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Synthesis and Antithrombotic Activity of Carbolinecarboxyl RGD Sequence

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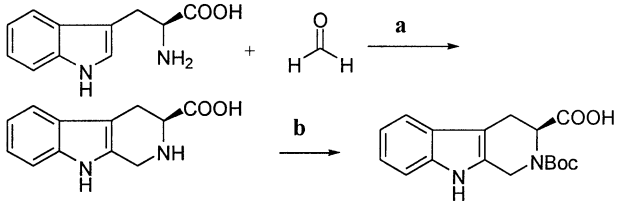
Abstract—3*S*-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid, RGDS, RGDV, RGDF and their linkers were synthesized. The anti-aggregation and adhesion of platelet indicated that the in vitro activities of the linkers remained at the same level as RGDS, RGDV, and RGDF ($p > 0.05$). The antithrombotic activities in vivo suggested, however, that the potencies of RGDS, RGDV and RGDF were enhanced by the introduction of 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxyl group into their alpha amino group ($p < 0.05$, 0.01 or 0.001).

In our previous papers, RGDS, RGDV and RGDF were used as the building blocks in the modification of the oligopeptides with antithrombotic and/or thrombolytic activity.[1,2] The results obtained indicated that the contribution of RGD sequence to the specific interaction of RGDS, RGDV and RGDF with GP II b/ III a receptor may result in the targeting ability for RGD-containing compounds.[3] 3*S*-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid isolated from A.Chinese G.Don is anti-aggregation active.[4] According to the HPLC analysis, it was understood that RGDS was degraded by amino peptidase from its N-terminal in the plasma of animals.[5] In the design of the targeting antithrombotic agents, 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid was linked with RGDS, RGDV and RGDF, respectively. By this kind of modification, we hope that with the targeting action of RGD sequence 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid may distribute to the thrombus forming area and with 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid blocking the N-terminal of RGDS, RGDV and RGDF may inhibit their amino peptidase based degradation.

Chemistry

The preparation of compounds **1** and **2** was carried out as outlined in Scheme 1. In the presence of sulfuric acid and water the Pictet–Spengler condensation of L-tryptophane and formaldehyde gave 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**1**)[4] in 100% yield. In the acylation **1** was treated with Boc-N_3 and the Boc group was introduced into the N_2 of **1** giving 3*S*-2-Boc-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**2**) in 50% yield.

The protective tetrapeptide intermediates (**3**–**5**) were prepared via the solution method according to the route depicted in Scheme 2. The stepwise synthesis (C→N in 83–96% yield) was carried out starting with benzyl ester of L-Ser (Bzl), L-Val and L-Phe as the C-terminal residue, respectively. Using the normal deprotective procedure **3**–**5** were treated with HF and **6**–**8**, RGDS (**6**, in 63% yield), RGDV (**7**, in 85% yield) and RGDF (**8**, in 83% yield),[3] were obtained. After the removal of Boc group of protective tetrapeptides **3**–**5** the corresponding N-terminal free protective tetrapeptides **9**–**11** were obtained in theoretical yield.



1 **2**

Scheme 1. (a) Sulfuric acid; (b) Boc-N_3.

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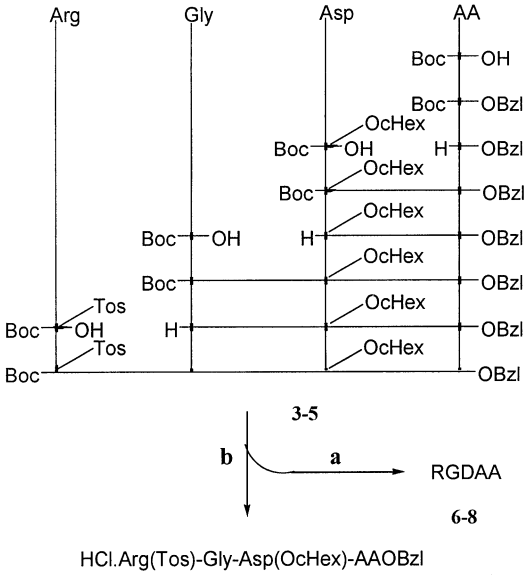


Scheme 2. (a) HF; (b) 4 N hydrochloride/ethyl acetate; AA represents the corresponding L-Ser, L-Val and L-Phe, respectively.

