Peptidyl (Acyloxy)methyl Ketones and the Quiescent Affinity Label Concept: The Departing Group as a Variable Structural Element in the Design of Inactivators of Cysteine Proteinases†

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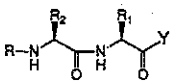
Received October 18, 1990; Revised Manuscript Received January 25, 1991

ABSTRACT: (Acyloxy)methyl ketones, of general structure Z-[AA_2]-[AA_1]-CH_2OCOAr, are potent inactivators of the cysteine proteinase cathepsin B. These reagents have been designed as affinity labels in which the dipeptidyl moiety serves as an affinity group (complementary to the S_1 and S_2 sites of the enzyme), while the (acyloxy)methyl ketone unit ($-COCH_2OCOR$), containing a weak leaving group in the form of a carboxylate nucleofuge, functions as the potentially reactive entity that labels the enzyme. The inhibition is time dependent, active site directed, and irreversible. The apparent second-order rate constant k_{inact}/K_{inact}, which characterizes the inhibition of cathepsin B by this series, spans several orders of magnitude and in certain cases exceeds 10^6 M^{-1} s^{-1}. The activity of this series of inhibitors was found to be exquisitely sensitive to the nature of the carboxylate leaving group as well as the affinity group. A strong dependence of second-order inactivation rate on leaving group pK_a was uncovered for Z-Phe-Ala (acyloxy)methyl ketones [$\log(k/K) = -1.1\ (\pm 0.1) \times pK_a + 7.2\ (\pm 0.4)$; $r^2 = 0.82$, $n = 26$]. Heretofore in constructing affinity labels the choice of leaving group was quite restricted. The aryl carboxylate group thus offers considerable variation as a design element in that both its binding affinity and reactivity can be controlled by substituent effects. Specific peptidyl (acyloxy)methyl ketones thus represent prime examples of highly potent, chemically stable enzyme inhibitors with variable structural elements in both the affinity and departing groups.

Affinity labeling of specific amino acid residues has been an invaluable technique for elucidating key aspects of enzyme structure, function, and reaction mechanisms (Jakoby & Wilchek, 1977). As originally conceived (Baker et al., 1961; Lawson & Schramm, 1962; Schoellmann & Shaw, 1962; Wofsy et al., 1962), affinity labels were attempts to concentrate "group-specific reagents" at the active sites of target enzymes in order to obtain reagents of enhanced specificity capable of establishing specific stable covalent linkages. In the realm of proteinase inhibition over the past 35 years, the reagents have evolved from analogues of functionalized amino acids bearing a reactive group to more specific alkylating agents possessing complex peptide-binding determinants complementary to the target enzyme active site (e.g., **1a–c**) (Shaw, 1980, 1990).



1a, Y = CH_2Cl **1d**, Y = CH_2F
1b, Y = CHN_2 **1e**, Y = CH_2OCOAr
1c, Y = $CHS(CH_3)_2$

† Contribution No. 322 from the Institute of Bio-Organic Chemistry.

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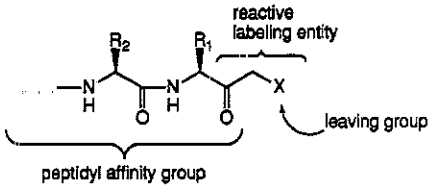


FIGURE 1: General structure of a peptide-based affinity label, containing the leaving group X, which is attached to a saturated center vulnerable to nucleophilic attack

Yet despite the considerable success of affinity labels as pharmacological and biochemical tools, their clinical utility has been severely restricted by the high reactivity of the labeling component, which often contains a leaving group or atom vulnerable to S_N2 displacements (Figure 1). Comparatively little progress has been made in designing affinity labels with difficultly displaceable groups, which might tap the potential reactivity of a specific enzyme target without affecting other macromolecules. The limited repertoire of practical departing groups is especially disappointing when set against the backdrop of the extraordinary diversity of proteinases representing numerous clinically attractive targets (Reich et al., 1975; Berlin et al., 1978; Barrett & Salvesen, 1986).

Theoretically, clinically useful affinity labels could be fashioned that are essentially inert chemically, if the huge rate accelerations observed for enzyme-catalyzed reactions such as amide hydrolysis (Jencks, 1969; Kahne & Still, 1988) could be achieved for displacement reactions between enzyme and chemically stable active-site-directed reagents. The discovery that peptidyl fluoromethyl ketones (**1d**) are potent inactivators of the enzyme cathepsin B (EC 3.4.22.1) (Rasnick, 1985; Rauber et al., 1986; Shaw et al., 1986) but exhibit merely a small fraction (0.2%) of the chemical reactivity of the corresponding peptidyl chloromethyl ketones (**1a**) toward the model thiol, glutathione, at pH 6.5 (Rauber et al., 1986) is tangible evidence that reagents of modest chemical reactivity can be useful inactivators of this enzyme.

Indeed, selective inhibitors of the cysteine proteinase cathepsin B (Barrett & Kirschke, 1981; Katunuma & Kominami, 1983) have potential therapeutic value in a variety of disease states including proteinuria in glomerular disease (Baricos et al., 1988), osteoclastic bone resorption (Delaissé et al., 1984; Kominami et al., 1985; Everts et al., 1988), tumor metastasis (Poole et al., 1978; Sloane et al., 1986; Lah et al., 1989), tissue damage in myocardial infarction (Prous, 1986b), and muscle wasting in Duchenne muscular dystrophy (Prous, 1986a).

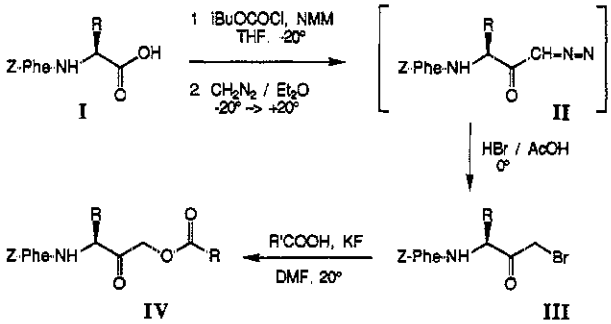
In this article we describe the use of peptidyl (acyloxy)methyl ketones (**1e**), conceived as affinity labels of low chemical reactivity that are potent and highly selective inactivators of cathepsin B (Smith et al., 1988a). These reagents incorporate the novel feature of an aryl carboxylate leaving group as a design element that can be systematically varied to provide a rational and exquisitely sensitive means of controlling the potency and chemical reactivity of an affinity label.

Experimental Procedures

Syntheses

Peptidyl (acyloxy)methyl ketones IV were synthesized via the analogous bromomethyl ketone III, as shown in Scheme I. In this procedure, N-protected peptides were prepared by standard procedures and then converted to the corresponding bromomethyl ketones III via the intermediate diazomethyl ketones II (Shaw & Ruscica, 1968; Green & Shaw, 1981).

Scheme I: Synthesis of Peptidyl (Acyloxy)methyl Ketones



Displacement of bromide by carboxylate, mediated by potassium fluoride (Clark & Miller, 1977), gave the desired product IV in good yield. All amino acid residues were of L-chirality, except where specified otherwise. Representative examples of synthetic procedures and additional experimental information are provided in the supplementary material.

