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The composition and sequence specificity of Pro-Ala-Lys-OH for the thrombolytic activities of P6A and related oligopeptides

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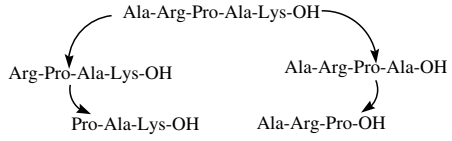
Abstract—The in vitro and in vivo thrombolytic activities of Ala-Arg-Pro-Ala-Lys-OH, its analogs and the related peptides were assayed. The results indicate that when ^{5}Lys of Ala-Arg-Pro-Ala-Lys-OH is changed into ^{5}Arg, and ^{3}Lys of Pro-Ala-Lys-OH is changed into ^{3}Arg the thrombolytic activities are collapsed; when Pro-Ala-Lys-OH is changed into Ala-Pro-Lys-OH, and Ala-Arg-Pro-Ala-Lys-OH is changed into Ala-Arg-Ala-Pro-Lys-OH the thrombolytic activities are also collapsed; when ^{5}Lys of Ala-Arg-Pro-Ala-Lys-OH is changed into ^{5}*n*Leu the thrombolytic activities are again collapsed. All of the results indicate that for the thrombolytic activities of Ala-Arg-Pro-Ala-Lys-OH and the related peptides Pro-Ala-Lys-OH exhibits either amino acid composition specificity or sequence specificity. The composition and sequence specificity of Pro-Ala-Lys-OH reflects its rule as the pharmacophore of P6A and the related peptides.

1. Introduction

It is known that the therapeutic use of thrombolytic agents usually associates with the release of the fibrinogen degradation products.[1] In certain cases some fibrinogen degradation products themselves may cause significant vasodilation. Among the fibrinogen degradation products a special sequence Ala-Arg-Pro-Ala-Lys (P6A, **1**) was firstly revealed as a permeability-increasing factor in 1989.[2] Afterwards the studies related to P6A were mainly focused on the reestablishment coronary blood flow in dogs,[3,4] the synergic effect with tPA or L-arginine,[5–7] and thus to thrombolytic effect.

Thrombus results in thromboembolic complications of arteriosclerosis, heart attacks, strokes, and peripheral vascular disease. The common and serious side effect such as hemorrhagenic tendency and immunogenic reactions, the inherent effect of macromolecular proteins for the clinical thrombolytic drugs, tPA, UK, and SK, attract a lot of attention to search of thrombolytic oligopeptides. Considering in certain cases some fibrinogen degradation products themselves may cause significant thrombolysis, the inherent advantages of oligopeptides, such as no hemorrhagenic tendency and immunogenic reactions, and the correlations of P6A and thrombus, P6A was used as the lead compound of thrombolytic drug. In the rigidization and cyclization based modifications of P6A ^{1}Gly-P6A and ^{1}Gln-P6A,[8,9] in the Arg-Gly-Asp based hybridization of P6A ^{1}Gly-P6A and ^{1}Gln-P6A,[10] particularly in the in vivo metabolism identification the importance of Pro-Ala-Lys-OH floated. For instance in the metabolism experiments we found that in vivo P6A degraded into four metabolites via two pathways (Scheme 1). The in vitro and in vivo thrombolytic assays showed that the metabolites, Arg-Pro-Ala-Lys-OH, and Pro-Ala-Lys-OH (**12**), exhibited thrombolytic activity. The thrombolytic potency of Arg-Pro-Ala-Lys-OH is comparable to that of P6A (**1**), and



Scheme 1. In vivo Ala-Arg-Pro-Ala-Lys-OH underwent stepwise metabolites and degraded from both of its C-terminal and N-terminal giving four metabolites Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala-Lys-OH, and Pro-Ala-Lys-OH.

Keywords: SAR; P6A; Pro-Ala-Lys-OH; Thrombolysis; Pharmacophore.
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of Pro-Ala-Lys-OH (**12**) is higher than that of P6A.[11] This observation suggests that even from such a five amino acid residue containing peptide it is still possible to have bioactive fragment. Based on the results drew from the studies on Ala-Arg-Pro-Ala-Lys-OH (**1**) itself it is conceivable that the studies on Pro-Ala-Lys-OH (**12**) will perhaps provide more interesting results.

In order to explore the necessity of the amino acid composition, the exclusiveness of the sequence and the importance of the positively charged side chain of Pro-Ala-Lys-OH (**12**) for the thrombolytic Ala-Arg-Pro-Ala-Lys-OH (**1**) and related peptides, in the present paper the ^{5}Lys of Ala-Arg-Pro-Ala-Lys-OH (**1**) was substituted by ^{5}nLeu, Pro-Ala-Lys-OH (**12**) sequence was alternated to Ala-Pro-Lys-OH (**7**), and the positively charged side chain was introduced into the N-terminal of Ala-Arg-Pro-Ala-Lys-OH (**1**). We hope that the differences of thrombolytic activities resulted from the structure modifications may reveal the possible pharmacophore of P6A and the related peptides.

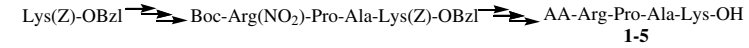
2. Results

2.1. Peptide synthesis

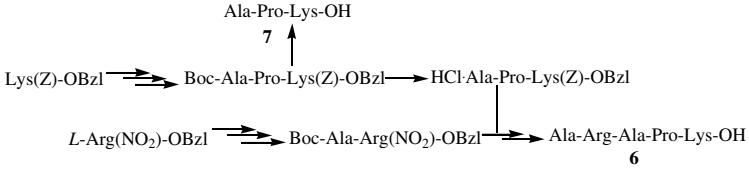
According to Scheme 2 the protective intermediate Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl was prepared via the solution method and stepwise synthesis (from C-terminal to N-terminal) with Lys(Z)-OBzl as the starting material in 62% total yield.[9] Coupling HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl and Boc-Ala-OH, Boc-Gln-OH, Boc-Lys(Z)-OH, Boc-Arg(NO_2)-OH or Boc-Gly-OH, Boc-Ala-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl, Boc-Gln-Arg-(NO_2)-Pro-Ala-Lys(Z)-OBzl, Boc-Lys(Z)-Arg-(NO_2)-Pro-Ala-Lys(Z)-OBzl, Boc-Arg(NO_2)-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl or Boc-Gly-Arg(NO_2)-Pro-Ala-Lys-(Z)-OBzl were obtained in 85–90% yield. In the presence of Pd/C (5%), formic acid in methanol (4.4%), and hydrogen they were converted into corresponding Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), Gly-Arg-Pro-Ala-Lys-OH (**3**), Lys-Arg-Pro-Ala-Lys-OH (**4**), and Arg-Arg-Pro-Ala-Lys-OH (**5**) in 88–90% yield.

According to Scheme 3 the protective intermediate Boc-Ala-Pro-Lys(Z)-OBzl, which was prepared with the procedure in the literature[11] was converted into HCl·Ala-Pro-Lys(Z)-OBzl. From Boc-Ala-OH and HCl·Arg(NO_2)-OBzl the intermediate Boc-Ala-Arg(NO_2)-OBzl was obtained in 86% total yield. After the condensation of Boc-Ala-Arg(NO_2)-OH and HCl·Ala-Pro-Lys(Z)-OBzl the protective intermediate Boc-Ala-Arg(NO_2)-Ala-Pro-Lys(Z)-OBzl was provided in 92% yield. In the presence of Pd/C (5%), formic acid in methanol (4.4%), and hydrogen Boc-Ala-Arg(NO_2)-Ala-Pro-Lys(Z)-OBzl and Boc-Ala-Pro-Lys(Z)-OBzl were converted into Ala-Arg-Ala-Pro-Lys-OH (**6**) and Ala-Pro-Lys-OH (**7**) in 92% and 90% yield, respectively.

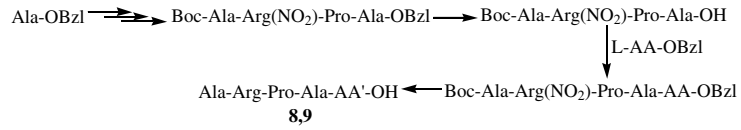
According to Scheme 4 the protective intermediate Boc-Ala-Arg(NO_2)-Pro-Ala-OBzl, which was prepared with the procedure in the literature[10] was converted into Boc-Ala-Arg(NO_2)-Pro-Ala-OH in 95% yield. This was coupled with HCl·*n*Leu-OBzl or HCl·Arg(NO_2)-OBzl to provide the protective intermediates Boc-Ala-Arg(NO_2)-Pro-Ala-*n*Leu-OBzl and Boc-Ala-Arg(NO_2)-Pro-Ala-



Scheme 2. Preparation of AA-Arg-Pro-Ala-Lys-OH. (1) General procedure for removal of benzyl group of the N-terminal component: At 0 °C the mixture of 0.80 mmol of the benzyl ester in 10 mL of methanol and 5 mL of the solution of NaOH in methanol (2 mol/L) was stirred for 2 h then neutralized to pH 7 and evaporated at room temperature to remove methanol. The residue was acidified to pH 1–2 with hydrochloric acid (2 mol/L). (2) General procedure for coupling of C-terminal and N-terminal components: At 0 °C the mixture of 0.20 mmol of the N-terminal component in 5 mL of anhydrous THF, 0.20 mmol of HOBt, and 0.25 mmol of DCC was stirred at 0 °C for 24 h. To the solution 0.20 mmol of C-terminal component and 0.26 mmol of *N*-methylmorpholine were added and stirred at room temperature for 24 h. (3) General procedure for removal of NO_2, Z, and Bzl: A suspension of 0.20 mmol of NO_2, Z, and Bzl protected peptides, 5 mg of Pd/C (5%), and 8 mL of formic acid in methanol (4.4%) was agitated with hydrogen at room temperature for 24 h. Wherein AA = Ala (**1**), Gln (**2**), Gly (**3**), Lys (**4**), Arg (**5**).



