Vinyl Sulfones as Mechanism-Based Cysteine Protease Inhibitors

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Proteases regulate a broad spectrum of physiological functions by the specific processing of proteins and peptides. Elevated levels of active proteases can result in an array of physiological processes ultimately leading to disease states. Therefore, compounds designed to restore the natural equilibrium of proteases present excellent opportunities for drug candidates.

Cysteine proteases, for example cathepsins B, L, and S, have been implicated in a number of diseases, including progressive cartilage and bone degradation associated with arthritis.[1] Inhibitors of these cathepsins have reduced inflammation and prevented joint destruction in animal models of arthritis.[2] Recently, human cathepsin O2 has been found to be highly expressed in osteoclastoma tissue.[3] Cathepsins B and L have been linked to metastasis and invasion by cancer cells.[4] The calcium-associated cysteine proteases calpains I and II have been associated with ischemia and hypoxia,[5] Alzheimer's disease,[6] and cataracts.[7] Inhibition of the trypanosomal cysteine protease cruzain has proven effective in models of Chagas's disease.[8]

Reversible inhibitors of cysteine proteases include peptide aldehydes,[9] nitriles,[10] and α-ketocarbonyl compounds.[11] Irreversible inhibitors include peptide halomethyl ketones,[12] diazomethyl ketones,[13] (acyloxy)-methyl ketones,[14] and ketomethylsulfonium salts,[15] believed to alkylate the active site thiol by formal S_N2 displacement, either directly or through a proposed hemithioketal–episulfonium ion pathway. Other irreversible inhibitors include various epoxysuccinyl compounds,[16] whose oxiranes are opened through nucleophilic attack by the thiol.

Previous work by Hanzlik and co-workers[17] demonstrated peptide Michael acceptors as inactivators of the plant protease papain, which displayed second-order rate constants of inactivation from 0.05 to 70 M^{-1} s^{-1}. However, to serve as disease modifying agents, inhibitors must inactivate target enzymes sufficiently *in vivo* as well as *in vitro*. Therefore, we designed potent inhibitors against *disease-associated* cysteine proteases such as the cathepsins B, L, S, and O2, calpains, and cruzain, concentrating on structures or scaffolds suitable for pharmaceutical development.

Molecular Design and Synthesis. Our approach required the catalytic mechanism of the targets to be vital for their inhibition. We designed inhibitors to be unreactive toward serine proteases, metalloproteases, and aspartyl proteases, nonactive site cysteines, and circulating thiols such as glutathione. We exploited a polarized yet inert double bond bearing functionality suitable for hydrogen bonding interactions with the active site of the target. The vinyl sulfone moiety (Figure 1) provided us with the necessary characteristics. Substituted vinyl sulfones are less reactive toward nucleophiles than the analogous vinyl ketones or esters[18] and thus are sufficiently inert without the target. They also are capable of hydrogen bonding with the active site; at lower pH values (4.5–6.5) where cysteine proteases are most active, the active site His residue is protonated. Molecular modeling studies using the Discover program (Biosym) suggest the proposed interactions are valid.

Known substrate selectivity for cathepsins B, L, and S, calpains, and cruzain afforded us templates for our first-generation compounds.[19] Although we recognized the potential loss of hydrogen bonding between active site elements and the carbonyl normally associated with protease substrates and enzyme–inhibitor transition states, we knew that the absence of a carbonyl group on papain inhibitors did not preclude time-dependent inactivation. Thus we constructed peptidomimetic structures containing "functional scaffolds" enabling both inhibitor–protease binding and mechanistic inactivation.

We required a versatile, stereospecific, and scaleable synthesis scheme with minimal purification steps. Chiral amino acid aldehydes, as reported by Fehrentz and Castro,[20] afforded us suitable building blocks. Reduction of *N,O*-dimethylhydroxamates **2** derived from commercially available α-amino acids **1** gave the requisite aldehydes **3** in excellent yields (Scheme 1). Wadsworth–Emmons[21] chemistry provided the vinyl sulfones **5** in good to excellent yields, with little or no chromatography needed. Removal of the BOC groups followed. Mixed anhydride coupling of the amine salts **6** with N-protected amino acids or peptide derivatives **7** gave the target peptidomimetic vinyl sulfones **8**. NMR spectroscopy confirmed the absence of possible aldehyde contamination in the vinyl sulfones, eliminating possible interference from aldehydes in the inhibition assays.

For our first series of enzyme kinetics, we assayed several compounds in time-based inactivation assays against cathepsins B, L, S, and O2, cruzain, and calpains I and II. The most potent inhibitors against cathepsin S displayed second-order rate constants approaching the diffusion limit.

