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Methylketone inhibitors of plasmin

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A series of peptide methylketones with general formula Y-Phe-Lys-CH_3 was prepared as potential inhibitors of plasmin.

1. Introduction

Recent studies show that plasmin plays an important role not only in the process of fibrinolysis, but also in a variety of biological processes like wound-healing, tissue repair, cell migration (Hervio et al. 2000) and also in pathological phenomena such as inflammation, tumor cell growth and metastasis (Markus 1984; Wanaka et al. 1996).
Epsilon-aminocaproic acid (EACA) and trans-aminomethylcyclohexanecarboxylic acid (AMCHA) are clinically used as plasmin inhibitors. These compounds inhibit the fibrinolytic activity of plasmin by blocking the lysine-binding sites. Their inhibitory activity on plasmin with respect fibrinogen, other proteins and small peptides is much weaker than towards fibrin. Because of this fact the synthesis of active center directed low molecular inhibitors of plasmin was undertaken. The optimal P_1-P_2 specificity for plasmin seems to be Phe-Lys. This cleavage sequence has been identified in many natural and synthetic substrates (Friberger 1982; Backes et al. 2000).
We focused our attention on small peptides derivatives with methylketone group on C-terminal lysine. Synthetic methylketones of peptides are reversible inhibitors of serine and cysteine proteases (Fittkau et al. 1984; Brömme et al. 1989). They are easy to synthesize and have higher chemical stability than other reversible peptide inhibitors, e.g. aldehydes. Ealier we obtained a series of plasmin inhibitors with tripeptide methylketone structure by the Dakin-West method, but their inhibitory activity was low (Midura-Nowaczek et al. 1997).

Table 1: Structure of peptide methylketones obtained

A_1-DL-Lys(X)-CH_3		
Compd.	A_1	X
1	Boc-L-Phe	Z
2	Boc-D-Phe	Z
3	Ac-L-Phe	Z
4	Ac-D-Phe	Z
5	H-LPhe	H
6	H-D-Phe	H
7	Ac-L-Phe	H
8	Ac-D-Phe	H
9	Z-D-Ala-L-Phe	Z
10	H-D-Ala-L-Phe	H

Boc = t-butoxycarbonyl
Z = benzyloxycarbonyl
Ac = acetyl

2. Investigations and results

In search for minimum peptide structure and higher activity of plasmin inhibitors with methylketone structure, we obtained now diastereoisomers of tripeptide methylketone H-Ala-Phe-Lys-CH_3 and its dipeptide analogs (Table 1). This tripeptide structure is present in synthetic substrats (H-L-Ala-L-Phe-L-Lys-pNA and H-D-Ala-L-Phe-Lys-pNA) (Kiss et al. 1985) and well known irreversible inhibitors of plasmin with chloro- and fluoromethylketone group (Ganu et al. 1987; Angliker et al. 1987).
The tripeptide H-D-Ala-L-Phe-DL-Lys is known as a trigger in some antitumor prodrugs. There are known antitumor prodrugs prepared by attaching the drug moiety (taxol, anthracyclines, camptothecin) to the spacer end of this tripeptide moieties. These prodrugs are selectively activated by the tumor-associated protease plasmin (de Groot et al. 1999, 2000, 2002). The tested compounds (Table 1) were obtained by the Dakin-West method with McMurray and Dyckes modification (Mc Murray et al. 1985), resulting in racemization of the lysine derivative on C^{α}. Because of this, we examine also antiplasmin activity of H-L-Ala-L-Phe-DL-Lys-CH_3 and two ealier obtained diastereoisomers: H-L-Ala-L-Phe-L-Lys-CH_3 and H-L-Ala-L-Phe-D-Lys-CH_3 (Midura-Nowaczek 1997).
The antifibrinolytic and antiamidolytic activity of the synthesized compounds were determined as described previously (Midura-Nowaczek et al. 1996), the results are given in Table 2.

3. Discussion

According to obtained results, L-configuration of C-terminal lysine derivative in tripeptide methyketone structure is necessary for inhibition of amidolytic and fibrinolytic activity of plasmin. Similar results were already obtained by Shaw (1975) for H-Ala-Phe-Lys-CH_2Cl, where diastereoisomers with L and D configuration of the lysine derivative had an effectiveness of 100 : 1 respectively. Racemization of the lysine residue on C^{α} of the tripeptide methylketone results in a reduction of inhibitory activity by 50%. The

Table 2: Inhibition of fibrinolytic and amidolytic activity of plasmin

Compd.	IC_{50} (M)	
	Fibrin	S-2251
H-L-Phe-DL-Lys-CH_3	0.006	>0.02
H-D-Phe-DL-Lys-CH_3	0.004	0.02
Ac-L-Phe-DL-Lys-CH_3	0.002	0.008
Ac-D-Phe-DL-Lys-CH_3	0.002	>0.02
H-D-Ala-L-Phe-DL-Lys-CH_3	0.004	0.012
H-L-Ala-L-Phe-DL-Lys-CH_3	0.004	0.002
H-L-Ala-L-Phe-L-Lys-CH_3	0.002	0.001
H-L-Ala-L-Phe-D-Lys-CH_3	n.i.	n.i.

n.i. = no inhibition was obsreved in maximum concentration (0.02M)

Dakin-West method is a simple and efficient method for a preliminary evaluation of peptide methyketones as potential plasmin inhibitors.

