Eur J Med Chem (1990) 25, 479-487

Original article

Irreversible inactivation of papain and cathepsin B by epoxidic substrate analogues

C Giordano[1], C Gallina[2*], V Consalvi[3], R Scandurra[3]

[1]*Centro di Studio per la Chimica del Farmaco;*
[2]*Dipartimento di Studi Farmaceutici;*
[3]*Dipartimento di Scienze Biochimiche, Università La Sapienza, Ple A Moro 5, 00185 Rome, Italy*

(Received 28 June 1989; accepted 5 December 1989)

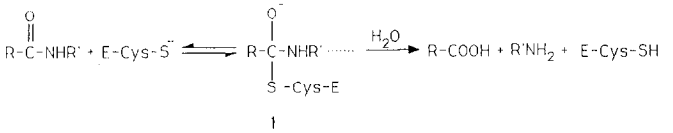
Summary — Epoxidic substrate analogues related to allylamine (**4a–4c**) and allyl alcohol (**5a–5f**) were synthesized and tested as models of cysteine-protease inhibitors. They proved to be irreversible inhibitors of papain and cathepsin B with pseudo-first-order inactivation rates ranging from 0.3 to 33 M^{-1} min^{-1}. The most active of the studied oxiranes **4a** bears *N*-acetyl-L-Phe as peptidyl unity. Most of the inhibitory activity was retained when the recognising moiety was extensively modified, provided that a phenyl group ∂ or the trapping epoxide ε was present. Specificity of peptidylepoxides for cysteine-proteases was confirmed, since no inhibitory activity was displayed toward serine or metallo-proteases.

Résumé — Inactivation irréversible de la papaïne et de la cathepsine B par des analogues de substrats époxidiques. *Les substrats époxydés correspondant à l'allylamine (**4a–4e**) et l'alcool allylique (**5a–5f**) ont été synthétisés et essayés en tant que modèles d'inhibiteurs des cystéine-protéases. Ils se sont montrés inhibiteurs irréversibles de la papaïne et de la cathepsine B avec des cinétiques d'inactivation de pseudo premier ordre de 0,3 à 33 M^{-1} min^{-1}. Le plus actif des oxiranes étudiés **4a** comporte le fragment peptidique* N-*acetyl* L-*Phe. La plus grande partie de l'activité inhibitrice est conservée lorsque la structure de reconnaissance est modifiée de façon notable, à condition qu'un groupe phényle ∂ ou époxyde ε soit présent. La spécificité des peptidylépoxydes pour les cystéine-protéases a été confirmée dans la mesure où aucune activité inhibitrice ne s'est manifestée vis-à-vis de sérine ou de métallo* protéases.

enzyme inhibitory activity / cysteine proteases / substrate analogues / peptidyloxiranes

Introduction

Proteases play a critical role in a number of biological transformations implicated in both physiological and pathological processes. Protein turnover, multiple sclerosis, bacterial and viral diseases, malignancy and muscular dystrophy, among others, are all initiated and/or sustained by well characterized cysteine-proteases found in mammalian cells [1, 2]. The active site of these enzymes contains a catalytic sulphydryl group (Cys-25 in the papain numbering system) and an imidazole unit (His-159). Addition of the deprotonated sulphydryl to an amide or ester carbonyl group to form a covalent intermediate (**1**) constitutes a key step of the enzymatic hydrolytic mechanism.



1

Scheme 1.

Several classes of irreversible inhibitors of cysteine-proteases have been developed from small peptide substrates by replacement of the carbonyl group of the scissile peptide bond by suitable trapping functions [3]. These include halomethyl-ketones, which attack both cysteine and serine proteases, diazomethyl-ketones and Michael acceptors that are specific for cysteine proteases. A complete different type of irreversible inactivator was isolated by Hanada [4] from cultures of *Aspergillus japonicus* and was called E-64, 1-[*N*-(L-3-*trans*-carboxyoxirane-2 carbonyl)-L-leucyl]amino-4-guanidinobutane (**2**).

E-64 and its analogues are among the most powerful inhibitors of several cysteine-proteases, having no effect on representative serine-proteases and metallo-proteases. The mechanism of inactivation by E-64 and its analogues has recently been demonstrated by ^{13}C NMR [6] and X-ray crystallographic analysis [7] of

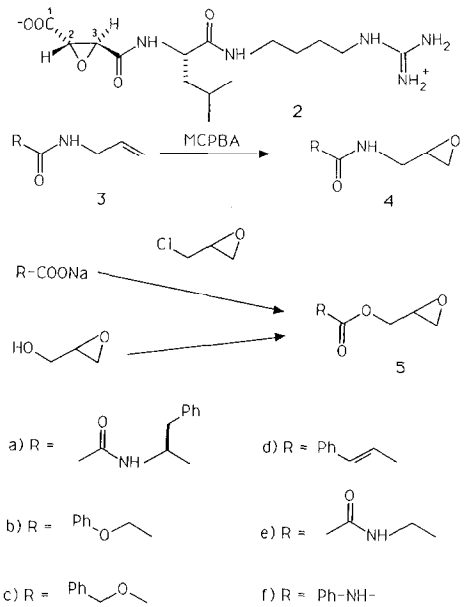
*Correspondence and reprints.

papain-inhibitor complexes: the epoxidic group of the epoxisuccinyl moiety alkylates the Cys-25 SH by forming a covalent bond with its C-2 atom.

The remarkable inhibitory properties of E-64 prompted us to investigate the epoxidic group as trapping moiety for cysteine-proteases. In this paper we describe the synthesis and evaluation of the biological activity of a series of epoxides designed to interact with the Cys-25 SH of papain. Epoxides related to allylamine (**4a–4e**) and to allyl alcohol (**5a–5e**) have been considered. According to the strong preference of papain for substrates with phenylalanine at P_2 [8], *N*-acetyl-L-Phe was chosen as the peptidyl moiety in **4a** and **5a**. Alternatively, a phenyl group has been introduced at the appropriate dis-tance from the trapping epoxide making use of a variety of reagents.

Chemistry

The oxiranes **4a–4e** have been prepared by epoxidation of the parent allylamides **3a–3e** with *m*-chloroperbenzoic acid (scheme 2).

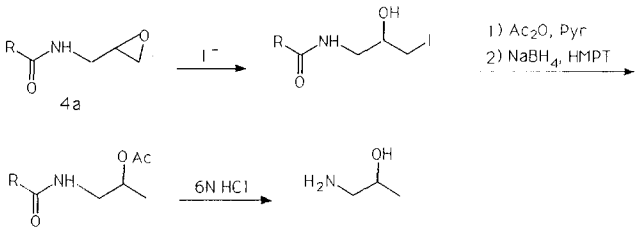


Scheme 2.

