Potent and selective inactivation of cysteine proteinases with *N*-peptidyl-*O*-acyl hydroxylamines

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A series of *N*-peptidyl-*O*-acyl hydroxylamines was synthesized and tested as inactivators of cysteine proteinases. Depending on the structure of the peptidyl residue of the inhibitors, rapid and complete irreversible inactivation of the lysosomal cathepsins, B, L and S, may be achieved. The most effective inhibitors display second-order rate constants of the inactivation in the range 10^5 – 10^6 M⁻¹·s⁻¹. By contrast, the activity of the aminoendopeptidase cathepsin H is only negligibly affected by the *N*-terminal-protected peptidyl inhibitors.

INTRODUCTION

The lysosomal cathepsins B, H, L and S are well characterized cysteine proteinases and play an important role in intracellular protein breakdown (Kirschke & Barrett, 1987). Furthermore, it has been shown previously that cathepsins B and L are involved in the degradation of extracellular-matrix proteins such as collagen and elastin (Burleigh *et al.*, 1974; Kirschke *et al.*, 1982; Mason *et al.*, 1986) and may take part in osteoporosis (Delaisé *et al.*, 1984), pulmonary emphysema (Mason, 1988) and tumour invasion (Denhardt *et al.*, 1987).

The design of specific irreversible inhibitors could therefore be a useful contribution in studying the role of these proteinases in disease.

Favoured inhibitors should be specific for one type of proteinase and should not induce non-specific side reactions. This goal may be reached by using enzyme-activated inhibitors that generate their inhibiting power only during the catalytic action of the target enzyme.

Searching for new inactivators of dipeptidyl peptidase IV (DP IV, EC 3.4.15.5) we developed *N*-peptidyl-*O*-aroyl hydroxylamines as potential mechanism-based inhibitors of serine and cysteine proteinases (Fischer *et al.*, 1982, 1983; Demuth *et al.*, 1988, 1989a,b). Diacyl hydroxylamine derivatives (R₁-CO-NH-O-CO-R₂) permit variations of the *N*-acyl and *O*-acyl residues and thus a selective control of their affinity and reactivity towards the enzymes.

Using DP IV as model enzyme we studied the inactivation reactions, enzyme-catalysed turnover and spontaneous degradation of a series of substrate-analogous *N*-Xaa-Pro-*O*-benzoyl hydroxylamines (Demuth *et al.*, 1988). The inhibitors have been successfully used in biological investigations to evaluate the participation of DP IV in human T-lymphocyte activation (Schön *et al.*, 1984, 1987, 1989).

Recently, Smith *et al.* (1988) have demonstrated the potential of this new inhibitor class to inactivate the cysteine proteinase cathepsin B.

In the present paper we report the rapid and selective inactivation, by several *N*-peptidyl-*O*-acyl hydroxylamines, of several lysosomal cysteine proteinases exhibiting different substrate specificities.

EXPERIMENTAL

Enzymes

Cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15) and cathepsin H (EC 3.4.22.16) were prepared from the lysosomal fraction of rat liver as described by Kirschke *et al.* (1977) and Barrett & Kirschke (1981). Bovine spleen cathepsin S (EC 3.4.22.-) was isolated as described by Kirschke *et al.* (1986). All cathepsins used were in an electrophoretically homogeneous form.

Substrates and inhibitors

Z-Phe-Arg-NHMec, Arg-NHMec·HCl and Z-Val-Val-Arg-NHMec were synthesized as in Brömme *et al.* (1989a,b).

N-Peptidyl-*O*-acyl hydroxylamines have been used from previous experiments or synthesized as described (Fischer *et al.*, 1983; Demuth *et al.*, 1988, 1989a). Boc- and Z-protected peptidyl methyl esters were built up according to standard procedures. Treatment of the ester with hydroxylamine in sodium methanolate solution gave the appropriate hydroxamic acids. Acylation using acid chlorides in dry tetrahydrofuran or in Schotten-Baumann reactions with *N*-methylmorpholine or triethylamine as base resulted the diacyl hydroxylamines. Yields of the final reaction step were between 60 and 85%. Final products, usually crystallized from ethanol/ethyl acetate or ethyl acetate/light petroleum (b.p. 30–60 °C), gave correct elemental (C,H,N) analyses, single spots on t.l.c. and were partially characterized by ¹³C-

Abbreviations used: Boc-, t-butyloxycarbonyl; Z-, benzyloxycarbonyl; -NHMe, 4-methyl-7-coumarylamide; -Nbz, 4-nitrobenzoyl; -Ac, acetyl; -Bz, benzoyl; -Ma, methacryl; -NHO- is the hydroxylamine moiety; ·HCl, hydrochloride; for the discussion of the interactions between proteinase and inhibitor the nomenclature of Schechter & Berger (1967) has been used; DP IV, dipeptidyl peptidase IV.