The fact that L-configuration of alanine in P_3 results in a more effective inhibition of amidolytic activity of plasmin is unexpected. According to the literature (Friberger 1982) compounds with D-configuration in P_3 have better affinity to plasmin. The peptide fragment H-D-Ala-Phe-Lys- is a part of a good synthetic substrate (Kiss et al. 1985) and prodrugs activable by plasmin (de Groot et al. 1999, 2000, 2002). However, H-L-Ala-L-Phe-L-Lys-CH_2Cl is the one of the most effective peptidyl lysine chloromethylketone for inactivating plasmin (Ganu et al. 1987).

The activity of dipeptide methylketones is observed mainly in the fibrinolysis test. Only Ac-L-Phe-DL-Lys-CH_3 was a more active inhibitor of amidolytic and fibrinolytic activity of plasmin than H-D-Ala-L-Phe-DL-Lys-CH_3. The activity of H-L-Phe-DL-Lys-CH_3 was lower. In the case of dipeptide methylketones with D-Phe in P_2, the presence of unsubstituted N-terminal amino group is prefered.

4. Experimental

4.1. Synthesis of the compounds

Classical coupling techniques were used to prepare all peptides. Methylketones group was introduced to acids of protected peptides by Dakin-West method (Mc Murray et al. 1985). The benzyloxycarbonyl group was removed by catalytic hydrogenation in acetic acid with the use of Pd/C and the t-butoxycarbonyl group with the HCl saturated solvents. Results are given in Tables 3 and 4. The synthesis of H-L-Ala-L-Phe-DL-Lys-CH_3, H-L-Ala-L-Phe-L-Lys-CH_3 and H-L-Ala-L-Phe-D-Lys-CH_3 was described earlier (Midura-Nowaczek 1997).

Organic solutions were dried over anh. $MgSO_4$. Reactions were monitored and homogeneity of products was examined on silica gel plates (Kieselgel 60 F_{254}, Merck) using the following solvent systems: 1: chloroform/acetone (7 : 1), 2: chloroform/ethanol (9 : 1), 3: benzene/methanol/acetic acid (12 : 5 : 1), 4: ethanol/water/25% ammonia solution (18 : 0.5 : 0.5). Spots were visualised with iodine and ninhidrin. The melting points were determined on Boetius 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 a Perkin-Elmer analyser and results were within ±0.4% of theoretical values.

Table 3: Yields and physicochemical properties of protected peptide methylketones

