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Synthesis and Thrombolytic Activity of Pseudopeptides Related to Fibrinogen Fragment

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Abstract—Two kinds of linkers consisting of 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, ARPAK, GRPAK and QRPAK were synthesized. The thrombolytic activities in vivo indicated that the coupling position of 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid in the peptides effected on the potencies significantly. When the C-terminal of the peptides was amidated by 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, the thrombolytic potency of the peptides was enhanced or kept. When the N-terminal of the peptides was acylated by 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, however, the thrombolytic effect of the peptides was banished. The expected specific β II′-turn conformation and the stability to trypsin in the pseudopeptides with 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid in its C-terminal may be responsible for the enhanced thrombolytic potency.

In the modification of ARPAK, the fragment from fibrinogen provided new sequences, QRPAK and GRPAK, with more potential thrombolytic activity than the mother sequence.[1] In a conformational study of ARPAK and its analogues with Discover program we indicated that in solution the β structure has the lowest energy and may be the active conformation.[2] The highly flexible conformations of ARPAK and the analogues told us that their conformational modification by increasing the rigidity may result in insightful knowledge related to their SAR. Our previous studies also suggested that 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid was a useful building block for construction of the pseudopeptides with antithrombolytic activity and has some conformational rigidity.[3,4] It is apparent that the two means of structural modification for ARPAK, QRPAK, and GRPAK provided by the carboxylic and aliphatic amino groups in 3-(*S*)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid may offer two kinds of pseudopeptides with obviously different conformations and perhaps different stability to proteases, such as trypsin. In accordance with Schemes 3 and 4 the C-terminal and N-terminal of the peptides were amidated and acylated by 3-(*S*)-1,2,3,4-tetrahydro-carboline-3-carboxylic acid, respectively.

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Chemistry

According to Scheme 1, in the presence of sulfuric acid and water, the Pictet–Spengler condensation of L-tryptophane and formaldehyde gave carboline-3-carboxylic acid (**1**) in 100% yield. In the acylation step of **1** with $(Boc)_2O$, *N*-Boc-carboline-3-carboxylic acid (**2**) was obtained in 76% yield. In the presence of Cs_2CO_3 and benzyl bromide, **2** was converted to *N*-Boc-carboline-3-carboxylic acid benzyl ester, which was without further purification deprotected directly with HCl/EtOAc to give the corresponding carboline-3-carboxylic acid benzyl ester (**3**) in excellent yield.

The protective oligopeptide intermediates (**4**–**6**) were prepared via the solution method by use of the stepwise synthesis from the C-terminal to the N-terminal of the peptides. After deprotection with HF the intermediates were converted into the corresponding sequences ARPAK (**7**), GRPAK (**8**), and QRPAK (**9**) in 77, 89 and 86% yield, respectively.

Removal of the Boc group or the benzyl group in **4**–**6** gave the corresponding N-terminal free intermediates (**10**–**12**) or C-terminal free intermediates (**13**–**15**). **10**–**12** were coupled with **2** to offer the protective pseudopeptide intermediates *N*-Boc-carboline-3-carboxyl Ala-Arg(Tos)-Pro-Ala-Lys(ClZ)-OBzl (**16**, in 84% yield), N-Boc-carboline-3-carboxyl Gly-Arg(Tos)-Pro-Ala-Lys(ClZ)-OBzl (**17**, in 72% yield) and N-Boc-carboline-3-carboxyl

