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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_2) 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 $M^{-1}s^{-1}$ for papain and 650–105 000 $M^{-1}s^{-1}$ for cathepsin B. For the inactivation of calpain I and II they ranged between 250 and 2000 $M^{-1}s^{-1}$ 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_2) < Tyr < Phe < Leu for papain and Phe < Tyr < Tyr(I) < Leu < Tyr(I_2) 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 **1**, 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.

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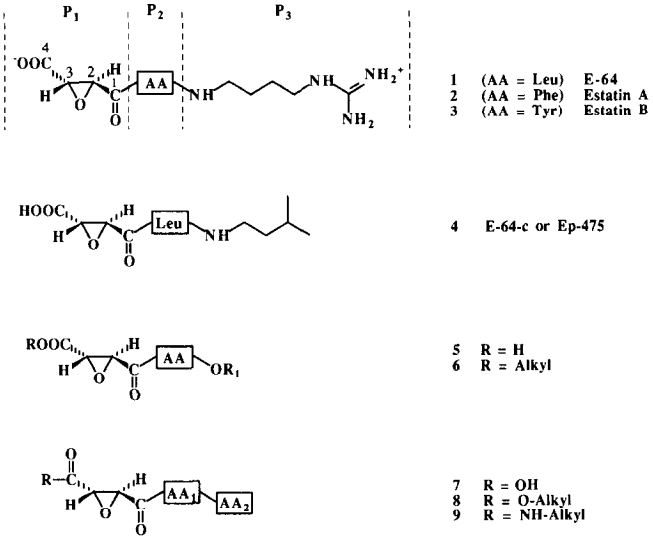
E-64 analogs are potential candidates for therapeutic application in those pathological states characterized by abnormally elevated levels of cysteine proteases activities, such as muscular dystrophy [21]

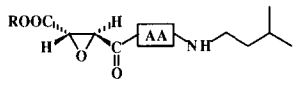


and myocardial ischemia [22, 23]. The most interesting properties of these inhibitors are: 1) remarkably fast reaction with the active site thiolate of cysteine proteases with second-order rate constants exceeding 10^5 $M^{-1}s^{-1}$ [6] under physiological conditions; 2) high specificity for cysteine 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].

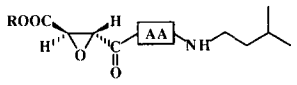
The main structural variations of L-*trans*-epoxysuccinyl inhibitors include: 1) modifications of the chain in P_3. It was shown that the charged guanidino group in P_3 was not essential for the onset of inhibitory activity [7–9]. In addition, the synthetic analog L-*trans*-epoxysuccinyl-leucinamido(3-methyl)butane **4**, named E-64-c or Ep-475 [6, 8, 9], containing the neutral *iso*-pentyl group in P_3, 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_2 and P_3 units by a selection of dipeptides (Ile-Pro-OH, Pro-Pro-OH and 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_3 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.



n°	R	AA
10a	Et	Phe
10b	H	Phe
11a	Et	Tyr
11b	H	Tyr
12a	Et	Tyr(I)
12b	H	Tyr(I)
13a	Et	Tyr(I_2)
13b	H	Tyr(I_2)



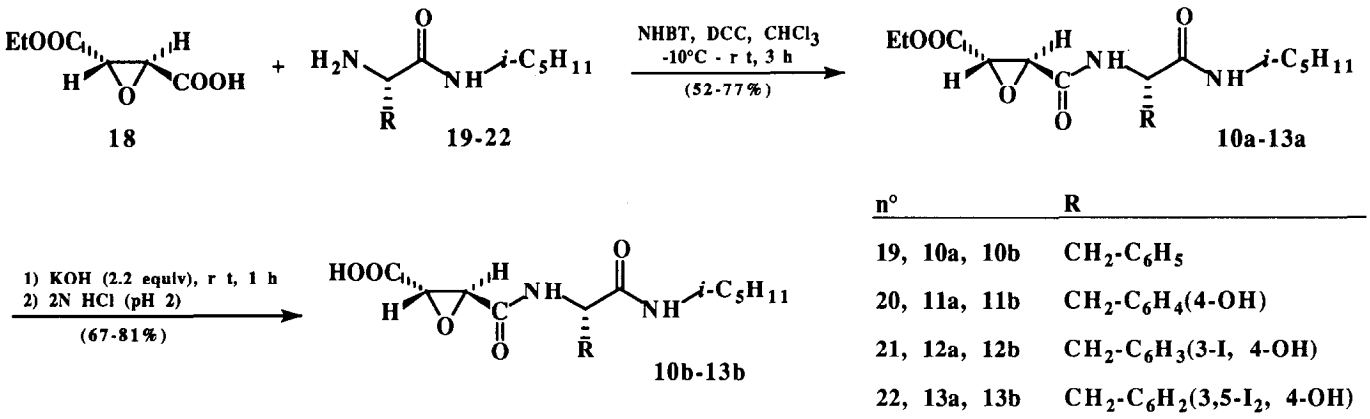
n°	R	AA
14a	Et	Phe
14b	H	Phe
15a	Et	Tyr
15b	H	Tyr
16a	Et	Tyr(I)
16b	H	Tyr(I)
17a	Et	Tyr(I_2)
17b	H	Tyr(I_2)

