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A class of novel nitronyl nitroxide labeling basic and acidic amino acids: Synthesis, application for preparing ESR optionally labeling peptides, and bioactivity investigations

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Abstract—Aimed at optional ESR label 2-(4′-hydroxyl)phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl was introduced into the guanido of L-Arg-OH, the ω-amino group of L-Lys-OH with methylcarboxyl as a linker, and into the β-carboxyl of L-Asp-OH and the γ-carboxyl of L-Glu-OH with ethylamino as a linker. It was explored that the synthetic 30 novel ESR labeling amino acid derivatives were stable enough to the reaction conditions of peptide synthesis. Their incorporation led to 12 novel ESR optionally labeling PAK, RGDS, RGDV, and ECG. A series of NO related chemical tests, the in vitro and in vivo assays of these peptides confirmed that this strategy was practical.

1. Introduction

Electronic spin resonance (ESR), as one of the most useful biological techniques, is able to provide information about conformation properties of peptides and their interaction with macromolecules and membrane of biological interest by the use of stable free radical spin labels.[1–4] So far most of the reports related to the use of ESR for labeling proteins and peptides involving chemically introducing the piperidine nitroxides to the side chains of their amino acid residues, their N- or C-terminal, or to any position of their sequences.[5,6] Unfortunately the synthesis of peptides with piperidine nitroxides as spin labels was of limited success because of the ability of the piperidine nitroxide moiety, which was decomposed during the deprotection steps required to remove the protecting groups of α-amino groups and side chains of each coupled amino acid residue, and to remove the protecting groups of α-carboxyl groups or cleave the peptides from the resin.[7] For instance in the presence of reagent K, the piperidine nitroxide moiety of 9-fluorenylmethyoxy-carbonyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid which was usually used to label peptides was decomposed to lose the electronic spin resonance signal and can not be recovered in basic solution. On the other hand even in the presence of HF the N–O of the piperidine nitroxide moiety was protonated though it can be reverted upon treatment with 0.02 M ammonium acetate at pH 9.0 for 3 h.[8] In the literature another effort was given to prepare ESR labeling peptides directly using (1,3-dioxyl-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine. This effort was also not successful because (1,3-dioxyl-4,4,5,5-tetramethyldihydroimidazol-2-yl)-L-alanine can be decomposed not only in strong acid media but also in the presence of anhydrous trifluoroacetic acid.[7] Thus there has been a long-standing interest to identify novel, more stable, and credible peptide spin labels especially ESR labeling amino acids to be directly used for the synthesis of peptide. The present paper describes fifteen novel nitronyl nitroxide modified L-Lys, L-Arg, L-Asp, and L-Glu derivatives, their synthesis, applications for preparing ESR labeling peptides, seven optionally ESR labeling peptides, and biological activities.

2. Results and discussion

2.1. Preparing nitronyl nitroxides 2g and 3g capable of labeling

To find a suitable nitronyl nitroxide capable of modifying L-Lys, L-Arg, L-Asp, and L-Glu a series of 2-alkyl,

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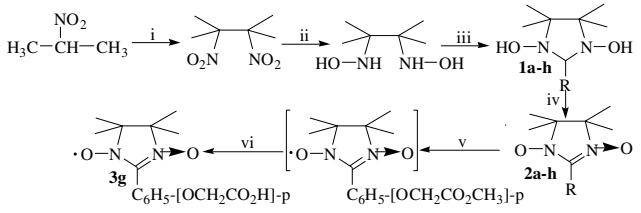
carboxyl, heteroaryl, and aryl substituted nitronyl nitroxides were prepared according to the synthetic route depicted in Scheme 1,[9] their NO free radical characteristic, their NO scavenging activity, and their chemical stability were tested and compared.

In the presence of Br_2 and NaOH 2-nitropropane was smoothly converted into 2,3-dimethyl-2,3-dinitrobutane which was reduced by zinc powder and NH_4Cl to give 2,3-dimethyl-2,3-dihydroxylaminobutane in 40% total yield. The condensation of hydroxylamine and eight substituted aldehydes, including methyl aldehyde, chloromethyl aldehyde, aldehydic acid, α-furaldehyde, β-pyridyaldehyde, benzaldehyde, 4-hydroxylbenzaldehyde, and 4-nitrobenzaldehyde, provided eight 2-substituted phenyl-1,3-dioxyl-4,4,5,5-tetramethylimidazolindines **1a**–**h** in 53%, 69%, 83%, 73%, 69%, 85%, 51%, and 63% yield, respectively. With PbO_2 as oxidation agent **1a**–**h** were converted into the corresponding nitronyl nitroxides **2a**–**h** in 99%, 38%, 88%, 83%, 79%, 80%, 52%, and 73% yield, respectively (Scheme 1).

In the determination of NO free radical characteristic the ESR spectra of **2a**–**h** were recorded on ESR spectrometer. It was found that **2a**–**h** consistently gave identical ESR spectrum as shown in Figure 1, which consists of a five-line pattern with 1:2:3:2:1 intensity ratios. This kind of spectrum obviously resulted from an electron interacting with two equivalent nitrogens in the nitronyl nitroxides. The spectra imply that **2a**–**h** are desirable nitronyl nitroxides with correct electronic structure.

In the determination of NO scavenging capacity the rat aortic strip assays of **2a**–**h** were performed in a perfusion bath with 5 ml of warmed (37 °C) and oxygenated (95% O_2, 5% CO_2) Krebs solution (pH 7.4) according to a standard procedure. The data are expressed by inhibition percentage of ACh-induced vasorelaxation which was recorded by the tension transducers and are listed in Table 1. The results indicated that though 100 μM **2a**–**h** consistently inhibited ACh-induced vasorelaxation, only 10 μM **2f**–**h** significantly inhibited ACh-induced vasorelaxation. Among nine compounds only **2g** and **3g** gave significant and dose-dependent inhibition for ACh-induced vasorelaxation.



Scheme 1. Route for preparing nitronyl nitroxides. Reagents: (i) Br_2, NaOH (6 M); (ii) Zn, NH_4Cl; (iii) Methyl aldehyde, chloromethyl aldehyde, aldehydic acid, α-furaldehyde, β-pyridyaldehyde, benzaldehyde, 4-hydroxylbenzaldehyde or 4-nitrobenzaldehyde; (iv) PbO_2; (v) $BrCH_2CO_2C_2H_5$, NaOEt, THF; (vi) NaOH (2 M) in **1a** and **2a** R = H, **1b** and **2b** R = –CH_2Cl, **1c** and **2c** R = –COOH, **1d** and **2d** R = fur-2-yl, **1e** and **2e** R = pyrid-3-yl, **1f** and **2f** R = phenyl, **1g** and **2g** R = 4′-hydroxylphenyl, **1h** and **2h** R = 4-nitrophenyl.

