Cysteine Protease Inhibition by Azapeptide Esters†

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Papain, a prototype cysteine protease, was inhibited in a time-dependent manner by azapeptide esters designed to deliver an azaglycine group to the active-site thiol. For example, the rate of inhibition was 18 M^{-1} s^{-1} for Ac-L-PheAglyOiBu (2) and >11 000 M^{-1} s^{-1} for Ac-L-PheAglyOPh (7). The rate of inhibition was slowed in the presence of substrate, and there was no reactivation of the inhibited enzyme after dialysis and incubation in the assay buffer. The inhibited enzyme was completely reactivated after the addition of valine methyl ester. The inhibited form of the enzyme is presumed to be acylated on the active-site thiol. An azaalanine-based peptide inhibited papain much more slowly. Azapeptide alkyl esters are unreactive with serine proteases; therefore, these inhibitors are selective for cysteine proteases.

Introduction

Cysteine proteases serve important functions in health and disease.[1] Examples of these functions include normal cellular protein turnover, cancer metastasis,[2] and viral replication.[3] As a result, inhibitors specific for each protease would be useful physiological probes and possess therapeutic potential. One of the challenges in developing selective cysteine protease inhibitors[4] is the resemblance between the catalytic mechanism of this class of proteases and that of serine proteases.[5] Both types of enzyme employ an imidazole as a proton shuttle and a nucleophile (a cysteine thiol or a serine hydroxyl) which is transiently acylated during the process. These similarities notwithstanding, inhibitors which are selective for one or the other of these types of enzymes are known. Examples include peptidyl trifluoromethyl ketones which are selective for serine proteases[6] and peptidyl diazomethyl ketones which react almost exclusively with cysteine proteases.[4c,d] Those differences are not easily explained, but it is intuitively reasonable that peptidyl (acyloxy)methyl ketones selectively inactivate cysteine proteases due to the greater nucleophilicity of the cysteine thiol(ate) compared with the serine hydroxyl.[7] It occurred to us that the nucleophilicity of the thiol might also lead to selective reactions with azapeptides.

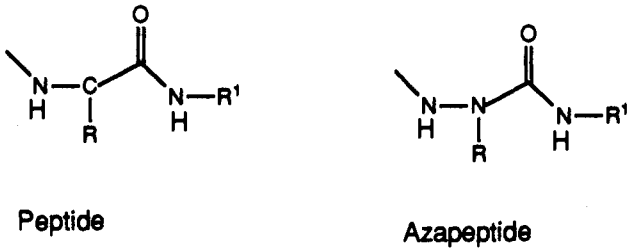

Figure 1. Structure of a peptide compared to an azapeptide.

Azapeptides are peptides in which one (or more) of the α-carbon(s) has been replaced by a nitrogen atom; formally, this is insertion of an azaamino acid (Figure 1).[8] Azapeptides that are designed to deliver the azaamino acid to the S_1[9] subsite of serine proteases, and which possess a good leaving group (e.g., phenol), are known to inhibit those proteases.[10] Presumably, the azapeptide acylates

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(1) For reviews, see: (a) Lowe, G. The Cysteine Proteinases. *Tetrahedron* 1976, *32*, 291–302. (b) Barrett, A. J.; McDonald, J. K. *Mammalian Proteases: A Glossary and Bibliography*, Vol. 1 *Endopeptidases*, Vol. 2 *Exopeptidases*; Academic Press: New York, 1980 and 1986. (c) Brocklehurst, K.; Willenbrock, F.; Salih, E. Cysteine Proteinases. In *Hydrolytic Enzymes*; Neuberger, A., Brocklehurst, E., Eds.; Elsevier: New York, 1987; p 139–58.

(2) For a review, see: Sloane, B. F.; Rozhin, J.; Hatfield, J. S.; Crissman, J. D.; Honn, K. V. Plasma Membrane-Associated Cysteine Proteinases in Human and Animal Tissues. *Exp. Cell Biol.* 1987, *55*, 209–24.

(3) For reviews, see: (a) Krausslich, H-G.; Wimmer, E. Viral Proteinases. *Annu. Rev. Biochem.* 1988, *57*, 701–54. (b) Hellen, C. U. T.; Krausslich, H-G.; Wimmer, E. Proteolytic Processing of Polyproteins in the Replication of RNA Viruses. *Biochemistry* 1989, *28*, 9881–90. (c) Lawson, M. A.; Semler, B. L. Picornavirus Protein Processing-Enzymes, Substrates and Genetic Regulation. *Curr. Top. Microbiol. Immun.* 1990, *161*, 49–87.

(4) For reviews, see: (a) Rich, D. H. Inhibitors of Cysteine Proteinases. In *Proteinase Inhibitors*; Barrett, A. J., Salvesen, G., Eds.; Elsevier: New York, 1986; pp 153–178. (b) Fischer, G. Trends in Protease Inhibition. *Nat. Prod. Rep.* 1988, 465–95. (c) Shaw, E. Cysteinyl Proteinases and Their Selective Inactivation. *Adv. Enzymol.* 1990, *63*, 271–347. (d) Rich, D. H. Peptidase Inhibitors. In *Comprehensive Medicinal Chemistry*; Hansch, C., Sammes, P. G., Taylor, J. B., Eds.; Pergamon: New York, 1990; Vol. 2, pp 209–24. (e) Demuth, H.-U. Recent Developments in Inhibiting Cysteine and Serine Proteases. *J. Enzyme Inhib.* 1990, *3*, 249–78. For a report of a new inhibitor, see: Singh, S. B.; Cordingley, M. G.; Ball, R. G.; Smith, J. L.; Dombrowski, A. W.; Goetz, M. A. Structure and Stereochemistry of Thysanone: a Novel Human Rhinovirus 3C-protease Inhibitor from Thysanophora Penicilloides. *Tetrahedron Lett.* 1991, *32*, 5297–82.

