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Synthesis and Bioactivities of Nitronyl Nitroxide and RGD Containing Pseudopeptides

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Abstract—1-(1′,3′-Dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2′-yl)-phenyl-4-yloxylacetic acid (**3**), and 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2′-yl)-phenyl-4-yloxylacetyl-RGDS (**13**), -RGDV (**14**), -RGDF (**15**) were synthesized. The ESR measurement gave the same spectroscopy for **3** and **13**–**15**. The NO scavenging tests in vitro, anti-platelet aggregation tests in vitro and the anti-thrombosis assay in vivo indicated that introducing **3** into the N-terminal of RGDS, RGDV and RGDF the corresponding bioactivities for both of **3** and RGD peptides can be remained completely. The present combinations provided a beneficial strategy for simultaneous scavenging NO and anti-thrombosis, and for the use of spin label of RGD peptides in the conformational researches.

Introduction

The data resulted from a number of investigations suggest that the impact of NO on ischemic brain injury depend on the stage of evolution of the tissue damage and glutamate–NO pathway may occur in ischemic penumbra, by which large amounts of NO are produced by nNOS and might contribute to metabolic deterioration of the penumbra leading to lager infarction.[1–3] The therapeutic strategies have attempted to reduce the damage either by intervening in the formation process of NO or by scavenging NO already formed. For the strategy of scavenging NO already formed and spin labeling of peptides nitronyl nitroxide attracts extensive attention.[4–6]

Platelet aggregation plays an essential role in normal homeostasis and is dependent on the interaction of membrane glycoprotein GPIIb/IIIa complex with plasma adhesive glycoproteins.[7,8] In addition to monoclonal antibodies the binding function of GPIIb/IIIa can be blocked by RGD containing peptides which can also prevent platelet-dependent thrombus formation in experimental models of coronary artery thrombosis.[9,10]

In our previous papers RGDS, RGDV and RGDF were used as the building block in the modification of the oligopeptides with anti-thrombotic and/or thrombolytic activity.[11–13] The results obtained indicated that the contribution of RGD sequence to the specific interaction of RGD containing peptides and pseudopeptides may result in the desirable lead compounds. Based on the abilities of nitronyl nitroxide as NO scavenging or spin labeling agent and of RGD peptide as anti-thrombotic agent a number of combinations of them were considered. As part of a program to develop thrombus related therapeutic psedopeptides in the present paper 1-(1′,3′-dioxy-4′,4′,5′,5′-tetramethyldi-hydroimidazol-2′-yl)-phenyl-4-yloxyacetic acid (**3**) was introduced into the N-terminal of RGDS, RGDV and RGDF and their ESR measurement, scavenging NO in vitro, anti-platelet aggregation in vitro, anti-platelet aggregation in vitro and anti-thrombotic in vivo were observed.

Chemistry

1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydromidazol-2′-yl)-phenyl-4-yloxylacetic acid (**3**) was synthesized according to Scheme 1. Treating 4′-hydroxy-2-phenyl-1,3-dioxyl-4,4,5,5-tetra-methyldihydroimida-zol (**1**), which was prepared by use of the same procedure as that in the literature[14] from 2,3-dihydroxyamino-2,3-dimethylbutane and *p*-hydroxylbenzaldehyde with $BrCH_2COOC_2H_5$ gave 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2′-yl)-phenyl-4-yloxylacetic ethyl ester (**2**) as an intense blue powder in 90% yield. In the presence of NaOH (2 mol/L), compound **2** was