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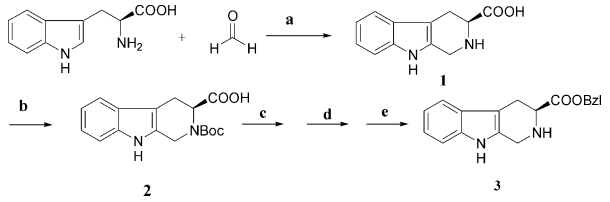
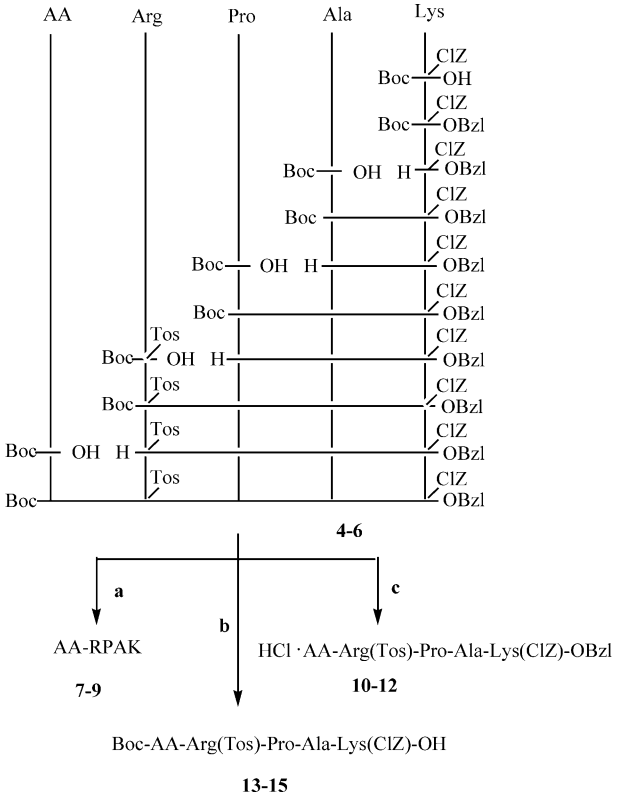
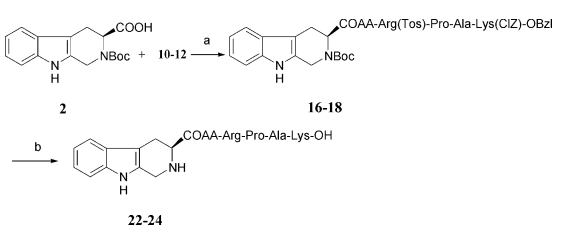
Gln-Arg(Tos)-Pro-Ala-Lys(ClZ)-OBzl (**18**, in 45% yield). **13–15** were coupled with **3** to offer the protective pseudopeptide intermediates Boc-Ala-Arg(Tos)-Pro-Ala-Lys(ClZ)-carboline-3-carboxylic acid benzyl ester (**19**, in 84% yield), Boc-Gly-Arg(Tos)-Pro-Ala-Lys(ClZ)-carboline-3-carboxylic acid benzyl ester (**20** in 72% yield), and Boc-Gln-Arg(Tos)-Pro-Ala-Lys(ClZ)-carboline-3-carboxylic acid benzyl ester (**21**, in 45% yield) (Scheme 2).



Scheme 1. (a) Sulfuric acid; (b) $(Boc)_2O$; (c) $Cs_2CO_3/C_6H_5CH_2Br$; (d) 4 N HCl/ethyl acetate; (e) 5% $NaHCO_3$.



Scheme 2. (a) HF; (b) 2N NaOH; (c) 4N HCl/ethyl acetate; AA represents the corresponding L-Ala, L-Gly, L-Gln, respectively.



Scheme 3. (a) DCC; (b) HF; AA represents the corresponding L-Ala, L-Gly, L-Gln, respectively.

In the presence of HF using the common procedure (Schemes 3 and 4), **16–21** were deprotected and the goal products carboline-3-carboxyl ARPAK (**22**, in 76% yield), carboline-3-carboxyl GRPAK (**23**, in 75% yield), carboline-3-carboxyl QRPAK (**24**, in 58% yield), *N*-ARPAK-carboline-3-carboxylic acid (**25**, in 84% yield), *N*-GRPAK-carboline-3-carboxylic acid (**26**, in 85% yield), and *N*-QRPAK-carboline-3-carboxylic acid (**27**, in 81% yield) were obtained.

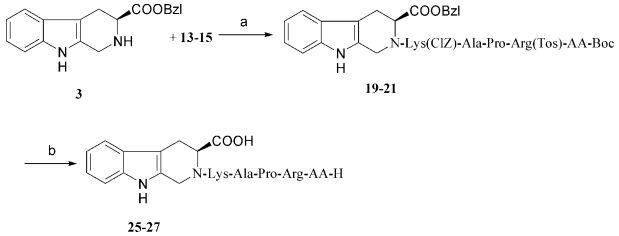
Thrombolytic activities in vivo[5]

Male Wistar rats weighing 200–300 g (purchased from Animal Center of Peking University) were anesthetized with pentobarbital sodium (80.0 mg/kg, ip). The right carotid artery and left jugular vein of the animals were separated. Into a 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 weighed exactly, then put into the middle polyethylene tube. The polyethylene tube was filled with heparin sodium (50 IU/mL of NS) and one end was inserted into the left jugular vein. Heparin sodium was injected via the other end of the polyethylene tube as the anticoagulant, following which the tested compound was injected. The blood was circulated through the polyethylene tube for 90 min, after which the helix was taken out and weighed accurately. The reduction of thrombolytic mass was recorded. The data are listed in Table 1. The statistical analysis of the data was carried out by use of ANOVA test; $p < 0.05$ is considered significant.

