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Selective Inhibitors of Plasmepsin II of *Plasmodium falciparum* on the Basis of Pepstatin

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Abstract—A number of new inhibitors of plasmepsin II (PlmII) *Plasmodium falciparum*, which was one of the key factors of survival of malarial parasite, was synthesized. The inhibitors were analogues of pepstatin with different substitutions for the alanine residue. Effects of the inhibitors on human PlmII and cathepsin D were studied. Inhibition of PlmII by the substrate was found. This discovery required modification of the Henderson method for determination of inhibition constants. Two synthesized inhibitors were shown to exhibit a pronounced selectivity to PlmII (K_i = 5.5 and 5 nM) in comparison with that of cathepsin D (K_i = 230 and 3000 nM, respectively).

Key words: malaria, proteases, inhibitors, plasmepsin II, Plasmodium falciparum

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INTRODUCTION

Elaboration of treatment modes of malaria is one of priority directions in the world. Urgency of this problem is determined by social factors and the absence of effective treatment[2].

According to World's Public Health Organization, malaria morbidity caused by the *Plasmodium* is 500 millions people every year, including 2 millions of fatal terminations (predominantly among children) [1]. Fast variability of the parasite and stability to the known medicines requires a search for novel antimalarial agents with a new mechanism of action. Four *Plasmodium* species pathogenic for humans are known: *vivax*, *ovale*, *malariae*, and *falciparum*. The latter is the most dangerous.

The key factor of survival of the etiological malarial agent is the presence of aspartyl proteases in its cells. They are called plasmepsins (PlmI–PlmV) and participate in catabolism of hemoglobin, which is a food resource of the parasite during intraerythrocyte development of the plasmodium. These enzymes (first of all PlmII, EC 3.4.23.39) are considered to be the most promising targets for therapy of malaria, because their specific activation by inhibitors results in the complete disorder of metabolism of the parasite cells and their fast death [2].

Degradation of hemoglobin proceeds in a food vacuole, the proteolytic compartment of *P. falciparum* with pH 5.0–5.4 [3, 4]. Plasmpepsins PlmI and PlmII recognize hemoglobin and hydrolyze initially single peptide bond between Phe33 and Leu34 [5] that is located in the region of a loop of the hemoglobin α-chain. Amino acid sequence of this chain is highly conservative in all hemoglobins of vertebrates: -EALERMF[33]-L[34]SFPTTK-. It is believed that the ordered decomposition of hemoglobin is initiated by aspartate proteases (PlmI and PlmII). The subsequent hydrolysis proceeds by the action of other enzymes of the food vacuole of the *P. falciparum*, for example by the cysteine protease, falcipain [5–7].

PlmI and PlmII, like other aspartate proteases, are synthesized as inactive precursors, but proareas of the plasmodium proteases are much longer than those of other zymogens: 123 and 124 amin acid residues, respectively [8]. A propeptides is cleaved in vitro as a result of autoprocessing of the precursors at acidification to pH 4.5–5.0 with the formation of mature active enzymes with molecular mass of 37 kDa. Their sizes are identical to those of proteases directly isolated from *P. falciparum* [9]. Activities of natural and recombinant forms of PlmII have been shown to be identical [4].

Specificity of the recombinant PlmII was studied using a number of synthetic chromogenic and fluorogenic substrates [10–14]. These substrates were chosen on the basis of the aforementioned amino acid sequence

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[2] Abbreviations: Abbreviations: CatD, cathepsin D; *CS*, the H-Leu-Glu-Arg-Ile-Phe-Phe(NO_2)-Ser-Phe-OH chromogenic substrate; PlmII–PlmV, plasmepsins II–V; Pst, pepstatin.

Table 1. Hydrolysis constants of the (**CS**) substrate by plasmepsin II and human cathepsin D in 0.1 M sodium formate buffer at 37°C

Enzyme	pH	[S], μM	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM min^{-1}	K'_S, μM
PlmII	4.4	3–10	9.53	466	48.90	–
		20–90	–	466	–	5.05
CatD	3.5	10–100	91.00	450	4.94	–

that surrounds the primary site of hydrolysis of the hemoglobine α-chain. The chromogenic substrates were prepared by substitution of the residue of *p*-nitrophenylalanine, Phe(NO_2) for Leu in the P1'-position. Easily oxidable Met residue in the P2-position was replaced by Thr [10], Ile, Val, or Nle [13]. We used the H-Leu-Glu-Arg-Ile-Phe-Phe(NO_2)-Ser-Phe-OH (**CS**) chromogenic substrate [11] in this study.

