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N-Haloacetyl-amino-acid amides as active-site-directed inhibitors of papain and cathepsin B

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Summary — A series of *N*-haloacetyl-amino-acid amides were synthesized and tested as models of cysteine-protease inhibitors. They irreversibly inactivated papain and cathepsin B *via* a reversible enzyme–inhibitor intermediate. Apparent second-order rate constants of inactivation ranging from 65 to 16 700 M^{-1} s^{-1} were observed. Reactivity against papain, as compared to glutathione, was increased 16 400-fold for *N*-bromoacetyl-leucine isopentylamide and 25 700-fold for the corresponding iodoacetyl derivative; these increases are probably due to proximity effects. No inhibition of trypsin, chymotrypsin and porcine pancreatic elastase was observed. Haloacetamides represent an interesting class of easily synthesized, efficient, irreversible inhibitors of cysteine proteases, which have low non-specific alkylating properties.

enzyme-inhibiting activity / cysteine proteases / haloacetamides

Introduction

Cathepsin B is a pathologically important cysteine protease which has been implicated in a number of diseases [1]. Very efficient and selective irreversible inhibitors of cysteine proteases have been developed from small peptide substrates by replacement of the carbonyl group of the scissile peptide bond with suitable trapping functions. Such active-site-directed reagents can covalently modify the enzyme by alkylation of the functional cysteine–thiol group. They include peptidyl chloromethanes, peptidyl diazomethanes, peptidyl methyl-sulfonium salts, peptidyl alcohol methansulfonates [1]. More recently, peptidyl fluoromethanes [2–6] and peptidyl acyloxymethylketones [7] have been studied in an attempt to avoid the toxic effects [8–11] observed with *in vivo* administration of the most powerful inhibitors, namely, chloromethyl and diazomethyl ketones, probably due to non-specific alkylation of bionucleophiles under physiological conditions.

Chloro-, bromo- and iodoacetic acid and some of their simple ester and amide derivatives have been used for long time to alkylate the catalytic thiolate of cysteine proteases as well as other nucleophilic groups of enzymes and proteins [12–14]. Kinetic deuterium-isotope effect studies [15–18] on the alkylation of papain with these reagents have been described recently as diagnostic tests for its catalytic-site chemistry. Inactivation of papain and other cysteine proteases by active-site-directed chloroacetic acid derivatives has been reported by Morihara and coworkers [19, 20]. They showed that papain was alkylated at the sulfydryl of the catalytic cysteine and succeeded in enhancing the apparent rate constants of inactivation at pH 7 and 25°C, from 0.38 M^{-1} s^{-1} for chloroacetamide to 5.24 M^{-1} s^{-1} for $ClCH_2CO$–D-Leu–OEt. On the other hand, even iodoacetamide [15] seemed to be a milder alkylating reagent than chloromethylketones [5] towards glutathione, a representative nucleophile likely to be encountered in biological environments. Pursuing our program of evaluation of trapping groups useful for the development of reversible [21] and irreversible [22] protease inhibitors, we started to study new haloacetyl-amino-acid and peptide derivatives with the aim of obtaining easily synthesized, efficient, irreversible inhibitors of cysteine proteases, with low non-specific alkylating properties.

The schematic representation of the papain–bromoacetamide complex reported by Brocklehurst *et al* [18] shows the possible hydrogen bonding of the reagent with C=O and NH groups of the backbone

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and presents the CH_2Br group in proximity to the Cys^{25} thiolate. Substitution of one of the hydrogens of the amide NH_2 with a recognizing moiety capable of extending into the hydrophobic cleft of the active site (fig 1) was expected to improve binding without an important unfavorable reorientation of the alkylating group relative to the Cys^{25} thiolate. On the basis of the mode of binding of the E-64 family of inhibitors at the papain active site [23], L-leucine isopentylamide was considered a useful candidate for preparation of active-site-directed haloacetamide inhibitors. The study was extended to haloacetyl derivatives of L-phenylalanine isopentylamide in consideration of the recent discovery of estatins [24], new epoxysuccinyl inhibitors of microbial origin, structurally related to E-64, containing L-phenylalanine or L-tyrosine in place of L-leucine. Benzylamides of L-leucine and L-phenylalanine were also included, as were two derivatives containing the unnatural D-enantiomers.

Chemistry

The intermediate leucine and phenylalanine amides were prepared using conventional methodologies of peptide synthesis. Haloacetyl groups can be attached to the free amino group by several convenient procedures, including use of haloacetyl-*N*-hydroxysuccinimide esters [14]. Acylation under Schotten–Baumann conditions with inexpensive chloroacetyl and bromoacetyl chlorides was employed in the present work and rapidly gave high yields under very mild conditions. The carbodiimide method, using 10% 1-hydroxybenzotriazole as the additive, was followed for preparation of iodoacetamido derivatives. All new compounds gave satisfactory elemental microanalyses and the proposed structures are in accordance with the spectroscopic data. Structures of the new *N*-halocetyl-amino-acid amides are reported in table I.

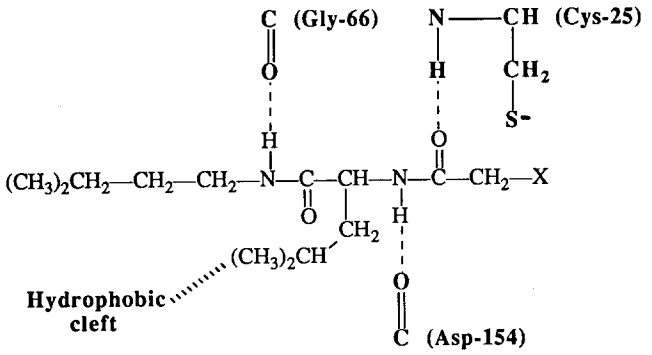

Fig 1. Diagrammatic representation of the proposed binding of haloacetamide inhibitors to the active site of papain. Groups belonging to the enzyme are in bold type.

Enzyme-inactivation measurements

Compounds **1a–6b** were tested as inhibitors of papain- and cathepsin B-catalyzed hydrolysis of *N*-carbobenzyloxyglycine-*p*-nitrophenylester (Z-Gly-ONp). All compounds irreversibly and completely inhibited the activity of both enzymes.

