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Peptidyl Diazomethylketones as Cysteine Protease Inhibitors Structurally Based Upon the Inhibitory Centers of Cystatins

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Six new putative cysteine protease inhibitors based upon sequences of the N-terminal binding fragments of rat cystatin A, bovine cystatin C and human cystatins D and S were synthesized. Inhibitory activities of these compounds against papain and bovine cathepsin B were tested. Additionally, agar well diffusion test of their antibacterial activity against *Streptococcus pyogenes* was performed.

Key words: cysteine proteases, cystatins, peptidyl diazomethanes

Cysteine proteases are a widely distributed class of enzymes in animals, plants and microorganisms. There are several classes of cysteine proteases. The largest and the best known class of these enzymes is the papain family, comprising plant cysteine proteases like actinidin, papain, chymopapain, ficin, bromelains, aleurain and lysosomal cathepsins B, H, K, L and S [1,2,3]. These enzymes show high sequence homology, similar folding pattern and active site arrangement what suggests that mechanism of the catalytic action is similar or the same for all members of the family [4,5].

Cysteine proteases are involved in the development and progression of a variety of human diseases such as muscular dystrophy [6], rheumatoid arthritis [7], osteoporosis [8,9] and tumor methastasis [10,11]. The activity of these enzymes is controlled by their endogenous inhibitors called cystatins. These inhibitors form tight, non-covalent complexes with the target enzymes. The superfamily of cystatins is subdivided into the following three families: stefins (family I), cystatins (family II) and kininogens (family III). These proteins show high sequential homology and similar pattern of folding what suggests a similar mechanism of the inhibition [12]. The X-ray studies of the chicken egg-white cystatin as well as the stefin B complex with papain showed that three small fragments of these proteins are involved in their binding to papain: N-terminal part interacting with S_4-S_1 binding sites of enzyme and two hairpin loops which binds to the positions S_1'-S_3' [13,14]. This cystatin–enzyme interaction model seems to be good as a starting point for the design of synthetic inhibitors with a low molecular weight. In our previous work, two effective irreversible

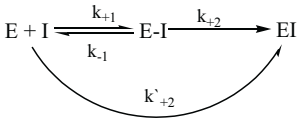
inhibitors based upon N-terminal binding fragment of cystatin C (-Arg-Leu-Val-Gly-) were synthesized [15,16]. These compounds, containing the diazomethylketone group as a reactive center, bind covalently to the catalytic sulfhydryl group of the enzyme following the addition – elimination mechanism [17]. The X-ray studies of the complex of the most active inhibitor of this group, Z-Arg-Leu-Val-Gly-DAM, with papain showed that the peptide part of this compound binds with the S_4-S_1 subsities of the enzyme, what was expected on the basis of postulated mechanism of cystatin – cysteine protease interaction (M. Jaskólski and coworkers, unpublished). More detailed results of these studies will be presented soon.

It was found that Z-Leu-Val-Gly DAM possesses selective bactericidal properties against *Streptococcus pyogenes*. It was assumed that the antibacterial properties of this compound were an effect of inhibition of the streptococcal cysteine protease – the enzyme, which is not a member of papain family. The association rate constant k'_{+2} of this inhibitor with streptococcal protease was equal to 102 $M^{-1}s^{-1}$ [18]. Surprisingly, elongation of the peptide chain of this inhibitor with arginine residue (Z-Arg –Leu-Val-Gly-DAM) led to the increase of inhibitory activity against papain and total downfall of the antibacterial properties. To test how the inhibitory activity and antibacterial properties depend on amino acid sequence, we decided to synthesize and examine a series of peptidyl diazomethanes modelled on N-terminal binding fragments of bovine cystatin C (-Arg-Leu-Leu-Gly-), human cystatin S (-Arg-Ile-Ile-Pro-Gly-), rat cystatin A (-Gly-Ile-Val-Gly-) and human cystatin D (-Arg-Thr-Leu-Ala-Gly-).