The highly pure pro-PlmII preparation was prepared by expression of the recombinant gene encoding the last 48 amino acid residues of the propeptides and all mature enzyme in *E. coli* [10]. The three-dimensional structure of PlmII was determined. It has a typical topography of aspartate proteases of eukaryotes [12].

It is very important to find compounds of selective action for prevention of possible inhibition of the activity of endogenous aspartate proteases, especially rennin and cathepsin D (EC 3.4.23.5) when inhibitors of plasmepsins are used. Highly selective inhibitors of PlmII, which are derivatives of pepstatin, were prepared in this study.

RESULTS AND DISCUSSION

Kinetic parameters of hydrolysis of the **CS** substrate by plasmepsin II and human cathepsin D are given in Table 1. Values of the kinetic constants of the hydrolysis of the **CS** substrate by PlmII determined by us (Table 1) are rather close to the literature data (k_{cat} 780 min^{-1}; K_m 10 μM, k_{cat}/K_m 78 $\mu M^{-1}min^{-1}$ [11]). However, we found that kinetics of hydrolysis of high concentrations of the substrate is deviated from the classic dependence of Michaelis–Menten (Fig. 1). Such kinetic dependence corresponds to inhibition by the substrate:

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P.$$
$$\Big\Updownarrow K'_S$$
$$ES_2$$

The equation of the starting reaction rate looks as follows:

$$v_0 = k_{cat}[E][S]/(K_m + [S] + [S]^2/K'_S). \quad (1)$$

The value of the inhibition constant by the substrate determined by us K'_S (Table 1) proved to be even lesser then the Michaelis constant, suggesting unusually strong inhibition of PlmII by the **CS** octapeptide at $[S] > K_m$. We did not observe the substrate inhibition in the case of cathepsin D in the studied concentration range (10–100 μM) (Table 1).

The known inhibitor of aspartate proteases (Iva-Val-Val-Sta-Ala-Sta) was taken as a basic structure of the inhibitors synthesized by us. Iva was the residue of isovaleric acid, and Sta was the residue of statin: –HN–$CH(CH_2CH(CH_3)_2)CH(OH)CH_2COOH$ [8]. We synthesized five modified peptide inhibitors (**I-1**)–(**I-5**) of the Y-Val-Val-Sta-X-Sta-OH general structure:

(**I-1**): X – –HN–$CH(CH_2$–O–$(CH_2)_2$–CH_2–OH)–CO–; Y – Iva;

(**I-2**): X – –HN–$CH((CH_2)_3$–CH_2–OH)–CO–; Y – Iva;

(**I-3**): X – –HN–$CH(CH_2$–O–$(CH_2)_2$–CH_2–OH)CO–; Y – $(CH_3)_2$CH–CH(CH_2–CH_2OH)–CO–;

(**I-4**): X –Ala; Y – $(CH_3)_2$CH–CH(OH)–CO–;

(**I-5**): X – –HN–$CH(CH_2$–O–$(CH_2)_2$–CH_2–OH)–CO–; Y – $(CH_3)_2$CH–CH(OH)–CO–.

Fragments X and Y for compounds (**I-1**)–(**I-5**) were prepared with the protective groups necessary for the further peptide synthesis. (*S*)-2-*tert*-Butyloxycarbonyl-

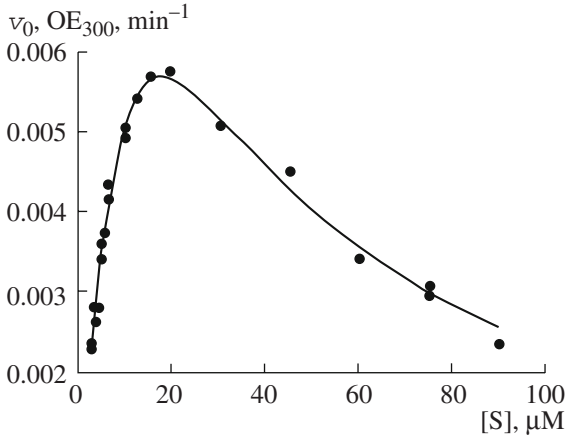

Fig. 1. Inhibition of plasmepsin II (17.6 nM) by the **CS** substrate; [**CS**] 3–90 μM, 0.1 M sodium formate buffer, pH 4.4, 37°C.

