Title	Preparation of Phosphatidyl-pantanol by phospholipase D-mediated transphosphatidylation and its anti-inflammatory activity on macrophage-like RAW264.7 cells
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Title: Preparation of phosphatidyl-panthenol by phospholipase D-mediated
transphosphatidylation and its anti-inflammatory activity on macrophage-like
RAW264.7 cells.

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ABSTRACT

Panthenol is known to be pro-vitamin B₅ and show several functions such as wound-healing, anti-oxidative, and anti-inflammatory activities. In this study, panthenol was phosphatidylated via phospholipase D (PLD)-mediated transphosphatidylation and its anti-inflammatory effects were investigated on macrophage-like cell RAW264.7 cells. By PLD-mediated transphosphatidylation from phosphatidylcholine (PC), hydroxy group of p1 position in panthenol molecule was alternatively formed phosphate ester bond even though panthenol has three of hydroxy groups in the molecule. The yield was 96 mol% under optimum reaction conditions: 50 μ mol of PC, 1.6 U of PLD, 500 μ mol of panthenol, 1.6 mL/1.6 mL of ethyl acetate/0.2 M acetate buffer (pH 5.6) ratio, 10 mM of CaCl₂ in the buffer, 37°C for 24 h. Phosphatidyl-panthenol (P-panthenol) suppressed mRNA expression level of pro-inflammatory mediators such as IL-6, IL-1 β , TNF- α and COX-2 more than precursor compounds, PC and panthenol, in activated RAW264.7 cells. P-panthenol also suppressed protein secretion levels of IL-6 and IL-1 β in the culture medium of RAW264.7 cells. These results demonstrate that P-panthenol, which is easily synthesized in high yield, is potential compound for novel anti-inflammatory phospholipid.

Key words: Phosphatidyl-panthenol, Phospholipid, Phospholipase D, Anti-inflammation

1. Introduction

D-Panthenol (panthenol, pro-vitamin B₅) is an essential compound in human body as a part of coenzyme A which supports a lot of biochemical reactions. Besides that, it also has several bio-functions such as wound-healing activity (Wolff and Kieser, 2007; Oztürk, et al., 2007), anti-oxidative activity in the cell (Li-Mei et al., 2016; Etense et al., 2007), and anti-inflammatory activity (Proksch and Nissen. 2002; Ebner et al., 2002). Therefore, panthenol is utilized as a component of a lot of formula for skin care purpose. In generally, topical use are solutions, aerosols, ointments, and creams. However, hydrophilic property of panthenol makes its application difficult, because hydrophilic compounds are often blended using some emulsifier to homogenize formula and to improve its penetration to skin. Wiesława and Ryszard (2004) have prepared panthenol-gel-formula with 2.5% hydroxyethylcellulose having good anti-inflammatory activity against ultra-violet induced irritation on guinea pigs.

Phosphatidylation is very useful technique for utilization of biological active compounds because phospholipids, especially glycerophospholipids have excellent biocompatibility. In addition, phosphatidylated compounds have the ability to form liposome which can be used as a substrate of drug delivery system (Komizu et al., 2006; Yagi et al., 2007), depending on their amphiphilic properties.

Phospholipase D (PLD), which hydrolyzes the distal phosphodiester bonds of phospholipids, is effective tool to synthesize phosphatidylated compounds through transphosphatidylation. Although chemical way to produce phospholipid derivatives requires several reaction steps, PLD requires only one step because it is able to transfer the phosphatidyl moiety of phospholipids to desired alcohol. Further, phosphatidylated

compounds often exerts superior bio-functions compared to precursor compounds. For example, genipin was phosphatidylated via PLD-catalyzed transphosphatidylation and phosphatidyl-genipin exerted superior cytotoxicity to precursor genipin against several cancer cell lines (Takami and Suzuki., 1994). 5-Fluorouridine, a known anticancer drug, was phosphatidylated and the synthesized phospholipid derivative exhibited higher anticancer activity against Meth A fibrosarcoma in mice compared to precursor 5-fluorouridine (Shuto et al., 1995). We also have previously reported on the synthesis of phosphatidyl-perillyl alcohol and phosphatidyl-nerol by PLD (Yamamoto et al., 2008a; Yamamoto et al., 2008b). The synthesized phosphatidyl-terpenes markedly reduced viability of human prostate cancer cells and leukemia (Yamamoto et al., 2008a).