Irreversible inactivation of proteases with substrate analog inhibitors proceeds according to 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 first-order rate constant of the formation of covalently modified enzyme. This rate constant is usually determined as described by Kitz and Wilson [25].

Preincubation of enzyme and inhibitor under pseudo-first-order conditions was followed by dilution of the incubation mixtures to stop the enzyme inhibitor reaction and by estimation of the residual activity in an assay with the substrate. Recently, Tian and Tsou [26] introduced a convenient method for the evaluation of inactivation rates in the presence of a substrate according to equation [2]:

$$\begin{array}{l} I \underset{}{\overset{}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{–}I \\ + \\ E \\ + \\ S \underset{}{\overset{}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \end{array} \qquad [2]$$

where substrate and inhibitor are competing for the enzyme's binding site. The decrease in enzyme concentration during the reaction obeyed pseudo-first-order kinetics. In practice, reactions were followed by measuring the decrease in the formation of *p*-nitrophenol at $[S] \gg K_m$, at various inhibitor concentrations. The time dependency of the absorbance change was exponential and fitted well to a first-order rate law [26]. Pseudo-first-order rate constants were determined by non-linear regression analysis of the absorbance *versus* time data using equation [3]:

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

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. Data were collected for no less than three half-times

Table I. Inactivation of papain and cathepsin B by *N*-haloacetyl-amino-acid amides determined using the method of Tian and Tsou [26][a].

No	*Enzyme* *Structure of inhibitor*	*Range* (μ*M*)	K_I (μ*M*)	k_2 (min^{-1})	k_2/K_I ($M^{-1}\ s^{-1}$)
Papain					
1a	Cl–CH_2CO–Leu–NH–*i*–C_5H_{11}	125–1 000	77	0.3	65
1b	Br–CH_2CO–Leu–NH-*i*-C_5H_{11}	2.5–20	1.7	0.6	5 900
1c	I–CH_2CO–Leu–NH-*i*-C_5H_{11}	1.0–10	0.6	0.5	13 900
2a	Cl–CH_2CO–Leu–NH–CH_2-Ph	125–1 000	154	0.7	76
2b	Br–CH_2CO–Leu–NH–CH_2–Ph	2.5–20	2.5	0.9	6 000
2c	I–CH_2CO–Leu–NH–CH_2–Ph	2.0–20	1.3	0.9	11 500
3a	Cl–CH_2CO–Phe–NH-*i*-C_5H_{11}	125–1 000	50	0.2	67
3b	Br–CH_2CO–Phe–NH-*i*-C_5H_{11}	2.5–20	0.9	0.4	7 400
3c	I–CH_2CO–Phe–NH-*i*-C_5H_{11}	2.0–20	0.4	0.4	16 700
4a	Cl–CH_2CO–Phe–NH–CH_2–Ph	125[b]	ND	ND	ND
4b	Br–CH_2CO–Phe–NH–CH_2–Ph	2.5–20	2.5	0.5	3 300
4c	I–CH_2CO–Phe–NH–CH_2–Ph	2.0–20	2.0	0.8	6 700
5b	Br–CH_2CO–D–Leu–NH–*i*–C_5H_{11}	200–1 000	182	0.8	73
6b	Br–CH_2CO–D–Phe–NH-*i*-C_5H_{11}	50–600	40	0.4	167
Cathepsin B					
1c	I–CH_2CO–Leu–NH-*i*-C_5H_{11}	5.9–66.7	6.7	0.4	1 000
2c	I–CH_2CO–Leu–NH–CH_2–Ph	5.9–66.7	20	1.0	830
3a	Cl–CH_2CO–Phe–NH–*i*–C_5H_{11}	176–1 330	83	0.3	60
3b	Br–CH_2CO–Phe–NH–*i*–C_5H_{11}	8.8–66.7	8.3	0.3	600
3c	I–CH_2CO–Phe–NH-*i*-C_5H_{11}	5.9–44.4	1.8	0.3	2 800
4c	I–CH_2CO–Phe–NH–CH_2–Ph	5.9–66.7	8.3	0.6	1 200

[a]Conditions: 100 mM phosphate buffer, pH 6.8; CH_3CN = 12% (v/v); [*E*] = 7 x 10^{-9} M; [*S*] = 125 mM. Replicate determinations indicate standard deviations for the kinetic parameters 20%. [b]Not soluble above this concentration.

to demonstrate the complete inactivation of the enzyme by the inhibitor tested. Non-linear regression analysis of the progress curve data using equation [3] is considered the method of choice for calculation of kinetic parameters because it yields accurate values for the inhibition constants under a variety of circumstances [27]. In case of complexing competitive inhibitors, it has been shown [26, 28, 29] that the following equation is valid:

$$k_{obs} = \frac{k_2 K_a}{1 + \frac{[S]}{K_m} + K_a [I]} \qquad [4]$$

where K_a is the inhibitor's association constant ($1/K_I$). Therefore, plotting $1/[I]k_{obs}$ *versus* $1/[I]$ gives k_2 as the reciprocal of the intercept on the ordinate and K_I from the slope $(1 + [S]/K_m)/K_a k_2$. Progress curves at constant substrate and different inhibitor concentrations yielded different k_{obs} values (fig 2). Kinetic parameters reported in table I were determined by fitting k_{obs} values in a plot of $1/[I]k_{obs}$ *versus* $1/[I]$ according to equation [5] which is a rearrangement of equation [4].

$$\frac{1}{k_{obs}[I]} = \frac{1 + [S]/K_m}{K_a k_2} \frac{1}{[I]} + \frac{1}{k_2} \qquad [5]$$

All progress curves examined reached a steady A_∞ absorbance within the time of the experiment indicating that inhibition was always complete. When concentrated solutions (20–50-fold that used in progress curves) of papain or cathepsin B were incubated (10–20 min) with the inhibitors in the concentration range reported in table I, enzymatic activity could not be restored by 20–50-fold dilution. These data suggest that haloacetamide derivatives **1a–6b** are not hydrolytically attacked to an appreciable extent, in the range of concentrations used, because complete inhibition was independent on [*E*] and that they behave as irreversible inhibitors because the final enzyme–inhibitor complex was not reversible upon dilution.