Table 1. *N*-Peptidyl-*O*-acyl hydroxylamines: analytical parameters

Under 'M.p. (°C)', (D) means that the compound decomposes. Under 'Elemental analysis', 'F' means 'found' and 'R' means 'requires'.

Compound	M_r	Formula	M.p. (°C)	Elemental analysis (%)		
				C	H	N
Boc-Phe-Gly-NHO-Bz	441.48	$C_{23}H_{27}N_3O_6$	118–119	F: 62.26 R: 62.57	6.15 6.15	8.80 9.52
Z-Phe-Phe-NHO-Bz	565.63	$C_{33}H_{31}N_3O_6$	192–194	F: 69.54 R: 70.08	5.96 5.53	7.51 7.43
Z-Phe-Phe-NHO-Ma	530.60	$C_{30}H_{32}N_3O_6$	180–182	F: 66.24 R: 67.91	6.09 6.08	7.18 7.92
Boc-Phe-Pro-Ala-NHO-Nbz	597.62	$C_{29}H_{35}N_5O_9$	111–113	F: 57.43 R: 58.28	6.02 5.90	11.09 11.72
Boc-Ala-Phe-Leu-NHO-Nbz	582.65	$C_{30}H_{38}N_4O_8$	115 (D)	F: 59.47 R: 61.84	6.06 6.57	10.28 9.62
Boc-Gly-Phe-Phe-NHO-Nbz	633.66	$C_{32}H_{35}N_5O_9$	128–129	F: 61.51 R: 60.66	5.59 5.57	11.03 11.05

Table 2. Spontaneous degradation of *N*-peptidyl-*O*-acyl hydroxylamines in aqueous solution

$t_{1/2}$ is the half-life. The standard deviation (S.D.) is given as a percentage value and is of the mean for three experiments. For further details, see the Experimental section.

Compound	$t_{1/2}$ (min)	k (min ⁻¹)	S.D. (%)
1 Boc-Ala-Pro-NHO-Nbz	209	0.0032	2.5
2 Boc-Ala-Phe-NHO-Nbz	178	0.0039	12.9
3 Boc-Phe-Ala-NHO-Nbz	365	0.0019	12.1
4 Boc-Phe-Gly-NHO-Bz	900	0.00077	5.9
5 Z-Phe-Phe-NHO-Bz	16	0.0439	2.2
6 Z-Phe-Phe-NHO-Ma	124	0.0056	9.8
7 Boc-Phe-Pro-Ala-NHO-Nbz	133	0.0052	7.3
8 Boc-Ala-Phe-Leu-NHO-Nbz	248	0.0028	24.0
9 Boc-Gly-Phe-Phe-NHO-Nbz	45	0.0153	3.4

n.m.r. spectroscopy. All reagents were obtained in Research Grade from commercial sources; organic solvents were dried before use.

Structural parameters of newly synthesized derivatives are listed in Table 1.

Non-enzymic decomposition of the inhibitors

The stability of the compounds in aqueous solution has been tested as described by Demuth *et al.* (1988) by means of u.v. spectroscopy. Inhibitors (10–200 μ M) were incubated in 0.04 M-Tricine buffer, pH 7.6, adjusted to I 0.125. In a typical experiment the total volume was 2.0 ml containing 5% (v/v) acetonitrile.

Kinetic runs were recorded in the range of 225–300 nm by using a Carl Zeiss (Jena, German Democratic Republic)-microprocessor-controlled spectrophotometer (M 40) equipped with a thermostatically controlled cell compartment at 30 ± 0.1 °C. The data were collected and stored in an internal RAM buffer and analysed by using software packages provided for the instrument on the ROM card REACTION KINETICS.

