Bioorganic & Medicinal Chemistry 19 (2011) 3096–3104

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and biological evaluation of unique stereodimers of sinomenine analogues as potential inhibitors of NO production

Peng Teng [a], Hai-Liang Liu [b], Zhang-Shuang Deng [a], Zhi-Bing Shi [a], Yun-Mian He [a], Li-Li Feng [b], Qiang Xu [b], Jian-Xin Li [a,*]

[a] Key Lab of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, PR China
[b] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Science, Nanjing University, Nanjing 210093, PR China

ARTICLE INFO

Article history:
Received 24 February 2011
Revised 2 April 2011
Accepted 4 April 2011
Available online 7 April 2011

Keywords:
Sinomenine
Stereodimer
Inhibitor
Nitric oxide
iNOS

ABSTRACT

Inhibition of the excessive NO production has been recognized as a potential means for the treatment of rheumatoid arthritis (RA). In order to discover more potent inhibitors and explore the preliminary structure activity relationship, a series of unique stereodimers of sinomenine analogues were designed and synthesized. Their inhibitory activity on NO production and cytotoxicity were evaluated using LPS-activated murine macrophages RAW264.7 assay and MTT method, respectively. Among these compounds, **1a**, **2**, **2a**, **2b**, and **4** showed potent inhibitory activity on NO production without obvious cytotoxicity. Furthermore, **2**, **2a**, and **2b** significantly suppressed mRNA expression of iNOS. Interestingly, (*S*)-dimers displayed a better bioactivity than (*R*)-dimers. These compounds may sever as lead candidates in the development of novel therapeutic drugs for RA treatment.

1. Introduction

Nitric oxide (NO) is an endogenous free radical generated from an NO-liberating compound L-arginine catalyzed by a family of nitric oxide synthase (NOS) including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). The short-life molecule NO has gained its reputation of being an important signaling mediator of vasorelaxation, neurotransmission, and host defence at low concentration.[1,2] The readily diffusible NO is involved in various signals, such as cardiovascular, pain, cancer, and inflammation.[3,4] Among the nitric oxide synthase family, iNOS can predominantly generate high expression of NO, which modulates the inflammation signals through multiple pathways and plays role of great importance in the regulation of immune reactions.[5] Aberrant NO synthesis catalyzed by iNOS, located in macrophage, has been involved in a variety of pathological signal pathways, such as stroke, hypertension, cancer, ischemia, inflammation, colitis, and rheumatoid arthritis.[6] Thus, blocking the excessive NO production has been recognized as a potential means for the treatment of these diseases.[7]

Rheumatoid arthritis (RA), a chronic and systemic inflammation disease has symptoms of progressive demolish of the articular cartilage, hyperplasia of the synovium, and damages to the joint tissue of bones, accompanied by the disability and limitation of bones' actions.[8,9] Abnormal or high expression of NO and pro-inflammation cytokines play an important role in the pathogenesis of several arthritis diseases.[10,11] Blocking the localized excess production of NO was identified as a way of treating RA.[12]

Sinomenine (**1**), a natural alkaloid isolated from Chinese medicinal plant *Sinomenium acutum*, has been employed clinically for the treatment of RA in China for a long time.[13] Furthermore, a variety of other bioactivities has also been reported, such as treating mesangial proliferative nephritis, adjuvant arthritis, osteoarthritis, inflammation mediated neurodegenerative disorders and other immune-related diseases.[14–16] However, the efficacy of sinomenine for the treatment of RA is quite weak. Additionally, certain adverse effects have also been reported.[17–19] Therefore, to get novel and better derivatives with sinomenine scaffold, a structure modification was conducted.

Dimers of sinomenine by C_1–C_1 coupling have been studied in the past 80 years, including some biocatalytic products,[20,21] however, so far neither synthesis of other dimer analogues, nor the bioactivities of the dimers have been emanated. It is reported that dimerization of two natural product moieties represents the promising and fundamental approaches in the filed of designing novel drug leads with high efficacy and low toxicity in medicinal chemistry,[22,23] which inspired us to dimerize the sinomenine analogues. We have already identified the stereochemistry of the (*S*)- and (*R*)-disinomenine and discovered that (*S*)-disinomenine possessed a more potent inhibitory activity on IL-6 production compared with sinomenine, while (*R*)-disinomenine exhibited a stimulative

* Corresponding author. Tel./fax: +86 25 83686419.
E-mail address: lijxnju@nju.edu.cn (J.-X. Li).

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.04.006

effect.[24,25] In the present study, as an effort to develop novel molecules with better bioactivity and understand the structure–activity relationships (SAR), a series of stereodimers of sinomenine analogues were synthesized and their inhibitory activity on NO production and cytotoxicity were evaluated using LPS-activated murine macrophages RAW264.7 assay and MTT method, respectively. Furthermore, the suppressive effect on mRNA expression of iNOS of the compounds with potent activity was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

2. Results and discussion

2.1. Syntheses of stereodimers of sinomenine

Dimerization of sinomenine was firstly conducted using potassium permanganate ($KMnO_4$) oxidation by Goto in 1929 and the existence of two dimers were confirmed.[20] However, after which, no reports were followed up on the stereostructures of the dimers due to the difficulty of the crystallization of the dimers.[26] Our previous work successfully identified the stereostructures of two sinomenine dimers by single crystal X-ray diffraction analysis (Scheme 1), (*S*)-disinomenine (**1a**) and (*R*)-disinomenine (**1b**) that possessed different angle around C1–C1 bond, and also discovered that in $KMnO_4$ aqueous solution the two dimers could be pH controlled stereoselectively synthesized.[25] Additionally, (*S*)-disinomenine can also be stereoselectively afforded by biocatalyst with a fungus *Antrodiella semisupina*.[24] These results encouraged us to expand the diversity of these dimers and discovery new molecules with better bioactivities, therefore, the other dimers were prepared via similar approaches to allow access to versatile analogues to investigate structure–activity relationship (SAR) of stereodimers of sinomenine analogues.

To more efficiently synthesize the dimers, an oxidation of sinomenine with manganese dioxide (MnO_2) in methanol solution was performed, as expected, compounds **1a** and **1b** were prepared under the mild conditions (Scheme 1) and could easily be separated by a silica gel column chromatography.

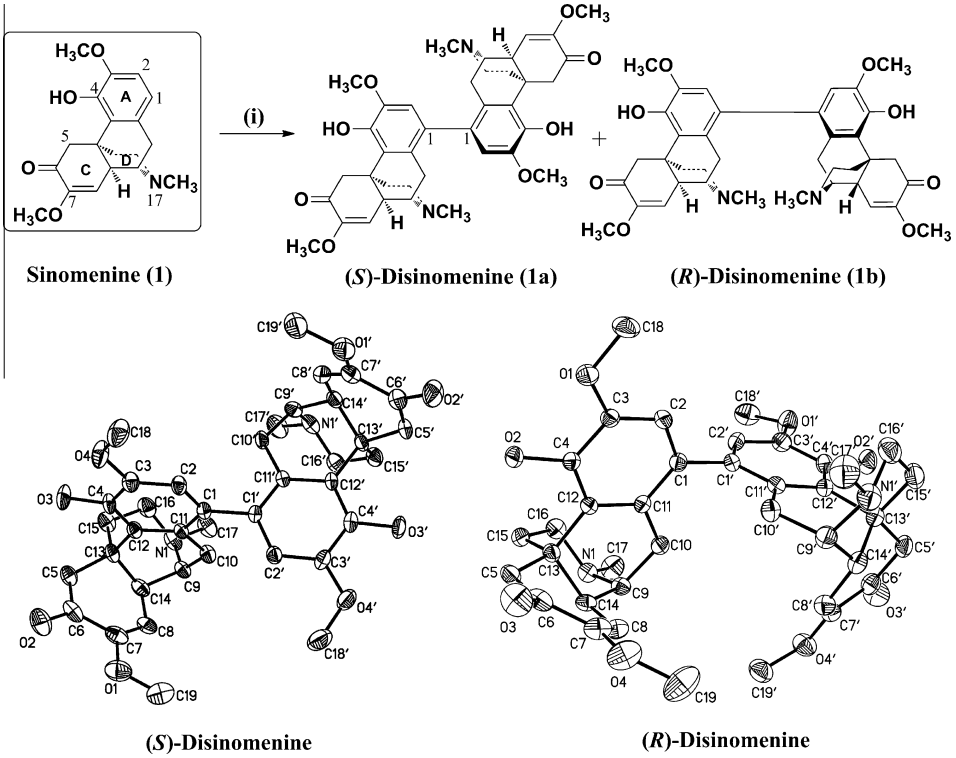
It should be noted that based on the results of single crystal X-ray diffraction analyses, the stereostructures of **1a** and **1b** could also be elucidated by circular dichroism (CD) spectra and ^1H NMR data.[24] In the CD spectrum, a positive Cotton effect was observed in (*S*)-disinomenine (**1a**) at 296.7 nm, while no Cotton effect was exhibited at same wavelength of the CD spectrum of (*R*)-disinomenine (**1b**), indicating the difference on regiochemistry of biphenyl linkage. Moreover, the protons signal at C-2 in **1a** (δ_H 6.27, s, 2H) displayed an upfield shift versus that of **1b** (δ_H 6.45, s, 2H).

