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Original article

# Microfluidics assisted synthesis and bioevaluation of sinomenine derivatives as antiinflammatory agents



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#### ABSTRACT

Sinomenine (1) is currently used for the treatment of rheumatoid arthritis (RA) in China and there is still room for the improvement of its efficacy. In present study, capillary based microfluidic system was effectively applied for the syntheses of two novel series of sinomenine derivatives. The Heck reactions in microreactor gave much higher conversions compared to the batch ones. The two-step synthesis of the isoxazoline in microreactor greatly shortened the reaction time without any isolation of intermediates. The inhibitory activity of synthesized compounds on the TNF- $\alpha$ -induced nuclear factor kappa B (NF- $\kappa$ B) activation was evaluated in vitro. Among the compounds, **3c** and **3g** showed the potent inhibitory activity. Furthermore, **3g** exhibited the antiinflammatory effect in vivo.

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# 1. Introduction

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, and irritants. Nuclear factor-kappa B (NF- $\kappa$ B), an ubiquitously expressed proinflammatory transcription factor, is intimately involved in the regulation of expression of numerous genes in the setting of the inflammatory responses including immune inflammatory responses, acute-phase inflammatory responses and so on [1,2]. NF- $\kappa$ B is frequently and constitutively activated in patients with chronic inflammatory conditions. Inappropriate regulation of NF- $\kappa$ B activity has been implicated in the pathogenesis of several diseases, such as inflammatory and rheumatic diseases including rheumatoid arthritis (RA), osteoarthritis (OA), chronic inflammatory demyelinating polyradiculoneuritis and inflammatory bowel disease [3]. Targeting the NF-*k*B signaling pathway may represent a therapeutic avenue for treating these inflammation related diseases.

As a tremendously powerful tool for drug discovery, the structure modification of natural products is continually needed for

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generating more useful molecules with better bioactivities and physical properties [4]. The Chinese medical plant Sinomenium acutum has been used for the treatment of various rheumatic diseases effectively in China for over 2000 years [5]. Sinomenine (1), a natural alkaloid isolated from the stem of S. acutum, was also used clinically for the treatment of rheumatic diseases including rheumatoid arthritis (RA) for a long time [6]. It was also demonstrated to possess a variety of pharmacological bioactivities, such as antiinflammation [7], immunosuppression [8], antiarthritis amelioration [9], protection against hepatitis induced by lipopolysaccharides [10], antirheumatic and antiarrhythmic effects [11,12]. However, the use of sinomenine was limited due to several problems such as weak efficacy, large dose and some side effects. To improve its efficacy and reduce the side effects, a number of sinomenine derivatives with modifications mainly focused on the ring C and D have been synthesized. However, no satisfactory results were achieved [13–15]. Previously, we discovered that the substitutions at C-1 position of A ring in sinomenine impacted the antiinflammatory activity of the derivatives remarkably [16-18]. This result encouraged us to target the sinomenine derivatives with C-1 substitutions for better activity.

There is an increasing demand for the research and development of new technologies that enable the rapid and efficient performance of the difficult reactions, especially in the construction of biologically interesting molecules [19]. In recent years, the use of

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microreactor as an alternative to round bottle flask chemistry for organic synthesis has attracted considerable interests, particularly within the biochemical reactions and fine chemicals industries [20]. Microreactor represents promising merits such as promoting highly effective chemical reactions, reducing reaction time and costs of chemical processes. Microreactor also exhibits great benefits that can not be achieved in traditional reactors, such as increased surface-to-volume ratios, excellent mass and heat transfer capabilities, significantly high yields and less waste. Up to date, it has been widely applied in the high temperature and long time reactions which can not progress well through conventional scale synthesis [21].

In this study, considering the advantages of the microreactor, a continuous flow capillary microreactor was applied in order to get a highly benign and efficient synthesis of sinomenine derivatives with different substitutions at C-1 position of ring A. The derivatives with cinnamate ester and isoxazoline moieties at C-1 position were prepared with satisfactory residence times and good conversions, the two isomers of isoxazoline derivatives were successfully separated by a normal silica gel column chromatography. The inhibitory activity of all synthesized compounds was assayed on NF- $\kappa$ B activation, a crucial aspect in the inflammatory response. Compound **3g** was further tested for its antiinflammatory effect in vivo.

# 2. Chemistry

#### 2.1. Synthesis of cinnamate ester derivatives

Cinnamate ester moiety represents an important framework in many bioactive compounds [22–24]. Incorporating the ring A of sinomenine, the reaction of aryl halogen with acrylic ester *via* Heck reaction was introduced to construct cinnamate esters in sinomenine skeleton. As the H-1 of sinomenine is easily halogenated by Niodosuccinimide (NIS), the preparation of the cinnamate esters was designed as shown in Scheme 1.

Treatment of **1** with NIS at room temperature afforded 1-idiosinomenine 2 in an excellent yield (92%). Pd(OAc)<sub>2</sub>/PPh<sub>3</sub> catalyzed Heck reactions of 2 with corresponding acrylic esters were then performed. In order to develop environmentally benign and efficient reaction conditions for C-C bond formation, a continuous flow capillary microreactor was employed (Fig. 1). The conversions of the reaction were determined by integration of the peaks arising from H-2 of both the 1-idiosinomenine and the cinnamate ester derivatives in <sup>1</sup>H NMR spectra. As shown in Table 1, the microreactor greatly enhanced the reaction speed compared with the traditional batch. In the microreactor, the conversions of the reactions reached 70-90% (entries 3a-3j) within 10 min, while they were just 5–20% (entries **3a–3h**) in the batch reaction. When the reaction time was prolonged to 20 min, all the conversions in the microreactor reached more than 90% (entries 3a-3i), however, only 30–50% (entries 3a–3h) were obtained in the batch.



Fig. 1. Continuous flow capillary microreactor.

### 2.2. Synthesis of isoxazoline derivatives

Isoxazoline scaffolds are important moieties in a variety of structurally complicated natural products and pharmacophores in medicinal chemistry [25–27]. With sinomenine cinnamate esters in hand, a 1,3-dipolar cycloaddition of nitrile oxide to the C=C bond was introduced to form the isoxazoline moieties at the ring A (Scheme 2). The isoxazoline derivatives of sinomenine were firstly synthesized by a two-step reaction in a batch condition: aromatic aldehyde oxime was reacted with N-chlorosuccinimide (NCS) to afford aryl hydroximino chloride. The alkene was then added into the aryl hydroximino chloride while the triethylamine (TEA) in DCM was added dropwise to provide the isoxazoline derivatives (**6a** and **6b**, 71.6% yield, Table 2).

Considering the regioselectivity and diastereofacial selectivity, the isoxazoline products may contain four configurations consisting of two regional isomers, F and N, and each isomer further includes two diastereomers,  $F_a$  and  $F_b$ ,  $N_a$  and  $N_b$ , respectively (Fig. 2). Because of the electron-withdrawing effect of the ester carbonyl group adjacent to the C=C bond, the F configurational isomer would be the main one [28]. Furthermore, sinomenine and aryl group were bulky substituents that can cause obvious steric hindrance in N configuration, so the F configurational isomer should also be preferred. HMBC spectrum of **6a** and **6b** supported the F isomers, in which a clear correlation between H-5' and C-2 of sinomenine was observed (see Supplementary data). Furthermore, when a chiral HPLC separation was conducted, only two isomers (**6a** and **6b**) were obtained. Similar evidence was also reported by Michele [28].

It is still ambiguous what the configurations at the C-5' and C-4' chiral centers in **6a** and **6b** were. The <sup>1</sup>H NMR spectra of the two isomers displayed clear differences in chemical shifts of H-4' and 5', while with the same splitting pattern (doublet signal) and constant value (J = 5.7 Hz). In isomer-**6a**, the chemical shifts of H-4' and H-5' were at 4.23 and 6.06 ppm, while those of **6b** were at 4.07 and 6.15 ppm, respectively (see Supplementary data). Attempts to get single crystal to determine their stereostructures unfortunately failed. Therefore, a detailed analysis of NOESY spectra was performed.

