Full Paper

Synthesis and Biological Activities of 2,4-Diaminopteridine Derivatives

Fei Ma, Gang Lü, Wei-Fen Zhou, Qiu-Juan Wang, Yi-Hua Zhang, and Qi-Zheng Yao

School of Pharmacy, China Pharmaceutical University, Nanjing, P. R. China

Substituted 2,4-diaminopteridine derivatives **10a–10l** were prepared in moderate to good yield. Their structures were confirmed by ^{1}H-NMR and MS spectroscopy, as well as by elemental analysis. Their inhibitory properties against inducible nitric oxide synthase (iNOS) were evaluated *in vitro*. Biological tests indicated that compound **10a**, **10d**, **10e**, **10h**, **10i**, and **10l** showed potent inhibitory activities similar to that of methotrexate (MTX), while the activities of compound **10b**, **10c**, **10f**, **10g**, **10j**, and **10k** are stronger than MTX. Two compounds, i. e., **10b** (IC_{50} = 18.85 μM) and **10i** (IC_{50} = 24.08 μM) were further studied for their effect on septic shock in rats and immunologically liver injured mice (*in vivo*). The results demonstrated that **10b** and **10i** had the capacity to increase the blood pressure in septic shock and showed notable protective activities on immunological hepatic injury.

Keywords: Inhibitor of nitric oxide synthase / Synthesis / Tetrahydrobiopterin

Received: December 9, 2008; accepted: December 12, 2008

DOI 10.1002/ardp.200800192

Introduction

Nitric oxide (NO) is a mediator with protean functions. It is involved in signalling in the cardiovascular, gastrointestinal, genitourinary, respiratory, and nervous systems, and disordered NO generation has been implicated in a wide range of diseases [1]. Nitric oxide synthase (NOS) catalyzes the oxidation of a guanidino N-atom of L-arginine to NO with concomitant formation of L-citrulline [2]. A critical aspect of NOS function is the requirement for the cofactor tetrahydrobiopterin (BH_4). Maintenance and stabilization of NOS dimers is dependent on BH_4, and BH_4 also plays a direct role in the multistep oxidation of arginine through the *N*-hydroxy-L-arginine intermediate and the subsequent generation of NO [3]. Thus, the BH_4 binding site of NOS may be an ideal target for selective pharmacological intervention [4].

Some structural analogs of BH_4 have been shown to inhibit the various forms of NOS, for example, the 4-aminopteridine nucleus, and the substitution pattern at the 2-, 4-, 5-, 6-, and 7-position was systematically varied [5]. Encouraged by these findings, we designed and synthesized a series of analogues (**10a–10l**) of 2,4-diamino pteridine, and determined the effects that twelve derivatives inhibited inducible nitric oxide synthase (iNOS). In addition, compounds **10b** and **10i** were tested for their capacity of increasing the blood pressure in septic-shock rats and their protective effects on immunological hepatic injured mice.

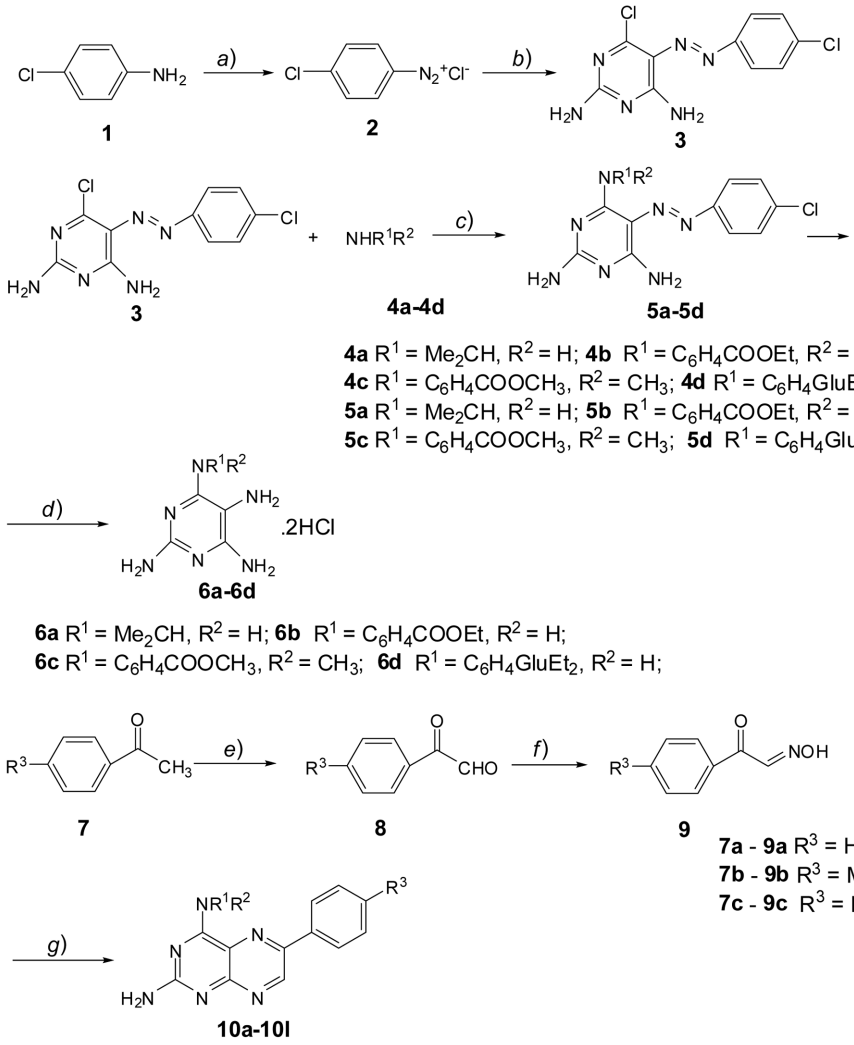
Results and discussion

Synthesis

Compounds **10a–10l** were prepared according to Scheme 1. The general principle for the synthetic approach of 2-amino-4-arylamino / isopropylamino-6-arylpteridines was based on the condensation of 2,5,6-triamino-4-arylamino / isopropylaminopyrimidine derivatives **6a–6d** with different substituted phenylglyoxal-

