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Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E_2 production in lipopolysaccharide-induced mouse macrophage cells

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Abstract—A series of natural stilbenoids, pinosylvin and its derivatives, were synthesized and evaluated for the inhibitory activity of prostaglandin E_2 production in lipopolysaccharide-induced RAW 264.7 cells. Potential inhibitors, including 3,5-dimethoxy-*trans*-stilbene and 3-hydroxy-5-benzyloxy-*trans*-stilbene, have been newly identified, and thus providing chemical leads for the further development of anti-inflammatory or cancer chemopreventive agents.

Naturally occurring stilbenoids have been reported to possess various biological and pharmacological activities such as antioxidant, anti-inflammatory, and anticarcinogenic effects.[1–4] In our continuous efforts to identify and develop the pharmacologically active stilbenoids from natural products or synthetic approaches, we have focused on a natural stilbenoid pinosylvin (3,5-dihydroxy-*trans*-stilbene) that possesses structural similarity with biologically active resveratrol. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), one of the representatives of natural stilbenoid and a major principle of French Paradox, abundant in grapes, peanuts, and pines, has demonstrated a variety of biological activities such as reduction in the incidence of coronary heart disease, chemoprevention of cancer, growth inhibition of many cancer cells, and anti-inflammatory effects.[5–8] One of the probable mechanisms for anti-inflammatory and cancer chemopreventive activity has been suggested with the inhibition of cyclooxygenase (COX) activity.[9,10]

COX is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs) and thromboxane A_2 (TXA_2) from arachidonic acid.[11,12] Two isozymes, designated as COX-1 and COX-2, have been identified: COX-1 is constitutively expressed in most of tissues, whereas COX-2 is negligible in normal conditions, but can be dramatically induced by pro-inflammatory cytokines, tumor promoters, growth factors, or oncogens.[13–15] Therefore, the overproduction of PGs mediated by COX-2 involves in the many pathophysiological processes including inflammation and carcinogenesis. On this line, reducing the levels of COX-2-mediated PG productions will be an effective strategy for inhibiting inflammation and carcinogenesis. Recently, we reported the styrylheterocyclic compounds might be new lead candidates for modulating COX-2 activity.[16] In this study, we further extended to envisage the procurement of natural stilbenoids with pinosylvin derivatives anticipating potential anti-inflammatory and cancer chemopreventive activity.

Pinosylvin (3,5-dihydroxy-*trans*-stilbene), a natural stilbenoid found in heartwood and leaves of pine, is a phytoalexin with antioxidant and antifungal activities, and inhibitory effects of tyrosinase, but it is relatively not much studied for the biological activity yet.[17–19]

Since the substitution patterns of the *trans*-stilbene template have been shown to be sensitive for various biological activities, we primarily investigated the substitutions of dihydroxy group in pinosylvin with several lipophilic derivatives. We report here the inhibition of COX-2-mediated PGE_2 production of synthesized pinosylvin

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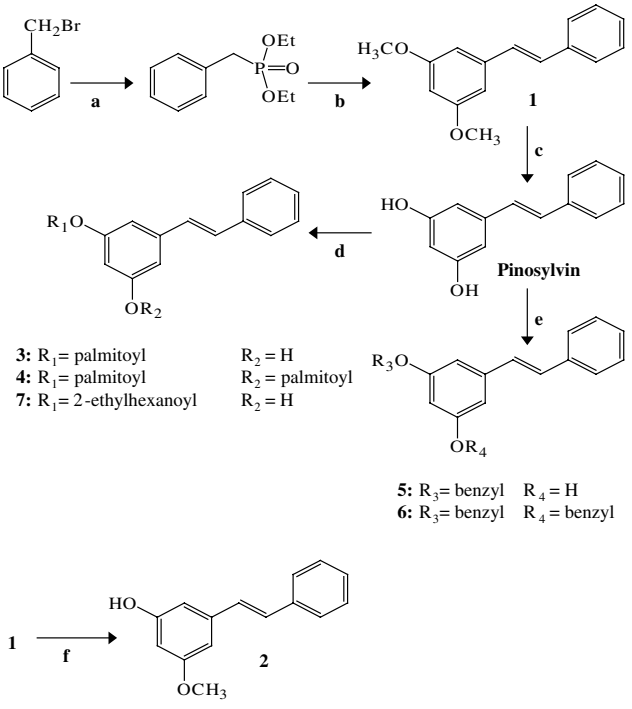
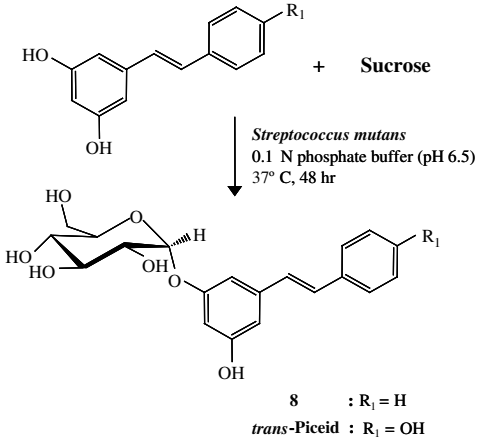
derivatives and our discovery of 3,5-dimethoxy-*trans*-stilbene and 3-hydroxy-5-benzyloxy-*trans*-stilbene as new lead compounds.