Peptidyl (Acyloxy)methyl Ketones (IV). In general, isolated yields for the conversion of bromomethyl to (acyloxy)methyl ketone were in the range 50–80%. Product purification was carried out by recrystallization (usually from EtOAc[1]/hexane) and/or by silica-gel column chromatography with EtOAc/hexane as eluant. Excess (5–10%) carboxylic acid was used in all cases to promote the consumption of all traces of bromomethyl ketone. HPLC analyses (Perkin-Elmer Pecosphere 3X3C C-8 or C-18 column, 0.46 × 3.3 cm, with an acetonitrile/H_2O gradient at 3.0 mL/min) at 250 nm indicated product purities of >90% and in most cases >96%. HPLC analyses at 220 nm permitted the detection of bromo- or chloromethyl ketone to a level equivalent to 0.2% contamination; all (acyloxy)methyl ketones were thereby confirmed to have no significant (i.e., <0.2%) halomethyl ketone contamination. Samples of inhibitors **6** and **15** were also further purified by preparative HPLC (Whatman Magnum 20 silica gel column, 30% EtOAc/hexane, 11 mL/min) to rigorously exclude any impurities; these samples were then reassayed against cathepsin B to confirm the integrity of the inhibition kinetic data. It should be noted that the very low inactivation potency of the Z-L-Phe-D-Ala derivatives **42** and **54** (Table II), relative to the Z-L-Phe-L-Ala diastereomers **6** and **15**, indicates that no significant diastereomeric impurities (epimeric at the P_1 α-carbon) exist in these (and presumably other) (acyloxy)methyl ketone samples. As well, these results indicate that epimerization at this position does not occur under our cathepsin B assay conditions. The following synthetic procedures are representative; information for additional compounds is provided in the supplementary material.

*N-(Benzyloxycarbonyl)-L-phenylalanyl-L-alanine [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone (**15**).* Anhydrous potassium fluoride (30 mmol, 1.75 g) was added to a solution of Z-L-phenylalanyl-L-alanine bromomethyl ketone (10 mmol, 4.48 g; see supplementary material) in 100 mL of anhydrous DMF. The mixture was stirred 3 min at room temperature,

[1] Abbreviations: THF, tetrahydrofuran; AcOH, acetic acid; EtOAc, ethyl acetate; Z, benzyloxycarbonyl; HPLC, high-pressure liquid chromatography; DMF, *N,N*-dimethylformamide; Boc, *tert*-butoxycarbonyl; DMSO, dimethyl sulfoxide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; NMM, *N*-methylmorpholine; LSIMS/FAB, liquid secondary ion mass spectrometry/fast atom bombardment; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BME, β-mercaptoethanol; DTP, 2,2′-dipyridyl disulfide; Nu, nucleophile; Ser(OBn), *O*-benzylserine; Cys(SBn), *S*-benzylcysteine; Sar, sarcosine or *N*-methylglycine; GABA, γ-aminobutyric acid; Lys(Boc), N^{ϵ}-Boc-lysine; TFA, trifluoroacetic acid; Im, imidazole.

Table I: Rates of Cathepsin B Inactivation by Peptidyl (Acyloxy)methyl Ketones: Variation of the Leaving Group[a]

no.	compound R	k/K (M^{-1} s^{-1})	K_{inact} (nM)	k_{inact} (s^{-1})	pK_a[b]
		Z-Phe-Ala-CH_2OCO-R (Benzoyloxy)methyl Ketones			
6	2,6-$(CF_3)_2$-Ph	1 600 000	28 ± 7	0.045 ± 0.008	0.58[c]
7	2,6-Cl_2-Ph	690 000	36 ± 11	0.025 ± 0.004	1.59
8	C_6F_5	520 000			1.48[d]
9	2,6-Me_2-4-COOMe-Ph	58 000	610 ± 150	0.036 ± 0.005	2.67[c]
10	2,5-$(CF_3)_2$-Ph	38 000[e]	880 ± 580	0.033 ± 0.013	2.63[c]
11	2,6-F_2-Ph	26 000	2100 ± 900	0.056 ± 0.017	2.24[f]
12	3,5-$(CF_3)_2$-Ph	22 000			3.18[c]
13	3,5-$(NO_2)_2$-Ph	~19 000[g]			2.79
14	2-CF_3-Ph	17 000[h]	770 ± 220	0.013 ± 0.002	2.49[c]
15	2,4,6-Me_3-Ph	14 000[h,i]	510 ± 110	0.0073 ± 0.0005	3.45
16	2,6-Me_2-Ph	14 000[j]			3.35
17	2,4,6-iPr_3-Ph	3800[j]			
18	3,4-F_2-Ph	630[j]			3.67,[c] 3.79[d]
19	4-NO_2-Ph	610			3.43
20	3-CF_3-Ph	420[j]			3.76,[c] 3.75[k]
21	2,6-$(OMe)_2$-Ph	300			3.44
22	4-F-Ph	290[h]			4.15
23	4-CN-Ph	280[j]			3.50[k]
24	4-CH_3-Ph	260[j]			4.37
25	3,5-$(OH)_2$-Ph	140[j]			4.04
26	Ph	90[j]			4.20
27	4-CF_3-Ph	80[j]			3.67[l]
28	3,5-Me_2-Ph	80[j]			4.30
29	4-OMe-Ph	ntd[m]			4.50
		Z-Phe-Ala-CH_2OCO-R (Alkanoyloxy)methyl Ketones			
30	CH_3	140			4.76
31	CMe_3	330[j]			5.03
32	$CH(CH_2CH_3)_2$	70			4.73
33	CH_2OCH_3	240			3.57
		Z-Phe-Lys-CH_2OCO-R			
34	2,6-$(CF_3)_2$-Ph (HCl salt)	>2 000 000			0.58[c]
35	2,4,6-Me_3-Ph (TFA salt)	230 000	170 ± 50	0.037 ± 0.008	3.45
36	Ph (TFA salt)	9200	2400 ± 800	0.022 ± 0.005	4.20
37	4-OMe-Ph (TFA salt)	660			4.50

[a] Conditions: bovine spleen cathepsin B, 100 mM potassium phosphate, 1.25 mM EDTA, and 1 mM dithiothreitol, pH 6.0, 25 °C, under argon. The parameter k/K is the second-order rate constant (k_{inact}/K_{inact}), except as noted (see Experimental Procedures). Standard errors for $k/K \leq 15\%$, except as noted. In those cases where saturation kinetics were observed, k_{inact}/K_{inact} and the individual parameters K_{inact} and k_{inact} are given, as determined from a hyperbolic fit. In the other cases, k_{inact}/K_{inact} was obtained from a linear fit. [b] pK_a (aqueous) of the acyloxy group (RCOOH); values are from Serjeant and Dempsey (1979) or Kortum et al. (1961), except as noted. [c] pK_a determined by UV measurement (or, in the case of **9**, by HPLC) (this work; see Experimental Procedures). [d] pK_a from Strong et al. (1987). [e] Standard error 20–30%. [f] pK_a from Strong et al. (1982). [g] Compound instability was evident. [h] Standard error 15–20%. [i] This value has been revised since our previous report (Smith et al., 1988a) (see supplementary material). [j] Second-order rate constant ($k/[I]$) determined at one inhibitor concentration near the solubility limit. [k] pK_a from Ludwig et al. (1986). [l] Calculated pK_a (Perrin et al., 1981). [m] ntd: no time dependence or significant inhibition observed near the compound solubility limit, over a 10–20-min period.

