Synthesis and Anti-inflammatory Activity of Phenylbutenoid Dimer Analogs

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Several phenylbutenoid dimer (PBD) analogs were synthesized and evaluated for their inhibitory activities against nitric oxide (NO) production and TNF-α release. The PBD analogs were synthesized via Diels–Alder and subsequent Schlosser reactions as key steps. Among the tested compounds, two analogs (**8c**, **8f**) exhibited much stronger inhibitory activity against LPS-stimulated NO production and TNF-α release in RAW 264.7 cells than that of wogonin.

Keywords: Phenylbutenoid dimers, Nitric oxide, TNF-α, Anti-inflammatory activity, Diels–Alder reaction

Introduction

Novel phenylbutenoid dimers (PBDs), (±)-*trans*-3-(4-hydroxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (**1**) and (±)-*trans*-3-(3,4-dimethoxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (**2**), were isolated from *Zingiber cassumunar* Roxb., the tropical ginger widely distributed in Southeast Asia.[1] The extracted oil of this herb, known as Plai oil in Thailand, is used as a massage oil by massage therapists. Naturally occurring PBDs (**1** and **2**, Figure 1) were reported to possess anti-inflammatory and cytotoxic activities.[1–3] The compounds were reported to possess strong inhibitory activity against LPS-induced PGE_2 production in RAW 264.7 cells,[1] which were comparable to that of wogonin (5,7-dihydroxy-8-methoxyflavone).[4] In this respect, phenylbutenoid dimer is considered as a potential target molecule by medicinal chemists who aimed to develop anti-inflammatory agents. The synthesis of optically active natural PBDs via Diels–Alder reaction with oxazolidine chiral auxiliaries has been reported.[5a,b] However, the effects of PBDs on other inflammatory mediators (NO, TNF-α, etc.) have not yet been studied. Therefore, we synthesized a series of PBD analogs and evaluated for their inhibitory activities against nitric oxide (NO) production and TNF-α release. RAW 264.7 cells were used as representative immune cells.

Structural alteration (deletion, addition, substitution) of functional group(s) in two benzene rings of natural PBD products (**1** and **2**) was conducted. Several PBD analogs (**8b**, **8j–m**) were designed to investigate the influence of physicochemical parameters of substituents in the benzene ring A of natural PBD products. Synthesized PBD analogs are displayed in Figure 2.

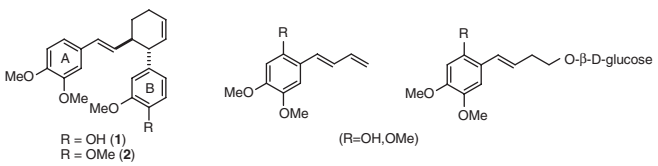

Figure 1. Structures of phenylbutenoid dimers and other phenylbutenoids isolated from *Z. cassumunar*.

Experimental

Synthesis (General). All chemicals were obtained from commercial suppliers and used without further purification. All solvents used for the reactions were freshly distilled from the appropriate dehydrating agent under nitrogen gas. All solvents used for chromatography were purchased and directly used without further purification. ^{1}H NMR spectra were recorded on a Varian Gemini 3000 instrument (300 MHz; Palo Alto, CA, USA) and a Bruker DPX 400 (400 MHz) spectrometer (Billerica, MA, USA). Chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane as an internal standard. Peak splitting patterns are abbreviated as m (multiplet), s (singlet), bs (broad singlet), d (doublet), bd (broad doublet), t (triplet), dd (doublet of doublets), and ddd (doublet of double doublet). ^{13}C NMR spectra were recorded on a Bruker DPX 400 (100 MHz) spectrometer, and fully decoupled and chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane as an internal standard. Mass spectra were recorded on a Voyager DE STR MALDI-TOF MS (Voyager DE STR, Applied BioSystems, USA). Analytical thin-layer chromatography (TLC) was performed using commercial glass plate with silica gel 60 F_{254} purchased from Merck (Darmstadt, Germany). Chromatographic purification was carried out by flash chromatography using Kieselgel 60 (230–400 mesh, Merck).

