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 B_5 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 μmol of PC, 1.6 U of PLD, 500 μmol of panthenol, 1.6 mL/1.6 mL of ethyl acetate/0.2 M acetate buffer (pH 5.6) ratio, 10 mM of $CaCl_2$ 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-1β, 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-1β 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 B_5) 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 cytotoxity 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 μmol) and 500 μmol 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, ^{1}H 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 $CDCl_3$:CD_3OD (3:1, v/v).

2.4 The yield of synthesized P-panthenol

121 The synthesized yield of P-panthenol was analyzed by a high-performance liquid
122 chromatography (HPLC) system equipped with a mobile phase delivery pump (L-7100,
123 Hitachi, Tokyo, Japan), diode array detector (L-7455, Hitachi) and Mightysil Si 60
124 column (250 x 4.6 mm (5 μm), Kanto Chemical Co., Inc., Tokyo, Japan). The lipid
125 fraction separated from the reaction mixture was analyzed by HPLC. Mobile phase was
126 solvent A (acetonitrile/sulfuric acid (100:0.5, v/v)) and solvent B (methanol) (98:2, v/v)
127 at 1.0 mL/min at 30°C. The P-panthenol was detected at 210 nm. The yield of P-
128 panthenol was calculated from the HPLC peak area using a calibration curve by purified
129 P-panthenol standard.
130
131 *2.5 Anti-inflammatory activities of P-panthenol*
132 *2.5.1. Cell culture*
133 Murine macrophage-like RAW264.7 cells (5×10^4 cells/well) were pre-incubated in
134 24-well plate with 1 mL RPMI 1640 supplemented with 10% fetal bovine serum, 100
135 μg/mL streptomycin and 100 U/mL penicillin, in 5% CO_2 at 37°C for 24 h. P-panthenol,
136 panthenol, and SoyPC were added into the culture medium, respectively, as an ethanol
137 solution. Final ethanol concentration was adjusted to 0.1% without cytotoxicity. After
138 incubation with each sample for 24 h, inflammation was induced by addition of
139 lipopolysaccharide (LPS, final concentration of 0.1 μg/mL) for 6-24 h in the presence of
140 P-panthenol, panthenol or SoyPC.
141
142 *2.5.2. Quantitative real-time RT-PCR*
143 The RAW264.7 cells were washed with phosphate-buffered saline three times after
144 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

168 Phosphatidylation of panthenol was carried out using SoyPC or DOPC as substrate
169 *via* PLD-mediated transphosphatidylation (Fig. 1). A new product was detected upper
170 side of PC on TLC plate after a 24 h reaction (Fig. 2). The negative HR-MS data of the
171 new product from DOPC and panthenol by PLD was m/z 886.62025 and coincided with
172 the predicted molecular formula ($C_{48}H_{89}N_1O_{11}P_1$) of P-panthenol. In both ^{1}H and ^{13}C
173 NMR analyses, panthenol signals were observed instead of the disappearance of choline
174 signals in the data of the synthesized compound (Table 1 and 2). The signal at δ_C 63.6
175 ppm and methylene signal at δ_H 3.91 ppm at p1 position were shifted to down-field
176 compared to δ_C 58.9 ppm and δ_H 3.63 ppm at p1 position of free panthenol (Fig.3, Table
177 1 and 2). On the other hand, other signals in panthenol moiety of the synthetic
178 compound showed similar δ_C and δ_H values of free panthenol. On the basis of HR-MS
179 and NMR analyses, the synthesized compound was identified as P-panthenol connected
180 with OH group at p1 position of panthenol moiety via phosphodiester linkage as shown
181 in Fig. 3.
182 It is well known that PLD recognizes hydroxy group with preference order at
183 primary, secondary, and tertiary alcohol (Ulbrich-Hofmann et al., 2005). Further, we
184 have reported that alcohols having bulky structure such as aromatic group near hydroxy
185 group is poor substrate for transphosphatidylation using PLD even though they are
186 primary alcohol group (Yamamoto et al., 2011). From these evidences, it was suggested
187 that secondary hydroxy group at p5 position and primary hydroxy group at p7 position
188 which has bulky structure near its position, respectively, were poorly recognized by
189 PLD.
190
191 *3.2. Optimization of transphosphatidylation with SoyPC and panthenol*

