SYNTHESIS OF ALKYLAMIDES OF DIPEPTIDES AS POTENTIAL PLASMIN INHIBITORS

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Abstract: Four dipeptide alkylamides with general formula H-D-Phe-L-Lys-NH-X, where X= cyclohexyl, -$(CH_2)_5NH_2$, -$(CH_2)_2$-OH and hexyl were obtained. Effect of these compounds on amidolytic and fibrinolytic activity of plasmin was examined.

Keywords: dipeptide alkylamides, plasmin inhibitor

Plasmin, a trypsin like serine protease, is a key enzyme for fibrinolysis. This enzyme plays also an important role in a variety of biological processes like wound healing, tissue repair, cell migration and also in pathological phenomena such as inflammation and tumor cell growth and metastasis. The one of optimal P_1-P_2 specificity for plasmin seems to be Phe-Lys [1]. The next amino acid ($P_1^{'}$ position) is often lysine or serine [2]. ε-Aminocaproic acid and tranexamic acid (trans-aminomethylcyclohexanecarboxylic acid) are clinically used as inhibitors of fibrinolysis. These compounds inhibit fibrinolytic activity of plasmin, but they practically do not influence amidolytic and proteolytic activity of the enzyme. Synthesis of active center directed inhibitor of plasmin is a research field with aim of obtaining an inhibitor of this enzyme to influence not only fibrinolysis but also amidolysis and proteolysis. The inhibitor controlling such plasmin activity will be very useful in defining the physiological and the pathological function of this enzyme and in treatment of plasmin-associated disorders. Some anilides of lysine and short peptides inhibited amidolytic and fibrinolytic activities of plasmin [3] but the heptylamide of L-alanyl-L-phenylalanyl-L-lysine showed only antifibrinolytic activity [4]. Several amides of D-leucyl-L-phenylalanine have been reported as inhibitors of chymotrypsin. Their inhibitory activity is probably a result of dipeptide side chain-side chain hydrophobic interaction [5]. It seems to be interesting whether this kind of derivatives may also inhibit plasmin activity. Previously, we examined activity of benzylamides with general structure H-X-Lys-NH-$CH_2C_6H_5$, where X = L-Phe, D-Phe, L-Leu, D-Leu [6]. Only H-D-Leu-Lys-NH-$CH_2C_6H_5$ inhibited amidolytic activity of plasmin. The lack of antiamidolytic activity of D-phenylalanyl-L-lysine benzylamide was unexpected.

In search of structure activity-relationship of reversible, active center inhibitors of plasmin, we prepared a series of dipeptide alkylamides with general structure: H-D-Phe-Lys-X, where X = -NHC_6H_{11} (cyclohexyl), -$NH(CH_2)_5NH_2$, -$NH(CH_2)_2OH$, -$NH(CH_2)_5CH_3$ (hexyl). Compound **2** contains cadaverine (decarboxylated derivative of lysine) and compound **3** – colamine (decarboxylated derivative of serine). Compound **4**, a hexylamide of D-phenylalanyl-L-lysine, is an analog of compound **2**, with -CH_3 instead of amine group in cadaverine residue.

EXPERIMENTAL

Synthesis
General

Z-D-Phe-OH and Boc-L-Lys(Z)-OH (IRIS Biotech), cyclohexylamine and cadaverine (Aldrich), benzyl chloroformate, hexylamine and colamine (Sigma). N-benzyloxycarbonylcadaverine was obtained by partial cleavage of synthesized earlier N,N'-dibenzyloxycarbonylcadaverine according to the literature method [7]. 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) using 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 Boëtius heating block and are uncorrected. The specific optical rotations were

