

## INHIBITORY EFFECT OF DI- AND TRIPEPTIDYL ALDEHYDES ON CALPAINS AND CATHEPSINS\*

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Eight different di- and tripeptidyl aldehyde derivatives, each having at its C-terminus an aldehyde analog of L-norleucine, L-methionine, or L-phenylalanine with a preceding L-leucine residue, were synthesized and tested for their inhibitory effects on several serine and cysteine endopeptidases. These compounds showed almost no inhibition of trypsin, and only weak inhibition of  $\alpha$ -chymotrypsin and cathepsin H, while they exhibited marked inhibition of cathepsin B < calpain II  $\approx$  calpain I < cathepsin L, being stronger in this order. The mode of inhibition of these cysteine proteinases was competitive for the peptide substrate used and inhibitor constants ( $K_i$ ) were calculated from the Dixon plot. The best inhibitors found were: 4-phenyl-butyryl-Leu-Met-H for calpain I ( $K_i$ , 36 nM) and calpain II ( $K_i$ , 50 nM); acetyl-Leu-Leu-nLeu-H for cathepsin L ( $K_i$ , 0.5 nM); acetyl-Leu-Leu-Met-H for cathepsin B ( $K_i$ , 100 nM).

**KEY WORDS:** Calpain, cathepsin, peptidyl aldehydes, proteinase inhibitor.

### INTRODUCTION

Cysteine endopeptidases, which include papain, calpains and cathepsins, are known to be strongly inhibited by leupeptin and antipain.<sup>1,2</sup> These *Actinomycete* products are tripeptide analogs, each having at its C-terminus an argininal residue (Arg-H), a residue in which the carboxylic acid group is reduced to a formyl group. In contrast to alkylating inhibitors such as peptidyl chloromethyl ketones<sup>3,4</sup> and diazomethanes,<sup>5,6</sup> these aldehyde analogs are essentially reversible inhibitors. By virtue of this, aldehyde inhibitors can be bound to the enzyme almost instantaneously and they can be used for reversible modification of the enzyme. Such usefulness could be amplified,

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**Abbreviations:** Z-, benzyloxycarbonyl-; Leu, L-leucine residue; nLeu, L-norleucine residue; Met, L-methionine residue; Phe, L-phenylalanine residue; PB-, 4-phenylbutyryl-; -OMe, methyl ester; AMC, 7-amino-4-methylcoumarine; MCA, 4-methylcoumaryl-7-amide; WSCD, *N*-ethyl-*N'*-diethylaminopropyl carbodiimide; MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; CDCl<sub>3</sub>, chloroform-d<sub>1</sub>; TMS, tetramethylsilane; t-BuOH, 2-methyl-2-propanol; DMSO, dimethylsulfoxide; Suc, succinyl.

if different peptidyl aldehyde derivatives could be synthesized each specifically acting on one of the different cysteine proteinases in a discriminative fashion.

We attempted to synthesize several di- and tripeptidyl aldehydes, aiming at obtaining some derivatives which would fulfill the specificity requirement of calpain.<sup>7</sup> Besides leupeptin and antipain, several synthetic peptide inhibitors for calpain have been reported,<sup>4</sup> but they are also effective on other cysteine proteinases. The peptidyl aldehydes we synthesized were found to inhibit strongly both calpain I (a low- $\text{Ca}^{2+}$ -requiring form) and calpain II (a high- $\text{Ca}^{2+}$ -requiring form), but they failed to be specific for calpains, also showing fairly strong inhibition of cathepsins L and B. Thus, the primary objective of our study was not reached, but the kinetic data obtained provided us with several new lines of information on the specificity of calpains and cathepsins.

## MATERIALS AND METHODS

### Materials

Calpains I and II were isolated from pig erythrocyte and kidney with specific activities of 185 units/mg and 217 units/mg, respectively, using the method of Kitahara *et al.*<sup>8</sup> Cathepsin B, H, and L were purified from rat tissues by the methods of Towatari *et al.*<sup>9</sup>, Kirschke *et al.*<sup>10</sup>, and Bando *et al.*<sup>11</sup>, respectively. Trypsin (Type I, 1100 units/mg) and  $\alpha$ -chymotrypsin (Type II, 59 units/mg) were purchased from Sigma, St. Louis, MO. Boc-Val-Leu-Lys-MCA, Z-Phe-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Arg-MCA were obtained from the Protein Research Foundation, Osaka, Japan. All other chemicals were of reagent grade.

