Title

Synthesis of novel phospholipids that bind phenylalkanols and hydroquinone *via* phospholipase D-catalyzed transphosphatidylation.

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Phenylalkanols such as tyrosol and hydroxytyrosol (h-tyrosol), which possess antioxidant and anticancer properties, were phosphatidylated by phospholipase D (PLD)-catalyzed transphosphatidylation. After a 24-h reaction of phosphatidylcholine (PC) and tyrosol with PLD, a new product was detected by TLC and identified to phosphatidyl-tyrosol by high resolution MS and NMR analyses. The optimum reaction conditions were as follows; soyPC 50 μmol, tyrosol 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h. Under the optimum reaction conditions, the yields of phosphatidyl-tyrosol, hydroquinone (HQ), 2-(4-aminophenyl)ethanol (4APE), h-tyrosol, and 2-phenylethanol (PEA) were 87±3.7, 13±1.3, 90±2.3, 64±5.5, and 85±1.0 mol%, respectively. Furthermore, from the results of transphosphatidylation of soyPC with several phenylethanols and phenylpropanols, we established the following details about the reaction specificity of transphosphatidylation by PLD from *Streptomyces* sp: (1) Para-amino and para-hydroxyl groups in the benzene ring of PEA derivatives do not affect the transphosphatidylation by PLD, whereas meta-hydroxyl group slightly inhibits the transphosphatidylation. (2) Meta and ortho-methyl groups in the benzene ring of PEA derivatives also slightly inhibit the transphosphatidylation. (3) Secondary and tertiary alcohols and hydroquinone are difficult to transphosphatidylate by PLD.

Introduction

Phospholipase D (PLD) (EC 3.1.4.4) is a lipolytic enzyme used for hydrolyzing the terminal phosphodiester bond of phospholipids (PLs) [1]. PLD can also be used as a catalyst in the transphosphatidylation in order to exchange the phosphatidyl moiety of PLs to various alcohols. Transphosphatidylation is an effective reaction for the application of functional alcohols in a wide variety of fields such as the production of fine chemicals, functional foods. In previous studies, 6-phosphatidyl-L-ascorbic acid was synthesized from egg yolk phosphatidylcholine (PC) and L-ascorbic acid [2], it exhibited a high anti-oxidative activity [3]. In addition, 5-fluorouridine [4], kojic acid and arbutin [5] were phosphatidylated by PLD-catalyzed transphosphatidylation, and the synthesized compounds exhibited antitumor effects [4] and inhibitory effects on tyrosinase [5]. We have also reported the synthesis of phosphatidyl-terpenes such as phosphatidyl-geraniol, phosphatidyl-myrtenol, and phosphatidyl-perillyl alcohol by PLD [6,7]. The synthesized phosphatidyl-perillyl alcohol significantly reduced viability of human leukemia and prostate cancer cells [7].

In this study, we focused on the transphosphatidylation of functional phenylalkanols by PLD. 2-(4-Hydroxyphenyl)ethanol (tyrosol; **1**) and 2-(3,4-dihydroxyphenyl)ethanol (h-tyrosol; **5**) (Fig. 1), which contained olive oil extracts, exhibit antioxidant activity [8] and apoptosis induction against colon cancer cells [9], and induce the differentiation of leukemia cells [10].

4-Hydroxybenzyl alcohol (4HBA; **2**) is a component of *Gastrodia elata* Blume (Orchidaceae) that has an anxiolytic-like effect on mice [11], and tyrosinase inhibitory activity [12]. In addition, we used hydroquinone (HQ; **3**), 2-(4-aminophenyl)ethanol (4APE; **4**), 2-phenylethanol (PEA; **6**) (Fig. 1) and other phenylalkanols as substrates to clarify the reaction specificity of the PLD from *Streptomyces* sp., because this information is important for the application of PLD-catalyzed transphosphatidylation to various fields. The present study provides critical information related to the production of novel PLs with an aromatic structure by PLD.