2,4,6-trimethylbenzoic acid (Aldrich, 11 mmol, 1.81 g) was added, and the mixture was stirred 3 h at room temperature. The mixture was diluted with ethyl ether, and then washed with water (5×) and saturated aqueous $NaHCO_3$ (2×). Ethyl acetate was added to redissolve organic material, and the solution was washed with brine (2×), dried ($MgSO_4$), and rotary evaporated to give a slightly yellow solid. Recrystallization from ethyl acetate/hexane provided 3.42 g (65%) of the product (**15**) as a white powder, mp 171–172 °C; $[\alpha]_D^{21}$ −35.0° (c = 1.13, acetone); IR (KBr) 1735, 1720, 1685, 1650 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.4–7.1 (m, 10 H, 2Ph), 6.9 (s, 2 H, $Me_3C_6H_2CO$), 6.4 (br d, J = 6.7 Hz, NH), 5.2 (br d, J = 8.0 Hz, NH), 5.1 (s, $PhCH_2O$), 4.8 (d, app J = 1.6 Hz, $COCH_2CO$), 4.8–4.2 (m, 2 H, 2NHCHCO), 3.2–3.0 (m, $PhCH_2CH$), 2.4 and 2.3 [2 s, $(CH_3)_3Ph$], 1.3 (d, J = 7.1 Hz, CH_3CH); anal. C, H, N.

N-(Benzyloxycarbonyl)-L-phenylalanyl-L-alanine [[2,6-Bis(trifluoromethyl)benzoyl]oxy]methyl Ketone (**6**). In a manner similar to that described for **15**, compound **6** was prepared. Recrystallization of the product from EtOAc/hexane gave a white powder (72%), mp 167.5–168.5 °C; $[\alpha]_D^{20}$ −27.1° (c = 0.95, acetone); IR (KBr) 1765, 1745, 1695, 1665 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.1–7.6 (m, 3 H, ArH), 7.5–7.0 (m, 10 H, 2Ph), 6.4 (br d, J = 6.7 Hz, NH), 5.3 (br d, J = 8.0 Hz, NH), 5.1 (s, $PhCH_2O$), 4.9 (s, $COCH_2CO$), 4.9–4.2 (m, 2 H, 2NHCHCO), 3.0–2.8 (m, $PhCH_2CH$), 1.3 (d, J = 7.1 Hz, CH_3CH); anal. C, H, N.

N-(Benzyloxycarbonyl)-L-phenylalanyl-L-lysine [(2,4,6-Trimethylbenzoyl)oxy]methyl Ketone, Trifluoroacetate Salt (**35**). A solution of Z-Phe-Lys(Boc)-CH_2OCO-(2,4,6-Me_3)Ph (75 mg, 0.11 mmol) in CH_2Cl_2 (2 mL) was cooled to 0 °C and then treated with trifluoroacetic acid (1.5 mL) over a 5-min period. The reaction mixture was allowed to warm to room temperature with stirring for 2.5 h and then rotary evaporated to remove all solvent. The product was precipitated from $EtOAc/Et_2O$, filtered, and dried at high vacuum to afford the product **35** as a white powder in 87% yield, mp 129–131 °C; ^1H NMR (CD_3OD) δ 7.5–7.1 (m, 10 H, 2Ph), 6.9 (s, 2 H, $Me_3C_6H_2CO$), 5.1 (s, 2 H, $PhCH_2O$), 4.8 (s, exchangeable protons), 4.7 (s, 2 H, $COCH_2O$), 4.7–4.3 (m, 2 H, 2NHCHCO), 3.3–2.7 (m, 4 H), 2.4 and 2.3 [2 s, $(CH_3)_3Ph$], 2.2–1.2 (br m, 6 H); LSIMS/FAB mass spectrum m/z 588 (MH^+, 74%), 570 (5%), 147 ($Me_3C_6H_2CO^+$, 100%), 126 (14%), 91 (36%); anal. C, H, N.

pK_a Determinations

The pK_a constants for various benzoic acid leaving groups (Table I) were determined by UV spectrophotometry (Albert

& Serjeant, 1984). UV spectra of the benzoic acids (20–100 μM solutions) were recorded (Perkin-Elmer 559A spectrophotometer) to ascertain the wavelength having the maximum difference in absorbance for the neutral and negatively charged species. Absorbance at this wavelength was then determined for the benzoic acid at various pH's. Buffers used in these measurements included 0.2 M KCl/HCl, pH 1–1.9; 0.2 M glycine/HCl, pH 2–3.4; 20 mM potassium citrate/potassium phosphate, pH 2.7–5.7; and 0.2 M sodium acetate, pH 4–4.5. For extreme acid and base conditions, 0.1 N HCl and 1 N NaOH were used. The absorbance (y) vs pH was fit by nonlinear regression to eq 1.

$$y = (Y_{acid} + Y_{base}K_a/[H^+])/(1 + K_a/[H^+]) \quad (1)$$

Compound **9** did not have a sufficient difference spectrum to permit determination of the pK_a by UV. The pK_a was estimated by fitting retention time on HPLC (aqueous mobile phase pH 2.7–5.25, reverse-phase C-18 cartridge) vs pH of eluant to eq 1 (Miyake et al., 1987).

Enzyme Assays

Enzyme Assay Materials. Cathepsin B was purified from bovine spleen by the procedure of Bajkowski and Frankfater (1983). The enzyme was stored at −70 °C in 25 mM acetate buffer, pH 5.1, containing 5 mM $HgCl_2$. The assay buffer (0.1 M potassium phosphate, 1.25 mM EDTA, and 1 mM DTT, pH 6.0) was made anaerobic by several cycles of evacuation and exchange with nitrogen or argon. Enzyme activity was monitored with a Perkin-Elmer 650-40 or 650-15 fluorometer by measuring the enzyme-catalyzed hydrolysis of one of two fluorogenic substrates: 7-(benzyloxycarbonyl-phenylalanyl-arginyl)-4-methylcoumarinamide (Peninsula Laboratories, San Carlos, CA) (fluorescence λ_{ex} = 370 nm, λ_{em} = 460 nm) or 7-(benzoyl-valyl-lysyl-lysyl-arginyl)-4-trifluoromethylcoumarinamide (Enzyme Systems Products, Livermore, CA) (fluorescence λ_{ex} = 400 nm, λ_{em} = 505 nm). Cathepsin B activity was titrated with DTP (Sigma), essentially following the procedure of Brocklehurst and Little (1973).

Continuous Enzyme Assay. An aliquot of assay buffer (2 mL) was placed in a fluorometer cuvette, which was thermostated at 25 °C and kept under an argon atmosphere. Cathepsin B enzyme, in the storage buffer, was then added to give a concentration of approximately 0.4 nM. After 2–5 min of incubation to allow enzyme activation, during which time a steady baseline level of fluorescence was measured, substrate (5 or 10 μL of 1 mM stock solution in DMSO) was added and the resulting increase in fluorescence was monitored continuously.

Inhibitors were added to the assay solution as 0.5–20 μL of a stock solution in DMSO or CH_3CN and the fluorescence was monitored. When inhibitors were tested at very low concentrations, the enzyme concentration was reduced to maintain a minimum of a 10-fold excess of inhibitor over enzyme. In the absence of inhibitor, a linear increase in fluorescence with time was observed. Upon addition of inhibitor, the rate of fluorescence increase was observed to decrease exponentially, to give a final rate essentially equal to zero (<2% of initial rate of fluorescence increase). A series of data points (generally 10–20 sets of fluorescence vs time) from the inhibition curve was analyzed by nonlinear regression to the exponential eq 2 (e.g., see Figure 3). The pseudo-

$$\text{fluorescence} = Ae^{-(k_{obs}t)} + B \quad (2)$$

first-order rate constant, k_{obs}, was thereby obtained for each concentration of inhibitor. In our assay, the inhibitor concentrations were limited to a minimum of 0.4 nM by the enzyme concentration required (≥0.04 nM) and to a maximum as defined by the inhibitor solubility. A number of our inhibitors have rather low solubility in aqueous media; to limit solubility problems, our measurements were conducted at inhibitor concentrations no greater than 4×10^{-6} M, at which all samples appeared to be homogeneous. Our continuous assay procedure does not allow for accurate measurement of k_{obs} values much greater than 0.016 s^{-1} ($t_{1/2}$ < 40 s). Also, k_{obs} values that are much less than 10^{-3} s^{-1} approach the linearity limitations of the assay.

