J. Biochem. 132, 327–334 (2002)

Formation of (*R*)-2,3-Dihydrogeranylgeranoic Acid from Geranylgeraniol in Rat Thymocytes[1]

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Received May 20, 2002; accepted June 10, 2002

In a metabolic study of [1-^{14}C]geranylgeranial involving rat thymocytes, the radioactivity was mainly incorporated into two metabolites, Z1 and Z2, the latter moving slower than the former on a silica-gel thin-layer plate. The time course of Z1 and Z2 formation superficially suggested a precursor-product relationship between Z1 and Z2. The two metabolites were chemically converted to their methyl esters on treatment with trimethylsilyl diazomethane. Z1 was cochromatographed with *E,E,E*-geranylgeranoic acid (GGA). Z2 was prepared in a large quantity from geranylgeranial using thymocytes, and purified by TLC followed by ESI (negative ion mode) or EI mass-spectrometry. The observation of a negative ion at *m/z* 305 on ESI and a molecular ion at *m/z* 306 ($C_{20}H_{34}O_2$) with fragments similar to GGA on EI implied that Z2 was dihydroGGA, which has been detected in the urine and serum of patients with Refsum disease. The EI mass spectrum of (*R*)-2,3-dihydroGGA was identical to that of Z2. The diastereomeric amide synthesized from metabolite Z2 with (*R*)-1-(1-naphtyl)ethylamine was cochromatographed with (*R* acid, *R*) amide, not with (*S* acid, *R*) amide, which were similarly synthesized from (*R*)- and (*S*)-2,3-dihydroGGAs, respectively. In another metabolic study on [1-^{14}C]geranylgeraniol (GGOH), the radioactivity was similarly incorporated into a metabolite corresponding to (*R*)-2,3-dihydroGGA. (*R*)-2,3-DihydroGGA induced DNA ladder formation with a maximum at 15 μM in thymocytes. However, 2,3-dihydrofarnesoic acid did not induce it at all.

Key words: apoptosis, dihydrogeranylgeranoic acid, geranylgeraniol, geranylgeranoic acid, isoprenoid, Refsum disease.

Geranylgeranylated proteins (Rho, Rac, and Rab) have been established to play an essential role in signal transduction, vesicle transportation, and cell proliferation (*1–2*). The prenyl-lipid precursor is GGPP, which is supplied from FPP and IPP through the action of GGPP synthase (*3*). Recently, it was found that GGPP is converted to GGOH through the action of a specific pyrophosphatase located in microsomes of rat liver (*4*). Also, labeling experiments have provided evidence that mammalian cells can utilize GGOH for protein isoprenylation (*5*), indicating that GGOH is converted to GGPP through the action of a kinase. In a metabolic study on mevalonic acid involving cell-free homogenates of bovine retinas, Fliesler and Schroepfer found *E,E,E*-GGA and its stereoisomer containing at least one internal *cis*-double bond as the metabolites (*6*), suggesting that GGOH derived from mevalonate is oxidized to GGA *via* geranylgeranial through the action of alcohol and aldehyde dehydrogenase.

Exogenously supplied GGOH has also been found to act as an apoptotic inducer in tumor cells (*7*), and as a negative regulator of LXR-RXR (*8*). Shidoji *et al.* have reported that GGA induces apoptosis in a human hepatoma cell line, HuH-7, but not in mouse primary cultured hepatocytes (*9*). Further, they have also described that GGA and chemically synthesized 4,5-didehydroGGA function as a potential agonist of retinoic acid, which is a differentiation inducer (*10*). Wang *et al.* more recently reported that GGA induces osteoblast differentiation and suppresses osteoclast formation (*11*). Judging from these studies, exogenously supplied C20 isoprenoids such as GGOH and GGA seem to operate as molecular signals for the regulation of cell-proliferation and cell-differentiation. However, it is also expected that the supplied isoprenoids are further metabolized to GGPP, GGA, or unknown derivatives.

Recently, it was reported that phytol, as a food source, is converted *via* phytenic acid to phytanic acid (*12*). Further, phytanic acid was recently found to act as an agonist of retinoic acid (*13*). It is unclear whether GGOH differs from phytol in the catabolic pathway. However, the two compounds have the same C20 carbon-chain length and are similar to each other in structure with an unsaturated isoprene unit at the α-terminal. Therefore, it is very important to establish the catabolic pathway of GGOH to see whether mevalonate-derived metabolites play an important role

[1] This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
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Abbreviations: IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; FOH, farnesol; FA, farnesoic acid; GGPP, geranylgeranyl diphosphate; GGOH, geranylgeraniol; GGA, geranylgeranoic acid; $TMSCHN_2$, trimethylsilyl diazomethane; *p*-tol-BINAP, 2,2′-bis (di-*p*-tolylphosphino)-1,1′-binaphthyl; PMHS, polymethyl hydrosiloxane; LXR, liver X receptor; RXR, retinoid X receptor; BSA, bovine serum albumin; TLC, thin-layer chromatography.

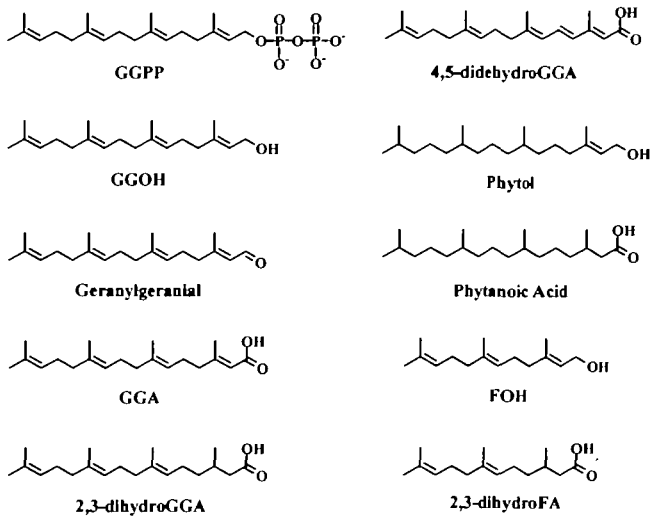


Fig. 1. **Chemical structures of C15 and C20 compounds.**

similarly to in the case of food-derived phytanic acid. In the present study, we pursued metabolites derived from geranylgeranial and GGOH in thymocytes and found two metabolites, Z1 and Z2, which were identical to *E,E,E*-GGA and (*R*)-2,3-dihydroGGA, respectively. These results are discussed in the context of C20 isoprenoid metabolism. The structures of several C15 and C20 compounds are shown in Fig. 1.