The stereostructures of **6a** and **6b** were analyzed with Material Studio 4.0 (Accelrys, USA). As shown in Fig. 3A, the NOE correlations



Table 2

Isoxazoline type compounds 4-11.

 Table 1

 Conversions of cinnamate ester derivatives in microreactor and batch

Entry R <sup>1</sup>		$-OR^2$	Conversion % (10 min) <sup>a</sup>		Conversion % (20 min) <sup>a</sup>	
			Microreactor	Batch	Microreactor	Batch
3a	Н	-0-	87.7	6.5	98.7	36.2
3b	Н	-0^	85.5	7.4	94.3	33.3
3c	Н	-0^	89.3	9.9	91.7	45.1
3d	Н	-0	76.9	5.7	90.1	24.2
3e	Н	-0	68.5	4.8	92.6	24.8
3f	н	-0~~0~	88.5	14.5	93.5	30.6
3g	Н	-0~~~~	78.1	18.7	93.5	52.8
3h	Н	-o-	84.7	16.7	92.6	46.8
3i	Н	-0	82.3 <sup>b</sup>	-	91.5 <sup>b</sup>	_
3j	СН	3 -0	80.6 <sup>b</sup>	_	92.7 <sup>b</sup>	-

<sup>a</sup> Determined by <sup>1</sup>H NMR. The conversion was calculated using the formula: [Int. (prod)/Int. (prod + 1-idiosinomenine)]  $\times$  100%.

<sup>b</sup> Isolated yield.

between signals of H-5' and H-10 $\beta$ , H-10 $\alpha$ ; H-4' and H-10 $\alpha$  in **6a** implied H-4' should locate at  $\alpha$  fashion, and H-5' at  $\beta$  fashion. In NOESY spectrum of **6b**, the correlation peaks (Fig. 3B) displayed a spatial proximity of H-5' to H-10 $\alpha$ , H-4' to H-10 $\beta$ , suggested that H-4' in **6b** should locate at  $\beta$  fashion, and H-5' at  $\alpha$  fashion. Thus, the structures of **6a** and **6b** were elucidated as shown in Table 2.

As described before, two-step reaction was an intermittent and time-consuming process and essential for the current synthetic route of isoxazoline derivatives. To overcome these defects, a continuous flow capillary microfluidic system was established (Fig. 4) and a practical, efficient and continuous synthesis of isoxazolines was performed.

As shown in Fig. 4, the continuous capillary microfluidic system consists of three microreactors, M1, M2 and M3. Sinomenine derivatives with cinnamate ester moieties at C-1 position were synthesized efficiently in microreactor M1 *via* Heck reaction as described before. The chlorination of aromatic aldehyde oxime was progressed in M2, where aryl aldehyde oxime and NCS were dissolved in DMF and then reacted at ambient temperature to afford the aryl hydroximino chloride. Before entering into M3, the crude



Scheme 2. Synthesis of isoxazoline derivatives. Reagents and conditions: (i) NCS, DMF, room temperature. (ii) 85  $^\circ\text{C}.$ 

H <sub>3</sub> CO HO	O <sup>N</sup> Ar COOCH <sub>3</sub> H H N-CH <sub>3</sub> OCH <sub>3</sub>	H <sub>3</sub> CO H <sub>3</sub> CO HO HO O CH <sub>3</sub>	N Ar H''COOCH3 H —N~CH3
	isomer-a	isor	ner-b
Entry	Ar	Yield % a	Ratio (a:b) <sup>b</sup>
4a, 4b		92	39:61
5a, 5b		93	37:63
6a, 6b		92	47:53
7a, 7b		96	34:66
8a, 8b	-CI	81	28:72
9a, 9b	-CI	84	43:57
10a, 10b	Br	87	34:66
11a, 11b	Br	86	31:69

<sup>a</sup> Isolated yield.

<sup>b</sup> Determined by <sup>1</sup>H NMR.

products of M1 and M2 were mixed in a T-mixer, where the aryl hydroximino chloride transformed into 1,3-diplor in the presence of TEA, the excess base of Heck reaction. In the reactor M3, the C=C of cinnamate ester was reacted with the in situ generated 1,3-diplor to afford the target isoxazoline products at 70 °C.

Because the whole experiment proceeded in a continuous process with no any isolation and purification conducted on any of the reaction intermediates, the protocol not only improved the reaction yields but also greatly shortened the reaction time. The whole reaction took about only 20 min.

Using this system, the cycloaddition reactions of various aryl aldehyde oximes were examined. As shown in Table 2, all of the reactions provided quite satisfactory yields. Consisted with the rule of 1,3-dipolar cycloaddition, when the aryl cycles were substituted by electron donor groups, such as methoxyl group, the 1,3-diplor was more active and prone to form isoxazoline products with higher yields. For those with electron-withdrawing group, such as chloro group, the 1,3-diplor reactions were a little sluggish and gave the products with lower yields. It was also found that the ratio



Fig. 2. Structures of possible isomers presented in the isoxazoline derivatives.

of isomer-a was smaller than that of isomer-b. This might be caused by the different steric hindrance when the 1,3-diplors were added to the different faces of C=C. For different substituents of cinnamate esters, no significant impact on the isomer selectivity was found.

### 3. Results and discussion

# 3.1. Cytotoxicity and inhibitory activity on TNF- $\alpha$ -induced NF- $\kappa$ B activation

Nuclear factor kappa B (NF- $\kappa$ B) has been proved to be a principle transcription factor with a crucial role in the immune response [29]. It is also known to be greatly activated in many inflammatory diseases such as asthma, multiple sclerosis, and rheumatoid arthritis [30]. Here, the sinomenine derivatives were exposed to mouse embryonic fibroblast (NIH/3T3) cells to assay their inhibitory activity on the TNF- $\alpha$ -induced NF- $\kappa$ B activation.

It is particularly important that the cytotoxicity of test compounds was screened before the bioactivity assay was carried out to avoid some erroneous conclusions. All compounds, at five different concentrations, were exposed to NIH/3T3 cells for 24 h without TNF- $\alpha$  treatment in 96 well plates, and their cytotoxicity were evaluated by MTT assay. As shown in Table 3, most cinnamate ester compounds displayed cytotoxicity IC<sub>50</sub> of near or more than 100  $\mu$ M, except compounds **3g** and **3i** with cytotoxicity IC<sub>50</sub> of 38.9 and 4.8  $\mu$ M respectively. The cytotoxicity IC<sub>50</sub> of parent compound sinomenine was also >100  $\mu$ M (data not shown). For the isoxazoline derivatives (**4a**, **b**–**11a**, **b**), the cytotoxicity IC<sub>50</sub> was more than 50  $\mu$ M. Based on the data of cytotoxicity, all the activity profiles were carried out below the cytotoxicity IC<sub>50</sub>.

The inhibitory activity of the compounds toward TNF- $\alpha$ -induced NF-*k*B activation was then investigated at 20 µM concentration. The binding activity of NF- $\kappa$ B to DNA was analyzed and expressed as relative light units (RLU, % of control) with a luciferase cell assay. Sinomenine was inactive at 20 µM (data not shown). As can be seen in Fig. 5, introductions of cinnamate moieties ameliorated the activity. Among the compounds, where a butyl group was introduced to cinnamate moiety, the cinnamate ester (3c) exhibited an enhancement in the inhibitory activity. Introduction of a long fatty chain substitute on the cinnamate ester moiety (3g) greatly improved the potency compared to sinomenine **1**. The isoxazoline derivatives, at 20 µM of concentration, exhibited the improved activity to some extent. The interesting thing is that all of the a-type isoxazoline derivatives excluding 5a and 6a were inactive, while btype ones displayed a better activity compared with those of a-type, the reason is still under investigation in our lab.