(5) For reviews, see: (a) Polgar, L. Current Problems in Mechanistic Studies of Serine and Cysteine Proteinases. *Biochem. J.* 1982, *207*, 1–10. (b) Baker, E. N.; Drenth, J. The Thiol Proteases: Structure and Function. In *Biological Macromolecules and Assemblies*; Wiley: New York, 1987; Vol. 3, pp 313–68. (c) Polgar, L. *Mechanisms of Protease Action*; CRC Press: Boca Raton, 1989; pp 123–156. (d) Reference 1, above. For a recent discussion, see: Arad, D.; Langridge, R.; Kollman, P. A. A Simulation of the Sulfur Attack in the Catalytic Pathway of Papain Using Molecular Mechanics and Semiempirical Quantum Mechanics. *J. Am. Chem. Soc.* 1991, *113*, 491–502.

(6) (a) Smith, R. A.; Copper, L. J.; Donnelly, S. L.; Spencer, R. W.; Krantz, A. Inhibition of Cathepsin B by Peptidyl Aldehydes and Ketones: Slow Binding Behavior of a Trifluoromethyl Ketone. *Biochemistry* 1988, *27*, 6568–73. (b) Angelastro, M.; Mehdi, S.; Burkhart, J. P.; Peet, N. P.; Bey, P. alpha-Diketone and alpha-Keto Ester Derivatives of N-Protected Amino Acids and Peptides As Novel Inhibitors of Cysteine and Serine Proteases. *J. Med. Chem.* 1990, *33*, 11–13.

(7) Krantz, A.; Copper, L. J.; Coles, P. J.; Smith, R. A.; Heard, S. B. Peptidyl (Acyloxy)methyl Ketones and the Quiescent Affinity Label Concept: The Departing Group as a Variable Structural Element in the Design of Inactivators of Cysteine Proteinases. *Biochemistry* 1991, *30*, 4678.

(8) For a review of azapeptide synthesis, see: Gante, J. Azapeptides. *Synthesis* 1989, 405–13. Azaamino acids are designated in a peptide sequence by a four-letter abbreviation starting with the letter "A" followed by the normal three-letter code for the corresponding amino acid. For example, azaglycine is abbreviated as Agly.

(9) We use the binding-site terminology of Berger and Schechter in which the carboxyl side of the scissile bond is termed the P_1 residue, and its corresponding enzyme subsite is called S_1: Berger, A.; Schechter, I. Mapping the Active Site of Papain with the aid of Peptide Substrates and Inhibitors. *Phil. Trans. R. Soc. London B* 1970, *257B*, 249–64.

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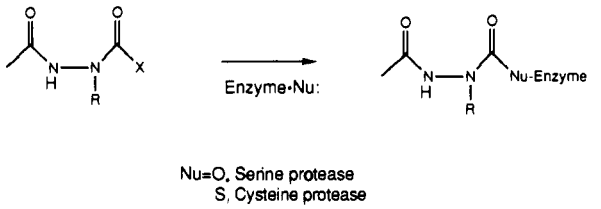
Scheme I. Generation of Acyl-enzyme from Azapeptide



the enzyme (Scheme I) but this intermediate is more resistant to hydrolysis, for electronic and steric reasons, than the normal acyl-enzyme intermediate. To date, the evidence for the putative acyl-enzyme intermediate formed from the azapeptides consists of the following: (1) slow reactivation (i.e., deacylation) of inhibited enzyme, and faster reactivation in the presence of added nucleophiles, has been observed, and (2) azapeptides with chromogenic leaving groups (e.g., *p*-nitrophenolates) release the chromophore in 1:1 stoichiometry with enzyme inactivation; indeed, they can serve as active-site titrants.

In contrast to azapeptides with good leaving groups (e.g., X = *p*-nitrophenolate in Scheme I), those with poor leaving groups (e.g., ethoxide) are poor inhibitors of serine proteases.[11] For comparison, four out of five attempts[10d,e,11a] to inhibit serine proteases with alkyl esters resulted in no reaction; the fifth case yielded an inhibition rate of 0.64 M^{-1} s^{-1} (see also ref 10b, in which an azapeptide ethyl ester inactivated chymotrypsin with a rate of 0.019 M^{-1} s^{-1} at 37 °C). Unfortunately, the good leaving groups that are required for reaction with serine proteases also result in high rates of undesired side reactions in buffer solution. For example, the half-life for azapeptide *p*-nitrophenolates at pH = 7 is on the order of a few (8–20) minutes.[10e] Moreover, the phenolic leaving groups should be of little use for inhibiting proteases, such as viral proteases, that are specific for certain amino acids in the S′ binding subsites. Azapeptide alkyl esters, on the other hand, could be designed with alkyl groups that satisfy the S′ binding requirements, making them the most peptide-like inhibitors available for cysteine proteases. They are said to be stable in solution[11a] and should be unreactive with serine proteases, but as mentioned above, they may react with cysteine proteases.