### Synthesis of inhibitors

The synthesis of Z-Leu-nLeu-H, Z-Leu-Met-H, PB-Leu-nLeu-H, and PB-Leu-Met-H was described earlier,<sup>12</sup> and the synthesis of Z-Leu-Phe-H and PB-Leu-Phe-H was carried out by the same methods. The following analytical data were obtained. Z-Leu-Phe-H,  $\text{C}_{23}\text{H}_{28}\text{N}_2\text{O}_4$  Calc.: C, 69.68; H, 7.12; N, 7.07. Found: C, 69.55; H, 7.06; N, 7.10;  $^1\text{H-NMR}$  ( $\delta$  – ppm from TMS in  $\text{CDCl}_3$ ) 0.88 (6H,m), 1.24 ~ 1.72 (3H,m), 3.12 (2H,d,J = 7Hz), 4.16 (1H,m), 5.08 (2H,s), 5.12 (1H,m), 5.64 (1H,m), 7.16 ~ 7.34 (10H,m), 9.56 (1H,s). PB-Leu-Phe-H,  $\text{C}_{25}\text{H}_{32}\text{N}_2\text{O}_3$ , Calc.: C, 73.50; H, 7.89; N, 6.86. Found: C, 73.33; H, 7.92; N, 6.88.  $^1\text{H-NMR}$  ( $\delta$  – ppm from TMS in  $\text{CDCl}_3$ ) 0.92 (6H,d,J = 6 Hz), 1.52 ~ 2.26 (7H,m), 2.57 ~ 2.72 (2H,m), 3.12 (2H,d,J = 7Hz), 4.40 ~ 4.76 (2H,m), 5.72 (1H,d,J = 7Hz), 6.68 (1H,d,J = 6Hz), 7.14 ~ 7.26 (10H,m), 9.58 (1H,s).

The derivatives of acetyl tripeptidyl aldehyde that were used in this study were Ac-Leu-Leu-nLeu-H and Ac-Leu-Leu-Met-H. The synthesis of Ac-Leu-Leu-R-H, with R representing nLeu or Met, was started by the deprotection of the Z-group of Z-Leu-R-OMe obtained in the course of the synthesis of Z-Leu-R-H.<sup>12</sup> The Z-group of Z-Leu-nLeu-OMe (4.0 g) was removed by catalytic reduction with a small amount of 5% palladium on carbon in absolute EtOH (100 ml) in an atmosphere of hydrogen to obtain H-Leu-nLeu-OMe quantitatively. H-Leu-nLeu-OMe (2.6 g) and Z-Leu-OH (2.6 g) were coupled by WSCD (2.0 g) in dry dichloromethane (100 ml) to give Z-Leu-Leu-nLeu-OMe in 85% yield (4.3 g) after purification by recrystallization from

