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Identification, Synthesis and Bioassay for the Metabolites of P6A

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Abstract—The metabolites Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH were identified by HPLC/ESI/MS from the in vivo blood of Ala-Arg-Pro-Ala-Lys-OH (P6A) received mice. The in vitro incubation of P6A in the blood of mice the same metabolites were also found by use of the Prep LC System. The protective intermediates of these metabolites were prepared via the solution method using the stepwise synthesis in 77.4, 90, 88, and 80% yield, respectively. After deprotection with catalytic hydrogenation the intermediates were converted into the corresponding sequences Arg-Pro-Ala-Lys-OH, Pro-Ala-Lys-OH, Ala-Arg-Pro-Ala-OH, and Ala-Arg-Pro-OH in 90, 95, 85% and 86% yield, respectively. In the thrombolysis in vivo assay the synthetic Ala-Arg-Pro-Ala-OH, and Ala-Arg-Pro-OH exhibited no activity. On the other hand the thrombolytic activity of Arg-Pro-Ala-Lys-OH was comparable to P6A, and an enhanced thrombolytic activity was observed for Pro-Ala-Lys-OH. In the in vitro fibrinolytic lysis tests the approximate results were obtained and an enhanced activity was also observed for Pro-Ala-Lys-OH. In the euglobulin clot lysis time tests P6A, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH gave significantly shorter time than that given by UK, demonstrating their fast action.

Introduction

P6A, one of the products obtained from the degradation of fibrinogen, was used as the lead compound in our previous study on thrombolytic agents.[1–3] Though a number of biological researches related to P6A were reported[4–6] only few was focused on its metabolism and no lead compound was obtained from the corresponding metabolite. It was known that the incubation of P6A with angiotension converting enzyme (ACE) in vitro gave metabolites Ala-Arg-Pro-OH and Ala-Lys-OH (Scheme 1).[7] Considering the importance of the metabolite for our investigations in the present paper the in vitro and in vivo metabolism of P6A was observed, the corresponding metabolites were identified and synthesized, and their related bioactivities were tested in vitro and in vivo.

Results

In the identification of the in vivo metabolites of P6A a solution of 0.4 or 4.0 mg of P6A in 0.3 mL of normal saline (NS) was injected into the tail vein of the mice (body weight, 40 g). Using the proper procedure the metabolites containing sample extracted from the plasma was injected on to the HPLC column of the HPLC/ESI/MS. From the plasma of 0.4 and 4.0 mg of P6A received mice a set of the same ions were monitored, based on which the same metabolites Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH were identified (Table 1, Scheme 2). If the whole blood from 0.3 mL of NS received mice (vehicle control) was treated by use of the same procedure not any mentioned sequence was observed.

In the identification of the in vitro metabolites of P6A the solution of 40.0, 4.0, or 0.12 mg of P6A in the mice blood was incubated. Using the proper procedure the metabolites containing sample extracted from the plasma was injected on to the Nova-Park HR C_{18} reverse phase column for preparation purpose or on to the HPLC column of the HPLC/ESI/MS for analytic purpose. For preparation the obtained fractions corresponding to the interested peaks (retention time, 4.95, 5.16, 5.78 and 5.99 min) gave four crystals in 4.2–0.24, 2.9–0.13, 3.3–0.17 and 5.0–0.31 mg yield, respectively. They were then tested by FAB/MS for sequence analysis. Based on the corresponding analytic ion and the resulted fragments the sequences of the four crystals

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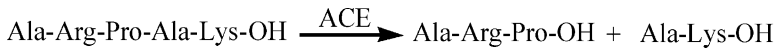


Scheme 1. P6A metabolized in the presence of ACE in vitro.

Table 1. The trapped ions and the corresponding sequences from in vivo metabolism of P6A

m/z	414	343	471	315
Sequence	Ala-Arg-Pro-Ala-OH	Ala-Arg-Pro-OH	Arg-Pro-Ala-Lys-OH	Pro-Ala-Lys-OH
Peak area	4.02×10^7	9.54×10^6	6.92×10^6	2.88×10^6

were assigned to Arg-Pro-Ala-Lys-OH, Pro-Ala-Lys-OH, Ala-Arg-Pro-OH and Ala-Arg-Pro-Ala-OH, respectively. For analysis the sequences of the metabolites were determined by retention time and analytic ion of Ala-Arg-Pro-Ala-OH, Ala-Arg- Pro-OH, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH.

In the examination of the stability of P6A the solution of 0.04 mg of P6A in 1.0 mL of water, or in 1.0 mL of buffer (0.1 mol/L of phosphate, pH 7.4), or in 1.0 mL of protein free plasma, or in 1.0 mL of plasma containing NaF (final concentration 2 mol/L), or in 1.0 mL of plasma containing EDTA (final concentration 1.5%), or in 1.0 mL of buffered ($NaHCO_3$–K_2CO_3, 3:2, pH 9.4) plasma was incubated at 37 °C. After the proper treatment the HPLC/ESI/MS analysis of all the obtained samples gave no degradation product of P6A.

