J Am Oil Chem Soc (2008) 85:313–320
DOI 10.1007/s11746-008-1206-1

ORIGINAL PAPER

Preparation of Phosphatidylated Terpenes via Phospholipase D-Mediated Transphosphatidylation

Yukihiro Yamamoto · Masashi Hosokawa · Hideyuki Kurihara · Kazuo Miyashita

Received: 4 December 2007 / Revised: 11 January 2008 / Accepted: 15 January 2008 / Published online: 20 February 2008

Abstract Terpenes such as geraniol, geranylgeraniol, farnesol, and phytol are known as functional compounds which exhibit anticancer effects and activate nuclear receptors. For the application of functional terpenes in various fields, including the cosmetic and food industries, we attempted to synthesize phosphatidylated terpenes (terpene-PLs) by using phospholipase D (PLD). Transphosphatidylation of phosphatidylcholine with terpenes was carried out using PLD in a biphasic system containing ethyl acetate/water or in an aqueous system without organic solvent. The yield of terpene-PL increased with the reaction time and the amount of PLD in both the biphasic and aqueous systems. Further, the yield of terpene-PL in the aqueous system was higher than that in the biphasic system. In addition, among four PLDs from *Streptomyces* sp., *Streptomyces chromofuscus*, cabbage, and peanut, only the PLD from *Streptomyces* sp. could synthesize terpene-PL. The reaction yield, based on substrate phospholipid, of phosphatidylgeraniol reached 90 mol% under the following optimal reaction conditions: 50 μmol soyPC; 2,000 μmol geraniol; 1.6 U PLD; 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6); temperature, 37 °C ; and reaction time, 24 h. The reaction yields of phosphatidylfarnesol, phosphatidylgeranylgeraniol, and phosphatidylphytol were 73, 54, and 17 mol%, respectively.

Keywords Phospholipid · Terpene · Transphosphatidylation · Phospholipase D · Aqueous system · Geraniol

Y. Yamamoto · M. Hosokawa (✉) · H. Kurihara · K. Miyashita
Faculty of Fisheries Sciences, Hokkaido University,
3-1-1 Minato, Hakodate, Hokkaido 041-8699, Japan
e-mail: hoso@fish.hokudai.ac.jp

Introduction

Phospholipase D (PLD) (EC 3.1.4.4) is a lipolytic enzyme that hydrolyzes the terminal phosphodiester bond on phospholipids. Due to its ability to transfer the phosphatidyl moiety of glycerophospholipids to various alcohols, PLD is also used to synthesize phospholipids with desired head groups that are poorly accessible via the chemical route. In previous studies, phosphatidylglycerol was produced from phosphatidylcholine (PC; 1) and glycerol through transphosphatidylation by PLD [1,2]. Moreover, the transphosphatidylation reaction has been shown to be useful for preparing phosphatidylserine and phosphatidylethanolamine [3–6]. In addition, novel types of phospholipids with various functional head groups have been synthesized. Nagao et al. [7] synthesized 6-phosphatidyl-L-ascorbic acid that had antioxidative activity. Phosphatidylnucleosides [8] and phospholipid-phytosterol conjugates [9] have also been synthesized via transphosphatidylation mediated by PLD. These phospholipids with functional polar head groups are expected to be used as fine chemicals or drugs.

Terpene is the generic term for compounds that are based on an isoprene unit, such as geraniol (2), farnesol (3), geranylgeraniol (4), and phytol (5) (Fig. 1). Geraniol and farnesol, found in the essential oils of rose, herb, lemongrass, and other plants, are well known to exhibit many pharmacological or chemopreventive effects. They exert antitumor activity against various cancer cells both in vitro and in vivo [10–13]. Antibacterial effects of geraniol and farnesol against *Anisakis* and *Staphylococcus aureus* have also been reported [14,15]. Geranylgeraniol, a carbon side chain of vitamin K_2, is known to induce apoptosis in various tumor cell lines [16,17] and inhibit osteoclast formation [18]. In addition, it has been recently reported

