Iodo and diiodotyrosine epoxysuccinyl derivatives as selective inhibitors of cathepsin B

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Summary — Eight new analogs of L-trans-epoxysuccinyl-L-leucylamido(3-methyl)butane (E-64-c) containing Phe, Tyr, Tyr(I) or Tyr(I,) in place of Leu, were synthesized and tested as inhibitors of papain, bovine spleen cathepsin B, calpain I and II from porcine red cells and porcine kidney, respectively. By use of kinetic methods, the new E-64 analogs proved to irreversibly inactivate both papain and cathepsin B via reversible enzyme-inhibitor intermediates EI. Second-order rate constants for inactivation were in the range 3500-55 100 M-1s-1 for papain and 650-105 000 M-1s-1 for cathepsin B. For the inactivation of calpain I and II they ranged between 250 and 2000 M-1s-1 and were similar to those of the known E-64-c. The effectiveness of the amino acid contained in the inhibitors tested increased in the order Tyr(I) = Tyr(I,) < Tyr < Phe < Leu for papain and Phe < Tyr < Tyr(I) < Leu < Tyr(I,) for cathepsin B inactivation. Replacement of the L with the D-trans-epoxysuccinyl unit caused a 10-100-fold decrease in inhibitor potencies.

enzyme inhibiting activity / cysteine proteases / E-64 analogs

Introduction

L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane I, named E-64, was first isolated [1] from extracts of solid cultures of Aspergillus japonicus TPR-64 and characterized [2] by Hanada et al. Its potent inhibiting activity toward papain, ficin fruit bromelain and stem bromelain [1], cathepsin L [3], cathepsin B [4], calpain [5] and cathepsin H [6] was soon revealed. Several E-64 analogs [7-13] have been synthesized and tested with the aim of increasing their potency and specificity for cathepsin B, H, L and calpains. The mechanism for irreversible inactivation of cysteine proteases by E-64 and its analogs and the mode of binding have been studied by NMR [14] and X-ray crystal analysis [15-20]. The L-trans-epoxysuccinyl unit represents the trapping function of E-64 and its analogs. They irreversibly inactivate the enzyme by formation of a covalent bond between the nucleophilic thiolate of the catalytic cysteine and the C-3 of the epoxysuccinyl unit.

L-64 analogs are potential candidates for therapeutic application in those pathological states characterized by abnormally elevated levels of cysteine proteases activities, such as muscular dystrophy [21]
and myocardial ischemia [22, 23]. The most interesting properties of these inhibitors are: 1) remarkably fast reaction with the active site thiolate of cysteine proteases with second-order rate constants exceeding $10^8 \text{M}^{-1}\text{s}^{-1}$ [6] under physiological conditions; 2) high specificity for cysteine proteases since no inhibition could be detected toward serine proteases and metallo proteases [1, 6]; 3) extremely low toxicity, presenting LD$_{50}$ > 2000 mg/kg of body weight in rats and mice and no toxic effects for treatments with 200-400 mg/kg in rats and dogs over 1 month [24]. In addition, the in vivo efficacy has been demonstrated and studies on their absorption, distribution and metabolism have given satisfactory results [24].

The main structural variations of L-trans-epoxysuccinyl inhibitors include: 1) modifications of the chain in P$_1$. It was shown that the charged guanidino group in P$_1$ was not essential for the onset of inhibitory activity [7-9]. In addition, the synthetic analog L-trans-epoxysuccinyl-leucinamido(3-methyl)butane 4, named E-64-c or Ep-475 [6, 8, 9], containing the neutral iso-pentyl group in P$_1$, proved to be substantially more efficient than E-64 as an inhibitor of cathepsin B and L; 2) replacement of the P$_2$ and P$_3$ units by esters of the natural α-amino acids as shown in 5. It was found that the amino acids bearing an alkyl side-chain like Leu, Ile and Val were the most effective toward papain, followed by the aromatic amino acids such as Phe and Tyr [10]; 3) replacement of the P$_3$ and P$_4$ units by a selection of dipeptides (Ile-Pro-OH, Pro-Thr-Ile-OH) as shown in 7. Important improvement of the selectivity toward cathepsin B with respect to cathepsin H and L and calpain was obtained in this case [12]; 4) conversion of the terminal carboxyl group of the L-trans-epoxysuccinyl moiety into an ester or an amido group. Esterification of the epoxysuccinyl moiety of L-trans-epoxysuccinyl amino-acid esters 5 to give methyl or ethyl esters 6 caused a decrease in the reactivity toward cathepsin B as large as 2 orders of magnitude [10]. The same effect was observed by conversion of E-64-c into its ethylester 11. A substantial retention of the activity has only been observed for particular esters such as 8 and amides like 9 of l-trans-epoxysuccinyl dipeptides [12, 13].

In addition, new natural analogs of E-64 containing Phe or Tyr in place of Leu were isolated [25] from culture filtrates of Myceliophtora thermophila and named estatin A and B [2 and 3]. They proved to be inhibitors of papain, ficin and bromelain although less potent than E-64.