2.2. Syntheses of stereodimers of sinomenine analogues

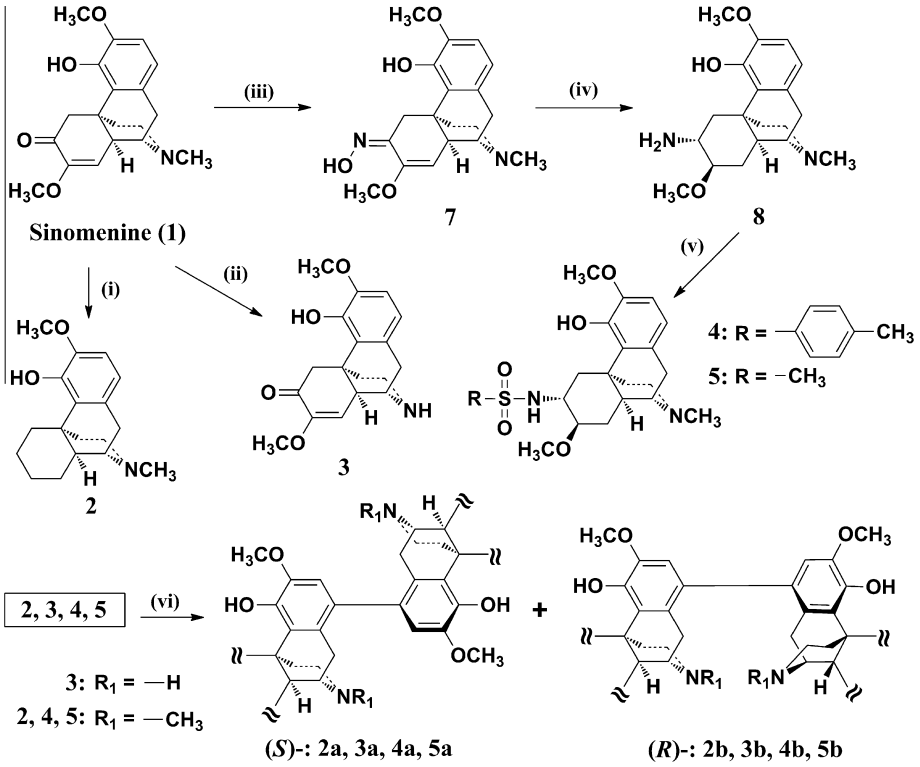
The treatment of sinomenine (**1**) with zinc amalgam afforded the ring-C reduced cyclohexane structure (**2**).[27] The dimerization of **2** was also carried out followed the sinomenine's dimerization, an oxidation with MnO_2 in methanol solution followed a column chromatography afforded the target dimers **2a** and **2b**.

The *N*-methyl group of sinomenine ring-D can be eliminated both by chemical and metabolic approaches.[28,29] In the present study, *N*-demethylsinomenine (**3**) was prepared in satisfactory yield by allowing a mixture of sinomenine and H_2O_2 to stand 24 h at room temperature and followed by a reduction in the presence of $FeSO_4$.[30] Oxidation of *N*-methyl group in the ring-D could facilely afford the intermediate oxynitride in an almost complete conversion (98.7%), which was then subjected to methanol solution in the presence of reducing agent $FeSO_4$, affording target molecule (**3**) in moderate yield (71.5%, Scheme 2).

Carbonyl group of sinomenine was reacted with hydroxylamine hydrochloride to afford oxime (**7**), followed by reduction with Pd/C to provide the compound 6β-amino-7β-methoxy-7,8-dihydro sinomenine (**8**). Due to the two additional chiral centers (C-6 and C-7), compound **8** should have four stereoisomers, however, the



Scheme 1. Synthesis of **1a** and **1b** and X-ray crystal structures from Ref. 25. Reagent and conditions: (i) MnO_2, MeOH, rt, 24 h.



Scheme 2. Synthesis of **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b**. Reagent and conditions: (i) zinc amalgam, concentrated HCl, reflux, 24 h; (ii) 15% H_2O_2, rt, 24 h; $FeSO_4$, MeOH, rt, 6 h; (iii) NH_2OH, EtOH, reflux, 8 h; (iv) NH_4HCO_3, Pd/C (10%), MeOH, reflux, 6 h; (v) MsCl or TsCl, Et_3N, CH_2Cl_2, rt, 5 h; (vi) MnO_2, MeOH, rt, 48–72 h.

reaction afforded only one pure compound rather than diastereomers based on chiral HPLC analysis (see Supplementary data). The configurations of C-6 and C-7 of **8** were determined based on the comparisons of coupling constant (J) with reported data.[31–33] In the ^{1}H NMR spectrum, the protons at C-5 (4.08, dd, J = 14.4, 3.0 Hz; 1.25, dd, J = 14.4, 3.0 Hz) and the proton at C-6 (3.38, dd, J = 6.9, 3.0 Hz) clearly revealed that the coupling constant of protons at C-6 and C-7 positions ($J_{6,7}$) was 6.9 Hz although the split pattern of 7-H was unreadable due to the signal overlapping. Because the preferential conformation of ring-C is a chair form, as can be seen in Figure 1, the $J_{6,7}$ at axial and equatorial bonds in four isomers of **8** displayed different values. Due to the $J_{6,7}$ was 6.9 Hz, the configurations of C-6 and C-7 were determined to be both 6H and 7H at axial bonds (Fig. 1).

Because the sulfonyl group manifests excellent effects in the field of medicinal molecules,[34] the amino group was further modified by several sulfonyl chlorides, aiming to get new compounds owning better bioactivity. Therefore, *p*-toluene sulfonyl chloride was firstly employed, compound **8** reacted with the sulfonyl chloride in the present of Et_3N gave the compound **4** with excellent yield (96.1%). The same approach was also employed in the small methyl sulfonyl group to afford corresponding substituted amino compound (**5**).

Reaction of **3**, **4**, and **5** with MnO_2 in dried methanol afforded corresponding dimers **3a**, **3b**, **4a**, **4b**, **5a**, and **5b** with different spatial configuration, respectively, and was outlined in Scheme 2.

It has been reported that quinoxaline moiety could provide an effective bioactivity.[35,36] Thus, a vicinal 6,7-diketone (**9**) and its quinoxaline derivative (**6**) were prepared followed the reported protocols (Scheme 3).[37,38] For the dimerization of **6**, although many efforts with different oxidants and pH controlled stereoselective syntheses, unfortunately, no targeted (*S*)- and (*R*)-dimers of **6** were obtained, mostly probably due to the strong steric hindrance. However, when a biotransformation of **6** by a strain of fungi (*A. semisupina*) was carried out, (*S*)-dimer (**6a**) was successfully afforded, while corresponding (*R*)-dimer was still failed to obtain (Scheme 3).

The stereostructures of (*S*)-dimers (**2a**, **3a**, **4a**, **5a**, **6a**) and (*R*)-dimers (**2b**, **3b**, **4b**, **5b**) were determined by comparison the CD spectra and ^{1}H NMR data with those of **1a** and **1b**. In the CD spectra of all (*S*)-dimers, a positive Cotton effect between 292 and 300 nm was clearly observed, while no Cotton effect was displayed in the same wavelength range of the CD spectra of all (*R*)-dimers (see Supplementary data). Additionally, the same as **1a** and **1b**, in ^{1}H NMR spectra, the proton signal at C-2 of all (*S*)-dimers showed an upfield shift compared with that of all (*R*)-dimers. Based on

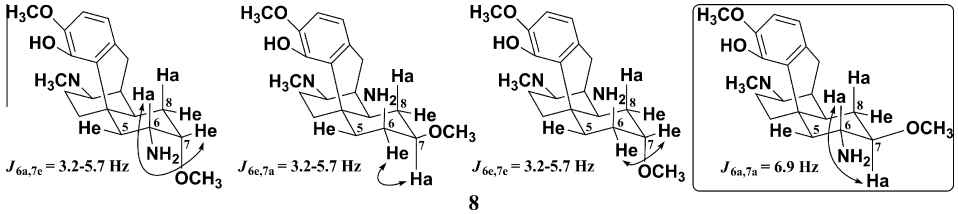
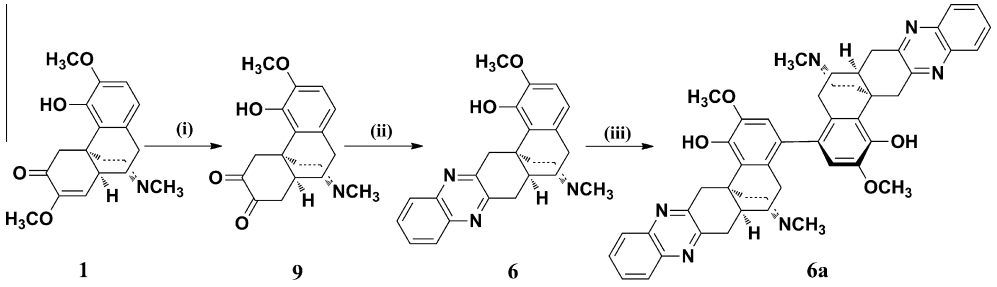


Figure 1. The possible stereoisomers of **8**.