10% EtOAc in ether. The  $R_f$  on TLC with 10% MeOH in chloroform was 0.80. The Z- group of Z-Leu-Leu-nLeu-OMe was removed by catalytic reduction with a small amount of 5% palladium on carbon in absolute EtOH (100 ml) in an atmosphere of hydrogen to give H-Leu-Leu-nLeu-OMe quantitatively. The H-Leu-Leu-nLeu-OMe (2.9 g) was acetylated by acetic anhydride (10 ml) in dry chloroform (50 ml) and dry benzene (20 ml) to afford Ac-Leu-Leu-nLeu-OMe in 90% yield (2.9 g) after purification by recrystallization from 10% EtOAc in ether. The  $R_f$  on TLC with 10% MeOH in chloroform was 0.75. Ac-Leu-Leu-nLeu-OMe (2.2 g) was then reduced to the alcohol using sodium borohydride (0.5g) in t-BuOH (50 ml) with absolute MeOH (8 ml) under reflux at 90°C in an atmosphere of nitrogen. The reaction mixture was quenched with 40 ml of water after 1h to give Ac-Leu-Leu-norleucinol in 42% yield (840 mg) after purification by recrystallization from EtOAc. The  $R_f$  on TLC with 10% MeOH in chloroform was 0.50. Ac-Leu-Leu-norleucinol (770 mg) was oxidized to the aldehyde by a sulfur trioxide pyridine complex (1.3 g) with triethylamine (800 mg) in anhydrous DMSO (16 ml). After quenching with water (100 ml), the final compound Ac-Leu-Leu-nLeu-H was obtained in 47% yield after purification by crystallization from EtOAc. The  $R_f$  on TLC with 10% MeOH in chloroform was 0.65. The analytical data for Ac-Leu-Leu-nLeu-H are as follows:  $C_{20}H_{37}N_3O_4$ , Calc.: C, 62.63; H, 9.72; N, 10.96. Found: C, 62.70; H, 9.81; N, 10.94.  $[\alpha]_D^{28} = -91.6^\circ$  (c = 1.6, MeOH); mp 182–184°C; MS (EI) (m/z)  $[M]^+ = 384$ ;  $^1H$ -NMR ( $\delta$  – ppm from TMS in  $CDCl_3$ ) 0.93 (15H,m), 1.16 ~ 1.89 (12H,m), 2.02 (3H,s), 4.37 ~ 4.66 (3H,m), 6.25 (1H,d,J = 8Hz), 6.89(1H,d,J = 8Hz), 7.00(1H,d,J = 8Hz), 9.62 (1H,s).

The synthesis of Ac-Leu-Leu-Met-H was carried out in the same manner as for Ac-Leu-Leu-nLeu-H except with the addition of boron trifluoride etherate during the deprotection of the Z-group of Z-Leu-Met-OMe of Z-Leu-Leu-Met-OMe because in the absence of boron trifluoride etherate, the sulfur atom of the Met residue would poison the hydrogenation catalyst. The analytical data for Ac-Leu-Leu-Met-H are as follows:  $R_f$  0.55 (10% MeOH in chloroform);  $C_{19}H_{35}N_3O_4S$ , Calc.: C, 56.69; H, 8.76; N, 10.44. Found: C, 56.33; H, 8.99; N, 10.81.  $[\alpha]_D^{28} = -74.3^\circ$  (c = 0.98, MeOH); mp 189–191°C; MS (EI) (m/z)  $[M]^+ = 402$ ;  $^1H$ -NMR ( $\delta$  – ppm from TMS in  $CDCl_3$ ) 0.95 (12H,m), 1.45 ~ 1.85 (8H,m), 2.02 (3H,s), 2.07 (3H,s), 2.42 ~ 2.62 (2H,m), 4.44 ~ 4.64 (3H,m), 6.30 (1H,d,J = 8Hz), 6.98 (1H,d,J = 8Hz), 7.29 (1H,d,J = 8Hz), 9.66 (1H,s).

#### *Assays for inhibitory activities*

**Calpains** : The reaction mixture (0.5 ml) contained 110 mM imidazole (pH 7.5), 5 mM cysteine, 5 mM  $CaCl_2$ , 0.01–0.5 mM Boc-Val-Leu-Lys-MCA, 0–0.4  $\mu$ M synthetic inhibitor, 6% DMSO, and 5.3  $\mu$ g of calpain (I or II). The reaction was started by adding the enzyme and continued for 5 min at 30°C. It was terminated by adding 50  $\mu$ l of 100 mM EGTA. AMC released was fluorimetrically determined at 460 nm for emission and at 380 nm for excitation. Control runs were performed by omitting  $Ca^{2+}$ .

**Cathepsins** : The method of Barrett and Kirschke<sup>13</sup> was employed. The reaction mixture (1 ml) contained 0.3 ng of cathepsin B or 2.5 ng of cathepsin L, 100 mM acetate (pH 5.5), 1 mM EDTA, 8 mM cysteine, 0.01–0.05 mM Z-Arg-Arg-MCA for cathepsin B or 0.01–0.05 mM Z-Phe-Arg-MCA for cathepsin L, and 0–0.4  $\mu$ M synthetic inhibitor. The reaction was allowed to proceed at 37°C for 6 min, and

terminated by adding a mixture of 1 ml of 100 mM acetate buffer (pH 4.3) containing 100 mM  $\text{ClCH}_2\text{COONa}$  and 0.5 ml of water. AMC released was fluorimetrically determined as previously. For cathepsin H (40 ng per assay), 100 mM sodium phosphate buffer (pH 6.8), containing 1 mM EDTA, 0.01–0.05 mM Arg-MCA, and 0–50  $\mu\text{M}$  synthetic inhibitor was used.

