Synthesis and Evaluation of Peptidyl Michael Acceptors That Inactivate Human Rhinovirus 3C Protease and Inhibit Virus Replication

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Received February 20, 1998

Human rhinovirus, the chief cause of the common cold, contains a positive-sense strand of RNA which is translated into a large polyprotein in infected cells. Cleavage of the latter to produce the mature viral proteins required for replication is catalyzed in large part by a virally encoded cysteine proteinase ($3C^{pro}$) which is highly selective for −Q∼GP− cleavage sites. We synthesized peptidyl derivatives of *vinylogous* glutamine or methionine sulfone esters (e.g., Boc-Val-Leu-Phe-vGln-OR: R = Me, **1**; R = Et, **2**) and evaluated them as inhibitors of HRV-14 3C protease ($3C^{pro}$). Compounds **1** and **2** and several related tetra- and pentapeptide analogues rapidly inactivated $3C^{pro}$ with submicromolar IC_{50} values. Electrospray mass spectrometry confirmed the expected 1:1 stoichiometry of $3C^{pro}$ inactivation by **1**, **2**, and several other analogues. Compound **2** also proved to be useful for active site titration of $3C^{pro}$, which has not been possible heretofore because of the lack of a suitable reagent. In contrast to **1**, **2**, and congeners, peptidyl Michael acceptors lacking a P_4 residue have greatly reduced or negligible activity against $3C^{pro}$, consistent with previously established structure−activity relationships for $3C^{pro}$ substrates. Hydrolysis of the P_1 vinylogous glutamine ester to a carboxylic acid also decreased inhibitory activity considerably, consistent with the decreased reactivity of acrylic acids vs acrylic esters as Michael acceptors. Incorporating a *vinylogous* methionine sulfone ester in place of the corresponding glutamine derivative in **1** also reduced activity substantially. Compounds **1** and **2** and several of their analogues inhibited HRV replication in cell culture by 50% at low micromolar concentrations while showing little or no evidence of cytotoxicity at 10-fold higher concentrations. Peptidyl Michael acceptors and their analogues may prove useful as therapeutic agents for pathologies involving cysteine proteinase enzymes.

Introduction

Many plant and animal viruses depend on virus-encoded proteinases for cleaving virus-specified polypeptides to generate proteins related to viral replication.[1] One of the largest families of human pathogenic RNA viruses is the *Picornaviridae*, which includes poliovirus, hepatitis A virus, rhinovirus, and others. Rhinovirus, the chief cause of the common cold, occurs in over 100 different serotypes; this makes immunization impractical as an approach to prevention, and it invites small-molecule chemotherapeutic approaches to treatment. Like other picornaviruses, rhinoviruses contain a positive-sense strand of RNA, translation of which in infected cells yields a large polyprotein. This polyprotein is cleaved co- and posttranslationally by virally encoded proteinases to produce mature viral enzymes and structural proteins. These cleavages are catalyzed in an organized cascade involving the viral cysteine proteinases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$. These enzymes are highly substrate-selective, highly specific in their choice of cleavage sites, and absent from normal (noninfected) host cells.[2,3] Since mutagenesis of their active site residues renders them incapable of processing polyprotein in vitro,[4] their inhibition by small-molecule inhibitors should stop viral replication in vivo. Thus these proteinases are attractive targets for development of new approaches to antiviral chemotherapy.

The 2A and 3C proteinases are susceptible to inhibition in vitro by a number of nonspecific thiol-proteinase inhibitors as well as by more specific agents such as elastatinal, leupeptin, and methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone.[5,6] The latter also reduces the yield of poliovirus type 1, coxsackievirus A21, and human rhinovirus 2 in infected HeLa cells.[5] Similarly, the peptidyl bromomethyl ketone (Cbz-Phe-azaglutamine-CH_2Br) is a moderately potent time-dependent inactivator of HRV $3C^{pro}$ (k_2/K_i > 2500 M^{-1} s^{-1}).[7] Interestingly the epoxysuccinyl derivative E-64, which is a potent active site-directed irreversible inhibitor and active site titrant toward cysteine proteinases of the papain family, does not inhibit 3C proteinases from HRV-14[6] or poliovirus.[8,9] Small molecules containing reactive carbonyl groups can be potent inhibitors of cysteine proteinases via reversible formation of covalent hemithioacetal or -ketal adducts with the active site thiol (cf. elastatinal and leupeptin above).[10−12] Peptide aldehydes tailored to resemble substrates for 2A and 3C proteinases have also provided quite potent in vitro inhibition[13−15] and have inspired the development of nonpeptidic carbonyl compounds including β-lactams,[16] isatins,[17] and homophthalimides[18] as HRV $3C^{pro}$ inhibi-

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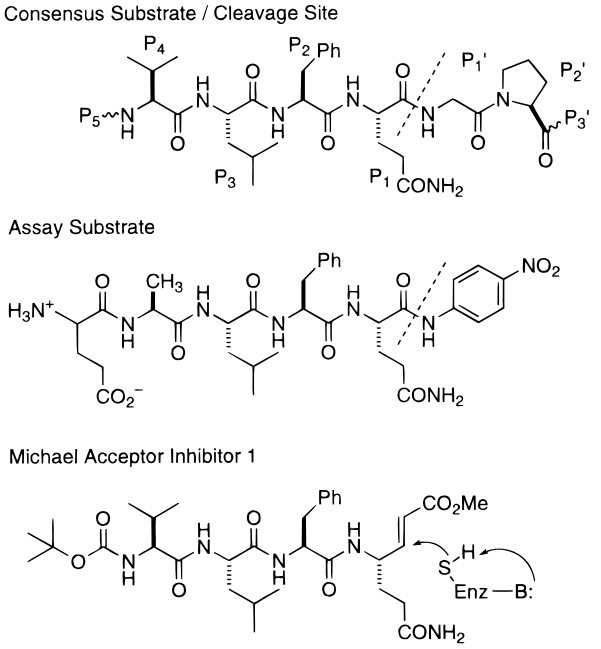
S0022-2623(98)00114-9 CCC: $15.00
Published on Web 06/16/1998

Scheme 1



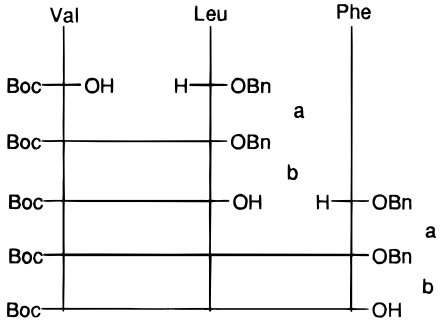
tors. Thus far, however, only the isatins have provided potent in vitro inhibitors, and results in cell culture have been disappointing.

Because peptidyl Michael acceptors based on *vinylogous* amino acids[19] inactivate cysteine proteinases selectively and specifically,[20−22] we became interested in exploring this approach to inhibition of the HRV 3C^{pro}. In this paper we report our results on the design, synthesis, and evaluation of peptidyl Michael acceptors as inhibitors of HRV 3C^{pro} and as inhibitors of viral replication in cell culture in vitro. We show that with appropriate peptidyl carrier moieties, *vinylogous* glutamine esters are extremely potent inactivators of HRV 3C^{pro} in vitro; in fact they can actually be used as active site titrants for this enzyme, for which no other titrant has been described. In addition, some of the compounds are also highly effective at inhibiting viral replication in cell culture while showing very low cytotoxicity.

Inhibitor Design

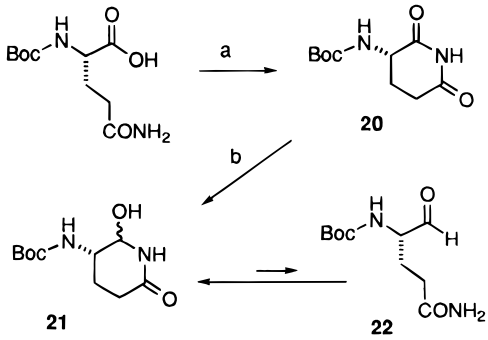
The 3C proteinase of HRV-14 has been cloned, expressed, and purified in a number of laboratories (see Birch et al.[23] and references therein). Its substrate requirements have been explored using synthetic peptides related to viral polyprotein cleavage sites and a consensus substrate has been defined.[6,24−26] A crystal structure of HRV-14 3C^{pro} and a hypothetical substrate docking model[27] rationalize the enzyme's observed kinetic specificity and highlight the following main points. The minimum consensus substrate which is efficiently cleaved is P_5-P_4-P_3-P_2-Q∼G-P, where Q∼G represents the scissile (P_1−P_1') bond (Scheme 1). Positions P_5 and P_3 are quite tolerant to substitution, and the docking model suggests these side chains project out into solution. On the other hand, kinetic studies indicate that P_4 should be small and nonpolar while P_2 may be large and hydrophobic, and the crystal structure of the enzyme reveals the existence of pockets complementary to such side chains. The stringent requirement

Scheme 2. Synthesis of Boc-VLF-OH[a]



[a] (a) DCC, NMM, NHS, CH_2Cl_2; (b) H_2, Pd/C, EtOH.

