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SHORT COMMUNICATION

SYNTHESIS AND BIOLOGICAL ACTIVITY OF DIPEPTIDES CONTAINING S-HEXYLCYSTEINE

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Pathological clot formation is the major cause of many serious diseases. A crucial step at the end of the coagulation cascade is the cleavage of soluble fibrinogen to fibrin clot by thrombin. A number of anticoagulants have been assessed clinically. Side effects such as excessive bleeding and difficulties of drug monitoring have led to considerable activity in the search of novel compounds with anticoagulant activity (1,2). During our earlier investigations on antifibrinolytic activity of S-substituted cysteine derivatives, we observed that some of ε-amino-caproil-(S-alkyl)-cysteines in the high concentration (20 mM), instead of the expected prolongation of fibrinolysis time in fibrinolytic test, prevented clot formation. ε-Aminocaproil-(S-hexyl)-cysteine was the most active compound (3). We observed also that glycyl-(S-hexyl)-cysteine shows similar activity in the range of the examined concentration (4). Anticoagulant or fibrinolytic activity of S-substituted cysteine derivatives was not earlier described. The garlic products (γ-glutamyl-S-alkylcysteines) can be an exception (5).

In order to explain this unexpected activity of S-hexyl- L-cysteine derivatives and in the search for new compounds with anticoagulant activity, we obtained eight dipeptides with general formula X-Gly-(S-hexyl)-L-Cys-Y and X- S-hexyl)-L-Cys-Gly-Y, where: X= H, Boc; Y= OH, OMe or OEt and examined their effect on: thrombin-clotting activity determined with the use of fibrinogen and plasma, fibrinolytic activity of plasmin and amidolytic activities of thrombin and plasmin.

EXPERIMENTAL

Synthesis

General

Boc-Gly-OH (IRIS Biotech), S-hexyl-L-Cys-OH was obtained according to the literature (5) and transformed into Boc- or ester derivative with the use of standard methods. Ethyl chloroformate and triethylamine were from Merck. Organic solutions were dried over anhydrous $MgSO_4$. Reactions were monitored and the homogeneity of the products was examined using silica gel plates (Kieselgel 60 F_{254}, Merck) and the following systems: 1. benzene/methanol/acetic acid (12:5:1, v/v/v); 2. ethanol/water/25% ammonia solution (18:0.5:0.5, v/v/v); 3. chloroform/methanol/water (3:3:0.5, v/v/v). Spots were visualized with tolidine/chlorine or iodine and ninhydrin. The melting points were determined on Boetius heating block and are uncorrected. The specific optical rotations were measured with a polarimeter (Optical Activity LTD AA-10R). ^{1}H NMR spectra were recorded with 200 MHz Bruker AC 200F spectrometer. Elemental analyses were performed on Perkin-Elmer analyzer and the results, indicated by symbols C, H, N, were within ± 0,4% of the theoretical values.

Synthesis of dipeptides

The protected dipeptides were obtained with the use of mixed anhydrides method (ethyl chloroformate and triethylamine). Boc group was removed with HCl/anhydrous methanol (ether) and ester

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Table 1. Analytical data of protected dipeptides.

Compound	Yield (%)	Molecular weight	R_f	m.p. (°C)	$[\alpha]_D^{20}$	IR cm^{-1}	^1H NMR (DMSO-d_6) [ppm.]
Boc-L-Cys-Gly-OEt | S-C_6H_{13}	82,3	390,54 ($C_{18}H_{34}0_5N_2S$)	$0,73_{(1)}$ $0,56_{(2)}$	54-56	-39,81 (c=1,MeOH)	3328, 1752, 1688, 1652, 1532	8.3 (t, 1H, NH), 6.87(d, 1H, NH), 4.03-4.14 (m, 3H, Cys CH^{α}, ethyl ester CH_2), 3.8-3.84 (m, 2H, Gly CH_2), 2.48-2.87 (m, 4H, Cys CH_2, hexyl S-CH_2) 1.15-1.5 (m, 20H, ethyl ester CH_3, hexyl 4×CH_2, Boc 3×CH_3), 0.86 (t, 3H, hexyl CH_3).
Boc-Gly-L-Cys-OMe | S-C_6H_{13}	71,5	376,51 ($C_{17}H_{32}0_5N_2S$)	$0,65_{(1)}$ $0,87_{(2)}$	oil	-28,67 (c=1,MeOH)	3320, 1728, 1688, 1656, 1548	8.18 (d, 1H, NH), 6.87 (t, 1H, NH), 4.45-4.56 (m, 1H, Cys CH^{α}), 3.65 (s, 3H, methyl ester), 3.58-3.61 (m, 2H, Gly CH_2), 2.46-2.96 (m, 4H, Cys CH_2, hexyl S-CH_2), 1.14-1.53 (m, 17H, hexyl 4×CH_2, Boc 3×CH_3), 0.86 (t, 3H, hexyl CH_3).
Boc-L-Cys-Gly-OH | S-C_6H_{13}	63,4	362,48 ($C_{16}H_{30}0_5N_2S$)	$0,61_{(1)}$ $0,48_{(2)}$	184-185	-9,33 (c=1,MeOH)	3304, 1696, 1652, 1560	7.72 (t, 1H, NH), 7.04 (d, 1H, NH), 4.0-4.22 (m, 1H, Cys CH^{α}), 3.43-3.64 (m, 2H, GlyCH_2),2.46-2.91 (m, 4H, Cys CH_2, hexyl S-CH_2), 1.26-1.48(m, 17H, hexyl 4×CH_2, Boc 3×CH_3), 0.86 (t, 3H, hexyl CH_3).
Boc-Gly-L-Cys-OH | S-C_6H_{13}	40,5	362,48 ($C_{16}H_{30}0_5N_2S$)	$0,38_{(1)}$ $0,45_{(2)}$	amorph.	-18,67 (c=1,MeOH)	3330, 1693, 1650, 1530	7.75 (d, 1H, NH), 7.03 (t, 1H, NH), 4.23-4.26 (m, 1H, Cys CH^{α}), 3.54-3.56 (m, 2H, Gly CH_2), 2.43-2.96 (m, 4H, Cys CH_2, hexyl S-CH_2), 1.12-1.47 (m, 17H, hexyl 4×CH_2, Boc 3×CH_3), 0.85 (t, 3H, hexyl CH_3).