Papain is often used as the prototype for cysteine proteases; herein we report that it is inhibited by azapeptide alkyl esters. This is the first report of inhibition of a cysteine protease by an azapeptide.

(10) (a) Elmore, D. T.; Smyth, J. J. A New Method for Determining the Absolute Molarity of Solutions of Trypsin and Chymotrypsin by Using p-Nitrophenyl N^2-Acetyl-N^1-benzylcarbazate. *Biochem. J.* **1968**, *107*, 103–7. (b) Barker, S. A.; Gray, C. J.; Ireson, J. C.; Parker, R. C. The Reaction of an alpha-Aza-amino Acid Derivative with Chymotrypsin and Its Use as a Ligand for Covalent Affinity Purification. *Biochem. J.* **1974**, *139*, 555–63. (c) Gray, C. J.; Al-Dulaimi, K.; Khoujah, A. M.; Parker, R. C. N-β-Ethoxycarbonyl-α-Azaornithine Phenyl Ester: a Stable Inhibitor of Trypsin and Thrombin. *Tetrahedron* **1977**, *33*, 837–40. (d) Gupton, B. F.; Carroll, D. L.; Tuhy, P. M.; Kam, C-M.; Powers, J. C. Reaction of Azapeptides with Chymotrypsin-like Enzymes. *J. Biol. Chem.* **1984**, *259*, 4279–87. (e) Powers, J. C.; Boone, R.; Carroll, D. L.; Gupton, B. F.; Kam, C-M.; Nishino, N.; Sakamoto, M.; Tuhy, P. M. Reaction of Azapeptides with Human Leukocyte Elastase and Porcine Pancreatic Elastase. *J. Biol. Chem.* **1984**, *259*, 4288–94.

(11) (a) Kurtz, A. N.; Niemann, C. The Interaction of Ethyl 1-Acetyl-2-benzylcarbazate with α-Chymotrypsin. *J. Am. Chem. Soc.* **1961**, *83*, 1879–82. (b) Dutta, A. S.; Giles, M. B. Polypeptides. Part XIV. A Comparative Study of the Stability Towards Enzymes of Model Tripeptides Containing α-Aza-amino-acids, L-Amino-acids and D-Amino-acids. *J. Chem. Soc., Perkin Trans. 1* **1976**, 244–8. (c) References 10b,d,e. (d) Dorn, C. P.; Zimmerman, M.; Yang, S. S.; Yurewicz, E. C.; Ashe, B. M.; Frankshun, R.; Jones, H. Proteinase Inhibitors. 1. Inhibitors of Elastase. *J. Med. Chem.* **1977**, *20*, 1464–8.

Table I. Rate of Inhibition of Papain by Azapeptide Esters

compd	k_i/K_i (M^{-1} s^{-1})	compd	k_i/K_i (M^{-1} s^{-1})
1	13	3	0.02[a]
2	18	7	>11000

[a] Estimated from the rate at 37 °C.

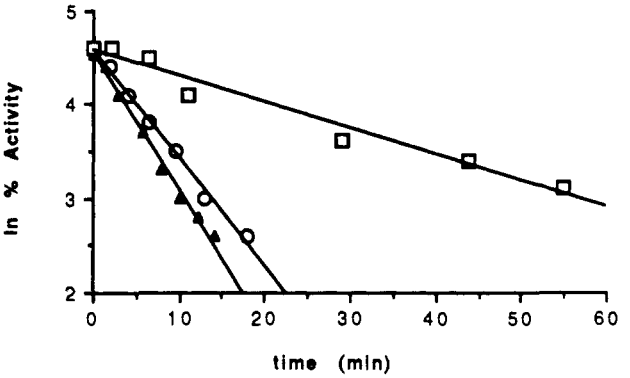


Figure 2. Inhibition of papain (2 μM) by Ac-L-PheAglyOiBu (2) at 25 °C (see Experimental Section for details). The enzyme was incubated with the inhibitor, and aliquots were withdrawn, at the indicated times, for activity assay. The inhibitor concentrations were 32 μM (squares), 126 μM (circles), and 158 μM (triangles).

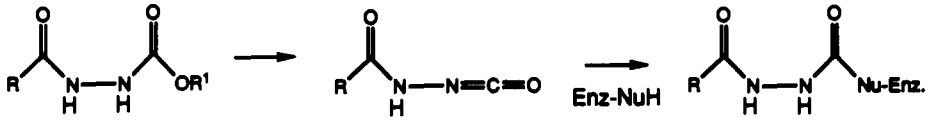
Results and Discussion

The prototype papain inhibitor Ac-L-PheAglyOMe (1) was designed to capitalize on the affinity of the papain S_2 subsite for a large hydrophobic residue, which delivers the azaglycine carbonyl to the active-site thiol. This compound inhibits papain in a time-dependent fashion with a second-order rate constant of 13 M^{-1} s^{-1} at 25 °C (the inhibitory rate constants for the compounds reported here are collected in Table I). Thus, 1 is a lead compound that could react selectively with cysteine proteases in the presence of serine proteases.