Scheme 3. Preparation of Ala-Pro-Lys-OH and Ala-Arg-Ala-Pro-Lys-OH. (1) General procedure for removal of benzyl group of the N-terminal component: At 0 °C the mixture of 0.80 mmol of the benzyl ester in 10 mL of methanol and 5 mL of the solution of NaOH in methanol (2 mol/L) was stirred for 2 h then neutralized to pH 7 and evaporated at room temperature to remove methanol. The residue was acidified to pH 1–2 with hydrochloric acid (2 mol/L). (2) General procedure for coupling of C-terminal and N-terminal components: At 0 °C the mixture of 0.20 mmol of the N-terminal component in 5 mL of anhydrous THF, 0.20 mmol of HOBt, and 0.25 mmol of DCC was stirred at 0 °C for 24 h. To the solution 0.20 mmol of C-terminal component and 0.26 mmol of *N*-methylmorpholine were added and stirred at room temperature for 24 h. (3) General procedure for removal of NO_2, Z, and Bzl: A suspension of 0.20 mmol of NO_2, Z, and Bzl protected peptides, 5 mg of Pd/C (5%), and 8 mL of formic acid in methanol (4.4%) was agitated with hydrogen at room temperature for 24 h.



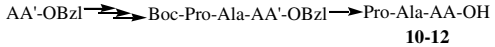
Scheme 4. Preparation of Ala-Arg-Pro-Ala-*n*Leu-OH and Ala-Arg-Pro-Ala-Arg-OH. (1) General procedure for removal of benzyl group of the N-terminal component: At 0 °C the mixture of 0.80 mmol of the benzyl ester in 10 mL of methanol and 5 mL of the solution of NaOH in methanol (2 mol/L) was stirred for 2 h then neutralized to pH 7 and evaporated at room temperature to remove methanol. The residue was acidified to pH 1–2 with hydrochloric acid (2 mol/L). (2) General procedure for coupling of C-terminal and N-terminal components: At 0 °C the mixture of 0.20 mmol of the N-terminal component in 5 mL of anhydrous THF, 0.20 mmol of HOBt, and 0.25 mmol of DCC was stirred at 0 °C for 24 h. To the solution 0.20 mmol of C-terminal component and 0.26 mmol of *N*-methylmorpholine were added and stirred at room temperature for 24 h. (3) General procedure for removal of NO_2, Z, and Bzl: A suspension of 0.20 mmol of NO_2, Z, and Bzl protected peptides, 5 mg of Pd/C (5%), and 8 mL of formic acid in methanol (4.4%) was agitated with hydrogen at room temperature for 24 h. Wherein AA = *n*Leu or Arg(NO_2), AA′ = *n*Leu (**8**) or Arg (**9**).

Arg(NO_2)-OBzl in 90% and 87% yield, respectively. In the presence of Pd/C (5%), formic acid in methanol (4.4%), and hydrogen they were converted into Ala-Arg-Pro-Ala-*n*Leu-OH (**8**) and Ala-Arg-Pro-Ala-Arg-OH (**9**) in 91% and 88% yield, respectively.

According to Scheme 5 using HCl·*n*Leu-OBzl, HCl·Arg(NO_2)-OBzl, and HCl·Lys(Z)-OBzl as the starting materials the protective intermediates Boc-Pro-Ala-*n*Leu-OBzl, Boc-Pro-Ala-Arg(NO_2)-OBzl, and Boc-Pro-Ala-Lys(Z)-OBzl, were obtained in 85%, 86%, and 75% total yield, respectively. In the presence of Pd/C (5%), formic acid in methanol (4.4%), and hydrogen they were converted into Pro-Ala-*n*Leu-OH (**10**), Pro-Ala-Arg-OH (**11**), and Pro-Ala-Lys-OH (**12**) in 94%, 87%, and 90% yield, respectively.

2.2. In vitro euglobulin clot lysis time of the peptides

The rabbit euglobulin clots were prepared by use of the method described in experimental section. To the rabbit euglobulin clots P6A or related peptide was added and the lysis time was determined and recorded using the standard procedure. The tested lysis times for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), and Pro-Ala-Arg-OH (**11**) are significantly different from that for Pro-Ala-Lys-OH (**12**). The tested lysis times for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), and Ala-Arg-Pro-Ala-Arg-OH (**9**) are significantly different from that for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**). The tested lysis times for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), and Pro-Ala-Arg-OH (**11**) are within 190.45 ± 15.24 to 208.16 ± 18.01 min, for Pro-Ala-Lys-OH (**12**) is 91.11 ± 14.57 min, for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), and Ala-Arg-Pro-Ala-Arg-OH (**9**) are within 178.56 ± 17.34 to 210.01 ± 18.42 min, for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**) are within 90.54 ± 15.32 to 95.01 ± 15.34 min, for NS is 202.32 ± 14.35 min, and for UK is 123.88 ± 23.86 min, respectively. The tested lysis time for Pro-Ala-Lys-OH (**12**) is significantly shorter than that for Pro-Ala-*n*Leu-OH, Ala-Pro-Lys-OH, Pro-Ala-Arg-OH, NS ($p < 0.001$), and UK ($p < 0.05$). The tested lysis times for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**) are significantly shorter than that for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), Ala-Arg-Pro-Ala-Arg-OH (**9**), NS ($p < 0.001$), and UK ($p < 0.05$).



Scheme 5. Preparation of Pro-Ala-*n*Leu-OH (**10**), Pro-Ala-Arg-OH (**11**), and Pro-Ala-Lys-OH (**12**). (1) General procedure for removal of benzyl group of the N-terminal component: At 0 °C the mixture of 0.80 mmol of the benzyl ester in 10 mL of methanol and 5 mL of the solution of NaOH in methanol (2 mol/L) was stirred for 2 h then neutralized to pH 7 and evaporated at room temperature to remove methanol. The residue was acidified to pH 1–2 with hydrochloric acid (2 mol/L). (2) General procedure for coupling of C-terminal and N-terminal components: At 0 °C the mixture of 0.20 mmol of the N-terminal component in 5 mL of anhydrous THF, 0.20 mmol of HOBt, and 0.25 mmol of DCC was stirred at 0 °C for 24 h. To the solution 0.20 mmol of C-terminal component and 0.26 mmol of *N*-methylmorpholine were added and stirred at room temperature for 24 h. (3) General procedure for removal of NO_2, Z, and Bzl: A suspension of 0.20 mmol of NO_2, Z, and Bzl protected peptides, 5 mg of Pd/C (5%), and 8 mL of formic acid in methanol (4.4%) was agitated with hydrogen at room temperature for 24 h. Wherein AA = *n*Leu, Arg(NO_2) or Lys(Z), AA′ = *n*Leu (**10**), Arg (**11**) or Lys (**12**).

2.3. In vitro fibrinolytic lysis of the peptides

The fibrinogen–agarose mixture was coagulated in the plastic dishes by thrombin according to the method described in experimental section. The fibrinolytic assay was carried out using the standard procedure. The fibrinolytic activity of P6A and related peptides were recorded and represented by the lysis area of the coagulated fibrinogen–agarose mixture in the plastic dishes. The tested lysis areas for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), and Pro-Ala-Arg-OH (**11**) are significantly different from that for Pro-Ala-Lys-OH (**12**). The tested lysis areas for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), and Ala-Arg-Pro-Ala-Arg-OH (**9**) are significantly different from that for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**). The tested lysis areas for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), and Pro-Ala-Arg-OH (**11**) are within 20.55 ± 9.26 to

$27.64 \pm 8.44\,mm^2$, for Pro-Ala-Lys-OH (**12**) is $236.11 \pm 10.01\,mm^2$, for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), and Ala-Arg-Pro-Ala-Arg-OH (**9**) are within 20.38 ± 9.44 to $29.81 \pm 10.52\,mm^2$, for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**) are within 213.47 ± 10.16 to $231.04 \pm 9.86\,mm^2$, for NS is $21.01 \pm 9.26\,mm^2$, and for UK is $222.99 \pm 10.99\,mm^2$, respectively. The tested lysis area for Pro-Ala-Lys-OH (**12**) is significantly larger than that for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), Pro-Ala-Arg-OH (**11**), and NS ($p < 0.001$), and substantially similar to that for UK ($p > 0.05$). The tested lysis are as for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**) are significantly larger than that for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), Ala-Arg-Pro-Ala-Arg-OH (**9**), and NS ($p < 0.001$), and substantially similar to that for UK ($p > 0.05$).

2.4. Thrombolytic activity in vivo

The thrombus prepared with artery blood of rat was fixed on a special helix according to the literature.[10] After the blood circulation outside the rat body was established the helix with thrombus was inserted into the polyethylene tube to stimulate the in vivo thrombus. The in vivo thrombolytic assay was carried out according to the mentioned method and the thrombolytic activities of peptides were tested using the standard procedure. The thrombolytic potencies of P6A (**1**) and related peptides were recorded and represented by the reduction of thrombus weight. The tested reduction of thrombus weight of the rat for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), and Pro-Ala-Arg-OH (**11**) are significantly different from that for Pro-Ala-Lys-OH. The tested reduction of thrombus weight for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), and Ala-Arg-Pro-Ala-Arg-OH (**9**) are significantly different from that for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**). The tested reductions of thrombus weight of the rat for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), and Pro-Ala-Arg-OH (**11**) are within 12.99 ± 2.87 to 16.03 ± 3.46 mg, for Pro-Ala-Lys-OH (**12**) is 26.98 ± 2.54 mg, for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), and Ala-Arg-Pro-Ala-Arg-OH (**9**) are within 13.81 ± 2.50 to 17.11 ± 3.40 mg, for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**) are within 18.84 ± 3.18 to 25.90 ± 2.05 mg, for NS is 15.31 ± 3.57 mg, and for UK is 24.10 ± 3.54 mg, respectively. The tested reduction of thrombus weight of the rat for Pro-Ala-Lys-OH (**12**) is significantly larger than that for Ala-Pro-Lys-OH (**7**), Pro-Ala-*n*Leu-OH (**10**), Pro-Ala-Arg-OH (**11**), and NS ($p < 0.001$), and substantially similar to that for UK ($p > 0.05$). The tested reductions of thrombus weight of the rat for Ala-Arg-Pro-Ala-Lys-OH (**1**), Gln-Arg-Pro-Ala-Lys-OH (**2**), and Gly-Arg-Pro-Ala-Lys-OH (**3**) are significantly larger than that for Lys-Arg-Pro-Ala-Lys-OH (**4**), Arg-Arg-Pro-Ala-Lys-OH (**5**), Ala-Arg-Ala-Pro-Lys-OH (**6**), Ala-Arg-Pro-Ala-*n*Leu-OH (**8**), Ala-Arg-Pro-Ala-Arg-OH (**9**), and NS ($p < 0.001$), and substantially similar to that for UK ($p > 0.05$).

