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Furoxan nitric oxide donor coupled chrysin derivatives: Synthesis and vasculoprotection

Xiao-Qing Zou [a,b], Sheng-Ming Peng [b], Chang-Ping Hu [a,*], Li-Feng Tan [b], Han-Wu Deng [a], Yuan-Jian Li [a]

[a] Department of Pharmacology, School of Pharmaceutical Sciences, Central South University, Changsha 410078, China
[b] Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, College of Chemistry, Xiangtan University, Xiangtan 411105, China

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ABSTRACT

A series of furoxan-based nitric oxide-releasing chrysin derivatives were synthesized. Pharmacological assays indicated that all chrysin derivatives exhibited in vitro inhibitory activities against aldose reductase and advanced glycation end-product formation. Some chrysin derivatives were also found to increase the glucose consumption of HepG2 cells. Furthermore, the compounds released a low amount of NO in the presence of L-cysteine (range from 0.20% to 1.89%). These hybrid furoxan-based NO donor chrysin derivatives offer a mutual prodrug design concept for the development of therapeutic or preventive agents for vascular complications due to diabetes.

Diabetes is characterized by hyperglycemia and the development of specific micro- and macro-vascular complications. Nitric oxide (NO), endothelium-derived vasodilator, plays a crucial role in the development of vascular complications.[1] NO has diverse potent physiological actions and helps maintain micro- and macrovascular homeostasis in the cardiovascular system through several mechanisms, including vasodilation, inhibition of platelet aggregation, and modulation of platelet and leukocyte adhesion to the endothelium.[2] Decreased endothelial NO bioavailability and production are critical contributors to endothelial dysfunction and vascular complications in diabetes mellitus.[3] Some NO donors are currently being used to treat certain cardiovascular diseases.[4] The most important NO donors are organic nitrates, such as glyceryl trinitrate. However, tolerance for these agents during chronic therapy is a cause for concern.[5] Furoxan (1,2,5-oxadiazole-2-oxides) derivatives are biologically active compounds that are capable of releasing nitric oxide in the presence of thiols and lack of tolerance.[6,7] Hybrid NO donor furoxan-based drugs are a novel type of drug that retains the pharmacological activity of the parent compound but also has the biological actions of NO.

Chrysin (5,7-dihydroxyflavone), a natural, widely distributed flavonoid, has diverse biologically active properties, including antioxidative,[8,9] anticancer,[10] anxiolytic,[11] anti-inflammatory,[12,13] anti-diabetic,[14] and anti-glucosidase characteristics.[15] Recently, several researchers attempted to modify chrysin properties and found that some chrysin derivatives have diverse activities, including anti-diabetic effects.[16–22] Based on the mutual prodrug concept, this study aims to investigate a synthesis approach based on grafting of an NO-releasing moiety furoxan to chrysin derivatives via a variable spacer. We suppose that the new pharmacodynamic hybrids may have combined advantages of hypoglycemic effect with a slow release of NO. This may mimic the effects of endogenous NO, which improve endothelial dysfunction and prevent the progression of diabetic complications.

Here, we report the synthesis of several NO-donating furoxan derivatives of chrysin. The NO-releasing capacities and glucose consumption promotion, as well as aldose reductase (AR) and advanced glycation end-product (AGE) inhibitory effects of these derivatives, were explored in vitro. The results will help in further understanding the potential uses of chrysin NO-donating derivatives for the development of therapeutic and preventive agents for the treatment of vascular complications of diabetes.

The preparation of furoxan derivatives **8a,b** and **9a,b** follows the synthetic routes illustrated in Scheme 1. For our study, hydroxybenzaldehyde was reduced with potassium borohydride to obtain hydroxybenzyl alcohols **4a** and **4b**. Sodium nitrate was added to cinnamyl alcohol under acidic conditions and thionyl chloride was used for chlorination to obtain **6**. Compounds **7a** and **7b** were synthesized through a reaction between **4a,b** and **6** in acetonitrile. Compounds **8a** and **8b** were then obtained through esterification of 7-carboxymethyl chrysin **2**. Finally, acetone was used as a solvent and potassium carbonate as a base to obtain **9a** and **9b** through a reaction with acetic anhydride.

The synthesis of compounds **10** and **11** was accomplished according to the general pathway illustrated in Scheme 2.

