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Synthesis and evaluation of nitric oxide-releasing derivatives of 3-*n*-butylphthalide as anti-platelet agents

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ABSTRACT

Most ischemic stroke results from brain blood vessel blockage by platelet-mediated thrombus, and anti-platelet therapy has been demonstrated clinical benefits in the treatment of this disease. In the present work, novel nitric oxide (NO)-releasing derivatives of an anti-ischemic stroke drug 3-*n*-butylphthalide (NBP) were synthesized. Compounds **7a** and **7c** exhibited more potent anti-platelet activity than NBP and aspirin, and released a moderate amount of NO, which is beneficial in improving cardiovascular and cerebral circulation. These findings provide an alternative approach to the development of drugs more potent than NBP for the intervention of ischemic stroke.

According to a report in 2004 from the World Health Organization (WHO), 15 million people are suffering from stroke worldwide annually. Of those, 5 million die, and another 5 million are permanently disabled. Ischemic stroke is the most common cerebrovascular disorder accounting for more than 80% of all strokes, a majority of which are caused by platelet-mediated thrombosis.[1] It is believed that thrombosis forms arterial blockage which leads to inadequate blood supply to the brain tissue, and eventually results in ischemic stroke.[2] It has been generally accepted that the inhibition of platelets aggregation and adhesion plays a crucial role in the treatment of ischemic stroke.[3]

The racemic 3-*n*-butylphthalide (NBP) received approval in China for the treatment of ischemic stroke in 2002. A large number of studies have demonstrated that NBP is able to inhibit platelet aggregation, decrease the brain infarct volume, and enhance microcirculation, thus benefiting patients with ischemic stroke.[4] However, the clinical application is limited due to its moderate potency. It has been found that the therapeutic effects of NBP could be improved by co-administration with another anti-platelet drug.[5]

It is well-known that nitric oxide (NO), a small endogenic gas molecule, plays an important role in regulating physiological functions, including the inhibition of platelet aggregation and thrombus formation in cerebrovascular and cardiovascular systems.[6] During the past few years, our group and others have employed a strategy by which an NO-donor moiety was connected to a 'native' molecule for the purpose of enhancing its therapeutic impact and/or mitigating its adverse effects.[7] Inspired by these successful studies, we coupled compound **1** with NO-donors via various linkers to generate NO-releasing derivatives of NBP (NO-NBP). Compound **1** is a ring-opening derivative of NBP, which could undergo a conversion to NBP very quickly in vivo.[8] The linkers are cinnamic acid derivatives such as ferulic acid and *p*-hydroxyl cinnamic acid with both antioxidant and anti-platelet aggregation activities.[9] We expected that the ester bonds of NO-NBP could be hydrolyzed in vivo by esterases to generate **1**, which would subsequently undergo ring closure to produce NBP, and cinnamic acid derivatives as well as NO (Scheme 1). These bioactive compounds would possess synergistical inhibitory effects on platelet aggregation.

The synthetic routes of NO-NBP are outlined in Scheme 2. Ferulic acid **2a** or *p*-hydroxyl cinnamic acid **2b** was treated with alkanolamine nitrates **3a**–**d**, respectively, in the presence of HOBT, EDCI and DMAP at room temperature to give the corresponding nitrates **4a**–**h** in 68–85% yields. Condensation of **4a**–**h** with

Abbreviations: NO, nitric oxide; HOBT, 1-hydroxybenzotriazole; EDCI, 1-(3-dimethyl aminopropyl)-3-ethylcarbodiimide hydrochloride; DMAP, 4-dimethylaminopyridine; DCC, dicyclohexylcarbodiimide ; ADP, adenosine 5′-diphosphate; PRP, platelet rich plasma.