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 ≈ 23-fold increase of the second-order rate constants of cathepsin B inactivation. Similar preference of cathepsin B for Tyr(I) in P_2 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 proteases 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-aminobutane, 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



n°	R
19, 10a, 10b	CH_2-C_6H_5
20, 11a, 11b	CH_2-C_6H_4(4-OH)
21, 12a, 12b	CH_2-C_6H_3(3-I, 4-OH)
22, 13a, 13b	CH_2-C_6H_2(3,5-I_2, 4-OH)

Scheme 1.

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 [29].

All new compounds were homogeneous by TLC and gave satisfactory elemental analyses. The proposed structures are in accordance with their IR and ^{1}H-NMR spectra.

Enzyme inactivation measurement

Compounds **10b–17b** completely and irreversibly inhibited the papain- and cathepsin B-catalyzed hydrolysis of *N*-carbobenzyloxyglycine *p*-nitrophenylester (*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]:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{-}I \qquad [1]$$

where EI, E-I, k_{+1} and k_{-1} represent the reversible complex, the irreversibly inactivated enzyme and the rate constants for the non-covalent reaction step respectively; k_2 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.

$$\begin{matrix} & +\,I \rightleftharpoons EI \xrightarrow{k_2} E\text{-}I \\ E & \\ & +\,S \rightleftharpoons ES \xrightarrow{k_{cat}} E + P \end{matrix} \qquad [2]$$

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_∞ values indicating that inhibition was always complete. Absorbance versus time data were computed by non-linear regression analysis to equation [3]:

$$A = A_\infty [1 - e^{-k_{obs}t}] \qquad [3]$$

where A and A_∞ are the absorbances at t and t_∞ 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_∞, 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

k_{obs} values at constant saturating substrate concentration. It has been shown [30, 32, 33] that equation [4]:

$$\frac{k_{obs}}{[I]} = \frac{k_2}{K_I} \cdot \frac{1}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_I}} \quad [4]$$

$$\frac{1}{k_{obs}} = \frac{K_I}{k_2}\left(1 + \frac{[S]}{K_m}\right)\frac{1}{[I]} + \frac{1}{k_2} \quad [5]$$

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 $1/k_{obs}$ *vs* 1/[I]. The reciprocal of the intercept on the ordinate is k_2 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_{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 (*ie*, 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 $k_{obs}/[I]$. From equation [4] it follows that:

$$\frac{k_2}{K_I} = \frac{k_{obs}}{[I]}\left(1 + \frac{[S]}{K_m}\right) = \left(\frac{k_{obs}}{[I]}\right)_{corr} \quad [6]$$

Table I. Inactivation of papain[a] and cathepsin B[a], calpain I and calpain II[b] by E-64 analogs.

