Synthesis and Biological Activity of Nitronyl Nitroxide Containing Peptides

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[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetic acid (**4**), a nitronyl nitroxide, and its peptide derivatives, *N*-[1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetyl-ARPAK (**9a**), -GRPAK (**9b**), and -QRPAK (**9c**), were synthesized and characterized. Judging from the results of electron spin resonance analysis, the newly synthesized nitronyl nitroxide containing peptides, **9a**, **9b**, and **9c**, demonstrated the characteristics of free radicals. The free radical scavenging activities of **9a**, **9b**, and **9c** were assessed using in vitro free radical scavenging tests. The thrombolysis effect of **9a**, **9b**, and **9c** was evaluated using an euglobulin clot lysis test, a fibrinolytic lysis test, and in vivo thrombolysis tests. Results indicated that these nitronyl nitroxide containing peptides possessed both free radical scavenging activity and thrombolytic activity.

Introduction

A number of previous studies suggested that the impact of nitric oxide (NO) on an ischemic brain injury was dependent on the stage of tissue damage.[1,2] In the early stages following cerebral ischemia (<2 h), NO was found to be beneficial by promoting collateral circulation and microvascular flow. However, a glutamate−NO pathway may occur in ischemic penumbra, the risky region for infarction, by which large amounts of NO are produced by neural nitric oxide synthase (nNOS) and might contribute to the metabolic deterioration of the penumbra leading to a larger infarction.[1] At later times (>6 h), the expression of inducible nitric oxide synthase (iNOS) is triggered and the large amounts of NO thus produced by this enzyme accelerates the progression of the tissue damage.[2] Considerable evidence suggests that NO is also involved in ischemia/reperfusion brain injury.[3] Therapeutic strategies have attempted to reduce the damage either by intervening in the formation process of NO or by scavenging NO already formed.

Nitronyl nitroxides, which were first synthesized more than 30 years ago,[4] have recently received considerable attention because of their capability to trap NO.[5,6] Among other biological activities, nitronyl nitroxides were found to be effective against various viral infections and endotoxin shock.[7−13] In addition, nitronyl nitroxides were found to react with free radicals such as $^{\bullet}OH$, $H_2O_2^{\bullet}$, and $O_2^{\bullet}$, protecting endothelial cells from the attack of free radicals.[14,15]

In addition to NO, thrombogenesis and thrombolysis are also known to play an important role in ischemic brain injuries.[1−4]

A pentapeptide, Ala-Arg-Pro-Ala-Lys (ARPAK), was isolated from products degraded by plasmin from fibrinogen β chain and identified as the active fraction responsible for increased microvascular permeability in rat and human skin.[16,17] We have since searched for other potential peptides of low molecular weight possessing the same biological effect, thrombolytic activity. Gly-Arg-Pro-Ala-Lys (GRPAK) and Gln-Arg-Pro-Ala-Lys (QRPAK) have been reported by our research group.[18−22]

Considering the NO scavenging activity exhibited by nitronyl nitroxides and the thrombolytic activity demonstrated by ARPAK, GRPAK, and QRPAK, the present study attempted to link a nitronyl nitroxide, [1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetic acid (**4**), to the respective thrombolytic oligopeptides hoping that the resulting nitronyl nitroxide containing peptides would exhibit both NO scavenging activity and thrombolytic activity and thus be beneficial for the prevention of ischemia/reperfusion brain injuries.

Results

Synthesis of [1-(1′,3′-Dioxyl-4′,4′-5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]- oxyacetic Acid (4). The compound 2,3-bis-(hydroxylamino)- 2,3-dimethylbutane was condensed with salicylaldehyde (c) according to the synthetic route depicted in Scheme 1 to provide 1,3-dihydroxy-2-(4′-hydroxy)-phenyl-4,4,5,5-tetramethylimidazolidine (**1**), and the chemical yield was 51%. Oxidation of **1** by lead dioxide resulted in **2** with a yield of 52%. The reaction of **2** and $BrCH_2COOC_2H_5$ provided [1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol- 2-yl)phenyl-4-yl]oxyacetic ethyl ester (**3**), and the yield was 90%. In the presence of NaOH (2 mol/L), **3** was converted to its corresponding acid **4**.

Synthesis of [(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]- oxyacetic Acid Containing ARPAK, GRPAK, and QRPAK (9a, 9b, and 9c). Protected peptide intermediates, **6a**, **6b**, and **6c**, respectively, were prepared as shown in Scheme 2 and the chemical yields were found to be in the range of 83−96%. Preparation of the intermediates started

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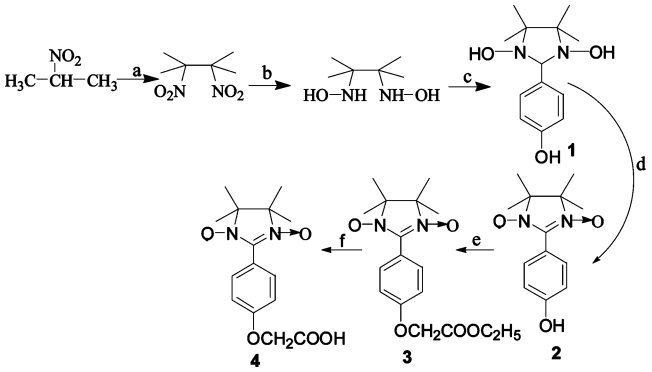
Scheme 1. Preparation of [1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetic Acid (**4**)[a]



[a] Reagents: (a) Br_2, NaOH (6 mol/L); (b) Zn, NH_4Cl; (c) HO−C_6H_4CHO; (d) PbO_2; (e) $BrCH_2COOC_2H_5$, NaOEt, THF; (f) NaOH (2 mol/L).

from the C-terminal, and *L*-Lys(Z)OBzl was the starting material. In the presence of hydrogen fluoride (HF), compounds **6a**, **6b**, and **6c** were converted to **7a**, **7b**, and **7c**, and chemical yields were 90%, 94%, and 94%, respectively. After removal of the Boc protecting group, **6a**, **6b**, and **6c** were coupled with compound **4** to give compounds **8a**, **8b**, and **8c**, and chemical yields were 92%, 90%, and 90%, respectively. The protective group on the side chain of the amino acid residue was removed with anhydrous HF. The resulting products were initially colorless. However, if left in air, they may turn into blue after a couple of hours. After purification by chromatography using Sephadex G-10, [*N*-(1′,3′-dioxyl-4′,4′,5′,5′- tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]-oxyacyl-ARPAK (**9a**), -GRPAK (**9b**), and -QRPAK (**9c**) were obtained and chemical yields were 84%,75%, and 80%, respectively.

Electron Spin Resonance (ESR) Spectra of 4, 8a−c, and 9a−c and Their Reactivity with NO. The ESR spectra of **4**, **8a−c**, and **9a−c** were obtained at two different concentrations, 10^{-7} and 10^{-5} mol/L, in water and phosphate buffer (pH 7.4), respectively. They all exhibited free radical characteristics with spectra showing a five-line pattern at a 1:2:3:2:1 ratio, and there was no difference observed among **4**, **8a−c**, and **9a−c**. The characteristic spectra resulted from the interaction of a free electron with the two nitrogens in the nitronyl nitroxides. The preparations of **4** and **9a−c** were then exposed to NO gas, and ESR spectra were recorded again. It was found that the spectra changed to a seven-line pattern, which suggests that the nitronyl nitroxide had been converted into its corresponding imino nitroxides. The ESR spectra of **9a** in water and phosphate buffer (pH 7.4), prior to and after NO gas was introduced, are provided in Scheme 3 as an example.