3. Discussion

3.1. Amino acid composition specificity of Pro-Ala-Lys-OH for thrombolytic activities of Ala-Arg-Pro-Ala-Lys-OH and its analogs

The in vitro and in vivo thrombolytic experiments demonstrated that when ^{5}Lys of Ala-Arg-Pro-Ala-Lys-OH (**1**) is changed into ^{5}Arg, and when ^{3}Lys of Pro-Ala-Lys-OH (**12**) is changed into ^{3}Arg and ^{3}*n*Leu, the thrombolytic activities are collapsed. This phenomenon suggests that the amino acid composition of Pro-Ala-Lys-OH is highly specific and this fragment is highly conservative. Additionally in our previous paper[10] we demonstrated that comparing to Ala-Arg-Pro-Ala-Lys-OH (**1**) its in vivo metabolites Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH exhibited no any activity, Arg-Pro-Ala-Lys-OH exhibited comparable thrombolytic activities, Pro-Ala-Lys-OH (**12**) exhibited enhanced thrombolytic activities. Pro-Ala-Lys-OH (**12**) is likely the active fragment of Ala-Arg-Pro-Ala-Lys-OH (**1**). The differences of the thrombolysis activities of Ala-Arg-Pro-Ala-Lys-OH (**1**) and Ala-Arg-Pro-Ala-Arg-OH (**9**), of Pro-Ala-Lys-OH (**12**) and Pro-Ala-Arg-OH (**11**) give also the evidence that Pro-Ala-Lys-OH (**12**) is likely the active fragment of Ala-Arg-Pro-Ala-Lys-OH (**1**) and its analogs.

3.2. Sequence specificity of Pro-Ala-Lys-OH for thrombolytic activities of Ala-Arg-Pro-Ala-Lys-OH and its analogs

The in vitro and in vivo thrombolytic experiments demonstrated that when the sequence of Pro-Ala-Lys-OH (**12**) is changed, for instance when Pro-Ala-Lys-OH (**12**) is changed into Ala-Pro-Lys-OH (**7**), and when Ala-Arg-Pro-Ala-Lys-OH (**1**) is changed into Ala-Arg-Ala-Pro-Lys-OH (**6**) the thrombolytic activities are collapsed. All the results mean that Pro-Ala-Lys (**12**) sequence exhibits high specificity for the thrombolytic activities of P6A (**1**) and its analogs.

3.3. The importance of the positively charged side chain of Pro-Ala-Lys-OH for the thrombolytic activities of Ala-Arg-Pro-Ala-Lys-OH and its analogs

In the interactions of peptides or proteins with receptors the positively charged side chain of Lys residue is generally considered as the most important group. In the present paper it is found that removal of the positively charged side chain of Pro-Ala-Lys-OH (**12**) will have negative influence upon the thrombolytic activities, for instance the substitution of ^{5}Lys of Ala-Arg-Pro-Ala-

Lys-OH (**1**) with ^{5}nLeu results in the collapse of the thrombolytic activities. In the interactions of peptides or proteins with receptors the positively charged side chain of Lys residue is generally considered as an important factor. In the present paper it is found that the introduction of additional positively charged side chain into the N-terminal of the peptides will also have negative influence upon the thrombolytic activities, for instance the substitution of ^{1}Ala of Ala-Arg-Pro-Ala-Lys-OH (**1**) with ^{1}Arg or ^{1}Lys results in also the collapse of the thrombolytic activities. The observed phenomena imply that the positively charged side chain of Pro-Ala-Lys-OH (**12**), P6A and its analogs has critical influence upon the thrombolytic activities.

3.4. Is Pro-Ala-Lys-OH the pharmacophore of Ala-Arg-Pro-Ala-Lys-OH and its analogs?

It is very clear that to retain the thrombolytic activities of Ala-Arg-Pro-Ala-Lys-OH (**1**) and its analogs the Lys residue in the C-terminal of Ala-Arg-Pro-Ala-Lys-OH (**1**) and Pro-Ala-Lys-OH (**12**) is not changeable, the Pro-Ala-Lys-OH (**12**) sequence in Ala-Arg-Pro-Ala-Lys-OH (**1**) is not alternatable, and the positively charged side chain of Lys residue in Ala-Arg-Pro-Ala-Lys-OH (**1**) is not removable. Since all of these requirements relate to retain the integrity of Pro-Ala-Lys-OH (**12**) perhaps it may be considered as the pharmacophore of Ala-Arg-Pro-Ala-Lys-OH (**1**), its analogs and the related peptides.

4. Experimental. Synthesis of the peptides

4.1. General

The protected amino acids were of L-configuration. The purity of the intermediates and the products was confirmed by TLC (Merck silica gel plates of type 60 F_{254}, 0.25 mm layer thickness) and HPLC (waters, C_{18} column 3.9×150 mm). Melting points were measured on a XT5 hot stage microscope (Beijing keyi electro-optic factory), and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 983 instrument. FAB-MS was determined by a VG-ZAB-MS and a HPES-5989x instrument; ^{1}H NMR spectra was determined by a Varian INOVA-500 MHz spectrometer. Optical rotations were determined at 20 °C on a Schmidt + Haensch Polartronic D instrument. The amino acid analysis was performed by a Hitachi 835-50 instrument.

4.2. 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 DCC were added. The reaction mixture was stirred at 0 °C for 24 h. Precipitated DCU was removed by filtration. The filtrate was evaporated under reduced pressure and the residue was titrated with petroleum ether to provide the corresponding active ester. To the solution of the active 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. On evaporation the residue was dissolved in 50 mL of ethyl acetate. The solution was 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, and purification by chromatography ($CHCl_3$/CH_3OH, 30:1) to provide the protective intermediates.

4.3. Boc-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

Using the general procedure for removal of Boc of the C-terminal component Boc-Pro-Ala-Lys(Z)-OBzl was converted into HCl·Pro-Ala-Lys(Z)-OBzl, which was coupled with Boc-Arg(NO_2)-OH according to the general procedure for coupling of C-terminal and N-terminal components to provide the title compound as a colorless powder in 77% yield (129 mg), mp 70–72 °C; FAB-MS (*m*/*e*): 840 $[M+H]^+$; $[\alpha]_D^{20}$ −7.5 (*c* 0.2, $CHCl_3$); IR (KBr): 3360, 3350, 3344, 3033, 3011, 1765, 1692, 1600,1590, 1504, 1462, 1395, 1385, 1365, 760, 705 cm^{-1}; ^{1}H NMR (DMSO-d_6): $\delta = 9.450$ (1H), 8.250 (1H), 8.126 (1H), 8.120 (1H), 8.009 (1H), 7.985 (1H), 7.905 (1H), 7.365 (2H), 7.360 (2H), 7.328 (1H), 7.324 (1H), 7.205 (2H), 7.200 (2H), 5.151 (2H), 5.150 (2H), 4.315 (1H), 4.190 (1H), 4.013 (1H), 4.101 (1H), 3.390 (2H), 3.280 (2H), 3.040 (2H), 2.750 (2H), 2.050 (2H), 2.012 (2H), 1.766 (2H), 1.682 (2H), 1.535 (2H), 1.528 (2H), 1.440 (9H), 1.264 (3H). Amino acid analysis: calcd, Arg/Pro/Ala/Lys = 1.0:1.0:1.0:1.0; found, Arg/Pro/Ala/Lys = 0.98:1.00:1.02:0.99. Anal. Calcd for $C_{40}H_{57}N_9O_{11}$: C, 57.20; H, 6.84; N, 15.01. Found: C, 57.41; H, 6.67; N, 15.08.

4.4. HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

The solution of 168 mg (0.20 mmol) of Boc-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl, which were prepared according to the literature,[10] in 2 mL of hydrochloride in ethyl acetate (6 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 dry. The resulted solid was used for coupling reaction directly.

4.5. Boc-Ala-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from Boc-Ala-OH and HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl the title compound was obtained as a colorless powder in 91% yield, mp 93–95 °C; FAB-MS (*m*/*e*): 911 $[M+H]^+$; $[\alpha]_D^{20}$ −9.5 (*c* 0.2, $CHCl_3$); IR (KBr): 3358, 3349, 3340, 3035, 3010, 1760, 1690, 1605, 1588, 1500, 1460, 1397, 1383, 1360, 764, 701 cm^{-1}; ^{1}H NMR (DMSO-d_6): $\delta = 9.454$ (1H), 8.200 (1H), 8.123 (1H), 8.120 (1H), 8.012 (1H), 7.989 (1H), 7.980 (1H), 7.366 (2H), 7.360 (2H), 7.329 (1H),

7.320 (1H), 7.205 (2H), 7.200 (2H), 5.153 (2H), 5.149 (2H), 4.324 (1H), 4.315 (1H), 4.311 (1H), 4.304 (1H), 3.988 (2H), 3.984 (1H), 3.552 (2H), 3.387 (2H), 3.031 (2H), 2.739 (2H), 2.045 (2H), 1.769 (2H), 1.701 (2H), 1.686 (2H), 1.530 (2H), 1.524 (2H), 1.442 (9H), 1.267 (3H), 1.254 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/Lys = 2.00:1.00:1.00:1.00; found, Ala/Arg/Pro/Lys = 2.03:0.99:1.00:0.97. Anal. Calcd for $C_{43}H_{62}N_{10}O_{12}$: C, 56.69; H, 6.86; N, 15.37. Found: C, 56.41; H, 6.99; N, 15.19.

