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Original article

Synthesis and biological evaluation of novel sinomenine derivatives as anti-inflammatory agents

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ABSTRACT

Sinomenine (**1**) is clinically available for the treatment of rheumatoid arthritis (RA), however, its efficacy is quite weak. In the present study, a library of novel sinomenine-based homodimers and monomers through variable-length linkers were designed and synthesized, and their bioactivities were evaluated using RAW264.7 cells and mice. Among the compounds, **2f** and **3b** possessed much more potent inhibitory effects on the production of nitric oxide (NO), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) than **1**. Preliminary mechanism investigation revealed that **3b** inhibited nuclear factor-κB (NF-κB) signaling pathway specifically, **2f** suppressed both NF-κB and mitogen-activated protein kinase (MAPK) cascades. Moreover, **3b** and **2f** significantly alleviated the lipopolysaccharide (LPS)-induced mortality. These two compounds might serve as valuable candidates for anti-inflammatory drug discovery.

1. Introduction

Inflammation is a complex process involving various mediators located in cellular or plasma. Nitric oxide (NO) is a radical with short half-life generated from L-arginine by NO synthesis isoenzymes, especially an isoform of NO synthase (iNOS) [1,2]. Overproduction of NO contributes to the pathogenesis of various human diseases, including inflammation, septic shock, pain, cardiovascular disease and cancer [3,4]. The NO inhibitors might allow us to identify potential therapeutic opportunities [5]. Besides, increasing evidences suggested that the aberrant proliferation and high expression of pro-inflammatory cytokines incubated by murine RAW264.7 macrophage plays a role of great importance in the pathogenesis and therapeutic process of a variety of pathological diseases, such as stroke, hypertension, cancer, colitis, and inflammation [6]. Specifically, overproduction of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) by macrophages and T cells predominantly have been involved in the pathogenesis of inflammatory diseases, including rheumatoid arthritis (RA) [7–9]. Suppression and blocking of excessive production of these cytokines and NO could be adopted as important criteria to evaluate anti-inflammatory effects of compounds. Although therapeutics neutralizing TNF-α, IL-6 and their receptors are already on the market or in late-phase development [10], suppressive agents of inflammatory diseases are still one of the hot spots in drug discovery.

Sinomenine (**1**), a bioactive natural alkaloid isolated from stem of a medicinal plant *Sinomenium acutum*, has been clinically available for the treatment of rheumatoid arthritis (RA) and inflammatory diseases in China for a long time [11,12]. It also possesses a variety of other bioactivities, such as inhibitory effects on mesangial proliferative nephritis, adjuvant arthritis, osteoarthritis, anti-inflammation mediated neurodegenerative disorders and immunosuppressive activity [13–15]. However, the clinical use of sinomenine has been limited because of its weak efficacy, a needed large dosage, and adverse effects [11,16,17]. To improve the efficacy, a number of structural modifications of **1** mainly focusing on rings C and D have been performed, nevertheless, only moderate

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improvements were achieved [18–21]. Therefore, there is a space to explore more active molecules with better anti-inflammatory effects.

Twin drug is defined as a compound that contains two pharmacophore components exerting pharmacological effects in a molecule. It is considered as a promising drug design strategy and has led quite interesting results [22]. For instance, "bivalent ligand" has been put forward by Portoghese and his co-workers to describe molecules containing two recognition moieties in 1982 [23,24], and has been widely subjected into the design of new lead structures in drug discovery as one of the most promising and fundamentally novel approaches [25]. Some morphine molecules targeted to binding with both μ- and δ-opioid receptors of G protein-coupled receptors (GPCRs) simultaneously were found to possess comparable analgesic benefit to morphine and have no side effects resulted from chronic exposure [26]. Homodimer derivatives also emerged as enzyme inhibitors because of the active site of the enzyme in a highly symmetrical fashion, for example, homodimers of a benzolactam showed a 200-fold high affinity for the protein kinase C-α (PKCα) enzyme than the corresponding monomer [27].

In our previous work, we have synthesized several sinomenine dimers in which sinomenine and its derivatives were directly coupled via a C–C bond at C-1 position, and found that the inhibitory activities of the (*S*)-dimers on the production of IL-6 and NO were significantly elevated compared with those of sinomenine, which however, still did not reach our expectation [28–30]. These results, together with the reports above gave us a hint that a bioactivity enhancement might be achieved via preparation of sinomenine dimers by the means of some flexible spacer. In the current study, a series of novel homodimers via sinomenine (**1**) as the pharmacophore connected through different linkers with various lengths (2–10 atoms), along with the corresponding monomeric derivatives were synthesized. The inhibitory activity on NO production and cytotoxicity of these derivatives were screened using RAW264.7 cells. The suppressive effect on IL-6 and TNF-α of the compounds with potent NO inhibitory activity and the preliminary mechanism were examined in vitro, and finally the anti-inflammatory effects of two compounds, **2f** and **3b**, were further investigated in vivo.

2. Chemistry

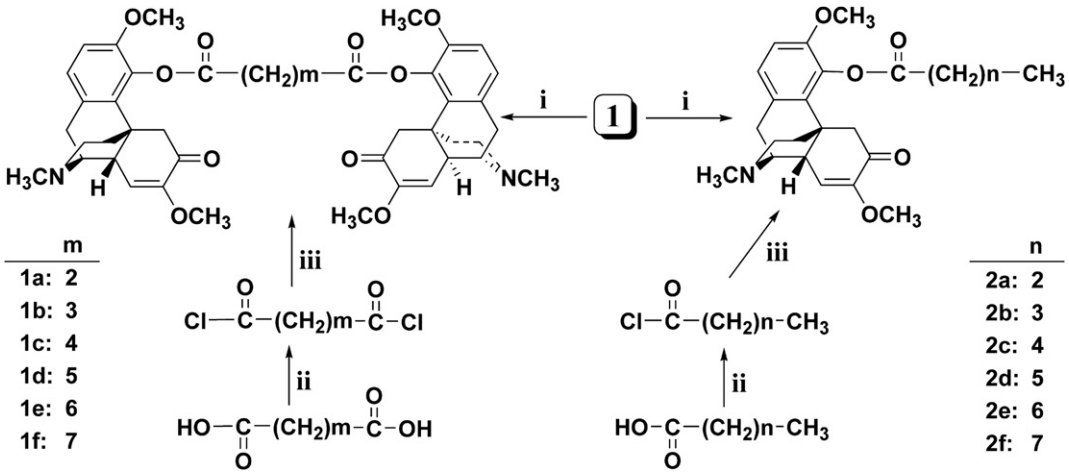
Efforts have been made to obtain sinomenine derivatives with improved bioactivity, and majority of the modifications concentrated on rings C and D due to the ease of synthesis [19,20], also several derivatives have been prepared recently in ring B [31]. For ring A, the main modifications were focused on the 1-position [32,33] and also a few derivatives were reported in 4-OH [34]. Despite the efforts, only some improvement of bioactivities was achieved [35]. In the present study, aiming at the development of sinomenine dimers, we chose the 4-OH as the connection site. In the preparations of dimers, various linkers, such as oligoethylene glycols, glycine oligomers and alkyl chains together with ester or ether bond linkages have been reported [23,36–40]. Therefore, alkyl chains, oligoethylene glycols with varying lengths and aromatic alcohols were employed via ester or ether linkages to prepare sinomenine dimers. The monomeric congeners were also synthesized to better understand the structure–activity relationship (SAR).

2.1. Synthesis of derivatives with ester linkage

Terminal dicarboxylic acids with chain length ranged from 4 to 9 were applied in the formation of sinomenine dimers via an ester linkage in our initial consideration. After converting the dicarboxylic acids to chlorides with $SOCl_2$ to improve the reactivity, the produced chlorides were reacted with sinomenine under Et_3N and then subjected to a column chromatography to afford expected dimers (Scheme 1). However, the same procedure did not give the desired compounds **1a** and **1c**, therefore, an alternative strategy was employed for the esterifications using a condensing agent *N*,*N*′-dicyclohexylcarbodiimide (DCC). Fortunately, **1a** and **1c** were successfully prepared when the dicarboxylic acids and sinomenine were treated with DCC in the presence of catalytic amount of 4-dimethylaminopyridine (DMAP). The DCC procedure was also fully applied for the synthesis of monomers. The related monocarboxylic acids were reacted with sinomenine to afford the target compounds **2b**–**f** except **2a**. Accordingly, **2a** was synthesized via *n*-butyric chloride prepared from *n*-butyric acid.

2.2. Synthesis of derivatives with ether linkage

In the synthesis of ether dimers of sinomenine, dibromoalkanes with different chain lengths (2–10 carbons) were employed. Conventionally, the formation of ether products is apt to use Williamson ether synthesis method, including the formation of aryl ether via aryl oxide ions [41,42]. Unfortunately, we found that the sinomenine was prone to decompose in the presence of sodium



Scheme 1. Synthesis of ester dimers and monomers. Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2, room temperature, for **1a**, **1c**, and **2b**–**f**. (ii) $SOCl_2$/DMF (cat.), CH_2Cl_2, reflux. (iii) Et_3N, CH_2Cl_2, room temperature, for **1b**, **1d**–**f**, and **2a**.

hydroxide under elevated temperature in *N*,*N*-dimethylformamide (DMF). Thus we turned our steps to other pathway, using potassium carbonate as a weaker base and acetone as a solvent to achieve mild reaction conditions, the target ether dimers (**3a**–**i**) were obtained in acceptable yields (Scheme 2). It should be noted that the reactions proceed quite slowly (generally about 1–3 days), however, byproducts were hardly detected except for mono-sinomenine substituted bromoalkane that was available for synthesis of dimers. Other solvents such as *N*,*N*-dimethylacetamide (DMA) with higher boiling point were also attempted to accelerate the rate of the reaction, which nevertheless, produced a mixture of unidentifiable materials.

Due to the low boiling point of bromoethane, monomeric derivative **4a** was synthesized via Mitsunobu reaction [EtOH, diisopropyl azodicarboxylate (DIAD), Ph_3P, tetrahydrofuran (THF)] in a nearly quantitative yield [43]. Other compounds, **4b**–**i** were prepared by reaction with bromoalkane directly under potassium carbonate at 60 °C. Compared with the preparation of dimers, all the reactions of monomers were finished within several hours in more than 96% yields.

Xylyl-disinomenines **3j** and **3k** were prepared through reactions of sinomenine with *p*- and *m*-xylylene glycols by Mitsunobu reaction as described before, respectively (Scheme 2). Glycol ethers of sinomenine were obtained as described in Scheme 2. In a THF–HMPA (5:1) mixed solvent, the reactions of sinomenine with glycol di-*p*-tosylates under sodium hydroxide afforded **3l**–**n**, respectively.