Scavenging Activities Determined in PC12 Cells. The free radical scavenging activities of **9a**, **9b**, and **9c** against NO, $H_2O_2^{\bullet}$, and $^{\bullet}OH$ were evaluated in PC12 cells and compared with that of **4** using a published method[23] with minor modifications. The results are expressed as EC_{50} (μM) values. As shown in Table 1, the EC_{50} values of **9a**, **9b**, and **9c** were found to be similar to that of **4**, suggesting that they were effective scavengers of NO, $H_2O_2^{\bullet}$, and $^{\bullet}OH$ as **4**.

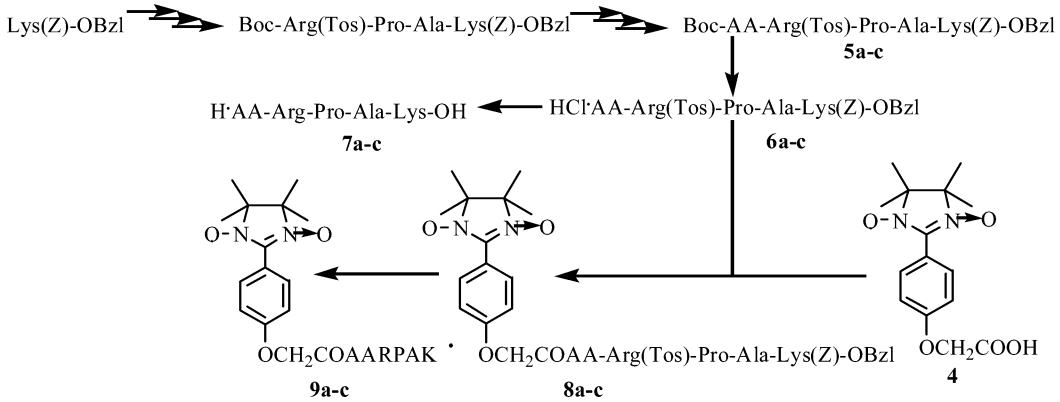
NO Scavenging Activity Determined Using Rat Aortic Strip. The NO scavenging activity of **4, 7a−c**, and **9a−c** was also determined using rat aortic strip according to a published method.[25] The results expressed as the percentage inhibition of acetylcholine (ACh)-induced vasorelaxation by test compounds are summarized in Table 2. The results indicated that **9a**, **9b**, and **9c** significantly inhibited ACh-induced vasorelaxation as **4**, while **7a**, **7b**, and **7c** had little effect.

Determination of Euglobulin Clot Lysis Time (ECLT). The ECLT of **9a−c** was determined using rabbit euglobulin clots prepared according to the literature[26,27] and was compared with that of **7a−c.** Normal saline (NS) and **4** were tested as the controls. As shown in Table 3, the results indicated that like **7a−c**, **9a−c** significantly shortened the ECLT in comparison with NS or **4**.

Fibrinolytic Activity. The fibrinolytic activities of **9a−c** were determined according to a published procedure[28] and expressed by lysis area. The results were compared with those of **7a−c** and urokinase (UK). NS and **4** were used as the controls. Data provided in Table 4 indicated that like UK and **7a−c**, **9a−c** significantly enlarged fibrinolytic lysis areas in comparison with NS and **4**.

Thrombolytic Activity. The thrombolytic activity of **9a−c** determined according to literature[19] was expressed as the reduction of thrombus mass and compared with that of **7a−c**. The results are summarized in Table 5. It was found that like **7a−c, 9a−c** significantly reduced thrombus mass and the effect is dose dependent.

Scheme 2. Preparation of **7a−c**, **8a−c**, and **9a−c**[a]



[a] AA = Ala in **7a**, **8a**, and **9a**; AA = Gly in **7b**, **8b**, and **9b**; AA = Gln in **7c**, **8c**, and **9c**.

Scheme 3. The ESR Spectra of 9a at 10^{-5} mol/L in Water (a) and in Phosphate Buffer (pH 7.4) (b), Prior to (Panel A) and After (Panel B) the Introduction of NO

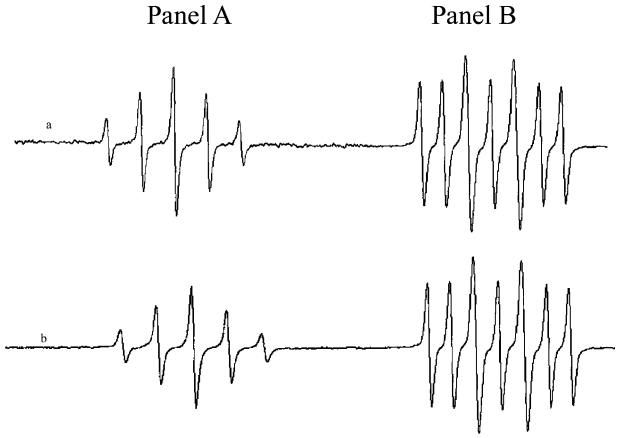


Table 1. The Free Radical Scavenging Activities of **4** and **9a**−**c**

compd	EC_{50} (μM) values (±SD)[a]		
	NO	H_2O_2•	•OH
4	91.20 ± 2.23	30.18 ± 2.12	99.87 ± 2.35
9a	89.09 ± 2.02	28.05 ± 1.99	98.08 ± 2.41
9b	92.34 ± 2.11	25.39 ± 2.04	95.38 ± 1.86
9c	91.76 ± 2.00	74.62 ± 1.97	94.17 ± 2.53

[a] SD = standard deviation, $n = 6$.

Table 2. Inhibition of ACh-Induced Vasorelaxation

compd	% $\bar{X}$ ± SD	compd	% $\bar{X}$ ± SD
NS	1.6 ± 1.4	**9a** (10^{-8} mol/L)	66.8 ± 2.7[a]
4 (10^{-6} mol/L)	99.2 ± 1.4[a]	**9b** (10^{-6} mol/L)	96.0 ± 1.4[a,b]
7a (10^{-6} mol/L)	9.5 ± 7.2	**9b** (10^{-7} mol/L)	80.9 ± 1.9[a,c]
7b (10^{-6} mol/L)	9.2 ± 8.5	**9b** (10^{-8} mol/L)	69.3 ± 2.2[a]
7c (10^{-6} mol/L)	8.9 ± 8.3	**9c** (10^{-6} mol/L)	95.6 ± 0.8[a,b]
9a (10^{-6} mol/L)	92.9 ± 3.2[a,b]	**9c** (10^{-7} mol/L)	80.1 ± 1.9[a,c]
9a (10^{-7} mol/L)	78.6 ± 3.0[a,c]	**9c** (10^{-8} mol/L)	68.3 ± 2.1[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. Euglobulin Clot Lysis Time

compd	dosage (μg)	$\bar{X}$ ± SD (min)
NS		204.03 ± 14.55
UK	5 IU	125.65 ± 16.76[a]
4	0.8	218.56 ± 15.34
7a	1.4	96.38 ± 14.84[a,b]
7b	1.3	91.09 ± 14.98[a,b]
7c	1.5	93.01 ± 13.97[a,b]
9a	2.2	104.12 ± 14.22[a−c]
9a	1.1	123.32 ± 12.25[a]
9a	0.6	145.61 ± 11.98[a,d]
9b	2.1	105.01 ± 14.40[a−c]
9b	1.1	126.51 ± 11.87[a,d]
9b	0.6	147.22 ± 12.37[a]
9c	2.3	100.09 ± 14.35[a−c]
9c	1.1	117.96 ± 13.01[a,d]
9c	0.6	136.00 ± 12.44[a]

[a] In comparison with NS and **4**, $p < 0.001$, $n = 6$. [b] In comparison with urokinase (UK), $p < 0.05$. [c] In comparison with 1.1 and 0.6 μg of the same compound, $p < 0.05$. [d] In comparison with 0.6 μg of the same compound, $p < 0.05$.