* Corresponding author. Tel.: +86 731 8235 5079; fax: +86 731 8235 5078.
E-mail address: huchangping@yahoo.com (C.-P. Hu).

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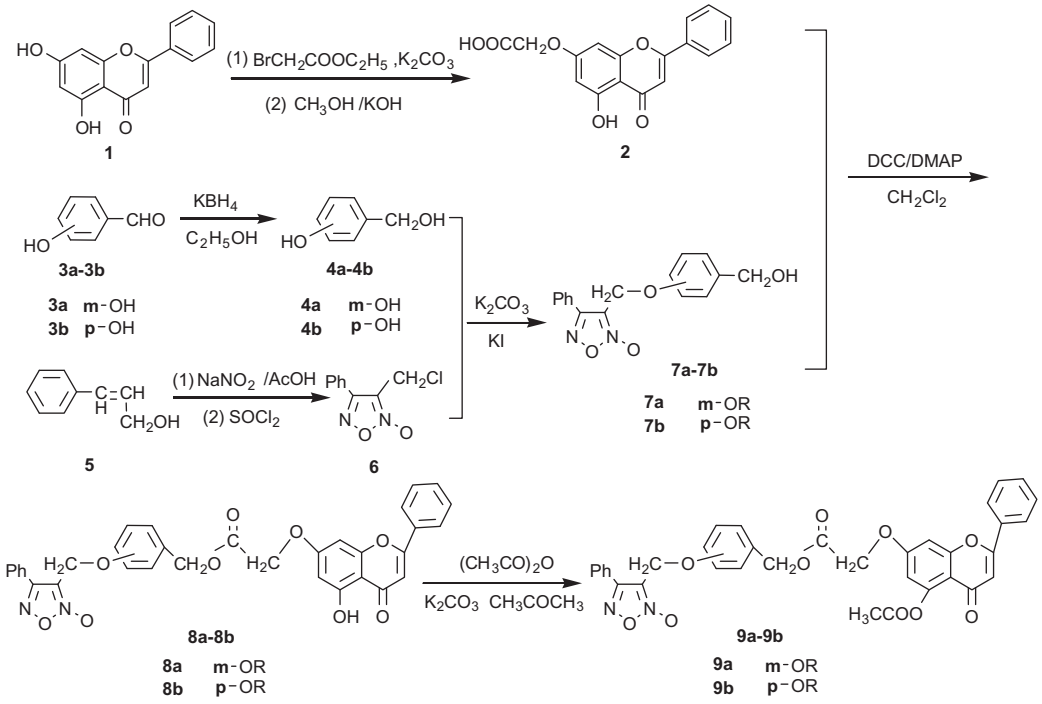
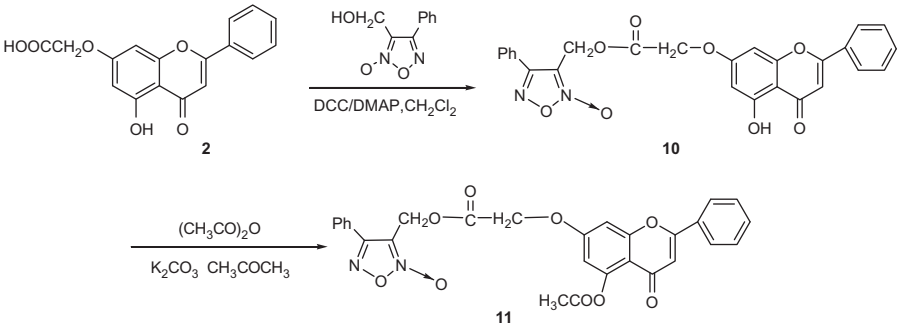


Scheme 1. Synthesis of target chemical compounds (**8a**,**b** and **9a**,**b**).



Scheme 2. Synthesis of target chemical compounds **10** and **11**.

7-Carboxymethyl chrysin **2** and furoxan were used to obtain the chemical compound **10** of chrysin coupled with furoxan through dehydration under DCC/DMAP action. With the use of potassium carbonate as base and acetone as solvent, **11** was obtained by reacting with acetic anhydride.

