chloro-6-(2,4-dichlorophenoxy)-*p*-benzoquinone, 76540-52-8; 2-chloro-*p*-benzoquinone, 695-99-8.

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# Inhibition of Cathepsin B by Peptidyl Aldehydes and Ketones: Slow-Binding Behavior of a Trifluoromethyl Ketone†


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ABSTRACT: Inhibition of the cysteine proteinase cathepsin B by a series of *N*-benzyloxycarbonyl-L-phenylalanyl-L-alanine ketones and the analogous aldehyde has been investigated. Surprisingly, whereas the aldehyde was found to be almost as potent a competitive reversible inhibitor as the natural peptidyl aldehyde, leupeptin, the corresponding trifluoromethyl ketone showed comparatively weak (and slow-binding) reversible inhibition. Evaluation of competitive hydration and hemithioketal formation in a model system led to a structure–activity correlation spanning several orders of magnitude in both cathepsin B inhibition constants ($K_i$) and model system equilibrium data ($K_{RSH,apparent}$).


It has been known for some time that peptidyl aldehydes are effective, reversible inhibitors of serine (Thompson, 1973; Thompson & Bauer, 1979; Dutta et al., 1984; Stein & Strimpler, 1987) and cysteine (Aoyagi et al., 1969; Westerik & Wolfenden, 1972; Mattis et al., 1977; Knight, 1980; Baici & Gyger-Marazzi, 1982; Ogura et al., 1985; Mackenzie et al., 1986) proteinases by forming hemiacetals and hemithioacetals at the active site (Scheme I). In recent years interest has focused on the potential of trifluoromethyl ketones to inhibit proteinases in a similar fashion, by the formation of

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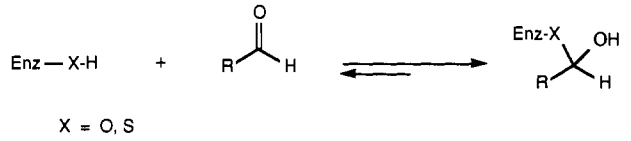
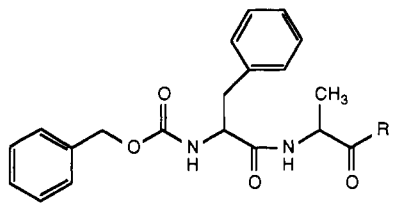
Scheme I: Hemiacetal Formation in the Inhibition of Serine and Cysteine Proteinases by Peptidyl Aldehydes




hemiketals. Trifluoromethyl ketones have been shown to be effective inhibitors of acetylcholinesterase (Brodbeck et al., 1979; Gelb et al., 1985), juvenile hormone esterase (Prestwich et al., 1984), and the serine proteinases $\alpha$-chymotrypsin, porcine pancreatic elastase (Imperiali & Abeles, 1986), and human leukocyte elastase (Dunlap et al., 1987; Stein et al., 1987). Difluoro and trifluoro ketones have also been exploited as inhibitors of other types of hydrolytic enzymes, including carboxypeptidase A, angiotensin converting enzyme, pepsin (Gelb et al., 1985), renin (Thaisrivongs et al., 1986; Fearon et al., 1987), and phospholipase $A_2$ (Gelb, 1986). In these cases, however, the hydrated ketone (*gem*-diol) has been proposed or demonstrated (Christianson & Lipscomb, 1986) to be the enzyme-bound species.

Most studies of the inhibition of cysteine proteinases by peptidyl aldehydes have been carried out with papain. Interactions of this class of inhibitors with the thiol proteinase cathepsin B have been studied to a lesser extent; cathepsin B is of particular interest because of its putative role in inflammation (Bayliss & Ali, 1978; Kominami et al., 1985), tumor metastasis (Sloane et al., 1986), myocardial tissue damage (Prous, 1986b), bone resorption (Delaissé et al., 1984), and muscular dystrophy (Prous, 1986a). Potent inhibition by the natural peptidyl aldehyde leupeptin is known (Knight, 1980; Baici & Gyger-Marazzi, 1982).

In recent years, peptidyl diazomethyl ketones (e.g., **1a**) (Green & Shaw, 1981; Shaw et al., 1983) and monofluoromethyl ketones (e.g., **1b**) (Rasnick, 1985; Rauber et al., 1986;




**1a**, R = CH=N=N **1c**, R = H **1e**, R = $CF_3$

**1b**, R = $CH_2F$ **1d**, R = $CH_3$

Angliker et al., 1987) have been developed as selective irreversible inhibitors of cathepsin B, and the *N*-benzyloxycarbonyl-L-phenylalanyl-L-alanine peptide structure has served as an effective enzyme recognition element. We felt that a study of the reversible inhibition by the analogous peptidyl aldehyde and ketone derivatives (**1c–1e**) was therefore in order.

