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In Vitro and Ex Vivo Inhibition of Hepatitis A Virus 3C Proteinase by a Peptidyl Monofluoromethyl Ketone†

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Abstract—Hepatitis A virus (HAV) 3C proteinase is the enzyme responsible for the processing of the viral polyprotein. Although a cysteine proteinase, it displays an active site configuration like those of the mammalian serine proteinases (Malcolm, B. A. *Protein Science* **1995**, *4*, 1439). A peptidyl monofluoromethyl ketone (peptidyl-FMK) based on the preferred peptide substrates for HAV 3C proteinase was generated by first coupling the precursor, *N,N*-dimethylglutamine fluoromethylalcohol, to the tripeptide, Ac-Leu-Ala-Ala-OH, and then oxidizing the product to the corresponding peptidyl-FMK (Ac-LAAQ′-FMK). This molecule was found to be an irreversible inactivator of HAV 3C with a second-order rate constant of 3.3×10^2 M^{-1} s^{-1}. ^{19}F NMR spectroscopy indicates the displacement of fluoride on inactivation of the enzyme by the fluoromethyl ketone. NMR spectroscopy of the complex between the ^{13}C-labeled inhibitor and the HAV 3C proteinase indicates that an (alkylthio)methyl ketone is formed. Studies of polyprotein processing, using various substrates generated by in vitro transcription/translation, demonstrated efficient blocking of even the most rapid proteolytic events such as cleavage of the 2A-2B and 2C-3A junctions. Subsequent ex vivo studies, to test for antiviral activity, show a 25-fold reduction in progeny virus production as the result of treatment with 5 μM inhibitor 24 h post-infection. © 1997 Elsevier Science Ltd.

Introduction

The hepatitis A virus (HAV), like all members of the picornaviral family, generates a single polyprotein of approximately 250 kD that undergoes multiple proteolytic cleavages, resulting in mature structural and nonstructural viral proteins. In HAV, these cleavages are apparently mediated solely by the 3C proteinase,[1] an enzyme present in all picornaviruses.[2,3] These 3C proteinases are structurally distinct from the papain family of proteinases and represent a new class of cysteine proteinase whose fold is similar to the chymotrypsin family of serine proteinases.[4]

A peptide aldehyde, Ac-Leu-Ala-Ala-(*N,N*-dimethylglutaminal), based on the preferred peptide substrate for HAV 3C proteinase, was synthesized and found to be a reversible, slow-binding inhibitor for HAV 3C with a K^*_i of 5.3×10^{-8} M.[5] This inhibitor showed a high degree of selectivity. Peptide aldehydes, although effective as enzyme inhibitors, are unlikely to be effective therapeutic agents because of metabolic degradation and transport problems. Peptidyl-fluoromethyl ketones,[6] however, have been shown to be effective in vivo in a number of systems,[7–9] and consequently a peptidyl monofluoromethyl ketone (peptidyl-FMK) inhibitor analogous to the peptide aldehyde was generated and tested for ability to arrest polyprotein processing in vitro and suppress viral replication ex vivo.

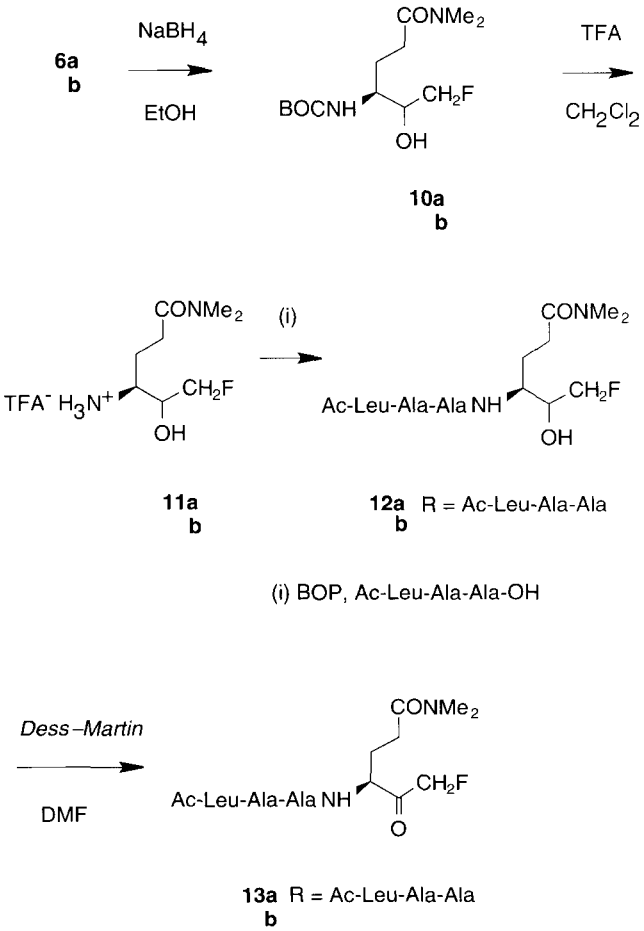
Chemistry

Peptide monofluoromethylketones (peptidyl-FMKs) have been of interest for some time in the field of enzyme inhibition[10] but their synthesis (via highly toxic fluoroacetic acid and with concomitant racemization) was only realized a decade ago.[11] They were subsequently found to be highly effective against cysteine proteinases,[7–9] but to be only marginally active against the mammalian serine counterparts.[12,13]

The picornaviral 3C proteinases, such as HAV 3C, are cysteine enzymes with a fold similar to the mammalian serine proteinases[14,15] and as such represent a new class of thiol enzymes distinct from the papain family.[4] Peptide aldehydes, in which the P1 amino acid residue[16] is replaced with the corresponding amino-aldehyde, have long been known to be unusually efficacious reversible inhibitors of the papain family of thiol proteinases[17] and were recently shown to be equally

effective against the HAV 3C enzyme.[5] Consequently, it was postulated that peptidyl-FMKs could also prove effective against the 3C proteinases. As peptidyl-FMKs are generally more stable than peptide aldehydes, this might permit the ex vivo investigation of proteinase inhibitors as antipicornaviral agents.

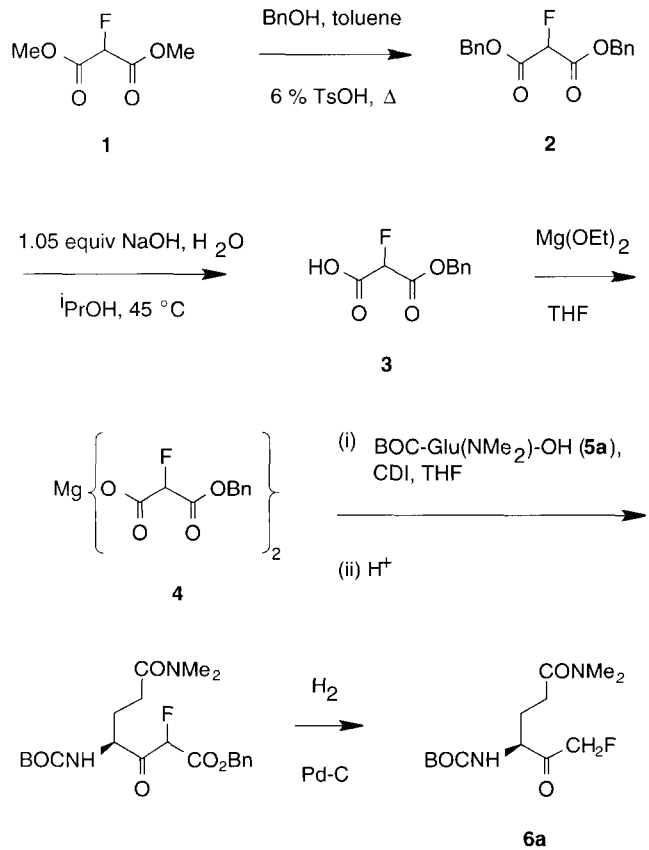
In order to synthesize a peptidyl-FMK homologous to the peptide aldehyde inhibitor of HAV 3C proteinase,[5] a conceptually similar strategy was employed; a chemically tractable precursor was generated, coupled to an appropriate peptide and subsequently converted to the reactive inhibitor. In order to generate a peptidyl-FMK tailored to the HAV 3C enzyme, the penultimate precursors were the fluoromethyl alcohol **10a** and the peptide, Ac-Leu-Ala-Ala-OH. Following coupling via standard procedures the tetrapeptide was converted to the inhibitor by oxidation with Dess-Martin periodinane (Scheme 1).[18] As with the synthesis of the peptide aldehyde inhibitor, the glutamine residue was replaced with *N,N*-dimethylglutamine (previously shown to have no impact on substrate peptide recognition or cleavage[5]) to avoid side-chain cyclization and the peptide fragment, Ac-Leu-Ala-Ala-OH, was substituted for the actual substrate sequence, Leu-Arg-Thr. This modified sequence greatly simplifies the synthesis by obviating deprotection and purification steps prior to oxidation of the fluoroalcohol **12a** and neither the arginine nor the threonine contribute significantly to the substrate specificity.[19]



Scheme 1.