Parameters of decomposition reactions with half-times

longer than 200 min were calculated by fitting the data collected at 30 min time intervals by non-linear regression programs running on an IBM-PC-compatible computer. The pseudo-first-order rate constants of the decompositions are compiled in Table 2.

Inactivation measurements

The inactivation of proteinases with substrate-analogous inhibitors proceeds according to eqn. (1):

where $E \cdot I$, $E-I$, k_1 and k_{-1} represent the enzyme-inhibitor complex, the inactivated enzyme and the rate constants of non-covalent reaction steps respectively. $k_{\text{inact.}}$ is the rate constant of the formation of covalent modified enzyme. This rate constant may be determined as described by Kitz & Wilson (1962) by preincubation of enzyme and inhibitor and subsequent estimation of residual activity in an assay with substrate. Tian & Tsou (1982) introduced a more convenient method of evaluation of inactivation rates in the presence of a substrate according to eqn. (2):

where substrate and inhibitor are competing for the enzyme's binding site. The decrease in enzyme concentration during incubation with inhibitor follows pseudo-first-order kinetics. Applying chromogenic or fluorogenic substrates and establishing steady-state conditions during the inactivation time, first-order rate constants may be obtained by fitting absorbance and time values to an exponential function. Measurements at constant inhibitor and different substrate concentrations give different $k_{\text{obs.}}$ values. Extrapolation of these values gives the rate constant at zero substrate concentration.

By fitting these rate constants evaluated at different inhibitor concentrations to a hyperbola, the inactivation parameters K_i ($\approx k_{-1}/k_1$) and $k_{\text{inact.}}$ may be obtained. This method was applied here.

The progress curves for the inactivation of the proteinases in the presence of substrate were monitored at 25 °C by using a Shimadzu spectrophotometer (UV-300) equipped with a fluorescence-detection unit at an excitation wavelength of 383 nm and with an emission filter of 450 nm. The kinetic experiments were performed with a constant enzyme concentration in 50 mM-acetate buffer, pH 5.5, for cathepsin L; in 50 mM-phosphate buffer containing 0.01 % (v/v) Triton X-100, pH 6.5, for cathepsin S; in 50 mM-phosphate buffer, pH 6.5, for cathepsin H; and in 50 mM-phosphate buffer, pH 6.0, for cathepsin B. In all experiments the enzyme concentrations were 0.7 nM, 2.3 nM, 2.4 nM and 0.9 nM for cathepsins L, S, H and B respectively. For the activation of the proteinases the cathepsins L, B and H were incubated for 5 min at 25 °C with 2.5 mM-dithioerythritol, 2.5 mM-EDTA · Na₂ and 0.005 % (v/v) Brij-35 in the assay buffer, whereas cathepsin S was activated for 15 min with 5 mM-dithioerythritol, 5 mM-EDTA · Na₂ and 0.01 % Triton X-100 in 50 mM-phosphate buffer, pH 6.5. The reaction was started by the addition of the activated enzyme (0.5 ml) to 1 ml of substrate in assay buffer containing different inhibitor concentrations (seven per substrate concentration).

Substrates were Z-Phe-Arg-NHMec (3 μM and 8 μM) for cathepsin L, Z-Phe-Arg-NHMec (10 μM and 50 μM) for cathepsin B, Z-Val-Val-Arg-NHMec (10 μM and 50 μM) for cathepsin S and Arg-NHMec (8 μM and 25 μM) for cathepsin H.

The exponential progress curves recorded were analysed by the method described above using non-linear regression programs running on an IBM-PC-compatible computer.

In cases where no saturation of the enzyme by the inhibitor was achieved ($[I] \ll K_i$), the second-order rate constants were calculated from $k_{\text{obs.}}/[I] (1 + [S]/K_m)$ as described by Crawford *et al.* (1988).

RESULTS AND DISCUSSION

N-Peptidyl-O-acyl hydroxylamines were shown to react irreversibly with different lysosomal cysteine proteinases. After incubation of cathepsin B with Boc-Phe-Ala-NHO-Nbz (compound 3 in Table 3) and cathepsin S with Boc-Ala-Phe-Leu-NHO-Nbz (compound 8) for 1 h in molar enzyme/inhibitor ratios of 1:1000 and 1:100 respectively, the enzymes were completely inactivated. No regeneration of activity within 15 h could be observed after removal of excess inhibitor by exhaustive ultrafiltration (up to an enzyme/free inhibitor ratio of < 1:0.05) or by chromatography on Sephadex G-10.