The second-order rate constant (k_{inact}/K_{inact}) was obtained from the dependence of pseudo-first-order inhibition rates (k_{obs}) on inhibitor concentration [I]. Where saturation kinetics were observed, the data were fit by nonlinear regression to eq 3 (Kitz & Wilson, 1962; Cleland, 1979). It should be noted that, due to the limitations described above, rates at fully saturating inhibitor concentrations could not usually be obtained. Therefore, the individual values of K_{inact} and k_{inact} from eq 3 are less well determined than the second-order rate constant k_{inact}/K_{inact}. In several cases, due to limitations of high inactivation rates (**8**, **12**, **34**, and **38**), limited solubility (**17–28**, **30–33**, and **37**), or both (**16**, **43**, **47**, and **48**), saturation kinetics could not be determined. In these cases the second-order rate constant k_{inact}/K_{inact} was obtained from linear regression to eq 4. With these limitations in mind, it was difficult at times

$$k_{obs} = k_{inact}[I]/(K_{inact} + [I]) \quad \text{(hyperbolic fit)} \quad (3)$$

$$k_{obs} = (k_{inact}/K_{inact})[I] \quad \text{(linear fit)} \quad (4)$$

to distinguish between saturating and nonsaturating kinetics. The second-order rate constants for slower inactivators with limited solubility are less accurate values and are determined as $k_{obs}/[I]$ values.

To standardize our cathepsin B assay, we evaluated the activity of certain related halomethyl ketones. Using our assay conditions as described above, we determined significantly higher inactivation rates for Z-Phe-Phe-CH_2Cl (k/K = 67 000 M^{-1} s^{-1}) and Z-Phe-Ala-CH_2Cl (k/K = 640 000 M^{-1} s^{-1}) than those previously reported for these compounds (Rauber et al., 1986; Rasnick, 1985); the related Z-Phe-Gly-CH_2Cl was also found to be highly potent (k/K = 1 700 000 M^{-1} s^{-1}). However, the rapid rate of decomposition of Z-Phe-Ala-CH_2Cl in the assay medium ($t_{1/2}$ = 27 min; see supplementary material) means that even these inactivation rate constants should be regarded as lower limits. The inactivation rate of Z-Phe-Ala-CH_2Br was too fast to measure, and due to the very rapid and competing decomposition of this inhibitor ($t_{1/2} \approx 10$ s), only partial inactivation occurred at low inhibitor concentrations; consequently, an apparent "IC_{50}" of 9 nM was obtained. Finally, our determination of the inactivation rate of cathepsin B by the fluoromethyl ketone Z-Phe-D,L-Ala-CH_2F (k/K = 21 000 M^{-1} s^{-1}; Smith et al., 1988b), which is stable in our assay ($t_{1/2}$ > 24 h), was found to be in accord with previously reported values (Rauber et al., 1986; Rasnick, 1985).

Dilution Assays. Enzyme, at approximately 0.4 nM, was incubated with inhibitor (at a concentration at least 10-fold higher than enzyme) in assay buffer as described above but in the absence of substrate. At various time points (t), a 50-μL aliquot was removed and added to 1.95 mL of buffer (i.e., 1:40 dilution) containing 2.5 μM fluorogenic substrate, and the reaction was monitored as described above. Values obtained from measurements of residual activity vs incubation time (t) were fit to eq 2. These experiments were carried out for compounds **6**, **15**, **16**, **17**, and **26**. In all cases, the kinetics resulting from these dilution experiments were in good

agreement with those of the continuous assay, as shown: k_{obs} (s^{-1}) for dilution assay vs k_{obs} (s^{-1}) for continuous assay were 1.14 (±0.07) × 10^{-2} vs 1.15 (±0.07) × 10^{-2} for 10 nM **6**, 4.0 (±0.4) × 10^{-3} vs 3.4 (±0.3) × 10^{-3} for 400 nM **15**, 7.7 (±0.4) × 10^{-4} vs 7.1 (±0.2) × 10^{-4} for 60 nM **16**, 1.1 (±0.1) × 10^{-3} vs 1.22 (±0.03) × 10^{-3} for 400 nM **17**, and 1.3 (±0.2) × 10^{-3} vs 1.36 (±0.09) × 10^{-3} for 300 nM **26**. This indicates that the k_{obs} value obtained from the continuous assay is equivalent to the rate of (irreversible) inactivation.

Dialysis Experiments. Cathepsin B, at ca. 0.4 nM, was incubated with inhibitor in assay buffer until virtually no enzyme activity remained (<5% of initial activity, as measured for aliquots of the incubation). A 2.5-mL sample of this incubation solution, and a control sample containing fully active enzyme, were each dialyzed against the assay buffer (24 h, 2 × 400 volumes, 25 °C, Spectrapor #2 10-mm dialysis tubing). Following dialysis, the activity of the inhibited sample was compared to that of the control sample. In the case of inhibitors **6**, **15**, and **16** there was no recovery of enzyme activity over the 24-h dialysis period, while experiments with compounds **17** and **26** showed 13% and 15% recovery, respectively.

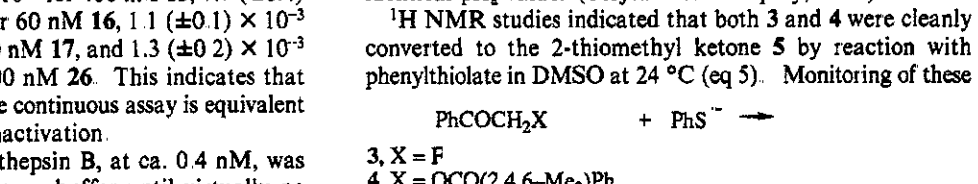
Stoichiometry of Inactivation with (Acyloxy)methyl Ketone ***15***. Cathepsin B stock solution (1 mL) was treated as described for the titration of enzyme activity (see supplementary material). The 1-mL protein fraction from the PD-10 gel filtration column was mixed with 4 μL of 0.5 M DTT to give a final concentration of 2 mM DTT. The 1-mL fraction was divided into a 200-μL control (uninhibited) sample and an 800-μL incubation sample to which incremental amounts of inhibitor were added. After each aliquot of inhibitor was added, enzyme activity was monitored (2-μL aliquots of incubation solution diluted into the standard assay system) until no further change in activity was observed. Another aliquot of inhibitor was then added, and the process was repeated until no further change in enzyme activity was detected. The *x*-intercept of the plot of $[I]/[E_0]$ vs $[E]/[E_0]$ from these results showed approximately 1.3 equiv of **15** were required to totally inactivate 1 equiv of cathepsin B.

