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Peptidyl and azapeptidyl methylketones as substrate analog inhibitors of papain and cathepsin B

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Summary — Peptidyl methylketones containing Phe, Tyr, Tyr(I), Tyr(I_2), Leu and Ile in P_2 were synthesized and tested as substrate analog reversible inhibitors of papain and bovine spleen cathepsin B. The most effective cathepsin B inhibitor contained Tyr(I_2) and displayed an inhibition constant of 4.7 μM at pH 6.8 and 25°C, while Leu or Ile gave practically inert analogs. Replacement of the amino acids in P_2 with the analogous α-azaamino acids, as well as the glycine in P_1 with α-azaglycine, led to complete loss of inhibiting activity. Introducing alkoxy substituents at the methyl adjacent to the ketone group generally resulted in more effective inhibitors, with inhibition constants in the micromolar range for both papain and cathepsin B.

enzyme inhibiting activity / cysteine protease / slow binding / peptidyl methylketone / azapeptidyl methylketone / papain / cathepsin B

Introduction

Cysteine proteases play important roles in numerous physiological processes in the cells of mammalian organisms [1] and imbalances in their regulation contribute to several pathological effects. In particular, cathepsin B has been implicated in various disease states such as inflammation [2, 3], tumour metastasis [4, 5], myocardial tissue damage [6], osteoclastic bone resorption [7, 8], and muscular dystrophy [9, 10]. Potent and selective, low molecular weight synthetic inhibitors of cathepsin B are thus of significant interest for *in vivo* biochemical investigations and development of potential therapeutical agents [11, 12].

The amino acid sequence, overall folding and catalytic mechanism of cathepsin B are closely related to those of papain and the other proteases of the papain family. These enzymes utilize a thiolate–imidazolium ion pair for their activity. Substrate analogue peptidyl aldehydes and peptidyl ketones form stable hemithioacetal and hemithioketal tetrahedral adducts incorporating the catalytic thiolate of the enzyme. Both naturally occurring [13] and synthetic [14, 15] peptidyl aldehydes were reported as powerful inhibitors of cysteine proteases. Experimental evidence for covalent tetrahedral complex formation was achieved by ^{13}C-NMR spectroscopy [16–18] and confirmed by X-ray crystallographic resolution of the papain–leupeptin complex [19].

The poor stability of the aldehyde functionality represents, however, a serious limitation for the *in vivo* use of peptidyl aldehydes. The more stable peptidyl methylketones have been studied and showed binding affinities 1500–3000-fold lower than that of the corresponding aldehydes [16], in accordance with the lower stability of ketone hemithioketals. Variation of the peptide chain length and of the amino acids in the P_1-P_4 positions [20] led to inhibitors displaying K_i in the micromolar range for papain and cathepsin B, with little or no effect on serine proteases. Contrary to serine proteases [20], replacement of the methyl with a trifluoromethyl group [22, 23] in peptidyl methylketone inhibitors has been reported to cause about a 10-fold decrease in their effectiveness. Only a small increase was observed when methyl was replaced by a trichloromethyl group [23].

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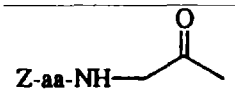
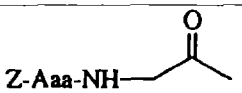
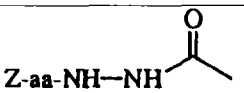
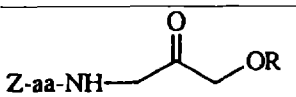
Table I. Types of structural variations applied to peptidyl methylketones.

Peptidyl ketones		*Peptidyl ketone analogs with α-azaamino acid in P_2*		*Peptidyl ketone analogs with α-azaamino acid in P_1*		*Peptidyl ketone analogs with structural variations in P'_1, P'_2*		
								
No	aa	No	Aaa	No	aa	No	aa	R
1a	Phe	**2a**	Aphe	**3a**	Phe	**4**	Phe	H
1b	Tyr	**2b**	Atyr	**3d**	Tyr(I_2)	**5**	Phe	CH_3
1c	Tyr(I)	**2c**	Atyr(I)			**6**	Phe	CH_2-O-CH_2-O-CH_2-Ph
1d	Tyr(I_2)	**2d**	Atyr(I_2)					
1e	Leu	**2e**	Aleu					
1f	Ile	**2f**	Aile					

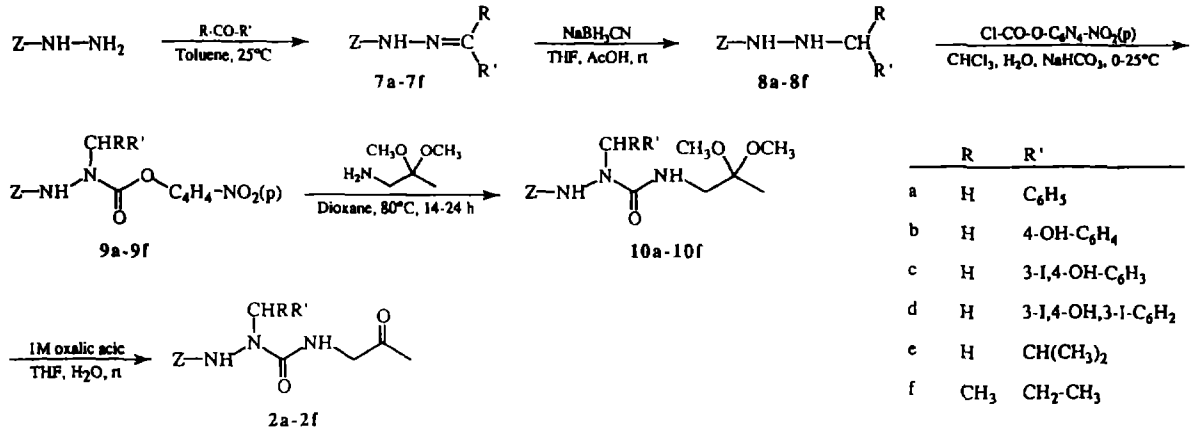
The peptidyl methylketones studied in the present paper contain variations at either the peptidyl affinity chain or the methyl group. The selection of amino acids in P_2 in the analogs **1a–f** (table I) reflects the strong preference of papain and cathepsin B for hydrophobic residues at the site of primary specificity S_2 [1]. Monoiodo and diiodo tyrosine present bulkier and more hydrophobic side chains than phenylalanine and tyrosine and could be useful when preparing radiolabeled inactivators. Further variation in the affinity unit includes replacement of the peptidyl with the corresponding azapeptidyl chain. Azapeptides [24] are peptide analogs where the α-CH group of one or more amino acid residues has been replaced by a nitrogen atom. The basic structure of a bioactive peptide is not substantially affected by this backbone modification, while receptor or enzyme interactions can be either significantly increased or diminished as a consequence of geometrical and electronic effects at the site of substitution. Generally, prediction of the results is difficult, since it would require precise evaluation of the physico-chemical variations caused by the α-CH/N replacement on binding. In order to assess these effects on the interactions between peptidyl methylketones and cysteine proteases, compounds **2a–f** containing an α-azaamino acid in P_2 and **3a–d**, with an α-azaglycine unit in P_1 position, have been synthesized.

Hydroxy or alkoxy groups were introduced at the methyl in **4–6** with the aim of increasing the nucleophilicity of the adjacent carbonyl carbon and allowing possible bonding interactions at the S'_1 and S'_2 subsites.