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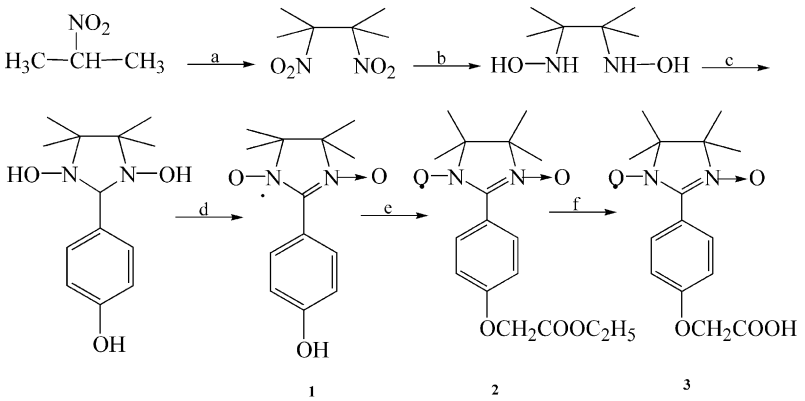
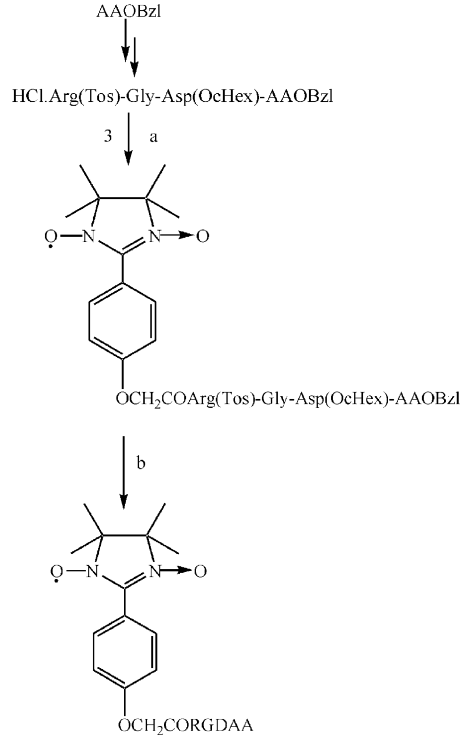


Scheme 1. Preparation of 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2′-yl)-phenyl-4-yloxylacetic acid **3**: (a) Br_2, NaOH (6 mol/l); (b) Zn, NH_4Cl; (c) *p*-HO–C_6H_4CHO; (d) PbO_2; (e) $BrCH_2COOC_2H_5$, $NaOC_2H_5$ THF; (f) NaOH (2 mol/L).

converted into the corresponding acid **3** in theoretical yield (Scheme 1).

With the same procedure as that in the literature[10,12] the protective tetrapeptides Boc-Arg(Tos)-Gly-Asp- (OcHex)-Ser(Bzl)-OBzl (**4**), Boc-Arg(Tos)-Gly-Asp-(OcHex)-Val-OBzl (**5**), Boc-Arg(Tos)-Gly-Asp-(OcHex)-Phe-OBzl (**6**) were prepared. After removal of their Boc protecting groups the protective peptides with N-terminal free were coupled with compound **3** to give the nitronyl nitroxide containing protective peptides (**10**–**12**) in 87, 90, 90% yield, respectively. The deprotection of the protective groups in the side chain of the amino acid residue with anhydrous HF and the deposition of the reaction residue in air or ether for 2 h the crude product, a colorless powder, may turn into blue. After purification by chromatography (Sephadex G_{10}) 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydro-imidazol-2′-yl)-phenyl-4-yloxylacetyl-RGDS (**13**),-RGDV (**14**), and-RGDF (**15**) were obtained in 73%, 70%, and 80% yield,[15] respectively (Scheme 2).



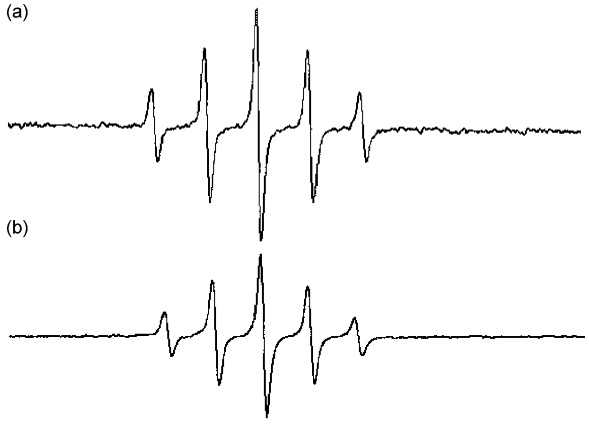
Scheme 2. Preparation of 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyl-dihydroimidazol-2′-yl)-phenyl-4-yloxylacetyl-RGDS (**13**), -RGDV (**14**), and -RGDF (**15**): (a) DCC and NMM; (b) HF.