According to Scheme 3 the protective intermediates of the in vivo metabolites of P6A, Boc-Arg (NO_2)-Pro-Ala-Lys(Z)-OBzl and Boc-Pro-Ala-Lys(Z)-OBzl, were prepared via the solution method and stepwise synthesis (from C-terminal to N-terminal) with L-Lys(Z)-OBzl as the starting material in 77.4 and 90.0% yield, respectively. After the removal of Boc group with hydrogen chloride in ethyl acetate, the removal of NO_2, Z, and Bzl with catalytic hydrogenation the protective intermediates were converted into the desirable products Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH in 90 and 95% yield, respectively.

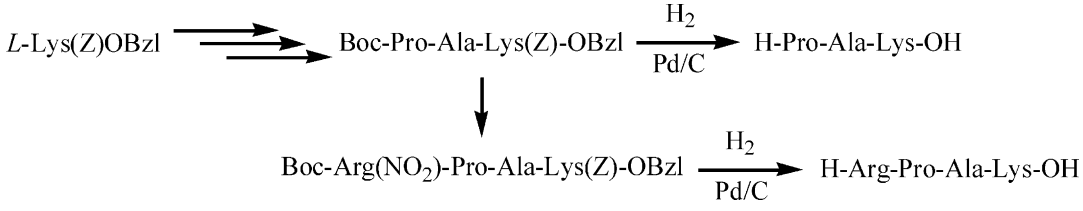
According to Scheme 4 via the solution method and stepwise synthesis (from C-terminal to N-terminal) with L-Pro-OBzl as the starting material the protective intermediates Boc-Ala-Arg(NO_2)-Pro-OBzl was prepared in 88.0% yield. After saponification of Boc-Ala-Arg(NO_2)-Pro-OBzl the resulting Boc-Ala-Arg(NO_2)-Pro-OH was coupled with L-Ala-OBzl to provide Boc-Ala-Arg(NO_2)-Pro-Ala-OBzl in 80% yield. Removing the Boc group with hydrogen chloride in ethyl acetate, removing NO_2, Z, and Bzl with catalytic hydrogenation the protective intermediates were converted into the desirable products Ala-Arg-Pro-OH and Ala-Arg-Pro-Ala-OH in 86.0 and 85.0% yield, respectively.

According to the literature[8] the in vitro fibrinolytic lysis test was performed and the quantified lysis areas of the regular fibrin plates for Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH were significantly smaller than that for Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH (Table 2).

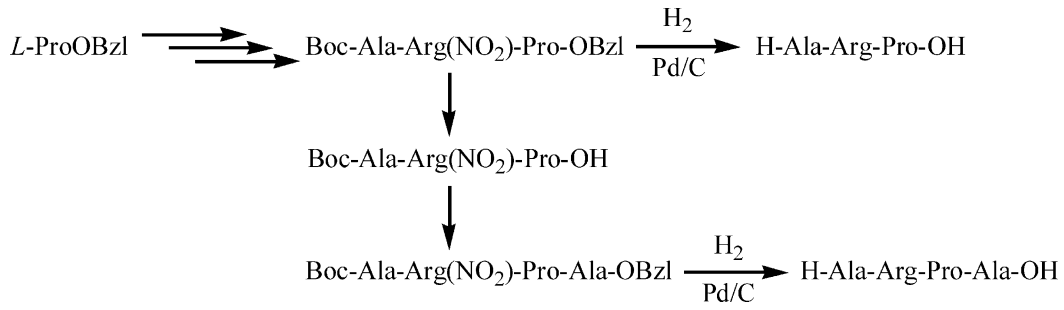
According to the literature[8–10] the in vitro euglobulin clot lysis test was performed and the resulting euglobulin clot lysis time for Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH were significantly shorter than that for Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH (Table 3).

According to the literature[8–10] the in vivo thrombolytic activity was tested and no difference between the reduction of thrombolytic mass for Ala-Arg-Pro-Ala-OH or Ala-Arg-Pro-OH and NS was observed, and a sig-

Scheme 2. The metabolism ways of P6A in vivo.



Scheme 3. Preparation of Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH. (1) General procedure for removal of Boc of the C-terminal component: The solution of 0.20 mmol of Boc protected compound in 2 mL of hydrogen chloride in ethyl acetate (4 mol/L) was stirred at room temperature for 3 h. (2) General procedure for coupling of 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. To the solution 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.



Scheme 4. Preparation of Ala-Arg-Pro-OH and Ala-Arg-Pro-Ala-OH. (1) General procedure for removal of benzyl group of the N-terminal component: At 0 °C to the solution of 0.80 mmol of protected peptide benzyl ester 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 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: 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. To the solution 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.

