methods

Novel *d*-γ-tocopherol derivative as a prodrug for *d*-γ-tocopherol and a two-step prodrug for *S*-γ-CEHC

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Abstract ***d*-γ-Tocopherol (γ-Toc) and its major metabolite, 2, 7, 8-trimethyl-2*S*-(β-carboxyethyl)-6-hydroxychroman (*S*-γ-CEHC), are currently receiving attention concerning their unique pharmacological activities. In order to achieve the efficient delivery of γ-Toc and *S*-γ-CEHC in vivo, we synthesized *d*-γ-tocopheryl *N,N*-dimethylglycinate hydrochloride (γ-TDMG) as a water-soluble prodrug of γ-Toc and a two-step prodrug of *S*-γ-CEHC. γ-TDMG is a solid (mp 161–163°C) and is quite soluble in water over 50 mM. The hydrolysis of γ-TDMG was effectively catalyzed by esterases in rat and human liver microsomes. The disposition of γ-TDMG after iv administration in rats was compared with that of γ-Toc solubilized with the surfactant, polyoxyethylene hydrogenated castor oil. The plasma and liver levels of γ-Toc rapidly increased after the iv administration of the γ-TDMG. The liver availability of γ-Toc after the administration of γ-TDMG was two times higher than that of the γ-Toc administration. The relative systemic availability of *S*-γ-CEHC after the γ-TDMG administration was an equivalent value (102%), and the mean residence time of *S*-γ-CEHC was eight times longer than the racemic γ-CEHC administration. Based on these results, γ-TDMG was identified as the most promising water-soluble prodrug of γ-Toc and the two-step prodrug of *S*-γ-CEHC.**—Takata, J., R. Hidaka, A. Yamasaki, A. Hattori, T. Fukushima, M. Tanabe, K. Matsunaga, Y. Karube, and K. Imai. **Novel *d*-γ-tocopherol derivative as a prodrug for *d*-γ-tocopherol and a two-step prodrug for *S*-γ-CEHC. *J. Lipid Res.* 2002.** 43: **2196–2204.**

Supplementary key words water-soluble prodrug • drug delivery • *d*-γ-tocopherol • two-step prodrug

d-γ-Tocopherol (2*R*,4′*R*,8′*R*-γ-Tocopherol, γ-Toc) is one of the major forms of natural tocopherols (vitamin E) and constitutes 70–80% of the vitamin E in the diets of people in the United States (1). For many years, *d*-α-tocopherol (2*R*,4′*R*,8′*R*-α-Tocopherol, α-Toc) was generally considered as the most active vitamin E and γ-Toc was mostly ignored because of its poor bioactivity as defined by the rat fetal resorption assay (2). However, γ-Toc is currently receiving attention concerning its beneficial effects. Several independent investigations have demonstrated that the plasma concentration of γ-Toc, not of α-Toc, was correlated to the incidence of coronary heart disease (3–5). γ-Toc is superior to α-Toc in its ability to trap reactive nitrogen species, mutagenic electrophiles generated during inflammation (6–9). Recently, it has been shown that γ-Toc was efficiently metabolized to 2,7,8-trimethyl-2*S*-(β-carboxyethyl)-6-hydroxychroman (*S*-γ-CEHC, *S*-LLU-α), and *S*-γ-CEHC exhibited a natriuretic activity (10–12). In addition, it has been shown that γ-Toc and *S*-γ-CEHC inhibited the generation of prostaglandin E_2 (PGE_2), an important mediator synthesized via the cyclooxygenase-2 (COX-2)-catalyzed oxidation of arachidonic acid during inflammation (13). Thus, γ-Toc and its metabolite, *S*-γ-CEHC, are also expected to exhibit important pharmacological activities as a drug.

Upon considering the therapeutic formulations of γ-Toc, we have to overcome the unavoidable problem that γ-Toc is a highly viscous oil, practically insoluble in water and readily oxidized by atmospheric oxygen. These physicochemical properties of γ-Toc limit its therapeutic applications, making difficult an efficient administration of γ-Toc to patients. Besides, *S*-γ-CEHC is also readily oxidizable as γ-Toc and the bioavailability of *S*-γ-CEHC is very low due to its rapid elimination rate (12, 14). Thus, it can be expected that an effective delivery of γ-Toc would be a meaningful method for achieving the adequate bioavailability of *S*-γ-CEHC. When administered in the form of an oil solution or some kind of oil emulsion, lipophilic compounds usually show a poor bio-

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Abbreviations: AUC, area under the concentration-time curve; DBD-PZ, 4-*N,N*-dimethylaminosulfonyl-7-piperazino-2,1,3-benzoxadiazole; FD-MS, field desorption mass spectrometry; HCO-60, polyoxyethylene hydrogenated castor oil; ^{1}H-NMR, proton nuclear magnetic resonance spectrometry; γ-Toc, *d*-γ-tocopherol; γ-TDMG, *d*-γ-tocopheryl *N,N*-dimethylglycinate hydrochloride; MRT, mean residence time; *R*-γ-CEHC, 2,7,8-trimethyl-2*R*-(β-carboxyethyl)-6-hydroxychroman; *S*-γ-CEHC, 2,7,8-trimethyl-2*S*-(β-carboxyethyl)-6-hydroxychroman; TFA, trifluoroacetic acid.

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availability regardless of their administration routes (e.g., parenteral, oral, or topical). In order to solubilize γ-Toc in water, a large amount of surfactant [e.g., polyoxyethylene hydrogenated castor oil (HCO-60)] would be needed for the solution formulation. The use of surfactants is, however, undesirable for a parenteral dosage because it generally induces toxicity such as an anaphylactoid reaction.

It is well known that the prodrug approach is a useful approach to improve the physicochemical properties of parent drugs. The phenolic functional group in γ-Toc is easily esterified and some of the ester derivatives are expected to provide the desired improvements in water-solubility and the stability to oxidation. An ideal prodrug in such applications should exhibit sufficient aqueous solubility and should be rapidly converted into the parent drug in vivo. In this regard, the most successful prodrugs of γ-Toc are those that exhibit sufficient water solubility and a reconversion characteristic to the parent drug after administration. We have already observed that the *N,N*-dimethylglycine esters of α-tocopherol and vitamin K hydroquinones exhibited a significant solubility in water and have a high susceptibility to hydrolysis catalyzed by esterase in rat and human livers (15–20). In this paper, we report that the *N,N*-dimethylglycine ester of γ-Toc was synthesized and evaluated as a water-soluble prodrug of γ-Toc for solution formulation and, in addition, as a two-step prodrug of *S*-γ-CEHC in vivo.