The fluoroalcohol precursor **10a** is available by reduction of *N,N*-dimethylglutamine-fluoroketone **6a**, which was synthesized using the patented procedure of Palmer.[20] Applying this approach, magnesium benzyl fluoromalonate **4** is coupled to an *N*-protected amino acid **5a** using 1,1′-carbonyldiimidazole (Scheme 2) without racemization of the fluoroketone-bearing amino acid. BOC-*N,N*-dimethylglutamine **5a** was synthesized from BOC-glutamic acid α-benzyl ester following standard procedures.

The efficacies of acetyl-leucylalanylalanyl *N,N*-dimethylglutamine fluoroketone (Ac-LAAQ′-FMK, **13a**) and the fluoroalcohol precursor (Ac-LAAQ′-FMA, **12a**) at inhibiting peptide hydrolysis by HAV 3C proteinase were determined by progress curve analysis (see Experimental). The alcohol precursor (Ac-LAAQ′-FMA) showed no significant inhibition at concentrations up to 20 μM over the length of the assay (3 h). However, the Ac-LAAQ′-FMK showed irreversible inactivation kinetics with a second-order rate constant ($k_{obs}/[I]$) = 3.3 × 10^2 M^{-1} s^{-1} ([E] = 0.07 μM, [I] = 1.0 μM). Although inhibition of HAV 3C (a thiol enzyme with a serine proteinase fold) is roughly 40-fold slower than the corresponding inactivation of cathepsin B (a member of the papain family of thiol proteinases)



Scheme 2.

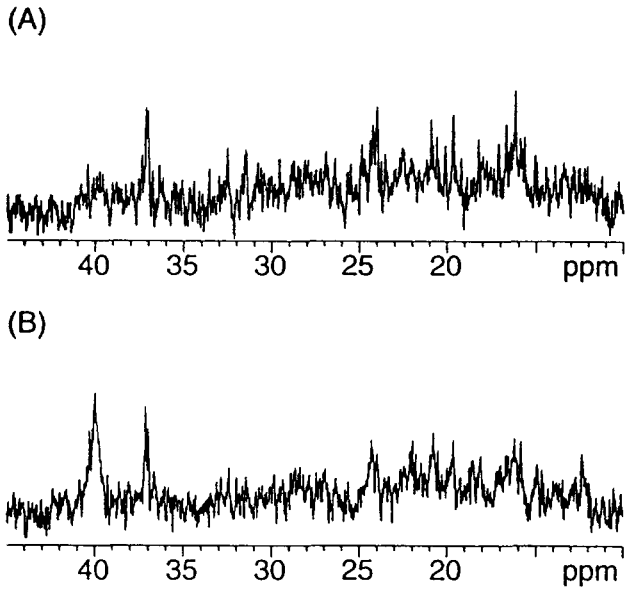


Figure 1. ^{13}C NMR spectra of HAV3C proteinase (A) and in complex with the ^{13}C-labeled inhibitor, Ac-LAAQ′-FMK (B). For experimental details see Experimental.

by a dipeptide-FMK inhibitor ($k_{obs}/[I] = 1.4 \times 10^4$ M^{-1} s^{-1}, 11), it is more than two orders of magnitude faster than the reaction of α-chymotrypsin (a serine proteinase) with an appropriate peptidyl-FMK ($k_{obs}/[I] = 1.7$ M^{-1} s^{-1}, 12).

Some studies have been conducted to establish the mechanism of action of peptidyl-FMKs and explain their unusual potency.[12] The mechanisms suggested are based on the inactivation of serine proteinases by peptidyl chloromethyl ketones (peptidyl-CMKs).[21-23] With peptidyl-CMKs, after the formation of the reversible Michaelis-type complex, a hemiacetal is formed with the serine nucleophile. Subsequent inactivation occurs either by direct displacement of the halogen by the $N^{\varepsilon 2}$ of the histidine (possibly even at the α-halo ketone stage), or alternatively, by conversion of the α-halo hemiacetal to an epoxide followed by alkylative ring-opening of this oxirane by the histidine.[10] Co-crystal structures of subtilisin BPN′[24] and thrombin[25] suggest that in addition to alkylation of the histidine a hemiacetal bond is also present in the inhibited complex. In contrast, in the papain-peptidyl-CMK complex, it appears that it is the sulfur of the cysteine residue which is alkylated.[26]

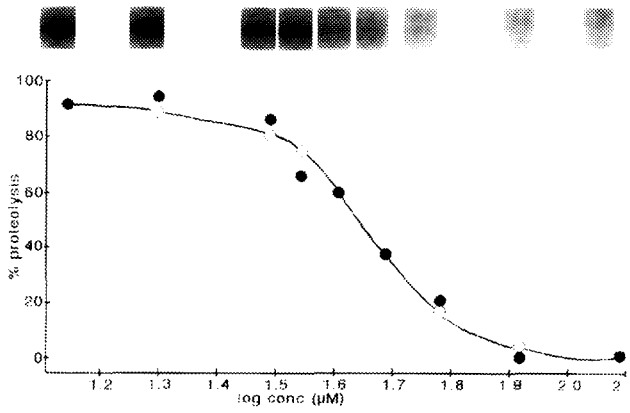
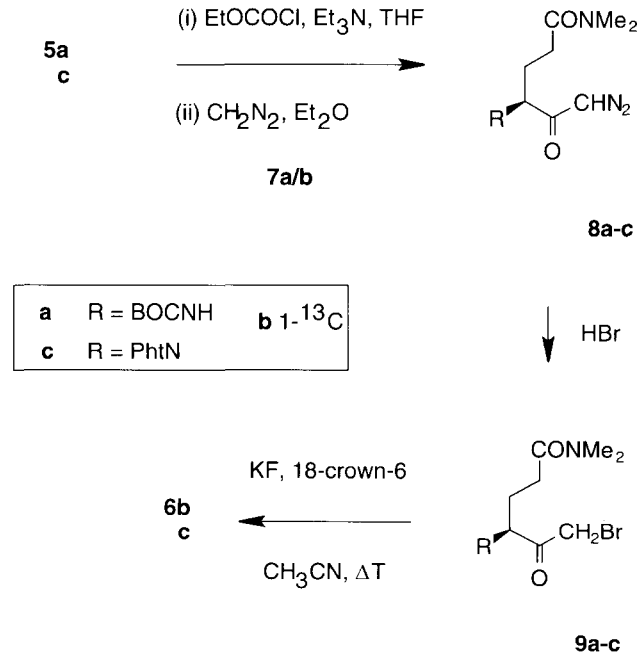


Figure 2. Inhibition of HAV 3C digestion of polyprotein substrate ΔVP1-P2-P3μ* by Ac-LAAQ′-FMK. Per cent proteolysis based on the level of the P3μ* product was estimated by densitimetry (see Experimental) and plotted against the log of inhibitor concentration (μM). HAV 3C concentration was 13 μM. Open circles represent points predicted values; filled circles represent actual datapoints.

Studies on peptidyl-FMK-enzyme complexes indicate that FMK inhibitors can also react differently with serine and cysteine proteinases. Amino acid analyses show that the mammalian serine proteinase, α-chymotrypsin, is alkylated on the histidine residue by a peptidyl-FMK,[12,13] whereas a recent crystal structure of the papain-like cysteine proteinase, cruzain, indicates that this enzyme appears to be alkylated on the cysteine residue.[7] In the former case the liberation of fluoride ion was observed by ^{19}F NMR.[12] The earlier studies suggest that fluorine hydrogen bonding and active site geometry may account for the alkylation of the histidine residue.[12,13] Picornaviral 3C proteinases, though utilizing cysteine nucleophiles, have active site geometries like those of the mammalian serine enzymes[14,15] and consequently the favored alkylation mechanism is difficult to predict a priori.

To determine experimentally the type of adduct formed between the peptidyl-FMK and the HAV 3C enzyme, the *N,N*-dimethyl glutamine-FMK, ^{13}C-labeled at the fluoromethyl group, was synthesized using a halogen exchange reaction (Scheme 3).[27] (As the yields in the final step with *N*-Pht derivatives were unsatisfactory, the synthesis was repeated with *N*-BOC-protected



Scheme 3.