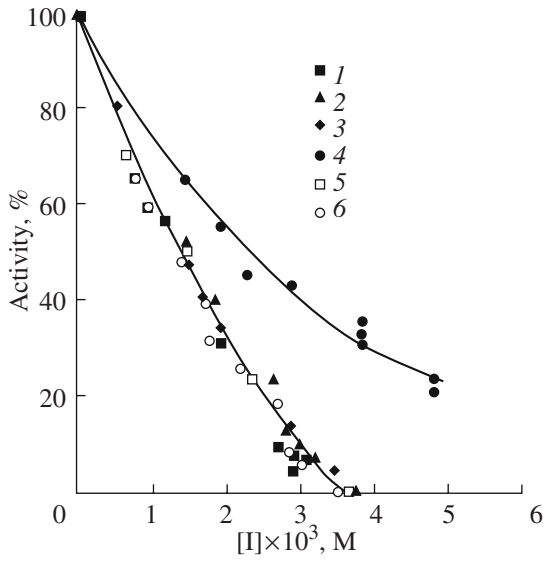

Fig. 2. Inhibition of plasmepsin II (30 nM) in the course of hydrolysis of the **CS** substrate (60 μM) by the following inhibitors: *1*, Pst; *2*, (**I-1**); *3*, (**I-2**); *4*, (**I-3**); *5*, (**I-4**); *6*, (**I-5**), in 0.1 M sodium formate pH 4.4 at 37°C.

6-benzyloxyhexanoic acid (for the X fragment in compound (**I-2**)) was prepared by deamination of N^{α}-*Z*-*L*-lysine with sodium nitroprusside followed by replacement of Z-protecting group by Boc-group and attachment of benzyl group to free ω–OH function. The overall yield of target product was 52%. For the synthesis of 2-*tert*-butyloxycarbonylamino-7-benzyloxy-4-heptanoic acid (for the X fragment in compounds (**I-1**), (**I-3**), an (**I-5**)), N-Boc-*L*-serine was converted into its methyl ester by the treatment with diazomethane. The obtained ester was alkylated with 3-benzyloxypropylbromide, and the free acid was prepared by alkaline hydrolysis. The overall yield was 41%. 4-Benzyloxy-2-isopropylbutanoic acid (for the Y fragment in compound (**I-3**) was synthesized by alkylation of diethylester of malonic acid with isopropylbromide, Saponification, and decarboxylation of the diethyl ester with 2-benzyloxy-2-isopropylmalonic acid. The target acid was prepared with the overall yield of 47%.

The dose-dependent inhibition of PlmII and CatD by compounds (**I-1**)–(**I-5**) and pepstatin A was studied using the **CS** substrate. The obtained titration curves correspond to the type of an inhibitor with a high affinity in all the cases (Fig. 2) that is characteristic of the inhibition of aspartate proteases by pepstatin [8]. The K_i values are usually determined by the Henderson equation [15]:

$$[I]/(1 - v_i/v_o) = [E] + (K_i \times \boldsymbol{A})v_0/v_i, \qquad (2)$$

where v_0 is the starting rate of hydrolysis of a substrate by an enzyme with the starting concentration [E] in the absence of an inhibitor, and v_i is the same rate in the presence of an inhibitor with the starting concentration [I]. Noefficient $\boldsymbol{A}$ depends on the type of inhibition: $\boldsymbol{A} = 1$ in the case of uncompetitive inhibition, and $\boldsymbol{A} = ([S] + K_m)/K_m$ for competitive inhibitors, including Pst [15].

The starting enzyme concentration is determined from the linear dependence of the $[I]/(1 - v_i/v_o$ on v_o/v_i according to intersection with the ordinate axis using the Henderson equation for strongly bound inhibitors. The K_i value is calculated from the tangent of the angle of the curve slope. All the studied inhibitors are the concurrent inhibitors, like pepstatin, because the tangents of the angles of slope for the inhibitors and the enzymes increase with the enhancement of the substrate concentration (see Table 2, inhibitor (**I-3**)). According to the Henderson equation [15], the value of corrective $\boldsymbol{A} = ([S] + K_m)/K_m$. We used high concentrations of the **CS** substrate for enhancement of the accuracy. The value of this increasing factor **A** is experimentally established and proved to be 2.65 at [**CS**] = 150 or 1.33 at [**CS**] = 30 μM in the case of cathepsin D (K_m = 91 μM) and 7.29 at [**SC**] = 60 μM in the case of plasmepsin II (K_m = 9.53 μM). The experimentally obtained values of $K_i \times \boldsymbol{A}$ (columns 2 and 5 for PlmII and CatD, respectively) and the values of inhibition constants calculated by the Henderson equation [15] (columns 3 and 6) are given in Table 3. The value of inhibition constant of CatD by pepstatin determined by us practically coincides with the literature data (3.8 nM, [16]). However, the calculated value of K_i for pepststin and plasmepsin (31 nM, Table 3) is one order higher than that known from the literature (3–5 nM) [8]. The effects of strong