Scheme 3[a]



[a] (a) DCC, NHS, THF, DMF; (b) $NaBH_4$, 90% EtOH, HCl.

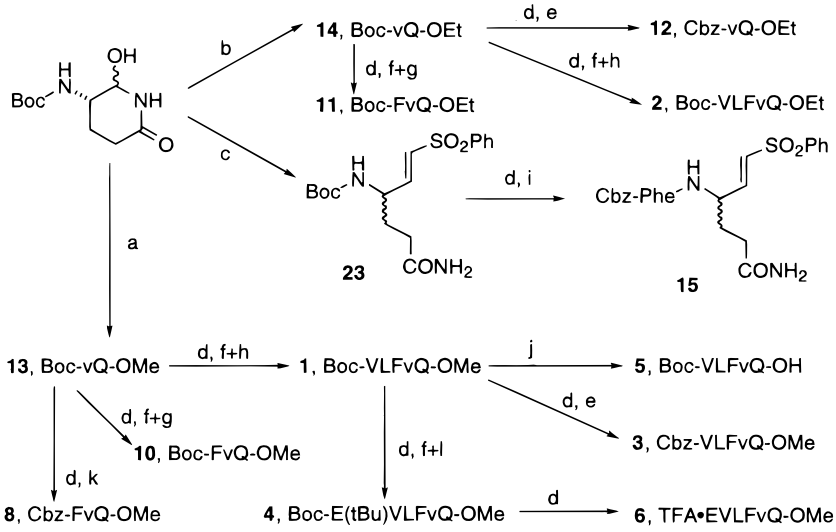
for P_1 glutamine is rationalized on the basis that its γ-carbonyl oxygen accepts two important hydrogen bonds from T141 and H160 of the enzyme. Thus asparagine, aspartate, and glutamate are poor substitutes for glutamine, although a dipeptide aldehyde with methionine sulfone at the P_1 position is a good competitive inhibitor or HRV-14 3C^{pro} (K_i = 0.47 μM),[15] and in the case of the related hepatitis A 3C^{pro}, *N*,*N*-dimethylglutamine derivatives are accepted.[13] The above structural requirements for substrates were translated into the design of the peptidyl Michael acceptors shown in Table 1.

Synthesis

The peptide Boc-V-L-F-OH, a common component of many of the inhibitors in Table 1, was synthesized using standard peptide-coupling chemistry as indicated in Scheme 2. The vinylogous glutamine derivatives were synthesized from Boc-L-glutamine by a sequence of cyclization to Boc-aminoglutarimide and reduction to Boc-glutaminal, which exists primarily as a cyclic hemiaminal (Scheme 3). The latter was converted to the vinylogous glutamine esters by Horner−Emmons chemistry[20−22] and on to the various peptidyl Michael acceptors as shown in Scheme 4. A similar approach starting with Boc-L-methionine was used for the synthesis of the vinylogous methionine sulfone derivatives **7** and **9** (Scheme 5).

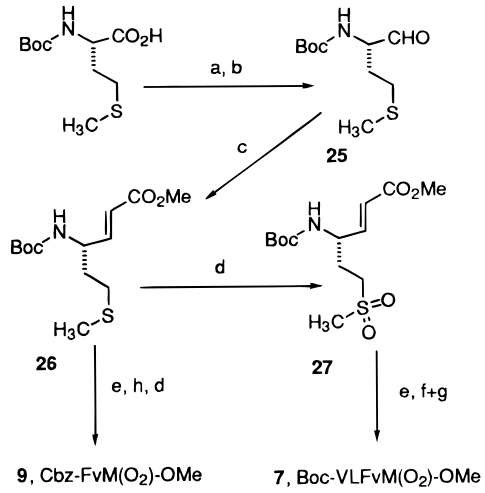
Since the racemization of peptide aldehydes can occur relatively easily, their potential epimerization during the Horner−Emmons olefination was of some concern. The fact that products **13** and **14**, which have a single chiral center, show appreciable optical activity indicates that *complete* racemization did not occur. However, other observations suggest that *some* epimerization

Scheme 4[a]



[a] (a) $(MeO)_2P(O)CH_2CO_2Me$, NaH, THF; (b) $(EtO)_2P(O)CH_2CO_2Et$, NaH, THF; (c) $(EtO)_2P(O)CH_2SO_2Ph$, NaH, THF; (d) TFA, CH_2Cl_2; (e) $PhCH_2OCOCl$, Et_3N, CH_2Cl_2; (f) DECP, Et_3N, THF; (g) Boc-F-OH; (h) Boc-VLF-OH; (i) Cbz-F-OSu, Et_3N; (j) aq NaOH, THF; (k) Cbz-F-OH; (l) Boc-E(tBu)-OH.

Scheme 5[a]



[a] (a) CDI, THF, then $MeONHCH_3$; (b) $LiAlH_4$, Et_2O; (c) $(MeO)_2P(O)CH_2CO_2Me$, NaH, THF; (d) Oxone, MeOH, H_2O; (e) TFA, CH_2Cl_2; (f) DECP, Et_3N, DMF; (g) Boc-VLF-OH; (h) Cbz-F-OH, EDC, HOBT, NMM.

probably did occur. This was most notable in the case of compound **7**, for which both HPLC and ^1H NMR clearly show the presence of two diastereomers in a 3:5 ratio. In the glutamine series (e.g., compounds **1, 2, 4, 10**, and **11**) diastereomeric purity tended to be significantly higher, typically 85−95% as determined by HPLC and/or ^1H NMR, and this was deemed adequate for initial evaluation of the compounds for enzyme inhibition and antiviral activity. That such epimerizations were not encountered during the synthesis of simple peptide derivatives (e.g., compounds **16**−**19**) further implicates the Horner−Emmons reaction as the source of epimerization, but efforts to minimize or control it were not undertaken.

Results and Discussion

Enzyme Inhibition. For evaluation as inhibitors of HRV-14 $3C^{pro}$ all compounds were tested initially at concentrations of 1, 5, 25, and 100 μg/mL. Representative results are shown in Table 1. A considerable range of inhibitory activities was observed, with compounds **1**−**4** giving complete inhibition even at the lowest concentration tested, while others gave only 18−40% inhibition at the highest concentration tested. For the more active compounds we next determined IC_{50} values using more narrowly spaced concentrations of inhibitor (chosen in light of the preliminary results) and recording progress curves for chromophore generation in each reaction. Progress curves obtained from the less active compounds showed curvature indicative of progressive enzyme inactivation during the observation period (data not shown). For the more active compounds the inactivation was so fast that it could not be observed in this way; instead linear progress curves were observed in all reactions in which enzyme inactivation was not complete. In addition, the IC_{50} values of the more active compounds were all very similar and approximately equal to one-half of the enzyme concentration used in the assays.

The above observations are consistent with a very rapid, stoichiometric, covalent inactivation of the enzyme by the Michael acceptors. We therefore used electrospray mass spectrometry to examine the enzyme after treatment with several peptidyl Michael acceptors. In each case the only protein species observed had the mass expected for a 1:1 protein:ligand adduct (Table 2). Moreover, these complexes were unaffected by dialysis for 4 h at 4 °C. In parallel with these experiments a control experiment was performed using the dipeptide aldehyde $3C^{pro}$ inhibitor LY338387 (Cbz-FM(O_2)-H).[15] In this case the undialyzed protein showed a MW of 20 446.4 ± 2.5 Da, as expected for a 1:1 hemithioacetal adduct, but after dialysis only the ligand-free native protein was observed. These results indicate that the inhibition of HRV-14 $3C^{pro}$ by the Michael acceptors is irreversible and thus covalent as well as stoichiometric.