MATERIALS AND METHODS

Materials—[1-^{14}C]IPP (specific activity, 55 mCi/mmol) was purchased from Amersham Pharmacia Biotech. (*S*)-*p*-tol-BINAP and (*R*)-1-(1-naphthyl)ethylamine were purchased from STREM and Kanto Kagaku, respectively. *E,E,E*-GGOH, ethyl *E,E,E*-geranylgeranoate, and ethyl *Z,E,E*- geranylgeranoate were generous gifts from Kuraray, Male Sprague-Dawley rats, RPMI 1640 medium, and newborn calf serum were obtained from Japan SLC, Nihon Pharmacol., and Gibco BRL, respectively. The TLC plates were purchased from Merck. All other chemicals were of reagent grade.

Analytical Method—NMR spectra were recorded on a JEOL LAMBDA 400 (400 MHz proton). EI-mass spectra were recorded on a Hitachi M-2500S mass spectrometer (Instrumental Analysis Center for Chemistry Graduate School of Science, Tohoku University). ESI- and GC-mass spectra were recorded on Micromass ZMD 4000 and Shimadzu GCMS-QP5050A mass spectrometers, respectively (Forensic Science Laboratory, Miyagi Prefectural Police Headquarters). Reverse-phase HPLC (TSK-GEL ODS-80-TS, 250 × 4.6 mm) was performed at a flow rate of 1.0 ml/min with a solvent system of CH_3CN/H_2O (85:15).

Synthetic Procedure for [1-^{14}C]GGOH and [1-^{14}C]Geranylgeranial—[1-^{14}C]GGOH was synthesized through two enzymatic reactions from FPP and [1-^{14}C]IPP. To obtain the recombinant GGPP synthase necessary for the first reaction, *E. coli* JM109 cells transformed with an expression vector (pTrc-99A) containing human GGPP synthase cDNA (*14*) were grown in 500 ml of LB medium to an A_{600} of 0.6. Isopropyl 1-thio-β-galactoside was added to a final concentration of 2.0 mM, and after 2-h culture the cells were collected and suspended in 50 ml of TE buffer (pH 8.0). The cell suspension was sonicated and centrifuged at 100,000 ×*g*. The supernatant was fractionated with $(NH_4)_2SO_4$, and the active 70–80% $(NH_4)_2SO_4$ fraction of GGPP synthase was dialyzed against TE buffer (pH 8.0) for 12 h at 4°C. The dialysate was used as a crude GGPP synthase. The first reaction mixture contained, in a final volume of 2 ml, 50 mM Tris-HCl buffer (pH 7.0), 2 mM dithiothreitol, 5 mM $MgCl_2$, 1 mg/ml BSA, 18 μM [1-^{14}C]IPP, 25 μM FPP, and 400 μl of the dialyzed GGPP synthase (0.35 μg/μl). The mixture was incubated at 37°C for 30 min, and the radioactive GGPP products were extracted with 10 ml of 1-butanol saturated with water. The 1-butanol extracts were subjected to the second enzymatic reaction with acid phosphatase (*15*). The reaction mixture contained, in a final volume of 2 ml, 280 μl of 1 M sodium acetate buffer (pH 5.6) containing 10 mg of acid phosphatase from potato, 400 μl of 1-butanol saturated with water containing [1-^{14}C]-GGPP, 800 μl of methanol, and 520 μl of water. The mixture was incubated at 37°C for 12 h, and then the hydrolysate was extracted with hexane (20 ml). The yield of [1-^{14}C]GGOH from [1-^{14}C]IPP was 50%.

[1-^{14}C]GGOH was chemically converted to [1-^{14}C]geranylgeranial according to the method of Corey and Schmidt (*16*). [1-^{14}C]GGOH (0.68 μmol) in DMF (100 μl) was mixed with pyridinium dichromate (3.19 mg) and then stirred for 4 h at 0°C. The reaction product was extracted with hexane (4 ml), and then the hexane extract was purified by silica-gel column chromatography with a solvent system of hexane/ethyl acetate (19:1). The yield of [1-^{14}C] geranylgeranial from [1-^{14}C]GGOH was 40%.

Cell Preparation and Culture—Thymic glands of 3–6-week-old male Sprague-Dawley rats were cut up and filtered through three layers of nylon mesh. Thymocytes were suspended to a final concentration of 3–8 × 10^6 cells/ml for the experiment with geranylgeranial and one of 5 × 10^7 cells/ml for the experiment with GGOH in an RPMI 1640 medium containing 0.1% newborn calf serum. The cells in the medium were incubated with [1-^{14}C]geranylgeranial (1.5 μM) or [1-^{14}C]GGOH (1.5 μM) for an appropriate time at 37°C under an atmosphere of 5% CO_2. Isoprenoid compounds were prepared as an ethanol solution and added to the cell suspension at a final concentration (v/v) of less than 1%.