Table 1. Analytical data of protected alkylamides of lysine

Compound	Yield (%)	Molecular Formula	R_f	m.p. (°C)	$[\alpha]_D^{20}$	¹H NMR (DMSO-d₆) [ppm]
Boc-L-Lys(Z)-NH-C_6H_{11}	50,55	$C_{25}H_{39}N_3O_5$	0,52 (1) 0,79 (2)	103-105	-10,67 (c=1, MeOH)	7.52-7.72 (d, 1H, NH), 7.16-7.47 (m, 6H ZC₆H₅, 1H, NH), 6.6-6.75 (d, 1H, NH), 4.84-5.03 (s, 2H, ZCH₂), 3.72-3.91 (m, 1H, Lys α CH), 3.42-3.59 (m, 1H, cyclohexyl CH) 2.89-3.15 (m, 2H, Lys εCH₂,), 1.02-1.91 (m, 25H, Lys βCH₂, γCH₂, δCH₂, cyclohexyl 5×CH₂, Boc 3CH₃)
Boc-Lys(Z)-NH-(C_5H_{10})-Z	51,94	$C_{32}H_{46}N_4O_7$	0,7 (1) 0,8 (2)	47-53	-4,4 (c=1, DMF)	7.65-7.82 (t, 1H, NH), 7.18-7.45 (m, 12H 2×ZC₆H₅, 2×NH), 6.71-6.82 (d, 1H, NH), 4.94-5.09 (s, 4H, 2×ZCH₂), 4.73-4.85 (m, 1H, Lys αCH), 2.82-3.09 (m, 6H, Lys εCH₂, cad. αCH₂, εCH₂), 1.01-1.67 (m, 21H, Lys βCH₂, γCH₂, δCH₂, cad βCH₂, γCH₂, δCH₂, Boc 3×CH₃)
Boc-L-Lys(Z)-NH-C_2H_4OH	48,5	$C_{21}H_{33}N_3O_6$	0,35 (3)	amorph.	-3,2 (c=1, DMF)	7,71-7,85 (t, 1H, NH), 7.12-7.42 (m, 6H ZC₆H₅, NH), 6,71-6,84 (d, 1H, NH), 4.92-5.08 (s, 2H, ZCH₂), 4.58-4.87 (t, 1H OH), 4.75-4.95 (m, 1H, Lys αCH), 3,25-3,51 (m, 2H, col. CH₂) 3,08-3,21 (m, 2H, col. CH₂) 2.82-3.07 (m, 2H, Lys εCH₂,), 1,08-1.68 (m, 15H, Lys βCH₂, γCH₂, δCH₂, Boc 3×CH₃)
Boc-L-Lys(Z)-C_6H_{13}	48,46	$C_{25}H_{41}N_3O_5$	0,46 (1) 0,32 (2)	70-72	+21 (c=1, ethyl acetate)	7,62-7,79 (t, 1H, NH), 7,15-7,41 (m, 6H, ZC₆H₅, NH), 6,63-6,79 (d, 1H, NH), 4,93-5,08 (s, 2H, ZCH₂), 3,7-3,9 (m, 1H, Lys αCH), 2.83-3.13 (m, 4H, Lys εCH₂, hexyl αCH₂), 1.09-1.62 (m, 23H, Lys βCH₂, γCH₂, δCH₂, hexyl 4×CH₂, Boc 3×CH₃), 0.7- 0.94 (t, 3H, hexyl CH₃)

cad. – cadaverine

col. – colamine

measured with a polarimeter (Optical Activity LTD AA-10R). ¹H NMR spectra were recorded with 200 MHz Brucker 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 lysine alkylamides

Boc-Lys(Z)-NH-$(CH_2)_5$-NH-Z

The compound was obtained from Boc-Lys(Z)-OH and NH_2-$(CH_2)_5$-NH-Z with the use of mixed anhydrides method.

Boc-Lys(Z)-NH-$(CH_2)_2$-OH

The compound was obtained from Boc-Lys(Z)-OPhCl₃ and NH_2-$(CH_2)_2$-OH (ratio of reagents – 1:5).

Boc-Lys(Z)-NH-C_6H_{11} and Boc-Lys(Z)-NH-C_6H_{13}

These compounds were obtained from Boc-Lys(Z)-OH and an excess of cyclohexyl- or hexylamine with the use of DCCI+HOBt method. The results are given in Table 1.

Synthesis of dipeptide alkylamides

Z-D-Phe-L-Lys(Z)-NH-C_6H_{11} and Z-D-Phe-L-Lys(Z)-NH-$(CH_2)_5$-NH-Z were obtained with the use of symmetrical anhydrides method.

Z-D-Phe-L-Lys(Z)-NH-$(CH_2)_2$-OH

The compound was obtained from Z-D-Phe-OPhCl₃ and hydrochloride of H-Lys(Z)-NH-$(CH_2)_2$-OH (ratio of reagents – 1:2) in the presence of N-methylmorpholine.

