Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 961–964

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Thrombolytic Activity of Fibrinogen Fragment Related Cyclopeptides

Ming Zhao, Na Lin, Chao Wang and Shiqi Peng*

College of Pharmaceutical Sciences, Peking University, Beijing 100083, PR China

Received 9 October 2002; accepted 3 December 2002

Abstract—In the modification of the fibrinogen fragment related sequences ARPAK, QRPAK GRPAK and KRPAK, the corresponding cyclo-ARPAK, cyclo-QRPAK, cyclo-GRPAK, and cyclo-KRPAK were prepared in the diluted solution. The bioassay in vivo indicated that the thrombolytic potencies of cyclo-ARPAK, cyclo-GRPAK, cyclo-QRPAK, and cyclo-KRPAK were significantly higher than that of ARPAK, QRPAK, GRPAK, and KRPAK. In water, the cyclopeptides were incubated with pepsin or trypsin at 37 °C for 64 h. There was no degradation product observed, on the other hand, with the same condition, the peptides were completely hydrolyzed in 8 h. The relationships among the rigidity or the conformation and the thrombolytic activity in vivo and the stability to enzyme-induced hydrolysis in vitro of the cyclopeptides were discussed.
© 2003 Published by Elsevier Science Ltd.

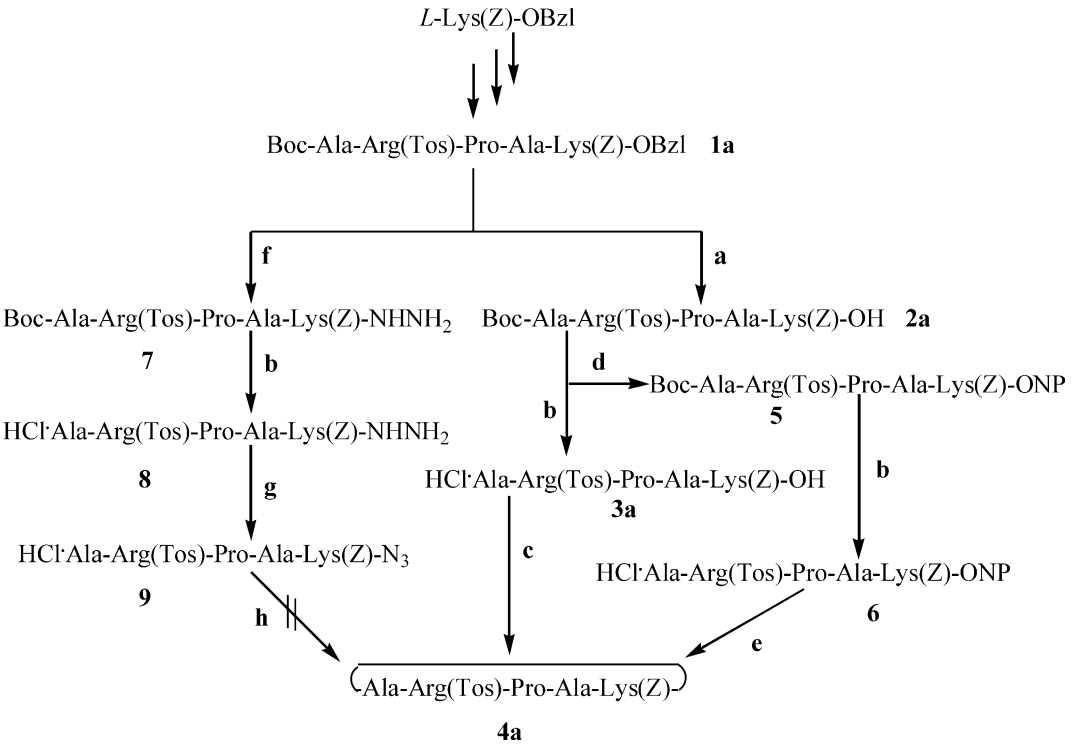
In the metabolism studies in vitro, it was found that when ARPAK, the fragment from fibrinogen, and its derivatives QRPAK and GRPAK, were incubated with pepsin or trypsin in water at 37 °C for 8 h, complete degradation was observed. Comparing to the unmodified peptide, the introduction of 3-(*S*)-1,2,3,4-tetrahydrocarboline- 3-carboxylic acid into their C-terminal improved the thrombolytic potency and the stability to pepsin or trypsin hydrolysis for *N*-ARPAK-, *N*-GRPAK-, and *N*-QRPAK-3-(*S*)-1,2,3,4-tetra- hydro-*beta*-carboline-3-carboxylic acid.[1] The results from the bioassay in vitro, in vivo, and the conformation analysis with discover program particularly by Sybyl version 6.4 suggested that the increase of the conformation rigidity of ARPAK, GRPAK, QRPAK and related sequences, resulted from the introduction of 3-(*S*)-1,2,3,4-tetrahydro-*beta*-carboline-3-carboxylic acid, was probably responsible for the enhanced potency in vitro and in vivo as mentioned above.[1–3] As the further modification aimed at the increase of the conformation rigidity, the corresponding cyclo-ARPAK, cyclo-GRPAK, cyclo-QRPAK and cyclo-QRPAK were prepared and their stability to enzyme-induced hydrolysis in vitro, the thrombolytic potency in vivo and the conformation were observed.