## Experimental Procedures

### *Synthesis*

The aldehyde **1c** and methyl ketone **1d** derived from *N*-CBZ-L-phenylalanyl-L-alanine[1] were synthesized by reaction of the corresponding *N,O*-dimethyl hydroxamate (**2**) with diisobutylaluminum hydride and methyllithium, respectively (Fehrentz et al., 1985). The general synthetic route to peptidyl fluoromethyl ketones developed by Imperiali and Abeles (1986) was utilized for the preparation of *N*-CBZ-L-phenylalanyl-D,L-alanine trifluoromethyl ketone (**1e**), which was then separated into its diastereomeric forms (**1e′**, **1e″**) by HPLC. Monofluoromethyl ketone **1b** (mixture of L,L and L,D isomers) (Rasnick, 1985) was obtained from Dr. David Rasnick of Enzyme Systems Products. NMR spectra were recorded with a Bruker WP80 spectrometer at 80 MHz for proton spectra and at 75 MHz for fluorine spectra. $^1$H NMR chemical shifts are reported relative to tetramethylsilane at 0 ppm, and $^{19}$F NMR chemical shifts are reported relative to $CFCl_3$ at 0 ppm (in $CDCl_3$) or to external $CF_3COOH$ assigned as −78.90 ppm (in $D_2O$).

*N-CBZ-L-Phenylalanyl-L-alanine N,O-Dimethyl Hydroxamate* (**2**). A solution of *N*-CBZ-L-Phe-L-Ala-OH (14 mmol, 5.21 g) and carbonyldiimidazole (15 mmol, 2.43 g) in 100 mL of anhydrous THF was stirred at room temperature for 3 h and then treated with *N,O*-dimethylhydroxylamine hydrochloride (42 mmol, 2.92 g). The mixture was stirred overnight, 10 mL of water was added, and the mixture was concentrated by rotary evaporation. Ethyl acetate (400 mL) was added, and the organic phase was washed with 1 N HCl (2×), aqueous $NaHCO_3$ (6×), and brine (1×), dried ($MgSO_4$), rotary evaporated, and dried at high vacuum to afford 4.93 g (85%) of *N*-CBZ-L-Phe-L-Ala-$NCH_3$-$OCH_3$ (**2**) as a white solid. Recrystallization from EtOAc gave material with mp 121–123 °C: $[\alpha]^{21}_D$ −20.5° (*c* 1.19, EtOH); IR (KBr) 1710, 1695, 1665, 1650 cm$^{-1}$; NMR (DMSO-$d_6$) includes $\delta$ 4.91 (s, $OCH_2Ph$), 3.72 (s, $OCH_3$), 3.11 (s, $NCH_3$), 1.22 (d, $J$ = 7.0 Hz, $CHCH_3$). Anal. Calcd for $C_{22}H_{27}N_3O_5$: C, 63.91; H, 6.58; N, 10.16. Found: C, 64.17; H, 6.64; N, 10.19.

*N-CBZ-L-Phenylalanyl-L-alanine Aldehyde* (**1c**). A solution of **2** (2.5 mmol, 1.05 g) in 30 mL of anhydrous THF at −78 °C was treated dropwise with diisobutylaluminum hydride (38 mmol, 1 M in hexane). After 40 min at −78 °C, methanol (2 mL) and 3 N HCl (10 mL) were successively added dropwise, and the mixture was allowed to warm to room temperature. The mixture was diluted with ether, washed with 3 N HCl (3×), aqueous $NaHCO_3$, and brine, dried ($MgSO_4$), rotary evaporated, and dried to provide a 70% yield of product. Recrystallization (EtOAc) gave 230 mg (26%) of **1c** as a white powder: mp 149–153 °C; $[\alpha]^{21}_D$ −24.7° (*c* 1.22, acetone); IR (KBr) 1730, 1680, 1640 cm$^{-1}$; NMR ($CDCl_3$) includes $\delta$ 9.37 (s, $CHO$), 5.10 (s, $OCH_2Ph$), 1.26 (d, $J$ = 7.4 Hz, $CHCH_3$). Anal. Calcd for $C_{20}H_{22}N_2O_4$: C, 67.78; H, 6.26; N, 7.90. Found: C, 67.69; H, 6.63; N, 8.12.

*N-CBZ-L-Phenylalanyl-L-alanine Methyl Ketone* (**1d**). A solution of **2** (1.0 mmol, 413 mg) in 50 mL of anhydrous THF at −78 °C was treated dropwise with methyllithium (15 mmol, 1.55 M in ether) and then stirred for 90 min at −78 °C. Quenching and workup as above for the aldehyde afforded 340 mg (92%) of the product. Recrystallization (EtOAc) gave **1d** as a white powder: mp 135–139 °C; $[\alpha]^{21}_D$ −16.3° (*c* 0.53, acetone); IR (KBr) 1720, 1690, 1650 cm$^{-1}$; NMR ($CDCl_3$) includes $\delta$ 5.10 (s, $OCH_2Ph$), 2.14 (s, $COCH_3$), 1.20 (d, $J$ = 9.0 Hz, $CHCH_3$). Anal. Calcd for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57; N, 7.60. Found: C, 68.32; H, 6.63; N, 7.55.

