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Short Communication

An enzymatic approach to the synthesis of phospholipid conjugates bearing polyunsaturated fatty acids, maltose and flavonoid glycosides

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ABSTRACT

An approach to the synthesis of phospholipid conjugates bearing both a polyunsaturated fatty acyl group and maltose or a flavonoid glycoside was studied. Under our established conditions, a linoleoyl-phosphatidyl derivative of naringin, a natural sugar flavonoid found in the inner peel of citrus fruits, as well as linoleoyl, icosapentaenoyl- or docosahexaenoyl-phosphatidyl derivatives of maltose could be synthesized via phospholipase D-catalyzed transphosphatidylation at 50 °C in chloroform.

KEYWORDS: phospholipase D, flavonoids, polyunsaturated fatty acids, eicosapentaenoic acid, docosahexaenoic acid

INTRODUCTION

Polyunsaturated fatty acids (PUFA) such as icosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exhibit multi-functional physiological functions including enhanced cell membrane permeability [1], cell death [2], cytotoxicity enhancement for anti-cancer drugs [3] and anti-malarial activity [4, 5]. They are also expected to show yet-unknown beneficial functions especially for human health. However, PUFA, especially DHA and EPA, are very sensitive to oxidation due to their all *cis* non-conjugated olefinic structure. The bis(allylic) hydrogens are easily abstracted by radical initiators to give carbon-centered radicals, which rapidly migrate to the conjugated dienyl allylic positions, and react with oxygen molecules to afford hydroperoxides. The unstable hydroperoxides thus formed decompose rapidly in the process of oxidative degradation of PUFA. The main final degradation products include reactive unsaturated aldehydes such as 4-hydroxy-2-nonenal. These aldehydes react with various biological nucleophiles such as amino groups in proteins and nucleic acids leading to the modification or inhibition of normal biological functions of the latter. In order to enhance their above-mentioned beneficial biological functions and to prevent their oxidative degradation, PUFA have been combined with different functional molecules as reviewed by Parang *et al.* [6]. For example, Siddiqui *et al.* [7] reported that the anticancer drug methotrexate, showed higher anti-proliferative activity against a murine leukemia cell line when conjugated to a docosahexaenoyl phosphatidylcholine (PC) than when conjugated to a PC bearing a stearic acid group,

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a typical saturated fatty acid in plant and animal fats. Piantadosi *et al.* [8] also claimed that their effectiveness as bioactive compounds could be amplified by combining them in one molecule.

Flavonoids that are produced by most plants also have a variety of pharmacological and medicinal functions such as anti-bacterial, anti-inflammatory and anti-viral effects. In addition to their wide utilization in various fields, their structural modifications have been studied extensively to expand their beneficial applications. The modifications have been done by chemical or enzymatic method, or combination of the two. Standard or common technologies have greatly contributed in these synthetic works. However, the optimal reaction conditions, especially for enzymatic synthesis, are different case by case. Therefore, synthetic approaches of these derivatives have extensively focused on the efficient utilization of enzymes. From this background, we examined the enzymatic preparation of novel conjugates of PUFA with sugars or sugar derivatives of flavonoids such as naringin and hesperidin according to the phosphatidyl transfer reaction depicted in Scheme 1. Naringin and hesperidin are constituents of citrus fruits, and are conjugates of saccharides and flavonoids. They are known to show a variety of nutraceutical and medicinal functions including antioxidant activity [9, 10].

MATERIALS AND METHODS

Phosphatidylcholine (**1**) was synthesized according to our reported method [11]. Phosphatidylchlines (**3**), (**4**) and (**5**) were also synthesized according to our reported method [12]. *N*-2-hydroxyethyl-*N*, *N*, *N*-triethylammonium bromide (TEAE) was prepared according to our method [13]. Naringin and hesperidin were obtained from Hayashibara Biochemical Laboratories, Inc. NMR spectra were done on a Varian INOVA UNITY 600 (^{1}H, 600 MHz) spectrophotometer. ESI-MS spectra were done on a Perkin Elmer Model API-III by positive and negative ion scan mode and direct infusion.