Scheme 3. Synthesis of **6a**. Reagent and conditions: (i) 10% HCl, reflux, 10 h; (ii) *o*-phenylenediamine, MeOH, N_2, 6 h; (iii) *Antrodiella semisupina*, mineral medium, 25 °C, incubation on rotary shaker.

those physiochemical and NMR data, the stereostructures of the dimers were elucidated as shown in Schemes 2 and 3.

2.3. Bioactivity evaluation

To evaluate the inhibitory effects on the excessive NO production, all the compounds were assayed using LPS-activated murine macrophage-like RAW264.7 cell culture systems.[39] Because the inhibitory activity of the compounds is sometimes a result of their toxic effect and consequently might cause an erroneous conclusion, prior to the following bioactivity analyses, the cytotoxicity of the synthesized compounds was determined by MTT assay and summarized in Table 1. The data showed that the most of the compounds had cytotoxicity IC_{50} of more than 100 μM, only compounds **2a** and **2b** displayed cytotoxicity IC_{50} of 9.5 and 14.2 μM, and **4a** and **4b** at 43.5 and 72.9 μM, respectively. Therefore, all the activity profiles were carried out below the toxic IC_{50} concentrations.

Sinomenine (**1**) showed a very weak activity on the production of NO at 100 μM, although no cytotoxicity was detected at this concentration (Tables 1 and 2). (*S*)-Disinomenine (**1a**) showed obvious increase on the inhibitory activity of NO compared with the parent compound **1**, while interestingly, (*R*)-disinomenine (**1b**) displayed no improvement of the activity. The same as **1a** and **1b**, (*R*)-dimer (**2b**) also had a good activity, but weaker than **2a**, while, the monomer (**2**) could also inhibit NO production at 100 μM. Compound **3** was an *N*-dimethyl group structure of **1** and exhibited a modest inhibitory effect, while, dimeric compounds **3a** and **3b** showed the similar results as **3**. Compound **4** displayed a prodigious enhancement in activity and a dose-dependence effect compared with parent compound **1**, and its dimers (**4a** and **4b**) both exhibited good inhibitory effect, while (*S*)-dimer (**4a**) was more potent than (*R*)-dimer (**4b**) (Table 3). Of all of compounds, (*S*)-dimer (**2a**) of **2** showed the most potent efficacy, a 41.6% inhibition at 6.25 μM, while no cytotoxicity was observed at this concentration. These results suggested that the deoxygenation and dimerization could elevate the activity.

Table 1
Cytotoxicity of the synthesized compounds

Compound	Cytotoxicity IC_{50} (μM)[a]
1	>100
1a	>100
1b	>100
2	>100
2a	9.5
2b	14.2
3	>100
3a	>100
3b	>100
4	>100
4a	43.5
4b	72.9
5	>100
5a	>100
5b	>100
6	57.9
6a	6.3

[a] Cytotoxicity IC_{50}: the concentration of inhibition of 50% cell growth with four experiments.

Table 2
The effect of compounds on LPS-induced NO production in RAW264.7 cells

Compound	Inhibition (%)		
	100 (μM)	50 (μM)	25 (μM)
1	12.8 ± 2.3	0.1 ± 2.9	−3.0 ± 5.5
1a	23.7 ± 2.1*	15.0 ± 3.1	−2.9 ± 5.4
1b	−2.1 ± 2.7	6.6 ± 3.2	−1.3 ± 3.1
2	30.6 ± 4.2*	28.1 ± 0.6*	12.1 ± 3.7
3	10.7 ± 2.8	−0.8 ± 1.6	−6.1 ± 4.1
3a	16.0 ± 2.2	−12.6 ± 5.4	−9.3 ± 2.3
3b	−7.1 ± 4.0	−11.0 ± 3.8	−11.6 ± 8.4
4	39.8 ± 4.1*	30.0 ± 4.6*	10.9 ± 6.7
5	−3.5 ± 14.4	−3.7 ± 5.1	−2.3 ± 1.5
5a	9.3 ± 6.6	0.2 ± 7.6	−0.2 ± 8.0
5b	9.9 ± 4.4	8.6 ± 6.6	−8.4 ± 6.6
AG	86.0 ± 0.3*	74.9 ± 2.5*	55.4 ± 0.5*
Control		0.0 ± 6.4	

The cells of control group were treated with LPS (1 μg/mL) only. The data of control group was pegged as 0.0%, while other data were calculated relative to it. Cells were treated with test compounds prior to LPS treatment. AG: aminoguanidine as a positive control. Data are expressed as mean ± SD, $n = 4$.
'−' A minus sign of the data means enhancement effect on NO production.
* Significant differences compared with control group, $p < 0.01$.

Table 3
The effect of compounds **2a**, **2b**, **4a** and **4b** on LPS-induced NO production in RAW264.7 cells

Compound	Inhibition (%)		
	6.25 (μM)	3.125 (μM)	1.5625 (μM)
2a	41.6 ± 1.3*	17.0 ± 7.0	2.7 ± 3.1
2b	35.2 ± 0.2*	16.9 ± 2.4	−4.4 ± 3.9
6a	NT	NT	8.4 ± 2.1
	50 (μM)	25 (μM)	12.5 (μM)
4a	NT	4.9 ± 2.4	2.6 ± 1.0
4b	−2.2 ± 6.2	−2.9 ± 6.2	−4.0 ± 2.9
6	NT	11.4 ± 3.8	1.3 ± 3.4
AG	76.5 ± 3.4*	52.5 ± 0.7*	–
Control		0.0 ± 6.6	

Cells were treated with test compounds prior to LPS treatment. AG: aminoguanidine as a positive control. Data are expressed as mean ± SD, $n = 4$.
'−' A minus sign of the data means enhancement effect on NO production.
NT: not tested due to cytotoxicity.
* Significant differences compared with control group (0.0%), $p < 0.01$.

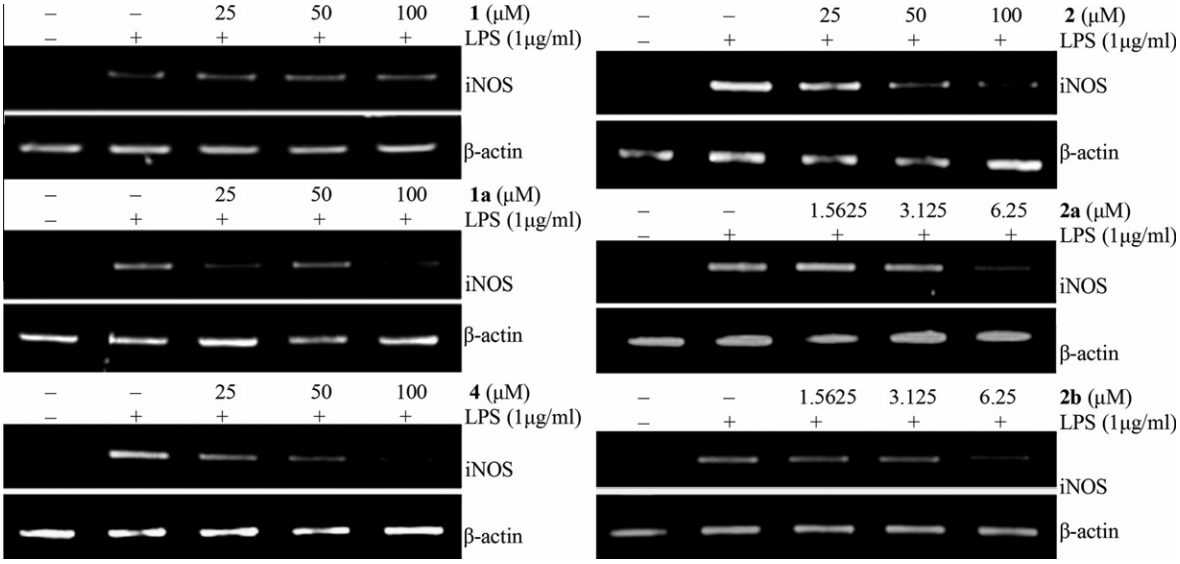


Figure 2. The effect of **1**, **1a**, **2**, **2a**, **2b** and **4** on the mRNA expression of iNOS by RT-PCR.

Above results revealed that the dimerization and the stereostructure did gave the impact on the activity. It is reported that phenolic compounds possess a capacity to trap free radicals.[40,41] Because the present dimers possess the same phenolic moiety (A ring), it is feasible that the dimers inhibit the NO production via scavenging NO directly. However, although (*R*)- and (*S*)-dimers have the same phenolic moiety, it is clear that the (*S*)-dimers with less steric hindrance possessed better inhibitory effect on NO production than the (*R*)-dimers, which was extraordinarily interesting for the further research on the SAR.

