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Inhibitory effect of resveratrol dimerized derivatives on nitric oxide production in lipopolysaccharide-induced RAW 264.7 cells

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ABSTRACT

Four types of resveratrol dimerized analogues were synthesized and evaluated in vitro on LPS-induced NO production in RAW 264.7 cells. The results showed that several compounds, especially those containing 1,2-diphenyl-2,3-dihydro-1*H*-indene core (type I), exhibited good inhibitory activities. Among 25 analogues, **12b** showed a significant inhibitory activity (49% NO production at 10 μM, IC_{50} = 3.38 μM). Further study revealed that compound **12b** could suppress LPS-induced iNOS expression, NO production, and IL-1β release in a concentration-dependently manner. The mechanism of action (MOA) involved for its anti-inflammatory responses was through signaling pathways of p38 MAPK and JNK1/2, but not ERK1/2.

Inflammatory reactions take place after injury, infection, or trauma and induce an accumulation of inflammatory immune cells. However, prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases. Inflammatory reactions are complex and multifactorial condition for which a number of mediators have been identified.[1] Among these, the nitric oxide (NO) radical generated by phosphorylated endothelial NO synthase (eNOS) is one of the most important mediators.[2] In addition, this phosphorylation is generated by an inducible enzymes called the inducible NO synthase (iNOS).[3,4] High-output NO by iNOS can provoke deleterious consequences and has been closely correlated with pathophysiology in a variety of diseases and inflammations.[5] Transcriptional induction of iNOS is largely dependent on cooperative activities of multiple transcription factors, including NF-κB and AP-1 which act important cis-elements for induction of iNOS gene transcription.[6] Many stimuli, such as lipopolysaccharides (LPS), can activate the transcription factor NF-κB and phosphorylation of its upstream signaling pathway mitogen-activated protein kinase (MAPK).[7,8] At present, three major MAPKs have been described, which are extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK)1/2, and p38 MAPK.[9] Thus, NO production, through iNOS induction by LPS, may reflect the degree of inflammation and can be one of the tools to assess the effect of drugs on the inflammatory process.

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, **1**, Fig. 1) is a natural polyphenol stilbene found in grapes and certain plants used as medicines. It has been reported to have a diverse range of pharmacological properties, including anti-inflammatory action,[10] preventing platelet aggregation[11] and antioxidant activities.[12] Resveratrol can be biotransformed by *Botrytis cinerea*, a fungal grapevine pathogen, into resveratrol oligomers such as isopaucifloral F (**2a**), quadrangularin A (*E*-**3a**), parthenocissin A (*Z*-**3a**), pallidol (**4a**) and (+)-α-viniferin (**5**) (Fig. 1).[13,14] Recent findings suggested that these oligomers also showed a variety of biological activities. Some of these derivatives demonstrated more potent biological activities than resveratrol due to their polyphenol motifs.[15] In particular, there were a few reports about anti-inflammation of resveratrol dimers.[15,16]

Since pallidol was isolated from *Caragana sinica* and determined to have estrogen-like activities by our colleagues in their early research,[17] we have developed methods to be capable of synthesizing resveratrol dimers including isopaucifloral F (**2a**), quadrangularin A (*E*-**3a**), pallidol (**4a**) and their derivatives.[18–20] Some of them have performed potent neuroprotection activities.[21] Recently, Shi and co-workers reported that resveratrol had the protective function against various neurological disorders in experimental models, including brain ischemia, seizures, and neurodegenerative disease models.[22] This work also showed the anti-inflammatory activities of resveratrol in the brain from both in vivo and in vitro studies and the relationship between the neuroprotection and anti-inflammation of resveratrol. Thus, it was of interest to screen our previous synthesized resveratrol dimers and derivatives to verify whether there is a particularly structural type rendering any