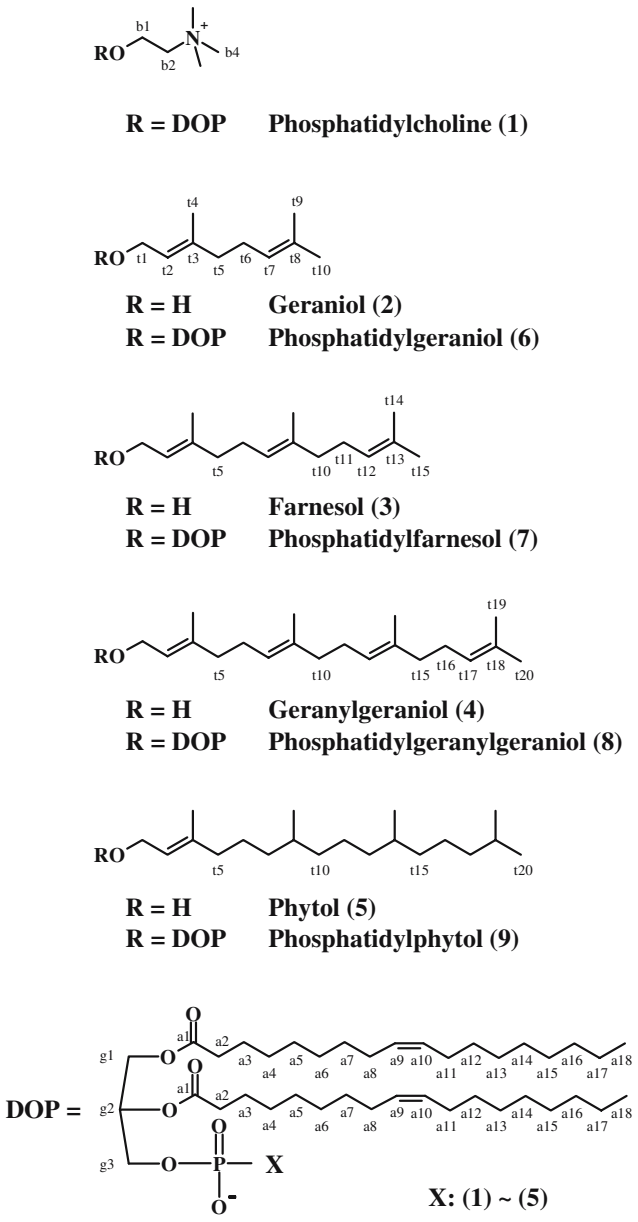

Fig. 1 Structures of terpenes and their phosphatidyl derivatives

that phytol and geranylgeraniol activate peroxisome proliferator-activated receptors (PPARs), thereby regulating lipid metabolism [19,20].

Therefore, we attempted to synthesize phospholipids containing terpene (terpene-PLs) in order to extend their application field. Generally, transphosphatidylation by PLD is carried out in a biphasic system containing water and an organic solvent such as diethyl ether or ethyl acetate. However, these organic solvents are not acceptable in the food industry. Therefore, an aqueous system is more desirable, and some researchers have attempted to synthesize phosphatidylglycerol and phosphatidylserine without using toxic organic solvents [21,22].

In the current study, the transphosphatidylation of PC with four terpenes, namely, geraniol, farnesol, geranylgeraniol, and phytol, was conducted in an aqueous system as well as in a biphasic system. At the optimum conditions determined in this study, the highest yield of 90 mol% was obtained in the case of transphosphatidylation with PC and geraniol in an aqueous system.

Experimental Procedures

Materials

PLDs from *Streptomyces* sp., *Streptomyces chromofuscus*, cabbage, and peanut and geranylgeraniol (>85%, MW 290.48) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SoyPC (soybean phosphatide extract; L-α-phosphatidylcholine, 95%) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; MW 786.15) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Farnesol (97%, MW 208.35) and bovine serum albumin were purchased from Merck (Darmstadt, Germany). Geraniol (98%, MW 154.25) was purchased from Aldrich (St. Louis, MO, USA). Phytol (80–90%, MW 296.54) was purchased from Extrasynthèse (Genay, France). All solvents and other chemicals used in this study were of analytical grade.

Transphosphatidylation of PC with Terpene

Either soyPC or DOPC (50 μmol) and 500 μmol terpene were dissolved in 1.6 ml ethyl acetate. To start the transphosphatidylation, 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 1.6 U PLD and 9 mg albumin was added to the above solution. In the case of PLDs from cabbage and peanut, 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 80 mM $CaCl_2$ was used, while for the PLD from *S. chromofuscus*, 0.8 ml of 0.2 M Tris–HCl buffer (pH 8.0) containing 80 mM $CaCl_2$ was used. Synthetic reaction of terpene-PL was carried out at 37 °C by stirring with magnetic stirrer at 350–400 rpm in the dark. The reaction was stopped by the addition of methanol. Subsequently, chloroform and water were added to the reaction mixture to obtain a chloroform–methanol–water ratio of 10:5:3 (v/v/v). The lipid fraction, including synthesized phospholipids, substrate terpene, and soyPC, was obtained from the chloroform layer.

In a typical aqueous system, 50 μmol soyPC was initially dissolved by sonication in 2,000 μmol terpene. The transphosphatidylation was started by the addition of 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6) containing 1.6 U PLD. The lipid fraction was extracted by the same procedure used in the biphasic system. For nuclear magnetic resonance (NMR) and mass spectra (MS) analyses

of synthesized phospholipids, DOPC was used as the substrate PC.

Thin-layer Chromatography of Terpene-PL

Lipid fractions extracted from the reaction mixture that contained DOPC and terpenes were applied onto a silica gel thin-layer chromatography (TLC) plate with a fluorescence dye (Silica gel 60 F254, Merck, Darmstadt, Germany) and developed by chloroform–methanol–water (65:25:4, v/v/v). After detection by UV at 254 nm and I_2, synthesized phospholipids were scraped off from TLC plate and then eluted using chloroform–methanol (3:7, v/v). The isolated phospholipids were used for structure analysis.

Spectral Analysis of Synthesized Phospholipids

To confirm the structure of synthesized terpene-PLs, MS and NMR analyses were performed. MS spectra were measured in the negative electrospray ionization (ESI) mode with JEOL JMS-700TZ (Japan Electronic Optics Laboratory Co., Tokyo, Japan). ^{1}H-and ^{13}C-NMR spectra were measured with a JOEL ECP400 FT NMR spectrometer at 399.78 and 100.53 MHz, respectively. Samples were dissolved in $CDCl_3$, and tetramethylsilane was used as an internal standard. The 2D-NMR spectra, namely, heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond coherence (HMBC), were also measured for the assignment of NMR data.