Table 1. Effect of the compounds on the platelet aggregation induced by ADP (10^{-5} mol/L)

Compd	Am % ($\bar{X}\pm$SD), at the dose of:		
	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS	56.72±5.15		
1	53.66±2.41	45.16±4.78[a]	24.60±1.99[a]
6	52.85±2.46	40.54±3.18[a]	14.22±2.49[a]
7	51.16±4.25[b]	29.40±2.95[a]	9.80±1.98[a]
8	35.70±2.14[a]	22.11±3.16[a]	7.36±1.10[a]
15	50.16±3.44[b]	43.05±3.90[a]	16.11±2.05[a,c]
16	50.20±4.01[b]	26.01±2.45[a,c]	10.75±1.56[a,c]
17	36.05±3.00[a,c]	21.05±3.06[a,c]	9.01±2.15[a,c]

$N=8$; NS, vehicle.
[a]Compared to NS, $p<0.001$.
[b]Compared to NS, $p<0.01$.
[c]Compared to **1**, $p<0.001$.

With the usual coupling procedure as indicated in Scheme 3, the N-terminal free protective tetrapeptides **9**–**11** were acylated by **2** to give 3*S*-2-Boc-1,2,3,4-tetrahydro - β - carboline - 3 - carbsoxyl - Arg(Tos) - Gly - Asp (OcHex)-Ser(Bzl)OBzl (**12**, in 76% yield), -Arg(Tos)-Gly-Asp(OcHex)-ValOBzl (**13**, in 72% yield) and -Arg(Tos)-Gly-Asp(OcHex)-PheOBzl (**14**, in 73% yield). After removal of all the protecting groups in compounds **12**–**14** the desirable products **15**–**17**, 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxyl-RGDS (**15**, in 63% yield), -RGDV (**16**, in 68% yield), and -RGDF (**17**, in 68% yield) were obtained.

Anti-platelet aggregation in vitro

Platelet-rich plasma was prepared by centrifugation of normal rabbit blood anticoagulation with sodium citrate at a final concentration of 3.8%. The platelet counts were adjusted to 2×10^5/μl by addition of autologous plasma. Platelet aggregation studies were conducted in an aggregometer using the standard turbidimetric technique. The agonists used were platelet activating factor (PAF, final concentration 10^{-7} mol/L) and adenosine diphosphate (ADP, final concentration 10^{-5} mol/L). The effects of compounds **1**, **6**, **7**, **8**, **15**, **16**, and **17** on PAF or ADP induced platelet aggregation were observed. The maximal rate of platelet aggregation (Am%) was represented by the peak height of aggregation curve. The data are listed in Tables 1 and 2 and the statistical analysis of the data was carried out by use of ANOVA test; $p<0.05$ is considered significant.

Table 2. Effect of the compounds on the platelet aggregation induced by PAF (10^{-7}mol/L)

Compd	Am% ($\bar{X}\pm$SD) , at the dose of:		
	10^{-7} mol/L	10^{-6} mol/L	10^{-5} mol/L
NS	58.90±4.65		
1	55.15±3.16	50.18±3.49	35.66±2.46[a]
6	57.20±3.90	36.15±3.45[a]	21.16±2.55[a]
7	54.36±4.11	34.17±2.99[a]	19.56±1.98[a]
8	39.18±2.55[a]	23.80±2.34[a]	7.81±1.85[a]
15	56.10±3.25	34.15±3.18[a,c]	28.07±2.58[a,c]
16	54.10±3.90[b]	35.05±3.12[a,c]	24.11±2.35[a,c]
17	36.05±3.60[a,c]	25.54±2.80[a,c]	20.05±2.41[a,c]

$N=8$; NS = vehicle.
[a]Compared to NS, $p<0.001$.
[b]Compared to NS, $p<0.01$.
[c]Compared to **1**, $p<0.001$.

Table 3. Effect of the compounds on the adhesion of SACC-LM and platelets

Compd	%, $\bar{X}\pm$SD
NS	78.3±4.0
1	45.6±2.4[a]
6	35.8±2.1[a]
7	32.1±1.9[a]
8	29.7±1.9[a]
15	36.1±1.4[a,b]
16	30.2±2.4[a,b]
17	28.1±1.6[a,b]

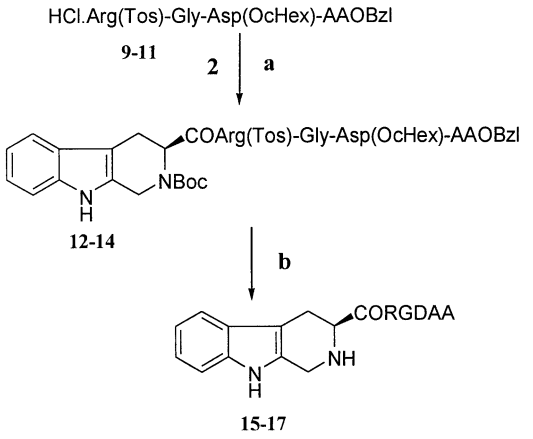
$N=8$; dosage, 5 mg/mL; NS = vehicle.
[a]Compared to NS, $p<0.001$.
[b]Compared to **1**, $p<0.001$.