The fact that the most potent inhibitors appeared to be literally "titrating" the enzyme suggested that they might actually be exploited for this purpose. This would be significant since E-64, the epoxysuccinyl derivative

Table 1. Structures, Numbering, Enzyme Inhibition, and Antiviral Activity of Peptidyl Michael Acceptors

		enzyme inhbition			
		rangefinding			plaque reduction
compd	structure	μg/mL	% inhib[a]	IC_{50} (μM)	IC_{50}[b] (μg/mL)
1	Boc-V-L-F-vQ-OMe	1	100	0.25 ± 0.02	0.74
2	Boc-V-L-F-vQ-OEt	1	100	0.13 ± 0.01	0.41
3	Cbz-V-L-F-vQ-OMe	1	100	0.17 ± 0.02	0.93
4	Boc-E(tBu)-V-L-F-vQ-OMe	1	100	0.2 ± 0.01	4.5
5	Boc-V-L-F-vQ-OH	25	43	17.7 ± 2.1	>10
6	E-V-L-F-vQ-OMe	1	100	0.49 ± 0.03	>10
7	Boc-V-L-F-vM(O_2)-OMe	5	48	13.6 ± 2.5	>10
8	Cbz-F-vQ-OMe	5	51	9.5 ± 1.9	3.2
9	Cbz-F-vM(O_2)-OMe	25	27	nd[c]	>10
10	Boc-F-vQ-OMe	100	42	nd	>10
11	Boc-F-vQ-OEt	100	39	nd	>10
12	Cbz-vQ-OEt	100	18	nd	>10
13	Boc-vQ-OMe	100	18	nd	nt[d]
14	Boc-vQ-OEt	100	27	nd	>10
15	(see Scheme 4)	100	42	nd	>10

[a] Average of duplicates which agreed within ±10%. [b] See the Experimental Section for details. [c] nd = not determined. [d] nt = not tested.

Table 2. Apparent Masses of HRV-14 3C Protease after Inactivation with Various Michael Acceptors

	MW		
ligand, structure	ligand	adduct[a]	ΔMW
none	0	19 998.4	
1, Boc-V-L-F-vQ-OMe	645	20 644.4	19 998.6
2, Boc-V-L-F-vQ-OEt	659	20 657.8	19 998.8
4, Boc-E(OtBu)-V-L-F-vQ-OMe	831	20 831.0	20 000.0
6, E-V-L-F-vQ-OMe	674	20 674.8	20 000.8
3, Cbz-V-L-F-vQ-OMe	679	20 465.2	19 997.2
8, Cbz-F-vQ-OMe	468	20 465.2	19 997.2
LY338387, Cbz-FM(O_2)-H	446	20 446.4	20 000.0

[a] Observed MW of protein (±2.5 Da). [b] The calculated mass of the native enzyme is 19 997 (ref 23).

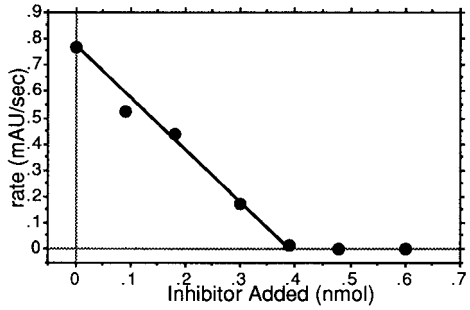


Figure 1. Active site titration of HRV 3C protease with Boc-V-L-F-vQ-OEt (compound **2**).

widely used for titrating many cysteine proteinases in the papain clan, does not inhibit HRV-14 $3C^{pro}$.[6] As shown in Figure 1, incremental addition of compound **2** to aliquots of HRV $3C^{pro}$ leads to proportional decreases in enzyme activity with complete loss of activity being reached once 0.4 nmol of **2** had been added. These results, coupled with the observation of 1:1 adducts by mass spectrometry, indicate that the enzyme preparation used actually contained only 47% active 3C protease, a result which agrees with estimates made by isoelectric focusing gel analysis of the purified recombinant protein followed by kinetic assays for $3C^{pro}$ activity (data not shown). Thus Michael acceptors such as **2** can indeed be used to determine the operational normality of solutions of HRV 3C protease.

Because the IC_{50} values for compounds **1**−**4**, and perhaps **6**, are very close to one-half of the enzyme concentration used in the assays, the IC_{50} data do not reveal potential differences in kinetic specificity among these compounds. In view of the extremely high reactivity of these inhibitors and the modest sensitivity of the photometric assay available to us, we did not attempt to determine apparent second-order rate constants (i.e., k_2/K_i) for the inactivation of HRV-14 $3C^{pro}$ by any of our compounds. Nevertheless the inhibition data presented in Table 1 allow some conclusions to be drawn about the effects of structure on the relative efficacy of the compounds. All of the potent compounds are tetra- or pentapeptides having a glutamine-like side chain at P_1 and a hydrophobic side chain at P_4; smaller compounds lacking the P_4 residue are very much less active (viz. compounds **10**−**14**). These features are consistent with the kinetic specificity of HRV-14 $3C^{pro}$ as delineated using peptide substrates comprised of only standard proteinogenic amino acids.[6,24−26] Although HRV-14 $3C^{pro}$ is highly selective for cleaving −Q∼GP− sites in protein substrates, it evidently tolerates replacement of the P_1'-P_2' Gly-Pro leaving group with nonphysiological groups including *p*-nitroaniline[28] or the acrylic ester moiety of the peptidyl Michael acceptors. As Michael acceptors, acrylate esters are much more reactive than the corresponding acrylic acids which are negatively charged at physiological pH values.[22,29,30] This may explain why compound **5** is much less effective as an inactivator of HRV-14 $3C^{pro}$ than its ester analogues **1** and **2**. At the P_5 position the enzyme accepts the lipophilic Boc or Cbz groups in **1**−**3**, the blocked glutamate derivative Boc-E(tBu) in **4**, or the unblocked zwitterionic glutamyl residue in **6**. However, replacement of the P_1 glutamine side chain ($-CH_2CH_2CONH_2$) by a methionine sulfone side chain ($-CH_2CH_2SO_2CH_3$) in **7** leads to substantial loss of activity. This is in contrast to the significant potency of the methional sulfone derivative Cbz-FM(O_2)-H as a competitive inhibitor of HRV-14 $3C^{pro}$.[15] Thus the ability of the sulfone group to function as a H-bond-accepting[17,27] surrogate for the amide side chain of glutamine evidently depends at least in part on the nature of other enzyme−ligand interactions and the degree to which these individual interactions may be cooperative.

Antiviral Activity and Cytotoxicity. Active $3C^{pro}$ is essential for viral replication, but for compounds which inactivate this enzyme in cell-free systems to exhibit antiviral activity in cells, they must be capable

of diffusion and/or transport into infected cells. Once inside they must not be inactivated (e.g., by conjugation with glutathione or hydrolysis of ester or peptide bonds required for enzyme inhibition) or transported back out of the cells before they have had a chance to react irreversibly with the viral $3C^{pro}$. Compounds **1**−**3** have very significant antiviral activity in cell culture assay, inhibiting viral replication and plaque formation by 50% at submicromolar concentrations (Table 1). Clearly they fulfill all the criteria for activity, cellular uptake, and metabolic stability stated above. The reduced activity of compound **6**, despite its potency as a $3C^{pro}$ inactivator in cell-free systems, is probably attributable to difficulty in entering cells, although the possibility that it is rapidly hydrolyzed intracellularly (e.g., by an aminopeptidase) cannot be ruled out. With the possible exception of compound **8**, the other Michael acceptor compounds showed only weak antiviral activity at best, consistent with their relatively low activity as enzyme inactivators.

To ensure that the observed antiviral activity was not due to a general cytotoxic effect, all compounds were evaluated for toxicity in uninfected cells using the XTT reduction assay. In this assay a tetrazolium dye (XTT) is metabolically reduced to form a colored product by metabolically viable cells. At the highest concentration of test compound examined, 10 μg/mL, compound **1** caused a 16% reduction in metabolic activity while compound **2** caused an 8% reduction; all other compounds showed no difference from controls, suggesting a very low level of cytotoxicity for these peptidyl Michael acceptors.

Conclusions

Peptidyl Michael acceptors comprising an appropriate recognition peptide and a vinylogous amino acid ester are extremely active as affinity-labeling inactivators of human rhinovirus 3C proteinase in vitro. They form 1:1 covalent adducts with the active site cysteine and can be used for active site titration of HRV-14 $3C^{pro}$. In cell cultures the more active inhibitors show excellent antiviral activity at concentrations far below those causing cytotoxicity. Peptidyl Michael acceptors and their analogues warrant further investigation as potential therapeutic agents for pathologies involving cysteine proteinase enzymes.

