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Carboxyl-Modified Amino Acids and Peptides as Protease Inhibitors

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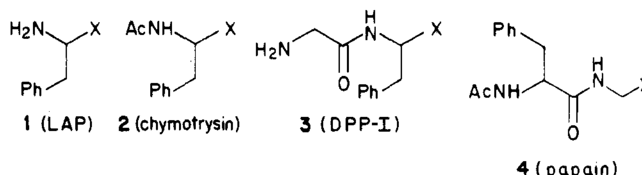
Several types of carboxyl-modified amino acids and peptides were prepared in forms having N-terminal modifications (carrier fragments) suitable for one of several representative protease enzymes, and their inhibitory action toward those enzymes were evaluated. The carboxyl modifications (inhibitory units) included (b) $CONH_2$, (c) $CSNH_2$, (d) CN, (e) *trans*-CH=$CHCO_2Me$, and (f) *trans*-CH=$CHSO_2Me$. The carrier fragments included $NH_2(PhCH_2)CHX$ (1), $AcNH(PhCH_2)CHX$ (2), $H_2NCH_2CONH(PhCH_2)CHX$ (3), and $AcNH(PhCH_2)CHCONHCH_2X$ (4). Compounds **1b**, **1d**, **1e**, and **1f** were competitive inhibitors of both microsomal and cytosolic leucine aminopeptidase (K_i = 14.8, 67, 61, and 3.7 mM with the former and 14.1, 26.4, 27.3, and 8.8 mM with the latter, respectively). Neither compound **1c** nor leucine thioamide had any detectable effect on either enzyme. Compounds **2b–f** were also competitive inhibitors toward chymotrypsin (K_i = 13.9, 23.0, 5.3, 30.8, and 29.4 mM, respectively). While **4b**, **4c**, and **4d** were competitive inhibitors of papain (K_i = 4.7, 0.095, and 0.0011 mM, respectively), **4e** proved to be an irreversible affinity label (K_i = 0.026 mM and k_2 = 0.0018 s^{-1}). Inactivation of papain by **4e** was retarded in the presence of **4d** and could not be reversed by dialysis. Similarly **3b** and **3d** were competitive inhibitors of dipeptidyl aminopeptidase I (DPP-I, EC 3.4.14.1) (K_i = 6.2 and 0.0027 mM, respectively), while **3e** and **3f** were irreversible affinity labels (K_i = 0.22 and 0.18 mM, and k_2 = 0.015 and 0.010 s^{-1}, respectively). Inhibition of DPP-I by **3d** provides only the second example of a cysteine protease which is strongly inhibited by a nitrile analogue of a specific substrate. Further studies are needed to determine the generality and potential utility of this finding. Compounds **3e**, **3f**, and **4e** exemplify a new class of specific affinity labels for cysteine proteases whose activity probably derives from irreversible Michael addition of the catalytic cysteine to the activated double bond.

It is becoming increasingly apparent that protease enzymes play critically important roles in initiating, sustaining, or terminating a wide variety of biological processes. Examples include blood coagulation, complement activation, inflammatory and tissue degenerative diseases, tumor metastasis, viral replication, and many others.[1,2] The central role of proteolysis in these processes makes the development of specific, selective, nontoxic protease inhibitiors an attractive challenge in drug design. Since the substrate specificity of many physiologically important proteases is often better known than other features of these enzymes, our approach to this problem has been to modify the structure of known specific substrates in the vicinity of the scissile bond in ways that might be expected to interfere, reversibly or not, with the normal catalytic process. This consideration led us recently to synthesize an analogue of a papain substrate containing a *vinylogous* amino acid residue. This compound proved to be a specific irreversible inactivator of papain, i.e. an affinity label.[3] Here we report that related compounds specifically and irreversibly inactivate another thiol protease, dipeptidyl aminopeptidase-I (DPP-I, EC 3.4.14.1), also known as cathepsin C, but not the serine protease chymotrypsin nor the microsomal or cytosolic forms of the metalloprotease leucine aminopeptidase. We also report on the inhibitory effects of *thioamide* and *nitrile* analogues of specific substrates on these enzymes.

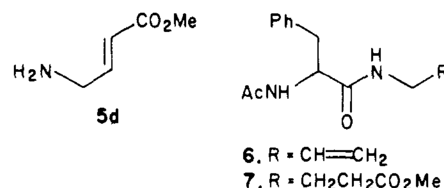
Design

Our purpose was to evaluate the inhibitory potential of functional groups incorporated into substrate analogues by testing them against proteases representing several different classes, while at the same time minimizing the amount of synthesis required. To meet this objective we selected five primary target enzymes which were commercially available and well characterized: (1) chymotrypsin (CT), a serine endoprotease preferring aromatic residues at P_1; (2) microsomal and (3) cytosolic leucine aminopeptidase (LAP-M and LAP-C), metalloproteases preferring unblocked N-terminal hydrophobic residues such as leucine or phenylalanine; (4) dipeptidyl aminopeptidase I (DPP-I, also called cathepsin C), a cysteine protease which cleaves dipeptides from proteins with unblocked N-termini and prefers hydrophobic residues at P_1; and (5) papain, a cysteine endoprotease which prefers aromatic residues at P_2. A further consideration in the choice of these enzymes was the availability[4] of crystal structures for chymotrypsin and papain, which would be useful for molecular graphics analysis of enzyme–inhibitor interactions.

Scheme I



A. Representative substrates: **1a–4a**, X = $CONHC_6H_4NO_2$
B. Inhibitors: **b**; X = $CONH_2$; **c**, X = $CSNH_2$; **d** = CN;
e, X = *trans*-CH=CHCOOMe; **f**, X = *trans*-CH=$CHSO_2Me$



Given the specificity of the above enzymes, the synthetic requirements could be streamlined as follows (viz. Scheme I). Carboxyl-modified phenylalanine analogues **1b–f** would be directly suitable for studies with LAP; N-terminal acetylation would render them suitable for chymotrypsin (**2b–f**), while appending an N-terminal glycine would ready them for DPP-I (**3b–e**). Comparable carboxyl-modified

(1) Reich, E.; Rifkin, D. B.; Shaw, E., Eds. "Proteases and Biological Control"; Cold Spring Harbor Laboratory: Cold Spring, New York, 1975.
(2) Berlin, R. D.; Herrmann, H.; Lepow, I. H.; Tanzer, J. M., Eds. "Molecular Basis of Biological Degenerative Processes"; Academic Press: New York, 1978.
(3) Hanzlik, R. P.; Thompson, S. A. *J. Med. Chem.* 1984, *27*, 711.
(4) Protein Data Bank. Bernstein, F. C.; Koetzle, T. F.; Williams, G. B.; Meyer, E. F., Jr.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* 1977, *112*, 535.

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glycine derivatives **4b–f** would be suitable for papain. The amide analogues **1b–4b** would serve as peptide-like alternate-substrate inhibitors and would thus provide a standard of comparison against which to judge the other types of inhibitors for a given enzyme.

Results

Synthesis. As indicated above, carboxyl-modified derivatives of L-phenylalanine (**1b–f**) were synthesized for LAP and derivatized further at the N-terminus for chymotrypsin and DPP-I. The Boc derivative of L-**1b** (Boc-L-PheCONH$_2$)[5] was dehydrated with trifluoroacetic anhydride (TFAA) and deprotected with trifluoroacetic acid (TFA) to give **1d**, isolated as its methanesulfonate salt, in 49% overall yield. Treatment of Boc-L-**1b** with Lawesson's reagent[6] in hexamethylphosphoric triamide (HMPA) formed Boc-**1c** in 70% yield, deprotection of which with TFA gave **1c** as a TFA salt in 63% overall yield.

