Acid-Sensitive Latent Inhibitors for Proteolytic Enzymes: Synthesis and Characterization

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The reaction between peptide aldehydes and acylhydrazones affords derivatives that represent potential prodrugs for selective inhibition of lysosomal enzymes. BzPheal=Ala, obtained from the reaction between *N*-benzoyl-L-phenylalaninal and *N*-acetyl-L-alanine hydrazide, has been most carefully studied. When BzPheal=Ala is introduced into ongoing reactions catalyzed by α-chymotrypsin or papain, the rate of these reactions diminishes more rapidly with time than do those of controls lacking BzPheal=Ala. Furthermore, the disparity between run and control is much greater at pH 5 than at pH 7. The extent of inhibition (defined as explained in the text) at pH 5 can exceed that at pH 7 by 25–40-fold. The data are quantitatively explained by a reaction scheme that recognizes three important properties of BzPheal=Ala: (1) It undergoes hydrolysis at pH 5–7 to regenerate *N*-benzoyl-L-phenylalaninal; (2) the aldehyde thus liberated is a far more potent inhibitor for serine or cysteine proteases than is BzPheal=Ala; and (3) the rate constant for hydrolysis of BzPheal=Ala at pH 5 greatly exceeds that at pH 7.

The "prodrug" concept posits that covalent attachment of a drug to a suitable carrier can afford materials with desirable pharmacological properties.[1,2] For example, a properly selected carrier can assist targeting or transport of the drug or can reduce its undesirable toxicological side effects. The prodrug itself may or may not display biochemical activity characteristic of the original drug. If it does not, enzymatic or simple chemical processing can liberate active drug from a prodrug precursor.

Lysosomes are subcellular organelles that contain an array of degradative enzymes in an environment of low pH (estimated as ~5).[3,4] A suitable prodrug, when introduced into the lysosome, could be activated by either those enzymes or the low-pH milieu itself.[5-7] The binding of daunomycin to carrier macromolecules via a pH-sensitive *cis*-aconityl link illustrates the latter approach[8,9] and bears on the preliminary efforts described here to develop a systematic approach to the design of pH-sensitive prodrugs directed toward lysosomes. Scheme I summarizes the principles underlying the present approach (each of the three reactions may be either irreversible or reversible). Intact prodrug IX exhibits little biochemical activity toward the target protein E. This is because the blocking group X, covalently bound to active inhibitor I, occupies a site essential to the inhibitor's interaction with, or recognition by, its target. However, the bond joining X to I undergoes hydrolysis at pH 5 far more rapidly than at pH 7. The encounter between compound IX and a region of low pH liberates free inhibitor, which subsequently inhibits the target protein.

Scheme I

$$E + IX \rightleftharpoons E{\cdot}IX$$
$$IX \rightarrow I + X$$
$$E + I \rightarrow E{\cdot}I$$

The choice of reagents for these initial studies was influenced by reports that peptide aldehydes affect lysosomal function[10-13] and by the observation that papain and lysosomal cathepsin B may be homologous enzymes.[14-16] Efforts to test the chemistry illustrated in Scheme I thus focused on studies of potential prodrugs derived from *N*-benzoyl-L-phenylalaninal (BzPheal) and other peptide aldehydes. These aldehydes reversibly inhibit proteases, such as α-chymotrypsin and papain, that possess an active site serine or cysteine residue which covalently binds to the aldehydo group.[17-22] This essential carbonyl group offers numerous opportunities for chemical attachment of a blocking group,[23] and the introduction of that blocking group should drastically reduce the inhibitory power of the aldehyde. For the purposes of the intended kinetic studies it was important that any IX derivative selected meet three criteria: (a) exhibit high solubility in aqueous solution; (b) undergo hydrolysis at pH 5 much more rapidly than at pH 7, with a half-life of the order of minutes for hydrolysis at pH 5, 35 °C; and (c) demonstrate a favorable hydrolytic equilibrium constant. Preliminary investigations established that reaction of *N*-acetyl-L-AlaNHNH$_2$ or nicotinic acid hydrazide with peptide aldehydes readily affords derivatives of the desired kind. The structures of the three substances that have proven most useful are shown. Each of these compounds satisfies the three basic requirements just enumerated.

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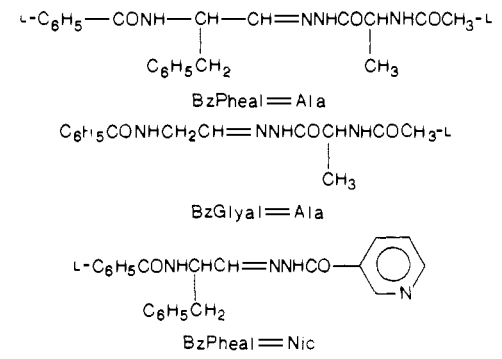

Experimental Section

General Synthetic Procedures. All compounds were tested for homogeneity by thin-layer chromatography (TLC) on Eastman silica gel Chromagram sheets. Iodine vapor was used for visualization. All reported R_f values refer to 9/1 benzene/ethanol. Melting points, obtained on a Mel-Temp apparatus, are uncorrected. Microanalyses were performed at the University of Massachusetts, Amherst. Values for proton NMR chemical shifts, obtained on samples dissolved in dimethyl-d_6 sulfoxide with a Varian EM-360 instrument, are reported in ppm from internal Me_4Si.

Nicotinic acid hydrazide was purchased from Aldrich Chemical Co. Other essential reactants were synthesized by reported procedures. *N*-Benzoyl-L-phenylalaninal (BzPheal) had mp 143–145 °C and $[\alpha]^{22}_D$ −109° (*c* 3, EtOH) [lit.[24] mp 143–144 °C, $[\alpha]^{20}_D$ −107° (*c* 2.5, EtOH)]. *N*-Benzoylglycinal (BzGlyal) had mp 64–65 °C (variable) (lit.[20] mp 66–72 °C). *N*-Acetyl-L-alanine hydrazide exhibited mp 138–147 °C and $[\alpha]^{22}_D$ −61° (*c* 1, MeOH) [lit.[25,26] mp 137–139 °C or 151–153 °C, $[\alpha]^{23}_D$ −60° (*c* 1, MeOH)].