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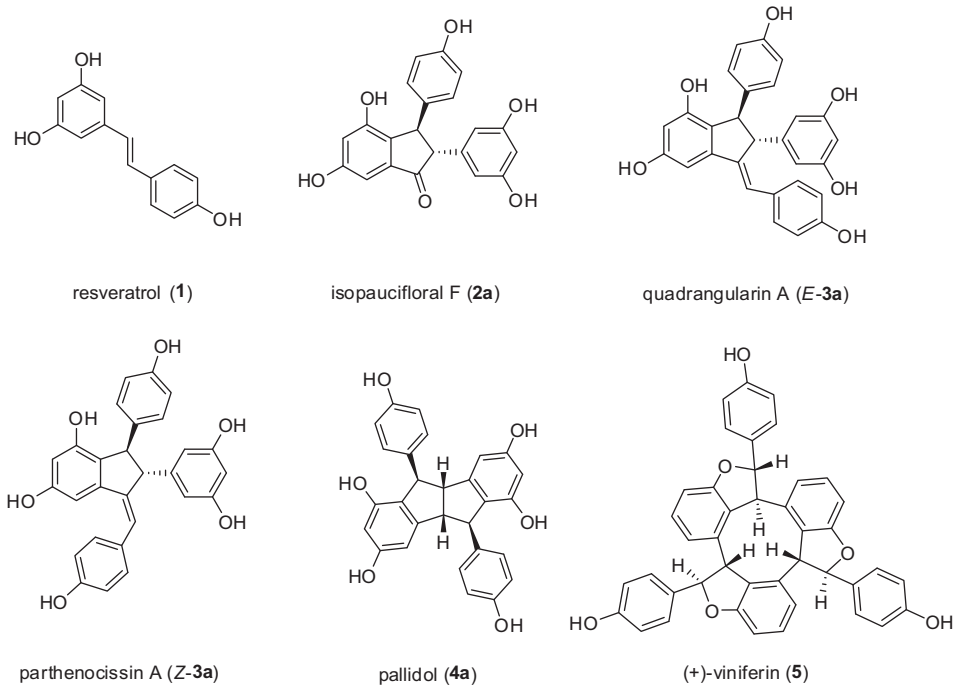


Figure 1. Natural resveratrol oligomers.

anti-inflammatory activity. Therefore, three natural products (**2a**, *E*-**3a**, **4a**) and their methylated precursors (**6a**, **7a**, **8a**, Fig. 2) were tested to inhibit LPS induced NO production in RAW 264.7 cells (Table 1). Interestingly, compound **6a** (indenone-type) and **7a** (indene-type) showed very good activity in RAW 264.7 cell with 56% and 61% NO production, respectively at 10 μM. In addition, methylated precursors were more effective in the NO production assay than their corresponding natural products (**6a** vs **2a**, **7a** vs *E*-**3a**, and **8a** vs **4b**).

The preliminary biological data encouraged us to continuously explore the structure–activity relationships (SARs) of resveratrol dimerized analogues by modifying the substituent patterns on the phenyl rings in various scaffolds. It was expected that functional groups on the benzene rings may play a pivotal role in providing their anti-inflammatory activity.[23] In order to make the discussion clearer, compounds were classified into four types of structures: type I (**6a**–**c** and **12a**–**c**), type II (**7a**–**g**), type III (**13a**–**g**), and type IV (**14a**–**g**) (Scheme 1). Thus, 25 novel resveratrol dimerized derivatives were synthesized[24] and evaluated for inhibition of NO production in LPS-induced RAW 264.7 cells. Meanwhile, it was reported that resveratrol exerted a regulatory effect on inflammatory reactions mediated through the down-regulation of the MAPK pathways.[25] Therefore, enzyme-linked immunosorbent assay (ELISA) and Western blot analysis were also carried out to attempt to identify the mechanisms underlying their anti-inflammatory effects.

The syntheses of these dimerized derivatives (types I–IV) were achieved by adopting our previous reported procedures which was starting from commercially available 3,5-dimethoxybenzoic acid (**9**) followed by a sequential process (Scheme 1).[18] The synthetic strategy involved a Wittig–Hornor reaction, Nazarov cyclization, and Ramberg–Backlund olefination. By incorporating different substituents on Wittig–Hornor reagents and benzyl mercaptans, total 25 dimerized compounds were synthesized and carefully characterized. Among them, 22 were novel compounds.

The anti-inflammatory activity of 25 compounds against LPS induced NO production in RAW 264.7 cells were evaluated and the results were shown in Table 1. In general, types I and IV compounds exhibited better inhibitory activity than other two types. For type I analogues (**6a**–**c**), there was no significant difference when the substituent changed on the ring A (R_1 = OMe, H, or F). All of them (**6a**–**c**) showed potent inhibitory activity against LPS

