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Design, synthesis, and anti-inflammatory activity of caffeoyl salicylate analogs as NO production inhibitors

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ABSTRACT

Chlorogenic acid (CGA) has been reported to exhibit potent anti-inflammatory activity. However, the development of anti-inflammatory agent based on CGA has not been investigated. In this paper, a series of caffeoyl salicylate compounds derived from CGA were designed, synthesized, and evaluated by LPS-induced nitric oxide synthase inhibition and QRT-PCR technique. Most compounds showed modest activity to inhibit production of nitric oxide (NO) in RAW 264.7 cells induced by lipopolysaccharides (LPS). Among these compounds, QRT-PCR and western blotting results indicated that compounds **6b**, **6c**, **6f, 6g** and **D104** that possess 5-member ring or 6-member ring caused a significant inhibition against expression of the iNOS2 in LPS-induced macrophages. In addition, cytotoxic assay displayed most derivatives have good safety in vitro. This new promising scaffold could be further exploited for the development of anti-inflammatory agent in the future.

1. Introduction

Chlorogenic acid (5-O-caffeoylquinic acid, CGA) is a phenolic compound widely distributed in plants, which has been extensively studied since it has been reported to display physiological activities such as anti-inflammatory [1], anti-oxidant [2], anticancer [3], antilipidemic [4], and anti-neurodegenerative activities [5]. Moreover, chlorogenic acid exhibits good activity against a wide range of microorganisms, and this property can be used for the food industry as a food additive. Therefore, chlorogenic acid could be regarded as a very promising starting point for the drug development.

Chlorogenic acid can be retrieved from the three bioactivity databases CHEMBL [6], PubChem [7], and BindingDB [8], respectively. In these small molecule databases, several drug targets such as aldose reductase, HIV type 1 integrase and 1,3-beta-glucan synthase, can be involved with various biological activities reported by many relevant references. Among these targets, only two molecular targets peroxisome proliferator-activated receptor gamma (PPARγ) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are closely related to the anti-inflammatory activity of CGA [9]. About fifty-seven anti-inflammatory molecules extracted from Selleck Chemicals website [10] are collected and curated carefully, in which three compounds are similar structurally with CGA (Fig. 1). It is worth noting that anti-inflammatory targets of these three compounds are also PPARγ and NF-κB. For this reason, we could attempt to design novel caffeoyl salicylate analogs as anti-inflammatory agent based on these two targets.

In this study, about 1300 kinds of drug-like fragments named Enamine_golden_fragment_library_sdf are downloaded from the Enamine website [11]. We would plan to select appropriate moiety in replacement of quinic acid group, and there are about twenty drug-like fragments screened by being docked into the PPARγ and NF-κB proteins. Subsequently, two hundred novel small molecules are carefully designed by the replacement of quinic acid portion of CGA using these fragments. Among these compounds, one caffeic acid phenethyl ester (CAPE) analog (compound **D104**) can be obtained that it was the precursor to CGA and CAPE, and Glide docking [12] screening (Fig. 2) reveals that compound **D104** would bind more tightly with PPARγ and NF-κB targets, than CGA or CAPE. Based on this, we sought to discover novel anti-inflammatory agent by modifying the promising scaffold using common chemicals in our lab.

2. Results and discussion

2.1. Chemistry

The intermediates (**3a-3d**) were prepared in the same way according to Scheme 1. The 3,4-dihydroxybenzaldehyde **1** was treated

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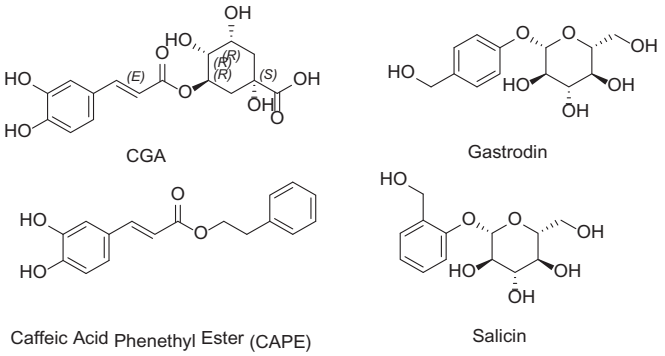
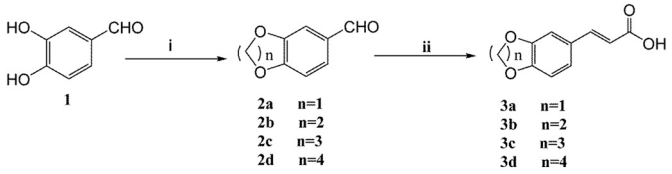
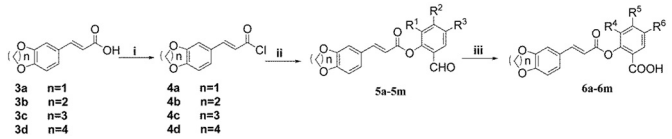


Fig. 1. Chlorogenic acid analogues in Selleck chemicals.

with the corresponding dibromoalkane and K_2CO_3 in acetone or N, N-Dimethylformamide (DMF) to afford the compounds **2a-2d** as the major products. Subsequently, the cyclized cinnamic acids (**3a-3d)** were synthesized by reacting **2a-2d** with malonic acid in the presence of pyridine and piperidine. The detailed mechanism of the reaction was involved with the Knoevenagel condensation reaction. The next step is that these cinnamic acids were converted to acyl chloride **4a-4d** before reacting with corresponding salicylic aldehyde in Scheme 2. Adding various salicylic aldehydes into the solution of acyl chloride **4a-4d** in CH_2Cl_2 or ethyl acetate at ice-bath under an inert (N_2) atmosphere would produce the required esters **5a-5m** (Table 1). The aldehyde group of compounds **5a-5m** was subsequently oxidized to acid group according to the Pinnick oxidation reaction: the designed acids **6a-6m** (Table 1) were obtained by the oxidation of sodium chlorite ($NaClO_2$) in tert-butyl alcohol and tetrahydrofuran (THF) (Scheme 2). The chemical structures of all new compounds were characterized by melting point and ^1H NMR spectra. Besides, most of the compounds were



Scheme 1. Synthesis of the cyclized cinnamic acids **3a**-**3d**. Reagents and conditions: (i) K_2CO_3, dibromoalkane, acetone or DMF, reflux, 4–5 h, yield 50–60%; (ii) malonic acid, pyridine, piperidine, 80 °C, 24 h, yield 70–80%.



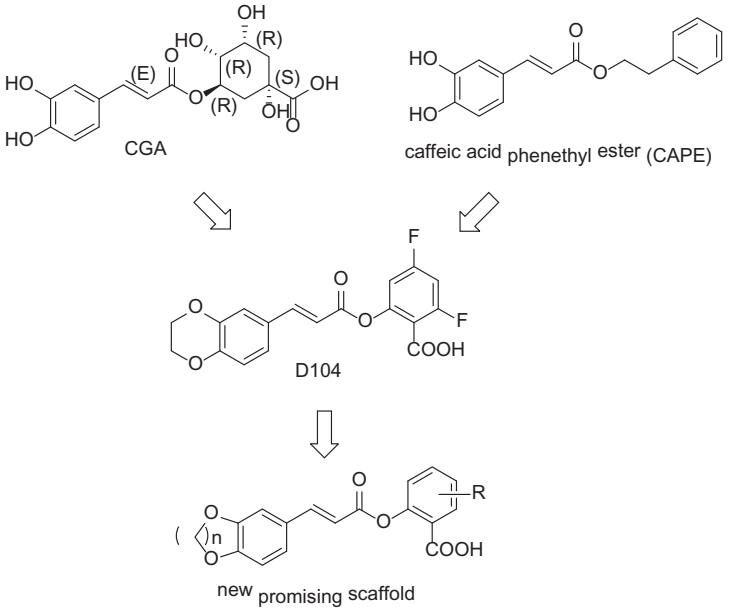
Scheme 2. Synthesis of required acids **6a**-**6m**. Reagents and conditions: (i) $SOCl_2$, 80 °C, reflux, 2 h, yield 93–95%; (ii) corresponding salicylic aldehyde, CH_2Cl_2 or ethyl acetate, pyridine, N_2, ice-bath, overnight, yield 40–60%; (iii) $NaClO_2$, NaH_2PO_4, 3-Methyl-1-butene, pH 3–4, stirred at rt., 5–6 h, yield 45–60%.

characterized by infra-red spectrum (IR spectrum). What is more, compound **3b** due to intermediate products and two compounds (**5a** and **6e**) as representative products of aldehydes and acids respectively were selected to give ^{13}C NMR spectra. In addition, a part of these compounds was also characterized by general mass spectra (MS) and high-resolution mass spectrum (HR-MS) (**5a**, **5f**, **6a** and **6f**).