For the synthesis of Michael acceptors **1e** and **1f**, Boc-L-PheCOOMe was converted to Boc-PheCHO by reduction with diisobutylaluminum hydride (Dibal) as described[7] by Rich et al. and condensed with (EtO)$_2$P(O)CHXNa (X = COOMe or SO$_2$Me) in THF to form the C=C bond, giving Boc-**1e** and Boc-**1f**, respectively. NMR analysis indicated that only the trans isomers were present, based on the coupling constant of 16 Hz observed in both cases for the vinylic protons. Deprotection with TFA gave **1e** and **1f**, respectively, which were isolated as TFA salts. It is noteworthy that despite the strongly basic conditions of the Horner–Emmons reaction and the general ease of racemization of α-amino aldehydes, **1e** and **1f** and their N-terminal derivatives showed substantial optical activity. However, no attempt was made to determine the enantiomeric purity of these compounds.[8]

For the synthesis of **4b** and **4d**, *N*-Ac-L-Phe was coupled to either glycinamide or aminoacetonitrile by using a standard mixed-anhydride method.[9] Thioamide **4c** was obtained by treating **4d** with H$_2$S in pyridine/Et$_3$N. For the synthesis of **4e**, Boc-allylamine was ozonized in methanol at −78 °C and the ozonide reduced with Me$_2$S to afford Boc-glycinal in 94% yield (crude). The latter was then condensed with (EtO)$_2$P(O)CHNaCOOMe in THF, giving *trans*-Boc-NHCH$_2$CH=CHCO$_2$Me (Boc-**5d**) in 62% yield; deprotection with TFA gave **5d** as the TFA salt in 94% yield. Coupling of **5d**, allylamine, and methyl 4-aminobutyrate with *N*-Ac-L-Phe gave **4e**, **6**, and **7**, respectively.

Inhibition Studies. Our approach to evaluating new potential inhibitors was to implement a standard photometric assay for the enzyme involved, to determine the K_m of the assay substrate for comparison to the reported values, and to evaluate the test compounds via Lineweaver–Burk plots. In most cases competitive inhibition was indicated, and K_i values were determined from replots of (K_m^{app}/V_{max}) vs. [I]. When irreversible inhibition was indicated, pseudo-first-order rate constants (k_{app}) were determined from the slopes of plots of ln (percent remaining activity) vs. time; replots of $1/k_{app}$ vs. $1/[I]$ gave the inhibition constants K_i and k_2 as described by Kitz and Wilson.[10] The K_m values observed with our assay substrates are collected in Table I, while the kinetic constants for the inhibitors are collected in Table II.

As expected, amides **1b–4b** proved to be competitive inhibitors of their respective enzymes. In all cases their K_i values are 2–30 times greater than the K_m values for the corresponding *p*-nitroanilides. For many serine and cysteine proteases, the turnover of amide substrates is limited primarily by the rate of enzyme acylation and K_m approximates the dissociation constant (K_s) for the enzyme–substrate complex. The *p*-nitrophenyl group is expected to lower K_m relative to the amides **b** through increased hydrophobic interactions and through electron withdrawal which facilitates the acylation step.[11] An extreme example of the latter effect can be seen by comparing the K_m values of **4a** and the much more reactive

Table I. Michaelis Constants for Assay Substrates

enzyme	substrate	K_m, mM obsd	K_m, mM lit.	ref
chymotrypsin	glutaryl-1a	0.44	0.65	30
LAP-M	L-Leu-pNA	0.60	0.50	33
			0.30	23
			0.52	31
LAP-C	L-Leu-pNA	2.10	2.20	23
			1.00	34
DPP-I	3a	3.1	3.3	35
papain	Z-Gly-ONP	0.0103	0.0070	36
			0.0052	37
	4a		0.24	37
			0.88	12

Table II. Inhibition Constants

enzyme	inhib	K_i,[a] mM
chymotrypsin	**2b**	13.9
	2c	23.0
	2d	5.3
	2e	30.8
	2f	29.4
LAP-M	**1b**	14.8
	1c	*b*
	1d	67
	1e	61
	1f	3.7
LAP-C	**1b**	14.1
	1c	*c*
	1d	26.4
	1e	23.7
	1f	8.8
DPP-I	**3b**	6.2
	3d	0.0027
	3e	0.22 (k_2 = 0.015 s^{-1})
	3f	0.18 (k_2 = 0.010 s^{-1})
papain	**4b**	4.7
	4c	0.095
	4d	0.0011
	4e	0.026 (k_2 = 0.0018 s^{-1})
	6	9.2
	7	19.5

[a] All K_i values are for competitive inhibition except those for **3e**, **3f**, and **4e**. [b] No inhibition with [**1c**] = 40 mM. [c] Slight stimulation was observed with [**1c**] = 5 mM but higher concentrations were not investigated.

(5) Our nomenclature of these compounds is based on naming the parent amino acid followed by the carboxyl modification. For example, phenylalanine methyl ester would be PheCOOMe (not PheOMe), phenylalaninal would be PheCHO, and *N*-Boc-aminoacetonitrile would be Boc-GlyCN.

(6) Clausen, K.; Thorsen, M.; Lawesson, S.-O. *Tetrahedron* **1981**, *37*, 3635.

(7) Rich, D. H.; Sun, E. T.; Boparai, A. S. *J. Org. Chem.* **1978**, *43*, 3624.

(8) All of the carboxy-modified phenylalanine derivatives synthesized and tested in this study were prepared from L-phenylalanine. Since there was no reason to expect substantial racemization to occur, particularly in the syntheses leading to amide (**b**), thioamide (**c**), or nitrile (**d**) analogues, no attempt was made to ascertain the optical purities of the products.

(9) Chen, F. M. F.; Steinauer, R.; Benoiton, N. L. *J. Org. Chem.* **1983**, *48*, 2941.

(10) Kitz, R.; Wilson, I. *J. Biol. Chem.* **1962**, *237*, 3245.

(11) Lowe, G.; Yuthavong, Y. *Biochem. J.* **1971**, *124*, 117.

ester Z-Gly-ONP (Table I). These arguments may or may not apply to LAP-M and LAP-C, since it is not clear whether acyl enzyme intermediates are formed by these enzymes.

The nitrile analogues **1d**–**4d** showed an interesting pattern of inhibitory effects. The two cysteine proteinases papain and DPP-I were strongly but reversibly inhibited by **4d** and **3d**, respectively. Toward these enzymes the K_i values of the nitriles were 3–4 × 10^3 lower than those of the corresponding amides. The K_i value found for **4d** (1.1 μM) is in good agreement with the value of 0.73 μM reported earlier by Westerick and Wolfenden.[12] Benzamidoacetonitrile (**8**), the nitrile analogue of another series of papain substrates, also inhibits papain reversibly, but less dramatically, with a K_i of 0.46 mM.[13] As far as we are aware, the inhibition of DPP-I by **3d** (K_i = 2.7 μM) is the only other example of inhibition of a cysteine protease by a specific nitrile. This inhibition was completely reversible by dialysis. The fact that the ratio of K_i values (amide/nitrile) is roughly the same for **3b**/**3d** as for **4b**/**4d** also suggests that the mechanisms of inhibition are similar in both cases. One must now consider the possibility that inhibition of cysteine proteinases by specific nitriles may be a *general* phenomenon with potential for exploitation.

Specific nitriles are reported not to be hydrolyzed by papain.[14,15] To rationalize their extraordinary potency, which is nearly as great as that of aldehyde analogues of specific substrates,[15] Lowe and Yuthavong[13] proposed that **8** bound to papain to give a Michaelis complex which is less strained than those for amides and peptides. Sluyterman and Wijdenes[14] proposed that **8** might be a transition-state analogue for papain. Lewis and Wolfenden[15] considered that nitriles may bind *covalently* at the active site of papain by means of sulfhydryl addition (to form a thioimidate ester linkage). We are presently investigating the possibility of thioimidate bond formation by means of ^{13}C NMR with papain and [nitrile-^{13}C]-enriched **4d**.

By comparison to their effect on cysteine proteinases, specific nitriles are mediocre inhibitors of chymotrypsin and leucine aminopeptidase, i.e. the ratio of K_i values (amide/nitrile) is 2.6 for chymotrypsin, 0.22 for LAP-M, and 0.53 for LAP-C. Similar results were obtained for both LAP-M and LAP-C with L-LeuCONH$_2$ and L-LeuCN as inhibitors (unpublished results). Too little is yet known to speculate whether covalent intermediates are involved in the catalytic action of LAP, but the results with nitrile **1d** would not favor a covalent mechanism for the interaction of these enzymes with nitrile inhibitors. On the other hand, chymotrypsin is well known to form acyl enzyme intermediates, and yet nitrile **2d** is hardly better than amide **2b** as an inhibitor. This would appear to be consistent with the relatively greater nucleophilicity of the imidazolium-thiolate ion pair at the active site of papain[16] (and perhaps DPP-I?) compared to the imidazole-hydrogen-bonded serine in chymotrypsin and with the recent suggestion by Asboth and Polgar[17] that electrophilic catalysis (by H-bond donors in the "oxyanion hole") may not be as important with cysteine proteinases as it is with serine proteases (see below). Since nitriles have linear sp geometry, as opposed to the trigonal sp^2 geometry of an amide carbon, the nitrile N might not be in a suitable position to accept H bonds from the oxyanion hole of chymotrypsin. Indeed this is borne out by molecular graphics analysis (see below).