3. Results and discussion

3.1. Biological activities

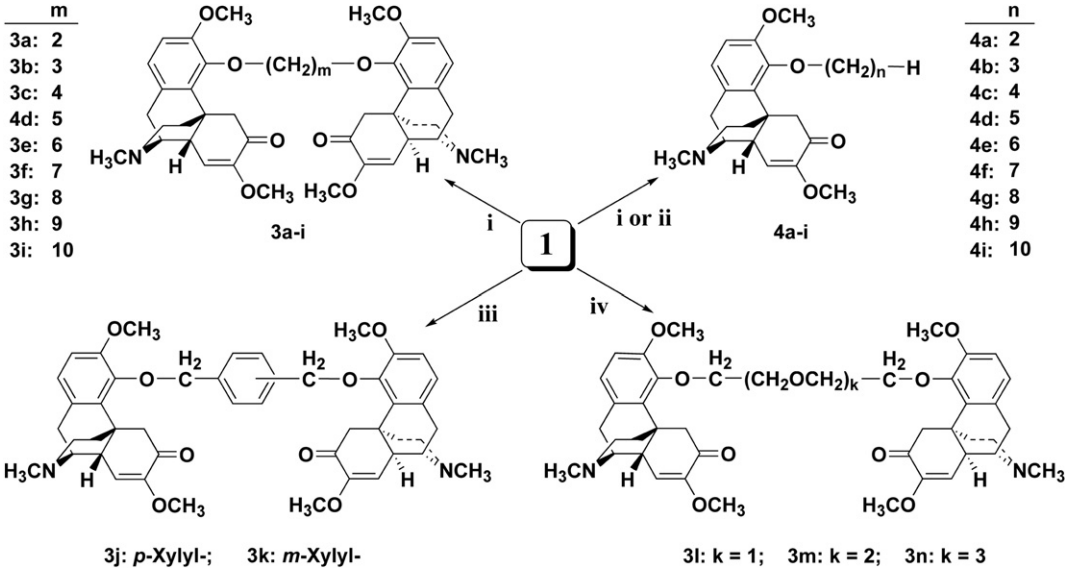
3.1.1. Inhibition of NO production and cytotoxicity in RAW264.7 cells

Initially, we evaluated the inhibitory activity on NO production of all the synthesized compounds using RAW264.7 cells in the absence or presence of 500 ng/ml LPS for 24 h to calculate their activity IC_{50}. The cytotoxicity against RAW246.7 cells was expressed as cytotoxicity IC_{50} which inhibited 50% growth of the LPS-untreated cells. As depicted in Table 1, sinomenine (**1**), together with some compounds (**1a**–**c**, **2a**, **2b**, **3a**, **3m**, **3n**, **4a**, and **4b**) displayed both weak activity and low cytotoxicity IC_{50} (more than 100 μM, for simomenine, both activity and cytotoxicity $IC_{50} > 200$ μM). Some compounds (**1d**, **1e**, **2c**, **2d**, **3e**, **3f**, **3i**–**k**, **4e**, **4h**, and **4i**) were quite cytotoxic, in which their cytotoxicity IC_{50} values were almost higher than activity IC_{50}. Among all the tested compounds, six compounds (**1f**, **2f**, **2e**, **3b**, **3c**, and **4f**) exhibited better inhibitory profiles on NO production without obvious cytotoxicity.

From the data above, a preliminary structure–activity relationship (SAR) for the compounds could be summarized. For the ester derivatives: (i) When the linker length is short (carbon number is 4–6 for dimers **1a**–**c**, 4 – 5 for monomers **2a**–**b**), almost no impacts on both activity and cytotoxicity were observed; (ii) When the linker length is middle (carbon number is 7–8 for dimers, 6 – 7 for monomers), clear negative effects were found, cytotoxicity IC_{50} was higher than activity IC_{50}; (iii) Compounds with longer linkers (**1f**, **2e**, and **2f**) showed good inhibitory profile on NO production without obvious cytotoxicity (Table 1). The results above revealed that the linker length in the ester-linked sinomenine derivatives greatly impacted the bio-profiles of the compounds, and the longer linkers could ameliorate the bioactivity on inhibition of NO production.

For the ether linked compounds, however, an opposite effect was observed compared with those of ester linked compounds. It was of great interest that the ether dimers with shorter linker, such as trimethylene and tetramethylene showed an obvious enhancement in the capacity of inhibition on NO production, especially compound **3b** and **4f** possessed IC_{50} values of 15.2 and 21.3 μM, respectively, while dimers with longer alkyl chain linkers (**4g**–**i**) induced obvious cytotoxicity (Table 1). The dimers with phenyl dimethyl spacers (**3j** and **3k**) could also lead to a visible inhibition in cell viability. Glycol scaffolds seemed to reduce the capacity of inhibition on NO production, serving little effects in the structural modification with neither visible activity nor obvious cytotoxicity.

From Table 1, both ester and ether linked dimers or monomers displayed an increased activity compared with sinomenine. It was interesting that the ether liked dimers showed more potent activity than ester ones, which gave us a useful direction for the further research.



Scheme 2. Synthesis of ether dimers and monomers. Reagents and conditions: (i) Dibromoalkane or bromoalkane, acetone, K_2CO_3, reflux. (ii) Alcohol, DIAD, Ph_3P, THF, room temperature. (iii) Xylylene glycol, DIAD, Ph_3P, THF, room temperature. (iv) Glycol di-*p*-tosylate, THF/HMPA (5:1, v/v), NaOH, reflux.

Table 1
The inhibitory effects on NO production and cytotoxicity of the synthesized compounds in RAW264.7 cells.

Compd	Cytotoxicity IC_{50} (μM)[a]	Activity IC_{50} (μM)	Compd	Cytotoxicity IC_{50} (μM)	Activity IC_{50} (μM)
1	>200	>200	**3f**	2.6	NS
1a	>100	>100	**3g**	5.4	3.8
1b	>100	>100	**3h**	3.7	2.9
1c	>100	>100	**3i**	2.8	NS
1d	52.7	NS	**3j**	12.5	NS
1e	17.3	NS	**3k**	14.2	NS
1f	50.8	29.5	**3l**	59.7	NS
2a	>100	>100	**3m**	>100	>100
2b	>100	>100	**3n**	>100	>100
2c	78.1	NS	**4a**	>100	>100
2d	38.6	NS	**4b**	>100	>100
2e	49.5	32.6	**4c**	68.1	59.6
2f	58.2	24.1	**4d**	53.4	38.8
3a	>100	>100	**4e**	29.1	NS
3b	41.1	15.2	**4f**	33.8	21.3
3c	36.7	24.6	**4g**	17.6	14.2
3d	4.2	3.6	**4h**	14.5	NS
3e	9.5	NS	**4i**	1.9	NS

NS: data not shown because activity IC_{50} is higher than cytotoxicity IC_{50}.
IC_{50} values are the mean values obtained from three separated experiments performed in triplicate.
Aminoguanidine was used as a positive control with activity IC_{50} of 22.6 μM.
[a] Cytotoxicity IC_{50}: the concentration to inhibit 50% growth of non-LPS activated cells.

3.1.2. Inhibition of TNF-α and IL-6 production in RAW264.7 cells

As TNF-α and IL-6 were two of crucial cytokines among the pro-inflammatory mediators, their blockers have been recognized as effective means for the treatment of inflammatory disease [44]. Six compounds (**1f**, **2f**, **2e**, **3b**, **3c**, and **4f**) with better inhibitory activity on NO overproduction were further exposed to RAW264.7 cells to investigate the inhibitory effects on TNF-α and IL-6 production. Time-dependent experiments were carried out at a fixed concentration of 10 μM (Table 2) in the current study.

The results revealed that only compound **3b** evidently inhibited LPS-induced TNF-α release at 6 and 12 h, while other compounds have unconspicuous effect on TNF-α. However, compounds **2e**, **2f**, and **4f**, especially **2f**, possessed remarkable inhibitory activity on LPS-induced IL-6 production, while the parent compound sinomenine (**1**) displayed no inhibitory effects on neither TNF-α nor IL-6 production at 10 μM (data not shown). These results suggested that these compounds probably worked through distinct pathways, which promoted us to investigate the possible anti-inflammatory mechanism.

Table 2
The inhibitory effects on TNF-α and IL-6 production of the selected compounds (10 μM) in RAW264.7 cells.

Compd	TNF-α (pg/ml)		
	6 h	12 h	24 h
Control	50.9 ± 0.4	52.1 ± 0.9	84.8 ± 4.1
LPS	2646.1 ± 499.8	4126.8 ± 282.0	10124.4 ± 1137.2
1f + LPS	2107.1 ± 133.2	3614.3 ± 162.2	9003.6 ± 769.8
2e + LPS	2298.9 ± 256.4	3357.8 ± 295.3[a]	7676.3 ± 1551.4
2f + LPS	2338.5 ± 108.2	4503.8 ± 138.6	9728.9 ± 524.1
3b + LPS	1817.6 ± 158.7[a]	3501.9 ± 251.3[a]	8303.2 ± 1347.6
3c + LPS	2018.0 ± 167.5	3732.8 ± 209.2	8744.4 ± 1126.2
4f + LPS	2248.7 ± 257.8	4127.2 ± 92.7	9134.7 ± 786.3
	IL-6 (pg/ml)		
Control	3.1 ± 1.8	7.2 ± 3.4	44.4 ± 7.8
LPS	1046.1 ± 111.2	2145.1 ± 368.7	13412.0 ± 1167.6
1f + LPS	825.5 ± 7.9[a]	1577.1 ± 148.7[a]	12863.3 ± 1760.7
2e + LPS	229.9 ± 39.5[a]	733.3 ± 88.4[a]	7143.8 ± 1287.7[a]
2f + LPS	148.5 ± 4.4[a]	223.7 ± 86.5[a]	486.3 ± 221.0[a]
3b + LPS	949.7 ± 114.3	1667.8 ± 231.6	13096.9 ± 1488.4
3c + LPS	853.3 ± 64.1[a]	1321.9 ± 246.2[a]	10038.7 ± 1192.6
4f + LPS	207.5 ± 23.8[a]	513.9 ± 34.3[a]	4370.2 ± 1519.0[a]

Results are shown as mean ± SD ($n = 3$).
[a] $p < 0.01$, significant difference vs control.

3.2. Possible mechanism of inhibition

The most important downstream signal transduction regulators that have been shown to mediate inflammatory responses include nuclear factor (NF-κB) and the mitogen-activated protein kinase (MAPK) family [45,46]. LPS-induced activation of NF-κB is correlated with the hyperphosphorylation and degradation of IκBα [47]. Normally in the cytoplasm, IκBα retains NF-κB through masking of the nuclear localization sequences. Upon macrophage activation, IκBα dissociates from the NF-κB/IκBα complex, leading to the translocation of NF-κB to the nucleus resulting in the transcription of specific pro- and anti-inflammatory genes. LPS also stimulates MAPK cascades, mainly including extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase (JNK), and p38 MAPK [48]. Upon activation of MAP kinases, the transcription factors located in the cytoplasm or nucleus are phosphorylated and then activated, resulting expression of target genes and corresponding biological responses. On the base of cellular viability and anti-inflammatory activity in vitro, we chose two compounds **2f** and **3b** for the further investigation of signaling pathway, Western blot analysis was applied to evaluate the effects on expression of pathway-related protein.