Typical progress curves for the cathepsin S-catalysed hydrolysis of Z-Val-Val-Arg-NHMec in the presence of the inhibitor Boc-Gly-Phe-Phe-NHO-Nbz are shown in Fig. 1.

The pseudo-first-order rate constants ($k_{\text{obs.}}$) obtained at fixed substrate and inhibitor concentrations are independent of proteinase concentrations varied over the range of one order of magnitude (cathepsin S: 2.3–23 nM, cathepsin B: 0.9–9 nM). This implies that the inactivation of both enzymes is not (or only to a very small extent)

Table 3. Inactivation of the cathepsins S, L, B and H with N-peptidyl-O-acyl hydroxylamines

Values are means $\pm$ S.E.M. For further details, see the Experimental section.

Compound	Cathepsin S			Cathepsin L			Cathepsin B			Cathepsin H		
	K_i (μM)	$k_{\text{inact.}}$ (s^{-1})	$k_{\text{inact.}}/K_i$ ($\text{M}^{-1}\text{s}^{-1}$)	K_i (μM)	$k_{\text{inact.}}$ (s^{-1})	$k_{\text{inact.}}/K_i$ ($\text{M}^{-1}\text{s}^{-1}$)	K_i (μM)	$k_{\text{inact.}}$ (s^{-1})	$k_{\text{inact.}}/K_i$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{inact.}}$ (s^{-1})	$k_{\text{inact.}}/K_i$ ($\text{M}^{-1}\text{s}^{-1}$)	$k_{\text{inact.}}/K_i$ ($\text{M}^{-1}\text{s}^{-1}$)
1 Boc-Ala-Pro-NHO-Nbz	100–200†		20*	100–200†		50*	100–200†		10*	N.D.		
2 Boc-Ala-Phe-NHO-Nbz	50–100†		30*	20–50†		600*	10–20†		NTD*§	N.D.		21*
3 Boc-Phe-Ala-NHO-Nbz	0.48 $\pm$ 0.06	0.02 $\pm$ 0.001	42000	0.32 $\pm$ 0.04	0.14 $\pm$ 0.01	437000	4.5 $\pm$ 0.7	0.063 $\pm$ 0.006	14000			
4 Boc-Phe-Gly-NHO-Nbz	1.6 $\pm$ 0.2	0.03 $\pm$ 0.001	19000	0.1 $\pm$ 0.5†		136000*	12.5 $\pm$ 2.1	0.032 $\pm$ 0.004	2600	N.D.		
5 Z-Phe-Phe-NHO-Nbz	0.4–1.0†		5600*	0.1 $\pm$ 0.5†		63000*	0.8–2.0†		600*	N.D.		19*
6 Z-Phe-Phe-NHO-Ma	1.9 $\pm$ 0.4	0.04 $\pm$ 0.005	21000	0.09 $\pm$ 0.01	0.11 $\pm$ 0.01	1222000	8.9 $\pm$ 2.5	0.025 $\pm$ 0.005	2800			
7 Boc-Phe-Pro-Ala-NHO-Nbz	27.0 $\pm$ 2.0	0.03 $\pm$ 0.003	1100		N.D.‡		8–30†		200*	N.D.		32*
8 Boc-Ala-Phe-Leu-NHO-Nbz	0.48 $\pm$ 0.07	0.11 $\pm$ 0.01	229000	0.23 $\pm$ 0.06	0.16 $\pm$ 0.03	696000	1.7 $\pm$ 0.1	0.020 $\pm$ 0.001	12000			
9 Boc-Gly-Phe-Phe-NHO-Nbz	0.15 $\pm$ 0.01	0.04 $\pm$ 0.002	267000	0.15 $\pm$ 0.02	0.12 $\pm$ 0.01	800000	1.8 $\pm$ 0.3	0.015 $\pm$ 0.002	8300	N.D.		

* Below saturation concentrations.

† Concentration range (μM).

‡ N.D., not determined.