MATERIALS AND METHODS

Melting points were determined using a Yazawa micromelting point BY-1 apparatus and are uncorrected. The microanalyses, the proton nuclear magnetic resonance spectrometry (^{1}H-NMR), and mass spectra measurements were carried out at the Central Microanalytical Department of Pharmaceutical Science, Fukuoka University. The ^{1}H-NMR spectra were recorded at 500 MHz in solutions of $CDCl_3$ using a JEOL JNM-A500 spectrometer. The chemical shifts are expressed in δ (ppm) using tetramethylsilane as the internal standard. The following abbreviations are used: s, singlet; m, multiplet. Mass spectra (MS) were obtained using a JEOL JMS-HX110 spectrometer.

A natural vitamin E mixture for food (EMix 80) and a synthesized 2,7,8-trimethyl-2-(β-carboxylethyl)-6-hydroxychroman (racemic γ-CE HC) were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan). Vitamin E reference standards consisting of α-Toc, *d*-β-tocopherol (2*R*,4′*R*,8′*R*-β-tocopherol, β-Toc), γ-Toc, and *d*-δ-tocopherol (2*R*, 4′*R*,8′*R*-δ-tocopherol, δ-Toc) was purchased from Eisai Co., Ltd. (Tokyo, Japan). Eserine (physostigmine hemisulfate) was purchased from Sigma Chemical Co. (St. Louis, MO). *N,N*-dimethylglycine hydrochloride was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). HCO-60 was purchased from Nikko Chemical (Tokyo, Japan). Male Sprague-Dawley (SD) rats (210–235 g), SD rat liver microsome, and human liver microsome were purchased from Charles River, Japan, Inc. (Kanagawa, Japan). Human plasma was obtained from healthy volunteers (age 21–22) after oral informed consent. All other chemicals were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Purification of *d*-γ-tocopherol fraction from a natural vitamin E mixture

The EMix 80 was fractionated by flash chromatography and normal phase HPLC, and γ-Toc was isolated without delay by solvent evaporation under vacuum then stored under argon at −30°C. The purity of the isolated γ-Toc was determined by normal phase HPLC analysis comparing with α-Toc, β-Toc, γ-Toc, and δ-Toc standards, and verified by mass spectra, ^{1}H-NMR, and microanalysis. α-Toc, and β-Toc, and δ-Toc were not found in the isolated γ-Toc fraction. The microanalytical data agreed with the calculated values. Thus, the purity of γ-Toc was confirmed as the same as the γ-Toc standard (100%). γ-Toc: colorless oil; field desorption mass spectrometry (FD-MS) *m/z* 416 (M^+). ^{1}H-NMR δ($CDCl_3$): 6.36 (1H, s), 2.67 (2H, m), 2.13 (3H, s), 2.11 (3H, s), 1.73, (2H, m), 1.59–1.03 [24H, m, including 1.24 (3H, s)], 087–0.83 (12H, m). *Anal.* Calcd for $C_{28}H_{48}O_2$: C, 80.71; H, 11.61. Found: C, 80.77; H, 11.57.

Synthesis of *d*-γ-tocopheryl *N,N*-dimethylglycinate hydrochloride

To a dry pyridine solution of γ-Toc (4.8 mmol), 5.7 mmol of *N,N*-dimethylglycine hydrochloride, and 5.7 mmol of dicyclohexylcarbodiimide were added. The reaction mixture was stirred at room temperature for 20 h and the dicyclohexylurea formed was removed by filtration. After the solvent was evaporated, the residue was treated with 100 ml of water and made alkaline by sodium bicarbonate. The solution was then extracted with ethyl acetate (100 ml × 3). The organic layer was dried over anhydrous sodium sulfate and evaporated. The residue was fractionated with a flash column packed Wakogel LP40, 60A using n-hexane ethyl acetate (8:2, v/v) as the eluent. The isolated ester was directly collected in isopropyl ether containing 3% HCl dioxane solution, and the precipitate was collected and recrystallized from acetone to give the hydrochloride salt of *d*-γ-tocopheryl *N,N*-dimethylglycinate (γ-TDMG). It was confirmed that the synthesized γ-TDMG was free of γ-Toc (starting material) by HPLC analysis as mentioned below. White solid, Yield 83%; mp 161-163°C; FD-MS *m/z*: 501 (M-HCl^+). ^{1}H-NMR δ($CDCl_3$): γ-tocopheryl moiety 6.63 (1H, s, 5-H), 2.71 (2H, m, 4-H_2), 2.11 (3H, s, 7-CH_3), 2.02 (3H, s, 8-CH_3), 1.76, (2H, m, 3-CH_2), 1.59–1.02 [24H, m, including 1.26 (3H, s, 2-CH_3)], 0.87–0.83 (12H, m). *N, N*-dimethylglycine moiety 4.21 (2H, s, NCH_2CO), 3.09 [6H, s, $(CH_3)_2N$]. *Anal.* Calcd for $C_{32}H_{56}NO_3Cl+0.2H_2O$: C, 70.93; H, 10.49; N, 2.58. Found: C, 70.89; H, 10.54; N, 2.60.

Water solubility

The aqueous solubility of γ-TDMG was determined by adding 50 μmol of the ester to 1 ml of water in amber vials maintained at 25 ± 0.1°C in a constant-temperature water bath. The vials were shaken for 24 h and the contents were filtered using membrane filters (Columnguard-LCR4, 0.5 μm, Nihon Millipore Kogyo K. K., Yonezawa, Japan). The ester concentration in the filtrates was determined by the HPLC method described below. The percentage of the γ-TDMG in the filtrate through the membrane (0.5 μm) was above 99.7%, while the percentage of the γ-TDMG remaining in the filter was below 0.3%. Thus, the adherence of γ-TDMG in the filter was ignored.