192 Using SoyPC (50 μmol) as a substrate, optimization of the transphosphatidylation
193 reaction was carried out with fixing the reaction solvent/buffer ratio (1.6 mL/0.8 mL),
194 the concentration of $CaCl_2$ (10 mM), the amount of PLD (1.6 U), reaction temperature
195 (37^{o}C), and reaction time (24 h). At first, the effect of the amount of panthenol (250-
196 5000 μmol) on the P-panthenol synthesis was examined (Fig. 4A). The yield increased
197 with the amount of panthenol and decreased more than 500 μmol of panthenol. It has
198 been reported that too much amount of substrate alcohol inhibits the PLD-mediated
199 transphosphatidylation (Yamamoto et al., 2008b; Yamamoto et al., 2011). The optimum
200 amount of panthenol was defined 500 μmol for our reaction system.
201 The effect of the reaction solvent/buffer ratio (v/v) was examined (Fig. 4B).
202 Reaction yield increased by changing the ratio from 1.6 mL/0.8 mL to 1.6 mL/1.6 mL.
203 However, the yield was almost the same at the ratio of solvent 3.2 mL and buffer 1.6
204 mL. Because the activity of transphosphatidylation by PLD is influenced with the
205 environment of water-solvent interphase such as particle size and interphase pressure
206 (Hirche and Ulbrich-Hofmann, 1999), the ratio of solvent and buffer will make adequate
207 interphase for the transphosphatidylation reaction in this study. From these results, the
208 solvent/buffer ratio of 1.6 mL/1.6 mL was defined as the optimum ratio.
209 Concentration of $CaCl_2$ was also affected the reaction yield of P-panthenol (Fig.
210 4C). By adding 10 mM of $CaCl_2$ in the buffer, reaction yield increased compare to the
211 reaction system without $CaCl_2$, although PLD form *Streptomyces* sp. doesn't require
212 Ca^{2+} for the activity (Juneja et al., 1987). The similar result was observed in our
213 previously study of the phosphatidyl-glycerol (PG) synthesis *via* PLD (*Streptomyces*
214 sp.)-mediated transphosphatidylation (Suzuri et al., 2009; Chen et al., 2020), although
215 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-1β, TNF-α and COX-2 in activated RAW264.7 cells. In addition, P-panthenol suppressed secretion of IL-6 and IL-1β 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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Figure legends

Fig. 1. Synthesis of phosphatisyl-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-1β mRNA, (C): TNF-α mRNA, (D): COX-2 mRNA. * $P < 0.01$ vs. LPS (+), # $P<0.01$ vs. Panthenol.

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

Position	Compounds		
	Panthenol	DOPC	Synthesized compound
g1		4.39 dd (12, 2.5)	4.38 dd (12, 2.5)
		4.12 dd (12, 7)	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

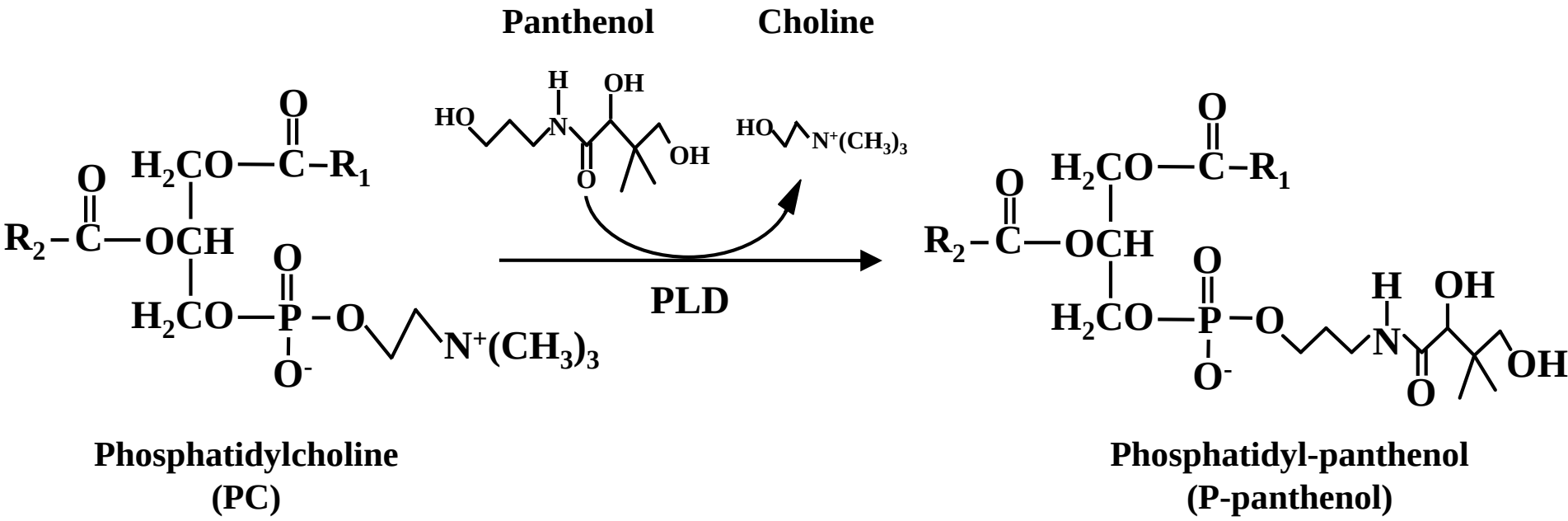
Table 2. ^{13}C NMR chemical shifts of dioleoyl-phosphatidylcholine (DOPC), panthenol, and synthesized compound