Table 2. Analytical data of alkylamides of protected dipeptides

Compound	Yield (%)	Molecular Formula	R_f	m.p. (°C)	$[\alpha]_D^{20}$	¹H NMR (DMSO-d₆) [ppm]
Z-D-Phe-L-Lys(Z)-NH-C₆H₁₁	69,07	C₃₇H₄₆N₄O₆	0,76 (1) 0,81 (2)	145-147	-5,4 (c=1, DMF)	8.18-8.22 (d, 1H, cyclohexyl NH), 7.6-7.65 (t, 1H, Lys εNH), 7.18-7.41 (m, 17H 2×ZC₆H₅, Phe C₆H₅, Lys αNH, Phe aNH), 4.84-5.03 (m, 4H, 2×ZCH₂), 4.03-4.29 (m, 2H, Phe αCH, Lys αCH), 3.50-3.59 (m, 1H, cyclohexyl CH) 2.65-3.15 (m, 4H, Lys εCH₂, Phe CH₂), 1.51-1.65 (m, 6H, Lys βCH₂, γCH₂, δCH₂), 1,15-1,20 (m, 10H, cyclohexyl 5×CH₂)
Z-D-Phe-L-Lys(Z)-NH-(CH₂)₅-Z	30,98	C₄₄H₅₃N₅O₈	0,61 (1) 0,73 (2)	131-136	-2,67 (c=1, DMF)	8.18-8.22 (d, 1H, NH), 7.72-7.80 (t, 1H, NH), 7.57-7.72 (d, 1H, NH), 7.18-7.41 (m, 22H 3×ZC₆H₅, Phe C₆H₅, Lys αNH, Phe αNH), 4.84-5.09 (s, 6H, 3×ZCH₂), 4.03-4.29 (m, 2H, Phe α CH, Lys αCH), 2.62-3.16 (m, 8H, Lys εCH₂, Phe CH₂, cad αCH₂ ε CH₂), 1.50-1.67 (m, 6H, Lys βCH₂, γCH₂, dCH₂), 0.94-1.50 (m, 6H, cad βCH₂, γCH₂, δCH₂)
Z-D-Phe-L-Lys(Z)-NH-C₂H₄OH	69,49	C₃₃H₄₀N₄O₇	0,25 (1) 0,42 (2)	amorph.	+12,3 (c=1, DMF)	8.18-8.22 (d, 1H, NH), 7.75-7.81 (t, 1H, NH), 7.51-7.68 (d, 1H, NH), 7.08-7.42 (m, 16H 2×ZC₆H₅, Phe C₆H₅, Lys αNH), 4.84-5.03 (s, 4H, 2×ZCH₂), 4.55-4.74 (t, 1H OH), 4.03-4.29 (m, 2H, Phe ⟨CH, Lys αCH), 2.65-3.26 (m, 8H, Lys εCH₂, Phe CH₂, col 2×CH₂), 0,92-1.72 (m, 6H, Lys βCH₂, γCH₂, δCH₂),
Z-D-Phe-L-Lys(Z)-C₆H₁₃	37,26	C₃₇H₄₈N₄O₆	0,76 (1) 0,81 (2)	165-170	-4,1 (c=1, DMF)	8.12-8.16 (d, 1H, NH), 7.61-7.69 (t, 1H, NH), 7.57-7.61 (d, 1H, NH), 7.16-7.36 (m, 16H, ZC₆H₅, Phe C₆H₅, Lys εNH), 4.87- 5.15 (s, 4H, ZCH₂), 4.10-4.32 (m, 2H, Lys αCH, Phe αCH), 2.83-3.1 (m, 4H, Lys εCH₂, Phe CH₂), 1.1-1.55 (m, 16H, Lys βCH₂, γCH₂, δCH₂, hexyl 5×CH₂), 0.8-0.9 (t, 3H, hexyl CH₃)

Z-D-Phe-L-Lys(Z)-NH-C₆H₁₃ was synthesized by mixed anhydrides method.

Boc group was removed with HCl/anhydrous methanol (ether) and Z group by hydrogenolysis (10% Pd/C) in acetic acid. Compounds 2 and 4 were transformed in hydrochlorides with HCl/anhydrous ether. Analytical data of the compounds are given in Table 2 and 3.