§ NTD, no time-dependence observed.

accompanied by enzyme-catalysed degradation of the inhibitor. Smith *et al.* (1988) found a partition ratio ($k_{\text{cat.}}/k_{\text{inact.}}$) of 5.5 in the reaction between cathepsin B and *N*-Z-Phe-Gly-*O*-mesitoyl hydroxylamine. In contrast, DP IV hydrolyses substrate-analogous diacyl hydroxylamines to a remarkable extent, exhibiting partition ratios between 8000 to 200000 (Demuth *et al.*, 1988).

The compounds 1, 2 and 7 (Table 4) are not suited to an effective inactivation of the lysosomal cathepsins. This is due to the non-specific peptide sequence of the inhibitors. Proline especially, in the P_1 or P_2 position prevents or disturbs interactions between the inhibitor and the target enzyme (Mason *et al.*, 1984; Brömme *et al.*, 1987). The same holds true for small amino acid residues in the P_2 position. A substitution of the P_2 residue by the specific subsite residue phenylalanine gives

very potent and rapid-acting inhibitors of cathepsins S, L and B (compounds 3–6, 8 and 9). The preference for aromatic hydrophobic residues in the S_2 subsite by cathepsins B and L had already been shown with various other inhibitors and substrates (Barrett & Kirschke, 1981; Kirschke & Shaw, 1981; Crawford *et al.*, 1988; Kirschke *et al.*, 1988).

Introduction of an additional hydrophobic residue such as phenylalanine or leucine into the P_1 position gives the most potent inhibitors of cathepsin L (compounds 6, 8 and 9). This is in agreement with the well-known high affinity of cathepsin L for bulky hydrophobic side chains in its subsites, S_1 and S_2 (Kirschke *et al.*, 1988). Z-Phe-Phe-NHO-Ma (compound 6 in Table 3; Fig. 2) reacts extremely rapidly with this enzyme ($k_{\text{inact.}}/K_i = 1\,222\,000\text{ M}^{-1}\cdot\text{s}^{-1}$), in contrast with the rather slow inactivation of cathepsin B by this compound ($k_{\text{inact.}}/K_i = 2800\text{ M}^{-1}\cdot\text{s}^{-1}$). This diacyl hydroxylamine should be well-suited to differentiate between cathepsins L and B.

Fig. 1. Progress curves of the cathepsin S-catalysed hydrolysis of Z-Val-Val-Arg-NHMec in the presence of Boc-Gly-Phe-Phe-NHO-Nbz

The substrate concentration was $10\text{ }\mu\text{M}$ and the following inhibitor concentrations were used: ■, 66 nM; △, 100 nM; ▲, 116 nM; ○, 133 nM; and ●, 200 nM.

Fig. 2. Dependence of the rate constant of inactivation (k_{app}) of cathepsin L on the concentration of Z-Phe-Phe-NHO-Ma

Cathepsin L was inactivated in 50 mM-acetate buffer, pH 5.5, containing 0.83 mM-dithioerythritol and 0.83 mM-EDTA at 25 °C in the presence of substrate (Z-Phe-Arg-NHMec; 3 and 8 μM). k_{app} values were obtained by extrapolation as described in the Experimental section.

Table 4. Comparison of second-order rate constants of inactivation of serine and cysteine proteinases

Abbreviations used: PSE, proline-specific endopeptidase; Ser, serine proteinase or serine peptidase; Cys, cysteine proteinase. References: a, Demuth *et al.* (1988); b, Demuth *et al.* (1989b); c, the present work.

Compound	$k_{\text{inact.}}/K_i$ ($\text{M}^{-1}\cdot\text{s}^{-1}$)	Enzyme	Reference
H-Ala-Pro-NHO-Nbz	1.9	DP IV (Ser)	a
Boc-Ala-Pro-NHO-Nbz	2.5	PSE (Ser)	b
Boc-Ala-Ala-NHO-Nbz	12	Elastase (Ser)	b
	166	Thermitase (Ser)	b
	226	Subtilisin (Ser)	b
Boc-Gly-Phe-NHO-Bz	652	Thermitase (Ser)	b
	1020	Subtilisin (Ser)	b
Z-Phe-Phe-NHO-Ma	1 222 000	Cathepsin L (Cys)	c
Boc-Ala-Phe-Leu-NHO-Nbz	696 000	Cathepsin L (Cys)	c
	229 000	Cathepsin S (Cys)	c

Fig. 3. Dependence of the rate constant of inactivation (k_{app}) of cathepsin S on the concentration of Boc-Ala-Phe-Leu-NHO-Nbz

Cathepsin S was inactivated in 50 mM-phosphate buffer, pH 6.5, containing 1.66 mM-dithioerythritol and 1.66 mM-EDTA at 0.01 % Triton X-100 at 25 °C in the presence of substrate (Z-Val-Val-Arg-NHMec; 10 μ M and 50 μ M). k_{app} values were obtained by extrapolation as described in the Experimental section.