## 3.2. In vivo assessment of antiinflammatory effect

The paw edema test has been widely employed to assay antiinflammatory agents. It works by measuring the ability of the tested samples in reducing edema-induced upon irritant carrageenan [31,32]. In order to determine the antiinflammatory activity in acute-phase inflammation in vivo, compound **3g** with relative potent inhibitory activity on NF- $\kappa$ B activation was selected and further evaluated with a carrageenan-induced mouse paw edema test. As can be seen in Fig. 6, obvious paw edema was witnessed 2 h after  $\lambda$ -carrageenan injection, and lasted for the whole experiment period. Indomethacin at 10 mg/kg, a positive control, significantly inhibited edema development from 2 h to 18 h. Administration (i.p.) of **3g** at both 15 and 30 mg/kg doses significantly attenuated



Fig. 3. Key NOE correlations observed in NOESY spectra of compounds 6a and 6b.



Fig. 4. Schematic continuous capillary microfluidic system.

edema formation at 6 h and the inhibitory effect lasted for 18 h. This result suggested that **3g** could be a potential anti-inflammatory agent. Because various chemicals and cytokines, such as histamine, serotonin, bradykinin and prostaglandins are involved in this carrageenan-induced paw edema [33,34], further effort is essential to clarify it.

#### 4. Conclusions

In this paper, continuous flow capillary microreactor was effectively used for the effective syntheses of cinnamate ester and isoxazoline derivatives at C-1 position of sinomenine. The microreactor not only greatly improved the reaction yield but also shortened the reaction time. Further, the isomers of isoxazoline derivatives were successfully separated by a normal silica gel column chromatography, and their structures were elucidated by NOESY spectra. The inhibitory activity of all synthesized compounds on the TNF- $\alpha$ -induced NF- $\kappa$ B activation was evaluated. Among the compounds, derivatives **3c** and **3g** displayed a better inhibitory activity, while **3g** also suppressed carrageenan-induced mouse paw edema. For the isoxazoline derivatives, b-type ones displayed better activity than those of a-type. The reason is under investigation. Further synthesis of related compounds with the microreactor are undergoing in our laboratory.

# 5. Experimental protocols

# 5.1. Chemistry

### 5.1.1. General

All reagents and solvents were commercially available and used without further purification. Melting points were determined on

Tabl	e 3
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Compound	Cytotoxicity $IC_{50} \ (\mu M)^a$	Compound	Cytotoxicity $IC_{50}$ ( $\mu M$ )
3a	>100	5a	77.96
3b	>100	5b	80.69
3c	79.96	6a	78.30
3d	>100	6b	>100
3e	>100	7a	83.90
3f	>100	7b	>100
3g	38.90	8a	75.12
3h	NT	8b	66.69
3i	4.80	9a	78.30
3j	>100	9b	71.31
4a	>100	10a	53.15
4b	>100	10b	55.70

NT: not tested because of poor solubility in DMSO or EtOH.

 $^{\rm a}$  Cytotoxicity IC\_{50}: the concentration to inhibit 50% growth of non-TNF- $\alpha$  activated cells.

a Taike X-4 digital micromelting point apparatus and uncorrected. <sup>1</sup>H and <sup>13</sup>C NMR spectra were measured on a Bruker Advance II 300 spectrometer using tetramethylsilane as internal standard. Chemical shift ( $\delta$ ) are reported in parts per million (ppm) and coupling constants *J* are reported in hertz (Hz), <sup>13</sup>C NMR spectra were fully decoupled, and the following abbreviations are used: singlet (s), doublet (d), triplet (t), double–double (dd) and multiplet (m). Chromatographic separations were performed on silica gel columns by column chromatography (Kieselgel 300–400 meshes) with CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>OH (15:1–9:1, v/v) as eluents. All reactions were monitored by TLC on GF<sub>254</sub> plates that were visualized under a UV lamp (254 nm). Evaporation of solvent was performed in vacuo with rotating evaporator.

#### 5.1.2. Idiosinomenine (2)

To a solution of sinomenine (**1**, 6.6 g, 20 mmol) in DCM (160 mL) was added NIS (4.7 g, 21 mmol) within 5 min. The reaction mixture was stirred at room temperature for 5 min and then quenched with saturated Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solution (120 mL). The organic phase was washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to afford the title compound **2**. Yield 92%; mp 106–108 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.16 (1H, s), 6.58 (1H, br), 5.44 (1H, s), 4.35 (1H, d, *J* = 15.6 Hz), 3.80 (3H, s), 3.50 (3H, s), 3.37 (1H, m), 3.14 (1H, m), 2.90 (1H, d, *J* = 18.9 Hz), 2.66 (1H, m), 2.49 (5H, m), 2.00 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 194.0, 152.6, 146.1, 145.5, 132.3, 124.7, 119.6, 115.0, 88.1, 57.4, 56.4, 55.2, 49.1, 47.2, 46.0, 43.0, 41.5, 35.8, 32.2. ESI-MS *m/z*: 454 [M – H]<sup>-</sup>.

# 5.1.3. General procedure for the synthesis of cinnamate ester derivatives of sinomenine (3a-j)

5.1.3.1. Synthesis with batch. A mixture of **2** (455 mg, 1 mmol), Pd(OAc)<sub>2</sub> (11.2 mg, 0.05 mmol), PPh<sub>3</sub> (26.2 mg, 0.1 mmol) was added in a 10 mL flask under N<sub>2</sub>, then acrylic ester (1 mmol) in 2 mL DMF/Et<sub>3</sub>N (5:1 v/v) was added. The solution was then stirred at 85 °C. After 10 min or 20 min, 200  $\mu$ L of the solution was added 1 mL H<sub>2</sub>O, and then extracted with ethyl acetate (3 × 2 mL). The organic phase was washed by brine (3 × 3 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuum to provide the crude product. The <sup>1</sup>H NMR spectrum of the crude mixture was recorded and the product conversion was determined by integration of the peaks arising from H-2 of both the 1-idiosinomenine and the cinnamate esters derivatives.

5.1.3.2. Synthesis with microreactor. A stock solution of the 1-idiosinomenine (455 mg, 1 mmol), Pd(OAc)<sub>2</sub> (11.2 mg, 0.05 mmol), PPh<sub>3</sub> (26.2 mg, 0.1 mmol), and acrylic ester (1 mmol) in DMF/Et<sub>3</sub>N (2 mL, 5:1, v/v) was prepared and taken up in an SGE gastight syringe. The syringe was placed on a TS2-60 syringe pump that was set to deliver 8  $\mu$ L/min and the oil bath was set at 85 °C. The output from the reactor was quenched with H<sub>2</sub>O immediately. After reaction, the solvent and



**Fig. 5.** Activity of synthesized compounds on TNF- $\alpha$ -induced NF- $\kappa$ B activation. Each data was expressed as mean  $\pm$  SD, n = 3. The data of the control group was pegged as 100%, while other data were calculated relative to it. Data were analyzed by student's *t* test. #p < 0.01 versus the blank (without any treatments). \*p < 0.05, \*\*p < 0.01 versus the control (treated with TNF- $\alpha$  only).

H<sub>2</sub>O were removed in vacuum and 10 mL ethyl acetate was added, washed by brine (3  $\times$  5 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuum to provide the crude product. The <sup>1</sup>H NMR spectrum of the crude mixture was recorded and the product conversion was determined by integration of the peaks arising from H-2 of both the 1-idiosinomenine and the cinnamate esters derivatives. The crude mixture was purified by silica gel column chromatography (DCM/MeOH) to provide the target compound **3a**–**j**.

