Eur J Med Chem (1993) 28, 297–311

Synthesis and inhibiting activities of 1-peptidyl-2-haloacetyl hydrazines toward cathepsin B and calpains

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(Received 2 August 1992; accepted 10 December 1992)

Summary — Twenty-four 1-peptidyl-2-haloacetyl hydrazines which can be considered azapeptide halomethanes were synthesized and tested as models of cathepsin B, calpain I and calpain II inhibitors. Reagents designed for cathepsin B inactivation include Z-Tyr, Z-Tyr(I) and Z-Leu-Leu attached to an α-azaglycine or α-azaalanine unit in P_1. By use of kinetic analysis, they proved to irreversibly inactivate cathepsin B *via* a reversible enzyme-inhibitor intermediate. Second-order rate constants in the range 725–306 000 $M^{-1}s^{-1}$ were found for cathepsin B inactivation, with no more than 7 500 $M^{-1}s^{-1}$ for calpain II. K_I for the reversible EI adducts ranged from 11 to 0.06 μM for cathepsin B. Structure of the possible reversible EI complex is proposed and used to discuss the effects of structural variation of the inhibitors on the kinetic parameters of inactivation. 1-Peptidyl-2-haloacetyl hydrazines designed for calpain inactivation include Boc-Val-(N_ε-carbomethoxy)Lys-Leu, Boc-Val-Lys(N_ε-Z)-Leu, Boc-Val-Lys(N_ε-Tos)-Leu and Z-Leu-Leu attached to an α-azatyrosine unit in P_1. They gave poor results. Title compounds proved to be selective for cysteine proteases, since no inhibiting activity could be detected toward trypsin, chymotrypsin and porcine pancreatic elastase at 0.1 mM concentration. Relatively low aspecific alkylating properties were also demonstrated in tests using glutathione as the nucleophile.

enzyme inhibitors / cathepsin B / calpains / halomethylketone analogs

Introduction

Cathepsin B, H and L and calpains are cysteine proteases found in mammalian tissues, which participate in numerous cellular functions [1]. Their protease activity is essential for the cell, while uncontrolled protease activity leads to random degradation of cellular proteins and is lethal to the cell. Selective inhibitors of cysteine proteinases are thus of significant interest as reagents for biological studies and in view of therapeutical applications [2].

We recently reported on haloacetyl amino-acid amides [3] as irreversible inhibitors of papain and cathepsin B and on 1-peptidyl-2-haloacetyl hydrazines [4] as a new model of inhibitor characterized by an azapeptide recognising moiety and a haloacetyl group as trapping function for the thiolate of the functional cysteine of the enzyme. These compounds proved to irreversibly inactivate papain and cathepsin B *via* a reversible enzyme-inhibitor intermediate and 1-peptidyl-2-haloacetyl hydrazines were found to be more effective for cathepsin B than for papain. In addition they were shown to be selective for cysteine proteases, since no inhibitory activity could be detected toward trypsin, chymotrypsin and porcine pancreatic elastase.

We now present the new 1-peptidyl-2-haloacetyl hydrazines **1a–8c** (table I), designed as effective and selective inhibitors toward cathepsin B and calpains. They can be regarded as peptidyl halomethylketones where the amino acid in P_1 has been replaced by an α-azaamino acid, and therefore as azapeptide derivatives. The azapeptidyl portions used to increase binding at the active site of the individual enzymes were derived from very effective and selective peptidyl diazomethylketones described by Shaw [5] for inactivation of cathepsin B, H and L and for calpain II. The inhibitors **1–4** designed for cathepsin B inactivation include Z-Tyr, Z-Tyr(I) and Z-Leu-Leu, followed by an α-azaglycine or α-azaalanine unit. Longer peptide chains including Boc-Val-(N_ε-carbomethoxy)Lys-

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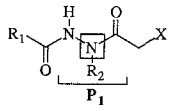
Table I. The new 1-peptidyl-2-haloacetyl hydrazines **1a–8c**.



1a-8c

X = Cl	*X = Br*	*X = I*	*R_1-CO*	*R_2*
1a	**1b**	**1c**	Z-Tyr(I)	H
2a	**2b**	**2c**	Z-Leu-Leu	H
3a	**3b**	**3c**	Z-Tyr	CH_3
4a	**4b**	**4c**	Z-Tyr(I)	CH_3
5a	**5b**	**5c**	Z-Leu-Leu	CH_2-C_6H_4-OH(*p*)
6a	**6b**	**6c**	Boc-Val-Lys(N_ε-Z)-Leu	CH_2-C_6H_4-OH(*p*)
7a	**7b**	**7c**	Boc-Val-Lys(N_ε-$COOCH_3$)-Leu	CH_2-C_6H_4-OH(*p*)
8a	**8b**	**8c**	Boc-Val-Lys(N_ε-Tos)-Leu	CH_2-C_6H_4-OH(*p*)

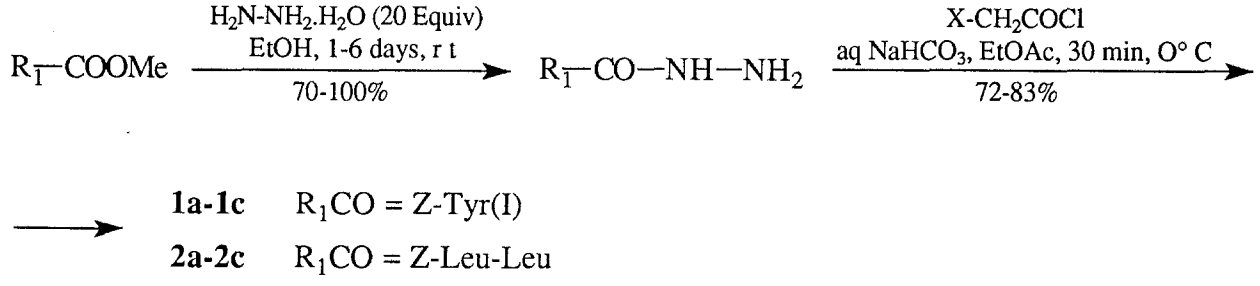
Leu, Boc-Val-Lys(N_ε-Z)-Leu, Boc-Val-Lys(N_ε-Tos)-Leu and Z-Leu-Leu followed by an α-azatyrosine unit in P_1 were considered for calpain inhibitors **4–8**.

Chemistry

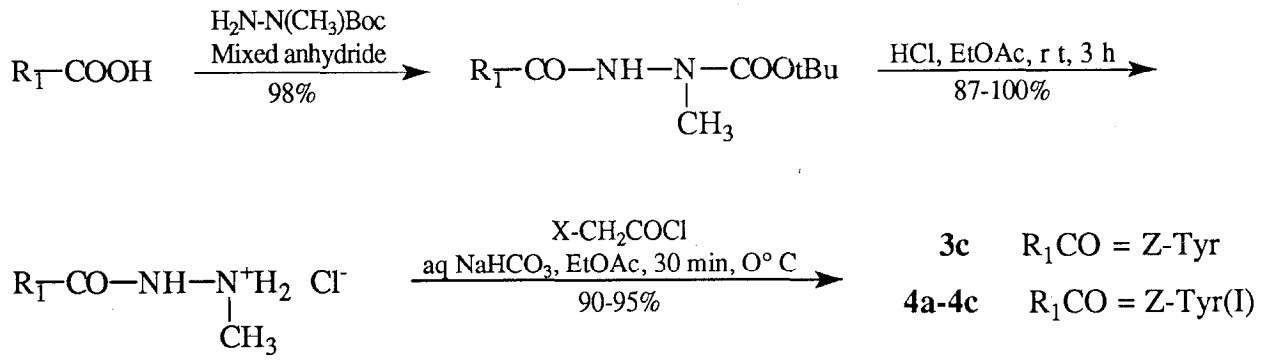
Three different synthetic schemes were required for the preparation of the inhibitors. Those presenting α-azaglycine in P_1 (**1a–2c**) were readily obtained by hydrazinolysis of the *N*-protected amino acid or peptide methylester, followed by acylation with haloacetyl chloride (scheme 1). According to our previous experience [4], this step was in most cases advantageously performed under very mild Schotten–Baumann conditions by employing commercial chloro and bromoacetyl chlorides. The method was extended to the iodoacetyl derivatives by using freshly prepared iodoacetyl chloride.

Preparation of the α-azaalanine derivatives **3a–4c** by employing the same scheme, required acylation of methylhydrazine. This step is poorly selective and is complicated by the formation of substantial amounts of both positional isomers 1-peptidyl-2-methylhydrazine and 1-peptidyl-1-methylhydrazine [4, 6, 7]. This disadvantage was overcome by using 1-methyl-1-*t*-butoxycarbonylhydrazine [8] in the acylation step (scheme 2). Removal of the protective group by treatment with hydrogen chloride in ethyl acetate gave the required 1-peptidyl-2-methylhydrazine.

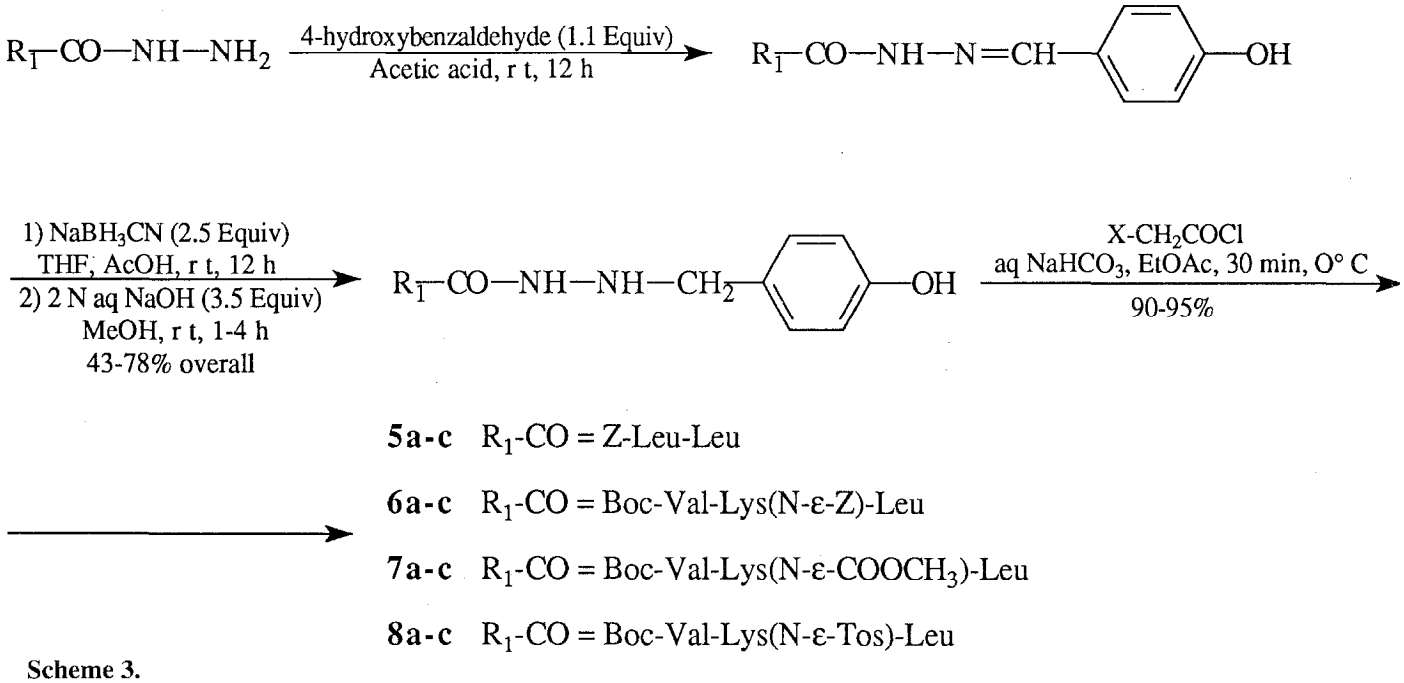
This very efficient route could not be applied to the preparation of the inhibitors presenting an α-azatyrosine unit in P_1 since no straightforward preparation of the required 1-(4-hydroxybenzyl)-1-*t*-butoxycarbonyl hydrazine was at hand. Selective alkylation of the proper hydrazine nitrogen with the 4-hydroxybenzyl group was therefore achieved according to scheme 3. Condensation of the required peptidyl hydrazines with 4-hydroxybenzaldehyde gave the expected hydrazones which were directly reduced with sodium cyanoborohydride, by using a modification of the recently described method [9]. Final acylation of the intermediate 1-peptidyl-2-(4-hydroxybenzyl)-hydrazines was carried out as usual under Schotten–



Scheme 1.