The excessive expression of NO is greatly dominated by iNOS, which is one of the most pivotal signals during the process of LPS-induced NO production. In an effort to study the preliminary mechanism of the compounds with potent inhibitory activity, the RT-PCR experiment was performed to assay the effect of five selected compounds on mRNA expression of iNOS. The RT-PCR results (Fig. 2) reveled that compounds **1a**, **2**, **2a**, **2b**, and **4** markedly inhibited the mRNA expression of iNOS in a dose-dependent manner, while the parent compound **1** only faintly restrained the expression at 100 μM. These results revealed that the sinomenine analogues might play crucial role in the pathway involved iNOS.

3. Conclusion

We have designed, synthesized and biologically evaluated a series of unique stereodimers of sinomenine analogues on the inhibitory effect of NO overproduction and mRNA overexpression of iNOS using LPS-induced murine RAW264.7 cells. Among the synthesized compounds, **1a**, **2**, **2a**, **2b**, and **4** displayed a potent inhibitory activity and also exerted the activity via suppressing mRNA expression of iNOS in a dose-dependent manner. Reduction of ring-C (**2**) could be beneficial to increase of the activity. The present results unfolded that dimerization of sinomenine analogues with weak steric hindrance (*S*-dimers) showed a better inhibitory potency both on NO overproduction and mRNA expression of iNOS, while those with a strong steric hindrance (*R*-dimers) showed almost no benefit on the enhancement of the activity, some of (*R*)-dimers (**3b**, **4b**) even promoted the secretion of NO. Why the (*R*, *S*)-dimers only with different angle around C1–C1 bond exhibited reversed activity clearly warrant further studies, and now the mechanism clarification and further expansion of the related compounds are under way in our laboratory.

4. Experimental section

4.1. Synthesis

4.1.1. General

^{1}H and ^{13}C NMR spectra were measured on a Bruker Advance II 300 spectrometer using tetramethylsilane as internal standard. Chemical shift (δ) are reported in parts per million (ppm) and coupling constants *J* are reported 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). The CD spectra were tested on a Model J-810 automatic recording spectropolarimeter in methanol. The optical rotations were recorded on Autopol I Automatic Polarimeter (Spectronic Camspec Ltd, UK) in CH_3OH or $CHCl_3$. Chromatographic separations were performed on silica gel columns by column 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). Evaporation of solvent was performed in vacuo with rotating evaporator. Commercially available chemicals were purchased from Alfa Aesar. 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 230–300 nm with a runtime of 35 min. The compound purity analysis data of final compounds (greater than 95%) can be seen in the Supplementary data.

4.1.2. General procedure for the synthesis of dimers

To a solution of mono compound in dried CH_3OH (50 mL) was added MnO_2 and the mixture was stirred at room temperature for 24–72 h. The solution was then filtered and washed by CH_3OH (10 mL × 3). The filtrate and the washes were combined, dried over anhydrous Na_2SO_4 and concentrated. The resulting brown solid was purified using silica gel column chromatography with CH_2Cl_2/MeOH as eluent to yield the title compounds.

4.1.2.1. 7-Demethoxy-6-desoxo-7, 8-dihydro sinomenine (2). A mixture of zinc power (52 g, 0.8 mol) and mercury(II) chloride (4 g, 15 mmol) in concentrated hydrochloric acid/distilled water (4 mL/40 mL) was stirred at room temperature for 5 min. The aqueous solution was decanted and the prepared amalgamated zinc was covered with concentrated hydrochloric acid (80 mL) and distilled water (40 mL) in sequence. Then the material sinomenine (13.2 g, 40 mmol) was added immediately and the reaction mixture was heated to 90 °C and stirred for 24 h. Concentrated hydrochloric acid (20 mL) was added every 8 h. After 24 h, the reaction mixture was cooled to room temperature, basified with concentrated ammonia solution, and extracted with dichloromethane (50 mL × 3). The combined organic phase were dried over Na_2SO_4 and concentrated in vacuum. The crude residue were purified by silica gel column chromatography (EtOAc/MeOH/NH_4OH, 470:20:10, v/v/v) to provide the target compound (5.16 g, 45%). ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.70 (d, J = 8.4 Hz, 1H), 6.62 (d, J = 8.4 Hz, 1H), 3.86 (s, 3H), 3.36 (dd, J = 10.2 and 4.2 Hz, 1H), 2.94 (d, J = 17.7 Hz, 1H), 2.78 (dd, J = 5.4 and 3.3 Hz, 1H), 2.67 (dd, J = 18.3 and 6.3 Hz, 1H), 2.44–2.50 (m, 1H), 2.39 (s, 3H), 2.09 (dt, J = 12.0 and 3.6 Hz, 1H), 1.84–1.93 (m, 1H), 1.55–1.78 (m, 5H), 1.07–1.45 (m, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 144.7, 144.5, 132.2, 125.9, 118.5, 108.2, 58.1, 56.2, 47.8, 46.8, 42.8, 38.4, 38.1, 37.1, 27.4, 26.8, 24.3, 23.3. Positive ESI-MS m/z: 288 $[M+H]^+$, positive HRESIMS m/z found 288.1935 $[M+H]^+$ (calcd for $C_{18}H_{26}NO_2$, 288.1964). $[\alpha]_D^{25} = +38.33$ (c 0.60, CH_3OH).

4.1.2.2. (*S*)-7-Demethoxy-6-desoxo-7, 8-dihydro disinomenine (2a) and (*R*)-7-demethoxy-6-desoxo-7, 8-dihydro disinomenine (2b). The title compounds **2a** and **2b** were prepared according the procedure described above; 7-demethoxy-6-desoxo-7, 8-dihydro sinomenine (**2**, 574 mg, 2.0 mmol) was dissolved in dried CH_3OH (50 mL) and MnO_2 (3000 mg, 34.5 mmol) was added with magnetic stirring, and the mixture was remained at room temperature for 3 days, and then filtered and washed by CH_3OH (10 mL × 3). The filtrate and the washes were combined, dried over anhydrous Na_2SO_4 and concentrated. The resulting brown solid was purified using silica gel column chromatography with chloroform/methanol (15:1, v/v) as eluent to yield **2a** (33%) and **2b** (45%).

Compound **2a**: ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.51 (s, 2H), 3.85 (s, 6H), 3.44 (d, J = 12.3 Hz, 2H), 2.74 (t, J = 3.3 Hz, 2H), 2.56 (d, J = 10.5 and 3.9 Hz, 2H), 2.42 (dd, J = 14.1 and 6.0 Hz, 2H), 2.39 (d, J = 6.3 Hz, 2H), 2.32 (s, 6H), 2.19–2.27 (m, 4H), 1.59–1.84 (m, 10H), 1.08–1.45 (m, 12H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 143.3, 131.3, 129.2, 126.1, 109.8, 57.7, 56.2, 47.7, 46.6, 42.9, 38.5, 38.2, 37.1, 27.3, 26.6, 23.7, 23.2. Positive ESI-MS m/z: 573 $[M+H]^+$, positive HRESIMS m/z found 573.3668 $[M+H]^+$ (calcd for $C_{36}H_{49}N_2O_4$, 573.3692). $[\alpha]_D^{25} = +65.00$ (c 0.60, CH_3OH). CD (c = 0.05 mM, CH_3OH): θ_{207} +9.81 mdeg, θ_{223} −10.39 mdeg, θ_{255} +6.00 mdeg, θ_{293} +3.62 mdeg.

Compound **2b**: ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.52 (s, 2H), 3.86 (s, 6H), 3.44(dd, J = 11.7 and 1.5 Hz, 2H), 2.69 (dd, J = 5.4 and 3.0 Hz, 2H), 2.57 (d, J = 18.9 Hz, 2H), 2.47–2.51 (m, 2H), 2.28 (s, 6H), 2.12 (dt, J = 12.6 and 3.3 Hz, 2H), 2.03 (d, J = 6.9 Hz, 2H), 1.86 (dd, J = 18.6 and 6.0 Hz, 2H), 1.75–1.78 (m, 2H), 1.58–1.70 (m, 8H), 1.32–1.42 (m, 4H), 1.26 (t, J = 7.2 Hz, 2H), 1.11–1.20 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 144.5, 143.4, 131.6, 129.9, 126.1, 108.8, 57.7, 56.0, 47.9, 46.7, 43.0, 38.3, 38.2, 37.2, 27.3, 26.8, 23.3, 22.5. Positive ESI-MS m/z: 573 $[M+H]^+$, positive HRESIMS m/z: found: 573.3668 $[M+H]^+$ (calcd for $C_{36}H_{49}N_2O_4$, 573.3692). $[\alpha]_D^{25} = +57.10$ (c 0.72, CH_3OH). CD (c = 0.05 mM, CH_3OH): θ_{208} +22.47 mdeg, θ_{234} +6.00 mdeg.