Compounds were prepared by following synthetic pathway and enzymatic biotransformation (Schemes 1 and 2). The synthesis of pinosylvin and 3-*O*-α-D-glucopyranosyl-(*E*)-pinosylvin were previously reported.[20–22] Briefly, Horner–Wadsworth–Emmons reactions between phosphonates and aromatic aldehydes were performed to yield 3,5-dimethoxy-*trans*-stilbene followed by producing pinosylvin in the catalytic condition of pyridine hydrochloride at 190 °C. After then, we prepared 3-(2-ethylhexanoyl)-, 3-palmitoyl-, and 3,5-dipalmitoyl-*trans*-stilbene from pinosylvin, corresponding acyl chloride, and pyridine in methylene chloride at room temperature.[20] In an attempt to obtain monobenzylate compound, the reaction with the corresponding stilbene and benzylbromide gave a 50% yield of the 3-benzyloxy-*trans*-stilbene. Further reaction for obtaining the second benzyl group gave 98% yield of 3,5-dibenzyloxy-*trans*-stilbene. In addition, 3-monomethoxy-*trans*-stilbene was synthesized by the reaction between 3,5-dimethoxy-*trans*-stilbene and boron tribromide (Scheme 1).[21] *trans*-Piceid and 3-*O*-α-D-glucopyranosyl-(*E*)-pinosylvin were synthesized by the biological reaction of *Streptococcus mutans* (Scheme 2).[22,23]



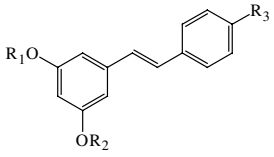
Scheme 1. Solution phase synthetic approach employed for the preparation of pinosylvin and its derivatives described in Table 1. Reagents and conditions: (a) triethyl phosphite, 130 °C; (b) dimethyl formamide, sodium methoxide, 0 °C, followed by 3,5-dimethoxy benzaldehyde, rt; (c) pyridine hydrochloride, 190 °C; (d) RCOCl, pyridine, methylene chloride, rt; (e) benzylbromide, potassium carbonate, acetone; (f) boron tribromide, −80 °C. Chemical yields of pinosylvin (50%); **1** (50%); **2** (40%); **3** (44%); **4** (28%); **5** (50%); **6** (98%); **7** (91%).



Scheme 2. Synthesis of glucosides of pinosylvin analogues by biotransformation. Chemical yields of *trans*-piceid (18%), **8** (11%).

Primarily, pinosylvin was prepared and evaluated for the inhibitory activity of PGE_2 production in LPS-induced mouse macrophage RAW 264.7 cells by PGE_2 enzyme immunometric assay (EIA).[24] In this assay, activity is defined as the difference between PGE_2 accumulation in the absence and in the presence of LPS for 20 h. Treatment of RAW 264.7 cells with LPS (1 μg/mL) increased dramatically the production of PGE_2 from endogeneous arachidonic acid up to 23 ng/mL from the basal level of 0.6 ng/mL without LPS. Pinosylvin was found to inhibit the COX-2-mediated PGE_2 production, and the potency (IC_{50} = 10.6 μM) was approximately twofold higher than that of resveratrol (IC_{50} = 20.8 μM). Based on the potency of pinosylvin, pinosylvin derivatives were prepared and evaluated. As shown in Table 1, the effects of substitution were revealed to be highly dependent on the position and substituents. For example, compound **1**, introduction of methoxy groups both 3- and 5-position, dramatically

Table 1. Inhibitory effects of pinosylvin analogues on the PGE_2 production in LPS-stimulated RAW264.7 cells



Compounds	R_1	R_2	R_3	IC_{50} (μM)[a]
Resveratrol	H	H	OH	20.8
Pinosylvin	H	H	H	10.6
trans-Piceid	Glycosyl	H	OH	13.5
1	Methyl	Methyl	H	0.1
2	H	Methyl	H	10.5
3	Palmitoyl	H	H	>50
4	Palmitoyl	Palmitoyl	H	>50
5	Benzyl	H	H	2.0
6	Benzyl	Benzyl	H	>50
7	2-Ethylhexanoyl	H	H	1.7
8	Glycosyl	H	H	12.1

[a] The IC_{50} values were determined by triplicate tests.