Typical Procedures for the Preparation of Compounds 3A–G. To a solution of 4-hydroxy-3-methoxybenzaldehyde (1 g, 6.6 mmol) in tetrahydrofuran (5 mL), allyl bromide

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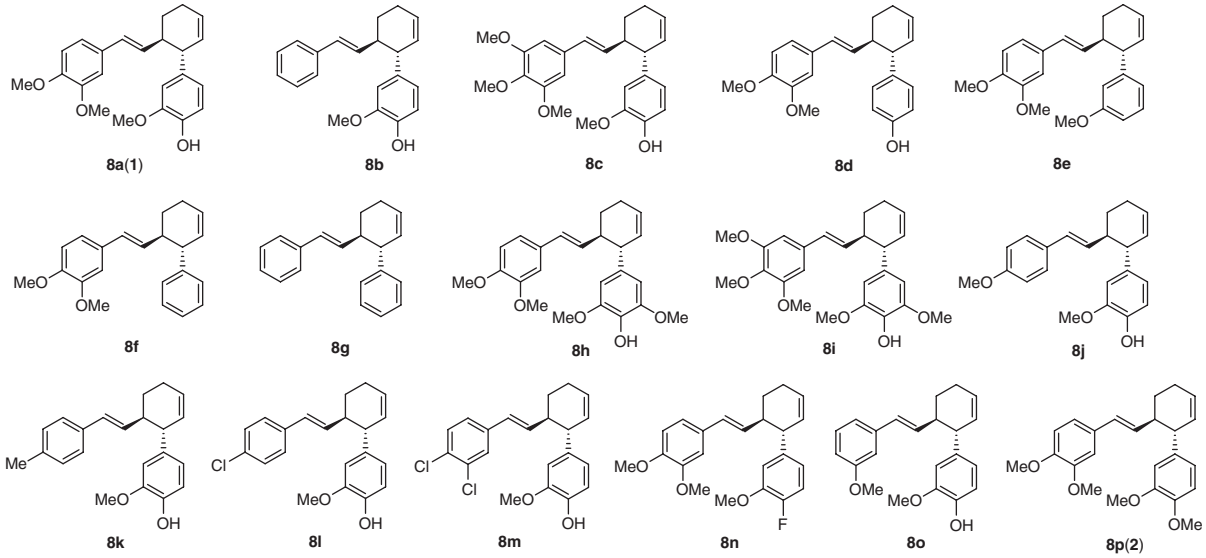

Figure 2. Structures of synthesized PBD analogs.

(1.7 mL, 20 mmol) and saturated aqueous NH_4Cl solution (25 mL) were added. To the reaction mixture was slowly added zinc power (2.56 g, 40 mmol) and the reaction mixture was stirred at room temperature for 30 min. After filtration on celite pad, the filtrate was extracted with ethyl acetate (EA) and the organic layer was washed with 3% HCl aqueous and brine and dried with $MgSO_4$. Evaporation to dryness yielded 1 g of crude compound **3A** as a white solid with 92% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.75–6.89 (m, 2H), 6.64–6.67 (d, J = 8.4 Hz, 1H), 6.07 (br, 1H), 5.71–5.82 (m, 1H), 5.25 (s, 1H), 5.06–5.14 (m, 1H), 4.93–5.02 (m, 1H), 4.02–4.65 (m, 1H), 3.81 (s, 3H), 2.25–2.68 (m, 2H). Other compounds (**3B–G**) were prepared following the same reaction conditions from the corresponding aldehydes, respectively. Analytical data of other compounds are available as Supporting Information.

Typical Procedures for the Preparation of Compounds 4A–G. To a solution of compound **3A** (1 g, 5.2 mmol) in toluene was added *p*-toluenesulfonic acid (8 mg) and the reaction mixture was refluxed for 1.5 h. The reaction mixture was diluted with CH_2Cl_2, washed with brine, dried with $MgSO_4$, and evaporated to dryness. Purification with column chromatography (hexane:EA = 10:1) yielded 0.66 g compound **4A** as a light yellow oil in 73 % yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.84–6.92 (m, 3H), 6.60–6.69 (dd, J = 9.8 Hz, 1.0 Hz, 1H), 6.41–6.54 (m, 2H), 5.79 (s, 1H), 5.25–5.30 (d, J = 17.3 Hz, 1H), 5.09–5.13 (d, J = 9.8 Hz, 1H), 3.88 (s, 3H). Other compounds (**4B–G**) were prepared following the same reaction conditions from the corresponding alcohols (**3B–G**), respectively.