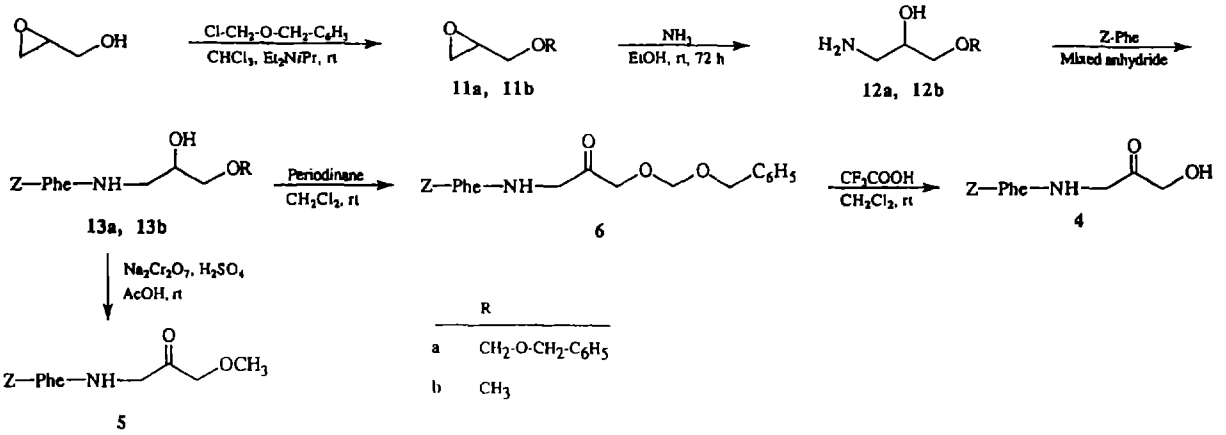
Chemistry

Peptidyl methylketones **1a–f** were prepared by mixed anhydride [25] acylation of 1-amino-2,2-dimethoxypropane with the required *N*-benzyloxycarbonylamino acid, followed by mild acid hydrolysis of the intermediate peptidyl dimethylketals. The α-azaanalogs **2a–f** (scheme 1) were obtained by condensation of the appropriate aldehyde with benzyl carbazate followed by reduction of the resulting hydrazones **7a–f** with sodium cyanoborohydride under acid conditions [26]. Acylation of the benzyloxycarbonyl hydrazines **8a–f** with *p*-nitrophenyl chloroformate gave the expected hydrazine dicarboxylic acids 1-benzylester, 2-*p*-nitrophenylesters **9a–f**, which were coupled with 1-amino-2,2-dimethoxypropane. Mild acid hydrolysis of the corresponding dimethylketals **10a–f** gave the required *N*-benzyloxycarbonyl-α-azaaminoacyl-1-aminopropan-2-ones **2a–f**.

The 3-methoxypropan-2-one **5** was obtained (scheme 2) by mixed anhydride coupling of *N*-benzyl-



Scheme 1.



Scheme 2.

oxycarbonyl-L-phenylalanine with 1-amino-2-hydroxy-3-methoxypropane **11b** followed by oxidation with $Na_2Cr_2O_7/H_2SO_4$ in acetic acid [27].

The 3-hydroxypropan-2-one **4** was prepared according to a similar route, provided that the 3-hydroxy group is adequately protected during the oxidation step. Glycidylbenzyloxymethyl ether **11a** was converted into the amine **12a** by epoxide opening with a saturated solution of ammonia in anhydrous ethanol, followed by mixed anhydride coupling with *N*-benzyloxycarbonyl-L-phenylalanine and oxidation with periodinane [28] to give the intermediate peptidyl ketone **6**. Removal of the protective benzyloxymethylene group, performed with 5% trifluoroacetic acid in dichloromethane, gave the *N*-benzyloxycarbonyl-L-phenylalanyl-1-amino-3-hydroxypropane-2-one **4**.

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

Enzyme inhibition measurements

Inhibition of papain and cathepsin B catalyzed hydrolysis of *N*-carbobenzyloxyglycine *p*-nitrophenylester (Z-Gly-ONp) was time dependent and the final steady-state velocity was a function of inhibitor concentration.

The observed decrease in the hydrolysis of Z-Gly-ONp was not due to irreversible inactivation or substrate depletion, but to an onset of activity loss

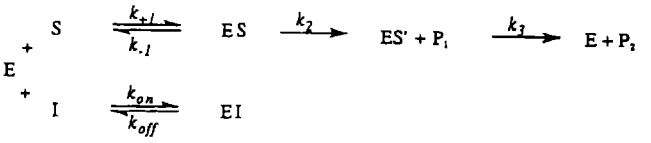
slower than the rate of diffusion, which is 'apparent' slow binding inhibition [29–31]. A quantitative description of this type of inhibition is possible if the substrate S is not depleted during the reaction and if the formation of the enzyme–inhibitor complex E·I does not change the inhibitor initial concentration [I] [32, 33]. For our competitive inhibitors, scheme 3 may be sufficient to describe the onset of inhibition, and equations [1] and [2] are valid:

$$K_i = k_{off}/k_{on} \quad [1]$$

$$k_{obs} = k_{on}[I] / (1 + [S] / K_m) + k_{off} \quad [2]$$

Equation [2] yields k_{obs}, the observed rate constant for the approach to steady-state, and a plot of experimentally determined k_{obs} *vs* [I] K_m / (K_m + [S]) results in a straight line whose slope and intercept are k_{on} and k_{off}, respectively. All inhibitors, tested under the same conditions, showed analogous behavior and are expected to follow the same putative mechanism. The results are reported in table II.

The observed linear dependence of k_{obs} from [I] K_m / (K_m + [S]) and the fact that initial velocities in the presence of the inhibitor are identical to those measured in its absence are in agreement with this simple minimal mechanism. If an intermediate E·I' complex intervenes in one of the steps prior to the formation of the stable E·I adduct, then k_{obs} would be a hyperbolic function of [I] and initial velocities would decrease with increasing [I] [30]. These data are not definitive, however, against the hypothesis that a preassociation complex E·I' is formed, because this complex may simply not be detectable under our experimental conditions. The most efficient cathepsin B inhibitors **1c**, **1d**, **5** and **6** showed inertness toward representative serine-proteases like trypsin, chymotrypsin and porcine pancreatic elastase.



Scheme 3. Minimal reaction mechanism for the inhibition of thiol proteases by substrate analog peptidyl methylketones in the presence of substrate. The second-order rate constant for the formation of E·I is represented by k_{on}; k_{off} is the first-order rate constant for its dissociation.

Results and discussion

Papain and cathepsin B are characterized by a large hydrophobic cleft in S_2, which represents the most important subsite for their specificity [1]. The amino acid in P_2 is therefore primarily responsible for the initial binding of the peptide component of the inhibitory ligand in the specificity sites, positioning the CO carbonyl correctly for the covalent reaction with the

Table II. Papain and cathepsin B inhibition[a] by peptidyl methylketones.

