Peptide Aldehyde Inhibitors of Hepatitis A Virus 3C Proteinase†

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ABSTRACT: Picornaviral 3C proteinases are a group of closely related thiol proteinases responsible for processing of the viral polyprotein into its component proteins. These proteinases adopt a chymotrypsin-like fold [Allaire et al. (1994) *Nature 369*, 72−77; Matthews et al. (1994) *Cell 77*, 761−771] and a display an active-site configuration like those of the serine proteinases. Peptide-aldehydes based on the preferred peptide substrates for hepatitis A virus (HAV) 3C proteinase were synthesized by reduction of a thioester precursor. Acetyl-Leu-Ala-Ala-(*N*,*N*′-dimethylglutaminal) was found to be a reversible, slow-binding inhibitor for HAV 3C with a K_i* of $(4.2 \pm 0.8) \times 10^{-8}$ M. This inhibitor showed 50-fold less activity against the highly homologous human rhinovirus (strain 14) 3C proteinase, whose peptide substrate specificity is slightly different, suggesting a high degree of selectivity. NMR spectrometry of the adduct of the ^{13}C-labeled inhibitor with the HAV-3C proteinase indicate that a thiohemiacetal is formed between the enzyme and the aldehyde carbon as previously noted for peptide-aldehyde inhibitors of papain [Lewis & Wolfenden (1977) *Biochemistry 16*, 4890−4894; Gamcsik et al. (1983) *J. Am. Chem. Soc. 105*, 6324−6325]. The adduct can also be observed by electrospray mass spectrometry.

The picornaviral family contains many closely related human pathogens: poliovirus (PV), human rhinovirus (HRV),[1] encephalomyocarditis virus (EMCV), and hepatitis A virus (HAV). All picornaviruses generate a single 250-kDa polyprotein that undergoes multiple proteolytic cleavages, resulting in mature capsid and viral proteins which subsequently assemble into infectious virions. These cleavages are mediated by the 3C proteinase, an activity found in all picornaviruses [reviewed in Kräusslich and Wimmer (1988)].

The picornaviral 3C processing enzymes are cysteine proteinases which catalyze peptide-bond cleavage through nucleophilic attack by the sulfur atom of the active-site cysteine residue upon the substrate carbonyl carbon atom of the scissile bond to form a covalent tetrahedral intermediate. The recently obtained crystal structures for the HAV and HRV 3C proteinases show that these thiol enzymes are structurally distinct from the papain family and represent a new class of cysteine proteinase whose fold is similar to the chymotrypsin family of serine proteinases [Allaire et al., (1994) and Matthews et al. (1994), respectively].

Peptide substrates for HAV have been previously identified (Petithory et al., 1991; Malcolm et al., 1992). Further experiments with the HAV system have identified only two essential positions on the P side [notation of Schechter and Berger (1967)] of the peptide substrate: P1 and P4 (Jewell et al., 1992). A series of peptides was subsequently synthesized using natural amino acids and some amino acid analogs to analyze side-chain requirements in these two positions. The results of these studies (unpublished) and the recently obtained crystal structures of HAV and HRV 3C proteinases [Allaire et al. (1994) and Matthews et al. (1994), respectively] suggest that an uncharged δ-carbonyl oxygen is required in the P1 position of the substrate for efficient recognition by the picornaviral enzymes. Results of substitutions at the P4 position (Jewell et al., 1992) suggest that any of several hydrophobic amino acids is sufficient to generate a fully active substrate for HAV 3C proteinase and that other picornaviral 3C enzymes show different preferences in this position (Kay & Dunn, 1990).

Peptide substrates in which the P1 residue is replaced with the corresponding amino aldehyde are known to be effective inhibitors of the papain family of enzymes (Westerik & Wolfenden, 1972; Baici, 1982; Mackenzie et al., 1986). Effective inhibitors of the interleukin 1β converting enzyme (ICE), a novel cysteine proteinase, were likewise generated by replacing the P1 residue of an optimal substrate peptide with the corresponding amino aldehyde (Thornberry et al., 1992; Mullican et al., 1994). An analogous strategy, using the appropriate substrate sequences, has generated effective and highly selective inhibitors of the 3C proteinase from HAV.

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[1] Abbreviations: HAV, hepatitis A virus; HRV, human rhinovirus; TNBS, trinitrobenzenesulfonate; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DMF, dimethylformamide; *t*-BOC, *tert*-butyloxycarbonyl; HMQC, heteronuclear multiple quantum coherence spectroscopy.

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MATERIALS AND METHODS

Proteinase Production and Purification. Recombinant C24S HAV 3C proteinase (a mutant in which the nonessential surface cysteine was replaced with serine and which exhibits catalytic parameters identical to those of wild-type enzyme; unpublished results) was expressed in *Escherichia coli* and purified as reported by Malcolm et al. (1992). Recombinant HRV14 3C was generated from an analogous construct containing the coding region as identified by Callahan et al. (1985). Edman degradation revealed that the N-terminal methionine was not removed from the protein, which is in agreement with the work by Cordingly et al. (1989, 1990). Expression and purification were performed as described by Cordingly et al. (1989, 1990). Purity of the enzyme samples was greater than 90% as determined by SDS−PAGE analysis (data not shown). Proteinase concentrations were determined spectrophotometrically with $\epsilon^{1\%}_{280nm} = 1.2$. Variation in the enzyme activity was corrected for by normalizing peptidase activity with respect to the substrate peptides Ac-ELRTQSFS-NH_2 and Ac-RPV-VVQGPN-NH_2 for HAV and HRV 3C, respectively.

Peptide Substrates. The peptide substrates were synthesized using solid-phase Fmoc chemistry (Atherton & Sheppard, 1989) on Rink resin (Rink, 1987). The N-termini were blocked using acetic anhydride. 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 spectroscopy.

Proteinase Assays. Substrate proteolysis was monitored using the trinitrobenzenesulfonate (TNBS) assay as previously described (Malcolm et al., 1992). Mixtures were incubated in reaction buffer at 25 °C. Aliquots (10 μL) were removed from the reaction mixture at timed intervals and peptide lysis was quenched with 50 μL of 0.25 M sodium borate, pH 10. A solution (12 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 room temperature. The color was stabilized by adding 225 μL of 3 mM Na_2SO_3/0.2 M KH_2PO_4. The concentration of free amine generated during peptide lysis was determined by measuring the absorbance at 405 nm using a microtiter plate reader (Bio-Rad, Richmond, CA).

