield), purified LA_2-DGA was obtained with an overall yield of 5.7%. We repeated the same procedure, and a total of 1.31 g of a colorless oily compound was obtained as possible LA_2-DGA. The gas chromatography analysis of this compound revealed 19.3 min and 94% of its retention time and purity, respectively(data not shown). The compound was identified as LA_2-DGA using ^{1}H NMR analysis(see **Table 1**).

3.2 Cytotoxicity testing

The cytotoxicity of LA_2-DGA was analyzed with NHDF and HME to evaluate the affinity of LA_2-DGA with skin because both types of cells are originally from dermal tissues. **Figure 1** illustrates the cytotoxic effect of LA_2-DGA on NHDF at concentrations from 0.0032 to 32 μM. Compared to the negative control, LA_2-DGA showed no cytotoxicity on cells cultured for 1 or 3 days. The cytotoxicity of LA_2-DGA was also evaluated using HME(**Fig. 2**). At the concentrations tested, LA_2-DGA showed no significant cytotoxicity in HME, indicating that LA_2-DGA has no cytotoxic effects to human dermal cells. The low solubility of LA_2-DGA in water limits further investigation at concentrations of LA_2-DGA higher than 32 μM; however, the low cytotoxicity to human dermal cells makes this compound appropriate as a material for skin care products and cosmetics if useful functions are found. Owing to the LA moiety in LA_2-DGA, it will be interesting to evaluate its responses and properties in an oxidative stress environment.

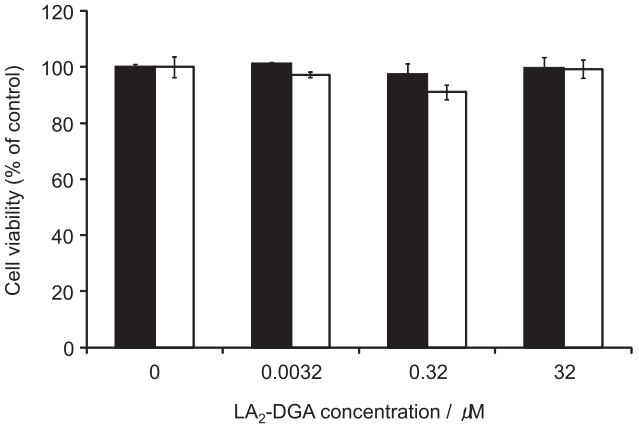

Fig. 1 Effects of dilinoleoyl-D-glyceric acid (LA_2-DGA) on normal human dermal fibroblast viability. The cells were cultured in Dulbecco's modified Eagle's medium containing both 10% fetal bovine serum and 1% penicillin-streptomycin solution with LA_2-DGA at defined concentrations for 1 day (black bar) and 3 day (white bar).

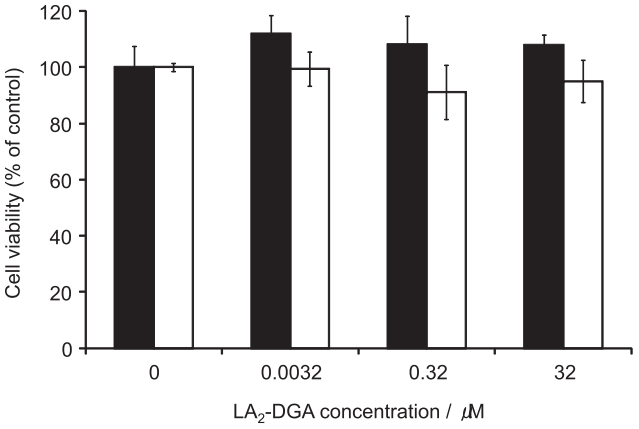

Fig. 2 Effects of dilinoleoyl-D-glyceric acid (LA_2-DGA) on cell viability of normal human dermal microvascular endothelial cells. The cells were cultured in CSC medium containing 10% fetal bovine serum, 1% penicillin-streptomycin solution, and 2% CultureBoost-R with LA_2-DGA at defined concentrations for 1 day (black bar) and 3 days (white bar).

4 CONCLUSIONS

We synthesized LA_2-DGA and evaluated its cytotoxicity in human dermal cells. The D-GA calcium salt was successfully converted to LA_2-DGA via 4-methoxybenzyl-D-glycerate. Cytotoxicity testing in NHDF and HME revealed that LA_2-DGA showed no cytotoxic effects in either of the cell types at concentrations between 0.0032 and 32 μM. These results suggest that LA_2-DGA could potentially be used as an ingredient in skin care products and cosmetics if useful properties and functions are discovered.

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