New Inhibitors of Cysteine Proteinases. Peptidyl Acyloxymethyl Ketones and the Quiescent Nucleofuge Strategy[1]

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Cathepsin B (EC 3.4.22.1)[3] is a clinically relevant cysteine proteinase that has been implicated in the pathogenesis of a number of diseases.[4] The most potent small molecule inhibitors of cysteine proteinases are the affinity labels with reactive leaving groups (Y),[4,5] many of which (i.e., Z-PheNHCHR(C=O)CH_2Y) have been developed by Shaw.[6] Recently, peptidyl fluoromethyl ketones have been introduced as inhibitors of cathepsin B and have proven to be affinity labels with low chemical reactivity.[7]

Conceptually, an ideal affinity label would be one in which the peptide moiety serves to transport a nucleofuge[8] on a carbon center that is uniquely reactive toward an active-site nucleophile of the target enzyme and quiescent in the presence of other bionucleophiles under physiological conditions. Hence, we sought to develop new inhibitors with difficultly displaceable leaving groups whose reactivity could be controlled by substituent effects and which might undergo rapid displacement in the enzyme inhibitor complex, by virtue of their proximity to a powerfully nucleophilic active site residue.

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Table I. Rates of Cathepsin B Inactivation by Peptidyl Acyloxymethyl Ketones (**1**) and Related Compounds

no.	compound	k/K^a (M^{-1} s^{-1})	pK_a^b
4	Z-Phe-Ser(OBn)-CH_2OCO-(2,6-$(CF_3)_2$)Ph	2 600 000[c]	<2 50[d]
5	Z-Phe-Ala-CH_2OCO-(2 6-$(CF_3)_2$)Ph	1 600 000	<2 50[d]
6	Z-Phe-Ala-CH_2OCO-(2 6-Cl_2)Ph	690 000	1 72
7	Z-Phe-Ala-CH_2OCO-(2 6-F_2)Ph	26 000	
8	Z-Phe-Ala-CH_2OCO-(3,5-$(CF_3)_2$)Ph	22 000	<3 00[d]
9	Z-Phe-Ala-CH_2OCO-(2 6-$(CH_3)_2$)Ph	3200[c]	3 31
10	Z-Phe-Ala-CH_2OCO-(2 4,6-$(CH_3)_3$)Ph	3000	3 45
11	Z-Phe-Gly-CH_2OCO-(2 4,6-$(CH_3)_3$)Ph	2700[c]	3 45
12	H-Phe-Ala-CH_2OCO-(2,4,6-$(CH_3)_3$)Ph·HCl	730[c]	3 45
13	Z-Phe-Ala-CH_2OCO-(4-NO_2)Ph	610[c]	3 43
14	Z-Phe-Ala-CH_2OCO-(2,6-$(CH_3O)_2$)Ph	300[c]	3 44
15	Z-Phe-Ala-CH_2OCO-Ph	90[e]	4 20
16	Z-Phe-Ala-CH_2OCO-(3 5-$(CH_3)_2$)Ph	80[e]	4 30
17	Z-Phe-Ala-CH_2OCO-(4-CH_3O)Ph	[f]	4 50
18	Z-Phe-Ala-CH_2OCO-$C(CH_3)_3$	330[c]	5 03
19	Z-Phe-Ala-CH_2OCO-CH_3	140[c]	4 76
20	Z-Phe-Gly-CH_2O-C_6F_5	180 000[c]	5 53
21	Z-Phe-Ala-CH_2O-C_6F_5	134 000	5 53
22	Z-Phe-CH_2OCO-(2,4,6-$(CH_3)_3$)Ph	[f]	3 45
23	Z-Phe-β-Ala-CH_2OCO-(2,4,6-$(CH_3)_3$)Ph	[f]	3 45
24	Z-Ala-Ala-Pro-Val-CH_2OCO-(2,6-$(CF_3)_2$)Ph	200[e]	<2 50[d]
25	Z-Phe-Ala-OCH_2CO-(2,4,6-$(CH_3)_3$)Ph	[f]	