We sought to increase the rate of inhibition by enhancing binding. It was hoped that the isobutyl ester Ac-L-PheAglyOiBu (2) would be more hydrophobic and would benefit from some increased binding in the S_1' subsite. A modest (40%) improvement in the second-order inactivation rate was recorded (18 M^{-1} s^{-1}) compared to 1; the data are shown in Figure 2. Over the accessible concentration range the slope of the lines is proportional to inhibitor concentration. Therefore K_i (reversible) for the inhibitor must be >1 mM. Inhibition is slowed by 48% in the presence of the substrate at a concentration approximately equal to K_M; that is, substrate protection is observed (data not shown).

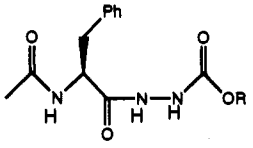
After determining the inhibition rate, presumably the rate of acylation of the active-site thiol, it was of interest to determine the reactivation (deacylation) rate of the inhibited enzyme. As described in the Experimental Section, the enzyme was inhibited 87% with approximately a 2-fold excess of 2. (The reaction was stopped at this point in order to furnish a positive control for the presence of enzyme after dialysis; it is possible to fully inactivate papain with 2 equiv of 2). Then the excess inhibitor was removed by dialysis. Over the course of 18 h, the inhibited enzyme activity remained constant compared to an identically treated control of active enzyme. Thus, the deacylation rate, if it occurs, has a half-time that is greater than or equal to 118 h (based on the conservative assumption that we could only detect a 10% increase in activity). In order to get some indication that the inhibited

Scheme II. Alternative Isocyanate Reaction Mechanism

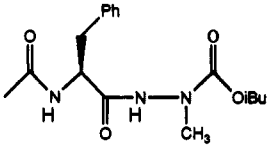


enzyme is acylated, we investigated the ability of added nucleophiles to reactivate the enzyme. We found that hydroxylamine, even at 90 mM, was unable to deacylate the enzyme; however, valine methyl ester (90 mM) could achieve complete reactivation in 2 h. This is in accord with Bender's early observations[12] concerning the reactivation of cinnamoylpapain. The greater effectiveness of the amine, compared with that of the prototypical strong nucleophile (hydroxylamine), is thought to be due to the binding of the hydrophobic moiety in the S_1' pocket which increases its effective concentration and poises it for reaction. Therefore, we feel that the observed reactivation of the inhibited enzyme is consistent with formation of an acyl enzyme which is too stable to be deacylated by water.

The next inhibitor that we tested was Ac-L-PheAalaOiBu (**3**). It was hoped that this compound would be significantly more hydrophobic than **1** or **2**, and it should benefit in its reaction with papain from the fact that papain has a small preference for alanine over glycine at S_1.[13] This



1 R=methyl
2 R=isobutyl
7 R=phenyl



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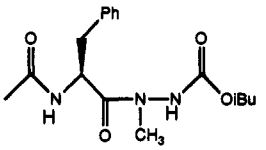

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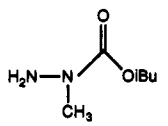

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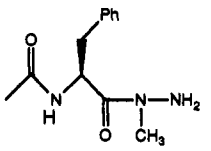

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compound, **3**, failed to inhibit papain detectably in 2 h under conditions in which compound **2** had a half-time for inhibition of a few minutes. Papain was slowly inhibited by **3**, with 45% inhibition observed in 48 h at 37 °C (from which we derive an approximate rate constant of 0.02 M^{-1} s^{-1} at 25 °C; data not shown). This result was unexpected, and we considered several possible explanations. First, we noted the possibility that the structure of **3** was incorrectly assigned; it could be the isomer with the methyl group on the β-nitrogen (an azasarcosine derivative, **4**). The structural assignment of **3** is fully consistent with precedent concerning its preparation, including the characterization of the intermediate carbazate **5**.[14] However, we prepared the isomer **4**, via the intermediate hydrazide **6**, for comparison. The preparations of **5** and **6** are based on the greater nucleophilicity of the substituted nitrogen of methylhydrazine. The structures are confirmed by the proton NMR chemical shifts of the *N*-methyl groups of the hydrazide and carbazate intermediates which are downfield (above 3.0) compared to the shifts expected for the other isomers (below 3.0). We also note that the NMR chemical shift for the α proton of phenylalanine in **4** and **6** at 5.2 ppm is downfield from the corresponding resonance for **1**, **2**, or **3** (ca. 4.8 ppm), which we feel is consistent with the assigned structure. We found that **4** does not inhibit papain either.[15] Thus, even in the unlikely event that the structures of **3** and **4** are reversed, we can still conclude that Ac-L-PheAalaOiBu is not an inhibitor of papain.

We attempted to accelerate the inhibition reaction by using a phenyl ester, which is inherently more reactive than the alkyl esters that we first investigated.[10c,d,e] As expected, Ac-L-PheAglyOPh (**7**) is a rapid inhibitor of papain (the rate was not accurately determined, but it is greater than 11 000 M^{-1} s^{-1}). The inhibited enzyme is thought to be the same as that derived from the isobutyl ester **2** because it does not reactivate spontaneously, but it is fully reactivated when treated with valine methyl ester. This observation was not investigated further because such esters could exhibit cross-reactivity with serine proteases. Moreover, this compound does not resemble a normal substituent at P_1' and was prone to decompose with the elimination of phenol.[10c]

Returning to the question of why the azaglycine-based peptide was more effective than the azaalanine-based peptide for inhibiting papain, we note that for azaglycine esters there is an alternative to the direct acylation mechanism which is not available to azaalanine. This is an isocyanate (E1cb) mechanism, which is depicted in Scheme II.[16] Such a reaction is observed in solution only for good leaving groups, whereas the alkyl esters involved here are poor leaving groups. However, we cannot dismiss the possibility that the enzyme is fortuitously able to

(12) (a) Brubacher, L.; Bender, M. L. The Preparation and Properties of *trans*-Cinnamoyl-Papain. *J. Am. Chem. Soc.* **1966**, *88*, 5871. See also: (b) Alecio, M. R.; Dann, M. L.; Lowe, G. The Specificity of the S_1' Subsite of Papain. *Biochem. J.* **1974**, *141*, 495.