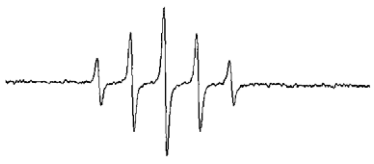


Figure 1. Typical ESR spectrum given by **2g**, **3g**, the modified amino acids, and the peptides.

It is well known that in preparation of ESR labeling peptides nitronyl nitroxide-amino acids will undergo a series of treatments of acidic and basic aqueous solution and may be reduced to imino nitroxide-amino acids. To observe the stability of **2a**–**h** in acidic and basic aqueous solution they were dissolved in the aqueous solutions of pH 2.0, 7.4, and 11.5 (final concentration 10^{-2} mM), kept at room temperature for up to 200 h, during which TLC ($CHCl_3/CH_3OH$, 20:1) monitor was conducted. The results are listed in Table 2. TLC monitor indicated that at the mentioned conditions **2a**–**c** were decomposed between 2 and 18 h, **2d**–**e** were decomposed between 15 and 30 h, and **2f**–**h** and **3g** did not decompose up to 200 h. According to the results of the ESR spectral, aortic strip assay and TLC analysis 2-(4′-hydroxylphenyl)nitronyl nitroxide (**2g**) should be the most suitable

Table 1. Inhibition of ACh-induced vasorelaxation of **2a**–**h** and **3g**[a]

	Inhibition potency		
	100 μM	10 μM	1 μM
NS		1.63 ± 1.45	
2a	31.2 ± 2.9[b]	8.6 ± 3.7[b]	1.7 ± 1.5
2b	25.0 ± 2.4[b]	7.7 ± 9.8	1.6 ± 1.5
2c	34.3 ± 6.5[b]	25.0 ± 17.8[b]	1.7 ± 1.6
2d	44.4 ± 5.9[b]	20.2 ± 4.2[b]	1.6 ± 1.5
2e	48.6 ± 2.9[b]	16.6 ± 2.8[b]	1.7 ± 1.7
2f	81.9 ± 8.9[c]	62.7 ± 5.5[c]	9.8 ± 7.8
2g	99.2 ± 1.4[c]	73.1 ± 5.9[c]	26.1 ± 7.0[b]
2h	98.0 ± 2.7[c]	71.3 ± 8.8[c]	−14.5 ± 14.5
3g	99.0 ± 1.8[c]	72.4 ± 5.2[c]	25.3 ± 6.2[b]

[a] Inhibition is expressed by $\overline{X} \pm$ SD%; NS (normal saline) = vehicle; $n = 6$.
[b] Compared to NS $p < 0.001$.
[c] Compared to NS $p < 0.001$ and to **2a**–**e**, $p < 0.01$.

Table 2. Stability of **2a**–**h** and **3g** in pH 2.0, 7.4, and 11.5 aqueous solution

Compound	Decomposition time (h)		
	pH 2.0	pH 7.4	pH 11.5
2a	2	18	2.5
2b	2	18	2.5
2c	2	18	2.5
2d	15	30	18
2e	15	30	18
2f	>200	>200	>200
2g	>200	>200	>200
2	>200	>200	>200
3g	>200	>200	>200

labeling block. The O-alkylation of **2g** and $BrCH_2CO_2H$ provided [1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)phenyl-4-yl]oxyacetic acid (**3g**) in theoretical yield. The stability evaluation indicated that **3g** should be suitable like **2g** as a labeling block.

2.2. Introducing 2g or 3g into L-Lys, L-Arg, L-Asp, and L-Glu

Considering the high appearance frequency and availability of labeling L-Lys-OH, L-Arg-OH, L-Asp-OH, and L-Glu-OH in peptides, **2g** or **3g** was introduced either into the side chain or into the α-amino group of these by use of the routes depicted in Schemes 2–7 and L-N^{α}-**3g**-Lys(Z)-OBzl (**4**, 65% yield), L-N^{α}-**3g**-Lys(Z)-OH (**5**, 91% yield), L-N^{α}-**3g**-Lys-OH (**6**, 90% yield), L-N^{ω}-**3g**-Lys-OCH_3 (**7**, 83% yield), L-N^{ω}-**3g**-Lys-OH (**8**, 93% yield), L-N^{α}-Boc-N^{ω}-**3g**-Lys-OH (**9**, 91% yield), L-N^{α}-**3g**-Arg-OMe (**10**, 87% yield), L-N^{α}-**3g**-Arg-OH (**11**, 73% yield), L-N^{α}-Boc-N^{G}-**3g**-Arg-OMe (**12**, 87% yield), L-N^{α}-Boc-N^{G}-**3g**-Arg-OH (**13**, 73% yield), L-N^{G}-**3g**-Arg-OMe (**14**, 98% yield), L-N^{α}-Boc-β-**2g**-Asp-OH (**17**, 39% yield), and L-N^{α}-Boc-β-**2g**-Asp-OCH_3 (**18**, 46% yield), L-β-**2g**-Asp-OH (**19**, 94% yield) and L-β-**2g**-Asp-OCH_3 (**20**, 94% yield), L-N^{α}-Boc-γ-**2g**-Glu-OH (**23**, 48% yield), and L-N^{α}-Boc-γ-**2g**-Glu-OCH_3 (**24**, 48% yield) were obtained. Their detailed data were provided as supporting information. The structures of the labeling amino acids **4**–**14**, **17**–**20**, **23**, and **24** are shown in Table 3. In contrast to the lack of ESR labeling amino acid previously, these derivatives of Table 1 not only form a family of ESR labeling amino acids but also provide structural diversity to meet the necessary selection in the preparation of optionally labeling peptides.

2.3. Incorporating 2g or 3g modified L-Lys, L-Arg, L-Asp or L-Glu into bioactive peptides

In order to clarify the application of the mentioned **2g** or **3g** modified L-Lys, L-Arg, L-Asp or L-Glu in the preparation of ESR label peptides they were incorporated into some bioactive peptides such as Pro-Ala-Lys (PAK), Glu-Cys-Gly (ECG), and Arg-Gly-Asp (RGD) peptides. The preparations were carried out according to the routes depicted in Schemes 8–13 and H-Pro-Ala-(N^{ω}-**3g**-Lys)-OH (**31**, 78% total yield), (N^{G}-**3g**-Arg)-Gly-Asp-Val-OH (**36**, 54% total yield), H-Arg-Gly-(β-**2g**-Asp)-Ser-OH (**43**, 25% total yield), and H-(γ-**2g**-Glu)-Cys-Gly-OH (**48**, 35% total yield) were obtained. Their detailed data were provided as supporting information. The structures of the labeling peptides **31**, **36**, **43**, and **48** are also shown in Table 3. In contrast to dull N-terminal labeling peptides previously, these derivatives of Table 3 not only form a family of ESR labeling peptides but also provide a strategy preparing optionally labeling peptides.