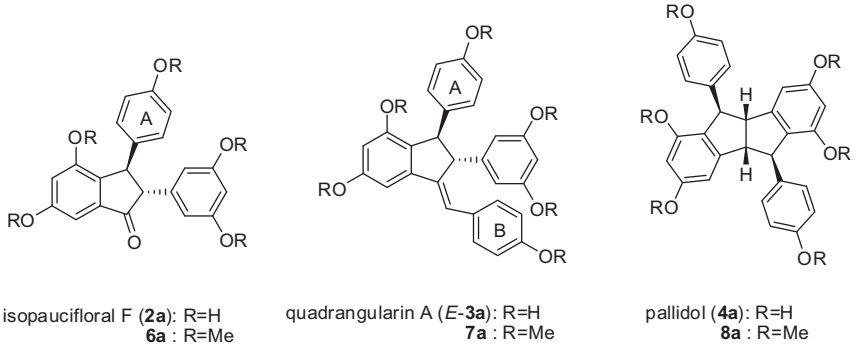
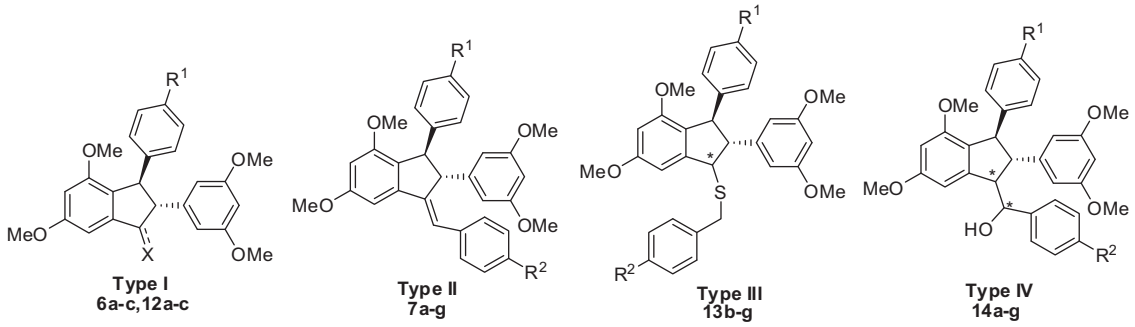


Figure 2. Natural resveratrol dimers and derivatives synthesized before.

Table 1
The effects of novel compounds on LPS induced NO production in RAW 264.7 cells



Entry	Compound	Type	NO production (%)[#]	IC_{50}[a] (μM)
1	**2a**	I	97.13 ± 0.89	>200
2	**3a**	II	70.00 ± 4.52*	NA[b]
3	**4a**	/	89.29 ± 2.23	>200
4	**6a**	I	60.86 ± 5.34*	>200
5	**6b**	I	65.95 ± 1.6*	27.1
6	**6c**	I	57.41 ± 0.25*	44.77
7	**7a**	II	56.10 ± 2.15*	6.26
8	**7b**	II	108.44 ± 2.39	>200
9	**7c**	II	84.35 ± 2.04	>200
10	**7d**	II	88.24 ± 0.42	>200
11	**7e**	II	99.24 ± 2.88	>200
12	**7f**	II	95.92 ± 1.62	>200
13	**7g**	II	90.72 ± 0.91	>200
14	**8a**	/	80.16 ± 0.15	>200
15	**12a**	I	58.32 ± 1.22*	7.87
16	**12b**	I	49.47 ± 2.11*	3.38
17	**12c**	I	54.01 ± 0.84*	NA[b]
18	**13a**	III	70.52 ± 0.87*	12.48
19	**13b**	III	75.94 ± 0.44	>200
20	**13c**	III	72.40 ± 0.35	>200
21	**13d**	III	78.12 ± 0.17	>200
22	**13e**	III	86.32 ± 0.34	>200
23	**13f**	III	92.48 ± 0.49	>200
24	**13g**	III	79.77 ± 0.48	>200
25	**14a**	IV	77.04 ± 2.02	>200
26	**14b**	IV	59.48 ± 0.50*	21.59
27	**14c**	IV	67.78 ± 0.92*	16.51
28	**14d**	IV	75.36 ± 1.05	>200
29	**14e**	IV	63.46 ± 0.61*	26.54
30	**14f**	IV	81.36 ± 1.06	>200
31	**14g**	IV	77.48 ± 1.32	>200