In this study, we focused on vitamin-binding phospholipids as novel compounds. Phosphatidyl-panthenol (P-panthenol) was synthesized via PLD-mediated transphosphatidylation and evaluated its anti-inflammatory effects *in vitro*.

2. Materials and methods

2.1. Materials

PLD from *Streptomyces* sp. and D-panthenol (panthenol) (> 98%) was purchased from Sigma-Aldrich (St. Louis, USA). 1,2-Dioleoyl-sn-glycero-3- phosphocholine (DOPC) and soybean phosphatidylcholine (SoyPC; PC > 95%) were obtained from Avanti Polar Lipids, Inc. (Alabaster, USA). Other chemicals and solvents were analytical grade.

2.2. Synthesis of P-panthenol

SoyPC (50 μ mol) and 500 μ mol panthenol were dissolved in 1.6 mL ethyl acetate. The ethyl acetate solution was mixed to 1.6 mL of 0.2 M acetate buffer (pH 5.6) dissolved PLD and 10 mM of CaCl_2 to start transphosphatidylation. The reaction was conducted at 37°C and stirred with magnetic bar at 250 rpm and terminated by methanol addition. Subsequently, chloroform and water were added to adjust a chloroform/methanol/water (10:5:3, v/v/v) and lipid fraction was recovered from lower layer. To separate the synthesized compound, lipid fraction was applied onto a silica gel thin-layer chromatography (TLC) plate (Silica gel 60 F254, Merck, Darmstadt, Germany) with a developing solvent, chloroform:methanol:water (65:25:4, v/v/v). The synthesized compound was scraped off from TLC plate detecting by UV at 254 nm and extracted from silica gel with chloroform/methanol (3:7, v/v). The synthesized compound separated on TLC plate was also detected by I_2 vapor.

2.3. Identification of the structure of synthesized compound

The molecular mass of synthesized compound from DOPC was estimated by high-resolution mass spectrometry (HR-MS) in the negative electrospray ionization (ESI) mode with JEOL JMS-T100LP (Japan Electronic Optics Laboratory Co., Tokyo, Japan). To identify the structure, ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of the synthesized compound, DOPC and panthenol were analyzed with a Varian UNITY INOVA 500 spectrometer (Varian, Inc., Palo Alto, CA, USA) at 500 and 126 MHz, respectively, by using tetramethylsilane as an internal standard. The samples were dissolved in CDCl_3 or $\text{CDCl}_3:\text{CD}_3\text{OD}$ (3:1, v/v).

2.4 The yield of synthesized P-panthenol

The synthesized yield of P-panthenol was analyzed by a high-performance liquid chromatography (HPLC) system equipped with a mobile phase delivery pump (L-7100, Hitachi, Tokyo, Japan), diode array detector (L-7455, Hitachi) and Mightysil Si 60 column (250 x 4.6 mm (5 μ m), Kanto Chemical Co., Inc., Tokyo, Japan). The lipid fraction separated from the reaction mixture was analyzed by HPLC. Mobile phase was solvent A (acetonitrile/sulfuric acid (100:0.5, v/v)) and solvent B (methanol) (98:2, v/v) at 1.0 mL/min at 30°C. The P-panthenol was detected at 210 nm. The yield of P-panthenol was calculated from the HPLC peak area using a calibration curve by purified P-panthenol standard.

2.5 Anti-inflammatory activities of P-panthenol

2.5.1. Cell culture

Murine macrophage-like RAW264.7 cells (5×10^4 cells/well) were pre-incubated in 24-well plate with 1 mL RPMI 1640 supplemented with 10% fetal bovine serum, 100 μ g/mL streptomycin and 100 U/mL penicillin, in 5% CO₂ at 37°C for 24 h. P-panthenol, panthenol, and SoyPC were added into the culture medium, respectively, as an ethanol solution. Final ethanol concentration was adjusted to 0.1% without cytotoxicity. After incubation with each sample for 24 h, inflammation was induced by addition of lipopolysaccharide (LPS, final concentration of 0.1 μ g/mL) for 6-24 h in the presence of P-panthenol, panthenol or SoyPC.

2.5.2. Quantitative real-time RT-PCR

The RAW264.7 cells were washed with phosphate-buffered saline three times after stimulation with LPS for 6 h. Total RNA was extracted from the cells using RNeasy

Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the user's manual. First-strand cDNAs were synthesized from total RNA with the High-Capacity cDNA Archive Kit (Applied Biosystems Japan Ltd, Tokyo, Japan) according to the manufacturer's protocol. Quantitative real time PCR analysis was conducted as our previous paper (Tian et al., 2017) with the ABI Prism 7500 (Applied Biosystems Japan Ltd, Tokyo, Japan) and TaqMan® Gene Expression Assays (Applied Biosystems Japan Ltd, Tokyo, Japan); IL-6: Mm00446190_ml, IL-1β: Mm00434228_ml, TFN-α: Mm00443258_ml, COX-2: Mm00478374_ml. GAPDH: Mm99999915_gl.