Experimental Section

The abbreviations which are used are as follows: Cbz, benzyloxycarbonyl; CDI, 1,1-carbonyldiimidazole; CI, chemical ionization; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DECP, diethyl cyanophosphonate; DMF, *N*,*N*-dimethylformamide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; EI, electron ionization; FAB, fast atom bombardment; FD, field desorption; G/TG, glycerol/thioglycerol; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HR, high resolution; MS, mass spectrometry; NBA, *m*-nitrobenzyl alcohol; NHS, *N*-hydroxysuccinimide; NMM, *N*-methylmorpholine; OSu, *N*-succinimidinyloxy; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Instruments and Materials. Chemicals, solvents, and reagents were obtained from commercial sources. The peptide *p*-nitroanilide EALFQ-pNA used for photometric assays was custom synthesized by American Peptide Co. (CA) and was characterized by amino acid analysis and mass spectrometry. THF was distilled from sodium and benzophenone, DMF was distilled from phthalic anhydride at reduced pressure, acetonitrile was distilled from CaH_2, and triethylamine was distilled from KOH pellets. Melting points were determined in capillary tubes using a Thomas-Hoover apparatus and are uncorrected. NMR spectra were recorded using a GE QE-Plus (300-MHz) or a Bruker DRX-400 (400-MHz) spectometer. Fast atom bombardment mass spectra were obtained using an AUTOSPEC-Q tandem hybrid mass spectrometer or a ZAB-HS mass spectrometer. Spectra were recorded from samples in matrixes of glycerol, glycerol/thioglycerol, or *m*-nitrobenzyl alcohol saturated with LiOAc. A Nermag R10-10b mass spectrometer was used for electron impact and desorption-chemical ionization mass spectra, the latter with ammonia as the reagent gas. Combustion analyses were performed using a Perkin-Elmer model 2400-CHN elemental analyzer. Glass plates coated with silica gel GLHF (250 μm, Analtech) were used for analytical thin-layer chromatography. HPLC analyses were performed using a Shimadzu gradient HPLC system with a Vydac C-18 column (218TP54, 5 μm, 4.6 × 250 mm). Depending on the compound being analyzed, the mobile phase contained either MeOH (40−60%, v/v) or MeCN (10−40%, v/v) in water containing 0.1% (v/v) TFA, but elution was always isocratic at a flow rate of 1.0 mL/min; peak detection was at 214 nm.

Synthesis. Boc-V-L-OBn (16). Boc-valine (1.0 g, 4.6 mmol), leucine benzyl ester *p*-toluenesulfonate salt (1.81 g, 4.6 mmol), CH_2Cl_2 (50 mL), NMM (1.52, mL, 13.8 mmol), and NHS (0.58 g, 5.06 mmol) were combined in a flask and cooled to 0 °C. DCC (1.04 g, 5.06 mmol) was added, and the reaction mixture was stirred for 3 h at 0 °C and for 18 h at room temperature. The solvent was evaporated and the residue dissolved in ethyl acetate (250 mL). The solution was washed successively with 1 M HCl, water, saturated $NaHCO_3$ solution, and brine (40 mL each), dried over $MgSO_4$, and filtered. Evaporation of the filtrate yielded 4.81 g of a white solid which was chromatographed over silica gel (CH_2Cl_2/MeOH, 19:1) to give compound **16** as a white solid (3.5 g, 70%). Mp: 88−89 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86−0.94 (m, 12H), 1.44 (s, 9H), 1.53−1.69 (m, 3H), 2.08 (m, 1H), 3.87 (dd, J = 6.4, 8.8 Hz, 1H), 4.68 (m, 1H), 5.04 (br d, J = 8 Hz, 1H), 5.15 (ABq, J = 12.3 Hz, $\Delta\nu$ = 9.0 Hz, 2H), 6.22 (br s, 1H), 7.30−7.40 (m, 5H).

Boc-V-L-OH (17). Compound **16** (3.46 g, 8.23 mmol) was dissolved in ethanol (100 mL) and hydrogenated over Pd/C (200 mg) at 30 psi H_2 for 12 h at room temperature. The catalyst was removed by filtration and the solvent evaporated yielding **17** as a white solid in quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.79−0.91 (m, 12H), 1.37 (s, 9H), 1.52 (m, 2H), 1.62 (m, 1H), 1.92 (m, 1H), 3.78 (m, 1H), 4.26 (m, 1H), 6.65 (d, J = 9.1 Hz, 1H), 7.96 (d, J = 7.9 Hz, 1H).

Boc-V-L-F-OBn (18). Compound **17** (2.58 g, 8.23 mmol), phenylalanine benzyl ester (2.10 g, 8.23 mmol), HOBt (1.22 g, 9.05 mmol), NMM (2.3 mL, 24.7 mmol), and 50 mL of CH_2Cl_2 were combined in a flask and cooled to 0 °C. EDC (1.85 g, 9.7 mmol) was added, and the mixture was stirred for 3 h at 0 °C and for 18 h at room temperature. The solvent was evaporated and the residue dissolved ethyl acetate (250 mL). The solution was washed successively with 1 M HCl, water, saturated $NaHCO_3$ solution, and brine (40 mL each), dried over $MgSO_4$, and filtered. Evaporation of the filtrate yielded 3.11 g of a white solid which was chromatographed over silica gel (CH_2Cl_2/MeOH, 19:1) to give compound **18** (3.11 g, 55%). Mp: 151−152 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89−0.95 (m, 12H), 1.43 (s, 9H), 1.47−1.53 (m, 1H), 1.55−1.65 (m, 2H), 1.89−1.97 (m, 1H), 2.05−2.15 (m, 1H), 3.09 (d, J = 6 Hz, 2H), 3.83−3.89 (m, 1H), 4.37−4.46 (m, 1H), 4.83−4.90 (m, 1H), 5.11 (ABq, J = 12.1 Hz, $\Delta\nu$ = 21.4 Hz, 2H), 7.00 (m, 2H), 7.21 (m, 3H), 7.35 (m, 2H), 7.75 (m, 3H). FABMS (NBA/LiOAc): m/z 574 (M + Li), 474 (M − Boc + Li). HRMS: calcd for $C_{32}H_{46}N_3O_6$, 568.3387; found (FAB, glycerol), 568.3375.

Boc-V-L-F-OH (19). Compound **18** was hydrogenated as described above for Boc-V-L-OBn giving Boc-V-L-F-OH as a white solid in quantitative yield. Mp: 124−125 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.82−0.92 (m, 12H), 1.43, (s, 9H), 1.53−1.62 (m, 2H), 1.71 (m, 1H), 1.92 (m, 1H), 2.05 (m, 1H), 3.03 (m, 1H), 3.17 (m, 1H), 3.89 (m, 1H), 4.58 (m, 1H), 4.77 (m,

1H), 5.20 (br s, 1H), 7.02 (br s, 2H), 7.13−7.28 (m, 5H). HRMS: calcd for $C_{25}H_{40}N_3O_6$, 478.2917; found (FAB, G/TG), 478.2900.

Boc-L-2-aminoglutarimide (20). Boc-L-glutamine (2.46 g, 10.0 mmol) and *N*-hydroxysuccinimide (1.5 g, 10 mmol) were dissolved in a mixture of DMF (2.25 mL) and THF (15 mL) and cooled to 0 °C. DCC (20.8 g, 10.0 mmol) was added, and the mixture was stirred at room temperature overnight and then filtered. The filtrate was concentrated in vacuo, and the residue was extracted with hot $CHCl_3$ several times. The extract was concentrated, and the residue was taken up in EtOAc. The latter was washed three times with water, dried over $MgSO_4$, filtered, and concentrated in vacuo. Chromatography of the solid residue over silica gel (EtOAc/hexanes, 1:1) provided Boc-L-2-aminoglutarimide (**20**) (752 mg, 34%) as a white solid. Mp: 153−155 °C. $[\alpha]_D$ −58.8 (c = 1.125, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.5 (s, 9H), 1.8−2.0 (m, 1H), 2.4−2.6 (m, 1H), 2.6−2.9 (m, 2H), 4.3 (m, 1H), 5.35 (br s, 1H), 8.2 (br s, 1H). CIMS: m/z 246 (M + NH_4), 229 (MH). IR (KBr): 3350 (sh), 1720, 1690 cm^{-1}. Anal. ($C_{10}H_{16}N_2O_4$) C, H, N.

(3*S*)-3-[(*tert*-Butoxycarbonyl)amino]-2-hydroxypiperidin-6-one (21). By analogy to reductions of peptidyl glutatimide derivatives reported by Kaldor et al.,[14] compound **20** (1.5 g, 6.6 mmol) was dissolved in 60 mL of EtOH/H_2O (9:1) and cooled to −15 °C. Sodium borohydride (965 mg, 25.4 mmol) was added in one portion followed by 3 drops of 2 N HCl. The solution was stirred at −10 °C while 2 drops of 2 N HCl was added every 15 min for 90 min. The mixture was poured into brine (50 mL) and extracted with $CHCl_3$. Removal of solvent and chromatography of the residue using EtOAc/MeOH (15:1) yielded 0.98 g (42.5%) of compound **21**, a mixture of C-2 epimers, as white crystals. Mp: 113−115 °C. $[\alpha]_D$ −6.8 (c = 0.5, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.40 (s, 9H), 1.7−1.9 (m, 2H), 2.20 (m, 1H), 2.45 (m, 1H), 3.77 (m, 1H), 4.7 (br s, 1H), 4.85 (m, 1H), 4.95 (s, 1H), 6.7 (br s, 1H). MS (EI): m/z 212 (M − H_2O). IR (KBr): 3300 (br), 1670 (br) cm^{-1}. This material was used in subsequent reactions without further characterization.