Synthesis of BzPheal=Ala. A room temperature reaction mixture contained 200 mg of BzPheal, 100 mg of Ac-L-AlaNHNH$_2$, and 2 drops of acetic acid dissolved in 6 mL of methanol. The precipitate that first appeared after 2 h was collected after a total of 5 h of reaction, washed with a little MeOH, and dried under vacuum (weight 180 mg). BzPheal=Ala had mp 253–255 °C and $[\alpha]^{22}_D$ −31° (*c* 2.5, Me_2SO). It was homogeneous on TLC (R_f = 0.48). The most characteristic features of its ^{1}H NMR are δ 1.2 (d, J = 8 Hz, 3 H, CH_3 of Ala), 1.8 (s, 3 H, CH_3 of CH_3CO), 3.1 (m, 2 H, CH_2 of $C_6H_5CH_2$), 8.7 (d, J = 8 Hz, 1 H, CH=N), and 11.0 (br, 1 H, =NNHCO) and a group of unresolved protons at δ 7.2–8.3 ppm. The α-protons of the Phe and Ala residues are so broad as to be barely discernible on the EM-360. Anal. ($C_{21}H_{24}N_4O_3$) C, H, N.

Comparable methods afforded samples of BzPheal=Nic and BzGly=Ala. The former had mp 199–201 °C, $[\alpha]^{22}_D$ −27° (*c* 4, Me_2SO), and R_f = 0.57 on TLC. ^{1}H NMR features are δ 3.1 (m, 2 H, CH_2 of Phe), 4.9 (br, 1 H, α-CH of Phe), 7.2–9.0 [m, 16(?) H], and 11.6 (s, 1 H, =NNHCO). Anal. ($C_{22}H_{20}N_4O_2$) C, H, N. BzGlyal=Ala showed mp 214–217 °C, $[\alpha]^{22}_D$ +13° (*c* 0.6, Me_2SO), and R_f = 0.31 on TLC. Its ^{1}H NMR exhibited resonances at δ 1.2 (d, J = 8 Hz, 3 H, CH_3 of Ala), 1.8 (s, 3 H, CH_3 of CH_3CO), 4.0 (t, J = 5 Hz, 2 H, CH_2 of Gly), 8.8 (br, 1 H, CH=N), and 11.1 (br, 1 H, =NNHCO) and an unresolved group of protons at δ 7.3–8.1. Anal. ($C_{14}H_{18}N_4O_3$+0.25 H_2O) C, H, N.

Substrates, Enzymes, and Buffers. Three enzyme substrates were used as purchased (from Sigma except where noted): *N*-benzoyl-L-tyrosine *p*-nitroanilide (BzTyr *p*-nitroanilide), *N*-acetyl-L-TrpOMe (AcTrpOMe, from Cyclo), and *N*-α-benzoyl-L-arginine *p*-nitroanilide (BzArg *p*-nitroanilide). Eight pH 7 total hydrolyses of 50–175 μM BzTyr *p*-nitroanilide with chymotrypsin afforded $\Delta\epsilon$ = +9260 ± 200 at 410 nm, in agreement with $\Delta\epsilon$ = 9044 calculated for pH 5–7 from the measured absorbances of reactants and products (cf. ref 27). All subsequent calculations used $\Delta\epsilon$ = 9125. AcTrpOMe showed a single spot on TLC (R_f = 0.9). Total hydrolysis with chymotrypsin gave $\Delta\epsilon_{300}$ = +242 ± 11 (11 runs, pH 5–7), in good agreement with a reported[28] value of +241. Tryptic hydrolysis of papain substrate BzArg *p*-nitroanilide at pH 7 afforded $\Delta\epsilon_{410}$ = +9056 ± 193 (four runs) as compared to values of 8834 (calculated) and 880 (lit.).[29] A value of 8800 was employed.

α-Chymotrypsin was purchased from Worthington Biochemicals (lot CDI OBF); its concentration was determined by titration with cinnamoyl imidazole.[30] Papain, obtained from Sigma (P-3125, lot 22F-8010), was freshly activated each day at a concentration of 0.16 mg/mL by exposing it for 30 min at room temperature to a solution containing 50 μM mercaptoethanol, 5 mM cysteine hydrochloride, and 1 mM EDTA (Worthington Enzyme Manual, 1977, p 233). The resultant enzyme solution, stored in ice, retained its full activity for a day. No experiment performed required accurately determining the active papain concentration in such stock solutions. One batch was estimated to hold ~1.9 μM enzyme (28% active) by measuring its ability, upon dilution, to cleave 0.25 mM BzArg *p*-nitroanilide at 25 °C, pH 5, and comparing these data to those of Mole and Horton[29] for purified enzyme under identical conditions. Determination of the activity for the same papain preparation and all subsequent activation mixtures under "standard" conditions (35 °C, pH 5) made it possible to estimate active papain concentrations in the latter samples.

Three buffers, employed in kinetic experiments, will be simply designated pH 5, 6, and 7. They were respectively pH 5.00 ± 0.05 sodium acetate (0.11 M), pH 6.09 ± 0.05 phosphate (0.1 M), and pH 7.05 ± 0.05 phosphate (0.1 M). Each buffer contained 1 mM EDTA and sufficient KCl to raise its final ionic strength to 0.3. Both phosphate buffers contained a mixture of Na and K salts.

Basic Spectrophotometric Kinetic Procedures. All experiments were performed at 35.0 ± 0.2 °C on a Cary 16 spectrophotometer equipped with Model 1626 interface, Fisher Recordall 5000 recorder, and thermostated cell holder and compartment. Rates of hydrolysis for BzPheal=Ala or BzGlyal=Ala were determined by recording at 245–260 nm the fall in absorbance that attends those reactions. Typical values for $\Delta\epsilon$ are −2570 for BzPheal=Ala at 260 nm and −3980 for BzGlyal=Ala at 255 nm. The rate of generation of BzPheal=Ala from BzPheal + Ac-AlaNHNH$_2$ and of BzGlyal=Ala from BzGlyal + AcAlaNHNH$_2$ can be studied at the same wavelengths. Hydrolysis of BzPheal=Nic was monitored at 265–285 nm ($\Delta\epsilon_{285}$ = −3280). The hydrolysis of these three IX derivatives is subject to minor buffer catalysis.