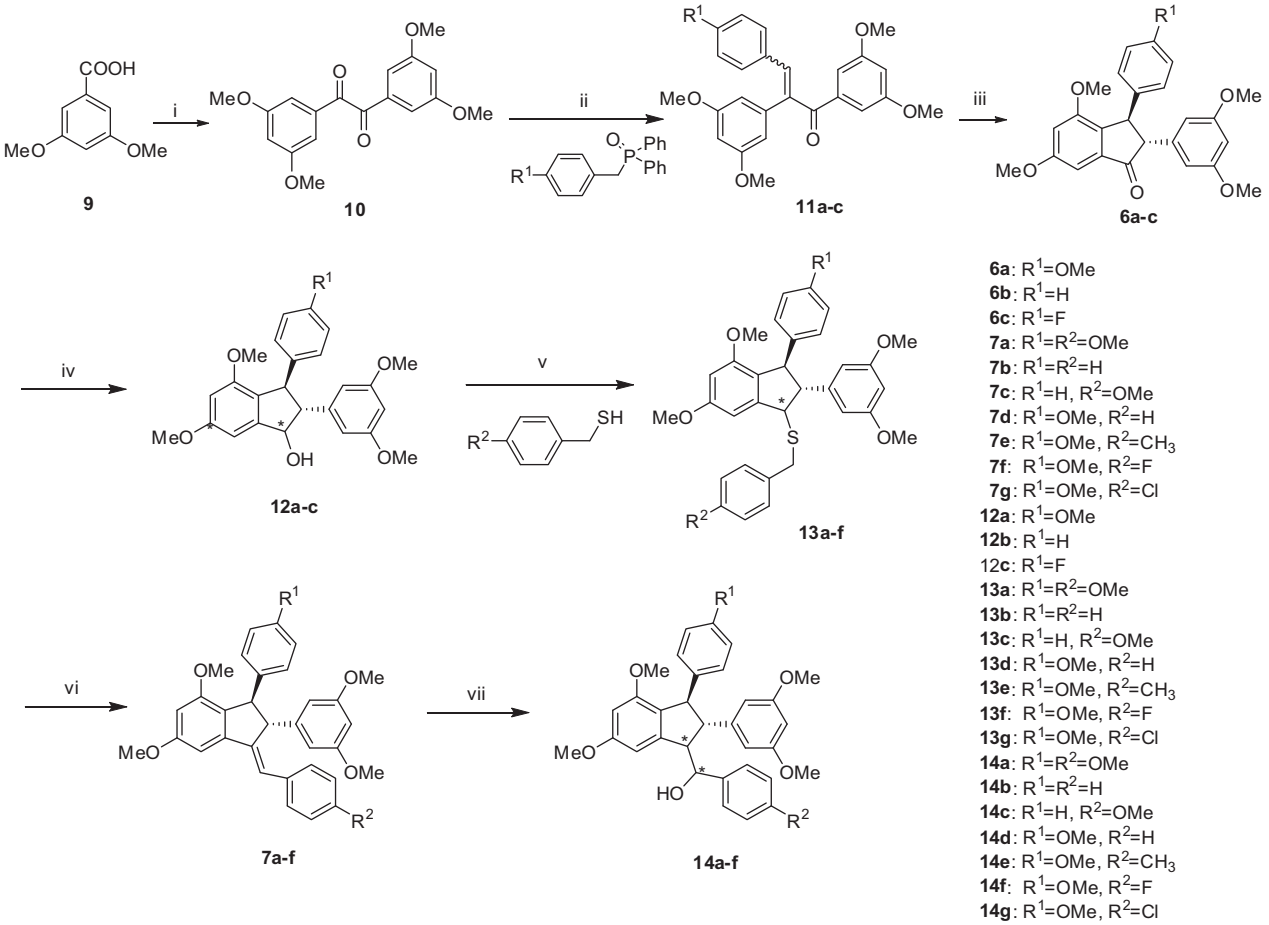
Data shown are means ± SEM, [#]P <0.05 compared with control cells (NO production percentage: 13.28 ± 0.19), *P <0.05 compared with LPS-stimulated cells (NO production percentage: 100 ± 1.46). Data were from at least three independent experiments, each performed in duplicate.

[a] IC_{50} value of each compound was defined as the concentration (μM) of indicated compound that caused 50% inhibition of NO production in LPS-stimulated RAW 264.7 cells.

[b] These compounds shows cytotoxic effect at high concentration.

induced NO production (61%, 66%, and 57% NO production at 10 μM, respectively). After reduced the carbonyl group of **6b**, the inhibitory activity of the corresponding hydroxyl analogue **12b** was further improved (49% NO production at 10 μM, IC_{50} = 3.38 μM). For types II and III, all analogues exhibited very weak inhibitory activity, though these analogues in which methoxyl group on ring A was replace by hydrogen showed slightly better activity (e.g., **7c** in type I and **13b–c** in type II). In type IV, the inhibitory activity was not increased when methoxyl group was replaced by hydrogen on ring B (**14d** vs **14a**). However, **14e**, with a methyl group instead of methoxyl group on ring B did improve the activity (63% NO production at 10 μM, IC_{50} = 26.54 μM). Interestingly, a most potent compound in this type, **14b**, was achieved when there was no substituent on both ring A and B. Among all the tested compounds, **12b** was emerged as the most active one on LPS-induced NO production assay. Accordingly, **12b** was chosen for further investigation to identify the mechanism responsible for it inhibitory effect on RAW 264.7 cells.