2.5.3. ELISA assay

After incubation with P-panthenol for 24 h, RAW264.7 cells were stimulated with 0.1 µg/mL LPS for additional 24 h in the presence of P-panthenol. IL-6 and IL-1β concentrations in the culture medium of RAW264.7 cells were measured by ELISA using commercialized kits (Thermo Scientific, Frederick, MD, USA) according to the manufacturer's protocol. Cell culture conditions are the same in 2.5.1. *Cell culture*.

2.6 Statistical analysis

All experimental values are expressed as the mean ± the standard deviation. Statistically significant differences are determined by *Scheffé's* method (Fig. 5) or *Dunnett's* test (Table 3). A value of $P < 0.01$ was considered significant.

3. Results and discussion

3.1. Synthesis of P-panthenol by PLD

Phosphatidylation of panthenol was carried out using SoyPC or DOPC as substrate *via* PLD-mediated transphosphatidylation (Fig. 1). A new product was detected upper side of PC on TLC plate after a 24 h reaction (Fig. 2). The negative HR-MS data of the new product from DOPC and panthenol by PLD was m/z 886.62025 and coincided with the predicted molecular formula ($C_{48}H_{89}N_1O_{11}P_1$) of P-panthenol. In both 1H and ^{13}C NMR analyses, panthenol signals were observed instead of the disappearance of choline signals in the data of the synthesized compound (Table 1 and 2). The signal at δ_C 63.6 ppm and methylene signal at δ_H 3.91 ppm at p1 position were shifted to down-field compared to δ_C 58.9 ppm and δ_H 3.63 ppm at p1 position of free panthenol (Fig.3, Table 1 and 2). On the other hand, other signals in panthenol moiety of the synthetic compound showed similar δ_C and δ_H values of free panthenol. On the basis of HR-MS and NMR analyses, the synthesized compound was identified as P-panthenol connected with OH group at p1 position of panthenol moiety via phosphodiester linkage as shown in Fig. 3.

It is well known that PLD recognizes hydroxy group with preference order at primary, secondary, and tertiary alcohol (Ulbrich-Hofmann et al., 2005). Further, we have reported that alcohols having bulky structure such as aromatic group near hydroxy group is poor substrate for transphosphatidylation using PLD even though they are primary alcohol group (Yamamoto et al., 2011). From these evidences, it was suggested that secondary hydroxy group at p5 position and primary hydroxy group at p7 position which has bulky structure near its position, respectively, were poorly recognized by PLD.

3.2. Optimization of transphosphatidylation with SoyPC and panthenol

Using SoyPC (50 μmol) as a substrate, optimization of the transphosphatidylation reaction was carried out with fixing the reaction solvent/buffer ratio (1.6 mL/0.8 mL), the concentration of CaCl_2 (10 mM), the amount of PLD (1.6 U), reaction temperature (37°C), and reaction time (24 h). At first, the effect of the amount of panthenol (250-5000 μmol) on the P-panthenol synthesis was examined (Fig. 4A). The yield increased with the amount of panthenol and decreased more than 500 μmol of panthenol. It has been reported that too much amount of substrate alcohol inhibits the PLD-mediated transphosphatidylation (Yamamoto et al., 2008b; Yamamoto et al., 2011). The optimum amount of panthenol was defined 500 μmol for our reaction system.

The effect of the reaction solvent/buffer ratio (v/v) was examined (Fig. 4B). Reaction yield increased by changing the ratio from 1.6 mL/0.8 mL to 1.6 mL/1.6 mL. However, the yield was almost the same at the ratio of solvent 3.2 mL and buffer 1.6 mL. Because the activity of transphosphatidylation by PLD is influenced with the environment of water-solvent interphase such as particle size and interphase pressure (Hirche and Ulbrich-Hofmann, 1999), the ratio of solvent and buffer will make adequate interphase for the transphosphatidylation reaction in this study. From these results, the solvent/buffer ratio of 1.6 mL/1.6 mL was defined as the optimum ratio.

