Targeting the Dimerization Interface of HIV-1 Protease: Inhibition with Cross-Linked Interfacial Peptides

Reena Zutshi, Jeff Franciskovich, Michael Shultz, Barbara Schweitzer, Patricia Bishop, Matt Wilson, and Jean Chmielewski*

Contribution from the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

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Abstract: Agents have been designed and synthesized which target the dimerization interface of HIV-1 protease. These agents, which contain cross-linked peptides from the N- and C-termini of the protease, both inhibit HIV-1 protease activity and decrease the amount of protease dimer in solution as measured by size exclusion chromatography, protein crosslinking, and protease fluorescence studies. Additionally we have shown that active site-targeted agents inhibit HIV-1 protease activity but have little effect on protease dimerization. These data support the claim that inhibition with the crosslinked agents is based on a decrease in the amount of protease homodimer in solution which in turn is responsible for a decrease in the activity of the protease.

Introduction

Enzyme inhibitors have traditionally been designed to bind into the active site of an enzyme and block the binding of substrate. A novel approach to inhibitor design for multiple-subunit enzymes would be based on dissociation of enzyme subunits which would ultimately destroy the active site and result in loss of biological activity. To date work in this area has focused on targeting the interface of the large and small subunits of ribonuclease reductase with small peptides and peptidomimetics[1] and targeting the dimerization interface of HIV-1 protease with peptides from its N- and C-termini.[2,3] In this paper we describe agents which contain N-terminally crosslinked, interfacial protease peptides designed to mimic the dimerization interface of the protease.

HIV-1 protease self assembles into a homodimeric structure[4] with K_d estimates in the range of 39 pM to 440 nM.[5] Dimerization of HIV-1 protease generates the catalytic center of the enzyme and also the substrate binding pocket. The dimerization interface is composed, to a large extent, by interdigitating the N- and C-terminal portions of the protease into a four-stranded β-sheet (Figure 1). By targeting this β-sheet portion of the protease, a region which is highly conserved among HIV-1 isolates,[6] agents may be generated which block the assembly of the homodimer or disrupt the dimeric interface, which should lead to a loss of biological activity.

Initial results had demonstrated that peptides corresponding to the N- and C-termini of HIV-1 protease inhibit protease activity.[2] The peptides with optimum activity[2c] (**1** and **2**) have been incorporated into novel structures containing a tether between the two peptide sequences (**4a**−**g**). These compounds are unique in that the N-termini of the protease peptides are covalently crosslinked to yield compounds capable of forming 1:1 complexes with a protease monomer (Figure 2). An alternative strategy developed by Babe *et al*.[3] generated a contiguous peptide sequence which contained the N- and C-terminal regions of HIV-1 protease. These agents linked C-terminal and N-terminal protease peptides with a 3.5 Å tether composed of three glycine residues (**11** and **12**). In the protease, however, the N- terminal ends of residues Pro(1) and Cys(95) are held at a distance of approximately 10 Å (Figure 1). Our agents, therefore, were designed to bridge this 10 Å gap while allowing the N- and C-terminal peptides to bind into the appropriate four stranded β-sheet arrangement with a protease monomer. This necessitated crosslinking the two N-termini of peptides **1** and **2** with tether molecules capable of spanning this 10 Å distance. Flexible tether molecules of a variety of lengths, therefore, were incorporated into agents **4a**−**g** to adequately assess the spacial requirements of the peptides when bound to a protease monomer.

* Author to whom correspondence should be addressed.