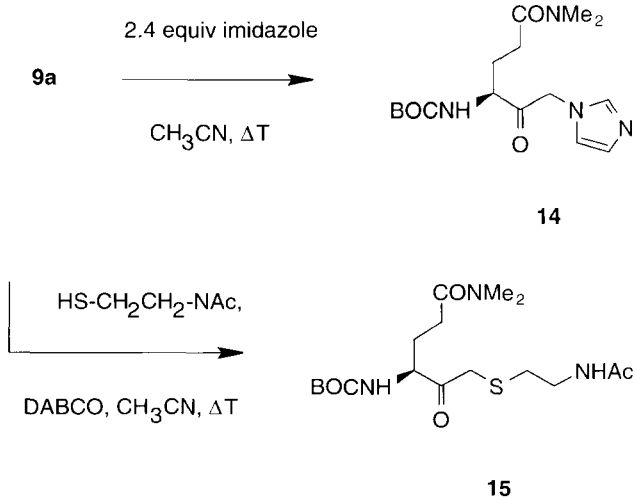
Table 1. Ex vivo inhibition of HAV replication by Ac-LAAQ′-FMK

Study	Log of viral titer[a] No inhibitor	5 μM inhibitor
HAV[b]		
Day 0	3.8	3.8
Day 1	5.3	3.9
Increase	32-fold	1.3-fold
VSV[c]		
Day 0	2.1	2.1
Day 1	5.1	4.8
Increase	1000-fold	500-fold

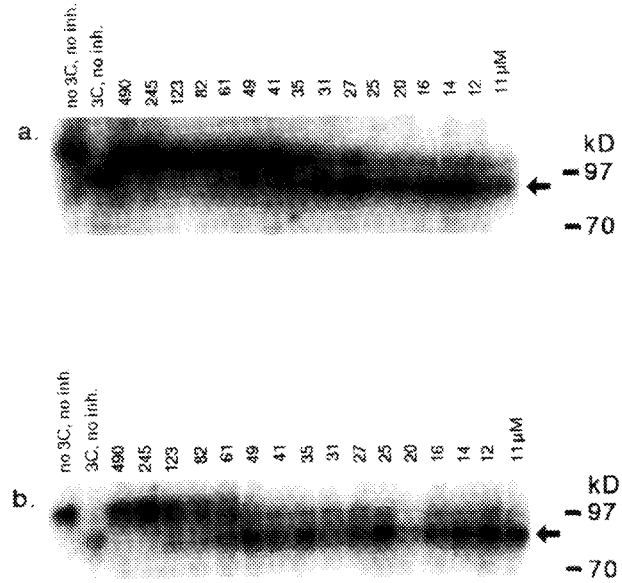
[a]Values calculated from the mean of triplicate wells.
[b]Titers from radioimmune focus assay (RIFA).
[c]Titers from neutral red staining.

derivatives.) Model compounds were also generated (Scheme 4) to assist in ascertaining whether alkylation occurs at the nitrogen or sulfur nucleophile. The chemical shifts of the methylene carbons adjacent to the heteroatoms in these model compounds are 51 ppm for the imidazoketone **14** and 38 ppm for the (alkylthio)ketone **15**. The latter value compares well with the observed shifts for similar compounds in the literature.[28] The methylene protons adjacent to the alkylated heteroatoms of both model compounds show rapid exchange in D_2O and consequently spectra were acquired in water.

The reaction of HAV 3C proteinase with the labeled peptidyl-FMK was monitored by ^{19}F NMR. Inactivation was accompanied by liberation of fluoride ions, as demonstrated by the characteristic shift of the fluoride signal at approximately −120 ppm, which is in good agreement with the literature.[12] To further corroborate the displacement of fluoride, the enzyme-inhibitor complex was analyzed by mass spectrometry. The difference in mass between the enzyme-inhibitor complex and the free enzyme is 471 Da, which matches the calculated value (data not shown).



Scheme 4.



c.

Cleavage Site	Aldehyde	FMK
2A-2B in P1-2ABC*	72	18
2A-2B in P1-2AB*	106	41
2C-3A in ΔVP1-P2-P3μ*	224	81

Figure 3. Inhibition of HAV 3C proteolysis of the 2A–2B junction of the P1-2AB* polyprotein substrate by the peptidyl-FMK (Ac-LAAQ′-FMK), panel (a) and the corresponding peptide aldehyde (Ac-LAAQ′-H; 5), panel (b). The arrow indicates the major product, P1-2A. HAV 3C proteinase concentration was 13 μM. Panel (c) summarizes the estimated IC_{50} values (μM) for both inhibitors against all three polyprotein substrates. HAV 3C concentration was 13 μM. For other experimental details see Experimental.

Following thorough dialysis (until no ^{19}F signal could be observed), the ^{13}C spectrum of the enzyme-inhibitor complex was obtained. Figure 1 shows the ^{13}C spectrum of HAV 3C alone and in complex with the inhibitor. In the enzyme complex, a new peak with a line width at half height of 78 Hz (corrected for line broadening) could be detected at 40 ppm, unequivocally indicating the formation of a (alkylthio)ketone. The broadness of the signal also agrees with previous studies on a similar inactivation product.[28] Hence, despite an active site geometry that resembles serine proteinases, HAV 3C is inactivated by a peptidyl-FMK in a manner analogous to cysteine proteinases of the papain family.

In vitro inhibition of polyprotein processing

In order to test whether this rather slow inactivator could measurably disrupt the processing cascade, protein substrates, representing various fragments of the HAV polyprotein, were synthesized by in vitro transcription/translation and subsequently digested by recombinant proteinase in the presence of various amounts of peptidyl-FMK. Multiprotein precursors containing multiple cleavage sites were used in an attempt to compare the effect of the inhibitor on the different proteolytic cleavages. The efficiency of proteolytic cleavage was estimated by the appearance of protein products.

In Figure 2, proteolysis of the P1-2AB* substrate at the 2A–2B junction was estimated by the level of P1-2A, the major product following cleavage.[1] Per cent proteolysis after 30 min has been plotted against the peptidyl-FMK concentration. Response was dose dependent and the concentration of inhibitor required to reduce proteolysis by 50% (IC_{50}) was calculated from the linear portion of the curve. The IC_{50} of the peptidyl-FMK was estimated to be roughly 41 μM (HAV 3C proteinase concentration 13 μM). For comparison, in the absence of inhibitor, the concentration of HAV 3C proteinase necessary to effect 50% proteolysis of this junction in 30 min (EC_{50}) was estimated to be 180 nM (data not shown).

Inhibition of cleavage of the 2A–2B junction was studied in parallel with both the peptide aldehyde and the peptidyl-FMK inhibitor (Fig. 3). The data, summarized as IC_{50} values, in panel (c), suggest that the peptidyl-FMK is the more effective inhibitor. Other proteinase concentrations were examined as well (data not shown). For all substrates and all proteinase concentrations tested, the peptidyl-FMK was the more effective inhibitor. This observation is consistent with the results obtained from parallel peptide proteolysis studies (in which an octapeptide representing the 2B–2C junction was the substrate) (data not shown).

The cleavage of the 2A–2B (EC_{50} = 180 nM) and 2C–3A junctions (EC_{50} = 380 nM) are the processing events that appear in the HAV system to be the most facile suggesting that they are primary steps in the processing cascade.[1,29] Hydrolysis of other sites in the polyproteins was also inhibited by the peptide aldehyde and the peptidyl-FMK (data not shown) with the apparent suppression tending to be inversely proportional to the rate of cleavage, as expected for ordered events (i.e., junctions that were cleaved later in the processing cascade appearing to be more sensitive to the inhibitor). Comparison of the cleavage of the 2A-2B junction in two slightly different polyprotein substrates, P1-2ABC* and P1-2AB*, gave comparable IC_{50} values (Fig. 3c).

Ex vivo inhibition of HAV replication

As the peptidyl-FMK appeared to have measurable effects on polyprotein cleavage in the reticulocyte lysate system, even at low micromolar concentrations, it was reasoned that viral replication should be effectively disrupted by similar dosages, as blockage of even one processing event would prevent the generation of a progeny virion from a given polyprotein. Consequently, an ex vivo study was initiated to determine whether HAV replication could be suppressed. Cells were preinfected with a cell culture-adapted strain of HAV and subsequently (24 h later) treated with the inhibitor. Ten percent DMSO was required to solubilize the inhibitor and to facilitate entry. Cells treated with DMSO only were followed and parallel experiments using VSV were conducted to ensure that the effects noted were due to the inhibitor and that the inhibitor was specific and did not simply affect the cells. Mock infected cells showed no loss in viability from either the DMSO or peptidyl-FMK treatment (data not shown).