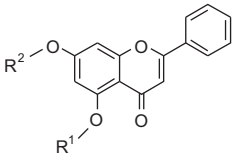
In the present study, we performed AR and AGE assays as described previously.[16] NO-releasing capacities were also determined for all the target compounds in vitro.[16] Structure–activity relationships acquired from the assays indicated that the 5-OH group was the key group responsible for the high inhibitory effects on AR (5-OH group: $IC_{50} = 0.22 \pm 0.01$ to 0.75 ± 0.06 μM vs 5-acetyl group: $IC_{50} = 0.90 \pm 0.02$ to 1.93 ± 0.07 μM). The potency of AR inhibition was over 10 times higher than the positive reference compound quercetin, the parent compound chrysin, the intermediate compounds 4-phenyl-3-methanol-furoxan and the NO releasing positive reference sodium nitroprusside (SNP). The potency of the AGE formation inhibition was 22.3–33 times higher than that of the positive reference compound aminoguanidine and was greater than nitrate-based chrysin derivatives reported recently,[16] meanwhile, 5-substituent did not affect the inhibitory effects on AGE formation. It has been found that SNP treatments significantly attenuates AGE-induced hypertrophic growth in human renal tubular cells[23] and ameliorates AGE-induced dysfunction of endothelial progenitor cells.[24] In our study, SNP was verified to inhibit the AGE formation effectively but the intermediate compounds 4-phenyl-3-methanol-furoxan showed no effect on in vitro AGE formation, the possible reason is that there is no sufficient sulfhydryl in the AGE preparation system which affects the release of NO from furazan. These results indicate that NO donors should play a protective role on chronic vascular complications induced by AGE in type 2 diabetes. NO was released from all the chrysin derivatives upon incubation with phosphate-buffered saline solution (PBS at pH 7.4) in the presence of L-cysteine. The percentage of released NO varied from $0.20 \pm 0.03\%$ to $1.89 \pm 0.03\%$, indicating a slow release. Correspondingly, the NO releasing from 4-phenyl-3-methanol-furoxan was also lower ($0.41 \pm 0.02\%$). In contrast, the amount of NO released from SNP was substantially higher ($10.42 \pm 1.80\%$). These results indicate that the release of adequate amounts of NO to provide protective effects was balanced with the concentration range required for the sufficient activity of chrysin derivatives.

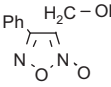
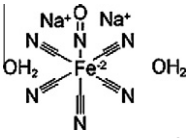
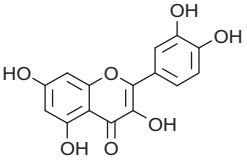
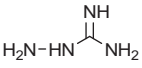
Turnbull et al. found that NO release from furoxan–aspirin hybrids was undetectable in buffer alone, but was accelerated in the presence of either plasma or plasma components, albumin, glutathione and ascorbate.[25] They therefore conclude that the

decomposition of furoxan–aspirin hybrids to generate biologically active NO is catalyzed by endogenous agents which may instil a potential for primarily intracellular delivery of NO. In our settings, the furoxan-based nitric oxide-releasing chrysin derivatives showed better inhibitory effect on AR and AGE in spite of their lower NO release rate, which is reasonable because we also found that chrysin itself had inhibitory activities on AR and AGE. Whether the decomposition of these furoxan–chrysin hybrids to generate biologically active NO also need to be catalyzed by endogenous agents deserves to be further investigated (Table 1).

Hyperglycemia increases the risk of both micro- and macro-vascular diseases in correspondingly diabetic patients.[26] The metabolism of excess glucose drives several damage pathways, such as oxidative stress, generation of AGE, increased AR-related polyol pathway flux, activation of protein kinase C, and increased hexosamine pathway flux, which leads to endothelial dysfunction. This indicates that hyperglycemia is a major cause of vascular complications in diabetes. To evaluate the hypoglycemic effects of the target compounds, we determined the glucose consumption of HepG2 cells treated with chrysin, chrysin derivatives, 4-phenyl-3-methanol-furoxan, SNP and a positive reference rosiglitazone. The results showed that **8a**, **8b**, **10**, 4-phenyl-3-methanol-furoxan, SNP and rosiglitazone significantly promoted glucose consumption of HepG2 cells compared to the blank vehicle 0.1% DMSO (P <0.05). The structure-activity relationships acquired for this assay indicated that the 5-OH group is the key influential factor on glucose consumption of HepG2 cells. In contrast, chrysin significantly reduced glucose consumption (P <0.05 vs 0.1% DMSO, Fig. 1). Compounds **9a, 9b**, and **11** had no effect on the glucose consumption of HepG2 cells. It has been shown that acute infusion of SNP increases