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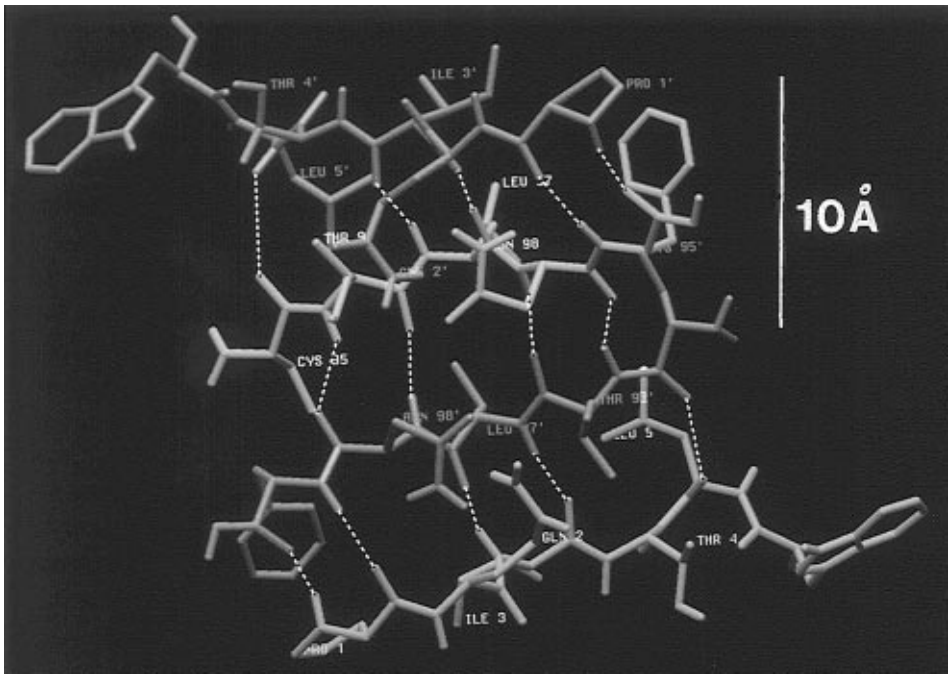

Figure 1. The four-stranded, β-sheet portion of the HIV-1 protease dimerization interface, showing one monomer in green and the other in pink.

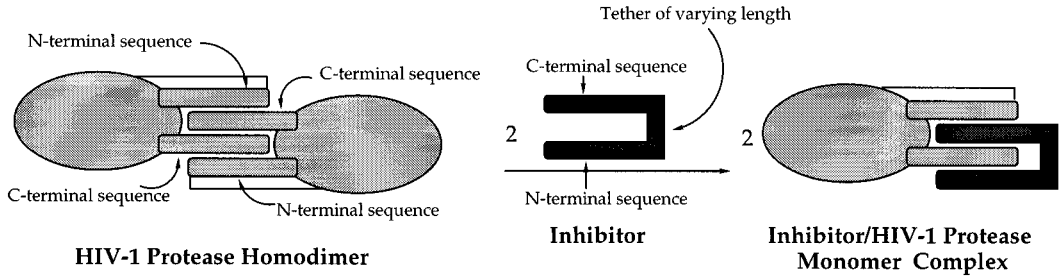

Figure 2. A schematic of the strategy employed for dimerization inhibition.

Results and Discussion

Peptides **1**−**3** were synthesized by a solid phase approach on the Wang resin[7] using an Fmoc-based strategy.[8] Agents **4** and **5** were synthesized either in solution by adding 1 equiv of di-*N*-hydroxysuccinimide esters **6a**−**e** to 1 equiv each of peptide **1** and peptide **2** or **3** (Scheme 1a), followed by HPLC purification of the desired products in approximately 5% overall yield, or on a solid support by treating peptide **2** with diesters **6a**−**e** followed by addition of peptide **1** on the Wang resin (Scheme 1b). Cleavage from the resin and HPLC purification provided the desired products in greater than 15% yield. The sequences of all compounds were confirmed by mass spectrometry (FAB) and amino acid analysis.