Synthesis of Peptide Aldehydes and Precursors. General procedure and instrumentation employed in chemical syntheses have been previously described (Abbott et al., 1994). The tripeptides Ac-Leu-Ala-Ala-OH and Ac-Leu-Ala-Thr-(O'Bu)-OH were prepared on a Rainin PS-3 solid-phase peptide synthesizer using standard Fmoc technology as described above and were purified by HPLC (C-18 reverse phase) before use.

H-Gln(NMe$_2$)-SEt, Trifluoroacetate Salt (**2**). Trifluoroacetic acid (1.7 mL) was added dropwise to a stirred solution of Boc-Glu(NMe$_2$)-SEt (**1**) (202 mg, 0.634 mmol) in dry distilled CH_2Cl_2 (5 mL) under argon at room temperature. The reaction mixture was stirred at room temperature for 2.5 h. The solvent was removed *in vacuo* and the residue was dried overnight under high vacuum to yield the title compound **2** (214 mg, quantitative): IR ($CHCl_3$ cast) 2938 (br), 1683, 1627, 1507, 1202, 1179, and 1139 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.38 (br s, 1 H), 3.02−2.95 (m, 8 H), 2.71 (br m, 6 H), 2.46−2.12 (2 × br m, 2 H), and 1.28 (t, 3 H, J = 7 Hz); MS (FAB) 219.00 ($M^+ - CF_3CO_2^-$, 100%).

Ac-Gln(NMe$_2$)-SEt (**3**). To a stirred solution of **2** (51.3 mg, 0.154 mmol) in CH_2Cl_2 (1 mL) under argon was added triethylamine (215 μL, 1.54 mmol), acetic anhydride (73 μL, 0.77 mmol) and 4-(dimethylamino)pyridine (DMAP) (catalytic quantity). The reaction mixture was stirred at room temperature for 5 h, then partitioned between EtOAc (15 mL) and water (5 mL). The organic phase was washed with saturated aqueous NH_4Cl solution (5 mL) and dilute aqueous $NaHCO_3$ solution (5 mL), dried ($MgSO_4$), filtered, and evaporated to yield **3** (17.5 mg, 44%) as an oil: ^1H NMR ($CDCl_3$, 360 MHz) δ 7.41 (d, 1 H, J = 6.5 Hz), 4.54 (dt, 1 H, J = 7.7 Hz), 3.00, 2.96 (2 × s, 2 × 3 H), 2.85 (quartet, 2 H, J = 7.5 Hz), 2.55−2.35 (m, 2 H), 2.17−2.12 (m, 2 H), 2.03 (s, 3 H), and 1.23 (t, 3 H, J = 7.5 Hz); exact mass 260.11917 (260.11948 calcd for $C_{11}H_{20}N_2O_3S$).

Ac-Gln(NMe$_2$)-H (**4**). Triethylsilane (210 μL, 1.31 mmol) and 10% Pd-C (6 mg) were added to a solution of the thioester **3** (17 mg, 0.065 mmol) in anhydrous acetone (800 μL) under argon at 0 °C. The reaction mixture was filtered through a small Celite pad and washed through with further acetone. The filtrate was evaporated *in vacuo* and purified by HPLC (Rainin Dynamax 300a 21.4 mm × 25 cm, C-18 reverse phase, gradient elution, 10−20% acetonitrile/water, 0.1% TFA) to yield **4** (11.5 mg, 88%): ^1H NMR ($CDCl_3$, 400 MHz) δ 9.56 (s, 1 H), 7.34 (br s, 1 H). 4.34−4.40 (br m, 1 H), 3.03, 2.97 (2 × 2 × 3 H), 2.60−2.35 (m, 2 H), 2.26−2.00 (m, 2 H), and 2.08 (s, 3 H).

Ac-Leu-Ala-Ala-Gln(NMe$_2$)-SEt (**5**). Triethylamine (11 μL, 0.079 mmol) was added to a solution of Ac-Leu-Ala-Ala-OH (12 mg, 0.038 mmol), **2** (13 mg, 0.039 mmol), and BOP reagent (17 mg, 0.038 mmol) in DMF (0.6 mL) under argon at room temperature. The solution was stirred at room temperature for 5 h, and then the solvent was evaporated *in vacuo* and the residue was dried under high vacuum. Purification by HPLC (Waters Resolve C-18 reverse phase, 8 mm × 200 mm, gradient elution, 17−24% acetonitrile/water, 0.1% TFA) gave the title compound **5** (8 mg, 41%) as a white powder: IR (KBr disk) 3273 (br), 1688, 1677, 1629, and 1536 cm^{-1}; ^1H NMR (D_2O, 400 MHz) δ 4.34−4.30 (m, 1 H), 4.19−4.08 (m, 3 H), 2.88 (s, 3 H), 2.73 (quartet, 2 H, J = 7.5 Hz), 2.36 (t, 2 H, J = 7.5 Hz), 2.12−2.04 (m, 1 H), 1.87 (s, 3 H), 1.83−1.73 (m, 1 H), 1.54−1.46 (m, 1 H), 1.44−1.40 (m, 2 H), 1.28 (d, 3 H, J = 7 Hz), 1.22 (d, 3 H, J = 7 Hz), 1.06 (t, 3 H, J = 7.5 Hz), 0.77 (d, 3 H, J = 6.5 Hz), and 0.73 (d, 3 H, J = 6.5 Hz); MS (FAB) 516.22 (MH^+, 32%).

Ac-Leu-Ala-Ala-Gln (NME$_2$)-H (**6**) was prepared analogous to **4**, using **5** (1.0 mg, 1.94 μmol), triethylsilane (15 μL, 93.9 μmol) and 10% Pd-C (1.0 mg) in anhydrous DMF (100 μL). The crude product was purified by HPLC (Waters Resolve C-18 reverse phase, 8 mm × 200 mm, gradient elution, 15−19% acetonitrile/water, 0.1% TFA) to give the title compound **6** (0.7 mg, 74%) as a white powder: IR (KBr disk) 3410 (br), 3310 (br), 1643 (br), 1536, and 1451 cm^{-1}; ^1H NMR (D_2O, 400 MHz) δ 4.81 (d, 1 H, J = 4.5 Hz), 4.15−4.09 (m, 3 H), 3.69−3.65 (m, 1 H), 2.88 (s, 3 H), 2.76 (s, 3 H), 2.27 (t, 2 H, J = 7.5 Hz), 1.86 (s, 3 H), 1.85−1.80 (m, 1 H), 1.56−1.45 (m, 2 H), 1.44−1.39 (m, 2 H), 1.26, 1.22 (2 × d, 2 × 3 H, J = 7 Hz), and 0.77, 0.73 (2 × d, 2 × 3 H, J = 6.5 Hz); MS (FAB) 456.34 (MH^+, 26%).