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

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

	IL-6 (pg/mL)	IL-1β (pg/mL)
LPS (0.1 μg/mL)	34.43 ± 0.43	10.38 ± 1.76
LPS + P-panthenol (25 μM)	24.41 ± 2.38*	8.22 ± 0.94
LPS + P-panthenol (50 μM)	26.85 ± 0.91*	6.63 ± 0.76*
LPS + P-panthenol (100 μM)	22.26 ± 0.36*	3.96 ± 0.33*

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



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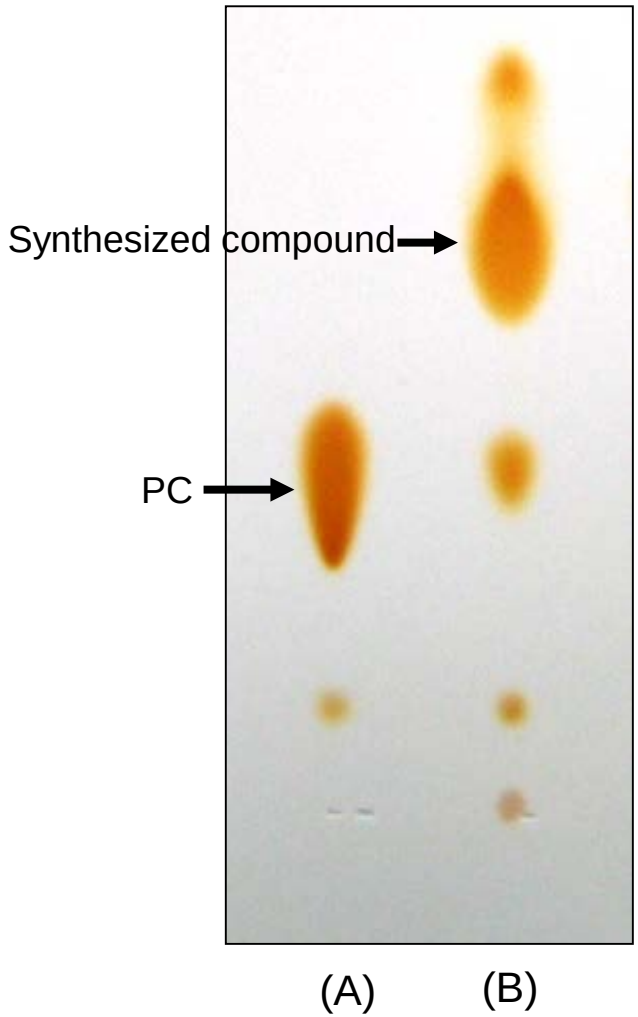

