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DESIGN, SYNTHESIS, AND EVALUATION OF AZAPEPTIDES AS SUBSTRATES AND INHIBITORS FOR HUMAN RHINOVIRUS 3C PROTEASE

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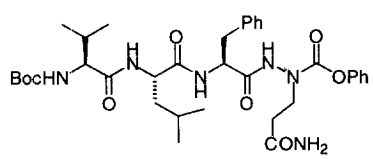
Abstract: A series of azapeptides was prepared and assessed as inhibitors of the human rhinovirus 3C protease. Boc–VLFaQ–OPh was a slow-turnover substrate that gave transient (ca. 1–2 h) inhibition as it underwent hydrolysis. Boc–VLFaG–OPh gave very slow but essentially irreversible inhibition.

Human rhinoviruses (HRVs) occur in over 100 different serotypes and are the major cause of the common cold. In infected cells their single strand of RNA is translated into a large polyprotein that is proteolytically processed by several virally-encoded cysteine proteinases to produce viral coat protein and other proteins essential for viral replication.[1] One of these enzymes, called $3C^{pro}$, is a 20 kDa protein that specifically recognizes and cleaves several glutamine–glycine (Q–G) bonds in the viral polyprotein.[2] Mutagenesis of the active site cysteine in HRV $3C^{pro}$ produces an inactive enzyme;[3] comparable changes in the related poliovirus $3C^{pro}$ also yield inactive enzyme and prevent viral replication in transfected cells.[4] These observations suggest that small-molecule inhibitors of $3C^{pro}$ enzymes might be attractive as potential antiviral agents.

Active site mapping studies indicate that the minimal consensus substrate for HRV $3C^{pro}$ contains a -P_4-P_3-P_2-Q—G-P- sequence in which P_4 is usually a small nonpolar residue. Peptidyl aldehydes,[5–7] carbonyl compounds,[8,9] halomethyl ketones,[10,11] and Michael acceptors[12–14] have been demonstrated to inhibit HRV $3C^{pro}$ in vitro and, in some cases, to inhibit viral replication in infected cells. Peptidyl carbazate esters (i.e., azapeptide esters, such as Boc–VLFaQ–OPh, below) constitute another generic group of proteinase inhibitors that inhibit serine and cysteine proteinases by carbamoylating the active site nucleophile.[15,16] In this paper, we report on the action of eight azapeptides as substrates and inhibitors of HRV-14 $3C^{pro}$.

Boc-VLFaQ-OPh (**4a**):



Chemistry. To generate the azaglutamine (aQ) skeleton the hydrazide derivative of Boc-phenylalanine (**1**) was combined with acrylamide (1.3 equiv) in a minimum volume of DMF (ca. 1.5 mL/g total reactants) and heated to 110 °C under nitrogen for 36 h (Scheme 1). Partitioning the products between brine and ethyl acetate afforded **2** as white crystals (mp 175–176 °C) in 35–40% yield after chromatography over silica gel using 5%

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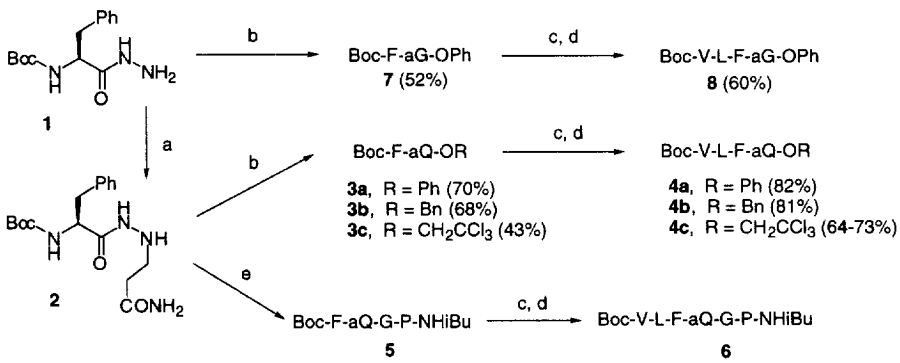
MeOH/EtOAc. Acylation of **2** with the appropriate chloroformate esters (ClCOOR) afforded azapeptide esters **3a–3c**. The latter were deblocked and coupled to Boc-VL-OH[12] using the diethylcyanophosphonate (DECP) method,[12] which furnished compounds **4a–4c**. Applying similar procedures directly to **1** rather than **2** furnished the azaglycine (aG) analogs **7** and **8** (Scheme 1).