Compound **3a** Yield 98%; mp 249–251 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.99 (1H, d, J = 15.6 Hz), 6.92 (1H, s), 6.23 (1H, d, J = 15.6 Hz), 5.47 (1H, d, J = 1.8 Hz), 4.36 (1H, d, J = 15.6 Hz), 3.84 (3H, s), 3.82 (3H, s), 3.49 (3H, s), 3.33 (1H, m), 3.12 (1H, d, J = 18.9 Hz), 3.09 (1H, m), 2.73 (1H, dd, J = 18.3, 6.0 Hz), 2.62 (1H, m), 2.50 (1H, d, J = 15.6 Hz), 2.45 (3H, s), 2.00 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.9, 168.0, 152.7, 147.4, 145.6, 142.2, 131.1, 123.5, 123.3, 116.8, 114.8, 107.0, 56.5, 56.2, 55.1, 51.9, 49.3, 47.3, 45.6, 43.1, 40.9, 35.7, 22.3. ESI-MS m/z: 412 [M – H]<sup>–</sup>, HRESIMS m/z: found: 414.1937 [M + H]<sup>+</sup> (calcd for C<sub>23</sub>H<sub>28</sub>NO<sub>6</sub>, 414.1917).

Compound **3b** Yield 94%; mp 166–168 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.98 (1H, d, J = 15.6 Hz), 6.92 (1H, s), 6.23 (1H, d, J = 15.6 Hz), 5.47 (1H, d, J = 1.8 Hz), 4.36 (1H, d, J = 15.6 Hz), 4.28 (2H, q, J = 7.2 Hz), 3.83 (3H, s), 3.49 (3H, s), 3.33 (1H, m), 3.12 (1H, d, J = 18.9 Hz), 3.07 (1H, m), 2.73 (1H, dd, J = 18.3, 6.0 Hz), 2.56 (1H, m), 2.51 (1H, d, J = 15.6 Hz), 2.43 (3H, s), 1.99 (3H, m), 1.36 (3H, t, J = 7.2 Hz). <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.8, 167.4, 152.4,



**Fig. 6.** Effect of **3g** on  $\lambda$ -carrageenan-induced paw edema in ICR mice. Indo, indomethacin was used as a positive control. The results were expressed as means  $\pm$  SD, n = 6. Data were analyzed by student's *t* test. \*p < 0.05; \*\*p < 0.01 versus the control (treated with  $\lambda$ -carrageenan only). Increase in paw thickness (%) = 100 × (compound – normal)/ normal.

147.2, 145.4, 141.7, 131.0, 123.3, 123.0, 116.9, 114.7, 106.8, 60.5, 56.2, 55.9, 54.9, 49.1, 47.1, 45.3, 42.8, 40.7, 35.4, 22.0, 14.4; ESI-MS m/z: 426  $[M - H]^-$ , HRESIMS m/z: found: 428.2111  $[M + H]^+$  (calcd for C<sub>24</sub>H<sub>30</sub>NO<sub>6</sub>, 428.2073).

Compound **3c** Yield 92%; mp 130–132 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.99 (1H, d, J = 15.6 Hz), 6.93 (1H, s), 6.23 (1H, d, J = 15.6 Hz), 5.48 (1H, d, J = 1.8 Hz), 4.37 (1H, d, J = 15.6 Hz), 4.23 (2H, t, J = 6.6 Hz), 3.84 (3H, s), 3.50 (3H, s), 3.32 (1H, m), 3.13 (1H, d, J = 18.9 Hz), 3.07 (1H, m), 2.72 (1H, dd, J = 18.3, 6.0 Hz), 2.58 (1H, m), 2.51 (1H, d, J = 15.6 Hz), 2.44 (3H, s), 2.00 (3H, m), 1.72 (2H, m), 1.48 (2H, m), 0.99 (3H, t, J = 7.2 Hz); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.9, 167.6, 152.4, 147.2, 145.4, 141.8, 131.1, 123.4, 123.1, 116.9, 114.8, 106.8, 64.5, 56.2, 56.0, 54.9, 49.2, 47.1, 45.5, 42.9, 40.7, 35.6, 30.9, 22.0, 19.3, 13.9. ESI-MS m/z: 454 [M – H]<sup>–</sup>, HRESIMS m/z: found: 456.2402 [M + H]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>34</sub>NO<sub>6</sub>, 456.2386).

Compound **3d** Yield 90%; mp 165–168 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 8.01 (1H, d, J = 15.6 Hz), 6.94 (1H, s), 6.25 (1H, d, J = 15.6 Hz), 5.48 (1H, d, J = 1.8 Hz), 4.37 (1H, d, J = 15.6 Hz), 4.02 (1H, d, J = 6.6 Hz), 3.84 (3H, s), 3.50 (3H, s), 3.34 (1H, m), 3.14 (1H, d, J = 18.9 Hz), 3.08 (1H, m), 2.69 (1H, dd, J = 18.3, 6.0 Hz), 2.58 (1H, m), 2.51 (1H, d, J = 15.6 Hz), 2.44 (3H, s), 2.04 (4H, m), 1.01 (6H, d, J = 6.9 Hz); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 167.5, 152.4, 147.3, 145.4, 141.8, 131.0, 123.4, 123.0, 116.7, 114.7, 106.8, 70.6, 56.1, 55.9, 54.9, 49.1, 47.0, 45.3, 42.8, 40.7, 35.4, 27.9, 22.0, 19.2. ESI-MS m/z: 454 [M – H]<sup>–</sup>, HRESIMS m/z: found: 456.2412 [M + H]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>34</sub>NO<sub>6</sub>, 456.2386).

Compound **3e** Yield 93%; mp 167–169 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.91 (1H, d, J = 15.6 Hz), 6.91 (1H, s), 6.16 (1H, d, J = 15.6 Hz), 5.46 (1H, d, J = 18. Hz), 4.36 (1H, d, J = 15.6 Hz), 3.81 (3H, s), 3.48 (3H, s), 3.31 (1H, m), 3.12 (1H, d, J = 18.9 Hz), 3.06 (1H, m), 2.71 (1H, dd, J = 18.3, 6.0 Hz), 2.57 (1H, m), 2.46 (1H, d, J = 15.6 Hz), 2.42 (3H, s), 1.99 (3H, m), 1.55 (9H, s); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.9, 166.9, 152.4, 147.0, 145.3, 140.8, 130.9, 123.2, 123.1, 118.5, 114.7, 106.7, 80.5, 56.2, 55.9, 54.9, 49.1, 47.0, 45.4, 42.9, 40.7, 35.5, 28.3, 22.0. ESI-MS *m*/*z*: 454 [M – H]<sup>-</sup>, HRE-SIMS *m*/*z*: found: 456.2421 [M + H]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>34</sub>NO<sub>6</sub>, 456.2386).

Compound **3f** Yield 93%; mp: 135–137 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 8.00 (1H, d, J = 15.6 Hz), 6.92 (1H, s), 6.30 (1H, d, J = 15.6 Hz), 5.45 (1H, d, J = 1.8 Hz), 4.38 (3H, t, J = 4.8 Hz), 4.36 (1H, d, J = 15.6 Hz), 3.84 (3H, s), 3.68 (3H, t, J = 4.8 Hz), 3.48 (3H, s), 3.44 (3H, s), 3.29 (1H, m), 3.11 (1H, d, J = 18.9 Hz), 3.04 (1H, m), 2.69 (1H, dd, J = 18.3, 6.0 Hz), 2.56 (1H, m), 2.48 (1H, d, J = 15.6 Hz), 2.43 (3H, s), 1.97 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.8, 167.5, 152.5, 147.2, 145.3, 142.3, 131.3, 123.3, 123.2, 116.6, 114.7, 106.9, 70.7, 63.6, 59.2, 56.3, 56.0, 54.9, 49.2, 47.1, 45.5, 43.0, 40.7, 35.7, 22.0. ESI-

MS m/z: 456 [M - H]<sup>-</sup>, HRESIMS m/z: found: 458.2203 [M + H]<sup>+</sup> (calcd for C<sub>25</sub>H<sub>32</sub>NO<sub>7</sub>, 458.2179).