Replacing the carbonyl oxygen of an amide or peptide unit by sulfur causes modest but well-characterized changes in chemical properties which can be useful for probing enzymic reaction mechanisms and/or creating enzyme inhibitors.[18,19] For example, X-ray studies have shown that except for the C=S bond being longer than C=O, there are no significant structural differences between oxopeptide and thiopeptide units.[20] Another key difference is that C=S sulfur, being a soft Lewis base, is a poorer hydrogen-bond acceptor but binds to transition metal ions better than C=O oxygen.[21] Recently several specific thioamides have been investigated as substrates and inhibitors of the zinc form of carboxypeptidase A.[19,22] The thiopeptide bound as well as the oxopeptides (i.e. $K_i \approx K_m$) but they hydrolyzed more slowly. The cadmium form of carboxypeptidase A, which does not hydrolyze peptides, was found to hydrolyze thiopeptide substrates.[22] This finding was interpreted to support a role for metal ion coordination to the substrate during catalysis. In this context it is interesting to note that thioamide **1c** had no detectable inhibitory effect on LAP-C or LAP-M, both of which are zinc metalloenzymes. Likewise, L-leucinethioamide was also without effect on these enzymes (unpublished results). This could be taken to imply that direct zinc-substrate coordination is not involved in LAP action. Other evidence consonant with this view is the finding[23] that whereas LAP hydrolyzes only L-amino acid derivatives with unblocked amino termini, hydroxamic acid derivatives of D-Leu and even isocaproic acid (i.e. desamino-Leu) inhibit LAP approximately as well as L-LeuCONHOH. On the other hand, it is possible that metal–substrate coordination does occur with L substrates, but that the D substrates and the desamino substrates bind from the S_1' position rather than S_1.

Lowe and Yuthavong found several thioamides to be competitive inhibitors of papain,[13] although it was not reported whether they were hydrolyzed. Earlier, Lowe and Williams found that methyl hippurate and methyl thionhippurate were hydrolyzed by papain with nearly equal k_{cat}/K_m values.[24] More recently Asboth and Polgar recorded similar observations for pairs of ester/thionoester analogues of specific substrates with papain.[16] With chymotrypsin on the other hand, thionoester analogues of specific ester substrates are hydrolyzed extremely slowly, although they are bound quite well and competitively inhibit the hydrolysis of other substrates.[17,18] Asboth and Polgar interpreted the differences between papain and chymotrypsin in behavior of ester/thionoester pairs of compounds to suggest that electrophilic catalysis (via an "oxyanion hole") was important with serine proteases but perhaps not for cysteine proteases. Our results with **2b**/**2c** and chymotrypsin are in accord with this. Although we did not determine if **2c** was hydrolyzed by chymotrypsin,

(12) Westerick, J. O.; Wolfenden, R. *J. Biol. Chem.* **1974**, *249*, 6351.
(13) Lowe, G.; Yuthavong, Y. *Biochem. J.* **1971**, *124*, 107.
(14) Sluyterman, L. A. Ae.; Wijdenes, J. *Biochim. Biophys. Acta* **1973**, *302*, 95.
(15) Lewis, C. A.; Wolfenden, R. *Biochemistry* **1977**, *16*, 4890.
(16) Polgar, L. *Eur. J. Biochem.* **1973**, *33*, 104.
(17) Asboth, B.; Polgar, L. *Biochemistry* **1983**, *22*, 117.
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(20) LaCour, T. F. M.; Hansen, H. A. S.; Clausen, K.; Lawesson, S.-O. *Int. J. Peptide Protein Res.* **1983**, *22*, 509.
(21) Hanzlik, R. P. "Inorganic Aspects of Biological and Organic Chemistry"; Academic Press: New York, 1976.
(22) Mock, W. L.; Chen, J.-T.; Tsang, J. W. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 389.
(23) Wilkes, S.; Prescott, J. M. *J. Biol. Chem.* **1983**, *258*, 13517.
(24) Lowe, G.; Williams, A. *Biochem. J.* **1965**, *96*, 189.

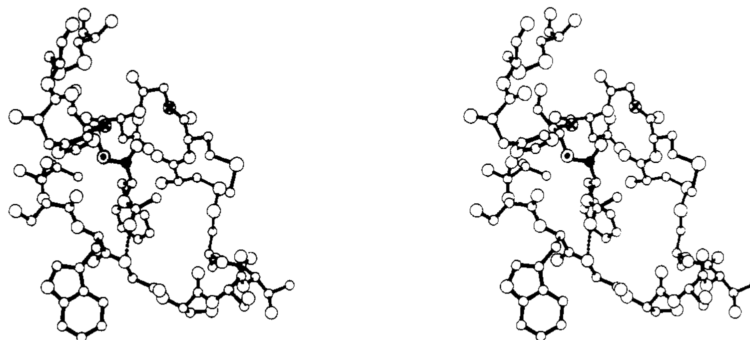

Figure 1. Stereopair view of hemiacetal adduct of *N*-acetylphenylalaninal with active site region of chymotrypsin. The phenyl of the inhibitor is in the center of the diagram, just below the aldehyde carbon (solid circle). The oxygen of Ser-195 is represented by a circle with a dot in the center. The OH of the hemiacetal is seen projecting toward the oxyanion hole (backbone NH of Ser-195 and Gly-193, which are represented by circles with crosses on them). The oxygen of the inhibitor acetyl group is hydrogen bonding (···) to the backbone NH of Gly-216. Compare to view of tosylchymotrypsin given in ref 25 and 27.

its K_i was within a factor of 2 of that of **2b**. However with papain the K_i of thioamide **4c** was considerably lower than that of amide **4b**. Furthermore, although papain readily hydrolyzes methyl and ethyl thionohippurate,[24] a 1 mM solution of thioamide **4c** was essentially unaffected when incubated for 16 h at room temperature with 0.1–0.2 μM papain, as determined by monitoring the thioamide chromophore at 265 nm. Further detailed studies of the interaction of serine and cysteine proteinases with thioamides and thiopeptides would clearly be useful, both in connection with elucidation of enzyme mechanisms and to evaluate better the prospect of using thioamide bonds in designing protease inhibitors and/or metabolically stable peptides.

The Michael acceptors **e** and **f** constitute a truly new group of protease inhibitors. Their design is based on the hope that the active-site nucleophile of a cysteine or serine protease might attack the β-carbon of the ethylenic unit as if it were the carbonyl carbon of a normal substrate, leading to irreversible covalent inactivation of the enzyme (i.e. affinity labeling). Even though it is unclear, possibly even unlikely, that anything analogous to an acyl enzyme forms during LAP catalysis, **1e** and **1f** were tested against LAP. They were found to have very modest activity as competitive inhibitors, and no evidence for inhibition which increased with time was found.

With chymotrypsin, **2e** and **2f** were found to be time-independent competitive inhibitors with K_i values similar to that of thioamide **2c** (i.e. roughly twice that of amide **2b**). We interpret this observation to indicate that these compounds can at least bind to the active site of chymotrypsin and that the increased bulk of the Michael acceptor relative to a carbonyl did not severely restrict binding. Inspection of hypothetical CT–Michael acceptor Michaelis complexes by means of molecular graphics techniques supports the lack of steric inhibition of binding despite the presumed "tightness" of the oxyanion hole (see below). Thus we conclude that the failure of **2e** or **2f** to inhibit chymotrypsin irreversibly may be due to the failure of the chemical step of addition of the serine nucleophile to the polarized C=C bond. However, with papain and **4e** this step evidently occurs quite readily, and **4e** behaves like a true irreversible affinity-labeling reagent toward papain.[3] This conclusion is supported by the following observations. The rate of papain inactivation by **4e** is concentration dependent, and Kitz-Wilson analysis[10] indicates rapid reversible E–I complex formation (K_i = 0.026 mM) followed by irreversible inactivation with first-order kinetics (k_2 = 0.0018 s^{-1}). Thus the apparent second-order rate constant (k_2/K_i) for inactivation of papain by **4e** is 70 M^{-1} s^{-1} at 25 °C. Overnight dialysis of papain inactivated by **4e** leads to no detectable reactivation, suggesting that the inactivation is indeed irreversible. The Michael acceptor moiety of **4e** is absolutely essential for activity as shown by the relatively weak activity of **6**, a des(methoxycarbonyl) analogue of **4e**, and **7**, the saturated analogue of **4e**, as competitive inhibitors of papain. Finally, the specificity of the inactivation process is reflected by the fact that incubation of papain with **1e** (1 mM for 20 min) led to no detectable loss of enzyme.