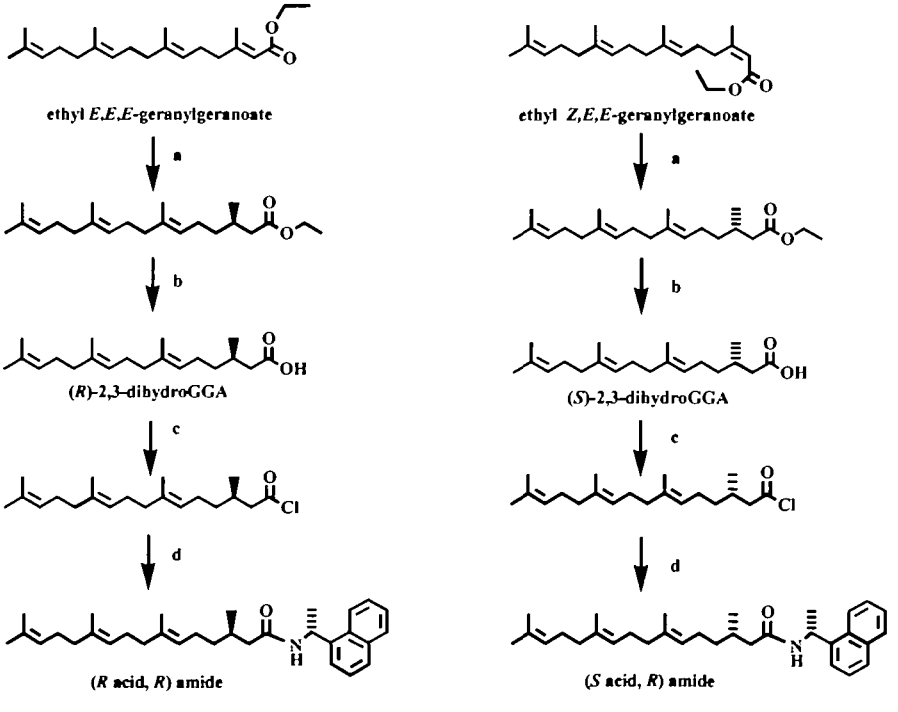
Extraction—The cell suspension after incubation was extracted by the Bligh-Dyer method (*17*). Proteins were obtained by acetone precipitation. Radioactivity in the lipid layer, aqueous layer, or protein pellet was analyzed with a liquid scintillation counter. The radioactive metabolites in the lipid and aqueous layers were analyzed by silica-gel TLC with $CHCl_3$ and a solvent system of 2-PrOH/NH_3/H_2O (6:3:1), respectively. On the other hand, radioactive proteins were analyzed by 10% SDS–polyacrylamide gel electrophoresis.

Synthetic Procedure for Rac-2,3-DihydroFA—*rac*-2,3-DihydroFA was chemically synthesized from FOH according to the method of Mankowski *et al.* (*18*), and Corey and Schmidt (*16*). Briefly, FOH (3.0 g) in 200 ml of ethanol was mixed with $NaBH_4$ (270 mg) and PtO_2 (IV) (150 mg), and then the mixture was stirred under hydrogen gas for 2.5 h at room temperature. Then the mixture was filtered to remove PtO_2 and excess $NaBH_4$, and the filtrate was dried

in vacuo. The residue was purified by C_{18}-silica-gel column chromatography with a solvent system of methanol/water (8:1). The obtained *rac*-2,3-dihydroFOH (400 mg) was next converted to *rac*-2,3-dihydroFA according to the method of Corey and Schmidt (*16*). The crude *rac*-2,3-dihydroFA was purified by silica-gel column chromatography with a solvent system of hexane/ethyl acetate (6:1). The yield was 56%. *rac*-2,3-DihydroFA; MS (EI, 70 eV) *m/z* (%) 238 (M^+, 25), 223 (31), 123 (77), 69 (100), ^1H NMR($CDCl_3$, δ) 0.95 (d, 3H, J = 6.6), 1.20–1.41 (m, 2H), 1.58 (s, 6H), 1.66 (s, 3H), 1.92–2.10 (m, 7H), 2.11–2.38 (m, 2H), 5.02–5.10 (m, 2H).