Ac-Ala-Ala-Ala-Gln(NMe$_2$)-SEt (**7**) was prepared analogously to **5**, using Ac-Ala-Ala-Ala-OH (26.7 mg, 0.098 mmol), **2** (32 mg, 0.096 mmol), BOP reagent (43.2 mg, 0.098 mmol), triethylamine (28 μL, 0.201 mmol), and DMF (1.5 mL). Purification of the crude product by HPLC (Waters Resolve C-18 reverse phase, 8 mm × 200 mm, gradient elution, 11−16% acetonitrile/water, 0.1% TFA) gave **7** (35 mg, 77%) as a white powder: IR (KBr disk) 3417 (br), 3278, 2414, 1673, 1625, and 1463 cm^{-1}; ^{1}H NMR (D_2O, 400 MHz) δ 4.40−4.37 (m, 1 H), 4.27−4.12 (m, 3 H), 2.93 (s, 3 H), 2.81 (s, 3 H), 2.79 (quartet, 2 H, J = 7.5 Hz), 2.43−2.39 (m, 2 H), 2.17−2.08 (m, 1 H), 1.92 (s, 3 H), 1.91−1.80 (m, 1 H), 1.35 (d, 3 H, J = 7 Hz), 1.29 (d, 3 H, J = 7 Hz), 1.26 (d, 3 H, J = 7 Hz), and 1.11 (t, 3H, J = 7.5 Hz); MS (FAB) 474.12 (MH$^+$, 7%).

Ac-Ala-Ala-Ala-Gln(NMe$_2$)-H (**8**) was prepared analogously to **4**, using **7** (20 mg, 0.042 mmol), triethylsilane (135 μL, 0.842 mmol) and 10% Pd-C (14 mg) in anhydrous DMF (0.5 mL). Purification of the crude product by HPLC (Waters Resolve C-18 reverse phase, 8 mm × 200 mm, gradient elution, 7−10% acetonitrile/water, 0.1% TFA) gave the aldehyde **8** (13.4 mg, 77%) as a white powder: IR (KBr disk) 3312 (br), 1731, 1651 (br), 1543, and 1453 cm^{-1}; ^{1}H NMR (D_2O, 400 MHz) δ 4.80 (d, 1 H, J = 5 Hz), 4.16−4.05 (m, 3 H), 3.68−3.63 (m, 1 H), 2.87 (s, 3 H), 2.75 (s, 3 H), 2.26 (t, 2 H, J = 7.5 Hz), 1.85 (s, 3 H), 1.84−1.79 (m, 1 H), 1.55−1.47 (m, 1 H), and 1.24−1.19 (m, 9 H); MS (FAB) 414.32 (MH$^+$, 36%).

*N-t-BOC-*L*-Glutamic Acid, α-t-Butyl Ester, γ-Benzyl Ester, 1,2-$^{13}C_2$* (**10**). A solution of *tert*-butyl trichloroacetimidate (256 mg, 1.172 mmol) in dry distilled cyclohexane (1.2 mL) was added to a solution of *N-t*-BOC-L-glutamic acid, γ-benzyl ester (**9**) (200 mg, 0.589 mmol) in CH_2Cl_2 (0.6 mL). BF_3•OEt_2 (15 μL) was then added and the reaction mixture was stirred at room temperature under argon for 21 h. Solid $NaHCO_3$ was added and the reaction mixture was filtered through a short silica gel plug to remove trichloroacetamide and washed through with further CH_2Cl_2. The solvent was removed *in vacuo* and the residue was purified by flash chromatography (SiO_2, 25 g, 30% EtOAc/hexane) to yield **10** (124 mg, 53%): IR ($CHCl_3$ cast) 2978, 1730, 1718, 1691, 1368, and 1150 cm^{-1}; ^{1}H NMR ($CDCl_3$, 360 MHz) δ 7.38−7.33 (m, 5 H), 5.13 (s, 2 H), 5.11−5.05 (m, 1 H0, 4.46−4.38, 4.07−3.98 (dm, 1 H, J_{C-H} = 142 Hz), 2.54−2.36 (m, 2 H), 2.24−2.12 (m, 1 H), 1.99−1.87 (m, 1 H), 1.47 (s, 9 H), and 1.44 (s, 9 H); exact mass 395.22179 (395.22186 calcd for $C_{19}H_{31}O_6N^{13}C_2$).

*N-t-BOC-*L*-Glutamic Acid, α-t-Butyl Ester, 1,2-$^{13}C_2$* (**12**). A solution of **10** (122 mg, 0.31 mmol) in MeOH (10 mL) was hydrogenated for 20 h in the presence of 10% Pd-C (15 mg). The solvent was removed *in vacuo* to furnish *N-t*-BOC-L-glutamic acid,α-*t*-butyl ester, γ-methyl ester, 1,2-$^{13}C_2$ (**14**): ^{1}H NMR ($CDCl_3$, 360 MHz) δ 5.08 (brs, 1 H), 4.45−4.36, 4.06−3.97 (dm, 1 H, J_{C-H} = 142 Hz), 3.68 (s, 3 H), 2.50−2.31 (m, 2 H), 2.22−2.08 (m, 1 H), 1.98−1.85 (m, 1 H), 1.47 (s, 9 H), and 1.44 (s, 9 H). A solution of **11** in MeOH/H_2O (3:1, 2 mL) cooled to 2 °C was treated with lithium hydroxide monohydrate (17 mg, 0.40 mmol) and the mixture was stirred at 5 °C for 19 h. The solvent was removed *in vacuo* and water (5 mL) was added to the residue, which was then acidified to pH 2.5 with 0.5 N HCl and immediately extracted with CH_2Cl_2 (5 mL). The acidification and extraction process was repeated a further five times. The combined organic layers were dried ($MgSO_4$), filtered, and evaporated *in vacuo* to yield **12** (82.3 mg, 88% from **10**) as a clear oil: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.17 (d, 1 H, J = 6 Hz), 4.52−4.40, 4.04−3.92 (dm, 1 H, J_{C-H} = 142 Hz), 2.55−2.35 (m, 2 H), 2.24−2.09 (m, 1 H), 2.01−1.83 (m, 1 H), 1.47 (s, 9 H), and 1.45 (s, 9 H); exact mass 306.18311 (MH$^+$) (306.18269 calcd for $C_{12}H_{26}NO_6{}^{13}C_2$).