Discussion

[(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetic acid (**4**) was successfully introduced into the N-terminal of ARPAK (**7a**), GRPAK (**7b**), and QRPAK (**7c**), resulting in **9a**, **9b**, and **9c**, respectively. The ESR spectrometry revealed the existence of unpaired electrons, suggesting that **9a**, **9b**, and **9c** were of the same free radical characteristics as **4**. In addition, it was shown that they are reactive with NO as demonstrated by the ESR results (Scheme 3). It is important to note that **9a**, **9b**, and **9c** retained the free radical scavenging activity of **4** against NO, H_2O_2•, and •OH and the thrombolytic activity of **7a**, **7b**, and **7c**. Compounds **9a**−**c** demonstrated similar free radical scavenging activity as **4** as demonstrated by the EC_{50} values. The euglobulin lysis test, fibrinolytic lysis test, ACh-induced vasorelaxation test, and thrombolysis tests indicated that **9a**, **9b**, and **9c** also possess thrombolytic activity similar to that of **7a**, **7b**, and **7c**.

The results showed that the coupling of a nitronyl nitroxide of free radical scavenging property with a peptide of thrombolytic activity provided a means of synthesizing compounds with dual properties, free radical scavenging property and thrombolytic activity.

It is known that both NO and thrombogenesis are important risk factors in ischemic brain injuries. The compounds synthesized in this study may be beneficial for the prevention of ischemia/reperfusion brain injuries.

Table 4. Determination of Lysis Areas

compd	dosage (μg)	$\bar{X}$ ± SD (mm^2)
NS		24.05 ± 9.98
UK	5 IU	224.05 ± 10.17[a]
4	0.8	23.96 ± 9.33
7a	1.4	212.78 ± 10.54[a]
7b	1.3	230.54 ± 9.95[a]
7c	1.5	223.99 ± 9.86[a]
9a	0.8	215.23 ± 9.65[a,b]
9a	0.4	194.54 ± 8.98[a,c]
9a	0.2	172.33 ± 10.22[a]
9b	0.7	217.35 ± 9.57[a,b]
9b	0.4	196.87 ± 10.34[a,c]
9b	0.2	175.04 ± 10.11[a]
9c	0.8	230.15 ± 9.68[a,b]
9c	0.4	215.12 ± 8.99[a,c]
9c	0.2	195.38 ± 10.14[a]

[a] In comparison with NS and **4**, $p < 0.001$, $n = 6$. [b] In comparison with 0.4 and 0.2 μg of the same compound, $p < 0.05$. [c] In comparison with 0.2 μg of the same compound, $p < 0.05$.

Table 5. Reduction of Thrombus Mass

compd	dosage	$\bar{X}$ ± SD (mg)
NS	(3 mL/kg)	15.11 ± 3.70
UK	20 000 IU/kg	25.40 ± 2.50[a]
4	10.0 (μmol/kg)	15.89 ± 2.53
7a	10.0 (μmol/kg)	18.54 ± 3.28[c]
7b	10.0 (μmol/kg)	25.82 ± 2.15[a]
7c	10.0 (μmol/kg)	21.17 ± 3.06[b]
9a	15.0 (μmol/kg)	26.88 ± 1.99[a,f]
9a	10.0 (μmol/kg)	19.91 ± 3.59[c,g]
9a	5.0 (μmol/kg)	16.27 ± 1.98
9b	15.0 (μmol/kg)	26.64 ± 1.85[a,f]
9b	10.0 (μmol/kg)	19.70 ± 3.76[c,g]
9b	5.0 (μmol/kg)	16.34 ± 1.96
9c	10 (μmol/kg)	28.85 ± 3.80[a,d,e,g]
9c	5 (μmol/kg)	20.43 ± 1.84[b,h]
9c	1 (μmol/kg)	16.40 ± 3.40

[a] In comparison with NS, $p < 0.001$, $n = 10$. [b] In comparison with NS, $p < 0.01$. [c] In comparison with NS, $p < 0.05$. [d] In comparison with **7a** and **7c**, $p < 0.001$. [e] In comparison with UK and **7b**, $p < 0.05$. [f] In comparison with 10 and 5 μmol/kg of the same compound, $p < 0.001$. [g] In comparison with 5 and 1 μmol/kg of **9c**, $p < 0.001$. [h] In comparison with 1 μmol/kg of **9c**, $p < 0.001$.

Experimental Section

General. The protected peptides were of the L-configuration. The purity of all of the products was checked by thin-layer chromatography (TLC) (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness) and high-performance liquid chromatography (HPLC) (Waters, C_{18} column, 3.9 mm × 150 mm). Melting points (mp) were measured using a XT5 hot stage microscope (Beijing keyi electrooptic factory), which was not calibrated. Infrared (IR) spectra were recorded with a Perkin-Elmer 983 instrument. Fast atom bombardment mass spectrometry (FAB-MS) was obtained by a VG-ZAB-MS and an HPES-5989x instrument. ^{1}H NMR spectra were obtained using a Varian INOVA-500 MHz spectrometer. Optical rotations were determined at 20 °C on a Schmidt + Haensch Polartronic D instrument. Amino acid analyses were performed using a Hitachi 835-50 instrument. Statistical analysis was carried out using an ANOVA test.

General Procedure for Coupling C-Terminal and N-Terminal Components. To a solution of 0.20 mmol of the N-terminal component in 5 mL of anhydrous THF at 0 °C, 0.20 mmol of HOBt and 0.25 mmol of dicyclohexylcarbodiimide were added. The reaction mixture was stirred at 0 °C for 24 h. Precipitated dicyclohexyl urea was removed by filtration. The filtrate was evaporated under reduced pressure, and the residue was triturated with petroleum ether to provide the corresponding ester. To the solution of the ester in 10 mL of anhydrous THF, 0.20 mmol of C-terminal component and 0.26 mmol of *N*-methylmorpholine were added. The reaction mixture was stirred at room temperature for 24 h. Upon evaporation, the residue was dissolved in 50 mL of ethyl acetate. The solution was washed with 5% sodium bicarbonate, followed by 5% citric acid and saturated sodium chloride, and the organic phase was separated and dried over anhydrous sodium sulfate. After filtration and evaporation under reduced pressure, purification by chromatography ($CHCl_3$/CH_3OH, 30:1) provided the desired product.

General Procedure for Removal of Boc of the C-Terminal Component. The solution of 0.20 mmol of Boc-protected compound in 2 mL of hydrogen chloride in ethyl acetate (4 mol/L) was stirred at room temperature for 3 h. The reaction mixture was evaporated to remove the solvent. The residue was dissolved in 10 mL of ethyl acetate, and the solution was evaporated to dryness. The resulting solid was used for the coupling reaction directly without further purification.

General Procedure for Removal of Side Chain Protective Groups of the Peptides. The mixture of 0.2 mmol of the protected intermediate of the peptide, 1 mL of phenyl methyl ether, 1 mL of dimethyl sulfide, and 4 mL of HF were stirred at 0 °C for 2 h. The reaction mixture was subjected to evaporation. The residue was mixed with 1 mL of phenyl methyl ether, 1 mL of dimethyl sulfide and 8 mL of HF and stirred at 0 °C for 2 h. The reaction mixture was evaporated in a vacuum and the residue was triturated with ether. For the preparation of **7a**−**c**, the triturated residue was directly purified on Sephadex G-10 and HPLC to provide the desired peptide. For the preparation of **9a**-**c**, the triturated residue was left in air for 2 h prior to purification on Sephadex G-10 and HPLC to provide the desired product.