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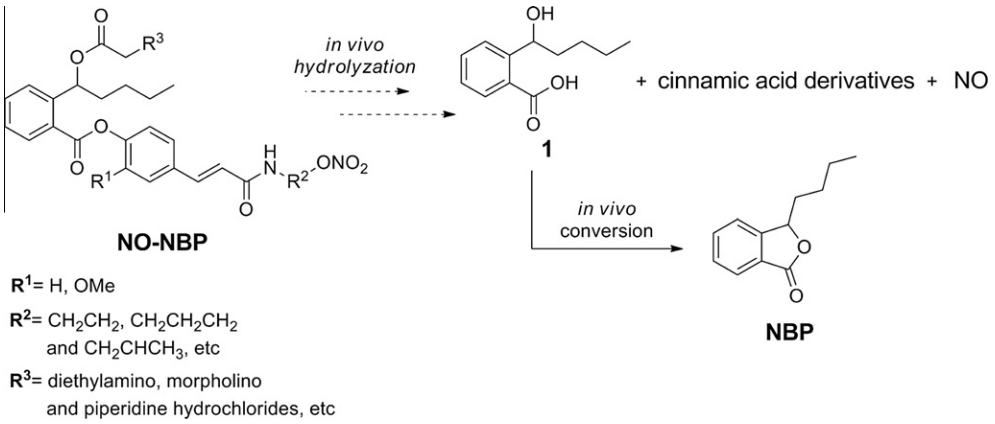
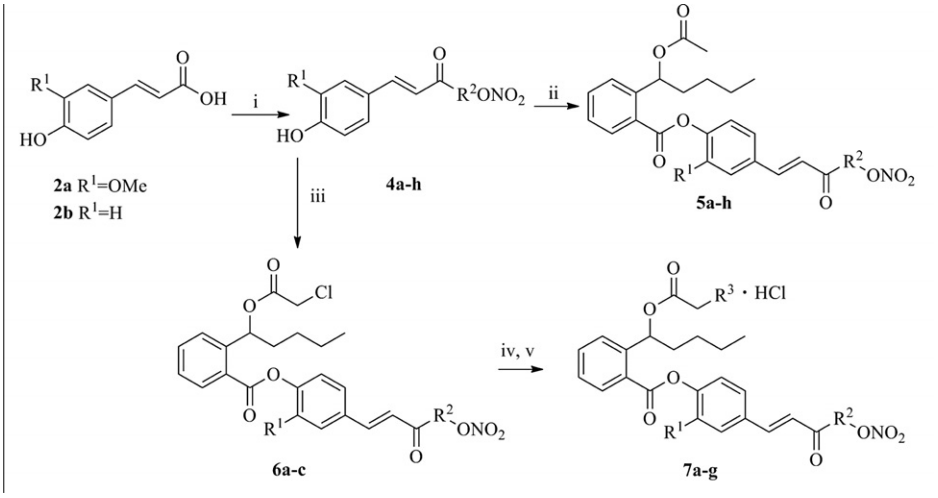


Scheme 1. Proposed metabolic route of NO-NBP.



4-5a R^1= OMe R^2= $NHCH_2CH_2$ **4-5e** R^1= H R^2= $NHCH_2CH_2$ **6a** R^1= H R^2= $NHCH_2CH_2$

4-5b R^1= OMe R^2= $NHCH_2CH_2CH_2$ **4-5f** R^1= H R^2= $NHCH_2CH_2CH_2$ **6b** R^1= H R^2= $NHCH_2CH_2CH_2$

4-5c R^1= OMe R^2= $NHCH_2CHCH_3$ **4-5g** R^1= H R^2= $NHCH_2CHCH_3$ **6c** R^1= OMe R^2= $NHCH_2CH_2$

4-5d R^1= OMe R^2= N CH **4-5h** R^1= H R^2= N CH

7a R^1= OMe R^2= $NHCH_2CH_2$ R^3= diethylamino **7e** R^1= H R^2= $NHCH_2CH_2$ R^3= morpholino

7b R^1= OMe R^2= $NHCH_2CH_2$ R^3= morpholino **7f** R^1= H R^2= $NHCH_2CH_2$ R^3= piperidino