The best inhibitors of cathepsin S are the tripeptide derivatives Boc-Ala-Phe-Leu-NHO-Nbz (Fig. 3) and Boc-Gly-Phe-Phe-NHO-Nbz, exhibiting one order of magnitude higher second-order inactivation rate constants compared with the most effective dipeptide derivatives. However, they do not allow one to differentiate between cathepsins S and L.

Compared with cathepsins L and S, cathepsin B is inhibited relatively slowly by the peptide inhibitors tested. Similar ratios of second-order inactivation rate constants have been found when cathepsins B and L were inactivated by peptidyl diazomethanes (Crawford *et al.*, 1988).

Smith *et al.* (1988) obtained somewhat higher second-order inactivation rate constants for comparable *N*-peptidyl-O-acyl hydroxylamines as inactivators of cathepsin B. This may be due to lower binding constants (K_i), whereas the saturation inactivation rate constants (k_{inact}) are similar to the values obtained in our experiments. The reason for this discrepancy may lie in the use of different *N*-protecting groups.

Cathepsin H, acting as aminoenkephalidase (Barrett & Kirschke, 1981), is only negligibly inhibited by the most potent inhibitors of the cathepsins B, L and S (compounds 3, 6 and 8; Table 3). Obviously those peptidyl derivatives are not able to bind productively to this enzyme. The same behaviour was found with peptidyl methyl ketones, known as potent reversible inhibitors of papain and the cathepsins B and L (Brömme *et al.*, 1989a). However, *N*-terminal unprotected aminomethyl ketones act as reversible inhibitors of cathepsin H. It is likely that unprotected *N*-amino acid-O-acyl hydroxylamines could serve as cathepsin H inhibitors.

Besides the *N*-peptidyl residue, the *O*-acyl part of the hydroxylamine inhibitor may also efficiently alter the second-order inactivation rate constants. Substitution of the benzoyl-O-acyl residue with the methacryl one (compounds 5 and 6, Table 3) leads to 5–20-times higher values for the inactivation of the cathepsins B, L and S.

Smith *et al.* (1988) were able to demonstrate a clear correlation between the efficiency of *N,O*-diacyl hydroxylamine derivatives and the lipophilicity of the *O*-acyl group. Thus the effect of systematic variation in the *O*-acyl residue requires investigation.

So far the mechanism of proteinase inactivation by diacyl hydroxylamines has not yet been established. However, in a preliminary report (Demuth *et al.* 1987), we published studies on the spontaneous degradation of *N*-peptidyl-O-(4-nitrobenzoyl) hydroxylamines in aqueous solution indicating that heterolytic *N*-O- bond fission of the hydroxylamine linkage is a rate-determining step of this reaction, as in a Lossen reaction (Bauer & Exner, 1974).

It is worth mentioning here that the more electron-withdrawing the *O*-acyl residues in *N*-peptidyl-O-acyl hydroxylamines are, the faster the enzyme inactivation occurs (Demuth *et al.*, 1988). Therefore this residue may also be used to preselect stability and efficacy of proteinase inhibitors in aqueous solution from a pharmacokinetic point of view. Comparing stability data given in Demuth *et al.* (1988, 1989a) and in Table 2, it becomes obvious that the nature of the amino acid side chain in the *N*-acyl residue (P_1 amino acid) also influences the decomposition rate of the compounds in solution. The empirical range of decomposition-promoting ability is: Gly < Pro, Ala < Leu, Val < Phe.

If one compares the second-order rate constants of the inactivation of target enzymes from the different proteinase classes (Table 4) the efficacy of the new inhibitor type in reactions with cysteine proteinases becomes obvious.

Similar differences in specificity have been described for the inactivation of serine and cysteine proteinases by peptidyl diazomethanes (Zumbrunn *et al.*, 1988). The *N*-peptidyl-O-acyl hydroxylamine inhibitors tested so far may therefore be considered to be specific for cysteine proteinases.

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