Compound **3g** Yield 94%; mp 118–119 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.98 (1H, d, J = 15.6 Hz), 6.92 (1H, s), 6.22 (1H, d, J = 15.6 Hz), 5.46 (1H, d, J = 1.8 Hz), 4.35 (1H, d, J = 15.6 Hz), 4.21 (2H, t, J = 6.6 Hz), 3.83 (3H, s), 3.48 (3H, s), 3.30 (1H, m), 3.11 (1H, d, J = 18.9 Hz), 3.05 (1H, m), 2.70 (1H, dd, J = 18.3, 6.0 Hz), 2.68 (1H, m), 2.49 (1H, d, J = 15.6 Hz), 2.42 (3H, s), 1.95 (3H, m), 1.71 (2H, m), 1.35 (6H, m), 0.91 (3H, t, J = 6.6 Hz); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 167.5, 152.4, 147.1, 145.3, 141.7, 131.0, 123.3, 123.1, 116.8, 114.7, 106.7, 64.7, 56.1, 55.9, 54.8, 49.1, 47.0, 45.4, 42.8, 40.6, 35.5, 31.4, 28.7, 25.6, 22.5, 21.9, 14.0. ESI-MS m/z: 482 [M – H]<sup>–</sup>, HRESIMS m/z: found: 484.2733 [M + H]<sup>+</sup> (calcd for C<sub>28</sub>H<sub>38</sub>NO<sub>6</sub>, 484.2699).

Compound **3h** Yield 93%; mp 158–160 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.96 (1H, d, J = 15.6 Hz), 6.92 (1H, s), 6.21 (1H, d, J = 15.6 Hz), 5.46 (1H, d, J = 1.8 Hz), 4.89 (1H, m), 4.35 (1H, d, J = 15.6 Hz), 3.83 (3H, s), 3.48 (3H, s), 3.31 (1H, m), 3.11 (1H, d, J = 18.9 Hz), 3.05 (1H, m), 2.71 (1H, dd, J = 18.3, 6.0 Hz), 2.57 (1H, m), 2.49 (1H, d, J = 15.6 Hz), 2.43 (3H, s), 1.94 (5H, m), 1.77 (2H, m), 1.48 (6H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 167.6, 152.4, 147.3, 145.4, 141.7, 131.0, 123.3, 123.0, 116.8, 114.7, 106.8, 67.0, 55.9, 54.8, 49.1, 47.0, 45.4, 42.8, 40.7, 38.9, 35.5, 30.5, 29.0, 23.9, 23.0, 22.0, 14.1, 11.1 ESI-MS m/z: 480 [M – H]<sup>-</sup>, HRESIMS m/z: found: 482.2572 [M + H]<sup>+</sup> (calcd for C<sub>28</sub>H<sub>36</sub>NO<sub>6</sub>, 482.2543).

Compound **3i** Yield 91%; mp 114–116 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.99 (1H, d, J = 15.6 Hz), 6.93 (1H, s), 6.22 (1H, d, J = 15.6 Hz), 5.47 (1H, d, J = 1.8 Hz), 4.36 (1H, d, J = 15.6 Hz), 4.14 (2H, t, J = 5.4 Hz), 3.83 (3H, s), 3.48 (3H, s), 3.31 (1H, m), 3.12 (1H, d, J = 18.9 Hz), 3.06 (1H, m), 2.71 (1H, dd, J = 18.3, 6.0 Hz), 2.57 (1H, m), 2.50 (1H, d, J = 15.6 Hz), 2.42 (3H, s), 1.99 (3H, m), 1.67 (1H, m), 1.40 (8H, m), 0.91 (6H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.8, 166.9, 152.5, 147.1, 145.4, 141.5, 131.1, 123.4, 123.2, 117.5, 114.8, 106.8, 72.8, 56.2, 56.0, 54.9, 49.2, 47.1, 46.8, 45.5, 42.9, 40.7, 35.6, 31.9, 31.7, 25.5, 25.4, 24.0, 23.8, 22.0. ESI-MS m/z: 510 [M – H]<sup>–</sup>, HRESIMS m/z: found: 512.3041 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>42</sub>NO<sub>6</sub>, 512.3012).

Compound **3j** Yield 92%; mp 189–191 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.63 (1H, s), 6.55 (1H, s), 5.43 (1H, d, *J* = 2.1 Hz), 4.34 (1H, d, *J* = 15.6 Hz), 3.81 (3H, s), 3.78 (3H, s), 3.46 (3H, s), 3.26 (1H, m), 3.06 (1H, m), 2.82 (1H, d, *J* = 18.9 Hz), 2.57 (1H, m), 2.48 (1H, m), 2.46 (1H, d, *J* = 15.6 Hz), 2.40 (3H, s), 2.03 (3H, m), 1.92 (3H, s); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.8, 168.9, 152.3, 144.8, 144.5, 138.1, 129.2, 128.1, 124.4, 122.7, 114.5, 109.8, 56.3, 55.9, 54.8, 52.0, 49.0, 47.1, 45.2, 42.7, 40.4, 35.4, 22.4, 14.0. ESI-MS *m*/*z*: 436 [M – H]<sup>-</sup>, HRESIMS *m*/*z*: found: 428.2099 [M + H]<sup>+</sup> (calcd for C<sub>24</sub>H<sub>30</sub>NO<sub>6</sub>, 428.2073).

# 5.1.4. General procedure for the synthesis of isoxazoline derivatives of sinomenine (**4a–11a** and **4b–11b**) using microreactor

Firstly, a stock solution of the 1-idiosinomenine (455 mg, 1 mmol), Pd(OAc)<sub>2</sub> (11.2 mg, 0.05 mmol), PPh<sub>3</sub> (26.2 mg, 0.1 mmol) and methylacrylate (1 mmol) in DMF/Et<sub>3</sub>N (2 mL, 5:1, v/v) was prepared and taken up in an SGE gastight syringe. The syringe was placed on a TS2-60 syringe pump that was set to deliver 8 µL/min and the M1 was set at 85 °C. Secondly, a solution of 1 mL aromatic aldehyde oxime solution (1 M in DMF) and a solution of 1 mL NCS solution (1 M in DMF) were prepared and taken up in two SGE gastight syringes. The syringes were placed on a TS2-60 syringe pump that was set to deliver 4  $\mu$ L/min, and the M2 was at room temperature. Thirdly, the M3 was set at 70 °C. The output from the reactor was quenched with H<sub>2</sub>O immediately. After reaction, the solvent and H<sub>2</sub>O were removed in vacuum and 10 mL ethyl acetate was added, washed by brine  $(3 \times 5 \text{ mL})$  and dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuum to provide the crude product. The crude mixture was purified by silica gel column chromatography (DCM/ MeOH) to provide the isomer-a and isomer-b, after determined the ratio by <sup>1</sup>H NMR, the diastereoisomers were separated by silica gel column chromatography (DCM/MeOH) to provide **4a**–**11a** and **4b**–**11b**.

5.1.4.1. Compound **4a** and **4b** yield 92%, isomer ratio: **4a**:**4b** = 39:61. Isomer **4a**, mp 151–153 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.71 (2H, m), 7.42 (3H, m), 6.74 (1H, s), 6.07 (1H, d, *J* = 5.7 Hz), 5.44 (1H, d, *J* = 1.8 Hz), 4.37 (1H, d, *J* = 15.6 Hz), 4.24 (1H, d, *J* = 5.7 Hz), 3.78 (3H, s), 3.77 (3H, s), 3.50 (3H, s), 3.29 (1H, m), 3.05 (1H, m), 2.84 (1H, d, *J* = 17.7 Hz), 2.60 (2H, m), 2.47 (3H, s), 2.45 (1H, d, *J* = 15.6 Hz), 1.95 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 170.2, 153.5, 152.6, 145.5, 144.9, 130.7, 129.0, 128.4, 127.1, 127.0, 126.2, 123.2, 114.4, 106.2, 84.6, 62.0, 56.4, 56.2, 55.0, 53.4, 49.3, 47.4, 45.5, 42.8, 40.7, 35.8, 21.7. ESI-MS *m/z*: 533 [M + H]<sup>+</sup>, HRESIMS *m/z*: found: 533.2307 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>33</sub>N<sub>2</sub>O<sub>7</sub>, 533.2288).