*N-t-BOC-*L*-Glutamic Acid, γ-Dimethylamide, 1,2-$^{13}C_2$* (**14**). BOP reagent (119 mg, 0.269 mmol) and triethylamine (38 μL, 0.273 mmol) were added to a solution of **12** (82 mg, 0.269 mmol) in CH_2Cl_2 (0.7 mL) under argon. The solution was stirred for 3 min and then dimethylamine hydrochloride (44 mg, 0.273 mmol) and triethylamine (75 μL, 0.538 mmol) were added. The reaction mixture was stirred for 3.5 h under argon and then the volatile material was removed *in vacuo*. The residue was dissolved in EtOAc (8 mL), washed with 10% aqueous $NaHCO_3$ solution (2 × 8 mL) and 10% aqueous citric acid (2 × 8 mL), dried ($MgSO_4$), filtered, and evaporated *in vacuo* to yield *N-t*-BOC-L-glutamic acid, α-*t*-butyl ester, γ-dimethylamide, 1,2-$^{13}C_2$ (**13**) as a clear oil: IR (CH_2Cl_2 cast) 3326 (br), 2978, 2934, 1714, 1698, 1639, 1506, 1368, 1252, and 1150 cm^{-1}; ^{1}H NMR ($CDCl_3$, 360 MHz) δ 5.26 (brs, 1 H), 4.41−4.32, 4.02−3.93 (dm, 1 H, J_{C-H} = 142 Hz), 3.00, 2.96 (2 × s, 2 × 3 H), 2.50−2.30 (m, 2 H), 2.24−2.11 (m, 1 H), 2.02−1.88 (m, 1 H), 1.47 (s, 9 H), and 1.45 (s, 9 H); exact mass 332.22170 (332.22217 calcd for $C_{14}H_{30}O_5N_2{}^{13}C_2$). A solution of **13** in CH_2Cl_2 (2 mL) and trifluoroacetic acid (2 mL) was stirred at room temperature for 2.5 h. The solvent was removed *in vacuo*, and the residue was evaporated *in vacuo* from toluene (2 × 5 mL) and further CH_2Cl_2 (5 mL). To a solution of this residue in dioxane (1 mL) and 1 M NaOH (0.75 mL) was added a solution of *tert*-butyl pyrocarbonate (100 mg, 0.458 mmol) in dioxane (0.4 mL). The reaction mixture was stirred at room temperature for 20 h at pH 8. Water (3 mL) was added and the reaction mixture was acidified to pH 2.5 with 5% aqueous $KHSO_4$ solution. The mixture was immediately extracted with CH_2Cl_2 (4 × 8 mL). The combined organic phases were dried ($MgSO_4$), filtered, and evaporated *in vacuo* to yield the title compound **14** (69 mg, 93% from **12**): IR ($CHCl_3$ cast) 3321 (br), 2977, 2931, 1694, 1633, 1610, 1505, 1367, and 1166 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.65 (brs, 1 H), 4.42−4.35, 4.07−4.00 (dm, 1 H, J_{C-H} = 142 Hz), 3.03, 2.96 (2 × s, 2 × 3 H), 2.77−2.66 (m, 1H), 2.49−2.39 (m, 1H), 2.29−2.17 (m, 1H), 2.05−1.93 (m, 1H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.7 (d), 156.0, 146.5, 79.6, 57.3 (d), 37.5, 35.4, 30.1, 27.3; exact mass 276.15905 (276.15958 calcd for $C_{10}H_{22}O_5N_2{}^{13}C_2$).

*N-t-BOC-*L*-Glutamic Acid, α-Ethyl Thioester, γ-Dimethylamide, 1,2-$^{13}C_2$* (**15**). A solution of **14** (67 mg, 0.2425 mmol) in dry CH_2Cl_2 (1 mL) under argon at 0 °C was treated with ethyl chloroformate (83 μL, 0.87 mmol) and triethylamine (242 μL, 1.74 mmol) for 15 min. Ethanethiol (180 μL, 2.43 mmol) and 4-(dimethylamino)pyridine (3.0 mg, 0.025 mmol) were added and the reaction mixture was stirred for a further 1.5 h at 0 °C. The reaction was quenched by the addition of glacial acetic acid (90 μL) and the volatile material was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 (15 mL), then washed with 5% aqueous $NaHCO_3$ solution (3 × 15 mL), 5% aqueous citric acid solution (2 × 15 mL) and brine (15 mL). The organic phase was dried ($MgSO_4$), filtered, and evaporated *in vacuo*. Purification by flash

chromatography (SiO_2, 20 g, 5% EtOH/CH_2Cl_2) afforded **15** (50 mg, 64%) as a white solid: mp 145−146 °C; $[\alpha]_D$ −16.3° (c = 1.3, $CHCl_3$); IR (CH_2Cl_2 cast) 3214, 2979, 1710, 1635, 1618, 1539, 1253, and 1162 cm^{-1}; ^1H NMR ($CDCl_3$, 360 MHz) δ 5.72 (d, 1 H, J = 6.5 Hz), 4.53−4.44, 4.13−4.05 (dm, 1 H, J_{C-H} = 142 Hz), 2.98, 2.94 (2 × s, 2 × 3 H), 2.85 (d quartet, 2 H, J = 7.5, 4 Hz), 2.50−2.33 (m, 2 H), 2.23−1.96 (m, 2 H), 1.44 (s, 9 H), and 1.23 (t, 3 H, J = 7.5 Hz); exact mass 320.16920 (MH^+) (320.16804 calcd for $C_{12}H_{26}N_2O_4S^{13}C_2$).

