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Design, synthesis and cardioprotective effect of a new class of dual-acting agents: Phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates

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Abstract—In this study, a new class of phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates was designed and synthesized. The radical scavenging activities of these newly synthesized compounds **12a–c** were evaluated in PC12 cell survival assays. The NO scavenging activities of these compounds were confirmed in the acetylcholine-induced vasorelaxation assay. Compounds **12a–c** were efficacious in a rat arterial thrombosis model, and were active in ADP- or PAF-induced in vitro platelet aggregation assays, which suggests these compounds also possess anti-thrombotic activity. The beneficial effects of dual-acting agent **12c** were demonstrated on the ischemia-reperfusion induced cardiac infarct size and oxidative change in an in vivo rat model.

1. Introduction

Cardiovascular disease is a major cause of disability and mortality in developed countries. Prolonged reduction in coronary blood flow due to atherosclerotic plaques or vasospasm can result in severe damage to the myocardium, leading to cellular injury and eventual cellular death due to apoptosis and/or necrosis. Myocardial reperfusion by thrombolytic therapy, transluminal angioplasty, and bypass surgery is the first line of treatment for acute myocardial infarction.[1] Although restoration of blood flow to the jeopardized myocardium is a prerequisite for myocardial salvage, reperfusion itself may lead to additional tissue damage, often referred to as reperfusion injury.[2,3] The mechanisms proposed to cause reperfusion injury include formation of oxygen free radicals, calcium overload, neutrophil mediated myocardial and endothelial injury, progressive decline in microvascular flow to the reperfused myocardium and depletion of high-energy phosphate stores.[2,3] Numerous studies suggest that a transient burst of free radicals is produced when the myocardium is reperfused following an episode of ischemia and that free radicals can injure myocytes and endothelial cells. The release of free radicals, in combination with ischemia induced decrease in antioxidant activity renders the myocardium extremely vulnerable.[1–3] Thus, targeting free radicals or improving overall antioxidant defense can be one of the important therapeutic strategies, whereby excess free oxy-radicals produced at the time of reperfusion could be scavenged. It has been reported that a variety of free radical scavengers and antioxidants are capable of ameliorating ischemia-reperfusion injury.[4–10]

Tetrahydro-β-carbolines are naturally occurring substances found in food, alcoholic and non-alcoholic drinks, and fruit-derived products. It has been reported that tetrahydro-β-carbolines are potent antioxidants and may thus be useful for the prevention of diseases associated with oxidative damage.[11–15] Furthermore, it is well recognized that phenolic compounds in foods possess several interesting biological and chemical properties, such as antioxidant activity and the ability to scavenge reactive oxygen species.[16,17] Hence, the phenolic compounds may prevent various diseases associated with oxidative stress, such as cancers, cardiovascular diseases, and inflammation. In addition, phenolic tetrahydro-β-carbolines have been identified as effective free radical scavengers and antioxidants.[13,24]

Keywords: Dual-acting agents; Phenolic tetrahydro-β-carboline RGD peptidomimetics conjugates; Free radicals scavenger; Antithrombotic agent.
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Since acute myocardial ischemia is usually due to a thrombus event in the coronary arteries, causal treatment and secondary prevention always include one or more forms of anti-thrombotic therapies, such as antiplatelet agents, heparins, or fibrinolytic drugs.[1] RGD (Arg-Gly-Asp) containing peptides have been investigated to develop therapeutic agents for treating thrombosis.[18–20] The mechanism of action of RGD peptides is inhibition cell adhesion to extracellular matrix proteins via binding of RGD peptides to the integrin receptors on the cell surface.[21] Several anti-thrombotic agents have been derived from RGD peptides.[18,22] For example, Aggrastat®, an RGD peptidomimetic, has been used clinically for treating patients with thrombosis.[23] In our previous studies, RGD-S, RGD-V, and RGD-F were used as building blocks for modification of the peptide conjugates with anti-thrombotic activity.[24]

However, one of the major potential problems limiting the use of natural peptides as therapeutic agents is their relatively poor stability. In general, such peptides are short-lived molecules that are rapidly degraded by proteases present in any biological fluid, particularly in an inflammatory environment. Therefore, it is important to develop stable peptide analogues that conserve their biological activity.

Recently, we have developed a new class of peptidomimetics containing aza-β^3-amino acid residues.[28–30] Aza-β^3-amino acid monomers, which can be considered as Nα-substituted hydrazino acetic acid monomers, have no chiral center. Substitution of α-amino acid residues by aza-β^3-amino acid residues overcomes stereochemical constraints and may contribute to enhanced proteolytic resistance. The resulting mixed α- and aza-β^3-peptide analogues have nitrogen-enriched peptide backbones, with nitrogen atoms carrying side chains that mimic proteinogenic amino acids.[25–30] More recently, we have investigated the possibility that these new peptidomimetics might replace the unstable natural peptides as therapeutic agents.[30]

Considering the free radical scavenging properties possessed by phenolic tetrahydro-β-carboline and the anti-thrombotic activity demonstrated by RGD peptidomimetics, we sought to link a phenolic tetrahydro-β-carboline moiety to the anti-thrombotic RGD peptidomimetics in the hope that the resulting phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates would exhibit free radical scavenging activity, anti-thrombotic activity, and enhanced stability.

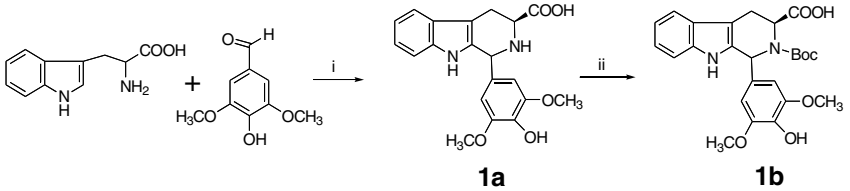
2. Results and discussion

2.1. Synthesis of phenolic tetrahydro-β-carboline

For the synthesis of the phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates, L-tryptophan was first reacted with phenolic aldehyde (Scheme 1). Specifically, syringaldehyde in acidic-aqueous conditions afforded **1** in 46% yield. This reaction occurred through a Pictet-Spengler intramolecular cyclization of the Schiff base to afford the 1,3-disubstituted tetrahydro-β-carbolines. 1,3-Disubstituted tetrahydro-β-carbolines that arise from L-tryptophan and syringaldehyde included two diastereoisomers (1*S*,3*S*-**1** and 1*R*,3*S*-**1**), which were then separated via RP-HPLC. Diastereoisomer assignments were based on acquired positive NOE spectra of H-1 and H-3 signals in the *cis*-isomer. H-1 signals corresponding to the *cis*-isomer appeared at higher field than in the *trans*-isomer, whereas C-1 and C-3 NMR signals corresponding to the *trans*-isomer appeared at higher field than those of the *cis*-isomer. This observation is in agreement with the expected pattern for 1,3-disubstituted tetrahydro-β-carbolines. Subsequent treatment of **1a** with Boc-N_3 gave the N-Boc protected **1b** in 50% yield.[24]