Methyl (2*E*,4*S*)-7-Amino-4-[(*tert*-butoxycarbonyl)amino]-7-oxo-2-heptenoate (Boc-vQ-OMe, 13). A solution of trimethyl phosphonoacetate (1.82 g, 10 mmol) in 25 mL of THF was added dropwise to a stirred suspension of NaH (330 mg, 13.75 mmol) in anhydrous THF (75 mL). After 1 h at room temperature a solution of compound **21** (1.15 g, 5 mmol) in 25 mL of THF was added. The mixture was stirred for 2.5 h, quenched with water (10 mL), and concentrated under reduced pressure. The residue was dissolved in $CHCl_3$, washed successively with 2% HCl, water, and saturated $NaHCO_3$ solution, and dried over $MgSO_4$. After concentration the crude product was triturated with Et_2O leaving a white crystalline product (1.19 g, 83%). Mp: 116−117 °C. $[\alpha]_D$ −27 (c = 0.4, MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H), 1.80 (m, 1H), 2.00 (m, 1H), 2.30 (m, 2H), 3.75 (s, 3H), 4.35 (br s, 1H), 4.90 (br d, 1H), 5.45 (br s, 1H), 5.95 (d, J = 16 Hz, 1H), 6.85 (dd, J = 16, 5 Hz, 1H). CIMS: m/z 287 (MH)$^+$. Anal. ($C_{13}H_{22}N_2O_5$) C, H, N.

Boc-vQ-OEt (14). This compound was prepared as described for compound **13** using triethyl phosphonoacetate in place of trimethyl phosphonoacetate. Yield: 81%. Mp: 123−124.5 °C. $[\alpha]_D$ −42 (c = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30 (t, 3H), 1.45 (s, 9H), 1.82 (m, 1H), 1.97 (m, 1H), 2.31 (m, 2H), 4.17 (q, 2H), 4.35 (br s, 1H), 4.96 (m, 1H), 5.78 (br s, 1H), 5.94 (d, J = 16 Hz, 1H), 6.10 (br s, 1H), 6.83 (dd, J = 16, 6 Hz, 1H). HRMS: calcd for $C_{14}H_{25}N_2O_5$, 301.1763; found (FABMS, G/TG), 301.1777. HPLC (MeOH/H_2O/TFA, 50:50:0.1) t_R = 5.91 min.

Cbz-vQ-OEt (12). Boc-vQ-OEt (500 mg, 1.74 mmol) was stirred in 2 mL of TFA/CH_2Cl_2 (1:1) for 30 min at room temperature. The mixture was evaporated to dryness and the residue dissolved in CH_2Cl_2 (10 mL) and Et_3N (0.5 mL) and cooled to 0 °C. Benzyl chloroformate (0.3 mL, 1.6 mmol) was added, and the mixture was stirred for 3 h and then diluted into CH_2Cl_2 (50 mL). After washing with water and saturated $NaHCO_3$ solution, the organic phase was dried over $MgSO_4$, filtered, and concentrated to yield compound **12** as a white solid (500 mg, 70%). Mp: 72−76 °C. $[\alpha]_D$ −58 (c = 1.0, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (t, 3H), 1.94 (m, 2H), 1.98 (m, 1H), 2.28 (m, 2H), 4.19 (q, 2H), 4.39 (m, 1H), 5.12 (m, 2H), 5.41 (br s, 1H), 5.54 (br s, 1H), 5.74 (br s, 1H), 5.94 (d, J = 16 Hz, 1H), 6.82 (dd, J = 6, 16 Hz, 1H), 7.32 (m, 5H). HPLC (MeOH/H_2O/TFA, 50:50:0.1),t_R = 5.60 min. Anal. ($C_{17}H_{22}N_2O_5$) C, H, N.

Boc-F-vQ-OEt (11). Boc-vQ-OEt (300 mg, 1.0 mmol) was stirred in 2 mL of TFA/CH_2Cl_2 (1:1) for 30 min at room temperature. The solution was evaporated to dryness, and the residue was combined with DMF (15 mL), Boc-F-OH (0.4 g, 1.5 mmol), Et_3N (0.5 mL), and DECP (0.25 mL, 1.5 mmol). After the mixture stirred for 44 h, EtOAc (250 mL) was added, and the solution was extracted with saturated $NaHCO_3$ solution, 0.1 N citric acid, and brine (40 mL each). The organic extract was dried over $MgSO_4$, filtered, and concentrated to give 600 mg of a white paste. The latter was chromatographed over silica gel (CH_2Cl_2/MeOH, 20:1) which provided ester **11** as a white solid. Mp: 86−89 °C, 70% yield. FABMS (G/TG): m/z 448 (MH), 348 (M − Boc + H). Anal. ($C_{23}H_{33}N_3O_6$) C, H, N. HPLC (MeOH/H_2O/TFA, 50:50:0.1) shows this material to be a mixture of two components, a minor one (20%) with t_R = 8.16 min and a major one (80%) with t_R = 15.8 min. In addition, in the ^{1}H NMR spectrum of this product several of the characteristically patterned peaks are accompanied by small peaks showing the same pattern but with a slight offset in chemical shift in a fashion suggestive of a diastereomeric impurity. However, preparative separation of the diastereomers was not pursued, and the spectrum reported below is that for the major diastereomer. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (t, 3H), 1.40 (s, 9H), 1.75 (m, 1H), 1.95 (m, 1H), 2.20 (m, 2H), 3.06 (d, 2H), 4.17 (q, 2H), 4.57 (m, 1H), 5.10 (br s, 1H), 5.45 (br s, 1H), 5.62 (d, J = 16 Hz, 1H), 5.97 (br s, 1H), 6.35 (br s, 1H), 6.65 (dd, J = 16, 6 Hz, 1H), 7.25 (m, 5H).

Boc-V-L-F-vQ-OMe (1). Boc-vQ-OMe (70 mg, 0.24 mmol) was dissolved in CH_2Cl_2 (1 mL), trifluoroacetic acid (1 mL) was added, and the solution was stirred for 2 h at room temperature and then concentrated in vacuo. The crude product was dissolved in DMF (5 mL), and Boc-V-L-F-OH (105 mg, 0.22 mmol), triethylamine (204 μL, 1.3 mmol), and diethyl cyanophosphonate (108 μL, 0.66 mmol) were added. After the mixture stirred for 44 h, ethyl acetate (250 mL) was added, and the solution was washed with saturated $NaHCO_3$ solution, 0.1 N citric acid, and brine (25 mL each). The organic layer was dried over $MgSO_4$ and filtered. After concentration, HPLC (MeOH/HOH/TFA, 60:40:0.1) showed two peaks at t_R = 10.3 and 11.2 min. Trituration of the residue with small portions of EtOAc selectively removed the latter and left behind white crystals of compound **1** (105 mg, 74%). Mp: 222 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.85 (m, 12H), 1.40 (s, 9H), 1.56 (m, 1H), 1.67 (m, 1H), 1.72 (m, 1H), 1.90 (m, 1H), 2.06 (m, 2H), 2.86 (m, 1H), 2.96 (m, 1H), 3.56 (s, 3H), 3.75 (m, 1H), 4.35 (m, 2H), 4.46 (m, 1H), 5.65 (d, J = 15 Hz, 1H), 6.67 (dd, J = 15, 6 Hz, 1H), 6.72 (m, 1H), 7.20 (m, 6H), 7.81 (m, 1H), 8.00 (m, 1H), 8.08 (m, 1H). EIMS: m/z 645 (M$^+$). FABMS (G/TG): m/z 646 (MH), 546 (M − Boc + H).

Boc-V-L-F-vQ-OEt (2). This compound was prepared as described for **1** using Boc-vQ-OEt in place of Boc-vQ-OMe. After trituration of the initial solid product with EtOAc, the yield of product was 50%. Mp: 142−146 °C. HPLC (MeCN/H_2O/TFA, 40:60:0.1) t_R = 9.3 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.80 (m, 12H), 1.25 (m, 3H), 1.40 (m, 9H), 1.55 (m, 1H), 1.66 (m, 1H), 1.75 (m, 1H), 1.90 (m, 1H), 2.07 (m, 2H), 2.88 (m, 1H), 2.95 (m, 1H), 3.75 (m, 1H), 4.12 (q, 2H), 4.35 (m, 2H), 4.49 (m, 1H), 5.62 (d, J = 6 Hz, 1H), 6.70 (m, 2H), 7.20 (m, 5H), 7 0.85 (m, 1H), 8.04 (m, 1H), 8.10 (m, 1H). FABMS (NBA/LiOAc): m/z 666 (M + Li), 566 (M − Boc + Li). HRMS: calcd for $C_{34}H_{53}N_5O_8Li$, 666.4037; found (FAB, NBA/LiOAc), 666.4054.