Every kinetic experiment with chymotrypsin contained 3.0 mL of buffer and 0.100 mL each of solutions of enzyme, substrate, and IX derivative (or the appropriate blanks). The reaction mixture contained 6% Me_2SO, since both substrate and IX derivative were dissolved in that solvent. Data for the hydrolysis of AcTrpOMe in the presence of BzPheal=Ala were corrected for a slight rise in absorbance at 300 nm contributed by cleavage of this prodrug. Kinetics with papain were performed in 3% *N*,*N*-dimethylformamide, the solvent in which the aldehyde derivative was dissolved. Cited pH values refer to those recorded for reaction mixtures that held buffer plus the amounts of organic solvent specified. Routine computer treatment of the kinetic data obtained afforded first-order kinetic curves, weighted Lineweaver–Burk plots,[31] etc. A VT-100 Retro-Graphics terminal allowed ready visual comparison of the match between theoretical curves and experimental data.

Results

The reactions shown in Scheme II are anticipated to be important for understanding how an IX derivative, whose rate of hydrolysis is linearly dependent upon [H$^+$], will affect an ongoing enzyme-catalyzed hydrolysis. The Mi-

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Table I. Properties of Three IX Prodrugs[a]

Scheme II parameter	BzPheal=Ala		BzPheal=Nic		BzGlyal=Ala	
	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7
K_{eq}, mM	0.65 ± 0.10[b]		0.66 ± 0.08[b]		0.40 ± 0.15	
k_f, min^{-1}	0.025 ± 0.001	0.0003	0.021 ± 0.001	0.0006	0.093 ± 0.008	
k_b, M^{-1} min^{-1}	40 ± 3		30 ± 3		63 ± 5	
K_i(Cht), μM	48 ± 3	31 ± 1[c]	48 ± 3	31 ± 1	≥8000	
K_{ix}(Cht), mM		2		0.7		≥10
K_i(pap), μM	0.5	0.7			38[d]	30
K_{ix}(pap), mM		0.15				1

[a] The data refer to pH 5.0, 6.1, or 7.1 and 35 °C. Solvents contained either 6% Me_2SO (chymotrypsin or Cht) or 3% $HCONMe_2$ (papain or pap). See texts for means of determination of values tabulated. [b] This average of six measurements includes one from pH 6. [c] Reference 17 reports K_i = 26 μM at pH 7.8. [d] Reference 19 gives K_i = 25 μM at pH 5.5.

chaelis–Menten constants K_m and k_c (eq 1) govern that enzymatic reaction while the reversible hydrolysis of precursor IX is the critical process that liberates active inhibitor (eq 2). The parameters K_{eq}, k_f, and k_b characterize hydrolysis of IX. The enzymatic reaction is posited to be slightly inhibited by intact IX (K_{ix}; eq 3) but strongly inhibited by free aldehyde I (K_i; eq 4). It is expected that as time passes, the rate of enzymatic hydrolysis will markedly diminish, relative to a suitable control, if substantial hydrolysis of the IX derivative has occurred. Evaluation of the six independent parameters that appear in Scheme II—K_{eq}, k_f, K_m, k_c, K_{ix}, and K_i—as explained below, has made it possible to decide if this scheme successfully describes the chemical behavior of these homogeneous reaction mixtures.

Scheme II

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_c} E + P \quad (1)$$

$$IX \underset{k_b}{\overset{k_f}{\rightleftharpoons}} I + X \qquad K_{eq} = k_f/k_b \quad (2)$$

$$E + IX \overset{K_{ix}}{\rightleftharpoons} E{\cdot}IX \qquad K_{ix} = [E][IX]/[E{\cdot}IX] \quad (3)$$

$$E + I \overset{K_i}{\rightleftharpoons} E{\cdot}I \qquad K_i = [E][I]/[E{\cdot}I] \quad (4)$$

Properties of IX. Evaluation of K_{eq}, k_f, and k_b. Under the experimental conditions employed, hydrolysis of each of the IX derivatives affords an equilibrium mixture in which significant concentrations of intact IX remain. Attainment of equilibrium was studied by starting either with IX or with a mixture of I + X. Six determinations of K_{eq} were made for BzPheal=Ala. Three derived from solutions that initially held 100, 200, or 400 μM BzPheal=Ala at pH 5, and three, from those that originally held equimolar concentrations of BzPheal and Ac-L-AlaNHNH$_2$ (200 and 400 μM at pH 5; 400 μM at pH 6). Calculation of K_{eq} from the experimental data relies solely on measured initial and final absorbances of reaction mixtures and on the values of ε for IX, I, and X at the relevant wavelength [see eq A2 or A6 in the Appendix (all equations labeled with an "A" appear there)]. Six comparable experiments were performed for BzPheal=Nic. Since the twelve values for K_{eq} obtained for the two derivatives are identical within experimental error, a grand average, K_{eq} = 0.66 ± 0.08 mM, has been used in all subsequent calculations. It has been assumed to be pH-independent for pH 5–7.

The rates of change of absorbance in the ten pH 5 equilibrations described determined k_f and k_b for BzPheal=Ala and BzPheal=Nic at pH 5 (eq A4 and A7). Values for K_{eq} calculated from k_f/k_b are 0.63 mM for BzPheal=Ala and 0.70 mM for BzPheal=Nic, in good agreement with those obtained directly. Initial rate measurements established k_f at pH 7 for the two compounds. The value of k_f for BzPheal=Ala depends upon $[H^+]^1$ for pH 4.0–7.0 (a plot of log k_f vs pH has slope = −0.96, intercept = 3.13, and correlation coefficient = 0.999 for four points).