*N-CBZ-L-Phenylalanine (3-Hydroxy-4,4,4-trifluoro-2-butyl)amide* (**3**). Nitroethane (90 mmol, 6.5 mL), trifluoroacetaldehyde ethyl acetal (80 mmol, 9.3 mL), and potassium carbonate (12 mmol, 164 mg) were combined at 0 °C and then heated under reflux for 6 h. After the mixture was stirred at room temperature overnight, it was diluted with ether, washed with water and brine, dried ($MgSO_4$), rotary evaporated, and distilled to provide 9.12 g (66%) of 3-nitro-1,1,1-trifluoro-2-butanol as a clear colorless liquid: bp 91.5–95 °C (15 mmHg);

[1] Abbreviations: CBZ, benzyloxycarbonyl; HPLC, high-pressure liquid chromatography; THF, tetrahydrofuran; EtOAc, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide.

NMR ($CDCl_3$) includes $\delta$ 1.67 (2d or dd, $J$ = 1.0 and 6.8 Hz); $^{19}F$ NMR ($CDCl_3$) −76.10 (d, $J$ = 6.7 Hz), −76.95 (d, $J$ = 6.7 Hz) ppm. A solution of this material (29 mmol, 5.0 g) in 50 mL of ethanol was then reduced with Raney nickel (0.5 g) under 80 psi of hydrogen for 5 h. After removal of the catalyst by filtration, concentrated HCl (6 mL) was added dropwise to the stirred filtrate. The mixture was rotary evaporated and the residue mixed with ether. Filtration and drying gave 4.95 g (95%) of 3-amino-1,1,1-trifluoro-2-butanol hydrochloride as a white powder: mp ~137 °C, broad (mixture of diastereomers); NMR ($D_2O$) includes $\delta$ 1.38 (d, $J$ = 6.5 Hz, $CH_3$), 1.33 (dd, $J$ = 1.0 and 6.8 Hz, $CH_3$); $^{19}F$ NMR ($D_2O$) −75.75 (d, $J$ = 7.1 Hz), −76.80 (d, $J$ = 7.1 Hz) ppm. A solution of this amine salt (17 mmol, 3.05 g) and *N*-methylmorpholine (17 mmol, 1.87 mL) in anhydrous 1:1 $CH_2Cl_2$–THF (250 mL) plus anhydrous DMF (25 mL) was treated with *N*-CBZ-L-phenylalanine (17 mmol, 5.09 g) followed by *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide hydrochloride (17 mmol, 3.25 g). The mixture was stirred for 3 days, concentrated by rotary evaporation, and mixed with EtOAc. This mixture was then washed with water, 1 N HCl (3×), aqueous $NaHCO_3$ (2×), and brine, dried ($MgSO_4$), and rotary evaporated. The oil residue was treated with ether–hexane to afford 6.13 g (85%) of peptidyl alcohol **3** (four diastereomers) as a white solid: mp 99–108 °C; IR (KBr) 1690, 1660 $cm^{-1}$; NMR ($CDCl_3$) includes $\delta$ 5.05 (s, $OCH_2Ph$), 4.6–3.6 (m, 3 H), 3.04 (pseudo d, $J$ = 7.3 Hz, $CHCH_2Ph$), 1.3–0.9 (four doublets, $J$ ~ 6–9 Hz, $CH_3$); $^{19}F$ NMR ($CDCl_3$) −75.70 (d, $J$ = 7.2 Hz), −75.86 (d, $J$ = 7.5 Hz), −77.07 (d, $J$ = 7.2 Hz), −77.17 (d, $J$ = 7.2 Hz) ppm. Anal. Calcd for $C_{21}H_{23}N_2O_4F_3$: C, 59.43; H, 5.46; N, 6.60. Found: C, 59.54; H, 5.48; N, 6.57.