As part of a project aimed at the development of new E-64 analogs of pharmacological interest, we synthesized and tested against papain, cathepsin B, calpain I and II the epoxysuccinyl amino-acid amides 10b–17b. They should more strictly be regarded as analogs of E-64-c, since the neutral iso-pentyl chain in P$_1$ has been retained to increase the inhibiting properties toward cathepsin B [6, 8, 9]. This modification brings, at the same time, a simplification of the molecular structure and of the chemical synthesis.

The central Leu unit of E-64 and E-64-c has been replaced by the aromatic amino acids Phe and Tyr which are present in the estatins A and B [25] and by Tyr(I) and Tyr(I$_2$). The introduction of iodinated tyrosines was suggested from the studies of Crawford et al. [26] which proved that replacement of Phe or Tyr by Tyr(I) in Z-Phe-Ala-CH=N$_2$ or Z-Tyr-Ala-CH=N$_2$ caused a $\approx$ 23-fold increase of the second-order rate constants of cathepsin B inactivation. Similar preference of cathepsin B for Tyr(I) in P$_1$ was also observed by us with 1-peptidyl-2-haloacetyl hydrazine inhibitors [27].

Owing to the variations of size and nature of the amino-acid residues included in the series 10b–13b with respect to E-64-c, fine effects on mode and strength of binding at the active site were not predictable with certainty. Thus the corresponding diastereoisomers 14b–17b containing a d-trans-epoxysuccinyl unit were also prepared and tested as cysteine protease inhibitors.

**Chemistry**

The E-64 analogs 10b–17b were synthesized according to scheme 1. The aminoacyl iso-pentylamides 20–22 were prepared by mixed anhydride or dicyclohexylcarbodiimide 1-hydroxybenzotriazole coupling of the protected amino acids with 3-methyl-amino-butan-1one, followed by removal of the protective group, as previously described for 19 [28]. The semi-ester 18 of L-trans-epoxysuccinic acid was prepared by partial alkaline hydrolysis of the diethylester obtained from d(-)diethyltartrate [29]. Acylation of the individual amino-acid iso-pentylamides with the semi-ester of
L-trans-epoxysuccinic acid to give the final inhibitors as the ethylesters 10a–13a was performed by the dicyclohexylcarbodiimide 1-hydroxybenzotriazole method. Conversion into the free acids 10b–13b was obtained by partial alkaline hydrolysis. The corresponding diastereoisomers 14b–17b containing the D-trans-epoxysuccinyl unit were synthesized through the same pathway by starting from L(+)diethyl tartrate 19. All new compounds were homogeneous by TLC and gave satisfactory elemental analyses. The proposed structures are in accordance with their IR and 1H-NMR spectra.

**Enzyme inactivation measurement**

Compounds 10b–17b completely and irreversibly inhibited the papain- and cathepsin B-catalyzed hydrolysis of N-carbobenzyloxyglycine p-nitrophenyl-ester (Z-Gly-ONp). The calpain I and calpain II-catalyzed hydrolysis of N-succinyl-leucyl-tyrosyl-amido-4-methylcoumarin was similarly inhibited by compounds 10b–13b and 16b.

Proteases are commonly inactivated by substrate analog, irreversible inhibitors according to a minimum kinetic mechanism which is in accordance with equation [1]:

\[
E + I \xrightleftharpoons[k_1]{k_2} EI \xrightarrow{k_2} E-I
\]

where EI, E-I, \(k_1\), and \(k_2\) represent the reversible complex, the irreversibly inactivated enzyme and the rate constants for the non-covalent reaction step respectively; \(k_3\) is the rate constant for the covalent modification of the enzyme.

Recently Tian and Tsou [30] introduced a convenient method for the calculation of inactivation rates in the presence of the substrate. Substrate and inhibitor compete for the enzyme binding site according to equation (2) and the decrease in enzyme concentration during the reaction obeys pseudo-first-order kinetics.

\[
E + I \xrightarrow{k_2} EI \xrightarrow{k_3} E-I
\]

The decrease in formation of p-nitrophenol at \([S] >> K_m\) was measured at various inhibitor concentrations; the temporal dependence of the change in absorbance was exponential and well fitting to a first-order rate law. Data were collected for no less than 3 half-lives to demonstrate the complete inactivation of the enzyme by the inhibitors and all progress curves reached steady \(A_\infty\) values indicating that inhibition was always complete. Absorbance versus time data were computed by non-linear regression analysis to equation [3]:

\[
A = A_\infty [1 - e^{-k_{obs}t}]
\]

where \(A\) and \(A_\infty\) are the absorbances at \(t\) and \(t_\infty\) respectively and \(k_{obs}\) is the pseudo-first-order rate constant for the formation of the inhibited enzyme. Non-linear regression analysis was used in place of manipulation of the progress curve data for enzyme inhibition or manual determination of \(A_\infty\), since it yields more reliable kinetic parameters under various circumstances [31].

Pseudo-first-order rate constants for enzyme inhibition, \(k_{obs}\) were dependent on inhibitor concentration and different inhibitor concentrations gave different
is valid for irreversible inhibitors which form preliminary reversible complexes. Equation [5], which is a rearrangement of [4], allows easy calculation of the kinetic parameters in a plot of $1/k_{\text{obs}}$ vs $1/[I]$. The reciprocal of the intercept on the ordinate is $k_1$, and $K_I$ may be calculated from the slope $K_I(1 + [S]/[K_m])/k_2$.