4.1.2.3. 17-Demethyl sinomenine (3). A solution of **1** (1.32 g, 4 mmol) in 15% H_2O_2 (20 mL) was allowed to warm to room temperature stirred for 24 h, basified with concentrated ammonia solution (to pH 9–10) and then extracted with $CHCl_3$ (30 mL × 3). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuum to afford the intermediate oxynitride (1.37 g, 98.7%), which was used in the next step without purification. The intermediate oxynitride (1.37 g, 3.95 mmol) was dissolved in methanol (30 mL) and added with $FeSO_4{\cdot}7H_2O$ (2.22 g, 8 mmol) under stirring at 0 °C and then warm to room temperature for 6 h. The solvent was removed and ethylenediaminetetraacetic acid (EDTA) aqueous solution (0.6 M, 40 mL) was added to remove excessive Fe^{2+} and Fe^{3+} under stirring for 4 h. The reaction was basified attentively with concentrated ammonia solution (to pH 9–10) and the extracted with $CHCl_3$ (50 mL × 4). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. Purification by column chromatography (CH_2Cl_2/MeOH, 15:1–6:1, v/v) afforded the title compound **3** as a gray solid (889 mg, 71.5%). ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.65 (d, J = 8.4 Hz, 1H), 6.54 (d, J = 8.4 Hz, 1H), 5.42 (d, J = 2.1 Hz, 1H), 4.33 (d, J = 15.6 Hz, 1H), 3.81 (s, 3H), 3.55 (t, J = 4.2 Hz, 1H), 3.48 (s, 3H), 3.18 (dd, J = 18.0 and 5.7 Hz, 1H), 3.02 (br s, 1H), 2.84 (dd, J = 14.1 and 3.9 Hz, 2H), 2.61 (dt, J = 12.6 and 3.6 Hz, 1H), 2.48 (d, J = 15.6 Hz, 1H), 1.93 (br d, J = 12.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 193.8, 152.4, 145.1, 144.8, 130.3, 122.7, 118.4, 114.7, 109.1, 56.0, 54.8, 50.0, 49.6, 45.9, 41.4, 38.9, 36.1, 33.7. Positive ESI-MS m/z: 316 $[M+H]^+$.

4.1.2.4. (*S*)-17-Demethyl disinomenine (3a) and (*R*)-17-demethyl disinomenine (3b). The titled compounds **3a** and **3b** were prepared according the procedure described above; 17-demethyl sinomenine (**3**, 630 mg, 2.0 mmol) was dissolved in dried CH_3OH (50 mL) and MnO_2 (3000 mg, 34.5 mmol) was added with magnetic stirring, and the mixture was remained at room temperature for 3 days and then filtered and washed by CH_3OH (10 mL × 3). The filtrate and the washes were combined, dried over anhydrous $MgSO_4$ and concentrated. The resulting brown solid was purified using silica gel column chromatography with chloroform/methanol/NH_4OH (60:40:1, v/v/v) as eluent to yield **3a** (75%) and **3b** (12%).

Compound **3a**: ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.30 (s, 2H), 5.38 (d, J = 2.4 Hz, 2H), 4.37 (d, J = 15.6 Hz, 2H), 3.75 (s, 6H), 3.49 (s, 6H), 3.36 (br t, J = 3.6 Hz, 2H), 2.90 (br s, 2H), 2.85 (dd, J = 13.0 and 3.9 Hz, 2H), 2.75 (dd, J = 18.4 and 5.6 Hz, 2H), 2.60 (dd, J = 13.0 and 3.9 Hz, 2H), 2.51 (d, J = 15.6 Hz, 2H), 2.19 (d, J = 18.4 Hz, 2H), 2.02 (d, J = 12.2 Hz, 2H), 1.72 (td, J = 13.0 and 3.9 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 193.8, 152.3, 145.1, 143.8, 131.0, 127.6, 122.9, 114.5, 110.4, 56.0, 54.8, 49.7, 49.4, 46.3, 41.6, 39.4, 36.3, 32.4. Positive ESI-MS m/z: 629 $[M+H]^+$, HRESIMS m/z: found: 629.2836 $[M+H]^+$ (calcd for $C_{36}H_{41}N_2O_8$, 629.2863). $[\alpha]_D^{25} = +91.43$ (c 0.53, CH_3OH). CD (c = 0.05 mM, CH_3OH): θ_{212} +12.91 mdeg, θ_{243} +12.22 mdeg, θ_{271} −5.32 mdeg, θ_{293} +0.59 mdeg.

Compound **3b**: ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.45 (s, 2H), 5.27 (d, J = 1.8 Hz, 2H), 4.38 (d, J = 15.6 Hz, 2H), 3.81 (s, 6H), 3.54 (s, 6H), 3.24 (br t, J = 4.5 Hz, 2H), 2.94 (brs, 2H), 2.78 (dd, J = 12.6 and 4.8 Hz, 2H), 2.63 (dt, J = 12.9 and 3.0 Hz, 2H), 2.50 (d, J = 15.3 Hz, 2H), 2.25–2.30 (m, 2H), 1.99–2.03 (m, 4H), 1.74 (dt, J = 12.6 and 5.1 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 194.1, 152.5, 145.4, 144.0, 131.0, 128.0, 123.1, 114.6, 110.0, 56.1, 54.6, 45.2, 41.5, 38.8, 35.5, 32.6, 31.0, 23.9. Negative ESI-MS m/z: 627 $[M-H]^-$, positive HRESIMS m/z: found: 629.2836 $[M+H]^+$ (calcd for $C_{36}H_{41}N_2O_8$, 629.2863). $[\alpha]_D^{25} = +6.49$ (c 0.15, CH_3OH). CD (c = 0.1 mM, CH_3OH): θ_{214} +24.39 mdeg, θ_{233} +23.23 mdeg, θ_{271} −14.62 mdeg.

4.1.2.5. 6-Hydroxyimino sinomenine (7). Sinomenine (329 mg, 1 mmol), hydroxylamine hydrochloride (700 mg, 1 mmol), and anhydrous NaOAc (410 mg, 5 mmol) were dissolved in absolute ethanol (20 mL), and the mixture was heated at reflux for 8 h and then concentrated to dryness. Water (20 mL) was added, and the mixture was made base with 15% ammonia water and

extracted with CH_2Cl_2 (50 mL × 3). The CH_2Cl_2 extract was washed with brine, dried over Na_2SO_4, filtered, concentrated and recrystallized from CH_2Cl_2/PE to give a white solid (327 mg, 95%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.58 (d, J = 9.0 Hz, 1H), 6.49 (d, J = 9.0 Hz, 1H), 5.10 (d, J = 15.0 Hz, 1H), 4.84 (s, 1H), 3.75 (s, 3H), 3.47 (s, 1H), 3.09 (t, J = 3.0 Hz, 1H), 2.92 (d, J = 18.0 Hz, 1H), 2.77 (br s, 1H), 2.72 (dd, J = 18.0 and 6.0 Hz, 1H), 2.53 (dd, J = 12.0 and 3.0 Hz, 1H), 2.39 (s, 1H), 1.91–2.08 (m, 3H), 1.79 (td, J = 12.0 and 3.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 150.9, 150.6, 144.8, 144.8, 130.9, 123.6, 117.9, 108.4, 104.4, 57.0, 55.9, 54.6, 47.7, 44.2, 42.6, 37.2, 35.8, 33.3, 24.1. Positive ESI-MS m/z: 345 $[M+H]^+$.

4.1.2.6. 6β-Amino-7β-methoxy-7,8-dihydro sinomenine (8). The oxime, **7** (344 mg, 1 mmol), and ammonium formate (630 mg, 10 mmol) was heated at reflux under magnetic stirring in the presence of 10% Pd/C (200 mg) and absolute methanol (20 mL) for 6 h, filtered through a pad of Celite (eluted with MeOH, 50 mL), and concentrated in vacuum to dryness. The resulting residue was dissolved in water (20 mL), made base (pH 9–10) with 15% ammonia water, extracted with CH_2Cl_2 (50 mL × 3). The combined CH_2Cl_2 extract was washed with brine, dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH, 9:1, v/v) providing **8** (305 mg, 92%) as a white solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.59 (d, J = 8.1 Hz, 1H), 6.52 (d, J = 8.1 Hz, 1H), 4.08 (dd, J = 14.4 and 3.0 Hz, 1H), 3.57 (s, 3H), 3.38 (dd, J = 6.9 and 3.0 Hz, 6α-H), 3.32 (s, 3H), 3.26–3.32 (overlapped, 7α-H), 2.82–2.93 (m, 2H), 2.72 (dd, J = 17.7 and 5.1 Hz, 1H), 2.42 (dd, J = 11.4 and 3.3 Hz, 1H), 2.35 (s, 3H), 1.95–2.03 (m, 2H), 1.71–1.77 (m, 1H), 1.43–1.64 (m, 3H), 1.29 (dd, J = 14.4 and 3.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 146.0, 145.7, 130.3, 126.2, 118.8, 108.9, 81.6, 57.8 (2C), 48.9, 47.6, 45.2, 42.8, 42.7, 38.3, 37.6, 35.3, 27.3, 24.2. Positive ESI-MS m/z: 333 $[M+H]^+$, positive HRESIMS m/z: found: 333.2157 $[M+H]^+$ (calcd for $C_{19}H_{29}N_2O_3$, 333.2178). $[\alpha]_D^{25} = +14.75$ (c 0.61, CH_3OH).