Table 2. Effect of synthetic metabolites of P6A on fibrin plate

Compound	Dosage (μg)	$\bar{X}\pm$SD (mm^2)
NS	—	21.01 ± 9.26
UK	5 IU	222.99 ± 10.99[a,b]
P6A	1.4	210.84 ± 12.08[a,b]
Ala-Arg-Pro-Ala-OH	1.1	31.01 ± 9.26
Ala-Arg-Pro-OH	0.9	29.81 ± 10.52
Arg-Pro-Ala-Lys-OH	1.2	211.12 ± 10.41[a,b]
Pro-Ala-Lys-OH	0.8	234.39 ± 9.39[a,b,c]

$n = 6$.
[a]Compare to NS $P < 0.001$.
[b]Compare to Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH $P < 0.001$.
[c]Compare to P6A and Arg-Pro-Ala-Lys-OH $P < 0.01$.

Table 3. Euglobulin clot lysis time of synthetic metabolites of P6A

Compound	Dosage (μg)	$\bar{X}\pm$SD (min)
NS	—	200.01 ± 15.26
UK	5 IU	122.19 ± 24.90[a]
P6A	1.4	94.84 ± 16.28[a,b]
Ala-Arg-Pro-Ala-OH	1.1	186.54 ± 19.16
Ala-Arg-Pro-OH	0.9	191.61 ± 14.52
Arg-Pro-Ala-Lys-OH	1.2	92.02 ± 16.21[a,b]
Pro-Ala-Lys-OH	0.8	90.98 ± 15.69[a,b]

$n = 6$.
[a]Compare to NS, Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH $P < 0.001$.
[b]Compare to UK $P < 0.05$.

nificant difference between the reduction of thrombolytic mass for Arg-Pro-Ala-Lys-OH or Pro-Ala-Lys-OH and NS was observed (Table 4).

Discussion

At the dose of 0.4 or 4.0 mg/kg the metabolites (Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg- Pro-Ala-Lys-OH and Pro-Ala-Lys-OH) of P6A in mice may be clearly identified by HPLC/ESI/MS. The peak area of the metabolites Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH identified by HPLC/MS directly from the plasma of P6A received mice was 4.02×10^7, 9.54×10^6, 6.92×10^6 and 2.88×10^6, respectively. The approximately equal peak

Table 4. The reduction of thrombolytic mass

Compound	Dosage (μg)	$\bar{X}\pm$SD (mg)
NS	3 mL	12.31 ± 2.57
UK	20 000 IU	22.10 ± 2.54[a,c,d]
P6A	5.4 mg	17.84 ± 2.18[a,b]
Ala-Arg-Pro-Ala-OH	4.2 mg	14.01 ± 2.26
Ala-Arg-Pro-OH	3.4 mg	13.81 ± 2.50
Arg-Pro-Ala-Lys-OH	4.7 mg	18.22 ± 2.40[a,c]
Pro-Ala-Lys-OH	3.1 mg	23.68 ± 2.34[a,c,d]

$n = 10$.
[a]Compare to NS $p < 0.001$.
[b]Compare to Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH $P < 0.01$.
[c]Compare to Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH $P < 0.001$.
[d]Compare to P6A $P < 0.001$.

area indicated that the metabolism pathways of P6A either from C-terminal or from N-terminal by cleavage of the corresponding peptide bond successively had approximately equal probability. The observation that the vehicle control gave no related sequences indicated that the metabolites did result from the degradation of P6A. The fact that when the mixture of P6A and the blood of mice was incubated and treated according to the procedure used for in vivo experiments as the metabolites Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala- Lys-OH and Pro-Ala-Lys-OH were also isolated by use of Waters Prep LC System indicated that the in vivo and in vitro metabolism took the same pathways. When the incubation of P6A was carried out in the solution without plasma or in the plasma containing enzyme inhibitor, for instance when the incubation of 0.04 mg of P6A was carried out in 1.0 mL of water, or in 1.0 mL of buffer (0.1 mol/L of phosphate, pH 7.4), or in 1.0 mL of plasma containing EDTA (final concentration 1.5%), or in 1.0 mL of buffered ($NaHCO_3$–K_2CO_3, 3:2, pH 9.4) plasma at the same condition and treated with the same procedure as mentioned above no any degradation of P6A was occured. The result suggested that the observed metabolism of P6A was plasma enzyme dependent.