Hydrolysis studies

The hydrolysis of the ester was studied at 37°C in an isotonic phosphate buffer (pH 7.4), rat plasma, rat liver microsome, human plasma, and human liver microsome. The stock solution of the ester was dissolved in water containing 5% methanol. The enzymatic reactions were initiated by adding 50 μl of an aqueous stock solution of the ester and 50 μl of the isotonic phosphate buffer to 900 μl of a preheated reaction medium in amber test tubes. The concentrations of the rat and human plasmas were 90%. Commercially available rat liver microsome and human liver microsome were used at 0.1 mg of protein/ml after being diluted with phosphate buffered saline (PBS). The initial con-

centration of the esters was 0.4–4.0 × 10^{-3} M. The solutions were incubated at 37°C. At appropriate intervals, 100 μl of the reaction solution were sampled and added to 350 μl of ethanol. After 2 min of vortex mixing, followed by centrifugation at 3,000 rpm for 5 min, 50 μl of the clear supernatant was analyzed by HPLC. The initial hydrolytic rate, in units of moles of γ-Toc formed per liter of reaction medium volume, was calculated from the initial slope of the formation plot of γ-Toc versus time. No measurable chemical hydrolysis of γ-TDMG occurred during the time span of these hydrolysis studies in the rat plasma, the rat liver microsome, the human plasma, or the human liver microsome, as demonstrated by the HPLC results mentioned below. In the phosphate buffer, the apparent first-order rate constants for the hydrolysis were obtained by linear regression analysis of the natural logarithm of concentration versus time (correlation coefficient >0.97).

The effects of eserine on the hydrolysis of the ester in the liver microsome were also studied. The procedure for the experiment was the same as that mentioned above, except that 50 μl of eserine aqueous solution was added in place of the PBS to the liver microsome solution at the beginning of the experiment. Eserine was used at a 0–2.0 mM concentration. The studies on the hydrolysis of γ-TDMG and the inhibition effect by eserine have been carried out in three separate experiments.

HPLC analysis

The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of a pump (LC6A), an auto sample injector (SIL 9A), a UV detector (SPD-10AV), a spectrofluorophotometer (RF-540) equipped with a 12 μl LC flow cell, and a peak integrator (C-R7A). The eluent was spectrophotometrically monitored at 283 nm, and spectrofluorometrically at an emission of 325 nm with excitation at 298 nm. For the analysis of γ-TDMG and γ-Toc, a reversed-phase column, CAPCELL PAK C18 UG120 (4.6 × 150 mm, Shiseido, Tokyo, Japan), with a mobile phase of CH_3OH-CH_3CN (7:3, v/v) at a flow rate of 0.7 ml/min was employed. Quantitation of these compounds was achieved using linear calibration curves constructed from the peak area versus the concentration of the standard compound.

Disposition study of γ-TDMG and γ-Toc in rats

All procedures regarding animal care and use were performed in compliance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University. The dose effect of γ-TDMG on the plasma disposition of γ-Toc was initially determined in rats. Male SD rats were fasted for 16 h prior to use, but water and sugar crystals were administered ad libitum. The solution of γ-TDMG was solubilized with distilled water contained 15% propylene glycol. The solution of γ-Toc was solubilized with water containing 10% HCO-60 and 15% propylene glycol. The drugs were administered via the left femoral vein exposed by means of a small incision under light ether anesthesia. The solution of γ-TDMG was injected at doses of 5, 10, and 25 mg/kg (equivalent for γ-Toc). The drug solution for injection was administered at 0.1 ml/100g of body weight. Blood (300 μl) was taken from the external jugular vein using heparinized syringes at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h. The plasma samples (100 μl) were added to 350 μl of ethanol, vortexed for 2 min, and then centrifuged at 3,000 rpm for 5 min. The supernatant layer (50 μl) was determined by the HPLC method as mentioned above.

The rats were treated and the drugs (γ-TDMG and γ-Toc) were administered according to the procedures mentioned above. The doses of the drugs were 25 mg/kg equivalent for γ-Toc. Under ether anesthesia, blood (4.5 ml) was taken from the abdominal artery using a syringe containing 0.5 ml of 3.2% sodium citrate, and the liver was removed at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h. The plasma was immediately separated by centrifugation at 5°C and stored at −80°C until the HPLC analysis. The tissues were homogenized with 3 vol of 1.15% KCl solution containing 1 mM of eserine using a POLYTRON homogenizer (Kinematica, Switzerland) and stored at −80°C until the HPLC analysis. The plasma and tissue homogenated samples (100 μl) were added to 350 μl of ethanol, vortexed for 2 min and then centrifuged at 3,000 rpm for 5 min. The supernatant layer (50 μl) was determined by the HPLC method described above.

Plasma disposition of *S*-γ-CEHC in rats

γ-TDMG or racemic γ-CEHC was administered to rats (8 weeks old) according to the procedures mentioned above. The solution of racemic γ-CEHC was solubilized with saline containing 33% polyethyleneglycol. Blood (300 μl) was taken from the external jugular vein using heparinized syringes. The plasma samples (50 μl) were subjected to the enantiometric determination of *S*-γ-CEHC and *R*-γ-CEHC mentioned below.

HPLC analysis of γ-CEHC enatiomers

The determination of γ-CEHC enantiomers (*S*-γ-CEHC and *R*-γ-CEHC) in rat plasma was carried out according to our HPLC system (14), which allowed the enantiometric determination of *S*-γ-CEHC and *R*-γ-CEHC without the manual isolation of precolumn fluorescent labeled γ-CEHC enantiomers (21). *S*-γ-CEHC and *R*-γ-CEHC for calibration standards were isolated from racemic γ-CEHC using the previously reported chiral HPLC system (21), and verified by CD spectrophotometry according to the method reported by Kantoci et al. (11). The chiral HPLC system was equipped with a chiral column Sumichiral OA-3100 (250 × 4.6 mm id, 5 mm)(Sumika Chemical Analysis Service, Co. Ltd.) and eluted with a mobile phase of 5.0 mM ammonium acetate in MeOH. The HPLC system for enantiometric determination of *S*-γ-CEHC and *R*-γ-CEHC consisted of three pumps, a L-7100 (Hitachi, Tokyo, Japan), two PU610-10s (Gl Science, Tokyo, Japan), two fluorescence detectors L-7480 (Hitachi), two integrators 807-IT (Jasco, Tokyo, Japan), and two 6-port valves HV-992-01 (Jasco, Tokyo, Japan). The mobile phase compositions and flow rates are as follows: H_2O-CH_3CN-TFA (650:350:1, v/v/v), 0.8 ml/min for phenyl column; H_2O-CH_3CN-TFA (400:600:1, v/v/v), 0.3 ml/min for ODS column; and CH_3OH-CH_3CN (95:5, v/v), 0.3 ml/min for chiral column. During the pretreatment procedure, briefly, γ-CEHC in the rat plasma (50 μl) was derivatized with a fluorescent reagent, 4-*N*,*N*-dimethylaminosulfonyl-7-piperazino-2,1,3-benzoxadiazole (DBD-PZ), and acetylated with acetyl chloride after deproteinization by adding CH_3CN-EtOH (4:1, v/v). Following purification with an Empore™ C_{18} cartridge, the sample was injected into a column-switching HPLC system (14) containing three different kinds of columns: TSKgel Super-Phenyl (100 × 4.6 mm, Tosoh, Tokyo, Japan), TSKgel ODS-80Ts (250 × 4.6 mm, Tosoh), and CHIRALCEL OD-RH (150 × 4.6 mm, Daicel Co. Ltd, Tokyo, Japan), which were connected through two 6-port valves equipped with a trapping column. The fraction including the γ-CEHC derivative, separated on the phenyl column, was introduced into the ODS column and the re-separated chiral column. The detection was made fluorometrically at 560 nm with a 450 nm excitation wavelength.