4.6. Boc-Gln-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from Boc-Gln-OH and HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl the title compound was obtained as a colorless powder in 86% yield, mp 78–80 °C; FAB-MS (*m*/*e*): 968 $[M+H]^+$; $[\alpha]_D^{20}$ −8.9 (*c* 0.2, $CHCl_3$); IR (KBr): 3358, 3352, 3341, 3035, 3014, 1761, 1689, 1662, 1602, 1588, 1500, 1464, 1392, 1387, 1360, 761, 700 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.452$ (1H), 8.207 (1H), 8.200 (1H), 8.126 (1H), 8.123 (1H), 8.012 (1H), 7.980 (1H), 7.912 (1H), 7.362 (2H), 7.358 (2H), 7.324 (1H), 7.321 (1H), 7.202 (2H), 7.199 (2H), 6.455 (2H), 5.153 (2H), 5.151 (2H), 4.312 (1H), 4.305 (2H), 4.191 (1H), 4.188 (1H), 3.986 (1H), 3.982 (1H), 3.393 (2H), 3.035 (2H), 2.753 (2H), 2.240 (2H), 2.053 (2H), 1.763 (2H), 1.687 (2H), 1.684 (2H), 1.665 (2H), 1.530 (2H), 1.526 (2H), 1.443 (9H), 1.265 (3H). Amino acid analysis: calcd, Gln/Arg/Pro/Ala/Lys = 1.00:1.00:1.00:1.00:1.00; found, Glu/Arg/Pro/Ala/Lys = 1.03:0.98:1.00:1.03:0.99. Anal. Calcd for $C_{45}H_{65}N_{11}O_{13}$: C, 55.83; H, 6.77; N, 15.92. Found: C, 55.66; H, 6.99; N, 15.87.

4.7. Boc-Gly-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from Boc-Gly-OH and HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl the title compound was obtained as a colorless powder in 93% yield, mp 80–82 °C; FAB-MS (*m*/*e*) 897 $[M+H]^+$; $[\alpha]_D^{20}$ −9.3 (*c* 0.2, $CHCl_3$); IR (KBr): 3365, 3352, 3340, 3036, 3010, 1767, 1690, 1604, 1591, 1500, 1460, 1396, 1383, 1360, 761, 707 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.452$ (1H), 8.206 (1H), 8.124 (1H), 8.125 (1H), 8.102 (1H), 8.011 (1H), 7.980 (1H), 7.700 (1H), 7.368 (2H), 7.363 (2H), 7.324 (1H), 7.320 (1H), 7.205 (2H), 7.202 (2H), 5.152 (2H), 5.150 (2H), 4.315 (1H), 4.311 (1H), 4.305 (1H), 4.300 (2H), 4.192 (1H), 3.992 (2H), 3.388 (2H), 3.082 (2H), 2.742 (2H), 2.038 (2H), 1.864 (2H), 1.760 (2H), 1.677 (2H), 1.675 (2H), 1.530 (2H), 1.444 (9H), 1.260 (3H). Amino acid analysis: calcd, Gly/Arg/Pro/Ala/Lys = 1.00:1.00:1.00:1.00:1.00; found, Gly/Arg/Pro/Ala/Lys = 1.03:0.97:1.02:1.03:0.98. Anal. Calcd for $C_{42}H_{60}N_{10}O_{12}$: C, 56.24; H, 6.74; N, 15.62. Found: C, 56.11; H, 6.89; N, 15.49.

4.8. Boc-Arg(NO_2)-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from Boc-Arg(NO_2)-OH and HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl the title compound was obtained as a colorless powder in 85% yield, mp 90–92 °C; FAB-MS (*m*/*e*): 1041 $[M+H]^+$; $[\alpha]_D^{20}$ −17.5 (*c* 0.2, $CHCl_3$); IR (KBr): 3362, 3347, 3340, 3038, 3018, 1760, 1690, 1607, 1595, 1500, 1460, 1392, 1382, 1360, 764, 700 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.454$ (1H), 9.450 (1H), 8.207 (1H), 8.200 (1H), 8.134 (1H), 8.129 (1H), 8.124 (1H), 8.013 (1H), 7.980 (1H), 7.985 (1H), 7.712 (1H), 7.360 (2H), 7.357 (2H), 7.323 (1H), 7.320 (1H), 7.209 (2H), 7.201 (2H), 5.152 (2H), 5.151 (2H), 4.311 (1H), 4.306 (1H), 4.192 (1H), 3.986 (1H), 3.981 (1H), 3.977 (2H), 3.968 (2H), 3.392 (1H), 3.389 (2H), 2.755 (2H), 2.046 (2H), 2.033 (2H), 1.768 (2H), 1.684 (2H), 1.682 (2H), 1.677 (2H), 1.532 (2H), 1.525 (2H), 1.438 (9H), 1.260 (3H). Amino acid analysis: calcd, Arg/Pro/Ala/Lys = 2.00:1.00:1.00:1.00; found, Arg/Pro/Ala/Lys = 1.96:1.01:1.03:0.98. Anal. Calcd for $C_{46}H_{68}N_{14}O_{14}$: C, 53.07; H, 6.58; N, 18.83. Found: C, 52.98; H, 6.79; N, 18.67.

4.9. Boc-Lys(Z)-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from Boc-Lys(Z)-OH and HCl·Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl the title compound was obtained as a colorless powder in 81% yield, mp 85–88 °C; FAB-MS (*m*/*e*): 1102 $[M+H]^+$, 1124 $[M+Na]^+$; $[\alpha]_D^{20}$ −12.5 (*c* 0.2, $CHCl_3$); IR (KBr): 3362, 3355, 3341, 3038, 3015, 1760, 1695, 1601, 1593, 1500, 1468, 1397, 1380, 1360, 765, 710 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.456$ (1H), 8.211 (1H), 8.199 (1H), 8.128 (1H), 8.122 (1H), 8.018 (1H), 8.016 (1H), 7.988 (1H), 7.711 (1H), 7.369 (2H), 7.364 (2H), 7.361 (2H), 7.348 (1H), 7.331 (1H), 7.327 (1H), 7.216 (2H), 7.210 (2H), 7.204 (2H), 5.153 (2H), 5.152 (2H), 5.150 (2H), 4.318 (1H), 4.309 (1H), 4.192 (1H), 4.173 (1H), 4.073 (1H), 3.986 (2H), 3.978 (2H), 3.975 (2H), 3.350 (2H), 2.756 (2H), 2.055 (2H), 1.768 (2H), 1.686 (2H), 1.683 (2H), 1.664 (2H), 1.538 (2H), 1.536 (2H), 1.524 (2H), 1.520 (2H), 1.446 (9H), 1.268 (3H). Amino acid analysis: calcd, Lys/Arg/Pro/Ala = 2.00:1.00:1.00:1.00; found, Lys/Arg/Pro/Ala = 1.97:0.96:1.02:1.02. Anal. Calcd for $C_{54}H_{75}N_{11}O_{14}$: C, 58.84; H, 6.86; N, 13.98. Found: C, 58.71; H, 6.70; N, 13.69.

4.10. Boc-Ala-Arg(NO_2)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 378 mg (2 mmol) of Boc-Ala-OH and 618 mg (2 mmol) of Arg(NO_2)OBzl 866 mg (90%) of the title compound were obtained as a colorless powder, mp 85–87 °C; FAB-MS (*m*/*e*): 481 $[M+H]^+$; $[\alpha]_D^{20}$ −12.0 (*c* 0.2, $CHCl_3$); IR (KBr): 3350, 3344, 3021, 3018, 1748, 1672, 1601, 1570, 1502, 1470, 1393, 1382, 1364, 760, 701 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.350$ (1H), 8.216 (1H), 8.122 (1H), 8.110 (1H), 8.105 (1H), 7.394 (2H), 7.310 (1H), 7.212 (2H), 5.153 (2H), 4.516 (1H), 4.405 (1H), 3.378 (2H), 2.104 (2H), 1.678 (2H), 1.421 (9H), 1.263 (3H). Amino acid analysis: calcd, Ala/Arg = 1.0:1.0; found, Ala/Arg = 1.02:0.97. Anal. Calcd for $C_{21}H_{32}N_6O_7$: C, 52.49; H, 6.71; N, 17.49. Found: C, 52.11; H, 6.93; N, 17.28.

4.11. Boc-Ala-Arg(NO_2)-OH

At 0 °C to the solution of 481 mg (1.0 mmol) of Boc-Ala-Arg(NO_2)-OBzl, which were prepared according to the literature,[10] in 10 mL of methanol 5 mL of the solution of NaOH in methanol (2 mol/L) were added. The reaction mixture was stirred at 0 °C for 2 h. The reaction mixture was neutralized to pH 7 and evaporated at room temperature to remove methanol. The residue was acidified to pH 1–2 with hydrochloric acid (2 mol/L) to provide the 375 mg (96%) of title compound as colorless powder, mp 145–147 °C; FAB-MS (*m*/*e*): 391 $[M+H]^+$; $[\alpha]_D^{20}$ −10.5 (*c* 0.2, $CHCl_3$); IR (KBr): 3430, 3360, 3332, 3265, 3070, 2969, 1668, 1553, 1395, 1388, 1362 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 10.991$ (1H), 9.372 (1H), 8.213 (1H), 8.122 (1H), 8.114 (1H), 8.135 (1H), 4.119 (1H), 4.111 (1H), 3.378 (2H), 2.151 (2H), 1.678 (2H), 1.426 (9H), 1.265 (3H). Amino acid analysis: calcd, Ala/Arg = 1.0:1.0; found, Ala/Arg = 1.00:0.98. Anal. Calcd for $C_{14}H_{26}N_6O_7$: C, 43.07; H, 6.71; N, 21.53. Found: C, 42.95; H, 6.90; N, 21.37.

4.12. Boc-Pro-Ala-Arg(NO_2)-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 430 mg (2.00 mmol) of Boc-Pro-OH and 654 mg (2.00 mmol) of HCl·Ala-Arg(NO_2)OBzl 833 mg (92%) of the title compound were obtained as a colorless powder, mp 105–107 °C; FAB-MS (*m*/*e*): 578 $[M+H]^+$; $[\alpha]_D^{20}$ −11.2 (*c* 0.2, $CHCl_3$); IR (KBr): 3359, 3340, 3025, 3010, 1750, 1666, 1604, 1567, 1507, 1472, 1395, 1380, 1365, 762, 706 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.454$ (1H), 8.216 (1H), 8.125 (1H), 8.012 (1H), 8.008 (1H), 7.390 (2H), 7.314 (1H), 7.210 (2H), 5.151 (2H), 4.306 (1H), 4.012 (1H), 4.115 (1H), 3.405 (2H), 3.064 (2H), 2.782 (2H), 2.061 (2H), 1.783 (2H), 1.689 (2H), 1.439 (9H), 1.268 (3H). Amino acid analysis: calcd, Pro/Ala/Arg = 1.0:1.0:1.0; found, Pro/Ala/Arg = 0.96:1.00:0.98. Anal. Calcd for $C_{26}H_{39}N_7O_8$: C, 54.06; H, 6.81; N, 16.97. Found: C, 53.92; H, 6.91; N, 16.88.