Viral titers on day 0 and day 1 (Table 1) show that HAV replication was reduced 25-fold in the presence of the peptidyl-FMK compared with the DMSO-only controls. In contrast, vesicular stomatitis virus (VSV) titers were essentially unaffected by the inhibitor (Table 1). Although it is impossible to estimate the actual concentration of peptidyl-FMK in the cytosol, it is unlikely that it exceeded the concentration applied (5 μM) and in all probability was significantly lower. These data suggest that despite the relatively slow second-order rate constant for inactivation as measured in vitro, the inhibitor was nonetheless capable of inactivating the enzyme rapidly enough to significantly impact on viral replication.

Conclusion

Although previous studies had demonstrated that a tetrapeptide aldehyde could inhibit rhinovirus replication ex vivo,[30] the IC_{50} = ~500 μM was rather high. Whether this was due to inhibitor potency, the reversible nature of aldehyde inhibition or lability of the aldehyde moiety (i.e., short half-life), is unclear. By comparison, the peptidyl-FMK appears to suppress HAV replication with an IC_{90} < 5 μM. This apparent efficacy may reflect, in part, the relative slowness of HAV replication or the relatively greater potency of the inhibitor itself, the irreversible nature of the inhibition/the greater stability of the fluoroketone functional group (i.e., longer half-life).

Peptidyl monofluoromethyl ketones are unlikely to be effective therapeutic agents for HAV or other picornaviral infections in vivo because of poor oral bioavailability. However, the demonstration of their efficacy ex vivo provides confirmation of the 'proteinase inhibitor as antiviral paradigm' with the HAV system. Development of more medicinally useful molecules based on the fluromethyl ketone functionality and other moieties are currently underway.

Experimental

Proteinase production and purification

Recombinant C24S HAV 3C proteinase (a mutant in which the nonessential surface cysteine was replaced with serine and which exhibits identical catalytic parameters to wild-type enzyme, unpublished results) was expressed in *Escherichia coli* and purified as reported previously.[31] Purity of the enzyme samples was greater than 90% as determined by SDS-PAGE analysis (data not shown). Proteinase concentrations were determined spectrophotometrically e = 1.2 mg mL^{-1}.

Peptide synthesis

The peptide substrates and fragments were synthesized using solid-phase Fmoc chemistry on Rink resin as previously described.[5] All peptides were purified by reverse-phase HPLC (C-18, 5 × 25 cm, Vydac, 2%/min linear gradient of 0.1% TFA:water adding 0.1% TFA:acetonitrile). Peptide structures were verified by NMR and mass spectrometry.

Synthesis of peptidyl monofluoromethyl ketones and precursors

General procedures and instrumentation employed in chemical syntheses have been previously described.[32] Dimethyl fluoromalonate (**1**) was purchased from PCR Inc., Gainesville, FL. (4*S*)-4-*tert*-butyloxycarbonylamino-6-fluoro-5-oxo-*N*,*N*-dimethylhexanamide (**6a**) was initially prepared by Prototek Inc., Dublin, CA.

Dibenzyl fluoromalonate (2). A mixture of dimethyl ester **1** (10.2 g, 67.9 mmol), toluene (28 mL), benzyl alcohol (34 mL, 330 mmol) and *p*-toluenesulfonic acid (760 mg, 4 mmol) in a 3-neck 100-mL round-bottom flask connected to a Dean-Stark apparatus was heated (75 °C oil bath) in vacuo (27 mm of Hg) until all of the toluene had distilled, then (75 mm Hg, 112 °C) for an additional 5 h. The mixture was allowed to cool to 75 °C and isopropanol (15 mL) was added, then hexane (30 mL). The product crystallized and was placed in the freezer overnight. The product was filtered, washed with hexane (2 × 30 mL), and dried overnight in vacuo, giving dibenzyl ester **2** (18.5 g, 90%) as a solid: mp 45.5–47.5 °C; IR (microscope) 1744, 1455, 1275, 1187, 1177, 741, 699 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.35 (10H, m), 5.36 (1H, d, J = 48 Hz), 5.25 (4H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.6 (d, $^2J_{C\text{-}F}$ = 24 Hz), 134.3, 128.7, 128.6, 128.4, 85.2 (d, $^1J_{C\text{-}F}$ = 198 Hz), 68.1; MS (CI, NH_3) m/z (relative intensity) 320.1 ($MH^+ + NH_3$, 100%).

Benzyl fluoromalonate (3). Dibenzyl ester **2** (6.20 g, 20.5 mmol) was suspended in isopropanol (34 mL) in a 200-mL Erlenmeyer flask. The mixture was heated to 45 °C, by which time the solids had dissolved. 1 M aqueous NaOH (21.5 mL, 21.5 mmol) was added dropwise over 1 h. After an additional 10 min, the solution was concentrated to 15 mL, then water was added (7.5 mL). The pH of the solution was adjusted to 9 using saturated $NaHCO_3$. The mixture was washed with CH_2Cl_2 (2 × 7.5 mL) to remove benzyl alcohol. The pH of the aqueous layer was adjusted to 2.2 with 5 M HCl. The mixture was extracted with diisopropyl ether (15 mL). The pH of the aqueous layer was adjusted to 1.9 with 5 M HCl and further extracted with disopropyl ether (15 mL). The combined extracts were washed with brine (10 mL), dried ($MgSO_4$), filtered, and evaporated in vacuo below 35 °C. The oily residue was triturated with hexane (20 mL) overnight. The solid was filtered and dried under vacuum to give monoester **3** (2.22 g, 51%) as a solid: mp 121 °C dec; IR (microscope) 3400–2500 (br), 1759, 1735, 1457, 1263, 1247, 1185, 1116, 754, 700 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 8.75 (1H, br s), 7.35 (5H, m), 5.39 (1H, d, J = 48 Hz), 5.31 (2H, s); ^{19}F NMR (376 MHz, $CDCl_3$) δ −195.3 (d, J = 48 Hz); MS (CI, NH_3) m/z (relative intensity) 230.1 ($MH^+ + NH_3$, 100%).

Benzyl fluoromalonate, magnesium salt (4). A solution of the monoester **3** (1.12 g, 5.28 mmol) in THF was treated with magnesium ethoxide (310 mg, 2.64 mmol). The mixture was stirred vigorously for 2 h and then filtered through a celite pad. The solid was washed with THF (2 × 2.5 mL). The filtrate was poured into hexane (55 mL) with vigorous stirring. The white precipitate was immediately filtered, washed with hexane (2 × 5 mL), and the filter cake was dried under vacuum overnight to give **4** (920 mg, 78%): IR (microscope) 1754, 1678, 1456, 1275, 1216, 1188, 1122, 738, 698 cm^{-1}; ^1H NMR (360 MHz, D_2O) δ 7.48 (10H, m), 5.37 (1H, d, J = 50 Hz), 5.34 (2H, s); ^{19}F NMR (376 MHz, $CDCl_3$) δ −186.0 (d, J = 50 Hz); MS (CI, NH_3) m/z (relative intensity) 230.1 ($MH^+ + NH_3$, 100%).

(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-fluoro-5-oxo-hexanamide (6a). CDI (370 mg, 2 mmol) was added to a solution of *N*-*t*-BOC-L-glutamic acid γ-dimethylamide (**5a**, 550 mg, 2 mmol) in THF (10 mL) and stirred for 1 h. Magnesium salt **4** (470 mg, 1 mmol) was added as a fine powder, and the mixture was stirred for 6 h. Then the mixture was washed with 1 N HCl (2 mL). The aqueous layer was extracted with toluene (2 × 4 mL), the combined organic extracts were washed with saturated $NaHCO_3$ (2 mL), brine (2 mL), dried ($MgSO_4$), and concentrated in vacuo to approximately 4 mL, at 30 °C or below. An analytical sample (ca. 40 μL) was evaporated in vacuo to give the α-fluoro-β-keto ester (3.0 mg, 35%) as an oil: ^1H NMR (360 MHz, $CDCl_3$, mixture of epimers ca. 1:1) δ 7.35 (5H, m), 5.69, 5.61, 5.58 (1.5 H, m and 2 × d, J = 48 Hz), 5.67 (0.5H, br d), 5.28 (1H, s), 5.17 (1H, q_{AB}, J_{AB} = 12 Hz), 4.66 (0.5H, m), 4.34 (0.5H, m), 2.97–2.90 (6H, 4s), 2.48–1.95 (2H, m), 1.41, 1.42 (9H, s). The rest of the solution in toluene was hydrogenated overnight at 1 atm H_2 in presence of palladium catalyst (100 mg, 10% on charcoal). The solution was filtered, washed with 1 N HCl (50 mL), $NaHCO_3$, brine, and dried ($MgSO_4$).