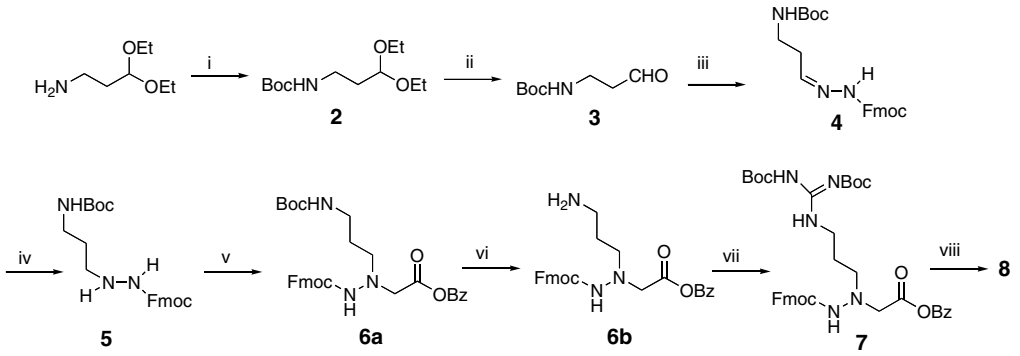
2.2. Synthesis of monomers

For the synthesis of Fmoc-aza-β^3-Arg $(Boc)_2$-OH, it was critical to protect the trifunctional guanidine group due to its strong nucleophilic character. The Goodman reagent *N*,*N*′-di-Boc-*N*″-trifluoromethane sulfonylguanidine was used as a versatile guanidinating reagent. Briefly, starting from the commercially available diethylacetal of 3-aminopropanal, after the treatment with Boc_2O, 3-*t*-butoxycarbonyl aminopropanal **2** was readily obtained in good yield (90%), which was then subjected to hydrolysis and condensation with Fmoc hydrazine to give hydrazone **4**. After the reduction with sodium cynanoborohydride ($NaBH_3CN$), hydrazone **4** was converted to *N*,*N*′-disubstituted hydrazine **5** in 82% yield. Subsequently, after the nucleophilic substitution with benzyl bromoacetate and removal of the NH-Boc protecting group, Fmoc-Aza-β^3-Orn-OBn was obtained in moderate yields (60%), which was then converted to **7** by treatment with guanidinating agent. Finally, deprotection of the benzyl-protecting group with Pd/C (10%) in ethanol proceeded successfully to give the expected Fmoc-aza-β^3-Arg$(Boc)_2$-OH **8** (Scheme 2). In addition, Fmoc-aza-β^3-Gly(Boc)-OH **9** and Fmoc-aza-β^3-Asp(O^{t}Bu)-OH **10** were successfully



1a **1b**

Scheme 1. Synthesis of phenolic tetrahydro-β-carboline. Reagents and conditions: (i) H_2SO_4, 70 °C; (ii) Boc-N_3, 0 °C → rt.



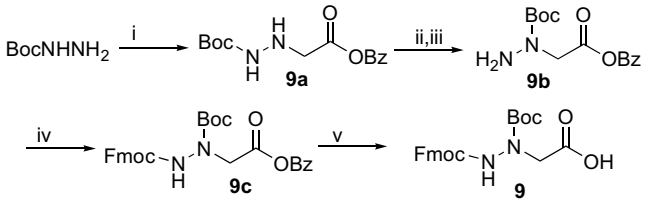
Scheme 2. Synthesis of Fmoc-aza-β^3-Arg $(Boc)_2$-OH. Reagents and conditions: (i) $(Boc)_2O/NEt_3$, 90%; (ii) AcOH, 98%; (iii) $FmocNHNH_2$, 89%; (iv) $NaBH_3CN$, 82%; (v) $BrCH_2CO_2Bn$, K_2CO_3, toluene, 48 h, 60%; (vi) CF_3CO_2H, NEt_3, 95%; (vii) $(BocNH)_2NTf$, NEt_3, 84%; (viii) H_2 (1 atm), Pd/C, 96%.

synthesized according to our previously reported method (Scheme 3).[28,29]

2.3. Solid-phase synthesis of phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates

The targeted phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates **12a–c** were manually synthesized according to our previously reported method. The general procedure for the solid-phase synthesis of hybrid peptides using the Fmoc/*tert*-butyl strategy was depicted in Figure 1.[28,29]

Briefly, the Fmoc-Val-OH Wang resin was first liberated upon treatment with 20% piperidine in dimethylformamide (DMF), for elongating the peptide chain, subsequently the resin was treated with a solution of 4 equiv of Fmoc-α-amino acid or Fmoc-aza-β^3-amino acid activated by diisopropyl-carbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) at room temperature for 30–80 min (depending on the amino acid). Coupling cycles for Fmoc-aza-β^3-amino acids and coupling cycles for α-amino acid with the analogue proceeded from 80 min to 2 h while those for Fmoc-α-amino acids proceeded for 30–60 min. Deprotection cycles were realized with 20% piperidine in DMF. The hybrid peptides were then deprotected and cleaved from the resin with TFA/phenol/TIS/H_2O (88:5:2:5), and after preparative HPLC, the hybrid peptides (25–45% yield) were isolated in >98% purity. All the peptidomimetics were characterized by MALDI-TOF MS.



Scheme 3. Reagents and conditions: (1) $BrCH_2CO_2Bz$, DIEA, toluene, 48 h, 61%; (ii) HClg, NEt_3, 85%; (iii) $(Boc)_2O/NEt_3$, CH_2Cl_2, 60%; (iv) FmocCl, 71%; (v) H_2 (1 atm), 10% Pd/C, 96%.

2.4. Scavenging activities determined by cell survival assay in PC12 cells

The free radical scavenging activities of **12a–c** against NO, H_2O_2, and •OH were evaluated in PC12 cells and compared with that of **1a** using our previously reported method.[24,31–35] The results were expressed as EC_{50} (μM) values.

As shown in Table 1, the EC_{50} values of **12a**, **12b**, and **12c** were found to be similar to that of **1a**, which suggested that they were as effective scavengers of NO, H_2O_2, and •OH as **1a**. The scavenging capacity (EC_{50}) among all of the tested compounds ranged from ~88 to 96 μM for NO, from ~34 to 75 μM for H_2O_2, and ~86 to 95 μM for •OH.

2.5. NO scavenging activity determined using rat aortic strip

The NO scavenging activities of **12a–c** were further evaluated in the acetylcholine (ACh)-evoked, endothelium-mediated relaxation assay according to our previously reported method.[24,33–35] The endothelium controls the tone of the underlying vascular smooth muscle through the production of vasodilator mediators.[36–41] In this experimental model, ACh acts on the endothelium to release nitric oxide (NO), a potent vasodilating mediator. The decreased relaxation response in a rat aortic strip could be attributed to a reduction in NO synthesis by the endothelium. Therefore, the present study was undertaken to assess the NO scavenging capability of these compounds. The results were expressed as the percentage inhibition of acetylcholine (ACh)-induced vasorelaxation by test compounds and are summarized in Table 2. It was observed that ACh-induced relaxation was reversed significantly by compounds **1a** and **12a–c**. At a concentration of 10^{-6} mol/L, compound **1a** showed good inhibition ability with 81.03 ± 4.12%, whereas compounds **12a–c** displayed higher inhibition activities at 90.12 ± 1.63, 95.77 ± 2.93, and 97.15 ± 1.15%, respectively. We hypothesized that the NO scavenging activity of compounds **12a–c** led to attenuation of the NO concentration in vitro, which then led to the inhibition of ACh-induced vasorelaxation.[42–44]