Table 2. Analytical data of dipeptide esters and dipeptides.

Compound	Yield (%)	Molecular weight	R_f	m.p. (°C)	$[\alpha]_D^{20}$	IR cm^{-1}
HCl·H-L-Cys-Gly-OEt | S-C_6H_{13}	54,4	362,88 ($C_{13}H_{27}0_3N_2SCl$)	$0,32_{(1)}$ $0,53_{(2)}$	oil	-6,21 (c = 1, MeOH)	3250, 1740, 1650, 1550
HCl·H-Gly-L-Cys-OMe | S-C_6H_{13}	42.4	312,85 ($C_{12}H_{27}0_3N_2SCl$)	$0,25_{(1)}$ $0,61_{(2)}$	oil	-7,21 (c = 1, MeOH)	3200, 1730, 1645, 1540
HCl·H-L-Cys-Gly-OH | S-C_6H_{13}	50,2	298,83 ($C_{11}H_{23}0_3N_2SCl$)	$0,15_{(1)}$ $0,38_{(3)}$	oil	+3,12 (c = 1, MeOH)	3200, 1645, 1535
HCl·H-Gly-L-Cys-OH | S-C_6H_{13}	38,5	298,83 ($C_{11}H_{23}0_3N_2SCl$)	$0,13_{(1)}$ $0,38_{(3)}$	amorph.	+5,43 (c = 1, MeOH)	3180, 1645, 1540

Table 3. Effect of S-hexylcysteine derivatives on clotting time and time of fibrinolysis.

Compound	Concentration of compound (mM)	Thrombin clotting time (s)		Time of fibrinolysis (s)
		Fibrinogen	Plasma	
Boc-L-Cys-Gly-OEt | S-C_6H_{13}	0,2 2,0 20,0	35 ± 0,18 60 ± 0,30 75 ± 0,40	15 ± 0,08 21 ± 0,11 60 ± 0,21	795 ± 5,56 700 ± 3,54 *
Boc-Gly-L-Cys-OMe | S-C_6H_{13}	0,2 2,0 20,0	36 ± 0,14 75 ± 0,32 252 ± 0,97	13 ± 0,06 15 ± 0,0,7 58 ± 0,25	886 ± 3,54 615 ± 4,30 *
Boc-L-Cys-Gly-OH | S-C_6H_{13}	0,2 2,0 20,0	34 ± 0,13 108 ± 0,38 >3600	12 ± 0,05 20 ± 0,12 112 ± 0,39	633 ± 4,43 * *
Boc-Gly-L-Cys-OH | S-C_6H_{13}	0,2 2,0 20,0	38 ± 0,17 1290 ± 5,6 >3600	10 ± 0,01 12 ± 0,03 265 ± 0,98	675 ± 4,72 * *
HCl·H-L-Cys-Gly-OEt | S-C_6H_{13}	0,2 2,0 20,0	58 ± 0,27 110 ± 0,32 > 3600	10 ± 0,01 12 ± 0,03 138 ± 0,42	932 ± 6,25 349 ± 2,41 *
HCl·H-Gly-L-Cys-OMe | S-C_6H_{13}	0,2 2,0 20,0	37 ± 0,15 49 ± 0,21 > 3600	9 ± 0,01 14 ± 0,04 145 ± 0,51	930 ± 6,27 449 ± 3,12 *
HCl·H-L-Cys-Gly-OH | S-C_6H_{13}	0,2 2,0 20,0	18 ± 0,09 228 ± 0,73 > 3600	10 ± 0,01 14 ± 0,04 619 ± 1,7	971 ± 6,71 * *
HCl·H-Gly-L-Cys-OH | S-C_6H_{13}	0,2 2,0 20,0	17 ± 0,08 33 ± 0,14 208 ± 0,41	13 ± 0,03 15 ± 0,06 191 ± 0,36	970 ± 6,70 625 ± 4,33 *
Control NaCl	15	16 ± 0,08	11 ± 0,01	961 ± 6,72

* clot was not formed

groups by alkaline hydrolysis. Analytical data of the compounds are given in Table 1 and 2.