Yield of Terpene-PL

The yield of synthesized terpene-PLs was measured using a high-performance liquid chromatography (HPLC) system (L-7100, Hitachi, Tokyo, Japan) equipped with a silica gel column (Mightysil Si 60, Kanto Chemical Co. Inc., Tokyo, Japan). The lipid fraction separated from the reaction mixture was injected into the HPLC system. We used acetonitrile/methanol (100:12, v/v) [solvent (A)] and methanol [solvent (B)] as the mobile phase at 1 ml/min. The elution program was as follows: 0–5 min: 100% solvent (A), 5–15 min: linear gradient (A) → (B) and 15–24 min: 100% solvent (B). The retention time of each terpene-PL was around 12 min. The synthesized terpene-PL was detected at 210 nm with a diode array detector (L-7455, Hitachi, Tokyo, Japan). The yield of terpene-PL synthesized from soyPC and the four terpenes, namely, geraniol, farnesol, geranylgeraniol, and phytol, was calculated based on the HPLC peak area and a calibration curve using synthesized standard terpene-PLs. The yield was estimated according to following equation.

$$\text{Yield (mol\%)} = (\text{synthesized phospholipids}/\text{soyPC}) \times 100$$

Results and Discussion

Synthesis of Terpene-PL

We attempted to synthesize terpene-PL via PLD-mediated transphosphatidylation of DOPC with geraniol. A new spot was detected between DOPC and geraniol on the silica gel TLC plate (Fig. 2). This spot gave a blue color with Dittmer's reagent [23], indicating the presence of phosphate and a red-purple color with $SbCl_3$, indicating the presence of the terpene moiety in the molecule. We also detected new spots in the reaction mixtures obtained after the transphosphatidylation of DOPC with farnesol, geranylgeraniol, and phytol (data not shown). Subsequently, the synthesized terpene-PLs were isolated from TLC plate, and their structures were identified by high resolution (HR) ESI-MS (Table 1) and NMR (Tables 2, 3) analyses.

Identification of Synthesized Phospholipids

Negative high resolution ESI-MS of terpene-PLs showed pseudo-molecular ions $(M\text{-}H)^-$ (Table 1). These data coincided with the predicted molecular formulas of the

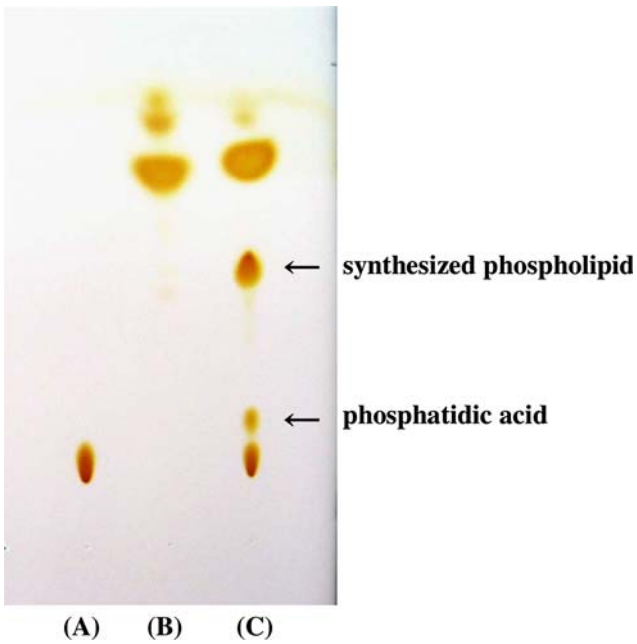

Fig. 2 TLC analysis of phosphatidylated terpene from phosphatidylcholine (*PC*) and geraniol by PLD. **a** DOPC, **b** geraniol, **c** reaction product, reaction condition; 50 μmol dioleoyl-PC, 500 μmol geraniol, 1.6 ml ethyl acetate, 9 mg albumin, 1.6 U PLD and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6) at 37 °C for 24 h. Spots were separated on the TLC plate by the development of chloroform–methanol–water (65:25:4, v/v) and detected by I_2

Table 1 Negative high resolution ESI-MS data of synthesized terpene-PLs

Terpene-PL	Observed $(M-H)^-$ (*m/z*) (theoretical value)	Elemental composition of M-H
Phosphatidylgeraniol (**6**)	835.6205 (835.6217)	$C_{49}H_{88}O_8P$
Phosphatidylfarnesol (**7**)	903.6825 (903.6843)	$C_{54}H_{96}O_8P$
Phosphatidylgeranylgeraniol (**8**)	971.7446 (971.7469)	$C_{59}H_{104}O_8P$
Phosphatidylphytol (**9**)	977.7928 (977.7938)	$C_{59}H_{110}O_8P$