4.1.2.7. 6β-NH-Ts-7β-Methoxy-7,8-dihydro sinomenine (4). To a solution of **8** (332 mg, 1 mmol) in dried CH_2Cl_2 (20 mL) was added p-toluene sulfonyl chloride (228 mg, 1.2 mmol) and Et_3N (0.21 mL, 1.5 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and after 5 h was quenched with water (5 mL), added more CH_2Cl_2 (50 mL) into the system. Then the organic layer was separated, washed with brine (20 mL × 5), dried over Na_2SO_4, filtered and concentrated. Purification by column chromatography (CH_2Cl_2/MeOH, 9:1, v/v) afforded the titled compound as a white solid (467 mg, 96.1%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.66 (d, J = 8.1 Hz, 2H), 7.23 (d, J = 8.1 Hz, 2H), 6.73 (d, J = 8.4 Hz, 1H), 6.62 (d, J = 8.4 Hz, 1H), 5.85 (br s, 1H), 4.11 (d, J = 8.7 Hz, 1H), 3.93–3.97 (m, 1H), 3.88 (s, 3H), 3.79 (dd, J = 14.7 and 3.3 Hz, 1H), 3.21 (dt, J = 12.0 and 4.2 Hz, 1H), 2.97 (s, 3H), 2.91 (d, J = 18.3 Hz, 1H), 2.82–2.85 (m, 1H), 2.67 (dd, J = 18.3 and 5.1 Hz, 1H), 2.40–2.44 (overlapped, 1H), 2.40 (s, 3H), 2.34 (s, 3H), 1.97 (td, J = 12.3 and 3.0 Hz, 1H), 1.85 (dt, J = 12.9 and 2.4 Hz, 1H), 1.73 (dt, J = 12.9 and 2.4 Hz, 1H), 1.59 (dt, J = 12.9 and 3.0 Hz, 1H), 1.22–1.50 (m, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.0, 144.5, 142.4, 139.0, 130.5, 129.0 (2C), 126.9 (2C), 124.3, 119.3, 109.7, 79.2, 57.1, 56.4, 55.7, 51.0, 47.1, 44.6, 42.4, 37.8, 37.4, 35.3, 28.9, 23.7, 21.4. Positive ESI-MS (+) m/z: 487 $[M+H]^+$, positive HRESIMS m/z: found: 487.2229 $[M+H]^+$ (calcd for $C_{26}H_{35}N_2O_5S$, 487.2267). $[\alpha]_D^{25} = +67.21$ (c 0.60, CH_3OH).

4.1.2.8. (*S*)-6β-NH-Ts-7β-Methoxy-7,8-dihydro disinomenine (4a) and (*R*)-6β-NH-Ts-7β-methoxy-7,8-dihydro disinomenine (4b). The title compounds **4a** and **4b** were prepared according the procedure described above. Specifically, 6β-NH-Ts-7β-methoxy-7,8-dihydro sinomenine (**4**, 486 mg, 1.0 mmol) was dissolved in dried CH_3OH (40 mL) and MnO_2 (1500 mg, 17.2 mmol) was added with magnetic stirring, and the mixture was remained at room temperature for 3 days and then filtered and washed by CH_3OH (10 mL × 3). The filtrate and the washes were combined, dried over anhydrous $MgSO_4$ and concentrated. The resulting yellow solid was purified using silica gel column chromatography with chloroform/methanol (15:1–10:1, v/v) as eluent to yield **4a** (160 mg, 33%) and **4b** (204 mg, 42%).

Compound **4a**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.74 (dd, J = 6.6 and 1.8 Hz, 4H), 7.30 (dd, J = 8.4 and 0.2 Hz, 4H), 6.66 (s, 2H), 4.24 (d, J = 9.0 Hz, 2H), 4.00 (td, J = 7.5 and 4.5 Hz, 2H), 3.94 (s, 6H), 3.88 (d, J = 3.3 Hz, 2H), 3.26 (td, J = 7.5 and 3.6 Hz, 2H), 3.04 (s, 6H), 2.72 (br d, J = 4.2 Hz, 2H), 2.43 (s, 6H), 2.32–2.41 (m, 2H), 2.25 (s, 6H), 2.09–2.15 (m, 4H), 2.03 (d, J = 7.2 Hz, 4H), 1.90 (d, J = 12.6 Hz, 2H), 1.76 (d, J = 12.0 Hz, 2H), 1.57–1.62 (m, 4H), 1.49 (dd, J = 13.2 and 4.8 Hz, 2H), 1.42 (d, J = 12.3 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.2, 143.8, 142.7, 138.9, 131.5, 129.2 (2C), 127.8, 127.1 (2C), 124.7, 112.0, 79.4, 57.0, 56.1, 51.0, 47.0, 44.4, 42.6, 38.0, 37.8, 35.8, 31.9, 29.7, 23.7, 22.7, 21.5. Positive ESI-MS m/z: 971 $[M+H]^+$, positive HRESIMS m/z: found: 971.4275$[M+H]^+$ (calcd for $C_{52}H_{67}N_4O_{10}S_2$, 971.4299). $[\alpha]_D^{25} = +121.67$ (c 0.60, CH_3OH). CD (c = 0.1 mM, CH_3OH): θ_{213} +10.68 mdeg, θ_{258} +4.17 mdeg, θ_{300} +0.87 mdeg.

Compound **4b**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.67 (d, J = 8.4 Hz, 4H), 7.27 (d, J = 8.4 Hz, 4H), 6.60 (s, 2H), 4.12 (d, J = 6.6 Hz, 2H), 3.94 (dd, J = 15.3 and 3.6 Hz, 2H), 3.89 (s, 6H), 3.76 (dd, J = 6.3 and 3.0 Hz, 2H), 3.37 (br t, J = 3.6 Hz, 2H), 3.21 (td, J = 8.1 and 3.6 Hz, 2H), 2.94 (br d, J = 13.5 Hz, 2H), 2.86 (s, 6H), 2.66 (br d, J = 18.6 Hz, 2H), 2.53 (s, 6H), 2.41 (s, 6H), 2.32 (br t, J = 6.0 Hz, 4H), 2.10 (d, J = 13.5 Hz, 2H), 1.77 (td, J = 12.9 and 3.9 Hz, 2H), 1.65 (d, J = 12.9 Hz, 2H), 1.18–1.34 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 146.9, 144.6, 143.4, 140.3, 131.6, 129.7 (2C), 129.0, 127.4 (2C), 126.1, 123.8, 111.0, 58.9, 56.9, 56.0, 50.4, 48.7, 41.3, 40.8, 36.1, 34.4, 29.8, 27.9, 22.8, 21.7, 19.3. Positive ESI-MS m/z: 971 $[M+H]^+$, positive HRESIMS m/z: found: 971.4289 $[M+H]^+$ (calcd for $C_{52}H_{67}N_4O_{10}S_2$, 971.4299). $[\alpha]_D^{25} = +120.51$ (c 0.72, CH_3OH). CD (c = 0.1 mM, CH_3OH): θ_{229} +18.19 mdeg, θ_{293} −1.71 mdeg.

4.1.2.9. 6β-NH-Ms-7β-Methoxyl-7,8-dihydro-sinomenine (5). To a solution of **8** (332 mg, 1 mmol) in dried CH_2Cl_2 (20 mL) was added mesyl chloride (137 mg, 1.2 mmol) and Et_3N (0.21 mL, 1.5 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature and quenched with water (5 mL), added more CH_2Cl_2 (50 mL) into the system 5 h later. Then the organic layer was separated, washed with brine (20 mL × 5), dried over Na_2SO_4, filtered and concentrated. Purification by silica gel column chromatography (CH_2Cl_2/MeOH, 10:1, v/v) afforded the title compound as a white solid (377 mg, 92%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.75 (d, J = 8.4 Hz, 1H), 6.64 (d, J = 8.4 Hz, 1H), 6.32 (s, 1H), 4.08 (br s, 2H), 3.83–3.89 (m, 1H), 3.86 (s, 3H), 3.37 (s, 3H), 3.27–3.33 (m, 1H), 2.95 (d, J = 18.3 Hz, 1H), 2.86 (s, 3H), 2.70 (dd, J = 18.3 and 5.4 Hz, 1H), 2.45 (dd, J = 12.3 and 3.0 Hz, 1H), 2.37 (s, 3H), 1.99 (td, J = 16.1 and 3.0 Hz, 1H), 1.87–1.93 (m, 1H), 1.67–1.80 (m, 2H), 1.51 (td, J = 12.6 and 4.5 Hz, 1H), 1.31–1.43 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.1, 144.6, 130.6, 124.2, 119.5, 110.0, 79.9, 57.3, 56.7, 56.2, 52.1, 47.2, 44.7, 42.5, 42.1, 38.0, 37.9, 35.5, 28.2, 23.9. Positive ESI-MS m/z: 411 $[M+H]^+$, positive HRESIMS m/z: found: 411.1922 $[M+H]^+$ (calcd for $C_{20}H_{31}N_2O_5S$, 411.1954). $[\alpha]_D^{25} = +27.91$ (c 0.43, $CHCl_3$).