To assess the validity of the continuous method of Tian and Tsou [26] which is based on equation [2], we

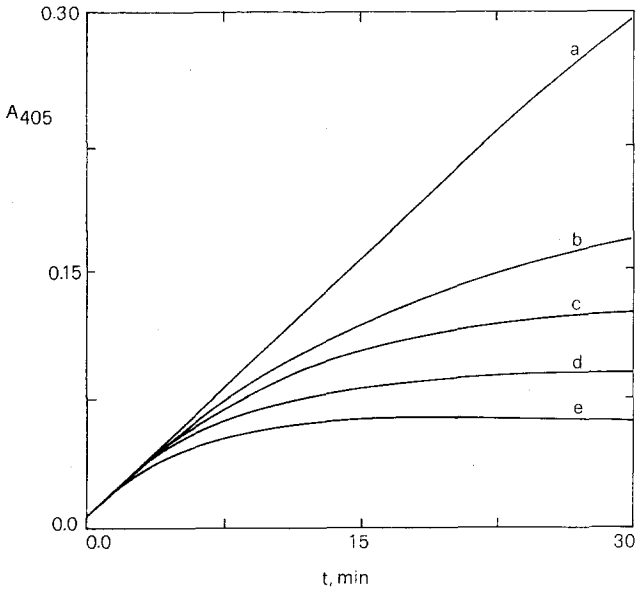

Fig 2. Papain inactivation by compound **5b**. Reaction conditions: [*E*] = 7 x 10^{-9} M, [Z-Gly-ONp] = 125 μM, 100 mM phosphate buffer, pH 6.8, 4 mM EDTA, 12% CH_3CN (v/v), 25°C. Inhibitor concentrations were 0.0, 0.2, 0.4, 0.6 and 1.0 mM for curves a, b, c, d and e respectively. Reactions were started by addition of the enzyme.

determined the inhibition parameters of three compounds (table II) according to the discontinuous method of Kitz and Wilson [25] in which the inhibition mixture of enzyme and inhibitor is prediluted in an activity assay with a fluorescent substrate.

Results and discussion

Kinetic experiments showed that the irreversible inactivation of papain and cathepsin B by haloacetamide derivatives **1a–6b** (table I) follows first-order kinetics at pH 6.8 and 25°C. The concentration dependence of inactivation revealed saturation kinetics proceeding *via* non-covalent enzyme–inhibitor complexes. For most of them, the strength is higher than that of typical enzyme–substrate complexes (K_m = 9 μM for papain-*Z*-Gly-*p*-nitrophenyl ester [30]). In fact, K_I determined for the bromo- and iodoacetyl derivatives **1b–4b** and **1c–4c** containing both L-leucine or L-phenylalanine ranged from 2.5 to 0.4 μM. Only chloroacetyl derivatives **1a–3a** gave appreciably higher values (50–154 μM), as well as bromoacetyl derivatives **5b** and **6b** containing D-leucine and D-phenylalanine. Binding at the active site (fig 1) is probably related to that reported for the peptidyl moiety of E-64 [23] and for bromoacetamide [18]. The covalent bond formed in the irreversible inhibition therefore likely involves the thiolate group of Cys^{25} as in the papain inactivation by $ClCH_2$–CO–Leu(*N*-hydroxy)–Ala-Gly–NH_2 [20].

The apparent rate constants for papain inactivation by chloroacetamide, bromoacetamide and iodoacetamide were determined by Polgar and Halasz [15–17] as $k_{(limit)}$ in studies of pH dependence. These constants, 0.039, 8.1 and 12.7 M^{-1} s^{-1}, respectively, correspond to the values determined at pH 5.5–6 and are some 2.5-fold lower than those obtained at pH 6.8. Enhancement of the activity by proximity effects, due to the contribution of the peptide moiety to *EI* complex formation, can be roughly estimated by comparison of these values with the apparent rate constants of papain inactivation by haloacetamide derivatives **1a–4c** reported in table I. The result is a 1 670–1 950-fold increase for chloroacetamide, 410–910-fold for bromoacetamide and 530–1310-fold for iodoacetamide derivatives.

Enhancement of reactivity against papain compared to glutathione (table III), under the same reaction conditions, was 16 400-fold for *N*-bromoacetyl-leucine isopentylamide and 25 700-fold for the corresponding iodoacetyl derivative. This effect, equally attributable to proximity effects, was only 1 850-fold against cathepsin B.

As expected, the efficiency of these active-site-directed inhibitors strongly depends upon the nature of the leaving group. In fact, for each peptidyl moiety,

Table II. Inactivation of papain by *N*-haloacetyl-amino-acid amides determined using the method of Kitz and Wilson [25][a].

No	*Structure of inhibitor*	*Range (μM)*	K_I *(μM)*	k_2 *(min^{-1})*	k_2/K_I *(M^{-1} s^{-1})*
Papain					
1a	Cl–CH_2CO–Leu–NH–*i*–C_5H_{11}	6–0.8	20	0.2	170
1b	Br–CH_2CO–Leu–NH-*i*-C_5H_{11}	0.09–0.01	0.70	0.6	14 300
1c	I–CH_2CO–Leu–NH-*i*-C_5H_{11}	0.06–0.01	0.24	0.4	27 800

[a]Conditions: 100 mM phosphate buffer, pH 6.8; CH_3CN = 2.5% (v/v); [*E*] = 5 x 10^{-11} M; [*S*] = 25 μM. Replicate determinations indicate standard deviations for the kinetic parameters < 20%.

Table III. Comparative reactivity of *N*-haloacetyl-amino-acid amides toward gluthathione.