Solvent was evaporated in vacuo at 30 °C or below, to give the fluoroketone (**6a**, 184 mg, 32% over both steps) as an oil: IR ($CHCl_3$ cast) 3293 (br), 1741, 1707, 1635, 1167 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.65 (1H, br s), 5.09 (2H, dq_{AB}, $J_{C\text{-}F}$ = 47 Hz, J_{AB} = 16 Hz), 4.51 (1H, m), 2.99 (3H, s), 2.94 (3H, s), 2.46 (1H, dt, J = 17 Hz), 2.38 (1H, dt, J = 17 Hz), 2.19 (1H, m), 1.97 (1H, m), 1.44 (9H, s); ^{13}C NMR (75 MHz, CDCl3) δ 205.1, 171.9, 155.8, 84.0 (d, $J_{C\text{-}F}$ = 184 Hz), 80.1, 56.3, 37.3, 35.6, 28.8, 28.3, 26.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ −232.2 (t, $J_{F\text{-}H}$ = 47 Hz); HRMS (EI) calcd for $C_{13}H_{23}O_4FN_2$ (M^+): 290.16418. Found: 290.16443.

(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-diazo-5-oxo-hexanamide (8a). Triethylamine (0.35 mL, 2.5 mmol) and ethyl chloroformate (0.25 mL, 2.5 mmol) were added to a cooled (0 °C) solution of *N*-*t*-BOC-l-glutamic acid-γ-dimethylamide (**5a**, 650 mg, 2.5 mmol) in THF (45 mL). The mixture was stirred at 0 °C for 5 min and then filtered into ca. 0.3 M ethereal diazomethane (**7a**) solution (17 mL, ca. 5 mmol) at 0 °C. The yellow mixture slowly paled and the product crystallized. After 1 h, additional ether (17 mL) was added and the flask was placed in the freezer overnight. The product was filtered, washed with ether (2 × 2 mL), and dried in vacuo, to give the diazoketone **8a** (482 mg, 65%) as off-white needles (mp 112–113 °C). This material contained approx. 4% *N*-*t*-BOC-l-glutamic acid-α-methyl ester-γ-dimethylamide, as evidenced by ^1H NMR. An analytical sample was purified by flash chromatography (ethyl acetate, SiO_2) to give a solid: mp 120–121 °C; $[\alpha]_D$ −1.7 (*c* 1.07; $CHCl_3$); IR (microscope) 3216 (br), 2114, 1708, 1628, 1614, 1545 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.68 (1H, br s), 5.61 (1H, br s), 4.19 (1H, br s), 2.99 (3H, s), 2.95 (3H, s), 2.51 (1H, ddd, J = 16, 6 Hz), 2.33 (1H, dt, J = 16, 6 Hz), 2.16 (1H, m), 1.98 (1H, ddt, J = 6 Hz), 1.43 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.2, 172.1, 155.7, 79.7, 57.5, 53.5, 37.1, 35.5, 29.1, 28.3, 27.7; MS (FAB, Cleland) *m/z* (relative intensity) 298.9 (MH^+, 11%); Anal. calcd for $C_{13}H_{22}O_4N_4$: C, 52.32; H, 7.44; N; 18.79. Found: C, 52.43; H, 7.39; N, 18.47.

[1-^{13}C]-(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-diazo-5-oxo-hexanamide (8b). Reaction of **5a** (954 mg, 3.5 mmol) and [^{13}C]-diazomethane solution (**7b**, 35 mL, ca. 3.5 mmol, >95% isotopic purity) as described for unlabeled **7a** gave labeled **8b** (437 mg, 42%): IR (microscope) 2110 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.61 (br d, $J_{C\text{-}H}$ = 202 Hz), remaining data as for **8a**; ^{13}C NMR (75 MHz, $CDCl_3$) δ 53.5; MS (FAB, Cleland) *m/z* (relative intensity) 300.1 (MH^+, 11%).

(4*S*)-*N*,*N*-Dimethyl-6-diazo-5-oxo-4-phthalimido-hexanamide (8c). Triethylamine (0.58 mL, 4.2 mmol) and ethyl chloroformate (1.1 mL, 4.2 mmol) were added to a cooled (0 °C) solution of *N*-phthaloyl-l-glutamic acid-γ-dimethylamide (**5c**, 1.05 g, 3.5 mmol) in THF (50 mL) . The mixture was stirred at 0 °C for 5 min and then filtered quickly into ca. 0.3 M ethereal diazomethane (**7a**) solution (47 mL, ca. 17 mmol) at 0 °C. The yellow mixture slowly paled on warming to 20 °C overnight. The solvent was removed in vacuo and the residue washed with water (10 mL) and ethyl acetate (3 × 10 mL). The sample was dried ($MgSO_4$) and concentrated in vacuo, giving the diazoketone **8c** (1.05 g, 92%) as an oil: IR (CH_2Cl_2 cast) 3093 (br), 2108, 1713, 1638, 1385, 1339, 720 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.88 (2H, m), 7.79 (2H, m), 5.61 (1H, s), 4.92 (1H, m), 2.95 (3H, s), 2.89 (3H, s), 2.59 (2H, m), 2.39 (2H, m); MS (FAB, Cleland) *m/z* (relative intensity) 329.9 (MH^+, 1.1%).

(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-bromo-5-oxo-hexanamide (9a). Aqueous hydrobromic acid (48%, 81 μL, 0.72 mmol) was added to a cooled (0 °C), vigorously stirred solution of the diazoketone **8a** (213 mg, 0.715 mmol) in CH_2Cl_2 (40 mL). After 1 h the mixture was washed with brine, dried ($MgSO_4$), and the solvent was evaporated in vacuo to give an oil. After trituration with hexane (10 mL) for 1 h, the solid was filtered and dried with vacuum to give the bromoketone **9a** (229 mg, 91%) as a solid (mp 61 °C): IR (microscope) 3227 (br), 1735, 1697, 1612, 1525, 1158 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.70 (1H, br s), 4.53 (1H, br s), 4.19 (2H, q_{AB}, J_{AB} = 13 Hz), 2.99 (3H, s), 2.94 (3H, s), 2.47 (1H, ddd, J = 17 Hz), 2.35 (1H, dt, J = 17 Hz), 2.22 (1H, m), 2.00 (1H, m), 1.44 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.1, 171.8, 155.6, 79.9, 57.4, 37.1, 35.5, 32.5, 28.7, 28.1, 26.3; HRMS (EI) calcd for $C_{13}H_{23}O_4N_2{}^{81}Br$ (M^+): 352.08206. Found: 352.08116.

[1-^{13}C]-(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-bromo-5-oxo-hexanamide (9b). Reaction of hydrobromic acid (113 μL, 1 mmol) and labeled diazoketone **8b** (285 mg, 0.95 mmol, >90% isotopic purity) as described for unlabeled **8a** gave an oil, which was purified by flash chromatography (SiO_2, 3.6 g), giving labeled bromomethyl ketone **9b** (300 mg, 72%) and the starting diazomethyl ketone **8b** (40 mg, 25% recovery) as a solid: ^1H NMR (360 MHz, $CDCl_3$) δ 4.19 (dq_{AB}, $J_{C\text{-}H}$ = 151.2 Hz), remaining data as for **9a**; ^{13}C NMR (75 MHz, $CDCl_3$) δ 32.5; HRMS (EI) calcd for $C_{12}H_{23}O_4N_2{}^{13}C^{81}Br$ (M^+): 351.08542. Found: 351.08514.

(4*S*)-*N*,*N*-Dimethyl-6-bromo-5-oxo-4-phthalimido-hexanamide (9c). Aqueous hydrobromic acid (48%) (0.23 mL, 2.05 mmol) was added to a cooled (0 °C), vigorously stirred solution of the diazoketone **8c** (561 mg, 1.71 mmol) in THF (20 mL). The solution was stirred for 1 h until gas evolution ceased, and then CH_2Cl_2 (20 mL) was added. The solution was washed with water (3 × 10 mL), dried over ($MgSO_4$), and the solvent evaporated in vacuo to give the bromoketone **9c** (0.638g, 98%) as an oil: IR ($CHCl_3$ cast) 2938 (br), 1774, 1714, 1641 1412, 1384, 721, 529 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.89 (2H, m), 7.78 (2H, m), 5.19 (1H, m), 4.07 (2H, q_{AB}, J_{AB} = 11 Hz), 2.91 (3H, s), 2.81 (3H, s), 2.62 (1H, m), 2.48 (1H, m), 2.38 (2H, m); MS (CI, NH_3) *m/z* (relative intensity) 380.8 (MH^+, 49%).

