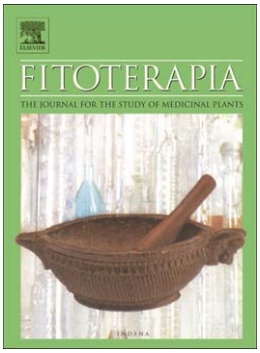

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Synthesis and Biological Evaluation of Pyranoisoflavone Derivatives as Anti-Inflammatory Agents

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Abstract

In this paper, barbigerone (**1a**) and its twenty-seven related structural analogues were synthesized via complementary synthetic routes and evaluated their anti-inflammatory effects on the expression of TNF-α in LPS-stimulated splenocytes. Among these compounds, **1a**, **1d**, **1f** and **1g** were found to remarkably inhibit TNF-α production. Furthermore, **1g** showed the most potent and dose-dependent manner inhibitory effect on TNF-α release, with better IC_{50} vaule (3.58 μM) than barbigerone (8.46 μM). Oral administration of **1g** at 20 mg/Kg/day for two weeks demonstrated obviously protective effect in adjuvant-induced arthritis models as evaluated by clinical score of paws, and histological examination of joint tissues from rats. Mechanism studies on mRNA and protein level suggested that **1g** inhibited the TNF-α production via depressing TNF-α converting enzyme **(**TACE) mRNA expression. In conclusion, these data show **1g** with potential therapeutic effects as an anti-inflammatory agent.

Key Words: Tumor necrosis factor α, inflammatory, pyranoisoflavone, TACE mRNA

1. Introduction

Inflammation is a primary response of an organism to various injurious stimuli, and the typical syndromes of inflammation are characterized by heat, redness, pain, even loss of function, and so on.[1] Cytokines are directly implicated in the processes of inflammation, such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α). TNF-α, a pro-inflammatory cytokine synthesized as a membrane-bound protein which can be cleaved by proteases to soluble form, is commonly released by macrophages and lymphocytes, and plays a very important role in the inflammatory cascades through activating nuclear factor-κB (NF-κB).[2, 3] Over expression of cytokines is responsible for a number of inflammatory pathological conditions such as asthma, colitis, hepatitis and rheumatoid arthritis (RA).

Rheumatoid arthritis, an autoimmune disease, is a synovial inflammatory disease of unknown etiology that occurs in about 1% population of the world, people of any age can be affected.[4-6] As a debilitating disease, RA causes chronic inflammation and involves the destruction of joints of the hands and feet, and it also causes inflammation of the tissue around the joints, as well as in other organs of the body.[7, 8] It has been proposed that the initiating event in RA involves an infectious agent or other environmental exposure.[9] Over the past decades, significant advances in understanding the pathogenesis of RA have led to the identification of novel therapeutic targets.[10] Cytokines are directly implicated in the processes of RA. Accordingly, cytokines modulation alters the outcomes in many rodent models of arthritis.[11] Hence, inhibition of TNF-α production is one

of the crucial aspects for the disease control and prognosis improvement, and now has been targeted in the standard treatment of patients with RA.[12, 13]

Barbigerone (**1a**, **Figure 1**), a naturally occurring pyranoisoflavone, was first isolated from the seeds of *Tephrosia barbigeria* and can be obtained from other plants.[14-17] Previous studies discovered that barbigerone exhibited various pharmacological properties, such as anti-plasmodia, antioxidant and 15-lipoxygenase inhibitory activity.[18, 19] Recently, barbigerone was also found to inhibit the proliferation of several cancer cell lines.[16, 20, 21]

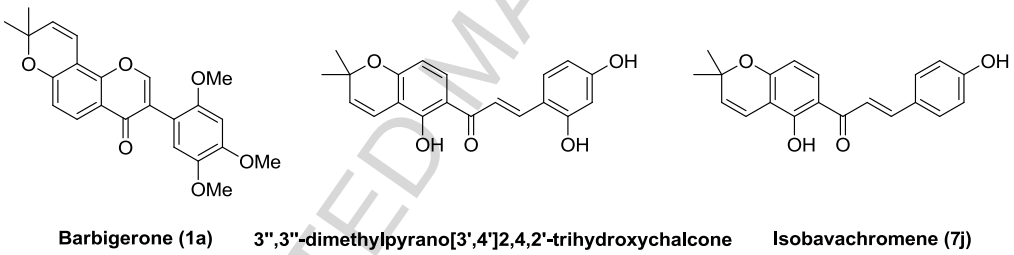


Figure 1. Structure of barbigerone and some typical bioactive pyranochalcones

In spite of the increasing interest in biological profiles of barbigerone, little attention has been paid to its structure-activity relationship (SAR) study. The first total synthesis of barbigerone presented by Pathak, however, was inefficient and low-overall-yielding (less than 4%).[22] On the other hand, isolation of barbigerone from natural sources was also proven in our hands to be inefficient and difficult to achieve enough amounts for in-depth biological and medicinal studies.[23] In our previous work, we have developed a concise and efficient route for the synthesis of barbigerone, which also proves to be viable for the synthesis of its several unnatural

analogues.[24] Herein, we report the complementary synthesis, SAR and mechanism study of barbigerone and its structural analogues as anti-inflammatory agents for the first time.

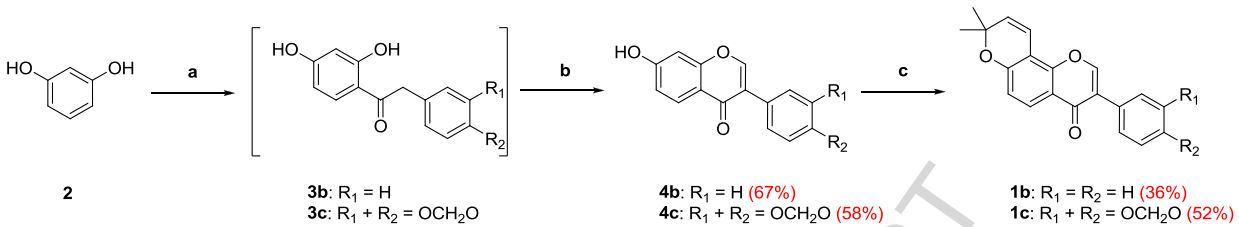
2. Results and discussion

2.1 Chemistry

In order to explore the SAR of barbigerone, its related structural analogues were prepared according to the complementary synthetic routes outlined in the **Schemes 1-6**. All of the target compounds were synthesized from simple and commercial available starting materials.

The synthesis of 7-hydroxy-isoflavones by using 'one-pot method' described by Singh and Pratap was involved in the synthesis of target compounds **1b** and **1c**.[25] Briefly, resorcinol **2** reacted with phenylacetic acid to generate intermediate deoxybenzoins **3** in the presence of anhydrous Lewis acid $ZnCl_2$. Based on Vilsmeier-Haack reaction, the isoflavone ring closing to form 7-hydroxy-isoflavone **4** was finished in reasonable yield. Treatment of **4b** and **4c** with 1,1-diethoxy-3-methylbut-2-ene to construct pyranoid ring to afford **1b** and **1c** proceed smoothly according to the known pyranoid formation method with the over-all yield of 24% and 30%, respectively (as shown in **Scheme 1**).[26]

Scheme 1 'One-pot' synthesis of **1b** and **1c**

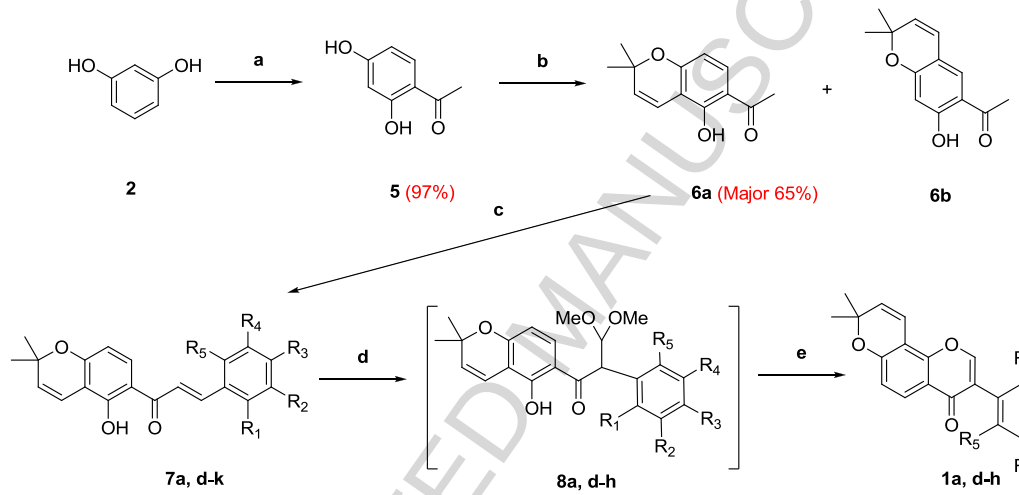


Reagents and conditions: (a) anhy. $ZnCl_2$, 2-phenylacetic acsid derivatives, 120 °C; (b) dry DMF, BF_3•OEt_2, 0 °C, $MeSO_2Cl$, 50-110 °C, 3 h; (c) 1,1-diethoxy-3-methylbut-2-ene, 3-picoline(cat. amount), dry xylene, reflux, 12h.