ENZYMATIC INVESTIGATION

General

Plasmin, S-2251 (H-D-Val-L-Leu-L-Lys-pNA×2HCl) and S-2238 (H-D-Phe-Pip-Arg-pNA·2HCl) (Chromogenix), thrombin and bovine fibrinogen (Lubelska Wytwórnia Szczepionek, Lublin, Poland). The inhibition of plasmin and thrombin activities was examined as described earlier [6]. Detailed descriptions of the methods are given below. Every value represents the average of triplicate determinations ± SD.

Inhibition of plasmin
Antiamidolytic assay (with the use of synthetic substrate S-2251).

The solution of examined compounds (0.2 mL) in the range of concentration 0.001-0.1 M (in control 0.2 mL 0.15 M NaCl) was added to the mixture of

Table 3. Analytical data of alkylamides of dipeptides

Compound	Yield (%)	Molecular Formula	R_f	m.p. (°C)	$[\alpha]_D^{20}$	¹H NMR (DMSO-d_6) [ppm]
H-D-Phe-L-Lys-NH-C_6H_{11}	46,9	$C_{21}H_{34}N_4O_2$ × 2 CH_3COOH	(1) 0,03 (3) 0,28	62-66	+5,3 (c=1, DMF)	7,75-8,12 (m, 2H Lys αNH, Phe α NH), 7.19-7.38 (m, 5H Phe C_6H_5), 2,53-4,27 (m, 13H, cyclo hexyl CH, Lys εCH₂, PheCH₂, Phe αCH, Lys αCH, Phe NH₃·, Lys NH₃·), 099-1,82 (m, 16H, Lys βCH₂, gCH₂, dCH₂, C_6H_{11})
H-D-Phe-L-Lys-NH-$(CH_2)_5$-NH_2	62,3	$C_{20}H_{35}N_5O_2$ × 3 HCl × 0.5 $(C_2H_5)_2O$	(1) 0,02 (3) 0,21	175-183	-2,1 (c=1, DMF)	7,95-8,83 (m, 11H, Lys αNH, Phe αNH, Phe NH₃·, Lys NH₃·, cad. NH₃·), 7.17-7.21 (m, 5H, Phe C_6H_5) 3.89-4.25 (m, 2H, Phe αCH, Lys αCH), 2.62-3.23 (m, 8H, Lys εCH₂, Phe CH₂, cad. αCH₂, εCH₂) 0,83-1,68 (m, 12H, Lys βCH₂, γCH₂, δCH₂, cad. βCH₂, γCH₂, δCH₂)
H-D-Phe-L-Lys-NH-C_2H_4OH	48,6	$C_{17}H_{28}N_4O_3$ × 2 CH_3COOH × 0.5 H_2O	(1) 0,04 (3) 0,25	amorph.	-4,8 (c=1, DMF)	7,92-8,21 (m, 2H Lys αNH, Phe αNH), 7.05-7.39 (m, 5H Phe C_6H_5), 4,57-5,11 (m, 7H, OH, Phe NH₃·, Lys NH₃·), 4,16-4,31 (m, 2H, Phe αCH, Lys αCH), 2,57-3,54 (m, 8H, Lys εCH₂, Phe CH₂, kol 2×CH₂·), 1,02-1,69 (m, 6H, Lys βCH₂, γCH₂, δCH₂)
H-D-Phe-L-Lys-NH-C_6H_{13}	69,3	$C_{21}H_{36}N_4O_2$ × 2 HCl × $(C_2H_5)_2O$	(1) 0,03 (3) 0,27	amorph.	+6,1 (c=1, DMF)	8,01-8,19 (m, 2H Lys αNH, Phe αNH), 7.11-7.19 (m, 5H Phe C_6H_5), 4,09-4,51 (m, 8H, Phe αCH, Lys αCH, Phe NH₃·, Lys NH₃·), 0,08-3,57 (m, 23H, Lys εCH₂, Phe CH₂, Lys βCH₂, γCH₂, δCH₂ hexyl 5×CH₂, hexyl CH₃)

Table 4. Inhibition of plasmin activity.

	Compound	IC$_{50}$ (M)	
		S-2251	fibrin
1	H-D-Phe-L-Lys-NH-C_6H_{11}	n.i.	n.i.
2	H-D-Phe-L-Lys-NH-$(CH_2)_5$-NH_2	0.01±0.0009	0.006±0.0004
3	H-D-Phe-L-Lys-NH-$(CH_2)_2$-OH	>0.02	0.008±0.0006
4	H-D-Phe-L-Lys-NH-$(CH_2)_5$-CH_3	0.014±0.0013	0.008±0.0006

n.i.- no inhibition was observed in concentration range 0.0002-0.02 M.