Initially, the cell viability experiment was performed at 5–20 μM concentration and there was no significantly cytotoxic of **12b** at the concentration up to 20 μM (data not shown). To investigate the anti-inflammatory effects of **12b** on LPS-induced inflammatory response, RAW 264.7 cells were first preincubated with **12b** for 2 h, and then stimulated with LPS for 24 h. As illustrated in Figure 3A, treatment with LPS (1 μg/mL) drastically increase the expression of iNOS protein; co-treatment with **12b** suppressed LPS-induced iNOS expression in a concentration dependent manner. In accordance with this, **12b** was reduced LPS-induced NO production in a concentration dependent manner (Fig. 3B). A further study revealed that **12b** could also inhibit LPS-induced inflammatory cytokine release. Inflammatory activity for cytokine IL-1β was measured in LPS-stimulated RAW 264.7 cells



Scheme 1. Synthesis of resveratrol dimerized derivatives. Reagents and conditions: (i) LiN reagent, THF, 0 °C; (ii) $(CH_3)_3COK$, toluene; (iii) $BF_3{\cdot}Et_2O$, 25 °C, 40 h; (iv) $NaBH_4$, THF, CH_3OH, 25 °C, 30 min; (v) *p*-CH_3O-$PhCH_2SH$, $In(OTf)_3$, CH_2Cl_2, 25 °C, 1.5 h; (vi) (a) *m*CPBA, $NaHCO_3$, CH_2Cl_2, 0–25 °C, 25 min, (b) KOH, CCl_4/*t*-BuOH/H_2O = 5/5/1, 80 °C, 12 h; (vii) (a) $BH_3{\cdot}THF$, THF, 25 °C, 18 h, (b) NaOH, 30% H_2O_2, Na_2SO_3, 25–0 °C.

by ELISA. As shown in Figure 3C, the LPS stimulation could significantly increase IL-1β release in RAW 264.7 cells. However, the IL-1β release in LPS-stimulated RAW 264.7 cells was suppressed by co-treatment with **12b** in a concentration-dependently manner. These results further confirmed the anti-inflammatory effect of **12b**.

In RAW 264.7 cells, the rapid activation of MAPK in response to LPS has been well documented and played an important role in the regulation with the expression of inflammatory mediators.[26] To investigate whether the anti-inflammatory response by **12b** was mediated by the inhibition of MAPK signaling pathway, the effects of **12b** on the LPS-induced phosphorylation of p38 MAPK, JNK1/2