2.4. Typical ESR spectra of 2g and 3g modified amino acids and peptides

To examine the free radical characteristics of the **2g** and **3g** modified amino acids and peptides their ESR spectra were tested. The testing samples were obtained by dissolving them in water or phosphate buffer (pH 7.4, final concentration 10 μM). The tests demonstrated that all of them gave similar ESR spectrum to that of **2g** and **3g**. The spectrum (Fig. 1) consists of a five-line pattern with 1:2:3:2:1 intensity ratios. This spectrum obviously resulted from an electron interacting with two equivalent nitrogens in the nitronyl nitroxides. Standard ESR spectrum of nitronyl nitroxide was recorded for **2g** and **3g** modified amino acids suggested that after a series of preparative reactions and treatments they were still desirable nitronyl nitroxide derivatives. This was also true for the peptides containing **2g** and **3g** modified amino acids. The standard ESR spectrum of nitronyl nitroxides gained from all of the labeling compounds themselves prepared here not only establishes a base for their structural determination but also provides a sensitive signal for their use as a probe. These are obviously impossible for the previous unstable ESR labeling compounds.

2.5. NO trapping activity of 2g and 3g modified amino acids and peptides

To examine the NO trapping activity of the **2g** and **3g** modified amino acids and peptides the NO trapping assay was performed. The testing samples were obtained by dissolving them in a deaerated phosphate buffer (pH 7.4, final concentration 10 μM), NO gas was bubbled and the ESR spectra were recorded. The tests explored that all **2g** and **3g** modified amino acids and peptides gave similar ESR spectrum of imino nitroxide (Fig. 2), which consists of a seven-line pattern attributing to an electron interacting with two equivalent nitrogenes. The formed imino nitroxide products obviously resulted from the oxidation–reduction reaction of nitronyl nitroxide and NO, suggesting that **2g** and **3g** modified amino acids and peptides are desirable NO scavengers. A desirable NO scavenging property was recorded for **2g** and **3g** modified amino acids, suggesting that after a series of preparative reactions and treatments they were still desirable free radicals. A desirable NO scavenging property was recorded for the peptides containing **2g** and **3g** modified amino acids, suggesting that after a series of preparative reactions and treatments they were also still free radicals. The results imply that these labeling methods are able to provide ESR optionally labeling peptides with typical NO scavenging property. The standard ESR spectrum of imino nitroxides gained from the deductive products of all the labeling compounds prepared here not only establishes a base for determining their capacity removing NO free radical but also provides a sensitive signal for their use as a probe. These are also obviously impossible for the previous unstable ESR labeling compounds.

2.6. Compounds 2g and 3g modified amino acids and peptides preventing PC12 cells from free radical damage

To examine the prevention function of **2g** and **3g** modified amino acids and peptides the PC12 cell damage assay was performed. In the experiment a method of Dawson with minor modifications was used,[10] NO, H_2O_2·, and ·OH were used as the damage radicals. The