Inhibitor	*Papain*				*Cathepsin B*			
	Range[b] *(μM)*	k_{on} *(M^{-1} min^{-1})*	k_{off} *(min^{-1})*	K_i *(μM)*	*Range (μM)*	k_{on} *(M^{-1} min^{-1})*	k_{off} *(min^{-1})*	K_i *(μM)*
1a	170–1300	1240	0.05	40.3	40–120	2540	0.09	35.4
1b	1000[c]	–	–	ni[d]	1000[c]	–	–	high[c]
1c	10–50	1340	0.03	22.4	45–500	2250	0.07	31.1
1d	50[c]	–	–	ni	9–50	10 700	0.05	4.7
1e	1000[c]	–	–	ni	1000[c]	–	–	high
1f	1000[c]	–	–	ni	1000[c]	–	–	high
2a-2f	1000	–	–	ni	1000	–	–	ni
3a	1000	–	–	ni	1000	–	–	ni
3d	85[c]	–	–	ni	85[c]	–	–	ni
4	200–1000	2140	0.03	14.0	50–290	742	0.06	80.9
5	190–550	2870	0.02	7.0	20–250	2260	0.07	31.0
6	10–50	12300	0.07	5.6	7–40	11 900	0.04	3.4

[a]Conditions were: 100 mM phosphate buffer, pH 6.8, at 25°C; [E] = 7 x 10^{-9} M; [S] = 125 μM; CH_3CN = 12% (v/v); standard deviation for replicate determinations of kinetic parameters were less than 20%; [b]range of inhibitor concentration; [c]maximum concentration available in the test conditions; [d]no significant inhibition observed; [e]the constant final velocity v_s was 30% lower than the initial velocity v_0, at the reported concentration.

catalytic thiolate of the enzyme. Other important interactions include P_1NH-OC(Asp-158), P_2CO-HN(Gly-66) and P_2NH-OC(Gly-66) hydrogen bonds (papain numbering, fig 1).

The peptidyl methylketone **1a** containing Phe in P_2 (table II) displays practically identical K_i values for papain and cathepsin B binding. Smaller and less lipophilic *i*-butyl and *s*-butyl side chains in P_2 strongly decrease the affinity of the inhibitors **1e** and **1f** for cathepsin B and even more for papain. This result is in accordance with the previous experience of Hanzlick *et al*, who found a 10-fold drop of papain affinity for peptidylaldehydes [15] and nitriles [35] by varying the side chain in P_2 from benzyl to *i*-butyl or methylthioethyl. Increasing the polarity of the P_2 side chain from benzyl (**1a**) to 4-hydroxybenzyl (**1b**) caused a similar decrease of the binding strength for both the enzymes. Increasing the size and lipophilicity of the chain from 4-hydroxybenzyl **1b** to 3-I-4-hydroxybenzyl **1c** led to more effective inhibitors for both the enzymes. In contrast, introducing the second I atom in 3,5-diiodo-4-hydroxybenzyl **1d** caused a further increase of binding affinity for cathepsin B and complete loss of observable inhibition for papain. Recent determination of X-ray crystal structure of human liver cathepsin B [36] allows a tentative rationalization of this result if we assume that a close resemblance between the active sites of the human liver and bovine spleen enzymes exists. The presumed S_2 subsite of cathepsin B has a similar construction, but is larger than that of papain. Thus, the 3,5-diiodo-hydroxybenzyl group of Tyr(I_2) can still be accommodated at the S_2 subsite of cathepsin B, but probably suffers serious steric strain at the S_2 papain subsite.

Methylketones **2a–f** were completely inactive as inhibitors of papain and cathepsin B (table II). They are characterized by the α-N atom of the azaamino acid in P_2. Delocalization of its electron pair leads to three main consequences: 1) trigonal rather than tetrahedral geometry of the α-atom in P_2, with extension of the coplanarity of the normal P_2 peptide bond to P_2CH_2 and P_2NH; 2) restriction of the free rotation around the $P_2\alpha N$–P_2CO bond; and 3) redistribution of the amide charges.

The consequences of charge redistribution following C/N replacement can possibly be compared with those reported by Storer *et al* [37] for C/O replacement in peptide substrates. By studying hydrolysis rates of the methylester CH_3-O-CO-Phe-Gly-OCH_3 *versus* CH_3-CO-Phe-Gly-OCH_3, they calculated a loss of specificity of $\Delta\Delta G_{obs}$ = 0.7 kcal/mol following substitution of the acetamido with the carbamate group. Furthermore, by *ab initio* self consistent field calculations on a simpler model (fig 2), they postulated that H-bonds involving carbamate NH would be less favourable due to the partial negative charge developed on the nearby sp^3 oxygen. A relatively small decrease in the strength of the P_1NH-OC(Asp-158) may therefore be envisaged, assuming that an analogous effect may intervene by C/N replacement at the P_2 position (fig 2).

The extension of coplanarity at the P_2 site and restriction of the free rotation are both involved in the arrangement of the P_2 side chain. Taken together,

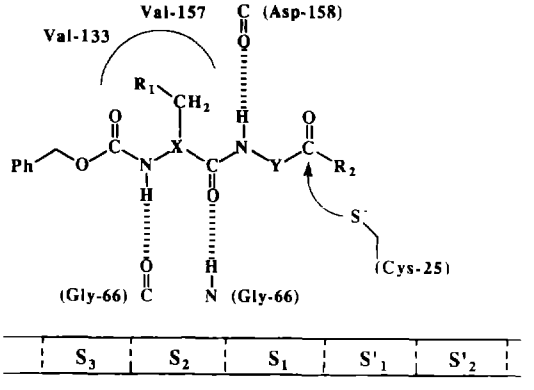


Fig 1. Schematic representation of binding of a peptidylketone inhibitor in the papain active site, as deduced from the X-ray crystallographic structure of the enzyme inhibited by Z-Phe-Gly-CH_2-Cl [34]. In the crystal complex, the side chain of the Phe residue is out of plane with its backbone atoms and establishes hydrophobic interactions with Val-133 and Val-157 residues in the S_2 subsite of the enzyme. The catalytic thiolate of Cys-25 and atomic groups of Gly-66 and Asp-158 intervening as hydrogen-bonding partners are also reported.

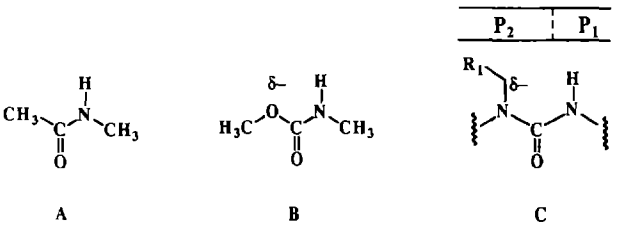


Fig 2. Electronic effects following C/O and C/N replacement in amides. *Ab initio* self consistent field calculations of the charge distribution [37] indicates that a partial negative charge on the sp^3 oxygen is the main difference between the electrostatic potential around the NH of a carbamate (**A**) with respect to an amide (**B**) model. This finding was used to assess why carbamates are worse H-bond donors than amides. An analogous effect may intervene in azapeptides as depicted in the partial structure **C** including an α-azaamino acid in P_2 and the weakened H-bond donor NH in P_1.

these two effects would require a considerable energetic cost to constrain the backbone and side chain of the α-azaamino acid in the same spatial arrangement as in the natural amino acid residue and to preserve the same bonding interactions in a hypothetical reversible adduct (fig 1, X = N, Y = CH_2). The consequent loss of cooperativity [37] between hydrogen bonding and hydrophobic interactions should significantly decrease the stability of the complex and is probably the main factor responsible for the loss of observable inhibition for peptidyl methylketones containing an α-azaamino acid in P_2. This hypothesis is in accordance with the high specificity of the S_2 subsite of papain as reported by Hanzlick *et al* [35]. They showed that inversion of the Phe chiral centre in Ac-L-Phe-NH-CH_2-CHO caused a 300-fold decrease in its binding affinity.