Table 2 ^{1}H-NMR chemical shifts of PC, terpenes, and synthesized terpene-PLs

Position	1	2	6	3	7
g1	4.40		4.40		4.40
	4.12		4.18		4.18
g2	5.19		5.24		5.25
g3	3.91		3.93		3.93
a2	2.28 (×2)		2.28, 2.26		2.29, 2.27
a3	1.58 (×2)		1.59		1.59
a4-a7, a12-a17	1.27 (×10)		1.27		1.27
a8, a11	2.00 (×2)		2.00		2.00
a9, a10	5.34 (×2)		5.32–5.37		5.32–5.37
a18	0.88		0.88		0.88
b1	4.25				
b2	3.70				
b4	3.37				
t1		4.15	4.38	4.15	4.38
t2		5.41	5.34	5.42	5.34
t4		1.68	1.64	1.68	1.65
t5		2.09	1.97	1.90–2.00	1.90–2.00
t6		2.03	2.30	1.96–2.10	1.99–2.10
t7		5.10	5.07	5.09–5.12	5.08
t9		1.60	1.59	1.60	1.59
t10		1.68	1.67	1.90–2.00	1.90–2.00
t11				1.96–2.10	1.99–2.10
t12				5.09–5.12	5.07
t14				1.60	1.60
t15				1.68	1.68

Position	4	8	5	9
g1		4.40		4.40
		4.19		4.19
g2		5.25		5.25
g3		3.93		3.93
a2		2.29, 2.27		2.28
a3		1.56		1.57
a4-a7, a12-a17		1.27		1.27
a8, a11		1.94–2.40		2.00
a9, a10		5.34		5.33
a18		0.88		0.88
t1	4.15	4.34	4.15	4.37
t2	5.42	5.34	5.41	5.34

Table 2 continued

Position	4	8	5	9
t4	1.68	1.65	1.67	1.63
t5	1.92–2.00	1.92–2.00	1.99	1.94
t6	1.95–2.15	1.96–2.10	1.20–1.30	1.20–1.30
t7	5.11	5.08	1.00–1.10[a]	1.00–1.10[c]
t8			1.33[b]	1.33[d]
t9	1.60	1.59	0.84	0.84
t10	1.92–2.00	1.92–2.00	1.20–1.30[a]	1.20–1.30[c]
t11	1.95–2.15	1.96–2.10	1.20–1.30	1.20–1.30
t12	5.11	6.08	1.00–1.10[a]	1.00–1.10[c]
t13			1.25[b]	1.25[d]
t14	1.60	1.59	0.84	0.84
t15	1.92–2.10	1.92–2.00	1.20–1.30[a]	1.20–1.30[c]
t16	1.95–2.15	1.96–2.10	1.20–1.30	1.20–1.30
t17	5.11	5.08	1.15	1.15
t18			1.52	1.50
t19	1.60	1.59	0.87	0.87
t20	1.68	1.68	0.87	0.87

[a–c] Exchangeable within same characters

terpene-PLs phosphatidylgeraniol (6), phosphatidylfarnesol (7), phosphatidylgeranylgeraniol (8), and phosphatidylphytol (9). The ^{1}H- and ^{13}C-NMR data of compounds 1–9 were assigned with consideration of 1D ^{1}H- and ^{13}C-NMR, and 2D-HSQC and HMBC spectra (Tables 2, 3). On comparing the NMR data of phosphatidylgeraniol (6) (Tables 2, 3) with the DOPC (1) data, it was observed that the choline moiety had disappeared, while the geraniol moiety had appeared. Methylene signals (δ_H 4.38 and δ_C 62.61) at the t1-position of 6 were shifted to down-field, compared with the data (δ_H 4.15 and δ_C 59.36) of 2. Other signals of 6 coincided with the corresponding data of DOPC (1) and geraniol (2). Additionally, carbon signals (δ_C 62.61 and 121.01) at the t1- and t2-position showed broad signals because of $^2J_{CP}$ and $^3J_{CP}$ couplings. These data suggested that phosphoryl and geraniol moieties were connected with phosphodiester linkage. Other terpene-PLs, namely, phosphatidylfarnesol (7), phosphatidylgeranylgeraniol (8), and phosphatidylphytol (9), showed similar NMR patterns (Tables 2, 3). Thus, the synthesized products 6–9 were identified as terpene-PLs.

Table 3 ^{13}C-NMR chemical shifts of PC, terpenes, and synthesized terpene-PLs

Position	1	2	6	3	7
g1	63.04		62.92		62.90
g2	70.57[a]		70.92[a]		70.94
g3	63.34[a]		63.66[a]		63.61
a1	173.52, 173.16		173.55 (2C)		173.56 (2C)
a2	34.34, 34.14		34.35, 34.11		34.36, 34.11
a3	24.98, 24.90		24.96, 24.89		24.97, 24.94
a4-a7, a12-a15	29.20–29.80		29.35–29.84		29.35–29.84
a8, a11	27.24–27.22		27.26 (4C)		27.26 (4C)
a9, a10	129.69–130.03		129.71–129.99		129.69–129.98
a16	31.92 (2C)		31.94 (2C)		31.94 (2C)
a17	22.69 (2C)		22.70 (2C)		22.70 (2C)
a18	14.12 (2C)		14.12 (2C)		14.12 (2C)
b1	59.34[a]				
b2	66.33[a]				
b4	54.37 (3C)				
t1		59.36	62.61[a]	59.37	62.61[a]
t2		123.41	121.01[a]	123.40	120.95[a]
t3		139.65	139.67	139.73	139.89
t4		16.27	16.37	16.28	16.44
t5		39.58	39.68	39.70[b]	39.79[d]
t6		26.42	26.57	26.74[c]	26.83[e]
t7		123.94	124.03	123.81	123.87
t8		131.74	131.49	135.36	135.26
t9		17.69	17.69	16.00	16.00
t10		25.68	25.69	39.57[b]	37.73[d]
t11				26.32[c]	26.64[e]
t12				124.34	124.36
t13				131.34	131.24
t14				17.68	17.69
t15				25.69	25.70