0.1 mL plasmin solution (0.05 units/mL) and 0.5 mL of Tris buffer (pH 7.7). After preincubation for 3 min at 37°C, 0.2 mL 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 mL of 50% acetic acid and absorbance at 405 nm was measured. The IC$_{50}$ value was taken as the concentration of inhibitor, which decreased the absorbance at 405 nm by 50% compared with the absorbance measured under the same conditions without inhibitor. The results are given in Table 4.

Antifibrinolytic assay

All examined substances were dissolved in 0.15 M NaCl solution and adjusted to pH 7.0 by addition 0.1 M HCl, other reagents were dissolved in Palitsch buffer pH 7.

The mixture of examined preparation (0.1 mL) in the range of concentration 0.001-0.1 M (in control 0.1 mL 0.15 M) and 0.1 mL of fibrinogen (0.5%) was added to 0.2 mL of plasmin (0.2%). After addition of 0.1 mL of thrombin (40 units/mL) the time of fibrinolysis was measured at 37^0C. The IC_{50} value was taken as the concentration of inhibitor which prolonged the time of fibrinolysis twofold in comparison with that without inhibitor. The results are given in Table 4. At the highest examined concentration of dipeptide alkylamides (0.02 M) the clot formation was not observed.

Inhibition of thrombin

The solution of examined compounds (0.2 mL) in the range of concentration 0.001-0.1 M (in control 0.2 mL 0.15 M NaCl) was added to the mixture of 0.1 mL thrombin solution (5 units/mL) and 0.5 mL of Tris buffer (pH 8.4). After preincubation for 3 min at 37^0C, 0.2 mL of S-2238 solution (0.75 mM) was added. The mixture was incubated for 15 min at 37^0C, then the reaction was stopped by the addition of 0.1 mL of 50% acetic acid and the absorbance at 405 nm was measured. The tested compounds were not inhibitors of thrombin in concentrations: 0.0002-0.02 M.

RESULTS AND DISCUSSION

Compounds **2**-**4** were weak inhibitors of amidolytic activity of plasmin and probably active center directed inhibitors of the enzyme. They inhibited also fibrinolytic activity of plasmin, although prevention of clot forming at higher concentration is difficult to explain. None of the examined derivatives was an inhibitor of thrombin in amidolytic test. The alkylamides of dipeptide (compounds **2** and **4**) with long aliphatic side chain are better inhibitors of this enzyme than the derivative containing colamine residue (compound **3**). Compound **2** was the most active inhibitor of plasmin between examined compounds. However, an effect of a presence of free amino group in the cadaverine residue was weak and the activity of this compound was similar to the inhibitory effect of hexylamide of D-phenylalanyl-L-lysine. According to obtained now and earlier [6] results, dipeptide amides with general structure H-D-Phe-L-Lys-X can be inhibitors of amidolytic activity of plasmin when X is the aliphatic chain but not a cyclic structure. The presence of cyclohexyl (compound **1**) or benzyl group [6] resulted in the loss of the inhibitory activity.

REFERENCES

1. Backes B.J., Harris J.L., Leonetti F., Craik C.S., Ellman J.A.: Nat. Biotechnol. 18, 187 (2000).
2. Hervio L.S., Coombs G.S., Bergstrom R.C., Trivedi K., Corey D.R., Madison E.L.: Chem. Biol. 7, 443 (2000).
3. Okada Y., Tsuda Y., Teno N., Wanaka K., Boghaki M., Hijikata-Okunomiya A., Naito T., Okamoto S.: Chem. Pharm. Bull. 36, 1289 (1988).
4. Midura-Nowaczek K., Roszkowska-Jakimiec W., Bruzgo I., Worowski K.: Acta. Pol. Pharm.-Drug Res., 47, 39 (1990).
5. Sakamoto H., Shimohigashi Y., Ogawa T., Kawano K., Ohno M.: Bull. Chem. Soc. Jpn., 64, 2519 (1991).
6. Midura-Nowaczek K., Roszkowska-Jakimiec W., Lepietuszko I., Bruzgo I.: Pharmazie 58, 687 (2003).
7. Lawson W.B., Leafer jr M.D., Tewes A., Rao G.J.S.: Hoppe-Seyler′s Z. Physiol. Chem. 349, 251 (1968).