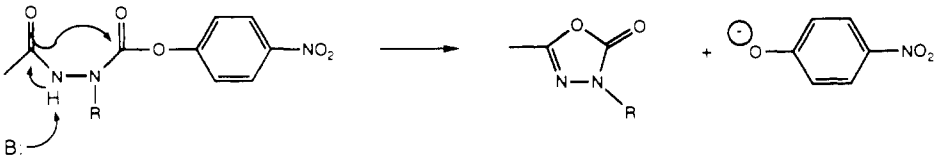
(13) (a) Reference 9. (b) For a recent comparison of alanine versus glycine at the P_1 site, see: Tonge, P. J.; Menard, R.; Storer, A. C.; Ruzsicska, B. P.; Carey, P. R. Markedly Different Acyl Papain Structures Deacylate at the Same Rates: Resonance Raman Spectroscopic and Kinetic Evidence. *J. Am. Chem. Soc.* **1991**, *113*, 4297–303.

(14) (a) Condon, F. E. Some Aspects of the Selective Acetylation of Methylhydrazine. 1-Acetyl-1-methyl- and 1-Acetyl-2-methylhydrazine. *J. Org. Chem.* **1972**, *37*, 3608–13. (b) Smith, P. A. S. *Derivatives of Hydrazine and Other Hydronitrogens Having N–N Bonds*; Benjamin/Cummings: Reading, MA, 1986.

(15) This was anticipated because replacement of a P_2–P_1 amide link with an ester can reduce V/K for a normal substrate by a factor of 10 due, presumably, to the loss of the hydrogen bond donating ability (for a recent discussion, see Berti, P. J.; Faerman, C. H.; Storer, A. C. Cooperativity of Papain–Substrate Interaction Energies in the S_2–S_2' Subsites. *Biochemistry* **1991**, 1394–1402). Furthermore, one would expect the methyl group to be sterically excluded from a site that is designed to provide an optimal hydrogen bond, thus preventing azasarcosine from binding to the enzyme with the scissile bond oriented correctly.

(16) (a) Bender, M. L.; Homer, R. B. The Mechanism of the Alkaline Hydrolysis of p-Nitrophenyl N-Methylcarbamate. *J. Org. Chem.* **1965**, *30*, 3975–8. (b) Williams, A. Alkaline Hydrolysis of Phenyl N-Phenylcarbamates. Structure–Reactivity Relationships Consistent With an E1cB Mechanism. *J. Chem. Soc., Perkin Trans. 2* **1972**, 808. (c) Hegarty, A. F.; Frost, L. N. Isocyanate Intermediates in E1cb Mechanism of Carbamate Hydrolysis. *J. Chem. Soc., Chem. Commun.* **1972**, 500.

Scheme III. Probable Decomposition Mode for Azapeptide *p*-Nitrophenylates in Aqueous Solution



catalyze the elimination. If the isocyanate mechanism is operative, then the enzyme is very efficient at capturing the reactive intermediate since we regularly inactivate papain wih small excess amounts of inhibitor.

Another explanation for lack of inhibition of papain by the azapeptide featuring azaalanine at P_1 derives from the structural differences between an azaamino acid and an amino acid.[10d,e,11a] In the first place, there should be a barrier to rotation around the α-nitrogen-to-carbonyl bond, by analogy to the rotation barrier in amides. The height of this barrier has never been measured, and it is expected to be less than that of an amide (18 kcal/mol); however, there is practically no barrier to rotation around the α-carbon-to-carbonyl bond in natural amino acids. Furthermore, whereas the α-carbon of an amino acid is tetrahedral, the α-nitrogen of an azaamino acid should be nearly planar.[17] Taken together, these differences can result in a considerable energetic cost when one tries to superimpose an azapeptide over the reactive conformation of a normal peptide. It has recently been demonstrated that separating two rapidly-reacting functional groups by 0.1 Å in the ground state resulted in a 10^{-4} decrease in the rate of a reaction.[18] Comparing azaglycine and azaalanine, the reactive (inhibiting) conformation of the azaglycine may be such that it directs its hydrogen atom toward a surface of the enzyme (rather than toward a binding pocket) where the bulkier methyl group will not fit. The importance of structural features, separated from electronic effects, has been demonstrated for serine proteases inhibited by azapeptides for which deacylation rates vary over 4 orders of magnitude.[10d,e] All of the azapeptides described above (that were made by us) were made from L-phenylalanine. We investigated the possibility that use of D-phenylalanine would realign the acylating group in the active site leading to a more rapid inactivation of papain. The D-antipodes of **2**, **3**, and **7** were made and tested. The rate of inactivation of papain by D-**7** was slower than that for **7**, and no inhibition was observed with D-**2** or D-**3** over the course of 2 h (data not shown).