2.2. Pharmacological screening

2.2.1. Assay for cytotoxicity to RAW264.7

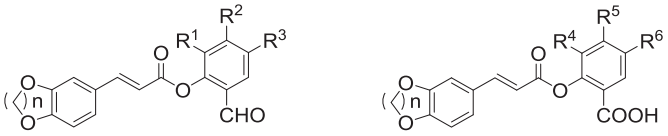
The cytotoxic activities of all these compounds were tested by the



COMPOUND	GLIDE Docking (SP) (kcal/mol)	
	PPARγ (PDB ID: 3U9Q)	NF-κB (PDB ID: 4KIK)
D104	-9.058	-6.755
CGA	-6.720	-6.970
CAPE	-7.005	-7.983

Fig. 2. New promising scaffold derived from CGA.

Table 1
Structures of caffeoyl salicylate analogs.



Compounds	n	R^1	R^2	R^3	Compounds	n	R^4	R^5	R^6
5a	1	H	H	H	**6a**	1	H	H	H
5b	1	H	H	Cl	**6b**	1	H	H	Cl
5c	1	Cl	H	Cl	**6c**	1	Cl	H	Cl
5d	1	H	$N(C_2H_5)_2$	H	**6d**	1	H	$N(C_2H_5)_2$	H
5e	2	H	H	H	**6e**	2	H	H	H
5f	2	H	H	Cl	**6f**	2	H	H	Cl
5g	2	Cl	H	Cl	**6g**	2	Cl	H	Cl
5h	2	H	$N(C_2H_5)_2$	H	**6h**	2	H	$N(C_2H_5)_2$	H
5i	3	H	H	H	**6i**	3	H	H	H
5j	3	Cl	H	Cl	**6j**	3	H	H	Cl
5k	3	H	H	Cl	**6k**	3	Cl	H	Cl
5l	3	H	$N(C_2H_5)_2$	H	**6l**	3	H	$N(C_2H_5)_2$	H
5m	4	H	H	Cl	**6m**	4	H	H	Cl

MTT method [13] using macrophage cell RAW264.7 cultured in DMEM medium. The cell monolayer was incubated with caffeoyl salicylate analogs (**5a-5m**, **6a-6m**, **pro-D104** and **D104**) at the concentration of 50 μM for 72 h, respectively. As shown in Fig. 3, it is obvious that most of these compounds could not exhibit any toxicity when compared with control and two positive molecules (CGA and aspirin). Only compounds **5b**, **5c**, and **5h** did significantly affect cell viability. This main reason could account much for toxicity of aldehyde group.

2.2.2. Anti-inflammatory activity

Lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria, can elicit strong immune responses in animals. In immune defense mechanism, the production of NO as a free radical with an unpaired electron is primarily regulated by the inducible nitric oxide synthase (iNOS), which is a key regulatory factor in immune response, particularly inflammation, and binds calmodulin at physiologically relevant concentrations [14]. Therefore, the inhibition of iNOS by these small molecules could to some extent reflect anti-inflammatory activities of these compounds. The chemical structures of caffeoyl salicylate analogs studied are listed in Table 1, and intuitively can be classified into two main types including caffeoyl salicylaldehyde (**5a-5m**) and caffeoyl salicylate (**6a-6b**) that contribute to follow-up structure-activity analysis. Meanwhile, the anti-inflammatory activities of caffeoyl salicylate analogs are presented in Fig. 4. As shown above, most of the caffeoyl salicylate analogs exhibited high-potency inhibitory activity against the nitrite production in LPS induced RAW 264.7 macrophage. Among these compounds, the inhibitory effects of compounds **6b**, **6c**, **6f**, **6g**, **6k**, **5b**, **5c**, **5d**, **5g, 5h** and **D104** against NO production were more potent in RAW264.7 than others. The structure-activity relationships (SARs) for inhibition of nitric oxide synthase could be demonstrated as follows: (1) compounds that possess 5-member ring or 6-member ring (compounds **6a-6h**) caused a more significant inhibition of the iNOS2 expression in LPS-induced macrophages than compounds with 7-member ring or 8-member ring (compounds **6i-6m**); (2) the analogs with electron withdrawing groups such as chlorine exhibited more effective inhibitory activity than the analogs with electron donating groups or non-substituted compounds; the compounds with electron donating groups and the molecular without substituted exhibited similar potency with non-substituted compounds; (3) compared to compounds with 5-member ring or 6-member ring, compounds with one-substituent on benzene ring displayed more effective inhibitory activity than compounds with double substituents (compounds **6c** and **6g**); (4) overall, the anti-inflammatory activity of caffeoyl salicylaldehyde exhibited better than that of caffeoyl salicylate.

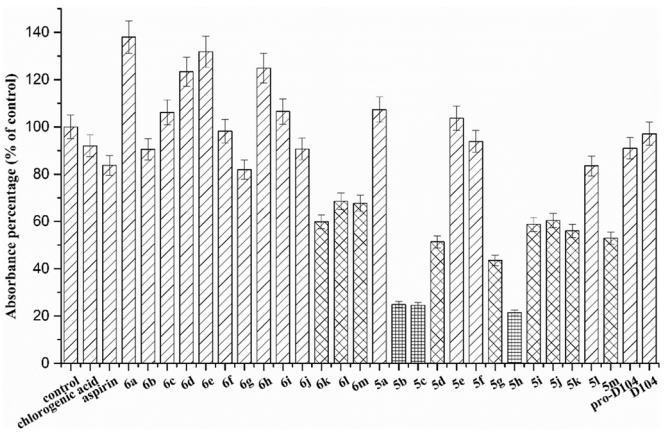


Fig. 3. Assay for cytotoxicity to RAW264.7 cells in vitro by compounds **5a-5m**, **6a-6m**, **pro-D104** and **D104** at the concentration of 50 μM for 72 h. Only compounds **5b**, **5c**, and **5h** did significantly affect cell viability. Data was represented by the mean ± SD of the three independent experiments.

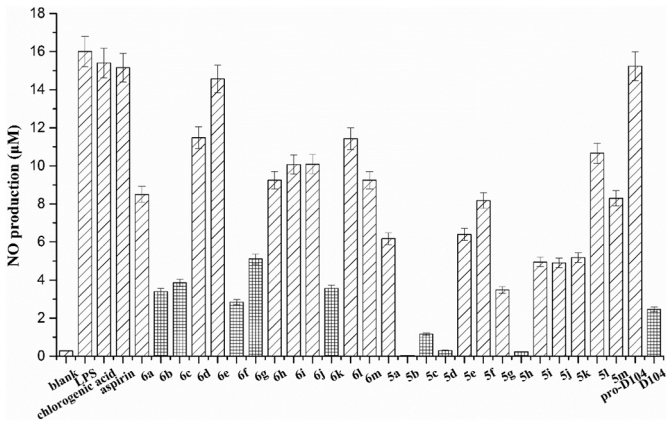


Fig. 4. The effect of inhibiting the production of NO in RAW 264.7 cells induced by LPS in vitro of compounds **5a-5m**, **6a-6m**, **pro-D104** and **D104** were compared with the controls of **chlorogenic acid** and **acetylsalicylic acid**. The inhibitory effects of compounds **6b**, **6c**, **6f**, **6g**, **6k**, **5b**, **5c**, **5d**, **5g, 5h** and **D104** against NO production were more potent in RAW264.7 than others. Data was represented by the mean ± SD of the three independent experiments.