Scheme 2.



Scheme 3.

Baumann conditions. The tripeptide methylester Boc-Val-Lys(N_ε-Z)-Leu-OMe was prepared by the mixed anhydride coupling using Boc-Val-Lys(N_ε-Z) [10] as the starting material. Structural variation at the ε lysine nitrogen leading to the analogous Boc-Val-Lys(N_ε-COOMe)-Leu-OMe and Boc-Val-Lys(N_ε-Tos)-Leu-OMe was readily achieved by hydrogenolysis of the carbobenzyloxy group followed by acylation with methylchloroformate or tosyl chloride.

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. Further structural evidence was obtained from ^{13}C-NMR spectroscopy for the bromoacetyl derivatives.

Enzyme inactivation measurements

Inhibition of cathepsin B catalyzed hydrolysis of *N*-carbobenzyloxyglycine *p*-nitrophenylester (Z-Gly-ONp) was tested with compounds **1a–8c**. All the compounds inhibited completely and irreversibly the activity of the enzyme.

Calpain I and calpain II hydrolysis of N-Suc-Leu-Tyr-7-amido-4-methylcoumarin was similarly inhibited by compounds **1a–8c**.

Substrate analog inhibitors inactivate proteases according to equation [1]:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{–}I \quad (1)$$

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; the rate constant for the covalent modification of the enzyme is k_2.

A convenient method for evaluation of inactivation rates in the presence of the substrate has been described by Tian and Tsou [11]. 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.

$$\begin{array}{lllll} + & I & \rightleftharpoons & EI \xrightarrow{k_2} & E\text{-}I \\ + & S & \rightleftharpoons & ES \xrightarrow{k_{cat}} & E + P \end{array} \qquad (2)$$

The decrease in formation of *p*-nitrophenol at [S]≫ K_m, was measured at various inhibitor concentrations and the temporal dependence of the absorbance change was found to be exponential and well fitting to a first-order rate law [11]. Pseudo-first-order rate constants, k_{obs}, for the formation of the inhibited enzyme, were computed by non-linear regression analysis of the absorbance *versus* time data to the equation [3], where A and A_∞ are the absorbances at t and t_∞ respectively.

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

To demonstrate the complete inactivation of the enzyme by the inhibitors, absorbance data were collected for no less than 3 $t_{1/2}$. Fitting of the progress curve data to the equation [3] by non-linear regression analysis is considered the method of choice for kinetic parameter calculations since it gives reliable values under a variety of circumstances [12].

It has been shown [11, 13, 14] that, in the presence of substrate, the following equation is valid for complexing competitive inhibitors:

$$\frac{k_{obs}}{[I]} = \frac{k_2}{K_I} \cdot \frac{1}{1 + \frac{[S]}{K_M} + \frac{[I]}{K_I}} \qquad (4)$$

At constant substrate and different inhibitor concentrations, progress curves give different k_{obs} values. According to equation [5], which is a rearrangement of equation [4], a plot of $1/k_{obs}$ *vs* 1/[I] will yield k_2 as the reciprocal of the intercept on the ordinate and K_I from the slope $K_I(1 + [S]/K_M)/k_2$.

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

All kinetic parameters reported in table II were determined accordingly. Inhibition was always complete for all progress curves examined, as indicated by the steady A_∞ absorbance reached within the time of the experiment. Enzymatic activity could not be restored by 20–50-fold dilution of concentrated solutions of cathepsin B (20–50-fold that used in progress curves) following 10–20 min incubation with the inhibitors in the concentration range shown in table II. These data suggest that compounds **1a–8c** under the range of concentrations used are not hydrolyzed to an appreciable extent by the enzyme, because complete inhibition was independent of [E] and that they inhibit the enzyme irreversibly, since dilution of the enzyme–inhibitor complex did not restore enzymatic activity.

The validity of the continuous method of Tian and Tsou has already been assessed for other inhibitors [3] under similar conditions by comparison of the inhibitor parameters obtained using the discontinuous method of Kitz and Wilson [15]. The kinetic parameters of compounds **1a** and **1c** were also determined in an activity assay with fluorescent substrate, because the lowest inhibitor concentration tested spectrophotometrically approached a 1:5 ratio with the enzyme.

In the case of calpain I and calpain II, the apparent second-order rate constant for inactivation was calculated as $k_{obs}/[I]$. This value, corrected for the presence of substrate, is practically equivalent to k_2/K_I, if [I] ≪ K_I. In fact, when this assumption is valid, equation [4] is practically equivalent to equation [6] and $k_{obs}/[I]$ will be independent of [I] [15, 16].

$$\frac{k_{obs}}{[I]} = \frac{k_2}{K_I} \cdot \frac{1}{1 + \frac{[S]}{K_M}} \qquad (6)$$

To check the validity of this assumption, a range of inhibitor concentrations was used toward calpain I and II. The use of the described procedure for calculation of kinetic parameters is limited in the case of calpains by their activity loss, due to autolysis at long assay times. This limitation does not affect the accuracy of the results if the inactivation rate constants are > 10 $M^{-1}s^{-1}$ [5].

Compounds **1a**, **1b**, **1c**, **3c**, **4c** and **5c** were tested as inhibitors of trypsin, chymotrypsin and porcine pancreatic elastase. These compounds, at the maximal inhibitor concentration of 100–400 μM and following 5 h incubation, did not inhibit any of the serine proteases tested.

Results and discussion

Cathepsin B inactivation

1-Peptidyl-2-haloacetyl-hydrazines **1a–4c** completely and irreversibly inactivated cathepsin B under pseudo-

Table II. Inactivation of cathepsin B[a] calpain I and calpain II[b] by 1-peptidyl-2-haloacetylhydrazines.

Inhibitor Structure No	*Cathepsin B*				*Calpain I*	*Calpain II*	*Conditions, Reference*
	Range (μM)	*K_I (μM)*	*k_2 (min^{-1})*	*k_2/K_I (M^{-1} s^{-1})*	*$k_{obs}/[I]^c$ (M^{-1} s^{-1})*	*$k_{obs}/[I]^c$ (M^{-1} s^{-1})*	
1a Z-Tyr(I)-Agly-CH_2-Cl	0.11–0.58	0.054	0.31	95700	700	900	
1b Z-Tyr(I)-Agly-CH_2-Br	0.033–0.26	0.10	1.6	267000	2300	7500	
1c Z-Tyr(I)-Agly-CH_2-I	0.033–0.26	0.060	1.1	306000	Fast[d]	5000	
2a Z-Leu-Leu-Agly-CH_2-Cl	1.56–12.5	2.7	1.2	7400	650	1300	
2b Z-Leu-Leu-Agly-CH_2-Br	0.19–1.56	0.25	0.81	54000	Fast[d]		
2c Z-Leu-Leu-Agly-CH_2-I	0.21–3.12	0.43	1.4	54300		5000	
3c Z-Tyr-Aala-CH_2-I	0.62–10	0.20	0.18	15000	70	280	
4a Z-Tyr(I)-Aala-CH_2-Cl	13.2–100	9.2	0.40	725	350		
4b Z-Tyr(I)-Aala-CH_2-Br	0.82–6.25	0.50	0.35	11700			
4c Z-Tyr(I)-Aala-CH_2-I	0.55–4.17	0.68	0.60	14700	Fast[d]	900	
5c Z-Leu-Leu-Atyr-CH_2-I	2.19–16.7	0.99	0.24	4000	20	450	
6c Boc-Val-Lys(N_ε-Z)-Leu-Atyr-CH_2-I	4.39–22.2	1.10	0.19	2900	< 10	350	
7c Boc-Val-Lys(N_ε-COOMe)-Leu-Atyr-CH_2-I	4.9–33.3	1.9	0.26	2300	< 10	300	
8c Boc-Val-Lys(N_ε-Tos)-Leu-Atyr-CH_2-I	3.3–25	1.8	0.29	2700	< 10	100	
9a Z-Phe-Agly-CH_2-Cl	1.5–16	2.40	0.96	6670			[a], 4
9b Z-Phe-Agly-CH_2-Br	0.2–2	0.46	0.84	30430			[a], 4
9c Z-Phe-Agly-CH_2-I	0.2–2	0.37	0.88	39640			[a], 4
10 Z-Phe-Aala-CH_2-I	6–50	11.0	1.3	1970			[a], 4
11 Z-Tyr(I)-Ala-CHN_2	0.2–1.0			27800	< 10		[e], 5
12 Z-Leu-Leu-Tyr-CHN_2	0.5–1.5			1300	230000		[e], 5
13 Boc-Val-Lys(ε-Z)-Leu-Tyr-CHN_2	0.8–2.5			900	20640		[e], 5
14 Z-Phe-Phe-CH_2-Cl		23	12.5	9000			[f], 17
15 Z-Phe-Ala-CH_2-F		0.55	1.8	53300			[f], 17
16 Z-Phe-Ala-CH_2-O-CO-$(CF_3)_2C_6H_4$		0.028	2.7	1600000			[g], 18

[a]Conditions: 100 mM phosphate buffer, pH 6.8, 25°C; CH_3CN = 12% (v/v); [E] = 7 x 10^{-9} M; [S] = 125 μM. Replicate determinations indicate standard deviations for the kinetic parameters less than 20%; [b]conditions: pH 7.5, 25°C; see text for further details; [c]values were corrected for the presence of substrate according to equation [6]; [d]fast indicates that the reaction of the inhibitor with the enzyme was > 90% complete in the 9 s necessary to mix enzyme and inhibitor in the presence of substrate, *ie* k_{obs} ~ 0.2 s^{-1}; [e]conditions: pH 5.5, 30°C for cathepsin B and pH 7.5, 22°C for calpain II; [f]conditions: pH 5.4, 37°C. [g]Conditions: pH 6, 25°C.

first-order conditions. The concentration dependence of inactivation revealed saturation kinetics in accordance with a preassociation step leading to a reversible, non-covalent enzyme–inhibitor complex EI according to equation [1]. Assuming that the rate of the second step (steps), independently of the nature of the reagent halogen, is relatively slow, so that the initial binding equilibrium is not perturbed, the kinetic parameters K_I, k_2 and k_2/K_I have been determined and are reported in table II. The corresponding values for the 1-peptidyl-2-haloacetyl hydrazines based on phenylalanine [4] and for peptidyl diazo [5], fluoro, chloro [17] and acyloxymethylketones [18] are included for comparison purposes.