HIV-1 protease inhibition was evaluated using a fluorogenic substrate assay developed by Toth and Marshall.[9] The effect of 30, 60, and 240 min preincubation of agents **4a**−**g** with HIV-1 protease upon the hydrolysis of substrate[9] was evaluated by monitoring the increase in fluorescence at 430 nm with respect to time. Maximum inhibition was obtained after 60 min preincubation and did not improve with longer times. Agents with tethers containing 10−12 methylene groups (**4a**−**c**) produced IC_{50} values which were greater than 15 μM (Table 1). Lengthening the tether within these compounds by one or two methylene units (**4d** and **4e**) led to a 8- to 10-fold increase in inhibition, with IC_{50} values in the low micromolar range (2 μM). Further extension of the tether by an additional one to two methylene units (**4f** and **4g**) led to small decreases in the inhibition obtained. All agents tested showed no proteolysis during the 1−4 h incubations with HIV-1 protease. A number of modifications were made in the peptide portion of these agents. Replacing the Trp residue in compound **4c** to Phe (**5**) led to a 2-fold increase in the observed inhibition for **5** as compared to **4c**. Removing the aromatic residues of **4c** either individually (**7** and **8**) or at the same time (**9**) led to a 3−4-fold decrease in inhibition for the single deletion compounds **7** and **8** and a larger decrease in inhibition with **9**, which demonstrates the importance of these residues in the current design. If both

Scheme 1. (a) Solution Phase and (b) Solid Phase Synthesis of Agents

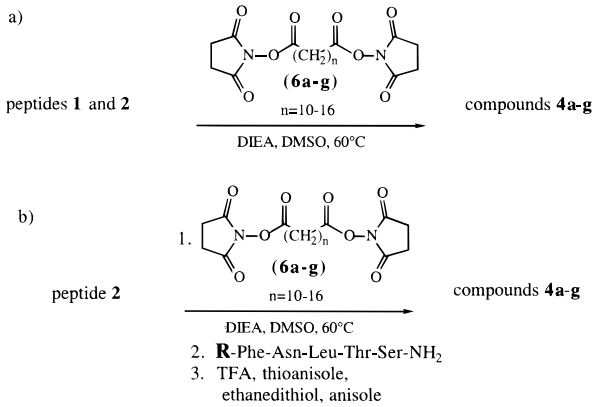

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Table 1. Inhibition of HIV-1 Protease with Cross-linked Peptides

compound	IC_{50} (μM)[a]	compound	IC_{50} (μM)[a]
4a (10 CH_2)	20	**4g** (16 CH_2)	4.0
4b (11 CH_2)	14	**5**	6.5
4c (12 CH_2)	13	**7**	37
4d (13 CH_2)	2.5	**8**	44
4e (14 CH_2)	2.0	**9**	>100
4f (15 CH_2)	3.2	**10**	57

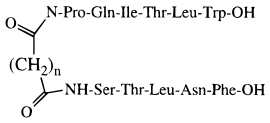
[a] All values are ±10%.

Chart 1

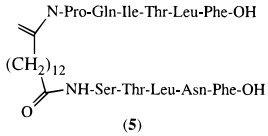
NH_2-Ser-Thr-Leu-Asn-Phe-OH (**1**)

NH-Pro-Gln-Ile-Thr-Leu-Trp-OH (**2**)

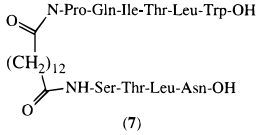
NH-Pro-Gln-Ile-Thr-Leu-Phe-OH (**3**)



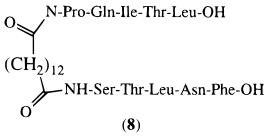
(**4a-g**) n = 10-16



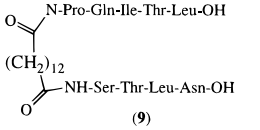
(**5**)



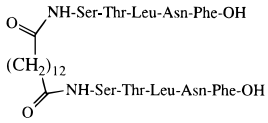
(**7**)



(**8**)



(**9**)



(**10**)

HN-Pro-Gln-Ile-Thr-Leu-$(Gly)_3$-Cys -Thr-Leu-Asn-Phe-OH (**11**)

H_2N-Met-Ser-Leu-Asn-Leu-$(Gly)_3$-Met -Ser-Leu-Asn-Leu-OH (**12**)

peptides of these agents are interdigitated into the protease monomer as depicted in Figure 2, we expected that an agent which contains two copies of peptide **1** (**10**) would be a poor inhibitor of HIV-1 protease because of the lack of a second interdigitating arm of the correct sequence. Agent **10** showed a significant decrease in HIV-1 protease inhibition as compared to agents with both, full peptide sequences, which lends support to an interdigitation, inhibition pathway.