**Trypsin and  $\alpha$ -chymotrypsin** : Trypsin (4.5  $\mu\text{g}$ ) or  $\alpha$ -chymotrypsin (0.3  $\mu\text{g}$ ) was incubated at 30°C for 5 min in 0.5 ml of the assay mixture which contained 100 mM imidazole (pH 7.5), 6 mM  $\text{CaCl}_2$ , 7% DMSO, 1 or 50  $\mu\text{M}$  synthetic inhibitor, 0.3 mM Z-Phe-Arg-MCA (for trypsin) or 0.3 mM Suc-Leu-Leu-Val-Tyr-MCA (for  $\alpha$ -chymotrypsin). The reaction was terminated by adding 50  $\mu\text{l}$  of 200 mM HCl. AMC released was fluorimetrically determined. Control runs were performed without enzyme.

## RESULTS AND DISCUSSION

The newly synthesized peptidyl aldehydes were all found to be inhibitory towards calpains I and II and cathepsins B and L. They showed only weak inhibition of cathepsin H. Lineweaver-Burk plots indicated that the mode of inhibition of calpains I and II is competitive for the substrate. The inhibitor constant,  $K_i$ , was calculated from the Dixon plot, several examples being shown in Figure 1. The  $K_i$  values obtained are listed in Table I. Table II shows that all the compounds tested were almost ineffective on trypsin and only weakly inhibitory on  $\alpha$ -chymotrypsin. Even with the best  $\alpha$ -chymotrypsin inhibitor, PB-Leu-Phe-H, the inhibitor concentration at which the enzyme activity was half of the uninhibited value ( $\text{IC}_{50}$ ) was estimated to be

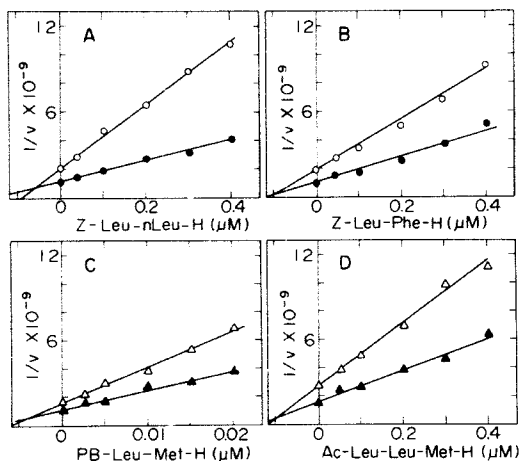


FIGURE 1 Dixon plots for the inhibition of calpains and cathepsins by peptidyl aldehydes. The assay was carried out at 30°C (for calpain) or 37°C (for cathepsin) and at two different concentrations of the substrate with varying amounts of the inhibitor in the assay system as described in "Materials and Methods." The rate of hydrolysis,  $v$  (mol/s/l), was calculated from the amount of AMC released in 5 min. A, calpain I; B, calpain II; C, cathepsin L; D, cathepsin B. Concentrations of the substrate used are:  $\circ$ , 0.3 mM;  $\bullet$ , 0.5 mM;  $\Delta$ , 0.04 mM;  $\blacktriangle$ , 0.08 mM.