GSH (*mM*)	*Inhibitor* *Structure*	*Conc (mM)*	*Org solvent* (*v/v*)	*pH*	*Temp* (°C)	k_2 ($M^{-1}s^{-1}$)
0.04	**1a**	0.4	13	7.4	37	2.9×10^{-2}
0.04	**1a**	0.4	13	6.8	25	0
0.04	**1b**	0.2	13	7.4	37	1.70
0.04	**1b**	0.2	13	6.8	25	0.36
0.04	**1c**	0.2	13	7.4	37	2.30
0.04	**1c**	0.2	13	6.8	25	0.54
0.05	Z–Phe–CH_2Cl[a]	0.1	0	7.4	37	6.16
3.3	Ala–Phe–Lys–CH_2F[a]	6.7	5	7.4	37	1.1×10^{-2}

[a]See [5].

the apparent rate constants of inactivation show about 100- and 200-fold increases by replacement of Br and I, respectively, with Cl. Most of this effect, however, appears to be related to a better binding of bromo- and iodoacetyl derivatives rather than to their increased alkylating activity. In fact, chloroacetamide derivatives **1a–3a** show K_I about 100-fold greater than the corresponding derivatives of bromo- and iodoacetamide, while the first-order rate constants, k_2, of enzyme inactivation of analogous chloro-, bromo- and iodoacetyl derivatives are all within the same order of magnitude. The increase of affinity in the series Cl < Br < I can probably be explained in terms of non-specific hydrophobic effects. It should be assumed that an appropriate hydrophobic area of the enzyme active site can accommodate substituents as bulky as a iodo group in the *EI* reversible complex shown in fig 1. Replacement of chlorine by bromine or iodine will simply increase the hydrophobic character of the inhibitory ligand, improving its removal from the water solvent to the hydrophobic active site. The lack of substantial enhancement of k_2 in the series Cl < Br < I probably reflects binding conformations which are increasingly unfavorable for C–S bond formation.

N-Haloacetyl-amino-acid amides **2a–6c** present their trapping function at the amino rather than at the carboxy-terminal end of the molecule. Thus the amino acid unit is oriented in the opposite direction, in the *EI* reversible complex, with respect to the amino acids of the enzyme substrate in the reversible *ES* complex. They can therefore be regarded as retro-isomers [31] with respect to substrate analog peptide inhibitors. Since retro-inverso [31] isomers bear a closer resemblance to the parent peptide, *N*-bromoacetyl-D-Leu-isopentylamide **5b** and *N*-bromoacetyl-D-Phe-isopentylamide **6b** were synthesized and tested for papain inactivation. The use of retro-inverso-modified peptides did not meet with success in this case, because they were found to be less effective than the L-enantiomers. On the other hand, this observation appears to substantiate the proposed mode of binding and mechanism of inactivation since the same effect was reported [32] for inhibitors of the E-64 family.

Minor effects on the kinetic parameters were obtained by changing the amino acid from leucine to phenylalanine and the alkyl substituent at nitrogen. The most active inhibitor contained the Leu–NH–CH_2Ph moiety in the series of chloroacetamides and the Phe–NH–*i*–C_5H_{11} in the series of bromo- and iodoacetamides.

The best papain inhibitors were also tested against cathepsin B. Maximum efficiency was shown by ICH_2–CO–Phe–NH-*i*–C_5H_{11} with an apparent rate constant of inactivation of 2 800 M^{-1} s^{-1} at 25°C and pH 6.8. A direct and precise comparison with the kinetic properties of chloromethyl and fluoromethyl ketones reported by Rasnick [2] (16 200 M^{-1} s^{-1} for Z–Phe–Ala–CH_2F and 45 300 M^{-1} s^{-1} for Z–Phe–Ala–CH_2Cl at 28°C and pH 6.5) and by Shaw *et al* [4] (9 000 M^{-1} s^{-1} for Z–Phe–PheCH_2Cl and 53 000 M^{-1} s^{-1} for Z–Phe–Ala–CH_2F at 37°C and pH 5.4) is complicated by some differences of temperature, pH and concentration and nature of the organic cosolvent used in the reaction conditions. If comparison is confined to the data of Rasnick under more similar conditions, the reactivity of the iodoacetamide **3c** against cathepsin B is about 1/6th and 1/16th that of a fluoromethylketone and a chloromethylketone, respectively.

The non-specific alkylation properties of the chloro-, bromo- and iodoacetamide derivatives were evaluated by measuring their rate of alkylation of glutathione according to Angliker *et al* [5]. The results listed in table III show that the reactivity of the chloroacetamide **1a** is about 3-fold higher than that of the fluoromethylketone, Ala–Phe–Lys–CH_2F. Bromo- and iodoacetamides, as expected, are considerably more powerful alkylating reagents. The reactivity of the iodoacetyl derivative **1c**, however, is less than one

half than that of the chloromethylketone Cbz–Phe–CH_2Cl.

Use of peptidyl halomethylketones as inhibitors of cysteine proteases may be complicated by their property of inactivating serine proteases by alkylation at the imidazole of the catalytic histidine unit. A similar behavior could not be excluded *a priori* for haloacetamide inhibitors **1a–4c**. All the iodoacetyl derivatives **1c–4c**, the most powerful alkylating reagents, were therefore tested as inhibitors of trypsin, chymotrypsin and porcine pancreatic elastase. At the maximal inhibitor concentration of 0.25–1 mM, no inhibition could be detected after 5 h incubation. This finding indicates a high degree of selectivity for cysteine proteases.

N-Haloacetyl-amino acid amides described in the present paper approximate the effectiveness of known peptidyl chloro- and fluoromethyl ketones against cysteine proteases. They represent an interesting class of inhibitors in view of their simple, direct and high yield chemical synthesis and of their low, non-specific, alkylating reactivity, especially for the chloroacetyl derivatives. Work is in progress to further improve kinetic parameters and to achieve enzyme selectivity among cysteine proteases.

Experimental protocols

Melting points (Büchi oil bath apparatus) are uncorrected. IR spectra (for $CHCl_3$ solutions) were obtained with a Perkin–Elmer 521 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian EM 390 spectrometer using tetramethylsilane (TMS) as the internal standard. $[\alpha]_D$ and $[\alpha]_{545}$ were determined with a Schmidt–Haensch 1604 polarimeter. Elemental analyses were within ± 0.4% of the calculated values.

Mixed anhydride coupling: general procedure A
A solution of the required protected amino acid (1 mmol) and *N*-methylmorpholine (1 mmol) in anhydrous CH_2Cl_2 (2 ml) was cooled to –15°C and *i*-butyl-chloroformate (1 mmol) was added dropwise under stirring. After 30 min, a solution of the required amine (1 mmol) in anhydrous CH_2Cl_2 (2 ml) was added slowly, while the temperature of –15°C was maintained. The resulting reaction mixture was stored for 15 h at 4°C in a refrigerator, allowed to warm to room temperature and filtered. The solution was concentrated under reduced pressure and the residue dissolved in EtOAc, sequentially washed with 1 N HCl (1 M $KHSO_4$ for Boc derivatives), saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed under reduced pressure to give the crude product.