EXPERIMENTAL

All amino acid derivatives used in the described syntheses were obtained from Nova-Biochem. Linear peptides, the substrates to compounds **1**, **3** and **5**, were prepared "step by step" using DCCI / HOBt activation method. C-terminal carboxyl groups of elongated peptide chains were protected by methyl ester group and finally deblocked with equimolar volume of 1N NaOH water/methanol solution after the synthesis. The *tert*-butyloxycarbonyl (Boc) group, applied to the temporary protection of α-amino functions, was removed by treatment with 4N HCl/dioxane solution before every coupling of the next amino acid derivative. The benzyloxycarbonyl (Z) group was applied as the N-terminal α-amino protection of the final linear peptides. Linear peptides, the substrates to the compounds **2** and **4**, were prepared in the similar manner as described above. Fluorenyloxycarbonyl (Fmoc) and *tert*-butyl groups were applied there as a temporary protection of α-amino and the C-terminal carboxy functions, respectively. 20% Piperidine solution in DMF has been used to remove of Fmoc moiety. Deblocking of the C-terminal carboxy function were carried out by treatment of the final linear peptides with 4N HCl/dioxane solution. Melting points are given uncorrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR spectra were recorded on a Bruker IFS 66. Elemental analyses were performed on EA 1108 (Carlo Erba Instrument). The structures of synthesized products were confirmed by FAB MS. TLC was carried out using silica gel plates (Merck) and following solvent systems: (A) chloroform – MeOH – 25% aq. ammonia (2:2:1), (B) AcOEt – pentane (3:1), (C) benzene – acetone (2 :1), (D) chloroform – MeOH (9 : 1). Purity of the final products was assessed by RP-HPLC on Kromasil 100-5-C8, 4.6 × 250 mm column, using triethylammonium phosphate buffer pH = 6.8 as ion-pairing agent and acetonirile as mobile phase organic modifier.

Determination of the enzyme inhibitory activity: The apparent second-order rate constants of inactivation (k''_{+2}) were determined by continuously monitoring the enzyme activity against a fluorogenic substrate before and after addition of the inhibitor [19].



where:
E – enzyme;
I – inhibitor;
E-I – non-covalent complex enzyme-inhibitor
EI – enzyme with irreversibly bonded inhibitor molecule

Scheme. Enzyme – irreversible inhibitor interactions.

The program used to calculate a substrate dependent rate constant (k''_{+2}), FLUSYS [20], was kindly provided by Neil Rawlings and Alan Barret, Strangeways Research Laboratory, Cambridge, England. The program calculates a pseudo-first order rate constant k_{obs} by the Guggenheim method and gives the apparent second-order rate constant, k''_{+2}, by dividing with inhibitor concentration ([I]). Michaelis constant (K_m) for the reaction between enzyme and substrate (S) under the given conditions was then used to calculate the substrate independent apparent second-order rate constant, k'_{+2}, according to:

$$k'_{+2} = k''_{+2}(1 + [S]/K_m) \quad (1)$$

Cysteine proteinase activities against Z-Phe-Arg-NHMec (10 μM) were assayed at 37°C in a Perkin Elmer LS 50 fluorimeter at an excitation wavelength 360 nm and emission monitored at 460 nm. The enzyme concentration in the assays was 0.01–0.03 nM. Papain was dissolved in 0.1 M sodium phosphate buffer, pH = 6.5, containing 1 mM dithiotreitol and 2 mM EDTA. Bovine cathepsin B was dissolved in the same buffer with pH adjusted to 6.0 and was preincubated for 20 min. at room temperature before use. All determinations of k''_{+2}, were based on assays with less than 2% substrate hydrolysis and [I] > 5x[E]. A number of 3.5–4.5 enzyme half-lives and non-linear correlation coefficient greater than 0.990 was obtained in all measurements used to calculate k''_{+2}. K_m values for papain and bovine cathepsin B hydrolysis of Z-Arg-Phe-NMec were estimated by plotting k''_{+2} values in reaction with Z-Arg-Leu-Val-Gly-DAM against substrate concentration (5–30 μM), at constant enzyme concentration. The k'_{+2} value for Z-Arg-Leu-Val-Gly-DAM was then determined by extrapolation to zero substrate concentration and K_m was calculated from the equation (1).