Table 3. Structures of **2g** and **3g** modified amino acids and peptides

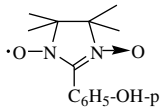
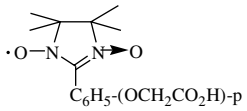
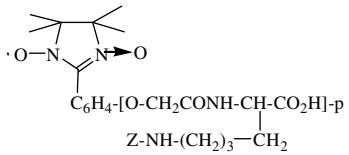
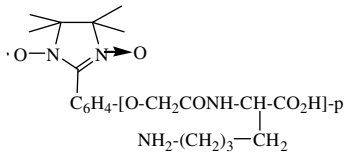
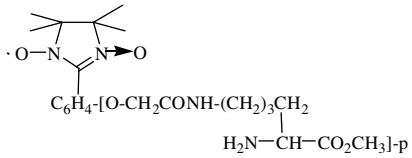
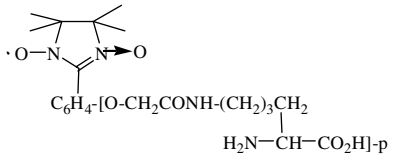
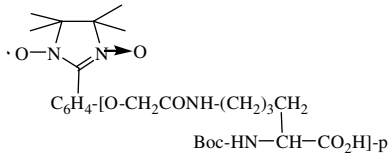
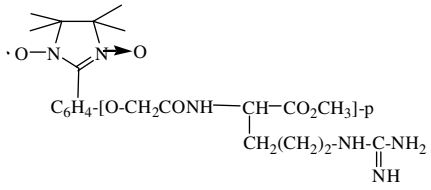
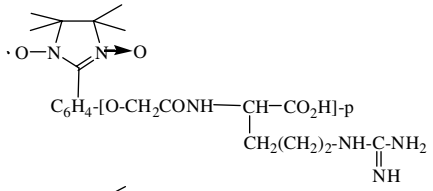
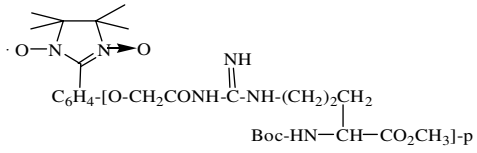
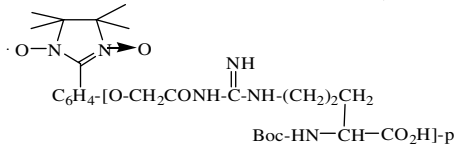
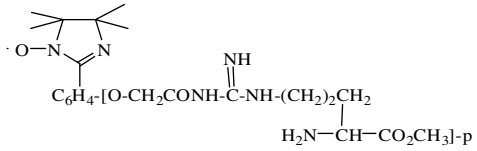
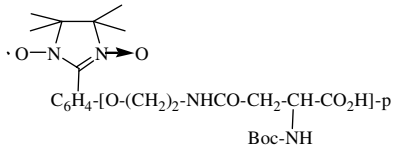
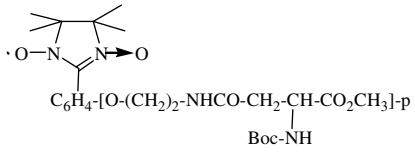
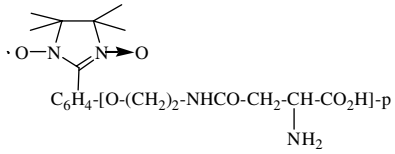
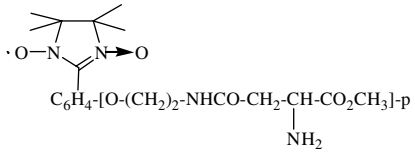
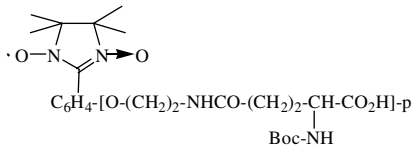
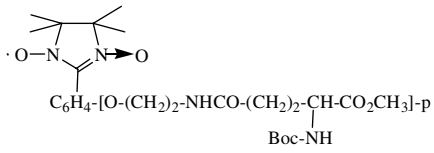
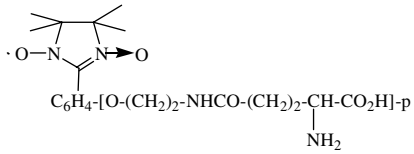
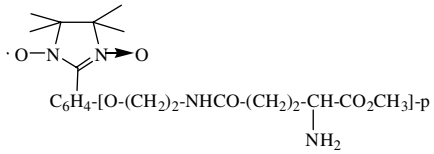
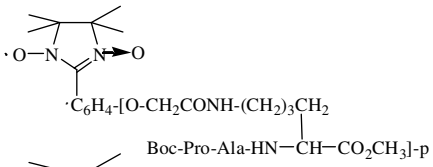
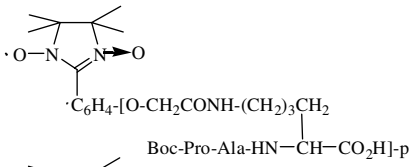
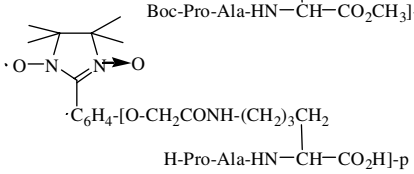
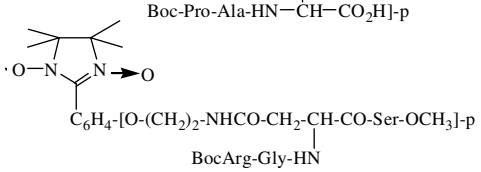
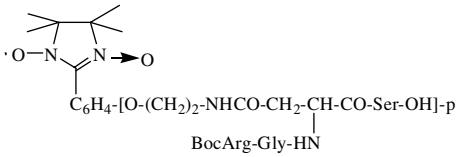
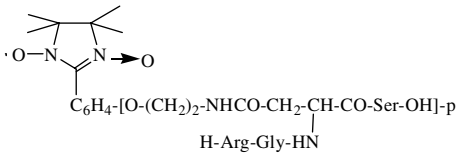
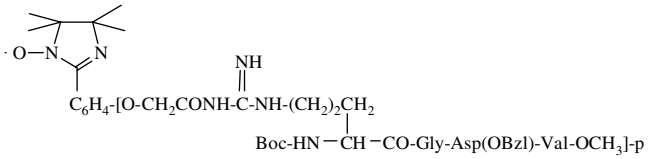
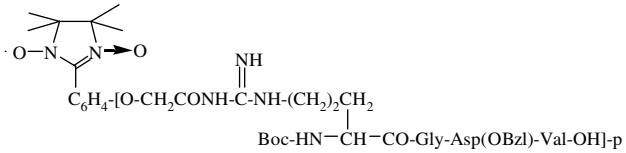
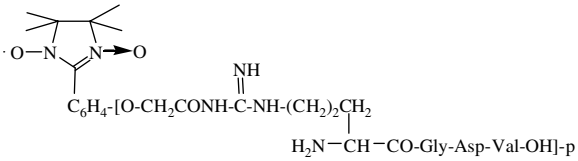
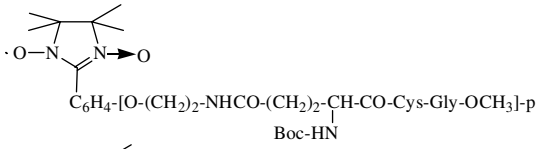
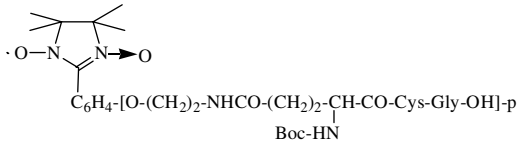
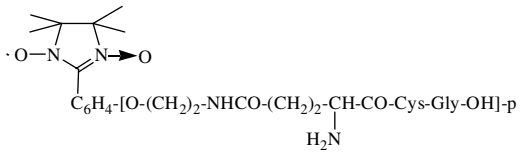
Compound	Chemical structure	Compound	Chemical structure
2g		**3g**	
5		**6**	
7		**8**	
9		**10**	
11		**12**	
13		**14**	
17		**18**	
19		**20**	
23		**24**	
25		**26**	

Table 3 (*continued*)

Compound	Chemical structure	Compound	Chemical structure
29		**30**	
31		**41**	
42		**43**	
34			
35			
36			
46			
47			
48			

potency is expressed by $EC_{50}(\mu M)$ and the data are listed in Table 4. The observation gave substantially similar EC_{50} values for **2g** and **3g** modified amino acids and peptides. The recorded capacity of **2g** and **3g** modified amino acids preventing PC12 cell from free radical damage suggested that after a series of preparative reactions and treatments they still had desirable free radical scavenging activity. The recorded ability of the peptides containing **2g** and **3g** modified amino acids preventing PC12 cell from free radical damage suggested that after a series of preparative reactions and treatments they still had desirable free radical scavenging activity. The capacity of all the labeling compounds themselves preventing PC12 cell from free radical damage not only establishes a base for directly using them in cell prevention but also provides a cell model for screening free radical scavengers with each the present compound as positive control. These are obviously impossible for the previous unstable ESR labeling compounds with bioactivity loss.

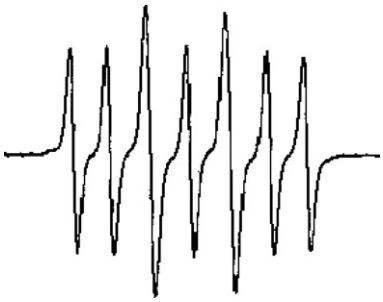


Figure 2. Typical ESR spectrum recorded from the reactions of **2g**, **3g**, the modified amino acids and the peptides with NO gas.