The fact that the synthetic metabolites and the in vitro metabolites gave substantially equal specific rotation indicated that in the metabolism of P6A no any configuration

change was occurred. Since in both of the HPLC/ESI/MS analysis and the HPLC preparation no metabolite Ala-Lys-OH was found the situation of ACE promoted in vitro metabolism of P6A (Scheme 1) was obviously different from the case of P6A in the blood of mice. The in vivo thrombolytic assay demonstrated that there was no significant difference between the thrombolytic activities of Ala-Arg-Pro-Ala-OH (the reduction of thrombolytic mass, 14.01±2.26 mg), Ala-Arg-Pro-OH (the reduction of thrombolytic mass, 13.81±2.50 mg) and NS (the reduction of thrombolytic mass, 12.31±2.57 mg). On the other hand the thrombolytic activity of Arg-Pro-Ala-Lys-OH (the reduction of thrombolytic mass, 18.22±2.40 mg) is comparable to that of P6A (the reduction of thrombolytic mass, 17.84±2.18 mg), and an enhanced thrombolytic activity was observed for Pro-Ala-Lys-OH (the reduction of thrombolytic mass, 23.68±2.34 mg). Combining the data of metabolism and bioassay one may consider Pro-Ala-Lys-OH as the pharmacophore of P6A and its analogues. Considering two of four metabolites of P6A (Ala-Arg-Pro-Ala-OH and Ala-Arg-Pro-OH) exhibited no thrombolytic effect the result that the thrombolytic activity of Pro-Ala-Lys-OH was higher than that of P6A became understandable. The quantified lysis area of the regular fibrin plate for Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH was 31.01±9.26, 29.81±10.52, 211.12±10.41 and 234.39±9.39 mm^2, respectively. Comparing to Arg-Pro-Ala-Lys-OH and P6A (the quantified lysis area, 210.84±12.08 mm^2) Pro-Ala-Lys-OH exhibited an enhanced activity. The euglobulin clot lysis time of Ala-Arg-Pro-Ala-OH, Ala-Arg-Pro-OH, Arg-Pro-Ala-Lys-OH and Pro-Ala-Lys-OH was 186.54±19.16, 191.61±14.52, 92.02±16.21 and 90.98±15.69 min, respectively, which exhibited significant difference ($P<0.001$). All of the fibrinolytic lysis tests of the metabolites suggested that the in vitro and in vivo results were parallel. These results demonstrate that the investigated oligopeptides may exhibite fast action as well.

Experimental

Identification of the in vivo metabolites of P6A

General. HP5989B MS engine mass spectrometer was used with analysis of Brandford LC/ESI/MS interfaces. A Hewlett-Packard Model 1090 system was used for HPLC separation of the metabolites of P6A and delivery of the eluant to the mass spectrometer. The HPLC column used was Agilent Zorbax SB-C_{18} reverse phase column (2.1×150 mm, 5 μm). The LC flow rate was 0.25 mL/min. The mobile phase consisted of acetic acid (A, 1%) and acetonitrile (B). The gradient program was that in 10 min 0% B was increased to 20% B and in another 10 min increased to 70% B. The temperature of the drying gas was 400 °C. The flow rate of the drying gas was 3.3l/min. The flow rate of the spray gas was 2.0l/min. The spray voltage was 4500 V. All the possible ions of the metabolites from P6A in vivo were monitored by ESI/MS.

(a) Identification of the metabolites from the plasma of high dose of P6A received mice. The solution of 4.0 mg of P6A in 0.3 mL of normal saline was injected into the tail vein of the mice (body weight 38–40 g, $n=4$). After 20 min the whole blood of the mice was centrifuged at 0 °C and 3500 *g* for 5 min to isolate the plasma. To 100 μL of the plasma 100 μL of perchloric acid (1 mol/L) were added in order to free the residual P6A and all its metabolites from the plasma proteins. The plasma mixture was centrifuged at 0 °C and 3500 *g* for 15 min. The separated upper layer from the plasma of 4.0 mg/kg of P6A received mice, as a clean solution, was used as the test sample and injected into the HPLC column of the HPLC/ESI/MS directly. According to the trapped ions the corresponding sequences were Ala-Arg-Pro-Ala-OH (*m*/*z*, 414), Ala-Arg-Pro-OH (*m*/*z*, 343), Arg-Pro-Ala-Lys-OH (*m*/*z*, 471), and Pro-Ala-Lys-OH (*m*/*z*, 315).

(b) Identification of the metabolites from the plasma of low dose of P6A received mice. Using the same procedure as that used in (a) with the solution of 0.4 mg of P6A in 0.3 mL of normal saline instead of the solution 4.0 mg of P6A in 0.3 mL of normal saline to inject into the tail vein of the mice (body weight 38–40 g, $n=4$). After the plasma was concentrated to 1/10 volume the separated upper layer, as a clean solution, was used as the test sample and injected on to the HPLC column of the HPLC/ESI/MS. According to the trapped ions the corresponding sequences were Ala-Arg-Pro-Ala-OH (*m*/*z*, 414), Ala-Arg-Pro-OH (*m*/*z*, 343), Arg-Pro-Ala-Lys-OH (*m*/*z*, 471), and Pro-Ala-Lys-OH (*m*/*z*, 315).

(c) Identification of the metabolites from the plasma of normal saline received mice. Using the same procedure as that used in (a) with 0.3 mL of normal saline instead of the solution 4.0 mg of P6A in 0.3 mL of normal saline to inject into the tail vein of the mice (body weight 38–40 g, $n=4$). According to the trapped ion only P6A was identified and no any metabolite was found.