2-O-Linoleoyl-1-O-palmitoyl-phosphatidylmaltose (6)

TLC R_f = 0.63 ($CHCl_3$:CH_3OH:CH_3COOH:H_2O = 45:20:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ ppm) 0.90 (6H, m, CH_3 x 2), 1.28 (38H, m, CH_2 x 19), 1.60 (4H, m, $OCOCH_2CH_2$), 2.06 (2H, m, CH_2CH=$CHCH_2CH$=$CHCH_2$), 2.32 (4H, m, $OCOCH_2$ x 2), 2.77 (2H, s, CH=$CHCH_2CH$=CH), 3.17-4.09 (12H, m, protons on sugar carbons), 4.00 (2H, m, -OCH_2-CH-CH_2O), 4.20(1H, m, one proton of -OCH_2-CH-CH_2O), 4.47 (1H, m, one proton of -OCH_2-CH-CH_2O), 4.99 and 5.09 (2H, m, anomeric protons), 5.23 (1H, m, -OC-CH-CO-), 5.35 (4H, m, olefinic protons). ESI MS: $[M+H]^+$ Calcd. for $[C_{49}H_{89}O_{18}P + NH_4]^+$: *m/z* 1014.5; Found: *m/z* 1014.6. $[M-H]^-$ Calcd. for $[C_{49}H_{89}O_{18}P]^-$: *m/z* 995.5; Found: *m/z* 995.6.

2-O-Eicosapentaenoyl-1-O-palmitoyl-phosphatidylmaltose (7)

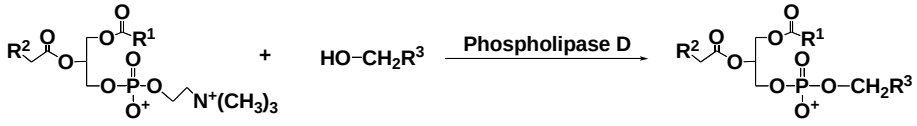
TLC R_f = 0.44 ($CHCl_3$:CH_3OH:CH_3COOH:H_2O = 45:20:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ ppm) 0.89 (3H, t, *J*=7.08 Hz, CH_3), 0.96 (3H, t, *J*=7.57, CH_3), 1.27 (24H, m, CH_2 x 12), 1.68 (4H, m, $OCOCH_2CH_2$ x 2), 2.35 (4H, m, $OCOCH_2$ x 2), 2.83 (2H, m, CH=$CHCH_2CH$=CH), 3.13-4.29 (12H, m, protons on sugar carbons), 4.00 (2H, m, -OCH_2-CH-CH_2O), 4.21 (1H, m, one proton of -OCH_2-CH-CH_2O), 4.44 (1H, m, one proton of -OCH_2-CH-CH_2O), 5.12 and 5.16 (2H, m, anomeric protons), 5.35 (10H, m, olefinic protons). ESI MS: $[M+H]^+$ Calcd. for $[C_{51}H_{86}O_{18}P + NH_4]^+$: *m/z* 1036.5; Found: *m/z* 1036.4. $[M-H]^-$ Calcd. for $[C_{51}H_{86}O_{18}P]^-$: *m/z* 1017.6; Found: *m/z* 1017.6.

2-O-Docosahexaenoyl-1-O-palmitoyl-phosphatidylmaltose (8)