In addition to testing for protease inhibition, size exclusion chromatography, protein crosslinking, and protease fluorescence assays were designed to monitor dissociation of HIV-1 protease directly with these agents. Due to the presence of two tryptophan residues within the protease, one of which is buried at the interface, the fluorescence of HIV protease was used as a means to monitor its dissociation. The fluorescence of the protease was monitored while adding increasing amounts of the agents to a constant protease concentration. A potent inhibitor from the substrate cleavage assay which lacks intrinsic fluorescence (**5**) produced a dramatic decrease in the fluorescence of the protease (Figure 3a). Upon addition of 8 μM of **5**, approximately 50% of the Trp fluorescence was quenched, presumably due to increased solvent exposure in the inhibitor/protease monomer complex as compared to the protease homodimer. No effect on the protease fluorescence was observed with similar concentrations of the weak inhibitor **7** (Figure 3c), as was also observed with the active site inhibitor MVT-101[10] (Figure 3b).

Apparent molecular weights were obtained for HIV-1 protease by size exclusion chromatography in the presence and absence

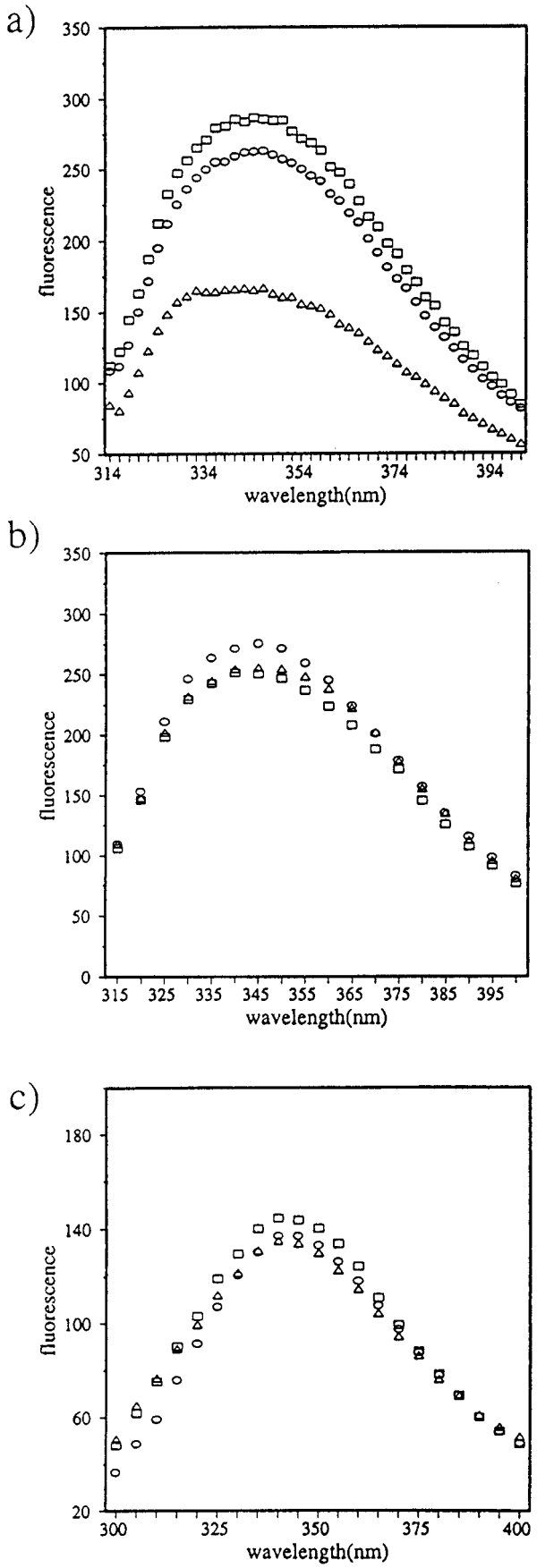


Figure 3. Fluorescence spectra of HIV-1 protease (300 nM) with agents (a) **5**, (b) MVT-101, and (c) **7**, □ - 0 μM, ○ - 1 μM, △ - 8 μM.