Similar experiments were performed with BzGlyal=Ala. Eight equilibrations of IX at pH 5 gave K_{eq} = 0.40 ± 0.15 mM and k_f = 0.093 ± 0.008 min^{-1}. Two experiments in which 400 μM BzGlyal reacted with 400 μM Ac-L-AlaNHNH$_2$ afforded K_{eq} = 2.0 ± 0.2 mM and k_b = 63 ± 5 M^{-1} min^{-1} (k_f/k_b = 1.5 mM). The two determinations of K_{eq} are only in fair agreement; K_{eq} = 2 mM has been used in analyzing all experiments with BzGlyal=Ala.

Table I summarizes the preceding data. These results, in conjunction with the physical data presented in the Experimental Section, establish that the structures assigned to the IX derivatives are correct. Two additional observations both confirm those assignments and document that the equilibrium and kinetic measurements are internally consistent. First, if hydrolysis of BzPheal=Ala and BzPheal=Nic liberates BzPheal, the concentration of BzPheal present at equilibrium is calculable from $[IX]_0$ and K_{eq}. Measurement of the ability of such solutions to inhibit chymotrypsin-catalyzed BzTyr *p*-nitroanilide hydrolysis should afford a value of K_i for BzPheal that agrees with the value of 48 ± 3 μM determined with bona fide aldehyde. This requirement has been met, for six such experiments at pH 5 (three with each derivative) gave K_i = 47 ± 3 μM. Second, it is readily shown that the slopes of suitable plots describing the rates of hydrolysis of IX (eq A4) and of regeneration of IX from its constituents, I + X (eq A7), should be identical if (a) all data pertain to the same experimental conditions and (b) $[IX]_0$ in the former equals $[I]_0$ and $[X]_0$ in the latter. Experiments with both BzPheal=Ala and BzPheal=Nic also successfully met this test, as Figure 1 demonstrates for one case.

Evaluation of K_m and k_c for Enzymatic Hydrolyses (Equation 1). Both chymotrypsin-catalyzed hydrolysis of BzTyr *p*-nitroanilide and papain-catalyzed cleavage of BzArg *p*-nitroanilide afford an increase in absorbance that is initially linear in time under the conditions employed. Weighted Lineweaver–Burk plots[31] of the initial rate data from these experiments provided the values for k_c and K_m recorded in Table II. Kinetic data for the α-chymotryptic cleavage of AcTrpOMe were analyzed both by the Lineweaver–Burk method and, because the extent of hydrolysis of AcTrpOMe was very great, by numerical integration of eq 5 (as will be explained shortly). The values for K_m obtained from the latter approach have been used in subsequent calculations. The values for k_c and K_m obtained for all three peptide derivatives agree well with those reported.[27,29,32]

Evaluation of K_i for I and K_{ix} for IX Derivatives (Equations 4 and 3). Measurement of the initial rate of

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Table II. Kinetic Constants for Enzymatic Hydrolyses[a,b]

reaction	pH	$[S]_0$, μM	$[E]_0$, nM	k_c, min^{-1}	K_m, mM
Cht + BTPNA	5	25–175	940	6.8 ± 1.9	1.05 ± 0.33
	6	25–175	190	25.0 ± 0.3	0.35 ± 0.06
	7	25–175	190	22.5 ± 1.2	0.12 ± 0.01
Cht + AcTrpOMe	5	100–1000	280	116 ± 3	0.150 ± 0.010
	5	100–1000	280	116	0.170[c]
	7	100–1000	7.4	3300 ± 500	0.165 ± 0.080
	7	100–1000	7.4	3300	0.175[c]
papain[d] + BAPNA	5	250–2000	57	74 ± 5	3.8 ± 0.3

[a] See footnote a, Table I. The substrates BzTyr and BzArg *p*-nitroanilide are abbreviated BTPNA and BAPNA, respectively. [b] Tabulated values derive from Lineweaver–Burk plots of initial velocities except where otherwise noted. [c] These values for K_m derive from a best fit of eq 5 to the total hydrolysis curve. [d] Since all papain runs at pH 7 incorporating IX employed 250 μM substrate, $K_m \gg [S]_0$. The measured value for k_c/K_m was 24 300 M^{-1} min^{-1} for the uninhibited reaction under those conditions, and that value was used in fitting the data for experiments with IX derivatives.

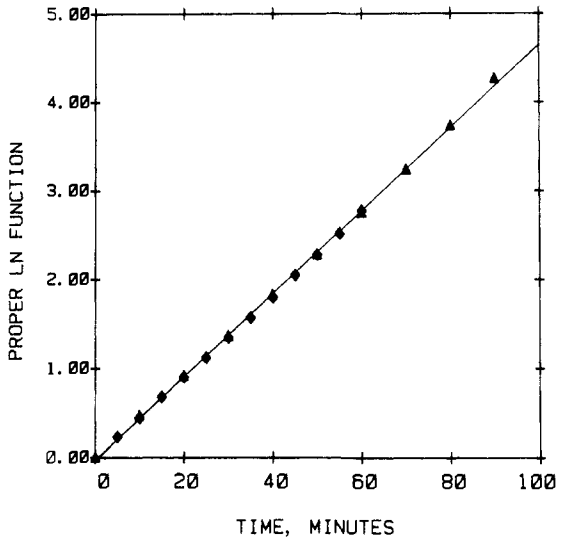

Figure 1. Kinetics of the interconversion of BzPheal=Ala and its constituents. The ▲'s represent data for the hydrolysis of 400 μM IX at pH 5 (obtained at 260 nm), plotted according to eq A4. The least-squares straight line through these points (not shown) has a slope of 0.047 min^{-1}. The ♦'s represent data (obtained at 255 nm) for regeneration of BzPheal=Ala from 400 μM BzPheal + 400 μM *N*-Ac-L-AlaNHNH$_2$ at pH 5, plotted according to eq A7. The line through these points (not shown) has slope = 0.045 min^{-1}. The least-squares line drawn through the pooled data has slope = 0.047 min^{-1} and correlation coefficient = 0.9997.

hydrolysis of 11–90 μM BzTyr *p*-nitroanilide by chymotrypsin in the absence and presence of BzPheal afforded a dozen values for the K_i characterizing dissociation of the chymotrypsin–BzPheal complex. Similar experiments established that BzGlyal inhibits chymotrypsin very poorly. Competitive inhibition of the papain-catalyzed hydrolysis of BzArg *p*-nitroanilide by the two aldehydes was evaluated at pH 5 and 7. The values for K_i in Table I agree with those reported where such comparisons are possible.