Synthetic Procedure for (R)- or (S)-2,3-DihydroGGA and Their Diastereomers—A mixture of ethyl *E,E,E*-geranylgeranoate and ethyl *Z,E,E*-geranylgeranote was completely purified by silica-gel column chromatography with a solvent system of hexane/diethyl ether (20:1). As shown in Scheme 1, (*R*)-2,3-dihydroGGA and (*S*)-2,3-dihydroGGA were synthesized from ethyl *E,E,E*-geranylgeranoate and ethyl *Z,E,E*-geranylgeranoate, respectively, and then converted to their diastereomers according to the method of Appella *et al.* (*19*), Durham *et al.* (*20*), and Mori *et al.* (*21*). Briefly, (*S*)-*p*-tol-BINAP (110 mg), *ter*-BuONa (8 mg), and then CuCl (8 mg) in dry toluene were mixed under nitrogen gas for 20 min at room temperature, and then ethyl *E,E,E*-geranylgeranoate (353 mg) was added after the addition of PMHS (0.36 ml) to the reaction mixture. The mixture was stirred for 60 h. EtOH (60 ml) was added to the stop reaction. The resulting solution was diluted with diethylether, and washed with water and then with brine, and then the organic layer was dried by the addition of anhydrous $MgSO_4$. The diethylether solution was concentrated and dried *in vacuo*. The product was purified by silica-gel column chromatography with a solvent system of hexane/ethyl acetate (40:1). To ethyl (*R*)-2,3-dihydrogeranylgeranoate was added 85% KOH (1 g) in EtOH (10 ml), and then the mixture was refluxed for 3.5 h. The crude (*R*)-2,3-dihydroGGA was purified by silica-gel column chromatography with a solvent system of $CHCl_3/Et_2O$ (10:1). The yield was 69.1%. (*R*)-2,3-DihydroGGA; ^1H NMR ($CDCl_3$, δ) 0.96 (d, 3H), 1.21–1.26 (m, 1H), 1.34–1.41 (m, 1H), 1.58 (s, 9H), 1.66 (s, 3H), 1.93–2.06 (m, 11H), 2.13 (q, 1H, J_{gem} = 14.9Hz, J_{vic} = 8.28Hz), 2.35(q, 1H, J_{gem} = 15.0 Hz, J_{vic} = 5.74 Hz), 5.07 (m, 3H). The enantiomeric excess (e.e.) of synthesized (*R*)-2,3-dihydroGGA was determined according to the method of Mori *et al.* (*21*). Briefly, oxalyl chloride (0.32 ml) was added to a solution of the (*R*)-2,3-dihydroGGA (50 mg) in dry benzene (5 ml). The mixture was heated under reflux for 3 h and then concentrated *in vacuo*. The residue was mixed with (*R*)-1-(1-naphthyl)ethylamine in dry diethylether (15 ml). The diastereomeric amide was analyzed by normal-phase HPLC [TSK-GEL, 250 × 4.6 mm, hexane/THF (6:1)]. The major (*R* acid, *R*) amide and the minor (*S* acid, *R*) one were eluted at the retention times of 9.2 and 10.5 min, respectively. The optical purity of (*R*)-2,3-dihydroGGA was estimated to be 86.4% e.e. Diastereomeric amide; MS (EI, 70 eV) *m/z* (%) 459 (M^+, 16), 304 (3), 170 (11), 155 (100), 69 (18). In the case of (*S*)-2,3-dihydroGGA, the yield of (*S*)-2,3-dihydroGGA was 50.1%. The enantiomeric excess of synthesized (*S*)-2,3-dihydroGGA was also determined similarly. In this case, the minor (*R* acid, *R*) amide and the major (*S* acid, *R*) one were eluted at the retention times of 9.3 min and 10.4 min, respectively. The optical purity of (*S*)-2,3-dihydroGGA was estimated to be 86.5% e.e.

DNA Extraction and Agarose Gel Electrophoresis—Cells (3–8 × 10^6 cells/ml, 400 μl) were lysed by incubation at 4°C for 10 min after the addition of a buffer (40 μl) containing 0.1 M Tris-HCl buffer (pH 7.4), 0.1 M EDTA (pH 8.0), and 5% (w/v) Triton X-100, and then centrifuged at 18,000 ×*g* for 15 min. The supernatant (400 μl) was treated with 0.5 μl of 10 mg/ml RNase A at 37°C for 2 h and then with 2.5 μl of 10 mg/ml Proteinase K under the same conditions. The mixture was added to a mixture of 2-propanol (500 μl)



Scheme 1. **Reagents: (a) (*S*)-*p*-tol-BINAP, *ter*-BuONa, CuCl, PMHS, toluene; (b) KOH, EtOH (95%); (c), $(COCl)_2$, benzene; (d) (*R*)-1-(1-naphtyl)ethylamine, Et_2O.**

and 5 M NaCl (100 μl), and then the solution was kept at −20°C overnight. The cold solution was centrifuged at 18,000 ×*g* for 15 min. The pellets were dissolved in 40–80 μl of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA (pH 8.0). Samples were analyzed by electrophoresis on a 1.5% agarose gel. DNA fragments were visualized under UV light after staining the gel with ethidium bromide.

RESULTS

Metabolism of Geranylgeranial—After thymocytes had been labeled with [1-^{14}C]geranylgeranial for 2 h, lipids were extracted by the Bligh-Dyer method and proteins were precipitated with acetone. The radioactivity in the lipid layer, aqueous layer, and protein pellet is shown in Table I. The majority of recovered radioactivity was detected in the lipid layer.

When the radioactive metabolites in the lipid layer were subjected to silica-gel TLC (Fig. 2a, left lane), radioactive geranylgeranial was not detected and two metabolites (Z1 and Z2) were detected, Z2 being the major one. Z1 moved with the spot of *E,E,E*-GGA as a reference. In addition, Z1 showed the same chromatographic behavior as *E,E,E*-GGA with other solvent systems. When the materials in the lipid layer were treated with $TMSCHN_2$, which is a methyl esterification-reagent for carboxylic acid, the Z1- and Z2-derived radioactivity was observed at the spot corresponding to methyl geranylgeranoate (Fig. 2a, right lane), indicating that each metabolite has a carboxylic acid group. We further examined the time-dependent formation of Z1 and Z2. As shown in Fig. 2b, Z1 increased, reaching a peak within 30 min, and then decreased slowly, whereas Z2 began to increase after 30 min. These results indicate that geranylgeranial is mainly metabolized to Z1 (*E,E,E*-GGA) and Z2, and that there is a precursor-product relationship between Z1 and Z2.

Concerning the aqueous layer, significant spots corresponding to GGPP and geranylgeranyl phosphate were not detected (data not shown). We also tried to analyze the protein pellet by SDS–polyacrylamide gel electrophoresis. Several radioactivity bands corresponding to 50–100, 30, 27, 18, and 3–6 kDa were detected (data not shown).