of agents **5** and MVT-101. The apparent molecular weight for the protease dimer (10 μM concentration of loaded sample) with no added inhibitor or with MVT-101 (100 μM) was determined

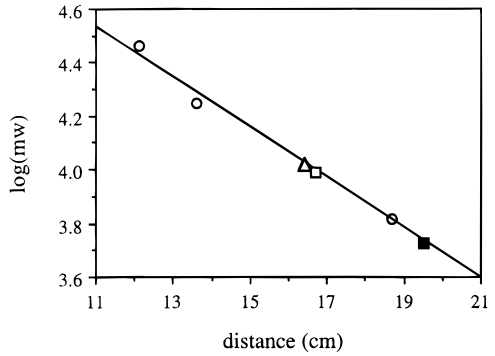


Figure 4. Size exclusion chromatography of HIV-1 protease (△), and HIV-1 protease with compound **5** (■) and MVT-101 (□). A standard curve was generated using carbonic anhydrase, myoglobin, and aprotinin (○).

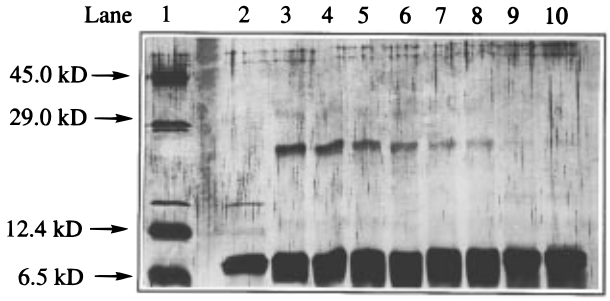


Figure 5. HIV-1 protease cross-linking reactions with BS^3 in the presence or absence of inhibitors. Lane 1: MW markers lane 2: protease; lane 3: protease and BS^3 (300 eq); lane 4: protease, BS^3 and MVT-101 (2 μM); lane 5: protease, BS^3 and **5** (5 μM); lane 6: protease, BS^3 and **5** (13 μM); lane 7: protease, BS^3 and **5** (30 μM); lane 8: protease, BS^3 and **5** (60 μM); lane 9: protease, BS^3 and **5** (120 μM); lane 10: protease, BS^3 and **5** (200 μM).

to be 11 000 by interpolation of a standard protein curve (Figure 4). Upon addition of agent **5** (100 μM), however, the peak at 10 500 was eliminated with the appearance of a new, broad peak at approximately one-half of the molecular weight of the dimer (5300). Cross-linking studies with HIV-1 protease (670 nM) and the crosslinker bis(sulfosuccinimidyl)suberate (BS^3) produced a higher molecular weight band with an approximate molecular weight in the dimer−trimer region of SDS PAGE gel (Figure 5). Addition of MVT-101 (2 μM) had little effect on the extent of protease oligomers crosslinked, whereas the addition of **5** (5−200 μM) at low concentrations decreased the amount of cross-linked protease, and the addition of higher concentrations of **5** eliminated the cross-linked band on the gel.

Modeling studies suggested that if both peptides of the inhibitor were incorporated into the β-sheet upon complexation with a protease monomer, the aliphatic chain in agents **4a**−**c** would lie within the van der Waals radius of Phe99 in the monomer. Modeling compounds with greater than 12 methylene units (**4d**−**g**), however, showed limited tether interactions with Phe99 upon complexation, which is in agreement with the inhibition data. In the modeled complexes, the aromatic residues in **4d**−**g** fit into two hydrophobic cavities within the protease monomer and may serve to anchor the agents within the protease, which would explain the poorer inhibition obtained with compounds **7** and **8** and the lack of inhibition obtained with agent **9**. Direct measurement of protease dissociation via fluorescence with **5** showed quenching of the protease fluorescence in a concentration range which is in good agreement with the inhibition of protease activity obtained for **5** with the substrate cleavage assay (IC_{50}, 6.3 μM). Both size exclusion and protein crosslinking studies also confirmed the decrease in the amount of protease dimer in solution with the addition of agent **5**, although the apparent molecular weight of the protease (approximately 6000) was lower than the expected molecular weight (approximately 11 000) using both techniques.