Table 1
Structure and in vitro AR and AGE inhibition and NO releasing properties of the target compounds



Compound	R^1	R^2	IC_{50} (μmol/L)		%NO released[c]
			AR inhibition[a]	AGE inhibition[b]	
0.1% DMSO			/	/	/
Chrysin	H	OH	7.78 ± 0.57	63.54 ± 6.42	/
8a	H	X=CH_2COOCH_2Ph(m)	0.22 ± 0.01	37.50 ± 7.92	0.76 ± 0.13
9a	CH_3CO	X=CH_2COOCH_2Ph(m)	1.62 ± 0.16	26.39 ± 2.05	1.89 ± 0.03
8b	H	X=CH_2COOCH_2Ph(p)	0.40 ± 0.05	36.59 ± 16.55	0.75 ± 0.08
9b	CH_3CO	X=CH_2COOCH_2Ph(p)	1.93 ± 0.07	25.38 ± 1.97	0.85 ± 0.01
10	H	X=$COCH_2$	0.75 ± 0.06	35.9 ± 11.3	0.24 ± 0.01
11	CH_3CO	X=$COCH_2$	0.90 ± 0.02	31.45 ± 8.95	0.20 ± 0.03
4-Phenyl-3-methanol-furoxan[d]			⩾10	⩾1000	0.41 ± 0.02
SNP[e]			7.71 ± 1.11	25.87 ± 0.37	10.42 ± 1.80
Quercetin[f]			2.85 ± 0.04		
Aminoguanidine[g]				826.22 ± 9.26	

Each value represents the mean ± SD (n = 3).
[a] The concentration required for a 50% inhibition of the decrease in the optical density of NADPH at 340 nm relative to 0.1% DMSO. IC_{50} values were calculated from the dose inhibition curve.
[b] The concentration required for a 50% inhibition of the fluorescence intensity of AGE relative to 0.1% DMSO. IC_{50} values were calculated from the dose inhibition curve.
[c] Percent of nitric oxide released based on a theoretical maximum release of 1 mol of NO/mol of the target compounds.
[d] 4-Phenyl-3-methanol-furoxan was the intermediate compounds for the synthesis of NO-releasing chrysin derivatives.
[e] Sodium nitroprusside (SNP) was used as NO releasing positive reference.
[f] Quercetin was used as positive control for AR inhibition test.
[g] Aminoguanidine was used as positive control for AGE inhibition test.

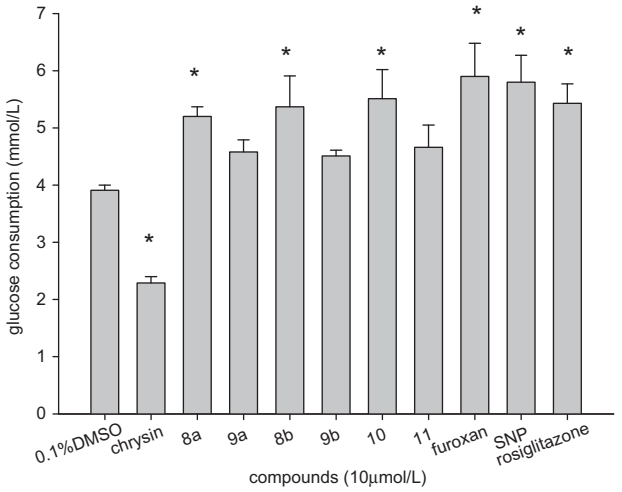


Figure 1. Effects of 10 μmol/L chrysin, chrysin derivatives (**8a**,**b**, **9a**,**b**, **10** and **11**), 4-phenyl-3-methanol-furoxan, SNP and rosiglitazone on HepG2 glucose consumption (values are mean ± SD; $n = 3$; $^{*}P < 0.05$ vs 0.1% DMSO. Differences between individual groups were analyzed by using ANOVA followed by Dunett's test).

leg glucose uptake at rest in patients with type 2 diabetes.[27] The present study indicated that the NO donor 4-phenyl-3-methanol-furoxan and SNP significantly promoted glucose consumption of HepG2 cells. These findings provide a rationale that NO may therefore be an important mediator of peripheral glucose disposal and the potential therapeutic role of NO donor on chronic vascular complications in diabetic patients warrants more attention.