Ac-Leu-Ala-Ala-Gln(NMe$_2$)(1,2-$^{13}C_2$)-SEt (**16**). Trifluoroacetic acid (0.35 mL) was added dropwise to a solution of **15** (42.4 mg, 0.132 mmol) in dry CH_2Cl_2 (1 mL) under argon at room temperature. The solution was stirred for 2.5 h at room temperature, and then the solvent was removed *in vacuo* and the residue was evaporated twice from toluene and again from CH_2Cl_2. To a solution of the residue in DMF (1.5 mL) at room temperature under argon was added Ac-Leu-Ala-Ala-OH (31.5 mg, 0.10 mmol), BOP reagent (44.2 mg, 0.10 mmol), and triethylamine (28 μL, 0.20 mmol). After being stirred for 5 h at room temperature, the solution was evaporated *in vacuo* and dried under high vacuum for 20 h. The residue was purified twice by HPLC (Rainin Dynamax 300a 21.4 mm × 25 cm C-18 reverse-phase, gradient elution, 25−30% acetonitrile/water, 0.1% TFA) to yield **16** (29.6 mg, 57%) as a white powder: IR (CH_2Cl_2 cast) 3270 (br), 1690, 1625 (br), and 1545 cm^{-1}; ^1H NMR (D_2O, 360 MHz) δ 4.55−4.49 (m, 0.5 H), 4.21−4.08 (m, 3.5 H), 2.88, 2.76 (2 × s, 2 × 3 H), 2.73 (d quartet, 2 H, J = 7.5, 4.5 Hz), 2.38−2.33 (m, 2 H), remaining data as for **5**; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.7 (d), 59.4 (d); MS (FAB) 518.35 (MH^+, 26%).

Ac-Leu-Ala-Ala-Gln(NME$_2$)(1,2-$^{13}C_2$)-H (**17**). To a solution of **16** (2.8 mg, 0.0054 mmol) in DMF (0.2 mL) at 0 °C was added triethylsilane (20 μL, 0.125 mmol) followed by 10% Pd-C (1 mg). The reaction mixture was stirred for up to 1.5 at 0 °C and then filtered through Celite and washed through with DMF, H_2O, and acetone. The filtrate was evaporated *in vacuo* and purified by HPLC (Rainin Dynamax 300a 21.4 mm × 25 cm) to yield the aldehyde **17** (2.2 mg, 89%) as a white powder: ^1H NMR (D_2O, 360 MHz) δ 4.81 (dd, 1 H, J = 4.5 Hz, J_{C-H} = 162 Hz), 4.18−4.07 (m, 3 H), 3.90−3.82, 3.51−3.43 (dm, 1 H, J_{C-H} = 142 Hz), remaining data as for **6**; ^{13}C NMR (CD_3CN, 100 MHz) δ 90.9 (d), 54.4 (d); MS (FAB) 458.20 (MH^+, 74%).

Progress Curve Analysis. Inhibitors were evaluated by progress curve analysis following the method of Morrison and Walsh (1988). Reactions were initiated with enzyme, and the extent of proteolysis was monitored by removing aliquots and measuring the liberated α-amino groups using TNBS. Absorbances were converted into micromolar product using a glycine standard curve.

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

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

where v_0 and v_s are the initial and steady-state velocities, respectively, and k is the apparent first-order rate constant for the formation of the tight complex (EI*). By varying the concentration of substrate at each inhibitor concentration, K_m^{app} was obtained based on either v_0 or v_s. In the former case $K_m^{app} = K_m (1 + [I]/K_i)$, where K_i is the dissociation constant for the EI complex, and in the latter case $K_m^{app} = K_m(1 + [I]/K_i^*)$, where K_i^* is the overall dissociation constant of the tight complex EI* (Morrison & Walsh, 1988). These values, together with the apparent first-order rate constant k for the formation of the EI* complex, can be used to calculate true rate constants for the formation (k_5) and the breakdown (k_6) of the tight complex:

$$k_6 = k \Big/ \left[\frac{1 + \dfrac{[I]}{K_i^*\left(1 + \dfrac{[S]}{K_m}\right)}}{1 + \dfrac{[I]}{K_i\left(1 + \dfrac{[S]}{K_m}\right)}} \right]$$

and

$$k_5 = k - k_6 \Big/ \left[\frac{\dfrac{[I]}{K_i}}{1 + \dfrac{[S]}{K_m} + \dfrac{[I]}{K_i}} \right]$$

Each inhibitor was analyzed at several concentrations. Appropriate data sets from independent trials, in which the nonlinear regression analysis yielded parameter estimates with standard errors of less than 20%, were used to calculate the kinetic constants. Parameter errors were propagated and used as weighting factors when the calculated constants were averaged to obtain final values.

NMR Spectroscopy of the Enzyme−Inhibitor Complex. $^1H/^{13}C$ Heteronuclear multiple quantum coherence spectra (HMQC; Summers et al., 1986) were recorded on a Varian Unity 600 MH_3 NMR spectrometer at 25 °C in D_2O with ^{13}C decoupling during acquisition. Spectra were obtained using 1344 complex data points in the t_2 dimension, 288 in t_1, and 144 transients/free induction decay (FID). The ^1H and ^{13}C sweep widths were 8000 and 15 000 Hz, respectively, with a ^1H 90° pulse width of 11 μs. Suppression of the HOD resonance was achieved using presaturation. FIDs were weighted using a cosine-squared function to increase the signal-to-noise ratio.

The solvent conditions for all HMQC spectra were 20 mM Na_2PO_4/D_2O at a pD of 6.85 with sodium 3-(trimethylsilyl)-1-propanesulfonate-2,2,3,3-d_4 (DSS) added as an internal standard. The inhibitor NMR sample concentration was 1.6 mM. The HAV 3C protease C-24S/inhibitor complex NMR sample initially had an enzyme concentration of 0.6 mM and an inhibitor concentration of 1.45 mM. Due to the presence of impurities, the protease/inhibitor NMR sample (500 μL) was subsequently dialyzed in 5 mL of 20 mM Na_2PO_4 at 5 °C for 5 h (10 kDa molecular weight cutoff membrane) lyophilized, and redissolved in 500 μL of Na_2PO_4/D_2O.