N-Benzyloxycarbonyl-leucyl-leucyl-glycyl-diazomethane, (Z-Leu-Leu-Gly-DAM) (1): Z-Leu-Leu-Gly-OH (0.90 g, 2 mmoles) and N-methylmorpholine (220 μl) were dissolved in dry THF (5 ml) and cooled to –20°C. Isobutyl chloroformate (260 μl, 2 mmoles) was added and the mixture was stirred during 15 min. at –20°C. The precipitated salts were filtered off, washed with anhydrous THF (2 ml) and the combined filtrates were added to a cold solution of diazomethane in diethyl ether. The reaction mixture was kept at 0°C for 2 hours and then overnight at room temperature. Solvents were removed under reduced pressure and the product was preliminary isolated by recrystallization from methanol – water to give 0.70 g of crude **1** (yield: 74%). The product was finally purified by gel filtration using Sephadex LH-20 (Pharmacia) column and methanol as an eluent. Yield: 0.30 g (32%); m.p. = 64–66°C; $[a_D^{20}] = -22.5°$ (c = 1, MeOH); TLC: $R_f = 0.42$ (C), 0.81 (D). Anal. calcd. for $C_{23}H_{33}N_5O_5$ (461.66): C, 59.83%; H, 7.20%; N, 15.17%; found: C, 60.58%; H, 7.07%; N, 14.33%. IR (nujol, cm^{-1}): 3200, 2100, 1695, 1680, 1640, 1250, 750, 705. FAB MS: $[M+H^+] = 463$.

N-Benzyloxycarbonyl-arginyl-leucyl-leucyl-glycyl-diazomethane acetate, (Z-Arg(AcOH)-Leu-Leu-Gly-DAM) (2): Fmoc-Leu-Leu-Gly-OH (1.57 g, 3 mmoles) was dissolved in methylene chloride (50 ml) containing triethylamine (0.303 g, 3 mmoles). The solution obtained was cooled to –30°C and isobutyl chloroformate (0.414 g, 3 mmoles) was added. The reaction mixture was stirred for 30 min. after which a diazomethane solution in diethyl ether was added in *ca*. 2-fold excess. The reaction was carried out overnight at 4°C. Solvents were evaporated. The resulting crude Fmoc-Leu-Leu-Gly-DAM was preliminary isolated by precipitation with water from its methanol solution. The product obtained was treated with 20% piperidine in DMF (30 ml) for 30 min. and then solvents were evaporated *in vacuo*. The residue was triturated three times with 100 ml portions of hexane. The resulted crude H-Leu-Leu-Gly-DAM (0.92 g) was purified by preparative RP-HPLC (Peptide & Protein Vydac C-18, 25–35 μm, 50 ×

500 mm column) using 3% isopropanol in 0.1 M triethylammonium phosphate buffer, pH 7.0. Then the isolated product was desalted by sorption on XAD-7 (25 × 200 mm column), washing with 1 M NaCl (100 ml), water (80 ml) and elution with 80% methanol (700 ml). DCCI (0.412 g, 2 mmoles) was slowly added to a solution of H-Leu-Leu-Gly-DAM (0.65 g, 2 mmoles), Z-Arg-OH × HCl (0.690 g, 2 mmoles) and HOBt (0.552 g, 4 mmoles) in DMF (20 ml) with stirring on ice bath. The reaction was carried out for 3 h. After DCU filtering off the filtrate was evaporated to dryness in *vacuo*. The crude product was preliminarily isolated on S-Sepharose Fast Flow cation exchanger (25 × 350 mm column, NH_4^+ form) using 0.5 N CH_3COONH_4/EtOH, pH = 6.5 as an eluent. The final purification was performed by preparative HPLC (Peptide & Protein Vydac C-18, 25–35 μm, 50 × 500 mm column) using 27% CH_3CN/0.1 N aq CH_3COONH_4, pH = 6.5 as an eluent with flow rate 50 ml/min. UV detection at λ = 226 nm was applied. Yield: 0.94 g (13.9%); $[\alpha]_D^{22}$ = –43.1° (c = 1, MeOH); TLC: R_f = 0.9 (A). IR (nujol, cm^{-1}): 3220–3180, 2100, 1690, 1680, 1650–1625, 1235, 750, 700. FAB MS: $[M+H^+]$ = 617.

N-Benzyloxycarbonyl-isoleucyl-prolyl-glycyl-diazomethane (Z-Ile-Pro-Gly-DAM) (3): This compound was synthesized and isolated in the same manner as described for **1.** Yield: 0.34 g (38%); m.p. = 158–161°C (decomp.); $[\alpha]_D^{20}$ = –21.2° (c= 1, MeOH); TLC: R_f = 0.67 (C), 0.71 (D). Anal. calcd. for $C_{22}H_{29}N_5O_5$ (445.62): C, 59.79% ; H, 6.56% ; N, 15.80%; found: C, 60.01% ; H, 6.75% ; N, 15.09% . IR (nujol, cm^{-1}): 3220, 2090, 1695, 1680, 1650–1620, 1230, 745, 695. FAB MS: $[M+H^+]$ = 447.