Correspondence: Qi-Zheng Yao, School of Pharmacy, China Pharmaceutical University, Nanjing 210009, P. R. China.
E-mail: qz_yao@yahoo.com.cn
Fax: +86 25 86634730

Abbreviations: aminoguanidine (AG); ceral ligation and perforation (CLP); cyclophosphamide (CY); glutamate pyruvate transaminase (GPT); glutamic oxalacetic transaminase enzyme (GOT); hepatic necrosis (HN); inducible nitric oxide synthase (iNOS); methotrexate (MTX); nitric oxide (NO); nitric oxide synthase (NOS)



4a R^1 = Me_2CH, R^2 = H; **4b** R^1 = C_6H_4COOEt, R^2 = H
4c R^1 = $C_6H_4COOCH_3$, R^2 = CH_3; **4d** R^1 = $C_6H_4GluEt_2$, R^2 = H
5a R^1 = Me_2CH, R^2 = H; **5b** R^1 = C_6H_4COOEt, R^2 = H
5c R^1 = $C_6H_4COOCH_3$, R^2 = CH_3; **5d** R^1 = $C_6H_4GluEt_2$, R^2 = H

6a R^1 = Me_2CH, R^2 = H; **6b** R^1 = C_6H_4COOEt, R^2 = H;
6c R^1 = $C_6H_4COOCH_3$, R^2 = CH_3; **6d** R^1 = $C_6H_4GluEt_2$, R^2 = H;

7a - **9a** R^3 = H
7b - **9b** R^3 = Me
7c - **9c** R^3 = MeO

10a NR^1R^2 = $HNCH(Me)_2$, R^3= H; **10b** NR^1R^2 = HNC_6H_4COOEt, R^3 = H;
10c NR^1R^2 = $MeNC_6H_4COOCH_3$, R^3= H; **10d** NR^1R^2 = $NHC_6H_4COGluEt_2$, R^3 = H;
10e NR^1R^2 = $HNCH(Me)_2$, R^3= Me; **10f** NR^1R^2 = HNC_6H_4COOEt, R^3 = Me;
10g NR^1R^2 = $MeNC_6H_4COOCH_3$, R^3= Me; **10h** NR^1R^2 = $NHC_6H_4COGluEt_2$, R^3 = Me;
10i NR^1R^2 = $HNCH(Me)_2$, R^3= OMe; **10j** NR^1R^2 = HNC_6H_4COOEt, R^3 = OMe;
10k NR^1R^2 = $MeNC_6H_4COOCH_3$, R^3 = OMe; **10l** NR^1R^2 = $NHC_6H_4COGluEt_2$, R^3 = OMe;

Reactions and conditions: a) $NaNO_2$, 22.0% HCl, Urea, 0–5°C, 0.5 h; b) 4-chloro-2,6-diaminopyrimidine, CH_3COONa, H_2O, r.t., 16 h, 81%; c) DMF, 0–5°C, 5 h, 86–93%; d) (1) EtOH, 5% HCl, zinc powder, 70°C, 0.5 h; (2) 70°C, 1 h, 78–85%; e) 1. SeO_2, 1,4-dioxane / H_2O (25 : 1), 50°C, 0.5 h; 2. reflux, 30 h, 62–68%; f) MeOH, H_2O, 3.6% HCl, pH 3–4, 60°C, 5 h, 56–64%; g) MeOH, N_2, reflux, 4 h, 30–62%.

Scheme 1. The synthesis of substituted 2,4-diaminopteridine derivatives.

monoximes **9a**–**9c** under refluxing MeOH [6]. *p*-Chloroaniline **1** was diazotized with $NaNO_2$ and 22% HCl, and subsequent coupling with 2,6-diamino-4-chloropyrimidine **2** gave **3** in 81% yield [7]. In **3**, the Cl-atom of the pyrimidine ring is sufficiently activated to be nucleophilically displaced by acyclic or arylic amines **4a**–**4d** in dehydrated alcohol to form **5a**–**5d**, which, on reduction with Zn powder, generated **6a**–**6d** [9]. The yields of these two steps were in the range of 86–93% and 78–88%, respectively.

Acetophenone derivatives **7a**–**7c** were treated with SeO_2 in 1,4-dioxane / H_2O (25 : 1) to provide the corresponding **8a**–**8c** in 62–68% yield; then, **8a**–**8c** treated with acetone oxime [10] in MeOH (pH 3–4) at 50°C led to **9a**–**9c** in 56–64% yields [8]. Finally, boiling a solution of the 2,5,6-triamino-4-arylamino / isopropylaminopyrimi-

Table 1. Structures and yields of substances **10a**–**10l**.

Compound	R^3	NR^1R^2	Yield (%)
10a	H	$HNCH(Me)_2$	62
10b	H	HNC_6H_4COOEt	35
10c	H	$MeNC_6H_4COOCH_3$	37
10d	H	$HNC_6H_4COGluEt_2$	32
10e	Me	$HNCH(Me)_2$	59
10f	Me	HNC_6H_4COOEt	32
10g	Me	$MeNC_6H_4COOCH_3$	51
10h	Me	$HNC_6H_4COGluEt_2$	30
10i	MeO	$HNCH(Me)_2$	33
10j	MeO	HNC_6H_4COOEt	35
10k	MeO	$MeNC_6H_4COOCH_3$	34
10l	MeO	$HNC_6H_4COGluEt_2$	40

Table 2. Inhibitive activities (IC_{50} in (M) of Pteridine derivatives **10a**–**10l** against iNOS.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
10a	31.01 ± 1.90	**10g**	16.77 ± 0.65
10b	18.85 ± 0.82	**10h**	37.21 ± 0.63
10c	18.22 ± 1.71	**10i**	24.08 ± 0.81
10d	42.37 ± 1.82	**10j**	14.02 ± 1.42
10e	28.14 ± 1.73	**10k**	15.08 ± 1.20
10f	16.85 ± 0.52	**10l**	33.77 ± 2.40
MTX	24.59 ± 1.43		

[a] Methotrexate (MTX) used as a positive control.