Synthesis of **5** required the isocyanoacetyl derivative of proline isobutylamide (i.e., O=C=N-CH_2CO-Pro-NH-*i*-Bu), which was generated in situ as follows. Boc-Gly-Pro-NH- *i*-Bu was deprotected by stirring with TFA in CH_2Cl_2 (1 h, rt) and evaporated to dryness. The residue and *i*-Pr_2NEt (2 equiv) were dissolved in CH_2Cl_2 and added to a stirred solution of triphosgene (0.35 mol/mol peptide) in CH_2Cl_2. After stirring for 10 min a solution of **2** in DMF was added and stirring continued for 10 min. Partitioning the mixture between brine and ethyl acetate afforded **5** as a colorless gum in 68% yield. The latter was deprotected and coupled to Boc-VL-OH as described above affording **6** in ca. 30% yield after flash chromotography over silica gel (5% MeOH/EtOAc).[17]



Conditions: (a) CH_2=CHCONH$_2$, DMF, 110 °C, 36 h; (b) ROCOCl (R = Ph, Bn, or CH_2CCl_3), MeOH, Et_3N; (c) TFA/CH_2Cl_2; (d) Boc-VL-OH, DECP, DMF, Et_3N; (e) TFA•GlyProNH- *i*-Bu, triphosgene, DMF, CH_2Cl_2, EtN(*i*-Pr)$_2$; followed by **2** in DMF.

Inhibitor Evaluation. HRV-14 $3C^{pro}$ was expressed, purified,[18] and assayed using the chromogenic *p*-nitroanilide substrate EALFQ-pNA as described previously.[12] Based on the expectation that the azapeptides would irreversibly inactivate $3C^{pro}$ by carbamoylation of its active site cysteine a two-stage incubation-dilution assay procedure was utilized. Azapeptides (50–150 μM) were incubated with $3C^{pro}$ (0.75–3.3 μM) in buffer (25 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.5) at 28–30 °C; aliquots of this mixture (50 μL) were withdrawn periodically and diluted into 600 μL buffer containing 250 μM EALFQ-pNA and absorbance at 405 nm was recorded. Control experiments in which the azapeptides were omitted showed little loss of enzyme activity over 24–30 h in assay buffer alone. To assess the hydrolytic stability of the azapeptides in the absence (or presence) of $3C^{pro}$, aliquots of solutions in buffer were analyzed directly by reverse phase HPLC (Vydac C-18 column, 4.6 x 250 mm, eluted isocratically with aqueous acetonitrile (30–60% v/v as needed) containing 0.1% v/v TFA.

Results and Discussion. Azapeptide **6** most closely resembles the normal consensus substrate for $3C^{pro}$, but after incubating **6** (142 μM) with $3C^{pro}$ (0.75 μM) for 60 min, a dilution assay indicated negligible (<5%) loss of enzyme activity and HPLC indicated that no hydrolysis of **6** had occurred. Azapeptide esters **4a–**

4c are the next most substrate-like compounds in the series. Initial experiments with **4a** indicated that it inhibits $3C^{pro}$ rapidly but incompletely. This is in contrast to related azapeptide esters which inactivate papain and cathepsin B not only rapidly but also completely and essentially irreversibly.

Closer examination of the time and concentration dependence of the inhibition of $3C^{pro}$ by **4a** generated the activity profiles shown in Figure 1. The transient nature of the inhibition, coupled with the dependence of both the initial degree and the duration of inhibition on inhibitor concentration suggests that **4a** is a slow-turnover substrate for $3C^{pro}$ (i.e., that a carbamoyl enzyme intermediate forms rapidly but hydrolyzes more slowly than the corresponding acyl enzyme from a normal substrate). HPLC examination of these reaction mixtures confirmed that **4a** is indeed extensively hydrolyzed by $3C^{pro}$ over the time course of these experiments.