[1-^{13}C]-(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-fluoro-5-oxo-hexanamide (6b). Potassium fluoride (58 mg, 1 mmol, dried at 100 °C under high vacuum for 24 h) and 18-crown-6 (5 mg, 0.02 mmol) were heated in

CH_3CN (0.5 mL) for 30 min with vigorous stirring (oil bath at 85 °C). Bromide **9b** (58 mg, 0.165 mmol, >95% isotopic purity) was added. The mixture was heated for an additional 2 h at 85 °C oil-bath temperature with vigorous stirring. The mixture was allowed to cool, and then the solvent evaporated in vacuo. Purification by flash chromatography (2 g SiO_2, ether) gave the fluoroketone **6b** (22 mg, 46%) as an oil, which solidified on storage: ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.19 (ddq_{AB}, J_{C-H} = 153 Hz), remaining data as for **6a**; ^{13}C NMR (75 MHz, $CDCl_3$) δ 84.0 (d, J_{C-F} = 184 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ −232.4 (dt, J_{C-F} = 184 Hz, J_{F-H} = 47 Hz); HRMS (EI) calcd for $C_{12}H_{23}O_4N_2{}^{13}CF$ (M^+): 291.16754. Found: 291.16759.

(4*S*)-*N*,*N*-Dimethyl-6-fluoro-5-oxo-4-phthalimido-hexanamide (6c). Potassium fluoride (145 mg, 2.5 mmol, dried at 100 °C under high vacuum for 24 h) and 18-crown-6 (16.4 mg, 0.06 mmol) were heated in CH_3CN (10 mL) for 30 min with vigorous stirring (oil bath at 90 °C). The mixture was allowed to cool, and then the bromide **9c** (237 mg, 0.622 mmol) was added. The mixture was heated for an additional 15 h at 90 °C oil-bath temperature with vigorous stirring. The mixture was allowed to cool, and then the solvent evaporated in vacuo. Purification by HPLC (Resolve C18 Prepak R 25 × 100 mm, 15 mL min^{-1} gradient elution, 8–44% acetonitrile:water) gave the fluoroketone **6c** (40.5 mg, 20%) as an oil: IR (CH_2Cl_2 cast) 3500 (br), 2933, 1778, 1740, 1737,1639, 1468, 1386, 724 cm^{-1}; ^{1}H NMR (360 MHz, $CDCl_3$) δ 7.88 (2H, m), 7.78 (2H, m), 5.09 (1H, m), 5.01 (2H, dq_{AB}, J_{F-H} = 47 Hz, J_{AB} = 16 Hz), 3.95 (3H, s), 3.85 (3H, s), 2.59 (1H, m), 2.41 (1H, m), 2.38 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.1 (d, $^2J_{C-F}$ = 18 Hz), 171.1, 167.7, 134.4, 131.6, 123.7, 84.4 (d, J_{C-F} = 185 Hz), 56.1, 37.1, 35.4, 29.2, 23.1; MS (CI, NH_3) *m/z* (relative intensity) 321.9 (MH^+, 18%).

(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-fluoro-5-hydroxy-hexanamide (10a). Sodium borohydride (13 mg, 0.34 mmol) in ethanol (1.5 mL) was added to a cooled (0 °C) solution of the ketone **6a** (240 mg, 0.83 mmol) in ethanol (1.5 mL). The mixture was then stirred at 20 °C for 1 h, concentrated in vacuo, dissolved in water (2 mL) and acidified to pH 1.5 with 1 N sulfuric acid. The mixture was immediately extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with brine (6 mL), then dried ($MgSO_4$), and the solvent evaporated in vacuo to yield the fluoroalcohol **10a** (215 mg, 89%) as a solid. The diastereomers (3:1) were separated by HPLC (Waters Resolve C-18 reverse phase, 8 × 200 mm, gradient elution, 8–32% acetonitrile:water); data for major diastereomer (mp 124–127 °C): IR (microscope) 3326 (br), 1699, 1625, 1526, 1162, 1048, 1030 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$), δ 5.28 (1H, br d), 4.47 (2H, ddq_{AB}, $^1J_{F-H}$ = 47 Hz, J_{AB} = 10 Hz, $^2J_{H-H}$ = 6 Hz), 3.91 (1H, br d), 3.83 (1H, br d, $^2J_{F-H}$ = 19 Hz), 3.68 (1H, m), 2.99 (3H, s), 2.93 (3H, s), 2.41 (2H, m), 1.92 (2H, m), 1.41 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 156.6, 84.8 (d, $^1J_{C-F}$ = 168 Hz), 79.7, 73.0 (d, $^2J_{C-F}$ = 19 Hz), 52.8, 37.3, 35.7, 29.8, 28.4, 25.4; ^{19}F NMR (376 MHz, $CDCl_3$) δ −229.7 (dt, $^1J_{F-H}$ = 47 Hz, $^2J_{F-H}$ = 19 Hz); HRMS (EI) calcd for $C_{13}H_{25}O_4FN_2$ (M^+): 292.17984. Found: 292.18009.

[1-^{13}C]-(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-fluoro-5-hydroxy-hexanamide (10b). Reduction of labeled **6b** (20 mg, 74 μmol, isotopic purity >90%) as described for unlabeled **6a** gave labeled **10b** (19 mg, 95%): ^{1}H NMR (360 MHz, D_2O) δ 4.54 ($dddq_{AB}$, J_{C-H} = 152 Hz), 3.80 (br d, $^2J_{H-F}$ = 19 Hz), remaining data as for **10a**; ^{13}C NMR (75 MHz, $CDCl_3$) δ 84.8 (d, J_{C-F} = 168 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ −230.2 (ddt, J_{C-F} = 168 Hz, J_{F-H} = 47, 19 Hz); HRMS (EI) calcd for $C_{12}H_{25}O_4FN_2{}^{13}C$ (M^+): 293.18320. Found: 293.18159.

(4*S*)-*N*,*N*-Dimethyl-4-amino-6-fluoro-5-hydroxy-hexanamide, trifluoroacetate salt (11a). Trifluoroacetic acid (0.3 mL) was added to a cooled (0 °C) solution of **10a** (34 mg, 0.116 mmol) in CH_2Cl_2 (1.2 mL). The solution was stirred and warmed to 12 °C over 1 h. The solvent evaporated in vacuo and the residue was dissolved in CH_2Cl_2 (1.2 mL) and cooled to 0 °C. Further trifluoroacetic acid (0.3 mL) was added and the mixture was allowed to warm to 20 °C over 1.5 h. The solvent was evaporated in vacuo, toluene and diethyl ether were added and evaporated, and the residue was dried under high vacuum to yield the trifluoroacetate salt **11a** (34 mg, 95%). The material was used without further purification; data for major diastereoisomer: IR ($CHCl_3$ cast) 3050 (br), 1677, 1625, 1505, 1180, 1132 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (3H, bs), 4.87 (1H, br), 4.56 (2H, ddq_{AB}, $^1J_{F-H}$ = 47 Hz, J_{AB} = 10 Hz, $^2J_{H-H}$ = 5 Hz), 4.23 (1H, d, $^2J_{H-F}$ = 19 Hz), 3.43 (1H, m), 3.01 (3H, s), 2.94 (3H, s), 2.78 (1H, dt, *J* = 18 Hz), 2.53 (1H, dt, *J* = 18 Hz), 2.01 (2H, m); ^{13}C NMR (75 MHz, $CDCl_3$, CF_3CO_2—signals too weak to assign) δ 173.3, 83.4 (d, $^1J_{C-F}$ = 170 Hz), 68.7 (d, $^2J_{C-F}$ = 20 Hz), 54.1, 37.4, 35.8, 30.8, 22.7; ^{19}F NMR (376 MHz, $CDCl_3$) δ −75.9 (3F, s), −230.6 (1F, dt, $^1J_{F-H}$ = 47 Hz, $^2J_{F-H}$ = 19 Hz); HRMS (EI) calcd for $C_8H_{18}O_2FN_2$ (M^+ $-CF_3CO_2-$): 193.13524. Found: 193.13523.