Table 2. Inhibition of plasmepsin II (12 nM) by compound (**I-3**) at different concentrations of the **CS** substrate (0.1 M sodium formate buffer, pH 4.4, 37°C)

[**CS**], μM	$K_i \times \boldsymbol{A}$, nM (tangent of the angle of the slope)	$\boldsymbol{A} = ([S] + K_m)/K_m$ [15]		$\boldsymbol{A} = (K_m + [S] + [S]^2/K'_S)/ K_m$	
		$\boldsymbol{A}$	K_i, nM	$\boldsymbol{A}$	K_i, nM
1	2	3	4	5	6
60	12.9	7.29	1.77	82.1	0.157
30	6.70	4.35	1.54	22.85	0.293
15	1.20	2.57	0.468	7.23	0.166

Table 3. Inhibition of plasmepsin II (pH 4.4) and human cathepsin D (pH 3.5) in the course of hydrolysis of the CS substrate; [S] 60 μM, 0.1 M sodium formate buffer, 37°C

Inhibitor	PlmII			CatD	
	$K_i \times A$, nM (tangent of the angle of the slope)	K_i, pM		$K_i \times A$, pM (tangent of the angle of the slope)	K_i, pM*
		A = 7.29*	A = 82.1**		
1	2	3	4	5	6
Pst	0.226	31.10	2.75	5.01***	3.77***
(I–1)	0.178	24.4	2.15	5.32***	4.00***
(I–2)	0.291	39.9	3.54	5.75***	4.32***
(I–3)	12.9	1770	157	3990***	3000***
(I–4)	0.451	61.9	5.49	623****	235****
(I–5)	0.413	56.6	5.03	8000****	3020****

Notes: * By the Henderson method.
** By our method.
*** [**CS**] = 30 μM; A = 1.33.
**** [**CS**] = 150 μM; A = 2.65.

substrate inhibition of PlmII by the **CS** substrate discovered by us (Table 1 and Fig. 1) allowed a proposal that the Henderson equation should be modified for calculations of the K_i values in experiments with a high concentration of a substrate (60 μM). Indeed, the substrate inhibition was absent I the case of cathepsin D, and the value of inhibition constant by pepstatin calculated by the Henderson equation was not distinguished from the literature data.

Dependence of the inhibition constants calculated by the Henderson equation on the substrate concentration (Table 2, column 4) is another argument in favor of inutility of the classic Henderson method for plasmepsin.

We discussed the modification of Henderson equation for the special case of strong substrate inhibition. The equation for calculation of the starting rate of hydrolysis in the presence of competitive inhibitor looks as follows:

$$v_i = k_{cat}[E][S]/\{(\mathbf{K_m} + [\mathbf{S}]) + K_m[I]/K_i\}. \quad (3)$$

Equation 3 results in the usual Henderson equation (2), where $\boldsymbol{A} = ([S] + K_m)/K_m$. Therefore, tangent of the angle of slope of the Henderson graph (2) is 1.33–2.65 × K_i for cathepsin D and 7.29 × K_i for plasmepsin II under our experimental conditions in the case of the strongly-binding competitive inhibitor.

However, equationfor the starting rate of hydrolysis in the presence of inhibitor becomes more complex with consideration of the substrate inhibition (1):

$$v_i = k_{cat}[E][S]/\{(\mathbf{K_m} + [\mathbf{S}] + [\mathbf{S}]^2/\mathbf{K'_S}) + K_m[I]/K_i\}. \quad (4)$$

The general view of the Henderson equation remains the same, but the increasing factor $\boldsymbol{A}$ looks as:

$$\boldsymbol{A} = (\mathbf{K_m} + [\mathbf{S}] + [\mathbf{S}]^2/\mathbf{K'_S})/K_m. \quad (5)$$

Evidently, this factor dramatically increases with the enhancement of the substrate concentration. Indeed, tangent of the angle of slope of the Henderson graph (2) in he case of plasmepsin II (calculated using kinetic parameters of Table 1, and the substrate concentration of 60 μM) is 82.1 × K_i instead of 7.29 × K_i calculated as usual:

$$\boldsymbol{A} = (9.53 + 60 + 3600/5.05)\ \text{мкМ}/9.53\ \text{мкМ} = 82.1.$$

It is evident that reliability of determination of the K_i values significantly increases in the case of high concentrations of substrate and inhibitor. True values of the inhibition constants of PlmII for compounds (**I-1**)–(**I-5**) and pepstatin calculated by our modification of the Henderson equation are presented in Table 2 (column 6) and Table 3 (column 4). The value of inhibition constant of PlmII by pepstatin coincides with the literature data [8].

