Department of Organic Chemistry[1] and Department of Instrumental Analysis[2], Medical University of Białystok, Poland

Synthesis of benzylamides of dipeptides as potential inhibitors of plasmin

K. MIDURA-NOWACZEK[1], W. ROSZKOWSKA-JAKIMIEC[2], I. LEPIETUSZKO[1], I. BRUZGO[1]

Received February 14, 2003, accepted April 29, 2003

Dr. K. Midura-Nowaczek, Department of Organic Chemistry, Medical University of Białystok, Kilińskiego 1, PL-15–089 Białystok, Poland

Pharmazie 58: 687–689 (2003)

Four benzylamides of dipeptides with the general formula: X-L-Lys-NH$-CH_2-C_6H_5$, where X = L-or D-Leu and L-or D-Phe were prepared as potential inhibitors of plasmin. All of them influenced on the fibrynolytic activity of plasmin, but only D-Leu-L-Lys-NH$-CH_2-C_6H_5$ inhibited the amidolytic activity of this enzyme. None of the tested compounds was an inhibitor of thrombin in an amidolytic test.

1. Introduction

Plasmin, a key enzyme for fibrynolysis, is a trypsin-like serine protease. The optimal $P_2–P_1$ substrate specificity for plasmin seems to be Phe-Lys or Leu-Lys. This cleavage sequence has been identified in many of natural and synthetic substrates [1, 2].

Recent studies indicate that this enzyme plays an important role in a variety of biological processes like wound-healing, tissue repair, cell migration [3] and also in pathologic phenomena such as inflammation and tumour cell growth and metastasis [4, 5].

At present ε-aminocaproic acid (EACA) and *trans*-aminomethylcyclohexanecarboxylic acid (AMCHA) are clinically used as plasmin inhibitors. These compounds inhibit the fibrinolytic activity of plasmin, but they practically do not influence amidolytic and fibrynogenolytic activities of the enzyme. This is because of the fact that these inhibitors act on plasmin by blocking the lysine-binding sites of this enzyme, which are not the catalytic site [6]. Synthesis of active centre directed inhibitors of plasmin is a research field with the object of obtaining inhibitors of this enzyme to influence not only fibrynolysis but also amidolysis and proteolysis. Substituted anilides of lysine and short peptides inhibited amidolytic and fibrynolytic activities of plasmin [7] but an heptylamide of the tripeptide with C-terminal lysine showed only antifibrynolytic activity [8]. Recently, several benzylamides of dipeptides, having the structure H-D-Xaa-Phe-NH$-CH_2-C_6H_5$ have been reported as potent chymotrypsin inhibitors. Inhibitory activity of these compounds was probably a result of side chain-side chain intramolecular CH/π interactions [9–11]. It seems to be interesting whether these kinds of dipeptide derivatives may be also plasmin inhibitors. Therefore, we were prepared benzylamides of L-leucyl-L-lysine (H-L-Leu-L-Lys-NH$-CH_2-C_6H_5$), D-leucyl-L-lysine (H-D-Leu-L-Lys-NH$-CH_2-C_6H_5$), L-phenylalanyl-L-lysine (H-L-Phe-L-Lys-NH$-CH_2-C_6H_5$) and D-phenylalanyl-L-lysine (H-D-Phe-L-Lys-NH$-CH_2-C_6H_5$) and examined their activity on amidolytic and fibrinolytic activities of plasmin and the amidolytic activity of thrombin.

2. Investigations, results and discussion

Dipeptide benzylamides were obtained according to standard synthetic procedures. Removal of the benzyloxycarbonyl group (Z) was carried out carefully by catalytic hydrogenation (Pd/C) with TLC monitoring. It was observed that too long reaction times may result in partial cleaving of the benzylamide bond.