Table 4. PC12 cell prevention activity of **2g** and **3g** modified compounds[a]

Compound	EC_{50} (μM)		
	NO	H_2O_2·	·OH
2g	40.02 ± 1.98	49.05 ± 2.75	56.90 ± 2.39
3g	92.30 ± 2.05	31.64 ± 2.20	100.08 ± 2.17
6	90.60 ± 2.79	30.07 ± 2.88	99.11 ± 3.09
8	90.56 ± 2.64	29.85 ± 2.66	98.77 ± 3.06
9	89.90 ± 2.78	29.33 ± 2.85	97.72 ± 3.22
11	92.88 ± 2.95	32.07 ± 2.69	100.92 ± 3.12
13	93.00 ± 2.90	32.41 ± 2.94	101.76 ± 2.99
14	93.61 ± 3.00	33.36 ± 2.93	102.85 ± 2.92
19	42.31 ± 3.04	48.62 ± 3.31	58.22 ± 3.11
20	38.99 ± 2.78	47.82 ± 2.93	55.63 ± 3.00
23	42.66 ± 2.18	49.89 ± 2.98	59.00 ± 2.99
25	41.77 ± 2.55	49.62 ± 2.80	58.06 ± 2.62
31	88.99 ± 3.22	28.83 ± 3.11	99.00 ± 3.03
36	92.86 ± 2.92	32.83 ± 2.87	101.78 ± 3.20
43	93.01 ± 3.00	32.04 ± 3.19	101.95 ± 3.24
48	37.84 ± 3.06	46.98 ± 2.91	54.90 ± 3.04

[a] EC_{50} is expressed by $\overline{X} \pm$ SDμM; $n = 6$.

2.7. Compounds 2g and 3g modified amino acids and peptides inhibiting ACh-induced vasorelaxation

To examine the in vitro NO scavenging ability of **2g** and **3g** modified amino acids and peptides the rat aortic strip assay was performed according to a published method.[11] The data are expressed by the percentage inhibition of ACh-induced vasorelaxation and listed in Table 5. The data demonstrated that except **11**, all **2g** and **3g** modified amino acids effectively inhibited ACh inducing vasorelaxation, suggesting that after a series of preparative reactions and treatments they had desirable in vitro NO scavenging activity. For L-N^{α}-**3g**-Arg-OH (**11**) only very weak vitro NO scavenging activity was tested. This observation should be understandable considering that the interaction of the guanido of **11** with the NOS in the rat aortic strip may generate NO to counteract the NO scavenging activity of **11** and thus the ACh-induced vasorelaxation may be retained. Compared to PAK, RGDV, RGDS, and ECG **3g** modified peptides **31**, **36**, **43**, and **48** significantly inhibited ACh-induced vasorelaxation. The capacity of all the labeling compounds themselves inhibiting ACh-induced vasorelaxation not only establishes a base for directly using them to inhibit ACh but also provides a tissue model for screening ACh antagonists with each the present compound as positive control. These are obviously impossible for the previous unstable ESR labeling compounds with bioactivity loss.

Table 5. Inhibition of **2g** and **3g** modified compounds to ACh-induced vasorelaxation[a]

	Inhibition		
	100 μM	10 μM	1 μM
NS	1.6 ± 1.4		
PAK	9.5 ± 7.2[b]	6.1 ± 5.4[b]	4.5 ± 4.8[b]
ECG	9.2 ± 8.5[b]	5.2 ± 6.5[b]	3.2 ± 4.1[b]
RGDS	8.9 ± 8.3[b]	4.3 ± 5.0[b]	2.9 ± 3.3[b]
RGDV	10.5 ± 8.0[b]	7.6 ± 6.2[b]	5.3 ± 4.1[b]
2g	98.3 ± 4.8[c]	74.5 ± 5.2[d]	27.8 ± 5.6
3g	99.2 ± 4.5[c]	73.1 ± 5.1[d]	26.7 ± 5.2
6	98.9 ± 4.9[c]	74.0 ± 5.7[d]	28.7 ± 5.4
8	98.6 ± 4.8[c]	72.3 ± 5.0[d]	25.4 ± 5.1
9	97.9 ± 4.2[c]	72.7 ± 4.9[d]	28.0 ± 5.5
11	15.9 ± 5.3[b]	10.7 ± 5.5[b]	7.8 ± 5.6[b]
13	95.0 ± 5.5[c]	71.2 ± 5.0[d]	24.1 ± 5.3
14	96.1 ± 4.9[c]	72.4 ± 5.3[d]	25.7 ± 5.6
19	97.5 ± 4.7[c]	72.8 ± 5.0[d]	26.7 ± 5.2
20	99.1 ± 5.0[c]	76.0 ± 5.1[d]	29.2 ± 5.3
23	96.9 ± 5.2[c]	72.4 ± 4.9[d]	26.0 ± 5.1
25	97.1 ± 4.9[c]	72.4 ± 5.0[d]	26.2 ± 5.2
31	99.1 ± 5.6[c]	73.9 ± 5.4[d]	26.6 ± 5.5
36	96.6 ± 5.4[c]	72.8 ± 5.3[d]	26.2 ± 5.6
43	95.9 ± 4.6[c]	71.3 ± 5.0[d]	24.0 ± 4.9
48	97.3 ± 5.1[c]	72.2 ± 4.9[d]	25.9 ± 5.0

[a] Inhibition is expressed by $\overline{X} \pm$ SD%; $n = 6$.
[b] Except **11**, compared to all **2g**, and **3g** modified amino acids and peptides $p < 0.01$.
[c] Compared to 10 μM group $p < 0.01$.
[d] Compared to 1 μM group $p < 0.01$.