Boc-E(tBu)-V-L-F-vQ-OMe (4). Boc-VLFvQ-OMe (100 mg, 0.15 mmol) was stirred with 36 μL of TFA and 20 μL of CH_2Cl_2 for 30 min at room temperature, and the mixture was

evaporated to dryness. DMF (5 mL) was added, followed by Boc-E(tBu)-OH (100 mg, 0.3 mmol), Et_3N (66 μL, 0.45 mmol), and DECP (75 μL, 0.45 mmol), and the mixture was stirred for 24 h at room temperature. EtOAc (25 mL) was added, and the solution was filtered and evaporated. The residue was triturated with THF (5 mL) and then with ether (20 mL) and dried under vacuum leaving 50 mg (33% yield) of a white solid. Mp: 189−194 °C. HPLC in two solvent systems showed a single peak (MeOH/H_2O/TFA, 50:50:0.1) t_R = 8.64 min; (MeCN/H_2O/TFA, 50:50:0.1) t_R = 8.06 min. Likewise the ^{1}H NMR spectrum (400 MHz, $CDCl_3$) showed no indication of diastereomeric impurities: δ 0.80 (m, 12H), 1.36 (s, 18H), 1.50 (m, 1H), 1.68 (m, 2H), 1.80 (m, 1H), 1.88 (m, 1H), 2.05 (t, 1H), 2.19 (t, 1H), 2.48 (s, 3H), 2.82 (dd, 1H, J = 8.82, 11.9 Hz), 2.92 (dd, 1H, J = 6.1, 11.9 Hz), 3.32 (s, 3H), 3.64 (s, 2H), 3.95 (m, 1H), 4.04 (m, 1H), 4.28 (m, 1H), 4.34 (m, 1H), 4.46 (m, 1H), 5.62 (d, J = 16 Hz, 1H), 6.66 (dd, J = 16, 6 Hz, 1H), 6.75 (s, 1H), 7.03 (d, 1H), 7.17 (m, 5H), 6.71 (d, 1H), 7.89 (d, 1H), 8.05 (d, 1H). HRMS: calcd for $C_{42}H_{67}N_6O_{11}$, 831.4868; found (FAB, glycerol), 831.4885.

Cbz-V-L-F-vQ-OMe (3). This compound was synthesized by removing the Boc protecting group of **1** (viz. **4** above) followed by introduction of the Cbz protecting group (viz. **12** above). The product, mp 228−242 °C dec, appeared as a single peak upon HPLC (MeCN/H_2O/TFA, 35:65:0.1) t_R = 20.3 min; (MeOH/H_2O/TFA, 60:40:0.1) t_R = 11.5 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.74−0.87 (m, 12H), 1.36 (m, 2H), 1.54 (m, 1H), 1.65 (m, 1H), 1.70 (m, 1H), 2.04 (t, 2H), 2.90 (dq, 2H), 3.64 (s, 2H), 3.82 (m, 1H), 4.30 (m, 2H), 4.65 (m, 1H), 5.02 (s, 2H), 5.62 (d, J = 15 Hz, 1H), 6.67 (dd, J = 15, 6 Hz, 1H), 6.75 (br s, 1H), 7.20 (m, 5H), 7.33 (m, 5H), 7.93 (d, 1H), 7.98 (d, 1H), 8.05 (d, 1H). HRMS: calcd for $C_{36}H_{49}N_5O_8Li$, 686.3741; found (FAB, NBA/LiOAc), 686.3740.

E-V-L-F-vQ-OMe·TFA (6). This compound was prepared by stirring Boc-E(tBu)-V-L-F-vQ-OMe (100 mg) with 50 μL of TFA for 30 min at room temperature and evaporating to dryness under vacuum. The resulting solid was used without further purification. FABMS (glycerol): m/z 673 $(M - H)^-$. HRMS: calcd for $C_{33}H_{51}N_6O_9$, 675.3718; found (FAB, glycerol), 675.3705. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.82−0.95 (m, 12H), 1.35−1.80 (m, 8H), 2.05 (t, 1H), 2.30 (t, 1H), 2.88 (dq, 2H), 3.60 (m, 1H), 3.88 (m, 1H), 4.14 (m, 1H), 4.34 (m, 1H), 4.48 (m, 1H), 5.62 (d, J = 16 Hz, 1H), 6.70 (dd, J = 6, 16 Hz, 1H), 6.78 (br s, 1H), 7.20 (m, 5H), 7.95 (d, 1H), 8.07 (m, 4H), 8.45 (d, 1H).

Cbz-F-vQ-OMe (8). Boc-vQ-OMe (49 mg, 0.17 mmol) was dissolved in 2 mL of CH_2Cl_2, and 2 mL of TFA was added. After the mixture stirred for 1 h at room temperature, CH_2Cl_2 was added, and the mixture was concentrated to a viscous oil. The latter was dissolved in DMF (3 mL) and triethylamine (102 μL, 0.73 mmol), Cbz-F-OSu (68 mg, 0.17 mmol) was added, and the mixture stirred for 2.5 h. Ethyl acetate was added, and the mixture was extracted with 10% aqueous $NaHCO_3$, 1% citric acid, and brine and dried over $MgSO_4$. After concentration the residue was fractionated by chromatotron (MeOH/CH_2Cl_2, 4:96) to give 34 mg (43%) of a white solid. FDMS: 468. ^{1}H NMR (300 MHz, $CDCl_3$/MeOH-d_4, 9:1): δ 1.52 (m, 1H), 2.00 (m, 2H), 2.80 (m, 2H), 3.58 (s, 3H), 4.15 (m, 1H), 4.30 (m, 1H), 4.84 (s, 2H), 5.42 (d, J = 16 Hz, 1H), 6.46 (dd, J = 16, 6 Hz, 1H), 7.20 (m, 5H), 7.62 (d, 1H). Anal. ($C_{25}H_{29}N_3O_6$), C, H, N.

Boc-F-vQ-OMe (10). This compound was prepared as described for Boc-F-vQ-OEt but using Boc-vQ-OMe in place of Boc-vQ-OEt. Yield: 50%. Mp: 74−77 °C. HPLC (MeCN/H_2O/TFA, 40:60:0.1) showed two peaks at t_R = 5.3 min (14%) and 6.65 min (86%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.30 (m, 9H), 1.70 (m, 1H), 1.77 (m, 1H), 2.10 (m, 2H), 2.75 (m, 1H), 2.92 (m, 1H), 3.65 (s, 1H), 4.15 (m, 1H), 4.40 (m, 1H), 5.70 (d, J = 15 Hz, 1H), 7.75 (m, 2H), 7.91 (m, 1H), 7.20 (m, 6H), 8.07 (m, 1H). FABMS (NBA/LiOAc): m/z 440 (M + Li), 340 (M − Boc + Li). Anal. ($C_{22}H_{31}N_3O_6$) C, H, N.

Boc-V-L-F-vQ-OH (5). Boc-V-L-F-vQ-OMe (0.5 g, 0.77 mmol) was dissolved in THF/H_2O (1:1, 4 mL), and 1.4 mL of 1 N NaOH solution was added. After the mixture stirred for 5 h at room temperature, the pH was brought to 3.0 using 1 M H_2SO_4, whereupon a white precipitate formed. The solid was collected by filtration, washed with ether, and dried under vacuum. Yield: 0.42 g (85%). Mp: 171−175 °C. HPLC (MeCN/H_2O, 40:60) t_R = 4.6 min; (MeOH/H_2O/TFA, 50:50:0.1) t_R = 29.0 min. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80 (m, 12H), 1.37 (s, 9H), 1.57 (m, 1H), 1.70 (m, 2H), 1.90 (m, 1H), 2.05 (t, 2H), 2.85 (dd, 1H, J = 8.40, 13.6 Hz), 2.93 (dd, 1H, J = 5.7, 13.6 Hz), 3.75 (m, 1H), 4.35 (m, 2H), 4.48 (m, 1H), 5.61 (d, J = 15 Hz, 1H), 6.61 (dd, J = 15, 6 Hz, 1H), 6.76 (br s, 1H), 7.19 (m, 5H), 7.84 (d, 1H), 8.00 (d, 1H), 8.08 (d, 1H). HRMS: calcd for $C_{32}H_{50}N_5O_8$, 632.3659; found (FAB, glycerol), 632.3636.