*N-CBZ-*L*-Phenylalanyl-*D,L*-alanine Trifluoromethyl Ketone* (**1e**). Peptidyl alcohol **3** (2.0 mmol, 849 mg) was mixed with 15 mL of THF and 20 mL of 0.3 N NaOH, and then $KMnO_4$ (2.4 mmol, 380 mg) was added portionwise with stirring at room temperature. After 1 h, additional $KMnO_4$ was added (ca. 150 mg), and the mixture was stirred another 15 min. The mixture was filtered, washing with water and THF. The filtrate was washed with water and brine and then dried ($MgSO_4$), evaporated, and dried at high vacuum to give 0.89 g of the crude product as a viscous oil: $^{19}F$ NMR ($CDCl_3$) −76.48 (s), −76.57 (s), −82.21 (s), −82.34 (s) ppm, plus signals for unreacted **3**; $^{19}F$ NMR (1:1 acetone–$D_2O$) −81.7 ppm. Preparative HPLC (Whatman Magnum 20 silica gel column, 35% EtOAc in hexane, 11 mL/min) afforded, by peak-shaving of the broad misshapen peak for product, samples of the two diastereomers of *N*-CBZ-L-phenylalanyl-D,L-alanine trifluoromethyl ketone, each with mp 132–136 °C: (a) **1e'**, less polar isomer, IR (KBr) 1770, 1690, 1665, 1525 $cm^{-1}$; NMR (acetone-$d_6$ + 1 drop of $D_2O$) includes $\delta$ 1.12 (dd, $J$ = 0.8 and 7.0 Hz, $CH_3$); $^{19}F$ NMR ($CDCl_3$) −76.47 (s, $COCF_3$), −82.25 [s, $C(OH)_2CF_3$] ppm. Anal. Calcd for $C_{21}H_{21}N_2O_4F_3 \cdot 1H_2O$: C, 57.27; H, 5.26; N, 6.36. Found: C, 57.42; H, 5.30; N, 6.35. (b) **1e''**, more polar isomer, IR (KBr) 1765, 1690, 1660, 1525 $cm^{-1}$; NMR (acetone-$d_6$ + 1 drop of $D_2O$) includes $\delta$ 1.23 (dd, $J$ = 0.8 and 7.0 Hz, $CH_3$); $^{19}F$ NMR ($CDCl_3$) −76.57 (s, $COCF_3$), −82.41 [s, $C(OH)_2CF_3$] ppm. Anal. Calcd for $C_{21}H_{21}N_2O_4F_3 \cdot 0.2H_2O$: C, 59.20; H, 5.06; N, 6.58. Found: C, 59.24; H, 5.22; N, 6.44.

*N-CBZ-*L*-Phenylalanylglycinal Dimethyl Acetal* (**4**). *N*-CBZ-Phenylalanine and aminoacetaldehyde dimethyl acetal were coupled by dicyclohexylcarbodiimide in THF for 3 h to give, after filtration and recrystallizations, the product as a white powder: mp 125–129 °C (EtOAc–hexane); $[\alpha]^{21}_D$ −7.5° (*c* 1.21, acetone); NMR ($CDCl_3$) includes $\delta$ 5.09 (s, $OCH_2Ph$), 4.18 [t, $J$ = 5.4 Hz, $CH(OCH_3)_2$], 3.29 and 3.28 (2s, 2 × $OCH_3$); IR (KBr) 1690, 1645 $cm^{-1}$. Anal. Calcd for $C_{21}H_{26}N_2O_5$: C, 65.27; H, 6.78; N, 7.25. Found: C, 65.19; H, 6.84; N, 7.22. Contrary to a published report (Mattis et al., 1977), we found the dilute acid hydrolysis (e.g., 1:1 acetone–0.1N HCl, room temperature) of **4** to be sluggish and to provide a mixture of products containing only minor amounts of the expected aldehyde.

*Equilibria Experiments*

1,1,1-Trifluoroacetone (Aldrich) in $D_2O$ (1.0 M) was examined by $^1H$ NMR (31 °C). The ketone was observed to be "completely" hydrated [$\delta$ 1.48 (q, $J_{HF}$ = 1.2 Hz)]. The signal for the free ketone [observed at $\delta$ 2.38 (q, $J$ = 0.9 Hz) in dioxane-$d_8$] was estimated to be <0.25% by comparison with the hydrate $^{13}C$ satellite signal at $\delta$ 2.28. This provides $K_{D_2O} \geq 7.2\ M^{-1}$ [=(.9975)/[(.0025)(55.27 *M*)]]. One equivalent of 3-mercaptopropionic acid (Aldrich) was then added, and the solution was maintained at 31 °C. NMR measurements at elapsed times ranging from 4 min to 24 h provided (by integration) values for the relative concentration of hemithioketal at $\delta$ 1.57 (q, $J$ = 0.9 Hz). Equilibrium was achieved within 3.5 h, giving [hemithioketal] ≈ 0.37 M and $K_{RSD}/K_{D_2O} \approx 47$. Calculation of the equilibrium rate constant (Laidler, 1965) for six time points as $k_{on}$ or $k_1 = x_e/[t(a_0^2 - x_e^2)]$ ln $[[x_e(a_0^2 - xx_e)]/[a_0^2(x_e - x)]]$, where $t$ is time, $x$ is [hemithioketal] at time $t$, $x_e$ is [hemithioketal] at equilibrium, and $a_0$ is initial [ketone + hydrate], gave $k_1 = (4.6 \pm 0.3) \times 10^{-4}\ M^{-1}\ s^{-1}$.