Typical Procedures for the Preparation of Intermediates 5A–G. To a solution of **4A** (0.87 g, 4.9 mmol) in CH_2Cl_2 (7 mL) was slowly added $BF_3{\cdot}OEt_2$ (0.63 mL, 4.9 mmol in CH_2Cl_2) and acrolein (0.7 mL, 10.4 mmol) in CH_2Cl_2 (3 mL) at −78 °C. The reaction mixture was stirred for 1 h, then warmed to 0 °C, quenched with 1 N aqueous HCl solution (2 mL), and extracted with CH_2Cl_2 (30 mL). The organic layer was washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL), dried over $MgSO_4$, and evaporated to dryness. Purification of the residue by column chromatography (hexane–acetone, 4:1) gave **5A** (0.16 g, 60%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.50 (s, 1H), 6.85–6.83 (d, J = 8.1 Hz, 1H), 6.71–6.74 (dd, J = 8.3 Hz, 1.9 Hz, 1H), 6.68–6.69 (d, J = 1.9 Hz, 1H), 5.95–6.00 (m, 1H), 5.78–5.83 (m, 1H), 5.54 (s, 1H), 3.89–3.92 (ddd, J = 5.7 Hz, 3.7 Hz, 2.1 Hz, 1H), 3.86 (s, 3H), 2.70–2.76 (dd, J = 5.9 Hz, 2.0 Hz, 1H), 2.24–2.33 (m, 1H), 2.09–2.20 (m, 1H), 1.83–1.88 (m, 2H). Other intermediates (**5B–G**) were prepared following the same reaction conditions from the corresponding dienes (**4B–G**), respectively. Analytical data of other compounds are available as Supporting Information.

Typical Procedures for the Preparation of Compounds 6A′–G′ and 7A′–H′. To a solution of 3,4-dimethoxybenzaldehyde (1 g, 6 mmol) in methanol was added $NaBH_4$ (0.27, 7.2 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 4 h, quenched with 3% aqueous HCl solution, and evaporated to dryness. The residue was extracted with CH_2Cl_2 and the organic layer was dried with $MgSO_4$. Evaporation of the dried organic layer yielded 0.96 g (3,4-dimethoxyphenyl)methanol (**6D′**) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.90–7.21 (d, J = 2.0 Hz, 1H), 6.85–6.88 (d, J = 8.2 Hz, 1H), 6.65–6.77 (dd, J = 8.2 Hz, 1.4 Hz, 1H), 4.63–4.66 (d, J = 5.0 Hz, 2H), 3.84 (s, 6H). Other compounds (**6A′–C′** and **6E′–H′**) were prepared following same reaction conditions from the corresponding aldehydes, respectively.

The solution of the crude **6D′** and $Ph_3P{\cdot}HBr$ (2.16 g, 6.3 mmol) in CH_3CN was refluxed overnight. Evaporation of the reaction mixture yielded 2.25 g of crude compound **7D′** as a white solid in 76% yield (2 steps) and used for the next reaction without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62–7.79 (m, 15H), 6.98–7.22 (m, 3H), 5.25 (s, 1H), 5.20 (s, 1H), 3.55 (s, 3H), 3.49 (s, 3H). Other