	Inhibitor	*Papain*				*Cathepsin B*				*Calpain I*	*Calpain II*
No	*Structure*	*Range (μM)*	K_I *(μM)*	k_2 *(min^{-1})*	k_2/K_I *($M^{-1}s^{-1}$)*	*Range (μM)*	K_I *(μM)*	k_2 *(min^{-1})*	k_2/K_I *($M^{-1}s^{-1}$)*	$k_{obs}/[I]$ *$M^{-1}s^{-1}$*	$k_{obs}/[I]$ *$M^{-1}s^{-1}$*
10b	L-*trans*-Eps-Phe-NH-*i*-C_5H_{11}	0.41–3.12	0.13	0.43	55 100	0.73–8.3	3.57	2.63	12 300	300	250
11b	L-*trans*-Eps-Tyr-NH-*i*-C_5H_{11}	0.62–3.12	0.44	1.16	43 900	0.41–3.12	0.55	0.81	24 100	800	500
12b	L-*trans*-Eps-Tyr(I)-NH-*i*-C_5H_{11}	1.7–12.5	0.56	0.91	27 000	0.21–1.56	0.34	1.39	68 100	1650	1300
13b	L-*trans*-Eps-Tyr(I_2)-NH-*i*-C_5H_{11}	0.93–6.25	0.23	0.40	29 000	0.1–0.78	0.068	0.43	105 000	1400	550
1	L-*trans*-Eps-Leu-NH-$(CH_2)_4$-NHC(NH)-NH_2				638 000[c]				89 400[d]		
4	L-*trans*-Eps-Leu-NH-*i*-C_5H_{11}	0.15–0.78	0.18	1.5	139 000[e]	0.14–1.04	0.27	1.24	76 500[f]	1000	2000
14b	D-*trans*-Eps-Phe-NH-*i*-C_5H_{11}	1.7–8.3	0.27	0.36	22 200	6.5–50	6.67	0.47	1200		
15b	D-*trans*-Eps-Tyr-NH-*i*-C_5H_{11}	1.5–11.1	0.48	0.42	14 600	8.9–67	11.1	0.43	650		
16b	D-*trans*-Eps-Tyr(I)-NH-*i*-C_5H_{11}	3.3–25	1.30	0.43	5500	2.2–25	4.76	1.12	3900	600	450
17b	D-*trans*-Eps-Tyr(I_2)-NH-*i*-C_5H_{11}	11.1–50	1.88	0.39	3500	13–100	6.25	0.28	750		

[a]100 mM phosphate buffer, pH 6.8; CH_3CN = 12% (v/v); [E] = 7 x 10^{-9} M; [S] = 125 μM; 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 μM; 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]357 000 $M^{-1}s^{-1}$ 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 $k_{obs}/[I]$ values, corrected for the presence of substrate according to equation [6], are equivalent to k_2/K_I if $[I] << K_I$ [26, 34, 35]. The observed $k_{obs}/[I]$ 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 $M^{-1}s^{-1}$ [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

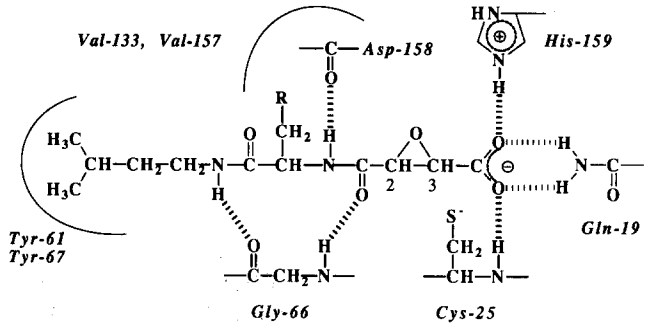


Fig 1. Schematic representation of the proposed binding of the L-*trans*-epoxysuccinyl amino acid amide analogs of E-64-c at the active site of papain according to X-ray crystal analysis and molecular dynamics studies [16–19]. Dotted lines represent possible hydrogen bonds. Curved lines indicate probable hydrophobic bonding interactions. Amino-acid residues connected with papain are given in italics. Analogous interactions are expected for binding at the cathepsin B active site, provided that the papain residues are replaced by the topologically equivalent residues of the enzyme.

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 mechanism 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 S_2 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(I_2) 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(I_2) 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(I_2) 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 k_2 values decreasing in the series **10b** > **12b** > **4** > **11b** > **13b**. The second-order inactivation rate constant k_2/K_I, 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-diiodotyrosylamido-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 $k_{obs}/[I]$. 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. ^{1}H-NMR spectra were recorded on a Varian EM 390 spectrometer using TMS as internal standard. $[\alpha]_D$ were determined with a Schmidt–Haensch 1604 polarimeter. Elemental analyses were within ± 0.4% of the calculated values.

N,N'-Dicyclohexylcarbodiimide coupling: general procedure A
A solution of the required amino and carboxylic reagents (1 mmol each) in anhydrous $CHCl_3$ (2 ml) in the presence of 1-hydroxy-benzotriazole (1 mmol) was cooled to –10°C. Dicyclohexylcarbodiimide (1.1 mmol) in $CHCl_3$ (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 $CHCl_3$ (20 ml) and washed with brine, 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, removal of the solvent under reduced pressure gave the crude product.