2.8. Compound 2g modified RGDS and 3g modified RGDV inhibiting in vitro platelet aggregation

To examine the in vitro anti-platelet aggregation activity of **2g** modified RGDS and **3g** modified RGDV the anti-platelet aggregation effect of **36** and **43** was examined with normal rabbit blood according to a published method,[12] platelet-activating factor (PAF, final concentration 10^{-7} M) and adenosine diphosphate (ADP, final concentration 10^{-5} M) were used as the activator. The maximal rate of platelet aggregation (Am%) was represented by the peak height of aggregation curve and the data are listed in Table 6. It was found that the in vitro anti-platelet aggregation activity of 10^{-6} M **43** equaled to the in vitro anti-platelet aggregation activity of 10^{-6} M RGDS and significantly higher than that of **2g**, while the in vitro anti-platelet aggregation activity of 10^{-6} M **2g** equaled to that of NS. This comparison suggested that **2g** contributed nothing to the in vitro anti-platelet aggregation activity of **43**. It was also found that the in vitro anti-platelet aggregation activity of 10^{-6} M **36** equaled to the in vitro anti-platelet aggregation activity of 10^{-6} M RGDV and significantly higher than that of **3g**, while the in vitro anti-platelet aggregation activity of 10^{-6} M **3g** equaled to that of NS. This comparison suggested that **3g** contributed nothing to the in vitro anti-platelet aggregation activity of **36**. Without anti-platelet aggregation activity loss for

Table 6. Inhibition of **36** and **43** to ADP and PAF-induced platelet aggregation[a]

	Inhibition to ADP			Inhibition to PAF		
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-7} M	10^{-6} M	10^{-5} M
NS		50.40 ± 5.23			56.70 ± 4.11	
2g	49.28 ± 2.63	50.39 ± 3.22	49.01 ± 3.08	54.00 ± 3.25	52.45 ± 3.01	53.04 ± 2.19
RGDS	51.50 ± 2.43	39.62 ± 3.00[b]	16.60 ± 2.61[c]	53.69 ± 3.10	36.47 ± 3.00[b]	22.03 ± 2.31[c]
43	50.00 ± 3.11	39.43 ± 3.14[b]	16.11 ± 2.51[c]	51.59 ± 3.11	34.33 ± 2.22[b]	21.52 ± 2.19[c]
3g	51.98 ± 2.45	50.65 ± 4.00	49.33 ± 4.02	53.80 ± 3.01	51.40 ± 3.11	52.45 ± 2.09
RGDV	49.78 ± 3.20	33.41 ± 2.83[b]	12.65 ± 1.84[c]	52.07 ± 3.27	34.84 ± 2.51[b]	20.01 ± 2.12[c]
36	50.20 ± 3.13	39.60 ± 3.03[b]	16.38 ± 2.50[c]	50.26 ± 3.22	32.80 ± 2.70[b]	19.88 ± 2.21[c]

[a] Inhibition is expressed by $\overline{X} \pm$ SD%, $n = 8$, NS = vehicle.
[b] Compared to NS and 10^{-7}M group $p < 0.01$.
[c] Compared to NS and 10^{-6}M group $p < 0.01$.

Table 7. In vivo anti-thrombotic activity of **36** and **43**[a]

Compound	Dose	Wet thrombus weight	Dry thrombus weight
NS	3 ml/kg	39.89 ± 3.60	6.95 ± 1.01
Asprin	20 mg/kg	24.16 ± 2.90[b]	4.04 ± 1.03[b]
2g	5 μmol/kg	38.90 ± 3.10	6.80 ± 0.88
RGDS	5 μmol/kg	27.90 ± 2.18[b]	4.22 ± 1.10[b]
43	5 μmol/kg	25.01 ± 2.12[b]	4.08 ± 1.11[b]
3g	5 μmol/kg	38.76 ± 3.18	6.67 ± 0.79
RGDV	5 μmol/kg	28.60 ± 3.20[b]	4.43 ± 1.06[b]
36	5 μmol/kg	26.11 ± 2.94[b]	4.18 ± 1.12[b]

[a] Thrombus weight is expressed by $\overline{X} \pm$ SDmg, $n = 10$, NS=vehicle.
[b] Compared to NS, **2g** and **3g** $p < 0.01$.

RGDS with ESR on its R guanido and RGDV with ESR on its D β-carboxyl not only establishes a base for directly using them to inhibit platelet aggregation but also provides an in vitro model for screening ESR containing anti-platelet aggregators with each the present compound as positive control. Though these were also true for previously reported RGD peptides with ESR on their N-termini,[9] this optional ESR labeling strategy provides a general means for structural diversity design.

2.9. Compound 2g modified RGDS and 3g modified RGDV possessing in vivo anti-thrombotic activity

To examine the in vivo anti-thrombotic activity of **2g** modified RGDS and **3g** modified RGDV **43** and **36** were evaluated with a rat model. In the evaluation a published method was followed,[13] and the thrombus weight was used to express the activity, which is listed in Table 7. It was found that the in vivo anti-thrombotic activity of 5 μmol/kg **43** equaled to the in vivo anti-thrombotic activity of 5 μmol/kg RGDS and significantly higher than that of **3g**, while the in vivo anti-thrombotic activity of 5 μmol/kg **3g** equaled to that of NS. This comparison suggested that **3g** contributed nothing to the in vivo anti-thrombotic activity of **43** and introducing **3g** into PAK resulted in no decrease of its in vivo anti-thrombotic activity. It was also found that the in vivo anti-thrombotic activity of 5 μmol/kg **36** equaled to the in vivo anti-thrombotic activity of 5 μmol/kg RGDV and significantly higher than that of **3g**, while the in vivo anti-thrombotic activity of 5 μmol/kg **3g** equaled to that of NS. This comparison suggested that **3g** contributed nothing to the in vivo anti-thrombotic activity of **36** and introducing **3g** into RGDV resulted in no decrease of its in vivo anti-thrombotic activity. Without anti-thrombotic activity loss for RGDS with ESR on its R guanido and RGDV with ESR on its D β-carboxyl not only establishes a base for directly using them to inhibit thrombosis but also provides an in vitro model for screening ESR containing anti-thrombotic agents with each the present compound as positive control. Though these were also true for previously reported RGD peptides with ESR on their N-termini,[9] this optional ESR labeling strategy provides a general means for structural diversity design.

2.10. Compound 3g modified PAK prolong euglobulin clot lysis time

To examine the in vitro thrombolytic activity of **3g** modified PAK the euglobulin clot lysis time (ECLT) assay was performed. In the determination the rabbit euglobulin clots were prepared in a 96-well microtiter plate according to a published method.[14,15] To the rabbit euglobulin clots, NS, UK, **3g**, PAK, and **31** were added and the ECLT was recorded. The data are listed in Table 8. It was found that the ECLT of 4.5 μM **31** equaled to the ECLT of 4.5 μM PAK and significantly shorter than that of 4.5 μM **3g**, while the ECLT of 4.5 μM **3g** equaled to that of NS. This comparison suggested that **3g** contributed nothing to the in vitro thrombolytic activity of **31** and introducing **3g** into PAK resulted in no decrease of its in vitro thrombolytic activity. The ECLTs of **31** at

Table 8. Euglobulin clot lysis time of **31**[a]