Electronic effects on the reactivity of the azapeptide carbonyl with respect to nucleophiles are difficult to assess. Ostensibly, it is the decreased susceptibility to nucleophilic attack of azapeptide alkyl esters, compared with aryl esters, that makes these compounds react selectively with cysteine proteases. However, two groups have given evidence to the effect that azaamino acid esters are as reactive with nucleophiles as their corresponding amino acid esters. The hydrolysis rate of the ethyl ester of acetylazaphenylalanine was determined by means of a pH stat at pH = 7.5 and found to be the same as that for the authentic phenylalanine ester.[11a] However, no product analysis was carried out and that leaves the nature of the observed reaction in question. In another case, the "hydrolysis" rates of some azapeptide *p*-nitrophenolates were compared with those for peptide *p*-nitrophenolates and found to be comparable on the basis of the rate of release of *p*-nitrophenol.[10d,e] Nonetheless, *p*-nitrophenol release is probably not due to solvent nucleophilic attack on the azapeptide carbonyl. Instead, it more likely represents a cyclization as depicted in Scheme III.[19] In support of this, we note that when the β-N-H is replaced by N-CH_3 or CH_2 the "hydrolysis" reaction rate is seriously curtailed.[10e] Given the questionable relevance of the foregoing experiments, the relative susceptibility of the azapeptide carbonyl to nucleophilic attack, thus its pertinence to enzyme inhibition, remains unknown.

Conclusion

Azapeptide esters inhibit papain in a time-dependent fashion; the rates of inhibition are summarized in Table I. The mechanism of inhibition is thought to be acylation of the active-site thiol. This is in accord with the facts that substrate slows the rate of inhibition (substrate protection), and enzyme activity can be fully recovered by allowing inhibited enzyme to react with an added nucleophile (specifically, the methyl ester of valine). The spontaneous rate of enzyme reactivation (i.e., in the absence of added nucleophiles) is negligible. Azapeptide alkyl esters exhibit much lower reactivity with serine proteases,[11] so these compounds should be selective inhibitors of cysteine proteases.

Experimental Section

^{1}H-NMR and ^{13}C-NMR spectra were obtained at 300 MHz and 75 MHz (respectively) with a Varian XL-300 spectrometer. Chemical shifts are reported in ppm from TMS, the spectra were referenced to TMS, the residual methyl proton in CD_3OD (3.35), the carbon of $CDCl_3$ (77.0) or the carbon of CD_3OD (49.9) as appropriate. Mass spectra were obtained with an HP-5985 instrument operating in chemical ionization mode. TLC was carried out on Merck silica gel plates with a fluorescent indicator; with UV or iodine vapor detection. Flash chromatography was done with Merck silica gel, grade 60, 230–400 mesh. Yields are reported for isolated, pure products, and were not optimized. Melting points were determined with a Thomas-Hoover capillary melting point apparatus. Solvents and pyridine were from Fisher Scientific and used as received. Reagents were from Aldrich Chemicals and were used as received. Papain (2× recrystallized, lyophilized powder) and (±)benzoyl arginine *p*-nitroanilide (BAPNA) were obtained from Sigma Biochemicals and were used as received.

***N*-Acetyl-L-phenylalanylazaglycine Methyl Ester (Ac-L-PheAglyOCH$_3$, 1).** *N*-Acetyl-L-phenylalanine (414 mg, 2 mmol) was dissolved in 20 mL of acetonitrile (at 25 °C) with a magnetic mixer. Mixing was continued while DCC (412 mg, 2 mmol) was added, followed immediately by anhydrous hydrazine (0.063 mL, 2 mmol). After 4 h at 25 °C, triethylamine (0.417 mL, 3 mmol) was added, followed by methyl chloroformate (0.170 mL, 2.2 mmol), and the mixture was left stirring for 16 h. The

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mixture was filtered and the solvent was removed in vacuo; the crude product was submitted to flash chromatography with 10% methanol in methylene chloride (R_f = 0.40) to yield 400 mg (72%) of 1, as a crystalline solid (mp = 169–171 °C): ^{1}H NMR (CD_3OD) 7.3 (m, 5 H, phenyl), 4.72 (dd, 1 H, α-H, J = 4.5, 9.5), 3.23 (br dd, 1 H, benzyl, J = 4.5, 13.5), 2.91 (dd, 1 H, benzyl, J = 9.5, 13.5), 1.91 (s, 3 H, acetyl); ^{13}CNMR (CD_3OD) 174.3, 173.9, 159.8, 139.2, 131.2, 130.3, 128.6, 55.3, 54.1, 39.8, 23.2. Anal. C, H, N.