[b] Inhibition of BH_4 (2 μM) stimulated NOS total activity at an inhibitor concentration of 100 μM.
The data were the mean ± SD obtained from three independent experiments.

dines hydrochloride **6a**–**6e** with **9a**–**9c** gave the corresponding 2-amino-4-arylamino/isopropylamino-6-aryl pteridines **10a**–**10l** in yields in the range of 30–62% (see Table 1) [9].

The structures of the newly synthesized compounds were characterized by ^{1}H-NMR and MS spectroscopy, as well as by elemental analysis.

Pharmacology

All of the prepared compounds **10a**–**10l** were evaluated for their inhibitive activities against iNOS according to the reported methods [10], and the results are listed in the Table 2.

The data in Table 2 indicated that **10e** and **10i** exhibited potent inhibitory activities similar to that of Methotrexate (MTX), while the activities of **10b**, **10c**, **10f**, **10g**, **10j**, and **10k** are stronger than that of MTX [10]. As compared with 6-phenyl-pteridine derivatives **10a**–**10d**, the corresponding 6-(4-methylphenyl)pteridine derivatives **10e**–**10h** showed a slight increase in inhibitive activities against iNOS, respectively, while 6-(4-methoxyphenyl)pteridine derivatives **10i**–**10l** had significantly more inhibitive activity than the former two series.

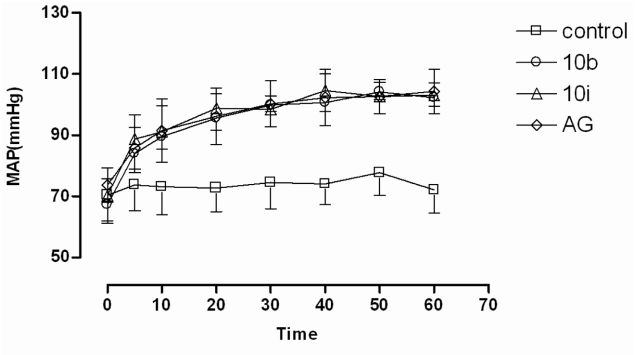


Figure 1. Effect of compound aminoguanidine (AG), **10b**, and **10i** on septic-shock rats.

Pharmacological results suggest that modification at the 4-amino substituents of pteridine by alkyl / benzoate appeared crucial for their bioactivities. The compounds with [*p*-(ethoxylcarbonyl)phenyl]amino or [*p*-(methoxycarbonyl)phenyl]methylamino functional groups in the 4-position displayed more potent inhibitive activities than those with isopropylamino or {*p*-[(*N*-L-glutamate)carbonyl]phenyl}amino groups. In the structure of the murine iNOS oxygenase domain, the BH_4 is axially co-oridinated to the haem and hydrogen bond bridges from the BH_4 N(3) (directly) and O(4) (through a H_2O molecule) through heme propionate to the L-Arg α-amino group [11, 12]. The cavity and the electron of the pterin with aminobenzoate may perhaps more easily interfere with hydrogen bonds between the BH_4 and the heme propionate.

Based on the *in-vitro* inhibitive activities of BH_4 derivatives against iNOS, compound **10b** and **10i** were selected for the determination of septic shock in rats and immunological liver injured mice. Septic shock was induced by topical application of ceral ligation and perforation (CLP) [13]. 12 h after laparotomy, all CLP animals demonstrated reduced activity, piloerection, and exudation around the eyes and nose. Generalized peritonitis was confirmed *post mortem* in all CLP animals. As shown in Fig. 1, **10b** and **10i** demonstrated a similar effect as aminoguanidine (AG) in elevating the MBP (mean blood pressure) significantly (compare control group $P < 0.01$, Fig. 1), while the MBP of the control rats tended to decline.

Immunological liver injury was induced by tail-vein injection of 0.25 mg/10g *Corynebacterium parvum* (CP), and ten day later, the mice became aggressive, irritable, they hit the cage, and nibble on the forage and trough; they are restless. [14]. Mucous membranes swelled all over the body and multi-ulcer developed, especially in

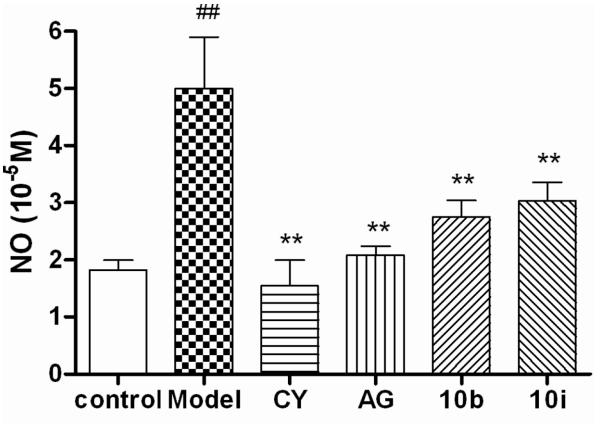


Figure 2. Effect of compound cyclophosphamide (CY), AG, **10b**, and **10i** on immunological liver injury in mice. ** $P < 0.01$ and ## $P < 0.01$.

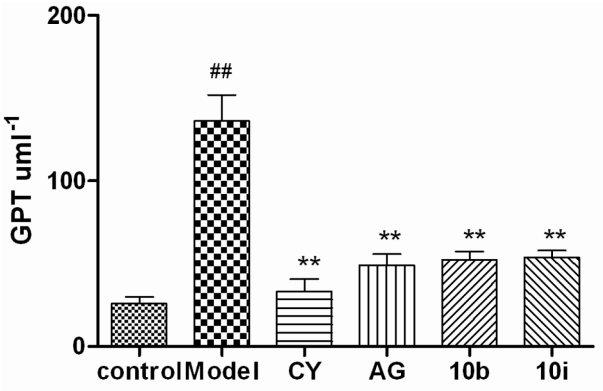


Figure 3. Effect of compound CY, AG, **10b** and **10i** on immunological liver injury in mice. ** $P < 0.01$ and ## $P < 0.01$.

the footpad and the tail. Treatment with cyclophosphamide (CY), AG, and compounds **10b** and **10i** alleviated these symptoms, while injection with lipopolysaccharide (LPS) promoted death. Serum glutamate pyruvate transaminase (GPT), glutamic oxalacetic transaminase enzyme (GOT), nitric oxide (NO) were assayed by commercially available kits. Significantly elevated NO, GPT, and GOT levels were reduced and immunological liver injury was alleviated by treatment with **10b** and **10i** (control group $P < 0.01$, Figs. 2, 3 ,4); these two compounds showed similar effects as CY and AG.