Previous studies by Babe *et al.*[3] used agents in which the interfacial peptides of HIV-1 protease were linked via three glycine residues (**11** and **12**). These agents were not designed to interdigitate using both halves and would not bind a protease monomer as in the present design (Figure 2), but would dissociate a protease dimer by binding half of the agent into each protease monomer. These agents showed selective inhibition of HIV-1 protease and decreased the amount of HIV-2 protease dimer in solution as determined by protein crosslinking but had IC_{50} values only in the 40−50 μM range (10 nM protease). By crosslinking the amino-termini of the C- and N-terminal peptides of HIV-1 protease much more effective inhibitors have been obtained with the best IC_{50} values in the range of 1−5 μM. The role of both peptides in inhibition was also evaluated with an agent which contained two copies of peptides **1**. Only agents which contain a copy of each peptide crosslinked at the N-terminus were effective inhibitors of HIV-1 protease.

Conclusion

In conclusion, agents were designed and synthesized which target the dimerization interface of HIV-1 protease, and both inhibit HIV-1 protease activity and decrease the amount of protease dimer in solution as measured by size exclusion chromatography, protein crosslinking, and protease fluorescence studies. Additionally we have shown that active site-targeted agents inhibit the HIV-1 protease activity but have little effect on the protease dimerization. Experiments with agent **10** demonstrated the necessity of two specific peptide sequences for optimum inhibition. These data support the claim that inhibition with these agents is based on a decrease in the amount of biologically active protease homodimer in solution which in turn is responsible for a decrease in the activity of the protease. This approach offers a unique means of inhibiting HIV-1 protease, and this strategy is currently being extended to irreversible protease inhibition and to other multisubunit enzymes and receptors.

Experimental Section

Materials and Methods. HIV-1 protease (affinity purified grade), amino acids, and resins used for peptide synthesis were purchased from Bachem Bioscience. MVT-101 was purchased from Novabiochem. The crosslinking agent (BS^3-bis(sulfosuccinimidyl)suberate) and the molecular weight markers were purchased from Pierce Chemical company. Sephadex G-50 was purchased from Sigma. All other chemicals were purchased from Aldrich. All peptides synthesized were purified by reverse phase-HPLC on a Waters Delta Prep 4000 using a UV detector. The fluorescence-based assays were done on a Hitachi F-2000 spectrofluorometer. For size exclusion chromatography, the chosen mode of detection was fluorescence for which Perseptive Biosystems' F-0304 fluorescence detector was used. Amino acid analysis was done on Beckman 7300 amino acid analyzer. Buffer A contains 20 mM phosphate, 20% glycerol, 0.1% CHAPS, 1 mM DTT, and 1 mM EDTA, at pH 5.5.

Synthesis of Peptides. Peptides were synthesized by a solid phase procedure using Wang's *p*-alkoxybenzyl alcohol resin.[7] Fluorenylmethyloxycarbonyl (Fmoc) was used as the semipermanent amine protecting group, and amino acid side chains were protected with *tert*-butyl-based functionality. Amino acid couplings were performed via the hydroxybenzotriazole method. The peptides were cleaved from

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the resin with trifluoroacetic acid (90%), thioanisole (5%), ethanedithiol (3%), and anisole (2%) and purified to homogeneity by reverse phase HPLC. The purified peptides were characterized by mass spectrometry and amino acid analysis:

1. FAB-MS (NBA matrix) calcd 580, found 581 ($M + H^+$). Amino acid analysis: Asx(1) 1.1, Thr(1) 1.0, Ser(1) 0.8, Leu(1) 1.1, Phe(1) 1.1.

2. FAB-MS (NBA matrix) calcd 756, found 757 ($M + H^+$). Amino acid analysis: Thr(1) 1.0, Glx(1) 1.1, Pro(1) 1.1, Ile(1) 1.0, Leu(1) 1.1, Trp (1).