Enzymatic investigations

General

Plasmin, S-2251 (H-D-Val-L-Leu-L-Lys-pNA × 2 HCl) and S-2238 (H-D-Phe-Pip-Arg-pNA × 2 HCl) (Chromogenix), thrombin and bovine fibrinogen (Lubelska Wytwórnia Szczepionek, Lublin, Poland). Platelet-poor plasma was obtained from human blood. Effects of the obtained compounds on clotting and amidolytic activities of thrombin and fibrinolytic and amidolytic activities of plasmin were examined with the use of standard methods. Detailed descriptions of the methods are given below. The results are given in Table 3. Every value represents the average of triplicate determinations ± SD.

Thrombin activity

Clotting activity determined with the use of fibrinogen

All examined substances were dissolved in 0,15 M NaCl solution, other reagents were dissolved in Palitsch buffer pH 7.4.

0.1 cm^3 of examined compounds in the range of concentrations 1-100 mM (in control 0.1 cm^3 of 0.15 M NaCl) was added to 0,3 cm^3 of fibrinogen (0,5%). After 1 min preincubation at 37°C, 0.1 cm^3 of thrombin (10 units/cm^3) was added and clotting time was measured.

Clotting activity determined with the use of plasma

The assay was performed as described above with the addition of 0.3 cm^3 of plasma instead of fibrinogen solution.

Amidolytic activity determined with the use of synthetic substrate S-2238.

0.2 cm^3 of examined compounds in the range of concentrations 1-100 mM (in control 0.2 cm^3 of 0.15 M NaCl) was added to the mixture of 0.1 cm^3 of thrombin solution (5 units/cm^3) and 0.5 cm^3 of Tris buffer (pH 8.4). After preincubation for 3 min at 37°C, 0.2 cm^3 of S-2238 solution (0.75 mM) was added. The mixture was incubated for 15 min at 37°C, then the reaction was stopped by the addition of 0.1 cm^3 of 50% acetic acid and absorbance at 405 nm was measured. The tested compounds in concentrations: 1-100 mM did not influence the thrombin amidolytic activity.

Plasmin activity

Fibrinolytic activity

All examined substances were dissolved in 0,15 M NaCl solution, other reagents were dissolved in Palitsch buffer pH 7.4.

0.1 cm^3 of examined compounds in the range of concentrations 1-100 mM (in control 0.1 cm^3 of 0.15 M NaCl) and 0.1 cm^3 of fibrinogen (0.5%) was added to 0.1 cm^3 of thrombin (40 units/cm^3). After clot formation, 0.2 cm^3 of plasmin (0.2%) was added and the time of fibrinolysis was measured at 37°C.

Amidolytic activity determined with the use of synthetic substrate S-2251

0.2 cm^3 of examined compounds in the range of concentrations 1-100 mM (in control 0.2 cm^3 of 0.15 M NaCl) was added to the mixture of 0.1 cm^3 plasmin solution (0.05 units/cm^3) and 0.5 cm^3 of Tris buffer (pH 7.4). After preincubation for 3 min at 37°C, 0.2 cm^3 of S-2251 solution (3 mM) was added. The mixture was incubated for 15 min at 37°C, then the reaction was stopped by the addition of 0.1 cm^3 of 50% acetic acid and absorbance at 405 nm was measured. The tested compounds, in the range of concentrations: 1-100 mM, did not influence plasmin amidolytic activity.

RESULTS AND DISSCUSION

All examined compounds prolonged thrombin clotting time. H-Cys(S-hexyl)-Gly-OH was the most active compound. Partially protected dipeptides were also effective inhibitors of thrombin clotting activity. There was no difference, which one from the groups: amino or carboxyl was substituted. The prolongation of the clotting time is lower with the use of plasma as a source of clottable fibrinogen than with the use of purified protein itself. The tested S-hexyl-L-cysteine derivatives inhibited only clotting activity of thrombin. They did not block thrombin's active site, as hydrolysis of synthetic substrate was not inhibited. But it is also rather difficult to suggest that the tested compounds can interact with thrombin exosites because they require peptides with acidic residues (6). The tested dipeptides showed fibrinolytic activity. However, the shortening of fibrinolysis time was observed only at the lower concentrations, at the higher concentrations of the examined compounds clot formation was not observed. The dipeptides did not influence amidolytic activity of plasmin. According to the obtained results, the examined S-hexylcysteine derivatives probably do not effect biological activities of thrombin and plasmin. Their anticlotting activity can be rather connected with interaction with fibrinogen or fibrin monomers but this problem needs further investigations.

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