*Enzyme Assays*

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 $HgCl_2$. Cathepsin B activity was titrated with 2,2'-dipyridyl disulfide (Sigma) following the procedure of Brocklehurst and Little (1973). The assay buffer (0.1 M potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 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-(benzyloxycarbonylphenylalanyl-arginyl)-4-methylcoumarinamide (Peninsula Laboratories, San Carlos, CA) (fluorescence $\lambda_{ex}$ = 370 nm, $\lambda_{em}$ = 460 nm) or 7-(benzoylvalyllysyllysylarginyl)-4-(trifluoromethyl)-coumarinamide (Enzyme Systems Products, Livermore, CA) (fluorescence $\lambda_{ex}$ = 400 nm, $\lambda_{em}$ = 505 nm).

*Continuous Enzyme Assay.* Two milliliters of assay buffer was placed in a fluorometer cuvette, thermostated at 25 °C and kept under an argon atmosphere. Enzyme was added to a concentration of approximately 0.4 nM, and after 2–5 min of incubation, substrate (5 or 10 $\mu$L of 1 mM stock solution in DMSO) was added and the increase in fluorescence followed continuously. Once an uninhibited rate was established (1–3 min), inhibitor (0.5–17 $\mu$L of a stock solution) was added. Fluorescence monitoring was typically continued for an additional 10–40 min. In the case of the very slow inhibition by **1e''**, lower cathepsin B activity (ca. 0.04 nM) and 200 $\mu$M **1e''** (close to its solubility limit) were used, and the reaction was monitored continuously for 2 h. Inhibition constants ($K_i$) were determined from the initial inhibited rates vs the inhibitor concentrations by using the program VKKI. This program, a special case of Cleland's (1979) program COMP for competitive inhibition, fits data to $v = (V_{max}/K_m)[S]/(1 + [I]/K_i)$, where

Table I: Inhibition of Cathepsin B by Peptidyl Ketones and Aldehydes (25 °C, pH 6.0)

| compound | $K_i$[a] ($\mu$M) |
|---|---|
| **1b**, R = $CH_2F$ | 1.5 ± 0.16[b] |
| | 0.57 ± 0.09[c] |
| **1c**, R = H | 0.021 ± 0.0026 |
| **1d**, R = $CH_3$ | 31 ± 8.1 |
| **1e′**, R = $CF_3$ (less polar isomer) | 470 ± 99 |
| **1e″**, R = $CF_3$ (more polar isomer) | 300 ± 32 |
| **1f**, R = $CH_2Cl$ | 1.9 ± 0.6[d] |
| leupeptin (Ac-Leu-Leu-Arginal) | 0.0041 ± 0.00075[e] |
| **2**, CBZ-Phe-Ala-$NCH_3$-$OCH_3$ | 120 ± 33 |
| **3**, CBZ-Phe-$NHCH(CH_3)CHOH-CF_3$ | 1100 ± 180 |
| **4**, CBZ-Phe-$NHCH_2CH(OCH_3)_2$ | 1300 ± 300 |

[a] As determined from initial rate measurements, except for leupeptin. [b] Inactivation rate $k/K = (21 \pm 2.4) \times 10^3$ $M^{-1}$ $s^{-1}$. [c] Data from Rasnick (1985), 28 °C, pH 6.5, $k/K = 16 \times 10^3$ $M^{-1}$ $s^{-1}$. [d] Data from Rasnick (1985), 28 °C, pH 6.5, $k/K = 45 \times 10^3$ $M^{-1}$ $s^{-1}$. [e] $k_{on} = (1.9 \pm 0.17) \times 10^5$ $M^{-1}$ $s^{-1}$ (lit. $K_i = 5.0$ nM, $k_{on} = 1.8 \times 10^5$ $M^{-1}$ $s^{-1}$ for human cathepsin B; Baici & Gyger-Marazzi, 1982).

$V_{max}/K_m$ is a single parameter as justified by the very low substrate concentrations used (Copp et al., 1987). Compound **1c** was also assayed by using 25, 100, and 200 $\mu$M substrate, and a Lineweaver–Burk analysis (using COMP) showed the inhibition to be competitive. In cases where inhibition was time dependent (due to slow binding or time-dependent inactivation), the rate constants for the time dependence were obtained by nonlinear regression of the fluorescence vs time traces to the equation (fluorescence) = $Ae^{-(k_{obsd}t)} + B + Ct$. The second-order rate constant for the time dependence ($k/K$) was obtained by regression to $k_{obsd} = (k/K)[I]$ [or $k_{obsd} = k_{max}[I]/(K_i + [I])$ for **1b**, which provides the inactivation parameters $k_{max} = 0.064 \pm 0.030$ $s^{-1}$, $K_i = 3.0 \pm 1.7$ $\mu$M, and $k/K = (2.1 \pm 0.24) \times 10^4$ $M^{-1}$ $s^{-1}$].