Introducing an α-azaamino acid residue (Agly) in P_1 led to the 1-peptidyl-2-acetyl hydrazines **3a** and **3d**, which proved to be completely inert as papain and cathepsin B inhibitors (table II). One reason for their inertness appears to be the amide rather than ketone nature of the P_1CO (fig 1, X = CH, Y = NH) and the consequent strong decrease in its tendency to add nucleophiles and to stabilize tetrahedral adducts [38], since development of a covalent bond at the P_1CO is considered an obligatory requirement for good substrate analog inhibitors for papain [35].

If one assumes that the acetyl CO group in **3a** and **3d** can be attacked by the Cys-25 thiolate, the structures **A** and **B** (fig 3) would represent the possible tetrahedral intermediates, in accordance with the mode of interaction of peptide aldehydes with papain [39]. In the mechanism [1] of papain-catalyzed hydrolysis of peptide substrates, the addition step leading to **C** is followed by the His-159-assisted cleavage of the CO-NH linkage, leading to the acyl-enzyme. In the putative adducts **A** and **B**, the NH group bound to the peptidyl affinity unit is oriented toward the S_1 subsite and the acylation step could hardly be assisted by His-159 catalysis. These circumstances seem to explain the inertness of 1-peptidyl-2-acetyl hydrazines **3a** and **3d** as substrates or substrate analogue inhibitors for cysteine proteases.

Various 1-peptidyl-2-acyl hydrazines (or azapeptide esters, Ac-Phe-NH-NH-CO-OR, R = methyl, *i*-butyl) have been reported by Abeles and Magrath [40] to inactivate papain by thiolate acylation. Second-order rate constants were in the range 13–18 M^{-1} s^{-1} at 37°C. In this case, their behaving as acylating substrate analogs cannot be excluded, since the acylation step could be assisted by the His-159 protonation of the alkoxylic leaving group, but an alternative isocyanate E1cb mechanism has also been proposed. The analog Ac-Phe-NH-N(CH_3)-CO-O*i*Bu, where the alternative mechanism is precluded, was reported to be completely inert. Restriction of the free rotation around the P_1αN-P_1CO bond and planarity of the P_1αN are among the arguments used to explain the failure of direct acylation. The same effects could also be involved in the loss of binding affinity of 1-peptidyl-2-acetyl hydrazines **3a** and **3d** for papain and cathepsin B.

Peptidyl (hydroxy) and (alkoxy)methylketones **4–6** were synthesized to investigate the effects of structural variations at the methyl group in P'_1 or P'_2. These peptidyl ketones were expected to form more stable thioacetal-type adducts E-I (fig 1, X = CH, Y = CH_2) as the electron-withdrawing effect of the oxygen makes the nearby carbonyl carbon more susceptible to nucleophilic attack. As shown in table II, an increase in the affinity for papain and a decrease in the affinity for cathepsin B is observed when the more polar hydroxy group is present (**4**). This suggests that, besides the electronic effect, different interactions of the hydroxyl group with the S'_1 subsite of the two enzymes might intervene in the stabilization of the enzyme–inhibitor complexes. Replacement of the hydroxy group with the less polar methoxy group (**5**) causes an increase of the affinity for both enzymes. A further increase, especially for cathepsin B, was obtained by introduction of a benzyloxymethoxy chain (**6**), suggesting possible hydrophobic interactions at the S'_2 subsite [41, 42].

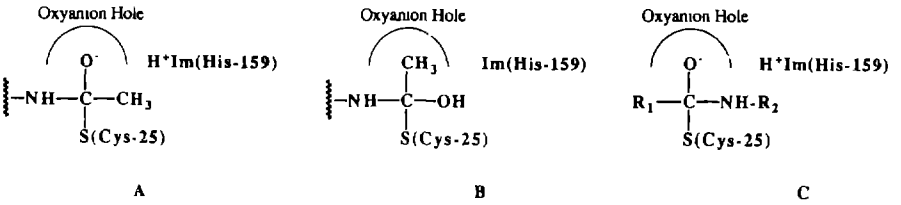


Fig 3. Postulated structures of tetrahedral intermediates for binding of 1-peptidyl-2 acetyl hydrazines **3a** and **3d** at the papain active site as substrate analogs (**A** and **B**) and for papain-catalyzed hydrolysis of peptidyl substrates (**C**). Protonation of the NH by His-159, which would facilitate cleavage of the amide linkage, can only easily intervene in the tetrahedral intermediate **C**.

In the peptidyl (acyloxy)methylketones (Z-Phe-Ala-CH_2-O-CO-Ar) studied by Krantz *et al* [43, 44], the oxygen atom is part of a carboxy rather than hydroxy or alkoxy group. These compounds behave as effective irreversible inhibitors by alkylation of the catalytic thiolate of cysteine proteases. The incorporation of the oxygen in a better leaving group may be one of the reasons for their different reactivity.

The present study indicates that incorporation of azaamino acid units in the P_1 or P_2 position of peptidyl methylketones causes complete loss of binding affinity for cysteine-proteases. Introducing Tyr(I_2) in P_2 or alkoxylic groups on the methylene α to the ketone carbonyl group led to more effective inhibitors of cathepsin B. Affinity can possibly be further increased by modifying the alkoxylic group to improve binding interactions at the S'_1, S'_2 subsites.

Experimental protocols

Chemistry

Melting points (Büchi oil-bath apparatus) are uncorrected. IR spectra were obtained with a Perkin-Elmer 521 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian EM 390 spectrometer using TMS as internal standard. The $[\alpha]_D$ were determined with a Schmidt-Haensch 1604 polarimeter. Analyses (Elemental analyzer Carlo Erba 1106) indicated by the symbols of the elements were within ±0.4% of theoretical values.

1-Amino-2,2-dimethoxypropane

The preparation of the dimethylketal was performed according to the method of Büchi *et al* [45]. A solution of 1-aminopropan-2-one hydrochloride (1.97 g, 18 mmol) and methyl orthoformate (2.29 g, 21.6 mmol) in anhydrous MeOH (20 ml) was refluxed for 24 h in the presence of *p*-toluenesulfonic acid monohydrate (342 mg, 1.8 mmol). The solvent was evaporated under reduced pressure, the residue was treated with ice-cooled 3 N NaOH and continuously extracted with ether overnight. After drying over anhydrous K_2CO_3, the solvent was removed under reduced pressure and the crude liquid product was purified by distillation under reduced pressure: 1.67g (78%); bp_{24} = 58°C; IR ($CHCl_3$) main peaks at 3391, 2990, 2956, 1601, 1461, 1379, 1100, 1046 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.28 (s, 3, CH_3CO), 2.75 (s, 2, CH_2N), 3.25 (s, 6, CH_3O).

*Preparation of N-benzyloxycarbonyl-L-aminoacyl-1-aminopropan-2-ones (**1a–f**). General procedure A*

A solution of the required *N*-benzyloxycarbonylamino acid (1 mmol) and *N*-methylmorpholine (1 mmol) in anhydrous THF (6.5 ml) was cooled to −15°C and *i*-butylchloroformate (1 mmol) was added dropwise, under stirring. After 30 min, a solution of 1-amino-2,2-dimethoxypropane (1 mmol) in THF (2 ml) was added slowly and the temperature of −15°C was maintained for 2 h. The reaction mixture was diluted with EtOAc and washed with brine, 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4 the solvent was removed under reduced pressure. The resulting *N*-benzyloxycarbonyl-L-aminoacyl-1-amino-2,2-dimethoxypropane was hydrolyzed by treatment with aqueous 1 M oxalic acid (2 ml) in THF (4 ml) at room temperature for 0.5–12 h. The reaction mixture was diluted with EtOAc and washed with saturated $NaHCO_3$ and brine. Drying over Na_2SO_4 and removal of the solvent under reduced pressure gave the crude product.