Pharmacokinetic analysis

The plasma and liver concentrations versus time data were analyzed using the model independent and statistical moment

methods (22, 23). Both the maximum concentration (C_{max}) and its corresponding time (t_{max}) were obtained directly from the observed data. The systemic availability and liver availability for γ-Toc after iv administration of γ-TDMG relative to the γ-Toc administration was determined from the ratio of area under the concentration-time curve (AUC) of γ-Toc, based on Eqs. 1 and 2, respectively. The selective advantage value (24) for γ-Toc in the liver was calculated using Eq. 3. The systemic availability for *S*-γ-CEHC after iv administration of γ-TDMG relative to the γ-Toc administration was determined from the ratio of AUC of *S*-γ-CEHC based on Eq. 4.

$$F = \frac{AUC^{Plasma}_{\gamma\text{-Toc},\gamma\text{-TDMG}} \cdot D_{\gamma\text{-Toc}}}{AUC^{Plasma}_{\gamma\text{-Toc},\gamma\text{-Toc}} \cdot D_{\gamma\text{-TDMG}}} \cdot 100\ (\%) \qquad (Eq.\ 1)$$

$$F_{Liver} = \frac{AUC^{Liver}_{\gamma\text{-Toc},\gamma\text{-TDMG}} \cdot D_{\gamma\text{-Toc}}}{AUC^{Liver}_{\gamma\text{-Toc},\gamma\text{-Toc}} \cdot D_{\gamma\text{-TDMG}}} \cdot 100\ (\%) \qquad (Eq.\ 2)$$

$$\text{selective advantage} = \left\{\frac{AUC^{Liver}_{\gamma\text{-Toc},\gamma\text{-TDMG}}}{AUC^{Liver}_{\gamma\text{-Toc},\gamma\text{-Toc}}}\right\} \Big/ \left\{\frac{AUC^{Plasma}_{\gamma\text{-TDMG},\gamma\text{-TDMG}}}{AUC^{Plasma}_{\gamma\text{-Toc},\gamma\text{-Toc}}}\right\} \qquad (Eq.\ 3)$$

$$F = \frac{AUC^{Plasma}_{S\text{-}\gamma\text{-CEHC},\gamma\text{-TDMG}} \cdot D_{\text{racemic }\gamma\text{-CEHC}}}{AUC^{Plasma}_{S\text{-}\gamma\text{-CEHC, racemic }\gamma\text{-CEHC}} \cdot D_{\gamma\text{-TDMG}}} \cdot 100\ (\%) \qquad (Eq.\ 4)$$

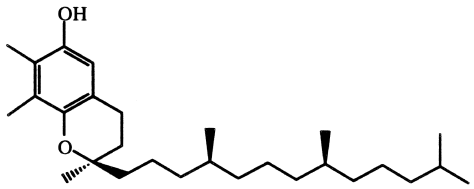
where $AUC^{Plasma}_{\gamma\text{-Toc},\gamma\text{-TDMG}}$, $AUC^{Plasma}_{\gamma\text{-Toc},\gamma\text{-Toc}}$, $AUC^{Liver}_{\gamma\text{-Toc},\gamma\text{-TDMG}}$, and $AUC^{Liver}_{\gamma\text{-Toc},\gamma\text{-Toc}}$ are the AUC values for γ-Toc in the plasma and tissues after the administration of γ-TDMG and γ-Toc, respectively. $AUC^{Plasma}_{\gamma\text{-TDMG},\gamma\text{-TDMG}}$ is the AUC value for γ-TDMG; $D_{\gamma\text{-TDMG}}$, $D_{\gamma\text{-Toc}}$, and $D_{\text{racemic }\gamma\text{-CEHC}}$ are doses of γ-TDMG, γ-Toc, and racemic γ-CEHC, respectively; $AUC^{Plasma}_{S\text{-}\gamma\text{-CEHC},\gamma\text{-TDMG}}$ and $AUC^{Plasma}_{S\text{-}\gamma\text{-CEHC, racemic }\gamma\text{-CEHC}}$ are the AUC values for the increased *S*-γ-CEHC in the plasma after the administration of γ-TDMG and racemic γ-CEHC, respectively.

RESULTS AND DISCUSSION

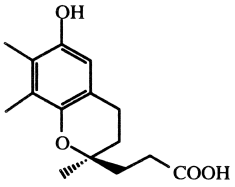
The γ-TDMG (**Fig.1**) was synthesized by procedures described in Materials and Methods, and characterized by mass spectrometric analysis, ^{1}H-NMR, and elemental analysis. The hydrochloride salt of the ester was isolated as a crystalline compound. The melting point of the ester was 161–163°C.