Conclusions

In search of novel pteridine compounds, three series of 2,4-diamino-pteridine derivatives were designed and synthesized. Compounds **10a**–**10g**, **10i**, **10j**, and **10k** are new compounds, while **10h** and **10l** had already been

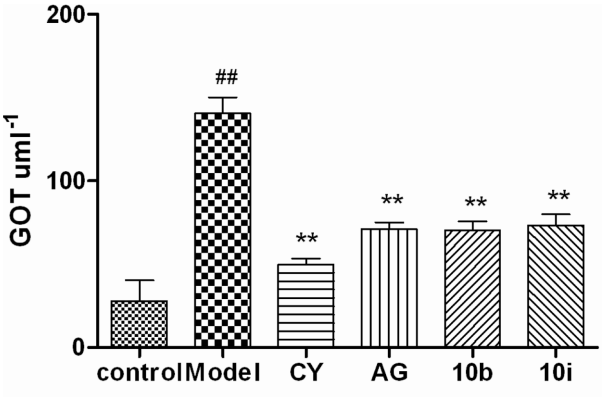


Figure 4. Effect of compound CY, AG, **10b**, and **10i** on immunological liver injury in mice.

reported by our group [10]. The inhibitory activities on iNOS of these compounds was evaluated *in vitro*. Within the series of compounds, **10b**, **10c**, **10e**, **10f**, **10g**, **10i**, **10j**, and **10k** showed potent inhibitive activities against iNOS similar to or better than that of MTX. Compound **10b** and **10i** showed similar effects as AG on rats with septic shock and immunological liver injury in mice. Our results may provide some guidance for the development of novel pteridine compounds as nitric oxide synthase inhibitor. Further, structure-activity relation studies and mechanistic studies on this new class of pteridine compounds are currently in progress.

The authors have declared no conflict of interest.

Experimental

Chemistry

Chemicals were bought from Shanghai Chemical Reagent Company (Shanghai, China) and were used without further purification. Melting points were determined with the capillary tube method, and the thermometer was uncorrected. Reaction kinetics was checked using silica gel 60 F254 plates (250 μm; Qingdao Ocean Chemical Company, China). Mass spectra were obtained with a Hewlett-Packard 1100 LC-MS (Agilent, Palo Alto, CA, USA). ^{1}H-NMR spectra were run on a Bruker ARX-300 instrument (Bruker Bioscience, Billerica, MA, USA), TMS as the internal standard. Elemental analysis was performed by a Elementar Vario EL III instrument (Heraeus GmbH, Hanau, Germany) for C, H, and N and the results are within ±0.5% of the theoretical values.

*2,6-Diamino-4-chloro-5-(p-chlorophenyl)azopyrimidine **3***

A solution of *p*-chloroaniline **1** (15.24 g, 0.12 mol) in 6 mol/L HCl (79 mL) was cooled to 0 – 5°C. Then, $NaNO_2$ (8.30 g, 0.12 mol) in H_2O (30 mL) was added dropwise with stirring. After the addition was completed, the solution was stirred for 15 min and checked by iodine-starch-paper to give a blue color. Urea (3.00 g) was

added to destroy the excess HNO_2. The diazonium salt solution **2** was then poured into a solution of 2,6-diamino-4-chloropyrimidine (15.60 g, 0.11 mol) in H_2O (300 mL) and stirred for 30 min. NaOAc (42.00 g) was added, and the mixture was stirred at room temperature for 16 h. The yellow precipitate was collected by filtration and washed with H_2O to afford **3** (26.40 g, 81%). M.p.: 268–270°C (dec.); ESI-MS: 282.1 $[M - H]^-$.

*General procedure for the synthesis of 2,6-diamino-4-substituted-5- (4-chlorophenyl)azopyrimidine **5a–5d***

A mixture of **3** (4.00 g, 14.20 mmol) and acyclic or arylic amines **4a–4d** (33.90 mmol) in DMF (15 mL) was heated at 70°C for 5 h. Then, the ice-water (20 mL) was added to the reaction and the final mixture was cooled at room temperature. The obtained solid was filtered, washed with H_2O, and crystallized from EtOH to afford **5a–5d**.

*2,6-Diamino-4-isopropylamino-5-(4-chlorophenyl)azopyrimidine **5a***

Yield: 86%. M.p.: 221–223°C. ^{1}H-NMR (DMSO-d_6) δ: 1.22 (d, J = 6.8 Hz, 6H), 4.08–4.28 (m, 1H), 5.52 (s, 2H), 6.17 (br, 2H), 7.45–7.47 (dd, J = 6.6 Hz, J = 6.6 Hz, 4H). ESI-MS: 306.1 $[M + H]^+$. Anal. calc. for $C_{13}H_{16}Cl_1N_7$ (305.77): C, 51.07; H, 5.27; N, 32.07. Found: C, 51.25; H, 5.47; N, 32.30.

*2,6-Diamino-4-[(4-ethoxylcarbonylphenyl)amino]-5-(p-chlorophenyl)azopyrimidine **5b***

Yield: 88%. M.p.: 285–287°C. ^{1}H-NMR ($CDCl_3$) δ: 1.34 (t, J = 6.0 Hz, 3H), 4.35 (q, J = 6.0 Hz, 2H), 5.48 (s, 2H), 6.15 (br, 2H), 7.45–7.47 (dd, J = 6.8 Hz, J = 6.8 Hz, 4H), 7.63–7.66 (m, 4H). ESI-MS: 412.2 $[M + H]^+$. Anal. calc. for $C_{19}H_{18}N_7O_2Cl_1$ (411.84): C, 55.41; H, 4.41; N, 23.81. Found: C, 55.25; H, 4.47; N, 23.38.