ESR Measurement

Center field: 3480 Gauss, Sweep Width: 100 Gauss, 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 and no any difference between compound **3** and **13**–**15** was observed. In phosphate buffer (pH 7.4) the ESR spectra of compound **3** and **13**–**15** may be monitored at 10^{-7} mol/L. At 10^{-7} mol/L–10^{-5} mol/L no any difference between the ESR spectra of compound **3** and **13**–**15** was observed. The typical spectroscopy is given in Scheme 3.

Scavenging NO In Vitro[16]

Immediately after decapitation, rat aortic strips were prepared and put in a perfusion bath with 5 mL of



Scheme 3. The ESR spectra of compound **3** (a) and **15** (b) in phosphate buffer (pH 7.4, final concentration 10^{-5} mol/L).

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 to the maximal level NE was washed[1] and the vessel strips were stabilized for 30 min. After renewal of the solution NE (final concentration 10^{-9} mol/L) was added. When the hypertonic contraction value of aortic strips reached the peak 15 μL of water (vehicle), or the solution of **3**, **13**–**15** in 15 μL of water (final concentration 10^{-6} mol/L) was added respectively. After stabilization, 1.5 μL of Ach (final concentration 10^{-6} mol/L) were added and the NO scavenging activities of the compounds are expressed with the inhibition percentage of Ach-induced vasorelaxation and listed in Table 1.

Anti-platelet Aggregation In Vitro[13]

Platelet-rich plasma was prepared by centrifugation of normal rabbit blood anticoagulation with sodium citrate at a final concentration of 3.8%. The platelet counts were adjusted to 2×10^5/μL by addition of autologous plasma. Platelet aggregation studies were conducted in an aggregometer using the standard turbidimetric technique. The agonists used were platelet-activating factor (PAF, final concentration 10^{-7} mol/L) and adenosine diphosphate (ADP, final concentration 10^{-5} mol/L). The effects of compounds **3**, **13**–**15** 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. The data are listed in Tables 2 and 3 and the statistical analysis of the date is carried out by use of ANOVA test, $p<0.05$ is considered significant.

Anti-thrombotic In Vivo[13]

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 anticoagulant, then the tested compounds were injected, which this end was inserted into the right carotid artery. In this case the tube was full of NS or tested compound containing NS. The blood was flowed

Table 1. Inhibition percentage of Ach-induced vasorelaxation

Compd	%, $\bar{X}\pm$SD
Water	1.6±1.4
3	99.2±1.4[a]
13	88.9±1.2[a,b]
14	91.2±0.5[a,b]
15	88.9±5.3[a,b]
RGDS	3.2±2.8
RGDV	2.4±1.9
RGDF	2.6±2.5

$n=6$; water = vehicle.
[a]Compare to water, $p<0.001$.
[b]Compare to **3**, $p>0.05$.

Table 2. Effect of the compounds on ADP induced platelet aggregation

Compd	Am % ($\bar{X}\pm$SD), at the dose of		
	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS	55.97±5.01		
Aspirin	54.89±2.88	42.58±2.99[a]	30.01±2.66[a]
3	54.68±2.40	51.96±4.18	51.60±3.99
RGDS	53.55±2.46	40.14±3.11[a]	17.02±2.69[a]
RGDV	50.06±3.29[b]	28.90±2.89[a]	12.07±1.89[a]
RGDF	33.76±2.44[a]	24.15±2.96[a]	10.13±1.02[a]
13	51.46±3.24[c]	40.05±3.21[a]	16.91±2.55[a]
14	49.98±3.81[b]	24.99±2.54[a]	11.85±1.65[a]
15	35.25±2.96[a]	20.66±2.96[a]	10.21±1.95[a]

$n=8$; NS, vehicle.
[a]Compare to NS, $p<0.001$.
[b]Compare to NS, $p<0.01$.
[c]Compare to NS, $p<0.0.^5$