Kinetic parameters reported in table I were determined accordingly, by fitting into the plot the experimental $k_{\text{obs}}$ values.

Cathepsin B activity was not restored by 20–50-fold dilution of concentrated solutions of the enzyme (20–50-fold that used in progress curves) after 10–20 min incubation with the inhibitors in the concentration range of table I. These results indicate that compounds 10b–17b under our experimental conditions are not appreciably hydrolyzed by cathepsin B, because complete inhibition was independent of [E], and that their inhibition is irreversible, because dilution of the enzyme–inhibitor complex did not restore the activity. The continuous method of Tian and Tsou has already been tested as valid for other protease inhibitors [28] by using the discontinuous method of Kitz and Wilson [34]. The kinetic parameters we obtained are fully comparable with that measured by Barrett et al [6] for papain and human liver cathepsin B inactivation by Ep-475 (i.e., E-64-c). The 2.6- and 3.9-fold enhanced reactivities reported [6] may be due to differences in the conditions for inactivation and to different enzyme source for cathepsin B.

The apparent second-order rate constants for inactivation of calpain I and II by compounds 10b–13b and 16b were evaluated as $k_{\text{obs}}/[I]$. From equation [4] it follows that:

\[
\frac{k_2}{K_1} = \frac{k_{\text{obs}}}{[I]} \left(1 + \frac{[S]}{K_m}\right) = \left(\frac{k_{\text{obs}}}{[I]}\right)_{\text{corr}}
\]

Table I. Inactivation of papain\(^a\) and cathepsin B\(^a\), calpain I and calpain II\(^b\) by E-64 analogs.

<table>
<thead>
<tr>
<th>No</th>
<th>Structure</th>
<th>Papain</th>
<th></th>
<th>Cathepsin B</th>
<th></th>
<th>Calpain I</th>
<th></th>
<th>Calpain II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range ((\mu)M)</td>
<td>(K_I) ((\mu)M)</td>
<td>(k_2) (min(^{-1}))</td>
<td>(k_2/K_1) (M(^{-1})s(^{-1}))</td>
<td>Range ((\mu)M)</td>
<td>(K_I) ((\mu)M)</td>
<td>(k_2) (min(^{-1}))</td>
<td>(k_2/K_1) (M(^{-1})s(^{-1}))</td>
</tr>
<tr>
<td>10b</td>
<td>L-trans-Eps-Phe-NH-i-C(<em>2)H(</em>{11})</td>
<td>0.41–3.12</td>
<td>0.13</td>
<td>0.43</td>
<td>55 100</td>
<td>0.73–8.3</td>
<td>3.57</td>
<td>2.63</td>
</tr>
<tr>
<td>11b</td>
<td>L-trans-Eps-Tyr NH i C(<em>2)H(</em>{11})</td>
<td>0.62</td>
<td>3.12</td>
<td>0.44</td>
<td>1.16</td>
<td>43 900</td>
<td>0.41</td>
<td>3.12</td>
</tr>
<tr>
<td>12b</td>
<td>L-trans-Eps-Tyr(I)-NH-i-C(<em>2)H(</em>{11})</td>
<td>1.7–12.5</td>
<td>0.56</td>
<td>0.91</td>
<td>27 000</td>
<td>0.21–1.56</td>
<td>0.34</td>
<td>1.39</td>
</tr>
<tr>
<td>13b</td>
<td>L-trans-Eps-Tyr(I)-NH-i-C(<em>2)H(</em>{11})</td>
<td>0.93-6.25</td>
<td>0.23</td>
<td>0.40</td>
<td>29 000</td>
<td>0.1-0.78</td>
<td>0.068</td>
<td>0.43</td>
</tr>
<tr>
<td>1</td>
<td>L-trans-Eps-Leu-NH-(CH(_2))(_2)NH-CN(=O)-NH(_2)</td>
<td>638 000(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>L-trans-Eps-Leu-NH-i-C(<em>2)H(</em>{11})</td>
<td>0.15–0.78</td>
<td>0.18</td>
<td>1.5</td>
<td>15 900(^d)</td>
<td>0.14–1.04</td>
<td>0.27</td>
<td>1.24</td>
</tr>
<tr>
<td>14b</td>
<td>n-trans-Eps-Phe-NH-i-C(<em>2)H(</em>{11})</td>
<td>1.7–8.3</td>
<td>0.77</td>
<td>0.36</td>
<td>77 700</td>
<td>6.5–50</td>
<td>6.67</td>
<td>0.47</td>
</tr>
<tr>
<td>15b</td>
<td>D-trans-Eps-Tyr-NH-i-C(<em>2)H(</em>{11})</td>
<td>1.5–11.1</td>
<td>0.48</td>
<td>0.42</td>
<td>14 600</td>
<td>8.9–67</td>
<td>11.1</td>
<td>0.43</td>
</tr>
<tr>
<td>16b</td>
<td>D-trans-Eps-Tyr(I)-NH-i-C(<em>2)H(</em>{11})</td>
<td>3.3–25</td>
<td>1.30</td>
<td>0.43</td>
<td>5500</td>
<td>2.2–25</td>
<td>4.76</td>
<td>1.12</td>
</tr>
<tr>
<td>17b</td>
<td>D-trans-Eps-Tyr(I)-NH-i-C(<em>2)H(</em>{11})</td>
<td>11.1–50</td>
<td>1.88</td>
<td>0.39</td>
<td>3500</td>
<td>13–100</td>
<td>6.25</td>
<td>0.28</td>
</tr>
</tbody>
</table>