Isomer **4b**, mp 147–149 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.66 (2H, m), 7.41 (3H, m), 6.76 (1H, s), 6.17 (1H, d, J = 6.0 Hz), 5.48 (1H, d, J = 2.1 Hz), 4.35 (1H, d, J = 15.6 Hz), 4.08 (1H, d, J = 6.0 Hz), 3.78 (3H, s), 3.76 (3H, s), 3.55 (3H, s), 3.32 (1H, m), 3.08 (1H, m), 2.97 (1H, d, J = 18.0 Hz), 2.60 (1H, m), 2.48 (5H, m), 2.06 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.8, 170.2, 153.6, 152.6, 145.6, 144.9, 130.7, 129.0, 128.3, 127.3, 127.0, 126.3, 123.5, 114.4, 106.1, 84.2, 61.9, 56.5, 56.2, 55.0, 53.4, 48.9, 47.3, 45.2, 42.9, 40.7, 35.4, 21.5. ESI-MS *m*/*z*: 533 [M + H]<sup>+</sup>, HRESIMS *m*/*z*: found: 533.2302 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>33</sub>N<sub>2</sub>O<sub>7</sub>, 533.2288).

5.1.4.2. Compound **5a** and **5b** yield 93%; isomer ratio: **5a**:**5b** = 37:63. Isomer **5a**, mp: 160–162 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.60 (2H, d, *J* = 8.4 Hz), 7.21 (2H, d, *J* = 8.4 Hz), 6.73 (1H, s), 6.05 (1H, d, *J* = 5.7 Hz), 5.45 (1H, d, *J* = 1.8 Hz), 4.37 (1H, d, *J* = 15.6 Hz), 4.21 (1H, d, *J* = 5.7 Hz), 3.78 (3H, s), 3.77 (3H, s), 3.50 (3H, s), 3.29 (1H, m), 3.04 (1H, m), 2.83 (1H, d, *J* = 17.7 Hz), 2.62 (2H, m), 2.47 (3H, s), 2.45 (1H, d, *J* = 15.6 Hz), 2.38 (3H, s), 1.94 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.6, 170.1, 153.3, 152.4, 145.3, 144.7, 140.8, 129.5, 127.0, 126.7, 126.0, 125.3, 123.0, 114.3, 106.0, 54.2, 61.9, 56.2, 56.0, 54.7, 53.2, 49.1, 47.2, 45.3, 42.6, 40.5, 35.6, 21.4. ESI-MS *m*/*z*: 547 [M + H]<sup>+</sup>, HRESIMS *m*/*z*: found: 547.2466 [M + H]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>35</sub>N<sub>2</sub>O<sub>7</sub>, 547.2444).

Isomer **5b**, mp 153–155 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.54 (2H, d, *J* = 8.4 Hz), 7.20 (2H, d, *J* = 8.4 Hz), 6.75 (1H, s), 6.15 (1H, d, *J* = 6.3 Hz), 5.48 (1H, d, *J* = 2.1 Hz), 4.35 (1H, d, *J* = 15.6 Hz), 4.06 (1H, d, *J* = 6.3 Hz), 3.77 (3H, s), 3.76 (3H, s), 3.55 (3H, s), 3.30 (1H, m), 3.05 (1H, m), 2.96 (1H, d, *J* = 18.3 Hz), 2.57 (1H, m), 2.47 (5H, m), 2.37 (3H, s), 2.00 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 170.1, 153.3, 152.4, 145.3, 144.7, 140.8, 129.5, 127.1, 126.7, 126.1, 125.2, 123.3, 114.4, 105.8, 83.8, 61.8, 56.2, 56.0, 54.8, 53.2, 48.7, 47.0, 45.1, 42.7, 40.5, 35.2, 21.4, 21.2. ESI-MS *m*/*z*: 547 [M + H]<sup>+</sup>, HRESIMS *m*/*z*: found: 547.2379 [M + H]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>35</sub>N<sub>2</sub>O<sub>7</sub>, 547.2444).

5.1.4.3. Compound **6a** and **6b** yield 92%; isomer ratio: **6a**:**6b** = 47:53. Isomer **6a**, mp 153–155 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.58 (1H, s), 7.45 (1H, d, *J* = 7.5 Hz), 7.28 (2H, m), 6.74 (1H, s), 6.06 (1H, d, *J* = 5.7 Hz), 5.44 (1H, d, *J* = 1.8 Hz), 4.38 (1H, d, *J* = 15.6 Hz), 4.23 (1H, d, *J* = 5.7 Hz), 3.78 (3H, s), 3.77 (3H, s), 3.50 (3H, s), 3.38 (1H, m), 3.14 (1H, m), 2.88 (1H, d, *J* = 18 Hz), 2.68 (2H, m), 2.52 (3H, s), 2.46 (1H, d, *J* = 15.6 Hz), 2.38 (3H, s), 1.97 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.5, 170.2, 153.6, 152.7, 145.6, 144.9, 138.8, 131.5, 128.9, 128.3, 127.5, 127.2, 125.7, 124.1, 123.0, 114.0, 106.4, 84.4, 62.0, 56.6, 56.2, 55.0, 53.4, 49.1, 47.5, 44.9, 42.6, 40.5, 35.4, 21.8, 21.5. ESI-MS *m*/*z*: 547 [M + H]<sup>+</sup>, HRESIMS *m*/*z*: found: 547.2473 [M + H]<sup>+</sup> (calcd for C<sub>31H35</sub>N<sub>2</sub>O<sub>7</sub>, 547.2444).

Isomer **6b**, mp 144–146 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.52 (1H, s), 7.40 (1H, d, J = 7.5 Hz), 7.27 (2H, m), 6.75 (1H, s), 6.15 (1H, d, J = 5.7 Hz), 5.48 (1H, d, J = 2.1 Hz), 4.35 (1H, d, J = 15.9 Hz), 4.07 (1H, d, J = 5.7 Hz), 3.78 (3H, s), 3.76 (3H, s), 3.56 (3H, s), 3.34 (1H, m), 3.10 (1H, m), 2.98 (1H, d, J = 18 Hz), 2.61 (1H, m), 2.47 (5H, m), 2.36 (3H, s), 2.05 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 170.2, 153.7, 152.6, 145.6, 144.9, 138.8, 131.5, 128.9, 128.1, 127.5, 127.3, 126.1, 124.1, 123.4, 114.4, 106.1, 84.0, 61.9, 56.5, 56.2, 55.0, 53.4, 48.8, 47.3, 45.1, 42.8, 40.6, 35.3, 29.8, 21.5. ESI-MS m/z: 547 [M + H]<sup>+</sup>, HRESIMS m/z: found: 547.2463 [M + H]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>35</sub>N<sub>2</sub>O<sub>7</sub>, 547.2444).