Michael acceptors **3e** and **3f** also proved to be affinity labels for DPP-I. Although these compounds did not bind as well to DPP-I as **4e** to papain, the irreversible reaction of their E–I complexes was faster (Table II). Thus the apparent second-order rate constants (k_2/K_i) for **3e** and **3f** were 68 and 55 M^{-1} s^{-1}, respectively, at 37 °C. The specificity of these processes is demonstrated by the fact that DPP-I showed no loss of activity after incubation for 90 min in a 1 mM solution of methyl *trans*-4-amino-2-butenoate (**5e**).

Molecular Graphics. In addition to biochemical evaluation of our compounds, the interactions of several of them with chymotrypsin and papain were examined by means of molecular graphics. Since this technique is relatively new, our main purpose was simply to determine the ways in which it might provide information complementary to or beyond that furnished by wet biochemical studies. We began by generating a stereoview of the active site of tosylchymotrypsin similar to that presented by Henderson,[25] using crystallographic coordinates provided by the Protein Data Bank.[4] Standard bond lengths and angles were used for generating structures of inhibitors.

The first inhibitor we examined by means of graphics was *N*-acetylphenylalaninal (**9**). This compound is thought to form a stable hemiacetal adduct with serine-195 of chymotrypsin. To fit this structure into CT, the hydrate of **9** (i.e. *N*-Ac-PheCH$(OH)_2$, **10**) was superimposed over the tosyl group by placing the aldehyde carbon at the locus of the tosyl sulfur, one of the oxygens of **10** over the oxygen of the tosyl group in the oxyanion hole, and the other oxygen of **10** essentially at the locus of the OH of serine-195. The tosyl moiety was then eliminated entirely and the conformation of the remainder of molecule **10** was adjusted until a "good" visual fit was obtained (Figure 1). Attempts to incorporate (**10**)–AcNH···O=C-Ser-214 hydrogen bonding, as discussed by various authors,[25-27] did

(25) Henderson, R. *J. Mol. Biol.* **1970**, *54*, 341.
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(27) Wipff, G.; Dearing, A.; Weiner, P. K.; Blaney, J. M.; Kollman, P. A. *J. Am. Chem. Soc.* **1983**, *105*, 997.

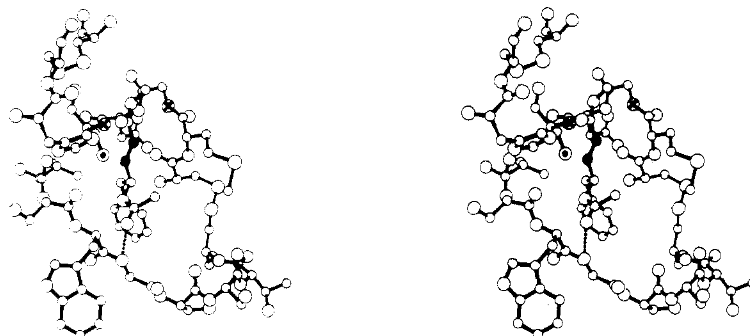

Figure 2. Stereopair view of inhibitor **2e** in the active site of chymotrypsin. The protein and the phenyl and acyl NH of the inhibitor are arranged as in Figure 1. The two solid circles represent the olefinic C=C bond of the inhibitor; other atoms are as in Figure 1.

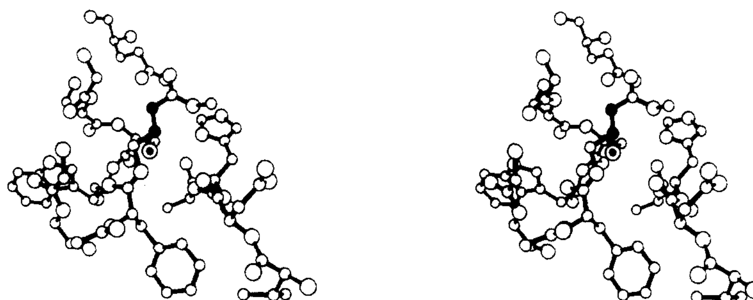

Figure 3. Stereopair view of inhibitor **4d** in the active site of papain. The sulfur of Cys-25 is represented by a circle with a dot in its center, and the two olefinic carbons of the inhibitor are represented by solid circles. Compare to crystal structures of covalent papain–inhibitor complexes given in ref 29.

not lead to satisfactory geometries. However, we did find it possible to incorporate a Gly-216-NH···O=C(CH_3)-NH–(**10**) hydrogen bond; this feature requires a cis amide bond in **10**, but the overall complex has otherwise favorable geometry and intermolecular interactions. The $CH(OH)_2$ group of the **10**–CT complex was then replaced, without changing the coordinates of the remaining portion of **10**, by the various groups **b–f** (see Scheme I); for **2b** and **2d** the carbonyl or thiocarbonyl groups were projected into the oxyanion hole. Visually it appeared that all of the groups, including the Michael acceptors **e** and **f**, fit well with no obvious steric problems. As an example, a potential Michaelis complex of **2e** with chymotrypsin is depicted in Figure 2. Actual calculation of distances from the NH units of the oxyanion hole to various atoms of the groups **b–f** corroborated the conclusions drawn from visual inspection of the structures (data not shown).

The examples shown in Figures 1 and 2 do not represent the only binding modes accessible to inhibitors **2b–2f** binding to chymotrypsin. Relaxation of the initial assumption that the carbonyl carbon equivalents of the inhibitors should be fixed at the same location as the tosyl sulfur in tosylchymotrypsin leads to an alternative binding mode similar to that proposed by Wipff et al.[27] for the interaction of D-*N*-acetyltryptophanamide with α-chymotrypsin. In this binding mode the inhibitors are capable of forming a hydrogen bond from the acyl nitrogen to the carbonyl group of Ser-214. Thus the major result of the molecular graphics studies with **2b–2f** and chymotrypsin, i.e. that all compounds appear able to bind to chymotrypsin, agrees with the biochemical finding that all are at least moderately effective competitive inhibitors. The *irreversible* inhibition of cysteine proteases but *not* chymotrypsin by the Michael acceptors is undoubtedly due to the *covalent* interaction of the active site cysteine with these inhibitors having no counterpart in the case of chymotrypsin. *Reversible* covalent E–I interaction *may* account for the enormously greater potency of nitriles toward cysteine proteases as compared to chymotrypsin, as it does in the case of aldehydes.[28]

A similar type of analysis was carried out for papain starting with the model structure of the *N*-Cbz-L-Phe-L-Ala- acyl enzyme given by Drenth et al.[4,29] Inhibitor **4e** was superimposed over the corresponding portions of the acyl enzyme structure, the Cbz-Phe-Ala moiety deleted, and the conformation of the ester group adjusted visually. The result is shown in Figure 3. The β-carbon of the Michael acceptor lies 180 pm directly above the sulfur of Cys-25; a normal C–S bond is ca. 172 pm. Thus the graphics supports the proposal, based on biochemical results, that **4e** is a specific affinity label for papain.

Experimental Section

Melting points were determined in open capillary tubes and are uncorrected. NMR spectra were run on Varian FT-80, T-60, and EM-360 spectrometers with tetramethylsilane as an internal standard. Elemental analyses were performed on a Hewlett-Packard Model 185B instrument and are within ±0.4% of the theoretical values. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Infrared spectra were taken on a Perkin-Elmer Model 33 spectrometer. Mass spectra were obtained on Ribermag R10-10 and Varian/Mat CH5 instruments by electron-impact ionization except where otherwise indicated. Spectrophotometric enzyme assays were performed on a Varian Cary 118 spectrophotometer.

Mixed-Anhydride Couplings (Method A).[9] The N-protected amino acid was dissolved in THF and *N*-methylmorpholine (1 equiv) was added. The solution was cooled to −15 °C, ethyl chloroformate (1 equiv) was added, and the solution was stirred for 20 min. The amine was dissolved in THF with *N*-methylmorpholine (1 equiv) and added to the reaction mixture while a temperature of −15 °C was maintained. Stirring was continued for 30 min at this temperature and a further 1–2 h while the reaction was allowed to warm to room temperature, after which

(28) Gamcsik, M. P.; Malthouse, J. P. G.; Primrose, W. U.; Mackenzie, N. E.; Boyd, A. S. F.; Russell, R. A.; Scott, A. I. *J. Am. Chem. Soc.* **1983**, *105*, 6324.
(29) Drenth, J.; Kalk, K. H.; Swen, H. M. *Biochemistry* **1976**, *15*, 3731.

the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2, washed with 2% HCl and saturated $NaHCO_3$, and dried ($MgSO_4$). The solvent was removed under reduced pressure to give the crude product.