Anti-adhesion in vitro

Effects of compounds **1**, **6**, **7**, **8**, **15**, **16**, and **17** on the adhesion of highly pulmonary metastatic SACC cell line SACC-LM and platelets were tested according to the literature.[6] The data are listed in Table 3.

Antithrombotic in vivo

Male Wistar rats weighing 250–300 g (purchased from Animal Center of Peking University) were used. The tested compounds were dissolved in NS just before use and kept in an ice bath. The rats were anesthetized with pentobarbital sodium (80.0 mg/kg, ip), and the right carotid artery and left jugular vein were separated. A 6-cm thread with exact weight was put into the middle of the polyethylene tube. The polyethylene tube was filled with heparin sodium (50 IU/mL of NS) and one end was inserted into the left jugular vein. From the other end of



Scheme 3. (a) DCC; (b) HF.

Table 4. Effect of the compounds on the thrombus weight ($\bar{X} \pm SD$)

Compd	Wet thrombus (mg)	Dry thrombus (mg)
NS	45.92 ± 6.23	8.95 ± 1.97
1	42.99 ± 3.80	7.17 ± 0.70^{a}
6	43.61 ± 5.78	7.68 ± 2.40
7	34.62 ± 3.20^{b}	6.03 ± 1.06^{b}
8	26.61 ± 3.44^{a}	4.14 ± 0.95^{a}
15	$28.09 \pm 4.42^{a,c}$	$4.98 \pm 1.21^{b,d}$
16	$29.49 \pm 4.30^{b,e}$	$4.87 \pm 1.04^{b,e}$
17	$22.30 \pm 2.43^{a,f}$	$2.76 \pm 0.45^{a,g}$

$N = 8$; dosage, 5 μmol/kg; NS = vehicle.
[a]Compared to NS (3 mL/kg), $p < 0.001$.
[b]Compared to NS (3 mL/kg), $p < 0.01$.
[c]Compared to **6**, $p < 0.01$.
[d]Compared to **6**, $p < 0.05$.
[e]Compared to **7**, $p < 0.05$.
[f]Compared to **8**, $p < 0.001$.
[g]Compared to **8**, $p < 0.01$.

the polyethylene tube heparin sodium was injected as anticoagulant, then the tested compounds were injected, when this end was inserted into the right carotid artery. In the case the tube was full of NS or tested compound containing NS. The blood was flowed from the right carotid artery to the left jugular vein via the polyethylene tube for 15 min. The thread was taken out and weighed and the weight of the wet thrombus was recorded. The thread was kept in a desiccator for 2 weeks and the weight of the dry thrombus was recorded. The data are listed in Table 4. The statistical analysis of the data was also carried out by use of ANOVA test; $p < 0.05$ is considered significant.

Discussion

By use of the usual procedure 3*S*-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**1**), RGD containing tetrapeptides (**6**, **7**, **8**) and the linkers of them, **15**, **16**, and **17**, were obtained successfully. The bioassay of **15**, **16**, and **17** in vitro suggested that when RGDS (**6**), RGDV (**7**), or RGDF (**8**) was amidated with **1** the potency of anti-aggregation and adhesion of platelets of **6**, **7**, and **8** remained at the same level as indicated in Tables 1–3 (compared to NS, $p < 0.001$; compared to **6**, **7**, and **8**, $p > 0.05$). The bioassay in vivo indicated, however, that antithrombotic activities of the linkers were obviously enhanced. The potency of **15**, **16**, or **17** was significantly higher than that of NS ($p < 0.001$, or 0.01), **1** ($p < 0.001$), **6** ($p < 0.05$ or 0.01), **7** ($p < 0.05$) and **8** ($p < 0.001$ or 0.01), respectively (Table 4). All of the results from the bioassay in vitro and in vivo showed that modification of **1**, **6**, **7**, and **8** by linking the carboxyl group of **1** and the amino group of **6**, **7**, and **8** was a successful way to find the related lead compounds.

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