\(^{a}\)100 mM phosphate buffer, pH 6.8; CH\(_3\)CN = 12% (v/v); [E] = 7 x 10\(^{-8}\) M; [S] = 125 \(\mu\)M; replicate determinations indicate SDs for the kinetic parameters < 20%.

\(^{b}\)50 mM Tris-HCl buffer, pH 7.5; 5 mM and 20 mM CaCl\(_2\) for calpain I and calpain II respectively; [E] = 6 x 10\(^{-8}\) M; [S] = 100 \(\mu\)M; replicate determinations indicate SDs for the kinetic parameters < 20%; \(^{c}\)at 40°C, pH 6.8 [6]; \(^{d}\)at 40°C, pH 6.0 [6]; \(^{e}\)557 000 M\(^{-1}\)s\(^{-1}\) at 40°C, pH 6.8 [6]; \(^{f}\)298 000 M\(^{-1}\)s\(^{-1}\) at 40°C, pH 6.0 for human liver cathepsin B [6].
when $[I]/K_i$ approaches zero. Thus $k_{\text{obs}}/[I]$ values, corrected for the presence of substrate according to equation [6], are equivalent to $k_i/K_i$ if $[I] \ll K_i$. The observed $k_{\text{obs}}/[I]$ values for calpain I and II inactivation were independent of $[I]$ under a range of inhibitor concentrations, confirming the validity of this assumption. The calculation of kinetic parameters for calpain inhibition is limited by activity loss, caused by autolysis at long assay times. This limitation does not affect the accuracy of the results for rate constants $> 10 \text{ M}^{-1}\text{s}^{-1}$ [26].

**Results and discussion**

All the epoxysuccinyl inhibitors 10b–17b completely and irreversibly inactivated papain and cathepsin B under pseudo-first-order conditions at pH 6.8 and 25°C. The minimal kinetic mechanism describing the inactivation process is indicated in equation [1]. The progress curves of inactivation showed pseudo-first-order kinetics in a concentration-dependent manner, in accordance with a preassociation step leading to a reversible, non-covalent enzyme-inhibitor complex EI. Assuming that the rate of the following irreversible step is relatively slow, so that the initial binding equilibrium is not perturbed, the kinetic parameters $K_i$, $k_2$ and $k_2/K_i$, which describe the enzyme inactivation, have been determined and are reported in table I. The corresponding values obtained for the known E-64-c were also included. Inhibition of papain by E-64-c was evaluated in order to calibrate results supplied by our assay with literature data [10]. Kinetic parameters for inactivation of bovine spleen cathepsin B by E-64-c were determined for a correct comparison with the corresponding values of the newly synthesized inhibitors 10b–17b, since literature data [10] deals with human liver cathepsin B. It is noteworthy that E-64-c, beyond the differences in the reaction conditions, inactivates human liver cathepsin B more rapidly and bovine spleen cathepsin B more slowly than papain.

Intervention of a preassociation step leading to a reversible EI intermediate is supported by studies [14–19] on the binding mode of E-64 and E-64-c to papain active site by X-ray crystal analysis of the complexes. In accordance with these findings, a possible structure of the EI reversible adduct between papain and the E-64 analogs 10b–13b is reported in figure 1. The L-trans-epoxysuccinyl derivatives 10b–13b showed dissociation constants for papain in the range 0.13–0.56 μM, for the most part slightly higher than that of E-64-c (0.18 μM). The first-order rate constants for papain inactivation were in the range 0.40–1.16 min$^{-1}$, slightly lower than that of E-64-c. As a consequence, the second order rate constant of inactivation $k_2/K_i$ decreases in the order $4 > 10b > 11b > 12b = 13b$, indicating a decreasing effectiveness of the amino acid involved in the order Leu > Phe > Tyr > Tyr(I) = Tyr(I$^*_2$).

The corresponding inhibitors 14b–17b, containing the D-trans-epoxysuccinyl unit, regularly showed higher $K_i$ and lower $k_2$, with respect to their diastereoisomers 10b–13b. This finding appears to exclude important changes in the mode of binding and inactivation mechanism of the newly synthesized inhibitors with respect to E-64 and E-64-c [16–19].