Amine Protection and Deprotection (Methods B and C). The amino acid was dissolved in dioxane/H_2O (2:1) with 1 N NaOH (1 equiv). Di-*tert*-butyl pyrocarbonate (1 equiv) was added and the solution was stirred overnight while CO_2 evolved. The dioxane was removed under reduced pressure and the residue was extracted into $CHCl_3$. The organic solution was washed with 2% HCl and dried ($MgSO_4$), and the residue was purified by chromatography or crystallization.

For removal of the *tert*-butoxycarbonyl group, the *N*-Boc compound was dissolved in trifluoroacetic acid and allowed to stand for 20 min. The trifluoroacetic acid was removed under reduced pressure and the trifluoroacetate salt was purified by crystallization. In cases where the TFA salt was hygroscopic, the crude product was dissolved in methanol, oxalic acid (1 equiv) or methanesulfonic acid (1 equiv) was added, and the solvent was removed in vacuo. Methanol was added three more times and removed in vacuo each time. The white product was purified by crystallization.

Acetylation of Amines (Method D). The amine compound was dissolved in THF, Et_3N (2 equiv) added, the solution cooled to 4 °C, and acetic anhydride (2 equiv) added. The solution was allowed to warm to room temperature (45 min) and the solvent removed in vacuo. The residue was dissolved in $CHCl_3$ and washed with 2% HCl and saturated $NaHCO_3$. After filtration and evaporation, the product was purified by crystallization.

Horner-Wadsworth-Emmons Reaction (Method E). NaH (50% in mineral oil) was washed twice with hexane, and THF was added. Trimethyl phosphonoacetate (or dimethyl [(methylsulfonyl)methyl]phosphonate) (1 equiv) was added and the solution stirred for 30 min while H_2 evolved. The aldehyde (1 equiv) was dissolved in THF and added to the reaction mixture, and the mixture was stirred for 1.5 h. The reaction was quenched with H_2O and the THF was removed under reduced pressure. The residue was dissolved in $CHCl_3$, washed with 2% HCl and saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated to a yellow product, which was purified by chromatography or crystallization.

L-Phenylalanine Nitrile Methanesulfonate (1d). *N*-Boc-L-phenylalaninamide (1.31 g, 4.97 mmol) was dissolved in dry THF (50 mL) and Et_3N (1.5 mL, 2.2 equiv) was added. The solution was cooled on an ice bath to 5 °C and trifluoroacetic anhydride (0.78 mL, 1.1 equiv), was added over 5 min. The solution was allowed to warm to room temperature. After 30 min, the reaction was quenched with H_2O (3 mL), the THF removed in vacuo, and the product extracted into ether. The ether was washed with dilute acid and base and dried ($MgSO_4$). The solvent was removed and the crude product (11) was purified (1.15 g, 94%) by silica gel chromatography (EtOAc-hexane): mp 117.5 °C; NMR ($CDCl_3$) δ 1.43 (s, 9), 3.07 (t, 2), 4.60-4.85 (br s, 1), 7.31 (s, 5); CI mass spectrum, *m/e* (relative intensity) 247 (34, M + 1) 208 (51), 191 (47), 163 (12), 120 (96), 91 (100). Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

Compound 11 (500 mg, 2.03 mmol) was deprotected (method C). The residue was dissolved in EtOH (10 mL), methanesulfonic acid (0.13 mL, 1 equiv) was added, and the solvent was evaporated. The crude product was crystallized from EtOH (10 mL) and Et_2O (90 mL), yielding 1d (250 mg, 52%): mp 162.5-163 °C dec; NMR (D_2O) δ 2.70 (s, 3, CH_3SO_3), 3.25 (d, 2, J = 8 Hz), 4.58-4.82 (m, 2), 7.35 (s, 5). Anal. ($C_{10}H_{14}N_2O_3S$) C, H, N.

***N*-Acetyl-L-phenylalanine Nitrile (2d).** Compound 1d (288 mg, 1.22 mmol) was dissolved in dry THF (15 mL) and acetylated (method D). The crude product was recrystallized from $CHCl_3$ (10 mL) and hexane (140 mL), yielding 160 mg (70%): mp 125-126 °C; NMR ($CDCl_3$) δ 1.99 (s, 3), 3.08 (d, 2, J = 6 Hz), 4.98-5.27 (m, 1), 5.69-5.94 (br s, 1), 7.33 (s, 5); mass spectrum, *m/e* (relative intensity), 188 (6, M^+), 162 (1), 144 (1), 129 (58), 91 (100), 65 (23). Anal. ($C_{11}H_{12}N_2O$) C, H, N.

Glycyl-L-phenylalanine Nitrile Methanesulfonate (3d). Boc-glycine (232 mg, 1.33 mmol) was coupled to compound 1d (1.33 mmol) by method A. The crude product (12) was recrystallized from CH_2Cl_2 (10 mL) and hexane (90 mL), which yielded 346 mg (86%): mp 100-100.5 °C; NMR ($CDCl_3$) δ 1.44 (s, 9), 3.07 (d, 2, J = 7 Hz), 3.74 (d, 2, J = 6 Hz), 4.90-5.20 (m, 2), 7.00 (d, 1, J = 8 Hz), 7.31 (s, 5); mass spectrum, *m/e* (relative intensity) 247 (13), 230 (22), 220 (9), 203 (6), 229 (71), 120 (9), 91 (62), 57 (100). Anal. ($C_{16}H_{22}N_3O_3$) C, H, N.

Compound 12 (343 mg, 1.13 mmol) was deblocked by method C. The crude product was dissolved in MeOH and 1 equiv of methanesulfonic acid was added. The solution was concentrated and the crude solid was recrystallized twice from hot EtOH to give 125 mg of 3d (37% yield): mp 234.5-235 °C; NMR (D_2O) δ 2.74 (s, 3, CH_3SO_3), 3.17 (d, 2, J = 8 Hz), 3.73 (s, 2), 5.05 (t, 1, J = 8 Hz), 7.34 (s, 5); mass spectrum, *m/e* (relative intensity) 204 (5, M^+), 159 (28), 129 (17), 104 (10), 91 (100), 77 (18), 65 (31). Anal. ($C_{12}H_{17}N_3O_4S$) C, H, N.

[*N*-(*N*-Acetyl-L-phenylalaninyl)amino]acetonitrile (4d). *N*-Acetylphenylalanine (5 g, 24.15 mmol) was coupled to aminoacetonitrile hydrochloride (2.23 g, 24.15 mmol) by method A. (Later experience showed that DMF was much preferred over THF or CH_2Cl_2 for this reaction.) Recrystallization from EtOH (100 mL), $CHCl_3$ (50 mL), and hexane (700 mL) gave 3.33 g of product (56% yield): mp 183.5-185.5 °C; NMR (MeOH-d_4) δ 1.68 (s, 3), 2.48-2.96 (m, 2), 3.86 (s, 2), 4.23-4.43 (m, 1), 7.01 (s, 5, Ar); mass spectrum, *m/e* (relative intensity) 2.45 (4, M^+), 186 (31), 162 (7), 131 (31), 120 (100), 112 (24), 103 (27), 91 (100), 77 (27). Anal. ($C_{13}H_{15}N_3O_2$) C, H, N.