Position	4	8	5	9
g1		62.93		62.87
g2		70.93[a]		70.02[a]
g3		63.65[a]		63.59[a]
a1		173.56 (2C)		173.54, 173.47
a2		34.32, 34.11		34.32, 34.11
a3		24.96, 24.86		24.95, 24.85
a4-a7, a12-a15		29.34–29.80		29.34–29.80
a8, a11		27.27 (4C)		27.35 (4C)
a9, a10		129.67–129.98		129.67–129.98
a16		31.93 (2C)		31.93 (2C)
a17		22.70 (2C)		22.69 (2C)

Table 3 continued

Position	4	8	5	9
a18		14.12 (2C)		14.12 (2C)
t1	59.40	62.61[a]	59.40	62.70[a]
t2	123.31	120.89[a]	123.14	120.66[a]
t3	139.79	139.89	140.23	140.38
t4	16.30	16.46	16.18	16.27
t5	39.74[b]	39.82[f]	39.90	40.12
t6	26.79[c]	26.83[g]	25.17[k]	25.43[p]
t7	124.20[d]	124.22[h]	39.39[l]	37.56[q]
t8	135.40[e]	135.37[i]	32.79[m]	32.86[r]
t9	16.02	16.03[j]	19.73[n]	19.73[s]
t10	39.71[b]	39.76[f]	37.40[l]	37.56[q]
t11	26.65[c]	26.80[g]	25.00[k]	24.63[p]
t12	123.82[d]	123.81[h]	37.34[l]	37.48[q]
t13	134.98[e]	134.93[i]	32.70[m]	31.93[r]
t14	16.02	16.00[j]	19.69[n]	19.65[s]
t15	39.58[b]	39.76[f]	37.31[l]	37.36[q]
t16	26.35[c]	26.70[g]	24.49[k]	24.58[p]
t17	124.41	124.41	39.39	39.40
t18	131.28	131.20	27.99	27.99
t19	17.69	17.68	22.74[o]	22.73[t]
t20	25.71	25.70	22.63[o]	22.64[t]

[a] Observed $^2J_{CP}$ or $^3J_{CP}$ coupling

[b–e] Exchangeable within same characters

Transphosphatidylation of Terpene-PL in the Biphasic System

We investigated the optimum conditions for the PLD-mediated transphosphatidylation of soyPC with terpenes in a biphasic system. Figure 3 shows the effect of the amount of

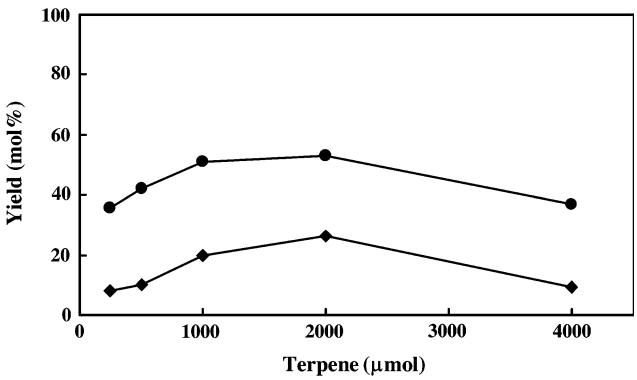

Fig. 3 Effect of the amount of substrate terpene on the terpene-PL synthesis in biphasic system. Reaction mixture: 50 μmol soyPC, 250–4000 μmol geraniol or farnesol, 1.6 ml ethyl acetate, 9 mg albumin, 1.6 U PLD and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6). The reaction was conducted at 37 °C for 24 h. (*filled circles*) phosphatidylgeraniol, (*filled diamonds*); phosphatidylfarnesol

the substrates geraniol and farnesol on the reaction yield of phosphatidylgeraniol and phosphatidylfarnesol, respectively. In each reaction, the yield of terpene-PLs gradually increased with the amount of substrate terpene, and after peaking at approximately 2,000 μmol of substrate, the yield decreased with the amount of substrate terpene. In the case of geraniol, the yield of phosphatidylgeraniol was 53 mol% at 2,000 μmol substrate in the biphasic system containing ethyl acetate and water. Further, the yield of phosphatidylfarnesol was lower than that of phosphatidylgeraniol.

We also studied the time course of the yield of terpene-PLs and the effect of the PLD amount on phosphatidylgeraniol synthesis. The yield increased with the reaction time and plateaued at 24 h at approximately 45 mol% (Fig. 4). The optimal amount of PLD was 16 U in the biphasic system (data not shown). Furthermore, the yield of phosphatidylgeraniol was higher than those of other terpene-PLs under the reaction conditions of 50 μmol soyPC; 2,000 μmol terpenes; 1.6 U PLD; 9 mg albumin; 1.6 ml 0.2 M sodium acetate buffer (pH 5.6); temperature, 37 °C; and reaction time, 24 h (Table 4). The terpene-PL yield decreased with the chain length of the substrate terpene in the biphasic system.