In the Western blot analysis, **2f** suppressed both LPS-induced activation of NF-κB and MAPK family in RAW264.7 cells. The phosphorylations of IκBα, p65 NF-κB, SAPK/JNK, ERK1/2, and p38 MAPK were obviously inhibited by 10 μM of **2f** from 4 to 6 h except for the phosphorylations of IκBα at 4 h (Fig. 1). Especially, the phosphorylations of p65 NF-κB and p38 MAPK were obviously inhibited after treatment with **2f**. Moreover, **2f** could antagonize

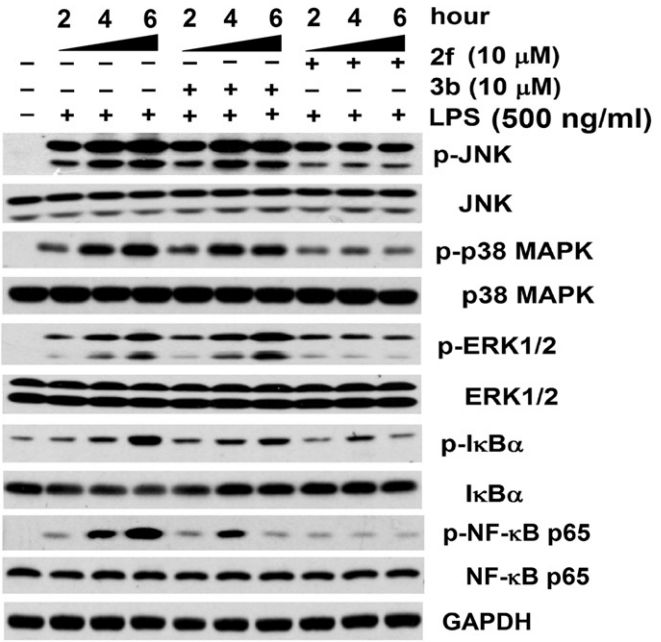


Fig. 1. The effects of compounds **2f** and **3b** on LPS-induced phosphorylations of JNK, ERK1/2, p38 MAPK, NF-κB p65, and IκBα in RAW264.7 cells. RAW264.7 cells were treated with **2f** and **3b** (10 μM) and LPS (500 ng/mL). The levels of JNK, ERK1/2, p38 MAPK, NF-κB p65, IκBα, and their phosphorylated forms (*p*-) at 2, 4 and 6 h incubations in the cellular lysates were analyzed using Western blotting.

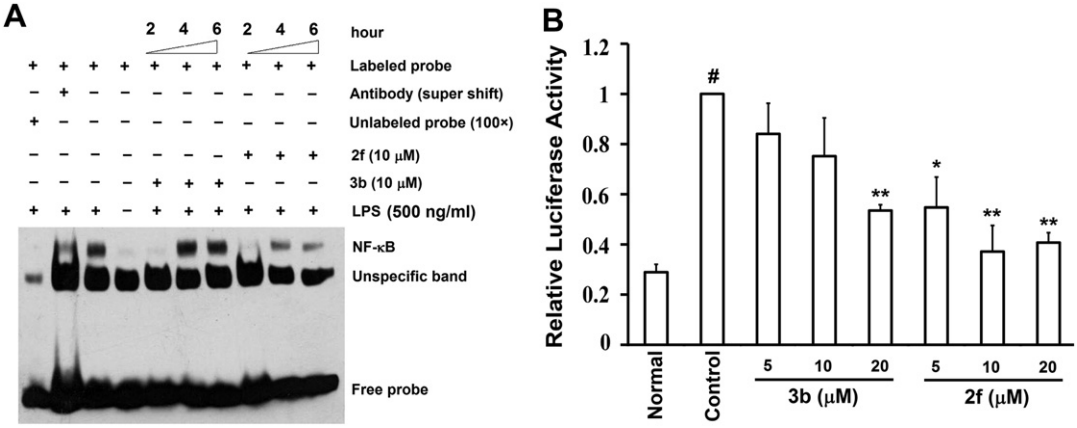


Fig. 2. Effects of **2f** and **3b** on NF-κB binding to nuclear protein–DNA. (A) Specificity of DNA–protein complex was confirmed with EMAS assay by competition with a 100× excess of unlabeled and p65 of NF-κB antibody supershift assay. (B) RAW264.7 cells transfected with the NF-κB-dependent reporter construct were incubated with LPS (500 ng/mL) in the presence of increasing concentrations of **2f** and **3b** (5, 10, or 20 μM), then the luciferase activity was measured. Values are means ± SD ($n = 3$). #$p < 0.01$ versus normal (without any treatments). *$p < 0.05$, **$p < 0.01$ versus control (treated with LPS only).

degradation of IκBα. Electrophoretic mobility shift assay (EMSA) result and luciferase activity showed that **2f** also significantly inhibited NF-κB-DNA interaction activity (Fig. 2). Interestingly, **2f** exhibited intensive effects on the phosphorylation of p65 NF-κB and the effect lasted permanently during the whole process. These data demonstrated that compound **2f** influenced multiple inflammatory signal transduction regulators.

For the compound **3b**, the results of Western blot analysis revealed that 10 μM of **3b** had no significant suppression on the LPS-induced activation of MAPK family, including the phosphorylations of SAPK/JNK and p38 during whole experiment period. Besides, **3b** could even activate the phosphorylation of ERK. It was reported that sinomenine augmented apoptosis of macrophages by activation of ERK [49], which might be associated with the current result. For the NF-κB pathway, the phosphorylation of p65 NF-κB after treatment with **3b** was decreased obviously especially at 6 h (Fig. 1), also the phosphorylation of IκBα at 6 h was suppressed moderately. Moreover, **3b** could antagonize degradation of IκBα from 4 to 6 h. EMSA result showed that 10 μM of **3b** had no obvious effect on NF-κB-DNA interaction activity (Fig. 2A), which was also confirmed by luciferase assay (Fig. 2B), however, 20 μM of **3b** could obviously suppress NF-κB-DNA interaction activity as shown in NF-κB-luc activity. These results revealed that **3b** suppressed LPS-induced inflammatory response specifically through inhibition of NF-κB and IκBα activation together with IκBα degradation, not MAPK pathway. Taken all together, these results above encouraged us to further explore the anti-inflammatory effect in vivo.

3.3. Treatment of LPS-induced septic shock

Sepsis, a leading cause of death in critically ill patients, is an infection-related syndrome with two or more features of systemic inflammation. As sepsis progresses to septic shock, the risk of death increases substantially [50]. LPS-induced septic shock model that mimics many of the initial clinical features of sepsis, including increases in pro-inflammatory cytokines such as TNF-α and IL-6, has been widely used for the evaluation of anti-inflammatory effect [51]. Thus, we studied whether compounds provide the protective effects against systemic inflammatory toxicity by LPS using septic shock mice model.

Balb/c mice were pretreated with **2f** and **3b** for 3 days (15 mg/kg/day, i.p.), and then exposed to LPS (20 mg/kg, i.p.) challenge. Pretreatment of **2f** elevated the survival rate from 30% (in LPS alone) to 80% at 19 h after LPS injection. At 24 h after LPS injection, survival rate of **2f** pretreated mice was 30% while that of untreated mice was only 10% (Fig. 3). Pretreatment of **3b** significantly alleviated the LPS-induced mortality. After LPS challenge, survival rate of **3b** pretreated mice was greatly improved to 70% compared with 10% of untreated mice, and **3b** displayed a much better effect compared with **2f**.

We further investigated whether **3b** and **2f** could inhibit the secretions of TNF-α and IL-6 in serum of LPS-induced septic shock mice. As shown in Fig. 4, after 3 h of LPS exposure, the serum TNF-α and IL-6 levels were markedly increased, pretreatments of **3b** and **2f** (15 mg/kg) significantly decreased the serum levels of both TNF-α and IL-6. It should be noted that in RAW246.7 cell assay, **3b** inhibited only TNF-α production while **2f** inhibited only IL-6 production, however, different from the in vitro data, **3b** and **2f** suppressed both of TNF-α and IL-6 in the experiment in vivo. The results might be an indirect effect of the compounds in animal body, to clarify it, further research is essential. The same as survival rate, **3b** showed a more potent inhibitory effect on TNF-

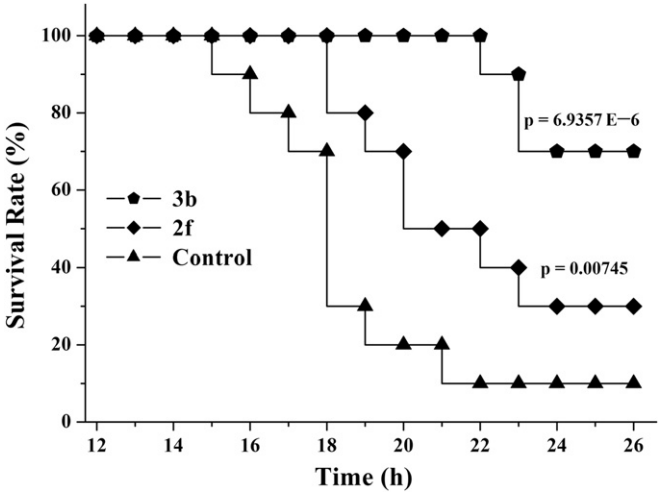


Fig. 3. Effects of **3b** and **2f** on LPS-induced lethality in mice ($n = 10$). Mice were injected (i.p.) with 20 mg/kg LPS. Compounds (**2f** or **3b**) were injected (i.p. 15 mg/kg) once per day for 3 days prior to LPS injection. Results were evaluated by the Kaplan–Meier method. The differences between the control group (LPS only) and the treatment groups are statistically significant.

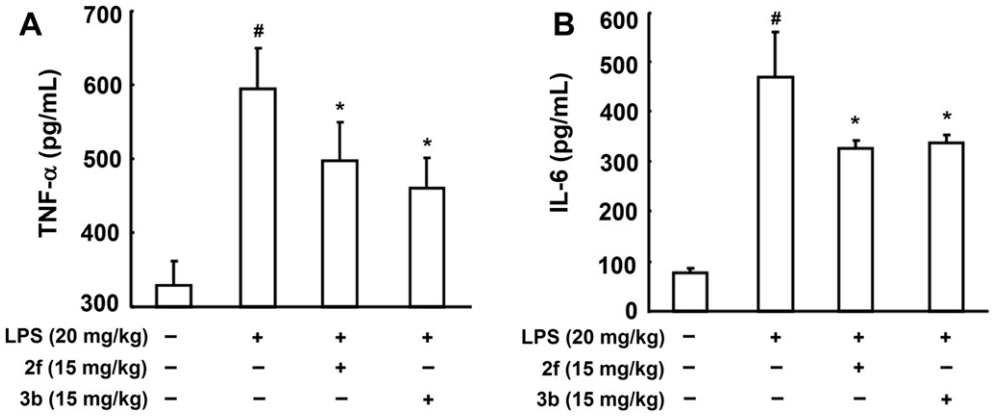


Fig. 4. Effects of **3b** and **2f** on LPS-induced TNF-α and IL-6 levels in serum ($n = 6$). Mice were injected (i.p.) with 20 mg/kg LPS. Compounds (**2f** and **3b**) were injected (i.p. 15 mg/kg) once per day for 3 days prior to LPS injection. $^{\#}p < 0.01$ versus blank (without any treatments). $^{*}p < 0.05$ versus control (treated with LPS only).

α production compared with **2f**, which might correlate with its better survival rate. As shown above, the consequences of LPS-induced septic shock model indicated that **2f** and **3b** were potential anti-inflammatory agents on antagonism of systemic inflammatory toxicity.

4. Conclusions

In this paper, sinomenine homodimers together with monomers that linked via ester and ether bonds were synthesized and their bioactivities were evaluated. The novel homodimer derivative (**3b**) and monomer compound (**2f**) effectively inhibited LPS-induced inflammatory mediators (NO, IL-6, or TNF-α) in RAW264.7 cells. Furthermore, **3b** specifically inhibited the phosphorylation of NF-κB and the degradation of IκBα in the NF-κB signaling pathway; while **2f** not only suppressed IκBα, p65 NF-κB phosphorylations in the NF-κB signaling pathway, but also ERK1/2, JNK, and p38 MAP kinase phosphorylations in the MAPK signaling pathway. Moreover, in vivo assay demonstrated that **3b** and **2f** displayed the protective effects against systemic inflammatory toxicity by LPS. These observations suggested that sinomenine homodimers with short ether linker (**3b**) and its monomers with long ester linker (**2f**) might have a great potential to be new leads for anti-inflammatory drug discovery. The current results might open new avenues of realizing mechanism for sinomenine derivatives and investigating new anti-inflammatory agents. Further synthesis of related compounds and detailed mechanism studies of **3b** and **2f** are undergoing in our laboratory.