Compound	Concentration	ECLT
NS	—	210.22 ± 15.31
UK	5 IU	130.34 ± 15.35[b]
3g	4.5 μM	223.11 ± 16.00
PAK	4.5 μM	101.60 ± 15.41[c]
	4.5 μM	102.95 ± 14.82[d]
31	2.2 μM	128.80 ± 12.77[e]
	1.1 μM	149.00 ± 13.19[b]

[a] ECLT is expressed by $\overline{X} \pm$ SDmin; NS = vehicle; $n = 6$.
[b] Compared to NS and **3g** $p < 0.01$.
[c] Compared to NS $p < 0.01$, to UK $p < 0.05$.
[d] Compared to NS $p < 0.01$, to 2.2 μM **31** $p < 0.05$.
[e] Compared to 1.1 μM **31** $p < 0.05$.

different concentration indicated that its in vitro thrombolytic activity depended on the dose. Without euglobulin clot lysis activity loss for PAK with ESR on its K ω-amino group not only establishes a base for directly using it as euglobulin clot lysis agent but also provides an in vitro model for screening ESR containing thrombolytic agents with it as positive control, and provides a general means for structural diversity design as well.

2.11. Compound 3g modified PAK enlarging fibrinogen–agarose lysis area

To further examine the in vitro thrombolytic activity of **3g** modified PAK the fibrinogen lysis area assay was also performed according to a published procedure.[16–18] In the determination the rabbit fibrinogen–agarose mixture was prepared and coagulated with thrombin in plastic dishes. To observe the fibrinolytic activity, 30 μL of NS and the solution of UK, PAK or **31** in NS were added to each well. The plate was incubated and areas of lysis were quantified. The data are listed in Table 9. It was found that the lysis area of 2.2 μM **31** equaled to the lysis area of 4.5 μM PAK and significantly larger than that of 4.5 μM **3g**, while the lysis area of 4.5 μM **3g** equaled to that of NS. This comparison suggested that **3g** contributed nothing to the in vitro thrombolytic activity of **31** and introducing **3g** into PAK resulted in no decrease of its in vitro thrombolytic activity. The lysis areas of **31** at different concentration indicated that its in vitro thrombolytic activity depended on the dose. Without fibrinogen–agarose lysis activity loss for PAK with ESR on its K ω-amino group not only establishes a base for directly using it as fibrinogen–agarose lysis agent but also provides an in vitro model for screening ESR containing thrombolytic agents with it as positive control, and provides a general means for structural diversity design as well.

2.12. Compound 3g modified PAK reducing thrombus weight in rat model

To examine the in vivo thrombolytic activity of **3g** modified PAK the thromboclasis assay was performed according to a published procedure.[12] In the assay the in vivo thrombolytic activity of **31** was tested on the pentobarbital sodium anesthetized male Wistar rats. The reduced weight of the thrombus in an inserted polyethylene tube is used to represent the thrombolytic activity. The data are listed in Table 10. It was found that the reduced thrombus weight of 10.0 μmol/kg **31** equaled to the reduced thrombus weight of 10.0 μmol/kg PAK and significantly higher than that of 10.0 μmol/kg **3g**, while the reduced thrombus weight of 10.0 μmol/kg **3g** equaled to that of 3 ml/kg NS. This comparison suggested that **3g** contributed nothing to the in vivo thrombolytic activity of **31** and introducing **3g** into PAK resulted in no decrease of its in vivo thrombolytic activity. The in vivo thrombolytic activities of **31** at different dose indicated that its in vivo thrombolytic activity depended on the dose. Without thrombolytic activity loss for PAK with ESR on its K ω-amino group not only establishes a base for directly using it as thrombolytic agent but also provides an in vivo rat model for screening ESR containing thrombolytic agents with it as positive control, and provides a general means for structural diversity design as well.

Table 9. Fibrinogen–agarose lysis areas of **31**[a]

Compound	Concentration	Lysis area
NS	—	25.09 ± 9.96
UK	5 IU	225.33 ± 11.02[b]
3g	4.5 μM	26.60 ± 9.85
PAK	4.5 μM	214.11 ± 10.20[b]
	4.5 μM	233.50 ± 10.00[c]
31	2.2 μM	216.23 ± 9.24[d]
	1.1 μM	197.02 ± 10.03[b]

[a] Fibrinogen–agarose lysis area is expressed by $\overline{X} \pm \mathrm{SD}\,\mathrm{mm}^2$; NS = vehicle; $n = 6$.
[b] Compared to NS and **3g** $p < 0.01$.
[c] Compared to NS and **3g** $p < 0.01$, to 2.2 μM **31** $p < 0.05$.
[d] Compared to NS and **3g** $p < 0.01$, to 1.1 μM **31** $p < 0.05$.

Table 10. Thrombolytic activity of **31**[a]

Compound	Dosage	Reduce thrombus weight
NS	5 ml/kg	12.02 ± 2.35
UK	20,000 IU/kg	23.62 ± 2.45[b]
3g	10.0 μmol/kg	12.11 ± 2.85
PAK	10.0 μmol/kg	24.11 ± 2.31[b]
	10.0 μmol/kg	24.20 ± 2.40[c]
31	5.0 μmol/kg	15.38 ± 2.25[d]
	2.5 μmol/kg	11.06 ± 2.30

[a] Reduced thrombus weight is expressed by $\overline{X} \pm \mathrm{SD}$mg; NS = vehicle; $n = 10$.
[b] Compared to NS and **3g** $p < 0.01$.
[c] Compared to NS and **3g** $p < 0.01$, to 2.2 μM **31** $p < 0.05$.
[d] Compared to NS and **3g** $p < 0.05$, to 1.1 μM **31** $p < 0.01$.

3. Conclusion

Using 2-(4′-hydroxyl)phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (**2g**) and [1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2-yl)-phenyl-4-yl]oxyacetic acid (**3g**) as ESR labeling block L-Asp-OH, L-Glu-OH, L-Lys-OH, L-Arg-OH, RGDS, RGDV, and ECG were successively modified and 30 related compounds with free radical property were obtained (Table 3). A series of NO free radical related chemical, cell and rat aortic strip tests confirmed that all these **2g** and **3g** labeling 18 amino acid derivatives were stable enough to the reaction conditions of peptide synthesis. A series of NO free radical related chemical, cell and rat aortic strip tests, as well as the in vitro and in vivo assays of 12 labeling peptides confirmed that the strategy of labeling bioactive peptides via incorporating **2g** and **3g** labeling amino acid was practical.

4. Experimental

4.1. General

Unless otherwise stated, all reactions were under a nitrogen atmosphere (1 bar). Melting points are uncorrected.

