Synthetic analogues of chymostatin*

Inhibition of chymotrypsin and *Streptomyces griseus* proteinase A

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A series of analogues of chymostatin, including Z-Arg-Leu-Phe-aldehyde (Z-Arg-Leu-Phe-H), have been synthesized. Analysis of the inhibitory potential of these analogues permits identification of residues and interactions that are important for inhibitory activity. Moreover, the structure–function relationship for Z-Arg-Leu-Phe-H and chymostatin inhibition of chymotrypsin and *Streptomyces griseus* proteinase A (SGPA) was probed further with the aid of molecular mechanics. This analysis identified interactions that provide an explanation for the enhanced activity of the natural product, chymostatin, over the synthetic analogues in the inhibition of chymotrypsin but not SGPA.

INTRODUCTION

Chymostatin, a tetrapeptide analogue from culture filtrates of *Streptomyces hygroscopicus* and *Streptomyces lavendulae*, is a powerful inhibitor of a number of proteinases including the serine proteinases, chymotrypsin and *Streptomyces griseus* proteinase A, and several cysteine proteinases (Umezawa *et al.*, 1979). The inhibitor has several unusual structural features (Fig. 1). A C-terminal aldehyde group is essential for inhibitory activity via formation of a hemiacetal or hemithioacetal adduct with the nucleophilic hydroxy or thiol group of the serine and cysteine proteinases. Secondly, a capreomycin residue occupies the position that would be defined as the P3 residue in a normal peptide, using the nomenclature of Schechter & Berger (1967). Additionally, a ureido linkage reverses the chain direction (Fig. 1). Although the main determinant of inhibitory activity is the C-terminal aldehydic group, it is not clear whether the other residues in chymostatin make major or minor contributions to the inhibitory potential of this molecule.

The chymostatin–*Streptomyces griseus* proteinase A (SGPA) complex has been analysed by X-ray crystallography (Delbaere & Brayer, 1985), and the main interactions between the proteinase and inhibitor have been identified. A covalent hemiacetal bond is formed between the active-site Ser-195 and the C-terminal aldehyde moiety, which contributes to the binding by its resemblance to the tetrahedral intermediate in proteolytic catalysis (Gorenstein & Shah, 1982). Secondly, the C-terminal phenylalanine residue of the inhibitor is bound to the primary specificity pocket of the enzyme. Thirdly, there is hydrophobic binding of the penultimate leucine to the small P2 pocket. Finally, hydrogen-bonding between the main chain of the inhibitor and part of the main chain of the enzyme stabilizes the interaction (Segal, 1972; Baumann *et al.*, 1973; Bauer *et al.*, 1976; Moffit & Means, 1978).

In common with several of the microbial proteinase inhibitors, chymostatin is relatively non-toxic and is well tolerated in a number of intact cell systems. It is an inhibitor of both the lysosomal and non-lysosomal pathways of protein degradation and can decrease rates of proteolysis in muscle fibres and hepatocytes (Grinde *et al.*, 1983; Mulligan *et al.*, 1985; Place *et al.*, 1987). Chymostatin is an inhibitor of the cytosolic Ca²⁺-

dependent proteinase calpain (Hopgood *et al.*, 1989), and some of the activities of the cytosolic multicatalytic proteinases (Mason, 1990; Dick *et al.*, 1991) that may play an essential role in antigen presentation (Ortiz-Navarette *et al.*, 1991; Martinez & Monaco, 1991; Kelly *et al.*, 1991) and in the degradation of ubiquitin–protein conjugates (Rivett, 1989).

We prepared a series of chymostatin analogues (Galpin *et al.*, 1984; Fig. 1). The C-terminal Phe-aldehyde was retained, but the remainder of the structure was modified. The hydrophobic phenylalanine residue would be expected to bind to the S1 pocket, in accordance with the known primary-sequence specificities of chymotrypsin and SGPA, favouring aromatic groups (phenylalanine, tyrosine, tryptophan) in P1 (Matthews *et al.*, 1967; Steitz *et al.*, 1969; Dupaix *et al.*, 1970). Additionally,

Fig. 1. Chymostatin and analogues prepared and used in this investigation

Abbreviations used: Z, benzyloxycarbonyl; SGPA *Streptomyces griseus* proteinase A; SAAPFNA, succinyl-Ala-Ala-Pro-Phe-4-nitroanilide; Phe-H, phenylalanyl aldehyde; Nle, norleucine; cyc, cyclic; note that 1 Å ≡ 0.1 nm.