2,3-Dimethyl-2,3-dinitrobutane. At −5 °C to the solution of 34.5 g (0.39 mol) of 2-nitropropane and 15.6 g (0.39 mol) of NaOH in 65 mL of water, 10 mL (0.19 mol) of Br_2 were added dropwise, which took about 1 h. Ethanol (128 mL) was added while stirring. The reaction mixture was stirred at 84 °C for 3 h and TLC (ethyl acetate/petroleum, 2:1) indicated the complete disappearance of 2-nitropropane. The reaction mixture was poured into 400 mL of icy water. Twenty five grams of 2,3-dimethyl-2,3-dinitrobutane (mp 110−112 °C) was collected by filtration as colorless crystals, and the chemical yield was 73%.

2,3-Bis(hydroxylamino)-2,3-dimethylbutane. The suspension of 7.0 g (40 mmol) of 2,3-dimethyl-2,3-dinitrobutane, 4.0 g of NH_4Cl, 40 mL of ethanol, and 40 mL of water was stirred at 0 °C. To the suspension, 16.0 g of zinc powder were slowly added which took about 3 h. The reaction mixture was stirred at room temperature for another 3 h and TLC (ethyl acetate/petroleum, 2:1) indicated the complete disappearance of 2,3-dimethyl-2,3-dinitrobutane. The reaction mixture was filtrated under reduced pressure, and the collected solids were washed with aqueous ethanol solution repeatedly. The combined filtrate was adjusted to pH 2 with concentrated hydrochloric acid and was subjected to evaporation under reduced pressure. The residue was mixed with 5 g of potassium carbonate and extracted with chloroform. The extracts were evaporated under reduced pressure to give 2.60 g (44%) of 2,3-bis(hydroxylamino)-2,3-dimethylbutane which was a colorless powder with a mp of 157−159 °C.

1,3-Dihydroxy-2-(4′-hydroxylphenyl)-4,4,5,5-tetramethylimidazolidine (1). The solution of 296 mg (2 mmol) of 2,3-bis(hydroxylamino)-2,3-dimethylbutane and 244 mg (2 mmol) of 4-hydroxylbenzaldehyde in 3 mL of methanol was stirred at room temperature for 8 h, and TLC ($CHCl_3$/CH_3OH, 6:1) indicated the complete disapperance of 4-hydroxylbenzaldehyde. The precipitate was collected, and the filtrate was evaporated under vaccum to dryness. The residue and the precipitate were combined to give 257 mg (51%) of the product which was directly used for the next reaction without purification. R_f value: 0.67 ($CHCl_3$/CH_3OH, 6:1). MS (EI) *m*/*z*: 252 $[M]^+$. IR (KBr): 3310, 1610, 1450, 830 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.03 (s, 6H), 1.05 (s, 6H), 4.39 (s, 1H), 6.70 (d, J = 6.9 Hz, 2H), 7.23 (d, J = 6.9 Hz, 2H), 7.63 (s, 1H), 7.85 (s, 2H).

2-(4′-Hydroxylphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (2). One hundred and twenty six milligrams (0.5 mmol) of **1** and 500 mg of lead dioxide in 30 mL of methanol were mixed and stirred at room temperature for 40 min, and TLC ($CHCl_3$/CH_3OH, 20:1) indicated the complete disapperance of **1**. The reaction mixture was filtrated, and the filtrate was evaporated under vacuum to provide 65 mg (52%) of **2** as dark blue crystals. mp: 134−135 °C. R_f value: 0.13 ($CHCl_3$/CH_3OH, 20:1). EI-MS (*m*/*z*): 249 $[M]^+$. IR (KBr) 3250, 1500, 1490, 1355, 840 cm^{-1}.

[1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetic Acid (4). The mixture of 250 mg (1.0 mmol) of **2**, 0.32 mL of ethyl bromoacetate, and 100 mg of sodium ethylate in 5 mL of anhydrous tetrahydrofuran was stirred at 60 °C for 5 h, and TLC ($CHCl_3$/CH_3OH, 20:1) indicated the complete disapperance of **2**. The reaction mixture was evaporated under vaccum, and the residue was purified on a silica gel chromatograph ($CHCl_3$ was the eluent, giving 300 mg (90%) of **3**). To the solution of 33 mg (1.0 mmol) of **3** in 3 mL of methanol, 0.5 mL of NaOH aqueous solution (2 mol/L) was added, and the mixture was stirred at room temperature for 30 min. TLC ($CHCl_3$/CH_3OH, 10:1) indicated the complete disapperance of **3**. The reaction mixture was evaporated under vaccum, and the residure was diluted with 2 mL of saturated NaCl aqueous solution. The solution was adjusted to pH 5−6 with HCl (2 mol/L) and extraced with $CHCl_3$ (3 × 3 mL). The organic phase was dried with anhydrous $MgSO_4$ and filtered. The filtrate was evaporated in vaccum to give 30 mg (100%) of the product as blue acicular crystals. mp: 155−157 °C. EI-MS: 307 $[M]^+$. IR (KBr): 1760, 1605, 1490, 1450; 830, 1260 cm^{-1}.

Boc-Lys(*Z*)OBzl. To the solution of 73 mg (0.20 mmol) of Boc-Lys(*Z*)-OH in 5 mL of anhydrous ethanol the solution of 33 mg (0.10 mmol) of Cs_2CO_3 in 2 mL of water was added. The mixed solution was stirred at room temperature for 1 h and then evaporated under reduced pressure to remove the solvent. The residue was kept in a desiccator overnight. The dried product was dissolved in 2 mL of anhydrous DMF. To the solution, 34 mg (0.2 mmol) of benzyl bromide was added. The reaction mixture was stirred at 50 °C for 16 h and then filtered to remove the resulting precipitate of CsBr. The filtrate was evaporated under reduced pressure to remove the solvent. The residue was dissolved in 30 mL of ethyl acetate and washed successively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride, and the organic phase was separated and dried over anhydrous sodium sulfate. After

filtration and evaporation under reduced pressure, 93 mg (99%) of the Boc-Lys(*Z*)OBzl were obtained.

HCl·Lys(*Z*)-OBzl. By use of the general procedure described above for the removal of the Boc of the C-terminal component, 91 mg (0.2 mmol) of Boc-Lys(*Z*)-OBzl was converted into HCl·Lys(*Z*)-OBzl. The resulting solid was used for the coupling reaction directly.

Boc-Ala-Lys(*Z*)-OBzl. By use of the general procedure described above for the coupling of C-terminal and N-terminal components from 38 mg (0.2 mmol) of Boc-Ala-OH and 81 mg (0.2 mmol) of HCl·Lys(*Z*)-OBzl, 98 mg of the title compound was obtained as a colorless powder (90% yield). mp: 88−90 °C. FAB-MS (*m/e*): 542 $[M + H]^+$. $[\alpha]^{20}_D$ −6.6 (*c* = 0.2, $CHCl_3$). IR (KBr): 3366, 3030, 3015, 1765, 1690, 1610, 1590, 1506, 1460, 1395, 1385, 1366, 760, 710 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.123 (d, *J* = 6.69 Hz, 1H), 8.102 (d, *J* = 6.70 Hz, 1H), 8.089 (t, *J* = 4.59 Hz, 1H), 7.365 (d, *J* = 7.86 Hz, 2H), 7.360 (d, *J* = 7.79 Hz, 2H), 7.310 (t, *J* = 7.86 Hz, 1H), 7.295 (t, *J* = 7.79 Hz, 1H), 7.208 (t, *J* = 7.86 Hz, 2H), 7.201 (t, *J* = 7.79 Hz, 2H), 4.315 (m, *J* = 6.69 Hz, 1H), 5.332 (s, 2H), 5.315 (s, 2H), 4.211 (dt, *J* = 6.70 Hz, *J* = 4.64 Hz, 1H), 2.950 (dt, *J* = 4.72 Hz, *J* = 4.59 Hz, 2H), 1.952 (dt, *J* = 4.77 Hz, *J* = 4.70 Hz, 2H), 1.529 (m, *J* = 4.74 Hz, 2H), 1.318 (m, *J* = 4.69 Hz, 2H), 1.469 (s, 3H), 1.450 (s, 9H).