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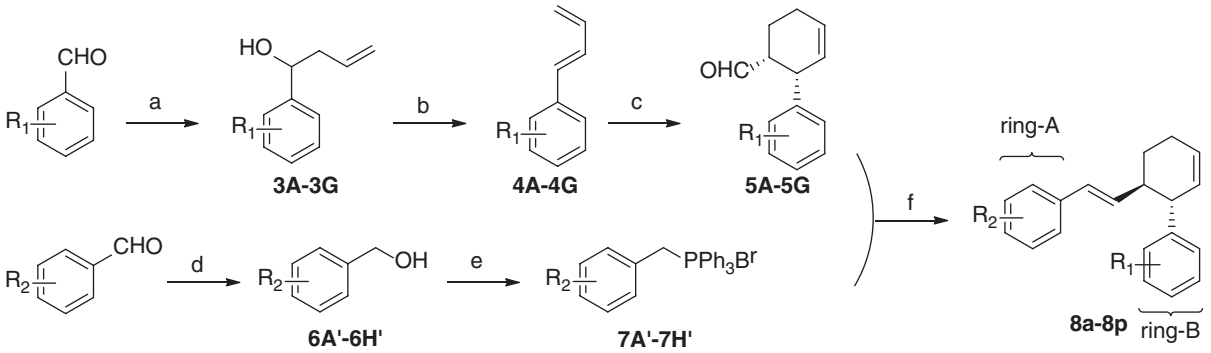
intermediates (**7A′–C′** and **7E′–H′**) were prepared following the same reaction conditions from the corresponding alcohols, respectively. Analytical data of other compounds are available as Supporting Information.

Typical Procedures for the Preparation of PBDs 8a–p. To a solution of compound **7D′** (0.59 g, 1.2 mmol) in toluene (10 mL) was slowly added *n*-BuLi (0.48 mL, 2.5 M in hexane) at −20 °C. The solution was stirred for 30 min at this temperature, then the compound **4A** (0.23 g, 1.0 mmol) in toluene (5 mL) was slowly added. The reaction mixture was heated to reflux and stirred for 3 h, quenched with saturated aqueous NH_4Cl (5 mL), extracted with ethyl acetate (2 × 50 mL), washed with saturated $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$, filtered and evaporated. The residue was purified by column chromatography (hexane:Acetone, 5:1) to afford compound **8a** (**1**, 0.14 g, 41%) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.75–6.84 (m, 4H), 6.66–6.71 (m, 2H), 6.10 (d, *J* = 16 Hz, 1H), 6.03 (dd, *J* = 16 Hz, 6.8 Hz, 1H), 5.90 (m, 1H), 5.67 (m, 1H), 3.81–3.88 (s, 3H × 3), 3.16 (m, 1H), 2.24 (m, 1H), 2.21 (m, 2H), 1.92 (m, 1H), 1.69 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.4, 148.7, 146.6, 144.3, 137.3, 132.6, 131.4, 130.7, 129.3, 127.9, 121.6, 119.2, 114.3, 111.6, 111.4, 109.2, 56.3, 56.3, 56.2, 48.5, 45.8, 28.2, 24.9. MS for $C_{23}H_{26}NaO_4$ (MALDI-TOF): $[M + Na]^+$ 389.19. Other compounds (**8b–8p**) were prepared following the same reaction conditions from the corresponding intermediates, respectively. Analytical data of other compounds (**8b–8p**) are available as Supporting Information.