Structural bases of human liver cathepsin B specificity have been recently investigated [19] by X-ray crystallography. The overall folding pattern of the enzyme and the arrangement of the active site residues were revealed to be similar to the related cysteine proteases papain, actinidin and calotropin DI. Analogous resemblance should probably include the active site of bovine spleen cathepsin B used in the present study. In accordance with this hypothesis, a

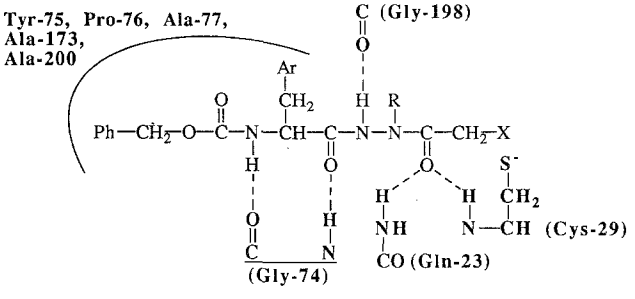


Fig 1. Schematic representation of the proposed binding of 1-peptidyl-2-haloacetyl inhibitors to the active site of cathepsin B (numbering according to [19]). Groups belonging to the enzyme are given in bold type.

possible structure of the EI reversible complex between this enzyme and azapeptide derivatives **1a–4c** based on α-azaglycine and α-azaalanine is reported in figure 1. All the hydrophobic bonding interactions and hydrogen bonds reported are inferred from the X-ray crystallographic analysis of the papain–Z-Phe-Ala-CH_2-Cl complex [20]. This hypothetical model is in accordance with the alkylation of Cys-25 thiolate and is reported as basis of discussion of the behaviour of reagents **1a–4c** as active site-directed inhibitors of cathepsin B.

The new series of haloacetyl derivatives Z-Leu-Leu-Agly-CH_2-X **2a–2c** revealed kinetic parameters for the inactivation of cathepsin B very similar to those previously reported for the Z-Phe-Agly-CH_2-X series **9a–9c** (table II). The range of variation of the term k_2 by variation of the halogen in the reagents was relatively small (0.81–1.4 min^{-1}) with respect to K_I (0.25–2.7 μM) which was thus mainly responsible for variances of the second-order rate constants. Analogous behaviour was previously observed for other 1-peptidyl-2-haloacetylhydrazines [4]. The trend of the affinities in the order Cl < Br < I for inhibitory ligands in the proposed EI reversible complex was explained in terms of hydrophobic considerations [4] while the smaller variations of the first-order rate constant k_2 were surprising in view of the large changes in leaving group ability. Minimal variation of the k_2 term (1.2–3.84 min^{-1}), however, is common to a variety of peptidyl diazomethylketones, fluoromethylketones, sulfonium methylylids, (acyloxy)methylketones and *O*-acylhydroxamates. According to Krantz [18] these values may represent the rate constant of a step (or steps) common to all of these reactions rather than the displacement step itself. We believe that this proposal could be extended to explain the k_2 trends for halogen variation in the 1-peptidyl-2-haloacetylhydrazines. At the same time it is clear that the interpretation of the kinetic constants K_I and k_2 beyond their precise determination in a complex multistep mechanism, without experimental evidence of structure of transient intermediates, is highly speculative.

It is noteworthy that replacement of Phe by Tyr(I) in the inhibitors based on α-azaglycine (**1a–1c**, **9a–9c** of table II) caused a 44-fold decrease of the dissociation constant K_I, only for the chloroacetyl derivative **1a**. The resulting value of 0.054 μM is lower than that of other chloroacetyl and even bromo and iodoacetyl derivatives studied in the present and previous works [3, 4]. The k_2 term, however, underwent a 3-fold decrease and the second-order rate constant for cathepsin B inactivation (k_2/K_I) for **1a** was 14-fold higher than that of the analog containing Phe **9a**. The bromoacetyl and iodoacetyl peptidylhydrazines **1b** and **1c** showed an 8.8 and 7.7 increase of the k_2/K_I ratio as a consequence of the same replacement of Phe by Tyr(I). For these derivatives, the increase of the second-order rate constant is the result of improvements in both K_I (4.6 and 6.2-fold) and k_2 (1.9 and 1.2-fold respectively). Replacement of Phe by Tyr(I) in the iodoacetyl azapeptide derivative **10** based on α-azaalanine, led to **4c** with a 16-fold decrease in the K_I term. The 7.5-fold increase in the second-order rate constant was practically identical to that achieved with the same replacement in the previously discussed analogs based on α-azaglycine **1c** and **9c**. In this series of inhibitors based on Aala, the effects of changing Phe by Tyr were also evaluated. The k_2/K_I constant observed for Z-Tyr-Aala-CH_2-I **3c** is substantially identical to that for Z-Tyr(I)-Aala-CH_2-I **4c** and both are some 7-fold higher than that for Z-Phe-Aala-CH_2-I **10**.

Replacement of Phe by a iodotyrosine unit in P_2 leads to an important decrease of the dissociation constant K_I toward cathepsin B for the inhibitory ligands presenting α-azaglycine in P_1. This effect can probably be explained on the basis of the proposed structure of the EI reversible adduct in figure 1. The active site of cathepsin B is constructed similarly to that in papain, but presents a larger hydrophobic cleft due to the bent chain of Gly-198 and lack of the topologically equivalent residue for papain residue Val-157 [19]. Thus cathepsin B would more easily accommodate at the S_2 subsite aromatic rings with even increased bulkiness like that of the Tyr(I) residue. In addition to the large iodine atom, probably involved in an increase of the bonding interactions at the hydrophobic S_2 subsite, the iodotyrosine residue also includes a phenolic hydroxyl group, whose acidity is enhanced by the presence of the electrophilic, vicinal iodine atom. Further stabilization of the EI reversible adduct may derive from a specific bonding interaction between the phenolic hydroxyl group of Tyr(I) and a complementary functional group of the hydrophobic cleft of cathepsin B.

The observed values of k_2/K_I of the chloro, bromo and iodoacetyl derivatives **1a–1c** based on α-azaglycine are at least 20-fold higher with respect to the analogs **4a–4c** based on α-azaalanine. The enhancement is mainly due to the improvement of the binding constant K_I. On the basis of the proposed structure of the EI reversible complex of figure 1, the presence of the methyl group at the α-N could decrease the affinity of the inhibitory ligand by means of 3 main factors: steric strain, conformational preferences of the free ligand and loss of a possible hydrogen bond. We do not think that the methyl group in P_1 would account for a serious enhancement of steric strain, since it is known that cathepsin B as well as papain can easily accommodate both Gly or Ala in P_1 and that bond lengths and bond angles in azapeptides are

not substantially modified with respect to the parent peptides. Conformational preferences of 1,2-diacyl hydrazines are strongly affected by further nitrogen substitution. For example, *N,N'*-di-L-phenylalanine hydrazide [21], whose structure is strictly related to that of our 1-peptidyl-2-haloacetyl hydrazines based on Agly shows an almost planar central hydrazide part, suggesting the existence of 2 fused 5-membered chelate rings with NH...OC intramolecular hydrogen bonds (fig 2). The coplanar disposition of the 2 R-CO-N planes should be turned to orthogonal in the 1-peptidyl-2-haloacetyl hydrazines based on Aala, in accordance with the X-ray structure deterrnination of N-substituted hydrazides bearing at least 1 alkyl group [22]. The third factor takes into account that a new H-bond productive for binding could be established between the αN-H of the inhibitors based on Agly and an appropriate electron donor group at the enzyme active site. Loss of the H-bond in the inhibitors based on Aala could explain their lowered affinity for the enzyme active site.

Independently of the precise effects of the structural variations in the inhibition mechanism, the contemporary presence of an iodotyrosine unit in P_2 and of the α-azaglycine in P_1 leads to inhibitors which are more effective against cathepsin B than the known peptidyl diazo (**11**), chloro (**14**) and fluoro (**15**) methylketones (see table II). The iodoacetyl derivative **1c** shows a second-order inactivation constant of 306 000 M^{-1} s^{-1} at pH 6.8 and 25°C. The corresponding chloroacetyl derivative **1a**, with very low inhibitory activity toward calpains and inertness toward serine proteases, displays 96 000 M^{-1} s^{-1} as a second-order inactivation constant. Only the most favourable structural variations in peptidyl-acyloxymethylketones like **16** [18] provide more potent inhibitors.

Calpain I and II inactivation

The subsite specificity of calpain II has been investigated by Shaw [5] in a study of peptidyldiazomethylketones presenting Leu in P_2 and different residues in P_1, P_3 and P_4. The most potent peptidyldiazomethylketones for calpain II inactivation presented Tyr in P_1 (**12** and **13** in table II). Their second-order rate constants for calpain II inactivation were 230 000 and 200 640 M^{-1} s^{-1} respectively, and their selectivity was excellent since cathepsin B was inactivated 177 and 23-fold more slowly. All the 1-peptidyl-2-haloacetyl hydrazines **5a–8c** were therefore based on an Atyr unit in P_1 and are structurally related to the peptidyldiazomethylketones **12** and **13**. Minor variations were introduced at the Lys ε-NH_2. A preliminary screening showed that their inhibitory activity against calpain I and calpain II was very low and only the results obtained for the more reactive iodocetyl derivatives **5c**, **6c**, **7c** and **8c** were measured and reported in table II. It is apparent that all these haloacetyl azatyrosine derivatives are by far less potent than the parent diazomethylketones and even weaker inhibitors toward calpains than azaglycine and azaalanine derivatives studied for cathepsin B. Particularly striking is the case of Z-Leu-Leu-Agly-CH_2-I (**2c**, $k_{obs}/[I]$ = 5000 M^{-1} s^{-1}). Conversion into the corresponding Atyr derivative **5c** by introduction of the 4-hydroxybenzyl group at the appropriate nitrogen atom caused a 10-fold decrease rather than an increase in the second-order rate constant for calpain II inactivation. Even weaker inactivators were obtained by applying the azapeptidyl-recognising unit based on Boc-Val-Lys(ε-Z)-Leu-Atyr and its variations.

These results extend to the calpains the previously observed strong preferences of papain and cathepsin B for an azaglycine unit in P_1 even when the corresponding Atyr derivatives are expected to be more productive for binding. This effect is probably related to the previously discussed inhibitors presenting Agly or Aala in P_1 and seems to reinforce the hypothesis of intervention of an H-bond productive for binding in the Agly derivatives.

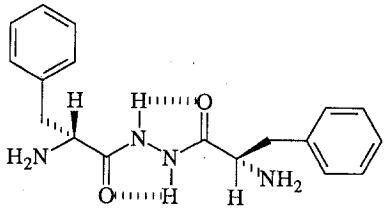


Fig 2. Representation of the structure of 1,2-di-L-phenylalanyl-hydrazine, determined by X-ray crystallography [21]. Probable CO...HN intramolecular hydrogen bonds are reported.