Concentration of CaCl_2 was also affected the reaction yield of P-panthenol (Fig. 4C). By adding 10 mM of CaCl_2 in the buffer, reaction yield increased compare to the reaction system without CaCl_2 , although PLD from *Streptomyces* sp. doesn't require Ca^{2+} for the activity (Juneja et al., 1987). The similar result was observed in our previously study of the phosphatidyl-glycerol (PG) synthesis via PLD (*Streptomyces* sp.)-mediated transphosphatidylation (Suzuri et al., 2009; Chen et al., 2020), although it is unclear why Ca^{2+} promotes PG and P-panthenol synthesis by PLD. As suggestion

by Yang and Roberts (2003), Ca^{2+} might reflect physical effects on the P-panthenol production. The optimum CaCl_2 concentration was determined at 10 mM in the buffer.

We also measured time course of the P-panthenol synthesis (Fig. 4D). The yield of P-panthenol increased in a time-dependent manner and reached a maximum yield 96% after 24 h reaction. From these results, we determined the following optimum reaction conditions: 50 μmol of SoyPC, 1.6 U of PLD, 500 μmol of panthenol, 1.6 mL/1.6 mL of solvent/buffer ratio, 10 mM of CaCl_2 in the buffer, 37°C for 24 h.

3.3. Anti-inflammatory effect of P-panthenol on RAW264.7 cells.

P-panthenol suppressed IL-6 mRNA expression rather than SoyPC and Panthenol in dose dependent manner in LPS-stimulated RAW264.7 cell line (Fig. 5). Even at 25 μM of low concentration of P-panthenol, significant suppression of IL-6 mRNA expression was observed compare to positive control (LPS+), although the same concentration of panthenol and SoyPC did not suppress. At 50 μM , P-panthenol, but not panthenol, down-regulated mRNA expressions of IL-6, IL-1 β , TNF- α and COX-2 in activated RAW264.7 cells. In addition, P-panthenol suppressed secretion of IL-6 and IL-1 β in the culture media of LPS-stimulated RAW264.7 cells (Table 3). The present results indicate that anti-inflammatory effect by panthenol was significantly improved by phosphatidylation of panthenol.

4. Conclusion

P-panthenol was successfully synthesized *via* PLD-mediated transphosphatidylation of phosphatidylcholine and panthenol in one-pot. In the reaction system examined in this study, hydroxy group of p1 position in panthenol molecule (Fig.3) was alternatively

formed phosphate ester bond. The reaction yield reached to quantitative (96%). Anti-inflammatory effect of P-panthenol was superior to precursor compounds, panthenol and SoyPC. It was suggested that P-panthenol has the potential as an alternative novel lipid for the treatment of inflammation.

Author statement

Conceptualization, Y.Y. and M.H.; methodology, Y.Y., M.H, H.K; investigation, Y.Y., K.S., T.K.; original draft preparation and writing, Y.Y; review and editing, H.K., and M.H.; supervision, K.M.

Declaration of competing interest

Authors declare no conflict of interest.

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References

- Chen, L., Beppu, F., Miyashita K., Hosokawa, M., 2020. Preparation of n-3 polyunsaturated phosphatidylglycerol from salmon roe lipids by phospholipase D and *in vitro* digestion. Eur. J. Lipid Sci. Technol. 122, 1900201.
- Ebner, F., Heller, A., Rippke, F., Tausch, I. 2002. Topical use of dexpanthenol in skin disorders. Am. J. Clin. Dermatol. 3, 427-433.
- Etense, B., Ozkisacik, S., Ozkara, E., Karul, A., Oztan, O., Yazici, M., Gürsoy, H. 2007. Dexpanthenol attenuates lipid peroxidation and testicular damage at experimental ischemia and reperfusion injury. Peditr. Surg. Int. 23, 177-181.

266 Hirche, F., Ulbrich-Hofmann, R., 1999. The interfacial pressure is an important parameter for
 267 the rate of phospholipase D catalyzed reactions in emulsion systems. *Biochim. Biophys.*
 268 *Acta* 1436, 383-389.

269 Juneja, L.R., Hibi, N., Inagaki, N., Yamane, T., Shimizu, S., 1987. Comparative study on
 270 conversion of phosphatidylcholine to phosphatidylglycerol by cabbage phospholipase D in
 271 micelle and emulsion systems. *Enzyme Microb. Technol.* 9, 350-354.

272 Komizu, Y., Matsumoto, Y., Ueoka, R., 2006. Membrane targeted chemotherapy with hybrid
 273 liposomes for colon tumor cells leading to apoptosis. *Bioorg. Med. Chem. Lett.*, 16, 6131-
 274 6134.