TABLE I  
Inhibition of calpains and cathepsins by di- and tripeptidyl aldehydes

| Inhibitor         | $K_i$ ( $\mu$ M) |            |             |             |             |
|-------------------|------------------|------------|-------------|-------------|-------------|
|                   | Calpain I        | Calpain II | Cathepsin L | Cathepsin B | Cathepsin H |
| Z-Leu-nLeu-H      | 0.067            | 0.062      | 0.0034      | 0.13        | > 10        |
| PB-Leu-nLeu-H     | 0.065            | 0.068      | 0.0033      | 0.22        | > 10        |
| Ac-Leu-Leu-nLeu-H | 0.19             | 0.22       | 0.00050     | 0.15        | > 10        |
| Z-Leu-Met-H       | 0.036            | 0.068      | 0.013       | 0.25        | > 10        |
| PB-Leu-Met-H      | 0.036            | 0.050      | 0.0045      | 0.16        | > 10        |
| Ac-Leu-Leu-Met-H  | 0.12             | 0.23       | 0.00060     | 0.10        | > 10        |
| Z-Leu-Phe-H       | 0.060            | 0.10       | 0.020       | 2.40        | > 10        |
| PB-Leu-Phe-H      | 0.038            | 0.078      | 0.0014      | 0.43        | > 10        |

TABLE II  
Inhibition of trypsin and  $\alpha$ -chymotrypsin by di- and tripeptidyl aldehydes

| Inhibitor         | Concentration<br>( $\mu$ M) | Activity remaining (%) |                        |
|-------------------|-----------------------------|------------------------|------------------------|
|                   |                             | Trypsin                | $\alpha$ -Chymotrypsin |
| Z-Leu-nLeu-H      | 1                           | 100                    | 100                    |
|                   | 50                          | 98.8                   | 57.4                   |
| PB-Leu-nLeu-H     | 1                           | 100                    | 97.4                   |
|                   | 50                          | 84.9                   | 18.2                   |
| Ac-Leu-Leu-nLeu-H | 1                           | 100                    | 100                    |
|                   | 50                          | 98.2                   | 71.7                   |
| Z-Leu-Met-H       | 1                           | 100                    | 88.8                   |
|                   | 50                          | 92.0                   | 60.8                   |
| PB-Leu-Met-H      | 1                           | 100                    | 95.3                   |
|                   | 50                          | 92.9                   | 23.8                   |
| Ac-Leu-Leu-Met-H  | 1                           | 100                    | 93.9                   |
|                   | 50                          | 97.6                   | 53.3                   |
| Z-Leu-Phe-H       | 1                           | 100                    | 75.1                   |
|                   | 50                          | 94.7                   | 14.0                   |
| PB-Leu-Phe-H      | 1                           | 100                    | 70.9                   |
|                   | 50                          | 92.5                   | 3.3                    |

approximately 4  $\mu$ M, a value much higher than the  $IC_{50}$  of this analog against calpain I or II (0.08  $\mu$ M or less).

The initial objective of the present study was to synthesize some potent inhibitor peptide analogs which were specific for calpain. The aldehyde derivatives were designed so as to match the substrate specificity of calpains I and II reported earlier.<sup>7</sup> A Leu residue was always present at  $P_2$  position, since this residue was known to be the most preferred by either calpain I or II.<sup>7</sup> Several kinds of bulky amino acid residues were placed at the  $P_1$  position, also conforming to the previous knowledge that such residues are favored by calpain both as peptide substrates<sup>4</sup> and as peptidyl chloromethylketone inhibitors.<sup>4</sup> Aldehyde derivatives were chosen in order to study steady state kinetics rather than the reaction rate which had earlier been studied with chloromethylketone derivatives.<sup>5</sup>

All of the 8 compounds were found, however, not to be specific for calpains, but to quite strongly inhibit also the cathepsins. From direct comparison of  $K_i$  values, the strength of affinity of the cysteine proteinases tested with these peptidyl aldehydes generally increases in the following order: cathepsin H < cathepsin B < calpain II  $\simeq$  calpain I < cathepsin L. However, when the  $K_i$  values are compared with the  $K_m$  values for the respective enzymes, some different views can be obtained. For example, the best (or the smallest)  $K_i$  values found for calpain I is  $0.036 \mu\text{M}$  with either Z-Leu-Met-H or PB-Leu-Met-H, and that for cathepsin L is  $0.0005 \mu\text{M}$  with Ac-Leu-Leu-nLeu-H (Table I). A  $K_m$  value of  $5.92 \text{ mM}$  for calpain I was reported with the substrate used in this study, Boc-Val-Leu-Lys-MCA,<sup>7</sup> and  $K_m$  of cathepsin L for Z-Phe-Arg-MCA was shown<sup>13</sup> to be  $7 \mu\text{M}$ . Thus, the ratio  $K_i/K_m$  calculated for calpain I ( $6.08 \times 10^{-6}$ ) is  $1(11.7)$  1.7-times smaller than that for cathepsin L ( $7.14 \times 10^{-5}$ ), implying higher relative specificity of inhibition of calpain I than of cathepsin L. Therefore, the apparent large difference between  $K_i$  values for calpain I and for cathepsin L, listed in Table I, may not be interpreted as showing much less capability of the peptide analogs to inhibit calpain I, but it may reflect inherent difference in affinity to the peptide moiety between these two enzymes.