Conclusions

The 1-peptidyl-2-haloacetylhydrazines **1a–1c** are easily synthesized and display very effective and selective inhibition of cathepsin B. No inhibitory activity could be detected toward trypsin chymotrypsin and porcine pancreatic elastase at 0.1–0.4 mM concentration after 5 h incubation. Their reactivity is at least 35-fold higher for cathepsin B than for calpain II. The ratio raises to 106-fold for the 1-peptidyl-2-chloroacetylhydrazine **1a** showing 96 000 M^{-1} s^{-1} as second-order inactivation constant for cathepsin B. Its aspecific alkylation properties are quite low and substantially similar to those reported by Shaw [23] for peptidylfluoromethylketones and by us for analogous 1-peptidyl-2-haloacetyl hydrazines [4]. They

Table III. Comparative reactivity of 1-peptidyl-2-haloacetyl-hydrazines toward glutathione[a].

GSH conc	*Inhibitor*		*CH_3CN conc*	*k*
(μM)	*Structure*	*[I] (μM)*	*(v/v)*	*(M^{-1} s^{-1})*
30	**1a**	200	13	0
20	**1b**	200	13	0.13
20	**1c**	100	13	0.14
40	**3c**	500	13	0.20
20	**4c**	100	13	0.14

[a]Conditions: 50 mM phosphate buffer, pH 6.8, 25°C. Rate of alkylation of glutathione was measured following the thiol disappearance using Ellman reagent [24].

were determined by measuring the second-order rate constant for glutathione alkylation according to Shaw [23], following the thiol disappearance by using Ellman reagent [24] and are reported in table III.

The success met with cathepsin B and the poor results obtained with calpains confirm that use of isosteres of pharmacologically active substances leads to rather large and unpredictable variations in the effects, particularly when replacement of carbon by a heteroatom concerns those regions of the molecule which are primarily involved in the recognition and binding.

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; ^{13}C-NMR spectra were recorded on a Varian XL 300 spectrometer; in all cases TMS was used as internal standard. Optical rotations were determined with a Schmidt–Haensch 1604 polarimeter. Elemental analyses were within ± 0.4% of the calculated values except where otherwise stated.

Preparation of peptidylhydrazones. General procedure A
A solution of the required peptidylhydrazine (1 mmol) and 4-hydroxybenzaldehyde (134 mg, 1.1 mmol) in AcOH (3 ml) was allowed to react overnight at room temperature (rt). The crude product was obtained by removal of the solvent at reduced pressure. EtOH/AcOH 10:1 (5.5 ml) was used as the solvent for Z-Leu-Leu-NH-NH_2.

Sodium cyanoborohydride reduction of peptidylhydrazones. General procedure B
To a solution of $NaBH_3CN$ (157 mg, 2.5 mmol) and the required hydrazone (1 mmol) in anhydrous THF (6 ml), AcOH (1.43 ml, 25 mmol) was added and the resulting solution was allowed to react overnight at rt. After removal of the solvent at reduced pressure, the residue was dissolved in EtOAc and sequentially washed with brine, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed under reduced pressure. The resulting residue, containing the peptidylhydrazine as the cyanoborane adduct, was hydrolyzed by treatment with 2 N NaOH (1.75 ml, 3.5 mmol) in MeOH (6 ml). After 4 h at rt, the reaction mixture was diluted with EtOAc (20 ml) and washed with brine. Drying over anhydrous Na_2SO_4 and removal of the solvent under reduced pressure gave the crude 1-peptidyl-2-(4-hydroxybenzyl) hydrazine.

Acylation of peptidylhydrazines. General procedure C
To an ice-cooled suspension of the required hydrazine (1 mmol) in EtOAc (10 ml) and 1 M aq $NaHCO_3$ (2.4 ml) a solution of the appropriate haloacetyl chloride (1.2 mmol) in EtOAc (1 ml) was added under vigorous stirring and the reaction mixture allowed to warm to room temperature while stirring was continued for 30 min. The crude product was recovered by dilution with EtOAc (10 ml), washing with brine, saturated $NaHCO_3$, 1 M $KHSO_4$ and brine, drying over anhydrous Na_2SO_4 and removal of the solvent under reduced pressure.When Z-Leu-Leu-NH-NH_2 was employed, the product separated as a white solid which was filtered, washed with water and EtOAc and dried.

Acylation of peptidylhydrazines. General procedure D
The peptidylhydrazine hydrochloride (1 mol) was dissolved in DMSO as co-solvent (1 ml) and acylated according to the previous procedure except that $CHCl_3$ (10 ml) was used in place of EtOAc.

Acylation of peptidylhydrazines. General procedure E
A solution of the required peptidylhydrazine (1 mmol) and haloacetyl *N*-hydroxysuccinimide ester (2 mmol) in DMF (10 ml) was allowed to react at room temperature for 30 min. The product was crystallized by addition of water, filtered, washed with water and dried *in vacuo*.

Mixed anhydride coupling. General procedure F
A solution of the required carboxylic acid (1.0 mmol) and *N*-methylmorpholine (1.0 mmol) in anhydrous THF (4 ml) was cooled to –15°C and *i*-butylchloroformate (1.0 mmol) was added dropwise under stirring. After 30 min, a solution of the required amine (1.0 mmol) was added slowly while the temperature of –15°C was maintained. The resulting reaction mixture was stirred for 2 h at the same temperature. After removal of the solvent at reduced pressure, the residue was dissolved in EtOAc (20 ml) and washed with brine, 1 M $KHSO_4$, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4, the solvent was removed under reduced pressure to give the crude product.

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-chloroacetyl-hydrazine ***1a***
Obtained from 1-(*N*-Z-3-iodo-L-tyrosyl)-hydrazine [25] (225 mg, 0.55 mmol) and chloroacetyl *N*-hydroxysuccinimide ester (212 mg, 1.1 mmol) according to *General procedure E*. The crude product was purified by crystallization from 1,2 dichloroethane/hexane: 278 mg (95%); mp: 193–196°C; $[\alpha]_D^{22} = -9°$ (1, DMF); IR (KBr) main peaks at 3271, 1693, 1606, 1488, 1268 cm^{-1}H-NMR (DMSO–d_6) δ 2.56–2.90 (m, 2H, Tyr CH_2), 4.14 (s, 2H, CH_2Cl), 4.15–4.40 (m, 1H, αCH), 4.95 (s, 2H, Z CH_2), 6.80 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.06–7.40 (m, 6H, Tyr and Z Ar), 7.71 (d, 1H, Tyr Ar, J = 1.5 Hz). Anal $C_{19}H_{19}N_3O_5Cl$ (C, H, N).

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-bromoacetyl-hydrazine ***1b***
Obtained from 1-(*N*-Z-3-iodo-L-tyrosyl)-hydrazine [25] (225 mg, 0.55 mmol) and bromocetyl *N*-hydroxysuccinimide ester (262 mg, 1.1 mmol) according to *General procedure E*. The product was purified by silica gel chromatography (EtOAc) and crystallized from dioxane/H_2O: 265 mg (92%); mp: 184–187°C; $[\alpha]_D^{22} = -5°$C (1, DMF); IR (KBr) main peaks at 3296,

1693, 1605, 1486, 1263 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.57–2.92 (m, 2H, Tyr CH_2), 3.93 (s, 2H, CH_2Br), 4.10–4.42 (m, 1H, αCH), 4.95 (s, 2H, Z CH_2), 6.80 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.03–7.43 (m, 6H, Tyr and Z Ar), 7.70 (d, 1H, Tyr Ar, J = 1.5 Hz); ^{13}C-NMR (DMSO–d_6) δ 26.99 (CH_2Br), 36.07 (Tyr CH_2), 54.95 (αCH), 65.11 (Z CH_2), 84.09, 114.42, 127.24, 127.52, 128.19, 130.25, 130.37, 136.88, 143.09 and 155.01 (Tyr and Z Ar), 155.76, 164.32 and 169.97 (C=O). Anal $C_{19}H_{19}N_3O_5Br$ (C, H, N).

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-iodoacetyl hydrazine ***1c***

Obtained from 1-(*N-Z*-3-iodo-L-tyrosyl)-hydrazine [25] (225 mg, 0.55 mmol) and iodoacetyl *N*-hydroxysuccinimide ester (331 mg, 1.1 mmol) according to *General procedure E*. The product was purified by silica gel chromatography (EtOAc) and crystallized from dioxane/H_2O: 280 mg (90%); mp: 192–196°C; $[\alpha]_D^{22}$ = –3° (1, DMF); IR (KBr) main peaks at 3264, 1691, 1599, 1488, 1261 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.58-3.08 (m, 2H, Tyr CH_2), 3.74 (s, 2H, CH_2I), 4.09–4.49 (m, 1H, αCH), 4.98 (s, 2H, Z CH_2), 6.84 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.08–7.84 (m 7H, Tyr and Z Ar). Anal $C_{19}H_{19}N_3O_5I$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-chloroacetyl hydrazine ***2a***

To a solution of Z-L-leucyl-L-leucine methylester (2.9 g, 7.4 mmol) in EtOH (30 ml), hydrazine hydrate (1.85 ml, 37 mmol) was added and the mixture was kept at rt for 24 h. After removal of the solvent and hydrazine excess at reduced pressure, *Z*-L-leucyl-L-leucyl hydrazine was obtained as a white solid by trituration with Et_2O: 2.5 g (95%) and was used without further purification.

Reaction of Z-L-leucyl-L-leucyl hydrazine (392 mg, 1 mmol) with chloroacetyl chloride (136 mg, 1.2 mmol) according to *Procedure C* and crystallization from DMF/H_2O gave the pure product as white crystals: 387 mg (83%); mp: 216–217°C; $[\alpha]_D^{22}$ = –25° (1; DMF); IR (KBr): main peaks at 3271, 3222, 2956, 1692, 1646, 1607, 1536, 1483, 1272, 1232 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.7–1.0 (m, 12H, Leu CH_3), 1.30–1.85 (m, 6H, Leu CH_2CH), 3.95–4.30 (m, 1H, αCH), 4.12 (s, 2H, CH_2Cl), 4.3–4.6 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 7.38 (s, 5H, Z C_6H_5). Anal $C_{22}H_{33}N_4O_5Cl$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-bromoacetyl hydrazine ***2b***

Reaction of Z-L-leucyl-L-leucyl hydrazine (196 mg, 0.5 mmol) with bromoacetyl chloride (95 mg, 0.6 mmol) according to *Procedure C* and crystallization from DMF/H_2O gave the pure product as white crystals: 211 mg (82%); mp: 209–211°C; $[\alpha]_D^{22}$ = –22° (1; DMF); IR (KBr) main peaks at 3263, 3217, 2956, 1692, 1645, 1606, 1537, 1483, 1269, 1235 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.7–1.0 (m, 12H, Leu CH_3), 1.30–1.80 (m, 6H, Leu CH_2CH), 3.90 (s, 2H, CH_2Br), 4.0–4.2 (m, 1H, αCH), 4.35–4.60 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 7.38 (s, 5H, Z C_6H_5); ^{13}C-NMR (DMSO–d_6) δ 21.54, 21.77, 23.09 and 23.21 (Leu CH_3), 24.13 and 24.27 (Leu γCH), 27.24 (CH_2Br), 40.85 and 41.33 (Leu βCH_2), 49.44 and 53.15 (αCH), 65.45 (Z CH_2), 127.74, 127.85, 128.42 and 137.18 (Ar), 155.98, 164.69, 170.76 and 172.22 (C=O). Anal $C_{22}H_{33}N_4O_5Br$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-iodoacetyl hydrazine ***2c***