Results and Discussion

Selection of the Leaving Group. A characteristic feature of cysteine proteinases of the papain family is their nucleophilicity (Bender & Brubacher, 1966; Whitaker & Perez-Villaseñor, 1968; Polgár, 1989). Even against very simple reagents such as iodoacetamide, papain exhibits powerful nucleophilicity at pH 6.0 rivaling the reactivity of the thiolate of glutathione at pH 11.0 (Halász & Polgár, 1976). Truly spectacular reactivity has been recorded for alkylations involving even sluggish nucleofuges, if the specificity requirements of cysteine proteinases are satisfied. For example, Ala-Phe-Lys-CH_2F (**2**) is a potent inactivator of cathepsin B, reacting essentially 10^8 times faster with this enzyme than with glutathione at pH 6.4 (Angliker et al., 1987).

To optimize the potency and selectivity of affinity labels targeted for cysteine proteinases, it would be advantageous to systematically vary the nucleofugality of a leaving group using substituent effects. Carboxylates are generally regarded as feeble leaving groups in S_N2 reactions unless highly nucleophilic conditions are employed (McMurry, 1976). To obtain an indication of the relative nucleofugalities of a carboxylate leaving group versus a notoriously sluggish nucleofuge in S_N2 displacements, such as fluoride (Streitwieser, 1962), reactions of 2-fluoro- and 2-(mesitoyloxy)acetophenone **3** and **4** with thiolate were investigated. We chose mesitoate as a standard for comparison because (1) the flanking ortho methyl groups shield the ester carbonyl of the substrate from nucleophilic attack (Newman, 1956), thus preventing "wasting" of the reagent, and (2) mesitoate and fluoride have nearly identical pK_a values (Serjeant & Dempsey, 1979).

^{1}H NMR studies indicated that both **3** and **4** were cleanly converted to the 2-thiomethyl ketone **5** by reaction with phenylthiolate in DMSO at 24 °C (eq 5). Monitoring of these



displacement reactions by HPLC afforded data which indicated that the fluoride reacts 12 times faster than the corresponding mesitoate (for **3**, k_2 = 0.84 ± 0.05 M^{-1} min^{-1}; for **4**, k_2 = 0.070 ± 0.005 M^{-1} min^{-1}).

These results clearly demonstrate that a simple (acyloxy)methyl ketone is less reactive than fluoromethyl ketone to displacement by thiolate, even under strongly nucleophilic conditions. (Acyloxy)methyl ketones, therefore, merit serious consideration as potential labeling entities of low chemical reactivity. Consequently, a systematic study of the effect of varying carboxylate nucleofugality on inhibitor potency was performed.

Structural Requirements for Inactivation. The Affinity Group. (Acyloxy)methyl ketones with peptide recognition elements that satisfy the specificity requirements of cathepsin B, span a range of 6 orders of magnitude in their ability to inactivate this enzyme (Tables I and II). Inhibitor potency is critically dependent on both the structure of the peptide moiety and the carboxylate leaving group. Our choice of peptidyl recognition elements has been dictated by the high affinity of papain-type cysteine proteinases for aromatic amino acids such as phenylalanine at the P_2 position of substrate (Schechter & Berger, 1967) and the work of Shaw (1990) specifying dipeptidyl affinity groups for cathepsin B. For dipeptidyl (acyloxy)methyl ketones (IV, Scheme I), a variety of L-α-amino acid residues are well tolerated at the S_1 position of cathepsin B (Table II). The same dipeptidyl frameworks appropriately linked to chloromethyl, diazomethyl, or (acyloxy)methyl ketone functions give rise to inactivators of cathepsin B, although there are differences in the rank order of potency within each series (Rich, 1986). Very dramatic inhibition is observed with Z-Phe-Lys-CH_2OCO-(2,4,6-Me_3)Ph (**35**), in accord with the affinity of cathepsin B for positively charged amino acids at the P_1 position.

A number of observations reinforce the notion that peptidyl (acyloxy)methyl ketones must be analogues of substrates and have favorable interactions with cathepsin B specificity subsites in order for inactivation to occur. For instance, mesitoyloxy compounds with an amino acid of D-configuration (**54**) or with methyl substitution at the P_1 amido nitrogen (i.e., the sarcosine analogue **53**) are not active against cathepsin B. The peptidyl ketones with β- or γ-amino acids at P_1, **51** and **52**, respectively, are also inactive. Furthermore, (acyloxy)methyl ketones **57** [Z-Pro-Val-CH_2OCO-[2,6-$(CF_3)_2$]Ph] and **58** [Z-Ala-Ala-Pro-Val-CH_2OCO-[2,6-$(CF_3)_2$]Ph] bearing peptidyl functionality that lack affinity for cathepsin B are not effective inactivators of this enzyme (k/K = 30 and 200 M^{-1} s^{-1}, respectively; see Table SI, supplementary material), despite the presence of a carboxylate leaving group of low pK_a (vide infra). The foregoing results and the fact that **50**, which possesses only a single amino acid residue, fails to inactive cathepsin B clearly demonstrate that a dipeptidyl complement to enzyme

Table II: Rates of Cathepsin B Inactivation by Peptidyl (Acyloxy)methyl Ketones: Variation of the Affinity Group[a]

no.	compound R′	k/K (M^{-1} s^{-1})	K_{inact} (nM)	k_{inact} (s^{-1})
		R′-CH_2OCO-[2,6-$(CF_3)_2$]Ph		
38	Z-Phe-Gly	4000000		
39	Z-Phe-Cys(SBn)	2900000	13 ± 2	0.039 ± 0.003
40	Z-Phe-Ser(OBn)	2600000	13 ± 15	0.033 ± 0.019
34	Z-Phe-Lys (HCl salt)	>2000000		
6	Z-Phe-Ala	1600000	28 ± 7	0.045 ± 0.008
41	Z-Phe-Thr(OBn)	>100000[b]		
42	Z-Phe-D-Ala (diastereoisomer of **6**)	7100[c]		
		R′-CH_2OCO-(2,4,6-Me_3)Ph		
35	Z-Phe-Lys (TFA salt)	230000	170 ± 50	0.037 ± 0.008
43	Z-Phe-Thr(OBn)	30000		
44	Z-Tyr(OMe)-Ala	19000[d]	280 ± 60	0.005 ± 0.0003
45	Z-Phe-Ser(OBn)	17000[d]	1300 ± 400	0.022 ± 0.002
15	Z-Phe-Ala	14000[d,e]	510 ± 110	0.0073 ± 0.0005
46	Z-Phe-Gly	9900[e]	1900 ± 900	0.019 ± 0.007
47	Z-Phe-Phe	4300		
48	Z-Phe-Cys(SBn)	4100		
49	H-Phe-Ala (HCl salt)	740		
4	PhCO	ntd[f]		
50	Z-Phe	ntd		
51	Z-Phe-β-Ala	ntd		
52	Z-Phe-GABA	ntd		
53	Z-Phe-Sar	ntd		
54	Z-Phe-D-Ala (diastereoisomer of **15**)	ntd		
		Isomer of **15**		
55	Z-Phe-Ala-OCH_2CO-(2,4,6-Me_3)Ph	ntd		

[a] Conditions: bovine spleen cathepsin B, 100 mM potassium phosphate, 1.25 mM EDTA, and 1 mM dithiothreitol, pH 6.0, 25 °C, under argon. The parameter k/K is the second-order rate constant (k_{inact}/K_{inact}), except as noted (see Experimental Procedures). Standard errors for $k/K \leq 15\%$, except as noted. In those cases where saturation kinetics were observed, k_{inact}/K_{inact} and the individual parameters K_{inact} and k_{inact} are given, as determined from a hyperbolic fit. In the other cases, k_{inact}/K_{inact} was obtained from a linear fit. [b] Compound instability was evident. [c] Second-order rate constant ($k/[I]$) determined at one concentration near the inhibitor solubility limit. This value represents an upper limit of $k/[I]$ for **42**, since it may be accounted for completely or in part by a slight amount (<0.5%) of the diastereomeric compound **6** (a potential contaminant). [d] Standard error 15–20%. [e] This value has been revised since our previous report (Smith et al., 1988a) (see supplementary material). [f] ntd: no time dependence or significant inhibition observed near the compound solubility limit, over a 10–20-min period.

