Journal of Medicinal Chemistry

Volume 27, Number 6 June 1984

Communications to the Editor

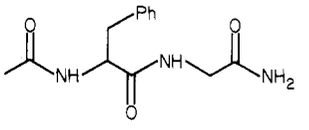
Vinylogous Amino Acid Esters: A New Class of Inactivators for Thiol Proteases

Sir:

The action of protease and peptidase enzymes plays a critical role in many diverse biological processes, including digestion, blood clotting, complement activation, tumor metastasis, viral replication, inflammation and tissue degenerative diseases, the biosynthesis and degradation of peptide hormones and neurotransmitters, and many others.[1,2] Extensive studies, chiefly with digestive and bacterial proteases, have revealed the major mechanistic features of several distinct classes of proteolytic enzymes, and a wide variety of naturally occurring, as well as synthetic, inhibitors of protease enzymes are known.[3] however, the fact still remains that very few therapeutic agents act primarily by modulating the activity of a proteolytic enzyme; notable exceptions would include captopril,[4] a synthetic inhibitor of angiotensin converting enzyme, and the penicillins and related β-lactams, which inhibit a transpeptidation step in bacterial cell wall synthesis.[5]

Our laboratory has been interested in applying knowledge of the mechanism of action of protease enzymes to the design of *new types* of inhibitors for these enzymes in the hope that it might eventually be possible to design effective, selective inhibitors for in vivo use against specifically targeted proteolytic enzymes. Such compounds could potentially be useful as therapeutic agents, investigational tools, or both. In this paper, we report on the design, synthesis, and characterization of one new type of inhibitor that shows selectivity for thiol proteases, a group of enzymes implicated in several pathological processes.

Our starting point is the fact that catalysis by serine and cysteine proteases involves attack by an enzymic nucleophile (either Ser-OH or Cys-SH) on the carbonyl group of the scissile peptide bond. We reasoned that replacement of this carbonyl group by a suitable "nucleophile trapping moiety" could convert a good substrate into an inhibitor (or inactivator) while still allowing maximum preservation of those structural features of the substrate responsible for enzyme–substrate recognition and binding. One specific example of this sort of change is illustrated in the comparison between **1**, a substrate for the thiol protease papain, and **2**, and analogue of **1** with potential for irreversible trapping of the SH group of Cys-25 at the active site of papain.



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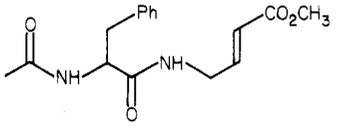

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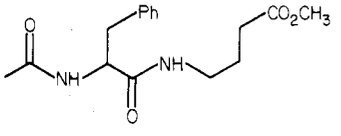

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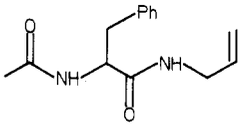

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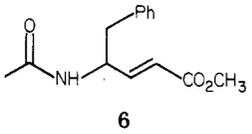

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Chemistry. The synthesis of **2** was achieved in five steps. Allylamine was protected with a *tert*-butoxycarbonyl (Boc) group (di-*tert*-butyl pyrocarbonate, NaOH, dioxane, H_2O) in 82% yield. Ozonolysis of the double bond (O_3, MeOH, −78 °C; then CH_3SCH_3, 25 °C) resulted in *N*-(*tert*-butoxycarbonyl)glycinal (94% yield), which was used without further purification. The aldehyde was converted to methyl *N*-(*tert*-butoxycarbonyl)-4-amino-2-butenoate (NaH, trimethylphosphonoacetate, THF) in 45% yield. Deprotection with trifluoroacetic acid (TFA), followed by coupling to *N*-acetylphenylalanine (isobutyl chloroformate, *N*-methylmorpholine, THF, −15 °C), resulted in **2** (23% overall yield). Compounds **3** and **4** were prepared by similar coupling of either methyl 4-aminobutyrate or allylamine, respectively, to *N*-acetylphenylalanine.[6]

Results and Discussion

Papain-catalyzed hydrolysis of *p*-nitrophenyl *N*-(benzyloxycarbonyl)glycinate (Z-Gly-NP, **5**, K_m = 10.3 μM) was followed photometrically at 347.5 nm.[7] Amide **1** was found