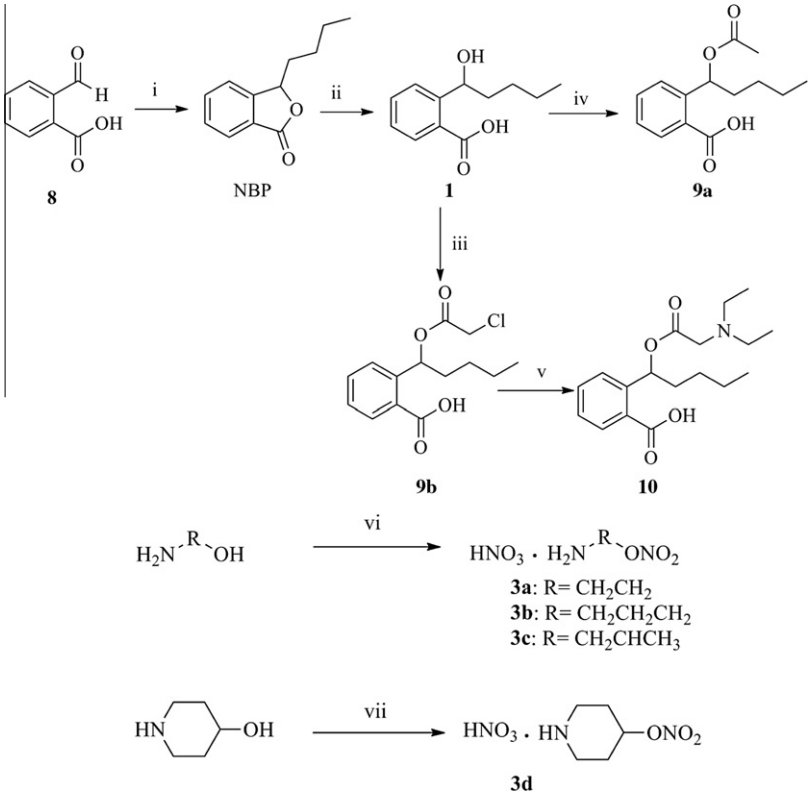
7c R^1= OMe R^2= $NHCH_2CH_2$ R^3= piperidino **7g** R^1=H R^2= $NHCH_2CH_2CH_2$ R^3= diethylamino

7d R^1= H R^2= $NHCH_2CH_2$ R^3= diethylamino

Scheme 2. Synthetic routes of compounds **5a**–**h** and **7a**–**g**. Reagents and conditions: (i) **3**, EDCI, HOBT, DMAP, CH_2Cl_2, rt, 5 h; (ii) **9a**, DCC, DMAP, CH_2Cl_2, rt, 5 h; (iii) **9b**, DCC, DMAP, CH_2Cl_2, rt, 5 h; (iv) diethylamine, piperidine or morpholine, Et_3N, DMF, rt, 8 h; (v) 1 M ethereal HCl, 0 °C, 1 h.

compound **9a** in the presence of DCC and DMAP provided esters **5a**–**h** in 78–83% yields.[10] Similarly, treatment of **4a**, **4e** and **4f** with **9b** offered esters **6a**–**c** in 75–85% yields. Compounds **7a**–**g** were obtained by reaction of **6a**–**c** with diethylamine, piperidine and morpholine, respectively, followed by treatment with anhydrous ethereal HCl below 0 °C.[11,12]

The alkanolamine nitrates **3a**–**d** were prepared by treatment of corresponding alkanolamine with fuming HNO_3 and Ac_2O at −10 °C (Scheme 3). Compounds **9a** and **9b** were obtained starting from reaction of 2-formylbenzoic acid **8** with Grignard reagent, *n*-BuMgBr, followed by acidification to give NBP in 85% yields. And subsequent ring-opening of NBP by saponification formed acid **1** in 92% yield. Finally, acylation of hydroxyl at the side chain of **1** with acetyl chloride or chloroacetyl chloride provided **9a** and **9b** in yields of 77% and 83%, respectively. In addition, compound **10** was obtained by condensation of **9b** with diethylamine in 62% yield



Scheme 3. Synthetic routes of compounds **3a–d**, **9a**, **9b** and **10**. Reagents and conditions: (i) (1) *n*-BuMgBr, Et_2O, –5–0 °C, 5 h; (2) 1 M HCl, RT, 0.5 h; (ii) (1) NaOH, CH_3OH–H_2O, reflux, 0.5 h; (2) 1 M HCl, −10 to 0 °C; (iii) $ClCH_2COCl$, Et_3N, DMAP, CH_2Cl_2, −10 °C; (iv) CH_3COCl, Et_3N, DMAP, CH_2Cl_2, −10 °C. (v) diethylamine, Et_3N, CH_2Cl_2, rt, 12 h; (vi) HNO_3, Ac_2O, CH_2Cl_2, −10 °C; (vii) HNO_3, Ac_2O, Et_2O, −10 °C.