5. Experimental protocols

5.1. Chemistry

5.1.1. General experimental information

Sinomenine was purchased from Great Dragon Natural Drug Biotechnological Ltd. and other chemicals were purchased from Alfa Aesar China (Tianjin) Co. or Acros Organics and were used without further purification. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Advance II 300 (300 MHz) spectrometer using tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) values are in hertz (Hz); ^{13}C NMR spectra were fully decoupled, and the following abbreviations are used: singlet (s), doublet (d), triplet (t), double–double (dd), double–triplet (dt), triplet–double (td), broad (br) and multiplet (m). Melting points were determined on a Taike X-4 digital micromelting point apparatus and were uncorrected. Chromatographic separations were performed on silica gel columns chromatography (Kieselgel 300–400 mesh) with CH_2Cl_2/CH_3OH (15:1–9:1, v/v) or EtOAc/CH_3OH/H_2O (37:2:1–17:2:1, v/v/v) as eluents. All reactions were monitored by TLC on GF_{254} plates that were visualized under a UV lamp (254 nm) or with I_2 detection. Evaporation of solvent was performed in vacuo with rotating evaporator. The purity of the final compounds was determined by HPLC on an Agilent 1200 system using a Agilent Eclipse XPB-C_{18} column (4.6 × 150 mm, 5 μm particle size) with a gradient mobile phase of CH_3OH/H_2O (45:55, v/v) with 0.125% of ethylenediamine (EDA) to CH_3OH/H_2O (80:20, v/v) with 0.125% of EDA to CH_3OH/H_2O (100:0, v/v) with 0.125% of EDA at a flow rate of 1 mL/min, with UV monitoring at the wavelength of 254 nm with a runtime of 35 min. The compound purity analysis data of final compounds (greater than 95%) were provided in the Supplementary data.

5.1.2. General procedure for the synthesis of ester homodimers (*1a–f*)

Method A (**1b**, **1d–f**): A mixture of dicarboxylic acid (0.6 mmol), $SOCl_2$ (10 mL) and two drops of dry DMF was refluxed for 4–6 h and then the excessive $SOCl_2$ was removed under vacuum. The afforded chloride was added dropwise to a solution of sinomenine (329 mg, 1 mmol) and Et_3N (1.5 mmol) in dry CH_2Cl_2 (10 mL) in an ice bath over 10 min. Then the ice bath was removed, the reaction solution was allowed to stir at ambient temperature overnight and then added with saturated $NaHCO_3$ (5 mL). The organic layer was separated and then washed with brine, dried over anhydrous Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography (EtOAc/MeOH/NH_4OH, 37:2:1–17:2:1, v/v/v), affording the title compounds as a white solid.

Method B (**1a**, **1c**): To a solution of sinomenine (**1**) (329 mg, 1 mmol) and dicarboxylic acid (0.6 mmol) in dichloromethane (10 mL) was added DCC (412 mg, 2 mmol) and DMAP (0.2 mmol), the mixture was stirred at ambient temperature for 2 days and then filtered and washed with CH_2Cl_2 (10 mL × 2). The filtrate was evaporated in vacuum and 10% ammonia (30 mL) was added and then extracted with CH_2Cl_2 (30 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. Purification by silica gel column chromatography (EtOAc/MeOH/NH_4OH, 37:2:1–17:2:1, v/v/v) afforded the title compounds.

*5.1.2.1. 4,4′-Succinyl-disinomenine (**1a**).* Yield 57%; white solid; mp 146–147 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.89 (d, $J = 8.4$ Hz, 2H), 6.73 (d, $J = 8.4$ Hz, 2H), 5.44 (d, $J = 1.5$ Hz, 2H), 3.89 (d, $J = 15.9$ Hz,

2H), 3.72 (s, 6H), 3.46 (s, 6H), 3.09–3.18 (m, 4H), 2.93–3.06 (m, 6H), 2.71 (dd, J = 18.3, 5.1 Hz, 2H), 2.39–2.46 (m, 4H), 2.39 (s, 6H), 1.99–2.10 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.6, 170.7, 152.3, 149.5, 139.3, 130.0, 129.6, 125.3, 114.8, 110.6, 56.2, 55.8, 54.7, 49.8, 46.5, 45.7, 42.5, 40.5, 36.6, 28.9, 24.1. Positive ESI–MS m/z: 741 $(M + H)^+$. HRMS: calcd for $C_{42}H_{49}N_2O_{10}$ (M + H), 741.3387; found, 741.3375.

*5.1.2.2. 4,4′-Glutaryl-disinomenine (**1b**).* Yield 51%; white solid; mp 138–140 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.89 (d, J = 8.4 Hz, 2H), 6.73 (d, J = 8.4 Hz, 2H), 5.45 (s, 2H), 3.71–3.84 (m, 2H), 3.74 (s, 6H), 3.46 (s, 6H), 3.16 (br, 2H), 2.97–3.06 (m, 4H), 2.81–2.91 (m, 2H), 2.74 (d, J = 5.7 Hz, 2H), 2.68 (d, J = 5.1 Hz, 2H), 2.52 (br, 2H), 2.46 (br, 2H), 2.41 (s, 6H), 2.22 (dt, J = 14.1, 6.6 Hz, 2H), 1.96–2.14 (m, 4H), 1.85 (td, J = 12.3, 4.2 Hz, 2H), 1.58 (d, J = 8.1 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.5, 170.6, 152.5, 149.8, 139.6, 130.0, 129.8, 125.5, 115.1, 110.8, 56.5, 56.0, 54.9, 50.2, 46.7, 46.0, 42.8, 40.7, 37.2, 33.4, 29.8, 24.3, 20.2. Positive ESI–MS m/z: 755 $(M + H)^+$. HRMS: calcd for $C_{43}H_{51}N_2O_{10}$ (M + H), 755.3544; found, 755.3548.

*5.1.2.3. 4,4′-Adipoyl-disinomenine (**1c**).* Yield 59%; white solid; mp 134–136 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.91 (d, J = 5.1 Hz, 2H), 6.76 (d, J = 5.1 Hz, 2H), 5.48 (d, J = 0.9 Hz, 2H), 3.87 (d, J = 9.6 Hz, 2H), 3.74 (s, 6H), 3.50 (s, 6H), 3.16–3.21 (m, 2H), 3.02–3.08 (m, 4H), 2.65–2.79 (m, 6H), 2.52–2.58 (m, 4H), 2.45 (s, 6H), 2.13 (t, J = 6.0 Hz, 2H), 1.87–2.06 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.2, 170.8, 152.3, 149.7, 139.5, 129.9, 129.6, 125.3, 114.8, 110.7, 56.3, 55.8, 54.7, 49.9, 47.0, 46.7, 46.5, 45.7, 42.5, 40.5, 36.9, 33.9, 24.1. Positive ESI–MS m/z: 769 $(M + H)^+$. HRMS: calcd for $C_{44}H_{53}N_2O_{10}$ (M + H), 769.3700; found, 769.3686.

*5.1.2.4. 4,4′-Pimeloyl-disinomenine (**1d**).* Yield 44%; white solid; mp 128–130 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.89 (d, J = 8.4 Hz, 2H), 6.63 (d, J = 8.4 Hz, 2H), 5.46 (d, J = 1.5 Hz, 2H), 3.83 (d, J = 15.3 Hz, 2H), 3.71 (s, 6H), 3.47 (s, 6H), 3.18 (t, J = 3.6 Hz, 2H), 2.99–3.06 (m, 4H), 2.47–2.77 (m, 10H), 2.41 (s, 6H), 1.98–2.13 (m, 4H), 1.78–1.91 (m, 6H), 1.54–1.62 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.5, 171.7, 152.5, 149.9, 139.6, 130.0, 129.8, 125.5, 115.0, 110.9, 56.4, 56.0, 54.9, 50.1, 46.7, 45.8, 42.7, 40.6, 37.1, 34.5, 28.6, 24.6, 24.3, 19.3. Positive ESI–MS m/z: 783 $(M + H)^+$. HRMS: calcd for $C_{45}H_{55}N_2O_{10}$ (M + H), 783.3857; found, 783.3906.

*5.1.2.5. 4,4′-Suberoyl-disinomenine (**1e**).* Yield 49%; white solid; mp 119–121 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.90 (d, J = 8.4 Hz, 2H), 6.75 (d, J = 8.4 Hz, 2H), 6.46 (d, J = 1.2 Hz, 2H), 3.81–3.86 (m, 2H), 3.72 (s, 6H), 3.48 (s, 6H), 3.20 (t, J = 3.9 Hz, 2H), 3.01–3.08 (br, 4H), 2.51–2.79 (m, 8H), 2.44 (s, 6H), 2.14 (dd, J = 12.0, 2.1 Hz, 2H), 2.01–2.07 (m, 4H), 1.90 (dd, J = 12.9, 4.5 Hz, 2H), 1.79–1.83 (m, 4H), 1.51–1.62 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.5, 171.7, 152.6, 150.0, 139.8, 130.1, 129.8, 125.4, 115.0, 111.0, 56.6, 56.1, 55.0, 50.2, 46.8, 45.9, 42.7, 40.7, 37.1, 34.5, 29.8, 28.9, 24.8, 24.4. Positive ESI–MS m/z: 797 $(M + H)^+$. HRMS: calcd for $C_{46}H_{57}N_2O_{10}$ (M + H), 797.4013; found, 797.3988.

*5.1.2.6. 4,4′-Azelaoyl-disinomenine (**1f**).* Yield 54%; white solid; mp 96–98 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.89 (d, J = 8.4 Hz, 2H), 6.73 (d, J = 8.4 Hz, 2H), 5.46 (d, J = 1.2 Hz, 2H), 3.84 (d, J = 15.9 Hz, 2H), 3.70 (s, 6H), 3.47 (s, 6H), 3.19 (t, J = 3.9 Hz, 2H), 3.00–3.06 (br, 4H), 2.74 (dd, J = 17.7, 5.4 Hz, 2H), 2.50–2.67 (m, 6H), 2.49 (d, J = 15.9 Hz, 2H), 2.42 (s, 6H), 1.99–2.14 (m, 4H), 1.86 (td, J = 12.6, 4.2 Hz, 2H), 1.75–1.80 (m, 2H), 1.43–1.60 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.5, 172.0, 152.5, 149.9, 139.7, 130.0, 129.7, 125.4, 115.0, 111.0, 56.4, 56.0, 54.9, 50.1, 46.7, 45.8, 42.7, 40.6, 37.1, 34.5, 29.1, 24.9, 24.3, 22.7. Positive ESI–MS m/z: 811 $(M + H)^+$. HRMS: calcd for $C_{47}H_{59}N_2O_{10}$ (M + H), 811.4170; found, 811.4150.