3. FAB-MS (NBA matrix) calcd 717, found 718 ($M + H^+$). Amino acid analysis: Thr(1) 0.9, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(1) 1.1, Phe(1) 1.0.

Solution Phase Synthesis of Inhibitors. Typically peptide **2** (50 mg, 0.07 mmol), DIEA (12 μl, 0.07 mmol), and 0.07 mmol of the appropriate bis(*N*-hydroxysuccinimide ester) (**6a**−**g**) were dissolved in 10 mL of DMSO and stirred at 60 °C for 12−18 h. Peptide **1** (41 mg, 0.07 mmol) and DIEA (12 μL, 0.07 mmol) were added to the reaction mixture, and stirring was continued for 24 h at 60 °C. The desired material was purified by reverse phase HPLC with a yield of approximately 5%.

Solid Phase Synthesis of Inhibitors. Typically peptide **2** (50 mg, 0.07 mmol), DIEA (12 μL, 0.07 mmol), and 0.07 mmol of the appropriate bis(*N*-hydroxysuccinimide ester) (**6a**−**g**) were dissolved in 10 mL of DMSO and stirred at 60 °C for 12−18 h. Peptide **1** on the Wang resin (300 mg, 0.13 mmol) was added to the reaction mixture and allowed to react at 60 °C for 24 h. The resin was filtered, washed with DMF, CH_2Cl_2, MeOH, and EtOH, and dried *in vacuo* for 4 h. The resin was treated with trifluoroacetic acid (90%), thioanisole (5%), ethanedithiol (3%) and anisole (2%) and precipitated in cold ether overnight. The precipitated crude inhibitor was purified to homogeneity by reverse phase HPLC with a yield of approximately 15%.

Characterization of Inhibitors. All inhibitors were purified on C_8 reverse phase HPLC using a linear gradient of 35% solvent A(CH_3-CN/0.1% TFA), 65% solvent B (H_2O/0.1% TFA) to 60% solvent A(CH_3CN/0.1% TFA), 40% solvent B (H_2O/0.1% TFA). The purified inhibitors were characterized by mass spectrometry and amino acid analysis.

4a: FAB-MS (NBA matrix) calcd 1530, found 1531 ($M + H^+$). Amino acid analysis: Asx(1) 1.0, Thr(2) 1.8, Ser(1) 0.6, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 1.9, Phe(1) 0.9, Trp (1).

4b: FAB-MS (NBA matrix) calcd 1544, found 1567 ($M + Na^+$). Amino acid analysis: Asx(1) 1.1, Thr(2) 1.8, Ser(1) 0.6, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 2.0, Phe(1) 1.0, Trp (1).

4c: FAB-MS (NBA matrix) calcd 1558, found 1559 ($M + H^+$). Amino acid analysis: Asx(1) 1.2, Thr(2) 2.0, Ser(1) 0.9, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 2.2, Phe(1) 1.2, Trp (1).

4d: FAB-MS (NBA matrix) calcd 1572, found 1595 ($M + Na^+$). Amino acid analysis: Asx(1) 1.3, Thr(2) 2.0, Ser(1) 1.0, Glx(1) 0.8, Pro(1) 0.8, Ile(1) 0.7, Leu(2) 2.1, Phe(1) 1.3, Trp (1).

4e: FAB-MS (NBA matrix) calcd 1586, found 1587 ($M + H^+$). Amino acid analysis: Asx(1) 1.1, Thr(2) 1.9, Ser(1) 0.6, Glx(1) 0.8, Pro(1) 0.9, Ile(1) 1.0, Leu(2) 2.5, Phe(1) 1.0, Trp (1).

4f: FAB-MS (NBA matrix) calcd 1600, found 1639 ($M + K^+$). Amino acid analysis: Asx(1) 1.3, Thr(2) 2.0, Ser(1) 1.0, Glx(1) 0.9, Pro(1) 0.9, Ile(1) 0.9, Leu(2) 2.1, Phe(1) 1.2, Trp (1).