RESULTS AND DISCUSSION

The common feature of substrates required for recognition by all 3C proteinases studied to date is a glutamine residue in the P1 position [Kräusslich and Wimmer (1988) and references therein]. Peptide substrates in which the glutamine has been replaced with an alanine no longer function as

Table 1: Kinetic Parameters of HAV 3C Proteinase Inhibitors

inhibitor	K_i (M)	K_i^* (M)	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6	$t_{1/2}$ for reactivation (h)
ac-Q′ aldehyde	$(2.5 \pm 0.4) \times 10^{-3}$ [a]					
ac-LAAQ′ aldehyde	$(2.8 \pm 0.5) \times 10^{-7}$	$(4.2 \pm 0.8) \times 10^{-8}$	$(2.2 \pm 0.1) \times 10^{-2}$	$(3.8 \pm 1.0) \times 10^{-3}$	5.8/1	3
ac-AAAQ′ aldehyde	$(8.4 \pm 1.0) \times 10^{-7}$	$(2.3 \pm 0.6) \times 10^{-7}$	$(6.6 \pm 0.1) \times 10^{-3}$	$(4.4 \pm 1.3) \times 10^{-3}$	1.5/1	2.6
ac-LAAQ′ thioester	ND[b]					

[a] No time-dependent behavior observed over the course of the assay (2 h). [b] No inhibition detected up to 50 μM.

substrates for the HAV-3C proteinase (Jewell et al., 1992). Replacement of glutamine with uncharged analogs such as the methyl ester of glutamate or dimethylamide of glutamine resulted in peptide substrates that were cleaved with equal efficiency (B.A.M., unpublished results). This observation was consistent with the predicted (Bazan & Fletterick, 1988; Gorbeleyna et al., 1989) and subsequently confirmed (Allaire et al., 1994; Matthews et al., 1994) structure of the S1 subsite of the picornavirus 3C proteinases. Modeling studies (manuscript in preparation) and published models of enzyme–substrate complexes (Matthews et al., 1994) have suggested that it was the δ carbonyl oxygen of the P1 residue that was responsible, through hydrogen bonding with the $N^{\epsilon 2}$ of a histidine at the bottom of the S1 pocket, for the essential recognition of a substrate by the 3C proteinase. It seemed that in order to obtain a reasonably effective and selective inhibitor of a 3C proteinase, this interaction would need to be preserved. This required the synthesis of a glutamine aldehyde (glutaminal) moiety.

Previously used approaches, such as oxidation of the corresponding alcohol (Thompson, 1973; Wilk & Orlowski 1983; Vinitsky et al., 1994), resulted in cyclization of the side chain of glutamine to form a six-membered cyclic aminal (data not shown). To circumvent this and subsequent synthetic problems, a strategy was devised for generating the aldehyde by reduction of a thioester precursor bearing two substituents on the side-chain nitrogen. (*N*,*N*-dimethyl)-glutamine, an amino acid analog in which the side-chain amide has been blocked, was used to generate the corresponding thioester precursor in a manner similar to the method reported by Fukugama et al. (1990). This thioester precursor was subsequently coupled to the unprotected peptide fragments using standard procedures prior to the reduction to the aldehyde. The peptide fragment Ac-LAA was substituted for the actual substrate sequence LRT, as it was found that neither the arginine nor the threonine contributed to substrate activity (Jewell et al., 1992), and the modified sequence simplified synthesis by obviating deprotection and purification steps prior to reduction of the thioester. This approach allows the rapid generation of a number of different peptide aldehydes tailored to the preferences of the different 3C proteinases in the P2, P3, and P4 positions [Kay and Dunn (1990) and references therein; Jewell et al., 1992].

Peptide aldehydes (in which the P1 residue is replaced with the corresponding aldehyde) were discovered to be unusually effective reversible inhibitors of papain almost a quarter of a century ago by Westerick and Wolfenden (1972). They were almost immediately found to be similarly effective at inhibiting serine proteinases (Thompson, 1973; Chen et al., 1979). Since then, many studies have been conducted to establish their mechanism of action and explain their unusual potency (Matts et al., 1977; Henes et al., 1979; Frankfater & Kuppy, 1981; Bone & Wolfenden, 1985; Hanzlick et al., 1991).

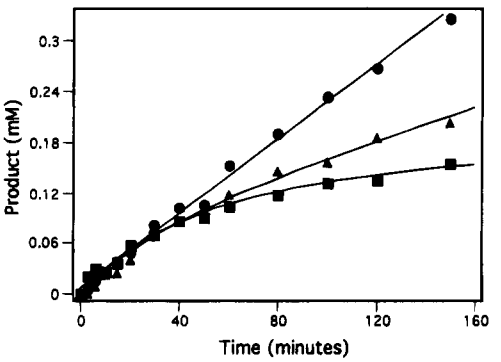


FIGURE 1: Time-dependent inhibition of HAV 3C proteinase by Ac-LAAQ′ aldehyde. Substrate peptide proteolysis was performed and monitored as described under Materials and Methods. Reactions were initiated with enzyme; substrate (Ac-ELRTQSFS-amide), 2.0 mM; HAV 3C proteinase, 0.07 μM; (▲) 0.25 μM Ac-LAAQ′ aldehyde and (■) 0.5 μM Ac-LAAQ′ aldehyde; (●) no inhibitor.

The 3C proteinases are thiol proteinases with a fold similar to the mammalian serine proteinases (Allaire et al., 1994, Matthews et al., 1994). As such they represent a new class of cysteine proteinase different from the papain family. To determine whether the target aldehydes would be effective inhibitors of these enzymes, peptide aldehydes based on a good and a mediocre substrate were synthesized and evaluated. The kinetic constants for the inhibition of HAV 3C proteinase by acetyl *N*,*N*-dimethylglutaminal (Ac-Q′-H), acetyl leucinylalanylalanyl *N*,*N*-dimethylglutaminal (Ac-LAAQ′-H), the thioester precursor (Ac-LAAQ′-SEt), and the related peptide aldehyde acetyl alanylalanylalanyl *N*,*N*-dimethylglutaminal (Ac-AAAQ′-H) are shown in Table 1.