*5.1.3. General procedure for the synthesis of ester monomers (**2a–f**)*

Method A (**2b–f**): To a solution of sinomenine (**1**) (329 mg, 1 mmol) and carboxylic acid (1.2 mmol) in dioxane (10 mL) was added DCC (412 mg, 2 mmol) and DMAP (0.2 mmol), the mixture was stirred under reflux for 20–48 h and then filtered and washed with CH_2Cl_2 (10 mL × 2). The filtrate was evaporated in vacuum and 10% ammonia (30 mL) was added and then extracted with CH_2Cl_2 (30 mL × 3). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. Purification by silica gel column chromatography (CH_2Cl_2/MeOH, 15:1, v/v) afforded the title compounds as a white solid.

Method B (**2a**): A mixture of carboxylic acid (0.6 mmol) and $SOCl_2$ (10 mL) was refluxed for 3–5 h and then the excessive $SOCl_2$ was removed under vacuum. The afforded acid chloride was added dropwise into a solution of sinomenine (329 mg, 1 mmol) and Et_3N (1.5 mmol) in dry CH_2Cl_2 (10 mL) over 10 min, then the reaction solution was kept at ambient temperature overnight, quenched with saturated $NaHCO_3$ (5 mL) and diluted with dichloromethane (20 mL). The organic layer was separated and then washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated. The residual was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 15:1, v/v), affording the title compound as a white solid at nearly quantitative yield.

*5.1.3.1. 4-Butyryl-sinomenine (**2a**).* Yield 94%; white solid; mp 79–82 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.90 (d, J = 8.4 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 5.46 (d, J = 1.8 Hz, 1H), 3.86 (d, J = 16.2 Hz, 1H), 3.71 (s, 3H), 3.48 (s, 3H), 3.21 (t, J = 4.2 Hz, 1H), 3.02–3.08 (br, 2H), 2.75 (dd, J = 18.0, 5.4 Hz, 1H), 2.46–2.71 (m, 4H), 2.44 (s, 3H), 2.13 (td, J = 12.0, 2.7 Hz, 1H), 1.75–1.92 (m, 3H), 1.58–1.67 (m, 1H), 1.05 (t, J = 4.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.2, 171.3, 152.3, 149.6, 139.5, 129.8, 129.6, 125.2, 114.9, 110.7, 56.1, 55.7, 54.7, 49.9, 46.4, 45.6, 42.4, 40.4, 36.8, 36.2, 24.1, 18.2, 13.5. Positive ESI–MS m/z: 400 $(M + H)^+$, HRMS: calcd for $C_{23}H_{30}NO_5$ (M + H), 400.2124; found, 400.2093.

*5.1.3.2. 4-Valeryl-sinomenine (**2b**).* Yield 86%; white solid; mp 85–87 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.91 (d, J = 8.4 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 5.46 (d, J = 1.8 Hz, 1H), 3.86 (d, J = 15.6 Hz, 1H), 3.72 (s, 3H), 3.48 (s, 3H), 3.25 (t, J = 3.9 Hz, 1H), 3.02–3.08 (m, 2H), 2.54–2.82 (m, 4H), 2.50 (d, J = 15.6 Hz, 1H), 2.45 (s, 3H), 2.14 (td, J = 12.3, 2.7 Hz, 1H), 1.89 (td, J = 12.6, 4.5 Hz, 1H), 1.73–1.78 (m, 2H), 1.60 (d, J = 12.6 Hz, 1H), 1.45 (td, J = 15.0, 4.5 Hz, 2H), 0.97 (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.1, 171.6, 152.3, 149.7, 139.5, 129.6, 129.3, 125.2, 114.4, 110.6, 56.1, 55.7, 54.7, 49.7, 46.4, 45.3, 42.2, 40.3, 36.6, 34.0, 26.7, 24.1, 22.0, 13.6. Positive ESI–MS m/z: 414 $(M + H)^+$, HRMS: calcd for $C_{24}H_{32}NO_5$ (M + H), 414.2280; found, 414.2250.

*5.1.3.3. 4-Caproyl-sinomenine (**2c**).* Yield 84%; white solid; mp 76–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.89 (d, J = 8.4 Hz, 1H), 6.73 (d, J = 8.4 Hz, 1H), 5.46 (d, J = 2.1 Hz, 1H), 3.85 (d, J = 16.2 Hz, 1H), 3.70 (d, 3H), 3.47 (s, 3H), 3.19 (t, J = 4.2 Hz, 1H), 2.98–3.07 (m, 2H), 3.49–2.78 (m, 4H), 2.47 (d, J = 16.2 Hz, 1H), 2.45 (s, 3H), 2.11 (td, J = 12.0, 2.7 Hz, 1H), 1.85 (td, J = 12.6, 4.5 Hz, 1H), 1.72–1.79 (m, 2H), 1.58 (d, J = 12.6 Hz, 1H), 1.34–1.43 (m, 4H), 0.92 (t, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.2, 171.6, 152.3, 149.6, 139.5, 129.8, 129.6, 125.1, 114.9, 110.7, 56.2, 55.7, 54.7, 49.9, 46.5, 45.6, 42.5, 40.4, 36.9, 34.2, 31.1, 24.3, 24.1, 22.2, 13.8. Positive ESI–MS m/z: 428 $(M + H)^+$, HRMS: calcd for $C_{25}H_{34}NO_5$ (M + H), 428.2437; found, 428.2406.

*5.1.3.4. 4-Heptanoyl-sinomenine (**2d**).* Yield 89%; white solid; mp 73–75 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.90 (d, J = 8.4 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 5.45 (d, J = 1.8 Hz, 1H), 3.85 (d, J = 15.9 Hz, 1H), 3.71 (s, 3H), 3.48 (s, 3H), 3.34 (t, J = 3.9 Hz, 1H), 3.04 (br, 2H),

2.51–2.87 (m, 4H), 2.50 (d, J = 15.9 Hz, 1H), 2.46 (s, 3H), 2.78 (t, J = 7.5 Hz, 1H), 2.17 (td, J = 12.0, 2.4 Hz, 1H), 1.92 (td, J = 12.6, 4.5 Hz, 1H), 1.71–1.81 (m 2H), 1.57–1.62 (m, 2H), 1.25–1.44 (m, 6H), 0.90 (t, J = 7.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.0, 171.6, 152.3, 149.9, 139.5, 129.4, 128.6, 125.3, 114.2, 110.9, 55.9, 55.7, 54.7, 49.5, 46.3, 44.7, 41.7, 40.0, 36.2, 34.3, 31.3, 28.6, 24.6, 24.2, 22.4, 13.9. Positive ESI–MS m/z: 442 $(M + H)^+$, HRMS: calcd for $C_{26}H_{36}NO_5$ (M + H), 442.2593; found, 442.2563.

*5.1.3.5. 4-Capryloyl-sinomenine (**2e**).* Yield 91%; white solid; mp 70–72 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88 (d, J = 8.4 Hz, 1H), 6.72 (d, J = 8.4 Hz, 1H), 5.46 (d, J = 1.8 Hz, 1H), 3.85 (d, J = 15.9 Hz, 1H), 3.70 (s, 3H), 3.47 (s, 3H), 3.17 (t, J = 4.2 Hz, 1H), 2.96–3.06 (br, 2H), 2.47–2.76 (m, 4H), 2.46 (d, J = 15.9 Hz, 1H), 2.41 (s, 3H), 2.09 (td, J = 12.0, 2.4 Hz, 1H), 1.71–1.89 (m, 3H), 1.58 (d, J = 12.0 Hz, 1H), 1.29–1.41 (m, 8H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.2, 171.5, 152.2, 149.5, 139.4, 129.8, 129.7, 125.1, 115.0, 110.6, 56.1, 55.6, 54.6, 49.9, 46.4, 45.7, 42.5, 40.4, 36.9, 34.2, 31.5, 28.8, 28.7, 24.6, 24.0, 22.4, 13.9. Positive ESI–MS m/z: 456 $(M + H)^+$, HRMS: calcd for $C_{27}H_{38}NO_5$ (M + H), 456.2750; found, 456.2714.

*5.1.3.6. 4-Nonanoyl-sinomenine (**2f**).* Yield 82%; white solid; mp 66–68 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.90 (d, J = 8.4 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 5.45 (d, J = 1.5 Hz, 1H), 3.85 (d, J = 15.9 Hz, 1H), 3.71 (s, 3H), 3.47 (s, 3H), 3.28 (t, J = 3.6 Hz, 1H), 3.03 (br, 2H), 2.79 (dd, J = 18.6, 5.4 Hz, 1H), 2.50–2.71 (m, 3H), 2.49 (d, J = 15.9 Hz, 1H), 2.45 (s, 3H), 2.15 (td, J = 12.3, 2.4 Hz, 1H), 2.02 (d, J = 4.8 Hz, 1H), 1.90 (td, J = 12.6, 4.5 Hz, 1H), 1.73–1.76 (m, 2H), 1.59 (d, J = 12.6 Hz, 1H), 1.23–1.40 (m, 9H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.0, 171.7, 152.3, 150.0, 139.5, 129.6, 129.1, 125.2, 114.4, 110.8, 56.1, 55.8, 54.7, 49.7, 46.4, 45.0, 42.0, 40.2, 36.5, 34.3, 31.7, 29.1, 29.0, 28.9, 24.7, 24.1, 22.5, 14.0. Positive ESI–MS m/z: 470 $(M + H)^+$, HRMS: calcd for $C_{28}H_{40}NO_5$ (M + H), 470.2906; found, 470.2872.

*5.1.4. General procedure for synthesis of ether homodimers (**3a–i**)*

To a solution of sinomenine (**1**) (329 mg, 1 mmol) and dibromoalkane (0.5 mmol) in dry acetone (2 mL) was added potassium carbonate (690 mg, 5 mmol). The mixture was stirred at 60 °C until the starting material was no longer detected by TLC. The reaction mixture was then filtered and solvent was removed under vacuum to afford the crude product, which was purified by gel column chromatography to give title compounds as a foamy pale-white solid.

*5.1.4.1. 4,4′-Dimethylene-disinomenine (**3a**).* Yield 36%; white solid; mp 210–212 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.19–6.25 (m, 4H), 5.41 (d, J = 1.5 Hz, 2H), 4.37 (d, J = 15.6 Hz, 2H), 3.64 (s, 6H), 3.62 (d, J = 1.5 Hz, 2H), 3.46 (s, 6H), 3.18 (t, J = 4.2 Hz, 2H), 3.00 (br, 2H), 2.75–2.81 (m, 2H), 2.54 (dd, J = 6.0, 2.7 Hz, 2H), 2.31–2.46 (m, 4H), 2.28 (s, 6H), 1.86–2.03 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.8, 152.3, 144.9, 143.0, 128.1, 127.7, 122.8, 114.9, 110.1, 56.4, 56.0, 54.6, 49.0, 47.1, 45.4, 42.5, 40.5, 35.7, 35.6, 21.5. Positive ESI–MS m/z: 685 $(M + H)^+$, HRMS: calcd for $C_{40}H_{49}N_2O_8$ (M + H), 685.3489; found, 685.3472.

*5.1.4.2. 4,4′-Trimethylene-disinomenine (**3b**).* Yield 53%; white solid; mp 101–103 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.99–6.66 (m, 4H), 5.46 (s, 2H), 4.35–4.14 (m, 6H), 3.78 (s, 6H), 3.47 (s, 6H), 3.16–3.14 (m, 2H), 2.95–2.76 (m, 4H), 2.72 (dd, J = 12.0, 6.0 Hz, 2H), 2.53–2.45 (m, 6H), 2.41 (s, 6H), 2.07–1.87 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.6, 152.3, 151.3, 148.0, 129.8, 122.7, 122.4, 115.1, 111.4, 69.5, 56.4, 55.7, 54.6, 49.8, 47.0, 45.9, 42.5, 40.7, 37.2, 31.3, 24.4. Positive ESI–MS m/z: 699 $(M + H)^+$, HRMS: calcd for $C_{41}H_{51}N_2O_8$ (M + H), 699.3645; found, 699.3625.