*2,6-Diamino-4-[(4-methoxylcarbonylphenyl)-methylamino]-5-(p-chlorophenyl)azopyrimidine **5c***

Yield: 85%. M.p.: 268–278°C. ^{1}H-NMR ($CDCl_3$) δ: 3.34 (s, 3H), 3.89 (s, 3H), 5.45 (s, 2H), 6.10 (br, 2H), 7.41–7.44 (dd, J = 7.8 Hz, J = 7.8 Hz, 4H); 7.63–7.67 (m, 4H). ESI-MS: 412.5 $[M + H]^+$. Anal. calc. for $C_{19}H_{18}N_7O_2Cl_1$ (411.84): C, 55.41; H, 4.41; N, 23.81. Found: C, 55.35; H, 4.40; N, 23.68.

*(S)-Diethyl2-{4-[2,6-diamino-5-[2-(4-chlorophenyl)-diazenyl]pyrimidin-ylamino]benzamido}pentanedioate hydrochloride **5d***

Yield: 93%. M.p.: 178–180°C. ^{1}H-NMR (DMSO-d_6) δ: 1.17 (t, J = 7.0 Hz, 3H), 1.22 (t, J = 7.0 Hz, 3H), 2.08–2.23 (m, 2H), 2.40–2.43 (m, 2H), 3.07 (bs, 2H), 4.04 (q, J = 7.0 Hz, 2H), 4.58–4.61 (m, 1H), 7.36 (d, J = 8.5 Hz, 2H), 7.53 (bs, 1H), 7.71–7.76 (m, 4H), 7.87 (d, J = 8.5 Hz, 2H), 8.16 (bs, 2H), 8.80 (bs, 1H). $[a]_D^{20}$ + 24, c = 1 in water; ESI-MS: 591.3 $[M + Na]^+$.

*General procedure for the synthesis of 2,5,6-triamino-4-substituted-pyrimidine dihydrochloride **6a–6d***

To a stirring mixture of **5a–5d** (4 mmol) in 20 mL alcohol, 5% HCl (w / v) solution (15 mL) was added and the mixture was heated at 70°C, then Zn powder (2.62 g, 40 mmol) was portionwise added over 30 min. After adding, the mixture was heated at 70°C for 1 h. The solution was filtered, and the filtrate was concentrated under reduced pressure. The precipitate was stirred with 10% HCl-ether (15 mL) for 1 h, then the solution was poured over and dried to afford **6a–6d**.

*2,5,6-Triamino-4-isopropylamino-pyrimidine dihydrochloride **6a***

Yield: 83%. M.p.: 233–236°C. ESI-MS: 205.2 $[M + Na]^+$, $C_7H_{14}N_6$ (182.23).

*2,5,6-Triamino-4-[(4-ethoxylcarbonylphenylamino]-pyrimidine dihydrochloride **6b***

Yield: 88%. M.p. 285–287°C. ESI-MS: 289.2 $[M + H]^+$, $C_{13}H_{16}N_6O_2$ (288.31).

*2,5,6-Triamino-4-[(4-methoxylcarbonylphenyl)-methylamino]pyrimidine dihydrochloride **6c***

Yield 78%. M.p.: 275–277°C. ESI-MS: 289.4 $[M + H]^+$, $C_{13}H_{16}N_6O_2$ (288.31).

*(S)-Diethyl2-[4-(2,5,6-triaminopyrimidin-4-ylamino)benzamido]pentanedioate dihydrochloride **6d***

Yield: 82%. M.p.: 254–257°C. $[a]_D^{20}$ + 22, c = 1 in water; ESI-MS: 446.5 $[M + H]^+$, $C_{20}H_{27}N_7O_5$ (445.47).

*General procedure for the synthesis of compounds **9a–9c***

Selenium dioxide (36.96 g, 0.33 mol) was added to a solution of H_2O (10 mL) in dioxane (250 mL), and the mixture was heated to 50(C for 0.5 h. Then, **7a–7c** (0.30 mol) was added, the solution was heated to reflux for 30 h. After cooling down, the solution was filtered to remove elemental selenium and the yellow-orange filtrate was concentrated under reduced pressure. The residue was recrystallized from H_2O to obtain pure **8a–8c**. Acetone oxime was prepared according to the method described elsewhere [10]. To a stirring mixture of **8a–8c** (0.10 mol) in MeOH (20 mL) was added H_2O (70 mL). After addition of acetoxime (8.76 g, 0.12 mol), the mixture was acidified to pH 3 to 4 with 3.6% (w / v) aq. HCL solution. Stirring was continued at 60°C for 5 h. After cooling down in an ice-bath, the solution was filtered and the residue was washed with water (3 × 10 mL) to afford **9a–9c**.

*2-Oxo-2-phenylacetaldehyde oxime **9a***

Yield: 64%. M.p.: 127–129°C. ESI-MS: 150.1 $[M + H]^+$, $C_8H_7N_1O_2$ (149.15).

*2-Oxo-2-(4-methylphenyl)acetaldehyde oxime **9b***

Yield: 56%. M.p. 98–100°C. ESI-MS: 164.5 $[M + H]^+$, $C_9H_9N_1O_2$ (163.17).

*2-Oxo-2-(4-methoxyphenyl)acetaldehyde oxime **9c***

Yield: 64%. M.p.: 119–100°C. ESI-MS: 202.1 $[M + Na]^+$, $C_9H_9N_1O_3$ (179.17).

*General procedure for the synthesis of compounds **10a–10l***

Compounds **6a–6d** (3.00 mmol) were dissolved in dry MeOH (15 mL). Then **9a–9c** (4.50 mmol) in dry MeOH (15 mL) was slowly added under N_2 atmosphere and the solution was stirred

at reflux (ca. 65°C) for 4 h. Then, the solution was evaporated to dryness *in vacuo*. The residue was purified by column chromatography (silica gel; $CHCl_3$ / MeOH 20 : 1, v / v) to afford compounds **10a – 10l**.