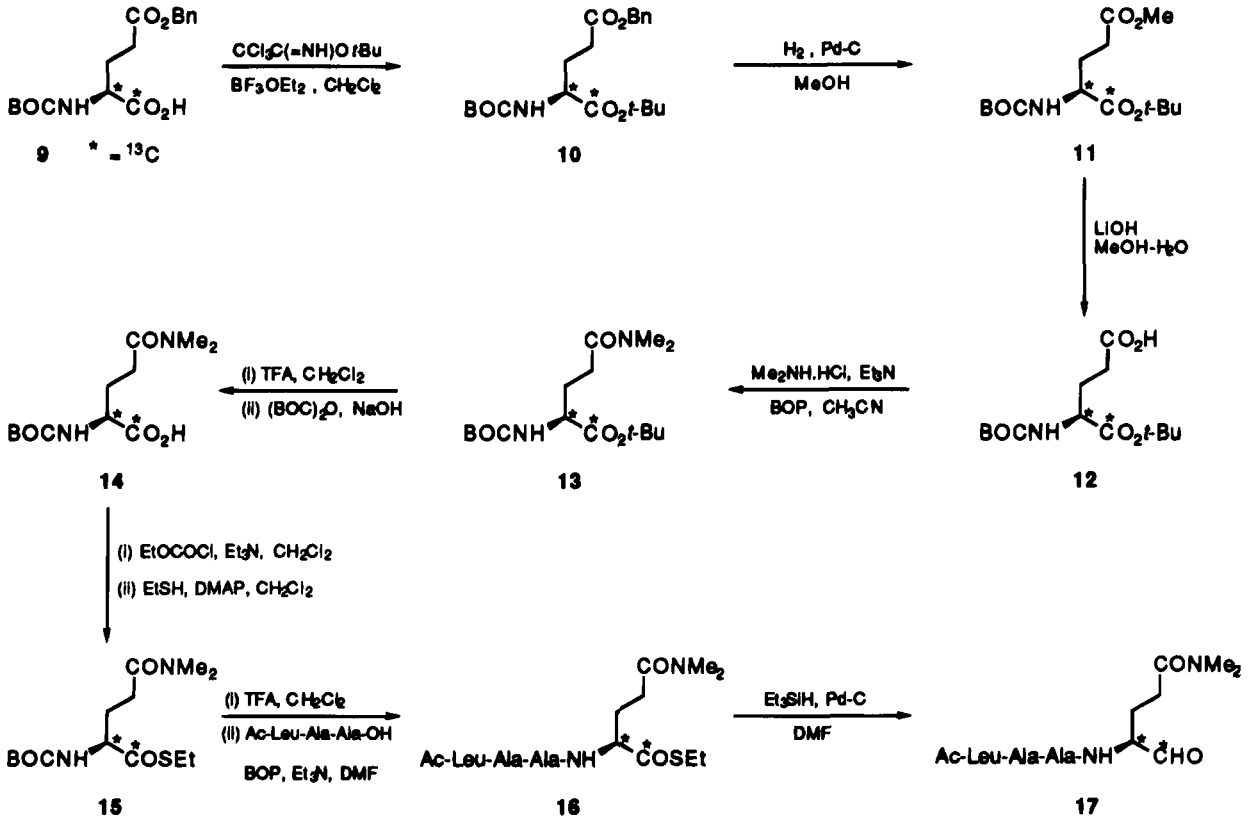
It is clear that the aldehyde based on the preferred substrate peptide, Ac-LAAQ′-H, is a potent, slow-binding inhibitor of the HAV 3C proteinase (Figure 1). None of the other molecules is as potent an inhibitor. The good peptide sequence LAAQ′-H yielded a slow tight-binding inhibitor with a K_i^* of $(4.2 \pm 0.8) \times 10^{-8}$ M (uncorrected for hydration). Estimation of the simple competitive K_i for the peptide aldehyde showed that it was roughly 9000 times more effective than the simple acetyl *N*,*N*-dimethylglutaminal (Table 1). The overall potency (K_i^*) is comparable to the K_i of 4.6×10^{-8} M originally obtained for Ac-phenylalanyl-aminoacetaldehyde with papain (Westerick & Wolfanden, 1972). The lack of inhibitory activity of the peptide thioester confirms that a neutral peptide fragment mimicking the N-terminal product is insufficient to obtain any significant inhibition of the enzyme as previously reported (Jewell et al., 1992).

To investigate the impact of amino acid residues on the activity of the effective Ac-LAAQ′-H inhibitor, an identical aldehyde inhibitor was synthesized in which the leucine residue in the P4 position was replaced with alanine (Ac-AAAQ′-H). Replacement of the preferred leucine with alanine results in a 5-fold loss in efficiency (Table 1)

Table 2: Activity of HAV 3C Proteinase Inhibitors against the HRV 3C Proteinase

inhibitor	K_i (M)	K_i* (M)	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6	$t_{1/2}$ for reactivation (min)
ac-LAAQ′ aldehyde	$(1.3 \pm 0.4) \times 10^{-5}$	$(2.7 \pm 0.2) \times 10^{-6}$	$(3.2 \pm 0.4) \times 10^{-2}$	$(2.6 \pm 0.2) \times 10^{-2}$	1.2/1	27
ac-AAAQ′ aldehyde	$(3.4 \pm 1.1) \times 10^{-7}$	$(1.1 \pm 0.3) \times 10^{-7}$	$(7.5 \pm 1.5) \times 10^{-2}$	$(1.4 \pm 0.4) \times 10^{-2}$	5.4/1	50

Scheme 1



comparable to the 10-fold drop in specific activity of a peptide substrate in which the same substitution was introduced (Jewell et al., 1992). To further confirm the importance of the P4 residue and to ascertain the selectivity of these peptide aldehydes, the HAV 3C inhibitor, Ac-LAAQ′-H, was tested against the highly homologous HRV14 3C proteinase in a parallel assay with its own substrate peptide. This enzyme has been shown to strongly prefer alanine in the P4 position of peptide substrates [reviewed in Kay and Dunn (1990)]. The inhibitor showed approximately 60-fold less activity (K_i*) against the HRV enzyme (Table 2), suggesting that the preference for alanine in the P4 site of peptide substrates carries over to peptide aldehydes, underscoring that these inhibitors are highly selective and can even distinguish between closely related members of the 3C proteinase family. Tests of the Ac-AAAQ′-H inhibitor against HRV14 3C proteinase (Table 2) yielded parameters similar to those obtained with this molecule against the HAV 3C enzyme, suggesting that the P4 subsite in the HRV enzyme, while capable of excluding inappropriate residues, does not appear to make significant interactions with the methyl side chain of the alanine. Further studies are underway to obtain peptide sequences tailored to the HRV14 3C proteinase.

Finally, to determine whether a covalent thiohemiacetal adduct was being formed between the inhibitor and the HAV 3C proteinase, an inhibitor uniformly labeled with ^{13}C in the α and carbonyl carbons of the *N,N*-dimethylglutaminal moiety was generated. In order to synthesize the ^{13}C-labeled inhibitor it was necessary to generate the thioester precursor from the ^{13}C-labeled, BOC-protected benzyl ester of glutamate (Scheme 1). After catalytic hydrogenation to remove the benzyl group, the free acid was generated by treatment with LiOH. Coupling of dimethylamine using BOP yielded the *N,N*-dimethylglutamine, which was subsequently converted to the thioester precursor as previously described.