Methyl 4-Amino-5-phenyl-2-pentenoate Trifluoroacetate (1e). Boc-phenylalaninal (13) was prepared from Boc-Phe-OMe as described by Rich et al. for Boc-L-leucinal.[7] The ester (10.6 g) was dissolved in dry toluene (200 mL) and cooled to −78 °C. Dibal-H (1 M in hexane, Aldrich, 75.5 mL) was added over 35 min and the solution was stirred. After 5 min the reaction was quenched with MeOH (3.2 mL) and allowed to warm to room temperature. The solvent was removed in vacuo and the residue was dissolved in Et_2O (1 L) and washed with saturated NaK tartrate solution. The ether was reduced to 200 mL, washed with dilute acid and base, dried ($MgSO_4$), and removed in vacuo, yielding a solid material. This was dissolved in 20 mL of EtOH and added dropwise to a cold solution of 1 N $NaHSO_3$ (300 mL) and allowed to stir overnight at 4 °C. The resulting solution was washed with $CHCl_3$ (200 mL), made alkaline with K_2CO_3, and extracted with $CHCl_3$ (600 mL). The $CHCl_3$ solution was dried ($MgSO_4$) and the solvent was evaporated to give 5.31 g (56%) of a white solid, Boc-phenylalaninal: mp 86 °C; $[\alpha]^{25}_D$ −42.5° (*c* 2.4, MeOH); NMR ($CDCl_3$) δ 1.36 (s, 9), 2.86-3.03 (m, 2), 4.22 (m, 1), 5.26 (d, 1, J = 7 Hz), 7.23 (s, 5), 9.40 (s, 1); mass spectrum, *m/e* (relative intensity) 200 (23), 164 (54), 120 (83), 91 (19), 57 (100). Anal. ($C_{14}H_{19}NO_3$) C, H, N.

Boc-phenylalaninal (2.49 g, 10 mmol) was condensed with trimethyl phosphonoacetate by method E. The product (14) was obtained by recrystallization from EtOH and H_2O (1:1) (200 mL) to give 2.01 g (66.5%): mp 68-70 °C; $[\alpha]^{25}_D$ 0.52° (*c* 1.1, CH_2Cl_2); NMR ($CDCl_3$) δ 1.39 (s, 9), 2.88 (d, 2, J = 7 Hz), 3.72 (s, 3), 4.35-4.75 (br, 2), 5.85 (d, 1, J = 16 Hz), 6.9 (dd, 1, J = 16, 5 Hz), 7.26 (s, 5); mass spectrum, *m/e* (relative intensity) 305 (1, M^+), 214 (31), 158 (45), 114 (78), 91 (24), 57 (100). Anal. ($C_{17}H_{23}NO_4$) C, H, N.

Compound 14 (2 g, 6.56 mmol) was deprotected with trifluoroacetic acid (15 mL) by method C. The product (1e) was recrystallized from $CHCl_3$ (3 mL) and Et_2O (40 mL): yield 1.85 g (88%); mp 137.5-138 °C dec; $[\alpha]^{25}_D$ 45.5° (*c* 1.0, CH_3OH); NMR (D_2O) δ 3.04 (d, 2, J = 7 Hz), 3.67 (s, 3), 4.23 (d, 1, J = 7 Hz), 5.88 (d, 1, J = 16 Hz), 6.68 (dd, 1, J = 16, 7 Hz), 7.28 (s, 5); mass spectrum, *m/e* (relative intensity), 206 (1), 174 (3), 144 (1), 128 (1), 114 (100), 91 (14), 54 (24). Anal. ($C_{14}H_{17}F_3NO_4$) C, H, N.

Methyl *N*-Acetyl-4-amino-5-phenyl-2-pentenoate (2e). Compound 1e (490 mg, 1.54 mmol) was acetylated with acetic anhydride (2 equiv) by method D. The crude product was recrystallized (284 mg, 75%) frm $CHCl_3$-hexane (1:20): mp 83-83.5 °C; NMR ($CDCl_3$) δ 1.94 (s, 3), 2.91 (d, 2, J = 7 Hz), 3.71 (s, 3), 4.95 (br, 1), 5.40 (br, 1), 5.82 (d, 1, J = 16 Hz), 6.93 (dd, 1, J = 16, 6 Hz), 7.25 (s, 5); mass spectrum, *m/e* (relative intensity) 247 (1, M^+), 204 (1), 188 (6), 174 (8), 156 (45), 129 (10), 114 (100), 91 (30), 65 (13), 54 (17), 43 (46). Anal. ($C_{14}H_{17}NO_3$) C, H, N.

Methyl *N*-Glycyl-4-amino-5-phenyl-2-pentenoate Oxalate (3e). Boc-glycine (87 mg, 0.5 mmol) was coupled to methyl 4-amino-5-phenyl-2-pentenoate by method A. The crude product (15) was purified by column chromatography (EtOAc-hexane) which gave 126 mg (70%) of a very viscous oil: NMR ($CDCl_3$) δ 1.45 (s, 9), 2.90 (d, 2, J = 7 Hz), 3.70 (s, 3), 3.73 (d, 2, J = 6 Hz),

4.90 (m, 1), 5.52 (t, 1, J = 6 Hz), 5.80 (dd, 1, J = 16, 2 Hz), 7.24 (s, 5), 6.70–7.32 (m, 2); mass spectrum, m/e (relative intensity) 363 (1, M + 1), 306 (1), 289 (1), 271 (7), 215 (16), 197 (5), 188 (7), 174 (5), 171 (7), 129 (15), 114 (100), 91 (27), 84 (44), 57 (51).

Compound **15** (279 mg, 0.77 mmol) was deprotected with trifluoroacetic acid (3 mL) by method C. The crude product was dissolved in MeOH (5 mL) and oxalic acid (107 mg, 0.85 mmol) was added. The solvent was removed in vacuo. MeOH was added and removed in vacuo a total of four times to remove residual trifluoroacetic acid. The crude product (185 mg, 66%) was precipitated from MeOH (8 mL) and Et_2O (100 mL): mp 172 °C; NMR (D_2O) δ 2.73–3.02 (m, 2), 3.65 (s, 5), 4.58–4.88 (br, 1), 5.84 (dd, 1, J = 16, 2 Hz), 6.91 (dd, 1, J = 16, 5 Hz), 7.24 (s, 5); mass spectrum, m/e (relative intensity) 263 (1, M + 1), 262 (1, M^+) 245 (1), 233 (1), 214 (3), 188 (6), 171 (60), 139 (8), 129 (23), 114 (100), 91 (49), 82 (11), 65 (15). Anal. ($C_{16}H_{20}N_2O_7$) C, H, N.

Methyl 4-Amino-2-butenoate Trifluoroacetate (5e). *N*-(*tert*-Butoxycarbonyl)-3-aminopropene (2.13 g, 13.57 mmol) was dissolved in 50 mL of MeOH and the mixture cooled to −78 °C. Ozone was bubbled through for 20 min (until the solution became blue). Nitrogen was passed through the solution to displace the excess ozone and dimethyl sulfide (1.5 mL, 20.4 mmol) was then added. The solution was allowed to warm to room temperature very slowly (6 h or more). The solution was concentrated and dissolved in $CHCl_3$, washed with dilute acid and base, and dried ($MgSO_4$). Evaporation gave 2.04 g (94%) and the product, *N*-(*tert*-butoxycarbonyl)glycinal, was used without further purification. It was condensed with trimethyl phosphonoacetate (1 equiv) by method E to produce the Boc derivative of **5e**. This compound was purified by medium pressure silica gel chromatography (EtOAc–hexane): yield 280 mg (62%); mp 45 °C; NMR ($CDCl_3$) δ 1.44 (s, 9), 3.69 (s, 3), 3.73–3.96 (m, 2), 6.63 (br s, 1), 5.87 (d, 1, J = 16 Hz), 6.88 (dt, 1 J = 16, 5 Hz); mass spectrum, m/e (relative intensity) 159 (23), 142 (15), 127 (10), 115 (8), 84 (31), 57 (100). Anal. ($C_{10}H_{17}NO_4$) C, H, N.

Boc-**5e** (327 mg, 1.52 mmol) was deprotected by method C. The crude product was recrystallized from MeOH (1 mL) and Et_2O (10 mL), yielding 327 mg of **5e** (94%): mp 120–120.5 °C; NMR (acetone-d_6) δ 3.69 (s, 3), 3.91 (d, 2, J = 6 Hz), 4.59 (d, 3, J = 6 Hz), 6.09 (dd, 1, J = 16, 8 Hz), 6.91 (dt, 1, J = 16, 6 Hz). Anal. ($C_7H_{10}F_3NO_4$) C, H, N.