Materials and methods

Materials

PLD from *Streptomyces* sp. was purchased from Sigma Chemical Co. (St. Louis, MO, USA). SoyPC (soybean phosphatide extracts; PC>95%) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; MW 786.15) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Egg yolk PC (COATSOME NC-50, >98%) was obtained from NOF Co. (Tokyo). Salmon roe PC was separated from total lipids extracted from salmon roe with preparative silica gel thin layer chromatograpy. All substrates, tyrosol (**1**), 4HBA (**2**), HQ (**3**), 4APE (**4**), h-tyrosol (**5**), PEA (**6**), benzyl alcohol (BA; **7**), 3-phenyl-1-propanol (3P1Pr; **8**), 4-phenyl-1-butanol (4P1Bu; **9**), 5-phenyl-1-pentanol (5P1Pe; **10**), 2-(2-methylphenyl)ethanol (2,2MPE; **11**),

2-(3-methylphenyl)ethanol (2,3MPE; **12**), 2-phenyl-1-propanol (2P1Pr; **13**), 1-phenyl-2-propanol (1P2Pr; **14**) and 2-methyl-1-phenyl-2-propanol (2M1P2Pr; **15**), were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo). All solvents and other chemicals used in this study were analytical grade.

Transphosphatidylation of phosphatidylcholine with aromatic alcohols

In the typical reaction, 50 μmol soyPC and 500 μmol phenylalkanol 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 was added to the ethyl acetate solution. The reaction was carried out at 37°C by stirring at 350–400 rpm in the dark. By the addition of methanol, the reaction was terminated. Then, chloroform and water were added to the reaction mixture to adjust a chloroform-methanol-water ratio of 10:5:3 (v/v/v). The lipid fraction, including synthesized phospholipids, and residual substrates was obtained from the chloroform layer. The lipid fractions were then 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). For analysis of synthesized PL structure, DOPC was used as substrate.

Identification of synthesized phospholipids structure

To determine the structure of synthesized PLs, mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses were performed. MS was measured in the negative atmospheric pressure chemical ionization (APCI) mode with JEOL JMS-T100LP (Japan Electronic Optics Laboratory Co., Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were obtained with a Varian UNITY INOVA 500 spectrometer (Varian, Inc., Palo Alto, CA, USA) at 500 and 126 MHz, respectively. Synthesized PLs, substrate DOPC and alcohols were dissolved in $CDCl_3$ or $CDCl_3$:CD_3D (3:1, v/v). Tetramethylsilane was used as an internal standard. The 2D NMR spectra, namely, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC), were also obtained for the assignment of NMR data.

Yield of phospholipids

The yield of synthesized PLs was measured by 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. Acetonitrile/methanol (100:12, v/v) [solvent (A)]

and methanol [solvent (B)] were used as the mobile phase at 1 ml/min. The elution program was as follows: 0–5 min: 100% solvent (A) and 5-15 min: linear gradient (A) → (B). Synthesized PLs from tyrosol (**1**), HQ (**3**), 4APE (**4**), h-tyrosol (**5**) and PEA (**6**) were detected at 224 nm, 226 nm, 213 nm, 241 nm and 223 nm, respectively (L-7455, Hitachi, Tokyo, Japan). The retention time of each synthesized PL was around 12 min. The yield of PLs was calculated based on the HPLC peak area and a calibration curve using synthesized PLs.

Results and discussion

Phosphatidylation of phenylalkanols and hydroquinone by phospholipase D

The phosphatidylation of tyrosol (**1**) was carried out *via* transphosphatidylation by PLD. After a 24-h reaction, a new spot was detected between soyPC and tyrosol (**1**) on silica gel TLC plate (Fig. 2). The synthesized PL was analyzed by high resolution (HR) MS and NMR analyses. The data of negative high resolution APCI-MS of the synthesized compound was 819.5540 and coincided with the predicted molecular formula ($C_{47}H_{80}O_9P$) of phosphatidyl-tyrosol. In both ^{1}H and ^{13}C NMR analyses, tyrosol signals attributed to tyrosol were observed instead of the disappearance of choline signals in the case of the synthesized compound **17** (Table 1). The ^{1}H signal at the P1 of compound **17** was shifted to the downfield, compared to the ^{1}H signal of tyrosol (**1**). In addition, $^{2}J_{CP}$ and $^{3}J_{CP}$ couplings were observed at the positions g2, g3, P1 and P2.

in ^{13}C NMR analysis. On the basis of these analytical data, the synthesized compound was identified as phosphatidyl-tyrosol (**17**) (Fig. 1). On the other hand, phosphatidyl-tyrosol that bound to para-hydroxyl group in the benzene ring and the phosphate group in PC was not detected by the NMR analysis.