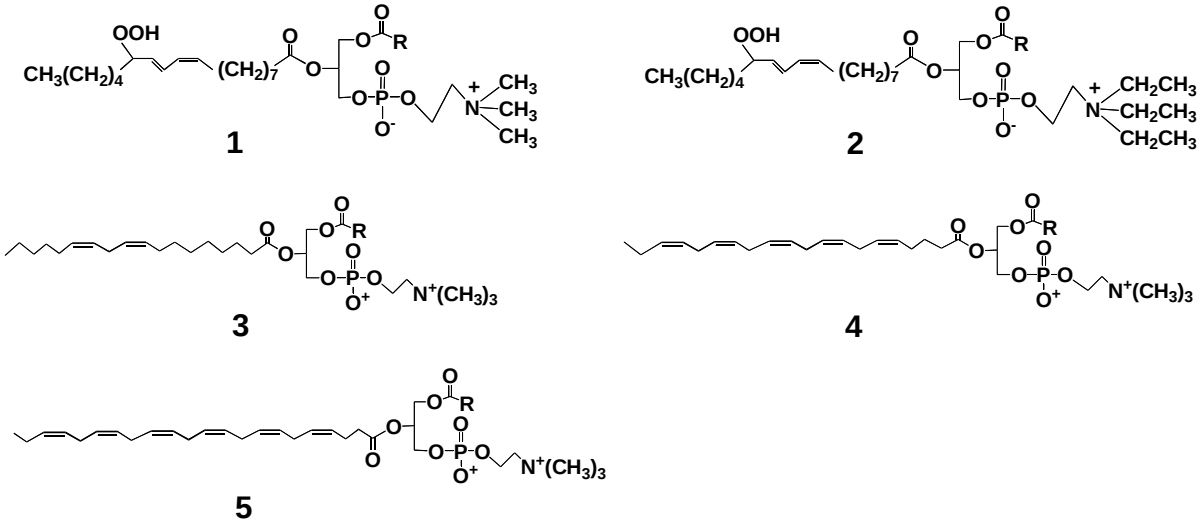
TLC R_f = 0.38 ($CHCl_3$:CH_3OH:CH_3COOH:H_2O = 45:20:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ ppm) 0.89 (3H, t, *J*=7.3 Hz, CH_3), 0.94 (3H, t, *J*=7.3 Hz, CH_3), 1.28 (28H, m, CH_2 x 14), 1.61 (4H, m, $OCOCH_2CH_2$ x 2), 2.06 (4H, m, CH_2CH=$CHCH_2CH$=$CHCH_2$), 2.34 (4H, m, $OCOCH_2$ x 2), 2.85 (2H, m, CH=$CHCH_2CH$=CH), 3.12-4.29 (12H, m, protons on sugar carbons), 4.09 (2H, m, -OCH_2-CH-CH_2O), 4.18 (1H, m, one proton of -OCH_2-CH-CH_2O), 4.47 (1H, m, one proton of -OCH_2-CH-CH_2O), 5.12 and 5.16 (2H, m, anomeric protons), 5.36 (10H, m, olefinic protons). ESI MS: $[M+H]^+$ Calcd. for $[C_{51}H_{86}O_{18}P + NH_4]^+$: *m/z* 1062.6; Found: *m/z* 1062.6. $[M-H]^-$ Calcd. for $[C_{51}H_{86}O_{18}P]^-$: *m/z* 1043.6; Found: *m/z* 1043.6.

2-O-Linoleoyl-1-O-palmitoyl-phosphatidyl-naringin (9)

TLC R_f = 0.83 ($CHCl_3$:CH_3OH:CH_3COOH:H_2O = 45:20:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ ppm)

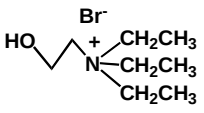


Phosphatidyl Transfer Reaction

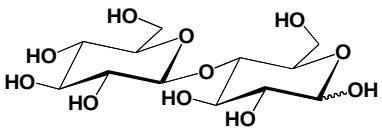


1 **2** **3** **4** **5**

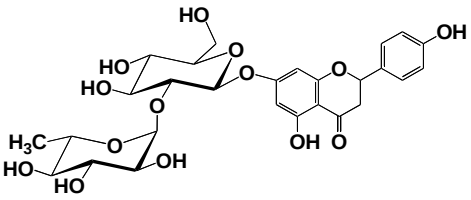
Phosphatidylcholines (R= $(CH_2)_{14}CH_3$)



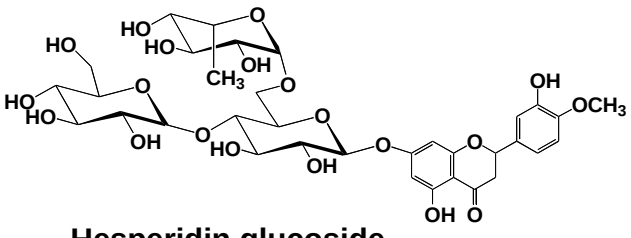
TEAE (a synthetic acceptor)