* This paper is dedicated to the memory of Ian Galpin.

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a hydrophobic amino acid such as leucine should contribute to favourable binding in P2, which can accommodate medium-sized aliphatic hydrophobic groups optimally (Moffit & Means, 1978). Fewer data are available for P3, P4 and beyond. In this study, we have examined in greater detail the inhibition kinetics of this series of analogues and have modelled the binding of some of them to chymotrypsin, on the basis of the known crystal structure of chymostatin bound to SGPA.

MATERIALS AND METHODS

Bovine α -chymotrypsin was purchased from Sigma Chemical Co., Poole, Dorset, U.K., and succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (SAAPFNA) from Cambridge Research Biochemicals, Cambridge, U.K. Pronase was supplied by BDH Ltd., Poole, Dorset, U.K. Chymostatin was supplied by the Peptide Research Institute, Osaka, Japan. General reagents were purchased from Fisons, Loughborough, Leics., U.K., and Aldrich Chemical Co., Gillingham, Dorset, U.K. The synthetic chymostatin analogues were prepared by I. Galpin, S. Norman, J. Chetland and A. Wilby as described previously (Norman, 1989).

Purification of SGPA

SGPA was purified from Pronase by the method of Narahashi (1970). Pronase is a partially purified isolate obtained from the culture broth of *Streptomyces griseus* K-1. It is a mixture of proteinases and as such is non-specific. The crude enzyme preparation was purified by two ion-exchange steps and the procedure was monitored by A_{280} to detect protein, and by assay of proteolytic activity against the substrate SAAPFNA (Jurávek *et al.*, 1971; Bauer *et al.*, 1976).

Assay of chymotrypsin

A stock of chymotrypsin ($1 \mu\text{M}$) was prepared in 1 mM-HCl by using the absorption coefficient $\epsilon_{280} = 50000 \text{ cm}^{-1} \cdot \text{M}^{-1}$. This solution ($20 \mu\text{l}$) was incubated for 10 min at 25°C with $20 \mu\text{l}$ of dimethyl sulphoxide as control or with $20 \mu\text{l}$ of inhibitor/dimethyl sulphoxide solution, in 1 ml of $50 \text{ mM-Hepes}/10 \text{ mM-CaCl}_2$ at pH 8.0 in a spectrophotometer cuvette. Dimethyl sulphoxide alone did not affect the rate of hydrolysis. The reaction was started by addition of one of five dilutions of substrate (SAAPFNA; Delmar *et al.*, 1979) in $20 \mu\text{l}$ of dimethyl sulphoxide, and the initial rate of 4-nitroaniline production was observed at 405 nm. The final concentration of enzyme was 20 nM , and the substrate concentration varied between 37.5 and $300 \mu\text{M}$. The substrate concentration was confirmed by the A_{405} of a completely hydrolysed sample.

Assay of SGPA

The assay was carried out as for chymotrypsin. A stock solution of SGPA ($2.3 \mu\text{M}$) was prepared in 10 mM-Hepes (pH 8.0)/ 10 mM-CaCl_2 by using $\epsilon_{280} = 15500 \text{ cm}^{-1} \cdot \text{M}^{-1}$ (Bauer & Peterson, 1974). A stock solution ($20 \mu\text{l}$) was added to the assay to give a final concentration of 46 nM . The stock solution was kept on ice throughout the assay.

Estimation of kinetic constants

Using the absorption coefficient for 4-nitroaniline, $\epsilon_{405} = 9960 \text{ cm}^{-1} \cdot \text{M}^{-1}$, the initial rates of absorbance change were converted into initial velocities of product formation. Values of K_m and V_{max} were obtained from the initial velocities, at a range of substrate concentrations, by using the non-linear curve-fitting program, P.Fit, published by Biosoft (22 Hills Road, Cambridge, U.K.). The values for K_i for each inhibitor were obtained from a

secondary plot of K_m/V_{max} as a function of inhibitor concentration.

Molecular modelling

All models were built from initial crystal structures by using the program QUANTA ver 3.0 by Polygen (Europe) Ltd. (Reading, Berks., U.K.) run on a Silicon-Graphics 4D/20G Personal Iris workstation.