The preparation of some special 2-phenylacetic acid needs several steps from simple starting material, 'one-pot method' is not fit for the synthesis of barbigerone,[27] the second method for the synthesis of barbigerone and main related analogues was designed and carried out as we described before.[24] As outlined in **Scheme 2**, our synthetic work began with Friedel-Crafts acylation of resorcinol **2** with acetic anhydride under the catalytic effect of BF_3•Et_2O, which afforded 1-(2,4-dihydroxyphenyl)ethanone **5** in 79% yield. Intermediate **6a** was produced from **5** and 1,1-dimethoxy-3-methylbut-2-ene with 65% yield, which was then subjected to 'Aldol reactions' with different aldehydes under basic condition in methanol to afford the key intermediate chalcones **7a, d-k** with moderate to good yields. To synthesize intermediates **8**, the oxidation-rearrangement reaction of **7** was examined under different conditions. TTN (Thallium(III) nitrate trihydrate) proved to be superior to other oxidants such as DIB (Iodosobenzene diacetate) and HTIB (Hydroxy(tosyloxy)iodobenzene), which drove the reaction to completion under mild conditions in 12 hours. However, the oxidation-rearrangement of **7i-k** with unprotected hydroxy group and electron-withdrawing group in the right phenyl ring was proven to be inefficient. After careful

work-up and purification, the crude products **8a, d-h** were directly treated with 1N HCl in methanol under reflux, construction of the main isoflavone skeleton was finished and the target compounds **1a, d-h** were obtained with yields ranging from 64% to 78% (based on chalcones).

Scheme 2 Synthesis of **1a, d-f** based on 'oxidation-rearrangement reaction'



7a: $R_2 = R_5 = H$, $R_1 = R_3 = R_4 = OMe$ (69%)
7d: $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OMe$ (72%)
7e: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = OMe$ (69%)
7f: $R_1 = R_4 = R_5 = H$, $R_2 = R_3 = OMe$ (73%)
7g: $R_2 = R_3 = R_5 = H$, $R_1 = R_4 = OMe$ (77%)
7h: $R_1 = R_5 = H$, $R_2 = R_3 = R_4 = OMe$ (81%)
7i: $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OH$ (76%)
7j: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = OH$ (72%)
7k: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = CF_3$ (43%)

8a: $R_2 = R_5 = H$, $R_1 = R_3 = R_4 = OMe$
8d: $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OMe$
8e: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = OMe$
8f: $R_1 = R_4 = R_5 = H$, $R_2 = R_3 = OMe$
8g: $R_2 = R_3 = R_5 = H$, $R_1 = R_4 = OMe$
8h: $R_1 = R_5 = H$, $R_2 = R_3 = R_4 = OMe$

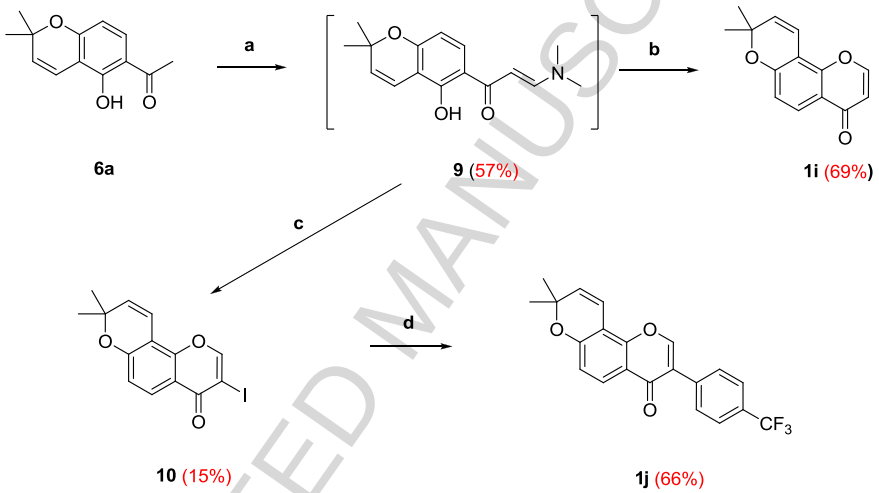
1a: $R_2 = R_5 = H$, $R_1 = R_3 = R_4 = OMe$ (72%)
1d: $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OMe$ (78%)
1e: $R_1 = R_2 = R_4 = R_5 = H$, $R_3 = OMe$ (78%)
1f: $R_1 = R_4 = R_5 = H$, $R_2 = R_3 = OMe$ (64%)
1g: $R_2 = R_3 = R_5 = H$, $R_1 = R_4 = OMe$ (68%)
1h: $R_1 = R_5 = H$, $R_2 = R_3 = R_4 = OMe$ (71%)

Reagents and conditions: (a) anhy. $AlCl_3$, AcCl, dry CS_2, reflux, 12 h; (b) 1,1-diethoxy-3-methylbut-2-ene, 3-picoline(cat. amount), dry xylene, reflux, 12h; (c) aq. 40% NaOH, various aldehydes, MeOH, 0 °C-r.t., 12-36 h; (d) Thallium(III) nitrate trihydrate(TTN), MeOH, r.t. overnight; (e) 1N HCl, MeOH, reflux, 6 h.

Treatment of **6a** with DMF-DMA under reflux, intermediate **9** was produced, which was then transformed to target compound **1i** and another key intermediate **10** by reacting with 1N HCl or

iodine in methanol.[28] Target compound **1j** was synthesized by coupling **10** to 4-(trifluoromethyl)phenylboronic acid smoothly with 82% yield by using Pd/C as catalyst (as shown in **Scheme 3**).[29]

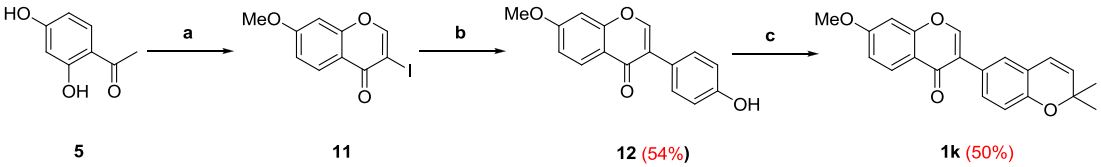
Scheme 3 Synthesis of **1i** and **1j**



Reagents and conditions: (a) DMF-DMA, reflux, 6 h; (b) 1N HCl, MeOH; (c) I_2, MeOH, r.t., overnight; (d) 4-(trifluoromethyl)phenylboronic acid, 10% Pd/C (cat. amount), K_2CO_3, MeCN:H_2O = 1:1, reflux, overnight.

Several special target compounds were also prepared in order to investigate the importance of pyranoid ring according to the **Schemes 4-6**. By using the same method as the synthesis of **1j** described above, target compound **1k** was prepared from **5** with the yield of 73% (last step).

Scheme 4 Synthesis of **1k**

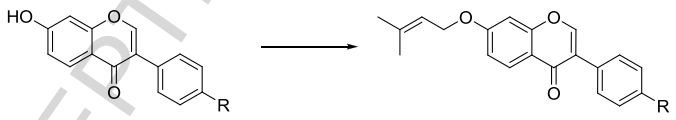


5 **11** **12** (54%) **1k** (50%)

Reagents and conditions: (a) DMF-DMA, reflux, 6 h; then I_2, MeOH, r.t., overnight; (b) 4-hydroxyphenylboronic acid, 10% Pd/C (cat. amount), K_2CO_3, MeCN:H_2O = 1:1, reflux, overnight; (c) 1,1-diethoxy-3-methylbut-2-ene, 3-picoline(cat. amount), dry xylene, reflux, 12h.