Base-catalyzed 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-aminoacyl-amido(3-methyl)butane ethylester (1 mmol) in EtOH (5 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 Na_2SO_4 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 $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was evaporated under reduced pressure and the residue purified by silica-gel chromatography ($CHCl_3$/EtOAc, 9:1). Crystallization from 1,2-dichloroethane/hexane gave the pure product: 6.45 g (yield: 82%); mp: 133–135°C; $[\alpha]_D^{22}$ = 9° (1, $CHCl_3$); IR (KBr) main peaks at 3428, 3065, 3010, 2956, 2398, 1711, 1670, 1514, 1210, 1047 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.79 and 0.84 (2 s, 6, Me_2C), 1.00–1.63 (m, 3, CH_2CH_2CH), 2.83–3.30 (m, 4, CH_2N and Tyr CH_2), 4.16–4.46 (m, 1, αCH), 5.08 (s, 2, Z CH_2), 6.75 (d, 2, C_6H_4, J = 9.0 Hz), 7.00 (d, 2, C_6H_4, J = 9.0 Hz), 7.34 (s, 5, C_6H_5).

N-tert-Butyloxycarbonyl-3-iodo-L-tyrosylamido(3-methyl)-butane

N-Boc-3-iodo-L-tyrosine [37] (3.3 g, 8.10 mmol) and 1-amino-3-methylbutane (0.71 g, 8.10 mmol) were coupled by *General procedure A*. Crystallization from 1,2-dichloroethane/hexane gave the pure product: 3.80 g (yield: 98%); mp: 137–139°C; $[\alpha]_D^{22}$ = 8° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3681, 3620, 3434, 3023, 2400, 1703, 1672, 1525, 1422, 1212, 1045 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.82 and 0.88 (2 s, 6, Me_2C), 1.10–1.64 (m, 12, CH_2CH_2CH and Boc CH_3), 2.77–3.37 (m, 4, CH_2N and Tyr CH_2), 4.05–4.45 (m, 1, αCH), 6.88 (d, 1, C_6H_3, J = 9.0 Hz), 7.07 (d, 1, C_6H_3, J = 9.0 Hz), 7.65 (s, 1, C_6H_3).

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

N-Boc-3,5-diiodo-L-tyrosine [38] (4.27 g, 8.00 mmol) and 1-amino-3-methylbutane (0.70 g, 8.00 mmol) were coupled by *General procedure A*. Crystallization from EtOAc gave the pure product: 3.50 g (yield: 73%); mp: 162–164°C; $[\alpha]_D^{22}$ = 9° (1, MeOH); IR ($CHCl_3$) main peaks at 3482, 3433, 2957, 1703, 1673, 1492, 1458, 1368, 1317, 1245, 1159 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.84 and 0.90 (2 s, 6, Me_2C), 1.10–1.64 (m, 12, CH_2CH_2CH and Boc CH_3), 2.90 (d, 2, Tyr CH_2, J = 7.5 Hz), 3.08–3.39 (m, 2, CH_2N), 4.08–4.40 (m, 1, αCH), 7.60 (s, 2, C_6H_2).

L-Tyrosylamido(3-methyl)butane ***20***

A solution of *N*-Z-L-tyrosylamido(3-methyl)butane (6.45 g, 16.73 mmol) in MeOH (30 ml) was submitted to hydrogenolysis in the presence of 10% Pd on charcoal (650 mg) at rt, under stirring, for 5 h. The catalyst was filtered through a short pad of celite and the solvent evaporated under reduced pressure. Silica-gel chromatography of the crude material ($CHCl_3$/*i*-PrOH 8:2) gave the pure product as a colorless oil which was used without further purification: 3.85 g (yield: 92%); $[\alpha]_D^{22}$ = 43° (1, MeOH); IR (KBr) main peaks at 3254, 2958, 1666, 1613, 1515, 1469, 1231 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.80 and 0.90 (2 s, 6, Me_2C), 1.06–1.60 (m, 3, CH_2CH_2CH), 2.84– 3.40 (bs, 4, CH_2N and Tyr CH_2), 3.73–4.20 (m, 1, αCH), 6.85 (d, 2, C_6H_4, J = 9.0 Hz), 7.17 (d, 2, C_6H_4, J = 9.0 Hz).