(Scheme 3). All target products were purified by column chromatography and their structures were identified by IR, MS, ^{1}H NMR, and HR-MS.

The inhibitory effects of the target compounds on adenosine 5′-diphosphate (ADP)-induced platelet aggregation in rabbit platelet rich plasma (PRP) were evaluated by using Born's turbidimetric method.[13] As shown in Figure 1, several compounds (**7a**, **7c**, **7f**, and **7g**) at 1.0 mM inhibited ADP-induced platelet aggregation by 83.0%, 83.0%, 82.4% and 82.1%, respectively, significantly stronger than control drugs, NBP (67.8%) and aspirin (ASP) (68.5%). And the most potent compounds **7a** and **7c** inhibited ADP-induced platelet aggregation with IC_{50} values of 54.44 μM and 39.40 μM, respectively.[14]

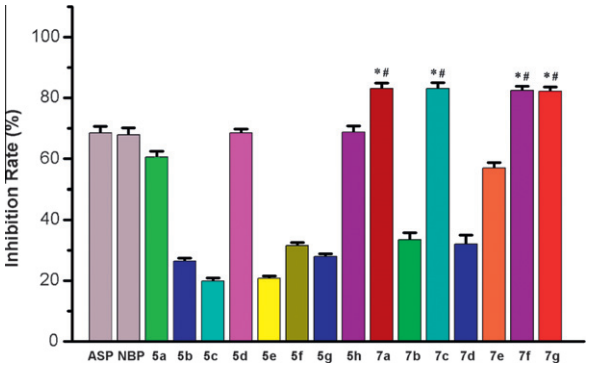


Figure 1. Inhibition of ADP-induced rabbit platelet aggregation by target compounds in vitro. Rabbit platelet suspensions were pre-incubated with tested compounds (1.0 mM) at 37 °C for 5 min and exposed to 10 μM of ADP, followed by continually monitoring. Rabbit platelet suspensions that had been treated with vehicle and exposed to ADP were used as positive controls. Data are expressed as mean ± S.D. of each group ($n = 4$) from several independent experiments. $^*P < 0.05$ versus NBP, $^\#P < 0.05$ versus ASP, determined by Student's *t* test.

Since the structure of compound **7a** is composed of two large moieties, NBP precursor **10** and NO donor **4a** as shown in Chart 1, we further investigated their individual contribution to the overall activity against ADP-induced platelet aggregation in vitro. We found that both **10** and **4a** displayed significant anti-platelet activity (67.2% and 51.3%, respectively), although each moiety was less active than the parent compound **7a** (81.2%), indicating that these two moieties in **7a** may synergistically inhibit the ADP-induced platelet aggregation. In addition, the anti-platelet effect of **7a** was dramatically attenuated by treatment with 20 μM of hemoglobin (He), an endogenous NO scavenger,[15] resulting in reduction in inhibition from 81.2% to 54.4% (Fig. 2). These results strongly support the important role of NO-releasing moiety for anti-platelet effects of NO-NBP.

In order to further explore the relationship between NO released by NO-NBP and their anti-platelet aggregation, the levels

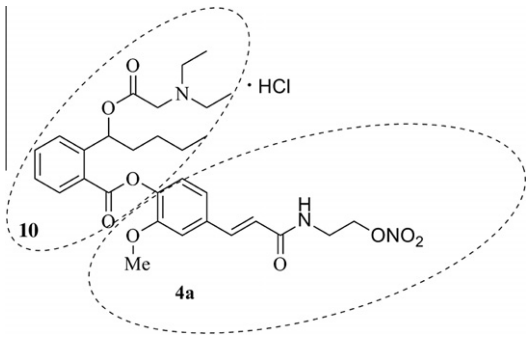


Chart 1. Structure of compound **7a**.