Carbobenzyloxy group removal: general procedure B
Hydrogen was bubbled into a solution of the required carbobenzyloxy derivative (1 mmol) in MeOH (15 ml), in the presence of 10% Pd on charcoal (50 mg) for 4 h, while the mixture was rapidly stirred. When conversion was complete thin-layer chromatography (TLC), the mixture was filtered through a celite layer and the solvent evaporated under reduced pressure to give the crude product.

tert-Butoxycarbonyl group removal: general procedure C
A solution of the required *N-tert*-butoxycarbonyl derivative (1 mmol) in anhydrous EtOAc (10 ml) was saturated with dry HCl gas at 0°C. After 2 h at room temperature, the mixture was concentrated at reduced pressure. The residue was dissolved by addition of $CHCl_3$ (10 ml) and 2 N Na_2CO_3 (5 ml). The organic phase was further washed with brine (5 ml) and dried over Na_2SO_4. Solvent was removed under reduced pressure to give the crude product.

Haloacetamide derivative preparation: general procedure D
To an ice-cooled solution of the required amino-acid amide (1 mmol) in $CHCl_3$ (10 ml), in the presence of 1 M aqueous Na_2CO_3 (1 ml), the appropriate haloacetyl chloride (1.1 mmol) was rapidly added under vigorous stirring. The reaction mixture was allowed to warm to room temperature while stirring was continued for 20 min. Dilution with $CHCl_3$ (20 ml) and sequential washing with saturated $NaHCO_3$, 1 N HCl and brine (10 ml each), followed by drying over Na_2SO_4 and removal of the solvent at reduced pressure gave the crude product.

Haloacetamide derivatives preparation: general procedure E
A solution of the required amino-acid amide (1 mmol), haloacetic acid (1 mmol) and dicyclohexylcarbodiimide (1.1 mmol) in anhydrous CH_2Cl_2 (10 ml) was allowed to react overnight, under stirring, in the presence of catalytic 1-hydroxybenzotriazole (0.1 mmol). The reaction mixture was cooled in a refrigerator for 2 h and solid dicyclohexylurea was removed by filtration. The CH_2Cl_2 solution was sequentially washed with 1 N HCl (10 ml), saturated $NaHCO_3$ (10 ml) and brine (10 ml). After drying over Na_2SO_4, the solvent was evaporated under reduced pressure to give the crude product.

N-Carbobenzyloxy-L- and D-leucine isopentylamide
Reaction of *N*-carbobenzyloxy-L-leucine (3.52 g, 13.5 mmol) with 1-amino-3-methylbutane (1.18 g, 13.5 mmol) according to *Procedure A* and crystallization of the crude material from hexane gave 4.24 g (95.5%) of the pure product: mp 95–6°C; $[\alpha]_D^{22} = -24°$ (1; $CHCl_3$); IR main peaks at 3435, 2957, 1711, 1672, 1502 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.84–0.98 (m, 12H, CH_3), 1.20–1.76 (m, 6H, CH_2CH), 3.10–3.29 (m, 2H, CH_2N), 4.02–4.32 (m, 1H, αCH), 5.11 (s, 2H, CH_2Ph), 5.36–5.66 (m, 1H, NH), 6.31 (bs, 1H, NH), 7.41 (s, 5H, C_6H_5). The D enantiomer was prepared accordingly and gave $[\alpha]_D^{22} = 24°$ (1; $CHCl_3$).

N-tert-Butoxycarbonyl-L-leucine benzylamide
N-tert-Butoxycarbonyl-L-leucine (2.49 g, 10 mmol) and benzylamine (1.07 g, 10 mmol) were reacted according to *Procedure A*. Purification of the crude material by silica gel chromatography ($CHCl_3$) and crystallization from hexane gave 2.87 g (85%) of the pure product: mp 81–3°C; $[\alpha]_D^{22} = 25°$ (1; MeOH); IR main peaks at 3434, 2959, 1678, 1497, 1368, 1162 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.84–0.96 (m, 6H, Leu CH_3), 1.35 (s, 9H, Boc CH_3), 1.50–1.74 (m, 3H, CH_2CH), 4.01–4.39 (m, 1H, αCH), 4.40 (d, 2H, CH_2N, J = 6 Hz), 5.20 (d, 1H, NH, J = 9 Hz), 6.85 (bs, 1H, NH), 7.30 (s, 5H, C_6H_5).

N-Carbobenzyloxy-L- and D-phenylalanine isopentylamide
N-Carbobenzyloxy-L-phenylalanine (6.15 g, 20.5 mmol) and 1-amino-3-methylbutane (2.39 g, 20.5 mmol) were reacted according to *Procedure A*. Crystallization of the crude material from 1,2-dichloroethane/hexane (1:3) gave the pure product: 7.51 g (92%); mp 122–4°C; $[\alpha]_D^{22} = -5°$ (2; benzene); IR main peaks at 3432, 2955, 1711, 1672, 1495 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.83 (d, 6H, CH_3, J = 7.5 Hz), 1.08–1.60 (m, 3H, CH_2CH),

2.90–3.34 (m, 4H, CH_2N and Phe CH_2), 4.24–4.56 (m, 1H, αCH), 5.09 (s, 2H, CH_2Ph), 5.57 (bs, 1H, NH), 5.83 (bs, 1H, NH), 7.15 (s, 5H, C_6H_5), 7.51 (s, 5H, C_6H_5).

The D enantiomer was prepared accordingly and gave $[\alpha]_D^{22} = 5°$ (2; benzene).

N-tert-Butoxycarbonyl-L-phenylalanine benzylamide

N-tert-Butoxycarbonyl-L-phenylalanine (2.65 g, 10 mmol) and benzylamine (1.07 g, 10 mmol) were reacted according to *Procedure A*. Purification of the crude material by silica gel chromatography ($CHCl_3$) and crystallization from EtOAc/hexane (1:1) gave the pure product: 2.90 g (82%); mp 130–1°C; $[\alpha]_{545}^{22} = 2°$ (1; MeOH); IR main peaks at 3438, 2982, 1660, 1492, 1350, 1178 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.35 (s, 9H, Boc CH_3), 3.10 (d, 2H, Phe CH_2, J = 7.5 Hz), 4.25–4.55 (m, 3H, CH_2N and αCH), 5.29 (d, 1H, NH, J = 9 Hz), 6.40 (bs, 1H, NH), 7.07–7.40 (m, 10H, C_6H_5).