*N-Benzyloxycarbonyl-L-phenylalanyl-1-aminopropan-2-one **1a**.* Product **1a** was obtained from *N*-benzyloxycarbonyl-L-phenylalanine (1.23 g, 4.1 mmol) and 1-amino-2,2-dimethoxypropane (0.49 g, 4.1 mmol) according to *Procedure A*. The hydrolysis step required 2 h. The crude material was crystallized from THF: 1.27 g (87%); mp = 124–127°C; $[\alpha]_D^{22}$ = −25° (1, DMF); IR ($CHCl_3$) main peaks at 3305, 2925, 1722, 1690, 1640, 1534, 1285 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.05 (s, 3, CH_3CO), 2.70–3.22 (m, 2, PheCH$_2$), 3.96 (d, 2, CH_2N, *J* = 4.5 Hz), 4.17–4.53 (m, 1, αCH), 4.98 (s, 2, Z CH_2), 7.33 (s, 10, Ar). Anal $C_{20}H_{22}N_2O_4$ (C, H, N).

*N-Benzyloxycarbonyl-L-tyrosyl-1-aminopropan-2-one **1b**.* Product **1b** was obtained from *N*-benzyloxycarbonyl-L-tyrosine (1.86 g, 5.9 mmol) and 1-amino-2,2-dimethoxypropane (0.70 g, 5.9 mmol) according to *Procedure A*. The hydrolysis step required 12 h. Purification of the crude material by silica gel chromatography (slurry, $CHCl_3$/EtOAc 6:4) and crystallization from THF/$CHCl_3$ gave the pure product as white crystals: 2.11 g (97%); mp = 165–168°C; $[\alpha]_D^{22}$ = −26° (1, DMF); IR (KBr) main peaks at 3340, 3171, 1730, 1685, 1640, 1529, 1267 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 2.04 (s, 3, CH_3CO), 2.60–3.13 (m, 2, TyrCH$_2$), 3.93 (d, 2, CH_2N, *J* = 4.5 Hz), 4.10–4.43 (m, 1, αCH), 4.98 (s, 2, Z CH_2), 6.70 (d, 2, C_6H_4, *J* = 9.0 Hz), 7.14 (d, 2, C_6H_4, *J* = 9.0 Hz), 7.33 (s, 5, C_6H_5). Anal $C_{20}H_{22}N_2O_5$ (C, H, N).

*N-Benzyloxycarbonyl-3-iodo-L-tyrosyl-1-aminopropan-2-one **1c**.* *N*-Benzyloxycarbonyl-3-iodo-L-tyrosine (310 mg, 0.7 mmol) and 1-amino-2,2-dimethoxypropane (83 mg, 0.7 mmol) were reacted according to *Procedure A*. The hydrolysis step required 12 h. Crystallization of the crude material from MeOH gave the pure product as white crystals: 301 mg (87%); mp = 168–170°C; $[\alpha]_D^{22}$ = −11° (1, DMF); IR (KBr) main peaks at 3373, 3340, 3195, 1691, 1644, 1512, 1248 cm^{-1}. ^{1}H-NMR (d_6-DMSO) δ 2.04 (s, 3, CH_3CO), 2.56–3.08 (m, 2, TyrCH$_2$), 3.95 (d, 2, CH_2N, *J* = 4.5 Hz), 4.07–4.43 (m, 1, αCH), 4.98 (s, 2, ZCH_2), 6.82 (d, 1, C_6H_3, *J* = 9.0 Hz), 7.17 (dd, 1, C_6H_3, *J* = 9.0 Hz and 1.5 Hz), 7.35 (s, 5, C_6H_5), 7.72 (dd, 1, C_6H_3, *J* = 1.5 Hz). Anal $C_{20}H_{21}N_2O_5I$ (C, H, N).

*N-Benzyloxycarbonyl-3,5-diiodo-L-tyrosyl-1-aminopropan-2-one **1d**.* Product **1d** was obtained from *N*-benzyloxycarbonyl-3,5-diiodo-L-tyrosine (816 mg, 1.44 mmol) and 1-amino-2,2-dimethoxypropane (171 mg, 1.44 mmol) according to *Procedure A*. The hydrolysis step required 4 h. Purification of the crude material by silica gel chromatography (slurry, $CHCl_3$/EtOAc 95:5) and crystallization from THF/hexane gave the pure product as white crystals: 550 mg (62%); mp = 161–163°C; $[\alpha]_D^{22}$ = −9° (1, DMF); IR (KBr) main peaks at 3307, 3064, 2923, 1690, 1650, 1531, 1457, 1262 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 2.05 (s, 3, CH_3CO), 2.33–3.10 (m, 2, TyrCH$_2$), 3.96 (d, 2, CH_2N, *J* = 4.5 Hz), 4.08–4.42 (m, 1, αCH), 4.98 (s, 2, ZCH_2), 7.35 (s, 5, C_6H_5), 7.80 (s, 2, C_6H_2). Anal $C_{20}H_{20}N_2O_5I_2$ (C, H, N).

*N-Benzyloxycarbonyl-L-leucyl-1-aminopropan-2-one **1e**.* *N*-Benzyloxycarbonyl-L-leucine (1.06 g, 4.0 mmol) and 1-amino-2,2-dimethoxypropane (474 mg, 4 mmol) were reacted according to *Procedure A*. The hydrolysis step required 3 h. Crystallization of the crude material from THF/hexane gave the

pure product as white crystals: 830 mg (65%), mp = 89–92°C; $[\alpha]_D^{22} = -14°$ (1, DMF); IR (KBr) main peaks at 3299, 2960, 1725, 1691, 1641, 1531, 1263 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 0.90 (d, 6, $LeuCH_3$, J = 4.5 Hz), 1.35–1.82 (m, 3, $Leu\beta CH_2\gamma CH$), 2.10 (s, 3, CH_3CO), 4.07 (d, 2, CH_2N, J = 4.5 Hz), 4.17–4.50 (m, 1, αCH), 5.10 (s, 2, Z CH_2), 7.35 (s, 5, C_6H_5). Anal $C_{17}H_{24}N_2O_4$ (C, H, N).

N-Benzyloxycarbonyl-L-isoleucyl-1-aminopropan-2-one ***1f***
N-Benzyloxycarbonyl-L-isoleucine (1.56 g, 5.9 mmol) and 1-amino-2,2-dimethoxypropane (0.7 g, 5.9 mmol) were reacted according to *Procedure A*. The hydrolysis step required 30 min. Under treatment with oxalic acid the product separated as white solid which was filtered and recrystallized from THF: 1.54 g (81%); mp = 174–177°C; $[\alpha]_D^{22} = -5°$ (1, $CHCl_3$); IR (KBr) main peaks at 3294, 2954, 1723, 1690, 1640, 1534, 1240 cm^{-1}; ^1H-NMR ($CDCl_3$), δ 0.75–1.00 (m, 6, Ile CH_3), 1.02–1.93 (m, 3, $Ile\beta CH\gamma CH_2$), 2.07 (s, 3, CH_3CO), 3.73–4.17 (m, CH_2N and αCH), 5.10 (s, 2, ZCH_2), 7.38 (s, 5, C_6H_5). Anal $C_{17}H_{24}N_2O_4$ (C, H, N).