Using this family of inhibitors, it became apparent that cathepsin S is the most readily inactivated enzyme, followed by cathepsin L and cathepsin O2, and then cathepsin B. Cruzain is homologous to cathepsin L;[23] we used the X-ray structure of cruzain to model inhibitors for cathepsin L. As anticipated, both enzymes show similar susceptibility to the inhibitors.

The calpains are decidedly resistant to most inhibitors in this series. Nevertheless, the incorporation of known binding determinants for calpain (Leu-Leu-Tyr or Leu-Leu-Met) into a series of vinyl sulfones resulted in significant inactivation (entries **8i–m**.)

Scope and Limitations. As stated earlier, the potential utility of this series of compounds depends on a number of factors, including (a) selectivity for cysteine proteases, (b) stability of target enzyme inactivation, and (c) their chemical stability, including that toward circulating thiols such as glutathione (GSH). To test this series of requirements, we performed the following experiments.

MeOSuc-Ala-Ala-Pro-ValVSPh, a vinyl sulfone derivative designed with specificity for human leukocyte elastase, was incubated with HLE in the presence of

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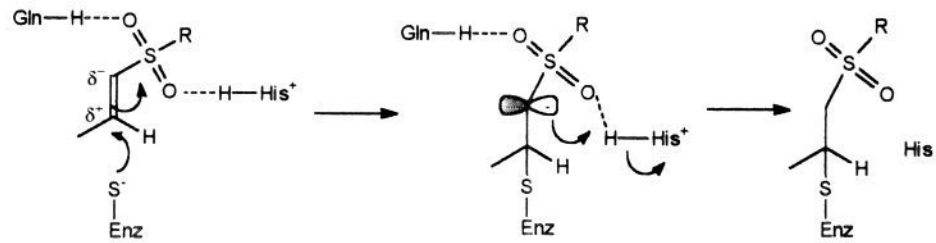
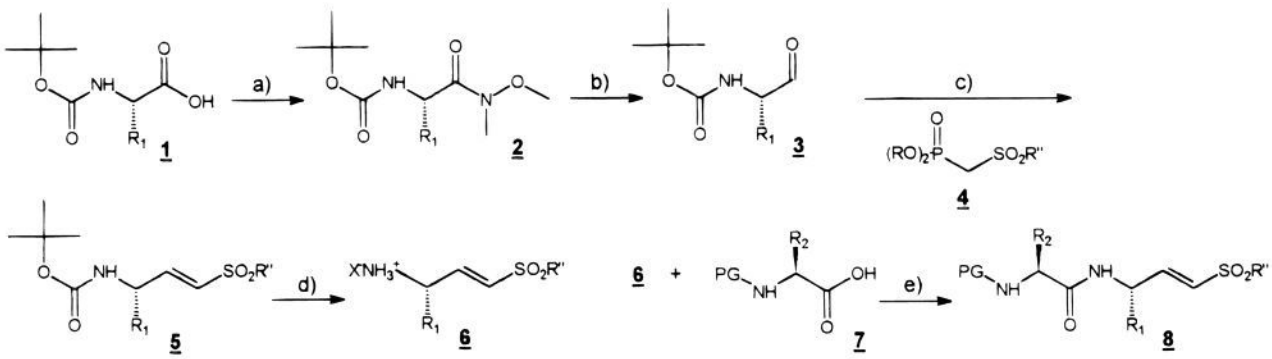


Figure 1.

Scheme 1[a]



[a] R_1, R_2 = amino acid side chain; R″ = alkyl or aryl; X = Cl or p-$CH_3C_6H_4SO_3^-$; PG = N-terminus protecting group. Reaction conditions: (a) HCl·HN(CH$_3$)OCH$_3$, DCC, Et$_3$N, CH_2Cl_2, 0−25 °C; (b) $LiAlH_4$, THF, 0 °C; (c) NaH, THF, 0−25 °C; (d) HCl/dioxane or p-TsOH/ether; (e) 4-methylmorpholine, isobutyl chloroformate, THF, −10 °C. **8a**−**m: 8a** = Mu-Phe-HphVSMe; **8b** = Mu-Phe-HphVSPh;[22] **8c** = Mu-Tyr(3,5-I$_2$)-HphVSPh; **8d** = Mu-Phe-LysVSPh.HBr; **8e** = Mu-Leu-HphVSPh; **8f** = Mu-Np2-HphVS-2Np; **8g** = Mu-Phe-HphVSEtPh; **8h** = Mu-2Np-HphVSEtPh; **8i** = Ac-Leu-Leu-MetVSPh; **8j** = Ac-Leu-Leu-NleVSPh; **8k** = Mu-Leu-Leu-TyrVSPh; **8l** = Ac-Leu-Leu-Met(O$_2$)VSPh; **8m** = Z-Leu-Leu-TyrVSPh. Abbreviations: Mu = morpholine urea, Z = benzyloxycarbonyl, Ac = acetyl. Where an amino acid abbreviation is used in reference to an R_2 or R_1 residue, it signifies the side chain portion of that amino acid. For example, "Hph" at R_1 = homophenylalanine side chain, $CH_2CH_2C_6H_5$; Mu-Phe at R_2 = morpholine urea of phenylalanine. Others: Np2 = 2-naphthylalanine, Tyr(3,5-I$_2$) = 3,5-diiodotyrosine, Nle = norleucine, Met(O$_2$) = methionine sulfone, VSMe = (vinylsulfonyl)methane, VSPh = (vinylsulfonyl)benzene, VS-2Np = (vinylsulfonyl)-2-naphthalene, VSEtPh = [(vinylsulfonyl)ethyl]benzene.