4.13. Boc-Pro-Ala-Lys(Z)-OBzl

Using the general procedure in the literature[10] from Lys(Z)-OBzl, Boc-Ala-OH, and Boc-Pro-OH the title compound was obtained as a colorless powder in 75% total yield (96 mg), mp 85–87 °C; FAB-MS (*m*/*e*) 639 $[M+H]^+$; $[\alpha]_D^{20}$ −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 (DMSO-d_6): $\delta = 8.118$ (1H), 8.105 (1H), 7.990 (1H), 7.370 (2H), 7.364 (2H), 7.341 (1H), 7.301 (1H), 7.210 (2H), 7.208 (2H), 5.153 (2H), 5.152 (2H), 4.309 (1H), 4.209 (1H), 4.011 (1H), 3.969 (2H), 3.048 (2H), 2.746 (2H), 1.768 (2H), 1.672 (2H), 1.532 (2H), 1.526 (2H), 1.442 (9H), 1.269 (3H). Amino acid analysis: calcd, Pro/Ala/Lys = 1.0:1.0:1.0; found, Pro/Ala/Lys = 1.00:1.03:0.96. Anal. Calcd for $C_{34}H_{46}N_4O_8$: C, 63.93; H, 7.26; N, 8.77. Found: C, 63.82; H, 7.37; N, 8.69.

4.14. Boc-Ala-Pro-Lys(Z)-OBzl

Using the general procedure for removal of Boc 567 mg (1.00 mmol) of Boc-Pro-Lys(Z)-OBzl was converted into HCl·Pro-Lys(Z)-OBzl, which was coupled with 189 mg (1.00 mmol) Boc-Ala-OH according to the general procedure for coupling of C-terminal and N-terminal components to provide 581 mg (91%) of the title compound as a colorless powder, mp 90–92 °C; FAB-MS (*m*/*e*) 639 $[M+H]^+$; $[\alpha]_D^{20}$ −7.9 (*c* 0.2, $CHCl_3$); IR (KBr): 3361, 3345, 3021, 3010, 1763, 1694, 1600, 1577, 1504, 1460, 1388, 1379, 1360, 761, 704 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 8.311$ (1H), 8.185 (1H), 7.976 (1H), 7.364 (2H), 7.358 (2H), 7.337 (1H), 7.322 (1H), 7.243 (2H), 7.237 (2H), 5.154 (2H), 5.152 (2H), 4.315 (1H), 4.228 (1H), 4.054 (1H), 3.325 (2H), 3.111 (2H), 2.727 (2H), 2.032 (2H), 1.753 (2H), 1.544 (2H), 1.531 (2H), 1.438 (9H), 1.263 (3H). Amino acid analysis: calcd, Ala/Pro/Lys = 1.0:1.0:1.0; found, Ala/Pro/Lys = 1.00:1.03:0.96. Anal. Calcd for $C_{34}H_{46}N_4O_8$: C, 63.93; H, 7.26; N, 8.77. Found: C, 63.77; H, 7.24; N, 8.67. Anal. Calcd for $C_{34}H_{46}N_4O_8$: C, 63.93; H, 7.26; N, 8.77. Found: C, 63.80; H, 7.35; N, 8.66.

4.15. Boc-Ala-*n*Leu-OBzl

Using the general procedure for coupling C-terminal and N-terminal components from 378 mg (2.00 mmol) of Boc-Ala-OH and 515 mg (2.00 mmol) of HCl·*n*Leu-OBzl to provide 721 mg (92%) of the title compound as a colorless powder, mp 79–81 °C; FAB-MS (*m*/*e*) 393 $[M+H]^+$; $[\alpha]_D^{20}$ −8.8 (*c* 0.2, $CHCl_3$); IR (KBr): 3347, 3342, 3032, 3021, 1752, 1675, 1605, 1574, 1500, 1474, 1380, 765, 704 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 8.120$ (1H), 8.114 (1H), 7.358 (2H), 7.286 (1H), 7.203 (2H), 5.152 (2H), 4.304 (1H), 4.217 (1H), 1.686 (2H), 1.549 (2H), 1.547 (2H), 1.429 (9H), 1.274 (3H), 1.201 (3H). Amino acid analysis: calcd, Ala/*n*Leu = 1.0:1.0; found, Ala/*n*Leu = 1.01:1.00. Anal. Calcd for $C_{21}H_{32}N_2O_5$: C, 64.26; H, 8.22; N, 7.14. Found: C, 64.18; H, 8.31; N, 8.09.

4.16. Boc-Pro-Ala-*n*Leu-OBzl

Using the general procedure for removal of Boc of the C-terminal component Boc-Ala-*n*Leu-OBzl was converted into HCl·Ala-*n*Leu-OBzl, which was coupled with Boc-Pro-OH according to the general procedure for coupling of C-terminal and N-terminal components to provide the title compound as a colorless powder in 92% yield, mp 83–85 °C; FAB-MS (*m*/*e*): 490 $[M+H]^+$; $[\alpha]_D^{20}$ −11.4 (*c* 0.2, $CHCl_3$); IR (KBr): 3355, 3341, 3338, 3030, 3014, 1756, 1670, 1602, 1570, 1504, 1470, 1380, 763, 702 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 7.980$ (1H), 7.975 (1H), 7.392 (2H), 7.320 (1H), 7.214 (2H), 5.150 (2H), 4.331 (1H), 4.215 (1H), 4.021 (1H), 3.059 (2H), 2.818 (2H), 1.890 (2H), 1.785 (2H), 1.554 (2H), 1.550 (2H), 1.418 (9H), 1.269 (3H), 1.221 (3H). Amino acid analysis: calcd, Pro/Ala/*n*Leu = 1.0:1.0:1.0; found, Pro/Ala/*n*Leu = 0.99:1.01:1.00. Anal. Calcd for $C_{26}H_{39}N_3O_6$: C, 63.78; H, 8.03; N, 8.58. Found: C, 63.69; H, 8.15; N, 8.50.

4.17. Boc-Ala-Arg(NO_2)-Ala-Pro-Lys(Z)-OBzl

Using the general procedure for coupling 287 mg (0.5 mmol) of C-terminal component HCl·Ala-Pro-Lys(Z)-OBzl and 195 mg (0.5 mmol) of N-terminal component Boc-Ala-Arg(NO_2)-OH 427 mg (93.8%) of the title compound was obtained as a colorless powder, mp 135–137 °C; FAB-MS (*m/e*): 911 $[M+H]^+$; $[\alpha]_D^{20}$ −9.7 (*c* 0.2, $CHCl_3$); IR (KBr): 3365, 3354, 3340, 3037, 3015, 1760, 1690, 1608, 1595, 1500, 1460, 1390, 1380, 1368, 768, 703 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.447$ (1H), 8.201 (1H), 8.169 (1H), 8.129 (1H), 8.127 (1H), 8.013 (1H), 7.980 (1H), 7.908 (1H), 7.361 (2H), 7.354 (2H), 7.332 (1H), 7.327 (1H), 7.201 (2H), 7.189 (2H), 5.152 (2H), 5.150 (2H), 4.377 (1H), 4.317 (1H), 4.310 (1H), 4.188 (1H), 4.032 (1H), 3.970 (2H), 3.758 (2H), 3.035 (2H), 2.747 (2H), 2.058 (2H), 2.012 (2H), 1.762 (2H), 1.685 (2H), 1.531 (2H), 1.526 (2H), 1.444 (9H), 1.267 (3H), 1.261 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/Lys = 2.00:1.00:1.00:1.00; found, Ala/Arg/Pro/Lys = 2.01:0.99:1.01:0.98. Anal. Calcd for $C_{43}H_{62}N_{10}O_{12}$: C, 56.69; H, 6.86; N, 15.37. Found: C, 56.58; H, 6.99; N, 15.25.

4.18. Boc-Ala-Arg(NO_2)-Pro-Ala-OBzl

Using the general procedure for removal of benzyl group of the N-terminal component Boc-Ala-Arg(NO_2)-Pro-OBzl was converted into Boc-Ala-Arg(NO_2)-Pro-OH, which was coupled with L-Ala-OBzl according to the general procedure for coupling of C-terminal and N-terminal components to provide the title compound as a colorless powder in 80% yield (104 mg), mp 86–88 °C; FAB-MS (*m/e*): 649 $[M+H]^+$; $[\alpha]_D^{20}$ −9.6 (*c* 0.2, $CHCl_3$); IR (KBr): 3364, 3336, 3031, 3009, 1760, 1695, 1600, 1580, 1500, 1466, 1390, 1382, 1360, 766, 700 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.454$ (1H), 8.212 (1H), 8.120 (1H), 8.110 (1H), 8.102 (1H), 7.994 (1H), 7.388 (2H), 7.308 (1H), 7.201 (2H), 5.151 (2H), 4.301 (1H), 4.295 (1H), 4.159 (1H), 4.145 (1H), 3.396 (2H), 3.059 (2H), 2.750 (2H), 2.044 (2H), 1.789 (2H), 1.679 (2H), 1.439 (9H), 1.278 (3H), 1.265 (3H). Amino acid analysis: calcd, Ala/Arg/Pro = 2.0:1.0:1.0; found, Ala/Arg/Pro = 1.98:0.97:1.00. Anal. Calcd for $C_{29}H_{44}N_8O_9$: C, 53.69; H, 6.84; N, 17.27. Found: C, 53.69496; H, 6.67; N, 17.55. Anal. Calcd for $C_{29}H_{44}N_8O_9$:C, 53.69; H, 6.84; N, 17.27. Found: C, 53.60; H, 6.93; N, 17.19.