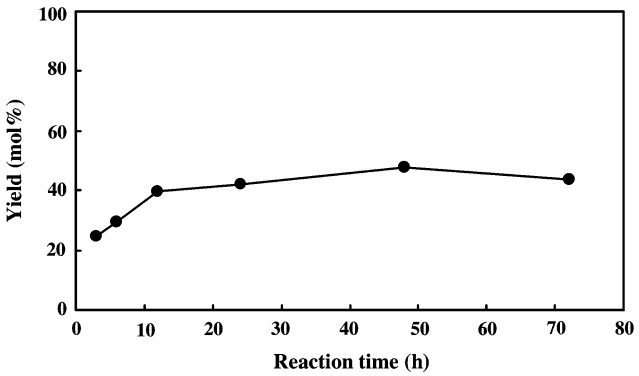

Fig. 4 Time course of phosphatidylgeraniol synthesis in biphasic system. Reaction mixture: 50 μmol soyPC, 500 μmol geraniol, 0.8 ml ethyl acetate, 1.6 U PLD, 9 mg albumin and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6). The reaction was conducted at 37 °C for 3–72 h

Table 4 The yield of terpene-PL synthesized by PLD in biphasic system or aqueous system

Terpene-PL	Yield (mol%)	
	Biphasic system	Aqueous system
Phosphatidylgeraniol	53	90
Phosphatidylfarnesol	26	73
Phosphatidylgeranylgeraniol	17	54
Phosphatidylphytol	14	17

Transphosphatidylation of Terpene-PL in the Aqueous System

Ethyl acetate is unsuitable for the production of terpene-PLs to be used in fields such as the nutraceutical and food industries. The synthesis of terpene-PLs was attempted in an aqueous system, without a toxic solvent.

Figure 5 shows the effect of the amounts of substrate geraniol and PLD on the synthesis of phosphatidylgeraniol in the aqueous system. With 2,000 μmol geraniol, 50 μmol soyPC, 1.6 U PLD, and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6), the yield of phosphatidylgeraniol reached 90 mol% at a temperature of 37 °C and reaction time of 24 h (Fig. 5a). At geraniol levels between 250 and 4,000 μmol geraniol, the yield of phosphatidylgeraniol was high. In contrast, this yield increased with PLD amounts and plateaued between 0.8 and 1.6 U (Fig. 5b). From these results, the optimal reaction conditions for phosphatidylgeraniol were estimated to be 50 μmol soyPC, 2,000 μmol geraniol, 1.6 U PLD, and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6). Furthermore, the time course of phosphatidylgeraniol synthesis was examined using the above-mentioned optimum reaction mixture (Fig. 6). The yield of

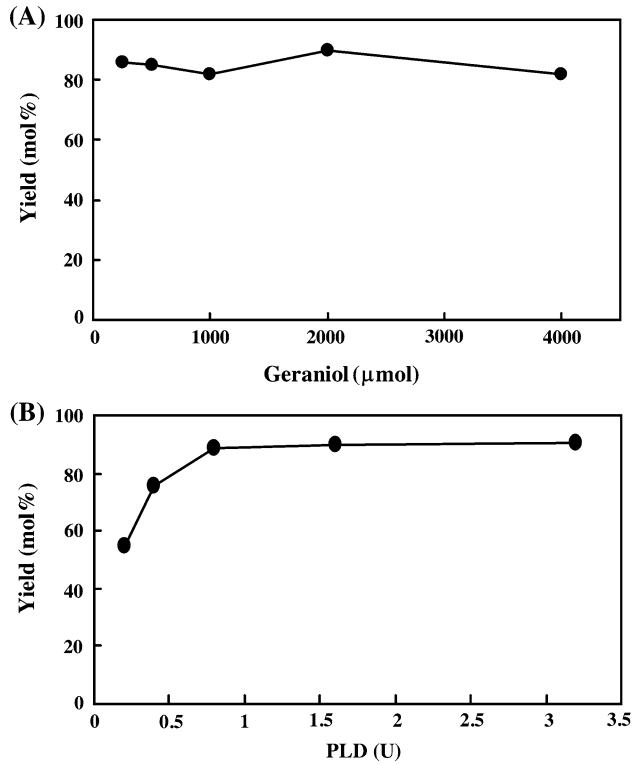

Fig. 5 Effect of the amount of geraniol and phospholipase D on the synthesis of phosphatidylgeraniol in aqueous system. Reaction mixture: 50 μmol soyPC, 250–2,000 μmol geraniol, 0.2–3.2 U PLD and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6). The reaction was conducted at 37 °C for 24 h

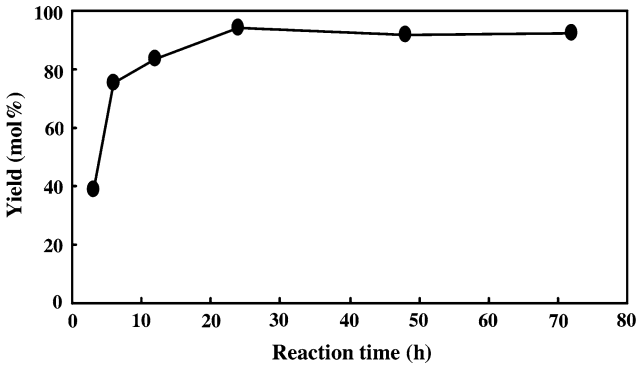

Fig. 6 Time course of phosphatidylgeraniol synthesis in aqueous system. Reaction mixture: 50 μmol soyPC, 2,000 μmol geraniol, 1.6 U PLD and 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6). The reaction was conducted at 37 °C for 3–72 h

phosphatidylgeraniol increased with the reaction time and was highest at 24 h.