N-Acetyl-L-phenylalanylazaglycine Isobutyl Ester (Ac-L-PheAglyOiC$_4$H$_9$, 2). This was made in the same way as compound 1, substituting isobutyl chloroformate (0.26 mL, 2 mmol) for methyl chloroformate. The product was obtained in 77% yield after flash chromatography (10% methanol in methylene chloride; R_f = 0.44) as a crystalline solid (mp = 157–9 °C); ^{1}H NMR ($CDCl_3$) 9.1 (br s, 1 H, NH), 7.2 (m, 6 H, phenyl and NH), 6.9 (br d, 1 H, NH, J = 6), 4.83 (dd, 1 H, α-H, J = 5.9, 8.3), 3.87 (d, 2 H, ester, J = 6.7), 3.18 (dd, 1 H, benzyl, J = 5.9, 13.9), 2.97 (dd, 1 H, benzyl, J = 8.3, 14.1), 1.89 (s/m, 4 H, acetyl and tertiary), 0.90 (d, 6 H, methyls, J = 6.6); ^{13}C NMR ($CDCl_3$) 171.5, 171.0, 156.5, 136.3, 129.2, 128.5, 126.8, 72.1, 52.6, 37.7, 27.8, 22.8, 18.8; MS (M + 1) 322. Anal. C, H, N.

N-Acetyl-L-phenylalanylazaalanine Isobutyl Ester (Ac-L-PheAalaOiC$_4$H$_9$, 3). Methylhydrazine (1.4 mL, 10 mmol) was dissolved in 10 mL of dry methylene chloride, and the solution was cooled in a dry ice/acetone bath. Vigorous stirring with a magnetic mixer was begun, and isobutyl chloroformate (0.65 mL, 5 mmol) was added dropwise. After the addition was complete, the cooling bath was removed, the mixture warmed to 25 °C, and the solvent was removed in vacuo. The crude product was submitted to flash chromatography with 2% methanol in methylene chloride (R_f = 0.34) to yield 681 mg (93%) of the carbazate, 5, an oil: ^{1}H NMR ($CDCl_3$) 4.2 (br s, 2 H, NH_2), 3.89 (d, 2 H, OCH_2, J = 6.6), 3.12 (s, 3 H, NCH_3), 1.95 (heptet, 1 H, CH, J = 6.7), 0.95 (d, 6 H, $C(CH_3)_2$, J = 6.7).

N-Acetyl-L-phenylalanine (206 mg, 1 mmol) was dissolved in dry THF and cooled below −5 °C in an ice/methanol bath. To this was added triethylamine (0.14 mL, 1 mmol) and ethyl chloroformate (0.096 mL, 1 mmol). The mixture was stirred for 30 min, after which time 146 mg (1 mmol) of 5 was added, the ice bath was removed, and mixing was continued while the flask contents warmed to 25 °C. The solvent was removed in vacuo, and the crude product was submitted to flash chromatography with 5% methanol in methylene chloride (R_f = 0.20) to furnish 170 mg (51% based on N-acetyl-L-phenylalanine) of 3, a crystalline solid (mp = 138–40 °C): ^{1}H NMR ($CDCl_3$) 7.15 (m, 7 H, phenyl and NH's), 4.8 (br m, 1 H, α-H), 3.75 (br m, 2 H, ester), 3.0 (br m, 5 H, benzyl and NCH_3), 1.83 (br s, 4 H, acetyl and tertiary), 0.81 (br s, 6 H, methyls); ^{13}C NMR ($CDCl_3$) 170.5 (2 coincident), 156.1, 136.3, 129.3, 128.3, 126.8, 72.3, 52.4, 37.9, 37.4 (br), 27.8, 22.7, 18.8; MS (M + 1) 336. Anal. C, H, N.

N-Acetyl-L-phenylalanylazasarcosine Isobutyl Ester (Ac-L-PheAsarOiC$_4$H$_9$, 4). N-Acetyl-L-phenylalanine (414 mg, 2 mmol) was dissolved in 20 mL of THF/acetonitrile 1:1 at 25 °C. The solution was stirred with a magnetic mixer while DCC (412 mg, 2 mmol) was added, followed by methylhydrazine (0.107 mL, 2 mmol). The mixture was stirred for 4 h, and then it was filtered and the solvent was removed in vacuo. The crude product was submitted to flash chromatography with 5% methanol in methylene chloride (R_f = 0.18) to yield 321 mg (68%) of the hydrazide, 6, a crystalline solid: ^{1}H NMR (CD_3OD) 7.3 (m, 5 H, phenyl), 5.7 (dd, 1 H, α-H, J = 4.9, 9.1), 3.17 (dd, 1 H, benzyl, J = 4.9, 13.6), 3.16 (s, 3 H, NCH_3), 2.82 (dd, 1 H, benzyl, J = 9.1, 13.5), 1.92 (s, 3 H, acetyl); ^{13}C NMR (CD_3OD) 176.3, 173.7, 139.9, 131.2, 130.1, 128.5, 53.3, 39.6, 39.4, 23.3.

Continued elution of the column with 10% methanol in methylene chloride yielded 30 mg (6%) of the regioisomer (R_f = 0.30) with the methyl group on the β-nitrogen of the hydrazide, as a crystalline solid which is useful for comparison: ^{1}H NMR (CD_3OD) 7.3 (m, 5 H, phenyl), 4.53 (t, 1 H, α-H, J = 7.5), 3.09 (dd, 1 H, benzyl, J = 7.3, 13.4), 2.94 (dd, 1 H, benzyl, J = 8.1, 13.6), 2.44 (s, 3 H, NCH_3), 1.95 (s, 3 H, acetyl); ^{13}C NMR (CD_3OD) 173.8, 172.5, 139.1, 131.2, 130.3, 128.7, 55.8, 40.0, 39.6, 23.3.