HCl·Ala-Lys(*Z*)-OBzl. By use of the general procedure described above for the removal of the Boc of the C-terminal component, 108 mg (0.2 mmol) of Boc-Ala-Lys(*Z*)-OBzl was converted into HCl·Ala-Lys(*Z*)-OBzl, which was used directly for the coupling reaction.

Boc-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure described above for the coupling of C-terminal and N-terminal components, 43 mg (0.2 mmol) of Boc-Pro-OH and 96 mg (0.2 mmol) of HCl·Ala-Lys(*Z*)-OBzl resulted in 115 mg of Boc-Pro-Ala-Lys(*Z*)-OBzl as a colorless powder and the chemical yield was 90%. mp: 85−87 °C. FAB-MS (*m/e*): 639 $[M + H]^+$. $[\alpha]^{20}_D$ −8.6 (*c* = 0.2, $CHCl_3$). IR (KBr): 3366, 3350, 3026, 3005, 1760, 1696, 1604, 1582, 1500, 1455, 1392, 1384, 1363, 769, 702 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.125 (d, *J* = 6.67 Hz, 1H), 8.106 (d, *J* = 6.71 Hz, 1H), 8.086 (t, *J* = 4.62 Hz, 1H), 7.367 (d, *J* = 7.88 Hz, 2H), 7.362 (d, *J* = 7.76 Hz, 2H), 7.312 (t, *J* = 7.87 Hz, 1H), 7.294 (t, *J* = 7.78 Hz, 1H), 7.215 (t, *J* = 7.84 Hz, 2H), 7.210 (t, *J* = 7.76 Hz, 2H), 4.321 (m, *J* = 6.67 Hz, 1H), 5.334 (s, 2H), 5.321 (s, 2H), 4.221 (dt, *J* = 6.72 Hz, *J* = 4.66 Hz, 1H), 3.442 (t, *J* = 4.78 Hz, 2H), 2.954 (dt, *J* = 4.75 Hz, *J* = 4.58 Hz, 2H), 2.228 (t, *J* = 4.69 Hz, 2H), 1.974 (m, *J* = 4.77 Hz, 2H), 1.953 (dt, *J* = 4.79 Hz, *J* = 4.72 Hz, 2H), 1.531 (m, *J* = 4.76 Hz, 2H), 1.321 (m, *J* = 4.71 Hz, 2H), 1.471 (s, 3H), 1.462 (s, 9H).

HCl·Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure described above for the removal of the Boc of the C-terminal component, 128 mg (0.2 mmol) of Boc-Pro-Ala-Lys(*Z*)-OBzl was converted into HCl·Pro-Ala-Lys(*Z*)-OBzl, which was used directly for the following coupling reaction.

Boc-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure described above for the removal of the Boc of the C-terminal component, Boc-Pro-Ala-Lys(*Z*)-OBzl was converted into HCl·Pro-Ala-Lys(*Z*)-OBzl, which was then coupled with Boc-Arg(Tos)-OH according to the general procedure for the coupling of C-terminal and N-terminal components to provide Boc-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl as a colorless powder in 79% yield. mp: 76−78 °C. FAB-MS (*m/e*): 977 $[M + H]^+$. $[\alpha]^{20}_D$ = −9.4° (*c* = 0.2, $CHCl_3$). IR (KBr): 3360, 3350, 3344, 3033, 3011, 1744, 1681, 1605, 1592, 1502, 1464, 1405, 1390, 1380, 762, 708 cm^{-1}. ^1H NMR (DMSO-*d6*): δ 9.356 (s, 1H), 8.230 (d, *J* = 6.72 Hz, 1H), 8.126 (d, *J* = 6.69 Hz, 1H), 8.120 (d, *J* = 6.68 Hz, 1H), 8.009 (t, *J* = 5.98 Hz, 1H), 7.855 (d, *J* = 7.81 Hz, 2H), 7.385 (d, *J* = 7.81 Hz, 2H), 7.325 (t, *J* = 7.54 Hz, 1H), 7.318 (t, *J* = 7.49 Hz, 1H), 7.228 (d, *J* = 7.62 Hz, 2H), 7.224 (d, *J* = 7.63 Hz, 2H), 7.205 (t, *J* = 7.55 Hz, 2H), 7.200 (t, *J* = 7.61 Hz, 2H), 5.342 (s, 2H), 5.331 (s, 2H), 4.785 (d, *J* = 6.76 Hz, 2H), 4.745 (dt, *J* = 6.72 Hz, *J* = 4.84 Hz, 1H), 4.691 (dt, *J* = 6.72 Hz, *J* = 4.68 Hz, 1H), 4.674 (m, *J* = 6.69 Hz, 1H), 4.421 (t, *J* = 5.64 Hz, 1H), 3.459 (t, *J* = 5.561 Hz, 2H), 2.954 (dt, *J* = 5.98 Hz, *J* = 4.84 Hz, 2H), 2.657 (dt, *J* = 6.78 Hz, *J* = 4.65 Hz, 2H), 2.382 (s, 3H), 2.254 (t, *J* = 5.64 Hz, 2H), 2.182 (t, *J* = 6.78 Hz, 1H), 2.15 (t, *J* = 6.76 Hz, 1H), 1.975 (m, *J* = 5.66 Hz, 2H), 1.922 (dt, *J* = 6.69 Hz, *J* = 4.88 Hz, 2H), 1.792 (m, *J* = 4.72 Hz, 2H), 1.612 (m, *J* = 4.72 Hz, 2H), 1.553 (m, *J* = 4.69 Hz, 2H), 1.511 (d, *J* = 6.69 Hz, 3H), 1.445 (9H), 1.301 (m, *J* = 4.72 Hz, 2H).

HCl·Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. A solution of 195 mg (0.20 mmol) of Boc-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl prepared according to literature[10] in 2 mL of hydrochloride in ethyl acetate (6 mol/L) was stirred at room temperature for 3 h. The mixture was subjected to evaporation to remove the solvent. The residue was dissolved in 10 mL of ethyl acetate, and the solution was evaporated to dryness. The resulting solid was used directly for the following coupling reaction.