Table 1. Vinyl Sulfone Inhibition Kinetics (k_{obs}/[I], M^{-1} s^{-1})

entry	cathepsin B	cathepsin L	cathepsin S	cathepsin O2	cruzain	calpain I,* calpain II
8a	5 100	8 700	1 200 000	6 540	22 000	
8d	11 300	220 000	10 700 000	83 300	149 000	0
8g	29 000	340 000	4 700 000	28 500	180 000	
8h	33 000	2 240 000	29 000 000		450 000	
8i	0	880 000		25 000	110 000	5 600
8j	0	930 000			133 000	6 900*
8k		28 700	25 100	815		7 600* 10 800**
8l	0	1 500 000		22 700	104 000	8 400*
8m	730		280 000	900	45 000	24 300* 6 400**

100 mM Tris (pH 7.5). This compound neither inhibited nor bound to the enzyme, suggesting that the vinyl sulfone series was indeed specific for cysteine proteases over serine proteases.

To explore the irreversibility of cysteine protease inactivation by this series, we performed the following. Cathepsin B was completely inactivated with Mu-Phe-HphVSPh under optimal conditions (50 mM phosphate, pH 6.0, 2.5 mM EDTA, 2.5 mM dithiothreitol (DTT). The inactivated enzyme was passed twice through a G-25 column and eluted with assay buffer to remove excess inhibitor. There was no recovery of enzymatic activity against the substrate when periodically assayed over the next 4 days. Control cathepsin B (treated similarly but without inhibitor) retained 37% of its activity during this period.

An examination of chemical stability of the various derivatives demanded a more rigorous kinetic analysis to compare the components of the second-order rate equation. Progress curves were computer-fit to a first-order equation to produce k_{obs} as previously described.[24] Kinetic constants for compounds listed in Table 1 are reported as k_{obs}/[I], whereby the k_{obs} values were determined from at least six inhibitor concentrations near K_i (for example, against cathepsin S, the concentrations of compound **8b** ranged from 1 to 20 nM of inhibitor, those of compound **8f** ranged from 0.2 to 2 nM, and those of compound **8e** ranged from 1 to 10 nM) and fitted to the equation

$$k_{obs} = \frac{k_{irr}[I]}{K_{i\ app} + [I]}$$

where $K_{i\ app}$ is the apparent K_i value, k_{irr} is the calculated first-order inactivation constant, and [I] is the inhibitor concentration. Since the inhibition was measured in the presence of substrate, the true K_i was

Table 2. Detailed Inhibition Kinetics

compound	k_{irr}[a] or k_{inact} (s^{-1})	K_i (μM)	(k_{irr}[a] or k_{inact})/K_i (M^{-1} s^{-1})
	Cathepsin B		
Mu-Phe-HphVSPh (**8b**)	0.17 ± 0.04	11 ± 3.5	16 400 ± 1300
Mu-Tyr(3,5-I_2)-HphVSPh (**8c**)	0.36 ± 0.01	0.34 ± 0.02	1 080 000 ± 40 000
Mu-Leu-HphVSPh (**8e**)	0.17 ± 0.06	39 ± 13	4 250 ± 71
Mu-Np2-HphVS-2Np (**8f**)	0.061 ± 0.006	0.15 ± 0.02	420 000 ± 20 000
	Cathepsin L		
Mu-Phe-HphVSPh (**8b**)	0.24 ± 0.08	1.0 ± 0.3	224 000 ± 30 000
Mu-Leu-HphVSPh (**8e**)	0.28 ± 0.15	0.72 ± 0.40	387 000 ± 4 000
Mu-Np2-HphVS-2Np (**8f**)	0.14 ± 0.03	0.018 ± 0.006	9 200 000 ± 500 000
	Cathepsin S		
Mu-Phe-HphVSPh (**8b**)	0.085 ± 0.021	0.011 ± 0.003	7 700 000 ± 200 000
Mu-Leu-HphVSPh (**8e**)	0.16 ± 0.01	0.0059 ± 0.0004	26 300 000 ± 500 000
Mu-Np2-HphVS-2Np (**8f**)	0.10 ± 0.02	0.0018 ± 0.0004	56 000 000 ± 8 000 000
	Cathepsin O2		
Mu-Phe-HphVSPh (**8b**)	0.31 ± 0.07	30.5 ± 9.7	10 164
Mu-Leu-HphVSPh (**8e**)	0.17 ± 0.05	0.23 ± 0.10	772 700
Mu-Np2-HphVS-2Np (**8f**)			<300 (k_{obs}/[I])
	Cruzain		
Mu-Phe-HphVSPh (**8b**)	0.072 ± 0.028	0.36 ± 0.018	203 000 ± 23 000
Mu-Leu-HphVSPh (**8e**)	0.13 ± 0.07	0.22 ± 0.13	620 000 ± 40 000
Mu-Np2-HphVS-2Np (**8f**)	0.046 ± 0.003	0.048 ± 0.01	1 040 000 ± 10 000