The first attempts to perform these conversions showed that excess of peracid in dichloromethane and long reaction times were needed, even under reflux, to complete the consumption of the alkene. Complex reaction mixtures and very low yields of the expected oxiranes were, however, obtained. Substantial improvements were achieved by carrying out the oxidation in the presence of 4,4'-thiobis-(6-*t*-butyl-3-methylphenol) according to Kishi [9]. Optimization of the yields was ultimately achieved at room temperature, in the presence of the same radical inhibitor, with dichloromethane as solvent for allylamides **4b** and **4c** and in THF for the other amides **4a**, **4d** and **4e**.

The oxiranes **5a**, **5b**, **5d** and **5e** were readily obtained by reaction of the appropriate sodium salts with 1-chloro-2,3-epoxipropane [10]. The other oxiranes of this series **5c** and **5f** were prepared by acylation of glycidol (scheme 2). The proposed structures of all new compounds are in accordance with the spectroscopic data (IR, ^{1}H NMR) and with elemental analyses.

The oxiranes **4b–4e** and **5b–5f** have been obtained as racemic compounds, while **4a** and **5a** are mixtures of diastereoisomers. Since examples of co-operative effects by polar groups like hydroxyl and alkoxyl in directing the steric course of epoxidation with *m*-chloroperbenzoic acid have been reported [11], a stereoselective outcome of the conversion, directed by the chiral center of the L-Phe, could not be excluded for **3a**. Attempts to resolve the purified product **4a** by TLC and HPLC, in order to determine the diastereoisomeric composition, were unsuccessful. However its transformation through the steps of scheme 3, which guarantees retention of the configuration at the internal carbon of the epoxide, gave completely racemic 1-amino-2-propanol. This result is in accordance with the enhanced complexity of the epoxidic proton signal in the ^{1}H NMR spectra of **4a** and **5a**.



Scheme 3.

It has therefore been assumed that **4a** represents a 50/50 mixture of diastereoisomers difficult to resolve by conventional chromatographic methods. As a consequence **4a**, as well as **5a**, were employed as such in the biological tests.

Results

Compounds **4a** to **4e** and **5a** to **5f** were tested as inhibitors of papain and cathepsin B catalyzed hydrolysis of *N*-carbobenzyloxy-glycine *p*-nitrophenyl ester (Z-Gly-ONp). All compounds, except **4e** and **5e**, irreversibly and completely inhibited the activity of both enzymes.

In all experiments we measured rates of inactivation with a large molar excess of inhibitor over

enzyme. Under these conditions, when [I]>>[E], the kinetics of inactivation follow equation (1) [12]:

$$\ln(v_t/v_c) = -k_{+2}t/(1 + K_i/[I]) \quad (1)$$

where v_c is the activity at 0 time, v_t the activity at time t and the scheme of the reaction is assumed to be as in the eqn (2):

$$E + I \overset{K_i}{\rightleftharpoons} EI \xrightarrow{k_{+2}} E - I \quad (2)$$

Semilogarithmic plots of v_t/v_c *versus* time were found to be linear and the pseudo-first-order rate constants, k_{obs} were calculated as $0.693/t_{1/2}$. For compounds **4a** and **4b** a replot of the reciprocal of k_{obs} *versus* the reciprocal of inhibitor concentration yielded the K_i and k_{+2} according to eqn 3 [12]:

$$1/k_{obs} = 1/[I]K_i/k_{+2} + 1/k_{+2} \quad (3)$$

For all the other compounds the apparent second order rate constant, $k_{obs}/[I]$, was calculated at $[I] \ll K_i$ with respect to the K_i determined for **4a** and **4b**. Under these conditions the $k_{obs}/[I]$ values are equal to k_{+2}/K_i [13].

The slope of the double logarithmic plot of the reciprocal of the half-times of inactivation toward compound **4a** and **4b** concentration [14] yielded a reaction order of 0.7 with respect to reagent concentration in both cases, indicating that interaction of at least one molecule of reagent with an enzyme site leads to inactivation.

The presence of a reversible competitive inhibitor such as *N*-acetyl-L-phenylalanyl-aminoacetaldehyde (A) in the inactivation mixture, quantitatively protects against enzyme irreversible modification. This evidence indicates that compound **4a** is an active-site-directed inhibitor. The pseudo-first-order rate constants for inactivation determined at A concentrations ranging from 25 to 125 nM were used to investigate the protection from inactivation by use of eqn (4) [15]:

$$v_a/v_o = k_3/k_2 + K_d(1 - v_a/v_o)/[A] \quad (4)$$

where v_a and v_o represent respectively the pseudo-first-order rate constants for inactivation in the presence and absence of the reversible competitive inhibitor; k_3 and k_2 are the fractional order rate constants for inactivation of free enzyme and enzyme-A complex in eqns (5) and (7), and I represents the irreversible inhibitor **4a**; K_d is the dissociation constant for enzyme-A reversible complex, eqn (6):

$$E + 0.7\ I \xrightarrow{k_2} E(0.7\ I) \quad (5)$$

$$E + A \overset{K_d}{\rightleftharpoons} EA \quad (6)$$

$$E + A + 0.7\ I \xrightarrow{k_3} EA(0.7\ I) \quad (7)$$

Table I. Inhibition of papain and cathepsin B by oxiranes derived from *N*-acetyl-L-phenylalanine and analogues. 100 mM phosphate buffer, pH 6.8; CH_3CN = 12% (v/v); [S] = 125 μM. Replicate determinations indicate standard deviation for kinetic parameters less than 20%.

Oxirane	*Papain*				*Cathepsin B*	
	[I] (mM)	$k_{obs}[I]^{-1}$ $(M^{-1}\ min^{-1})$	K_i *(mM)*	k_2/K_i $(M^{-1}\ min^{-1})$	*[I] (mM)*	$k_{obs}[I]^{-1}$ $(M^{-1}\ min^{-1})$
4a	1–8[a]		3.0	47.0	5.0	3.5
4b	4–20[a]		33.0	6.4	4.0	1.1
4c	10.0	1.8	ND	ND	10.0	0.3
4d	10.0	0.8	"	"	10.0	0.7
4e	10.0	NI[b]	"	"		
5a	2.0	8.0	"	"	4.0	0.9
5b	4.0	1.9	"	"	4.0	0.5
5c	4.0	0.8	"	"	4.0	0.3
5d	2.5	2.6	"	"	2.5	1.3
5e	20.0	NI[b]	"	"		
5f	8.0	0.6	"	"	8.0	0.5
Phenyl-	10.0	NI[b]	"	"		
Ethyl-	10.0	NI[b]	"	"		

[a]Inhibitor concentrations were 1, 2, 3, 4, 6, 8 mM and 4, 6, 8, 12, 18, 20 mM for compounds **4a** and **4b** respectively. [b]No inhibition observed for 180 min incubation at the concentration indicated.