Target compound **1l** and **1m** without pyranoid ring were prepared from **4b** and **13** by treating them with 1-bromo-3-methylbut-2-ene with good yields under mild condition, 68% and 72%, respectively.[30]

Scheme 5 Synthesis of **1l** and **1m**



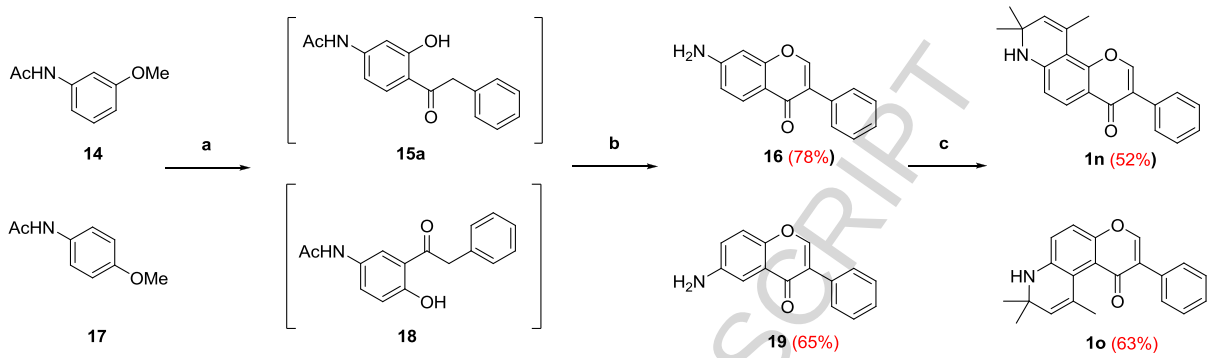
4b: R = H
13: R = OMe

1l: R = H (69%)
1m: R = OMe (72%)

Reagents and conditions: 1-bromo-3-methylbut-2-ene, K_2CO_3, acetone, reflux, overnight.

Intermediates 7-amino isoflavone **16** and 6-amino isoflavone **19** were prepared from compounds **14** and **17** by using the same method as the synthesis of 7-hydroxy isoflavone **4**. By using silicotungstic acid as catalyst, intermediates **16** and **19** reacted with acetone under reflux for overnight afforded target compounds **1n** and **1o** with acceptable yield, 52% and 62%, respectively.[31]

Scheme 6 General route for the synthesis of aza-isoflavone **1n** and **1o**



Reagents and conditions: (a) 2-phenylacetic acid, anhy. $AlCl_3$, dry CS_2, reflux, 12 h; (b) dry DMF, BF_3•OEt_2, 0 °C, $MeSO_2Cl$, 50-110 °C, 3 h; then 6N HCl, MeOH, reflux, 6 h; (c) acetone, silicotungstic acid (cat. amount), reflux, 12 h.

2.2 Inhibitory effect of TNF-α production *in vitro* and SAR analysis

Activated inflammatory cells play crucial roles in the initiation and maintenance of inflammation. LPS promotes the secretion of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, in many cell types especially in lymphocytes and macrophages. Reduction of TNF-α production could serve as a therapy for inflammatory diseases. Barbigerone and its twenty-seven derivatives were evaluated for their anti-inflammatory activity of inhibiting the TNF-α release in LPS-stimulated mouse splenocyte. The result was shown in **Figure 2**, dexamethasone (DEX), a steroidal anti-inflammatory drug, was chosen as a positive control. Among these compounds, **1a**, **1d**, **1f** and **1g** effectively suppressed LPS-induced TNF-α production at 10 μM (the inhibitory rates were more than 65.0%) and closed to positive control DEX. **1g** showed the most potent inhibitory effect on

LPS-induced TNF-α production and its inhibitory rates reached 78.3%, compared to the LPS control.

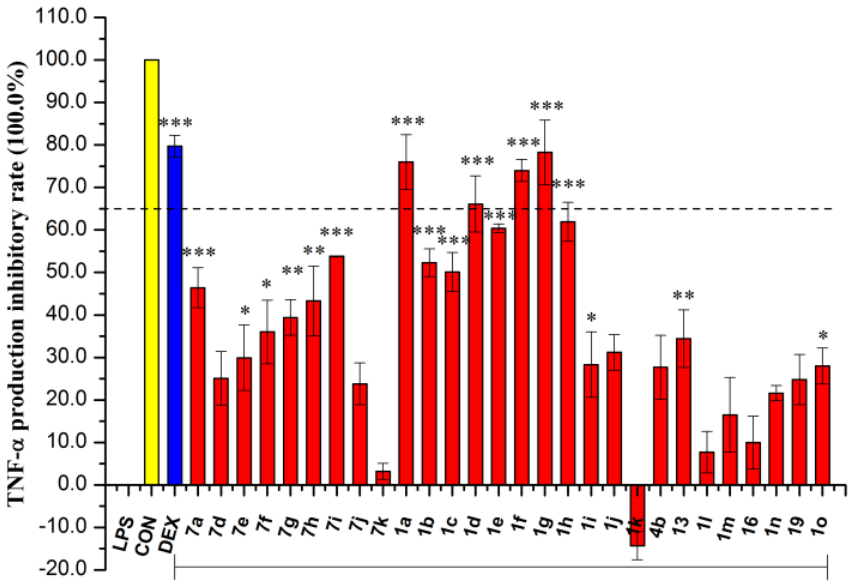


Figure 2. Inhibitory effects on TNF-α production at a concentration of 10 μM in spleen cells. The amount of TNF-α with LPS-treated group was set as 100.0% and the vehicle control as 0.0%. Inhibition (%) = [LPS(OD450) - compounds(OD450)]/[LPS(OD450) - control(OD450)] × 100%. Values (means ± SD) were combined from three independent experiments (* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).

Compared with **1b**, inhibitory potency decreased dramatically when substituent of electron-withdrawing group was added (para-trifluoromethyl for **1j**) on the right phenyl ring. However, the addition of electron-donating group obviously showed positive effect on inhibitory potency (different types of methoxy for compounds **1a**, **c-h**). Compound **1d** with meta-methoxy on the right phenyl ring showed a little better potency than that of compound **1e** with para-methoxy.

Addition of methoxy groups helped increasing the potency (78.3% for **1g**, 76.0% for **1a** and 61.9% for **1h** were better than 52.3% for **1b**). A conclusion that dimethoxy on position C2 and C5 of right phenyl ring was the dominant factor for maintaining and enhancing the inhibitory potential could be drawn. These results also indicated that the properties of inhibitory potency may contribute to the bulk and electron density of the right aromatic ring. Taking some natural pyranochalcones possess anti-inflammation property into consideration (**Figure 1**),[32, 33] chalcones **7a**, **d-k**, precursors of isoflavones, were also tested for their TNF-α production inhibitory effect. However, chalcones **7** showed week to moderate inhibitory effect, and all of them were not as good as the corresponding target compounds. For example, 66.1% for **1d** was better than that of 25.0% for **7d**, 78.3% for **1g** was better than 39.4% for **7g**. This indicated that chromone structure was very important for their activity against TNF-α production. Removal of pyranoid ring on the left phenyl ring clearly had negative effect on inhibitory potency (53.2% for **1b** vs 27.7% for **4b**, 60.4% for **1e** vs 34.4% for **13**). The presence of prenyl seemed to reduce the capacity of 7-hydroxyl isoflavones **4b** (27.7% vs 7.7% for **1l**) and **13** (34.4% vs 16.5% for **1m**). On the other hand, the migration of pyranoid ring from the left phenyl ring to the right phenyl ring or replacement of pyranoid ring with quinolin ring also reduced the inhibitory activity dramatically (60.4% for **1e** vs -14.3% for **1k**, 52.3% for **1b** vs 21.6% for **1n** or 28% for **1o**). These results suggested that pyranoid ring on the left phenyl ring was very important for the activity and could not be modified.

Furthermore, compounds **1a**, **1d**, **1f** and **1g** were chosen to investigate both the inhibitory activity against TNF-α production and the cytotoxicity in spleen cells at different concentrations. As

exhibited in **Table 1**, **1g** and **1a** presented remarkable inhibitory effect on TNF-α production, with IC_{50} of 3.58 and 8.46 μM respectively. To check whether the inhibitory effect against TNF-α production was related to cell viability, a MTT assay was also adopted. As the result shown, the two compounds exerted little cytotoxicity in splenocyte (without or with LPS, IC_{50} > 70.00 μM).

Table 1. IC_{50} values on TNF-α production and cytotoxicity in mouse spleen cells.

		cytotoxicity IC_{50} (μM)	
CPD	TNF-α IC_{50} (μM)	without LPS	with LPS
1a	8.46	82.14	85.99
1d	> 10	-	-
1f	> 10	-	-
1g	3.58	77.78	74.39

*Cytotoxicity = [1 - Compounds(OD570)/Control(OD570)] × 100%.