2-Amino-4-isopropylamine-6-phenylpteridine ***10a***

Yield: 62%, yellow solid. M.p.: 269 – 271°C. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (d, *J* = 6.5 Hz, 6H), 4.56 (m, 1H), 6.22 (bs, 2H), 7.52 (m, 1H); 7.59 (m, 3H), 8.02 (m, 2H), 9.23 (s, 1H). ESI-MS: 281.1 $[M + H]^+$. Anal. calc. for $C_{15}H_{16}N_6$ (280.32): C, 64.27; H, 5.75; N, 29.98. Found: C, 64.37; H, 5.69; N, 29.89.

2-Amino-4-[(4-ethoxylcarbonylphenyl)amino]-6-phenylpteridine ***10b***

Yield: 35%, yellow solid. M.p.: 275 – 277°C. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (t, *J* = 6.9 Hz, 3H) 4.39 (q, *J* =6.9 Hz, 2H), 6.06 (bs, 2H), 7.41 – 7.51 (bs, 3H), 7.64 (d, *J* = 8.1 Hz, 2H), 7.67 (d, *J* = 8.1Hz, 2H), 8.13 (d, *J* = 8.1 Hz, 2H), 9.23 (s, 1 H), 9.32 (s, 1H). ESI-MS: 387.2 $[M + H]^+$. Anal. calc. for $C_{21}H_{18}N_6O_2$ (386.40): C, 65.27; H, 4.70; N, 21.75. Found: C, 65.47; H, 4.69; N, 21.89.

2-Amino-4-[(4-methoxylcarbonylphenyl)methylamino]-6-phenylpteridine ***10c***

Yield: 37%, yellow solid. M.p.: 268 – 270°C. ^{1}H-NMR ($CDCl_3$) δ: 3.68 (s, 3H), 3.96 (s, 3H), 5.36 (bs. *s,* 2H), 7.16 – 7.56 (m, 7H), 8.11 (d, *J* = 8.0 Hz, 2H), 9.10 (s, 1H). ESI-MS: 387.2 $[M + H]^+$. Anal. calc. for $C_{21}H_{18}N_6O_2$ (386.40): C, 65.27; H, 4.70; N, 21.75. Found: C, 65.17; H, 4.51; N, 22.12.

(S)-Diethyl2-[4-(2-amino-6-phenylpteridin-4-ylamino)benzamido]pentanedioate ***10d***

Yield: 32%, yellow solid. M.p.: 228 – 231°C. ^{1}H-NMR ($CDCl_3$) δ: 1.21 (t, *J* = 7.0 Hz, 3H), 1.32 (t, *J* = 7.0 Hz, 3H), 2.09 – 2.11 (m, 2H), 2.45 – 2.53 (m, 2H), 4.07 (q, *J* = 7.0 Hz, 2H), 4.18 (q, *J* = 7.0 Hz, 2H), 4.55 – 4.68 (m, 1H), 5.78 (bs. s, 2H), 7.18-7.31 (m, 3H), 7.85-7.91 (m, 7H), 9.01 (bs. s, 1H), 9.14 (s, 1H). ESI-MS: 544.2 $[M + H]^+$. $[a]_D^{20}$ + 20, c = 1 in acetic acid; Anal. calc. for $C_{28}H_{29}N_7O_5$ (543.57): C, 61.87; H, 5.38; N, 18.04. Found: C, 61.65; H, 5.41; N, 18.22.

2-Amino-4-isopropylamine-6-(4-methylphenyl)-pteridine ***10e***

Yield: 59%, yellow solid. M.p.: 318 – 320°C. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (d, *J* = 6.5 Hz, 6H), 4.01 (s, 1H), 4.55 (m, 1H), 5.91 (bs, 2H), 7.39 (d, *J* = 8.1 Hz, 2H), 7.51 (bs, 1H), 7.91 (d, *J* = 8.1 Hz, 2H), 9.19 (s, 1 H). ESI-MS: 295.1 $[M + H]^+$. Anal. calc. for $C_{16}H_{18}N_6$ (294.35): C, 65.74; H, 5.52; N, 28.75. Found: C, 65.57; H, 5.69; N, 28.89.

2-Amino-4-[(p-ethoxylcarbonylphenylamino]-6-(4-methylphenyl)pteridine ***10f***

Yield: 32%, yellow solid. M.p.: 262 – 264°C. ^{1}H-NMR ($CDCl_3$) δ: 1.38 (d, *J* = 7.0 Hz, 3H), 2.94 (s, 3H), 4.34 (q, *J* = 7.0 Hz, 2H), 5.96 (bs, 2H), 7.26 (d, *J* = 4.6 Hz, 2H), 7.84 (d, *J* = 7.3 Hz, 2H), 7.87 (d, *J* = 7.3 Hz, 2H), 8.00 (d, *J* = 7.3 Hz, 2H), 8.99 (bs. s, 1H), 9.16 (s, 1H). ESI-MS: 401.2 $[M + H]^+$. Anal. calc. for $C_{22}H_{20}N_6O_2$ (400.43): C, 65.99; H, 5.03; N, 20.99. Found: C, 65.54; H, 4.74; N, 21.29.

2-Amino-4-[(p-methoxylcarbonylphenyl)methylamino]-6-(4-methylphenyl)pteridine ***10g***

Yield: 51%, yellow solid. M.p.: 273 – 275°C. ^{1}H-NMR ($CDCl_3$) δ: 2.45 (s, 3H), 3.68 (s, 3H), 3.97 (s, 3H), 5.41 (bs. s, 2H), 7.23 (d, *J* = 8.0 Hz, 2H), 7.37 (d, *J* = 8.0 Hz, 2H), 8.03 (d, *J* = 8.0 Hz, 2H), 8.11 (d, *J* = 8.0 Hz, 2H), 9.08 (s, 1 H). ESI-MS: 401.1 $[M + H]^+$. Anal. calc. for $C_{22}H_{20}N_6O_2$ (400.43): C, 65.99; H, 5.03; N, 20.99. Found: C, 65.87; H, 5.29; N, 20.85.