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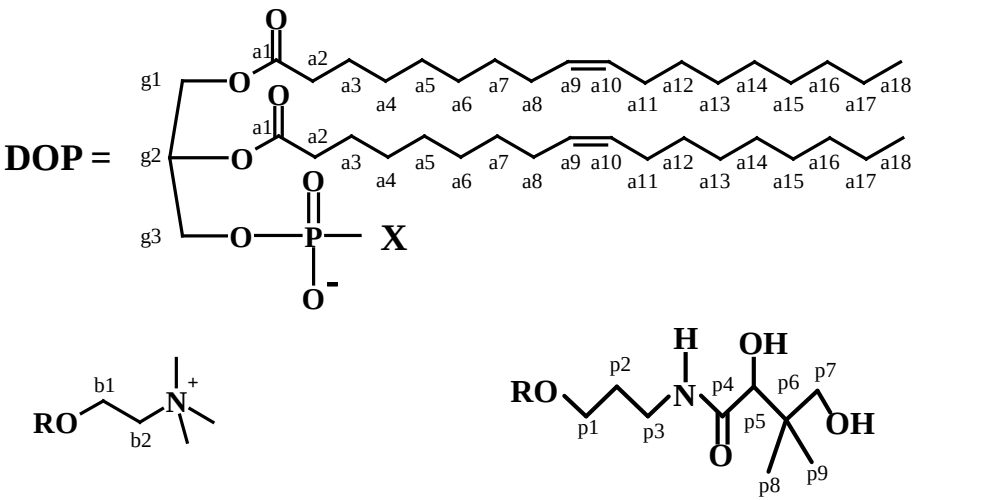

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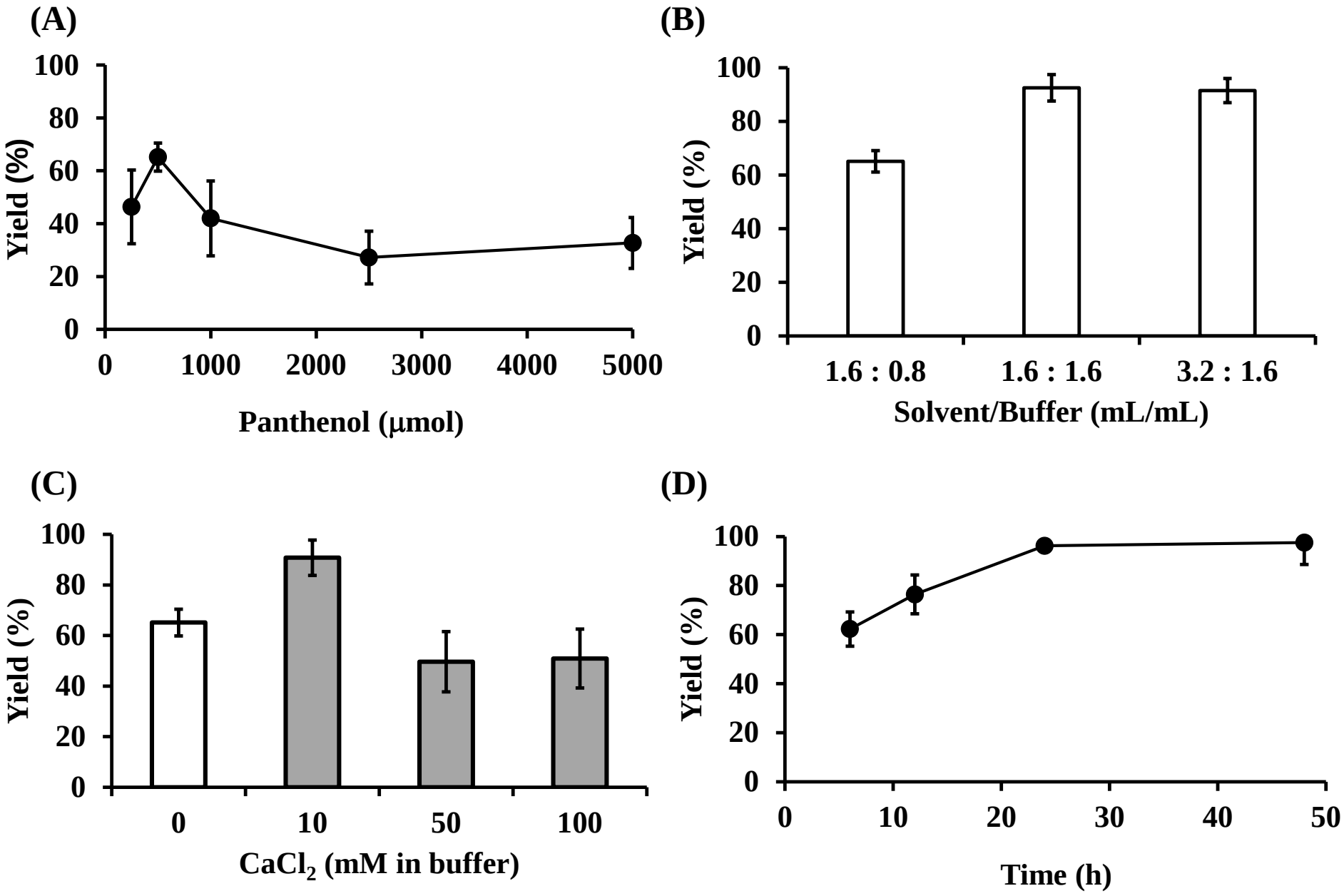