An O^7-furoxan-based NO donor moiety was attached to chrysin. The AR, AGE formation, and glucose consumption assays, as well as NO-releasing capacities, showed that: (i) the parent compound chrysin and the NO releasing positive reference SNP are potent AGE inhibitors; (ii) the intermediate compounds 4-phenyl-3-methanol-furoxan and the NO releasing positive reference SNP are strong promoter on glucose consumption of HepG2 cells; (iii) all the hybrid prodrugs release NO slowly upon incubation with PBS in the presence of L-cysteine; (iv) all the furoxan based derivatives have high inhibitory effects on AR and AGE formation; (v) the 5-OH group is the key group that determines the inhibition of AR and promotion of glucose consumption; (vi) O^7-[3-(methylol phenoxymethyl)-4-phenyl-1,2,5-oxadiazole-2-oxide carboxymethyl acylation] **8a**, **8b** and O^7-[3-methylol-4-phenyl-1,2,5-oxadiazole-2-oxide carboxymethyl acylation] analog **10** significantly promote glucose consumption of HepG2 compared to the blank vehicle 0.1% DMSO.

In conclusion, these hybrid furoxan-based NO donor derivatives offer a mutual prodrug design concept for the development of therapeutic or preventive agents for vascular complications due to diabetes.

Acknowledgments

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Further reading

28. 3-(Methylol phenoxymethyl)-4-phenyl-1,2,5-oxadiazole-2-oxide acylation coupling-carboxymethyl chrysin (**8a**, **8b**): **8a** (69.2% yield, mp: 134–135 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 12.70 (s, 1H), 7.76–7.82 (m, 4H), 7.45–7.51 (m, 6H), 7.27–7.29 (d, 2H), 6.91–6.93(m, 2H), 6.62 (s, 1H), 6.43–6.44 (d, 1H), 6.29–6.30 (d, 1H), 5.15(s, 2H), 5.02(s, 2H), 4.67 (s, 2H). EI-MS (*m*/*z*): 592. Anal. Calcd for $C_{33}H_{24}N_2O_9$: C, 66.89; H, 4.08; N, 4.73. Found: C, 66.58; H, 4.42; N, 4.37. Compound **8b** (68% yield, mp: 155–156 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 12.76 (s, 1H), 7.87 (s, 4H), 7.55 (s, 6H), 7.32 (s, 1H), 6.97–7.02 (d, 3H), 6.69 (s, 1H), 6.53 (s, 1H), 6.39 (d, 1H), 5.25 (s, 2H), 5.10 (s, 2H), 4.80 (s, 2H), EI-MS (*m*/*z*): 592. Anal. Calcd for $C_{33}H_{24}N_2O_9$: C, 66.89; H, 4.08; N, 4.73; Found: C, 66.51; H, 4.45; N, 4.34. Characteristic of O^5-acetyl-O^7-Chrysin acetic acid[3-(4-phenyl-1,2,5-oxadiazole-2-oxide-3-)methoxyl-]benzyl ester (**9a**, **9b**): **9a** (31.5% yield, mp: 119–121 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.83–7.84 (m, 4H), 7.50–7.57 (m, 6H), 7.30–7.32 (d, 2H), 6.95–6.97 (d, 2H), 6.81–6.82 (d, 1H), 6.65 (s, 2H), 5.21 (s, 2H), 5.05 (s, 2H), 4.75 (s, 2H), 2.43 (s, 3H). EI-MS (*m*/*z*): 634. Anal. Calcd for $C_{35}H_{26}N_2O_{10}$: C, 66.24; H, 4.13; N, 4.41. Found: C, 66.62; H, 4.29; N, 4.05. Compound **9b** (47.3% yield, mp: 165–166 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.83–7.84 (m, 4H), 7.50–7.56 (m, 6H), 7.29–7.31(d, 1H), 7.00 (s, 2H), 6.93–6.95(d, 1H), 6.85 (s, 1H), 6.67 (s, 1H), 6.60 (s, 1H), 5.24 (s, 2H), 5.08 (s, 2H), 4.80 (s, 2H), 2.43 (s, 3H), EI-MS (*m*/*z*): 634. Anal. Calcd for $C_{35}H_{26}N_2O_{10}$: C, 66.24; H, 4.13; N, 4.41. Found: C, 66.64; H, 4.26; N, 4.03. Characteristic of O^7-chrysin acetic acid(4-phenyl-1,2,5-oxadiazole-2-oxide-3-)methyl ester **10** (1.53 g, 63% yield, mp: 174–175 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 12.76 (s, 1H), 7.87–7.89 (m, 2H), 7.63–7.65 (m, 2H), 7.49–7.57 (m, 6H), 6.68 (s, 1H), 6.46–6.47 (d, 1H), 6.32–6.33 (d, 1H), 5.31 (s, 2H), 4.76 (s, 2H), EI-MS (*m*/*z*): 486; Anal. Calcd for $C_{26}H_{18}N_2O_8$: C, 64.20; H, 3.73; N, 5.76. Found: C, 64.07; H, 3.98; N, 5.38. Characteristic of O^5-acetyl-O^7-Chrysin acetic acid(4-phenyl-1,2,5-oxadiazole-2-oxide-3-)methyl ester **11** (0.23 g, 43.5% yield, mp: 179–180 °C). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.86–7.88 (m, 2H), 7.65–7.66 (d, 2H), 7.50–7.53 (m, 6H), 7.26 (s, 1H), 6.64–6.66 (d, 2H), 5.33 (s, 2H), 4.78 (s, 2H), 2.43 (s, 3H), EI-MS (*m*/*z*): 528. Anal. Calcd for $C_{28}H_{20}N_2O_9$: C, 63.64; H, 3.81; N, 5.30. Found: C, 63.93; H, 3.97; N, 4.96.
29. Glucose consumption assays: HepG2 cells were plated into 96-well tissue culture plates with some wells left blank. After the cells reached 80–90% confluence, the medium was removed and the same culture medium containing target compounds, chrysin or rosiglitazone (standard product) was added to wells. After 24 h of treatment, glucose was assayed with diagnostic kits, a MTT assay was used to monitor the cell number and to adjust the glucose consumption values. [Zou, X. Q.; Peng, S. M.; Hu, C. P.; Tan, L. F.; Yuan, Q.; Deng, H. W.; Li, Y. J. *Bioorg. Med. Chem.* **2010**, *18*, 3020.]
30. AR inhibitory activity: The inhibitory activity of the compounds on aldose reductase was carried out using a 200 μL volume optimized amount of enzyme (750 μg/mL protein), and different concentrations of the compounds (0.1–10 μmol/L) in 50 mM PBS (pH 6.0) containing 5 mM β-mercaptoethanol, 0.24 mM NADPH, 0.4 M Li_2SO_4 and 2.5 mM of glyceraldehyde (substrate) were added. The reaction was initiated by addition of glyceraldehyde and the