Isolation and identification of the in vitro metabolite of P6A

General. The separation was proceeded on the Nova-Park HR C_{18} reverse phase column (40×100 mm, 6 μm) with the support of Waters PrepLe System. The LC flow rate was 18 mL/min. The mobile phase consisted of acetic acid (A, 1%) and acetonitrile (B). The grant program was that in 15 min 0% B was increased to 20% B and in another 15 min increased to 70% B.

Isolation of the metabolites from the incubation of P6A in mouse blood. To 10 mL of the mouse blood 40.0 or 4.0 mg of P6A were added. The solution was incubated at 37 °C for 20 min and then was centrifuged at °C and 3500 *g* for 5 min to isolate the plasma. To the obtained plasma 2 mL of perchloric acid (1 mol/L) were added to free the residual P6A and all its metabolites from the plasma proteins. The plasma was centrifuged at 0 °C and 3500 *g* for 15 min and the separated upper layer, as a clean solution, was frozen and lyophilized. The resulted crystals were dissolved in 1 mL of water and the obtained solution was injected on to the column for

separation. The fractions corresponding to the interested peaks were collected, frozen and lyophilized. The resulted 4 crystals were analyzed with FAB/MS. The *m*/*z*, retention time, sequence and $[\alpha]_D^{24}$ of the metabolites was 471 $[M+H]^+$, 4.95 min, Arg-Pro-Ala-Lys-OH, $-46.5°$ (c = 0.31, H_2O); 315 $[M+H]^+$, 5.16 min, Pro-Ala-Lys-OH, $-34.6°$ (c = 0.22, H_2O); 343 $[M+H]^+$, 5.78 min, Ala-Arg-Pro-OH, $-43.0°$ (c = 0.24, H_2O) and 414 $[M+H]^+$, 5.99 min, Ala-Arg-Pro-Ala-OH $-54.7°$ (c = 0.35, H_2O), respectively.

The incubation of 40.0 mg of P6A in 10 mL of mouse blood yielded 4.2 mg of Arg-Pro-Ala-Lys-OH, 2.9 mg of Pro-Ala-Lys-OH, 3.3 mg of Ala-Arg-Pro-OH and 5.0 mg of Ala-Arg-Pro-Ala-OH.

The incubation of 4.0 mg of P6A in 10 mL of mouse blood yielded 0.24 mg of Arg-Pro-Ala-Lys-OH, 0.13 mg of Pro-Ala-Lys-OH, 0.17 mg of Ala-Arg-Pro-OH and 0.31 mg of Ala-Arg-Pro-Ala-OH.

Identification of the metabolites from the incubation of P6A in mouse blood. To 2 mL of the mouse blood 0.4 mg of P6A were added. The solution was incubated at 37 °C for 20 min and then was centrifuged at 0 °C and 3500 *g* for 5 min to isolate the plasma. To 1 mL of the plasma 0.5 mL of perchloric acid (0.25 mol/L) were added to free the residual P6A and all its metabolites from the plasma proteins. The plasma mixture was centrifuged at 0 °C and 3500 *g* for 15 min and the separated upper layer, as a clean solution, was frozen and lyophilized to provide 20 μg of crystals. The resulted crystals were dissolved in 0.01 mL of water and the obtained solution was injected on to the column of HPLC/MS for analysis. According to the trapped ions and LC the corresponding sequences were also Ala-Arg-Pro-Ala-OH (*m*/*z*, 414), Ala-Arg-Pro-OH (*m*/*z*, 343), Arg-Pro-Ala-Lys-OH (*m*/*z*, 471), and Pro-Ala-Lys-OH (*m*/*z*, 315) and the relative proportion for them was approximately 2.0:1.0:1.2:2.2.

Stability of P6A in solution

(a) Stability of P6A in phosphate buffer (pH = 7.4). To 2 mL of phosphate buffer (pH = 7.4) 0.4 mg of P6A were added. The solution was incubated at 37 °C for 20 min and then was centrifuged at 0 °C and 3500 *g* for 5 min. To the solution 0.5 mL of perchloric acid (0.25 mol/L) were added. The solution was centrifuged at 0 °C and 3500 *g* for 15 min and the separated upper layer, as a clean solution, was frozen. The resulting residue was dissolved in 0.01 mL of water and the resulting solution was injected on to the column of HPLC/MS for analysis. According to the trapped ion the corresponding sequence was only Ala-Arg-Pro-Ala-Lys-OH (*m*/*z*, 541) no metabolite was found.

(b) Stability of P6A in enzyme inhibitor containing solution. To 2 mL of water 0.4 mg of P6A and 150 mg of EDTA were added. The solution was incubated at 37 °C for 20 min and then was centrifuged at 0 °C and 3500 *g* for 5 min. To the solution 0.5 mL of perchloric acid (0.25 mol/L) were added. The solution was centrifuged at 0 °C and 3500 g for 15 min and the separated upper layer, as a clean solution, was frozen. The resulted residue was dissolved in 0.01 mL of water and the resulted solution was injected on to the column of HPLC/MS for analysis. According to the trapped ion the corresponding sequence was only Ala-Arg-Pro-Ala-Lys-OH (*m*/*z*, 541) no metabolite was found.