Chemistry

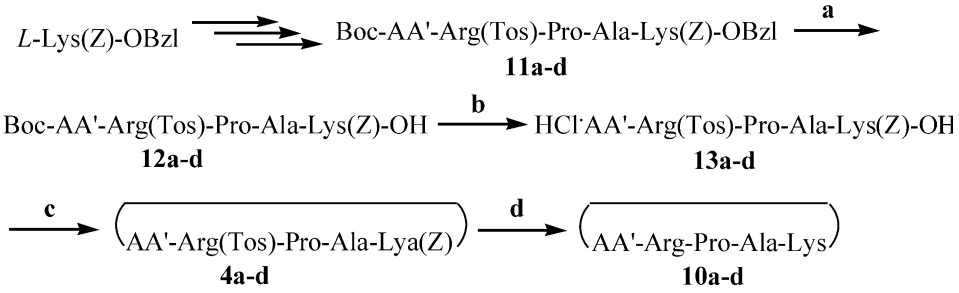
The protected intermediates (**1a–d** and **11a–d**) were prepared via the solution method mentioned in the literature.[4] The stepwise synthesis (from C-terminal to N-terminal, in 83–96% yield) was carried out starting with L-Lys(*Z*)-OBzl and L-Ala-OBzl, respectively. According to Scheme 1, by use of the suitable procedures, **1a** was converted into HCl·Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-N_3 (**9**) via HCl·Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-NH_2NH_2 in 70% total yield, into HCl·Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-ONP (**6**) via Boc-Ala-Arg (Tos)-Pro-Ala-Lys(Z)-OH in 76% total yield, and into HCl·Ala-Arg(Tos)-Pro-Ala-Lys(Z)-OH (**3a**) in 80% yield. The cyclization of **9** in the presence of DIEA, of **3a** in the presence of TBTU/HOBT/NMM, HBTU/HOBt/NMM, BOP/HOBt/NMM, or BOP/DIEA/NMM gave no desirable product, cyclo-Ala- Arg(Tos)-Pro-Ala-Lys(*Z*) (**4a**). Cyclization of **3a** in the presence of DCC/NMM or **6** in the presence of NMM afforded **4a** in 18 and 5% yield, respectively. Thus, the cyclization of the diluted solution of the protective peptides (**3a–d**, 10^{-3} mol/L) was carried out by use of the routes described in Schemes 2 and 3. The yields of **4a–d** were

*Corresponding author. Tel.: +86-10-6209-2482; fax: +86-10-6209-2311; e-mail: sqpeng@mail.bjmu.edu.cn

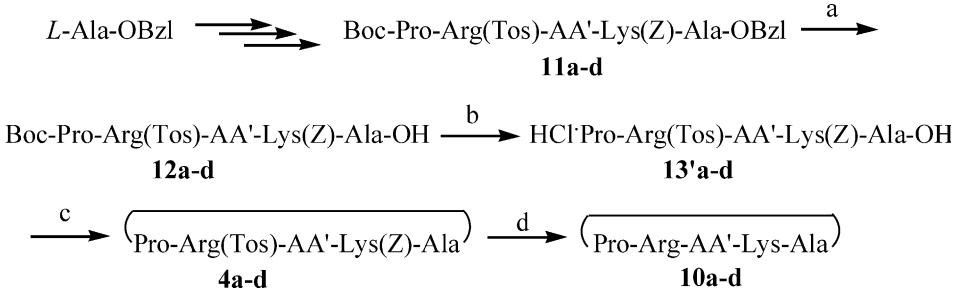
0960-894X/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
doi:10.1016/S0960-894X(02)01072-7



Scheme 1. Selection of the reaction conditions for cyclization: (a) 2 mol/L of NaOH; (b) 4 mol/L of HCl in EtOAc; (c) TBTU/HOBt/NMM, HTBU/HOBt/NMM, BOP/HOBt/NMM, BOP/DIEA/NMM, DCC/NMM; (d) *p*-NO_2-phenol/DCC; (e) NMM; (f) $NH_2NH_2{\cdot}H_2O$; (g) $HCl/NaNO_2$; (h) DIEA.