*Incubation/Dilution Assays.* Cathepsin B (at 5-fold higher concentration than in the standard assay) was mixed with 20, 50, or 100 $\mu$M inhibitor **1e″** in 2 mL of assay buffer containing 0.5% DMSO, each in triplicate. At various incubation times, 50-$\mu$L aliquots were diluted into 2 mL of buffer (i.e., 1:40) containing substrate, and the reaction was monitored as described above. Remaining activity vs incubation time were fit to the equation (activity) = $Ae^{-(k_{obsd}t)} + B$. Linear regression to $k_{obsd} = (k/K)[I]$ gave the second-order rate constant $k/K$ (or $k_{on}$) ($M^{-1}$ $s^{-1}$), while final (steady-state) enzyme activity vs [I] fit by using the VKKI program gave $K_{i(final)}$. Due to the instability of cathepsin B in these lengthy incubations (up to 45 h) as judged by the controls, these assays only gave approximate values for $K_{i(final)}$ and $k/K$ (or $k_{on}$).

## Results and Discussion

Inhibition constants ($K_i$'s) were determined from initial rate measurements for the derivatives **1b–1e** and related compounds and are collected in Table I. Kinetic parameters for the competitive slow tight-binding inhibition by the aldehyde leupeptin (Baici & Gyger-Marazzi, 1982) were also reevaluated with bovine spleen cathepsin B. The inhibition of cathepsin B by the aldehyde **1c** was found to be both potent and competitive in nature, with a $K_i$ approaching that of leupeptin. This demonstrates the CBZ-Phe-Ala peptidyl group to be an effective cathepsin B active-site recognition element, as has been shown in the case of the irreversible inhibitors **1a**, **1b**, and **1f**. It is very likely that the inhibition by the related CBZ-Phe-Ala ketones **1d** and **1e** is also active-site-directed and therefore competitive. Quite intriguingly, while trifluoromethyl ketones are known to be ca. 10-fold more potent than the corresponding aldehydes in the inhibition of serine proteinases (Imperiali & Abeles, 1986), we found the inhibition of cathepsin B by **1e** in these measurements to be more than 4 orders of magnitude less effective than **1c**.

Scheme II: Competitive Equilibria: Hemithioacetal/Ketal Formation and Hydration

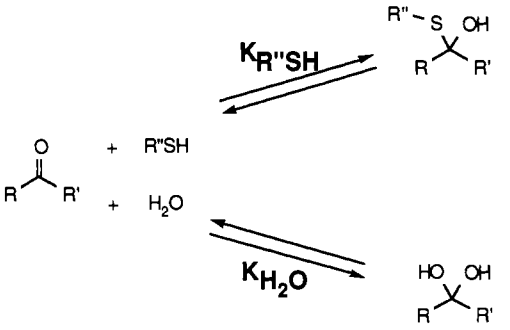



Table II: Hydration and Hemithioketal Formation for $CH_3CO$–R and HS–$CH_2CH_2COOH$ (34 °C, $D_2O$)

| R | $K_{D_2O}$[a] ($M^{-1}$) | $K_{RSD}$[a] ($M^{-1}$) | $K_{RSD,app}$[b] ($M^{-1}$) |
|---|---|---|---|
| H | $1.6 \times 10^{-2}$ | $5.4 \times 10^{1}$ | $2.9 \times 10^{1}$ |
| $CH_3$ | $2.3 \times 10^{-5}$ | $5.2 \times 10^{-3}$ | $5.2 \times 10^{-3}$ |
| $CH_2Cl$ | $7.2 \times 10^{-4}$[c] | $3.9 \times 10^{-2}$[c] | $3.8 \times 10^{-2}$ |
| $CH_2F$ | $2.0 \times 10^{-3}$ | $3.3 \times 10^{-1}$ | $3.0 \times 10^{-1}$ |
| $CF_3$ | ≥7[d] | ≥$3.3 \times 10^{2}$[d] | $8.5 \times 10^{-1}$ |

[a] Data from Burkey and Fahey (1983), except as noted otherwise. [b] Calculated as $K_{RSD,app} = K_{RSD}/(1 + 55.3K_{D_2O})$ (see text). [c] Calculated by using regression equations in Burkey and Fahey (1983) and $\sigma^*$ and $E_s$ from Hansch and Leo (1979). [d] This work (31 °C).