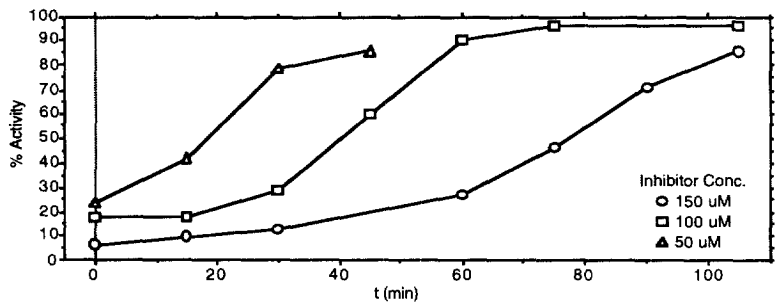


Figure 1. Effect of azapeptide **4a** on the activity of HRV-14 $3C^{pro}$. Enzyme was incubated with the test compound at the concentrations indicated and aliquots were withdrawn and diluted 13-fold into assay buffer containing the chromogenic substrate EALFQ-pNA. Enzyme activity is reported relative to controls in which inhibitor was omitted. For each inhibitor concentration the earliest data were obtained ca. 10–15 s after mixing.

Since **6** and **4a** would generate the same carbamoyl enzyme, the failure of **6** to be hydrolyzed by $3C^{pro}$ can be attributed to its semicarbazide moiety being insufficiently reactive to carbamoylate the enzyme's active site thiol. In agreement with this, azapeptide amides and *p*-nitroanilides are neither substrates nor inhibitors of papain. In contrast to **4a**, compound **3a** shows no activity as an inhibitor of $3C^{pro}$, whereas the closely related *N*-acetyl analog (i.e. Ac-FaG-OPh) is a very potent affinity label for papain (k_{inact}/K_i = 67,600 $M^{-1}s^{-1}$).[15] This result reflects the importance of the P_4 and P_3 residues for substrate recognition by $3C^{pro}$ and the dependence of the inactivation (carbamoylation) process on the same intermolecular interactions which contribute to catalysis with normal peptide substrates.

Azapeptide benzyl- and trichloroethyl esters analogous to **4b** and **4c** are very reactive inactivators of papain and cathepsin B and can even be used for active site titration of these enzymes. Surprisingly, however, **4b** and **4c** do not inactivate and are not hydrolyzed by $3C^{pro}$. As with **6**, this must be ascribed to the failure of these compounds to carbamoylate the active site of $3C^{pro}$, because they would generate the same intermediate as **4a** which does turn over with $3C^{pro}$.

In view of its lack of P_4 and P_3 residues, and its lack of a P_1 glutamine side chain, the failure of azaglycine derivative **7** (150 μM) either to inactivate or to be hydrolyzed by $3C^{pro}$, despite its having a reactive leaving group, is perhaps not surprising. However, incubating its larger analog **8** (150 μM) with $3C^{pro}$ results in a slow but kinetically first-order loss of enzyme activity ($k \approx 0.009$ min^{-1}, data not shown) with no sign of reactivation at later times as seen in Figure 1. Because of the very slow inactivation rate with **8** we did not

pursue this observation further. However, comparing results with **4a** to those with **8** suggests that as a determinant of enzyme specificity, the glutamine side chain may be more important for deacylation than for acylation when the substrate contains a reactive leaving group.

In conclusion, small azapeptides which resemble the consensus substrate for $3C^{pro}$ and contain a reactive leaving group (-OAr but not -OR, -NHR) inactivate the enzyme by carbamoylating its active site thiol. A reactive leaving group is insufficient in the absence of P_4 and P_3 residues and, conversely, the lack of a reactive leaving group can not be overcome by the presence of these residues. A P_1 glutamine side chain may be required for efficient acylation *and deacylation* of the enzyme.

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