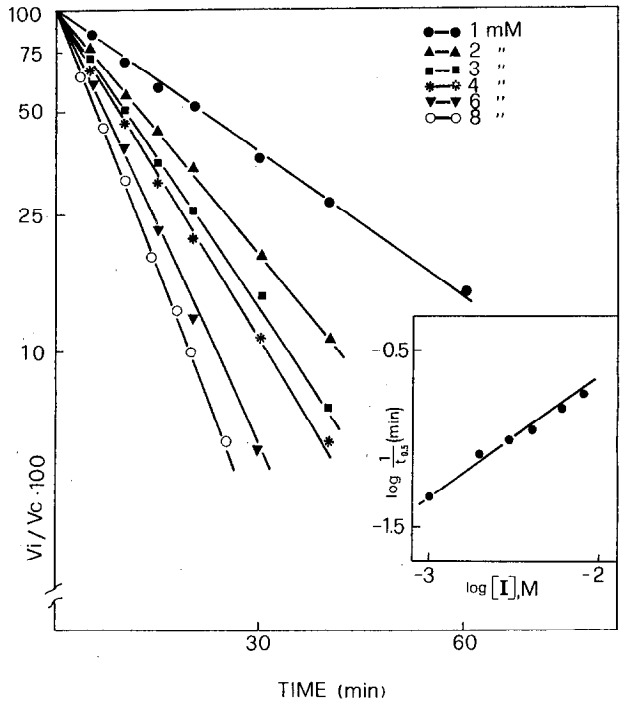


Fig 1. Time course of irreversible papain inactivation by oxirane (**4a**). Papain was incubated at room temperature in 50 mM phosphate buffer pH 6.8 with increasing concentrations of inhibitor. At the indicated times, samples were diluted 50-fold in the activity assay to measure residual activity as described in *Materials and Methods*. Inset: order of papain inactivation with respect to **4a**. The data of the time course are replotted in the form of log $1/t_{0.5}$ (min) as a function of log [I]. The slope of the line yields a reaction order of 0.7.

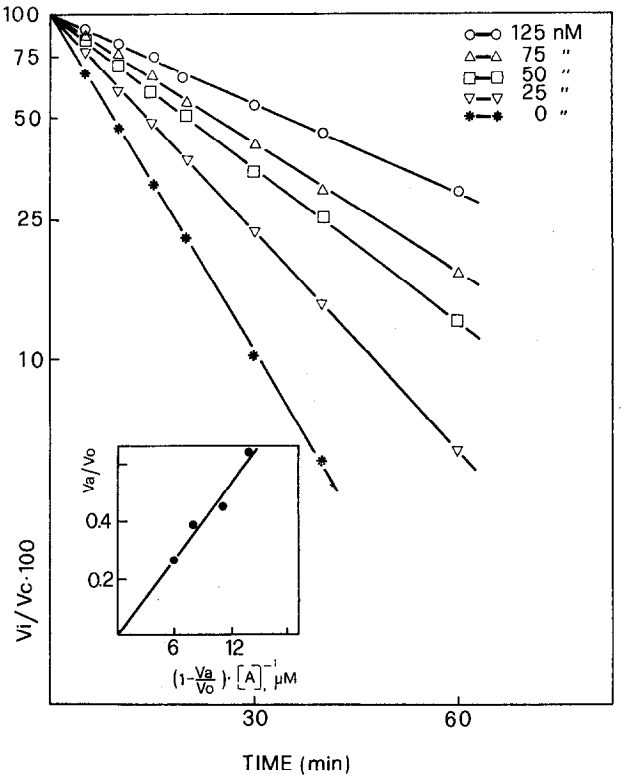


Fig 2. Protection by *N*-acetyl-L-phenylalanyl-aminoacetaldehyde (A) of papain irreversible inactivation by oxirane (**4a**). The enzyme was inactivated at 4 mM concentration of **4a** as in figure 1 and protected with various initial concentrations of A. The replot of the data according to eqn (4) yields a straight line passing through the origin; v_a and v_0 are the reciprocal of the inactivation half-times (min) for protected and unprotected enzyme, respectively.

The inset of figure 2 shows a plot of v_a/v_0 *versus* $(1-v_a/v_0)/[A]$. A straight line passing through origin is obtained demonstrating that EA complex is totally protected against epoxide inactivation; *ie* k_3 is very small compared to k_2. The slope of the line yields an apparent dissociation constant of 45 nM close to the K_i value for A [16]. The apparent dissociation constant calculated for the EA complex is affected by a very low level of accuracy owing to the condition of mutual depletion. A specific concentration of enzyme $[E]_{tot}/K_i$ [17] of 60 was indeed used in this experiment. No correction was made for the aldehyde concentration value in eqn (4), since our experiments were addressed to the demonstration of the complete protection from inactivation rather than to the calculation of the aldehyde K_i. The serine-proteases chymotrypsin and porcine pancreatic elastase and the metallo-protease leucine aminopeptidase were unaffected by 2 h incubation with 5 mM **4a**.

Discussion

The results obtained in the present study (table I) demonstrate that peptidylepoxides based on *N*-acetyl-L-phenylalanine and their analogues behave as irreversible inhibitors of papain and cathepsin B. The simultaneous presence of the recognising moiety and of the oxirane group are required for the onset of the biological activity. In fact ethyl and phenyloxirane, as well as the peptidyl oxiranes **4e** and **5e** lacking the hydrophobic group were completely devoid of inhibitory activity in the range of concentration tested. The active site directed nature of the interaction with papain has been confirmed by protection of the enzyme in the presence of *N*-acetyl-L-phenylalanyl-aminoacetaldehyde [16] which is a reversible competitive inhibitor of papain.

The most active of the studied oxiranes was **4a**, bearing the well established *N*-acetyl-L-phenylalanyl

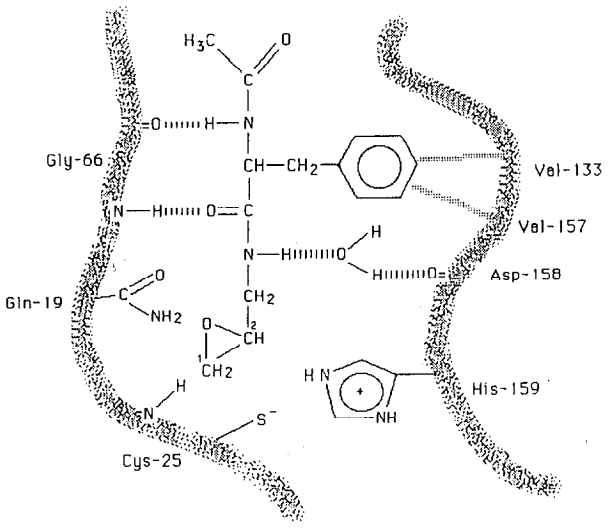


Fig 3. Schematic diagram showing the expected bonding interactions of the oxirane **4a** at the active site of papain.

recognising moiety. It is assumed to be reversibly bonded at the papain active site [18] according to the schematic diagram of figure 3.