Boc-Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure for the coupling C-terminal and N-terminal components, Boc-Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl was obtained as a colorless powder. Boc-Ala-OH and HCl·Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl were obtained in 91% yield. mp: 98−101 °C. FAB-MS (*m/e*) 1020 $[M + H]^+$. $[\alpha]^{20}_D$ = −8.8° (*c* = 0.2, $CHCl_3$). IR (KBr): 3363, 3351, 3339, 3031, 3012, 1754, 1685, 1608, 1592, 1507, 1462, 1408, 1385, 762, 707 cm^{-1}. ^1H NMR (DMSO-*d6*): δ 9.362 (s, 1H), 8.318 (d, *J* = 6.70 Hz, 1H), 8.286 (d, *J* = 6.69 Hz, 1H), 8.130 (d, *J* = 6.68 Hz, 1H), 8.123 (d, *J* = 6.67 Hz, 1H), 8.012 (t, *J* = 5.89 Hz, 1H), 7.861 (d, *J* = 7.75 Hz, 2H), 7.391 (d, *J* = 7.77 Hz, 2H), 7.332 (t, *J* = 7.56 Hz, 1H), 7.321 (t, *J* = 7.54 Hz, 1H), 7.228 (d, *J* = 7.64 Hz, 2H), 7.218 (d, *J* = 7.61 Hz, 2H), 7.211 (t, *J* = 7.49 Hz, 2H), 7.199 (t, *J* = 7.64 Hz, 2H), 5.345 (s, 2H), 5.335 (s, 2H), 4.748 (dt, *J* = 6.70 Hz, *J* = 4.83 Hz, 1H), 4.694 (dt, *J* = 6.74 Hz, *J* = 4.67 Hz, 1H), 4.664 (m, *J* = 6.68 Hz, 1H), 4.672 (m, *J* = 6.65 Hz, 1H), 4.430 (t, *J* = 5.65 Hz, 1H), 3.461 (t, *J* = 5.57 Hz, 2H), 2.957 (dt, *J* = 5.96 Hz, *J* = 4.87 Hz, 2H), 2.661 (dt, *J* = 6.76 Hz, *J* = 4.67 Hz, 2H), 2.390 (s, 3H), 2.262 (t, *J* = 5.62 Hz, 2H), 2.203 (t, *J* = 6.78 Hz, 1H), 2.157(t, *J* = 6.74 Hz, 1H), 1.977 (m, *J* = 5.68 Hz, 2H), 1.924 (dt, *J* = 6.66 Hz, *J* = 4.85 Hz, 2H), 1.794(m, *J* = 4.70 Hz, 2H), 1.614 (m, *J* = 4.70 Hz, 2H), 1.551 (m, *J* = 4.71 Hz, 2H), 1.508 (d, *J* = 6.67 Hz, 3H), 1.489 (d, *J* = 6.67 Hz, 3H), 1.447 (s, 9H), 1.312 (m, *J* = 4.70 Hz, 2H).

HCl·Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure for the removal of the Boc of the C-terminal component, 204 mg (0.2 mmol) of Boc-Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl was converted to HCl·Ala-Arg(Tos)-Pro-Ala-Lys-(*Z*)-OBzl. The resulting solid was used for the coupling reaction directly without further purification.

Ala-Arg-Pro-Ala-Lys-OH (7a). By use of the general procedure for the removal of side chain protective groups of the peptides from 204 mg (0.2 mmol) of Boc-Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl, 98 mg (90%) of (**6a**) was obtained as colorless crystals. mp: 224−226 °C. FAB-MS (*m/e*): 542 $[M + H]^+$. $[\alpha]^{20}_D$ = −40.4° (*c* = 2, H_2O). IR (KBr): 3434, 3368, 3245, 3083, 2965, 1667, 1550, 1386 cm^{-1}. ^1H NMR (DMSO-*d*$_6$): δ 9.982 (s, 1H), 8.432 (s, 2H), 8.282(d, *J* = 6.68 Hz, 1H), 8.224 (d, *J* = 6.72 Hz, 1H), 8.011 (d, *J* = 6.70 Hz, 1H), 7.587 (d, *J* = 6.65 Hz, 1H), 7.160 (d, *J* = 6.68 Hz, 1H), 4.266 (1H), 4.215 (dt, *J* = 6.69 Hz, *J* = 4.84 Hz, 1H), 4.223 (dt, *J* = 6.72 Hz, *J* = 4.65 Hz, 1H), 4.022 (m, *J* = 6.67 Hz, 1H), 3.671 (m, *J* = 6.67 Hz, 1H), 3.535 (t, *J* = 6.74 Hz, 2H), 3.349 (t, *J* = 5.55 Hz, 2H), 3.076 (t, *J* = 5.93 Hz, 2H), 2.732 (t, *J* = 5.64 Hz, 2H), 2.249 (t, *J* = 6.75 Hz, 1H), 2.242 (m, *J* = 5.66 Hz, 2H), 2.021 (dt, *J* = 6.64 Hz, *J* = 4.83 Hz, 2H), 2.006 (m, *J* = 4.72 Hz, 2H), 1.861 (m, *J* = 4.72 Hz, 2H), 1.674 (m, *J* = 4.73 Hz, 2H), 1.534 (m, *J* = 4.73 Hz, 2H), 1.533 (m, *J* = 6.64 Hz, 2H), 1.282 (d, *J* = 6.65 Hz, 3H), 1.268 (d, *J* = 6.64 Hz, 3H).

Boc-Gln-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure for the coupling C-terminal and N-terminal components from Boc-Gln-OH and HCl·Arg(Tos)-Pro-Ala-Lys-(*Z*)-OBzl, Boc-Gln-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl was obtained as a colorless powder in 86% yield. mp: 90−92 °C. FAB-MS (*m/e*): 1077 $[M + H]^+$. $[\alpha]^{20}_D$ = −8.9° (*c* = 0.2, $CHCl_3$). IR (KBr): 3361, 3355, 3345, 3033, 3010, 1758, 1682, 1660, 1600, 1590, 1506, 1460, 1394, 1385, 1361, 760, 702 cm^{-1}. ^1H NMR (DMSO-*d*$_6$): δ 9.401 (s, 1H), 8.332(d, *J* = 6.72 Hz, 1H), 8.314

(d, J = 6.64 Hz, 1H), 8.138 (d, J = 6.66 Hz, 1H), 8.126 (d, J = 6.65 Hz, 1H), 8.025 (t, J = 5.92 Hz, 1H), 7.859 (d, J = 7.73 Hz, 2H), 7.396 (d, J = 7.75 Hz, 2H), 7.341 (t, J = 7.60 Hz, 1H), 7.325 (t, J = 7.60 Hz, 1H), 7.230 (d, J = 7.60 Hz, 2H), 7.221 (d, J = 7.60 Hz, 2H), 7.215 (t, J = 7.50 Hz, 2H), 7.196 (t, J = 7.62 Hz, 2H), 6.458 (t, J = 6.69 Hz, 2H), 5.346 (s, 2H), 5.338 (s, 2H), 4.744 (dt, J = 6.71 Hz, J = 4.81 Hz, 1H), 4.692 (dt, J = 6.72 Hz, J = 4.66 Hz, 1H), 4.666 (m, J = 6.66 Hz, 1H), 4.670 (m, J = 6.67 Hz, 1H), 4.432 (t, J = 5.66 Hz, 1H), 3.463 (t, J = 5.60 Hz, 2H), 2.961 (dt, J = 5.98 Hz, J = 4.85 Hz, 2H), 2.664 (dt, J = 6.74 Hz, J = 4.70 Hz, 2H), 2.392 (s, 3H), 2.264 (t, J = 5.64 Hz, 2H), 2.205 (t, J = 6.76 Hz, 1H), 2.191 (t, J = 6.69 Hz, 1H), 2.155 (t, J = 6.72 Hz, 1H), 2.075 (t, J = 6.67 Hz, 1H), 1.978 (m, J = 5.66 Hz, 2H), 1.926 (dt, J = 6.67 Hz, J = 4.85 Hz, 2H), 1.930 (dt, J = 6.65 Hz, J = 4.83 Hz, 2H), 1.796 (m, J = 4.74 Hz, 2H), 1.621 (m, J = 4.72 Hz, 2H), 1.556 (m, J = 4.70 Hz, 2H), 1.511 (d, J = 6.66 Hz, 3H), 1.445 (s, 9H), 1.309 (m, J = 4.72 Hz, 2H).

HCl·Gln-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure for the removal of the Boc of the C-terminal component, 216 mg (0.2 mmol) of Boc-Gln-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl was converted to HCl·Gln-Arg(Tos)-Pro-Ala-Lys-(*Z*)-OBzl. The resulting solid was used directly for the coupling reaction without further purification.