We also examined the relation between terpene-PL yield and the chain length of substrate terpenes in the aqueous system (Table 4). The yields of terpene-PLs decreased with the chain length of substrate terpenes, similar to the findings in the biphasic system. Furthermore, the yield of phosphatidylphytol was lower than that of phosphatidylgeranylgeraniol.

In previous studies, a large variety of phospholipids were synthesized through transphosphatidylation by PLD. It is known that primary hydroxyl group is good as accepter compared to secondary hydroxyl group, while tertiary hydroxyl group can not be acceptors. In addition, sugars [24], phenols [25], nucleotides [8], ascorbic acid [7], and 1,8-octandiol [26] have been also reported to act as acceptors. In the current study, we indicated for the first time that terpenes such as geraniol, farnesol, geranylgeraniol, and phytol can also act as acceptors in PLD-mediated transphosphatidylation. The preference of PLD for geraniol and farnesol was higher than that for geranylgeraniol and phytol. In addition, the yields of terpene-PLs in the aqueous system were higher than those in the biphasic system for each terpene. The emulsion state of the substrate may affect the PLD-mediated transphosphatidylation of PC with terpene. In the aqueous system, substrate PC was suspended in a buffer and terpene mixture, while it was dissolved in an organic solvent phase in the biphasic system. Dittrich and Ulbrich-Hofman [21] reported that the addition of Triton X-100 enhances the conversion of PC to phosphatidylglycerol mediated by immobilized PLD in an aqueous system. Their results showed that PLD-mediated transphosphatidylation was strongly influenced by the physical state of substrate phospholipids in the reaction system. Furthermore, the contact frequency of the substrate and PLD in the aqueous system was supposedly higher than that in the biphasic system because the substrate in the aqueous system was not diluted by the organic solvent.

It is known that PLDs show different activities and specificities depending on their origin [27]. We therefore compared the activities of terpene-PL synthesis using some PLD isozymes under the following reaction conditions: 50 μmol soyPC; 2,000 μmol geraniol; 1.6 U PLD; 0.8 ml of each buffer; temperature, 37 °C; and reaction time, 24 h. Of the four enzymes from *Streptomyces* sp., *S. chromofuscus*, cabbage, and peanut, only the PLD from *Streptomyces* sp. could catalyze the transphosphatidylation of soyPC with geraniol in both biphasic and aqueous systems (data not shown). This result indicates that PLD from *Streptomyces* sp. is a suitable enzyme for terpene-PL synthesis.

Because of their interfacial activity, phospholipids have many applications such as in food emulsifiers and cosmetics. In particular, the ability of liposome formation is one of the important characteristics of phospholipids that can be applied in the medical field. Further studies are required to investigate the functions of synthesized terpene-PLs.

In conclusion, the present study showed that PLD from *Streptomyces* sp. drives the transphosphatidylation of PC with geraniol, farnesol, geranylgeraniol, and phytol in both biphasic and aqueous systems. In particular, the aqueous system was superior to the biphasic system for terpene-PL production. Furthermore, the yield of terpene-PL decreased with the chain length of the substrate terpene. The yield of phosphatidylgeraniol reached 90 mol% in the aqueous system, under the following optimum conditions: 50 μmol soyPC; 2,000 μmol geraniol; 1.6 U PLD; 0.8 ml of 0.2 M sodium acetate buffer (pH 5.6); temperature, 37 °C; and reaction time, 24 h.

Acknowledgments This work was supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan and Kieikai Research Foundation. We thank Mrs. Seiko Oka and Mr. Tomohiro Hirose (Center for Instrumental Analysis Hokkaido University) for skillful assistance with the MS analyses and NMR analyses.

References

1. Lee SY, Hibi N, Yamane T, Shimizu S (1985) Phosphatidylglycerol synthesis by phospholipase D in a microporous membrane bioreactor. J Ferment Technol 63:37–44
2. Juneja LR, Hibi N, Yamane T, Shimizu S (1987) Repeated bath and continuous operations for phosphatidylglycerol synthesis from phosphatidylcholine with immobilized phospholipase D. Appl Microbiol Biotechnol 27:146–151
3. Juneja LR, Kazuoka T, Goto N, Yamane T, Shimizu S (1989) Conversion of phosphatidylcholine to phosphatidylserine by various phospholipases D in the presence of L- or D-serine. Biochim Biophys Acta 1003:277–283
4. Hosokawa M, Shimatani T, Kanada T, Inoue Y, Takahashi K (2000) Conversion to docosahexaenoic acid-containing phosphatidylserine from squid skin lecithin by phospholipase