Maltose



Naringin



Hesperidin glucoside

Phosphatidyl Acceptors

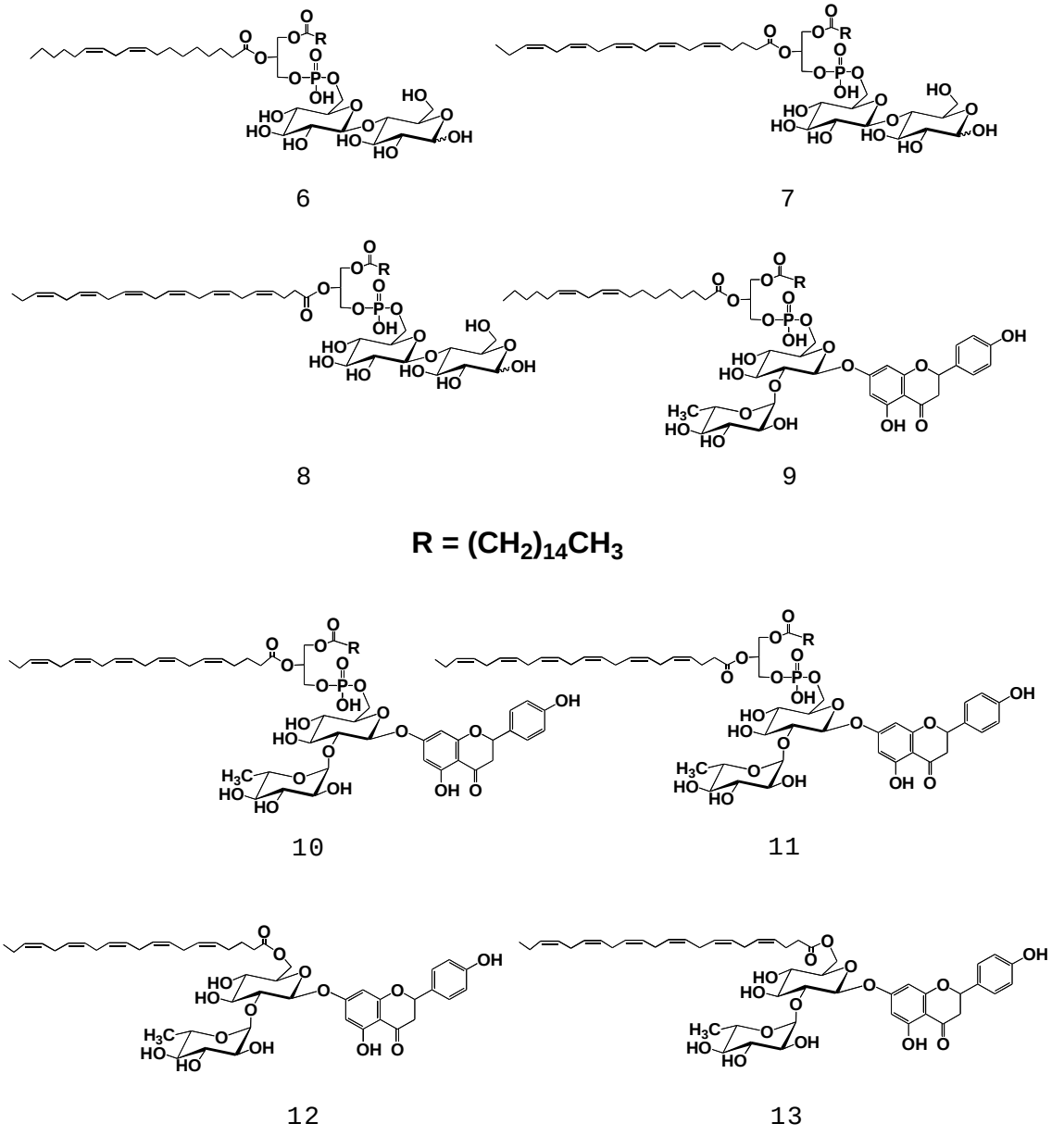
Scheme 1

0.86 (6H, br, CH_3 x 2), 1.22 (3H, br, CH_3 on rhamnose ring), 1.25 (38H, m, CH_2 x 19), 1.57 (4H, m, $OCOCH_2CH_2$ x 2), 2.02 (4H, m, $CH_2CH{=}CHCH_2CH{=}CH$ CH_2), 2.32 (4H, m, $OCOCH_2$ x 2), 2.67 (2H, m, $CH{=}CHCH_2CH{=}CH$), 2.73 (2H, m, CH_2–CO in ring C), 3.00-4.19 (14H, m, protons on the sugar part of naringin and glycerol carbons), 4.99 and 5.09 (2H, m, anomeric protons), 5.21 (1H, m, -OC-CH-CO-), 5.30 (4H, m, olefine protons), 6.11 (2H, m, protons at α, γ-carbons from C-OH in ring A), 6.77 (2H, m, protons at α, α'-carbons from C=O in ring B), 7.26 (2H, m, protons at α, α'-carbons from C-OH in ring C). ESI MS: $[M\text{-}H]^-$ Calcd. for $[C_{64}H_{99}O_{21}P]^+$: *m/z* 1233.6; Found: *m/z* 1233.6

RESULTS AND DISCUSSION

Our aim in the present study was to find possible synthetic conditions for novel conjugates (**8**)–(**12**) shown in Scheme 2. These conjugates carry a PUFA moiety and a sugar flavonoid in one glycerophospholipid molecule. They can be synthesized via chemical, enzymatic or combined chemical and enzymatic methods. However, chemical methods usually require multi-step reactions including protection, deprotection and activation of functional groups such as hydroxyl groups. Therefore, enzymatic method was examined in the present study. There are two classes of lipid related enzymes, lipases and phospholipases, which can be used for the synthesis of a variety of conjugates such as glycosyl lipids, glycosyl phospholipids [14], and various lipid conjugates of drugs [15]. Phospholipase D (PLD) is an enzyme that is ubiquitously