Results and Discussion

Synthesis. PBD analogs (**8a–p**) were synthesized following a concise synthetic route outlined in Scheme 1. Zinc-mediated allylation of aldehydes yielded the corresponding alcohols **3A–G** (90–95%). Dehydration of the alcohols in the presence of *p*-toluenesulfonic acid gave the corresponding butadienes **4A–G** (46–75%). Cyclohexenecarbaldehydes (**5A–G**), key intermediates for Diels–Alder reaction, were synthesized from the butadienes **4A–G**, acrolein, and catalytic amount of BF_3-Et_2O.[6–9] The stereochemistry of two chiral centers in cyclohexene ring of **5A–G** was identified as *cis* based on the chemical shift values of two protons on chiral carbons (H-3, 2.73 ppm; H-4, 3.90 ppm) by comparing the chemical shift data in ^{1}H NMR spectra from precedent literatures.[5a,b] We found that *cis* cyclohexene intermediates possessed different chemical shift values of two protons on chiral carbons (H-3, 2.73 ppm/H-4, 3.92 ppm[5a] and H-3, 2.74 ppm/H-4, 3.91 ppm[5b]) compared with those of the *trans* cyclohexene intermediate (H-3, 2.58 ppm; H-4, 3.71 ppm).[5b] Ylides (**7A′–H′**) were prepared from the corresponding aldehydes in two steps. Reduction of aldehydes with sodium borohydride in methanol (93–100%) followed by reactions with Ph_3P·HBr and *n*-BuLi in toluene (70–94%) gave ylides (**7A′–H′**). Reactions in Schlosser conditions[10] between aldehydes **5A–G** and ylides **7A′–H′** yielded PBD analogs **8a–p** (40–55%) with *E*-stereochemistry. The stereochemistry of the double bond was decided as "*E*" based on the coupling constant (*J* = 16 Hz) of two vinyl protons. The stereochemistry of two chiral centers of synthesized PBD analogs **8a–8p** was identified as *trans* based on the chemical shift values and the large chemical shift difference of two protons on chiral carbons (H-3, 3.16 ppm; H-4, 2.24 ppm). By comparing the chemical shifts in ^{1}H NMR spectra from precedent literatures,[5a,b] we found that *trans* PBD analogs possessed different chemical shift values and relatively large chemical shift difference of two protons on chiral carbons (H-3, 3.18 ppm/H-4, 2.35 ppm[5a] and H-3, 3.16 ppm/H-4, 2.14 ppm[5b]) compared with those of the *cis* PBD analog (H-3, 3.52 ppm/H-4, 2.80 ppm).[5a] Our results imply that the "*cis*" stereochemistry of aldehydes **5A–G** seemed to be changed to *trans* during olefination reaction in strong base (*n*-BuLi). Thus, we successfully synthesized 16 PBD analogs with *E* and *trans* stereochemistry without any further elaboration from *cis* cyclohexene intermediates.

NO and TNF-α Assay. Inhibitions of *i*NOS-mediated NO production and TNF-α release by synthetic PBD analogs were determined in LPS-stimulated RAW 264.7 cells.[11] Briefly,



(**A**=3-OCH_3, 4-OH; **B**=4-OH; **C**=3-OCH_3; **D**=4-H; **E**=3,5-$(OCH_3)_2$, 4-OH; **F**=3-OCH_3, 4-F; **G**=3,5-$(OCH_3)_2$)
(**A'**=4-OCH_3 ; **B'**=4-H; **C'**=3,4,5-$(OCH_3)_3$; **D'**=3,4-$(OCH_3)_2$; **E'**=4-CH_3; **F'**=4-Cl; **G'**=3,4-Cl_2; **H'**=3-OCH_3)

Scheme 1. Synthesis of PBD analogs. Reagents and conditions: (a) allyl bromide, Zn, aqueous NH_4Cl–THF (5:1), 0 °C, 2 h, 90–95%; (b) TsOH, reflux, 2–3 h, 46–75%; (c) acrolein, BF_3-Et_2O, 55–80%; (d) $NaBH_4$, MeOH, 0 °C, 4 h, 93–100%; (e) Ph_3P·HBr, CH_3CN, reflux, overnight, 70–94%; (f) *n*-BuLi, toluene, −20 °C → reflux, 3 h, 40–55%.

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RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and penicillin-streptomycin (Gibco BRL), at 37 °C in 5% CO_2. Cultured cells were pretreated with PBD analogs for 1 h and stimulated with LPS (200 ng/mL) (Sigma, St Louis, MO, USA) for another 24 h. NO production was measured in the culture supernatant using the Griess reaction. TNF-α release was quantified using ELASA assay according to the manufacturer's instructions (R&D Systems). Cell viability was assessed using MTT assay. Data obtained at nontoxic concentrations were analyzed and the results are shown in Table 1 and Figure 3.