L- and D-Leucine isopentylamide

N-Carbobenzyloxy-L-leucine isopentylamide (4.24 g, 12.68 mmol) was hydrogenated according to *Procedure B*. Purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc, 1:1) and bulb-to-bulb distillation gave the pure product as a colorless oil: 2.24 g (90%); bp 150°C (oven temperature) at 0.2 mm Hg; $[\alpha]_D^{22} = -12°$ (1; benzene); IR main peaks at 3373, 2959, 1658, 1516, 1369, 1138 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.84–1.10 (m, 12H, CH_3), 1.20–1.91 (m, 8H, CH_2CH and NH_2), 3.11–3.49 (m, 2H, CH_2N), 3.80–4.00 (m, 1H, αCH), 7.28 (bs, 1H, NH).

The D enantiomer was prepared accordingly and gave $[\alpha]_D^{22} = 12$ (1; benzene).

L-Leucine benzylamide

N-tert-butoxycarbonyl-L-leucine benzylamide (2.00 g, 5.90 mmol) was treated according to *Procedure C*. The crude material was purified by bulb-to-bulb distillation to give the pure product as a colorless oil: 1.3 g (93%); bp 180°C (oven temperature) at 0.2 mm Hg; $[\alpha]_D^{22} = 14°$ (1; MeOH); IR main peaks at 3229, 2960, 1672, 1268, 1082 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.70–1.08 (m, 6H, CH_3), 1.30–1.95 (m, 3H, CH_2CH), 3.36 (s, 2H, NH_2), 3.43–3.72 (m, 1H, αCH), 4.43 (d, 2H, CH_2N, J = 6 Hz), 7.30 (s, 5H, C_6H_5), 7.70–7.86 (m, 1H, NH).

L- and D-Phenylalanine isopentylamide

N-Carbobenzyloxy-L-phenylalanine isopentylamide (4.20 g, 11.4 mmol) was hydrogenated according to *Procedure B*. Bulb-to-bulb distillation of the crude material gave the pure product as a colorless oil: 2.40 g (90%); bp 180°C (oven temperature) at 0.2 mm Hg; $[\alpha]_D^{22} = -52°$ (1; benzene); IR main peaks at 3372, 2959, 1656, 1525, 1100 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.87 (d, 6H, CH_3, J = 7.5 Hz), 1.25–1.42 (m, 3H, CH_2CH), 1.65 (s, 2H, NH_2), 2.52–2.86 (m, 2H, Phe CH_2), 3.04–3.44 (m, 2H, CH_2N), 3.48–3.76 (m, 1H, αCH), 7.23 (s, 5H, C_6H_5).

The D enantiomer was prepared accordingly and gave $[\alpha]_D^{22} = 52°$ (1; benzene).

L-Phenylalanine benzylamide

Treatment of *N-tert*-butoxycarbonyl-L-phenylalanine benzylamide (1.75 g, 6.90 mmol) according to *Procedure C* gave the crude product as a white solid. Crystallization from EtOAc gave the pure product: 1.45 g (82%); mp 169–71°C; $[\alpha]_D^{22} = 35°$ (1; MeOH); IR main peaks at 3411, 2924, 1755, 1672, 1602, 1079 cm^{-1}; ^{1}H-NMR (d_6-DMSO) 3.14 (d, 2H, Phe CH_2, J = 7.5 Hz), 4.01–4.38 (m, 3H, αCH and CH_2N), 7.04–7.39 (m, 10H, C_6H_5), 8.24–8.84 (bs, 2H, NH_2), 9.26 (t, 1H, NH, J = 6).

N-Chloroacetyl-L-leucine isopentylamide ***1a***

L-Leucine isopentylamide (300 mg, 1.5 mmol) and chloroacetyl chloride (166 mg, 1.5 mmol) were reacted according to *Procedure D*. Crystallization from EtOAc gave the pure product: 340 mg (82%); mp 145–6°C; $[\alpha]_D^{22} = -32°$ (1; MeOH); IR main peaks at 3020, 2403, 1669, 1522, 1206, 1046 cm^{-1}; ^{1}H-NMR ($CDCl_3$), δ 0.80–1.00 (m, 12H, CH_3), 1.22–1.80 (m, 6H, CH_2CH), 3.04–3.50 (m, 2H, CH_2N), 4.08 (s, 2H, CH_2Cl), 4.38–4.70 (m, 1H, αCH), 6.60 (bs, 1H, NH), 7.35 (bs, 1H, NH).

N-Bromoacetyl-L-leucine isopentylamide ***1b***

L-Leucine isopentylamide (300 mg, 1.5 mmol) and bromoacetic acid (208 mg, 1.5 mmol) were reacted according to *Procedure E*. Purifiction of the crude material by silica gel chromatography (CH_2Cl_2/EtOAc, 95:5) and crystallization from EtOH/H_2O (1:1) gave the pure product: 366 mg (76%); mp 140–2°C; $[\alpha]_D^{22} = -36°$ (1; MeOH); IR main peaks at 2957, 1665, 1518, 1244, 1091 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.89–1.05 (m, 12H, CH_3), 1.25–1.80 (m, 6H, CH_2CH), 3.11–3.49 (m, 2H, CH_2N), 3.90 (s, 2H, CH_2Br), 4.44–4.73 (m, 1H, αCH), 6.98 (t, 1H, NH, J = 3 Hz), 7.80 (d, 1H, NH, J = 9 Hz).

N-Bromoacetyl-D-leucine isopentylamide ***5b***

Prepared according to the procedure used for the L-enantiomer **1b**. $[\alpha]_D^{22} = 36°$ (1; MeOH).