S_1 and S_2 subsites is a critical component of these type of inactivators. The inertness of **50** toward cathepsin B stands in sharp contrast to the action of the corresponding fluoromethyl ketone (Rauber et al., 1986), which is reported to inactivate both cathepsin B and the serine proteinase chymotrypsin.

It is also noteworthy that Z-Phe-Lys-CH_2OCO-(2,4,6-Me_3)Ph (**35**), a powerful inactivator of cathepsin B, binds to trypsin with a $K_i = 17 \pm 1$ μM without inactivating this enzyme. As well, **57** and **58** (vide supra), which bear affinity groups complementary to human leukocyte elastase (Stein, 1985), do not exhibit significant time-dependent activity ($k/K < 15$ M^{-1} s^{-1}) toward this elastase (see Table SI, supplementary material). Apparently, serine proteinases lack the nucleophilicity required for facile expulsion of a carboxylate leaving group in an S_N2-like displacement. This point underscores the ability of (acyloxy)methyl ketones as enzyme inactivators to discriminate between serine and cysteine proteinases despite their similar catalytic mechanisms. We have also noted that (acyloxy)methyl ketone inactivators of cathepsin B do not exhibit time-dependent activity against either aspartyl proteinases or metalloproteinases (D. H. Pliura & Z. Yuan, unpublished results), indicating that such compounds are specific inactivators of cysteine proteinases.

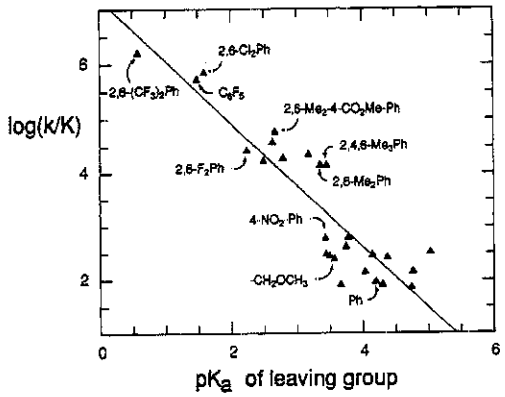


FIGURE 2: Correlation of the rate of cathepsin B inactivation [log (k/K)] by Z-Phe-Ala-CH_2OCOR with pK_a of the leaving group RCOOH. Data are from Table I, for all Z-Phe-Ala compounds except **17** and **29**.

Variation of the Carboxylate Leaving Group. Carboxylates are generally regarded as feeble leaving groups in S_N2 reactions unless highly nucleophilic conditions are employed. Larsen and Shaw (1976), who investigated the possibility of employing departing groups other than bromide or chloride to limit side reactions of affinity labeling reagents, could not observe any time-dependent activity against chymotrypsin with Z-PheCH_2OAc. It seemed reasonable to us that a threshold value for enzyme inactivation might be achieved by systematically varying the pK_a of the carboxylate departing group in the presence of a powerfully nucleophilic cysteine proteinase.

Consequently, we have investigated Z-Phe-Ala (acyloxy)methyl ketones with leaving groups spanning a range of pK_a values from 0.6 to 5.0 (Table I). The activity of these inhibitors was found to be exquisitely sensitive to the nature of the carboxylate leaving group, as a strong relationship between the logarithm of the second-order inactivation rate constant and carboxylate pK_a was uncovered by linear regression (Figure 2, eq 6). The great majority of the leaving groups

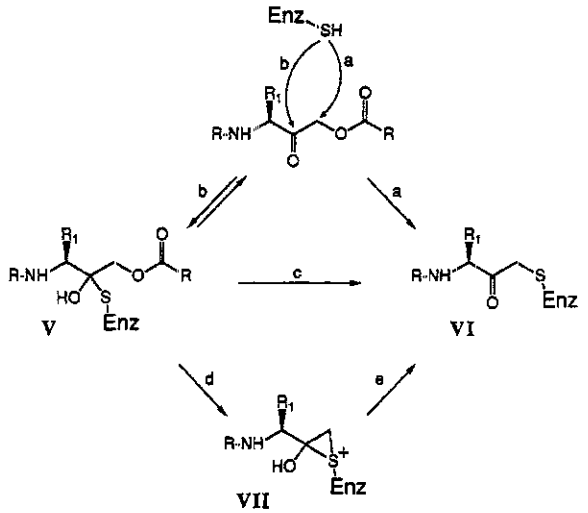
$$\log (k/K) = -1.1 (\pm 0.1) \times pK_a + 7.2 (\pm 0.4) \qquad r^2 = 0.82 \quad n = 26 \quad (6)$$

in this series are substituted benzoates. Some of the most potent inactivators of cathepsin B are (acyloxy)methyl ketones containing 2,6-disubstituted benzoate leaving groups. The unique structural feature of these inactivators, which sets them apart from other compounds in the series, is that there is a large dihedral angle between the ring and the ester carbonyl in the equilibrium conformation (e.g., 55° for mesitoic acid; Baumstark et al., 1987).

There are two obvious ways by which ring substituents could affect the second-order rate constant, k/K, and produce the linear regression shown in Figure 2 (eq 6). Substitution on the benzoate framework clearly affects the strength of the RCH_2–OCOAr bond, but the sensitivity of the rate to substituent effects depends upon whether this bond is broken in an "early" or "late" rate-determining transition state.

Intuitively, if cleavage of the CH_2–OCOR bond is rate determining, one would expect that (1) inhibitors with the better leaving groups (i.e., those with lower pK_a values) would

Scheme II: Mechanistic Considerations for the Inactivation Process



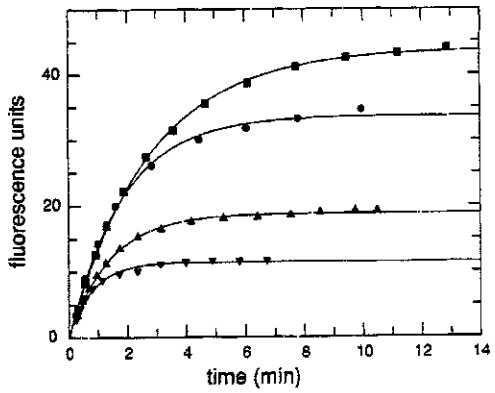


FIGURE 3: Examples of progress curves for the inhibition of cathepsin B by compound **6**. The data points are taken from the continuous fluorescence measurement, while the solid lines are the nonlinear regression fit to the exponential equation 2 Conditions are as described under Experimental Procedures. Inhibitor concentrations and inactivation half-lives, respectively, are 4 nM, 1.91 min (■); 8 nM, 1 36 min (●); 12 nM, 0.97 min (▲); and 20 nM, 0.57 min (▼)

react more rapidly and (2) for a given peptide frame, a threshold leaving group pK_a value would be reached beyond which the inactivation rate would be immeasurably slow. Indeed, that is generally what is observed, although leaving group pK_a is clearly not the only determinant of the inactivation rate. (Subtle or specific effects can also influence inhibitor potency, as evidenced by the broad range of cathepsin B inactivation rates (k/K = 80–14 000 M^{-1} s^{-1}) observed for Z-Phe-Ala (acyloxy)methyl ketones with leaving group pK_a values of ca 3 5)

From the large slope in the regression (Figure 2), it is tempting to propose that bond breaking has proceeded extensively in the transition state (Lowry & Richardson, 1987). However, this assumes that the environment of the enzyme active site is very much like the medium in which the carboxylic acid pK_a values have been determined (water) In fact, it is known that the relative magnitudes of pK_a values within a series of carboxylic acids can vary dramatically in going from water to nonaqueous environments (Ritchie, 1969).