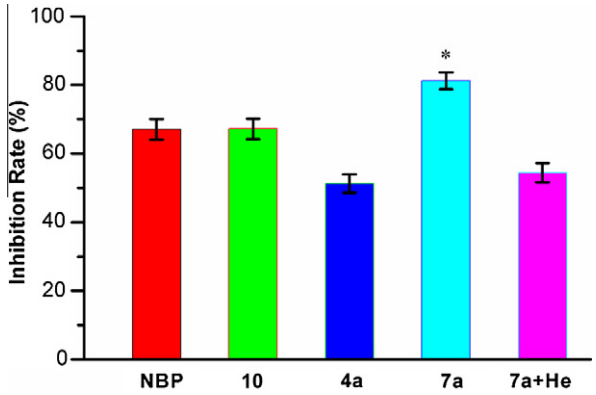


Figure 2. Inhibition of ADP-induced rabbit platelet aggregation by selected compounds in vitro. Rabbit platelet suspensions were pre-incubated with tested compounds (1.0 mM) at 37 °C for 5 min followed by addition of ADP (10 μM). Hemoglobin (20 μM) was added and incubated with the drug-platelet suspension in the indicated group. Data are expressed as mean ± SD from several independent experiments. $^{*}P < 0.05$ versus NBP, determined by Student's *t* test.

of NO produced by **5c**, **5e**, **7a**, **7c** and **7e** were evaluated by Griess assay.[16] It is shown in Figure 3 that the active compounds **7a**, **7c** and **7e** released moderate amount of NO (0.057, 0.060, and 0.043 μg/ml, respectively). In contrast, the less active compounds **5c** and **5e** under the same conditions released lower levels of NO (0.027 and 0.022 μg/ml, respectively). These date suggest that the NO-releasing ability of NO-NBP may be correlated to their inhibitory effects on platelet aggregation ($R^2 = 0.98$, $P < 0.01$, determined by logistic regression analysis).

Analysis of structure–activity relationships (SAR) revealed that the target compounds with different acylation of hydroxyl in the side chain of NO-NBP exhibited varied inhibitory activities on platelet aggregation. Acylation by diethylaminoacetyl or piperidinoacetyl functional group generally led to higher activity than acetylation (**7a** vs **5a**, **7f** vs **5e, 7g** vs **5f**), probably owing to enhanced aqueous solubility and improvement of cell permeability, since our previous studies showed that some NO-NBP compounds structurally similar to **7a** had a greatly improved aqueous solubility in comparison with NBP, and displayed significant capacity to penetrate across blood–brain barrier to retard cerebral injury after ischemia–reperfusion.[17] On the other hand, the carbon chain length of aminoalkyl connecting to carboxyl of ferulic acid or *p*-hydroxyl cinnamic acid moiety of NO-NBP seems correlated to their

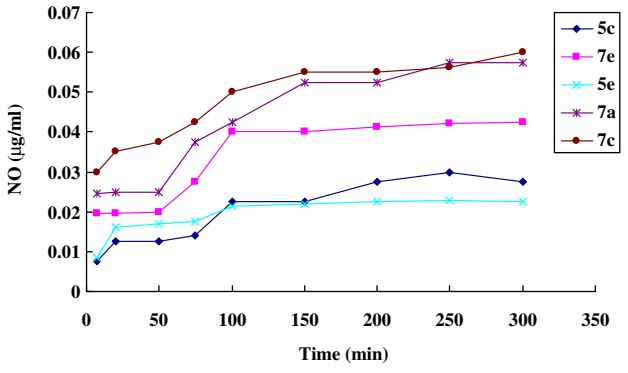


Figure 3. NO-release assay of selected compounds in vitro. NO_2^- concentrations which represent the quantity of NO were determined by Griess assay. Griess reagent can combine with NO_2^- and form chromospheres after 10 min at 30 °C. The absorbance then was measured at 540 nm. The levels of NO produced by individual compounds were calculated, according to the standards of different concentrations of nitrate. Data are expressed as means of individual compounds tested at each time point and intra-group variations were less than 10%.