Compound (**I-3**) is the weakest inhibitor of plasmepsin from all the studied compounds. The K_i value for it is two orders higher than that for pepstatin and compounds (**I-1**), (**I-2**), (**I-4**), and (**I-5**) (Table 3). The K_i values for these inhibitors are of the same order than that for pepstatin (≈pM). It should be especially noted that the inhibition of human cathepsin D by compounds (**I-4**) and (**I-5**) is two and three orders lower in comparison with plasmepsin II, respectively. The selectivity of the inhibitors of the target enzymes appears o be one of

the important factors for creation of therapeutic agents along with their high inhibiting ability. The plasmepsin inhibitors (**I-4**) and, especially (**I-5**) studied by us are evidently promising for pharmacology.

EXPERIMENTAL

Synthesis of inhibitors and substrate. TLC analysis was performed on Silufol plates (Chemapol, Czech Republic). Chromatographic purification was carried out on columns filled with SiO_2 (Merck G-60, Germany) eluted with the mixture of chloroform and methanol (7 : 1). ^{1}H NMR spectra were recorded on a Unity Inova spectrometer (400 MHz) in $CDCl_3$. The residual protons of solvent were used as an internal standard. Melting points were determined on a Boetius device.

Fragment Y, 2-hydroxy-3-methylbutanoic acid used for synthesis of compounds (**I-4**) and (**I-5**) was purchased from Aldrich company (United States).

(2*S*)-6-Benzyloxy-2-*tert*-butyloxycarbonylaminohexanoic acid (for fragment X in (**I-2**)). ***(2S)-2*-benzyloxycarbonyl-amino-6-hydroxyhexanoic acid** was prepared by deamination of N^{α}-*Z*-*L*-lysine with sodium nitroprusside [17] with the yield of 80%. Water (10 ml) and 10% Pd/C (0.5 g) were added to the solution of (2*S*)-2-benzyloxycarbonyl-amino-6-hydroxyhexanoic acid in methanol (100 ml), and the reaction mixture was subjected to hydrogenolysis in the hydrogen current until the disappearance of the starting compound. The catalyst was filtered off and washed with water on a filter. The joined filtrate was evaporated to dryness. Water (10 ml) and ethanol (110 ml) were added to the residue. The precipitated crystals were separated, washed with anhydrous ethanol, and dried in vacuum over alkali. **(2*S*)-2-amino-6-hydroxyhexanoic acid** was obtained with the yield of 2.63 g (95%); mp 251°C; $[\alpha]_D^{20}$ + 23° [18]; ^{1}H NMR spectrum (D_2O): 1.45 (2 H, m, H4), 1.62 (2 H, m, H5), 1.91 (2 H, m, H3), 3.64 (2 H, t, *J* 7.5, H6), 3.78 (H, t, *J* 6.8, H2).

Triethylamine (2.5 ml, 18 mmol) and di(*tert*-butyl)pyrocarbonate (4.44 g, 20.5 mmol) were added to the solution of (2*S*)-2-amino-6-hydroxyhexanoic acid (2.50 g, 17 mmol) in the mixture of THF and water (1 : 1, 30 ml). The reaction mixture was stirred for 6 h, diluted with water (50 ml), and THF was evaporated in vacuum. The aqueous solution was washed with hexane (2 × 10 ml) and acidified to pH 3. The target product was extracted with ethyl acetate (5 × 20 ml) and dried over Na_2SO_4. Ethyl acetate was evaporated. The residue was purified by chromatography (ethyl acetate was used as an eluent). **(2*S*)-2-*tert*-butyloxycarbonylamino-6-hydroxyhexanoic acid** was prepared with the yield of 3.70 g (88%); mp 112°C, $[\alpha]_D^{20}$ −6.36° [19]; ^{1}H NMR: 1.43 (2 H, m, H4), 1.50 (9 H, s, CH_3), 1.68 (2 H, m, H5),1.95 (2 H, m, H3),3,66 (2 H, t, *J* 7.5, H6), 3.80 (H, m.s, OH), 4.48 (H, t, *J* 6.9, H2), 5.20 (H, m.s, NH), 11.80 (H, m.s, COOH).