All three IX derivatives inhibit chymotrypsin and papain weakly at pH 7. Values for K_{ix} were estimated by measuring the initial rates of reaction between chymotrypsin and BzTyr *p*-nitroanilide or papain and BzArg *p*-nitroanilide in the absence and presence of 400 μM IX at pH 7, where hydrolysis of IX is slow. Experiment also established that inhibition of chymotrypsin-catalyzed hydrolysis of BzTyr *p*-nitroanilide by liberated *N*-Ac-L-AlaNHNH$_2$ or nicotinic acid hydrazide is negligible (K_i = >5 and >2 mM, respectively).

Investigation of the Complete Reaction System: Scheme II and Equation 5. Differential eq 5 explicitly

$$-d[S]/dt = k_c[E]_0/\{1 + (K_m/[S])(1 + [I]_t/K_i + [IX]_t/K_{ix})\} \quad (5)$$

defines the rate of substrate hydrolysis in an enzymatic reaction to which Scheme II pertains, if both the IX derivative initially present and the I product formed from it act as competitive inhibitors.[33] Numerical integration of eq 5 was by the Runge–Kutta method[34] with increments of 0.1 min/cycle; values for [S] calculated for each minute were converted to fraction of substrate reacted ($1 - [S]/[S]_0$), and that fraction was plotted versus time. Trial calculations with 0.01-min increments generated theoretical curves that were indistinguishable from those obtained with the larger increments. The computer program first calculates $[I]_t$ and $[IX]_t$ from entered values for $[IX]_0$, k_f, and K_{eq} (cf. eq A2–A4), next numerically integrates eq 5, and finally plots the theoretical curve and the experimental data for the experiment under study (see Figures 2–5). To obtain theoretical curves for runs containing enzyme, substrate, and IX compound, it is necessary to input nine values: initial stoichiometries ($[S]_0$, $[E]_0$, and $[IX]_0$) and values for the six independent parameters of Scheme II (k_c, K_m, k_f, K_{eq}, K_i, and K_{ix}). The preceding sections have explained how these parameters were evaluated. With one exception, the values recorded in Tables I and II were used; it was assumed that the values for K_{ix} at pH 5 and 7 are identical (the data for pH 5 are consistent with values for K_{ix} that are as much as 50% smaller than those at pH 7). The exception was made because runs where hydrolysis of an IX derivative afforded significant inhibition by liberated aldehyde showed a degree of inhibition that modestly but consistently exceeded that predicted when tabulated values for K_i were used.[35] All computations for such runs therefore employed the following smaller values for K_i: for chymotrypsin–BzPheal, 40 μM at pH 5; for papain–BzPheal, 0.4 and 0.6 μM at pH 5 and pH 7, respectively; and for papain–BzGlyal, 15 μM at pH 5 and pH 7.

Data from enzymatic reaction mixtures that initially held I rather than IX or that lacked both were analyzed with the same computer program. The version of eq 5 appropriate to a particular experiment is obtained by entering suitable values for the several variables that appear in the equation. For example, $[IX]_0 = 10^{-8}$ M, $k_f = 10^{-8}$

(33) The treatment assumes [E·I] and [E·IX] are stoichiometrically insignificant as far as [I] and [IX], respectively, are concerned. The latter assumption is invariably valid in these experiments, while the former will at most introduce an error of a few percent in the early stages of pH 7 papain runs incorporating low initial [BzPheal=Ala].

(34) Kells, L. M. *Elementary Differential Equations*; McGraw-Hill: New York, 1947; pp 214–216.

(35) Hydrolysis of IX should liberate unhydrated RCHO, the active inhibitory species (see ref 21 and Temerk, Y. M. et al. *J. Chem. Soc., Perkin Trans.* **1984**, *2*, 337). During the period of IX hydrolysis, the steady-state concentration of effective inhibitor may exceed that predicted by assuming equilibrium conditions, causing a reduction in the apparent K_i.

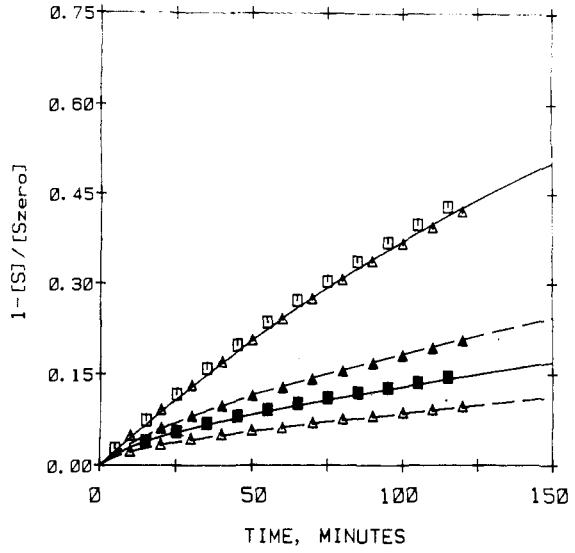

Figure 2. Hydrolysis of 74 μM *N*-benzoyl-L-tyrosine *p*-nitroanilide at pH 5 by 0.76 μM α-chymotrypsin in the presence and absence of BzPheal=Ala. The experimental data represent (from top to bottom) duplicate runs in the absence of IX (△, □) and single runs containing 100 (▲), 200 (■), and 400 μM (bottom △) IX. All experiments described in this figure and Figures 3–5 refer to 35 °C. All theoretical curves shown were generated by numerical integration of eq 5, with the introduction of appropriate numerical values for the relevant quantities from Tables I and II. The text provides a full description of this procedure.