Isolation and analysis of metabolite Z2—In order to identify metabolite Z2 by mass spectrometry, we prepared a sufficient amount of Z2 from geranylgeranial using thymocytes and purified it by silica-gel TLC. The negative ion peak of the purified metabolite Z2 on ESI was observed at *m/z* 305 ([M–H]$^-$) (data not shown). Figure 3 shows the EI mass spectrum of Z2 with a molecular peak at *m/z* 306 (M$^+$). Since the calculated molecular weight of GGA is 304 and GGA has four carbon-carbon double bonds in its structure, we assumed that Z2 is a derivative of GGA, one of the four double bonds being saturated. The observation of the mass peak at *m/z* 69 and 136 suggested the presence of a geranyl group at the ω-end of Z2. Furthermore, the absence of a mass peak at *m/z* 100, a fragment derived from the structure of conjugated carboxylic acid in GGA (Fig. 4a), suggested the presence of a non-conjugated carboxylic acid at the α–end of Z2. The structure was also supported by that the peak at *m/z* 109 was stronger than that at m/z 136 in comparison with in the case of GGA. We chemically synthesized (*R*)-2,3-dihydroGGA from ethyl *E,E,E*-geranylgeranoate. As expected, the mass spectrum (Fig. 4b) was identical to that of Z2 (Fig. 3). On analysis with a GC-mass spectrometer, esters Z2 and (*R*)-2,3-dihydroGGA showed the same retention time of 17.9 min, and same mass spectrogram with respect to the molecular ion at *m/z* 320 and subsequent fragmentation (data not shown). Moreover, on analysis by reverse-phase HPLC, radioactive metabolite Z2 was coeluted with (*R*)-2,3-dihydroGGA with a retention

TABLE I. **Distribution of [1-^{14}C]geranylgeranial-derived metabolites.** A thymocyte suspension (5×10^6 cells/ml, 3 ml) was labeled with [1-^{14}C]geranylgeranial (5.5×10^5 dpm) for 2 h and then treated as described under "MATERIALS AND METHODS," except that chloroform/methanol extraction and acetone precipitation were performed using 2 ml and 400 μl of 3 ml of thymocyte suspension, respectively. Each fraction was analyzed with a liquid scintillation counter.

Fraction	Radioactivity (dpm)	Recovery (%)
Chloroform/methanol extraction		
Lipid layer	359,000	65.3
Aqueous layer	63,300	11.5
Acetone precipitation		
Protein pellet	<27,500	<5

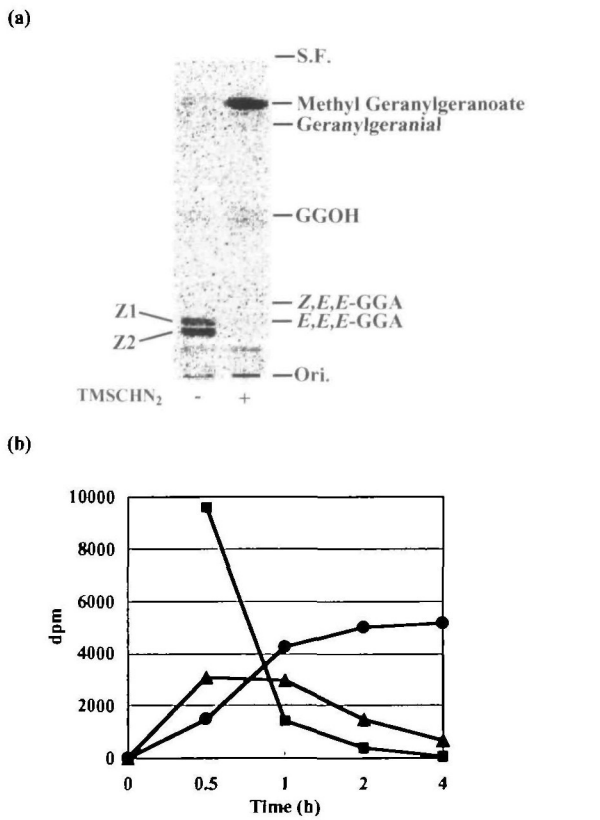


Fig. 2. **Analysis of [1-^{14}C]geranylgeranial-derived lipids in thymocytes.** (a) The radioactive materials in the lipid layer in Table I were analyzed by TLC in $CHCl_3$ (left lane). The materials were also treated with $TMSCHN_2$ and analyzed similarly (right lane). S.F. solvent front; Ori., origin. (b) Thymocytes were labeled with 1.5 μM [1-^{14}C]geranylgeranial for 0.5, 1, 2, and then 4 h, and then radioactive materials in each lipid layer were analyzed by silica gel TLC in $CHCl_3$ as described under "MATERIALS AND METHODS." Each stop of radioactivity on the developed thin-layer plate corresponding to geranylgeranial (■), Z1 (▲), and Z2 (●) was evaluated with a BAS 1000 image analyzer.

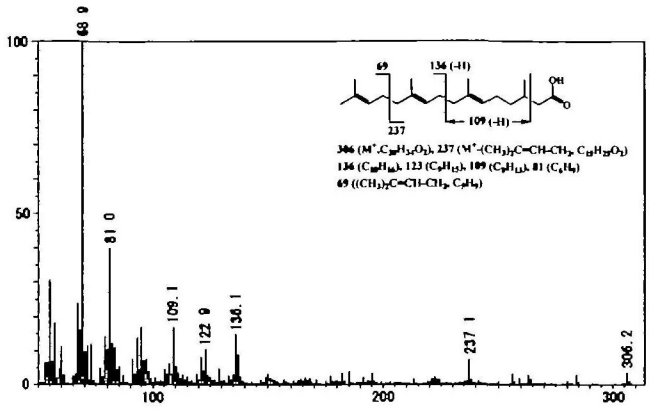


Fig. 3. **EI mass spectrogram of geranylgeranial-derived metabolite Z2.** The thymocytes (5×10^7 cells/ml) in 108 ml of medium were cultured in the presence of geranylgeranial (3 μM) for 8 h under an atmosphere of 5% CO_2 and then treated as described under "MATERIALS AND METHODS". Metabolite Z2 in the lipid layer was purified by TLC. The purified metabolite Z2 (about 30 μg) was analyzed by EI mass spectrometry. The potential of the ionizing beam was 70 eV.