The benzylamide of D-leucyl-L-lysine is a weak inhibitor of the amidolytic activity of plasmin and probably an active centre directed inhibitor of this enzyme. Although a decrease in fibrinolysis time by lower concentrations and a prevention of clot forming in higher concentrations of this compound is difficult to explain. None of the tested compounds was an inhibitor of thrombin in an amidolytic test. The other examined benzylamides of dipeptides (H-L-Leu-L-Lys-NH$-CH_2-C_6H_5$, H-L-Phe-L-Lys-NH$-CH_2-C_6H_5$ and H-D-Phe-L-Lys-NH$-CH_2-C_6H_5$) inhibited the fibrinolytic but not the amidolytic activity of plasmin, similar to observed ealier activity of the heptylamide of L-alanyl-L-phenylalanyl-L-lysine [8]. This fact can be connected with an interaction of these compounds with weak lysine binding sites (AH-sites) as *N*-acetyl-L-lysine-methylester proposed by Christensen [12].

Table 1: Structure of synthesized compounds

	A-L-Lys(X)-NHBzl	
Compd.	A	X
1	Z-L-Leu	Z
2	Z-D-Leu	Z
3	Boc-L–Phe	Z
4	Boc-D-Phe	Z
5	H-L-Leu	H
6	H-D-Leu	H
7	H-L-Phe	H
8	H-D-Phe	H

Z = benzyloxycarbonyl ($C_6H_5CH_2OCO$)
Boc = t-butoxycarbonyl ($(CH_3)_3COCO$)
Bzl = benzyl

Table 2: Analytical data of protected benzylamides of dipeptides

Compd.	Yield (%)	Molecular formula	R_f	M.p. (°C)	$[\alpha]_D^{20}$ (C = 1, DMF)	^{1}H NMR (DMSO-d_6) δ [p.p.m.]
1	26	$C_{35}H_{44}O_6N_4$	0.77 (2) 0.81 (3)	168–176	−5.0	8,39 (t, J = 5.7 Hz, 1 H, BzlNH), 7.93 (d, J = 8.2 Hz, 1 H, CONH), 7.46 (d, J = 8.2 Hz, 1 H, CONH), 7.09–7.42 (m, 16 H, 3 × C_6H_5, CONH), 5 (s, 4 H, 2 × Z CH_2), 4.15–4.39 (m, 3 H, BzlCH_2, CH^{α}), 4.06 (m, 1 H, CH^{α}), 3.05 (m, 2 H, Lys CH_2^{ε}), 1.06–1.83 (m, 9 H, LeuCH_2^{β}, CH^{δ}, Lys $CH_2^{\beta,\gamma,\delta}$), 0.7–1 (m, 6 H, Leu 2 × CH_3)
2	25	$C_{35}H_{44}O_6N_4$	0.71 (2) 0.92 (3)	162–170	−15.2	8.08–8.36 (m, 2 H, 2 × NH), 7.5 (d , J = 7.1 Hz, 1 H, CONH), 7.08–7.4 (m, 16 H, 3 × C_6H_5, CONH), 4.74–5.1 (m, 4 H, 2 × ZCH_2), 4.12–4.38 (m, 3 H, BzlCH_2, CH^{α}), 4.01 (m, 1 H, CH^{α}), 3.07 (m, 2 H, Lys CH_2^{ε}), 1.18–1.68 (m, 9 H, Leu CH_2^{β}, CH^{δ}, Lys $CH_2^{\beta,\gamma,\delta}$) 0.68–1.05 (m, 6 H, Leu 2 × CH_3)
3	30	$C_{35}H_{44}O_6N_4$	0.68 (1) 0.8 (2)	173–175	−11.1	8.39 (t, J = 5.8 Hz, 1 H, BzlNH), 7.95 (d, J = 7.9 Hz, 1 H, CONH), 7.12–7.44 (m, 16 H, 3 × C_6H_5, CONH), 6.98 (d, J = 8.4 Hz, 1 H, CONH), 5 (s, 2 H, ZCH_2), 4.04–4.42 (m, 4 H, BzlCH_2, 2 × CH^{α}), 2.63–3.09 (m, 4 H, Lys CH_2^{ε}, PheCH_2), 0.89–1.82 (m, 15 H, Lys $CH_2^{\beta,\gamma,\delta}$, Boc 3 × CH_3)
4	27.5	$C_{35}H_{44}O_6N_4$	0.65 (1) 0.79 (2)	155–157	−14.1	8.33 (t, 1 H, BzlNH), 8.14 (d, 1 H, J = 8 Hz, CONH), 7.06–7.39 (m, 17 H, 3 × C_6H_5, 2 × CONH), 4.99 (s, 2 H, ZCH_2), 4.06–4.42 (m, 4 H, BzlCH_2, 2 × CH^{α}), 2.72–3.03 (m, 4 H, Lys CH_2^{ε}, PheCH_2), 0.85–1.88 (m, 15 H, Lys $CH_2^{\beta,\gamma,\delta}$, Boc 3 × CH_3),