RESULTS AND DISCUSSION

Seventeen inhibitors were tested, along with chymostatin (Fig. 1). For all inhibitors, kinetic constants were determined at five substrate concentrations and four inhibitor concentrations; each velocity measurement was made in duplicate or triplicate. Representative data for a relatively good inhibitor (chymostatin inhibition of SGPA) and a relatively weak inhibitor (Z-Nle-Leu-Phe-H inhibition of chymotrypsin) are shown in Fig. 2. The data are plotted with superimposed fitted hyperbolic curves derived from non-linear curve fitting. Similar-quality data were derived from all other inhibitors (results not shown). Except for chymostatin inhibition of chymotrypsin, the data are clearly indicative of pure competitive inhibition. This is illustrated by plots of

Fig. 2. Inhibition of (a) SGPA by chymostatin and (b) chymotrypsin by Z-Nle-Leu-Phe-H

The data are representative of the analysis of each inhibitor that has been used in this study. At each of five substrate and four inhibitor concentrations, two or three determinations of reaction velocity were made in a 1.0 ml reaction volume. The average velocities were plotted, and superimposed on these data are the hyperbolic curves corresponding to the values of V_{max} and K_m estimated by non-linear curve fitting. The fitted values of V_{max} and K_m were then used in secondary plots to derive K_i .

Fig. 3. Inhibition of chymotrypsin by Z-Nle-Leu-Phe-H
Chymotrypsin (50 nM) was preincubated with Z-Nle-Leu-Phe-H at 0–4 μM for 10 min, at which time the assay of uninhibited enzyme was initiated by addition of substrate to different final concentrations. The reaction velocity (v) was determined, and the data are plotted as [substrate]/velocity ($[S]/v$) as a function of inhibitor concentration ($[I]$) at each substrate concentration. Superimposed on the data are the lines derived by linear regression of the $[S]/v$ (f) $[I]$ data.

Fig. 4. Determination of the inhibition constant for inhibition of SGPA by chymostatin and of chymotrypsin by Z-Nle-Leu-Phe-H
Chymotrypsin (50 nM) was preincubated with Z-Nle-Leu-Phe-H at 0 to 4 μM for 10 min, at which time the assay of uninhibited enzyme was initiated by addition of substrate to different final concentrations (■). K_m and V_{max} were determined at each inhibitor concentration, and used to generate secondary plots of K_m/V_{max} as a function of $[I]$. Linear-regression analysis of the data yielded K_i . A similar inhibition analysis is shown for SGPA and chymostatin (□).

[substrate]/velocity as a function of inhibitor concentration at each of the five substrate concentrations (Fig. 3). The family of best-fit lines are clearly parallel to each other, confirming that the inhibitor is acting as a purely competitive ligand. Similar data were obtained for all other inhibitors (results not shown). For chymostatin inhibition of chymotrypsin there is evidence for a small contribution of non-competitive binding, and the inhibition constants for both competitive (K_i) and non-competitive components ($K'_i = 0.02 \mu\text{M}$) were derived. No other inhibitor showed any evidence of mixed inhibition. The values for K_i were determined by linear-regression analysis of a plot of K_m/V_{max} as a function of inhibitor concentration (Fig. 4).

Inhibition equilibrium constants (K_i) for the binding of the inhibitors to chymotrypsin and SGPA are summarized in Table 1. All non-aldehydes bind less strongly than their aldehydic equivalents, confirming the contribution of the aldehyde to serine proteinase inhibition and to efficient binding. For example, Z-Arg-Leu-Phe-H binds to chymotrypsin ($K_i = 0.24 \mu\text{M}$ approx.

Table 1. Inhibition constants for chymostatin and synthetic analogues towards chymotrypsin and SGPA

n.d., not determined.

Inhibitor	K_i (μM)	
	Chymotrypsin	SGPA
Chymostatin	0.015	0.26
Z-Phe-H	30.5	n.d.
Z-Leu-Phe-H	5.21	2.83
Z-Ile-Phe-H	5.84	4.70
Z-Val-Phe-H	20.0	4.44
Z-Arg-Leu-Phe-H	0.24	0.2
Z-Arg-Ile-Phe-H	0.36	0.30
Z-Arg-Val-Phe-H	1.24	n.d.
Z-Nle-Leu-Phe-H	0.82	n.d.
Z-Nle-Ile-Phe-H	2.0	n.d.
Z-Nle-Val-Phe-H	1.21	n.d.
Z-Arg-Leu-Phe-semicarbazone	10.5	n.d.
Z-Arg-Val-Phe-semicarbazone	55.1	n.d.
Z-Nle-Leu-Phe-amide	14.7	n.d.
Z-Nle-Leu-Phe-alcohol	13.6	n.d.
Z-Arg-Leu-Phe-imidate	8.14	n.d.
Z-Asp-Leu-Phe-imidate	39.0	n.d.
Z-Nle-Leu-cyc-o-Tyr	7.11	n.d.