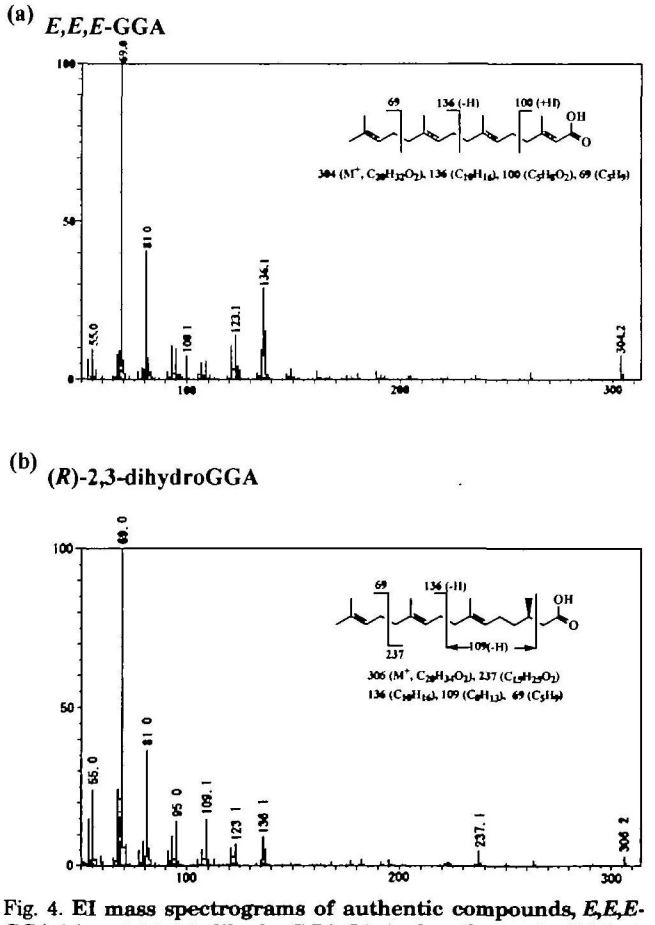


Fig. 4. **EI mass spectrograms of authentic compounds, *E,E,E*-GGA (a) and (*R*)-2,3-dihydroGGA (b).** As described under "MATERIALS AND METHODS." *E,E,E*-GGA and (*R*)-2,3-dihydroGGA were obtained by alkaline treatment of ethyl *E,E,E*-geranylgeranoate and by reduction of ethyl *E,E,E*-geranylgeranoate followed by alkaline treatment, respectively. The potential of the ionizing beam was 70 eV.

time at 13.9 min. Considering all these results, we concluded that metabolite Z2 was 2,3-dihydroGGA.

We next tried to determine whether Z2 (2,3-dihydroGGA) has the *R*- or *S*-configuration at the C3-chiral center. As shown in Scheme 1, we chemically synthesized two kinds of diastereomeric amides, (*R* acid, *R*) amide and (*S* acid, *R*) amide. Radioactive metabolite Z2 was also similarly converted to the corresponding diastereomeric amide. Also, radioactive amide was analyzed with the two standard amides by normal-phase TLC (Fig. 5). (*R* acid, *R*) amide and (*S* acid, *R*) amide were separated from each other (lane 1). The radioactive amide comigrated with (*R* acid, *R*) amide (lane 2), although a band of radioactivity corresponding to the intermediate (2,3-dihydrogeranylgeranoyl chloride) in the reaction was detected near the solvent front. These results indicate that metabolite Z2 is (*R*)-2,3-dihydroGGA.

Metabolism of GGOH—Having established that geranylgeranial is metabolized to (*R*)-2,3-dihydroGGA as well as *E,E,E*-GGA, we next examined whether GGOH is simi-

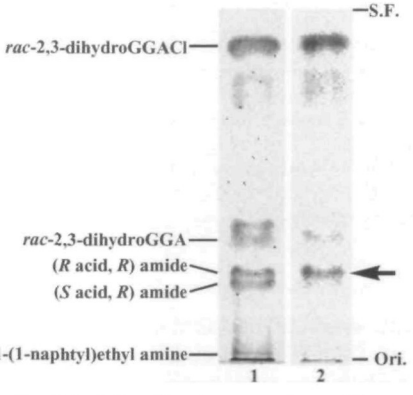


Fig. 5. **Silica gel TLC of the diastereomeric amide synthesized from radioactive metabolite Z2.** Purified radioactive metabolite Z2 (5,000 dpm) was mixed with 50 μg each of (*R*)-2,3-dihydroGGA and (*S*)-2,3-dihydroGGA, and then treated with $(COCl)_2$ and (*R*)-1-(1-naphtyl)ethylamine as described under "MATERIALS AND METHODS." The reaction products were analyzed by silica gel TLC with a solvent system of hexane/THF (6:1). The developed plate was exposed to iodine vapor (lane 1) and the radioactivity was analyzed with a BAS 1000 image analyzer (lane 2) S.F., solvent front; Ori., origin; GGACl, geranylgeranoyl chloride; (*R* acid, *R*) amide, (*R*)-2,3-dihydroGGA (*R*)-1-(1-naphtyl)ethylamine amide; (*S* acid, *R*) amide, (*S*)-2,3-dihydroGGA (*R*)-1-(1-naphtyl)ethylamine amide. Arrow, radioactive diastereomeric amide.

TABLE II. **Distribution of [1-^{14}C]GGOH-derived metabolites.** A thymocyte suspension (5×10^7 cells/ml, 3 ml) was labeled with [1-^{14}C]GGOH (5.5×10^6 dpm) for 16 h and then treated as described under "MATERIALS AND METHODS," except that chloroform/methanol extraction and acetone precipitation were performed using 2 ml and 400 μl of 3 ml of thymocyte suspension, respectively. Each fraction was analyzed with a liquid scintillation counter.