Gln-Arg-Pro-Ala-Lys-OH (7c). By use of the general procedure for the removal of the side chain protective groups of the peptides, 215 mg (0.2 mmol) of Boc-Gln-Arg(Tos)-Pro-Ala- Lys(*Z*)-OBzl was converted to 134 mg (95% of yield) of **6c** as colorless crystals. mp: 180−182 °C. FAB-MS(*m/e*): 599 [M + H]$^+$. $[\alpha]^{20}_D = -35.0°$ (c = 2.01, H_2O). IR (KBr): 3436, 3367, 3254, 3073, 2964, 1662, 1558, 1385 cm^{-1}.^{1}H NMR (DMSO-d_6): δ 9.989 (s, 1H), 8.446 (s, 2H), 8.283 (d, J = 6.74 Hz, 1H), 8.214 (d, J = 6.66 Hz, 1H), 8.157 (d, J = 6.68 Hz, 1H), 7.575 (d, J = 6.66 Hz, 1H), 7.164 (t, J = 5.94 Hz, 1H), 6.401 (s, 2H), 4.283 (dt, J = 6.73 Hz, J = 4.84 Hz, 1H), 4.273 (dt, J = 6.70 Hz, J = 4.68 Hz, 1H), 4.218 (m, J = 6.68 Hz, 1H), 4.112 (m, J = 6.65 Hz, 1H), 4.241 (t, J = 5.67 Hz, 1H), 3.541 (dt, J = 5.96 Hz, J = 4.87 Hz, 2H), 3.359 (t, J = 5.62 Hz, 2H), 3.072 (t, J = 5.64 Hz, 2H), 2.742 (t, J = 5.62 Hz, 2H), 2.393 (t, J = 6.67 Hz, 2H), 2.291 (t, J = 6.65 Hz, 2H), 2.233 (t, J = 6.64 Hz, 2H), 2.191 (t, J = 6.70 Hz, 2H), 2.031 (m, J = 5.68 Hz, 2H),1.912 (dt, J = 6.65 Hz, J = 4.83 Hz, 2H), 1.765 (dt, J = 6.67 Hz, J = 4.81 Hz, 2H), 1.672 (m, J = 4.76 Hz, 2H), 1.535 (m, J = 4.74 Hz, 2H), 1.527 (m, J = 4.72 Hz, 2H), 1.267 (d, J = 6.64 Hz, 3H).

Boc-Gly-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure for the coupling C-terminal and N-terminal components, Boc-Gly-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl was generated from Boc-Gly-OH and HCl·Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl as a colorless powder, and the chemical yield was 93%. mp: 93−95 °C. FAB-MS (*m/e*): 1006 [M + H]$^+$. $[\alpha]^{20}_D = -10.6°$ (c = 0.2, $CHCl_3$). IR (KBr): 3368, 3350, 3346, 3032, 3008, 1765, 1688, 1602, 1561, 1501, 1462, 1401, 1387, 1361, 765, 701 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 9.355 (s, 1H), 8.320 (d, J = 6.69 Hz, 1H), 8.279 (d, J = 6.70 Hz, 1H), 8.141 (d, J = 6.70 Hz, 1H), 8.128 (d, J = 6.66 Hz, 1H), 8.015 (t, J = 5.87 Hz, 1H), 7.864 (d, J = 7.74 Hz, 2H), 7.394 (d, J = 7.79 Hz, 2H), 7.335 (t, J = 7.58 Hz, 1H), 7.324 (t, J = 7.52 Hz, 1H), 7.232 (d, J = 7.65 Hz, 2H), 7.220 (d, J = 7.58 Hz, 2H), 7.214 (t, J = 7.47 Hz, 2H), 7.196 (t, J = 7.66 Hz, 2H), 5.346 (s, 2H), 5.336 (s, 2H), 4.746 (dt, J = 6.72 Hz, J = 4.81 Hz, 1H), 4.692 (dt, J = 6.76 Hz, J = 4.65 Hz, 1H), 4.667 (d, J = 6.69 Hz, 2H), 4.659 (m, J = 6.67 Hz, 1H), 4.432 (t, J = 5.67 Hz, 1H), 3.458 (t, J = 5.60 Hz, 2H), 2.956 (dt, J = 5.94 Hz, J = 4.85 Hz, 2H), 2.663 (dt, J = 6.74 Hz, J = 4.65 Hz, 2H), 2.392 (s, 3H), 2.260 (t, J = 5.64 Hz, 2H), 2.205 (t, J = 6.76 Hz, 1H), 2.155 (t, J = 6.72 Hz, 1H), 1.974 (m, J = 5.69 Hz, 2H), 1.921 (dt, J = 6.67 Hz, J = 4.86 Hz, 2H), 1.793 (m, J = 4.71 Hz, 2H), 1.616 (m, J = 4.72 Hz, 2H), 1.554 (m, J = 4.74 Hz, 2H), 1.487 (d, J = 6.64 Hz, 3H), 1.445 (s, 9H), 1.321 (m, J = 4.72 Hz, 2H).

HCl·Gly-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl. By use of the general procedure for the removal of the Boc of the C-terminal component, 202 mg (0.2 mmol) of Boc-Gly-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl was converted into the title compound. The product obtained was used for the coupling reaction directly without further purification.

Gly-Arg-Pro-Ala-Lys-OH (7b). By use of the general procedure for the removal of side chain protective groups of the peptides from 201 mg (0.2 mmol) of Boc-Gly-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl, 99 mg (94%) of the title compound was obtained as colorless crystals. mp: 168−170 °C. FAB-MS (*m/e*): 528 [M + H]$^+$. $[\alpha]^{20}_D = -20.0°$ (c = 1.98, H_2O). IR (KBr): 3440, 3373, 3256, 3071, 2946, 1667, 1558, 1384 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 9.994 (s, 1H), 8.563 (s, 2H), 8.104 (d, J = 6.67 Hz, 1H), 7.985 (d, J = 6.72 Hz, 1H), 7.973 (d, J = 6.71 Hz, 1H), 7.627 (d, J = 6.67 Hz, 1H), 7.077 (t, J = 5.86 Hz, 1H), 4.625 (dt, J = 6.70 Hz, J = 4.83 Hz, 1H), 4.525 (dt, J = 6.75 Hz, J = 4.64 Hz, 1H), 4.256 (d, J = 6.67 Hz, 2H), 4.228 (m, J = 6.65 Hz, 1H), 4.106 (t, J = 5.65 Hz, 1H), 3.217 (t, J = 5.62 Hz, 2H), 3.385 (dt, J = 5.96 Hz, J = 4.82 Hz, 2H), 3.068 (t, J = 5.48 Hz, 2H), 2.736 (dt, J = 6.71 Hz, J = 4.63 Hz, 2H), 2.534 (t, J = 5.65 Hz, 2H), 2.531 (t, J = 5.66 Hz, 2H), 2.046 (m, J = 5.67 Hz, 2H), 1.985 (dt, J = 6.65 Hz, J = 4.84 Hz, 2H), 1.865 (m, J = 4.74 Hz, 2H), 1.675 (m, J = 4.74 Hz, 2H), 1.531 (m, J = 4.75 Hz, 2H), 1.532 (m, J = 4.71 Hz, 2H), 1.243 (d, J = 6.61 Hz, 3H).