4.19. Boc-Ala-Arg(NO_2)-Pro-Ala-OH

At 0 °C to the solution of 518 mg (0.80 mmol) of Boc-Ala-Arg(NO_2)-Pro-Ala-OBzl, which were prepared according to the literature,[10] in 10 mL of methanol 5 mL of the solution of NaOH in methanol (2 mol/L) were added. The reaction mixture was stirred at 0 °C for 2 h. The reaction mixture was neutralized to pH 7 and evaporated at room temperature to remove methanol. The residue was acidified to pH 1–2 with hydrochloric acid (2 mol/L) to provide the 424 mg (95%) of title compound as colorless powder, mp 146–148 °C; FAB-MS (*m/e*): 559 $[M+H]^+$; $[\alpha]_D^{20}$ −30.1 (*c* 0.2, $CHCl_3$); IR (KBr): 3424, 3364, 3336, 3260, 3070, 2960, 1660, 1550, 1390, 1382, 1360 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 10.254$ (1H), 9.454 (1H), 8.212 (1H), 8.120 (1H), 8.110 (1H), 8.102 (1H), 7.994 (1H), 4.301 (1H), 4.295 (1H), 4.198 (1H), 4.101 (1H), 3.541 (2H), 3.059 (2H), 2.396 (2H), 2.044 (2H), 1.789 (2H), 1.685 (2H), 1.439 (9H), 1.278 (3H), 1.265 (3H). Amino acid analysis: calcd, Ala/Arg/Pro = 2.0:1.0:1.0; found, Ala/Arg/Pro = 1.97:0.98:1.01. Anal. Calcd for $C_{22}H_{38}N_8O_9$: C, 47.30; H, 6.86; N, 20.06. Found: C, 47.18; H, 7.00; N, 19.95.

4.20. Boc-Ala-Arg(NO_2)-Pro-Ala-*n*Leu-OBzl

Using the general procedure from 112 mg of Boc-Ala-Arg(NO_2)-Pro-Ala-OH and 44 mg of *n*Leu-OBzl 137 mg (90%) of the title compound was obtained as a colorless powder, mp 105–107 °C, $[\alpha]_D^{20}$ −32.1 (*c* 0.2, $CHCl_3$); FAB-MS (*m/e*): 762 $[M+H]^+$; IR (KBr): 3370, 3346, 3025, 3011, 1758, 1692, 1605, 1584, 1509, 1460, 1392, 1380, 1365, 769, 708 cm^{-1}; ^1H NMR (DMSO-*d*): $\delta = 9.450$ (1H), 8.214 (1H), 8.125 (1H), 8.113 (1H), 8.107 (1H), 8.015 (1H), 8.105 (1H), 7.384 (2H), 7.311 (1H), 7.211 (2H), 5.152 (2H), 4.305 (1H), 4.292 (1H), 4.256 (1H), 4.189 (1H), 3.393 (2H), 3.055 (2H), 2.747 (2H), 2.041 (2H), 2.010 (2H), 1.783 (2H), 1.686 (2H), 1.532 (2H), 1.530 (2H), 1.436 (9H), 1.273 (3H), 1.267 (3H), 1.263 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/*n*Leu = 2.00:1.00:1.00:1.00; found, Ala/Arg/Pro/*n*Leu = 2.02:0.98:0.99:1.01. Anal. Calcd for $C_{35}H_{55}N_9O_{10}$: C, 55.18; H, 7.28; N, 16.55. Found: C, 55.06; H, 7.41; N, 16.42.

4.21. Boc-Ala-Arg(NO_2)-Pro-Ala-Arg(NO_2)-OBzl

Using the general procedure from 112 mg of Boc-Ala-Arg(NO_2)-Pro-Ala-OH and 62 mg of Arg(NO_2)-OBzl 148 mg (87%) of the title compound was obtained as a colorless powder, mp 126–128 °C, $[\alpha]_D^{20}$ −42.1 (*c* 0.2, $CHCl_3$); FAB-MS (*m/e*): 850 $[M+H]^+$; IR (KBr): 3366, 3341, 3032, 3016, 1762, 1688, 1602, 1590, 1515, 1464, 1390, 1382, 1364, 764, 702 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.452$ (1H), 9.450 (1H), 8.215 (1H), 8.215 (1H), 8.214 (1H), 8.124 (1H), 8.114 (1H), 8.105 (1H), 7.995 (1H), 7.967 (1H), 7.389 (2H), 7.310 (1H), 7.206 (2H), 5.152 (2H), 4.307 (1H), 4.298 (1H), 4.195 (1H), 4.167 (1H), 4.298 (1H), 3.398 (2H), 3.394 (2H), 3.060 (2H), 2.050 (2H), 2.048 (2H), 2.046 (2H), 1.686 (2H), 1.786 (2H), 1.684 (2H), 1.441 (9H), 1.280 (3H), 1.270 (3H). Amino acid analysis: calcd, Ala/Arg/Pro = 2.00:2.00:1.00; found, Ala/Arg/Pro = 2.02:1.97:0.99. Anal. Calcd for $C_{35}H_{55}N_{13}O_{12}$: C, 49.46; H, 6.52; N, 21.42. Found: C, 49.37; H, 6.60; N, 21.31.

4.22. General procedure for removal of NO_2, Z, and Bzl

A suspension of 0.20 mmol of NO_2, Z, and Bzl protected peptides, 5 mg of Pd/C (5%) and 8 mL of formic acid in methanol (4.4%) was agitated with hydrogen at room temperature for 24 h. The reaction mixture was filtrated. The filtrate was evaporated and the residue was titrated

with ether and the resulted solid was purified on the Sephadex G-10 column. The collected fractions were lyophilized to provide the corresponding peptide.

4.23. Ala-Arg-Pro-Ala-Lys-OH (1)

Using general procedure for removal of NO_2, Z, and Bzl from 182 mg (0.2 mmol) of Boc-Ala-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl 98 mg (90%) of the title compound was obtained as colorless crystals, mp 224–226 °C; FAB-MS (m/e): 542 $[M+H]^+$, $[\alpha]_D^{20}$ −40.0 ($c = 1.95$, H_2O); IR (KBr): 3433, 3370, 3243, 3085, 2966, 1666, 1549, 1384 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.987$ (1H), 8.432 (2H), 8.280 (1H), 8.220 (1H), 8.007 (1H), 7.598 (1H), 7.152 (1H), 4.266 (1H), 4.205 (1H), 4.219 (1H), 4.015 (1H), 3.673 (1H), 3.535 (2H), 3.353 (2H), 3.076 (2H), 2.730 (2H), 2.248 (2H), 2.242 (2H), 2.023 (2H), 2.006 (2H), 1.863 (2H), 1.676 (2H), 1.532 (2H), 1.531 (2H), 1.280 (3H), 1.267 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/Lys = 2.00:1.00:1.00:1.00; found, Ala/Arg/Pro/Lys = 2.02:0.98:1.00:0.97. Anal. Calcd for $C_{23}H_{43}N_9O_6$: C, 51.00; H, 8.00; N, 23.27. Found: C, 50.92; H, 8.11; N, 23.19.

4.24. Gln-Arg-Pro-Ala-Lys-OH (2)

Using general procedure for removal of NO_2, Z, and Bzl from 193 mg (0.2 mmol) of Boc-Gln-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl 134 mg (95%) of the title compound was obtained as colorless crystals, mp 180–182 °C; FAB-MS (m/e): 599 $[M+H]^+$, $[\alpha]_D^{20}$ −35.0 (c 2.01, H_2O); IR (KBr): 3434, 3368, 3252, 3075, 2962, 1664, 1556, 1382 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.989$ (1H), 8.446 (2H), 8.285 (1H), 8.211 (1H), 8.155 (1H), 7.578 (1H), 7.162 (1H), 6.405 (2H), 4.281 (1H), 4.272 (1H), 4.216 (1H), 4.106 (1H), 4.239 (1H), 3.541 (2H), 3.361 (2H), 3.075 (2H), 2.740 (2H), 2.395 (2H), 2.196 (2H), 2.034 (2H), 1.908 (2H), 1.768 (2H), 1.674 (2H), 1.532 (2H), 1.529 (2H), 2.290 (2H), 2.231 (2H), 1.265 (3H). Amino acid analysis: calcd, Gln/Arg/Pro/Ala/Lys = 1.00:1.00:1.00:1.00:1.00; found, Gln/Arg/Pro/Ala/Lys = 0.99:1.01:1.02:1.02:1.00. Anal. Calcd for $C_{25}H_{46}N_{10}O_7$: C, 50.15; H, 7.74; N, 23.40. Found: C, 50.03; H, 7.82; N, 23.29.

4.25. Gly-Arg-Pro-Ala-Lys-OH (3)

Using general procedure for removal of NO_2, Z, and Bzl from 179 mg (0.2 mmol) of Boc-Gly-Arg(NO_2)-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 $[M+H]^+$, $[\alpha]_D^{20}$ −20.0 (c 1.98, H_2O); IR (KBr): 3442, 3371, 3259, 3074, 2948, 1664, 1556, 1380 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.996$ (1H), 8.563 (2H), 8.106 (1H), 7.987 (1H), 7.971 (1H), 7.629 (1H), 7.076 (1H), 4.624 (1H), 4.528 (1H), 4.254 (2H), 4.226 (1H), 4.108 (1H), 3.215 (2H), 3.388 (2H), 3.068 (2H), 2.739 (2H), 2.531 (2H), 2.530 (2H), 2.049 (2H), 1.988 (2H), 1.868 (2H), 1.677 (2H), 1.531 (2H), 1.530 (2H), 1.245 (3H). Amino acid analysis: calcd, Gly/Arg/Pro/Ala/Lys = 1.00:1.00:1.00:1.00:1.00; found, Gly/Arg/Pro/Ala/Lys = 1.00:0.99:1.02:1.03:0.99. Anal. Calcd for $C_{22}H_{41}N_9O_6$: C, 50.08; H, 7.83; N, 23.89. Found: C, 49.99; H, 7.95; N, 23.77.