275 Li-Mei, W., Jie, T., Shan-He, W., Dong-Mei, M., Peng-Jiu, Y., 2016. Anti-inflammatory and
 276 anti-oxidative effects of dexpanthenol on lipopolysaccharide induced acute lung injury in
 277 mice. *Inflammation*. 39, 1757-1763.

278 Oztürk, N., Korkmaz, S., Oztürk, Y., 2007. Wound-healing activity of St. John's Wort
 279 (*Hypericum perforatum* L.) on chicken embryonic fibroblasts. *J. Ethnopharmacol.* 111, 33-
 280 39.

281 Proksch, E., Nissen, H.P., 2002. Dexpanthenol enhances skin barrier repair and reduces
 282 inflammation after sodium lauryl sulphate-induced irritation. *J. Dermatolog. Treat.* 13, 173-
 283 178.

284 Shuto, S., Itoh, H., Sakai, A., Nakagami, K., Imamura, S., Matsuda, A., 1995. Nucleosides and
 285 nucleotides--CXXXVII. Antitumor phospholipids with 5-fluorouridine as a cytotoxic
 286 polar-head: synthesis of 5'-phosphatidyl-5-fluorouridines by phospholipase D-catalyzed
 287 transphosphatidylation. *Bioorg. Med. Chem.* 3, 235-43.

288 Suzuri, K., Yamamoto, Y., Hosokawa, M., Miyashita, K., 2009. Effect of alpha-tocopherol on
 289 the synthesis of phosphatidylglycerol catalyzed by phospholipase D in an aqueous system
 290 *Biotechnol. Lett.* 31, 719-723.

291 Takami, M., Suzuki, Y., 1994. Enzymatic synthesis of novel phosphatidylgenipin, and its
 292 enhanced cytotoxicity. *Biosci. Biotechnol. Biochem.* 58, 1897-1898.

293 Tian, Y., Katsuki, A., Romanazzi, D., Miller, M.R., Adams, S.L., Miyashita, K., Hosokawa, M.,
 294 2017. Docosapentaenoic acid (22:5n-3) downregulates mRNA expression of pro-
 295 inflammatory factors in LPS-activated murine macrophage like RAW264.7 cells. *J. Oleo*
 296 *Sci.* 66,1149-1156.

297 Ulbrich-Hofmann, R., Lerchner, A., Oblozinsky, M., Bezakova, L., 2005. Phospholipase D and
 298 its application in biocatalysis. *Biotechnol. Lett.* 27, 535-544.

- Wiesława, S.; Ryszard, P. 2004. Investigation of some topical formulations containing dexpantenol. *Acta Poloniac. Pharmaceutica*. 61, 433-437.
- Wolff, H.H., Kieser, M., 2007. Hamamelis in children with skin disorders and skin injuries: results of an observational study. *Eur. J. Pediatr.* 166, 943-948.
- Yagi, N., Yano, Y., Hatanaka, K., Yokoyama, Y., Okuno, H., 2007. Synthesis and evaluation of a novel lipid-peptide conjugate for functionalized liposome. *Bioorg. Med. Chem. Lett.* 17, 2590-2593.
- Yamamoto, Y., Hosokawa, M., Kurihara, H., Maoka, T., Miyashita, K., 2008a. Synthesis of phosphatidylated-monoterpene alcohols catalyzed by phospholipase D and their antiproliferative effects on human cancer cells. *Bioorg. Med. Chem. Lett.* 18, 4044-4046.
- Yamamoto, Y., Hosokawa, M., Kurihara, H., Miyashita, K., 2008b. Preparation of phosphatidylated terpenes via phospholipase D-mediated transphosphatidylation. *J. Am. Oil Chem. Soc.* 85, 313-320.
- Yamamoto, Y., Kurihara, H., Miyashita, K., Hosokawa, M., 2011. Synthesis of novel phospholipids that bind phenylalkanols and hydroquinone via phospholipase D-catalyzed transphosphatidylation *New Biotechnol.* 28, 1-6.
- Yang, H., Roberts, M.F., 2003. Phosphohydrolase and transphosphatidylation reactions of two *Streptomyces* phospholipase D enzymes: covalent versus noncovalent catalysis. *Protein Sci.* 12, 2087-2098.

Figure legends

Fig. 1. Synthesis of phosphatidyl-panthenol by PLD-mediated transphosphatidylation of PC and panthenol.