***tert*-Butyl *N*-[(*E*)-1-(3-Amino-3-oxopropyl)-3-(phenylsulfonyl)-2-propenyl]carbamate (23).** A solution of diethyl (phenylsulfonyl)methylphosphonate (254 mg, 0.87 mmol) in THF was added to a suspension of NaH (35 mg, 0.87 mmol) in THF. After the mixture stirred for 30 min at room temperature, a THF solution of Boc-L-glutarimide (200 mg, 0.87 mmol) was added, and stirring was continued for 2.5 h. A saturated solution of NH_4Cl was added, and the mixture was extracted with ethyl acetate. The extract was dried over $MgSO_4$ giving an oil which was fractionated by chromatotron (EtOAc/MeOH, 90:10) to give 100 mg (31%) of a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.38 (s, 9H), 1.90 (m, 2H), 2.30 (m, 2H), 4.38 (m, 1H), 5.38 (d, J = 8 Hz, 1H), 5.82 (br s, 1H), 6.12 (br s, 1H), 6.45 (d, J = 16 Hz, 1H), 6.83 (dd, J = 15, 5 Hz, 1H), 7.5−7.6 (m, 3H), 7.8−8.0 (m, 2H). Anal. ($C_{17}H_{24}N_2O_5$) C, H, N.

Benzyl *N*-((1*S*)-2-{[(1*S*,2*E*)-1-(3-Amino-3-oxopropyl)-3-(phenylsulfonyl)-2-propenyl]amino}-1-benzyl-2-oxoethyl)carbamate (15). Compound **23** (90 mg, 0.24 mmol) was dissolved in 2 mL of CH_2Cl_2, and 2 mL of TFA was added. After the mixture stirred for 1 h, CH_2Cl_2 was added, and the mixture was concentrated to a viscous oil which was used immediately. DMF (3 mL) and triethylamine (102 μL, 0.73 mmol) were added, followed by Cbz-F-OSu (97 mg, 0.24 mmol), and the mixture was stirred for 2 h. Ethyl acetate was added, and the mixture was extracted with 10% aqueous $NaHCO_3$, 1% citric acid, and brine and dried over $MgSO_4$. After concentration the white solid residue was fractionated by chromatotron (MeOH/CH_2Cl_2, 4:96) to give 28 mg (21%) of a white solid. ^{1}H NMR (300 MHz, $CDCl_3$/MeOH-d_4, 9:1): δ 1.58 (m, 1H), 2.03 (m, 2H), 2.81 (m, 2H), 4.15 (t, J = 7 Hz, 1H), 4.42 (m, 1H), 4.91 (s, 2H), 5.82 (d, J = 16 Hz, 1H), 6.60 (dd, J = 16, 5 Hz, 1H), 7.0−7.2 (m, 5H), 7.4−7.8 (m, 5H). Anal. ($C_{25}H_{29}N_3O_6$·0.5H_2O) C, H, N.

Boc-M-N(CH_3)OCH_3 (24). Boc-L-methionine (5.0 g, 20 mmol) was dissolved in THF (30 mL), 1,1-carbonyldiimidazole (3.25 g, 20 mmol) was added, and the solution was stirred for 2 h at room temperature. *N*,*O*-Dimethylhydroxylamine hydrochloride (5.85 g, 60 mmol) was introduced, and the reaction was stirred for 18 h at room temperature and concentrated. The residue was dissolved in 250 mL of EtOAc and the latter extracted with 50-mL portions of 1 M HCl, water, 1 M $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed over silica gel (EtOAc/hexanes, 1:1) to give 4.4 g (75%) of amide **24** as a white solid. TLC: R_f = 0.6 (CH_2Cl_2/MeOH, 20:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (s, 9H), 1.80 (m, 2H), 2.12 (s, 3H), 2.54 (m, 2H), 3.10 (s, 3H), 3.78 (s, 3H), 4.78 (m, 1H), 5.71 (br d, 1H). This material was used without further purification.

Boc-methional (25). Amide **24** (4.4 g, 15 mmol) was dissolved in ether (100 mL) and cooled in an ice bath. $LiAlH_4$ (0.72 g, 18.8 mmol) was added cautiously, and the mixture was stirred and monitored by TLC. After 35 min the reaction was quenched by cautious addition of 0.5 M $KHSO_4$ solution (40 mL) while maintaining vigorous stirring. Ether (100 mL) was added, and the aqueous phase was separated and extracted with ether (5 × 50 mL). The ether phases were combined, extracted with 3 M HCl (3 × 30 mL), saturated $NaHCO_3$ solution (3 × 20 mL), and brine (3 × 20 mL), dried over $MgSO_4$, and concentrated to give Boc-methional as a translucent white oil (3.1 g, 88%). TLC: R_f = 0.37 (hexanes/EtOAc, 3:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H), 2.05 (s, 3H), 2.08 (m,

2H), 2.54 (m, 2H), 4.30 (m, 1H), 5.21 (m, 1H), 9.52 (s, 1H). This material was used immediately for the next reaction.

Boc-vM-OMe (26). Trimethyl phosphonoacetate (5.40 mL, 33.4 mmol) was dissolved in THF (20 mL) and added to a stirred suspension of NaH (1.11 g, 27.85 mmol) in THF (20 mL). After 30 min at room temperature a solution of Boc-methional (2.60 g, 11.1 mmol) in THF (25 mL) was added, and stirring continued for 1 h. Water (5 mL) was cautiously added dropwise with stirring, the mixture concentrated, and the residue partitioned between $CHCl_3$ (200 mL) and water (20 mL). The $CHCl_3$ layer was extracted with dilute HCl, $NaHCO_3$ solution, and brine, dried over $MgSO_4$, and concentrated to give an off-white solid. Chromatography of the latter over silica gel (EtOAc) gave Boc-vM-OMe (2.22 g, 70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (s, 9H), 2.05 (s, 3H), 2.08 (m, 2H), 2.47 (m, 2H), 3.58 (s, 3H), 4.38 (m, 1H), 4.61 (br d, 1H), 5.88 (d, J = 15 Hz, 1H), 6.68 (dd, J = 6, 15 Hz, 1H).

Boc-vM(O_2)-OMe (27). Compound **26** (1.5 g, 5.35 mmol) was dissolved in MeOH (15 mL) and water (5 mL) and cooled in an ice bath, and a cold solution of Oxone (9.21 g, 14.98 mmol) in water (10 mL) was added. The solution was allowed to come to room temperature and stirred for 3 h while monitoring by TLC (EtOAc). Chloroform (25 mL) was added and the aqueous phase removed. The chloroform was dried over $MgSO_4$ and concentrated to yield a transparent oil (1.72 g). Chromatography of the latter over silica gel (EtOAc/hexanes, 10:1) afforded Boc-vM-OMe (0.61 g, 32%) as a white solid. Mp: 125−131 °C dec. $[\alpha]_D$ 3.2 (c = 1.1, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.43 (s, 9H), 2.05 (m, 1H), 2.20 (m, 1H), 2.94 (s, 3H), 3.08 (s, 3H), 3.75 (s, 3H), 4.43 (m, 1H), 4.67 (br d, 1H), 5.97 (d, J = 5 Hz, 1H), 6.84 (dd, J = 5, 15 Hz, 1H). FABMS (NBA/LiOAc): m/z 328 (M + Li).

Boc-V-L-F-vM(O_2)-OMe (7). Boc-vM(O_2)-OMe was deprotected and coupled to Boc-V-L-F-OH as described for compound **1** above. The product, a white solid (mp 180 °C), was evidently a mixture of two diastereomers whose preparative separation was not attempted. HPLC (MeCN/H_2O/TFA, 40:60:0.1) showed two peaks in a 3:5 ratio, t_R = 24.9 and 26.3 min, respectively. The ^{1}H NMR spectrum (400 MHz, DMSO-d_6) also indicated the presence of two diastereomers in a 3:5 ratio. Major isomer: δ 2.94 (s), 3.64 (s), 3.75 (m), 5.62 (d, J = 6 Hz), 6.78 (dd, J = 6, 16 Hz). Minor isomer: δ 2.90 (s), 3.62 (s), 4.00 (m), 5.87 (d, J = 6 Hz), 6.67 (dd, J = 6, 16 Hz). In addition the following common resonances were observed: (δ) 0.75−0.85 (m, 12H), 1.06−2.00 (m, 8H), 2.80−3.10 (m, 3−4H), 4.25−4.55 (m, 3H), 5.05 (d, 1H), 7.15−7.28 (m, 5H), 7.72 (t, 1H), 8.08−8.20 (m, 2H). HRMS: calcd for $C_{33}H_{52}N_4O_9SLi$, 687.345; found (FABMS, NBA/LiOAc), 687.3638.