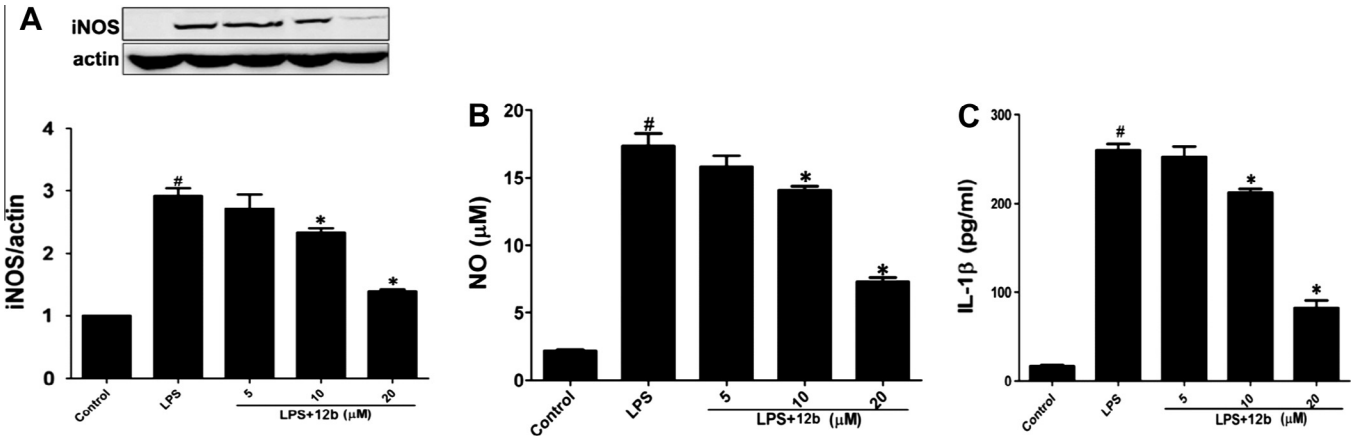


Figure 3. Compound **12b** inhibited LPS-induced inflammatory response in RAW 264.7 cells, After **12b** (5–20 μM) pretreatment, RAW 264.7 cells were stimulated with LPS (1 μg/mL) for 24 h. Western blot analysis for iNOS expression, nitrite and nitrate assay for NO production, and ELISA for IL-1β release were carried out. (A) Quantitative analysis of iNOS expression, β-actin was used as loading control; (B) quantitative analysis of NO production; (C) quantitative analysis of IL-1β release; $^{\#}P$ <0.05 compared with unstimulated cells, $^{*}P$ <0.05 compared with LPS-stimulated cells; data were from at least three independent experiments, each performed in duplicate.

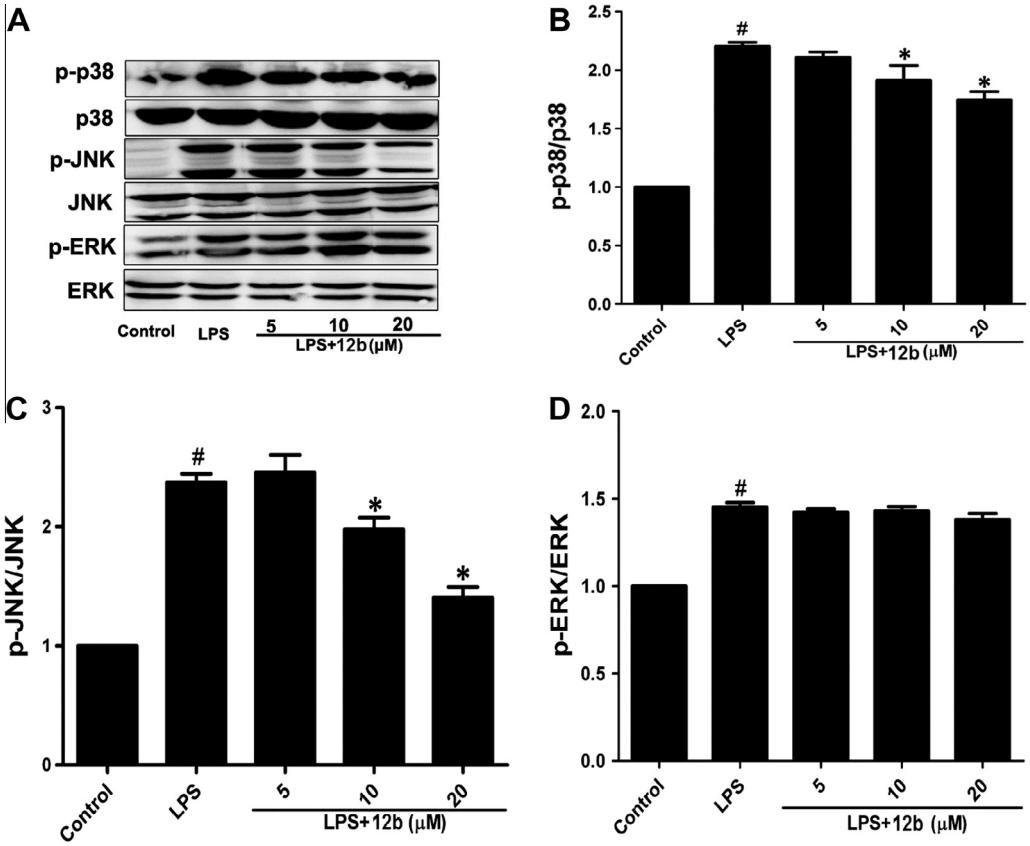


Figure 4. Compound **12b** suppressed LPS-induced activation of MAPK signaling pathway in RAW 264.7 cells, After **12b** (5–20 μM) pretreatment, RAW 264.7 cells were stimulated with LPS (1 μg/mL) for 45 min. The total and phosphorylation levels of MAPK were detected by Western blot. (A) Showing the total and phosphorylation levels of MAPK detected by Western blot; (B) quantitative analysis of p-p38. Total p38 was used as loading control; (C) quantitative analysis of p-JNK1/2. Total JNK1/2 was used as loading control; (D) quantitative analysis of p-ERK1/2. Total ERK1/2 was used as loading control; $^{\#}P$ <0.05 compared with unstimulated cells, $^{*}P$ <0.05 compared with LPS-stimulated cells; data were from at least three independent experiments, each performed in duplicate.