Figure 2 shows HMQC spectra of the Ac-LAAQ′-H inhibitor alone and in complex with the HAV 3C proteinase. The appearance of a new cross peak around 79 ppm on the carbon chemical shift axis upon addition of enzyme is a clear indication of the formation of a thiohemiacetal adduct to the inhibitor (Mackenzie et al., 1986). The reduced signal strength is due to losses incurred during a 6-h dialysis of the enzyme−inhibitor complex to remove small molecular weight impurities introduced with the enzyme preparation, in particular, the diastereomeric adducts of dithiothreitol (a buffer constituent) with the aldehyde inhibitor. As observed by Gamcsik et al. (1983) and later confirmed by Mackenzie et al. (1986), there is an upfield shift of the aldehydic carbon (which exists in the hydrate form in aqueous solution) upon formation of the thiohemiacetal from 92 ppm to approximately 80 ppm when enzyme is added to a labeled peptide aldehyde (Figure 2). This observed shift with HAV is in good agreement with that observed for papain and Ac-phenylalanylaminoacetaldehyde (viz. 88 ppm → 74 ppm). Mass spectrometry further confirmed the formation of a

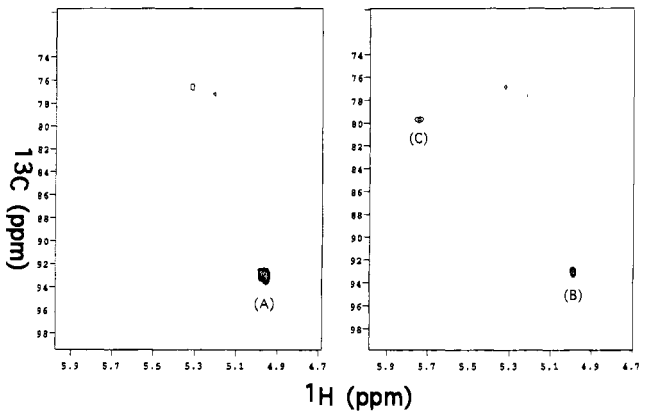


FIGURE 2: ^{13}C HMQC spectra of the ^{13}C-labeled Ac-LAAQ′ aldehyde inhibitor alone (panel 1) and together with HAV 3C proteinase; 0.6 mM HAV 3C/1.45 mM, (panel 2). The spectra were acquired at 600 MHz, 25 °C in 20 mM Na_2PO_4/D_2O with a pD of 6.7. Cross peaks (A) and (B) show the proton−carbon correlation of the unreacted inhibitor, which exists as an aldehyde hydrate in aqueous solution. Peak (C) is the cross peak due to the thiohemiacetal adduct, *i.e.*, enzyme−inhibitor complex. A 6-h dailysis of the complex was performed to remove small molecular weight impurities, in particular, the diastereomeric adducts of dithiothreitol (a buffer constituent) with the aldehyde inhibitor. This resulted in a signal reduction of 80−90% of these species, but the bound inhibitor signal was only reduced by 50%.

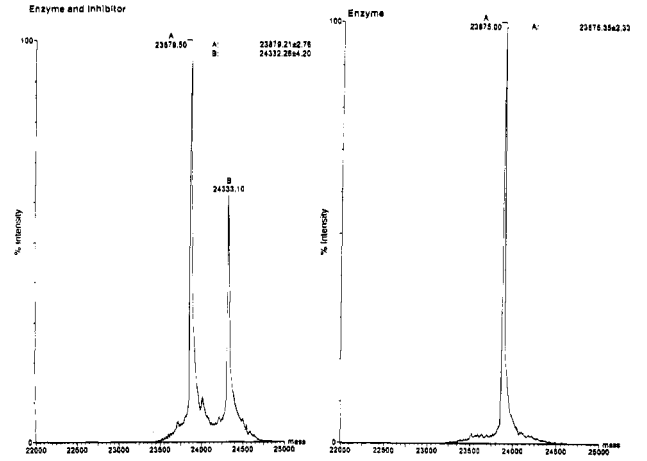


FIGURE 3: Mass spectrometry of C245 HAV 3C (panel 2) and C245 HAV 3C in complex with Ac-LAAQ′ aldehyde (panel 1). Mass analysis of the protein and complex were performed on a Fisons VG Quattro triple-quadrupole mass spectrometer (Manchester, England) fitted with an electrospray ionization source operating in positive ion mode. Injections, 10 μL of the samples, in aqueous acetonitrile containing 0.05% trifluoroacetic acid at an approximate concentration of 50 pmol/μL, were made into a carrier solutions composed of 1:1 (v/v) water/acetonitrile containing 0.05% trifluoroacetic acid at a rate of 10 μL/min into the electrospray source. The quadrupoles were scanned from 600 to 1600 mass over charge ratio at 10 s/scan. Data were acquired in the MCA mode with typically 10−15 scans being summed to produce a spectrum.

covalent adduct. The spectra of the uncomplexed enzyme and the HAV 3C−peptide aldehyde complex are shown in Figure 3. The difference in mass between the complex and free enzyme, 453 Da, is in good agreement with the calculated mass of the inhibitor, 456 Da.

To test whether the formation of this adduct was reversible, attempts were made to measure the rate and extent of enzyme reactivation. Fully inhibited enzyme was separated from excess inhibitor by passing the mixture over a small G-25 spin column. The enzyme−inhibitor complex was then added to an excess of substrate (2.5 × K_m) and product formation was monitored over the next 2 h. The observed reactivation, 5% of initial activity, was in rough agreement with that calculated (12%) on the basis of total protein recovered. However, direct accurate assessment of the $t_{1/2}$ for reactivation was difficult due to substrate solubility (2.5 × K_m) and the subsequent low levels of product formation during the course of the investigation. The $t_{1/2}$ for reactivation was subsequently estimated following the methods of Morrison and Walsh (1988) to be approximately 3 h (Table 1).

The picornaviral 3C proteinases, as their biochemical and structural studies have shown, are a hybrid between a thiol proteinase of the papain family and the well-studied mammalian serine proteinases. Peptide aldehydes inhibitors will be useful tools for the comparison of 3C proteinase specificities and study of their mechanism of action. Although they are unlikely to be effective therapeutic agents becuase of metabolic degradation and transport problems, they provide a basis for the development of more medicinally useful molecules. The design of second-generation substrate analogs as *in vivo* inhibitors and tools for further structure−function investigations is currently underway. Such molecules should block polyprotein processing and picornaviral replication.

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