found in biological systems and catalyzes hydrolysis of phosphate esters in phospholipids to produce phosphatidic acids. It has been documented in recent years to be involved in a variety of important biological signal transactions [16]. The enzyme also catalyzes transphosphatidylation from phosphate esters to different primarily alcohols affording different PC molecular species. From this background, PLD has been employed as a biocatalyst for the preparation of various bioactive compounds having a phosphate group [17, 18]. Although a number of PLD-catalyzed transphosphatidylation reactions have been reported so far, the reaction conditions were not always similar in the synthetic studies. Therefore, our own conditions such as reaction temperature, solvent, concentration of enzymes, substrates and reaction time should be newly determined. Especially, the phosphatidyl transfer reaction to naringin and hesperidin glucoside might not be easy due to the presence of flavonoid structure that may show inhibitory activity against phospholipase D. Our previous study [19] demonstrated that phosphatidylcholines such as (**1**) having hydroperoxy-linoleoyl group at *sn*-2 position could serve well as a substrate for PLD-catalyzed transphosphatidylation reaction, and it was employed for our synthesis of phosphatidylserine and phosphatidyletanolamine hydroperoxides. Another study of ours [14] also showed that the same phosphatidyl group could be transfered to a synthetic acceptor, TEAE, shown in Scheme 1 affording unnatural phosphatidylcholine hydroperoxide which has *N*, *N*, *N*-triethylammonium group at the polar head group and was easily detected by mass spectroscopy.