Hydrazide 6 (43 mg, 0.18 mmol) was dissolved in 5 mL of acetonitrile at 25 °C and stirred with a magnetic mixer. To this was added triethylamine (0.028 mL, 0.2 mmol) and isobutyl chloroformate (0.026 mL, 0.2 mmol), and mixing was continued for 2 h. The solvent was removed in vacuo and the crude product was submitted to flash chromatography with 5% methanol in (R_f = 0.22) to yield 30 mg (50%) 4, as a brittle-glassy solid (mp = 37–38 °C): ^{1}H NMR (CD_3OD) 7.25 (m, 5 H, aromatic), 5.2 (br s, 1 H, NCHC=O), 4.0 (br s, 2 H, OCH_2), 3.17 (s, 3 H, NCH_3), 3.1 and 2.8 (br m, 2 H total, benzyl), 2.0 (br m, 1 H, CH), 1.90 (s, 3 H, O=CCH_3), 1.01 (d, 6 H, $C(CH_3)_2$, J = 6.6); ^{13}C NMR (CD_3OD) 176.5, 173.4 (br), 158.6, 139.6, 131.0, 130.3, 128.6, 74.1, 53.1 (br), 39.3 (br d), 36.8, 30.1, 23.2, 20.2; MS (M + 1) 336. Anal. C, H, N: calcd 60.9, 7.51, 12.5; found 60.2, 7.64, 12.4.

N-Acetyl-L-phenylalanylazaglycine Phenyl Ester (Ac-L-PheAglyOC$_6$H$_5$, 7). This was made in the same way as compound 1, substituting phenyl chloroformate (0.25 mL, 2 mmol) for methyl chloroformate and pyridine (0.16 mL, 2 mmol) for triethylamine. The product was obtained in 82% yield after flash chromatography (5% methanol in methylene chloride; R_f = 0.27), as a crystalline solid (mp = 137–40 °C); ^{1}H NMR ($CDCl_3$) 9.2 (br, 1 H, NH), 7.2 (m, 10 H, phenyls), 6.9 (br d, 1 H, NH, J = 3), 4.89 (dd, 1 H, α-H, J = 6.1, 8.1), 3.40 (dd, 1 H, benzyl, J = 6.1, 14.2), 3.03 (dd, 1 H, benzyl, J = 8.1, 14.2), 1.91 (s, 3 H, acetyl); ^{13}C NMR ($CDCl_3$) 171.5, 171.3, 154.5, 150.4, 136.1, 129.4, 129.2, 128.6, 127.0, 125.8, 121.4, 52.7, 37.6, 22.8; MS decomposition in probe to phenol (M + 1) 95 and ((M + 1) − 94) = 248. Anal. C, H, N.

Papain Assay. The activity of papain was measured spectrophotometrically as the rate of release of p-nitroaniline from BAPNA at pH = 7.0, 25 °C, in 50 mM potassium phosphate buffer with 5 mM EDTA and 5 mM cysteine, at a chart speed of 1 cm/min and a scale of 0.3 absorbance unit with the detector set at 410 nm. A stock solution of papain was prepared by dissolving it in the buffer at a concentration of approximately 1 mg (of lyophilized powder as received)/mL. This was incubated for 30 min at 25 °C to activate the enzyme. The substrate (BAPNA) was dissolved in DMSO at a concentration of 50 mg/mL. A typical assay consisted of 0.98 mL buffer, 10 μL of enzyme stock, and 10 μL of substrate.

Inhibition of Papain with Azapeptides. Inhibitors were dissolved in acetonitrile or DMSO as appropriate for their solubility. A volume of the inhibitor solution was added to an appropriate dilution of papain stock solution in the assay buffer to achieve the desired inhibitor concentration. Typically, 10–50 μL of inhibitor was added per mL of papain stock. This was incubated at 25 °C, and aliquots were taken for assay over the course of time (usually 30–180 min for azapeptide concentrations in the range of 10–800 μM).

Reactivation of Papain Inhibited with Azapeptides. (A) Deacylation with Water. Papain stock (0.1 mL) was incubated with 85 μM 1 (greater than a 2-fold excess of inhibitor) until the activity of the papain had decreased to 13%. The solution was diluted to 1 mL and dialyzed with 1 L of phosphate buffer (pH = 7) at 4 °C for 3 h, and then the buffer was changed and dialysis repeated for another 3 h. After this, the activity of the enzyme was assayed (100-μL aliquots) and compared with an identically treated control (without inhibitor) over the course of 18 h during which the samples were stored at 25 °C. The activity of the inhibited enzyme remained constant at ca. 13% for the duration of the experiment.

(B) Deacylation with Added Nucleophiles. A papain stock was made with 1.2 mg of lyophilized powder in 100 μL of buffer (approximately 435 μM papain). A control sample of 10 μL was withdrawn and diluted in 90 μL of buffer. The remainder was inhibited with 700 μM 1; after 2 h of incubation at 25 °C, the enzyme was totally inactivated. Aliquots (10 μL) of inhibited enzyme were removed and diluted into 90 μL of solutions containing nucleophiles at pH = 7.0, as well as a control without a nucleophile. The nucleophiles were hydroxylamine and valine methyl ester (90 mM each). Aliquots (10 μL) were removed and diluted to 1 mL with buffer for assay at various times. No enzyme reactivation was observed with hydroxylamine over the course of 16 h. At the first time point (2 h) the valine ester-treated enzyme was fully reactivated.

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