4.1.2.10. (*S*)-6β-NH-Ms-7β-Methoxy-7,8-dihydro disinomenine (5a) and (*R*)-6β-NH-Ms-7β-methoxy-7,8-dihydro disinomenine (5b). 6β-NH-Ms-7β-Methoxy-7,8-dihydro sinomenine (**8**, 410 mg, 1.0 mmol) was dissolved in dried CH_3OH (40 mL) and MnO_2 (1500 mg, 17.2 mmol) was added with magnetic stirring, and the mixture was remained at room temperature for 3 days and then filtered and washed by CH_3OH (10 mL × 3). The filtrate and the

washes were combined, dried over anhydrous $MgSO_4$ and concentrated. The resulting yellow solid was purified using silica gel column chromatography with chloroform/methanol (15:1, v/v) as eluent to afford **9a** (147 mg, 36%) and **9b** (164 mg, 40%).

Compound **5a**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.64 (s, 2H), 4.20 (d, J = 12.0 Hz, 2H), 4.10 (br t, J = 4.2 Hz, 2H), 3.97 (d, J = 14.8 and 3.3 Hz, 2H), 3.90 (s, 6H), 3.41 (s, 6H), 3.33–3.39 (m, 2H), 2.91 (s, 6H), 2.79 (s, 2H), 2.39–2.48 (m, 4H), 2.29 (s, 6H), 2.15 (t, J = 9.3 Hz, 2H), 1.95 (d, J = 12.9 Hz, 2H), 1.83 (d, J = 12.6 Hz, 2H), 1.72 (d, J = 12.9 Hz, 2H), 1.59 (td, J = 12.9 and 4.2 Hz, 2H), 1.35–1.43 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.5, 143.7, 132.1, 127.7, 124.7, 110.6, 79.9, 57.2, 56.6, 56.5, 52.0, 47.5, 43.9, 42.4, 42.1, 37.7, 37.4, 35.8, 28.1, 22.2. Positive ESI-MS m/z: 819 $[M+H]^+$, positive HRESIMS m/z: found: 819.3644 $[M+H]^+$ (calcd for $C_{40}H_{59}N_4O_{10}S_2$, 819.3673). $[\alpha]_D^{25} = +116.13$ (c 0.43, CH_3OH). CD (c = 0.1 mM, CH_3OH): θ_{205} +14.98 mdeg, θ_{257} +10.27 mdeg, θ_{297} +12.48 mdeg.

Compound **5b**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ: ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 6.57 (s, 2H), 4.09 (br s, 4H), 3.94 (d, J = 14.4 Hz, 2H), 3.87 (s, 6H), 3.39 (s, 6H), 3.33 (d, J = 12.0 Hz, 2H), 2.91 (s, 2H), 2.87 (s, 6H), 2.57–2.63 (m, 4H), 2.29(s, 6H), 2.08–2.15 (m, 2H), 1.99 (br d, J = 14.4 Hz, 2H), 1.89 (d, J = 12.6 Hz, 2H), 1.72 (d, J = 12.6 Hz, 2H), 1.62 (td, J = 12.2 and 4.2 Hz, 2H), 1.43 (d, J = 13.8 Hz, 2H), 1.24–1.36 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 145.3, 144.0, 131.4, 127.7, 124.5, 112.1, 79.6, 59.6, 57.1, 56.5, 52.0, 47.0, 43.8, 42.5, 42.0, 38.1, 37.7, 35.8, 28.4, 23.9. Positive ESI-MS m/z: 819 $[M+H]^+$, HRESIMS m/z: found: 819.3652 $[M+H]^+$ (calcd for $C_{40}H_{59}N_4O_{10}S_2$, 819.3673). $[\alpha]_D^{25} = +101.38$ (c 0.61, CH_3OH). CD (c = 0.1 mM, CH_3OH): θ_{211} +23.00 mdeg, θ_{290} −1.67 mdeg.

4.1.2.11. 6,7-Quinoxaline sinomenine (6). Sinomenine (9.9 g, 0.03 mol) was dissolved in 10% HCl (50 mL) under N_2 and stirred at reflux. After 10 h, the mixture was cooled to ambient temperature followed by base workup (pH 10) with 15% ammonia water and then extracted with CH_2Cl_2 (80 mL × 3). The CH_2Cl_2 extract was washed with brine, dried over Na_2SO_4, filtered, concentrated and recrystallized from MeOH to give a flavescent solid **9** (7.65 g, 81%). A solution of compound **9** (315 mg, 1 mmol) and *o*-phenylenediamine (130 mg, 1.2 mmol) in MeOH (30 mL) was stirred under N_2 atmosphere for 12 h. The mixture was filtered and washed with MeOH (10 mL × 3), and the filter cake was collected as a white solid (329 mg, 85%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.98–8.01 (m, 1H), 8.84–8.87 (m, 1H), 7.56–7.63 (m, 2H), 6.59 (s, 1H), 6.58 (s, 1H), 6.07 (s, 1H), 5.02 (dd, J = 15.0 Hz, 1H), 3.70 (s, 3H), 3.25 (dd, J = 6.0 Hz, 1H), 3.19 (dd, J = 6.0 Hz, 1H), 3.08 (brs, 1H), 2.88–3.02 (m, 3H), 2.58–2.62 (m, 2H), 2.48 (s, 3H), 2.20 (td, J = 12.0 and 3.0 Hz, 1H), 2.10 (d, J = 12.0 Hz, 1H), 1.94 (td, J = 12.0, and 6.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 153.9, 153.5. 144.7, 144.5, 141.5, 131.1, 128.8, 128.8 (2C), 128.3, 123.1, 118.8, 109.0, 56.7, 56.0, 47.0. 44.6, 43.1, 42.8, 38.2, 36.3, 33.7, 23.4. Positive ESI-MS m/z: 388 $[M+H]^+$, positive HRESIMS-ESI m/z: found: 388.2000 $[M+H]^+$ (calcd for $C_{24}H_{26}N_3O_2$, 388.2025).

4.1.2.12. (*S*)-6,7-Quinoxaline disinomenine (6a). After 4 days of incubation of mycelia in mineral medium (50 mL, consists of potato extract 200 g, glucose 20 g, KH_2PO_4 3.0 g, $MgSO_4{\cdot}7H_2O$ 1.5 g, trace vitamin B_1, per liter, and with pH 6.0) on a rotary shaker at 160 rpm at 25 °C, 6,7-quinoxaline sinomenine (**6**, 387 mg, 1.0 mmol) was added into the fermentation flask, and the mixture was maintained under the same conditions as described above for an additional 4 days. Mycelia were harvested by filtration, and then the filtrate was adjusted to pH 9 by NH_4OH, extracted with CH_2Cl_2 (50 mL × 3), dried over anhydrous Na_2SO_4, filtrated and then concentrated under vacuum to afford the crude yellow product, which was purified using silica gel column chromatography with chloroform/methanol (15:1, v/v) as eluent to yield **6a** (243 mg, 63%).

Compound **6a**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.00 (br t, J = 3.0 Hz, 2H), 7.87 (br t, J = 3.0 Hz, 2H), 7.61 (dd, J = 6.0 and 3.0 Hz, 4H), 6.33 (s, 2H), 5.13 (dd, J = 15.0 Hz, 2H), 3.69 (s, 6H), 3.21 (dd, J = 18.0 and 6.0 Hz, 2H), 2.96–3.07(m, 6H), 2.53–2.65 (m, 6H), 2.38 (s, 6H), 2.21–2.33 (m, 2H), 2.19 (d, J = 12.0 Hz, 2H), 2.00 (td, J = 12.0 and 6.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 154.0, 153.3, 145.0, 143.8, 141.6, 141.4, 131.2, 128.9, 128.8 (2C), 128.4, 128.1, 123.6, 110.4, 56.3 (2C), 47.1, 44.9, 43.3, 42.8, 38.7, 36.7, 33.9, 23.0. Positive ESI-MS m/z: 773 $[M+H]^+$, HRESIMS m/z: found: 773.3788 $[M+H]^+$ (calcd for $C_{48}H_{49}N_6O_4$, 773.3815). $[\alpha]_D^{25} = +359.22$ (c 0.42, $CHCl_3$). CD (c = 0.05 mM, CH_3OH): θ_{208} +17.46 mdeg, θ_{244} +20.00 mdeg, θ_{292} +4.15 mdeg.

4.2. Biological assay

4.2.1. RAW264.7 cell culture

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

In all cell cultures, each compound was prepared in dimethyl sulphoxide (DMSO) followed by dilution with culture medium to desired 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.

4.2.2. Measurement of nitrite levels in the culture supernatants

RAW264.7 cells were plated at a density of 5×10^4 cells in a 96-well plate, and compounds at various concentrations (0.5–200 μM) were added to each plate with or without LPS (1 μg/mL). 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. Aminoguanidine (AG) was used as positive control.