Table 3: Analytical data of benzylamides of dipeptides

Compd.	Yield (%)	Molecular formula	R_f	M.p. (°C)	$[\alpha]_D^{20}$ (C = 1, DMF)	^{1}H NMR (DMSO-d_6) [p.p.m.] −CH_2Bzl (benzylamide) (d, 2 H)
5	88	$C_{19}H_{32}O_2N_4 \times CH_3OH \times 1.5\ H_2O$	0.12 (2) 0.1 (3)	amorph.	−5.0	4.29
6	94	$C_{19}H_{32}O_2N_4 \times CH_3OH \times H_2O$	0.1 (2) 0.09 (3)	amorph.	−15.2	4.29
7	32.9	$C_{22}H_{30}O_2N_4 \times 1.5\ CH_3OH$	0.14 (1) 0.11 (2)	84–87	−11.1	4.28
8	23.7	$C_{22}H_{30}O_2N_4 \times 1.5\ CH_3OH$	0.19 (1) 0.18 (2)	amorph.	−14.1	4.28

3. Experimental

3.1. Synthesis of the compounds

3.1.1. General

Organic solutions were dried over anh. $MgSO_4$. Reactions were monitored and the homogeneity of the products was examined using the silica gel plates (Kieselgel 60 F_{254}, Merck) using the following systems: 1: benzene/methanol/acetic acid (12 : 5 :1); 2: ethanol/water/25% ammonia solution (18 : 0.5 : 0.5); 3: chloroform/methanol/water (3 : 3 : 0.5). Spots were visualised with tolidine/chlorine or iodine and ninhydrin. The melting points were determined on Boëtius 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 Brucker AC 200F spectrometer. Elemental analysis were performed on a Perkin-Elmer analyser and the results, indicated by symbols C, H, N, were within ±0.4% of the theoretical values.

3.1.2. N^{α}-t-Butoxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine benzylamide

The compound was obtained from Boc-Lys(Z)-OH and benzylamine with the use of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in the presence of *N*-methylmorpholine in ethyl acetate. Yield 55%, m.p. 103–108 °C, $[\alpha]_D^{20} = -20,9$ (c = 1, MeOH); Rf = 0.4 (1), 0.76 (2), 0.93 (3); ^{1}H NMR: 8.3 (t, J = 5.9 Hz, 1 H, BzlNH), 7.12–7.42 (m, 11 H, ZC_6H_5, $BzlC_6H_5$, CONH), 6.85 (d, J = 8 Hz, 1 H, CONH), 5.0 (s, 2 H, ZCH_2), 4.28 (d, J = 5.9 Hz, 2 H, BzlCH_2), 3.93 (m, 1 H, CH^{α}), 2.97 (m, 2 H, CH_2^{ε}), 1.12–1.8 (m, 15 H, $CH_2^{\alpha,\beta,\gamma}$, Boc 3 × CH_3).

3.1.3. Benzylamides of dipeptides

The compounds (structures are given in Table 1) were obtained by coupling of hydrochloride of N^{ε}-benzyloxycarbonyl-L-lysine benzylamide with respective Boc- or Z-aminoacid with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole in the presence of *N*-methylmorpholine in DMF. The Boc group was removed with HCl/anhydrous methanol and Z group with hydrogenolysis (10% Pd/C) in methanol. Analytical data of the compounds are given in Tables 2 and 3.