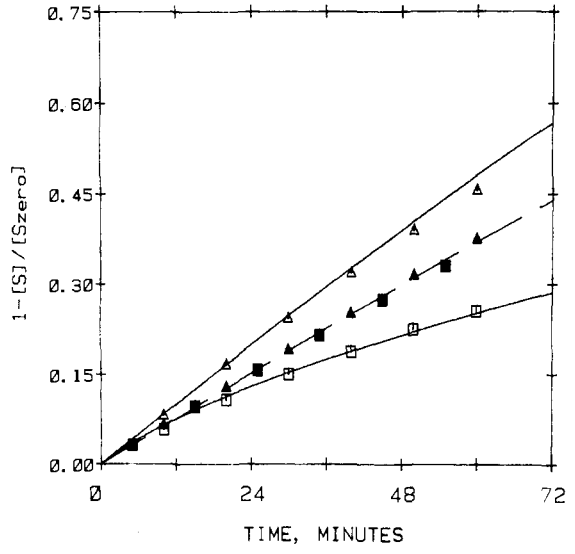

Figure 3. Hydrolysis of 1 mM *N*-acetyl-L-tryptophan methyl ester by chymotrypsin in the presence and absence of BzPheal=Ala. The experimental data for pH 5 refer to 85 nM $[E]_0$ and no (△) or 600 μM $[IX]_0$ (□). Similarly, those for pH 7 refer to 2.3 nM $[E]_0$ and no (▲) or 400 μM $[IX]_0$ (■).

min^{-1}, and $K_{eq} = K_{ix} = K_i = 1$ M are suitable values for generating theoretical curves for experiments incorporating just enzyme and substrate, as in the case of AcTrpOMe hydrolysis by chymotrypsin, discussed previously. There, k_c and K_m were treated as variables in matching theory to experiment. The entered values for these two quantities were varied until visual observation indicated that a "best" fit between experimental data and theoretical curve had been achieved.

Discussion

Because eq 5 is derived from Scheme II, the question of whether Scheme II adequately represents what transpires in reaction mixtures containing enzyme, substrate,

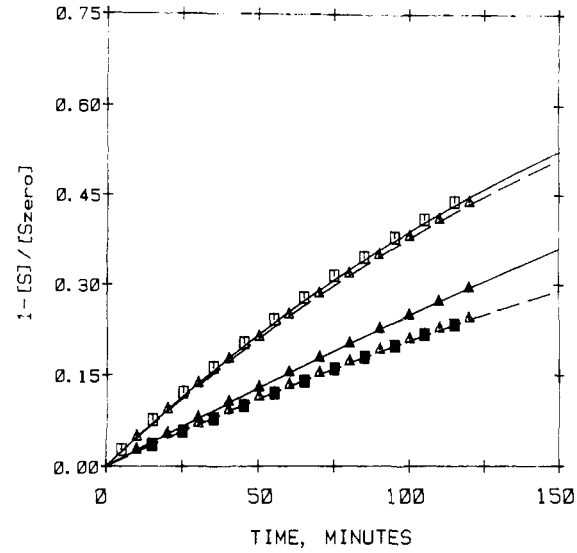

Figure 4. Chymotryptic cleavage of BzTyr *p*-nitroanilide in control experiments. The upper pair of runs reflect hydrolysis at pH 5 of 74 μM $[S]_0$ by 0.78 μM enzyme in the absence (△; solid line) and presence of 400 μM BzGlyal=Ala (□; broken line). The three lower runs refer to hydrolysis at pH 7 of 161 μM substrate by 35 nM chymotrypsin in the absence of any IX (▲) or in the presence of 200 μM BzPheal=Nic (■) or 400 μM BzPheal=Ala (△). The lower broken line was calculated for the BzPheal=Nic run.

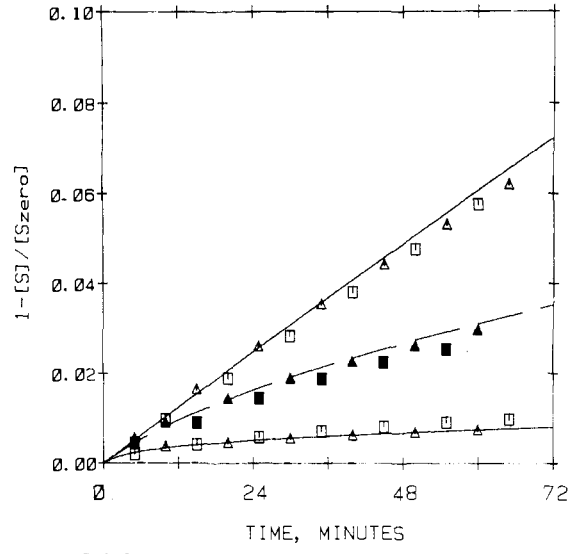

Figure 5. Inhibition of the papain-catalyzed hydrolysis of 0.3 mM α-*N*-benzoyl-L-arginine *p*-nitroanilide by BzPheal=Ala. Runs at pH 5 employed 80 nM $[E]_0$; those at pH 7, 64 nM $[E]_0$. From top to bottom, the data for pH 5 pertain to no (△), 1.0 (▲), and 10 (△) μM $[IX]_0$. Similarly, those for pH 7 refer to no (□), 41 (■), and 250 μM (□) $[IX]_0$. The theoretical curves shown were generated to fit the runs at pH 5.

and IX prodrug is really one of whether eq 5 satisfactorily describes the time dependence of substrate disappearance in those mixtures. Data from a few of the experimental tests of eq 5 are illustrated in Figures 2–5, where the observed fraction of $[S]_0$ consumed as a function of time is compared to the theoretical curves obtained by numerical integration of eq 5. First, we focus on studies with α-chymotrypsin. When *N*-benzoyl-L-tyrosine *p*-nitroanilide is the substrate, $K_m \gg [S]_0$, while when AcTrpOMe is the substrate, $[S]_0 \geq K_m$. In both instances the introduction of BzPheal=Ala results in substantial inhibition of the rate of substrate cleavage at pH 5 (Figures 2 and 3). Furthermore, the degree of inhibition at pH 7 is far less than