4.26. Lys-Arg-Pro-Ala-Lys-OH (4)

Using general procedure for removal of NO_2, Z, and Bzl from 225 mg (0.2 mmol) of Boc-Lys(Z)-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl 96 mg (80%) of the title compound was obtained as colorless crystals, mp 168–170 °C, FAB-MS (m/e): 599 $[M+H]^+$, $[\alpha]_D^{20}$ −15.0 (c 1.87, H_2O). IR (KBr): 3429, 3372, 3261, 3073, 2962, 1665, 1554, 1380 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.885$ (1H), 8.445 (2H), 8.283 (1H), 8.214 (1H), 8.105 (1H), 7.565 (1H), 7.159 (1H), 4.269 (1H), 4.267 (1H), 4.224 (1H), 4.015 (1H), 4.174 (1H), 3.539 (2H), 3.360 (2H), 3.081 (2H), 3.078 (2H), 2.736 (2H), 2.295 (2H), 2.241 (2H), 2.229 (2H), 2.030 (2H), 2.011 (2H), 2.005 (2H), 1.868 (2H), 1.544 (2H), 1.541 (2H), 1.534 (2H), 1.530 (2H), 1.515 (2H), 1.258 (3H). Amino acid analysis: calcd, Lys/Arg/Pro/Ala = 2.00:1.00:1.00:1.00; found, Lys/Arg/Pro/Ala = 2.02:1.00:1.03:1.01. Anal. Calcd for $C_{26}H_{50}N_{10}O_6$: C, 52.16; H, 8.42; N, 23.39. Found: C, 52.03; H, 8.55; N, 23.28.

4.27. Arg-Arg-Pro-Ala-Lys-OH (5)

Using general procedure for removal of NO_2, Z, and Bzl from 208 mg (0.2 mmol) of Boc-Arg(NO_2)-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl 106 mg (85%) of the title compound was obtained as colorless crystals, mp 168–170 °C; FAB-MS (m/e): 627 $[M+H]^+$, $[\alpha]_D^{20}$ −13.3 (c 1.92, H_2O). IR (KBr): 3433, 3365, 3252, 3068, 2952, 1663, 1556, 1381 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 9.899$ (1H), 8.437 (2H), 8.435 (2H), 8.282 (1H), 8.280 (1H), 8.210 (1H), 7.602 (1H), 7.599 (1H), 7.212 (1H), 7.167 (1H), 4.277 (1H), 4.214 (1H), 4.210 (1H), 4.109 (1H), 4.096 (1H), 3.549 (2H), 3.537 (2H), 3.356 (2H), 3.078 (2H), 2.620 (2H), 2.256 (2H), 2.239 (2H), 2.136 (2H), 2.069 (2H), 2.025 (2H), 1.867 (2H), 1.670 (2H), 1.668 (2H), 1.536 (2H), 1.534 (2H), 1.265 (3H). Amino acid analysis: calcd, Arg/Pro/Ala/Lys = 2.00:1.00:1.00:1.00; found, Arg/Pro/Ala/Lys = 1.98:1.00:1.02:1.02. Anal. Calcd for $C_{26}H_{50}N_{12}O_6$: C, 49.82; H, 8.04; N, 26.82. Found: C, 49.70; H, 8.17; N, 26.70.

4.28. Ala-Arg-Ala-Pro-Lys-OH (6)

Using general procedure for removal of NO_2, Z, and Bzl from 182 mg (0.2 mmol) of Boc-Ala-Arg(NO_2)-Ala-Pro-Lys(Z)-OBzl 100 mg (92%) of the title compound was obtained as colorless crystals, mp 215–217 °C; FAB-MS (m/e): 542 $[M+H]^+$, $[\alpha]_D^{20}$ −30.0 (c 1.99, H_2O). IR (KBr): 3439, 3373, 3249, 3088, 2957, 1664, 1552, 1382 cm^{-1}; ^1H NMR (DMSO-d_6): $\delta = 10.050$ (1H), 8.214 (1H), 8.184 (2H), 8.183 (1H), 8.101 (1H), 7.535 (1H), 7.106 (1H), 4.492 (1H), 4.381 (1H), 4.339 (1H), 4.125 (1H), 4.010 (1H), 3.356 (2H), 3.324 (2H), 3.059 (2H), 2.750 (2H), 2.051 (2H), 1.866 (2H), 1.668 (2H), 2.493 (2H), 2.491 (2H), 1.988 (2H), 1.535 (2H), 1.533 (2H),

1.315 (3H), 1.278 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/Lys = 2.00:1.00:1.00:1.00; found, Ala/Arg/Pro/Lys = 2.03:0.99:1.00:0.98. Anal. Calcd for $C_{23}H_{43}N_9O_6$: C, 51.00; H, 8.00; N, 23.27. Found: C, 50.89; H, 8.13; N, 23.15.

4.29. Ala-Pro-Lys-OH (7)

Using general procedure for removal of NO_2, Z, and Bzl from 128 mg (0.2 mmol) of Boc-Ala-Pro-Lys(Z)-OBzl 57 mg (90%) of the title compound was obtained as colorless crystals, mp 165–167 °C; FAB-MS (*m*/*e*): 315 $[M+H]^+$, $[\alpha]_D^{20}$ −30.3 (*c* 1.95, H_2O). IR (KBr): 3430, 3262, 3060, 2954, 1663, 1550 cm^{-1}; ^1H NMR (DMSO-d_6): δ = 10.056 (1H), 8.214 (1H), 4.498 (1H), 4.127 (1H), 4.015 (1H), 3.345 (2H), 3.062 (2H), 2.782 (2H), 2.324 (2H), 2.331 (2H), 1.864 (2H), 2.015 (2H), 1.498 (2H), 1.491 (2H), 1.224 (3H). Amino acid analysis: calcd, Ala/Pro/Lys = 1.00:1.00:1.00; found, Ala/Pro/Lys = 1.03:1.01:1.00. Anal. Calcd for $C_{14}H_{26}N_4O_4$: C, 53.49; H, 8.34; N, 17.82. Found: C, 53.38; H, 8.46; N, 17.70.

4.30. Ala-Arg-Pro-Ala-*n*Leu-OH (8)

Using general procedure for removal of NO_2, Z, and Bzl from 152 mg (0.2 mmol) of Boc-Ala-Arg(NO_2)-Pro-Ala-*n*Leu-OBzl 96 mg (91%) of the title compound was obtained as colorless crystals, mp 201–203 °C; FAB-MS (*m*/*e*): 527 $[M+H]^+$, $[\alpha]_D^{20}$ −22.0 (*c* 1.95, H_2O). IR (KBr): 3433, 3368, 3258, 3069, 2957, 1662, 1556, 1382 cm^{-1}; ^1H NMR (DMSO-d_6): δ = 9.988 (1H), 8.601 (2H), 8.196 (1H), 8.064 (1H), 8.049 (1H), 7.501 (1H), 7.209 (1H), 4.527 (1H), 4.335 (1H), 4.261 (1H), 4.190 (1H), 4.086 (1H), 3.599 (2H), 3.098 (2H), 2.769 (2H), 2.684 (2H), 2.034 (2H), 1.982 (2H), 1.854 (2H), 1.684 (2H), 1.342 (2H), 1.335 (2H), 1.248 (3H), 1.204 (3H), 1.132 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/*n*Leu = 2.00:1.00:1.00:1.00; found, Ala/Arg/Pro/*n*Leu = 2.03:0.99:0.97:1.00. Anal. Calcd for $C_{23}H_{42}N_8O_6$: C, 52.46; H, 8.04; N, 21.28. Found: C, 53.38; H, 8.46; N, 17.70.

4.31. Ala-Arg-Pro-Ala-Arg-OH (9)

Using general procedure for removal of NO_2, Z, and Bzl from 170 mg (0.2 mmol) of Boc-Ala-Arg(NO_2)-Pro-Ala-Arg(NO_2)-OBzl 100 mg (88%) of the title compound was obtained as colorless crystals, mp 214–216 °C; FAB-MS (*m*/*e*): 570 $[M+H]^+$, $[\alpha]_D^{20}$ −34.3 (*c* 1.95, H_2O). IR (KBr): 3440, 3365, 3254, 3064, 2955, 1660, 1553, 1380 cm^{-1}; ^1H NMR (DMSO-d_6): δ = 9.968 (1H), 8.440 (2H), 8.436 (2H), 8.286 (1H), 8.284 (1H), 8.010 (1H), 7.562 (1H), 7.441 (1H), 7.260 (1H), 7.118 (1H), 4.270 (1H), 4.207 (1H), 4.115 (1H), 4.103 (1H), 4.025 (1H), 3.556 (2H), 3.540 (2H), 3.062 (2H), 2.741 (2H), 2.250 (2H), 2.034 (2H), 2.026 (2H), 1.865 (2H), 1.687 (2H), 1.681 (2H), 1.278 (3H), 1.269 (3H). Amino acid analysis: calcd, Ala/Arg/Pro/*n*Leu = 2.00:2.00:1.00; found, Ala/Arg/Pro/*n*Leu = 2.00:1.96:0.97. Anal. Calcd for $C_{23}H_{43}N_{11}O_6$: C, 48.49; H, 7.61; N, 27.05. Found: C, 48.38; H, 7.75; N, 26.96.

4.32. Pro-Ala-*n*Leu-OH (10)

Using general procedure for removal of NO_2, Z, and Bzl from Boc-Pro-Ala-*n*Leu-OBzl the title compound was obtained as colorless crystals in 94% yield, mp 186–188 °C; FAB-MS (*m*/*e*): 300 $[M+H]^+$, $[\alpha]_D^{20}$ −25.7 (*c* 1.95, H_2O). IR (KBr): 3431, 3355, 3266, 3065, 2954, 1662, 1557 cm^{-1}; ^1H NMR (DMSO-d_6): δ = 9.986 (1H), 8.265 (1H), 8.161 (1H), 4.601 (1H), 4.329 (1H), 4.023 (1H), 3.044 (1H), 2.719 (2H), 2.276 (1H), 2.011 (2H), 1.762 (2H), 1.556 (2H), 1.552 (2H), 1.296 (3H), 1.210 (3H). Amino acid analysis: calcd, Pro/Ala/*n*Leu = 1.00:1.00:1.00; found, Pro/Ala/*n*Leu = 1.03:1.01:0.97. Anal. Calcd for $C_{14}H_{25}N_3O_4$: C, 56.17; H, 8.42; N, 14.04. Found: C, 56.05; H, 8.61; N, 13.96.