50 times more efficiently than does the corresponding semicarbazone or imidate ($K_i = 10.5 \mu\text{M}$ and $8.1 \mu\text{M}$ respectively). Similar behaviour is seen with Z-Nle-Leu-Phe-H ($K_i = 0.8 \mu\text{M}$) and the corresponding amide or alcohol ($K_i = 14.7 \mu\text{M}$ and $13.6 \mu\text{M}$ respectively). Semicarbazones, which also bind cysteine proteinases more weakly, are effective affinity ligands (Rich *et al.*, 1986).

The tripeptide aldehydes are at least an order of magnitude more active than the dipeptides. The difference in activity between the P3 norleucyl-tripeptides and the dipeptides may be attributable to main-chain hydrogen-bonding between the CO and NH groups of the third inhibitor residue (norleucine) and the peptide backbone of the enzyme. This is inferred from results obtained by Moffitt & Means (1978) and Segal (1972) that showed a difference in binding constants of a similar magnitude between P3 glycyl-tripeptide and -dipeptide substrates, indicating the lack of any contribution to binding from the P3 side-chain residue. However, replacement of norleucine in P3 by arginine leads to a small but significant increase in binding, when the P2 group is either leucine or isoleucine. Interestingly, this does not seem to be the case when the P2 residue is valine. The data for Z-Arg-Leu-Phe-imidate and Z-Asp-Leu-Phe-imidate show that aspartic acid in the P3 position is detrimental to inhibition, indicating that the enhanced binding on replacement of a norleucine by arginine in P3 is probably not due to simple hydrophilicity of the arginine group but that side-chain length or basicity may be responsible. A study of lysine or glutamate derivatives should be informative in this context. Finally, the Z-Nle-Leu-cyc-o-Tyr structure, although reasonably inhibitory ($K_i = 7 \mu\text{M}$), is not much superior to Z-Nle-Leu-Phe-alcohol or Z-Nle-Leu-Phe-amide. Constraint of the carbonyl group by incorporation into a cyclic structure did not seem to favour binding or inhibition.

Chymostatin behaves as a significantly more potent inhibitor of chymotrypsin than does Z-Arg-Leu-Phe-H, the closest structural analogue. Moreover, the inhibition data are consistent with

Fig. 5. Conformations of chymostatin and Z-Arg-Leu-Phe-H fitted to the active sites of chymotrypsin and SGPA

The crystallographic structure of chymostatin bound to SGPA was used to generate the conformation of chymostatin and as a basis on which to build the structure of Z-Arg-Leu-Phe-H. For the remaining three structures, the inhibitor was docked into the active site of the corresponding proteinase and minimized. (a) Chymostatin bound to chymotrypsin; (b) Z-Arg-Leu-Phe-H bound to chymotrypsin; (c) chymostatin bound to SGPA; (d) Z-Arg-Leu-Phe-H bound to SGPA. (c) is derived from a crystallographic study; the other three are generated by molecular mechanics. The inhibitor structure is highlighted in bold, and Lys-175 in chymotrypsin is indicated by a dashed line.

chymostatin being a mixed inhibitor, whereas Z-Arg-Leu-Phe-H is purely competitive. By contrast, the same two inhibitors are equally potent against SGPA and both are pure competitive inhibitors of this enzyme ($K_i = 0.24 \mu\text{M}$ and $0.20 \mu\text{M}$ respectively). This implies that there are interactions between chymostatin and chymotrypsin that enhance the binding and which are not characteristic of binding to SGPA. Whatever is responsible for the strong chymostatin–chymotrypsin interaction over and above that present in Z-Arg-Leu-Phe-H–chymotrypsin must result from a feature(s) unique to chymostatin (i.e. missing from Z-Arg-Leu-Phe-H) interacting with corresponding structural feature(s) on the chymotrypsin molecule. This additional interaction must be unique to chymotrypsin, as chymostatin is no better an inhibitor