N-Benzyloxycarbonyl-arginyl-isoleucyl-isoleucyl-prolyl-glycyl-diazomethane acetate, (Z-Arg(AcOH)-Ile-Ile-Pro-Gly-DAM) (4): This compound was synthesized and isolated in the same manner as described for **2**. Yield: 83 mg (10.7%); $[\alpha]_D^{22}$ = –61.4° (c = 0.4, MeOH); TLC: R_f = 0.55 (A). FAB MS: $[M+H^+]$ = 714.

N-Benzyloxycarbonyl-isoleucyl-valyl-glycyl-diazomethane, (Z-Ile-Val-Gly-DAM) (5): This compound was synthesized and isolated in the same manner as described for **1**. Yield: 0.34 g (33%); m.p. = 160–163°C; $[\alpha]_D^{20}$ = –25.1° (c = 0.5, MeOH); TLC: R_f = 0.36 (B). Anal. calcd. for $C_{22}H_{31}N_5O_5$ (447.64): C, 59.03%; H, 6.97%; N, 15.64%; found: C, 60.40%; H, 7.05%; N, 15.03%. IR (nujol, cm^{-1}): 3195, 2100, 1695, 1680, 1650–1625, 1240, 750, 706. FAB MS: $[M+H^+]$= 449.

N-Benzyloxycarbonyl-arginyl-threonyl-leucyl-alanyl-diazomethane acetate, (Z-Arg(AcOH)-Thr-Leu-Ala-DAM) (6): Fmoc-Leu-Ala-DAM (1.5 g, 3.35 mmol), obtained according to the procedure for Fmoc-Leu-Leu-Gly-DAM in the preparation of the compound **2**, was stirred with 30 ml 30% piperidine/DMF during 30 min. at 20°C. The solvents were then evaporated *in vacuo* and the residue was triturated three times with 100 ml portions of *n*-hexane. Yield: 0.72 g (95%) of crude H-Leu-Ala-DAM (oil). TLC: R_f = 0.90 (A). Compound **6** was synthesized from Z-Arg-Thr-OH (1.1 g, 2.6 mmol) and H-Leu-Ala-DAM (0.59 g, 2.6 mmol) in DMF using HBTU/HOBt activation method. The crude product was isolated from the reaction mixture in the same manner like the compound **2**. The final HPLC purification was performed using 25% CH_3CN / 0.1 N aq CH_3COONH_4 as an eluent for preparative HPLC. Yield: 0.13 g (7.4%). FAB MS: $[M+H^+]$ = 619.

RESULTS AND DISCUSSION

Syntheses of peptidyl-diazomethanes were performed according to the procedures described previously [15,16]. Due to low chemical and photochemical stability of the compounds, the main challenge in their preparation is the isolation and purification of the crude products. The protected peptidyl-diazomethanes, containing the arginine residue, were isolated from the reaction mixture using ion-exchange chromatography on S-Sepharose Fast Flow in nearly neutral conditions. The procedure allowed us to remove all non-cationic impurities. Ammonium acetate present in the mobile phase was removed by lyophilization. The final purification was performed by preparative RP–HPLC on Vydac Peptide & Protein C-18 column in neutral conditions, in the presence of ammonium acetate as ion-pairing agent. The purity of the compounds purified with this procedure was higher than 97% (HPLC). Better purity

was achieved using 0.1–0.2 M triethylammonium phosphate buffer, pH = 6.8 as ion-pairing agent, but this procedure needed an additional desalting step after purification. For this purpose reversed phase solid phase extraction (RP-SPE) was used successfully. Due to high photosensitivity of diazoketones, exposure of these compounds to the direct light should be avoided during all steps of the preparation procedure and especially during purification step. Some decomposition (up to 3%) was observed even during flow of an eluate containing peptidyl-diazomethane through the UV monitor cell. In the case of highly hydrophobic peptidyl-diazomethanes (without arginine residue or an unblocked amino group), the RP-HPLC was less suitable, due to their low solubility in water–organic solvent mixtures. Good results (purity about 95%) were achieved by separation (in darkness) on Sephadex LH-20, using methanol as an eluent. Results of the inhibition of papain, cathepsin B and antibacterial activity tests are presented in the Table.