Based on this, we could surmise: (1) the potent inhibitory activity of compounds **6b**, **6c**, **6f**, **6g** and **D104** would be closely related to stable structure of 5-member ring and 6-member ring; (2) the contribution of the inductive effect of the halogens to anti-inflammatory activity is demonstrated by the differences in activity of compounds **6a** and **6e** with chlorinated compounds **6b**, **6c**, **6f** and **6g**; (3) the N,N-diethylamine group reduces activity due to steric effects and does not take into consideration electronic factors; (4) the number of electron withdrawing groups has also a modest effect on activity while comparing compounds **6b, 6f** with **6c**, **6g**; (5) although compounds **5b**, **5c**, **5d**, **5g** and **5h** displayed obvious inhibitory activity, the main reason could account for toxicity of aldehyde group.

Seven compounds (**6b**, **6c**, **6f**, **6g**, **5a**, **5e** and **5f**) were subsequently selected to investigate the IC_{50} values [15] of anti-inflammatory activity against nitric oxide synthase. IC_{50} values can be calculated by formula $IC_{50} = 1 - [(C_{experimental\ group} - C_{control})/(C_{LPS} - C_{control})]$ through non-linear fitting in origin software. $C_{experimental\ group}$ C_{LPS} and $C_{control}$ are nitric oxide mean concentration of experimental group, only LPS added group and only cell sap added group, respectively. The results were determined from replicates of 6 wells from at least three independent experiments (Table 2). In accordance with the analysis mentioned above, the most potent compounds are **6f** and **D104**, and it is obviously noted that the inhibitory activity of acid compounds (**6b**, **6c**, **6f**, and **6g**) were superior to that of aldehyde compounds (**5a**, **5e**, and **5f**).

2.2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR

In order to explore anti-inflammatory activity at the mRNA molecular level, we carried out mRNA microarray analyses and quantitative reverse transcription-PCR (QRT-PCR) [16] on iNOS2 in this study. The PCRs were amplified using β-actin as standard. Sequences of the PCR primers are listed in Table 3. We chose three concentrations (10 μM, 20 μM and 40 μM) around IC_{50} value to test iNOS2 expression (Fig. 5). The obtained results showed that the compounds **6b, 6c, 6f, 6g** and **D104** caused a significant reversal of the iNOS2 inhibition in LPS-induced macrophages with expression values of 0.7297 to 28.43741 in percentage (%) based on a no-additions (NA) control. Among these compounds, compounds **6f** and **D104** displayed the most significant inhibitory activities. The inhibitory activities of **6f** and **D104** were approximately 200-fold greater than the LPS group.

2.2.4. Western blotting analysis

Based on western blot analysis, we determined whether the NO inhibitory effects of compounds **6b**, **6c**, **6f** and **6g** are related to the regulation of the protein expression of iNOS. As shown in Fig. 6, pretreatment with 100 μM of the four compounds led to a decrease in iNOS protein level. These results indicate that compounds **6b**, **6c**, **6f** and **6g** exert attenuate LPS-induced pro-inflammatory responses in RAW 264.7 cells through down regulation of NO. Among them, compound **6f** was the most potent derivative showing the strongest inhibitory effect on iNOS protein expression. Different concentrations (10, 30, 100 μM) of compounds **6f** with or without LPS (500 ng/mL) were subsequently selected to explain **6f** work through the iNOS signaling pathway to downregulate the cytokines. NO regulation of compounds **6b**, **6c**, **6f** and **6g** might be attributed to the iNOS signaling pathway.

Table 2
Inhibitory effect on NO production of compounds **6b, 6c, 6f, 6g, 5a, 5e, 5f** and **D104** in LPS-activated RAW264.7 cells.

Compounds	IC_{50}(μM)
Chlorogenic acid	> 100
Aspirin	> 100
6b	23.44 ± 1.57
6c	28.84 ± 1.98
6f	19.95 ± 2.47
6g	31.62 ± 2.02
5a	41.78 ± 0.42
5e	46.81 ± 0.90
5f	65.06 ± 4.75
D104	17.59 ± 1.64

Table 3
Primer used in Real time-PCR assay.

Gene	Primer sequence
Mus-β-actin	Forward 5′-GGTGTGATGGTGGGAATGGG-3′ Reverse 5′-ACGGTTGGCCTTAGGGTTCAG-3′
Mu-iNOS2	Forward 5′-CAGCTGGGCTGTACAAACCTT-3′ Reverse 5′-CATTGGAAGTGAAGCGTTTGG-3′

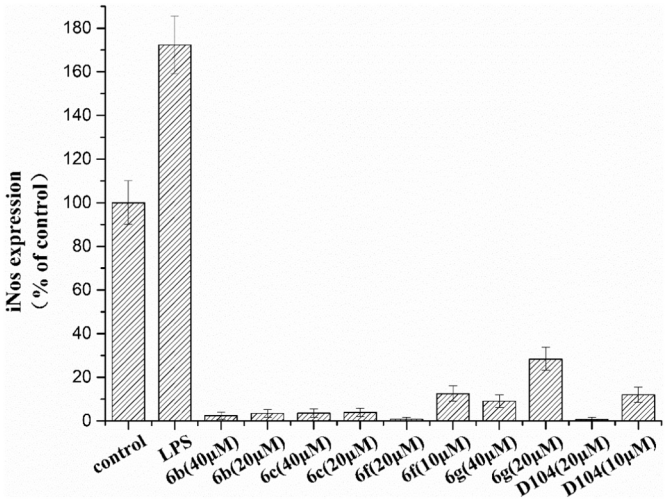


Fig. 5. The LPS-stimulated macrophages were treated with compounds **6b, 6c, 6f 6g** and **D104**, incubated for 24 h, following which RNA was isolated and subjected to the RT-PCR analysis for the expression of iNOS2 mRNA. Compounds **6b, 6c, 6f** and **6g** and **D104** caused a significant reversal of the iNOS2 inhibition in LPS-induced macrophages with expression values of 0.7297 to 28.43741 in percentage (%) based on a no-additions (NA) control. Data was represented by the mean ± SD of the three independent experiments.

3. Conclusions

In summary, our design and synthesis have led to a series of caffeoyl salicylate analogs derived from CGA. Bioassays showed that compounds **6b**, **6c**, **6f, 6g** and **D104** displayed significant activity to inhibit production of nitric oxide (NO) in RAW 264.7 cells induced by lipopolysaccharides (LPS). Compounds **6b**, **6c**, **6f, 6g** and **D104** can therefore be used as promising treatment candidate compounds for diseases related with excess NO production. Structure-activity relationships are showed as follows. (1) The anti NO synthase activity of compounds with 5-member ring or 6-member ring is higher than that of the seven and eight elements ring. (2) The electron-withdrawing inductive effect and steric effect have great effect on anti-inflammatory activity. (3) The amount and position of substitution groups must attract our attention. (4) The assay for cytotoxic activity on RAW 264.7 cells demonstrate that most of the caffeoyl salicylate analogs have good safety in vitro. Based on this, compounds **6b**, **6c**, **6f, 6g** and **D104** will be studied in the next step and we also give the relevant bioactivity evaluation to these compounds. Further investigation is needed to determine mechanisms underlying this effect and the most effective concentrations. What is more, the compounds with different groups should be synthetized to intensive study structure-activity relationships of caffeoyl salicylate analogs derived from chlorogenic acid.