Table 1. The reduction of thrombolytic mass ($\overline{X} \pm SD$)

Compd	Dosage (mg/kg)	$\overline{X} \pm SD$ mg
NS	3 mL	15.31 ± 3.57
UK	20,000 Iu	24.10 ± 3.54^{a}
7	5.4	18.84 ± 3.18^{a}
8	5.3	25.90 ± 2.05^{a}
9	6.0	21.28 ± 3.46^{a}
22	7.4	16.13 ± 7.83
23	7.2	19.70 ± 3.70^{a}
24	8.0	17.27 ± 3.57
25	7.4	$26.43 \pm 3.84^{a,b}$
26	7.2	$24.02 \pm 5.21^{a,c}$
27	8.0	$22.88 \pm 9.21^{a,d}$

$N = 9$; dosage = 10 μmol/kg; NS = vehicle; UK = urokinase.
[a]Compared to NS, $p < 0.05$.
[b]Compared to **7** and **22**, $p < 0.05$.
[c]Compared to **23**, $p < 0.05$.
[d]Compared to **24**, $p < 0.05$.



Scheme 4. (a) DCC; (b) HF; AA represents the corresponding L-Ala, L-Gly, L-Gln, respectively.

Stability to trypsin in vitro

10 mg of the tested compound were dissolved in 1 mL of phosphate buffer (pH 8). To the solution 0.5 mg of trypsin was added. The reaction mixture was kept at 37 °C and the concentration of the tested compound was monitored every 1 h using HPLC; the mobile phase was 25% of CH_3OH in water containing 0.1% CF_3COOH. The results indicated that the depletion of **22**, **23**, and **24** was observed after the enzyme promotion hydrolysis had proceeded for 5, 8, and 9 h, respectively. On the other hand, however, the concentrations of **25**, **26** and **27** were not changed, even when the enzyme promotion hydrolysis had proceeded for more than 24 h.

Conformational Analysis

Based on the calculated results using Sybyl version 6.4 the conformations of **22**–**27** were analyzed. The conformational analysis indicated that in the used conditions the lowest conformational energy for **22**, **23**, **24**, **25**, **26**, and **27** was 8.853, 10.787, 1.779, 10.640, 9.399, and 1.834 kcal/mol, respectively, under which **23**, **24**, **25** take the stretch conformation and **26**, **27**, **28** exhibited βII′ turn with intramolecular hydrogen bond consisting of the NH of Ala^3 residue and the C=O of the carboline. The exact data are listed in Table 2.

Discussion

Using the usual procedure (*S*)-tetrahydro-β-carboline-3-carboxylic acid (**1**), peptides **7**–**9** and the pseudopeptides **22**–**27** were obtained in good yield. The bioassay of **7**, **8**, **9**, **22**, **23**, **24**, **25**, **26** and **27** in vivo suggested that in general the thrombolytic activities of **7**–**9** were not changed, at least when their C-terminals were amidated by **1**. On the other hand, however, if their N-terminals were acylated by **1** the thrombolytic activities of **7**–**9** were banished completely. In accordance with the conformational analysis using Sybyl version 6.4 it may be clear that after the introduction of **1** into the N-terminal of the peptides the flexibility of the conformations were not improved at all and the stretch conformations were formed. After introduction of **1** into the C-terminal of the peptides the flexibility of the conformations was obviously changed with the formation of rigid βII′ turn in which the hydrogen bond between the NH of Ala^3 residue and the C=O of carboline resulted in the desired conformational rigidity. In the enzyme promotion hydrolysis experiments **25**, **26** and **27** exhibited higher stability to trypsin than **22**, **23** and **24**. Perhaps in the used conditions the stretch conformation is easy to access for trypsin; in contrast the βII′ turn is difficult to access for trypsin. This kind of βII′ turn with free NH_2 of Lys residue as the head may be responsible for the enzymatic stability and the thrombolytic activity. Considering the synthetic difficulty of the corresponding cyclic peptides, this special modification, such as **7**–**9** by introducing **1** at their C-terminals to increase their conformational rigidity, may be another way of accessing the cyclic peptide. The detailed conformational analysis based on the NMR data is proceeding now.

Table 2. Key data for conformation analysis

Compd	Distance between O and H (Å)	The lowest energy (kcal/mol)	Conformation
22	7.504	10.640	Stretch
23	7.614	9.399	Stretch
24	7.579	1.834	Stretch
25	2.064	8.853	βII′ turn
26	1.938	10.787	βII′ turn
27	1.857	1.777	βII′ turn

Acknowledgements

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