[1-^{13}]-(4*S*)-*N*,*N*-Dimethyl-4-amino-6-fluoro-5-hydroxy-hexanamide, trifluoroacetate salt (11b). Reaction of trifluoroacetic acid (200 μL) and labeled **10b** (16.7 mg, 57 μmol, >90% isotopic purity) as described for unlabeled **10a** gave **11b** (16.2 mg, 95%). Data for major diastereoisomer: ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.78, 4.50–4.17 (1H and 3H, ddq_{AB} and m, J_{C-H} = 152 Hz), remaining data as for **11a**; ^{13}C NMR (75 MHz, $CDCl_3$) δ 83.5 (d, J_{C-F} = 170 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ −75.9 (3F, s), −231.1 (1F, ddt, J_{C-F} = 170 Hz, J_{F-H} = 47, 19 Hz); HRMS (EI) calcd for $C_7H_{18}O_2FN_2{}^{13}C$ (M^+ $-CF_3CO_2-$): 194.13858. Found: 194.13810.

(4*S*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)amino-6-fluoro-5-hydroxy-hexanamide (12a). Triethylamine (8 μL, 56 μmol) was added to a solution of *N*-acetylleucylalanylalanine (17.5 mg, 56 μmol) and BOP (24.5 mg, 55 μmol) in DMF (450 μL) at 0 °C. The solution was stirred at 0 °C for 5 min, then added dropwise over 10 min to a solution of the trifluoroacetate salt **11a** (17 mg,

56 μmol) and triethylamine (8 μL, 56 μmol) in DMF (450 μL) also at 0 °C. The mixture was stirred at 0 °C for 2 h, then the cold bath was removed and stirring was continued 3 h. The mixture was dried in vacuo overnight, and the residue was purified by HPLC (Waters Resolve C-18 reverse phase, 8 × 200 mm, gradient elution, 0–20% acetonitrile:water) to yield **12a** (13.7 mg, 50%) as a solid: IR (KBr disk) 3410 (br), 3315 (br), 2960, 2932, 1654, 1541, 1457, 1203, 1141 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 4.60–4.65, 4.48–4.53 (1H, br d, J = 47 Hz), 4.52–4.58, 4.40–4.46 (1H, br d, J = 47 Hz), 4.23–4.36 (3H, m), 3.74–3.99 (2H, m), 3.08 (3H, s), 2.95 (3H, s), 2.41–2.50 (2H, m), 2.08–2.17 (1H, m), 2.06 (3H, s), 1.56–1.79 (4H, m), 1.43 (6H, dd, J = 7, 7 Hz), 0.97 (3H, d, 7 Hz), 0.93 (3H, d, 7 Hz); MS (FAB, Cleland) 490.35 (MH^+, 100%).

[1-^{13}C]-(4*S*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)-amino-6-fluoro-5-hydroxy-hexanamide (12b). Reaction of *N*-acetylleucylalanylalanine (13 mg, 41 μmol) and labeled **11b** (15 mg, 41 μmol, >90% isotopic purity) as described for unlabeled **11a** gave labeled **12b** (12 mg, 60%): ^1H NMR (360 MHz, $CDCl_3$) δ 4.45–4.21 (4H, m), 3.99–3.75 (3H, m) remaining data as for **12a**; ^{13}C NMR (75 MHz, CD_3CN) δ 85.9, 85.9, 86.0 (each d, $J_{C\text{-}F}$ = 167 Hz); $^{19}F\{^1H\}$ NMR (376 MHz, D_2O) δ −231.5, −231.7, −231.9, −232.0 (each d, $J_{C\text{-}F}$ = 170 Hz); MS (FAB, Cleland) *m/z* (relative intensity) 491.3 (MH^+, 21%).

(4*S*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)amino-6-fluoro-5-oxo-hexanamide (13a). Dess–Martin periodinane (40 mg, 56 μmol) was added to a solution of the fluoroalcohol **12a** (15 mg, 31 μmol) in DMF (800 μL) at 20 °C. The mixture was stirred for 2 h, then evaporated in vacuo and dried under high vacuum. The residue was triturated with Et_2O and purified by HPLC (Waters Resolve C-18 reverse phase, 8 × 200 mm, gradient elution, 0–20% acetonitrile:water) to yield the fluoroketone **13a** (13.5 mg, 85%) as a solid: IR (microscope) 3281 (br), 1741, 1641, 1540 cm^{-1}; ^1H NMR (500 MHz, CD_3CN) δ 7.72–6.86 (4H, m), 5.26–5.19 and 5.16–4.93 (2H, 8 dt, each $J_{H\text{-}F}$ =47 Hz, J_{AB} = 17 Hz), 4.39–4.29 (1H, m), 4.25–3.98 (3H, m), 2.94–2.90 (3H, 4 s), 2.86–2.82 (3H, 3 s), 2.45–2.28 (2H, m), 2.12–1.89 (m, obscured by solvent), 1.72–1.63 (1H, m), 1.54–1.45 (2H, m), 1.35–1.28 (6H, m), 0.94–0.91 (3H, m), 0.90–0.88 (3H, m); ^1H NMR (500 MHz, D_2O) δ 5.17 (0.8H, dd, J = 47, 18 Hz, ketone), 4.42–4.24 (4.6H, m), 3.82–3.88 (0.6H, m, *CH*$CH(OH)_2CH_2F$), 3.11–3.08 (1H, m), 3.04–3.03 (3H, 4 s), 2.91 (3H, s); 2.51–2.44 (0.6H, m), 2.44–2.36 (0.6H, m), 2.25–2.17 (0.4H, m), 2.16–2.08 (0.4H), 2.02, 2.01 (3H, 2 s), 1.91–1.85, 1.76–1.52 (5H, m), 1.42–1.30 (6H, m), 0.94–0.91 (3H, m), 0.90–0.88 (3H, m); {^1H, ^{13}C} HMQC (500 MHz, CD_3CN), ^{13}C dimension, δ 86.5 (d, $J_{C\text{-}F}$ = ca. 47 Hz), 58.5, 57.0, 52.5, 43.0, 39.0, 37.5, 31.5, 28.0, 27.5, 25.0, 23.5, 19.0; {^1H, ^{13}C} HMBC (500 MHz, CD_3CN), ^{13}C dimension, δ 200, 176.5, 174.5; ^{19}F NMR (470 MHz, CD_3CN) δ −232.5, −232.9, −233.1, −233.3 (2.0:1.3:1.1:1.0, each t, $J_{F\text{-}H}$ = 47 Hz); MS (FAB, Cleland) *m/z* (relative intensity) 488.0 (MH^+, 73%).

[1-^{13}C]-(4*S*)-*N*,*N*-Dimethyl-4-(acetylleucylalanylalanyl)-amino-6-fluoro-5-oxo-hexanamide (13b). Oxidation of labeled **12b** (10.2 mg, 20 μmol, >90% isotopic purity) as described for unlabeled **12a** gave labeled **13b** (7.0 mg, 78%) as a solid: ^1H NMR (500 MHz, D_2O) δ 5.17 (0.8H, ddd, $J_{C\text{-}H}$= 154 Hz, $J_{F\text{-}H}$ = 47 Hz, J_{AB} = 18 Hz); 4.58–4.37, 4.28–4.04 (4.6H, 2 m), remaining data as for **13a**; $^{19}F\{^1H\}$ NMR (376 MHz, D_2O) δ −231.5, −231.7, −231.9, −232.0 (each d, $J_{C\text{-}F}$ = 170 Hz); MS (FAB, Cleland) *m/z* (relative intensity) 489.2 (MH^+, 0.6%).

(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-(imidazol-2-yl-methyl)-5-oxo-hexanamide (14). A solution of imidazole (17 mg, 0.25 mmol) and the bromoketone **9a** (35 mg, 0.1 mmol) in CH_3CN was heated to 50 °C for 4 h. The mixture was allowed to cool, and the solvent evaporated in vacuo. Purification by flash chromatography (SiO_2, 10% methanol in chloroform with 0.1% triethylamine) gave the imidazoketone **14** (32 mg, 95%) as an oil: IR (microscope) 3258 (br), 3114 (br), 1736, 1701, 1636, 1508, 1168 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.48 (1H, s), 7.07 (1H, s), 6.91 (1H, s), 5.90 (1H, br d), 5.03 (2H, q_{AB}, J_{AB} = 18 Hz), 4.34 (1H, m), 2.99 (3H, s), 2.94 (3H, s), 2.48 (1H, ddd, J = 17 Hz), 2.36 (1H, dt, J = 17 Hz), 2.22–2.01 (2H, m), 1.43 (9H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5, 172.0, 155.6, 138.0, 128.7, 120.2, 80.4, 57.6, 53.1 ($COCH_2N$), 37.1, 35.6, 28.3, 25.9; HRMS (EI) calcd for $C_{16}H_{26}O_4N_4$ (M^+): 338.19540. Found: 338.19551.