The agents used in this work were purchased from Sigma Chemical Co (USA). Chromatography was performed on Qingdao silica gel H (Qingdao of China). The purities of the intermediates and the products were confirmed by TLC (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness, Germany) and HPLC (Waters, C_{18} column 4.6 × 150 mm, USA). NMR spectra were recorded at 300 MHz on a VXR-300 instrument or at 500 MHz on a Bruker Am-500 instrument in $CDCl_3$ with tetramethylsilane as internal standard. EI-MS was determined by Trace MS System (Thermo Finnigan, USA). Optical rotations were determined with a Schmidt + Haensch Polartromic D instrument (Germany). The statistical analysis of all the biological data was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

4.2. Preparing compounds 1a–h, 2a–h, 3g, and 4–48

Compounds **1a**–**h**, **2a**–**h**, **3g**, and **4**–**48** were prepared according to the procedures described in supporting information which also provides their physical chemical data.

4.3. Determination of ESR spactrum

Center field 3480 G, sweep width 100 G, sweep time 100 s, modulation amplitude 1.0×10^{-1} G, time constant 1.6×10^{-1} s, modulation frequency 100 kHz, microwave frequency 9.72 GHz, and microwave power 10 MW were used for ESR measurement. The ESR spectra of nitronyl nitroxide modified L-Lys, L-Arg-OH, L-Asp-OH, L-Glu-OH, ECG, PAK, RGDS, RGDV peptides and the related intermediates at two different concentrations, 10^{-7} M and 10^{-5} M, in deaerated water and phosphate buffer (pH 7.4), were recorded. The deaerated phosphate buffer containing nitronyl nitroxide modified L-Lys-OH, L-Arg-OH, L-Asp-OH, L-Glu-OH, ECG, PAK, RGDS, RGDV and the related intermediates (10^{-5} M) were bubbled with NO gas for 30 s and the ESR spectra of reaction products were recorded.

4.4. NO, H_2O_2· and ·OH induced PC12 cell damage assay

Free radical scavenging activities were evaluated in PC12 cells using a method of Dawson with minor modifications. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated horse serum (Hyclone), 5% 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. During the exponential phase of growth, PC12 cells were seeded in 96-well plates coated with poly-L-lysine at a density of 20,000 cells per well. After 24 h attachment period fresh media containing 12.5 μM, 25 μM, 50 μM, 100 μM, 200 μM of nitronyl nitroxide modified L-Lys, L-Arg-OH, L-Asp-OH, L-Glu-OH, ECG, PAK, RGDS, and RGDV 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. 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 of H_2O_2 followed by 1 h of incubation, while ·OH damage was induced by 1 mM of H_2O_2/30 μM Fe (II) followed by 1 h of incubation.

4.5. Rat aortic strip relaxation assay

Immediately after decapitation rat aortic strips were prepared 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 connected to the tension transducers and their relaxation–contraction curves were recorded. The noradrenaline (NE) solution (final concentration 10^{-9} mol/L) was added for inducing the contraction of the strips, and when the hypertonic contraction reached the maximal level NE was washed and the vessel strips were stabilized for 30 min. After renewal of the solution NE (final concentration 10^{-9} M) was added. When the hypertonic contraction value of aortic strips reached the peak 15 μl of water (vehicle), or the solution of nitroxide modified L-Lys-OH, L-Arg-OH, L-Asp-OH, L-Glu-OH, ECG, PAK, RGDS, and RGDV in 15 μl of water (final concentration 10^{-6} M), was added, respectively. After stabilization 1.5 μl of Ach (final concentration 10^{-6} M) was added and the NO scavenging activities of the compounds are expressed with the inhibition percentage of Ach-induced vasorelaxation.

4.6. In vitro platelet aggregation assay

Platelet-rich plasma was prepared by centrifugation of normal rabbit blood anti-coagulation 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 agonists used were platelet-activating factor (PAF, final concentration 10^{-5}–10^{-7} M) and adenosine diphosphate (ADP, final concentration 10^{-5}–10^{-7} M). The effects of **36** and **43** on PAF or ADP induced platelet aggregation were observed. The maximal rate of platelet aggregation (Am%) was represented by the peak height of aggregation curve.

4.7. ECLT assay

The rabbit euglobulin clots were prepared according to a published method. 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 of $CaCl_2$, 0.68 mM of $MgCl_2$ and 93.96 mM of NaCl). To the rabbit euglobulin clots, NS, UK, PAK or **31** was added and the ECLT or time to clot lysis was determined in a 96-well microtiter plate.

4.8. Fibrinogen–agarose lysis area assay

The fibrinogen–agarose mixture was prepared and coagulated with thrombin in plastic dishes according to a

published procedure. 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 × 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, PAK, and **31** was added to each well. The plate was incubated and areas of lysis were quantified by lysis area. The statistical analysis of the data was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

4.9. In vivo anti-thrombosis assay

Male Wistar rats weighing 250–300 g (purchased from Animal Center of Peking University) were used. The tested compounds were dissolved in NS just before use and kept in an ice bath. The rats were anesthetized with pentobarbital sodium (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 full with heparin sodium (50 IU/ml of NS) and one end was inserted into the left jugular vein. From the other end of the polyethylene tube heparin sodium was injected as anti-coagulant, then the tested compounds were injected, which was inserted into the right carotid artery. In the case the tube was full of NS or tested compound dissolved in NS. 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, weighed to record the weight of the wet thrombus was recorded, kept in a desiccator for 2 weeks, and weighed to record the weight of the dry thrombus. The statistical analysis of the data was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

4.10. In vivo thrombolytic assay

Male Wistar rats weighing 200–300 g (purchased from Animal Center of Peking University) were anesthetized with pentobarbital sodium (80.0 mg/kg, ip). The right carotid artery and left vein jugular of the animals were separated. To the glass tube filled with artery blood (1.0 ml) from the right carotid artery of the animal a stainless steel filament helix (15 circles; L, 15 mm; D, 1.0 mm) was put immediately. After 15 min the helix with thrombus was carefully taken out and weighted exactly, which was put into the middle polyethylene tube. The polyethylene tube was full 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 anti-coagulant, following which the tested compound was injected. The blood was circulated through the polyethylene tube for 90 min, after which the helix was taken out and weighted accurately. The reduction of thrombus mass was recorded. The statistical analysis of the data was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

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Supplementary data

Synthetic routes, preparing procedures, physical, analytical, and spectrometric data of all compounds. This material is available free of charge via the Internet at http://www.siencedirect.com/science/journal/09680896. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.01.022.

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