3-Iodo-L-tyrosylamido(3-methyl)butane ***21***

A solution of *N*-Boc-3-iodo-L-tyrosylamido(3-methyl)butane (3.5 g, 7.34 mmol) in anhydrous EtOAc (35 ml) was saturated with dry HCl at 0°C. After 3 h at rt, the mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc (30 ml) and washed with 2 N Na_2CO_3 (20 ml) and brine. The solution was dried over Na_2SO_4 and the solvent removed under reduced pressure to give the product as a colorless oil, which was used without further purification. Crystallization from 1,2-dichloroethane/hexane gave the pure product: 2.35 g (yield: 85%); $[\alpha]_D^{22}$ = –23° (1, MeOH); IR ($CHCl_3$) main peaks at 3337, 2956, 1644, 1516, 1461, 1291, 1228 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.82 and 0.90 (2 s, 6, Me_2C), 1.10–1.74 (m, 3, CH_2CH_2CH), 2.78 (d, 2, Tyr CH_2, J = 7.5 Hz), 3.00–3.66 (m, 2, CH_2N), 3.83–4.20 (m, 1, αCH), 6.82 (d, 1, C_6H_3, J = 9.0 Hz), 7.15 (dd, 1, C_6H_3, J = 9.0 Hz and 1.5 Hz), 7.65 (d, 1, C_6H_3, J = 1.5 Hz).

3,5-Diiodo-L-tyrosylamido(3-methyl)butane ***22***

N-Boc-3,5-diiodo-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: 0.98 g (yield: 76%); mp: 146–148°C; $[\alpha]_D^{22}$ = 21° (1, MeOH); IR (KBr) main peaks at 3391, 2956, 1656, 1531, 1428, 1294, 1207 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.82 and 0.89 (2 s, 6, Me_2C), 1.11–1.68 (m, 3, CH_2CH_2CH), 2.72 (bs, 2, Tyr CH_2), 2.95–3.25 (m, 2, CH_2N), 3.35–3.58 (m, 1, αCH), 7.59 (s, 2, C_6H_2).

L-trans-Epoxysuccinyl-L-phenylalanylamido(3-methyl)butane ethylester ***10a***

L-Phenylalanylamido(3-methyl)butane [28] (703 mg, 3.00 mmol) and L-*trans*-epoxysuccinic acid monoethylester (480 mg, 3.0 mmol) were coupled according to *General procedure A*. Fractionation by silica-gel chromatography ($CHCl_3$) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 650 mg (yield: 57%); mp: 118–120°C; $[\alpha]_D^{22}$ = –31° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3393, 2933, 1747, 1666, 1518, 1315, 1197 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.80 and 0.85 (2 s, 6, Me_2C), 1.10–1.50 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.90–3.30 (m, 4, CH_2N and Phe CH_2), 3.50 and 3.66 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.50–4.84 (m, 1, αCH), 7.25 (s, 5, C_6H_5).

L-trans-Epoxysuccinyl-L-tyrosylamido(3-methyl)butane ethylester ***11a***

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; $[\alpha]_D^{22}$ = –28° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3389, 2956, 1747, 1666, 1513, 1315, 1172 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.79 and 0.86 (2 s, 6, Me_2C), 1.07–1.48 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.80–3.30 (m, 4, CH_2N and Tyr CH_2), 3.53 and 3.65 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.42–4.76 (m, 1, αCH), 6.78 (d, 2, C_6H_4, J = 9.0 Hz), 7.05 (d, 2, C_6H_4, J = 9.0 Hz).

L-trans-Epoxysuccinyl-3-iodo-L-tyrosylamido(3-methyl)butane ethylester ***12a***

3-Iodo-L-tyrosylamido(3-methyl)butane (426 mg, 1.13 mmol) and L-*trans*-epoxysuccinic acid monoethylester (181 mg, 1.13 mmol) were coupled according to *General procedure A*. Fractionation by silica-gel chromatography ($CHCl_3$/EtOAc, 9:1) and crystallization from THF/hexane gave the pure product: 351 mg (yield: 60%); mp: 170–172°C; $[\alpha]_D^{22}$ = –20° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3391, 2955, 1747, 1671, 1516, 1289, 1179 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.80 and 0.87 (2 s, 6, Me_2C), 1.13–1.42 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.83 (d, 2, Tyr CH_2, J = 4.5 Hz), 3.06–3.29 (m, 2, CH_2N), 3.56 and 3.69 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.28 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.46–4.72 (m, 1, αCH), 6.90 (d, 1, C_6H_3, J = 9.0 Hz), 7.10 (dd, 1, C_6H_3, J = 9.0 Hz and 1.5 Hz), 7.55 (d, 1, C_6H_3, J = 1.5 Hz).