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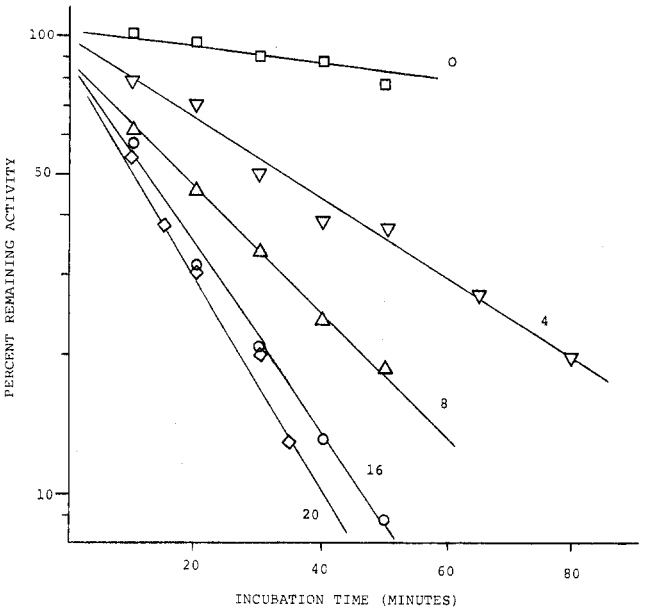

Figure 1. Inactivation of papain by methyl 4-[(*N*-acetylphenylalanyl)amino]but-2-enoate (**2**). Papain was incubated at 23 °C, pH 6.2, with the following concentrations of inhibitor **2**: (□) uninhibited control; (▽) 4 μM; (△) 8 μM; (○) 16 μM; (◇) 20 μM. At various times, aliquots were removed and diluted with an equal volume of a 50 μM solution of substrate (**5**) for assay. A replot of the regression slopes of these lines according to ref 8 (r = 0.9998) gives estimates for K_i and k_2 of 26 μM and 1.82 × 10^{-3} s^{-1}, respectively.

to inhibit the hydrolysis of **5** competitively with a K_i of 4.7 mM. In the presence of **2** (1 μM), the hydrolysis of **5** was markedly inhibited, and preincubation of papain with **2** (4–20 μM) was found to lead to a progressive loss of enzyme activity, which followed pseudo-first-order kinetics (Figure 1). Analysis[8] of these data revealed a K_i of 26 μM and a k_2 of 1.82 × 10^{-3} s^{-1}. These data are consistent with **2** being an active-site-directed irreversible inhibitor or affinity-labeling reagent for papain. To investigate this possibility further, we completely inactivated papain by incubation with excess **2** (200 μM for 4 h) and then subjected it to exhaustive dialysis. Control enzyme maintained a high degree of activity throughout this experiment, whereas the enzyme inactivated with **2** showed no detectable recovery of activity, confirming that the inactivation is essentially irreversible.

Several other experiments were performed to characterize the action of **2** as an inactivator of papain. First, compounds **3** and **4** were prepared and found to be weak *competitive* inhibitors of papain (K_i > 5 mM; cf. **1**), indicating that the Michael acceptor moiety of **2** was essential for activity. To evaluate the specificity of the inactivation of papain by **2**, we prepared analogue **6**. In **6**, the benzyl side chain is in position P_1,[9] whereas papain prefers such residues at position P_2 (cf. **1**–**5**). Thus, the binding of **6** to the hydrophobic pocket of papain (i.e., S_2) should position the Michael acceptor too far from the active site SH for effective interaction. In accordance with this expectation, compound **6** (1 mM for 0.3 h) did not detectably inactivate papain.

We have also evaluated the activity of Michael acceptors analogous to **2** toward several other proteases of differing mechanistic classes and substrate specificities. These results, to be described more fully elsewhere,[10] indicate that the thiol protease cathepsin C is also efficiently and irreversibly inactivated by Michael acceptor analogues of its substrates. However, Michael acceptors designed for chymotrypsin (e.g., **6**) and leucine aminopeptidase (both cytosolic and microsomal forms) failed to show any inhibition or inactivation of these enzymes. Thus, the Michael acceptors exemplified by **2** constitute a new class of protease inhibitors that may prove to be specific for thiol proteases.

Acknowledgment. Financial support for this work was provided by a grant from the University of Kansas General Research Fund.

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Received November 3, 1983