Preparation of Z-hydrazones ***7a–f****. General procedure B*
A solution of benzylcarbazate (1 mmol) and the appropriate carbonyl compound (1.1 mmol) in toluene (2 ml) was kept for 30 min at 50°C and allowed to cool overnight to rt. The crude product separated as a white solid, was collected by filtration and used without further purification.

Preparation of Z-hydrazines ***8a–f****. General procedure C*
A solution of the hydrazone (1 mmol) and $NaBH_3CN$ (2.5 mmol) in anhydrous THF (5 ml) and AcOH (3 ml) was kept overnight at rt. The reaction mixture was diluted with EtOAc (10 ml) and water (10 ml) and made basic by addition of solid $NaHCO_3$. The organic phase was washed with brine, saturated $NaHCO_3$, brine and dried over Na_2SO_4. The solvent was removed under reduced pressure and the resulting residue containing the hydrazine derivative as the cyanoborane adduct was hydrolyzed by treatment with 2 N NaOH (3 ml) in MeOH (5 ml). The hydrolysis was complete within 2 h. The reaction mixture was concentrated (when the phenol function was present, 1 M HCl was added to neutrality and the solution made basic again by addition of aqueous $NaHCO_3$), ether was added and the resulting solution washed with brine. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give the crude hydrazines **8a–f** which were used without further purification.

Preparation of N-benzyloxycarbonyl-α-azaaminoacyl-p-nitrophenyl esters ***9a–f****. General procedure D*
To an ice-cooled solution of the required hydrazine **8a–f** (1 mmol) in $CHCl_3$ (3 ml) (EtOAc was used to dissolve **8b**) and 0.5 M aqueous $NaHCO_3$ (4 ml), a solution of *p*-nitrophenyl chloroformate (1 mmol) in $CHCl_3$ (1 ml) was added under vigorous stirring and the reaction mixture allowed to warm to rt while stirring was continued for 30 min. The product was recovered by addition of $CHCl_3$ (10 ml), washing with brine, saturated $NaHCO_3$, and brine, drying over Na_2SO_4 and removal of the solvent under reduced pressure. The crude *p*-nitrophenyl-esters were crystallized from 1,2-dichloroethane (**9a–c**), EtOAc (**9d**) and EtOAc/hexane (**9e**, **9f**).

Preparation of N-Z-α-azaaminoacyl-1-aminopropan-2-ones ***5a–f****. General procedure E*
A solution of 1-amino-2,2-dimethoxypropane (2 mmol) and the appropriate *N*-benzyloxycarbonyl-α-azaaminoacyl-*p*-nitrophenylester **9a–f** (1 mmol) in dioxane (4 ml) was allowed to react for 14–24 h at 80°C. The solution was diluted with EtOAc and washed with brine, 2 N Na_2CO_3, 1 N $KHSO_4$ and brine. After drying over Na_2SO_4, the solvent was removed under reduced pressure. The crude *N*-benzyloxycarbonyl-α-azaaminoacyl-1-amino-2,2-dimethoxypropane was hydrolyzed by treatment with aqueous 1 M oxalic acid (2 ml) in THF (5 ml) at room temperature for 2–12 h. The conditions required are reported with the physical constants of the different products. The reaction mixture was diluted with EtOAc and washed with saturated $NaHCO_3$ and brine. Drying over Na_2SO_4 and evaporation of the solvent under reduced pressure gave the crude product.

N-Benzyloxycarbonyl-α-azaphenylalanyl-1-aminopropan-2-one ***2a***
N-Benzyloxycarbonyl-α-azaphenylalanyl-*p*-nitrophenylester **9a** (1.12 g, 2.66 mmol) and 1-amino-2,2-dimethoxypropane (0.63 g, 5.32 mmol) were reacted according to *Procedure E*. The acylation and the hydrolysis steps required 24 and 2 h respectively. Purification of the crude material by silica-gel chromatography ($CHCl_3$) and crystallization from EtOAc/ hexane gave the pure product as white crystals: 0.67 g (70%); mp = 111–113°C; IR ($CHCl_3$) main peaks at 3431, 3154, 3003, 1742, 1641, 1518, 1232 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 2.12 (s, 3, CH_3CO), 4.12 (d, 2, CH_2N, J = 4.5 Hz), 4.72 (bs, 2, $PhCH_2$), 5.15 (s, 2, ZCH_2), 7.10–7.53 (m, 10, Ar). Anal $C_{19}H_{21}N_3O_4$ (C, H, N).

N-Benzyloxycarbonyl α-azatyrosyl-1-aminopropan-2-one ***2b***
Product **2b** was obtained from *N*-benzyloxycarbonyl-α-azatyrosyl-*p*-nitrophenylester **9b** (3.26 g, 7.64 mmol) and 1-amino-2,2-dimethoxypropane (1.77 g, 14.92 mmol) according to *Procedure E*. The acylation and the hydrolysis steps required 22 and 5 h, respectively. Crystallization of the crude material from THF/$CHCl_3$ gave the pure product as pale yellow crystals: 1.67 g (61%); mp = 165–167°C; IR ($CHCl_3$) main peaks at 3418, 3262, 3011, 1746, 1726, 1632, 1530, 1279 cm^{-1}; ^1H-NMR (d_6-DMSO) δ 2.08 (s, 3, CH_3CO), 3.82 (d, 2, CH_2N, J = 4.5 Hz), 4.47 (bs, 2, $PhCH_2$), 5.08 (s, 2, Z CH_2), 6.73 (d, 2, C_6H_4, J = 9.0 Hz), 7.08 (d, 2, C_6H_4, J = 9.0 Hz), 7.37 (s, 5, C_6H_5). Anal $C_{19}H_{21}N_3O_5$ (C, H, N).

N-Benzyloxycarbonyl-3-iodo-α-azatyrosyl-1-aminopropan-2-one ***2c***
N-Benzyloxycarbonyl-3-iodo-α-azatyrosyl-*p*-nitrophenylester **9c** (2.5 g, 4.44 mmol) and 1-amino-2,2-dimethoxypropane (1.05 g, 8.88 mmol) were reacted according to *Procedure E*. The acylation and the hydrolysis steps required 22 and 4 h respectively. Purification of the crude material by silica-gel chromatography ($CHCl_3$/*i*-PrOH 95:5) and crystallization from MeOH gave the pure product as white crystals: 1.22 g (56%); mp = 182–184°C; IR ($CHCl_3$) main peaks at 3396, 3276, 2925, 1744, 1723, 1632, 1537, 1216 cm^{-1}; ^1H-NMR (d_6-DMSO) δ 2.03 (s, 3, CH_3CO), 3.82 (d, 2, CH_2N, J = 4.5 Hz), 4.48 (bs, 2, $PhCH_2$), 5.08 (s, 2, ZCH_2), 6.85 (d, 1, C_6H_3, J = 9.0 Hz), 7.13 (dd, 1, C_6H_3, J = 9.0 and 1.5 Hz), 7.37 (s, 5, C_6H_5), 7.61 (d, 1, C_6H_3, J = 1.5 Hz). Anal $C_{19}H_{20}N_3O_5I$ (C, H, N).