Fig. 2. TLC analysis of reaction mixture after PLD-mediated transphosphatidylation of PC and panthenol. (A): Substrate SoyPC, (B): Reaction mixture. Developing solvent for TLC analysis was chloroform/methanol/water (65:25:4, v/v), and spots were detected by I_2 .

Fig. 3. Structure of dioleoyl-phosphatidylcholine, panthenol and phosphatidyl-panthenol.

Fig. 4. Optimum conditions on PLD-mediated P-panthenol synthesis. A: Amount of panthenol, B: Ratio of reaction solvent and buffer, C: $CaCl_2$ concentration in the buffer, D: Reaction time.

Fig. 5: Down-regulation of pro-inflammatory factor mRNA expression by phosphatidyl-panthenol. (A): IL-6 mRNA, (B): IL-1 β mRNA, (C): TNF- α mRNA, (D): COX-2 mRNA. * $P < 0.01$ vs. LPS (+), # $P < 0.01$ vs. Panthenol.

Table 1. ¹H NMR chemical shifts of dioleoyl-phosphatidylcholine (DOPC), panthenol, and synthesized compound

Position	Compounds		
	Panthenol	DOPC	Synthesized compound
g1		4.39 dd (12, 2.5) 4.12 dd (12, 7)	4.38 dd (12, 2.5) 4.15 dd (12, 7)
g2		5.18 m	5.21 m
g3		3.94 and 3.89 m	3.93 m
a1			
a2		2.27 t (6.5)	2.29 t (6.5)
a3		1.58 m	1.58 m
a4-a7, a12-a17		~ 1.29	~1.29
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)	0.88 t (6.5)
b1		3.76 br	
b2		4.28 br	
NCH ₃		3.34 s	
p1	3.63 t (6.5)		3.91 br
p2	1.74 quintet (6.5)		1.80 m
p3	3.37 m		3.55 and 3.31 br
p4			
p5	3.95 s		4.06 s
p6			
p7	3.48 and 3.43 d (10.5)		3.44 and 3.40 d (11)
p8	0.93		0.97 s
p9	0.96		0.90 s
NH	7.66		7.48 br s

378 Table 2. ¹³C NMR chemical shifts of dioleoyl-phosphatidylcholine (DOPC), panthenol,
379 and synthesized compound

Position	Compounds		
	Panthenol	DOPC	Synthesized compound
g1		62.9	62.8
g2		70.3	70.5
g3		63.2	63.6
a1		173.0	173.6
a2		34.0	34.1
a3		24.8	24.9
a4-a7, a12-a17		31~ 27	~ 29
a8, a11		27.1	27.1
a9, a10		130.0	130.0
a18		14.0	14.1
b1		66.1	
b2		59.2	
NCH ₃		54.2	
p1	58.9		63.6
p2	31.4		29.9
p3	35.5		35.7
p4	174.3		174.8
p5	76.3		76.5
p6	38.7		39.1
p7	69.8		69.8
p8	20.1		21.8
p9	19.9		20.7

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389 Table 3. Effect of P-panthenol on secretion of IL-6 and IL-1 β into the culture medium
 390 of LPS-stimulated RAW264.7 cells.

	IL-6 (pg/mL)	IL-1 β (pg/mL)
LPS (0.1 μ g/mL)	34.43 $\pm$ 0.43	10.38 $\pm$ 1.76
LPS + P-panthenol (25 μ M)	24.41 $\pm$ 2.38*	8.22 $\pm$ 0.94
LPS + P-panthenol (50 μ M)	26.85 $\pm$ 0.91*	6.63 $\pm$ 0.76*
LPS + P-panthenol (100 μ M)	22.26 $\pm$ 0.36*	3.96 $\pm$ 0.33*

391 P-panthenol: phosphatidyl-panthenol, * P <0.01 vs LPS

Fig. 1. Synthesis of phosphatidyl-panthenol by PLD-mediated transphosphatidylation of PC and panthenol.

Fig. 2. TLC analysis of reaction mixture after PLD-mediated transphosphatidylation of PC and panthenol.
(A): Substrate SoyPC, (B): Reaction mixture
Developing solvent for TLC analysis was chloroform-methanol-water (65:25:4, v/v), and spots were detected by I₂.

R = DOP Phosphatidylcholine (DOPC)

R = H Panthenol

R = DOP Phosphatidyl-panthenol

Fig. 3. Structure of dioleoyl-phosphatidylcholine, panthenol and synthesized phosphatidylpanthenol.

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