Substituents could also influence the second-order rate by altering the position of equilibrium (or steady state) between free enzyme and inhibitor versus the tetrahedral intermediate V (Scheme II) Electron withdrawal should destabilize the ketone and thus promote the formation of the tetrahedral intermediate V Such an effect on a dissociation constant, K_i, has been documented by McMurray and Dyckes (1986) for the action of α-substituted ketones on trypsin Whether arylacyl ring substituent effects can be transmitted to a remote keto group and give rise to the large changes observed in Table I by virtue of their effect on a dissociation constant is open to question Although a mechanistic rationale for the effects of ring substituents on the second-order rate of inactivation of cathepsin B remains to be established, the relationship of leaving group pK_a and rate in Figure 2 provides a useful predictive framework.

For Z-Phe-Ala (acyloxy)methyl ketones, the second-order rate of inactivation falls off to very low values when leaving group pK_a > 4, yet this threshold can be overcome dramatically by using a tighter binding peptidyl moiety For example, the 4-methoxybenzoate **29** does not exhibit time-dependent activity in our assay, but the 4-methoxybenzoate having the Z-Phe-Lys framework (**37**) inactivates cathepsin B at a rate essentially identical with that observed for the 4-nitrobenzoate in the Z-Phe-Ala series (**19**) This ability to exploit a tighter binding affinity group, to compensate for the effect of modifying the leaving group, is a useful design element for fine-tuning the properties of an inhibitor lead structure.

Kinetics and Mechanistic Considerations. A minimal kinetic mechanism describing the general features of the inactivation is indicated in eq 7, where E = free enzyme, I =

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E{-}I \qquad (7)$$

inhibitor, E·I = a reversible complex, and E–I = stable adduct (Kitz & Wilson, 1962; Silverman, 1988). The physical significance of E·I can, in principle, vary with the (acyloxy)methyl ketone inhibitor: E·I may correspond to a noncovalent (Michaelis–Menten) complex or a tetrahedral intermediate (hemithioketal V, Scheme II), or it may not be a minimum along the reaction pathway to E–I (i.e., E + I → E–I)

The irreversibility of the inhibition has been established by both dialysis and dilution assays (see Experimental Procedures for details of assays), as well as by NMR characterization of the stable enzyme adduct as a thiomethyl ketone VI (Scheme II) (Smith et al , 1988a) In addition, the stoichiometry of inactivation was determined to be ca 1:1 by titration of enzyme activity with **15** Leupeptin, a competitive inhibitor of cathepsin B, protects the enzyme from inactivation by **6** [an extremely potent peptidyl (acyloxy)methyl ketone], with a K_i of 5 nM (Baici & Gyger-Marazzi, 1982; $K_i \approx 5$ nM), providing confirmatory evidence for the active-site-directed nature of the inactivation The progress curves of the inhibition show pseudo-first-order kinetics in a concentration-dependent manner, as illustrated in Figure 3. Further analysis of these kinetics provides the second-order rate constant k_{inact}/K_{inact}, which describes the reaction of free enzyme and inhibitor to give adduct E–I. Where saturation kinetics have been observed, the individual parameters k_{inact} and K_{inact} have been determined (see Tables I and II), although the error limits in some cases owing to experimental difficulties are substantial (see Experimental Procedures).

Nevertheless, the lack of variation of the k_{inact} term is striking By contrast, K_{inact} ranges over 2 orders of magnitude and thus is mainly responsible for variances in the second-order rate. Other than the fact that K_{inact} represents the concentration of inhibitor that gives a half-maximal rate of inactivation ($K_{inact} = (k_{-1} + k_2)/k_1$; Silverman, 1988), its physical significance in this study is obscure Indeed, the formation of the E·I species and/or its conversion to adduct E–I may be quite complex mechanistically (i.e., not single-step processes).

Even with a precise determination of k_1, k_{-1}, and k_2, without physical evidence of structure of transient intermediates the interpretation of these kinetic constants and the E·I species in molecular terms is highly speculative.

It is intriguing, however, that for cathepsin B inactivation by a variety of peptidyl diazomethyl ketones, fluoromethyl ketones, sulfonium methyl ylids, (acyloxy)methyl ketones, and *O*-acylhydroxamates (Rasnick, 1985; Zumbrunn et al., 1988a,b; Shaw, 1988; Rauber et al., 1986; Smith et al., 1988b,c), the rate-limiting constant k_{inact} falls within a narrow range (0.002–0.064 s^{-1}), while K_{inact} spans more than 4 orders of magnitude. An implication of the data is that k_{inact} represents the rate constant of a step (or steps) common to all of these enzyme reactions, which is not the displacement step itself.

It has been known for some time that a common feature of the chemistry of serine and cysteine proteinases is their ability to form tetrahedral adducts with complementary peptidyl aldehydes (Aoyagi et al., 1969; Schultz et al., 1989; Mackenzie et al., 1986). In fact, McMurray and Dyckes (1986) have shown that the serine proteinase trypsin undergoes hemiketal formation with a series of peptide ketones (Lys-Ala-Lys-CH_2X), in which X is a poor or ineffective leaving group, and that there is a linear relationship between the free energy of binding and the electron-withdrawing power of X. As well, we have demonstrated that, for a small set of compounds, the inhibition of cathepsin B by Z-Phe-Ala aldehyde and ketones correlates well with hemithioketal formation in a model system (Smith et al., 1988b).

Two mechanisms can be envisaged by which tetrahedral adducts might mediate the formation of product, that differ only in the timing of bond-breaking and bond-making processes (Scheme II). One possibility, involving neighboring group participation by the sulfur atom, features a discrete episulfonium ion intermediate VII en route to product (two-step migration). The other is characterized by migration of the sulfur group in a single step concomitant with loss of carboxylate and regeneration of the ketone carbonyl (step c, Scheme II). The pathway involving the formation of the episulfonium ion intermediate VII would seem to be the less likely alternative (unless steps d and e are enzyme activated) because of the known chemical stability of simple α-acyloxy thioethers (Sunko et al., 1987). Speculation then centers around whether the tetrahedral intermediate V lies on the reaction coordinate leading to adduct VI (steps b and c) or whether direct displacement of carboxylate occurs (step a, Scheme II).

The latter mechanistic point bears on the classification of (acyloxy)methyl ketones as inhibitors of cysteine proteinases. If enzyme inactivation proceeds via the tetrahedral intermediate V, then (acyloxy)methyl ketones must be regarded as suicide inhibitors. These reagents would then fulfill the criteria employed for suicide inhibitors (Silverman, 1988) in that (1) *their reactivity is latent*, a consequence of the reactive possibilities inherent in the α-substituted hemithioketal intermediate, and (2) *the enzyme inactivation pathway parallels that of normal substrate turnover* by initially producing a tetrahedral intermediate. However, if the system bypasses the tetrahedral intermediate and inactivation proceeds by direct displacement of carboxylate from the inhibitor, then (acyloxy)methyl ketones are not suicide reagents, since their reactivity is not unmasked during the course of catalysis. Neither would they be adequately described as classical affinity labels, for they lack the intrinsic chemical reactivity associated with such reagents, which are known to undergo facile chemical reactions with bionucleophiles at physiological temperatures and pH.