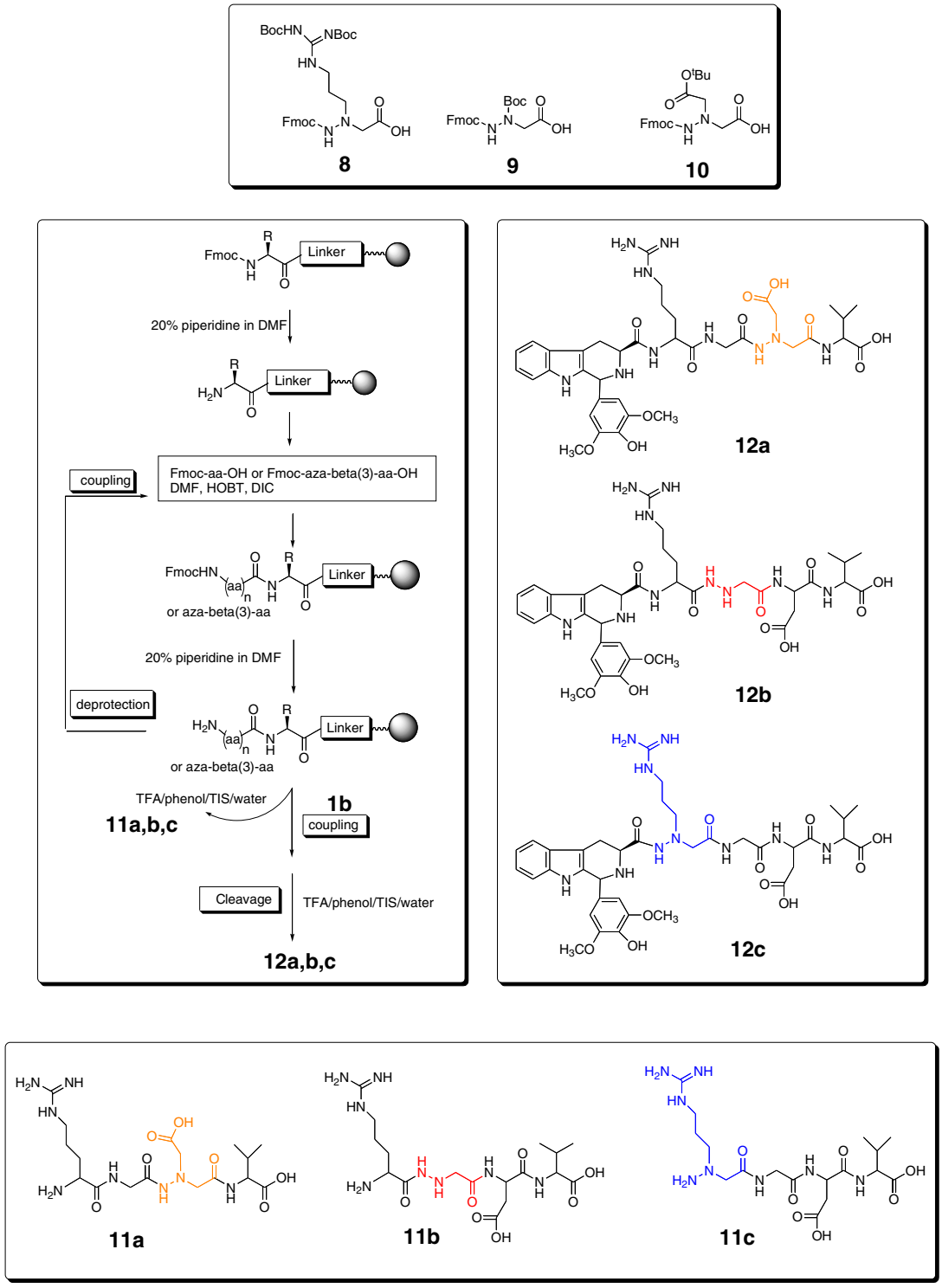


Figure 1. Solid phase synthesis of compounds **11a–c** and **12a–c**.

Table 1. Free radicals scavenging activities in PC12 cell survival assays

Compound	EC_{50} (μM) values ($\bar{x} \pm SD$)		
	EC_{50}/NO	EC_{50}/H_2O_2	EC_{50}/.OH
1a	88.40 ± 1.39	34.52 ± 2.19	85.90 ± 1.59
12a	90.15 ± 2.01	45.68 ± 3.10	93.21 ± 1.67
12b	91.20 ± 1.90	41.20 ± 2.00	90.30 ± 3.09
12c	96.46 ± 3.56	75.35 ± 1.90	95.12 ± 2.03

2.6. Antiplatelet aggregation in vitro

In vitro anti-platelet aggregation activities of compounds **1a**, **11a–c**, and **12a–c** were again evaluated according to our previously reported method.[24,33–35] Platelet-rich plasma was prepared by centrifugation of normal rabbit blood anti-coagulated with sodium citrate at a final concentration of 3.8%. The platelet counts

Table 2. Inhibition of ACh-induced vasorelaxation

Compound	Inhibition percentage ($\bar{x} \pm$ SD%)		
	10^{-6} mol/L	10^{-7} mol/L	10^{-8} mol/L
NS		1.53 ± 1.31	
1a	81.03 ± 4.12[a,b]	55.32 ± 1.52[a,c]	10.81 ± 2.40[a]
12a	90.12 ± 1.63[a,b]	63.72 ± 3.54[a,c]	12.98 ± 2.16[a]
12b	95.77 ± 2.93[a,b]	71.03 ± 0.95[a,c]	14.07 ± 3.92[a]
12c	97.15 ± 1.15[a,b]	75.31 ± 2.45[a,c]	28.30 ± 1.91[a]

[a] In comparison with normal saline NS, $p < 0.001$, $n = 6$.
[b] In comparison with 10^{-7} and 10^{-8} mol/L of the same compound, $p < 0.001$.
[c] In comparison with 10^{-8} mol/L of the same compound, $p < 0.001$.

Table 3. Effect of**1a**, **RGDV**, **11a–c**, and **12a–c** on ADP-induced platelet aggregation

Compound	Am% ($\bar{x} \pm$ SD)		
	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS		55.80 ± 4.32	
1a	54.67 ± 1.88	47.83 ± 3.58[a]	25.35 ± 1.90[a]
RGDV	51.00 ± 2.01	28.76 ± 2.23	11.79 ± 1.34
11a	49.10 ± 2.43	40.08 ± 1.29[a]	18.03 ± 1.32[a]
11b	45.00 ± 2.15[b]	28.20 ± 1.03[a]	12.09 ± 1.47[a]
11c	35.89 ± 0.23[a]	20.20 ± 1.41[a]	6.58 ± 1.55[a]
12a	44.86 ± 2.56[b]	27.46 ± 4.79[a,c]	10.65 ± 2.09[a,c]
12b	52.16 ± 4.02[b]	28.32 ± 2.16[a,c]	15.32 ± 1.29[a,c]
12c	36.95 ± 2.16[a,c]	25.29 ± 1.09[a,c]	10.89 ± 4.01[a,c]

$N = 8$; NS = vehicle.
[a] Compared to NS, $p < 0.001$.
[b] Compared to NS, $p < 0.01$.
[c] Compared to **1a**, $p < 0.001$.

Table 4. Effect of **1a**, **RGDV**, **11a–c**, and **12a–c** on PAF-induced platelet aggregation

Compound	Am% ($\bar{x} \pm$ SD)		
	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS		57.93 ± 3.57	
1a	55.03 ± 1.29	53.40 ± 2.13	37.95 ± 3.56[a]
RGDV	54.23 ± 2.10	34.00 ± 3.23[a]	20.03 ± 2.89[a]
11a	56.15 ± 2.63	37.32 ± 1.69[a]	22.36 ± 2.96[a]
11b	54.93 ± 1.93	36.43 ± 2.94[a]	20.54 ± 2.46[a]
11c	41.03 ± 2.18[a]	22.31 ± 3.25[a]	8.39 ± 1.67[a]
12a	53.01 ± 2.11[b]	33.29 ± 4.10[a]	21.61 ± 2.14[a,c]
12b	50.26 ± 1.79[b]	32.46 ± 2.15[a]	26.30 ± 1.49[a,c]
12c	35.10 ± 1.23[a,c]	24.31 ± 3.39[a,c]	9.18 ± 1.64[a,c]

$N = 8$; NS = vehicle.
[a] Compare to NS, $p < 0.001$.
[b] Compared to NS, $p < 0.01$.
[c] Compared to **1a**, $p < 0.001$.

were adjusted to 2–10^5 per μL through addition of autologous plasma. Platelet aggregation studies were conducted in an aggregometer using the standard turbidimetric technique. The agonists used were platelet activating factor (PAF, final concentration 10^{-7} mol/L) and adenosine diphosphate (ADP, final concentration 10^{-5} mol/L). The effects of compounds **1a**, **11a–c**, **12a–c** on PAF- or ADP-induced platelet aggregation were studied. The maximal rate of platelet aggregation (Am%) was represented by the peak height of the aggregation curve. The obtained data are listed in Tables 3 and 4, respectively.