Reaction of Z-L-leucyl-L-leucyl hydrazine (392 mg, 1 mmol) with iodoacetyl chloride (245 mg, 1.2 mmol) according to *Procedure C* and crystallization from DMF/H_2O gave the pure product as white crystals: 402 mg (72%); mp: 206–210°C; $[\alpha]_D^{22}$ = –20° (1; DMF); IR (KBr) main peaks at 3271, 3219, 2955, 1692, 1644, 1605, 1537, 1481, 1269, 1235 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.75–0.95 (m, 12H, Leu CH_3), 1.30–1.80 (m, 6H, Leu CH_2CH), 3.70 (s, 2H, CH_2I), 3.95–4.25 (m, 1H, αCH), 4.25–4.55 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 7.37 (s, 5H, Z C_6H_5). Anal $C_{22}H_{33}N_4O_5I$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-tyrosyl)-2-methyl-2-tert-butoxycarbonyl hydrazine

Obtained from Z-L-tyrosine (3.15 g, 10 mmol) and 1-Boc-1-methylhydrazine (1.46 g, 10 mmol) according to *Procedure F*. The solid crude material was triturated with hexane and used without further purifications: 4.3 g (100%), mp: 104–105°C; $[\alpha]_D^{22}$ = –20° (1; DMF); IR ($CHCl_3$) main peaks at 3298, 2976, 1681, 1503, 1368, 1255 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.43 (s, 9H, Boc), 3.05 (bs, 5H, Tyr CH_2 and NCH_3), 4.30–4.64 (m, 1H, αCH), 5.05 (s, 2H, Z CH_2), 6.68 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.00 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.35 (s, 5H, Z C_6H_5). Anal $C_{23}H_{29}N_3O_6$ (C, H, N).

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-methyl-2-tert-butoxycarbonyl hydrazine

Obtained from Z-3-iodo-L-tyrosine (2.4 g, 5.41 mmol) and 1-Boc-1-methylhydrazine (0.79 g, 5.41 mmol) by *Procedure F*. The oily residue was purified by filtration through a short pad of SiO_2 ($CHCl_3$). The crystalline product obtained after solvent evaporation was triturated with hexane and used without further purification: 3.02 g (100%); mp: 89–91°C; $[\alpha]_D^{22}$ = –18° (1; DMF); IR ($CHCl_3$) main peaks at 3419, 2982, 1711, 1487, 1369, 1249 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.52 (s, 9H, Boc), 3.61 (bs, 5H, Tyr CH_2 and NCH_3), 4.38–4.74 (m, 1H, αCH), 5.12 (s, 2H, Z CH_2), 6.85 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.14 (dd, 1H, Tyr Ar, J = 9.0 and 1.5 Hz), 7.40 (s, 5H, Z C_6H_5), 7.61 (unr d, 1H, Tyr Ar). Anal $C_{23}H_{29}N_3O_6I$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-tyrosyl)-2-methylhydrazine hydrochloride

A solution of 1-(*N*-Z-L-tyrosyl)-2-methyl-2-Boc hydrazine (3.2 g, 7.22 mmol) in anhydrous EtOAc (25 ml) was saturated with dry HCl at room temperature. After 5 h the resulting white crystals were collected by filtration and washed with anhydrous EtOAc and anhydrous Et_2O and dried under high vacuum: 2.39 g (87%); mp: 182–185°C; $[\alpha]_D^{22}$ = 6° (1; MeOH); IR (KBr) main peaks at 3300, 3211, 2320, 1689, 1531, 1273, 1233 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.70 (s, 3H, NCH_3), 2.77–3.08 (m, 2H, Tyr CH_2), 4.14–4.52 (m, 1H, αCH), 5.05 (s, 2H, Z CH_2), 6.77 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.20 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.37 (s, 5H, Z C_6H_5). Anal $C_{18}H_{22}N_3O_4Cl$ (C, H, N).

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-methylhydrazine hydrochloride

1-(*N*-Z-3-iodo-L-tyrosyl)-2-methyl-2-Boc-hydrazine (2.42 g, 4.72 mmol) was subjected to the previous procedure to give 2.37 g (100%) of the hydrochloride as white crystals: mp: 130–132°C; $[\alpha]_D^{22}$ = –14° (1; DMF); IR (KBr) main peaks at 3280, 2960, 1680, 1530, 1259, 1223 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.44 (s, 3H, NCH_3), 2.56–2.85 (m, 2H, Tyr CH_2), 3.95–4.31 (m, 1H, αCH), 4.94 (s, 2H, Z CH_2), 6.89 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.00–7.41 (m, 6H, Tyr and Z Ar), 7.63 (d, 1H, Tyr Ar, J = 1.5 Hz). Anal $C_{18}H_{21}N_3O_4ClI$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-tyrosyl)-2-methyl-2-chloroacetyl hydrazine ***3a***

Obtained from 1-(*N*-Z-L-tyrosyl)-2-methylhydrazine hydrochloride (300 mg, 0.78 mmol) and chloroacetyl chloride (132 mg, 1.17 mmol) according to *Procedure D*. The product

was purified by silica gel chromatography (CH_2Cl_2/*i*-PrOH 93:7) and crystallized from 1,2-dichloroethane/hexane: 213 mg (65%); mp: 138–141°C; $[\alpha]_D^{22} = 8°$ (1; MeOH); IR (KBr) main peaks at 3313, 1703, 1644, 1514, 1256 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.70–2.99 (m, 5H, Tyr CH_2 and CH_3N), 3.85–4.25 (m, 3H, αCH and CH_2Cl), 5.04 (s, 2H, Z CH_2), 6.74 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.12 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.37 (s, 5H, Z C_6H_5). Anal $C_{20}H_{22}N_3O_5Cl$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-tyrosyl)-2-methyl-2-bromoacetyl hydrazine ***3b***

Obtained from 1-(*N*-Z-L-tyrosyl)-2-methylhydrazine hydrochloride (300 mg, 0.78 mmol) and bromoacetyl chloride (184 mg, 1.17 mmol) according to *Procedure D* and directly crystallized from 1,2-dichloroethane/hexane: 334 mg (72%); mp: 171–175°C; $[\alpha]_D^{22} = 13°$ (1; MeOH); IR (KBr) main peaks at 3459, 3279, 1707, 1655, 1517, 1263 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.95 (s, 3H, CH_3N), 3.10 (d, 2H, Tyr CH_2, J = 7.5 Hz), 4.05 (s, 2H, CH_2Br), 4.30–4.64 (m, 1H, αCH), 5.08 (s, 2H, Z CH_2), 7.00–7.50 (m, 9H, Tyr and Z Ar); ^{13}C-NMR (DMSO–d_6) δ 27.78 (CH_2Br), 35.57 and 35.93 (CH_3N and Tyr CH_2), 55.37 (αCH), 65.65 (Z CH_2), 115.15, 127.15, 127.83, 127.93, 128.45, 130.36, 136.97 and 156.22 (Tyr and Z Ar), 157.25, 161.83 and 171.37 (C=O). Anal $C_{20}H_{22}N_3O_5Nr$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-tyrosyl)-2-methyl-2-iodoacetyl hydrazine ***3c***

Obtained from 1-(*N*-Z-L-tyrosyl)-2-methylhydrazine hydrochloride (300 mg, 0.78 mmol) and iodoacetyl chloride (240 mg, 1.17 mmol) according to *Procedure D*. The crude product was purified by silica gel chromatography (CH_2Cl_2/*i*-PrOH 93:7) and crystallized from 1,2-dichloroethane/hexane: 260 mg (63%); mp: 182–187°C; $[\alpha]_D^{22} = -9°$ (1; MeOH); IR (KBr) main peaks at 3457, 3271, 1707,1647, 1517, 1262 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.80 (bs, 5H, Tyr CH_2 and CH_3N), 3.61 (bs, 2H, CH_2I), 4.00–4.33 (m, 1H, αCH), 5.01 (s, 2H, Z CH_2), 6.72 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.12 (d, 2H, Tyr C_6H_4, J = 9.0 Hz), 7.38 (s, 5H, Z C_6H_5). Anal $C_{20}H_{22}N_3O_5I$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-3-iodo-tyrosyl)-2-methyl-2-chloroacetyl hydrazine ***4a***

Obtained from 1-(*N*-Z-3-iodo-L-tyrosyl)-2-methylhydrazine hydrochloride (250 mg, 0.50 mmol) and chloroacetyl chloride (68 mg, 0.60 mmol) according to *Procedure D*. The crude product was purified by silica gel chromatography (CH_2Cl_2/EtOAc 70:30) and crystallized from 1,2-dichloroethane/hexane: 230 mg (84%); mp: 128–131°C; $[\alpha]_D^{22} = -18°$ (1; CH_3CN); IR (KBr) main peaks at 3285, 1678, 1534, 1284, 1038 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.85 (bs, 5H, Tyr CH_2 and CH_3N), 3.90–4.33 (m, 3H, αCH and CH_2Cl), 5.03 (s, 2H, Z CH_2), 6.82 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.16 (dd, 1H, Tyr Ar, J = 9.0 Hz and 1.5 Hz), 7.36 (s, 5H, Z C_6H_5), 7.67 (d, 1H, Tyr Ar, J = 1.5 Hz). Anal $C_{20}H_{21}N_3O_5ClI$ (C, H, N).

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-methyl-2-bromoacetyl hydrazine ***4b***

Obtained from 1-(*N*-Z-3-iodo-L-tyrosyl)-2-methylhydrazine hydrochloride (250 mg, 0.50 mmol) and bromoacetyl chloride (94 mg, 0.60 mmol) according to *Procedure D*. The crude product was purified by silica gel chromatography (CH_2Cl_2/EtOAc 70:30) and crystallized from 1,2-dichloroethane/hexane: 218 mg (74%); mp: 139–143°C; $[\alpha]_D^{22} = -12°$ (1; CH_3CN); IR (KBr) main peaks at 3271, 1693, 1502, 1260, 1024 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.86 (bs, 5H, Tyr CH_2 and CH_3N), 3.9 (s, 2H, CH_2Br), 4.01–4.33 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 6.85 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.15 (dd, 1H, Tyr Ar, J = 9.0 Hz and 1.5 Hz), 7.38 (s, 5H, Z C_6H_5), 7.65 (d, 1H, Tyr Ar, J = 1.5 Hz); ^{13}C-NMR (DMSO–d_6) δ 27.65 (CH_2Br), 35.23 (CH_3N), 35.59 (Tyr CH_2), 55.27 (αCH), 65.64 (Z CH_2), 84.44, 114.71, 127.75, 127.90, 128.44, 129.79, 130.54, 136.90, 139.33 and 155.44 (Tyr and Z Ar), 156.18, 163.80 and 171.17 (C=O). Anal $C_{20}H_{21}N_3O_5BrI$ (C, H, N).