N-Iodoacetyl-L-leucine isopentylamide ***1c***

L-Leucine isopentylamide (300 mg, 1.5 mmol) and iodoacetic acid (279 mg, 1.5 mmol) were reacted according to *Procedure E*. Purification of the crude material by silica gel chromatography ($CHCl_3$) and trituration of the resulting oil with hexane gave the pure product as a white solid: 425 mg (77%); mp 125–7°C; $[\alpha]_D^{22} = -36°$ (1; MeOH); IR main peaks at 2958, 1660, 1512, 1162, 1091 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.88–1.02 (m, 12H, CH_3), 1.22–1.78 (m, 6H, CH_2CH), 3.13–3.74 (m, 2H, CH_2N), 3.75 (s, 2H, CH_2I), 4.32–4.70 (m, 1H, αCH), 7.00 (t, 1H, NH, J = 3 Hz), 7.90 (d, 1H, NH, J = 9 Hz).

N-Chloroacetyl-L-leucine benzylamide ***2a***

L-Leucine benzylamide (238 mg, 1.0 mmol) was reacted with chloroacetyl chloride (124 mg, 1.1 mmol) according to *Procedure D*. Crystallization of the crude material from EtOAc gave the pure product: 258 mg (82%); mp 148–50°C; $[\alpha]_D^{22} = -30°$ (2; $CHCl_3$); IR main peaks at 3408, 2959, 1666, 1519, 1083, 1016 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.72–1.00 (m, 6H, CH_3), 1.35–1.80 (m, 3H, CH_2CH), 3.84 (s, 2H, CH_2Cl), 4.39 (d, 2H, CH_2N, J = 6 Hz), 4.47–4.82 (m, 1H, αCH), 7.30 (m, 7H, C_6H_5 and NH).

N-Bromoacetyl-L-leucine benzylamide ***2b***

L-Leucine benzylamide (238 mg, 1.0 mmol) was reacted with bromoacetyl chloride (173 mg, 1.1 mmol) according to *Procedure D*. Crystallization of the crude material from EtOAc gave the pure product: 262 mg (73%); mp 157–60°C; $[\alpha]_D^{22} = -28°$ (2; $CHCl_3$); IR main peaks at 3402, 2961, 1666, 1517, 1250 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 0.72–1.00 (m, 6H, CH_3), 1.35–1.74 (m, 3H, CH_2CH), 3.95 (s, 2H, CH_2Br), 4.16–4.55 (m, 3H, αCH and CH_2N), 7.30 (s, 5H, C_6H_5), 8.35–8.78 (m, 2H, NH).

N-Iodoacetyl-L-leucine benzylamide ***2c***

L-Leucine benzylamide (238 mg, 1.0 mmol) was reacted with iodoacetic acid (186 mg, 1.0 mmol) according to *Procedure E*. Crystlllization of the crude material from 1,2-dichloroethane gave the pure product: 211 mg (52%); mp 163–5°C; $[\alpha]_D^{22} = -30°$ (1; $CHCl_3$); IR main peaks at 3432, 2934, 1660, 1516, 1162 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 0.77–0.97 (m, 6H, CH_3),

1.38–1.70 (m, 3H, CH_2CH), 3.70 and 3.72 (two s, 2H, CH_2I), 4.20–4.45 (m, 3H, αCH and CH_2N), 7.30 (s, 5H, C_6H_5), 8.40–8.70 (m, 2H, NH).

N-Chloroacetyl-L-phenylalanine isopentylamide ***3a***
L-Phenylalanine isopentylamide (351 mg, 1.5 mmol) was reacted with chloroacetyl chloride (166 mg, 1.5 mmol) according to *Procedure D*. Crystallization of the crude material from EtOAc gave the pure product: 431 mg (91%); mp 120–1°C; $[\alpha]_D^{22}$ = 6° (1; MeOH); IR main peaks at 3434, 2957, 1665, 1513, 1262, 1086 cm^{-1}; ^{1}H-NMR δ 0.85 (d, 6H, CH_3, J = 6 Hz), 1.08–1.45 (m, 3H, CH_2CH), 3.00–3.25 (m, 4H, CH_2N and Phe CH_2), 4.02 (s, 2H, CH_2Cl) 4.44–4.80 (m, 1H, αCH), 5.80 (bs, 1H, NH), 7.35 (s, 5H, C_6H_5).

N-Bromoacetyl-L-phenylalanine isopentylamide ***3b***
L-Phenylalanine isopentylamide (351 mg, 1.5 mmol) and bromoacetic acid (208 mg, 1.5 mmol) were reacted according to *Procedure E*. Purification of the crude material by silica gel chromatography ($CHCl_3$) and crystallization from EtOAc/hexane (1:1) gave the pure product: 340 mg (64%); mp 130–2°C; $[\alpha]_D^{22}$ = –2° (2; CH_3CN); IR main peaks at 3433, 2957, 1660, 1513, 1198, 1085 cm ^{1}; ^{1}H-NMR ($CDCl_3$) δ 0.80 (d, 6H, CH_3, J = 6 Hz), 1.10–1.50 (m, 3H, CH_2CH), 3.00–3.30 (m, 4H, CH_2–N and Phe CH_2), 3.85 (s, 2H, CH_2Br), 4.50–4.80 (m, 1H, αCH), 5.96 (bs, 1H, NH), 7.33 (s, 5H, C_6H_5), 7.52 (d, 1H, NH, J = 9 Hz).

N-Bromoacetyl- D-phenylalanine isopentylamide ***6b***
Prepared according to the procedure used for the L-enantiomer **3b**. $[\alpha]_D^{22}$ = 2° (2; CH_3CN).

N-Iodoacetyl-L-phenylalanine isopentylamide ***3c***
L-Phenylalanine isopentylamide (351 mg, 1.5 mmol) and iodoacetic acid (279 mg, 1.5 mmol) were reacted according to *Procedure E*. The crude material was purified by silica gel chromatography ($CHCl_3$). Trituration of the resulting oil with hexane gave the pure product as a white solid: 548 mg (91%), mp 140–2°C; $[\alpha]_D^{22}$ = –7° (1; MeOH); IR main peaks at 3432, 2956, 1661, 1502, 1160, 1090 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.85 (d, 6H, CH_3, J = 6 Hz), 1.20–1.45 (m, 3H, CH_2–CH), 3.00–3.30 (m, 4H, CH_2N and Phe CH_2), 3.78 (s, 2H, CH_2I), 4.56–4.90 (m, 1H, αCH), 6.38 (bs, 1H, NH), 7.33 (s, 5H, C_6H_5), 7.85 (d, 1H, NH, J = 9 Hz).