*5.1.4.3. 4,4′-Tetramethylene-disinomenine (**3c**).* Yield 61%; white solid; mp 124–125 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (s, 4H), 5.47 (s, 2H), 4.11–4.23 (m, 6H), 3.77 (s, 6H), 3.48 (s, 6H), 3.15 (s, 2H), 2.98 (d, J = 16.8 Hz, 4H), 2.76 (td, J = 17.7, 4.8 Hz, 2H), 2.46–2.52 (m, 4H), 2.41 (s, 6H), 1.91–2.04 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.9, 152.6, 151.6, 148.3, 130.2, 130.1, 122.6, 115.6, 111.6, 72.1, 56.7, 55.9, 54.9, 50.0, 47.2, 46.1, 42.8, 41.0, 37.4, 27.0, 24.8. Positive ESI–MS m/z: 713 $(M + H)^+$. HRMS: calcd for $C_{42}H_{53}N_2O_8$ (M + H), 713.3802; found, 713.3782.

*5.1.4.4. 4,4′-Pentamethylene-disinomenine (**3d**).* Yield 65%; white solid; mp 100–102 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 5.47 (s, 2H), 4.18 (d, J = 15.9 Hz, 2H), 4.05 (dt, J = 19.8, 6.9 Hz, 4H), 3.76 (s, 6H), 3.47 (s, 6H), 3.13 (t, J = 3.9 Hz, 2H), 2.97 (d, J = 17.1 Hz, 4H), 2.71 (dd, J = 18.0, 4.8 Hz, 2H), 2.44–2.51 (m, 4H), 2.39 (s, 6H), 1.86–2.03 (m, 10H), 1.66 (d, J = 15.0, 7.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.0, 152.6, 151.6, 148.2, 130.1, 130.0, 122.6, 115.5, 111.4, 72.3, 56.7, 55.9, 54.9, 49.9, 47.1, 46.1, 42.8, 40.8, 37.4, 30.4, 24.7, 22.4. Positive ESI–MS m/z: 727 $(M + H)^+$. HRMS: calcd for $C_{43}H_{55}N_2O_8$ (M + H), 727.3958; found, 727.3941.

*5.1.4.5. 4,4′-Hexamethylene-disinomenine (**3e**).* Yield 63%; white solid; mp 68–69 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (d, J = 8.4 Hz, 2H), 6.68 (d, J = 8.4 Hz, 2H), 5.49 (s, 2H), 4.25 (d, J = 16.5 Hz, 2H), 4.03–4.11 (m, 4H), 3.77 (s, 6H), 3.48 (s, 6H), 3.16 (s, 2H), 2.99 (d, J = 18.0 Hz, 4H), 2.73 (dd, J = 18.0, 4.8 Hz, 2H), 2.50 (t, J = 4.8 Hz, 2H), 2.41 (s, 6H), 2.35 (d, J = 6.3 Hz, 2H), 1.90–1.99 (m, 8H), 1.57–1.70 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.9, 152.5, 151.5, 148.1, 130.1, 130.0, 122.5, 115.5, 111.4, 72.2, 56.5, 55.8, 54.8, 49.9, 47.1, 46.1, 42.7, 40.8, 37.3, 30.3, 25.9, 24.6. Positive ESI–MS m/z: 741 $(M + H)^+$. HRMS: calcd for $C_{44}H_{57}N_2O_8$ (M + H), 741.4115; found, 741.4106.

*5.1.4.6. 4,4′-Heptamethylene-disinomenine (**3f**).* Yield 66%; white solid; mp 57–59 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (t, J = 9.0 Hz, 4H), 5.48 (d, J = 1.5 Hz, 2H), 4.18 (d, J = 15.9 Hz, 2H), 4.00–4.07 (m, 4H), 3.77 (s, 6H), 3.49 (s, 6H), 3.20 (br t, 2H), 2.96–3.02 (br, 4H), 2.77 (dd, J = 18.0, 5.4 Hz, 2H), 2.56 (d, J = 11.1 Hz, 2H), 2.48 (d, J = 15.9 Hz, 2H), 2.45 (s, 6H), 1.86–2.07 (m, 10H), 1.50 (br, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.6, 152.4, 151.5, 148.1, 130.0, 122.3, 115.0, 111.3, 72.2, 56.6, 55.6, 54.7, 49.6, 47.1, 45.7, 42.5, 40.6, 37.0, 30.2, 29.4, 25.8, 24.5. Positive ESI–MS m/z: 755 $(M + H)^+$, HRMS: calcd for $C_{45}H_{59}N_2O_8$ (M + H), 755.4271; found, 755.4262.

*5.1.4.7. 4,4′-Octamethylene-disinomenine (**3g**).* Yield 70%; white solid; mp 58–60 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.68 (d, J = 8.1 Hz, 2H), 6.65 (d, J = 8.1 Hz, 2H), 5.46 (d, J = 1.5 Hz, 2H), 4.16 (d, J = 16.2 Hz, 2H), 3.98–4.07 (m, 4H), 3.74 (s, 6H), 3.46 (s, 6H), 3.12 (t, J = 4.2 Hz, 2H), 2.96 (d, J = 17.1 Hz, 4H), 2.70 (dd, J = 18.0, 5.4 Hz, 2H), 2.46–2.50 (m, 4H), 2.39 (s, 6H), 1.97 (t, J = 4.5 Hz, 2H), 1.84–1.87 (m, 8H), 1.42–1.48 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.0, 152.6, 151.6, 148.3, 130.2, 130.1, 122.5, 115.5, 111.5, 72.4, 56.6, 55.9, 54.9, 50.0, 47.2, 46.2, 42.8, 40.9, 37.4, 30.5, 29.7, 26.0, 24.7. Positive ESI–MS m/z: 769 $(M + H)^+$. HRMS: calcd for $C_{46}H_{61}N_2O_8$ (M + H), 769.4428; found, 769.4419.

*5.1.4.8. 4,4′-Nonamethylene-disinomenine (**3h**).* Yield 68%; white solid; mp 75–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (t, J = 8.7 Hz, 4H), 5.47 (d, J = 1.8 Hz, 2H), 4.18 (d, J = 15.9 Hz, 2H), 4.03–4.09 (m, 4H), 3.77 (s, 6H), 3.50 (s, 6H), 3.18 (t, J = 4.5 Hz, 2H), 2.93–3.02 (m, 4H), 2.75 (dd, J = 18.3, 4.8 Hz, 2H), 2.46–2.56 (m, 4H), 2.44 (s, 6H), 1.86–2.05 (m, 12H), 1.39–1.46 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.7, 152.4, 151.4, 148.0, 130.0, 129.9, 122.3, 115.3, 111.2, 72.2, 56.4, 55.6, 54.6, 49.7, 47.0, 46.0, 42.6, 40.7, 37.2, 30.2, 29.5, 29.4, 25.8, 24.5.

Positive ESI–MS *m/z*: 783 $(M + H)^+$, HRMS: calcd for $C_{47}H_{63}N_2O_8$ (M + H), 783.4584; found, 783.4583.

*5.1.4.9. 4,4′-Decamethylene-disinomenine (**3i**).* Yield 67%; white solid; mp 70–72 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 5.47 (d, J = 1.8 Hz, 2H), 4.18 (d, J = 16.2 Hz, 2H), 3.99–4.11 (m, 4H), 3.76 (s, 6H), 3.49 (s, 6H), 3.16 (t, J = 3.6 Hz, 2H), 2.96–3.02 (br, 4H), 2.73 (dd, J = 18.3, 5.4 Hz, 2H), 2.51 (br, 2H), 2.46 (d, J = 16.2 Hz, 2H), 2.42 (s, 6H), 1.83–2.04 (m, 12H), 1.35–1.44 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.6, 152.4, 151.4, 148.0, 129.9, 129.8, 122.3, 115.2, 111.3, 72.2, 56.4, 55.6, 54.7, 49.7, 47.0, 45.9, 42.6, 40.7, 37.1, 30.2, 29.5, 29.4, 25.8, 24.5. Positive ESI–MS *m/z*: 797 $(M + H)^+$, HRMS: calcd for $C_{48}H_{65}N_2O_8$ (M + H), 797.4741; found, 797.4787.

*5.1.5. 4,4′-p-Xylyl-disinomenine (**3j**)*

To a solution of sinomenine (658 mg, 2 mmol), *p*-xylylene glycol (138 mg, 1 mmol) and triphenylphosphine (768 mg, 3 mmol) in dried THF (10 mL) was added a solution of diisopropyl azodicarboxylate (0.59 mL, 3 mmol) in dry THF (2 mL) over a period of 30 min. The reaction mixture was stirred at room temperature for 12 h and then the solvent THF was removed under vacuum. Purification by silica gel column chromatography (EtOAc/CH_2Cl_2/MeOH, 100:0:0–0:6:1, v/v/v) afforded the title compounds (403 mg, 53%); white solid; mp 140–142 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62 (s, 4H), 6.77 (d, J = 8.4 Hz, 2H), 6.74 (d, J = 8.4 Hz, 2H), 5.50 (d, J = 1.5 Hz, 2H), 5.30 (d, J = 11.4 Hz, 2H), 5.10 (d, J = 11.4 Hz, 2H), 4.18 (d, J = 15.9 Hz, 2H), 3.81 (s, 6H), 3.51 (s, 6H), 3.17 (t, J = 3.6 Hz, 2H), 2.98–3.04 (br, 4H), 2.75 (d, J = 12.6, 5.4 Hz, 2H), 2.44–2.49 (m, 4H), 2.41 (s, 6H), 1.97 (td, J = 11.7, 3.9 Hz, 2H), 1.77–1.90 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.2, 152.6, 151.7, 147.6, 137.8, 130.3, 130.2, 128.2, 127.1, 123.0, 115.5, 111.3, 73.1, 56.6, 55.8, 54.9, 49.9, 47.1, 46.1, 42.8, 41.0, 37.2, 24.7. Positive ESI–MS *m/z*: 761 $(M + H)^+$, HRMS: calcd for $C_{46}H_{53}N_2O_8$ (M + H), 761.3802; found, 761.3790.

*5.1.6. 4,4′-m-Xylyl-disinomenine (**3k**)*

Compound **3k** was obtained following the same procedure described for compound **3j**. Purification was performed by means of silica gel column chromatography (EtOAc/CH_2Cl_2/MeOH, 100:0:0–0:9:1, v/v/v). The title compound was obtained as a pale-yellow solid (372 mg, 49% yield); mp 133–134 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.71 (s, 1H), 7.56 (dd, J = 7.2, 1.2 Hz, 2H), 7.42 (t, J = 7.8 Hz, 1H), 6.75 (d, J = 8.4 Hz, 2H), 6.72 (d, J = 8.4 Hz, 2H), 5.49 (d, J = 1.8 Hz, 2H), 5.26 (d, J = 11.4 Hz, 2H), 5.14 (d, J = 11.4 Hz, 2H), 4.19 (d, J = 15.9 Hz, 2H), 3.80 (s, 6H), 3.49 (s, 6H), 3.15 (t, J = 4.2 Hz, 2H), 2.96–3.03 (br, 4H), 2.73 (dd, J = 18.0, 5.1 Hz, 1H), 2.43–2.49 (m, 4H), 2.40 (s, 6H), 1.77–2.00 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.9, 152.4, 151.5, 147.5, 138.3, 130.2, 130.0, 128.3, 127.5, 127.2, 122.7, 115.2, 111.2, 73.1, 56.4, 55.6, 54.7, 49.7, 47.0, 46.0, 42.6, 40.8, 37.0, 24.5. Positive ESI–MS *m/z*: 761 $(M + H)^+$, HRMS: calcd for $C_{46}H_{53}N_2O_8$ (M + H), 761.3802; found, 761.3782.