***N*-[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetyl-Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl (8a).** By use of the general procedure for coupling of C-terminal and N-terminal components from 61 mg (0.2 mmol) of **4** and 191 mg (0.2 mmol) of HCl·Ala-Arg(Tos)-Pro-Ala-Lys-(ClZ)-OBzl 222.3 mg (92%) of the title compound was obtained as a colorless powder. mp: 92−94 °C. FAB-MS (*m/e*): 1209 [M + H]$^+$. $[\alpha]^{20}_D$ = 24.0 (c = 2, $CHCl_3$).

***N*-[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetyl-Gly-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl (8b).** By use of the general procedure for the coupling of C-terminal and N-terminal components from 61 mg (0.2 mmol) of **4** and 188 mg (0.2 mmol) of HCl·Gly-Arg(Tos)-Pro-Ala-Lys(ClZ)OBzl, 219.7 mg (92%) of **8b** was obtained as a colorless powder. mp: 90−92 °C. FAB-M (*m/e*): 1195 [M + H]$^+$. $[\alpha]^{20}_D$ = 13.0 (c = 2, $CHCl_3$).

***N*-[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetyl-Gln-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl (8c).** By use of the general procedure for the coupling of C-terminal and N-terminal components from 61 mg (0.2 mmol) of **4** and 203 mg (0.2 mmol) of HCl·Gln-Arg(Tos)-Pro-Ala-Lys-(ClZ)OBzl, 225.4 mg (89%) of compound **8c** was obtained as a colorless powder. mp: 96−98 °C. FAB-MS (*m/e*) 1267 [M + H]$^+$. $[\alpha]^{20}_D$ = 29.0 (c = 2, $CHCl_3$).

***N*-[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetyl-ARPAK (9a).** By use of the general procedure for the removal of side chain protective groups, 136 mg (0.1 mmol) of **8a** was converted to 70 mg of compound **9a** and was obtained as a blue powder. The chemical yield was 84%. mp: 170 °C(dec). ESI-MS (*m/e*): 831 [M]$^+$. $[\alpha]^{20}_D$ = 35.0 (c = 1, H_2O).

***N*-[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetyl-GRPAK (9b).** By use of the general procedure for the removal of side chain protective groups and from 130 mg (0.1 mmol) of **8b**, 60 mg of **9b** was obtained as a blue powder and the chemical yield was 75%. mp: 174 °C(dec). ESI-MS (*m/e*): 817[M]$^+$. $[\alpha]^{20}_D$ = 21.0 (c = 1, H_2O).

***N*-[1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetyl-QRPAK (9c).** By use of the general procedure for the coupling of C-terminal and N-terminal components and from 136 mg (0.1 mmol) of **8c**, 70 mg of **9c** were obtained as a blue powder and the chemical yield was 80%. mp: 178 °C(dec). ESI-MS (*m/e*): 889 [M + 2 H]$^+$. $[\alpha]^{20}_D = -27.0$ (c = 1, H_2O).

Determination of ESR. A center field of 3480 G, sweep width of 100 G, sweep time of 100 s, modulation amplitude of 1.0×10^{-1} G, time constant of 1.6×10^{-1} S, modulation frequency of 100 kHz, microwave frequency of 9.72 GHz, and microwave power of 10 MW were all used for the ESR measurement. The ESR spectra of **4**, **8a**−**c**, and **9a**−**c** at two

different concentrations, 10^{-7} and 10^{-5} mol/L, and in deaerated water and phosphate buffer (pH 7.4), respectively, were recorded.

To study the reactivity of respective compounds with NO, the deaerated water or phosphate buffer containing **4, 8a−c,** and **9a−c** (10^{-5} mol/L) were bubbled with NO gas for 30 s, following which the ESR spectra were then recorded.

Evaluation of 9a−c as Scavengers of NO, H_2O_2·, and ·OH in PC12 Cells. Free radical scavenging activities were evaluated in PC12 cells using a method of Dawson[23] 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 24 h, attachment period fresh media containing 12.5 , 25 , 50 , 100 , or 200 μM of **4**, **9a**, **9b**, or **9c**, respectively, were added to each well and were incubated for 1 h. NO damage was then induced by adding 2 mM of sodium nitroprusside 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.[24] 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.

NO Scavenging Activity Determined Using Rat Aortic Strip. The NO scavenging activity of **4, 7a−c**, and **9a−c** in the rat aortic strip was examined according to a published method.[25] 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 to the maximum level, NE was washed and the vessel strips were stabilized for 30 min. After the renewal of the solution, NE (final concentration 10^{-9} mol/L) was added. When the hypertonic contraction value of aortic strips reached the peak, 15 μL of NS, or the solution of **4**, **7a−c**, and **9a−c** in 15 μL of water (final concentration 10^{-6} mol/L) were added, respectively. Upon stabilization, 1.5 μL of ACh (final concentration 10^{-6} mol/L) were added and the percentage inhibition of ACh-induced vasorelaxation by test compounds was determined.

Determination of ECLT. The rabbit euglobulin clots were prepared according to a published method.[26,27] Plasma diluted at 1:20 in distilled water was precipitated at pH 5.7 with acetic acid (0.25%). After 30 min at 4 °C, the suspension was centrifuged at 2000*g* for 15 min and the precipitate was resuspended to the initial plasma volume with 50 mM sodium barbiturate buffer (pH 7.8, containing 1.66 mM $CaCl_2$, 0.68 mM $MgCl_2$, and 93.96 mM NaCl). To the rabbit euglobulin clots, NS, UK, **4**, **7a−c**, or **9a−c** was added, and the ECLT or time to clot lysis was determined in a 96-well microtiter plate.

Determination of Fibrinolytic Activity. The fibrinogen−agarose mixture was prepared and coagulated with thrombin in plastic dishes according to a published procedure.[28] The fibrinogen−agarose mixture was prepared by mixing equal volumes of 0.3% rabbit fibrinogen and 0.95% agarose solutions, both dissolved in 50 mM sodium barbiturate buffer (pH 7.8). The fibrinogen−agarose mixture was coagulated with 100 mL of thrombin (100 IU/ mL) in plastic dishes (90 mm diameter x 1 mm depth). After 30 min at 4 °C, an adequate number of wells, 5 mm in diameter, were perforated. To determine fibrinolytic activity, 30 μL of NS, UK, **4**, **7a−c**, or **9a−c** was added to each well. The plate was incubated and areas of lysis were quantified by the lysis area.

The thrombus was prepared, supported on a stainless steel filament helix, weighted, and put into the circulation polyethylene tube outside male Wistar rat according to the standard procedure.[19] After administration of NS, UK, **4**, **7a−c**, or **9a−c,** the blood was circulated through the polyethylene tube for 90 min and the helix was taken out and weighed accurately. The reduction in thrombus mass was recorded. The results are summarized in Table 5. The effect of three different dosages of **9a−c** was compared, and the results are also listed in Table 5. Statistical analysis of the results was carried out using an ANOVA test. The results indicated that **9a−c** significantly reduced thrombus mass, and the effect appeared to be dose dependent.

Male Wistar rats weighing 200−300 g (purchased from the Animal Center of Peking University) were anesthetized with pentobarbital sodium (80.0 mg/kg, intraperitoneally). The right carotid artery and left jugular vein of the animals were separated. To the glass tube containing 1.0 mL of blood obtained from the right carotid artery of the animal, a stainless steel filament helix (15 circles; 15 mm × 1.0 mm) was added immediately. Fifteen minutes later, the helix with thrombus was carefully taken out and weighed. It was then put into a polyethylene tube which was filled with heparin sodium (50 IU/mL of NS), and one end was inserted into the left jugular vein. Heparin sodium was injected via the other end of the polyethylene tube as the anticoagulant, following which the test compound was injected. The blood was circulated through the polyethylene tube for 90 min, after which the helix was taken out and weighed. The reduction of the thrombolytic mass was recorded.

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