In order to interpret these results, we considered the equilibria given in Scheme II. For hemithioketal (or -acetal) formation in aqueous media, competitive hydration of a ketone (or aldehyde) results in the observation of an "apparent $K_{R"SH}$" defined as (Sander & Jencks, 1968)

$$K_{R"SH,app} = \frac{[\text{hemithioketal}]}{[\text{ketone} + \text{hydrate}][R"SH]} = \frac{K_{R"SH}}{1 + [H_2O]K_{H_2O}}$$

That is, in cases where hydration is minimal (<5% hydrate; $K_{H_2O} < 0.001$ $M^{-1}$), $K_{R"SH,app} \approx K_{R"SH}$; where hydration is substantial (>85% hydrate; $K_{H_2O} > 0.1$ $M^{-1}$), $K_{R"SH,app} \approx 0.02(K_{R"SH}/K_{H_2O})$. If the inhibition of the thiol proteinase cathepsin B by aldehydes and ketones conforms to this scheme (in which case R″SH represents the active-site cysteine thiol group), then it should be possible to correlate cathepsin B *inhibition constants* ($K_i$) with *equilibrium constants* ($K_{R"SH,app}$) in a model system.

Burkey and Fahey (1983) have recently investigated the hydration and hemithioacetal/ketal formation of several acetyl derivatives with 3-mercaptopropionic acid in $D_2O$; relevant data are given in Table II. In the case of 1,1,1-trifluoroacetone ($CH_3COCF_3$), no free ketone could be detected; however, a value for $K_{RSD}/K_{D_2O} = 31$ was obtained (Burkey & Fahey, 1983). We also examined the properties of $CH_3COCF_3$ ($^1H$ NMR, $D_2O$, 31 °C) and found that hydration was >99.75%, providing $K_{D_2O} \geq 7$ $M^{-1}$. However, it was very interesting to note that hemithioketal formation with 3-mercaptopropionic acid was quite *slow*, with $k_{on} = 4.6 \times 10^{-4}$ $M^{-1}$ $s^{-1}$, $K_{RSD}/K_{D_2O} \approx 47$ at equilibrium. Our observation of a slow rate of hemithioketal formation prompted suspicions that the competitive inhibition of cathepsin B by the peptidyl trifluoromethyl ketone **1e** might also be time dependent.

Reexamination of the inhibition of cathepsin B by the diastereomers of **1e** (19-h incubation, 100 $\mu$M inhibitor) indi-

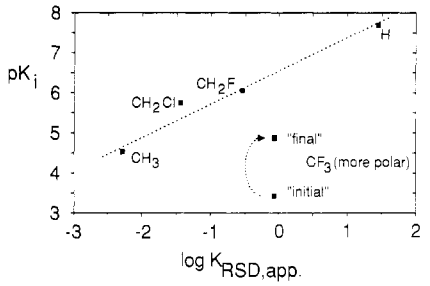



FIGURE 1: Correlation of cathepsin B inhibition ($pK_i$) by **1** (CBZ-Phe-Ala–R) with hemithioketal formation (log $K_{RSD,app}$) of $CH_3CO$–R and HS–$CH_2CH_2COOH$.

cated no time-dependent inhibition for the less polar isomer **1e′**; in sharp constrast, the experiment with **1e″** (more polar isomer) exhibited time dependence resulting in >90% inhibition (relative to controls). This phenomenon was sufficiently slow that, rather than representing slow reversible inhibition by **1e″**, it could conceivably be due to the presence of a small amount of a time-dependent irreversible inhibitor. However, incubation/dilution experiments with **1e″** demonstrated that both the rate of inhibition and the final (steady-state) enzyme activity were dependent on the inhibitor concentration. The observation that final enzyme activity was dependent on inhibitor concentration is not consistent with irreversible inhibition. Approximations for kinetic constants ($k_{on} \approx 9$ $M^{-1}$ $s^{-1}$, $K_{i(final)} \approx 8$ $\mu M$) were also obtained. A modified continuous assay (200 $\mu M$ inhibitor, 2 h) provided the values $k_{on} = 4.9$ $M^{-1}$ $s^{-1}$ and $K_{i(final)} = 24$ $\mu M$.

These observations provide support for characterization of the inhibition by **1e″** as slow-binding competitive reversible. On the basis of the significantly poorer activity of **1e′**, we tentatively assign the configuration L,L to **1e″** (more polar isomer) and L,D to **1e′** (less polar isomer).

The time-dependent inhibition of cathepsin B by **1e″** is remarkably slow; however, slow reversible inhibition is not unprecedented: competitive inhibition of cathepsin B by the aldehyde leupeptin occurs with $k_{on} \approx 2 \times 10^5$ $M^{-1}$ $s^{-1}$ (Table I), and inhibition of serine proteinases by certain peptidyl trifluoromethyl ketones (Imperiali & Abeles, 1986; Dunlap et al., 1987; Stein et al., 1987) occurs with $k_{on}$ ranging from $\sim 3 \times 10^2$ to $\sim 8 \times 10^4$ $M^{-1}$ $s^{-1}$. It may be possible that the initial inhibition by **1e″** [$K_{i(initial)}$], which is almost as poor as that of the alcohol precursor **3** and the glycinal dimethyl acetal **4** (Table I), represents noncovalent Michaelis binding. The time-dependent phase of the inhibition would likely be due to conformational changes and covalent addition of the active-site cysteine thiol to the carbonyl functionality.