Scheme 2. Preparation of cyclo-AA′-Arg-Pro-Ala-Lys from Boc-AA′-Arg(Tos)-Pro-Ala-Lys(*Z*)-OBzl: (a) 2 mol/L of NaOH; (b) 4 mol/L of HCl in EtOAc; (c) DCC/NMM; (d) HF; for **4a** AA' = Ala, for **4b** AA′ = Gln, for **4c** AA′ = Gly, for **4d** AA′ = Lys, for **10a** AA = Ala, for **10b** AA = Gln, for **10c** AA = Gly, for **10d** AA = Lys.



Scheme 3. Preparation of cyclo-Pro-Arg-AA′-Lys-Ala from Boc-Pro-Arg(Tos)-AA′-Lys(*Z*)-Ala-OBzl: (a) 2 mol/L of NaOH; (b) 4 mol/L of HCl in EtOAc; (c) DCC/NMM; (d) HF; for **4a** AA′ = Ala, for **4b** AA′ = Gln, for **4c** AA′ = Gly, for **4d** AA′ = Lys, for **10a** AA = Ala,for **10b** AA = Gln, for **10c** AA = Gly, for **10d** AA = Lys.

listed in Table 1. In the presence of HF, **4a–d** were converted into the target cyclopeptides, **10a–d**, in 53, 64, 82 and 80% yield, respectively.

Stability to pepsin or 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 pepsin or trypsin were added. The reaction mixture was kept at 37 °C and the concentration of the tested compound was monitored every 1 h by HPLC, the mobile phase is 25% of CH_3OH in water containing 0.1% of CF_3COOH. Results indicated that the depletion of ARPAK (**14**), QRPAK (**15**), GRPAK (**16**), and KRPAK (**17**) was observed after the enzyme promotion hydrolysis was proceeded for 8 h. On the other hand, however, the concentrations of **10a**, **10b**, **10c** and **10d** were not changed at all even the enzyme promotion hydrolysis was proceeded for 64 h. The related data are listed in Table 2.

Table 1. Yield in cyclization[a]

Material	Condensation agents	Product	Yield (%)
3a	TBTU, HOBt, NMM	—	—
3a	TBTU, HOBt, NMM	—	—
3a	BOP, HOBt, NMM	—	—
3a	BOP, DIEA, NMM	—	—
3a	DCC, NMM	**4a**	18
6	NMM	**4a**	5
9	DIEA	—	—
3b	DCC, NMM	**4b**	15
3c	DCC, NMM	**4c**	31
3d	DCC, NMM	**4d**	10
13a	DCC, NMM	**4a**	9
13b	DCC, NMM	**4b**	14
13c	DCC, NMM	**4c**	29
13d	DCC, NMM	**4d**	10

[a](1) Solvent = anhydrous DMF; (2) concentration of the material was 10^{-3} mol/L; (3) deprotection of **4a–d** with HF gave **10a–d** in 53, 61, 64 and 60% yield, respectively; (4) there is no significant difference of the yield was observed between the cyclization **13a–d** and **13′a–d**.

Table 2. Stability of the peptides to enzyme promoting hydrolysis

Compd	Incubation time (h)	%Degradation	
		In pepsin	In trypsin
10a	64	0	0
10a	84	40	38
10b	64	0	0
10b	84	25	20
10c	64	0	0
10c	84	10	20
10d	64	0	0
10d	84	30	35
14	1	10	79
14	2	50	95
14	8	98	99
15	1	45	20
15	2	78	72
15	8	92	95
16	1	56	25
16	2	75	77
16	8	93	98
17	1	50	30
17	2	80	70
17	8	95	96

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 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 accurately, which was 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, followed by the injection of the tested compound. The blood was circulated through the polyethylene tube for 90 min, after which the helix was taken out and weighted accurately. The reduction of thrombus mass was recorded. The data are listed in Table 3. The statistical analysis of the date was carried out by use of ANOVA test, $p < 0.05$ is considered significant.