The epoxysuccinyl derivatives 10b–13b, when tested as inhibitors toward bovine spleen cathepsin B, dramatically changed their behavior with respect to papain. Replacement of Leu in 4 by Phe in 10b caused a 13-fold increase in $K_i$. This structural variation which did not affect the binding for papain, was strongly unfavorable for cathepsin B. On the other hand, the first-order rate constant was increased from 1.24 min$^{-1}$ for E-64-c to 2.63 min$^{-1}$ for 10b. On the whole, the $k_2/K_i$ ratio changed from 76 540 to 12 280 M$^{-1}$s$^{-1}$ with a 6-fold decrease. Further replacement of Phe by Tyr, Tyr(I) and Tyr(I$^*_2$) caused a progressive decrease in $K_i$. The inhibitor 12b containing Tyr(I) showed $K_i$, $k_2$ and $k_2/K_i$ values similar to those of E-64-c and 13b, containing Tyr(I$^*_2$), attained a dissociation constant of 0.068 μM, one-fourth that of E-64-c. Thus the active site of cathepsin B presents increasing binding affinity for the E-64 analogs considered in the present paper in the order: $10b > 11b > 12b > 4 > 13b$. This trend of the aromatic amino acids, which is practically opposite to that found for
papain inactivation, can probably be explained on the basis of the special architecture of the hydrophobic cleft of cathepsin B. The complete X-ray crystal structure of human liver cathepsin B has been recently determined [36]. The overall folding pattern of the enzyme, the arrangement of the active site residues, the principal bonding interactions with the substrate molecule and the hydrolytic mechanisms are similar to that of papain and related cysteine proteases. Assuming that the active site of bovine spleen cathepsin B used in the present study is substantially similar to that of the human liver enzyme, the EI reversible complex between cathepsin B and the epoxysuccinyl inhibitors 4 and 10b–13b will resemble that proposed for papain in figure 1, except that the represented papain residues should be replaced by the topologically equivalent residues of cathepsin B. In particular, the S2 subsite of human liver cathepsin B shows a larger hydrophobic cleft with respect to papain owing to the variation of the interested amino-acid sequence.

The relevant increase in binding affinity (3.57–0.55 μM) in the reversible complex EI by replacement of Phe by Tyr suggests a specific bonding interaction of the phenolic hydroxyl group with some complementary functional group of the hydrophobic cleft, which should characterize cathepsin B with respect to papain. Further increase of the binding affinity for 12b and 13b containing Tyr(I) and Tyr(I2) can be explained by a reinforcement of this special bonding interaction when 1 or 2 flanking iodine atoms increase the acidity of the phenolic hydroxyl group. In addition, the enhanced hydrophobic character and the increased size of the Tyr(I) and Tyr(I2) residues account for a stronger bonding interaction only with the larger hydrophobic cleft of cathepsin B.

On the other hand, the binding mode of the Tyr(I2) derivative 13b seems to place the trapping epoxide group in a position which is unfavorable for the attack by the functional cysteine thiolate as shown by the k3 values decreasing in the series 10b > 12b > 4 > 11b > 13b. The second-order inactivation rate constant k3/K3, however, is greater for 13b than for E-64-c as a consequence of the strong improvement of the binding affinity. Thus l-trans-epoxysuccinyl-diiodotyroosylamido-3-methylbutane 13b represents an E-64 analog more efficient than E-64-c for bovine spleen cathepsin B inactivation.

The analogs 14b–17b containing the D-trans-epoxysuccinyl moiety gave rate constants for cathepsin B inactivation 10–140-fold lower with respect to the l-trans-epoxysuccinyl derivatives 10b–13b. This decrease of the reactivities is entirely attributable to a strong decrease of their binding affinities. Since the corresponding decrease observed for papain is much smaller, the cathepsin B active site appears to be more sensible to stereochemical changes of these inhibitory ligands.

Rate constants for calpain I and calpain II inactivation by a selection of the newly synthesized inactivators were also measured and are reported in table I as koff/[I]. Precise comparison of these data with the rate constants reported for papain and cathepsin B are not possible, since they were necessarily determined under different conditions. Their behavior is essentially similar to that of E-64-c. The l-trans-epoxysuccinyl derivatives 12b and 13b, which are the most effective toward cathepsin B, showed to be less reactive toward calpain II and moderately more reactive toward calpain I with respect to E-64-c.

Work is in progress to develop new more effective and more selective E-64 analogs based on iodotyrosine and diiodotyrosine.

**Experimental protocols**

Melting points (Büchi oil bath apparatus) are uncorrected. IR spectra were obtained with a Perkin–Elmer 521 spectrophotometer. 1H NMR spectra were recorded on a Varian EM 390 spectrometer using TMS as internal standard. [α]D were determined with a Schimdt–Haensch 1604 polarimeter. Elemental analyses were within ±0.4% of the calculated values.

**N,N’-Dicyclohexycarbodiimide coupling: general procedure A**

A solution of the required amino and carboxylic reagents (1 mmol each) in anhydrous CHCl3 (2 ml) in the presence of Dicyclohexylcarbodiimide (1.1 mmol) in CHCl3 (2 ml) was added dropwise under stirring, over a 10-min period. The cooling bath was removed and stirring was continued at rt. After 3 h a few drops of acetic acid were added and the reaction mixture was filtered, diluted with CHCl3 (20 ml) and washed with brine. Then the crude product.