(S)-Diethyl2-[4-(2-amino-6-(4-methylphenyl)pteridin-4-ylamino)benzamido]-pentanedioate ***10h***

Yield: 30%, yellow solid. M.p.: 235 – 237°C. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (t, *J* = 7.0 Hz, 3H), 1.31 (t, *J* = 7.0, 3H), 2.10 – 2.38 (m, 2H), 2.41 (s, 3H), 2.47 – 2.55 (m, 2H), 4.12 (q, *J* = 7.0 Hz, 2H), 4.25 (q, *J* = 7.0 Hz, 2H), 4.77 – 4.80 (m, 1H), 5.88 (bs. s, 2H), 7.21 – 7.35 (m, 3H), 7.87-7.96 (m, 7H), 9.05 (bs. s, 1H), 9.19 (s, 1H). $[a]_D^{20}$ +19 c = 1 in acetic acid; ESI-MS: 558.3 $[M + H]^+$. Anal. calc. for $C_{29}H_{31}N_7O_5$ (557.60): C, 62.46; H, 5.60; N, 17.58. Found: C, 62.06; H, 5.59; N, 17.52.

2-Amino-4-isopropylamine-6-(4-methoxyphenyl)-pteridine ***10i***

Yield: 33%, yellow solid. M.p.: 298 – 300°C. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (d, *J* = 6.5 Hz, 6H), 3.91 (s, 1H), 4.55 (m, 1H), 5.91 (bs, 2H), 7.39 (d, *J* = 8.8 Hz, 2H), 7.48 (bs, 1H), 7.97 (d, *J* = 8.8 Hz, 2H), 9.17 (s, 1H). ESI-MS: 311.1 $[M + H]^+$. Anal. calc. for $C_{16}H_{18}N_6O_1$ (310.35): C, 61.92; H, 5.85; N, 27.08. Found: C, 61.77; H, 5.69; N, 28.29.

2-Amino-4-[(p-ethoxylcarbonylphenylamino]-6-(4-methoxyphenyl)pteridine ***10j***

Yield: 35%, yellow solid. M.p.: 262 – 264°C. ^{1}H-NMR ($CDCl_3$) δ: 1.40 (d, *J* = 7.0 Hz, 3H), 3.89 (s, 3H), 4.37 (q, *J* = 7.0 Hz, 2H), 5.60 (bs, 2H), 7.05 (d, *J* = 7.5 Hz, 2H), 7.86 (d, *J* = 7.5 Hz, 2H), 7.98 (d, *J* = 7.5 Hz, 2H), 8.07 (d, *J* = 7.5 Hz, 2H), 9.11 (bs, 1H), 9.24 (s, 1H). ESI-MS: 417.3 $[M + H]^+$. Anal. calc. for $C_{22}H_{20}N_6O_3$ (416.43): C, 63.45; H, 4.84; N, 20.18. Found: C, 63.55; H, 4.69; N, 20.14.

2-Amino-4-[(p-methoxylcarbonylphenyl)methylamino]-6-(4-methoxyphenyl)pteridine ***10k***

Yield: 34%, yellow solid. M.p.: 240 – 242°C. ^{1}H-NMR ($CDCl_3$) δ: 3.70 (s, 3H), 3.87 (s, 3H), 3.96 (s, 3H), 5.66 (bs. s, 2H), 6.75 (d, *J* = 8.4 Hz, 2H), 7.16 (d, *J* = 8.4 Hz, 2H), 7.31 (d, *J* = 8.4 Hz, 2H), 8.12 (d, *J* = 8.4 Hz, 2H), 9.06 (s, 1H). ESI-MS: 439.1 $[M + Na]^+$. Anal. calc. for $C_{22}H_{20}N_6O_3$ (416.43): C, 63.45; H, 4.84; N, 20.18. Found: C, 63.53; H, 4.69; N, 20.13.

(S)-Diethyl2-[4-(2-amino-6-(4-methoxyphenyl)pteridin-4-ylamino)benzamido]-pentanedioate ***10l***

Yield: 40%, yellow solid. M.p.: 237 – 239°C. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (t, *J* = 7.0 Hz, 3H), 1.30 (t, *J* = 7.0 Hz, 3H), 2.16 – 2.29 (m, 2H), 2.39 – 2.48 (m, 2H), 3.71 (s, 3H), 4.12 (q, *J* = 7.0 Hz, 2H), 4.24 (q, *J* = 7.0 Hz, 2H), 4.70 – 4.75 (m, 1H), 6.50 (bs. s, 2H), 7.21 (bs. s, 1H), 7.45 – 7.60 (m, 3H), 7.85 – 8.16 (m, 6H), 8.79 (bs. s, 1H), 9.12 (s, 1H). $[a]_D^{20}$ +19, c=1 in acetic acid; ESI-MS: 574.2 $[M + H]^+$. Anal. calc. for $C_{29}H_{31}N_7O_6$ (573.60): C, 60.72; H, 5.45; N, 17.09. Found: C, 60.53; H, 5.51; N, 17.21.

Pharmacology

Enzyme activity

The inhibitory activities of compounds **10a** – **10l** against the NOS-II (i-NOS) enzyme were evaluated *in vitro* according to the manufacturer's (Jiancheng, Nanjing, China) instructions, with 2,4-diamino-biopterin (ABP) as the positive control. NOS-II was incubated for 20 min at pH = 7.2 and 37°C in a mixture containing 2 μM tetrahydro-L-biopterin, 50 nM CaM, 1 mM $CaCl_2$, 5 μM FAD, 10 μM FMN, 250 μM 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonat, 50 mM triethanolamine (TEA), 1 mM NADPH, 7 mM GSH, and 50 μM L-arginine. Then, the reaction was stopped by adding ice-cold acetate buffer (pH = 5.5) and the optical density was measured at 530 nm. The IC_{50} values were calculated according to the Logit method after getting the inhibitory rate. NOS-II and tetrahydro-L-biopterin was purchased from from Sigma-Aldrich (St. Louis, MI, USA). All other chemicals used were of analytical grade or higher purity. IC_{50} values were expressed in μM. The inhibitory activities of 2,4-diamino biopterin derivatives **10a** – **10l** against NOS-II were summarized in Table 2.