(4*S*)-*N*,*N*-Dimethyl-4-*tert*-butyloxycarbonylamino-6-(acetylaminoethylthio)-5-oxo-hexanamide (15). A solution of DABCO (8.0 mg, 71 μmol), the bromoketone **9a** (25 mg, 71 μmol) and *N*-acetylcysteamine (9.0 μL, 84 μmol) in CH_3CN was heated to 50 °C for 4 h. The mixture was allowed to cool, and then the solvent evaporated in vacuo. Purification by flash chromatography (SiO_2, 2% methanol in chloroform) gave the (alkylthio)ketone **15** (27 mg, 97%) as an oil: IR (microscope) 3299 (br), 1705, 1634, 1523 cm^{-1}; ^1H NMR (500 MHz, CD_3CN) δ 6.65 (1H, br s), 6.08 (1H, br d), 4.32 (1H, m), 3.52 (1H, d, J_{AB} = 14 Hz), 3.44 (1H, br d, J_{AB} = 14 Hz), 3.27 (2H, tq_{AB}, J_{AB} = 14 Hz, J = 7 Hz), 2.93 (3H, s), 2.85 (3H, s), 2.59 (2H, t, J = 7 Hz), 2.37 (2H, m), 2.06 (1H, dddd), 1.77 (1H, m), 1.43 (9H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 205.2, 171.8, 170.4, 155.9, 80.1, 57.6, 38.3 ($COCH_2S$), 38.1 37.1, 35.6, 32.9, 29.0, 28.3, 26.4, 23.1; HRMS (EI) calcd for $C_{17}H_{31}O_5N_3S$ (M^+): 389.19846. Found: 389.19854.

NMR spectroscopy of the inhibitor-enzyme complex

NMR samples were 2.5 mM peptidyl-FMK, 0.7 mM HAV 3C proteinase in 20 mM phosphate buffer at a pH of 7.5 with 10% D_2O. HDO was used as an internal standard. Tris-buffer and DTT were removed from the enzyme preparation (to below 0.5 μmol) by dialysis with 20 mM phosphate buffer (pH adjusted to 7.5). The resulting solution of enzyme was inactivated with inhibitor (4 μmol). A dialysis was performed to remove small molecule impurities and excess inhibitor.

^{13}C NMR spectra of the dialyzed sample and of a sample containing enzyme alone were obtained using 32,768 data points, a sweep width of 25,000 Hz, a 90° pulse width of 10.6 μs, a relaxation delay of 2 s and 26,000 transients on a Varian Unity 500 NMR spectrometer with ^{13}C decoupling during acquisition. A line broadening of 10 Hz was applied.

^{19}F NMR spectra were recorded on a Bruker WH-400 NMR spectrometer at 25 °C in H_2O:D_2O (9:1) with a triple 5-mm probe and with composite pulse decoupling during acquisition using 32,768 data points and 22,500 transients/free induction decay (FID). The sweep width was 75,000 Hz, with a 45° pulse width of 20 μs. Chemical shifts are reported relative to $CFCl_3$ as external standard. A line broadening of 2 Hz was applied.

Proteinase assays

Peptide proteolysis was monitored using the trinitrobenzene sulfonate (TNBS) assay as previously described.[31] Reaction mixtures were incubated in reaction buffer at 20 °C. Aliquots (10 μL) were removed from the reaction mixture at timed intervals and peptide hydrolysis was quenched with 50 μL of 0.25 M sodium borate, pH 10. A solution (12.5 μL) of freshly prepared 0.14 M TNBS (Johnson–Matthey, Ward Hill, MA) in 0.25 M sodium borate solution was added to the quenched reaction mixture and incubated for 10 min at 20 °C. The color was stabilized by adding 200 μL of 3.5 mM Na_2SO_3, 0.2 M KH_2PO_4. The concentration of free amine generated during peptide hydrolysis was determined by measuring the absorbance at 405 nm using a microtiter plate reader (Biorad, Richmond, CA).

Progress curve analysis

Proteinase inactivation was quantitatively evaluated by progress curve analysis[32] as previously described.[5] The extent of peptide proteolysis (release of α-amino groups) was monitored using the TNBS assay as described above (see Proteinase assays). Peptidyl-FMK concentration was varied from 0.3 to 1.0 μM; substrate (Ac-ELRTQSFS-amide) concentration was 2 mM and HAV 3C proteinase (C24S mutant) concentration was 0.07 μM. Enzyme was dialyzed against reaction buffer to remove DTT immediately prior to use. Reactions were initiated with enzyme and absorbances were converted into μmoles of product using a glycine standard curve. All determinations were performed in triplicate with different enzyme and inhibitor preparations.

Progress curves were fitted using least squares nonlinear regression analysis using Mac Curve Fit 1.0.7, (K. Raner) to:

$$P = \frac{v_0(1 - e^{-kt})}{k}$$

where v_0 is the initial velocity and k is the apparent first-order rate constant (k_{obs}) for the inactivation process. Parameter estimates from individual experiments (weighted by standard error) were averaged to obtain the final value.

Inhibition of polyprotein processing

Polyprotein substrates P1-2ABC*, P1-2AB*, and ΔVP1-P2-P3μ* were generated in a DNA-directed, reticulocyte lysate-based transcription/translation system (Promega) as previously described.[1,29] Translation reactions were divided and used for both the peptide aldehyde and the peptidyl-FMK studies. For inhibition experiments 2.5 μL of translation mix, containing the radiolabeled substrate, was incubated with 1 μL of inhibitor for 30 min at 37 °C prior to addition of proteinase (final concentrations 6, 10, 13, or 16 μM). The peptide aldehyde (Ac-Leu-Ala-Ala-(*N,N*-dimethylglutaminal); **5**) and the peptidyl FMK inhibitors were tested at several concentrations between 10 and 600 μM at each enzyme concentration. Proteolysis was allowed to proceed for 30 min at 37 °C. Reactions were stopped by adding Lämmli sample buffer and heating to 95 °C for 5 min. Products were separated by 12% SDS-PAGE and subsequently analyzed by autoradiography.[34] IC_{50} and EC_{50} values were derived from densitometry of the autoradiograms. Band densities were normalized to the film background (i.e., no protein) and 100% proteolysis defined by product formation in the absence of inhibitor. Plots of percent proteolysis versus inhibitor concentration were fitted using nonlinear regression analysis (SAS, Berkeley, CA).

Inhibition of viral replication

Monolayers of fetal rhesus monkey kidney cells, subclone 11-1 (FRhK-4-cells),[35] grown in 96-well plates using modified Eagle's medium (MEM) with 5% fetal calf serum, were infected, at a mutiplicity of infection of 4 radioimmune focus units/cell, with a chimeric virus derived from the HAV/7 cell culture adapted strain of HAV and a large plaque mutant, 24a.[36] At 3 h post-inoculation, a 'zero time' control was obtained by harvesting the medium and releasing the cells by trypsinization. The supernatant and cell suspension were pooled and alternately frozen and thawed three times to lyse cells and release virus. Lysates were stored at −70 °C and titered at the end of the experiment as described below.

At one day post-infection, wells were washed with serum-free MEM and each monolayer covered with 100 μL of MEM with 5% fetal calf serum. Ten μL of a 50 μM stock of the FMK inhibitor in DMSO was added to each well. Controls received 10 μL of pure DMSO. Mock infected cells were also treated with the inhibitor solution or pure DMSO and later infected with

vesicular stomatitis virus (VSV) as controls for cell damage. After 3 h of incubation at 37 °C, an additional 100 μL of fresh MEM with 5% fetal calf serum was added to each well. Sample wells of HAV and VSV-infected cells were harvested immediately (day 0) and 24 h post-treatment (day 1) and stored as described above. For VSV studies supernatants were simply removed and frozen for later analysis as VSV is efficiently released from cells.

The quantification of infectious HAV by radioimmunofocus assay (RIFA) has been described.[35] Briefly, FRhK-4 cell monolayers were infected with half-log dilutions of the harvested lysates and the monolayers subsequently overlaid with MEM agarose (0.5% agarose). The overlay was allowed to solidify at room temperature before incubation for 10 days at 34.5 °C. Cells were fixed with acetone and viral foci stained with antibodies. The primary antibody was anti-HAV chimpanzee hyperimmune serum and the secondary antibody was anti-human Ig I^{125}, $F(ab')_2$, fragment from sheep (Amersham, 100 μCi mL^{-1}). Antibody-stained foci were detected by autoradiography.

FRhK-4 cell monolayers were infected with log dilutions of VSV and incubated in a similar manner but as VSV is cytolytic, a standard plaque assay was performed in which 0.05% neutral red was used to visualize plaques for quantification.[37]

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