[a] Bovine spleen cathepsin B[14] was assayed essentially as described by Rasnick[7a] (100 mM potassium phosphate, 1.25 mM EDTA, 1 mM dithiothreitol, pH 6.0, 25 °C, under argon). The rate constants for inactivation (k_{obsd}) at each inhibitor concentration were obtained by nonlinear regression: fluorescence = $Ae^{-(k_{obsd})t} + B$. The second-order rate constants (k/K) were obtained by regression to $k_{obsd} = k_{max}[I]/(K_i + [I])$, by using the program HYPER,[15] except as noted. Standard errors for $k/K \leq 15\%$. [b] pK_a of acyloxy or aryloxy group; values from ref 16, except as noted. [c] No saturation observed; k/K determined from linear regression: $k_{obsd} = (k/K)[I]$. Standard errors for $k/K \leq 15\%$. [d] Experimental limit (this work) determined by HPLC mobility versus pH. [e] Due to experimental limitations, k/K estimated by $k_{obsd}/[I]$ at a single $[I]$. [f] No time dependence observed.

We now report our observations on peptidyl acyloxymethyl ketones **1**, a class of compounds designed in accordance with the above principles[9] which have proven to be potent inactivators of cathepsin B. Peptidyl acyloxymethyl ketones were prepared by the KF-mediated condensation (DMF, 21 °C)[10] of the requisite carboxylic acid R′COOH and peptidyl bromomethyl ketone **2** (obtained via the corresponding diazomethyl ketone **3**).[11,12]

$$\underset{\mathbf{3}}{\text{RCOCHN}_2} \rightarrow \underset{\mathbf{2}}{\text{RCOCH}_2\text{Br}} \xrightarrow[\text{KF/DMF}]{\text{R'COOH}} \underset{\mathbf{1}}{\text{RCOCH}_2\text{OCOR'}}$$

To minimize the possibility of reaction at the acyloxy carbonyl group, sterically hindered 2,6-disubstituted benzoates were preferred as leaving groups,[13] and peptides bearing these functions are especially potent (Table I). For example, the $t_{1/2}$ for cathepsin B inactivation by 0.1 μM Z-Phe-Ala-CH_2OCO-(2,6-$(CF_3)_2$)Ph (**5**) is less than 5 s; this derivative is one of the most rapid cathepsin B inactivators yet reported. For the corresponding mesitoyloxy species **10** at 1 μM, $t_{1/2}$ is still less than 4 min. Inhibition by compounds **5** or **10** is irreversible, since exhaustive dialysis (24 h; 2 × 400 vol; 25 °C) does not restore activity to the enzyme. Leupeptin, a competitive inhibitor of cathepsin B, protects the enzyme from inactivation by **5** with a K_i of 5 nM (lit.[17] K_i 5 nM), providing evidence for the active-site directed nature of the inactivation.

The apparent second-order rate constant k/K (Table I) is critically dependent on both the nature of the peptide moiety and the carboxylate leaving group. Significant time-dependent inhibition of cathepsin B by acyloxymethyl ketones requires that the peptide component contain high affinity recognition elements for this enzyme (e.g., **4**, **5**, **10**, **11**; cf. **22**, **23**).[4] However, note that the acyloxymethyl ketone **25**, which is isomeric with **10**, does *not* exhibit time-dependent activity. Interestingly, a peptide (**24**) specifically designed to inhibit human leukocyte elastase (EC 3.4.21.11)[18] is a feeble inhibitor of this enzyme ($k/[I] \leq 15$ M^{-1} s^{-1}, pH 7.8, 25 °C) as well as cathepsin B, despite the fact that (2,6-$(CF_3)_2$)PhCOO$^-$ is the nucleofuge of choice for maximal potency in our series.