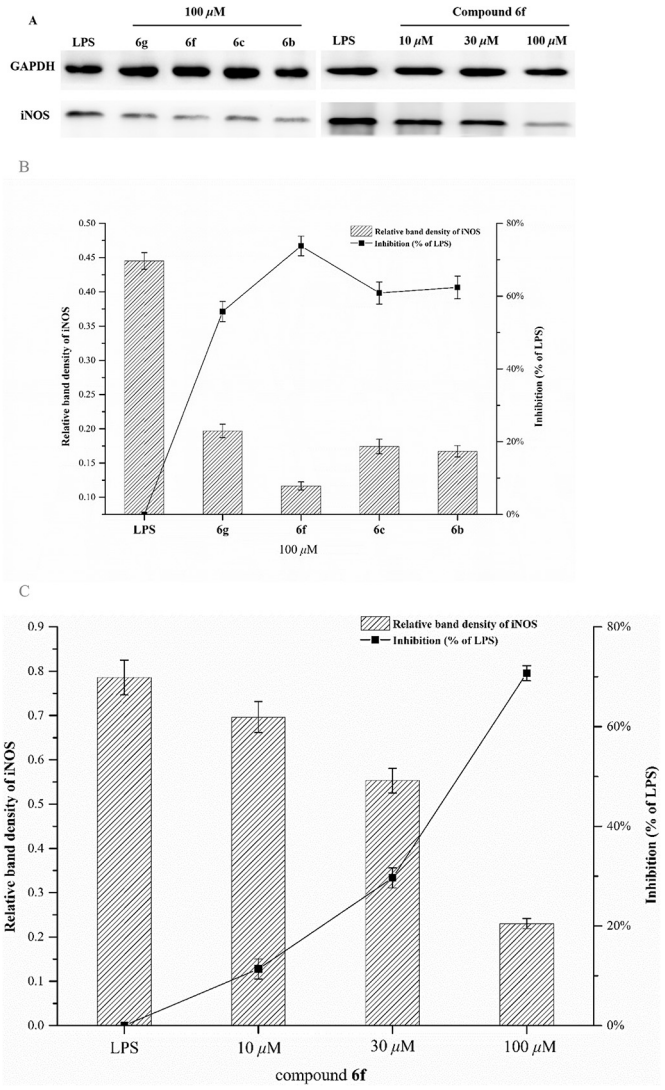


Fig. 6. Effects of compounds **6b**, **6c**, **6f** and **6g** on NO regulation. (A) RAW 264.7 cells were treated with compounds **6b, 6c, 6f** and **6g** (100 μM) for 24 h. LPS-induced iNOS and GAPDH protein levels were determined by western blotting. (B—C) Image J software was used to analyze the levels of iNOS with GAPDH as the reference. Data was represented by the mean ± SD of the three independent experiments.

4. Experimental section

4.1. Chemistry

4.1.1. Reagents and general methods

^{1}H and ^{13}C NMR spectra were recorded on Bruker AM 101, 400 and 600 MHz spectrometers with tetramethylsilane (TMS) as the internal standard. Melting points (M.p.) were recorded on SRS OptiMelt-100 full automatic micro melting point instrument. Electrospray ionization mass spectra (ESI-MS) and high-resolution mass spectrum (HR-MS) were recorded by Agilent 6520B Q-TOF. FT-IR spectra were carried out on Nicolet 380 fourier transformation infrared spectrometer using KBr pellets in the 400–4000 cm^{-1} range. Column chromatography (CC): silica gel (200–300 mesh; Qingdao Makall Group Co., Ltd.; Qingdao; China). All reactions were monitored using thin layer chromatography (TLC) on silica gel plates. Reaction reagents were analytical reagent grade and purchased from Aladdin.

4.1.2. General procedure for the preparation of ***2a-2d***

A mixture of 3,4-dihydroxybenzaldehyde (3.04 g, 22 mM) and acetone or DMF (100 mL) was stirred at room temperature until clear, and then dibromoalkane (26.4 mM) and K_2CO_3 (3.64 g, 26.4 mM) were added. The mixture was heated at reflux about 60 °C for 4–5 h. After completion of the reaction as indicated by TLC, the solution was evaporated under reduced pressure. Then the resulting solid was poured into H_2O (50 mL) and extracted with ethyl acetate for column chromatography. Column chromatography was performed using silica gel (200–300 mesh) eluting with ethyl acetate and petroleum ether to give **2a-2d** (Yield: 50–60%).

*4.1.2.1. benzo[d][1,3]dioxole-5-carbaldehyde **(2a)***. White solid was obtained. ESI-MS m/z: 150.03. M.p. 36–38 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.79 (s, 1H), 7.39 (dd, J = 7.9, 1.5 Hz, 1H), 7.31 (d, J = 1.4 Hz, 1H), 6.91 (d, J = 7.9 Hz, 1H), 6.06 (s, 2H). Purity: 98%.

*4.1.2.2. 3,4-dihydro-2H-benzo[b][1,4]dioxepine-7-carbaldehyde **(2c)***. White solid was obtained. ESI-MS m/z: 178.06. M.p. 49–50 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.81 (s, 1H), 7.61–7.34 (m, 2H), 7.17–6.83 (m, 1H), 4.35–4.29 (m, 2H), 4.25 (t, J = 5.9 Hz, 2H), 2.42–2.03 (m, 2H). Purity: 98%.

*4.1.2.3. 2,3,4,5-tetrahydrobenzo[b][1,4]dioxocine-8-carbaldehyde **(2d)***. White solid was obtained. ESI-MS m/z: 192.08. M.p. 77–78 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.80 (s, 1H), 7.48 (dq, J = 3.8, 2.0 Hz, 2H), 7.00 (d, J = 8.8 Hz, 1H), 4.61–4.48 (m, 2H), 4.30–4.17 (m, 2H), 2.02–1.92 (m, 2H), 1.81 (td, J = 11.0, 5.8 Hz, 2H). Purity: 98%.

4.1.3. General procedure for the preparation of ***3a-3d***

The cyclized cinnamic acids were synthesized using Doebner-Knoevenagel modification. Cinnamic acids were prepared by mixing cyclized benzaldehydes (4.43 g, 27 mmol), malonic acid (3.37 g, 32.4 mM), pyridine (20 mL) and piperidine (340 μL) together and stirring 80 °C on a magnetic stirrer for 24 h. The reaction mixture was evaporated under reduced pressure. Precipitates obtained were filtered, washed with cold water repeatedly, and dried. Finally, the mixtures were recrystallized with dichloromethane to give these acids. The yields were between 70% and 80%.

*4.1.3.1. (E)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylic acid **(3b)***. Faint yellow solid was obtained. ESI-MS m/z: 206.06. M.p. 191–193 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.23 (s, 1H), 7.47 (d, J = 16.0 Hz, 1H), 7.34–7.05 (m, 2H), 6.87 (d, J = 8.3 Hz, 1H), 6.35 (d, J = 16.0 Hz, 1H), 4.26 (qd, J = 3.7, 2.1 Hz, 4H)·^{13}C NMR (101 MHz, DMSO- d_6) δ 167.72 (s), 145.35 (s), 143.64 (s), 143.51 (s), 127.68 (s), 121.89 (s), 117.40 (s), 117.13 (s), 116.68 (s), 64.31 (s), 63.92 (s). Purity: 97%.

4.1.4. General procedure for the preparation of ***5a-5m*** and ***pro-D104***

The starting materials (compounds **3a-3d**) for the synthesis of esters should be activated in the first procedure: the compounds **3a-3d** (12 mM) and $SOCl_2$ (20 mL) were mixed and stirred at reflux 80 °C for 2 h. The reaction mixture was cooled and evaporated to give reactive acyl chloride obtained as an oil, which would be dissolved in CH_2Cl_2 or ethyl acetate (10–15 mL) in the next step. A solution of acyl chloride (12 mM) in CH_2Cl_2 or ethyl acetate was added dropwise to corresponding salicylic aldehyde (10 mM) in CH_2Cl_2 or ethyl acetate containing pyridine (1.6 mL, 20 mM) under an inert (N_2) atmosphere and at 0 °C with constant stirring overnight. The reaction mixture was then poured in excess of diluted NaOH and extracted with CH_2Cl_2 or ethyl acetate. The extraction liquid was purified by a flash chromatography with ethyl acetate/petroleum ether to give these compounds. The yields were between 40% and 60%.

*4.1.4.1. (E)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acryloyl chloride **(4b)***. Faint yellow solid was obtained. ESI-MS m/z: 224.02. ^{1}H NMR

(400 MHz, $CDCl_3$) δ 7.70 (dd, J = 19.1, 15.7 Hz, 1H), 7.13–7.02 (m, 2H), 6.89 (dd, J = 10.0, 8.2 Hz, 1H), 6.38 (dd, J = 76.0, 15.7 Hz, 1H), 4.39–4.21 (m, 4H). Purity: 98%.