1-(N-Benzyloxycarbonyl-3-iodo-L-tyrosyl)-2-methyl-2-iodoacetyl hydrazine ***4c***

Obtained from 1-(*N*-Z-3-iodo-L-tyrosyl)-2-methylhydrazine hydrochloride (300 mg, 0.78 mmol) and iodoacetyl chloride (195 mg, 0.93 mmol) according to *Procedure D*. The product was purified by silica gel chromatography (CH_2Cl_2/EtOAc 70:30) and crystallized from 1,2-dichloroethane/hexane: 260 mg (63%); mp: 112–115°C; $[\alpha]_D^{22} = -11°$ (1; CH_3CN); IR (KBr) main peaks at 3246, 1693, 1502, 1257, 1019 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 2.81 (bs, 5H, Tyr CH_2 and CH_3N), 3.65 (bs, 2H, CH_2I), 4.04–4.30 (m, 1H, αCH), 5.01 (s, 2H, Z CH_2), 6.83 (d, 1H, Tyr Ar, J = 9.0 Hz), 7.18 (dd, 1H, Tyr Ar, J = 9.0 Hz and 1.5 Hz), 7.34 (s, 5H, Z C_6H_5), 7.67 (d, 1H, Tyr Ar, J = 1.5 Hz). Anal $C_{20}H_{21}N_3O_5I_2$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine

Reaction of Z-Leu-Leu hydrazine (942 mg, 2.4 mmol) with 4-hydroxybenzaldehyde (330 mg, 2.7 mmol) according to *Procedure A* gave the expected hydrazone as a pale yellow oil: 1.19 g (100%) which was employed without further purification. Reduction of 1-(*N*-Z-L-leucyl-L-leucyl)-2-(4-hydroxybenzal) hydrazone (2.23 g, 4.5 mmol) with $NaBH_3CN$ (706 mg, 11.25 mmol) was performed according to *Procedure B*. The crude product was washed with $CHCl_3$/EtOAc 8:2 (20 ml) and crystallized from EtOH to give 1-(*N*-benzyloxycarbonyl-L-leucyl-L-leucyl)-2-(4-hyroxybenzyl) hydrazine as white crystals: 967 mg (43%); mp: 161–163°C; $[\alpha]_D^{22} = -50°$ (1; MeOH); IR (KBr) main peaks at 3271, 2957, 1696, 1656, 1639, 1546, 1516, 1278, 1264, 1237 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.65–0.95 (m, 12H, Leu CH_3), 1.20–1.70 (m, 6H, Leu CH_2CH), 3.73 (bs, 2H, $C_6H_4CH_2$), 3.95–4.55 (m, 2H, αCH), 5.05 (s, 2H, Z CH_2), 6.73 (d, 2H, C_6H_4, J = 9.0 Hz), 7.15 (d, 2H, C_6H_4, J = 9.0 Hz), 7.39 (s, 5H, Z C_6H_5). Anal $C_{27}H_{38}N_4O_5$ (C, N, N).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl)-2-chloroacetyl hydrazine ***5a***

Reaction of 1-(*N*-Z-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (200 mg, 0.4 mmol) with chloroacetyl chloride (54 mg, 0.48 mmol) according to *Procedure C* and purification by silica gel chromatography ($CHCl_3$/EtOAc 9:1) gave the pure product as a colourless oil: 193 mg (84%); $[\alpha]_D^{22} = -24°$ (1; DMF); IR (KBr) main peaks at 3285, 2958, 1692, 1649, 1515, 1267, 1239 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–0.95 (m, 12H, Leu CH_3), 1.25–1.80 (m, 6H, Leu CH_2CH), 3.90–4.50 (m, 5H, $C_6H_4CH_2$, CH_2Cl, αCH), 4.7–4.9 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 6.73 (d, 2H, C_6H_4, J = 9.0 Hz), 7.08 (d, 2H, C_6H_4, J = 9.0 Hz), 7.42 (s, 5H, Z C_6H_5). Anal $C_{29}H_{39}N_4O_6Cl$ (C, H, N, Cl).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl)-2-bromoacetyl hydrazine ***5b***

Reaction of 1-(*N*-Z-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (200 mg, 0.4 mmol) with bromoacetyl chloride (76 mg, 0.48 mmol) according to *Procedure C* and purification by silica gel chromatography ($CHCl_3$/EtOAc 9:1) gave the pure product as a colourless oil: 223 mg (90%); $[\alpha]_D^{22} = -20°$ (1;

DMF); IR (KBr) main peaks at 3281, 2958, 1692, 1649, 1515, 1266, 1237 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–0.95 (m, 12H, Leu CH_3), 1.25–1.80 (m, 6H, Leu CH_2CH), 3.85–4.45 (m, 5H, $C_6H_4CH_2$, CH_2Br, Leu αCH), 4.7–4.9 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 6.73 (d, 2H, C_6H_4, J = 9.0 Hz), 7.12 (d, 2H, C_6H_4, J = 9.0 Hz), 7.40 (s, 5H, Z C_6H_5); ^{13}C-NMR (DMSO–d_6) δ 21.38, 21.61, 22.58 and 22.99 (Leu CH_3), 24.00 and 24.03 (Leu γCH), 28.47 (CH_2Br), 40.25 and 40.49 (Leu βCH_2), 49.74 and 52.64 (αCH), 50.40 ($C_6H_4CH_2$), 65.22 (Z CH_2), 114.96, 125.55, 126.86, 127.43, 128.19, 130.04, 136.98 and 155.81 (Ar), 156.92, 171.15, 171.61 and 172.66 (C=O). Anal $C_{29}H_{39}N_4O_6Br$ (C, H, N, Br).

1-(N-Benzyloxycarbonyl-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl)-2-iodoacetyl hydrazine ***5c***

Reaction of 1-(*N*-Z-L-leucyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (200 mg, 0.4 mmol) with iodoacetyl chloride (98 mg, 0.48 mmol) according to *Procedure C* and purification by silica gel chromatography ($CHCl_3$/EtOAc 9:1) gave the pure product as a pale yellow oil: 162 mg (61%); $[\alpha]_D^{22}$ = –9° (1; DMF); IR (KBr) main peaks at 3278, 2958, 1692, 1644, 1515, 1263, 1237 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–0.95 (m, 12H, Leu CH_3), 1.25–1.80 (m, 6H, Leu CH_2CH), 3.5–4.4 (m, 5H, $C_6H_4CH_2$, CH_2I, αCH), 4.5–4.7 (m, 1H, αCH), 5.04 (s, 2H, Z CH_2), 6.73 (d, 2H, C_6H_4, J = 9.0 Hz), 7.07 (d, 2H, C_6H_4, J = 9.0 Hz), 7.43 (s, 5H, Z C_6H_5). Anal $C_{29}H_{39}N_4O_6I$ (C, H, N, I).

N-tert-Butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucine methylester

Obtained from Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysine [10] (1.63 g, 3.4 mmol) and L-leucine methylester hydrochloride (617 mg, 3.4 mmol) according to *Procedure F*. Crystallization from 1,2-dichloroethane/hexane gave the pure product as white crystals: 1.81 g (88%); mp: 137–139°C; $[\alpha]_D^{22}$ = –33° (1; $CHCl_3$); IR (KBr) main peaks at 3312, 2958, 1727, 1691, 1640, 1536, 1276, 1248, 1173 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.80–1.10 (m, 12H, Leu CH_3, Val CH_3), 1.3–1.9 (m, 9H, Lys $\beta\gamma\delta CH_2$, Leu CH_2CH), 1.40 (s, 9H, Boc), 1.95–2.30 (m, 1H, Val CH), 3.10–3.35 (m, 2H, Lys εCH_2), 3.68 (s, 3H, OCH_3), 3.90–4.20 (m, 1H, αCH), 4.40–4.80 (m, 2H, αCH), 5.12 (s, 2H, Z CH_2), 7.47 (s, 5H, Z C_6H_5). Anal $C_{31}H_{50}N_4O_8$ (C, H, N).

N-tert-Butyloxycarbonyl-L-valyl-L-lysyl-L-leucine methylester

Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucine methylester (3 g, 4.95 mmol) in anhydrous THF (30 ml) was hydrogenated in the presence of 10% Pd/C (300 mg) for 3 h. MeOH (15 ml) was added to dissolve the solid formed during the reaction and the solution filtered on celite. The solvent was removed under reduced pressure and the resulting material was crystallized from $CHCl_3$/hexane to give the pure product as white crystals: 2.34 g (100%); mp: 110–112°C; $[\alpha]_D^{22}$ = –45° (1; $CHCl_3$); IR (KBr) main peaks at 3292, 2960, 1746, 1690, 1641, 1552, 1524, 1247, 1172 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–1.00 (m, 12H, Leu CH_3, Val CH_3), 1.20–1.80 (m, 9H, Lys $\beta\gamma\delta CH_2$, Leu CH_2CH), 1.37 (s, 9H, Boc), 1.80–2.20 (m, 1H, Val CH), 2.4–2.7 (m, 2H, Lys εCH_2), 3.59 (s, 3H, OCH_3), 3.6–4.0 (m, 1H, αCH), 4.20–4.50 (m, 2H, αCH). Calcd for $C_{23}H_{44}N_4O_6$: C 58.45, H 9.38, N 11.85; found: C 56.82, H 9.22, N 11.19.

N-tert-Butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucine methylester

Reaction of Boc-L-valyl-L-lysyl-L-leucine methylester (2 g, 4.23 mmol) and methylchloroformate (0.358 ml, 4.65 mmol) in $CHCl_3$ (25 ml) according to *Procedure C* and purification of the crude product by silica gel chromatography (CH_2Cl_2/*i*-PrOH 95:5) gave the pure product as a white solid which was triturated with Et_2O: 1.58 g (70%); mp: 155–157°C; $[\alpha]_D^{22}$ = –38° (1; $CHCl_3$); IR (KBr) main peaks at 3317, 3286, 2960, 1727, 1691, 1640, 1549, 1530, 1276, 1171 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.80–1.10 (m, 12H, Leu CH_3, Val CH_3), 1.30–1.80 (m, 9H, Lys $\beta\gamma\delta CH_2$, Leu CH_2CH), 1.40 (s, 9H, Boc), 1.90–2.30 (m, 1H, Val CH), 3.0–3.3 (m, 2H, Lys εCH_2), 3.67 and 3.73 (two s, 6H, Lys OCH_3, Leu OCH_3), 3.85–4.15 (m, 1H, αCH), 4.35–4.75 (m, 2H, αCH). Anal $C_{25}H_{46}N_4O_8$ (C, H, N).