[a] k_{irr} for cathepsin B only.

determined by the relationship

$$K_i = \frac{K_{i\ app}}{1 + [S]/K_m}$$

The second-order rate constant k_{irr}/K_i was independently determined from the slope of the plot of $1/k_{obs}$ vs 1/[I] and compared to that computed from the ratio of k_{irr} and K_i.[25] Results comparing inhibition of the target enzymes by several vinyl sulfones are depicted in Table 2. The sulfonyl moiety clearly plays a significant role in binding. Enhanced hydrophobicity in progressing from the phenyl to the 2-naphthyl series (**8b**, **8f**) may explain these results.

Variability between compounds for a particular enzyme is clearly controlled by the degree of binding to the enzyme. Inactivation of the cysteine proteases by the inhibitors was performed in the presence of substrate, which slowed down the inactivation sufficiently to allow reliable measurement of rates. For example, while the first order rate constant k_{irr} varies only slightly across the entire series of enzymes, the K_i values range from mid-micromolar to low nanomolar. This suggests the electrophilicity of the vinyl sulfone moiety is of less importance to the enzyme's inactivation than are the vital hydrophobic interactions.

To test this idea further, we measured the stability of selected inhibitors toward glutathione (GSH) using the method described by Shaw and co-workers.[26] After incubation with GSH (2.5 mM) in the cathepsin S assay buffer at pH 6.2, Mu-Phe-HphVSPh (**8b**, 10 μM) lost no inhibitory potency after 22 h incubation. Similarly, the second-order rate constant for the loss of GSH was 5.5 $\times$ 10^{-4} M^{-1} s^{-1} for Mu-Phe-LysVSPh·HBr (**8d**). Given the second-order inhibition constants for these and similar compounds against the target enzymes are at least 10^7 times greater, one may reasonably assume that peptide-based vinyl sulfones are effectively inert in the absence of the enzymes' catalytic machinery. As such, they represent a novel class of therapeutic candidates that may be employed against those diseases manifested by the upregulation of these proteases. At the same time, their specificity and stability may alleviate many of the traditional concerns associated with potentially toxic, mutagenic, non-enzyme-selective, or highly reactive substituted methyl ketones and epoxides previously reported.

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(22) Mp: 143−144 °C. TLC (50% ethyl acetate/CH_2Cl_2): R_f = 0.45. ^{1}H NMR ($CDCl_3$): δ 1.68−1.93 (2H, m, C**H**$_2$CH$_2$C$_6$H$_5$), 2.58 (2H, CH$_2$C**H**$_2$C$_6$H$_5$), 3.03 (2H, d, J = 7 Hz, PhC**H**$_2$CH), 3.27 (4H, m, 2 × NC**H**$_2$CH$_2$O), 3.62 (4H, m, 2 × NCH$_2$C**H**$_2$O), 4.44 (1H, q, J = 7 Hz, C**H**NH (Phe)), 4.62 (1H, m, C**H**NH (Hph)), 4.93 (1H, d, J = 7 Hz, N**H**CH (urea)), 6.08 (1H, dd, J = 2, 15 Hz, trans SO$_2$C**H**=CH), 6.23 (1H, d, J = 7 Hz, N**H**CH (amide)), 6.77 (1H, dd, J = 5, 15 Hz, trans C**H**=CHSO$_2$), 7.07−7.87 (15H, m, aromatic). MS (fast-atom bombardment, high resolution): calculated for $C_{31}H_{35}N_3O_5S$, (m + H) 562.2376, found 562.2362. Anal. Calcd for $C_{31}H_{34}N_3N_3O_5S$: C, 66.29%; H, 6.10; N, 7.48; S, 5.70. Found: C, 66.03; H, 6.45; N, 7.45; S, 5.83.

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