**Base-catalysed ethylester hydrolysis: general procedure B**

A 1 M solution of KOH in EtOH (2.2 ml) was added dropwise at rt to a stirred solution of trans-epoxysuccinyl-aminoacetyl-amido(3-methyl)butane ethylester (1 mmol) in EtOH (2 ml). After 1 h the reaction mixture was concentrated under reduced pressure, the residue taken up with water (20 ml) and the solution washed with EtOAc (2 x 10 ml). The aqueous layer was cooled in ice, acidified (pH = 2) by careful addition of 2 N HCl under stirring and extracted with EtOAc (3 x 10 ml). The pooled extracts were washed with brine (2 x 10 ml), dried over Na2SO4 and evaporated under reduced pressure to give the crude product.

**N-Carbobenzyloxy-L-tyrosylamido(3-methyl)butane**

A solution of N-Z-L-tyrosine (6.50 g, 20.54 mmol) and N-methylmorpholine (2.07 g, 20.54 mmol) in anhydrous THF (40 ml) was cooled to −15°C and i-butylchloroformate (2.80 ml, 20.54 mmol) was added dropwise under stirring. After 30 min, a solution of 1-amino-3-methylbutane (2.26 ml, 20.54 mmol) in THF (10 ml) was added slowly while stirring and the temperature of −15°C was maintained. The reaction mixture was stored overnight at 4°C in a refrigerator, allowed to warm to rt and filtered. The solvent was removed under
reduced pressure, the residue was dissolved in EtOAc (30 ml), washed with 1 N HCl, saturated NaHCO₃ and brine. After drying over Na₂SO₄, the solvent was evaporated under reduced pressure and the residue purified by silica-gel chromatography (CHCl₃/EtOAc, 9:1). Crystallization from 1,2-dichloroethane/hexane gave the pure product: 6.45 g (yield: 87%); mp: 133-135°C; [α]₀° = 9° (1, CHCl₃); IR (KBr) main peaks at 3428, 3065, 3010, 2938, 1711, 1670, 1514, 1210, 1047 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.79 and 0.84 (2 s, 6, Me₂C), 1.00-1.63 (m, 3, CH₂CH₂CH and Boc CH₂), 2.77-3.37 (m, 4, CH₂N and Tyr CH₂), 4.05-4.45 (m, 1, αCH), 6.88 (d, 1, CH₃), 7.07 (d, 1, CH₂), J = 9.0 Hz), 0.70 (d, 2, CH₃, J = 9.0 Hz). 7.34 (s, 5, CH₅).

N-tert-Butyloxycarbonyl-3,5-diiodo-L-tyrosine [38] (4.27 g, 8.00 mmol) and N-tert-Butyloxycarbonyl-3-iodo-L-tyrosylamido(3-methyl)butane (1.57 g, 2.6 mmol) in anhydrous EtOAc (20 ml) was submitted to removal of the protective group according to the previous procedure. Crystallization from EtOAc/hexane gave the pure product: 6.45 g (yield: 82%); mp: 133-135°C; [α]₀° = -23° (1, MeOH); IR (CHCl₃) main peaks at 3393, 2956, 1747, 1666, 1518, 1315, 1197 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.80 and 0.85 (2 s, 6, Me₂C), 1.10-1.50 (m, 6, CH₂CH₂CH and CH₂CH₂CH), 2.90-3.30 (m, 4, CH₂N and Phe CH₂), 3.50 and 3.66 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH₃, J = 7.5 Hz), 4.50-4.84 (m, 1, αCH), 7.25 (s, 5, CH₅).

N-tert-Butyloxycarbonyl-3,5-diido-L-tyrosylamido(3-methyl)butane 22

N-Boc-3,5-diido-L-tyrosine [28] (703 mg, 3.00 mmol) and L-trans-epoxysuccinic acid monoethylester (346 mg, 2.16 mmol) were coupled according to General procedure A. Fractionation by silica-gel chromatography (CHCl₃) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 600 mg (yield: 57%); mp: 118–120°C; [α]₀° = -31° (1, CHCl₃); IR (CHCl₃) main peaks at 3393, 2933, 1747, 1666, 1518, 1315, 1197 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.80 and 0.85 (2 s, 6, Me₂C), 1.07-1.48 (m, 6, CH₂CH₂CH and CH₂CH₂CH), 2.90-3.30 (m, 4, CH₂N and Phe CH₂), 3.50 and 3.66 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH₃, J = 7.5 Hz), 4.50-4.84 (m, 1, αCH), 7.25 (s, 5, CH₅).

L-Trans-Epoxy succinyl-L-phenylalanylamido(3-methyl)butane ethylester 10a

L-Tyrosylamido(3-methyl)butane (540 mg, 2.16 mmol) and L-trans-epoxysuccinic acid monoethylester (346 mg, 2.16 mmol) were coupled according to General procedure A. Crystallization from 1,2-dichloroethane/hexane gave the pure product: 485 mg (yield: 57%); mp: 150–152°C; [α]₀° = -28° (1, CHCl₃); IR (CHCl₃) main peaks at 3399, 2933, 1747, 1666, 1513, 1315, 1172 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.79 and 0.86 (2 s, 6, Me₂C), 1.07–1.48 (m, 6, CH₂CH₂CH and CH₂CH₂CH), 2.80–3.30 (m, 4, CH₂N and Phe CH₂), 3.50 and 3.66 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH₃, J = 7.5 Hz), 4.42–4.76 (m, 1, αCH), 6.78 (d, 2, CH₃, J = 9.0 Hz), 7.05 (d, 2, CH₃, J = 9.0 Hz).
**L-trans Epoxysuccinyl 3,5-diido L-tyrosylamido(3-methyl)butane ethylester 13a**