Preparation of septic-shock rats

Sepsis was induced by cecal ligation and puncture (CLP) as previously described [13]. Briefly, SD rats (Male Sprague Dawley rats were purchased from Vital River, Beijing, China.), weighing 150 – 300 g, were anesthetized with ethylether. Then, a 2 cm midline abdominal incision was cut at the level of the cecum, which was then extracted and ligated with 3-0 silk just below the ileocecal valve. The cecum was punctured several times with a 22-gauge needle, and a small amount of cecal content was expressed. The perforated cecum was returned to the abdominal cavity, and the abdomen was suture-closed. After the surgical procedure, all animals were housed in cages with access to food and water *ad libitum*.

Measurement of blood pressure

The rats were randomly divided into four groups: Group 1, CLP + normal saline (control); Group 2, CLP + aminoguanidine (AG); Group 3, CLP + compound **10b**; Group 4, CLP + compound **10i**. At 18 h after operation, carotid artery and jugular vein catheterizations were performed. Briefly, rats were anesthetized with pentobarbital (35 mg/kg, i.p.) and left carotid artery and right jugular vein were exposed after a middle incision on the neck. After that, one catheter connected with a pressure transducer was inserted into the left carotid artery to record the blood pressure (BP) while another catheter containing normal saline or the test agents was inserted into the right jugular vein. When BP recoding was stable, normal saline, aminoguanidine, **10b**, or **10i** (50 mg/kg) was infused into the body through the jugular vein. The systolic BP (SBP) and diastolic BP (DSP) were recorded before and 5, 10, 20, 30, 40, 50, 60 min after infusion. The mean blood pressure (MBP) was calculated as:

$$MBP = DSP + (SDP - DSP)/3.$$

Preparation of Corynebacterium parvum-primed and lipopolysaccharide-induced hepatic necrosis (HN)

Male Kunming Mice (Mice were obtained from Qinglong Mountain Farm, Nanjing, China.), weighing 18 – 22 g, were randomly divided into six groups: Group 1, control; Group 2, model (HN); Group 3, HN + cyclophosphamide (CY); Group 4, HN + aminoguanidine (AG); Group 5, HN + compound **10b**; Group 6, HN + compound **10i** [14]. On the first day, a dose of 0.5 mg *Corynebacterium parvum* was injected into each mouse of group 2 to group 6 via the tail vein. On the 5th, 7th, and 9th day, the mice in the treated groups were injected with cyclophosphamide, aminoguanidine, compound **10b**, or compound **10i** (30 mg/kg, i.p.), respectively. On the 10th day, a dose of 10 μg LPS was injected into each mouse. For the control group, we injected normal saline. 12 h later, mice were sacrificed and blood samples were collected. The serum content of glutamic-pyruvic transaminase (GPT), glutamic-oxalacetic transaminase (GOT), and nitric oxide (NO) were determined. All data were expressed as mean ± S.D. Differences between the groups were compared with Student's *t*-test.

References

[1] P. Vallance, J. Leiper, *Nat. Rev. Drug Discov.* **2002**, *1*, 939 – 950.

[2] S. Moncada, R. M. Palmer, E. A. Higgs, *Pharmacol. Rev.* **1991**, *43*, 109 – 142.

[3] K. M. Channon, *Trends Cardiovasc. Med.* **2004**, *14*, 323 – 327.

[4] T. P. Misko, W. M. Moore, T. P. Kasten, G. A. Nickols, *et al.*, *Eur. J. Pharmacol.* **1993**, *233*, 119 – 125; W. M. Moore, R. K. Webber, G. M. Jerome, F. S. Tjoeng, *et al.*, *J. Med. Chem.* **1994**, *37*, 3886 – 3888; O. W. Griffith, S. S. Gross in *Methods in Nitric Oxide Research*, John Wiley and Sons, New York, **1996**, p. 187 – 208.

[5] H. M. Bömmel, A. Reif, L. G. Fröhlich, A. Frey, *et al.*, *J. Biol. Chem.* **1998**, *273*, 33142 – 33149; E. R. Werner, E. Pitters, K. Schmidt, G. Werner-Felmayer, B. Mayer, *Biochem. J.* **1996**, *320*, 193 – 196.

[6] E. C. Bigham, G. K. Smith, J. F. Reinhard, W. R. Mallory, *et al.*, *J. Med. Chem.* **1987**, *30*, 40 – 45.

[7] W. R. Boon, T. Leigh, to Imperial Chemical Industries Ltd, Brit. Pat. 677, 342 (*Chem. Abstr.* **1953**, *47*, 9369).

[8] E. C. Taylor, P. A. Jacobi, *J. Am. Chem. Soc.* **1974**, *96*, 6781 – 6782.

[9] Q.-Z. Yao, Q.-J. Wang, G. Lu, W.-F. Zhou, *et al.*, to China Pharmaceutical University, C. N. Pat. 1,546,491 (*Chem. Abstr.* **2005**, *143*, 211928t).

[10] F. Tang, G.-H. Zheng, Q.-Z. Yao, G. Lü, *et al.*, *Acta Chimi. Sin.* **2006**, *64*, 249.

[11] W. K. Alderton, C. E. Cooper, R. G. Knowles, *Biochem. J.* **2001**, *357*, 593 – 615.

[12] B. R. Crane, A. S. Arvai, D. K. Ghosh, C.-Q. Wu, *et al.*, *Science* **1998**, *279*, 2121 – 2126.

[13] R. D. Piper, F. Y. Li, L. M. Myers, W. J. Sibbald, *Am. J. Respir. Crit. Care Med.* **1997**, *156*, 1473 – 1482; Y. Kadoi, F. Goto, *Anesth. Analg.* **2004**, *99*, 212 – 220.

[14] T. Sato, H. Schinzawa, Y. Abe, T. Takahashi, *et al.*, *J. Leukoc. Biol.* **1993**, *53*, 144 – 150.