In the case of the transphosphatidylation of DOPC with other hydroquinone (3) and phenylalkanols such as 4HBA (**2**), 4APE (**4**), h-tyrosol (**5**), and PEA (**6**), the synthesized PLs were also identified to be phosphatidyl-4HBA, phosphatidyl-HQ, phosphatidyl-4APE, phosphatidyl-h-tyrosol and phosphatidyl-PEA by TLC, MS and NMR analyses (data not shown).

Optimization of transphosphatidylation with soyPC and tyrosol

We examined the effect of the tyrosol content on the transphosphatidylation by PLD. The yield of phosphatidyl-tyrosol (17) increased with an increase in the amount of tyrosol (**1**) and reached to 87±3.7 mol% at 500 μmol of tyrosol (**1**) (Fig. 3). However, the yield of phosphatidyl-tyrosol reduced to less than 20% at 2000 μmol of tyrosol. The same results were observed in our previous research in which we investigated the transphosphatidylation between soyPC and terpene alcohols [6]. Therefore, it is suggested that an excessive amount of alcohol inhibits the transphosphatidylation by PLD. On the other hand, the yield of phosphatidyl-tyrosol increased in a time-dependent manner and reached a plateau after a 12-h reaction (Fig. 4). Based upon these

results, we could determine the following optimum reaction conditions: soyPC 50 μmol, tyrosol (**1**) 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h.

Further, we attempted the transphosphatidylation using different substrate PCs with tyrosol by PLD. In the case of egg yolk PC containing mainly saturated palmitic acid and stearic acid, and salmon roe PC containing eicosapentaenoic acid and docosahexaenoic acid, the yield of phosphatidyl-tyrosol in these two cases was 86±2.8 and 80±4.2 mol%, respectively, and was almost the same as that from soyPC. PLD from *Streptomyces* sp. used in this study did not discriminate the substrate PCs having different fatty acid composition.

Effect of substrate alcohol structure on the transphosphatidylation by PLD

In order to investigate the specificity of PLD-catalyzed transphosphatidylation, we attempted reactions using several phenylalkanols as shown in Fig. 5, under the following reaction conditions: soyPC 50 μmol, phenylalkanol 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h.

When 4APE (**4**) and PEA (**6**) were used as substrate alcohols, the yields of the synthesized PLs were 90 ± 2.3 and 85 ± 1.0 mol%, which were almost the same as the yield of phosphatidyl-tyrosol (Fig. 5). These data show that para-hydroxyl and para-amino groups in the

benzene ring of PEA derivatives do not affect the transphosphatidylation by PLD, while the para-hydroxyl group cannot be an acceptor. On the other hand, the yield of phosphatidyl-h-tyrosol was approximately 20% lower than that of phosphatidyl-tyrosol. Therefore, the meta-hydroxyl group in the benzene ring is suggested to slightly inhibit the transphosphatidylation by PLD. The degree of synthesis of phosphatidyl-4HBA as estimated by the TLC analysis was low (Fig. 5). HQ (3) was low yield for the transphosphatidylation catalyzed by PLD from *Streptomyces* sp.

Furthermore, the degrees of transphosphatidylation of phenylalkanols such as phosphatidyl-BA, phosphatidyl-3P1Pr, phosphatidyl-4P1Bu and phosphatidyl-5P1Pe were almost the same moderate levels (60%-80%) as estimated by a qualitative TLC analysis. Their reaction yields were lower than that of phosphatidyl-PEA (>80%). From these results, the optimum distance from the benzene ring to the primary hydroxyl group is suggested to be two carbon chains length. Ortho-methyl group (2,2MPE (11)) or meta-methyl group (2,3MPE (12)) in the benzene ring also decreased the transphosphatidylation by PLD as compared to PEA (6) (Fig. 5). Moreover, when compared with the yields from phenylpropyl alcohol isomers, it was revealed that primary alcohol (2P1Pr (13)) was a better acceptor for transphosphatidylation than secondary alcohol (1P2Pr (14)) and tertiary alcohol (2M1P2Pr (15)) (Fig. 6). These results indicate that the hydroxyl group close to bulky alkyl groups is difficult to be transphosphatidylated by PLD.