It was earlier reported<sup>7</sup> that the substrate specificity of calpain I is closely similar to but not identical with that of calpain II. This was shown also to be true when the peptidyl aldehydes are used as inhibitors (Table I). For example, about twice the difference in  $K_i$  values between calpains I and II were noted with Z-Leu-Met-H, Ac-Leu-Leu-Met-H, Z-Leu-Phe-H, and PB-Leu-Phe-H, whereas almost equal  $K_i$  values were obtained with Z-Leu-nLeu-H, PB-Leu-nLeu-H, and Ac-Leu-Leu-nLeu-H. No general rule may be deduced from these variations.

The data shown in Table I also gives some information on the subsite specificity of cathepsins L and B. With a Met or Phe residue at  $P_1$  position, the nature of the  $P_3$  residue showed a profound influence of  $K_i$ , while such an influence was not seen when  $P_1$  was nLeu. Besides, elongation from di- to tripeptide analogs by inserting one Leu residue at  $P_3$  position strongly increased the affinity of the peptide analogs to cathepsin L, but not to cathepsin B. All these lines are in accord with the known importance

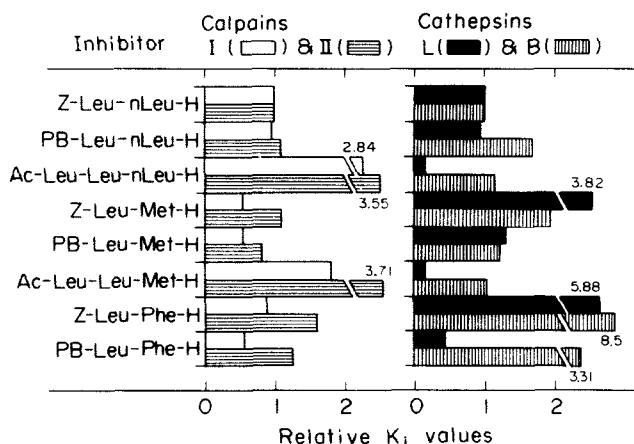


FIGURE 2 Profiles of inhibitory potencies of several peptidyl aldehydes on calpains and cathepsins.  $K_i$  values for Z-Leu-nLeu-H were taken as unity.



of the P<sub>3</sub> residue in determining the specificity of these cathepsins.<sup>14</sup> The nature of the P<sub>3</sub> residue also influenced calpains I and II, but not always in the same ways as those for cathepsins. The presence of a Leu at P<sub>3</sub> always resulted in a remarkable decrease in affinity of the inhibitor to calpains.

The fact that the aldehyde derivatives used for the present study all showed stronger inhibition of cathepsin L and calpains I and II compared with cathepsin B (Table II) is in full accord with the recent report by Crawford *et al.*<sup>6</sup> who showed that several peptidyl diazomethanes inactivated cathepsin L and calpains rapidly, but cathepsin B only slowly. However, this may not necessarily be interpreted as representing closer similarity of specificities between calpains (I and II) and cathepsin L than between calpains and cathepsin B. Thus, if the profile of affinities (in terms of K<sub>i</sub> values) is depicted in such a way after normalization by placing K<sub>i</sub> for Z-Leu-nLeu-H for each enzyme as unity (Figure 2), comparative degrees of dissimilarity can be seen between any one of the three pairs out of calpain (I or II), cathepsin L and cathepsin B. It should be noted, however, that cathepsin B also exhibits a peptidyl-dipeptidase activity<sup>15</sup> whereas the action of calpains is thought to be exclusively endopeptidatic<sup>7</sup> as that of cathepsin L is.<sup>16</sup>

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