Fraction	Radioactivity (dpm)	Recovery (%)
Chloroform/methanol extraction		
Lipid layer	386,000	70.1
Aqueous layer	55,500	10.1
Acetone precipitation		
Protein pellet	<9,900	<1.8

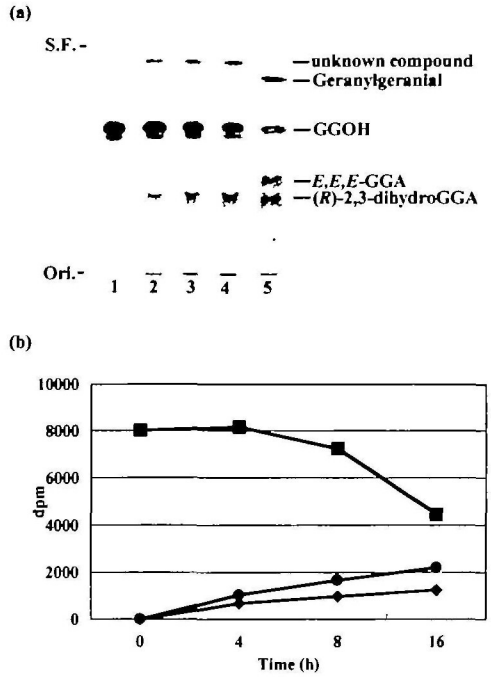


Fig. 6. **Analysis of [1-^{14}C]GGOH-derived lipids in thymocytes.** Thymocytes (5×10^7 cells/ml) were labeled with 1.5 μM [1-^{14}C]GGOH for 0, 4, 8, and 16 h (lanes 1–4) at 37°C, and then the radioactive materials in each lipid layer were analyzed by silica-gel TLC with a solvent system of $CHCl_3/Et_2O$ (10:1) as described under "MATERIALS AND METHODS." Authentic standards are shown in lane 5. S.F., solvent front; Ori., origin. (b) Each radioactivity band corresponding to an unknown metabolite (♦), GGOH (■), and (*R*)-2,3-dihydroGGA (●) in (a) was evaluated with a BAS 1000 image analyzer.

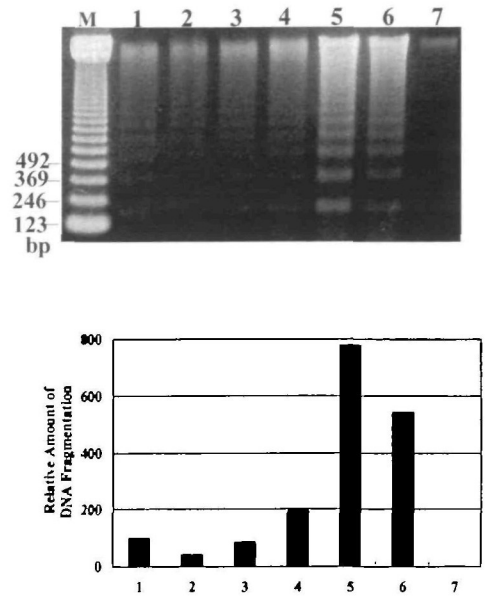


Fig. 7. **Agarose gel electrophoresis of nucleosomal DNA obtained from thymocytes treated with (*R*)-2,3-dihydroGGA.** Thymocytes were treated with 0–40 μM (*R*)-2,3-dihydroGGA for 2 h and then nucleosomal DNA was extracted as described under "MATERIALS AND METHODS." The upper panel shows agarose gel electrophoresis and the lower panel shows the relative amount of DNA fragmentation compared to the control. M, 123 bp ladder. Lanes 1, 2, 3, 4, 5, 6, and 7 indicate 0, 1, 5, 10, 15, 20, and 40 μM, respectively.

larly metabolized to carboxylic acids. Thymocytes were labeled with [1-^{14}C]GGOH and then treated similarly to in the case of [1-^{14}C]geranylgeranial. Table II summarizes the results obtained on a 16-h culture with [1-^{14}C]GGOH. The distribution was almost the same as in the case of [1-^{14}C]geranylgeranial-derived metabolites, and the radioactivity was mainly recovered in the lipid layer. Figure 6a shows the time-dependent formation of [1-^{14}C]GGOH-derived metabolites in the lipid layer. Two metabolites were detected after 2 h labeling. One was a slower-moving radioactive metabolite corresponding to (*R*)-2,3-dihydroGGA and the other an unknown compound. No radioactive band corresponding to *E,E,E*-GGA was detected. In further experiments involving a thymocyte suspension of $3–8 \times 10^6$ cells/ml instead of 5×10^7 cells/ml, two radioactivity bands corresponding to *E,E,E*-GGA and (*R*)-2,3-dihydroGGA were detected (data not shown). Concerning the minor radioactive materials in the aqueous layer and protein pellet, further analysis was not performed. These results suggest that GGOH is also metabolized to (*R*)-2,3-dihydroGGA, presumably *via E,E,E*-GGA.

Apoptotic Induction by (R)-2,3-DihydroGGA—In preliminary experiments, we confirmed that GGOH and FOH have the ability to induce DNA-ladder formation in rat thymocytes. As described above, GGOH is metabolized to (*R*)-2,3-dihydroGGA, implying that FOH might be metabolized to (*R*)-2,3-dihydroFA. We chemically synthesized *rac*-2,3-dihydroFA from FOH. Using (*R*)-2,3-dihydroGGA and *rac*-2,3-dihydroFA, thymocytes were treated for 2 h at 37°C, and then DNA fragmentation was analyzed by agarose gel electrophoresis. As shown in Fig. 7, (*R*)-2,3-dihydroGGA induced concentration-dependent (1–40 μM) DNA fragmentation with a maximum at 15 μM. On the other hand, *rac*-2,3-dihydroFA did not induce concentration-dependent (2.5–40 μM) DNA fragmentation (data not shown).