N-Benzyloxycarbonyl-3,5-diiodo-α-azatyrosyl-1-aminopropan-2-one ***2d***
Product **2d** was obtained from *N*-benzyloxycarbonyl-3,5-diiodo-α-azatyrosyl-*p*-nitrophenylester **9d** (1.67 g, 2.42 mmol) and 1-amino-2,2-dimethoxypropane (0.57 g, 4.84 mmol) according to *Procedure E*. The acylation and the hydrolysis steps required 15 and 12 h, respectively. Purification of the crude material by silica-gel chromatography ($CHCl_3$/*i*-PrOH

98:2) and crystallization from EtOAc/hexane gave the pure product as white crystals: 0.35 g (23%); mp = 160–162°C; IR (KBr) main peaks at 3389, 3271, 2925, 1722, 1645, 1630, 1535, 1293 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 2.12 (s, 3, CH_3CO), 4.10 (d, 2, CH_2N, J = 4.5 Hz), 4.60 (bs, 2, $PhCH_2$), 5.17 (s, 2, ZCH_2), 7.38 (s, 5, C_6H_5), 7.59 (s, 2, C_6H_2). Anal $C_{19}H_{19}N_3O_5I_2$ (C, H, N).

N-Benzyloxycarbonyl-α-azaleucyl-1-aminopropan-2-one ***2e***
N-Benzyloxycarbonyl-α-azaleucyl-*p*-nitrophenylester **9e** (1.69 g, 4.36 mmol) and 1-amino-2,2-dimethoxypropane (1.03 g, 8.72 mmol) were reacted according to *Procedure E*. The acylation and the hydrolysis steps required 24 and 3 h, respectively. Crystallization of the crude material from EtOAc gave the pure product as white crystals: 0.90 g (65%); mp = 130–132°C; IR ($CHCl_3$) main peaks at 3441, 3175, 2884, 1729, 1633, 1536, 1223 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.88 (d, 6, CH_3CH, J = 6.0 Hz), 1.57–2.03 (m, 1, $CHCH_3$), 2.08 (s, 3, CH_3CO), 3.35 (d, 2, CH_2CH, J = 7.5 Hz), 4.05 (d, 2, CH_2N, J = 4.5 Hz), 5.18 (s, 2, ZCH_2), 7.38 (s, 5, C_6H_5). Anal $C_{16}H_{23}N_3O_4I_2$ (C, H, N).

N-Benzyloxycarbonyl-α-azaisoleucyl-1-aminopropan-2-one ***2f***
N-Benzyloxycarbonyl-α-azaisoleucyl-*p*-nitrophenylester **9f** (1.02 g, 2.64 mmol) and 1-amino-2,2-dimethoxypropane (0.63 g, 5.28 mmol) were reacted according to *Procedure E*. The acylation and the hydrolysis steps required 14 and 2 h, respectively. The crude material was purified by silica-gel chromatography ($CHCl_3$/EtOAc 8:2) and crystallized from EtOAc/hexane to give the pure product as white crystals: 0.25 g (29%); mp = 119–122°C; IR (KBr) main peaks at 3441, 3187, 2985, 1738, 1626, 1514, 1219 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.83 (t, 3, CH_3CH_2, J = 7.5 Hz), 1.05 (m, 2, CH_2CH_3), 2.08 (s, 3, CH_3CO), 4.05 (d, 2, CH_2N, J = 4.5 Hz), 4.20–4.52 (m, 1, $CHCH_3$), 5.17 (s, 2, ZCH_2), 7.38 (s, 5, C_6H_5). Anal $C_{16}H_{23}N_3O_4$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-phenylalanyl)-2-acetylhydrazine ***3a***
To an ice-cooled suspension of *N*-benzyloxycarbonyl-L-phenylalanylhydrazine (312 mg, 1.0 mmol) in $CHCl_3$ (5 ml) and 1 M aqueous $NaHCO_3$ (2.4 ml) under vigorous stirring, acetic anhydride (408 mg, 4.0 mmol) was rapidly added and stirring continued for 2 h at rt. The reaction mixture was diluted with $CHCl_3$ (20 ml), washed with brine, saturated $NaHCO_3$, 1 M HCl and brine. Drying over Na_2SO_4 and removal of the solvent under reduced pressure gave the crude product which was crystallized from MeOH/Et_2O: 301 mg (85%); mp = 212–213°C; $[\alpha]_D^{22}$ = –20° (1, DMF); IR (KBr) main peaks at 3275, 3198, 1692, 1610, 1542, 1488, 1285, 1260 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 1.87 (s, 3, CH_3CO), 2.72–3.23 (m, 2, Phe CH_2), 4.24–4.60 (m, 1, αCH), 4.95 (s, 2, ZCH_2), 7.34 (s, 10, Ar). Anal $C_{19}H_{21}N_3O_4$ (C, H, N).

1-(N-Benzyloxycarbonyl-L-3,5-diiodotyrosyl)-2-acetylhydrazine ***3d***
A solution of *N*-benzyloxycarbonyl-L-3,5-diiodotyrosylhydrazine (300 mg, 0.5 mmol), *N*-methylmorpholine (206 mg, 2 mmol) and acetic anhydride (208 mg, 2 mmol) in anhydrous DMF was allowed to react at rt, overnight. Aqueous 25% NH_3 (1 ml) was added to remove the ester acetyl group from the *N,O*-diacetyl intermediate. The crude 1-(*N*-benzyloxycarbonyl-L-3,5-diiodotyrosyl)-2-acetylhydrazine was crystallized by addition of water. Recrystallization from dioxane/water gave the pure product as a white solid: 292 mg (91%); mp = 199–201°C; $[\alpha]_D^{22}$ = –6° (1, DMF); IR (KBr) main peaks at 3441, 3276, 3198, 1691, 1610, 1537, 1482, 1271, 1245 cm^{-1}; ^{1}H-NMR (d_6-DMSO) δ 1.90 (s, 3, CH_3CO), 2.52–2.95 (m, 2, Tyr CH_2), 4.11–4.35 (m, 1, αCH), 4.98 (s, 2, ZCH_2), 7.21–7.46 (m, 5, C_6H_5), 7.77 (s, 2, C_6H_2). Anal $C_{19}H_{19}N_3O_5I_2$ (C, H, N).

N-Benzyloxycarbonyl-L-phenylalanyl-1-amino-3-methoxypropan-2-one ***5***
N-Benzyloxycarbonyl-L-phenylalanine (1.5 g, 5.0 mmol) and 1-amino-3-methoxypropan-2-ol [46] (0.53 g, 5.0 mmol) were reacted according to *Procedure B*. The crude material was crystallized from 1,2-dichloroethane/hexane to give *N*-benzyloxycarbonyl-L-phenylalanyl-1-amino-3-methoxypropan-2-ol **13b** as a white solid. The alcohol (1.72 g, 4.4 mmol) in AcOH (8.8 ml) was oxidized by treatment with a solution of $Na_2Cr_2O_7 \cdot 2H_2O$ (715 mg, 2.2 mmol) and H_2SO_4 (862 mg, 8.8 mmol) in AcOH (11 ml) under stirring at rt. After 10 min the reaction was quenched by addition of *i*-PrOH (1 ml) and further stirred for 10 min. The reaction mixture was poured in EtOAc and the solution sequentially washed with brine, saturated $NaHCO_3$ and brine. After drying over anhydrous Na_2SO_4, the solvent was removed under reduced pressure. Crystallization of the crude material from toluene/hexane gave the pure product as a white solid: 1.24 g (73%); mp = 84–86°C; $[\alpha]_D^{22}$ = –13° (1, acetone); IR ($CHCl_3$) main peaks at 3417, 2971, 1710, 1678, 1497, 1361 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.05 (d, 2, $PheCH_2$, J = 7.5 Hz), 3.39 (s, 3, CH_3), 4.00 (s, 2, CH_2CO), 4.16 (bs, 2, CH_2N), 4.35–4.67 (m, 1, αCH), 5.05 (s, 2, ZCH_2), 7.26 and 7.34 (two s, 10, Ar). Anal $C_{21}H_{24}N_2O_5$ (C, H, N).