4.1.4.2. 2-formylphenyl (E)-3-(benzo[d][1,3]dioxol-5-yl)acrylate ***(5a)***. Faint yellow solid was obtained. HR-MS m/z ($[M + Na]^+$): $C_{17}H_{12}O_5Na$, calculated 319.0577, found 319.0578. M.p. 119–121 °C. IR KBr (cm^{-1}): 3434, 3077, 2914 (C—H), 1735 (CHO), 1709 (C=O), 1628, 1599, 1497, 1450 (C=C). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.19 (s, 1H), 7.91 (dd, J = 7.7, 1.7 Hz, 1H), 7.81 (d, J = 15.9 Hz, 1H), 7.67–7.57 (m, 1H), 7.38 (t, J = 7.5 Hz, 1H), 7.24 (s, 1H), 7.10 (d, J = 1.6 Hz, 1H), 7.07 (dd, J = 8.0, 1.6 Hz, 1H), 6.83 (d, J = 8.0 Hz, 1H), 6.49 (d, J = 15.9 Hz, 1H), 6.02 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 188.68 (s), 165.41 (s), 152.49 (s), 150.45 (s), 148.67 (s), 147.64 (s), 135.42 (s), 130.03 (s), 128.50 (s), 128.40 (s), 126.44 (s), 125.43 (s), 123.63 (s), 114.10 (s), 108.83 (s), 106.82 (s), 101.88 (s). Purity: 98%.

4.1.4.3. 5-chloro-2-formylphenyl (E)-3-(benzo[d][1,3]dioxol-5-yl)acrylate ***(5b)***. Faint yellow solid was obtained. ESI-MS m/z: 330.03. M.p. 181–183 °C. IR KBr (cm^{-1}): 3342, 3074, 2911 (C—H), 1723 (CHO), 1685 (C=O), 1625, 1595, 1501, 1477, 1450 (C=C), 1401 (C—Cl). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.15 (s, 1H), 7.89 (d, J = 2.6 Hz, 1H), 7.83 (d, J = 15.9 Hz, 1H), 7.60 (dd, J = 8.7, 2.7 Hz, 1H), 7.25 (d, J = 8.7 Hz, 1H), 6.86 (d, J = 7.9 Hz, 1H), 6.48 (d, J = 15.8 Hz, 1H), 6.05 (s, 2H). Purity: 98%.

4.1.4.4. 3,5-dichloro-6-formylphenyl (E)-3-(benzo[d][1,3]dioxol-5-yl) acrylate ***(5c)***. Faint yellow solid was obtained. ESI-MS m/z: 363.99. M.p. 152–154 °C. IR KBr (cm^{-1}): 3071, 2908 (C—H), 1729 (CHO), 1691 (C=O), 1622, 1596, 1501, 1494, 1450, 1414 (C=C), 1401 (C—Cl). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.06 (s, 1H), 7.88 (d, J = 15.8 Hz, 1H), 7.80 (s, 1H), 7.71 (s, 1H), 7.11 (d, J = 11.3 Hz, 2H), 6.86 (d, J = 7.9 Hz, 1H), 6.52 (d, J = 15.8 Hz, 1H), 6.05 (s, 2H). Purity: 98%.

4.1.4.5. 4-(diethylamino)-2-formylphenyl (E)-3-(benzo[d][1,3]dioxol-5-yl)acrylate ***(5d)***. Faint yellow solid was obtained. ESI-MS m/z: 367.14. M.p. 128–131 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (s, 1H), 7.79 (d, J = 15.9 Hz, 1H), 7.72 (d, J = 8.9 Hz, 1H), 7.06 (dd, J = 12.1, 4.1 Hz, 2H), 6.82 (d, J = 8.0 Hz, 1H), 6.55 (dd, J = 8.9, 2.3 Hz, 1H), 6.48 (d, J = 15.9 Hz, 1H), 6.34 (d, J = 2.4 Hz, 1H), 6.01 (s, 2H), 3.40 (q, J = 7.1 Hz, 4H), 1.20 (t, J = 7.1 Hz, 6H). Purity: 96%.

4.1.4.6. 2-formylphenyl (E)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylate ***(5e)***. Faint yellow solid was obtained. ESI-MS m/z: 310.08. M.p. 117–119 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.19 (s, 1H), 7.91 (d, J = 7.7 Hz, 1H), 7.79 (d, J = 15.9 Hz, 1H), 7.63 (t, J = 7.7 Hz, 1H), 7.37 (t, J = 7.5 Hz, 1H), 7.24 (s, 1H), 7.14–7.06 (m, 2H), 6.88 (d, J = 8.2 Hz, 1H), 6.50 (d, J = 15.8 Hz, 1H), 4.28 (d, J = 4.7 Hz, 4H). Purity: 98%.

4.1.4.7. 5-chloro-2-formylphenyl (E)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylate ***(5f)***. Faint yellow solid was obtained. HR-MS m/z ($[M + Na]^+$): $C_{18}H_{13}O_5ClNa$, calculated 367.0344, found 367.0340. M.p. 182–184 °C. IR KBr (cm^{-1}): 3309, 2928, 2857 (C—H), 1682 (C=O), 1491, 1439 (C=C), 1411 (C—Cl). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.05 (s, 1H), 7.94 (d, J = 2.7 Hz, 1H), 7.87–7.77 (m, 2H), 7.46 (d, J = 8.7 Hz, 1H), 7.40 (d, J = 2.0 Hz, 1H), 7.32 (dd, J = 8.4, 2.0 Hz, 1H), 6.94 (d, J = 8.4 Hz, 1H), 6.77 (d, J = 16.0 Hz, 1H), 4.29 (td, J = 5.2, 3.7 Hz, 4H). Purity: 98%.

4.1.4.8. 3,5-dichloro-6-formylphenyl (E)-3-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylate ***(5g)***. Faint yellow solid was obtained. ESI-MS m/z: 378.01. M.p. 174–176 °C. IR KBr (cm^{-1}): 3028, 2991, 2934 (C—H), 1726 (CHO), 1691 (C=O), 1625, 1599, 1506 (C=C), 1408 (C—Cl). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.06 (s, 1H), 7.86 (d, J = 15.9 Hz, 1H), 7.80 (d, J = 2.3 Hz, 1H), 7.71 (d, J = 2.3 Hz, 1H), 7.17–7.10 (m, 2H), 6.91 (d, J = 8.3 Hz, 1H), 6.54 (d, J = 15.9 Hz, 1H), 4.31 (dd, J = 10.3, 4.9 Hz, 4H). Purity: 98%.

4.1.4.9. 4-(diethylamino)-2-formylphenyl (E)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylate ***(5h)***. Faint yellow solid was obtained. ESI-MS m/z: 381.16. M.p. 122–124 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.85 (s, 1H), 7.76 (dd, J = 19.7, 12.4 Hz, 2H), 7.20–7.03 (m, 2H), 6.89 (d, J = 8.3 Hz, 1H), 6.57 (dd, J = 8.9, 2.4 Hz, 1H), 6.51 (d, J = 15.9 Hz, 1H), 6.35 (d, J = 2.4 Hz, 1H), 4.29 (q, J = 5.1 Hz, 4H), 3.42 (q, J = 7.1 Hz, 4H), 1.21 (t, J = 7.1 Hz, 6H). Purity: 98%.

4.1.4.10. 2-formylphenyl (E)-3-(3,4-dihydro-2H-benzo[b][1,4]dioxepin-7-yl)acrylate ***(5i)***. Faint yellow solid was obtained. ESI-MS m/z: 324.10. M.p. 110–112 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.22 (s, 1H), 7.95 (dd, J = 7.7, 1.7 Hz, 1H), 7.83 (d, J = 15.9 Hz, 1H), 7.67 (ddd, J = 8.1, 7.6, 1.8 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.31–7.27 (m, 1H), 7.25 (d, J = 2.1 Hz, 1H), 7.20 (dd, J = 8.3, 2.1 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 6.56 (d, J = 15.9 Hz, 1H), 4.30 (dt, J = 11.5, 5.7 Hz, 4H), 2.32–2.19 (m, 2H). Purity: 98%.