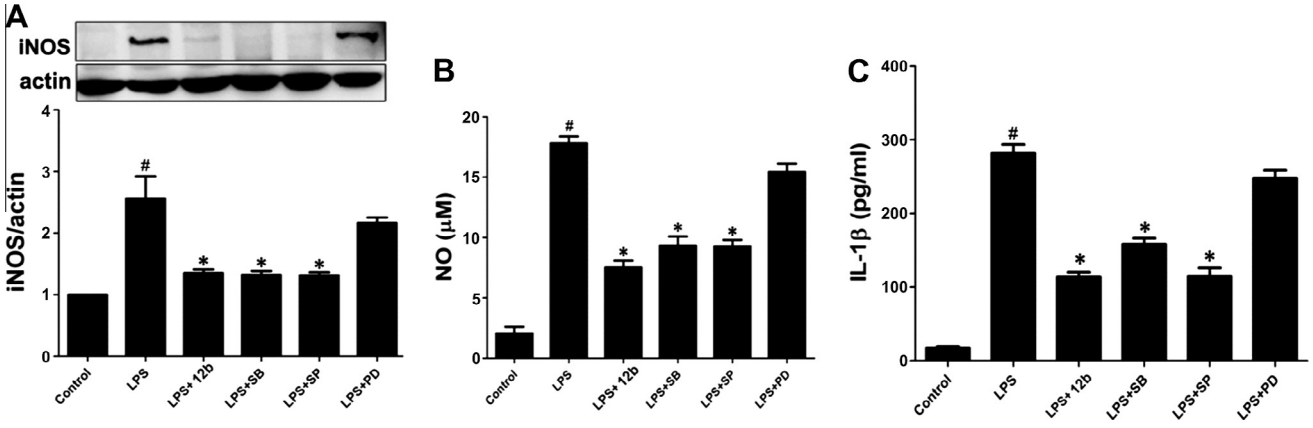


Figure 5. Compound **12b** suppressed LPS-induced inflammatory responses through inhibition of p38 MAPK and JNK1/2 signaling pathway, After preincubation with **12b** (20 μM), SB203580 (p38 inhibitor, 10 μM), SP60012 (JNK1/2 inhibitor, 10 μM), or PD98059 (ERK1/2 inhibitor, 10 μM), RAW 264.7 cells were stimulated with LPS (1 μg/mL) for 24 h. Western blot analysis for iNOS expression, nitrite and nitrate assay for NO production, and ELISA for IL-1β release were carried out. (A) Quantitative analysis of iNOS expression. β-Actin was used as loading control; (B) quantitative analysis of NO production; (C) quantitative analysis of IL-1β release; $^{\#}P$ <0.05 compared with unstimulated cells, $^{*}P$ <0.05 compared with LPS-stimulated cells; data were from at least three independent experiments, each performed in duplicate.

and ERK1/2 were examined in RAW 264.7 cells. LPS could cause a significant phosphorylation of MAPK (p38 MAPK, JNK1/2 and ERK1/2) after stimulation for 45 min detected by Western blot analysis (Fig. 4). Compound **12b** concentration-dependently diminished LPS-induced phosphorylation of p38 MAPK (Fig. 4B) and JNK1/2 (Fig. 4C). However, it had a little effect on phosphorylation of ERK1/2 (Fig. 4D). These results strongly suggested that inhibition of p38 MAPK and JNK1/2 phosphorylation by **12b** may be responsible for achieving LPS-induced inflammatory response.

To further verify that anti-inflammatory effect of **12b** was mediated through inhibition of p38 MAPK and JNK1/2 signaling pathway, RAW 264.7 cells were preincubated with **12b** (20 μM), SB203580 (p38 inhibitor, 10 μM), SP60012 (JNK1/2 inhibitor, 10 μM), or PD98059 (ERK1/2 inhibitor, 10 μM). The LPS

stimulation induced a markable increase in the expression of iNOS and release of NO and IL-1β compared with unstimulated cells. Co-treatment with **12b** (20 μM) significantly reduced the expression of iNOS and release of NO and IL-1β in LPS-stimulated cells (Fig. 5). In addition, the effect of **12b** on LPS-induced inflammatory responses was mimicked by SB203580 (p38 inhibitor) or SP60012 (JNK1/2 inhibitor), but not PD98059 (ERK1/2 inhibitor) (Fig. 5). Overall, the results further confirmed that **12b** suppressed LPS-induced inflammatory responses through inhibition of p38 MAPK and JNK1/2, but not ERK1/2 signaling pathway.