L-trans-Epoxysuccinyl-3,5-diiodo-L-tyrosylamido(3-methyl)-butane ethylester ***13a***

3,5-Diiodo-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 ($CHCl_3$/EtOAc, 9:1) and crystallization from EtOH gave the pure product: 450 mg (yield: 56%); mp: 159–161°C; $[\alpha]_D^{22}$ = – 23° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3435, 2935, 1747, 1666, 1515, 1317, 1153 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.83 and 0.90 (2 s, 6, Me_2C), 1.16–1.43 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.95 (d, 2, Tyr CH_2, J = 7.5 Hz), 3.00–3.40 (m, 2, CH_2N), 3.53 and 3.72 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.21 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.38–4.68 (m, 1, αCH), 7.72 (s, 2, C_6H_2).

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

L-Phenylalanylamido(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 ($CHCl_3$) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 575 mg (yield: 51%); mp: 142–144°C; $[\alpha]_D^{22}$ = 25° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3680, 3392, 3029, 1747, 1672, 1514, 1231 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.83 and 0.90 (2 s, 6, Me_2C), 1.07–1.73 (m, 6, CH_2CH_3 and CH_2CH_2CH), 3.00–3.44 (m, 5, CH_2N, Phe CH_2 and epoxide CH), 3.65 (unresolved d, 1, epoxide CH), 4.29 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.48–4.80 (m, 1, αCH), 7.40 (s, 5, C_6H_5).

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

L-Tyrosylamido(3-methyl)butane (500 mg, 2.00 mmol) and D-*trans*-epoxysuccinic acid monoethylester (320 mg, 2.00 mmol) were coupled according to *General procedure A*. Fractionation by silica-gel chromatography ($CHCl_3$/EtOAc, 9:1) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 408 mg (yield: 52%); mp: 68–70°C; $[\alpha]_D^{22}$ = 31° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3389, 2957, 1747, 1666, 1516, 1308, 1172 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.81 and 0.90 (2 s, 6, Me_2C), 1.15–1.63 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.93 (d, 2, Tyr CH_2, J = 7.5 Hz), 3.03–3.25 (m, 2, CH_2N), 3.30 and 3.65 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.21 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.44–4.74 (m, 1, αCH), 6.75 (d, 2, C_6H_4, J = 9.0 Hz), 7.00 (d, 2, C_6H_4, J = 9.0 Hz).

D-trans-Epoxysuccinyl-3-iodo-L-tyrosylamido(3-methyl)butane ethylester ***16a***

3-Iodo-L-tyrosylamido(3-methyl)butane (426 mg, 1.13 mmol) and D-*trans*-epoxysuccinic acid monoethylester (181 mg, 1.13 mmol) were coupled according to *General procedure A*. Fractionation by silica-gel chromatography ($CHCl_3$/EtOAc, 9:1) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 340 mg (yield: 58%); mp: 114–116°C; $[\alpha]_D^{22}$ = 18° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3615, 3389, 2975, 1748, 1666, 1514, 1245, 1044 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.80 and 0.86 (2 s, 6, Me_2C), 1.10–1.60 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.91 (d, 2, Tyr CH_2, J = 7.5 Hz), 3.06–3.34 (m, 2, CH_2N), 3.49 and 3.68 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.43–4.78 (m, 1, αCH), 6.85 (d, 1, C_6H_3, J = 9.0 Hz), 7.10 (d, 1, C_6H_3, J = 9.0 Hz), 7.53 (s, 1, C_6H_3).

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

3,5-Diiodo-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 ($CHCl_3$/EtOAc, 9:1) and crystallization from 1,2-dichloroethane/hexane gave the pure product: 580 mg (yield: 77%); mp: 146–148°C; $[\alpha]_D^{22}$ = 5° (1, $CHCl_3$); IR ($CHCl_3$) main peaks at 3389, 2933, 1748, 1670, 1516, 1457, 1097 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.84 and 0.91 (two s, 6, Me_2C), 1.10–1.47 (m, 6, CH_2CH_3 and CH_2CH_2CH), 2.90 (d, 2, Tyr CH_2, J = 7.5 Hz), 3.03–3.33 (m, 2, CH_2N), 3.46 and 3.65 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.25 (q, 2, CH_2CH_3, J = 7.5 Hz), 4.41–4.65 (m, 1, αCH), 7.66 (s, 2, C_6H_2).