N-Benzyloxycarbonyl-L-phenylalanyl-1-amino-3-(benzyloxy)-methoxypropan-2-one ***6***
To an ice-cooled solution of glycidol (2.61 g, 39.0 mmol) and ethyldiisopropylamine (11.3 g, 87.5 mmol) in $CHCl_3$ (48 ml) benzylchloromethylether [47] (7.3 g, 46.6 mmol) was added dropwise under stirring and the mixture was allowed to stand at rt overnight. After washing with brine, the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude oily residue was purified by silica-gel chromatography (CH_2Cl_2) to give glycidylbenzyloxymethylether **11a** as a colourless oil: 4.19 g (56%).

A solution of glycidylbenzyloxymethylether in EtOH (42 ml) was saturated with anhydrous NH_3 under ice cooling and the reaction mixture was kept at rt for 3 d. After removal of the solvent under reduced pressure, the resulting 3-(benzyloxy)methoxy-2-hydroxypropylamine **12a** was dissolved in 0.2 N oxalic acid (48.5 ml) and washed with ether. The aqueous phase was made alkaline by addition of 2 N NaOH and the amine recovered by continuous overnight ether extraction. After drying over Na_2SO_4 the solvent was removed under reduced pressure and the oily amine (2.76 g, 13.0 mmol) acylated with *N*-benzyloxycarbonyl-L-phenylalanine (3.9 g, 13.0 mmol) according to *Procedure A* to give *N*-benzyloxycarbonyl-L-phenylalanyl-1-amino-3-(benzyloxy)-methoxypropan-2-ol **13a** as a white solid: 4.95 g (77%).

Oxidation of the alcohol (1.22 g, 2.48 mmol) in CH_2Cl_2 (20 ml) was performed by treatment with periodinane [21] (3.7 g, 12.4 mmol) for 2 h at rt. After removal of the solvent and addition of saturated $NaHCO_3$ (50 ml), the mixture was extracted with ether. The organic phase was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Silica gel chromatography ($CHCl_3$/hexane 8:2) and crystallization of the crude material from benzene/hexane gave the pure ketone **6** as a white solid: 630 mg (52%); mp = 96–98°C; $[\alpha]_D^{22}$ = –11° (1, acetone); IR ($CHCl_3$) main peaks at 3419, 2971, 1710, 1678, 1496, 1363 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.07 (d, 2, $PheCH_2$, J = 7.5 Hz), 4.11–4.26 (m, 4, CH_2N and CH_2CO), 4.33–4.58 (m, 1, αCH), 4.65 (s, 2, OCH_2O), 4.81 (s,

2, OCH_2Ph), 5.09 (s, 2, ZCH_2), 7.25, 7.30 and 7.35 (3 s, 15, Ar). Anal $C_{28}H_{30}N_2O_6$ (C, H, N).

N-Benzyloxycarbonyl-L-phenylalanyl-1-amino-3-hydroxypropan-2-one **4**

To a solution of *N*-benzyloxycarbonyl-L-phenylalanyl-1-amino-3-(benzyloxy)methoxypropan-2-one (490 mg, 1 mmol) in CH_2Cl_2 (22 ml) trifluoroacetic acid (0.77 ml) was added and the mixture kept overnight at rt. After washing with saturated $NaHCO_3$ and brine, the organic phase was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure. Silica-gel chromatography ($CHCl_3$/EtOAc 7:3) of the crude oily residue and crystallization from 1,2-dichloroethane/hexane gave the pure product as a white solid: 230 mg (62%); mp = 106–108°C; $[\alpha]_D^{22} = -19°$ (1, acetone); IR ($CHCl_3$) main peaks at 3303, 2925, 1683, 1649, 1532, 1285 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 3.08 (d, 2, $PheCH_2$, J = 7.5 Hz), 4.06 (bs, 2, CH_2N), 4.21 (s, 2, CH CO), 4.40–4.70 (m, 1, αCH), 5.05 (s, 2, ZCH_2), 7.26 and 7.33 (two s, 10, Ar). Anal $C_{20}H_{22}N_2O_5$ (C, H, N).

Pharmacology

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 rt in 25 ml of 50 mM phosphate buffer pH 6.8 containing 2 mM EDTA and 0.5 mM L-cysteine according to Thompson *et al* [48] with minor modifications. Approximate enzyme concentration was determined from the absorbance at 280 nm (E = 58.5 mM^{-1} cm^{-1}) [49]. 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 [50] 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 the kinetic parameters of papain-catalyzed hydrolysis of Z-Gly-ONp [50].

Reactions were started by addition of enzyme solution to the cuvette. A substrate concentration of 125 μM ($K_m \times 12.5$) avoided substrate depletion due to spontaneous and enzymatic hydrolysis during the assays. Absorbances *vs* time data were measured continuously, stored in the computerized spectrophotometer and transferred to a Data System 450 Personal Computer (Kontron) equipped with a mathematical coprocessor. Progress curves were composed of 180–360 (absorbance, time) pairs. Non-linear regression analysis by using Enzfitter [51] was employed to fit data pairs to the equation [3], where P is the concentration of the product, as the increase of the absorbance at 405 nm.

$$P = \frac{v_s t + (v_0 - v_s)(1 - e^{-k_{obs} t})}{k_{obs}} + d \qquad [3]$$

v_0 is the initial velocity at $t = 0$, v_s is the final velocity at steady state, k_{obs} is the first-order rate constant for the approach to steady state and d is the displacement of absorbance from zero at $t = 0$. Collection and handling of progress curves data was made possible with software supplied by Kontron.

Cathepsin assay

Cathepsin B EC 3.4.22.1 from bovine spleen, product No C-6286 was purchased from Sigma and solutions were made fresh daily by incubating the enzyme under the same conditions as for papain. Approximate enzyme concentration was determined from absorbance at 280 nm ($E^{1\%}$ = 20 cm^{-1}) [52]. Enzyme activity was tested as described for papain [53], as well as analysis of kinetic data.

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 approximate concentration determined from the absorbance at 280 nm (E = 50 mM^{-1} cm^{-1}) [54]. Chymotrypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester at 256 nm [55]. Trypsin EC 3.4.21.1 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 [56]. Trypsin was assayed by monitoring the hydrolysis of *N*-benzoyl-L-arginine ethyl ester at 253 nm [57]. Preincubation at 25°C for 6 h with 2 mM peptidyl methylketones **5** and **6** or 50 and 500 μM respectively of **1c** and **1d** with both the enzymes did not inhibit their activity.

Porcin 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 approximate concentration determined from $E^{1\%}$ = 22 cm^{-1} at 280 nm [58]. Activity was monitored by following the hydrolysis of Boc-L-Ala-ONp at 347 nm [57] in 50 mM phosphate buffer, pH 7.0 at 25°C. No inactivation was observed following 6 h incubation with peptidyl methylketones **8**, **9**, **4c** and **4d** under the same conditions as for chymotrypsin and trypsin.

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