We then examined the relation of the cathepsin B inhibition by peptidyl derivatives **1** (CBZ-Phe-Ala–R) with hemithioketal formation in the model system $CH_3CO$–R + HS-$CH_2CH_2C$-OOH (in $D_2O$), as proposed above. Linear regression of the data for R = H, $CH_3$, $CH_2F$, and $CH_2Cl$ provides the correlation $pK_i = 0.80$ (±0.10) log $K_{RSD,app}$ + 6.54 (±0.15), ($r^2$ = 0.97). Inclusion of the averaged $K_{i(final)}$ data for **1e″** (R = $CF_3$, more polar isomer) gives a weak correlation ($r^2$ = 0.64) of $pK_i = 0.70$ (±0.30) log $K_{RSD,app}$ + 6.2 (±0.42). Note that this correlation spans several orders of magnitude for both $K_i$ and $K_{RSD,app}$. Graphical representation of the relationship is given in Figure 1.

This correlation prompts us to conclude that competitive inhibition of cathepsin B by derivatives **1c** and **1d** and the initial inhibition (given by $K_i$) for the irreversible inhibitors **1b** and **1f** are due to formation of a hemithioacetal/ketal with the active-site cysteine thiol. McMurray and Dyckes (1986) have also concluded that the initial $K_i$ of peptidyl ketone inhibitors of trypsin, both reversible and irreversible, are a measure of reversible hemiketal formation. Although the $K_{i(final)}$ for **1e″** is higher than predicted from our correlation, it is reasonable to consider that inhibition by **1e″** also represents hemithioketal formation (vide infra). It is apparent that the hemithioacetal/ketal formation of simple acetyl derivatives with 3-mercaptopropionic acid provides a good model for the competitive inhibition of cathepsin B by peptidyl aldehydes and ketones.

It remains interesting that the trifluoromethyl ketone **1e″** is a significantly poorer inhibitor of cathepsin B than the corresponding aldehyde **1c**, in view of the superior inhibition of serine proteinases by trifluoromethyl ketones. In the case of serine proteinases, potent inhibition by trifluoromethyl ketones could *not* be predicted simply on the basis of hemiketal formation equilibrium data (Imperiali & Abeles, 1986; Stein et al., 1987). Particularly favorable interactions of the acidic hemiketal hydroxyl group with the oxyanion hole at the active site have been proposed as an explanation for the enhanced stability of the enzyme–inhibitor complex. For the thiol proteinase cathepsin B, our correlation correctly predicts that the inhibition by **1e″** should be significantly less potent than that of the analogous aldehyde **1c** (Figure 1), although the $K_i$ for **1e″** (at equilibrium) is higher than expected. Burkey and Fahey (1983) found that steric effects were important in the hemithioketal formation of a variety of ketones and that 1,1,1-trifluoroacetone exhibited considerable steric hindrance on the basis of its very low $K_{RSD}/K_{D_2O}$ ratio. We observed a somewhat slow *rate* of hemithioketal formation for $CH_3CO$-$CF_3$, and it follows that similar steric hindrance in **1e″** could result in a very slow rate of hemithioketal formation at the active site. This is consistent with the unusually slow time-dependent inhibition determined in our experiments. Significant deviation of the steady-state $K_i$ for **1e″** from that predicted by our correlation may indicate further steric (e.g., antagonistic effects from binding of the alanine side chain) or other (e.g., electrostatic) problems in the active-site hemithioketal that are not adequately represented in the model system. Indeed, experimental values of $K_{D_2O}$ and $K_{RSD}$ for $CH_3COCF_3$ are also *not* predictable from Burkey and Fahey's (1983) regression equations.

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**Registry No.** **1b**, 96922-64-4; **1c**, 115363-75-2; **1d**, 115363-76-3; **1e**, 115363-77-4; **1f**, 96922-72-4; **2**, 115363-74-1; **3**, 115363-78-5; **4**, 102579-45-3; $CH_3COH$, 75-07-0; $CH_3COCH_3$, 67-64-1; $CH_3COCH_2Cl$, 78-95-5; $CH_3COCH_2F$, 430-51-3; $CH_3COCF_3$, 421-50-1; $HSCH_2CH_2COOH$, 107-96-0; *N*-CBZ-L-Phe-L-Ala-OH, 21881-18-5; cathepsin B, 9047-22-7; nitroethane, 79-24-3; trifluoroacetaldehyde ethyl acetal, 31224-45-0; 3-nitro-1,1,1-trifluoro-2-butanol, 434-39-9; 3-amino-1,1,1-trifluoro-2-butanol hydrochloride, 115363-79-6; *N*-CBZ-L-phenylalanine, 1161-13-3; aminoacetaldehyde dimethyl acetal, 22483-09-6; *N,O*-dimethylhydroxylamine hydrochloride, 6638-79-5.

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