2.3 Anti-arthritic effects of 1g on AIA models

Adjuvant-induced arthritis (AIA) is a form of chronic arthritis and commonly used to test agents for anti-inflammatory activity. As the most potent target compound **1g** was chosen to examine the ability of anti-inflammation on AIA rats. AIA Lewis rats were treated orally with **1g** at a dose of 10 mg/kg/day and 20 mg/kg/day following the appearance of first AIA signs. Results showed that the severity of AIA was dose-dependently improved in **1g** treated animal models (**Figure 3**).

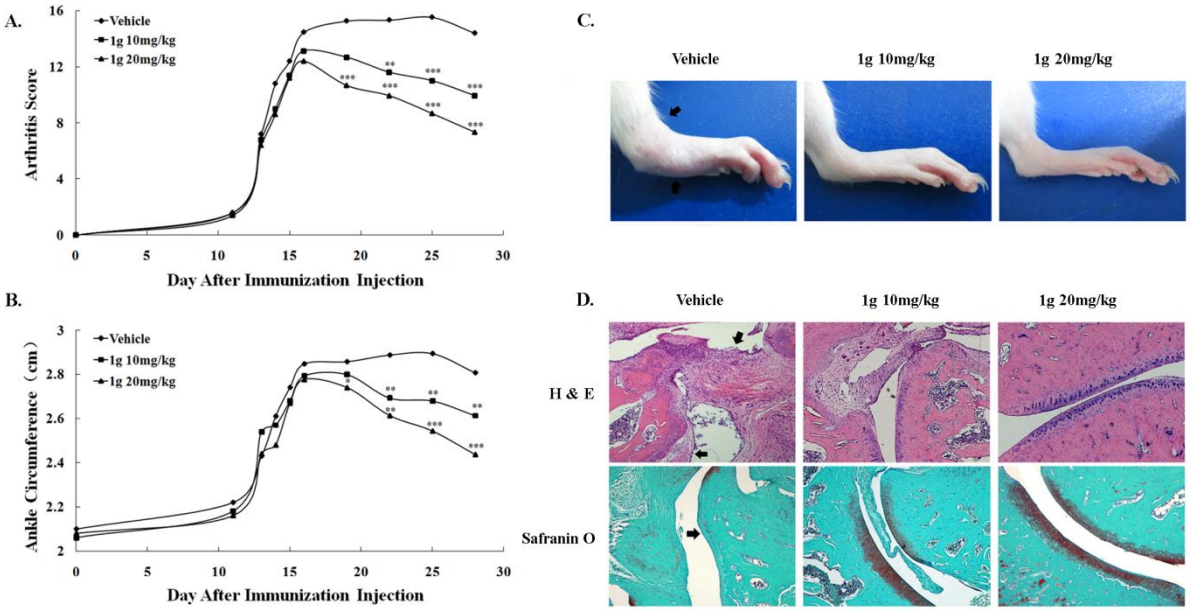


Figure 3. Anti-arthritic effects of **1g** on AIA models. **1g** was orally administered once daily at a dose of 10 mg/kg and 20 mg/kg. (A) Clinical disease activity score of **1g**. The result were expressed as the means (n = 5; ***P < 0.001). (B) Ankle circumference after adjuvant injection. The result were expressed as the means (n = 5; ***P < 0.001). (C) Macroscopic observations of the ankles on day 30. (D) Representative histopathologies of the knee joints stained with H&E and Safranin O fast green (× 40).

The time course of adjuvant arthritic development, expressed as arthritic scores and ankle circumference, were significantly reduced in the therapeutic process (**Figure 3A and 3B**). This effect was further confirmed with macroscopic observations of the ankles and joint histology. Compared to the vehicle control groups, **1g** significantly reduced the paw edema of rats after 17 days continuous treatment at a dose of 20 mg/kg/day (**Figure 3C**). Histological evaluation by H&E and Safranin O staining were carried out from vehicle and **1g** treated mice too. A destroyed joint

space with large amounts of inflammatory cell infiltration and cartilage destruction were observed in AIA vehicle groups (showed as arrows in **Figure 3D**). Treatment of **1g** at a dose of 20 mg/kg/day led to significantly and obviously suppression of these histopathological changes with few signs of inflammation and cartilage was almost completely preserved in histological state of joints (**Figure 3D**).

To explore whether **1g** had the ability of reducing pro-inflammatory cytokines expression, joint tissues were collected and the relative mRNA levels of TNF-α, IL-1β and IL-6 were measured by RT-PCR. Treatment with **1g** significantly reduced the levels of these pro-inflammatory cytokines in the joint tissues (**Figure 4A**). To define whether **1g** had a systemic effect, quantity of TNF-α, IL-1β and IL-6 in collected serum samples as described above were measured by ELISA. Treatment with **1g** showed a significant effect on the systemic level of these cytokines (**Figure 4B, 4C and 4D**).

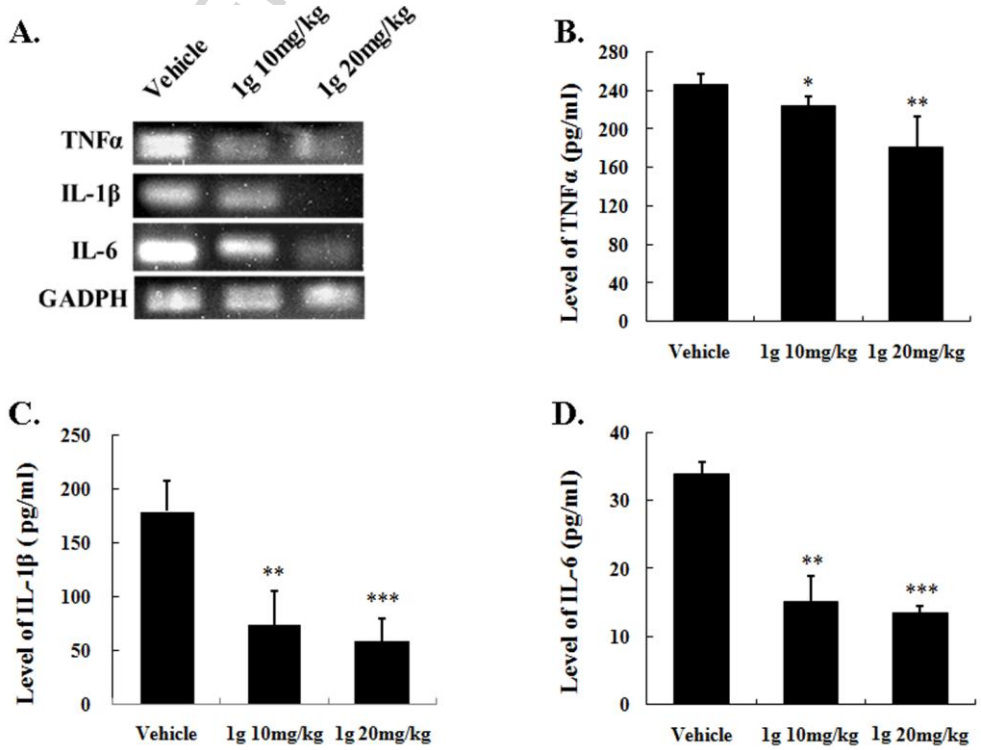


Figure 4. Inhibitory effects on the mRNA levels of TNF-α, IL-1β and IL-6 in joint tissues of animal vehicle. The inflammatory cytokine mRNA levels in joint tissues were measured by RT-PCR (A). Treatment with **1g** significantly inhibited expression of inflammatory cytokine. Serum samples on day 30 were measured for the quantity of TNF-α (B), IL-1β (C) and IL-6 (D). Values (means ± SD, n = 5 for each group) were from one representative experiment of three independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001).

2.4 Mechanism study of 1g

Since compound **1g** significantly suppressed LPS-triggered TNF-α production on splenocytes, we also investigated whether **1g** inhibited LPS-induced TNF-α production in Raw 264.7 cells. As shown in **Figure 5A**, **1g** reduced LPS-induced TNF-α production in Raw 264.7 cells in a dose-dependent manner.