anti-platelet activity. For example, compounds containing aminoethyl are generally more active than those containing aminopropyl (**5a** vs **5b**, **7a** vs **7g**). It is likely that those various substituents and linkers may have different abilities to modulate the structure, stability, metabolism and penetrability, which affect the NO production and NBP releasing, leading to varied bioactivities of NO-NBP. Indeed, Compound **7a** containing both diethylamino and aminoethyl moieties produced higher level of NO and displayed strongest inhibitory activity among the compounds tested. These preliminary SAR results provide a guideline for further design and development of NO-releasing derivatives of NBP.

Taken together, a novel class of NO-NBP hybrids were synthesized based on our previous studies, and all the target compounds exhibited inhibitory effects on platelet aggregation to various extents. Apparently, the synergic effects of NBP and NO production contribute to their potent activity against platelet aggregation. Compounds **7a** and **7c**, which had a great potency superior to ASP and NBP, may be promising candidates for further intensive study. Furthermore, several NO-NBP produced moderate levels of NO, which are correlated with their anti-platelet aggregation activities. Interestingly, our previous pharmacokinetic study showed that the compound structurally similar to **7a** and **7c** could produce the parent molecule NBP under physiological conditions, which is in accordance with our working assumption.[18] Notably, the two moieties of **7a**, NBP precursor **10** and NO donor **4a**, had moderate anti-platelet aggregation activity, and treatment with NO quencher mitigated the inhibitory effect of **7a** on the ADP-induced platelet aggregation. These results indicate that these two moieties may perform synergistical inhibition on platelet aggregation. As a result, our novel findings may provide a novel framework for the design of new NO-releasing NBP hybrids for the intervention of ischemic stroke.

Acknowledgments

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References and notes

1. Lloyd, J. D.; Adams, R.; Carnethon, M. *Circulation* **2009**, *119*, e21.
2. Stoll, G.; Kleinschnitz, C.; Nieswandt, B. *Blood* **2008**, *112*, 3555.
3. Aleviadou, B. R.; Moake, J. L. *Blood* **1993**, *81*, 1263.
4. (a) Liu, C.; Liao, S.; Zeng, J.; Lin, J.; Li, C.; Xie, L.; Shi, X.; Huang, R. *J. Neurosci.* **2007**, *260*, 106; (b) Zhu, X.; Li, X.; Liu, J. *Eur. J. Pharmacol.* **2004**, *500*, 221; (c) Yan, C.; Feng, Y.; Zhang, J. *Acta Pharmacol. Sin.* **1998**, *19*, 117; (d) Peng, Y.; Zeng, X.; Feng, Y.; Wang, X. J. *Cardiovasc. Pharmacol.* **2004**, *43*, 876.
5. Xu, H. L.; Feng, Y. P. *Acta Pharm. Sin.* **2001**, *36*, 329.
6. Koh, K. K. *Int. J. Cardiol.* **2003**, *87*, 1.
7. (a) Fang, L.; Appenroth, D.; Decker, M.; Kiehntopf, M.; Zhang, Y.; Lehmann, J. *J. Med. Chem.* **2008**, *51*, 713; (b) Huang, Z.; Zhang, Y.; Zhao, L. *Org. Biomol. Chem.* **2010**, *8*, 632; (c) Ling, Y.; Ye, X.; Zhang, Y. *Bioorg. Med. Chem.* **2010**, *18*, 3448.
8. Zhang, Y.; Wang, L.; Zhang, L.; Wang, X. *Drug Dev. Res.* **2004**, *63*, 174.
9. (a) Fang, J.; Zhang, Y.; Chang, X.; Zhao, B.; Jiang, D.; Saito, M.; Li, Z. J. *Agric. Food. Chem.* **2009**, *57*, 8683; (b) Maurya, D. K.; Devasagayam, T. P. *Food Chem.Toxicol.* **2010**, *48*, 3369.
10. *General procedure for the synthesis of the target compounds* **5a–h**: DMAP (0.1 mmol) and appropriate nitrates **4a–h** (0.31 mmol) were added to a mixture solution of acetyl ester **9a** (0.26 mmol) and DCC (0.33 mmol) in dry CH_2Cl_2 (25 mL). The reaction mixture was stirred at room temperature for 24 h. Filtration and removal of the solvent in vacuo afforded the crude product, which was subsequently purified by column chromatography using (PE/EtOAc = 2:1) to give pure **5a–h** in 78–83% yields.
11. *General procedure for the synthesis of the target compounds* **7a–g**: A solution of chloroacetyl ester **9b** (1.0 mmol), DCC (1.25 mmol), DMAP (0.125 mmol) and appropriate nitrates **4a**, **4e** and **4f** (1.1 mmol) in dry CH_2Cl_2 (25 mL) was stirred at room temperature for 24 h. After filtration, the filtrate was evaporated in vacuo and the crude product was purified by column chromatography (PE/EtOAc = 3:1) to afford esters **6a–c**. A solution of appropriate amines (0.45 mmol), Et_3N (0.45 mmol) and **6a** (0.15 mmol) in DMF (15 mL) was stirred at room temperature for 8 h. The solvent was reconstituted in EtOAc and water, and the organic phase was washed with water and brine dried over

anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude product was dissolved in EtOAc (5 mL) and 1 M ethereal HCl (0.5 mL), and left stirring at 0 °C for 1 h to yield pure hydrochlorides **7a–c**. The target compound **7d–g** was obtained under the same procedure.

12. *Analytical data for* **7a**: mp 103–105 °C. ESI–MS: *m*/*z* 586 $[M+H]^+$. IR (KBr): 757, 1035, 1630, 1743, 2957, 3067 cm^{-1}. ^1H NMR ($CDCl_3$, 500 Hz, δ): 0.78 (t, 3H, CH_3, *J* = 6.6 Hz), 1.02 (m, 7H, 2× CH_2 and CH_3), 1.22 (t, 3H, CH_3, *J* = 7.2 Hz), 1.81 (m, 2H, CH_2), 3.22 (m, 4H, 2× NCH_2), 3.33 (m, 2H, NCH_2), 3.63 (m, 2H, $COCH_2N$), 3.78 (s, 3H, OCH_3), 4.51 (t, 2H, CH_2ONO_2, *J* = 7.0 Hz), 6.29 (d, 1H, CH═, *J* = 16.0 Hz), 6.57 (m, 1H, CH), 6.75 (m, 1H, CONH), 7.03 (m, 3H, ArH), 7.35–7.55 (m, 3H, ArH), 7.60 (d, 1H, CH═, *J* = 15.9 Hz), 8.07 (d, 1H, ArH, *J* = 7.8 Hz), 10.58 (br s, 1H, NH^+). ^{13}C NMR ($CDCl_3$, 500 Hz, δ): 165.8, 165.4, 164.3, 151.0, 144.2, 140.1, 138.3, 134.3, 133.5, 130.5, 128.1, 126.6, 126.4, 123.2, 122.4, 120.2, 111.7, 74.1, 72.4, 64.8, 55.9, 51.2, 48.2, 43.3, 40.9, 35.7, 27.2, 21.6, 13.6, 8.7. HR-MS for $C_{30}H_{40}N_3O_9$ ($[M+H]^+$) calcd 586.2765, found: 586.2768. **7c**: mp 113–114 °C. ESI–MS: *m*/*z* 598 $[M+H]^+$. IR (KBr): 755, 1037, 1623, 1739, 2970, 3069 cm^{-1}. ^1H NMR ($CDCl_3$, 500 Hz, δ): 0.85 (t, 3H, CH_3, *J* = 6.3 Hz), 1.15 (m, 4H, 2× CH_2), 1.86∼1.92 (m, 8H, 4× CH_2), 3.5 (m, 4H, 2× NCH_2), 3.68 (m, 2H, NCH_2), 3.81 (m, 2H, $COCH_2N$), 3.85 (s, 3H, OCH_3), 4.60(t, 2H, CH_2ONO_2, *J* = 6.8 Hz), 6.37 (d, 1H, CH = , *J* = 16.0 Hz), 6.65 (m, 1H, CH), 6.78 (m, 1H, CONH), 7.05–7.20 (m, 3H, ArH), 7.39 (m, 1H, ArH), 7.56 (d, 1H, CH = , *J* = 15.9 Hz), 7.62–7.67 (m, 2H, ArH), 8.15 (d, 1H, ArH, *J* = 7.7 Hz), 10.20 (br s, 1H, NH^+). ^{13}C NMR ($CDCl_3$, 500 Hz, δ): 165.3, 165.1, 164.2, 150.9, 142.1, 139.9, 138.3, 134.2, 133.5, 130.5, 128.0, 126.6, 126.3, 123.1, 122.3, 120.2, 111.7, 73.9, 64.8, 55.9, 55.5, 52.8, 48.2, 43.4, 40.8, 35.7, 27.2, 21.9, 21.6, 21.0, 13.6. HR-MS for $C_{31}H_{40}N_3O_9$ ($[M+H]^+$) calcd 598.2765, found: 598.2762.
13. (a) Born, G. V.; Cross, M. J. *J. Physiol.* **1963**, *168*, 178; (b) Jones, M.; Inkielewicz, I.; Medina, C.; Santos-Martinez, M. J.; Radomski, A.; Radomski, M. W.; Lally, M. N.; Moriarty, L. M.; Gaynor, J.; Carolan, C. G.; Khan, D.; O'Byrne, P.; Harmon, S.; Holland, V.; Clancy, J. M.; Gilmer, J. F. *J. Med. Chem.* **2009**, *52*, 6588.