N-Chloroacetyl-L-phenylalanine benzylamide ***4a***
L-Phenylalanine benzylamide (253 mg, 1 mmol) and chloroacetyl chloride (124 mg, 1.1 mmol) were reacted according to *Procedure D*. Crystallization of the crude material from EtOAc gave the pure product: 257 mg (78%); mp 177–9°C; $[\alpha]_{545}^{22}$ = –3° (2; MeOH); IR main peaks at 3401, 2995, 1665, 1516, 1248, 1031 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 2.82–3.14 (m, 2H, Phe CH_2), 4.10 (s, 2H, CH_2Cl), 4.35 (d, 2H, CH_2N, J = 6 Hz), 4.50–4.85 (m, 1H, αCH), 7.30 (s, 10H, C_6H_5), 8.47–8.83 (m, 2H, NH).

N-Bromoacetyl-L-phenylalanine benzylamide ***4b***
L-Phenylalanine benzylamide (253 mg, 1.0 mmol) and bromoacetyl chloride (173 mg, 1.1 mmol) were reacted according to *Procedure D*. Crystallization of the crude material from 1,2–dichloroethane gave the pure product: 314 mg (84%); mp 205–7°C; $[\alpha]_{545}^{22}$ = –7° (2; MeOH); IR main peaks at 3431, 2931, 1665, 1511, 1260, 1081 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 2.73– 3.17 (m, 2H, Phe CH_2), 3.80 (s, 2H, CH_2Br), 4.25 (d, 2H, CH_2N, J = 6 Hz), 4.30–4.82 (m, 1H, αCH), 7.18 (s, 10H, C_6H_5), 8.55 (bs, 2H, NH).

N-Iodoacetyl-L-phenylalanine benzylamide ***4c***
L-Phenylalanine benzylamide (253 mg, 1.0 mmol) and iodoacetic acid (186 mg, 1.0 mmol) were reacted according to *Procedure E*. Crystallization of the crude material from 1,2-dichloroethane gave the pure product: 253 mg (60%); mp 189–91°C; $[\alpha]_D^{22}$ = –9° (1; MeOH); IR main peaks at 3431, 2934, 1661, 1515, 1206 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 2.89–3.05 (m, 2H, Phe CH_2), 3.71 (s, 2H, CH_2I), 4.27 (s, 2H, CH_2N), 4.46–4.70 (m, 1H, αCH), 7.30 (s, 10H, C_6H_5).

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 ethylenediaminetetraacetic acid (EDTA) and 0.5 mM L-cysteine according to Thompson *et al* [33] with minor modifications. Approximate enzyme concentration was determined from the absorbance at 280 nm (E = 58.5 mM^{-1} cm^{-1}) [34]. 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 buffers 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 [30] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. A cuvette containing everything but 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 does not affect kinetic parameters of papain-catalyzed hydrolysis of *Z*-Gly-ONp [30].

Reactions were started by addition of the 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, the non-linear regression data analysis program from Elsevier-Biosoft (Cambridge), or the non-linear regression program of Duggleby [35]. Progress curves were composed of 180–360 (absorbance, time) pairs. Software for collection of progress curves in the computer was supplied by Kontron. A substrate concentration of 125 μM (K_m x 12.5) avoided substrate depletion due to spontaneous and enzymatic hydrolysis during the assays.

The papain fluorimetric assay was performed using 25 μM of *Z*–Phe–Arg–NMec as the substrate. The reaction was followed continuously for 3–5 min by monitoring the fluorescence of amino-methylcoumarin in a SFM-25 Kontron fluorimeter with excitation set at 380 nm and emission at 460 nm. The final concentration of acetonitrile in the cuvette was 2.5% (v/v) in 100 mM phosphate buffer, pH 6.8, 4 mM EDTA and 0.01% Brij 35. The temperature was maintained at 25°C by a circulating wather bath. Data were collected and analyzed by a Data System 450 personal computer (Kontron) using a computer program for SFM-25 supplied by Kontron. The ratio between enzyme and inhibitor during the inactivation reaction performed as in [22] was always at least 1:10. To test residual activity in the fluorimeter cuvette the inactivation mixture was diluted at least 10-fold.

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 condi-

tions as those described above for papain. The approximate enzyme concentration was determined from absorbance at 280 nm ($E^{1\%}$ = 20 cm^{-1}) [36]. Enzyme activity was tested as described for papain [37].

Chymotrypsin and trypsin assays

Chymotrypsin (EC 3.4.21.1) from bovine pancreas (product No 27270) was obtained from Fluka. The enzyme was dissolved in 50 mM phosphate buffer, pH 6.8, and its approximate concentration determined from the absorbance at 280 nm (E = 50 mM^{-1} cm^{-1}) [38]. Chymotrypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester at 256 nm [39]. Trypsin (EC 3.4.21.4) from bovine pancreas (code TRTPCK), was from Worthington Enzyme (Cooper Biomedical). The enzyme was dissolved in 0.001 N HCl and concentration determined from the absorbance at 280 nm [40]. Trypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-arginine ethyl ester at 253 nm [41]. Preincubation at 25°C for 5 h with 1 mM iodoacetyl derivative **1c** and 0.25 mM iodoacetyl derivatives **2c**, **3c**, and **4c** with both the enzymes did not inhibit their activities.

Porcine pancreatic elastase assay

Porcine pancreatic elastase (EC 3.4.21.36) (product No E-0127), was obtained from Sigma. Enzyme was dissolved in 50 mM phosphate buffer, pH 7.0, and its approximate concentration determined from $E^{1\%}$ = 22 cm^{-1} at 280 nm [42]. Activity was monitored by following the hydrolysis of Boc-L-Ala-ONp at 347 nm [43] in 50 mM phosphate buffer, pH 7.0, at 25°C. No inactivation was observed following 5 h incubation with iodoacetyl derivatives **1c**–**4c** under the same conditions as for chymotrypsin and trypsin.

Alkylation of glutathione

The rate of alkylation of glutathione was measured by following the thiol disappearance using Ellman's reagent [44]. Observations were made in phosphate buffer at pH 6.8 and 7.4 and at 25 and 37°C to obtain information relative to both physiological and experimental conditions. The concentration of organic solvent and all other conditions were similar to those used in the experiments for enzyme inactivation. Results are given in table III.

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