An anti-platelet aggregation assay was used to evaluate the effects of compounds **12a–c** utilizing in vitro rabbit platelet aggregation induced by adenosine 5-diphosphate (ADP) or platelet activating factor (PAF). The in vitro assays indicated that this new class of phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates was very likely to have potent platelet aggregation inhibition based on in vitro induced platelet aggregation. From Tables 3 and 4, it was noted that compounds **12a–c** displayed a remarkable dual anti-platelet aggregation activity in both ADP- and PAF-induced platelet aggregation assays. For the ADP-induced platelet assay, with **12a–c** at a concentration of 10^{-6} mol/L, the platelet aggregation rates were collectively decreased to ~28–30% (NS: 55.80 ± 4.32; **1a**: 47.83 ± 3.58, $n = 8$, $p < 0.001$). A similar observation was observed in the PAF-induced platelet aggregation assay.

2.7. Evaluation of anti-thrombotic effect in vivo

The anti-thrombotic effects of compounds **11a–c** and **12a–c** were evaluated using an in vivo rat model according to our previously reported methods,[24,33–35] and were expressed as the reduction of thrombus mass. The obtained results are summarized in Table 5. As shown in Table 5, the thrombus weight after treatment with **12a–c** was 4.85 ± 0.93, 3.79 ± 0.29, and 2.23 ± 0.16 mg, respectively (NS: 9.28 ± 1.32, $p < 0.01$). Thus, the anti-thrombotic activities of **12a–c** were higher than those of **11a–c** at a dosage of 5 μmol/kg.

2.8. Stability of 12a–c to trypsin in vitro

Ten milligrams of each of tested compounds **12a–c** were dissolved in 1 mL of phosphate buffer (pH 8), and 0.5 mg of trypsin was added. The reaction mixture was kept at 37 °C and the concentration of the tested compound was monitored every 1 h by HPLC; the mobile phase was 25% CH_3OH in water containing 0.1% CF_3COOH. The results indicated that the depletion of **12a**, **12b**, and **12c** observed after the enzyme promoted hydrolysis was postponed for 4, 3, and 4 h, respectively.

Table 5. Effect of the compounds **1a**, **11a–c** and **12a–c** on thrombus weight ($\bar{x} \pm$ SD)

Compound	Dosage	Wet thrombus (mg)	Dry thrombus (mg)
NS	3 mL/kg	46.03 ± 3.12	9.28 ± 1.32
1a	5 μmol/kg	44.01 ± 2.06	8.03 ± 0.57
RGDV	5 μmol/kg	35.75 ± 2.19	6.78 ± 2.43
11a	5 μmol/kg	41.80 ± 3.23	7.83 ± 1.32[b]
11b	5 μmol/kg	34.98 ± 2.47[b]	7.05 ± 1.25[b]
11c	5 μmol/kg	27.04 ± 1.50[a]	4.39 ± 1.65[b]
12a	5 μmol/kg	25.46 ± 3.19[a]	4.85 ± 0.93[b]
12b	5 μmol/kg	24.54 ± 4.46[a]	3.79 ± 0.29[b]
12c	5 μmol/kg	21.05 ± 2.14[a]	2.23 ± 0.16[a]

$N = 10$.
[a] In comparison with normal saline (NS), $p < 0.001$.
[b] In comparison with NS, $p < 0.01$.

Table 6. Effects of **12c** on myocardial MDA content after reperfusion

Treatment	MDA (nmol/mg prot.)
Sham I/R + vehicle	2.69 ± 0.53
Sham I/R + **12c**	2.52 ± 0.39
I/R + vehicle	4.90 ± 0.87
I/R + **12c**	2.91 ± 0.63[a]

$N = 8$.
[a] Compared with I/R + vehicle ($p < 0.05$).

2.9. Effects of dual-acting agent 12c on MDA formation

Malondialdehyde (MDA), a stable metabolite of the free radical-mediated lipid peroxidation cascade, is widely used as marker of oxidative stress. Recent studies have demonstrated that ischemia-reperfusion of the tissue is associated with lipid peroxidation, which is an autocatalytic mechanism leading to oxidative destruction of the cellular membranes. The destruction can lead to the production of toxic, reactive metabolites and cell death. Lipid peroxidation, as the free radicals generating system, is related to I/R-induced tissue damage, and MDA is a typical indicator of the rate of lipid peroxidation.[45,46] We therefore investigated the MDA levels so as to evaluate the effect of this dual-acting agent (i.e., **12c**) on myocardial I/R damage. As shown in Table 6, after 120 min of reperfusion, MDA content was low in sham-I/R hearts with **12c** treatment. In the I/R groups, without **12c** pretreatment, the MDA content was 4.90 ± 0.87 nmol/mg protein. **12c** reduced the MDA content to 2.91 ± 0.63 nmol/mg protein. Clearly, the levels of MDA significantly increased due to I/R. After administration of the dual-acting agent **12c**, the levels of MDA significantly decreased suggesting that **12c** might cause reduction in lipid peroxidation and cellular injury. We speculated that the protective effect of this newly synthesized dual-acting agent might occur, in part, due to the scavenging of any highly reactive •OH and $OONO^-$.

2.10. Reduction of infarct size with the dual-acting agent 12c

In this study, hearts were arrested by global ischemia for 30 min. The whole ventricle was therefore considered as the area of risk. Percentage of infarct size was significantly reduced for the **12c** treated group compared with the I/R+ vehicle group (Table 7).

I/R increased MDA levels comparative to control and dual-acting agent **12c** administration significantly reduced the increased MDA levels. The cardiac infarct size resulting from I/R was also significantly reduced by the administration of **12c**. These results suggest that this dual-acting agent may reduce ischemia-reperfusion injury induced damage.

Table 7. Summary of infarct size data

Groups	Risk zone (cm^3)	Infarct size (cm^3)	Infarct size/risk zone (%)
I/R + vehicle	46 ± 3	22 ± 3	50 ± 4
I/R + **12c**	48 ± 2	17 ± 2[a]	35 ± 2[a]

$N = 8$.
[a] Compared with I/R + vehicle ($p < 0.05$). Dose = 10 mg/kg.

3. Conclusion

In conclusion, we have demonstrated that phenolic tetrahydro-β-carboline RGD peptidomimetic conjugates **12a–c** have as good free radicals scavenging activity as parental compound **1a** as shown by the EC_{50} values in the PC12 cell survival assay. We have further confirmed the potent NO scavenging activity of compounds **12a–c** in the ACh-induced vasorelaxation assay. In addition, we have shown that **12a–c** are efficacious in a rat arterial thrombosis model and active in ADP- or PAF-induced in vitro platelet aggregation assay, which suggest that compounds **12a–c** also possess anti-thrombotic activity. To explore the possible cardioprotective effect of one of these dual-acting pharmacological agents against oxidative stress during I/R injury of the heart, we have further explored the beneficial effects of **12c** on the I/R-induced cardiac infarct size and oxidative change in an in vivo rat model.

Taken together, the overall beneficial effect of the dual-acting agent probably can be attributed to its activity as a potent free radical scavenger and anti-thrombotic agent. In particular, the protective action of the dual-acting agent most likely can be attributed to the reduction of free radical-mediated lipid peroxidation, thereby enhancing anti-oxidative defense system. In addition, the function of the dual-acting agent as antithrombotic agent may also play a major synergistic role in aiding the recovery of I/R injured tissue.