decrease in the optical density of NADPH at 340 nm was recorded for 3 min. [Zou, X. Q.; Peng, S. M.; Hu, C. P.; Tan, L. F.; Yuan, Q.; Deng, H. W.; Li, Y. J. *Bioorg. Med. Chem.* **2010**, *18*, 3020.]

31. Inhibitory activity of AGE formation: To prepare the AGE reaction solution, 10 mg/mL of bovine serum albumin in 50 mM PBS (pH 7.4) was added to 0.2 M glucose, and 0.02% sodium azide was added to prevent bacterial growth. The reaction mixture (950 μL) was then mixed with various concentrations of the target compounds (1–300 μmol/L). After incubating at 37 °C for 14 d, the fluorescence intensity of AGE was determined by a LS-55 fluorospectrophotometer (PE, USA) with excitation and emission wavelengths at 350 nm and 420 nm. [Zou, X. Q.; Peng, S. M.; Hu, C. P.; Tan, L. F.; Yuan, Q.; Deng, H. W.; Li, Y. J. *Bioorg. Med. Chem.* **2010**, *18*, 3020.]
32. Detection of nitrite: A solution of the appropriate compound was added to 2 mL of 1:1 v/v mixture of 50 mM PBS (pH 7.4) with MeOH, containing of 5×10^{-4} M L-cysteine. The final concentration of target compounds was 10^{-4} M. After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of Griess reagent. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 nmol/mL) were used to construct the calibration curve. The results were expressed as the percentage of NO released ($n = 3$) relative to a theoretical maximum release of 1 mol NO/mol of test compound. [Zou, X. Q.; Peng, S. M.; Hu, C. P.; Tan, L. F.; Yuan, Q.; Deng, H. W.; Li, Y. J. *Bioorg. Med. Chem.* **2010**, *18*, 3020.]