Table. The inhibition rate (k'_{+2}) constants and the antistreptococcal activity of cysteine proteinase inhibitors structurally modelled on the N-terminal binding segment of various cystatins.

Comp. No.	Inhibitor	k'_{+2} [$M^{-1}s^{-1}$]		*Streptococcus pyogenes* growth inhibition zone*
		Papain	Cathepsin B (bovine)	
1	Z-Leu-Leu-Gly-DAM	15 600	n.d.**	++
2	Z-Arg-Leu-Leu-Gly-DAM	54 700	19 398	–
3	Z-Ile-Pro-Gly-DAM	3 521 ± 332	inactive	–
4	Z-Arg-Ile-Ile-Pro-Gly-DAM	1 468 ± 134	inactive	–
5	Z-Ile-Val-Gly-DAM	297 000	3 634 ± 527	++
6	Z-Arg-Thr-Leu-Ala-DAM	26 194 ± 5 250	n.d.	–
7	Z-Leu-Val-Gly-DAM [15]	448 000 ± 17 000	24 000 ± 1 200	+++
8	Z-Arg-Leu-Val-Gly-DAM [16]	559 000 ± 23 000	39 600 ± 800	–
9	Z-Leu-Ala-Gly-DAM [21]	10 900	1 980	+
	E-64 [16] ***	813 000 ± 27 000	53 500 ± 2 000	n.d.

*Inhibition zone diameter: – = no zone; (+) = 6–7 mm; + = 8–11 mm; ++ = 12–15 mm; +++=>15 mm;
** n.d. = not determined; *** E-64 – 1-[L-N-trans-(epoxysuccinyl)leucyl]amino-4-(guanidino)butane.

All newly obtained peptidyl-diazomethanes (compounds **1**–**6** of the table) inhibited papain irreversibly, although their inhibition rate constants were lower than those for compounds modelled on human cystatin C (Z-Leu-Val-Gly-DAM and Z-Arg-Leu-Val-Gly-DAM). Interestingly, the papain inhibition rate constants of the peptidyl-diazomethanes based on N-terminal binding fragment of bovine cystatin C (compounds **1** and **2** of the Table) were lower than the ones measured for corresponding inhibitors based on the structure of human variant of this protein (Z-Leu-Val-Gly-DAM and Z-Arg-Leu-Val-Gly-DAM, respectively). In the case of com-

pounds **3**–**6** the observed inhibition rate constants were lower than those for peptidyl-diazomethanes based on human cystatin C (compounds **7** and **8**). For the elongated peptides (**2**, **8**) the inhibition rate constants measured against cathepsin B are much lower than those against papain, although in the case of the compound **2** the difference is much less pronounced.

In the case of peptidyl-diazomethanes derived from N-terminal binding fragment of human cystatin S, (compounds **3** and **4**) elongation of the peptide chain led to the decrease of the inhibition rate constants against papain. Additionally, both compounds did not inhibit cathepsin B.

As it was mentioned above, the cystatin C–derived peptidyl diazomethane – Z-Leu-Val-Gly-DAM possessed a very narrow antibacterial activity against the cysteine protease producing *Streptococcus* gr. A. A similar compound, based on the same cystatin – Z-Arg-Leu-Val-Gly-DAM – was more efficient inhibitor of papain, however, it was completely devoid of antibacterial properties. Therefore, we decided to test whether diazomethylketones other than those based on cystatin C inhibited streptococcal cysteine protease and/or possessed antibacterial activity. Compounds **1**, **2**, **5** and **6** showed the activity against streptococcal protease, although their action seemed to be rather slow. Interestingly, compounds containing arginine moiety (**2** and **6**) were completely devoid of the antistreptococcal activity. Additionally, compound **3**, containing the proline residue in the position P_2, was inactive in agar well diffusion test, whereas the similar compound with valine in this position (Z-Ile-Val-Gly-DAM, **5**) inhibited growth of the bacteria. On the other hand, in the case of cysteine protease inhibitors based on cystatin C, only peptidyl-diazomethanes possessed antibacterial activity [22]. These results seem to suggest that the bactericidal properties are not directly related with inhibition of streptococcal cysteine protease.

Abbreviations: The symbols of amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*European J. Biochem*., **138**, 9 (1984)). Other symbols are used as follows: DAM, diazomethyl; HBTU, O-benzotriazol-1-yl-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate; –NHMec – 7-(4-methyl)-coumarylamide.

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