increased the inhibitory activity with 100 times more potent than that of pinosylvin and exhibited the most potent activity among test compounds ($IC_{50} = 0.1\,\mu M$). However, substitution with only one methoxy group **2** showed the similar activity with pinosylvin. In order to further elucidate the substitution effects on hydroxy group the introduction of various aliphatic chains or benzyl groups were employed. One benzyl-substituent **5** enhanced the activity with fivefold ($IC_{50} = 2.0\,\mu M$) compared to pinosylvin, but dibenzyl-substituent **6** did not show the inhibitory activity ($IC_{50} > 50.0\,\mu M$), indicating that the introduction of bulky groups might hinder the penetration of the compound into cells. In the introduction of aliphatic chains the short chain of aliphatic groups exampled by 2-ethylhexyl group **7** increased the activity ($IC_{50} = 1.7\,\mu M$) with the similar potency of compound **6**, whereas the introduction of long chain aliphatic group **3** and **4** did not show the inhibitory activity ($IC_{50} > 50.0\,\mu M$). In another variation, the introduction of glucose moiety on the hydroxy group **8** was found to be similar activity ($IC_{50} = 12.1\,\mu M$) with pinosylvin and the inhibitory potential was also relevant to *trans*-piceid ($IC_{50} = 13.5\,\mu M$), which is a glycoside form of resveratrol.

To clarify the possible underlying molecular mechanisms of pinosylvin derivatives on PGE_2 production in LPS-induced cells, further studies were performed by examining COX-2 gene expression, using reverse transcription-polymerase chain reaction (RT-PCR) analysis.[25] As a result, compound **1** and **7** demonstrated the suppressive effects of COX-2 mRNA expression (Fig. 1), indicating that the inhibitory effects of PGE_2 production by pinosylvin derivatives are possibly in part related to the suppression of COX-2 gene expression.

In conclusion, a series of pinosylvin derivatives has been prepared and evaluated as to their effects on the activity of COX with the goal of identifying a potent inhibitor. We found for the first time that pinosylvin is also one of active natural stilbenoids exhibiting an appreciable inhibitory activity against the overproduction of the inflammatory mediator PGE_2, and could serve as a new lead for further chemical optimization. We have also gained an insight into the preliminary structure–activity relationships of pinosylvin derivatives, which is valuable in the design and development of a new class of COX inhibitors. Further studies for more potent inhibitors, based on the above findings, are in progress in our laboratory.

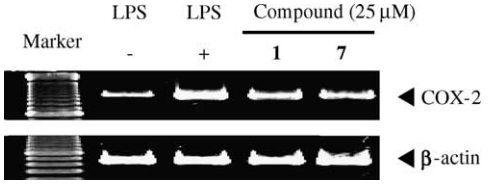


Figure 1. Effects of compound **1** and **7** on the expression of COX-2 mRNA in LPS-stimulated RAW 264.7 cells using RT-PCR analysis.

Acknowledgements

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24. Production of PGE_2 by LPS-induced COX-2 in RAW 264.7 cells: RAW 264.7 mouse macrophage cells applied to this study were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). PGE_2 production was measured by an enzyme-immunometric assay (EIA), as

described previously. Briefly, the cells (5×10^5/mL) were incubated in 96-well culture plate for 24 h. Then, the cells were washed twice with phosphate buffered saline (PBS), and changed in the fresh medium with test compounds and 1 μg/mL of LPS. After additional 20 h incubation, the PGE_2 in the media were analyzed by the PGE_2 enzyme immunometric assay (Cayman Co., Ann Arbor, MI, USA). The percentage of inhibition was calculated as $[1 - (PGE_2$ level of compound/PGE_2 level of vehicle treated-control)] × 100.

25. Effects of some compounds on COX-2 mRNA expression in RAW 264.7 cells by reverse transcription-polymerase chain reaction (RT-PCR) analysis: RAW 264.7 cells (5×10^5/mL) were grown in 6 cm^2 dishes for 24 h. Then, compound **1**, or **7** was treated with 1 μg/mL of LPS for 6 h. After washing with PBS, total RNA was isolated from the cells. Briefly, cells were lysed by Tri-reagent and separated into three phases by chloroform. The aqueous phase was transferred to a fresh tube, and added the same amount of isopropanol to precipitate RNA pellet. RNA pellet was washed with 75% ethanol and dissolved in nuclease free water. The concentration of RNA was determined by absorbance at 260 nm. RNA (1 μg) was performed reverse transcription (RT) using avian myeloblastosis virus (AMV) reverse transcriptase. The cDNA products of RT were amplified by PCR using Taq DNA polymerase. The sense and antisense primers for COX-2 were 5′-GGAGAGACTATCA-AGATAGTGATC-3′ and 5′-ATGGTCAGTAGAC-TTTTACAGCTA-3′, respectively. The sense and antisense primers for β-actin were 5′-TGTGATGG-TGGGAATGGGTCAG-3′ and 5′-TTTGATGTCA-CGCACGATTTCC-3′, respectively. The PCR products were run on a 2% agarose gel and visualized by SYBR Gold staining.