Cbz-F-vM-OMe (28). Compound **26** (432 mg, 1.5 mmol) was dissolved in 3 mL of CH_2Cl_2 and cooled to 0 °C. TFA (3 mL) was added, the ice bath was removed, and the reaction was stirred for 3 h and concentrated. The residue was dissolved in THF (8 mL) and cooled to 0 °C. HOBt (201 mg, 1.5 mmol), EDC (286 mg, 1.5 mmol), Cbz-F-OH (447 mg, 1.5 mmol), and NMM (0.5 mL, 4.5 mmol) were added, the ice bath was removed, and the mixture was stirred for 18 h at room temperature. EtOAc (200 mL) was added, and the solution was extracted with dilute HCl, $NaHCO_3$ solution, and brine and dried over $MgSO_4$. After evaporation the residue was fractionated by chromatotron (EtOAc) and concentrated to give compound **28** as a white solid (360 mg, 51%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.63−1.88 (m, 2H), 2.03 (s, 3H), 2.40 (t, J = 5 Hz, 2H), 3.01 (dd, J = 5, 7 Hz, 1H), 5.11 (q, J = 8 Hz, 2H), 5.26 (m, 1H), 5.71 (d, J = 16 Hz, 1H), 5.95 (br d, J = 8 Hz, 1H), 6.65 (dd, J = 5, 16 Hz, 1H), 7.2−7.4 (m, 10 H). Anal. ($C_{25}H_{30}N_2O_5S$) C, H, N.

Cbz-F-vM(O_2)-OMe (9). Compound **28** (190 mg, 0.4 mmol) was dissolved in MeOH (3 mL) and cooled to 0 °C. A solution of Oxone (339 mg, 1.2 mmol) in H_2O (3 mL) was added, and the mixture was stirred vigorously for 3 h at room temperature. The mixture was extracted with $CHCl_3$ (2 × 100 mL) and the latter dried over $MgSO_4$ and concentrated giving a white solid which was fractionated by chromatotron (MeOH/CH_2Cl_2, 4:96) yielding 45 mg (22%) of pure **9** (TLC R_f = 0.45; MeOH/CH_2Cl_2, 5:95), plus an additional 110 mg of **9** contaminated with a small amount of sulfoxide. FDMS: 502. ^{1}H NMR (300 MHz, $CDCl_3$/MeOH-d_4, 9:1): δ 1.81 (m, 1H), 2.00 (m, 1H), 2.78 (s, 3H), 2.8−3.0 (m, 4H), 3.75 (s, 3H), 4.20 (m, 1H), 4.52 (m, 1H), 4.98 (s, 2H), 5,43 (d, J = 16 Hz, 1H), 6.18 (br d, J = 8 Hz, 1 H), 6.52 (dd, J = 5, 16 Hz, 1H), 7.0−7.3 (m, 10H), 7.65 (br d, J = 8 Hz, 1H). Anal. ($C_{25}H_{30}N_2O_7S$) C, H, N.

Enzyme Purification and Assay. Recombinant HRV-14 3C^{pro} was expressed and purified as described previously.[23] The chromogenic substrate EALFQ-pNA was used to measure its activity.[28] A typical assay (200 μL) contained 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 250 μM substrate (EALFQ-pNA), and 3C^{pro} (ca. 0.4 μM). Assay substrate and inhibitors were added as DMSO solutions, keeping the total DMSO concentration in each incubation constant at 10% (v/v). Control assays were identical but with pure DMSO added in place of inhibitor solution. Assays were run in duplicate at 30 °C; A_{405} readings were taken every 2 min for 20 min, and reaction rates were calculated from $\Delta A/\Delta t$ using $\Delta\epsilon$ = 10 300 M^{-1} cm^{-1}. All reactions were performed in microtiter plate wells and monitored using a temperature-controlled microplate reader (Molecular Devices). Rates were proportional to substrate concentration over the range 0−250 μM (the upper limit of solubility), and the apparent second-order catalytic constant (k_{cat}/K_m) was 840 ± 11 M^{-1} s^{-1}.[28]

For initial evaluation, each inhibitor was tested at concentrations of 1, 5, 25, and 100 μg/mL. Substrate solution, buffer, and inhibitor solution were combined, and reactions were initiated by adding an aliquot of 3C^{pro} enzyme solution. A_{405} readings were recorded every 2 min to generate progress curves for each reaction. The percent inhibition was determined after 20 min by comparing A_{405} for the test case to that of the uninhibited control reaction. For the more active compounds IC_{50} values were determined using a total of eight concentrations of inhibitor (controls and assay conditions as described above). A_{405} readings were taken after 20 min of reaction, and IC_{50} values were determined from plots of percent inhibition vs inhibitor concentration.

Active Site Titration of HRV 3C Protease with Michael Acceptor 2. To each of seven polystyrene microcuvettes (1-mL capacity, 1-cm path length) was added, in order, assay buffer (525 μL), enzyme solution (32.5 μL, 0.52 mg of protein/mL), DMSO (13, 18, 20, 23, 27, 30, or 33 μL), and a solution of Michael acceptor **2** in DMSO (30 μM; 20, 15, 13, 10, 6, 3, or 0 μL, respectively). After the last addition each cuvette was inverted several times for mixing and allowed to stand at room temperature for 60 min. The amount of enzyme activity was then determined by adding a solution of assay substrate (EALFQ-pNA, 5.11 mM in DMSO, 32.5 μL) to each cuvette and recording $\Delta A/\Delta t$ as described above.

Protein Mass Spectrometry. Purified HRV-14 3C^{pro} (0.8 nmol) was incubated with a given Michael acceptor (10 nmol) in a volume of 0.4 mL for 60 min at room temperature, with other conditions as described for enzyme assays. Samples were then subjected to electrospray ionization mass spectrometry analysis using a PESciex API III triple stage quadrupole mass spectrometer. Sample was continuously infused into the interface at a rate of 10−20 μL/min. The instrument was operated in the positive ion detection mode with an ion spray voltage of 3500 V and an inlet orifice potential of 55 V. Mass spectra were collected over a mass range of 300−2000 U at 0.1 U intervals with a dwell time of 1 ms/interval. The final spectrum was obtained by averaging a total of 5−10 scans.

Plaque Reduction Assay for HRV-14. Confluent monolayers of HeLa cells were infected using 100 plaque-forming units/plate. Controls consisted of mock-infected plates. After an adsoprtion period of 30 min the innocula were replaced by 1.5 mL of a maintenance medium overlay containing 0.5% agarose and supplemented with various concentrations of test compound. The plates were incubated at 35 °C for 48 h, and the infected monolayers were then fixed with buffered 10% formalin and stained with crystal violet after removal of the overlay. The mean plaque number was calculated from a duplicate series of counts, converted to a percentage of

untreated controls, and plotted against the log concentration of test compound to determine the 50% inhibitory concentration (IC_{50}). Each experiment was repeated two or three times with results agreeing within ±25%. For each compound the value reported in Table 1 is the *highest* of the interpolated IC_{50} values, which thus gives the most conservative estimate of antiviral potency. As a control on consistency of the plaque reduction assay, the antirhinoviral compound enviroxime[31] was routinely included among the other test compounds assayed. In the three series of assays which affoded the data in Table 1, enviroxime showed IC_{50} concentrations of 0.045, 0.054, and 0.047 μg/mL.

Cytotoxicity Assay. HeLa cells were cultured without virus infection but with the test compounds as described above, and cell viability was determined by the metabolism of the tetrazolium dye XTT.[32] In this assay metabolically active cells transform XTT to a soluble, colored formazan product which is measured spectrophotometrically. Microtiter plates (96-well) were seeded with 10^4 cells/well in medium 199 containing Earl's balanced salt solution, 1% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and varying concentrations of test compound. Plates were incubated under 5% CO_2 at 37 °C for 3 days. Each well received 0.05 mL of XTT solution (1 mg/mL in phosphate-buffered saline containing 0.025 mM phenazine methosulfate). Plates were then incubated for an additional 4 h at 37 °C, and A_{450} was measured. The absorption was proportional to the number of viable cells in the well. The concentration of compound which produced a 50% reduction in the number of viable cells was designated the TC_{50} concentration.

Acknowledgment. We thank Dr. Elcira Villareal for antiviral activity testing, Mark Wakulchik for performing cytotoxicity testing, John Richardson for mass spectral analyses, and Drs. Carlos Lopez, Steven Kaldor, and Louis Jungheim for their advice and interest in this work.

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JM980114+