(c) Stability of P6A in $NaHCO_3$–K_2CO_3 (3:2, pH 9.4) buffer. To 5 mL of saturated solution of $NaHCO_3$–K_2CO_3 (3:2, pH 9.4) in water 0.4 mg of P6A were added. The solution was incubated at 37 °C for 20 min and then was centrifuged at 0 °C and 3500 g for 5 min. To the solution 0.5 mL of perchloric acid (0.25 mol/L) were added. The solution was centrifuged at 0° and 3500 *g* for 15 min and the separated upper layer, as a clean solution, was frozen. The resulted residue was dissolved in 0.01 mL of water and the resulted solution was injected on to the column of HPLC/MS for analysis. According to the trapped ion the corresponding sequence was only Ala-Arg-Pro-Ala-Lys-OH (*m*/*z*, 541) no any metabolite was found.

Synthesis of the in vivo metabolite of P6A

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. ^1H 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.

General procedure for removal of Boc of the C-terminal component. The solution of 0.20 mmol of Boc protected compound in 2 mL of hydrogen chloride in ethyl acetate (4 mol/L) was stirred at room temperature for 3 h. The reaction mixture was evaporated to remove the solvent. The residue was dissolved in 10 mL of ethyl acetate and the solution was evaporated to dry. The resulted solid was used for coupling reaction directly.

General procedure for coupling of 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 triturated 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 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.

General procedure for removal of benzyl group of the N-terminal component. At 0 °C to the solution of 0.80 mmol of protected peptide benzyl ester 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 protected peptide carboxylic acid.

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

Boc-Arg(NO_2)-Pro-OBzl. Using the general procedure for coupling of C-terminal and N-terminal components from Boc-L-Arg(NO_2)-OH and L-Pro-OBzl the title compound was obtained as a colorless powder in 86% yield (87 mg), Mp 70–72 °C FAB-MS (*m*/*e*): 507 $[M+H]^+$; $[\alpha]_D^{20} = -7.8°$ (c = 0.2, $CHCl_3$); IR (KBr): 3340, 3030, 3010, 1750, 1692, 1600, 1580, 1500, 1463, 1394, 1382, 1364, 760, 700 cm^{-1}; ^1H NMR ($CDCl_3$): δ = 9.465 (1H), 8.225 (1H), 8.115 (1H), 8.111 (1H), 7.401 (2H), 7.332 (1H), 7.201 (2H), 4.301 (1H), 4.124 (1H), 3.905 (1H), 3.561 (2H), 3.402 (2H), 2.746 (2H), 1.886 (2H), 1.465 (9H), amino acid analysis: calcd Arg:Pro = 1.0:1.0; found, Arg:Pro = 0.97:1.00. Anal. calcd for $C_{23}H_{34}N_6O_7$: C, 54.53; H, 6.77; N, 16.59. Found: C, 54.25; H, 6.44; N, 16.35.

Boc-Ala-Arg(NO_2)-Pro-OBzl. Using the general procedure for removal of Boc of the C-terminal component Boc-Arg(NO_2)-Pro-OBzl was converted into HCl·Arg(NO_2)-Pro-OBzl which was coupled with Boc-L-Ala-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 87% yield (100 mg). Mp 82–84 °C; FAB-MS (*m*/*e*) 578 $[M+H]^+$; $[\alpha]_D^{20} = -7.0°$ (c = 0.2, $CHCl_3$), IR (KBr): 3355, 3348, 3025, 3015, 1758, 1677, 1605, 1575, 1509, 1472, 1396, 1387, 1369, 765, 704 cm^{-1}, ^1H NMR ($CDCl_3$): δ = 9.354 (1H), 8.218 (1H), 8.125 (1H), 8.114 (1H), 8.109 (1H), 7.399 (2H), 7.315 (1H), 7.214 (2H), 3.884 (1H), 3.778 (1H), 3.533 (2H), 3.382 (2H), 3.100 (2H), 2.698 (2H), 2.102 (2H), 1.764 (2H), 1.426 (9H), 1.269 (3H). Amino acid analysis: calcd Ala:Arg:Pro = 1.0:1.0:1.0; found, Ala:Arg:Pro = 1.02:0.97:1.00. Anal. calcd for $C_{26}H_{39}N_7O_8$: C, 54.06; H, 6.81; N, 16.97. Found: C, 54.30; H, 6.55; N, 16.70.

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 ($CDCl_3$): δ = 9.454 (1H), 8.212 (1H), 8.120 (1H), 8.110 (1H), 8.102 (1H), 7.664 (1H), 7.388 (2H), 7.308 (1H), 7.201 (2H), 4.301 (1H), 4.295 (1H), 3.884 (1H), 3.778 (1H), 3.541 (2H), 3.396 (2H), 3.059 (2H), 2.750 (2H), 2.044 (2H), 1.789 (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.