5.1.4.4. Compound **7a** and **7b** yield 96%; isomer ratio: **7a**:**7b** = 34:66. Isomer **7a**, mp 158–160 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.66 (2H, m), 6.92 (2H, m), 6.74 (1H, s), 6.04 (1H, d, *J* = 5.7 Hz), 5.45 (1H, d, *J* = 2.1 Hz), 4.38 (1H, d, *J* = 15.6 Hz), 4.20 (1H, d, *J* = 5.7 Hz), 3.84 (3H, s), 3.79 (3H, s), 3.78 (3H, s), 3.50 (3H, s), 3.30 (1H, m), 3.05 (1H, m), 2.82 (1H, d, *J* = 17.7 Hz), 2.62 (2H, m), 2.47 (3H, s), 2.45 (1H, d, *J* = 15.6 Hz), 1.95 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.5, 170.1, 161.3, 152.8, 152.4, 145.3, 144.6, 128.4, 127.1, 125.9, 123.0, 120.6, 114.2, 106.0, 84.0, 62.0, 56.2, 56.0, 55.3, 54.7, 53.2, 49.1, 47.2, 45.2, 42.6, 40.5, 35.6, 21.4. ESI-MS *m/z*: 563 [M + H]<sup>+</sup>, HRESIMS *m/z*: found: 563.2416 [M + H]<sup>+</sup> (calcd for C<sub>31H35N2O8</sub>, 563.2393).

Isomer **7b**, mp 150–152 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.60 (2H, d, J = 9 Hz), 6.91 (2H, d, J = 9 Hz), 6.76 (1H, s), 6.14 (1H, d, J = 6.3 Hz), 5.48 (1H, d, J = 1.8 Hz), 4.35 (1H, d, J = 15.6 Hz), 4.04 (1H, d, J = 6.3 Hz), 3.83 (3H, s), 3.79 (3H, s), 3.77 (3H, s), 3.56 (3H, s), 3.33 (1H, m), 3.09 (1H, m), 2.97 (1H, d, J = 18.3 Hz), 2.61 (1H, m), 2.47 (5H, s), 2.06 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.6, 170.1, 161.2, 152.9, 152.3, 145.4, 144.8, 128.3, 127.2, 125.9, 123.3, 120.5, 114.3, 114.2, 105.9, 83.6, 61.9, 56.2, 55.9, 55.3, 54.8, 53.2, 48.7, 47.1, 44.9, 42.6, 40.4, 35.1, 29.6, 21.3. ESI-MS m/z: found: 563.2428 [M + H]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>35</sub>N<sub>2</sub>O<sub>8</sub>, 563.2393).

5.1.4.5. Compound **8a** and **8b** yield 81%; isomer ratio: **8a**:**8b** = 28:72. Isomer **8a**, mp 157–159 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.66 (2H, m), 7.39 (2H, m), 6.73 (1H, s), 6.09 (1H, d, *J* = 5.7 Hz), 5.44 (1H, d, *J* = 2.1 Hz), 4.38 (1H, d, *J* = 15.6 Hz), 4.25 (1H, d, *J* = 5.7 Hz), 3.79 (6H, s), 3.51 (4H, m), 3.28 (1H, m), 2.95 (1H, m), 2.78 (2H, m), 2.59 (3H, s), 2.58 (1H, d, *J* = 15.6 Hz), 2.04 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 192.9, 169.7, 152.6, 152.5, 145.6, 144.8, 136.6, 129.1, 128.1, 126.9, 126.7, 124.8, 122.4, 112.9, 106.4, 84.4, 61.5, 56.8, 56.1, 54.9, 53.4, 48.6, 47.5, 44.0, 42.2, 40.0, 34.5, 21.8. ESI-MS *m/z*: 567, 569 [M + H]<sup>+</sup>, HRESIMS *m/z*: found: 567.1904 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>ClN<sub>2</sub>O<sub>7</sub>, 567.1898).

Isomer **8b**, mp 151–153 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.60 (2H, m), 7.38 (2H, m), 6.72 (1H, s), 6.19 (1H, d, J = 6.3 Hz), 5.47 (1H, d, J = 2.1 Hz), 4.35 (1H, d, J = 15.6 Hz), 4.05 (1H, d, J = 6.3 Hz), 3.78 (3H, s), 3.77 (3H, s), 3.55 (3H, s), 3.32 (1H, m), 3.08 (1H, m), 2.97 (1H, d, J = 18 Hz), 2.60 (1H, m), 2.46 (5H, m), 2.01 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.6, 169.7, 152.5, 152.4, 145.4, 144.8, 136.5, 129.1, 128.0, 126.8, 126.6, 126.0, 123.3, 114.2, 105.8, 84.2, 61.4, 56.2, 26.0, 54.8, 53.3, 48.6, 47.1, 44.9, 42.6, 40.4, 35.1, 21.3. ESI-MS m/z: 567, 569 [M + H]<sup>+</sup>, HRESIMS m/z: found: 567.1932 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>ClN<sub>2</sub>O<sub>7</sub>, 567.1898).

5.1.4.6. Compound **9a** and **9b** yield 84%; isomer ratio: **9a**:**9b** = 43:57. Isomer **9a**, mp: 152–154 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.66 (2H, m), 7.39 (2H, m), 6.71 (1H, s), 6.09 (1H, d, *J* = 6.0 Hz), 5.43 (1H, s), 4.37 (1H, d, *J* = 15.6 Hz), 4.22 (1H, d, *J* = 6.0 Hz), 3.79 (3H, s), 3.78 (3H, s), 3.51 (3H, s), 3.38 (1H, m), 3.15 (1H, m), 2.92 (1H, d, *J* = 17.7 Hz), 2.69 (2H, m), 2.52 (3H, s), 2.47 (1H, d, *J* = 15.6 Hz), 1.99 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.2, 169.6, 152.5, 145.4, 144.8, 134.9, 130.5, 130.1, 130.0, 126.8, 126.7, 125.5, 124.9, 122.8, 113.6, 106.1, 84.6, 61.3, 56.4, 56.1, 54.8, 53.3, 48.8, 47.3, 44.6, 42.4, 40.3, 35.0, 29.6, 21.6. ESI-MS *m*/*z*: 567, 569 [M + H]<sup>+</sup>, HRE-SIMS *m*/*z*: found: 567.1906 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>ClN<sub>2</sub>O<sub>7</sub>, 567.1898). Isomer **9b**, mp: 146–148 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.60 (2H, m), 7.37 (2H, m), 6.72 (1H, s), 6.19 (1H, d, J = 6.3 Hz), 5.47 (1H, m), 4.35 (1H, d, J = 15.6 Hz), 4.05 (1H, d, J = 6.3 Hz), 3.79 (3H, s), 3.77 (3H, s), 3.55 (3H, s), 3.33 (1H, m), 3.10 (1H, m), 2.97 (1H, d, J = 17.7 Hz), 2.62 (1H, m), 2.48 (5H, m), 2.06 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.6, 169.5, 152.4, 145.5, 144.9, 134.8, 130.4, 130.0 1299, 126.8, 126.7, 126.0, 124.8, 123.5, 114.2, 105.9, 84.3, 61.9, 56.2, 56.0, 54.8, 53.3, 48.6, 47.2, 47.1, 44.8, 42.5, 40.4, 35.0, 29.6, 21.3. ESI-MS m/z: 567, 569 [M + H]<sup>+</sup>, HRESIMS m/z: found: 567.1932 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>ClN<sub>2</sub>O<sub>7</sub>, 567.1898).

5.1.4.7. Compound **10a** and **10b** yield 87%; isomer ratio: **10a**:**10b** = 34:66. Isomer **10a**, mp 158–160 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.57 (4H, m), 6.70 (1H, s), 6.10 (1H, d, *J* = 6.0 Hz), 5.45 (1H, d, *J* = 2.1 Hz), 4.37 (1H, d, *J* = 15.6 Hz), 4.20 (1H, d, *J* = 6.0 Hz), 3.79 (3H, s), 3.77 (3H, s), 3.50 (3H, s), 3.30 (1H, m), 3.05 (1H, m), 2.81 (1H, d, *J* = 17.7 Hz), 2.60 (2H, m), 2.46 (3H, s), 2.45 (1H, d, *J* = 15.6 Hz), 1.95 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.6, 169.7, 152.5, 152.4, 145.4, 144.9, 132.0, 128.2, 127.2, 126.5, 126.0, 124.8, 123.1, 114.3, 105.9, 84.6, 61.4, 56.1, 56.0, 54.7, 53.3, 49.1, 47.2, 45.2, 42.6, 40.5, 35.5, 21.5. ESI-MS *m/z*: 611, 613 [M + H]<sup>+</sup>, HRESIMS *m/z*: found: 611.1433 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>BrN<sub>2</sub>O<sub>7</sub>, 611.1393).