Table III. Evaluation of Efficiency Ratios (ER) for IX Prodrugs[a]

substrate	$[S]_0$, μM	IX	reaction fit $[IX]_0$, μM	reaction fit pH	matched by $[IX]_0$, μM	matched by pH	ER
BAPNA[b]	300	BzPheal=Ala	1	5	41	7	41
BAPNA[b]	300	BzPheal=Ala	10	5	250	7	25
BTPNA[c]	74	BzPheal=Ala	100	5	2000	7	20
BTPNA	74	BzPheal=Ala	400	7	25	5	16
BTPNA	74	BzPheal=Nic	100	5	800	7	8
BTPNA	74	BzPheal=Nic	400	7	50	5	8
AcTrpOMe	1000	BzPheal=Ala	600	5	5000	7	8
AcTrpOMe	200	BzPheal=Ala	600	5	>5000[d]	7	>8

[a] Text supplies details of analyses. Substrates are abbreviated as in Table II. [b] Papain reactions. See Figure 5 for illustrations of these two runs. [c] This and all subsequent entries pertain to chymotrypsin experiments. Values for the efficiency ratio were obtained by computer simulations only. [d] The computer-generated curve fit the initial portion of the pH 5 data but did not demonstrate enough inhibition for $t \geq 30$ min. Unhydrolyzed IX contributes much inhibition to the pH 7 simulation, and this does not increase with time.

that at pH 5 (Figure 3 and the three lower experiments in Figure 4). BzPheal=Nic provided similar data (unshown). The two upper plots in Figure 4 establish that BzGlyal=Ala, although it suffers rapid hydrolysis at pH 5, affords no significant inhibition. This accords with expectation; BzGlyal so liberated is an ineffective inhibitor for chymotrypsin (Table I). Scheme II is demonstrably satisfactory, since it affords theoretical curves that accurately reproduce the experimental data under all conditions examined. Alternative interpretations for the data have not been examined. According to Scheme II, hydrolysis of a derivative such as BzPheal=Ala liberates free aldehyde (BzPheal); the result is increasing enzymatic inhibition as time advances. The extent of inhibition rises more rapidly at pH 5 than at pH 7 because the rate of hydrolysis of the aldehyde derivative is greater at the lower pH.

The effect of the aldehyde derivatives upon papain-catalyzed hydrolysis of *N*-α-benzoyl-L-arginine *p*-nitroanilide was also investigated. With BzGlyal=Ala significant inhibition is expected and found (in contrast to the chymotrypsin example) since $K_i \simeq 38$ μM for the papain–BzGlyal complex at pH 5 (data unshown). Figure 5 illustrates the data obtained in experiments on the effect of BzPheal=Ala upon the papain-catalyzed hydrolyses. This last combination of reactants is particularly interesting, for the following reason.

The intent of the present research was to develop latent protease inhibitors that generate active inhibitor more effectively at low pH than at high. The qualitative comparisons above demonstrate some success in meeting that goal has been realized. One way to evaluate the success more quantitatively is to ask what concentration of IX derivative at pH 7 is required to afford the same degree of inhibition as is observed with a specified [IX] at pH 5. The ratio of those two concentrations represents a kind of "effectiveness" that will be termed an "efficiency ratio":

$$\text{efficiency ratio} = [IX]_7/[IX]_5 \quad \text{for equal inhibition}$$

Because of the high affinity of papain for BzPheal, it has been possible to establish an efficiency ratio for the papain-catalyzed hydrolysis of BzArg *p*-nitroanilide in the presence of BzPheal=Ala by direct experimentation. First, three enzymatic reactions were performed at pH 5, in the presence of 0, 1, and 10 μM BzPheal=Ala. Second, computer simulations of eq 5 were used to estimate what [BzPheal=Ala] at pH 7 would afford data comparable to those obtained in the last two experiments. This was done by generating hypothetical curves reproducing the data from pH 5, but employing values for the variables that appear in eq 5 appropriate to pH 7. The first simulation, for [BzPheal=Ala] = 0, established what $[E]_0$ would afford an uninhibited rate of substrate hydrolysis at pH 7 identical with that observed for the uninhibited run at pH 5. The other two estimated, with $[E]_0$ now fixed, what values for $[IX]_0$ were required at pH 7 to match the experimental data for 1 and 10 μM $[IX]_0$ at pH 5. Finally, experiments under the conditions predicted by the simulations were performed at pH 7. As Figure 5 shows, the match between the data obtained at pH 5 and at pH 7 is good. The values of 25–40 for the efficiency ratio that result mean that BzPheal=Ala is 25–40 times more effective as a papain inhibitor at pH 5 than at pH 7. These are the highest values realized.

Values for the efficiency ratio in chymotrypsin studies were estimated solely by computer simulations. Data for two experiments of identical $[E]_0$ and $[S]_0$ at pH 5 were selected, one without and one with an IX derivative. Theoretical curves were then generated for the former and, subsequently, the latter in the manner just described. These simulations used the appropriate pH 7 parameters and defined what $[IX]_0$ was expected to afford the same degree of inhibition at pH 7 as had been observed at pH 5. Alternatively, data for two experiments at pH 7 were fit with curves generated from parameters appropriate to pH 5. Table III summarizes the results from some simulations. Not unexpectedly, inhibition by the intact IX derivative becomes significant at high $[IX]_0$ and reduces the advantage of IX hydrolysis. For much the same reason, BzPheal=Nic, which displays a value for K_{ix} that is significantly smaller than that for BzPheal=Ala, affords an appreciably smaller efficiency ratio.

The preceding experiments demonstrate that acylhydrazide derivatives of aldehydes offer a potentially useful device for achieving selective release of biochemically active substances in cellular regions of low pH. Indeed, Storrie and Ferris[36] have used chemistry resembling that described above for probing endocytic traffic patterns in fibroblasts (cell surface → prelysosomal vesicles → lysosomes). While various considerations dictated that in vivo studies with acylhydrazide-based prodrugs be deferred, experiments with an immobilized chymotrypsin–collodion membrane were undertaken in order to investigate the behavior of BzPheal=Ala in a microenvironment of low pH (in what may be regarded as a primitive model lysosome). The results of those experiments will be described elsewhere.