L-trans-Epoxysuccinyl-L-phenylalanylamido(3-methyl)butane ***10b***

L-*trans*-Epoxysuccinyl-L-phenylalanylamido(3-methyl)butane ethylester (271 mg, 0.72 mmol) was hydrolyzed according to *General procedure B*. Crystallization from THF/hexane gave the pure product: 238 mg (yield: 95%); mp: 113–115°C; $[\alpha]_D^{22}$ = –59° (1, DMF); IR (KBr) main peaks at 3331, 2956, 1731, 1626, 1549, 1453, 1216 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.83 and 0.91 (2 s, 6, Me_2C), 1.07–1.72 (m, 3, CH_2CH_2CH), 2.90–3.25 (m, 4, CH_2N and Phe CH_2), 3.47 and 3.60 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.50–4.75 (m, 1, αCH), 7.27 (s, 5, C_6H_5).

L-trans-Epoxysuccinyl-L-tyrosylamido(3-methyl)butane ***11b***

L-*trans*-Epoxysuccinyl-L-tyrosylamido(3-methyl)butane ethylester (385 mg, 0.98 mmol) was hydrolyzed according to *General procedure B*. Crystallization from MeOH gave the pure product: 298 mg (yield: 83%); mp: 212–214°C; $[\alpha]_D^{22}$ = –60° (1, DMF); IR (KBr) main peaks at 3316, 2964, 1736, 1655, 1555, 1450, 1234 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.82 and 0.89 (2 s, 6, Me_2C), 1.04–1.54 (m, 3, CH_2CH_2CH), 2.73– 3.26 (m, 4, CH_2N and Tyr CH_2), 3.50 and 3.62 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.40–4.70 (m, 1, αCH), 6.77 (d, 2, C_6H_4, J = 9.0 Hz), 7.05 (d, 2, C_6H_4, J = 9.0 Hz).

L-trans-Epoxysuccinyl-3-iodo-L-tyrosylamido(3-methyl)butane ***12b***

L-*trans*-Epoxysuccinyl-3-iodo-L-tyrosylamido(3-methyl)butane ethylester (252 mg, 0.48 mmol) was hydrolyzed according to *General procedure B*. The crude material was triturated with hexane to give the pure product as a white solid: 210 mg (yield: 89%); mp: 78–80°C; $[\alpha]_D^{22}$ = – 41° (1, DMF); IR (KBr) main peaks at 3321, 2958, 1738, 1644, 1537, 1443, 1225 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.80 and 0.89 (2 s, 6, Me_2C), 1.10–1.56 (m, 3, CH_2CH_2CH), 2.73–3.27 (m, 4, CH_2N and Tyr CH_2), 3.53 and 3.64 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.40–4.67 (m, 1, αCH), 6.81 (d, 1, C_6H_3, J = 9.0 Hz), 7.07 (dd, 1, C_6H_3, J = 9.0 Hz and 1.5 Hz), 7.60 (d, 1, C_6H_3, J = 1.5 Hz).

L-trans-Epoxysuccinyl-3,5-diiodo-L-tyrosylamido(3-methyl)-butane ***13b***

L-*trans*-Epoxysuccinyl-3,5-diiodo-L-tyrosylamido(3-methyl)-butane ethylester (420 mg, 0.65 mmol) was hydrolyzed according to *General procedure B*. Crystallization from MeOH/H_2O gave the pure product: 324 mg (yield: 81%); mp: 149–151°C; $[\alpha]_D^{22}$ = – 39° (1, DMF); IR (KBr) main peaks at 3343, 2954,

1758, 1633, 1556, 1460, 1237 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.83 and 0.91 (2 s, 6, Me_2C), 1.10–1.50 (m, 3, CH_2CH_2CH), 2.80–3.00 (m, 2, Tyr CH_2), 3.02–3.43 (m, 2, CH_2N), 3.60 and 3.70 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.45–4.70 (m, 1, αCH), 7.70 (s, 2, C_6H_2).