*5.1.7. 4,4′-Diethylene glycol-disinomenine (**3l**)*

To a solution of sinomenine (329 mg, 1 mmol) and diethylene glycol di-*p*-tosylate (207 mg, 0.5 mmol) in dry THF (15 mL) and HMPA (3 mL) was added sodium hydroxide (60 mg, 1.5 mmol), then the reaction mixture was refluxed for 16 h and solvent was removed under vaccum, the residue was added with chloroform (50 mL) and washed with water (20 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and then concentrated. Purification by silica gel column chromatography (EtOAc/MeOH/NH_4OH, 460:25:15, v/v/v) afforded the title compound (229 mg, 63%); white solid; mp 96–98 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 5.44 (d, J = 1.5 Hz, 2H), 4.15–4.38 (m, 6H), 3.97 (t, J = 4.8 Hz, 4H), 3.75 (s, 6H), 3.46 (s, 6H), 3.16 (t, J = 4.2 Hz, 2H), 2.94–2.99 (m, 4H), 2.72 (dd, J = 18.3, 5.1 Hz, 2H), 2.45–2.50 (m, 4H), 2.40 (s, 6H), 1.98–2.00 (m, 6H), 1.87 (td, J = 12.9, 3.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.8, 152.4, 151.3, 147.6, 129.9, 129.8, 122.5, 115.0, 111.3, 71.0, 70.6, 56.3, 55.6, 54.6, 49.6, 46.9, 45.8, 42.4, 40.8, 36.7, 24.5. Positive ESI–MS *m/z*: 729 $(M + H)^+$, HRMS: calcd for $C_{42}H_{54}N_2O_9$ (M + H), 729.3751; found, 729.3774.

*5.1.8. 4,4′-Triethylene glycol-disinomenine (**3m**)*

Compound **3m** was obtained following the same procedure described for compound **3l**. Purification was performed by means of silica gel column chromatography (EtOAc/MeOH/NH_4OH, 460:25:15, v/v/v). The title compound was obtained as a white solid (278 mg, 72% yield); mp 90–92 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 5.44 (d, J = 1.2 Hz, 2H), 4.17–4.32 (m, 6H), 3.77–3.93 (m, 8H), 3.74 (s, 6H), 3.36 (s, 6H), 3.17 (br t, J = 4.2 Hz, 2H), 2.94–2.98 (br, 2H), 2.73 (dd, J = 18.6, 5.1 Hz, 2H), 2.44–2.53 (m, 4H), 2.40 (s, 3H), 1.96–2.03 (m, 4H), 1.86 (td, J = 12.9, 3.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.8, 152.4, 151.3, 147.6, 129.8, 129.7, 122.6, 115.0, 111.3, 70.9, 70.7, 70.5, 56.4, 55.6, 54.7, 49.6, 47.0, 45.7, 42.4, 40.7, 36.6, 24.5. Positive ESI–MS *m/z*: 773 $(M + H)^+$, HRMS: calcd for $C_{44}H_{58}N_2O_{10}$ (M + H), 773.4013; found, 773.3984.

*5.1.9. 4,4′-Tetraethylene glycol-disinomenine (**3n**)*

Compound **3n** was obtained following the same procedure described for compound **3l**. Purification was performed by means of silica gel column chromatography (EtOAc/MeOH/NH_4OH, 460:25:15, v/v/v). The title compound was obtained as a white solid (310 mg, 76% yield); mp 70–72 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 5.46 (d, J = 1.8 Hz, 2H), 4.16–4.33 (m, 6H), 3.67–3.80 (m, 12H), 3.75 (s, overlapped, 6H), 3.47 (s, 6H), 3.15 (t, J = 3.9 Hz, 2H), 2.95–3.00 (m, 4H), 2.72 (dd, J = 18.3, 5.4 Hz, 2H), 2.43–2.52 (m, 4H), 2.41 (s, 6H), 1.94–2.03 (m, 6H), 1.85 (td, J = 13.2, 4.2 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.9, 152.3, 151.2, 147.4, 130.0, 129.9, 122.6, 115.3, 111.2, 70.8, 70.6, 70.5, 70.4, 56.3, 55.6, 54.6, 49.6, 46.9, 45.9, 42.5, 40.8, 36.8, 24.4. Positive ESI–MS *m/z*: 817 $(M + H)^+$, HRMS: calcd for $C_{46}H_{62}N_2O_{11}$ (M + H), 817.4275; found, 817.4315.

*5.1.10. Procedure for synthesis of ether monomer (**4a**)*

To a solution of sinomenine (329 mg, 1 mmol) and triphenylphosphine (768 mg, 3 mmol) in dry THF (10 mL) was added a solution of alcohol (3 mmol) and diisopropyl azodicarboxylate (0.59 mL, 3 mmol) in dry THF (2 mL) over a period of 30 min. The reaction mixture was stirred at room temperature for 12 h and then the solvent THF was removed under vacuum. Purification by silica gel column chromatography (EtOAc/CH_2Cl_2/MeOH, 100:0:0–0:15:1, v/v/v) furnished the target compounds.

*5.1.10.1. 4-Ethyl-sinomenine (**4a**).* Yield 99%; white solid; mp 77–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 5.47 (d, J = 1.5 Hz, 1H), 4.09–4.20 (m, 3H), 3.77 (s, 3H), 3.49 (s, 3H), 3.22 (t, J = 4.5 Hz, 1H), 2.99 (br d, J = 18.0 Hz, 2H), 2.77 (dd, J = 18.4, 4.6 Hz, 1H), 2.56 (dt, J = 11.7, 2.4 Hz, 1H), 2.49 (d, J = 16.2 Hz, 1H), 2.43 (s, 3H), 1.89–2.06 (m, 3H), 1.43 (t, J = 6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 193.6, 152.4, 151.5, 147.9, 129.6, 129.5, 122.4, 115.0, 111.3, 67.8, 56.3, 55.6, 54.7, 49.6, 46.9, 45.5, 42.3, 40.5, 36.8, 24.6, 15.7. Positive ESI–MS *m/z*: 358 $(M + H)^+$, HRMS: calcd for $C_{21}H_{28}NO_4$ (M + H), 358.2018; found, 358.1986.

*5.1.11. General procedure for synthesis of ether monomers (**4b–i**)*

To a solution of sinomenine (**1**) (329 mg, 1 mmol) and bromoalkane (2 mmol) in dry acetone (2 mL) was added potassium carbonate (690 mg, 5 mmol). The mixture was stirred at 60 °C until the starting material was no longer detected by TLC. The reaction

mixture was then filtered and solvent was removed under vacuum to afford the crude product, which was purified by gel column chromatography to give title compounds.

*5.1.11.1. 4-Propyl-sinomenine (**4b**).* Yield 98%; white solid; mp 126–128 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (t, J = 8.4 Hz, 2H), 5.47 (d, J = 1.8 Hz, 1H), 4.18 (d, J = 15.9 Hz, 1H), 3.96–4.08 (m, 2H), 3.76 (s, 3H), 3.49 (s, 3H), 3.25 (t, J = 4.2 Hz, 1H), 2.96–3.04 (m, 2H), 2.79 (d, J = 18.0, 5.4 Hz, 1H), 2.59 (dt, J = 11.7, 2.7 Hz, 1H), 2.48 (d, J = 15.9 Hz, 1H), 2.44 (s, 3H), 1.95–2.08 (m, 2H), 1.78–1.94 (m, 4H), 1.04 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ: 193.5, 152.5, 151.5, 148.0, 129.6, 129.4, 122.4, 114.6, 111.4, 73.7, 56.3, 55.6, 54.7, 49.5, 46.9, 45.3, 42.1, 40.4, 36.7, 24.5, 23.4, 10.3. Positive ESI–MS m/z: 372 $(M + H)^+$, HRMS: calcd for $C_{22}H_{30}NO_4$ (M + H), 372.2175; found, 372.2148.

*5.1.11.2. 4-Butyl-sinomenine (**4c**).* Yield 96%; white solid; mp 143–145 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 5.48 (d, J = 2.1 Hz, 1H), 4.18 (d, J = 16.2 Hz, 1H), 4.07 (dt, J = 6.9, 3.0 Hz, 2H), 3.77 (s, 3H), 3.49 (s, 3H), 3.17 (t, J = 4.2 Hz, 1H), 2.96–3.02 (m, 2H), 2.74 (dd, J = 18.3, 5.1 Hz, 1H), 2.52 (dt, J = 9.0, 2.7 Hz, 1H), 2.47 (d, J = 16.2 Hz, 1H), 2.43 (s, 3H), 1.78–2.05 (m, 5H), 1.45–1.57 (m, 2H), 1.00 (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ: 193.6, 152.4, 151.4, 148.0, 129.9, 129.8, 122.3, 115.2, 111.3, 56.4, 55.6, 54.7, 49.7, 47.0, 45.9, 42.6, 40.6, 37.1, 32.3, 24.5, 19.0, 13.9. Positive ESI–MS m/z: 386 $(M + H)^+$, HRMS: calcd for $C_{23}H_{32}NO_4$ (M + H), 386.2331; found, 386.2305.

*5.1.11.3. 4-Amyl-sinomenine (**4d**).* Yield 96%; white solid; mp 50–52 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (d, J = 8.4 Hz, 1H), 6.69 (d, J = 8.4 Hz, 1H), 5.47 (d, J = 2.1 Hz, 1H), 4.17 (d, J = 15.9 Hz, 1H), 4.05 (td, J = 7.2, 2.1 Hz, 2H), 3.76 (s, 3H), 3.49 (s, 3H), 3.17 (t, J = 3.9 Hz, 1H), 2.96–3.02 (br, 2H), 2.74 (dd, J = 18.3, 5.4 Hz, 1H), 2.53 (dt, J = 11.4, 2.7 Hz, 1H), 2.47 (d, J = 15.9 Hz, 1H), 2.43 (s, 3H), 1.81–2.05 (m, 5H), 1.36–1.51 (m, 4H), 0.94 (t, J = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ: 193.6, 152.4, 151.4, 148.0, 129.9, 129.8, 122.3, 115.2, 111.3, 72.1, 56.4, 55.6, 54.6, 49.7, 46.9, 45.8, 42.5, 40.6, 37.1, 29.9, 27.9, 24.5, 22.5, 13.9. Positive ESI–MS m/z: 400 $(M + H)^+$, HRMS: calcd for $C_{24}H_{34}NO_4$ (M + H), 400.2488; found, 400.2457.