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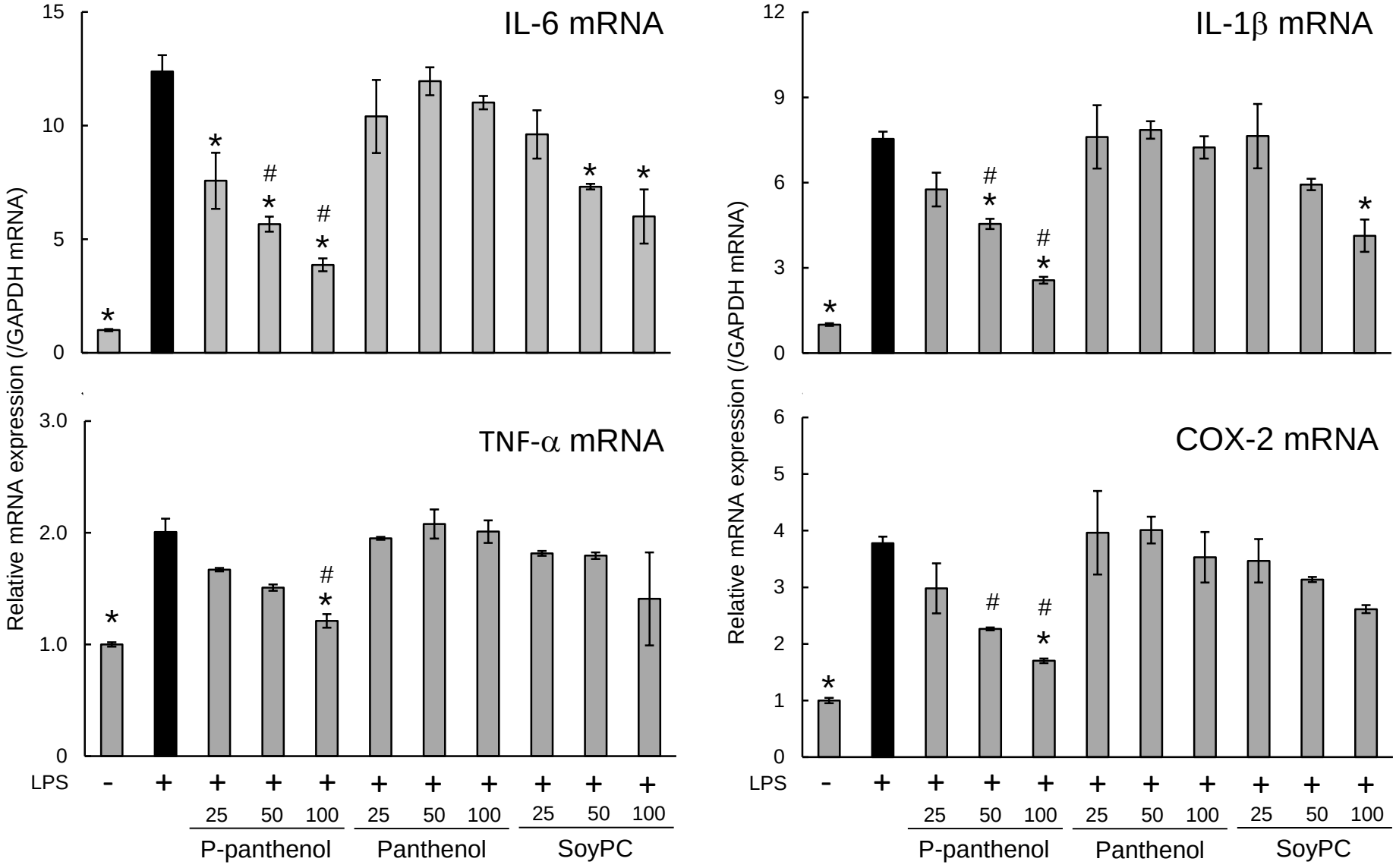

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