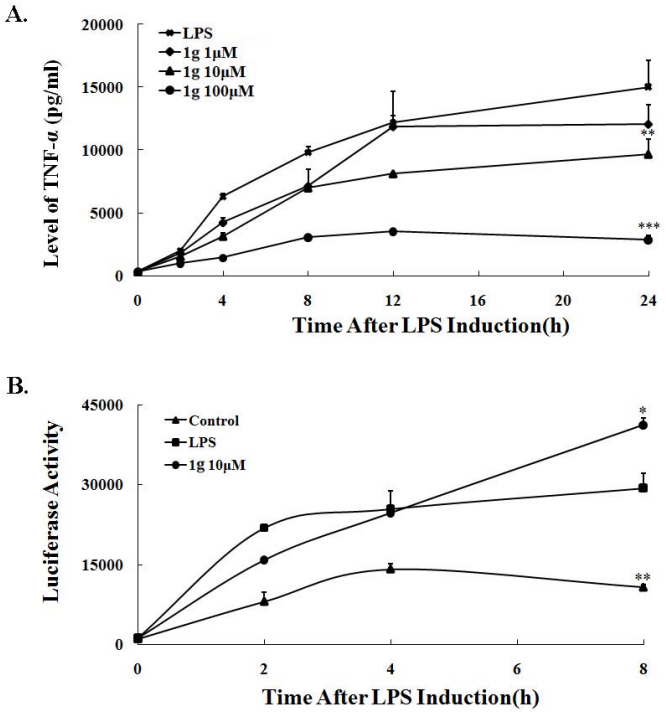


Figure 5. (A) RAW 264.7 were treated in the presence or absence of different dose of **1g** culturing with LPS (1 μg/ml). Supernatants at 2, 4, 8, 12, 24h post LPS addition were collected. Data (means ± SD) combined from three independent experiment (**$P < 0.01$). (B) Luciferase assay was performed in the presence or absence of **1g** (10 μM) as described in Materials and Methods. Values (means ± SD) combined from three independent experiments (**$P < 0.01$).

To clarify the underlying molecular mechanisms against TNF-α production of **1g**, we tested whether **1g** affected TNF-α mRNA level *in vitro* at first. RAW 264.7 cells were treated in the presence or absence of **1g** followed by LPS stimulation, and cells were collected for the detection of TNF-α mRNA level by RT-PCR analysis. Result showed that **1g** almost had no effect on the transcription of TNF-α mRNA (**Figure 6**). Then we decided to examine whether **1g** affected the TNF-α production at protein level. TNF-α-luciferase transgenic mouse splenocytes were collected and luciferase activity was measured as described previously to detect the intracellular TNF-α expression. Interestingly, results indicated that treatment with **1g** had no clearly effects on the intracellular protein level of TNF-α (**Figure 5B**). However, to some extent, it even enhanced the intracellular protein level after 4h LPS-induction. Our results showed that **1g** had the ability of inhibition of TNF-α secretion, but could not inhibit the TNF-α expression at mRNA and protein level. We supposed that this compound may influence the processing of pro-TNF-α to a soluble form. Consequently, we chose the most important TNF-α converting enzyme (TACE) for further study. As we all known, TACE is a membrane-bound disintegrin metalloproteinase that processes the membrane-associated cytokine pro-TNF-α to a soluble form. According to the result showed in

Figure 6, TACE mRNA is abundantly expressed in RAW264.7 cells stimulated by LPS. The 1g-treated group than in LPS-treated group decreased expression, and as the dose increased gradually, TACE mRNA expression decreased. High dose group was significantly reduced compared with the other three groups expression. The result showed that 1g decreased TACE mRNA level in RAW 264.7 cells in a dose-dependent manner.1g decreased TACE mRNA level in RAW 264.7 cells in a dose-dependent manner. So, we could draw a conclusion that **1g** inhibited TNF-α production through depressing the TACE mRNA expression.

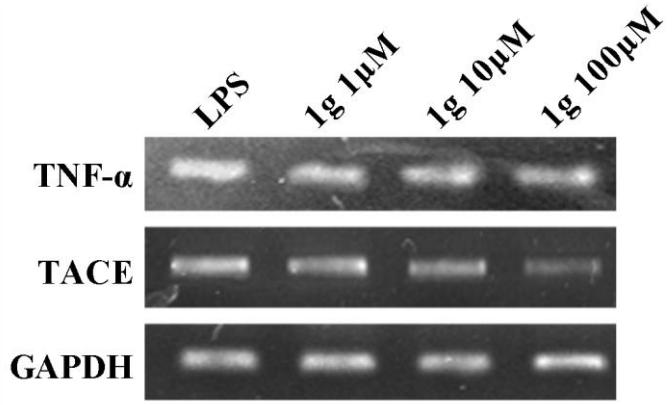


Figure 6. 1g inhibited TNF-α production through effects on TACE mRNA level. The TNF-α and TACE mRNA levels in RAW 264.7 were measured by RT-PCR as described before.

3. CONCLUSION

Inflammation is a response of host to inside and outside stimuli. TNF-α, a pro-inflammation cytokine mainly produced by macrophages and lymphocytes, plays a very important role in the inflammatory cascades, and inhibition of TNF-α production serves as an effective therapy for chronic inflammatory disease, such as rheumatoid arthritis.

In this study, barbigerone and its 27 related structural analogues were designed, synthesized, and evaluated for their pharmacological activity. At 10 μM, barbigerone (**1a**) and **1g** significantly reduced the production of TNF-α, with IC_{50} of 8.46 and 3.58 μM, respectively. Both of the compounds also exerted low cytotoxicity (without or with LPS, IC_{50} > 70 μM). SAR analysis indicated that: (1) pyranoid ring on the left phenyl ring and chromone structure of isoflavone are very important for the inhibitory activity on TNF-α production; (2) the bulk and electron density of right phenyl ring shows even more regularity with respect to the appropriate typical activity, and suitable electron-donating substitutions on C2 and C5 are the dominant factor for maintaining or enhancing the activity. Furthermore, oral administration of **1g**, at a dose of 20 mg/Kg/day for about two weeks, obviously led to improve the inflammatory response such as significantly reduction of footpad swelling and the improvement of pathology from joints of AIA rats. Mechanism studies suggested that **1g** inhibits the TNF-α production by means of depressing TACE mRNA expression. In summary, above results indicate that **1g** might be a potential agent for the treatment of chronic inflammatory diseases.

4. Experimental

4.1 Chemistry methods. All the reagents and solvents of analytical grade were purchased from commercial and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F-254 thin-layer plates and spots of desired products were located by UV lamp and Iodine. Intermediates and target compounds were purified by silica gel column. The purities of all compounds in biological assays were higher than 95%, which were

confirmed by HPLC analysis with a phodediode array detector (Waters, Milford, MA, USA) and the chromatographic column was an atlantis C_{18} (150 mm × 4.6 mm, I.D. 5 μm, Waters, Milford, Ireland). Samples were supplied as 0.1 mg/ml in methanol with 10 μl injected on a partial loop fill at a flow rate of 1 ml/min for 20 min. Melting points were measured on X-4 digital microscopic melting point apparatus and are not correct. ^{1}H spectra were recorded on Bruker AV-600, 400 and 300 MHz, ^{13}C spectra were record on Bruker AV-150 and 100 MHz. MS (low resolution) spectra were measured by Waters Quattro Premier XE and HRMS (high resolution) spectra were measured by Bruker Daltonics micrOTOF-Q II Electrospray Ionization Mass Spectrometer and Waters Q-TOF Premier mass spectrometer utilizing electrospray ionization (ESI) (Micromass, Manchester, UK).

General experimental procedure for the synthesis of 4b-c. To the molten anhydrous $ZnCl_2$ (3.3 mmol), phenylacetic acid (3.6 mmol) was added with vigorous stirring and heating at about 120-130 °C followed by the slow addition of resorcinol **2** (3 mmol). After completion of the reaction according to TLC monitor, the mixture was cooled to room temperature, followed by the addition of dry DMF (2 ml), and BF_3•OEt_2 (12 mmol) at 0 °C. To this mixture was added a solution of $MeSO_2Cl$ (9 mmol) in 5ml DMF at 50 °C and then the temperature was raised to 110 °C and stirred for about 2 hours. After completion, the reaction mixture was cooled and poured into ice water. The separated oil was extracted with ethyl acetate, and the organic layer was combined, washed with brine, and dried by $MgSO_4$, then the solvent was removed. The residue was

recrystallized from methanol. 7-hydroxy-isoflavones **4b** and **4c** were obtained as white needle solids.

General experimental procedure for the synthesis of 1b-c. Under N_2 atmosphere, intermediates **4b** or **4c** (0.5 mmol) was dissolved into 10 ml dried toluene, then catalytic 3-picoline was added, followed by the addition of 1,1-diethoxy-3-methyl-2-butene (1 mmol), stirred for a while and then the mixture was refluxed at 130 °C for about 18 hours. After the disappearance of reactant according to TLC, the solvent was evaporated and the residue was chromatographed over silica gel using petroleum/ethyl acetate = 3/1 as eluent, **1b** and **1c** were obtained with the yield of 24% and 30% in two steps, respectively.