In this enzyme–inhibitor complex, the trapping moiety is placed in a region where both the C-1 and C-2 of the epoxide can form a covalent bond with the Cys-25 sulphur. In addition to the expected bonding interactions represented in the figure, other H-bonds could be established by the oxirane oxygen to the side chain of Gln-19 and to backbone NH of Cys-25. In particular, protonation by His-159, promoting a hypothetical acid catalyzed epoxide opening [19] by cysteine-25 thiolate attack, would effectively improve the rate of formation of the irreversible adduct. A more detailed discussion of this point would require separation of the **4a** diastereoisomers, assignment of the configuration and determination of the individual inhibitory activities; a time-consuming work which has been delayed in consideration of the rather slow rate of inactivation shown by the mixture of diastereoisomers. This finding, however, seems to exclude effective transition states where the epoxide oxygen successfully encounters the imidazolium side chain of His-159 while the nucleophile attacks one of the oxirane carbons.

Imidazolium protonation of the epoxidic group, on the other hand, has been recently excluded by Huber [7] in the mechanism of inactivation of papain by E-64. The exceptional activity of this inhibitor (apparent second-order rate constant some 10^5 times higher than **4a**) seems therefore to be attributable to the complex network of H-bonds which involves its carboxylic acid group and probably approaches and favorably orients the epoxidic group for the thiolate attack. The strong decrease in the activity observed [20] by esterification of the carboxylic group of E-64 and its analogues is also in accordance.

The other oxiranes tested as papain inhibitors are derived from **4a** by modifying structural details which are considered important for the strength and mode of absorption of the molecule.

N-Acetyl-L-phenylalanine-2,3-epoxipropyl ester **5a** is an isoster of **4a**. It has been prepared in view of the strong resemblance to **4a** and of the simplicity of the synthetic method required. Since the reactivity of the epoxide group of **5a** towards nucleophiles should not be changed significantly, the 4-fold decrease in the inhibitory activity can be chiefly attributed to the loss of the H-bond with the Asp-158 backbone. Analogous decrease of activity has been observed in the other cases where an O-atom was introduced in place of NH, except that for the derivatives of cinnamic acid **4d** and **5d**.

The *N*-acetylglycine derivative **4e**, where the usual sequence of peptide bonds has been retained, while the phenyl group is lacking, and its analogue **5e** showed to be completely inactive as papain inhibitors, confirming that the hydrophobic interactions of the phenyl group with Val-133 and Val-157 are more important than the H-bonds at Gly-66 and Asp-158.

Oxiranes **4b–4d** and **5b–5d**, which are not derived from L-Phe, and however present a phenyl group ∂ to the epoxidic trapping moiety, still retain an inhibitory activity lower than that of **4a**, but within the same order of magnitude. Shift of the aromatic group to the ε position as in **5f** suggests important changes in the distances and orientation of the oxirane carbons and bonds relative to Cys-25 thiolate in the enzyme–inhibitor reversible complex. Inhibitory activity of the same order was, however, observed while a complete loss occurred when the phenyl group was placed on the epoxidic carbon.

According to the behaviour of several other irreversible inhibitors of cysteine proteases [5], the rates of inactivation of cathepsin B (table I) were shown to be about one order of magnitude lower than that obtained for papain. The sequence homology around the catalytically important residues, Cys-25 and His-159 (papain numbering) suggests that papain and cathepsin B share the same catalytic mechanism [21]. Our results are not in contradiction to this, but possibly reflect some of the differences around the specificity sites like replacement of Val-133 by Gly and Ser-205 by Glu [22]. In contrast, the serine-proteases chymotrypsin and porcine pancreatic elastase and a metallo protease, cytosolic leucine aminopeptidase, were unaffected by **4a** confirming [5] that peptidyloxiranes are selective inhibitors of cysteine proteases.

Experimental protocols

Melting points (Büchi oil bath apparatus) are uncorrected. Spectra were obtained as follows: IR spectra (for $CHCl_3$ solutions) on a Perkin–Elmer 521 spectrophotometer and ^1H NMR spectra on a Varian EM 390 spectrometer (the reported assignments are for $CDCl_3$ solutions relative to TMS as an internal standard, unless otherwise stated). $[\alpha]_D$ were determined with a Schmidt–Haensch 1604 polarimeter. Elemental analyses were performed by the Servizio Microanalisi del CNR, Area della Ricerca di Roma and were within ± 0.4% of the theoretical values.

Mixed anhydride coupling (Method A)
The appropriate carboxylic acid (1 mmol) and *N*-methylmorpholine (1 mmol) were dissolved in dry THF (2.5 ml) and the solution cooled to –15°C. *i*-Butyl-chloroformate (1 mmol) was added dropwise and the solution stirred for 30 min. Allylamine (1 mmol) in THF (1.5 ml) was added under stirring, while the temperature of –15°C was maintained. The reaction mixture was stored for 15 h at 4°C in a refrigerator, allowed to warm to room temperature and filtered. Solvent was removed under reduced pressure and the residue dissolved in EtOAc (30 ml), washed with 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed at reduced pressure to give the crude product.

N-*Phenoxyacetyl-3-aminopropene* ***3b***
Phenoxyacetic acid (2.7 g, 18.3 mmol) was coupled with allylamine (1.37 ml, 18.3 mmol) by *Method A*. The crude product was purified by bulb to bulb distillation (85–90°C, oven temperature, at 1 mm Hg) and crystallized from benzene/*n*-hexane to give 2.00 g (57%) of a white solid: mp 44–45°C; ^1H NMR ∂ 4.03 (m, 2, CH_2N), 4.53 (s, 2, CH_2O), 5.06–5.40 (m, 2, CH_2=C), 5.68–6.20 (m, 1, CH=C), 6.90–7.20 (m, 3, C_6H_5), 7.20–7.50 (m, 2, C_6H_5).

N-*Cinnamoyl-3-aminopropene* ***3d***
Cinnamic acid (2.2 g, 15 mmol) was coupled with allylamine (1.12 ml, 15 mmol) by *Method A*. The crude product was purified by silica gel chromatography ($CHCl_3$/*n*-hexane 1:1) and crystallized from 1,2-dichloroethane/*n*-hexane to give 1.71 g (61%) of a white solid: mp 88–89°C; ^1H NMR ∂ 3.95–4.10 (m, 2, CH_2N), 5.10–5.35 (m, 2, CH_2=C), 5.70–6.20 (m, 1, CH=C), 6.53 (d, 1, J = 15 Hz, CHCO), 7.33–7.62 (m, 5, C_6H_5), 7.71 (d, 1, J = 15 Hz, CHPh).