4.33. Pro-Ala-Arg-OH (11)

Using general procedure for removal of NO_2, Z, and Bzl from 115 mg (0.2 mmol) of Boc-Pro-Ala-Arg(NO_2)-OBzl 60 mg (87%) of the title compound was obtained as colorless crystals, mp 195–197 °C; FAB-MS (*m*/*e*): 343 $[M+H]^+$, $[\alpha]_D^{20}$ −31 (*c* 1.95, H_2O). IR (KBr): 3437, 3346, 3262, 3060, 2951, 1665, 1550, 1380 cm^{-1}; ^1H NMR (DMSO-d_6): δ = 9.986 (1H), 8.610 (2H), 8.071 (1H), 8.051 (1H), 7.323 (1H), 7.110 (1H), 4.310 (1H), 4.252 (1H), 4.035 (1H), 3.350 (2H), 3.070 (2H), 2.818 (2H), 2.511 (1H), 2.036 (2H), 1.764 (2H), 1.669 (2H), 1.265 (3H). Amino acid analysis: calcd, Pro/Ala/Arg = 1.00:1.00:1.00; found, Pro/Ala/Arg = 1.00:1.02:0.97. Anal. Calcd for $C_{14}H_{26}N_6O_4$: C, 49.11; H, 7.65; N, 24.54. Found: C, 49.02; H, 7.81; N, 24.33.

4.34. Pro-Ala-Lys-OH (12)

Using the general procedure in the literature[10] after removal of NO_2, Z, and Bzl from 128 mg (0.2 mmol) of Boc-Pro-Ala-Lys(Z)-OBzl 60 mg (95%) of the title compound was obtained as colorless crystals in 95% yield (60 mg), mp 150–152 °C; FAB-MS (*m*/*e*): 315 $[M+H]^+$; $[\alpha]_D^{20}$ −35.6 (*c* 2.02, H_2O); IR (KBr): 3421, 3259, 3063, 2950, 1660, 1554 cm^{-1}; ^1H NMR (DMSO-d_6): δ = 9.987 (1H), 8.461 (1H), 8.132 (1H), 4.350 (1H), 4.086 (1H), 3.847 (1H), 3.335 (2H), 3.003 (2H), 2.745 (2H), 2.650 (2H), 2.509 (1H), 2.108 (1H), 1.710 (2H), 1.509 (2H), 1.307 (2H), 1.220 (3H). Amino acid analysis: calcd, Pro/Ala/Lys = 1.0:1.0:1.0; found, Pro/Ala/Lys = 1.00:1.01:0.96. Anal. Calcd for $C_{14}H_{26}N_4O_4$: C, 53.49; H, 8.34; N, 17.82. Found: C, 53.36; H, 8.43; N, 17.73.

5. Bioassay of the peptides

5.1. In vitro euglobulin clot lysis time of the peptides

The rabbit euglobulin fraction was prepared according to the literature.[12] Plasma diluted 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 barbital buffer

Table 1. Euglobulin clot lysis time of the peptides

Compound	Dosage (μg)	$\overline{X} \pm$ SD (min)
NS	—	202.32 ± 14.35
UK	5 IU	123.88 ± 23.86[a]
Ala-Arg-Pro-Ala-Lys-OH (**1**)	1.4	95.01 ± 15.34[a,b]
Gln-Arg-Pro-Ala-Lys-OH (**2**)	1.5	92.22 ± 13.19[a,b]
Gly-Arg-Pro-Ala-Lys-OH (**3**)	1.3	90.54 ± 15.32[a,b]
Lys-Arg-Pro-Ala-Lys-OH (**4**)	1.5	178.56 ± 17.34
Arg-Arg-Pro-Ala-Lys-OH (**5**)	1.6	201.22 ± 17.60
Ala-Arg-Ala-Pro-Lys-OH (**6**)	1.4	189.99 ± 16.66
Ala-Pro-Lys-OH (**7**)	0.8	196.77 ± 15.66
Ala-Arg-Pro-Ala-*n*Leu-OH (**8**)	0.9	191.61 ± 14.52
Ala-Arg-Pro-Ala-Arg-OH (**9**)	1.4	210.01 ± 18.42
Pro-Ala-*n*Leu-OH (**10**)	0.7	190.45 ± 15.24
Pro-Ala-Arg-OH (**11**)	0.8	208.16 ± 18.01
Pro-Ala-Lys-OH (**12**)	0.8	91.11 ± 14.57[a,b]

$n = 6$.

[a] Compare to NS and peptides **4**–**11** $p < 0.001$.

[b] Compare to UK $p < 0.05$. The dosage of the peptides and UK were chosen based on the literature.[11]

Table 2. Effect of the peptides on fibrin plate

Compound	Dosage (μg)	$\overline{X} \pm$ SD (mm^2)
NS	—	23.21 ± 9.77
UK	5 IU	224.05 ± 10.17[a]
Ala-Arg-Pro-Ala-Lys-OH (**1**)	1.4	213.47 ± 10.16[a]
Gln-Arg-Pro-Ala-Lys-OH (**2**)	1.5	224.78 ± 9.79[a]
Gly-Arg-Pro-Ala-Lys-OH (**3**)	1.3	231.04 ± 9.86[a]
Lys-Arg-Pro-Ala-Lys-OH (**4**)	1.5	22.44 ± 9.16
Arg-Arg-Pro-Ala-Lys-OH (**5**)	1.6	27.38 ± 9.76
Ala-Arg-Ala-Pro-Lys-OH (**6**)	1.4	20.41 ± 9.44
Ala-Pro-Lys-OH (**7**)	0.8	23.23 ± 9.57
Ala-Arg-Pro-Ala-*n*Leu-OH (**8**)	0.9	29.81 ± 10.52
Ala-Arg-Pro-Ala-Arg-OH (**9**)	1.4	20.38 ± 9.44
Pro-Ala-*n*Leu-OH (**10**)	0.7	27.64 ± 8.44
Pro-Ala-Arg-OH (**11**)	0.8	20.55 ± 9.26
Pro-Ala-Lys-OH (**12**)	0.8	236.11 ± 10.01[a]

$n = 6$.

[a] Compare to NS, and peptides **4**–**11** $p < 0.001$. The dosage of the peptides and UK were chosen based on the literature.[11]

(pH 7.8, contained 1.66 mM of $CaCl_2$, 0.68 mM of $MgCl_2$, and 93.96 mM of NaCl). To the rabbit euglobulin clots the peptide was added and the euglobulin clot lysis time (ECLT) was determined by the method in a 96 well microtiter plate.[12] The data are listed in Table 1 and the statistical analysis of the date was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

5.2. In vitro fibrinolytic lysis of the peptides[13]

The plates were prepared by mixing equal volumes of 0.3% rabbit fibrinogen and 0.95% agarose solutions, both dissolved in 50 mM of barbital buffer (pH 7.8, contained 1.66 mM of $CaCl_2$, 0.68 mM of $MgCl_2$, and 93.96 mM of NaCl). The fibrinogen–agarose mixture was coagulated with 100 mL thrombin (100 IU: mL) in the plastic dishes (its diameter is 90 mm and the depth of the fibrin plate is 1 mm). After 30 min at 4 °C an adequate number of wells, 5 mm in diameter, were perforated. To determine fibrinolytic activity, 30 μL aliquots of the peptide to be tested were added to each well, the plate was incubated, and areas of lysis were quantified as described for the regular fibrin plates. The data are listed in Table 2 and the statistical analysis of the date was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

5.3. Thrombolytic activity in vivo[14]

Male wistar rats weighing 200–300 g (purchased from Animal Center of Perking University) were anesthetized with pentobarbital sodium (80.0 mg/kg, ip), and the right carotid artery and left vein jugular were separated. Artery blood (1.0 mL) from the right carotid artery was rapidly injected into the glass tube, then a stainless steel filament helix (15 circles; *L*, 15 mm; *D*, 1.0 mm) was put into the glass tube immediately. The helix with thrombus was carefully pulled out in 15 min later and weighted exactly. This helix was put into the middle polyethylene tube. The polyethylene tube was fully injected with heparin. Both ends of the tube were connected with another polyethylene tube, which end was pulled to change thin to inserted into artery and vein. The blood and the tested peptide was flowed from the right carotid artery to the left jugular vein via the polyethylene tube for 90 min, after which the helix was taken out and weighted accurately. The difference of the helix weight, namely the reduction of thrombolytic mass, was recorded. The date are listed in Table 3 and the statistical analysis of the date was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

Table 3. The reduction of thrombolytic mass

Compound	Dosage (μg)	$\overline{X} \pm$ SD (mg)
NS	3 mL	15.31 ± 3.57
UK	20,000 IU	24.10 ± 3.54[a]
Ala-Arg-Pro-Ala-Lys-OH (**1**)	5.4	18.84 ± 3.18[a]
Gln-Arg-Pro-Ala-Lys-OH (**2**)	6.0	21.28 ± 3.46[a]
Gly-Arg-Pro-Ala-Lys-OH (**3**)	5.3	25.90 ± 2.05[a]
Lys-Arg-Pro-Ala-Lys-OH (**4**)	6.0	17.11 ± 3.40
Arg-Arg-Pro-Ala-Lys-OH (**5**)	6.3	14.87 ± 3.09
Ala-Arg-Ala-Pro-Lys-OH (**6**)	5.4	15.08 ± 3.22
Ala-Pro-Lys-OH (**7**)	3.1	16.03 ± 3.46
Ala-Arg-Pro-Ala-*n*Leu-OH (**8**)	5.3	13.81 ± 2.50
Ala-Arg-Pro-Ala-Arg-OH (**9**)	5.7	15.00 ± 3.11
Pro-Ala-*n*Leu-OH (**10**)	3.0	14.22 ± 3.00
Pro-Ala-Arg-OH (**11**)	3.4	12.99 ± 2.87
Pro-Ala-Lys-OH (**12**)	3.1	26.98 ± 2.54[a]

$n = 10$.

[a] Compare to NS, and peptides **4**–**11** $p < 0.001$. The dosage of the peptides and UK were chosen based on the literature.[11]

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