Boc-Ala-Lys(Z)-OBzl. Using the general procedure for coupling of C-terminal and N-terminal components from Boc-L-Ala-OH and L-Lys(Z)-OBzl the title compound was obtained as a colorless powder in 90% yield (98 mg). Mp 88–90 °C; FAB-M (*m*/*e*) 542 $[M+H]^+$; $[\alpha]_D^{20} = -6.6$ (c = 0.2, $CHCl_3$), IR (KBr): 3366, 3030, 3015, 1765, 1690, 1610, 1590, 1506, 1460, 1395, 1385, 1366, 760, 710 cm^{-1}, ^1H NMR ($CDCl_3$): δ = 8.123 (1H), 8.102 (1H), 8.089 (1H), 7.365 (2H), 7.360 (2H), 7.310 (1H), 7.295 (1H), 7.208 (2H), 7.201 (2H), 4.315 (1H), 4.211 (1H), 3.988 (2H), 3.565 (2H), 3.550 (2H), 1.655 (2H), 1.529 (2H), 1.520 (2H), 1.450 (9H), 1.269 (3H). Amino acid analysis: calcd Ala:Lys = 1.0:1.0; found, Ala:Lys = 1.00:0.97. Anal. calcd for $C_{29}H_{39}N_3O_7$: C, 64.31; H, 7.26; N, 7.76. Found: C, 64.10; H, 7.08; N, 7.54.

Boc-Pro-Ala-Lys(Z)-OBzl. Using the general procedure for removal of Boc of the C-terminal component Boc-Ala-Lys(Z)-OBzl was converted into HCl·Ala-Lys(Z)-OBzl which was coupled with Boc-L-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 90% yield (115 mg), mp 85–87 °C, FAB-M (*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 ($CDCl_3$): δ = 8.118 (1H), 8.105 (1H), 7.690 (1H), 7.370 (2H), 7.364 (2H), 7.341 (1H), 7.301 (1H), 7.210 (2H), 7.208 (2H), 7.201 (2H), 4.309 (1H), 4.209 (1H), 3.969 (2H), 3.546 (2H), 3.540 (2H), 3.048 (2H), 2.746 (2H), 1.768 (2H), 1.672 (2H), 1.532 (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, 63.68; H, 7.20; N, 8.54.

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}, ^1H NMR ($CDCl_3$): δ = 8.450 (1H), 8.205 (1H), 8.126 (1H), 8.120 (1H), 8.009 (1H), 7.985 (1H), 7.705 (1H), 7.365 (2H), 7.360 (2H), 7.328 (1H), 7.324 (1H), 7.205 (2H), 7.200 (2H), 4.315 (1H), 4.301 (1H), 4.190 (1H), 3.989 (1H), 3.980 (2H), 3.548 (2H), 3.544 (2H), 3.390 (2H), 3.040 (2H), 2.750 (2H), 2.050 (2H), 1.766 (2H), 1.681 (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.

H-Ala-Arg-Pro-OH. Using the general procedure for removal of NO_2, Z and Bzl from Boc-Ala-Arg(NO_2)-Pro-OBzl the title compound was obtained as colorless crystals in 86% yield (59 mg), mp 150–152 °C, FAB-MS (*m*/*e*) 343 $[M+H]^+$, $[\alpha]_D^{20} = -43.7°$ (c = 2.04, H_2O), IR (KBr): 3424, 3260, 3070, 2960, 1660, 1550 cm^{-1}, ^1H NMR (DMSO-d_6): δ = 8.825 (3H), 8.415 (2H), 8.200 (1H), 8.195 (1H), 8.065 (1H), 4.023 (1H), 4.016 (1H), 4.001 (1H), 3.405 (2H), 2.745 (2H), 1.796 (2H), 1.365 (3H). Amino acid analysis: calcd Ala:Arg:Pro = 1.0:1.0:1.0; found, Pro:Ala:Lys = 1.00:0.97:1.01. Anal. calcd for $C_{14}H_{26}N_6O_4$: C, 49.11; H, 7.65; N, 24.54. Found: C, 49.28; H, 7.42; N, 24.33.