3-phenyl-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1b): white powder, over-all yield 24%; HPLC: 99.4%; ^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm) = 1.55 (s, 6H), 5.77 (d, *J* = 12.0 Hz, 1H), 6.87 (d, *J* = 12.0 Hz, 1H), 6.91 (d, *J* = 12.0 Hz, 1H), 7.42-7.61(m, 5H), 8.01 (s, 1H), 8.12 (d, *J* = 12.0 Hz, 1H). MS (ESI), *m/z*: 305.07 $[M + H]^+$. NMR spectra match the reference.[34]

3-(1',3'-dioxolphenyl-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1c): white powder, over-all yield 30%; HPLC: 98.4%; ^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm) = 1.50 (s, 6H), 5.72 (d, *J* = 12.0 Hz, 1H), 5.99 (s, 2H), 6.81 (d, *J* = 12.0 Hz, 1H), 6.86 (t, *J* = 6.0 Hz, 2H), 6.96 (d, *J* = 6.0 Hz, 1H), 7.10 (s, 1H), 7.92 (s, 1H), 8.06 (d, *J* = 12.0 Hz, 1H). MS (ESI), *m/z*: 349.12 $[M + H]^+$. NMR spectra match the reference.[35]

General experimental procedure for the synthesis of 7a, d-k and 1a, d-h. Compound **6a** (2 mmol) was dissolved into 10 ml methanol and cooled to 0 °C, a solution of 40% NaOH (3.5 ml) was added to the previous mixture and stirred at the temperature for about 30 minutes, then different substituted aldehydes (2.4 mmol) was added slowly. The mixture was allowed raise to room temperature and stirred for about 24 to 36 hours. After the disappearance of **6a**, the mixture was acidified with 2N HCl (about 10 ml), chalcones **7a**, **d-k** came into appearance as yellow precipitates. The precipitates were collected and recrystallized from methanol, high purity of **7a**, **d-k** were obtained with the yield ranging from 64%-78%. To the stirred suspension of chalcones **7a**, **d-h** (0.5 mmol) in methanol (5 ml) were added thallium (III) nitrate (0.75 mmol) carefully, and stirred continued for overnight at room temperature. After the disappearances of chalcones, enough volume of saturated sodium sulfite (about 30 ml) was added, and stirred for about 1 hour, then the mixture was extracted with ethyl acetate for three times. The organic phase were combined and dried with $MgSO_4$, then the solvent were removed *in vacuo* and the stick yellowish oil was purified by silica gel chromatography using DCM as eluent. The purified products were dissolved into methanol (15 ml) and treated with 2N HCl (3 ml), and the mixture was refluxed for about 3 hours, as the time went by, white or pale yellow solid precipitated. After completion of the reagents, the precipitates were collected and recrystallized from methanol, target compounds **1a**, **d-h** were obtained with yields of 64%-78% (based on chalcones).

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(2,4,5-trimethoxyphenyl)prop-2-en-1-one (7a): yellowish powder, yield 69%; HPLC: 99.5%.[24]

Barbigerone (1a): pale yellowish powder, yield 76%; HPLC: 99.6%.[24]

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(3-methoxyphenyl)prop-2-en-1-one (7d): yellowish powder, yield 72%; HPLC: 99.5%.[24]

3-(3'-methoxyphenyl)-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1d): white powder, yield 78%; HPLC: 96.1%.[24]

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(4-methoxyphenyl)prop-2-en-1-one (7e): yellowish powder, yield 69%; HPLC: 99.6%.[24]

3-(4'-methoxyphenyl)-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1e): white solid, yield 78%; HPLC: 98.8%.[24]

(*E*)-3-(3,4-dimethoxyphenyl)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)prop-2-en-1-one (7f): yellowish powder, yield 73%; HPLC: 99.7%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 1.47 (s, 6H), 3.94 (s, 3H), 3.96 (s, 3H), 5.59 (d, J = 12.0 Hz, 1H), 6.38 (d, J = 9.0 Hz, 1H), 6.75 (d, J = 12.0 Hz, 1H), 6.90 (d, J = 9.0 Hz, 1H), 7.15 (d, J = 1.5 Hz, 1H), 7.22 (d, J = 1.5 Hz, 1H), 7.42 (d, J = 15.4 Hz, 1H), 7.73 (d, J = 8.6 Hz, 1H), 7.84 (d, J = 15.4 Hz, 1H), 13.79 (s, 1H). MS (ESI), *m/z*: 367.17 $[M + H]^+$. NMR spectra match the reference.[33]

3-(3',4'-dimethoxyphenyl)-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1f): off-white solid, yield 64%; HPLC: 98.3%; ^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm) = 1.50 (s, 6H), 3.91 (s, 3H), 3.93 (s, 3H), 5.72 (d, J = 12.0 Hz, 1H), 6.81 (d, J = 12.0 Hz, 1H), 6.87 (d, J = 6.0 Hz,

1H), 6.92 (d, *J* = 6.0 Hz, 1H), 7.05 (d, *J* = 6.0 Hz, 1H), 7.21 (s, 1H), 7.96 (s, 1H), 8.02 (d, *J* = 6.0 Hz, 1H). MS (ESI), *m/z*: 365.22 $[M + H]^+$. NMR spectra match the reference.[36]

(*E*)-3-(2,5-dimethoxyphenyl)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)prop-2-en-1-one (7g): yellowish powder, yield 77%; HPLC: 98.3%.[24]

3-(2',5'-dimethoxyphenyl)-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1g): pale white solid, yield 68%; HPLC: 98.4%.[24]

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (7h): yellowish powder, yield 81%; HPLC: 99.4%.[24]

3-(3',4',5'-trimethoxyphenyl)-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1h): pale white solid, yield 71%; HPLC: 99.6%.[24]

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(3-hydroxyphenyl)prop-2-en-1-one (7i): yellowish powder, yield 76%; HPLC: 99.6%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 1.47 (s, 6H), 5.60 (d, *J* = 12.0 Hz, 1H), 6.39 (d, *J* = 6.0 Hz, 1H), 6.76 (d, *J* = 12.0 Hz, 1H), 6.89 (t, *J* = 6.0 Hz, 1H), 7.11 (s, 1H), 7.22 (d, *J* = 12.0 Hz, 1H), 7.28-7.30 (t, *J* = 12.0, 6.0 Hz, 1H), 7.53 (d, *J* = 6.0 Hz, 1H), 7.71 (d, *J* = 6.0, 1H), 7.80 (d, *J* = 18.0 Hz, 1H), 13.62 (s, 1H). MS (ESI), *m/z*: 323.19 $[M + H]^+$. NMR spectra match the reference.[37]

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(4-hydroxyphenyl)prop-2-en-1-one (7j): yellowish soild, yield 72%; HPLC: 99.7%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 1.47 (s, 6H),

5.59 (d, J = 6.0 Hz, 1H), 6.38 (d, J = 3.0 Hz, 1H), 6.76 (d, J = 6.0 Hz, 1H), 6.88 (d, J = 6.0 Hz, 1H), 7.09 (d, J = 6.0 Hz, 1H), 7.42-7.46 (dd, J = 6.0, 3.0 Hz, 1H), 7.57 (d, J = 3.0 Hz, 1H), 7.70-7.72 (dd, J = 3.0, 3.0 Hz, 1H), 7.72-7.86 (dd, J = 6.0, 3.0 Hz, 1H), 13.75 (d, J = 3.0 Hz, 1H). MS (ESI), *m/z*: 323.17 $[M + H]^+$. NMR spectra match the reference.[38]

(*E*)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-(4-(trifluoromethyl)phenyl)prop-2-en-1-one (7k): yellow powder, yield 43%; HPLC: 97.0%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) = 1.48 (s, 6H), 5.61 (d, J = 8.0 Hz, 1H), 6.40 (d, J = 8.0 Hz, 1H), 6.75 (d, J = 12.0 Hz, 1H), 7.62 (d, J = 16.0 Hz, 1H), 7.67-7.75 (m, 5H), 7.86 (d, J = 16.0 Hz, 1H), 13.52 (s, 1H). HRMS (ESI) exact mass calcd. for $(C_{21}H_{17}F_3O_3\text{-}H)^-$ requires *m/z* 373.1130, found *m/z* 373.1054.