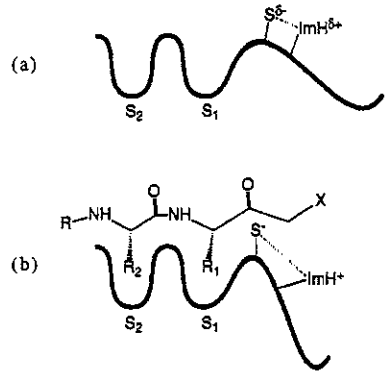


FIGURE 4: Schematic representation of a hypothesis for enhanced active-site thiolate reactivity resulting from tighter inhibitor binding

It may be envisaged that this new type of affinity label, like an enzyme substrate, might exploit intrinsic binding (Jencks, 1975) to effect chemical transformations that would otherwise be excruciatingly slow without some form of catalysis. Such inactivators could be designed to react facilely only upon binding to the target enzyme and, ideally, would be "quiescent" in the presence of other bionucleophiles lacking a proper complementary surface. Whereas suicide substrates utilize binding and normal catalysis to produce reactive intermediates that cause enzyme inactivation, quiescent affinity labels would exploit intrinsic binding to lower the overall free energy of activation of an "aberrant" chemical path, leading to facile enzyme inactivation. Perhaps another example that can be encompassed by the quiescent affinity label principle has been reported by Poulter et al. (1989), who have described an allyl fluoride inactivator of the enzyme isopentenyldiphosphate: dimethylallyldiphosphate isomerase.

In the case of cysteine proteinases, in addition to factors normally associated with intrinsic binding (Jencks, 1975), one can envisage a viable model that accounts for enhanced reactivity with tighter binding, assuming that thiolate nucleophilicity is a critical determinant of enzyme reactivity. A reasonable postulate is that tighter binding leads to increased separation of charge in the thiolate imidazolium ion pair (Figure 4). The freer the thiolate, the greater is the nucleophilicity of the enzyme and the more rapidly displaced is a given leaving group from the inactivator. The implication of this hypothesis is that the stabilization derived from affinity groups that are perfect active-site complements to enzyme active sites can be used to drive chemical reactions under ambient conditions that are unprecedented in the annals of organic chemistry and distinct from those which the enzyme has evolved to catalyze.

Summary

Peptidyl (acyloxy)methyl ketones are noteworthy because they represent prime examples of chemically stable, highly potent, and specific enzyme inactivators with variable structural elements in both the affinity and departing groups. A broad range of cathepsin B inactivation rates, in some cases exceeding 10^6 M^{-1} s^{-1}, are obtainable by systematically varying these structural elements. Such peptidyl (acyloxy)methyl ketones are specific inactivators of papain-type proteinases, by virtue of the extraordinary ability of these enzymes to displace weak nucleofuges such as carboxylate leaving groups. This type of reagent extends the boundaries of inhibitor design to a new generation of affinity labels and holds forth the prospect of a practical clinical end point.

ACKNOWLEDGMENTS

We are indebted to Dr. Robin W. Spencer for initial contributions to the biochemistry, to Valerie J. Robinson for NMR investigations, and to Dr. Henry W. Pauls for synthetic contributions and helpful discussions We are grateful to students from the University of Waterloo cooperative program (S Donnelly, A. Erhardt, L. Stait-Gardner, K Streutker, S Lukovits, and H. LeBlanc) for their assistance with the chemistry and enzymology

SUPPLEMENTARY MATERIAL AVAILABLE

Experimental procedures for the synthesis of precursors, bromomethyl, chloromethyl, and (acyloxy)methyl ketones, and model compounds **3–5**; information for model reaction kinetics and competition experiments; stability studies; titration of cathepsin B, and additional enzyme assay information; Table SI [Rates of Cathepsin B and HLE Inactivation by Peptidyl (Acyloxy)methyl Ketones **56–58** Having Affinity Groups for Human Leukocyte Elastase]; Table SII (Rates of Cathepsin B Inactivation by Peptidyl Halomethyl Ketones); and physical data for all bromomethyl, chloromethyl, and (acyloxy)methyl ketones (20 pages) Ordering information is given on any current masthead page

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^{2}H Nuclear Magnetic Resonance of the Gramicidin A Backbone in a Phospholipid Bilayer†

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Received December 5, 1990; Revised Manuscript Received January 23, 1991

ABSTRACT: Solid-state ^{2}H NMR spectroscopy has been employed to study the channel conformation of gramicidin A (GA) in unoriented 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine (DMPC) multilayers. Quadrupolar echo spectra were obtained at 44 °C and 53 °C, from gramicidin A labels in which the proton attached to the α carbon of residue 3, 4, 5, 10, 12, or 14 was replaced with deuterium. Because of the nearly axially symmetric electric field gradient tensor, the quadrupolar splittings obtained from an unoriented multilamellar dispersion of DMPC and singly labeled GA directly yield unambiguous orientational constraints on the C–^{2}H bonds. The average of the ratios of the quadrupolar splittings of the left-handed amino acids to those of the right-handed amino acids, $\langle \Delta\nu_{QL}/\Delta\nu_{QD} \rangle$, is expected to be 0.97 ± 0.04 for a relaxed right-handed $\beta^{6.3}_{LD}$ helix, while a ratio of 0.904 ± 0.003 is expected for a left-handed $\beta^{6.3}_{LD}$ helix. Since we have experimentally determined this ratio to be 1.01 ± 0.04, we conclude that the helix sense of the channel conformation of GA is right-handed. Assuming that the dominant motions are fast axial diffusion of the gramicidin molecule and reorientation of the diffusion axis with respect to the local bilayer normal, then the theoretical splittings may all be scaled down by a constant motional narrowing factor. In this case, a relaxed right-handed $\beta^{6.3}_{LD}$ helix, whose axis of motional averaging is roughly along the presumed helix axis, gave the best fit to experimental results. The reasonably uniform correspondence between the splittings predicted by the relaxed right-handed $\beta^{6.3}_{LD}$ helix and the observed splittings, for labels from both the inner and outer turn of GA, did not reflect a peptide backbone flexibility gradient, since an outer turn (i.e., the turn of the helix closest to the interface with the water) with greater flexibility would show additional motional narrowing for labels located there.

(Val$_1$)gramicidin A (GA)[1] is a pentadecapeptide consisting of 15 alternating L- and D-amino acids (Sarges & Witkop, 1965) and has the following chemical formula:

HCO-L-Val$_1$-Gly$_2$-L-Ala$_3$-D-Leu$_4$-L-Ala$_5$-D-Val$_6$-L-Val$_7$-D-Val$_8$-L-Trp$_9$-D-Leu$_{10}$-L-Trp$_{11}$-D-Leu$_{11}$-L-Trp$_{13}$-D-Leu$_{14}$-L-Trp$_{15}$-NHCH$_2$CH$_2$OH

Pure GA can be isolated in gram quantities from commercially

† This work has been supported by grants from the Natural Sciences and Engineering Research Council. R.S.P. would also like to acknowledge NSERC for graduate studies scholarships.

0006-2960/91/0430-4687$02.50/0