3.2. Enzymatic investigations

3.2.1. General

Plasmin (Novo Industri A/S Copenhagen, Denmark), thrombin and bovine fibrinogen (Lubelska Wytwórnia Szczepionek, Lublin, Poland), S-2251 and S-2238 (Kabi-Virtum, Stockholm, Sweden). Every value represents the average of triplicate determination.

3.3.2. Inhibition of plasmin

Antifibrinolytic and antiamidolytic activities (with the use of S-2251) were determined as described previously [13]. Results are given in Table 4.

3.3.3. Inhibition of thrombin

The enzyme assay was performed according to the literature [14]. 0.2 mL of solution of examined compounds in the concentration range of 0.001–0.1 mol/mL (in control 0.1 mL 0.15 mol/L 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 °C, 0.2 mL of S-2238 solution (0.75 mmol/L) was added. The mixture was incubated for 15 min at

Table 4: Inhibition of fibrinolytic and amidolytic activity of plasmin

Compd.	IC_{50} (M)	
	Fibrin	S-2251
5	0.0018	n.i.
6	− *	0.01
7	0.002	n.i.
8	0.0019	n.i.

n.i. – no inhibition was observed in maximum concentration (0.02 M)

* – compound in concentration 0.0002 M decreased complete lysis time twofold in comparison with that without inhibitor but in concentration 0.02 M formation of clot was not observed

37 °C, then the reaction was stopped by the addition of 0.1 mL of 50% acetic acid and absorbency was measured. All examined compounds were not inhibitors of thrombin in the concentration range tested.

References

1 Friberger, P.: Scand. J. Clin. Lab. Invest. **42** (Suppl.162), 17 (1982)
2 Backes, B. J.; Harris, J. L.; Leonetti, F.; Craik, C. S.; Ellman, J. A.: Nat. Biotech. **18**, 187 (2000)
3 Hervio, L. S.; Coombs, G. S.; Bergstrom, R. C.; Trivedi, K.; Corey, D. R.; Madison, E. L.: Chem. Biol. **7**, 443 (2000)
4 Markus, G.: Semin. Thromb. Hemost. **10**, 61 (1984)
5 Wanaka, K.; Okamoto, S.; Horie, N.; Hijikata-Okunomija A.; Okamoto, U.; Naito, T.; Ohno, N.; Boghaki, M.; Tsuda,Y.; Okada, Y.: Thromb. Res. **82**, 79 (1996)
6 Iwamoto, M.: Thrombos. Diathes. Haemorrh. **33**, 573 (1975)
7 Okada, Y.; Tsuda, Y.; Teno, N.; Wanaka, K.; Boghaki, M.; Hijikata-Okunomija A.; Naito, T.; Okamoto, S.: Chem. Pharm. Bull. **36**, 1289 (1988)
8 Midura-Nowaczek, K.; Bruzgo, I.; Roszkowska-Jakimiec, W.; Worowski, K.: Acta Polon. Pharm.-Drug Research **47**, 39 (1990)
9 Shimohigashi, Y.; Maeda, I.; Nose, T.; Isekue, K.; Sakamoto, H.; Ogawa, T.; Ide, Y.; Kawahara, M.; Nezu, T.; Terada, Y.; Kawano, K.; Ohno, M.: J. Chem., Perkin Trans. 1, 2479 (1996)
10 Kashima, A.; Inoue, Y.; Sugio, S.; Maeda, I.; Nose, T.; Shimohigashi, Y.: Eur. J. Biochem. **255**, 12 (1998)
11 Shimohigashi, Y.; Nose, T.; Yamaguchi, Y.; Maeda, I.: Biopolymers (Peptide Science) **51**, 9 (1999)
12 Christensen, U.: Biochem. J. **223**, 413 (1984)
13 Midura-Nowaczek, K.; Bruzgo, I.; Dubis E.; Roszkowska-Jakimiec, W.; Worowski, K.: Pharmazie **51**, 775 (1996)
14 Gaffney, P. J.; Lord, K.; Brasher, M.; Kirkwood, T. B. L.: Tromb. Res. **10**, 549 (1977)