In conclusion, several functional phenylalkanols and hydroquinone were phosphatidylated in

a one-pot synthesis by PLD. The characterization of the transphosphatidylation by PLD from *Streptomyces* sp. was also shown by using several phenylalkanols. Takami et al. reported the transphosphatidylation of PC with phenols by PLD [13]. However, there is little information about the synthesis of PLs with aromatic alcohols by PLD. The present results provide beneficial data related to the substrate specificity for PLD-catalyzed transphosphatidylation of phenylalkanols.

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FIGURE LEGEND

FIGURE 1 Structure of dioleoyl-phosphatidylcholine (16), phenylalkanols, hydroquinone and their PL derivatives.

FIGURE 2 TLC analysis of phosphatidyl-tyrosol from PC and tyrosol by PLD. (A): soyPC, (B): tyrosol (**1**), (C): reaction product. Reaction conditions: soyPC 50 μmol, tyrosol 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h. Developing solvent for TLC analysis was chloroform-methanol-water (65:25:4, v/v), and spots were detected by I_2.

FIGURE 3 Effect of amount of tyrosol on transphosphatidylation by PLD. Reaction conditions: soyPC 50 μmol, tyrosol 50-2000 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h.

FIGURE 4 Time course of phosphatidyl-tyrosol synthesis by PLD. Reaction conditions: soyPC 50 μmol, tyrosol 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 3-48 h.

FIGURE 5 Synthesis of phenylalkanol- and hydroquinone-phospholipids by PLD. Degree of synthesis of novel phospholipids was qualitatively measured by TLC analysis. (A): High (yield >80%), (B):Moderate (50%~80%); (C): Low (20%~50%); and (D): Poor (<20%). Reaction conditions: soyPC 50 μmol, phenylalkanols or hydroquinone 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h.

FIGURE 6 TLC analysis of phosphatidyl-phenylpropyl alcohol isomers by PLD. Reaction conditions; soyPC 50 μmol, phenylpropanol 500 μmol, ethyl acetate 1.6 ml, PLD 1.6 U, 0.2 M sodium acetate buffer (pH 5.6) 0.8 ml, 37^{o}C for 24 h. Developing solvent for TLC analysis was chloroform-methanol-water (65:25:4, v/v), and spots were detected by I_2.

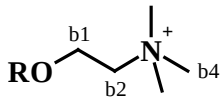
Table 1. ^{1}H NMR chemical shifts of DOPC (**16**), tyrosol (**1**), and synthesized phospholipid (**17**)

Position	Compounds		
	1	**16**	**17**
g1		4.39 dd (12, 2.5)	4.31 br
		4.12 dd (12, 7)	4.12 br
g2		5.18 m	5.19 br
g3		3.94 and 3.89 m	3.90 br m
a1			
a2		2.27 t (6.5)	2.26 t (6.5)
a3		1.58 m	1.57 m
a4-a7, a12-a17		~ 1.29	~1.26
a8, a11		2.00 m (x2)	2.00 m (x2)
a9, a10		5.33 (x2)	5.33 m (x2)
a18		0.88 t (6.5)	0.87 t (6.5)
b1		3.76 br	
b2		4.28 br	
b4		3.34 s	
p1	3.67 t (7)		3.83 br
p2	2.71 t (7)		2.67 br
p3			
p4	7.02 d (8.2)		6.75 br
p5	6.69 d (8.5)		6.68 br
p6			
p7	6.69 d (8.5)		6.68 br
p8	7.02 d (8.2)		6.75 br

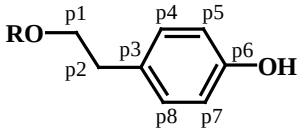
Table 2. ^{13}C NMR chemical shifts of DOPC (**16**), tyrosol (**1**), and synthesized phospholipid (**17**)