Water solubility of the γ-TDMG

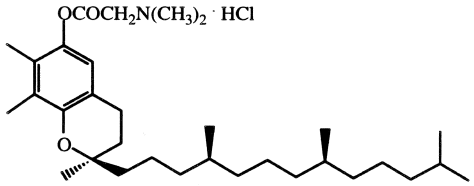
The hydrochloride salt of γ-TDMG showed a drastic increase in its water solubility and gave a turbid solution up to 50 mM. The transparent solution of the ester was prepared (25 mg/ml equivalent for γ-Toc) when dissolved in water containing 15% propylene glycol. It was thought that the introduction of an ionizable *N,N*-dimethylglycine group in the ester moiety to γ-Toc made it possible to obtain a crystalline and water-soluble derivative of γ-Toc.



d-γ-Tocopherol (2*R*,4'*R*,8'*R*-γ-Tocopherol, γ-Toc)



2,7,8-Trimethyl-2*S*-(β-carboxyethyl)-6-hydroxychroman (*S*-γ-CEHC, *S*-LLU-α)



d-γ-Tocopheryl *N,N*-dimethylglycinate hydrochloride
(2*R*,4'*R*,8'*R*-γ-Tocopheryl *N,N*-dimethylglycinate hydrochloride, γ-TDMG)

Fig. 1. Chemical structures of *d*-γ-Tocopherol (γ-Toc), 2,7,8-trimethyl-2*S*-(β-carboxyethyl)-6-hydroxychroman (*S*-γ-CEHC), and *d*-γ-tocopheryl *N,N*-dimethylglycinate hydrochloride (γ-TDMG).

Enzymatic hydrolysis of the γ-TDMG

In developing a useful prodrug, the linkage between the parent drug and the promoiety should be stable in the formulations but rapidly cleaved in vivo. The most successful prodrug of γ-Toc necessitates reconverting it into the parent drug by enzyme(s) encountered after administration. To determine its in vivo behavior, the kinetics of hydrolysis of the ester were investigated in an isotonic phosphate buffer (pH 7.4), rat plasma, rat liver microsome preparation, human plasma, and human liver microsome preparation at 37°C. HPLC analysis showed a significant acceleration of the hydrolytic rates of the ester to produce γ-Toc in rat plasma, rat liver, and human liver microsome, but not in the human plasma. The kinetics of the hydrolysis can be represented by the Michaelis-Menten model. This result obviously indicates that the hydrolysis of the ester enzymatically proceeded. The kinetic data were analyzed using the Lineweaver-Burk equation (25),

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{S_0} + \frac{1}{V_{max}} \qquad (Eq.\ 5)$$

where v_0 is the initial rate of the hydrolysis, S_0 is the initial concentration of the ester, K_m is the Michaelis constant, and V_{max} is the maximal hydrolytic rate for a saturating substrate concentration at a given enzyme concentration.

Representative Lineweaver-Burk plots are shown in **Fig. 2**, which depict the hydrolysis in the rat and human liver

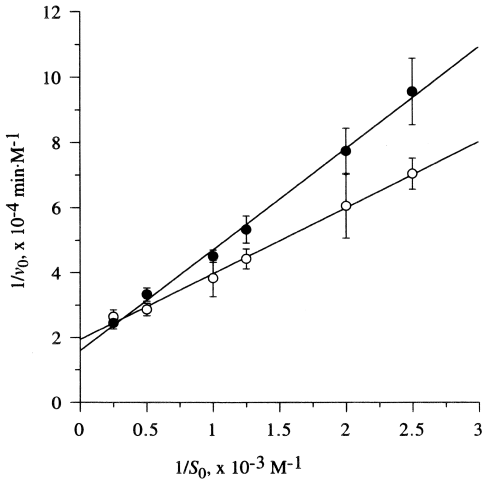


Fig. 2. Representative Lineweaver-Burk plots of initial rates for the hydrolysis of γ-TDMG in the rat liver microsome and the human liver microsome at pH 7.4 and 37°C. Open circle, rat liver microsome; closed circle, human liver microsome. Each point represents the mean ± SD of three experiments. The lines represent least-square regression lines ($r > 0.98$).

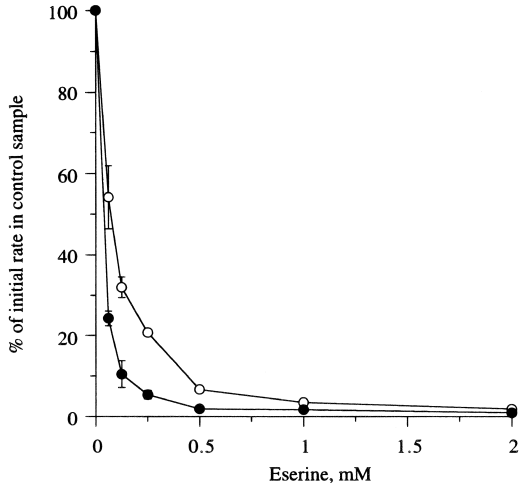


Fig. 3. Effect of eserine on the rat liver and the human liver enzymatic hydrolysis of γ-TDMG. Open circle, in the rat liver microsome; closed circle, in the human liver microsome. The initial concentration of γ-TDMG was adjusted at 0.4 mM. Each point represents the mean ± SD of three experiments.

microsome preparations. The kinetic parameters of V_{max} and K_m generated from the initial-rate data and a linear regression analysis of Eq. 5 are listed in **Table 1** along with V_{max}/K_m.

The results obtained from the kinetic analysis demonstrated that the hydrolysis of the ester was catalyzed by an enzyme in rat plasma and rat liver. The concentrations of rat plasma preparation and rat liver microsome preparation used in this test are equivalent for 90% and 0.87% of tissues, respectively. Although the rat liver microsome preparation was diluted more than the rat plasma, the K_m/V_{max} in the rat liver preparation gave a higher value. This result strongly suggested that the ester would be more effectively hydrolyzed in rat liver than in rat plasma.

Considering the therapeutic application of the ester as a prodrug, it is an important criteria that the ester can be readily cleaved by a human enzyme. A significant acceleration of the hydrolytic rate of the ester was found in the human liver microsome preparation, but not in the human plasma preparation (Table 1). This in vitro result suggested that the hydrolysis of the ester could be catalyzed by enzyme(s) located in humans. In humans, the hydrolysis of γ-TDMG has been studied only in vitro.

As described above, γ-TDMG was mainly cleaved by an enzyme contained in the liver to release the parent drug. To assess whether the observed catalytic cleavage of γ-TDMG in the liver could be attributed to liver esterase, we investigated the effects of eserine, an esterase inhibitor, on the release of γ-Toc in the rat and human liver microsome preparations. The catalytic hydrolysis of γ-TDMG by both rat and human liver microsomes was prominently inhibited in the presence of eserine (**Fig. 3**). It has been postulated that carboxylesterase is present in both the rat (26) and human (27) liver microsomes and that eserine is an inhibitor of carboxylesterase (28). Thus, the results suggested that the rat and human liver carboxylesterases mainly catalyzed the reconversion of the prodrug.