14. Antiplatelet aggregation assays: Blood samples were withdrawn from rabbit carotid artery and mixed with 3.8% trisodium citrate (9:1 v/v), followed by centrifuging at 500 rpm for 10 min. The supernatants were collected and used as platelet rich plasma (PRP). Additional samples were centrifuged at 3000 rpm for 10 min and the supernatants were collected as platelet poor plasma (PPP). The effect of individual compounds on the ADP-induced platelet aggregation was measured by the Born's turbidimetric method using a Platelet-Aggregometer (LG-PABER-I Platelet-Aggregometer, Beijing). Briefly, PRP (240 l) was pre-treated in duplicate with vehicle, different concentrations of individual compounds or the reference drugs for 5 min and exposed to 10 μM of ADP incubated at 37 °C for 5 min. The formation of platelet aggregation was monitored longitudinally by optical density. Platelet aggregation was induced by ADP (final concentration 10 μM). Compounds under study or vehicle alone were added to the PRP samples 5 min before addition of the aggregating agent. The antiplatelet aggregation activity of tested compound individual compounds was evaluated as percent inhibition of platelet aggregation compared to positive controls that had been pre-treated with vehicle alone and exposed with the inducer samples. For most active compounds, The IC_{50} values of most compounds were determined by nonlinear regression analysis and the percent inhibition at the maximal concentration tested (1.0 mM) was calculated.
15. Gong, P.; Cederbaum, A. I.; Nieto, N. *Mol. Pharmacol.* **2004**, *65*, 130.
16. Nitrate/nitrite measurement in vitro: Briefly, 0.1 mM of each compound in phosphate buffer solution (PBS) containing 2% dimethyl sulfoxide and 5.0 mM L-cysteine at pH 7.4 was incubated at 37 °C for 15–300 min and were sampled every 15 min for 120 min and then every 30 min for the remaining time. The collected samples (2 mL) were mixed with 0.5 ml of Griess reagent and incubated at 37 °C for 10 min, followed by measuring at 540 nm. The different concentrations of nitrite were used as standards to calculate the concentrations of NO formed by individual compounds.
17. Zhuang, P.; Ji, H.; Zhang, Y. *Clin. Exp. Pharmacol. Physiol.* **2010**, *37*, e121.
18. Li, N.; Wang, X.; Li, T.; Ji, H.; Zhang, Y.; Qiu, Z.; Zhao, D.; Chen, X. *Xenobiotica* **2011**, **accepted**, DOI: 10.3109/00498254.2011.580385.