N-tert-Butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucine methylester

Reaction of Boc-L-valyl-L-lysyl-L-leucine methylester (2 g, 4.23 mmol) with tosyl chloride (886 mg, 4.65 mmol) according to *Procedure C*, using $CHCl_3$ as the solvent, and purification of the crude product by silica gel chromatography (CH_2Cl_2-*i*-PrOH 95:5) gave the pure product as white crystals after trituration in the presence of Et_2O: 1.81 g (68%); mp: 111–113°C; $[\alpha]_D^{22}$ = –32° (1; $CHCl_3$); IR (KBr) main peaks at 3291, 2960, 1743, 1690, 1642, 1547, 1524, 1246, 1160 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.75–1.10 (m, 12H, Leu CH_3, Val CH_3), 1.35–1.90 (m, 9H, Lys $\beta\gamma\delta CH_2$, Leu CH_2CH), 1.40 (s, 9H, Boc), 1.90–2.20 (m, 1H, Val CH), 2.42 (s, 3H, Tos CH_3), 2.8–3.1 (m, 2H, Lys εCH_2), 3.72 (s, 3H, OCH_3), 3.9–4.2 (m, 1H, αCH), 4.40–4.75 (m, 2H, αCH), 7.37 (d, 2H, C_6H_4, J = 9.0 Hz), 7.83 (d, 2H, C_6H_4, J = 9.0 Hz). Anal $C_{30}H_{50}N_4O_8S$ (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine

A solution of Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucine methylester (2 g, 3.3 mmol) in EtOH (30 ml) and hydrazine hydrate (4.1 ml, 82.5 mmol) was kept at rt for 4 d under stirring. The crystalline *N-tert*-butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl hydrazine was collected by filtration, washed with cold EtOH and dried under vacuum: 1.79 g (89.5%). It was used without further purification. Reaction of *N*-Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl hydrazine (1.29 g, 2.13 mmol) with 4-hydroxybenzaldehyde (286 mg, 2.34 mmol) according to *Procedure A* gave 1-(*N*-Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzal) hydrazone as a colourless oil: 1.5 g (100%) which was used without further purification. Reduction of 1-(*N*-Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzal) hydrazone (2.99 g, 4.2 mmol) with $NaBH_3CN$ (660 mg, 10.5 mmol) according to *Procedure B* and purification of the crude material by silica gel chromatography (EtOAc/$CHCl_3$ 7:3) gave the 1-(N-Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine as a colourless oil: 1.80 g (78.5%); $[\alpha]_D^{22}$ = –50° (1; MeOH); IR (KBr) main peaks at 3279, 2959, 1692, 1641, 1515, 1247, 1170 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.7–1.0 (m, 12H, Leu CH_3, Val CH_3), 1.25–1.80 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.37 (s, 9H, Boc), 1.8–2.2 (m, 1H, Val CH), 2.85–3.20 (m, 2H, Lys εCH_2), 3.6–4.0 (m, 1H, αCH), 3.73 (s, 2H, $C_6H_4CH_2$), 4.1–4.5 (m, 2H, αCH), 5.04 (s, 2H, Z CH_2), 6.76 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.17 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.37 (s, 5H, Z C_6H_5). Anal $C_{37}H_{56}N_6O_8$ (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine

A solution of Boc-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucine methylester (1.38 g, 2.6 mmol) in EtOH (30 ml) and hydrazine hydrate (3.2 ml, 65 mmol) was kept at rt for 60 h under stirring. The crystalline *N-tert*-butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl hydrazine was collected by filtration, washed with cold EtOH and dried under vacuum: 1.38 g (100%). It was used without further purification.

Reaction of Boc-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl hydrazine (1.6 g, 3 mmol) with 4-hydroxybenzaldehyde (403 mg, 3.3 mmol) according to *Procedure A* gave 1-(*N-tert*-butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzal) hydrazone as a colourless oil: 1.9 g (100%) which was used without further purification. Reduction of 1-(Boc-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzal) hydrazone (1.92 g, 3 mmol) with $NaBH_3CN$ (471 mg, 7.5 mmol) according to *Procedure B*, followed by hydrolysis of the cyanoborane adduct with 2 N Na_2CO_3 (5 ml) in place of NaOH and purification by silica gel chromatography (EtOAc/$CHCl_3$ 7:3) gave 1-(*N-tert*-butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine as a colourless oil: 1.31 g (68%); $[\alpha]_D^{22} = -56°$ (1; MeOH); IR (KBr) main peaks at 3290, 2959, 1693, 1639, 1517, 1248, 1172 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.70–0.95 (m, 12H, Leu CH_3, Val CH_3), 1.20–1.60 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.37 (s, 9H, Boc), 1.75–2.20 (m, 1H, Val CH), 2.80–3.20 (m, 2H, Lys εCH_2), 3.53 (s, 3H, Lys OCH_3), 3.7–4.0 (bs, 3H, $C_6H_4CH_2$, αCH), 4.10–4.45 (m, 2H, αCH), 6.73 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.18 (d, 2H, C_6H_4OH, J = 9.0 Hz). Anal $C_{31}H_{52}N_6O_8$ (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine

A solution of Boc-L-valyl-N_ε-tosyl-L-lysyl-L-leucine methyl-ester (1.63 g, 2.6 mmol) in EtOH (30 ml) and hydrazine hydrate (3.2 ml, 65 mmol) was kept at rt for 60 h. After removal of the solvent at reduced pressure, the residue was dissolved in $CHCl_3$ and washed with brine. Drying over Na_2SO_4 and removal of the solvent at reduced pressure gave *N-tert*-butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl hydrazine as a colourless oil: 1.63 g (100%) which was used without further purification. Reaction of Boc-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl hydrazine (1.45 g, 2.31 mmol) with 4-hydroxybenzaldehyde (310 mg, 2.44 mmol) according to *Procedure A* gave 1-(*N-tert*-butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-idroxybenzal) hydrazone as a colourless oil: 1.65 g (100%). Reduction of 1-(Boc-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzal) hydrazone (1.69 g, 2.3 mmol) with $NaBH_3CN$ (361 mg, 5.75 mmol) according to *Procedure B* and purification of the crude product by silica gel chromatography (EtOAc/$CHCl_3$ 7:3) gave the 1-(*N-tert*-butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine as a colourless oil: 1.31 g (77.5%); $[\alpha]_D^{22} = -47°$ (1; MeOH); IR (KBr) main peaks at 3285, 2960, 1688, 1641, 1515, 1246, 1159 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.65–1.00 (m, 12H, Leu CH_3, Val CH_3), 1.20–1.60 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.32 (s, 9H, Boc), 1.8–2.1 (m, 1H, Val CH), 2.34 (s, 3H, Tos CH_3), 2.50–2.85 (m, 2H, Lys εCH_2), 3.60–3.90 (bs, 3H, $C_6H_4CH_2$, αCH), 4.10–4.45 (m, 2H, αCH), 6.70 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.13 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.40 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz), 7.72 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz). Anal $C_{36}H_{56}N_6O_8S{\cdot}H_2O$ (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-chloroacetyl hydrazine ***6a***

Reaction of 1-(Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (200 mg, 0.28 mmol) with chloroacetyl chloride (38.5 mg, 0.34 mmol) according to *Procedure C* and purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1) gave the pure product as a colourless oil: 170 mg (77%); $[\alpha]_D^{22} = -32°$ (1; $CHCl_3$); IR (KBr) main peaks at 3289, 2960, 1679, 1642, 1515, 1247, 1171 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.65–1.00 (m, 12H, Leu CH_3, Val CH_3), 1.15–1.65 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.35 (s, 9H, Boc), 1.80–2.20 (m, 1H, Val CH), 2.80–3.20 (m, 2H, Lys εCH_2), 3.70–4.00 (m, 1H, αCH), 4.00–4.55 (bs, 5H, $C_6H_4CH_2$, CH_2Cl, αCH), 4.70–5.00 (m, 1H, αCH), 5.02 (s, 2H, Z CH_2), 6.75 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.10 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.38 (s, 5H, Z C_6H_5). Anal $C_{39}H_{57}N_6O_9Cl$ (C, H, N, Cl).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-bromoacetyl hydrazine ***6b***

Reaction of 1-(Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (200 mg, 0.28 mmol) with bromoacetyl chloride (53 mg, 0.34 mmol) according to *Procedure C* gave the pure product as a colourless oil: 233 mg (100%); $[\alpha]_D^{22} = -28°$ (1; $CHCl_3$); IR (KBr) main peaks at 3285, 2962, 1692, 1641, 1514, 1248, 1171 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.65–1.00 (m, 12H, Leu CH_3, Val CH_3), 1.10–1.60 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.35 (s, 9H, Boc), 1.80–2.10 (m, 1H, Val CH), 2.80–3.10 (m, 2H, Lys εCH_2), 3.60–4.50 (bs, 6H, $C_6H_4CH_2$, CH_2Br, αCH), 4.75–5.00 (m, 1H, αCH), 5.02 (s, 2H, Z CH_2), 6.72 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.09 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.38 (s, 5H, Z C_6H_5); ^{13}C-NMR (DMSO-d_6) δ 18.16 and 19.29 (Val CH_3), 21.63 and 22.83 (Leu CH_3), 22.55 (Lys γCH_2), 24.17 (Leu γCH), 28.11 (CH_2Br), 28.38 (CMe_3), 29.29 (Lys βCH_2), 30.43 (Val βCH), 32.04 (Lys δCH_2), 38.75–40.43 (Lys εCH_2 and Leu βCH_2, obscured by DMSO), 49.83, 51.97 and 59.88 (αCH), 50.40 ($C_6H_4CH_2$), 65.20 (Z CH_2), 78.11 (CMe_3), 114.94, 115.26, 127.82, 128.41, 130.06, 130.30, 137.35 and 155.48 (Ar), 156.10, 157.07, 169.60, 171.15, 171.35 and 171.96 (C=O). Anal $C_{39}H_{57}N_6O_9Br$ (C, H, N, Br).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-iodoacetyl hydrazine ***6c***

Reaction of 1-(Boc-L-valyl-N_ε-benzyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (200 mg, 0.28 mmol) with iodoacetyl chloride (69.5 mg, 0.34 mmol) according to *Procedure C* and purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1) gave the pure product as a colourless oil: 150 mg (61%); $[\alpha]_D^{22} = -27°$ (1; $CHCl_3$); IR (KBr) main peaks at 3288, 2961, 1691, 1641, 1514, 1249, 1169 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.65–1.00 (m, 12H, Leu CH_3, Val CH_3), 1.20–1.65 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.34 (s, 9H, Boc), 1.80–2.15 (m, 1H, Val CH), 2.80–3.15 (m, 2H, Lys εCH_2), 3.60–4.00 (m, 4H, $C_6H_4CH_2$, CH_2I), 4.1–4.5 (m, 2H, αCH), 4.75–5.00 (m, 1H, αCH), 5.02 (s, 2H, Z CH_2), 6.72 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.07 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.38 (s, 5H, Z C_6H_5). Anal $C_{39}H_{57}N_6O_9I$ (C, H, N, I).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-chloroacetyl hydrazine ***7a***

Reaction of 1-(Boc-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (197 mg, 0.31 mmol) wilh chloroacetyl chloride (42 mg, 0.37 mmol) according to *Procedure C* and purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1) gave the pure product as a colourless oil: 176 mg (80%); $[\alpha]_D^{22} = -26°$ (1; $CHCl_3$); IR (KBr) main peaks at 3287, 2962, 1687, 1641, 1515, 1249, 1171 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ 0.60–0.95 (m, 12H, Leu CH_3, Val CH_3), 1.10–1.60 (m, 9H, Leu CH_2CH, Lys $\beta\gamma\delta CH_2$), 1.34 (s, 9H, Boc), 1.80–2.15 (m, 1H, Val CH), 2.80–3.10 (m, 2H, Lys εCH_2), 3.50 (s, 3H, Lys OCH_3), 3.65–4.00 (m, 1H, αCH), 4.00–4.45 (m, 5H, $C_6H_4CH_2$, CH_2Cl, αCH), 4.70–5.10