4.2.3. Cell viability assay

Cells were plated at a density of 5×10^4 cells in a 96-well plate, and compounds were added to each plate at the indicated concentrations. After a 24 h incubation period, 20 μL MTT reagent (5 mg/mL) was added, and the cells were incubated for 4 h. The supernatants were aspirated, and the formazan crystals in each well were dissolved in 200 μL of dimethyl sulfoxide for 30 min at 37 °C. The absorbance value was monitored by microplate reader at 570 nm and the cytotoxicity (%) was calculated using the following formula:

$$\text{The cytotoxicity } (\%) = [1 - (\text{Compounds } (OD_{570\,nm}) - \text{Background } (OD_{570\,nm}))/(\text{Control } (OD_{570\,nm}) - \text{Background } (OD_{570\,nm}))] \times 100$$

The potency results were expressed as the 50% inhibitory concentration (IC_{50}).

4.2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression

To examine iNOS mRNA expression levels in RAW264.7 cells, total RNA was extracted using the Trizol reagent, and cDNA was synthesized from 1 μg RNA utilizing M-MLV reverse transcriptase according to manufacturer's instruction (TOYOBO, JAPAN). The

PCR reaction was performed by the following reaction conditions: 94 °C for 5 min and 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with a final elongation step of 72 °C for 5 min. The PCR products were run on a 1% agarose gel and were visualized by ethidium bromide staining. The produced bands in the gel were then photographed. The primer sequences used in this study were as follows: iNOS: forward, 5′-CAACATCAGGTCGGCCATCACT-3′; reverse, 5′-ACCAGAG GCAGCACATCAAAGC-3′; β-actin: forward, 5′-TGCTGTCCCTGTATGCCTCT-3′; reverse, 5′-TTTGATGTCACG CAC GATTT-3′.

Acknowledgments

This work was supported by National Natural Science Foundation of China (20872060, 90913023), the 973 Program (2007CB714504) and the National Natural Science Fund for Creative Research Groups of China (20821063). We thank Dr. Yin Ding (Nanjing University, China) for HRMS measurements.

Supplementary data

Supplementary data (CD spectra of dimers, ^{1}H NMR spectra and HPLC analysis results of the compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.006. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Bruckdorfer, R. *Mol. Aspects Med.* **2005**, *26*, 3.
2. Bruene, B. *Pharmacol. Res.* **2010**, *61*, 265.
3. Huang, Z. H.; Huang, P. L.; Panahian, N.; Dalkara, T.; Fishman, M. C.; Moskowitz, M. A. *Science* **1994**, *265*, 1883.
4. Huang, P. L.; Huang, Z. H.; Mashimo, H.; Bloch, K. D.; Moskowitz, M. A.; Bevan, J. A.; Fishman, M. C. *Nature* **1995**, *377*, 239.
5. Groves, J. T.; Wang, C. C. Y. *Curr. Opin. Chem. Biol.* **2000**, *4*, 687.
6. Maccallini, C.; Patruno, A.; Besker, N.; Ali, J. I.; Ammazzalorso, A.; De Filippis, B.; Franceschelli, S.; Giampietro, L.; Pesce, M.; Reale, M.; Tricca, M. L.; Re, N.; Felaco, M.; Amoroso, R. *J. Med. Chem.* **2009**, *52*, 1481.
7. Vallance, P. *Fund. Clin. Pharmacol.* **2003**, *17*, 1.
8. Smolen, J. S.; Steiner, G. *Nat. Rev. Drug Disc.* **2003**, *2*, 473.
9. Strand, V.; Kimberly, R.; Isaacs, J. D. *Nat. Rev. Drug Disc.* **2007**, *6*, 75.
10. Ohtsuka, M.; Konno, F.; Honda, H.; Oikawa, T.; Ishikawa, M.; Iwase, N.; Isomae, K.; Ishii, F.; Hemmi, H.; Sato, S. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 52.
11. Hu, X. D.; Yang, Y.; Zhong, X. G.; Zhang, X. H.; Zhang, Y. N.; Zheng, Z. P.; Zhou, Y.; Tang, W.; Yang, Y. F.; Hu, L. H.; Zuo, J. P. *J. Ethnopharmacol.* **2008**, *120*, 447.
12. Van't Hof, R. J.; Ralston, S. H. *Immunology* **2001**, *103*, 255.
13. Xu, M.; Liu, L.; Qi, C.; Deng, B.; Cai, X. *Planta Med.* **2008**, *74*, 1423.
14. Wang, Y.; Fang, Y. F.; Huang, W. H.; Zhou, X.; Wang, M. H.; Zhong, B.; Peng, D. Z. *J. Ethnopharmacol.* **2005**, *98*, 37.
15. Huang, B. D.; He, A. S.; Fu, M.; Sheng, P. Y.; Liao, W. M. *J. Med. Plants Res.* **2010**, *4*, 1830.
16. Liu, Z. Q.; Chan, K.; Zhou, H.; Jiang, Z. H.; Wong, Y. F.; Xu, H. X.; Liu, L. *Life Sci.* **2005**, *77*, 3197.
17. Song, S. H.; Shen, X. Y.; Tang, Y.; Wang, Z. X.; Guo, W. Y.; Ding, G. S.; Wang, Q. X.; Fu, Z. R. *Int. Immunopharmacol.* **2010**, *10*, 679.
18. Huang, G. D.; Li, J. B.; Huang, Y. H.; Huang, D. F.; Tang, L. J.; Zhang, S.; Li, Q. *J. Emerg. Tradit. Chin. Med.* **2007**, *16*, 416.
19. Yamasaki, H. *Acta Med. Okayama* **1976**, *30*, 1.
20. Goto, K. S. H. *Bull. Chem. Soc. Jpn.* **1929**, *4*, 107.
21. Goto, K. *Bull. Chem. Soc. Jpn.* **1929**, *4*, 129.
22. Zuo, Z.; Jimenez, X.; Witte, L.; Zhu, Z. P. *Protein Eng.* **2000**, *13*, 361.
23. Amara, J. F.; Clackson, T.; Rivera, V. M.; Guo, T.; Keenan, T.; Natesan, S.; Pollock, R.; Yang, W.; Courage, N. L.; Holt, D. A.; Gilman, M. P. *Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10618.
24. Deng, Z. S.; Li, J. X.; Teng, P.; Li, P.; Sun, X. R. *Org. Lett.* **2008**, *10*, 1119.
25. Deng, Z. S.; Zhao, Y.; He, C. C.; Jin, J.; He, Y. M.; Li, J. X. *Org. Lett.* **2008**, *10*, 3879.
26. Minamikawa, J. I.; Tijima, I.; Brossi, A. *Heterocycles* **1978**, *10*, 79.
27. Goto, K. I. R. *Chem. Pharm. Bull.* **1931**, 93.
28. Christians, U. K., V. W. U.S. Patent, 6372756B1, 2002.
29. Cheng, W. M.; Qiu, F.; Yao, X. S. *J. Asian Nat. Prod. Res.* **2007**, *9*, 13.
30. McCamley, K.; Ripper, J. A.; Singer, R. D.; Scammells, P. J. *J. Org. Chem.* **2003**, *68*, 9847.
31. Iijima, I.; Minamikawa, J. I.; Jacobson, A. E.; Brossi, A.; Rice, K. C. *J. Org. Chem.* **1978**, *43*, 1462.
32. Sayre, L. M.; Portoghese, P. S. *J. Org. Chem.* **1980**, *45*, 3366.
33. Weller, D. D.; Rapoport, H. *J. Med. Chem.* **1976**, *19*, 1171.
34. Bachi, M. D.; Korshin, E. E.; Hoos, R.; Szpilman, A. M.; Ploypradith, P.; Xie, S. J.; Shapiro, T. A.; Posner, G. H. *J. Med. Chem.* **2003**, *46*, 2516.
35. Portoghese, P. S.; Nagase, H.; Maloneyhuss, K. E.; Lin, C. E.; Takemori, A. E. *J. Med. Chem.* **1991**, *34*, 1715.
36. Li, J. F.; Zhao, Y.; Cai, M. M.; Li, X. F.; Li, J. X. *Eur. J. Med. Chem.* **2009**, *44*, 2796.
37. Goto, K. S. H. *Bull. Chem. Soc. Jpn.* **1935**, *10*, 597.
38. Lou, Y. T.; Zhou, H. B.; Zou, J.; Yan, L. C.; Bi, E. G.; Sun, B.; Yao, Z. J. *Tetrahedron Lett.* **2010**, *51*, 485.
39. Xie, Q. W.; Whisnant, R.; Nathan, C. *J. Exp. Med.* **1993**, *177*, 1779.
40. Ciz, M.; Pavelkova, M.; Gallova, L.; Kralova, J.; Kubala, L.; Lojek, A. *Physiol. Res.* **2008**, *57*, 393.
41. Kang, K. S.; Yamabe, N.; Kim, H. Y.; Okamoto, T.; Sei, Y.; Yokozawa, T. *J. Ethnopharmacol.* **2007**, *113*, 225.