4.1.4.11. 5-chloro-2-formylphenyl(E)-3-(3,4-dihydro-2H-benzo[b][1,4] dioxepin-7-yl)acrylate ***(5j)***. Faint yellow solid was obtained. ESI-MS m/z: 358.06. M.p. 174–176 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.14 (s, 1H), 7.89 (d, J = 2.6 Hz, 1H), 7.81 (d, J = 15.9 Hz, 1H), 7.60 (dd, J = 8.7, 2.7 Hz, 1H), 7.25–7.22 (m, 2H), 7.18 (dd, J = 8.3, 2.1 Hz, 1H), 6.99 (d, J = 8.3 Hz, 1H), 6.52 (d, J = 15.9 Hz, 1H), 4.29 (dt, J = 11.9, 5.7 Hz, 4H), 2.30–2.16 (m, 2H). Purity: 98%.

4.1.4.12. 3,5-dichloro-6-formylphenyl(E)-3-(3,4-dihydro-2H-benzo[b] [1,4]dioxepin-7-yl)acrylate ***(5k)***. Faint yellow solid was obtained. ESI-MS m/z: 392.02. M.p. 165–167 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.00 (s, 1H), 8.18 (d, J = 2.6 Hz, 1H), 7.97 (d, J = 2.6 Hz, 1H), 7.87 (d, J = 16.0 Hz, 1H), 7.49 (d, J = 2.1 Hz, 1H), 7.45 (dd, J = 8.3, 2.1 Hz, 1H), 7.02 (d, J = 8.3 Hz, 1H), 6.85 (d, J = 16.0 Hz, 1H), 4.20 (dt, J = 13.7, 5.6 Hz, 4H), 2.19–2.08 (m, 2H). Purity: 98%.

4.1.4.13. 4-(diethylamino)-2-formylphenyl(E)-3-(3,4-dihydro-2H-benzo [b][1,4]dioxepin-7-yl)acrylate ***(5l)***. Faint yellow solid was obtained. ESI-MS m/z: 395.17. M.p. 115–118 °C. IR KBr (cm^{-1}): 3071, 2973, 2922 (C—H), 1735 (CHO), 1664 (C=O), 1608, 1499, 1406 (C=C). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.66 (s, 1H), 7.18 (d, J = 2.1 Hz, 2H), 7.13 (d, J = 2.1 Hz, 1H), 7.12 (d, J = 2.1 Hz, 1H), 6.97 (s, 1H), 6.96 (s, 1H), 6.32 (s, 1H), 6.30 (s, 1H), 4.29–4.27 (m, 4H), 4.25 (t, J = 5.8 Hz, 4H), 2.25–2.20 (m, 4H), 1.25 (s, 2H). Purity: 97%.

4.1.4.14. 5-chloro-2-formylphenyl (E)-3-(2,3,4,5-tetrahydrobenzo[b][1,4] dioxocin-8-yl)acrylate ***(5m)***. Faint yellow solid was obtained. ESI-MS m/z: 372.08. M.p. 96–98 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.14 (s, 1H), 7.88 (d, J = 2.6 Hz, 1H), 7.81 (d, J = 15.9 Hz, 1H), 7.59 (dd, J = 8.7, 2.7 Hz, 1H), 7.24 (dd, J = 5.4, 3.2 Hz, 2H), 7.22 (dd, J = 8.3, 2.2 Hz, 1H), 6.98 (d, J = 8.3 Hz, 1H), 6.51 (d, J = 15.9 Hz, 1H), 4.51–4.47 (m, 2H), 4.33–4.26 (m, 2H), 1.97 (m, J = 11.4, 5.9 Hz, 2H), 1.92–1.85 (m, 2H). Purity: 98%.

4.1.4.15. 3,5-difluoro-2-formylphenyl (E)-3-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylate ***(pro-D104)***. Faint yellow solid was obtained. ESI-MS m/z: 346.07. M.p. 179–181 °C. IR KBr (cm^{-1}): 3433, 3086, 2891 (C—H), 1751 (CHO), 1699 (C=O), 1641, 1607, 1586, 1508 (C=C), 1438 (C—F). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.13 (s, 1H), 7.77 (d, J = 15.9 Hz, 1H), 7.52–7.46 (m, 1H), 7.41 (d, J = 2.0 Hz, 1H), 7.33 (d, J = 2.0 Hz, 1H), 7.32 (d, J = 1.9 Hz, 1H), 6.94 (d, J = 8.4 Hz, 1H), 6.75 (d, J = 16.0 Hz, 1H), 4.31 (m, 2H), 4.29–4.27 (m, 2H). Purity: 98%.

4.1.5. General procedure for the preparation of ***6a-6m*** *and* ***D104***

A mixture of **5a-5m** and **pro-D104** (0.15 g, 0.5 mmol) and tert-butyl alcohol (8 mL) and THF (6 mL) was stirred at room temperature until clear, and 3-methyl-1-butene (0.84 mL, 10 mM) what was cooled at 0 °C was added. Sodium dihydrogen phosphate (0.6 g, 5 mM) and $NaClO_2$

was dissolved in 2 mL aqueous solution and acidified with hydrochloric acid aqueous solution (3 mM) to pH 3–4. The solution was slowly added into the prepared aldehyde. Then the reaction mixture was stirred at room temperature for 5–6 h. The solvent was evaporated away and purified by a flash chromatography in pleasing yield (Yield: 45–60%).

4.1.5.1. (E)-2-((3-(benzo[d][1,3]dioxol-5-yl)acryloyl)oxy)benzoic acid ***(6a)***. Yellow solid was obtained. HR-MS m/z ($[M + Na]^+$): $C_{17}H_{12}O_6Na$, calculated 335.0526, found 335.0527. M.p. 153–155 °C. IR KBr (cm^{-1}): 3077, 3018, 2917 (C—H), 1735 (COOH), 1697 (C=O), 1625, 1604, 1500 (C=C). ^1H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, J = 7.9 Hz, 1H), 7.78 (d, J = 15.9 Hz, 1H), 7.62 (t, J = 7.8 Hz, 1H), 7.34 (d, J = 7.6 Hz, 1H), 7.20 (d, J = 8.1 Hz, 1H), 7.10–7.05 (t, 2H), 6.83 (d, J = 7.9 Hz, 1H), 6.48 (d, J = 15.9 Hz, 1H), 6.03 (s, 2H). Purity: 98%.

4.1.5.2. (E)-2-((3-(benzo[d][1,3]dioxol-5-yl)acryloyl)oxy)-5-chlorobenzoic acid ***(6b)***. Yellow solid was obtained. ESI-MS m/z: 346.02. M.p. 189–191 °C. IR KBr (cm^{-1}): 3080, 2991, 2902 (C—H), 1720 (COOH), 1679 (C=O), 1602, 1503 (C=C), 1452 (C—Cl). ^1H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, J = 2.6 Hz, 1H), 7.78 (d, J = 15.6 Hz, 1H), 7.57 (d, J = 11.2 Hz, 1H), 7.15 (d, J = 8.6 Hz, 1H), 7.09 (s, 1H), 7.06 (d, J = 7.9 Hz, 1H), 6.84 (d, J = 7.9 Hz, 1H), 6.46 (d, J = 15.9 Hz, 1H), 6.04 (s, 2H). Purity: 98%.

4.1.5.3. (E)-2-((3-(benzo[d][1,3]dioxol-5-yl)acryloyl)oxy)-3,5-dichlorobenzoic acid ***(6c)***. Yellow solid was obtained. ESI-MS m/z: 379.99. M.p. 172–175 °C. ^1H NMR (600 MHz, DMSO-d_6) δ 8.08 (d, J = 2.6 Hz, 1H), 7.89 (d, J = 2.6 Hz, 1H), 7.82 (d, J = 15.9 Hz, 1H), 7.56 (d, J = 1.5 Hz, 1H), 7.32 (dd, J = 8.1, 1.5 Hz, 1H), 7.00 (d, J = 8.0 Hz, 1H), 6.81 (d, J = 15.9 Hz, 1H), 6.11 (s, 2H). Purity: 98%.