of SGPA than is Z-Arg-Leu-Phe-H. Initial docking studies suggested that a site of this interaction could be the 170–175 helical loop on chymotrypsin and the *N*-terminal phenylalanine residue on chymostatin; these are in close proximity in the modelled chymostatin–chymotrypsin structure. The loop is not present in SGPA, and the *C*-terminal phenylalanine residue, with its carboxylate group, is absent from Z-Arg-Leu-Phe-H. All the other binding sites from S1 to S3 appear to be similar for both enzymes. In both enzymes, S1 is a deep hydrophobic pocket of optimal size for binding six-membered aromatic rings, S2 is a shallow hydrophobic pocket capable of accepting isopropyl or similar-sized side chains and S3 is primarily exposed to solvent. Main-chain hydrogen-bonding between enzyme and ligand is

also similar for both SGPA and chymotrypsin binding of tripeptides.

Loop 170–175 is a relatively basic peptide, the sequence being Lys(169)-Lys(170)-Tyr(171)-Trp(172)-Gly(173)-Thr(174)-Lys(175)-Ile(176)-Lys(177), which might suggest an interaction between one or more of these basic residues with the distal carboxylate group of chymostatin. Although this would be mainly a surface interaction surrounded by water, it is the most likely explanation for the increased strength of binding. This is supported by studies showing that acidic *N*-terminal groups favour substrate binding and acylation in substrates of similar length to these inhibitors (Delmar *et al.*, 1979; Fischer *et al.*, 1984; Tözsér *et al.*, 1986).

To investigate the binding of chymostatin to chymotrypsin further, a docking study was performed, using published X-ray structures and starting from the co-ordinates of chymotrypsin (Tsukada & Blow, 1985; Tulinsky & Blevins, 1987; 4CHA, 6CHA, October 1990 version) and the co-ordinates of SGPA–chymostatin (Delbaere & Brayer, 1985; 1SGC, October 1990 version) obtained from the Protein Data Bank (Bernstein *et al.*, 1977; Abola *et al.*, 1987). The structure of chymostatin was excised from chymostatin–SGPA and docked into the crystal structure of chymotrypsin. It was not necessary to modify the enzyme in order to dock the P1, P2 and P3 side chains and the main chain of the inhibitor to chymotrypsin. The P1–S1 interaction and the hemiacetal were modelled first, avoiding close contacts. The P2 and P3 groups were then fitted, again using the chymostatin–SGPA crystal structure as a guide. However, the P4 side chain could not adopt the same conformation as in chymostatin–SGPA owing to the presence of Trp-172 blocking this position. Rotation about the CA–CB and CB–CG bonds of the P4 phenylalanine side chain brought the benzene ring up and forward away from close contacts. This was aided by a small rotation of the CB–CG bond of Trp-172. There was no obstruction to this movement.

This structure was refined by energy minimization by using the molecular mechanics package, CHARMM (version 21.2; Brooks *et al.*, 1983) with the commercial parameter set PARM30.PRM. The structure was inspected and plotted by using the molecular graphics package, QUANTA (version 3.0) which also acted as the interface to CHARMM. Constraints between the P2 leucine and residue Ile-95 of chymotrypsin [CD5(Leu)–CG2(Ile) 3.5 ± 0.1 Å (0.35 ± 0.01 nm in S.I. units), CD4(Leu)–CD(Ile) 4.0 ± 0.1 Å] and between the hemiacetal oxygen and the corresponding oxyanion pocket hydrogens [residues 193 and 195 N–H (2.5 ± 0.3 Å)] (scale 20.0, temperature 300 K) were necessary to maintain the docked structure in the initial stages of the minimization. These constraints were gradually decreased to zero during the minimization. The protein was held in place in the early stages using a force constant of 84 kJ/mol per Å but this was also gradually decreased to zero during the minimization. After 500 steps of unconstrained Adopted Basis-set Newton–Raphson (ABNR) minimization using a dielectric permittivity $\epsilon = r$ (the interatomic distance in Å), and a shifted non-bonded cut-off of 12.0 Å and updating the non-bonded list every 20 steps, the energy of the molecule was -24229.4 kJ and the derivative of the energy was less than 2.1 kJ/mol per Å.