Since this novel class of potential dual-action agents has anti-thrombotic, free radical scavenging capabilities, and enhanced stability, we expect they may provide beneficial effects in ischemia-reperfusion related injuries.

4. Experimental

4.1. Chemistry

4.1.1. Synthesis of the monomers

4.1.1.1. (1*S*,3*S*)- and (1*R*,3*S*)-1-(4′-hydroxy-3′,5′-dimethoxyphenyl)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (*cis*-1 and *trans*–1). L-Tryptophan (306 mg, 1.5 mmol) dissolved in 0.05 N H_2SO_4 (13 mL) was stirred with the syringaldehyde (1.65 mmol) (Aldrich). The reaction mixture was allowed to react at 70 °C for 6 days, and a precipitate of **1** was obtained (1.1:1) (254 mg, 46%). *cis*-**1**: ^{1}H NMR (CD_3OD + TFA) δ 3.77 (m, 2 H, H-4), 3.97–4.02 [2s, 6H, $(OCH_3)_2$], 4.80 (dd, 1H, H-3, $J_{3,4a}$ = 12.1 Hz, $J_{3,4b}$ = 5.3 Hz), 6.01 (s, 1H, H-1), 6.85, 6.98 (2s, 2H, Ph), 7.2–7.65 (m, 4H, indol); ^{13}C NMR (CD_3OD + TFA) δ 23.73 (C-4), 56.91 (OCH_3), 57.69 (C-3), 60.58 (C-1), 108.30 (C-4a), 108.47 (Ph), 112.56 (C-8), 119.25 (C-5), 120.84 (C-6), 124.04 (C-7), 124.45 (Ph), 127.16 (C-4b), 129.43

(C-9a), 138.78 (C-8a), 149.73, 158.84, 160.24 (Ph), 171.06 (COOH); RP-HPLC-ESI-MS (t_R = 15.95 min) $(M+H)^+$ 369, (M+H-73) 296. *trans*-**1**: ^{1}H NMR (CD_3OD + TFA) δ 4.72 (m, 1H, H-3), 6.22 (s, 1H, H-1); ^{13}C NMR (CD_3OD + TFA) δ 53.60 (C-3), 57.37 (C-1); other signals as *cis*-**1**: RP-HPLC-ESI-MS (t_R = 16.8 min) $(M+H)^+$ 369, (M+H-73) 296.

4.1.1.2. 1-Boc-amino-3,3-diethoxypropane (2). A mixture of Boc_2O (9 g, 40 mmol) in dioxane (40 mL) was added dropwise to a stirred solution of 1-amino-3,3-diethoxypropane (5.52 g, 37 mmol) and Et_3N (4.04 g, 40 mmol) in dioxane (5 mL) at 0 °C. After 2 h, the mixture was allowed to warm to rt. Stirring was continued overnight, and the solvent was evaporated. The residual oil was taken up in water (10 mL), and the mixture was acidified with an aqueous solution of 1 N HCl (pH 3–4) and extracted with EtOAc (60 mL × 3). The organic layer was dried (Na_2SO_4) and evaporated to give a crude oil of 1-Boc-amino-3,3-diethoxypropane 8.89 g (90%) suitable for further work without further purification. ^{1}H NMR ($CDCl_3$) δ 1.25 (t, 6H, J = 8.8 Hz, CH_3), 1.48 (s, 9H, CH_3), 1.85 (q, 2H, J = 6.3 Hz, CH_2), 3.26 (q, 2H, J = 6.1 Hz, CH_2), 3.22–3.77 (m, 6H, CH_2), 4.58 (t, 1H, J = 6.3 Hz, CH), 4.95 (br s, 1H, NH).

4.1.1.3. 3-Boc-aminopropanal (3). A solution of 1-Boc-amino-3,3-diethoxypropane (8.89 g, 36 mmol) in AcOH (15 mL) and H_2O (4 mL) was stirred at rt for 10 h, neutralized with Na_2CO_3, taken up in ether, and washed with brine. The organic phase was evaporated under vacuum to give a yellow oil used as such in the next step (6.31 g, 98%). ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H, CH_3), 2.75 (t, 2H, CH_2), 3.46 (m, 2H, CH_2), 4.95 (br s, 1H, NH), 9.85 (s, 1H, CHO).

4.1.1.4. Fmoc-protected ornithine hydrazine (5). Fmoc carbazate (9.24 g, 36.4 mmol) was added to a stirred solution of 3-Boc-aminopropanal (6.31 g, 36.4 mmol) in CH_2Cl_2 (150 mL) at rt The reaction mixture was stirred for 12 h and concentrated under vacuum to give a crude solid that was triturated with petroleum ether to afford the hydrazone (**4**) as a white solid (13.2 g, 89%). ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H, CH_3), 2.31 (m, 2H, CH_2), 3.11 (m, 2H, CH_2), 4.28 (t, 1H, J = 6.8 Hz, CH), 4.41 (d, 2 H, J = 6.8 Hz, CH_2), 6.91 (br, 1H, NH), 7.28–7.93 (m, 9H, Ar + CH), 10.84 (s, 1H, NH). ^{13}C NMR (DMSO) δ 155.5 (s), 153.2 (s), 146.2 (s), 143.7 (s), 140.72 (s), 127.6 (d), 127.0 (d), 125.1 (d), 120.1 (d), 77.5 (s), 65.4 (t), 46.6 (d), 37.3 (t), 28.8 (t), 28.2 (q).

Fmoc-protected hydrazone (**4**) (3 g, 7.31 mmol) was then reduced with sodium cyanoborohydride (0.55 g, 1.2 equiv). The crude solid was triturated with petroleum ether to afford a white solid of **5** (2.48 g 82%): mp 101 °C; ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H, CH_3), 1.50 (m, 2H, CH_2), 2.68 (m, 2H, CH_2), 2.98 (m, 2H, CH_2), 4.24 (t, 1H, J = 6.9 Hz, CH), 4.32 (d, 2H, J = 6.9 Hz, CH_2), 4.70–4.85 (br s, 1H, NH), 6.78 (br s, 1H, NH), 6.80 (br s, 1H, NH), 7.25–7.90 (m, 8H, Ar). ^{13}C NMR (DMSO) δ 157.2 (s), 155.9 (s), 144.2 (s), 142.9 (s), 128.0 (d), 127.6 (d), 125.6 (d), 120.5 (d), 77.7 (s), 65.8 (t), 47.1 (d), 38.4 (t), 28.6 (q), 28.2 (t). Anal. Calcd for $C_{23}H_{29}N_3O_4$ (411.22): C, 67.12; H, 7.11; N, 10.22. Found: C, 67.22; H, 7.19; N, 10.24.

4.1.1.5. Fmoc-Aza-β^3-Orn(NBoc)-OBn (6a). A mixture of Fmoc-protected ornithine hydrazine (**5**) (3.7 g, 9 mmol), benzyl 2-bromoacetate (2.66 g, 11.6 mmol), toluene (20 mL), and dry K_2CO_3 (870 mg, 0.7 equiv) was refluxed under stirring for 28 h. After the workup followed by flash column chromatography (EtOAc/hexane, 1:3), 3.02 g (60%) of the title compound was obtained as a colorless oil that slowly crystallized in ether: mp 107 °C; ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 9H, CH_3), 1.62 (m, 2H, CH_2), 2.97 (m, 2H, CH_2), 3.24 (m, 2H, CH_2), 3.75 (m, 2H, CH_2), 4.20 (t, 1H, J = 6.9 Hz, CH), 4.50 (d, 2H, J = 6.9 Hz, CH_2), 5.19 (s, 2H, CH_2), 6.88 (br s, 1H, NH), 7.25–7.90 (m, 13H, Ar). ^{13}C NMR (DMSO) δ 171.23 (s), 156.55 (s), 155.50 (s), 144.13, 141.77 (s), 135.54 C (s), 129.13, 129.04, 128.84 (d), 128.14, 127.47, 125.44, 120.40 (d), 79.38 (s), 67.09 (t), 64.47 (t), 57.63 (t), 54.56 (t), 47.67 (d), 38.83 (t), 28.85 (q), 27.83 (t). Anal. Calcd for $C_{32}H_{37}N_3O_6$ (559.27): C, 68.66; H, 6.67; N, 7.51. Found: C, 68.59; H, 6.86; N, 7.28.