4.1.5.4. (E)-2-((3-(benzo[d][1,3]dioxol-5-yl)acryloyl)oxy)-4-(diethylamino)benzoic acid ***(6d)***. Yellow solid was obtained. ESI-MS m/z: 383.14. M.p. 160–162 °C. ^1H NMR (600 MHz, $CDCl_3$) δ 7.92 (d, J = 9.1 Hz, 1H), 7.75 (d, J = 15.9 Hz, 1H), 7.11–7.06 (m, 1H), 7.04 (dd, J = 8.1, 1.6 Hz, 1H), 6.81 (d, J = 8.0 Hz, 1H), 6.47 (d, J = 15.9 Hz, 2H), 6.30 (d, J = 2.0 Hz, 1H), 6.02 (s, 2H), 3.38 (q, J = 7.1 Hz, 4H), 1.18 (t, J = 7.1 Hz, 6H). Purity: 98%.

4.1.5.5. (E)-2-((3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acryloyl)oxy) benzoic acid ***(6e)***. Yellow solid was obtained. ESI-MS m/z: 326.08. M.p. 102–105 °C. ^1H NMR (400 MHz, DMSO-d_6) δ 7.92 (dd, J = 7.8, 1.7 Hz, 1H), 7.71 (d, J = 16.0 Hz, 1H), 7.63 (td, J = 7.8, 1.7 Hz, 1H), 7.41–7.35 (m, 2H), 7.30 (dd, J = 8.4, 2.0 Hz, 1H), 7.24 (dd, J = 8.1, 0.9 Hz, 1H), 6.92 (d, J = 8.4 Hz, 1H), 6.72 (d, J = 16.0 Hz, 1H), 4.28 (dd, J = 9.9, 5.1 Hz, 4H). ^{13}C NMR (101 MHz, DMSO) δ 165.94 (s), 165.08 (s), 149.98 (s), 145.90 (s), 143.60 (s), 133.40 (s), 131.26 (s), 127.38 (s), 125.94 (s), 125.12 (s), 123.79 (s), 122.48 (s), 117.53 (s), 117.15 (s), 115.31 (s), 64.39 (s), 63.93 (s). Purity: 98%.

4.1.5.6. (E)-5-chloro-2-((3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl) acryloyl)oxy)benzoic acid ***(6f)***. Yellow solid was obtained. HR-MS m/z ($[M + Na]^+$): $C_{18}H_{13}O_6ClNa$, calculated 383.0293, found 383.0292. M.p. 157–159 °C. IR KBr (cm^{-1}): 3071, 2997, 2881 (C—H), 1726 (COOH), 1685 (C=O), 1625, 1599, 1506 (C=C), 1436 (C—Cl). ^1H NMR (600 MHz, DMSO-d_6) δ 7.89 (d, J = 2.7 Hz, 1H), 7.74 (d, J = 2.8 Hz, 1H), 7.72–7.71 (m, 1H), 7.38 (d, J = 2.0 Hz, 1H), 7.34 (d, J = 8.6 Hz, 1H), 7.31 (dd, J = 8.4, 2.0 Hz, 1H), 6.92 (d, J = 8.4 Hz, 1H), 6.73 (d, J = 16.0 Hz, 1H), 4.30 (dd, J = 5.6, 2.2 Hz, 2H), 4.27 (dd, J = 5.4, 2.0 Hz, 2H). Purity: 98%.

4.1.5.7. (E)-3,5-dichloro-2-((3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl) acryloyl)oxy)benzoic acid ***(6g)***. Yellow solid was obtained. ESI-MS m/z: 394.00. M.p. 153–155 °C. ^1H NMR (400 MHz, DMSO-d_6) δ 7.88 (s, 1H), 7.72 (s, 1H), 7.65 (d, J = 15.9 Hz, 1H), 7.37 (s, 1H), 7.14 (d, J = 8.1 Hz, 1H), 6.82 (d, J = 8.0 Hz, 1H), 6.63 (d, J = 15.9 Hz, 1H), 5.95 (s, 2H), 2.35 (s, 2H). Purity: 98%.

4.1.5.8. (E)-4-(diethylamino)-2-((3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acryloyl)oxy)benzoic acid ***(6h)***. Yellow solid was obtained. ESI-MS m/z: 397.15. M.p. 107–109 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, J = 15.9 Hz, 1H), 7.74 (d, J = 8.9 Hz, 1H), 7.13 (d, J = 1.9 Hz, 1H), 7.10 (dd, J = 8.3, 2.0 Hz, 1H), 6.89 (d, J = 8.3 Hz, 1H), 6.57 (dd, J = 8.9, 2.4 Hz, 1H), 6.51 (d, J = 15.9 Hz, 1H), 6.35 (d, J = 2.4 Hz, 1H), 4.29 (q, J = 5.1 Hz, 4H), 3.42 (q, J = 7.1 Hz, 4H), 1.21 (t, J = 7.1 Hz, 6H). Purity: 98%.

4.1.5.9. (E)-2-((3-(3,4-dihydro-2H-benzo[b][1,4]dioxepin-7-yl)acryloyl) oxy)benzoic acid ***(6i)***. Yellow solid was obtained. ESI-MS m/z: 340.09. M.p. 163–164 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, J = 7.8 Hz, 1H), 7.76 (d, J = 15.9 Hz, 1H), 7.64–7.58 (m, 1H), 7.33 (t, J = 7.5 Hz, 1H), 7.21 (d, J = 3.5 Hz, 2H), 7.15 (dd, J = 8.3, 1.8 Hz, 1H), 6.97 (d, J = 8.3 Hz, 1H), 6.51 (d, J = 15.9 Hz, 1H), 4.28 (dt, J = 11.2, 5.7 Hz, 4H), 2.25–2.21 (m, 2H). Purity: 98%.

4.1.5.10. (E)-5-chloro-2-((3-(3,4-dihydro-2H-benzo[b][1,4]dioxepin-7-yl)acryloyl)oxy)benzoic acid ***(6j)***. Yellow solid was obtained. ESI-MS m/z: 374.06. M.p. 210–212 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 7.76 (d, J = 15.9 Hz, 1H), 7.57 (dd, J = 8.6, 2.5 Hz, 1H), 7.21 (d, J = 1.7 Hz, 1H), 7.15 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 8.4 Hz, 1H), 6.49 (d, J = 15.9 Hz, 1H), 4.28 (dt, J = 11.4, 5.8 Hz, 4H), 2.25–2.21 (m, 2H). Purity: 96%.

4.1.5.11. (E)-3,5-dichloro-2-((3-(3,4-dihydro-2H-benzo[b][1,4]dioxepin-7-yl)acryloyl)oxy)benzoic acid ***(6k)***. Yellow solid was obtained. ESI-MS m/z: 408.02. M.p. 198–199 °C. ^1H NMR (400 MHz, DMSO-d_6) δ 8.07 (d, J = 2.6 Hz, 1H), 7.89 (d, J = 2.6 Hz, 1H), 7.81 (d, J = 16.0 Hz, 1H), 7.48 (d, J = 2.0 Hz, 1H), 7.43 (dd, J = 8.4, 2.1 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 6.82 (d, J = 16.0 Hz, 1H), 4.24–4.14 (m, 4H), 2.20–2.07 (m, 2H). Purity: 98%.

4.1.5.12. (E)-4-(diethylamino)-2-((3-(3,4-dihydro-2H-benzo[b][1,4] dioxepin-7-yl)acryloyl)oxy)benzoic acid ***(6l)***. Yellow solid was obtained. ESI-MS m/z: 411.17. M.p. 145–147 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 7.92 (d, J = 9.0 Hz, 1H), 7.80–7.68 (m, 1H), 7.21 (d, J = 2.0 Hz, 1H), 7.15 (dd, J = 8.3, 2.1 Hz, 1H), 6.97 (d, J = 8.3 Hz, 1H), 6.57–6.46 (m, 2H), 6.31 (d, J = 2.1 Hz, 1H), 4.27 (dt, J = 9.5, 5.7 Hz, 4H), 3.39 (q, J = 7.0 Hz, 4H), 2.34–2.10 (m, 2H), 1.19 (t, J = 7.1 Hz, 6H). Purity: 98%.