DISCUSSION

We recently studied the effect of several isoprenoid compounds on thymocytes, and preliminarily found that GGOH, geranylgeranial, and GGA induce DNA ladder formation, and that geranylgeranial differs from the other two compounds in the inductive effect in the presence of phosphatidylcholine. Geranylgeranial has a reactive aldehyde group, and similar aldehyde compounds such as 4-hydroxynonenal, malondialdehyde and fatty aldehyde have been found to react with lysine residues of LDL (*22*) and with phosphatidylethanolamine (*23*). Therefore, we tried to analyze the metabolites derived from geranylgeranial to detect geranylgeranial-phosphatidylethanolamine Shiff-base or geranylgeranial-modified proteins. However, such metabolites were too small to analyze. Instead, we found two metabolites, Z1 and Z2. In the present study, metabolite Z1 and metabolite Z2 were found to be identical to *E,E,E*-GGA and (*R*)-2,3-dihydroGGA, respectively. To our surprise, (*R*)-2,3-dihydroGGA, the main geranylgeranial-derived metabolite, had the ability to induce DNA-ladder formation at 15 μM in rat thymocytes, and *rac*-2,3-dihydroFA, shorter by one isoprene unit than 2,3-dihydroGGA, did not act as a similar inducer. The result concerning 2,3-dihydroFA may be associated with the fact that 2,3-dihydroFA was used as

a racemic mixture. We do not know at present whether the geranylgeranial-induced DNA ladder formation in thymocytes is induced by (*R*)-2,3-dihydroGGA, the main geranylgeranial-derived metabolite. However, it is likely that (*R*)-2,3-dihydroGGA is harmful to thymocytes at a higher concentration.

On analysis of Z2, it showed slower mobility than GGA on a silica-gel thin-layer plate. Because Z2 is more polar than GGA, we at first speculated that Z2 is not a dihydroderivative of GGA. However, the molecular ion (*m/z* 306) in the EI mass spectrum of Z2 suggested that it is a dihydroderivative of GGA. So, we became aware that GGA, being a conjugated carboxylic acid, showed much higher chromatographic mobility than dihydroGGA. The mass fragmentation with a base peak at 69, two peaks at 109 and 136, and no peak at 100 ruled out the possibility of 14,15-dihydroGGA, 10,11-dihydroGGA, or 6,7-dihydroGGA, and supported that the possible structure is 2,3-dihydroGGA. The mass fragmentation of chemically synthesized (*R*)-2,3-dihydroGGA was in fact identical to that of Z2. Also, further analysis with respect to the chirality at the C3 position of Z2 supported the structure of (*R*)-2,3-dihydroGGA.

2,3-DihydroGGA, together with phytanic acid and phytenic acid, has already been found by Dulaney *et al.* (*24*), and Evans *et al.* (*25*) to occur in the serum and urine of patients with Refsum disease, which leads to peripheral nerve damage, atypical retinitis pigmentosa, cerebellar ataxia, and skin and bone changes. Since Refsum disease is caused by mutations in the gene encoding phytanoyl-CoA hydroxylase, it is expected that such metabolites are virtually undetected except for in patients. Therefore, we were very surprised to detect the compound as a GGOH- or geranylgeranial-derived metabolite in rat thymocytes. There might be a difference in the catabolic pathway between humans and rats. Alternatively, the detection might be limited to thymocytes released from thymic gland, which are known to be destined to spontaneous apoptosis. Concerning the biosynthetic pathway leading to 2,3-dihydroGGA, the C20 carbon skeleton is synthesized as GGPP from mevalonate. A specific pyrophosphatase for GGPP has also been found (*4*). As the present study shows, 2,3-dihydroGGA is synthesized from GGOH. Therefore, it is probably true that in mammalian cells 2,3-dihydroGGA is *de novo* synthesized from mevalonic acid. Apart from in mammalian cells, Vandewaa *et al.* (*26*) have reported that an unidentified non-sterol isoprenoid compound other than dolichol stimulates egg production in *Schistosoma mansoni*, which is unable to synthesize sterol *de novo*. Then, Foster *et al.* (*27*) reported that a metabolite, which they tentatively assigned as 2,3-dihydoGGA, occurs in the blood-fluke. They also described that radioactive mevalonate was incorporated into the saponifiable lipids, with the putative 2,3-dihydroGGA, accompanied by much less GGA, being detected.

We have shown that [1-^{14}C]GGOH (specific activity, 55 mCi/mmol) exogenously added to thymocytes was partly metabolized to 2,3-dihydroGGA. However, it was not easy to detect radioactive protein bands on SDS-polyacrylamide gel electrophoresis. In preliminary experiments involving [1-^{3}H]GGOH (specific activity, 20 Ci/mmol), several radioactivity bands corresponding to 20–30 kDa were easily detected on SDS-electrophoresis. Therefore, the exogenously added GGOH seems to be metabolized partly to GGPP for isoprenylated protein biosynthesis and partly to 2,3-dihydroGGA, the latter metabolic flow being predominant. Exogenously added GGOH acts as a negative regulator of LXR-RXR, and GGPP is also a possible candidate (*8*). Therefore, it might be important to investigate whether 2,3-dihydroGGA acts as a negative regulator.

Muto *et al.* (*28*) have reported that 2,3-dihydroGGA synthesized as a synthetic analog of all-*trans*-retinoic acid exhibites high binding affinity for cellular retinoid binding proteins from rat testis, but exhibites no ligand activity in the chloramphenicol acetyltransferase assay with retinoic acid response element β or retinoid X response element. Lemotte *et al.* (*13*) recently reported that phytanic acid, fully saturated GGA, acts as an RXR ligand. 2,3-DihydroGGA as well as phytanic acid accumulates in Refsum disease cases, implying that (*R*)-2,3-dihydroGGA also functions as a ligand for nuclear orphan receptors other than RXR.

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