Position	Compounds		
	1	**16**	**17**
g1		62.9	62.8
g2		70.3 br[a]	70.5 br[a]
g3		63.2 br[a]	66.8 br[a]
a1		173.0	173.9
a2		34.0	34.0
a3		24.8	24.9
a4-a7, a12-a17		31~ 27	31~ 27
a8, a11		27.1	27.1
a9, a10		130.0	130.0
a18		14.0	14.1
b1		66.1 br[a]	
b2		59.2 br[a]	
b4		54.2	
p1	63.4 br[a]		64.6 br[a]
p2	35.9 br[a]		39.4 br[a]
p3	131.0		130.0
p4	130.9		130.1
p5	116.1		115.6
p6	156.7		155.0
p7	116.1		115.6
p8	130.9		130.1

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

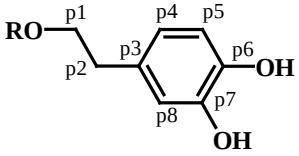


R = DOP **Phosphatidylcholine (16)**



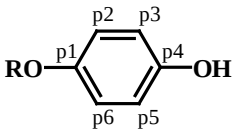
R = H **Tyrosol (1)**

R = DOP **Phosphatidyl-tyrosol (17)**



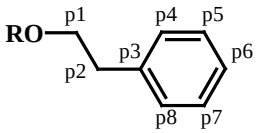
R = H **h-Tyrosol (5)**

R = DOP **Phosphatidyl-h-tyrosol (20)**



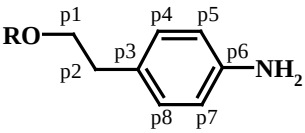
R = H **HQ (3)**

R = DOP **Phosphatidyl-HQ (18)**



R = H **PEA (6)**

R = DOP **Phosphatidyl-PEA (21)**



R = H **4APE (4)**

R = DOP **Phosphatidyl-4APE (19)**

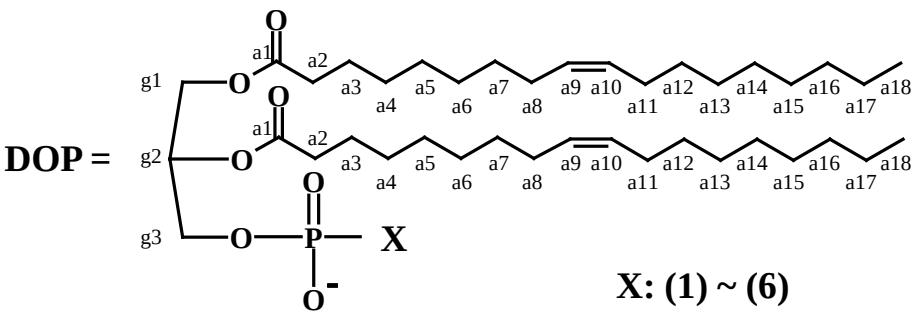


FIGURE 1 Yamamoto et al.