3,5-Diido-L-tyrosylamido(3-methyl)butane (627 mg, 1.26 mmol) and L-trans-epoxysuccinic acid monoethylester (200 mg, 1.25 mmol) were coupled according to General procedure A. DMSO (2 ml) was required to dissolve the amino reagent in this case. Fractionation by silica-gel chromatography (CHCl₃/MeOH, 9:1) and crystallization from EtOH gave the pure product: 298 mg (yield: 83%); mp: 212–214°C; [α]D = +28° (1, CHCl₃); IR (CHCl₃) main peaks at 3615, 3389, 2958, 1731, 1526, 1225 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.84 and 0.91 (two s, 6, Me₂C), 1.03-1.58 (m, 6, CH₂CH₃ and CH₂CH₂CH), 3.46 and 3.65 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.50-4.75 (m, 1, αCH), 7.27 (s, 5, C₆H₅).

**D-trans-Epoxysuccinyl-L-phenylalanlylamido(3-methyl)butane ethylester 14a**

L-Phenylalanlylamido(3-methyl)butane (703 mg, 3.00 mmol) and d-trans-epoxysuccinic acid monoethylester (480 mg, 3.0 mmol) were coupled according to General procedure A. Silica-gel chromatography (CHCl₃) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 408 mg (yield: 52%); mp: 68-70°C; [α]D = 31° (1, CHCl₃); IR (CHCl₃) main peaks at 3389, 2957, 1747, 1516, 1317, 1153 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.83 and 0.90 (2 s, 6, Me₂C), 1.16-1.43 (m, 6, CH₂CH₃ and CH₂CH₂CH), 3.10-3.72 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.21 (q, 2, CH₂CH₂J, J = 7.5 Hz), 4.38-4.68 (m, 1, αCH), 7.72 (s, 2, C₆H₅).

**D-trans-Epoxysuccinyl-L-phenylalanlylamido(3-methyl)butane ethylester 15a**

L-Phenylalanlyamido(3-methyl)butane (700 mg, 3.00 mmol) and d-trans-epoxysuccinic acid monoethylester (320 mg, 2.00 mmol) were coupled according to General procedure A. Fractionation by silica-gel chromatography (CHCl₃/MeOH, 9:1) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 468 mg (yield: 56%); mp: 159-161°C; [α]D = 18° (1, DMF); IR (KBr) main peaks at 3471, 3321, 2958, 1738, 1644, 1537, 1443, 1225 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.80 and 0.85 (two s, 6, Me₂C), 1.07-1.73 (m, 6, CH₂CH₃ and CH₂CH₂CH), 2.90-3.44 (m, 5, CH₂N, Phe CH₃ and epoxide CH), 3.65 (unresolved d, 1, epoxide CH), 4.29 (q, 2, CH₂CH₂J, J = 7.5 Hz), 4.25 (q, 2, CH₂CH₂J, J = 7.5 Hz), 4.41-4.63 (m, 1, αCH), 7.66 (s, 2, C₆H₅).

**D-trans-Epoxysuccinyl-3,5-diido-L-tyrosylamido(3-methyl)butane ethylester 16a**

L-trans-Epoxysuccinyl-3,5-diido-L-tyrosylamido(3-methyl)butane (500 mg, 2.00 mmol) and d-trans-epoxysuccinic acid monoethylester (200 mg, 1.25 mmol) were coupled according to General procedure A. DMSO (2 ml) was required to dissolve the amino reagent in this case. Fractionation by silica-gel chromatography (CHCl₃/MeOH, 9:1) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 340 mg (yield: 58%); mp: 142-144°C; [α]D = 25° (1, CHCl₃); IR (CHCl₃) main peaks at 3435, 2935, 1747, 1516, 1308, 1172 cm⁻¹; ¹H-NMR (CDCl₃) δ 0.83 and 0.90 (2 s, 6, Me₂C), 1.07-1.73 (m, 6, CH₂CH₃ and CH₂CH₂CH), 3.00-3.44 (m, 5, CH₂N, Phe CH₃ and epoxide CH), 3.65 (unresolved d, 1, epoxide CH), 4.29 (q, 2, CH₂CH₂J, J = 7.5 Hz), 4.48-4.80 (m, 1, αCH), 7.40 (s, 5, C₆H₅).