Compd.	Yield (%)	Molecular formula	Rf: 2 3	m.p. (°C)	$[\alpha]^{20}_D$ (C=1, AcOH)	^{1}H NMR (d_6DMSO) δ ppm
1	68	$C_{29}H_{39}N_3O_6$	0.79 0.68	109–111	−2.34	8.22–8.15 (m, 1 H, CONH), 7.35–7.17 (m, 11 H, 2 × C_6H_5, CONH), 6.98–6.94 (d, J = 8.2 Hz, 1 H, CONH), 4.99 (s, 2 H, ZCH_2), 4.21–4.08 (m, 2 H, 2 × CH^{α}), 2.99–2.58 (m, 4 H, LysCH_2^{ε}, PheCH_2), 2.05 (s, 3 H, Lys$COCH_3$), 1.96 (s, 3 H, Lys$COCH_3$), 1.75–1.02 (m, 6 H, Lys $CH_2^{\beta,\gamma,\delta}$), 1.31 (s, 9 H, Boc$(CH_3)_3$)
2	59	$C_{29}H_{39}N_3O_6$	0.79 0.68	110–112	−36.12	8.29–8.20 (m, 1 H, CONH), 7.37–7.20 (m, 11 H, 2 × C_6H_5, CONH), 7.05–7.01 (d, J = 7.6 Hz, 1 H, CONH), 4.99 (s, 2 H, ZCH_2), 4.20–4.13 (m, 2 H, 2 × CH^{α},), 2.98–2.92 (m, 2 H, LysCH_2^{ε}), 2.87–2.75 (m, 2 H, PheCH_2), 2.04 (s, 3 H, Lys$COCH_3$), 1.95 (s, 3 H, Lys$COCH_3$), 1.80–1,12 (m, 6 H, Lys-$CH_2^{\beta,\gamma,\delta}$), 1.31 (s, 9 H, Boc$(CH_3)_3$)
3	56	$C_{26}H_{33}N_3O_5$	0.45 0.51	150–152	−8.67	8.34 (d, J = 7.4 Hz, 1 H, CONH), 8.17 (d, J = 8.3 Hz, 1 H, CONH), 7.34–7.17 (m, 11 H, 2 × C_6H_5, CONH), 4.99 (s, 2 H, ZCH_2), 4.60–4.49 (m, 1 H, CH^{α}), 4.09–4.07 (m, 1 H, CH^{α}), 3.00–2.86 (m, 2 H, LysCH_2^{ε}), 2.82–2.69 (m, 2 H, PheCH_2), 2.03 (s, 3 H, Lys$COCH_3$), 1.91 (s, 3 H, Lys$COCH_3$), 1.75 (s, 3 H, AcCH_3), 1.38–1.05 (m, 6 H, Lys$CH_2^{\beta,\gamma,\delta}$)
4	57	$C_{26}H_{33}N_3O_5$	0.45 0.51	175–178	−16.71	8.34 (d, J = 7.4 Hz, 1 H, CONH), 8.15 (d, J = 8.3 Hz, 1 H, CONH), 7.35–7.19 (m, 11 H, 2 × C_6H_5, CONH), 7.09–7.01 (d, J = 7.6 Hz, 1 H, CONH), 4.99 (s, 2 H, ZCH_2), 4.75–4.44 (m, 1 H, CH^{α}), 4.21–4.12 (m, 1 H, CH^{α}), 3.01–2.69 (m, 4 H, LysCH_2^{ε}, PheCH_2), 2.04 (s, 3 H, Lys$COCH_3$), 1.92 (s, 3 H, Lys$COCH_3$), 1.75 (s, 3 H, AcCH_3), 1.38–1.05 (m, 6 H, Lys$CH_2^{\beta,\gamma,\delta}$)
9	65	$C_{35}H_{42}N_4O_7$	0.62 0.65	115–118	+6.67	8.28–8.17 (m, 2 H, 2 × CONH), 7.44–7.16 (m, 17 H, 3 × C_6H_5, 2 × CONH), 4.99 (s, 4 H, 2 × ZCH_2), 4.57–4.50 (m, 1 H, CH^{α}), 4.14–4.06 (m, 1 H, CH^{α}), 4.02–3.96 (m, 1 H, CH^{α}), 3.05–2.90 (m, 2 H, Lys CH_2^{ε}), 2.83–2.79 (m, 2 H, Phe CH_2), 2.05 (s, 3 H, Lys$COCH_3$), 1.96 (s, 3 H, Lys$COCH_3$), 1.80–1.12 (m, 6 H, Lys $CH_2^{\beta,\gamma,\delta}$), 1.00–0.94 (m, 3 H, Ala CH_3)

Table 4: Yields and physicochemical properties of peptide methylketones

Compd.	Yield (%)	Molecular formula	Rf: 4 5	m.p. (°C)	$[\alpha]^{20}_D$ (C=1, CH_3COOH)	^1H NMR (d_6DMSO) δ ppm – $COCH_3$
5	68	$C_{16}H_{25}N_3O_2 \times 2\ CH_3COOH$	0.78 0.74	150–154	+7.34	2.10, 1.88
6	64	$C_{16}H_{25}N_3O_2 \times 2\ CH_3COOH$	0.78 0.74	175–178	−38.21	2.11, 1.90
7	79	$C_{17}H_{27}N_3O_3 \times CH_3COOH$	0.68 0.63	amorphic	+11.34	1.85, 1.77
8	82	$C_{17}H_{27}N_3O_3 \times CH_3COOH$	0.68 0.63	amorphic	−30.14	1.83, 1.76
10	63	$C_{19}H_{30}N_4O_3 \times 2\ CH_3COOH$	0.69 0.62	167–170	−33.34	1.84, 1.82

4.2. Enzymatic investigations

Plasmin (Chromogenix), bovine fibrinogen (Lubelska Wytwórnia Szczepionek, Lublin, Poland), S-2251 (Chromogenix) were used. Antifibrinolytic and antiamidolytic activity was determined as described previously (Midura-Nowaczek et al. 1996). Antifibrinolytic assay: IC_{50} value was considered as the concentration of inhibitor which prolonged the complete lysis time twofold in comparison with that without inhibitor. Antiamidolytic assay (with the use of synthetic substrate S-2251): IC_{50} value was considered 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.

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