PLD-catalyzed transphosphatidylation reaction is generally conducted in a mixture of ether and buffer solution. However, since PLD from *Actinomodula* sp. that was used in our present study has an optimum temperature of 60 °C, diethyl ether (bp. 35 °C) could not be used for the reaction. Accordingly, we tested other solvents such as chloroform (bp. 61 °C) and diisopropyl ether (bp. 69 °C) instead. To evaluate the catalytic activity of PLD in these solvents, we applied our novel and quick method [13] using synthetic phospholipid acceptor, TEAE, and electrospray ionization mass spectrometry. The reaction was conducted at 50 °C for 2 hours in a mixture of an acetate buffer (60 μL, pH 6.5, 0.1 M calcium chloride) and chloroform (200 μL) or diisopropyl ether (200 μL) using 2-(13-hydroperoxy)-linoleoyl-1-palmitoyl-phosphatidylcholine (1, 0.2 mg, 0.3 μmol), *N*-2-hydroxyethyl-*N*, *N*, *N*-triethylammonium bromide (TEAE, 32 mg, μmol) and PLD (5 mg, 1500 u/g) from *Actinomodula* sp. in nitrogen atmosphere. The reaction was stopped by addition of EDTA aq. (80 μL, 0.1 M) and the solution was washed with saturated aqueous hydrogen carbonate solution, followed by extraction of the product with a mixture of methanol/chloroform (2:1). After drying with anhydrous sodium sulfate, the extract was analyzed by neutral loss scan mode at neutral loss of mu 574.2 in collision induced tandem electrospray ionization mass spectrometry. Two signals were



Scheme 2

observed at *m/z* 758.4 and at *m/z* 800.4 for unreacted phosphatidylcholine (**1**) and the product (**2**), respectively. The ratio of these two signals represents the extent of the PLD-catalyzed reaction. In this analysis, the signal intensity ratio, reactant (**1**)/product (**2**) was 49/6 in chloroform and 61/17 in diisopropylether. Thus, chloroform was found to be a better solvent than diisopropylether.

Some flavonoid compounds like naringin and hesperidine glycoside have been reported to show inhibitory activity against phospholipid related enzymes including phospholipase A_2 and D [20, 21]. This inhibitory nature has been discussed in regard to the mechanism of their anti-inflammatory and anti-cancer activities [21]. Therefore, we conducted the same PLD-catalyzed reaction using **1** and TEAE in the presence of naringin and hesperidin glycoside, and the result was analyzed by the neutral loss scan mode, which indicated that the reaction proceeded well with no inhibition by the flavonoid compound.

As per the report by Comfuries and Zwaal [22], a portion of PLD was added three times to the reaction mixture.

We next examined the reaction of PC (3) with maltose to form linoleoyl maltose 5 under the preliminary examined conditions. Typically, a mixture of 2-*O*-linoleoyl-1-*O*-palmitoylphosphatidylcholine **3** (50 mg, 0.65 mmol), maltose (180 mg, 0.53 μmol), phospholipase D (PLD, 10 mg, *Actinomodura* sp., 1500 u/g), ethanol-free distilled chloroform (5 ml), trace of butylated hydroxytoluene as an antioxidant and an acetate buffer (0.5 ml, 0.1 M, pH 6.5) containing $CaCl_2$ (0.1 M) was stirred at 50 °C in a round-bottomed flask (50 ml) for 2 h in a nitrogen atmosphere. During this period, additional PLD (10 mg) was supplemented two times after 40 and 80 min from the starting time. After the reaction period, aqueous EDTA solution (2 ml, 0.1 M) was added to inactivate the enzyme, and the organic solvent was evaporated off under reduced pressure. The product-containing fraction was extracted with a mixture of $CHCl_3$ and CH_3OH (2:1, 3.4 vol. eq. to the aqueous phase), and the enzyme was removed by filtration. Water (1 vol. eq.) and $CHCl_3$ (3.7 vol. eq.) were added to this solution, and the mixture was stirred for 10 min. To separate the water phase and the organic phase, centrifuging was conducted at 3000 r.p.m for 10 min. The $CHCl_3$ phase was concentrated and the residue was chromatographed on silica gel eluted with a mixture of $CHCl_3$: CH_3OH:CH_3COOH:H_2O (45:20:9:1). Since the elution of all the desired product from the column and complete removal of the water from the product fraction were difficult, net product weight could not be obtained. The approximate product yield was estimated as 13% based on the amount of phosphatidylcholine used. TLC R_f = 0.63 ($CHCl_3$: CH_3OH:CH_3COOH:H_2O = 45:20:9:1). The following spectral data confirm the product structure. ^{1}H NMR (600 MHz, CD_3OD, δ ppm) 0.90 (6H, m, CH_3 x 2), 1.28 (38H, m, CH_2 x 19), 1.60 (4H, m, $OCOCH_2CH_2$), 2.06 (2H, m, CH_2CH=$CHCH_2CH$=$CHCH_2$), 2.32 (4H, m, $OCOCH_2$ x 2), 2.77 (2H, s, CH=$CHCH_2CH$=CH), 3.17-4.09 (12H, m, protons on sugar carbons), 4.00 (2H, m, -OCH_2-CH-CH_2O), 4.20 (1H, m, one proton of -OCH_2-CH-CH_2O), 4.47 (1H, m, one proton of -OCH_2-CH-CH_2O), 4.99 and 5.09 (2H, m, anomeric protons), 5.23 (1H, m, -OC-CH-CO-), 5.35 (4H, m, olefinic protons). ESI MS: $[M+H]^+$ Calcd. for $[C_{49}H_{89}O_{18}P + NH_4]^+$: *m/z* 1014.5; Found: *m/z* 1014.6. $[M-H]^-$ Calcd. for $[C_{49}H_{89}O_{18}P]^-$: *m/z* 995.5; Found: *m/z* 995.6.