Table 3. Effect of the compounds on PAF induced platelet aggregation

Compd	Am % ($\bar{X}\pm$SD), at the dose of		
	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS	57.82±4.55		
Aspirin	53.42±3.44	36.98±2.95[a]	29.99±2.95[a]
3	54.25±3.16	52.98±4.39	52.92±4.19
RGDS	54.21±3.19	37.10±3.25[a]	22.66±2.36[a]
RGDV	52.66±4.91	35.11±2.70[a]	20.50±2.01[a]
RGDF	51.88±2.65[a]	25.18±2.28[a]	10.22±2.15[a]
13	52.10±3.25[c]	35.11±2.48[a]	22.05±2.33[a]
14	50.88±4.10[b]	33.22±3.09[a]	20.45±2.26[a]
15	50.02±3.98[b]	24.26±2.42[a]	9.98±2.27[a]

$n=8$; NS = vehicle.
[a]Compare to NS, $p<0.001$.
[b]Compare to NS, $p<0.01$.
[c]Compare to NS, $p<0.05$.

Table 4. Effect of the compounds on the thrombus weight

Compd	Wet thrombus ($\bar{X}\pm$SD mg)	Dry thrombus ($\bar{X}\pm$SD mg)
NS (3 mL/kg)	40.19±4.13	6.95±2.01
Aspirin	28.11±2.99[c]	3.99±1.030[b]
3	38.10±3.18	6.17±0.70
RGDS	36.96±3.78	6.68±1.54
RGDV	32.62±3.20[a]	5.13±1.06[a]
RGDF	25.61±3.14[c]	3.94±0.95[b]
13	32.09±3.42[a]	5.08±1.21[a]
14	29.49±3.30[c]	4.07±1.00[b]
15	24.30±2.43[c]	3.16±0.45[c]

$n=8$; dosage: 170 μmol/kg for aspirin, 5 μmol/kg for others; NS, vehicle.
[a]Compare to NS, $p<0.05$.
[b]Compare to NS, $p<0.01$.
[c]Compare to NS, $p<0.001$.

from the right carotid artery to the left jugular vein via the polyethylene tube for 15 min. The thread was taken out and weighed and the weight of the wet thrombus was recorded. The thread was kept in a desiccator for 2 weeks and the weight of the dry thrombus was recorded. The data are listed in Table 4. The statistical analysis of the date is also carried out by use of ANOVA test, $p < 0.05$ is considered significant.

Discussion

As the NO scavenging agent block **3** can be smoothly introduced into the N-terminal of RGDS, RGDV and RGDF. In the ESR measurements **3**, **13**–**15** give the same spectroscopy means that as the NO scavenging block **3** was stable enough to the deprotection of the protective groups on the side chain of the amino acid residue in **3** containing peptides with CF_3SO_3H/TFA (1:4, v/v) or anhydrous HF as the deprotection system. The special stability of **3** to the conditions of peptide synthesis indicates that as peptide spin label block **3** may have potential use.

Even though RGDS, RGDV and RGDF exhibited no any NO scavenging activity the inhibition percentage of Ach-induced vasorelaxation for **3**, **13**, **14**, and **15** was 99.2 ± 1.4, 88.9 ± 1.2, 91.2 ± 0.5, and 88.9 ± 5.3, respectively, which was significantly different from that (1.6 ± 1.4) for water (Table 1). The approximate equal ability of NO scavenging for **3**, **13**, **14**, and **15** demonstrated that when RGDS, RGDV or RGDF was introduced into **3** the formed linker as the substrate may be still suitable for NO scavenging reaction and NO scavenging activity of **3** was consequently remained.