Conformational Analysis

Based on the calculated results by Sybyl version 6.4, the conformations of **10a–d** were analyzed. The conform-

Table 3. The thrombolytic activity of the peptides

Compd	Dosage (mg)	$\overline{X} \pm$SD mg
NS	3 mL	0.76 ± 2.40
UK	20,000 IU	12.81 ± 3.15[a]
14	5.0	6.07 ± 2.14[a]
10a	5.0	10.62 ± 3.10[a,b]
15	5.0	7.02 ± 2.25[a]
10b	5.0	9.68 ± 2.20[a,c]
16	5.0	9.31 ± 2.14[a]
16	10.0	13.17 ± 3.13[a]
16	20.0	16.81 ± 3.40[a,e]
10c	5.0	11.86 ± 2.16[a,c]
10c	10.0	17.31 ± 2.39[a,f]
10c	20.0	18.88 ± 2.08[a,g]
17	5.0	0.28 ± 2.13
10d	5.0	6.13 ± 2.31[a,d]

$N = 8$, NS = vehicle
[a]Comparing to NS, $p < 0.001$.
[b]Comparing to **14**, $p < 0.01$.
[c]Comparing to **15** or **16**, $p < 0.05$.
[d]Comparing to **17**, $p < 0.001$.
[e]Comparing to UK, $p < 0.05$.
[f]Comparing to UK, $p < 0.01$.
[g]Comparing to UK, $p < 0.001$.

Table 4. Key data for conformation analysis

Compd	Distance between O and H (Arg)	The lowest energy (kcal/mol)	Conformation
10a	2.004	0.619	*Beta* II′ turn
10b	1.930	−2.610	*Beta* II′ turn
10c	1.619	−8.317	*Beta* II′ turn
10d	2.550	5.219	*Beta* II′ turn

ation analysis indicated that at the used conditions, the lowest conformation energy for **10a–d** was 0.619, −2.610, −5.20, and 5.219 kcal/mol, respectively, under which **10a–d** exhibited beta II′ turn with intramolecular hydrogen bond consisted of the alpha NH of Lys^1 residue and the C=O of Arg^4. The exact data were listed in Table 4.

Discussion

The stepwise synthesis (from C-terminal to N-terminal) and the related procedures starting from L-Lys(Z)-OBzl or L-Ala-OBzl, may be provide all of the desirable protective intermediates in good yields. Using the dilute solution (10^{-3} mmol/L) of HCl·Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-N_3, HCl·Ala-Arg(Tos)-Pro-Ala- Lys(*Z*)-ONP and HCl·Ala-Arg(Tos)-Pro-Ala- Lys(*Z*)-OH in DMF, the cyclization resulted in different yield and only the cyclization for HCl·Ala-Arg(Tos)-Pro-Ala-Lys(*Z*)-OH gave acceptable yield. On the other hand, however, when the cyclization was carried out in the presence of TBTU/HOBT/NMM, HBTU/HOBt/NMM, BOP/HOBt/NMM, BOP/DIEA/NMM, or DCC/NMM, only the latter gave acceptable yields for all of the protective cyclopeptides. The solutions of cyclo-ARPAK, cyclo-GRPAK, cyclo-QRPAK, or cyclo-KRPAK in phosphate buffer (pH 8) showed excellent enzymatic stability. At 37 °C in the presence of pepsin or trypsin, the solutions were kept even for 64 h not any degradation product was observed.

The bioassay of **14–17** and **10a–d** in vivo suggested that in general the thrombolytic activities of **14–17** were enhanced. In accordance with the conformation analysis using of Sybyl version 6.4, it may be clear that the flexibility of the fibrinogen fragment related peptides were significantly reduced after the cyclization. The *beta* II′ turn resulted from the intramolecular hydrogen bond consisted of the alpha NH of Lys^1 residue and the C=O of Arg^4 resulted not only the increase of the conformation rigidity, but also the enhance of the stability of the enzyme promotion hydrolysis and the thrombolytic potency.

Acknowledgements

The author Shiqi Peng wishes to thank the key Basic Research Project (G1998051111) of China for financial support.

References and Notes

1. Wu, Y.; Zhao, M.; Wang, C.; Peng, S. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 2331.
2. Zhao, M.; Peng, S. *J. Prakt. Chem* **1999**, 668.
3. Zhao, M.; Zhang, L.; Yu, T.; Peng, S. *Chinese J. Med. Chem. 6,* 125*; Chem. Abstr*. **1999**, *124*, 290228y.
4. Lin, N.; Zhao, M.; Wang, C.; Peng, S. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 585.
5. Lin, N.; Yang, J.; Wang, C.; Zhao, M.; Peng, S.; Song, D.; Wong, J. *J. Beijing Med. Uni*. **2000**, *32*, 283.