We previously reported that the hydrolysis of the aminoalkylcarboxylic acid esters of *d*-α-tocopherol was catalyzed by esterase in rat liver, but not by esterase in both the rat and human plasma (15). It has been shown that the enzymes located in rat liver, rat plasma, and human liver, but not in human plasma, catalyzed the hydrolysis of the aminoalkylcarboxylic acid esters of menahydroquinone-4 (17, 19). On the other hand, a catalytic hydrolysis of the aminoalkylcarboxylic acid esters by the human plasma enzyme has been shown in the case of metronidazol (29) and 1-(hydroxymethyl) allopurinol (30). These observations led us to the idea that not only the structure of the promoieties, but also the structure of the parent drugs, affected the susceptibilities of the ester prodrugs to plasma enzymatic hydrolysis.

For the purpose of developing the prodrug for a solu-

TABLE 1. Kinetic parameters for the hydrolysis of γ-TDMG in vitro at pH 7.4 and 37°C

	K_m[a]	V_{max}[a]	V_{max}/K_m
	$\times 10^{-3}$ M	$\times 10^{-6}$ M/min	$\times 10^{-3}$ min^{-1}
In buffer[b]	—	—	k = 0.0231 × 10^{-2} h^{-1}
In rat liver microsome[c]	1.06 ± 0.03	52.2 ± 4.7	49.3 ± 5.4
In human liver microsome[d]	2.04 ± 0.15	64.7 ± 2.2	31.8 ± 2.3
In rat plasma[e]	4.78 ± 0.29	1.37 ± 0.04	0.288 ± 0.018
In human plasma[e,f]	—	—	—

[a] Values are obtained from the Lineweaver-Burk plots of three experiments.
[b] Isotonic phosphate buffer (pH 7.4); rate was obtained from the first order plot.
[c] Protein concentration was 0.1 mg/ml (equivalent for 0.87% of rat liver).
[d] Protein concentration was 0.1 mg/ml 90% plasma.
[e] Ninety percent plasma.
[f] Significant acceleration was not observed.

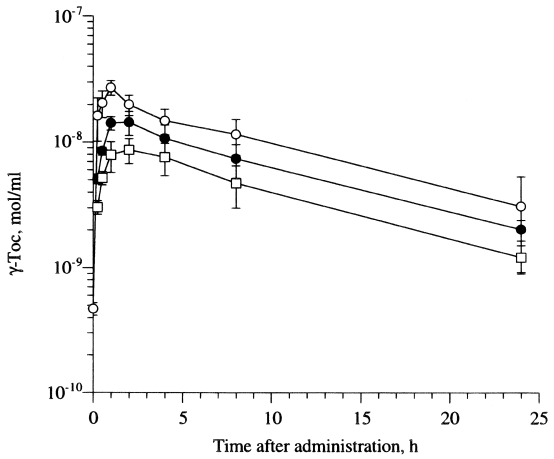


Fig. 4. Mean plasma concentration of γ-Toc after the intravenous administration of γ-TDMG in the rats. The doses are 25 mg/kg (open circle), 10 mg/kg (closed circle), 5 mg/kg (square) equivalent for γ-Toc. Each point represents the mean ± SD of three rats.

tion formulation, a prodrug with a high water solubility and a high reconversion rate into γ-Toc appeared the most promising for further in vivo studies. γ-TDMG would be a suitable water-soluble prodrug of γ-Toc for iv administration, because γ-TDMG was a crystalline compound and soluble in water (over 50 mM) and γ-TDMG is converted into γ-Toc catalyzed by esterases in both the rat and human livers. Based on these findings, further in vivo studies are now being carried out.

Disposition of the prodrug after iv administration in rats

The dose effect of γ-TDMG on the plasma disposition of γ-Toc was preliminarily determined in rats in the dose range from 5 to 25 mg/kg equivalents to γ-Toc (**Fig. 4**). The rapid appearance and dose dependently increased level of γ-Toc in plasma after γ-TDMG administration indicated that γ-Toc was regenerated from γ-TDMG in vivo. The AUC values of γ-Toc from 0 to 24 h after the administration of γ-TDMG were also increased dose dependently and a linear correlation was found between the AUC of γ-Toc and the dose of γ-TDMG (data not shown). This result indicated that there was no saturated process during the regeneration of γ-Toc in the dose range tested in this study. Thus, further studies were carried out using a dose of 25 mg/kg equivalent to γ-Toc.

In order to evaluate the utility of γ-TDMG as a prodrug, the disposition of the intrinsic ester and γ-Toc in the plasma and liver after the iv administration of γ-TDMG was compared with that after the iv administration of the γ-Toc solubilized with HCO-60. The tissue concentration-time profiles after the iv administration of γ-TDMG or γ-Toc are shown in **Fig. 5**. The pharmacokinetic parameters for γ-Toc and the intrinsic ester are summarized in **Table 2**. Following the iv administration, γ-TDMG was rapidly eliminated from the plasma and significantly accumulated in the liver, in which the maximum accumulation was achieved at 0.25 h after the administration of γ-TDMG. The liver level of γ-Toc achieved a maximum at 2 h after the administration of γ-TDMG. The relative systemic availability for γ-Toc (F) after the γ-TDMG and γ-Toc administrations were 26.8 ± 3.4 and 100 ± 6.0%, respectively. Compared with γ-Toc, γ-TDMG showed an improvement in the liver availability of γ-Toc; the relative liver availabilities of γ-Toc (F_{Liver}) were 303 ± 47.0 (γ-TDMG) and 100 ± 8.8% (γ-Toc).