**D-trans-Epoxysuccinyl-3,5-diido-L-tyrosylamido(3-methyl)butane ethylester 17a**

L-trans-Epoxysuccinyl-3,5-diido-L-tyrosylamido(3-methyl)butane (622 mg, 1.24 mmol) and d-trans-epoxysuccinic acid monoethylester (198 mg, 1.24 mmol) were coupled according to General procedure A. DMSO (2 ml) was required to dissolve the amino reagent in this case. Fractionation by silica-gel chromatography (CHCl₃/MeOH, 9:1) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 324 mg (yield: 81%); mp: 149-151°C; [α]D = 39° (1, DMF); IR (KBr) main peaks at 3433, 2954,
d-trans-Epoxysuccinyl-l-phenylalanlamido(3-methyl)butane ethylester (645 mg, 1.71 mmol) was hydrolyzed according to General procedure B. Crystallization from CHCl₃/hexane gave the pure product: 291 mg (yield: 81%); mp: 191-193°C; [α]₂₀⁰ = 52° (1, MeOH); IR (KBr) main peaks at 3316, 2962, 1754, 1659, 1450, 1240 cm⁻¹; ¹H-NMR (CD,OD) δ 0.83 and 0.91 (2 s, 6, Me₂), 1.08-1.60 (m, 3, CH₂CH₂CH), 2.94-3.10 (m, 2, CH₂N), 3.35 and 3.70 (two d, 2, epoxide CH, J = 2.0 Hz), 4.50-4.69 (m, 1, αCH), 7.30 (s, 5, C₆H₅).

d-trans-Epoxysuccinyl-l-tyrosylamido(3-methyl)butane ethylester (554 mg, 0.90 mmol) was hydrolyzed according to General procedure B. The crude material was triturated with hexane to give the pure product as a white solid: 170 mg (yield: 89%); mp: 105-108°C; [α]¹⁷¹⁵ = 35° (1, DMF); IR (KBr) main peaks at 3359, 2956, 1738, 1644, 1536, 1444, 1228 cm⁻¹; ¹H-NMR (CD,OD) δ 0.84 and 0.91 (2 s, 6, Me₂), 1.03-1.52 (m, 3, CH₂CH₂CH₂CH₂), 2.76-3.07 (m, 2, Tyr CH₃), 2.94-3.20 (m, 2, CH₂N), 3.35 and 3.59 (two d, 2, epoxide CH, J = 2.0 Hz), 4.31-4.52 (m, 1, αCH), 7.56 (s, 2, C₆H₅).

Papain assay
Papain EC 3.4.22.2 was obtained from Sigma, product No P-4762, and solutions were made fresh daily by incubating the enzyme (1.5-2 mg) for 45 min at room temperature in 25 ml of 50 mM phosphate buffer pH 6.8 containing 2 mM EDTA and 0.5 mM l-cysteine according to Thompson [39] with minor modifications. Approximate enzyme concentration was determined from the absorbance at 280 nm (E = 58.5 mM⁻¹ cm⁻¹) [40]. The activated enzyme solution was stored at 4°C and papain fully retained its activity for at least 10 h. All reagents used were from Sigma unless otherwise indicated. All buffer and solutions were made with ultra-high quality water (Filga USP).

The rate of hydrolysis of Z-Gly-ONp was monitored continuously at 405 nm [41] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. A cuvette containing everything but the enzyme was used in the reference cell to correct for non-enzymatic hydrolysis of the substrate. The final concentration of the organic solvent in the activity mixture was 12% (v/v) acetonitrile. This concentration of organic solvent is ineffective towards kinetic parameters of papain-catalyzed hydrolysis of Z-Gly-ONp [41].

Reactions were started by addition of enzyme solution in the cuvettes. Absorbances were continuously monitored and stored in the computerized spectrophotometer. Absorbance data were transferred to a Data System 450 Personal Computer (Kontron) equipped with a mathematical coprocessor and fitted to equation [3] by using Enzfitter [42], non-linear regression data analysis program from Elsevier-Biosoft (Cambridge). Progress curves were composed of 180-300 (absorbance, time) pairs. Software for collection of progress curves on computer was supplied by Kontron. A substrate concentration of 125 μM (Km × 12.5) avoided substrate depletion during the assays.

Cathepsin assay
Cathepsin B EC 3.4.22.1 from bovine spleen, product No C-6286 was purchased from Sigma and solutions were made fresh daily by incubating the enzyme under the same conditions as for papain. Approximate enzyme concentration was determined from absorbance at 280 nm (E¹⁰ = 20 cm⁻¹) [43]. Enzyme activity was tested as described for papain [44].

Calpain I and calpain II assay
Calpain I and II EC 3.4.22.17 from porcine red cells and porcine kidney respectively were purchased from Nakalay Tesque, Kyoto, Japan. The hydrolysis rate of 0.1 mM Suc-Leu-Tyr-NHMe was recorded fluorimetrically at 25°C in 1 ml of 50 mM Tris–HCl buffer pH 7.5, containing 10 mM dithiothreitol and 5 mM or 20 mM CaCl₂ for calpain I and calpain II respectively. Aminomethylcoumarin produced by enzyme-catalyzed hydrolysis was recorded continuously with a Kontron SFM 25 spectrofluorimeter with excitation set at 360 nm and emission at 460 nm. Data pairs (relative fluorescence vs time) in the presence and absence of inhibitors were analyzed as described for papain and cathepsin B. Software for fluorimeter progress curves recording was supplied from Kontron. Calpain was added at 60 nM final concentrations followed by the inhibitor. The limits to the magnitude of the apparent second-order rate constant measurable by this procedure were 10 M⁻¹ s⁻¹ as indicated in [26].

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