Isomer **10b**, mp 153–155 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.53 (4H, m), 6.72 (1H, s), 6.19 (1H, d, J = 6.3 Hz), 5.47 (1H, d, J = 2.1 Hz), 4.35 (1H, d, J = 15.6 Hz), 4.05 (1H, d, J = 6.3 Hz), 3.78 (3H, s), 3.77 (3H, s), 3.55 (3H, s), 3.33 (1H, m), 3.10 (1H, m), 2.97 (1H, d, J = 18.3 Hz), 2.61 (1H, m), 2.47 (5H, m), 20.2 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.7, 169.6, 152.6, 152.3, 145.6, 145.1, 132.0, 128.2, 127.1, 126.5, 126.2, 124.7, 123.7, 114.5, 105.8, 84.2, 61.3, 56.0, 55.9, 54.8, 53.3, 48.7, 47.0, 44.8, 42.5, 40.5, 35.0, 29.6, 21.3. ESI-MS *m*/*z*: 611, 613 [M + H]<sup>+</sup>, HRESIMS *m*/*z*: found: 611.1361 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>BrN<sub>2</sub>O7, 611.1393).

5.1.4.8. Compound **11a** and **11b** yield 86%; isomer ratio **11a**:**11b** = 31:69. Isomer **11a**, mp 157–159 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.91 (1H, m), 7.59 (2H, m), 7.29 (1H, m), 6.69 (1H, s), 6.10 (1H, d, *J* = 6.0 Hz), 5.44 (1H, d, *J* = 1.8 Hz), 4.37 (1H, d, *J* = 15.6 Hz), 4.20 (1H, d, *J* = 6.0 Hz), 3.80 (3H, s), 3.78 (3H, s), 3.51 (3H, s), 3.32 (1H, m), 3.08 (1H, m), 2.83 (1H, d, *J* = 17.7 Hz), 2.63 (2H, m), 2.48 (3H, s), 2.46 (1H, d, *J* = 15.6 Hz), 1.96 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.5, 169.6, 152.4, 152.2, 145.4, 144.8, 133.3, 130.3, 130.2, 129.7, 126.5, 125.9, 125.3, 123.0, 122.9, 114.1, 105.9, 84.7, 61.3, 56.2, 56.0, 54.8, 53.3, 49.0, 47.2, 45.0, 42.5, 40.4, 35.4, 21.5. ESI-MS *m/z*: 611, 613 [M + H]<sup>+</sup>, HRESIMS *m/z*: found: 611.1367 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>BrN<sub>2</sub>O<sub>7</sub>, 611.1393).

Isomer **11b**, mp 149–151 °C; <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 7.85 (1H, m), 7.56 (2H, m), 7.28 (1H, m), 6.74 (1H, s), 6.19 (1H, d, J = 6.0 Hz), 5.48 (1H, d, J = 1.8 Hz), 4.36 (1H, d, J = 15.6 Hz), 4.07 (1H, d, J = 6.0 Hz), 3.80 (3H, s), 3.79 (3H, s), 3.56 (4H, m), 3.27 (1H, m), 3.02 (1H, d, J = 18.3 Hz), 2.79 (1H, m), 2.63 (1H, m), 2.56 (3H, s), 2.52 (1H, d, J = 15.6 Hz), 2.08 (3H, m); <sup>13</sup>C NMR (75 MHz; CDCl<sub>3</sub>; TMS)  $\delta$ : 193.2, 169.5, 152.5, 152.3, 145.6, 145.0, 133.4, 130.3, 130.0, 129.7, 126.8, 125.3, 125.1, 123.0, 122.9, 113.3, 106.1, 84.1, 61.1, 56.4, 56.0, 54.9, 53.4, 48.2, 47.2, 43.9, 42.1, 40.1, 34.4, 29.6, 21.5. ESI-MS *m/z*: 611, 613 [M + H]<sup>+</sup>, HRESIMS *m/z*: found: 611.1412 [M + H]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>32</sub>BrN<sub>2</sub>O<sub>7</sub>, 611.1393).

#### 5.2. Biological activity

#### 5.2.1. Cultivation of fibroblasts

The mouse embryonic fibroblast (NIH/3T3) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

In cell cultures, each compound was prepared in DMSO followed by dilution with culture medium to the indicated concentrations, and DMSO final concentration was 0.1%. DMSO at 0.1% was added into control (the cells treated with TNF- $\alpha$  only) and blank (the cells without any treatments) groups and showed no effects on the cells.

#### 5.2.2. MTT assay

Cell cytotoxicity was assessed by MTT assay and expressed as IC<sub>50</sub>. NIH/3T3 cells were plated at 1  $\times$  10<sup>4</sup> cells/well in 96-well plates, and compounds were added to each plate as the indicated concentrations. Untreated cells were served as blank. After a 24 h incubation period at 37 °C in 5% CO<sub>2</sub>, culture medium was replaced with fresh medium and 20  $\mu$ L MTT reagents (5 mg/mL) were added. Four hours later, the supernatants were aspirated, and the formazan crystals in each well were dissolved in 200  $\mu$ L of dimethyl sulphoxide (DMSO) for 30 min at 37 °C. The absorbance value was monitored by microplate reader at 570 nm. The density of formazan formed in blank (medium alone) cells was set as 100% of viability. The IC<sub>50</sub> values were determined graphically from the concentration–activity curve.

#### 5.2.3. Measurement of luciferase activity

NIH/3T3 were plated and cultured overnight in 100 mm dish  $(4 \times 10^5 \text{ cells/well})$ . The pNF- $\kappa$ B-luc containing NF- $\kappa$ B binding motifs (GGGAATTTCC) was transfected into cells using Lipofect-amine 2000 reagents (Invitrogen, USA). At 24 h post-transfection, the cells were plated in 24-well plates and then treated with 20  $\mu$ M of test compounds in presence of 20 ng/mLTNF- $\alpha$  for 6 h. The luciferase activity of cell extracts from each sample was measured using a luciferase activity of each test compound was reported as relative light units (RLU).

### 5.2.4. $\lambda$ -Carrageenan-induced paw inflammation suppression test

The ICR mice (body weight about 20 g) were procured from Model Animal Research Center of Nanjing University. Animals were housed in a climate-controlled room, 12 h light/dark photoperiod. All the mice had free access to food and water.

Mice were marked with a permanent marker on the ankles of their right hind paws to define the area of the paws to be monitored. Animals were randomly divided into 4 groups (6 mice per group): control group (treated with  $\lambda$ -carrageenan), Indo group (positive control,  $\lambda$ -carrageenan + indomethacin i.p. at a dose of 10 mg/kg, 2 h before  $\lambda$ -carrageenan challenge) and compound **3g** groups ( $\lambda$ -carrageenan + **3g** i.p. at 15 mg/kg or 30 mg/kg, 2 h before  $\lambda$ -carrageenan (1% w/v in saline, 50 µL) was injected into the plantar surface of the right hind paw to induce acute inflammation. The thickness of the paw was measured pre-injection (as normal data) and at 2, 6, and 18 h after the  $\lambda$ -carrageenan treatment, using a thickness gauge. Percentage of increase in paw thickness = 100 × (tested compound – normal)/ normal.

The present animal studies were approved by the Institutional Animal Care and Use Committee and all of the protocols complied with the Guide for the Care and Use of Laboratory Animals.

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# Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.12.051.

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