4.1.1.6. Fmoc-Aza-β^3-Arg(Boc)$_2$OBn (7). To a solution of **6a** (1 g, 1.79 mmol) in CH_2Cl_2 (5 mL), 5 mL of TFA was added. After stirring at rt overnight, 25 mL of CH_2Cl_2 and 10 mL of water were added. The mixture was neutralized with solid Na_2CO_3 (pH 8–9), and the organic layer was washed with brine and dried over Na_2SO_4. After concentration, without further purification, the crude product **6b** was directly subjected to the next step. **6b** was diluted with 10–15 mL of CH_2Cl_2, and then NEt_3 (1.1 equiv, 251 L) and $(BocNH)_2C$ = NTf (0.9 equiv, 0.64 g) were added. After stirring at rt for 15 h, the solution was sequentially washed with a 2 N aqueous solution of $NaHSO_4$ (10 mL), Na_2CO_3 (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4. After concentration, the crude product was further purified by flash chromatography (hexane/EtOAc, 7:3) to afford the title compound **7** (1.00 g, 80%). ^{1}H NMR ($CDCl_3$) δ 1.39 (s, 18H, CH_3), 1.60 (m, 2H, CH_2), 2.80 (m, 2H, CH_2), 3.32 (m, 2H, CH_2), 3.66 (m, 2H, CH_2), 4.24 (t, 1H, J = 7.1 Hz, CH), 4.45 (d, 2H, J = 7.1 Hz, CH_2), 5.05 (s, 2H, CH_2), 6.95 (br s, 1H, NH), 7.27–7.82 (m, 13H, Ar), 8.28 (br s, 1H, NH), 11.45 (br s, 1H, NH). ^{13}C NMR (DMSO) δ 171.45 (s), 170.80 (s), 163.97 (s), 156.57 (s), 153.59 (s), 144.17 (s), 141.73 (s), 135.67 (d), 129.06, 128.91, 128.78 (d), 128.09, 127.45, 125.47, 120.34 (d), 83.35 (s), 79.48 (s), 66.93 (t), 60.74 (t), 58.04 (t), 53.89 (t), 47.64 (d), 38.83 (t), 28.67 (q), 28.44 (q), 27.45 (t). Anal. Calcd for $C_{38}H_{47}N_5O_8$ (701.34): C, 65.02; H, 6.75; N, 9.98. Found: C, 65.10; H, 6.83; N, 9.98.

4.1.1.7. Fmoc-Aza-β^3-Arg(Boc)$_2$-OH (8). Catalytic hydrogenolysis with 10% Pd/C (50 mg) of **7** (0.86 g, 0.5 mmol) afforded 0.72 g (96%) of the title compound **8** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H, CH_3), 1.39 (s, 9H, CH_3), 1.62 (m, 2H, CH_2), 2.89 (m, 2H, CH_2), 3.38 (m, 2H, CH_2), 3.60 (m, 2H, CH_2), 4.14

(t, 1H, J = 7.1 Hz, CH), 4.40 (d, 2H, J = 7.1 Hz, CH_2), 7.27–7.82 (m, 8H, Ar), 7.95 (br s, 1H, NH), 8.50 (br s,1H, NH), 11.50 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ 171.35 (s), 170.70 (s), 163.90 (s), 157.45 (s), 153.57 (s), 143.96 (s), 141.75 (s), 128.18 (d), 127.52 (d), 125.44 (d), 120.38 (d), 83.35 (s), 79.60 (s), 67.42 (t), 58.04 (t), 53.89 (t), 47.61 (d), 38.83 (t), 28.59 (q), 28.46 (q), 27.47 (t). HRMS m/z calcd for $C_{31}H_{41}N_5O_8$ (M^+): 611.2955, found: 611.2960.

4.1.1.8. Boc-Aza-β^3-Gly-OBn (9a). To a solution of Boc carbazate (2.00 g, 15.13 mmol) in toluene (25 mL) were added diisopropylethylamine (DIEA) (1.96 g, 1 equiv) and benzyl 2-bromoacetate (3.47 g, 1 equiv). The mixture was stirred at 75 °C for 4 days. After filtration and concentration, the residue was further purified by flash chromatography (EtOAc/CH_2Cl_2, 1:9) to give the title compound as a colorless oil (2.60 g, 61%) that slowly crystallized; mp 40 °C. ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H, CH_3), 3.72 (s, 2H, CH_2), 5.19 (s, 2H, CH_2), 6.43 (br s, 1H, NH), 7.31–7.42 (m, 5H, CHar). ^{13}C NMR ($CDCl_3$) δ 171.5 (s), 156.7 (s), 135.8 (s), 129.0 (d), 128.8 (d), 128.7 (d), 80.9 (s), 67.0 (t), 53.2 (t), 28.7(q). HRMS (ESI) m/z calcd for $C_{14}H_{20}N_2O_4Na$ $[M+Na]^+$: 303.1321, found: 303.1336.

4.1.1.9. Aza-β^3-Gly(Boc)-OBn (9b). A solution of Boc-Aza-β^3-Gly-OBn (2.23 g, 7.95 mmol) in CH_2Cl_2 30 mL was saturated by HClg and stirred at rt overnight. The mixture was concentrated and taken up with ether, giving after filtration the hydrochloride of **9a** as a white powder (1.47 g), which was added into a stirred solution of Et_3N (0.76 g, 1.1 equiv) in 20 mL of CH_2Cl_2. Boc_2O (1.47 g, 1 equiv) was added, and the mixture was stirred overnight, concentrated, and taken up with ether. After cooling for 4 h, the hydrochloride of Et_3N was removed by filtration, and the filtrate was concentrated. The crude oil was purified by chromatography on silica gel (EtOAc/hexane, 3:7) to give the title compound (1.15 g, 52%) as colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H, CH_3), 4.17 (s, 2H, CH_2), 5.17 (s, 2H, CH_2), 7.34–7.41 (m, 5H, CHar). ^{13}C NMR ($CDCl_3$) δ 170.4 (s), 158.0 (s), 136.1 (s), 129.2 (d), 129.0 (d), 128.9 (d), 81.9 (s), 67.4 (t), 54.1 (t), 28.8 (q). HRMS (ESI) m/z calcd for $C_{14}H_{20}N_2O_4Na$ $[M+Na]^+$: 303.1320, found: 303.1335.

4.1.1.10. Fmoc-Aza-β^3-Gly(Boc)-OBn (9c). To a stirred solution of **9b** (1.83 g, 6.52 mmol) in THF/water (15/15 mL) was first added solid $NaHCO_3$ (1.09 g, 2 equiv) and then a mixture of FmocCl (2.02 g, 1.2 equiv) in THF (15 mL) was added dropwise. The reaction mixture was stirred overnight at rt, and then ether (50 mL) was added. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated to afford an oil that was further purified by chromatography on silica gel (hexane/EtOAc, 9:1 and 7:3) to give 2.31 g (71%) of the title compound as a white powder: mp 140 °C; ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9 H, CH_3), 4.26 (br s, 2H, CH_2), 4.35 (br s, 1H, CH), 4.47 (br, 2H, CH_2), 5.21 (s, 2H, CH_2), 6.96 (br s, 1H, NH), 7.30–7.79 (m, 13H, CHar). ^{13}C NMR ($CDCl_3$) δ 170.0 (s), 156.5 (s), 155.4 (s), 144.1 (s), 141.9 (s), 135.8 (s), 129.3 (d), 129.2 (d), 129.0 (d), 128.9 (d), 128.5 (d), 125.6 (d), 120.7 (d), 83.1 (s), 68.6 (t), 67.8 (t), 53.5 (t), 47.7 (d), 28.7 (q). HRMS (ESI) m/z calcd for $C_{29}H_{30}N_2O_6Na$ $[M+Na]^+$: 525.2001, found: 525.2016.