N-*(*N*-Acetyl-glycyl)-3-aminopropene* ***3e***
N-Acetyl-glycine (2.15 g, 18.3 mmol) was coupled with allylamine (1.37 ml, 18.3 mmol) by *Method A*. The crude product was purified by silica gel chromatography (EtOAc/iPrOH 7:3) and crystallized from EtOAc to give 2.03 g (71%) of a white solid; mp 131–132°C; ^1H NMR (CD_3OD) ∂ 2.00 (s, 3, CH_3), 3.80–4.10 (m, 4, CH_2CO and CH_2N), 5.05–5.40 (m, 2, CH_2=C), 5.55–6.15 (m, 1, CH=C).

N-*(*N*-Acetyl-L-phenylalanyl)-3-aminopropene* ***3a***
N-Acetyl-L-phenylalanine (2.07 g, 10 mmol) was coupled with allylamine (0.75 ml, 10 mmol) by *Method A*. The crude product was crystallized from 1,2-dichloroethane to give 1.43 g (58%) of a white solid; mp 160–162°C, Lit [23] 160–161°C.

*Benzyl-*N*-allyl-carbamate* ***3c***
To a solution of allylamine (0.94 ml, 12.5 mmol) and $NaHCO_3$ (2.62 g, 31.2 mmol) in water (62 ml) benzyl chloroformate (3.06 ml, 15 mmol) was added dropwise under efficient stirring at room temperature. After further stirring for 1 h the reaction mixture was extracted with ether (3 x 30 ml). The ethereal phases were washed with 1 N HCl and brine, combined, dried over Na_2SO_4 and evaporated under reduced pressure. The yellow crude material was distilled under vacuum to give the pure product as a colorless oil, 2.05 g (86%); bp 115–118°C at 0.5 mm Hg, Lit [24] 104–106°C at 0.3 mm Hg.

m-*Chloroperbenzoic acid epoxidation of allylamides* ***3b–3d*** *(Method B)*
A solution of the appropriate allylamide (1 mmol) and MCPBA (2 mmol) in dry CH_2Cl_2 (THF) (5 ml) was allowed to react overnight in the presence of 3–4 mg of 4,4'-thiobis (6-*t*-butyl-3-methylphenol). The reaction mixture was concentrated at reduced pressure to about 1/5 of its original volume and diluted with EtOAc (80 ml). The solution was washed with 3% $Na_2S_2O_5$, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed at reduced pressure to give the crude oxirane.

N-*(*N*-Acetyl-L-phenylalanyl)-2,3-epoxipropanamide* ***4a***
N-(*N*-Acetyl-L-phenylalanyl)-3-aminopropene (1.47 g, 6 mmol) and MCPBA (4.2 g, 1.2 mmol) were allowed to react overnight in dry THF (30 ml) in the presence of 3 mg of 4,4'-thiobis-(6-*t*-butyl-3-methylphenol). Addition of *n*-hexane to the reaction mixture gave an oil which spontaneously solidified. Further crystallization from 1,2-dichloroethane/*n*-hexane gave the oxirane as a white solid, 0.97 g (62%): mp 133–134°C; $[\alpha]_D$ = + 11° (c = 1, MeOH); ^1H NMR ∂ 1.95 (s, 3, CH_3), 2.20–2.50 (m, 1, epoxide CH_2), 2.50–2.75 (m, 1, epoxide CH_2), 2.80–3.15 (m, 3, epoxide CH and PheCH_2), 3.15–3.75 (m, 2, CH_2N), 4.63–4.93 (m, 1, PheCH), 7.27 (s, 5, C_6H_5).

Control of the diastereoisomeric composition
The epoxide **4a** could not be resolved by TLC (EtOAc) and by HPLC (4.6 x 250 mm column filled with 10 μ spherical lichrosorb RP 18, CH_3CN/H_2O 90:10).

A solution of the epoxide **4a** (262 mg, 1 mmol), NaI (149 mg, 10 mmol) and AcOH (60 mg, 1 mmol) in MeOH (12 ml) was allowed to react overnight. The reaction mixture was diluted with EtOAc (30 ml) and extracted with 3% $Na_2S_2O_5$ (2 x 10 ml) and brine. After drying over Na_2SO_4, the solution was concentrated at reduced pressure and stripped in high vacuum to give the expected iodidrine, 366 mg (94%) as a thick oil, homogeneous by TLC (EtOAc/*i*- PrOH 3:1).

A solution of the iodidrine (310 mg, 0.8 mmol) and Ac_2O (0.66 ml, 96 mmol) in pyridine (1.5 ml) was allowed to react for 6 h at room temperature. The reaction mixture was diluted with EtOAc (30 ml) and the solution washed with saturated $NaHCO_3$ (20 ml), 1 N HCl (3 x 20 ml), brine (20 ml) and dried over Na_2SO_4. After removal of the solvent at reduced pressure, the residue was stripped in high vacuum to give 305 mg (88%) of the iodidrine acetate as a thick oil, homogeneous by TLC (EtOAc/*i* PrOH 95:5).

A solution of the iodidrine acetate (256 mg, 0.6 mmol) and $NaBH_4$ (46 mg, 1.2 mmol) in anhydrous HMPT (4 ml) was allowed to react at room temperature for 4 h [25]. The reaction mixture was diluted with EtOAc (20 ml), washed with H_2O (3 x 20 ml) and dried over Na_2SO_4. After evaporation of the solvent at reduced pressure, the residue was purified by filtration through a short pad of silica gel (EtOAc/*i*-PrOH 95:5). The expected acetate, 161 mg (86%) was obtained as a thick oil, homogeneous by TLC (EtOAc/*i* PrOH 95:5). The structure of all the intermediates was in accordance with their ^1H NMR spectra.

The acetate (126 mg, 0.4 mmol) was hydrolyzed by treatment with 6 N HCl in 50% MeOH (5 ml) for 6 h at reflux

temperature. The HCl excess and solvents were carefully removed at reduced pressure. A water solution of the residue was passed through a column filled with a strongly basic ion exchange resin (Dowex 21 K) and the column eluted with water. Fractions positive to ninhydrin were combined, acidified with 1 N HCl and concentrated to give 41 mg (92%) of D,L-2-hydroxypropylamine hydrochloride. The identity of the product was confirmed by comparison with an authentic sample (TLC, *n*BuOH/AcOH/H_2O 60:20:20 and ^1H NMR). A solution of the sample in MeOH (2.45 ml) showed $[\alpha]_D = 0$, Lit $[\alpha]_D = 31.5°$ (c = 1, MeOH) for the L enantiomer [26].