*5.1.11.4. 4-Hexyl-sinomenine (**4e**).* Yield 97%; white solid; mp 42–44 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (d, J = 8.4 Hz, 1H), 6.68 (d, J = 8.4 Hz, 1H), 5.47 (d, J = 1.8 Hz, 1H), 4.18 (d, J = 15.9 Hz, 1H), 4.05 (td, J = 6.9, 2.4 Hz, 1H), 3.76 (s, 3H), 3.49 (s, 3H), 3.16 (t, J = 3.9 Hz, 1H), 2.96–3.02 (m, 2H), 2.74 (d, J = 18.3, 5.1 Hz, 1H), 2.44–2.55 (m, 2H), 2.42 (s, 3H), 1.79–2.04 (m, 5H), 1.33–1.49 (m, 6H), 0.89–0.93 (m, 3H). ^{13}C NMR ($CDCl_3$) δ: 193.6, 152.4, 151.4, 148.0, 129.9, 129.8, 122.3, 115.2, 111.3, 72.2, 56.4, 55.6, 54.6, 49.7, 47.0, 45.9, 42.5, 40.6, 37.1, 31.6, 30.1, 25.4, 25.0, 22.5, 14.0. Positive ESI–MS m/z: 414 $(M + H)^+$, HRMS: calcd for $C_{25}H_{36}NO_4$ (M + H), 414.2644; found, 414.2622.

*5.1.11.5. 4-Heptyl-sinomenine (**4f**).* Yield 96%; white oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (d, J = 8.7 Hz, 1H), 6.68 (d, J = 8.7 Hz, 1H), 5.47 (d, J = 2.1 Hz, 1H), 4.18 (d, J = 15.9 Hz, 1H), 4.05 (td, J = 6.9, 2.4 Hz, 2H), 3.77 (s, 3H), 3.49 (s, 3H), 3.18 (t, J = 4.5 Hz, 1H), 2.96–3.02 (m, 2H), 2.75 (dd, J = 18.3, 5.1 Hz, 1H), 2.53 (dt, J = 11.4, 3.0 Hz, 1H), 2.47 (d, J = 15.9 Hz, 1H), 2.43 (s, 3H), 1.80–2.03 (m, 5H), 1.31–1.46 (m, 8H), 0.90 (t, J = 3.6 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 193.6, 152.4, 151.4, 148.0, 129.9, 129.8, 122.3, 115.2, 111.3, 72.2, 56.4, 55.6, 54.6, 49.6, 47.0, 45.8, 42.5, 40.6, 37.0, 31.7, 30.2, 29.1, 25.7, 24.5, 22.5, 14.0. Positive ESI–MS m/z: 428 $(M + H)^+$, HRMS: calcd for $C_{26}H_{38}NO_4$ (M + H), 428.2801; found, 428.2778.

*5.1.11.6. 4-Octyl-sinomenine (**4g**).* Yield 99%; white oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (d, J = 8.4 Hz, 1H), 6.68 (d, J = 8.4 Hz, 1H), 5.47 (d, J = 2.1 Hz, 1H), 4.18 (d, J = 15.9 Hz, 1H), 4.05 (dt, J = 7.2, 3.3 Hz, 2H), 3.77 (s, 3H), 3.49 (s, 3H), 3.17 (t, J = 3.3 Hz, 1H), 2.96–3.01 (m, 2H), 2.74 (dd, J = 18.4, 5.4 Hz, 1H), 2.52 (br, 1H), 2.47 (d, J = 15.9 Hz, 1H), 2.43 (s, 3H), 1.79–2.05 (m, 5H), 1.26–1.47 (m, 10H), 0.89 (t, J = 6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 193.6, 152.3, 151.3, 148.0, 130.0, 129.8, 122.3, 115.3, 111.2, 72.1, 56.3, 55.5, 54.6, 49.7, 46.9, 45.9, 42.5, 40.6, 37.1, 31.7, 30.1, 29.3, 29.1, 25.7, 24.4, 22.5, 14.0. Positive ESI–MS m/z: 442 $(M + H)^+$, HRMS: calcd for $C_{27}H_{40}NO_4$ (M + H), 442.2957; found, 442.2924.

*5.1.11.7. 4-Nonyl-sinomenine (**4h**).* Yield 98%; white oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (t, J = 8.7 Hz, 2H), 5.46 (d, J = 1.8 Hz, 1H), 4.17 (d, J = 15.9 Hz, 1H), 4.06 (t, J = 6.9 Hz, 2H), 3.77 (s, 3H), 3.50 (s, 3H), 3.29 (t, J = 3.9 Hz, 1H), 2.96–3.08 (m, 2H), 2.82 (dd, J = 18.6, 5.4 Hz, 1H), 2.63 (br, 1H), 2.49 (d, J = 15.9 Hz, 1H), 2.46 (s, 3H), 1.79–2.11 (m, 5H), 1.29–1.46 (m, 12H), 0.89 (t, J = 6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 193.4, 152.4, 151.5, 148.0, 129.4, 129.2, 122.4, 114.7, 111.4, 72.2, 56.2, 55.5, 54.6, 49.4, 46.8, 45.1, 41.9, 40.3, 36.5, 31.7, 30.1, 29.4, 29.3, 29.1, 25.7, 24.6, 22.5, 14.0. Positive ESI–MS m/z: 456 $(M + H)^+$, HRMS: calcd for $C_{28}H_{42}NO_4$ (M + H), 456.3114; found, 456.3081.

*5.1.11.8. 4-Decyl-sinomenine (**4i**).* Yield 96%; white oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.71 (t, J = 8.7 Hz, 2H), 5.46 (d, J = 1.8 Hz, 1H), 4.17 (d, J = 15.9 Hz, 1H), 4.06 (t, J = 6.9 Hz, 2H), 3.77 (s, 3H), 3.49 (s, 3H), 3.29 (t, J = 3.9 Hz, 1H), 2.96–3.07 (m, 2H), 2.82 (dd, J = 18.3, 5.1 Hz, 1H), 2.50–2.66 (m, 2H), 2.46 (s, 3H), 1.80–2.10 (m, 5H), 1.27–1.46 (m, 14H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 193.4, 152.4, 151.5, 148.0, 129.5, 129.2, 122.4, 114.6, 111.4, 72.2, 56.2, 55.6, 54.6, 49.4, 46.8, 45.1, 42.0, 40.3, 36.5, 31.8, 30.2, 29.5 (2H), 29.4, 29.2, 25.8, 24.6, 22.5, 14.0. Positive ESI–MS m/z: 470 $(M + H)^+$, HRMS: calcd for $C_{29}H_{44}NO_4$ (M + H), 470.3270; found, 470.3237.

5.2. Biological assays and procedures

5.2.1. Cell culture

Murine monocyte-macrophage RAW264.7 cells maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

5.2.2. Cell viability assay

Cell cytotoxicity was evaluated by MTT method and expressed as IC_{50}. RAW264.7 cells were plated at a density of 1×10^5 cells in a 96-well plate, 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, 5% CO_2, medium on cells was replaced with fresh medium, 20 μL MTT reagents (5 mg/mL) were added, and then the cells were incubated for another 4 h. The supernatants were aspirated, and the formazan crystals in each well were dissolved in 200 μL of dimethyl sulphoxide (DMSO) for 30 min at 37 °C. The absorbance value was monitored by microplate reader at 570 nm (OD_{570}). The density of formazan formed in blank (medium alone) cells was set as 100% of viability.

5.2.3. Nitrite assay

The nitrite assay was performed following the reported method [52]. In brief, RAW264.7 cells were plated at a density of 1×10^5 cells in a 96-well plate. RAW264.7 cells were treated with compounds at various concentrations (0.5–100 μM) and LPS (500 ng/mL) simultaneously. After a 24 h incubation period, 100 μL of culture medium was mixed with an equal volume of Griess reagent (0.2% naphthylethylenediamine dihydrochloride and 2% sulfanilamide in 5% phosphoric acid). After 10 min incubation at

room temperature with protection from light, the absorbance value was measured at 540 nm (OD_{540}) with a microplate reader.

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 (RAW264.7 cells were treated with LPS only) and blank (RAW264.7 cells only, without any treatments) groups and showed no effects on cells. Aminoguanidine was used as positive control.

5.2.4. ELISA assays of TNF-α and IL-6 in RAW264.7 cells

The cell culture procedure was the same as that described in Nitrite Assay, only 10 μM of compounds was added. At indicated incubation time points 6, 12 and 24 h, the TNF-α and IL-6 levels of cell supernatant were measured using ELISA kits from R&D systems (Minneapolis, MN) following kit instructions.

5.2.5. Western blot assay

The cell culture procedure was the same as ELISA Assays of TNF-α and IL-6. The cells incubated for 2, 4 and 6 h after LPS treatment were used for the assay. Western Blot was performed as described in literature [53]. Briefly, cells were washed with phosphate-buffered saline and lysed in the lysis buffer containing Triton X-100. After 10,000 g centrifugation at 4 °C for 10 min, the protein content of the supernatant was determined by a BCA™ protein assay Kit (Pierce, Rochford, IL). The protein lysates were separated by 10% SDS-PAGE and subsequently electro-transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% nonfat milk for 1–2 h at room temperature. The blocked membrane was incubated with the indicated primary antibodies. Primary antibodies used were anti-phosphorylation of SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, anti-phosphorylation of ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phosphorylation of p38 (Thr180/Tyr182), anti-p38, anti-phosphorylation of IκBα (Ser32/36), anti-IκBα, anti-phosphorylation of NF-κB, anti-NF-κB (Cell Signaling Technology, Danvers, MA) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), and the secondary antibody were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (1:10,000, KPL, Gaithersburg, MD). Protein bands were visualized using Western blotting detection system according to the manufacturer's instructions.

5.2.6. Electrophoretic mobility shift assay (EMSA) assay

Nuclear extracts of the cells were subjected to EMSA assay. Consensus biotin-labeled double-strand oligo-deoxynucleotide probes for NF-κB (AGTTGAGGGGACTTTCCCAGGC) were obtained from Beyotime Institute of Biotechnology (China). Reactions for nuclear protein–DNA binding were performed using the Light Shift Chemiluminescent EMSA kit. Briefly, 5 μg of nuclear protein was incubated in a 20 μL reaction volume at room temperature for 20 min and then loaded onto a 6% nondenaturing polyacrylamide gel in 0.5 × tris-borate-ethylenediaminetetraacetic acid (TBE) buffer at 4 °C, and then the binding reactions were transferred to nylon membrane. After cross-link transferred DNA to membrane, the bands were detected by chemiluminesecence. Specificity of DNA–protein complex was confirmed by competition with a 100-fold excess of unlabeled and p65 of NF-κB antibody supershift assay (Cell Signaling Technology, Danvers, MA).

5.2.7. Luciferase reporter assay

RAW264.7 cells were plated and cultured overnight in 100 mm dish (1×10^7 cells/well). The pNF-κB-luc containing NF-κB binding motifs (GGGAATTTCC) was transfected into cells using Lipofectamine 2000 reagents (Invitrogen, USA). At 24 h post-transfection, the cells were plated in 24-wells plates and then treated with increasing concentrations of **2f** or **3b** (5, 10, and 20 μM) in presence of 500 ng/ml LPS for 6 h. The luciferase activity of cell extracts from each sample was measured using a luciferase assay kit according to the manufacturer's protocol.

5.2.8. LPS-induced septic shock model

The Balb/c 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 (10 mice per group) were injected intraperitoneally (i.p.) with 20 mg/kg LPS. Compounds, dissolved in olive oil, were injected intraperitoneally (15 mg/kg) for 3 days prior to LPS injection. Cumulative proportions of mice surviving after injecting LPS were observed. After injection of LPS for 3 h, blood samples were collected from mice and serum samples were separated, then the levels of TNF-α and IL-6 were analyzed by ELISA methods.

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. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2012.01.036. These data include MOL files and InChiKeys of the most important compounds described in this article.

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