The solution of (2*S*)-2-*tert*-butyloxycarbonylamino-6-hydroxyhexanoic acid (2.47 g, 10 mmol) in anhydrous ether (30 ml) was treated with the solution of CH_3N_2 in ether (20 ml). After completion of the reaction, ether was evaporated in vacuum. The residue was dissolved in anhydrous dimethylformamide (20 ml), cooled to 0°C and mixed with 55% suspension (0.5 g) of NaH in oil (11.4 mmol of NaH). After termination of the H_2 liberation, benzyl bromide (1.71 g, 10 mmol) was added. The reaction mixture was poured out into water (100 ml). The pH value was brought up to 7, and the precipitated oil was extracted with ethyl acetate (5 × 25 ml). The extracts were washed, the solvent was evaporated, and NaOH was added to the residue dissolved in methanol (60 ml). The free acid was prepared by the treatment of the salt with the DOWEX 50 × 8 ion-exchange resin in H^+-form and purified by chromatography. ***(2S)-6*-Benzyloxy-2-*tert*-butyloxycarbonylaminohexanoic acid** was obtained as colorless oil with the yield of 2.60 g (77%); ^{1}H NMR: 1.41 (2 H, m, H4), 1.49 (9 H, s, CH_3), 1.66 (2 H, m, H5), 1.95 (2 H, m, H3), 3.30 (2 H, t, *J* 7.5, H6), 4.50 (H, t, *J* 7.0, H2), 4.58 (2 H, s, C H_2Ph), 5.18 (H, broadened s, NH), 7.10–7.40 (5 H, m, Ph), 11.40 (H, s, COOH).

Found, %: C 63.91, H 8.00, N 4.06. Calcd. for $C_{18}H_{27}NO_5$, %: C 64.07, H 8.07, N 4.15.

***2-tert*-Butyloxycarbonylamino-7-benzyloxy-4-oxaheptanoic acid** (for fragment X in compounds (**I-1**), (**I-3**), and (**I-5**). The solution of *N*-Boc-*L*-Ser-OH (4.1 g, 20 mmol) in methanol (20 ml) was treated with CH_3N_2 in ether. After completion of the reaction, acetic acid (0.5 ml) was added to the reaction mixture. The solvents were evaporated, and the residue was purified by flesh-chromatography (SiO_2, Merck G-60) in the mixture of chloroform and ethyl acetate (1 : 1). *N*-Boc-*L*-Ser-OMe was prepared as colorless oil with the yield of 3.94 g (90%). It was dissolved in anhydrous dimethylformamide (30 ml) and cooled to 0°C. The 55% suspension (0.87 g) of NaH (20 mmol) in oil was added, and the reaction mixture was stirred for 1 h. $PhCH_2O(CH_2)_3Br$ (4.85 g) was added dropwise, and the reaction mixture was stirred for 2 h at 0°C and kept for a night at room temperature. The free acid was isolated as described above. Chromatographic purification yielded 3.18 g (45%) of 2-*tert*-butyloxycarbonyl-7-benzyloxy-4-oxaheptanoic acid as colorless oil; ^{1}H NMR: 1.49 (9 H, s, CH_3), 1.83 (2 H, m, H6), 3.45 (2 H, m, H3), 3.65 (4 H, m, H5 and H7), 4.38 (H, m, H2), 4.50 (2 H, s, C H_2Ph), 5.80 (H, broadened s, NH), 7.18–7.23 (5 H, m, Ph), 11.20 (H, s, COOH).

Found, %: C 61.00, H 7.74, N 4.01. Calcd. for $C_{18}H_{27}NO_6$, %: C 61.17, H 7.70, N 3.96.

***4*-Benzyloxy-*2*-isopropylbutanoic acid** for fragment Y in compound (**I-3**). Isopropylmalonic ester (5.05 g, 25 mmol) [20] was added to the 55% suspension of NaH (1.1 g) in anhydrous dimetgylformamide (30 ml) at room temperature on stirring. The reaction mixture was stirred for 1 h, and 2-benzyloxyethyl bromide (5.38 g, 25 mmol) [21] was added. The reaction mixture was stirred for 6 h at 75°C and 12 h at 50°C, cooled, and poured out into water (150 ml). The pH value was brought out to 7, and the precipitate was extracted with ethyl acetate (4 × 40 ml). The joined extracts was washed and dried over Na_2SO_4 and evaporated. The yield of diethyl ester of **2-benzyloxyethyl(isopropyl)malonic acid** was 6.30 g (75%); ^{1}H NMR: 0.95 (6 H, d, *J* 6.8, CHC$\underline{H}_3$), 1.18 (6 H, t, *J* 6.5, $CH_2C\underline{H}_3$), 2.19 (2 H, t, *J* 6.5, $OCH_2C\underline{H}_2$), 2.30 (H, m, CH), 3.50 (2 H, t, *J* 6.0, $OC\underline{H}_2CH_2$), 4.10 (4 H, q, *J* 6.5, $C\underline{H}_2CH_3$), 4.41 (2 H, s, $C\underline{H}_2Ph$), 7.18–7.25 (5 H, m, Ph).