4.1.1.11. Fmoc-Aza-β^3-Gly(Boc)-OH (9). Catalytic hydrogenolysis with 10% Pd/C (100 mg) of compound **9c** (0.75g, 1.5 mmol) afforded 0.60 g (95%) of the title compound (**9**) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.47 (m, 9H, CH_3), 4.05–4.38 (br m, 3H, CH + CH_2), 4.52 (d, 2H, J = 6.8 Hz, CH_2), 5.21 (s, 2H, CH_2), 6.96 (br s, 1H, NH), 7.30–7.79 (m, 8H, CHar). ^{13}CNMR ($CDCl_3$) δ 169.8 (s), 156.5 (s), 155.2 (s), 144.0 (s), 141.7 (s), 135.6 (s), 129.1 (d), 128.9 (d), 128.8 (d), 128.3 (d), 127.6 (d), 125.5 (d), 120.5 (d), 82.9 (s), 68.4 (t), 67.6 (t), 53.3 (t), 47.4 (d), 28.49 (q). HRMS (ESI) m/z calcd for $C_{22}H_{24}N_2O_6Na$ $[M+Na]^+$: 435.1532, found: 435.1538.

4.1.1.12. Fmoc-Aza-β^3-Asp(O^{t}Bu)-OH (10). The synthetic procedure was similar to the synthesis of **9**: ^{1}H NMR ($CDCl_3$) δ ppm: 1.51 (s, 9H, CH_3), 3.63 (s, 2H, CH_2), 3.71 (s, 2H, CH_2), 4.23 (t, 1H, CH), 4.56 (d, 2H, CH_2), 7.29–7.80 (m, 8H, CHar). ^{13}C NMR ($CDCl_3$) δ ppm: 171.8, 169.5, 157.2, 143.4, 141.3, 127.8, 127.2, 125.1, 120.1, 82.9, 67.6, 59.2, 58.6, 47.0, 28.1. HRMS (ESI) m/z calcd for $C_{23}H_{25}N_2O_6Na$ $[M+Na]^+$: 449.1689, found: 449.1702.

4.1.2. General procedure for the synthesis of hybrid peptides 11a–c. The hybrid peptide was prepared from 1 g of resin loaded with the Fmoc-Val-OH (0.60 mmol/g) in a manual synthesizer using nitrogen bubbling. The resin was first swelled with DMF for 3 min at a 1.5 mL/min flow rate. After cleavage of the Fmoc group using 20% piperidine in DMF (5 min, 5 mL/min), the resin was washed with DMF for 7 min (5 mL/min). Subsequently, 4 equiv of the appropriate amino acid was dissolved in HOBt/DMF (1.4 mL, 0.6 M) and DIC/DMF (1.4 mL, 0.6 M), and the activated amino acid was then transferred to the reaction vessel. After 30–80 min, the reaction vessel was washed with DMF for 4 min (5 mL/min). The peptide backbone was elongated step by step as described in Figure 1. Finally, the Fmoc protective group was removed by treatment with 20% piperidine in DMF (1 and 5 min, 5 mL/min), and the resin was washed with DMF (7 min, 5 mL/min), removed from the reaction vessel, washed with DCM and ether, and dried in vacuum. The anchored hybrid peptide thus obtained was cleaved from the solid support by treatment with TFA/phenol/TIS/water (88:5:2:5) for 2 h. The mixture was then filtered, and the resin was washed thoroughly with TFA and then with DCM. The total filtrate was concentrated in vacuum to a volume of approximately 1–2 mL, and then cold ether (10 mL) was added to precipitate the peptide. The precipitated peptide was collected by filtration through a fritted glass funnel and dried in vacuum. The crude product was purified by reversed-phase preparative HPLC (XTerra RP18 19 × 300 10 μm). The HPLC fraction was freeze-dried to give the target hybrid peptide as a white fluffy solid in 35–46% yield.

4.1.2.1. H_2N-Arg-Gly-Aza-β^3-Asp-Val-OH (11a). HRMS (ESI) *m/z* calcd for $C_{17}H_{32}N_8O_7Na$ $[M+H]^+$: 484.2292, found: 484.2297.

4.1.2.2. H_2-NArg-Aza-β^3-Gly-Asp-Val-OH (11b). HRMS (ESI) *m/z* calcd for $C_{17}H_{32}N_8O_7Na$ $[M+H]^+$: 484.2292, found: 484.2301.

4.1.2.3. H_2N-Aza-β^3-Arg-Gly-Asp-Val-OH (11c). HRMS (ESI) *m/z* calcd for $C_{17}H_{32}N_8O_7Na$ $[M+H]^+$: 484.2292, found: 484.2295.

4.1.3. General procedure for the synthesis of hybrid peptides 12a–c. The hybride peptide was prepared from 1 g of resin loaded with the Fmoc-Val-OH (0.60 mmol/g) in a manual synthesizer using nitrogen bubbling. The resin was first swelled with DMF for 3 min at 1.5 mL/min flow rate. After cleavage of the Fmoc group using 20% piperidine in DMF (5 min, 5 mL/min), the resin was washed with DMF for 7 min (5 mL/min). Subsequently, 4 equiv of the appropriate amino acid was dissolved in HOBt/DMF (1.4 mL, 0.6 M) and DIC/DMF (1.4 mL, 0.6 M), and the activated amino acid was then transferred to the reaction vessel. After 30–80 min, the reaction vessel was washed with DMF for 4 min (5 mL/min). The peptide backbone was elongated step by step as described in Figure 1. The last Fmoc protective group was removed by treatment with 20% piperidine in DMF (5 min, 5 mL/min), and the resin was washed with DMF (7 min, 5 mL/min). 5 equiv of N-Boc protected phenolic tetrahydro-β-carboline was dissolved in HOBt/DMF and DIC/DMF (1.4 mL, 0.6 M), and the activated Boc protected compound **1b** was then transferred to the reaction vessel. After 80 min of coupling, the targeted hybrid peptide analogues thus obtained were cleaved from the solid support by treatment with TFA/phenol/TIS/water (88:5:2:5) for 2 h. The mixture was then filtered, and the resin was washed thoroughly with TFA and then with DCM. The total filtrate was concentrated in vacuum to a volume of approximately 1–2 mL, and then cold ether (10 mL) was added to precipitate the peptide. The precipitated peptide was collected by filtration through a fritted glass funnel and dried in vacuum. The crude product was purified by reversed-phase preparative HPLC (XTerra RP18 19×300 10μm). The HPLC fraction was freeze-dried to give the target hybrid peptide **12a–c** as a white fluffy solid in 30–38% yield.

4.1.3.1. Phenolic tetrahydro-β-carboline-Arg-Gly-Aza-β^3-Asp-Val-OH (12a). HRMS (ESI) *m/z* calcd for $C_{37}H_{50}N_{10}O_{11}Na$ $[M+H]^+$: 834.3558, found: 834.3560.

4.1.3.2. Phenolic tetrahydro-β-carboline-Arg-Aza-β^3-Gly-Asp-Val-OH (12b). HRMS (ESI) *m/z* calcd for $C_{37}H_{50}N_{10}O_{11}Na$ $[M+H]^+$: 834.3558, found: 834.3572.