Phenoxyacetyl-2,3-epoxipropanamide ***4b***
N-Phenoxyacetyl-3-aminopropene (1.91 g, 10 mmol) and MCPBA (3.55 g, 20 mmol) were allowed to react in CH_2Cl_2 according to *Method B*. The crude material was purified by silica gel chromatography ($CHCl_3$/hexane 1:1). Bulb to bulb distillation gave the pure product as a colorless oil, 1.39 g (67%): bp 140°C (oven temp) at 0.1 mm Hg; ^1H NMR ∂ 2.52 (dd, 1, J = 4.8 and 2.4 Hz, epoxide CH_2), 2.74 (t, 1, J = 4.8 Hz, epoxide CH_2), 3.00–3.25 (m, 1, epoxide CH), 3.25–4.00 (m, 2, CH_2N), 4.50 (s, 2, CH_2O), 6.85–7.20 (m, 3, C_6H_5), 7.25–7.45 (m, 2, C_6H_5).

*Benzyl-*N*-2,3-epoxipropylcarbamate* ***4c***
Benzyl-*N*-allylcarbamate (1.91 g, 10 mmol) and MCPBA (3.55 g, 20 mmol) were reacted in CH_2Cl_2 according to *Method B*. The crude material was purified by silica gel chromatography ($CHCl_3$). High vacuum distillation gave the pure product as a colorless oil, 1.90 g (92%): bp 122–125°C at 0.2 mm Hg, Lit [27] 150–151°C at 0.5 mm Hg; ^1H NMR ∂ 2.54 (dd, 1, J = 4.8 and 2.1 Hz, epoxide CH_2), 2.72 (t, 1, J = 4.8 Hz, epoxide CH_2), 3.00–3.25 (m, 1, epoxide CH), 3.25–3.80 (m, 2, CH_2N), 5.15 (s, 2, CH_2O), 7.40 (s, 5, C_6H_5).

Cinnamoyl-2,3-epoxipropanamide ***4d***
N-Cinnamoyl-3-aminopropene (1.12 g, 6 mmol) and MCPBA (2.13 g, 12 mmol) were reacted in THF according to *Method B*. The crude material was purified by silica gel chromatography ($CHCl_3$). Crystallization from 1,2-dichloroethane/*n*-hexane gave the pure product as a white solid, 0.58 g (48%): mp 108–109°C; ^1H NMR ∂ 2.66 (dd, 1, J = 4.5 and 3.0 Hz, epoxide CH_2), 2.77 (t, 1, J = 4.5 Hz, epoxide CH_2), 3.10–3.30 (m, 1, epoxide CH), 3.30–4.10 (m, 2, CH_2N), 6.51 (d, 1, J = 15 Hz, CHCO), 7.30–7.60 (m, 5, C_6H_5), 7.72 (d, 1, J = 15 Hz, CHPh).

N-*(N-Acetylglycyl)-2,3-epoxipropanamide* ***4e***
N-(*N*-Acetylglycyl)-2,3-aminopropene (1.56 g, 10 mmol) and MCPBA (3.55 g, 20 mmol) were allowed to react overnight in THF (40 ml) in the presence of 4,4'-thiobis-(6-*t*-butyl-3-methylphenol). The reaction mixture diluted with water (80 ml) was extracted with ether (2 x 50 ml). Ethereal phases were discarded, while the aqueous solution was concentrated at reduced pressure. The crude residue was purified by silica gel chromatography (EtOAc/*i*PrOH 7:3). Crystallization from acetone gave the pure product as a white solid, 1.12 g (65%): mp 125–126°C; ^1H NMR ∂ 1.98 (s, 3, CH_3), 2.63 (dd, 1, J = 4.8 and 3.0 Hz, epoxide CH_2), 2.73 (t, 1, J = 4.8 Hz, epoxide CH_2), 2.95–3.23 (m, 1, epoxide CH), 3.25–3.53 (m, 2, CH_2N), 4.75 (s, 2, CH_2CO).

General procedure for the preparation of the oxiranes ***5a, 5b, 5d and 5e*** *(Method C)*
The appropriate carboxylic acid sodium salt (1 mmol) and 1-chloro-2,3-epoxipropane (10 mmol) contained in a flask equipped with a reflux condenser protected from moisture ($CaCl_2$) were heated at 80°C for 12 h under efficient magnetic stirring in the presence of tetrabutylammonium bromide (0.5 mmol). The reaction mixture was filtered and the 1-chloro-2,3-epoxipropane excess removed at reduced pressure. The EtOAc (30 ml) solution of the residue was washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated at reduced pressure to give the crude epoxide.

N-*Acetyl-*L*-phenylalanyl-2,3-epoxipropyl ester* ***5a***
N-Acetyl-L-phenylalanine sodium salt (1.14 g, 5 mmol) was reacted with 1-chloro-2,3-epoxipropane (4.5 ml, 50 mmol) according to *Method C*. The crude material was purified by silica gel chromatography (EtOAc) and bulb to bulb distillation under reduced pressure. The pure product was obtained as a colorless oil, 1.08 g (83%): bp 190°C (oven temp) at 0.2 mm Hg; $[\alpha]_D = 0°$ (c = 1, MeOH); ^1H NMR ∂ 1.95 (s, 1, CH_3), 2.40–2.70 (m, 1, epoxide CH_2), 2.70–2.95 (m, 1, epoxide CH_2), 2.95–3.40 (m, 1, epoxide CH), 3.12 (d, 2, J = 7.0 Hz, $PheCH_2$), 3.83–4.12 (m, 1, CH_2O), 4.40 (dd, 1, J = 12.0 and 3.0 Hz, CH_2O), 4.80–5.05 (m, 1, PheCH), 7.15–7.30 (m, 5, C_6H_5).

Phenoxyacetyl-2,3-epoxipropyl ester ***5b***
Phenoxyacetic acid sodium salt (2.15 g, 12.5 mmol) and 1-chloro-2,3-epoxipronane (11.4 ml, 125 mmol) were reacted according to *Method C*. The crude material was purified by silica gel chromatography (*n*-hexane/$CHCl_3$ 1:1) and bulb to bulb distillation at reduced pressure. The pure product, 2.03 g (78%) was obtained as a colorless oil: bp 140°C (oven temperature) at 0.1 mm Hg; ^1H NMR ∂ 2.60 (dd, 1, J = 4.8 and 3.0 Hz, epoxide CH_2), 2.81 (t, 1, J = 4.8 Hz, epoxide CH_2), 3.10–3.23 (m, 1, epoxide CH), 4.05 (dd, 1, J = 12.3 and 6.3 Hz, CH_2O), 4.58 (dd, 1, J = 12.3 and 3.0 Hz, CH_2O), 4.70 (s, 2, CH_2CO), 6.85–7.20 (m, 3, C_6H_5), 7.25–7.45 (m, 2, C_6H_5).