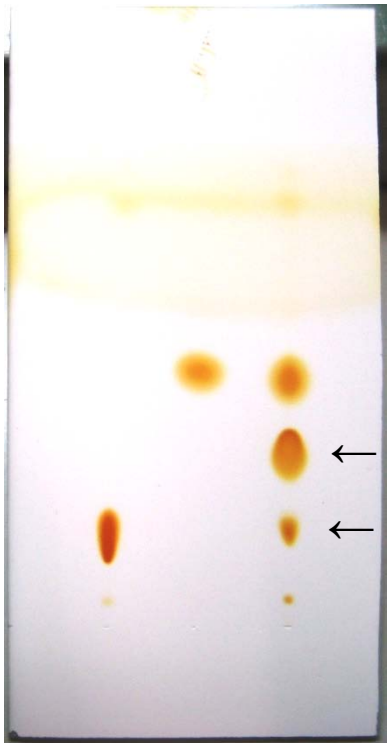


FIGURE 2 Yamamoto et al

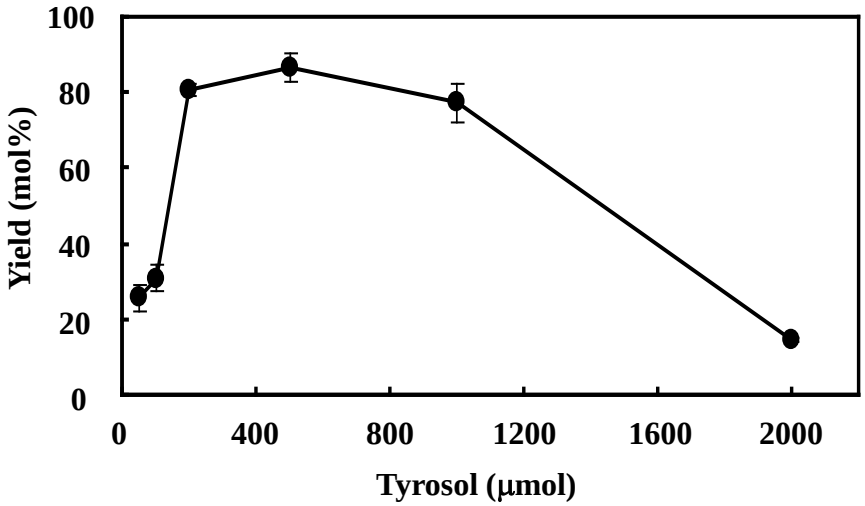


FIGURE 3 Yamamoto et al

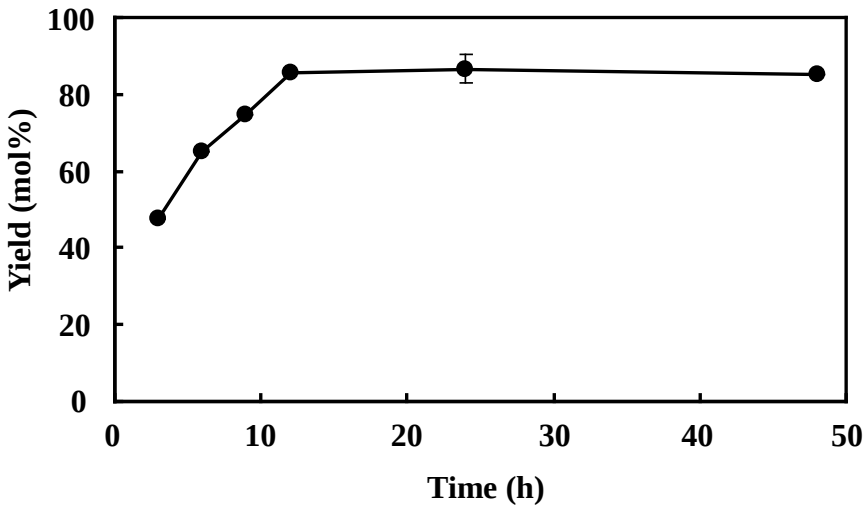
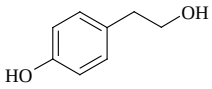


FIGURE 4 Yamamoto et al

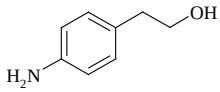
(A) High

Tyrosol (1)



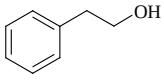
(87±3.7 mol%)

4APE (4)



(90±2.3 mol%)

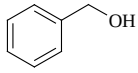
PEA (6)



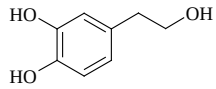
(85±1.0 mol%)

(B) Moderate

BA (7)

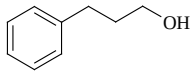


h-Tyrosol (5)

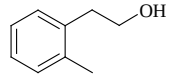


(64±5.5 mol%)

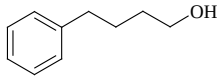
3P1Pr (8)



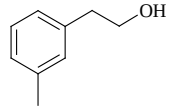
2,2MPE (11)



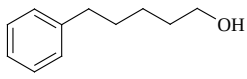
4P1Bu (9)



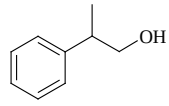
2,3MPE (12)



5P1Pe (10)

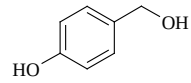


2P1Pr (13)

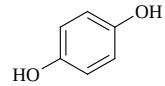


(C) Low

4HBA (2)