This structure was modified to give Z-Arg-Leu-Phe-H–chymotrypsin which was then minimized. In both enzymes the Z-group benzene ring of Z-Arg-Leu-Phe-H could fit into the space occupied by the P4 phenylalanine ring of chymostatin. The structure of chymostatin–SGPA was modified to give Z-Arg-Leu-Phe-H–SGPA. These two structures were also minimized. This gave four structures; the crystal structure of chymostatin–SGPA and the modelled structures of Z-Arg-Leu-Phe-H–SGPA, chymostatin–chymotrypsin and Z-Arg-Leu-Phe-H–chymo-

Fig. 6. Interaction between chymostatin and chymotrypsin

The minimized structure of chymostatin bound to chymotrypsin is presented in greater detail to show the potential salt bridge between the carboxylate group of the reversed phenylalanine residue in the inhibitor and Lys-175. This interaction is indicated with an arrow. The main chain of chymotrypsin is indicated by the shaded spheres; the interaction between the P1 phenylalanine residue of the inhibitor and the specificity pocket of chymotrypsin is clear.

trypsin. Of these, chymostatin–chymotrypsin alone contains the interaction between the *N*-terminal carboxy group of the inhibitor and the ammonium group of Lys-175, which improved binding (Fig. 5).

Fig. 6 shows the docked and minimized chymostatin–chymotrypsin structure in greater detail. The *N*-terminal carboxy group sits in a cleft formed by residues 216–218 and Trp-172. Lys-175 can be manipulated to within 2.0 Å of this acidic group. The CO_2^- – NH_3^+ interaction is arrowed. This interaction is not present in the other three structures owing to the absence of the 170–175 loop (including Lys-175) in SGPA and the *N*-terminal carboxy group in Z-Arg-Leu-Phe-H. This single interaction may be responsible for the greater stability of chymostatin–chymotrypsin over and above that of Z-Arg-Leu-Phe-H–chymotrypsin.

The inhibition of chymotrypsin by chymostatin can be broken down into four subsite interactions plus main-chain hydrogen-bonding and covalent hemiacetal formation. The synthetic inhibitors presented here can form these interactions in various stages of completeness. The most active of the synthetic inhibitors is Z-Arg-Leu-Phe-H, which can form all the interactions that chymostatin can except for the presence of an *N*-terminal (P4) carboxy group that can interact with lysine residue(s) on the enzyme. These residues are not present in SGPA and consequently chymostatin is not such a powerful inhibitor of this enzyme.

All other interactions appear to be the same for the two enzymes, chymotrypsin and SGPA, and for the two best inhibitors, chymostatin and Z-Arg-Leu-Phe-H. Therefore a suggestion for a good chymotrypsin inhibitor would be *N*-succinyl-Ala-Leu-Phe-H. This would have the most important features for chymotrypsin inhibition, namely the *C*-terminal aldehyde, the aromatic P1 group, the isopropyl P2 group, a tripeptide

backbone for hydrogen-bonding to the enzyme and the *N*-terminal P4 carboxy group, and would avoid the synthetically troublesome arginine residue, which only contributes marginally to inhibition. The mixed inhibition of chymotrypsin by chymostatin may be due to the resemblance of the *N*-terminal phenylalanine to the hydrolysis product resulting in pseudo-product inhibition at a non-competitive site. This is possibly the same site for non-competitive inhibition observed by Smith & Hansch (1973) for hydrophobic molecules with ionic substituents. Whatever is responsible for this non-competitive inhibition is clearly not present in SGPA.

One of the phenylalanine aldehyde inhibitors examined in this study, X-Val-Phe-H, has been synthesized by another group (and termed MD 28170) and is reported to be an effective inhibitor of calpain (Medhi *et al.*, 1988) and a weak inhibitor of chymotrypsin (54 μM ; Medhi, 1991). Our data yield a K_i of 20 μM for this interaction, but the assay conditions are not identical. MS 28170 is thought to be a cell-penetrant inhibitor and thus offers an opportunity for inhibition *in vivo* of intracellular proteolysis. We expect that some of our analogues might also be sufficiently hydrophobic to be cell-permeant and thus may also find utility in such applications. Indeed, our data on inhibition of hepatocyte (Grinde *et al.*, 1983; Place *et al.*, 1987) and muscle (Mulligan *et al.*, 1985) proteolysis lend weight to this argument.

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