Cinnamoyl-2,3-epoxipropyl ester ***5d***
Cinnamic acid sodium salt (2.12 g, 12.5 mmol) and 1-chloro-2,3-epoxipropane (11.4 ml, 125 mmol) were reacted according to *Method C*. Bulb to bulb distillation of the crude material at reduced pressure, gave the pure product as a colorless oil, 1.60 g (63%): bp 140°C (oven temp) at 0.2 mm Hg; ^1H NMR ∂ 2.68 (dd, 1, J = 4.5 and 3.0 Hz, epoxide CH_2), 2.86 (t, 1, J = 4.5 Hz, epoxide CH_2), 3.22–3.30 (m, 1, epoxide CH), 4.07 (dd, 1, J = 12.0 and 6.0 Hz, CH_2O), 4.57 (dd, 1, J = 12.0 and 3.0 Hz, CH_2O), 6.50 (d, 1, J = 16.5 Hz, CHCO), 7.33–7.76 (m, 5, C_6H_5), 7.80 (d, 1, J = 16.5 Hz, CHPh).

N-*Acetylglycyl-2,3-epoxipropyl ester* ***5e***
N-Acetylglycine sodium salt (1.73 g, 12.5 mmol) and 1-chloro-2,3-epoxipropane (11.4 ml, 125 mmol) were reacted according to *Method C*. The crude material was purified by silica gel chromatography ($CHCl_3$) and crystallization from EtOAc. The pure product was obtained as a white solid, 1.19 g (55%): mp 68–69°C; ^1H NMR ∂ 2.03 (s, 1, CH_3), 2.66 (dd, 1, J = 4.5 and 2.7 Hz, epoxide CH_2), 2.85 (t, 1, J = 4.5 Hz, epoxide CH_2), 3.12–3.22 (m, 1, epoxide CH), 3.90–4.20 (m, 1, CH_2N), 4.01 (dd, 1, J = 12.3 and 5.4 Hz, CH_2O), 4.48 (dd, 1, J = 12.3 and 3.0 Hz, CH_2O).

*2,3-Epoxipropyl-*N*-phenyl-carbamate* ***5f***
Freshly distilled phenyl isocyanate (2.38 g, 12 mmol) and 2,3-epoxipropanol (1.48 g, 20 mmol) were allowed to react for 48 h at room temp. The crude material was purified by silica gel chromatography ($CHCl_3$) and crystallization from toluene. The pure product was obtained as a white solid, 2.60 g (62%): mp 57–58°C, Lit [28] 59–60°C; ^1H NMR ∂ 2.63 (dd, 1, J = 4.5

and 2.7 Hz, epoxide CH_2), 2.83 (t, 1, J = 4.5 Hz, epoxide CH_2), 3.15–3.30 (m, 1, epoxide CH), 4.00 (dd, 1, J = 12.3 and 6.6 Hz, CH_2O), 4.55 (dd, 1, J = 12.3 and 3.0 Hz, CH_2O), 6.95–7.45 (m, 5, C_6H_5).

2,3-Epoxipropyl, benzyl carbonate ***5c***
Benzyl chlorocarbonate (2.56 g, 30 mmol) was added dropwise, *via* syringe, under stirring, to a vial closed by a rubber serum cap, containing freshly distilled 2,3-epoxipropanol (1.11 g, 30 mmol) and dry pyridine (1.37 ml, 60 mmol) cooled in an ice bath. After standing for 30 min at room temp, the reaction mixture was diluted with ether (50 ml) and the solution extracted with saturated $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4, the solvent removed at reduced pressure and the remaining pyridine stripped in high vacuum. The crude residue was purified by Al_2O_3 chromatography (*n*-hexane/$CHCl_3$ 7:3) and by bulb to bulb distillation to give 2.81 g (45%) of the pure product as a colorless oil: bp 140°C (oven temp) at 0.2 mm Hg; ^1H NMR ∂ 2.65 (dd, 1, J = 4.5 and 3.0 Hz, epoxide CH_2), 2.83 (t, 1, J = 4.5 Hz, epoxide CH_2), 3.15–3.35 (m, 1, epoxide CH), 4.08 (dd, 1, J = 12.0 and 6.0 Hz, CH_2O), 4.42 (dd, 1, J = 12.0 and 3.6 Hz, CH_2O), 7.40 (s, 5, C_6H_5).

Papain assay and inactivation
Papain was obtained from Sigma (type IV), and solutions were made fresh daily by incubating the enzyme (1.5-2 mg) for 45 min at room temp in 25 ml of 50 mM phosphate buffer pH 6.8 containing 2 mM EDTA and 0.5 mM L-cysteine according to Thompson [23] with minor modifications. Enzyme concentration was determined from the absorbance at 280 nm (E = 58.5 mM^{-1} cm^{-1} [29]. Activated enzyme solution was stored at 4°C and, under these conditions, papain fully retained its activity at least for 10 h. All buffers and solutions were made with ultra high quality water (Elga UHQ). All reagents used were from Sigma unless otherwise stated. The rate of hydrolysis of Z-Gly-ONp was monitored continuously at 405 nm [30] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. A cuvette containing buffer and substrate 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 10% (v/v) acetonitrile. This concentration of organic solvent is uneffective towards kinetic parameters of papain catalysed hydrolysis of Z-Gly-ONp [31]. Dilution of the enzyme (50-fold) from the inactivation mixture to the activity assay initiated the reaction. Absorbances were continuously measured every second for 2 min and the activities were calculated from the computerized spectrophotometer. Software was supplied by Kontron. A substrate concentration of 125 μM (12.5 K_m) was used in the experiments. All kinetic parameters indicated in the text were determined by linear regression analysis, but all data were plotted for inspection.

The inhibitors were dissolved in water for **4a**, **4e**, **5e** and in acetonitrile for **4b–4d**, **5a–5d** and **5f** respectively. The reaction of the enzyme with the inhibitors was in the same buffer used for its activation. The concentration of the acetonitrile required to dissolve inhibitors with the enzyme did not exceed 5% (v/v). This concentration of organic solvent did not affect enzyme activity over the time required to follow inactivation. The enzyme concentration was generally ranging from 2 to 3 μM and the inhibitor concentration was at least 300-fold greater. Inactivations were performed at room temperature. Samples were removed at suitable times and diluted 50-fold into the assay mixture.