Synthesis of 8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1i): under N_2 atmosphere, compound **6a** (3 mmol) was dissolved into 3 ml DMF-DMA and refluxed for about 3 hours, after completion of the reaction and cooled down, 10 ml ethanol was added to the mixture, yellowish needles (**9**) came into appearance. Collected and dried *in vacuo*, 579 mg **9** was obtained. At room temperature, **9** (0.5 mmol) was dissolved into 3 ml methanol followed by the addition of 3 ml 2N HCl. The mixture was refluxed for about 6 hours. The acidic solution was neutralized by 20 ml saturated Na_2CO_3 solution. Extracted by ethyl acetate for 3 times, combined and dried with $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel column, using petroleum/ethyl acetate = 4/1 as eluent, and 79 mg yellowish solid was obtained, yield 69%; HPLC: 99.2%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) = 1.49 (s, 6H), 5.71 (d, J = 8.0 Hz, 1H), 6.27 (d, J = 4.0 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 7.80 (d, J = 4.0 Hz,

1H), 7.97 (d, J = 8.0 Hz, 1H). MS (ESI), *m/z*: 228.04 $[M + H]^+$. NMR spectra match the reference.[34]

Synthesis of

3-(4'-(trifluoromethyl)phenyl)-8,8-dimethyl-4-oxo-*4H*,*8H*-benzo[1,2-b:3,4-b']dipyran (1j): 400 mg (1.46 mmol) compound **9** was dissolved into 10 ml methanol, then 444.4 mg (1.75 mmol) I_2 was added to the mixture and stirred at room temperature for overnight. After disappearance of the reactant, 30 ml saturated Na_2SO_3 solution was added, extracted with ethyl acetate for 3 times, the organic phase was combined and dried with $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel column, and 272 mg compound **10** as off-white powder was obtained. 70.8 mg (0.2 mmol) compound **10** and 57 mg (0.3 mmol) 4-(trifluoromethyl)phenylboronic acid were dissolved into 3 ml acetonitrile and 3 ml water, followed by the addition of catalytic amount 10% Pd/C and 41 mg (0.3 mmol) K_2CO_3. The mixture was refluxed for about 6 hours. After the completion of the reaction, 20 ml ethyl acetate and 30 ml water was added to the reaction mixture, the organic phase was collected and dried with $MgSO_4$, the solvent was removed under reduced pressure. The residue was purified by silica gel column, using petroleum/ethyl acetate = 3/1 as eluent, and 49.3 mg white solid was obtained, clarified as titled compound, yield 66%; HPLC: 98.7%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) = 1.51 (s, 6H), 5.74 (d, J = 8.0 Hz, 1H), 6.81 (d, J = 8.0 Hz, 1H), 6.89 (d, J = 8.0 Hz, 1H), 7.69 (s, 4H), 8.01 (s, 1H), 7.16 (s, 1H), 8.07 (d, J = 8.0 Hz, 1H). HRMS (ESI) exact mass calcd. for $(C_{21}H_{15}F_3O_3\text{-}H)^-$ requires *m/z* 371.0973, found *m/z* 371.0895.

Synthesis of 7-methoxy-2',2'-dimethyl-2'H,4H-3,6'-bichromen-4-one (1k): Starting from compound **5**, the synthetic methods of **1j** and **1b-c** were used for the synthesis of **1k**, yield 50.2% (final step); HPLC: 98.8%; ^{1}H NMR (DMSO-d_6, 600 MHz) δ (ppm) = 1.26 (s, 6H), 3.89 (s, 3H), 6.80 (d, *J* = 6.0 Hz, 2H), 7.07 (m, 1H), 7.13 (d, *J* = 6.0 Hz, 1H), 7.38 (d, *J* = 6.0 Hz, 2H), 8.02 (d, *J* = 6.0 Hz, 1H), 8.35 (s, 1H), 9.50 (s, 1H). MS (ESI), *m/z*: 335.12 $[M + H]^+$. NMR spectra match the reference.[39]

Synthesis of 7-(3-methylbut-2-enyloxy)-3-phenyl-4H-chromen-4-one (1l): compound **4b** (0.5 mmol) was dissolved into 10 ml acetone, 138 mg K_2CO_3 was added and followed by the addition of 1-bromo-3-methylbut-2-ene (0.6 mmol, 71 μl), the mixture was refluxed for about 3 hours. Filtrated and the solvent was removed, the residue was purified by silica gel column, using petroleum/ethyl acetate = 6/1 as eluent, 96 mg white solid was obtained and then clarified as titled compound, yield 69%; HPLC: 99.1%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) = 1.70 (s, 3H), 1.74 (s, 3H), 4.53 (d, *J* = 4.0 Hz, 2H), 5.42 (t, *J* = 8.0, 8.0 Hz, 1H), 6.77 (s, 1H), 6.91 (d, *J* = 8.0, 1H), 7.28 (t, *J* = 4.0, 8.0 Hz, 1H), 7.34 (t, *J* = 8.0, 8.0 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 2H), 7.85 (s, 1H), 8.12 (d, *J* = 8.0 Hz, 1H). MS (ESI), *m/z*: 329.11 $[M + Na]^+$. NMR spectra match the reference.[40]

Synthesis of 3-(4-methoxyphenyl)-7-(3-methylbut-2-enyloxy)-4H-chromen-4-one (1m): according to the synthetic method as **1l**, 112 mg white solid was obtained from **13**, which was then clarified as titled compound, yield 72%; HPLC: 98.4%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) = 1.71 (s, 3H), 1.75 (s, 3H), 3.76 (s, 3H), 4.54 (d, *J* = 8.0 Hz, 2H), 5.43 (t, *J* = 4.0, 8.0 Hz, 1H), 6.78

(s, 1H), 6.88 (s, 1H), 6.91 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.83 (s, 1H), 8.12 (d, J = 8.0 Hz, 1H). MS (ESI), *m/z*: 359.12 $[M + Na]^+$. NMR spectra match the reference.[41]

7-amino-3-phenyl-4H-chromen-4-one (16): By using the same method as the synthesis of 7-hydroxy-isoflavones **4**, to the molten anhydrous $ZnCl_2$ (3.3 mmol), phenylacetic acid (3.6 mmol) was added with vigorous stirring and heating at about 120-130 °C followed by the slow addition of *N*-(3-methoxyphenyl)acetamide (3 mmol). After completion according to TLC, the reaction mixture was cooled to room temperature, followed by the addition of dry dimethylformamide (DMF) (2 ml), and BF_3•OEt_2 (12 mmol) at 0 °C. To this mixture was added a solution of methanesulfonyl chloride ($MeSO_2Cl$, 9 mmol) in 5ml DMF at 50 °C and then the temperature was raised to about 110 °C for about 2 hours. After completion, the reaction mixture was cooled and poured into ice water. The separated oil was extracted with ethyl acetate for 3 times, and the organic layer washed with brine, combined and dried with $MgSO_4$, then the solvent was removed. The residue was purified by silica gel column, 286 mg *N*-(5-methoxy-2-(2-phenylacetyl)phenyl)acetamide (pale yellowish powder, discarded) and 158 mg *N*-(4-oxo-3-phenyl-4H-chromen-7-yl)acetamide as pale brown powder were obtained, which was then dissolved into 5 ml MeOH, followed by the addition of 6N HCl (3 ml), the mixture was refluxed for about 6 hours, then neutralized by enough volume saturated K_2CO_3 solution, extracted by ethyl acetate for 3 times, combined and dried, the organic phase was removed under reduced pressure. The residue was recrystallized from methanol, 106 mg titled compound was obtained as pale brown solid, yield 78% (last step); HPLC: 99.2%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) = 6.32 (s, 2H), 6.52 (s, 1H), 6.70 (d, J = 8.0 Hz, 1H), 7.35-7.43 (m, 3H), 7.55 (d, J =

8.0 Hz, 2H), 7.79 (d, J = 8.0 Hz, 1H), 8.24 (s, 1H). MS (ESI), *m/z*: 238.10 $[M + H]^+$. NMR spectra match the reference.[42]

8,8,10-trimethyl-3-phenyl-7,8-dihydro-4H-pyrano[2,3-f]quinolin-4-one (1n): 90 mg compound **16** was dissolved into 10 ml acetone, followed by the addition of catalytic amount silicotungstic acid, the mixture was refluxed for about 12 hours, filtered and removed of the solvent, the residue was purified by silica gel column, using petroleum/ethyl acetate = 1/1 as eluent, 66 mg compound **1n** was obtained as pale brown solid, yield 52%; HPLC: 99.1%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 1.34 (s, 6H), 2.32 (d, J = 1.5 Hz, 3H), 5.32 (d, J = 1.5 Hz, 1H), 7.36-7.45 (m, 3H), 7.57 (t, J = 1.5, 7.5 Hz, 2H), 7.91 (s, 1H), 8.00 (d, J = 1.5 Hz, 1H). HRMS (ESI) exact mass calcd. for $(C_{21}H_{19}NO_2+H)^+$ requires *m/z* 318.1416, found *m/z* 318.1487.