The liver and plasma distributions of γ-Toc and γ-TDMG at 0.5 h after the γ-TDMG administration were 21.4 ± 1.9 (γ-Toc in liver), 76.5 ± 3.2 (γ-TDMG in liver), 1.4 ± 0.4 (γ-Toc in plasma), and 0.7 ± 0.3% (γ-TDMG in plasma) of the dose. At this time, the distributions of γ-Toc after the γ-Toc administration were 30.7 ± 2.0 (γ-Toc in liver)

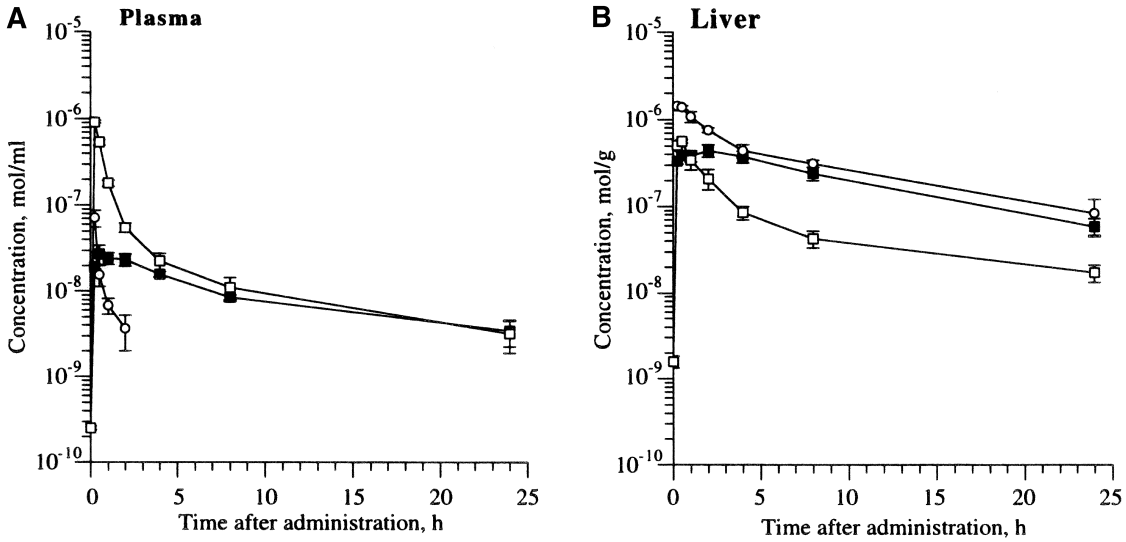


Fig. 5. Mean plasma and liver concentration of γ-TDMG and γ-Toc after the intravenous administration of γ-TDMG and γ-Toc in the rats. Open circle, γ-TDMG; closed circle, γ-Toc after γ-TDMG administration; square γ-Toc after γ-Toc administration. Each point represents the mean ± SD of three rats. The dose is 25 mg/kg equivalent for γ-Toc.

TABLE 2. Pharmacokinetic parameters in plasma and liver after the intravenous administration of γ-Toc and γ-TDMG in the rats[a]

	γ-Toc		γ-TDMG	
	Plasma	Liver	Plasma	Liver
For intrinsic *d*-γ-TDMG				
C_{max} (μmol · ml^{-1} or g^{-1})	—	—	1.42 ± 0.141	1.42 ± 0.141
T_{max} (h)	—	—	0.25	0.25
AUC (μmol · h · ml^{-1} or g^{-1})	—	—	0.031 ± 0.007	7.95 ± 1.01
MRT (h)	—	—	0.53 ± 0.02	6.24 ± 0.51
For *d*-γ-Toc				
C_{max} (μmol · ml^{-1} or g^{-1})	0.915 ± 0.003	0.565 ± 0.029	0.027 ± 0.007	0.027 ± 0.007
T_{max} (h)	0.25	0.50	0.50	2.0
AUC (μmol · h · ml^{-1} or g^{-1})	0.844 ± 0.051	1.720 ± 0.152	0.226 ± 0.029	5.210 ± 0.809
MRT (h)	2.60 ± 0.35	4.98 ± 0.29	7.09 ± 0.42	7.10 ± 0.27
F (%)[b]	100 ± 6.0		26.8 ± 3.4	
F_{Liver} (%)[c]	100 ± 8.8		303 ± 47.0	
Selective advantage[d]	1.0		83.8	

[a] The values are the mean and SD of three rats at a dose of 25 mg/kg equivalent for γ-Toc.
[b] Calculated from Eq. 1.
[c] Calculated from Eq. 2.
[d] Calculated from Eq. 3.

and 28.0 ± 2.0% (γ-Toc in plasma) of the dose. The rapid and liver-specific uptake of γ-TDMG and the rapid appearance of γ-Toc in the liver indicated that the regeneration of γ-Toc might thus mainly occur in the liver. It appeared that these characteristics of γ-TDMG might provide a specific delivery system for γ-Toc to the liver. A remarkable liver-specific delivery of γ-Toc was observed after the administration of γ-TDMG. The selective advantages of the γ-TDMG and γ-Toc administrations were 83.8 and 1.0, respectively.

These disposition studies clearly indicated that γ-TDMG would be a useful candidate for the parenteral prodrug of γ-Toc. The effective delivery of γ-Toc with the iv administration of γ-TDMG is a meaningful method for achieving a rapid and accurate onset of action of γ-Toc and might alter the several prospective biological activities of γ-Toc. In preliminary experiments, it was found that the iv administration of the prodrug to the middle cerebral artery occlusion of mice afforded a lesser degree of cerebral brain damage compared to administration of α-Toc solubilized in DMSO. The results of the preventative effect in cerebral infarction will be the subject of a subsequent paper.

Plasma disposition of *S*-γ-CEHC after iv administration of the prodrug

Since *S*-γ-CEHC contains the same cromanol structure as γ-Toc, *S*-γ-CEHC is readily oxidized by atmospheric oxygen as well as γ-Toc. *S*-γ-CEHC exhibits a 20-fold more potent natriuretic activity than *R*-γ-CEHC (12), but shows a

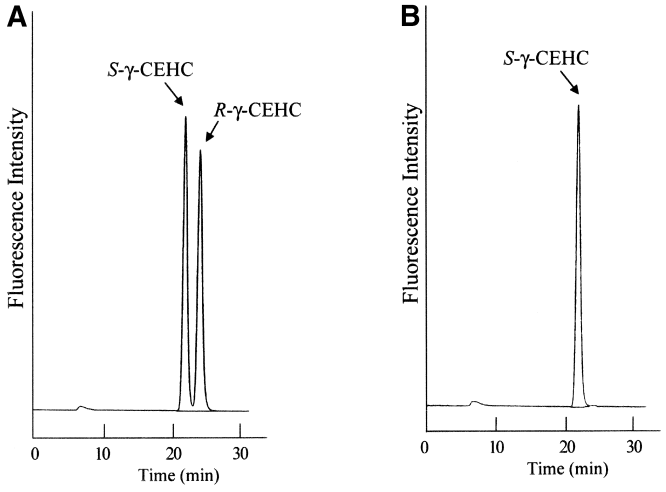


Fig. 6. Representative chromatograms of enantiometric separation of fluorescent γ-CEHC derivative. A: Racemic γ-CEHC standard. B: Rat plasma sample at 1 h after iv administration of γ-TDMG.