Acknowledgment. This research was supported in part by a grant from the National Science Foundation.

Appendix

Kinetic Analysis. Characterization of IX Derivative Hydrolysis. When intact IX was the starting ma-

(36) Storrie, B.; Ferris, A. L. *J. Histochem. Cytochem.* **1985**, *33*, 1145.

terial, initial conditions were $[IX] = [IX]_0$ and $[I]_0 = [X]_0 = 0$. Let $[IX] = [IX]_e$ at equilibrium. At intermediate times, $[IX] = [IX]_t$ and $[X]_t = [I]_t = [IX]_0 - [IX]_t$. Measured absorbances are conveniently designated A_0, A_e, and A_t. Equation A1 provides $[IX]_e$ in terms of measured or known quantities, where the ϵ's are appropriate to the wavelength employed and A_{tot} is a calculated hypothetical absorbance corresponding to total conversion of IX into I + X. Equation A2 defines K_{eq}. In order to extract k_f

$$[IX]_e = (A_e - A_{tot})/(\epsilon_{ix} - \epsilon_i - \epsilon_x) \quad (A1)$$

$$K_{eq} = ([IX]_0 - [IX]_e)^2/[IX]_e \quad (A2)$$

from rate data for the preceding equilibration, time-dependent absorbance data and values for $[IX]_0$ and K_{eq} were entered into the computer. The program calculates $[IX]_e$ (eq A2 rearranged) and $[IX]_t$ (eq A3). The slope obtained when the resultant values for $[IX]_t$ are plotted according to eq A4 defines k_f.[37] Treatment of reactions in which IX

$$[IX]_t = [(A_t - A_e)/(A_0 - A_e)]([IX]_0 - [IX]_e) + [IX]_e \quad (A3)$$

$$\ln \frac{[IX]_0^2 - [IX]_e[IX]_t}{[IX]_0([IX]_t - [IX]_e)} = k_f \frac{[IX]_0 + [IX]_e}{[IX]_0 - [IX]_e} t \quad (A4)$$

was generated from equimolar concentrations of I and X ($[IX]_0 = 0$, $[I]_0 = [X]_0$) was similar. Equations A5 and A6 define $[IX]_e$ and K_{eq}, the increase in $[IX]_t$ is proportional to the rise in absorbance from A_0 to A_e, and the slope of a plot of eq A7 plot defines k_b.

$$[IX]_e = (A_e - A_0)/(\epsilon_{ix} - \epsilon_i - \epsilon_x) \quad (A5)$$

$$K_{eq} = ([I]_0 - [IX]_e)^2/[IX]_e \quad (A6)$$

$$\ln \frac{[IX]_e([I]_0^2 - [IX]_t[IX]_e)}{[I]_0^2([IX]_e - [IX]_t)} = k_b \frac{([I]_0^2 - [IX]_e^2)}{[IX]_e} t \quad (A7)$$

(37) Frost, A. A.; Pearson, R. G. *Kinetics and Mechanism*, 2nd Ed.; John Wiley: New York, 1961; pp 186–187.

Synthesis, Opioid Receptor Binding Profile, and Antinociceptive Activity of 1-Azaspiro[4.5]decan-10-yl Amides

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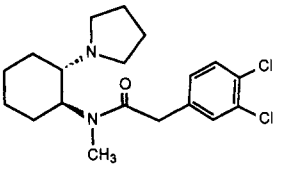
A series of azaspiro[4.5]decanyl amides were prepared by a novel cyclization route and examined for opiate receptor binding and antinociceptive activity. Selected tertiary amides in this series showed potent selective μ-receptor binding and antinociceptive activity, in contrast to the less conformationally restricted secondary amides, which showed relatively weak activity. Although structurally similar to the κ-agonist U-50488H (1), these compounds showed virtually no tendency to bind to the κ-receptor. An X-ray crystal structure of compound (21) confirms that the spirocyclic amine does not cause distortion away from the chair conformation of the cyclohexane ring. Either this receptor has very specific requirements for the orientation of the two nitrogens of these compounds or this ring system fills a portion of space more readily tolerated by the μ- and δ-receptors.

The characterization of multiple opiate receptors[1,2] has given rise to the concept that it may be possible to design novel opiate analgesics lacking the side effects associated with morphine and its congeners. As a result, extensive research has been done to identify new types of compounds and to probe the structural differences between the various opiate receptors.

Research with the selective κ-agonist U50,488 (1)[3] has suggested that such an analgesic will not cause respiratory depression or constipation[4] normally associated with morphine. In addition, this compound appears not to be self-administered,[5] does not cause tolerance to morphine,[6] and may not have as severe withdrawal problems.[7] However, κ-agonists may cause diuresis[8] and undesirable central nervous system (CNS) effects such as dysphoria.



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U-50,488

An examination of the known cyclohexanediamine analgesics shows that, like the benzomorphans, subtle variations in structure will alter their binding profile.[3,9] It is thought that a key feature of most opiate analgesics is the relative orientation of the aromatic ring to a basic nitrogen. In order to more rigidly define this orientation, we have prepared a series of amino azaspirodecanes to compare with the cyclohexanediamines. The orientation of the basic amine is fixed relative to the cyclohexane ring in this type of structure. Thus only a subset of conformations available to the cyclohexanediamines can be adopted by the azaspirodecanes. This has resulted in the identification of potent analgesics which, while structurally related to 1, do not show affinity for the κ-receptor. In this report, we

(1) Martin, W. R.; Eades, C. G.; Thompson, J. A.; Huppler, R. E.; Gilbert, P. E. *J. Pharmacol. Exp. Ther.* **1976**, *197*, 517.
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(9) Cheney, B. V.; Szmuszkovicz, J.; Lahti, R. A.; Zichi, D. A. *J. Med. Chem.* **1985**, *28*, 1853.

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