An important factor that influences inhibitory activity is the pK_a of the leaving group. It is apparent that a necessary, but not sufficient, condition for impressive cathepsin B inhibition by acyloxymethyl ketones is that the nucleofuge possess a $pK_a < 4$.

Convincing evidence that peptidyl acyloxy- and, as well, aryloxymethyl (see Table I) ketones are thiol-specific active-site-directed alkylating agents is derived from NMR experiments with papain (EC 3.4.22.2), which serves as a model for the closely homologous cathepsin B enzyme.[19] The covalent adducts obtained by inactivation of papain by Phe-Ala and Phe-Gly chloromethyl ketones have been established by X-ray crystallographic analyses[20] to be cysteine-25 thiomethyl ketones **26**. That our acyloxy- and aryloxymethyl ketone inhibitors give the same type of adduct (**26**)

(9) Competition experiments monitored by NMR spectroscopy establish a lower limit of 10:1 for the relative rates of displacement of X = F$^-$ versus (2,4,6-Me_3)PhCOO$^-$, respectively, for the reaction of PhS$^-$Na$^+$ with Ph(C=O)CH_2X in DMF, thereby characterizing mesitoate as a very weak nucleofuge.

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(12) (a) All new compounds gave appropriate spectroscopic and/or elemental analyses. Representative data, **5**: 167.5–168.5 °C; $[\alpha]^{21}_D$ −27.1° (*c* 0.954, acetone); IR (KBr) 1765, 1745, 1695, 1665 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$) δ 8.1–7.6 (m, 3 H, Ar*H*), 7.5–7.0 (m, 10 H, 2 × Ph), 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_2O), 4.9–4.2 (m, 2 H, 2 × NHC*H*CO), 3.0–2.8 (m, PhCH_2CH), 1.3 (d, *J* = 7.1 Hz, CH_3CH). Anal. C, H, N. **10**: mp 171–172 °C; $[\alpha]^{21}_D$ −35.0° (*c* 1.13, acetone); IR (KBr) 1735, 1720, 1685, 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.4–7.1 (m, 10 H, 2 × Ph), 6.9 (s, 2 H, Ar*H*), 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_2O), 4.8–4.2 (m, 2 H, 2 × NHC*H*CO), 3.2–3.0 (m, PhCH_2CH), 2.4 and 2.3 (2 s, $(CH_3)_3$Ar), 1.3 (d, *J* = 7.1 Hz, CH_3CH); Anal. C, H, N.

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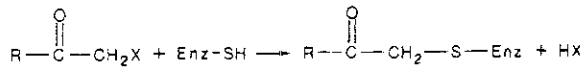

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can be ascertained by comparison of the ^{13}C NMR spectra of papain inactivated with 1 equiv of Z-Phe-Gly-CH_2X (X = Cl, $OCO(2,4,6\text{-}Me_3)Ph$, or OC_6F_5), labeled as $-^{13}C(=O)-CH_2X$ and as $-C(=O)-^{13}CH_2X$. The resonances of the inactive enzyme adduct (C=O, 214.7 ppm; CH_2, 38.1 ppm) are identical for all three inactivators Z-Phe-Gly-CH_2X and are entirely consistent with an active-site bound thiomethyl ketone structure (**26**).

In summary, peptidyl acyloxymethyl ketones can be designed to be potent and specific cysteine proteinase inhibitors that are active-site-directed and irreversible in their action. The aryl carboxylate leaving group offers considerable variation as a design element and with the appropriate peptide component, affinity labels possessing exquisite specificity can, in principle, be constructed. This type of reagent bearing a quiescent nucleofuge lends scope to the affinity label concept and holds forth the prospect of a practical clinical endpoint.

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Supplementary Material Available: Physical data for all compounds and synthetic information for **20** and **21** (1 page). Ordering information is given on any current masthead page.