D-mediated transphosphatidylation. J Agric Food Chem 48:4550–4554
5. Kanda P, Wells MA (1986) Dihexanoylphosphatidylethanolamine: effect of head group charge on rates of alkaline and phospholipase A_2 catalyzed hydrolyses. Chem Phys Lipids 39:31–39
6. Juneja LR, Kazuoka T, Yamane T, Shimizu S (1988) Kinetic evaluation of conversion of phosphatidylcholine to phosphatidylethanolamine by phospholipase D from different sources. Biochim Biophys Acta 960:334–341
7. Nagao A, Ishida N, Terao J (1991) Synthesis of 6-phosphatidyl-L-ascorbic acid by phospholipase D. Lipids 26:390–394
8. Shuto S, Itoh H, Ueda S, Imamura S, Fukukawa K, Tsujino M, Matsuda A, Ueda T (1988) A facile enzymatic synthesis of 5′-(3-*sn*-phosphatidyl)nucleosides and their antileukemic activities. Chem Pharm Bull 36:209–217
9. Hossen M, Hernandez E (2004) Phospholipase D-catalyzed synthesis of novel phospholipid-phytosterol conjugates. Lipids 39:777–782
10. Shoff SM, Grummer M, Yatvin MB, Elson CE (1991) Concentration-dependent increase of murine P388 and B16 population doubling time by the acyclic monoterpene geraniol. Cancer Res 51:37–42
11. Yu SG, Hildebrandt LA, Elson CE (1995) Geraniol, an inhibitor of mevalonate biosynthesis, suppresses the growth of hepatomas and melanomas transplanted to rats and mice. J Nutr 125:2763–2767
12. Adany I, Yazlovitskaya EM, Haug JS, Voziyan PA, Melnykovych G (1994) Differences in sensitivity to farnesol toxicity between neoplastically- and non-neoplastically-derived cells in culture. Cancer Lett 79:175–179
13. McAnally JA, Jung M, Mo H (2003) Farnesyl-*O*-acetylhydroquinone and geranyl-*O*-acetylhydroquinone suppress the proliferation of murine B16 melanoma cells, human prostate and colon adenocarcinoma cells, human lung carcinoma cells, and human leukemia cells. Cancer Lett 202:181–192
14. Hierro I, Valero A, Pérez P, González P, Cabo MM, Montilla MP, Naverro MC (2004) Action of different monoterpenic compounds against *Anisakis simplex* s.l. L3 Larvae. Phytomedicine 11:77–82
15. Inoue Y, Shiraishi A, Hada T, Hirose K, Hamashima H, Shimada J (2004) The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. FEMS Microbiol Lett 237:325–331
16. Ohizumi H, Masuda Y, Nakajo S, Sakai I, Ohsawa S, Nakaya K (1995) Geranylgeraniol is a potent inducer of apoptosis in tumor cells. J Biochem 117:11–13
17. Miquel K, Pradines A, Favre G (1996) Farnesol and geranylgeraniol induce actin cytoskeleton disorganization and apoptosis in A549 lung adenocarcinoma cells. Biochem Biophys Res Commun 225:869–876
18. Hiruma Y, Nakahama K, Fujita H, Morita I (2004) Vitamin K2 and geranylgeraniol, its side chain component, inhibited osteoclast formation in a different manner. Biochem Biophys Res Commun 314:24–30
19. Takahashi N, Kawada T, Goto T, Yamamoto T, Taimatsu A, Matsui N, Kimura K, Saito M, Hosokawa M, Miyashita K, Fushiki T (2002) Dual action of isoprenols from herbal medicines on both PPARγ and PPARα in 3T3-L1 adipocytes and HepG2 hepatocytes. FEBS Lett 514:315–322
20. Goto T, Takahashi N, Kato S, Egawa K, Ebisu S, Moriyama T, Fushiki T, Kawada T (2005) Phytol directly activates peroxisome proliferator-activated receptor α (PPARα) and regulates gene expression involved in lipid metabolism in PPARα-expressing HepG2 hepatocytes. Biochem Biophys Res Commun 337:440–445
21. Dittrich N, Ulbrich-Hofmann R (2001) Transphosphatidylation by immobilized phospholipase D in aqueous media. Biotechnol Appl Biochem 34:189–194
22. Iwasaki Y, Mizumoto Y, Okada T, Yamamoto T, Tsutsumi K, Yamane T (2003) An aqueous suspension system for phospholipase D-mediated synthesis of PS without toxic organic solvent. JAOCS 80:653–657
23. Dittmer JC, RL Lester (1964) A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J Lipid Res 7:126–127
24. Kokusho Y, Tsunoda A, Kato S, Machida H, Iwasaki S (1993) Production of various phosphatidylsaccharides by phospholipase D from *Actinomadura* sp. strain no. 362. Biosci Biotechnol Biochem 57:1302–1305
25. Takami M, Hidaka N, Suzuki Y (1994) Phospholipase D-catalyzed synthesis of phosphatidyl aromatic compounds. Biosci Biotechnol Biochem 58:2140–2144
26. Koketsu M, Nitoda T, Sugino H, Juneja LR, Kim M, Yamamoto T, Abe N, Kajimoto T, Wong CH (1997) Synthesis of a novel sialic acid derivative (sialylphospholipid) as an antirotaviral agent. J Med Chem 40:3332–3335
27. Ulbrich-Hofmann R, Lerchner A, Oblozinsky M, Bezakova L (2005) Phospholipase D and its application in biocatalysis. Biotechnol Lett 27:535–544