At the concentrations of 10^{-7}, 10^{-6}, and 10^{-5} mol/L **3** exhibited no any effect on ADP and PAF induced platelet aggregation but at the concentration of 10^{-6} and 10^{-5} mol/L **13**, **14** and **15** obviously inhibited platelet aggregation. When ADP activated platelets were treated with 10^{-6} mol/L, and 10^{-5} mol/L of **13**, **14** or **15** the platelet aggregation rate (%) was lowered from 55.97 ± 5.01 (for NS, control) to 40.05 ± 3.21, 24.99 ± 2.54, 20.66 ± 2.96, and 16.91 ± 2.55, 11.85 ± 1.65, 10.21 ± 1.95, respectively. The treatment potency for **13**, **14** or **15** was substantially same as that for RGDS, RGDV or RGDF (Table 2). Approximately same effects of **13**, **14**, **15**, RGDS, RGDV or RGDF on PAF induced platelet aggregation was also observed (Table 3). The results indicated that when **3** was introduced into the N-terminal of RGDS, RGDV or RGDF the formed linker as the ligand may be still suitable for its binding to GP IIb/IIIa receptor and the anti-platelet aggregation activity of RGDS, RGDV or RGDF was consequently remained.

The anti-thrombolitic in vivo of **3** and **13**–**15** indicated that **3** exhibited no any effect on the thrombus weight but at the dose of 5 μmol/kg **13**, **14**, or **15** did exhibited anti-thrombolitic effect. The thrombus weight after the treatment with **13**, **14**, or **15** was 32.09 ± 3.42, 29.49 ± 3.30, and 24.30 ± 2.43 mg, respectively. The treatment potency for **13**, **14** or **15** was again substantially same as that for RGDS, RGDV or RGDF (Table 4). The results demonstrated that when **3** was introduced into the N-terminal of RGDS, RGDV or RGDF the formed linker as the the ligand may be still suitable for its binding to GP IIb/IIIa receptor and the anti-thrombolitic activity of RGDS, RGDV or RGDF was consequently remained.

Scavenging NO is an important strategy in the prevention of ischemic brain injury. The combination of nitronyl nitroxide and RGD peptide provides a possibility to scavenge NO and prevent thrombus formation simultaneously. On the other hand the label of RGD peptides with nitronyl nitroxide may provide new path for further biological investigation as well.

Acknowledgements

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126 °C, $[\alpha]_D^{20} = -40$ (*c* 0.2, 6 N HCl), FAB-MS (*m*/*e*): 494 $[M+H]^+$; 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyldihydroimidazol-2′ - yl) - phenyl - 4 - yloxylacetylArg(Tos) - Gly - Asp(OcHex)-Ser(Bzl)-OBzl, mp 76–78 °C, $[\alpha]_D^{20} = +50$ (*c* 1, CH_3OH), FAB-MS (*m*/*e*): 1109 $[M+H]^+$; 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyl-dihydroimidazol-2′-yl)-phenyl-4-yloxylacetylArg-Gly-Asp-Ser-OH (**13**), mp 154 °C (decomp.), $[\alpha]_D^{20} = 40$ (*c* 1, H_2O), FAB-MS (*m*/*e*): 723 $[M+H]^+$; 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyl-dihydroimidazol-2′-yl)-phenyl-4-yloxylacetylArg-(Tos)-Gly-Asp(OcHex)-Val-OBzl, mp 92–94 °C $[\alpha]_D^{20} = +55$ (*c* 1, CH_3OH), FAB-MS (*m*/*e*): 1031 $[M+H]^+$; 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyl-dihydroimidazol-2′-yl)phenyl-4-yloxylacetylArg-Gly-Asp-Val-OH (**14**), mp 160 °C (decomp.), $[\alpha]_D^{20} = +17$ (*c* 1, H_2O), FAB-MS (*m*/*e*): 735 $[M+H]^+$; 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyl-dihydroimidazol-2′-yl)phenyl-4-yloxylacetylArg-(Tos)-Gly-Asp(OcHex)-Phe-OBzl, mp 108–110 °C, $[\alpha]_D^{20} = -38$ (*c* 1, CH_3OH), FAB-MS (*m*/*e*): 1079 $[M+H]^+$; 1-(1′,3′-dioxyl-4′,4′,5′,5′-tetramethyl - dihydroimidazol-2-yl)phe-nyl-4-yloxylacetylArg-Gly-Asp-Phe-OH (**15**), mp 158 °C (decomp.), $[\alpha]_D^{20} = +20$ (*c* 1, H_2O), FAB-MS 783$[M+H]^+$.

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