4.1.5.13. (E)-5-chloro-2-((3-(2,3,4,5-tetrahydrobenzo[b][1,4]dioxocin-8-yl)acryloyl)oxy)benzoic acid ***(6m)***. Yellow solid was obtained. ESI-MS m/z: 388.07. M.p. 80–82 °C. ^1H NMR (400 MHz, DMSO-d_6) δ 7.89 (d, J = 2.7 Hz, 1H), 7.77–7.67 (m, 2H), 7.48–7.40 (m, 2H), 7.31 (d, J = 8.6 Hz, 1H), 6.98 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 16.0 Hz, 1H), 4.40 (t, J = 5.4 Hz, 2H), 4.23 (t, J = 5.3 Hz, 2H), 1.88 (dd, J = 12.5, 6.8 Hz, 2H), 1.80–1.73 (m, 2H). Purity: 98%.

4.1.5.14. (E)-2-((3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acryloyl)oxy)-4,6-difluorobenzoic acid ***(D104)***. Yellow solid was obtained. ESI-MS m/z: 362.06. M.p. 154–156 °C. IR KBr (cm^{-1}): 3428, 2959, 2880 (C—H), 1727 (COOH), 1686 (C=O), 1626, 1600, 1573, 1506 (C=C), 1435 (C—F). ^1H NMR (600 MHz, DMSO-d_6) δ 8.39 (d, J = 2.4 Hz, 1H), 8.12 (d, J = 2.4 Hz, 1H), 7.85 (d, J = 15.9 Hz, 1H), 7.43 (d, J = 2.1 Hz, 1H), 7.35 (dd, J = 8.4, 2.1 Hz, 1H), 6.94 (d, J = 8.3 Hz, 1H), 6.82 (d, J = 15.9 Hz, 1H), 4.32–4.30 (m, 2H), 4.30–4.27 (m, 2H). Purity: 98%.

4.2. Biological evaluation

4.2.1. Reagents and cell culture

RAW 264.7 cell line was a gift from Nanjing University.

Lipopolysaccharides (LPS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) were from Sigma. 6-well and 96-well plates were from Beyotime biotechnology.

RAW 264.7 cell were grown in High glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin and propagated at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

Compounds **5a-5m, 6a-6m, pro-D104**, **D104**, chlorogenic acid and acetylsalicylic acid were dissolved in dimethyl sulfoxide to make stock solutions, respectively, and kept at −20 °C. The final concentration of the vehicle in the solution never exceeded 0.1% and had no effects on NO production and cell viability.

4.2.2. Anti-inflammatory assay

Accumulation of nitrite (NO_2^-), an indicator of NO synthase activity, in culture supernatant fluids was measured based on Griess reaction [17]. Briefly, Cells (2×10^4) were seeded in 100 μL of DMEM into 96-well plates and co-incubated with different concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50, 100 μM) of compounds **5a-5m**, **6a-6m**, **pro-D104** and **D104** in the absence or presence of LPS (500 ng/ml) for 48 h. Meanwhile, chlorogenic acid and acetylsalicylic acid also were tested as positive controls. Culture supernatant fluids were mixed with 100 μL Griess reagent at room temperature for 5 min. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an absorbance reading at 540 nm.

4.2.3. Total RNA isolation

The concentration and purity of RNA were detected. The operation was as follows [18]. Put cells in tripure isolation reagent and incubate samples at 15–25 °C for 5 min. Then 200 μL chloroform was added. The mixture were shook vigorously with vortex for 15 s and incubated at 15–25 °C for 2–15 min. After centrifuged at 12000*g* at 4 °C for 15 min, aqueous phase was pipetted into a clean screw-cap centrifuge tube carefully and lower phases were discarded. Aqueous phase was mixed with an equal volume of isopropyl alcohol and incubated at 15–25 °C for 5–10 min. Then the mixture was centrifuged at 12000*g* at 4 °C for 10 min and the precipitate was washed with 1 ml 75% ethanol by spinning briefly. After centrifuged at 7500*g* at 4 °C for 5 min, the supernatant was aspirated and precipitate was dried. Then diethyl pyrocarbonate (DEPC/ddH_2O) was added and incubated at 55–60 °C for 10–15 min to dissolve precipitate. Total RNA concentration and the absorbance 260/280 ratios were measured.

4.2.4. QRT-PCR analysis

Quantitative RT-PCR assays [19] to investigate differences in the expression of the mRNAs of interest were performed on a 7300 Sequence Detection System (Applied Biosystems) using EvaGreen Dye (Biotium, Hayward, CA). Briefly, 2 μL total RNA (1 μg/μL) was reverse transcribed into cDNA using PrimeScript RTase (Takara Bio, Shiga, Japan) and a Oligo dT primer (Invitrogen) under the following conditions:30 °C for 10 min, 42 °C for 20 min, and 99 °C for 5 min. The conditions for the PCR were as follows: 95 °C for 5 min, 95 °C for 30 s, and 60 °C for 45 s, for 40 cycles. All reactions, including the no template controls, were run in triplicate. After the reactions were complete, the C_T values were determined using fixed threshold settings. The mRNA expression was normalized to β-actin expression in this study. The amount of mRNA to relative to the internal control β-actin was calculated using the eq. $1000 * 2_T^{-\Delta C}$, in which $\Delta C_T = C_{T,mRNA}\text{-}C_{T,\beta\text{-actin}}$.

4.2.5. Protein extraction and western blotting analysis

The RAW 264.7 cells on 6-well plates were rinsed twice with cold PBS and lysed in 100 μL RIPA lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 1 mM PMSF) on ice for 30 min. Insoluble components of cell lysates were removed by centrifugation at 12000*g* for 5 min at 4 °C, and protein concentrations were measured using the BCATM protein quantification kit. The iNOS protein level was determined by western blotting analysis of 50 μg of cell extract using the antibody against iNOS (D6B6S). Briefly, the protein samples were centrifuged (4 °C, 12000 g, 10 min) and boiled for 10 min, then subjected to a 10% SDS-polyacrylamide gel electrophoresis at a constant 20 mA current for 1 h. The resolved proteins were transferred to a PVDF membrane by wet rotation at 70 mV and blocked with a blocking buffer (2% free fat milk, 10 mM Tris-Cl, 50 mM NaCl, 0.1% Tween 20, pH 7.4) at room temperature for 1 h. The membrane was incubated with primary antibodies against iNOS and GAPDH overnight on the converter at low temperature (4 °C). The next day, the membrane was washed using the washing buffer (10 mM Tris-Cl, 50 mM NaCl, 0.1% Tween 20, pH 7.4) three times, twice for 5 min and once for 10 min, to remove any nonspecific primary antibody binding. Then the membrane was incubated with an appropriate dilution of secondary antibody at room temperature for 2 h. After washing three times with washing buffer, the membrane was illuminated with ECL reagent according to the manufacturer's instructions. A photograph of the gel was taken, and the relative band density was analyzed by optical densitometry using Image J.

4.2.6. Assay for cytotoxic activity

Non-toxic concentrations of compounds **5a-5m**, **6a-6m**, **pro-D104** and **D104** were determined according to MTT test [20] and concentration of 50 μM were chosen to test the effects of caffeoyl salicylate analogs on nitric oxide production. MTT was dissolved at 4 mg/ml in PBS and used essentially as previously described. Briefly, cell lines in logarithmic phase were seeded at a density of 3×10^3 cells/well in 100 μL of DMEM into 96-well microtiter plates. After 24 h, exponentially growing cells were exposed to the indicated compounds at various concentrations. After 48 h in final volumes of 200 μL, cell survival was determined by the addition of an MTT solution (20 μL of 4 mg/mL MTT in PBS) for 4 h. After carefully removing the medium, the precipitates were dissolved in 200 μL of DMSO, shaken mechanically for 10 min, and then absorbance values at a wavelength of 540 nm were taken on an LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentages with respect to untreated cells.

Conflict of interest

The authors confirm that this article content has no conflict of interests.

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