Evidence for Activation of the C–O Bond of Methanol on the Pd{111} Surface after Low-Temperature Adsorption

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Direct evidence, using secondary ion mass spectroscopy (SIMS) supported by X-ray photoelectron spectroscopy (XPS), has been obtained for the activation of the C–O bond of methanol after adsorption at 110 K on the Pd{111} surface. In our experiments we find that the C–O bond dissociates after heating to 175 K leaving CH_{3ads} (methyl) and H_2O_{ads}, as evidenced by prominent SIMS signals at m/e 15 and 18, respectively. Methoxide (CH_3O_{ads}) is produced simultaneously and has been identified by its carbon 1s photoelectron binding energy. In addition to reporting the first observation of methanolic C–O bond activation on a well-defined transition-metal surface[1] we also report the discovery of a stable methyl species on Pd{111} as the surface is heated from 175 to 400 K. This observation has mechanistic implications for the uniquely selective formation of methanol from CO and H_2 on Pd catalysts.[2] Previously, only CH_3O_{ads} has been found on single-crystal transition-metal surfaces[3-6] exposed to CH_3OH at low temperatures and subsequently heated between 140 and 200 K. Additionally, the formation of CH_3O_{ads} at low temperatures was found to be enhanced by predosing the transition-metal surface with O_2 at 300 K.[7-9]

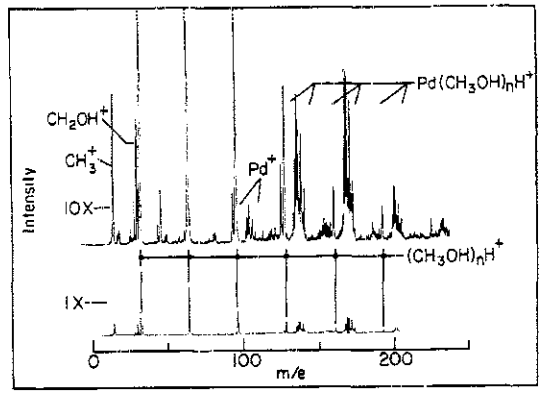

Figure 1. The SIMS spectrum for a 5 L methanol exposure to Pd{111} at 110 K. The primary ion current is 0.4 nA/cm^2.

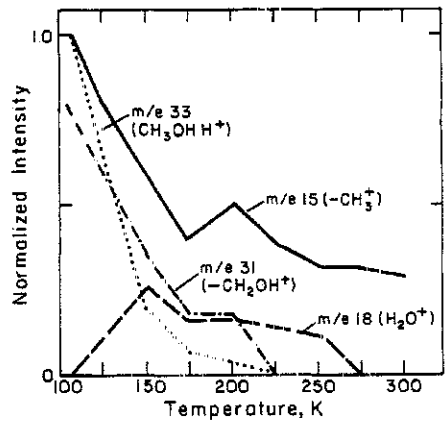

Figure 2. Secondary ion intensity versus surface temperature for a 2 L methanol exposure to Pd{111} initially at 110 K. The primary ion current is 0.4 nA/cm^2.

The apparatus used for these experiments has been described in detail previously.[10] The Pd{111} surface was rigorously cleaned by cycles of argon ion sputtering (2 μA cm^{-2}) for 5 min followed by annealing to 1200 K prior to each experiment. After this treatment the surface then proved to be free of impurities as determined by SIMS and XPS. The methanol was distilled and subjected to several freeze–pump–thaw cycles before use. The exposures listed throughout this letter are in units of Langmuirs (1 L = 1 × 10^{-6} Torr·s) and are reported as corrected ion gauge readings.

The SIMS spectrum for 5 L CH_3OH on Pd{111} at 110 K is shown in Figure 1. The prominent peaks, marked in the figure, are assigned to CH_3^+ at m/e 15, CH_2OH^+ at m/e 31,[11] and CH_3OHH^+ at m/e 33 as well as to additional hydrogenated n-mers observed at higher mass. In Figure 2, the intensities of

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