Methyl *N*-(*N*-Acetyl-L-phenylalanyl)-4-amino-2-pentenoate (4e). *N*-Acetyl-L-phenylalanine (501 mg, 2.42 mmol) was coupled to compound **5e** by method A. Precipitation from EtOH (5 mL) and $CHCl_3$ (25 mL) with hexane (400 mL) yielded pure **4e** (70%): mp 156.5–157 °C; NMR ($CDCl_3$) δ 1.95 (s, 3), 3.05 (d, 2, J = 8 Hz), 3.70 (s, 3), 3.80–3.97 (m, 2), 4.66 (q, 1, J = 8 Hz), 5.66 (d, 1, J = 16 Hz), 6.69 (dd, 1, J = 16, 6 Hz), 6.20–6.60 (br, m, 2), 7.2 (s, 5); mass spectrum, m/e (relative intensity) 304 (4, M^+), 261 (3), 245 (4), 213 (2), 190 (5), 162 (22), 120 (100), 114 (26), 91 (26). Anal. ($C_{16}H_{20}N_2O_4$) C, H, N.

3-Amino-4-phenyl-1-(methylsulfonyl)-1-butene Trifluoroacetate (1f). Boc-phenylalaninal (**13**) was condensed with dimethyl [(methylsulfonyl)methyl]phosphonate (6.3 g, 31.2 mmol) by method E. The product (**16**) was recrystallized from $CHCl_3$ (30 mL) and hexane (100 mL): yield 1.25 g (58%); mp 143–144 °C; $[\alpha]^{25}_D$ 6.68° (c 0.9, CH_2Cl_2); NMR ($CDCl_3$) δ 1.36 (s, 9), 2.83 (s, 3), 2.93–3.02 (d, 2, J = 4 Hz), 4.66 (br, 1), 6.06–5.52 (br, 1), 6.30 (d, 1, J = 16 Hz), 6.9 (br d, 1, J = 16 Hz), 7.27 (s, 5); mass spectrum, m/e (relative intensity) 270 (8), 252 (12), 246 (15), 234 (15), 190 (60), 178 (21), 134 (92), 91 (100). Anal. ($C_{16}H_{23}NO_4S$) C, H, N.

Compound **16** (502 mg, 1.54 mmol) was deprotected with trifluoroacetic acid (6 mL) by method C. The product (**1f**) was recrystallized from MeOH (10 mL) and Et_2O (50 mL): yield 480 mg (92%); mp 196–200 °C dec; $[\alpha]^{25}_D$ 33.9° (c 1.0, MeOH); NMR (D_2O) δ 2.95 (s, 3), 2.98–3.23 (m, 2), 4.15–4.47 (m, 1), 6.55 (d, 1, J = 16 Hz), 6.82 (dd, 1, J = 16, 6 Hz), 7.3 (s, 5); mass spectrum, m/e (relative intensity) 226 (1), 146 (12), 134 (56), 91 (31), 72 (20), 54 (100). Anal. ($C_{13}H_{16}F_3NO_4S$) C, H, N.

***N*-Acetyl-3-amino-1-(methylsulfonyl)-4-phenyl-1-butene (2f).** Compound **1f** (410 mg, 1.21 mmol) was treated with acetic anhydride (2 equiv) by method D. The product was recrystallized from EtOH (10 mL) and hexane (50 mL): yield 291 mg (90%); mp 164 °C; NMR ($CDCl_3$) δ 1.96 (s, 3), 2.88 (s, 3), 2.95 (d, 2, J = 6 Hz), 4.94 (br t, 1, J = 6 Hz), 5.57 (br d, 1, J = 6 Hz), 6.78 (d, 1, J = 15 Hz), 6.83 (dd, 1, J = 15, 6 Hz), 7.26 (s, 5); mass spectrum, m/e (relative intensity) 268 (2, M + 1), 208 (6), 188 (16), 176 (12), 146 (12), 134 (80), 129 (20), 128 (23), 91 (70), 65 (26), 54 (63), 43 (100). Anal. ($C_{13}H_{17}NO_3S$) C, H, N.

Boc-glycine (291 mg, 1.66 mmol) was coupled with **1f** (563 mg, 1.66 mmol) by method A. The product (**17**) was recrystallized from EtOAc (18 mL) and hexane (80 mL): yield 476 mg (75%); mp 91 °C; NMR ($CDCl_3$) δ 1.42 (s, 9), 285 (s, 3), 2.91 (d, 2, J = 7 Hz), 3.69 (d, 2, J = 6 Hz), 4.78–5.13 (br, 1), 5.43 (t, 1, J = 6 Hz), 6.39 (d, 1, J = 15 Hz), 7.20 (s, 5), 6.68–7.3 (m, 2); mass spectrum, m/e (relative intensity) 309 (1), 303 (2), 247 (3), 235 (5), 155 (10), 134 (24), 91 (22), 57 (100). Anal. ($C_{18}H_{26}N_2O_5S$) C, H, N.

Compound 17 (44.7 mg, 0.12 mmol) was deprotected by method C and the counterion was changed to oxalate as with compound **3f**. The product was precipitated cleanly (40.7 mg, 91%) from warm MeOH (10 mL) and Et_2O (10 mL): mp 198–199 °C; NMR (D_2O) δ 2.87 (d, 2, J = 8 Hz), 2.97 (s, 3), 3.65 (s, 3), 4.60–5.00 (br, 1), 6.53 (d, 1, J = 16 Hz), 6.93 (dd, 1, J = 16, 4 Hz), 7.25 (s, 5); mass spectrum, m/e (reltive intensity) 283 (3, M + 1), 282 (3, M^+), 203 (84), 246 (25), 134 (49), 120 (70), 111 (100), 91 (100), 83 (65), 65 (74), 56 (85). Anal. ($C_{15}H_{20}N_2O_7S$) C, H, N.

***N*-Glycyl-L-phenylalaninamide Methanesulfonate (3b).** *N*-Boc-glycine (363 mg, 2.07 mmol) was coupled to phenylalaninamide hydrobromide (508 mg, 2.07 mmol) by method A. The product **18** was recrystallized from EtOH (2 mL), $CHCl_3$ (20 mL), and hexane (50 mL): yield 518 mg (78%); mp 167–168 °C; NMR ($CDCl_3$) δ 1.41 (s, 9), 3.05–3.16 (m, 2), 3.73 (d, 2, J = 5.8 Hz), 4.70 (q, 1, J = 7.2 Hz), 5.14 (br, t, 1, J = 5.6 Hz), 5.53 (br, s, 1), 6.14 (br, s, 1), 6.79 (br, d, 1, J = 7.8 Hz), 7.26 (s, 5). Anal. ($C_{16}H_{23}N_3O_4$) C, H, N.

The product of the above reaction was deprotected by method C and the counterion was changed to mesylate by the method used for compound **1d**. The product was recrystallized from MeOH (60 mL) and Et_2O (60 mL): yield 359 mg (90%); mp 199–201 °C; NMR (D_2O) δ 2.72 (s, 3), 2.93–3.09 (m, 2), 3.69 (s, 2), 4.49–4.63 (m, 1), 7.28 (s, 1). Anal. ($C_{12}H_{19}N_3O_5S$) C, H, N.

***N*-(*N*-Acetyl-L-phenylalaninyl)glycinamide (4b).** *N*-Acetyl-L-phenylalanine (1.0 g, 4.83 mmol) was coupled to methyl glycinate hydrochloride (0.607 g, 4.83 mmol) by method A. The product (**19**) was precipitated from CH_2Cl_2 (50 mL) with hexane (170 mL): yield 851 mg (63%); mp 137–138.5 °C; NMR ($CDCl_3$) δ 1.91 (s, 3), 3.03 (d, 2, J = 7 Hz), 3.68 (s, 3), 3.93 (d, 2, J = 6 Hz), 4.80 (q, 1, J = 7 Hz), 6.55–7.10 (m, 2), 7.23 (s, 5); mass spectrum, m/e (relative intensity) 2.78 (2, M^+), 247 (1), 219 (16), 190 (6), 162 (16), 145 (33), 131 (27), 120 (100), 91 (25). Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

Compound **19** (315 mg, 1.13 mmol) was dissolved in MeOH (10 mL) and cooled to 5 °C. NH_3 was bubbled through the solution for 2.5 h. The solution was allowed to warm to room temperature and stirred for 8 h. The solvent was evaporated and the white product was recrystallized (239 mg, 81%) from MeOH (4 mL), EtOAc (20 mL), and Et_2O (2 mL): mp 184 °C; NMR (CD_3OD) δ 1.70 (s, 3), 2.80 (t, 2), 3.55 (d, 2, J = 6 Hz), 4.25 (dd, 1, J = 8, 7 Hz), 7.00 (s, 5); CI mass spectrum, m/e (relative intensity) 264 (36, M + 1), 247 (10), 204 (11), 190 (43), 162 (35), 146 (68), 131 (27), 120 (100), 91 (27). Anal. ($C_{13}H_{17}N_3O_3$) C, H, N.