D-trans-Epoxysuccinyl-L-phenylalanylamido(3-methyl)butane ***14b***

D-*trans*-Epoxysuccinyl-L-phenylalanylamido(3-methyl)butane ethylester (645 mg, 1.71 mmol) was hydrolyzed according to *General procedure B*. Crystallization from $CHCl_3$/hexane gave the pure product: 418 mg (yield: 70%); mp: 142–145°C; $[\alpha]_D^{22}$ = 52° (1, DMF); IR (KBr) main peaks at 3287, 2958, 1715, 1688, 1549, 1454, 1228 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.84 and 0.90 (2 s, 6, Me_2C), 1.09–1.60 (m, 3, CH_2CH_2CH), 2.77–3.36 (m, 5, CH_2N, Phe CH_2 and epoxide CH), 3.61 (d, 1, epoxide CH, J = 2.0 Hz), 4.50–4.86 (m, 1, αCH), 7.30 (s, 5, C_6H_5).

D-trans-Epoxysuccinyl-L-tyrosylamido(3-methyl)butane ***15b***

D-*trans*-Epoxysuccinyl-L-tyrosylamido(3-methyl)butane ethylester (354 mg, 0.90 mmol) was hydrolyzed according to *General procedure B*. Crystallization from dichloroethane/hexane gave the pure product: 220 mg (yield: 67%); mp: 200–202°C; $[\alpha]_D^{22}$ = 54° (1, MeOH); IR (KBr) main peaks at 3316, 2962, 1754, 1659, 1547, 1450, 1240 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.83 and 0.91 (2 s, 6, Me_2C), 1.13–1.62 (m, 3, CH_2CH_2CH), 2.80–3.24 (m, 4, CH_2N and Tyr CH_2), 3.34 and 3.60 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.45–4.70 (m, 1, αCH), 6.78 (d, 2, C_6H_4, J = 9.0 Hz), 7.05 (d, 2, C_6H_4, J = 9.0 Hz).

D-trans-Epoxysuccinyl-3-iodo-L-tyrosylamido(3-methyl)butane ***16b***

D-*trans*-Epoxysuccinyl-3-iodo-L-tyrosylamido(3-methyl)butane ethylester (206 mg, 0.39 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; $[\alpha]_D^{22}$ = 35° (1, DMF); IR (KBr) main peaks at 3359, 2956, 1738, 1644, 1536, 1444, 1228 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.84 and 0.91 (2 s, 6, Me_2C), 1.08–1.60 (m, 3, CH_2CH_2CH), 2.74–3.34 (m, 4, CH_2N and Tyr CH_2), 3.43 and 3.65 (two d, 2, epoxide CH, J = 2.0 Hz), 4.40–4.69 (m, 1, αCH), 6.82 (d, 1, C_6H_3, J = 9.0 Hz), 7.10 (dd, 1, C_6H_3, J = 9.0 Hz and J = 1.5 Hz), 7.61 (d, 1, C_6H_3, J = 1.5 Hz).

D-trans-Epoxysuccinyl-3,5-diiodo-L-tyrosylamido(3-methyl)-butane ***17b***

D-*trans*-Epoxysuccinyl-3,5-diiodo-L-tyrosylamido(3-methyl)-butane ethylester (377 mg, 0.58 mmol) was hydrolyzed according to *General procedure B*. Crystallization from $CHCl_3$/hexane gave the pure product: 291 mg (yield: 81%); mp: 191–193°C; $[\alpha]_D^{22}$ = 39° (1, MeOH); IR (KBr) main peaks at 3266, 2954, 1739, 1638, 1563, 1457, 1231 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 0.84 and 0.90 (2 s, 6, Me_2C), 1.03–1.52 (m, 3, CH_2CH_2CH), 2.76–2.87 (m, 2, Tyr CH_2), 2.94–3.20 (m, 2, CH_2N), 3.35 and 3.55 (2 d, 2, epoxide CH, J = 2.0 Hz), 4.31–4.55 (m, 1, αCH), 7.56 (s, 2, C_6H_2).

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 (Elga UHQ).

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 cuvette. 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 180–360 (absorbance, time) pairs. Software for collection of progress curves on computer was supplied by Kontron. A substrate concentration of 125 μM (K_m x 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 ($E^{1\%}$ = 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-NHMec was followed 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 $CaCl_2$ 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 constants measurable by this procedure were 10 $M^{-1}s^{-1}$ as indicated in [26].

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