4.1.3.3. Phenolic tetrahydro-β-carboline-Aza-β^3-Arg-Gly-Asp-Val-OH (12c). HRMS (ESI) *m/z* calcd for $C_{37}H_{50}N_{10}O_{11}Na$ $[M+H]^+$: 834.3558, found: 834.3564.

4.2. Biology

4.2.1. NO scavenging activity determined using rat aortic strip.[24,33–35] The NO scavenging activity of **12a–c** in the rat aortic strip was examined according to our previously published method. In brief, immediately after decapitation rat aortic strips were obtained and put in a perfusion bath with 5 mL of warmed (37 °C), oxygenated (95% O_2, 5% CO_2) Krebs solution (pH 7.4). The aortic strips were mounted to the tension transducers and the relaxation–contraction curves were recorded. Noradrenaline (NE, final concentration 10^{-9} mol/L) solution was added to induce contraction. When the hypertonic contraction reached the maximum level, the NE was washed away and the vessel strips were stabilized for 30 min. After renewal of the solution, NE (final concentration 10^{-9} mol/L) was added. When the hypertonic contraction value of aortic strips reached their peak, 15 μL of NS or solutions of **12a–c** in 15 μL of water (final concentration 10^{-6} mol/L) were added. Upon stabilization, 1.5 μL ACh (final concentration 10^{-6} mol/L) was added and the percentage inhibition of ACh-induced vasorelaxation by the test compounds was determined.

4.2.2. Evaluation of 12a–c as scavengers of NO, H_2O_2, and •OH in PC12 cells.[24,33–35] Free radical scavenging activities of these newly synthesized compounds **12a–c** were evaluated in PC12 cells using the method of Dawson with minor modifications. In brief, PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% of heat-inactivated horse serum (Hyclone), 5% of fetal bovine serum (GIBCO), 1.0 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C, in 5% CO_2 atmosphere. PC12 cells were seeded in 96-well plates coated with poly-L-lysine at a density of 20,000 cells per well during the exponential phase of growth. After a 24 h attachment period fresh media containing 12.5, 25, 50, 100, or 200 μM of **12a–c**, respectively, were added to each well and were incubated for 1 h. NO damage was then induced by adding 2 mM of sodium nitroprusside (SNP) followed by 2 h of incubation. The media were replaced with fresh media and cells were incubated for 14 h, following which cell survival was measured by a colorimetric assay with MTT according to the method of Mosmann. Similarly, H_2O_2 damage was induced by 1 mM H_2O_2 followed by 1 h of incubation, while •OH damage was induced by 1 mM H_2O_2/30 μM Fe(II) followed by 1 h of incubation. The statistical analysis of the data was carried out by use of the ANOVA test, $p < 0.05$ being considered significant.

4.2.3. In vitro anti-platelet aggregation activity assay.[24,33–35] Platelet-rich plasma was prepared by centrifugation of normal rabbit blood anticoagulation with sodium citrate at a final concentration of 3.8%. The platelet counts were adjusted to 2×10^5/μL by addition of autologous plasma. Platelet aggregation tests were conducted in an aggregometer using the standard turbidimetric technique. The effects of **1a**, **11a–c** and **12a–c** on PAF (final concentration 10^{-5}– 10^{-7} mol/L) or ADP (final concentration 10^{-5}–10^{-7} mol/L) induced platelet aggregation were observed. The maximal rate of platelet aggregation (Am%) was represented by the peak height of the aggregation curve.

4.2.4. In vivo antithrombotic activity in rat model.[24,33–35] The assessments described herein were performed based on a protocol reviewed and approved by the Ethics Committee of HeBei Medical University. The committee assured that the welfare of the animals was maintained in accordance with the requirements of the animal welfare act and according to the guide for care and use of laboratory animals. The tested compound was dissolved in NS just before use and kept in an ice bath. Male Wistar rats weighing 250–300 g were anaesthetized with sodium pentobarbital (80.0 mg/kg, ip) and the right carotid artery and left jugular vein were separated. A 6 cm thread with exact weight was put into the middle of the polyethylene tube. The polyethylene tube was filled with sodium heparin (50 IU/mL of NS) and one end was inserted into the left jugular vein. From the other end of the polyethylene tube sodium heparin was injected as anticoagulant, NS or the solution of the tested compound in NS was injected and the polyethylene tube was inserted into the right carotid artery. The blood was flowed from the right carotid artery to the left jugular vein via the polyethylene tube for 15 min. The thread was taken out and weighed and the weight of the wet thrombus was recorded. The thread was kept in desiccator for 2 weeks and the weight of the dry thrombus was recorded. The data are listed in Table 5.

4.2.5. Ischemia–reperfusion model preparation.[8,9,46] Male Wistar rats weighing 250–300 g underwent myocardial ischemia by a temporary occlusion of the left coronary artery as previously described. Briefly, rats were anaesthetized with sodium pentobarbital (80.0 mg/kg, ip) and placed on an operating table. Polyethylene catheters were inserted into the common femoral artery for the measurement of blood pressure and heart rate, and from the internal carotid artery into the left ventricle for measurement of left ventricular maximum systolic pressures (LVSP) and the maximal first derivative of developed pressure (dP/dt_{max}). After tracheotomy, the animals were ventilated with room air using small rodent respirator. The chest was opened and the ribs were gently spread. The heart was quickly expressed out of the thoracic cavity, inverted and a 4–0 silk ligature was placed under the left coronary artery. The heart was repositioned in the chest and the animal was allowed to recover for 15 min. A small plastic snare was threaded through the ligature and placed in contact with the heart. Tightening the ligature could occlude the artery and reperfusion was achieved by releasing the tension applied to the ligature (I/R groups). Sham I/R animals underwent all the above described surgical procedures, apart from the fact that the 4–0 silk, passing around the left coronary artery, was not tied (Sham I/R group). Animals were infused with **12c** (10 mg/kg) by vehicle (dimethyl sulfoxide −0.9% NaCl, 1:103; v/v) via the transjugular route 15 min before coronary occlusion. The coronary artery was occluded for 30 min followed by 120 min reperfusion and the animals were randomized in the following groups: (1) Sham I/R + vehicle; (2) Sham I/R + **12c**; (3) I/R + vehicle; (4) I/R + **12c**.

4.2.6. Evaluation of infarct size.[8,9] Hearts to be used for infarct size calculations were taken at the end of the experiment. The coronary artery was again briefly occluded through tightening the ligature that remained at the site of the previous occlusion. Immediately after the ligation, 1% Evans blue solution was infused through the catheter into the beating left ventricular cavity to delineate the ischemic area at risk (underperfused and then reperfused area) of the left ventricle. Hearts were quickly taken out and put at −20 °C for subsequent processing. Frozen hearts (including only ventricular tissue) were sliced transversely in a plane perpendicular to the apical–basal axis into 1 mm-thick sections, the sections were then placed in 1% triphenyltetrazolium (TTC) and incubated for 15 min at 37 °C in a dark room. The heart was divided into ischemic but viable (TTC-stained) and infarcted (TTC-unstained) zones within the underperfused and then reperfused area (Evans blue-unstained) and the non-ischemic area (Evans blue-stained).

4.2.7. Estimation of malondialdehyde (MDA) formation.[8,9,46] Malondialdehyde (MDA) was assayed as described previously to monitor the development of oxidative stress.[8,9,46] Hearts to be used for MDA content detection were taken at the end of the experiment and quickly put into ice saline to flush residual blood. Ischemic myocardial tissues were sheared and a 10% homogenate was produced in the ice bath. An improved Grass technique and TBA technique were partly adopted to detect myocardial MDA content.

4.3. Statistical analysis

A two-way ANOVA followed by Scheffé's test was first carried out using the Origin Program to test for differences between groups. If differences were established, the values were compared using Student's *t*-test for paired data. The values were expressed as means ± SE. The results were considered significant if p was <0.05.

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