Likewise, phosphatidylmaltoses (**7**) and (**8**) having eicosapentaenoyl group and docosahexaenoyl group, respectively could be synthesized under the same conditions.

The procedure for the synthesis of 2-*O*-linoleoyl-1-*O*-palmitoylphosphatidyl-naringin (**8**) was similar to that of (**6**) except for some parts. A mixture of 2-*O*-linoleoyl-1-*O*-palmitoylphosphatidylcholine (145 mg, 0.19 mmol), naringin (500 mg, 0.53 mmol), PLD (20 mg, *Actinomodura* sp., 1500 u/g), ethanol-free distilled chloroform (5 ml), trace of butylated hydroxytoluene and an acetate buffer (1.0 ml, 0.1 M, pH 6.5) containing $CaCl_2$ (0.1 M) was stirred at 50 °C in a round-bottomed flask (50 ml) for 7 h in the nitrogen atmosphere. During this period, PLD (20 mg) was added two times after 3 and 5 h from the starting time. After the reaction period, aqueous EDTA solution (1.5 ml, 0.1 M) was added to inactivate the enzyme, and the organic solvent was evaporated off under reduced pressure. The product-containing aqueous fraction was extracted with a mixture of $CHCl_3$ and CH_3OH (2:1, 4.3 vol. equivalent to the aqueous phase) by shaking for 30 min, and the enzyme was removed by filtration. After removing the organic solvent, the residual water was removed by azeotropic distillation with diisopropyl ether followed by toluene. The residue was dissolved in a small amount of $CHCl_3$. The solution was chromatographed on silica gel eluted with a mixture of $CHCl_3$:CH_3OH: CH_3COOH:H_2O (45:20:9:1). Approximate yield was 18% based on the phosphatidylcholine used. Structural integrity of this product was confirmed by proton NMR and MASS spectra. This constitutes a first example of demonstration in which PLD from *Actinomodula* sp. catalyzes transphosphatidylation from a phospholipid bearing linoleic acd, a PUFA having linoleic acid with two double bonds, to a sugar flavonoid, naringin.

Unfortunately however, products (**10**)-(**13**) that were expected to be formed through transphosphatidylation reactions from EPA-PA-PC (**4**) and DHA-PA-PC (**5**) to naringin and hesperidine glucoside could not be detected on TLC plate after reaction under the same conditions.

Since there is no decisive reason for the unsuccessful results, other reaction conditions may be explored as well as use of other enzymes.