2 M NaOH (20 ml) was added to the solution of diethyl ester of 2-benzyloxyethyl(isopropyl)malonic acid (5.38, 16 mmol) in ethanol (100 ml). The reaction mixture was boiled for 8 h, cooled, acidified to pH 3, and evaporated to dryness. ***2*-Benzyloxyethyl(isoporopyl)malonic acid** was extracted from the residue with ether, ether was evaporated, and the acid was decarboxylated by heating for 1 h at 180°C. The product was distilled in a vacuum. ***4*-Benzyloxy-2-isopropylbutanoic acid** was obtained as colorless oil in yield of 2.64 g (70%); mp 116–120°C/0.1 mm of Hg column; ^{1}H NMR: 0.92 (6 H, d, *J* 6.8, C$\underline{H}_3$), 1.90 (2 H, m, CHC$\underline{H}_2$), 2.16 (H, m, C$\underline{H}$COOH), 2.32 (H, m, C$\underline{H}(CH_3)_2$), 3.43 (2 H, m, $OC\underline{H}_2CH_2$), 4.44 (2 H, s, $C\underline{H}_2Ph$), 7.18–7.25 (5 H, m, Ph), 10.0 (H, s, COOH).

Found, %: C 71.00, H 8.50. Calcd. for $C_{14}H_{20}O_3$, %: C 71.16,H 8.53.

Synthesis of peptide inhibitors (I-1)–(I-5) and the H-Leu-Glu-Arg-Ile-Phe-Phe(NO_2)-Ser-Phe-OH substrate (**CS**) was performed by the solid phase method according to the Boc-strategy on a modernized Beckman 990 synthesizer (United States). Reagents and amino acid derivatives were purchased from Reanal (Hungary), PRF (Japan), Advanced Chem. Tech., Aldrich (United States), and Fluka (Switzerland). The starting polymeric carrier of the peptide synthesis was prepared by attachment of Boc-Sta-OH (Neosystem, France) to the chlormethylated polymer (Advanced Chem. Tech., United States) according to the standard procedure via cesium salt of Boc-Sta-OH [22]. The amino acid content on the polymer was 0.25 mmol/g. Condensations were carried out by DCC in the presence of HOBu (1 : 1) with 10-min preactivation at 0°C. The reagents were taken in 3.5-fold excess. Temporary Boc-group was removed by the treatment with 30% solution of trifluoroacetic acid in chloroform. The peptides were cleaved from the resin and deprotected by the treatment with liquid HF in the presence of *p*-cresol (10/1) at 0°C for 1 h. When reaction was finished, HF was removed in vacuum. The peptides were precipitated from reaction mixtures by the addition of diethyl ether (15 ml). The resin and a deprotected peptide were filtered off, washed with ether (5 × 10 ml), and dried on a filter. The peptides were extracted with 50–95% solution of acetic acid depending on their hydrophobicity, frozen, and lyophilized.

Gel filtration was performed on a column (800 × 25 mm) with Sephadex LH-20 (Pharmacia, Sweden) in methanol at a flow rate of 0.8 ml/min at 226 nm (Multirac 2158, LKB, Sweden). The preparative HPLC was carried out on a System Gold chromatograph (Beckman, Sweden) equipped with an Ultrasphere ODS column (7 μm, 25 × 2.0 cm) in a gradient of acetonitrile (from 10 to 90%) in 0.23 M $(NH_4)_2NaPO_4$ (pH 6.1) at a flow rate of 6 ml/min at 220 nm. A fraction containing a target product was evaporated, and another gel filtration on the column with Sephadex LH-20 in 80% methanol was performed.

Analytical HPLC was carried out on a System Gold chromatograph (Beckman, Sweden) equipped with an Ultrasphere ODS column (5 μm, 250 × 2.0mm) in the same gradient regime at a flow rate of 0.25 ml/min at 220 nm.

Purity of the synthesized peptides was 95% according to the analytical HPLC. The peptides were characterized by mass spectrometry on a MALDI-TOF Vision 2000 mass spectrometer (United States). The determined molecular masses were: 760 Da for (**I-1**), 744 Da for (**I-2**), 804 for (**I-3**), 702 for (**I-4**), 776 for (**I-5**), and 1103 for the substrate.