In our study, substituent effects on bioactivity of the benzene ring-A were explored. Following batchwise Topliss operational scheme,[12] five PBD analogs with substituents (4-H, 4-Cl, 4-CH_3, 4-OCH_3, and 3,4-Cl_2) possessing different chemo-physical parameters (π and σ) values were initially prepared (**8b**, **8j–8m**) to monitor the relationship between parameters and bioactivity. Analogs **8k** (4-CH_3: +π, −σ) and **8l** (4-Cl: +π, +σ) exhibited equivalent to more potent inhibitory activity of NO production compared with those of wogonin (IC_{50} = 20.80 μM) and two natural PBDs (**8a** and **8p**) as shown in Table 1. Wogonin, a naturally occurring anti-inflammatory flavonoid,[4,13] was used as a reference compound. The potency order of substituents is 4-CH_3 > 4-Cl > 4-OCH_3 > 3,4-Cl_2 and 4-H. Our present results imply that the positive π value contributed to bioactivity. Mostly, alteration on the benzene B-ring resulted in loss of bioactivity (**8d–8i**, **8n**) except the analog **8f** (IC_{50} = 7.41 μM). These results imply that the benzene B-ring plays very important role in bioactivity and, therefore, is worth to be further modified for better bioactivity. The compound with additional 5-OCH_3 group on the benzene ring-A (**8c**) of the natural PBD analog **8a** (**1**) resulted in strong bioactivity (IC_{50} = 7.62 μM).

Table 1. IC_{50} values for inhibition of NO production of synthesized PBD analogs.

Chemicals		IC_{50} (μM)	Chemicals		IC_{50} (μM)
PBDs	**8a**	25.78 ± 3.14	PBDs	**8i**	n.d.
	8b	n.d.		**8j**	60.59 ± 4.34
	8c	7.62 ± 1.23∗		**8k**	14.22 ± 1.22
	8d	n.d.		**8l**	19.79 ± 3.78
	8e	n.d.		**8m**	n.d.
	8f	7.41 ± 0.89∗		**8n**	n.d.
	8g	n.d.		**8o**	n.d.
	8h	n.d.		**8p**	18.06 ± 2.19
Wogonin		20.85 ± 3.35			

Values were expressed as mean ± SD of three replicates. n.d. (not determined): cellular toxicity of chemical compounds prevented the measurement of IC_{50} values.
∗PDB analogs with statistically stronger activity ($p < 0.05$, student t-test) than wogonin.

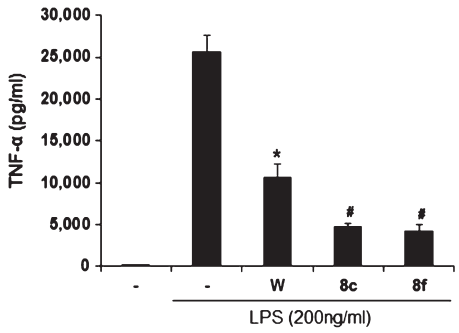

Figure 3. TNF-α release inhibition of **8c**, **8f**, and wogonin stimulated by LPS in RAW 264.7 cells. ∗$p < 0.05$ and #$p < 0.05$ indicate statistically significant differences by student t-test for the treatment with LPS alone and with LPS plus wogonin, respectively.

To further compare the pharmacological profiles of selected PBDs (**8c** and **8f**) and wogonin, TNF-α releases stimulated by LPS in RAW 264.7 cells of these compounds were determined. The values are expressed as mean ± SD for three independent experiments. PBD analogs exhibited much stronger bioactivity compared with that of wogonin in the inhibitory potency of TNF-α release as shown in Figure 3. These results demonstrate that PBD analogs **8c** and **8f** have better anti-inflammatory profiles than wogonin. Further pharmacological research for compounds **8c** and **8f** is currently under way to evaluate the potential therapeutic uses as anti-inflammatory agents.

Conclusion

In summary, an efficient synthetic route for PBD analogs was developed and 16 PBD anaolgs were synthesized. Our synthetic route involves Diels–Alder reaction and a subsequent Schlosser reaction as key steps. Among the 16 PBD analogs, two analogs (**8c**, **8f**) exhibited much stronger inhibitory activity against LPS-stimulated NO production and TNF-α release in RAW 264.7 cells than those of natural PBD products (**8a** and **8p**) and wogonin. Two synthetic PBDs (**8k** and **8l**) exhibited equivalent to more potent inhibitory activity of NO production than that of wogonin. Overall, we discovered two PBD analogs (**8c** and **8f**) with good biological profiles as anti-inflammatory agents.

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Supporting Information. Additional supporting information is available in the online version of this article.

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