L-Phenylalanine Thioamide (1c). Boc-L-phenylalaninamide (4.0 g, 15.15 mmol) was dissolved in HMPA (20 mL). Lawesson's reagent[6] (Aldrich, 3.1 g, 7.6 mmol) was added and the solution was stirred at 80 °C for 1 h. The solution was allowed to cool to room temperature and the reaction mixture was partially purified by silica gel chromatography (EtOAc, hexane). The product (**20**) was twice recrystallized from Et_2O (10 mL) and hexane (500 mL) to yield 2.95 g (70%): mp 104–105 °C; NMR ($CDCl_3$) δ 1.33 (s, 9), 3.07 (d, 2, J = 8 Hz), 4.70 (q, 1, J = 8 Hz), 5.53 (d, 1, J = 8 Hz), 7.27 (s, 5), 7.77–8.07 (br 2); mass spectrum, m/e (relative intensity) 280 (4, M^+), 224 (13), 220 (8), 207 (5), 163 (32), 130 (8), 120 (68), 91 (54), 57 (100). Anal. ($C_{14}H_{20}N_2O_2S$) C, H, N.

Compound **20** (201 mg, 0.72 mmol) was deprotected by method C. The product was recrystallized from EtOH (1 mL), Et_2O (5 mL), and hexane (30 mL) to yield 197 mg (93%): mp 168–169.5 °C dec; NMR (D_2O) δ 3.17 (d, 2, J = 7.4 Hz), 4.37 (t, 1, J = 7.4 Hz), 7.31 (s, 5); mass spectrum, m/e (relative intensity) 180 (4, M^+), 163 (40), 130 (7), 120 (100), 104 (19), 91 (36). Anal. ($C_{11}H_{13}F_3N_2O_2S$) C, H, N.

***N*-Acetyl-L-phenylalanine Thioamide (2c).** Compound 1c (112 mg, 0.38 mmol) was acetylated by method D. The product was recrystallized from $CHCl_3$ (10 mL) and hexane (50 mL): yield 64 mg (76%); mp 163–164 °C; NMR (CD_3OD) δ 1.66 (s, 3), 2.73–2.97 (m, 2), 4.45–4.73 (m, 1), 7.02 (s, 5); mass spectrum, *m/e* (relative intensity) 222 (14, M^+), 162 (35), 129 (43), 120 (72), 91 (100), 89 (300), 77 (14), 65 (26). Anal. ($C_{11}H_{14}N_2OS$) C, H, N.

***N*-(*N*-Acetyl-L-phenylalaninyl)glycine Thioamide (4c).** Compound 4d (1.25 g, 5.10 mmol) was dissolved in 15 mL of pyridine and 10 mL of Et_3N with mild heating. H_2S was bubbled through for 30 min and the solution was allowed to stir overnight. The solution was diluted with 300 mL of ether and a precipitate separated. The supernatant was evaporated and extracted into EtOAc. The solution was washed with dilute acid and base and dried ($MgSO_4$). The solvent was removed and the product was recrystallized from EtOH (10 mL), EtOAc (10 mL), and hexane (400 mL): yield 441 mg (38%); mp 178 °C; NMR (MeOH-d_4) δ 1.68 (s, 3), 2.60–2.95 (m, 2), 3.86 (d, 2, J = 6 Hz), 4.06–4.30 (m, 1), 6.98 (s, 5); mass spectrum, *m/e* (relative intensity) 279 (10, M^+) 220 (17), 162 (16), 132 (22), 120 (100), 91 (63). Anal. ($C_{13}H_{17}N_3O_2S$) C, H, N.

***N*-(*N*-Acetyl-L-phenylalaninyl)-3-aminopropene (6).** *N*-Acetyl-L-phenylalanine (2.5 g, 12.08 mmol) was coupled to allylamine (0.91 mL, 12.08 mmol) by method A. The product was crystallized to fine white needles from hot EtOH (20 mL) and hexane (200 mL): yield 957 mg (32%); mp 160–161 °C; NMR ($CDCl_3$) δ 1.96 (s, 3), 3.06 (d, 2, J = 6.7 Hz), 3.75 (t, 2, J = 5.5 Hz), 4.65 (q, 1, J = 7 Hz), 4.80–5.15 (m, 2), 5.40–6.00 (m, 1), 6.00 (br, 1), 6.38 (br d, 1, J = 7 Hz), 7.24 (s, 5). Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

Methyl *N*-(*N*-Acetyl-L-phenylalaninyl)-4-aminobutanoate (7). *N*-Acetyl-L-phenylalanine (0.5 g, 2.42 mmol) was coupled to methyl 4-aminobutanoate (370 mg, 2.42 mmol) by method A. The product was crystallized from $CHCl_3$ (5 mL), Et_2O (60 mL), and hexane (20 mL): yield 276 mg (37%); mp 120–120.5 °C; NMR ($CDCl_3$) δ 1.56–1.75 (m, 2), 1.97 (s, 3), 2.11–2.28 (m, 2), 2.97–3.21 (m, 4), 3.65 (s, 3), 4.51 (q, 1, J = 6.4 Hz), 5.88–6.43 (br, 2), 7.23 (s, 5). Anal. ($C_{16}H_{22}N_2O_4$) C, H, N.

Assay and Inhibition of Chymotrypsin. Chymotrypsin (Worthington, crystallized three times) was assayed with use of *N*-glutaryl-L-phenylalanine *p*-nitroanilide essentially as described by Erlanger et al.,[30] except that the rate of substrate hydrolysis at 25 °C was monitored continuously at 410 nm ($\Delta\epsilon$ = 8600 M^{-1} cm^{-1}). Test compounds were added in small aliquots of methanol (<5% of final volume).

Assay and Inhibition of Cytosolic Leucine Aminopeptidase. LAP-C (EC 3.4.11.1) was obtained from Sigma (type III-CP) and activated as described by Anderson et al.[31] The hydrolysis of L-leucine *p*-nitroanilide (Sigma) at 25 °C was monitored continuously at 405 nm ($\Delta\epsilon$ = 9330 M^{-1} cm^{-1}). Inhibitors were dissolved in buffer and added to the enzyme prior to addition of substrate.

Assay and Inhibition of Microsomal Leucine Aminopeptidase. LAP-M (EC 3.4.11.2) was obtained from Sigma (type VI-S). It was diluted to 1 unit/mL of phosphate buffer (50 mM, pH 7.2) and assayed as described for LAP-C.

Assay and Inhibition of Papain. Papain was obtained from Sigma (type IV), and solutions were prepared fresh daily by incubating the enzyme (6 mg) for 45 min at 25 °C in 100 mL of phosphate buffer (50 mM, pH 6.2) containing 1 mM EDTA and 0.25 mM cysteine. The rate of hydrolysis of *N*-Cbz-glycine *p*-nitrophenyl ester at 25 °C was monitored continuously at 340 nm, using $\Delta\epsilon$ = 6000 M^{-1} cm^{-1} as described by Bajkowski and Frankfater.[32] Small corrections were made for the rate of nonenzymic hydrolysis of the substrate. Both substrate and inhibitors were dissolved in acetonitrile or DMF, the final concentration of which was kept below 2.5% v/v, although concentrations up to 10% or more had no adverse effects.

Assay and Inhibition of Dipeptidyl Aminopeptidase-I. DPP-I (EC 3.4.14.1) was obtained from Boehringer-Mannheim in 50% glycerol solution. This preparation was diluted 1:200 daily with ice-cold buffer (50 mM citrate, pH 5) containing 10 mM cysteamine and was stored on ice until just before use. The hydrolysis of glycylphenylalanine *p*-nitroanilide (Enzyme System Products) at 37 °C was monitored at 405 nm ($\Delta\epsilon$ = 9900 M^{-1} cm^{-1}), as recommended by the enzyme supplier. Substrate and inhibitors were dissolved in buffer. Reactions were initiated by adding substrate to the prewarmed enzyme/inhibitor solution.

Kinetic Methods. Lineweaver–Burk double-reciprocal plots were used to determine the K_m value of the assay substrate for each enzyme and the K_i value of the reversible inhibitors. To assess irreversible inhibition the enzyme was incubated with varying concentrations of inhibitor, and at various times aliquots were diluted with substrate for measurement of residual activity. These data were plotted by the method of Kitz and Wilson[10] to determine K_i and k_2. All data were plotted for inspection, but in all cases slopes and intercepts were determined by linear regression analysis.

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