(m, 1H, αCH), 6.74 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.10 (d, 2H, C_6H_4OH, J = 9.0 Hz). Anal $C_{33}H_{53}N_6O_9Cl$ (C, H, N, Cl).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-bromoacetyl hydrazine ***7b***
Reaction of 1-(Boc-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (197 mg, 0.31 mmol) with bromoacetyl chloride (58 mg, 0.37 mmol) according to *Procedure C*, purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1), and crystallization from CH_2Cl_2 gave the pure product as white crystals: 197 mg (84%); mp: 117–119°C; $[\alpha]_D^{22} = -26°$ (1; $CHCl_3$); IR (KBr) main peaks at 3291, 2960, 1688, 1641, 1516, 1248, 1171 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–1.05 (m, 12H, Leu CH_3, Val CH_3), 1.10–1.80 (m, 9H, Leu CH_2CH, Lys βγδCH_2), 1.37 (s, 9H, Boc), 1.85–2.20 (m, 1H, Val CH), 2.75–3.15 (m, 2H, Lys εCH_2), 3.52 (s, 3H, Lys OCH_3), 3.75–4.50 (bs, 6H, $C_6H_4CH_2$, CH_2Br, αCH), 4.60–5.10 (m, 1H, αCH), 6.72 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.08 (d, 2H, C_6H_4OH, J = 9.0 Hz); ^{13}C-NMR (DMSO–d_6) δ 17.91 and 19.01 (Val CH_3), 21.36 and 22.25 (Leu CH_3), 22.54 (Lys γCH_2), 23.87 (Leu γCH), 27.80 (CH_2Br), 27.98 (CMe_3), 29.01 (Lys βCH_2), 30.17 (Val βCH), 31.77 (Lys δCH_2), 39.97 (Leu βCH_2), 40.20 (Lys εCH_2), 49.65 (CH_3O), 50.12 ($C_6H_4CH_2$), 50.92, 51.72 and 59.61 (αCH), 77.89 (CMe_3), 114.85, 127.99, 129.95, 155.24 (Ar), 156.44, 156.80, 169.87, 170.95, 171.10 and 171.71 (C=O). Anal $C_{33}H_{53}N_6O_9Br$ (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-iodoacetyl hydrazine ***7c***
Reaction of 1-(Boc-L-valyl-N_ε-methyloxycarbonyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (140 mg, 0.22 mmol) with iodoacetyl chloride (53 mg, 0.26 mmol) according to *Procedure C* and purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1) gave the pure product as a colourless oil: 155 mg (88%); $[\alpha]_D^{22} = -30°$ (1; $CHCl_3$); IR (KBr) main peaks at 3285, 2960, 1692, 1641, 1515, 1249, 1170 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–1.10 (m, 12H, Leu CH_3, Val CH_3), 1.20–1.70 (m, 9H, Leu CH_2CH, Lys βγδCH_2), 1.36 (s, 9H, Boc), 1.90–2.20 (m, 1H, Val CH), 2.80–3.10 (m, 2H, Lys εCH_2), 3.53 (s, 3H, Lys OCH_3), 3.68–4.00 (m, 3H, CH_2I, αCH), 4.10–4.55 (bs, 3H, $C_6H_4CH_2$, αCH), 4.70–5.10 (m, 1H, αCH), 6.72 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.10 (d, 2H, C_6H_4OH, J = 9.0 Hz). Anal $C_{33}H_{53}N_6O_9I$ (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-chloroacetyl hydrazine ***8a***
Reaction of 1-(Boc-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (198 mg, 0.27 mmol) with chloroacetyl chloride (37 mg, 0.33 mmol) according to *Procedure C* and purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1) gave the pure product as a colourless oil: 177 mg (80%); $[\alpha]_D^{22} = -17°$ (1; $CHCl_3$); IR (KBr) main peaks at 3276, 2961, 1643, 1516, 1247, 1158 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–0.95 (m, 12H, Leu CH_3, Val CH_3), 1.20–1.60 (m, 9H, Leu CH_2CH, Lys βγδCH_2), 1.34 (s, 9H, Boc), 1.80–2.10 (m, 1H, Val CH), 2.38 (s, 3H, Tos CH_3), 2.60–2.85 (m, 2H, Lys εCH_2), 3.65–3.95 (m, 1H, αCH), 4.0–4.4 (bs, 5H, $C_6H_4CH_2$, CH_2Cl, αCH), 4.60–4.90 (m, 1H, αCH), 6.73 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.10 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.43 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz), 7.75 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz). Anal $C_{38}H_{57}N_6O_9S$ Cl (C, H, N).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-bromoacetyl hydrazine ***8b***
Reaction of 1-(Boc-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (198 mg, 0.27 mmol) with bromoacetyl chloride (52 mg, 0.33 mmol) according to *Procedure C* gave the pure product as a colourless oil: 230 mg (100%); $[\alpha]_D^{22} = -21°$ (1; $CHCl_3$); IR (KBr) main peaks at 3289, 2962, 1642, 1514, 1246, 1158 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–0.95 (m, 12H, Leu CH_3, Val CH_3), 1.10–1.60 (m, 9H, Leu CH_2CH, Lys βγδCH_2), 1.33 (s, 9H, Boc), 1.80–2.20 (m, 1H, Val CH), 2.37 (s, 3H, Tos CH_3), 2.55–2.82 (m, 2H, Lys εCH_2), 3.55–4.40 (bs, 6H, $C_6H_4CH_2$, CH_2Br, αCH), 4.60–4.90 (m, 1H, αCH), 6.74 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.10 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.43 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz), 7.75 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz); ^{13}C-NMR (DMSO–d_6) δ 18.22 and 19.29 (Val CH_3), 21.65 and 22.78 (Leu CH_3), 22.58 (Lys γCH_2), 24.02 (Leu γCH), 27.85 (CH_2Br), 28.12 (CMe_3), 28.93 Lys βCH_2), 30.55 (Val βCH), 31.95 (Lys δCH_2), 38.76–40.43 (Leu βCH_2 and Lys εCH_2, obscured by DMSO), 42.65 (Tos CH_3), 49.91 ($C_6H_4CH_2$), 50.06, 51.86 and 59.89 (αCH), 78.11 (CMe_3), 114.98, 115.31, 126.72, 129.67, 130.12, 130.35, 137.65, 142.55 and 155.50 (Ar), 157.11, 162.07, 171.08, 171.38 and 171.91 (C=O). Anal $C_{38}H_{57}N_6O_9SBr$ (C, H, N, Br).

1-(N-tert-Butyloxycarbonyl-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl)-2-iodoacetyl hydrazine ***8c***
Reaction of 1-(Boc-L-valyl-N_ε-tosyl-L-lysyl-L-leucyl)-2-(4-hydroxybenzyl) hydrazine (198 mg, 0.27 mmol) with iodoacetyl chloride (67 mg, 0.33 mmol) according to *Procedure C* and purification of the crude material by silica gel chromatography ($CHCl_3$/EtOAc 1:1) gave the pure product as a colourless oil: 183 mg (75%); $[\alpha]_D^{22} = -17°$ (1; $CHCl_3$); IR (KBr) main peaks at 3282, 2962, 1642, 1515, 1248, 1158 cm^{-1}; ^{1}H-NMR (DMSO–d_6) δ 0.70–0.95 (m, 12H, Leu CH_3, Val CH_3), 1.10–1.70 (m, 9H, Leu CH_2CH, Lys βγδCH_2), 1.36 (s, 9H, Boc), 1.80–2.00 (m, 1H, Val CH), 2.37 (s, 3H, Tos CH_3), 2.55–2.70 (m, 2H, Lys εCH_2), 3.60–3.95 (m, 3H, CH_2I, αCH), 4.00–4.35 (m, 3H, $C_6H_4CH_2$, αCH), 4.70–4.90 (m, 1H, αCH), 6.71 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.02 (d, 2H, C_6H_4OH, J = 9.0 Hz), 7.37 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz), 7.65 (d, 2H, $C_6H_4SO_2$, J = 9.0 Hz). Anal $C_{38}H_{57}N_6O_9SI$ (C, H, N).

Cathepsin B 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 incubating the enzyme for 45 min at rt in 50 mM phosphate buffer, pH 6.8, containing 2 mM EDTA and 0.5 mM cysteine, according to Thompson [26], with minor modifications. Approximate enzyme concentrations were determined from the absorbance at 280 nm ($E^{1\%}$ = 20 cm^{-1}) [27]. The activated enzyme solution was stored at 4°C and fully retained its activity for at least 10 h. Reagents were from Sigma unless otherwise indicated. Buffers and solutions were made with ultra high-quality water (Elga UHQ).

The rate of hydrolysis of the Z-Gly-ONp was monitored continuously at 405 nm [28] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. In the reference cell was placed a cuvette containing everything but enzyme 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. Reactions were started by addition of the enzyme solution to the cuvette. The computerized spectrophotometer continuously stored absorbances *versus* time data. These data pairs were transferred to a personal computer equipped with a mathematical coprocessor and fitted to equation [3] by Enzfitter, a non-linear regression data analysis program from Elsevier Biosoft (Cambridge) [29]. Progress curves were composed of 180 to 360 data pairs. A substrate concentration of 125 μM prevented substrate depletion during the assays.

Cathepsin B fluorimetric assay was performed using 25 μM of Z-Phe-Arg-NHMec as the substrate. Reaction progress curves were followed continuously for 20 min by monitoring the fluorescence of aminomethylcoumarin in a SFM-25 Kontron fluorimeter with excitation set at 380 nm and emission at 460 nm. Final concentration of acetonitrile in the cuvette was 7% (v/v) in 100 mM phosphate buffer, pH 6.8, 4 mM EDTA and Brij 35 0.01%. Enzyme concentration was at least 1:100 with respect to inhibitor.

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, Kioto, 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–Cl 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 analysed as described for cathepsin B. Software for fluorimeter progress curves recording was from Kontron. Calpains were 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 [5].

Chymotrypsin and trypsin assay

Chymotrypsin EC 3.4.21.1 from bovine pancreas, product No 27270 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}) [30]. Chymotrypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester at 256 nm [31]. 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 [32]. Trypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-arginine ethyl ester at 253 nm [33]. Compounds **1a**, **1b**, **1c**, **3c**, **4c** and **5c** following 5 h incubation at the maximal concentration of 100–400 μM did not inhibit their activity.

Porcine pancreatic elastase assay

Porcin pancreatic elastase EC 3.4.21.11, product No E-0127, was obtained 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 [34]. Activity was monitored by following the hydrolysis of Boc-L-Ala-ONp at 347 nm [35] in 50 mM phosphate buffer, pH 7.0 at 25°C. No inactivation was observed following 5 h incubation with compounds **1a**, **1b**, **1c**, **3c**, **4c** and **5c** under the conditions described for the other serine proteases.

Alkylation of glutathione

The rate of alkylation of glutathione was measured following the thiol disappearance by using Ellman reagent [24]. Observations were made in phosphate buffer at pH 6.8 and 25°C. Concentration of organic solvent and all other conditions were similar to that employed in the experiments for enzyme inactivations. Results are reported in table III.

Acknowledgments

Elemental microanalyses were performed by L Petrilli and F Dianetti, Servizio Microanalisi del CNR, Area della Ricerca di Roma, Montelibretti. This study was supported by the Italian CNR Progetto Finalizzato Chimica Fine II, the Italian MURST and Telethon.

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