Cathepsin B assay and inactivation
Cathepsin B from bovine spleen was purchased from Sigma and solutions were made fresh daily by incubating the enzyme under the same conditions as for papain. Enzyme concentration was determined from absorbance at 280 nm ($E^{1\%}$ = 20 cm^{-1}) [32]. Cathepsin activity was tested as described for papain [33]. General conditions of cathepsin B inactivation were as described for papain. Samples were removed at suitable times and diluted 20-fold into assay mixture.

Chymotrypsin assay and inactivation
Chymotrypsin from bovine pancreas was obtained from Fluka. Enzyme was dissolved in 50 mM phosphate buffer pH 6.8 and concentration determined from the absorbance at 280 nm (E = 50 mM^{-1} cm^{-1}) [34]. Chymotrypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester at 256 nm [35]. Compound **4a** was tested for 2 h inactivation at 5 mM concentration in the buffer used to dissolve the enzyme.

Porcine pancreatic elastase assay and inhibition
Enzyme (type III) was from Sigma. Enzyme was dissolved in 50 mM phosphate buffer pH 7.0 and concentration determined from $E^{1\%}$ = 22 cm^{-1} at 280 nm [36]. Activity was monitored by following the hydrolysis of BOC-L-Ala-*p*-nitrophenyl ester at 347 nm [37] in 50 mM phosphate buffer pH 7.0 at 25°C. Inactivation was performed for 2 h at 5 mM concentration of oxirane **4a** in the buffer used for activity.

Cytosolic leucine aminopeptidase (LAP-C) assay and inhibition
LAP-C from hog kidney was from Fluka. The enzyme was activated according to Anderson [38] and the hydrolysis of L-Leu-*p*-nitroanilide was monitored at 405 nm. Inactivation was performed at 5 mM concentration of oxirane **4a** for 2 h in the same buffer used for LAP-C activation.

Acknowledgments

We wish to thank CNR (Centro di Studio per la Chimica del Farmaco and Progetto Finalizzato Chimica Fine II) for financial support and CNR Microanalyses Service, Area della Ricerca di Roma, for useful co-operation.

References

1 Rich DH (1986) *In: Proteinase Inhibitors* (Barrett AJ, Salvesen G, eds) Elsevier, Amsterdam
2 Fischer G (1988) *Natural Product Rep* 465–495
3 Rich DH (1986) *In: Proteinase Inhibitors* (Barrett AJ, Salvesen G, eds) Elsevier, Amsterdam 156-161; Fischer G (1988) *Natural Product Rep* 476–477
4 Hanada K, Tamai M, Yamagishi M, Ohmura S, Sawada J, Tanaka I (1978) *Agric Biol Chem* 42, 523–528
5 Barrett AJ, Kembhawi AA, Brown MA, Kirschke H, Knight CG, Tamai M, Hanada K (1982) *Biochem J* 201, 189–198
6 Yabe Y, Guillaume D, Rich DH (1988) *J Am Chem Soc* 110, 4043–4044
7 Varughese KI, Ahmed FR, Carey PR, Hasmain S, Huber CP, Storer AC (1989) *Biochemistry* 28, 1330–1332
8 Schechter I, Berger A (1968) *Biochem Biophys Res Comm* 32, 889–902
9 Kishi Y, Aratani M, Tanino H, Fukuyama T, Goto T (1972) *J Chem Soc Chem Commun* 64–65

10 Kester RB, Gaiser CJ, Lazar ME (1943) *J Org Chem* 8, 550–555
11 Johnson MR, Kishi Y (1979) *Tetrahedron Lett* 4347–4350
12 Kitz R, Wilson IB (1962) *J Biol Chem* 237, 3245–3249
13 Knight CG (1986) *In: Proteinase Inhibitors* (Barrett AJ, Salvesen G, eds) Elsevier, Amsterdam 23–51
14 Levy HM, Leber PD, Ryan EM (1963) *J Biol Chem* 238, 3654–3659
15 Scrutton MC, Utter MF (1965) *J Biol Chem* 240, 3714–3723
16 Westerik JO, Wolfenden R (1972) *J Biol Chem* 247, 8195–8197
17 Webb JL (1963) *In: Enzymes and Metabolic Inhibitors* Academic Press, New York, 1, 66–79
18 Drenth J, Kalk KH, Swen HM (1976) *Biochemistry* 15, 3731–3738
19 Parker RE, Isaak NS (1959) *Chem Rev* 59, 737–799
20 Tamai M, Yokoo C, Murata M, Oguma K, Sota K, Sato E, Kamaoka Y (1987) *Chem Pharm Bull* 35, 1098–1104
21 Kamphuis IG, Drenth J, Baker EN (1985) *J Mol Biol* 182, 317–329
22 Barrett AJ, Nicklin MJH, Rawlings ND (1984) The papain superfamily of cysteine-proteases and their protein inhibitors. *Symp Biol Hung* 25, 203–217
23 Thompson SA, Andrews PR, Hanzlik RP (1986) *J Med Chem* 29, 104–111
24 Butler DN, Soloway AH (1966) *J Am Chem Soc* 88, 484–487
25 Hutchins RO, Kandasamy D, Dux III F, Maryanoff CA, Rotsein D, Goldsmith B, Burgoyne W, Cistone F, Dalessandro J, Puglis J (1978) *J Org Chem* 43, 2259–2267
26 Clark RL, Jones WH, Raich WJ, Folkers K (1954) *J Am Chem Soc* 76, 3995–3997
27 Schleppnik AA, Gutsche CD (1960) *J Org Chem* 25, 1378–1386
28 Iwakura Y, Taneda Y (1959) *J Org Chem* 24, 1992–1994
29 Glazer AN, Smith EL (1971) *In: The Enzymes* (Boyer PD, ed) Academic Press, New York, 3, 501–516
30 Kirsch JF, Ilgestrom M (1966) *Biochemistry* 5, 783–791
31 Brocklehurst K, Little G (1973) *Biochem J* 133, 67–70
32 Barret AJ, Kirschke H (1981) *In: Methods in Enzymology* (Lorand L, ed) Academic Press, New York, Part C, 80, 535–561
33 Bajkowski AS, Frankfater A (1975) *Anal Biochem* 68, 112–127
34 Kumar S, Hein GE (1969) *Anal Biochem* 30, 203–211
35 Liang TC, Abeles RH (1987) *Arch Biochem Biophys* 252, 627–634
36 Feinstein G, Kupfer A, Sokolovsky M (1973) *Biochem Biophys Res Commun* 50, 1020–1026
37 Visser L, Blout LK (1972) *Biochim Biophys Acta* 268, 257–260
38 Anderson L, Isley TC, Wolfenden R (1982) *Biochemistry* 21, 4177–4180