H-Ala-Arg-Pro-Ala-OH. Using the general procedure for removal of NO_2, Z and Bzl from Boc-Ala-Arg(NO_2)-Pro-Ala-OBzl the title compound was obtained as colorless crystals in 85% yield (70 mg), mp 157–159 °C, FAB-MS (*m*/*e*) 414 $[M+H]^+$, $[\alpha]_D^{20} = -55.5°$ (c = 1.75, H_2O), IR (KBr): 3450, 3266, 3240, 3069, 2966, 1655, 1558 cm^{-1}, ^1H NMR (DMSO-d_6): δ = 8.621 (3H), 8.401 (2H), 8.211 (1H), 8.185 (1H), 8.010 (1H), 7.605 (1H), 4.206 (1H), 3.901 (1H), 3.820 (1H), 3.816 (1H), 3.360 (2H), 2.740 (2H), 1.855 (2H), 1.276 (3H), 1.275 (3H), amino acid analysis: calcd, Ala:Arg:Pro = 2.0:1.0:1.0; found, Pro:Ala:Lys = 1.98:0.98:1.00. Anal. calcd for $C_{17}H_{31}N_7O_5$: C, 49.38; H, 7.56; N, 23.71. Found: C, 49.16; H, 7.80; N, 23.49.

H-Pro-Ala-Lys-OH. Using the general procedure for removal of NO_2, Z and Bzl from Boc-Pro-Ala-Lys(Z)-OBzl 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 = 8.650 (3H), 8.461 (1H), 8.132 (1H), 4.350 (1H), 4.086 (1H), 3.847 (1H), 3.335 (2H), 3.003 (2H), 2.745 (2H), 2.108 (1H), 1.710 (4H), 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, 53.65; H, 8.54; N, 17.61.

H-Arg-Pro-Ala-Lys-OH. Using the general procedure for removal of NO_2, Z and Bzl from Boc-Arg(NO_2)-Pro-Ala-Lys(Z)-OBzl the title compound was obtained as a colorless crystals in 90% yield (85 mg), mp 158–160 °C, FAB-MS (*m*/*e*) 471 $[M+H]^+$, $[\alpha]_D^{20} = -47.5°$ (c = 1.95, H_2O), IR(KBr): 3425, 3377, 3256, 3070, 2955, 1668, 1552 cm^{-1}, ^1H NMR (DMSO-d_6): δ = 8.560 (3H), 8.172 (1H), 7.955 (1H), 7.600 (1H), 7.231 (2H), 6.898 (1H), 4.433 (1H), 4.253 (1H), 4.155 (4H), 3.489 (2H), 3.097 (2H), 2.744 (2H), 1.837 (2H), 1.817 (2H), 1.736 (2H), 1.530 (2H), 1.304 (2H), 1.255 (3H), amino acid analysis: calcd Arg:Pro:Ala: Lys = 1.0:1.0:1.0:1.0; found, Arg:Pro:Ala:Lys = 0.96:1.01:1.00:0.97. Anal. calcd for $C_{20}H_{38}N_8O_5$: C, 51.05; H, 8.14; N, 23.81. Found: C, 51.28; H, 8.30; N, 23.57.

Bioassay of the synthetic metabolites of P6A

In vitro euglobulin clot lysis time of the synthetic metabolites of P6A. The rabbit euglobulin fraction was prepared according to the literature.[9] 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 re-suspended to the initial plasma volume with 50 mM barbital buffer (pH 7.8, contained 1.66 mM of $CaCl_2$, 0.68 mM of $MgCl_2$ and 93.96 mM of NaCl). Euglobulin clot lysis time (ECLT) was measured using a 96 well microtiter plate.[8,10]

In vitro fibrinolytic lysis of the synthetic metabolites of P6A. 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).[8] 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 metabolites 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.

In vivo thrombolytic activities of the synthetic metabolites of P6A.[2] Male Wistar rats weighing 200–1300 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 anticoagulant, 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 thrombolytic mass was recorded. The data are listed in Table 4. The statistical analysis of the date was carried out by use of ANOVA test, $P<0.05$ is considered significant.

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References and Notes

1. Zhao, M.; Peng, S. *J. Prakt. Chem.* **1999**, *341*, 668.
2. Lin, N.; Zhao, M.; Wang, C.; Peng, S. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 585.
3. Wu, Y..; Zhao, M.; Wang, C.; Peng, S. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2331.
4. Mehta, J. L.; Nichols, W. W.; Saldeen, T. G. P. *J. Cardiovasc. Pharmacol.* **1989**, *13*, 803.
5. Saldeen, K.; Lawson, D. L.; Mehta, J. L. *Thrombosis Research* **1991**, *61*, 81.
6. Saldeen, T. G. P.; Nichols, W. W.; Saldeen, K.; Wallin, R.; Mehta, J. L. *J. Cardiovasc. Pharmacol.* **1994**, *23*, 103.
7. Saldeen, T.; James, W.; Berryer, R.; Berryer, P. *Thrombosis Research* **1981**, *23*, 465.
8. Kluft, C. *Thromb. Haemost.* **1979**, *41*, 356.
9. Urano, T.; Sakakibara, K.; Rydzewski, A.; Urano, S.; Takada, Y.; Takada, A. *Thromb. Haemost.* **1990**, *63*, 82.
10. Lin, N.; Yang, J.; Wang, C.; Zhao, M.; Peng, S.; Song, D.; Wong, J. *J. Beijing Mwed. Univ.* **2000**, *32*, 283.