Expression and isolation of PlmII. Gene for the target protein encoding the short form of pro-PlmII (the 76-453 fragment according to the numeration of the full-size zymogen) were earlier cloned in the pET-23a(+) vector (Invitrogen, United States). Cells of the BL21 (DE3) strain were transformed, grown at 37°C for a night in the presence of 100 mg/ml of ampicillin, and reseeded into preliminary agarized medium to achieve the 100-fold dilution of the night culture. The culture was induced at the suspension density of 0.5 OU_{600} by the addition of 1 mM of isopropyl-β-*D*-thiogalactopyranoside and incubated in a regime of intensive stirring for 3 h under the same temperature. The cell biomass (10 g from 5 l of the culture) was separated by centrifugation and used for protein isolation.

The cell paste (5 g) was resuspended in 20 ml of buffer A (50 mM Tris-HCl, pH 8.0, o.1 M NaCl, 2 mM EDTA) treated with ultrasound, diluted with the cooled buffer A in 6 times, and centrifuged for 1 h at 100000 g. Insoluble fraction of lysate that contained the target protein was resuspended in 60 ml of buffer B (50 mM Tris-HCl, pH 8.0, 1% Triton X-100). The suspension was stirred for 30 min and centrifuged at 100000 g for 20 min. The supernatant was removed, and the residue was washed by centrifugation with buffer B (120 ml) without the detergent two times. Incorporation corpus-

cles were dissolved in 20 ml of the denaturing buffer (6 M urea, 0.5 M Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM β-mercaptoethanol), stirred for a night, and centrifuged at 100000 g for 30 min. The supernatant was diluted with buffer C (50 mM Tris-HCl, pH 8.5) in 50 times by dropping through a peristaltic pump at a flow rate of 0.5 ml/min. The protein refolding was performed for 16 h (25°C) at slow stirring of the solution. The solution of the enzyme after the refolding (1 l) was filtered through a membrane (pore size of 0.45 μm), concentrated in 20 times on a cellulose membrane that retained proteins with molecular masses higher than 10 kDa, and used for anion-exchange chromatography.

The protein solution was applied onto Q-Sepharose (10 ml) equilibrated with buffer C (pH 8.0) at a flow rate of 1 ml/min. The column was washed with ten volumes of the same buffer and eluted with a linear gradient of NaCl (from 0 to 1 M) in buffer C (pH 8.0) (ten volumes of the column) at the same flow rate. Fraction of 2 ml were collected and analyzed by electrophoresis. Fractions containing the target protein were joined and concentrated to the volume of 2 ml using Amicon Ultra-15 centrifuge concentrators.

Gel filtration was performed on a column with Superdex 200 16/60 (Amersham Biosciences) in 20 mM Tris-HCl-buffer (pH 8.0) containing 0.2 M NaCl at a flow rate of 0.2 ml/min. Fractions of the target protein were joined and used in further studies. The average yield of the enzyme was 20 mg per 10 g of the cellular biomass. The purity of final preparation was no less than 95%.

Kinetic measurements. The rate of hydrolysis of the **CS** chromogenic substrate by PlmII was registered on a Gilford 2400-2 spectrophotometer (United States) at 37°C according to a decrease in optical absorption at 300 nm in 0.1 M sodium formate buffer (pH 4.4) containing 3% dimethylsulfoxide. $\Delta\varepsilon_{300}$ 1700 M^{-1} cm^{-1}. Active PlmII were prepared by incubation of a reserve solution of pro-PlmII 5-fold diluted with 0.1 M sodium formate buffer (pH 4.4) for 30 min at 37°C.

The enzyme concentration was determined by titration of its active site with pepstatin A (Sigma, United States).

Activity of human cathepsin D (Sigma, United States) was evaluated using the same substrate at pH 3.5.

Inhibition of PlmII and CatD by compounds (I-1)–(I-5) was studied at 37°C in 0.1 M sodium formate buffer (pH 4.4 and 3.5 for the first and the second enzyme, respectively). Pepstatin was sued as inhibitor under the same conditions for comparison.

The values of kinetic parameters of hydrolysis of the substrate (k_{cat} and K_m) for CatD were obtained by nonlinear regression directly from the michaelis–Menten equation. In the case of PlmII, the values of these parameters and the inhibition constants by the substrate (K'_S) were calculated by equation (1). The inhibition constants were determined form the Henderson equation (2) with the corrective of the competitive character of the inhibition in the case of CatD and from the Henderson equation modified by us with the corrective of the substrate inhibition (5). The standard deviation was not higher than 20% in all the cases.

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