4g: FAB-MS (NBA matrix) calcd 1614, found 1637 ($M + Na^+$). Amino acid analysis: Asx(1) 1.3, Thr(2) 2.0, Ser(1) 1.0, Glx(1) 0.9, Pro(1) 0.9, Ile(1) 0.9, Leu(2) 2.1, Phe(1) 1.2, Trp (1).

5: FAB-MS (NBA matrix) calcd 1519, found 1542 ($M + Na^+$). Amino acid analysis: Asx(1) 1.2, Thr(2) 1.8, Ser(1) 0.7, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 2.2, Phe(2) 2.2.

7: FAB-MS (NBA matrix) calcd 1412, found 1435 ($M + Na^+$). Amino acid analysis: Asx(1) 1.2, Thr(2) 1.8, Ser(1) 0.7, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 2.2.

8: FAB-MS (NBA matrix) calcd 1373, found 1396 ($M + Na^+$). Amino acid analysis: Asx(1) 1.2, Thr(2) 1.8, Ser(1) 0.7, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 2.2, Phe(1) 1.2.

9: PD-MS calcd 1225, found 1228 ($M + H^+$). Amino acid analysis: Asx(1) 1.2, Thr(2) 1.9, Ser(1) 0.7, Glx(1) 1.0, Pro(1) 1.0, Ile(1) 0.9, Leu(2) 2.1.

10: FAB-MS (NBA matrix) calcd 1382, found 1404 ($M + Na^+$). Amino acid analysis: Asx(2) 2.2, Thr(2) 2.0, Ser(2) 1.5, Leu(2) 2.2, Phe(2) 2.2.

Enzyme Assay. Fifty microliters of a 200 nM HIV-1 protease solution in a buffer containing 20 mM phosphate, 20% glycerol, 1 mM DTT, 1 mM EDTA, and 0.1% CHAPS at pH 5.5 (Buffer A) was preincubated with 10 μL of the inhibitor solution in DMSO for 1 h. This solution was added to 40 μL of the substrate solution (65 μM, Buffer A with 10% DMSO) at 25 °C. The final concentration of DMSO was kept constant at 14%. The change in fluorescence at 430 nm (λ_{ex} = 355 nm) was monitored over a period of 5 min at 18 °C.

Size Exclusion Chromatography. Size exclusion chromatography was done at 4 °C using a 1.8 cm × 98 cm column of Sephadex G-50 equilibrated with a buffer containing 20 mM phosphate, 1 mM DTT, and 1 mM EDTA at pH 5.5. A flow rate of 0.26 mL/min was maintained, and the eluant was detected via fluorescence. A standard curve was generated using bovine serum albumin, carbonic anhydrase, myoglobin, and aprotinin. Seventy-five microliters of 0.1 mg/mL HIV protease solution was loaded on the column, and the molecular weight of the eluent peaks was calculated from the standard curve. Similarly, 75 of 0.1 mg/mL HIV protease solution was preincubated with either 1.5 μL of a 5 mM solution of **5** (DMSO) or 2 μL of a 0.4 mM solution of MVT-101 (DMSO) for 1.5 h at room temperature, this solution was loaded on the column, and the molecular weight of the eluting species calculated.

Crosslinking of HIV Protease. A 670 nM solution of HIV-1 protease (26 μL) in a 20 mM phosphate buffer, pH 7, was incubated with **5** (2 μL, 0.1−2.0 mM) or MVT-101 (2 μL, 0.4 mM) in DMSO for 2 h at 25 °C. The concentrations of **5** used were 5, 13, 30, 60, 120, 200, and 2 μM of MVT-101. The reaction mixtures were treated with the crosslinking agent BS^3 (300 equiv) for 4 h at 37 °C. A control containing protease with no inhibitor was also treated with BS^3. The reaction mixtures were denatured and analyzed by SDS PAGE on 17% polyacrylamide gels at a constant current of 30 mA. The protein bands were visualized by silver staining.

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Supporting Information Available: Densitometry readings from the crosslinking gel (2 pages). See any current masthead page for ordering and Internet access instructions.

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