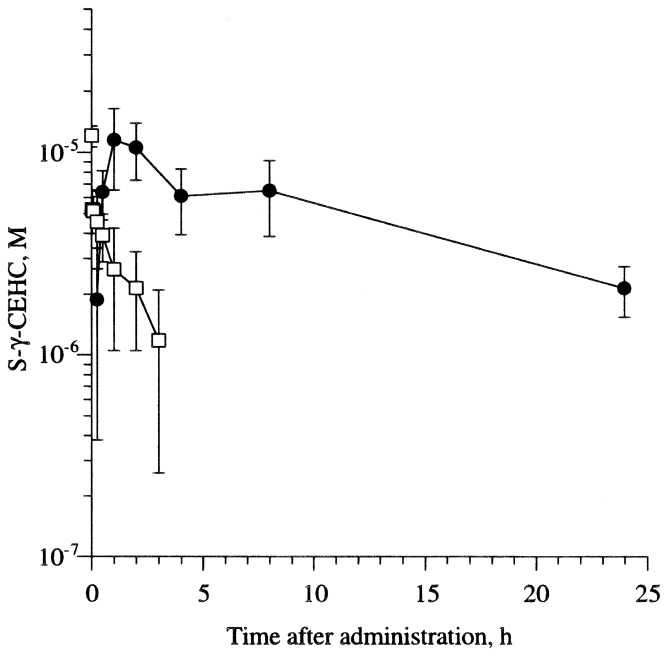


Fig. 7. Mean plasma concentration of *S*-γ-CEHC after the intravenous administration of γ-TDMG and racemic γ-CEHC in the rats. Circle, γ-TDMG; square, racemic γ-CEHC. Each point represents the mean ± SD of four rats. The doses are shown in Table 3.

TABLE 3. Pharmacokinetic parameters for *S*-γ-CEHC after iv administration of γ-TDMG and racemic γ-CEHC in the rats

Compounds	γ-TDMG	Racemic γ-CEHC
Dose (μmol/kg)	60	3.8[a]
C_{max} (μmol/l)	11.5 ± 4.97	12.1 ± 1.42
T_{max} (h)	1	0.003
AUC (μmol · h · l^{-1})	128 ± 36.9	7.93 ± 4.34
MRT (h)	8.32 ± 0.99	1.00 ± 0.25
F (%)	102[b]	100

[a] γ-CEHC was not soluble in distilled water for injection. Therefore, γ-CEHC was solubilized with water containing 33% polyethyleneglycol and used for iv administration. The low solubility of γ-CEHC compelled us to adopt a low dose of γ-CEHC for the disposition study.
[b] Calculated from Eq. 4.

rapid elimination rate after iv administration (14). These characteristics limit the therapeutic applications of *S*-γ-CEHC. It has been shown that γ-Toc is mainly metabolized to *S*-γ-CEHC without epimerization at C2 (10, 11), and the conversion to *S*-γ-CEHC was catalyzed by cytochrome P450 3A (CYP3A) in a cell culture (31). Therefore, it seems that the use of γ-TDMG as a two-step prodrug for *S*-γ-CEHC is a meaningful method for overcoming the delivery problems of *S*-γ-CEHC because γ-TDMG can efficiently deliver γ-Toc to the liver.

To evaluate the γ-TDMG as a two-step prodrug of *S*-γ-CEHC, the plasma disposition of *S*-γ-CEHC after the iv administration of γ-TDMG was compared with that of the racemic γ-CEHC. As a result of the HPLC analysis, only *S*-γ-CEHC was detected in the plasma during the experiment interval after the γ-TDMG administration (**Fig. 6**). The time-course of the plasma concentration of *S*-γ-CEHC is shown in **Fig. 7**. The pharmacokinetic parameters for *S*-γ-CEHC are summarized in **Table 3**. Following the iv administration of γ-TDMG, the plasma *S*-γ-CEHC level was rapidly increased and achieved the maximum accumulation at 1 h after the administration, and the mean residence time (MRT) of *S*-γ-CEHC was prolonged by eight times compared to the racemic γ-CEHC administration. The relative systemic availabilities for *S*-γ-CEHC (*F*) after the γ-TDMG and racemic γ-CEHC administration have equivalent values of 102% and 100%, respectively. This result clearly indicated that γ-TDMG can act as a two-step prodrug of *S*-γ-CEHC. Although the mechanism for the delivery of *S*-γ-CEHC with γ-TDMG administration could not be confirmed, the selective appearance of *S*-γ-CEHC suggested that γ-TDMG was first reconverted to γ-Toc and metabolized to *S*-γ-CEHC. It has already been shown that γ-Toc was metabolized to γ-CEHC by CYP3A (31). Thus, when γ-TDMG is taken together with drugs that are principally metabolized by CYP3A, the disposition kinetics of γ-CEHC after γ-TDMG administration may be altered due to competitive inhibition between γ-Toc and the drugs on the CYP3A.

In conclusion, the hydrochloride salt of the *N*, *N*-dimethylglycinate of γ-Toc displayed a high melting point, a sufficient solubility in water, and high susceptibility to the enzymatic hydrolysis by rat and human liver enzymes. Since the aim of the present prodrug development is to overcome the problems of crystallization and solubilization of γ-Toc in aqueous solution, γ-TDMG is a desirable prodrug of γ-Toc. The animal experiments suggested that γ-TDMG was a potentially useful prodrug of γ-Toc and also useful as a two-step prodrug of *S*-γ-CEHC for iv administration. The prodrug could also avoid the toxicity induced by the solubilizing agent, HCO-60. It appears that the effective and selective delivery of γ-Toc to the liver and prolonged delivery of *S*-γ-CEHC might lead to an enhanced pharmacological efficacy of γ-Toc and *S*-γ-CEHC.

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