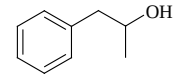


HQ (3)



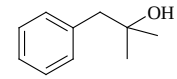
(13±1.3 mol%)

1P2Pr (14)



(D) Poor

2M1P2Pr (15)



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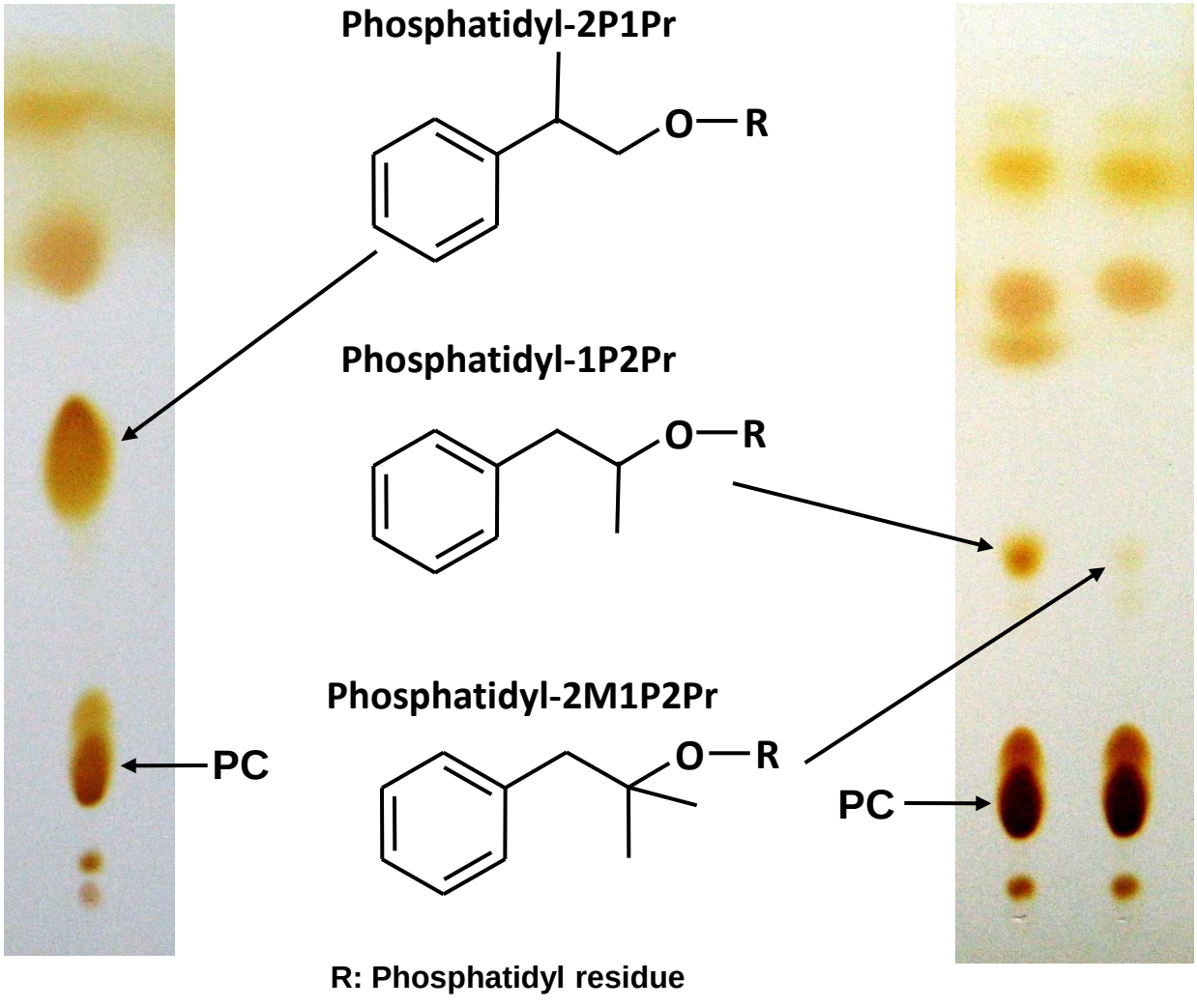

FIGURE 6 Yamamoto et al.