6-amino-3-phenyl-4H-chromen-4-one (19): Starting from *N*-(4-methoxyphenyl)acetamide (3 mmol), by using the same method as the synthesis of compound **16**, 462 mg titled compound was obtained as pale brown powder, yield 65%; HPLC: 99.1%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm) = 5.52 (s, 2H), 7.07 (d, J = 8.0 Hz, 1H), 7.21 (s, 1H), 7.37-7.45 (m, 4H), 7.58 (d, J = 8.0 Hz, 2H), 8.04 (s, 1H). MS (ESI), *m/z*: 238.08 $[M + H]^+$. NMR spectra match the reference.[42]

8,8,10-trimethyl-2-phenyl-7,8-dihydro-1H-pyrano[3,2-f]quinolin-1-one (1o): Starting from compound **19** (90 mg), and by using the same method as the synthesis of **1n**, 79 mg titled compound was obtained as pale brown powder, yield 63%; HPLC: 97.7%; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) = 1.29 (s, 6H), 2.04 (d, J = 1.5 Hz, 3H), 5.48 (d, J = 1.5 Hz, 1H), 6.79-6.81 (m, 1H),

6.92 (d, J = 9.0 Hz, 1H), 7.15 (d, J = 9.0 Hz, 1H), 7.35-7.45 (m, 3H), 7.58 (dd, J = 3.0, 3.0 Hz, 2H), 7.87 (s, 1H). HRMS (ESI) exact mass calcd. for $(C_{21}H_{19}NO_2 + H)^+$ requires *m/z* 318.1416, found *m/z* 318.1487.

4.2 Biological evaluation

Cell Culture. Suspension of Balb/c mouse splenocytes and RAW 264.7 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium containing 10% FBS in a 95% air, 5% CO_2 humidified atmosphere at 37 °C. Cells were treated in the presence or absence of compounds with LPS (1 μg/ml, Escherichia coli serotype 0111:B4) stimulation for 24 hours, the supernatants were collected for TNF-α cytokine analysis by ELISA Kit.

Cell Cytotoxicity. Cell cytotoxicity was evaluated by MTT assay and expressed as IC_{50}. Splenocytes were treated with or without compounds for 24 hours. Cells were incubated in 0.5 mg/ml MTT reagent dissolved in H_2O for 4 hours, and the formazan product dissolved in 150 μl of DMSO. The optical density was measured using MTT at 570 nm.[43]

Luciferase assay. Splenocytes of TNF-α-luciferase reporter mouse were stimulated by LPS (1 μg/ml) in the presence or absence of **1g** (10 μM) for 8 hours.[44] Cells were lysed and analyzed for luciferase activity using the Luciferase Reporter Assay kit (Promega, Cat. #E4550).

Assay of mRNA level of TNF-α and TACE. RAW 264.7 cells were seeded in wells and incubated with different dose of **1g** in presence of LPS (1 μg/ml) for 24 hours. The procedures of RNA extraction and RT-PCR were same as before. Primer sequences for the PCR are in Table 2.

Induction of adjuvant-induced arthritis. Female Lewis rats (6-8 weeks old, Tengxin Biotech Company, Chongqing, P. R. China) were injected subcutaneously with 0.5mg heat-killed inactivated Mycobacterium tuberculosis emulsified in 0.1 mL incomplete Freund's adjuvant (Sigma, F5506) at the base of the tail.[43] The day of adjuvant injection is considered day 0. **1g** was orally administered once daily at a dose of 10 mg/kg and 20 mg/kg. The treatment was initiated from day 14. All rats were treated continuously up to day 30 and then sacrificed.

Clinical evaluation of arthritis. The clinical disease activity score was initiated from day 14. The animals were inspected every day for the development of AIA. Clinical parameters measured included articular index score and ankle circumference. Scoring was performed using a 0-4 scale, 0, normal; 1, slight swelling or redness of toe/finger joints; 2, mild swelling and redness of ankle or wrist joints; 3, involvement of the ankle and metatarsal; 4, severe swelling and redness of the entire paw. Each paw was graded and the four scores were totaled. The circumference of the ankle of each hind paw was measured with a caliper.

Histological Examination. Hind paws were removed, fixed in 4% paraformaldehyde in PBS, decalcified in EDTA buffer, subjected in a series progression of dehydration, and then embedded in paraffin. The paraffin section was stained with hematoxylin-eosin and Safranin O-fast green. The histological changes were examined under microscope.

Measurement of serum cytokines. Sera were collected before postmortem on day 30. Cytokines that TNF-α, IL-1β and IL-6 were measured by ELISA kit (Boster Biological Technology, EK0527)

according to the manufacturer's specifications. The 96-well plates were monitored at 450nm by ELISA plate reader.

RNA extraction and semiquantitative RT-PCR. The hind paw joints were collected, dissected free of soft tissue and bones, snapped frozen in liquid nitrogen and pulverized to achieve a fine powder. For each data point, tissue from three rats was pooled. Total RNA was prepared by extracting the powder with Trizol (Ambion, LOT.36008) according to the manufacturer's instructions. The final RNA pellet was dissolved in 20 μL of water and the concentration was measured spectrophotometrically. cDNA were reverse-transcribed by Premix Ex Taq (TAKARA, D332A). PCR of the cDNA was performed under the following conditions: denaturation at 94 °C for 5 seconds, primer annealing at 60 °C for 30 seconds, and primer extension at 72 °C for 30 seconds. Primer sequences for the PCR are in Table 2.[45-48] PCR products were run on a 1% agarose gel.

Table 2. The primer sequences of mouse and rat gene.

Gene	Forward primer	Reverse primer
TNF-α (rat)	5'-CGGGGGCCACCACGCTCTTC-3'	5'-GGCAAATCGGCTGACGGTGTG-3'
TNF-α (mouse)	5'-ATCCGCGACGTGGAACTG-3'	5'-ACCGCCTGGAGTTCTGGAA-3'
IL-1β	5'-TCAAGGCATAACAGGCTCATC-3'	5'-CCACGGGCAAGACATAGGTAG-3'
IL-6	5'-CTTGGGACTGATGTTGTTGAC-3'	5'-GAAGTTGGGGTAGGAAGGAC-3'
TACE	5'-CTTGCCAAGATCCAAGCA-3'	5'-AGCAATAAAGTTTGTGGG

		AA-3'
GADPH(mouse and rat)	5'-CCATGTTCGTCATGGGTGTGAACCA-3'	5'-GCCAGTAGAGGCAGGGATGTTC-3'

Supporting information

Synthesis and analytical data of all intermediates not described in the Experimental Section and detailed information of compounds **7g**, **7k**, **1d**, **1g**, **1h**, **1j**, **1n** and **1o**. This material is available as Supporting Information.

Author contributions

Zhe Wei was responsible for the biological study and Youzhe Yang was responsible for the synthetic work.

Notes

These authors claim no competing finical interests.

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Abbreviations

RA, rheumatoid arthritis; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; TACE, TNF-α converting enzyme; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme linked immunosorbent assay; DEX, dexamethasone; LPS, lipopolysaccharide; AIA, adjuvant-induced arthritis; IC_{50}, half maximal inhibitory concentration; GADPH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; DMSO, dimethylsulfoxide; TLC, thin layer chromatography.

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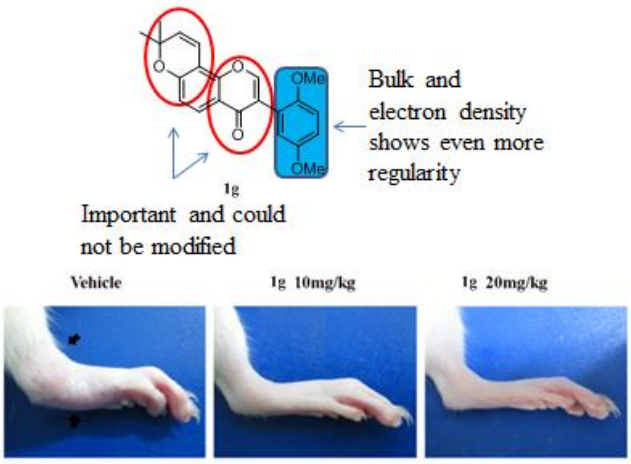

Graphical abstract