In conclusion, four-types (types I–IV) of resveratrol dimerized analogues were synthesized and evaluated for anti-inflammatory activity. In general, analogues in both types I and IV were more potent than other two types. Among these, the methylated precursors (e.g., **6b**, **6c**, **7a**, and **12b**) were found to be more effective than their corresponding natural products (e.g., **2a**, **3a**, **4a**). Of the compounds synthesized, compound **12b** could significantly suppress LPS-induced iNOS expression and NO production through p38 MAPK and JNK1/2 signaling pathways. Accordingly, our findings provide a partial description of the mechanism underlying the anti-inflammatory effect of **12b**. It is believed that this compound can be a good lead for further design of iNOS inhibitors.

Acknowledgements

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24. *Typical procedures for the synthesis of derivatives of type I. Preparation of 1,2-bis(3,5-dimethoxyphenyl)ethane-1,2-dione (**10**):* To a solution of naphthalene (23.3 g, 181 mmol) in anhydrous THF (100 mL) under nitrogen was added lithium (1.26 g, 181 mmol) at room temperature for 8 h. The mixture was add dropwise to a solution of 3,5-dimethoxybenzoic acid **9** (15.0 g, 82.3 mmol) in anhydrous THF (300 mL) at 0 °C and quenched with water when dropping complete. The reaction mixture was concentrated. Then the organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was recrystallized in ethyl acetate to give **10** as a yellow sold (12.0 g, 44%).
*Preparation of 1,2-bis(3,5-dimethoxyphenyl)-3-phenylprop-2-en-1-one (**11b**):* To a solution of **10** (1.00 g, 3.03 mmol) and benzyl-diphenyl-phosphate oxygen (0.89 g, 3.03 mmol) in anhydrous THF (100 mL) under nitrogen was added potassium *tert*-butoxide (0.56 g, 4.54 mmol) at room temperature for 1 h. The reaction mixture was concentrated. Then the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford the liquid which was purified by silica gel chromatography, utilizing ethyl acetate and hexane (1:10) as mobile phase, to afford **11b** as a yellow oil (0.87 g, 71%).
*Preparation of trans-2-(3,5-dimethoxyphenyl)-4,6-dimethoxy-3-phenyl-2,3-dihydroinden-1-one (**6b**):* To a solution of chalcones **11b** (0.40 g, 1.00 mmol) in CH_2Cl_2 (15 mL) was added dropwise $BF_3{\cdot}OEt_2$ (0.12 mL, 1.00 mmol) via syringe. After the reaction mixture was stirred at room temperature for 40 h under a nitrogen atmosphere, the reaction was quenched by the addition of H_2O (5 mL). The resulting mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic phases were washed with brine and dried over anhydrous Na_2SO_4. After removal of solvent, the resulting yellow residue was recrystallized in methanol to afford **6b** as a white solid (0.38 g, 94%).
Preparation of trans-2-(3,5-dimethoxyphenyl)-4,6-dimethoxy-3-phenyl-2,3-dihydro-1H-inden-1-ol (12b): To a solution of **6b** (0.40 g, 1.00 mmol) in THF (10 mL) was added NaBH4 (0.23 g, 6.00 mmol) at room temperature. Then the reaction mixture was added methanol (10 mL) and continuously stirred at room temperature for 30 min. Upon completion, the reaction mixture was concentrated. After separated the organic layer, the aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were washed with brine (15 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo to give **12b** (0.40 g, 99%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 3.17 (t, J = 6.8 Hz, 1H), 3.53 (s, 3H), 3.73 (s, 6H), 3.86 (s, 3H), 4.30 (d, J = 7.0 Hz, 1H), 5.20 (d, J = 6.3 Hz, 1H), 6.33 (d, J = 9.8 Hz, 3H), 6.42 (s, 1H), 6.66 (s, 1H), 7.02–7.06 (m, 2H), 7.13–7.25 (m, 3H); ^{13}C NMR ($CDCl_3$,100 MHz) δ: 54.12, 55.21, 55.26, 55.57, 67.36, 82.18, 98.57, 99.31, 99.71, 105.84, 123.08, 125.92, 127.34, 127.96, 144.11, 144.21, 157.05, 160.77, 160.86, 161.63; ESI-MS m/z: 389.2 $[M-H_2O]^+$; IR (KBr) ν_{max} = 3441, 2935, 2836, 1596, 1511, 1455, 1203, 1148 cm^{-1}.
Typical procedures for the synthesis of derivatives of types II–IV can be found in early research article by our group: Zhong, C.; Liu, X.-H.; Hao, X.-D.; Chang, J.; Sun, X. *Eur. J. Med. Chem.* **2013**, *62*, 187.
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