CONCLUSION

Reaction conditions for phospholipase D-catalyzed synthesis of phospholipids bearing polyunsaturated fatty acyl group and sugar or sugar flavonoid were established. Under these conditions, phosphatidylmaltose derivatives bearing linoleoyl, eicosapentaenoyl or docosahexaenoyl groups, and a phosphatidylnaringin bearing a linoleoyl group were successfully synthesized. However, the conjugate of eicosapentaenoyl- or docosahexaenoyl-phosphatidylcholine with naringin or hesperidine could not be obtained. Further survey for the reaction conditions as well as finding different enzyme species is important.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

REFERENCES

1. Stillwell, W., Ehringer, W. and Jenski, L. J. 1993, Lipids, 28, 103-108.
2. Cristea, I. M. and Esposti, M. D. 2004, Chem. Phys. Lipids, 129, 133-160.
3. Germain, E., Chajès, V., Sophie Cognault, S., Claude Lhuillery, C. and Bougnou, P. 1998, Int. J. Cancer, 75, 578-583.
4. Kumaratilake, L. M., Robinson, B. S., Ferrante, A. and Poulos, A. 1992, J. Clin. Invest., 89, 961-967.
5. Carballeira, N. M. 2008, Progress in Lipid Research, 47, 50-61.
6. Parang, K., Wiebe, L. L. and Knaus, E. E. 2000, Current Medicinal Chemistry, 7, 995-1039.
7. Siddiqui, R. A., Zerouga, M., Wu, M., Castillo, A., Harvey, K., Zaloga, G. P. and Stillwell, W. 2005, Breast Cancer Res., 7, R645-R654.
8. Piantadosi, C., Marasco, Jr. C. J., Morris-Natschke, S. L., Meyer, K. L., Gumus, F., Surles, J. R. and Ishaq, K. S. 1991, J. Med. Chem., 34, 1408-1414.
9. Xu, G., Ye, X., Chen, J. and Liu, D. 2007, J. Agric. Food. Chem., 55, 330-335.
10. Semalty, A., Semalty, M., Singh, D. and Rawat, M. S. M. 2010, J. Inclusion Phenomena and Macrocyclic Chem., 67, 253-260.
11. Baba, N., Yoneda, K., Tahara, S., Iwasa, J., Kaneko, T. and Matsuo, M. 1990, J. Chem. Soc. Chem. Commun., 1281-1282.
12. Baba, N., Alam, Md. K., Mori, Y., Haider, S. S., Tanaka, M., Nakajima, S. and Shimizu, S. 2001, J. Chem. Soc. Perkin Trans., 1, 221-223.
13. Oda, K., Imura, M., Ueda, Y., Hosokawa, M., Kobayashi, M., Matsubara, J., Sato, M., Kumura, N., Izumi, M., Nakajima, S., Sugio, T. and Baba, N. 2009, Biosci. Biotechnol. Biochem., 73, 1233-1237.
14. Kitamoto, D., Isoda, H. and Nakahara, T. 2002, J. Biosci. Bioeng., 94, 187-201.
15. Blanchfield, J. and Toth, I. 2004, Current Medicinal Chem., 11, 2375-2382.
16. Jenkins, G. M. and Frohman, M. A. 2005, Cellular and Molecular Life Sience, 36, 903-914.
17. Ulbrich-Hofmann, R., Lerchner, A., Oblozinsky, M. and Bezakova, L. 1995, Biotechnol. Lett., 27, 535-543.
18. Ulbrich-Hofmann, R., Haftendorn, R., Dittrich, N., Hirche, F. and Aurich, I. 1998. Eur. J. Lipid Sci. Technol., 100, 96-120.
19. Onyango, A. N., Inoue, T., Nakajima, S., Baba, N., Takao Kaneko, T., Matsuo, M. and Shimizu, S. 2001, Angewandte Chemie International Edition, 40, 1755-1757.
20. Santos, M. L., Toyama, D. O., Oliveira, S. C. B., Cotrim, C. A., Diz-Filho, E. B. S., Fagundes, F. H. R., Soares, V. C. G., Aparicio, R. and Toyama, M. H. 2011, Molecules, 16, 738-761.
21. Manthey, J. A., Guthrie, N. and Grohmann, K. 2001, Curr. Med. Chem., 8, 135-153.
22. Comfuries, P. and Zwaal, P. F. A. 1977, Biochim. Biophys. Acta, 488, 36-42.